Artificial stripping and embryonic development of the common gudgeon (*Gobio gobio* L.) and its use in embryo-larval tests – a pilot study

M. Palíková[1], R. Krejčí[2]

[1]Department of Veterinary Ecology and Environmental Protection, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
[2]Department of Fishery and Hydrobiology, Mendel University of Agriculture and Forestry, Brno, Czech Republic

ABSTRACT: Artificial reproduction with and without hormonal treatment was performed in females of the common gudgeon (*Gobio gobio* L.) to assess the suitability of this cyprinid species as a fish model for embryo-larval cytotoxic tests. Considering sperm immobilisation tests, the solution for tench was utilizable. The clearing solution designed for the observation of embryonic development in the European catfish was also found suitable for the common gudgeon embryos. The egg and embryonic mortality, embryonic development, hatching and survival rate were examined. Ovulation occurred in 89% of females after the application of the carp pituitary (5 mg/kg) but embryonic mortality reached nearly 100%. The highest embryonic mortality in females with hormonal treatment was observed within 24 hours of development. On the other hand, the reproduction of females without the application of hormonal stimulation was nearly completely successful with mean mortality 60%. The hatching of embryos of females without hormonal stimulation occurred (average values) 71 hours after fertilization and lasted 65 hours. Embryonic development finished within 176 hours. Our pilot study demonstrated that the eggs of common gudgeon were suitable for embryo-larval tests from the 7th to the 8th embryonic developmental stage.

Keywords: hormonal stimulation; mortality; embryonic development; hatching; survival

Various aspects of embryonic, larval and juvenile development in the common gudgeon (*Gobio gobio* L.)* as well as hatching and the use of hormonal stimulation of spawning were already described by Peňáz and Prokeš (1978). Prokeš and Peňáz (1979) also published a description of the larval and juvenile period. Kouřil et al. (2000) studied hormonally induced ovulation in gudgeon females using the carp pituitary and GnRH analogue. Similarly, Kestemont (1988) carried out artificial stripping in the gudgeon. Data on the rate of fertilization, unsticking of fish eggs and subsequent rearing of the gudgeon are, however, missing.

The aim of this study was to assess the suitability of common gudgeon in embryo-larval tests. Even though OECD (1992 or 1998) methods 210 and 212 do not mention the gudgeon as a model species for embryonic and embryo-larval tests of toxicity, the importance of its use lies in cytogenetic studies evaluating chromosomal aberrations. Regarding the relatively large chromosomes (2*n* = 50, Ráb and Collares-Pereira, 1995) and the

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* As presently recognized (Kottelat and Persat, 2004), the nominal term *G. gobio* is a "catch-all" collective group of morphologically similar species. *G. gobio* inhabits rivers feeding into the Channel, North and Baltic Sea only. The diversity of "common" gudgeons in the Danube basin is not well understood.

broad geographic distribution, the gudgeon (*Gobio gobio* L.) is a suitable species for cytogenetic studies of chromosomal changes.

MATERIAL AND METHODS

Parent fish were collected in two locations in the Svratka and Svitava river (Danube River basin) using an electric aggregate unit during early June.

1. Fish from the first group were used to test immobilization solutions for sperm collection and the method of unsticking the eggs. Sperm was collected into immobilization solutions for silurid fish (8 g NaCl, 5 g KCl, 10 g glycine per litre of water) and for tench (5 g NaCl, 2 g KCl, 10 g glycine per litre of water), (Linhart et al., 2000). We evaluated spermatozoal motility using light microscopy in a qualified survey after adding Ringer's fertilization solution. We used Ringer's fertilization solution (Linhart, 1985), (6 g NaCl, 0.075 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.1 g $NaHCO_3$/l water) to activate the sperm collected into immobilization solutions. Eggs were unsticky using alcalase (Alcalase, Merck EC 3.4.21.14.) in the concentration of 6 ml/l of water together with 1 g of NaCl for 3 min (Linhart et al., 2000), milk diluted at the ratio of 1 to 3 for 2 h, talc in the concentration of 20 g/l of water together with 1 g NaCl for 2.5 h and milk diluted at the ratio of 1 to 3 for 40 min with the subsequent use of talc in the concentration of 20 g/l of water together with 1 g NaCl for 20 min.

