

# First report of the occurrence and different types of conjoined twins in common whitefish *Coregonus maraena* larvae originating from the Baltic Sea

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**ABSTRACT:** This paper describes for the first time various forms of conjoined twins in the common whitefish *Coregonus maraena* originating from the brackish waters of the Baltic Sea. The occurrence of conjoined twins was observed during the hatching of whitefish under controlled conditions. Two types of twins were identified: symmetric (parapagus) and asymmetric (parasitic form). The percentage of conjoined twins among the whitefish did not exceed 0.01 % of the hatched larvae. The forms of twins observed in this study were similar to conjoined twins occurring among humans. One main type, parapagus, was identified in the group of symmetric twins with 3 subtypes: diprosopus parapagus, dicephalic parapagus and dithoracic parapagus. In its parasitic form, the parasitic twin occurred as a 'cyclops' or just as an eye growing out of the other twin's head. We conclude that fish, as well as their fertilised eggs, can be used as a model system to observe how such twins are joined in humans because of the similarity with conjoined twins occurring in humans. Because of internal fertilisation in fish, as well as the size and transparency of egg cells in these animals, it is possible to observe the process of fertilisation, cell division and formation of an embryo and subsequently of twins, as a model for humans.

**KEY WORDS:** Deformation · Hatching · Larvae · Parapagus twins · Parasitic twins

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## 1. INTRODUCTION

One very interesting phenomenon in the natural world is the occurrence of organisms with developmental anomalies, such as conjoined ('Siamese') twins. There are numerous possible causes of such anomalies, including genetic and environmental factors and the action of toxins (Roberts 2012). However, science still cannot identify the causes of all developmental anomalies or the mechanisms or the time (stage of embryonic development) of their formation (Mian et al. 2017).

Although usually associated with human populations (Mian et al. 2017), the occurrence of conjoined twins has been noted increasingly in the animal world (Nowosad et al. 2018). Anomalies of this type occur extremely rarely in the animal world, but they

have been observed in humans and in other mammals, including dolphins *Tursiops truncatus* (Kompanje 2005), leopard cats *Prionailurus bengalensis* (Kompanje & Hermans 2008), buffalo *Bubalus bubalis bubalis* (De La Ossa & Altahona 2010) and gorillas *Gorilla gorilla* (Langer et al. 2014), as well as in other groups of animals such as reptiles, including snapping turtles *Chelydra serpentina* (Cederstrom 1931) and crocodiles (Platt et al. 2012), as well as in fish (e.g. Von Bonde & Marchand 1929).

The reasons behind the formation of conjoined twins are not clear. To date, conjoined twins have been found to occur among ovoviviparous and viviparous fishes (sea horse, guppy, sharks), and in oviparous farmed salmonid and cichlid fishes, in which genomic manipulations are often conducted during culture protocols (Leach et al. 2014, Fjellidal et al.

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2016). Such anomalies may be caused by genetic defects brought about by a high degree of inbreeding. Various manipulations made by farmers in fish hatcheries can also stimulate the formation of conjoined twins and other developmental malformations (Owusu-Frimpong & Hargreaves 2000, Shirak et al. 2013, Leach et al. 2014). Studies on fish conducted by Samarin et al. (2015) and Nowosad et al. (2018) indicate that chromosomal and developmental anomalies may occur in offspring developed from ageing oocytes. Toxic substances, such as heavy metals (cadmium, mercury), pesticides or petrochemical substances, may also be the cause of developmental malformations by affecting gametes (mainly oocytes) or embryos at an early stage of development in fishes and other animals (Jezierska et al. 2000, Šebelová et al. 2002, Pecínková et al. 2005, Witeska et al. 2014). One species in which the impact of all of these factors may accumulate is the common whitefish *Coregonus maraena*, which occurs in the Baltic Sea basin. This whitefish species has traditionally been known as *C. lavaretus* in the Baltic basin countries (Froese & Pauly 2018).

