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SHORT COMMUNICATION

Variable occurrence of apoptosis in the testes of diploid and sterile allotetraploid *Cobitis* (Teleostei, Cobitidae) males during the reproductive cycle

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ABSTRACT

Cobitis species exist in both diploid and diploid–polyploid (d–p) populations, but mostly occur in the latter. They are considered an important model organism to study the biology and physiology of natural hybrid and polyploid vertebrates. Indeed, polyploidization causes a huge stress for in terms of cell physiology and alters spermatogenesis in polyploid fish. The most extensively studied mode of germ cell death during spermatogenesis in vertebrates is apoptosis. The aim of this study was to examine caspase-3 immunoexpression in the testes of *Cobitis taenia* from a diploid population as well as *C. taenia* and sterile tetraploid *Cobitis* from d–p populations before, during and after spawning. The obtained results suggest a different performance of apoptosis in the testes of *C. taenia* from the two studied populations and seems to be conditioned by their role as the only sperm donors in d–p populations. Moreover, apoptosis was an active cell death process in the testes of tetraploid *Cobitis*.

KEY WORDS: Caspase-3, *Cobitis taenia*, Reproduction, Gonad maturation

INTRODUCTION

Cobitis taxa are small-sized, bottom-dwelling fishes indigenous to Europe and Asia and are considered an important model to study the biology, functioning and physiology of natural polyploid organisms. The spined loach, *Cobitis taenia* Linnaeus 1758, protected under the Natura 2000 Network (Annex II to the European Habitat Directive), exists in exclusively diploid bisexual populations. However, mostly *C. taenia* occur in mixed diploid–polyploid (d–p) hybrid populations where they co-exist with *Cobitis* hybrid triploid (3n) asexual females and hybrid tetraploids (4n) of both sexes (Vasil'ev et al., 1989; Boroń, 2003; Janko et al., 2012). The triploid females, which dominate (90%) in d–p populations, reproduce mainly via gynogenesis, where sperm is required to trigger development of the egg (Choleva et al., 2012; Juchno et al., 2014). Under experimental conditions, 33% (Saat, 1991) or 66% (Juchno et al., 2014) of eggs laid by triploid females were fertilized by spermatozoa of *C. taenia* and developed into tetraploids. Importantly, tetraploid progeny were less viable than triploid ones and their number significantly decreased, reaching about 6% (Juchno et al., 2014), similar to levels that occur naturally (Boroń, 2003; Juchno et al., 2007). There is evidence from *C. taenia* and mixed *Cobitis* species populations that in the reproduction processes of d–p populations, only *C. taenia* males are the sperm donors (Choleva et al., 2012; Juchno et al., 2014), as *Cobitis* tetraploid males were sterile (Juchno and Boroń, 2006; Juchno et al., 2017). Therefore, the existence of allopolyploid *Cobitis* forms seems to be dependent upon the presence of *C. taenia*.

Generally, polyploidization is rare in animals and mostly occurs in fish (Arai and Fujimoto, 2013). Polyploid *Cobitis* complexes have attracted interest for studying 'evolution in action' via hybridization and polyploidization (Choleva et al., 2012; Juchno et al., 2007; 2014; Janko et al., 2018; Bartoš et al., 2019). It seems that *Cobitis* loaches achieve various stages of the evolutionary theater, where diploid hybrids and triploid unisexual lines (Janko et al., 2012; Juchno et al., 2007, 2014; Vasil'ev et al., 2007) are intermediate stages in the process of forming tetraploid bisexual forms (Arai and Fujimoto, 2013; Saitoh et al., 2010).

The influence of polyploidy on fish reproduction has previously been examined primarily using artificially induced triploids and, in most cases, sterility was a common outcome (for review, see Piferrer et al., 2009). Naturally occurring tetraploid males of *Misgurnus anguillicaudatus* produced diploid gametes (Zhao et al., 2014), whereas the ultrastructure analysis of tetraploid *Cobitis* testes revealed the presence of cysts with spermatogonia and spermatocytes and a lack of spermatids or spermatozoa (Juchno et al., 2017). Moreover, many cells within the cyst displayed degeneration, e.g. pyknotic nuclei, chromatin condensation and/or fragmentation (Juchno et al., 2017), indicating the process of cell death via apoptosis.