2. Fish from the second group were used for artificial stripping and egg incubation. Fish eggs were incubated at 21 ± 0.3°C in incubation Zug jars of 1 l with water recirculation. Fish eggs from individual breeder females were incubated separately. Females releasing fish eggs spontaneously (3 individuals) after transport and calming down were stripped immediately, other females (9 individuals) were treated intraperitoneally with a single dose of the carp pituitary (5 mg per 1 kg of body weight). Fish eggs from nine females were used to incubate in the recirculation system, fish eggs from individual females being set for incubation in vials separately. Fish eggs from two females were not incubated due to poor quality. One female did not ovulate. No such treatment was performed in males. Sperm was collected into immobilization solutions for tench and stored for a short period (up to 3 hours) at 4°C. Ringer's fertilization solution was used to activate the sperm. Fish eggs were unsticked enzymatically using alcalase for three minutes. The size and mass of fish eggs after stripping and swelling were determined.

Regarding the opacity of egg envelopes it was necessary to use clearing solutions for making them transparent and suitable for embryogenetic studies. Two kinds of clearing solutions were used to study the development by light microscopy – the first solution was composed of 60 ml of ethanol, 30 ml of 36–38% formaldehyde and 10 ml of acetic acid, the second one was composed of 5 ml of acetic acid per 100 ml of saline (Linhart et al., 2000).

RESULTS AND DISCUSSION

Testing a suitable immobilization solution, methods of unsticking and clearing the eggs

Probably due to contamination by urine, spermatozoal motility was observed in the collected sperm without immobilization solutions. No motility was found in the sperm collected into both immobilization solutions. The motility of the sperm collected into the immobilization solution for silurid fish was visually lower than that in the immobilization solution for tench.

The use of alcalase led to nearly 100% egg unsticking. Lower efficacy was reached for the combination of whole milk and talc subsequently (80–90%) and then whole milk only (10–70%). The lowest

Table 1. Mean reproductive parameters of females (mean ± SD)

	Hormonally untreated (n = 3)	Hormonally treated (n = 9)
Body weight (g)	13.4 ± 3.6	19.9 ± 7.9
Gonad mass (g)	1.1 ± 0.3	0.8 ± 0.4
Number of eggs per female	1589 ± 460	822 ± 551
Number of eggs per g of body weight	117.8 ± 14.7	65.6 ± 33.2
Percentage of stripping	100	89
Percentage of hatching	40.0 ± 21.6	0.6 ± 1.7

efficacy of egg unsticking was found out after the application of talc only (5%). The method with alcalase was selected for further use because it provided good results of unsticking, time consumption and duration of egg handling.

Fish eggs and embryos were made transparent for only about 2 minutes when the second clearing solution was used. After this short time the embryos started to get darker and it was not possible to distinguish individual structures. The use of the second solution resulted in no changes in the opacity of fish eggs thus not making the embryo clearly visible.

Artificial stripping and studies of embryonic development

Table 1 presents the mean reproductive parameters of females. A total of eight females representing 89% of all hormonally treated females ovulated within 12 hours.

Fish eggs from four females showed 100% mortality within 15 hours. Mortality in other cases amounted up to 10% within the first 15 hours and started to get higher at the eyed egg stage and during hatching. Fish eggs from two females had high, nearly 100% mortality, due to degeneration after the eyed egg stage.

By hormonal stimulation we achieved a higher percentage of ovulating females than reported by Kouřil et al. (2000). The hatching of low numbers of larvae occurred only in one case. Fish eggs were probably collected in an inappropriate stage of oogenesis. The administration of the pituitary probably resulted in a release of eggs unable to develop or fertilise or showing a high mortality of eyed embryos. Females without hormonal treatment provided eggs with a higher percentage of hatching (10–60%) and the ovulated oocytes were suitable for fertilization and further development.

