Umwelt Bundes Amt
Für Mensch und Umwelt

Background Paper

on

FISH EMBRYO TOXICITY ASSAYS

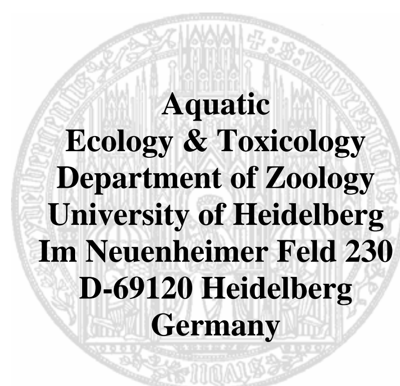
(UBA Contract Number 203 85 422)

March 30, 2006

Prepared for

GERMAN FEDERAL ENVIRONMENT AGENCY
PO Box 1406 · D-06813 Dessau

Thomas Braunbeck & Eva Lammer



Aquatic
Ecology & Toxicology
Department of Zoology
University of Heidelberg
Im Neuenheimer Feld 230
D-69120 Heidelberg
Germany

TABLE OF CONTENTS

		Page
1.0	**Executive summary**	**7**
2.0	**Introduction**	**9**
2.1	Fish acute toxicity testing	9
2.2	Why tests on fish?	9
2.3	Fish *in vitro* tests	10
2.4	Fish embryo tests	12
2.5	Purpose of the present document	13
3.0	**Methodological basis for fish embryo toxicity testing**	**14**
3.1	Fish, fish maintenance and spawning procedure	14
3.1.1	Zebrafish	14
3.1.2	Fathead minnow	36
3.1.3	Japanese medaka	38
3.1.4	Other fish species used for embryo toxicity tests	45
3.2	General description of endpoints used for fish embryo toxicity tests	46
3.2.1	Endpoints used for the acute zebrafish embryo whole effluent toxicity test in Germany ("fish egg test")	46
3.2.2	Additional endpoints used for acute zebrafish embryo toxicity tests	48
3.2.3	Further potential endpoints in fish embryo toxicity endpoints	50
3.3	Validity criteria	50
4.0	**Embryo toxicity tests with the zebrafish (*Danio rerio*)**	**51**
5.0	**Embryo toxicity tests with the fathead minnow (*Pimephales promelas*)**	**91**
6.0	**Embryo toxicity tests with the Japanese medaka (*Oryzias latipes*)**	**92**
7.0	**Embryo toxicity tests with other fish species**	**96**
8.0	**Correlation between embryo toxicity tests with different fish species**	**99**
9.0	**Statistical considerations**	**102**
9.1	Inter-laboratory transferability of the zebrafish embryo toxicity test	102
9.2	Correlation of selected zebrafish embryo toxicity data to conventional acute fish toxicity data according to a statistical analysis by Ratte and Hammers-Wirtz (2003)	104
9.3	Correlation of all fish embryo toxicity data to conventional acute fish toxicity data on the basis of data compiled in the present review	105
10.0	**Scientific and ethical considerations as to the duration of exposure**	**116**
11.0	**Recommended protocol and testing strategy**	**119**
12.0	**Additional fields of application for the fish embryo test**	**119**
13.0	**References**	**123**
	ANNEX	**135**

List of tables

		Page
Table 1	Maintenance, breeding and typical conditions for embryo toxicity tests with the common OECD test fish species	15
Table 2	Stages of embryonic development of the zebrafish (*Danio rerio*) at 26 ± 1 °C (Nagel, 2002)	17
Table 3	Endpoints of acute toxicity originally discussed by the German DIN working group for whole effluent toxicity tests (DIN 38415-6; “fish egg test”)	47
Table 4	Lethal and sublethal endpoints for evaluating the toxicity and teratogenicity of chemicals on zebrafish embryos as listed by Nagel (2002, modified; according to Schulte and Nagel, 1994; Nagel, 1998; Bachmann, 2002)	48
Table 5	Embryo toxicity data from experiments with zebrafish (*Danio rerio*)	53
Table 6	Embryo toxicity data from experiments with fathead minnow (*Pimephales promelas*)	89
Table 7	Embryo toxicity data from experiments with Japanese medaka (*Oryzias latipes*)	93
Table 8	Embryo toxicity data from experiments with fish other than zebrafish, medaka or fathead minnow	97
Table 9	EC_{50} data for selected reference compounds from embryo tests with zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*). Toxicological endpoints as listed by German DIN standards	99
Table 10	LC_{50} data (mg/L) for lethal effects by 20 selected substances in a German national inter-laboratory calibration exercise.	102
Table 11	EC_{50} data (mg/L) for lethal and sublethal effects by 21 selected substances in a German national inter-laboratory calibration exercise.	103
Table 12	Comparison of critical parameters for the correlation between fish embryo toxicity data and conventional acute fish toxicity (LC_{50})	108
Table 13	Comparison of critical parameters for the correlation between fish embryo toxicity data and conventional acute fish toxicity (LC_{50}) following log-transformation	109

List of figures (incl. credits for figures)

		Page
Fig. 1	Adult zebrafish (*Danio rerio*) female (upper individual) and male (lower individual)	14
Fig. 2a - d	Selected stages of early zebrafish (*Danio rerio*) development (from Kimmel et al., 1995).	18
Fig. 3a - f	Normal development of zebrafish (*Danio rerio*) embryos	22
Fig. 3g	Mortality in early zebrafish (*Danio rerio*) eggs: coagulation (I), heavy infestation with fungi (II) and invasion of *Vorticella* spec. (III).	27
Fig. 4	Setup of the tanks used for breeding zebrafish in the Heidelberg laboratory	28
Fig. 5	Setup of the tanks used for breeding zebrafish in the Berlin laboratory (courtesy of Dr. T. Meinelt, IGB)	29
Fig. 6	Oxygen consumption of control zebrafish (*Danio rerio*) embryos in relation to the amount of oxygen provided in the medium	30
Fig. 7	Oxygen consumption of control zebrafish (*Danio rerio*) embryos in dependence from the stocking density in given volume of medium	30
Fig. 8	Toxicity of 3,4-dichloroaniline to zebrafish (*Danio rerio*) embryos in various volumes of medium	31
Fig. 9	Toxicity of 2,4-dinitrophenol to zebrafish (*Danio rerio*) embryos in various volumes of medium	32
Fig. 10	Effect of the exposure start (time lapse after fertilization) on the cumulative mortality of 2,4-dinitrophenol. After 24 and 48 h, it is evident that mortalities are increased with early exposure start.	32
Fig. 11	Effect of the exposure start (time lapse after fertilization) on the cumulative mortality of 3,4-dichloroaniline. After 24 and 48 h, it is evident that mortalities are increased with early exposure start.	33
Fig. 12	Effects of potassium chromate to zebrafish (*Danio rerio*) embryos w and w/o dechorionation	34
Fig. 13	Toxicity of 4-chloroaniline to zebrafish (*Danio rerio*) embryos w and w/o dechorionation	35
Fig. 14	Fathead minnow (*Pimephales promelas*)	36
Fig. 15	Normal development of normal fathead minnow (*Pimephales promelas*) embryos and embryos exposed to 15 mg/L 3,4-dichloroaniline	37
Fig. 16	Japanese medaka (*Oryzias latipes*)	39
Fig. 17	Normal development of Japanese medaka (*Oryzias latipes*) embryos I - IV	40
Fig. 18	Development of control Japanese medaka (*Oryzias latipes*) controls and after exposure to 9 mg/L 2,4-dinitrophenol	44
Fig. 19	Development of rosy barb (*Puntius* = *Barbus conchonius*) controls and after exposure to 3,4-dichloroaniline	45
Fig. 20	Development of rosy barb (*Puntius* = *Barbus conchonius*) controls and after exposure to 3,4-dichloroaniline	46
Figs. 21a, b	Synchronicity of zebrafish (*Danio rerio*) embryo development (courtesy of Dr. T. Meinelt)	49

Page

Fig. 21c	Dechorionated 48 h old zebrafish (*Danio rerio*) embryo	49
Fig. 22	Sublethal endpoints (morphological malformation) *versus* acute lethality in zebrafish (Bachmann 2002)	50
Fig. 23	Acute toxicity of sodium dodecylsulphate (LC_{50}) to selected fish species *in vivo* as well as to embryos of zebrafish, medaka and fathead minnow *in ovo*	23
Fig. 24	Correlation between zebrafish embryo tests for 56 chemicals and the corresponding 48 h acute fish LC_{50} test data (various species; n = 56; Ratte & Hammers-Wirtz, 2003)	104
Fig. 25	Correlation between zebrafish (*Danio rerio*) embryo toxicity LC_{50} and fish *in vivo* acute toxicity LC_{50} data from IUCLID (2000)	106
Fig. 26	Correlation between general fish embryo toxicity LC_{50} and fish *in vivo* acute toxicity LC_{50} data from IUCLID (2000)	107
Fig. 27	Correlation between zebrafish (*Danio rerio*) embryo toxicity LC_{50} and fish *in vivo* acute toxicity LC_{50} data from IUCLID (2000) plus additional validated literature sources	107
Fig. 28	Correlation between general fish embryo toxicity LC_{50} and fish *in vivo* acute toxicity LC_{50} data from IUCLID (2000) plus additional validated literature sources	108
Fig. 29	Minimum and maximum acute fish LC_{50} data taken from IUCLID (2000) *versus* acute zebrafish embryo data LC_{50}	110
Fig. 30	Minimum and maximum acute fish LC_{50} data taken from various validated literature sources other than IUCLID (2000) *versus* acute zebrafish embryo data LC_{50}	112
Fig. 31	Minimum and maximum acute fish LC_{50} data taken from IUCLID (2000) *versus* acute fish embryo data LC_{50}	113
Fig. 32	Minimum and maximum acute fish LC_{50} data taken from various validated literature sources other than IUCLID (2000) *versus* acute fish embryo data LC_{50}	115
Fig. 33	Overview of different terminologies of a fish “embryo” and a fish “larva”	118
Fig. 34	Effects of 48 h exposure to organic extracts (a, b) or native samples (c, d) of Danube river sediments in zebrafish embryos (a, b) or dechorionated zebrafish embryos (c, d).	119
Fig. 35	Embryos of zebrafish (*Danio rerio*) after 24 and 48 h incubation with native sediments from different locations in the Neckar river catchment area	120
Fig. 36	Induction of micronuclei in the liver of a 48 h old zebrafish embryos exposed *in ovo* to 190 µg/L 4-nitroquinoline-*N*-oxide	121
Fig. 37	Genotoxic effects of hydrogen peroxide in a heterogeneous cell suspension obtained from zebrafish (*Danio rerio*) embryos after 1 h of incubation	122

1.0 Executive summary

This document provides information about embryo toxicity tests conducted in different fish species. The review has been designed as a source of information and as an attachment to a proposal for an OECD test guideline with embryos of zebrafish as an alternative for the conventional acute fish toxicity test (OECD 203). Although most embryo toxicity tests have been conducted with zebrafish, other typical OECD fish species, namely fathead minnow and medaka, were given particular emphasis; however, albeit scarce in the scientific literature, information about other species was also included.

This document has been compiled after validation of approx. 200 reports in the peer-reviewed scientific literature. Given the considerable body of information collected on especially zebrafish embryo toxicity in non-peer-reviewed sources, data from selected master and PhD theses have also been included, provided they had been carried following a standardized protocol.

The contents of this document can be subdivided into 6 major chapters:

(1) consideration of methodological aspects in fish embryo toxicity testing including a description and illustration of the normal development of zebrafish, fathead minnow and Japanese medaka;
(2) description of the toxicological endpoints used for the determination of toxicity in fish embryos;
(3) in-depth analysis of embryo toxicity tests that have been conducted with zebrafish, fathead minnow and Japanese medaka as well as other fish species;
(4) statistical analyses of the correlation between fish embryo toxicity tests and data from corresponding conventional *in vivo* acute fish toxicity tests;
(5) considerations on both the scientific and ethical background of fish embryo toxicity testing; and
(6) the recommended OECD test guideline.

In the Annexes of this document, information can be found about:

(1) the normal development of the medaka;
(2) physicochemical properties of substances tested in embryo toxicity tests with zebrafish, fathead minnow and Japanese medaka as well as other fish species;
(3) the full version of an in-depth statistical evaluation of the existing data base from fish embryo toxicity tests by Ratte & Hammers-Wirtz (2003); and
(4) a tabular compilation of acute fish toxicity data from conventional *in vivo* fish tests for all substances that have been tested in fish embryo toxicity tests so far.

Since 2005, fish embryo toxicity testing has been made mandatory for routine sewage surveillance in Germany; since then, conventional fish tests are no longer accepted for routine whole effluent testing. On the basis of the protocol originally designed for whole effluent testing, modifications have been introduced to make the protocol fit for chemical testing. This review provides a detailed illustration of the normal development of zebrafish (as well as the Japanese medaka in the Annex) and provides a detailed description of fish care and breeding in zebrafish, fathead minnow and Japanese medaka.

The zebrafish embryo toxicity test is based on a 48 h exposure of newly fertilized eggs in a static or semi-static system. As toxicological endpoints, coagulation of eggs and embryos, failure to develop somites, lack of heart-beat as well as non-detachment of the tail from the yolk are recorded after 24 and 48 h and used for the calculation of an LC_{50} value. Additional (mainly sublethal) endpoints may be recorded, but are not an integral part of the guideline proposed.

By far, most embryo toxicity tests have been conducted with zebrafish in the laboratories of Dr. Roland Nagel (Universities of Mainz and Dresden, Germany). Within an inter-laboratory calibration exercise, the transferability has been demonstrated, and inter-laboratory variability has been analyzed. According to an analysis of an independent biostatistician, there is a reliable correlation between the fish embryo test and the acute fish test ($R^2 = 0.854$; $\alpha = 0.05$). The confidence belt of the regression line was found to be relatively small, which was regarded due to the large sample size of 56. In contrast, the prediction range was relatively wide (2.36 to 2.5 logarithmic units), resulting in possible deviations by factors of 229 to 320. As a consequence, the regression function was regarded to be appropriate to describe the average relationship between the acute fish test LC_{50} and the embryo test LC_{50} with good confidence, but to be less appropriate as a prediction model. The inter-laboratory comparison was found to meet the requirements for a prevalidation study.

In an independent approach to analyze the correlation between fish embryo toxicity data and acute fish data, the embryo data were not correlated to LC_{50} data from specific fish species, but were compared to the entire range of LC_{50} data available from certified sources (IUCID, 2000). Only for 5 out of 100 substances, the fish embryo LC_{50} value was not within the range documented for conventional acute fish LC_{50}s; for none of these outliers, the maximum deviation from the mean did not exceed a factor of 10.

In a preliminary inter-species comparison of embryo toxicity data generated from experiments with zebrafish, fathead minnow and the Japanese medaka using analogous protocols, the transferability of the zebrafish protocol to the other OECD species could be demonstrated. For 7 substances coming from different chemical classes and covering several orders of magnitude of K_{OW}, the differences in LC_{50} values between the three different species did not extend a factor of 10.

Within the chorion, fish embryos are not subject to Directive 86/609/EEC, which regulates the use of animals in scientific experiments. However, since there is no scientific consensus as to where the transition from an embryo to a larva should be set, an extension of the exposure period to the onset of external feeding (i.e., including the eleutheroembryo stage) has been proposed especially for the Japanese medaka, since the eleutheroembryo stage is particularly long and well-defined for this species. Given the good correlation between embryo and adult fish LC_{50} data, an extension does not seem necessary for the standard fish embryo test protocol. However, should there be any evidence of delayed effects, exposure of, e.g., zebrafish to up to 5 days seems possible without interfering with present animal welfare legislation.

As a conclusion, the replacement of the acute fish toxicity test according to OECD TG 203 seems possible. For this end, a proposal for a future OECD test guideline with fish embryos to assess the acute toxicity of chemicals is made.

