

RESEARCH OUTPUTS / RÉSULTATS DE RECHERCHE

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

Żarski, Dąniel; Ben Ammar, Imen; Bernáth, Gergely; Baekelandt, Sébastien; Bokor, Zoltán; Palińska-Żarska, Katarzyna; Fontaine, Pascal; Horváth, Ákos; Kestemont, Patrick; Mandiki, Syaghalirwa N.M.

Published in:
Fish & Shellfish Immunology

DOI:
[10.1016/j.fsi.2020.03.057](https://doi.org/10.1016/j.fsi.2020.03.057)

Publication date:
2020

Document Version
Peer reviewed version

[Link to publication](#)

Citation for pulished version (HARVARD):

Żarski, D, Ben Ammar, I, Bernáth, G, Baekelandt, S, Bokor, Z, Palińska-Żarska, K, Fontaine, P, Horváth, Á, Kestemont, P & Mandiki, SNM 2020, 'Repeated hormonal induction of spermiation affects the stress but not the immune response in pikeperch (*Sander lucioperca*)', *Fish & Shellfish Immunology*, vol. 101, pp. 143-151.
<https://doi.org/10.1016/j.fsi.2020.03.057>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

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

3

4 Daniel Źarski^{1*}, Imen Ben Ammar², Gergely Bernáth³, Sébastien Baekelandt², Zoltán Bokor³,
5 Katarzyna Palińska-Źarska⁴, Pascal Fontaine⁵, Ákos Horváth³, Patrick Kestemont²,
6 Syaghalirwa N.M. Mandiki²

7

8 ¹Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish
9 Academy of Sciences, Olsztyn, Poland

10 ²URBE, Institute of Life, Earth & Environment, Université de Namur, Namur, Belgium

11 ³Department of Aquaculture, Szent István University, Gödöllő, Hungary

12 ⁴Department of Fisheries, Faculty of Animal Bioengineering, University of Warmia and Mazury, Olsztyn, Poland

13 ⁵University of Lorraine, INRAE, UR AFPA, Nancy, France

14

15 *Corresponding author: Phone: +48895393165, Fax: +48895357421, e-mail: d.zarski@pan.olsztyn.pl

16

17

18 **Abstract**

19 Hormonal induction of spermiation, previously reported to be immunogenic in fishes, is a
20 common hatchery practice in pikeperch, *Sander lucioperca*. The aim of the present study was
21 to investigate the effects of repeated induction of spermiation in pikeperch, following
22 application of either human chorionic gonadotropin (hCG) or salmon gonadoliberine analog
23 (sGnRHa) on sperm quality indices as well as on immune and stress response. Mature males of
24 pikeperch (n=7 per group) were stimulated twice with five days between injections of either
25 hCG (hCG; 500 IU kg⁻¹), sGnRHa (sGnRHa; 50 µg kg⁻¹) or NaCl (control group; 1 ml kg⁻¹) to
26 assess spermatozoa motility with a computer-assisted sperm analysis (CASA) system. During
27 second sampling, blood plasma was sampled for humoral innate immune (peroxidase and
28 lysozyme activities, ACH50), stress (cortisol, glucose) and endocrine (testosterone) markers.
29 In addition, the head kidney was dissected to assay the expression of several immune genes
30 (such as *ill*, *c3*, *hamp*, *tnf-α* and *lys* genes). The results indicate that hormonal treatment

31 significantly increased sperm production. Sperm sampled after the hormonal treatment
32 maintained its quality throughout the study, regardless of the sampling time. However, it
33 appears that the application of hCG induced elevated cortisol and glucose plasma levels
34 compared to the control group. Almost all immune markers, except the relative expression of
35 hepcidin (*hamp* gene), were unaffected by the two hormones applied. The results showed that
36 the induction treatment of spermiation processes in pikeperch resulted in an important
37 physiological stress response for which the intensity varied according to the hormonal agent
38 used. However, this stress response (more profound following application of hCG) was weakly
39 associated with innate immune functions. On the other hand, a significant negative correlation
40 between the expression of several important immune markers (peroxidase activity, relative
41 expression of *c3* and *ill* genes) and sperm quality indices indicates significant involvement of
42 immune status on sperm quality. The results obtained shed light on immune-system-induced
43 modifications to sperm quality. The data presented here highlight the need for careful revision
44 of broodstock management and selection practices where welfare status as well as individual
45 predispositions of fish to cope with the stress should be taken under the consideration.

46

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

48 1. Introduction

49 Expansion of the aquaculture sector relies on domestication and selective breeding
50 programs that allow preservation of desired traits to promote high survival and growth rates of
51 progeny [1,2]. This, however, requires implementation of advanced breeding technologies in
52 which controlled reproduction is among the most important steps [3,4]. This especially applies
53 to newly domesticated fish species, such as pikeperch (*Sander lucioperca*), whose production
54 technology, despite having established commercial production, is still being optimised [5].

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

64 Another bottleneck in controlled reproduction of percid fishes is the synchronisation of
65 final maturation. Intensively cultured percid males are usually not spermiating when females
66 are ready to spawn [3,5]. In addition, those males, from which it is possible to obtain sperm,
67 usually release small volumes of milt, further lowering its fertilisation capacity. Consequently,
68 in controlled reproduction of percid fishes, hormonal stimulation is practiced to synchronise
69 ovulation and spermiation as well as to enhance the quality and quantity of sperm obtained
70 [8,9].

71 Hormonal stimulation of ovulation and spermiation in percids usually uses two types of
72 spawning agents: gonadotropins (GtH; human chorionic gonadotropin [hCG]) and

73 gonadoliberins (usually in the form of pure gonadoliberine analogs) [4]. Both types act at
74 different levels of the hypothalamic-pituitary-gonadal (HPG) axis. Application of GtH
75 stimulates the gonads for production of sex steroids (directly influencing maturation of the
76 gonads), whereas application of gonadoliberine analogs induces the secretion of endogenous
77 GtH [10]. Both spawning agents have been tested in percids for over a decade, with similar
78 efficiency in the stimulation of ovulation [4] and spermiation [9].