The hatching of eggs from females not treated hormonally started on average 71 hours after fertilization with the range of 66 to 79 hours. Most eggs hatched within 39 hours from the start of hatching. All embryos hatched within 136 hours after fertilization. The hatching of fish eggs from

Table 2. Comparison of the length of individual stages of embryonic development

Stage of embryonic development	Period (h) Interval in hours after fertilisation, our study Mean water temperature 21°C	Period (h) Interval (h after fertilisation) according to Peňáz and Prokeš (1978) Mean water temperature 19.5°C
Development of perivitelline space and blastodisk	1 0–1	1 0–1
Cleavage	6 2–7	6 2–7
Blastula	5 8–12	6 8–13
Gastrulation	11 13–23	12 14–25
Origin of head and trunk organs	9 24–32	15 25–40
Completion of trunk segmentation, development of tail section, rudimentary pectoral fins	13 33–45	35 40–75
Completion of the tail section, onset of eye pigment, separation of the anterior part of head from yolk sac	26 46–71	30 72–105
Separation of head from yolk sac, development of 1st melanophores on body surface, hatching	38 72–110	30 102–135
Development of air bladder, transition to active life habits	66 110–176	45 132–180

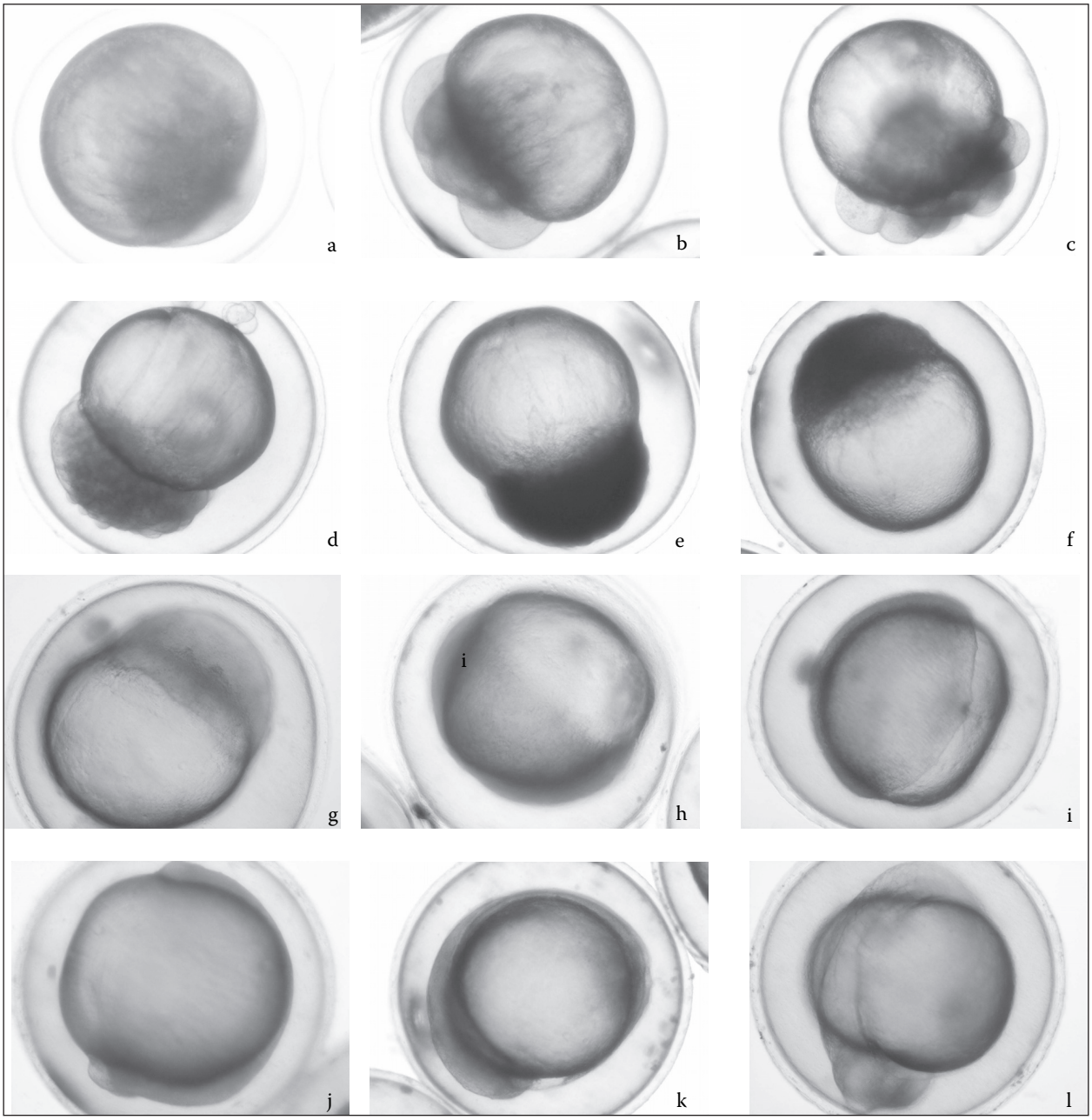

Figure 1. 1 h after fertilization – development of the blastodisk (a), 2 h after fertilization – 4 blastomeres (b), 3 h after fertilization – 16 blastomeres, 4 h, 5 h and 6 h after fertilization – morula (d–f), 9 h after fertilization – blastula (g), 12 h and 13 h and 16.5 h after fertilization – gastrula (h–j), 24 h and 27 h after fertilization – embryo (k–l)

hormonally treated females started after 97 hours and ended within 145 hours. As compared to females not treated hormonally the onset of hatching was 26 hours later and the process of hatching was finished some 9 hours later.

Comparing our results with those of Peňáz and Prokeš (1978), the hatching of eggs from females not treated hormonally started earlier (71 hours after fertilization compared to 109 hours) and finished earlier (136 hours after fertilization compared to 164 hours). The hatching of 90% of eggs occurred within 110 h after fertilization compared to 131 h and the incubation duration in daily degrees was 119 D.D. instead of 133.4 D.D. This acceleration was caused by the higher temperature of incubation (21°C instead of 19.5°C) and conditions of incubation (flow-through system instead of Petri dishes). Table 2 presents the comparison of duration of individual developmental stages given by Peňáz and Prokeš (1978).

