



PROOF

Post-Ovulatory Oocyte Ageing in Pikeperch (*Sander lucioperca* L.) and its Effect on Egg Viability Rates and the Occurrence of Larval Malformations and Ploidy Anomalies

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Abstract

The effect of post-ovulatory oocyte ageing on egg quality was assessed in pikeperch *Sander lucioperca* to identify the reliable and the best post-ovulatory stripping time. Ovulated eggs were retained in the ovarian cavity for 0-3, 3-9, 6-12 and 12-18 hours post-ovulation (HPO) prior to fertilization. Fertilization, hatching and embryo mortality rates as well as the occurrence of larval malformations and ploidy anomalies were considered as indices for egg quality. The results indicated that the fertilization and hatching rates remained nearly constant, at approximately 80%, for the eggs retained in the ovarian cavity between 0-18 hours after ovulation. Post-ovulatory oocyte ageing did not affect the embryo mortality or the occurrence of larval malformations at least during the 12-hour experimental period. However, the incidence of triploidization in the larvae increased over time from 1.6% at 0-3 HPO to 5% in more aged oocytes at 6-18 HPO. Egg quality retention for at least 12 hours after ovulation appears to be helpful for pikeperch breeding programs by providing synchronous artificial egg insemination and thereby easing hatchery management.

Keywords: Pikeperch, ovulation, oocyte ageing, egg quality.

Introduction

In fish, matured eggs are released from follicle cells into the ovarian or body cavity during ovulation while they are in metaphase of the second meiotic division stage (Bobe and Labbe, 2010) and remain there until the stimulation of spawning by environmental stimulates or hand-stripping. Under farming conditions, environmental and social stimuli are absent (Aegerter and Jalabert, 2004) and stressors provide conditions that can inhibit reproduction (Schreck, 2010). Thus, oocytes usually remain in the body cavity until they are manually stripped by fish farmers (Aegerter and Jalabert, 2004). Delayed spawning in nature, delayed stripping in capture and even delayed fertilization after egg stripping can result in excessive oocyte ageing and finally over-ripening phenomenon. In the artificial propagation of many cultured fishes accordingly, the females are examined for ovulation time to time to avoid the ageing of ovulated eggs. Oocyte ageing has been reported to be the most important factor affecting egg quality of several fish species after ovulation (e.g., McEvoy, 1984; Rime *et al.*, 2004; Policar *et al.*, 2010). Through over-ripening, major morphological,

physiological, biochemical, histological, cellular and molecular changes occur inside the eggs and ovarian fluid that negatively affect the egg fertilizing ability and larval developmental stages (e.g., Nomura *et al.*, 1974; Craik and Harvey, 1984; Formacion *et al.*, 1993; Lahnsteiner, 2000, Aegerter *et al.*, 2005). The time period during which the eggs remain viable after ovulation differs from species to species and is largely dependent on temperature (e.g., Piper *et al.* 1982; Espinach *et al.* 1984; Harvey and Kelly 1984; Formacion *et al.* 1993; Legendre *et al.* 2000; Rizzo *et al.* 2003; Samarin *et al.* 2008; Bahrekazemi *et al.* 2010).

Pikeperch *Sander lucioperca* has recently been considered to be involved in European aquaculture with the purpose of diversification in freshwater aquaculture (Zarski *et al.*, 2012a). In the artificial reproduction of pikeperch, brood females are checked for their oocyte maturation stage after anaesthetization. This is done by sampling of eggs using a catheter and clearing them into a solution (e.g. Serra's solution). Then fish are divided into the separate groups depending on their egg maturity stage. Females with the eggs at stages III to V are induced for ovulation using different hormones. The time interval between hormone injection and the

occurrence of ovulation varies between a few hours to 4.5 days depending on the egg maturational stage, water temperature, hormone type and its dosage (Kucharczyk *et al.*, 2007). One of the most important problems in the artificial breeding of this fish species is that the females may release the eggs after ovulation and therefore the eggs cannot be easily obtained by stripping for artificial insemination. Indeed, egg deposits in the tank are very frequent in artificial spawning (Zarski *et al.*, 2012b). On the other hand, in most pikeperch hatcheries, it is believed that the eggs will be over-ripened and lose their fertilizing ability soon after ovulation. Thus, in the artificial propagation of this fish species, brood fish are examined for ovulation using short time intervals. This provides stress for the fish and is also a time-, labour- and cost-intensive process. Successful *in vivo* retention of eggs using a suture on the female genital papilla not only provides the synchronous fertilisation of brood fishes but is also worthwhile when a completely mature male brood fish is unavailable for fertilisation. A better understanding and refinement of such egg retention methods is strongly desirable to maximize the efficacy of mass production of this fish species. The present study was performed to identify the reliable and the best post-ovulatory stripping time in pikeperch when females were retained at 15 °C. The effect of delayed spawning caused by suturing female genital papilla on fertilization, hatching and embryo mortality rates as well as the occurrence of larval malformations and ploidy anomalies were assessed for the eggs fertilized between 0-18 hours after ovulation.