INTRODUCTION

2.1 Fish acute toxicity testing

For individual substances, there are extensive regulatory requirements for fish acute toxicity data in support of both environmental risk assessment and also hazard classification. For example, in the European Union, data requirements for the notification of new substances are listed in the annexes to the Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances (EC, 1967). The amount of data required increases according to the quantity of substance put on the market (European Commission, 1992): The "base set" data are stipulated for all substances for which the marketing quantity exceeds one ton per year per manufacturer (Annex VII.A of the Directive). These requirements include acute toxicity for freshwater fish (96h LC_{50}), acute toxicity for daphnids (48h EC_{50}) and growth inhibition test on freshwater algae (growth rate: 72h E_rC_{50} and/or biomass: 72h E_bC_{50}). After submission by industry, these "base set" data contained in summary notification dossiers are entered into the New Chemicals Database (NCD) hosted by the European Chemicals Bureau at Ispra, Italy. The reported data are used for deciding on the classification and labeling as well as for hazard and risk assessment (calculation of Predicted No Effect Concentration – PNEC) of the substance. Likewise, acute aquatic toxicity data are required for evaluation of biocides and plant protection products (EC, 1998; EC, 1991).

Fish toxicity testing is also an important element of Whole Effluent Toxicity (WET) testing in North American (Grothe et al., 1996) and its European counterpart, termed Whole Effluent Assessment (WEA; OSPAR Commission, 2000) or Direct Toxicity Assessment (DTA; Tinsley et al., 2004). Although differences between these concepts existed in particular during the early days, each approach has benefited from the experience with the other one. However, each of these included a recommendation to perform short-term toxicity testing with fish (Wharfe & Heber, 1998). In an international review of this topic, Power and Boumphrey (2004) reported that many countries still do include fish testing in WEA; however, some European countries are now moving away from fish LC_{50} testing and adopting alternative test methods such as the fish embryo test (Braunbeck et al., 2005; Nagel, 2002). Scientific drivers to move towards the use of sublethal effects assessment in fish exposed to rivers receiving effluents include concern over developmental and reproductive effects due to endocrine disrupters (Jobling et al., 1998; Vos et al., 2000). In summary, regulators in Europe and many other regions may require the provision of aquatic ecotoxicity data for either environmental risk assessment of single substances, for hazard classification of substances or, in some cases, for the purpose of Whole Effluent Assessment. In technical terms, this requires an assessment of acute and chronic toxicity to fish and also the potential of substances to bioconcentrate in fish tissues. Increasingly, mechanistic data are seen as an important basis for guiding and prioritizing fish testing (Escher and Hermans, 2002; Hutchinson et al., 2005).

2.2 Why tests on fish?

Fish as a taxonomic group are the only primarily aquatic vertebrate class and have, thus, traditionally been regarded as an indispensable component of integrated toxicity testing strategies. In fact, fish may differ not only from vertebrates, but also from most invertebrates in terms of their metabolic capacities, e.g. in biotransformation competence for certain chemical classes. Moreover, given the occasionally high pollution levels and frequencies of chemical spills, fish have frequently been the targets of overt chemical pollution. From both politi-

cal and emotional points of view, fish kills provided a straightforward tool to communicate the need for reduction of chemical discharge via direct spills and by continuous release from sewage treatment plants. Last, but not least, from an anthropogenic perspective, fish have traditionally been used as sentinels for the quality of waters that serve as sources for human drinking water.

Given the importance of fish in aquatic pollution monitoring, both at the national and international levels, fish have thus intensively been implemented in aquatic toxicity testing regulations. At the OECD level, a whole set of test guidelines using fish as test organisms has been established for the testing of acute toxicity (OECD 203), early life-stage toxicity (OECD 210), short term toxicity test on embryo and sac-fry stages (OECD 212), and juvenile growth test (OECD 215). However, given the recent progress in the improvement of water quality in numerous countries, focus has been redirected from the need to assess acute toxicity (mainly of single compounds) to the identification of more subtle toxic effects (by complex mixtures of compounds, each at much lower concentration levels), since – despite all efforts to reduce chemical pollution – fish populations have not recovered in many regions. With respect to potentially adverse effects following long-term exposure to sublethal concentration of chemicals (chemical mixtures), more emphasis has been given to the development of methodologies to identify more specific modes of toxic action, e.g. endocrine disruption. Thus, OECD expert groups are currently developing modified test guidelines, which incorporate such more sophisticated endpoints.

On the other hand, considerations of animal welfare have increasingly questioned ecotoxicity testing with fish and stimulated efforts to develop various alternatives and/or refinements based on primary and permanent fish cell cultures as well as early developmental stages of fish embryos. Another approach to reduce the number of fish used for toxicological purposes is the use of Quantitative Structure-Activity Relationships (QSARs) especially for the prediction of the inherent bioaccumulation potential of chemical substances.

2.3 Fish *in vitro* tests

In vitro assays with permanent fish cell lines have been used in ecotoxicology for screening, for toxicity ranking of chemicals, chemical mixtures, environmental samples and in Toxicity Identifications Evaluations (T.I.E.) during the last 30 years (for review, see Castaño et al., 2003). A number of *in vitro* cytotoxicity assays using fish cells have been developed, the majority of them employing cells derived from salmonid and cyprinid species. The endpoints used for *in vitro* cytotoxicity assays with fish cells included mainly measurements of basal cytotoxicity such as membrane integrity or energy metabolism (Segner, 2004). As a general rule, good correlations were found among different cell lines, endpoints and among different laboratories. The good correlation found between the *in vitro* data and the *in vivo* fish data in ranking chemical acute toxicity over a large number of chemicals from a variety of chemical classes, has lead to suggest them as an alternative to acute fish bioassays (Kilemade & Quinn, 2003). Among the advantages count the fastness and the cost-effectiveness of their use, on the limitations the lack of sensitivity. On average, the absolute sensitivity of *in vitro* test is one or two orders of magnitudes less than that of the acute lethality of *in vivo* fish bioassays (Castaño et al., 2003; Segner & Lenz, 1993). Therefore, *in vitro* tests do not have the risk to indicate false positives, but include a certain risk of false negatives (Babich and Borenfreund, 1987; Castaño et al., 2003).

Since basal cytotoxicity reflects adverse effects on cell structures and processes that are intrinsic to virtually all cells, most cell systems should show similar reactions and also respond similarly when toxicity is measured by various viability criteria (Babich and Borenfreund 1991). It has been argued that mammalian cells, which are cultured at higher temperatures and proliferate faster than fish cells, might be more sensitive and, therefore, should provide a better *in vitro* system to predict acute fish lethality, at least if cell growth is considered as an endpoint (Segner 2004; Castaño et al. 2003). Recently, two studies have compared cytotoxicity data from fish and mammalian cell lines. Gülden and coworkers (2005) compared fish and mammalian cell lines exposed to 26 reference chemicals within the Multicenter Evaluation of *In Vitro* Cytotoxicity program (MEIC). They concluded that the cytotoxic potencies of fish and mammalian cell lines were almost equally sensitive. The mammalian cell line assay, however, becomes more sensitive than the fish cell line assays, if cell growth instead of cell survival is used as an endpoint after prolonged exposure periods; nonetheless, the increase of sensitivity did never exceed one order of magnitude.

In the other study, Castaño and Gómez-Lechón (2005) compared basal cytotoxicity as evidenced by cell survival in mammalian and fish cell lines for a set of 51 chemicals. Linear correlation of IC_{50} values between fish cells and mammalian cells after 24 h of treatment was good ($r = 0.915$), and both fish and mammalian cell lines showed similar sensitivities for most chemicals after 24 h treatment.

In either study, however, there are remarkable exceptions: Paraquat was clearly more toxic for mammalian cell lines than for fish cell lines (Castaño and Gómez-Lechón 2005; Gülden et al. 2005), whereas sodium chloride, the mechanism of action of which is an increase in osmotic pressure, was 200 times more toxic to fish cell lines than to mammalian cell lines (Castaño and Gómez-Lechón 2005).

Increasing knowledge about fish cells has shown that there are many fundamental similarities between fish and mammalian cells with respect to cellular mechanisms, but that fish cells also reflect a number of fish-specific traits that cannot be assessed with mammalian cells (Castaño et al. 2003, Segner, 2004; Wolf and Quimby 1969). Moreover, fish cells have many practical advantages over mammalian cells: They can be incubated at room temperature (20 °C) and in ambient atmosphere. Thus, sophisticated incubators are not needed. Fish cells can be stored for long periods at 4 °C, circumventing the need for freezing/thawing the cultures. They can be exposed to various aquatic environmental samples at varying osmolarities, which, with mammalian cells, can only be done with renal cells. As a consequence, from a practical point of view, fish cell lines seem a more promising alternative than mammalian cells.

A major drawback of the studies cited above is the chemicals database used for the comparisons: most chemicals were drugs for human use and only a few of them are of ecotoxicological relevance. Likewise, the comparisons between fish *in vivo* data and *in vitro* data from both fish and mammalian cell lines suffer from a severe lack of data for relevant substances; in fact, most data that can be used come from the MEIC reference chemicals list. Segner (2004) made such a comparison and confirmed that the *in vitro* cytotoxicity assay with fish cells was less sensitive than the fish test *in vivo*; however, the mammalian cell lines responded fairly similarly as the fish cell line, i.e., the data set used did not provide evidence that mammalian cytotoxicity assays are more sensitive or are better predictors of fish lethality than fish cell cytotoxicity assays (Segner 2004).

In order to increase comparability of results and to go deeper into the detection of species-specific differences, chemicals with higher aquatic (fish) toxicity relevance should be tested. The comparison of more environmentally relevant data versus reliable *in vivo* fish acute data would confirm whether or not fish cells are able to detect fish-specificity for different chemical classes (Castaño and Gómez-Lechón 2005).

2.4 Fish embryo tests

From a review on approx. 150 toxicological studies using different life-stages of fish, McKim (1977) arrived at the conclusion that in at least 80 % of the cases long-term toxicity could be predicted by results from studies with early life-stages. This conclusion was later corroborated by other studies, e.g. by Woltering (1984) or Chorus (1987). In a study comparing the fish cytotoxicity test with the permanent fish cell line RTG-2 (Castaño et al. 1994, 1996) and an early version of the embryo toxicity test with zebrafish (*Danio rerio*) as two competing alternatives to the acute fish toxicity test, the embryo test proved to be more sensitive (Lange et al., 1995), and Nagel (2002) documented that the zebrafish embryo assay is a very promising tool to replace the acute fish test. It should be noted, however, that for a small set of particular substances, cytotoxicity tests may be more sensitive than fish embryo tests (e.g., Zabel and Peterson, 1996).

Most importantly, in contrast to fish cytotoxicity tests, fish embryo tests have been shown to detect not only samples characterized by strong fish toxicity, but also samples, which induce only minor toxicity in conventional acute *in vivo* fish tests (Nagel, 2002, Braunbeck et al., 2005). This is in line with conclusions drawn by Lange et al. (1995), who compared fish cytotoxicity tests with the permanent rainbow trout cell line RTG-2 to the zebrafish embryo test and found that the embryo test was more sensitive than the cell test.

In an independent statistical analysis, Ratte and Hammers-Wirtz (2003) analyzed existing data from zebrafish embryo tests with respect to the correlation to existing data from acute *in vivo* fish tests. On the basis of tests with 56 substances, the authors arrived at the conclusion that there is a reliable correlation between the fish embryo test and the acute fish test ($R^2 = 0.854$; $\alpha = 0.05$). Due to the large sample size, the confidence belt of the regression line was found to be relatively small, but the prediction range was relatively wide (2.36 to 2.5 logarithmic units) corresponding to possible deviations by a factor of 229 and 320. As a consequence, the regression function seems appropriate to describe the average relationship between the acute fish test LC_{50} and the embryo test LC_{50} with good confidence, but less appropriate as a prediction model (Ratte and Hammers-Wirtz, 2003). The results of this statistical review will be reviewed in more detail later in this document.

In an attempt to establish an alternative method for the routine testing of whole effluent discharges, in 2002 Germany took the initiative and established a 48 h toxicity test starting from fertilized zebrafish eggs as an alternative to the acute fish test. By 2005, this alternative has become mandatory and thus replaced the acute fish test.

Most recently, Braunbeck et al. (2005) provided data substantiating that an optimized test protocol can equally be applied to the early embryonic stages of other OECD species such as the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*). However, given its superior high number of eggs per spawning act, the rapid development, the perfect transparency of its eggs, and, last but not least, the immense body of already existing information on zebrafish development (Braunbeck et al., 2005; Nagel, 2002), the zebra-

fish seems to be first choice for routine embryo toxicity testing. Moreover, Braunbeck et al. (2005) listed several modifications to the standard zebrafish embryo test protocol that extend its use to fields other than acute toxicity testing, including *in situ* sediment toxicity evaluation, genotoxicity and mutagenicity testing as well as histopathological studies within the framework of laboratory and field studies.

2.5 Purpose of the present document

Given the lack of data for numerous (especially existing) industrial chemicals, in June 1999 the EC council adopted a new strategy on chemical testing in the European Community known under the acronym of REACH (Registration, Evaluation and Authorization of Chemicals). The future EU chemicals policy has been designed to ensure a high level of protection of human health and the environment as enshrined in the Treaty both for the present and future generations, while also ensuring the efficient functioning of the internal market and the competitiveness of the chemical industry. Fundamental to achieving these objectives is the *Precautionary Principle*. Whenever reliable scientific evidence is available that a substance may have an adverse impact on human health and/or the environment, but there is still scientific uncertainty about the precise nature or the magnitude of the potential damage, decision-making must be based on precaution in order to prevent damage to human health and the environment. Another important objective is to encourage the substitution of dangerous by less dangerous substances where suitable alternatives are available (White paper; EC, 2001).

Another important feature of the White Paper is that, whenever possible, toxicity and ecotoxicity testing should be done on the basis of non-animal assays, thus giving credit to growing concern by animal welfare organizations. Thus, in a further initiative, Germany has taken the lead to submit a proposal for an OECD test guideline for a fish embryo test as an alternative to the acute fish test in chemical registration (cf. Braunbeck et al., 2005). As a basis for future discussions at the OECD level, the present document has been prepared as a review of the existing database on fish embryo toxicity tests. It not only includes data from peer-reviewed scientific publications, but also incorporates data from master and PhD theses that were regarded valid by the authors of this review.

This review also summarizes the results of an independent statistical analysis on behalf of the German Federal Environmental Agency (UBA) by Ratte and Hammers-Wirtz (2003), who analyzed the existing data from zebrafish embryo tests, most of which were carried out within various diploma and PhD theses at Dresden University as well as within a comparative laboratory study organized by Prof. Nagel (Dresden), with respect to their correlation to existing data from acute *in vivo* fish tests. In this context, embryo toxicity testing approaches different to the German zebrafish embryo assay protocol will be discussed with respect to both the correlation to *in vivo* data and animal welfare considerations.

Finally, an attempt will be made to provide a proposal for the integration of existing embryo toxicity testing approaches into a strategy to maximally reduce the number of fish required for acute toxicity testing of chemicals.

3.0 Methodological basis for fish embryo toxicity testing

3.1 Fish, fish maintenance and spawning procedure

It should be noted that – except for water quality criteria – maintenance conditions (and partly also test conditions) can be modified within relatively wide ranges and are, thus, subject to discussion and refinement. Data given in Table 1 are those typically relevant for the maintenance facilities of the authors of this document.

3.1.1 Zebrafish

Fish

The zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) is a small benthopelagic cyprinid fish originating from the Ganges River system, Burma, the Malakka peninsula and Sumatra (Eaton and Farley, 1974; Talwar and Jhingran, 1991) with a mean adult length between 3 and 5 cm (Table 1). In both soft and hard waters, zebrafish grows quickly at 26 °C and completes its life-cycle within three months. The species is easily obtainable, inexpensive, readily maintainable and, under appropriate conditions, yields a large number of non-adherent, fully transparent eggs (Laale, 1977). The zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Dooley & Zon, 2000; Ekker and Akimenko, 1991; Goolish et al., 1999; Hisaoka and Battle, 1958; Kimmel et al., 1995; Laale, 1977; Lele and Krone, 1996; Nüsslein-Vollhard, 1994; Roosen-Runge, 1938; Sander and Baumann, 1983; Westerfield, 2000; Wixon, 2000).

Care should be taken to select a wild-type zebrafish strain with continuously high egg production and high fertilization rate; many strains developed for molecular biological purposes may be unsuitable for toxicological experiments. Under spawning conditions, males can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body (Fig. 1). Due to the large number of eggs produced, females can be recognized by their swollen bellies. One female spawns between 50 and 200 eggs on a daily basis. Egg production can be significantly stimulated by additional rations of natural food (*Artemia* spec. nauplii; *Daphnia*). The fish used for producing eggs should be between 4 and 15 months of age.

