The fathead minnow embryo as a model for the development of alternative testing methods in ecotoxicology

Diplomarbeit

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Ich erkläre hiermit, dass ich die vorliegende Diplomarbeit selbständig unter Anleitung verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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Für meine Mami

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1 Introduction

About 5 million chemicals are known so far, 100,000 of which are in current use (Fent 2007). In 2010, 339 million tons of chemicals were produced in the European Union (EC 2010b). This vast amount of chemicals has major impact on terrestrial and aquatic environments: Various chemicals show acute effects, while others are more persistent, and chronic effects can be detected after several months or even years. Hence, it is very important to know and to assess the risk potential of all of these chemicals.

In February 2001, the European Commission proposed a new system to deal both with existing and new chemicals produced, used and imported into the European Union (Combes et al. 2003, EC 2001). In October 2003, the first draft of REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) was published (EC 2003), classifying chemicals *via* the quantities they are produced, used and imported. REACH came into force in 2006 (EC 2006) with the aim to systematically evaluate the risk of approximately 30,000 chemical compounds produced, used or imported in quantities of one or more tons per year (Hengstler et al. 2006). However, with the introduction of REACH, the number of animals used for toxicity testing is likely to increase significantly (Combes et al. 2003), and new testing strategies are warranted. Therefore, REACH also offers an opportunity to optimize and innovate testing strategies for chemicals (Hengstler et al. 2006).Closely linked with REACH is the 3R strategy introduced by Russell and Burch (1959), a landmark in animal welfare and protection. Firstly, they proposed a replacement of higher animals by lower animals, tissue cultures, or nowadays computer modelling. Secondly, they called for a reduction of the number of animals used to obtain information, and thirdly, a refinement of the methods used for testing was requested to minimize suffering.

However, in aquatic toxicology, fish are still very important vertebrate test models and are used in high numbers to determine the impact of toxicity fish over several life stages. OECD guidelines cover acute toxicity (OECD 1992a), early life-stage toxicity (OECD 1992b), short-term toxicity test on embryo and sac fry stages (OECD 1998), and the so-called juvenile growth test (OECD 2000). All those tests have negative impacts on fish welfare, resulting eventually in the death of animals. Several authors have hypothesized that fish suffer from pain and distress (Braithwaite 2010, Braunbeck et al. 2005, Chandroo et al. 2004, Huntingford et al. 2006, Sneddon 2003, Sneddon et al. 2003a, b), but proof yet needs to be found.

The new EU Directive 2010/63/EU on the protection of animals used for scientific purposes (EC 2010a) states that all live non-human animals, including independently feeding larvae and foetal forms of mammals as from the last third of the normal development, as well as cephalopods are considered test animals and are, thus, to be classified as protected. According to this classification, all current OECD fish tests fall under that directive and all animals used are considered as protected test animals.

Therefore, with regard to REACH and the 3R-strategy, new approaches in ecotoxicology need to be developed. One example is the substitution of the acute fish test (OECD 1992a) with a fish embryo toxicity (FET) test (OECD 2006). Comparison and evaluation of existing data of both, acute toxicity test and FET show that neither one is neither better nor worse than the other one (Lammer et al. 2009, Ratte and Hammers-Wirtz 2003).

However, the introduction of the FET as an alternative testing method started before the onset of REACH. Since 2001, the 48 h zebrafish FET has been used for waste water treatment in Germany (DIN 2000) and became mandatory in 2005; an ISO standard has been available since 2007 (ISO 2007).

Braunbeck et al. (2005) stated that the fish embryo toxicity test can also be carried out with other OECD fish species commonly used in aquatic acute toxicity testing, such as the Japanese medaka (*Oryzias latipes*) or the fathead minnow (*Pimephales promelas*). Hence, a first draft for an OECD test guideline was published in May 2006 (OECD 2006). Currently, interlaboratory tests are being carried out with zebrafish (*Danio rerio*) eggs to gain data to validate the test as a precondition for the FET to become an OECD guideline in the near future.

Overall, the FET gives advantage for both, the fish and the researcher: the pain inflicted upon the fish is minimized during the test due to a not yet or only poorly developed nervous system, the test system including the eggs/embryos and the observation is simple, and the duration of a test is as short as the conventional acute fish test. Though, there are still problems. For example, how long should a fish embryo toxicity test last? In accordance with the EU directive 2010/63/EU on the protection of animals used for scientific purposes (EC 2010a), free-feeding larvae are already considered as test animals. However, hatching and onset of feeding are dependent on several criteria: species, water temperature, reduction rate of the yolk sac, etc. Belanger et al. (2010) carried out extensive research with the zebrafish. They proposed that zebrafish embryo tests should be terminated between 24 and 48 hours after hatching. Strähle et al. (2011) analyzed yolk consumption, feeding and swimming behavior of zebrafish, and concluded that zebrafish older than 120 hours post fertilization should be re-

garded as independently feeding. Limited data for the fathead minnow (Belanger et al. 2010) suggested determination of the tests at 24 hours or less after hatching to avoid the transition of the fish from the eleutheroembryo to the free-feeding larval phase, and thus into animal testing. After feeding studies with the fathead minnow, Belanger (Belanger 2012)(personal communication, 2012) states that feeding initiates somewhere between 120 and 144 hr post fertilization at 25C independently of food size, type and swimming speed.

Another question concerning the fish embryo test is whether the chorion functions as an important barrier that significantly prevents chemicals from entering into the egg and thus not harming the embryo. Braunbeck et al. (2005) found out that in zebrafish the barrier function of the chorion may increase with lipophilicity.

The chorion is an acellular envelope surrounding the fish embryo, varying in thickness and with species: the zebrafish chorion is between 1.5 and 2.5 µm (Hart and Donovan 1983, Rawson et al. 2000), the fathead minnow chorion about 10 µm (Manner et al. 1977), and the medaka chorion about 15 µm thick (Yamamoto and Yamagami 1975).

Extensive research has been carried out to understand the structure and properties of both the medaka and the zebrafish chorion. Besides the considerable amount of data available for zebrafish and medaka dealing with the chorion ultrastructure and thus with embryonic development, little is known about the fathead minnow, the most common laboratory fish in the United States of America. The minnow has been used in laboratories since the 1950ies (Ankley and Villeneuve 2006) to gather information about acute toxicity for many chemicals, but data concerning the embryo is scarce.

Manner and colleagues (Manner and Dewese 1974, Manner and Muehleman 1976, Manner et al. 1977) investigated the fathead minnow embryo, especially the chorion, in the 1970ies: Manner and Muehleman (1975) studied the permeability of the fathead minnow chorion and found out that the permeability increases while embryogenesis progresses. In 1977, Manner studied the fathead minnow chorion by means of light and electron microscopy and found out that it comprises an outer layer with ridges covering a middle substructure which is in turn composed of 19 lamellar layers. All of those appear to have different fiber orientation. The inner layer is composed of a more electron dense material. In 1974, Manner and Dewese also studied the normal development of the fathead minnow at 23°C water temperature. A new, more detailed study was conducted by (Devlin et al. 1996) at 25°C water temperature to allow applicability for the U.S. Environmental Protection Agency standard procedures for rearing fathead minnow (Denny 1987, 1988). Both studies give an overview of embryonic develop-

ment, but are by far not as comprehensive as the work conducted in zebrafish (Hisaoka and Battle 1958, Kimmel et al. 1995, Westerfield 2007) or the Japanese medaka (Iwamatsu 1993, 2004, Yamamoto and Yamagami 1975).

The fish embryo and the knowledge of its exact development are another major advantage: it can be used as an *in vivo* toxicity test compared to *in vitro* toxicity tests. Otte et al. (2010) developed a method to identify the activity of the cytochrome P450 1 (CYP1) family in early life-stages of zebrafish. These enzymes are activated *via* the aryl hydrocarbon receptor (AhR) to biotransform planar halogenated and polycyclic aromatic hydrocarbons when present in the organism. This study gave rise to the development of an *in-vivo* EROD (ethoxyresorufin-*O*-deethylase) assay to detect even very low concentrations of planar halogenated hydrocarbons in complex environmental samples *via* life imaging in zebrafish embryos as early as 48 hours post-fertilization (Kais et al., in preparation).

It is important for the development of alternative test methods to transfer already existing protocols from one test species to another, to obtain new data and to look at existing data from a new perspective. Thus, a more detailed study into the fathead minnow embryo is required: moreover, a comparison of the fathead minnow with zebrafish and the Japanese medaka embryo is due.

This study serves several purposes:

- optimization of the rearing conditions and the egg production of the fathead minnow in the laboratory of the ecotoxicology group at Heidelberg University;
- detailed description of the normal development of the fathead minnow embryo from fertilization to hatching and beyond;
- based on these results, adaptation and progressive refinement of the embryo toxicity test protocol for the fathead minnow embryo to ensure equal conditions during testing to allow for immediate comparability and reproducibility;
- conduction of tests for several substances to validate the fatheads minnow embryo test protocol;
- development of a protocol for dechorionation of fathead embryos;
- analysis of the fathead minnow chorion itself and comparison of new toxicity data using different preparation, staining and microscopic methods with existing data.

2 Methods & Materials

2.1 Fathead minnow (*Pimephales promelas*)

2.1.1 Natural habitat

The fathead minnow (*Pimephales promelas*, Rafinesque, 1820) belongs to the largest fish family with over two thousand species, the Cyprinidae. The binomial name is of Greek ancestry: *pimephales* meaning 'fat head' and describing the head shape of male minnows, and *promelas* meaning 'forward and black' describing the colorations of the males during the breeding period. The fathead minnow is a small freshwater fish, which can be found throughout eastern and northern parts of North America, including Mexico. They live close to the bottom in muddy pools of headwaters, small rivers and creeks, where they feed on detritus, algae and small insects. The fathead minnow is also able to tolerate unsuitable conditions such as high temperatures, high turbidity and low oxygen levels. The adult fathead minnow is between four and ten centimeters in length. The coloration outside the breeding season ranges from olive to brown on the upper and silvery-white on the lower body, separated by a dark mid-lateral stripe (Fig. 1). In the breading season, male fathead minnows become considerably larger than females, showing a black coloration of the head, and growing nuptial tubercles on the snout and a pad of spongy tissue on the nape. Males also have distinct vertical black bands on both sides of the body. The spawning period is between May and August, in which a single female is able to spawn up to twelve times and lay up to 10,000 eggs. Before spawning, the male fathead minnow has to build a nest side in calm shallows of streams or along the shoreline of ponds which is normally located on the under surfaces of submerged stones and branches. The male defends the nest site from intruders, guards the eggs after spawning and ventilates them from time to time. With the spongy tissue on the nape containing chemo sensors, the male fathead is able to check the condition of the eggs. It is assumed that this tissue produces a substance with fungicidal properties. After four to ten days, depending on the water temperature, the eggs hatch.

Fig. 1: Male and female fathead minnow (Source: Joseph Tomelleri)

2.1.2 Fathead minnow maintenance and breeding

All fish used for breeding were taken from the fathead minnow stock maintained at the Aquatic Ecology and Toxicology group at the Centre for Organismal Studies (COS), University of Heidelberg. Originally, the fathead minnow stock was a gift from R. Länge (Schering AG, Berlin, Germany). The fish stock was kept in two 150 L tanks under winter conditions (light regime: 8 hours light/ 16 hours dark, with a water temperature between 10 and 12 °C) in a semi-static system. Water and filters were changed every two weeks to maintain healthy conditions. Before the start of the study, fathead minnows from the stock were transferred into flow-through spawning tanks (Fig. 2) containing 40 L or 20 L of water, respectively. These tanks were covered with black construction paper on all for sides to keep the disturbance level from the surroundings to a minimum and to ensure optimal spawning conditions. Each 40 L spawning tank contained two male and four female minnows, as well as two spawning substrates (2.1.4). Each 20 L tank contained one male and one female fathead minnow plus a single spawning substrate. The light regime was held at 14h light/10 hours dark cycle, the water temperature was maintained between 23 and 25°C , the oxygen saturation was above 90 %, pH was between 7.8 and 8.1, and conductivity ranged between 600 and 700 µS/cm^2. Water criteria such as temperature, oxygen saturation, conductivity and pH were checked regularly using a WTW Multi 350i parameter instrument (WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) to ensure water quality parameters were within range. After hatching, the offspring of the fathead minnows was transferred to 40 L tanks maintained under the same conditions as the adult spawners to ensure rapid growth. They were fed special food (2.1.3). After three to four months, when fish were close to maturity, they were transferred to the stock tanks, where they were kept until needed.

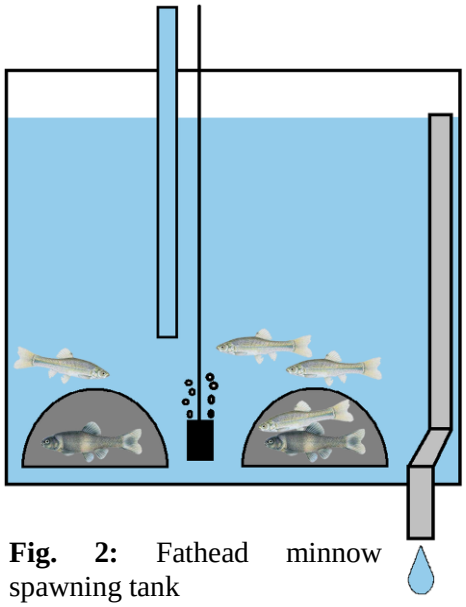


Fig. 2: Fathead minnow spawning tank

Excess feces was removed at least every second day to keep the disturbance level at a minimum. When fish died in the spawning tanks, the spawning groups were restocked with fish from the stock held at winter conditions to maintain the sex ratio.

2.1.3 Feeding regime

The feeding regime had to be adjusted to the different sizes/age of the fish and to the maintenance conditions. For larvae, liquid rearing and powder food was used (NobilFluid *Artemia* and NovoTom *Artemia* Staubfutter, JBL, Neuhofen, Germany). For older stages, fresh larvae of *Artemia* (Great Salt Lake Artemia Cysts, Sander Brine Shrimp Company, Ogden, USA), flake food, and frozen brine shrimp (Kordon's Golden Gate ® Frozen Brine Shrimp, Kordon LLC, Hayward, U.S.A.; *ad libidum*) were used.

2.1.4 Spawning substrate

The spawning substrates should simulate crevices, where fathead minnows normally spawn in the wild. Therefore, three sides of the tanks had to be covered to ensure that the male fathead minnow is able to protect the nest side against other males and egg-eating females. Two different kinds of spawning substrates were tested: red clay pot halves and small houses made of a grey PVC tube half and a plastic tray.

To ensure that no harmful substance entered the tanks, the plastic parts of the houses were cooked in deionised water for an hour; clay pot halves were thoroughly cleaned under hot water with a clean dishwashing brush. After removal of eggs, the spawning substrates were thoroughly cleaned with hot water and a brush.

2.1.5 Handling of eggs

The spawning substrates were checked every morning after feeding (approx. 9.30 a.m.) and in the afternoon (approx. 3 p.m.) for eggs using a flashlight if the behavior of the male fathead minnow indicated that it was guarding eggs.

If eggs were present, the house was removed from the tank. Eggs, in clutches and singles, were detached from the spawning substrate, either by rolling the eggs with a thumb off the surface or by using a featherweight forceps half formed into a spatula to scrape off the surface. Eggs were then transferred from the spawning substrate with a finger or a spatula directly into a petri dish containing 25 ± 1 °C warm dilution water with adjusted pH (7.7-7.8).

These eggs were separated and counted using a stereo microscope (Stemi 2000-C with Canon Power Shot G7, Carl Zeiss MicroImaging, Göttingen, Germany). The developmental stage of the eggs was also determined and recorded. Eggs younger than the 128-cell stage were used for the fish embryo tests, older eggs were used for other purposes or were discarded.

2.1.6 Measurements of the fathead minnow egg

To gain statistically relevant data concerning the dimensions of the fathead minnow egg, a total number of 80 eggs (20 eggs per batch) were photographed at an age of 24 h at 4 x magnification using an inverted microscope (Olympus CKX41, Olympus, Germany). The eggs were analyzed using ImageJ (Image Processing and Analysis in Java, free tool from the U.S. Department of Health & Human Services) and the mathematical formulas stated below. The diameter of the fathead minnow eggs was determined using a burned-in metering bar as a reference. The measurements were taken considering the orientation of the embryo in each picture to allow comparison.

- The surface area (A) of the fathead minnow egg was determined using the diameter (d) calculated with ImageJ, as well as the formula $A = 4 * \pi * r^2 = \pi * d$ to calculate the surface area of a sphere.
- The thickness of the chorion (h) was determined *via* transmission electron microscopy photographs. (2.8).
- Volume of the total egg was calculated using this formula: $V = 4/3 * \pi * r^3$
- The egg mass (m) was determined by weighing 3 x 100 eggs with the following procedure: A piece of weighing paper is placed on a precision balance, the balance was cleared, and100 fathead minnow eggs were placed on the weighing papers after excess water had been removed as well and as quickly as possible using filter paper. Due to a constant weight loss of the eggs as soon as they were placed on the balance, the weight was taken after 5 min. The eggs were left under the extractor hood for 24 hours, and were then weighted again to determine the dry weight of the embryo. The density (ρ) was then calculated using the formula: $\rho = m / V$.

2.1.7 Dechorionation procedure

A dechorionation procedure for fathead minnow eggs was developed during this thesis. Mechanical and chemical methods were tried to dechorionate fathead minnow embryos, as well as combinations of both. The embryos used for dechorionation were of 24 hpf (hours post fertilization). For mechanical dechorionation, older embryos (48 and 72 hpf) were used as well.

In the mechanical approach, it was tried to tear apart the chorion of eggs on a 2 % agarose bed using sharply pointed forceps, e.g. Dumont^{TM} no. 5 forceps (Henn and Braunbeck 2011).

In later experiments, a small hollow needle (Sterican® size 20, 0.4 x 20 mm, Braun, Melsungen, Germany) was additionally used to pierce the chorion before mechanical dechorionation to release pressure inside the chorion.

In the chemical approach, it was tested whether a pronase solution is more suitable for dechorionation. A stock solution of 40 mg/ml was prepared using Pronase XIV (activity approximately 4 units per mg solid, Sigma Aldrich, Deisenhofen, Germany). A pre-test was done to determine whether the concentration used for the zebrafish (Henn and Braunbeck 2011) works for the fathead minnow embryo. Additional tests were carried out to determine an appropriate pronase concentration.

A combination of both methods, mechanical and chemical dechorionation, was performed as well. Embryos were incubated in a pronase solution, rinsed in dilution water to remove excess pronase, and mechanically dechorionated using forceps.

Dechorionated embryos were transferred by means of a pipette into 24-well plates containing 2 ml dilution water. Survival was checked shortly after transfer, as well as after 24 and 48 h. It was also tested if a coating of the well plates with 300 µl agarose helps to improve survival of dechorionated embryos.

2.2 Embryonic development

There is a lack of data on the embryonic development of the fathead minnow: Except for a detailed brochure from the Environmental Protection Agency (Devlin et al. 1996) describing pre-hatching development at 25 °C and a developmental study by Manner and Dewese (1974) describing 12 different developmental stages up to the 13-somite stage at 23 °C, nothing has been published on fathead minnow development. This is even more surprising, if the frequent use of this species in aquatic ecotoxicology is considered. Thus, it was a major aim of this thesis to document the pre-hatching development and to establish a timeline. A testing temperature of 25 ± 1 °C was chosen to match the maintenance temperature used by EPA(Denny 1987) and the testing temperature used for early life-stage and larval tests with the fathead minnow (Ankley et al. 2001).

2.2.1 Procedure

Fathead minnow embryos were observed from fertilization under an Olympus CKX 41 culture microscope until hatching in order to determine the time frame for certain morphological characteristics such as specific cell divisions, different embryological stages, somite devel-

opment, and the onset of heartbeat and circulation, gill development, and hatching time.

A magnification of 4x was used to document the development. Photos were taken using an Olympus C-5060 Wide Zoom camera (Olympus, Hamburg, Germany) and the program AnalySIS (Olympus Soft Imaging Solutions, Münster, Germany). During the first 90 minutes of observation, one photo was taken every 5 minutes, then the interval was changed to 30 min for the next 10.5 hours, afterwards the time interval was set to 1 hour. To maintain a constant water temperature at 25 ± 1 °C, a control unit HT 200 W for heating systems (Minitüb GmbH, Tiefenbach, Germany) was used to heat the centre stage of the microscope.

The eggs were removed from the spawning tanks as soon as they were detected and transferred into a petri dish (diameter 9 cm) filled with artificial water (temperature 25 ± 1 °C); alternatively, embryos were obtained by *in vitro* fertilization (2.2.2)

Two ama-digit 15th thermometers (Amarell, Kreuzwertheim, Germany) were used to control the water temperature inside the petri dish as well as the air temperature close to the microscope to allow immediate adjustments to ensure constant temperature.

Fathead minnow embryos older than 24h were dechorionated to improve observation of the whole animal. With the onset of spontaneous movements, the embryos were anaesthetized with 0.016% tricaine solution, while pictures were taken. Anaesthesia was kept as short as possible, because tricaine may interfere with development (Kimmel et al. 1995).

The pictures were processed with Adobe Photoshop CS5 (Adobe Systems, Munich, Germany) to ensure the same positioning of the embryos to get a better understanding about the changes in morphology during development and to facilitate comparisons.

2.2.2 Egg production by *in vitro* fertilization

In order to obtain eggs prior to fertilization and to start the observation of the early development right from the start, eggs and sperm were collected from females and males and fertilized *in vitro.* The protocol used was adapted from Walker and Streisinger (Westerfield 2007).

A male fathead minnow was anesthetized with 100 mg/L tricaine buffered with 0.5 M sodium bicarbonate (Sigma-Aldrich) according to (Hala et al. 2009), rinsed in water and placed belly up in a slit within a damp dishwashing sponge. Afterwards, fish were placed under a stereo microscope with the illumination highlighting the anal region. Anal fins were pushed aside to expose the anus; excess water was removed using filter paper, because water makes handling of the sperm difficult and activates sperm motility in advance (Hala et al. 2009). The belly of

the male minnow was gently stroked from the pectoral fins to the anal fins using either fingers or smooth forceps. The white and viscous ejaculated milt was collected using a capillary tube. The sperm was added to ice-cold Hank's solution (Sigma-Aldrich) to prolong viability; afterwards, the sperm was placed on ice. Finally, the male was placed into a small tank (approx. 2 L) filled with tank water for recovery from the procedure.

Female fathead minnows were anesthetized like the male minnows, rinsed in water and blotted damp-dry on paper towels; excess water would make eggs swell and will prevent fertilization. The fish were placed into a petri dish. The fish were squeezed firmly, but gently on the belly with damp fingers. If female minnows are prepared to spawn, they will release their eggs quite easily. Afterwards, the fish were transferred into a recovery container.

In order to fertilize the eggs *in vitro*, sperm was added to the eggs and mixed by gentle stirring. After mixing, 1 ml of dilution water was added to activate the sperm.

2.3 Embryo toxicity test

The embryo toxicity test with the fathead minnow (*Pimephales promelas*) was run according to the standard operation procedure for the zebrafish (*Danio rerio*), another cyprinid (OECD 2011) with minor adjustments.

2.3.1 Test concentrations and controls

For each test compound a stock solution was prepared using the analytical standard (Pestanal, Sigma Aldrich) and dilution water.

Cadmium chloride

Cadmium chloride is available in colorless crystal. It is produced as a by-product in lead and zinc mining. It is used in galvanic techniques, for microscopy and photography. Cadmium chloride is highly toxic. It can be cancerogenic, mutagenic and can impair reproduction.

Tab. 1: Chemical properties of cadmium chloride

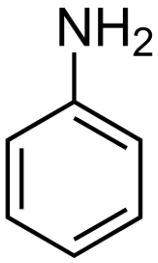
CAS No:	10108-64-2
Molecular weight:	183,32 g/mol
Partition coefficient log Pow	N/A
Water solubility	1400 g/l at 20 °C
Physical state:	solid at 20 °C

Aniline

Aniline is an organic compound consisting of a phenyl group attached to an amino group. Aniline is a precursor to many industrial chemicals, for example polyurethane. It is also used to synthesize herbicides, dyes and drugs like paracetamol.

Tab. 2: Chemical properties and structure of aniline

CAS No:	62-53-3
IUPAC name:	benzeneamine
Molecular weight:	93.13 g/mol
Partition coefficient log Pow	0.90*
Water solubility	34 g/l at 20 °C
Physical state:	liquid at 20 °C



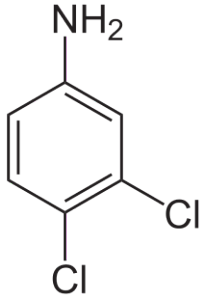
3,4-Dichloroaniline

Every year, about 15,000 tonnes of 3,4-dichloroaniline are produced in Europe by only two firms. 3,4-dichloroaniline is a phenylamine which is an intermediate for the synthesis of the herbicide propanil, azo dyes and phenylurea herbicides, e.g. the anti-fouling agent diuron. It can also occur as a degradation product of various chemicals in the environment.

3,4-Dichloroaniline is very toxic for aquatic organisms and may cause long-term adverse effects Since the introduction of the FET, 3,4-dichloroaniline has been used as the positive control for toxicity to zebrafish eggs and embryos.

Tab. 3: Chemical properties and structure of 3,4-DCA

CAS No:	95-76-1
IUPAC name:	3,4-dichlorophenylamine
Molecular weight:	162 g/mol
Partition coefficient log Pow	2.68*
Water solubility	580 mg/l at 20 °C
Physical state:	solid at 20 °C

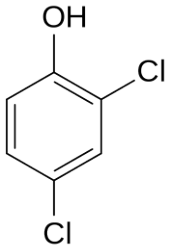


2,4-Dichlorophenol

2,4-dichlorophenol is a flammable solid. At 20 °C it is a colorless crystal. Water solubility is quite low, and 2,4-DCP is better soluble in organic solvents.

Tab. 4: Chemical properties and structure of 2,4-DCP

CAS No:	120-83-2
Molecular weight	163.0 g/mol
Partition coefficient log Pow	3.17* LOGKOW database
Waters solubility	4,5g/ L at 20 °C
Physical state:	solid at 20 °C



For each test compound, five concentrations were prepared using the stock solution and dilution water. Dilution water was used as negative control, and 3,4-dichloroaniline was used at a concentration of 12 mg/l as a positive control. Each test was carried out with 10 eggs per tests concentration and controls; an internal control was not used due to the usually small size of clutches. Before the start of the fish egg test, crystallization dishes and 24-well plates used during the test were pre-exposed with 20 ml or 2 ml, respectively, to ensure coating of all possible binding sites inside the wells. Because fathead minnow spawning cannot be predicted, a new set of 24-well plates were prepared as soon as another test started. For the same purpose glass vessels for each chemical were covered with parafilm and stored for the duration of the experiment.

Tab. 5: Components of the dilution water

Volume	Compound	Concentration
20 ml	CaCl2 2H2O (14.7 g in 500 ml)	294.0 mg/L
20 ml	MgSO4 7 H2O (6.165 g in 500 ml)	123.3 mg/L
20 ml	NaHCO3 (3.235 g in 500 ml)	64.7 mg/L
20 ml	KCl (0.285 g in 500 ml)	5.7 mg/L
1920 ml	Distilled water	

Exposure of fish embryos

If eggs were present in the spawning tanks, these were removed and separated as stated above (2.1.5). Only eggs younger than the 128-cell stage were used for the fish egg test; others were either used for other purposes or discarded.

Separated eggs were transferred as soon as possible into crystallization dishes containing the five different test solution concentrations, as well as the positive (3,4-dichloroaniline) and the negative control (dilution water).

In the meantime, the test concentrations in the pre-exposed 24-well plates were renewed and the eggs were transferred into the wells from the crystallization dishes into the wells using a 1,000 ml Gilson pipette with a cut-off tip.

Plates were covered with self-adhesive foil (Nunc Sealing Tape, Thermo Fisher Scientific, Langenselbold, Germany) and matching lids, and were incubated at 25 ± 1 °C. Eggs were observed every 24h with an inverted microscope (Olympus CKX 41) and rated according to OECD standards. Any effects were recorded using an Olympus E330 camera and program (Cell A). The 2 ml of the test concentrations and control were renewed each day after observation. Lethal endpoints included non-detachment of the tail, lack of heart beat, non-formation of somites and coagulated embryos.

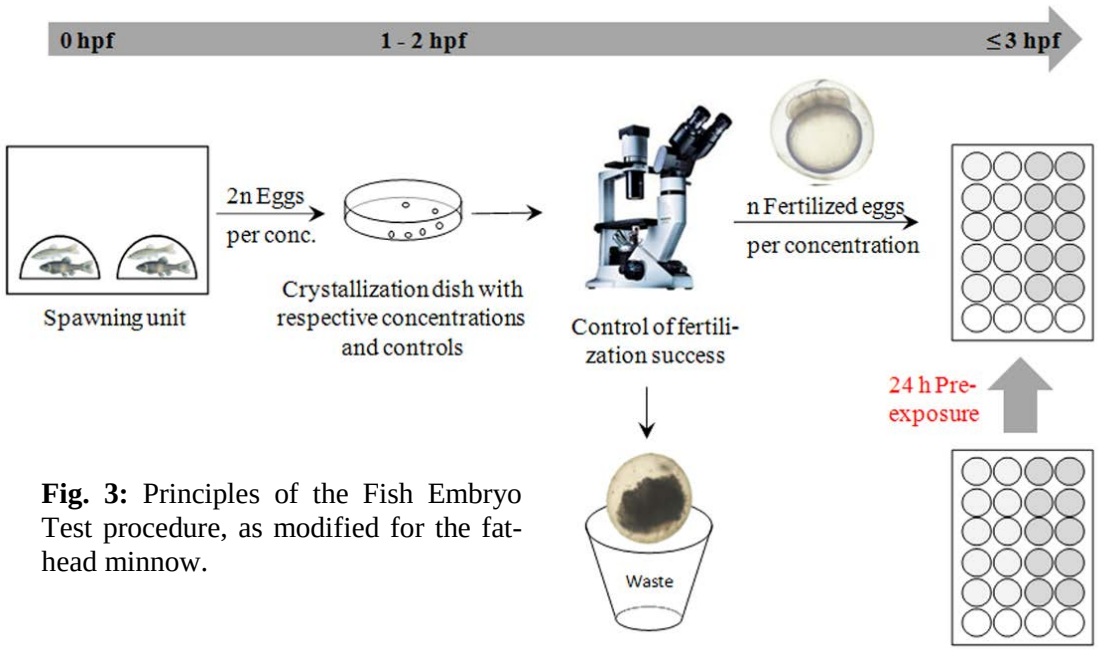


Fig. 3: Principles of the Fish Embryo Test procedure, as modified for the fathead minnow.

Non-lethal endpoints included formation of edemas, malformations, reduction of blood circulation and of heartbeat(OECD 2011). Based on the observation data, LC50- (lethal concentration for 50 % of eggs/embryos tested) and EC50-values (concentration at which 50 % of eggs/embryos show effects of any kind including lethal ones) were determined using ToxRat (Toxicity Response Analysis & Testing) where values were calculated using probit analysis. Graphs were plotted using SigmaPlot.

2.4 Histology of the fathead minnow and zebrafish egg with focus on the chorion

2.4.1 Fixation

Fathead minnow and zebrafish eggs were transferred into small glass tubes containing modified Davidson's fixative (Fournie et al. 2000) and were stored until usage, however, for at least 24 hours at 4 °C.

Tab. 6: Composition of Davidson's fixative

Volume	Compound
200 ml	Formaldehyde (37 – 40 %)
115 ml	Glacial acetic acid
300 ml	Ethanol (95 %)
335 ml	Distilled water (aq.dist)

2.4.2 Dehydration and embedding

After fixation, the eggs were transferred into modified embedding cassettes (Simport, Beloeil, Canada) which were covered with a 200 µm gauze (Clear Edge, Geldern-Walbeck, Germany) to prevent loss of eggs during the dehydration and embedding procedure. The gauze was attached to the plastic cassettes by the use of a soldering gun. The cassettes were processed in an automatic tissue processor (TP1020, Leica Microsystems, Wetzlar, Germany). In this automated process (Tab. 7), the samples were firstly dehydrated using a gradient series of ethanol concentrations and finally embedded in paraffin (HistoplastS, Serva, Heidelberg, Germany). It is necessary to extract all water from the samples in order to allow paraffin to permeate the sample and to ensure a uniform hardness to simplify further processing. Afterwards, samples were embedded in paraffin using a heated paraffin embedding module (EG 1140 H, Leica Mircosystems)to enclose the paraffin-permeated samples in a paraffin block of uniform hardness. The blocks were cooled down using a cold plate (EG 1040 C, Leica Microsystems) and stored at room temperature.

Tab. 7: Dehydration and permeation of the samples (duration 24 h)

Step	Solvent	Concentration	Time
1	Ethanol	80 %	1 h
2	Ethanol	90 %	1 h
3	Ethanol	90 %	1 h
4	Ethanol	96 %	1 h
5	Ethanol	96 %	1 h
6	Isopropyl alcohol	100 %	1 h
7	Isopropyl alcohol	100 %	1 h
8	Xylene		1 h
9	Xylene		12 h
10	Xylene		4 h
11	Histoplast		12 h
12	Histoplast		

2.4.3 Sectioning

For sectioning, the embedded eggs were cooled at -20°C and cut into slides of 4 µm thickness using a microtome (HN 40-Schlittenmikrotom, Reichert-Jung, Heidelberg, Germany). Each section was transferred for stretching into a 40 °C warm water bath by means of a fine brush. The sections were mounted on a slide (Neolab, Heidelberg, Germany) and coated with protein-glycerin (Serva, Heidelberg, Germany), which ensured that the sample was securely attached to the slide during staining. Slides were dried at 38 °C overnight.

Due to the water solubility of the following staining solutions, it was necessary to remove the Histoplast with X-tra Solv (Medite GmbH, Burgdorf, Germany) with subsequent rehydrating with a graded ethanol series.

After staining, the slides were coverslipped in DePeX (Serva, Heidelberg, Germany) and dried for at least one day at room temperature.

2.4.4 Staining

Three different staining methods were performed: the hemalum-eosin (HE) and the Masson-Goldner-stain, and the Alcian Blue 8GS - PAS-stain.

Haematoxylin-Eosin-stain (HE-stain) (Romeis 2010)

HE stain is the most important staining procedure in histology and gives a good overview of the structures of the sample in a short time.

Tab. 8: Composition of Mayer's hemalum solution

Volume	Ingredient
1 g	Hematoxylin is dissolved in
1000 ml	Distilled water
0.2 g	Sodium iodate
50 g	Potash alum
50 g	Chloral hydrate
1 g	Citric acid

Tab. 9: HE-staining procedure

Step	Solution	duration
De-paraffinizing	X-tra Solv (Merck)	3 x 10min
Dehydration	100 % isopropanol	2 x 5 min
	96 % ethanol	3 min
	90 % ethanol	3 min
	80 % ethanol	3 min
	70 % ethanol	3 min
	Distilled water (aq. dist)	briefly
Staining	Mayer’s hemalum solution	15 min
	Blueing under flowing tap water	10 min
	Aqua dist.	briefly
	0.1 % eosin	5 min
	Aqua dist.	briefly
Dehydration	70 % ethanol	briefly
	80 % ethanol	briefly
	90 % ethanol	briefly
	96 % ethanol	briefly
	100 % isopropanol	2 x 5 min
	X-tra Solv (Merck)	3 x 10 min

Mayer's hemalum solution stains negatively charged as well as basophilic substances blue like the nucleus. Eosin, on the other hand, stains cytoplasm, connective tissue and acidophilic substances red.

Masson–Goldner stain (Romeis 2010)

Reagents:

- Phosphomolybdic acid – Orange G:

  Dissolve 8 g phosphomolybdic acid and 4 g Orange G in 200 ml distilled water.

- Lightgreen SF yellowish:

  0.4 g lightgreen and 0.4 g glacial acidic acid to 200 ml distilled water

Tab. 10: Composition of Weigert's hematoxylin

Compound	Ingredient
Solution A	1 g hematoxylin dissolved in
	100 ml ethanol
Solution B	1.16 g iron trichloride (FeCl3) dissolved in
	99 ml distilled water, add
	1 ml hydrochloric acid
Usage solution	1:1 ration of both solutions, prepare freshly

Tab. 11: Acid fuchsin – xylidine Ponceau – azophloxine-staining solution

Compound	Ingredient
Solution A	0.2 g xylidine Ponceau
	0.1 g acid fuchsin
	0.6 ml glacial acid dissolved in
	300 ml distilled water
Solution B	0.5 g azophloxine dissolved in
	100 ml distilled water, add
	0.2 ml glacial acidic acid
Usage solution	16 ml solution A
	4 ml solution B
	176 ml 0.2 % acidic acid

This overview stain colors nuclei brown-black, cytoplasm and muscle red, erythrocytes orange and collagenous tissue green.

Tab. 12: Masson-Goldner stain – timetable

Step	Solution	Duration
De-paraffinizing	X-tra Solv (Merck)	3 x 10 min
Hydration	100 % isopropanol	2 x 5 min
	96 % ethanol	3 min
	90 % ethanol	3 min
	80 % ethanol	3 min
	70 % ethanol	3 min
	Aqua dist.	briefly
Staining	Weigert's hematoxylin	15 min
	Blueing under flowing tap water	10 min
	Acid fuchsin – xylidine ponceau – azophloxine	5 min
	1 % acetic acid	briefly
	Phosphomolybdic acid – Orange G	5 min
	1 % acetic acid	briefly
	Lightgreen	5 min
	1 % acetic acid	briefly
Dehydration	70 % ethanol	
	80 % ethanol	
	90 % ethanol	
	96 % ethanol	5 min
	100 % isopropanol	2 x 5 min
	X-tra Solv (Merck)	3 x 10 min

Alcian blue 8 GS - periodic acid - Schiff stain (PAS-stain)(Romeis 2010)

Alcian blue 8 GS at pH 2.5 is used to stain acid mucins and other negatively charged macromolecules. Mucins appear turquoise, the background pink. PAS stain is used to detect glycogen, glycolipids, hyaline, chitin, cellulose, collagen and neutral mucins which all appear bright red.

