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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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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 analog (sGnRHa) 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}), sGnRHa (sGnRHa; 50 µg 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.

Keywords: percids; immunity; sperm quality; hCG, sGnRHa

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

gonadoliberins (usually in the form of pure gonadoliberine 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 gonadoliberine 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. Żarski, 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.

121 The aim of this study was to investigate whether repeated hormonal induction of
122 spermiation improves sperm quality and volume in pikeperch males and to assess the effects of
123 different spawning agents on the stress and immune response in this species.
124
125 **2. Materials and methods**
126 The experiment was performed in compliance with European legislation for fish welfare
127 and approved by the local Ethics Committee (APAFIS-2016022913149909).
128 **2.1. Broodstock management**
129 Pikeperch broodstock (85 females and 110 males; age 6+; average weight 2.84±0.72 kg)
130 was reared in a recirculating aquaculture system (RAS), consisting of 8000 L rectangular tanks.
131 The stocking density did not exceed 35 kg m^{-3}. When the females started to mature, the males
132 were separated from the females, although both sexes were kept in the same RAS. The system
133 was supplied with tap water. Broodstock management protocols throughout the entire life of
134 the fish was developed by the fish farm (Asialor SARL, Pierrevillers, France) where the fish
135 were coming from. The fish used in this study were reproduced already three times before the
136 experiment was carried out indicating that they were fully 'functional' spawners with spawning
137 experience. Fish were fed according to the typical, commercially relevant practice with
138 compound-extruded feed (50% protein, 11% fat, 10% moisture, 1.55% crude fibre, 1.35%
139 phosphorus, 9.5% ash and 17.9% nitrogen-free extract; Le Gouessant, France) with a daily
140 feeding rate ranging between 0.2 and 1.0% of biomass, depending on temperature and apparent
141 satiation. Briefly, the fish were offered manually small portions of feed 8-12 times a day until
142 the staff of the farm noticed typical foraging behavior (fish were swimming up for the feed and
143 were ingesting it). After the foraging behavior was not evident anymore, the fish were still given
144 small portions of feed twice more (in order to insure the satiety). During the experiment the fish
145 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 Żarski 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 Żarski 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 gonadoliberine analog (sGnRHa; each time at a dose of 50 µg 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 anesthetised, and then blood was collected with heparinised syringes from the caudal vein. Blood sampling was performed separately for each group within five minutes 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 undisrupted 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 euthanized 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 µm, pixel to µm ratio: 151:100, pMOT, %), curvilinear velocity (VCL, µm s^{-1}), straight linear velocity (VSL, µm 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 Żarski 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 to 100 μm^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, DIAsource, 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 Ca^{2+} or Mg^{2+}. As a substrate, 25 µl of reactional solution (20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride and 5 mM H_2O_2) 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 a round-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 (Na_2HPO_4, 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 DIAsource 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 tumour 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.

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% ($p < 0.05$).

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 sGnRHa-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).

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*, *tnfα*, *c3* and *lyz*) did not significantly differ between groups (Fig. 3).

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).

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

342 research aiming at optimization of the protocol. Nevertheless, the data presented in this study
343 are the first successful multiple sperm collections in pikeperch to be reported.

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

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

365

366 *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 Falahatkar 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 Żarski 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.

392

393 *4.4.Potential involvement of testosterone in immune response*

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

404

405 *4.5.Potential involvement of testosterone in stress response*

406 The increased levels of cortisol and glucose after injection with hCG, as recorded in our
407 study are in accordance with the findings of Falahatkar and Poursaeid [16]. In their study, the
408 changes observed were linked with immune response as well as with stress induced by the
409 spawning act. In this study, males injected with NaCl spermiated only slightly, but fish treated
410 with sGnRHa released an ample amount of sperm, suggesting that the hormonal treatment was
411 effective. So, it cannot be confirmed that spawning readiness, considered an important stress-
412 inducing factor by Falahatkar and Poursaeid [16], may be responsible for the elevated cortisol
413 or glucose levels. Additionally, typical immune markers, such as *tnf-α* and *il-1*, usually
414 downregulated by elevated levels of glucocorticoids [44–46], were not affected by the applied
415 hormonal treatment. It can, thus, be suggested that other pathways contributed to the increase
416 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.