The whitefish is one of the most valuable species of European freshwater ichthyofauna. However, the species is becoming increasingly rare because of the deteriorating state of the environment, as evidenced by a decrease in the percentage of whitefish caught over the last 20 yr (Szczepkowski et al. 2010). Currently, the species is listed on the IUCN Red List of Threatened Species as Vulnerable (VU) (CITES & UNEP-WCMC 2017). The factors blamed for this decline include eutrophication of lakes, destruction of natural spawning grounds, pressure from birds of prey (e.g. cormorants) as well as a shortage of stocking material (Szczepkowski et al. 2010, Wunderlich et al. 2011, Vonlanthen et al. 2012). Populations of this fish are kept alive mainly through stocking (Sebesta et al. 2018). However, stocking material is often produced from a small parent pool, which favours inbreeding. Whitefish offspring are often obtained from breeders caught in natural aquatic environments (including the Baltic Sea basin) during the spawning period. The Baltic Sea is one of the most polluted waters in Europe (Lang et al. 2018). Negative effects of eutrophication on the whitefish population and the formation of developmental anomalies in the gonads have been previously reported (Bittner et al. 2010, Hliwa et al. 2011, Vonlanthen et al. 2012). However, there have been no reports on the occurrence of conjoined twins in *Coregonus* spp.

The aim of this study was to verify the occurrence (and identify the types) of conjoined twins in com-

mon whitefish, a species susceptible to developmental anomalies.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Whitefish spawners ( $n = 125$ ) were obtained from Szczecin Lagoon in the Odra River in north-western Poland. Gametes were stripped manually (without hormonal stimulation) by commercial fishermen in December shortly after the fish were captured and transported to local hatcheries for fertilization and incubation. The eggs were incubated at the ambient water temperature ( $0.3\text{--}3.5^\circ\text{C}$ ) of the river. In February, the eggs were taken to the Department of Lake and River Fisheries (Olsztyn, Poland) where they were distributed among five 8 l Weiss jars and incubated at  $3.5 \pm 0.1^\circ\text{C}$  ( $4\text{ l eggs jar}^{-1}$ ). After 60 d, the eggs were transferred to a second set of 8 l Weiss jars and incubated at  $8\text{--}9^\circ\text{C}$  to accelerate development and hatching. Hatched larvae were allowed to swim up and out of the jars, and were collected in a holding tank (total volume 1000 l) lined with a 0.2 mm mesh. Most larvae hatched within 24 h, and were transferred to tanks (250 l) in a recirculation aquaculture system (Sebesta et al. 2018).

Unhatched embryos or hatched embryos with deformations remained in the jars and were transferred to a bowl. Eight larvae with visible anomalies were caught and then euthanized with MS-222 (dose:  $0.1\text{ g l}^{-1}$ ). Analysis of larval deformation was performed using a Leica MZ16 A stereomicroscope and a digital colour camera with 5 MP resolution (Leica DFC420) for image analysis. After the photographs were taken, the larvae were fixed for histological examinations. An additional 3 larvae (conjoined twin type) were reared further in an aquarium (2 l) and fed with freshly-hatched nauplii (*Artemia* sp.). The larvae were reared at a temperature of  $15 \pm 0.1^\circ\text{C}$  and the water oxygenation was over 80% of saturation.

The present study did not require a permit from the local ethics committee.

### 2.2. Classification of twins

Conjoined whitefish twins were classified according to an equivalent of a system applied in the classification of conjoined twins in humans based on the site of attachment (Mian et al. 2017; Fig. 1).

### 2.3. Histological analysis

The samples (fish) were fixed in Bouin's fluid. The fixed material was put into biopsy cartridges and then into a tissue processor (Leica TD 1020) for 21 h, where it was dehydrated in ethanol at increasing concentrations (75, 80, 90, 95 %), then soaked in acetone, xylene and finally liquid paraffin at 54°C. The obtained study material was then sealed in paraffin blocks and sliced in a rotating microtome (Leica RM 2155) into 5–6 µm thick sequences. The preparations were made with Mayer's haematoxylin and eosin (HE) (Zawistowski 1986). Subsequently, the stained preparations were sealed with cover slips and histokitt (Glaswarenfabrik Karl Hecht). After drying, the histological preparations were analyzed under a light microscope (Olympus BX51) with Cell^D software.

