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Use of ultrasonography to determine sex in sexually immature European river lamprey *Lampetra fluviatilis* (L.)

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ABSTRACT

In this study, there was the first attempt to sex immature European river lampreys *Lampetra fluviatilis*, classified as Agnatha using ultrasonography. This species starts a spawning migration from seas to rivers in the autumn and reproduction is initiated in the late spring. It is recommended to collect breeders soon after the beginning of the spawning migration, however, to date no method has been developed for distinguishing the sex of individuals during this developmental period. The lampreys for the present study were caught in autumn (November) in the Vistula River (northern Poland) during the period of spawning migration and transported to the laboratory. The lampreys were anaesthetised (MS–222, dose: 0.1 g/dm^3 prior to sex determinations (n = 100) using ultrasonography. The images obtained using ultrasonography were verified with post–mortem and histological examinations. The findings with this study confirmed that the sex of the European river lamprey can be effectively ascertained much earlier than can occur with assessment of external secondary sexual characteristics (e.g., sexual papilla, ovipositor, skinfold). The advantages of the method include: 100% effectiveness, survival of the fish after examination, non–invasiveness, rapid verification of the lamprey sex and the possibility of determining extent of gonadal development.

1. Introduction

There are animals living in both fresh and salt waters which resemble eels that are actually Agnatha. One of these is the European river lamprey *Lampetra fluviatilis* (L.), with adult individuals growing to be as long as 50 cm, although lengths usually range from 30 to 45 cm (Staff, 1950; Witkowski, 2000). The life cycle of lampreys is very interesting, but little is known in this regard. The European river lamprey spawn only once in their lifetime, usually at the age of 5 or 6 years and then die within 2–3 weeks after spawning. Before spawning, adult individuals swim to rivers where spawning occurs in the spring of the year (Kujawa et al., 2017; 2018).

The river lamprey was of great economic importance in the Baltic Sea region in the last century (Birzaks and Abersons, 2011). These lamprey were caught in large volumes in seas, bays and river estuaries. Its meat was regarded as a delicacy because of its taste (similar to that of eel) and for its lack of small bones (unlike fish). It was smoked, salted, fried and pickled. For example, 50 tonnes of lamprey a day were caught in Poland in the early 20th century and there was a large export market for lamprey meat. The habitat

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area for lampreys started to rapidly decrease in the late 20th century and about 900 kg of lamprey a year was caught (i.e., 1/20,000 of the previous amount; Bartel, 1992). The reduction of the lamprey population resulted mainly from river pollution, river flow regulation and hydro-technical structures built in the river current areas (dams, locks, weirs) which restricted or even prevented the migration of lamprey to their spawning sites. These hydrotechnical structures in rivers do not often have proper fish ladders, thereby inhibiting the lampreys migration to the spawning sites (Aronsuu and Virkkala, 2014; Fopp-Bayat et al., 2018). Excessive catching of lampreys and poaching, especially when the lamprey congregate before spawning also had an effect on the lamprey population size. As a result of these adverse factors, the habitat areas of river lamprey started to markedly decrease, not only in Polish rivers, where it has become an endangered species, but also in European waters (Nunn et al., 2008). Studies of the ichthyofauna status in Poland have provided evidence of the catastrophic reduction of the river lamprey population (Kuszewski and Witkowski, 1995; Witkowski and Kuszewski, 1995; Witkowski, 1996; Danilkiewicz, 1997). Unless effective protection programmes are implemented, including the reproduction and rearing of the lamprey stocks under controlled conditions, the status of this species may change from "endangered" to "extinct". Developing reproduction technologies and then rearing river lamprey larvae is essential to maintain and enhance numbers for this endangered species. Considering the information that has been accrued, a range of studies were undertaken to understand the biology of the species and in develop protocols for reproduction techniques and larviculture of the lamprey in controlled conditions (Aronsuu and Virkkala, 2014; Cejko et al., 2016; Fopp-Bayat et al., 2018; Kujawa et al., 2018, 2019). To accomplish this goal, it is necessary to obtain a sufficient number of males and females in a controlled environment. Lampreys are usually caught during the time of their upstream spawning migration. Such migrations usually start in the autumn season preceding the time of spawning and lasts (with a break) until spring. The lamprey sex can be determined, as in other animals of the genus, only during the short pre–spawning period. During the migration period, the lamprey vary in terms of their size and colour, but these features cannot be used to determine their sex (Kujawa, 2017; Kujawa et al., 2019). Only during a short time period before spawning changes can be observed in the body structure of both male and female lampreys. A spongy swelling appears just above the sexual opening in females, at the caudal fin and at the foot of the dorsal fin, whereas a 1–2 cm ovipositor develops in the males (Kujawa, 2017; Kujawa et al., 2019).

