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Reproduction of endangered river lamprey (*Lampetra fluviatilis*) in controlled conditions

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ABSTRACT

The research reported focuses on reproduction of the river lamprey *Lampetra fluviatilis,*(Linnaeus, 1758) in controlled conditions. There was specific emphasis on fish harvesting dates (autumn and spring), holding conditions and reproduction in a controlled environment. Attempts were also made to synchronize the time of ovulation among river lampreys, egg and sperm collections. Hormonal stimulation was conducted using carp pituitary homogenate (CPH) at a total dose of 4 mg/kg which allowed for shortening of the egg-laying period from 2 to 3 weeks to a few days while sustaining embryo survival rates and larvae quality. River lamprey males were found to not require hormonal treatment to yield good-quality sperm, as measured using the CASA system. River lamprey broodstocks adapted well to different manipulations in hatchery conditions when harvested in the autumn and spring. The results of the present study may be used to restore endangered natural populations of the river lamprey (egg and sperm collection, fertilization or gamete preservation) because ovulation and spermiation synchronization is very difficult to achieve without hormonal treatment in controlled conditions.

1. Introduction

The river lamprey *Lampetra fluviatilis* (Linnaeus, 1758) is an endangered species in Europe, including Poland. This is a migratorous and diadromous agnathan which spawns only once in a lifetime (Brylińska, 2000). For many years, the river lamprey was regarded as a ubiquitous and commercially significant species of freshwater fish (Valtonen, 1980; Birzaks and Abersons, 2011). According to Bartel (1992), there were 80 tons of river lamprey captured per year in Poland until the end of the 1970s, after which there was a decrease to approximately 900 kg per year towards the end of the 1980s. In many Baltic countries, lampreys were widely appreciated for the nutritional value of the meat, which is characterized by a high protein (up to 16%) and fat (up to 30%) content and relatively greater concentrations of vitamins A and B12 in comparison with other fish species (Filipiak and Raczyński, 1999). In the past, river lampreys colonized major rivers, lake catchment areas, the Baltic Sea and the North Sea (a vast territory that stretched from Norway through the British Isles to France and Portugal). In the Mediterranean Sea region, the river lamprey have a natural habitat along the

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western coast of the Apennine Peninsula (Brylińska, 2000).

There has been a dramatic reduction of geographic range of the natural habitat of river lamprey in recent years. For example, results from research with the ichthyofauna species of Polish rivers indicate a catastrophic decrease in populations of river lamprey during the last 20 years (Kuszewski and Witkowski, 1995; Witkowski and Kuszewski, 1995; Danilkiewicz, 1997). The main causes of the reduction in lamprey populations are hydraulic constructions (dams, sluices, and weirs), river pollution and river regulation (Maitland, 1980; Fopp-Bayat et al., 2018). With many hydraulic constructions, there are not fish ladders, which restricts lamprey migrations to spawning areas. Overfishing and poaching also pose a significant threat to lamprey populations, particularly in spawning aggregation sites. According to the IUCN/WCU classification, the river lamprey is considered an endangered species (EN). It is listed as an endangered species in the Polish Red Data Book of Animals (Wieser, 1992). River lamprey populations have been decreasing steadily not only in Poland, but also throughout Europe (Maitland, 2003; Nunn et al., 2008; Silva et al., 2015; Fopp-Bayat et al., 2018). The river lamprey, brook lamprey (*Lampetra planeri*) and sea lamprey (*Petromyzon marinus*) are listed in Annex II to the Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora of 21 May 1992 (EU Habitats Directive). The Directive imposed on EU Member States the obligation to maintain protected habitats and restore endangered species of flora and fauna. There was introduction of year-round protection of the river lamprey and a fishing ban in Poland to counteract the steady decrease in the Polish population of the species. Prior to 2004, there was protection of the river lamprey only during the larval developmental stage.