Tab. 13: Alcian blue 8GS - PAS staining procedure

Step	Solution	Duration
Deparaffination	X-tra Solv (Merck)	3 x 10 min
Rehydration	100% isopropanol	2 x 5 min
	96 % ethanol	3 min
	90 % ethanol	3 min
	80 %, ethanol	3 min
	70 % ethanol	3 min
	Aqua dist.	briefly
Alcian blue 8 GS	3 % acetic acid	3 min
	Alcian blue solution	30 min
	3 % acetic acid	3 min
	Aqua dist.	5 min
PAS reaction	0.5 % periodic acid	10 min
	Tap water	briefly
	Aqua dist.	briefly
	Schiff's reagent	15 min
	Sulphite water	3 x 2 min
	Blueing under flowing tap water	15 min
	Aqua dist	briefly
Counterstaining	Mayer's hemalum solution	briefly
	Blueing under flowing tap water	10 min
	Aqua dist	briefly
Dehydration	70 % ethanol	briefly
	80 % ethanol	briefly
	90 % ethanol	briefly
	96% ethanol	briefly
	100 % isopropanol	2 x 5 min
	X-tra Solv (Merck)	3 x 10 min

Reagents:

- Alcian blue 8 GS:

  1 % Alcian blue 8 GS in 3 % acetic acid (pH 2.5)

- Periodic acid solution:

  Solve 1g periodic acid in 100 ml Aqua dist.

- Sulphite water:

  Add 10 ml 1 N HCl and 10 ml 10 % aqueous solution of sodium and potassium pyrosulphate. Always prepare freshly.

2.4.5 Analysis

The stained slides were examined with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) and photographed with an associated digital camera (ColorView, Soft Imaging Systems, Münster, Germany). They were also examined with a Nikon Eclipse 90i microscope (Nikon Instruments Europe B.V., Amstelveen, Netherlands) using the DIC (differential interference contrast) function and a DS-Ri-1 camera (Nikon Instruments Europe B.V., Amstelveen, Netherlands). This technique, invented in the 1950ies, is used to image living or stained specimen containing little optical contrast if bright field illumination is used. The advantage of this technique of light microscopy is the brilliancy of colors, the high resolution, and the occurring three dimensional effects of the images.

2.5 Native preparation of the chorion

Fathead minnow and zebrafish eggs were dechorionated mechanically and small parts of the chorion were transferred at different time stages of the embryo development (24, 48, 72, and 96 hpf for the fathead minnow and 24, 48 and 72 hpf for the zebrafish) onto gelatin-coated slides and dried at 40°C in an incubator. The chorion was orientated in order to expose either the outer or the inner surface. These slides were stained in the same way as the paraffin sections (2.4.4).

Gelatin-coated slides were prepared by submerging them for several days in 96 % ethanol, washing them in distilled water, and immersing them briefly in 0.5 % gelatin solution at 40 °C. Excess gelatin solution was removed, and the slides were dried over night at 60°C.

Cross sections were examined with a Nikon Eclipse 90i microscope (Nikon Instruments Europe B.V., Amstelveen, Netherlands) using the differential interference contrast (DIC) function. Pictures were taken using a DS-Ri-1 camera (Nikon Instruments Europe B.V., Amstelveen, Netherlands).

2.6 Dichloroflurescein staining

2.6.1 2,7-Dichloroflurescein

2,7-Dichlorofluorescein (DCF, Fig.4) is a derivative of fluorescein and quite lipophilic (log Pow: 4.7). It is a dye and very sensitive to pH. Therefore, the excitation and absorption spectra are pH-dependent. DCF has an excitation maximum of 504 nm and an emission maximum of 529 nm (in 0.1 M Tris pH 8.0, Sigma Aldrich). Its fluorescence emission intensity is reduced quickly, if exposed to light, a process known as photo bleaching (Von der Golz 2009). Therefore, it is necessary to protect stock solutions and exposed specimen from light.

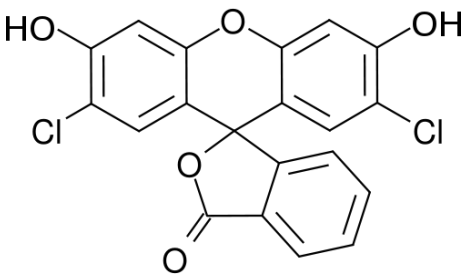


Fig. 4: Chemical structure of 2,7-dichlorofluorescein

2.6.2 Procedure of DCF staining

24 and 48 h old fathead minnow eggs were incubated for 24 hours in 25 mg/ml dichlorofluorescein (0.1 % DMSO). Exposed embryos and eggs were rinsed in 24-well plates at least 2 times with dilution water to avoid external signals. Each well contained one individual egg. Then, the embryos were transferred into 1 ml 0.016 % tricaine solution for anesthesia. For observation, the eggs were put into glass bottom culture dishes™ (MatTek Corporation, Ashland, USA) containing dilution water (eggs < 24hpf) or 0.016 % tricaine solution (eggs > 24hpf) given the onset of spontaneous movements. Dechorionated embryos were embedded in 1 % low melting agarose (SeaPlaque® GTG® Agarose, FMC Bioproducts, Rockland, USA).

Images of all specimen were obtained using the confocal function of a Nikon ECLIPSE 90i microscope (Nikon Instruments B.V., Amstelveen, Netherlands) equipped with a 10x water-immersion objective (CFI Plan Fluor 10x W, NA 0.3, W. D. 3.5 mm). The characteristic feature of confocal microscopy is its ability to generate in-focus images of thick specimen at selected depths, a process known as optical sectioning. Images are obtained point by point and are reconstructed using a computer, thus allowing three-dimensional reconstructions of topologically complex objects. 2,7-dichlorofluorescein (DCF) was filtered by FITC with an excitation spectrum of 480 ± 15 nm, an emission spectrum of 535 ± 20 nm and with a dichroic mirror of 505 nm.

2.7 *In vivo*-EROD assay

The *in vivo* EROD-assay (Ethoxyresorufin-*O*-deethylase) is used to detect dioxin-related substances in complex environmental samples by using live imaging. Dioxin-related substances are able to bind to the aryl hydrocarbon (Ah)-receptor due to their chemical structure. The binding induces the assembly of cytochrome P450-dependent mono-oxygenases (CYPs) which play an important role in detoxification. The presence of dioxin-related substances and, therefore, the presence of CYPs in the body of the fathead minnow can be detected by using ethoxyresorufinm which is deethoxylated into the fluorescent resorufin by the CYPs.

As a negative control, dilution water (2.3.1), and, as a positive control, 10µg/L ß-naphthoflavone was used, given to its strong binding abilities to the Ah-receptor. The fathead minnows were incubated in dilution water at 25 ± 1°C. After 48, 72, and 96 hpf, the embryos were transferred for dechorionation into a 2 mg/L pronase solution. After dechorionation and rinsing in dilution water, the embryos were transferred into 24 well plates containing either 2 ml dilution water per well as a negative control or 2 ml ß-naphthoflavone (10 µg/L, prepared from 0.1 g/L DMSO stock solution) as a positive control. 120 hpf and 144 hpf old embryos were transferred without dechorionation into 24-well plates.

The embryos were either incubated in the positive control for 3 hours or 24 hours. After the incubation time, the dechorionated embryos were transferred into another 24-well plate for washing. The first two rows of the plate contained 2 ml dilution water to wash off excess ß-naphthoflavone, the third row contained 1 ml Ethoxyresorufin, in which the embryos were incubated for 20 minutes. From this step onward, the plates needed to be kept in darkness because ethoxyresorufin is light-sensitive. The last row contained 1 ml tricaine solution to anaesthetize the embryos. While incubating the embryos in ethoxyresorufin, a 1 % low melting agarose with tricaine was prepared. The anaesthetized embryos were transferred into glass bottom culture dishes™ (MatTek, Ashland, USA), and excess water was removed. The embryos were then covered with 1 % agarose at 30 ± 3 °C and orientated on the right side (embryos looking to the left) using featherweight forceps.

For analysis, a Nikon Eclipse 90i microscope with a CFI Plan Fluor 10x/0.30 W objective (Nikon Instruments) using both the confocal and the epifluorescence functions, was used to examine the embryo. For confocal images, the resorufin was filtered by Texas Red HYQ with an excitation spectrum of 560 ± 20 nm, an emission spectrum of 630 ± 30 nm and a dichroic mirror of 595 nm. Image software NIS-Elements 4.0 and a DS-Ri-1 camera (Nikon Instruments) were used to obtain images.

The purpose of an epifluorescence microscopy is to irradiate the specimen with light of specific wavelength(s), and to separate the emitted fluorescence from the excitation light using a dichroic mirror. In a properly adjusted microscope, only the emitted fluorescence should reach detector resulting in an image which shows the fluorescent structures in high contrast against a dark background.

2.8 Transmission electron microscopy (TEM)

2.8.1 Fixation, dehydration and embedding

Eggs of the fathead minnow were transferred into 1 ml 2.5 % glutaraldehyde solution in 0.1 M cacodylic acid buffer (Tab. 14) at 24, 48, 72 and 96 hpf, , and incubated at 4°C at least overnight.Following the fixation in glutaraldehyde, a second fixation in 1 % reduced osmium tetroxide solution (Tab. 15) in cacodylate buffer at pH 7.4 (Karnovsky 1967) is performed.

The en bloc-contrasting is achieved by using a 1 % solution of uranyl acetate in 0.05 M maleic acid buffer at a pH of 5.2 at 4°C. Subsequent dehydration of the samples is achieved by using a graded series of ethanol. Samples were embedded in Spurr's medium (Spurr 1969). All procedures concerning Spurr's medium were performed at 4 °C in a rotator. Compounds of the Spurr's medium were purchased at Serva (Tab. 17). Samples were adjusted in embedding moulds (doubled end mould; Ted Pella, Redding, USA). The overall timeline for the sample preparation is shown in Tab. 18.

Tab. 14: Composition of 0.1 M cacodylic acid buffer

Volume	Ingredient
5.56 g	Cacodylic acid $Na(CH_3)2AsO_2$ 3 H20 are dissolved in
200 ml	Aqua dist.
5.4 ml	0.2 M HCl and
200 ml	Aqua dist. are added and
pH = 7.4	are adjusted with 1 M HCl

Tab. 15: Composition of 1 % osmium tetroxide solution

Volume	Ingredient
50 ml	4 % osmium tetroxide (OsO4) stock solution
50 ml	Aqua dist.
100 ml	1 % potassium ferrocyanide

Tab. 16: Composition of 0.05 M maleic acid buffer

Compound	Ingredient
Solution A	23.2 g maleic acid and 8 g sodium hydroxide (NaOH) are dissolved in 1 L Aqua dist.
Solution B	0.2 N sodium hydroxide (NaOH)
Usage solution	100 ml solution A and 14.4 ml solution B are added to 85.6 ml Aqua dist. and pH= 5.2 is adjusted with 1 N NaOH

Tab. 17: Composition of Spurr's medium

Weight	Compound
26.0 g	Nonenylsuccinicanhydride (NSA)
10.0 g	Epoxycyclohexylmethyl (ERL 4221)
6.0 g	Diglycid ether (DER 736)
0.4 g	Diethylaminoethanol (DMAE)

Tab. 18: Overall timeline for sample preparation

Step	Solution	Duration	Temperature
Fixation	2,5 % glutaraldehyde solution	at least overnight	4 °C
	0.1 M cacodylic acid buffer	3 x 10 min	4 °C
	1 % reduced OsO4 solution	2 h	20 °C
	0.1 M cacodylic acid buffer	3 x 10 min	4 °C
En bloc-contrasting	0.05 M maleic acid buffer	3 x 10 min	4° C
	1 % uranyl acetate solution	overnight	4 °C
	0.05 M maleic acid buffer	3 x 10 min	4 °C
Dehydration	30 % ethanol	15 min	4 °C
	40 % ethanol	15 min	4 °C
	50 % ethanol	15 min	4 °C
	60 % ethanol	15 min	4 °C
	70 % ethanol	3 x 10 min	4 °C
	80 % ethanol	3 x 10 min	4 °C
	90 % ethanol	3 x 10 min	4 °C
	100 % ethanol	3 x 10 min	4 °C
Embedding	1:1 Spurr / 100 % ethanol	overnight	4 °C
	3:1 Spurr / 100 % ethanol	overnight	4 °C
	Spurr	overnight	4 °C
	Spurr	16 h	75 °C

2.8.2 Sectioning and staining

Sections were obtained with a Reichert-Jung Ultracut microtome (Leica). A diamond knife (Diatome, Biel, Switzerland) was used to cut ultrathin sections. The thickness of the sections was determined using interference colors according to (Robinson et al. 1985). To obtain good quality electron microscopy pictures, only sections that appeared silver-gray were used (approx. 70 nm).

Ultrathin sections were transferred onto copper grids (100 meshes, hexagonal) and dried on filter paper. Copper grids were coated before use with 0.6 % Formvar (polyvinyl formal, Monsanto Chemical Company, St. Louis, USA) in chloroform.

Staining of ultrathin sections was performed by 3 % uranyl acetate in 70 % methanol and lead citrate solution according to (Reynolds 1963). The sections on the copper grids were also incubated for 3 min with uranyl acetate and lead citrate solutions (Tab. 19). Between both staining steps, the grids were washed once in 50 % methanol, and two times in double-distilled water. After staining, the grids were rinsed with double-distilled water and dried on filter paper.

Tab. 19: Compounds of lead nitrate solution

Portion	Compound
3.52 g	Trisodium citrate dehydrate
2.66 g	Lead-II-nitrate
16 ml	1 N NaOH
84 ml	Double distilled water

Contrasted and dried sections were examined with a Philips CM10 and with a JEOL JEM-1400 transmission electron microscope (JEOL Germany GmbH, München, Germany). Images taken with the CM10 needed to be developed; JEM-1400 images were already digitalized. Negatives were scanned; all images were analysed using ImageJ.

3 Results

3.1 Behavior of the mature fathead minnows in the breeding tanks

The minnows in the spawning tanks were found to be prone to disturbances. Therefore, the tanks were covered on all four sides with black paper; the front cover was created as a removable blind to maintain good observability of fish, to detect eggs and to clean the tanks. In order to reduce stress, cleaning was only performed once a week. However, it still took months for the fish to adapt to the conditions in the spawning tanks. At the beginning of the experiment, they often chose the weekend for spawning, because it was the quietest time in the aquaria rooms. Fish, which were kept at winter conditions, needed some time to adjust to the new surroundings and the higher level of disturbance (persons in the room, lifting of the blinds, cleaning, etc.) when transferred to the spawning tanks. If disturbed, they swam quickly, whereas fish that were already used to the conditions stayed mostly calm and uninterested, except when guarding eggs.

Males inside the spawning tanks showed distinctive breeding colors in black and gold most of the time. Sometimes, the coloration was faint or not visible, but the males were still distinguishable from the females due to their bulky head and black spot on the dorsal fin. The females, on the other hand, had a slender body and head, and displayed a rather dull coloration. If the fish were exposed to stress, the males discolored within seconds. The minnows in all the tanks stayed mostly near the bottom, males inside their territory with the spawning substrate as centre, and females in the surrounding areas. They only swam further up during feeding time, and sometimes the males during fighting. Younger mature fish were much smaller than the old ones (age approx. 3 years). Especially the older males were twice or even three times the size and displayed a more prominent pad on their nape.

The male minnows chose their spawning substrate quite soon after the transfer into the spawning tank. They protected the small PVC houses and the surrounding water against other males and females in the tank by swimming vigorously to the opponent and if the other fish was not giving up it hit the other one in the side with its snout or the dorsal pad. If male fish were guarding eggs, they displayed an even more aggressive behavior and attacked anything that could be a threat to the spawn; they even attacked the hand of the person who removed the spawning substrate to collect the eggs. The minnows that were kept in pairs in smaller tanks (approx. 20 L) showed the same behavior as fish in larger tanks. The male stayed close to or inside its spawning substrate at the rear of the tank, whereas the female stayed behind

the PVC houses close to the air supply or underneath the drain pipe, either alone or, in the 40 L tanks, in groups.

Observed behavior was considered as spawning behavior, when a male and a female fish swam closely side by side inside the spawning substrate with their sides touching each other constantly. However, duration of the procedure varied greatly and sometimes, even though spawning behavior was observed, the fish did not spawn necessarily.

The minnows mainly spawned inside the small PVC houses, in which the female attached the eggs to the lower surface of the house. Nonetheless, sometimes the male minnows tried to establish their territory with the drainpipe as centre, even though proper spawning substrate was available. If this behavior was detected, it was prohibited by placing small sponges underneath the drain pipes. Detection and removal of eggs spawned at the drain pipe or the glass wall was difficult. Therefore, the embryos could hatch inside the spawning tanks and the offspring was not eaten by adult fish. However, a distribution of the minnows according to their size could be observed. The more recently hatched larvae preferred the area right underneath the surface, whereas older larvae stayed in the middle of the water column, while the adult, as stated above, stayed near the bottom of the tank.

Sometimes, the old females displayed some male phenotypes after they were kept in the spawning tanks for several months: they became as big as the males, showed the distinct black spot on the dorsal fin, which is normally only displayed in breeding males, and sometimes they even showed barely visible stripe coloration. Nonetheless, the head shape always remained slender in comparison of the bulky head of the male, and the females never displayed breeding tubercles or a dorsal pad. When the females displayed those phenotypes, they also became quite dominant: They build like the males a territory around a spawning substrate and stayed most of the time within the small PVC house. Less dominant, younger and therefore smaller males had a hard time to establish a territory of their own at the same time.

3.2 Husbandry & breeding

At the beginning, the spawning tanks were stocked with a total of 36 fathead minnows. Each tank contained two male and four female minnows. All detected eggs were counted. Egg number and stage at detection were recorded. Over six months, approximately 18,000 fertilized eggs were counted, ranging between 1,000 eggs in spawning tank B up to nearly 5,000 eggs in spawning tank D. The highest number of eggs in a single spawn was 748 eggs. Usually, the number of fertilized eggs per spawn ranged between 70 and 200. The mean value per

female was calculated as 107 eggs per spawn with a standard deviation of 101. The time span for spawning for each female ranged between 1.5 and 2 months. However, occasionally, females only spawned once or twice before they stopped again. The mean fecundity for female minnows was 242 ± 150 eggs. The overall fecundity ranged from just below 100 up to 500 eggs per female. If eggs were older than 24 hours at detection, for example after weekends, male minnows had already got rid of unfertilized eggs within the clutches.

To compare size of spawning groups and spawning success, couples of fathead minnows (one male and one female) were transferred into a 20 l tank with a spawning substrate and egg production was recorded. Out of six couples, only three spawned. Of these three, two couples only spawned four times within 12 and 14 days, respectively. The first spawn of this group was the largest with 260 and 460 eggs, respectively. Only one group managed to spawn over one and a half month, spawning between 140 and 235 eggs per spawning incident, which were approximately four days apart.

During the experimental time (six months, June to December 2011), 57 fish died: 42 female and 15 male minnows. Fish were either restocked from the minnow stock kept at 12 °C, or groups were rearranged. Rearranging was done twice, once in August and once in December. A total number of 94 adult fish were used during the experimentation time. Prior to death, fish displayed discoloration of the body as well as protruding eyes. Both females and males changed color, and had a dark back and a whitish-silvery belly. The belly was often swollen in comparison to healthy fish. They all suffered from severe signs of disorientation and had problems of maintaining neutral buoyancy: they were not able to swim down to the bottom any longer and stayed in the upper part of the water column. If their condition became more severe, they started swimming in spirals. They seemed apathetic, showed accelerated breathing and were easy to catch. Fish that showed these conditions were taken out of the tank and killed using benzocaine. The mortality of the minnows was 1:3, for one male three females died. In comparison, the sex ratio in the spawning tanks was 1:2. Water criteria (Tab. 20) were monitored and recorded for the six months of the experiments as well.

Tab. 20: Water criteria in the spawning tanks (n = 9)

	Tank A	Tank B	Tank C	Tank D	Tank E	Tank F
Temperature [°C]	23.5 ± 0.9	23.4 ± 0.8	23.4 ± 0.8	23.6 ± 0.7	23.6 ± 0.8	23.6 ± 0.8
pH	8.1 ± 0.2	8.1 ± 0.2	8.0 ± 0.2	8.0 ± 0.2	7.9 ± 0.3	7.9 ± 0.3
Oxygen content [%]	96 ± 5	95 ± 5	95 ± 4	95 ± 3	96 ± 3	96 ± 3
Conductivity [$\mu S/cm^2$]	668 ± 49	655 ± 43	663 ± 57	653 ± 41	660 ± 45	654 ± 52

3.2.1 Feeding regime

The feeding regime applied to the fathead minnows at all stages worked properly. Small larvae just after hatching were fed liquid rearing food containing grounded brine shrimp *Artemia* and other important nutrients (NobilFluid *Artemia*). For a few days, the diet was enriched with powder food also containing *Artemia* (NovoTom *Artemia*) three times daily. After a month, the young fish were fed with fresh *Artemia* twice a day plus a third time with crushed flake food (TetraMin^{TM}). After three months, the food was enriched with frozen brine shrimp (*ad libidum*) substituting *Artemia* once daily. The fathead minnow stock held at 12 °C was fed every other day with flake food to simulate winter conditions where food is scarce in nature.

The fish in the spawning groups were fed three times a day: with frozen brine shrimp in the morning and flake food at noon and at night. Then, the amount of frozen adult brine shrimp was reduced in comparison to the suggestion of the Environmental Protection Agency (Denny 1987). To enrich food variety and due to logistical problems, feeding with frozen brine shrimp was reduced to once daily. Minnows were fed with frozen brine shrimp in the morning and with flake food at noon and for the night feeding. This change in the feeding regime did not seem to impair reproduction success.

3.2.2 Spawning substrate

Two different spawning substrates were tested: clay pot halves and small houses. They both simulated crevices, the fathead minnow normally spawn into in the wild with three sides. The houses were made of PVC tube halves with matching trays. It turned out that the PVC houses were better to use than the clay halves.

The PVC halves had a smoother surface which prevented the accumulation of limescale and algae, providing a more hygienic substrate for spawning. Even if there was debris inside the

house, it could be cleaned more easily under running hot tab water. The only problem was that debris accumulated underneath the trays. The smoother surface also allowed an easier detection of eggs attached to it because the light of the flashlight was reflected. The grey color of the PVC halves, also allowed better detection of the eggs. Due to the smoother surface it was much easier to remove the spawned eggs from the substrate. The trays attached to the spawning tanks proved very useful because some of the female fish laid most of their eggs at the bottom or the eggs were not properly attached to the surface and fell down. It was observed that the eggs on the surface of the trays were always scattered, whereas the eggs attached to the lower surface were always in clutches, and therefore had to be separated with featherweight forceps.

3.2.3 Handling of fathead minnow eggs

Egg clutches could be detached from the spawning substrate with both methods without any difficulties. Scraping eggs off the substrate using the spatula proved to be an easy and time-saving method to detach eggs, but eggs could only be detached in clutches and needed to be separated in a second step. Rolling the eggs with the thumb carefully off the substrate needed training to be carried out without difficulties. If the pressure applied was too much, the eggs burst. If rolling was performed, the egg clutches were separated to a certain degree during the process. This saved time during the subsequent separation. Normal loss in egg number during the separation process was about 2 %, when egg quality was good. Eggs could be separated by mechanical means immediately after collecting using a stereo microscope, the spatula and featherweight forceps. Egg stages, as early as 4-cell stages, were separated without any major losses (< 5 %). Then, eggs could be used immediately for fish embryo toxicity tests or other experiments.

3.2.4 Measurements of 24 h old fathead minnow eggs

The egg of the fathead minnow was measured on the assumption that both the chorion and the embryo have a spherical shape (Tab. 21). The thickness of the chorion was determined using transmission electron microscopy pictures.

Tab. 21: Measurements of the 24 h old fathead minnow egg (n = 80; at 25 ±1 °C)

Egg diameter	1.378 ± 0.066 mm
Egg surface	5.98 ± 0.578 mm^2
Perimeter of the egg	4.33 ± 0.208 mm
Thickness of the chorion	7.1 ± 1.8 µm
Wet weight of the egg	1.1192 ± 0.0302 mg
Liquid content	0.956 ± 0.021 mg
Liquid content [%]	85.4 ± 1.6 %
Dry weight of embryo	0.1629 ± 0.0209 mg
Density of the egg	~ 810 g/L
Total volume of the egg	1.380 ± 0.201 µl

For each geometrical value, 80 eggs were measured. A fathead minnow egg is around 1.4 mm in diameter. The perimeter of the egg is approximately 4.33 mm long, and the surface measures nearly 6 mm^2. The volume of the egg is approximately 1.38 µl.

For weight measurements, 300 eggs were measured in three batches of 100 eggs each. The wet weight for each egg was 1.119 ± 0.030 mg and the dry weight after 24 hours dehydration under the extraction hood was 0.1629 ± 0.021 mg. This resulted in a water content of 85.4 %, when both measurements were compared. Egg density which was calculated using the wet weight and the total volume of the egg was about 810 g/L.

3.2.5 Dechorionation

Mechanical approaches

Firstly, dechorionation by mechanical means only proved to be difficult. The procedure was carried out on petri dishes coated with 2 % agarose. It was very challenging to get a hold on the chorion with the Dumont forceps. Small dents in the agarose proved helpful in which the eggs were pressed against the rim to grip the egg. It was important not to lacerate the chorion because as soon as the chorion was damaged by the forceps the yolk sac, mostly, or another part of the embryo in close proximity to the laceration exploded through the opening into the surrounding medium. This did not only damage the embryo; it made it even more difficult to remove the chorion. Some damages resulted in the instant death of the embryo, others survived with a malformed yolk sac, spine or tail (Fig. 6). There was a correlation between the age of the embryos and the success of the mechanical dechorionation (Fig. 5). At 24 hpf, 20

out of 60 embryos survived the procedure, at 48 hpf 78 %, and at 72 hpf 88 % survived. 24 hours after dechorionation (hpd), only one out of twenty remaining embryos had survived (1.7 %), at 48 hpf more than 75 % survived the first 24 hpd, and no mortality could be observed for embryos 24 hpd, which were dechorionated at 72 hpf.

In a second step, it was tried to reduce the pressure inside the egg before mechanical dechorionation. Two different methods were tried: The egg was carefully pierced either with a glass patch pipette or a hollow needle (Sterican® size 20, 0.4 x 20mm, Braun, Melsungen, Germany). If the opening in the chorion made by the sharp object was too large or made too quickly and forcefully, the embryo was squeezed out of the chorion as described above. However, this procedure improved the survival rate up to 62.5 % at an embryo age of 24 hours, but the embryos were still damaged either during the pressure release or while the chorion was torn open. After 24 hpd, 40 % of the transferred embryos had survived. The total survival rate compared to the start of the experiment was 8.4 %. That meant an increase of 6.7 % compared to a exclusively mechanical approach. Additionally, the procedure proved to be very labour- and time-intensive.

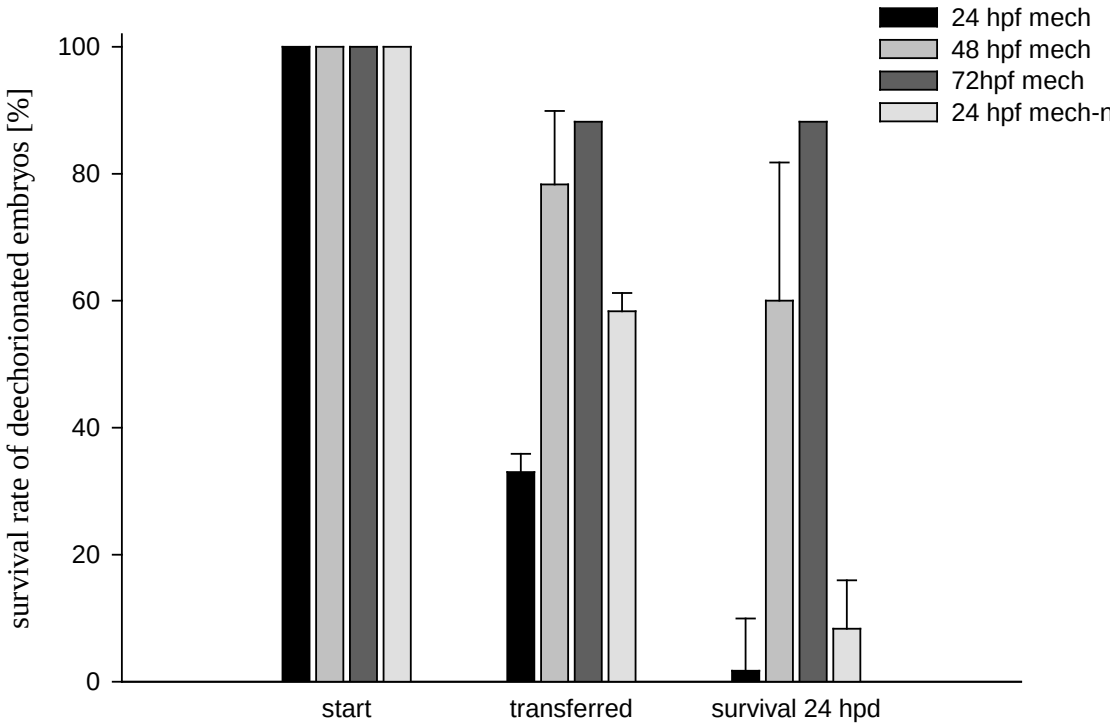


Fig. 5: Success rate of mechanical dechorionation after 24, 48 and 72 hpf, as well as after pressure release.

Fig. 6: 72 hpf embryos that were dechorionated mechanically after 48 hours. Arrows show damages of the spine that occurred during the dechorionation procedure.

Chemical and combined (mechanical and chemical) approaches

The first chemical approach was carried out using 1 mg/L pronase according to Henn et al. (2011). This concentration proved insufficient to dechorionate zebrafish within minutes. Fathead minnow eggs incubated with 1 mg/L pronase showed no change in chorion structure or hardness even after two hours incubation. After a literature search, the pronase concentration was increased by a factor twenty thousand. A stock solution with a concentration of 20 mg/ml was prepared in order to find the most effective pronase concentration.

Embryos were exposed for 5, 7, 10, 15 min and until they dropped out of the chorion, respectively. Three different concentrations were tested (5, 10 and 20 mg/ml), as well as the influence of agarose as coating of the wells. After each time interval, 4 embryos were transferred into dilution water and rinsed several times. Then embryos were dechorionated by mechanical means using Dumont forceps, when they were still inside the chorion. At 5 mg/ml, the embryos dropped out of the chorion by occasional swirling after 22 minutes, at 10 mg/ml after 18 minutes, and at 20 mg/ml after 15 minutes. All fish survived the dechorionation procedure at all three pronase concentrations. After 24 hours, only one embryo out of 138 did not survive. At 48 hpd, there were still 133 embryos alive. The mortalities were observed at 5 minutes incubation with 5 mg/ml with agarose, where two embryos died, one at 7 minutes and one at 15 minutes incubation with 20 mg/ml without agarose, and one died at 15 minutes incubation 20 mg/ml with agarose. No change in survival was observed after 48 hpd until the experiment was terminated at 120 hpd. Less than 10 % of the fish showed effects. The most common effect was curvature of the tail, but also cardiac edema, reduction of heart beat and circulation could seldom be observed.

In order to reduce the amount of pronase even further, eggs were incubated with 2 mg/ml pronase. Incubation time of five minutes proved to be too short. Embryos still exploded though the chorion when chorion was removed with forceps. Therefore, incubation time was

raised to seven minutes. After the pronase treatment, the embryos were transferred into dilution water and left there with occasional swirling for another seven minutes, before they were dechorionated using forceps. The transfer rate was 100% compared to 33% of the embryos that were dechorionated mechanically at 24 hpf and 62.5 % of the embryos that were dechorionated mechanically with pressure release. The survival rate at 24 hpd was 84 % compared to 1.7 % and 8.4 %, respectively (Fig. 7).

A chemical approach with 2 mg/ml pronase was also carried out with 24 h old embryos. Constant swirling was applied. It took 8.5 minutes until the first embryo dropped out of the chorion, the last one dropping out after 12.5 minutes. They were transferred into dilution water and rinsed thoroughly for at least three times with dilution water. 100 % of the embryos survived the procedure, 97.5 % of the embryos were still alive 24 hours after the procedure. There was no change in the survival rate until 192 hpf, when the tests were terminated.

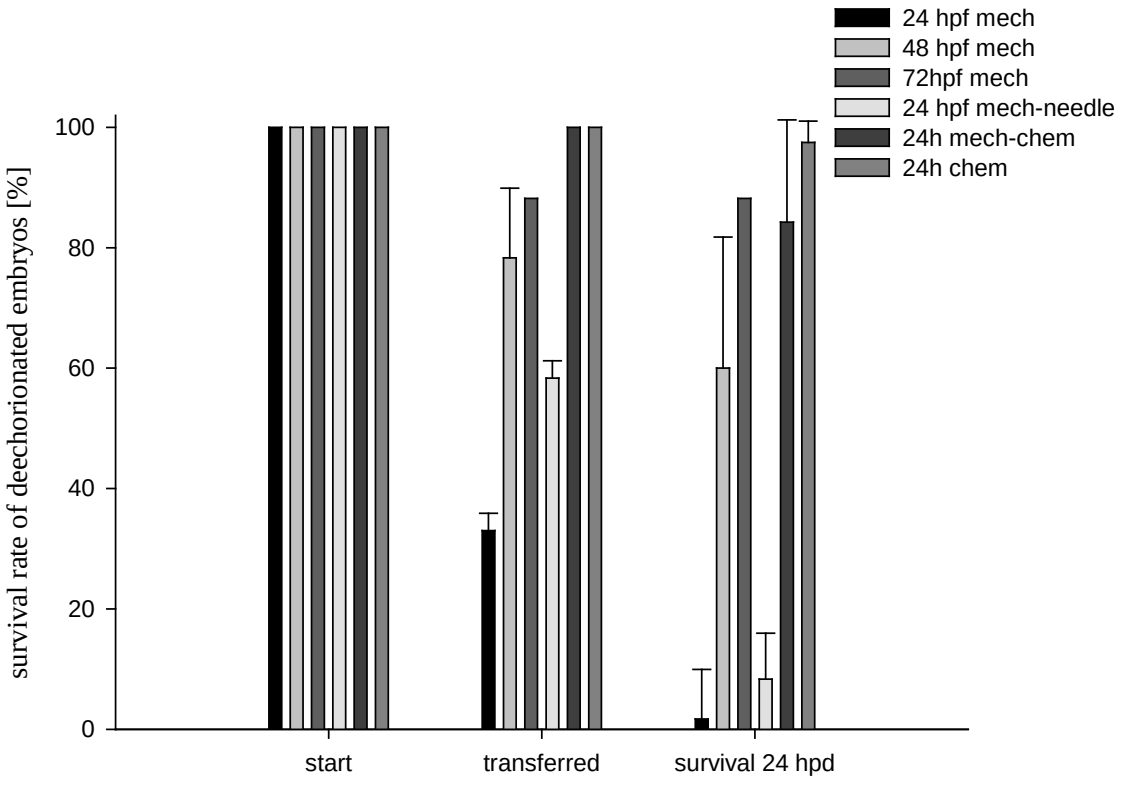


Fig. 7: Overview of all dechorionation procedures used to dechorionate fathead minnow eggs

Combined experiments were carried out to determine whether and which sort of agarose is needed as coating for embryos during and after the dechorionation process. The age of the embryos was 24 hours post fertilization. 2 % high-melting agarose (HMA) was more effective for the coating of the petri dishes than low melting agarose (LMA), because it was more sta-

ble and did not tear as easily as the LMA, but embryos older than 24 hours post fertilization did not need a coating to survive the procedure. With younger stages, coating should be used. The coating of the wells in the 24-well plates was also tested high-melting agarose proved again to be as effective as the low-melting agarose. However, coating of the wells had no influence on survival of the dechorionated embryos which were older than 24 hpf. The diameter of the petri dish used for dechorionation was reduced from18 to 9 centimeters, which proved handier. Agarose-coated petri dishes could be kept for at least two weeks at 4 °C without change in quality. ´The use of cut-off plastic tips or Pasteur pipettes did not impair the overall survival rate of 24 hour old embryo.

3.3 Embryonic development

It was not possible to obtain eggs with the *in vitro* fertilization method. The fish used to obtain eggs were quite stressed due to the procedure. Therefore, it was not possible to observe a recently fertilized egg or a one-celled blastodisc, because eggs could not be detected as early as desired.

