



UMEÅ UNIVERSITY

# Fish on Drugs

Behaviour Modifying Contaminants in Aquatic Ecosystems

Annelie Lagesson

**Department of Ecology and Environmental Science**  
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*Till pappa*

*"Here and there awareness is growing that man, far from being the overlord of all creation, is himself part of nature, subject to the same cosmic forces that control all other life. Man's future welfare and probably even his survival depend upon his learning to live in harmony, rather than in combat, with these forces."*

Rachel Carson, 1958

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## Sammanfattning

Vattenföroreningar är ett omfattande globalt problem. Läkemedel är en grupp av föroreningar som når framförallt akvatiska ekosystem via t.ex. reningsverk och avrinningar från jordbruk. Den finns en ökande oro för att läkemedel kan påverka vattenlevande organismers beteenden med negativa ekologiska konsekvenser som följd. Syftet med den här doktorsavhandlingen var att öka vår förståelse för upptag samt effekter av beteendeförändrande läkemedel i akvatiska ekosystem.

Min avhandling visar att i ett naturligt system där läkemedel tillsatts, tog de bentiska arterna i botten av födoväven upp mest läkemedel (högst bioackumuleringsfaktor; BAF) medan fisken i toppen av födoväven ackumulerade minst. Det verkar därför som att överföringen av läkemedel mellan olika trofiska nivåer är lägre än den för mer "traditionella" föroreningar. Upptaget i förhållande till vattenkoncentrationen av det ångestdämpande läkemedlet oxazepam, ökade dock i abborre (*Perca fluviatilis*) över studieperioden. Detta tyder på att vissa läkemedel kan transporteras genom födovävar upp till högre trofinivåer. För att undersöka om oxazepam kunde påverka tillväxten och/eller överlevnaden av abborre, gjorde jag ett replikerat damm-experiment. I denna studie testade jag hypotesen att exponerade fiskar skulle växa snabbare men också drabbas av högre mortalitet från predation. Hypotesen baserades på tidigare laboratorieresultat som visat att oxazepam ökar abborrars födointag samt risktagande. I motsats till vad jag predikerade, hittade jag inte några signifikanta skillnader i ökad tillväxt och dödlighet hos exponerade abborrar. I studien framkom dock att abborrens naturliga predator (gädda; *Esox lucius*) blir sämre på att fånga byten när den är exponerad för oxazepam. Denna exponeringseffekt på predationseffektiviteten, bidrog troligen till den uteblivna predationseffekten i dammarna. I två följande laboratoriestudier undersökte jag effekterna av beteendeförändrande läkemedel (oxazepam och ett tillväxthormon, 17 $\beta$ -trenbolone) i kombination med ytterligare miljöfaktorer (temperatur och predationsrisk). Läkemedel- och temperaturinteraktioner fanns för 17 $\beta$ -trenbolone, där vattentemperatur tillsammans med 17 $\beta$ -trenbolone framkallade effekter på bland annat flyktbeteende och risktagande hos moskitfisk (*Gambusia holbrooki*). I den andra studien, fann vi att oxazepam, temperatur och predationsrisk alla påverkade beteenden förknippade med ångest (t.ex. risktagande), men oberoende av varandra.

Sammanfattningsvis, drar jag slutsatsen att läkemedel kan påverka ekologiskt viktiga beteenden hos fisk, åtminstone över kortare tidsramar, samt att vissa läkemedel kan ackumuleras i akvatiska födovävar. Enligt mina resultat verkar det som att *in situ*-effekter av beteendeförändrande läkemedel i akvatiska ekosystem beror både på artspecifika reaktioner och abiotiska interaktioner. Därför är det långt ifrån enkelt att prediktera miljöeffekter baserat på laboratoriestudier gjorda med enskilda arter under kontrollerade förhållanden. Framtida studier bör därför inkludera och utreda effekterna av läkemedel i mer komplexa system för att vi ska få en djupare förståelse för vilka konsekvenser läkemedel kan orsaka i miljön.

## Abstract

Contamination of surface waters is a worldwide problem. One group of emerging contaminants that reach aquatic ecosystems via sewage treatment plant effluents and agricultural run-offs is pharmaceuticals. Impacts of pharmaceuticals on the behaviour of aquatic organisms can have important ecological and evolutionary consequences because behaviour is directly linked to fitness. The aim of my doctoral thesis was to increase our understanding of the fate and effects of behaviour modifying drugs in aquatic ecosystems.

While studying an aquatic ecosystem spiked with pharmaceuticals, I found that the benthic species at the bottom of the food chain were the main receivers (highest bioaccumulation factor; BAF) while fish at the top of the food web had the lowest uptake of the studied drugs. Interestingly, the BAF of the anxiolytic pharmaceutical oxazepam, increased in fish (perch; *Perca fluviatilis*) over the study period, suggesting that this drug can be transferred between trophic levels in food webs. To assess whether oxazepam could affect growth and survival in perch, I exposed perch populations to oxazepam for 2-months in a replicated pond experiment. In this study, I tested the hypothesis that oxazepam exposed perch would grow faster but also suffer from increased predation. Oxazepam has been shown previously to induce 'anti-anxiety' behaviours that improve foraging but may also make individuals more exposed to predators. In contrast, I found no statistically significant increase in growth and mortality in the exposed perch. However, the study revealed that the natural predator of perch (pike; *Esox lucius*) became less effective at catching prey when exposed to oxazepam. This exposure effect on predation efficiency likely contributed to the absence of predation effects in the exposed ponds. In two following laboratory studies I investigated effects of behaviour modifying drugs (oxazepam and a growth hormone, 17 $\beta$ -trenbolone) in combination with additional stressors (temperature and predator cues). Drug and temperature interactions were found for 17 $\beta$ -trenbolone, where water temperature interacted with treatment to induce changes in predator escape behaviour, boldness, and exploration in mosquitofish (*Gambusia holbrooki*). However, in the other study, we found that oxazepam, temperature, and predator cue all affected perch 'anti-anxiety' behaviours, but independently.

I conclude that pharmaceuticals can alter ecologically important behaviours in fish, and that at least some, can accumulate in aquatic food webs. It seems that *in situ* effects of behaviour modifying drugs in aquatic ecosystems depend on both species-specific responses and abiotic interactions. As such, it is far from straightforward to predict net ecosystem effects based on experiments conducted using single species and static conditions. Future studies should assess the effects of pharmaceuticals in aquatic ecosystems under more complex conditions for us to gain a better understanding of what consequences behaviour modifying drugs have in the environment.

**Keywords:** Aquatic ecosystems, Behavioural effects, Ecotoxicology, Endocrine disruptors, Pharmaceuticals, Interaction effects

## List of papers

This thesis is based on the following four studies, referred to in the text by their respective Roman numerals:

- I. Lagesson, A.,** Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., and Klaminder, J. 2016. **Bioaccumulation of Five Pharmaceuticals at Multiple Trophic Levels in an Aquatic Food Web – Insights from a Field Experiment.** *Science of the Total Environment*. 568: 208-215.
- II. Lagesson, A.,** Brodin, T., Fahlman, J., Fick, J., Jonsson, M., Persson, J., Byström, P., and Klaminder, J. 2018. **No Evidence of Increased Growth or Mortality in Fish Exposed to Oxazepam in Semi-Natural Ecosystems.** *Science of the Total Environment*. 615: 608-614.
- III. Saaristo, M., Lagesson, A.,** Bertram, M.G., Fick, J., Klaminder, J., Johnstone, C., Wong, B.B.M, and Brodin, T. 2018. **Behavioural effects of temperature, predation-risk and anxiolytic exposure on the European perch (*Perca fluviatilis*).** Manuscript.
- IV. Lagesson, A.,** Saaristo, M., Brodin, T., Fick, J., Martin, J.M., Klaminder, J., and Wong, B.B.M 2018. **Fish on steroids: temperature dependent effects of 17 $\beta$ -trenbolone on anti-predator, risk-taking and exploratory behaviours.** *In revision*, *Environmental pollution*.

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## Author contributions

### *Paper I*

JoF and JK designed the study; JoF collected the field data; JeF did the chemical analysing; AL did the statistical analysis and wrote the paper under supervision from JK; All co-authors contributed with comments on the manuscript.

### *Paper II*

AL and JK designed the study; AL and JoF collected the field data; JeF did the chemical analysing; TB and JP did the additional predator experiment; AL extracted the data and did the statistical analysis with support from JK; AL wrote the manuscript with comments from all co-authors.

### *Paper III*

TB and AL designed the study; AL, TB, MS and MB collected the data; JeF did the chemical analysing; MS extracted the data; CJ did the statistical analyses; MS wrote the manuscript with contribution from AL and comments from all co-authors.

