

The influence of ploidy level on ultrastructure and motility of tench *Tinca tinca* (L.) spermatozoa

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Abstract The ultrastructure of diploid and triploid tench *Tinca tinca* (L.) spermatozoa were examined using electron microscopy focusing on parameters that influence movement. Triploid tench were produced artificially using a cold shock. Spermatozoa of triploid males in comparison with diploids featured significantly larger head ($P < 0.01$), higher amount of mitochondria ($P < 0.05$), and, surprisingly larger widths of the peripheral doublets and central pair of microtubules and the single microtubule ($P < 0.01$). However, the diameters of the flagellum were without significant differences as well as the length of the flagellum and length and width of the midpiece. Also motility parameters of spermatozoa did not significantly differ between diploid and triploid males, but the total velocity (summary of spermatozoa velocity and duration of movement) positively correlated with the flagellum length and negatively with the head diameter of tench spermatozoa with a high significant influence ($P < 0.01$).

Keywords Microtubule · Mitochondria · Ploidy level · Spermatozoa · *Tinca tinca* · Ultrastructure

Introduction

The tench is an interesting fish from the viewpoint of polyploidy and related atypical reproduction aspects. Polyploidization, gynogenesis with control of sex has been intensively studied in tench (Flajshans et al. 1993a, b, 2004; Linhart and Billard 1995). Among Czech strains, natural triploids have been recorded several times and growth in a triploid tench was significantly higher than in a diploid tench (Flajshans et al. 1993b, 2004; Linhart et al. 2006; Buchtova et al. 2003).

Ploidy level can be manipulated and gonadal sex differentiation disrupted by triploidization in many fish species (e.g. Thorgaard 1983; Purdom 1983; Donaldson and Devlin 1996; Devlin and Nagahama 2002). In salmonids, triploid females have poorly developed gonads with very few developing oocytes. Spermatogenesis was reported in triploid males, but sterility occurs due to random segregation of trivalents, which produces aneuploid sperm (Lincoln and Scott 1983; Benfey et al. 1986; Ueda et al. 1987; Hussain et al. 1996; Devlin and Nagahama 2002).

Gonads of triploid tench were reported to be rudimentary with fat deposits. Microscopic observation of ovaries reveals mainly oogonia and oocytes in protoplasmatic growth. Rudimentary testes contained all developmental stages of spermatogenesis (Buchtova et al. 2003). Weight of testes, gonadosomatic index, volume of sperm and motility were significant

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higher in diploid tench males than in triploids. In addition, fertilization and hatching tests revealed that sperm of triploid tench was able to fertilize the eggs of diploid females, but with significantly less efficacy compared to diploid males, resulting in mostly unviable larvae (Linhart et al. 2006).

Humphries et al. (2008) and Stoltz and Neff (2006) generally describe possible links between morphology of spermatozoa and their speed. Psenicka et al. (2008b) found a correlation between the flagellum length and velocity and motility in Siberian sturgeon and sterlet especially at the end of motility period. It can give an advantage in the case for external fertilizers in which females have relatively limited opportunity to influence a sperm's motility. Oviparous fishes as tench have external reproduction strategy with the so-called "aqua sperm" (Jamieson, 1991). Spermatozoa exhibit a hyper motile behavior for 60–90 s (Zuromska and Markowska 1984; Linhart et al. 2003).

According to Psenicka et al. (2006), tench spermatozoa of 26.1 μm total length possess typical simple structure without acrosomal head structures. It is probably the smallest described spermatozoon among cyprinid fishes. Heads of the spermatozoa are mostly composed of dense and slightly granular material, which appeared to be fairly homogeneous except for the occasional presence of nuclear vesicles. These vesicles originate from incomplete dehydration of nucleus during spermiogenesis in teleost spermatozoa. It can be species-specific and also nutrition-dependent, such as in tench (Psenicka et al. 2006) and barb *Barbus barbus* (Alavi et al. 2008a, b). The cylindrical/cone-shaped midpiece remains separated from the flagellum by the cytoplasmic channel. The distal centriole appears almost tangential to the nucleus and it serves as a basal body for the flagellum. It has an angle orientation of 140° with respect to the proximal centriole. The angle is species-specific in the cyprinid fish (Baccetti et al. 1984; Psenicka et al. 2006). The sperm flagellum has no fin, which is found for example in sturgeon (Psenicka et al. 2007, 2008a, b). A flagellar vesicle is attached to the most basal region of the flagellum and is located just under plasma membrane of the flagellum.