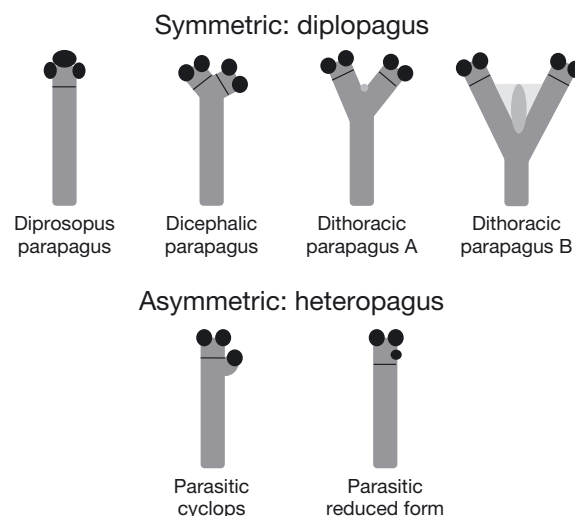


Fig. 1. Types of connections in conjoined whitefish twins (0 d post hatching). Black dots: eyes; line: posterior limit of head

Table 1. Characteristics of different forms of conjoined twins in whitefish *Coregonus maraena* larvae

Type (no. individuals found)	Place of conjoining	Heads/ eyes	Hearts	Digestive systems	Neural cords/ pectoral fins	Comments	Figure
Normal (~150 000)	Not applicable	1 / 2	1	1	1 / 2		Fig. 2
<b>Symmetric conjoined twins: diplopagus</b>							
Diprosopus parapagus (1)	Head, common eye	1 / 3	1	1	1 / 2	One trunk and 1 head, 3 eyes, 1 large eye located centrally in front of the head.	Fig. 3
Dicephalic parapagus (2)	Laterally, by the whole section of the trunk and tail	2 / 4	2	1	2 later 1 / 2	Common trunk, 2 separate heads, 2 separate hearts, 2 oesophaguses conjoined to 1 common intestine, and 2 neural cords conjoined to 1.	Fig. 4 Fig. 5
Dithoracic parapagus type A (3)	Laterally, by a common posterior trunk and tail	2 / 4	2	2 later 1	2 later 1 / 4	Each twin had a separate head and front part of the trunk. They had 2 separate oesophaguses, with middle intestines that connected into 1 common intestine in the posterior segment, and 2 neural cords conjoined to 1.	Fig. 6a Fig. 7
Dithoracic parapagus type B (1)	Laterally, by a common tail	2 / 4	2	2 later 1	2 later 1 / 4	Each twin had a separate head and the trunk. Twins conjoined laterally, with a common yolk sac and tail. They had 2 separate oesophaguses and part of the intestine, which conjoined into 1 common intestine in the posterior part, and 2 neural cords conjoined to 1.	Fig. 6b
<b>Asymmetric conjoined twins: heteropagus</b>							
Parasitic cyclops (3)	Just behind the head, beginning of the trunk	2 / 3	2	1	2 later 1 / 2	One twin had a head with 1 eye (cyclops) and grew from the front part of the trunk of the main twin. The parasitic form had or lacked a heart. The parasitic twins did not have a patent oesophagus, but did have 2 neural cords conjoined to 1.	Fig. 8
Parasitic reduced form (1)	Head	1 / 3	1	1	1 / 2	Reduced parasite in the form of third smaller eye, growing in the upper part of the head (parietal bone).	Fig. 9

### 3. RESULTS

In this study, both symmetrical (diplopagus) and asymmetrical (heteropagus) twins were found (Table 1). All of the conjoined twins were similar in overall shape to non-conjoined individuals, i.e. larvae with normal appearance (Fig. 2). One main type, parapagus, was identified in the group of symmetric twins, with 3 subtypes: diprosopus parapagus (Fig. 3), dicephalic parapagus (Figs. 4 & 5), dithoracic parapagus (Figs. 6 & 7). A single diprosopus parapagus individual had 3 eyes at the central position at the front of the head: it consisted of 2 joined ocular lenses (Fig. 3b,c). In the dicephalic parapagus subtype (Fig. 4;  $n = 2$ ), the individuals had 2 heads, 2 separate oesophaguses which joined to form 1 common intestine, a common liver and yolk (Fig. 5a), and 2 separate neural cords, which joined only in the tail section (Fig. 5b). Two types of individuals were identified within the dithoracic parapagus subtype, differentiated by the site where the digestive tracts and notochords joined to make the common organ (Fig. 6): type A (Figs. 6a & 7) and type B (Fig. 6b). Dithoracic parapagus type B twins had additional swelling between the intestine and the common gall bladder (Fig. 6b).

