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Time of response to hormonal treatment but not the type of a spawning agent affects the reproductive effectiveness in domesticated pikeperch, *Sander lucioperca*

Daniel Żarski^{1,2*}, Pascal Fontaine¹, Jennifer Roche¹, Maud Alix³, Mirosław Blecha³, Coralie Broquard¹, Jarosław Król⁴, Sylvain Milla¹

1. Université de Lorraine, INRA, UR AFPA, F-54000 Nancy, France
2. Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, ul. Tuwima 10, 10-748 Olsztyn, Poland
3. Asialor, 22 Rue Roger Husson, 57260, Dieuze, France
4. Department of Ichthyology, University of Warmia and Mazury, ul. Oczapowskiego 2, 10-719 Olsztyn, Poland

* corresponding author: Tel. (+48 89) 539 31 65, e-mail: d.zarski@pan.olsztyn.pl (D. Żarski)

Abstract

Improving reproductive protocols is one crucial step towards aquaculture expansion of pikeperch (*Sander lucioperca*), which is still characterised by variable and/or low spawning effectiveness. One of the main challenges is to synchronise ovulation at a precisely planned time with a consistently satisfactory reproductive outcome. To this end, the present study examined the effect of different spawning agents (human chorionic gonadotropin [hCG] and salmon gonadotropin-releasing hormone analogue [sGnRHa]) with different doses and application modes (including double injection). The study covered three consecutive spawning seasons, which corresponded to three distinct experiments, where domesticated pikeperch broodstock, commercially grown in a recirculating aquaculture system, was used. In the first year of the study, the effect of different doses of sGnRHa (10, 25 and 50 µg kg⁻¹) and hCG (250, 500 and 1000 IU kg⁻¹) on the reproductive performance of the domesticated broodstock was evaluated. The results were also compared with literature data for wild fish. During the second and third years, typical indicators of spawning performance (ovulation rate, latency time and egg quality) were followed when a double sGnRHa injection was compared to a single 50 µg kg⁻¹ or 500 IU kg⁻¹ injection of sGnRHa or hCG, respectively; the best results were

obtained in the first and second experiments. The results of the present study clearly indicate that various hormonal treatments effectively induced domesticated pikeperch ovulation, although highly variable egg quality was observed throughout the three spawning seasons (maximum hatching rates were 60.6 ± 11.5 , 37.7 ± 28.9 and $49.1 \pm 24.7\%$ in the first, second and third years of the study, respectively). However, additional analysis of the data from the entire study revealed for the first time that a significant proportion of the lower-quality eggs came from fish that responded 'early' to hormonal treatment (<120 h after injection) regardless of the hormone used. This group represented approximately 40% of the population each year. Further, most of the fish that responded to hormone treatment early exhibited this trait during all three consecutive spawning seasons. This finding indicates that early hormone response is a potential selection trait. The present study showed that controlled domesticated pikeperch broodstock reproduction may involve application of either hCG or sGnRH α , with no clear difference in their effectiveness, although the recommended doses are 500 IU kg⁻¹ and 50 μ g kg⁻¹, respectively.

Keywords: GnRH; hCG; egg quality; controlled reproduction; percids

1. Introduction

Controlled reproduction involves a set of specific protocols that allow larvae acquisition from mature fish (Żarski et al., 2017a). It directly affects subsequent production steps by influencing the number of obtained larvae as well as the time and period when spawning occurs. Since spawning time is an indispensable element in production planning, asynchrony in this process in intensive culture usually results in increased offspring size heterogeneity, which subsequently leads to cannibalism (Kestemont et al., 2003; Mélard et al., 1996). This phenomenon is especially important in percid fish, such as pikeperch (*Sander lucioperca*), a highly valued commercial fish species where size heterogeneity and cannibalism are serious obstacles during commercial production (Kestemont et al., 2007). Pikeperch typically spawn for a long period (up to 1 month) when they mature spontaneously (Müller-Belecke and Zienert, 2008). This period may easily be shortened and synchronised with hormonal stimulation (Křišť'an et al., 2013; Ljubobratović et al., 2017; Żarski et al., 2012b, 2013), a procedure that is already practised at several commercial pikeperch farms. However, applying hormonal stimulation very often leads to lower and/or variable egg quality (Schaerlinger and Żarski, 2015; Żarski et al., 2015); this occurrence is still one of the main bottlenecks in intensive aquaculture for this species.

In practice, pikeperch broodstock is held in recirculating aquaculture systems (RASs) where gonadal development is controlled by application of specific annual photo-thermal fluctuations (Fontaine et al., 2015). In temperate fish such as pikeperch, reduced photoperiod and temperature trigger gonadal development until the end of vitellogenesis (Fontaine et al., 2015; Hermelink et al., 2016, 2011), after which final oocyte maturation (FOM) commences (King et al., 1995; Nagahama and Yamashita, 2008). In percids, FOM involves many cellular changes, including migration and disintegration of the germinal vesicle, formation of the oil globule and reorganisation and homogenisation of yolk constituents (Żarski et al., 2012b).

FOM in pikeperch directly precedes ovulation. It does not occur simultaneously in all the females, which is a direct reason for the prolonged spawning season (during which particular females attempt to spawn at different times) that is also recorded in wild females (Żarski et al., 2012b). In domesticated fish at the beginning of FOM, a vast majority of fish exhibit a similar oocyte maturation stage (stage I), when considering the classification provided by Żarski et al. (2012b). Therefore, the beginning of the FOM may be considered as a very good starting point for hormonal stimulation. Hormonally treating wild percids at oocyte maturation stage I, however, lowers egg quality (Żarski et al., 2015, 2012b, 2011a). Therefore, common practice has been to delay hormonal treatment until the fish reached at least oocyte maturation stage III, and this practice usually resulted in high egg quality (Żarski et al., 2012b). Such a procedure (i) prolonged the entire spawning operation, (ii) made impossible precise planning of the spawning time, (iii) was highly laborious and (iv) was highly stressful to fish, due to the need for frequent handling. Thus, the most reasonable way towards commercialisation of controlled pikeperch reproduction is to develop a protocol that allows high egg quality from fish treated at oocyte maturation stage I.

Fish gonadogenesis and FOM is controlled by the hypothalamic-pituitary-gonadal axis (HPG axis) (Mylonas et al., 2010). For hormonal induction of ovulation, either gonadotropin-releasing hormone (GnRH) analogues or gonadotropins (GtHs) are applied (Donaldson, 1996; Zohar and Mylonas, 2001). For pikeperch, either human chorionic gonadotropin (hCG) or GnRHs are recommended (Zakęś and Demska-Zakęś, 2009; Żarski et al., 2015). Human chorionic gonadotropin has a very long half-life (up to 5 days) following injection (Ohta and Tanaka, 1997) in comparison to GnRHs analogues (as short as a few hours) (Zohar et al., 1990). One could hypothesise that the surge of steroids following hCG injection is much higher than that following GnRHs, and consequently this intense surge may have negative effects on oocytes since they could continue acting for too long. A similar phenomenon was described in

mammals, and termed ovarian hyperstimulation syndrome (OHSS) (Youssef et al., 2014). This process could partially explain why hCG injection at oocyte maturation stage I (when oocytes are exposed to high steroid levels for a few more days compared to stage III) reduced percid egg quality (Żarski et al., 2012b, 2011a). Moreover, hCG application enhanced cortisol production in pikeperch (Falahatkar and Poursaeid, 2013), a result that suggests another strong physiological reaction potentially involved in FOM that would presumably affect egg quality. Until now there has been no information available on the optimisation of dosage of either hCG or GnRH in induction of ovulation in domesticated pikeperch.

Research on hormonal stimulation of ovulation in pikeperch has mostly been conducted with wild fish (Żarski et al., 2015), and only a few studies have been performed with domesticated fish grown fully in RAS (Ljubobratović et al., 2017; Roche et al., 2018; Zakęś et al., 2013). Because the domestication process, rearing conditions and life history (Khendek et al., 2017, 2018) influence fish reproductive physiology and performance, there is an urgent need to develop hormonal treatment protocols for fish commercially grown in RAS, where both hCG and GnRH should be tested.

