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Mid-autumn spermiation in outdoor-cultured pikeperch (*Sander lucioperca*) using different gonadoliberin application strategies

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ABSTRACT

This study aimed to find the optimal strategy for the application of salmon gonadotropin-releasing hormone analogue (sGnRHa) in outdoor-cultured pikeperch males in an exceptionally early term in early November. According to the histological examination, on the day of hormonal treatment, pikeperch testes were in the late stadium of spermatogenesis, with 78.7% of germ cells attributed to sperm cells. Five experimental groups were established varying on hormonal preparations and water temperature: (1) sGnRHa (25 μg kg^{-1}) was applied to fish at pre-warming at water temperature of 11 °C (GnRH-LOW); (2) sGnRHa (25 μg kg^{-1}) was applied to fish post-warming at water temperature of 15 °C (GnRH-HIGH); (3) priming group (5 μg kg^{-1} at 11 °C +25 μg kg^{-1} at 15 °C); (4) reference group – fish were injected with human chorionic gonadotropin; (5) control group - fish were injected with saline solution. Sperm was obtained from all fish in hormonally treated groups, whereas the fish treated with saline did not spermiate. The greatest values of the straight-line velocity (VSL), the curvilinear velocity (VCL), and the angular path velocity (VAP) of sperm cells, as well as the amounts of Mg^{2+}, K^+, and Na^+ in the sperm seminal plasma, were obtained in GnRH-HIGH, whereas the GnRH-LOW group yielded the greatest sperm volume. Among the ions in seminal plasma, K^+ and Na^+ dominated and showed a relationship with sperm kinematic parameters. According to the obtained data, sGnRHa can be considered as an appropriate hormonal preparation to induce spermiation at an early preseason. Either before or after warming, sGnRHa application can yield sperm of proper quantity and quality as early as 5 months before the natural spawning season. A comprehensive study is recommended to evaluate the full period of sperm availability in outdoor-reared males, followed by direct evaluation of the fertilizing capacity.

1. Introduction

Pikeperch (*Sander lucioperca* L.) is a highly valuable commercial fish for inland aquaculture in Europe. Seasonal and pre-seasonal hormonal induction of artificial reproduction has, so far, been evaluated in wild (Rónyai, 2007; Křišťan et al., 2013; Żarski et al., 2013) and outdoor cultured breeds (Müller-Belecke and Zienert, 2008; Zakeś et al., 2013; Ljubobratović et al., 2019). These studies focused on egg quality and showed that pikeperch can produce high-quality gametes for a prolonged period of the year. A recent study found that the threshold of oocyte maturational competence is obtained as early as in January (Ljubobratović et al., 2021), leading to the assumption that ovarian gametogenesis in outdoor-reared pikeperch reaches the level to be artificially induced to final maturation and ovulation the earliest in January, about 3 months before the natural spawning season. Nevertheless, to the best of our knowledge, there are no reports on the earliest moment in the season to obtain viable sperm. Concerning the differences between oogenesis and spermatogenesis in teleost fish (Lubzens et al., 2010; Schulz et al., 2010) and in percids particularly (Fontaine et al., 2015), it might be hypothesized that testes mature earlier during the season. Concerning recent aquaculture tendencies towards intensification through a recirculation aquaculture system (RAS) and its demand

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for a constant supply of fingerlings, it is crucial to fully understand the reproductive cycle in both genders and, with it, to fully define the timespan for obtaining high-quality gametes. To define the state of readiness of males for hormonal stimulation, assessment of testes histology is of great importance since it can reveal the maturation stage of male gonads before final induction by determining the volume of cysts in each spermatogenic cycle (Leal et al., 2009; Kiros et al., 2011; De Melo Dias et al., 2017). Subsequently, hormonal induction needs to be applied and spermiation confirmed, whereas the quantity and quality of obtained sperm require thorough evaluation. Thus, to fully assess the testes' status at the given moment of the season, two data sets are crucial: (1) pre-stimulation histological evaluation of testis; (2) sperm quality and quantity parameters.

Estimation of sperm quality in fish is an important tool for establishing its potential fertility. To fully define sperm quality, a specific set of parameters needs to be quantified, namely motility (described via several kinematic parameters), pH, spermatocrit, and seminal plasma contents of sperm (Rurangwa et al., 2004; Kowalski and Cejko, 2019). That is so for pikeperch as well, where main parameters of thus far studies were conducted regularly on motility and kinematics (Křišťan et al., 2014; Blecha et al., 2015, 2016; Schaefer et al., 2016), while the issues of kinematics and plasma ions were rarely in focus (Dziewulska, 2020). Sperm quantity and quality may vary depending on different factors such as nutrition, environment, hormonal induction, and season (Bobe and Labbé, 2010). Hormonal manipulations are often practiced to induce spermiation in many cultured fish species, especially at the time of year other than the natural spawning season. With respect to their mode of action and the organs in the reproductive axis they influence (Lubzens et al., 2010), external hormones are usually classified as gonadotropins or gonadoliberins. While gonadotropins target the gonads, gonadoliberins affect the organism at a higher level of pituitary, leading to increased internal secretion of gonadotropins. The physiological state of the fish changes constantly throughout the year (Schulz et al., 2010). Considering the reproductive cycle in percids, critical changes take place in the period from autumn to the natural spawning season in spring (Fontaine et al., 2015) With this respect, the answer of fish to different preparations may differ depending on whether the hormones are administered during the natural spawning season or in an earlier period (Zohar et al., 2010; Żarski et al., 2017; Ljubobratović et al., 2019). Although gonadotropin preparations are still widely used, the efficacy of gonadotropin-releasing hormone analogs (GnRHa) lays in their advantage to be used repetitively over more spawning seasons since they can reduce endocrine disruption (Zohar and Mylonas, 2001). Different GnRHa application strategies improve sperm features in various cultured species (Agulleiro et al., 2007; Cabrita et al., 2008; Alavi et al., 2012). Nevertheless, the human chorionic gonadotropin (hCG) is a commonly used agent for out-of-season spermiation induction in pikeperch (Blecha et al., 2015; Żarski et al., 2019; Ljubobratović et al., 2019) and positively influences the ability of males to produce sperm during the natural spawning season (Blecha et al., 2016). However, a recent study aiming to prolong the spermiation period in indoor-reared pikeperch found salmon GnRH analogue (sGnRHa) as more favorable for fish welfare compared to hCG (Żarski et al., 2020). Likewise, in the case of the related percid species Eurasian perch (*Perca fluviatilis*), a slight improvement in sperm quality was obtained using sGnRHa compared to hCG when stimulation took place during winter (Żarski et al., 2017). Finally, regarding pikeperch egg quality, sGnRHa showed benefits compared to hCG for pre-season reproduction (Ljubobratović et al., 2019). Thus, it might be assumed that a similar beneficial effect of gonadoliberins would be found in pikeperch sperm after autumn injection.

