Studies of Fish Responses to the Antifoulant Medetomidine

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TILL

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ABSTRACT

Growth of marine organisms, fouling, on man-made constructions submerged in the water is regarded as a major problem. For vessels, fouling increases drag and thereby fuel consumption, wherefore antifouling paints are used. Traditionally, they contain toxic compounds, and several of these have unwanted effects in the environment. Today the search for environmentally acceptable and efficient alternatives is intense.

Medetomidine, originally used as a veterinary sedative, inhibits barnacle settling at nanomolar concentrations. It is presently under evaluation for use as an antifouling agent. The studies within this thesis were performed to investigate medetomidine responses in fish. The focus was to identify early effects, occurring from low concentrations. Studies have been performed in the species rainbow trout, Atlantic cod, turbot, Atlantic salmon and three spined stickleback. Exposure time vary from 1 up to 54 days, and a set of parameters have been investigated including biochemical biomarkers, growth and related parameters, behaviour and large scale gene expression.

Paleness is the most obvious effect of medetomidine in fish and appears from 0.5 to 50 nM, depending on species. Colour was observed and quantified, and the function of melanophores (pigment cells) after long term exposure to medetomidine was investigated. It is suggested that melanophores are functional after treatment, and thus the colour change may be reversible. Although not lethal per-see, paleness may have consequences for fish predator-prey interactions (camouflage), social signalling and UV protection.

Medetomidine also showed to affect the activity of the hepatic enzyme Cytochrome P4501A (CYP1A), measured as EROD activity. A minor increase in activity was observed *in vivo* in several of the investigated species. *In vitro*, medetomidine showed instead to be a potent inhibitor of EROD activity with median inhibition values (IC50) in the nanomolar range. An inhibited CYP1A activity may interfere with fish detoxification of toxicants abundant in the aquatic environment.

No significant effects were found on growth rate, but the results indicate lowered blood glucose levels and decreased liver size after medetomidine treatment and thus a shift in carbohydrate metabolism. The large scale gene expression study revealed no significant differences among treatments. We found no effects on glutathione or glutathione dependent enzymes in any of the studies. In the behavioural studies, fish were less active and had less appetite in medetomidine treatments compared to control. Medetomidine had no effects on investigated antioxidant enzymes and showed no cytotoxicity.

Among the responses studied within this thesis, paleness and inhibition of EROD activity are perhaps the most important. These effects appear early and are clear and consistent among several species.

Keywords: Fish, Medetomidine, Antifouling, Ecotoxicology, Biomarker

POPULÄRVETENSKAPLIG SAMMANFATTNING

Livet i havet är aldrig stilla. Därför föredrar tusentals växter och djur att sitta fast på en yta. Till exempel havstulpaner, musslor, sjöpungar och alger. För sjöfarten är denna *marina påväxt* ett stort problem, och långt tillbaks i historien hittar man bevis för hur människor försökt skydda sina fartygsskrov från den. Det största problemet med påväxten är att friktionen mot vattnet ökar markant, och därmed också bränsleförbrukningen. Påväxten är på så sätt ett miljöproblem, likväl som ett praktiskt och ekonomiskt problem.

För att bli av med påväxten har man traditionellt använt sig av giftig färg som målats på skrovet. Gifterna håller undan påväxten, men problemet är att även den omgivande havsmiljön skadas av gifterna. Ett viktigt exempel är färger innehållande TBT, tennorganiska föreningar. Dessa föreningar är svårnedbrytbara och ackumuleras genom näringskedjorna. Många organismer far illa av dessa substanser och stora skador har upptäckts hos blötdjur som får problem med att föröka sig. Sedan 2008 finns ett världsomfattande förbud mot TBT-innehållande färg. TBT har till stora delar ersatts av koppar och så kallade booster biocider, men dessa nya färger är sällan lika effektiva som TBT-färgerna, och dessutom har även många av dessa oönskade effekter i miljön.

De senaste tjugo åren har sökandet efter nya alternativ intensifierats. Fortfarande finns det ingen självklar lösning, men flera lovande idéer. Denna avhandling ingår som en del i forskningsprojektet Marine Paint, som fokuserar på dessa frågor. Medetomidine är en substans som används som sövningsmedel inom veterinärmedicin, men som också visat sig ha förmågan att hindra havstulpanens larver från att fästa vid en yta, redan vid mycket låga koncentrationer. Dessutom skadas inte larverna, utan kan slå sig ner någon annanstans För närvarande genomgår medetomidine en utvärdering enligt EU:s biociddirektiv rörande användningen av medetomidine som aktiv substans i båtbottenfärg.

Syftet med avhandlingen har varit att undersöka effekter av medetomidine på fisk, och fokus har varit på på tidig påverkan, som uppträder vid låga koncentrationer. I avhandlingen har fiskarterna regnbåge, torsk, piggvar, storspigg och atlantlax studerats. Fisken har behandlats med låga koncentrationer av medetomidine under perioder från ett dygn upp till 54 dygn och en rad olika parametrar har studerats. Däribland beteende, tillväxt, hormoner, avgiftningsenzymer och genetiska fingeravtryck.

Den mest uppenbara effekten av medetomidine är förmågan att göra fiskar bleka. Medetomidine påverkar de hudceller som innehåller pigment (melanoforer) och omfördelar pigmentet i dessa. Färgförändingarna har studerats och även funktionen hos pigmentcellerna efter längre tids exponering för medetomidine. Det verkar inte som att pigmentcellerna skadas under långvarig behandling med medetomidine, men däremot påverkas deras känslighet något. Blekheten är inte skadlig i sig, men en fungerande pigmentering är mycket viktig för t.ex kamouflage, kommunikation och UV-skydd.

En annan effekt som påträffats i flera av studierna är att aktiviteten av ett avgiftningsenzym i levern (CYP1A) påverkas. Vid studier på isolerade leverfraktioner har vi kunnat konstatera att enzymets verkan förhindras av medetomidine. Detta skulle kunna innebära att fiskars nedbrytning av skadliga substanser i miljön försämras av medetomidine.

I en långtidsstudie med regnbåge undersöktes om fiskens tillväxt påverkades av medetomidine. Dessutom undersöktes tillväxthormoner och andra faktorer relaterade till tillväxt. Vi kunde inte finna några effekter på själva tillväxten, men en påverkan på blodsockerhalt och leverstorlek. En storskalig analys av vilka gener som uttryckts i leverprover från försöket visade inte på några statistiska skillnader.

I beteendestudier sågs en något lägre aktivitet och aptit hos fiskar som behandlats med medetomidine, detta kan förklaras som en lätt sövningseffekt. Flera studier visar att medetomidine vid de aktuella koncentrationerna troligtvis inte ökar effekter av skadliga syreradikaler och dessutom visar studier på isolerade, odlade celler att medetomidine inte är giftigt för cellerna.

Avsikten med avhandlingen har varit att försöka identifiera en del av de effekter på fisk som skulle kunna uppträda vid användning av medetomidine i båtbottenfärg. De effekter vi sett har uppträtt tidigast vid koncentrationer från 0.5-50 nM. Att avgöra hur troligt det är att dessa koncentrationer, och därmed effekter, uppstår i miljön ligger utanför ramen för den här avhandlingen.

ABBREVIATIONS

ABC	ATP binding cassette
AhR	aryl hydrocarbon receptor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CDNB	chlorodinitrobenzene
CF	condition factor
CYP1A	cytochrome P450 1A
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
DTNB	5,5´-dithiobis-(2-nitrobenzoic acid)
EROD	ethoxyresorufin-O-Deethylase
GH	growth hormone
GR	glutathione reductase
GST	glutathione-S-transferase
HIS	heartsomatic index
IC50	median inhibition value
i.p.	intra peritoneal
IGF-1	insulin-like growth factor I
LSI	liver somatic index
MCH	melanophore concentrating hormone
MDR	multi drug resistance
mRNA	messenger RNA
MSH	melanophore stimulating hormone
MXR	multi xenobiotic resistance
nM	nanomolar
PAH	polyaromatic hydrocarbons
PCB	polychlorinated biphenyls
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
TBT	tributyltin

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following manuscripts and published papers. Published papers are reproduced with permission from Elsevier Ltd.

I Lennquist A, Hilvarsson A, Förlin L. 2010. *Responses in fish exposed to medetomidine, a new antifouling agent*. Marine Environmental Research. In press.

II Lennquist A, Lindblad Mårtensson LGE, Björnsson BTh, Förlin L. 2010. *The effects of medetomidine, a new antifouling agent, on rainbow trout physiology*. Manuscript.

III Lennquist A, Celander MC, Förlin L. 2008. *Effects of mdetomidine on hepatic EROD activity in three species of fish*. Ecotoxicology and Environmental safety 69, 74-79.

IV Lennquist A, Hedberg D, Lindblad Mårtensson LGE, Kristiansson E, Förlin L, 2009. *Colour and melanophore function in rainbow trout after long term exposure to the new antifoulant medetomidine*. Under revision.

V Lennquist A, Asker N, Kristiansson E, Brenthel A, , Björnsson BTh, Kling P, Larsson DGJ, and Förlin L. 2010. *Physiology and gene expression in rainbow trout (Oncorhynchus mykiss) after long term exposure to the new antifoulant medetomidine*. Manuscript.

INTRODUCTION

Fouling and antifouling

For shipping, colonisation of marine organisms on underwater surfaces has always been regarded as a major problem. Many, over 2000, marine species, prefer to live their life settled onto a hard surface in the turbulent aquatic environment. When a surface is submerged into the water, it is only a matter of minutes until the colonisation begins. Organic macromolecules attach first, and within hours or days, bacteria and unicellular algae. This *microfouling* is often referred to as biofilm or slime. *Macrofouling* starts when spores and larvae from larger species arrive to the surface, attach and metamorphose into sessile adults (Wahl 1989; Chambers *et al*. 2006) (figure 1).

Figure 1. Fouling is the growth of marine organisms on surfaces submerged in the water. More than 2000 species prefer to live settled to a hard substrate. This is the hull of a small leisure boat on the Swedish west coast.

The major problem with fouling is that it increases the drag and thereby the fuel consumption dramatically. Only the first layer of bacteria and unicellular algae may increase fuel consumption with up to 11 percent, and a heavily fouled vessel will eventually be very difficult to move (Schultz 2007) (figure 2). Other problems associated with fouling are corrosion, impaired manoeuvrability and spreading of invasive species. Also other types of underwater constructions such as oil rigs, fishing gear and cooling water systems need to be protected from fouling organisms.

Methods to prevent fouling have been used since early history. The traditional way to prevent fouling is to use toxic substances on the ship hull to kill the fouling organisms. Copper is one of the oldest antifouling agents, and it is still the one most

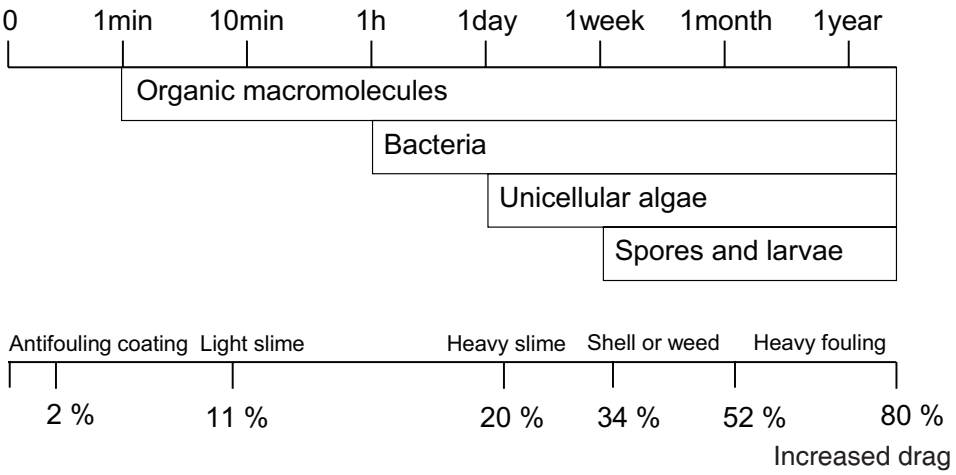


Figure 2. Marine biofouling starts within minutes after a surface is submerged in the water. The major problem with fouling is increased drag and thereby increased fuel consumption. The figure is redrawn from Wahl (1989) and Schultz (2007). The total cost of antifouling paints in the world has been estimated to 5 billions SEK per year (Ahlbom and Duus 2003).

commonly used. In the 1970´s, a revolutionary antifouling technique was introduced. This was self-polishing (SPC) paint containing organic tin compounds (TBT's). In a self-polishing paint, the surface layer is being hydrolysed over time, and thus a fresh layer of biocides is continuously being presented. By application of many layers of paint, the antifouling effect can last for 4-6 years. Additionally, the organic tin compounds are efficient towards the whole spectrum of fouling organisms in every part of the world.

Environmental impact of antifouling paints

Unfortunately, the compounds designed to prevent fouling also showed to have unwanted effects in other marine organisms. For example, reproduction of marine gastropods is severely disturbed in areas contaminated with TBT´s (Bryan and Gibbs 1991; Champ 2000; Ketata *et al*. 2007). France was the first country to ban the use of TBT´s in 1989. Since 2008 there is a worldwide ban on TBT´s by the International Maritime Organisation (IMO). However, there are still TBT compounds present in the environment, exerting effects. TBT´s are persistent and have accumulated in the sediments for a long time. They are also bioaccumulated and biomagnified in the food web (Strand and Jacobsen 2005). Also, TBT paints are available and used illegally, especially in developing countries (Gipperth 2009).

The last decades, the search for new antifouling alternatives has been intense. The most common substitutes for TBT containing paints are self-polishing paints containing mainly copper compounds. However, copper is not as efficient in preventing fouling as TBT, especially regarding algae, and several fouling organisms develop tolerance to copper. Therefore, so called booster biocides are added to copper paints. Examples of booster biocides are Irgarol, Seanine and copper pyrithione. Additionally, many paints contain large amounts of zinc, often registered as pigment, but with additional antifouling properties. One of the problems with booster biocides is that they are not chemically compatible with the paint polymer, and thus the release from the paint is uncontrolled and too quick. Also, many of the booster biocides, as well as copper, have adverse effects in the environment (Thomas *et al.* 2001; Konstantinou and Albanis 2004; Thomas and Brooks, 2010).

New perspectives of antifouling solutions

There is an inbuilt challenge in the development of antifouling systems. By definition the hull environment must be very hostile to all fouling organisms, but it shall not have any impact on the surrounding aquatic environment. Also, the paint should not interfere with desired hydrodynamic properties. Large-scale production is necessary as well as fair price, and minimal maintenance requirements (figure 3).

