

The influence of physiological activity parameters and protein composition of spermatozoa of first-time and repeat spawning males of Antonino-Zozulenets carp on egg fertilization

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Abstract. In Ukrainian aquaculture, Antonino-Zozulenets carps are a structural unit of Ukrainian breeds and are represented by two types - scaly and framed ones. The main method of creating initial selective breeding stocks was through continuously improving (mass) selection, based on the assessment of carp productivity, by a set of traits and the selection of the most adapted individuals to growing conditions, biologically complete, with noticeable changes in useful traits. The focus was on the description of biological and productive parameters from a number of scientific works. Therefore, this work is devoted to the study of the physiological and biochemical parameters of milt, which will allow providing a comprehensive characterization of males and to develop the main directions of selective breeding works with Antonino-Zozulenets carps. The average ejaculate volume in the experimental fish was in the range of 7.6-21.2 cm³, and the duration of active movement was 48.6-68.3 s. Repeat-spawning males of both experimental groups had the largest number of spermatozoa with straight line movement. However, the velocity between the initial and final points of the path in scaly fish was at the same level, while in framed fish it was higher in repeat spawners compared to first-time spawners. The analysis of the protein content proved that the sperm of scaly males had a higher content of prealbumins, while the sperm of framed males had a higher content of haptoglobins (Hp-globins), β -globulins and albumins. The study of catalase isozymes showed that six catalytically active catalase bands appeared in the sperm of first-time and repeat-spawning males, but their relative content was different and depended on the genetic origin of the males. The average rate of fertilization of all experimental groups was at a high level and exceeded 90%. The maximum value of 93.1% was recorded in repeat-spawning framed males.

Key Words: general sperm activity, straight line movement, curvilinear movement.

Introduction. In selective breeding works, it is important to select males with high quality parameters of not only productive traits (Vandeputte et al 2008; Tang et al 2020), but also reproductive ones, such as the quality of sperm production and the fertilizing ability of sperm (Kvarnemo 2022; Billard et al 1995). The qualitative characteristics of sperm reflect the relationship between the fertilization process and the further development of a free embryo (Ciereszko et al 2017). At the same time, it is worth noting that physiological and biochemical parameters are very variable and can be characterized by individual and seasonal changes, even at the individual level, and depend on a number of biotic and abiotic factors (Zhang et al 2023; Alavi & Cosson 2005; Dzyuba & Cosson 2014). At the same time, feeding during the prespawning period is important (Izquierdo et al 2001; Syrovatka et al 2023; Iurchak et al 2018) and, accordingly, it is possible to influence the composition of sperm proteins by increasing their fertilizing ability through the adjustment of the diet composition (Mohammadi & Mesbah 2018). These factors should be taken into account during practical works, because they can have both positive and negative consequences for the development of germ cells (Cabrita et al 2014).

For a long time, the evaluation of sperm density, concentration, activity and survival of spermatozoa was used to determine the qualitative characteristics of ejaculates (Kuts et al 2023). However, these parameters do not provide full characteristics of milt and do not indicate the fertilizing ability of sperm. Therefore, more extensive studies should be conducted to assess the sperm quality. The physiological parameters of sperm activity and enzymes can be used for the most sensitive and genetically determined tests (Li et al 2011), and also for studying the physiological characteristics of sperm during long-term storage (Dietrich et al 2015; Dietrich et al 2021; Babiak et al 2006a).

The membranes of spermatozoa are susceptible to peroxidation due to an excess of reactive oxygen species, therefore it is appropriate to conduct studies on the status of one of the enzymes of the enzymatic pathway of antioxidant protection, which is the catalase. Its main function is the neutralization of hydrogen peroxide by splitting H_2O_2 with the formation of water and oxygen (Shaliutina-Kolešová et al 2014; Sadiqul & Akhter 2011). Previous studies proved the positive effect of catalase activity on motility, viability and lipid peroxidation (Kankofer et al 2004; Pagl et al 2006; Bansal & Cheema 2016; Singh et al 2020).