### *Paper IV*

MS designed the study; AL, MS, JM, TB, and BW collected the data; JeF did the chemical analysing; AL extracted the data, did the statistical analysis and wrote the manuscript under supervision of JK and TB; All co-authors contributed with comments on the manuscript.

## Author abbreviations

**AL:** Annelie Lagesson, **JK:** Jonatan Klaminder, **TB:** Tomas Brodin, **JeF:** Jerker Fick, **MJ:** Micael Jonsson, **JoF:** Johan Fahlman, **PB:** Per Byström, **JP:** Josefin Persson, **MS:** Minna Saaristo, **MB:** Michael Bertram, **JM:** Jake Martin, **BW:** Bob Wong, **CJ:** Christopher Johnstone.

## Abstracts of the papers

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### *I. Science of the Total Environment (2016). 568: 208-215.*

#### **Bioaccumulation of Five Pharmaceuticals at Multiple Trophic Levels in an Aquatic Food Web – Insights from a Field Experiment**

**Annelie Lagesson**, Johan Fahlman, Tomas Brodin, Jerker Fick, Micael Jonsson, Per Byström, and Jonatan Klaminder

Pharmaceuticals derived from manufacturing and human consumption contaminate surface waters worldwide. To what extent such pharmaceutical contamination accumulates and disperses over time in different compartments of aquatic food webs is not well known. In this study we assess to what extent five pharmaceuticals (diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine) are taken up by fish (European perch) and four aquatic invertebrate taxa (damselfly larvae, mayfly larvae, waterlouse, and ramshorn snail), by tracing their bioconcentrations over several months in a semi-natural large-scale (pond) system. The results suggest both significant differences among drugs in their capacity to bioaccumulate and differences among species in uptake. While no support for *in situ* uptake of diclofenac and trimethoprim was found, oxazepam, diphenhydramine, and hydroxyzine were detected in all analyzed species. Here, the highest bioaccumulation factor (tissue:water ratio) was found for hydroxyzine. In the food web, the highest concentrations were found in the benthic species ramshorn snail and waterlouse, indicating that bottom-living organism at lower trophic positions are the prime receivers of the pharmaceuticals. In general, concentrations in the biota decreased over time in response to decreasing water concentrations. However, two interesting exceptions to this trend were noted. First, mayfly larvae (primarily grazers) showed peak concentrations (a fourfold increase) of oxazepam, diphenhydramine, and hydroxyzine about 30 days after initial addition of pharmaceuticals. Second, perch (top-predator) showed an increase in concentrations of oxazepam throughout the study period. Our results show that drugs can remain bioavailable for aquatic organism for long time periods (weeks to months) and even re-enter the food web at a later time. As such, for an understanding of accumulation and dispersion of pharmaceuticals in aquatic food webs, detailed ecological knowledge is required.

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### *II. Science of the Total Environment (2018). 615: 608-614.*

#### **No Evidence of Increased Growth or Mortality in Fish Exposed to Oxazepam in Semi-Natural Ecosystems**

**Annelie Lagesson**, Tomas Brodin, Johan Fahlman, Jerker Fick, Micael Jonsson, Josefin Persson, Per Byström, and Jonatan Klaminder

An increasing number of short-term laboratory studies on fish reports behavioral effects from exposure to aquatic contaminants or raised carbon dioxide levels affecting the GABA<sub>A</sub> receptor. However, how such GABAergic behavioral modifications (GBMs) impact populations in more complex natural systems is not known. In this study, we induced GBMs in European perch (*Perca fluviatilis*) via exposure to a GABA agonist (oxazepam) and followed the effects on growth and survival over one summer (70 days) in replicated pond ecosystems. We hypothesized that anticipated GBMs, expressed as anti-anxiety like

behaviors (higher activity and boldness levels), that increase feeding rates in laboratory assays, would; i) increase growth and ii) increase mortality from predation. To test our hypotheses, 480 PIT tagged perch of known individual weights, and 12 predators (northern pike, *Esox lucius*) were evenly distributed in 12 ponds; six control (no oxazepam) and six spiked ( $15.5 \pm 4 \mu\text{g l}^{-1}$  oxazepam [mean  $\pm$  1 S.E.]) ponds. Contrary to our hypotheses, even though perch grew on average 16% more when exposed to oxazepam, we found no significant difference between exposed and control fish in growth (exposed:  $3.9 \pm 1.2 \text{ g}$ , control:  $2.9 \pm 1 \text{ g}$  [mean  $\pm$  1 S.E.], respectively) or mortality (exposed:  $26.5 \pm 1.8 \text{ individuals pond}^{-1}$ , control:  $24.5 \pm 2.6 \text{ individuals pond}^{-1}$ , respectively). In addition, we show that reduced prey capture efficiency in exposed pike may explain the lack of significant differences in predation. Hence, our results suggest that GBMs, which in laboratory studies impact fish behavior, and subsequently also feeding rates, do not seem to generate strong effects on growth and predation-risk in more complex and resource limited natural environments.

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### III. Manuscript (2018).

#### **Behavioural Effects of Temperature, Predation-risk and Anxiolytic Exposure on the European Perch (*Perca fluviatilis*)**

Minna Saaristo, **Annelie Lagesson**, Michael Bertram, Jerker Fick, Jonatan Klaminder, Christopher Johnstone, Bob Wong, and Tomas Brodin

With the ability to resist biodegradation and exert therapeutic effects at low concentrations, emerging contaminants have become environmental stressors for wildlife. One such contaminant is the anxiolytic oxazepam, a psychoactive pharmaceutical which is frequently detected in surface waters globally. Despite the growing interest in understanding how wildlife responds to such contaminants, the synergistic fitness effects of pharmaceuticals and increased variability in temperature remain unclear. Here, by using a multi-stressor approach, we investigated the effects of 7-d oxazepam exposure ( $6.5 \mu\text{g/L}$ ) on anxiety-related behaviours in juvenile European perch (*Perca fluviatilis*). The multi-stressor approach was achieved by exposing perch to oxazepam at either low ( $10^\circ\text{C}$ ) or high ( $18^\circ\text{C}$ ) temperature, with or without a predation cue, generating 8 treatments. Our exposures resulted in a successful uptake of the drug from the water, i.e. oxazepam was measured at muscle tissue concentrations around  $50 \pm 17 \text{ ng/g}$  (mean  $\pm$  SD). We found significant effects on boldness induced by the studied drug: 92.8 % of the fish in the 'oxazepam and predation and high temperature' treatment entered the white background (representing a novel area where exposure to presumed risks are higher) within the first 5 min, compared to 79.3 % of the 'control and predation and high temperature' fish. We also found a significant effect on temperature on the total time freezing (i.e. staying motionless). Specifically, fish in the low temperature treatments (oxazepam, predation and control) froze for longer than fish in the high temperatures, respectively. Our study is the first to show altered anxiety-related behaviours in a native juvenile fish resulting from oxazepam, predation and high temperature. As adaptation to a range of biotic and abiotic pressures is essential to living organisms, our study highlights the need to focus on multiple stressors to improve understanding of how organisms not only survive, but adapt to human-induced environmental change.

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IV. *Environmental Pollution, in revision (2018).*

**Fish on Steroids: Temperature Dependent Effects of 17 $\beta$ -trenbolone on Anti-predator, Risk-taking and Exploratory Behaviors**

**Annelie Lagesson**, Minna Saaristo, Tomas Brodin, Jerker Fick, Jake Martin, Jonatan Klaminder, and Bob Wong

Hormonal growth promoters (HGP), widely used in beef cattle production globally, make their way into the environment as agricultural effluent—with potential impacts on aquatic ecosystems. One HPG of particular concern is 17 $\beta$ -trenbolone, which is persistent in freshwater habitats and can affect the development, morphology and reproductive behaviors of aquatic organisms. Despite this, few studies have investigated impacts of 17 $\beta$ -trenbolone on non-reproductive behaviors linked to growth and survival, like boldness and predator avoidance. None consider the interaction between 17 $\beta$ -trenbolone and other environmental stressors, such as temperature, although environmental challenges confronting animals in the wild seldom, if ever, occur in isolation. Accordingly, this study aimed to test the interactive effects of 17 $\beta$ -trenbolone and temperature on organismal behavior. To do this, eastern mosquitofish (*Gambusia holbrooki*) were subjected to an environmentally-relevant concentration of 17 $\beta$ -trenbolone ( $\leq 5.1 \pm 0.5$  ng/L) or freshwater (i.e. control) for 21 days under one of two temperatures (20 and 30°C), after which the predator escape, boldness and exploration behavior of fish were tested. Predator escape behavior was assayed by subjecting fish to a simulated predator strike, while boldness and exploration were assessed in a separate maze experiment. We found that 17 $\beta$ -trenbolone exposure increased boldness behavior. Interestingly, some behavioral effects of 17 $\beta$ -trenbolone depended on temperature, sex, or both. Specifically, significant effects of 17 $\beta$ -trenbolone on male predator escape behavior were only noted at 30°C, with males becoming less reactive to the simulated threat. Further, in the maze experiment, 17 $\beta$ -trenbolone-exposed fish had a higher activity and explored the maze faster than control fish, but only at 20°C. We conclude that field detected concentrations of 17 $\beta$ -trenbolone can impact ecologically important behaviors of fish, and such effects can be temperature dependent. Such findings underscore the importance of considering the potentially interactive effects of other environmental stressors when investigating behavioral effects of environmental contaminants.