Fig. 1: Adult zebrafish (*Danio rerio*) female (upper individual) can easily be differentiated from male (lower individual) by their extended bellies and the lack of reddish tint along the silver longitudinal lines.

Table 1: Maintenance, breeding and typical conditions for embryo toxicity tests with the common OECD test fish species *

		Zebrafish (*Danio rerio*)	Fathead minnow (*Pimephales promelas*)	Japanese medaka (*Oryzias latipes*)
Maintenance of parental fish	Origin of species	India, Burma, Malakka, Sumatra	Temperate zones of central North America	Japan, China, South Korea
	Sexual dimorphism	Females: protruding belly, when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)	Females: more plump, when carrying eggs, ovipositor Non-spawning males: black spot on dorsal fin Spawning males: black coloration of head, dorsal nuptial pad and nuptial tubercles in spawning season, vertical black bands along body sides	Females: generally more plump, carrying sticky eggs at anal fin Males: anal fin larger, papillary processes on posterior dorsal fin rays
	Feeding regime	Dry flake food (max. 3 % fish weight per day) 3 - 5 times daily; from three days before spawning, plus frozen adult brine shrimp (*Artemia* spec.) or *Daphnia* twice daily (*ad libitum*). To guarantee for optimal water quality, excess feces should be removed approx. one hour after feeding.		
	Approximate weight of adult fish	Females: 0.65 ± 0.13 g Males: 0.5 ± 0.1 g	Females: 1.5 ± 0.3 g Males: 2.5 ± 0.5 g	Females: 0.35 ± 0.07 g Males: 0.35 ± 0.07 g
	Illumination	Fluorescent bulbs (wide spectrum); 10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels); 12 - 16 hours photoperiod		
	Water temperature	26.0 ± 1.0 °C	16.5 ± 1.5 °C	24.0 ± 0.5 °C
	Water quality	O_2 ≥ 80 % saturation, hardness: e.g., 3 - 20°dH (~ 30 - 215 mg/L $CaCO_3$), NO_3^-: ≤ 48mg/L, NH_3 and NO_2^-: < 0.001mg/L, total organic carbon < 2 mg/L, residual chlorine < 10 µg/L, total organochlorine pesticides plus polychlorinated biphenyls < 50 ng/L, total organophosphorus pesticides < 50 ng/L, total organic chlorine < 25 ng/L, pH = 7.8 ± 0.2		
	Further water quality criteria	Particulate matter < 20 mg/L, total organic carbon < 2 mg/L, unionized ammonia < 1 µg/L, residual chlorine < 10 µg/L, total organophosphorus pesticides < 50 ng/L, total organochlorine pesticides plus polychlorinated biphenyls < 50 ng/L, total organic chlorine < 25 ng/L		
	Tank size for maintenance	180 L (max. 180 individuals)	180 L (max. 80 individuals)	50 L (max. 60 individuals)
	Water purification	Permanent (charcoal filtered); possible are combinations with semi-static maintenance or flow-through system with continuous water renewal		

* Data given explicitly are those relevant for the maintenance facilities of the authors of this document.

Table 1: Maintenance, breeding and typical conditions for embryo toxicity tests with the common OECD test fish species (cont´d.)

	Zebrafish (*Danio rerio*)	**Fathead minnow (*Pimephales promelas*)**	**Japanese medaka (*Oryzias latipes*)**
Male to female ratio for breeding	2:1 (4:2)	2:4	15:15
Breeding tanks	4 L tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats (cf. Fig. 4)	30 L tanks with black glass walls maintained at 24 °C and equipped with 2 clay tiles divided into two halves as spawning substrate	30 L tanks with black glass walls equipped with several plant dummies or *Ceratophyllum* spec. as substrate for spawning
Egg structure and appearance	Stable chorion, highly transparent, non-sticky, diameter: ~0.8 mm	Chorion only hardens in multicellular stage, transparent, sticks to surfaces, diameter < 1 mm	Stable chorion with spiny hooks (adheres to anal fin of female), moderately transparent, diameter < 1 mm
Embryo development at 26 °C	18 h: Development of somites 21 h: Tail detachment 26 h: Heart-beat visible 28 h: Blood circulation 72 h: Hatching	22 h: Development of somites 25 h: Tail detachment 27 h: Heart-beat visible 30 h: Blood circulation 120h: Hatching	30 h: Development of somites 54 h: Heart-beat visible 78 h: Blood circulation 78 h: Tail detachment 160h: Hatching
Test type	Static or semi-static, 26 °C, 24-well plates (2 ml per cavity)	Static or semi-static, 25 °C, 24-well plates (2 ml per cavity)	Static or semi-static, 26 °C, 24-well plates (2 ml per cavity)
Major toxicological endpoints at 25°C	24 h: Coagulation, tail detachment, somite development 48 h: Heart-beat visible	28 h: Coagulation, tail detachment, somite development 3 d: Blood circulation 3 d: Blood circulation 4 d: Blood circulation	30 h: Coagulation, tail detachment, somite development 78 h: Blood circulation 4 d: Pectoral fins; 30 % tail detachment 5 d: Blood circulation; movement of pectoral fins; tail detached and pigmented 7 d: Hatch; blood circulation

Normal zebrafish development

The embryonic development of zebrafish has been described in most detail (Hisaoka and Battle, 1958; Kimmel et al., 1988, 1995; Laale, 1977; Roosen-Runge, 1938; Thomas and Waterman, 1978). The zebrafish egg is telolecithal, cleavage is meroblastic and discoidal. Selected major stages of zebrafish development are given in Figs. 2 and 3; for more details, see Table 2 as well as Kimmel et al. (1995).

Table 2: Stages of embryonic development of the zebrafish (*Danio rerio*) at 26 ± 1 °C (Nagel, 2002)

Time (h)	Stage	Characterization (after Kimmel et al., 1995)
0	Fertilization	Zygote
0	Zygote period	Cytoplasm accumulates at the animal pole, one-cell stage
0.75	Cleavage period	Discoidal partial cleavage: 1. (median vertical) division: two-cell-stage
1		2. (vertical) division: four-cell-stage
1.25		3. (vertical and parallel to the plane of the first) division: 8-cell-stage
1.5		4. (vertical and parallel to the second) division: 16-cell-stage
2	Blastula period	Start of blastula stage
3		Late cleavage; blastodisc contains approximately 256 blastomers
4		Flat interface between blastoderm and yolk
5.25	Gastrula period	50 % of epibolic movements; blastoderm thins and interface between periblast and blastoderm become curved
8		75 % of epibolic movement
10		Epibolic movement ends, blastopore is nearly closed
10.5	Segmentation period	First somite furrow
12		Somites are developed, undifferentiated mesodermal component of the early trunk, tail segmented or metameric
20		Muscular twitches; sacculus; tail well extended
22		Side to side flexures; otoliths
24	Pharyngula period	Phylotypic stage, spontaneous movements, tail is detached from the yolk; early pigmentation
30		Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1 visible
36		Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating
72 - 96	Hatching period	Heart-beat regular; yolk extension beginning to taper; dorsal and ventral pigmentation stripes meet at tail; segmental blood vessels detectable: thickened sacculus with two chambers visible; foregut development; neuromasts

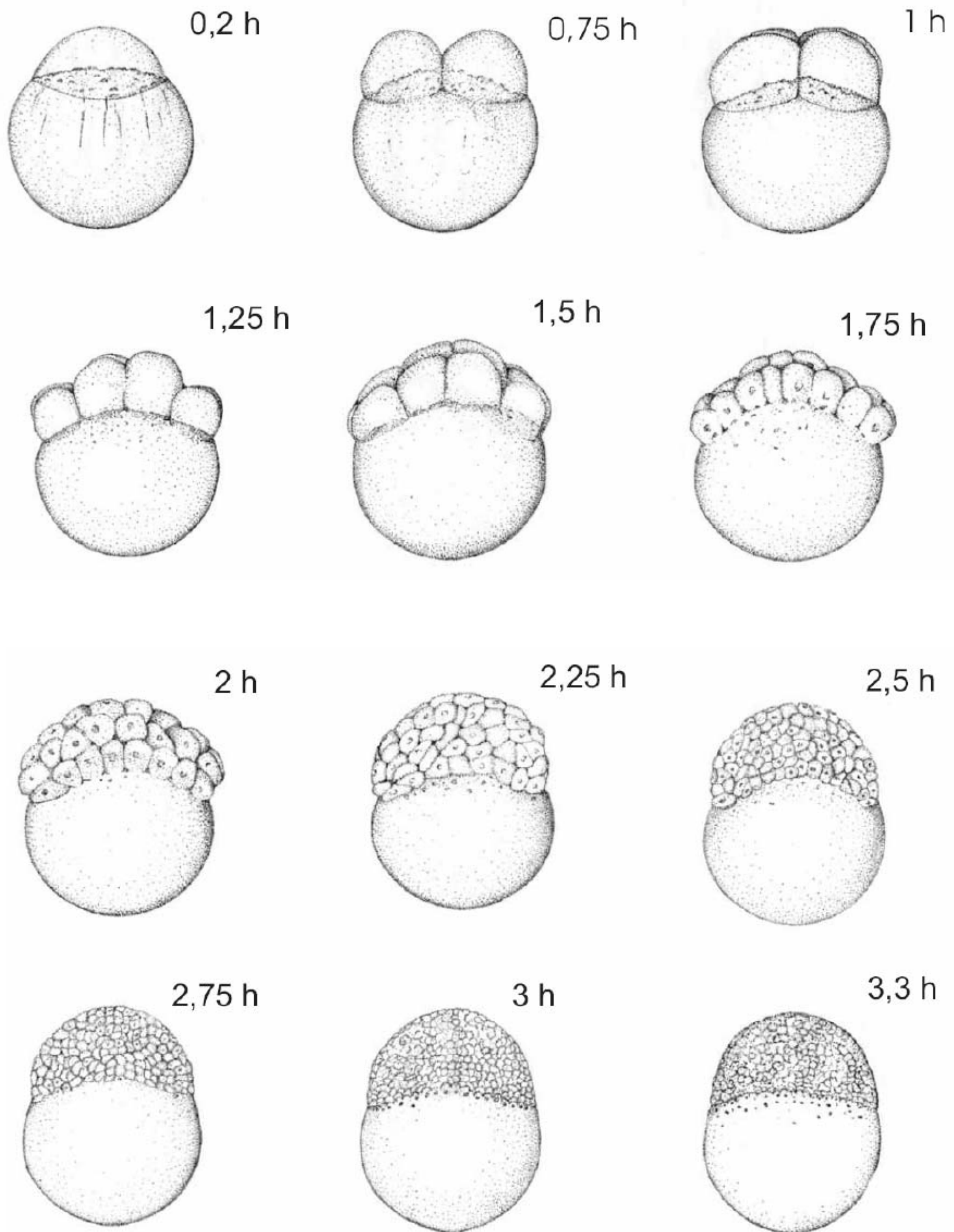


Fig. 2a: Selected stages of early zebrafish (*Danio rerio*) development: 0.2 - 3.3 h post-fertilization (from Kimmel et al., 1995).

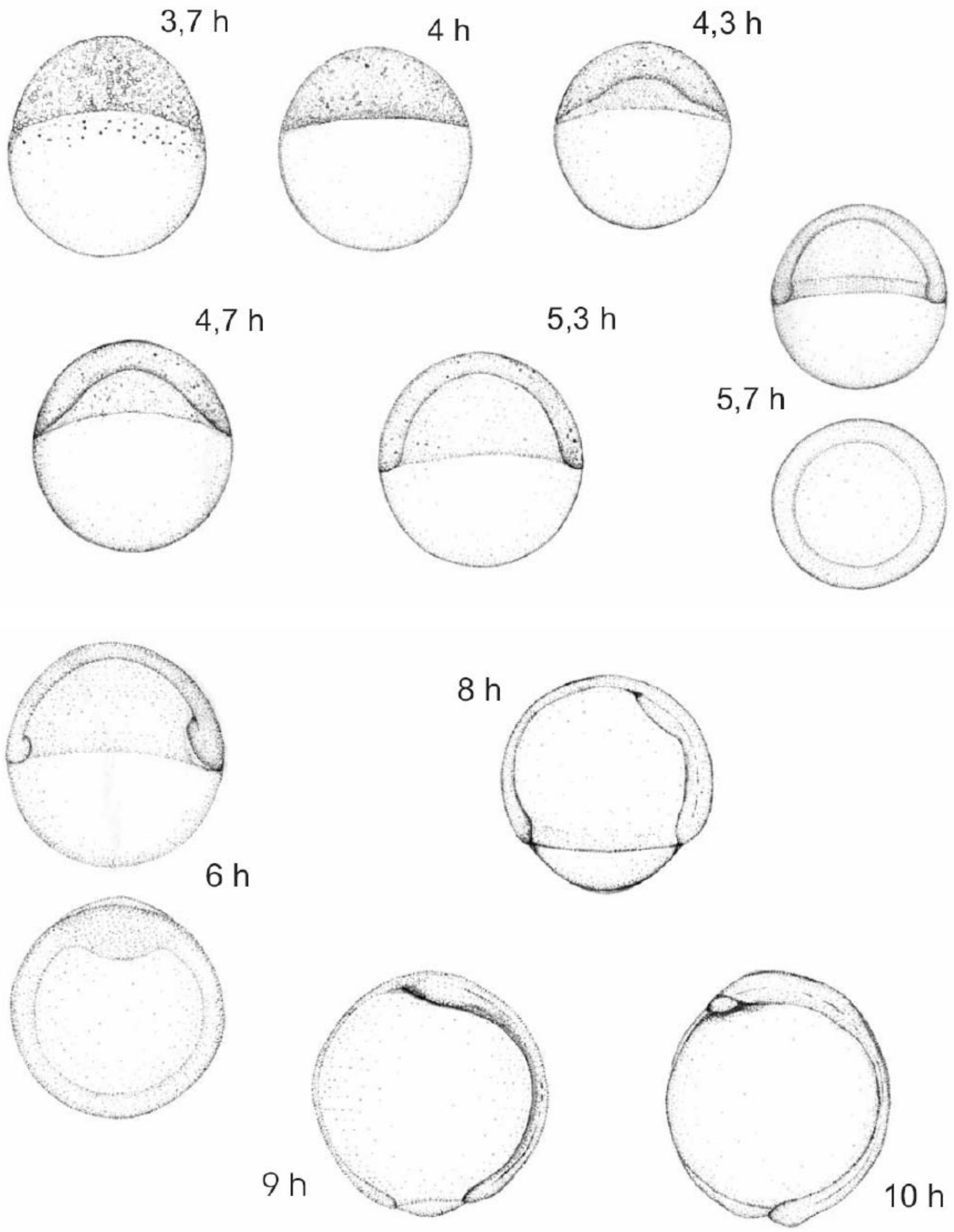


Fig. 2b: Selected stages of intermediate zebrafish (*Danio rerio*) development: 3.7 - 10 h post-fertilization (from Kimmel et al., 1995).