Evaluation of sperm motility as a research method has become widely used in reproductive technology because it allows establishing the quality of the obtained germ cells, detecting abnormalities and preventing inefficiency of fertilization (Browne 2015; Gallego & Asturiano 2018). Motility is one of the most important characteristics associated with the fertilizing ability of spermatozoa, indicating their viability and structural integrity (Alavi et al 2019; Bondarenko & Cosson 2019). Evaluation of motility through kinetic parameters such as curvilinear, straight line and average path velocity, is one of the main directions of studies of milt in many fish species (Alavi et al 2010; Gallego et al 2014; Babiak et al 2006b). Literature suggest that the active movement of spermatozoa should last at least 30 seconds, while the survival period can reach two days (Billard et al 1995; Cejko et al 2022). A detailed analysis of the motility, evaluating the dynamics of the spermatozoa of brood fish, classifies sperm according to the categories of motility: fast and straight linear movement, slow and straight linear movement, curvilinear movement and completely immobile spermatozoa (Fauvel et al 2010). One of the most important factors responsible for successful reproduction is the quality of germ cells. Therefore, the aim of the study was to analyze the dynamic parameters of sperm and the enzymatic activity. The study of the protein composition of milk and the activity of spermatozoa will contribute to the deepening of knowledge about the ability to fertilize and contribute to the improvement of reproduction technology through an enhancement of productive parameters of the new inbred types of carp.

Material and Method

The objects of the study were 4-10 years aged sexually mature males of the Antonino-Zozulenets intrabreed type of the Ukrainian framed and scaly carp (Figure 1). According to their sexual maturity, they were divided into two experimental groups: first-time spawners (age 4) and repeat spawners (age 4). All experimental groups of males were clinically healthy and corresponded to the "elite" class in terms of growth and development and later were used for spawning.



Figure 1. Males of the Antonino-Zozulenets intrabreed type of the Ukrainian scaly and framed carp breed.

Sperm were collected during the 2023 spawning campaign. The collection of milt samples was carried out in the conditions of the Stara Sinyava Pond of PJSC "Khmelnyskrybhosp" (Figure 2). Milt was collected in 2 mL Eppendorfs (Figure 3) and sent for further studies to the laboratory of reproduction biotechnology of the Institute of Animal Biology of the National Academy of Sciences, Lviv. Reproduction was carried out artificially. Egg incubation was conducted in 8 L Weiss incubators.

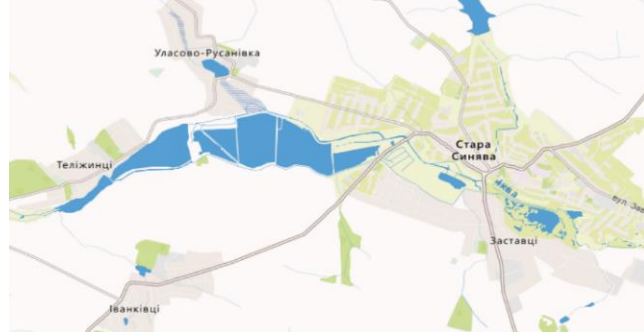


Figure 2. Location of the Stara Sinyava Pond Unit of PrJSC "Khmelnyskrybhosp".



Figure 3. Sperm selection.

