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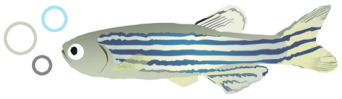

THESIS SUBJECTS ZEBRAFISHLAB

Our thesis subjects are grouped within 3 broad themes, which are described first and are then followed by specific topics. Promoters of these thesis projects are prof. dr. Dries Knapen and/or dr. Lucia Vergauwen. Most of the subjects described below are suited for both MP and IP.

THEME 1.
Unravelling the mechanisms underlying narcosis: does this toxicity mechanism affect mitochondrial function?

At least **60%** of the most commonly used **chemicals** in industrial applications are thought to **act by narcosis**. In a toxicological context, the term narcosis refers to lipophilic chemicals partitioning into cellular membranes as a function of their degree of lipophilicity, thereby causing toxicity. It is assumed that with increasing lipophilicity, chemicals have an increasing tendency to accumulate in membranes and disrupt their integrity and function. These chemicals can adversely impact a wide range of species, including humans, when they enter into the environment. Generally, loss of reaction to external stimuli, loss of equilibrium, decline in respiratory rate and death are expected in fish. However, the **mechanistic details** of how these chemicals cause toxic effects at the molecular and cellular level are **largely unknown**. Environmental risk assessment for this large array of chemicals is therefore currently mainly based on standard toxicity tests and lethality estimates using quantitative structure activity relationships (QSARs), which predominantly rely on lipophilicity estimates.

At this point, it is **unclear which membrane types** and which proteins **are functionally impaired** by narcotics. We hypothesize that the **cell membrane** is the **first target** of lipophilic compounds, which may then further **partition into different organelle membranes.** Many membrane-bound processes such as the mitochondrial electron transport chain are vital to cell survival and may be affected. We have found that narcotic compounds inhibit electron transport chain activity in 3 day old zebrafish embryos. Therefore, we further hypothesize that narcotic compounds affect the function of the mitochondrial electron transport chain. The thesis subjects related to this theme will focus on testing this hypothesis and further unravelling the mechanism of narcosis by investigating mitochondrial effects in the zebrafish embryo model at different levels of biological organisation (molecular, biochemical, physiological) and using a variety of techniques.

Thesis subject 1: **Characterization of physiological effects of narcotics with direct ecological relevance in zebrafish embryos**

In this thesis the effects of narcotics will be investigated at the physiological level which is of **direct ecological relevance for risk assessment**. Zebrafish embryos will be exposed to dilution series of narcotic compounds. Heart rate, swimming behaviour, oxygen consumption, growth and survival will be observed, and related to what we already know about the narcosis mechanism. We will observe **loss of equilibrium**, perform automated **swimming behaviour** analysis using a Zebrabox (Viewpoint), measure **growth** as larval length, monitor **survival** and determine **heart rate** in 1 day old embryos. **Oxygen consumption** measurements will be performed using small respiration chambers with optical oxygen probes suitable for zebrafish embryos. This knowledge is vital to understanding the mechanism and the impact of narcotics on the normal function of organisms.

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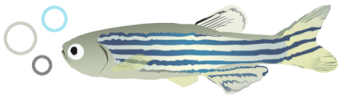

Thesis subject 2: **Identifying direct protein inhibitory potential of narcotics using *in vitro* techniques**

It is unclear **whether and how narcotics interact** with the mitochondrial membrane and/or the (membrane) proteins involved in the electron transport chain. Using specific *in vitro* tests interactions between narcotics and mitochondria and mitochondrial proteins can be investigated. For example, the fraction of a homogenate of control zebrafish embryos containing all mitochondrial components can be used as a test system to **measure electron transport chain activity** after addition of narcotic compounds. A second example is an *in vitro* test in which specific complexes of the electron transport chain are captured from a mitochondrial homogenate in a test vessel using antibodies. Subsequently the **activity of each specific complex** can be measured after addition of narcotic compounds. This offers the opportunity to specifically investigate whether narcotics can directly inhibit these proteins. This has never been investigated before and would significantly advance international understanding of the narcosis mechanism.

Thesis subject 3: **Investigating molecular and biochemical effects of narcotics on mitochondria of zebrafish embryos**

In this thesis, zebrafish embryos will be exposed to narcotic chemicals. At the biochemical level, the effects of narcotics on **electron transport chain activity** in exposed zebrafish embryos will be further explored. Inhibition of proteins in toxicity processes is often related to changes in transcript levels of those proteins. At the molecular level **transcriptional expression of** components of **all respiratory complexes will be analysed** using QPCR to identify such relations. In cases where aerobic cellular respiration is disrupted, there may be a shift in the balance between aerobic and anaerobic ATP synthesis. By measuring lactate levels and lactate dehydrogenase (LDH) activity in exposed zebrafish embryos, the **relative contribution of anaerobic and aerobic ATP synthesis** can be evaluated.

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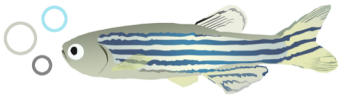

THEME 2.
The role of steroid hormones in vertebrate embryonic and larval development

The role of endogenous steroid hormones in adult fish in general, and zebrafish in particular, **is fairly well characterized** and is similar to their role in other vertebrates with a few exceptions. For example, comparable to mammals, estradiol regulates ovarian function and additionally, in fish, estradiol regulates vitellogenin (yolk protein) synthesis and yolk formation. Similarly, there has been a large focus on the potential of exogenous factors (e.g. endocrine disrupting compounds) to disrupt adult reproductive function. In contrast, **surprisingly little is known about the role of steroid hormones in early vertebrate development**. For some hormones and their receptors maternal transfer has been shown, i.e. the presence of hormones and mRNA coding for receptors in unfertilized oocytes. This indicates that these hormones are essential for early development, but overall the current understanding of the role of steroid hormones in embryonic development remains fragmented. The importance of the zebrafish as a vertebrate model in developmental biology calls for further research of the time-dependent profiles of the steroid hormone pathway and the functions of steroid hormones during embryonic development.