The river lamprey is at serious risk of extinction unless effective protection and conservation programs are designed for re-establishing populations. Biotechnology options for reproducing and rearing lamprey larvae in controlled conditions provide an important approach for enhancing natural stream populations. Controlled reproduction and rearing larvae in artificial, protected conditions and stocking endangered fish species in their natural habitat is a popular method for preserving endangered species and populations (Nowosad et al., 2014, 2016; Cejko and Kucharczyk, 2015; Kucharczyk et al., 2016, 2018, 2019). There could be reproduction of different lamprey species in controlled conditions. For example, Pacific lamprey (*Lampetra tridentata*) breeders might be reproduced spontaneously or artificially without hormonal treatment (Wade and Beamish, 2012; Lampman et al., 2016; Kujawa et al., 2018). In some other species (i.e., sea lamprey; *Petromyzon marinus*), hormonal treatments were evaluated for males (Young et al., 2007). The objective of the present study was to gain a greater understanding of the reproduction of the river lamprey in controlled conditions with or without hormonal treatment. Spawners harvested from their natural habitat in autumn and in spring were observed and hormonally stimulated. Attempts were also made to synchronize the time of ovulation among river lampreys to shorten the natural egg-laying period from 2 to 3 weeks to 2 to 3 days.

2. Materials and methods

2.1. River lamprey origin

River lamprey were harvested in autumn (October) and spring (April) during spawning migrations in the Vistula Lagoon (Vistula delta region – northern Poland) using tunnel nets. The river lamprey were carefully removed from the nets and placed in containers with aerated water. The experimental animals were then transported in plastic bags with oxygen to a hatchery at the Department of Lake and River Fisheries of the University of Warmia and Mazury in Olsztyn where the Aquaculture and Ecological Engineering Center is located in Olsztyn, Poland.

Although experiments with river lamprey do not need any Animal Care Commision approval, the permission of General Director for Environmental Protection for obtaining adult lampreys from environment was obtained (Decision number DOP-OZ.6401.10.3.2013.ls).

2.2. Broodstock management

In the hatchery, lampreys were placed in plastic spawner tanks with a volume of 1000 dm^3 each (Kujawa et al., 1999). All tanks were equipped with an independent oxygen supply and temperature control system, biological filters and UV lamps. The water temperature was controlled with the temperature imposed being that determined in the Vistula Lagoon during sampling (i.e., 8 °C, both in October and April). After 2 weeks, the river lamprey were transferred to flow-through aquaria with a volume of 500 dm^3 each. The tanks were tightly covered to prevent the fish from escaping. Each aquarium was provided with independent water inlets and outlets, aeration pumps and EHEIM 2260 filters (Eheim, Germany). The filters maintained optimal water variable values and created water currents that were similar to those in the river ecosystem.

2.3. Sex determination

The river lamprey were handled with the utmost care ($n = 60$). Sex determination was practically impossible in adults collected in the autumn, although lampreys had distinguishing sex characteristics in the spring immediately before spawning (Fig. 1). The abdomen was visibly enlarged in females, and a distinctive, elongated urogenital papilla was noted in males (Fig. 2). In lampreys, because sexual dimorphism is minimally expressed outside the breeding season, sex determination based on body size alone can be misleading. River lamprey characteristics of fish captured in the autumn and spring are provided in Table 1.

Fig. 1. River lamprey (*Lampetra fluviatilis*) spawners captured from the Vistula river (north Poland) during autumn (a) and spring (b): male (top) and female (bottom).

2.4. Hormonal treatment

The water temperature was maintained at 8 °C in the aquaria, and it was increased at the beginning of March to reach 13 °C in mid-April. Spawners collected in the autumn and spring were kept in separate and unconnected tanks. In April, before the water temperature was increased, lampreys were divided into six groups, including four groups of ten females each and two groups of ten males each. When certain characteristic features were observed in lampreys prior to spawning (urogenital papilla in males, enlarged anal fin in females), hormonal injections with carp pituitary homogenate (CPH; Argent, USA) were administered to synchronize the time of ovulation among females. The CPH was ground in a mortar and combined with a 0.9% NaCl solution. The CPH was administered twice, at 24-hour intervals, intramuscularly under the base of the first dorsal fin (Table 2). Females injected with saline solution were considered to be the control group (Table 2).

Injections were not administered to males, which released small quantities of sperm when light pressure was applied to the region of the urogenital papilla. Lampreys are very difficult to immobilize without anaesthesia, and muscle tension prevents hormonal injection and gamete sampling. The lampreys, therefore, were anesthetized with Propiscin (2% etomidate, i.e. ethyl-3[(1R)-phenylethyl]imidazole-4-carboxylate), which is commonly used to anesthetize cyprinids and salmonid fish (Lambooij et al., 2009). The dose that was administered was several times larger than that recommended for lampreys (i.e., 2.0–3.5 ml dm^{-3}) because they failed to respond to smaller doses of the anaesthetic.

