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RESEARCH ARTICLE

# Comparison of egg and offspring size of karyologically identified spined loach, *Cobitis taenia* L., and hybrid triploid *Cobitis* females (Pisces, Cobitidae)

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**Abstract**. The aim of this study was to determine and compare for the first time the size of eggs laid by *C. taenia* (2n=48) and its naturally occurring triploid (3n=74) hybrid females and the total length of offspring of these females obtained from experimental crosses, in the period from hatch to 22 days post hatch. The eggs laid by triploid females had an average diameter of 1.18 mm and were significantly larger than the eggs spawned by *C. taenia* females, which averaged 0.93 mm in diameter. The size of the triploid female eggs was associated with their level of ploidy. Similarly, the average total length of triploid female progeny from one to ten days post hatch was statistically significantly higher than the average total length of *C. taenia* offspring in the same period of life. After two weeks, the offspring of diploid and triploid females reached average total lengths of 13.27 mm and 13.60 mm, respectively, which was not a significant difference. The possible causes of triploid *Cobitis* female domination in diploid-polyploid populations in terms of egg size and other traits associated with polyploid functioning was also investigated.

**Keywords**: diploids, loach, egg size, larvae, triploids

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## Introduction

Spined loach, *Cobitis taenia* L., identified by the karyotype comprising 2n = 48 chromosomes, is not as widespread as was previously believed (Vasil'ev et al. 1989). The geographical range of this species includes Europe: the Atlantic drainages from the River Seine northward, the Baltic basin south of 61°N, the upper Volga drainages, and the northern Black Sea basin (excluding the Danube) (Freyhof 2011, Vasil'ev et al. 2011). In Poland, spined loach is found in rivers and lakes with sandy or muddy and sandy bottoms, with the exception of mountain rivers. It attains a small size, and the average body length of an adult fish is about 10 cm. *C. taenia* females are slightly larger than males and live an average of one year longer (Boroń et al. 2008). Spined loach spawn in the spring and summer months (May to July), and the eggs are laid in two or three batches (Juchno and Boroń 2006).

The reason for identifying *C. taenia* based on karyotype (or other genetic traits) is that it crosses with several other European species of the genus *Cobitis,* forming polyploid, and sometimes diploid hybrids (Vasil'ev et al. 1989, Boroń 2003, Janko et al. 2003, 2012). Because of this phenomenon, *C. taenia* is a model species in studies on hybridization and polyploidization. Hybrids of *Cobitis* found in Poland contain genomes of *C. taenia*, the Danubian loach *Cobitis elongatoides* Bacescu & Maier, and *Cobitis tanaitica* Bacescu & Maier or *Cobitis taurica* Vasil'eva, Vasil'ev, Janko, Ráb & Rabová (Boroń 2003, Janko et al. 2003, 2012, Maciak et al. 2011). The occurrence of the latter two species has not been confirmed among Polish fish fauna. Parental species and hybrid progeny are not distinguishable based on external morphological characteristics; thus, it is necessary to apply genetic and cytogenetic discriminatory techniques.

Species of *Cobitis* can be found in diploid populations, but they are much more common in mixed diploid-polyploid populations. In the latter case, females and males of *C. taenia* and/or *C. elongatoides* coexist with allotriploid, gynogenetic *Cobitis* females, which predominate numerically, as well as with rare tetraploid hybrids of both sexes (Boroń 1999, 2003). These chromosome studies showed that among the 24 populations of *Cobitis* fish, only four – from lakes Klawój, Głębokie, Wigry, and Legińskie, comprised solely *C. taenia* individuals.

Triploid *Cobitis* females are a phenomenon as they are dominant in mixed populations, and can comprise as much as 95% of all individuals collected (Boroń 2003). The dominance of allotriploid *Cobitis* females in mixed diploid-polyploid populations has been analyzed extensively, and the results show that: a) they reproduce primarily gynogenetically (Vasil'ev et al. 1989, Saat 1991); b) they live a year longer than parental diploid loach females (Jeleń 2005); c) they produce fewer but larger oocytes than diploids (Juchno et al. 2007, Juchno and Boroń 2010); d) their eggs are characterized by significantly higher yolk content in comparison with those of diploids (Pavlov et al. 2004).