The quality analysis of the physiological characteristics of spermatozoa was carried out under a microscope, using a digital video camera connected to a computer equipped with the SpermVision software, by determining the total activity, the percentage of sperm cells with straight line movement and curvilinear movement, as well as the proportion of dead spermatozoa. The computer system CASA (Computer Assisted Sperm Analysis)-Sperm Vision was used for determining the kinetic parameters of spermatozoa: VAP –average path velocity, $\mu\text{m sec}^{-1}$; VSL – straight line velocity, $\mu\text{m sec}^{-1}$; VCL – curvilinear velocity, $\mu\text{m sec}^{-1}$; STR – straightness (VSL/VAP), %; LIN –linearity (VSL/VCL), % (Özgür 2019; Yaremchuk 2012). The content of soluble protein fractions was evaluated by vertical electrophoresis in 7.5% polyacrylamide gel (PAAG) plates (Vlizlo 2012). Samples for electrophoresis were prepared as follows: 0.1 mL of sperm were diluted 1:12 with electrode buffer (pH 8.5), 0.1 mL of the sample were mixed with a similar amount of 40% sucrose, 0.02 mL were placed into the concentrating wells with gel ($\sim 150\text{--}200 \mu\text{g}$ of protein). The marker stain (0.01% solution of bromophenol blue) was added to the electrode buffer before diluting the samples. After electrophoresis, the gels were fixed and simultaneously stained in 12.5% trichloroacetic acid with 0.25% aqueous coomassie R 250.

Catalase isozymes (CAT) were detected after electrophoresis in 7.5% PAAG. The gel plates were stained according to W. Woodbury (Wodbury et al 1971): they were kept for 45 min in distilled water, saturated with 0.003% hydrogen peroxide solution for 10 min, washed three times with water and incubated at room temperature in the dark for 15 min, in a medium containing a 1% solution of potassium ferricyanide (III) and ferric chloride. After the PAAG staining, the locations of catalase proteins appear as bright yellow bands on a blue-green background. Analysis of the content of soluble protein fractions and the content of CAT isozymes (%) was performed using the TotalLab TL120.

Statistical analysis was performed using ANOVA in SPSS Statistics 23.0 (IBM Knowledge Center, USA, 2020). The significance of the obtained data was assessed at the level of $P < 0.05$. Data are presented as $x \pm SD$ (mean \pm standard deviation).

Results. The average ejaculate volume in the experimental fish was in the range of 7.6-21.2 cm^3 , with the average ejaculate volume of $7.6 \pm 1.8 \text{ cm}^3$ in framed and $19.8 \pm 2.9 \text{ cm}^3$ in scaly first-time spawning males, while the spermatozoa activity was 57.2 s and 68.3 s, respectively. In repeat spawning males, these values were exceeded by 12.6% in framed and 17.6% in scaly carps, with an average ejaculate volume of $18.3 \pm 5.2 \text{ cm}^3$ and $21.2 \pm 6.3 \text{ cm}^3$, respectively (Figure 4).

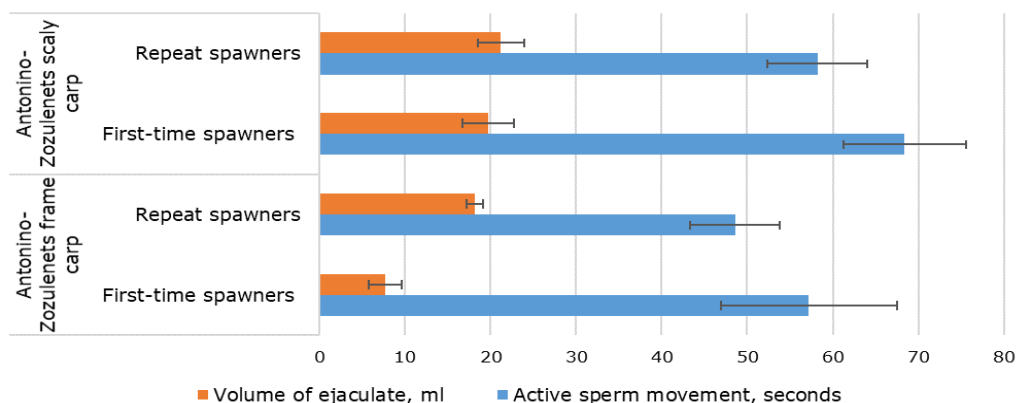


Figure 4. Reproductive parameters of first-time spawning and repeat spawning males of the Antonino-Zozulenets intrabreed carp by type: the duration of active spermatozoa movement (s), end ejaculate volume (mL) ($x \pm SD$, $n=5$).