We also found heteropagus (asymmetrical) twins, where one of the

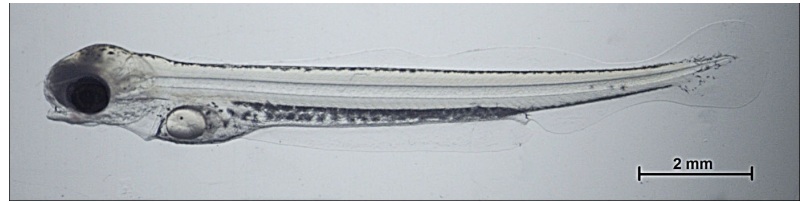


Fig. 2. Normal whitefish larva at 0 d post-hatch (DPH)

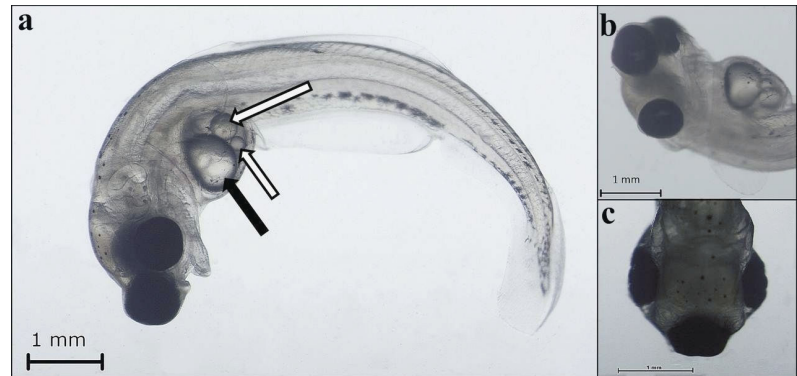


Fig. 3. Diprosopus parapagus whitefish larvae (0 DPH). (a) Longitudinal, (b) bottom and (c) top view of large head with 4 eyes. Black arrow shows a large lipid droplet, and white arrows show small lipid droplets

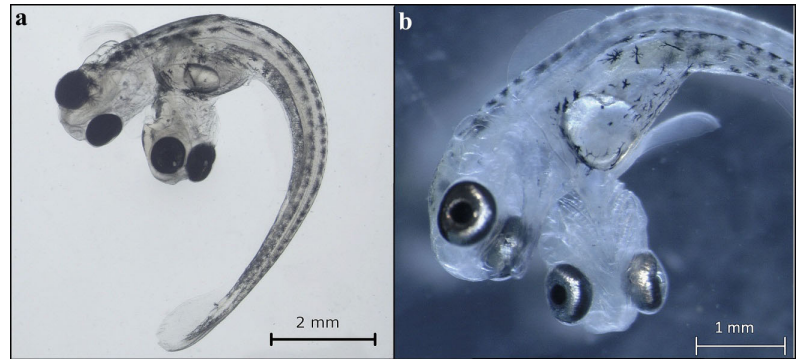


Fig. 4. Dicephalic parapagus whitefish (0 DPH). View of (a) the whole larva and (b) magnification of the 2 conjoined heads