79 Sperm is usually collected for analysis only once from each fish when the total amount
80 of sperm possible to obtain is stripped out [9,11]. This is done in order to prevent testicular
81 sperm ageing [12], a symptom also noticeable in Eurasian perch, *Perca fluviatilis*, after
82 injection with hCG [9]. Preliminary observations revealed that further sperm collection (up to
83 3 days after first sperm stripping) in pikeperch is possible, although in very small volumes
84 (below 0.3 ml per kg of body weight) and of lower quality (below 60% of motility) (D. Źarski,
85 unpublished), confirming the findings of Grozea et al. [13]. This could be associated with the
86 slow progression of final maturation of the sperm (from spermatids to spermatozoa, for details
87 see Schulz et al. [14]) and consequently, spermiation within a few days following first stripping.
88 This could in turn be related to low levels of sex steroids responsible for maturation of the
89 spermatids that remained in the testes following first sperm collection [14,15]. It can be
90 assumed that, before the second sperm collection, additional hormonal stimulation should be
91 performed that may enhance spermiation, by influencing the production of sex steroids as is
92 practiced in other cultured species (see the review by Mylonas et al. [15]). Such a strategy of
93 hormonal induction of spermiation in pikeperch was proposed by Grozea et al. [13], who
94 recommend repeating the hormonal treatment at least three days following the first hormonal
95 injection in order to collect high volumes of sperm 10 h later at 17 °C. However, Grozea et al.
96 [13] considered the combination of different hormonal preparations (different types of
97 hormones used for the first and second injection) and only a single parameter (sperm volume)

98 was investigated without providing any information on sperm quality or physiological response
99 of the fish. Therefore, more detailed study investigating the possibility and impact of repeated
100 hormonal treatments on the induction of spermiation in pikeperch is needed.

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

113 Hormonal treatment in fishes, by stimulating excessive secretion of sex steroids [18],
114 such as testosterone in males may activate humoral immune response [19]. Therefore, while
115 considering the effect of different type of hormonal preparations on the stress and the immune
116 response in males testosterone level in the blood plasma should also be investigated along with
117 various stress (including cortisol and glucose [16,20]) and immune markers (including
118 lysozyme and peroxidase activities [21]). However, to address the research question in more
119 complex manner expression level (in the head kidney) of genes being identified as robust stress
120 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

146 gametes with either feces or urine. For the experiment 21 randomly chosen males ($n = 21$; age
147 6+; average weight 2.65 ± 0.52 kg) were used.

148 The fish were exposed to a photo-thermal program simulating annual fluctuations as
149 described by Żarski et al. [4]. The light intensity was fixed at 20 lx (provided by neon tubes) at
150 the water surface. After the wintering period (during which fish were exposed to a temperature
151 below 10 °C and a photoperiod of 9 and 15 h of light and dark periods, respectively; see Żarski
152 et al. [4]), temperature and photoperiod were increased, reaching 12 °C and 14 h of light within
153 6 weeks. Then the photo-thermal variations were stopped and both factors remained constant
154 until the end of the experiment. Seven days later, the maturation stage of females was checked,
155 and they were found to enter into the final oocyte maturation process heralding the
156 commencement of the spawning period. At this time, the males were randomly assigned to one
157 of three groups, each treated with a different spawning agent. Each fish was tagged individually
158 (with passive integrated transponders, i.e., PIT-tags). Males assigned to the same group were
159 kept together in separate cube-shaped cages placed in the tanks. Each manipulation (injection
160 and sperm stripping) was performed under anesthesia (MS-222 at a dose of 150 mg L^{-1} ; [26]).
161 At the end of the experiment, the anaesthetised fish were euthanised by overexposure to the
162 anesthetics (MS-222, 300 mg L^{-1} , [27]).

163 **2.2. Experimental design and sampling**

164 For the experiment, three groups were distinguished ($n=7$ for each group) and each group was
165 treated twice, at five-day intervals, with either 0.9% NaCl (control group), salmon
166 gonadolibertine analog (sGnRHa; each time at a dose of $50 \mu\text{g kg}^{-1}$) (Bachem, Switzerland) or
167 hCG (each time at a dose of 500 IU kg^{-1}) (Chorulon, Intervet, France) (Fig. 1). The fish were
168 injected intraperitoneally at the base of the ventral fin. The hormones were dissolved/diluted in
169 0.9% NaCl solution so that each fish received 1 ml of the solution per kg of body weight, each

170 time. The doses of the spawning agents were those recommended, and applied during the
171 commercial reproduction of pikeperch [4].

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

190 **2.3. Sperm quality evaluation**

191 Sperm motility in the three groups was recorded with a CASA system (Sperm Vision™
192 v. 3.7.4., Minitube of America, Verona, USA). Spermatozoa were activated using an ionic
193 solution (50 mM NaCl, 30 mM Tris, pH: 8.0±0.2, [29]) with approximately 0.01 g ml⁻¹ of BSA
194 (bovine serum albumin). Motility parameters, such as progressive motility (criteria according

195 to Sperm Vision™ v. 3.7.4. straight line distance > 5 μm, pixel to μm ratio: 151:100, pMOT,
196 %), curvilinear velocity (VCL, μm s⁻¹), straight linear velocity (VSL, μm s⁻¹), linearity of
197 movement (LIN, %), amplitude of lateral head displacement (ALH, μm) and beat cross
198 frequency (BCF, Hz) were studied to describe pikeperch spermatozoa movement (after Żarski
199 et al. [9]). Motility assessment was carried out in duplicate (at a ratio of 1:99 v/v sperm-to-
200 activating solution), and moving cells were identified (1 to 100 μm²) with a digital camera (JAI
201 CV-A10 CL, Minitube of America, Verona, USA) using a frame rate of 60 frames s⁻¹.