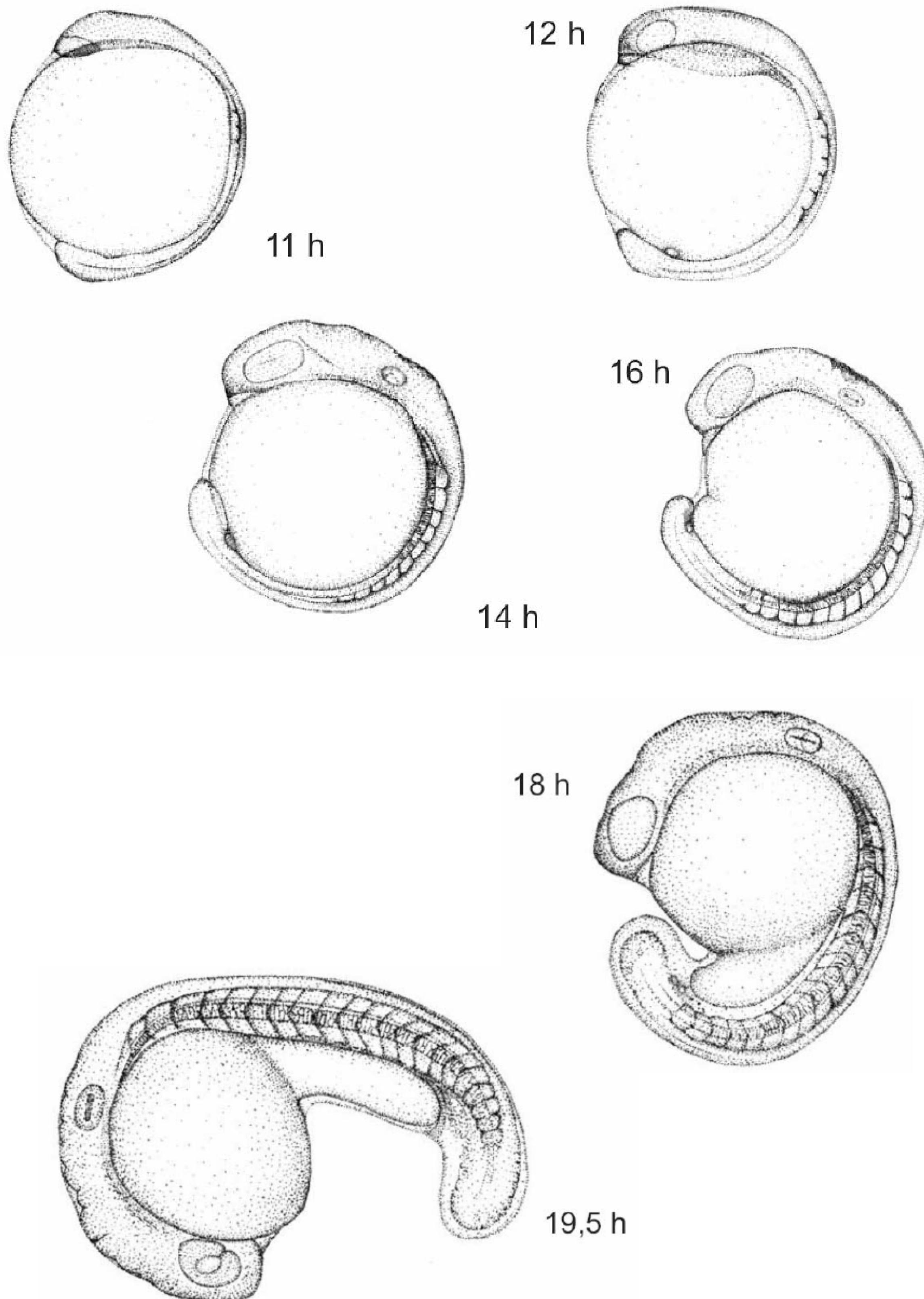


Fig. 2c: Selected stages of intermediate zebrafish (*Danio rerio*) development: 11 – 19.5 h post-fertilization (from Kimmel et al., 1995).

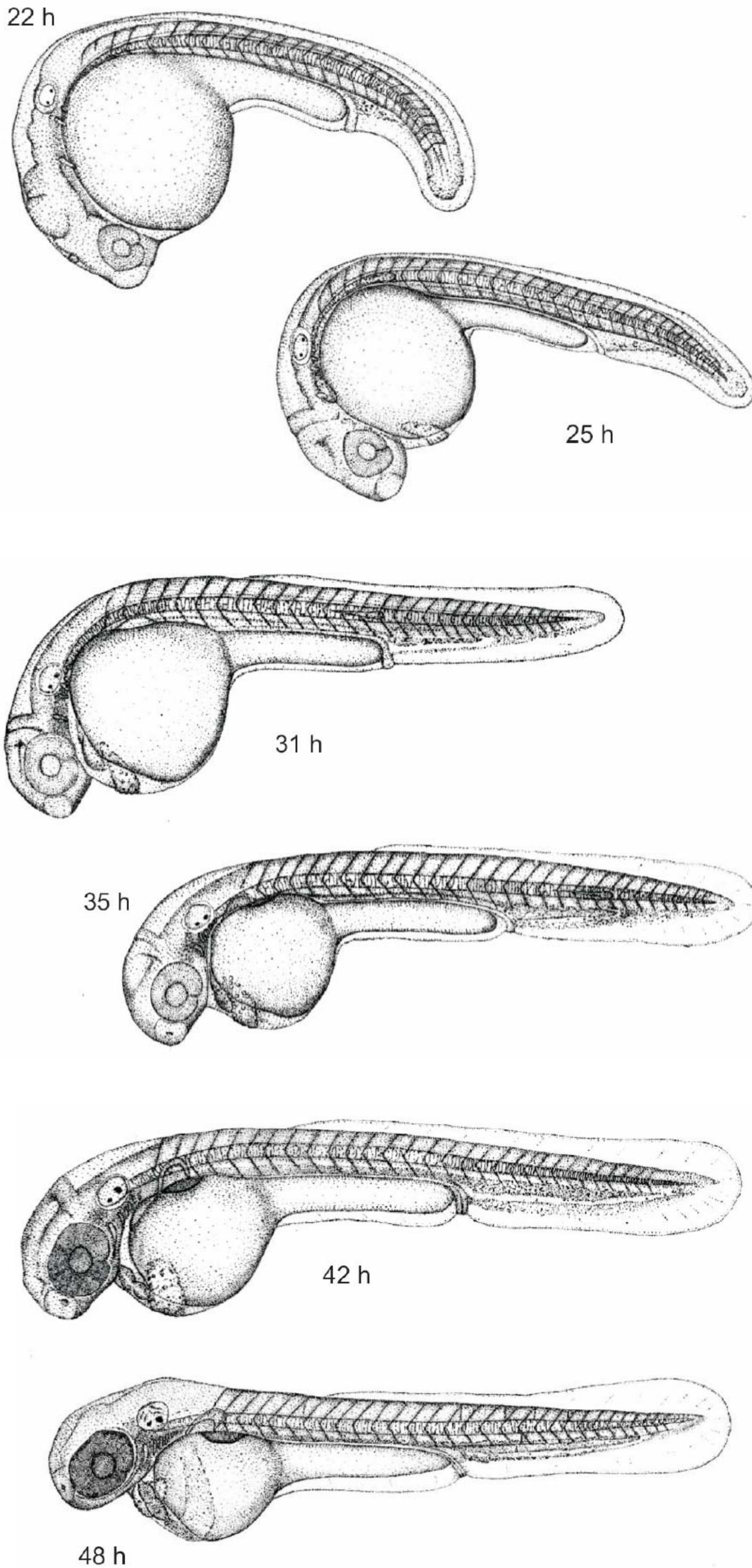


Fig. 2d: Selected stages of late zebrafish (*Danio rerio*) development: 22 - 48 h after fertilization (from Kimmel et al., 1995).

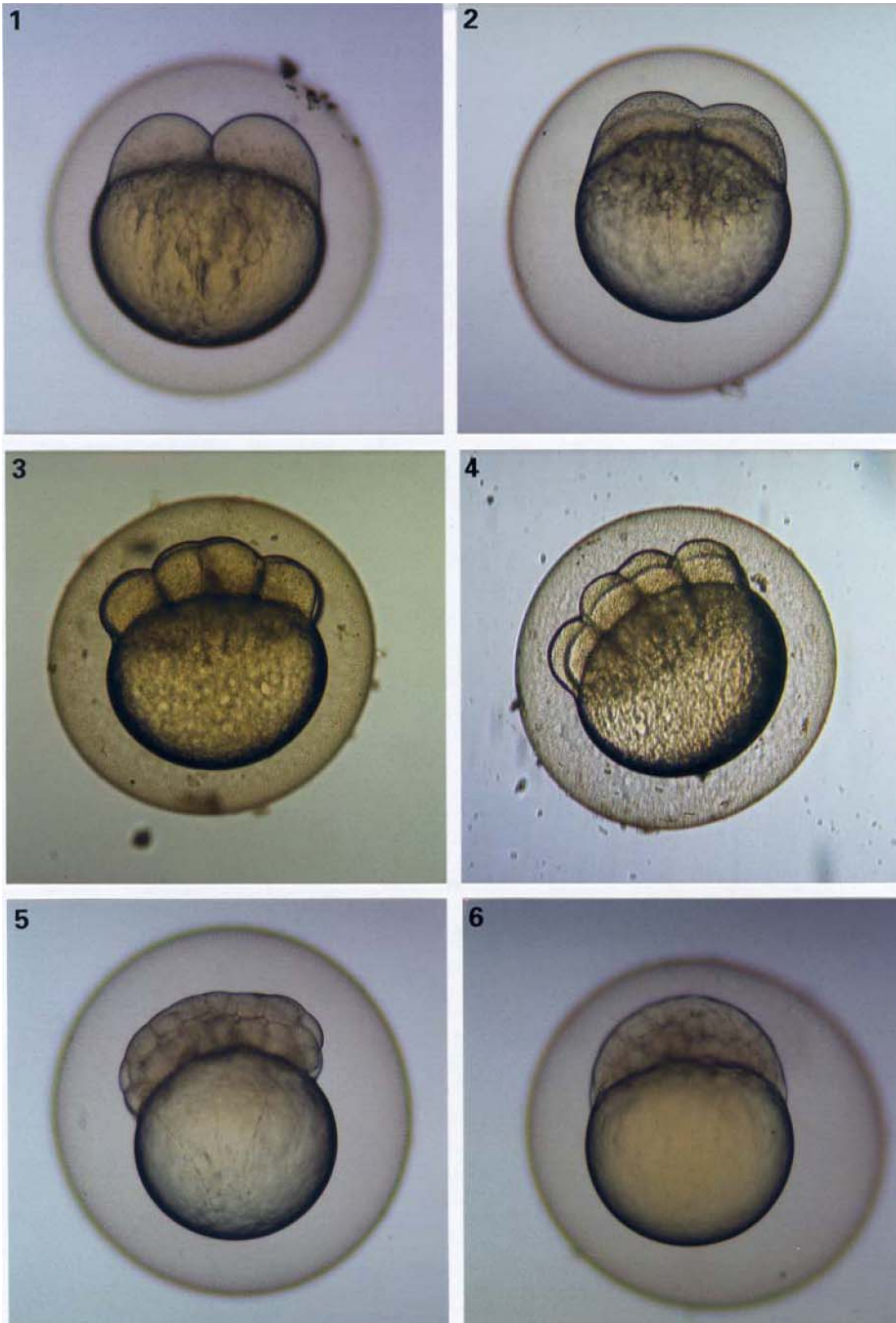


Fig. 3a: Normal development of zebrafish (*Danio rerio*) embryos I: (1) 0.75 h; (2) 1 h; (3) 1.2 h; (4) 1.5 h; (5) 4.7 h; (6) 5.3 h.

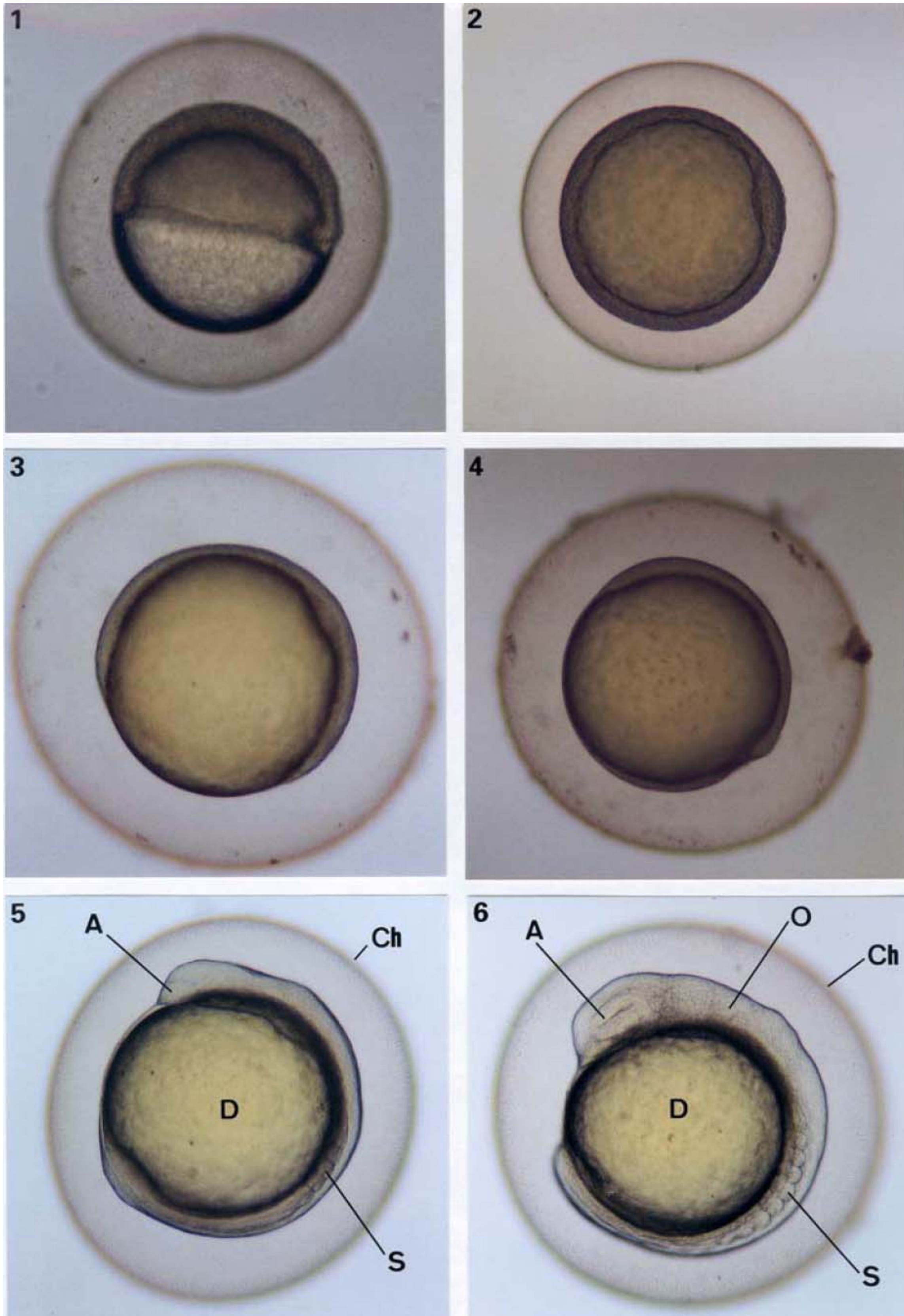


Fig. 3b: Normal development of zebrafish (*Danio rerio*) embryos II: (1) 6 h; (2) 6 h; (3) 8 h; (4) 9 h; (5) 12 h; (6) 14 h. A – eye anlage; Ch – chorion; O – ear bud; S – somites (muscle segments).