To prolong the period of gamete availability, males are artificially photo-thermally conditioned, which is commonly achieved by constant broodstock maintenance in fully controlled conditions (Schaefer et al., 2016; Żarski et al., 2020). Nevertheless, the extension of gamete availability can also be obtained using outdoor-reared broodstock. With this respect, a rather informative study was performed (Blecha et al., 2016), controlling the thermal conditions in pond-reared fish to obtain the sperm later than in the natural spawning season. The study concluded that prolonged cooling of the males does not lead to impaired sperm quality and prolongs the gamete accessibility in outdoor-reared fish as long as few months post-season, what was found not possible in case of females (Müller-Belecke and Zienert, 2008). This leads us to infer that pikeperch testes can be more effective in gamete production. However, it is still unclear to which extent the testes and ovaries can differ in terms of the earliest possible time to obtain viable gametes? High-quality eggs in outdoor-reared domesticated females are available as early as in mid-January. Thus, respecting the described disagreements in terms of postponed gamete production, it may be hypothesized that the testes can produce mature sperm earlier. If so, the availability of outdoor-reared males could be extended to about half of the year, usable for the fertilization of out-of-season obtained eggs in the period from autumn to summer.

The main objective of this study was to evaluate the aptness of different injection strategies in means of post-injection thermal regime and priming injection for the mid-autumn spermiation induction with sGnRHa in domesticated pikeperch cultured in outdoor conditions.

2. Materials and methods

Fish handling was performed according to the regulations of the Animal Ethical Panel of the Institute, which were established according to Hungarian State law (10/1999.I.27.). Before each manipulation (injection, stripping), fish were anesthetized in 2-phenoxyethanol (300 μL L^{-1}).

2.1. Experimental facilities, fish and broodstock management

On 4 November 2019, 36 male breeders (mean weight 1.08 ± 0.19 kg) were transported into the indoor recirculation system of the Research Institute for Fisheries and Aquaculture NAIK-HAKI (Szarvas, Hungary). Upon transport, all experimental fish were inserted with a passive integrated transponder (PIT) in the cheek muscle according to the recommendations given by Zakęś and Hopko (2013). At the time of transportation, the water temperature in the RAS was equalized to the outdoor temperature of 11 °C. Water temperature was increased daily by 1 °C until it reached 15 °C. This rate of thermal increase has recently been recently described as appropriate for out-of-season either after (Ljubobratović et al., 2021) or before hormonal induction (Ljubobratović et al., 2020). The stable temperature of 15 °C was maintained further. The recirculation loop was composed of three 5-m^3 tanks, bead filter, UV lamp, and oxygen dispenser units inside the tanks.

2.2. Experimental design

Two groups of fish were hormonally treated immediately upon transportation with sGnRHa ([D-Arg6, Trp7, Leu8, Pro9-NEt]-GnRH; Ova-RH; Syndel Laboratories Ltd., Canada). The hormone was administered to two groups, GnRH-LOW and GnRH-Priming, which received a priming dose. At the same time, a 0.65% saline solution was administered to group Control-NaCl. Four days later, when the water temperature reached 15 °C, sGnRHa was administered to two groups, GnRH-HIGH and GnRH-Priming, which received a resolving dose, whereas group REF-hCG received hCG (Choragon, Ferring International Center S. A., Switzerland). The procedure using a stable temperature upon treatment with hCG was selected as a reference concerning the recent successful application in out-of-season, pre-seasonal, and seasonal artificial reproduction (Ljubobratović et al., 2018, 2019, 2020). The used dosages of both hormonal preparations, sGnRHa, and hCG, were set at 25 μg kg^{-1} and 250 IU, respectively, twice as low as the dosage recommended for females in out-of-season reproduction (Żarski et al., 2019). Before application, all inducing substances were diluted in a 0.65% NaCl

solution, and hormonal solution was applied to the dorsal muscle of fish at a rate of 1 mL kg^{-1} of body weight (Rónyai, 2007). Thus, according to the thermal strategy, priming dose, and hormonal substance, the groups were as follows:

- GnRH-LOW – induction at the day of transport at 11 °C with 25 μg kg^{-1} of sGnRHa;
- GnRH-HIGH – induction four days upon transport at 15 °C with 25 μg kg^{-1} of sGnRHa;
- GnRH-Priming – induction at the day of transport at 11 °C with 5 μg kg^{-1} of sGnRHa; followed by resolving treatment 4 days upon transport at 15 °C with 25 μg kg^{-1} of sGnRHa;
- REF-hCG – induction 4 days upon transport at 15 °C with 250 IU kg^{-1} of HCG (human chorionic gonadotropin);
- Control-NaCl – induction at the day of transport at 11 °C with 1 mL kg^{-1} of 0.65% NaCl solution.

Each group consisted of six male specimens, totaling 30 experimental animals.