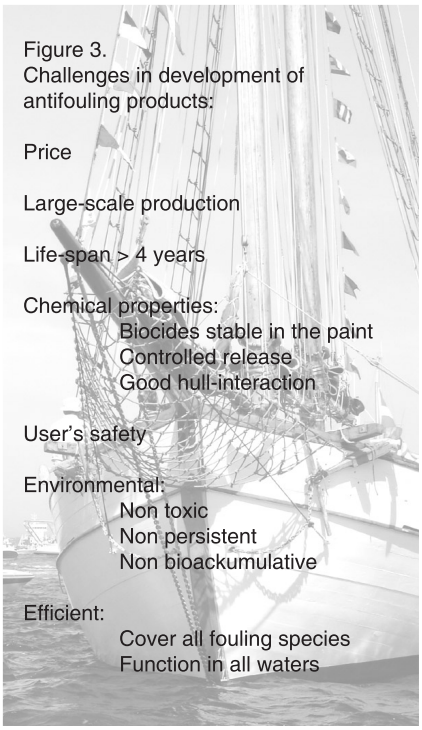


Figure 3.
Challenges in development of antifouling products:

There are today many good ideas how to solve this issue (table 1). However, it is very difficult to find a universal method. It is likely that we will not find a "new TBT", but there will be different solutions for different situations; different types of vessels and different waters. One of the most wide-spread more environmentally acceptable methods in use today, are low surface energy paints, such as silicon paints. They function simply by being slippery. To function well, however, the vessel needs to attain high speed and being frequently used. For this reason they are not suitable in all situations. Also, to paint a ship is very complicated and costly. Other methods that are commercially available, at least to a limited extent, are paints containing enzymes

Method	Reference	In use	+	-
SPC copper paint with booster biocides		Most common	Functions fairly good Cheap	Environmental impact Premature release of boosters
Low surface energy, silicon		In use worldwide	Free from biocides	Expensive, requires high speed and regular use, fragile
Cleaning, robots and stations	www.cleanhull.no www.boatwasher.se	Limited use so far	Free from biocides	No continous protection, toxic waste
Oxygen depleted surfaces	www.ekomarine.se	Limited use in the Baltic sea	Free from biocides	Short life span
Nanostructured surfaces	www.ambio.bham.ac.uk www.sharklet.com	Under evaluation	Free from biocides	Possibly expensive and difficult to produce in large scale
Natural products	Reviewed in Raveendran & Mol, 2009	Under evaluation	Possibly repellent rather than toxic	Difficult to produce in large scale
New biocides with non-lethal target; medetomidine, quorom sensing inhibition	www.marinepaint.se Dobretsov et al. 2009	Limited use	Non-lethal mechanism, potent at low concentrations	Environmental impact needs to be evaluated
Enzymes	www.biolocus.com Reviewed by Olsen et al., 2007	One commersial paint	Commercially produced Non-lethal mechanism	Life-span, storing, toxic metabolites?

Table 1. Strategies to prevent fouling.

to prevent fouling (Olsen *et al.* 2007), and paints containing proteins to create oxygen-depleted surfaces (Lindgren *et al.* 2009).

An interesting and promising field is the use of nanostructured surfaces to prevent fouling. There is quite a lot of research in this field, for example the Ambio-project (www.ambio.bham.ac.uk). Another area where research has been extensive is the search for "natural" antifouling agents. Many marine organisms have developed substances to prevent themselves from being fouled. There is a general idea that these natural substances should be more environment friendly, since marine organisms may have strategies to handle them. Today more than 145 natural substances have been identified as antifouling agents (Raveendran and Mol 2009), however to my knowledge no commercial products have yet been introduced. One major problem is that these molecules are often very complex and difficult to produce in large-scale. Also natural occurring substances need to be evaluated according to current legislation; many of these are very toxic.

Over the last years, sophisticated methods to clean hulls have been developed. For smaller boats there are cleaning stations similar to automatic car wash stations, and for large ships there are robots "walking" the hull, cleaning it under water. This is a good method since no toxic agents are used, however there may be toxic waste from

previous layers of paint. This thesis work lies within the project Marine Paint, which is one of few projects evaluating new types of repellent biocides. Such a biocide does not function by being toxic, or lethal, but by interfering with some process involved in settling and metamorphosis of a fouling organism.

Defined by the European Union Biocidal Products Directive (BPD), biocidal products are "Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means". Antifouling products sort under the BPD. The directive was implemented in law in May 2000, and all biocidal products are supposed to be evaluated according to the directive. The directive also promotes the principle of substitution, meaning that when two products are equally well functioning, the more environmentally acceptable should always be used (www.kemi.se). The BPD together with national and international legislation provides a pressure to speed up the development of new antifouling technologies.

The Marine Paint project

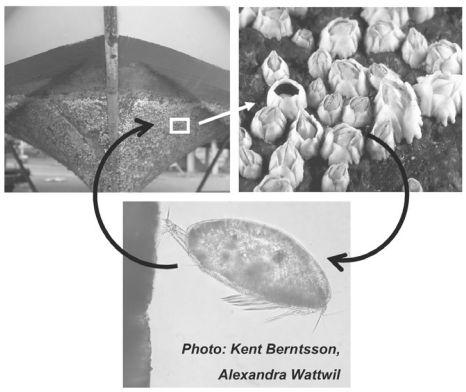


Figure 4. The barnacle lifecycle includes a mobile cyprid larvae stage. It is the cyprid that seeks a surface to settle down on. Medetomidine inhibits settling of cyprid larvae at nanomolar concentrations.

Based on the finding that a substance, normally used within veterinary medicine, could inhibit the settling of barnacle larvae at very low concentrations, the Marine Paint project started in 2003.

The barnacle *Amphibalanus improvisius* is by many regarded as the toughest fouling organism in our waters. To be able to spread to new surfaces, adult sessile barnacles release planktonic larvae. The final of seven larval stages is called cyprid, and it is the cyprid that has the task to find a suitable surface to settle on. Therefore this larval stage is of high interest in antifouling research (figure 4).

In 2000, the substance medetomidine was shown to reversibly inhibit settling of cyprids at nanomolar concentrations, hundred thousand times lower than the lethal concentrations (Dahlström *et al.* 2000). To evaluate the use of medetomidine as an antifouling agent, a multidisciplinary research program was formed, funded by the Swedish Foundation for Strategic Environmental Research, MISTRA. The focus of the first four years was entirely on medetomidine and barnacles. Much remained to be learned about the cyprid and the settling mechanisms, and to find the target for medetomidine in the cyprid. Chemists faced the challenge to develop a paint matrix enabling controlled medetomidine release. Medetomidine was also further tested in the field. To answer the question if medetomidine is safe to use in the environment, an ecotoxicology group was formed.

After 2006, the research program was broadened, to target all fouling organisms. Other substances are evaluated, since medetomidine is only efficient towards barnacles and tubeworms. The concept, developed within Marine Paint, is to use intelligent mixtures of biocides to gain maximum antifouling effect at minimum environmental impact. These mixtures can then be optimised for different uses. To chemically handle this cocktail of substances, and to control release rate, a microencapsulation technique has been developed (www.marinepaint.se).

This thesis has its major focus in the first part of Marine Paint, investigating the effects of medetomidine in fish.

Medetomidine

The chemical

Medetomidine (4-[1-(2, 3-dimethylphenyl) ethyl)-imidazole) is an imidazole. It has a molecular weight of 200.28g/mole and is molecule with a chiral centrum. It has two optical enantiomers, dex-medetomidine and levo-medetomidine (figure 5). It was introduced in the 1980´s as a sedative and analgesic drug and is today widely used in veterinary medicine and wildlife management. To a

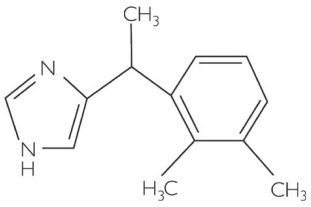


Figure 5. Medetomidine was originally introduced as a veterinary sedative. It is an imidazole, and has molecular weight of 200.28 g/mole.

limited extent it is also used within human medicine. Commonly the dexmedetomidine hydrochloride is used for clinical applications. Domitor, Cepetor, Sedator and Precedex are examples of names on products based on medetomidine. The first paper suggesting medetomidine as an antifoulant was published in 2000 (Dahlström *et al.* 2000). Medetomidine in the use as an antifoulant, may also be called Catemine I.

Biological mechanism

Medetomidine is known to be a selective and potent α_2-adrenoceptor agonist (Savola *et al.* 1986). Adrenoceptors are receptors for the catecholamines adrenaline and noradrenaline. The receptor is situated in the cell membrane and consists of seven transmembrane amino acid helixes and extracellular and intracellular loops. All adrenoceptors are G-protein coupled. Stimulation of the receptor activates G-proteins and second messenger signals including cAMP, $Ca^{2+,}$ diacylglycerol and inositol-triphosohate (IP3) (Hein 2006)(figure6). The subtypes α and β were classified already in 1948 by Ahlquist (Ahlquist 1948). This classification has remained the base for further classification. Today several subgroups are described: α_1 (A, B, C); α_2 (A, B, C) and β_1, β_2, β_3. The α_2-adrenoceptors are essential in presynaptic inhibition of noradrenaline release. Examples of body functions regulated by α_2-adrenoceptors are analgesia, sedation, behaviour and cardiovascular control (Hein 2006).

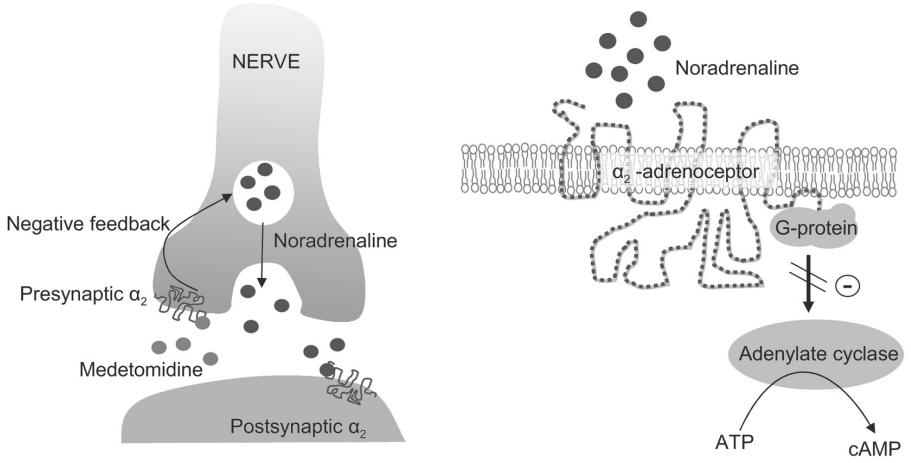


Figure 6. Medetomidine is known as an α_2-adrenoceptor agonist. This receptor is G-protein coupled and when stimulated by noradrenaline, production of cAMP is inhibited. Presynaptic α_2-adrenoceptors regulate noradrenaline release via negative feedback.

The classification from mammals is not necessarily applicable in fish (Fabbri *et al.* 1998). There are studies showing for example unexpected responses to selective antagonists or agonists known from mammalian literature. One example is the classic α_2-adrenoceptor agonist clonidine, showing antagonistic properties in cod (Johansson 1979). A more recent study focusing on beta receptors also address the lack of knowledge of adrenoceptors in important fish species (Owen *et al.* 2007). However, in zebrafish five different subtypes of α_2-adrenoceptors have been identified, where three show great similarity to the mammalian receptors regarding structural, functional and pharmacological properties (Ruuskanen *et al.* 2005). Also from cuckoo wrasse (*Labrus ossifagus*) the α_2- adrenoceptor has been cloned and characterised (Svensson *et al.* 1993). To our knowledge, in rainbow trout, there are no sequence data on α_2-adrenoceptors. α_1-adrenoceptor paralogs have been characterised, suggesting that the role of α_1 is different in fish than in mammals (Chen *et al.* 2007). Also, studies of the β_2-receptor suggests that control and signaling in rainbow trout differs from mammalian $\beta_2$receptors (Nickerson *et al.* 2001). It is possible that medetomidine also exerts effects via special imidazoline receptors. Imidazoline receptors and α_2-adrenoceptors may share many features and interact with one another. The existence of imidazoline receptors is debated and they are not very well studied (Hieble and Ruffolo1995; Szabo 2002).

Effects of medetomidine

Because of its medical use, numerous studies have been performed evaluating the acute effects of medetomidine during sedation, in a variety of species. The central nervous effects include decreased turnover of noradrenaline and also serotonin (Scheinin *et al.* 1989). The sedative effect is explained by noradrenaline decrease in the brain nuclei *locus coeruleus* (Scheinin and Schwinn 1992). Medetomidine has also an analgesic effect, which is favorable in the clinical application. Commonly reported side effects of medetomidine sedation are bradycardia, hypotension and hypertension (Scheinin and Schwinn 1992; Sinclair 2003; Murrell and Hellebrekers 2005; Gerlach and Dasta 2007). Medetomidine also increases the level of growth hormone (Venn *et al*. 2001). Medetomidine is closely related to an other α_2-adrenoceptor agonist, clonidine, Clonidine is not as α_2 specific as medetomidine, but it has been around for a long time and is well studied.

The knowledge of effects of medetomidine in aquatic organisms is limited. In a few studies medetomidine has been used as a sedative in fish (Horsberg *et al*. 1999; Fleming *et al*. 2003; Williams *et al*. 2004). Within the Marine Paint project, larval

turbot (*Psetta maxima*), Atlantic cod *(Gadus morhua)* and lumpfish (*Cyclopterus lumpus*) have been studied for oxygen consumption and respiration rate after medetomidine exposure. In turbot and lumpfish there was a dose-dependent decrease in respiration rate and oxygen consumption *(*Bellas *et al.* 2005; Hilvarsson *et al.* 2007). Medetomidine has also been used as a tool in studies of fish pigment cells, melanophores, since medetomidine has a skin lightening effect (Karlsson *et al.* 1989; Ruuskanen *et al.* 2004).

Even more limited is the knowledge in invertebrates. In the mussel *Abra nitida*, medetomidine reduced burrowing behaviour (Bellas *et al*. 2006), and in the amphipod *Corophium volutator* mate search behaviour was reduced (Krång and Dahlström 2006). Studies in the marine organisms *Mytilus edulis*, *Abra nitia*, *Crangon crangon* and periphyton communities have shown bioconcentration factors ranging from 2.8 to 1195 l/kg FW (Hilvarsson *et al.* 2009).

Ecotoxicology

The studies within this thesis were performed as part of ecotoxicological evaluation for medetomidine. The term ecotoxicology was coined in the 1970´ s and can be defined as the study of effects of chemicals of anthropogenic origin on the ecosystem (Truhaut 1977). Ecotoxicological studies involve studies on all levels of organisation, but since it is almost impossible to study a whole ecosystem, investigations are most commonly performed in single species or micro or mesocosms.

The aquatic environments are the ultimate sink for many pollutants, and aquatic organisms are highly vulnerable to pollutants in the water they breathe and live in. Fish toxicology is an important field in ecotoxicology. Fish are top predators and accumulate toxicants from lower trophic levels. Fish health is also of commercial interest, as an important food source all over the world. The knowledge base from fish physiology, ecology and morphology is rather extensive, and fish can be handled and studied both in field and laboratory environments (Van der Oost *et al*. 2003; Di Giulio and Hinton 2008).

Two major areas of ecotoxicology are *biomonitoring* and *risk assessment*. In biomonitoring, the health of the ecosystem in a geographical area is assessed. Risk assessment is performed for specific chemicals, or classes of chemicals, to predict the risk of introducing new chemicals or estimate impact of existing chemicals. In ecotoxicological studies, chemical analyses are important, but has limitations. Levels of chemicals can fluctuate and even though present in the water, they are not necessarily

bioavailable -taken up by biota. Therefore, studying biological endpoints is important. These can be studied in all levels of biological organisation. Impact on individual or species level is of high ecological relevance, but when such responses are observed, the ecosystem is already damaged. Effects on cellular, biochemical or genetic levels, on the other hand, may serve as "early warning signals". The development and usage of these early warning signals, or *biomarkers* is an important field within ecotoxicology (Peakall and Walker 1994; Sanchez and Porcher 2009) (figure 7).

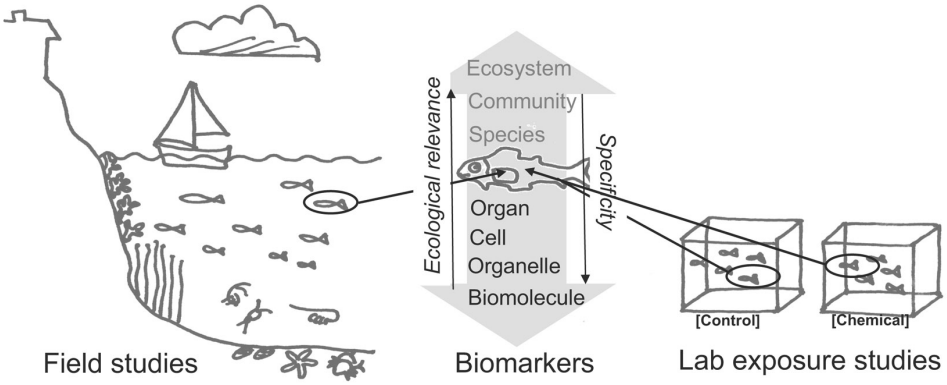


Figure 7. Biomarkers are important tool in ecotoxicological studies. Biomarkers on lower levels of organisation can serve as early warning signals, and indicate exposure and response to chemicals before damage has reached individual or ecosystem level.