202 **2.4. Stress indicators analysis**

203 *2.4.1. Cortisol assay*

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

218 *2.4.2. Plasma glucose assay*

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

227 **2.5. Immune parameters analysis**

228 *2.5.1. Peroxidase activity*

229 The total peroxidase activity in plasma was assessed according to Quade and Roth [31].
230 The samples and negative control (distilled water) were assayed in triplicate. In a flat-bottomed
231 96-well plate, 7 µl of plasma were diluted in 68 µl of Hanks' Balanced Salt Solution (HBSS)
232 without Ca²⁺ or Mg²⁺. As a substrate, 25 µl of reactional solution (20 mM 3,3',5,5'-
233 tetramethylbenzidine hydrochloride and 5 mM H₂O₂) was added. The reaction was stopped
234 after 2 min by adding 50 µl of 4 M sulphuric acid and the absorbance was measured at 450 nm.
235 One unit (U) of peroxidase activity was defined as the amount producing an absorbance change
236 of 1 optical density (OD).

237 *2.5.2. Plasma alternative complement pathway*

238 The plasma alternative complement pathway (ACH50) procedure was used to measure
239 the haemolytic activity in plasma samples using rabbit red blood cells (RRBC) as targets [32].
240 A serial dilution from 1/20 to 1/480 into a veronal buffer (IDVert, France) was performed in
241 duplicate for each plasma sample in a round-bottomed 96-well plate. Then, 10 µl of RRBC
242 (Biomerieux) suspension (3% in veronal buffer) were added to each well and the plate was
243 incubated at 25 °C for 120 min at 300 rpm using the orbital shaker (KS 4000 ic control, IKA®-

244 Werke GmbH & Co. KG, Germany). The total haemolysis was obtained by mixing 10 μ l of
245 RRBC lysed with bi-distilled water and the spontaneous haemolysis was obtained by adding
246 veronal buffer to 10 μ l of RRBC (total volume = 70 μ l). After the incubation, the turbidity
247 (inversely proportional to the haemolysis) was measured using the 96-well plate reader
248 (FLUOstar[®] Omega, BMG LABTECH, Germany) at 650 nm. The ACH50 value (unit ml⁻¹ of
249 plasma) is the reciprocal of the plasma dilution which induces the haemolysis of 50% of the
250 RRBC.

251 2.5.3. *Lysozyme activity*

252 The lysozyme activity protocol was adapted from Siwicki and Studnicka [33] and
253 Douxfils et al. [34]. In flat-bottom 96-well plates, samples were assayed in triplicate by mixing
254 7 μ l of pikeperch plasma with 130 μ l of lyophilized *Micrococcus lysodeikticus* (Sigma)
255 suspension at 0.6 g L⁻¹ in phosphate buffer (Na₂HPO₄, 0.05 M, pH 6.2). A negative control
256 (phosphate buffer) and a positive control (*M. lysodeikticus*) were also assayed in triplicate in
257 the same plate (total volume = 137 μ l). The absorbance (OD) at 450 nm was monitored between
258 0 min and 15 min (linearity range) using the 96 well-plate reader. Lysozyme activity represents
259 the amount of enzyme decreasing the turbidity by 0.001 OD per min.

260 2.6. Testosterone assay

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

267 2.7. Immune gene expression

268 Total RNA from the head kidney was extracted using Total RNA Mini kit (A&A
269 Biotechnology, Gdynia, Poland) according to the producer's protocol. The concentration of
270 RNA was analysed with NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA).
271 The quality of the RNA samples was checked using a 2100 Bioanalyzer (Agilent Technologies
272 Inc., Santa Clara, USA) and samples with RIN value higher than 9.0 were used for further
273 analysis. Next, the RNA was treated with TURBO DNase (Cat. No. AM2238, Invitrogen, Life
274 Technologies Corporation, Carlsbad, CA, USA) in order to remove contamination of samples
275 with genomic DNA. Reverse transcription was performed using MAXIMA First Strand cDNA
276 Synthesis Kit (Thermo Fisher Scientific Inc.). The real-time quantitative polymerase chain
277 reaction (RT-qPCR) was performed with LightCycler 480 II (Roche, Bazylea, Switzerland)
278 using DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific Inc). Enzyme activation
279 and denaturation was performed for 10 min at 95 °C, followed by 40 cycles of denaturation at
280 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min [35].

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

287 **2.8. Data analysis and statistics**

288 Data were analysed with MS Excel 2016 and STATISTICA 13 (TIBCO Software Inc.,
289 Palo Alto, CA, USA), tested for homogeneity of variance with Levene's test and further tested
290 for normal distribution with the Shapiro-Wilk test. Data meeting the criteria of normality were
291 analysed with either t-test (sperm quality indicators) or one-way ANOVA followed by Tukey's
292 post-hoc test (blood parameters, gene expression data). Additionally, Pearson's correlation

293 coefficients were calculated for sperm quality indices between the 1st and 2nd sampling in order
294 to compare the trend of sperm quality in the same individuals between the two samplings.
295 Besides, correlation analysis was performed between stress, immune and endocrine markers
296 against sperm quality indices. All statistical tests were performed at a significance level of 5%
297 ($p < 0.05$).

298

299 **3. Results**

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

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

315 Application of hCG significantly increased the level of plasma cortisol when compared
316 to the level assayed in control fish ($p < 0.05$). In addition, this spawning agent significantly
317 increased glucose level ($p < 0.05$) compared to both remaining groups ($p > 0.05$), among which

318 similar levels of plasma glucose were recorded (Table 3). The immune parameters measured
319 (lysozyme, peroxidase activities and ACH50) and testosterone levels showed similar levels
320 among all the groups ($p>0.05$; Table 3).