In freshwater non-salmonid fish species (e.g. cyprinids), GnRH-based spawning agents are typically applied in two doses: initial and resolving, a protocol that usually leads to improved spawning efficiency. The initial dose is generally 10–20% of the resolving one (Kucharczyk et al., 2008; Podhorec and Kouril, 2009). This administration strategy of GnRH has also been reported to improve the out-of-season spawning effectiveness of Eurasian perch, *Perca fluviatilis*, (Żarski et al. 2017). However, there is no information on such an injection strategy of GnRH in pikeperch.

The aim of the study was to verify the effectiveness of hCG and sGnRH α for induction of ovulation in RAS-grown pikeperch. In a three-year study, performed with the use of commercially grown broodstock, different doses and treatment modes (including double

injection of sGnRHa) were considered from the perspective of the effect on spawning performance (synchronisation of ovulation and egg quality).

2. Materials and Methods

All experiments carried out in this study were performed with the compliance of the European and French legislations for fish welfare and approved by the institutional Ethics Committee (APAFIS-2016022913149909).

2.1. Broodstock management

Pikeperch broodstock (60 females and 60 males that originated from the Czech Republic) were RAS-grown from the juvenile stage until maturity at the ASIALOR (Pierrevillers, France) fish farm. Fish, between the experiments, were randomly distributed in two separate 8000 L rectangular tanks. Females were separated from the males before each experiment. The system was equipped with biological and mechanical filtration and automatic temperature regulation (cooling and heating). The oxygen level (always >80% saturation) and temperature were monitored constantly, whereas ammonia (0.2–1.1 mg L⁻¹) and nitrite (0.05–0.70 mg L⁻¹) concentrations were measured twice a week. Before the first gonadal cycle induction, the fish were kept at a constant 22 °C and a constant photoperiod of 14 h of light at an intensity of 20 lux (provided with neon tubes). Next, the fish were exposed to first induction of gonadal cycle by the use of a specific, year-round photo-thermal programme involving reduction of temperature and photoperiod followed by increase of both factors, simulating natural annual photo-thermal fluctuations. This photo-thermal regime was established according to Fontaine et al. (2015) for Eurasian perch, with modification involving application of a higher temperature (i.e., between 8–9 °C) during the wintering period (the period of lowest

temperatures), in accordance with the findings of Hermelink et al. (2013). In total, fish were exposed to a temperature below 10 °C for 4 months.

Fish were fed compound extruded feed (50% protein, 11% fat, 10% moisture, 1.55% crude fibre, 1.35% phosphorus, 9.5% ash and 17.9% nitrogen-free extract; Le Gouessant, France). Fish were fed between 0.2 and 1.0% biomass daily, depending on the temperature and appearance of satiation.

In 2014, fish were first exposed to the photo-thermal programme that allowed gonadal cycle induction, and the first spawning was performed in December 2014. Next, fish were exposed to the photo-thermal programme (as described above) and were used for the experiments starting from 2016 for three consecutive years (three spawning operations). Thus, the beginning of the first, second and third experimental spawning trials were commenced at the beginning of February 2016, 2017 and 2018, respectively. All fish used in this study were at the same age (they were 4+, 5+ and 6+ during the first, second and third spawning season, respectively).

2.2. General experimental design

During the study, three separate spawning operations (hereafter referred to as Experiment 1, Experiment 2 and Experiment 3, conducted in 2016, 2017 and 2018, respectively) were performed each year with the same broodstock. Each fish was tagged individually (with passive integrated transponder tags). Spawning operations always started 7 days after the water temperature reached 12 °C and the photoperiod was 14 h of light per day. At the beginning of the spawning operation, the fish were always checked for oocyte maturation stage, and only females who exhibited maturation stage I were taken for further procedures. The day after maturation stage selection, all chosen females were divided randomly into different groups and treated separately with a different hormone, dose and/or treatment mode

(including double injection), as shown in Fig. 1. Pikeperch males were hormonally treated (250 IU kg⁻¹ hCG) at the time of female injections. After hormonal treatment, the fish were separated according to sex (males were kept in a separate tank). During the study, fish were always handled following MS-222 anaesthesia (150 mg L⁻¹).

2.3. Oocyte maturation stage determination

Maturation stage determination in pikeperch females was performed as described by Żarski et al. (2012b). Briefly, an oocyte sample from each female was taken with a flexible catheter (infant feeding tube, size CH-06, Galmed, Poland) and then immediately immersed in clearing solution (70% ethanol, 38% formaldehyde and glacial acetic acid in the proportions 6:3:1). After 5 min, the oocytes were classified under a stereoscopic microscope at 4× magnification (Motic® SFC-11 Series, Motic Asia, Hong Kong, China) according to the seven-stage classification, with stage I coinciding with the beginning of FOM and stage VII representing the ovulated egg.

2.4. Experiment 1

The aim of this experiment was to evaluate the effectiveness of different hormonal preparations at different doses on pikeperch female reproduction and oocyte maturation kinetics, and to investigate hormonal response (steroids) to applied hormone treatment in domesticated female pikeperch (average body weight 2.41 ± 0.44 kg). Fish were divided into seven groups (n = 7 per group). Apart from the control group, which was treated with 0.9% NaCl solution, fish were treated with either sGnRH α (10, 25 or 50 μ g kg⁻¹ for the GnRH-10, GnRH-25 and GnRH-50 groups, respectively; Bachem, Switzerland) or hCG (250, 500 or 1000 IU kg⁻¹ for the hCG-250, hCG-500 and hCG-1000 group, respectively; Chorulon, Intervet, France). The sGnRH α peptide sequence was: Pyr-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-

Pro-NHEt. The doses were chosen based on those most commonly used for controlled percid reproduction (Żarski et al., 2015). Fish were injected intraperitoneally at the base of the left ventral fin. The spawning agents were always diluted so that each fish received 1 mL of solution per kg body weight. Following injection, fish were monitored for oocyte maturation stage (as described by Żarski et al., 2012b) every second day until they reached stage V, and then every day until they reached stage VI. Next, the fish were checked for ovulation every 6 h. The oocyte maturation stage data were used to evaluate the differences in maturation kinetics of domesticated fish (studied here) and wild fish, for which the oocyte maturation stages were developed and maturation kinetics considered as a 'model' (taken from Żarski et al., 2012b). This selection was performed in order to evaluate whether the response to hormonal treatment varied between wild fish and the broodstock studied. Further, it allowed verification of the robustness of applying oocyte maturation stages developed for wild fish to domesticated ones, as the information on such kinetics exists only for wild fish. The details of this analysis are described in the 'Statistical Analysis' section.

When ovulation was certified by stripping (hereinafter referred to as spawning), the time was recorded, the eggs collected, the weight of eggs measured and controlled fertilisation was performed in order to evaluate egg quality (as described below). Temperature during spawning was constant (12 °C). Photoperiod was identical to the photo-thermal programme described above.

During this experiment, at the time of injection (0 h) blood was sampled with a heparinised syringe from the caudal vein from 10 randomly selected fish. The next samplings were performed from each fish at 24 h and 48 h following injections. The last sampling was performed at the time of spawning, or 14 days following injection if the fish did not ovulate (i.e., 5 days after the last certified ovulation in the experimental groups). The fish were not checked later as no (or very minor) oocyte maturation progression was observed, and additional

observation would require excessive manipulation and cause unnecessary stress. Following collection, the blood was placed in a 2 mL tube and centrifuged (15 min at 6700×g). The blood plasma was then stored at $-80\text{ }^{\circ}\text{C}$ for future sex steroid analysis.