By definition, biomarkers should reflect adverse biological responses towards anthropogenic environmental toxins. Biomarkers should preferably be dose-dependent specific to a class of pollutants or to a mode of action to be useful in the ecological interpretation (Van der Oost *et al*. 2003). Biomarkers are important tools in biomonitoring and risk assessment. The studies within this thesis were performed as part of the risk assessment for medetomidine, using a biomarker approach.

Biomarkers used within this thesis

Most biochemical defences respond to injury by increasing defence levels through self-regulating signalling mechanisms. Several important biochemical biomarkers are part of metabolic or oxidative defence systems. The Cytochrome P450 enzyme family subgroup 1A, **CYP1A,** is involved in transformation of xenobiotic molecules and is commonly induced by classical environmental pollutants such as polyaromatic

hydrocarbons and PCB:s. CYP 1A can be measured at transcriptional, protein or catalytic levels (EROD activity)(Arellano-Aguilar *et al*. 2009) . The glutathione-S-transferase, **GST**, family is also involved in xenobiotic transformation and can be measured at several levels. The enzymatic assay measure many different forms of the enzyme. The small molecule **glutathione** is of large importance in the antioxidant defence system and can reduce radicals by being oxidized itself. The enzyme glutathione reductase, **GR**, reduces glutathione and restores its reducing capacity (Stephensen *et al.* 2002). Important biochemical biomarkers not used within this thesis are metallothioneins, stress proteins, vitellogenin (egg yolk protein), multidrug resistance proteins and genotoxic markers.

Physiological biomarkers are often less specific to contaminants, but give an overall health estimation. Hematological indexes such as **hematocrit** (volume of red blood cells) and **haemoglobin** content have been used in our studies, as well as the metabolic markers blood **glucose** and **lactate**. Circulating hormones can be used as biomarkers and within this thesis levels of growth hormone (**GH**), insulin-like growth factor 1 (**IGF-I**) and **leptin** have been measured (Reinecke *et al*. 2005). Condition indexes are very simple measurements that index the physical proportions of the fish or a specific organ. We have used **condition factor** expressed as weight/lenght^3. We have also calculated the organosomatic indexes liver somatic index (**LSI**), sometimes referred to hepatosomatic index, and heart somatic index (**HSI**), calculated as organ weight/body weight×100 (Di Giulio and Hinton 2008)**. Growth** itself can also be used as a biomarker (Amara *et al.* 2009) expressed for example as specific growth rate, as in this thesis. We have also used **behaviour** as biomarker. We have not used any pathological biomarkers or morphological abnormalities.

"Omic" studies refer to large scale screening such as proteomics, metabolomics, **genomics** and transcriptomics. We have performed a large scale gene expression analysis using an oligonucleotid microarray.

Environmental Risk assessment

Risk assessment has developed as a tool for chemical legislation authorities. In Europe, risk assessment is described in Technical Guidance Document on Risk Assessment, TGD from 2003 (http://ecb.jrc.ec.europa.eu/tgd/). Very simplified, to be able to perform an environmental risk assessment, two things need to be known. One is the predicted environmental concentration, PEC, and the other is the predicted no effect concentration, PNEC. PEC values are predicted using models that into account important processes, such as leakage, degradation, bioaccumulation, water solubility,

evaporation, hydrolysis etc. For antifouling paints, there is a specialised model called MAMPEC, Marine Antifoulant Model to Predict Environmental Concentrations. To estimate a PNEC value, laboratory exposure studies are performed using a standardized set of organisms and endpoints. Lack of knowledge is compensated using assessment (uncertainty) factors. If the PEC/PNEC ratio is less than 1, the risk of using the chemical is regarded as small, since the environmental concentrations are estimated not to reach levels were the ecosystem is affected (figure 8).

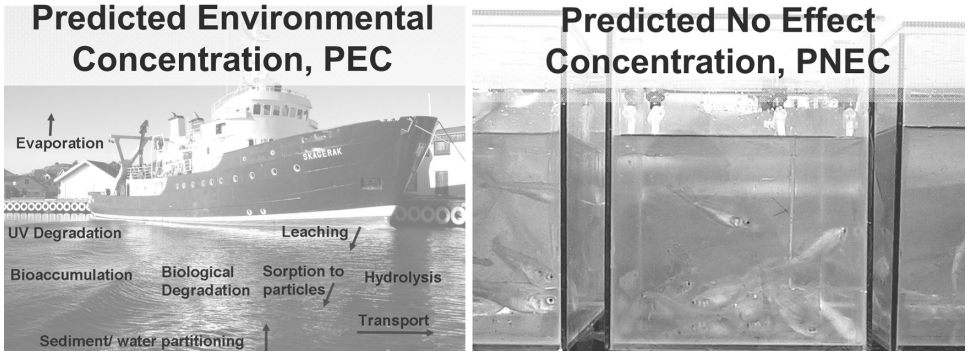


Figure 8. In environmental risk assessment models can be used to predict environmental concentrations (PEC). These include important processes affecting the fate and distribution of the chemical. To find the no effect concentration (PNEC), most commonly exposure studies are performed in relevant species, investigating different endpoints. If the PEC/PNEC ratio exceeds 1, there is a risk that the chemical will influence the ecosystem.

The TGD document is still new, and several weak points and needs of improvements have been presented. For example, the tested species should be of relevance to the area where the chemical is to be used, and species as well as endpoints studied should be chosen with respect to the type of chemical investigated (Guerit *et al.* 2008; Gunnarsson *et al.* 2008). Another important point is how to handle risk assessment for the enormous variety of combinations of toxicants present in the environment (http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf).

AIMS OF THIS THESIS

The overall aim of this thesis was to identify and study early responses of medetomidine in fish, occurring from low, non-sedative concentrations. The specific aims were:

To use a broad approach to screen effects after medetomidine exposure in rainbow trout, turbot and Atlantic cod. Screening studies were performed after short term *intra peritoneal* injection and after two weeks of water exposure.

To study effects of medetomidine on fish colouration and also the density and function of pigment cells after medium and long term medetomidine exposure.

To investigate effects of medetomidine on the hepatic detoxification enzyme Cytochrome P450 1A (CYP1A) activity in several fish species *in vivo*. To study *in vitro* effects of medetomidine on CYP1A activity in rainbow trout, turbot and Atlantic cod. To study the effects of medetomidine *in vivo* on glutathione and glutathione-dependent enzymes in the same species.

To study effects on growth and growth related hormones and investigate gene expression in rainbow trout after long term medetomidine exposure.

To study the effects of medetomidine on basic behavioural parameters in three-spined stickleback and Atlantic salmon.

To study the cytotoxicity of medetomidine in hepatic zebrafish cells.

METHODOLOGY

Fish experiments

Within this thesis, the fish species rainbow trout, turbot, Atlantic cod, Atlantic salmon and three-spined stickleback have been used.

Figure 9. ***Oncorhynchus mykiss***

Rainbow trout (*Oncorhynchus mykiss*) (figure 9) is very suitable for laboratory studies since it is one of the most well studied model fish species. It is commercially available from hatcheries in Sweden and easily kept in the laboratory environment. Many of the studies in this thesis were performed in rainbow trout. This was for practical reasons, but also some of the important studies; measuring growth hormone levels and the microarray gene expression analysis, required the use of a well studied species. The rainbow trout experiments were performed in fresh water.

Atlantic cod (*Gadus morhua*) (figure 10) and turbot (*Psetta maxima*) (figure 11) are two commercially important marine species. Cod is a schooling pelagic species, while turbot is bottom dwelling, strongly relying on camouflage. The studies in this thesis were performed in Sandgerdi, Iceland, using hatchery reared fish in a water salinity of 32 psu.

Figure 10. ***Gadus morhua***

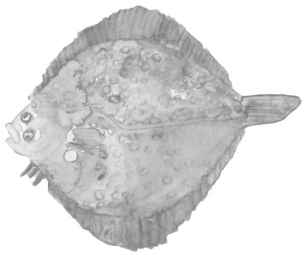


Figure 11. Psetta maxima

The behavioural studies were performed in three-spined stickleback, which is also a well studied model species, and in Atlantic salmon, an important commercial species. These experiments were performed in natural seawater around 30 psu and brackish water of 9 psu respectively. As an antifoulant, medetomidine would be used in waters of varying salinity from full ocean salinity to coastal zones, estuaries and in the Baltic Sea.

We used two different routes of exposure for the studies (figure 12). For short term exposures we injected medetomidine or sham *intra peritoneal* (into the gut cavity). The major advantage of this method is that it is easy to perform, and you can control the dose given to each individual. Since the substance is metabolised over time this method is consequently best suited for short term studies.

For studies more than five days, we added medetomidine to the water using peristaltic pumps in a controlled flow-through system. In this way we could keep a continuous exposure for up to 54 days. The third option, which we did not use in this thesis, is to add the substance via food. This may very well be an important route of exposure in nature, especially for lipophilic substances. However, to control the exposure of each individual, the fish should be force-fed or kept in individual aquaria.

One problem when keeping fish in groups in aquaria is the formation of hierarchies. Dominant fish can be very aggressive and cause physical damage on the subordinate fish. Also, there may be a very uneven distribution of food supply in a strong hierarchy. There are reports showing that during water exposure to contaminants, subordinate fish may have higher uptake of toxicants. This has been shown in experiments with copper and silver (Sloman *et al*. 2002, 2003).

Figure 12. Within this thesis, long term water exposures have been performed using flow-through systems controlled by peristaltic pumps (left). For short exposures, *intra peritoneal* injection of medetomidine have been used (right).

In the beginning of our long-term water exposure, we had one obvious problem with social structure. In one of the aquaria one individual allowed no one else to eat, resulting in bad water quality from the leftover food. We removed that individual, which in this case solved the problem.

In several of our studies, we have used one aquarium per treatment. However, many of the studies have been repeated and performed in several species. The important results from this thesis are consistent among several experiments. For the behavioural studies and the long term exposures, we chose to replicate aquaria of the same treatment.

All studies performed were made according to legislation and guidelines for animal welfare, and after permission from the Ethical Committee on Animal Experimentation in Gothenburg.

Sampling

At sampling we followed routines used in the extensive biomonitoring programmes executed in our lab. Fish were sacrificed by a sharp blow to the head. Blood was drawn from the caudal vein using a heparinised syringe (figure 13). The fish was weighed and measured. A small amount of the blood was immediately analysed for haemoglobin and glucose content, and hematocrit measurement was performed after centrifugation. Plasma was separated and immediately frozen on dry ice. Organs of interest were dissected from the fish (figure 13), weighed and snap frozen in liquid nitrogen for further biochemical analysis. Heart and liver somatic indexes were calculated as (organ weight/body weight)×100.

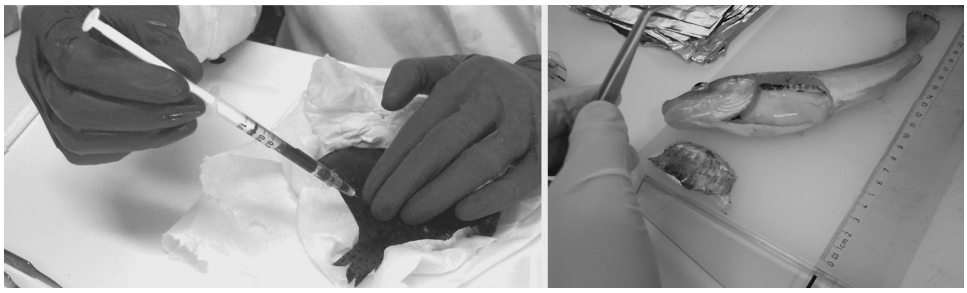


Figure 13. At sampling, blood is drawn from the caudal vein and organs of interest are dissected and snap frozen in liquid nitrogen.

Medetomidine concentrations

It may be difficult to choose the most optimal concentrations for an exposure study. We wanted to find early effects of medetomidine in fish. The starting point was taken from the concentrations needed to inhibit barnacle settling in a Petri dish, which was 1 nM. In early *intra peritoneal* exposure studies we used 0.5 and 5.0 μmol/kg fish, which would roughly correspond to 0.5 and 5.0 nM when using an estimated log K_{ow} of 3. We found some effects at the higher dose, and found none at the lower dose. After this we used 5 nM as a starting point and added lower and sometimes higher concentrations, and this showed to be a successful strategy. We often found some responses in the higher concentrations and none in the lower.

Water samples for chemical analysis were taken from all water exposure studies, but unfortunately, due to unforeseen technical problems, it has not yet been possible to analyse very low concentrations in a reliable way. This is of course now a prioritised issue within the Marine Paint project. The lack of chemical data has also limited the ability to gather data for Prediction of Environmental Concentrations, PEC values. For these reasons, we use nominal concentrations for presentation, and are not able to relate effect data presented within this thesis to Predicted Environmental Concentrations.

Paleness

In most of our studies, the fish colour has just been observed and documented, but not quantified (summarised in *Paper I*). However in the long-term exposure study, we chose to put effort in making a good quantification of the paleness (see *Paper IV*). At the sampling occasion, within 30 s after death, we took a picture of each fish, using a "studio" with light, background and camera fixed. These pictures could then be analysed for "paleness" using the Image J software. A specified area of each fish was analyzed and a background value was obtained and accounted for in each picture. This method was easily performed, did not interfere with the sampling procedure and was sensitive.

There is in every sampling situation a risk that stress in the fish interferes with the results, and stress could well interfere with fish colour, since colour is regulated partly by catecholamines. Such stress effects may mask minor effects, but in our case the differences were large. Also, we had carefully observed the live fish in the aquaria every day during exposure, and the obtained results were consistent with our ocular observations.

Melanophore studies

Pigment containing cells are inclusively called chromatophores. Melanophores are one type of chromatophores, containing dark brown or black pigment. The pigment can be either dispersed throughout the cell, which gives the fish a dark appearance, or it can be aggregated around the nucleus, which gives a pale appearance (figure 14).

We have studied melanophores from medetomidine-exposed rainbow trout *in vitro*. At the end of exposure, just after death, scales have been scraped of from the area under the dorsal fin and put in a cell culturing medium. The melanophores have then been exposed to different substances. In the first experiment (*see Paper*

Figure 14. To the left are two pictures of a fish scale seen through a microscope. In the upper picture the pigment is dispersed within each melanophore (pigment cell), which gives the fish a dark appearance. In the lower picture the pigment is aggregated around the nucleus, which gives the light appearance.

II), medetomidine was added to the melanophores to see how melanophores from fish exposed to medetomidine reacted to further medetomidine treatment compared to control. In the second study, described in *Paper IV*, we also added melanophore stimulating hormone (MSH) to disperse the melanophores before adding the medetomidine.

To assess the degree of aggregation, we used an index called the Hogben-Slome index, where 1 stands for maximum aggregation and 5 for maximum dispergation (Hogben and Slome 1931). This is a very straight forward method where pictures of the melanophores are blindly assessed by a second person. We found this method to be quick and convenient for the large amount of pictures we had. There are other methods to assess pigment aggregation. One is to use software (e.g. Image J) to analyse the amount of pigment in each cell. This requires that you can identify the membrane of each cell, which was not possible in our samples. Spectrophotometrical assays can also be used, but this requires the same number of cells in each sample, and thus this method is better suited for cultured cells (Hedberg 2009, Aspengren *et al.* 2006).