It is, therefore, impossible to determine the lamprey sex until the external secondary sexual characteristics develop. There have been a number of descriptions in the literature, which help to externally distinguish males from females (Witkowski, 2000). The external dimorphisms in colour and size of the animals are generally used to sex lamprey, however, these are highly subjective differences and cannot be used for ascertaining the sex of lampreys with 100% confidence before the spawning season. Obviously, assessments of the gonads would allow for precise determination of the lamprey sex. Intravital sex verification is difficult with traditional methods (e.g., with a catheter), which is why the use of an ultrasonic device would be an effective non–invasive solution for assessing the sex of lampreys. This method has, however, not been used in animals of the *Petromyzontidae* family. Ultrasonography (USG) is currently used frequently in human and veterinary medicine (Hildebrandt and Saragusty, 2015). In most cases, this method does not require anaesthesia and there is not a convalescence period after its use. It is increasingly used in animal breeding (Evans et al., 2004; Kucharczyk et al., 2016). Ultrasonography is a diagnostic method which is widely used and constantly developing with the major advantages being safety and non–invasiveness (Evans et al., 2004; Novelo and Tiersch, 2012). Compared to other non–-invasive imaging techniques, an ultrasonic examination does not involve use of any ionising radiation and does not expose the body to strong electromagnetic fields. Combined with a relatively small ultrasonic unit cost, these advantages contribute to the popularity in use of this method. Considering the advantages in use of ultrasonography, the present study focused on effectiveness of using this technique for identifying the sex of immature lampreys.

2. Material and methods

2.1. River lamprey origin

River lamprey *Lampetra fluviatillis* (L.) specimens (n = 100) were used in the study. The lamprey were caught randomly with an entrapment device in November, at the beginning of their spawning migration, in the Vistula river, near the village of Czatkowy (54°13′N, 18°82′E). The river water temperature was about 8 °C during the period when the lamprey were caught. The animals were transported in plastic bags containing water and oxygen to the laboratory of the Centre for Aquaculture and Environmental Engineering at the University of Warmia and Mazury Olsztyn. The lamprey were then placed in a pool (capacity 1 m^3, temperature 8 °C) where fish brooders are routinely maintained (Kujawa et al., 1999) in which a knitted net (5 mm mesh) was installed to catch the lampreys (Kujawa et al., 2019). All of the lampreys were then weighed, measured and labelled with electronic PIT markers (Biomark, USA) (Nowosad et al., 2015). The appropriate water movement imitating a river current and proper water variables were ensured using an external EHEIM CLASSIC 2260 filter (1500 L; Germany) as well as by intensive aeration using a pipe diffuser. Low water temperature in the pool was ensured through use of an automatic water cooling system. The values for water variables were recorded using a central OxyGuard Pacific system. Because lampreys do not feed during the spawning migration, they were not fed during this study.

Although experiments with river lamprey do not need any Animal Care Commission approval, a permit from the General Director for Environmental Protection in Poland for capturing adult lampreys from their natural habitat was obtained (Decision number DOP-OZ.6401.10.3.2013.ls).

Table 1
Pre-spawning characteristics of river lamprey *Lampetra fluviatilis* spawners collected in the spring; Data (mean ± SD) in columns with different superscripts differ ($P < 0.05$).

	Body weight [g]	Body length [cm]	GSI* [%]
Female (n =10)	108.30 ± 21.99	38.91 ± 2.66	6.69 ± 0.64^{a}
Male (n = 10)	99.52 ± 22.50	38.38 ± 3.50	4.45 ± 1.03^{b}

* Gonadosomatic index [GSI = (gonad weight/body weight)*100].

