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NOTE

Survival rates and the occurrence of larval malformations, including Siamese twins, following fertilization of post-ovulatory aged oocytes in ide *Leuciscus idus*

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ABSTRACT: Embryonic development of lower vertebrates can be influenced by many factors, especially when outside of the mother's body and under the influence of variable abiotic and biotic factors. In this study, the effects of fertilization of post-ovulatory aged (overmature) oocytes of ide *Leuciscus idus* on the risk of developing anomalies in ide larvae were assessed under controlled conditions. There was a negative effect of delayed fertilization of post-ovulatory aged (overmature) oocytes in the ovary on the quality of offspring, which caused reduced survival 3 days post-fertilization and at the hatching stage (68.3 vs. 81.2% and 47.2 vs. 79.7% for the overmature and mature groups, respectively). It also increased the occurrence of numerous developmental defects (19.3 vs. 0.8% for the overmature and mature groups, respectively), including the development of Siamese twins. This is the first reported case of the occurrence of Siamese twins in ide. The ide Siamese twins look like hen eggs with 2 heads and are different from 'typical' Siamese twins in fish.

KEY WORDS: Anomalies · Siamese twins · Artificial reproduction · Fertilization · Ide

INTRODUCTION

Progressive water eutrophication and anthropopression have contributed to a reduction in the number of naturally occurring fish populations in the environment. Therefore, the main goal of conservative aquaculture is to restore or enhance the naturally occurring fish population, most often through restocking. The material to be stocked should be of high quality, condition and health. Animals that are in poor condition or deformed are susceptible not only to diseases, but also to becoming easy victims of a potential predator (Nowosad & Kucharczyk 2017). The main bottleneck of commercial fish production is artificial reproduction, because its success is influenced by the quality of gametes, embryos and larvae. When using artificial reproduction of fish for restocking purposes, the ability to obtain high-quality gametes and to appropriately carry out fertilization and incubation of eggs is of key importance (Kucharczyk et al. 2016a, Nowosad et al. 2016).

Numerous scientific studies have reported that increased mortality and the occurrence of numerous deformities in newly hatched fish larvae are caused by inadequate or sublethal conditions during the development of eggs, such as too low or too high temperature, too low or too high pH or low oxygen content (Jezierska et al. 2000, Kupren et al. 2011).

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However, the major cause of deformation in larvae is exposure to toxic substances, including heavy metals, such as lead (Pb) or copper (Cu) (Jezierska et al. 2000), and petroleum derivatives (Sisman et al. 2016). Therefore, most studies on the occurrence of this phenomenon in larvae involve testing different concentrations of heavy metals or other toxic substances on the eggs.

In addition to abiotic factors, biotic factors can also affect the quality of gametes, embryos and larvae. Unfortunately, artificial reproduction under controlled conditions can be complicated by the overmaturing of eggs (often referred to as 'egg-ageing' in the literature), e.g. due to asynchronous ovulation (Samarin et al. 2015). These studies show that fertilization of overmature eggs causes not only deformation in the offspring, but also a change in ploidy level (Samarin et al. 2017). Such research, targeted at egg ageing and embryo development, has not yet been conducted in cyprinid fishes. The purpose of this study was to determine the effect of fertilization of mature and overmature eggs of the cyprinid fish species ide *Leuciscus idus* L. on the occurrence of developmental anomalies in offspring.

MATERIALS AND METHODS

The breeders (a wild-coloured form of ide *Leuciscus idus*, n = 22: 12 females and 10 males) originated from the Czarci Jar Fish Farm (north-eastern Poland), where they were kept in natural ponds (cultured generation F_1). Just before spawning (water temperature: 8°C), they were transported in oxygen bags to the Department of Lake and River Fisheries (University of Warmia and Mazury in Olsztyn). The average weight (±SD) of the fish was 943.43 ± 68.9 g. Artificial reproduction was carried out using the method described by Targońska et al. (2012) with a modification of the fertilization process as described by Kucharczyk et al. (2016a). After transport, the breeders were kept in 1 m^3 tanks at 10°C. Fish from both sexes were stimulated hormonally with an intraperitoneal injection of Ovopel (mGnRH + metoclopramide, Unic-trade) at the base of the pelvic fin. The first dose was 0.2 pellet kg^{-1} of body weight (1 pellet of Ovopel contains 18 to 20 g mammalian analogue of GnRH and 8 to 10 mg metoclopramide; Horvath et al. 1997) at 10°C. After 24 h, the second injection was performed with the administration of 1 pellet kg^{-1} of body weight. The pellets were pulverized before application and then dissolved in saline solution (0.9% sterile NaCl). After the second injection, the water temperature was raised to 12.5°C. Fish were first checked 32 h after the second injection and then at 2 h intervals. The sperm was collected separately from the males with sterile syringes, while care was taken to prevent contamination with urine, faeces or blood. The oocytes were collected twice from the same females (n = 7): shortly after ovulation (T_0) and 8 h after the first stripping (T_8). Between oocyte collections, the water temperature was a constant 12.5°C.

