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Repeated hormonal induction of spermiation affects the stress but not the immune response in pikeperch (*Sander lucioperca*)

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Percids, immunity, sperm quality, hCG, sGnRH α .

Abstract

Hormonal induction of spermiation, previously reported to be immunogenic in fishes, is a common hatchery practice in pikeperch, *Sander lucioperca*. The aim of the present study was to investigate the effects of repeated induction of spermiation in pikeperch, following application of either human chorionic gonadotropin (hCG) or salmon gonadoliberine analogue (sGnRH α) on sperm quality indices as well as on immune and stress response. Mature males of pikeperch ($n = 7$ per group) were stimulated twice with five days between injections of either hCG (hCG; 500 IU kg⁻¹), sGnRH α (sGnRH α ; 50 μ g kg⁻¹) or NaCl (control group; 1 ml kg⁻¹) to assess spermatozoa motility with a computer-assisted sperm analysis (CASA) system. During second sampling, blood plasma was sampled for humoral innate immune (peroxidase and lysozyme activities, ACH50), stress (cortisol, glucose) and endocrine (testosterone) markers. In addition, the head kidney was dissected to assay the expression of several immune genes (such as *il1*, *c3*, *hamp*, *tnf- α* and *lys* genes). The results indicate that hormonal treatment significantly increased sperm production. Sperm sampled after the hormonal treatment maintained its quality throughout the study, regardless of the sampling time. However, it appears that the application of hCG induced elevated cortisol and glucose plasma levels compared to the control group. Almost all immune markers, except the relative expression of hepcidin (*hamp* gene), were unaffected by the two hormones applied. The results showed that the induction treatment of spermiation processes in pikeperch resulted in an important physiological stress response for which the intensity varied according to the hormonal agent used. However, this stress response (more profound following application of hCG) was weakly associated with innate immune functions. On the other hand, a significant negative correlation between the expression of several important immune markers (peroxidase activity, relative expression of *c3* and *il1* genes) and sperm quality indices indicates significant involvement of immune status on sperm quality. The results obtained shed light on immune-system-induced modifications to sperm quality. The data presented here highlight the need for careful revision of broodstock management and selection practices where welfare status as well as individual predispositions of fish to cope with the stress should be taken under the consideration.

1. Introduction

Expansion of the aquaculture sector relies on domestication and selective breeding programs that allow preservation of desired traits to promote high survival and growth rates of progeny [1,2]. This, however, requires implementation of advanced breeding technologies in which controlled reproduction is among the most important steps [3,4].

This especially applies to newly domesticated fish species, such as pikeperch (*Sander lucioperca*), whose production technology, despite having established commercial production, is still being optimised [5].

Although some semi-controlled reproduction methods have been developed in pikeperch, based on spawning in artificial nets, the optimisation of percid fish breeding requires application of *in vitro* fertilisation to enable the production of specific crossbreeds. Considering the high fecundity of females (up to 2 million eggs per fish [6]), obtaining a suitable amount of high-quality sperm remains challenging. That is why sperm from several males is often pooled for fertilisation in the hatchery practice [4]. Recent findings suggest that this may constitute a huge problem due to sperm competition and may lead to the loss of genetic variability [7]. Therefore, control over sperm quality and quantity obtained from a single male with desirable traits is a crucial element of a successful selective-breeding program.

Another bottleneck in controlled reproduction of percid fishes is the synchronisation of final maturation. Intensively cultured percid males are usually not spermiating when females are ready to spawn [3,5]. In addition, those males, from which it is possible to obtain sperm, usually release small volumes of milt, further lowering its fertilisation capacity. Consequently, in controlled reproduction of percid fishes, hormonal stimulation is practiced to synchronise ovulation and spermiation as well as to enhance the quality and quantity of sperm obtained [8,9].

Hormonal stimulation of ovulation and spermiation in percids usually uses two types of spawning agents: gonadotropins (GtH; human chorionic gonadotropin [hCG]) and gonadoliberin (usually in the form of pure gonadoliberin analogs) [4]. Both types act at different levels of the hypothalamic-pituitary-gonadal (HPG) axis. Application of GtH stimulates the gonads for production of sex steroids (directly influencing maturation of the gonads), whereas application of gonadoliberin analogs induces the secretion of endogenous GtH [10]. Both spawning agents have been tested in percids for over a decade, with similar efficiency in the stimulation of ovulation [4] and spermiation [9].

Sperm is usually collected for analysis only once from each fish when the total amount of sperm possible to obtain is stripped out [9,11]. This is done in order to prevent testicular sperm ageing [12], a symptom also noticeable in Eurasian perch, *Perca fluviatilis*, after injection with hCG [9]. Preliminary observations revealed that further sperm collection (up to 3 days after first sperm stripping) in pikeperch is possible, although in very small volumes (below 0.3 ml per kg of body weight) and of lower quality (below 60% of motility) (D. Źarski, unpublished), confirming the findings of Grozea et al. [13]. This could be associated with the slow progression of final maturation of the sperm (from spermatids to spermatozoa, for

details see Schulz et al. [14]) and consequently, spermiation within a few days following first stripping. This could in turn be related to low levels of sex steroids responsible for maturation of the spermatids that remained in the testes following first sperm collection [14,15]. It can be assumed that, before the second sperm collection, additional hormonal stimulation should be performed that may enhance spermiation, by influencing the production of sex steroids as is practiced in other cultured species (see the review by Mylonas et al. [15]). Such a strategy of hormonal induction of spermiation in pikeperch was proposed by Grozea et al. [13], who recommend repeating the hormonal treatment at least three days following the first hormonal injection in order to collect high volumes of sperm 10 h later at 17 °C. However, Grozea et al. [13] considered the combination of different hormonal preparations (different types of hormones used for the first and second injection) and only a single parameter (sperm volume) was investigated without providing any information on sperm quality or physiological response of the fish. Therefore, more detailed study investigating the possibility and impact of repeated hormonal treatments on the induction of spermiation in pikeperch is needed.