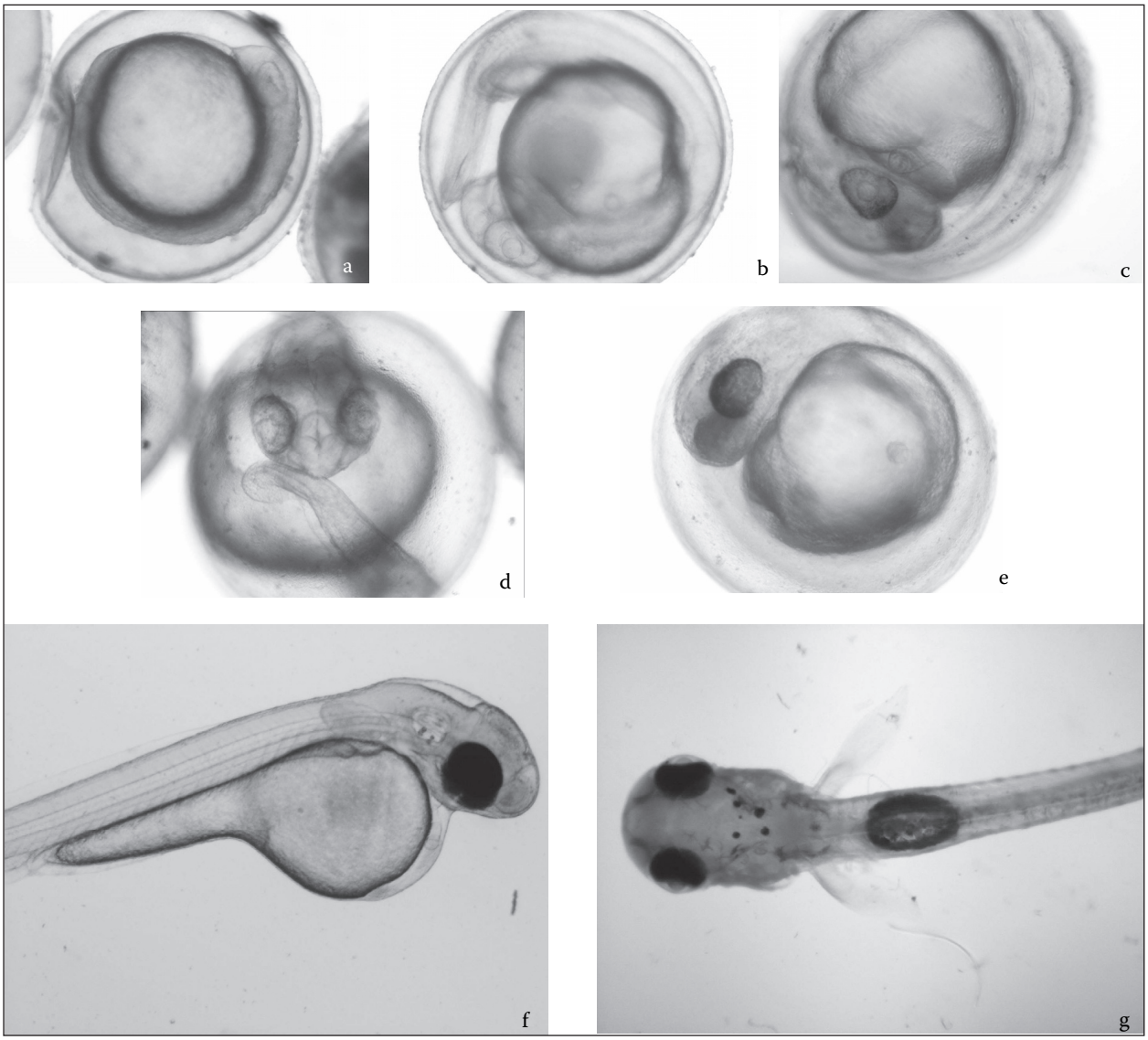

Figure 2. Embryos 29.5 h, 45 h, 51 h, 56.5 h and 63 h after fertilization (a–e), embryo after hatching (f) and embryo with the air bladder filled (g)

Comparison of our data and those of Peňáz and Prokeš (1978) showed that the embryonic development was faster in the second half of its duration, especially from the stage of gastrula, while in the early phase our data are compatible. Contrary to this, the late stages of development (after hatching, in particular) lasted for a longer time, so that the air bladder got filled in approximately the same period. Figure 1 shows the embryonic development and its individual stages. Figure 2 demonstrates older embryos, an embryo after hatching and another one with the filled air bladder. Figure 3 shows the occurrence of various malformations of the embryonic development. Apart from changes during the first hours of development such as irregular cleavage, damage to the outer envelope of the egg, cracking of the yolk membrane, there were numerous deformed embryos later in the development, i.e. in the stage of eye pigmentation.

Unfertilized and not swollen fish eggs measured 1.24 mm on average, with the range from 1.17 to 1.32 mm, and their mass averaged to 0.70 mg (0.67 to 0.78). Fertilized fish eggs in a swollen state had 1.51 mm (1.42–1.62) in diameter and their mass was 1.48 mg on average (1.22–1.87).

Embryos had the filled air bladder within 176 hours and were able to take up feeds.

It can be concluded that the gudgeon may be used as an experimental model for embryo-larval tests from the 7^{th} –8^{th} embryonic stage. For the methods