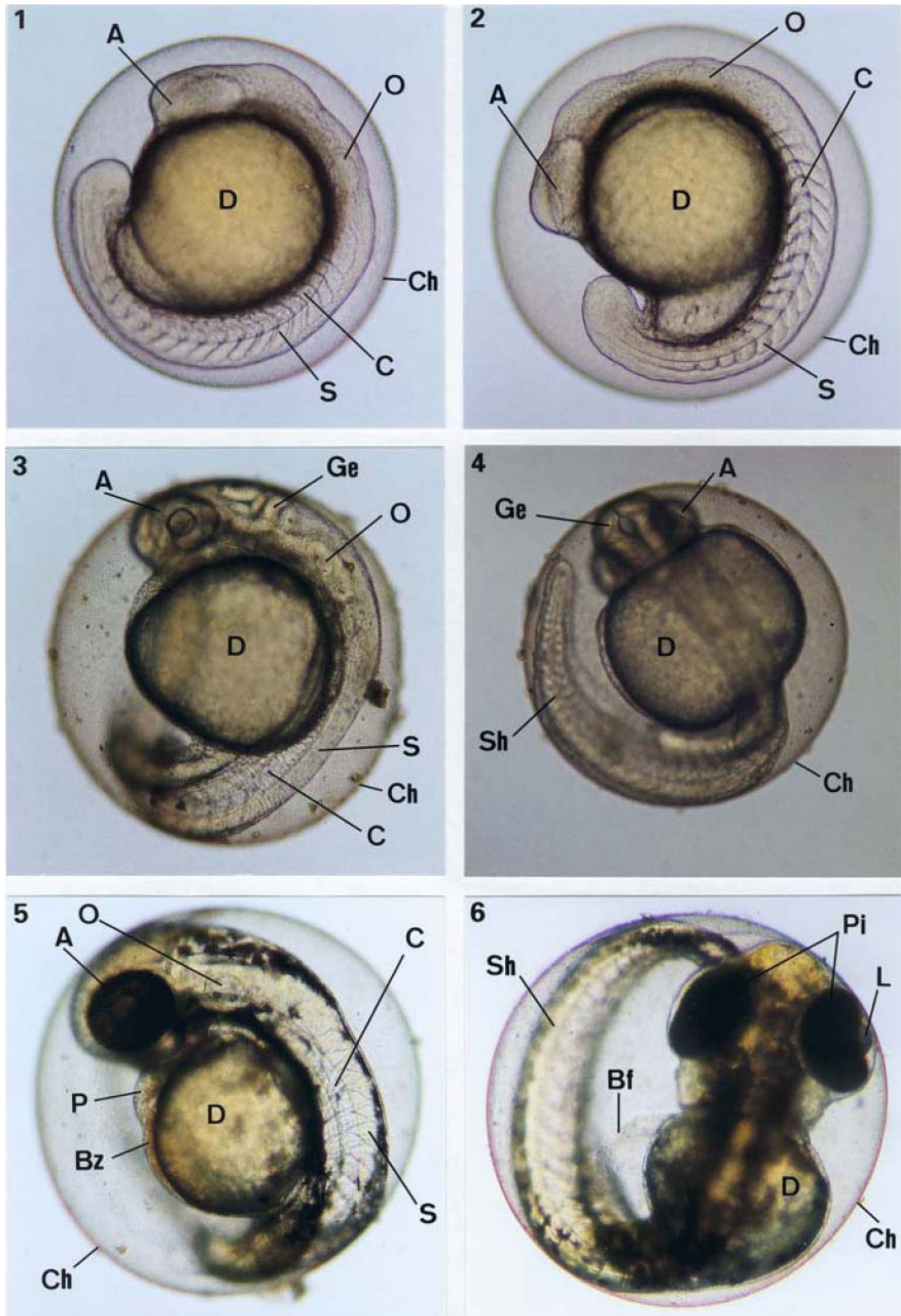


Fig. 3c: Normal development of zebrafish (*Danio rerio*) embryos III: (1) 16 h; (2) 18 h; (3) 25 h; (4) 25 h; (5) 48 h; (6) 72 h. A – eye anlage; Bf – pectoral fin; Bz – blood cells; C – chorda; Ch – chorion; Ge – brain anlage; L – optical lens; O – ear bud; P – pericard; S – somites; Sh – tail.

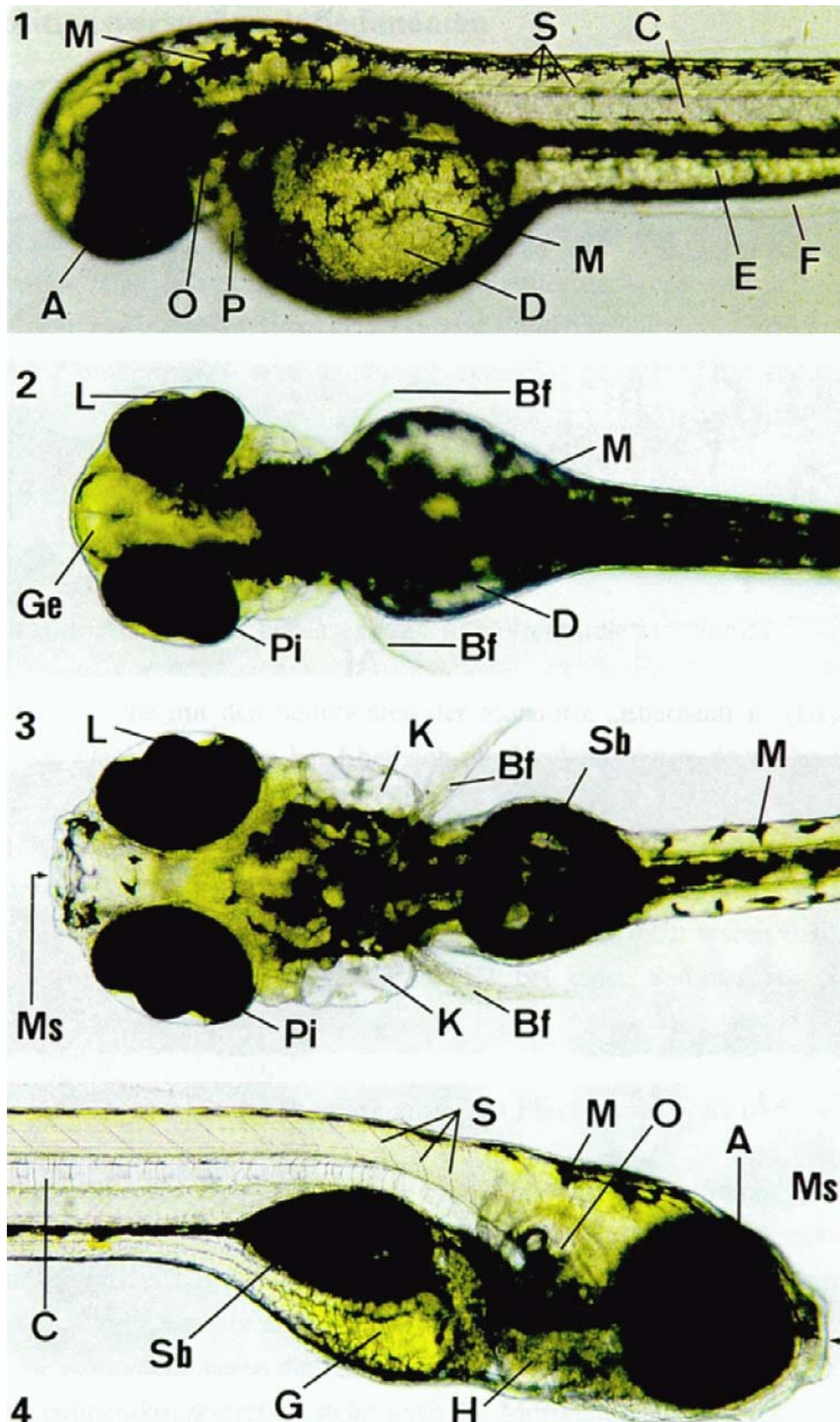


Fig. 3d: Normal development of zebrafish (*Danio rerio*) embryos IV: (1) 48 h; (2) 72 h; (3) 144 h; (4) 144 h. A – eye anlage; Bf – pectoral fin; Bz – blood cells; C – chorda; Ch – chorion; D – yolk sac; E – gut; F – fin; Ge – brain; H – heart; K – gills; L – eye lens; M – melanophores; Ms – mouth slit; O – ear; P – pericard; Pi – ocular pigment layer; S – somites (muscle segments); Sb – swimming bladder; Sh – tail.

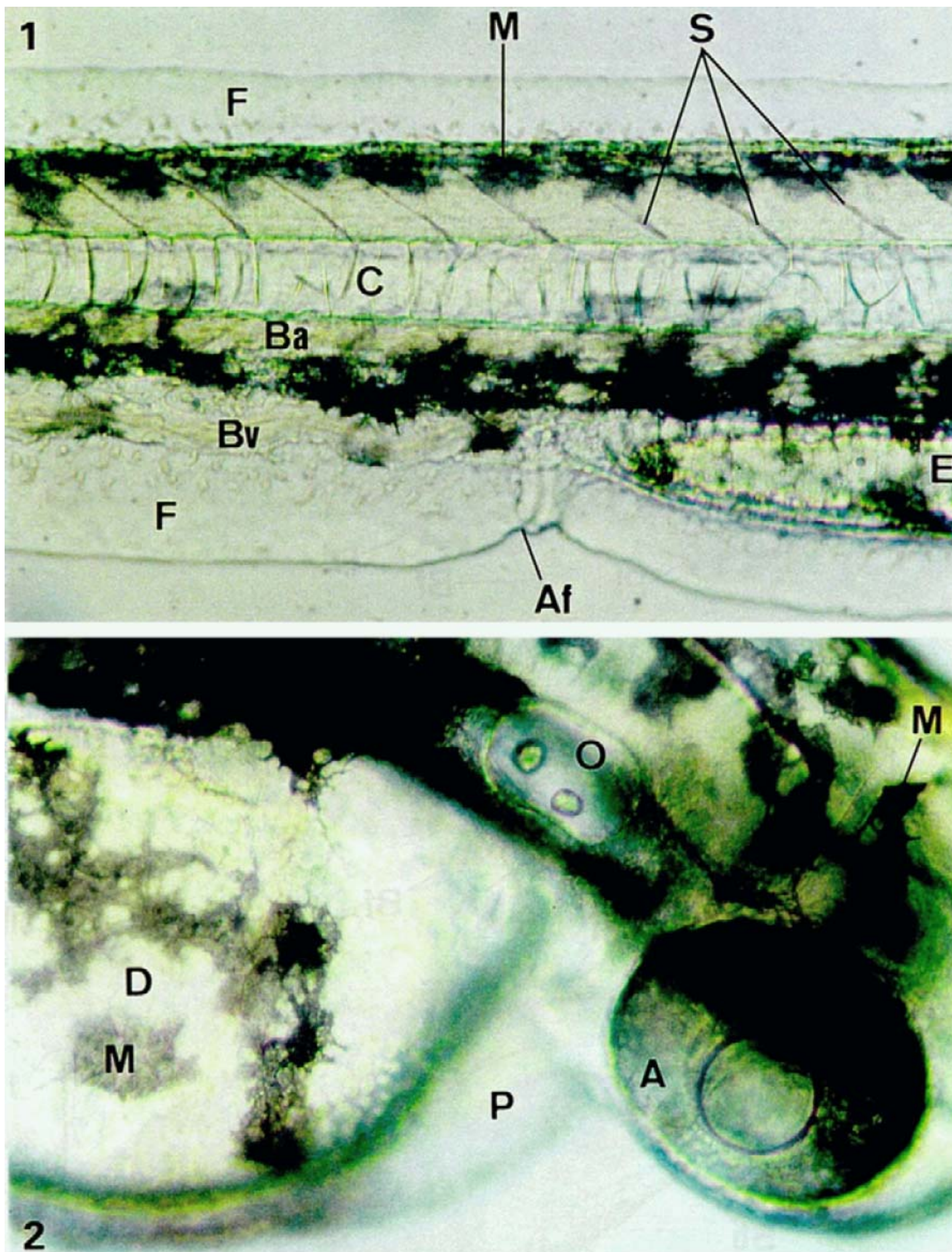


Fig. 3e: Normal development of zebrafish (*Danio rerio*) embryos V (following dechorionation): (1) 48 h, anal region; (2) 48 h, ear region. A – eye anlage; Af – position of the anus; Ba – dorsal aorta; Bv – central ventral axial vein; D – yolk sac; E – peritoneum; F – fin; H – heart; M – melanophores; P – pericard; O – ear; S – somites (muscle segments).

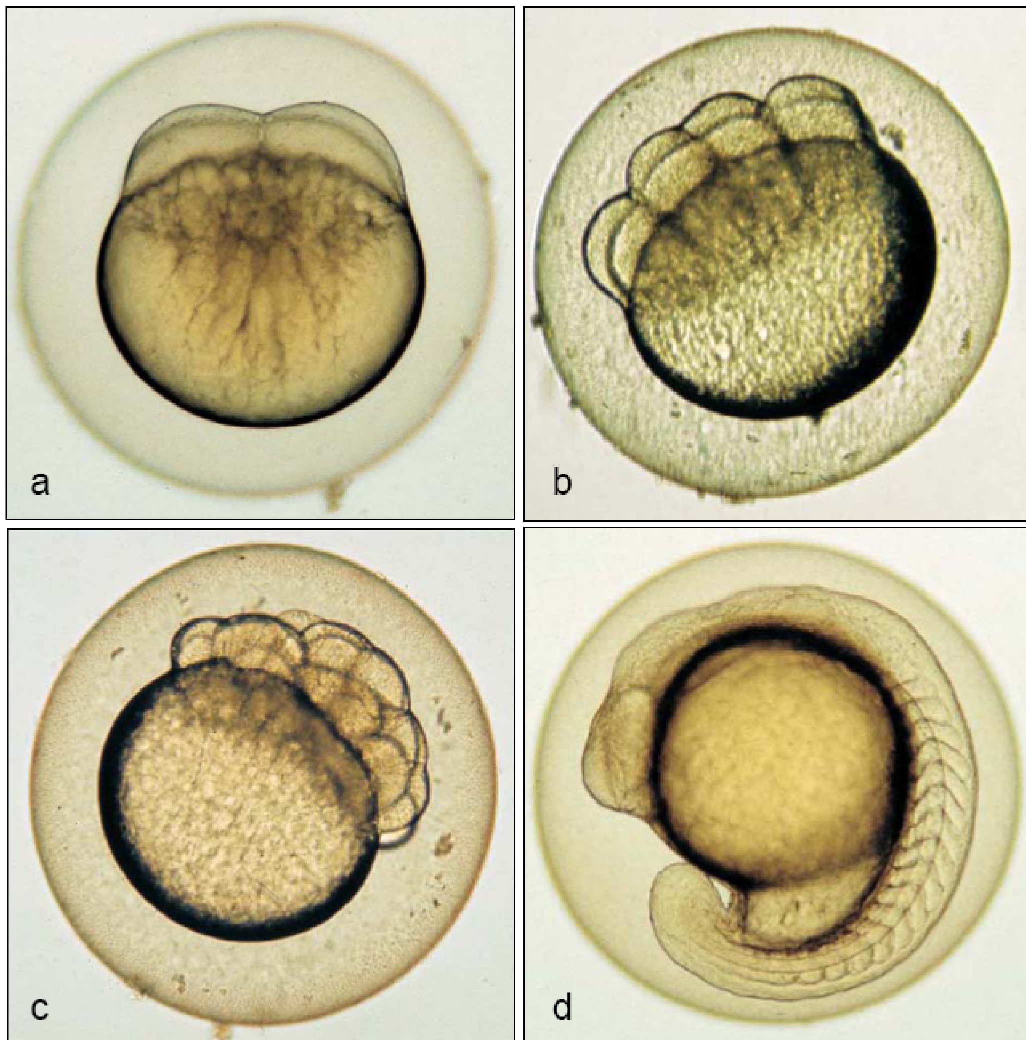


Fig. 3f: Selected stages of zebrafish (*Danio rerio*) development: (a) 4-cell stage (approx. 1 h); (b) 16-cell stage (approx. 1.3 h); (c) 64-cell stage (approx. 1.8 h); (d) detachment of tail (approx. 17.5 h).

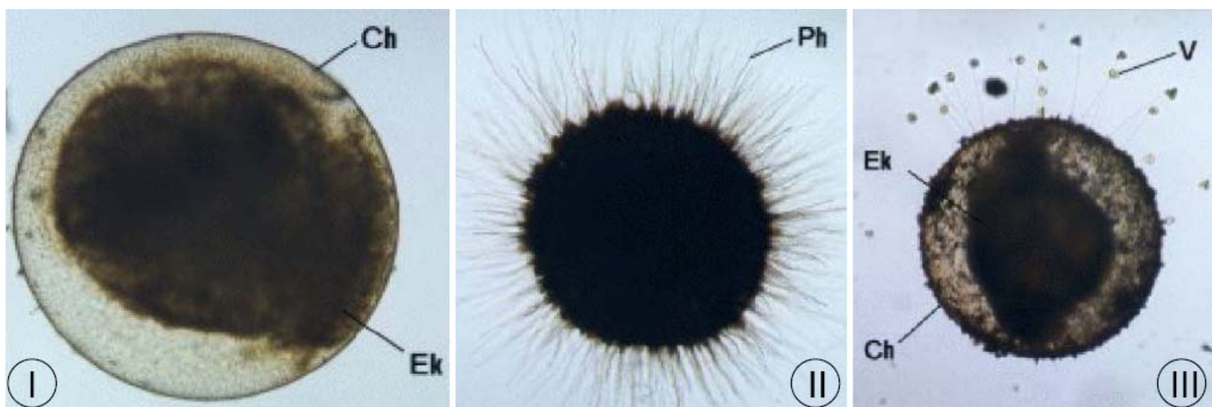


Fig. 3g: Mortality in early zebrafish (*Danio rerio*) eggs: coagulation (I), heavy infestation with fungi (II) and invasion of *Vorticella* spec. (V; III). Ch – chorion; Ek – coagulated egg; Ph – fungi.