This study shows detailed structure and relationship with motility parameters in diploid and triploid tench spermatozoa.

Materials and methods

Samples source

Four-year old diploid and triploid tench populations were established in USB RIFCH Vodnany by mass artificial propagation of tench breeds (Vodnany, Hluboka, Marianske Lazne, Tabor, Hungarian, and Romanian; Flajshans et al. 2004). Triploidy ($3n$) was induced by means of cold shock following the protocols of Flajshans et al. (1993a, b). Ploidy level was verified in fresh sperm and blood cells collected in an immobilizing solution (180 mM NaCl, 2.68 mM KCl, 1.36 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.38 mM NaHCO_3 ; Rodina et al. 2004) and physiological solution, respectively. Sperm and blood samples were prepared according to Vindelov and Christensen (1990) and examined using Partec CCA I flow cytometer. The erythrocytes and spermatozoa of diploid tench male gave the relative DNA content of $2n$ as the diploid and $1n$ as the haploid standards. In addition, genotyping of six microsatellite loci was used to verify ploidy level of observed individuals. Protocol of PCR amplification on microsatellite loci and fragment analysis by automated sequencer were performed as in Kohlman and Kersten (2006).

Electron microscopy

Sperm was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 days at 4°C and postfixed and washed repeatedly for 2 h at 4°C in 4% osmium tetroxide and dehydrated through an acetone series. Samples for SEM were dehydrated at the Critical Point Dryer Pelco CPD 2 (Ted Pella, Inc., Redding, CA, USA). The samples were coated with gold under vacuum with an SEM Coating Unit E5100 (Polaron Equipment Ltd, England) and studied using a JSM 6300 scanning electron microscope for morphological parameters and JSM 7401 S (JEOL Ltd, Akishima, Tokyo, Japan, for both equipment) in cryo-regime for fine details. Samples for TEM were embedded in resin (Polybed 812). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Mikrosysteme GmbH, Austria), double-stained with uranyl acetate and lead citrate and viewed in a JEOL 1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) operated at 80 kV.

Sperm samples intended for TEM visualization of mitochondria according to Psenicka et al. (2008a) were briefly fixed in glutaraldehyde and incubated at room temperature (RT) for 15 min in medium prepared by mixing of 0.2 M Na₂HPO₃ (6.5 cm³), 0.2 M succinic acid (0.2 cm³), 0.2 M potassium sodium tartrate (5 cm³), distilled water (4 cm³), polyvinylpyrrolidone (PVP; 1.5 g), 0.1 M CuSO₄ (1 cm³) and 0.02 M potassium ferricyanide (1.5 cm³). After incubation, sperm samples were washed in 20 cm³ of a solution containing 0.2 M NaH₂PO₄, 2.5 M Na₂HPO₄ and PVP (1.5 g) and finally processed for TEM as mentioned above.

Micrographs were evaluated using Olympus MicroImage software (version 4.0.1. for Windows) to measure morphological parameters of spermatozoa.

Sperm motility

Sperm velocity ($\mu\text{m s}^{-1}$) and percentage of motile sperm after activation (%) were measured using dark-field microscopy (200 \times magnification). For measurement of sperm motility, sperm was diluted in distilled water. To avoid sperm sticking to the slide, 0.1% BSA was added. Sperm motility was analyzed from a video recording taken after activation with a three CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (NIKON Optiphot 2, Japan). The successive positions of the recorded sperm heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP, Japan) and analyzed, from five successive frames each, with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows).

Data analysis

Analysis of differences in 30 times repeated measurement of morphological parameters of spermatozoa of six diploid and five triploid individuals was carried out by *t*-test (Statistica 8).

The area of the midpiece occupied by mitochondria and vesicles and the area of the nucleus occupied by nuclear vesicles are presented as percentages and thus had to be subjected to arcsin transformation prior to any further processing.