CYP1A activity

CYP1A is involved in metabolism of many toxicants, and altered activity of this enzyme has become a commonly used biomarker. CYP1A enzyme activity can be measured easily using the artificial substrate ethoxyresorufin, which by CYP1A is converted to a fluorescent product. The rate of product formation, ethoxyresorufin-*O*-deethylase (EROD) activity gives a direct measure of CYP1A activity.

We have measured EROD activity in liver microsomes as previously described (Förlin *et al.* 1994) in a number of studies (*Paper III, Paper V*, Ekvall 2004; Hilvarsson *et al*. 2007b).For the *in vitro* inhibition studies we used pooled liver microsomes from β-naphthoflavone treated fish with an initially high EROD activity, which was then inhibited by medetomidine. The EROD measurements were performed using a 96 well plate (Yawetz *et al*.1998). Medetomidine was added in a dilution series to the reaction mixture containing microsomes, NADPH and ethoxyresorufin. From the results one could graphically determine the concentration of medetomidine inhibiting activity with 50% (IC50 value).

Growth

Growth gives an integrated measure of fish health. We examined if medetomidine affects growth rate, and therefore we performed a long-term study for totally 54 days (*see Paper V*). To be able to measure growth, fish were weighed and individually marked before the experiment started. We decided to feed the fish at the same rate as at the fish farm, which was 1% of the body weight per day. We added 1 % of the total initial body weight of all the fish in one aquarium and fed them simultaneously. We observed the fish to assure that all individuals gained access to the food. In spite of this there was probably an uneven distribution of food among the fish. As previously encountered, we had a situation with an extremely aggressive individual in one aquarium. After removal of this individual, however, all fish had access to food. Since the amount of food was constant through the experiment, in the end the fish were actually fed about 0.6 % of the final body weight. We could also see that the growth rate was higher at the first sampling occasion after 31 days, than at the final sampling. Growth rate was calculated as specific growth rate as *(SGR = 100×(log (final weight/initial weight))/days)* according to Ricker (1979). Because of the daily feeding it was important to clean the aquaria to assure a good water quality. Over time we began to notice that the amount of faeces produced varied among the aquaria, also among aquaria of the same treatment. This difference could not be correlated with any other parameter. If the experiment was to be repeated, we would probably increase the food allotment over time along with fish growth.

Levels of plasma growth hormone (GH) were determined in duplicate samples using a GH radio immune assay specific for salmon (Björnsson *et al.* 1994), validated for rainbow trout. Levels of insulin-like growth factor-I (IGF-I) was measured, using radio immunoassay according to Moriyama *et al.* (1994). Leptin levels were assessed by homologous radioimmunoassay (Kling *et al.* 2009).

Gene expression

Gene expression analysis was performed using DNA microarray technique, where the gene expression (mRNA level) of several thousands of genes can be analysed simultaneously (*see Paper V*). Liver samples from the sampling occasion after 31 days of exposure were selected for the gene expression analysis. mRNA was extracted from liver frozen in liquid nitrogen, and the purity, quality and concentration was checked. Through reversed transcription, mRNA was converted to more stable cDNA (complementary DNA). This was then converted again to biotinylated RNA, with a biotin molecule attached to some of the bases. During the hybridization process, where the biotinylated RNA strands line up with the corresponding probes on the array, fluorescent molecules with high affinity for biotin was added. The expression of each gene was then visible as a fluorescent spot on the array (Figure 15).

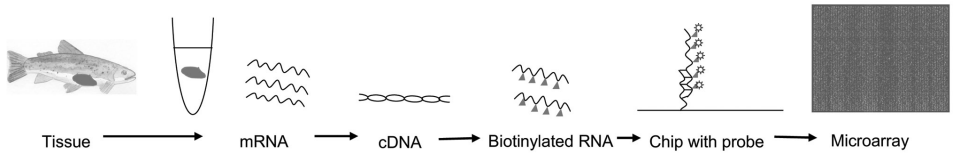


Figure 15. The large scale gene expression analysis was performed using an oligonucleotid microarray. Liver samples from 31 days of exposure to 5 nM of medetomidine were used for mRNA extraction. The mRNA was then converted to cDNA using reverse transcriptase. After this the cDNA was converted to biotinulated mRNA. During the hybridisation of the mRNA on the microarray chip, fluorescent molecules with high affinity for biotin were added. Finally the expression of each selected gene was visible as a fluorescent spot on the array. The spots could then be analysed according to intensity, and to compare expression between the treatments.

We used an oligonucleotid microarray and the platform Geniom (febit, Heidelberg, Germany). The array was based on the rainbow trout gene indices EST database version 7.0 (http://compbio.dfci.harvard.edu/tgi/) (Quackenbush *et al*. 2001). The microarray design is described in more detail in paper V and in (Kristiansson *et al.* 2009).

The probes included on the microarray were selected as followed:

- Rainbow trout transcript of the α-adrenoceptors and the down-stream pathways.
- Rainbow trout transcripts associated with growth and metabolism.
- Drug targets reported in (Gunnarsson *et al.* 2008).
- Genes associated with the pathways in Pharmacogenetics and Pharmacogenomics Knowledge database (PharmGKB) (Hernandez-Boussard *et al*. 2008).
- Rainbow trout homologs of genes described in the Comparative Toxicological Database.
- Homologs to all Cytochrome P450 annotated in zebrafish *Danio rerio*.

In total 15205 probes were selected by these criteria, and the rest of the array was filled up with random well-annotated rainbow trout probes. The microarrays were generated by *in situ* synthesis. Each biochip consists of eight individually-accessible microarrays.

The statistical analysis of the results from the array was performed as described in Paper V, using the language R. The data was normalised to remove technical artefacts and a comparison of gene expression between treatments was made using moderated t-statistics.

The results were further explored using the gene ontology database GO Gorilla (http://cbl-gorilla.cs.technion.ac.il/), for identification of biological processes, functions or cellular components with a possible difference in gene expression between the treatments.

Glutathione and glutathione dependent enzymes

Glutathione is a small molecule of great importance in the antioxidant defence system. Glutathione can be measured using the molecule DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), which binds to the sulphydryl group of reduced glutathione to form a colored molecule that can be measured. Glutathione levels were measured in the liver cytosolic fraction using the method of Baker *et al.* (1990) adapted to a microplate reader by Vandeputte *et al.* (1994).

The glutathione dependent enzymes were mearsured using enzyme kinetics, where the accumulation of a coloured enzyme product over time was measured spectrophotometrically. Glutathione-S-transferase (GST) activities were measured in the liver cytosolic fraction using 1-chloro 2, 4-dinitrobenzene (CDNB) as a substrate,

according to the method described by Habig *et al*. (1974) modified by Stephensen *et al.* (2002). This method measures multiple forms of GST, both conjugating and reducing forms, which is a limitation of the method.

Glutathione reductase activity was measured in the liver cytosolic fraction according to Cribb *et al.* (1989). Oxidized glutathione was added to the reaction solution and the formed reduced glutathione reacted with DTNB as described above. All these methods are well described and have previously been used in these species.

Behaviour

Two different behavioural studies in fish have been performed, one in Atlantic salmon (Ekvall 2004), and one in three-spined stickleback (Hilvarsson *et al*. 2007b). In the Atlantic salmon study, fish were exposed to medetomidine for one and two days and were held in individual aquaria. The tests conducted were an appetite test and a mirror image stimulation (MIS) test, used to assess aggressiveness (Johnsson *et al.* 2003).

For the appetite test the fish were given one pellet every five minutes, in total three pellets, and it was noted whether the fish took the pellet or not. For the MIS test, a mirror was placed on one of the sides of the tank and covered by a PVC sheet. Immediately before the observations commenced the PVC sheet was removed. The fish's behavioural response to the mirror image was visually monitored for a period of 10 min. The behaviours recorded were: SAM (swim against mirror), FD (frontal display), LD (lateral display), I (inactive) and S (swimming). The distance from the mirror was also noted. Aggression was then measured as seconds performing aggressive acts (SAM, FD, LD).

In the three-spined stickleback test (Hilvarsson *et al.* 2007b) fish were held in threes in aquaria and were exposed to medetomidine for 7 days before the behavioural studies were performed. After seven days, each aquarium was filmed on two separate occasions for 20 minutes each time. Films were later analysed using the software EthoLog. The first period was filmed in the morning and was used to evaluate the spontaneous swimming activity of one randomly selected fish per aquarium. Time spent swimming was recorded during the 20 minute trial period. At the start of the second period, which was filmed in the afternoon, each aquarium received 300 mg of frozen red mosquito larvae and response time (time until the first fish, out of the three, struck the food) to food was registered. Following filming the remaining mosquito larvae were collected and weighed and food consumption was calculated.

Studies of cytotoxicity

The cytotoxicity of medetomidine was evaluated in a zebrafish hepatoma cell-line, ZFL. The test used was a conventional LDH test (Roche), measuring cell leakage of lactate dehydrogenase as measure of viability. Cells were cultivated in 96 well plates and exposed to medetomidine for 48 hours. The LDH leakage was measured using an enzymatic reaction where LDH converted formazan salt to yellow tetrazolium salt. The activity of LDH could then be measured spectrophotometrically.

FINDINGS AND DISCUSSION

Paleness and melanophore function

Paleness is one of the earliest effects of medetomidine in fish, appearing from relatively low concentrations (figure 16). Body paleness appears within 30 minutes after exposure to 0.5-50 nM of medetomidine, depending on species (table 2). Medetomidine has earlier been used as a tool in pigment cell studies (Karlsson *et al*. 1989). In a study of medetomidine as a sedative in rainbow trout, fish did not regain colour until after 5 days (Horsberg *et al*. 1999). In zebrafish, medetomidine induced paleness has been used as a marker for α_2-adrenergic function (Ruuskanen *et al.* 2005). Within the Marine Paint project, paleness and the ability to adapt to a new background colour has been studied in early life stages of turbot and lumpfish, showing a dose-dependent impairment of the ability to colour adapt (Hilvarsson *et al.* 2007; Bellas *et al.* 2005). Table 2 shows the fish species investigated within Marine Paint and the concentrations where paleness has been observed.

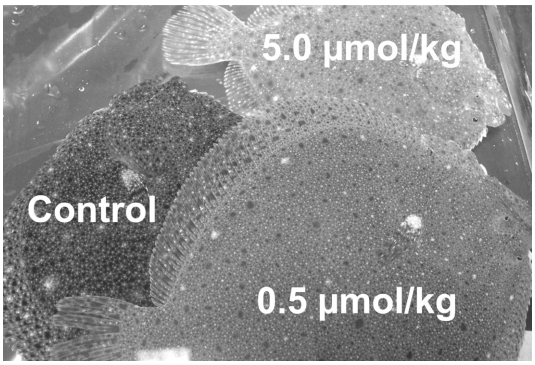


Figure 16. Paleness is one of the earliest effects of medetomidine in fish. Here are *i.p*. injected live turbot.

In the rainbow trout exposure studies for 31 and 54 days paleness was quantified from pictures of each fish as described in the method section. This revealed a significant difference in paleness in rainbow trout exposed to 0.5 nM of medetomidine for 31 days. But, after 54 days, only the fish from the 5 nM treatment were significantly paler than control. This may indicate desensitization over time, which is discussed later. In the earlier mentioned studies by Bellas and Hilvarsson (Bellas *et al*. 2005; Hilvarsson *et al.* 2007a), paleness after short term exposure was to a large extent reversible. We have not performed any recovery studies after our medium and long term exposures.

In vitro studies of the pigment cells from exposed fish where performed to further investigate the effects on pigmentation. Several types of chromatohopores exist, for example melanophores (black and brown pigment), irridophores/leucophores (white/metal), xantophores (yellow), and erythorphores (red). The melanophore is the most common type, and it is responsible for much of the dorsal pigmentation in

Species	Year of the study	Paleness appears from
Lumpfish, fry *Cyclopterus lumpus*	2005, 2002	4 nM (colour adaptation impaired)
Atlantic cod, juvenile *Gadus morhua*	2004, 2005	5.0 µmol/kg fish, 50 nM
Turbot, fry *Psetta maxima*	2005, 2006	4.2 nM (colour adaptation impaired)
Turbot, juvenile *Psetta maxima*	2004, 2005	0.5 µmoll/kg fish, 0.5 nM
Rainbow trout, juvenile *Oncorhynchus mykiss*	2003, 2004, 2005, 2006, 2007	0.5 nM
Atlantic salmon, fry *Salmo salar*	2003	5.0 nM
Three-spined stickleback, adult *Gasterostus aculeatus*	2005	50-100 nM
Two-spotted goby, adult *Gobiusculus flavescens*	2004	50 nM

Table 2. Summary of observed paleness in different fish species exposed to medetomidine.

many species as well as for distinct patterns. The melanin pigment in melanophores is contained within vesicles, melanosomes, and transported via the microtubule network. In fish, this transport is under both neural and hormonal control, where the neural control is the more rapid. The pigment can be either dispersed throughout the cell, which gives a dark appearance, or it can be aggregated around the nucleus, which gives a pale appearance (reviews by Bagnara and Hadley 1973; Fujii 2000; Aspengren *et al.* 2008).

The neural regulation of pigment movement within melanophores is accomplished by sympathetic postganglionic nerve fibers. In several species this has shown to be mediated via α_2-adrenoceptors (Andersson *et al*. 1984; Svensson *et al*. 1993; Aspengren *et al.* 2003). Noradrenaline binds to the receptor and induces a decrease in cAMP (see the chapter on medetomidine in the introduction), which in turn induces aggregation. The hormones involved in pigment movement are melatonin, melanophore concentrating hormone (MCH) and melanophore stimulating hormone (MSH). All these hormones binds to G-protein coupled receptors and regulate cAMP levels. Additionally, isolated melanophores respond to light with dispersion.

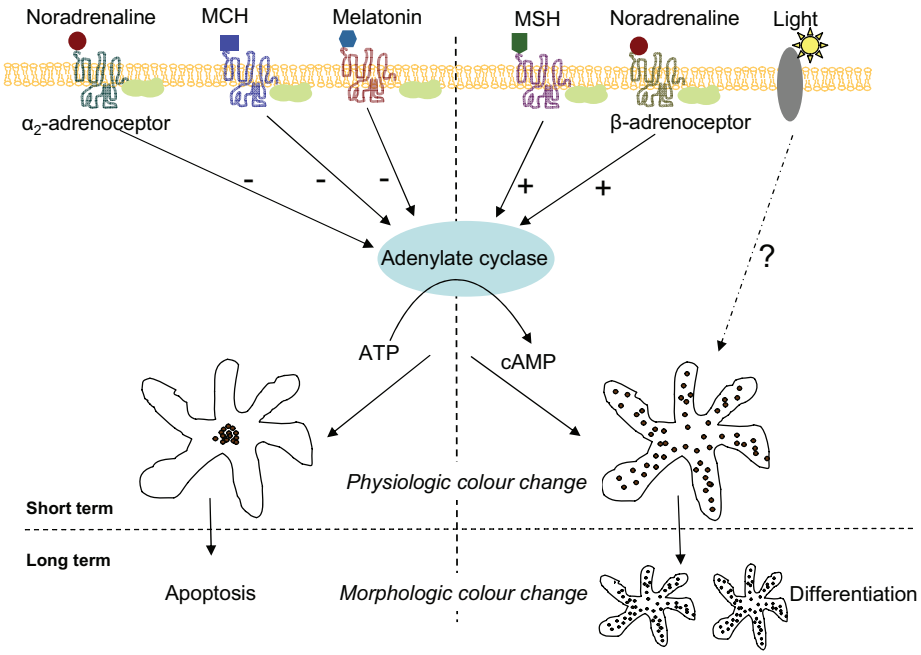


Figure 17. Signal pathways for melanophore aggregation and dispergation. It has been suggested that the same signals stimulating physiological colour change also induces morphological colour change during long term acclimatisation. MCH is melanophore concentrating hormone, MSH is melanophore stimulating hormone, cAMP is cyclic AMP.