The larger genome causes larger poliploid cells, and slower cell divisions. This can affect the development and production of gametes (Pandian and Koteeswaran 1998, Kozlowski et al. 2003). Allopolyploids, like triploid *Cobitis* females, are more vigorous than their diploid parents (Comai 2005, Pandian and Koteeswaran 1998), possibly because of higher resistance, higher survival, and faster growth, and also because of the greater genetic variation among individuals.

In the current study, we continued attempts to further clarify the dominance of hybrid triploid *Cobitis* females in natural populations. We investigated whether the potentially larger eggs laid by the triploid females produced larger offspring, which in the early stages of life would potentially have better chances of survival. The aim of this study was to determine and compare the size of eggs laid by *C. taenia* (2n = 48) and naturally occurring hybrid triploid females of *Cobitis* (3n = 74) and the length of offspring of these females obtained from experimental crosses.

## Materials and methods

The research material comprised eggs and offspring obtained from the following crosses: a) triploid *Cobitis* females and *C. taenia* males collected from a mixed diploid-polyploid population of the Bug River (52°09 N, 23°309 E); b) *C. taenia* males and females from diploid populations of this species in Lake Legińskie (52°509 N, 20°559 E). The diploid-polyploid population from the Bug River includes males and females of *C. taenia* as well as triploid *Cobitis* females and tetraploid males and females. The study used progeny derived from crosses between *C. taenia* males and females from Lake Legińskie; despite efforts to acquire female and male *C. taenia* from the mixed population in the Bug River, we failed to do so because of insufficient numbers.

In order to induce gamete maturation, both males and females underwent double hormonal injections with Ovopel (an analog containing GnRH and metoclopramide) (Horvath et al. 1997, Kujawa et al. 2002), at a dose of 0.05 ml per 10 g of fish body weight. Eggs and sperm were obtained by gently massaging the abdominal integument. The gametes were incubated initially in Petri dishes, then in tanks with flowing water at a temperature of 24°C. A total of seven crosses were made, including three crosses of diploid females and males and four crosses of triploid females and diploid males. The ploidy of all parental individuals was determined based on chromosome counts of chromosome slides made from fish kidney and stained with Giemsa solution according to Boroń (1999).

One hour after fertilization the eggs were imaged under a stereoscopic microscope (Nikon SMZ 800) and measured to the nearest 0.01 mm (Matsubara et al. 1995). Two measurements were taken and then averaged for each egg. A total of 708 eggs were measured, including 234 eggs obtained from diploid and 474 eggs obtained from triploid females.

The offspring obtained were kept in tanks with flowing water and a constant temperature of 24°C and fed *ad libitum* with *Artemia* sp. naupli twice daily. Offspring were collected at hatch and on one day post hatch (dph) (appearance of external gills), 10 dph (operculum covers the gills) and 22 dph (juveniles, after complete resorption of the yolk sac). The fish were anesthetized with MS 222, and then fixed in 4% formaldehyde solution. Standard length (SL) and total length (TL) were measured in 45 offspring individuals of diploid and 93 offspring individuals of triploid females. Only total length (TL) was used in further analyses.

The measurements of eggs and offspring individuals were performed with CoolView 1.6.1 software (Precoptic 2009). The size distributions of the eggs of diploid (Shapiro-Wilk test, W = 0.97956, P < 0.01) and triploid (Shapiro-Wilk test, W = 0.99132, P < 0.01) females were not normal, and the Mann-Whitney test was used to compare them. The Mann-Whitney and Student's t test were used to compare the offspring size obtained from diploid and triploid females. In order to analyze differences in offspring sizes (TL), regression analysis was used and the hypothesis about the equality of *b* values ($H_0$: $b_d=b_t$) for diploid and triploid specimens was tested. Analysis of covariance (ANCOVA) was applied to determine whether the size of the female and ploidy level affected the size of the eggs (Stanisz 1998). Statistical analyses were performed using Statistica software (StatSoft Inc., Tulsa, OK, USA), version 10 and Microsoft Excel software. Loach is a protected species in Poland, and catches of the fish were allowed by permit from the Ministry of the Environment DOP-OZGiZ.6401.10.2011.1s. The study was conducted with consent from the local Commission of Ethics; individual consent no. 04/11.