Apoptosis, or programmed cell death, occurs during the proper development and aging processes, and plays a homeostatic role in many physiological processes (Elmore, 2007). It has been well documented that it is essential during testicular development and spermatogenesis in mammals and takes place mostly in spermatogonia and spermatocytes (for review, see Sharma et al., 2015). Germ cell apoptosis occurs spontaneously during spermatogenesis but it can be induced by both internal and external environmental stressors such as hormones (Wang et al., 2019), an additional set of chromosomes (Cal et al., 2010), toxicants (McClusky, 2008), heat shock (Yabu et al., 2001) or pH changes (Iger and Wendelaar Bonga, 1994). Although the number of studies about the role of spontaneous (Prisco et al., 2003; Chaves-Pozo et al., 2005; Corriero et al., 2007; McClusky et al., 2008; Kaptaner and Kankaya, 2013; Zupa et al., 2013; Ribeiro et al., 2017) as well as excessive apoptosis (for reviews, see Takle and Andersen, 2007; Krumschnabel and Podrabsky, 2009) during fish spermatogenesis is growing, this area of research still remains poorly known.

The key role in the apoptotic signal transduction pathway is played by caspase-3. It is essential for activation of the endonuclease (caspase-activated deoxyribonuclease) responsible

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for DNA fragmentation. This leads to irreversible morphological and biochemical changes including reorganization of the cell into apoptotic bodies which *in vivo* undergo rapid phagocytosis (Saraste and Pulkki, 2000).

To the best of our knowledge, information on the role of apoptosis during spermatogenesis in polyploid fish is scarce and limited to the study of Cal et al. (2010). Here, we attempted to verify the hypothesis that apoptotic cell death occurs in the testes of *C. taenia* and natural tetraploid *Cobitis* hybrid males during various stages of the reproductive cycle, and that the incidence of apoptosis in tetraploid germ cells might be higher than that in diploid ones. Thus, the present study focused on detecting apoptosis by immune expression of caspase-3 in the testes of *C. taenia* from an exclusively diploid population as well as *C. taenia* and tetraploid *Cobitis* hybrids (with genomes of *C. taenia*, *Cobitis elongatoides* and *Cobitis tanaitica*) from a d–p population during the pre-spawning, spawning and post-spawning period.

MATERIALS AND METHODS

Ethics statement

Fish sampling was performed with the permission of the Polish Ministry of Environment (DOP-OZGIZ.6401.10.12.2011.ls) and all the experiments were conducted in accordance with the principles of Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn, Poland (no. 04/2011).

Fish specimens and tissue preparation

The study was carried out on a total of 36 fish, which were divided into three groups: (1) 12 males of diploid *C. taenia* (2n=48) from an exclusively diploid population in Legińskie Lake (53°58′4″N; 21° 08′20″E), (2) 12 males of diploid *C. taenia* (2n=48) from a mixed d–p population in Pilica River (51°34′27″N; 20°20′3″E) and (3) 12 males of tetraploid *Cobitis* (4n=98) from a mixed d–p population in Pilica River (51°34′27″N; 20°20′3″E). From each group, four fish were collected during three periods of the reproductive cycle: pre-spawning (May), spawning (June/July) and post-spawning (September) in 2014 and 2015 by net and then were transported alive to the laboratory. Fish were anesthetized with MS 222 and the testes quickly removed and fixed in 10% formalin in phosphate-buffered saline [PBS; at room temperature (RT), 4 h], dehydrated and then paraffin embedded. Sections of 5 µm thickness were floated on a distilled water bath (40°C), and collected on superfrost plus slides coated with 3-aminopropyltriethoxysilane (Sigma Aldrich, St Louis, MO, USA). Chromosome counting and comparative analysis of the chromosomal location of 28S and 5S rDNA sequences using double-color fluorescence *in situ* hybridization (FISH) were previously used to determine ploidy and genome composition of analyzed tetraploid *Cobitis* males (Boroń et al., 2006, 2015).

Histology and proportion of testicular germ cells

Deparaffinized and rehydrated sections were subjected to hematoxylin and eosin (HE) staining to define maturation of the specimens according to Juchno and Boroń (2006) as well as to calculate the ratio of each type of testicular germ cell (spermatogonia, spermatocytes, spermatids, spermatozoa), interstitium and lumen. Images for HE were recorded using a Nikon eclipse 80i light microscope at 200× magnification. For the percentage of germ cells, three randomly chosen digital fields (825-point grid) obtained from all *C. taenia* specimens under study (24 males) were analyzed using ImageJ software (Nascimento et al., 2017).