Sperm was collected 6 days after the hormonal treatment in group GnRH-LOW and 5 days after the final hormonal application in other groups. The mean water temperature after hormonal treatment in the GnRH-LOW group was 13.3 °C, whereas in the other groups, it was 15.0 °C. Considering that latency times in females differ at about 1 day for the given two temperatures (Żarski et al., 2013), a day difference between the groups in the present study was due to the thermal regime difference between these treatments. After anesthesia in 2-phenoxyethanol (Teletchea et al., 2009), sperm samples were collected by catheterization to prevent contamination with blood, feces, or urine (Sarosiek et al., 2016). Sperm samples were collected from six males per treatment in all hormonally treated groups as all the fish spermiated. In the case of Control-NaCl, spermiation was assessed at both sampling times. Only one fish spermiated in the first sampling in an amount insufficient for any analysis, whereas the other five specimens did not produce sperm. Thus, this group was excluded from further analyzes. Sperm samples were kept on ice until conducting the analysis.

2.3. Histology of testes and stereological analysis

To assess the gonad maturity status of fish prior to hormonal treatment, histological analysis of pikeperch testes was performed. Six fish were randomly chosen on the injection day, anesthetized in 2-phenoxyethanol (300 μL L^{-1}), and sacrificed by severing the spinal cord. After ventral incision, one testis was carefully removed from each specimen, and the central part of the randomly chosen lobe (either left or right) was sampled for histological analysis. Testicular tissue was fixed in neutral buffered formalin solution (4%) for 48 h and later processed, using the standard histological protocol in an automated tissue processor Leica TP 1020 (Nussloch, Germany): dehydration in increasing ethanol gradient (70%, 80%, 90%, 96%, and 100%), clearing in xylene, and embedding in paraffin. Subsequently, paraffin blocks were sectioned to the nominal thickness of 5 μm using a microtome Leica SM 2000R (Nussloch, Germany), and three sections were randomly selected from each tissue block for evaluation. Sections were stained using a combination of hematoxylin and eosin (HE; Sigma-Aldrich) in an automated tissue stainer Leica ST 4040 (Nussloch, Germany).

The histological assessment aimed to estimate volume densities (Vv) of cysts in different stages of spermatogenesis, applying the criteria published elsewhere (Blazer, 2002; Schulz et al., 2010), with four types of germ cysts were quantified – spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 1a, b). Quantification was conducted by the systematic sampling of each tissue section using a Leica DM2000 microscope (Wetzlar, Germany) equipped with a Leica DFC320 digital camera (Wetzlar, Germany). Micrographs were taken using constant steps in the x and y direction; the first micrograph was taken in a random manner (Fig. 2a). Later, a counting grid of 35 crosses (7 × 5 crosses; Fig. 2b) was superimposed to each micrograph in the ImageJ software (Schneider et al., 2012), and the point-counting method was used (Weibel et al., 1996). Each point falling on a cyst containing germ cells was counted, and volume densities were calculated as follows:

$$V_v(n, germ\ cells) = P_n \times P_T^{-1}$$

where n is the spermatogenesis stage (spermatogonia, spermatocytes, spermatids, sperm cells), P_n represents the points hitting cysts in one of the mentioned stages, P_T is the total points hitting all cysts containing germ cells. For the purpose of this study, only cysts containing germ cells in different phases of spermatogenesis were assessed, whereas volume density of tunica albuginea, interstitium, efferent ducts, and large blood vessels were not quantified. Using this unbiased methodology, around 31% of total reference space was covered, which resulted in an average of 1088 points falling at germ cells per each of the six fish. A similar methodology has already been used to assess testis development or reproductive cycles of fish (Tomkiewicz et al., 2011; Huszno and Klag, 2012).

2.4. Sperm cell kinematics

The sperm was diluted with saline solution to inhibit sperm cell motility (IMS): 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$ $2H_2O$, and 2.38 mM $NaHCO_3$ at pH 8.0 (Blecha et al., 2015) at a ratio of 1:10 (sperm: IMS). After dilution, sperm cells were activated with activation solution (AS) containing 100 mM sucrose, 1 mM $CaCl_2$, 20 mM Tris, pH

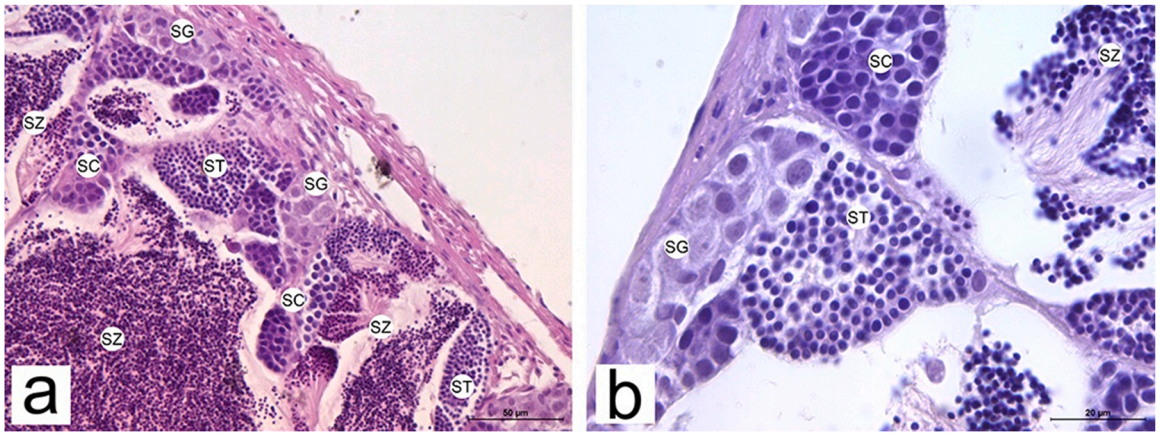

Fig. 1. Representative histological micrographs showing cysts with germ cells at different stages of maturation in pikeperch (*Sander lucioperca*) testis: spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ) at two magnifications **(a)** × 400; **(b)** × 1000; sections were stained with HE.