The signaling pathways for this response are not completely understood (Aspengren *et al*. 2008) (figure 17). This control of pigment movement is called physiological colour change. During long time adaption, morphological changes occur. Long term adaptation to a white background induces apoptosis of melanophores, while long term adaptation to a black background increases the number of melanophores. The mechanisms behind apoptosis and differentiation are suggested to be the same as during the physiological colour change (Uchida-Oka and Sugimoto 2001; Sugimoto 2002; Sugimoto *et al.* 2005).

In the study with medetomidine exposure for 17 days (*Paper II*), we investigated how the melanophores responded to further medetomidine treatment and found that melanophores from medetomidine exposed fish were desensitisised to medetomidine in a dose-dependent manner. In the second study, after medetomidine exposure for 54 days (*Paper IV*), we additionally investigated the response to melanophore stimulating hormone (MSH). In this study there was also a tendency towards desensitization,

but overall the melanophores were well functioning. Additionally, we investigated the density of melanphores and found that it did not differ between the treatments, which suggest that the colour change at least to some extent is reversible, even though the sensitivity may be lowered. It would be interesting to investigate the suggested desensitization further. It is well known that G-protein-coupled receptors can be internalised in membrane vesicles and "deactivated" during exposure to an agonist (Takano and Yaksh 1993; Clark *et al*. 1999; Tsao *et al.* 2001).

Flexible colouration is important for camouflage as well as for communication and UV-protection. Hatchery reared fish released into the wild are more vulnerable to predators than wild fish, being conspicuous in the natural environment (Donnelly and Whoriskey 1993). An experiment with two species of juvenile flatfish also showed that fish mismatched to the sediment were more vulnerable to predation. More surprisingly, when fish had the possibility to chose sediment, sediment preferences were rather weak, suggesting that coloration is not so well integrated into behavioural decision making processes (Ryer *et al*. 2008). Albinism among fish is quite common in hatcheries, but very rarely seen in the wild, which has also been explained by increased predation risk (Bolker and Hill 2000). An example of a natural cause of impaired pigmentation is infection. Rainbow trout infected by eye-fluke becomes darker than healthy fish, and is more susceptible to avian predators (Seppälä *et al.* 2004, 2005).

One other important function of melanin pigment in fish is UV protection, in a similar way as in human skin. When exposed to UV-light, the melanophores of the species red seabream *(Pagrus major)* increase in size and number (Adachi *et al*. 2005). Fish coloration is also important in social signaling and affects behavioural patterns, for example during sexual selection and in social hierarchies (Kodric-Brown 1998; Höglund *et al.* 2000). One interesting aspect of colour is that fish cultured in a light environment grow faster than dark adapted fish (Papoutsoglou *et al.* 2000). There is a suggested interaction between melanophore concentrating hormone (MCH) and food intake, but several studies also show also that melanin production is costly, taking energy from other body functions (Takahashi *et al*. 2004). It is likely, that if medetomidine in nature would appear in doses high enough to induce paleness in fish, this would have consequences for individual fish survival.

Altered CYP1A activity

As reviewed in *Paper I*, altered activity of the hepatic detoxification enzyme CYP1A, measured as EROD activity, is one of the earliest effects of medetomidine and it has been observed in a number of species. *Paper III* summarises these effects in

rainbow trout, turbot and Atlantic cod. In addition to this, EROD was measured in the rainbow trout long term exposure study, *Paper V*, and in three-spined stickleback (Hilvarsson *et al.* 2007b) and Atlantic salmon (Ekvall 2004).

Enzymes within the cytochrome P450 family are involved in the metabolism of endogenous and foreign compounds in the body. For lipophilic compounds to be excreted via urine or bile, these need to be transformed into more water soluble molecules. This transformation can be divided into two phases. During the first phase a functional group, commonly a hydroxyl group is added. In the second phase, conjugation with small, polar molecules makes the compound water soluble and thus extractable. Cytochrome P450 monooxygenases (CYP´s) are first phase enzymes, associated with the following reaction (RH is an organic compound undergoing biotransformation):

$$\mathbf{RH + NADPH + O_2 + H^+ \rightarrow ROH + NADP^+ + H_2O}$$

At least 34 isoforms of cytochrome P450 has been described in fish (Arellano-Aguilar *et al.* 2009), and activity of the subfamily CYP1A is one of the most commonly used biomarkers within ecotoxicology. CYP1A is typically induced by classical pollutant groups such as PAH´s and PCB´s, and is evolutionary well conserved among species. Activity changes are often dose-dependent and one of the earliest effects of exposure (Stegeman 1992; Whyte *et al.* 2000; Arellano-Aguilar *et al*. 2009).

The suggested regulatory pathway of CYP1A is via a cytosolic receptor, the Aryl hydrocarbon (Ah)-receptor. The ideal Ah-receptor ligand should be a co-planar molecule matching a theoretical box model of 12*14*5 Å (Whyte *et al*. 2000). Once the ligand is bound, the Ah-receptor is activated, loosing its two additional heat shock proteins and the AhR inhibitory protein (AIP) and thereafter translocated into the nucleus. In the nucleus the Ah-ligand complex is associated with a nuclear translocator (ARNT), and the ARNT/Ah-complex binds to an area of the DNA called xenobiotic responsive element (XRE), including, among other genes, the CYP1A gene, inducing transcription (figure 18) (Whyte *et al*. 2000). However, an increasing number of substances that are theoretically not able to be Ah-receptor ligands have shown to induce CYP1A. This has lead to search for alternative ways of induction. Navas and coworkers have studied a number of imidazole compounds, using both computational structural studies and rainbow trout hepatocytes. They found that these compounds are structurally unable to obtain the right form for Ah-receptor ligand binding, and that they are able to induce CYP1A even when the Ah-receptor is blocked by the

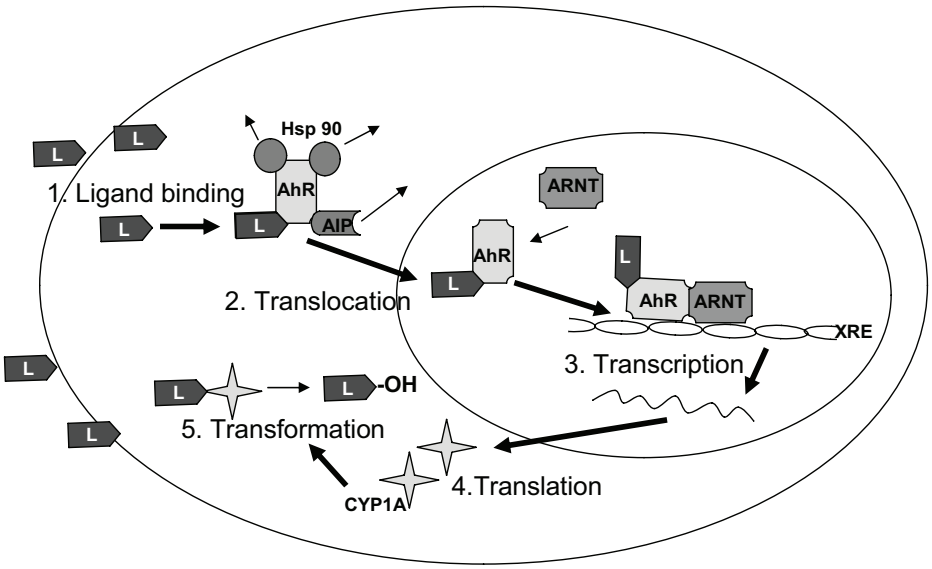


Figure 18. The most established pathway for CYP1A induction. 1. A ligand (L) binds to the cytosolic Ah-receptor (AhR) which is then activated, loosing heat shock proteins (Hsp90) and AhR inhibitory protein (AIP). 2. The ligand - receptor-complex is then translocated to the nucleus, associating with the nuclear translocator (ARNT). 3. The ARNT/Ah-complex binds to an area of the DNA called xenobiotic responsive element (XRE), and transcription of the CYP1A gene is initiated. 4. The CYP1A protein is then synthesised in the cytosol and 5. catalyses first phase reactions. It is not clear how medetomidine increases CYP1A activity.

agonist α-naphthoflavone (Navas *et al.* 2003, 2004; Babin *et al*. 2005). However, in one study using 1-phenylimadazole they found that this induced CYP1A in a similar way as the classical Ah-receptor ligand β-naphthoflavone. This concluded that in spite of structural similarities, different imidazoles compounds could induce CYP1A through different pathways (Jos *et al*. 2007).

Possible alternative ways of CYP1A induction have been discussed. These could be either totally Ah-receptor independent, involving neural and hormonal signalling pathways (Delescluse *et al.* 2000; Konstandi *et al.* 2005), or they could be mediated via the Ah-receptor. This could be via a different binding site, or by altering for example ATP levels, causing disassociation of the heat shock proteins and thus activating the receptor (Lesca *et al*. 1995; Korashy *et al*. 2007).

The use of increased EROD activity as a biomarker in field studies and complex mixture exposures needs to be interpreted with caution. One major reason for this

is that many pollutants have the ability to *inhibit* CYP1A activity. Interpretation may improve with additional data from transcriptional or protein measurements of CYP1A, chemical analysis of the water or results from other biomarkers (Hasselberg *et al*. 2005, 2008).

We found an increased EROD activity in rainbow trout (2 and 14 days) and *i.p.* injected turbot (2 and 5 days) after medetomidine exposure. This increase ranged from 1.5 to 7 times, which is referred to as a weak induction *(Whyte et al.* 2000). We found no significant differences in Atlantic cod and an inhibited activity in turbot after 14 days. In a study in three-spined stickleback we found an increased EROD activity after 7 days water exposure (Hilvarsson *et al.* 2007b) and in Atlantic salmon a significant increase in EROD activity was observed after 2 days of water exposure (Ekvall 2004). In the long-term study with rainbow trout, however, we found no significant effects on EROD activity after 31 or 54 days.

In vitro inhibition studies of medetomidine showed that medetomidine is a very potent inhibitor of CYP1A activity with median inhibition values (IC50) in the nanomolar range in rainbow trout, Atlantic salmon and Atlantic cod. Simular medetomidine inhibition has earlier been observed in human liver microsomes (Pelkonen *et al*. 1991; Kharasch *et al.* 1992).

Inhibition of CYP1A activity by imidazoles has been explained partly by interactions between the non-bonded electrons of the imidazole nitrogen and the hemegroup in the CYP1A protein (Levine and Oris 1997; Hegelund *et al.* 2004). Additionally, competitive inhibition at the substrate binding site has been shown in human liver microsomes (Kharasch *et al.* 1992).

Several imidazole compounds have shown a biphasic dose-response-curve caused by a balance between induced CYP1A transcription and enzyme activity inhibition (Sturm *et al*. 2001; Hegelund *et al.* 2004). This could probably also be true for medetomidine and explain the different results *in vivo* and *in vitro*, but it remains to be tested. Balancing between induction and inhibition might also explain that in turbot, after *i.p.* injection we observed an increased EROD activity, but after water exposure we observed a decreased EROD activity. To clarify the effects of medetomidine on CYP1A in fish, future studies should involve additional measurements of protein and mRNA levels, and combination exposures, for example combining medetomidine and well known Ah-receptor ligands or antagonists.

From an environmental perspective the fact that medetomidine is such a potent inhibitor of EROD activity is probably the most important aspect. Since CYP1A has an important role in metabolism of xenoboiotics, inhibition of this enzyme

activity would increase the risk of damages following exposure to other pollutants abundant in the marine environment. A study by Hasselberg *et al.* (2008) shows that exposure to ketokonazole increases the negative effects of xenoestrogens in rainbow trout, and another study showed that ketokonazole increased bioconcentration of benzo(a)pyrenes in gizzard shad (Levine *et al*. 1997).

Growth and metabolism

The growth of an individual integrates many different processes, and may therefore be a good measure of overall health.

An early pilot study in rainbow trout with *intra peritoneal* injected medetomidine indicated an effect on levels of growth hormone (GH). Therefore we continued measuring GH in the rainbow trout studies and decided to perform a study of actual growth (*Paper V*).

GH is a pluripotent hormone, involved in almost every important event in the life of a fish; behaviour, osmoregulation, immune function, reproduction and growth (Björnsson 1997; Björnsson *et al*. 2002). There is no direct dose-dependent relationship between GH and growth. The dual somatomedin hypothesis states that GH exerts its effect on growth via insulin-like growth factor-I (IGF-I or somatomedin) (figure 19). But this process is also more complex, involving several actors, such as the hormone grehlin. Also, GH has a direct effect in target tissues (Reinecke *et al*. 2005; Björnsson *et al.* 2002). GH is produced in the pituitary chromaffin cells and released into the blood stream. The release of GH is controlled by the hypothalamus. In mammals, this control is exerted via growth hormone releasing hormone in

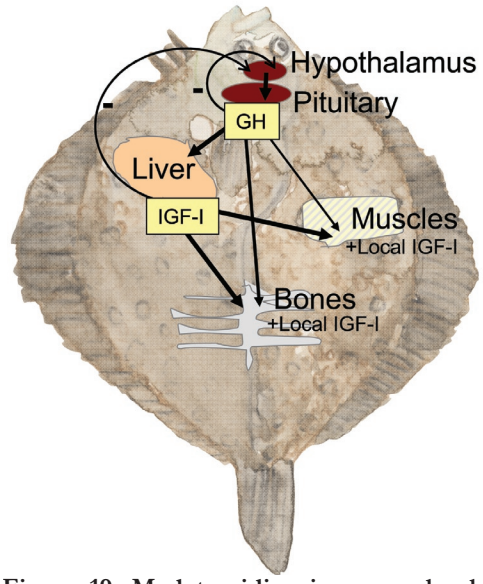


Figure 19. Medetomidine increases levels of growth hormone in human and rat. Growth hormone regulates growth via a number of pathways, but the most important is the GH-IGF-I axis. We have studied growth and growth hormone in rainbow trout, but have not found any significant effects.

the portal blood vessels, while in fish, there is a direct neural connection between the hypothalamus and the pituitary.

GH receptors are located in the liver, and in target tissues such as in bones and muscles. In the liver, GH binding stimulates the release of IGF-I. This is secreted into the blood to reach target tissues. There is also a local production of IGF-I in target tissues after GH stimulation (Canosa *et al*. 2007). IGF-1 regulates the release of GH via negative feedback and this relationship has shown to be very strong in rainbow trout (Blaise *et al.* 1995). GH has also a negative feedback mechanism on it self (Ágústsson and Björnsson 2000) .

Noradrenaline and α_2-adrenoceptors have shown to be involved in GH release. Clonidine, an α_2-adrenoceptor agonist closely related to medetomidine, increases levels of GH in humans (Gil-Ad *et al*. 1979), and the Clonidine Growth Hormone Test (CGHT) is used to diagnose neural disorders such as Parkinson's and Alzheimer's diseases (Herrmann *et al.* 2004; Pellecchia *et al.* 2006). Clonidine has also been tested as a growth stimulating agent in fish farming, and showed to increase growth and GH levels in Coho salmon (Bates and McKeown 1990). This has also been observed in goldfish (Lee *et al.* 2000). Medetomidine has shown to increase GH levels in humans (Venn *et al*. 2001; Scheinin *et al*. 1989) and rat (Idänpään-Heikkilä *et al.* 1995).

In the rainbow trout water exposure study described in *Paper II*, we found no significant effects on levels of GH or IGF-I, but a trend towards increased GH levels and decreased IGF-I levels, and thus an increase in GH:IGF-I ratio. The growth study described in *Paper V* was performed using individually marked fish sampled after 31 and 54 days. We did not see any significant effects on growth rate, however we observed an insignificant trend towards *decreased* GH:IGF-I ratio.