Hence, the eggs were collected as early as possible from the spawning substrate as soon as spawning behavior was observed. It took approximately ten minutes from the detection of the eggs until they could be observed and photographed under the microscope. The earliest observed stage was a 2-celled blastodisc (Fig. 8) which marked the beginning of the experiment. The 2-celled blastodisc stage occurred to Devlin et al. (1996) one hour after the fertilization. To allow comparison with Devlin (1996), the timeline of the present observation was adjusted. It was assumed that the two-celled blastodisc stage occurred one hour after fertilization. In the 2-celled blastodisc stage, two equally sized blastomeres are formed on the yolk sac. Ten minutes after the appearance of the cleavage furrow, which marked the transformation into a four-celled blastodisc. This progress was completed within five minutes. The 4-celled blastodisc stage lasted for ten minutes. Half an hour after the start of the observation, the egg transformed further from a 4-celled into an 8-celled blastodisc. It consisted of 2 rows of four blastomeres each. The 8-cell stage was reached five minutes later, 35 minutes after the start of the observation. The 16-celled blastodisc was reached ten minutes later consisting of four rows of four blastomeres. 55 Minutes after the start, the blastomeres had doubled again into a 32-cell blastodisc consisting of four rows of eight blastomeres. After that stage, it became quite difficult to define the time when the blastomeres doubled again. The next observable stage was recognized two and a half hours later. It was determined due to the shape of the

cells which had formed an elevated cup upon the yolk mass. It was termed late cleavage stage. The outermost layer was closely packed with blastomeres and was defined as early epidermal stratum. The high blastula stage was reached an hour later and lasted for about 30 minutes. The epidermal stratum was now distinct; and between the blastoderm and the yolk sac the periblast corona consisting of syncytial cytoplasm could be seen. The next stage was termed flat blastula. The blastoderm became flatter and looked smoother than the high blastula. The periblast corona became more distinct. An hour later, gastrulation started. The yolk became slightly depressed at the side due to a lateral growth of the blastoderm. The 25 % epiboly was reached after 7 hours, 50 % after 8.5 hours, and 75 % epiboly after 10.5 hours. In this time, the yolk was covered more and more by the germ ring. Both ends of the blastoderm appeared to be more prominent than the rest forming the anterior and posterior end of the embryo. After 12 hours, the germ ring had closed over the yolk. Another three hours later, the first five somites had developed. The pericardial coelom, the optic anlagen, and even the three brain regions could be distinguished. After 17 hours after the start, the optic vesicles were clearly observed. The number of somite pairs increased to ten. The notochord is now visible. During the next three hours, the number of somite pairs, as well as the size of the embryo increased. After 19 hours, the first neuromers were observed and the optic cup became more prominent. Two and a half hours later, the otic vesicles formed. The tail started to detach from the yolk, and the Kuppfer's vesicle which is important for the left-right development of the brain, heart and gut, could be observed for the first time. Nearly a day after the start of the observation, the tail finally detached from the yolk and the Kuppfer's vesicle became smaller. Lenses form inside the optic cups, and otic vesicle developed further. The first movement of the embryo was observed after 25.5 hours. The tail was completely detached an hour later, and the size of the pericardial coelom had increased. After 48 hours, the eyes and the blood were pigmented compared to the rest of the body. Heart beat and circulation were present. The yolk sac was the most prominent characteristic of the embryo. After 72 hours, the yolk sac was reduced. Curvature of the embryo around the yolk sac decreased. The fish body started to be lightly pigmented. Tail fin and breast fins started to grow. After 96 hours, the body of the embryo became quite straight, breast fins elongated, and the yolk sac was nearly absorbed giving the fish the ability to swim. After 144 hours, the swim bladder in the free-swimming larvae got coated with guanine crystals. Movement of the eye lenses and ventilation of the gills and could be observed.

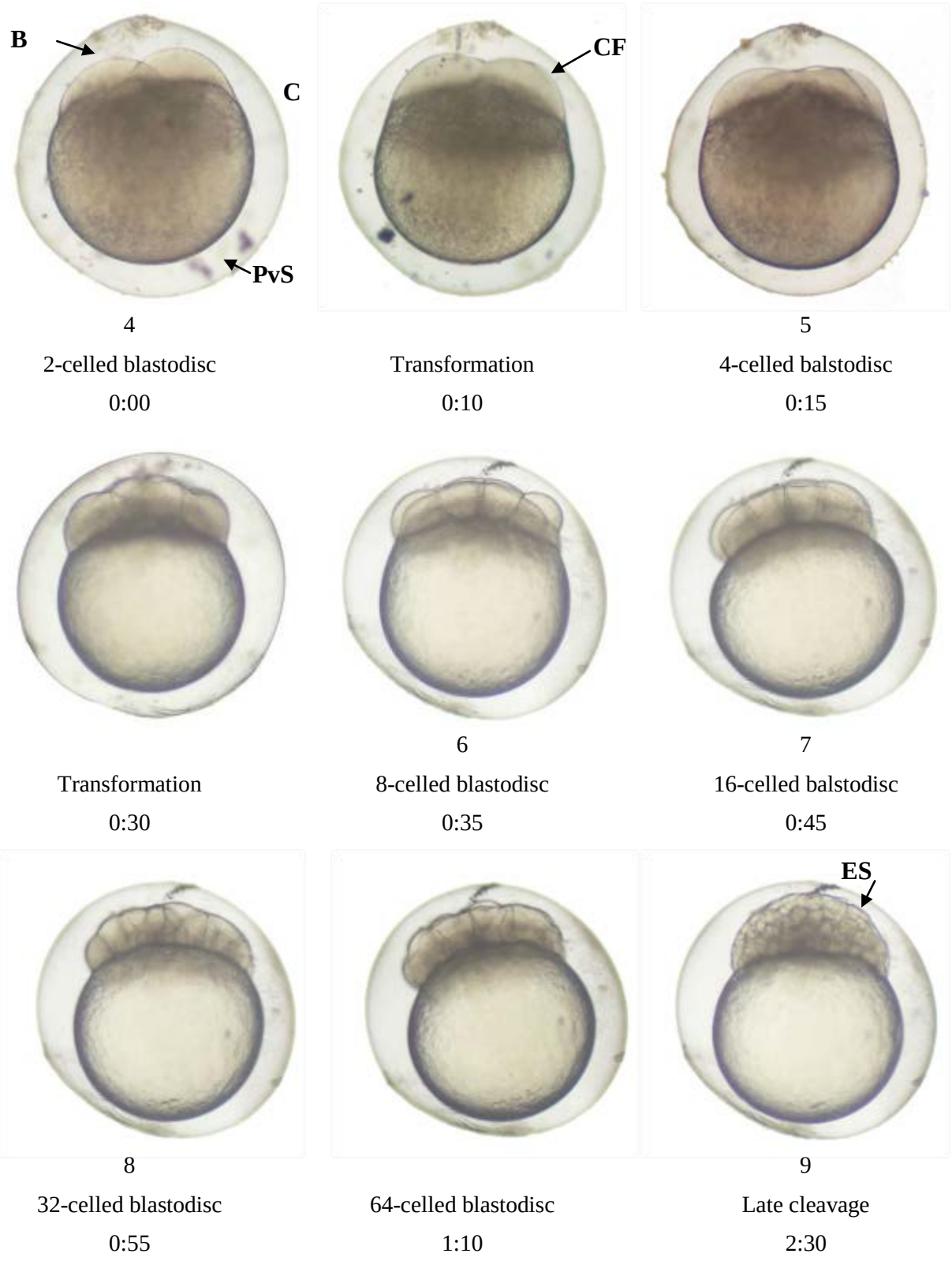


Fig. 8: Normal development of the fathead minnow embryo; number and name of embryo stage according to Devlin et al. (1996) for better comparison; B blastomere, C chorion, CF cleavage furrow, ES epidermal stratum, PvS perivitelline fluid, Y yolk.

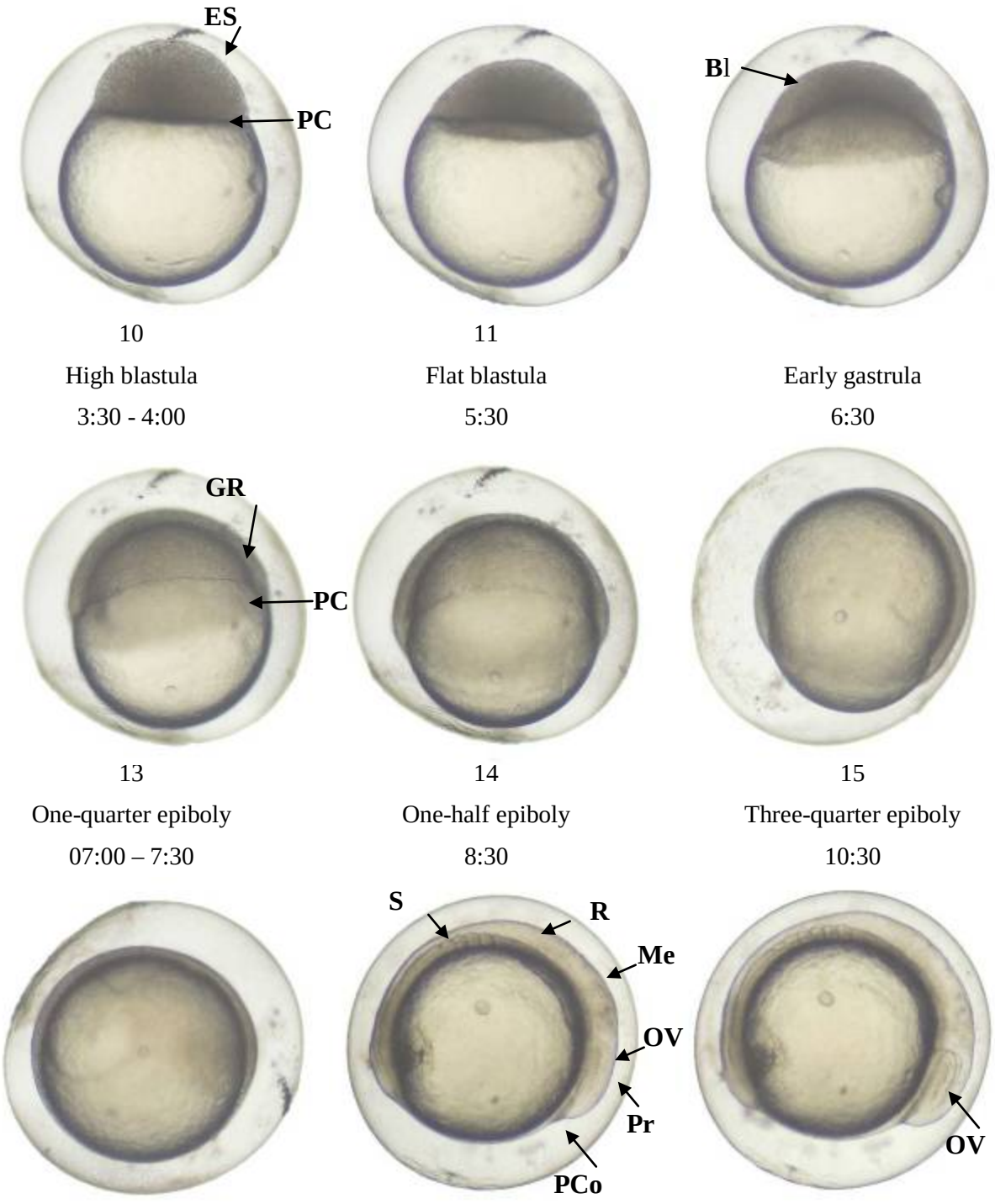


Fig. 9: Normal development of the fathead minnow embryo; number and name of embryo stage according to Devlin et al. (1996) for better comparison. Bl blastoderm, ES epidermal stratum, GR germ ring, Me Mesencephalon, N notochord, OV optic vesicle, PC periblast corona, PCo pericardial coelom, Pr proencephalon, R rhomobencephalon, S somites.

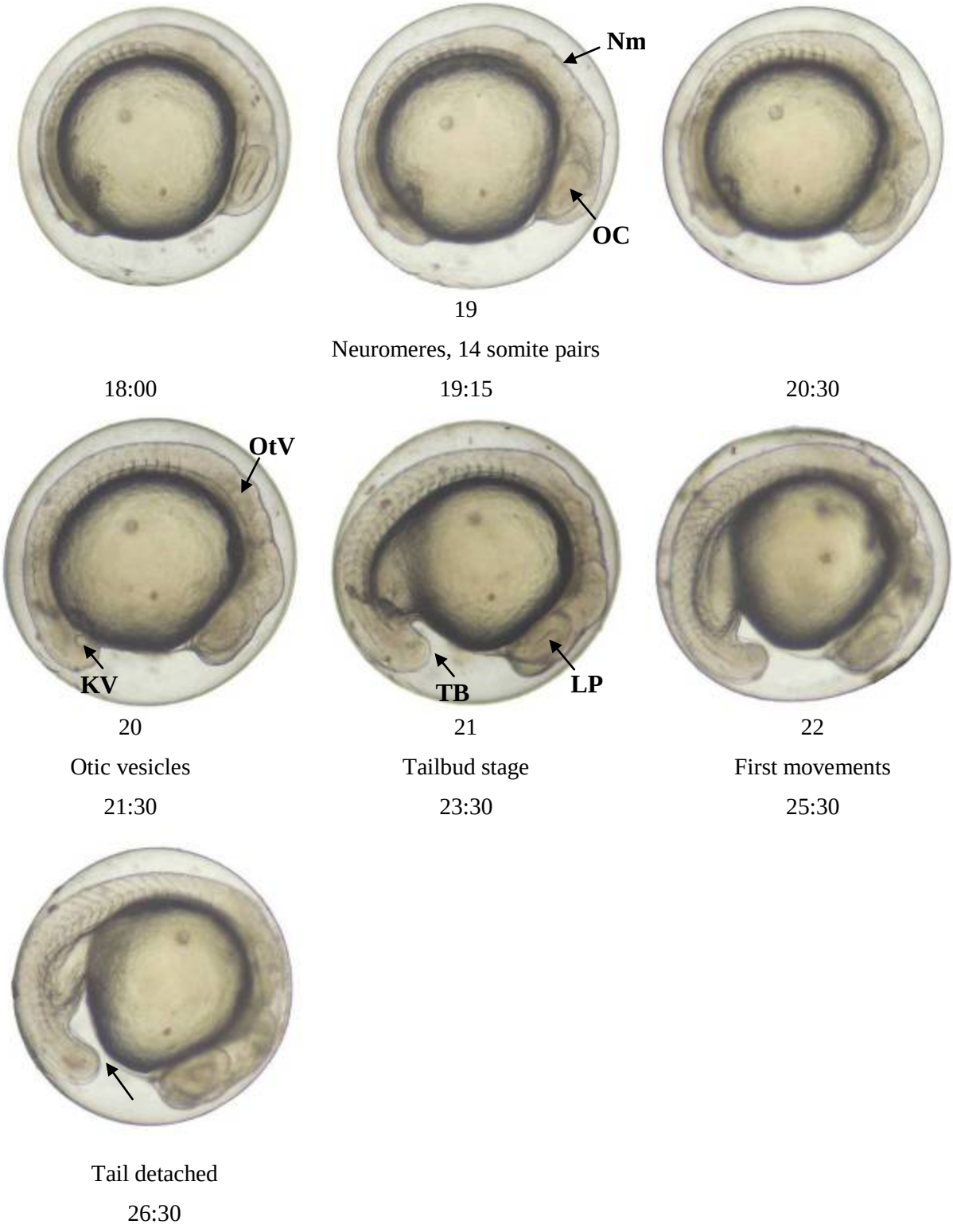


Fig. 10: Normal development of the fathead minnow embryo; number and name of embryo stage according to Devlin et al. 1996 for better comparison; KV Kupffer's vesicle, LP lense placode, Nm neuromeres, OC optic cup, OtV otic vesicle, TB tailbud.

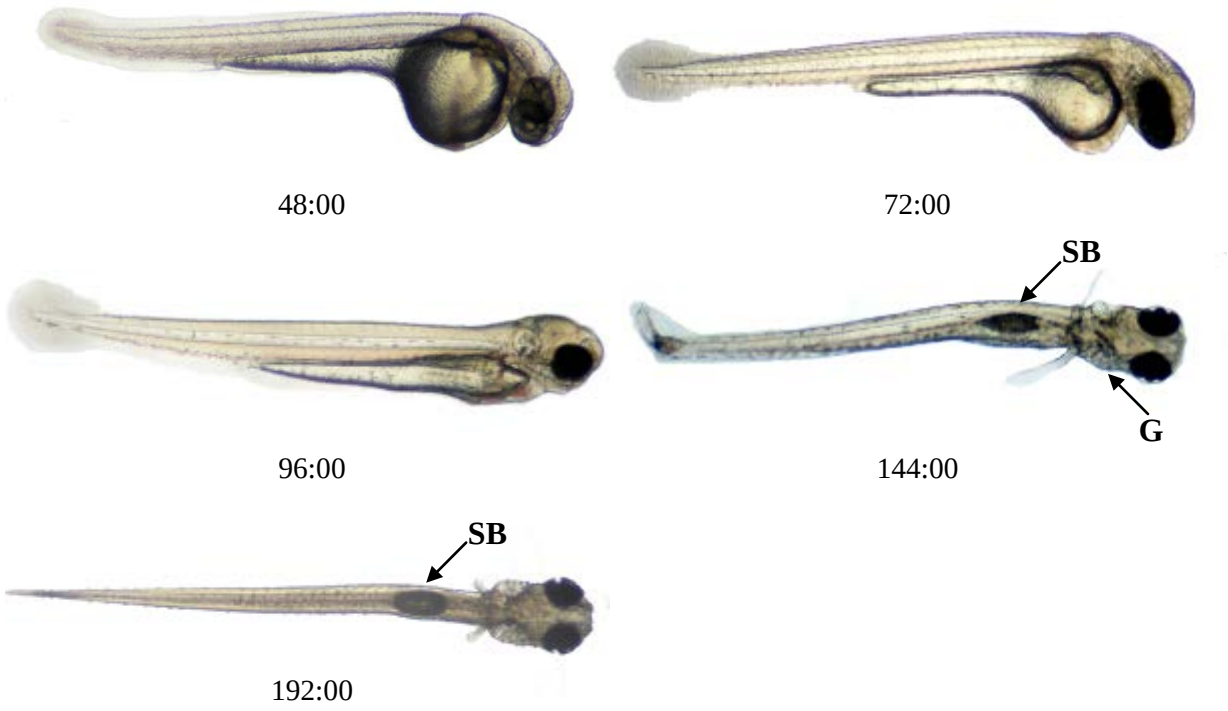


Fig. 11: Normal development of the fathead minnow embryo; G gills, SB swim bladder.

Tab. 22: Overview of the normal development of fathead minnow embryo. Time adjustments made to allow comparison with Devlin et al. (1996). 2-celled blastodisc stage occurs one hour after fertilization.

Stage	Time	Adjusted time	Characteristics of the embryo stage
1	-	0:00	Unfertilized ovum
2	-	0:10	Recently fertilized ovum
3	-	0:40	1-celled blastodisc
4	0:00	1:00	2-celled blastodisc
5	0:15	1:15	4-celled blastodisc
6	0:35	1:35	8-celled blastodisc
7	0:45	1:45	16-celled blastodisc
8	0:55	1:55	32-celled blastodisc
	1:10	2:10	64-celled blastodisc
9	2:30	3:30	Late cleavage
10	3:30 – 4:00	4:30 – 5:00	High blastula

11	4:30	5:30	Flat blastula
12	5:30	6:30	Early gastrula
13	7:00 – 7:30	8:00 – 8:30	One-quarter epiboly
14	8:30	9:30	One-half epiboly
15	10:30	11:30	Three-half epiboly
16	12:30	13:30	Closure of the germ ring
17	15:45	16:45	Neurula stage, 4-5 somite stairs
18	16:55	17:55	Optic vesicles, 9-10 somite pairs
19	19:15	20:15	Neuromeres, 14 somite pairs
20	21:30	22:30	Otic vesiclces, 16 somite pairs
21	23:30	24:30	Tailbud stage, 18-20 somite pairs
22	25:30	26:30	First movements, lens formation
	26:30	27:30	Tail completely detached

3.4 Fish embryo toxicity test

3.4.1 Cadmium chloride ($CdCl_2$)

The pre-tests to confirm the best five test concentrations were carried out in range-finding tests: 0.625, 1.25, 2.5, 5 and 10 mg/L, respectively. Three runs were carried out to determine the toxicity of cadmium chloride to the fathead minnow embryo. The embryos were exposed to the different cadmium chloride concentrations as soon as they were detected, but in different stages of development: at a 128-cell stage in run 1, at an 8 – 32-cell stage in run 2, and at an 8-cell stage in run 3. All runs were within the temperature range of 25 ± 1°C.

Effects (Fig. 12) were observed every 24 hours, starting 24 hours after fertilization (hpf). All embryos showed no signs of effects for all concentrations at 24 hpf. After 48 hpf, the embryos in all cadmium concentrations showed nearly 100 % sublethal effects, namely reduced heartbeat, reduced blood circulation and the formation of cardiac edema. Moreover, the heads of exposed embryos were less developed, and the eyes were less pigmented than those in the negative controls. An accumulation of blood cells in the pericardium could be observed at 1.25 and 2.5 mg/L. After 72 and, 96 hpf, all embryos had the same effects than 24 hours earli-

er just more severe and increasing with concentration. Various embryos exposed to 0.625and 1.25 mg/L developed an accumulation of blood cells in the brain region, sometimes located around the eyes. Embryos exposed to 2.5 mg/L cadmium chloride and more, showed no signs of blood circulation at 96 hpf. Exposed embryos started hatching about 72 hpf, around 24 hours earlier than the negative controls. Hatched embryos (eleutheroembryos) showed a curvature of the spine, problems with balance, convulsion of the body and enlarged somites. Swimming movements, when observed, were not directed. Some embryos were able to rip the chorion but were not able to succeed in hatching and died during the process. This occurred especially at higher concentrations. At 120 hpf, eleutheroembryos showed the same effects as mentioned at 72 and 96 hpf, the only new one was the occurrence of eye edemas. Then, after nearly all of the embryos had hatched, even fish at low concentrations of 0.625 and 1.25 mg/L died due to lack of heartbeat. After 144 hpf, some embryos developed an edema along the yolk sac in the visceral cavity. All embryos, eleutheroembryos and larvae that still lived, showed retardation in development: reduced tail length, less eye pigmentation, no or little reduction of the yolk sac, no or insufficient development of the swim bladder and no or little pigmentation of the body, compared to the negative controls.

The LC_{50} values (Fig. 13, Fig. 14) were determined for all three runs based on the mortality of the fathead minnow embryo throughout the tests using ToxRat. The LC_{50}-values for four different points in time are shown for each run: 48 hpf for comparison with the 48 h zebrafish FET (DIN 2000), 96 hpf for comparison with the zebrafish FET (OECD 2011), 144 hpf for comparison with the prolonged zebrafish FET and 192 hpf as the current endpoint of the fathead minnow FET. After 24 hpf, no mortality could be observed in all three runs. After 48 hpf, lethal effects were only present at the highest test concentration of 10 mg/L, ranging from 30 % in run 2, to 70 % and 100 % in run 3 and 1. Therefore, an LC_{50}-value after 48 hours could only be determined for run 2 and run 3 (7.05 and 9.38 mg/L). Lethality at 72 hpf was 100 % at the highest concentration (10 mg/L), but could not be observed in the others. At 96 hpf, the lethality at 5 mg/L and 2.5 mg/L increased, whereas in run 1 no significant increase in mortality could be observed. The determined LC_{50} values ranged from 5.9 mg/L in run 1, over 3.79 mg/L in run 3, to 2.65 mg/L in run 2. At 120 hpf, first lethal effects were present at 1.25 mg/L, and only in run 3 mortality was observed in the lowest concentration (0.625 mg/L). At 144 hpf, mortality increased further, leading to LC_{50} values ranging between 3.40 mg/L in run 1, 1.62 mg/L in run 2 and 0.95 mg/L in run 3.

At 168 and 192 hpf, the mortality increased slightly for all cadmium chloride concentrations resulting in 100 % mortality at both 10 and 5 mg/L in all three runs. LC_{50}-values determined at the last day of the runs were 1.37 mg/L for run 1, 0.77 mg/L for run 2 and 0.64 mg/L for run 3.

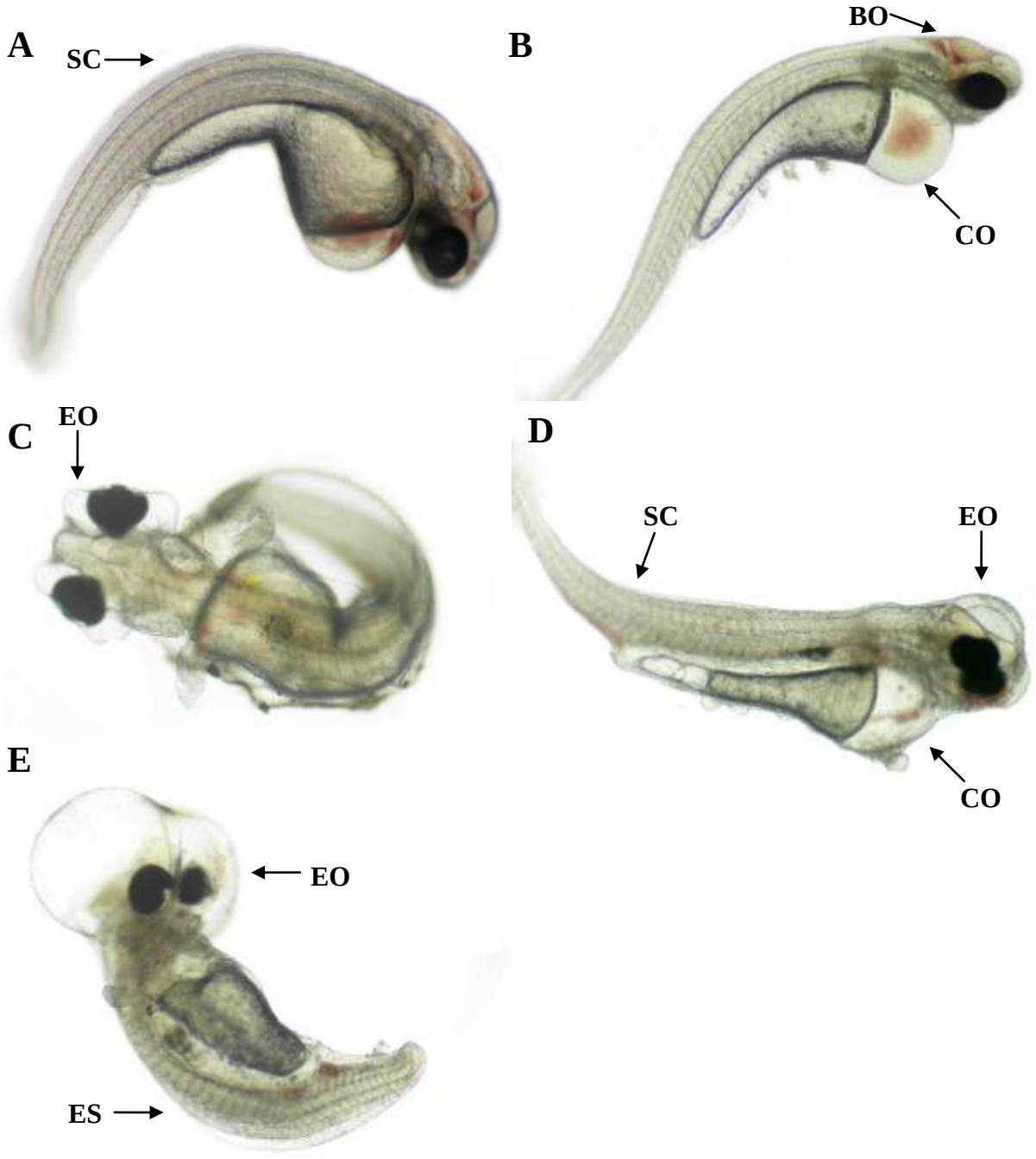


Fig. 12: Embryos treated with cadmium chloride. A) 72 hpf treated with 0.625 mg/L $CdCl_2$ showing brain hemorrhage (BH) and cardiac edema (CO); B) 96 hpf embryo treated with 0.625 mg/L $CdCl_2$ showing more severe effects than 24 hours earlier; C) 144 hpf embryo treated with 2.5 mg/L $CdCl_2$ trying to hatch and showing eye edema; D) 144 hpf embryo treated with 2.5 mg/L $CdCl_2$ showing spine curvature (SC), eye and cardiac edema, and yolk sac malformation; E) 192 hpf embryo treated with 2.5 mg/L $CdCl_2$ showing reduced tail, enlarged somites (ES), huge eye edema and overall malformations.

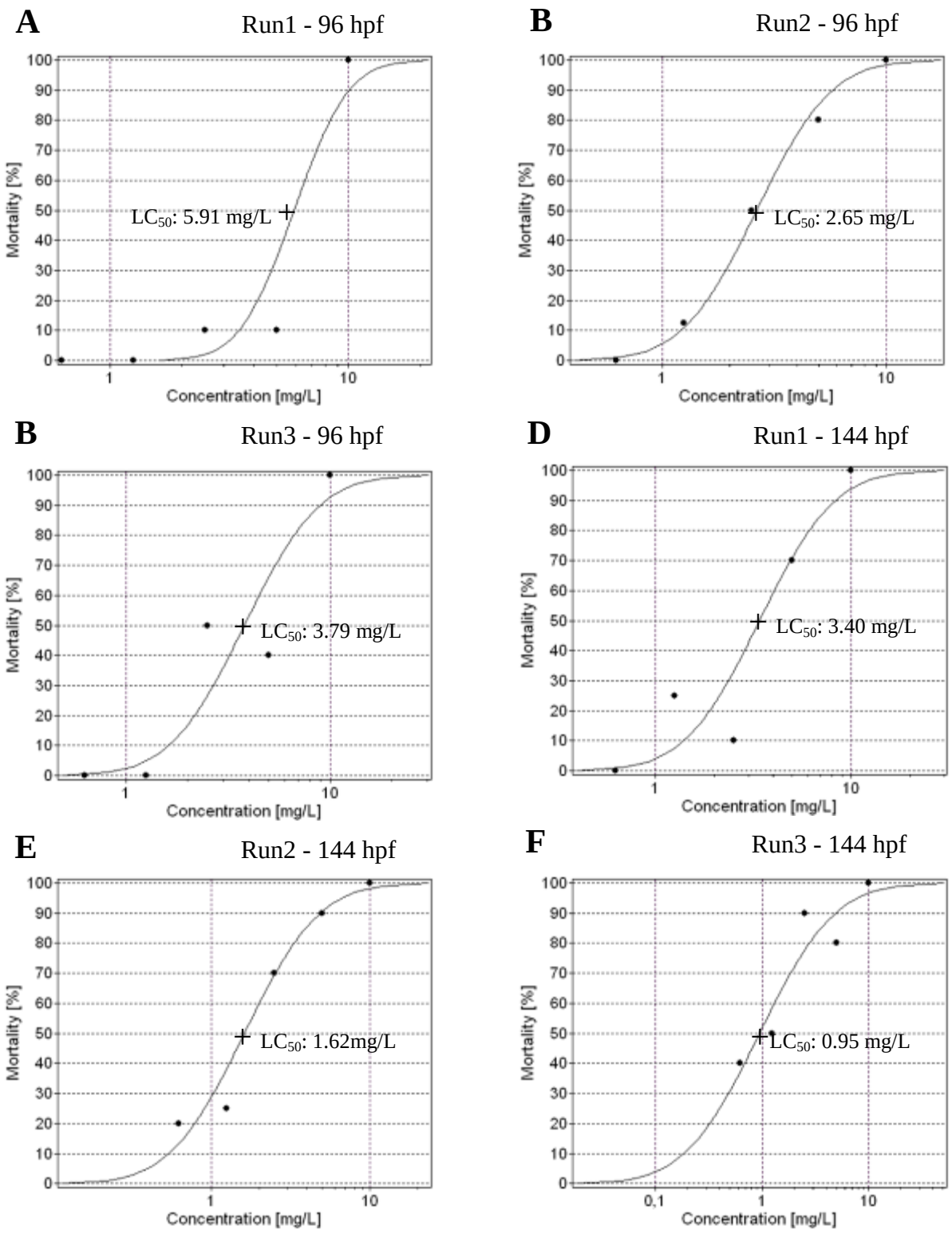


Fig. 13: Dose-response relationships at 96 and 144 hpf of the $CdCl_2$ runs; no value for 48 hpf could not be determined.

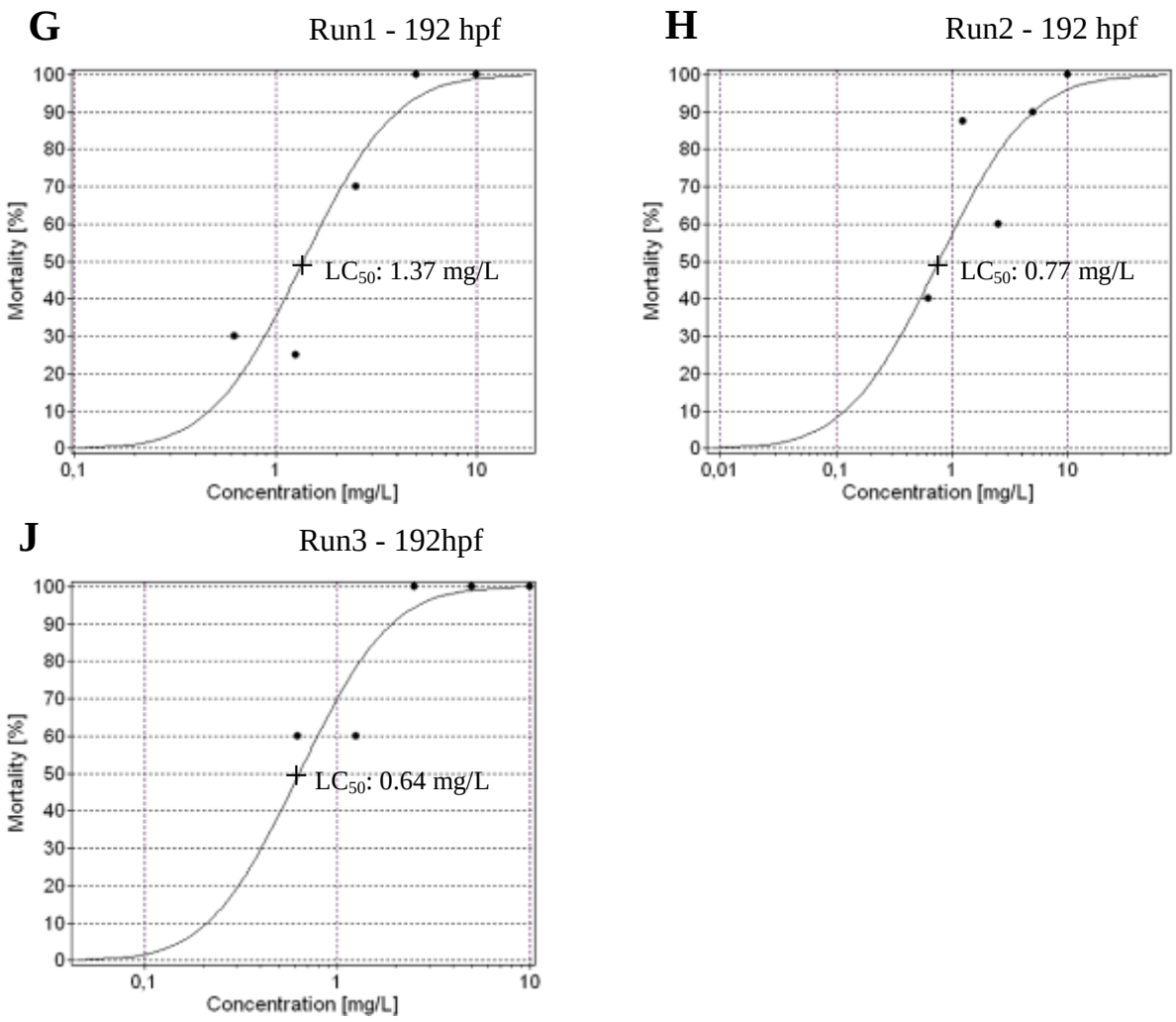


Fig. 14: Dose-response relationships at 192 hpf of the $CdCl_2$ runs

The bar diagram (Fig. 15) shows the mean LC_{50} values and the change of the LC_{50} over time from 48 hpf to 192 hpf. At 48 hpf, the mean LC_{50} value was 8.22 mg/L. It was calculated only run 2 and run3, because no value could be determined for run1. The LC_{50} decreased in the same intervals of nearly 2 mg/L for the next two days, resulting in 6.24 mg/L at 72 hpf and 4.11 mg/L at 96 hpf. It dropped further to 2.77 at 120 hpf, to 1.99 mg/L at 144 hpf, to 1.51 mg/L at 168 hpf, resulting in a final LC_{50} value of 0.92 mg/L at 192 hpf.

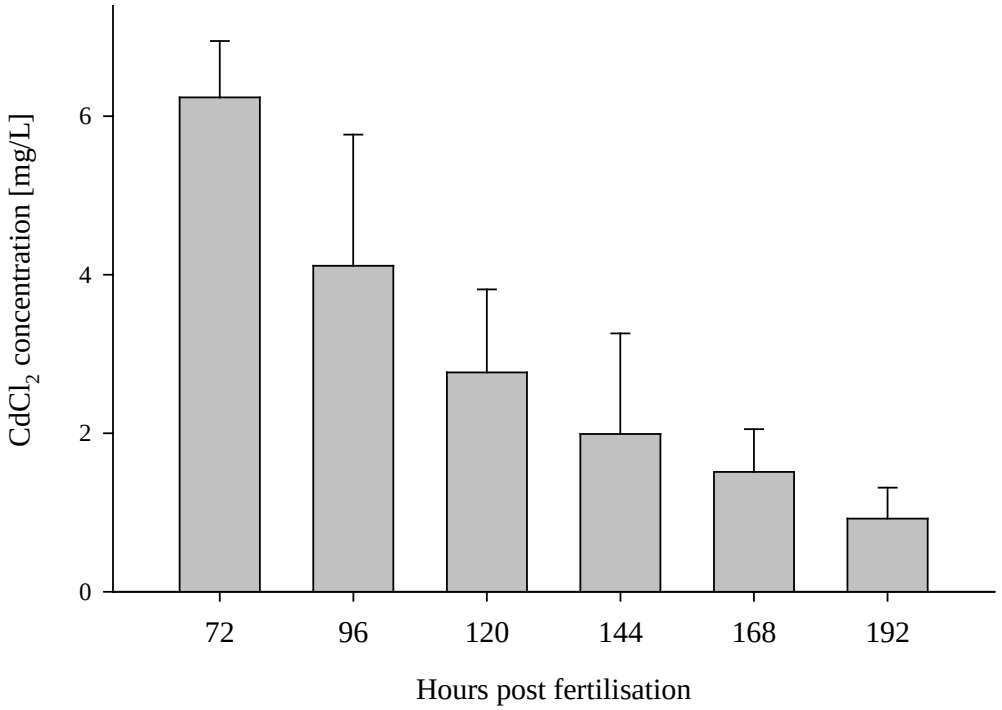


Fig. 15: Overview of the mean LC_{50}-values of $CdCl_2$, and their change over time.