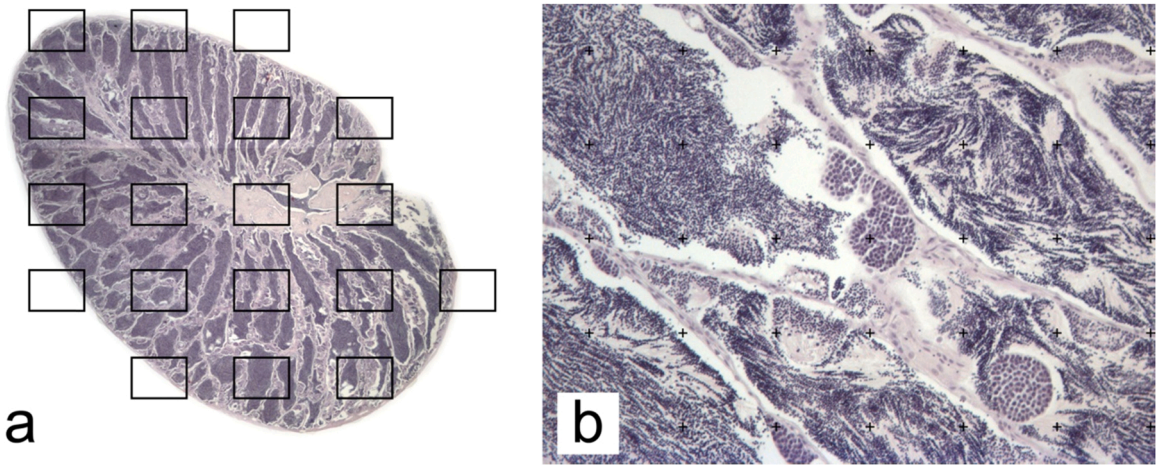

Fig. 2. (a) Illustration of the systematic uniform sampling method at one histological section from fish no. 3: 19 micrographs were systematically taken using identical steps in both x and y directions (× 25 magnification; HE); **(b)** one of the taken micrographs showing a superimposed counting grid of 35 points (5 × 7) in the ImageJ software for estimating volume densities of cysts at different stages of spermatogenesis (× 200 magnification; HE).

8.5 (Křišťan et al., 2014) at a ratio of 1:20 (sperm:AS). The sperm was diluted to facilitate the evaluation of sperm kinematics, according to the recommendations of by Křišťan et al. (2014) and Blecha et al. (2015).

Kinematic parameters of sperm cells of pikeperch were determined using a computer-assisted sperm analysis (CASA) system. All sperm seminal plasma ions were analyzed as well, using the same samples. Sperm activity was captured using an Olympus BX51 microscope (× 400 magnification) equipped with a negative-phase objective. The system was programmed to capture images at 30 frames per second, using a Sony CCD camera. The samples were evaluated with image-processing and video-tracking software of the BASA-Sperm Aqua software package (Merk Biotechnology, Turkey). The following sperm cell motility parameters were evaluated:

- straight-line velocity (VSL; μm s^{-1});
- curvilinear velocity (VCL; μm s^{-1});
- angular path velocity (VAP; μm s^{-1});
- straightness = VSL/VAP (STR; %);
- linearity = VSL/VCL (LIN; %);
- amplitude of lateral displacement of the sperm head (ALH; μm);
- beat cross frequency (BCF; Hz);
- mean angular displacement (MAD; °);
- wobble = VAP/VCL (WOB; %).

as explained by Özgür et al. (2019a), (2019b). The mean of three independent activation trials from each fresh sperm sample was used for statistical analysis.

This approach is not applicable to measure the percentage of motile sperm, which was therefore not subject of the study.

2.5. Ions of seminal plasma

Seminal plasma was collected after centrifugation of the sperm at 3400 g for 10 min in a Beckman L-8-70M ultracentrifuge (Rotor SW-28, Munich, Germany). The plasma was centrifuged twice to avoid contamination with sperm cells and was stored at − 20°C until analysis. The sperm samples were digested in an ultrasonic bath (VWR, USC 300TH; Ultrasound cleaning baths, USC) for 20 min at 30 °C, using a solution of HNO_3: H_2O_2 (4:2 mL). Digested solutions were diluted with deionized water until being adjusted to 10 mL. After dilution, solutions were analyzed for the presence of Na^+, K^+, Ca^{2+}, and Mg^{2+} ions (Slivkova et al., 2009; Özgür et al., 2015).

2.6. Statistical analysis

All experimental data were tested for normality and homogeneity of variance with Shapiro-Wilk's and Levene's test, respectively, using the SPSS 17 software (IBM, New York, NY, USA). Comparison between groups was conducted using Multiple Variance Analysis (MANOVA), followed by Duncan's post-hoc test. Possible correlations between data sets were determined using bivariate Pearson's correlation coefficients. Data in the manuscript are presented as mean ± standard error of the mean (SE); the significance level (α) was set at 5%. Graphs were created by Graph Pad Prism 5 (Software, Inc., San Diego, CA).

3. Results

Histological parameters of pikeperch testes on the day of injection were quantified using differential point counting, and it was estimated that 75% or more cysts with germ cells present in the testis of each fish contained sperm cells (Table 1). Spermatogenesis was fairly synchronized among sampled fish since sperm cells occupied between 75.3% and 78.9% of total germ cells in five out of six fish, whereas only one specimen had a higher volume fraction of sperm cells (89.7%). Volume densities of other cysts are also presented in Table 1, while no pathologies were present in assessed fish testes.

The sperm volume per kg of fish and the pH values are shown in Fig. 3. The volume of sperm in the group of sGnRHa-LOW was significantly higher compared to that of the REF-hCG group, whereas there were no statistically significant differences in sperm pH.

The sperm cell kinematics is shown in Fig. 4. The highest straight-line velocity (VSL; 29.04 ± 1.05 μm s^{-1}) of sperm cells, the highest curvilinear velocity (VCL, 132.30 ± 5.72 μm s^{-1}), and the highest angular path velocity (VAP; 61.37 ± 2.02 μm s^{-1}) were found in the group sGnRHa-HIGH. The sperm in both single-injection sGnRHa groups was characterized by a significantly higher straight-line, whereas only sGnRHa-HIGH sperm was of significantly higher curvilinear velocity compared to REF-hCG. The values of STR, LIN, and BCF were significantly higher in the sGnRHa-HIGH group compared to REF-hCG. The values of VAP, ALH, MAD, and WOB did not differ significantly among groups.