All manipulations of fish were made under the influence of anaesthesia using a MS-222 solution (150 ppm, Argent). Oocytes were fertilized for 10 min after their collection. The eggs collected from 1 female were fertilized with semen collected from a minimum of 3 males (each sperm motility above 70%). The sperm was added to eggs 3 times at 0, 30 and 60s after adding water (Kucharczyk et al. 2016a). The fertilized eggs were incubated in Weiss jars at 12.5°C, which is optimal for the embryonic development of this species (Kupren et al. 2011) until hatching. The larvae hatched over 15 h. After hatching, the survival (hatching) rate was analysed. From each group of incubated eggs, 3 subsamples (about 300 eggs each) were taken for analysis of embryo survival (at 3 days post-fertilization: live embryos × 100% / all embryos), hatching (hatched embryos × 100% / all eggs) and abnormal embryo (abnormal embryos × 100% / all hatched embryos) rates.

Larval deformities analysis

Deformation analysis of larvae was performed using a Leica MZ16 A stereomicroscope and a digital colour camera with 5 MP resolution for Leica DFC420 Image Analysis.

Data analyses

Data are presented as mean ± SD. The data were analysed using a *t*-test ($\alpha = 0.05$). The statistical analysis was conducted with Microsoft Excel and Statistica v. 12.5 (StatSoft).

RESULTS

The survival rate of mature eggs (collected shortly after ovulation) during the hatching stage was >79%, whereas that of overmature groups was between 45.1 and 49.3% (Table 1). After fertilization of aged

Table 1. Survival, hatching and abnormal larvae rates (mean ± SD) following incubation of mature and overmature ide *Leuciscus idus* eggs. The survival and hatching rates are given as percentages of all eggs, while the abnormal larvae rate is a percentage of all hatched larvae. Data marked with different letters in rows differ significantly (*t*-test; α = 0.05)

	Mature eggs	Overmature eggs
Survival rate (3 day post-fertilization)(%)	81.2 ± 2.1[a]	68.3 ± 2.0[b]
Hatching rate (%)	79.7 ± 2.1[a]	47.2 ± 2.1[b]
Abnormal (deformed) larvae rate (%)	0.8 ± 0.2[a]	19.3 ± 2.5[b]

overmature eggs, about 20% of the larvae showed macroscopical anomalies, whereas the developmental deviation of the offspring from the mature eggs did not exceed 1%. In all cases, significant differences between the groups were observed ($p < 0.05$). The total body length of hatched larvae with developmental anomalies was up to 5 times shorter than that of normally developing larvae at the same age (0 days post-hatching; Fig. 1). In spite of the various deformities, deformed individuals were strong and hatched spontaneously. Beating of hearts and blood flow through blood vessels were observed in all abnormal larvae. Siamese twins were also found for the first time in ide, with 2 heads and 1 (Figs. 2 & 3C) or 2 hearts (Fig. 3B, right).

The Siamese twin ide, in comparison with normal larvae (Fig. 3A), had a spherical balloon body shape (like a ball) and lacked a spine and tail (Fig. 2). In extreme cases, they had no eyes (Fig. 3B). Most deformed individuals displayed kyphosis (Fig. 3D) and lordosis (Fig. 3E,F). In some specimens, ocular lenses were lacking or single eyes occurred (Fig. 3E). In addition, many larvae were found to have cardiac oedema (Fig. 3D–F) and an irregularly shaped yolk sac (Fig. 3B–E).

Fig. 1. Normal and deformed ide *Leuciscus idus* larvae of the same age (0 days post-hatching)

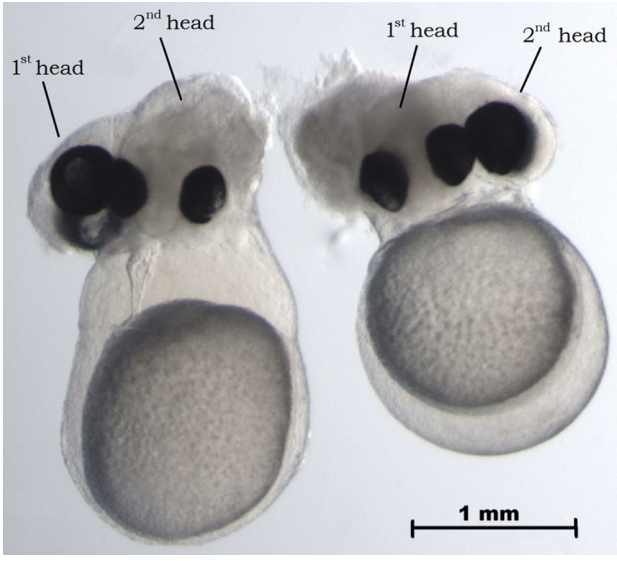

Fig. 2. Siamese twins of ide *Leuciscus idus* larvae on the first day after hatching (0 days post-hatching)