4.7.Conclusions

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 optimized 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

442 data obtained suggest immune-system-induced modifications of sperm quality highlighting the
443 need for careful revision of broodstock management and selection practices taking into account
444 welfare status as well as individual predispositions of fish to cope with the stress.
445
446 ## 5. References

447 [1] T. Gjedrem, N. Robinson, M. Rye, The importance of selective breeding in aquaculture
448 to meet future demands for animal protein: A review, Aquaculture. 350–353 (2012)
449 117–129. doi:10.1016/j.aquaculture.2012.04.008.

450 [2] F. Teletchea, P. Fontaine, Levels of domestication in fish: implications for the
451 sustainable future of aquaculture, Fish Fish. 15 (2014) 181–195.
452 doi:10.1111/faf.12006.

453 [3] D. Żarski, A. Horváth, J.A. Held, D. Kucharczyk, Artificial Reproduction of Percid
454 Fishes, in: P. Kestemont, K. Dąbrowski, R.C. Summerfelt (Eds.), Biol. Cult. Percid
455 Fishes, 1st ed., Springer Netherlands, Dordrecht, 2015: pp. 123–161. doi:10.1007/978-
456 94-017-7227-3_4.

457 [4] D. Żarski, P. Fontaine, J. Roche, M. Alix, M. Blecha, C. Broquard, J. Król, S. Milla,
458 Time of response to hormonal treatment but not the type of a spawning agent affects
459 the reproductive effectiveness in domesticated pikeperch, Sander lucioperca,
460 Aquaculture. 503 (2019) 527–536. doi:10.1016/j.aquaculture.2019.01.042.

461 [5] T. Policar, F.J. Schaefer, E. Panana, S. Meyer, S. Teerlinck, D. Toner, D. Żarski,
462 Recent progress in European percid fish culture production technology—tackling
463 bottlenecks, Aquac. Int. (2019). doi:10.1007/s10499-019-00433-y.

464 [6] J. Lappalainen, H. Dorner, K. Wysujack, Reproduction biology of pikeperch (Sander
465 lucioperca (L.)) - A review, Ecol. Freshw. Fish. (2003). doi:10.1034/j.1600-
466 0633.2003.00005.x.

467 [7] J. Beirão, T.B. Egeland, C.F. Purchase, J.T. Nordeide, Fish sperm competition in
468 hatcheries and between wild and hatchery origin fish in nature, Theriogenology. 133
469 (2019) 201–209. doi:10.1016/j.theriogenology.2019.03.034.

470 [8] D. Żarski, Á. Horváth, G. Bernáth, S. Krejszeff, J. Radóczi, K. Palińska-Żarska, Z.
471 Bokor, K. Kupren, B. Urbányi, Stimulation of Ovulation and Spermiation, in: Control.
472 Reprod. Wild Eurasian Perch - A Hatch. Man., 2017: pp. 33–40. doi:10.1007/978-3-
473 319-49376-3_5.

474 [9] D. Żarski, G. Bernáth, J. Król, B.I. Cejko, Z. Bokor, K. Palińska-Żarska, S. Milla, P.
475 Fontaine, S. Krejszeff, Effects of hCG and salmon gonadoliberine analogue on
476 spermiation in the Eurasian perch (*Perca fluviatilis*), Theriogenology. 104 (2017) 179–
477 185. doi:10.1016/j.theriogenology.2017.08.022.

478 [10] Y. Zohar, C.C. Mylonas, Endocrine manipulations of spawning in cultured fish: from
479 hormones to genes, Aquaculture. 197 (2001) 99–136. doi:10.1016/S0044-
480 8486(01)00584-1.

481 [11] B. Sarosiek, K. Dryl, S. Krejszeff, D. Żarski, Characterization of pikeperch (Sander
482 lucioperca) milt collected with a syringe and a catheter, Aquaculture. 450 (2016) 14–

16. doi:10.1016/j.aquaculture.2015.06.040.