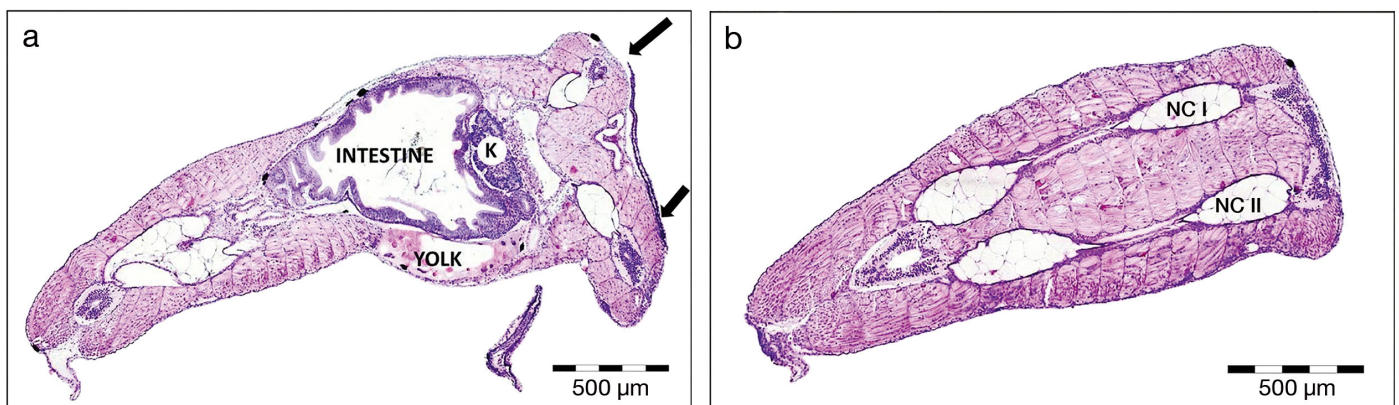


Fig. 5. Histology sections of dicephalic parapagus whitefish larvae at 0 DPH: (a) intestinal region; (b) neural cord region. K: kidney; NC: neural cord of first (I) and second (II) twin. Black arrows indicate the head regions of the twins





Fig. 6. Different subtypes of dithoracic parapagus whitefish: (a) type A (neural cords conjoined in trunk) and (b) type B (neural cords conjoined posterior to anus). Inset: histological section of the second form. Arrow indicates connection of 2 intestines into one

twins is present as a parasitic form, differing from the other in terms of its structure and appearance (Fig. 8), or there was only part of it left, for example, as an eye growing out of the head (Fig. 9). In the first case, the parasitic (smaller) twins had only 1 eye ('cyclops') and did not have a working digestive tract, and they only had residual elements of the muscular system and a skeleton; however, they had their own heart, and the vascular system was connected with the larger twin. Such twins had other defects, such as the curvature of the spine (Fig. 8b) and abdominal swelling (Fig. 8c).

Our study has shown that conjoined whitefish twins can take up food and survive for some time ( $n = 3$ ). Two conjoined individuals, left alive, survived for about 2 wk. One individual of the heteropagus type (the larger and the smaller individual: 10.8 and 1.8 mm, respectively, at 0 d post hatching) survived 5 wk and reached a total body length (TL) of 21.9 mm (larger twin) and 3.0 mm (smaller twin). The smaller parasitic individual grew slightly more slowly than its twin; it was 6 times smaller than its twin after hatching and 7 times smaller after 5 wk (Fig. 10). Its death was caused by difficulties in moving, loss of strength and difficulty in absorbing food.

#### 4. DISCUSSION

This is the first report which describes the occurrence of conjoined twins and their various forms in

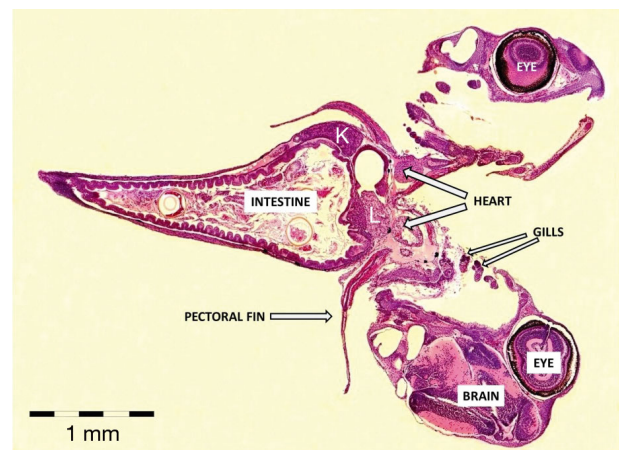


Fig. 7. Histological section of dithoracic parapagus whitefish type A (8 DPH). L: liver; K: kidney

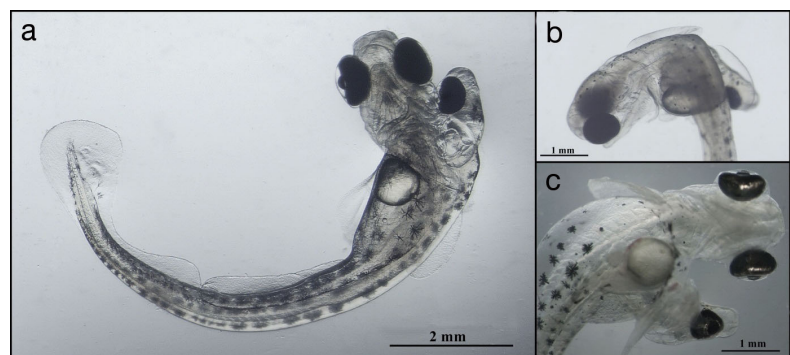


Fig. 8. Different forms of parasitic 'cyclops' whitefish (0 DPH). View of (a) the whole larva, and individuals with subtle anomalies in (b) spine curvature and (c) abdominal swelling

common whitefish. Developmental abnormalities, such as conjoined twins, are very rare in animals, and their cause(s) remains unclear despite advancements in science. Conjoined twins have been found among