Sperm seminal plasma ions are presented in Fig. 5. The highest levels of Mg^{2+} (2.00 ± 0.26 mg L^{-1}), K^+ (64.07 ± 5.13 mg L^{-1}), and Na^+ (362.09 ± 31.84 mg L^{-1}) were found in the sGnRHa-HIGH group, whereas the highest level of Ca^{2+} (8.62 ± 0.29 mg L^{-1}) was found in the sGnRHa-LOW group. Significant differences were established for K^+ and Na^+ between the sGnRHa-HIGH and REF-hCG groups, whereas the Mg^{2+}

Table 1
Volume densities (%) of different types of germ cells in testes of each sampled pikeperch (*Sander lucioperca*) males (Fish 1–6) about 5 months prior to the natural spawning season; mean values are given in the last column.

Volume densities	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Mean ± SE
Vv (spermatozoa)	75.5	89.7	75.3	75.3	78.9	77.4	78.7 ± 2.3
Vv (spermatids)	9.8	4.2	8.5	10.3	7.3	7.1	7.9 ± 0.9
Vv (spermatocytes)	5.0	2.3	4.9	5.6	4.8	4.4	4.5 ± 0.5
Vv (spermatogonia)	9.7	3.7	11.4	8.8	9.0	11.1	9.0 ± 1.0

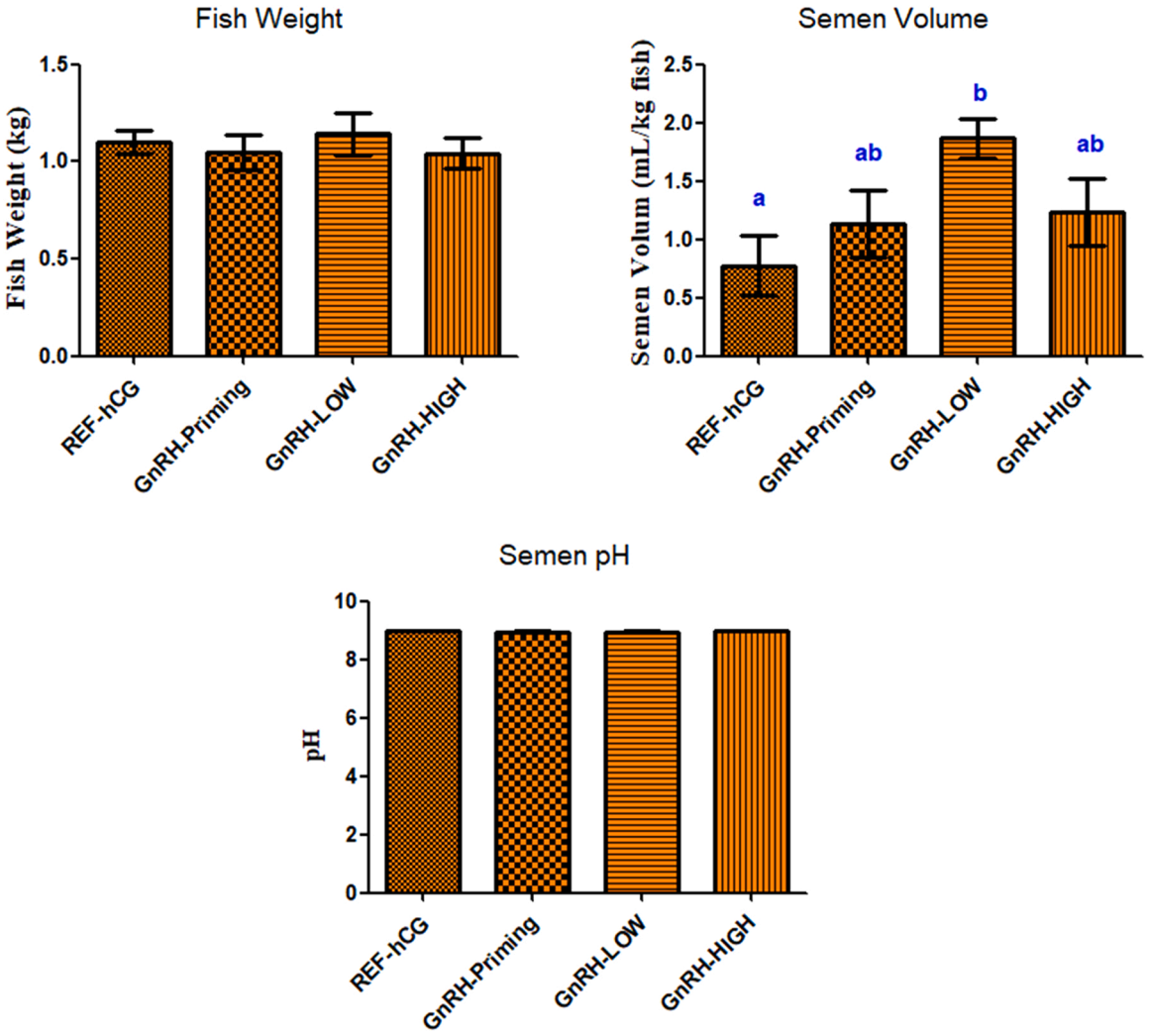

Fig. 3. Fish weight, volume of milt per kg of fish, and pH of semen in pikeperch (*Sander lucioperca*) in different hormonally treated groups about 5 months prior to the natural spawning season. Different letter superscripts indicate significant differences among experimental groups (Duncan's post hoc test; mean ± *SE*; $p < 0.05$). Control NaCl group was not evaluated due to the absence of spermiation.

concentrations were significantly higher in sGnRHa-HIGH compared to the sGnRHa-LOW group. The level of Ca^{2+} was similar among the tested groups. Sodium was the predominant ion of pikeperch sperm seminal plasma (Fig. 5). Positive but weak relationships were found between K^{+}, Na^{+}, Ca^{2+}, and Mg^{2+} in sperm seminal plasma and the values of VSL, VCL, and VAP in sperm cell velocities (Table 2). However, while the Na^{+} ion correlated ($r = 0.521$, $p < 0.01$) with the VCL value of sperm cells, there was no correlation between other ions (Ca^{2+}, Mg^{2+}, and K^{+}) and sperm cell velocities.