[12] C. Fauvel, M. Suquet, J. Cosson, Evaluation of fish sperm quality, J. Appl. Ichthyol. 26 (2010) 636–643. doi:10.1111/j.1439-0426.2010.01529.x.

[13] A. Grozea, A. Telea, B. Korbuly, I. Bănăţean, A. Osman, Preliminary Study Regarding the Efficiency of Different Hormones on Pikeperch Spermiation, 41 (2008) 59–64.

[14] R.W. Schulz, L.R. de França, J.J. Lareyre, F. LeGac, H. Chiarini-Garcia, R.H. Nobrega, T. Miura, Spermatogenesis in fish, Gen. Comp. Endocrinol. 165 (2010) 390–411. doi:10.1016/j.ygcen.2009.02.013.

[15] C.C. Mylonas, N.J. Duncan, J.F. Asturiano, Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality, Aquaculture. (2016). doi:10.1016/j.aquaculture.2016.04.021.

[16] B. Falahatkar, S. Poursaeid, Effects of hormonal manipulation on stress responses in male and female broodstocks of pikeperch Sander lucioperca, Aquac. Int. 22 (2013) 235–244. doi:10.1007/s10499-013-9678-x.

[17] G. Van Der Kraak, N.W. Pankhurst, R.E. Peter, H.R. Lin, Lack of antigenicity of human chorionic gonadotropin in silver carp (Hypophthalmichthys molitrix) and goldfish (Carassius auratus), Aquaculture. 78 (1989) 81–86. doi:10.1016/0044-8486(89)90007-0.

[18] J. Roche, D. Żarski, A. Khendek, I. Ben Ammar, C. Broquard, A. Depp, Y. Ledoré, T. Policar, P. Fontaine, S. Milla, D1, but not D2, dopamine receptor regulates steroid levels during the final stages of pikeperch gametogenesis, Animal. 12 (2018) 2587–2597. doi:10.1017/S1751731118000824.

[19] A. Cuesta, L. Vargas-Chacoff, A. García-López, F.J. Arjona, G. Martínez-Rodríguez, J. Meseguer, J.M. Mancera, M.A. Esteban, Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream, Fish Shellfish Immunol. 23 (2007) 693–700. doi:10.1016/j.fsi.2007.01.015.

[20] A. Khendek, A. Chakraborty, J. Roche, Y. Ledoré, A. Personne, T. Policar, D. Żarski, R. Mandiki, P. Kestemont, S. Milla, P. Fontaine, Rearing conditions and life history influence the progress of gametogenesis and reproduction performances in pikeperch males and females, Animal. (2018) 1–12. doi:10.1017/S1751731118000010.

[21] S. Baekelandt, S.N.M. Mandiki, P. Kestemont, Are cortisol and melatonin involved in the immune modulation by the light environment in pike perch Sander lucioperca?, J. Pineal Res. (2019). doi:10.1111/jpi.12573.

[22] B.B. Aggarwal, Signalling pathways of the TNF superfamily: a double-edged sword, Nat. Rev. Immunol. 3 (2003) 745–756. doi:10.1038/nri1184.

[23] C.A. Dinarello, Overview of the IL-1 family in innate inflammation and acquired immunity, Immunol. Rev. 281 (2018) 8–27. doi:10.1111/imr.12621.

[24] R. Harikrishnan, J.-S. Kim, M.-C. Kim, C. Balasundaram, M.-S. Heo, Molecular characterization, phylogeny, and expression pattern of c-type lysozyme in kelp grouper, Epinephelus bruneus, Fish Shellfish Immunol. 31 (2011) 588–594. doi:10.1016/j.fsi.2011.06.019.

[25] D. Ricklin, E.S. Reis, D.C. Mastellos, P. Gros, J.D. Lambris, Complement component C3 - The “Swiss Army Knife” of innate immunity and host defense, Immunol. Rev. 274 (2016) 33–58. doi:10.1111/imr.12500.