2.5. Sperm collection and characterization

Semen samples were collected from adults harvested in the autumn (n = 10) and spring (n = 10) by gently massaging the abdomen. Before manipulation, the males were anesthetized, weighed and measured. Sperm motility was activated in 25 µl of a 20 mM Tris buffer containing 40 mM $NaHCO_3$ (pH 8.5 and osmolality of 100 mOsm/kg) with the addition of 0.5% BSA (Cejko et al., 2015). Next, 1 µl of the mixture of sperm and activating fluid was placed on a Teflon-coated slide (12 wells of 30-µl depth and 5-mm-diameter Teflon-coated slide glass, Tekdon, Inc., Myakka City, FA, USA). Approximately 6 s after movement activation, a recording of movement was initiated using a Basler a202 K digital camera (Basler, Germany), integrated with an Olympus BX51 microscope (Plan FL N 20x/0.5 NH ph1 lens; Olympus, Tokyo, Japan). The motility variables of at least 50 tracks in each of the samples were used to estimate the mean the computer-assisted sperm analysis (CASA) values. The recording speed was 46.6 frames per second and 200 frames from each recording were analysed using the CRISMAS program (Image House Ltd., Denmark). Values for sperm motility variables were analysed with the use of CASA system. Those analysed values included: percentage of motile sperm [MOT, %], progressively motile sperm [PRG, %], curvilinear velocity [VCL, µm/s], straight-line velocity [VSL, µm/s], movement linearity [LIN, %], wobbling index [WOB, %], amplitude of lateral head displacement [ALH, µm] and beat cross frequency [BCF, Hz].

Sperm motility was measured twice (repeated) for each sperm sample, after which the average was taken from the two measurements in every group and for each of the analysed samples. Samples of sperm from several males were stored on ice before