Zebrafish maintenance and breeding

The maintenance and breeding of zebrafish has been described in detail by Nagel (2002). A breeding stock of non-treated, mature zebrafish is used for egg production. Females and males are kept at a ratio of 1:2 in a glass aquarium filled with charcoal filtered tap water with an oxygen saturation of more than 80%. The culture conditions are 26 ± 1 °C at a 12 hour day/night light regime. Optimal filtering rates should be adjusted using a filter system. The fish are fed with dry flakes twice per day, and *ad libitum* with larvae of *Artemia* spec. once a day. To ensure optimal water quality remaining food should be removed daily. To prevent the eggs from cannibalism by the adult zebrafish the spawn traps are covered with a stainless steel mesh. Plant imitations made of green glass are used as spawning substrate. As modifications, green plastic wire materials were successfully used as spawning stimulants instead of glass plant imitates (Fig. 4).

Spawning and fertilization take place within 30 minutes after light has been turned on in the morning. About 30 - 60 minutes after spawning, the egg traps can be removed, and the eggs are collected by means of a plastic mesh sieve or pipettes. For collection of eggs, the bottom of the 3 L breeding tanks should be replaced by a stainless steel grid with a mesh size of 1.25 mm in order to prevent predation of eggs by the parent fish. In the authors´ laboratory, the breeding tanks are placed on rectangular full-glass dishes of similar dimensions as the tanks themselves. To collect the eggs after spawning, the dishes are removed from the breeding facility and placed under a temperature-controlled dissecting microscope for counting and sorting of viable (i.e., fertilized) eggs.

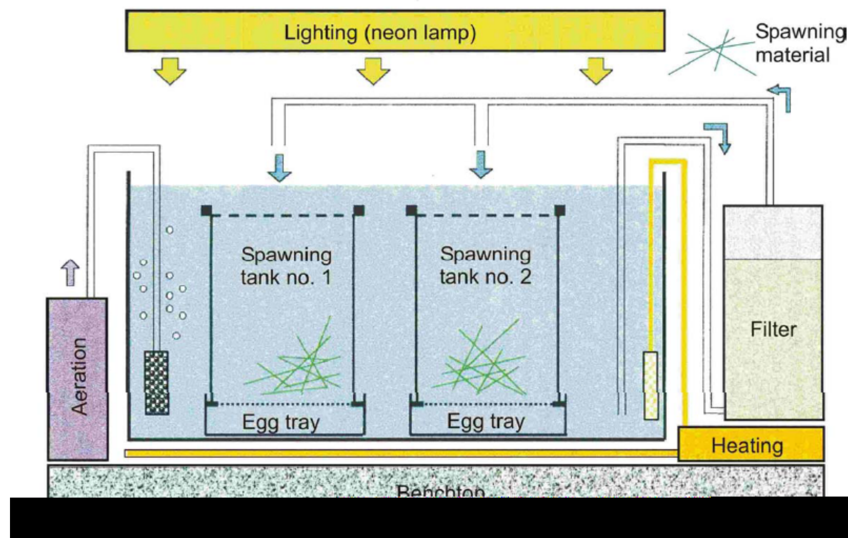


Fig. 4: Setup of the tanks used for breeding zebrafish in the Heidelberg laboratory: Up to 10 tanks, the bottom of which had been replaced by a stainless steel grid, were placed on top of spawning dishes of similar dimensions. All spawning tanks were immersed into one bigger tank equipped with fully conditioned aquarium water. To collect the eggs after spawning, the dishes can easily be removed from the breeding facility.

Alternatively, eggs may be collected by larger trays covered with a stainless steel grid and placed at the bottom of the normal maintenance tanks; as a consequence of mass spawning procedures, however, it should be noted that the eggs recovered from a higher number of individuals are characterized by higher genetic diversity than those derived from small spawning groups. Finally, as a further alternative, big tanks with glass funnels placed below tank bottoms made of stainless steel grid have successfully been used for large-scale mass spawning of zebrafish (Fig. 5).

Fig. 5: Setup of the tanks used for breeding zebrafish in the Berlin laboratory (Leibniz-Institute of Freshwater Ecology and Inland Fisheries): Large groups of zebrafish are maintained in tanks the bottom of which has been replaced by a stainless steel grid to avoid predation of the eggs by the parent fish. The tanks are sitting on a big funnel, which allows simple release of the newly spawned eggs (courtesy of Dr. T. Meinelt, IGB).

A single mature female lays 50-200 eggs per day. At the culture conditions described above, fertilized eggs undergo the first cleavage after approximately 15 min and consecutive synchronous cleavages form 4, 8, 16, and 32 cell blastomeres. At these stages (4 - 32 cells), eggs can be identified clearly as fertilized, and only these should be used for the experiments.

The principle of the zebrafish embryo toxicity test

The embryo test procedure itself has been described by Schulte and Nagel (1994) as well as Nagel (1998) in detail. In brief, following initial range-finding experiments, the toxicity of a chemical substance can be determined by using 24-well-plates. After preparing a stock solution of the test substance, typically five concentrations are tested. Information about the use of solvents can be found in Maiwald (1997) as well as in Nagel (1998).

In the original version of the zebrafish embryo test, 40 eggs were transferred to the test solutions at latest 60 minutes after light had been turned on to initiate spawning. Fertilized eggs were separated from the non-fertilized ones and placed in the 24-well-plates with a pipette using a stereo microscope. 20 Fertilized eggs were placed individually in 2 ml of the respective test solutions to exclude mutual influences. The remaining four wells of each plate were used as internal control filled with dilution water amounting to a total of 20 controls per test. The dilution water corresponded to the reconstituted water according to ISO-standard 7346/3, which was diluted 1:5 using deionized water. After this procedure, the 24-well-plates were covered with a self-adhesive foil and incubated at 26°C ± 1°C. Lethal, sublethal and teratogenic endpoints were recorded using a dissecting microscope within 48h. A test was classified as valid, if 90% of the embryos in the control treatments showed neither sublethal nor lethal effects. For a discussion of lethal and sublethal effects, see below.

Oxygen requirements of zebrafish (*Danio rerio*) embryos

In order to determine the oxygen requirements and consumption by zebrafish embryos, eggs were incubated and exposed to 3,4-dichloroaniline and 2,4-dinitrophenol under low oxygen concentrations varying between 3 and 6 mg/l, which were prepared by selectively fumigating the solutions with nitrogen (Braunbeck et al., 2005). For incubation, the 24-well microtiter plates were kept within closed chambers under a pure nitrogen atmosphere. In a second series of experiments, zebrafish eggs (embryos) were maintained at densities between 10 and 60 individuals per ml medium under similar conditions.

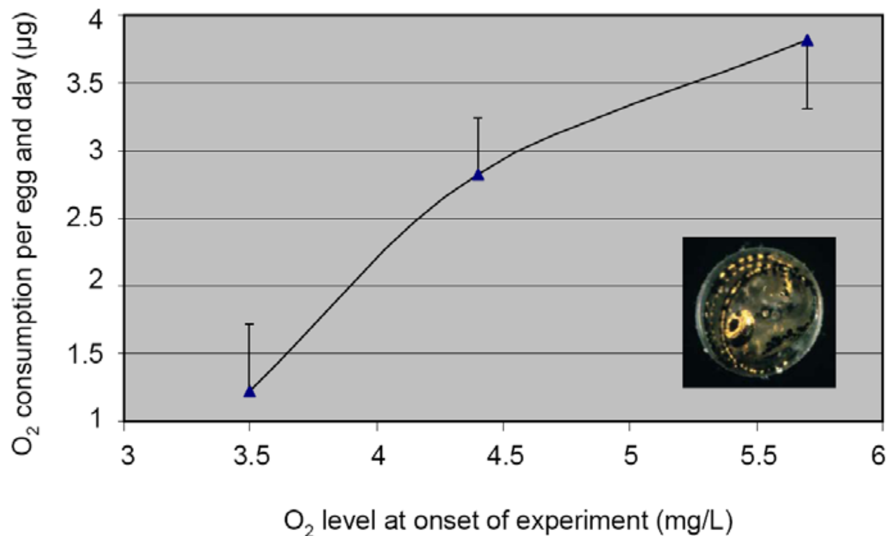


Fig. 6: Oxygen consumption of control zebrafish (*Danio rerio*) embryos in relation to the amount of oxygen provided in the medium. Apparently, zebrafish embryos are capable of adapting to low oxygen levels without any symptoms of developmental effects. Data are given as means from 4 independent experiments ± S.D. (Braunbeck et al., 2005).

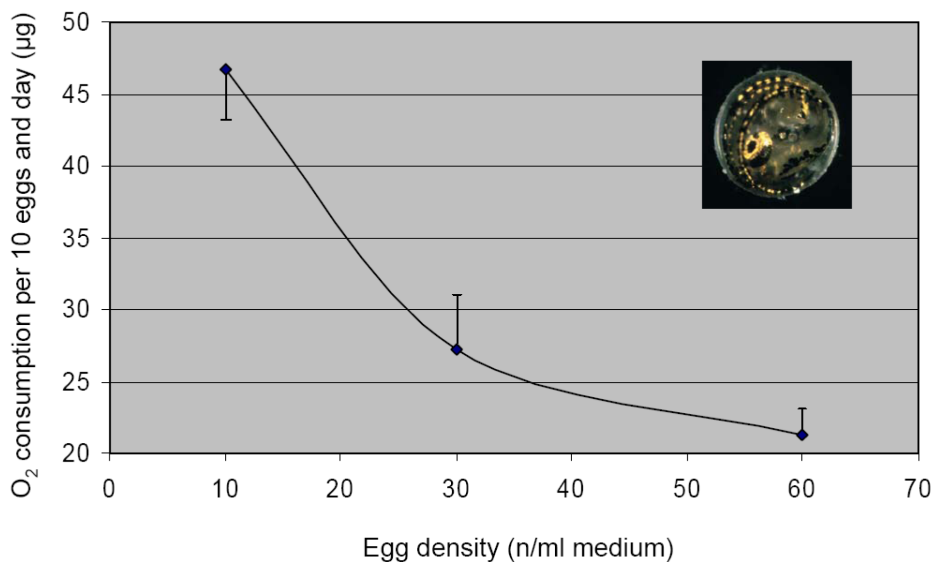


Fig. 7: Oxygen consumption of control zebrafish (*Danio rerio*) embryos in dependence from the stocking density in given volume of medium. As could be expected from data presented in Fig. 6, the embryos adapt to low oxygen levels by reducing their oxygen consumption. Data are given as means from 5 independent experiments ± S.D. (Braunbeck et al., 2005).

Zebrafish embryos incubated at varying levels of oxygen in the medium were apparently well capable of adapting to low oxygen tension. The more oxygen was provided in the medium, the more oxygen the embryos consumed (Fig. 6). Even at oxygen concentrations as low as 2 mg/l, which should be expected to be lethal to adults of most other cyprinid fish species, zebrafish embryos did not show any symptom of malformation or even growth retardation (additional experiments; data not shown in Fig. 6). This observation is of particular importance for the routine testing of whole effluents, since in sewage there may be severe oxygen depletion due to bacterial breakdown. As could be expected from the adaptive reduction of oxygen consumption under conditions of low oxygen levels, zebrafish embryos are also able to react to increasing stocking densities in a given volume of medium (Fig. 7).

Space requirements of zebrafish embryos

To elucidate the minimum space required by a zebrafish embryo, eggs were incubated in 96-well microtiter plates filled with 100, 200 and 300 µl water or toxicant (3,4-dichloroaniline, 2,4-dinitrophenol) concentrations and compared to eggs incubated in a total volume of 2 ml in 24-well microtiter plates (Braunbeck et al., 2005).

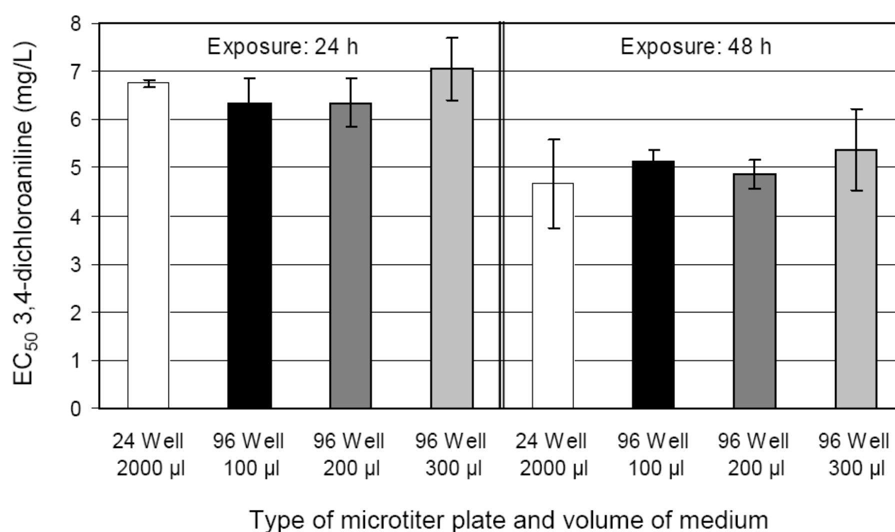


Fig. 8: Toxicity of 3,4-dichloroaniline to zebrafish (*Danio rerio*) embryos in various volumes of medium (2 ml in 24-well microtiter plates as well as 100, 200 and 300 µl in 96-well microtiter plates) after 24 h (left) and 48 h of exposure (right). Endpoints as listed by DIN standards. The incubation volume does not seem to take any influence on the toxicity of 3,4-dichloroaniline (Braunbeck et al., 2005).

In fact, exposure experiments with 3,4-dichloroaniline and 2,4-dinitrophenol did not show any change with respect to the exposure volume (Figs. 8, 9). As a consequence, zebrafish embryos cannot only be exposed in 24-well microtiter plates in a total volume of 2 ml medium (as suggested in the OECD TG proposal), but also in even lower volumes of 300, 200 and even 100 µl within the cavities of 96-well microtiter plates. Thus, the fish embryo test provides a tool to test even smallest volumes of test substances, which may be of particular relevance to the testing of, for example, metabolites of pesticides. On the other hand, extremely lipophilic substances may require modification of the test protocol in that microtiter plates should be replaced by glassware. In such cases, the possibility to expose a higher number of embryos within one vessel to a small volume might also be a significant advantage.

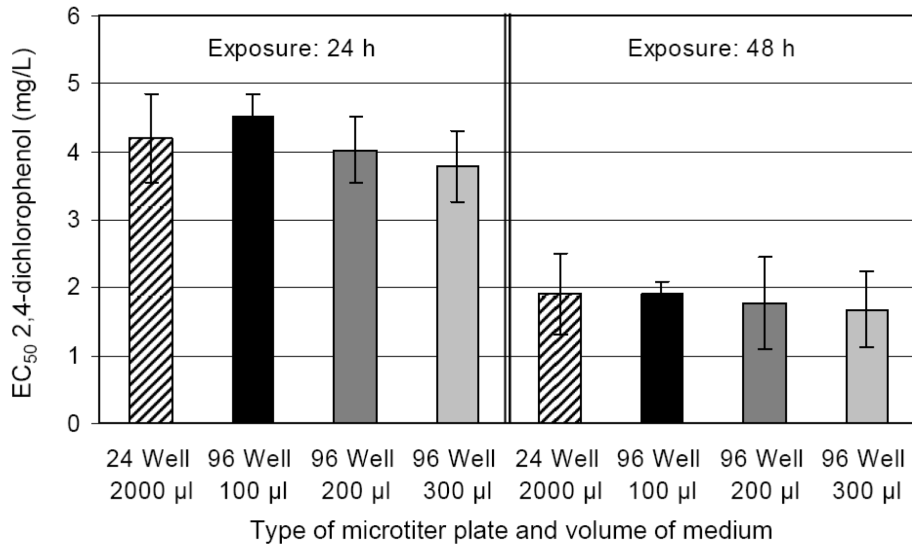


Fig. 9: Toxicity of 2,4-dinitrophenol to zebrafish (*Danio rerio*) embryos in various volumes of medium (2 ml in 24-well microtiter plates as well as 100, 200 and 300 µl in 96-well microtiter plates) after 24 h (left) and 48 h of exposure (right). Endpoints as listed by DIN standards. The incubation volume does not seem to take any influence on the toxicity of 2,4-dinitrophenol (Braunbeck et al., 2005).