[26] J. Kristan, A. Stara, J. Turek, T. Policar, J. Velisek, Comparison of the effects of four anaesthetics on haematological and blood biochemical profiles in pikeperch (Sander lucioperca L.)., Neuro Endocrinol. Lett. 33 Suppl 3 (2012) 66–71. http://www.scopus.com/inward/record.url?eid=2-s2.0-84874802973&partnerID=tZOtx3y1.

[27] N. Topic Popovic, I. Strunjak-Perovic, R. Coz-Rakovac, J. Barisic, M. Jadan, A. Persin Berakovic, R. Sauerborn Klobucar, Tricaine methane-sulfonate (MS-222) application in fish anaesthesia, J. Appl. Ichthyol. 28 (2012) 553–564. doi:10.1111/j.1439-0426.2012.01950.x.

[28] S. Milla, C. Mathieu, N. Wang, S. Lambert, S. Nadzialek, S. Massart, E. Henrotte, J. Douxfils, C. Mélard, S.N.M. Mandiki, Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, Perca fluviatilis, Fish Shellfish Immunol. 28 (2010) 931–941. doi:10.1016/j.fsi.2010.02.012.

[29] F. Lahnsteiner, Spermatozoa of the teleost fish Perca fluviatilis (perch) have the ability to swim for more than two hours in saline solutions, Aquaculture. 314 (2011) 221–224. doi:10.1016/j.aquaculture.2011.02.024.

[30] P. Trinder, Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor, Ann. Clin. Biochem. 6 (1969) 24–27.

[31] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, Vet. Immunol. Immunopathol. (1997). doi:10.1016/S0165-2427(97)00048-2.

[32] V. Cornet, A. Ouaach, S.N.M. Mandiki, E. Flamion, A. Ferain, M. Van Larebeke, B. Lemaire, F.E. Reyes López, L. Tort, Y. Larondelle, P. Kestemont, Environmentally-realistic concentration of cadmium combined with polyunsaturated fatty acids enriched diets modulated non-specific immunity in rainbow trout, Aquat. Toxicol. 196 (2018) 104–116. doi:10.1016/j.aquatox.2018.01.012.

[33] A. Siwicki, M. Studnicka, The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, Cyprinus carpio L., J. Fish Biol. (1987). doi:10.1111/j.1095-8649.1987.tb05293.x.

[34] J. Douxfils, M. Deprez, S.N.M. Mandiki, S. Milla, E. Henrotte, C. Mathieu, F. Silvestre, M. Vandecan, C. Rougeot, C. Mélard, M. Dieu, M. Raes, P. Kestemont, Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication - Clues to physiological acclimation and humoral immune modulations, Fish Shellfish Immunol. 33 (2012) 1112–1122. doi:10.1016/j.fsi.2012.08.013.

[35] D. Żarski, T. Nguyen, A. Le Cam, J. Montfort, G. Dutto, M.O. Vidal, C. Fauvel, J. Bobe, Transcriptomic Profiling of Egg Quality in Sea Bass (Dicentrarchus labrax) Sheds Light on Genes Involved in Ubiquitination and Translation, Mar. Biotechnol. 19 (2017). doi:10.1007/s10126-017-9732-1.

[36] M. Blecha, J. Kristan, A.M. Samarin, M. Rodina, T. Policar, Quality and quantity of pikeperch (Sander lucioperca) spermatozoa after varying cold water treatments, J. Appl. Ichthyol. 31 (2015) 75–78. doi:10.1111/jai.12853.

[37] B. Korbuly, A. Grozea, A. Cean, I. Banatean-Dunea, N. Pacala, A. Valean, Milt dilution effectiveness on pikeperch (Sander lucioperca) sperm DNA inactivation, Zooteh. Şi Biotehnol. 42 (2009) 65–70.

[38] B.I. Cejko, K. Targońska, R.K. Kowalski, D. Żarski, B. Sarosiek, D. Kucharczyk, J. Glogowski, The effectiveness of hormonal preparations (Ovopel, Ovaprim, LHRHa, hCG and CPE) in stimulating spermiation in dace Leuciscus leuciscus (L.), J. Appl. Ichthyol. 28 (2012) 873–877. doi:10.1111/jai.12054.