Repeat spawning framed males, despite the low active movement of their spermatozoa, of 48.6 s, had the highest rate of total activity, of 92.6%, and the highest relative content of spermatozoa with straight line movement, of 84.4%. At the same time, this experimental group was characterized by the smallest number of dead spermatozoa, 6.23%. According to the relative content of spermatozoa with straight line movement, the repeat spawning males were superior over the first-time spawners in both experimental groups, namely by 13.0% ($P < 0.01$) for the scaly carps and by 24.4% ($P < 0.05$) for the framed carps. First-time spawning scaly males, having the highest activity index of 68.3 s, were inferior in terms of total activity, relative spermatozoa content with straight line movement and with curvilinear movement to first-time spawning framed individuals and repeat spawning framed and scaly individuals (Table 1).

Table 1
Physiological parameters of sperm quality after their activation in phosphate buffer, ($x \pm SD$, $n=5$)

Experimental group	Total activity, %	Relative content of spermatozoa with straight line movement, %	Relative content of spermatozoa with curvilinear movement, %	Relative content of dead spermatozoa, %
Scaly males				
First-time spawners	89.49 ± 3.20^a	69.80 ± 2.28^a	19.68 ± 3.22^a	10.51 ± 3.20^a
Repeat spawners	88.76 ± 3.17^a	82.76 ± 3.17^b	6.00 ± 0.96^b	11.24 ± 3.17^a
Framed males				
First-time spawners	79.01 ± 4.42^a	60.01 ± 5.36^a	19.00 ± 3.55^a	20.99 ± 4.42^a
Repeat spawners	92.57 ± 2.79^a	84.36 ± 1.88^b	9.41 ± 1.52^b	6.23 ± 1.79^b

Values that share a common letter are not significantly different. Differences between experimental groups were considered $P < 0.05$.

The straight line velocity in the studied groups was in the range of 31.7-46.7 $\mu\text{m sec}^{-1}$. At the same time, it is worth noting that the VSL of scaly first-time and re-spawning males was at the same level and amounted to 40.2 $\mu\text{m sec}^{-1}$. On the other hand, in individuals with a framed type of scaly cover, there was a significant difference in the velocity values, with a spread of 14.9 $\mu\text{m sec}^{-1}$ ($P < 0.05$) between the maximum and minimum values.

The average curvilinear velocity (VCL), which characterizes the average velocity of spermatozoa movement over time along its real path, was significantly higher in repeat spawning framed males, by 27.7% ($P < 0.05$), and in scaly males, by 5.2% ($P < 0.05$) in comparison with first-time spawners. At the same time, the velocity was higher by 8.3 $\mu\text{m sec}^{-1}$ (10.1%) in framed repeat spawning males compared to repeat spawning scaly ones. For individuals of the same experimental group and for scaly first-time spawners, higher results were obtained for the straightness of spermatozoa movement (STR) - 71.6 and 71.8 $\mu\text{m sec}^{-1}$, respectively, that indicated a better fertilizing capacity of sperm obtained from individuals of these experimental groups. A similar pattern was observed for the average path velocity (VAP), where the superiority of repeat spawning scaly males over first-time spawners was of 3.5%, and 29.9% for framed males, respectively (Table 2).

Table 2
Evaluation of carp spermatozoa motility using CASA ($x \pm \text{SD}$, $n=5$)

Groups	VAP, $\mu\text{m sec}^{-1}$	VCL, $\mu\text{m sec}^{-1}$	VSL, $\mu\text{m sec}^{-1}$	STR, %	LIN, %
Scaly males					
First-time spawners	55.5 \pm 1.30 ^a	107.5 \pm 2.23 ^a	40.2 \pm 1.18 ^a	71.8 \pm 0.95 ^a	37.0 \pm 0.57 ^a
Repeat spawners	57.5 \pm 2.26 ^{ac}	113.5 \pm 5.94 ^{ac}	40.2 \pm 1.65 ^{ac}	69.4 \pm 0.73 ^a	35.2 \pm 0.9 ^a
Framed males					
First-time spawners	44.9 \pm 3.32 ^{bc}	88.1 \pm 7.00 ^{bc}	31.7 \pm 2.49 ^{dc}	69.8 \pm 0.52 ^a	35.6 \pm 0.46 ^a
Repeat spawners	64.1 \pm 2.6 ^a	121.8 \pm 2.56 ^a	46.6 \pm 2.97 ^a	71.6 \pm 1.73 ^a	37.6 \pm 1.82 ^a

Values that share a common letter are not significantly different. Differences between experimental groups were considered $P < 0.05$.