321 Gene expression analysis showed that the injection of hCG significantly lowered the
322 relative expression of hepcidin (*hamp*) compared to the remaining groups ($p<0.05$), among
323 which no significant differences were observed ($p>0.05$). The relative expression level of other
324 genes (*ill*, *tnfa*, *c3* and *lyz*) did not significantly differ between groups (Fig. 3).

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

329

330 **4. Discussion**

331

332 *4.1. The effect of hormonal stimulation on spermiation in domesticated pikeperch*

333 In the present study significant enhancement of amount of sperm obtained from
334 pikeperch following hormonal treatment was recorded. It should be noted that the sperm
335 volumes obtained in both hormonal-treated groups (on average $0.9\text{--}1.1\text{ ml kg}^{-1}$) were very close
336 to average values reported so far for pond-reared fish ($0.5\text{--}1.5\text{ ml kg}^{-1}$ by Blecha et al. [36] and
337 $0.4\text{--}1.1\text{ ml kg}^{-1}$ by Korbuly et al. [37]). It also appeared that the latency time tested (five days
338 between hormonal injection and sperm collection) was suitable for the collection of high-quality
339 sperm as reported for Eurasian perch [9]. However, considering the fact that sperm can be
340 collected after an additional five days indicates the possibility of the development of specific
341 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*

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

379

380 *4.3. The effects of hCG and sGnRHa on stress response in pikeperch*

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

417

418 *4.6. Correlation between sperm quality and immune and stress markers*

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

434

435 *4.7. Conclusions*

436 Hormonal stimulation was found to be essential to obtain high quality and quantity of
437 sperm during the out-of-season reproduction of domesticated pikeperch. It was also found, for
438 the first time, that multiple, hormonally controlled sperm collection is possible in this species,
439 though optimized hatchery-applicable protocols remains to be developed. Due to the fact that
440 application of hCG induced a stress response and decreased expression of the *hamp* gene its
441 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. Radóczy, 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–

- 483 16. doi:10.1016/j.aquaculture.2015.06.040.
- 484 [12] C. Fauvel, M. Suquet, J. Cosson, Evaluation of fish sperm quality, *J. Appl. Ichthyol.* 26
485 (2010) 636–643. doi:10.1111/j.1439-0426.2010.01529.x.
- 486 [13] A. Grozea, A. Telea, B. Korbuly, I. Bănăţean, A. Osman, Preliminary Study Regarding
487 the Efficiency of Different Hormones on Pikeperch Spermiation, 41 (2008) 59–64.
- 488 [14] R.W. Schulz, L.R. de França, J.J. Lareyre, F. LeGac, H. Chiarini-Garcia, R.H.
489 Nobrega, T. Miura, Spermatogenesis in fish, *Gen. Comp. Endocrinol.* 165 (2010) 390–
490 411. doi:10.1016/j.ygcen.2009.02.013.
- 491 [15] C.C. Mylonas, N.J. Duncan, J.F. Asturiano, Hormonal manipulations for the
492 enhancement of sperm production in cultured fish and evaluation of sperm quality,
493 *Aquaculture.* (2016). doi:10.1016/j.aquaculture.2016.04.021.
- 494 [16] B. Falahatkar, S. Poursaeid, Effects of hormonal manipulation on stress responses in
495 male and female broodstocks of pikeperch *Sander lucioperca*, *Aquac. Int.* 22 (2013)
496 235–244. doi:10.1007/s10499-013-9678-x.
- 497 [17] G. Van Der Kraak, N.W. Pankhurst, R.E. Peter, H.R. Lin, Lack of antigenicity of
498 human chorionic gonadotropin in silver carp (*Hypophthalmichthys molitrix*) and
499 goldfish (*Carassius auratus*), *Aquaculture.* 78 (1989) 81–86. doi:10.1016/0044-
500 8486(89)90007-0.
- 501 [18] J. Roche, D. Źarski, A. Khendek, I. Ben Ammar, C. Broquard, A. Depp, Y. Ledoré, T.
502 Policar, P. Fontaine, S. Milla, D1, but not D2, dopamine receptor regulates steroid
503 levels during the final stages of pikeperch gametogenesis, *Animal.* 12 (2018) 2587–
504 2597. doi:10.1017/S1751731118000824.
- 505 [19] A. Cuesta, L. Vargas-Chacoff, A. García-López, F.J. Arjona, G. Martínez-Rodríguez, J.
506 Meseguer, J.M. Mancera, M.A. Esteban, Effect of sex-steroid hormones, testosterone
507 and estradiol, on humoral immune parameters of gilthead seabream, *Fish Shellfish*
508 *Immunol.* 23 (2007) 693–700. doi:10.1016/j.fsi.2007.01.015.
- 509 [20] A. Khendek, A. Chakraborty, J. Roche, Y. Ledoré, A. Personne, T. Policar, D. Źarski,
510 R. Mandiki, P. Kestemont, S. Milla, P. Fontaine, Rearing conditions and life history
511 influence the progress of gametogenesis and reproduction performances in pikeperch
512 males and females, *Animal.* (2018) 1–12. doi:10.1017/S1751731118000010.
- 513 [21] S. Baekelandt, S.N.M. Mandiki, P. Kestemont, Are cortisol and melatonin involved in
514 the immune modulation by the light environment in pike perch *Sander lucioperca*?, *J.*
515 *Pineal Res.* (2019). doi:10.1111/jpi.12573.
- 516 [22] B.B. Aggarwal, Signalling pathways of the TNF superfamily: a double-edged sword,
517 *Nat. Rev. Immunol.* 3 (2003) 745–756. doi:10.1038/nri1184.
- 518 [23] C.A. Dinarello, Overview of the IL-1 family in innate inflammation and acquired
519 immunity, *Immunol. Rev.* 281 (2018) 8–27. doi:10.1111/imr.12621.
- 520 [24] R. Harikrishnan, J.-S. Kim, M.-C. Kim, C. Balasundaram, M.-S. Heo, Molecular
521 characterization, phylogeny, and expression pattern of c-type lysozyme in kelp grouper,
522 *Epinephelus bruneus*, *Fish Shellfish Immunol.* 31 (2011) 588–594.
523 doi:10.1016/j.fsi.2011.06.019.
- 524 [25] D. Ricklin, E.S. Reis, D.C. Mastellos, P. Gros, J.D. Lambris, Complement component
525 C3 - The “Swiss Army Knife” of innate immunity and host defense, *Immunol. Rev.*
526 274 (2016) 33–58. doi:10.1111/imr.12500.