[39] F.J. Schaefer, J.L. Overton, J. Bossuyt, D. Żarski, W. Kloas, S. Wuertz, Management of pikeperch *Sander lucioperca* (Linnaeus, 1758) sperm quality after stripping, J. Appl. Ichthyol. (2016). doi:10.1111/jai.13144.

[40] Z. Zakęś, M. Szczepkowski, K. Partyka, K. Wunderlich, Effect of gonadotropin hormonal stimulation on out-of-season propagation success of different year classes of indoor-reared pikeperch (Sander lucioperca (L.)), Aquac. Int. 21 (2013) 801–810. doi:10.1007/s10499-012-9562-0.

[41] S. Dhindsa, H. Ghanim, M. Batra, N.D. Kuhadiya, S. Abuaysheh, K. Green, A. Makdissi, A. Chaudhuri, P. Dandona, Effect of testosterone on hepcidin, ferroportin, ferritin and iron binding capacity in patients with hypogonadotropic hypogonadism and type 2 diabetes, Clin. Endocrinol. (Oxf). 85 (2016) 772–780. doi:10.1111/cen.13130.

[42] T. Ganz, E. Nemeth, Hepcidin and Disorders of Iron Metabolism, Annu. Rev. Med. 62 (2011) 347–360. doi:10.1146/annurev-med-050109-142444.

[43] H. Lodish, J. Flygare, S. Chou, From stem cell to erythroblast: Regulation of red cell production at multiple levels by multiple hormones, IUBMB Life. 62 (2010) 492–496. doi:10.1002/iub.322.

[44] L. Tort, Stress and immune modulation in fish, Dev. Comp. Immunol. 35 (2011) 1366–1375. doi:10.1016/j.dci.2011.07.002.

[45] E.M. Sternberg, Neural regulation of innate immunity: A coordinated nonspecific host response to pathogens, Nat. Rev. Immunol. (2006). doi:10.1038/nri1810.

[46] R. Castro, J. Zou, C.J. Secombes, S.A.M. Martin, Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line, Fish Shellfish Immunol. (2011). doi:10.1016/j.fsi.2010.10.010.

[47] C.B. Schreck, W. Contreras-Sanchez, M.S. Fitzpatrick, Effects of stress on fish reproduction, gamete quality, and progeny, Aquaculture. 197 (2001) 3–24. doi:10.1016/S0044-8486(01)00580-4.

[48] S. Losdat, H. Richner, J.D. Blount, F. Helfenstein, Immune Activation Reduces Sperm Quality in the Great Tit, PLoS One. 6 (2011) e22221. doi:10.1371/journal.pone.0022221.

[49] T. Janevic, L.G. Kahn, P. Landsbergis, P.M. Cirillo, B.A. Cohn, X. Liu, P. Factor-Litvak, Effects of work and life stress on semen quality, Fertil. Steril. 102 (2014) 530–538. doi:10.1016/j.fertnstert.2014.04.021.

[50] A. Kowalska, A.K. Siwicki, R.K. Kowalski, Dietary resveratrol improves immunity but reduces reproduction of broodstock medaka Oryzias latipes (Temminck & Schlegel), Fish Physiol. Biochem. 43 (2017) 27–37. doi:10.1007/s10695-016-0265-8.

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619

620 **7. Tables and Figures caption:**

621

622 **Tab. 1.** Names, symbols, accession numbers and primer sequences of the genes
623 analyzed in the present study. All the primers were used according to Baekelandt et al. [21].

624

625 **Tab. 2.** Sperm motility analysis performed with the computer assisted sperm analysis
626 (CASA) system on pikeperch sperm obtained each time 5 days after 1st or 2nd hormonal
627 injection.

628

629 **Tab. 3.** Levels (mean ±SD) of stress and immune response markers recorded in the
630 blood plasma of pikeperch males treated twice (with 5-days interval) with different hormonal
631 preparations prior to blood sampling performed at the end of the experiment.