Motility and velocity values estimated by analysis of more than 100 spermatozoa per each of male were

analyzed by Factorial ANOVA. In addition, the stepwise regression with forward selection comparing average parameters of spermatozoa and ploidy level with “total velocity” was performed. Total velocity is average values from 15th, 30th and 45th s of spermatozoa movement \times percentage of motile spermatozoa/100, dead spermatozoa evaluated at the beginning of activation were subtracted). Influences of individual parameters were evaluated by means of standardized partial regression coefficient (β).

General formula of total velocity

$$V_t = \frac{V_o + \sum_1^{\infty} V_n(M_n + D_o)}{n + 1}$$

V_t —total velocity, V_o —velocity of motile spermatozoa in first time of evaluation, V_n —velocity of motile spermatozoa in other time of evaluation, M_n —percentage of motile spermatozoa in the time of evaluation/100, D_o —percentage of dead spermatozoa evaluated at the first time of evaluation/100, n —number of evaluation.

Results

Spermatozoon of triploid tench males (similarly to diploids) were found to be differentiated into three main parts: a head devoid of an acrosome with nuclear vesicles in nucleus; a midpiece containing a centriolar complex, mitochondria and midpiece vesicles; and a flagellum consisting of an axoneme and flagellar vesicles in a basal part (Figs. 1a, b, 2a, b, 3, 4a, b).

Sperm heads of diploid and triploid males were 1.65 ± 0.03 (mean \pm SD) vs. 1.71 ± 0.09 μm in width and 1.27 ± 0.05 vs. 1.58 ± 0.12 μm in length, respectively. Highly significant differences ($P < 0.01$) were found between both parameters. However, no significant differences were found among the parameters of the midpiece (length 1.04 ± 0.08 vs. 1.08 ± 0.17 , width 0.87 ± 0.07 vs. 0.94 ± 0.08 μm in diploid and triploid males, respectively) as was the length of the flagellum (25.86 ± 1.59 μm in diploid vs. 26.85 ± 0.99 μm in triploid males). The diameters of the flagellum were also almost identical among diploid ($172.47 \pm 5.24 \times 196.02 \pm 6.13$ nm) and triploid males ($173.67 \pm 5.88 \times 200.71 \pm 7.21$ nm). On the contrary, within the inner ultrastructure of the midpiece (Fig. 2a) the staining of mitochondria revealed

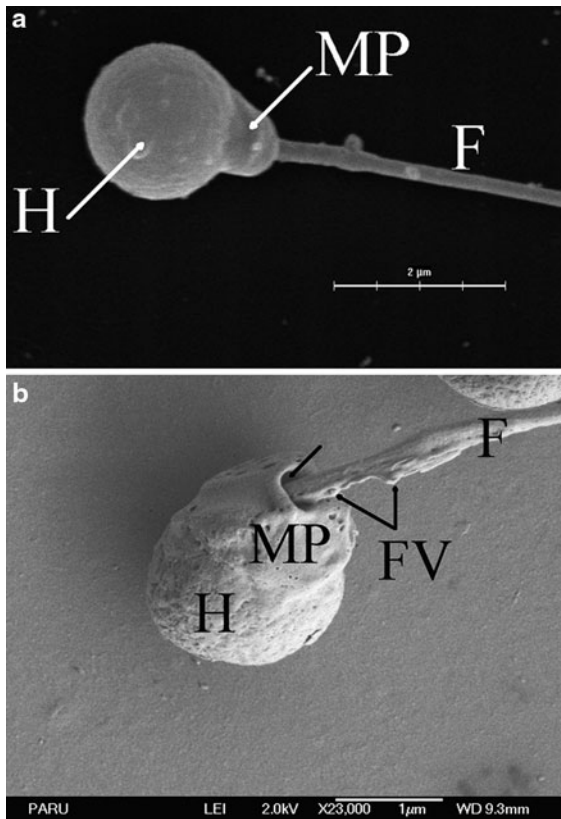


Fig. 1 **a, b** Scanning electron micrographs taken by means of **a** JSM 6300 showing spermatozoon of diploid male (*scale bar* = 2 µm) and JSM 7401 S **b** showing spermatozoon of triploid male (*scale bar* = 1 µm) with head (*H*), midpiece (*M*), flagellum (*F*), cytoplasmic channel (*arrow*) and flagellar vesicle (*FV*)