Materials and Methods

Fish

Seven pairs of pikeperch brood fish, females weighing 1543 ± 225 g (mean \pm SEM) and males weighing 1392 ± 184 g, were selected for the experiment. The fish were captured from the pond near Nove Hradky village and then transferred to the South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic, from April-May 2013. Pond-cultured brood fish adapted to the controlled conditions were then transferred to indoor holding tanks (600 L capacity) supplied with water from a recirculating system. The storage temperature was gradually increased to 15 °C, which is the required temperature for pikeperch spawning.

Egg Retention in the Ovarian Cavity and Spawning

After one week of adaptation, non-ovulated females that were expected to ovulate in a few days (regarding to the soft and swollen belly) as well as males were treated with a single muscular injection of

Human Chorionic Gonadotropin HCG (Chorulon preparation) (500 IU/kg). Subsequently, the genital papilla of females were sutured. Each pair of injected fish was then placed into a separate tank, which was previously covered at the bottom with an artificial grass-made spawning nest and kept at 15 ± 0.5 °C until spawning. Sperm motility was assessed before introducing the males into the spawning tanks. Females were examined for ovulation every 3 hours starting at 24 hours after injection. The state of ripeness was judged by gentle palpation of the abdomen without cutting the suture but only loosening it a little. Fish in which high number of eggs could be easily removed by applying gentle pressure on the abdomen were considered to have already ovulated. The fish were not fed during the entire experiment. For injection and to examine whether ovulation occurred, the fish were anaesthetized with a 0.03 ml/L clove oil water bath to minimize stress and to make them easier to handle (Kristan *et al.*, 2012). When ovulation occurred, the eggs were retained inside the fish body for 0-3, 3-9, 6-12 and 12-18 hours post-ovulation (HPO) prior to fertilization by adjusting the time of suture cutting. Unexpectedly, some of the fishes didn't release the eggs immediately after removing the sutures. However, the exact time during which eggs were retained inside the fish body between ovulation and spawning was calculated for each fish by checking the nests. The occurrence of spawning was determined by observing the nests every 3 hours after ovulation.

Incubation and Examination of Egg Developmental Success

After the detection of spawning and its confirmation by egg sampling, 3 batches of spawned eggs were collected from each tank by cutting small parts of the substrate and placed into separate small rectangular-shaped incubators (0.25 L capacity) at 15 °C until determining the considered egg quality parameters. The number of eggs which were placed into each incubator was counted while transferring them into the incubators. During this period, the water in the incubators was changed every 6 hours. The ratios of the number of live embryos and hatched larvae to the number of initially fertilized eggs (fertilization and hatching rates, respectively) were used as indices of egg viability. The Fertilization rate was determined the day after spawning because after this time, it was easily possible to distinguish between the live and dead embryos. For this purpose, a stereo microscope was used. The hatching percentage was examined by counting the number of hatched larvae 7 days after fertilization. The embryo mortality rate was also used as an index of egg quality. The latter was measured as the percentage of embryos died between the measurement of fertilization rate and hatching stage in the total number of fertilized eggs. Malformed larvae (spinal cord torsion, yolk sac, eye

deformations, etc.) were quantified using a stereomicroscope to determine the incidence of malformation rates.

Measurement of Ploidy Levels

The ploidy level of each larvae specimen was verified as the relative DNA content using flow cytometry (Table 1) (Partec CyFlow Cube 8, Partec GmbH, Münster, Germany). The larvae were randomly sampled and processed according to the protocol of Lecommandeur *et al.* (1994) using a CyStain DNA 2-Step Kit (Partec GmbH, Germany) containing 4',6-diamidino-2-phenylindol (DAPI; excitation/emission maximum 358/461 nm) for nuclear DNA staining (Otto, 1994). Larvae from the control group (i.e., from eggs fertilized at 0-3 HPO) were used as a diploid standard. The samples were analysed individually with a flow rate of $0.4 \mu\text{l.s}^{-1}$.