The study of soluble proteins of spermatozoa of framed and scaly Antonino-Zozulenets carps showed typical (relative to blood serum) zones: globulins (γ -, β - and α), albumin and prealbumin. At the same time, a significant share (24.3-28.8%) consisted of proteins localized in the concentrating gel (Figure 5).

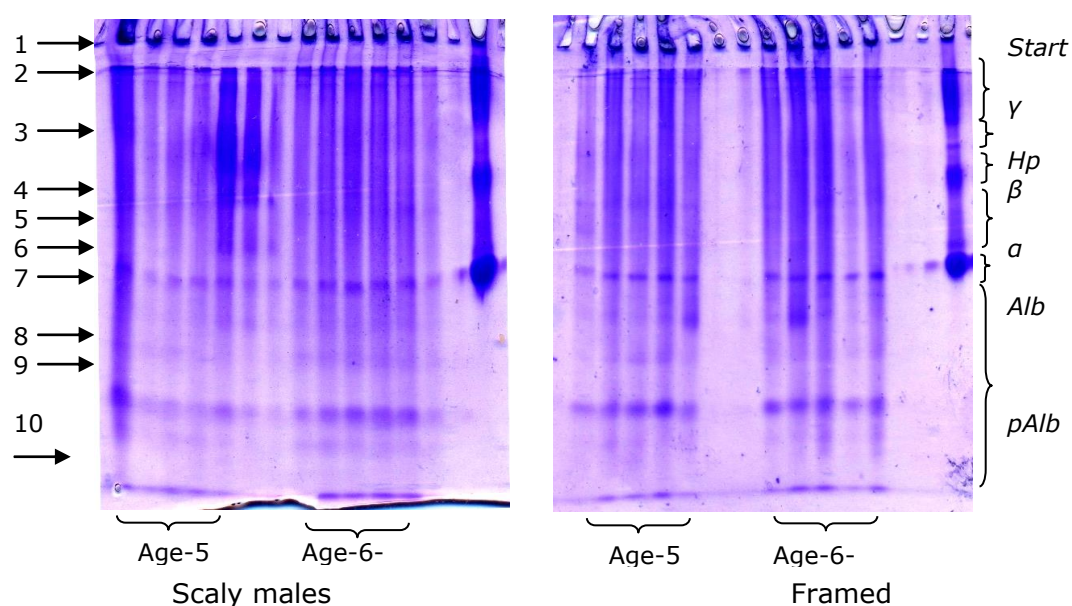


Figure 5. Soluble proteins in the sperm of Antonino-Zozulenets first-time and repeat spawning carp males.

The values of the soluble proteins of the γ - and α -globulin zones were the most variable. Scaly first-time spawners had a 0.81% higher content of γ -globulin zone proteins than first-time framed spawners. On the other hand, repeat spawners were superior over the scaly ones of the same age category by 1.18%. According to the content of α -globulins, repeat spawners had higher values compared to first-time spawners, by 0.48% for the scaly-shaped and by 0.94% for the frame-shaped. However, the scaly first-time spawners were superior (by 0.19%) over the framed ones, and the framed repeat spawners were superior by 0.23% over the scaly ones (Table 3).