632

633 **Tab. 4.** Pearson's correlation coefficients (r values) calculated between stress, immune
634 and endocrine markers against sperm quality indices recorded in pikeperch males treated twice
635 with different hormonal preparations (n=7 treated with hCG and n=7 with sGnRHa) with 5 day
636 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.

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

Fig. 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 ($p<0.05$).

Fig. 3. Sperm motility parameters (measured by CASA system), with Pearson's correlation coeficient (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 gonadoliberine 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 (×4).

Fig. 4. Relative immune genes expression (mean ±SD) (normalized to geometric mean of two housekeeping genes: beta actin [*β-actin*] and elongation factor alpha [*ef1-α*]) 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;

662 sGnRHa – salmon gonadoliberine analogue; hCG – human chorionic gonadotropin. Different
663 letters showed significant differences among the hormonal treatments ($p<0.05$).

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

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

Tab. 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.

		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 (μm 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 (μm 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 gonadotropin; pMOT – progressive spermatozoa motility; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; VSL – straightline velocity; LIN – linearity of movement; BCF – beat cross frequency.

Tab. 3. Levels (mean ±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 ($p<0.05$).

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

sGnRHa – salmon gonadoliberine analogue; hCG – human chorionic gonadotropin

Tab. 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 [µm s^{-1}]	VSL [µm 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 [µg ml^{-1}]	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
Il-1 [relative expression]	-0.30	**-0.61**	**-0.64**	0.12	-0.01	0.36
tnf-α [relative expression]	0.02	-0.15	-0.27	-0.06	-0.41	**-0.57**
hamp [relative expression]	-0.15	-0.42	-0.12	0.40	-0.39	0.29
lys [relative expression]	-0.13	-0.42	-0.53	-0.01	-0.08	-0.05
c3 [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 gonadoliberine analogue (applied dose: 50 µg kg^{-1})