Application of hCG in Teleosts was reported to be highly immunogenic [10]. Therefore, repeated administration or subsequent attempts of hormonal stimulation over repeated reproductive seasons will make the same specimens unresponsive to hCG. In addition, it was also reported that the application of different hormonal preparations affects the level of cortisol circulating in the blood plasma at different intensities [16]. This may suggest that improper hormonal therapy may negatively affect the physiological stress and/or immune response, and thereby the welfare of the fish. However, the hCG-stimulated immune response seems to be species-specific as no antibodies were detected in freshwater cyprinids following injection with hCG [17]. It should be emphasised that this aspect, never studied in percids, should be carefully reconsidered before making recommendations to fish farmers regarding hormonal therapies to be applied in commercial production. Especially, when studies suggest that negative effects may accumulate over time in cultured fishes [10,17].

Hormonal treatment in fishes, by stimulating excessive secretion of sex steroids [18], such as testosterone in males may activate humoral immune response [19]. Therefore, while considering the effect of different type of hormonal preparations on the stress and the immune response in males testosterone level in the blood plasma should also be investigated along with various stress (including cortisol and glucose [16,20]) and immune markers (including lysozyme and peroxidase activities [21]). However, to address the research question in more complex manner expression level (in the head kidney) of genes being identified as robust stress and immune markers (e.g. *tnf-α* [22], *il-1* [23], *lys* [24] and *c3* [25]) is of high importance.

The aim of this study was to investigate whether repeated hormonal induction of spermiation improves sperm quality and volume in pike-perch males and to assess the effects of different spawning agents on the stress and immune response in this species.

2. Material and Methods

The experiment was performed in compliance with European legislation for fish welfare and approved by the local Ethics Committee (APAFIS-2016022913149909).

2.1. Broodstock management

Pikeperch broodstock (85 females and 110 males; age 6+; average weight 2.84 ± 0.72 kg) was reared in a recirculating aquaculture system (RAS), consisting of 8000 L rectangular tanks. The stocking density did not exceed 35 kg m^{-3} . When the females started to mature, the males were separated from the females, although both sexes were kept in the same RAS. The system was supplied with tap water. Broodstock management protocols throughout the entire life of the fish was developed by the fish farm (Asialor SARL, Pierrevillers, France) where the fish were coming from. The fish used in this study were reproduced already three times before the experiment was carried out indicating that they were fully 'functional' spawners with spawning experience. Fish were fed according to the typical, commercially relevant practice with compound-extruded feed (50% protein, 11% fat, 10% moisture, 1.55% crude fibre, 1.35% phosphorus, 9.5% ash and 17.9% nitrogen-free extract; Le Gouessant, France) with a daily feeding rate ranging between 0.2 and 1.0% of biomass, depending on temperature and apparent satiation. Briefly, the fish were offered manually small portions of feed 8–12 times a day until the staff of the farm noticed typical foraging behavior (fish were swimming up for the feed and were ingesting it). After the foraging behavior was not evident anymore, the fish were still given small portions of feed twice more (in order to insure the satiety). During the experiment the fish were not fed what is typical hatchery practice at the farm aiming at avoiding contamination of gametes with either feces or urine. For the experiment 21 randomly chosen males ($n = 21$; age 6+; average weight 2.65 ± 0.52 kg) were used.

The fish were exposed to a photo-thermal program simulating annual fluctuations as described by Źarski et al. [4]. The light intensity was fixed at 20 lx (provided by neon tubes) at the water surface. After the wintering period (during which fish were exposed to a temperature below 10°C and a photoperiod of 9 and 15 h of light and dark periods, respectively; see Źarski et al. [4]), temperature and photoperiod were increased, reaching 12°C and 14 h of light within 6 weeks. Then the photo-thermal variations were stopped and both factors remained constant until the end of the experiment. Seven days later, the maturation stage of females was checked, and they were found to enter into the final oocyte maturation process heralding the commencement of the spawning period. At this time, the males were randomly assigned to one of three groups, each treated with a different spawning agent. Each fish was tagged individually (with passive integrated transponders, i.e., PIT-tags). Males assigned to the same group were kept together in separate cube-shaped cages placed in the tanks. Each manipulation (injection and sperm stripping) was performed under anesthesia (MS-222 at a dose of 150 mg L^{-1} [26]). At the end of the experiment, the anaesthetised fish were euthanised by overexposure to the anesthetics (MS-222, 300 mg L^{-1} [27]).

2.2. Experimental design and sampling

For the experiment, three groups were distinguished ($n = 7$ for each group) and each group was treated twice, at five-day intervals, with either 0.9% NaCl (control group), salmon gonadolibertine analogue (sGnRHa; each time at a dose of $50 \mu\text{g kg}^{-1}$) (Bachem, Switzerland) or hCG (each time at a dose of 500 IU kg^{-1}) (Chorulon, Intervet, France) (Fig. 1). The fish were injected intraperitoneally at the base of the ventral fin. The hormones were dissolved/diluted in 0.9% NaCl solution so that each fish received 1 ml of the solution per kg of body weight, each time. The doses of the spawning agents were those recommended, and applied during the commercial reproduction of pikeperch [4].

On day five after the first injection, the sperm was collected from each male into the Eppendorf tubes with a catheter (as described by Sarosiek et al. [11]). From each male, as much sperm as possible was collected. Next, the total volume of sperm collected was recorded and its quality was evaluated (for details see section 2.3). Then the males were injected for the second time with their respective spawning agent and returned to the tanks. The second sampling was performed five days after the second injection. The time interval was chosen because, in percids, the positive effect of hormonal stimulation was recorded after a minimum of 4 and maximum of 10 days post injection [9]. During the second sampling, each fish was anaesthetised, and then blood was collected with heparinised syringes from the caudal vein. Blood sampling was performed separately for each group within 5 min of the moment the fish net was put in the water as required for further evaluation of cortisol levels in blood plasma [28]. The sampling was performed for each group separately. Each group was left undisturbed for 24 h prior to sampling in order to avoid additional stress. Next, the sperm was stripped (in the same way as the first sampling) from each male separately and sperm quality was further evaluated. At the end, fish were euthanised and the head kidney was sampled for the assay of immune gene expression (Fig. 1). Blood samples were centrifuged immediately after collection (15 min at 6700 g). Plasma and head kidney samples were snap frozen in liquid nitrogen and stored at -80°C prior to further analyses.