As for other proteins in fractions, the prealbumin content in the sperm of scaly first-time spawners was by 5.92% higher than for the framed first-time spawner carps, while in the sperm of repeat spawners it was by 6.89% higher in scaly than in framed carps. At the same time, the superiority of first-time spawners over repeat spawning was of 1.2% for the scaly specimens and of 2.17% for the framed ones. On the other hand, both framed first-time and repeat spawning males had higher albumin contents compared to scaly ones. The superiority of framed first-time spawners over scaly ones was of 4.62%, and over repeat spawners it was of 5.51%. The prealbumin content in sperm of framed repeat spawners was by 2.39% higher compared to that in first-time spawners, while its content in the sperm of repeat spawner scaly carps was by 1.5% higher than in the first-time spawner scaly carps. Similar dynamics were observed for Hp-globins and β -globulins. As for Hp-globins, the superiority of framed first-time spawners over scaly first-time spawners was of 2.64% and that of framed repeat spawners over scaly repeat spawners was of 2.93%. As for β -globulins, the superiority of framed first-time spawners over scaly first-time spawners for was of 2.34% and that for repeat spawners was of 1.51% (Table 3).

Table 3

The spectrum of proteins in the sperm of first-time and repeat spawning males of the Antonino-Zozulenetsk intrabreed type of carp, % ($x \pm SD$, $n=5$)

Protein fractions	Scaly males		Framed males	
	First-time spawners	Repeat spawners	First-time spawners	Repeat spawners
Start globulin	27.04 \pm 1.67 ^a	28.77 \pm 3.15 ^a	24.36 \pm 5.24 ^a	24.28 \pm 3.12 ^a
γ - globulin	4.93 \pm 2.93 ^a	2.43 \pm 0.78 ^a	4.12 \pm 0.45 ^b	3.61 \pm 0.59 ^a
Hp-globins	1.59 \pm 0.24 ^{ac}	1.32 \pm 0.2 ^a	4.23 \pm 0.91 ^b	4.25 \pm 0.80 ^{bc}
β - globulin	1.29 \pm 0.34 ^{ac}	1.53 \pm 0.23 ^a	3.63 \pm 0.98 ^b	3.04 \pm 0.31 ^{bc}
α - globulin	2.73 \pm 0.86 ^a	3.25 \pm 0.68 ^a	2.54 \pm 0.52 ^a	3.48 \pm 0.70 ^a
Albumin	6.35 \pm 0.85 ^a	7.85 \pm 0.8 ^a	10.97 \pm 1.52 ^b	13.36 \pm 2.48 ^a
Prealbumin	56.07 \pm 5.03 ^a	54.87 \pm 4.68 ^b	50.15 \pm 3.18 ^a	47.98 \pm 2.48 ^a

Values that share a common letter are not significantly different. The differences between experimental groups were considered at $P < 0.05$

The study of CAT isozymes showed that six catalytically active bands of catalase appeared in the spermatozoa of first-time and repeat spawning males of the Antonino-Zozulenets carps, but their relative contents were different and depended on the genetic affiliation of the males. Accordingly, no pattern of distribution was found. Thus, in the spermatozoa of the first-time and repeat spawning males, the maximum values were observed in CAT1 and CAT2. Similarly, the sperm of repeat spawning scaly males had the highest CAT1 and CAT2 contents, however, the maximum value (30.5%) was for CAT6 in framed repeat spawning males. The minimum content of CAT3 was observed in three of the four experimental groups, however, the minimum content of CAT4 was found in the framed first-time spawners. At the same time, the content of CAT3 and CAT4 in the sperm of scaly first-time spawning males was almost at the same level and amounted to 7.29% and 7.37%, respectively.

An insignificant difference, within one percent, was observed between CAT1 and CAT2 in framed repeat spawners as well as between CAT5 and CAT6 in framed first-time and scaly repeat spawners (Figure 6).