2.5. Experiment 2

In Experiment 2, the fish (average body weight $3.16 \pm 0.60\text{ kg}$) were divided randomly into six groups ($n = 7$ per group) with NaCl-treated fish again serving as the negative control. The remaining five groups were assigned either to two positive control groups following Experiment 1 (see Fig. 1; $50\text{ }\mu\text{g kg}^{-1}$ of sGnRH α or 500 IU kg^{-1} of hCG) or to the three new experimental groups treated with double injection of sGnRH α , with 48 h between injections (Fig. 1). The doses (presented in Fig. 1) were determined according to the typically recorded doses in double-injection hormonal stimulation in freshwater teleosts (Krejszeff et al., 2009; Kucharczyk et al., 2008). The time interval was determined on the basis of Experiment 1 data and was chosen as that in which the fish on average exhibited oocyte maturation stage III, which was considered the most suitable for the resolving injection due to the lack of negative effects when pikeperch were hormonally treated at this stage (Żarski et al., 2012b). The resolving injection was fixed at $25\text{ }\mu\text{g kg}^{-1}$ sGnRH α , which is a commonly used resolving dose in freshwater finfish. The final dose did not exceed that applied in the GnRH-50 group. Oocyte maturation kinetics evaluation and blood sampling were not performed in this experiment. The remaining procedures were the same as in Experiment 1.

2.6. Experiment 3

Following the findings of Experiments 1 and 2, in Experiment 3 pikeperch females (average body weight $3.5 \pm 0.8\text{ kg}$) were randomly assigned to only three groups: a control group treated with 0.9% NaCl ($n = 7$) and two treated groups with either hCG (hCG-500) or

sGnRHa (GnRH-50) (n = 20 for each group). This experiment was performed in order to validate the usefulness of the chosen treatment methods with larger fish numbers per type of spawning agent. All the other procedures and data collection, except evaluation of oocyte maturation kinetics and blood sampling, were performed in the same way as in Experiment 1.

2.7. Gamete management, fertilisation and incubation procedure

Eggs were collected into a separate dry container with a sealed lid. The eggs were then weighed (for calculation of pseudogonadosomatic index [PGSI], according to the formula: $[\text{weight of eggs}] \times 100 / [\text{weight of fish before ovulation}]$), and stored for no longer than 30 min prior to fertilisation at 10 °C. In the meantime, fresh sperm was collected into dry disposable syringes from five randomly chosen males from among 60 available males (0.5 mL fresh sperm per male). Next, spermatozoa motility was checked subjectively under the microscope at 400× magnification (Motic® B3 Series, Motic, Hong Kong, China), as described by Cejko et al. (2010). For subsequent procedures, only sperm from the three males with the highest motility was used; the lowest threshold was 80% motility. The sperm was pooled (in equal proportions) in an Eppendorf tube just prior to fertilisation. Next, the eggs were fertilised according to the method described by Roche et al. (2018): the so-called ‘wet method’ on glass Petri dishes (diameter 50 mm), to which the fertilised eggs adhered. Briefly, to each individually labelled Petri dish, 5 mL of hatchery water was added and a small portion of eggs (approximately 100) was placed on it simultaneously with 50 µl of the pooled sperm sample. The Petri dish was stirred vigorously for about 15 s and then incubated at 12 °C in plastic cups that contained 500 mL of fresh water.

During incubation, the survival rate of embryos at 72 h post fertilisation (by direct counting under the stereoscopic microscope of the live and dead embryos from among all the eggs) and hatching rates were determined; these indices are most commonly used in

reproductive studies (Bobe and Labbé, 2010; Żarski et al., 2011b). Additionally, every hatched larva was verified for occurrence of deformity, including deformations typically reported in percids, following observations made by Alix et al. (2017), and the deformity rate ($= 100 \times$ number of larvae showing deformity / total number of larvae) was determined.

2.8. Sex steroid hormone levels in blood plasma

Obtained in Experiment 1, blood plasma was used to evaluate sex steroid hormone concentrations; namely, 17β oestradiol (E2) and testosterone (T), using commercially available competitive ELISA kits (Diasource, Louvain-La-Neuve, Belgium, catalogue numbers: KAP0621 and T KAPD1559 for E2 and T, respectively) following the manufacturer's protocols. The sensitivity limit and the intra- and inter-assay coefficients of variation (CV) were, respectively: 0.005 ng/mL (measuring range: 0.013 to 0.935 ng/mL), <4% and <5% for E2; and 0.083 ng/mL (measuring range: 0.2 to 16 ng/mL), <10% and <9% for T.

2.9. Data analysis and statistics

In Experiment 1, oocyte maturation kinetics were followed for each fish separately, from maturation stage I to VII. These data were used to plot the oocyte maturation kinetic curves (oocyte maturation stage versus time in h following injection) and to calculate the regression coefficient for each curve separately using MS Excel 2016 for Windows with the LINEST function. These data were then used to analyse the equality of regression coefficients between the experimental fish curve and the model curve obtained from Żarski et al. (2012b) for wild fish. During this comparison, a statistical method adapted from Clogg et al. (1995) and Paternoster et al. (1998) was used, where the z values that characterised differences ($z = 0$ for identical regression coefficients) between two regression coefficients were calculated as follows:

$$z = \frac{b_1 - b_2}{\sqrt{SEb_1^2 + SEb_2^2}}$$

In the above equation, b_1 is the regression coefficient of a model (in this case, the regression coefficient calculated from the wild fish model) and b_2 is the regression coefficient of the experimental group (for details, see methods for Experiment 1). Next, the calculated z value for each female was used for statistical analysis, as described below.

Data obtained from Experiment 3 were additionally analysed for verification of relatedness of latency time to spawning performance. To this end, the results obtained for all fish were sorted according to latency time. Next, three groups were distinguished: early responders (ovulation occurring <120 h following hormonal injection), timely responders (ovulation occurring 120–150 h) and late responders (ovulation certified >150 h following injection). These groups were distinguished on the basis of typical responses evaluated in Experiment 1 and recorded for wild fish (Żarski et al., 2012b), where ovulation typically occurred 120–150 h post injection at oocyte maturation stage I. These groups were then compared statistically. Further, the proportions between different groups are presented. Additional analysis involved evaluation of time of response to hormonal treatment considering the three-year history for all individuals, as well as Pearson’s correlation analysis between all the zootechnical parameters recorded.

All the data expressed in percentages (i.e., ovulation, fertilisation, hatching and deformity rates) were arcsine-root-square-transformed prior to statistical analysis. Next, the data were checked for homogeneity of variance with the Levene test. If the data were normally distributed, they were subsequently tested with analysis of variance (ANOVA) to determine potential statistical differences, followed by Duncan’s post-hoc test (Experiments 1 and 2) or the t-test (Experiment 3). Otherwise, the data were analysed with the Kruskal–Wallis non-parametric test followed by the Mann–Whitney test whether the comparisons were significant.

Data were compared at the significance level of $P < 0.05$. The data were analysed with MS Excel for Windows and Statistica 13 software (StatSoft).

3. Results

3.1. Experiment 1

During Experiment 1, no control group fish ovulated, and only one female exhibited oocyte maturation progression (reached maturation stage II at the end of the experiment, i.e., 14 days after injection). Therefore, this group was excluded from most of the analysis, except that for sex steroid levels.

There were no differences in ovulation rate (always 100%), latency time, PGSI (average $12.7 \pm 3.7\%$) or egg quality indices (fertilisation, hatching and deformity rates) between the experimental groups (Table 1). Therefore, in the succeeding study (Experiment 2) two treatments were designated to represent each hormone type, where the main criterion was the lowest coefficient of variation (in terms of fertilisation and hatching rates) recorded in the GnRH-50 and hCG-500 groups (Table 1).

Analysis of oocyte maturation kinetics showed that fish from the GnRH-50 group reacted to the hormone much more slowly than those treated with 1000 IU kg^{-1} hCG. Additionally, the fish oocyte maturation kinetics were generally very similar to the model developed based on wild fish, as the z values were usually very close to 0, indicative of high similarity (Fig. 2 and 3).

Sex steroid analysis revealed that hCG and sGnRH α had similar effects on plasma E2 and T levels at respective sampling points (Fig. 4; $P > 0.05$). Only the hCG-250 group had higher plasma E2 levels 24 and 48 h post injection compared to the control group. Also, group hCG-500 had significantly higher E2 plasma levels 48 h post injection as compared to fish injected with NaCl ($P < 0.05$). In the remaining groups, E2 levels were always similar to control

fish ($P > 0.05$). A similar trend was recorded for T, where it was higher in all experimental groups 24 and 48 h following injection compared to the control group ($P < 0.05$). Control fish showed no changes in E2 or T levels during the experimental period. However, just after ovulation (at the time when the eggs were stripped), T concentrations were lower than that recorded in the NaCl-treated fish at 14 days post injection (Fig. 4; $P < 0.05$).