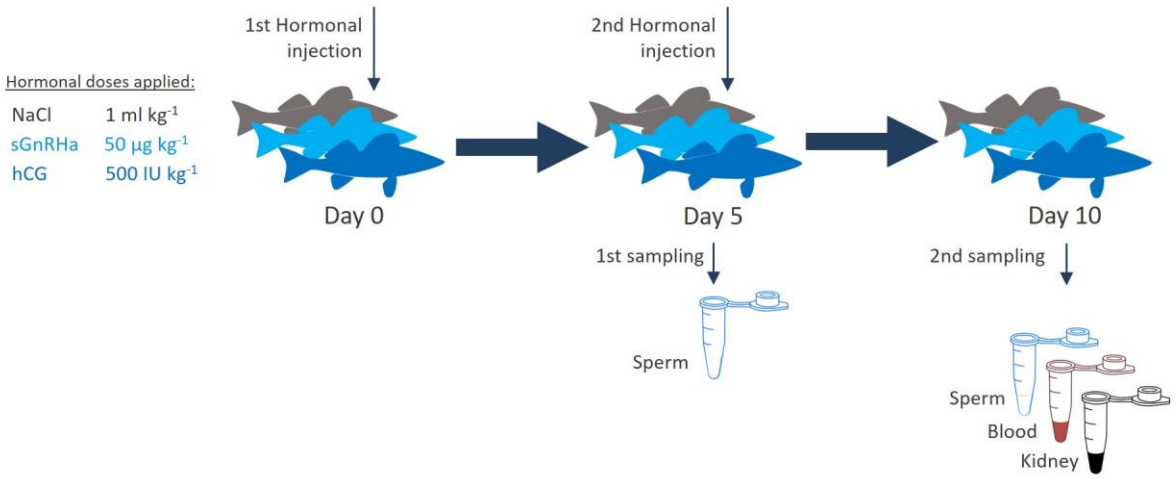

Figure 1 Zarski et al

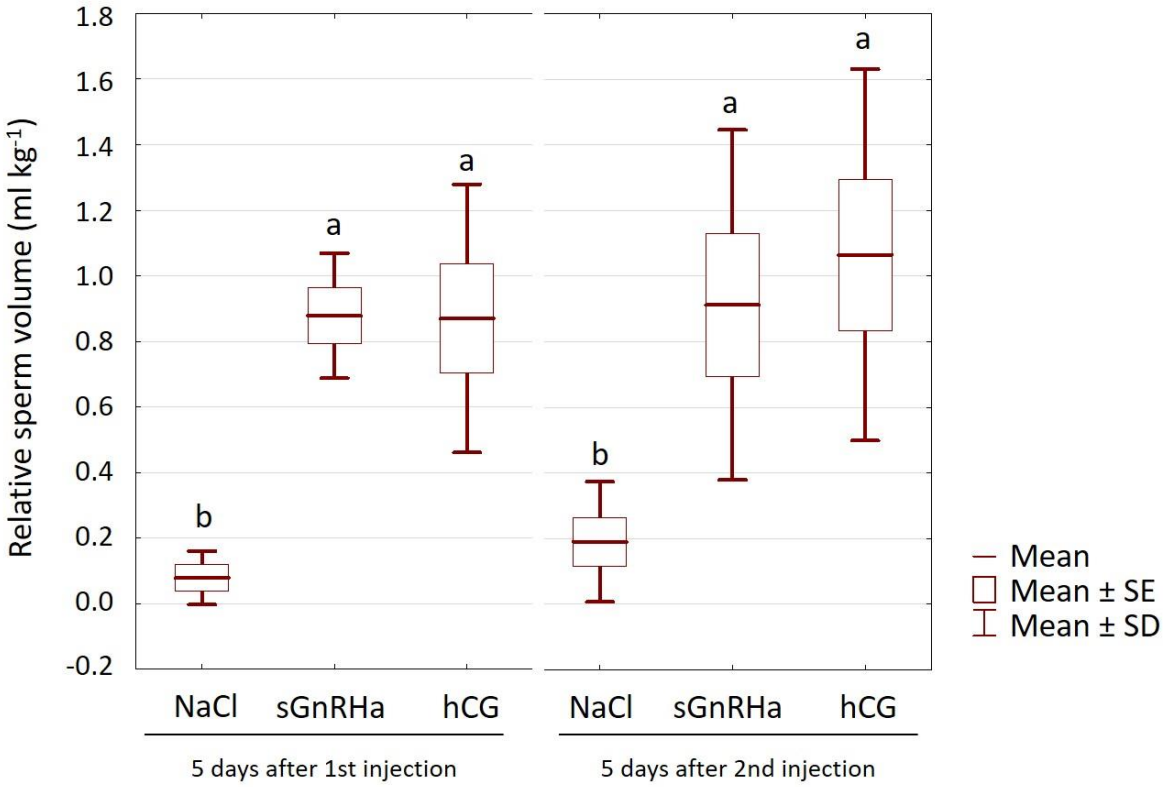

Figure 2 Zarski et al