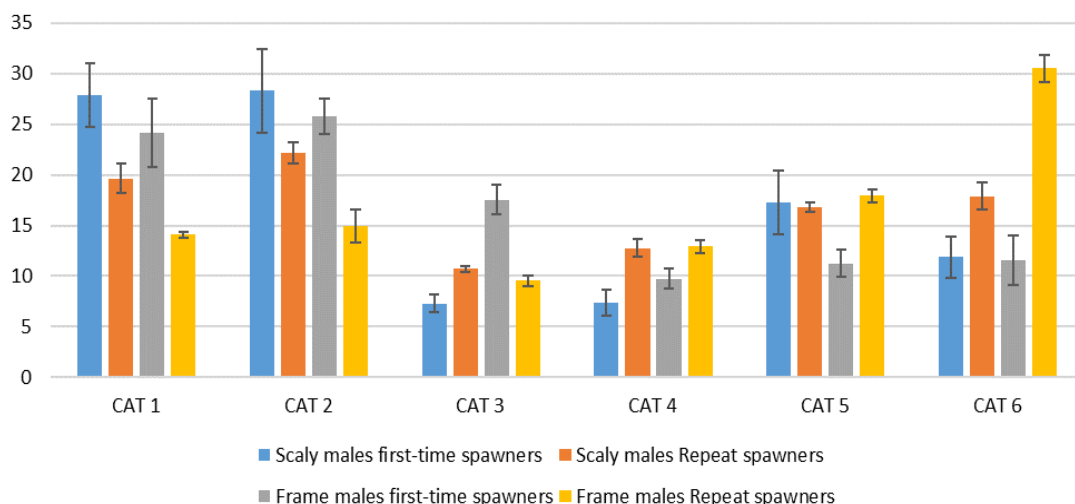


Figure 6. The content of CAT isozymes in the sperm of first-time and repeat spawning males of the Antonino-Zozulenets inbred type of carp, %.

The average rate of fertilization of all experimental groups was at a high level and exceeded 90%. The maximum value of 93.1% was recorded in repeat spawning framed males. Taking into account that the active movement of sperm in this experimental group had the shortest duration compared to other experimental groups, we can assume that the protein content of sperm affects their structural integrity and, accordingly, the degree of survival, but the velocity and direction of spermatozoa movement depends only partially on this composition. At the same time, it is worth noting that the survival rate of free embryos, where fertilization took place with the sperm of framed repeat spawning males, also had a maximum rate of 85.1%, but the difference in this rate compared to the rest of the experimental groups was within 0.5-1.7%, which is quite probable in fish farming (Table 4).

Table 4
Fertilization rate and survival rate of free embryos of the Antonino-Zozulenets intrabreed type of carp obtained during artificial reproduction ($x \pm SD$, $n = 5$)

Parameters	Framed carp		Scaly carp	
	Age-5	Age-6-8	Age-5	Age-6-8
Fertilization rate, %	91.3±0.4	93.1±0.8	92.6±0.5	92.8±0.9
Survival rate of free embryos, %	83.4±0.6	85.1±0.7	83.6±0.8	84.6±0.6
Survival rate of 3-day larvae, %	82.9±0.8	82.5±0.7	80.6±1.3	81.8±1.7

Therefore, the obtained results indicate that brood carps of a new intra-breed type are characterized by high reproductive rates and can be recommended for obtaining new promising lines for industrial cultivation at different levels of aquaculture intensification.

Discussion. For a long time, scientists have been conducting an intensive search for various biological methods that will allow them to fully characterize the reproductive functions of breeding individuals (Martínez Páramo et al 2014; Vandeputte 2003; Vesna et al 2010). Since spermatozoa motility is one of the main parameters affecting fertilization (Cosson 2019; Tichopád et al 2020; Cheng et al 2022), it was used to assess the viability and structural integrity of carps' sperm. For example, the ejaculate volume was at a high level in both experimental groups, in the range of 7.6-21.2 cm³, while the duration of active movement was in the range of 48.6-68.3 s.