## Abbreviations and glossary

**Additive effect** – an increased effect, greater than the effects produced by either substance alone, each substance contributes to the effect in accordance with its own potency.

**Agonist** – a chemical that binds to a receptor and activates the receptor to produce a biological response.

**Benthic** – occurring at the bottom of a body of water.

**Bioaccumulation** – the uptake and accumulation of a substance in an organism from all sources combined (e.g. water and food). Bioaccumulation occurs, within a trophic level, when an organism absorbs a substance at a rate faster than that at which the substance is lost by metabolism and excretion.

**Bioavailability** – the amount of a compound that is accessible to an organism for uptake (absorption) across its cellular membrane.

**Bioconcentration** – the uptake and accumulation of a substance from water alone.

**Biomagnification** – the increasing concentration of a substance in the tissue of organisms at successively higher levels in a food chain, i.e. occurs across trophic levels.

**Biomarker** – a molecular or cellular expression indicating a biological response to an external stressor.

**Contamination** – the act of contaminating, or of making something impure or unsuitable, but with unknown effects (in contrast to pollutants where negative effects are implicit).

**Ecotoxicology** – an interdisciplinary field that uses knowledge and techniques from the fields of ecology and toxicology to study the effects of toxic chemicals on organisms, especially at the population, community or ecosystem level.

**EDC** – Endocrine-disrupting chemical.

**Emerging contaminants** – compounds that are not commonly monitored but have the potential to enter the environment and cause adverse ecological and human health effects.

**Endocrine-disrupting chemical** – a substance interfering with the body's endocrine (hormone) system that may produce e.g. adverse developmental, reproductive, neurological, and immune effects.

**Endocrine system** – a network of glands that produce, store, and secrete hormones. The system controls the bodily metabolic activity and serve as chemical messengers that allow cells to communicate with one another.

**Excretion** – the process by which metabolic waste is eliminated from an organism. In vertebrates this is primarily carried out by the lungs, kidneys and skin.

**Food chain** – a linear sequence of organisms through which nutrients and energy pass as one organism eats another.

**Food web** – many interconnected food chains. A more realistic representation of consumption relationships in ecosystems.

**GABA** – *gamma*-Aminobutyric acid, the chief inhibitory neurotransmitter in the mammalian central nervous system.

**GABA<sub>A</sub>-receptor** – a class of receptors that respond to the neurotransmitter GABA.

**GABAergic** – A substance that produces its effects via interactions with the GABA system, by stimulating or blocking neurotransmission.

**GBM** – GABAergic behavioural modification.

***In situ*** – in the original place instead of being moved to another place.

**Metabolism** – the sum of the physical and chemical processes in an organism by which its material substance is produced, maintained, and destroyed, and by which energy is made available.

**Pesticide** – a substance that is meant to kill plant, fungal, or animal pests.

**PIT-Tag** – Passive integrated transponder (a small radio transponder that contains a specific code).

**Pollution** – the discharge of material, in any physical state, that is dangerous to the environment or human health.

**Synergetic effect** – an exaggerated, diminished or new effect that neither substance produces on its own.

**Toxicokinetics** – the study of absorption, distribution, metabolism, and excretion of toxicants i.e. how a substance gets into the body and what happens to it in the body, in relation to time.

**Trophic level** – the position an organism occupies in a food chain.

**Trophic transfer** – the transfer of contaminants between trophic levels via ingestion.

**Xenobiotic** – a substance, typically a synthetic chemical, that is foreign to the body or to an ecological system.

# Introduction

## 1. Pharmaceuticals in the environment

In the 1960's, Rachel Carson challenged the idea that humans could master nature by using chemicals. With the publication of her book *Silent Spring*, in 1962, she warned of the devastating consequences from the ongoing carefree usage of pesticides and subsequently boosted the environmental movement and the onset of ecotoxicological research. Today, the subject is more relevant than ever with an estimated 25-fold increase of chemicals sold on the global market between 1970 and 2013 (from \$171 billion to \$4.1 trillion), and a total global chemical production increase of 54% between 2000 and 2010. Further, the production increase is expected to continue (Kemf 2013) and few parts of the world are being spared from chemical contamination (Travis and Hester 1991, Hong et al. 1994, Pozo et al. 2007).

One group of contaminants of concern is pharmaceuticals. As the human population is growing and ageing, so are the needs for medical and veterinary healthcare. Today, more than 4000 pharmaceuticals are being used to medicate humans and animals and to enhance livestock production around the world. With an increased consumption, comes of course increased excretion and discharge (Boxall et al. 2012, Arnold et al. 2014).

Pharmaceuticals often enter the environment still biochemically active through pathways derived from, for example, sewage treatment plant effluents, agricultural run-offs, aquaculture, landfill run-offs and manufacturing industry effluents (Arnold et al. 2014). Today, more than 600 pharmaceutical substances have been detected in surface waters worldwide (aus der Beek et al. 2016). Once in the environment, abiotic and biotic transformation processes may over time degrade the substances to products with less biological impacts or, sometimes, transform it into products with similar or different biological impacts (La Farre et al. 2008). Furthermore, a mixture of drugs can have both additive, antagonistic and synergistic effects (Backhaus 2014, Vasquez et al. 2014). Pharmaceuticals will only cause biological effects however, if it enters the biota and thereafter arrive at a target site or interfere with other biological processes. This uptake, and biological effects, depends on the bioavailability of the substance (Semple et al. 2004) and the toxicokinetic processes within the organism (Stehly et al. 1999). The probability of drugs affecting exposed wildlife seems likely since pharmaceuticals are highly bioactive and receptors and the endocrine system are highly conserved across vertebrates (Campbell et al. 2004, Gunnarsson et al. 2008, Brown et al. 2014). As aquatic organisms do not only get exposed to pharmaceuticals through the water, but also through their diet, it means that many pharmaceuticals also have the potential to bioaccumulate in food chains. However, relatively little is known to what extent drugs bioaccumulate in aquatic food webs.

The toxicity of pharmaceuticals on non-human organisms have been studied in laboratories for long, but with rather high exposure concentrations, often using physiological changes or mortality as endpoints (e.g. Organisation for Economic Co-operation and Development protocols). During the last couple of decades, the interest for sub-lethal effects and behavioural studies has increased (Zala and Penn 2004, Hellou 2011, Brodin et al. 2014). As behaviour is a physical indicator of several complex internal processes, it reflects the health of an organism (Wong and Candolin 2015). Therefore, it is not surprising that behavioural changes have been reported to occur at much lower concentrations compared to those affecting reproduction or development (Melvin and Wilson 2013) or those causing mortality (Gerhardt 2007) and are equally sensitive as established biomarker responses (Söffker and Tyler 2012). However, to what extent exposure-induced behavioural changes affect the fitness of organisms living in natural ecosystems are not well known.

## **2. Animal behaviour**

Understanding the potential impacts of pharmaceuticals on ecologically relevant behaviours, such as boldness, predator escape and exploration, is important because both single behaviours and suites of correlated behaviours are known to be directly linked to fitness correlates such as reproduction, growth and survival (Brodin and Johansson 2004, Sih et al. 2004, Smith and Blumstein 2008, Wilson et al. 2010). Behaviours can therefore have important ecologically and evolutionary consequences (Sih et al. 2012, Saaristo et al. 2018).