significant differences in numbers and area occupied by them in the TEM sections (3.60 ± 0.55 , $50.02 \pm 2.16\%$ in diploid vs. 4.85 ± 0.57 , $62.86 \pm 3.66\%$ in triploid). Highly significant differences ($P < 0.01$) were surprisingly found also in the diameter of microtubules. The peripheral microtubules had 24.45 ± 1.18 vs. 27.06 ± 1.46 nm and the central microtubules 28.42 ± 0.79 vs. 30.77 ± 0.92 nm in diameter between diploid and triploid males, respectively (Fig. 4a, b). Other ultrastructural parameters of spermatozoa, such as nucleus vesicles (1.42% in diploid vs. 1.50% in triploid), midpiece vesicles (18.36% in diploid vs. 16.98% in triploid) and diameter of centrioles (285 nm in diploid vs. 290 nm in triploid), did not significantly differ between diploid and triploid males. The values of main parameters are listed in Table 1.

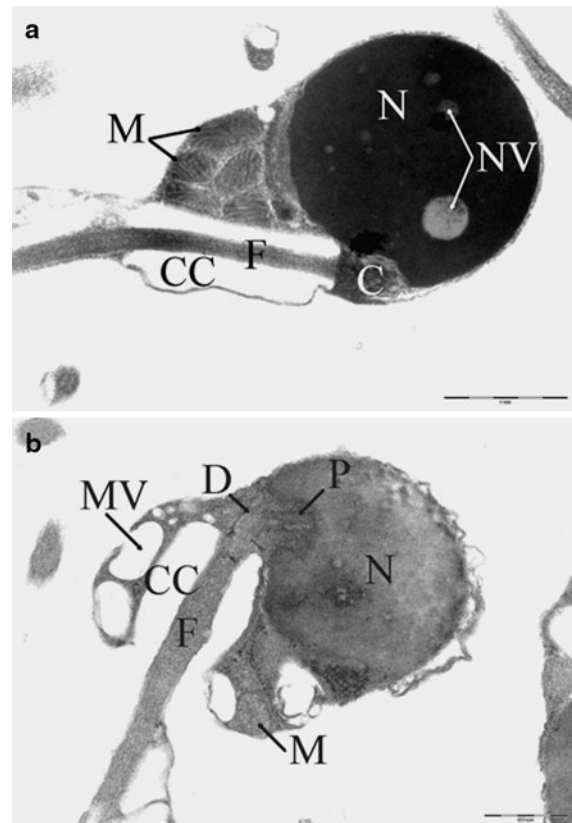


Fig. 2 **a** Transmission electron micrograph shows a longitudinal section of spermatozoon of triploid male with nucleus (*N*), nuclear vesicle (*NV*), centriolar complex (*C*), flagellum (*F*), cytoplasmic channel (*CC*) and mitochondria (*arrow*), which were stained according to protocol for evidence of succinic dehydrogenase. *Scale bar* = 1 µm. **b** Transmission electron micrograph shows a longitudinal section of spermatozoon of triploid male with nucleus (*N*), proximal and distal centriole (*P* and *D*), mitochondria without any staining (*M*), midpiece vesicles (*MV*), cytoplasmic channel (*CC*) and flagellum (*F*). *Scale bar* = 500 nm

The percentages of motility differed significantly between times of evaluation and ploidy levels. Contrariwise the values of velocity differed only between times, but not in ploidy levels ($P < 0.05$, Table 2). The percentage of motile spermatozoa and their velocity within the motility period was summarized together as one parameter; the total velocity. The stepwise regression selected significant variables from all evaluated values. The order according to influence on total velocity was: the flagellum length with positive correlation and the head diameter with negative correlation (beta = 0.784 and -0.57 , respectively; $R^2 = 0.89$, $P < 0.01$).