- 527 [26] J. Kristan, A. Stara, J. Turek, T. Policar, J. Velisek, Comparison of the effects of four
528 anaesthetics on haematological and blood biochemical profiles in pikeperch (*Sander*
529 *lucioperca* L.), *Neuro Endocrinol. Lett.* 33 Suppl 3 (2012) 66–71.
530 [http://www.scopus.com/inward/record.url?eid=2-s2.0-](http://www.scopus.com/inward/record.url?eid=2-s2.0-84874802973&partnerID=tZOtx3y1)
531 [84874802973&partnerID=tZOtx3y1](http://www.scopus.com/inward/record.url?eid=2-s2.0-84874802973&partnerID=tZOtx3y1).
- 532 [27] N. Topic Popovic, I. Strunjak-Perovic, R. Coz-Rakovac, J. Barisic, M. Jadan, A. Persin
533 Berakovic, R. Sauerborn Klobucar, Tricaine methane-sulfonate (MS-222) application
534 in fish anaesthesia, *J. Appl. Ichthyol.* 28 (2012) 553–564. doi:10.1111/j.1439-
535 0426.2012.01950.x.
- 536 [28] S. Milla, C. Mathieu, N. Wang, S. Lambert, S. Nadzialek, S. Massart, E. Henrotte, J.
537 Douxfils, C. Mélard, S.N.M. Mandiki, Spleen immune status is affected after acute
538 handling stress but not regulated by cortisol in Eurasian perch, *Perca fluviatilis*, *Fish*
539 *Shellfish Immunol.* 28 (2010) 931–941. doi:10.1016/j.fsi.2010.02.012.
- 540 [29] F. Lahnsteiner, Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the ability
541 to swim for more than two hours in saline solutions, *Aquaculture.* 314 (2011) 221–224.
542 doi:10.1016/j.aquaculture.2011.02.024.
- 543 [30] P. Trinder, Determination of glucose in blood using glucose oxidase with an alternative
544 oxygen acceptor, *Ann. Clin. Biochem.* 6 (1969) 24–27.
- 545 [31] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine
546 neutrophil primary granules, *Vet. Immunol. Immunopathol.* (1997).
547 doi:10.1016/S0165-2427(97)00048-2.
- 548 [32] V. Cornet, A. Ouaach, S.N.M. Mandiki, E. Flamion, A. Ferain, M. Van Larebeke, B.
549 Lemaire, F.E. Reyes López, L. Tort, Y. Larondelle, P. Kestemont, Environmentally-
550 realistic concentration of cadmium combined with polyunsaturated fatty acids enriched
551 diets modulated non-specific immunity in rainbow trout, *Aquat. Toxicol.* 196 (2018)
552 104–116. doi:10.1016/j.aquatox.2018.01.012.
- 553 [33] A. Siwicki, M. Studnicka, The phagocytic ability of neutrophils and serum lysozyme
554 activity in experimentally infected carp, *Cyprinus carpio* L., *J. Fish Biol.* (1987).
555 doi:10.1111/j.1095-8649.1987.tb05293.x.
- 556 [34] J. Douxfils, M. Deprez, S.N.M. Mandiki, S. Milla, E. Henrotte, C. Mathieu, F.
557 Silvestre, M. Vandecan, C. Rougeot, C. Mélard, M. Dieu, M. Raes, P. Kestemont,
558 Physiological and proteomic responses to single and repeated hypoxia in juvenile
559 Eurasian perch under domestication - Clues to physiological acclimation and humoral
560 immune modulations, *Fish Shellfish Immunol.* 33 (2012) 1112–1122.
561 doi:10.1016/j.fsi.2012.08.013.
- 562 [35] D. Źarski, T. Nguyen, A. Le Cam, J. Montfort, G. Dutto, M.O. Vidal, C. Fauvel, J.
563 Bobe, Transcriptomic Profiling of Egg Quality in Sea Bass (*Dicentrarchus labrax*)
564 Sheds Light on Genes Involved in Ubiquitination and Translation, *Mar. Biotechnol.* 19
565 (2017). doi:10.1007/s10126-017-9732-1.
- 566 [36] M. Blecha, J. Kristan, A.M. Samarin, M. Rodina, T. Policar, Quality and quantity of
567 pikeperch (*Sander lucioperca*) spermatozoa after varying cold water treatments, *J.*
568 *Appl. Ichthyol.* 31 (2015) 75–78. doi:10.1111/jai.12853.
- 569 [37] B. Korbuly, A. Grozea, A. Cean, I. Banatean-Dunea, N. Pacala, A. Valean, Milt
570 dilution effectiveness on pikeperch (*Sander lucioperca*) sperm DNA inactivation,
571 *Zooteh. Și Biotehnol.* 42 (2009) 65–70.