3.2. Experiment 2

In the second year of the study, only one fish from the negative control group ovulated, and thus the results presented for this group considered only one female. This group was not considered in the statistical analyses. All the hormonal treatment modes were comparable ($P > 0.05$) in terms of their effectiveness in ovulation stimulation (all fish ovulated) and latency time (eggs from all fish were stripped between 82 and 232 h following injection, but there was high variability in all groups) and their effect on egg quality indices (Table 2). Additionally, PGSI was similar between all fish (average $14.2 \pm 2.6\%$). Overall, the single injection mode was considered to be more convenient for commercial hatchery use because only one manipulation is necessary. Since double injection had no significant positive effect, it was discarded from further consideration, and GnRH-50 and hCG-500 treatments were used for commercial-scale validation in Experiment 3.

3.3. Experiment 3

Experiment 3 confirmed the results of previous years; no differences in latency time, PGSI (average $13.2 \pm 3.4\%$) or egg quality were detected (Table 2).

3.4. Analysis of response time to hormonal treatment and spawning quality

Analysis of response time to hormonal treatment indicated that, regardless of the type of hormone used, fish who responded to the hormone earlier (less than 120 h post injection, considered the earliest time for ovulation based on Experiment 1 findings) spawned lower-quality eggs ($P < 0.05$) compared to timely responders and late responders (Fig. 5). This was also confirmed when the fish were split into the response groups and then sub-grouped into the different hormonal preparations tested (Fig. S1). Interestingly timely responders (the group who yielded the highest-quality eggs) had greater body weight than the remaining groups (Fig. 6). However, correlation analysis did not reveal significant relationships between body weight or PGSI of the fish and egg quality indices. Positive significant correlation was recorded between latency time and fertilisation and hatching rates, whereas negative correlation was found between latency time and deformity rate (Tab. S1). Notably, among all the fish monitored over three years, early responders represented about 40% of the entire population (Fig. 7a). Retrospective analysis of the same fish over three years identified 17 fish who in Experiment 1 were recognised as fast responders (Fig. 7b). From those 17 fish, only two were later found to respond to hormonal treatment in a ‘timely’ way, and two of the fish died during that period. The remaining fish (recognised in Experiment 1 as timely and late responders) exhibited the same response pattern as the early responders, i.e. ~70% of the fish were classified each time to the same group. None of the fish representing ‘timely’ or ‘late’ responders were found to become ‘early responders’ in Experiments 2 and 3.

4. Discussion

The results presented in this study indicate, for the first time, that ‘early responders’ to hormonal treatment yield lower-quality eggs. This trait is suspected to be specimen-specific (as the ‘early responders’ mostly responded early to hormones three years in a row), a phenomenon never previously reported in this species. Additionally, the same domesticated pikeperch

broodstock reproduced every year with consistent effectiveness at a precisely planned period. Each year, a subset of the fish spawned satisfactory-quality eggs within three days, regardless of hormonal treatment, a finding reported here for the first time.

Fish domestication, considered for teleosts as a process of adaptation to a cultured environment (Teletchea and Fontaine, 2014), is known to affect not only behaviour and exhibition of particular commercially-relevant traits (such as growth rate) (Molnár et al., 2018; Teletchea and Fontaine, 2014), but also to influence reproductive physiology (Khendek et al., 2017, 2018; Krejszefz et al., 2009). A recent study reported that pikeperch life history affects gametogenesis and physiology during reproductive cycle induction, where domesticated fish exhibited significantly lower sex steroid levels, namely E2 and T, when compared to wild fish just at the end of vitellogenesis. Further, domesticated fish progressed to oogenesis more asynchronously, and very few of them (<20%) progressed to the end of vitellogenesis; approximately 60% of the wild fish reached the late vitellogenesis stage (Khendek et al., 2018). The findings of our study, however, suggest that latency time is very close to the model developed based on wild fish (see Fig. 2 and 3). It must be emphasised that initial sex steroid levels differed significantly from those reported for wild fish. For instance, the initial E2 level in this study (<5 ng mL⁻¹; Fig. 4) was quite low compared to pre-spawning wild fish (68 ng mL⁻¹; Pourhosein Saramah et al., 2012). On the other hand, the T level observed in this study was at least four times higher than those reported for wild females. Interestingly, the E2 level recorded during this study reached an average maximum of less than 30 ng mL⁻¹, half the wild fish level, even after hormonal stimulation. In contrast, the T level was nearly 20 times higher after injection compared to wild fish (Pourhosein Saramah et al., 2012). A similar discrepancy in terms of sex steroid levels was reported by Roche et al. (2018), where pond-reared fish (originating from a wild-like environment) had much lower T levels compared to domesticated fish. Therefore, the results obtained here clearly show that hormonal stimulation, irrespective

of the type of spawning agent, very effectively stimulated T production in domesticated broodstock. A lower E2 level, which can be converted directly from T (Ishikawa et al., 2006), suggests that the T-to-E2 conversion was somehow slow or ineffective, as its level did not reach those observed in wild pikeperch females (Pourhosein Saramah et al., 2012). Fast E2 metabolism stemming from its use in promoting FOM cannot be ruled out, as FOM progression was actually rapid when compared to the natural course of FOM observed in wild fish (Długosz, 1986). Further consideration of this phenomenon requires additional studies that should include measuring plasma T and E2 levels, other steroids and the expression of aromatases (such as cytochrome P450 aromatase involved in E2 biosynthesis) and gonadal oestrogen receptors. These data would potentially explain any differences between the mode of action of particular spawning agents and reveal mechanisms related to the administration of exogenous hormonal preparations and their effect(s) on FOM.

Application of different hormonal preparations in freshwater teleosts usually affects latency time and spawning quality, both of which may be related to different modes of action (at either the brain or gonadal level in the HPG axis), the half-life in blood circulation and/or spawning agent potency (Kucharczyk et al., 2008; Targońska et al., 2010). However, in the case of percids, differences in latency time following hCG- or GnRH-based applications were never reported (Zakęś and Demska-Zakęś, 2009; Żarski et al., 2015). The results of the present study confirm this finding; namely, that latency time cannot be significantly modulated by application of different spawning agents.

The results presented in this study did not confirm improved efficiency upon repeated sGnRH α administration, as has been reported for other freshwater non-salmonid teleosts (Kucharczyk et al., 2008). For pikeperch, repeated administration was previously tested with hCG (Zakęś et al., 2013), acetone-dried carp pituitary homogenate (Rónyai, 2007) or a mammalian GnRH analogue administered alone or together with dopamine antagonists

(Křišť'an et al., 2013; Źarski et al., 2013). The results following application of those repeated injections had either negative or no effect (see Źarski et al., 2015). Considering these previous findings, the fact that repeated injection did not improve spawning effectiveness in the present study and that double injection is a highly laborious procedure that will subject fish to undue stress, a single-dose hormone treatment is recommended for commercial reproduction of RAS-grown pikeperch.

Egg quality is a huge concern for commercial pikeperch farms (Schaerlinger and Źarski, 2015; Źarski et al., 2012 a); high intragroup variability makes precise production planning difficult. Variable egg quality was observed in this study. Nonetheless, the average hatching rate of approximately 50%, as recorded in the final year of study, may be considered as satisfactory, but large deviations, along with >20% larval deformity, remain a concern. Lower egg quality can be associated with diet quality (Henrotte et al., 2010) or environmental conditions provided throughout the annual cycle that have direct effects on the course of gametogenesis (Fontaine et al., 2015; Hermelink et al., 2016). However, in this study there were some cases of very high fertilisation (>90%) and hatching (>85%) rates coupled with very low (<8%) rates of larval deformities. These results indicate that the problem of low-quality eggs does not stem only from different diet or an improper photo-thermal programme, since all fish were exposed to the same conditions. Of course, individual differences that result from specimen-specific foraging behaviour/effectiveness, as well as vulnerability to stress or social interactions, cannot be excluded as a factor that conditions their overall performance. Nevertheless, the induced spawning protocol was unlikely to be the reason for such extreme results. Considering the fact that the reproductive protocol used in this study yields more consistent results in wild fish (see Źarski et al., 2011a), it can be hypothesised that the problem of spawning quality in the studied broodstock was related either to better adaptation abilities of

particular specimens to the conditions or to genetic determinants. To determine the culprit, additional studies that address these aspects are urgently required.