Tab. 23: Mean LC_{10} and LC_{50} values for $CdCl_2$ in fathead minnow

	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf	168 hpf	192 hpf
LC_{10} [mg/L]		5.00 ± 1.54	2.10 ± 1.2	1.00 ± 0.71	0.70 ± 0.62	0.64 ± 0.59	0.27 ± 0.18
LC_{50} [mg/L]	8.22 ± 1.64	6.24 ± 0.71	4.11 ± 1.65	2.77 ± 1.05	1.99 ± 1.27	1.51 ± 0.54	0.92 ± 0.39

Hatching was observed for all three runs. Percentages of fish hatched at certain time intervals were determined (Fig. 16). In the negative control, hatching was observed for the first time at 96 hpf, when over 20 % of the embryos hatched. After 120 hpf, 90 % had hatched, leading to a maximum of 97 % (29 out of 30) after 144 hpf. All embryos exposed to cadmium chloride started hatching 24 hours earlier than embryos in the negative control: at 72 hpf. The hatching success of embryos exposed to cadmium chloride was impaired. The impairment was concentration dependent: the higher the concentration of cadmium chloride, the less fish were able to hatch during the time of the experiment. At 10 mg/L, 13 % of the embryos (4 out of 30) hatched at 72 hpf, but the number of hatched embryos did not increase further due to the death of all fish from 72 hpf onwards. At 5 mg/L, only 3 % of the embryos hatched at 72 hpf, the last fish hatched at 192 hpf resulting in a final 77 % of hatched fish. At 2.5 mg/L, 20 % of the

embryos hatched at 72 hpf reaching 83 % of hatched fish at 192 hpf. At 1.25 mg/L, 37 % of fish hatched at 72 hpf; it stopped at 144 hpf, when 87 % of fish had hatched. At the lowest concentration (0.625 mg/L), hatching started with 30 % (72 hpf) resulting in 100 % hatched fish at 144 hpf.

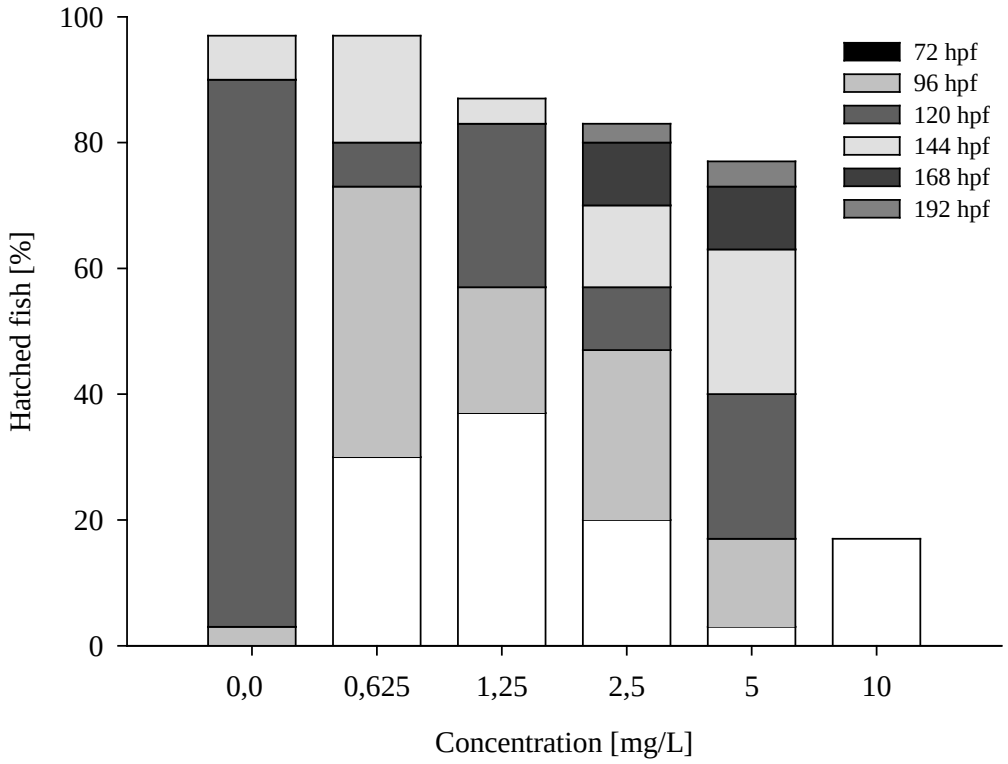


Fig. 16: Hatching times and hatching success for the fathead minnow embryos exposed to different cadmium chloride concentrations. Number of embryos per concentration n = 30.

3.4.2 Aniline

No pre-tests with the fathead minnow were carried out for aniline. The concentration range was adapted from A. Keth (in preparation), who tested aniline with the zebrafish embryo at18.75, 37.5, 75, 150 and 300 mg/L. Two runs were carried out to determine the toxicity of aniline using the fathead minnow embryo. For the first run, the embryos were exposed to the different aniline concentrations as soon as they were detected, at an 8-to 32-cell stage. In the second run, the embryos were exposed at 24 hpf. The temperature was within the range of 25 ± 1°C.

Effects were observed every 24 hours, starting 24 hours after fertilization. At 24 hpf, no effect could be observed at all five aniline concentrations. At 48 hpf, the only apparent effect was

differences in the pigmentation of the eyes which appeared more amber with higher aniline concentration (Fig. 17). Embryos appeared hyperactive after exposure to aniline compared to the negative control. At 72 hpf, the first embryos hatched. They swam fast compared to the fish in the negative control, and it was hard to photograph them. No difference in eye pigmentation was observed. There were no changes in observed effects throughout the experiment. Nonetheless, embryos at lower concentrations (18.75, 35.5, and 75 mg/L) showed a change from amber to black pigmentation in the eyes at 120 hpf. No change was observed at the two highest concentrations (150 and 300 mg/L). Agility of eleutheroembryos, and then larvae, remained high. It appeared that the higher the aniline concentration, the higher the agility. After 192 hpf, the embryos were anaesthetized in tricaine and observed again. The swim bladder of all larvae exposed to aniline was not present, too small or not coated with crystals. Sometimes, larvae also had blood accumulation in the gastrointestinal tract. Exposure of the embryos at 24 hpf led to the same effects; the onset was just 24 hours later than in run 1. No mortality was observed in either test over the entire experimental period.

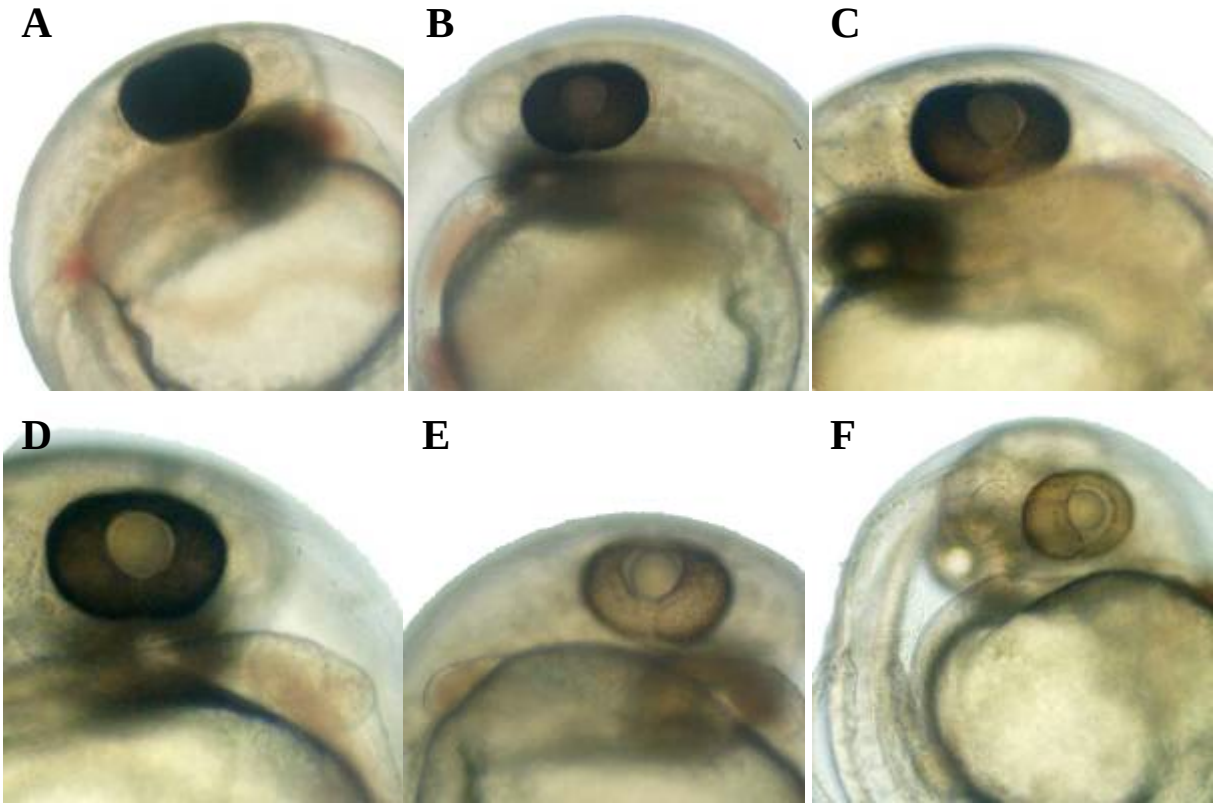


Fig. 17: Comparison of 72 hpf embryos treated with different aniline concentrations: A) negative control; B) 18.75 mg/L; C) 37,5 mg/L; D) 75 mg/L; E) 150 mg/L; F) 300 mg/L.

Hatching was observed for both runs. Percentages of fish hatched at certain time intervals were determined. The first embryos hatched at 72 hpf: in the negative control and the two highest concentrations. Embryos in other concentrations (18.75, 37.5 and 75mg/L) started 24 hours later. At 120 hpf, in all concentrations all embryos had hatched.

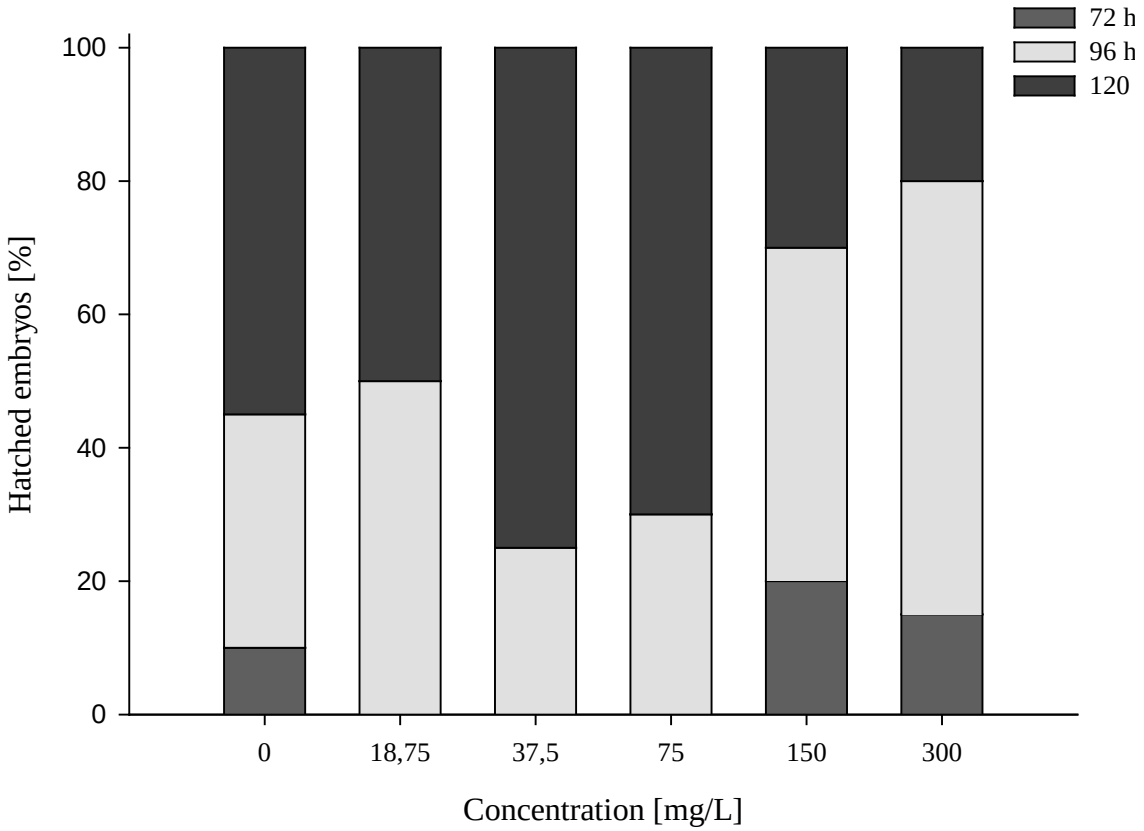


Fig. 18: Hatching times and hatching success for fathead minnow embryos exposed to different aniline concentrations; number of embryos per concentration n = 20.

3.4.3 3,4-Dichloroaniline (3,4-DCA)

No pre-test was carried out for 3,4-DCA; instead, the existing concentration range for the zebrafish was used: 1, 2, 4, 8 and 16 mg/L. Four runs were carried out to determine the toxicity of 3,4-dichloroaniline using the fathead minnow embryo. The embryos were exposed to the different 3,4-dichloroaniline concentrations as soon as they were detected, but at slightly different stages of development: at an 8 to 16-cell stage in run 1, and at an 8- to 32-cell stage in runs 2 and 3. The temperature was within the range of 25 ± 1°C. Two additional test were carried out to determine if exposure later in the development (24 hpf) or the chorion play an important role for 3,4-dichloroaniline toxicity. Embryos of the dechorionation test were exposed at 8- to 32-cell stages and dechorionated at 24 hpf.

Effects were observed every 24 hours, starting 24 hpf. At 24 hpf, only the embryos in the two highest concentrations (8 mg/L and 16 mg/L) had reduced tail length (Fig. 19) in comparison to controls. The heads were slightly smaller and showed a darker color. At 48 hpf, the embryos at 16 mg/L showed all effects, mainly cardiac edema, tail curvature, reduced blood circulation and heartbeat, and absent eye pigmentation. Eyes in the negative controls were already black. Some embryos at 8 mg/L showed reduced heartbeat and blood circulation as well as reduced eye pigmentation. No effects could be observed for the other three concentrations except for embryos in run 2. At 72 and 96 hpf, embryos at 16 mg/L showed extremely reduced heartbeat, tail curvature was more severe, blood circulation had stopped and blood cells were colorless or already absent due to degradation; edemas grew bigger. At 8 mg/L, and after 96 hpf at 4 mg/L, blood cells accumulated in the pericardium and tail. First effects were now observed at 2 and 4 mg/L. Overall, all exposed embryos showed tail curvature and reduction in pigmentation of the eyes and the body compared to the negative control. At 120 hpf, embryos at the lowest concentration (1 mg/L) showed edemas and reduced blood circulation. At 4 and 8 mg/L, edemas in the brain region were observed. Somites were enlarged and formation of edemas in the visceral cavity developed, especially at higher concentrations. All eleutheroembryos, even at low concentrations, showed loss in balance and directed swimming movements. Pigmentation was still less than negative control but dependent on exposure concentration. At 144, 168 and 192 hpf, listed effects became severe. Somite enlargement also observed at lower concentrations (1 - 4 mg/L). No difference in effects or onset of effects could be detected for the dechorionation approach. In the fourth run, in which embryos were exposed to DCA after 24 hours, effects started to show up 24 hours later than in the other three runs.

After 24 hours, the first embryos had died except for the first run, where no mortality was detected. For the following three days, the mortality rate remained low. Even at the highest concentration (16 mg/L), the mortality did not exceed 50 %. Therefore, no LC_{50} values could be determined for 48 and 96 hpf. Mortality increased after 120 hpf, and 100 % mortality was finally reached at the highest concentration for run1 and run 3 (run 2 90%) at 144 hpf (Fig. 20). LC_{50} values ranged from 4.25 mg/L in run 1, 5.53 mg/L in run 3, to 9.55 mg/L in run 2. Mortality at 2 mg/L was the lowest observed. LC_{50} values for the last day of the experiment were 0.92 mg/L in run 1, 1.35 mg/L in run 2 and 1.71 mg/L in run 3.

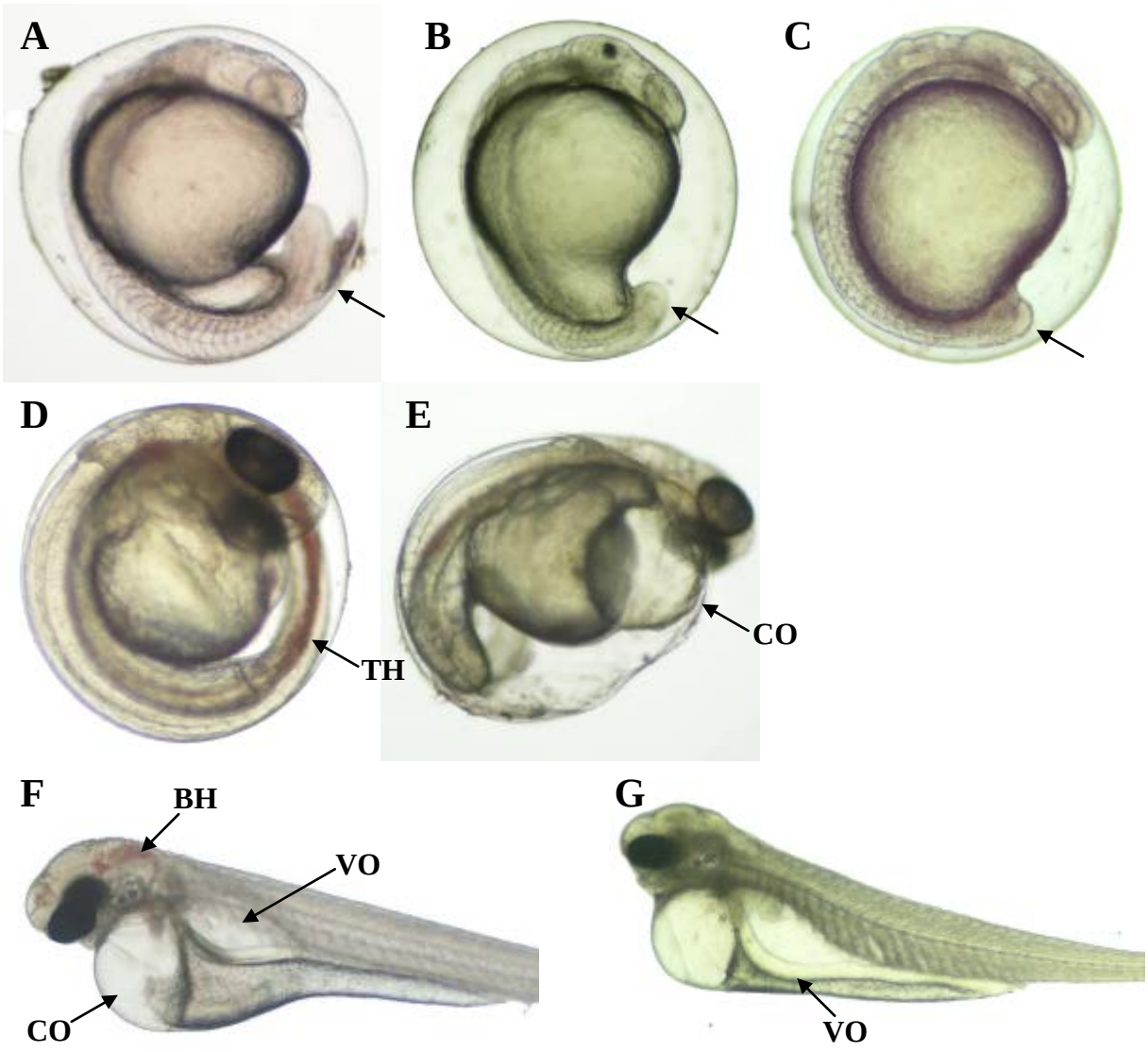


Fig. 19: Embryos treated with 3,4-dichloroaniline. A – C) retardation in development (arrow) in 24 hpf embryos: A) 4 mg/L DCA; B) 8 mg/L DCA; C) 16mg/L DCA; D) 72 hpf embryo treated with 8 mg/L DCA shows hemorrhage in tail (TH); E) 72 hpf embryo treated with 16 mg/L DCA with cardiac edema (CO) tried to hatch; F) 120 hpf embryo treated with 4 mg/L DCA shows hemorrhage in the brain (BH), cardiac and visceral edema (VO); G) 168 hpf embryo treated with 2 mg/L DCA showing large edemas and enlarged somites (SE)

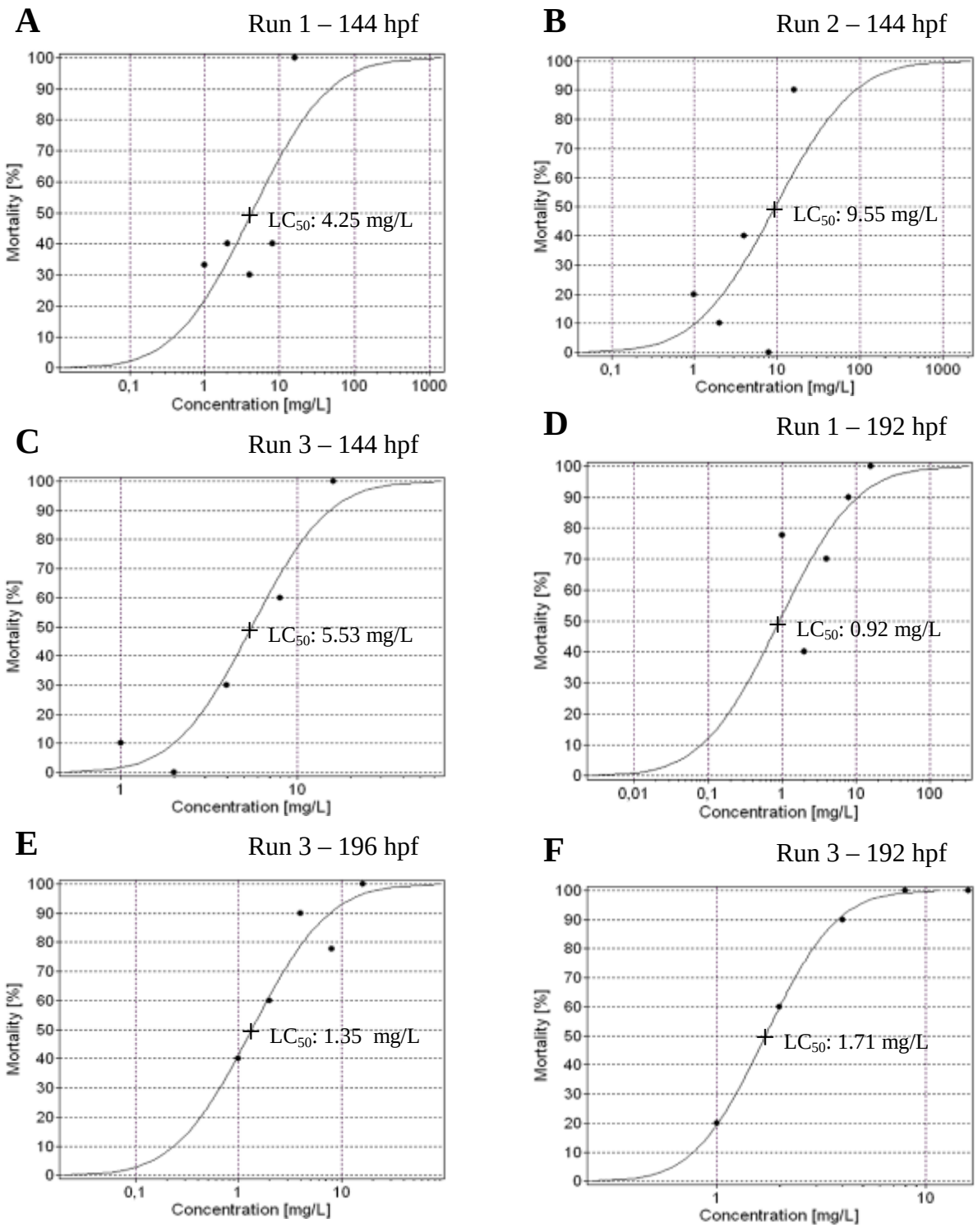


Fig. 20: Dose-response relationships of the 3,4-DCA runs.

The bar diagram (Fig. 21) shows the mean LC_{50} values and the change of the LC_{50} values over time from 120 hpf to 192 hpf. At 120 hpf, the mean LC_{50} was 24.16 mg/L. After 24 hours, the value dropped to 6.44 mg/L. At 168 hpf, the value halved down to 3.38 mg/L, resulting in a final of 1.33 mg/L after 192 hpf.

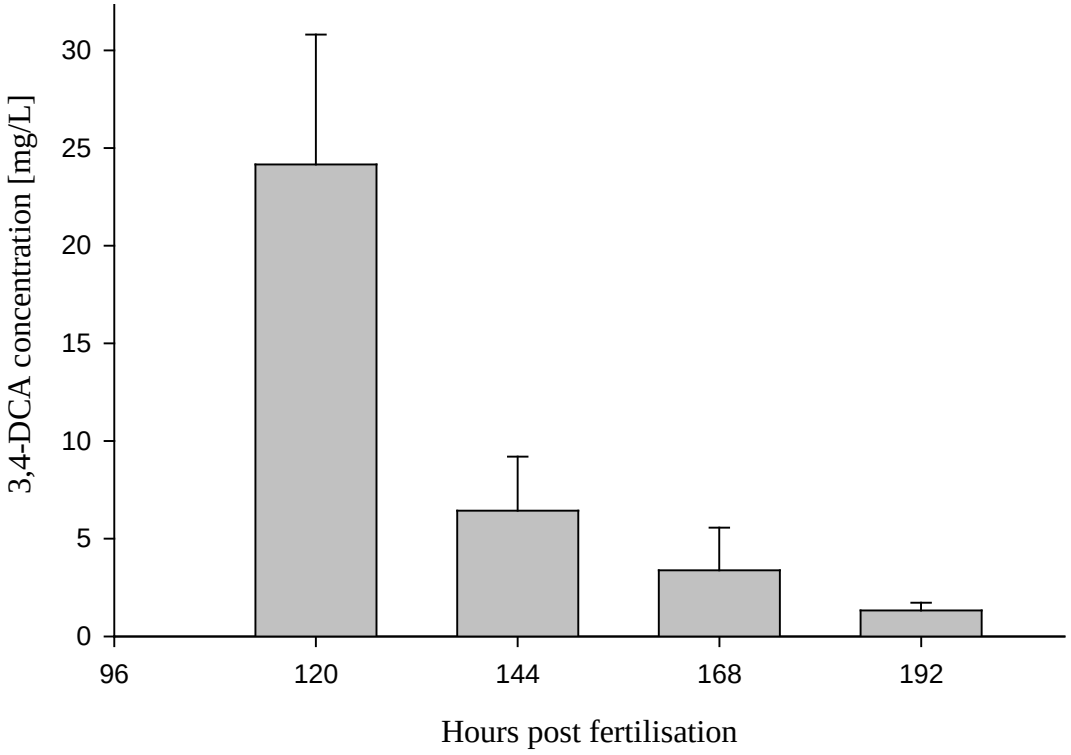


Fig. 21: LC_{50} values of 3,4-dichloroaniline over time.

Hatching was observed in all three runs. Percentages of fish hatched at certain time intervals were determined (Fig. 22). In the negative controls, hatching was observed for the first time at 96 hpf, when 25 % of the embryos hatched; 80 % had hatched after 120 hpf leading to a maximum of 98 % (39 out of 40) after 144 hpf. All embryos exposed to 3,4-DCA started hatching 24 hours earlier than embryos in the negative control: 3 % in all concentrations except 18 % at the highest concentration (16 mg/L). Approximately 80 % hatched at 96 and 120 hpf. Hatching was only impaired at the highest concentration (16 mg/L) leading to a decrease of 25%. Hatching was completed after 144 hpf. Only in the negative controls and at 4 mg/L did the last few fish hatch at 168 hpf.

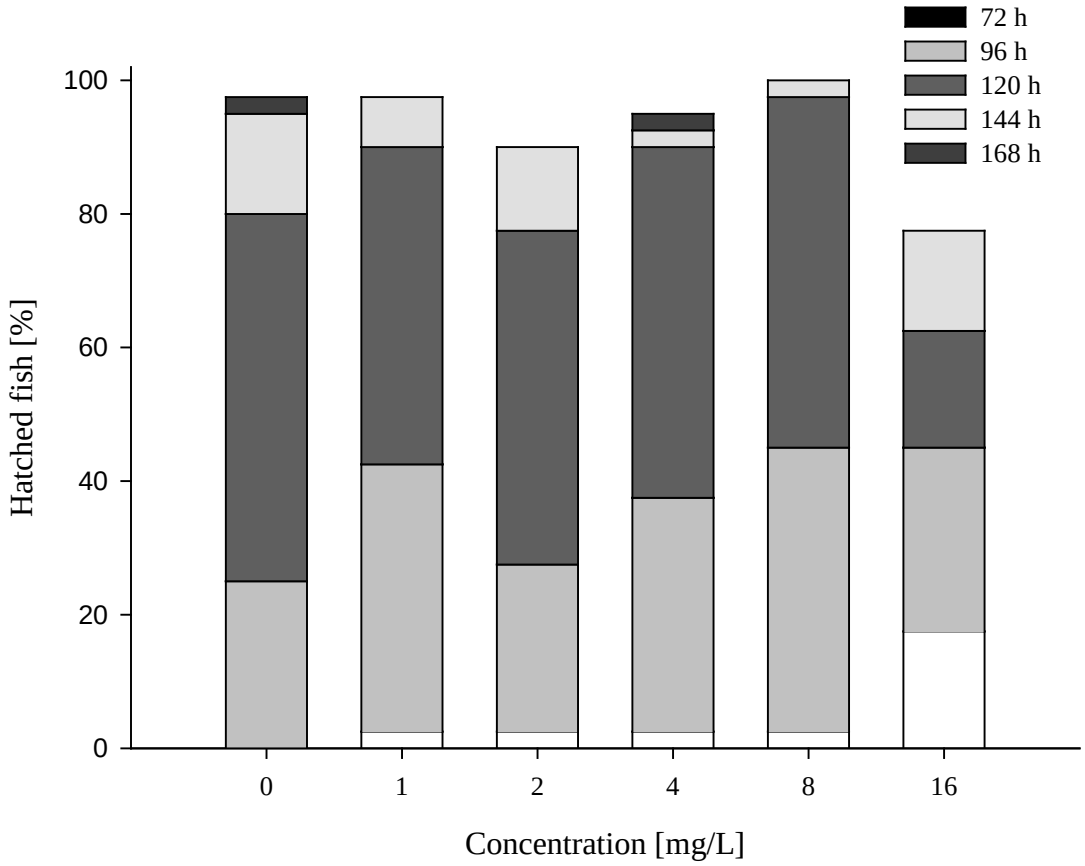


Fig. 22: Hatching times and hatching success for fathead minnow embryos exposed to different 3,4-dichloroaniline concentrations, number of embryos per concentration n = 30.

3.4.4 2,4-Dichlorophenol (2,4-DCP)

No pre-tests were carried out. The concentration range was adapted from A. Keth (in preparation) who tested 2,4-dichlorophenol with the zebrafish embryo at 2.5, 5, 7.5, 10 and 15 mg/L. Three runs were carried out to determine the toxicity of 2,4-DCP using the fathead minnow embryo. The embryos were exposed to the different 2,4-dichlorophenol concentrations as soon as they were detected, but at slightly different stages of development: at 32 cell stage in run 1 and 2, and at a 64 cell stage in run 3. The temperature was within the range of 25 ± 1°C.

Effects were observed every 24 hours, starting 24 hours after fertilization. At 24 hpf, 2,4-DCP exposed embryos showed a retardation in development (Fig. 23) compared to the negative control. The stage of development was concentration-dependent: the higher the DCP concentration, the lesser the development. After 48 hpf, all embryos in the highest concentration had died. At the second highest concentration (10 mg/L), all embryos had no blood circulation and

showed reduced heartbeat and cardiac edemas. The eyes were not pigmented. Spontaneous movement was restricted and slowed. At 7.5 mg/L, only small effects like reduced heartbeat and blood circulation, as well as edemas were observed, but in run 2 no effects were present. No effects were also detected at the two lowest concentrations (2.5 and 5 mg/L), except in run1 were several embryos showed signs of reduced heart beat and circulation. At 72 hpf, at 10 mg/L edemas in the brain region were present; other effects observed were reduction heartbeat, absence of circulation, cardiac edema, tail curvature, and somite enlargement. Eye pigmentation was reduced and blood cells appeared colorless when still present. The same effects were observed at 7.5 mg/L; additionally, accumulation of blood cells in the pericardium was detected. No or only few effects were present in the two lowest concentrations, namely the sparse or not present coloration of the blood cells and at run 1 at 5 mg/L the accumulation of blood cells in the pericardium and the dorsal aorta. At 96 hpf, 10 mg/L the observed effects stayed the same, just became more severe. At 7.5 mg/L, accumulation of blood cells in the pericardium was still an issue re, furthermore tail curvature increased. No effects were observed for run 2. Most embryos showed still no effects at 5 mg/L. A few embryos developed a reduction of circulation and heartbeat. Cardiac edema and accumulation of the blood cells in the dorsal aorta was also observed. At 2.5 mg/L no effects could be detected. At 120 hpf, there were still no effects at 2.5 mg/L. At 10 mg/L the effects were the same, as well as at 7.5 mg/L. At 5 mg/L, hatched embryos showed a strange orientation of the breast fins, which showed either both to the head or one to the head and one to the rear end of the larvae. For the remaining observing times, 144, 168 and 192 hpf, respectively, no great change in effects could be observed.

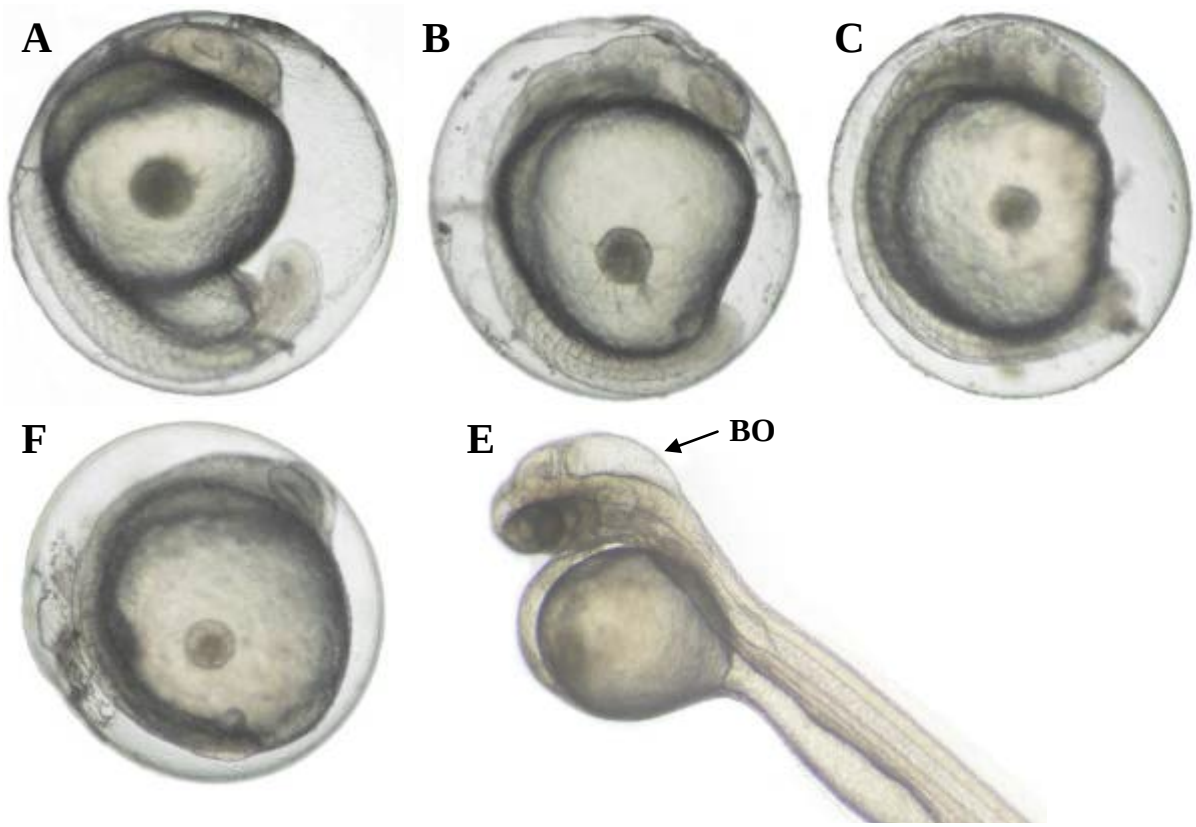


Fig. 23: Embryos treated with 2,4-dichlorophenol. Retardation in development in 24 hour old embryos: A) 5 mg/L DCP; B) 7.5 mg/L DCP; C) 10 mg/L DCP; D) 15 mg/L DCP; E) 72 hpf embryo treated with 10 mg/L DCP showing brain edema (BO) and no pigmentation.