Cleaved caspase-3 immunohistochemistry

Testis sections (see 'Histology and proportion of testicular germ cells', above) were subjected to fluorescence immunohistochemistry (F-IHC) to detect caspase-3 expression in fish testes. Before F-IHC staining, an antigen retrieval procedure was carried out by heating sections in a microwave at 400–500 W in 10 mmol l^{-1} sodium citrate buffer (pH 6) with 0.05% Triton X-100 for 15 min. The sections were then left for 30 min in the buffer to cool. Non-specific binding of IgG was eliminated by incubating the sections in 50 mmol l^{-1} NH_4Cl for 30 min, followed by blocking in PBS supplemented with 10% normal goat serum (NGS; Cell Signaling Technology, Beverly, MA, USA), 0.1% Triton X-100 and 0.2% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at RT. Next, the sections were incubated in a humidified chamber with a rabbit polyclonal antibody to cleaved caspase-3 (17/19 kDa fragment of activated caspase-3; Cell Signaling Technology) diluted 1:400 in PBS supplemented with 0.1% BSA and 0.3% Triton X-100 (overnight, 4°C). The primary antibody was used based on previous studies performed on fish (McClusky et al., 2008; McClusky, 2013). After three washes for 10 min in PBS supplemented with 0.1% BSA, 0.05% saponin and 0.2% gelatin, the sections were incubated with anti-rabbit IgG (H+L), F(ab')2 Fragment conjugated to Alexa Fluor 488 fluorescent dye (Cell Signaling Technology) diluted 1:2000 (1 h, RT, darkness). Following three washes in PBS (each of 15 min), the sections were stained with propidium iodide (1 µg ml^{-1}) for 10 min in RT in the dark and mounted in Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Negative controls were generated by omitting the primary antibody. SignalSlide Cleaved Caspase-3 (Asp175) IHC Controls (Cell Signaling Technology) were used as a positive control.

Images for F-IHC analysis were recorded using a DM 5500 B fluorescence microscope with a digital camera (DFC 360 FX, Leica, Wetzlar, Germany). The analysis of caspase-3 immunoexpression was examined on three sections corresponding to three different regions (anterior, middle and posterior) of each testis obtained from four fish in each studied group during three periods of the reproductive cycle. Each section was photographed at 200× magnification. All images were analyzed using Leica Application Suite (LAS) software version 4.0.0 (Leica Microsystems, Switzerland). Caspase-3 immunoexpression data were expressed as ratio of mean grey value (optical density, OD) of caspase-3 immunodetection in each optical field to the total area of the testis section (mm^2).

Statistical analyses

All data are presented as the mean±s.e.m. All data were tested for normality by the Shapiro–Wilk test at the 5% level of significance. Non-normal data were log transformed and retested. Two-way analysis of variance (ANOVA) followed by *post hoc* NIR Fisher's test was used to assess statistical significance. Data expressed as a percentage of the testicular germ cells were arcsine transformed before ANOVA. Differences with a probability of $P<0.05$ were considered significant. Data analysis was performed using Statistica version 13.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Maturation of the male gonads was determined according to Juchno and Boroń (2006); as they described, four basic types of germ cells (spermatogonia, spermatocytes, spermatids, spermatozoa) were observed in the histological sections of *C. taenia* testes from both populations (diploid and d–p) before, during and after spawning (Fig. 1). Interestingly, different ratios of particular germ cells in *C. taenia* from the diploid and d–p populations (Fig. 2) indicated that those males exhibited no parallel development of spermatogenic

