Aquaculture 450 (2016) 431–438

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Post-ovulatory and post-stripping oocyte ageing in northern pike, *Esox lucius* (Linnaeus, 1758), and its effect on egg viability rates and the occurrence of larval malformations and ploidy anomalies

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ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form 10 July 2015
Accepted 12 August 2015
Available online 15 August 2015

Keywords:
Egg quality
Esox lucius
Oocyte ageing
Over-ripening
Ploidy anomalies

ABSTRACT

To identify the effects of oocyte ageing on egg quality in northern pike, *Esox lucius*, two experiments were performed as follows: in experiment I, partial volumes of the eggs from 7 pike females were stripped and fertilised with 2-day intervals for 8 days post-ovulation (DPO). In experiment II, the stripped eggs of 3 females were stored in ovarian fluid at 10 °C and fertilised separately with 12-hour intervals for 96 hours post-stripping (HPS). In both experiments, the eggs from each batch were fertilised with mixed milt obtained from 5 males. Eyeing, hatching and eyed-egg mortality rates as well as the occurrence of larval malformations and ploidy anomalies were considered as indices for egg quality. The results of experiment I indicated that the best and most reliable post-ovulatory stripping times in pike were at 2 DPO and up to 4 DPO, respectively, when females were retained at 10 °C. Over-ripening of the eggs occurred from 8 days after ovulation. The eyed-egg mortality and larval malformation rates did not show any significant increase up to 6 DPO, but then increased significantly and reached 36% and 43% at 8 DPO, respectively. The incidence of triploid larvae increased over time, from 0% at 0 and 2 DPO to 11% and 14% in more aged oocytes at 4 and 6 DPO, respectively. The second experiment clarified that the best post-stripping fertilisation time in pike was at 0 HPS. However, stored eggs in ovarian fluid could retain almost 50% of their initial fertilising ability for 24 h after stripping. Complete loss of egg viability occurred after 96 HPS, and the highest eyed-egg mortality rate (27%) and larval malformation rate (33%) were detected in this group. The obtained results suggest that for commercial artificial reproduction procedures in northern pike, the time interval between successive fish examinations for ovulation should not exceed 4 days. In addition, the eggs[1] stripped from the female should be fertilised immediately following collection to secure the highest possible fertilisation rate.

Statement of relevance: Knowledge of in vivo and in vitro storage of fish eggs can improve hatchery techniques as well as better broodstock management, which are the most important factors affecting synchronization of the artificial fertilisation and consequently the effectiveness of the entire operation. The duration of egg viability after ovulation and stripping has not yet been determined for the pike. A better understanding and refinement of egg storage methods are strongly desirable to maximize the efficacy of mass production in pike hatcheries.

1. Introduction

Northern pike, *Esox lucius* L., is one of the most valuable freshwater fish species. As a top predator in inland waterbodies, it is ecologically one of the most precious teleost species that regulates populations of omnivorous finfishes (Craig, 2008; Zarski et al., 2013). Additionally, northern pike is one of the most valuable commercial fish species for aquaculture (Kucska et al., 2007) and recreational fishing (Milardi et al., 2014). These activities create the risk of significant overexploitation of this species, which creates the necessity of artificial reproduction to support aquaculture facilities as well as restocking programs. In the artificial reproduction of northern pike a common practice is to catch the females from the wild or earthen ponds just before or after ovulation and strip the eggs for in vitro fertilisation. However, more often the injection of brood fish with the homogenate of acetone-dried carp pituitary is applied which provides more predictable time for egg ovulation,

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[1] DPO: days post-ovulation. HPS: hours post-stripping.

http://dx.doi.org/10.1016/j.aquaculture.2015.08.017
0044-8486/

often on the 4th day after the injection (Szabo, 2003). The larviculture of this species is still a real bottleneck toward commercial intensive production due to the high cannibalistic behaviour starting on the 5th–7th days of rearing and may last until the late juvenile stage (Giles et al., 1986; Kucharczyk et al., 1998; Mamcarz et al., 1998). That is why, the aquaculture commercial production is mainly limited to the production of larvae which are starting feed exogenously and restock them to the open water bodies or to the earthen ponds for extensive production. Considering the fact that such a production technology is characterized by a low efficiency more intensive research on hatchery techniques is needed, including research on controlled reproduction and fertilisation protocols. At the moment, however, knowledge of hatchery protocols in northern pike is very limited.

Egg quality is influenced by a significant number of external factors and broodstock management practices (Bobe and Labbe, 2010). Oocyte ageing has been reported to be one of the most important factors affecting egg quality of several fish species (e.g., McEvoy, 1984; Rime et al., 2004). Ageing of oocytes refers to the time period between ovulation and fertilisation. During this time period, eggs are retained inside the fish body (in vivo storage of eggs) or are kept outside it (in vitro storage of eggs). In fish, matured eggs are released from follicle cells into the ovarian or body cavity during ovulation and remain there until the stimulation of spawning by environmental stimulates or hand stripping. At the moment of ovulation, eggs are at metaphase of the second meiotic division stage (Bobe and Labbe, 2010). During the post-ovulatory period, eggs are bathed in a fluid known as ovarian fluid. Delayed spawning in nature, delayed stripping in captivity and even delayed fertilisation after egg stripping can lead to excessive oocyte ageing and, eventually, an over-ripening phenomenon. During oocyte ageing and over-ripening, some morphological, physiological, biochemical, histological, cellular and molecular changes occur inside the eggs and the ovarian fluid (e.g., Aegerter et al., 2005; Craik and Harvey, 1984; Lahnsteiner, 2000; Nomura et al., 1974). These changes negatively affect egg fertilising ability and larval developmental success (e.g., Formacion et al., 1993; Gisbert and Williot, 2002; Lahnsteiner, 2000; Lahnsteiner et al., 2001). The duration of successful egg storage has been reported to be temperature- and species-dependent and varies between a few minutes to a few weeks in vivo (e.g., Legendre et al., 2000; Piper et al., 1982; Samarin et al., 2008, 2011a) and in vitro (e.g., Niksirat et al., 2007a,b; Rizzo et al., 2003; Samarin et al., 2011b). Currently, there are no data on the period of time during which ovulated eggs (during storage in vivo or in vitro) retain their fertilising ability in northern pike.