2.3. Sperm quality evaluation

Sperm motility in the three groups was recorded with a CASA system (Sperm VisionTM v. 3.7.4., Minitube of America, Verona, USA). Spermatozoa were activated using an ionic solution (50 mM NaCl , 30 mM Tris , pH: 8.0 ± 0.2 [29]) with approximately 0.01 g ml^{-1} of BSA (bovine serum albumin). Motility parameters, such as progressive motility (criteria according to Sperm VisionTM v. 3.7.4. straight line distance $> 5 \mu\text{m}$, pixel to μm ratio: 151:100, pMOT, %), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight linear velocity (VSL, $\mu\text{m s}^{-1}$), linearity of movement (LIN, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) were studied to describe pikeperch spermatozoa movement (after Źarski et al. [9]). Motility assessment was carried out in duplicate (at a ratio of 1:99 v/v sperm-to-activating solution), and moving cells were identified ($1\text{--}100 \mu\text{m}^2$) with a digital camera (JAI CV-A10

CL, Minitube of America, Verona, USA) using a frame rate of 60 frames s⁻¹

2.4. Stress indicators analysis

2.4.1. Cortisol assay

Cortisol was assayed in duplicate using a cortisol ELISA kit (KAPDB270, DIALsource, Belgium), based on a typical competitive binding scenario, following the manufacturer's instructions (as described by Khendek et al. [20]). Briefly, into each well (on 96-well plate delivered by the manufacturer) 20 µl of blood plasma (in duplicates) as well as provided calibrators were pipetted. Next, to each well conjugate working solution was added using a multichannel pipette. Next, the plate was incubated for 45 min at room temperature on a plate shaker (200 rpm). After incubation plate was washed three times with wash buffer (provided with the kit). Next, to each well 150 µl of TMB substrate (provided with the kit) was pipetted and further incubated for 20 min at room temperature on a plate shaker. After incubation to each well 50 µl of stopping solution (provided with the kit) was added in order to terminate the reaction. The optical density (OD) was measured at 450 nm wavelength (FLUOstar® Omega, BMG LABTECH, Germany). The assay dynamic range was between 0 and 600 ng ml⁻¹. The intra-assay coefficient of variation and the analytical sensitivity were 5.8%, and 4 ng ml⁻¹, respectively.

2.4.2. Plasma glucose assay

Plasma glucose, assayed in triplicate, was determined calorimetrically based on a glucose oxidase/ peroxidase method described by Trinder [30]. Briefly, 20 µl of samples and standards were deproteinised using perchloric acid (0.33 M) and centrifuged 10 min at 850 g (Centrifuge 5424, Eppendorf, Belgium). In a flat-bottomed 96-well plate, 10 µl of each sample and standard were mixed with a glucose oxidase/ peroxidase reactional solution (glucose oxidase type X-S, peroxidase type 1, ABTS, phosphate buffer 0.1 M, pH 7.5) after incubation for 15 min at 38 °C, the absorbance was measured at 436 nm using the 96-well plate reader (FLUOstar® Omega, BMG LABTECH, Germany).

2.5. Immune parameters analysis

2.5.1. Peroxidase activity

The total peroxidase activity in plasma was assessed according to Quade and Roth [31]. The samples and negative control (distilled water) were assayed in triplicate. In a flat-bottomed 96-well plate, 7 µl of plasma were diluted in 68 µl of Hanks' Balanced Salt Solution (HBSS) without Ca²⁺ or Mg²⁺. As a substrate, 25 µl of reactional solution (20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride and 5 mM H₂O₂) was added. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and the absorbance was measured at 450 nm. One unit (U) of peroxidase activity was defined as the amount producing an absorbance change of 1 optical density (OD).

2.5.2. Plasma alternative complement pathway

The plasma alternative complement pathway (ACH50) procedure was used to measure the haemolytic activity in plasma samples using rabbit red blood cells (RRBC) as targets [32]. A serial dilution from 1/ 20 to 1/480 into a veronal buffer (IDVert, France) was performed in duplicate for each plasma sample in around-bottomed 96-well plate. Then, 10 µl of RRBC (Biomerieux) suspension (3% in veronal buffer) were added to each well and the plate was incubated at 25 °C for 120 min at 300 rpm using the orbital shaker (KS 4000 ic control, IKA®-Werke GmbH & Co. KG, Germany). The total haemolysis was obtained by mixing 10 µl of RRBC lysed with bi-distilled water and the spontaneous haemolysis was obtained by adding veronal buffer to 10 µl of RRBC (total volume = 70 µl). After the incubation, the turbidity (inversely proportional to the haemolysis) was measured using the 96-well plate reader (FLUOstar® Omega, BMG LABTECH, Germany) at 650 nm. The ACH50 value (unit ml⁻¹ of plasma) is the reciprocal of the plasma dilution which induces the haemolysis of 50% of the RRBC.

2.5.3. Lysozyme activity

The lysozyme activity protocol was adapted from Siwicki and Studnicka [33] and Douxfils et al. [34]. In flat-bottom 96-well plates, samples were assayed in triplicate by mixing 7 µl of pikeperch plasma with 130 µl of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension at 0.6 g L⁻¹ in phosphate buffer (Na₂HPO₄, 0.05 M, pH 6.2). A negative control (phosphate buffer) and a positive control (*M. lysodeikticus*) were also assayed in triplicate in the same plate (total volume = 137 µl). The absorbance (OD) at 450 nm was monitored between 0 min and 15 min (linearity range) using the 96 well-plate reader. Lysozyme activity represents the amount of enzyme decreasing the turbidity by 0.001 OD per min.