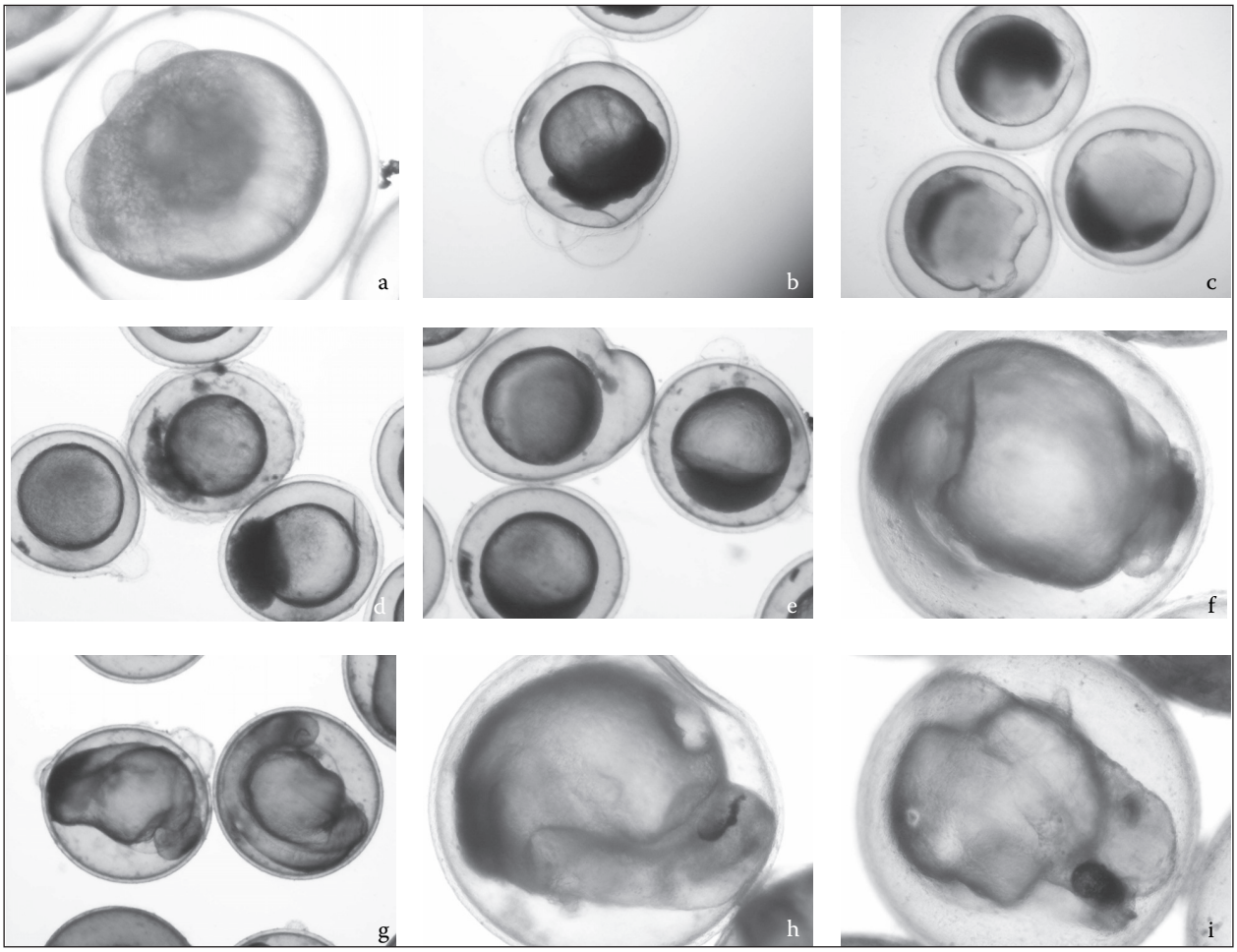

Figure 3. Developmental anomalies: 2 h after fertilization – irregular cleavage (a), 4 h after fertilization – "buble-like" structures on the outer envelope of the fish egg (b), 8.5 h – cracking of the yolk membrane (c), 10.5 h – "wave-like" irregularities on the outer envelope of the fish egg (d), 12 h – "swelling" (e), 54 h, 55.5 h, 59.5 h and 71 h – degenerated embryos at the stage of eye spots (f–i)

of embryonic and embryo-larval tests of toxicity it is required that the mortality in control groups be up to 10%. Because it is necessary to use a clearing solution for the study of development (fertilization), it is not possible to distinguish fertilized and developing eggs from unfertilized ones without killing the embryo. That is why the gudgeon can be used only from the 7th to the 8th stage of development of noticeable eyed eggs. High mortality would otherwise distort the results of these tests.

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Corresponding Author

MVDr. Miroslava Palíková, PhD., Department of Veterinary Ecology and Environmental Protection, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, Brno, Czech Republic
Tel. +420 541 562 654, e-mail: palikovam@vfu.cz