Due to retardation in development, all embryos were dead after 24 hours. At 48 hpf, only the embryos in the highest concentrations (15 mg/L) had died (Fig. 24). Mortality at 10 mg/L ranged between 10 and 60 %; embryos in the lower concentrations remained unaffected. LC50-value, determined at 48 hpf, ranged from 9.52 mg/L in run 1, over 10.92 mg/L in run 2 up to 12.23 mg/L. Over the next two days, mortality did not increase significantly. Therefore, LC50-values at 96 hpf were similar to the 48 hpf-values: 9.96 mg/L in run 1, 10.92 mg/L in run 2 and 10.38 mg/L in run3. Mortality at 7.5 and 10 mg/L slowly increased leading to 9.15 mg/L in run 1, 11.12 mg/L in run 2 and 8.66 mg/L in run 3 as LC50 values at 144 hpf (Fig. 25). At the end of the experiment (192 hpf), LC50 values were 6.64 mg/L in run 1, 8.06 mg/L in run 2 and 6.94 mg/L in run 3. An overview of all LC50 values gives Fig. 26.

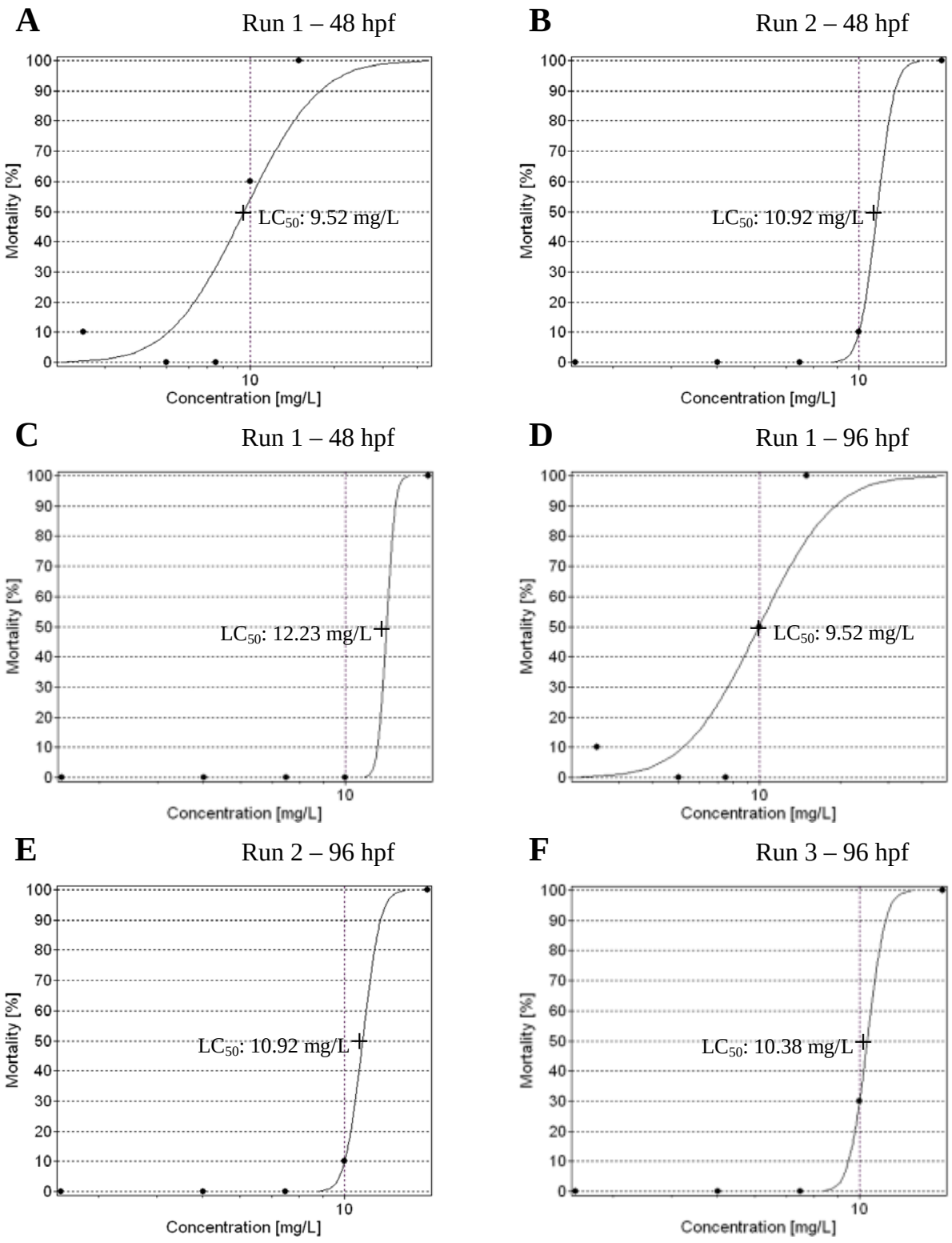


Fig. 24: Dose-response relationships after 48 and 96 hpf of the 2,4-DCP runs.

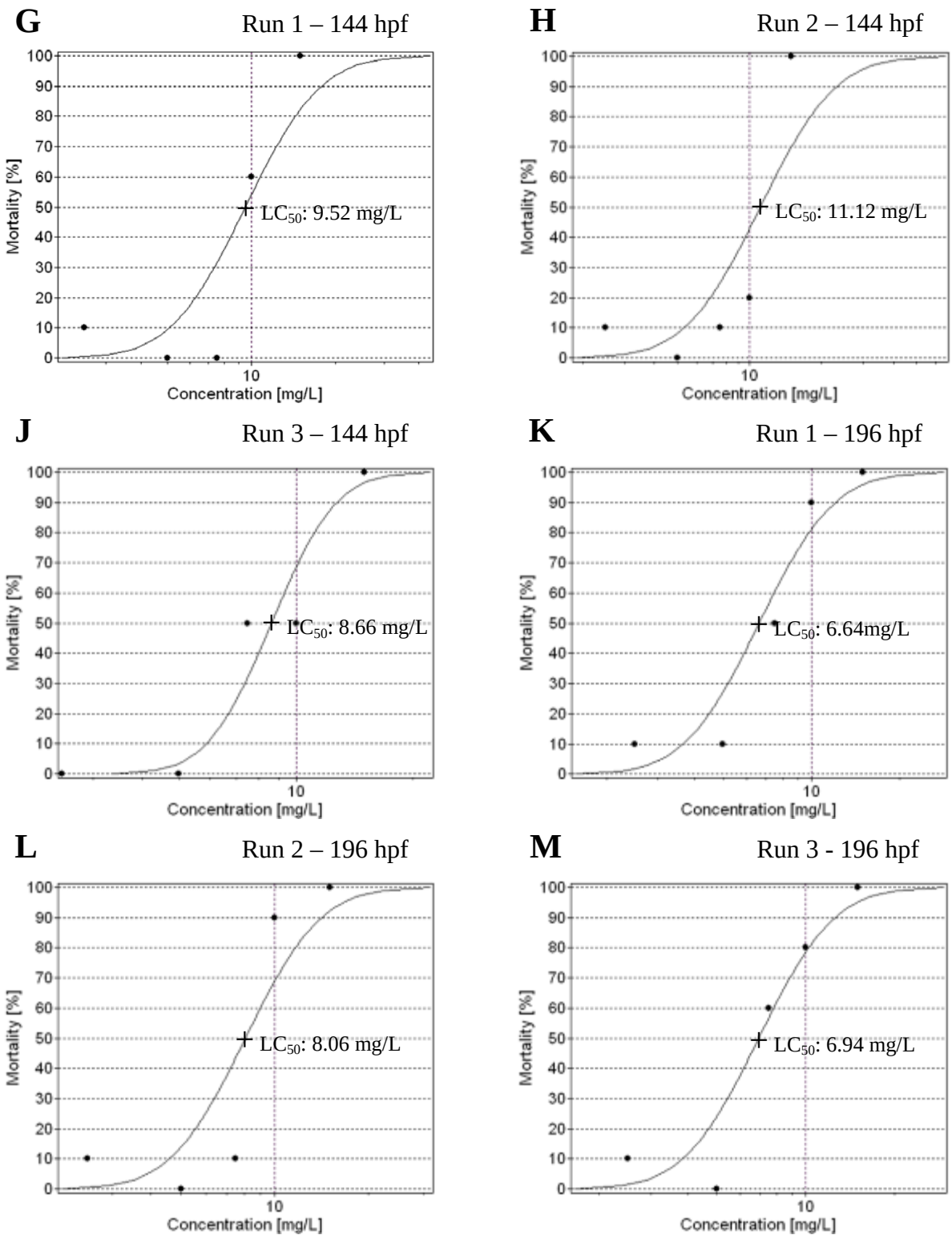


Fig. 25: Dose-response relationships after 144 and 192 hpf of the 2,4-DCP runs.

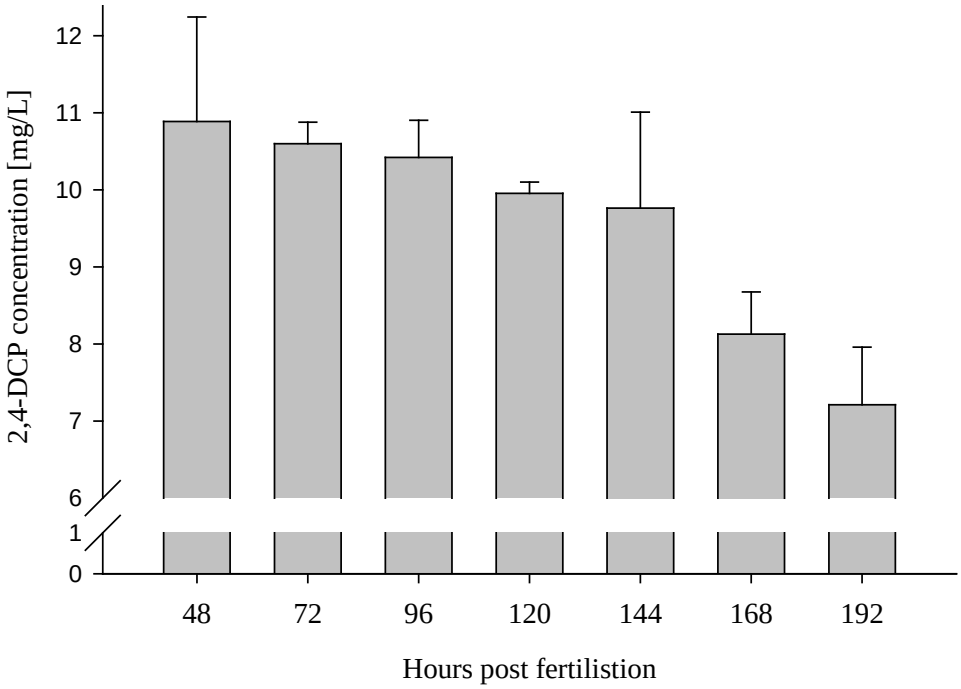


Fig. 26: Overview of the LC_{50}-values 2,4-dichlorophenol, and their change over time.

Tab. 24: Overview of the mean LC_{10} and LC_{50} values of 2,4-DCP

	48h		72h		96h		120h		144h		168h		192h	
LC10	8.92	±	7.81	±	8.22	±	5.84	±	5.43	±	4.29	±	4.02	±
	3.44		2.34		2.64		5.43		0.42		0.38		0.50	
LC50	10.89	±	10.60	±	10.42	±	9.95		9.76	±	8.13	±	7.21	±
	1.36		0.28		0.48		± 0.15		1.25		0.55		0.75	

Hatching was observed for all three runs. Percentages of fish hatched at certain time intervals were determined (Fig. 27). In the negative controls hatching was observed for the first time at 96 hpf, when 30 % of the embryos hatched. After 144 hpf all 100 % of the embryos had hatched. All embryos exposed to 2,4-DCP started hatching 24 hours earlier than embryos in the negative controls: at 72 hpf (3 to 10 %). The higher the concentration of 2,4-DCP, the less fish were able to hatch during the time of the experiment. All embryos, except one, were able to hatch at the two lowest concentrations (2.5 and 5 mg/L). At 7.5 mg/L 83 % hatched between 72 and 144 hpf. At 10 mg/L, 47% of embryos hatched between 72 and 168 hpf. At 15 mg/L, the highest concentration, minnows hatched only after 168 hpf resulting in a hatching success of 10 %.

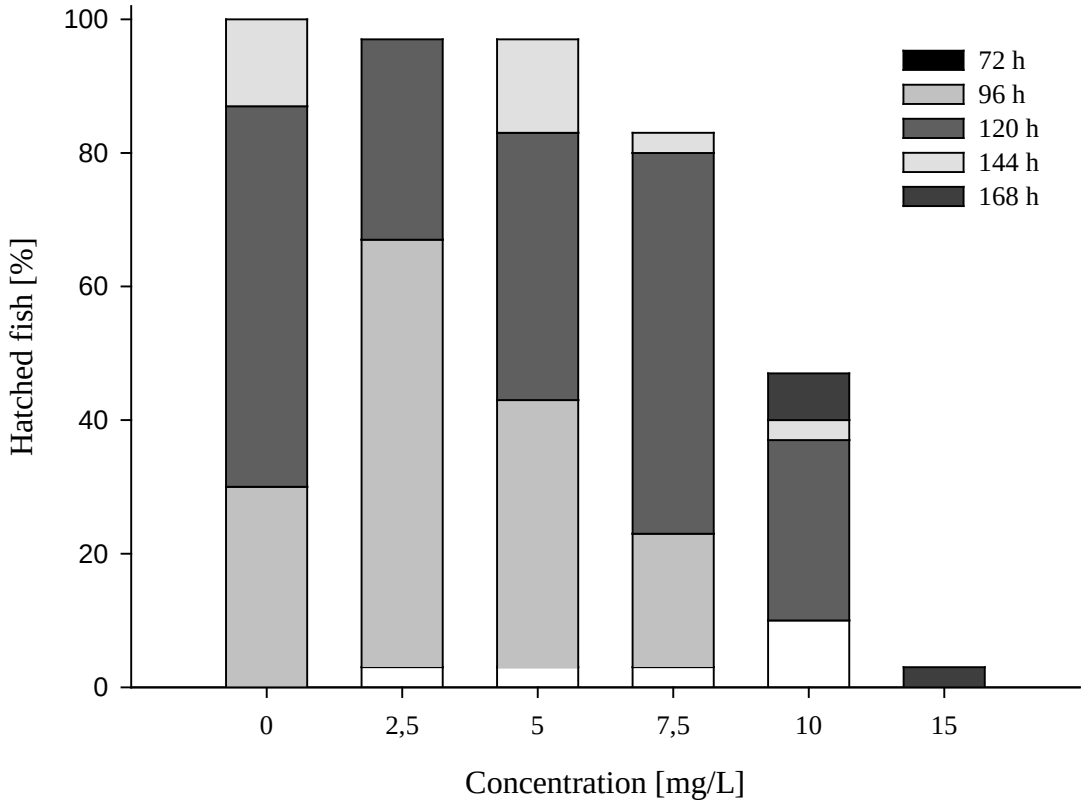


Fig. 27: Hatching times and hatching success for fathead minnow embryos exposed to different 2,4-dichlorophenol concentrations; number of embryos per concentration n = 30.

3.5 Histology of the fathead minnow and the zebrafish egg

Fathead minnow and zebrafish embryos developed in a similar way. At the animal pole, the embryo developed after fertilization protected by a membrane; in unfertilized eggs there was a granular structure. The vegetative pole was abounded in yolk cells. Yolk cells were big, irregular cells without nuclei that were also surrounded by a membrane which separated the yolk from the animal pole. Both developing embryo and yolk sac were surrounded by another layer which separated them from the perivitelline fluid, which filled the space between the embryo and the chorion. The different morphological structures like somites, the brain or the eye were often well preserved. The area where the ear placode or the spine was located was often just a white mesh-like structure.

3.5.1 Zebrafish

Transverse sections of unfertilized eggs, as well as 24 and 72 hour old embryos were used (Fig. 28). While the embryo could be observed quite well depending on the quality of the sec-

tions, no transverse section of the chorion could be observed in the zebrafish because the chorion bent and gave way to a top view of either the outer or the inner side of the chorion. The orientation of the chorion was determined by the shape of the pores (3.6). In the brain region of the developing 72 hpf zebrafish, there were dusky pink particles without structure between the purple tissue. The HE stain gave the whole transverse section a pinkish color, but all components were colored in a slightly different shade of pink. The brightest pink was displayed by the yolk cells, the cells of the embryo tissue being a little less bright, followed by the chorion, and the less colored were the components of the perivitelline fluid. If treating the transverse sections of zebrafish eggs with Masson-Goldner, the chorion is stained orange-red, and the embryo tissues purple, while the yolk cells and the compounds of the perivitelline fluid are turquoise. The PAS stain colored the embryo tissues purple. The perivitelline fluid components which were the most colored appeared bright red in contrast with the pink of the yolk cells and the chorion.

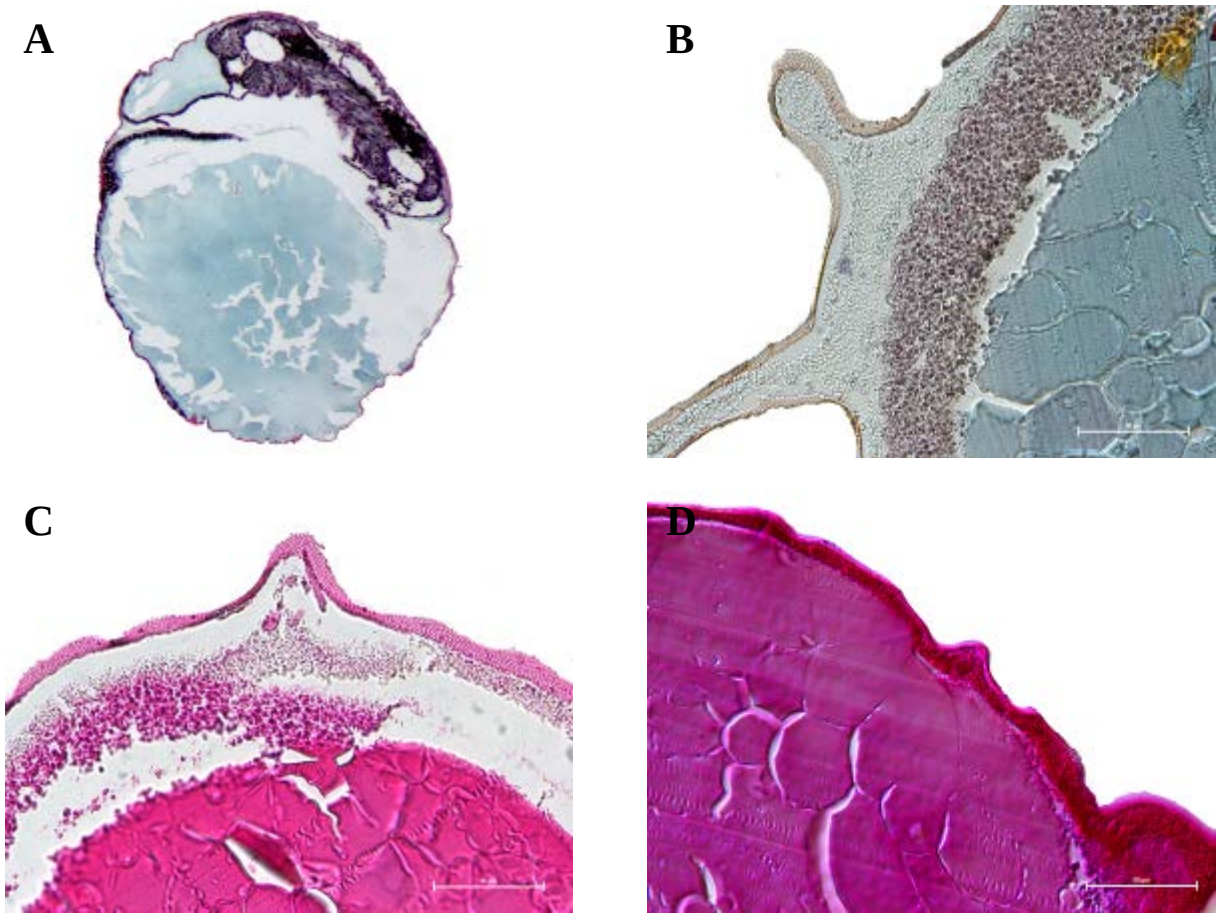


Fig. 28: Histology of the zebrafish. A) 72hpf zebrafish embryo stained with Masson-Goldner (4X); B) section of an unfertilized zebrafish chorion (MG, 40X); C) unfertilized zebrafish egg stained with HE; D) unfertilized zebrafish egg stained with PAS, the perivitelline fluid is bright red (40X).

3.5.2 Fathead minnow

Fathead minnow embryo sections were prepared for multi-cell, 24, 48, 72 and 96 hour old embryos (Fig. 30). It was hard to detect a proper transverse section of the chorion, because it often bent like the zebrafish chorion, but most of the time without revealing its structure or pore shape. However a few sections were found (Fig. 29), where the chorion seemed to have a thick outer layer and a part, where many layers laid on top of each other giving it a striped appearance. The pigmentation of the eyes was visible after 48 hpf; the overall structure of the eye could also be observed several times. The swim bladder was detected in 96hpf fathead minnows for the first time. In the brain region of 72 and 96 hpf fathead minnow embryos, two structures, a purple granular and a dusky pink unstructured, were observed. Eggs stained with HE appeared pink like the zebrafish. The colors of the tissues were slightly different from one another. Masson-Goldner stain colored the fathead minnow egg in a similar way than the zebrafish. The only difference was that the muscular tissue of the fathead minnow was more blue than purple and that the chorion was nearly not stained at all. In one of the sections an orange colored indentation that could be a micropyle. The PAS-stained sections exhibited the same color than the sections of the zebrafish.

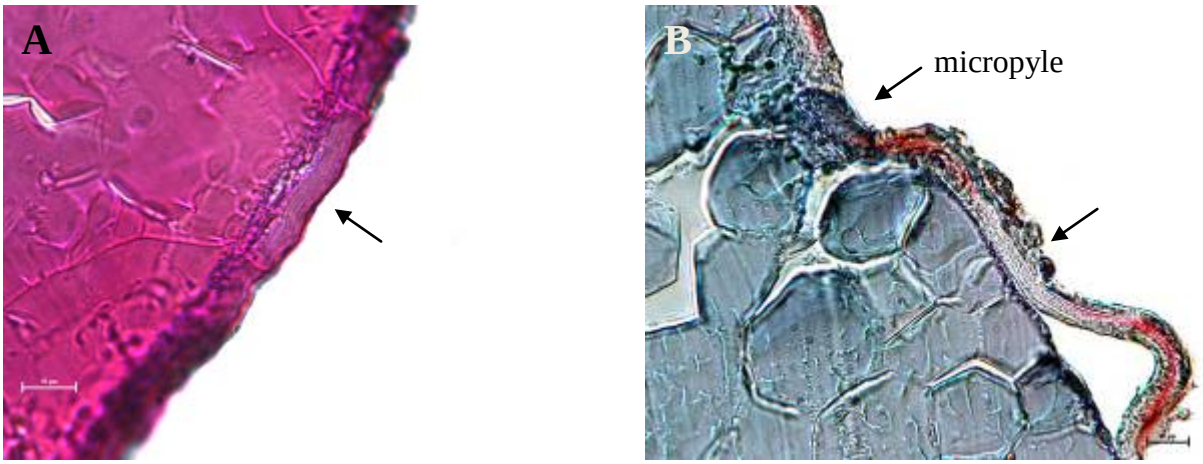


Fig. 29: Transverse sections of the zebrafish egg revealing the striped appearance of the chorion (arrow). A) chorion and yolk cells (HE-stain, 60X); B) yolk cells and chorion showing the micropyle (MG-stain, 60X).

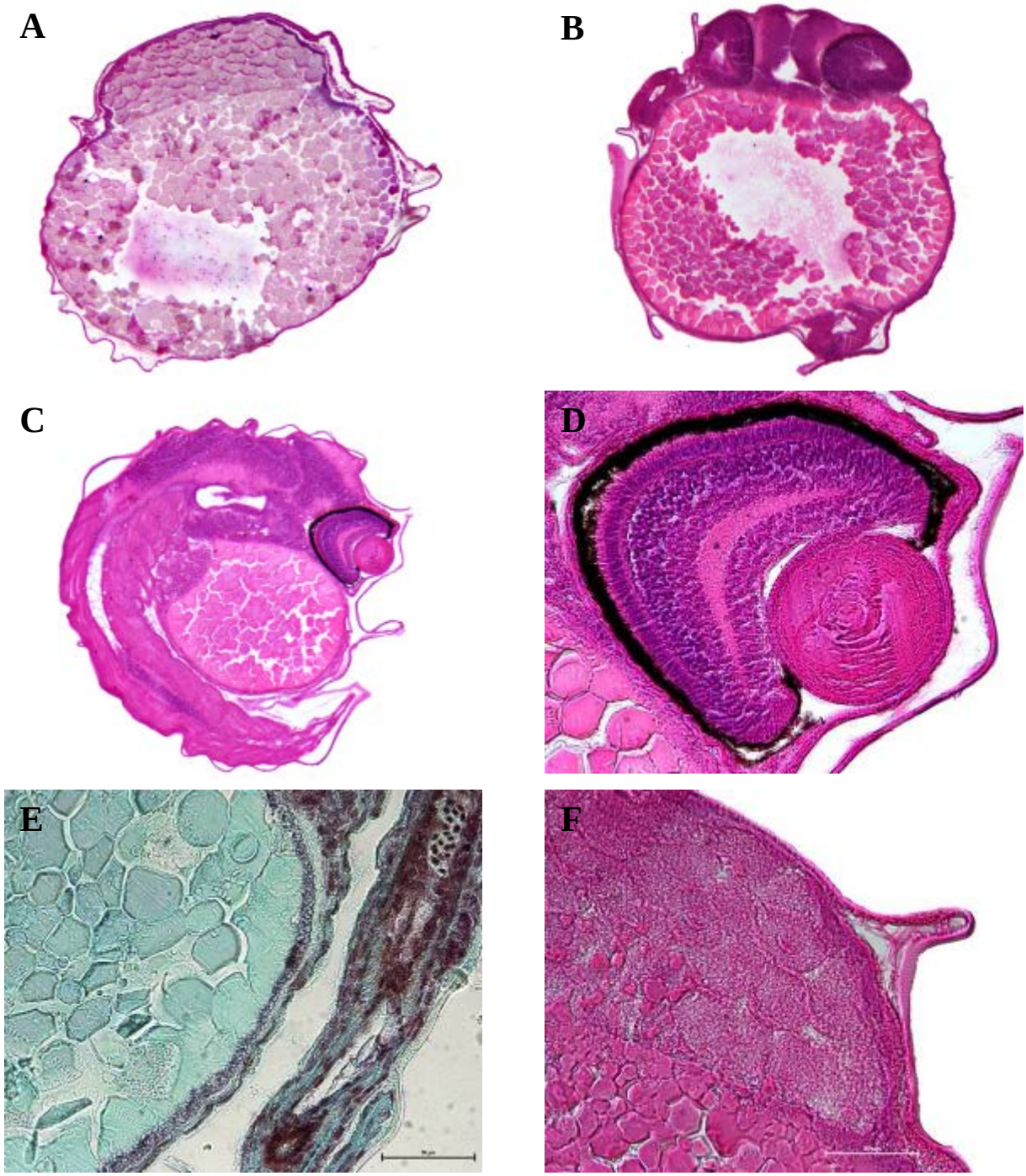


Fig. 30: Histology of the fathead minnow embryo. A) multicell-stage (PAS,4X); B) 48 hpf embryo (HE, 4X); C) 96 hpf embryo, swim bladder present (HE,4X); D) transverse section of the eye of a 96 hpf embryo (HE, 40X); E) section of a 72 hpf embryo revealing the different layers surrounding yolk and embryo including the chorion (MG, 40X); F) section of a multicell stage (HE, 40X).

3.6 Native preparation of the chorion

3.6.1 Zebrafish

Even with 20x magnification, the pores were already visible and translucent. On the outside of the chorion, distinctive projections were observed. The edges, where the chorion was lacerated, appeared quite smooth (Fig. 31a). It seems like it was torn along a perforated line, because pore halves were visible at the edges. The pores on the outer surface of the chorion appeared to have a circular shape, whereas the pores of the inner surface appeared to be polygonal. They had three, four and five vertexes (Fig. 31d). Nearly no bacteria could be found on top of the zebrafish chorion. However, a very fine layer on the outer surface associated with bacteria was detected. It was opaquely smooth on the ripped edges. To measure the number of pores per square millimeter, the pore diameter and the distances between the pores, the chorion of different stages (24, 48 and 72 hpf) were focused on the outer and inner surface (Fig. 31 c +d). The number of pores per square millimeter was slightly reduced in 48 hpf zebrafish embryos compared to 24 and 72 hpf old embryos. The area of the outer pores were smaller than the ones on the inside, pore distance was also slightly greater between the inner pores compared to the outer ones. The difference between outer and inner pore area was greatest in 24 hpf embryos and was reduced with time. The pore radius was the smallest in 24 hours old embryos. No sessile microorganisms were found on the zebrafish chorion, but an amoeba and probably some green algae were observed was on the gelatine slides stained.

The HE-stained chorion of the zebrafish appeared magenta (Fig. 31b). The distinctive projections showed an even darker magenta than the rest of the chorion. No micropyle could be detected on the HE stained zebrafish chorion. Chorion that was stained with Masson-Goldner showed two different colors. If the outside of the chorion was exposed to the stain, the chorion appeared more green-blue, when the inside was exposed it appeared more orange-red (Fig. 31c + d) but color was not quite distinguishable sometimes. Distinctive projections appeared turquoise. Edges of the chorion appeared always red. Only one micropyle (Fig. 31e) was observed; it was stained in a bright orange. With the Alcian Blue-PAS-stain, the chorion was irregularly colored in blue and purple (Fig. 31f). No micropyle could be detected. The distinctive projections were stained in the same color than the rest of the chorion.

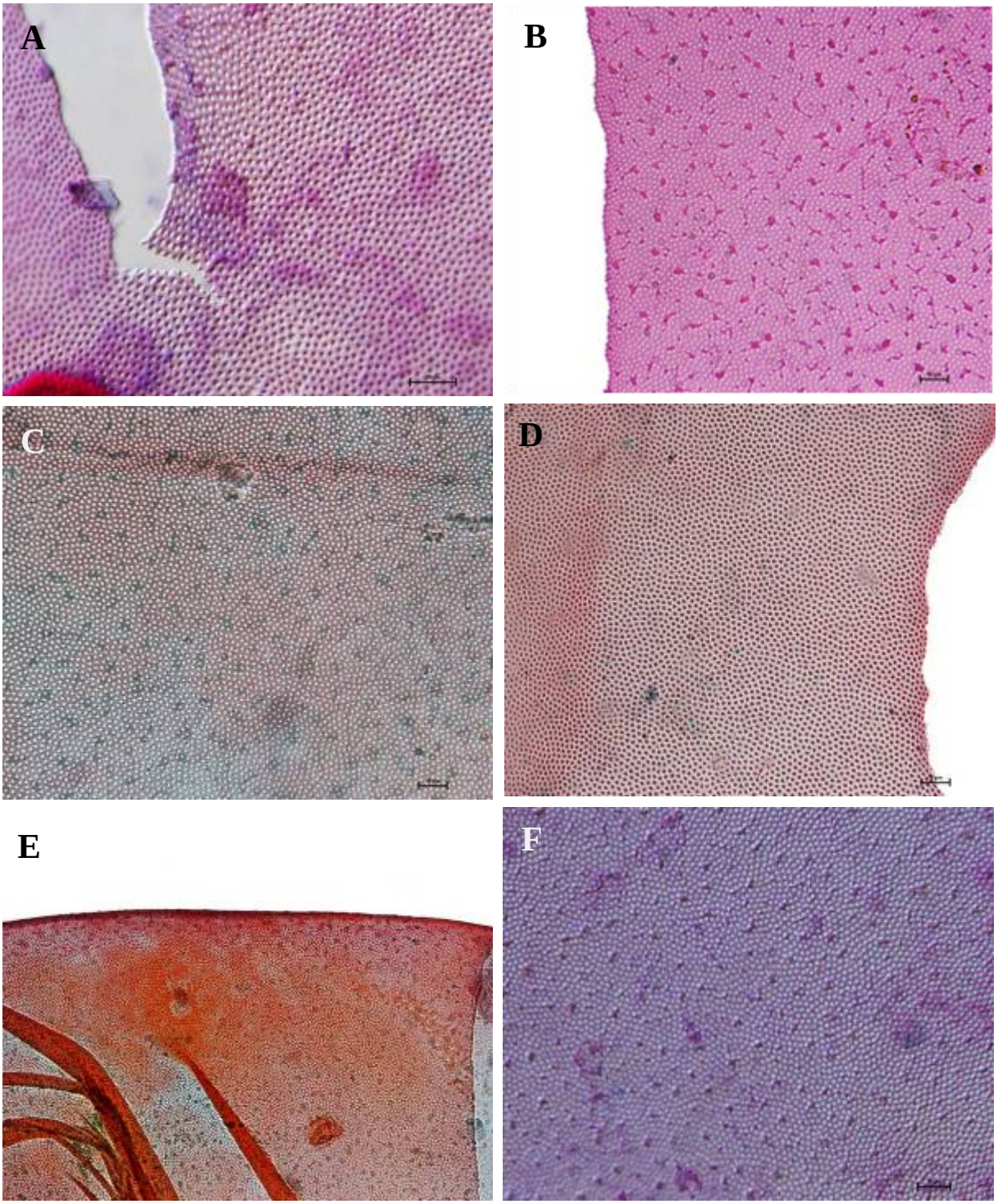


Fig. 31: Native preparation of the zebrafish chorion: A) the chorion of a 48 hpf embryo was torn apart along the pores (Alcian blue-PAS stain, 40X); B) outer surface of the chorion with distinctive projections brightly colored (HE-stain, 60X); C) outer chorion surface of a 72 hpf zebrafish embryo (Masson-Goldner, 60X); D) inner chorion surface of a 72 hpf zebrafish embryo (Masson-Goldner, 60X); E) micropyle (Masson-Goldner, 40X); F) outer chorion surface (Alcian blue-PAS-stain, 60X).

Tab. 25 Measurements of the outer and inner zebrafish chorion at different time intervals

	Number of pores per µm^2	Pore radius [µm]	Area of a pore [µm^2]	Distance between pores [µm]
24hpf-outside	3.16 ± 0.21	0.355	0.793 ± 0.163	1.909 ± 0.192
24hpf-inside	3.21 ± 0.17		1.335 ± 0.203	1.916 ± 0.195
48hpf-outside	2.79 ± 0.10	0.416	1.087 ± 0.163	1.925 ± 0.243
48hpf-inside	2.84 ± 0.17		1.465 ± 0.331	2.024 ± 0.257
72hpf-outside	3.20 ± 0.20	0.403	1.020 ± 0.309	1.871 ± 0.162
72hpf-inside	3.17 ± 0.21		1.270 ± 0.233	1.898 ± 0.207

3.6.2 Fathead minnow

The chorion of the fathead minnow appeared to be thicker than that of the zebrafish. The pores were only detected at magnifications ≥40x. The pores were not translucent as those of the zebrafish. Edges, where the chorion was ripped apart, were frayed and revealed several layers that all tore differently. Every layer looked as if it consisted of many fibers. The fiber orientation of each layer appeared to be different. After 96 hpf, the chorion seemed slightly less colorful after staining than the earlier stages. The micropyle of the fathead minnow egg is quite a prominent structure and was found quite often (, Fig. 33c). The entrance in the middle is surrounded by a slightly elevated wall. Several rays originated from the wall giving the whole structure a sun-like appearance. The rays seemed to be covered with a stretched opaque layer. The pores seemed to have a round shape on the outside and a polyangular shape on the inside like the pores of the zebrafish chorion (Fig. 33a + b). Several sessile water microorganisms are associated with the fathead minnow chorion. Vorticellae and rotifers could be found attached to the chorion. The outer surface of the chorion was often closely colonized with bacteria (Fig. 33d). To measure the number of pores per square millimeter, the pore diameter and the distances between the pores, the chorion of the different stages (24, 48, 72, and 96 hpf) were focused on the outer and inner surface (Tab. 26). The number of pores per square millimeter increased over time hpf and older)48hpf and older embryos. Therefore, theThe chorion pieces stained with HE (Fig. 32) showed the same magenta color than the zebrafish chorion, but it was more vivid. The prominent micropyle has the same color than the surrounding area. In contrast to the vividly Masson-Goldner stained zebrafish chorion, the fathead minnow chorion appeared to be light blue (Fig. 33) on the outer and inner surface. With

aging of the chorion, the stain became less strong. Only the wall of the micropyle surrounding the sperm entry side was vividly colored in orange indicating that it is made out of a different material than the rest of the chorion. Chorion stained with Alcian blue-PAS stain appeared magenta like the HE-stained chorion, but micropyle and adherend were darker colored than the rest of the chorion (Fig. 32). The lacerated chorion stained with PAS did not show the same sharp edges as the one stained with Masson-Goldner indicating that the chorion was less hard than the other and therefore easier to rip apart even though they were of the same age.