In order to maximize fitness, individuals continuously react to their environment. Many studies have shown that, for example, individuals that come from an area with one level of predation-pressure, on average differ in boldness compared to individuals (of the same species) from areas with higher or lower predation-risk (Brown et al. 2005, Magnhagen and Borcherting 2008, Harris et al. 2010). What is even more interesting, however, is the within population variation of behaviours. Individuals within a population show consistent (largely maintained) variations in behaviour over time and contexts, i.e. there are different behavioural types, or personalities, in similarity with humans (Wilson et al. 1994, Gosling 2001, Ward et al. 2004, Reale et al. 2007). Within a population there are individuals that express behaviour ranging from, e.g., very shy to very bold. That is, behaviour variables are not bimodal, but a continuum where shy and bold is two extremes of personalities (Wilson et al. 1994, Reale et al. 2007). This means that individuals from the same population behave more or less “correct” according to e.g. shifting environmental conditions such as predation pressure (Réale and Festa-Bianchet 2003) and food availability (Dingemanse et al. 2004). That is, a constrained behavioural plasticity results in negative fitness consequences in certain contexts (Wilson et al. 1993, Sih et al. 2003, Brodin and Johansson 2004, Dingemanse et al. 2004, Bell 2005, Wilson and Stevens 2005, Smith and Blumstein 2010, Pearish et al. 2013). For example, a shy

individual may be too cautious when searching for food, resulting in slow food acquisition and hence slow growth, whereas a bold individual may grow fast when predation risk is low, but may take too many risks and being eaten when predation risk is high (Biro et al. 2006). Further, individuals that are for example, bolder in risky situations, also tend to be more active and more aggressive towards conspecifics (Huntingford 1976, Bell and Sih 2007, Dingemanse et al. 2007). Such positive correlations between two or more personality traits are called behavioural syndromes (Sih et al. 2004). Given the ecological and evolutionary importance of behaviours, it seems highly relevant to study the effects that pharmaceuticals might evoke on them.

### 3. Behaviour modifying drugs

The risk of pharmaceuticals affecting the environment and wildlife was not generally considered until the 1990's. In mid-1990's, researchers found a cocktail of chemicals, including synthetic estrogens found in contraceptive pills, to be the cause of large numbers of intersex individuals (feminized males) of fish, downstream sewage treatment plants in the UK (Purdom et al. 1994, Harries et al. 1997, Jobling et al. 1998). A later full-lake field-study, concluded that exposure to a synthetic estrogen (17 $\alpha$ -ethinylestradiol) could collapse entire fish populations (Kidd et al. 2007). Another major ecological effect generated by pharmaceutical exposure was discovered in the early 2000's when researchers found an anti-inflammatory drug (diclofenac) to be the cause of a devastating 95-99% decline of several vulture species in Asia (Pain et al. 2003).

Today, a plethora of studies have demonstrated effects of pharmaceuticals found in the environment on a wide range of organisms (Söffker and Tyler 2012, Brodin et al. 2014, Sehonova et al. 2018), and many of them have reported effects on behaviours. For example, a common antidepressant (fluoxetine) affects diurnal feeding habits (Bean et al. 2014) and courtship behaviours (Whitlock et al. 2018) of starlings (*Sturnus vulgaris*). Many birds get exposed to pharmaceuticals when foraging invertebrates on fields fertilized with sewage sludge or at wastewater treatment plants. Antihistamines (hydroxyzine and fexofenadine) has been shown to negatively affect fleeing response in damselfly larvae (Jonsson et al. 2014) and the main component of classical contraceptives (17 $\alpha$ -ethinylestradiol), can disrupt amphibian mating behaviour (Hoffmann and Kloas 2012).

The effects and accumulation of pharmaceuticals in aquatic wildlife, such as fish, are of particular interest because of their inevitable exposure to contaminated water, direct uptake via gills or skin, and consumption of other exposed organisms. Two pharmaceutical contaminants of interest that has been shown to reach aquatic environments and to affect fish, are a commonly prescribed anxiolytic pharmaceutical named oxazepam, and a growth hormone widely used in agriculture, called trenbolone. These two compounds are the main substances studied in this thesis (see Box 1 for details).

### Box 1. Drugs of central importance for this thesis

**Oxazepam** is a pharmaceutical used to treat, foremost, anxiety and belongs to a larger group of anxiolytics referred to as benzodiazepines (Wishart et al. 2006). Oxazepam is the most commonly prescribed benzodiazepine in Sweden and is also a metabolite of another common benzodiazepine named diazepam (i.e. Valium). Benzodiazepines are one of the most persistent psychoactive substances in the environment (Loffler et al. 2005), and field experiments indicate that the dissipation rate can be several months in deep waters and at low temperatures, or many decades in lake sediments (Klaminder et al. 2015). Benzodiazepines are GABA (the main inhibitory neurotransmitter in the brain) agonists reducing anxiety via a chain of events as the drugs target the GABA<sub>A</sub>-receptor and depolarize the neuron leading to a calming effect as it reduces the communication between the neurons (Argyropoulos and Nutt 1999). Oxazepam has been found to alter important ecological behaviours in fish, e.g., boldness, activity, sociality, feeding rate (Brodin et al. 2013, Brodin et al. 2017), and even migratory behaviour (Hellstrom et al. 2016).

**Trenbolone acetate** is the most common androgen in hormonal growth promoters administered to beef cattle in many beef-producing countries (Hunter 2010). Although growth promoters are banned in the European Union, due to human health concerns (Johnson and Hanrahan 2010), they are heavily used in other parts of the world, including Australia, New Zealand, Japan, the United States, Canada, Mexico, Chile, and South Africa (Hunter 2010, Kolodziej et al. 2013). Trenbolone acetate is a synthetic androgenic anabolic steroid used to promote growth by specifically targeting the endocrine system (i.e. an endocrine disrupting chemical; EDC), and is known to bind to androgen receptors with 15-50 times the affinity of testosterone (Neumann 1976, Yarrow et al. 2010, Kolodziej et al. 2013). Its metabolite, **17 $\beta$ -trenbolone**, is highly stable in animal waste (~260 days) (Schiffer et al. 2001) and enters aquatic environments through run-off from feedlots or direct discharge of livestock urine and faeces (Cavallin et al. 2014). 17 $\beta$ -trenbolone has well documented effects on physiology and morphology in fish, for example, abnormal growth in males (Bertram et al. 2015) and complete sex reversal in females (Galvez et al. 1996, Ankley et al. 2003, Orlando et al. 2004, Sone et al. 2005, Seki et al. 2006, Örn et al. 2006, Larsen and Baatrup 2010, Morthorst et al. 2010, Baumann et al. 2014, Li et al. 2015). Although studies on behaviour are much more sparse, it is clear that it can affect both reproductive (Saaristo et al. 2013, Bertram et al. 2015, Tomkins et al. 2017, Tomkins et al. 2018) and non-reproductive behaviours (Heintz et al. 2015, Bertram et al. 2018) in fish at low levels of exposure (ng/l).

#### **4. Current knowledge gaps and the aim of this thesis**

Risk assessment tests in laboratory settings usually expose organisms to chemicals under constant and favourable experimental conditions. Many experiments are also single species studies, often using model organisms bred for many generations in laboratory environments, where the natural behavioural repertoire may have been lost. In these simplified model systems, uptake only from water are usually assessed and effects are measured on an individual level. In the wild, countless of biotic and abiotic stressors can influence and alter, e.g., behaviour of organisms and as such, these stressors have the potential to interact with contaminants. Surprisingly few studies consider the impacts of pharmaceuticals in combination with other environmental stressors, e.g. in more complex environments, or look at population or even ecosystem effects. This means that uptake and effects are often greatly oversimplified and/or underestimated.

The aim of this thesis was to increase our understanding about the fate and effects of behavioural modifying drugs in complex aquatic ecosystems. Study I and II were conducted in semi-natural aquatic ecosystems (i.e., ponds) with natural fluctuations in abiotic and biotic factors. In study I, I looked at the fate of pharmaceuticals in a natural aquatic food web. In study II, I investigated population effects of a pharmaceutical known to alter fish behaviours. Study III and IV were conducted in the laboratory, but in addition to pharmaceutical exposure, both studies also included one (study IV) or two (study III) additional stressors (specifically, temperature and predator cues).

## Objectives

The main objective of the first study (I) was to determine the fate of five pharmaceuticals in an aquatic food web, whereas study II-IV, assessed the effects that pharmaceuticals evoke on fish behaviour. The pharmaceutical effects on behaviour were studied in environments with higher complexity than most laboratory studies. More specifically, my thesis addressed the following research questions:

- I. To what extent are five commonly prescribed pharmaceuticals (diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine) taken up by biota at different trophic levels in an aquatic food web?
- II. How do induced behavioural changes, caused by exposure to an anxiolytic drug (oxazepam), affect growth and overall survival of European perch (*Perca fluviatilis*) in a semi-natural environment?
- III. How are anxiety-related behaviours in European perch affected by the exposure to an anxiolytic drug (oxazepam) at two different temperatures (10 and 18 °C) and in interaction with predator cues?
- IV. How does exposure to an environmental relevant concentration of the growth promoter 17 $\beta$ -trenbolone, at two different temperatures (20 and 30°C), affect boldness, exploration, and predator escape in the eastern mosquitofish (*Gambusia holbrooki*)?