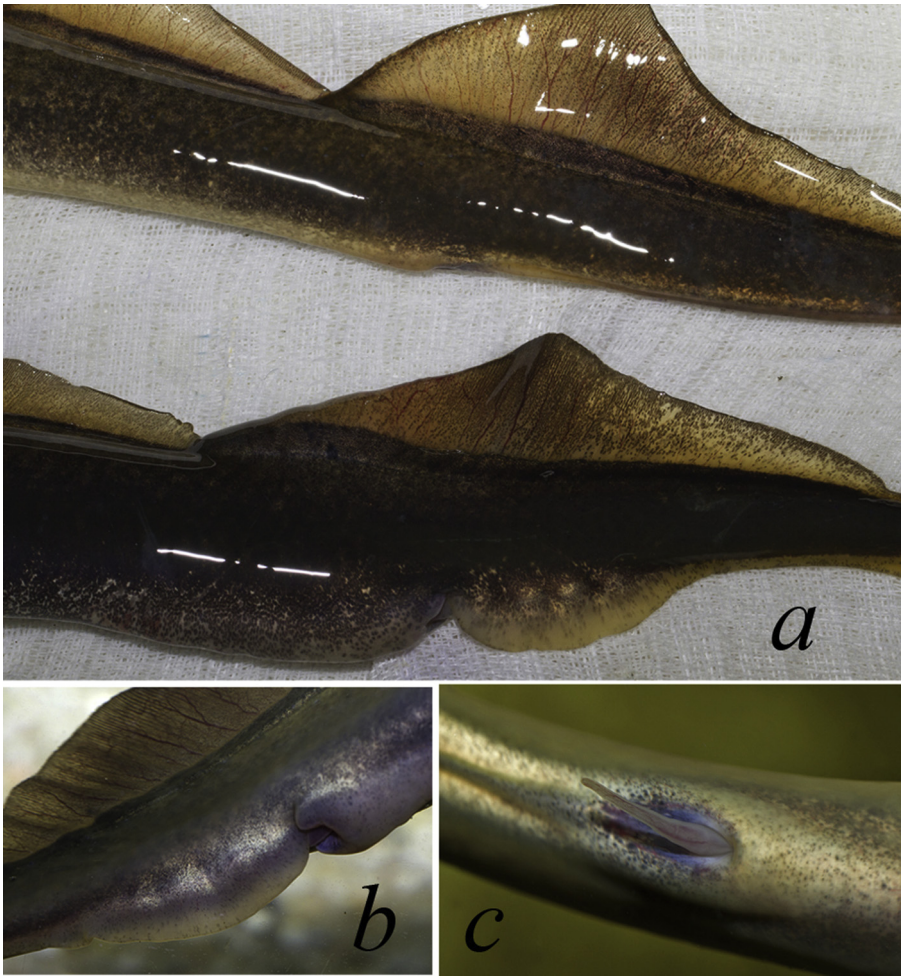

Fig. 2. Male and female sex tissues (a); sexual genital characteristics of mature female (b); and male (c) during reproductive season of river lamprey (*Lampetra fluviatilis*) collected from Vistula river (north Poland).

Table 1
Characteristics of river lamprey (*Lampetra fluviatilis*) broodstocks harvested in autumn (October) and in spring (April) from Vistula river (north Poland); Data (mean ± SD) in rows (separately females and males) did not differ ($P > 0.05$).

Variables	Autumn		Spring	
	Females (n = 20)	Males (n = 10)	Females (n = 20)	Males (n = 10)
Weight (g)	121.7 ± 13.7	83.6 ± 6.3	120.8 ± 18.2	90.6 ± 15.2
Length (cm)	40.7 ± 11.9	36.5 ± 7.6	36.5 ± 7.6	36.8 ± 14.8

Table 2
Preparations used for river lamprey (*Lampetra fluviatilis*) stimulation of ovulation in controlled conditions in females harvested in autumn (October) and in spring (April) from Vistula river (north Poland).

	Autumn		Spring	
Hormone	CPH	0.9% NaCl	CPH	0.9% NaCl
Number of females	10	10	10	10
Primary doses (mg kg^{-1})	0.4	1.0	0.4	1.0
Resolving dose (mg kg^{-1})	3.6	2.0	3.6	2.0