Analysis of the latency time in relation to egg quality clearly showed that earlier-spawning females produced much lower-quality eggs compared to the timely- or late-spawning fish. This has been also confirmed by the correlation analysis, where significant relationships between latency time and egg quality indices were found (Tab. S1). The relation of spawning time to egg quality was also studied in wild Eurasian perch, where late-ovulating fish yielded lower-quality eggs (Kucharczyk et al., 1998). However, that study considered wild fish, and thus the lower egg quality of late-spawning fish could be related to the higher stress load the fish had to cope with for a prolonged time spent in the hatchery. In this study, fish responsiveness to hormonal treatment (regardless of the hormone type; see also Fig. S1) seems to be a specimen-specific trait, a finding not previously reported. It can be speculated that such a phenomenon stems from the different responsiveness of the fish organism to the application of exogenous hormones. Applied hormonal therapy affects a wide range of organism functions by affecting gene transcription, peptide secretion or induction of feedback mechanisms (Zohar et al., 2010), which makes the identification of the main mechanisms that condition different responsiveness a highly challenging task for future research.

Results of the present study suggest either that ‘early responders’ should be excluded from the broodstock, since fish responsiveness to hormones appears to be a potentially important selection trait – especially since the ‘early responders’ had lower body weight than the best performing group (‘timely responders’) – or that only fish with the highest body weight should be selected for the broodstock. Considering the fact that selective breeding for fast growth may have deleterious effects on teleost reproductive capacity (Mylonas et al., 2016), special care should be taken when deciding on such a selection strategy. Nonetheless, it can be speculated that fish exhibiting lower growth rate are probably having problems in the

production of highest-quality eggs. However, the mechanisms behind this phenomenon remain unclear and require further studies. In particular, there was no clear correlation between the weight of the fish and egg quality indices (Table S1), and only when fish were grouped according to their ‘responsiveness’ to hormonal stimulation was it possible to detect statistical differences for ‘timely responders’ (Fig. 6). On the other hand, it should be also reconsidered that ‘early responders’ possibly require an alternative spawning protocol whenever they exhibit some important trait that is attractive for selective breeding. To confirm this hypothesis, more specific research is required.

The results of the present study show for the first time that controlled reproduction of domesticated pikeperch broodstock may involve application of either hCG or sGnRH α without clear differences in their effectiveness. This is the case even if different responsiveness of fish to the hormonal treatment were considered (Fig. S1). Results presented in Figs. 1 and 2 suggest that the CV of the latency time was lower following hCG treatments compared to GnRH treatments. This might indicate that hCG is more efficient than GnRH in synchronising the timing of ovulation, potentially explained by different targeted tissue (gonad vs pituitary, respectively) or different half-life in the blood (longer vs shorter) between the two spawning agents. However, this observation is not so evident in view of the results presented in Fig. S1, which in some cases suggest the opposite. Therefore, further research would be needed to draw strong conclusions on the differences, if any, between results recorded in fish treated with different types of hormones. However, huge intragroup variability in spawning effectiveness indicates an urgent need for additional studies that allow identification of the factors that condition egg quality, including ‘time of response to the hormone’ as a specific variable to be tested. Additionally, the testing of other spawning protocols, such as alternative thermal protocols, including lower and/or changeable temperature (since temperature is an important modulatory factor for FOM; Pourhosein Saramah et al., 2012; Źarski et al., 2013) that allow

control over oocyte maturation speed and, presumably, egg quality, is recommended for future studies.

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Table 1. Results of induced spawning (mean \pm standard deviation and coefficient of variation [%]) for domesticated pikeperch; n indicates the number of females used. For explanation of groups, see Materials and Methods. Data in rows, between the groups, were not statistically different ($P > 0.05$). Control group is not presented since there was no observed ovulation.

Group	n	Latency time (h)			Fertilisation rate (%)			Hatching rate (%)			Deformity rate (%)		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
GnRH-10	6	139	40	28	30.9	27.4	89	18.1	25.3	140	31	28	89
GnRH-25	7	137	53	39	14.9	26.2	176	11.8	25.5	216	41	47	115
GnRH-50	6	169	50	29	67.6	9.6	14	60.6	11.5	19	22	6	27
hCG-250	7	146	31	21	28.3	28.9	102	19.4	19.6	101	28	7	23
hCG-500	7	130	14	11	46.9	19.6	42	32.0	18.4	57	23	9	40
hCG-1000	7	113	23	20	48.5	37.8	78	37.4	34.4	92	16	4	25

Table 2. Reproduction results for pikeperch females for Experiments 2 and 3 (see Materials and Methods for details) after different hormonal treatments. Differences in the data between the treatment groups for the same experiment were not statistically different ($P > 0.05$).

Group	Number of females ovulated (ovulated/treated)	Latency time (h)		Fertilisation rate (%)		Hatching rate (%)		Deformity rate (%)	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Experiment 2									
NaCl (control)*	1/7	157	-	21.5	-	15.0	-	23.5	-
GnRH-5+25	7/7	146	25	35.9	12.9	15.4	12.2	4.7	2.1
GnRH-10+25	7/7	126	7	41.1	11.2	27.3	8.6	4.9	1.3
GnRH-25+25	7/7	113	8	52.6	12.4	37.7	10.9	4.6	1.3
GnRH-50	7/7	137	13	51.2	9.2	27.8	5.1	3.3	0.9
hCG-500	7/7	128	7	62.1	4.7	27.3	8.1	1.8	0.6
Experiment 3									
NaCl (control)	0/7	-	-	-	-	-	-	-	-
GnRH-50	20/20	135	8	65.1	5.0	48.1	5.1	24.4	5.3
hCG-500	20/20	125	5	73.0	4.8	49.1	5.5	26.5	4.7

* This group was excluded from statistical analysis as only one female were found to ovulate.

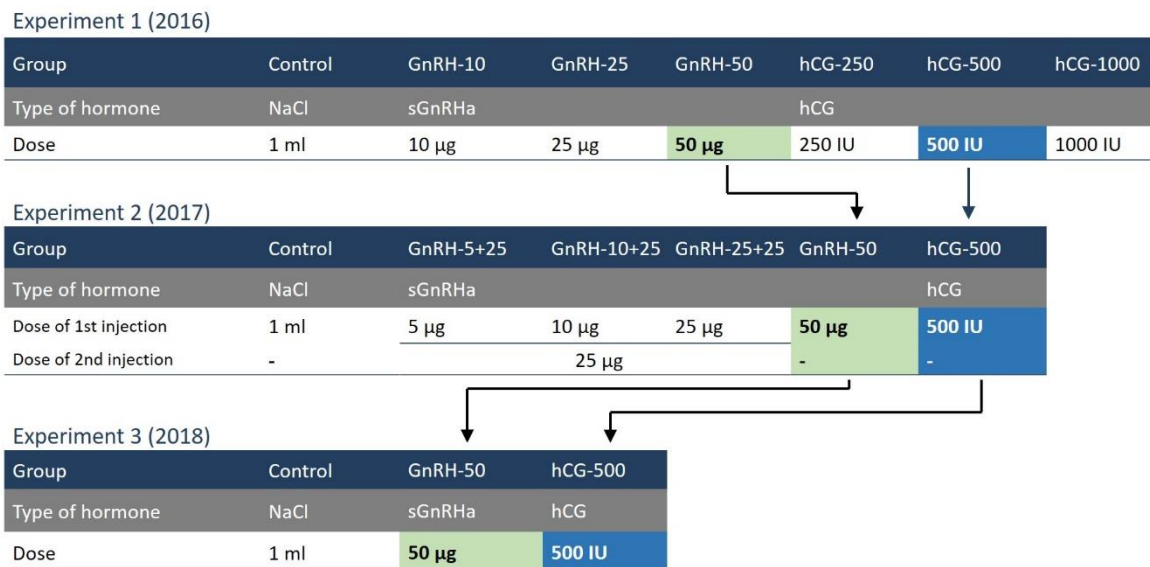


Fig. 1. Experimental design and definition of the groups in each experiment. Each spawning agent at a specified dose (NaCl, sGnRH α or hCG) per kg body weight was given. In Experiment 2, the interval between injections was 48 h. Each hormonal preparation was dissolved in sterile 0.9% NaCl solution, and the same solution was used for control group injections. The groups highlighted in green or blue were chosen as the ‘best’ from GnRH- and hCG-treated fish, respectively, and were thus used for subsequent experiments.