# Material and Methods

## 1. The ponds

Both study I and II were conducted at Röbbäck (63°48.572'N 20°14.584'E), Umeå, Sweden. The pond used for study I is approximately 40 × 10 m, with a mean depth of 1.3 m. It contained natural populations of aquatic invertebrates and perch had been introduced one year before the study started. On the starting day of the experiment, the pond was spiked with trimethoprim (antibiotic), diclofenac (anti-inflammatory), oxazepam (anxiolytic), hydroxyzine (antihistamine), and diphenhydramine (antihistamine) by dispensing a stock solution evenly over the pond with a pipette. Water was sampled weekly and aquatic organisms were sampled on a daily to weekly basis, over a total of 66 days, to analyse the uptake of the studied substances in the food web. In short, the sampled organisms were: **European perch** (*P. fluviatilis*), that feed on fish, zooplankton, and benthic macroinvertebrates; **damselfly larvae** (Zygoptera: Coenagrion), that prey on zooplankton and benthic macroinvertebrates; **mayfly larvae** (Ephemeroptera: Cloeon) that mainly graze on algae and detritus; **waterlouse** (*Asellus aquaticus*), which are grazing omnivores feeding on bacteria, fungi, detritus, macrophyte tissue, and algae, and; **ramshorn snails** (Planorbidae) that are grazers and detritivores (paper I: Fig. 1).

For study II, we used a replicated ( $n = 12$ ) pond system where two larger ponds (36 × 10 m) were divided into eight smaller ponds each (4.5 × 10 m) with a maximum water depth of 90 cm and slight inflow of municipality groundwater to each smaller pond (Fig. 1). The four outermost ponds (at the ends of each large pond) were excluded from the experiment. The ponds contained natural populations of aquatic invertebrates. On the start of the experiment, 40 PIT-tagged perch, with known weight, were introduced to each pond. Seven days later, one PIT-tagged pike (*Esox Lucius*), with known weight, was introduced to each pond and oxazepam was added to six of the ponds whereas the other six were left as untreated controls. Degradation losses were compensated for by adding additional oxazepam to the ponds every fourth day. On day 70, surviving fish was collected with a beach sein net, identified by PIT-tag scanning and then weighed before they were euthanized.



**Figure 1.** The replicated ponds at R  b  ck, Ume  , Sweden, used to assess the effects of oxazepam exposure on perch growth and mortality in a semi-natural environment (study II).

## 2. Animal collection and housing

For study I, macro invertebrates were collected by randomly hand netting in the littoral zone, and fish were caught using umbrella traps. For study II and III, perch were caught using a beach sein net with a mesh size of 5 mm. This was dragged slowly through the water column along the bottom by two persons, one on each end. For study IV, fish were caught using dip-nets.

In study II and III, fish were held in a large holding tank (85 × 150 × 150 cm) with oxygenated, aged tap water (~13°C) and a tap water flow through, up until the start of the experiment. The light/dark regime was kept at 12/12 h and fish were fed with thawed red Chironomidae larvae daily during the acclimation period. In study IV, fish were kept in same sex glass holding tanks (80 × 45 × 45 cm), with oxygenated, aged tap water (20–23°C) and a light/dark regime of 12/12 h. Fish were fed daily with commercial fish food.

During the exposure period in study III, fish were held in individual exposure containers (14 × 19 × 28 cm), with 4 L of oxygenated aged tap water at the corresponding treatment temperature (10 or 18°C). They were fed thawed red Chironomidae larvae every second day. The exposure system in study IV comprised of 16 tanks (60 × 30 × 30 cm), with approximately 30 same sex individuals in each. Each tank was supplied with either carbon-filtered freshwater (control treatments) or 17  -trenbolone spiked water from a stock solution, and they were heated to the corresponding temperature treatment (20 or 30°C). The light/dark regime was kept at 12/12 hours and fish were fed daily with commercial fish food.

### 3. Pit-tagging

In study II, the fish were PIT-tagged (Biomark PIT-tag: 8.4 mm 134.2 kHz ISO FDX-B) making it possible to follow individual growth and survival in the replicated populations. The fish were anesthetized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate) after which a small incision was made with a scalpel and the PIT-tag was inserted into the abdominal cavity (Fig. 2). The total time for tagging and handling each individual was ~50 sec, after which the fish were allowed to recover for 15 days before being released into the ponds. Noteworthy, only one fish died during the 15 day-recovery period and fish also started to feed quickly after the PIT-tagging, indicating a successful PIT-tagging procedure.



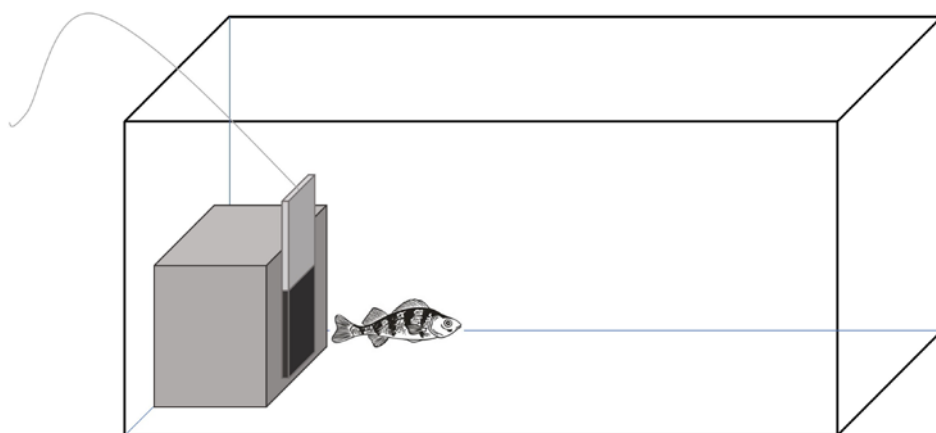
**Figure 2.** A PIT-tag being inserted into the abdominal cavity of a perch (*P. fluviatilis*) (study II).

### 4. Behavioural experiments

Given the importance of behaviour, it seems crucial for studies to assess the important behavioural traits in a correct manner. The terminology in behavioural studies can be confusing and often different names are used for measuring the same trait. In this thesis I have done behavioural experiments to assess boldness/shyness (study III, IV, and background data for study II), general activity (study IV, and background data for study II), exploration (study IV), and predator avoidance (study IV). Below follow the definitions used for the different behaviours and the methods used for measuring the corresponding traits.

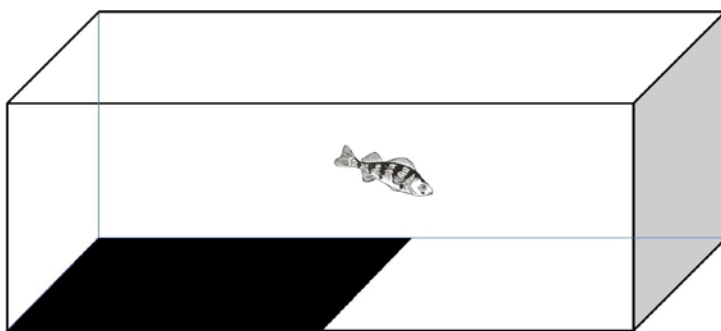
**Boldness-shyness:** how an individual reacts to a risky situation (Reale et al. 2007). This can be tested in different ways. In study IV, boldness was measured as latency to leave a

shelter (sec), a method commonly found in the literature (Fraser et al. 2001, Brown and Braithwaite 2004, Chapman et al. 2011). In study II, the assumption that perch increase their risk-taking behavior (i.e. boldness) when exposed to oxazepam, was based on previous results from our research group where the same method was used to assess boldness (Brodin et al. 2013). More specifically, the test individual was placed in an enclosed opaque refuge (a small box), after a five min acclimation period a door was remotely opened, and the fish was allowed to leave the shelter and swim into an unfamiliar environment (Fig. 3; for study IV, this was measured in the same arena as the assessment of exploration, Fig. 5). The test individual was given 20 min in total to leave the refuge from the moment the door was opened. This gave each fish a time score, where a low score indicates a bold individual and a high score a shy individual.



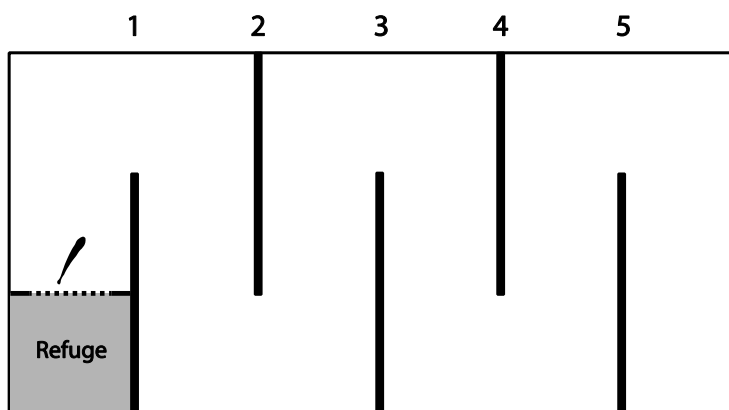
**Figure 3.** Boldness set-up. The test individual was placed in a small refuge with a remotely removable door that was opened after five min of acclimation, allowing the fish to enter the novel environment. Each test individual had 20 min, from the moment the door was opened, to leave the refuge (study II (background data) and III).