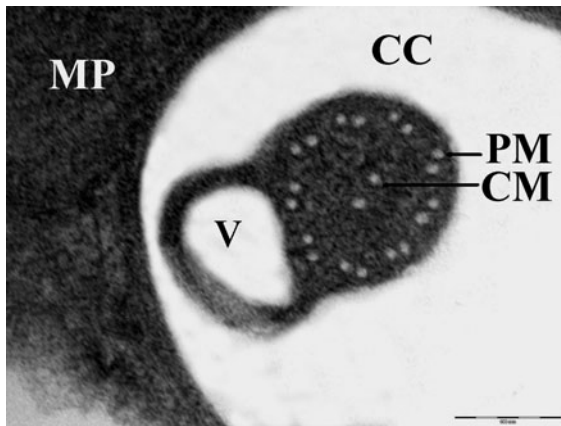


Fig. 3 Transmission electron micrograph shows a cross section of spermatozoon of diploid male with midpiece, where the flagellum (*F*) containing the flagellar vesicle (*FV*) and peripheral doublets and central pair of microtubule (*PM* and *CM*), passes through the cytoplasmic channel (*CC*). Scale bar = 100 nm

Discussion

The changes of some general parameters, such as live weight, weight of testes, gonadosomatic index, volume of sperm, motility, velocity, outer structures and viability of spermatozoa, fertilization and hatching rate, caused by ploidy level have been already described by Linhart et al. (2006). In the present study we followed up and further investigated detailed ultrastructure and compared it with motility. Initially the increased size of heads of spermatozoon of triploid fish has to be mentioned. That logically comes from increased DNA content in nucleus of individuals with higher ploidy level. Chourrout et al. (1986) suggested that low fertilization rate in artificially induced tetraploid males of rainbow trout was caused by the head size of spermatozoa, which would be an obstacle during penetration to the micropyle.

Surprisingly, in spite of the larger head, motility parameters of spermatozoa were without significant differences, from which we presumed higher potential in terms of sperm movement. Following this hypothesis we focused on the amount of mitochondria, which provide energy for the movement of spermatozoa and on the length of flagellum as a motility apparatus. Significantly higher amount of mitochondria in spermatozoa of triploid tench was found. Similarly, Dong et al. (2005) described this feature in tetraploids of Pacific oysters in comparison

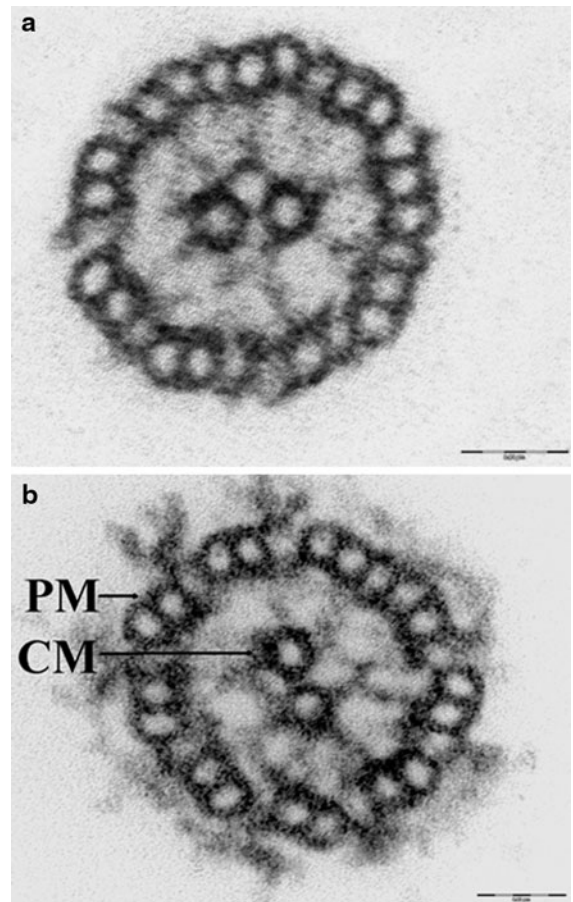


Fig. 4 Transmission electron micrograph shows a cross section of axoneme of diploid (a) and triploid (b) male spermatozoa with the peripheral doublets and central pair of microtubule (*PM* and *CM*). Scale bar = 0.05 μ m

with diploid ones. Probably the amount of mitochondria accurately balanced the increase in diameter of spermatozoa heads in individuals with higher ploidy level. As triploid tench balance a lower number of erythrocytes in the body with higher a content of hemoglobin in erythrocytes (Svobodova et al. 1998), spermatozoa of triploid males can compensate larger heads with greater amounts of mitochondria. In addition a larger, although nonsignificant, size of the spermatozoa midpiece of triploids was found. According to Cosson et al. (1999) the spermatozoa with smaller midpiece tend to swim for a shorter time than spermatozoa with larger midpiece, but the number or amount of mitochondria should be less important for short period of spermatozoa movement. The decrease of spermatozoa velocity as well as that