Statistical Analysis

The normality of the data was ascertained using SPSS software version 18. Differences between the means of the groups for each measured parameter were evaluated using ANOVA, followed by Duncan's multiple range test. $P < 0.05$ was considered to be

significant.

Results

The fertilization and hatching rates for the eggs spawned and fertilized immediately after ovulation (0–3 HPO) were $85.9 \pm 2.2\%$ and $79.6 \pm 2.1\%$ (mean \pm SEM), respectively. The values remained nearly constant, at approximately 80%, for the eggs retained in the ovarian cavity between 0-18 hours after ovulation (Figure 1).

Post-ovulatory oocyte ageing did not significantly affect the embryo mortality as well as the occurrence of the larval malformations during the 18-hour experimental period (Figure 2). The lowest embryo mortality ($1.7 \pm 1\%$) was observed for the eggs spawned and fertilized at 3-9 hours after ovulation, while no larval malformation and ploidy anomalies were detected in this group. Most of the measured larvae specimens from different HPOs were diploids (channel number or centre of the distribution graph: around 50) (Table 1). However, the incidence of ploidy anomalies with potentially triploidization (channel number: around 75) in the larvae increased over time from 1.6% at 0-3 HPO to 5% in more aged oocytes at 6-18 HPO.

Table 1. Hours post-ovulation, number of specimens, their relative DNA contents (the mean channel number) and coefficients of variation, c.v. (%), verified using a flow cytometer

HPO	No. of fish analysed, Ploidy level	Channel no.	c.v. (%)
0-3	59 (2n)1 (3n)	48.4 ± 3.4^a 78.5	$8.6 \pm 5.63.5$
3-9	39 (2n)0 (3n)	47.5 ± 3.4^a	8.3 ± 6.1
6-12	19 (2n)1 (3n)	46.2 ± 3.3^b 67.6	6.2 ± 1.64
12-18	19 (2n)1 (3n)	46.7 ± 1.2^b 70.5	3.9 ± 23.9

Approximately 4000 cells were analysed for each measurement. Data with the same alphabetic superscript are not significantly different.

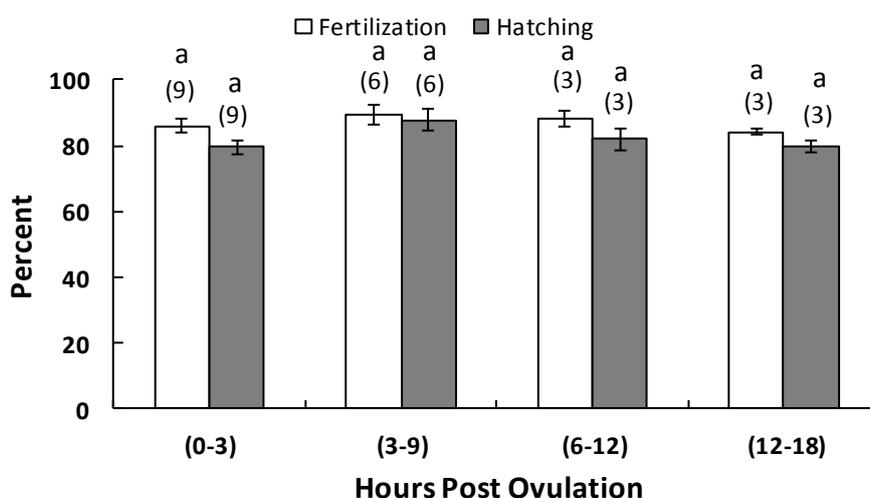


Figure 1. Effects of post-ovulatory oocyte ageing (Hours Post Ovulation-HPO) on the fertilization and hatching rates, which are shown as the mean \pm SEM. The number of batches for which the data were measured is shown in parentheses. For each measured parameter, the means sharing a common alphabetical symbol do not significantly differ.

Discussion

In this study, the egg viability rates did not show any marked reduction during the experimental period of 0-18 HPO. The time period during which the eggs remain viable inside the fish body after ovulation, which guarantees egg fertility, has been reported for a variety of species (Table 2). The results of the present study indicated that the *in vivo* retention of pikeperch eggs for at least 12 HPO does not have any adverse effect on the egg viability rates. Thus, to identify the time interval between ovulation and loss of egg quality in pikeperch, the egg retention needs to be studied for a longer time period after ovulation.