In study III, we used the scototaxis test to assess boldness, which is a well-documented protocol used to measure an individual's anxiety-like behaviours (Maximino et al. 2010). More specifically, an individual's light/dark preference was measured, by placing the test individual in the centre of a half-white/half-black bottomed aquaria (Fig. 4). After five min of acclimation, the number of entries between white and black and the duration in each compartment (white or black) was recorded for 25 min. Several fish species have been shown to perceive the dark side as safer (Maximino et al. 2010), and hence, an increase in usage of the white compartment was interpreted as increased boldness and an increase in usage of the dark compartment as reduced boldness.



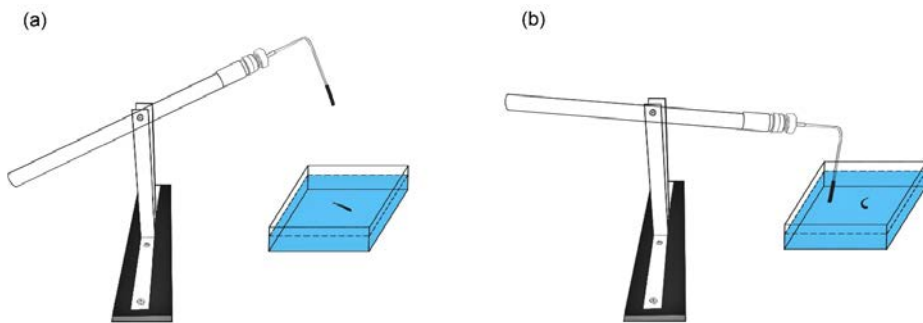
**Figure 4.** Scototaxis set-up. The fish was placed in the centre of the arena and then allowed to swim freely for 30 min. The number of entries and duration in each compartment (white or black) was recorded (study III).

**Exploration-avoidance:** how an individual reacts to and investigate new situations, such as new food, new habitats or novel objects (Reale et al. 2007). We chose to measure this as how fast an individual explored a novel environment, i.e., swam through a maze (sec). This was measured in the same arena and at the same time as boldness. More specifically, when (if) leaving the small shelter used to quantify boldness, the individual entered a maze, i.e. an aquarium with six “corridors”, defined by opaque internal walls, extending two-thirds of the width of the aquarium (Fig. 5). We quantified the time taken for the fish to reach each maze corridor, i.e., a shorter time indicating a more exploratory individual.

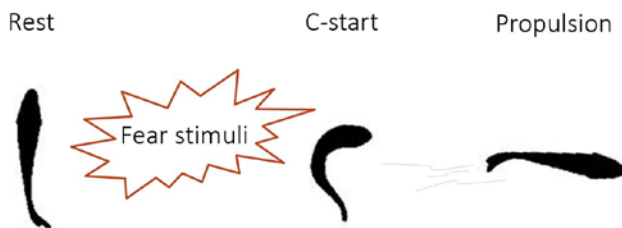


**Figure 5.** Exploration (and boldness) set-up. The test individual was placed in a small refuge (grey) with a remotely removable door (dotted line) that was opened after five min of acclimation, allowing the fish to enter the maze. The maze had six corridors defined by five internal walls (denoted 1-5). Each test individual was given 20 min to leave the refuge and explore the maze (figure from paper IV).

**Predator escaping:** how an individual reacts to and escape a predator strike. We measured this predator escape response by subjecting the test individual to a simulated predator strike, i.e., a metal probe was remotely dropped into the water in proximity to the fish to provoke an escape response (Fig. 6). Fast-starts, including the C-start, are burst accelerations initiated by a fear stimuli (e.g. a predator strike) used by many fish species to evade predators (Domenici and Blake 1997). The C-start response is characterized by three distinct stages: rest (just before the fear stimulus), C-bend (the individual turns away from the stimulus bending into a C-shape) and propulsion (the individual executes a burst acceleration swimming away from the stimulus; Fig.7; Hale et al. 2002). We studied if the fish did the C-bend response or not, and if yes, we calculated the speed of the response (both C-start and propulsion speed).



**Figure 6.** Predator escape set-up. The metal probe was remotely dropped within three cm of the fish to provoke an escape response. Fish were allowed five min to acclimate before the metal probe (a) was dropped (b) to stimulate a predator strike. Each fish was filmed for one min following the drop and during this time general activity was measured (figure from paper IV).



**Figure 7.** The C-start predator escape response. Rest; before a fear stimulus, C-start; the individual turns away from the stimulus bending into a C-shape, and Propulsion; the individual executes a burst acceleration swimming away from the fear stimulus (study IV).

**Activity:** an individual's general activity level, usually, time in motion. For study **IV**, activity was measured as the total distance swam (cm) during the one min following the simulated predator strike in the predator escape experiment (Fig. 6). A longer distance swam was interpreted as a more active individual. Our predictions for study **II** were based on previous results from our research group showing altered activity levels in perch exposed to oxazepam (Brodin et al. 2013). In said study, activity was measured (simultaneously as sociality) as time spent actively swimming (during ten min).

## 5. Video analysing

All behavioural trials were video recorded (study **III**: SONY Handycam HDR-PJ50VE, study **IV**: Canon Powershot S110) from above allowing for quantification of the studied behaviours after completed experiments. Videos from study **III**, were analysed manually by quantifying the latency of fish to first enter the white and/or black compartment respectively, the total time fish spent in either compartment, and duration and frequencies of freezing events. Videos from study **IV**, were analysed both manually (boldness: time taken to leave refuge and exploration: time taken to reach each maze corridor) and by using a point of mass tracking software (Tracker 4.97; Open Source Physics, USA) to analyse fish movements in the predator escape experiment. C-start speed (cm/sec) was calculated as the distance travelled between picture frame two and three, and propulsion speed (cm/sec) was calculated as the distance travelled between picture frame three and four.

## 6. Statistics

We used a **one-way analysis of variance (ANOVA)** to test for differences among species in tissue concentrations (bioaccumulation; study **I**); if oxazepam induced behavioural changes resulted in higher growth rates in perch populations; and if mortality was higher in the oxazepam exposed perch populations (study **II**). A **two-way ANOVA** was used to test for differences in the mean tissue:water ratio (bioaccumulation factor) between biota and between substances (study **I**); effects of oxazepam treatment on zoobenthos biomass and pike weight; and oxazepam exposure effects on pike attack efficiency (study **II**). A **Pearson's rank correlation analysis** was used to test if there were interspecific differences in recovery rates (study **I**); and relationship between perch weight gain and zoobenthos biomass (study **II**).

In study **III**, we used a **principal component analysis (PCA)** followed by a **three-way ANOVA** to examine treatment effects on anxiety-related and boldness behaviours, using a behavioural syndrome approach. The same method was used to examine treatment effects on morphology and physiology. A **Kruskal-Wallis** and **post hoc Dunn's tests** (with Bonferroni corrections) were used to compare time of first freezing event among

treatments. A **survival regression analysis**, using weibull distribution, was used to compare the time it took for fish to cross into the white and/or black background for the first time.

In study **IV**, we used a **chi-square test** to compare the number of performed C-starts among treatments. **Generalized linear models (GLM)** with gamma distribution and log-link (positive skewed data) were used to test for differences in C-start and propulsion speed in mosquitofish between different treatments; effects of different treatments on boldness (i.e. time to exit the refuge); and treatment effects on exploration (time to reach the last maze corridor). A **general linear model** was used to test for differences in activity levels (i.e. total distance swam) in mosquitofish across treatments; if treatments altered exploration behaviour (i.e. time to reach first maze corridor); and differences in  $^{17}\beta$ -trenbolone uptake (BCF).

The statistical analyses done for study **I**, **II** and **IV** were conducted using the statistical software IBM SPSS Statistics 24 (IBM Corp. 2016). The statistical analyses for study **III**, were conducted using R (version 3.5.0 (2018-04-23) R Core Team (2018)) and RStudio (version 1.0.153, RStudio Team (2016)).