We found also a dose-dependent decease in blood glucose and a decrease in liver somatic index. In many fish species, including rainbow trout, catecholamines have shown to increase blood glucose levels (Fabbri *et al.* 1998). Since medetomidine is known to decrease noradrenaline levels, this may explain the effect. Glycogen, which can be converted to blood glucose is stored in liver hepatocytes (Enes *et al.* 2009), the decreased liver size could be due to depleted glycogen storages, however this remains to be proved.

Because of the insignificant decrease in GH/IGF-I ratio and the observed effect on blood glucose we can not exclude that under other circumstances; such as other fish species, life stages or feeding regimes, medetomidine may effect growth. However, from the studies within this thesis, no such responses can be shown.

Gene expression

The development of DNA microarray techniques for performance of large scale gene expression analysis has been very fast during the last decade. Sequencing of transcriptomes, or Expressed Sequence Tags (EST:s), have also generated increasing amounts of genetic information available in databases. It is now possible to perform large scale gene expression analysis in species not fully sequenced, also in species of interest in ecotoxicology (Denslow *et al*. 2007; Steinberg *et al.* 2008)

A microarray consists of a chip, usually made of glass. On the chip are thousands of probes for genes spaced out regularly. The length and origin of the probes vary between different types of arrays. On a cDNA array, the probes typically origin from cloned cDNA, spotted onto the chip. Commonly samples from two different treatments, labelled with different dyes, (visible as red and green) hybridise on the same array. The colour of each spot then tells which one of the samples has the highest expression of that specific gene. Oligonucleotid arrays contain shorter probes that are designed *in silco* and synthesised either direct on the chip or spotted on the chip after synthesis. Only one sample per array is analysed. The results from oligonucleotide arrays have shown to be more accurate when comparing to RNA measurements using quantitative PCR, than results from cDNA arrays (Kuo *et al*. 2006).

The most common use of microarray data is to generate hypotheses. These hypotheses need to be followed up in studies linking gene expression to phenotypic trays or modes of action (Aardema and MacGregor 2002; Steinberg *et al.* 2008).

In our case we performed the microarray study late, when we had already some knowledge about responses of medetomidine in fish. The reason for this, perhaps backward strategy, was simply that this study was not possible to perform until recently, and we wanted to see what additional information could be obtained from this extensive analysis.

The microarray study used liver from rainbow trout sampled after 31 days of exposure. We found no genes to be significantly regulated between the treatments. This was a bit unexpected. Further gene ontology analysis revealed some interesting areas. Among the biological processes were *regulation of bone mineralisation*, *amine metabolic process*, *response to chemical stimulus*, *developmental growth* and *glutathione biosynthetic process.* Also the molecular functions *oxidoreductase activity* and *glutathione transferase activity* as well as the cellular component *MHC protein complex* were suggested as altered by the treatment. Among the top 5 genes was adenylate cyclase, which is involved in the signal pathway of medetomidine. These results are further discussed in *paper V*.

It is possible that a similar gene expression analysis performed after only a few days of exposure would give very different results. Traditionally, stress responses can be divided into three phases, an alarm phase, a resistance phase and an exhaustion phase. During these different phases probably different gene profiles are expressed. Also, gene responses do not seem to be directly dose-dependent, but the expression profile may vary for the same substance at different concentrations (Steinberg *et al.* 2008). It would be very interesting to perform an acute study and to compare the gene expression results with this long term study.

Oxidative stress markers

In all processes demanding oxygen, there is also formation of reactive oxygen species (ROS). The most common one is the super oxide radical O_2-. The production of radicals may be enhanced by disease, aging, physical exercise and xenobiotica. Oxygen radicals may also be produced as a result of phase I metabolism. The oxygen radicals are very reactive and cause damages on biomolecules, such as proteins, lipids and DNA. When the antioxidant defence system is unable to scavenge all radicals formed, the organism is put at "oxidative stress".

The antioxidant defence system consists of enzymes, vitamins, caretonoids and low weight molecular substances. One of the most important and abundant substances is glutathione. It consists of three amino acids, and is itself able to reduce and detoxify ROS, but is also used by glutathione-S-transferases (GST) and glutathione peroxidases (GPX) for phase II conjugation of xenobiotics. Once oxidised, glutathione can be reduced by glutathione reductase (GR), and re-used as an antioxidant. Glutathione in reduced (GSH) and oxidised form (GSSG) as well as GR and GST are used as biomarkers for oxidative stress in ecotoxicological investigations (Stephensen *et al.* 2002; Berglund *et al.* 2007; Carney Almroth *et al.* 2008; Sturve *et al.* 2008). We have measured glutathione reductase (GR) and glutathione (GSH) or glutathione-S-transferases (GST) in all our studies in rainbow trout (*Paper II, V),* Atlantic cod and turbot (unpublished), and we have not seen any effects on these parameters from the medetomidine treatment. When analysing data from the microarray studies there were indications that genes coupled to activity of glutathione transferases had been altered during medetomidine exposure (*Paper V*). Possibly these are other transferases than the ones picked up by the catalytic assay.

One interesting aspect of medetomidine is that it is sometimes used in medicine as a neuroprotective agent during ischemia and surgery. It has been shown that medetomidine reduces neural damage during these events. Several studies have shown

a connection with reduced production of ROS and increases in antioxidants, including glutathione (Eser *et al*. 2008; Aslan *et al*. 2009). The mechanisms behind this are not clear. It would be interesting to perform a combination study with medetomidine and a substance known to induce oxidative stress, and see whether medetomidine could be protective also in fish.

Tolerance to medetomidine?

The results, or lack of significant results, from the gene expression study reveals the question if fish during long term exposure to medetomidine develop resistance or tolerance to the substance. Indications of this may be observed when comparing results from the different studies within this thesis. Altered EROD activity, suggested as one of the consistent and early responses, is no longer observed after 31 and 54 days (*paper V*). Desensitization, receptor deactivation was suggested as an explanation for the results from the two melanophore studies. When studying paleness after 31 and 54 days, fish from the 0.5 nM treatment are only significantly pale at the first occasion and fish from the 5.0 nm treatment are also less pale (insignificant) at the second occasion.

The development of tolerance to medetomidine is known from mammalian studies where it has been observed that the level of analgesia or hypnosis diminishes over time (few days) in spite of the same dosage of medetomidine. It has been observed in mice that tolerance for analgesia and hypnosis does not occur simultaneously, suggesting diverse mechanisms behind the tolerance (Reid *et al.* 1997; Davies *et al.* 2001, 2003). Clonidine is well known to induce tolerance in patients. It has been shown that by having un- continuous administration, i.e. only during daytime or nigh time, tolerance development is inhibited (El-Mas and Abdel-Rahman 2007). Desensitization is a common phenomenon from G-protein coupled receptors. These can be detached from the G-protein and internalised in the cell in a clathrin coated membrane vesicle. Since medetomidine interferes with catecholamines that are regulated via negative feedback systems it might also be possible that the tolerance to medetomidine in our study could be due to a shift in noradrenaline turnover.

Tolerance development can also be due to induction of ATP-binding cassette (ABC) super family of membrane transporters. Induction of these transporters was first discovered in cancer patients developing resistance towards a variety of drugs (multidrug resistance, MDR), and has later shown also to be an important reason for tolerance development in aquatic organisms from contaminated sites (multixenobiotic resistance, MXR) (Bard 2000). P-glycoproteins serve as a first line

of defence, being very unspecific, transporting a wide variety of organic compounds with only a moderate hydrophobicity in common, while MRP proteins transport mainly phase 1 and 2 metabolites. Multidrug resistance is used as a biomarker and tool in biomonitoring (Minier *et al.* 1999, 2000), and several proteins associated with MXR have been identified in rainbow trout (Fischer *et al.* 2009). It would be interesting to investigate if ABC proteins are regulated by medetomidine treatment and thus may be involved in potential tolerance development.

Behaviour

When evaluating the impact of environmental pollutants, behavioural studies in fish were first suggested to be used in the 1960´s (Warner *et al.* 1966). Behavioural changes are the combined result of several biochemical and physiological processes in an animal, and are often of high ecological relevance. Behavioural studies may function as more sensitive early warning signals than standard test methods. Medetomidine inhibits noradrenaline release, and substances that interfere with neurotransmitters are likely to alter behaviour (Scott and Sloman 2004). It was therefore of interest to perform behavioural studies.

Behavioural responses to medetomidine have earlier been studied in the bivalve *Abra nitida* (Bellas *et al.* 2006), where medetomidine showed to reduce burrowing behaviour. It has also been studied in the amphipod *Corophium volotator*, where it shown to reduce mate search behaviour (Krång and Dahlström 2006). In fish, studies of locomotor inhibition in response to medetomidine has been studied in zebrafish (Ruuskanen *et al.* 2005), but was investigated only from 1000 nM.

In our studies with Atlantic salmon (Ekvall 2004) we found no significant effects on the studied behaviours, the concentrations studied were 5 and 50 nM. There were however dose dependent trends toward lower aggressiveness and lower appetite (figure 20).

In the stickleback study, a decreased locomotor activity was observed in the three highest concentrations (50-500 nM). An increasing response time to food with increasing concentrations of medetomidine was also seen. The fish in the highest concentration (500 nM) did not seem to show any interest in food during the 20 minutes trial period. There was consequently also a decrease in food consumption by the fish in the highest concentration and a slight increase in food consumption in fish exposed to lower concentrations of medetomidine (Hilvarsson *et al*. 2007b).

In both of these studies, EROD activity was measured and paleness observed.

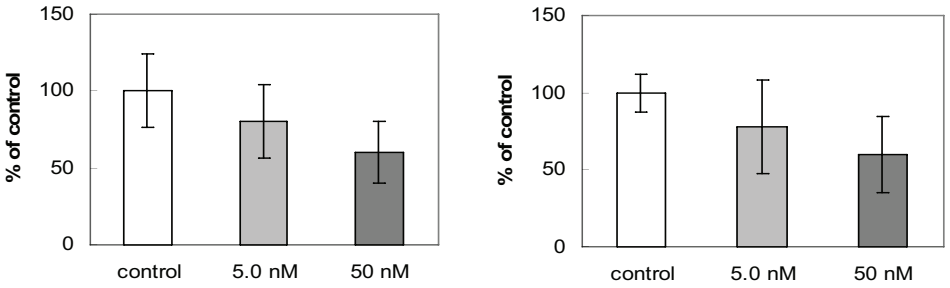


Figure 20. Appetite (left) and aggression (right)
Summary of behavioural studies in Atlantic salmon as percent of control. Appetite is measured as number of pellets consumed, while aggression is measured as seconds performing aggressive acts. Error bars show standard errors.

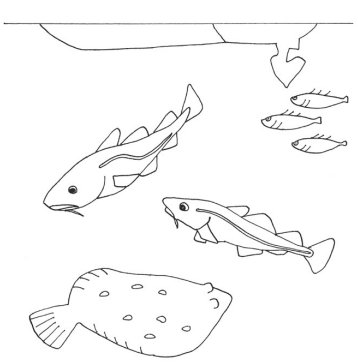
In Atlantic salmon we found a significant EROD induction, indicating that the behavioural parameters measured were not more sensitive than EROD activity. However, in the stickleback we saw an increased EROD activity first at 500 nM, while behaviours were altered already from 50 nM. Paleness was also observed, and was altered from 5 nM in salmon and from 50 nM in stickleback (unquantified observations).

The observed behavioural effects of medetomidine can be interpreted as slight sedation. In the test, aggressiveness was possibly also affected by the fish appearance, where the medetomidine exposed fish were clearly pale. We can conclude that altered behaviours belong to the early responses of medetomidine exposure.

Cytotoxicity and viability

We suggest that medetomidine is not cytotoxic. The medium EC50 (50% effect) value for tests was 150 mg/l, which is corresponding to 750 μM. It was difficult to dissolve the amount of medetomidine needed to obtain full toxicity using the solvent DMSO. During our *in vivo* studies we have never observed mortality following medetomidine exposure, but of course that was never our intention. In previous studies using medetomidine to sedate fish, high concentrations have been used, without lethal effects. For zebrafish 100 μM was used (Ruuskanen *et al.* 2005), and for rainbow trout 25 μM was used (Horsberg *et al.* 1999). Thus in rainbow trout, the span between effects on pigmentation and EROD activity and death is more than 50.000 times.

SUMMARY AND CONCLUSIONS



We have studied the effects of medetomidine in fish, as part of a large evaluation campaign investigating aspects of using medetomidine in antifouling paint. Medetomidine is presently in use in veterinary medicine as a sedative, and it is known to be a potent α_2-adrenoceptor agonist.

Within the scope of this thesis a number of different studies have been performed. In total five species of fish have been exposed to medetomidine either via *intra peritoneal* injection or via water for time periods ranging from 1 to 54 days. The studies were designed to pick up early effects occurring from low, non-sedative concentrations. Additionally, to better understand some of the responses, isolated pigment cells and hepatic microsomes have been studied *in vitro*.

The most prominent effect of medetomidine in fish is skin colour lightening. This effect was observed in all species tested, and was further quantified in rainbow trout after 31 and 54 days of exposure. It was observed that rainbow trout exposed to 5 nM of medetomidine was significantly paler than control at both sampling occasions, while after 31 days also fish exposed to 0.5 nM were paler than control. Melanophores were studied in rainbow trout after 17 and 54 days of exposure. After 54 days of exposure, the melanophores were functional, and the density of the melanophores was unchanged. This suggests that the paleness induced by medetomidine is reversible. However, these two studies also suggest a difference in sensitivity to medetomidine after long-term exposure.

Medetomidine also showed to be a very strong inhibitor of CYP1A activity *in vitro*. An inhibited EROD activity was also observed in turbot after 14 days of exposure *in vivo*. However, increased EROD activity was observed in turbot after short term exposure *in vivo*. Increased EROD activity was also observed in Atlantic salmon and three-spined stickleback. In rainbow trout, there was an increased EROD activity after 2 and 17 days, while after 31 and 54 days there were no differences from the control. These results suggest from a balance between CYP1A protein induction and enzyme inhibition.

In rainbow trout exposure experiment for 17 days we observed a non-significant trend in the levels of growth hormone and insulin-like growth factor 1. Since medetomidine is known from literature to affect growth hormone in mammals, this was important to investigate further. However, after 31 and 54 days of exposure we found no significant effects on growth or growth hormone. However, there were lowered blood glucose levels and decreased liver size, indicating a carbohydrate metabolic response to the treatment.

When exploring the changes in gene expression after medetomidine exposure for 54 days, we found no significant changes, which was a bit surprising. Analysing gene ontology (GO) terms, we could however identify some biological pathways suggested to be altered by the medetomidine treatment. These include enzymes involved in glutathione transferase activity, MHC protein complex and developmental growth. Among the top 5 regulated genes was adenylate cyclase type II, which is a key component in the signal cascade from α_2-adrenoceptors.

We have studied glutathione and glutathione dependent enzymes in the majority of the experiments, without finding any effects. There is literature suggesting that medetomidine reduces production of reactive oxygen species, something that has been used within therapy.

In the behavioural studies, we saw trends towards decreased appetite, decreased swimming activity and decreased aggression. These effects could be explained as a slight sedation and belong to the early effects of medetomidine.

According to the studies in hepatic zebrafish cells, medetomidine has very low cytotoxicity in fish cells.

Concluding all studies in rainbow trout, it seams like some of the responses observed after short term exposure, declines over time, suggesting tolerance development. EROD activity is most regulated after 2 days of exposure, still significant after 17 days but no different from control after 31 and 54 days. Also, when quantifying paleness, this effect is stronger after 31 days than after 54 days. The results from the melanophore study indicate also desensitization over time.

From a risk perspective, the most important effects to consider are suggested to be paleness and the strong inhibition of EROD activity observed *in vitro*. Paleness could be a large problem in intra- and interspecies situations, especially predator-prey situations, which has been discussed. Inhibition of EROD activity may be a problem in contaminated areas where decreased detoxification would lead to damages and bioaccumulation of toxicants. These effects appear from nominal concentrations of

0.5-5 nM. Overall we can conclude that from these low concentrations only few of the investigated parameters were clearly affected by medetomidine treatment.