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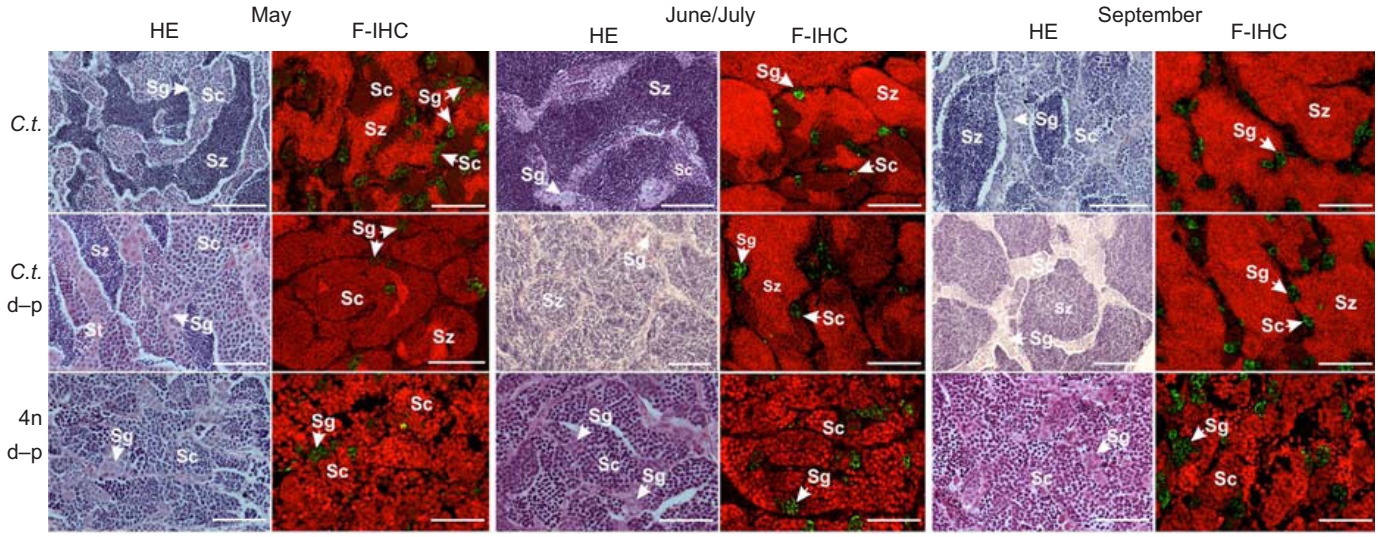

Fig. 1. Histological and apoptotic evaluation of *Cobitis* male gonads. Representative images of testis sections obtained from *Cobitis taenia* from an exclusively diploid population (*C.t.*) as well as *C. taenia* from a diploid–polyploid (d–p) population (*C.t.* d–p) and tetraploid *Cobitis* hybrids (4n d–p) from a diploid–polyploid (d–p) population in May (pre-spawning), June/July (spawning) and September (post-spawning), subjected to hematoxylin and eosin (HE) staining as well as fluorescence immunohistochemistry (caspase-3 expression; F-IHC). Green (Alexa Fluor 488 fluorescent dye-labeled anti-caspase-3 antibody) – positive signal; red (propidium iodide fluorescent dye) – negative signal. Sg, cyst of spermatogonia; Sc, cyst of spermatocytes; St, cyst of spermatids; Sz, spermatozoa. Scale bars: 100 µm.

cells and documented biological differences between the two populations. The significantly higher number of spermatozoa in testes of *C. taenia* from the diploid population (P<0.05; 61±2.39%) in comparison with those from the d–p population (29±3.54%) provided evidence that the steroidogenic signals triggering spermatogenesis were initiated earlier in males from the diploid population than from the d–p one. Moreover, histological sections of tetraploid *Cobitis* testes confirmed our previous studies (Juchno and Boroń, 2006; Juchno et al., 2017) where only spermatogonia and spermatocytes were visible during the entire reproductive cycle (Fig. 1).

In the present study, we showed that apoptosis occurred in testes of *C. taenia* from both diploid and d–p populations as well as in natural tetraploid *Cobitis* hybrids before, during and after spawning (Fig. 1). Apoptosis affected spermatogonia and spermatocytes in testes with normal spermatogenesis in *C. taenia* or only spermatogonia in infertile tetraploid *Cobitis* (Fig. 1). Similarly,

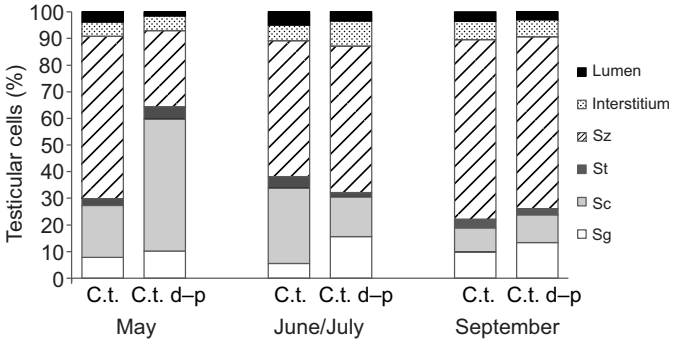

Fig. 2. Proportion of *Cobitis taenia* testicular cells during the reproductive cycle. Percentage of germ cells, interstitium and lumen in testes of *C. taenia* from exclusively diploid (C.t.) and d–p populations (C.t. d–p) in May (pre-spawning), June/July (spawning) and September (post-spawning). Values represent the mean±s.e.m. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; Sz, spermatozoa.