4. Discussion

The natural reproductive cycle of percids is well understood and described in the literature (Hermelink and Wang, 2015). However, the majority of published studies monitored the reproductive status of pikeperch (in wild or pond-reared fish) only by calculating the gonadosomatic index (Saulamo et al., 2005; Khendek et al., 2018; Marenkov, 2018), whereas only a couple of studies used histological assessment for determining the maturity of germ cells. As mentioned before, pikeperch in Central and Eastern Europe naturally spawns in April, whereas spermatogenesis in testes is completed long before the spawning time, at the end of autumn, when cysts containing spermatids and sperm cells dominate the parenchyma of the testis (Hermelink and Wang, 2015). This is in line with the results from the current study since cysts containing spermatids and sperm cells account for 86%, on average, at the beginning of November. Malison et al. (1994) showed that in September, cysts containing sperm cells in the North American percid, walleye (*Sander vitreum*), accounted for only 2% of the total germ cells, whereas

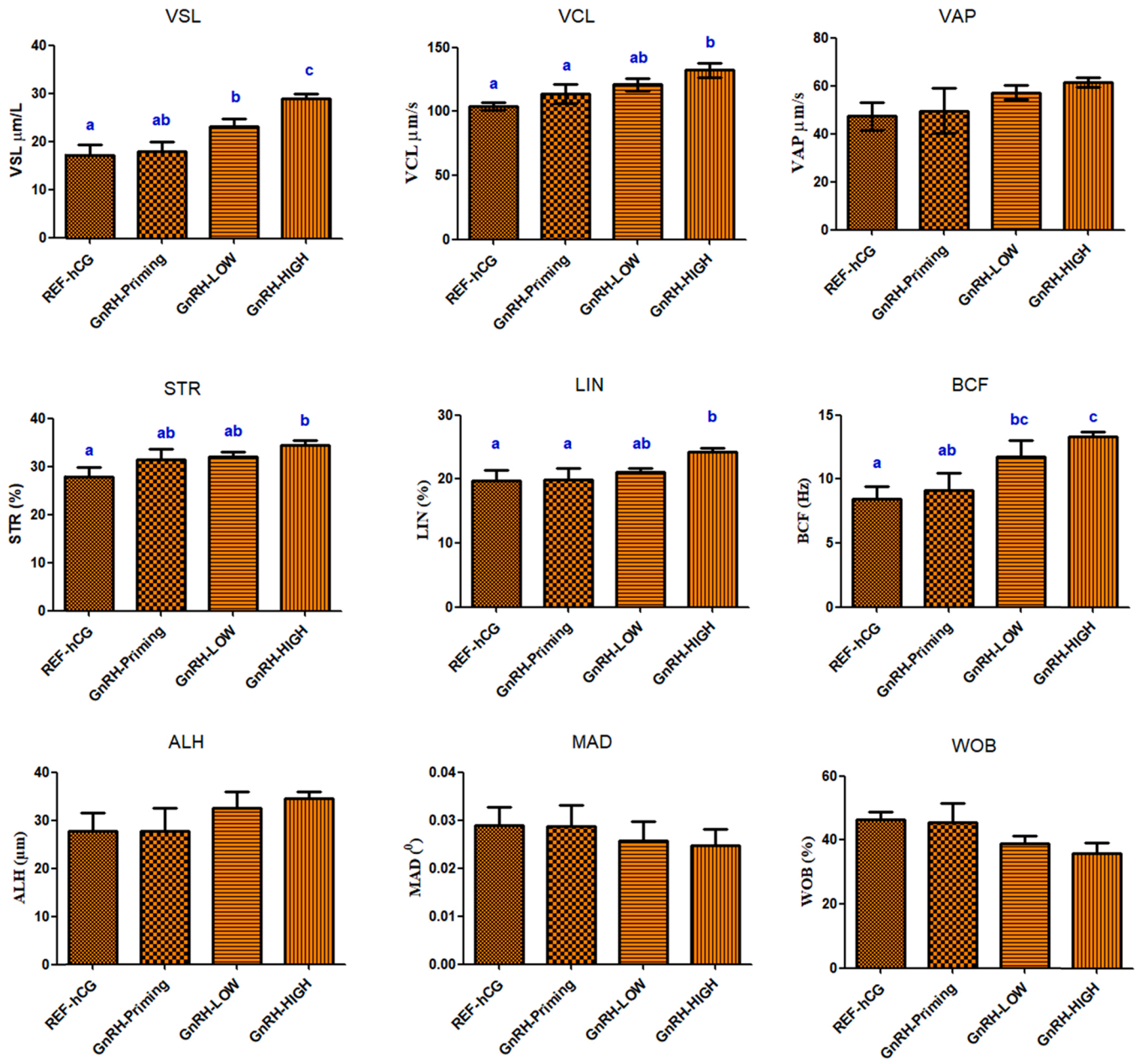

Fig. 4. Sperm cell kinematics of pikeperch (*Sander lucioperca*) in different hormonally treated groups about 5 months prior to the natural spawning season. Different letter superscripts indicate significant differences among experimental groups (Duncan's post hoc test; mean ± *SE*; $p < 0.05$). Control NaCl group was not evaluated due to the absence of spermiation.

by October, they reached 50%. Furthermore, in January, that number exceeded 95%, and fish were able to spermiate from January to April. Similar results were obtained in European perch (*Perca fluviatilis*), although the authors performed semi-quantitative scoring, and quantification of different stages was not conducted (Sulistyo et al., 2000). In any case, pikeperch males from the present study were in the late phase of the spermatogenic cycle as cysts with sperm cells were dominating, according to the classification given by Blazer (2002). Spermatogenesis was fairly synchronized within the sampled fish, as only one fish showed higher levels compared to the average proportion of sperm cells in testes. Thus histological examination confirmed the readiness of outdoor-cultured males for hormonal treatment as early as at the beginning of November.