Tab. 26: Measurements of the outer and inner fathead minnow chorion at different time intervals

	Number of pores per µm^2	Pore radius [µm]	Area of a pore [µm^2]	Distance between pores [µm]
24 hpf-outside	2.37 ± 0.04	0.278	0.488 ± 0.055	2.042 ± 0.158
24 hpf-inside	2.42 ± 0.08		1.107 ± 0.122	2.061 ± 0.250
48 hpf-outside	2.97 ± 0.26	0.184	0.212 ± 0.027	1.749 ± 0.254
48 hpf-inside	3.20 ± 0.04		0.714 ± 0.136	1.805 ± 0.247
72 hpf-outside	2.92 ± 0.12	0.185	0.215 ± 0.059	1.918 ± 0.288
72 hpf-inside	2.91 ± 0.19		1.001 ± 0.215	1.926 ± 0.259
96 hpf-outside	2.67 ± 0.07	0.243	0.370 ± 0.081	1.949 ± 0.154
96 hpf-inside	2.87 ± 0.04		0.820 ± 0.171	1.903 ± 0.169

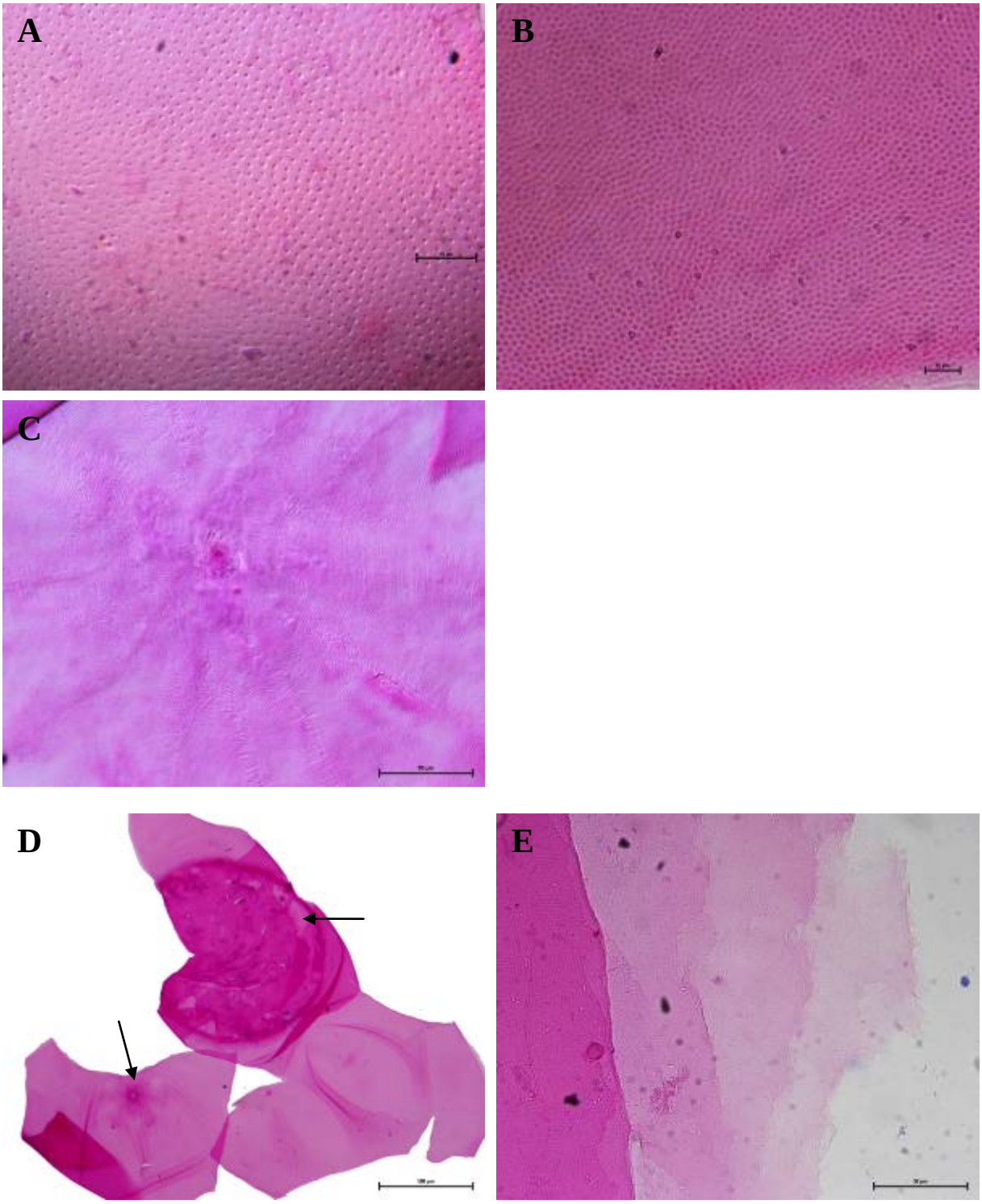


Fig. 32: The fathead minnow chorion stained with HE (A-C) and with Alcian blue-PAS (D, E): A) outer surface of the chorion (24 hpf embryo, 60X); B) inner surface of the chorion (48hpf, 60X); C) overview of a micropyle with sperm entry site and rays (72 hpf, 40X); D) top view of the chorion with micropyle and adherend (arrows, 24 hpf, 4X); E) lacerated chorion (72 hpf, 40X).

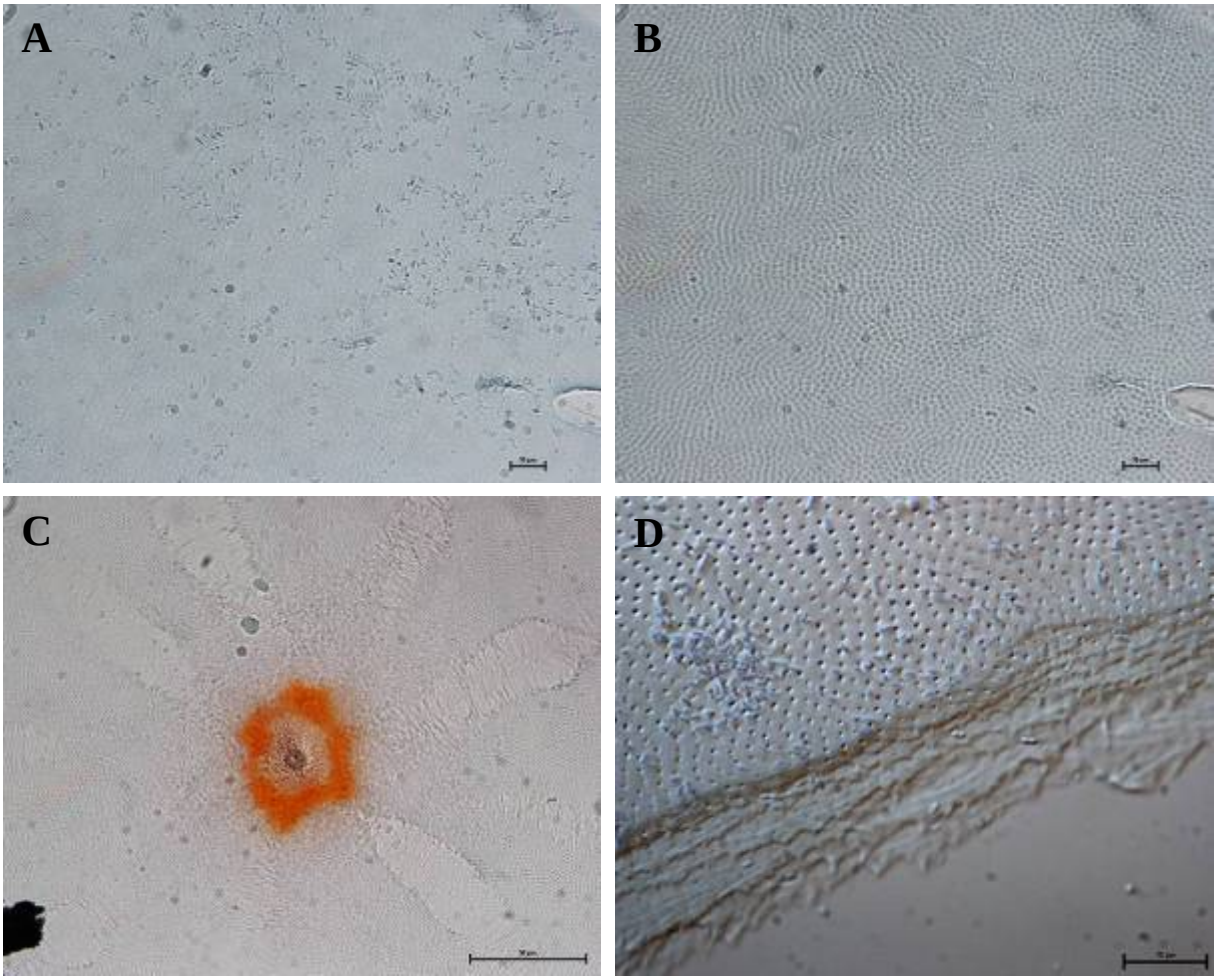


Fig. 33: Fathead minnow chorion stained with Masson-Goldner: A) outer surface of the chorion colonized with bacteria (72hpf, 60X); B) inner surface of the chorion (72 hpf, 60X); C) micropyle, wall surrounding the sperm entry side vividly colored, and rays prominent (72 hpf, 40X); D) lacerated chorion with at least nine visible layers, and bacteria on the outer surface (72 hpf, 60X).

3.7 2,7-Dichloroflurescein (DCF)

48 hour old fathead minnow eggs, incubated in 25mg/ml DCF in 0.1 % DMSO for 24 hours, only showed a distinct coloration on the chorion (Fig. 34) when examined with a confocal laser scanning microscope. The laser was set to full power; the high voltage was set to 27 and the transmitted light to 45 by the use of the auto exposure function. No signal of DCF could be detected within the egg and inside the embryo. Dechorionated embryos showed no evidence of 2,7-dichlorofluorescein. A three-dimensional picture revealed that the DCF was only apparent at sides where the eggs were attached to neighboring eggs at the nest site. No signal could be detected on other parts of the chorion, like the pores or the micropyle.

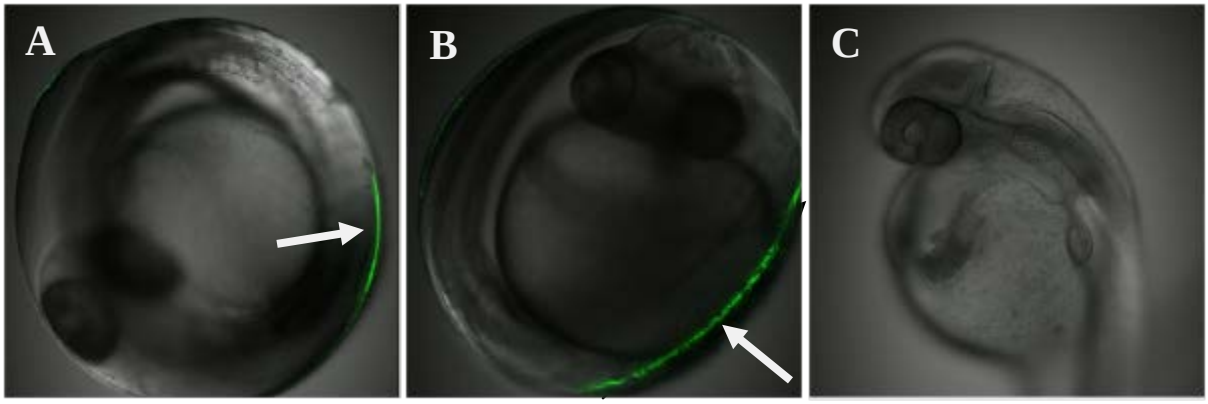


Fig. 34: 48 hpf embryos that were incubated for 24 hours with DCF; A + B) embryo in egg with signal on small area of the chorion (arrow); C) dechorionated embryo displays no DFC signal.

In 72 hours old embryos, detected effects were the same (Fig. 35). Incubation with DCF only highlighted the adherends on the chorion. Dechorionated embryos showed no DCF signal.

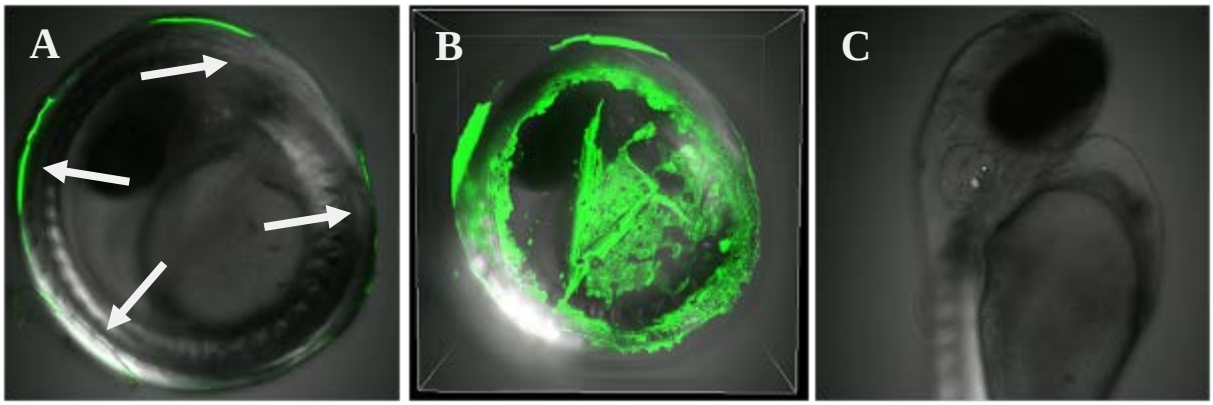


Fig. 35: 72 hpf embryos that were incubated for 24 hours with DCF; A) chorion of the embryo displays four signals of DCF (arrows) on the chorion; B) front view of the adherend of the same egg; C) dechorionated embryo displays no DCF signal.

3.8 *In vivo* EROD assay with the fathead minnow

Embryos were dechorionated and incubated after 48 hpf for three hours with β-naphthoflavone as positive control and dilution water as a negative control (Fig. 36). In the negative control, a signal was only detected in the pericardium, whereas in the embryos that were incubated for 3 hours with β-naphthoflavone a diffuse signal was detected in the circulatory system. Only the major blood vessels and the pericardium, as well as the ear placode displayed a weak signal in the embryos However, no signal could be detected in the intestinal tract. 48 hpf embryos that were incubated for 24 hours showed no signal at all in the positive control but the iridescent ear placode. The before-detected weak signal in the vessels and the pericardium had vanished.

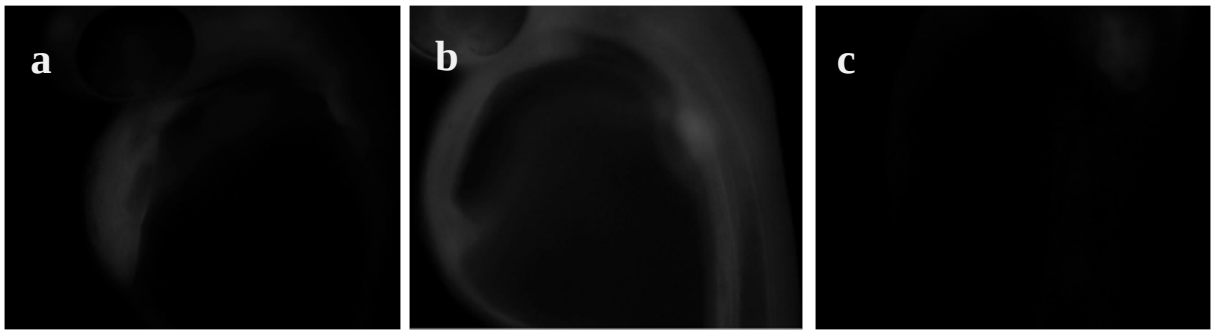


Fig. 36: Epifluorescence images of 48 hour old fathead minnow embryo; a) negative control, b) incubated for three hours with 10 µg/L β-β-naphthoflavone, c) incubated for 24 hours with 10 µg/L β-naphthoflavone(all images shutter time 20 ms).

72hpf embryos that were incubated for three hours (Fig. 37) exhibited a strong signal in the intestinal tract indicating that the CYP1A activity in this region was induced, and that the ethoxyresorufin was converted by CYP1A into the fluorescent resorufin. The gall bladder and the duct of Cuvier were also highlighted due to the resorufin signal in the positive control when examined with epifluorescence. When looked at with confocal laser scanning microscopy, a weaker signal was additionally detected in all the visible blood vessels including the heart due to signal enhancing. However, 72hpf embryos that were incubated for 24 hours (Fig. 38) did not display a strong signal, although some fluorescence could still be detected in the intestinal tract. For visualization of the signal, the shutter time had to be extended from 60 ms to 100 ms.

Fig. 37 Images show 72 hours old embryos that were incubated for three hours; a) negative control (20 ms), b) positive control, epifluorescence microscopy (60 ms), c) positive control, confocal laser scanning microscopy (CLSM) with transmitted light (laser power 100, high voltage 40).

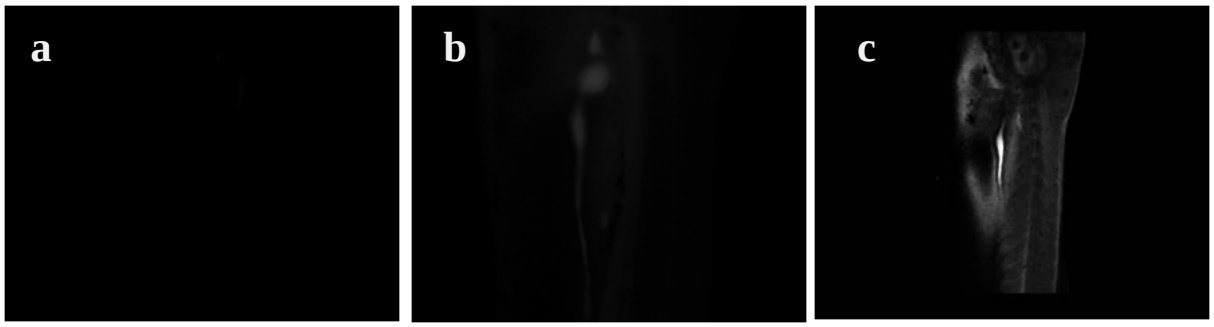


Fig. 38: Images show 72 hours old embryos that were incubated for 24 hours; a) negative control (100 ms), b) positive control, epiflourescence microscopy image (100 ms), c) positive control, confocal laser scanning microscopy (L100, HV40).

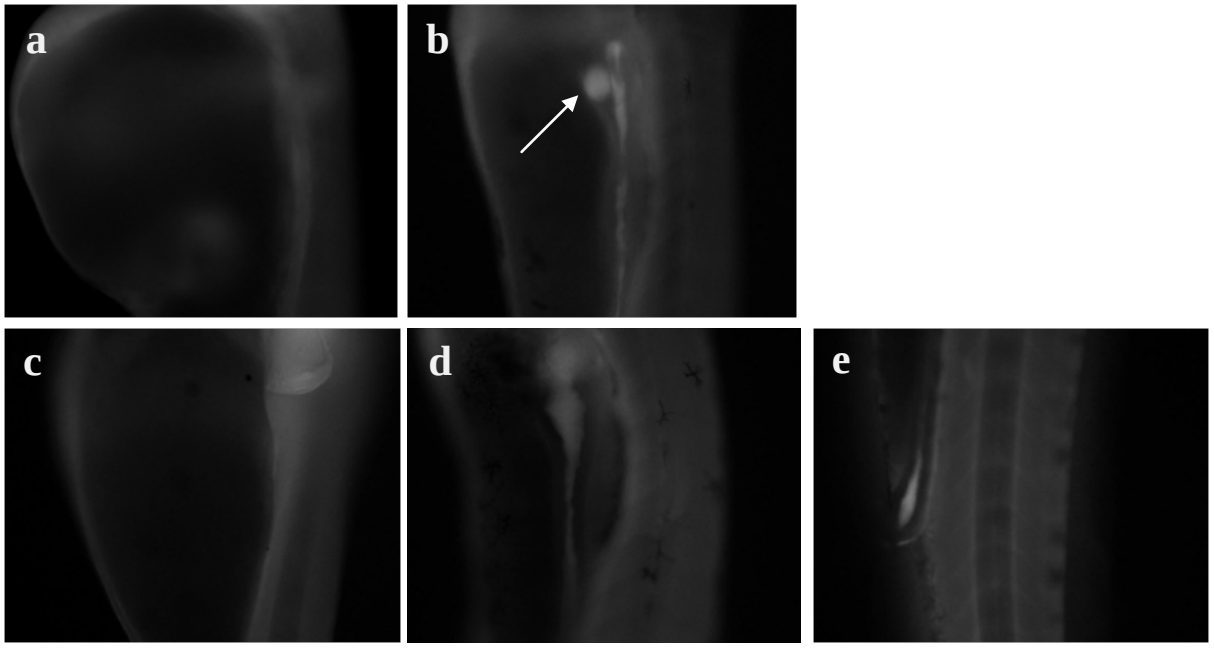


Fig. 39: The epifluorescence microscopy images show 96 hours old embryos that were exposed for three hours (a + b) and for 24 hours (c – e); a) negative control; b) positive control (arrow shows gall bladder); c) negative control; d) positive control; e) positive control, anal region (all images 100 ms).

96 hpf embryos that were exposed for 3 hours (Fig. 39) displayed the same signals than the 72hpf embryos. If exposed for 24 hours, the signal had gotten weaker but the gall bladder and the intestinal tract were still visible. The fluorescence signal was also detected at the anal region For the 120 hpf embryos (Fig. 40), only half of the normal ethoxyresorufin concentration was used due to a mistake (0.6 instead of 1.2 mg/L). This resulted in a more defined signal in the intestinal tract, and reduced the background signal. The appearance of the intestinal tract had changed but still exhibited a strong signal. The front part had grown larger compared to the previous days and the smooth lining had become serrated. Confocal laser scanning images revealed that the resorufin was again present in the circulatory system, and the intestine and the gall bladder which is located above the developing swim bladder. The signal was again

weaker if incubated for 24 hours (Fig. 40). And due to the growth of the swim bladder the intestine region was bent towards the ventral side.

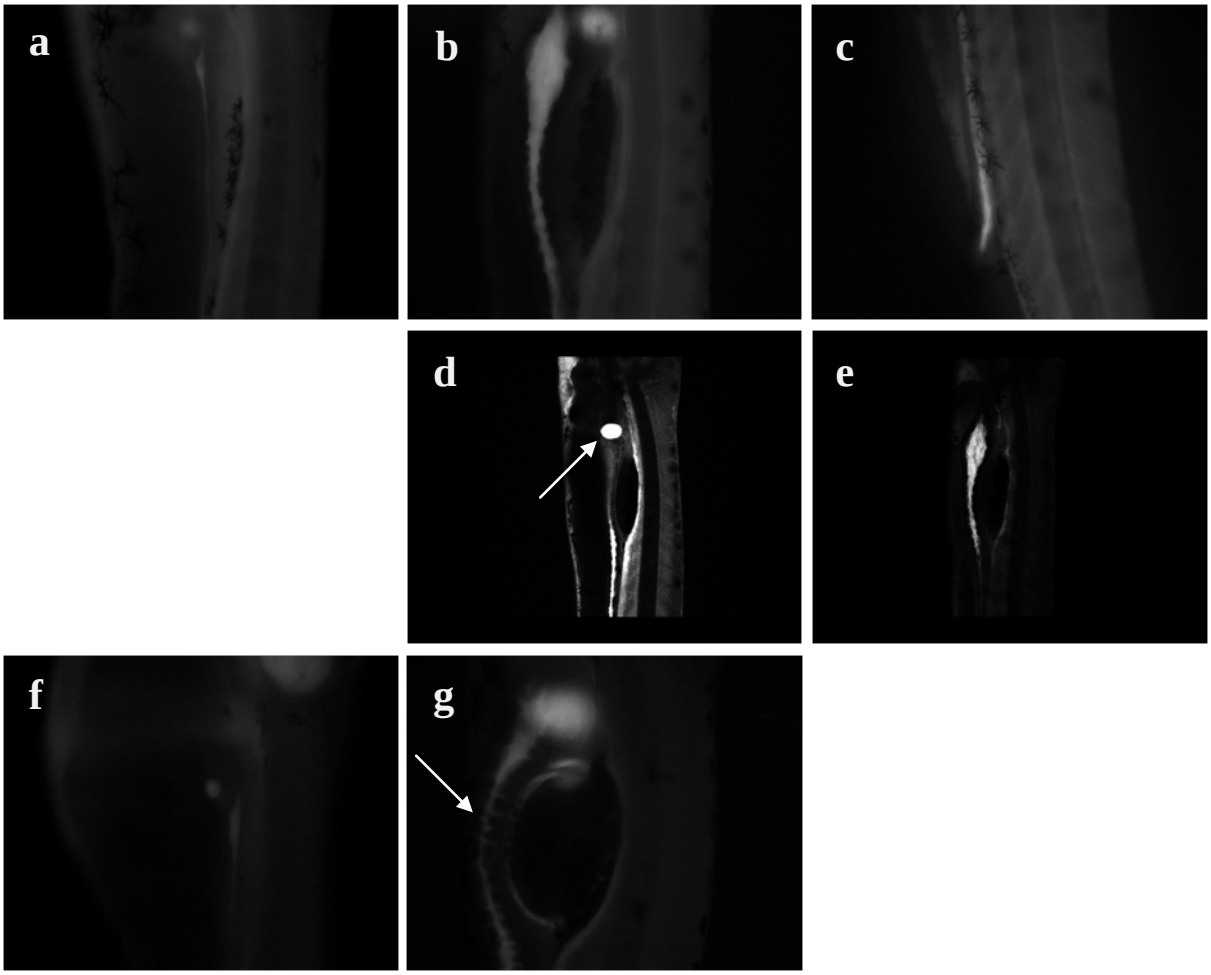


Fig. 40 The images show 120 hours old embryos incubated for 3 hours (a-e) and 24 hours (f-g): a) negative control; b + c) positive control (0.6 mg/L ethoxyresorufin), highlighting the gastrointestinal tract and the anal region, epifluorescence microscopy (all 100 ms); d) positive control, intestinal tract with focus on the gall bladder (arrow), CLSM (L 100, HV 40); e) positive control, intestinal tract, CLSM (L 100, HV 40); f) negative control for 24 hour incubated embryos; g) positive control, gastrointestinal tract has bent due to the growth of the swim bladder (arrow, both 100 ms).

In summary, the first signal of CYP1A induction could be detected in 72 hpf embryos that were exposed to β-naphthoflavone for three hours. The last good signal was detected in 120 hpf embryos incubated for three hours. The signal exhibited after 3 hours of incubation was much stronger than after a 24 hour incubation time. The accidental reduction of the ethoxyresorufin concentration reduced the background signal that was present most of the time.

3.9 Transmission electron microscopy

Six different stages of the fathead minnow chorion were analyzed: the chorion from an unfertilized egg, from a multi-cell, 24 hpf, 48 hpf, 72 hpf and 96 hpf embryo. The overall comparison of the stages revealed the chorion is composed of three different structures. The innermost structure was the most prominent one and was approximately 10 times as thick as the middle structure (Tab. 27). It is composed of several layers with each layer having a different fiber orientation. The number of layers of the innermost structure was variable and ranged between ten and twenty. Layers, that had a horizontal fiber orientation, seemed to be smaller than the other ones. It was observed that the layers merged and separated. The surface of the innermost layer facing the perivitelline fluid was loosely packed and showed a wave like structure. It was covered by a narrow middle structure which was the most electron-dense of the three and had no distinct additional features. The third structure was located on top of the middle one facing the surrounding environment. It was quite electron dense, but appeared fringy and loosely at the same time. That structure was not observed in unfertilized eggs (Fig. 41). However, a structure of rough appearance was found on top of the chorion of an unfertilized egg. The most electron dense structure in unfertilized eggs was twice as thick as in fertilized eggs. The inner structure appeared to be loosely packed and riddled with lacunae and canals especially in the layers right beneath the electron dense structure. The layers closer to the inside of the egg appeared to be more homogenous; thirteen layers were observed. The small outward protruding curvatures of the outer layer were interpreted as pores. In a multi-cell stage (Fig. 42), approximately four hours after fertilization, the chorion still consists of two structures: the inner one is loosely packed and consisted of thirteen layers; the different layers with their fiber orientation are clearly visible. The outer, electron dense layer is as thick as in the unfertilized stage. The indentations were found in the outer layers; these were covered by a plug and were interpreted as pores. The electron density of this plug was different from the chorion structures. The position of a canal from the indentation to the inner side of the chorion could be observed. It ended in an indentation of the innermost layer of the inner structure. A third structure, that covered the electron dense layer, was absent in multi-cell stage eggs.

In 24 hpf eggs, the chorion consisted of the three different structures (Fig. 43). The inner most striped layer was more homogenous than in unfertilized eggs but still riddled with lacunae and canals lacunae that showed no distinctive orientation. They could be found on top or underneath the horizontal orientated layers; sometimes, it seemed that those layers blocked the way of the canals through the chorion so lacunae could form. Between ten and thirteen of these

layers were counted. On top of the electron dense layer was a granular structure which was twice as high covering the whole structure with exception of the indentations. These indentations were observed in the middle structure; these were still blocked by round plugs that protruded into the inner and outer layer of the chorion. The innermost structure was riddled with lacunae and parts of pores canals but no whole canal was detected. 48 hpf chorions (Fig. 44) had the same appearance as 24 hours old ones. Three different structures were apparent as mentioned before. In 72 hpf eggs, the overall structure of the chorion remained the same (Fig. 45). Inner, middle and outer structure could be clearly observed. Indentations in the middle layer were now covered with the outer layer. Plugs that covered these indentations before were missing. Layers of the inner structure seemed to have moved closer together the nearer they were to the middle structure, layers close to the perivitelline fluid were wider in diameter. Twenty layers were observed. Lacunae and canals were still present. Sometimes, a lacuna was no longer closely surrounded by the chorion.

In 96 hpf eggs, still three structures could be observed (Fig. 46), although the outer one had decreased in height and coverage. It was only found in spots on top of the middle structure. The inner structure of the chorion seemed to be torn. In this stage the inner structure was composed out of 18 layers.

Tab. 27: Overview of the length [µm] of the different chorion structures

	Unfertilized egg	Multicell-stage	24 hpf egg	48 hpf egg	72 hpf egg	96 hpf egg
Total [µm]	7.010 ± 0.410	4.545*	6.734 ± 0.442	7.755 ± 0.474	10.009*	6.523 ± 0.740
Outer structure [µm]	/	/	0.130 ± 0.026	0.114 ± 0.024	0.087 ± 0.028	/
Middle structure [µm]	0.134 ± 0.008	0.140 ± 0.025	0.069 ± 0.009	0.130 ± 0.023	0.092 ± 0.152	0.115 ± 0.026
Inner structure [µm]	6.574 ± 0.773	7.578 ± 0.485	6.560 ± 0.448	4.433*	9.842*	6.022 ± 1.174

Explanatory notes: / - data not available, * - only one measurement could be taken, for outer/ middle structure n = 20, for inner structure and total n= 3.

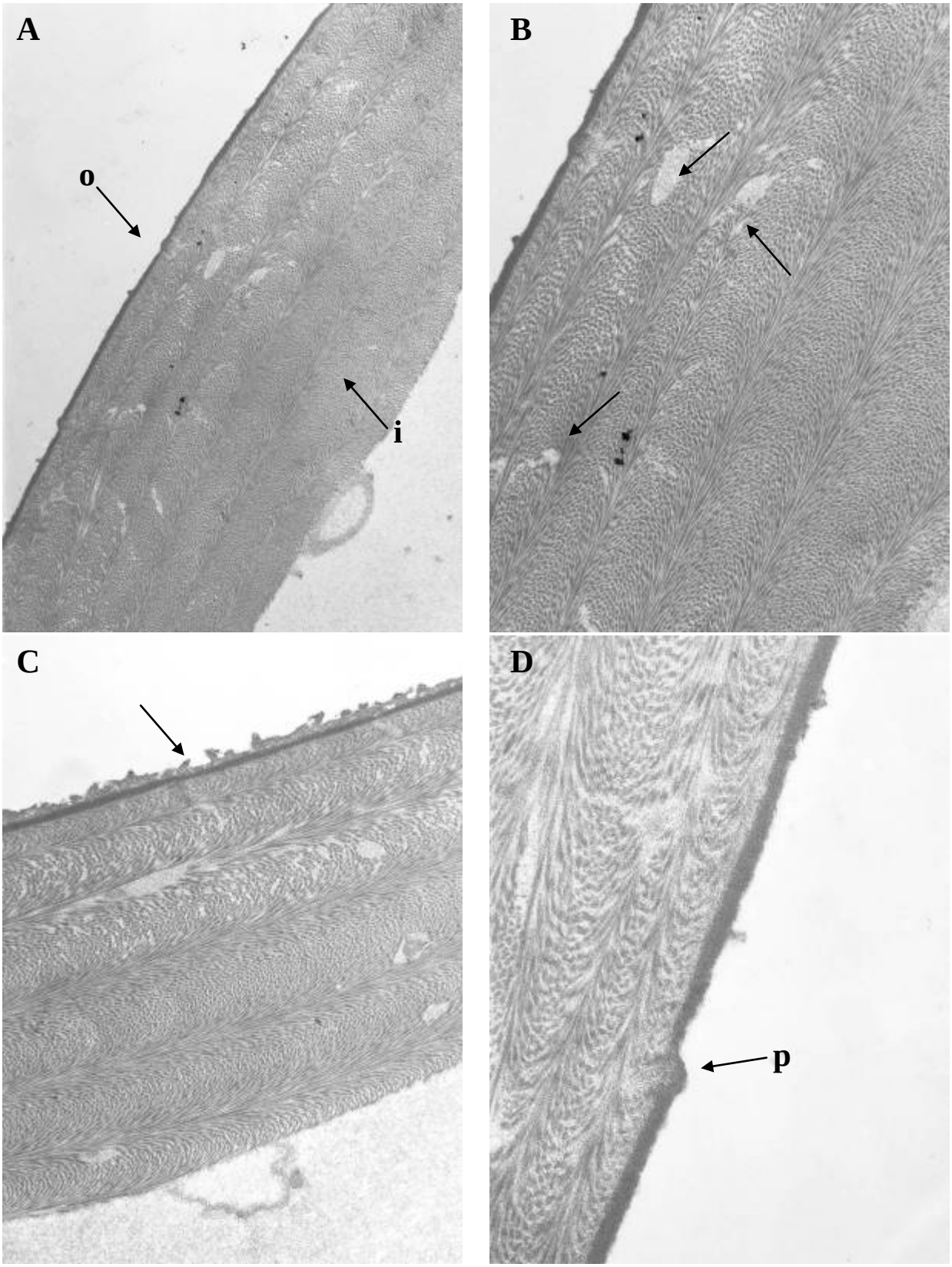


Fig. 41: Images of the chorion of a unfertilized fathead minnow egg taken with a Philips CM10 transmission electron microscope; A) Overview of the chorion, o – outer structure of the chorion, i – inner structure of the chorion (4400X); B) Close-up view, layers of the inner chorion structure merge (M), arrows show lacunae and canals in the less dense part of the inner structure (8700X); C) Chorion with a structure on top of electron dense one (Arrow, 6200X); D) Close-up view of the outer structure of the chorion revealing a pore (p, 16000X).

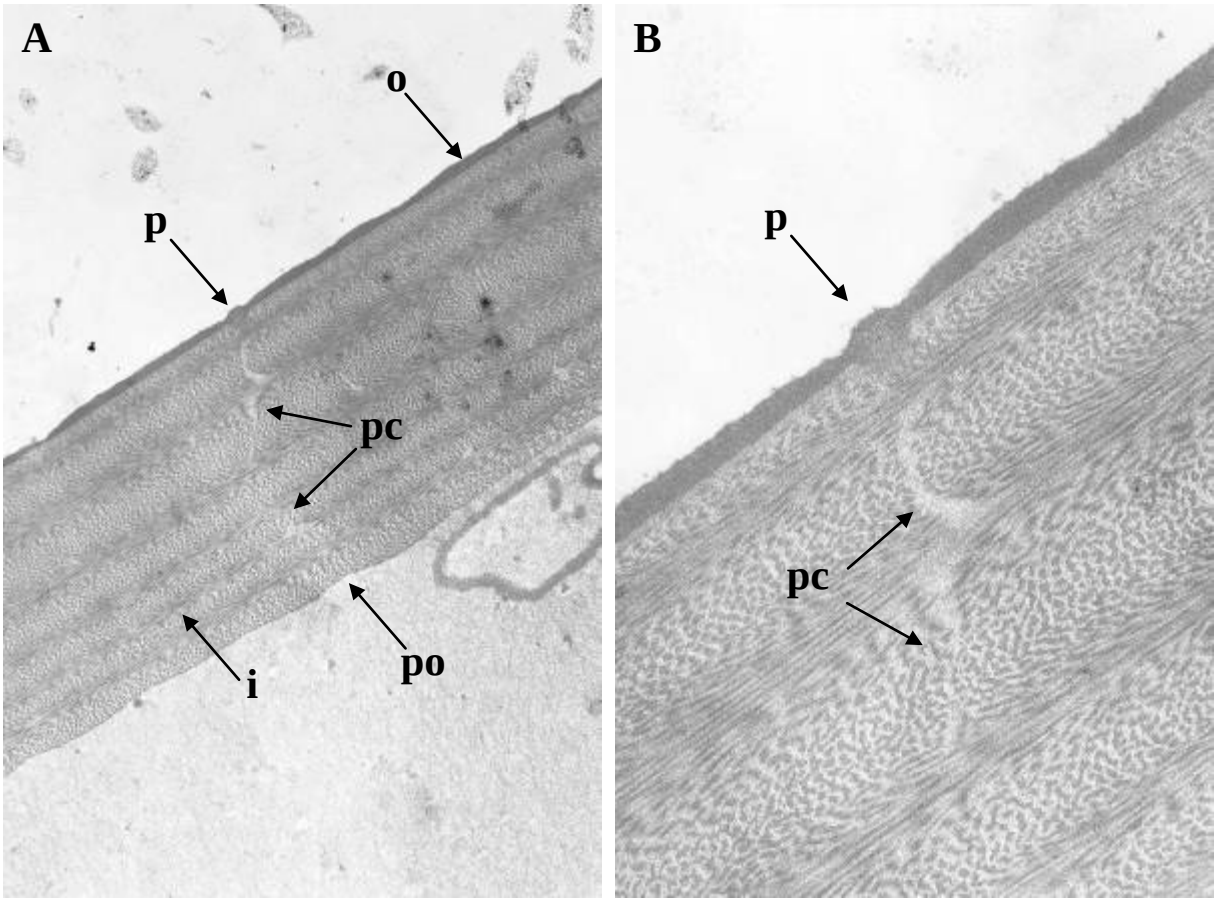


Fig. 42: Images of the chorion of a multicell fathead minnow egg taken with a Philips CM10 transmission electron microscope; A) Overview of the chorion, outer (o) and inner (i) structure of the chorin clearly visible, as well a pore (p) with subjacent pore canal (pc) that is protruding trough the whole chorion where it ends in an indentation of the wavy innermost layer (4400X); B) Close-up view of the thick outer layer with the pore (p) and the pore canal (pc) (16000X).