Although do not differ significantly the highest egg viability rates and the lowest embryo mortality, larval malformation and ploidy anomalies were observed for the eggs fertilized 3-9 hours after ovulation. Such an initial increase in the egg viability rates after ovulation has been documented in some studies (Sakai *et al.*, 1975; Bry, 1981; Springate *et al.*, 1984; Mylonas *et al.*, 1992; Linhart and Billard, 1995; Aegerter and Jalabert, 2004; Samarin *et al.* 2008; Rasines *et al.*, 2012), while it has not been found in other reports (e.g., Espinach *et al.*, 1984; Lahnsteiner, 2000; Rizzo *et al.*, 2003; Samarin *et al.*, 2011). We found the same trend in common carp (*Cyprinus carpio*) and pike (*Esox lucius*) post-ovulatory oocytes

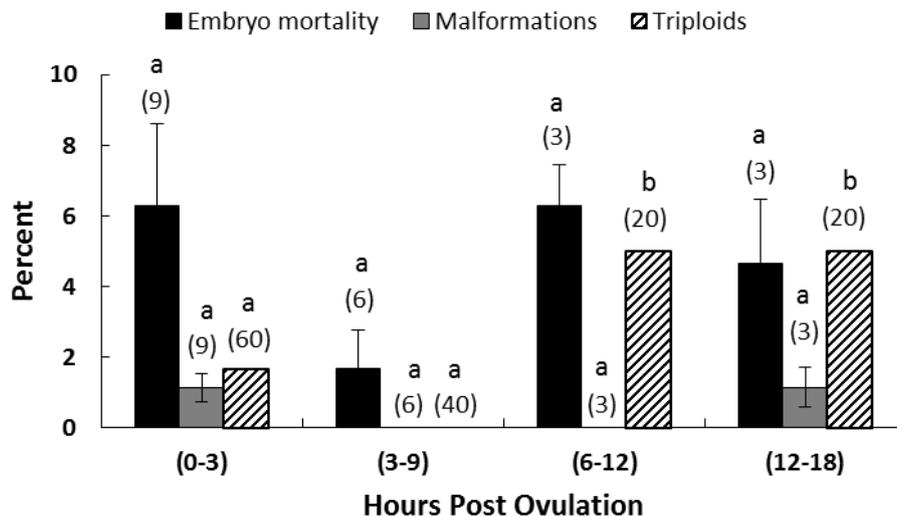


Figure 2. Effects of post-ovulatory oocyte ageing (Hours Post Ovulation-HPO) on embryo mortality, larval malformation and triploid percentages, which are shown as the mean \pm SEM. The number of batches for which the data were measured is shown in parentheses. For each measured parameter, means sharing a common alphabetical symbol do not significantly differ.

Table 2. Successful egg *in vivo* storage time in different fish species

Species	Successful egg <i>in vivo</i> storage time	Temperature (°C)	Reference
Striped bass (<i>Morone saxatilis</i>)	30 minutes	-	Piper <i>et al.</i> 1982
Tilapia (<i>Sarotherodon mossambicus</i>)	1.5 hours	18–20	Harvey and Kelley 1984
Asian catfish (<i>Pangasius hypophthalmus</i>)	2 hours	28–29	Legendre <i>et al.</i> 2000
Curimata (<i>Prochilodus marggravii</i>)	2 hours	18 and 26	Rizzo <i>et al.</i> 2003
Senegalese sole (<i>Solea senegalensis</i>)	3 hours	16	Rasines <i>et al.</i> , 2012
South American catfish (<i>Rhamdia sapo</i>)	9 hours	20	Espinach <i>et al.</i> 1984
	5 hours	24	Formacion <i>et al.</i> 1993
Goldfish (<i>Carassius auratus</i>)	12 hours	-	Samarin <i>et al.</i> , 2011a
Kutum (<i>Rutilus frisii kutum</i>)	72-96 hours	-	
	60-72 hours	11	Sakai <i>et al.</i> 1975; Bry
Rainbow trout (<i>Oncorhynchus mykiss</i>)	5-15 days	14	1981; Springate <i>et al.</i> 1984;
		8-17	Lahnsteiner 2000; Azuma
			<i>et al.</i> 2003; Bonnet <i>et al.</i>
			2003; Aegerter and Jalabert
			2004; Samarin <i>et al.</i> 200
Caspian brown trout (<i>Salmo trutta caspius</i>)	30-40 days		Bahrekazemi <i>et al.</i> 2010

as well (unpublished data). The lack of the initial increasing trend in these studies might be attributed to a masking effect due to the relatively longer time intervals between successive strippings used. It is likely that this initial increasing trend can be observed only when the time intervals between successive strippings are sufficiently short regarding to the fish species. A slight asynchrony between the processes of meiotic maturation and ovulation has been reported to be the most likely reason for this trend (Mylonas *et al.*, 1992). The reason for observing this increasing trend in egg quality after ovulation is interesting for future studies.