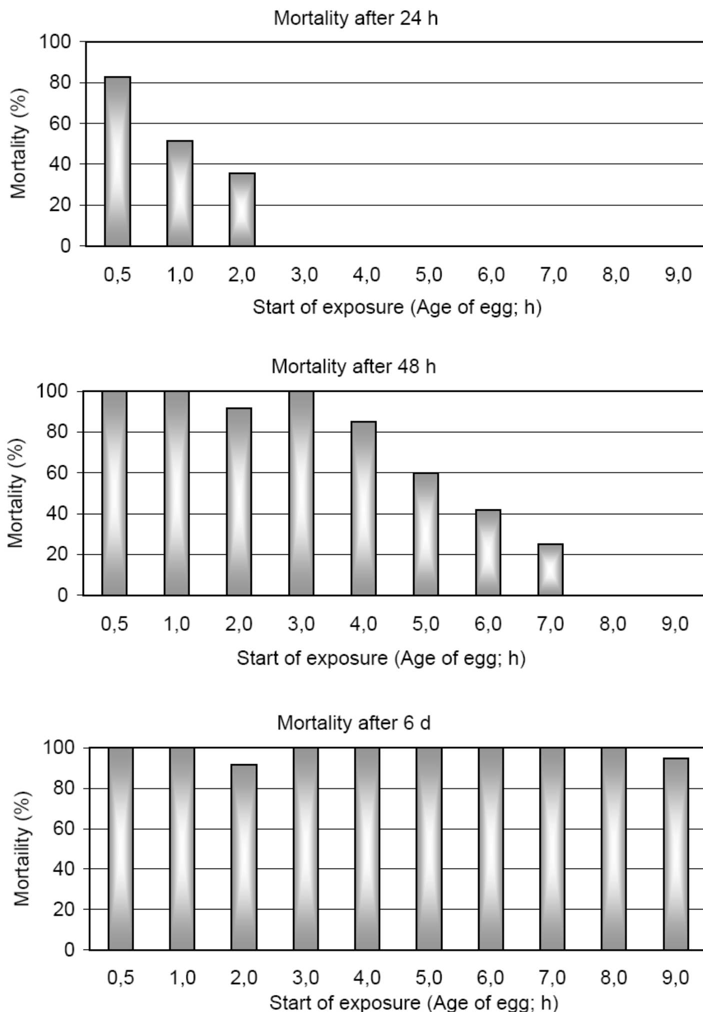


Fig. 10: Effect of the exposure start (time lapse after fertilization) on the cumulative mortality of 2,4-dinitrophenol. After 24 and 48 h, it is evident that mortalities are increased with early exposure start.

Importance of an early onset of exposure of zebrafish (*Danio rerio*) eggs

In order to investigate the role of the start of the exposure, zebrafish eggs were exposed to the model compounds 2,4-dinitrophenol and 3,4-dichloroaniline (Figs. 10, 11) with different starting points from 1 to 9 h of egg age. For either substance, a clear relationship between the age of the eggs at onset of exposure and the cumulative mortality after 24 and 48 h of exposure was found. After 6 days of exposure, however, mortality had accumulated to such a rate that the effect could no longer be seen (Figs. 10, 11). As a conclusion, the eggs should be transferred to the test solutions at latest 1 h post-fertilization.

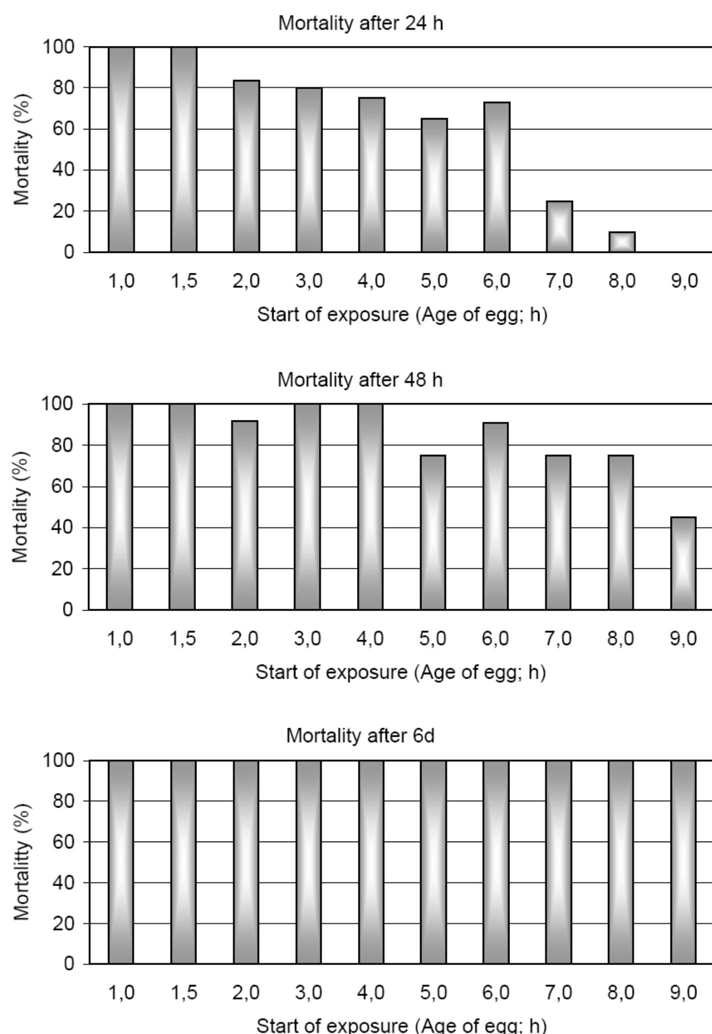


Fig. 11: Effect of the exposure start (time lapse after fertilization) on the cumulative mortality of 3,4-dichloroaniline. After 24 and 48 h, it is evident that mortalities are increased with early exposure start.

The chorion of zebrafish (*Danio rerio*) embryos as a barrier for the uptake of chemicals

In order to elucidate the effect of the chorion as a barrier, the chorion of 6 h old zebrafish embryos (pre-exposed from 30 min post fertilization) was softened by incubation in a 2 mg/l pronase solution (protease from *Streptomyces griseus*, Westerfield, 2000) with an activity of 4 units per mg in dilution water for 1 ± 0.5 min at 28.5° C and mechanically disrupted by means of two pairs of forceps or dissection needles. Control experiments were carried out to ensure that pronase treatment did not have any effect on embryonic development. Since the optimal incubation time depends on the developmental stage, the optimal duration should be checked in range-finding experiments. Without enzymatic digestion, the mechanical stress frequently resulted in destruction of the embryo. Disruption of the chorion without

enzymatic softening of the egg shell was only possible in embryos > 48 h. Dechorionated embryos were incubated in 24-well microtiter plates and exposed to potassium chromate (hydrophilic; Fig. 12), 4-chloroaniline (moderately lipophilic; Fig. 13) and lindane (lipophilic) and compared to non-dechorionated embryos exposed under similar conditions. In order to avoid excessive adsorption, the wells had been pre-incubated with toxicant 24 h prior to addition of the eggs.

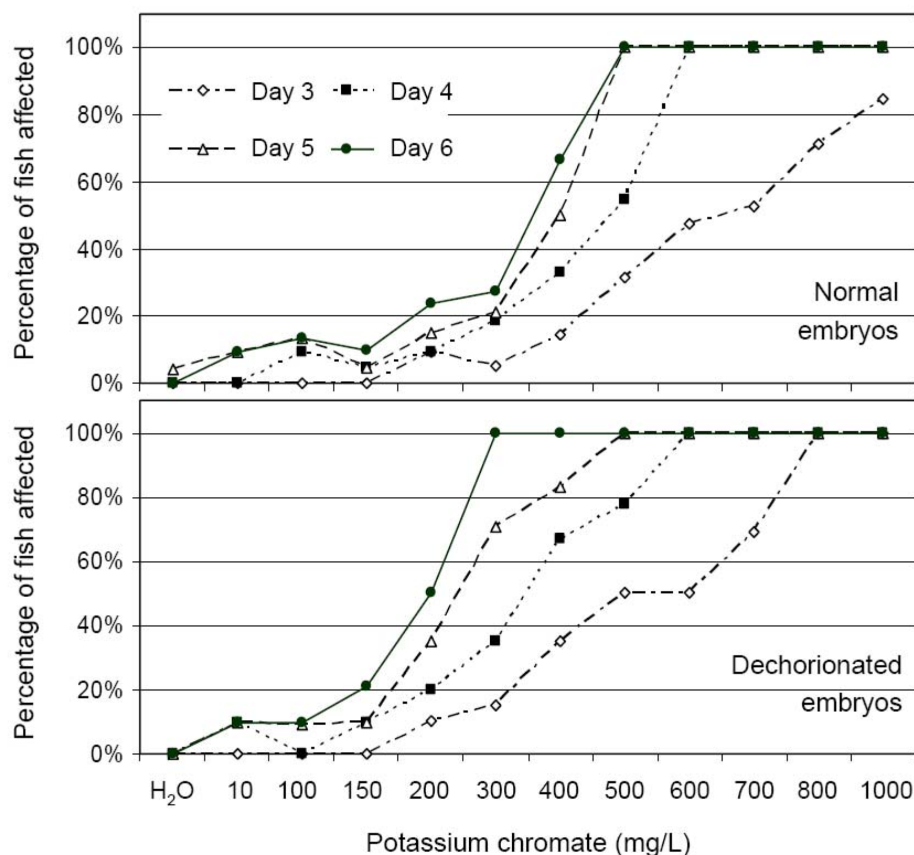


Fig. 12: Effects of potassium chromate to zebrafish (*Danio rerio*) embryos depending on the presence of a chorion (upper panel) or after dechorionation (lower panel) on the basis of disturbances of the equilibrium after various periods of exposure. For potassium chromate, dechorionation does not affect acute embryo toxicity, but clearly shows sublethal effects (Braunbeck et al., 2005).

Whereas exposure to the relatively hydrophilic potassium chromate did not result in any change of the core endpoints of the embryo toxicity test (details not shown), prolonged exposure of dechorionated embryos over 4 days (i.e., until hatching) produced severe disturbances to swimming equilibrium in hatched larvae (Fig. 12), thus indicating that the chorion did act at least as some form of barrier, even for hydrophilic substances. In contrast, for 4-chloroaniline, a significant increase in toxicity could already be recorded for the core endpoints of the fish embryo test as defined by the current DIN standards (Fig. 13). This increase in toxicity is even more pronounced for more lipophilic substances such as lindane: Whereas the EC_{50} value of lindane for normal embryos could be identified as 26.5 mg/l, the corresponding value for dechorionated embryos is 11.3 mg/L. Albeit significant, this difference in lindane embryo toxicity becomes relative when compared with the broad range of acute conventional (*in vivo*) fish toxicity from 2 mg/L in bluegill sunfish over 12 mg/L in fathead minnow, 14 mg/L in rainbow trout (*Oncorhynchus mykiss*) and 23 mg/L in golden ide (*Leuciscus idus melanotus*) to 26 mg/L in guppy (*Poecilia reticulata*; Verschueren, 1983). Nevertheless, results indicate that the barrier function of the chorion may increase with lipophilicity, a fact

that should be taken into consideration in the interpretation of correlations between fish embryo and conventional acute fish toxicity (Braunbeck et al., 2005).

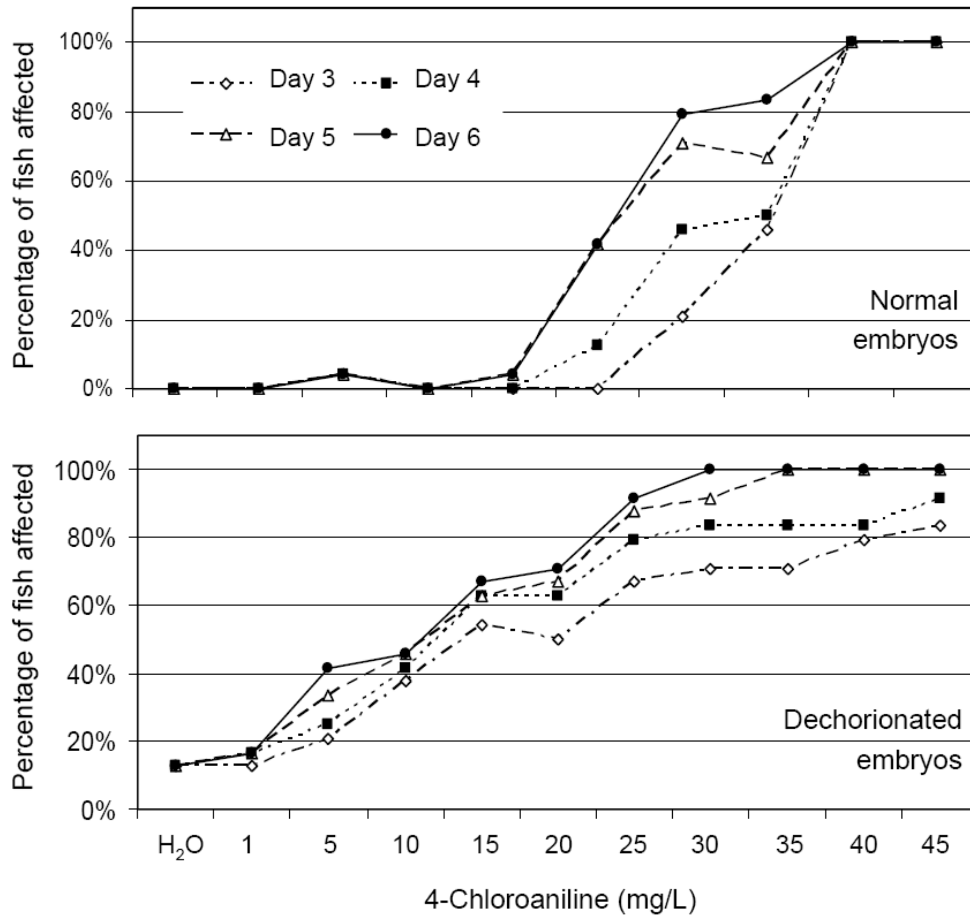


Fig. 13: Toxicity of 4-chloroaniline to zebrafish (*Danio rerio*) embryos depending on the presence of a chorion (upper panel) or after dechorionation (lower panel) based on acutely toxic effects as specified by German DIN (DIN, 2001) standards after various periods of exposure. For 4-chloroaniline, dechorionation results in an increase of acute embryo toxicity (Braunbeck et al., 2005)

3.1.2 Fathead minnow

The fathead minnow (*Pimephales promelas*, Rafinesque, 1820) is another demersal cyprinid species originating from the temperate waters of central North America (Page and Burr, 1991); it inhabits muddy pools of headwaters, creeks and small rivers. Among the three primary OECD species, fathead minnow is most likely the one with the largest toxicological database (Ankley et al., 2001; Gray et al., 2002; Keddy et al., 1995; Miracle et al., 2003; Sinks and Schultz, 2001).

Whereas females grow up to 1.5 ± 0.3 g, males may reach 2.5 ± 0.5 g in weight. Water quality parameters are essentially identical to those described for zebrafish; for water details of maintenance, see Table 1. In order to prevent permanent stress, parental fathead minnow can be maintained at temperatures of approx. 16.5 ± 1.5 °C ("winter conditions"); prior to spawning, the temperature should then be gradually raised to 24 °C at a rate of 1 - 2 °C/day, and fish should be given increasing ratios of live food (Table 1). During spawning, dominant males are not only considerably larger than females, but are also characterized by a pronounced black coloration of their heads, a thick dorsal nuptial pad and nuptial tubercles (Fig. 14), as well as conspicuous vertical black bands along body sides. In contrast, spawning females can easily be identified by an ovipositor. For optimal spawning, fathead minnow should be between 6 and 12 months of age; spawning can be stimulated by means of extra rations of brine shrimp.