The number of possible methods for storing eggs following stripping is relatively limited. There are difficulties associated with the unsuccessful cryopreservation of fish eggs, including unsuccessful removal of intercellular water during cooling and toxicity of cryoprotectants (e.g., Guan et al., 2008; Lubzens et al., 2005; Rana, 1995; Stoss and Donaldson, 1983). Therefore, protocols for the short-term storage of eggs have been developed. These protocols are especially important in species such as northern pike because the reproductive season is quite long (up to 5 weeks) and usually few females are spawned simultaneously. It is a common practice for females to be examined for ovulation periodically and after the fish is ovulated, the eggs are stripped and an in vitro fertilisation protocol is applied. Such a practice is very stressful for the fish and makes the entire operation a time-, labour- and cost-intensive process. Moreover, such a practice often causes high mortality among the spawners and decreased egg quality. Another problem in pike hatcheries is that only a limited amount of milt can be stripped from males (Hulak et al., 2008; Lahnsteiner et al., 1998). Thus, knowledge of in vivo and in vitro storage of eggs can improve hatchery techniques as well as better broodstock management, which are the most important factors affecting synchronization of the artificial fertilisation and consequently the effectiveness of the entire operation.

It should be emphasized that the duration of egg viability after ovulation and stripping has not yet been determined for the pike. A better understanding and refinement of egg storage methods are strongly desirable to maximize the efficacy of mass production in pike hatcheries. The present study was conducted to identify the period of time during which eggs retain the highest fertilisability following ovulation, during in vivo and in vitro storage.

2. Materials and methods

2.1. Fish

In March 2014, 20 male and 20 female northern pike brood fish were captured from an earthen pond (when the average daily temperature of water reached 5–7 °C) near the Nove Hrady region (Nove Hrady Fish Farm, Ltd.) and then transferred to the South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. The fish were then transferred to indoor holding tanks (600 l capacity) supplied with water from a recirculating aquaculture system. Photoperiod was adjusted at 14L:10D and the storage temperature was gradually increased to 10 °C and was fixed for the whole experimental period. After 2–3 weeks of acclimation, females were checked for their oocyte maturation before the injection. To determine the oocyte maturational stage, a few eggs were sampled from each female using a plastic catheter via the genital pore (Szabo, 2003). Then the position of the germinal vesicle (GV) was observed under the stereomicroscope (Nikon SMZ745T, Japan) after clearing them with Serra solution (60 ml of 96% ethanol, 30 ml of 40% formaldehyde and 10 ml of glacial acetic acid in the proportion of 6:3:1 v/v). Males and non-ovulated females that were expected to ovulate in the near future regarding to their oocyte GV position, soft and swollen belly were treated with a single muscular injection of common carp pituitary homogenate (CPH; 3 mg kg^{-1} body weight) (Szabo, 2003). CPH was provided by the Genetic Fisheries Center, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Starting 48 h after injection, the females were examined for ovulation every 12 h. The state of ripeness was judged by gentle palpation of the abdomen. If eggs could be removed by applying gentle pressure on the abdomen, the fish were considered to have already ovulated. To check the female's maturity stage, to examine if ovulation occurred and to collect gametes, the fish were anaesthetized with a 0.02 ml l^{-1} clove oil water bath to minimize stress and to make them easier to handle (Zaikov et al., 2008). Two experiments were performed as follows.

2.2. Experiment I, egg retention inside the ovarian cavity (in vivo storage)

Seven females weighing 876 ± 126 g (mean ± SD) were randomly selected for the experiment, and coloured tags were attached to the dorsal fin for individual identification. Females were not ovulated simultaneously; five of them ovulated 72 h and two of them 96 h after hormone injection. When ovulation occurred, a partial volume (3 g, equivalent to ca. 370 eggs) of eggs was stripped individually from each female and fertilised (0 days post-ovulation, DPO). The rest of the eggs were left inside the ovarian cavity of each female before fertilisation. The eggs were then stripped (3 g of eggs each time) at 2, 4, 6 and 8 DPO. In total, five batches of 3-g eggs from each of the seven females were separately stripped with 2-day intervals and fertilised during the experimental period of 8 days after ovulation.

2.3. Experiment II, egg storage outside the ovarian cavity (in vitro storage)

Three ovulated females weighing 1185 ± 170 g were randomly selected for the experiment. Females were not ovulated simultaneously; two of them ovulated 72 h and one of them 96 h after hormone injection. The eggs and ovarian fluid were collected separately for each individual. Nine batches of 3-g egg aliquots from each of the three females were stored with the ovarian fluid in 6-chamber cell culture plates. The plates were transferred to an incubator with an adjusted constant temperature at 10 °C and kept in the dark. The batches of eggs