2.6. Testosterone assay

The testosterone was assayed in duplicate on 25 µl of plasma using the DIALsource Testosterone ELISA Kit (KAPD1559) according to the manufacturer's instructions (as described by Roche et al. [18]). A dilution at a ration 1:2 of the plasma samples was performed. The assay dynamic range was between 0 and 16 ng ml⁻¹. Sensitivity was 0.083 ng ml⁻¹, coefficient of variation (CV) intra-assay varied between 1.5 and 9.5% for low and high levels, respectively.

2.7. Immune gene expression

Total RNA from the head kidney was extracted using Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland) according to the producer's protocol. The concentration of RNA was analysed with NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The quality of the RNA samples was checked using a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA) and samples with RIN value higher than 9.0 were used for further analysis. Next, the RNA was treated with TURBO DNase (Cat. No. AM2238, Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) in order to remove contamination of samples with genomic DNA. Reverse transcription was performed using MAXIMA First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). The real-time quantitative polymerase chain reaction (RT-qPCR) was performed with LightCycler 480 II (Roche, Bazylea, Switzerland) using DyNAmo HS SYBR Green qPCR Kit

(Thermo Fisher Scientific Inc). Enzyme activation and denaturation was performed for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min [35].

In the head kidney, the relative expression of immune-related genes was investigated by RT-qPCR. The chosen genes are involved in bactericidal defence (C-type lysozyme [lys], hepcidin c [hamp], complement C3 [c3]), pro-inflammatory action (interleukin-1b [il-1] and tumor necrosis factor alpha [tnf- α]). In addition, expression levels of reference genes, β -actin and elongation factor alpha (ef1- α) used for the normalisation of data were analysed (after Baekelandt et al. [21]). The primers used are specified in Table 1.

3. Results

Only two males were found to slightly spermiate (less than 0.1 ml was obtained from those males) at the beginning of the experiment – one from control group and one from sGnRH α -treated group. During both samplings, most of the fish from hormonal-treated groups (except 1 fish treated with hCG) were found to spermiate, while in control groups, small amounts of sperm were stripped from four and six fish during the 1st and 2nd samplings, respectively. From control fish, significantly lower volumes of sperm (0.1–0.2 ml of sperm per kg of body weight on average) were stripped at each sampling event compared to the hormonal-treated groups (Fig. 2). There were no differences ($p > 0.05$) in terms of the amount of sperm collected between the two hormonal-treated groups and between the two samplings (Fig. 2).

The low amount of sperm collected from control fish and one fish from hCG-treated group did not allow us to perform robust analysis of spermatozoa motility. Sperm obtained from the remaining fish were characterised by similar quality markers ($p > 0.05$) (Table 2). The analysis of correlation between the 1st and 2nd samplings (based on full data obtained from 13 fish) revealed high and significant ($p < 0.05$) positive correlation of all sperm quality markers evaluated (Fig. 3).

4. Discussion

4.1. The effect of hormonal stimulation on spermiation in domesticated pikeperch

In the present study significant enhancement of amount of sperm obtained from pikeperch following hormonal treatment was recorded. It should be noted that the sperm volumes obtained in both hormonal-treated groups (on average 0.9–1.1 ml kg⁻¹) were very close to average values reported so far for pond-reared fish (0.5–1.5 ml kg⁻¹ by Blecha et al. [36] and 0.4–1.1 ml kg⁻¹ by Korbuly et al. [37]). It also appeared that the latency time tested (five days between hormonal injection and sperm collection) was suitable for the collection of high-quality sperm as reported for Eurasian perch [9]. However, considering the fact that sperm can be collected after an additional five days indicates the possibility of the development of specific hatchery protocols for multiple sperm collections in this species following more specific research aiming at optimisation of the protocol. Nevertheless, the data presented

2.8. Data analysis and statistics

Data were analysed with MS Excel 2016 and STATISTICA 13 (TIBCO Software Inc., Palo Alto, CA, USA), tested for homogeneity of variance with Levene's test and further tested for normal distribution with the Shapiro-Wilk test. Data meeting the criteria of normality were analysed with either t-test (sperm quality indicators) or one-way ANOVA followed by Tukey's post-hoc test (blood parameters, gene expression data). Additionally, Pearson's correlation coefficients were calculated for sperm quality indices between the 1st and 2nd sampling in order to compare the trend of sperm quality in the same individuals between the two samplings. Besides, correlation analysis was performed between stress, immune and endocrine markers against sperm quality indices. All statistical tests were performed at a significance level of 5% ($p < 0.05$).

Application of hCG significantly increased the level of plasma cortisol when compared to the level assayed in control fish ($p < 0.05$). In addition, this spawning agent significantly increased glucose level ($p < 0.05$) compared to both remaining groups ($p > 0.05$), among which similar levels of plasma glucose were recorded (Table 3). The immune parameters measured (lysozyme, peroxidase activities and ACH50) and testosterone levels showed similar levels among all the groups ($p > 0.05$; Table 3).

Gene expression analysis showed that the injection of hCG significantly lowered the relative expression of hepcidin (hamp) compared to the remaining groups ($p < 0.05$), among which no significant differences were observed ($p > 0.05$). The relative expression level of other genes (il1, tnfa, c3 and lyz) did not significantly differ between groups (Fig. 4).

Six negative correlations between immune status and sperm quality were recorded. An additional significant negative correlation was detected between testosterone and VSL. Lack of significant correlation between stress markers and sperm quality indices was recorded ($p > 0.05$, Table 4).

in this study are the first successful multiple sperm collections in pikeperch to be reported.

Previous studies have noted that hormonal induction may enhance sperm volume but its effect on sperm quality indices, such as motility rate, remains debatable [15]. In the common dace, *Leuciscus leuciscus*, VCL and VSL were found to be higher after application of sGnRH α as compared to hCG [38]. In the present study, there were no differences in obtained sperm volume or sperm motility indices in any of the samplings, regardless of the type of hormone. Motility as well as average VCL and VSL values recorded in this study, were similar to those already reported for wild pikeperch by Sarosiek et al. [11] and VCL values were nearly twice as high as those reported for domesticated pikeperch by Schaefer et al. [39]. This indicates, that the quality of sperm obtained in the present study was high and was not related to the type of hormone applied. Therefore, both types of spawning agents may be recommended for induction of spermiation in pikeperch.