We have obtained **transcriptional expression profiles** of 20 genes coding for **enzymes involved in steroid hormone synthesis and hormone receptors** at 25 time points during the **first 32 days of zebrafish development**. This time frame covers embryonic to juvenile development. In these natural profiles we observe maternal transfer and increases or decreases of mRNA levels of specific genes during specific developmental stages. The thesis subject related to this theme will use this dataset as a starting point and will attempt to answer questions on the biological function of these profiles.

Thesis subject 4: **Disruption of natural hormone profiles in developing zebrafish to clarify their function in early vertebrate development**

For this thesis, **transcriptional expression changes of interest** - of which the function in early development is currently unknown - will be selected based on the profiles obtained during normal development. A suitable technique to **disrupt these profiles** will be selected. Examples include exposure of zebrafish embryos to endocrine disrupting chemicals which are known to inhibit enzymes involved in hormone synthesis, bind to hormone receptors, etc. Another example is the use of knockdown technology to silence a specific gene and. By **observing changes in early development** after disrupting the normal profile, the **function of these profiles** can be investigated. Observations can include molecular analyses (verification of the disruption of a transcriptional expression peak using QPCR, analysis of altered expression of related genes), biochemical characteristics (altered hormone levels, altered enzyme activity), morphological characteristics (morphology of eyes, ears, body shape, etc.), and physiological characteristics (heart rate, swimming behaviour, metabolic rate).

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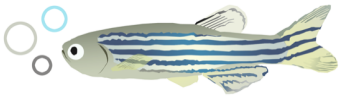

THEME 3.
Using the zebrafish embryo to investigate endocrine disrupting potential of pharmaceuticals

Pharmaceutical companies are required to perform an environmental risk assessment for every drug that is launched on the market. The mandatory tests for potential endocrine disrupting compounds require a lot of time and laboratory animals, which is not consistent with the **3R principle** of refinement, reduction and replacement of animal tests. Therefore, the goal of this study is to develop a zebrafish embryo test, which is not considered an animal test according to European regulations.

Endocrine disruption has long been a major concern for the health of wildlife populations. Although many studies have shown reproductive impairment as a consequence of endocrine disruption in adult fish, knowledge of the **consequences of endocrine disruption for vertebrate embryonic and larval development** is scarce. Therefore, a number of aspects related to this theme can be investigated.

Thesis subject 5: **Nano-injection in the zebrafish embryo as an alternative exposure route for environmental risk assessment of endocrine disrupting pharmaceuticals**

It is often **difficult to expose fish aquatically** to endocrine disrupting pharmaceuticals because of their lipophilicity (these compounds tend to adsorb to the test vessel walls instead of dissolving in the water). **Nano-injection in the yolk** is therefore proposed as an alternative exposure route because the yolk of zebrafish embryos contains many lipids in which these chemicals may dissolve. Secondly, this exposure route mimics maternal transfer which is a realistic exposure route in the field when female fish are exposed to lipophilic compounds which are accumulated and deposited in the oocytes. We developed a technique to inject 0.5 nL of a solution containing a pharmaceutical into the yolk of zebrafish embryos. To use nano-injection as an alternative exposure route it needs to be **characterised and compared to the classical exposure route via water**. Differences and similarities between effects observed after nano-injection and those observed after exposure of zebrafish embryos via water will be investigated and compared. Potential effects to compare include transcript levels of genes involved in endocrine disruption (QPCR), organismal morphology including malformations (e.g. yes, ears, body shape) and swim bladder inflation (stereomicroscope), heart rate and swimming behaviour (automated behaviour tracking) during early zebrafish development.

Thesis subject 6: **Distinguishing among different endocrine disruption modes of action using the zebrafish embryo**

Endocrine disrupting chemicals (e.g. pharmaceuticals) can influence the endocrine system through a variety of different mechanisms. For disruption of the sex hormone pathway, **5 main modes of action** are distinguished: estrogen receptor (ER) agonism and antagonism, androgen receptor (AR) agonism and antagonism and aromatase inhibition (aromatase is the key enzyme in estrogen synthesis). This variety of mechanisms complicates the development of a screening assay for identifying endocrine disrupting chemicals. Ideally, such assay should be able to **differentiate among these different mechanisms**. In this thesis, effects of model chemicals with known and differing endocrine disrupting mode of action will be compared. The goal is to **identify endpoints that are**

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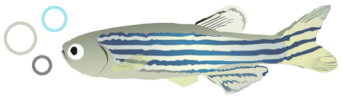

unique for a specific mode of action and thus allow for distinguishing among different modes of action when screening new and unknown compounds. Candidate endpoints include transcript levels of genes involved in endocrine disruption (QPCR), enzyme activities, protein levels, morphological profiles (profile of observed morphological malformations), behavioural profiles, etc.

Thesis subject 7: **The use of transgenic zebrafish to screen for endocrine disrupting properties of chemicals**

The zebrafish is a model organism in many disciplines and its entire genome has been sequenced. This allows for the development of transgenic models containing gene constructs that aid in visualizing responses to chemicals among others. We have several transgenic models which **express a green fluorescent protein (GFP) upon activation of a receptor** (e.g. estrogen receptor) initiated through binding of a chemical. Such models **may be ideal components of a screening assay** to detect endocrine disrupting pharmaceuticals and distinguish different modes of action. In this thesis the potential of transgenic fish in this context will be further investigated.

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