## Results

The size of eggs obtained from diploid females ranged from 0.80 mm to 1.01 mm and averaged 0.93 mm (± 0.04), whereas the diameter of triploid female eggs ranged from 1.08 mm to 1.29 mm and averaged 1.18 mm (± 0.04). The eggs obtained from triploid females were significantly (Mann-Whitney, Z = 21.65, P < 0.05) larger than the eggs obtained from diploids.

The size distributions of eggs laid by the diploid and triploid females formed two separated groups (Fig. 1). Approximately 73% of the eggs laid by diploid females had diameters of 0.91 mm to 0.98 mm (Fig. 1). Among the eggs laid by triploid females, eggs with diameters of 1.17 mm to 1.22 mm, of which most had a diameter of 1.19 mm, were predominant. Overall, they constituted approximately 62% of all eggs (Fig. 1). Covariance analysis revealed that the size of the eggs was influenced significantly by the female ploidy level (F = 1600.20, df = 1, P < 0.0001), but not by female standard length (F = 0.397, df = 1, P = 0.5287).

The average total length (TL) of *C. taenia* one dph was 5.21 mm (± 0.17 mm) (Table 1), which was statistically significantly lower (t test, t = -4.07456, df = 35, P < 0.05) than that of the triploid females, which was 5.51 mm (± 0.24 mm). The average TLs of triploid female offspring one (Mann-Whitney, Z = -2.20250, P < 0.05) and ten dph (t test, t = -5.67592, df = 27, P < 0.05) were significantly statistically greater than those of diploid offspring. By contrast, the total length of progeny of diploid and triploid females 22 dph (Mann-Whitney, Z = -1.07044, P > 0.05) did not differ significantly.

Regression analysis showed that there was significant difference between total length (TL) on different days of life for diploid (2n) and triploid (3n) progeny. The hypothesis about the equality of b values ($H_0$: $b_d=b_t$, P <

**Table 1**

Total length (TL, mm) of progeny of spined loach *C. taenia* and triploid *Cobitis* females; dph – days post hatch

| | | Progeny of *C. taenia* × *C. taenia* | | | | | Progeny of 3n *Cobitis* × *C. taenia* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age (dph) | n | min | max | mean | SD | n | min | max | mean | SD |
| hatching | 14 | 4.87 | 5.45 | 5.21 | 0.17 | 23 | 4.99 | 5.84 | 5.51 | 0.24 |
| 1 | 10 | 5.09 | 5.61 | 5.43 | 0.18 | 30 | 4.81 | 6.41 | 5.82 | 0.46 |
| 10 | 9 | 9.94 | 11.88 | 11.06 | 0.54 | 20 | 11.05 | 14.18 | 12.78 | 0.84 |
| 22 | 12 | 12.38 | 14.15 | 13.27 | 1.25 | 20 | 10.06 | 16.93 | 13.60 | 1.72 |