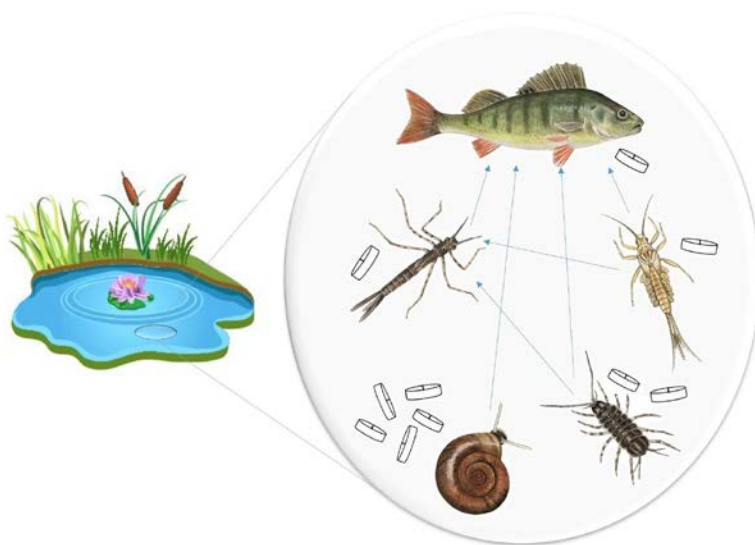
## Results and discussion

### 1. Bioaccumulation of pharmaceuticals in an aquatic food webs

Study **I** show that uptake of pharmaceuticals are species and substance specific. Two of the substances, trimethoprim and diclofenac, could not be detected in any of the sampled biota during the study period (likely due to their hydrophilic properties and/or sedimentation and sorption processes). Oxazepam, hydroxyzine and diphenhydramine were detected in all sampled biota during the experiment. Concentrations of both hydroxyzine and diphenhydramine in the biota decreased over time in response to decreasing water concentrations (paper **I**: fig 2). However, one of the most interesting findings, was that the bioaccumulation factor increased in perch over the study period (paper **I**: fig. 4a). Hence, the bioavailability of the drug or exposure via the diet seemed to increase over time. This may indicate that there was a trophic transfer of oxazepam in the studied food web that allowed tissue concentrations to remain unchanged despite decreasing water concentrations of the drug. Although a previous laboratory study succeeded in demonstrating a trophic transfer of oxazepam to one top consumer (dragonfly larvae), they could not find a significant trophic transfer to fish (perch) (Heynen et al. 2016). This finding illustrates the difficulties in comparing exposure via natural diets to simplified diets in artificial settings. Our result also shows that previous laboratory studies assessing uptake of oxazepam in fish, when neglecting dietary uptake (Brodin et al. 2013, Klaminder et al. 2014, Heynen et al. 2016), have underestimated the 'true' uptake of oxazepam in aquatic ecosystems by a factor ranging between 3-10 (paper **I**: fig.

4a). These findings highlight the difficulties in mimicking uptake in artificial settings. As such, for some pharmaceutical, it is reasonable to think that uptake in top consumers, such as perch, via lower trophic levels eventually could lead to critical levels that can cause behavioural changes, even if water concentrations are lower than those critical levels. Such a considerable trophic transfer has previously been shown for terrestrial animals (Bean et al. 2014). The potential for oxazepam to bioaccumulate in fish gets further support from a recent study in which wild carp (*Cyprinus carpio*) had the highest BAF (927 L/kg) of  $\Sigma$ Diazepam (diazepam and oxazepam) amongst a broad array of personal care product chemicals measured in an urban wetland (Muir et al. 2017).

In general, the benthic species (i.e. the ramshorn snail and water louse) had the highest bioaccumulation factors of the studied pharmaceuticals and perch had the lowest (fig. 7; paper I: fig. 3). This pattern indicates that, at least during the time frame of this study, none of the studied drugs became enriched higher up in the food-web. We argue that differences in physiology and ecology, such as diet and habitat use, play a major role explaining differences in uptake amongst the sampled organisms. The ramshorn snail and waterlouse have a similar diet, (i.e. detritivores and herbivores), they feed to a large extent on, for example, periphyton, which have been shown to readily take up pharmaceuticals (Rodríguez-Mozaz et al. 2015). Further, these grazing taxa use the sediment surface as habitat, and an uptake of pharmaceuticals released from the sediment could also explain their generally higher BAFs. However, the rapid (within days) uptake and recovery rate in response to concentrations in surrounding water clearly shows that uptake from water also is an important route for pharmaceuticals into these organisms.



**Figure 7.** Conceptual illustration of the results from study I. Differences in calculated bioaccumulation factors (BAF) of pharmaceuticals in the studied biota are shown by the number of pills (in grey). The number of pills illustrates the general trend in measured BAFs in the food web, i.e. higher BAFs at the bottom (snail) and lower BAFs at the top (fish) of the food web (modified from paper I). Diclofenac and trimethoprim were not detected in any of the sampled organisms, whereas oxazepam, diphenhydramine and hydroxyzine were detected in all organisms.

## 2. Figuring out the effects of oxazepam: “it’s complicated”

Based on previous results from laboratory studies, showing altered behaviour in perch exposed to oxazepam in simplified laboratory environments (Brodin et al. 2013, Brodin et al. 2014) as well as during the first days when released into a lake (Klaminder et al. 2016), I hypothesized in study II that we would see effects of oxazepam on perch growth and survival in a natural setting. The rationale for this hypothesis was that perch become more risk-taking (bolder), more active and have a higher feeding rate when exposed to oxazepam. As such, they are predicted to be more exposed to predators but also grow faster due to higher food encountering and feeding rate. However, we did not find effects of oxazepam exposure on neither mortality nor growth (paper II; fig. 1). Noticeably, oxazepam exposed perch had grown on average 16 % more than control fish, but this difference was not significant. If the data would have been analysed on an individual level (i.e. exposed vs. control perch) and not on pond level (a statistical approach sometimes used in OECD ecotoxicological tests) the difference would have been significant. However, as this by definition is pseudoreplication, we rejected our hypothesis that exposed perch would grow more. Our results indicate that, for at least these endpoints and in this system, other environmental factors affected growth and mortality more than

oxazepam. As perch growth in most, if not all, freshwater-systems is limited by resource availability, this could potentially have hampered any positive effect of increased foraging efficiency. That is, resource limitation could have led to a lower increase in growth-rate than expected based on the laboratory results. It is possible that in a more resource dense system, the benefits would have been higher. In an additional laboratory study, we found that exposed pike experienced reduced predation efficiency (paper II: fig. S1), despite the prey increasing their vulnerability through increased boldness and activity. Thus, this could explain the non-significant result in mortality, i.e. negative effects on the predators may have counteracted the cost of increased predation-risk of the prey. As discussed below (study III and study IV), there might also have been interaction effects between induced behaviour modifications and abiotic factors, such as water temperature. This may have contributed to the mismatch between my laboratory-based predictions and the results from a natural environment.

In study III, we returned to the controlled conditions in the laboratory, but added two additional stressors (temperature and predator cues) to the oxazepam treatment, in an attempt to further examine the mechanism at work in systems with higher complexity than traditional ecotoxicological experiments. In line with previous studies, we found that oxazepam induced an anti-anxiety like behaviour. More specifically, during the first five min of the trials, fish exposed to oxazepam showed increased boldness, i.e. entered the white compartment in the scototaxis trial faster than control fish (paper III: fig. 3). Hence, we were able to demonstrate anti-anxiety effects of oxazepam using a new independent behavioural assay. As such, these results together with previous findings (Brodin et al. 2013) suggest that oxazepam induce, at least, a short-term (minute-scale) anxiolytic effect on perch behaviour. However, this study also highlighted an important factor to consider when extrapolating these results to natural ecosystems: temperature has also profound effects on anxiety-related behaviours (such as boldness) in perch (paper III: fig. 2; 3) and should therefore be considered in addition to effects induced by oxazepam. Fish in the lower temperature treatment froze (stayed motionless) for longer than fish in the higher temperature treatment. Moreover, fish in the warmer treatment entered the white compartment faster (i.e. higher boldness) than fish in the lower temperature treatment. It is well-known that, for example, activity is temperature dependent due to decreasing e.g. metabolism, heart rate and respiration at cooler temperatures (Farrell 1997, Pörtner 2002, Jensen et al. 2017), which could explain longer times of freezing in the colder treatment. Further, increased temperature has previously also been shown to increase boldness (Biro et al. 2010).

### 3. Temperature dependent effects of 17 $\beta$ -trenbolone on non-reproductive fish behaviours

In study IV, we show that very low ( $\leq 5$  ng/l), environmentally relevant, concentrations of the growth hormone 17 $\beta$ -trenbolone, can alter ecologically important behaviours in fish. This was in line with our hypothesis, based on previous studies assessing 17 $\beta$ -trenbolone effects on non-reproductive fish behaviours (Heintz et al. 2015, Bertram et al. 2018). The most interesting finding however, was that the behavioural effects were temperature dependent, a discovery not previously shown. More specifically, 17 $\beta$ -trenbolone exposed mosquitofish (regardless of temperature and sex) became bolder (i.e. time to leave refuge) compared to control fish (paper IV: fig. 2). Further, 17 $\beta$ -trenbolone exposure altered the predator escape response in mosquitofish negatively, but only at the higher temperature (30°C), with significantly fewer individuals performing C-starts when subjected to a simulated predator strike, compared to control fish at 30°C (paper IV: fig. S3). This discrepancy might be linked to higher metabolic demands in fish at the higher temperature, resulting in a higher motivation for fish to take risks in search for food (Biro et al. 2007). Exposed males also performed slower escape responses compared to control males at 30°C, an effect not seen in females. However, in 20°C, exposed fish (regardless of sex) explored the maze (i.e. reached last maze corridor) faster than control fish, an effect not seen at the higher (30°C) temperature (paper IV: Table 1; fig. 1). One possible explanation is that the temperature optimum for mosquitofish is closer to 30°C than 20°C (Pyke 2005), meaning that fish at 20°C could have been under a higher physiological stress, resulting in a higher food search motivation (i.e. a higher boldness and exploratory behavior) due to higher energy demands (Killen et al. 2011).