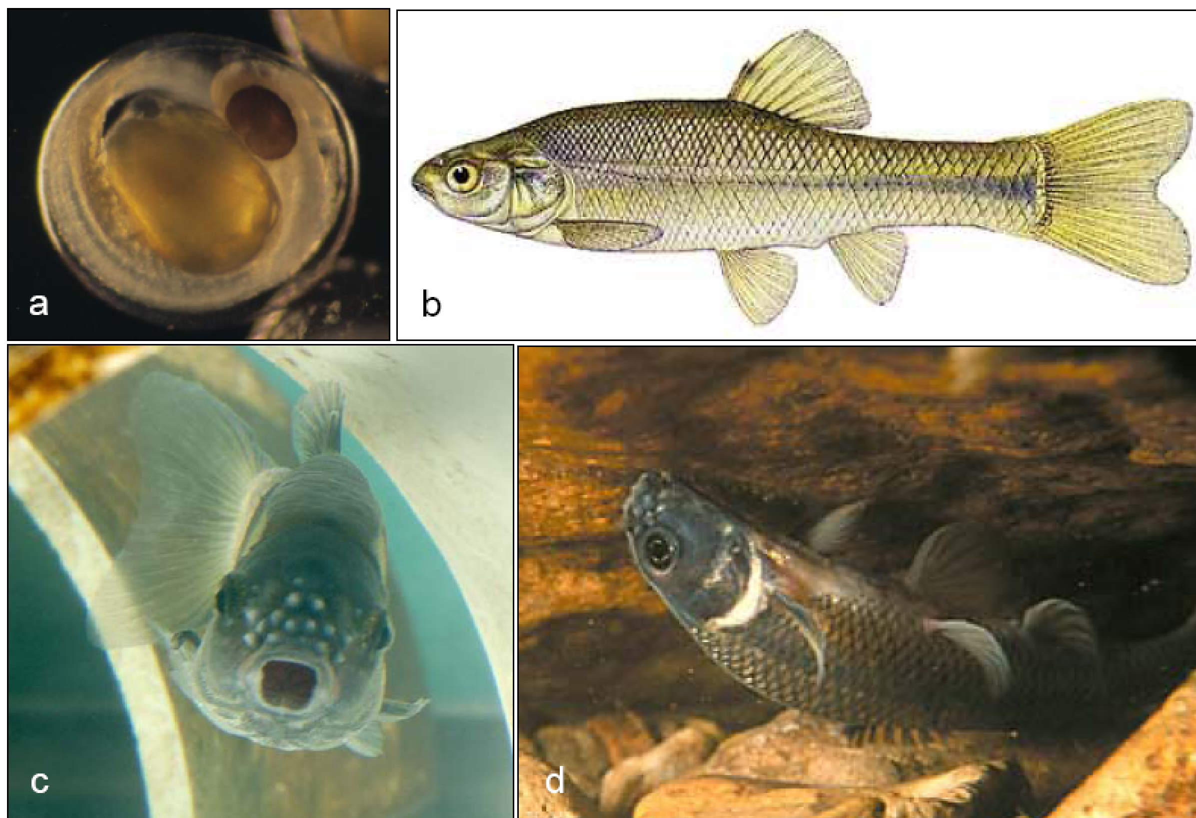


Fig. 14: Fathead minnow (*Pimephales promelas*): (a) newly hatched embryo; (b) typical female minnow; (c, d) male fathead minnow are characterized by pronounced nuptial tubercles on the snout (from various www-sources).

For spawning, fathead minnow are normally kept in small groups of two males and four females in tanks of about 30 L volumes. Since fathead minnow are quite sensitive to disturbance during spawning, the spawning tanks should be kept under quiet conditions. As soon as, e.g., a clay tile divided into two halves is added to each spawning group as a spawning ground, male fathead minnow build up individual territories with the tiles as their centers. During the spawning period, the tiles should be inspected for eggs at intervals of at latest 60 minutes post fertilization. Usually, 100 - 250 eggs are laid per spawning act. Since the chorion only hardens at the first multicellular stages, the transparent, sticky eggs should be given about two hours, before they are removed and directly transferred to 6- or 24-well plates by means of a flexible forceps.

The development of fathead minnow is basically similar to that of zebrafish (Manner and Dewese, 1974); at least partly due to the lower temperature, however, the time schedule of development is slightly slower (Fig. 15; cf. Table 1).

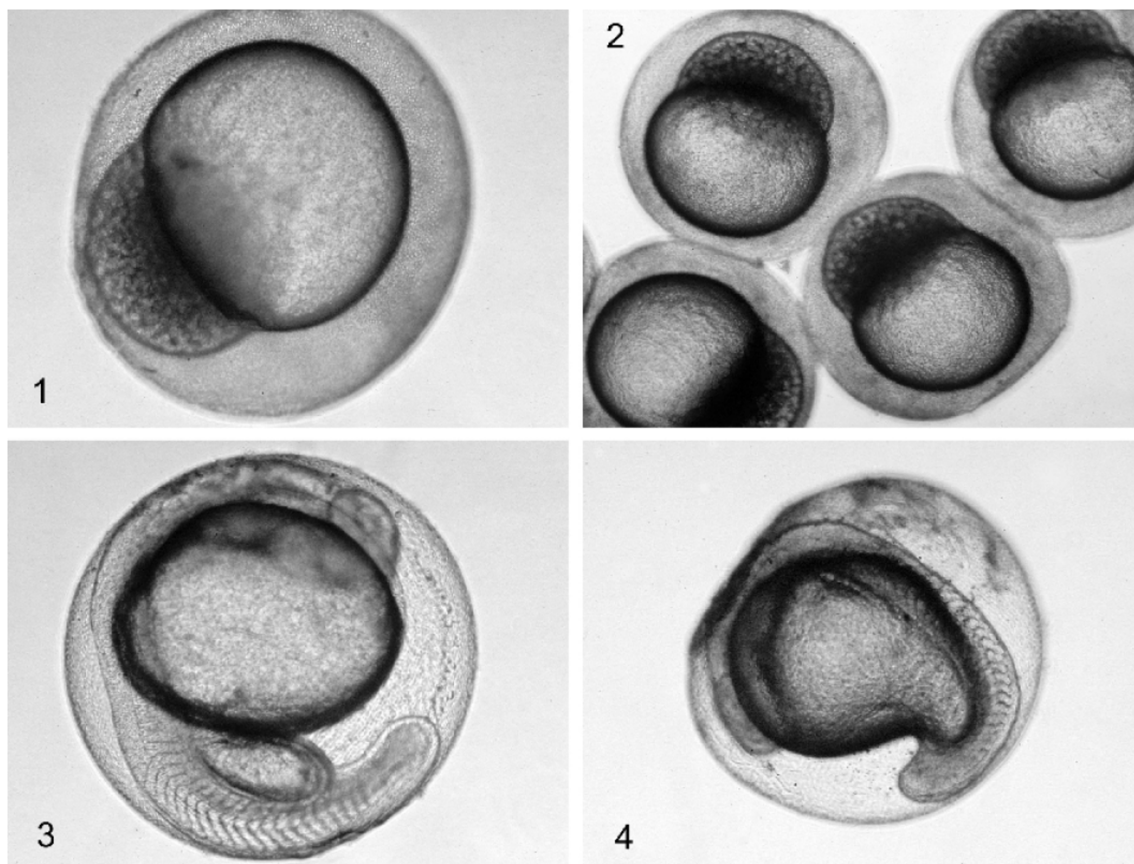


Fig. 15: Normal development of fathead minnow (*Pimephales promelas*) embryos (1 - 3) in comparison to fathead minnow embryos exposed to 15 mg/L 3,4-dichloroaniline (4): (1, 2) 2 h post-fertilization, normal development; (3) 30 h post-fertilization, normal development: tail has detached from yolk sac, and somites are clearly detectable; (4) following exposure to 15 mg/L 3,4-dichloroaniline, the entire embryo appears deformed, and the tail has not yet detached from the yolk sac.

3.1.3 Japanese medaka

Similar to zebrafish, the Japanese medaka (*Oryzias latipes*, Temminck & Schlegel, 1846) has become a favorite model in developmental biology and molecular genetics (Furutani-Seikia and Wittbrod, 2004; Winn, 2002; Wittbrod et al., 2001) as well as in ecotoxicology (Arcand-Hoy and Benson, 1998; De Koven et al., 1992; Hatanaka et al., 1982; Ishikawa et al., 1984; Seki et al., 2002, 2003). The medaka belongs to the ricefishes (Adrianichthyidae, Huber, 1996; Ishizaki, 1994) within the order of the Beloniformes and originates from Japan, China, Vietnam and South Korea (Jordan and Snyder, 1906; Shima and Mitani, 2004). In its original habitats, the medaka is severely threatened.

The biology of the medaka is well documented (Hyodo-Taguchi and Egami, 1985; Kirchen and West, 1976; Yamamoto, 1975), and its requirements with respect to maintenance are comparable to those of zebrafish; in fact, both species can easily be raised side by side in one aquatic system (Furutani-Seikia and Wittbrod, 2004). Most of the standard experimental procedures can be applied to both species with slight modifications, including the observation of embryos, gynogenesis, sperm freezing and *in vitro* fertilization, cell transplantation, as well as RNA and DNA injection, *in situ* hybridization using riboprobes and immunohistochemistry. Once dechorionated, handling of the softer Medaka embryos requires some practice. Medaka embryos tolerate a wide temperature range: 4 - 35 °C until the onset of heart beating and 18 - 35 °C thereafter, compared to 25 - 33 °C in the case of zebrafish (Westerfield, 2000).

Since the original habitats of the Japanese medaka are still waters, for optimal maintenance care should be taken to avoid excessive turbulence. Water characteristics are basically identical to those described above for zebrafish. E.g., up to 60 individuals of either sex can be kept in 50 L tanks at an ambient temperature of 24 ± 0.5 °C (Table 1). Whereas during the spawning period females (0.35 ± 0.07 g) can readily be identified by a generally more plump body shape and the sticky eggs attached to the anal fins (Fig. 16), male individuals (0.35 ± 0.07 g) are characterized by larger anal fins and the so-called papillary processes on posterior dorsal fin rays. For optimal stimulation of spawning, 5 to 12 months old medaka should be at best be kept under normal daylight conditions with extra ratios of frozen adult brine shrimp (*Artemia* spec.). Moreover, spawning success can be further improved by means of natural plants (*Ceratophyllum* spec.) in the aquaria. The female medaka spawns between 20 and 40 eggs every day within an hour after the onset of light. Again, care should be taken to select a suitable strain (cf. Wakamatsu and Ozato, 2002).

Fig. 16: Japanese medaka (*Oryzias latipes*; from various www-sources).

Medaka eggs measure about 1 mm in diameter; they are transparent and characterized by an orange color and conspicuous spiny hooks, which allow firm adhesion to the anal fin of the female (Figs. 17, 18), but – to some degree – reduce the visibility of malformations during development. On the other hand, the egg clutch attached allows instant identification of reproductively active females. If required, attachment filaments on the chorion can be easily removed by rolling eggs on a piece of Whatman filter paper or by proteinase K digestion. Since the medaka chorion is quite stable, the eggs can be removed from the anal fin as soon as 30 minutes post fertilization and directly transferred to 6- or 24-well plates by means of thin metal wire loop.

If compared to zebrafish, the development of medaka is slightly slower; normally developing embryos only hatch after approx. 7 days of incubation. Developmental stages and corresponding morphological characteristics have been described in detail by Iwamatsu (1994, 2004; see Annex 1); an in-depth comparison of developmental staging with zebrafish has been published by Furutani-Seikia and Wittbrod (2004). Representative figures of developmental stages of Japanese medaka are given in Figs. 19a - d. As also outlined in Table 1, the time schedule for the toxicological endpoints needs to be modified for the medaka:

(1) After 30 h, the somites 3 to 10 have been developed. The optic vesicles are clearly datable.
(2) After 54 h, the eye development allows the differentiation of iris and pupils. Heartbeat is visible, but blood circulation is not yet regular.
(3) After 78 h, blood circulation is regular (can best be seen on yolk vessels). Occasionally, spontaneous tail movements can be recorded. In the cranial area, some pigment cells are visible. Tail tip has detached from the yolk.
(4) After 4 d, pectoral fins are detectable. Tail tip reaches eyes and moves regularly; about 30 % of the tail are detached from the yolk.
(5) After 5 d, a regular row of pigment cells along the tail are clearly discernable. Pectoral fins actively moving. Tail has detached completely.
(6) After 6 d, when bent forward, the tail tip appears longer than head. Increasing number of pigment cells.
(7) After 7 d, the medaka embryo hatches.

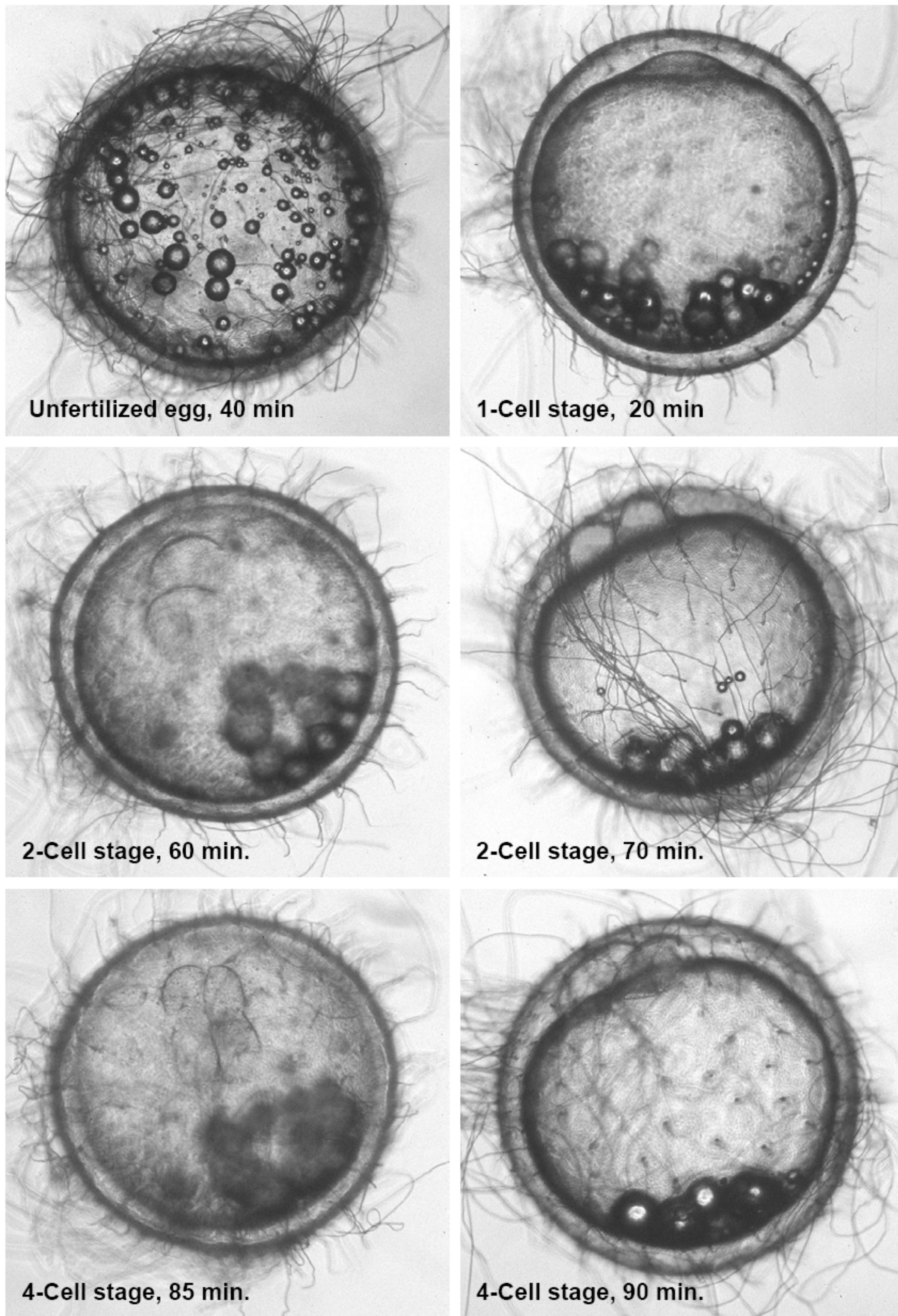


Fig. 17a: Normal development of Japanese medaka (*Oryzias latipes*) embryos I: from unfertilized eggs to the 4-cell stage (90 min).