apoptosis was observed during the whole reproductive cycle in the testes of many other Teleostei (Prisco et al., 2003; Corriero et al., 2007; Kaptaner and Kankaya, 2013; Zupa et al., 2013; Ribeiro et al., 2017). In turn, some data indicated that the appearance of apoptosis during the reproductive cycle differed, taking place in fish testes only during pre-spawning (Cal et al., 2010) and/or post-spawning (Cal et al., 2010; Chaves-Pozo et al., 2005). Similar to the present results, previous studies indicated that the process occurred mainly in spermatogonia (Cal et al., 2010; Chaves-Pozo et al., 2005) or spermatogonia and spermatocytes (McClusky, 2008; Corriero et al., 2007, 2009; Zupa et al., 2013; Ribeiro et al., 2017), and rarely in spermatocytes and/or spermatids (Prisco et al., 2003; Kaptaner and Kankaya, 2013). So, apoptotic germ cell death shows a varying contribution to spermatogenesis in fish testes that may depend on the species, age, reproductive season or environmental conditions.

In the current study, caspase-3 immunoexpression in testes of *C. taenia* from the diploid population did not differ (P>0.05) before, during and after spawning while in testes of *C. taenia* from the d–p population and tetraploid *Cobitis* males the process increased (P<0.05) during spawning and post-spawning in comparison to pre-spawning (Fig. 3). Similar to our findings, higher levels of germ cell apoptosis were observed during spawning in comparison to pre-spawning in the testes of *Chalcalburnus tarichi* (Kaptaner and Kankaya, 2013). In contrast, a higher occurrence of apoptosis during pre-spawning versus spawning was found in Atlantic bluefin tuna (Zupa et al., 2013) and swordfish *Xiphias gladius* (Corriero et al., 2007). This discrepancy between our results and those of others may be caused by species-specific features, e.g. the type of spawning, the state of gonad maturation. However, the present findings confirm that apoptosis is an integral component of testicular function in *Cobitis*. Moreover, this process may eliminate the undesirable, unknown or damaged germ cells formed in excess in spawning during spermatogenesis and eliminate residual cells after spawning.

The level of apoptosis in testes of *C. taenia* from the two studied populations differed before spawning (Fig. 3). It cannot be excluded that the higher caspase-3 immunoexpression (P<0.05) observed in

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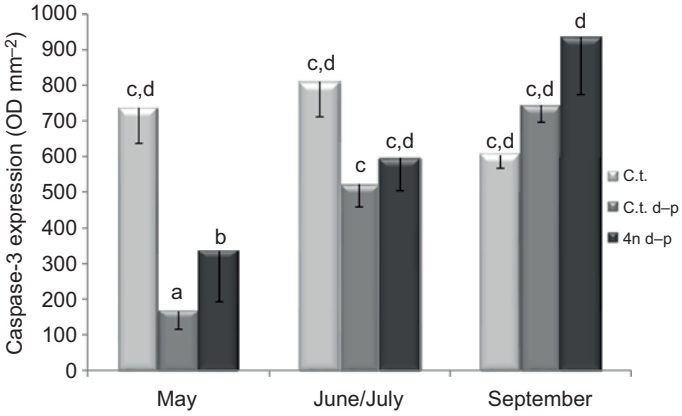

Fig. 3. Seasonal changes in caspase-3 immunoexpression in *Cobitis* testes. Expression levels in testes of *C. taenia* from an exclusively diploid population (C.t) as well as a diploid–polyploid (d–p) population (C.t. d–p) and tetraploid *Cobitis* hybrids (4n d–p) in May (pre-spawning), June/July (spawning) and September (post-spawning). Values represent the mean ±s.e.m. Different letters indicate significant differences (P<0.05).