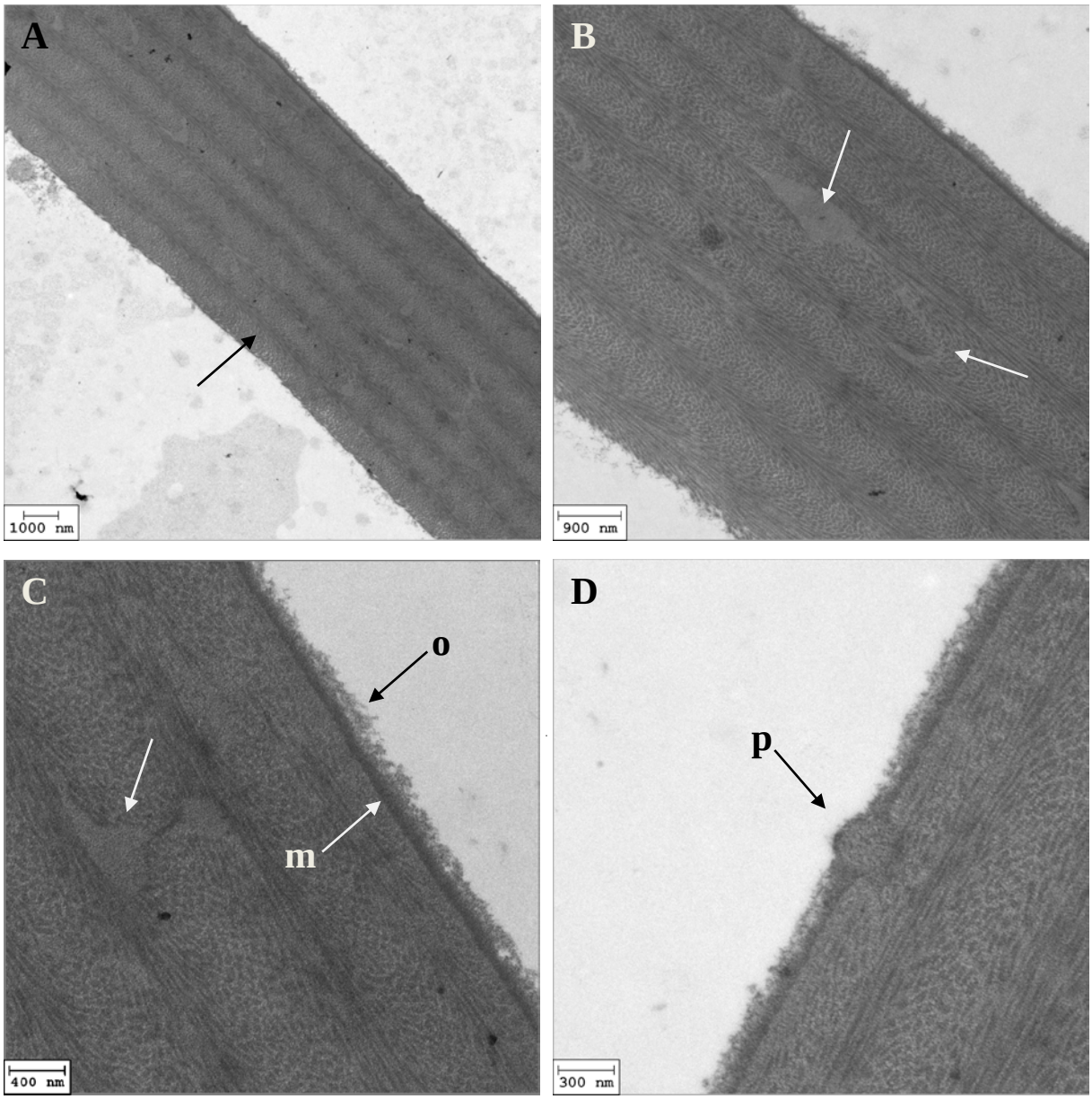


Fig. 43: Images of the chorion of a 24 hpf fathead minnow egg taken with a JOEL JEM-1400 transmission electron microscope; A) Overview of the chorion (arrow striped inner structure); B) Closer view of the chorion (arrows - lacunae and canals through inner structure of the chorion); C) Close-up view of the outer (o)and middle (m) structure of the chorion (arrow - lacuna); D) Close-up view of a pore (p) in the chorion which is filled with a round plug.

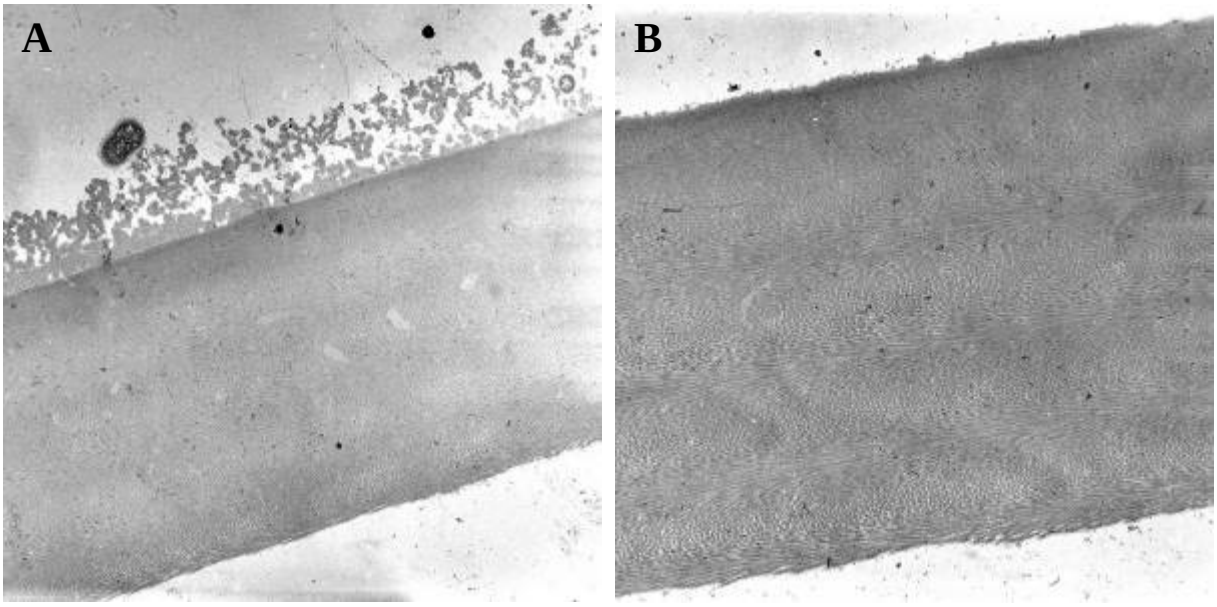


Fig. 44: Images of the chorion of a 48 hpf fathead minnow egg taken with a Philips CM10 transmission electron microscope; A) Overview of the chorion (3400X); B) Closer view of the chorion (6200X).

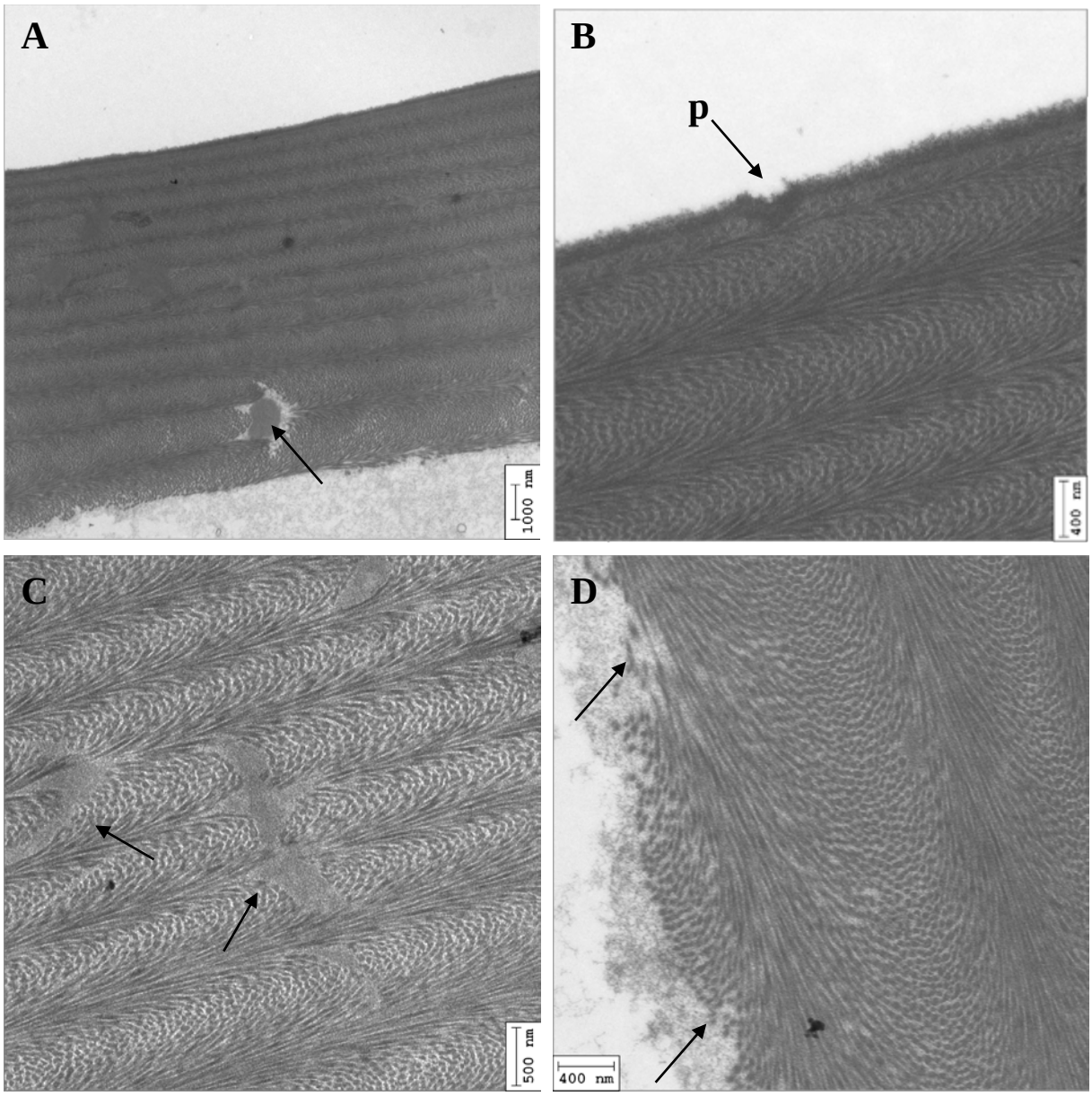


Fig. 45: Images of the chorion of a 72 hpf fathead minnow egg taken with a JOEL JEM-1400 transmission electron microscope; A) Overview of the chorion (arrow – lacuna); B) Close-up view of the outer parts of chorion showing pore (p) without plug; C) Close-up view of the inner structure of the chorion; different layer became wider and looser packed the closer to the inside (arrow – lacuna and canals); D) Close-up view of the inner most layers of the inner structure facing the peri-vitelline fluid; its appearance is fringy.

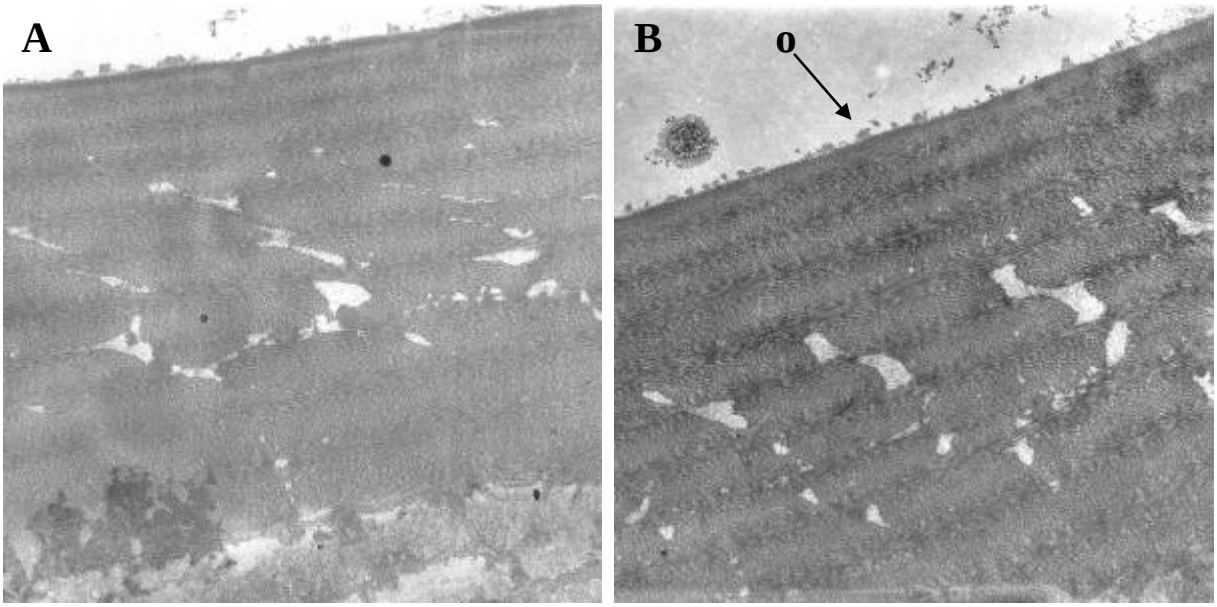


Fig. 46: Images of the chorion of a 96 hpf fathead minnow egg taken with a Philips CM10 transmission electron microscope; A) Overview of the chorion (4400X); B) Close-up view of the chorion showing the disintegration of the outer (o) structure (6200X).

4 Discussion

4.1 Behavior of fathead minnows in breeding tanks

Extensive ethological research has been carried out in order to understand fathead minnow behavior and male-female interaction (Cole and Smith 1987, McMillan and Smith 1974, Sargent 1988, 1989, Unger 1983, Unger and Sargent 1988). Observations of the spawning tanks revealed that behavior of the minnows was identical to observation made by the researchers mentioned above indicating that overall behavior of fish in spawning tanks was not impaired. Disturbances due to cleaning, feeding or water quality checks did change behavior even though the fish held at the United States Environmental Protection Agency (Denny 1987) do not get any special treatment to avoid disturbance induced stress. Breeding pairs are only separated from each other by stainless steel meshes inside the tanks (Denny 1987). In other laboratories (e.g. BASF), fathead minnows are held in stainless steel tanks with matching lids to keep disturbance to a minimum.

Differences in breeding color of male fathead minnow were explained by Unger (1983) with different level of rivalry. Dominant fish display very colorful breeding color. Fish, that are less dominant but living close to a rival mimic this breeding color to avoid nest predation and territory loss. However, solitary males remain pale. At the end of the spawning season, male fish again became pale because displaying breeding color is quite energy consuming, besides caring for eggs and defending the nest site.

The behavior of observed male fish that were defending their territory by pushing opponents, both male and female, with their anterior end of the snout, directed at various parts of the opponent fish, was termed ‘butting’ by McMillan and Smith (1974). The authors pointed out that territorial behavior became more aggressive when guarding eggs. Fish even started to attack much bigger objects like fingers in order to defend their nest site against intruders. Cole and Smith (1987) observed that female minnows normally signaled disinterest in males inside spawning tanks; they stayed close to the bottom and away from the spawning substrate. Males on the other hand stayed inside their territory with the spawning substrate as their ethological centre (McMillan and Smith 1974).

Spawning behavior was described in McMillan and Smith (1974) but sometimes spawning behavior did not necessarily led to spawning (Cole and Smith 1987). After spawning, male minnows cared for eggs that are attached in a single layer to the surface by removing dead or

fungal eggs with its mouth (Johnston 1999, Johnston and Page 1992, McMillan and Smith 1974). Due to this parental care, it could happen that embryos could hatch from eggs that were not spawned inside the spawning substrate. Parental care of fathead minnow males is rather interesting and has been the subject of several studies (Green et al. 2008, Sargent 1988, 1989, Unger 1983, Unger and Sargent 1988).

Why female minnows display male characteristics could not be determined but it might be related to a decrease of female hormones in the fish after it had 'spawned out'.

4.1.1 Husbandry & Breeding

The number of spawning pairs or groups maintained must meet the demands of the researcher; however spawning success might not only be attributed to the number of spawning groups but also to the age of the fish. The U.S. EPA (Denny 1987, 1988) maintains 96 breeding pairs which produce between 1000 and 2000 eggs a day which led to 400-500 juvenile fish for acute testing. The twelve groups maintained in the fish room at the University of Heidelberg did sometimes spawn not within a week which may indicate that the age of the spawning fish may have impaired the egg production. Even though, the number of eggs per spawn recorded was nearly as high as the numbers known from studies with wild female fathead minnows during breeding season (Denny 1987): 242 compared to 258, but fecundity was often only half as high. This fact and that sometimes female minnows only spawned once or twice could indicate impairment due to age.

Fish kept in spawning pairs did not show an increased rate in egg production as it is reported by the EPA (Benoit et al. 1982). Separation of fish into spawning pairs is not essential (Denny 1987), although keeping fish in pairs reduces fighting and competition between males (Denny 1987) which was often observed inside the spawning tanks with two males and four females, and therefore the fish could use their energy for spawning. Furthermore, spawning pairs allow a more precise documentation on fecundity and 'spawned out' fish could easily be detected and replaced.

The high number of fish dying in the spawning tank might be related to the high age of the fish (2.5 - 3.5 years) and the high energy consumption needed during breeding. Males must defend their territory from intruding males and females and had to care for their nest site and female must produce eggs. Therefore, it is not surprising that male fish die after a spawning season in the wild (Unger 1983). Even though, fish in the spawning tanks had not to defeat their nest but their territory which may have been too much for the aged fish, the same applies

to the female minnow where energy consumption for egg production might have been too much for the fish.

4.1.2 Feeding

Even though, protein rich adult brine shrimp were only fed once a day, it did not seem to impair the adult minnows and their ability to produce eggs. Variety in diets for the fathead minnow was observed, but no one else used TetraMin as a supplement. Fathead minnow in the breeding stock of the U.S. EPA are fed with frozen adult brine shrimp; other common diets for fathead minnows were mixtures of frozen adult brine shrimp mixed with trout pellets (Chiu and Abrahams 2010, Unger 1983, Unger and Sargent 1988) or a diet consisting of trout pellets (Sargent 1989). It could be considered to reduce feeding times from three to two times a day to minimize overfeeding like it is done by the U.S. EPA (1987, 1988).

4.1.3 Spawning substrate

It made no difference to the fish whether they were provided with clay pot halves or PVC houses but for collecting eggs, the houses with the adjoining trays should be preferred by the collectors because sometimes eggs fell on the ground. If proper saucer could be provided for clay pot halves, they could be used as well. It seemed that fish did not mind about their spawning substrates as long as there is a visual presence of an overhanging ceiling (Cole and Smith 1992, McMillan and Smith 1974). This explains why fish also spawned underneath the drain pipe inside the tanks, and why multiple spawning substrates beside clay pot halves and PVC pipes are used to gain eggs. Unger (1983, 1988) and Sargent (1988, 1989) used suspended spawning substrate made of clay bricks and a glass spawning surface, McMillan and Smith (1974) and Cole and Smith used C-shaped concrete blocks (1992) as well as clay pot halves (1987) as spawning substrate. Benoit (1982) used semicircular arched stainless steel glued on quartz sand as spawning surface; the US EPA suggests in their guideline (Denny 1987, 1988) different possibilities: clay pot, PVC pipe, and fiber cement water pipe halves.

Even though the EPA stated in their guideline for the culture of fathead minnow (Denny 1987, 1988) that threadable PVC does not contain leachable substances and can be used inside aquaria. The PVC house parts were cooked in order to remove any substance from the material which may interfere with the development of the embryo attached to the surface or may impair the health of adult fish.

4.1.4 Handling of eggs

Removal of eggs from the substrate with both methods did not interfere with egg yield, leaving the choice with which methods the eggs should be removed in the hands of the handler. But, rolling eggs of the surface needed more training because wrongly applied pressure could easily lead to damage of the egg. Change of detachment method may also be considered when a different spawning substrate is used because change in surface texture could lead to differences in egg attachment. Rolling of eggs should have led not only to a detachment from the surface but also to a separation of the eggs from one another. However, this did not happen to full extend when eggs were rolled of the PVC houses probably due to the smooth surface where the eggs could detach more easily than from a rougher surface. Success rate of detaching eggs from substrate and separating them from one another is rather dependent on chorion thickness, and therefore egg quality, and not necessarily on age because eggs as early as four cell stages (approx. one hour after fertilization) could be separated without losses.

However, preparing fathead eggs for the use in the fish embryo toxicity test or for other purposes, takes a considerable amount of time which is dependent on number of eggs, egg quality and clutch size. Zebrafish eggs, on the other hand, just need to be collected from the spawning tanks, and can then directly be used (OECD 2011). Handling stress on the egg is therefore minimized to the transference of the eggs with pipettes into beakers.

4.1.5 Measurements

The measurements concerning the fathead minnow egg are approximations made under the assumption that the fathead minnow egg is spherical but it is not. Compared to the zebrafish egg, the egg of the fathead minnow is slightly more laterally depressed. This more ellipsoidal form may occur because the eggs are attached to one another in clutches and to the spawning substrate. The adhesive parts are flatter than the rest of the egg due to space conflicts with neighboring eggs. However, the obtained measurements still allow a comparison with the zebrafish. Measurements concerning the dimension of the zebrafish egg are mostly taken from Von der Golz (2007); measurements concerning the chorion are taken out of Hart and Donovan (1983) and Rawson (2000) and calculated anew. The total volume of the zebrafish egg was also newly calculated because there was an error in the original data (Von der Golz 2007). The new value for the zebrafish chorion was nearly twice as high as before, the chorion thickness was set to 2 microns and the chorion volume to 0.008 mm3. The first measurements indicated that the fathead minnow egg is by 0.2 millimeters bigger in diameter than

the zebrafish. This led to a one third bigger surface area, to an increase in egg and chorion volume. The zebrafish chorion is between 1.5 and 2.5 microns thick, whereas the fathead minnow is 7 microns thick on average, according to Manner et al. (1977) even 10 microns, which is a 3 to 5-fold difference. The calculated volume led to a 6-fold difference between zebrafish and fathead minnow. However, the total volume of the egg did only double. No measurements for the embryo volume could be taken because the embryo was already lying close to the chorion due to its big size compared to the smaller zebrafish embryo. Von der Golz (Von der Golz 2007) calculated embryo volume under the assumption that the embryo has a spherical shape like the egg.

Due to the greater surface area, the contact surface for substances may by greater as well, but it is possible, that the adhesive surfaces where the eggs were attached to surface and neighboring eggs might restrict uptake of chemicals. The increased chorion thickness and volume may also restrict the uptake and the fathead minnow chorion could be a better barrier than the zebrafish chorion. Due to this increase in volume, the chorion could also serve as a much better sink for substances assuming a comparable composition of the chorions.

The wet weight measurements should not be considered accurate due to constant water loss and probably excess water still attached to the eggs. Therefore, it was refrained to use these measurements. The dry rate is more accurate because all changeable substance had been evaporated but there were no data available for the zebrafish. However, density was still calculated and compared. The density of the fathead minnow egg is approximately twice as much as zebrafish egg. They are both heavier than water explaining why the zebrafish eggs after spawning and the fathead minnow eggs after detaching sink to the bottom of the dish.

4.2 Embryonic development

Recording and determining of different developmental stages of the fathead minnow is very important in order to understand and to classify effects of chemicals that may harm the embryos. Therefore, it is important to gain data obtained under the same exposure conditions as water composition, temperature and light cycle. Only two studies are available for fathead minnow development (Devlin et al. 1996, Manner and Dewese 1974), but only one met the criteria of 25 °C water temperature and 16 hours light: 8 hours dark light cycle.

Manner oriented the fathead minnow development temperature on 23°C, the temperature at which adult minnows where kept for breeding. His staging proved to be useful to determine the age of the eggs detected in the spawning tanks which normally had a water temperature

between 23 and 24 °C. However, the more detailed study from Devlin et al. (1996) was carried out at 25 °C, and because the testing conditions of the fish toxicity test were adjusted to 25 °C. This was more helpful in order to detect developmental differences that may occur due to exposure to chemicals. Comparison of the present observation with Devlin revealed certain differences, although the overall timeline was quite similar. Due to a lack of very early stages, and to allow comparison of both studies the timeline was adjusted: the two-cell stage according to Devlin et al. (1996) occurred after 1 hour. This was also assumed for the 2-cell stages in the present study. The change from 8 to 16 cells (16 to 32 cells) occurred 0.5 h (1 h) earlier than recordings of Devlin et al. (1996) suggest. Late cleavage, high and flat blastula stages were observed in both studies within the same time frame. The first stage of epiboly occurred later than recorded by Devlin et al. (1996), but the last stage occurred earlier. Somite stages, however, were observed within the same time frame. There might have been an influence of changing water temperature on development. Even though the temperatures were kept within 25 ± 1 °C; the one degree more or less might have already slowed or accelerated development. Manner's observation showed that normal development of the fathead minnow embryo was slowed down to a great extend compared to Devlin et al.(1996). The 2-cell stage was observed already an hour later than in the present study, two hours after fertilization. Later cleavage stages, where cell counting proved to be hard, occurred at similar times compared to Devlin et al. (1996) and to the present observation indicating that exact determination of stages is rather difficult. However, greater time differences in development due to lower temperature only became apparent after gastrulation. The neurula stage occurred with eight hours delay, and the neuromere stage even with 13 hours compared to Devlin et al. (1996) and the present observation.

In comparison, the zebrafish development progresses more quickly than the fathead minnows (Hisaoka and Battle 1958, Kimmel et al. 1995): Again water temperature was of the essence. While Hisaoka and Battle studied zebrafish development at 26 °C, Kimmel et al. used 28.5 °C water, therefore, zebrafish in Kimmel's study developed faster. Embryos in both studies developed faster than the fathead minnow up to the closure of the sperm ring. Then, developmental stages (Hisaoka and Battle 1958) were observed at similar times in both zebrafish and fathead minnow. Neurula stages between 14 and 16 hours in zebrafish and at 16 hours in fathead minnow, neuromere stage after 20 hours in both fish species indicating that speed of development of different fish at similar temperatures align to a certain degree.

However, due to a good timeline from 2-cell to first movement of the embryo, there are no recordings of an unfertilized egg, a recently fertilized egg and the one-cell stage. *In-vitro* fertilization as it is performed with the zebrafish (Westerfield 2007) did not seem to work. Neither eggs nor milt could be obtained which leads to the assumption that the used method is not feasible for the fathead minnow or that the researcher was not experienced enough. It might also be that especially the females were not gravid enough to release the eggs when pressure was applied to the belly. Devlin, on the other hand, killed fathead minnows to obtain eggs directly from the ovary and milt from testes, this procedure was not carried out due to a low number of fish available.

4.3 Fish embryo toxicity test

The fish embryo test with the fathead minnow is performable as stated by Braunbeck et al.(2005), but minor adjustments needed to be made to the existing zebrafish protocol (OECD 2011). For one, the cultivating and testing temperature for the fathead minnow embryo test was decreased by 1 °C to 25 °C, which is the maintenance temperature of the fathead minnow in the United States (Devlin et al. 1996), where the minnow is the most important laboratory fish species (Ankley and Villeneuve 2006), and at which a proper description of normal embryonic development is available (Devlin et al. 1996). Furthermore, number of eggs used for every single test concentration needed to be halved and an internal negative control was set aside due to a lower egg yield per spawning incident (70 - 150 eggs), in comparison with the zebrafish embryo test, in which twenty eggs per concentration and controls are used. Higher yields of eggs may be obtained by using younger minnows (0.5 – 1.5 years). The egg number and the sometimes lesser quality of the eggs may be owed, on one hand, to the progressed age of the fish (2.5 – 3.5 years).

Another problem that frequently occurred while working with minnows, were the poor prediction and identification of spawning incidents. Fathead minnows spawned in different time intervals (2 - 5 days, sometimes even longer) and at different times of the day. If disturbance level was higher than usual, no eggs could be obtained at all. Sometimes, the minnows displayed fake spawning behavior as described in Cole and Smith (1987). At other times, eggs were removed too early, which meant they were either not properly fertilized or that the number of eggs was quite low (< 70). Therefore, it was quite difficult to obtain enough eggs as early as possible. Due to all this obstacles, it was also difficult to pre-expose beakers and 24-well plates prior to the experiment. Normally, plates were pre-exposed as soon as another test

had started. This gave the opportunity to run a second test as soon as new eggs were available. But, if no eggs were detected and depending on the substance and their degradability, chemical solutions and well plates were discarded after a week of pre-exposure.

Compared to the fish embryo test with the zebrafish (OECD 2011), it took much longer to expose the eggs in the test concentrations. While time zebrafish spawning is highly predictable, and pre-exposure and other preparations can be performed the day before and in the morning of the start of the experiment, nearly no predictions can be made for the fathead minnow. Furthermore, fathead minnow eggs need to be detached from the spawning substrate and separated from each other, while zebrafish eggs are ready to use. After detection and separation, the test solutions need to be prepared as well. The use of substances with a high log K_{ow} which need some time to dissolve before it can be used to expose eggs. Therefore, it is not favorable for tests with the fathead minnow egg. Due to this time consuming procedures, it is also difficult to expose as early stages in development as it is common with the zebrafish. The normal developmental stages exposed ranged between 8 and 64-cell stage.

Another problem addressed was the duration of the fish embryo toxicity test with the fathead minnow. While zebrafish test are carried out for 96 hours, fathead minnow tests lasted for 192 hours to determine, for how long the tests can be carried out without violating the laws (EC 2010a). It was observed that the minnows in the negative control had a fully developed swim bladder at 144 hpf, which enabled them to swim freely. Belanger (2012) stated that feeding initiates between 120 and 144 hours in the fathead minnow which marks the transition between eleutheroembryo and larvae. In the future, tests with the fathead minnow embryo should to be terminated after 144 hours except in cases where an extension of maximum two days seems preferable. The overall procedure of the fathead minnow test corresponds to the zebrafish test, but after 120 hours exposure is switched from semi-static to static to avoid damaging the eleuthroembryos.

4.3.1 Cadmium chloride

Apparently, the free cadmium ion is the toxic form of cadmium (Suedel et al. 1997). Cadmium chloride dissociates in water and can build complexes with carbonate or associate with dissolved organic matter (Meinelt et al. 2001). Therefore, the amount of cadmium ions available is dependent on water quality(Sherman et al. 1987) and more bioavailable and toxic the lower the alkalinity, hardness (Brinkman and Hansen 2007) and dissolved organic content (Witeska and Jezierska 2003). Dilution water at an adjusted pH does not give the cadmium

ions many possibilities to form complexes and hence it remains toxic.

Cheng et al. (2000) recorded six different types of deformities in zebrafish development due to cadmium exposure that increased with cadmium concentration. These effects, namely head hypoplasia, less pigmentation, cardiac edema, yolk sac abnormalities, altered axial curvature and tail malformation, all occurred during fathead minnow development as well. (Cheng et al. 2000) also stated that altered axial curvature and tail malformations in zebrafish occurred due to deformities in the somites, and that the nervous system was impaired to a great extend. This explains the insufficient development of the head region which was confirmed by Chow and Cheng (2003) and Chow et al. (2008, 2009). Furthermore, Chow found out that cadmium exposure impaired axon growth, notochord development and eye development in zebrafish embryos. Microarray studies (Sawle et al. 2010) showed, that cadmium chloride influenced cytoskeletal parameters and cell cycle phases. Fish took up cadmium over the gills, where it competes with calcium ions (Galvez et al. 2008, Glynn et al. 1994). Calcium uptake does not only occur at the fish gills. In embryonic development, calcium transport is elevated for optimal growth, inducing also the uptake of cadmium. Cadmium is believed to be taken up by the same pathway than calcium shown by (Galvez et al. 2008) in a study with zebrafish. (Hallare et al. 2005) also recorded head edema and, in hatched embryos, convulsions of the body, as well as reduced swimming activity and subsequent immobilization. Disturbances of the swimming behavior due to cadmium chloride were also observed by (Fraysse et al. 2006).

The toxicity of cadmium chloride is dependent on the age of the fish and the test species: While the LC_{50}-value of cadmium chloride after 48 hours for the fathead minnow embryo was 12.2 mg/L, the value for adult fathead minnows exposed for 48 hours in an acute static test was 8.9 mg/L (Suedel et al. 1997). After 96 hours of exposure the difference between the LC_{50}-values was less than a milligram per litre: 4.11 mg/L in the fathead minnow embryo test and 4.8 mg/L in the acute test. Overall, the LC_{50} (96 h) values for the fathead minnow acute test ranged from 0.63 mg/L up to 73.5 mg/L mostly due to differences in water hardness, pH, and alkalinity, but the high values of 73.5 mg/l (Pickering et al. 1996) are nominal values, and as (Sherman et al. 1987) stated, there was a great difference between measured and nominal values of cadmium chloride in the acute fathead minnow tests. Early larval stage tests with the zebrafish led to two different LC_{50}-values: 0.65 mg/L at pH 7 (Dave 1985) and 30.1 mg/L (Hallare et al. 2005). Overall, the fathead minnow embryo is slightly less toxic to cadmium chloride than adult fish. This is probably because the chorion acts as a sink for cadmium (Galvez et al. 2008). It is suggested that cadmium binds to an anionic charged group, possib-

liy sufhydryl, in the chorion (Wendler 2012). Dave et al. (1985) found out that accumulation of cadmium is strongly pH dependent: the lower the pH, the more metal is bound to the chorion. Meinelt et al. (2001) calculated that 61 % of the cadmium accumulated in the chorion, 38 % reached the perivitelline fluid and only one 1 % was found in the embryo. This would explain why the mortality of the embryos increased after 72 hours when cadmium treated embryos started to hatch.

Hatching success was impaired due to mortality that increased with cadmium concentration. In the negative control most fish (90 %) had hatched after 120 hours, whereas in higher concentrations fish still hatched after 168 and even 192 hours. This delay in hatching time was also observed by (Fraysse et al. 2006) who conducted cadmium tests with zebrafish embryos and (Gauthier et al. 2006) who stated that fathead minnow hatching time was elevated in cadmium contaminated lakes whereas laboratory control did not show any influences of cadmium on hatching. Other sources (Hallare et al. 2005) also could not detect impairment in hatching of exposed zebrafish embryos but said that hatching success was reduced due to cadmium exposure, because fish were trapped inside the chorion.

4.3.2 Aniline

LC_{50} data for acute toxicity test of several fish species are: 36.2 mg/L (96h, pH 7.8) for trout (Hodson 1985), 57.0mg/l (96 h, pH 8.6) for zebrafish (Zok et al. 1991), 77.9 mg/l (96 h) for fathead minnow and 187 mg/L (96 h, pH 7.4) for the goldfish (Holcombe et al. 1987). But in the fish embryo test with the fathead minnow, even within the concentration range (18.75 to 300 mg/L), no mortality was observed. Keth (in preparation) came to the same results when testing aniline with zebrafish embryos. This indicated that fish embryos are less sensitive to aniline than adult fish. The effects observed for aniline were also not as severe as for the other aniline compound. Exposed fish were all able to hatch within the same time as the negative controls; developmental retardation was not observed. There is no evidence that aniline was hindered from entering the egg or the embryo in order to produce no mortality because the pigmentation of the eyes was definitely affected by aniline. Fish exposed to higher aniline concentration exhibited amber eyes, and even fish exposed to lower concentration displayed a slightly less black pigmentation. The impairment of eye pigmentation was not irreparable. Fish exposed to lower aniline concentration showed a change to black pigmentation after 120 hpf, the eleutheroembryo was then either able to biotransform or degrade the toxic compound or the pathway leading to eye pigmentation had changed during development. However, fish exposed to higher concentrations showed no change to black in eye color implying

that the fish could not get rid of higher yields of aniline. All fish exposed to aniline showed hyperactivity which level was again dose dependant. Due to the absence or retarded development of the swim bladder, the larvae probably tried to gain buoyancy by swimming. It also seemed that the fathead minnows ingested aniline because hemorrhaging in the intestinal tract was observed which could be caused by aniline ingestion.

4.3.3 3,4-Dichloroaniline

Effects of 3,4-dichloroaniline to the chorionated embryo showed that the compound was not immediately lethal but did damage and weaken the embryo. Effects were mainly edemas and spine curvatures. Theses effects concur with observations made by (Ensenbach and Nagel 1995) who stated that the most common effects were skeletal deformities and edemas, which both impaired overall activity of the embryo. (Sawle et al. 2010) who studied the effects of 3,4-dichloroniline by microarrays found out, that even small doses of 3,4-dichloroaniline affect the cytoskeleton, the response to hypoxia and oxidative stress, and protein folding amongst other things. Accumulation of blood cells in tail and brain occurred probably because the circulatory system was no longer able to transport the cells through the body, and the blood cells got stuck in the fine capillaries, where they were then degraded. The heart was stretched due to the huge size of the cardiac edema and the pump performance was reduced greatly.