Table 1 The average values of selected parameters of each male

Ploidy level	Total velocity ($\mu\text{m/s}$)	Flagellum length (μm)	Flagellum diameter (nm)	Area of mitochondria (%)	Number of mitochondria	Head diameter (μm)	Midpiece length (μm)	Midpiece width (μm)	Peripheral microtubule diameter (nm)	Central microtubule diameter (nm)
2n	57.02	27.30	193	0.52	3.40	1.39	1.01	0.79	24.36	28.90
2n	30.43	23.81	184	0.47	4.41	1.50	1.07	0.89	24.85	27.57
2n	57.32	28.10	179	0.53	3.29	1.37	0.96	0.93	26.17	29.95
2n	51.87	25.41	177	0.48	4.17	1.44	1.20	0.82	25.25	28.12
2n	38.85	24.05	188	0.49	3.56	1.43	0.98	0.99	22.54	27.86
2n	55.53	26.50	183	0.51	2.78	1.42	1.01	0.80	23.52	28.14
3n	30.51	25.15	188	0.59	5.81	1.74	1.26	0.86	25.23	29.94
3n	44.03	26.97	180	0.62	4.78	1.70	0.91	0.92	28.12	31.98
3n	53.85	28.12	194	0.68	4.30	1.60	0.87	0.97	29.65	31.13
3n	46.13	27.45	177	0.67	5.11	1.68	1.26	1.08	28.39	32.62
3n	43.22	26.58	194	0.60	4.27	1.69	1.11	0.87	27.46	30.84

Table 2 The average values of velocity ($\mu\text{m s}^{-1}$) and percentage of motile spermatozoa of diploid and triploid males

Time	15 s	30 s	45 s
Velocity			
Diploids	99.06	64.19	38.52
Triploids	91.13	58.02	40.41
Motility			
Diploids	98.37	95.36	86.45
Triploids	94.27	92.78	73.03

of the beat frequency and wave amplitude of flagella corresponds to the decrease of intracellular ATP concentration. ATP synthesis rate of mitochondria is too slow if related to energy consumption by the motility mechanics of fish spermatozoa during their movement. This is consistent with results showing that respiration is at the same level before and after initiation of motility in rainbow trout spermatozoa (Christen et al. 1987). Energy consumed by flagellum motion is generated mainly from the ATP accumulated in the flagellum before the onset of activation. The amount of phosphocreatine needed to cover the ATP degradation never fulfils sufficiently the ATP requirement necessary to balance the need during the motility period. Finally, the amount of mitochondria might not be as important for velocity as the content of ATP accumulated in flagellum before triggering the sperm motility. The energy of midpiece mitochondria is used for spermatozoa movement mainly in case of spermatozoa re-activation (Linhart et al. 2008).

Total velocity used in the present study has purpose to summarize the spermatozoa velocity and the duration of their movement for statistical analyses. The main idea was to devise an average parameter of velocity, where is counted with dead spermatozoa at the beginning and spermatozoa, which consume energy during evaluation. Total velocity and the length of spermatozoa flagellum were similar within both ploidy levels of males, but only the head diameter and the flagellum length were significantly correlated with the total velocity in the present study. Humphries et al. (2008) suggest to use the ratio of head to tail length to assess the possible link between morphology and speed of spermatozoa. The present study confirmed this opinion. We suppose that mitochondria content has a function to balance changes in ploidy level, but the head

diameter as a higher load for spermatozoon and the flagellum length as main tool for movement, in which more ATP can be accumulated, have the main influence on spermatozoa velocity.

The unusual increasing of axoneme microtubules diameter of diploid/triploid tench spermatozoa was found in the present study. Although this fact needs deeper study with using methodology for microtubules visualization, we suppose that there can be a change in count of protofilaments number, those from the microtubules. Davis and Gull (1983) described differences in the number of protofilaments within several species of nematodes with all the examined tissues (nerve, intestinal, pharyngeal, and hypodermal cells) containing the same number of microtubule protofilaments within one species. In future studies, the examination of microtubules from several tissues of tench with different ploidy level should be done.

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