DISCUSSION

In this study, the formation of Siamese twins and the numerous developmental anomalies in newly hatched ide larvae are thought to be due to the fertilization of overmature eggs. The success of fish reproduction under controlled conditions is influenced by many important factors, such as the spawner storage conditions, water temperature and its fluctuations (Nowosad et al. 2014, Kucharczyk et al. 2016b) and appropriate nutrition (Król et al. 2014, Nowosad et al. 2017), but also the age of fish (Targońska et al. 2012) and their origin and degree of domestication (Kujawa et al. 2011). During the reproduction of certain species of fish under controlled conditions (such as barbel *Barbus barbus* L.) or ovulation induction outside of the natural spawning season, environmental stimulation itself may not be sufficient to cause final gamete maturation, and, therefore, it may be necessary to use hormonal stimulation (Nowosad et al. 2014, 2016). These reproductive and non-reproductive treatments both require high-quality gametes, which leads to high rates of fertilization and larval development without deformation (Kucharczyk et al. 2008). Unfortunately, reproduction under controlled conditions may cause complications due to the retrieval of overmaturing eggs, which may even lead to ploidy level changes and the development of many different deformities in the offspring (Samarin et al. 2015, 2017).

In the present study, fertilization of over-matured egg was found to increase the occurrence of developmental anomalies in ide larvae. The hatching rates of