Mortality did only occur after hatching, implying that the chorion could act as a barrier and protecting the developing embryo as stated by (Ensenbach and Nagel 1995). This might be true due to its higher log K_{ow}-value. On the other hand, 3,4-Dichloroaniline is used as positive control in the fish embryo toxicity test with zebrafish where it is lethal to zebrafish embryos quite soon after exposure indicating that 3,4-DCA is toxic to very early embryonic stages.. This would explain why fathead minnow embryos do not die from exposure, as long as they are in the chorion, because they were exposed at later developmental stages compared to the zebrafish, which are exposed within one hour after fertilization (4-cell stage, (Kimmel et al. 1995). Another explanation why 3,4-dichloroaniline is more toxic to zebrafish than to fathead minnows is probably the build of the chorion. While the chorion of the zebrafish is only 1.5 µm in height and has a sieve like structure with see through pores, the chorion is thicker and the pore openings smaller. Therefore, compounds entering the egg may face greater difficulties when entering the fathead minnow eggs than zebrafish eggs. Due to the late occurrence of mortality in the fathead minnow embryo, the first LC_{50}-values were determined at 120 hpf. Data for comparison was either derived after 48 or 96 hpf. LC_{50} (96 h) values for adult fat-

head minnows (TG 203), excerpted from the Fathead Minnow Database (Russom et al. 1997), range between 7.0 and 8.06 mg/L compared to 24.16 mg/L which was calculated for 120 hpf for the fish embryo test with the fathead minnow. (Call et al. 1987) states a similar LC_{50} of 7.6 after 96 hours for adult fathead minnow. Data for adult zebrafish (Ensenbach and Nagel 1995, Nagel et al. 1991, Zok et al. 1991) and guppy (Adema and Vink 1981) show similar values as those for the adult fathead minnows: 8.4 to 8.9 mg/L. Only adult trout and perch are more sensitive: 1.92 mg/L (Hodson 1985) and 2.1 - 5.3 mg/L (Water Research Centre, 1987) for trout, and 3.1 mg/L (Schaefers and Nagel 1993) for perch. This shows that the fathead minnow embryo is less sensitive for DCA than adult fish. Comparisons with the zebrafish embryo (LC_{50} 48 h): 2.86 mg/L (Busquet et al. 2010), show that the fathead minnow embryo is also less sensitive due to the causes stated above.

Hatching was not impaired by exposure to 3,4-Dichloroaniline, even though, exposed embryos started to hatch 24 hours before the embryos in the negative control. Over ninety percent of the fish hatched between 96 and 144 hpf, with the most hatching around 120 hpf and only a few at 144 hpf. No influence on hatchability corresponds with data recorded by Call et al. (1987) for fathead minnow embryos and by Ensenbach and Nagel (1995) for zebrafish embryos.

4.3.4 2,4-Dichlorophenol

2,4-Dichlorophenol interfered with developmental processes of the fathead minnow embryo within the first 24 hours, which could be due to an impact in neurogenesis in general and the development of synapses as(Sawle et al. 2010) stated. The retardation in neurogenesis may also have led to the observed edemas in the brain region, that some of the fish displayed. Visual perception was also found to be impaired, explaining the insufficient development of the eyes and the lack of eye pigmentation. Accumulation of blood cells in different parts of the fish body (pericardium, dorsal aorta) may be owed to an insufficiency of circulation and heart beat where the blood could no longer be transported to the body. Hatched fish showed problems of maintaining their equilibrium which may be related to strange fin orientation or to the weakened stage due to exposure. Exposed, hatched and living fish showed a decrease in activity. Problems to maintain the equilibrium was also reported by (Holcombe et al. 1980).

Even though, Helmstetter and Alden (Helmstetter and Alden 1995)found out that the amount of organic compounds that penetrate the fish egg is controlled by their lipophilicty, no differences in sensitivity between acute and embryo toxicity test could be observed: fish acute

LC_{50}-values for 96 and 192 hours, determined by (Holcombe et al. 1980), were exactly the same than the ones determined for the fish embryo toxicity test with the fathead minnow in the present study: 10.42 and 7.21 mg/L. Phipps et al. (Phipps et al. 1981) had slightly lower values (8.25 and 6.5 mg/L) which were owed to a slightly elevated pH. The LC_{50}-value at 96 hours in the fathead minnow database (Russom et al. 1997) is with 7.5 mg/L lower than the other data (Holcombe et al. 1980, Phipps et al. 1981). While there was no difference in sensitivity between embryos and adult fathead minnows, there was a relative large difference in inter-species sensitivity: rainbow trout was more sensitive (1.16 mg/L, Holcombe et al. 1987), while goldfish were not so much affected (23 mg/L). When fish embryo toxicity tests were compared, the zebrafish reacted slightly more sensitive than the fathead minnow with a LC_{50} of 7.31 and 5.96 mg/L compared to 10.24 and 9.76 mg/L. Between 48 and 144 hours the LC_{50} did not significantly change (11.89 and 9.76 mg/L), but after hatching was completed and all fish had hatched. The mortality increased and the LC_{50}-values decreased confirming Holcombe et al. (1982) who stated that fathead minnow embryos are more resistant than larvae and juvenile stages.

Hatching was affected by exposure to 2,4-dichlorophenol at concentrations higher than 7.5mg/L. Embryos exposed to lower concentrations showed a similar hatching pattern compared with the negative control. In 10 mg/L 2,4-DCP, the last embryos hatched after 168 hours. This may be caused by a lower developmental rate and weaker state of the embryos exposed to higher concentrations. The hatching success was affected as well: the number of embryos hatched at 7.5, 10.0 and 15.0 mg/L decreased dose dependant, correlating with the increasing effects and mortality at higher 2,4 DCP concentrations. Affected hatching due to 3,4-dichloroaniline exposure was also observed by Holcombe et al (1982).

4.4 Histology of the fathead minnow and the zebrafish egg

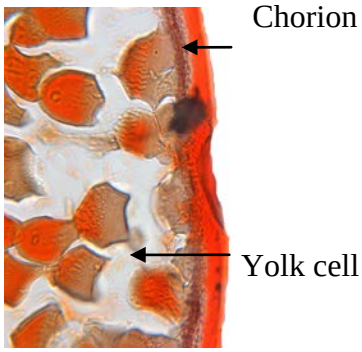


Fig. 47: Close-up view of a 48 hpf fathead minnow embryo stained with Masson-Goldner (63x).

Zebrafish and fathead minnow embryos stained with different histological stains, revealed no differences in tissues and coloring. Yolk stained with the Masson-Goldner should be colored in red according to the color table (Romeis 2010). In earlier trial stains, the red color was observed not only in the cytoplasm of the yolk cells but also on the chorion (Fig. 47). In later stains was

the yolk and chorion no longer red, but showed a blue color. This color change might be owed to the age of the dyes which may have been subject to oxidation or to an overlong washing step which may have washed away the red dye molecules. However, the transverse sections revealed the striated appearance of the chorion for the first. This composition of the fathead minnow chorion was confirmed by the transmission electron microscopy images obtained by Manner (1977) showing an electron dense outer and striated inner layer.

4.5 Native preparation of the chorion

The staining of the natively prepared chorion revealed several aspects. The outer surface of the HE stained zebrafish chorion was overall pink, even the distinctive projections indicated, that the chorion consists of positively charged proteins stained by the eosin component of the stain. This finding is supported by Nakano (1969) and Makeeva and Mikodina (1977) stating that the chorion of different Cyprinidae contains proteins and mucopolysaccharids. Wang and Gong (1999) characterized two chorion proteins in zebrafish, ZP2 and ZP3, which can also be found as homologous zona pellucida proteins of mammalians and in the chorion of other fish species.

When the chorion was stained with Masson-Goldner, both inner and outer surface, were stained differently. While the outer surface, including the distinctive projections, was stained green, indicating the presence of mucus, the inner surface was red indicating the presence of cytoplasm. In transmission electron microscope images Rawson et al.(2000) revealed, that the chorion was covered with an outer most granular layer, in which the distinctive projections laid. This layer could have mucous properties which could protect the chorion from outside influences. Makeeva and Mikodina (1977) stated that acid mucopolysaccharids are localised along the outer edge of cyprinid chorions. Bonsignorio (1996) found out that the zebrafish chorion consists partly of N-linked glycoproteins. And Wendler (2012)stated that glycoproteins constitute the outer layer of the chorion. Why the inner surface was stained red, needs yet to be determinated. However, it does not seem to be associated with either cytoplasm, but probably with the perivitelline fluid which may have similar properties compared to cytoplasm. Solid remains of the perivitelline fluid could have still been attached to the inner surface of the chorion when it was stained. This could explain the red color at least to an extent. The micropyle, especially the wall surrounding the entry site, was brightly orange colored, indicating that it was build out of a different substance than the rest of the chorion. In Masson-Goldner stains, orange is normally associated with cytoplasm, why the micropyle is stained

orange needs to be investigated. Alcian blue-PAS stained chorion appeared purple on the outside, including the distinctive projections. Purple is associated with mucus backing the hypothesis that the outermost granular layer has mucous properties. The inner surface on the other hand, was only lightly colored, indicating that it consisted of another, not PAS positive substance compared to the outside of the chorion. This was confirmed by several ultra structural studies of the zebrafish chorion (Hart and Donovan 1983, Rawson et al. 2000). However, with all staining methods, no plugs covering the pores, could be observed. The chorion seemed to be sievelike and the pores see-through. The plugs are probably opaque, if investigated under light microscopy.

The fathead minnow chorion, on the other hand, showed a different color palette when treated with the different stains. The HE-stain colored the chorion as pink as the zebrafish one indicating the presence of positively charges proteins. As mentioned above, zona pellucida homologous proteins were found not only in the zebrafish chorion but in the chorion of other teleosts as well. Therefore, they could also be present in the fathead minnow chorion. Alcian blue-PAS stained chorion did not show a difference between inside and outside, but it was brightly pink colored, indicating the presence of PAS-positive substances. The adherend, however, was even darker stained than the rest of the chorion which can be explained by an accumulation of PAS positive substances contributing to the gluing substance and could belong to either sugars or proteins. While the zebrafish chorion showed a variety of colors when stained with Masson-Goldner, the fathead minnow chorion was only lightly blue stained on inner and outer surface. This indicates, that the surfaces were not covered with mucus or solid remains of the perivitelline fluid as assumed for the zebrafish. It rather seemed that the structure of the chorion was so densely packed, that the different dyes had no change to properly penetrate the many layers that were revealed when the chorion was torn. However, the wall surrounding the micropyle was stained in the same orange than that of the zebrafish meaning that it is probably made of the same substance.

The distance between the pores of the zebrafish and the fathead minnow chorion revealed no differences between inner and outer surface indicating that each outer pore opening was associated with one inner pore opening. This assumption is backed up by the number of pores present per square micron of the outer and inner surface. However, the pore openings on the outer and inner surface of the fathead minnow chorion seemed to be a little scarcer than the ones on the zebrafish chorion: 2.8 compared to 3.1 per square micron on average. The radius of the outer pore openings was also different; the pores of the zebrafish chorion were 1.7

times bigger compared to the fathead minnow chorion. Difference in pore radius and pore area is related. The pore area of the fathead minnow was two thirds smaller compared to zebrafish. However, the distance between pore centre and pore centre was on both sides of the chorion as well as in both cyprinids similar meaning that the interstices between the pores varied. The interstices on the inner surface were always smaller than the outer ones, and zebrafish interstices were smaller than the fathead minnow ones due to larger pore area. Comparison of the overall appearance of the chorion indicates that the fathead minnow chorion is thicker than the zebrafish chorion. This was already stated in literature (Hart and Donovan 1983, Manner et al. 1977, Rawson et al. 2000). However, calculations of distances and errors should only elucidate the differences between the two teleost chorions, but actual values might not be correct, because the chorion could have been stretched or otherwise damaged when it was arranged on the slides and therefore the orientation could be impaired.

These observations result in the assumption that the fathead minnow chorion plays a more prominent role as a barrier between the surroundings of the embryo compared to zebrafish. Substances must be able to both fit in the pores and progress through the longer pore canals without getting stuck to have an effect on the embryo. Diffusion of substances might also be impaired due to the thickness of the chorion and the composition of the chorion layers. Substances may be hindered by size, complexity, charge, and lipophilicity to pass the chorion. This will result in a decrease of toxicity in chorionated fathead minnow embryos compared to chorionated zebrafish. This was shown for 2,4-dichlorophenol and 3,4-dichloroaniline in the fish embryo toxicity test with the fathead minnow but not for cadmium chloride. However, not only might the structure of the chorion constrict the uptake of substances, but the composition of chorion proteins and polysaccharides, as well as their characteristics, may also interfere.

4.6 2,7-Dichlorofluorescein

A signal of 2,7-dichloroflurescein was only found on the adherend of the fathead minnow egg in comparison with the zebrafish where the whole chorion was emitting a signal (Von der Golz 2009) after 24 hours of exposure. This suggests that dichlorofluorescein has binding sites all over the zebrafish chorion, whereas no binding sites on the fathead minnow chorion could be detected. The only area emitting a signal is the adherend which is probably composed of glycoproteins or mucopolysaccharides with side chains or functional groups that might interact with dichlorofluorescein. This may indicate that either both chorions are com-

posed of different proteins, or more probable, that the proteins of the chorion are covered by the outermost granular layer of the zebrafish chorion. This layer might interact with the dichlorofluorescein indicating that the layer covering the zebrafish chorion and the adherend of the fathead minnow chorion may both consist of substances with similar binding sites.

A signal was detected in the zebrafish embryo inside the circulatory system mainly in the aorta and brain region (Von der Golz 2009). In zebrafish, a difference in signal strength between a 0.01 and 0.1 %, but not between 0.1 and 1 % DMSO was detected (Kais et al., in prep.). The fathead minnow embryo on the other hand, did not emit any signal. This indicates that dichlorofluorescein was not able to pass the fathead minnow chorion. The molecule was either hindered by size or sterical composition which may have inhibited entering and passing through the pore or permeating over the chorion. However, the zebrafish chorion may be a weaker barrier, because it is much thinner and has wider pores, and bigger pore areas, than the fathead minnow chorion. Therefore, it would be interesting to see if longer exposure to DCF, different DMSO (dimethylsulfoxide) concentrations or dechorionated exposed embryos may lead to an increased uptake of dichlorofluorescein in the embryo.

However, dichlorofluorescein might not have reached diffusion equilibrium between the surrounding medium and the egg due to the thickness and probably impaired permeability of the fathead minnow chorion. Therefore, it is necessary to study the exposure of DCF over a longer period. Kais (2009) found out that DCF concentration exhibits a stronger signal in the embryo after 48 hours of exposure. Dechorionated embryos should be exposed to DCF additionally to make sure that the embryo is able to take up the chemical.

4.7 *In-vivo* EROD assay

Fathead minnow embryos (48 hpf) did not display an EROD signal after exposure to β-naphthoflavone in the liver even though liver tissue was already present (Devlin et al. 1996). However, basal EROD activity in the pericardium could be detected in the negative control; additionally a signal in circulatory system was displayed in the positive control. Otte et al. (2010)who investigated spatio-temporal development of CYP1A activity in early life stages of zebrafish, detected basal EROD activity in embryos exposed to artificial water as negative controls, especially in the heart and the dorsal aorta. Basal EROD activity in the negative control as described by Otte et al. (2010) was observed in the all developmental stages, but only in the circulatory system and not in the intestine, liver or the vascular tissue surrounding the yolk sac except at an age older than 120 hours.

The first signal was detected in the gastrointestinal region of embryos which were exposed for three hours which have now a noticeable enlarged liver (Devlin et al. 1996). Otte et al. (2010) also observed in β-naphthoflavone treated zebrafish embryos (80 hpf) a more intense signal in the intestine and an EROD activity in all vessels compared to the remaining tissue. The strong signal in the intestine occurs because metabolized ethoxyresorufin reaches the intestine *via* the gall bladder where it accumulates. EROD activity in the blood explains the strong signal emitted by the duct of Cuvier, and the signal in heart, dorsal and ventral aorta, as well as in the brain region. The signal intensified in 96 and 120 hpf embryos due to the growth of the liver and intestine (Devlin et al. 1996). Mattingly and Toscano (2001) observed that in zebrafish the CYP1A activity increased prior to hatching. Andreasen et al. (2002) found out that CYP1A activity also along intestine, in the liver and around the anal pore after hatching and identified the mucosa and endothelia of the gut as well as hepatocytes as locations of CYP1A expression in zebrafish embryos and adults.

EROD activity was reduced or not present in fathead minnow embryos exposed to β-naphthoflavone for 24 hours indicating that EROD activity might be inhibit. Zebrafish embryos show a reduction in signal due to a 48 hours exposure to β-naphthoflavone. Why the signal in the fathead minnow is reduced already after 24 hours of exposure needs further investigation.

Comparing zebrafish with fathead minnow embryos shows that the functional blood circulation in the liver of the zebrafish started at 72 hpf (Korzh et al. 2008), whereas it started after 85 hpf in fathead minnow (Devlin 1996). Therefore, the liver cells of zebrafish are earlier included in the detoxification system and thus exposed to chemicals that might induce CYP1A activity in the liver and intestine (Kais and Braunbeck 2011). The highest CYP1A activity in the zebrafish is analyzed after 72 and 96 hpf (Kais and Braunbeck 2012). Later stages are not recommended due to the start of exogenous feeding (Belanger et al. 2010). Fathead minnow embryos, however, should be imaged at 96 and 120 hpf to obtained good signal intensities. Afterwards, a reduction in resorufin signal might be observed due to a competing reaction. It has been reported (Jones 2010) that resorufin can undergo glucuronidation which prevents the detection of the fluorescent product.

However, for the present EROD test with the fathead minnow embryo the ß-naphthoflavone concentration determined for the zebrafish was used. This may have resulted in a weaker EROD signal of the fathead minnow due to differences in chorion and development, as well as different uptake rate and toxicity of ß-naphthoflavone, compared to the zebrafish. There-

fore, fish embryo toxicity test must be carried out to determine the exact ß-naphthoflavone concentration (EC_{10}-value) to induce comparable EROD activity in the fathead minnow.

4.8 Transmission electron microscopy

The thickness of the fathead minnow chorion was approximately 7.1 ± 1.8 µm, close to the ten microns calculated by Manner et al. (1977). The zebrafish chorion on the other hand is between 3 and 5 times thinner than the fathead chorion (1.5 - 2.5 µm, Rawson et al. 2000) The suggestion of Manner et al. (1977) that the chorion of the fathead minnow consists of three different structures was confirmed but only for eggs older than 24 hpf, although the overall orientation of the chorion as well as the structure classification proofed to be wrong: In unfertilized eggs, only two distinctive layers were found. A small electron dense structure, being only one tenth of the height of the underlying structure, was the outer part, the underlying structure composed of layers with different fiber orientation giving the structure a striped appearance, was the inner part of the chorion. After 24 hours, an additional granular structure was detected that covered the electron dense structure. This structure was not mentioned by Manner (1977). It seemed that this structure was synthesized after the fertilization of the egg, and that this process needed some time because chorion of multicell-stage eggs (approx. four hours old, (Kimmel et al. 1995) still showed no sign of this structure. It covered the whole electron dense structure, probably to cover pore openings and to hinder substances from entering the egg after fertilization, either by diffusion or through the pores. A similar structure was also found associated with the zebrafish chorion, where it is termed the outermost granular layer (Rawson et al. 2000). However, the covering of the pore opening by the granular structure only seems to be an additional feature to protect the embryo because the pore openings were covered with a round plug. The electron density of the plugs and the granular structure was not the same indicating a second mechanism to close pore openings. The plugs were observed in all stages. Flügel (1967) found out that the pores of the brook trout *Salvelinus fontinalis* were clogged by the end of egg maturation with material secreted by the follicle cells. Furthermore, Flügel (1967) showed that the matrix of the chorion became homogenous after ovulation and no pore canals could be observed in the chorion of the mud minnow *Fundulus heteroclitus*. The composition of the innermost striped structure changed, in contrast to Manner (1977) who stated that the number of layers of the inner structure was constant and that it was composed of 19 layers. Even though the chorion of 72 and 96 hpf eggs showed more layers (approx.18) than younger ones (approx. 13), no evidence that change of the inner structure was dependent on the age of the eggs was found. However, eggs used for transmission elec-

tron microscopy were collected from different spawning incidents and, therefore, different females which may have contributed to a difference in quantity. The composition of the chorion is determined during ovulation where it is developed between the growing oocyte and follicle cells in the ovary (Guraya 1978), and therefore, not an object to change over time. It is well established that the materials of the chorion are deposited between the oocyte surface and the follicle epithelium during the growth of the follicle forming microvilli and follicle cell processes respectively (Flügel 1967, Guraya 1978). Number of layers did not only vary with different stages, they even varied within the same section due to the merging and separation of the different layers. It is possible that a higher number of layers is increasing the egg quality because it is increasing the stability of the chorion and, therefore, the protection of the embryo. However, one difference over time was observed in the fathead minnow chorion. The distance of the layers and the closeness of the fibers did change over time. In unfertilized eggs and still in multicell-stage eggs, the fibers of the layers were further apart and appeared more electron lucent than in later stages when the layers narrowed and condensed. In 96 hpf eggs, the appearance of the chorion again changed. The outer granular structure was reduced, and the inner structure became wider again and big openings formed. Whether these openings already contribute to the enzymatic decomposition of the chorion due to hatching or if they are just artifacts needs to be investigated. In addition, in transverse section of an unfertilized egg a granular layer was observed but electron density and structure were different compared to the later granular structure. It was not as electron dense and the fringes were rougher than in later stages. It is believed that this structure was part of the adherend which rough appearance was well observed with the 2,7-dichlorofluorescein experiment.

But certain differences of the fathead minnow chorion became apparent when compared to the zebrafish chorion. The fertilized zebrafish chorion is composed of four structures: the outermost granular structure, the outer electron dense structure (zona radiata externa), a middle fibrillar structure (superficial zona radiata interna) and the inner striped structure (deep zona radiata interna, (Hart and Donovan 1983).

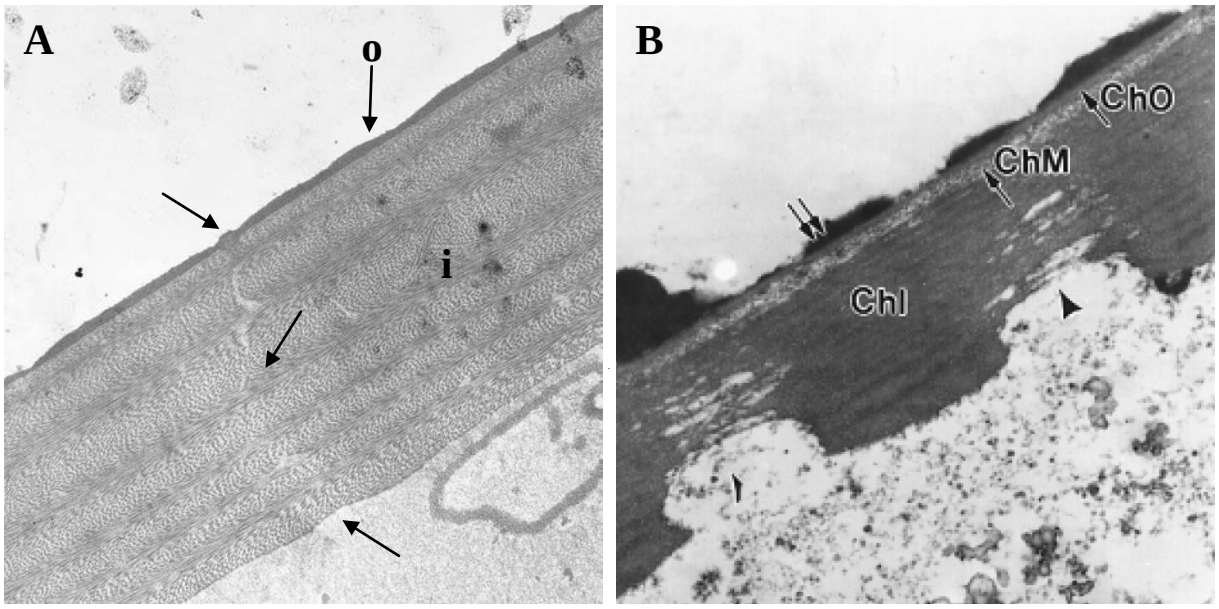


Fig. 48: A) chorion of a fathead minnow egg (approx. 4 hours), outer granular structure not yet present, o – outer structure, i - inner structure , arrows - pore; B) chorion of a gastrula stage embryo (Rawson et al. 2000), ChO - outer layer, ChI - innermost layer, ChM - middle layer, arrow - pores, double arrow - outermost granular layer.

The fathead minnow chorion is void of the fibrillar structure. The deep zona radia interna follows directly the electron dense zona radiata externa. The diameter of the electron dense structure in the fathead minnow is about 0.1 µm, whereas it is of the same thickness (Hart and Donovan 1983) or between 0.2 and 0.3 µm (Rawson et al. 2000) in the zebrafish. The deep zona radiata interna of the fathead minnow is about seven times thicker than the one of the zebrafish (6.8 µm compared to 1 – 1.6 µm). Another difference are the pores of the zebrafish chorion which are clearly visible as openings in the inner and middle structure confirming the assumptions made by using light microscopy namely that the zebrafish pores are quite big and see through. The pore canals of the fathead minnow chorion, in contrast, are hardly being seen. They are narrow in diameter, except where lacunae were built, and meander through the inner structure of the chorion till they reach the wavy innermost layer where they open at shallow indentations into the perivitelline space. This also confirms the assumptions drawn from the light microscopy images. That pore openings are present on the outer and inner surface of the chorion, but that the pores canals see through and observable.

5 Conclusions

The purpose of this study was the adaption and the refinement of the fish embryo toxicity test protocol to the fathead minnow. Therefore, it was important to optimize rearing condition and egg production. In order to allow detection of effects induced by chemicals, the normal development as well as the structure and permeability of the chorion of the fathead minnow had to be investigated. Additionally, the *in-vivo* EROD assay developed for the zebrafish was carried out to examine its applicability for the fathead minnow embryo.

Rearing and egg production. Fathead minnow displayed normal behavior according to McMillan and Smith (1974) under the adapted conditions in the spawning tanks. The preferred spawning substrate (PVC houses) made sure that all eggs could be removed from the tanks. Water temperatures between 23 and 25°C did not impair reproduction. Constant water flow-through and occasional checks ensured good water criteria. Egg yield and egg quality was good despite the high age of the fish used. However, the stress of the fish in order to produce eggs led to a high mortality after they had 'spawned out', which would probably have been avoided with younger fish. The 40L-spawning tanks provided breeding groups with a surplus of space. In order to allow better recording of fecundity and to save space and water, the fathead breeding facility will have to be remodeled. In the future, one male and two females or a pair of fish will share a 10L-tank for spawning. No further adjustments need to be made with respect to feeding, and handling with the eggs. It turned out that improvements were gained with experience. However, more experience is needed to improve the spawning frequency and to detect spawned eggs as early as possible. One way to gain eggs as early as possible might be the development of a method to fertile eggs by *in-vitro* fertilization. Until now, the known method for the zebrafish did not seem to work for the fathead minnow, but this might be due to the inexperience of the researcher.

Normal development and *in vitro* fertilization. Recordings of the normal development of the fathead minnow embryos at 25°C as far as it was carried out, matched the findings of Devlin et al. (1996) to a great extent. However, further research is needed in order to gain data for older embryonic stages until hatching. In case *in vitro* fertilization does not work, it should be considered to obtain ripe eggs and sperm from killed fish as performed by Devlin et al. (1996) to establish an exact timeline for the normal development starting with fertilization.

Fish embryo toxicity test. The fish embryo toxicity test (FET) originally developed for zebrafish can be modified to fit the requirements of the fathead minnow. However, the age of eggs at detection and the uncertainty of spawning frequency does make preparations for the test difficult, because solutions and pre-exposed material must always be available. So far, this uncertainty restricts the use of substances with a high log K_{ow} due to water solubility and therefore longer preparation time. Use of substances that degrade quickly might also be restricted. The overall duration of the test must be reduced from 192 hours to 120 hours in order to meet animal rights regulations (EU 2010), because (Belanger 2012) states that exogenous feeding starts between 120 and 144 h. In addition, the concentration of the used positive control (4 mg/L 3,4-dichloroaniline) must be elevated to 12 mg/L to achieve 30 % mortality at the end of the test as required by the OECD protocol. Mortality due to DCA is further restricted by the age of eggs at the start of exposition. Furthermore, the role of the fathead minnow chorion as a barrier needs to be investigated. Data gained so far allows the conclusion that the chorion hinders substances from entering the egg and might also work as a sink for substances. To what extent this happens still needs to be investigated. At the moment, it seems that chorionated fathead minnows are less sensitive to chemicals than chorionated zebrafish embryos. It might proof useful to perform two tests per substance: one with chorionated and one with dechorionated fathead minnows. A method to dechorionate fathead minnow embryos was therefore developed. Incubation with 2 mg/ml pronase for eight minutes was performed until the first embryos dropped out of their chorions. To avoid damages of the embryo, extensive washing is needed afterwards to remove the enzyme properly. So far, embryos that were 24 hours or older were dechorionated, but experiments with the zebrafish (Schneider, personal communication) show that chemical dechorionation of younger stages is possible without losses. One major goal concerning the fish embryo toxicity test with the fathead minnow must be the gain in more data for a comparison with the zebrafish embryo toxicity test which is already close to the end of the validation phase. However, in overall comparison, the FET with the zebrafish seems to be more feasible than that with the fathead minnow: the start of the test can be regulated and therefore preparation and pre-exposure can be carried out in advance; due to easy egg production a high number of already isolated eggs can be exposed at an early age in development.

***In vivo* EROD assay.** It was also shown that CYP1A in the embryo of the fathead minnow was induced by ß-naphthoflavone, the positive control in the zebrafish *in vivo* EROD assay. However, the signal in the fathead minnow embryo was weaker than in the zebrafish. To allow the expansion of the *in vivo* EROD assay with the fathead minnow as an alternative to the

zebrafish, it is necessary to investigate the toxicity of ß-naphthoflavone to the fathead minnow embryo in order to use the right exposure concentration (EC_{10}-value).

Fathead minnow chorion. In addition to the adaption of the fish embryo toxicity test and the *in vivo* EROD originally developed for the zebrafish, a detailed description of the fathead minnow chorion was a major goal of this thesis. Planar views stained with three different histological stains revealed that the chorion is thicker than the zebrafish chorion. It consisted of many different layers, but the overall structure with small round pore openings on the outside and bigger polygonal openings on the inside shows similarity to the pores in the zebrafish chorion. Histological staining showed that the fathead minnow chorion has no mucous layer on the outer surface like the chorion of the zebrafish Transverse sections confirmed the first indications of a stratified structure of the chorion. Investigations of the ultra structure of the chorion with transmission electron microscopy revealed that the fathead minnow chorion is composed of two or three structures depending on age. Unfertilized eggs and multi-cell staged eggs consisted of an outer electron-dense structure and an inner structure made up of many lamellar layers. Other stages had an additional granular layer on top of the electron-dense layer. The outermost granular layer of the zebrafish chorion which has probably mucous properties, is not present in the chorion of the fathead minnow. Furthermore, a translucent middle layer could not be observed.

A test with 2,7-dichlorofluorescein in 0.1 % DMSO showed that it did not enter the egg and did not precipitate on the whole chorion. Only on the adherend of the fathead minnow egg, there was a signal indicating that the outer surface of the fathead minnow chorion must be of a composition different to that of the zebrafish. Further investigations are needed to extend our knowledge of the chorion properties. Ultrastructural planar views of the inner and outer surface of the chorion should be obtained and freeze-fracture techniques should be used to explore the structure and course of the pore canal through the chorion. In addition, permeability and chorion composition, also in comparison with the zebrafish, need to be studied to allow better comparison between the two fish species and allow further adaption of the fish embryo toxicity test to increase comparability and reproducibility.

In conclusion, the fathead minnow embryo proved to be a useful model for the development of alternative ecotoxicological testing protocols, although the handling of the eggs is more time consuming. For the moment, tests with fathead minnow embryos cannot be started as early as with zebrafish embryos. Furthermore, the planning of tests is more difficult than with the zebrafish. Much more research is needed in order to establish test protocols like the zebra-

fish FET and the *in vivo* EROD assay for the fathead minnow. Likewise, there is still a need to investigate the structure and composition of the chorion for a better understanding in permeability of chemicals through the chorion. Profound experimental data and elaborated test protocols can prepare the ground in the US and Canada for the use of fathead minnow embryos as an alternative to the acute fish test (OECD TG 203) to obtain ecologically relevant data on chemicals and to reduce the use of animals.

Summary

The fathead minnow, *Pimephales promelas*, a small cyprinid from North America, is widely used as laboratory test fish. In the present study, rearing and breeding conditions for the fathead minnow were optimized in our laboratory to produce eggs on a regular basis for testing. Firstly, these eggs were observed and a timeline for normal development of the embryo was established. Results showed only minor differences to already existing studies (Devlin et al. 1996, Manner and Dewese 1974).

Based on fathead minnow-specific developmental data, the embryo toxicity test was adapted and refined. Using the fathead minnow embryo test, LC_{50} data were generated for a heavy metal (cadmium chloride), a substituted aniline (3,4-dichloroaniline), which is currently used as the positive control in the fish embryo toxicity test with the zebrafish (ZFET) and a substituted phenol (2,4-dichlorophenol). Fathead minnow embryos proved to be slightly less sensitive for cadmium chloride and clearly less sensitive for 3,4-dichloroaniline than adult fathead minnow (OECD TG 203), whereas for 2,4-dichlorophenol no difference in sensitivity could be detected. However, the comparison with the ZFET showed that zebrafish embryos are significantly more sensitive. For all three substances, toxicity increased after hatching (≥ 96 and 120 hpf). Differences in toxicity between the fathead minnow and the zebrafish embryo tests might be due to differences in the chorion.

Examinations of the chorion with light, confocal laser scanning and transmission electron microscopy revealed significant differences to the zebrafish chorion. The fathead minnow chorion was thicker and had less and smaller pores on the outside of the chorion. Furthermore, the TEM images showed a layer composition different to the zebrafish chorion. Histological stains and a test with the fluorescent dye 2,7-dichlorofluorescein supported the assumption that at least the outer layer of the fathead minnow chorion is composed of a material different to the zebrafish.

A preliminary attempt with the fathead minnow embryo instead of the zebrafish embryo in the newly developed *in vivo* EROD assay (Kais) was performed successfully. However, first signals of EROD activity were detected at least 24 hours later than in the zebrafish, and the signal intensity was weaker than in zebrafish.

Zusammenfassung

Die Dickkopfelritze, *Pimephales promelas,* ein kleiner Cyprinid aus Nordamerika, wird häufig als Laborfisch verwendet. In der vorliegenden Studie wurden die Hälterungs- und Zuchtmethoden für die Elritze optimiert um eine regelmäßige Eiproduktion für Testzwecke zu gewährleisten. Zuerst wurde mittels der produzierten Eier die Normalentwicklung der Dickkopfelritze beobachtet und der zeitliche Ablauf dokumentiert. Die Ergebnisse zeigen, dass die aktuelle Studie nur wenig von den bereits existierenden Studien (Devlin et al. 1996, Manner and Dewese 1974) abweicht.

Ausgehend von den genauen Entwicklungsdaten der Dickkopfelritze, wurde der Fischembryotoxizitätstest angepasst und weiterentwickelt. Mit dem Dickkopfelritzen-Embryo Test wurde LC_{50}-Werte für ein Schwermetall (Cadmiumchlorid), ein substituiertes Anilin (3,4-Dichloranilin) und ein substituiertes Phenol (2,4-Dichlorphenol) erzeugt. Die Embryonen der Dickkopfelritze erwiesen sich als etwas weniger empfindlich gegenüber Cadmiumchlorid und deutlich unempfindlicher gegenüber 3,4-Dichloranilin als adulte Dickkopfelritzen, wohingegen kein Unterschied für 2,4-Dichlorphenol festgestellt werden konnte. Im Vergleich mit Daten des ZFET konnte jedoch festgestellt werden, dass die Zebrafischembryonen deutlich empfindlicher reagieren. Für alle drei Substanzen erhöhte sich die Toxizität nach dem Schlüpfen ($\geq$ 96 und 120 h nach der Befruchtung). Die Unterschiede in der Toxizität zwischen Embryonen von Zebrafisch und Dickkopfelritze treten vermutlich auf Grund von verschiedenen Chorionbestandteilen auf.

Untersuchungen des Chorions mit verschiedenen Mikroskopiermethoden ergaben große Unterschiede im Vergleich mit dem Zebrafischchorion. Das Chorion der Elritze ist wesentlich dicker und besitzt weniger und kleinere Poren auf der Außenseite. Zudem zeigten elektronenmikroskopische Aufnahmen eine andere Schichtung als im Zebrafischchorion. Histologische Färbungen und ein Versuch mit dem Fluoreszenzfarbstoff 2,7-Dichlorfluorescein bestätigten die Annahme, dass zumindest die äußere Schicht des Dickkopfelritzenchorions aus einem anderen Material besteht als das des Zebrafisches.

Ein Versuch die Embryonen der Dickkopfelritzen an Stelle von Zebrafischembryonen im *in vivo* EROD Assay einzusetzen verlief erfolgreich, allerdings wurde das erste Signal mindestens 24 Stunden später detektiert, und die Signalstärke war schwächer als im Zebrafisch.

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