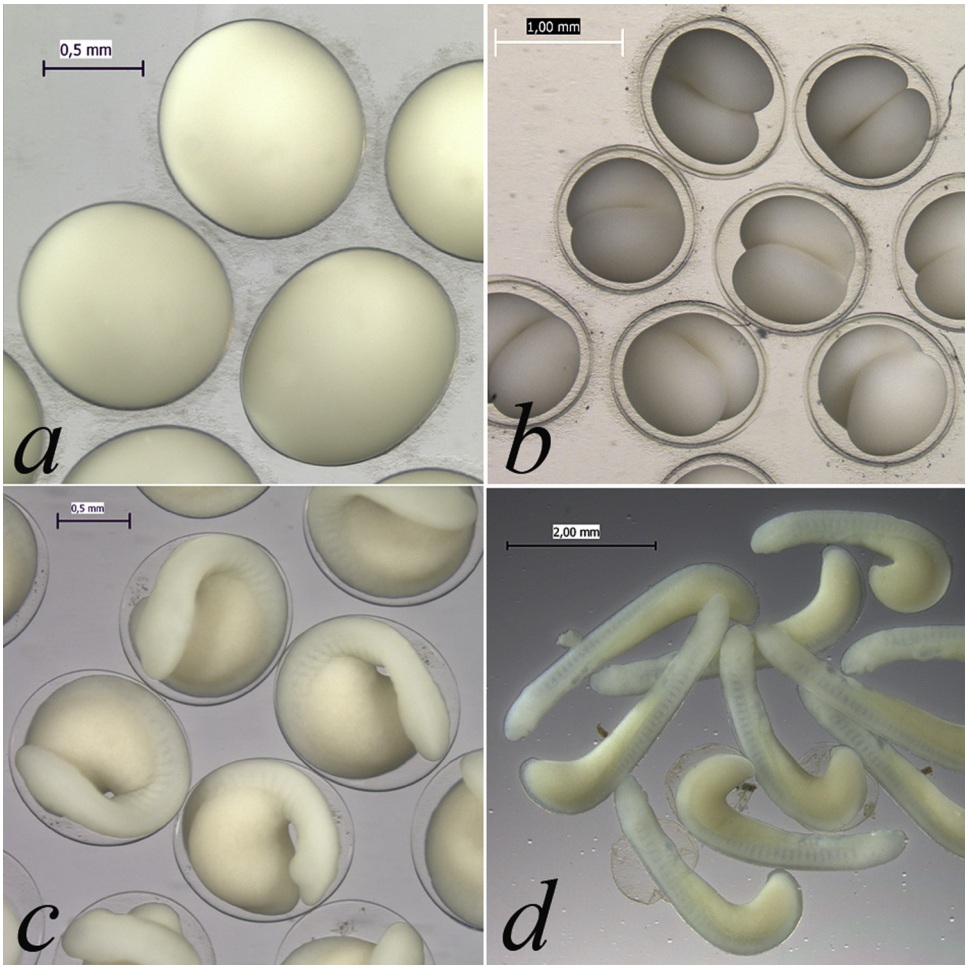

Fig. 3. Eggs (a); cleavage: stage of the two blastomers (b); growing embryos (c); and newly hatched larvae (d) of river lamprey (*Lampetra fluviatilis*) collected from Vistula river (north Poland).

fertilization.

2.6. Egg collection and fertilization

After the entire CPH dose had been administered to females, the water temperature was increased to 14 °C (± 0.5 °C). Starting 36 h after the second injection, the females were monitored for the presence of eggs every 12 h. The following variables were monitored:number of females having ovulations relative to the number of stimulated females [%], female survival after hormonal treatment [%] latency time after second injection, which includes the time between hormonal stimulation and ovulation [h], weight of eggs collected from each female having ovulations – with the use of electronic weight scales accurate to 0.01 g. The eggs deposited by females from each group were sampled separately in plastic containers and were fertilized with sperm collected from several males.

2.7. Egg incubation and embryo characteristics

The eggs collected from each female were rinsed several times with hatchery water and placed in separate 2.0 dm^3 Weiss jars in a closed circulation system (Kujawa et al., 2000). Lamprey eggs are somewhat sticky and have to be rinsed several times with water. The water temperature during egg incubation was set at 16 °C. Eggs were randomly sampled from each jar to determine: the ratio of eggs with viable embryos to total eggs [%], the ratio of free-floating hatchlings to total eggs [%] (Fig. 3).

2.8. Statistical analysis

The one-way analysis of variance (ANOVA) and Tukey's test as a *post-hoc* procedure ($P < 0.05$) were applied to indicate

Table 3
Values for sperm motility variables of river lamprey (*Lampetra fluviatilis*) males ($n = 10$) harvested in autumn (October) and in spring (April) from Vistula river (north Poland) analysed using the computer-assisted sperm analysis (CASA); Data (mean ± SD) in rows did not differ ($P > 0.05$).

Variables	Autumn	Spring
MOT (%)	69.8 ± 14.3	59.6 ± 20.6
PRG (%)	36.8 ± 14.8	25.1 ± 13.5
VCL (μm s^{-1})	163.5 ± 38.2	149.8 ± 46.8
VSL (μm s^{-1})	130.1 ± 42.7	103.1 ± 47.2
LIN (%)	72.0 ± 11.5	61.2 ± 14.3
WOB (%)	102.6 ± 7.8	82.5 ± 9.1
ALH (μm)	1.3 ± 0.2	1.2 ± 0.2
BCF (Hz)	12.5 ± 2.9	13.0 ± 1.9

statistically significant differences between groups of females in relation to egg and sperm variables measured in each group (autumn/spring). The statistical analyses were performed with the use of Statistica 10.0 PL software.

3. Results

3.1. Spermiation rate and reproductive performance of males

Sperm from river lamprey were collected from all males harvested in the autumn and spring. The values of MOT (60%–70%) and PRG (25%–37%) variables were similar between both groups of river lamprey ($P > 0.05$; Table 3). There were similar results in sperm velocities where the average value of VCL and VSL were at the level of 163 (autumn) and 130 μm/s (spring) and 149 (autumn) and 103 μm/s (spring), respectively. The linearity of sperm and wobbling index were not significantly different between groups of river lamprey and the values for ALH and BCF variables were also similar ($P > 0.05$; Table 3). Although there were no differences in sperm characteristics between river lamprey harvested in the autumn and spring, there were greater values for CASA variables for males harvested in the autumn (Table 3).