In the present study, four injection strategies were applied in early November, without additional photo-thermal conditioning, and all males treated with hormones spermiated, yielding a large volume of high-quality sperm. To the best of our knowledge, such an early term to obtain gametes has not been documented thus far in outdoor-reared pikeperch male specimens. While gonadotropin treatment is usually preferred in seasonal reproduction (Blecha et al., 2016), it appears that GnRH treatment might be beneficial for the application of reproductive hormones in pre-season. This outcome agrees with a study conducted in a similar advanced term on Eurasian perch (Żarski et al., 2017). Mid-autumn is far-term from the natural spawning season, which, in the given area takes place in late March and early April (Rónyai, 2007). Considering this, the levels of internal gonadotropin responsible for final sperm maturation, luteinizing hormone (LH), is most likely rather low, while the follicle-stimulating hormone (FSH) is rather active in the pre-maturational stages of spermatogenesis (Schulz et al., 2010). Thus, the mode of action of hCG is mainly mimicking the increase of LH to artificially facilitate final sperm maturation (Ezcurra and Humaidan, 2014). On the other side, the action of GnRH leads to a pituitary increase

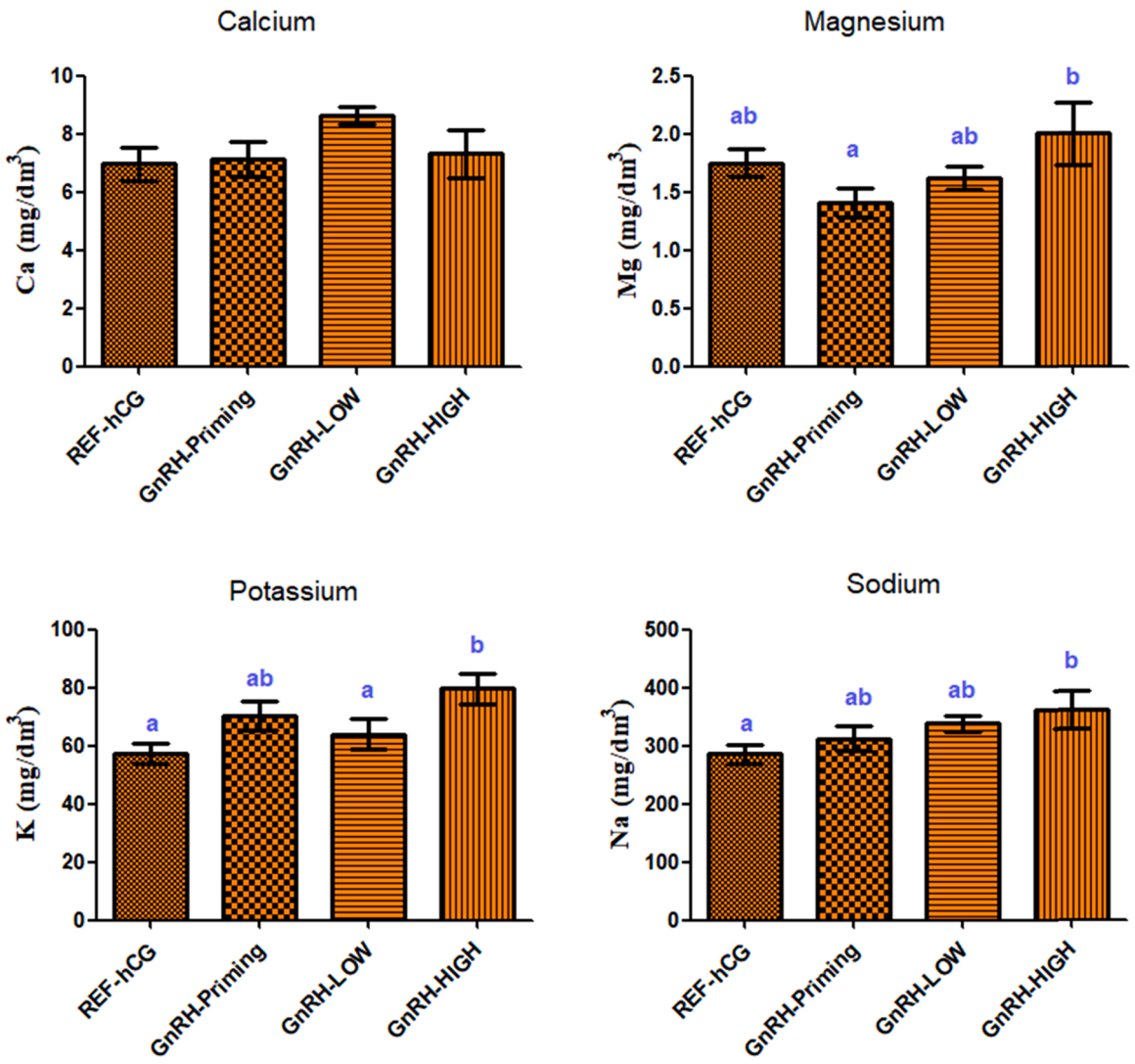

Fig. 5. Semen seminal plasma ions of pikeperch (*Sander lucioperca*) in different hormonally treated groups about 5 months prior to the natural spawning season. Different letter superscripts indicate significant differences among experimental groups (Duncan's post hoc test; mean ± *SE*; $p < 0.05$). Control NaCl group was not evaluated due to the absence of spermiation.

Table 2
Pearson correlations between sperm cell kinematics and semen seminal plasma ions in hormonally treated pikeperch (*Sander lucioperca*) males about 5 months prior to the natural spawning season.

	VSL	VCL	VAP	BCF	ALH	MAD	Ca^{+2}	Mg^{+2}	K^{+}
VCL	0.595(**)								
VAP	0.634(**)	0.502(*)							
BCF	0.280	0.318	-0.203						
ALH	0.473(*)	0.458(*)	0.792(**)	-0.301					
MAD	0.104	0.352	0.561(**)	-0.680(**)	0.671(**)				
Ca^{+2}	0.087	0.152	0.107	0.011	0.159	0.178			
Mg^{+2}	0.144	0.241	0.024	0.309	0.044	-0.039	0.460(*)		
K^{+}	0.181	0.381	-0.054	0.458(*)	-0.031	-0.164	0.054	0.409(*)	
Na^{+}	0.254	0.521(**)	0.029	0.441(*)	0.235	-0.035	0.320	0.547(**)	0.543(**)

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

in both gonadotropins (LH and FSH), depending on the developmental status of the gonad (Zohar et al., 2010). Such a wider range of action caused by GnRH could be more appropriate for the not fully matured testes to produce sperm than the plain increase in maturation gonadotropin level via hCG. This might be the reason why, at such an early term of hormonal application, GnRHs were more beneficial compared to gonadotropins.