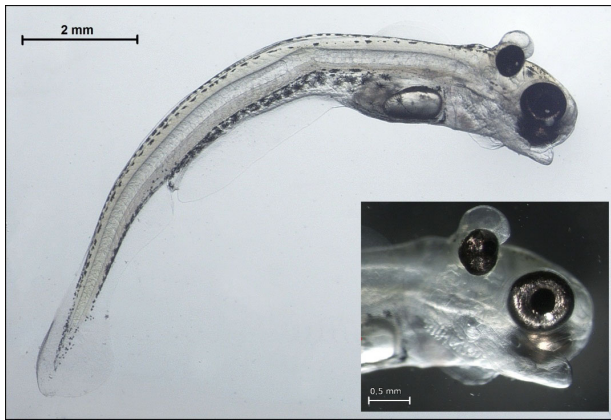


Fig. 9. Parasitic reduced form of asymmetric (heteropagus) conjoined whitefish twins, showing 1 small eye (0 DPH). Inset: magnification of the head of the same specimen

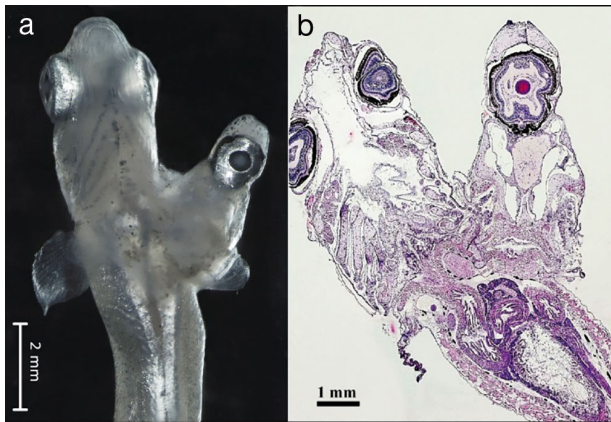


Fig. 10. (a) Normal view and (b) histological section of a parasitic 'cyclops' form of asymmetric (heteropagus) conjoined whitefish twins at age 35 DPH

viviparous animals, e.g. mammals and viviparous fish (Kompanje 2005, Moreno-Valcarcel et al. 2011, Langer et al. 2014), and among oviparous animals, such as birds, reptiles and fish (Cederstrom 1931, Corti & Vogelaar 2008, Platt et al. 2012, Fjellidal et al. 2016). Eight types and 3 subtypes of conjoined twins have been identified in humans on the basis of the location where they are joined. Such anomalies are formed only in monozygotic, monoamniotic twins, where twins are genetically identical (Mian et al. 2017). Genetic studies conducted on parasitic conjoined fish twins formed by hybridisation in catfish (female *Ictalurus punctatus* × male *I. furcatus*) have pointed to identical genomes of both twins (Leach et al. 2014). The same situation has also been noted in humans (Mian et al. 2017). Conjoined twins are born at an approximate rate of 1 per 200 000 live births in humans (Mian et al. 2017). The majority of conjoined

pregnancies (60%) are miscarried spontaneously or children born from such pregnancies are stillborn (Sliwa & Macura 2015, Mian et al. 2017). In the case of fish, the rate of occurrence of conjoined twins did not exceed 0.4 and 0.1–0.7% among Atlantic salmon *Salmo salar* (Fjellidal et al. 2016) and several species of tilapia (Huang et al. 1987), respectively. In our study, the rate of occurrence of conjoined twins in whitefish was also ca. 0.01%. Both this study and others have found that conjoined twins in fish die within 6 wk—and usually within a few days—of hatching (Huang et al. 1987). However, there have been reports documenting the existence of adult conjoined twins in fish: in rainbow trout *Oncorhynchus mykiss* (Behnke & Kloppel 1975), guppy *Poecilia wingei* (Arbuatti et al. 2011), hybrid catfish *I. punctatus* × *I. furcatus* (Leach et al. 2014) and hybrid Atlantic salmon × Arctic char (Fjellidal et al. 2016). The high mortality rate among conjoined twins may not be directly linked to the function of common organs, but it may be the result of congenital defects, which occur more often in these individuals (Sliwa & Macura 2015).