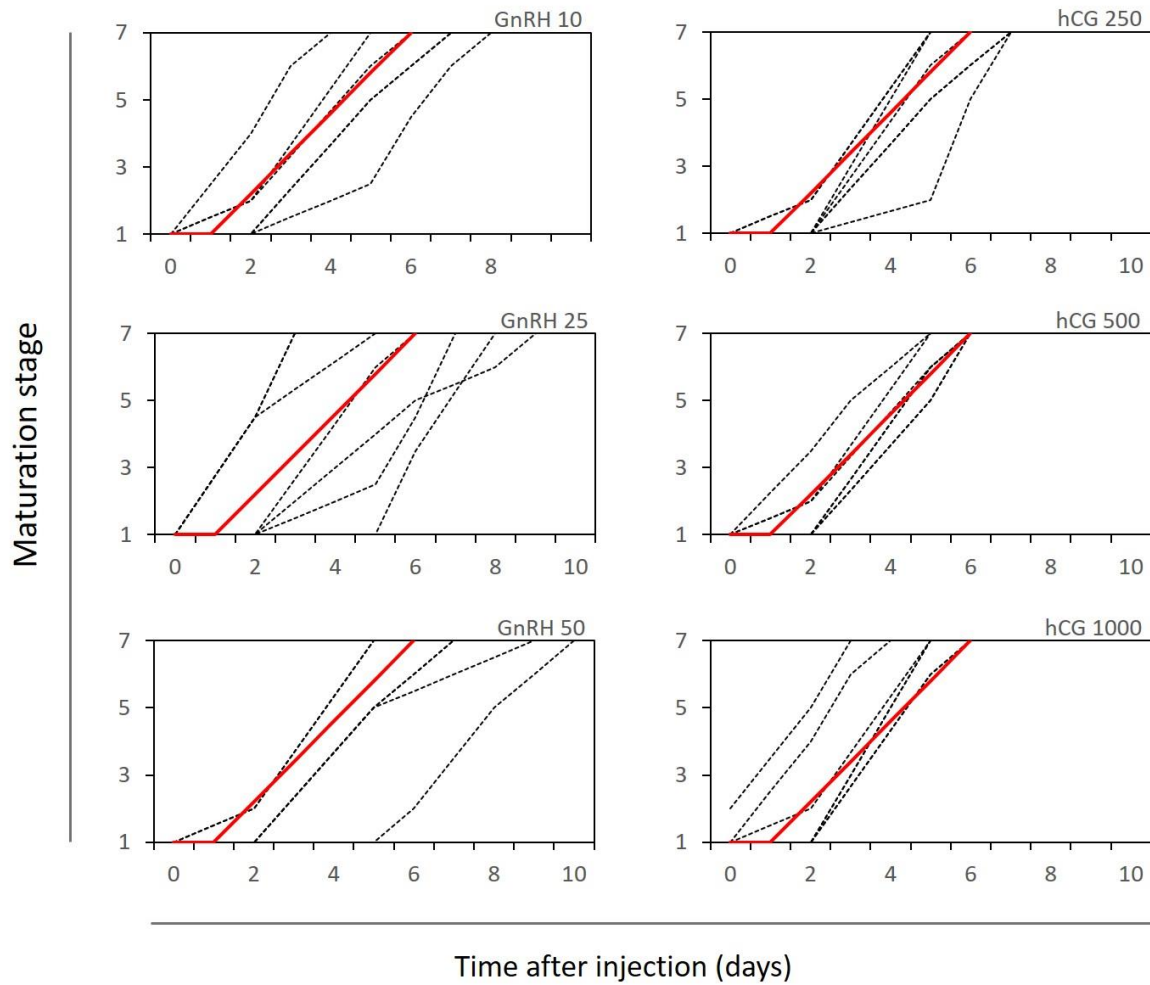


Fig. 2. Maturation kinetics of pikeperch females, based on classification from Źarski et al. (2012), after hormonal treatments (see Fig. 1 for details) recorded in Experiment 1 (see Materials and Methods for details). The red line represents an estimation ('model') of maturation kinetics in wild fish adopted from Źarski et al. (2012). The dashed black lines represent particular specimens in each group.

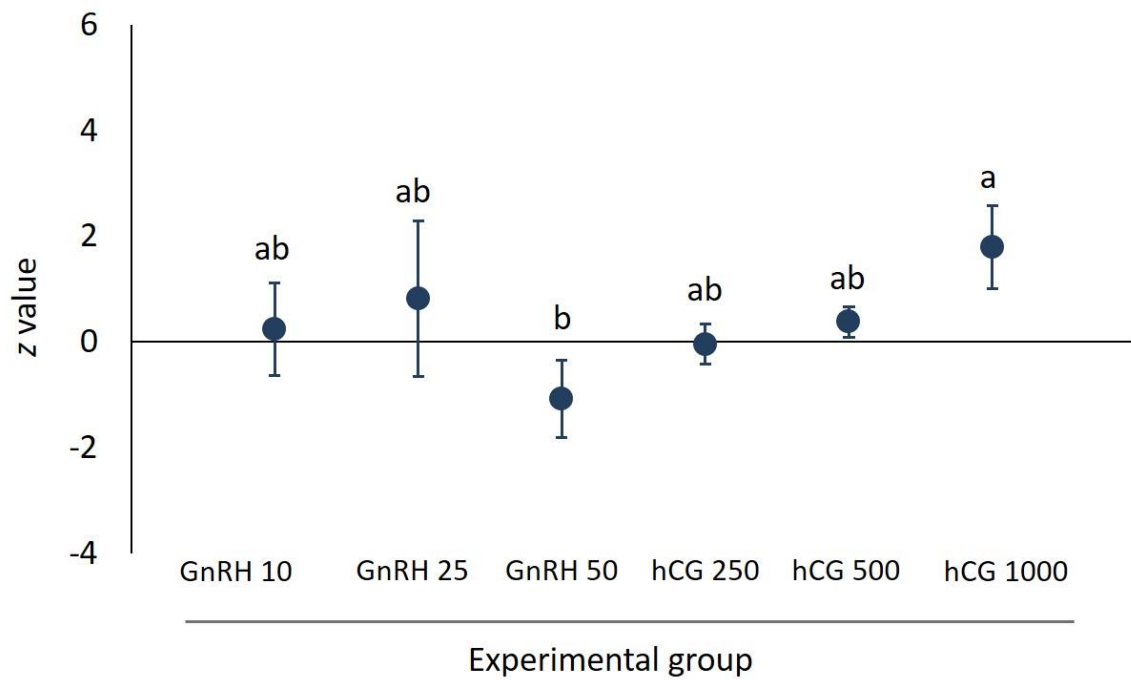


Fig. 3. Analysis of equality of maturation kinetics regression coefficients (based on regression curves calculated for each specimen) recorded in Experiment 1 (see Materials and Methods for details). z represents the statistical value that describes equality of these coefficients; 0 represents identical regression coefficients and thus identical regression curves. Further, $z = 0$: maturation kinetics are the same as the model kinetics; $z < 0$: maturation kinetics are lower than model; and $z > 0$: maturation kinetics are faster than model. The reference coefficient was calculated for wild fish according to Źarski et al. (2012). Data are presented as mean \pm SEM.