This value is typical for the Ukrainian and European breeds of carp (not lower than 30 s), but it is lower than that of eastern breeds, where the activity ranges from 80 to

110 s (Verma et al 2009a; Cejko et al 2022), which may be a genetic feature and a consequence of the keeping conditions. Along with the analysis of the active movement of spermatozoa, it is important to assess the velocity and direction of spermatozoa movement (Okumus et al 2021). The repeat spawning males of both experimental groups had the largest number of spermatozoa with straight line movement. However, the velocity of the straight linear movement in scaly carps was at the same level, but, in the case of framed carps, the repeat spawning carps were superior over the first-time spawners. This indicates that the spermatozoa of the framed repeat spawning males had a shorter period of time to reach the micropyle (Gallego et al 2013).

The study of soluble proteins of spermatozoa of framed and scaly Antonino-Zozulenets carps showed zones of globulins characteristic for the electrophoretic separation of proteins (relative to blood serum): γ -, β - and α -, albumin and prealbumins. However, their relative contents were different and a significant part (24.3–28.8%) accounted for starter proteins. Since the brood carps were kept in the same conditions, the difference in the relative values of different globulin zones can be explained by the genetic origin of the experimental individuals. At the same time, it is worth paying attention to the fact that scaly carps, having higher search abilities, primarily consume natural feed, while framed carps are more adapted to consume artificial feeds. Accordingly, the differences in the composition of proteins could be due to the level of nutrition and quantity composition of proteins and carbohydrates in the diet (Mohammadi & Mesbah 2018; Verma et al 2009b).

At the same time, the content of isozymes in the first and second catalytically active bands of catalase in first-spawning males exceeded those of repeat spawners of both scaly and framed forms. A higher content of the fourth and sixth isozymes of catalase was found in repeat spawning males in both scaly and framed forms. The content of the third and fifth catalytically active bands of CAT was the most unstable. For example, the sperm of repeat spawning males of the scaly form had a higher content of isozymes compared to that of first-time spawners. However, in the case of the framed form, the third catalytically active band of the enzyme in the sperm of first-time spawners had a higher isozyme content than in repeat spawners. As for the fifth band, on the contrary, its content was 6.69% higher than that in first-time spawners compared to repeat spawning males. Such diversity and the presence of catalytically active isozymes indicates the high ability of catalase to split H_2O_2 with the formation of water and oxygen. The high variability of the protein structure of sperm contributes to the development of spermatozoa's ability to move, strengthening their structural integrity and, accordingly, increases the fertility rate (Li et al 2009; Li et al 2017; Binner et al 2021).

Conclusions. The obtained results allowed providing a full evaluation of the ejaculates of first-time and repeat spawning males of Antonino-Zozulenets carps. It has been proven that physiological and biochemical parameters were very variable and depended on a number of biotic and abiotic factors such as genetic origin, age category, feeding. For example, the average ejaculate volume of the experimental individuals was in the range of 7.6-21.2 mL³, where framed first-time spawning and repeat spawning males produced a smaller volume of ejaculate compared to peers of the scaly form. The observed active movement of spermatozoa was of 48.6-68.3 s. At the same time, framed repeat spawning males, despite the lowest rate of spermatozoa movement activity (48.6 s), had the largest percentage of active spermatozoa, of 92.6%, including those with straight line movement, of 84.4%. Despite the short duration of the active movement of spermatozoa in the males of this experimental group, due to a significant number of spermatozoa with high VAP, VCL and VSL values, they had the maximum rate of fertilization. At the same time, the relative content of spermatozoa with curvilinear movement (within 20%, in first-time spawning males of both framed and scaly forms), indicated the expediency of increasing the period of pre-spawning keeping, and the use of broodstock of this age category at the end of the spawning campaign, as a last resort. The analysis of the protein content showed that spermatozoa of scaly males had a higher content of prealbumins, while spermatozoa of framed males had a higher content of Hp-globulins, β -globulins and albumins. Such a variety of the content of protein fractions contributes to

the strengthening of the structural integrity and, accordingly, increases fertilization rates. In general, the obtained results indicate high reproductive rates and high fertilization capacity of first-time and repeat spawning males and the possibility of their use in the selective breeding process.

Conflict of interest. The authors declare no conflict of interest.

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