Further analyses revealed a very strong correlation for sperm motility indices between the two sampling times (Fig. 3), which, to the best of our knowledge, has not been reported thus far in freshwater fishes. These results clearly indicate that sperm quality is not affected by subsequent handling, hormonal treatment and sperm collection. In other words, males that produce low-quality sperm upon first collection will produce low-quality sperm upon second collection. This is extremely important information for fish farmers and may help the selection process by removing fish that yield low-sperm quality from the broodstock. However, the final recommendation can only be given if the sperm quality produced by a given male can be maintained over subsequent reproductive seasons, confirming the hypothesised robustness of using sperm quality as a specific trait for selection.

4.2. The effects of hCG and sGnRHa on immune response in pikeperch

It was previously suggested that application of hCG, being considered an antigen for fish, may induce an immune response in fish that affects its effectiveness over consecutive treatments [10,17]. However, despite being treated twice within the 10-day period, fish did not show any innate humoral immune response. None of the immune markers investigated in this study, namely lysozyme and peroxidase activities as well as alternative complement pathway (ACH50), were affected by any of the tested hormonal preparations. It was previously reported that domesticated pikeperch may successfully be induced to reproduce with application of hCG over several years without negative effect on its reproductive performance [4,40]. Along with the lack of differences in the expression levels of genes considered to be immune response markers (i.e., *tnf- α* , *il-1*, *lys* and *c3*), it can be concluded that intraperitoneal injection with either sGnRHa or hCG does not affect the immune response in this species, as previously suggested for pikeperch by Fala-hatkar and Poursaeid [16].

4.3. The effects of hCG and sGnRHa on stress response in pikeperch

In the present study, significant increments of stress indices were associated with a lowered expression of the *hamp* gene following hCG treatment. One hypothesis explaining this could be linked with increased levels of testosterone as previously reported [41]. Consequently, the lowered expression of *hepcidin* could cause excessive erythropoiesis [42] which was also reported to be linked with increased levels of cortisol [43]. However, neither Roche et al. [18] nor Źarski et al. [4] found any difference between hCG and sGnRHa in terms of increase in sex steroid levels within the first few days after injection. A lack of significant increase in testosterone levels was also observed in our study. Therefore, the pathway linking treatment of hCG and increased stress response through testosterone-*hamp* mediated processes cannot be confirmed, and the elevated cortisol and glucose levels following injection with hCG remains unclear and requires further research.

4.4. Potential involvement of testosterone in immune response

In pikeperch females, as in other species, increased levels of testosterone following injection of hCG and sGnRHa were

observed until 48 h after hormonal treatment [4,18], and to our knowledge, no information on further kinetics of sex steroid levels in pikeperch is available. It has been shown that injection of testosterone in gilthead seabream, *Sparus aurata*, increased ACH50 and peroxidase activities three days after treatment and decreased them to a basal level seven days later [19]. This may be related to a strong testosterone-induced immune response that was not observed in our study. A very low level of testosterone was recorded in all groups in the present study, similar to the basal level reported for pikeperch in Roche et al. [18] and Źarski et al. [4]. This indicates that the testosterone-induced immune response, if any, could already have repressed upon sperm collection.

4.5. Potential involvement of testosterone in stress response

The increased levels of cortisol and glucose after injection with hCG, as recorded in our study are in accordance with the findings of Falahatkar and Poursaeid [16]. In their study, the changes observed were linked with immune response as well as with stress induced by the spawning act. In this study, males injected with NaCl spermated only slightly, but fish treated with sGnRHa released an ample amount of sperm, suggesting that the hormonal treatment was effective. So, it cannot be confirmed that spawning readiness, considered an important stress-inducing factor by Falahatkar and Poursaeid [16], may be responsible for the elevated cortisol or glucose levels. Additionally, typical immune markers, such as *tnf- α* and *il-1*, usually downregulated by elevated levels of glucocorticoids [44–46], were not affected by the applied hormonal treatment. It can, thus, be suggested that other pathways contributed to the increase observed in cortisol and glucose greater than those suggested by Falahatkar and Poursaeid [16].

4.6. Correlation between sperm quality and immune and stress markers

It is widely known that stress affects reproductive performance, including gamete quality in fishes [47]. However, correlation analysis did not confirm a significant effect of stress indices on spermatozoa motility. Interestingly, there was a significant ($p < 0.05$) and always negative correlation between some sperm quality parameters (*pMOT*, *VCL*, *VSL* and *BCF*) and important immune status markers (*peroxidase*, *il-1*, *tnf- α* , *c3*). The activation of immune response generates some physiological costs that can negatively affect reproductive performance [48]. It has been reported that immune responses have detrimental effects on the sperm quality in mammals [49] and birds [48]. It was also reported that diet-modulated immune responses negatively affected sperm motility as well as embryonic survival in medaka (*Oryzias latipes*) [50]. Such differences were not confirmed in our study; however, correlation analysis revealed significant relationship between immune status and sperm quality. To the best of our knowledge, the significant effect of several important immune indices on sperm quality parameters is reported here for the first time for Teleostei. However, the evidence should be confirmed and mechanisms standing behind immune-response-induced decrease in sperm quality remains to be explored in future studies.

5. Conclusion

Hormonal stimulation was found to be essential to obtain high quality and quantity of sperm during the out-of-season reproduction of domesticated pikeperch. It was also found, for the first time, that multiple, hormonally controlled sperm collection is possible in this species, though optimised hatchery-applicable protocols remains to be developed. Due to the fact that application of hCG induced a stress response

and decreased expression of the hamp gene its application in controlled reproduction of pikeperch should be re-considered. Additionally, the data obtained suggest immune-system-induced modifications of sperm quality highlighting the need for careful revision of broodstock management and selection practices taking into account welfare status as well as individual predispositions of fish to cope with the stress.