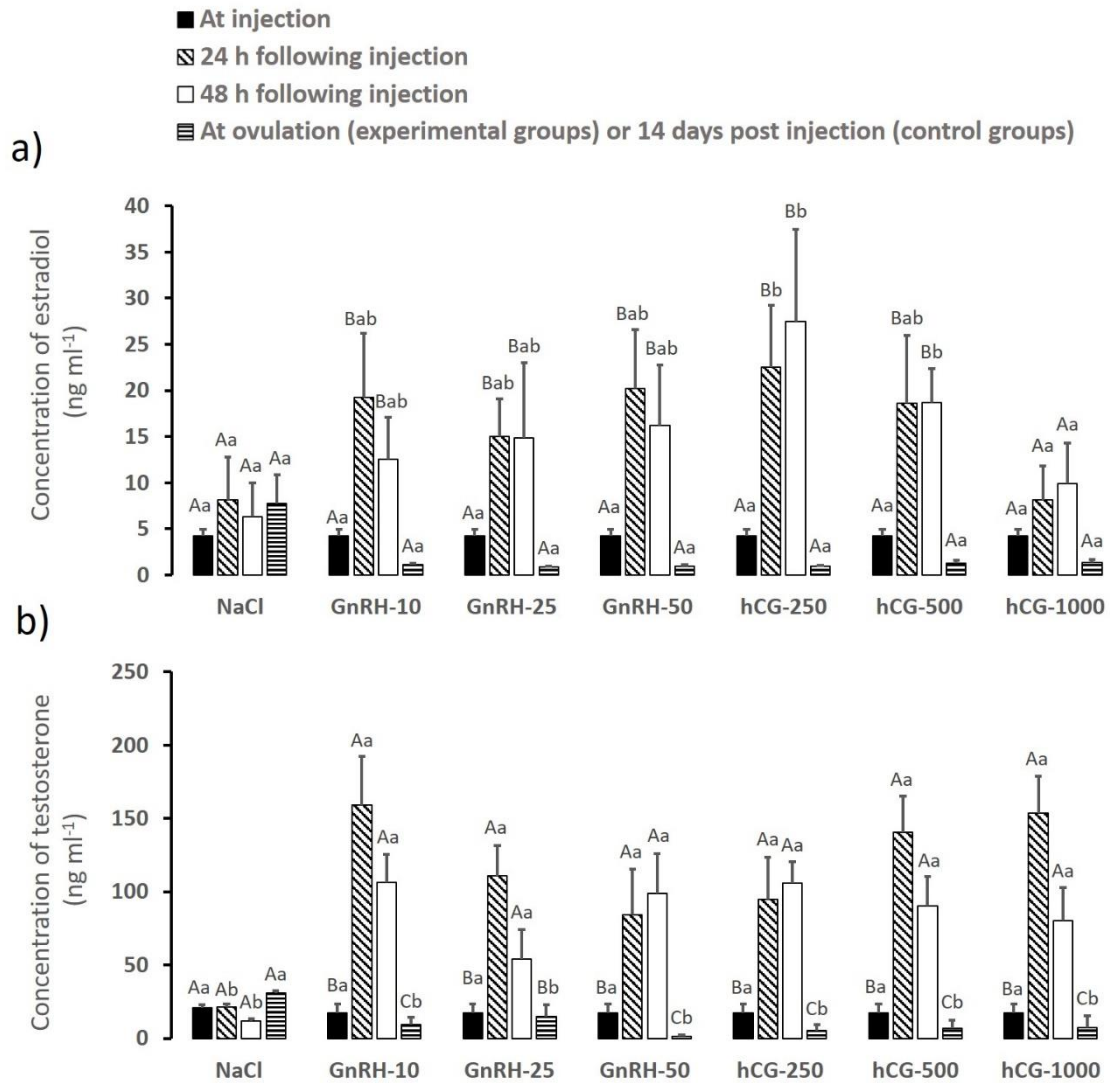


Fig. 4. The concentration of (a) oestradiol and (b) testosterone in female pikeperch plasma following hormonal treatments (see Fig. 1 for treatment details) at different times following injection recorded in Experiment 1 (see Materials and Methods for details). Data (mean \pm SEM) marked with different lower-case letters indicate statistical differences between the groups at the same sampling time. Data marked with different upper-case letters indicate statistical differences within the same group, but between different sampling times. Significance was set at $P < 0.05$.

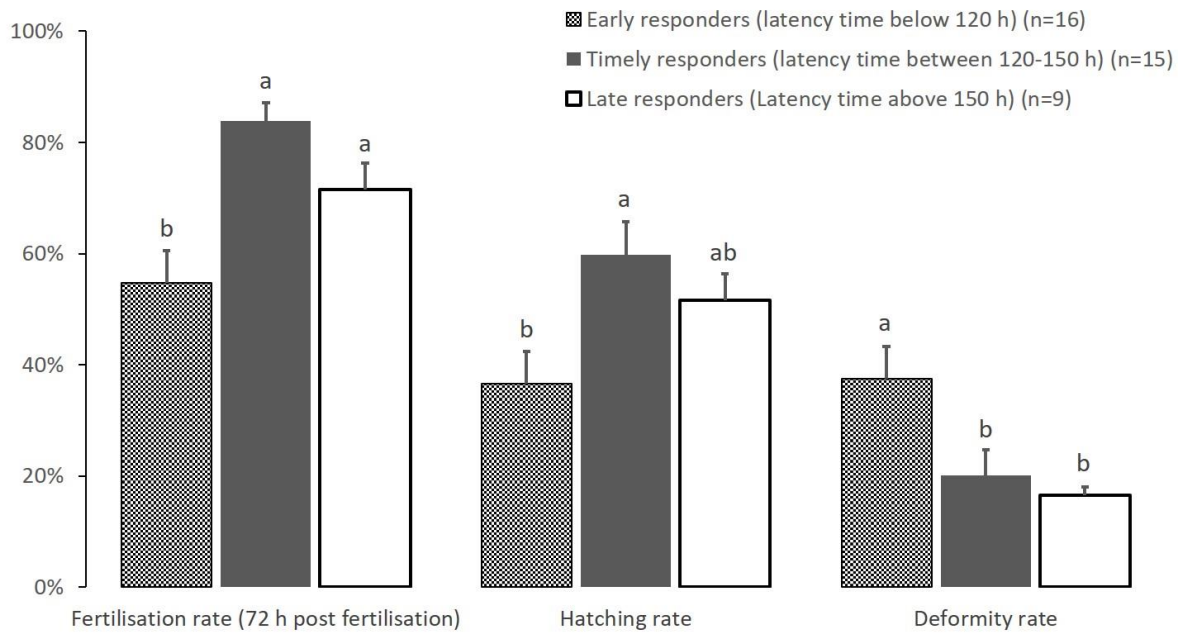


Fig. 5. Egg quality indicators recorded during the third year of the study (Experiment 3) with fish grouped into ‘early’, ‘timely’ or ‘late’ responders. Data (mean \pm SEM) marked with different letters among the respective parameters and between the groups differed significantly ($P < 0.05$).

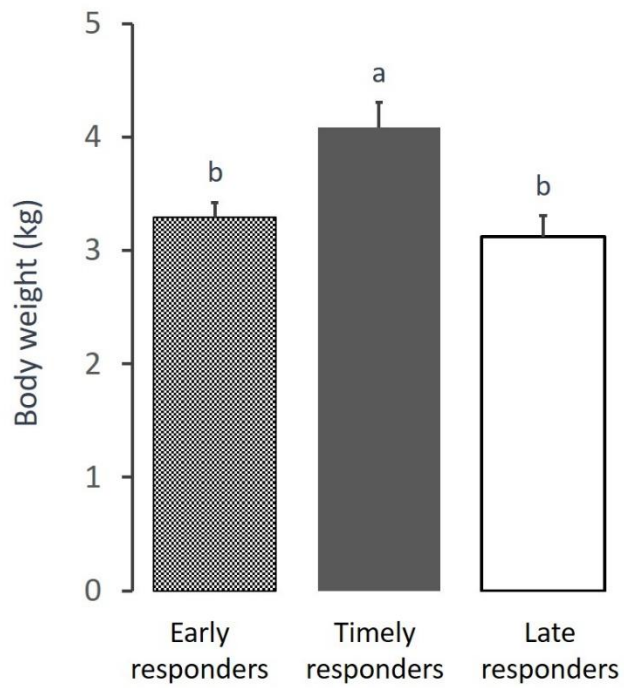


Fig. 6. Body weight (kg) of fish that were ‘early’, ‘timely’ or ‘late’ responders to hormonal treatment recorded in Experiment 3 (see Materials and Methods for details). Data (mean \pm SEM) marked with different letters were statistically different ($P < 0.05$).