3.2. Ovulation rate and reproductive performance of females

Eggs were collected from all hormonally stimulated and non-stimulated river lamprey females. Furthermore, hormonal treatment had no effect on river lamprey mortality. The shortest latency time was noted in hormonally-stimulated females that were harvested in the autumn, whereas there was the longest latency time in females harvested in the spring which were not hormonally stimulated ($P < 0.05$; Table 4). In general, hormonally stimulated females yielded eggs in about half the time in comparison to the females from the control group. The time between the first and the last ovulation was 8.5 days. The weight of eggs collected from individual females from different experimental groups ranged from 23.0 to 41.5 g, however, there were no significantly differences in the average weight of eggs sampled from various groups ($P > 0.05$; Table 4). The quantity of sampled eggs was not determined by harvesting season or hormonal stimulation, and there was only a correlation with the body size of the females.

3.3. Embryo survival and viability

There were differences in embryo survival rates before hatching between experimental groups. There was the least embryo survival in the control group of females harvested in spring, whereas there was the greatest survival rate in hormonally stimulated

Table 4
Ovulation rate and reproductive performance of river lamprey (*Lampetra fluviatilis*) females harvested in autumn (October) and in spring (April) spawned artificially in controlled conditions; Number of females are presented in Table 2; Data (mean ± SD) in rows with different letters are different ($P < 0.05$).

Variables	Autumn		Spring	
	CPH	Control	CPH	Control
Female survival[a] (%)	100	100	100	100
Ovulation rate (%)	100	100	100	100
Latency time (h)	57.6 ± 21.0***a***	118.8 ± 63.1***ab***	74.4 ± 37.0***a***	141.6 ± 73.5***b***
Weight of eggs (g)	33.9 ± 3.6	32.2 ± 5.4	31.7 ± 4.7	31.1 ± 3.8
Survival embryos (%)	86.8 ± 1.1***a***	84.8 ± 0.6***b***	85.6 ± 0.6***a***	83.5 ± 0.5***c***
Swimming larvae (%)	83.8 ± 0.6***a***	82.9 ± 0.8***ab***	82.1 ± 0.6***b***	81.7 ± 0.9***b***

[a] - to the end of ovulation period.

females harvested in the autumn ($P < 0.05$; Table 4). There were similar results as for embryo survival for the percentages of free-floating hatchlings ($P < 0.05$; Table 4). In general, embryos collected from non-hormonally stimulated females were characterized by a lesser viability than embryos collected from hormonally-stimulated females.

4. Discussion

The presented results indicate, for the first time, that ovulation of river lamprey when there is controlled conditions may be achieved using carp pituitary homogenate, which when administered there is a decrease in the natural egg-laying period from 2 to 3 weeks to several days. Furthermore, there was no mortality of the females after hormonal treatment at any stage in the captive females. Results of previous research indicate that hormonal injections are not required for gamete maturation and collection of gametes from both genders of lampreys. When there was controlled conditions, ovulation and spermiation of this species, however, was difficult to achieve at the same time (Cejko et al., 2016). Results from the present study indicate that hypophysation of the females can be applied due to its beneficial role in synchronization of time of ovulation among females and the possibility for harvesting good quality eggs and embryos within a short period of time. Results of the present study also confirm that river lamprey males do not require hormonal treatment to yield high quality sperm.

The administration of hormonal injections during the period of spawning readiness (i.e., 2 weeks after development of urogenital papillae in males and enlarged anal fins in females) resulted in a synchronized time of spawning among river lampreys. It is a significant aspect of the reproduction of adults housed in captivity because the same conditions (i.e., water flow, oxygen concentration or ambient temperature) do not always ensure synchronization of egg and sperm collections of the river lamprey (Cejko et al., 2016). In the case of fish, hormonal treatment is recommended when large quantities of sperm are required, however, the amount of males available for reproduction is limited (Cejko and Krejszeff, 2016). Such solutions are primarily used in the reproduction of high-economic value (Brzuska, 2000; Judycka et al., 2015) or endangered species (Kowalski et al., 2012; Cejko and Kucharczyk, 2015). In the case of river lamprey in the present study, sperm were collected without hormonal administrations and sperm quality, as measured using the CASA system, was optimal for ensuring the success of fertilization. This indicated there were no negative effects of captivity on gonad development and sperm maturation of the river lamprey when there was maintenance of animals in controlled conditions. A negative effect of temperature (14 °C) on sperm quality indicators such as MOT, PRG, VCL and VSL, however, was previously observed (Cejko et al., 2016). Sperm motility (MOT) is an indicator of sperm maturity in fish (Lahnsteiner and Mansour, 2012), whereas sperm velocity (VCL) directly affects the capacity of sperm to fertilize eggs (Gage et al., 2004). In the case of river lamprey, therefore, values for such variables should exceed 80% and 150 μm/s, respectively. In a previous study, males harvested from open water were housed in captivity for half a year. There could be variability in testes maturation between individual river lamprey males when there are controlled conditions, unlike sperm production and quality of the sperm from animals residing in their natural habitat (Cejko et al., 2016).