Table 1. Names, symbols, accession numbers as well as primer sequences of the genes analysed in the present study. All the primers were used according to Baekelandt et al. (2019).

Gene name	Gene symbol	Accession number	Forward (5'-3')	Reverse (5'-3')
Beta actin (reference gene)	<i>β-actin</i>	MF472627	CGACATCCGTAAGGACCTGT	GCTGGAAGGTGGACAGAGAG
Elongation factor 1 (reference gene)	<i>ef1-α</i>	MF472628	TGATGACACCAACAGCCACT	AAGATTGACCGTCGTTCTGG
C-type lysozyme	<i>lys</i>	MF472629	AGCCAGTGGGAGTCGAGTTA	CATTGTCGGTCAGGAGCTCA
Hepcidin c	<i>hamp</i>	MK036790	CCGTCGTGCTCACCTTTATT	GCCACGTTTGTGTCTGTTGT
Complement component 3	<i>c3</i>	MF472630	TGGTGATGTGAGAGGAGCAG	GACGTCATGGCAACAGCATA
Interleukin 1b	<i>il-1</i>	MK036791	TTTCCCATCATCCACTGACA	ATTCACACACGCACACCATT
Tumor necrosis factor alpha	<i>tnf-α</i>	MK167462	CTGATTCGCCTCAACGTGTA	GGAGATGGGTCATGAGGAGA

Table 2. Sperm motility analysis performed with the computer assisted sperm analysis (CASA) system on pikeperch sperm obtained each time 5 days after first (1st sampling) or second (2nd sampling) hormonal injection

Empty Cell	Empty Cell	sGnRHa		hCG	
		Mean	SD	Mean	SD
pMOT (%)	1st sampling	69.9	19.5	75.9	22.8
	2nd sampling	78.1	13.5	77.1	19.0
VCL ($\mu\text{m s}^{-1}$)	1st sampling	143.5	23.0	157.5	19.9
	2nd sampling	152.9	18.0	166.6	19.0
ALH (μm)	1st sampling	2.39	0.35	2.41	0.36
	2nd sampling	2.22	0.27	2.28	0.32
VSL ($\mu\text{m s}^{-1}$)	1st sampling	83.0	7.9	89.4	7.3
	2nd sampling	88.6	9.9	91.0	9.0
LIN (%)	1st sampling	0.58	0.05	0.57	0.08
	2nd sampling	0.58	0.04	0.55	0.06
BCF (Hz)	1st sampling	29.2	1.3	28.9	1.0
	2nd sampling	29.6	1.0	29.0	1.1

sGnRHa – salmon gonadoliberine analogue; hCG – human chorionic gonado-tropin; pMOT – progressive spermatozoa motility; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; VSL – straightline velocity; LIN – linearity of movement; BCF – beat cross frequency.

Table 3. Levels (mean \pm SD) of stress and immune response markers recorded in the blood plasma of pikeperch males treated twice (with 5-days interval) with different hormonal preparations prior to blood sampling performed at the end of the experiment. Different letters showed significant differences among the hormonal treatments ($p < 0.05$).

Empty Cell	Cortisol [ng ml^{-1}]	Glucose [$\mu\text{g ml}^{-1}$]	Lysozyme [U ml^{-1}]	Peroxidase [U ml^{-1}]	ACH50	Testosterone [ng ml^{-1}]
NaCl	148.2 \pm 45.7 ^b	55.1 \pm 12.9 ^b	3130 \pm 527	76.1 \pm 20.8	110.1 \pm 28.0	10.0 \pm 7.5
GnRHa	214.4 \pm 84.0 ^{ab}	43.4 \pm 20.9 ^b	3614 \pm 610	60.4 \pm 20.2	124.2 \pm 34.6	6.6 \pm 3.8
hCG	268.5 \pm 99.4 ^a	71.1 \pm 16.6 ^a	3214 \pm 249	62.3 \pm 24.7	115.4 \pm 37.0	10.6 \pm 6.3

sGnRHa – salmon gonadoliberine analogue; hCG – human chorionic gonadotropin

Table 4. Pearson's correlation coefficients (r values) calculated between stress, immune and endocrine markers against sperm quality indices recorded in pikeperch males treated twice with different hormonal preparations (n = 7 treated with hCG and n = 7 with sGnRHa) with 5-days interval. For calculations values recorded 5 days following the second hormonal injection were used. Fields shadowed with orange color indicate significant (p < 0.05) negative correlation. No significant positive correlation was detected.

	pMOT [%]	VCL [$\mu\text{m s}^{-1}$]	VSL [$\mu\text{m s}^{-1}$]	LIN [%]	ALH [μm]	BCF [Hz]
Peroxidase [U ml^{-1}]	-0.61	-0.58	-0.37	0.43	-0.38	0.03
ACH50	0.11	0.00	0.17	0.17	0.48	0.23
Cortisol [ng ml^{-1}]	-0.24	-0.01	0.16	0.22	0.05	-0.02
Lysozyme [U ml^{-1}]	0.28	0.07	0.28	0.17	0.22	0.35
Glucose [$\mu\text{g ml}^{-2}$]	0.14	0.43	0.44	-0.07	0.35	-0.16
Testosterone [ng ml^{-1}]	-0.13	-0.48	-0.61	-0.05	-0.05	0.45
<i>Il-1</i> [relative expression]	-0.30	-0.61	-0.64	0.12	-0.01	0.36
<i>tnf-α</i> [relative expression]	0.02	-0.15	-0.27	-0.06	-0.41	-0.57
<i>hamp</i> [relative expression]	-0.15	-0.42	-0.12	0.40	-0.39	0.29
<i>lys</i> [relative expression]	-0.13	-0.42	-0.53	-0.01	-0.08	-0.05
<i>c3</i> [relative expression]	-0.03	-0.39	-0.59	-0.15	0.02	0.17

hCG - human chorionic gonadotropin (applied dose: 500 IU kg^{-1}); sGnRHa - salmon gonadoliberrine analogue (applied dose: 50 $\mu\text{g kg}^{-1}$)