Samarin *et al.* (2011) found an increase in embryo mortality rates with oocyte ageing in kutum, and concluded that the mortality of embryos can be indicative of over-ripening progression caused by the storage period. Oocyte ageing has also been reported to cause several malformations in the larvae of different fish species (Table 3). Increasing eyed-egg mortality and malformation rates can be interpreted as post-ovulatory oocyte ageing is accompanied by biochemical changes inside the eggs (Craik and Harvey, 1984; Lahnsteiner, 2000) as well as by the leakage of a number of oocyte components, such as protein fragments, into the ovarian fluid (Rime *et al.*, 2004). These components are most likely required not only for survival to the hatching stage but also for the development of the embryo into normal larvae, as the yolk nutritionally supports the embryo. However, in the present study, we did not find any changes in the embryo mortality rates as well as in larval malformations during 0-18 HPO. This might be attributed to the time duration of the experiment, i.e., longer time intervals between ovulation and spawning might result in increases in these values, as any significant decrease in egg viability rates was also not detected during the experimental period.

The incidence of ploidy anomalies larvae increased with elapsed time after ovulation, from 1.6% at 0-3 HPO to 5% at 6-18 HPO. Reports in European catfish (Varkonyi *et al.*, 1998), salmonids (Yamazaki *et al.*, 1989; Aegerter and Jalabert, 2004; Aegerter *et al.*, 2005) and tench (*Tinca tinca*) (Flajshans *et al.*, 2007) also indicated increases in the occurrence of ploidy anomaly larvae in more aged oocytes. In European catfish, 3-20% of the eggs fertilized 6 hours post-ovulation produced larvae with

errors in chromosome distribution during fertilization such as aneuploidy, triploidy and tetraploidy, while no chromosomal abnormality was found in the larvae fertilized immediately after ovulation (Varkonyi *et al.*, 1998). The incidence of triploid larvae increased with the post-ovulatory ageing time in rainbow trout with higher frequency and earlier at 17 °C compared to 12 °C (Aegerter and Jalabert, 2004). In tench, the incidence of triploid larvae significantly increased after 5 hours of *in vitro* storage at 24 °C, as well as after 3 hours of *in vitro* storage at 21.9 and 17 °C, while during *in vivo* storage, a significant triploid larval yield appeared after 5 hours of storage at 21.9 °C only (Flajshans *et al.*, 2007). Our recent experiments indicated that the rate of larval triploidization increased with oocyte ageing in pike, while all measured larvae from different HPOs were diploids in common carp (unpublished data). Changes in the oocyte cytoskeletal organization during ageing have been reported to be associated with a failure of second polar body extrusion and finally the occurrence of ploidy anomalies in larvae (Aegerter and Jalabert, 2004; Flajshans *et al.*, 2007). Complete loss of egg viability rates in this study would be more clearly accompanied by increasing ploidy anomalies. Since the malformed larvae may include a number of ploidy anomalies (Varkonyi *et al.*, 1998; Aegerter and Jalabert, 2004), measurement of the larval ploidy levels should be performed during the initial days of hatching while malformed larvae are still alive, as performed in the present study.

Conclusions

Based on the results obtained in this study, unfertilized eggs of pikeperch could retain their viability up to 80% at least 12 hours after ovulation when they are stored in the parental fish body at 15 °C. Thus, the reliable time interval between successive fish examined for ovulation can be considered for up to 12 hours. The long egg quality retention time appears to be helpful for pikeperch breeding programs by providing synchronous artificial egg insemination using a suture on the female genital papilla and eventually easing hatchery management.

Table 3. Larval malformation rates caused by *in vivo* egg storage in different fish species

Species	Malformation%	Storage time (<i>in vivo</i>)	Temperature (°C)	Reference
African catfish (<i>Heterobranchus longifil</i>)	20	2 hours	30	Legendre and Oteme, 1995
European catfish (<i>Silurus glanis</i>)	50	6 hours	22-24	Linhart and Billard, 1995
Asian catfish (<i>Pangasius Hypophthalmu</i>)	100	5 hours	28	Legendre <i>et al.</i> 2000
Curimata (<i>Prochilodus marggravii</i>)	60	2 hours	26	Rizzo <i>et al.</i> , 2003
Rainbow trout (<i>Oncorhynchus mykiss</i>)	50	16 days	12	Bonnet <i>et al.</i> , 2007

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