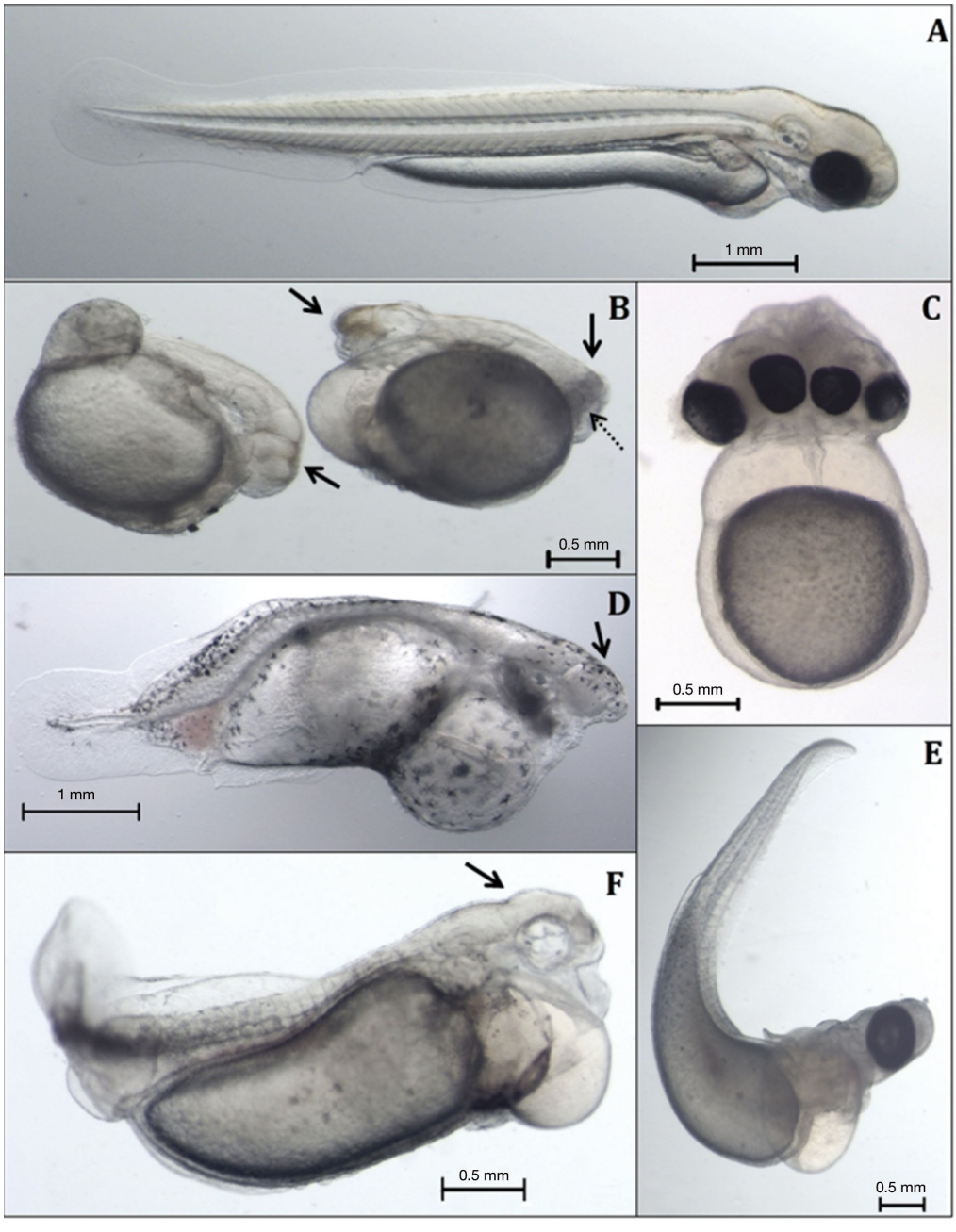

Fig. 3. Types of body malformations in ide *Leuciscus idus* larvae (0 days post-hatching): (A) normal larvae; (B) larvae with shortened body, absent tail, deformation of the yolk sac, and 1 (left) or 2 (right) heads (Siamese twins) without eyes; (C) Siamese twins with 2 heads and 1 heart, with shortened body, absent tail and deformation of the yolk sac; (D) larvae with kyphosis, deformed yolk sac with oedema, and lacking eyes; (E) larvae with lordosis, deformed yolk sac with oedema, and distorted head with a single eye; (F) larvae with lordosis, deformed yolk sac with oedema, and eye sockets without eyeballs. (Arrows) head, (broken arrow) outline of the eye. All larvae were of the same age

eggs collected shortly after ovulation was over 79%, in contrast to the overmature groups, in which the hatching rate was between 45.1 and 49.3% with a high percentage of abnormal larvae (~20%). This confirms observations made by Targońska et al. (2012); who found a very small amount (0.1%) of deformed larvae when eggs were fertilized shortly after ovulation. Siamese twins were also found in the present study, with 2 heads and 1 heart (Figs. 2 & 3C) or 2 hearts (Fig. 3B, right). Such individuals are generally very rare among animals and especially in fish. There are reports on the occurrence of Siamese twins

in eastern mosquitofish *Gambusia holbrooki* (Moreno-Valcarcel et al. 2011), black-bar Endler guppy *Poecilia wingei* (Arbuatti et al. 2011), Salmonidae (Fjelldal et al. 2016), Cichlidae (Shirak et al. 2013) and seahorses *Hippocampus guttulatus* (Blanco et al. 2012). Numerous development anomalies in newly hatched larvae and the occurrence of 1 set of Siamese twins in cyprinid fish were reported by Jezierska et al. (2000), but these anomalies were mainly due to the exposure of developing common carp *Cyprinus carpio* embryos to heavy metals (Cu and Pb).

The Siamese twins described in the literature do not differ much in appearance from normally developing larvae, except that they are fused, one of the siblings with a 'sister-brother', and have a shared digestive system (e.g. 2 heads, 2 hearts, 2 intestines and 1 anus as a common part), as seen in spiny dogfish *Squalus fernandinus* (von Bonde & Marchand 1929), seahorse (Blanco et al. 2012), Atlantic salmon *Salmo salar* (Fjelldal et al. 2016), and other parts of the body, e.g. Atlantic salmon (Fjelldal et al. 2016), blue tilapia *Oreochromis aureus* and Nile tilapia *O. niloticus* (Owusu-Frimpong & Hargreaves 2000). In this study, the ide Siamese twins did not closely resemble typical fish larvae, and many of them looked like hen eggs with 2 heads.

There are no typical explanations of the cause of Siamese twins in the literature, but they are believed to be due to disturbances in the early development of the embryo as a result of heat shock (Owusu-Frimpong & Hargreaves 2000) or chemical impurities, such as insecticides (Manna & Sadhukhan 1986). In this study, the formation of Siamese twins and the numerous developmental anomalies in newly hatched ide larvae are thought to be due to the fertilization of overmature eggs. This may indicate that changes take place when eggs mature too long in the ovary that result in the occurrence of numerous deformations, including chromosomal abnormalities. Similar observations were made by Samarin et al. (2015, 2017) while studying the fertilization of overmatured eggs of Eurasian perch *Perca fluviatilis* and pikeperch *Sander lucioperca*. In other finfish species, such as kutum *Rutilus frissi kutum* or some sturgeons, the eggs lost their viability within a few hours after ovulation (Samarin et al. 2015). Another developmental disruption following fertilization of overmature eggs is larval malformation, which results in a significant increase in the number of hatched abnormal larvae, e.g. between 20 and 50% abnormal larvae was noted in African *Heterobranchus longifilis* and European catfish *Silurus glanis*, respectively (Samarin et al. 2015).

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