The predator escape behaviour examined can be described as a reflex response to an immediate perceived risk (i.e. the predator strike), whereas the maze experiment represents a situation that might be dangerous, but not containing any direct information about the risk-level. Consequently, we suggest that the former measured an acute risk-response, while the latter was measuring the chronic level of boldness behaviour of the fish. Seemingly, the effects of 17 $\beta$ -trenbolone on these two types of behaviours/reactions depend on temperature. Further, as the reflex response (predator escape) was more affected at 30°C, whereas the chronic boldness was more affected at 20°C, effects of 17 $\beta$ -trenbolone-exposure appears to be context dependent.

The importance of a fast escape response is especially high amongst bolder individuals (McCormick et al. 2018) due to a higher risk of e.g. predator encounters (Houston et al. 1993). Our results, showing that 17 $\beta$ -trenbolone can make fish more risk-taking but also affect the predator escape response negatively, therefore seem rather alarming. Even small changes in risk perception can result in individuals behaving suboptimal in risky situations, potentially leading to lowered fitness and survival (Slabbekoorn et al. 2010, Ferrari et al. 2012, Halfwerk and Slabbekoorn 2015). If fish are taking more risks in general but also in combination getting slower at escaping predators,

it is not unrealistic to think that this could possibly result in population declines that in turn, could lead to alterations in both community and food web structures (Kidd et al. 2014), but this of course, need further investigations.

Study IV clearly highlights that abiotic fluctuations, such as in temperature, can determine how behavioural modifications, caused by pharmaceuticals, are expressed. This abiotic dependence might be important for the effects of some pharmaceuticals, but not for others. It seems like behavioural modifications caused by growth hormones (study IV) may be more sensitive to temperature changes in comparison to behavioural changes caused by drugs acting on the GABA<sub>A</sub>-receptor (study III). It is of course too early to draw any major conclusions regarding this discrepancy in temperature dependence based on only these studies. What is obvious though, is the potency of 17 $\beta$ -trenbolone, inducing behavioural changes at concentrations 4-7 times lower than what has been measured in aquatic environments, and the importance of including environmental fluctuations when doing risk assessments.

## Concluding remarks and the way forward

It is evident that pharmaceuticals can alter ecologically important behaviours in fish, at least over short time-scales. It is also clear that some drugs accumulate in aquatic food webs, potentially leading to concentrations in organisms high enough to induce behavioural or other effects. Organisms at the lower trophic positions seem to be the main receivers of pharmaceuticals in aquatic ecosystems (Du et al. 2014, Du et al. 2015, Rodríguez-Mozaz et al. 2015, Boström et al. 2017, Xie et al. 2017). Support for this was also found in in this thesis (study I), where the benthic invertebrate species at the lowest position in the studied food web, had the by far highest bioaccumulation factors. These species are rarely tested in laboratory assays however, and more research focused on pharmaceutical uptake and effects in invertebrate species seems highly warranted. As shown in study I, the uptake of pharmaceuticals is often underestimated and predicted no-effect concentration based solely on bioconcentration studies performed in the laboratory can result in inaccurate assessment of environmental risks. Field-based experiments are therefore needed for assessing true exposures and potential effects in natural systems. Further, a delay in reaching steady state when assessing BAFs in natural ecosystems, must be considered for an accurate environmental risk assessment. Subsequently, to identify general patterns of pharmaceutical uptake and transfer in aquatic biota, long-term studies of pharmaceutical uptake in controlled aquatic systems are necessary.

It is very difficult to predict net ecosystem effects based on laboratory experiments alone. According to ecological theory, behavioural alterations could result in altered

species interactions, population dynamics, and changed community structures (Candolin and Wong 2012, Wong and Candolin 2015). However, as seen in study II, nature is much more complex than controlled laboratory settings and my theoretical predictions could not be validated by my empirical data. The non-significant results in study II should not be seen as evidence that oxazepam will not cause effects in populations in natural environments however, but rather highlights that the complexity of prey and predator interactions as well as interactions with abiotic factors, needs to be recognised when predicting effects occurring in aquatic ecosystems. Nevertheless, oxazepam-induced behavioural changes in fish under controlled laboratory conditions have been shown to correspond to similar behavioural modifications expressed in a natural environment, with subsequent alterations of habitat-use and changes in home range size (Klaminder et al. 2016). Further, oxazepam-exposure seem to increase downstream migration intensity in salmon smolt (*Salmo salar*; Hellström et al. 2016). In a follow-up study, the exposed smolt experienced an increased mortality during migration when released into a natural stream (Klaminder et al., *in prep*), possibly due to higher risk-taking behaviour. Interestingly, this is in line with what we predicted and expected but did not find in the pond study (study II). Noteworthy is that most, if not all studies so far, assess behavioural effects caused by oxazepam at short time-scales (minutes to days). As we did not find effects on growth or mortality in study II, which we predicted based on oxazepam induced behavioural changes, I cannot exclude that behavioural modifications of this drug cease over time. As such, to what extent the effects of oxazepam are time-dependent should be assessed in future studies.

Long-term whole lake studies, like the impressive experiment by Kidd and colleagues (2007), are complex and take a lot of time and resources. Nevertheless, I think whole-lake experiments are the next step to be taken for us to gain a better understanding of the environmental effects of pharmaceuticals. As shown in study IV, abiotic factors, such as temperature, can indeed determine the outcome of an exposure. Effects of both additional stressors and additional contaminants are complex as they can be additive, antagonistic or even synergistic (Heugens et al. 2001, Coors and De Meester 2008, Ding et al. 2016, Alton and Franklin 2017). At a laboratory-scale, investigations of the effect of one stressor at the time and various substances alone is indeed a good first step, but interactive effects need to be prioritized. Here, species interactions should be accounted for. As shown in study II, one of the natural predators of perch (i.e. pike) also gets less effective at catching prey when exposed to oxazepam. It has also been shown that roach (*Rutilus rutilus*), which is a species that commonly occur in the same systems as perch and pike, is even more sensitive to oxazepam exposure than perch, showing similar behavioural changes as exposed perch but at lower concentrations (Brodin et al. 2017). Contrary, a fourth study species, crucian carp (*Carassius carassius*), does not seem to be affected by oxazepam exposure to the same degree (Brodin et al., *in prep*). As ecosystem effects are highly dependent on individual species responses to contaminants,

it seems urgent for future studies to focus on quantifying symmetric and/or asymmetric effects within aquatic food webs.

Research addressing “reduction” and “replacement” of experimental organism also needs to be prioritised. Millions of fish are used annually around the world for experimental and scientific purposes (Taylor et al. 2008, Home office, 2016). Even though fish experiments are of high scientific value, I think we need to push toward other methods, limiting animal experiments, foremost to reduce the suffering of living organisms.

Pharmaceuticals in the environment are of concern, and we still do not know what the net ecological effects are or will be. We have gained a lot of knowledge since the 1960’s about human effects on wildlife and the environment, and many things have changed for the better. However, we are still facing big challenges. There is hope for a cleaner future with new technologies on the rise (Ahmed et al. 2017), but just like Rachel Carson did question the use of pesticides, I think we need to review our production and usage of pharmaceuticals and work hard towards decreasing the risk of drugs ending up in the environment.

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## *Thank you / Tack!*

I am writing this (with some later editing) sitting in a couch in Berkeley, CA, USA. It is the 7<sup>th</sup> of October, 2016, 7:50 pm local time. I am trying to write on my second paper and am wondering if I will reach that day when I actually need to write a “thank you”-part for a doctoral thesis. Instead of writing the paper, I am writing this “thank you”. Procrastination, I think it is called.

The crazy thing is though, since you are reading this it means that I made it, the whole way! And, except for my own part in this, it is all due to the following people that have been there for and with me (both professionally and privately) along the way (warning! this might be the longest “thank you” in history):

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All of my gratitude!

/H

Now, another chapter begins. That's why I'm easy, I'm easy like Sunday morning.