- 572 [38] B.I. Cejko, K. Targońska, R.K. Kowalski, D. Żarski, B. Sarosiek, D. Kucharczyk, J.
573 Glogowski, The effectiveness of hormonal preparations (Ovopel, Ovaprim, LHRHa,
574 hCG and CPE) in stimulating spermiation in dace *Leuciscus leuciscus* (L.), *J. Appl.*
575 *Ichthyol.* 28 (2012) 873–877. doi:10.1111/jai.12054.
- 576 [39] F.J. Schaefer, J.L. Overton, J. Bossuyt, D. Żarski, W. Kloas, S. Wuertz, Management
577 of pikeperch *Sander lucioperca* (Linnaeus, 1758) sperm quality after stripping, *J. Appl.*
578 *Ichthyol.* (2016). doi:10.1111/jai.13144.
- 579 [40] Z. Zakęś, M. Szczepkowski, K. Partyka, K. Wunderlich, Effect of gonadotropin
580 hormonal stimulation on out-of-season propagation success of different year classes of
581 indoor-reared pikeperch (*Sander lucioperca* (L.)), *Aquac. Int.* 21 (2013) 801–810.
582 doi:10.1007/s10499-012-9562-0.
- 583 [41] S. Dhindsa, H. Ghanim, M. Batra, N.D. Kuhadiya, S. Abuaysheh, K. Green, A.
584 Makdissi, A. Chaudhuri, P. Dandona, Effect of testosterone on hepcidin, ferroportin,
585 ferritin and iron binding capacity in patients with hypogonadotropic hypogonadism and
586 type 2 diabetes, *Clin. Endocrinol. (Oxf)*. 85 (2016) 772–780. doi:10.1111/cen.13130.
- 587 [42] T. Ganz, E. Nemeth, Hepcidin and Disorders of Iron Metabolism, *Annu. Rev. Med.* 62
588 (2011) 347–360. doi:10.1146/annurev-med-050109-142444.
- 589 [43] H. Lodish, J. Flygare, S. Chou, From stem cell to erythroblast: Regulation of red cell
590 production at multiple levels by multiple hormones, *IUBMB Life*. 62 (2010) 492–496.
591 doi:10.1002/iub.322.
- 592 [44] L. Tort, Stress and immune modulation in fish, *Dev. Comp. Immunol.* 35 (2011) 1366–
593 1375. doi:10.1016/j.dci.2011.07.002.
- 594 [45] E.M. Sternberg, Neural regulation of innate immunity: A coordinated nonspecific host
595 response to pathogens, *Nat. Rev. Immunol.* (2006). doi:10.1038/nri1810.
- 596 [46] R. Castro, J. Zou, C.J. Secombes, S.A.M. Martin, Cortisol modulates the induction of
597 inflammatory gene expression in a rainbow trout macrophage cell line, *Fish Shellfish*
598 *Immunol.* (2011). doi:10.1016/j.fsi.2010.10.010.
- 599 [47] C.B. Schreck, W. Contreras-Sanchez, M.S. Fitzpatrick, Effects of stress on fish
600 reproduction, gamete quality, and progeny, *Aquaculture*. 197 (2001) 3–24.
601 doi:10.1016/S0044-8486(01)00580-4.
- 602 [48] S. Losdat, H. Richner, J.D. Blount, F. Helfenstein, Immune Activation Reduces Sperm
603 Quality in the Great Tit, *PLoS One*. 6 (2011) e22221.
604 doi:10.1371/journal.pone.0022221.
- 605 [49] T. Janevic, L.G. Kahn, P. Landsbergis, P.M. Cirillo, B.A. Cohn, X. Liu, P. Factor-
606 Litvak, Effects of work and life stress on semen quality, *Fertil. Steril.* 102 (2014) 530–
607 538. doi:10.1016/j.fertnstert.2014.04.021.
- 608 [50] A. Kowalska, A.K. Siwicki, R.K. Kowalski, Dietary resveratrol improves immunity but
609 reduces reproduction of broodstock medaka *Oryzias latipes* (Temminck &
610 Schlegel), *Fish Physiol. Biochem.* 43 (2017) 27–37. doi:10.1007/s10695-016-0265-8.

611

612

6. Acknowledgements

613 The study was funded by the projects No. TÉT_14_VL1-2015-0001 and GINOP-2.3.2-15-
614 2016-00025. Additional funding was provided by the by funds appropriated to the Institute of
615 Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland
616 The Authors would like to thank to SARL Asialor for providing fish for the study. Also, sincere
617 thanks to Dr Jarosław Król for his valuable technical assistance during the realization of the
618 experiment.

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 \pm 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 sGnRH α) with 5 day
636 interval. For calculations values recorded 5 days following the second hormonal injection were

637 used. Fields shadowed with orange color indicate significant ($p < 0.05$) negative correlation. No
638 significant positive correlation was detected.

639

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

642

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

648

649 **Fig. 3.** Sperm motility parameters (measured by CASA system), with Pearson's
650 correlation coefficient (r value) and p -values provided separately for each set of data recorded
651 in pikeperch treated with either human chorionic gonadotropin (hCG; dark blue triangles) or
652 salmon gonadoliberine analogue (sGnRHa; light blue circles) with 5 day interval. Samples were
653 collected 5 days after 1st injection (x axis) and 5 days after 2nd injection (y axis). On the plot
654 for pMOT points encircled indicates overlaid data-points and the number of data-points plotted
655 in this area is indicated ($\times 4$).