FUTURE PERSPECTIVES

This thesis is an important contribution to the knowledge of effects of medetomidine in fish, but it is not a complete base for risk assessment. Important parts outside the scope of this thesis are studies of effects on reproduction and early life stages, and information about predicted environmental concentrations.

It would be of interest with further studies of medetomidine and CYP1A. First, CYP1A mRNA levels and perhaps also protein levels after medetomidine exposure should be investigated. Today it is not clear how imidazoles can induce CYP1A activity *in vivo*, and further studies with medetomidine may contribute to fill this gap of knowledge. It would also be of interest with a combination study of medetomidine and a classical Ah-receptor ligand, such as β-naphthoflavone to investigate consequences of the inhibitory properties of medetomidine *in vivo*. Also other members of the CYP family, such as CYP3 would be interesting to study.

Our studies of melanophores suggest that colour change is reversible also after long term exposure, but this reversibility remains to be tested experimentally. Also, these experiments were performed with scales from fish adapted to a dark background. It would be interesting to perform dose-response studies to find out if medetomidine is able to induce apoptosis and at which concentration, and how responses to medetomidine differ between light and dark adapted fish.

The gene expression analysis revealed no significant changes between the treatments. It would be of interest to compare this long term study with gene expression after acute exposure.

There are some indications among the early response studies within this thesis, that during long term exposure, the observed effects decline. This tolerance aspect is interesting to investigate further, for example by performing time-series studies for chosen parameters and additional measurements of α_2-adrenoceptors.

The results from the long term study indicate also effects on carbohydrate metabolism, an effect that would be interesting to further investigate.

To answer the question if medetomidine is an environmentally acceptable antifoulant lies without the scope of this thesis. That answer is dependent on leakage rates and degradation rates for the substance.

However, I believe that the extensive research and interest in this field today will generate a spectra of good solutions to limit the environmental impact of antifouling paints in the future.

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REFERENCES

Ahlbom, J., Duus, U., 2003. Rent skepp kommer lastat : med möjligheter till en miljöanpassad sjöfart . Länstyrelsen Västra Götalands Län.

Aardema, M.J., MacGregor, J.T., 2002. Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 499, 13–25.

Adachi, K., Kato, K., Wakamatsu, K., Ito, S., Ishimaru, K., Hirata, T., Murata, O., Kumai, H., 2005. The histological analysis, colorimetric evaluation, and chemical quantification of melanin content in'suntanned'fish. Pigment Cell Research 18, 465.

Ágústsson, T., Björnsson, B.T., 2000. Growth hormone inhibits growth hormone secretion from the rainbow trout pituitary *in vitro*. Comparative Biochemistry and Physiology. Part C: Comparative Pharmacology and Toxicology 126, 299–303.

Ahlquist, R.P., 1948. A study of the adrenotropic receptors. Am J Physiol 153, 586–600.

Amara, R., Selleslagh, J., Billon, G., Minier, C., 2009. Growth and condition of 0-group European flounder, *Platichthys flesus* as indicator of estuarine habitat quality. Hydrobiologia 627, 87–98.

Andersson, R.G.G., Karlsson, J.O., Grundstrom, N., 1984. Adrenergic nerves and the alpha 2-adrenoceptor system regulating melanosome aggregation within fish melanophores. Acta Physiol Scand 121, 173–179.

Arellano-Aguilar, O., Montoya, R.M., Garcia, C.M., 2009. Endogenous Functions and Expression of Cytochrome P450 Enzymes in Teleost Fish: A Review. Reviews in Fisheries Science 17, 541–556.

Aslan, A., Cemek, M., Eser, O., Altunbaş, K., Buyukokuroglu, M.E., Cosar, M., Baş, O., Ela, Y., Fidan, H., 2009. Does dexmedetomidine reduce secondary damage after spinal cord injury? An experimental study. European Spine Journal 18, 336–344.

Aspengren, S., Hedberg, D., Sköld, H.N., Wallin, M., 2008. New insights into melanosome transport in vertebrate pigment cells. International Review of Cell and Molecular Biology, 245–302.

Aspengren, S., Hedberg, D., Wallin, M., 2006. Studies of pigment transfer between *Xenopus laevis* melanophores and fibroblasts *in vitro* and *in vivo*. Pigment Cell Research 19, 136.

Aspengren, S., Sköld, H.N., Quiroga, G., Mårtensson, L., Wallin, M., 2003. Noradrenaline-and melatonin-mediated regulation of pigment aggregation in fish melanophores. Pigment Cell Research 16, 59–64.

Babin, M., Casado, S., Chana, A., Herradón, B., Segner, H., Tarazona, J.V., Navas, J.M., 2005. Cytochrome P4501A induction caused by the imidazole derivative Prochloraz in a rainbow trout cell line. Toxicology *in vitro* 19, 899–902.

Bagnara, J.T., Hadley, M.E., 1973. Chromatophores and color change: the comparative physiology of animal pigmentation. Prentice-Hall Englewood Cliffs, NJ.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360–365.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48, 357–389.

Bates, D.J., McKeown, B.A., 1990. Clonidine Mects Growth Hormone and Growth in Juvenile Coho Salmon *Oncorhynchus kisutch*. Journal of the World Aquaculture Society 21, 210–215.

Bellas, J., Hilvarsson, A., Birgersson, G., Granmo, Å., 2006. Effects of medetomidine, a novel antifouling agent, on the burrowing bivalve *Abra nitida* (Müller). Chemosphere 65, 575–582.

Bellas, J., Hilvarsson, A., Granmo, Å., 2005. Sublethal effects of a new antifouling candidate on lumpfish (*Cyclopterus lumpus L.*) and Atlantic cod (*Gadus morhua L.*) larvae. Biofouling 21, 207–216.

Berglund, Å., Sturve, J., Förlin, L., Nyholm, N.E.I., 2007. Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environmental Research 105, 330–339.

Björnsson, B.T., 1997. The biology of salmon growth hormone: from daylight to dominance. Fish Physiology and Biochemistry 17, 9–24.

Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jönsson, E., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiology and Biochemistry 27, 227–242.

Björnsson, B.T., Taranger, G.L., Hansen, T., Stefansson, S.O., Haux, C., 1994. The interrelation between photoperiod, growth hormone, and sexual maturation of adult Atlantic salmon (*Salmo salar*). General and comparative endocrinology 93, 70.

Blaise, O., Weil, C., Le Bail, P.Y., 1995. Role of IGF-I in the control of GH secretion in rainbow trout (*Oncorhynchus mykiss*). Growth regulation 5, 142.

Bolker, J.A., Hill, C.R., 2000. Pigmentation development in hatchery-reared flatfishes. Journal of Fish Biology 56, 1029–1052.

Bryan, G.W., Gibbs, P.E., 1991. Impact of low concentrations of tributyltin (TBT) on marine organisms: a review. Metal ecotoxicology: concepts and applications, 323–361.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. General and comparative endocrinology 151, 1–26.

Carney Almroth, B., Albertsson, E., Sturve, J., Förlin, L., 2008. Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. Ecotoxicology and Environmental Safety 70, 370–378.

Chambers, L.D., Stokes, K.R., Walsh, F.C., Wood, R.J.K., 2006. Modern approaches to marine antifouling coatings. Surface & Coatings Technology 201, 3642–3652.

Champ, M.A., 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. Science of the Total Environment, The 258, 21–71.

Chen, X., Perry, S.F., Aris-Brosou, S., Selva, C., Moon, T.W., 2007. Characterization and functional divergence of the alpha1-adrenoceptor gene family: insights from rainbow trout (*Oncorhynchus mykiss*). Physiological Genomics 32, 142.

Clark, R.B., Knoll, B.J., Barber, R., 1999. Partial agonists and G protein-coupled receptor desensitization. Trends in Pharmacological Sciences 20, 279–286.

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of glutathione reductase using 5, 5'-dithiobis (2-nitrobenzoic acid). Analytical biochemistry 183, 195–196.

Dahlström, M., Mårtensson, L.G., Jonsson, P.R., Arnebrant, T., Elwing, H., 2000. Surface active adrenoceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. Biofouling 16, 191–203.

Davies, M.F., Haimor, F., Lighthall, G., Clark, J.D., 2003. Dexmedetomidine fails to cause hyperalgesia after cessation of chronic administration. Anesthesia & Analgesia 96, 195.

Davies, M.F., Reid, K., Guo, T.Z., Agashe, G.S., Amin, Y.K., Maze, M., 2001. Sedative but Not Analgesic [alpha] 2 Agonist Tolerance Is Blocked by NMDA Receptor and Nitric Oxide Synthase Inhibitors. Anesthesiology 95, 184.

Delescluse, C., Lemaire, G., De Sousa, G., Rahmani, R., 2000. Is CYP1A1 induction always related to AHR signaling pathway? Toxicology 153, 73–82.

Denslow, N.D., Garcia-Reyero, N., Barber, D.S., 2007. Fish 'n'chips: the use of microarrays for aquatic toxicology. Molecular BioSystems 3, 172–177.

Di Giulio, R.T., Hinton, D.E., 2008. The toxicology of fishes. CRC.

Donnelly, W.A., Whoriskey Jr, F.G., 1993. Transplantation of Atlantic salmon (*Salmo salar*) and crypsis breakdown. Production of juvenile Atlantic salmon, *Salmo salar*, in natural waters, 25–34.

Ekvall, H., 2004. Effects of medetomidine, a candidate antifouling compound and a silicone based antifouling paint on behaviour and EROD activity of Atlantic salmon (*Salmo salar*) fry
. M.Sc. Thesis in Zoological Ecology 20 p. Department of Zoology, Göteborg University.

El-Mas, M.M., Abdel-Rahman, A.A., 2007. Intermittent clonidine regimen abolishes tolerance to its antihypertensive effect: A spectral study. Journal of cardiovascular pharmacology 49, 174.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiology and Biochemistry 35, 519–539.

Eser, O., Fidan, H., Sahin, O., Cosar, M., Yaman, M., Mollaoglu, H., Songur, A., Buyukbas, S., 2008. The influence of dexmedetomidine on ischemic rat hippocampus. Brain research 1218, 250.

Fabbri, E., Capuzzo, A., Moon, T.W., 1998. The role of circulating catecholamines in the regulation of fish metabolism: An overview. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 120, 177-192.

Fischer, S., Pietsch, M., Schirmer, K., Luckenbach, T., 2009. Identification of multi-drug resistance associated proteins MRP1, Marine Environmental Research, in press.

Fleming, G.J., Heard, D.J., Floyd, R.F., Riggs, A., 2003. Evaluation of propofol and medetomidine-ketamine for short-term immobilization of Gulf of Mexico sturgeon (*Acipenser oxyrinchus de soti*). Journal of Zoo and Wildlife Medicine 34, 153–158.

Fujii, R., 2000. The regulation of motile activity in fish chromatophores. Pigment Cell Res 13, 300–319.

Förlin, , L., Goksoyr, A., Husoy, A.M., 1994. Cytochrome P450 monooxygenase as indicater of PCB/Dioxin like compounds in fish. I: Biomonitoring of coastal waters and estuaries. . Kramer, K.J.M. (Ed.), ss. 135-150.

Gerlach, A.T., Dasta, J.F., 2007. Dexmedetomidine: an updated review. The Annals of pharmacotherapy 41, 245.

Gil-Ad, I., Topper, E., Laron, Z., 1979. Oral clonidine as a growth hormone stimulation test. Lancet 2, 278.

Gipperth, L., 2009. The legal design of the international and European Union ban on tributyltin antifouling paint: Direct and indirect effects. Journal of Environmental Management 90, 86–95.

Guerit, I., Bocquene, G., James, A., Thybaud, E., Minier, C., 2008. Environmental risk assessment: A critical approach of the European TGD in an in situ application. Ecotoxicology and Environmental Safety 71, 291–300.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol 42, 5807-5813.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. Journal of biological Chemistry 249, 7130.

Hasselberg, L., Grosvik, B.E., Goksoyr, A., Celander, M.C., 2005. Interactions between xenoestrogens and ketoconazole on hepatic CYP 1 A and CYP 3 A, in juvenile Atlantic cod(*Gadus morhua*). Comparative Hepatology 4, 2.

Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C., 2008. Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17alpha-ethynylestradiol exposure. Aquatic Toxicology 86, 256–264.

Hedberg, D., 2009. Melanosome transfer, photoreception and toxicity assays in melanophores. Doctoral thesis. Dep of Zoology, University of Gothenburg. ISBN 978-91-628-7890-0

Hegelund, T., Ottosson, K., Raadinger, M., Tomberg, P., Celander, M.C., 2004. Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. Environmental Toxicology and Chemistry 23, 1326–1334.

Hein, L., 2006. Adrenoceptors and signal transduction in neurons. Cell and tissue research 326, 541–551.

Hernandez-Boussard, T., Whirl-Carrillo, M., Hebert, J.M., Gong, L., Owen, R., Gong, M., Gor, W., Liu, F., Truong, C., Whaley, R., others, 2008. The pharmacogenetics and pharmacogenomics knowledge base: accentuating the knowledge. Nucleic acids research 36, D913.

Herrmann, N., Lanctôt, K.L., Eryavec, G., van Reekum, R., Khan, L.R., 2004. Growth hormone response to clonidine predicts aggression in Alzheimer's disease. Psychoneuroendocrinology 29, 1192–1197.

Hieble, J.P., Ruffolo Jr, R.R., 1995. Possible structural and functional relationships between imidazoline receptors and alpha 2-adrenoceptors. Annals of the New York Academy of Sciences 763, 8.

Hilvarsson, A., Halldórsson, H.P., Granmo, Å., 2007a. Medetomidine as a candidate antifoulant: Sublethal effects on juvenile turbot (*Psetta maxima L.*). Aquatic Toxicology 83, 238–246.

Hilvarsson, A., Lennquist, A., Backhaus, T., 2007b. Behavioutal and biochemical effects of medetomidine, a new antifoullant, in the three-spined stickleback (*Gasterosteus aculeatus L.*). PhD Thesis Dep of Marine Ecology, University of Gothenburg.

Hilvarsson, A., Ohlauson, C., Blanck, H., Granmo, Å., 2009. Bioaccumulation of the new antifoulant medetomidine in marine organisms. Marine Environmental Research 68, 19–24.

Hogben, L., Slome, D., 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 10–53.

Horsberg, T.E., Burka, J.F., Tasker, R.A.R., 1999. Actions and pharmacokinetic properties of the alpha2-adrenergic agents, medetomidine and atipamezole, in rainbow trout (*Oncorhynchus mykiss*). Veterinary Anaesthesia and Analgesia 26, 18–22.

Höglund, E., Balm, P.H., Winberg, S., 2000. Skin darkening, a potential social signal in subordinate Arctic charr (*Salvelinus alpinus*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. The Journal of Experimental Biology 203, 1711–1721.

Idänpään-Heikkilä, J.J., Rauhala, P., Männistö, P.T., 1995. Neuroendocrine Effects of Dexmedetomidine: Evidence of Cross-Tolerance Between a mu-Opioid Agonist and an alpha 2-Adrenoceptor Agonist in Growth Hormone Secretion of the Male Rat. Pharmacology & toxicology 78, 136–142.

Johansson, P., 1979. Antagonistic effects of synthetic alpha adrenoceptor agonists on isolated artery strips from the cod, *Gadus morhua*. Comparative biochemistry and physiology. C: Comparative pharmacology 63, 267.

Johnsson, J.I., Parkkonen, J., Förlin, L., 2003. Reduced territorial defence in rainbow trout fry exposed to a paper mill effluent: using the mirror image stimulation test as a behavioural bioassay. Journal of Fish Biology 62, 959–964.

Jos, A., Segner, H., Herradón, B., Repetto, G., Navas, J.M., 2007. Induction of EROD activity by 1-phenylimidazole and beta-naphthoflavone in rainbow trout cultured hepatocytes: A comparative study. Toxicology *in vitro* 21, 1307–1310.