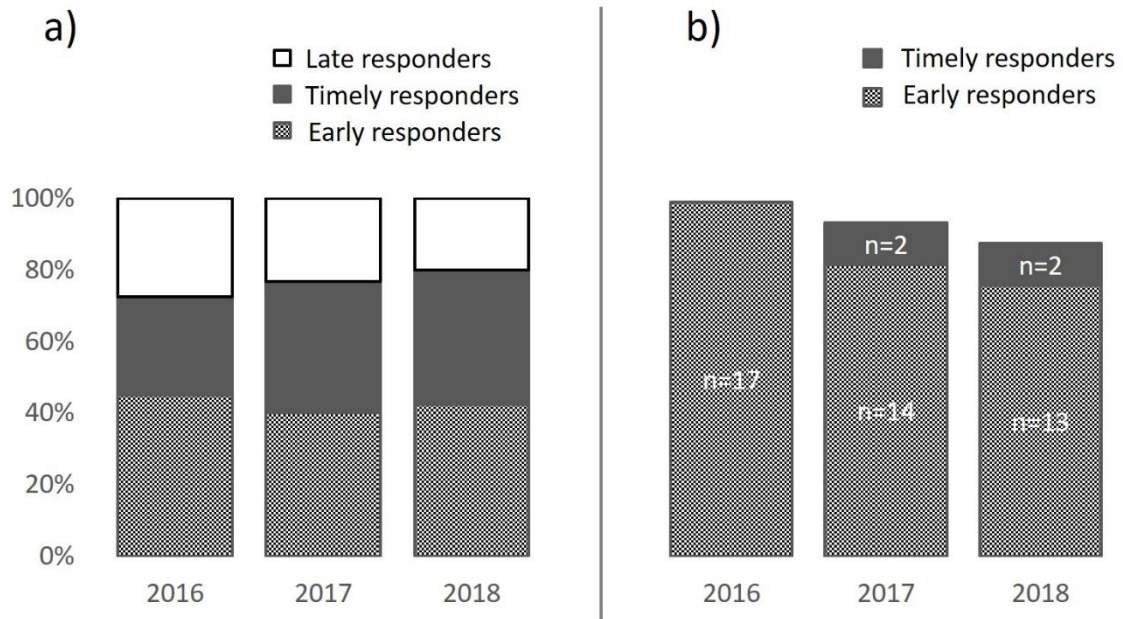


Fig. 7. (a) Percentage of fish recognised as ‘early responders’ (ovulation <120 h following injection), ‘timely responders’ (ovulation 120–150 h following injection) and ‘late responders’ (ovulation >150 h following injection) over the three-year study (n = 42, 35 and 40 fish in the first, second and third year of the study, respectively) and (b) history of fish initially recognised as ‘early responders’ over the course of entire three-year study.

Table S1. Correlation matrix, with Pearson's correlation coefficients provided, of different zootechnical parameters recorded during Experiment 3.

	(1)	(2)	(3)	(4)	(5)	(6)
(1) Latency time						
(2) PGSI	-0.13					
(3) Weight of eggs	-0.03	0.64** *				
(4) Body weight of females	0.05	-0.09	0.72** *			
(5) Fertilization rate at 72 h post fertilization	0.41* *	0.03	0.23	0.32 *		
(6) Hatching rate	0.38*	0.09	0.21	0.24	0.87** *	
(7) Deformity rate	- 0.40*	0.01	-0.07	-0.12	-0.57	- 0.62***

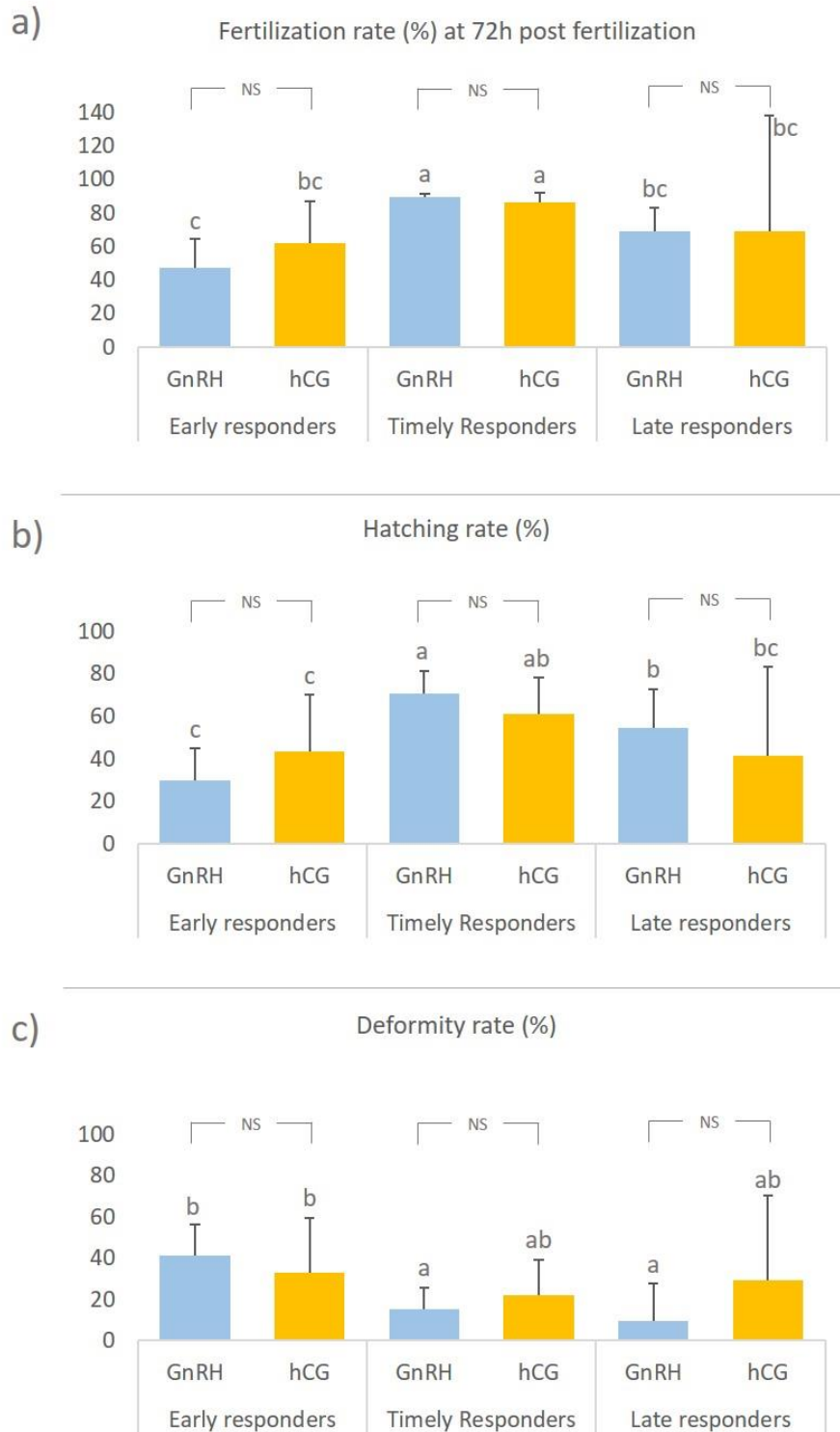


Fig. S1. Egg quality indicators (a – fertilisation rate; b – hatching rate; c – deformity rate) obtained during Experiment 3 (for details see Materials and Methods) following application of either hCG and GnRH in stimulation of ovulation in domesticated pikeperch females. Fish were grouped into ‘early’, ‘timely’ and ‘late’ responders (i.e. eggs were stripped <120 h, 120–150 h and >150 h following injection in early-, timely- and late-responding fish, respectively) and later sub-grouped according to different hormone applied. Data (mean \pm SD) marked with different letters indicate statistical differences ($P < 0.05$) between different sub-groups. No significant differences (NS) were recorded between different hormones among respective responsiveness groups (i.e. early, timely and late responders) ($P > 0.05$).