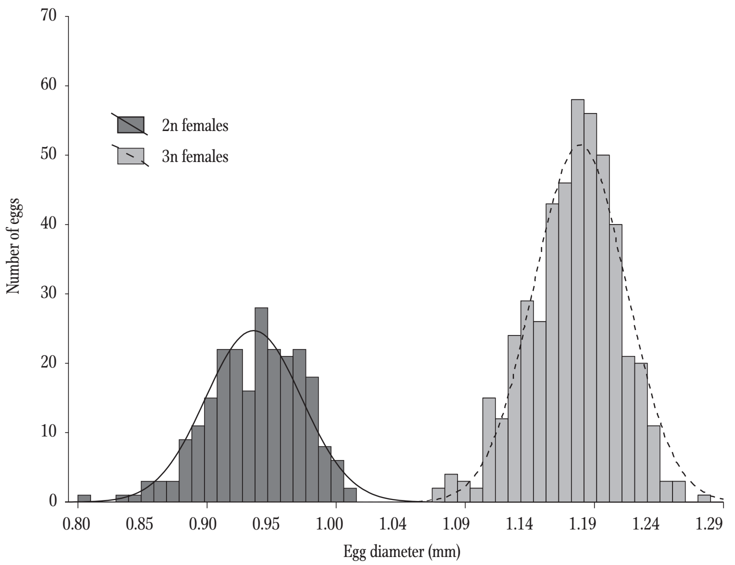


Figure 1. Size distribution of eggs laid by *C. taenia* (2n) and triploid *Cobitis* females (3n).

0.05) of the corresponding regression equations was rejected. So the equations for diploid and triploid larvae were, respectively, y = 0.3788x + 5.5142, $r^2$ = 0.9230 and y = 0.3985x + 6.0420, $r^2$ = 0.7954.

## Discussion

Hybridization and polyploidy in fish might be important in the formation of new species that are better adapted to the environment and changes therein. Polyploidy in fish is associated with traits such as large body size, faster growth rate, longevity, and environmental adaptability (Schulz 1979, Schlupp 2005). An increased amount of genetic material in the cell causes polyploid organisms to have larger cells. However, most polyploid animals are not larger than diploids, because the number of cells in poliploids is typically fewer than that in diploids (Benfey 1999, Leggatt and Iwama 2003). The genetic diversity of polyploid fish is caused by frequent polyploidization events, which determine the high percentage of polyploid occurrence (Janko et al. 2012).

The reason for the dominance of *Cobitis* triploid females is probably their reproductive success, which consists primarily of gynogenetic reproduction (Vasil'ev et al. 1989, Saat 1991). It has also been shown (Juchno et al. 2007) that in mixed diploid-polyploid populations, the breeding period of triploid (3n = 74) females is longer than that of the coexisting *C. taenia* species (2n = 48). In addition, the results presented in this study confirmed previous findings concerning the size of oocytes (Juchno et al. 2007) in gonad histology, i.e., triploid females lay significantly larger eggs than diploids. The average size of eggs laid was 1.18 and 0.93, respectively. Similar results were reported by Pavlov et al. (2004) for *Cobitis melanoleuca* Nichols and its triploid forms. Triploid females laid significantly larger eggs than did diploids. Moreover, larger eggs have larger stores of yolk, which is used in the early life larval stages. It has also been proven that larger offspring develop from larger eggs, which could potentially translate into increased opportunities and chances of survival in changing environments. This is particularly important for fish spawning in batches such as loach, the breeding season of which is prolonged and the offspring of which appears both in June and July. The hatch of triploid *Cobitis* was significantly larger than that of diploid loach, and their total lengths were 5.51 mm and 5.21 mm, respectively. Similar total length in diploid hatch, at an average of 5.03 mm, was also obtained under laboratory conditions in a study by Bohlen (1999). However, the larvae of the triploid form were larger than that of *C. melanoleuca*, but the growth rate of the latter increased substantially from the ray formation stage in the dorsal and anal fins. So, the average total length of *C. melanoleuca* juveniles and triploids of this species at the age of 58 days post hatch was 33.1 and 18.4 mm, respectively (Pavlov et al. 2004). According to our data, the average total length of *C. taenia* larvae was not significantly different from the total length of triploid female larvae on 22 dph. Keckeis et al. (2000) analyzed the effect of egg size on embryo mortality and the resistance of broods to starvation. It has been shown that the size of the eggs produced by female common nase, *Chondrostoma nasus* (L.), did not affect the mortality of embryos during development or immediately after hatching, but it was positively correlated with resistance to starvation. The larger eggs produce larger and potentially stronger fry, which is more resistant to starvation, and thus has a greater chance of survival (Keckeis et al. 2000). The larvae of triploid *Cobitis* females could benefit from the fact that they are larger during the first three weeks of life. They seem to have greater chances of survival, since, for example, they are more effective in feeding in comparison with the larvae of diploid *C. taenia*.