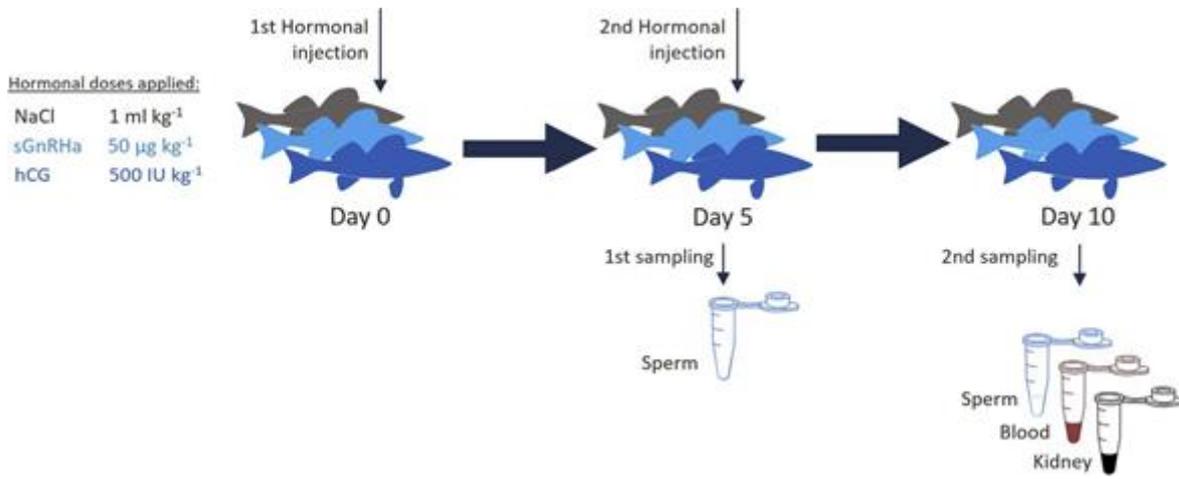


Figure 1. A scheme of the design of the experiment and sampling strategy undertaken in the present study.

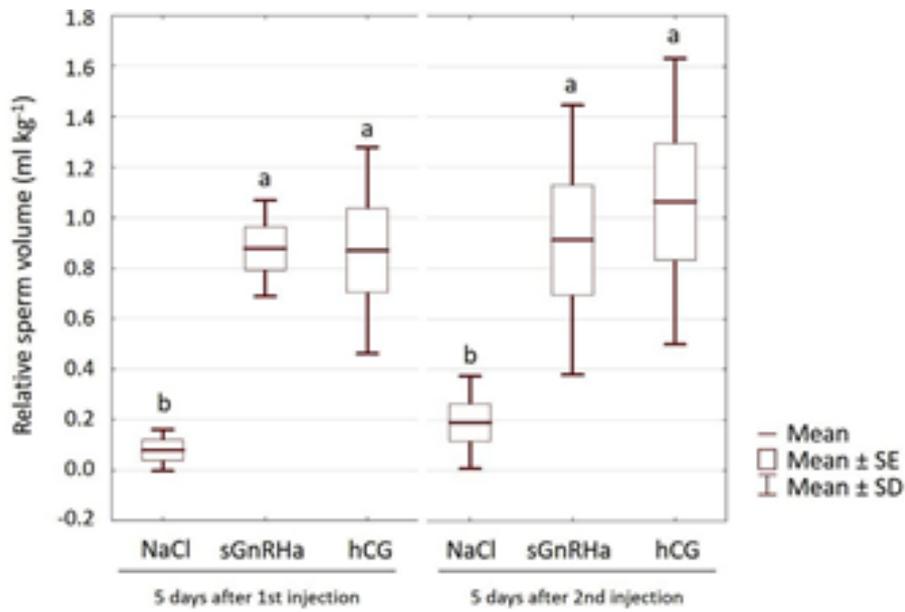


Figure 2. Relative sperm volume obtained (per kg of body weight of the fish) from pikeperch males (n = 7 for each group) treated with different spawning agents with 5-day interval. Sperm sampling was performed each time 5 days after the injection. sGnRHa – salmon gonadoliberine analogue; hCG – human chorionic gonadotropin. Different letters showed significant differences among the hormonal treatments (p < 0.05).