656

657 **Fig. 4.** Relative immune genes expression (mean \pm SD) (normalized to geometric mean
658 of two housekeeping genes: beta actin [β -actin] and elongation factor alpha [$ef1-\alpha$]) in head
659 kidney of pikeperch males treated twice (with 5-days' interval) with different spawning agents
660 before the final sampling (5 days after 2nd injection). $c3$ – complement C3; lys – C-type
661 lysozyme; $hamp$ – hepcidin c ; $il-1$ – interleukin-1b; $tnf-\alpha$ – 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	Accession number	Forward (5'-3')	Reverse (5'-3')
Beta actin (<i>reference gene</i>)	<i>β-actin</i>	MF472627	CGACATCCGTAAGGACCTGT	GCTGGAAGGTGGACAGAGAG
Elongation factor 1 (<i>reference gene</i>)	<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	TTCCCATCATCCACTGACA	ATCACACACGCACACCATT
Tumor necrosis factor alpha	<i>tnf-α</i>	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 (%)	1 st sampling	69.9	19.5	75.9	22.8
	2 nd sampling	78.1	13.5	77.1	19.0
VCL ($\mu\text{m s}^{-1}$)	1 st sampling	143.5	23.0	157.5	19.9
	2 nd sampling	152.9	18.0	166.6	19.0
ALH (μm)	1 st sampling	2.39	0.35	2.41	0.36
	2 nd sampling	2.22	0.27	2.28	0.32
VSL ($\mu\text{m s}^{-1}$)	1 st sampling	83.0	7.9	89.4	7.3
	2 nd sampling	88.6	9.9	91.0	9.0
LIN (%)	1 st sampling	0.58	0.05	0.57	0.08
	2 nd sampling	0.58	0.04	0.55	0.06
BCF (Hz)	1 st sampling	29.2	1.3	28.9	1.0
	2 nd 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 \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 ($p < 0.05$).

	Cortisol [ng ml ⁻¹]	Glucose [μ 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

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 [$\mu\text{m s}^{-1}$]	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 [$\mu\text{g ml}^{-1}$]	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
<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 gonadoliberine analogue (applied dose: 50 $\mu\text{g kg}^{-1}$)