It is known that a portion of the eggs, which is as high as 33% according to Saat (1991), laid by triploid females is fertilized, giving rise to the development of tetraploid individuals. The chromosomal analyses of the experimental crosses of *Cobitis* triploid females with diploid males described in this work and elsewhere indicated that both triploid and tetraploid offspring were obtained. The proportion of tetraploid individuals among the larvae was shown to decrease significantly after the first days of life (Jablońska et al. 2012). Therefore, the greater body length of triploid female offspring examined in this work at hatch and on one and ten dph might correspond to the presence of tetraploid individuals. Whereas the absence of statistically significant differences in the total lengths of the two groups of progeny on 22 dph can be explained by the previously documented decrease in relation to clonal triploids in the number of tetraploid individuals, this was likely due to reduced survival. These theoretical considerations are not supported by the results of the measurements of the total length of sexually mature individuals of *C. taenia* and triploid and tetraploid *Cobitis* fish obtained from the natural environment (including *Cobitis* fish populations in the Bug River), among which there were no statistically significant differences (Jeleń 2005).

The size and quality of fish eggs is influenced by many factors. These include, among others, nutrient content and physicochemical properties of the water the eggs are incubated in, female size (usually larger females produce larger eggs), and the genomic composition of parental hybrid individuals (Brooks et al. 1997, Bobe and Labbé 2010). The size of eggs produced by *Cobitis* seems to be dependent primarily on their ploidy. The diameter of the eggs produced was not affected by the standard length of the female among either diploids or triploids. In addition, the frequency distribution of egg diameter of both triploid and diploid females revealed two different groups. However, eggs of different ploidy (haploid and triploid) produced by triploid females of the loach *Misgurnus anguillicaudatus* (Cantor) (Yoshikawa et al. 2008) and triploid females of *Iberocypris (Squalius) alburnoides* (Steindachner) (Alves et al. 2001) differed in size. One can assume that the triploid *Cobitis* females lay eggs of equal ploidy, presumably triploid, as was established in previous studies (Saat 1991).

Einum and Fleming (2000) developed a model which shows that the phenomenon occurs where fish somehow adapt the optimal size of eggs laid, and this adaptation affects the number and viability of the resulting offspring. It turned out that the optimum size of the eggs varied only slightly from the average of the observed values for the population, suggesting that it was mainly due to reactions to maternal selection rather than offspring adaptation. The model shows that the maximization of maternal fitness by sacrificing offspring survival could be a general phenomenon among highly fecund organisms (Einum and Fleming 2000). What is more, if offspring size is dependent on egg size, then an increase of parental investment in offspring will be possible until the higher vigor of juveniles compensates for reduced fecundity (McGinley 1989, Ojanguren et al. 1996). Triploid *Cobitis* females are less fecund than diploid *C. taenia* (Juchno et al. 2007), which is likely compensated for by larger offspring developing from larger eggs. Even, as is documented by the present study, larvae are larger only during the short period of time from hatch until 22 dph, after which they attain the same size as larvae of diploid *C. taenia*. Since the dominance of triploid *Cobitis* is potentially associated with other polyploid traits, e.g., larger erythrocytes (Juchno et al. 2010), resulting in a reduced metabolic rate (Maciak et al. 2011), the probable advantages and disadvantages of unisexual forms of *Cobitis* over bisexual species in the utilization of natural resources were analyzed precisely by Vasil'ev et al. (2011).

The results of the present study indicate for the first time based on the example of *Cobitis* fish, that in addition to laying larger eggs, triploid organisms can produce offspring that, within the first few weeks of life or longer, benefits from polyploidy, a process that has played and plays an important role in the evolution of fish.

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