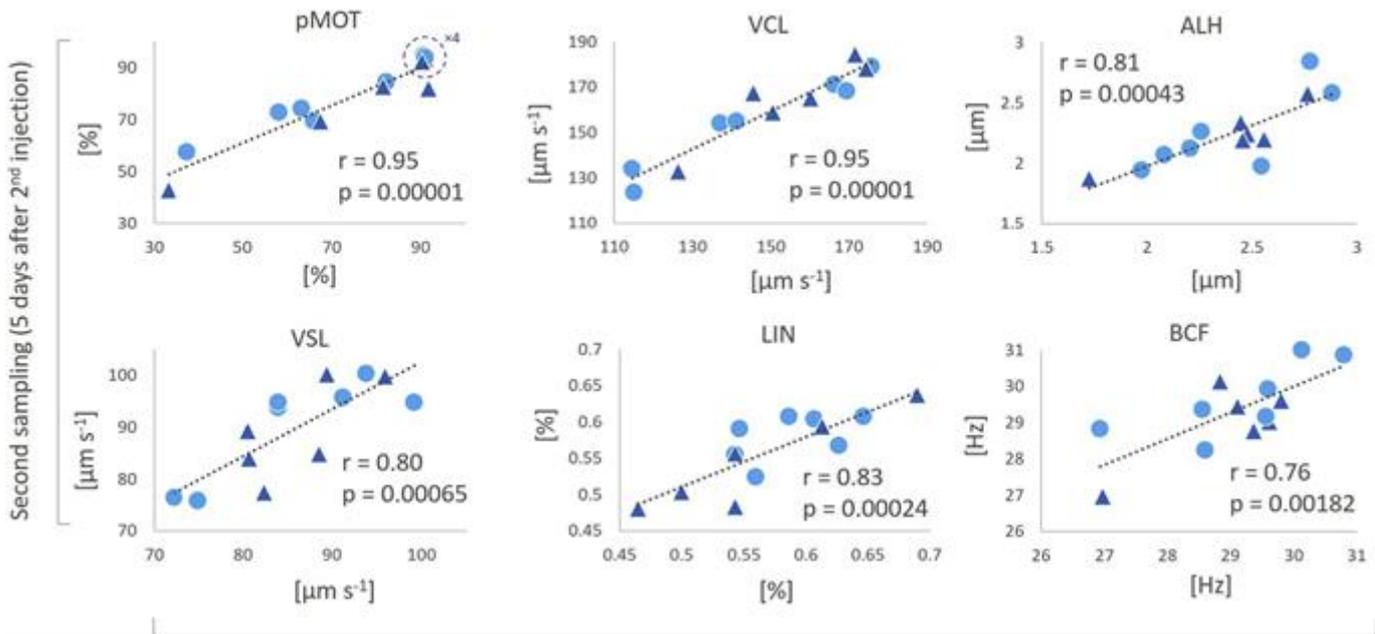


Figure 3. Sperm motility parameters (measured by CASA system), with Pearson's correlation coefficient (r value) and p-values provided separately for each set of data recorded in pikeperch treated with either human chorionic gonadotropin (hCG; dark blue triangles) or salmon gonadoliberrine analogue (sGnRHa; light blue circles) with 5 day interval. Samples were collected 5 days after 1st injection (x axis) and 5 days after 2nd injection (y axis). On the plot for pMOT points encircled indicates overlaid data-points and the number of data-points plotted in this area is indicated (x 4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

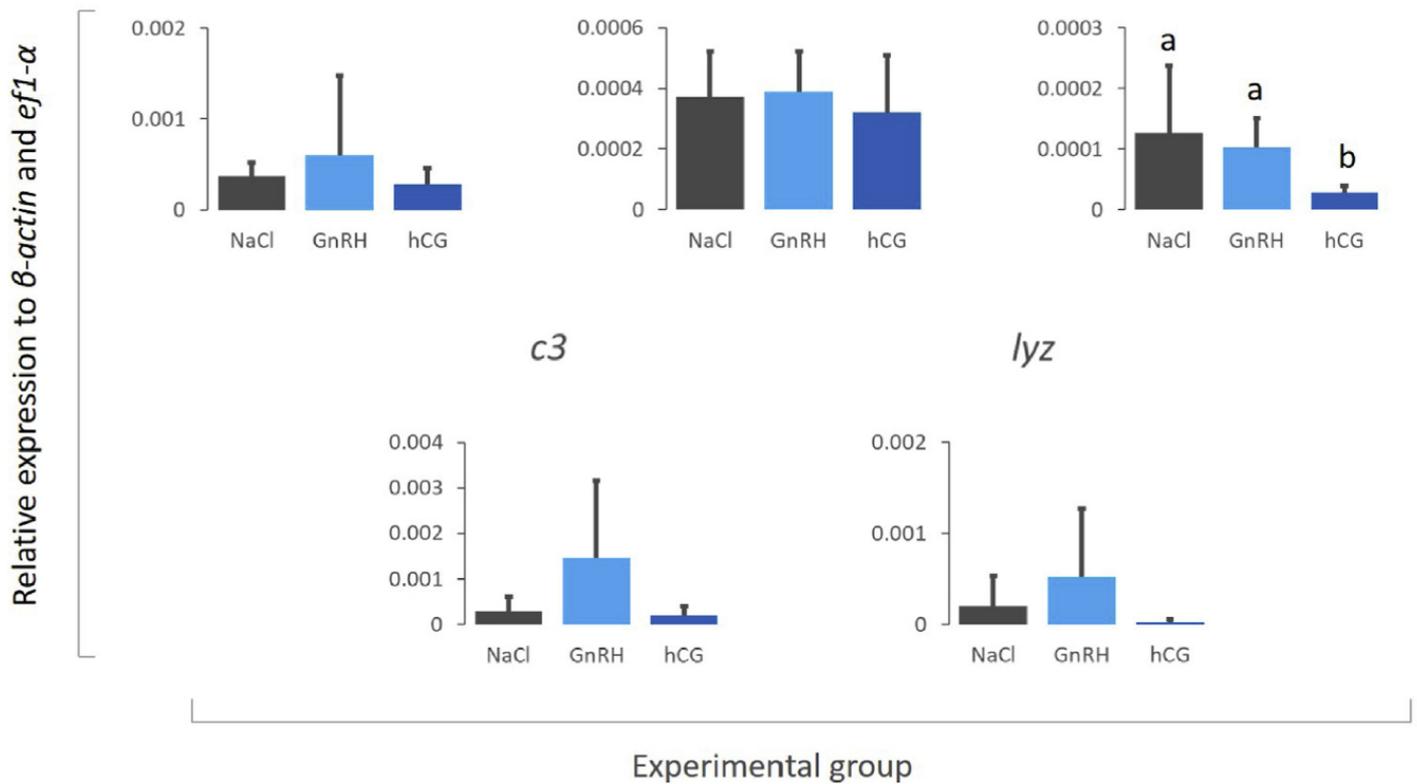


Figure 4. Relative immune genes expression (mean \pm SD) (normalized to geometric mean of two housekeeping genes: beta actin [β -actin] and elongation factor alpha [$ef1-\alpha$]) in head kidney of pikeperch males treated twice (with 5-days' interval) with different spawning agents before the final sampling (5 days after 2nd injection). *c3* – complement C3; *lys* – C-type lysozyme; *hamp* – hepcidin c; *il-1* – interleukin-1b; *tnf- α* – and tumor necrosis factor alpha; *sGnRHa* – salmon gonadoliblerine analogue; *hCG* – human chorionic gonadotropin. Different letters showed significant differences among the hormonal treatments ($p < 0.05$).

6. References

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