High semen volumes were obtained in all treatments, and it appears that all tested sGnRHa application strategies were effective as the mean sperm volume was in the range of 0.7–1.7 mL kg^{-1}, which is in line with previously obtained data (Blecha et al., 2015, 2016; Malinovskyi et al., 2021). Regarding sperm cell kinematics, there was only one

disagreement between the single-injection GnRH groups; the VSL was significantly higher in GnRH-HIGH compared to GnRH-LOW. Otherwise, GnRH-Priming seems to be an inferior strategy to GnRH-HIGH in terms of four analyzed parameters (VSL, VCL, LIN, BCF). Therefore, priming seems to be unnecessary, and both single-injection GnRH strategies, pre- and post-warming, can be recommended. Although the differences in values of the same parameters might differ due to different CASA systems used (Boryshpolets et al., 2013), with respect to the aims of the present study, it is necessary to review the sperm kinematics achieved in earlier studies and relate them to the present results. Thus, with group means of VSL and VCL in the range 17–29 and 96–117 μm s^{-1}, the results appear to fit the so far published kinematics of pikeperch sperm in seasonally stripped wild pikeperch (Sarosiek et al., 2016; Dziewulska, 2020) as well as those obtained for indoor-reared domesticated pikeperch (Schaefer et al., 2016; Żarski et al., 2020). Both Na^+ and K^+ ions are the main electrolytes involved in the maintenance of the osmolality of sperm seminal plasma (Alavi and Cosson, 2006), which has recently also been confirmed for pikeperch (Dziewulska, 2020). In agreement with the previously mentioned study, the present study showed that Na^+ and K^+ ions were predominant compared to other ions in sperm seminal plasma of pikeperch. Likewise, these ions were positively correlated with sperm kinematics (Table 2). Although the composition of seminal plasma might change throughout the reproductive season (Marshall, 1986; Christ et al., 1996; Cejko et al., 2009), the ion composition of milt obtained at this early term agrees with that of milt from wild breeders during the spawning season (Dziewulska, 2020).

Our results highlight that the sperm of outdoor-reared breeders is applicable already in November, about 2 months earlier before the first oocytes in females achieve maturation competence (Ljubobratović et al., 2021). Therefore, the applicability of the present results is still unclear. Complementing our findings with those obtained for postponed spermiation by Blecha et al. (2016), it appears that outdoor-reared pikeperch males can be used for gamete production from mid-November to mid-June, about twice as long than females (Rónyai, 2017; Ljubobratović et al., 2019, 2021). This knowledge can be used for out-of-season larvae production. Namely, to obtain eggs in the summer and autumn periods, it is necessary to culture breeders under fully controlled conditions (Żarski et al., 2019; Ljubobratović et al., 2020). This implies thermal conditioning of the water, and the energy investment for this purpose is directly influenced by the volume of the water, which, again, is a function of the fish biomass (Dalsgaard et al., 2013). Therefore, the utility of the single stock of males over the multiple female spawning batches can lead to a reduction in the biomass of males, thus reducing costs via reducing the size of fully controlled broodstock climate rooms. Although the values of all sperm quality parameters assessed in the present study indicate that the can be used for fertilization, two important parameters were not evaluated. Therefore, sperm concentration and motility, together with direct fertilization of off-season-obtained eggs, should be a topic of future studies to reliably determine the full period of gamete production ability in pikeperch males.

Temperature is the primary environmental factor affecting the development of a reproductive system and gonadal maturation in fish (Van Der Kraak and Pankhurst, 1997). While in Chondrostei, the effect of rearing water thermal conditions on sperm quality is significant (Williot et al., 2000) in Teleostei, thermal increment was only hypothesized as beneficial, as in the case of ide (*Leuciscus idus*) (Jamróz et al., 2008; Cejko et al., 2009). However, the only study which approached this issue found improvement in sperm quantity and quality with post-injection thermal increase insignificant (Cejko et al., 2010). This agrees with our findings of no fully conclusive differences between GnRH-LOW and GnRH-HIGH. Therefore, concerning the obtained results, both tested single-injection-GnRH strategies can be assumed as effective, and perhaps this plasticity of pikeperch males can be a useful tool to extend the period of high-quality gamete availability. In this sense, the recently explained multiple-injection strategy (Żarski et al., 2020) could be a subject of interest for future studies on pre-seasonally treated pikeperch males. As these males were induced to spermiate 5 months before the season, future studies should evaluate the length of the spermiation period if extended via repeated injections.

In the climatic conditions of Hungary, spermiation in outdoor-reared pikeperch males can be hormonally induced about 5 months prior to the natural spawning season. The assessed sperm quality parameters seem favorable compared to earlier reports on wild and domesticated pikeperch. Both applied GnRH strategies – pre-warming and post-warming injection – led to improved spermiation compared to the use of gonadotropin. Thus, gonadoliberin injections at different times post-transport can be applied to prolong the period of gamete availability. Sodium is the most abundant ion in pikeperch sperm, and its concentration directly correlates with sperm kinematics. A comprehensive study is recommended to determine the full period of quality sperm availability in outdoor-reared breeders.

CRediT authorship contribution statement

Mustafa Erkan Özgür: Conceptualization, Methodology, Software, Formal analysis, Writing – original draft, Funding acquisition. **Selim Erdoğan**: Investigation, Formal analysis, Writing – review & editing, Visualization. **Božidar Rašković**: Methodology, Formal analysis, Writing – original draft, Visualization. **Georgina Fazekas**: Investigation, Resources. **Uroš Ljubobratović**: Conceptualization, Validation, Writing – original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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