Obtaining eggs and sperm of high quality is the most important aspect of reproduction of fish and other animals under controlled conditions (Bobe and Labbé, 2010). Various hormones at different doses, therefore, have been tested and applied in reproductive biotechnology for many years (Mylonas et al., 2010). In addition, the lack of synchronization in the timing of ovulation among river lamprey poses a significant problem in hatcheries where the maturation cycle may last for as long as 3 weeks (Cejko et al., 2016). Furthermore, because females that are not ready to spawn are often harmed by males, broodstocks should be kept in separate tanks in captivity. In addition, skin wounds may cause spawners to die prematurely before eggs can be harvested. The proper method of reproductive stimulation, gamete collection and management, therefore, are required to achieve fertilization success. Research into the controlled breeding of lampreys with hormonal stimulation was based on studies of the reproduction of rheophilic cyprinids (Kucharczyk et al., 2008; Krejszeff et al., 2008; Cieśla et al., 2014). There, therefore, was stimulation of females using CPH, a hormonal product that is widely used in the controlled breeding of this species and directly influences the maturation of gametes during the latter developmental stages (Mylonas et al., 2010). The results of the current study indicate that CPH injections can be used to effectively synchronize time of ovulations among river lamprey females harvested from their natural habitat. Furthermore, there was no mortality of river lamprey after hormonal treatment. The capacity of liver lamprey to adapt to different manipulations in hatchery conditions may facilitate protection programs, such as reproduction under artificial conditions, short-term sperm preservation or cryopreservation.

The results of the present study indicated that river lamprey eggs can be successfully collected after CPH administration in a controlled environment. Furthermore, even though there was a lack of differences in the weight of egg collections between hormonally and non-hormonally stimulated females, CPH administration resulted in a greater survival of embryos in comparison to the control group. Similar results were also observed in barbel (*Barbus barbus*), where the percentage of hatched embryos after CPH administration was greater in comparison to the control group (Targońska et al., 2011). The results from the present study regarding river lamprey ovulation and embryo quality using CPH appear be very satisfactory for application consideration in the field. Because CPH is a foreign protein when administered into the recipient body during the hypophysation process (Yaron, 1995), and there is no standardization of CPE administration protocols (Jaczó et al., 1989), other hormonal preparations should be tested in the future. Among these, Ovopel, which contains the mammalian GnRHa (D-Ala^6, Pro^9-NEt-mGnRH), and metoclopramide, a dopamine receptor antagonist (Horvath et al., 1997), and Ovaprim, which contains the salmon GnRHa (D-Arg^6, Pro^9-NEt-sGnRH), and domperidone, a dopamine receptor antagonist (Peter et al., 1993), should be taken into consideration. The GnRHa are particularly recommended in fish because of the potent action of inhibiting the release of gonadotropins from the pituitary gland. This situation is typical for cyprinids (Mikołajczyk et al., 2004), catfish (Silverstein et al., 1999) and mullets (Glubokov et al., 1994). Furthermore, GnRHa indirectly influences endocrine glands that produce gonadoliberins and induce the maturation of eggs and sperm (Mylonas et al.,

2010).It has also been confirmed that the administration of synthetic analogues is associated with greater embryo survival compared to the CPH application in some cyprinid species (Krejszeff et al., 2008; Targońska et al., 2012).

5. Conclusions

The negative changes in environment, such as migration barriers, pollutions, river regulation, and several other factors have affected and continue to affect lamprey populations. Before there is initiation of conservation programs of restocking river lamprey, the effects of these factors should be minimized as much as possible. River lamprey should subsequently be reproduced artificially and larvae should be mass-reared in controlled conditions. There can be use of the resulting lamprey as stocking material to re-populate selected populations. River lampreys migrating to the sea (and then back to spawning grounds) will increase for several years after the species has been restocked in optimal sites.

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Conflict of interest

The authors declare no conflict of interest.

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