Previous studies have confirmed that *Coregonus* spp., including whitefish, are very sensitive to changes in the environment and chemical environmental pollution (eutrophication), with consequent abnormal gonad morphology, including their narrowing or atrophy and probably formation of hermaphroditic individuals (Bernet et al. 2009, Hliwa et al. 2011). In this study, twins of 2 major groups were found among whitefish: symmetrical, parapagus type (subtypes: diprosopus, dicephalic and dithoracic parapagus) and asymmetrical, where 1 of the twins is present as a parasitic form. The diprosopus parapagus subtype has also been observed in guppies (Moreno-Valcarcel et al. 2011, Petrescu-Mag et al. 2011) and tilapia *Oreochromis aureus* (Shirak et al. 2013); the dicephalic parapagus subtype has been reported in dolphins (Kompanje 2005), turtles (Cederstrom 1931), tilapia (Owusu-Frimpong & Hargreaves 2000) and Atlantic salmon (Fjellidal et al. 2016); and the dithoracic parapagus subtype (types A and B combined) has been found in red tilapia (Huang et al. 1987), sea horse *Hippocampus guttulatus* (Blanco et al. 2012) and hybrid Atlantic salmon × Arctic char (Fjellidal et al. 2016). Studies conducted with human conjoined twins have confirmed that in most cases, such twins are formed in females (Mutchinick et al. 2011, Mian et al. 2017). However, the parapagus form occurs more often in males and among parasitic twins in humans (Mutchinick et al. 2011, Sliwa & Macura 2015). In the present study, the sex of whitefish larvae could not be determined because sexual differ-

entiation in fish takes place at a later stage of their development (Długosz & Demska-Zakes 1992).

The formation of abnormal animals, including conjoined twins, may result from fertilisation of low-quality oocytes, as well as over-mature oocytes, which was indicated in reports by Nowosad et al. (2018). A study conducted by Zarski et al. (2011) on the Eurasian perch *Perca fluviatilis* showed a strong correlation between agglomeration of lipid droplets into 1 large drop and the quality of oocytes and, later, of larvae. A lower degree of agglomeration of lipid droplets thus means a lower survival rate and a higher percentage of deformed larvae. In this study, we also observed a lack of agglomeration of lipid droplets into 1 large drop (Fig. 3)

Despite advances in science, the pathogenesis of developmental abnormalities, such as conjoined twins, remains unexplained, likely because of insufficient knowledge of the embryonic processes and teratogenic factors (Sliwa & Macura 2015). However, according to many researchers, there are genetic factors underlying these developmental abnormalities (Petrescu-Mag et al. 2011, Roberts 2012), although some researchers have reported the teratogenic effect of some factors, such as hypothermia or hypoxia, on the formation of such anomalies at early stages of human embryonic development (Mutchinick et al. 2011). Sliwa & Macura (2015) described several theories explaining the formation of human conjoined twins. All of them were based on disruptions at an early stage of embryonic development, e.g. during early gastrulation, when incomplete division of the embryonic disc takes place. Fragmentation of the embryonic disc and separation of the tissue elements may be caused by environmental factors, disruption of cellular adhesion or apoptosis (Mutchinick et al. 2011, Sliwa & Macura 2015).

The use of fish as model organisms can help to understand the process of formation of conjoined twins and to develop diagnostic tools for treating humans; this is possible when there is a considerable similarity between the human and fish genomes (e.g. in zebrafish) or when the walls of fish egg cells are transparent (Bruneel & Witten 2015, Shi et al. 2015). Since some of the factors which stimulate the formation of conjoined twins in fish are known, e.g. insecticides (Manna & Sadhukhan 1986), hypothermia (Yamamoto et al. 1996), polyploidisation (Owusu-Frimpong & Hargreaves 2000), gynogenesis (Shirak et al. 2013) and hybridisation (Leach et al. 2014, Fjellidal et al. 2016), such a type of development can be stimulated *in vitro* and the embryogenesis can be observed.

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