testes of *C. taenia* from the diploid population in comparison to *C. taenia* from the d–p population (Fig. 3) is caused by a different male reproductive potential forced by the different male:female ratio in those two populations. Our previous results showed that *C. taenia* males from the diploid population (male:female ratio 1:3) showed a temporary rest from sperm maturation whereas males of this species from the d–p population that have to meet the reproductive challenge to fertilize more females (male:female ratio 1:18) were characterized by a continually maintained sperm production (Juchno and Boroń, 2006). The higher amount of spermatogonia during spawning (P<0.05; 16±2.32%) and after spawning (P<0.05; 13±0.85%) in *C. taenia* testes from the d–p population compared with *C. taenia* testes from the diploid population (6±0.61% and 10±0.9%, respectively) also supports the hypothesis of constant reproductive readiness of those males breeding in an environment with excess females. Both previous and current studies indicated the dynamic role of apoptosis in the quantitative control of germ cell population in *C. taenia*.

The present results show that, in contrast to findings in diploid *C. taenia*, apoptosis eliminates only spermatogonia from tetraploid *Cobitis* testes. It is known that polyploidy in animal cells causes problems for the completion of mitosis as a result of the presence of supernumerary centrosomes following multipolar mitotic spindle formation, which is a source of abnormal segregation of chromatids and production of aneuploid cells (Woodhouse et al., 2009). Previously, Juchno et al. (2017) suggested that spermatogenesis of tetraploid *Cobitis* was arrested during meiosis I but the present study gives insight into errors in mitotic divisions of spermatogonia. The cause of chromatid/chromosome difficulties in mitotic/meiotic segregation in the testes of tetraploid *Cobitis* seems to be a mismatch of chromosomes from different genomes that create their genome (4n=98; two genomes of *C. taenia*, one genome of *C. elongatoides* and one of *C. tanaitica*; Boroń et al., 2015). It would be worth checking in the future what the signal is that triggers the spermatogonial mitotic errors in tetraploid *Cobitis* hybrids.

The expected higher level of apoptosis in *Cobitis* tetraploid germ cells in comparison to diploid ones was observed only before spawning (P<0.05) and the difference was limited to *C. taenia* from the same population (Fig. 3). So it seems that in sterile tetraploid *Cobitis* testes, apoptosis is not the only process for germ cell elimination, as it was observed in testes of sterile triploid-induced turbot (Cal et al., 2010). It is possible that some of the spermatogonia and spermatocytes in testes of tetraploid *Cobitis* might also undergo other types of cell death at the same time. Juchno et al. (2017) observed ultrastructural changes in the tetraploid *Cobitis* germ cells such as swollen or abnormally large crescent-shaped mitochondria, dilated smooth endoplasmic reticulum or large vacuole-like structures in the cytoplasm that might indicate autophagy. During this process, the cellular components are degraded through lysosomal machinery. It has been reported that autophagy can act as an alternative death pathway to apoptosis; however, interaction between apoptosis and autophagy is complicated and depends on the cellular context (Gonzalez et al., 2018). Information concerning the role of autophagy in the testis is scarce and limited to rodents (Gonzalez et al., 2018). Nevertheless, Huang et al. (2018) reported that increased autophagy in ovaries of triploid female rainbow trout *Oncorhynchus mykiss* caused gonadal regression and remodeling. It cannot be excluded that similar processes may take place in the testes of tetraploid *Cobitis* hybrids.

In summary, the present studies add new insight into the physiology and reproductive biology of *C. taenia* and natural tetraploid *Cobitis* hybrid males, underlining the role of apoptosis during spermatogenesis. The obtained results confirm the hypothesis that apoptosis takes place in testes of *C. taenia* as well as tetraploid *Cobitis* hybrids during the entire reproductive season. The variable presence of programmed cell death in testes of *C. taenia* from diploid versus d–p populations supports a possible role for apoptosis as a regulator of germ cell stock and as an eliminator of damaged or not-recognized germ cells. Moreover, it seems that some of the germ cells in the testes of tetraploid *Cobitis* hybrids might also undergo other types of cell death, which would be worth investigating in future studies.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: O.J., A.L.; Methodology: O.J., D.J., K.K.; Formal analysis: O.J.; Writing - original draft: O.J.; Writing - review & editing: D.J., A.L., A.B.; Visualization: D.J.; Supervision: A.B.; Funding acquisition: A.B.

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