Karlsson, J.O., Andersson, R.G., Grundström, N., 1989. Characterization of pigment aggregating alpha 2-adrenoceptors of fish melanophores by use of different agonists after partial irreversible receptor inactivation. British journal of pharmacology 97, 222.

Ketata, I., Denier, X., Hamza-Chaffai, A., Minier, C., 2007. Endocrine-related reproductive effects in molluscs. Comparative Biochemistry and Physiology, Part C.

Kharasch, E.D., Herrmann, S., Labroo, R., 1992. Ketamine as a probe for medetomidine stereoisomer inhibition of human liver microsomal drug metabolism. Anesthesiology 77, 1208.

Kodric-Brown, A., 1998. Sexual Dichromatism and Temporary Color Changes in the Reproduction of Fishes 1. Integrative and Comparative Biology 38, 70–81.

Konstandi, M., Kostakis, D., Harkitis, P., Marselos, M., Johnson, E.O., Adamidis, K., Lang, M.A., 2005. Role of adrenoceptor-linked signaling pathways in the regulation of CYP1A1 gene expression. Biochemical Pharmacology 69, 277–287.

Konstantinou, I.K., Albanis, T.A., 2004. Worldwide occurrence and effects of antifouling paint booster biocides in the aquatic environment: a review. Environment international 30, 235–248.

Kristiansson, E., Asker, N., Förlin, L., Larsson, D.G., 2009. Characterization of the *Zoarces viviparus* liver transcriptome using massively parallel pyrosequencing. BMC genomics 10, 345.

Krång, A.S., Dahlström, M., 2006. Effects of a candidate antifouling compound (medetomidine) on pheromone induced mate search in the amphipod *Corophium volutator*. Marine pollution bulletin 52, 1776–1783.

Kuo, W.P., Liu, F., Trimarchi, J., Punzo, C., Lombardi, M., Sarang, J., Whipple, M.E., Maysuria, M., Serikawa, K., Lee, S.Y., McCrann, D., Kang, J., Shearstone, J.R., Burke, J., Park, D.J., Wang, X., Rector, T.L., Ricciardi-Castagnoli, P., Perrin, S., Choi, S., Bumgarner, R., Kim, J.H., Short, G.F., Freeman, M.W., Seed, B., Jensen, R., Church, G.M., Hovig, E., Cepko, C.L., Park, P., Ohno-Machado, L., Jenssen, T., 2006. A sequence-oriented comparison of gene expression measurements across different hybridization-based technologies. Nat. Biotechnol 24, 832-840.

Lee, E.K.Y., Chan, V.C.C., Chang, J.P., Yunker, W.K., Wong, A.O.L., 2000. Norepinephrine Regulation of Growth Hormone Release from Goldfish Pituitary Cells. I. Involvement of a2 Adrenoreceptor and Interactions with Dopamine and Salmon Gonadotropin-Releasing Hormone. Journal of Neuroendocrinology 12, 311–322.

Lesca, P., Peryt, B., Larrieu, G., Alvinerie, M., Galtier, P., Daujat, M., Maurel, P., Hoogenboom, L., 1995. Evidence for the ligand-independent activation of the AH receptor. Biochemical and Biophysical Research Communications 209, 474–482.

Levine, S.L., Czosnyka, H., Oris, J.T., 1997. Effect of the fungicide clotrimazole on the bioconcentration of benzo [a] pyrene in gizzard shad (Dorosoma cepedianum): *in vivo* and i*n vitro* inhibition of cytochrome P4501A activity. Environmental Toxicology and Chemistry 16, 306–311.

Lindgren, J.F., Haeffner, M., Ericsson, C.T., Jonsson, P.R., 2009. Oxygen-depleted surfaces: a new antifouling technology. Biofouling 25, 455.

Minier, C., Eufemia, N., Epel, D., 1999. The multi-xenobiotic resistance phenotype as a tool to biomonitor the environment. Biomarkers 4, 442–454.

Minier, C., Levy, F., Rabel, D., Bocquené, G., Godefroy, D., Burgeot, T., Leboulenger, F., 2000. Flounder health status in the Seine Bay. A multibiomarker study. Marine environmental research 50, 373–377.

Moriyama, S., Swanson, P., Nishii, M., Takahashi, A., Kawauchi, H., Dickhoff, W.W., Plisetskaya, E.M., 1994. Development of a homologous radioimmunoassay for coho salmon Insulin-like Growth Factor-I. General and comparative endocrinology 96, 149.

Murrell, J.C., Hellebrekers, L.J., 2005. Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. Vet Anaesth Analg 32, 117–27.

Navas, J.M., Chana, A., Herradón, B., Segner, H., 2003. Induction of CYP1A by the N-imidazole derivative, 1-benzylimidazole. Environmental Toxicology and Chemistry 22, 830–836.

Navas, J.M., Chana, A., Herradón, B., Segner, H., 2004. Induction of cytochrome P4501A (CYP1A) by clotrimazole, a non-planar aromatic compound. Computational studies on structural features of clotrimazole and related imidazole derivatives. Life Sciences 76, 699–714.

Nickerson, J.G., Dugan, S.G., Drouin, G., Moon, T.W., 2001. A putative 2-adrenoceptor from the rainbow trout (*Oncorhynchus mykiss*): Molecular characterization and pharmacology. FEBS Journal 268, 6465–6472.

Olsen, S.M., Pedersen, L.T., Laursen, M.H., Kiil, S., Dam-Johansen, K., 2007. Enzyme-based antifouling coatings: a review. Biofouling 23, 369–383.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J.A., Winter, M.J., Sumpter, J.P., 2007. Comparative physiology, pharmacology and toxicology of beta-blockers: Mammals versus fish. Aquatic Toxicology 82, 145–162.

Papoutsoglou, S.E., Mylonakis, G., Miliou, H., Karakatsouli, N.P., Chadio, S., 2000. Effects of background color on growth performances and physiological responses of scaled carp (*Cyprinus carpio L.*) reared in a closed circulated system. Aquacultural Engineering 22, 309–318.

Peakall, D.B., Walker, C.H., 1994. The role of biomarkers in environmental assessment (3). Vertebrates. Ecotoxicology 3, 173–179.

Pelkonen, O., Puurunen, J., Arvela, P., Lammintausta, R., 1991. Comparative effects of medetomidine enantiomers on *in vitro* and *in vivo* microsomal drug metabolism. Pharmacol Toxicol 69, 189–194.

Pellecchia, M.T., Pivonello, R., Colao, A., Barone, P., 2006. Growth Hormone Stimulation Tests in the Differential Diagnosis of Parkinson; s Disease. Clinical Medicine and Research 4, 322.

Quackenbush, J., Cho, J., Lee, D., Liang, F., Holt, I., Karamycheva, S., Parvizi, B., Pertea, G., Sultana, R., White, J., 2001. The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucleic Acids Res 29, 159-164.

Raveendran, T.V., Mol, V.P., 2009. Natural product antifoulants. Current Science 97, 508–520.

Reid, K., Guo, T.Z., Davies, M.F., Maze, M., 1997. Nifedipine, an L-type calcium channel blocker, restores the hypnotic response in rats made tolerant to the alpha-2 adrenergic agonist dexmedetomidine. Journal of Pharmacology and Experimental Therapeutics 283, 993.

Reinecke, M., Björnsson, B.T., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., Gutiérrez, J., 2005. Growth hormone and insulin-like growth factors in fish: where we are and where to go. General and comparative endocrinology 142, 20–24.

Ricker, W.E., 1979. Growth Rates and Models, Fish Physiology. Bioenergetics and Growth, Acad. Press, pp, 677–743.

Ruuskanen, J.O., Laurila, J., Xhaard, H., Rantanen, V.V., Vuoriluoto, K., Wurster, S., Marjamäki, A., Vainio, M., Johnson, M.S., Scheinin, M., 2005a. Conserved structural, pharmacological and functional properties among the three human and five zebrafish alpha2-adrenoceptors. British Journal of Pharmacology 144, 165.

Ruuskanen, J.O., Peitsaro, N., Kaslin, J.V., Panula, P., Scheinin, M., 2005b. Expression and function of [alpha] 2-adrenoceptors in zebrafish: drug effects, mRNA and receptor distributions. Journal of neurochemistry 94, 1559.

Ruuskanen, J.O., Xhaard, H., Marjamaki, A., Salaneck, E., Salminen, T., Yan, Y.L., Postlethwait, J.H., Johnson, M.S., Larhammar, D., Scheinin, M., 2004. Identification of Duplicated Fourth alpha2-Adrenergic Receptor Subtype by Cloning and Mapping of Five Receptor Genes in Zebrafish. Molecular biology and evolution 21, 14.

Ryer, C.H., Lemke, J.L., Boersma, K., Levas, S., 2008. Adaptive coloration, behavior and predation vulnerability in three juvenile north Pacific flatfishes. Journal of Experimental Marine Biology and Ecology 359, 62–66.

Sanchez, W., Porcher, J.M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. Trends in Analytical Chemistry 28, 150–158.

Savola, J.M., Ruskoaho, H., Puurunen, J., Salonen, J.S., Kärki, N.T., 1986. Evidence for medetomidine as a selective and potent agonist at alpha 2-adrenoreceptors. Journal of autonomic pharmacology 6, 275.

Scheinin, H., Virtanen, R., MacDonald, E., Lammintausta, R., Scheinin, M., 1989. Medetomidine: a novel alpha2 -adrenoceptor agonist: a review of its pharmacodynamic effects. Progress in neuro-psychopharmacology & biological psychiatry 13, 635–651.

Scheinin, M., Schwinn, D.A., 1992. The Locus Coeruleus Site of Hypnotic Actions of [alpha] 2-Adrenoceptor Agonists? Anesthesiology 76, 873.

Schultz, M.P., 2007. Effects of coating roughness and biofouling on ship resistance and powering. Biofouling 23, 331–341.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic toxicology 68, 369–392.

Seppälä, O., Karvonen, A., Tellervo Valtonen, E., 2004. Parasite-induced change in host behaviour and susceptibility to predation in an eye fluke–fish interaction. Animal Behaviour 68, 257–263.

Seppälä, O., Karvonen, A., Valtonen, E.T., 2005. Impaired crypsis of fish infected with a trophically transmitted parasite. Animal Behaviour 70, 895–900.

Sinclair, M.D., 2003. A review of the physiological effects of alpha2-agonists related to the clinical use of medetomidine in small animal practice. The Canadian Veterinary Journal 44, 885.

Sloman, K.A., Baker, D.W., Wood, C.M., McDonald, G., 2002. Social interactions affect physiological consequences of sublethal copper exposure in rainbow trout, *Oncorhynchus mykiss.* Environmental Toxicology and Chemistry 21, 1255–1263.

Sloman, K.A., Morgan, T.P., McDonald, D.G., Wood, C.M., 2003. Socially-induced changes in sodium regulation affect the uptake of water-borne copper and silver in the rainbow trout, *Oncorhynchus mykiss.* Comparative Biochemistry and Physiology, Part C 135, 393–403.

Stegeman, J.J., 1992. Nomenclature for hydrocarbon-inducible cytochrome P450 in fish. Marine Environmental Research 34, 133–138.

Steinberg, C.E., Stürzenbaum, S.R., Menzel, R., 2008. Genes and environment—striking the fine balance between sophisticated biomonitoring and true functional environmental genomics. Science of the Total Environment 400, 142–161.

Stephensen, E., Sturve, J., Förlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comparative Biochemistry and Physiology, Part C 133, 435–442.

Strand, J., Jacobsen, J.A., 2005. Accumulation and trophic transfer of organotins in a marine food web from the Danish coastal waters. Science of the Total Environment, The 350, 72–85.

Sturm, A., Cravedi, J.P., Perdu, E., Baradat, M., Segner, H., 2001. Effects of prochloraz and nonylphenol diethoxylate on hepatic biotransformation enzymes in trout: a comparative *in vitro/in vivo*-assessment using cultured hepatocytes. Aquatic Toxicology 53, 229–245.

Sturve, J., Almroth, B.C., Förlin, L., 2008. Oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to sewage treatment plant effluent. Ecotoxicology and Environmental Safety 70, 446–452.

Sugimoto, M., 2002. Morphological color changes in fish: regulation of pigment cell density and morphology. Microscopy research and technique 58.

Sugimoto, M., Yuki, M., Miyakoshi, T., Maruko, K., 2005. The influence of long-term chromatic adaptation on pigment cells and striped pigment patterns in the skin of the zebrafish, *Danio rerio*. Journal of Experimental Zoology Part A: Comparative Experimental Biology.

Svensson, S.P., Bailey, T.J., Pepperl, D.J., Grundström, N., Ala-Uotila, S., Scheinin, M., Karlsson, J.O., Regan, J.W., 1993a. Cloning and expression of a fish alpha 2-adrenoceptor. Br. J. Pharmacol 110, 54-60.

Svensson, S.P., Bailey, T.J., Pepperl, D.J., Grundström. 1993b. Cloning and expression of a fish alpha 2-adrenoceptor. British journal of pharmacology 110, 54.

Szabo, B., 2002. Imidazoline antihypertensive drugs: a critical review on their mechanism of action. Pharmacology and Therapeutics 93, 1–35.

Takahashi, A., Tsuchiya, K., Yamanome, T., Amano, M., Yasuda, A., Yamamori, K., Kawauchi, H., 2004. Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder. Peptides 25, 1613–1622.

Takano, Y., Yaksh, T.L., 1993. Chronic spinal infusion of dexmedetomidine, ST-91 and clonidine: spinal alpha 2 adrenoceptor subtypes and intrinsic activity. Journal of Pharmacology and Experimental Therapeutics 264, 327.

Thomas, K.V., Fileman, T.W., Readman, J.W., Waldock, M.J., 2001. Antifouling paint booster biocides in the UK coastal environment and potential risks of biological effects. Marine pollution bulletin 42, 677–688.

Thomas, K.V., Brooks, S., 2009. The environmental fate and effects of antifouling paint biocides. Biofouling 26, 73–88.

Truhaut, R., 1977. Ecotoxicology: objectives, principles and perspectives. Ecotoxicology and Environmental Safety 1, 151.

Tsao, P., Cao, T., von Zastrow, M., 2001. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends in pharmacological sciences 22, 91–96.

Uchida-Oka, N., Sugimoto, M., 2001. Norepinephrine Induces Apoptosis in Skin Melanophores by Attenuating cAMP-PKA Signals Via [alpha] 2-Adrenoceptors in the Medaka, *Oryzias Latipes.* Pigment Cell Research 14, 356.

Wahl, M., 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. Marine ecology progress series. Oldendorf 58, 175–189.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13, 57–149.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell biology and toxicology 10, 415–421.

Warner, R.E., Peterson, K.K., Borgman, L., 1966. Behavioural pathology in fish: a quantitative study of sublethal pesticide toxication. Journal of Applied Ecology 3, 223–247.

Venn, R.M., Bryant, A., Hall, G.M., Grounds, R.M., 2001. Effects of dexmedetomidine on adrenocortical function, and the cardiovascular, endocrine and inflammatory responses in post-operative patients needing sedation in the intensive care unit. British Journal of Anaesthesia 86, 650–656.

Whyte, J.J., Jung, R.E., others, 2000. EROD Activity. Critical Reviews in Toxicology 30, 347–570.

Williams, T.D., Rollins, M., Block, B.A., 2004. Intramuscular anesthesia of bonito and Pacific mackerel with ketamine and medetomidine and reversal of anesthesia with atipamezole. Journal of the American Veterinary Medical Association 225, 417–421.

Yawetz, A., Woodin, B.R., Stegeman, J.J., 1998. Cytochromes P450 in liver of the turtle *Chrysemys picta picta* and the induction and partial purification of CYP1A-like proteins. BBA-General Subjects 1381, 12–26.