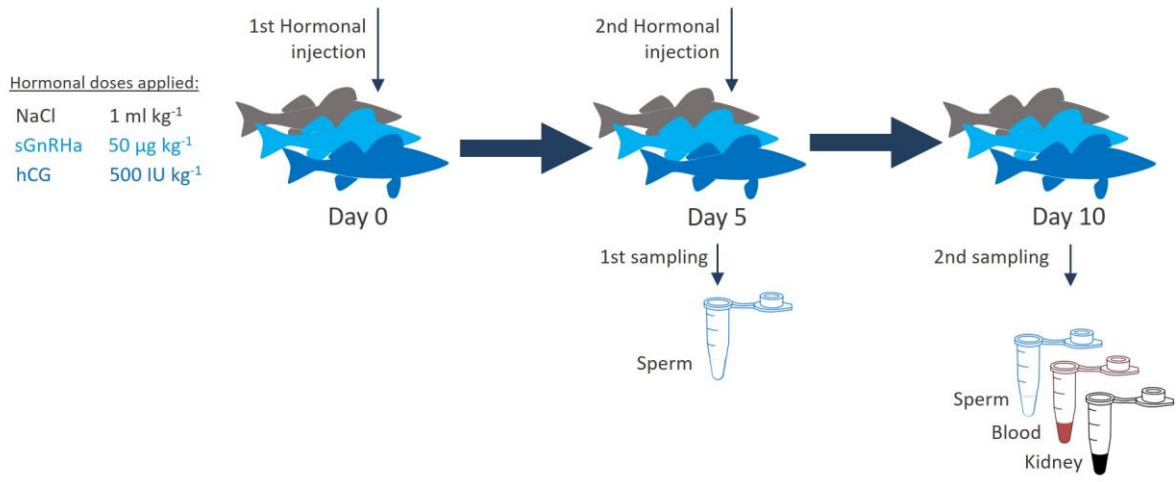


Figure 1 Zarski et al

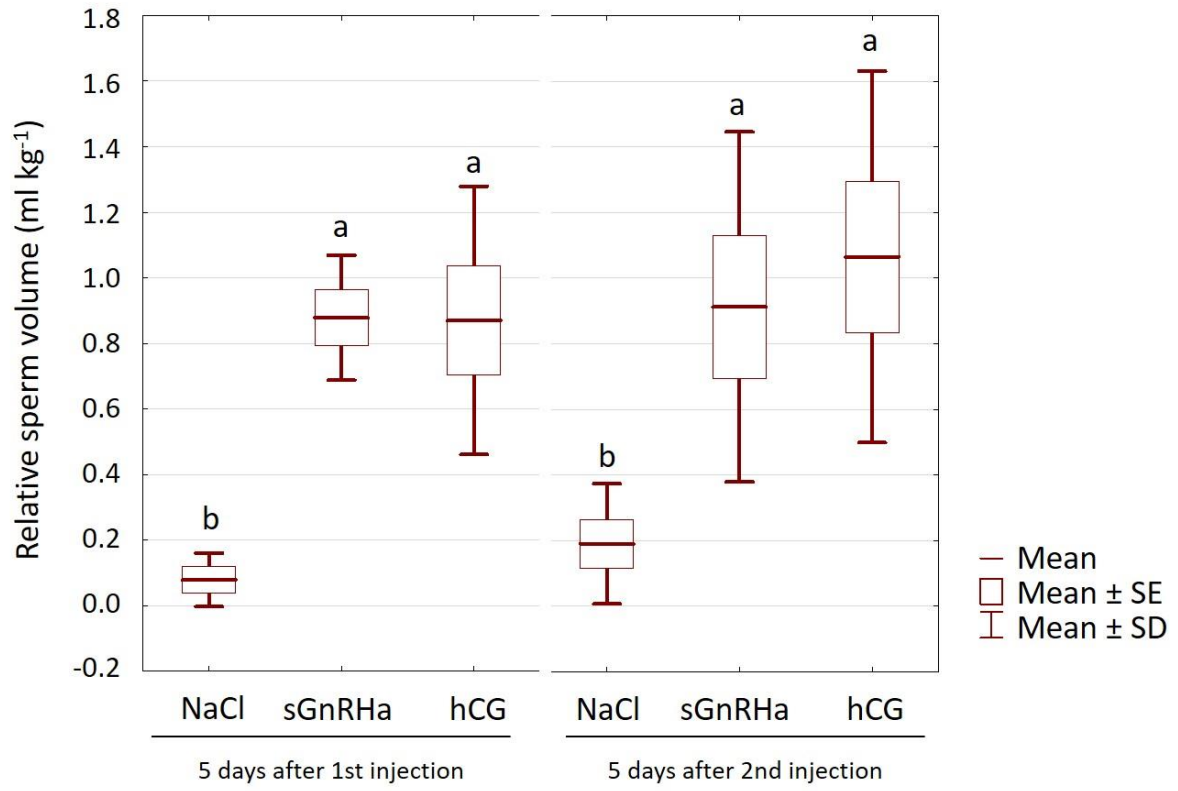


Figure 2 Zarski et al

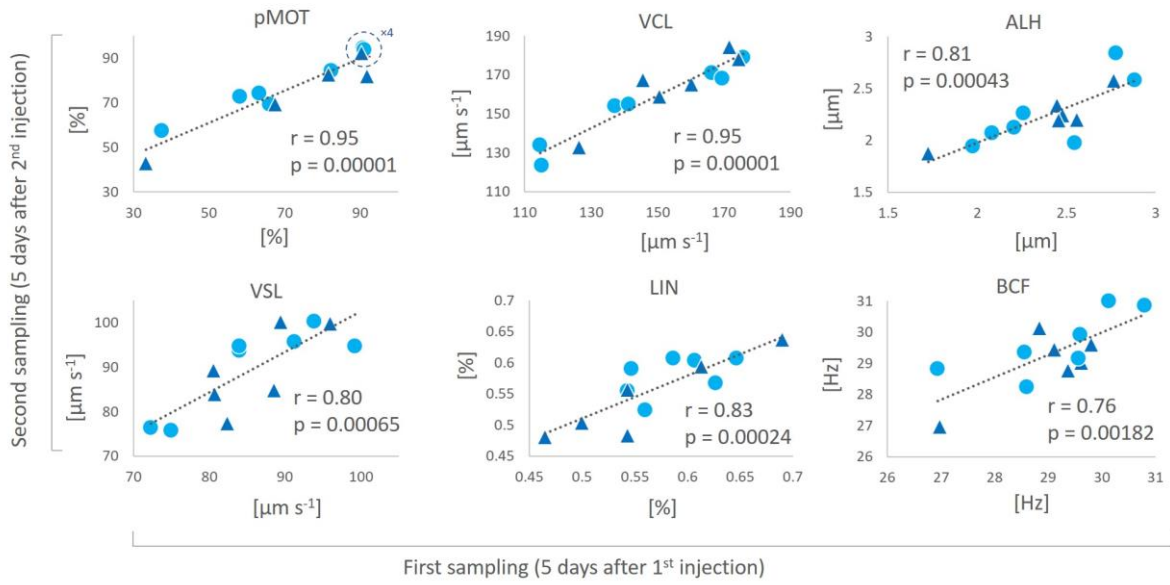


Figure 3 Zarski et al

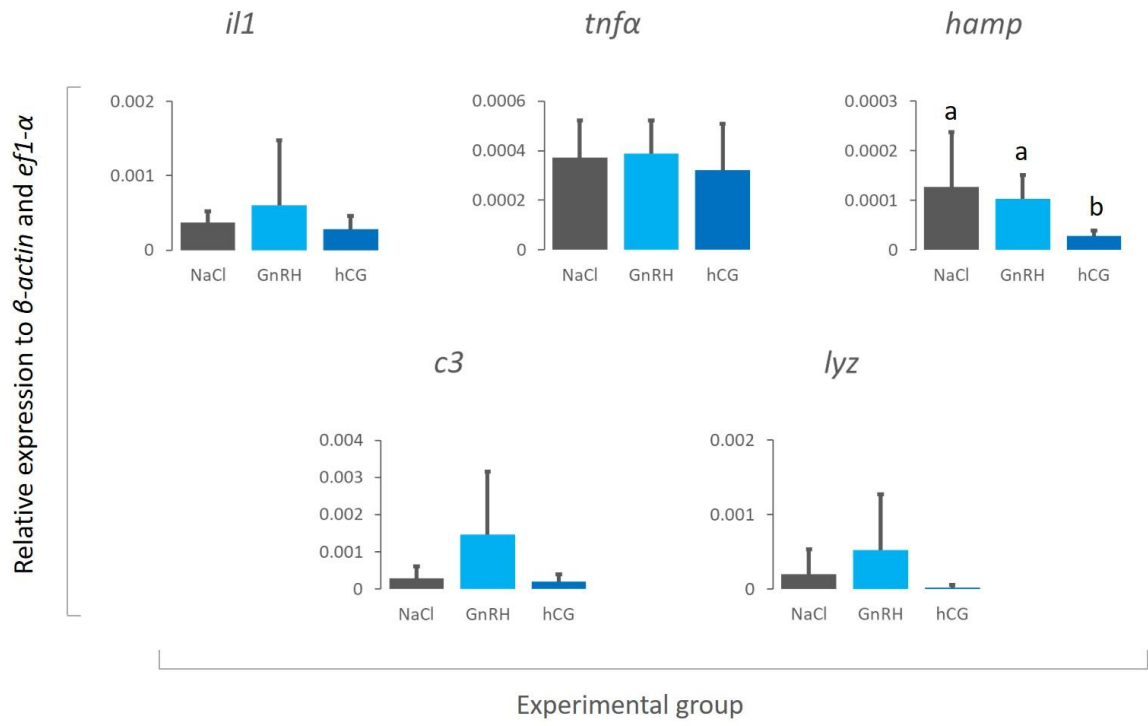


Figure 4 Zarski et al