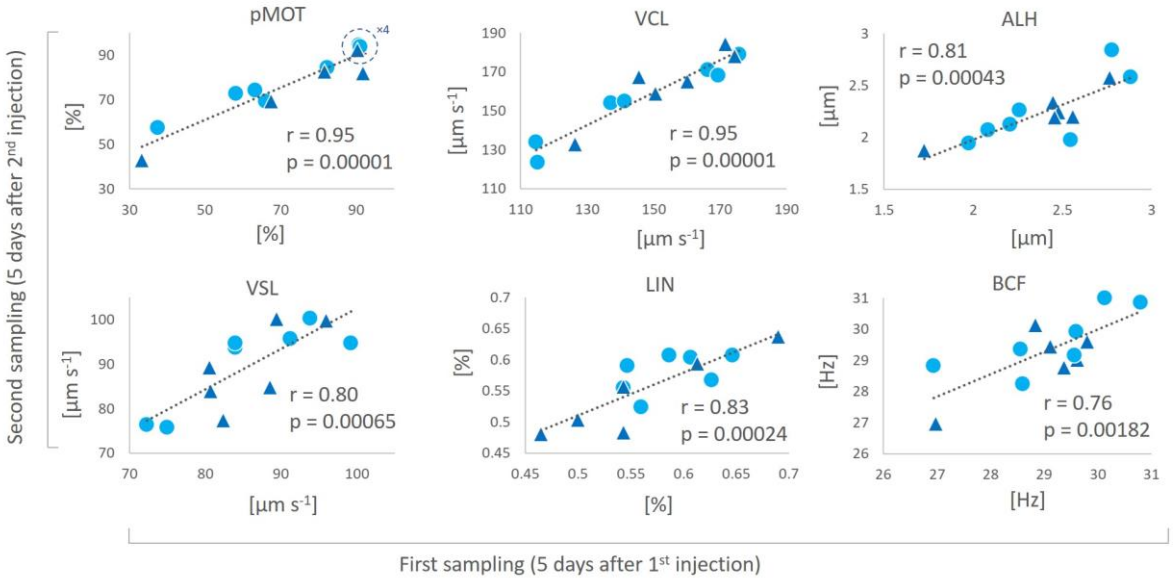

Figure 3 Zarski et al

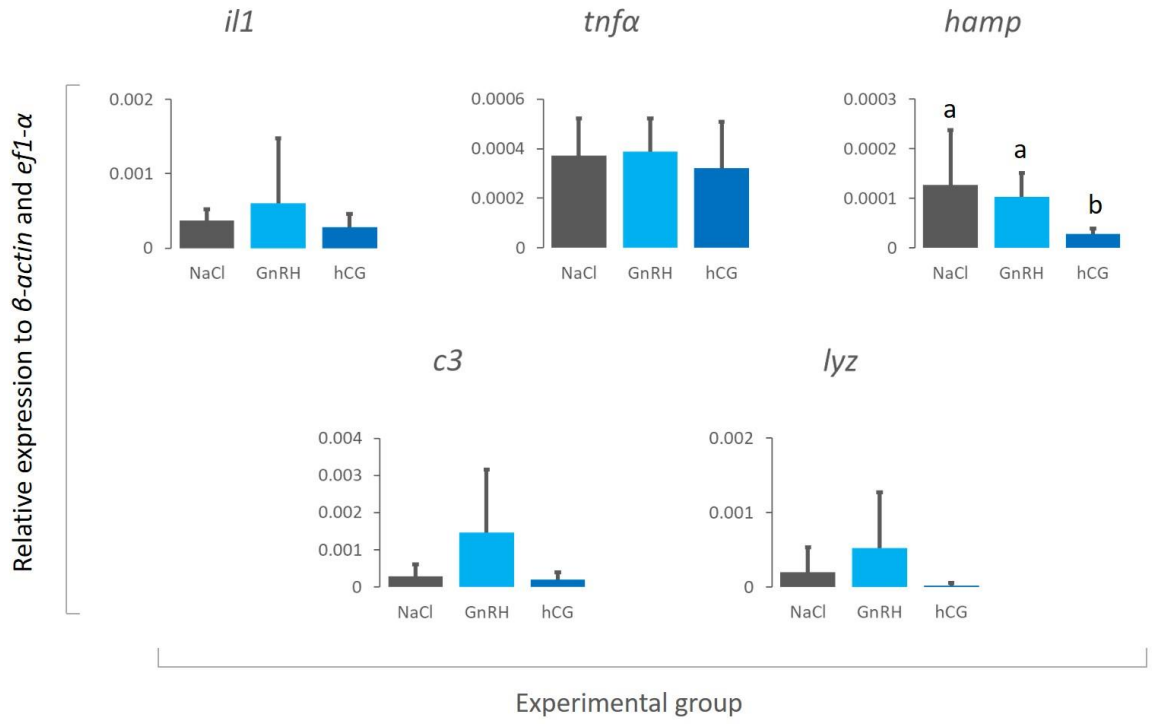

Figure 4 Zarski et al