2.2. Methods

Prior to lamprey sex identification, they were caught and placed in a solution of MS–222 (Finquel, USA) at a dose of 0.10 g/dm, which was determined to be effective in an earlier study (Czerniak et al., 2017). After being anaesthetised, the lengths of the lampreys were measured (± 0.1 mm) and weights (± 0.01 g) were determined (Table 1) and there was visual estimations of the sex of each individual based on their colour, size, and shape of abdomen (Witkowski, 2000). The sex was also determined using an A DRAMIŃSKI 4Vet ultrasonograph with a linear probe at a frequency of 6–14 MHz with a touch screen. The device provided black–and–white imaging and can be uitilised with multiple types of multi–frequency probes. The ultrasonic examination was conducted using the following parameters: probe frequency 10–14 MHz, probe penetration depth 40 mm, focus set at the depth of 5–15 mm. The probe was pressed against the body 5 cm behind the gills. Additionally, photographic imaging documentation of selected lamprey individuals was performed. Prior to determining differences between gonad images of males and females, each individual was examined, the image was recorded and, subsequently, to confirm the observation, a post–mortem assessment of 10 random animals of each sex was performed. The gonads of those animals were used for histological analyses. The gonadosomatic index (GSI) represents the weight of the gonads as a percent of the body weight and was calculated as: GSI = (gonad weight/body weight)*100. Significant differences were observed during the examination of male and female images.

2.3. Sex verification

Sex verification in a group of 100 animals was conducted twice: in the autumn (W_1), after the animals were taken to the laboratory (November) and after 5 months (W_2) (April). The first evaluation of the lamprey sex (in November) was based on visual examination (Fig. 1), using such literature characterizations as: size and colour of the animals (Witkowski, 2000) and an ultrasonic examination. To confirm the results of a visual assessment and an ultrasonic examination, a post–mortem examination of 20 random animals was performed (10 of each sex). These animals were prepared, weighed (± 0.01 g) and the gonads were preserved for histological examination. The other animals (W_2; n = 80) were placed in clean running water and then in two separate pools, where they were divided into males (n = 39) and females (n = 41). The pools were placed in one RAS system and values of all physicochemical variables were the same for all groups (sexes). After 5 months (April), when all the secondary sexual characteristics were visible, another sex verification was performed in the lampreys (n = 80), separately for each group (pool). Sex verification was based on external secondary sexual characteristics, such as ovipositor and swollen sexual papilla, as well as a skinfold, typical of males and females, respectively (Kujawa, 2017).Furthermore, when the abdomen was pressed lightly, spawning or semen grains could be observed.

Fig. 1. River lamprey *Lampetra fluviatilis* spawning specimens caught in November; First three individuals from the top are males and others are females (three from below).

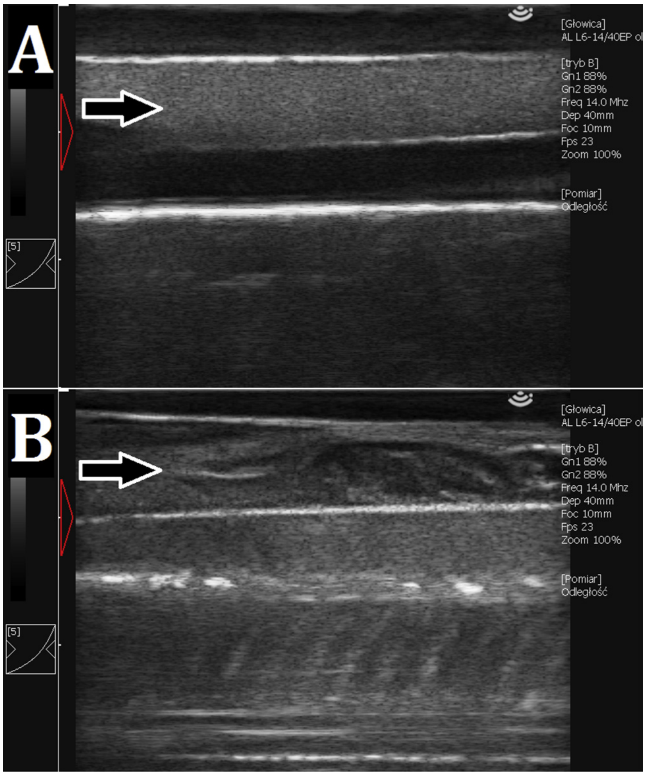

Fig. 2. Ultrasonic image of a lengthwise ventral (between the head and the outline of the dorsal fin) section of a female (A) and male (B) body cavity (Arrow indicates gonad).

2.4. Histological analysis

Samples of gonads were fixed in Bouin solution, dehydrated in ethanol, cleared in xylene and embedded in paraffin blocks. The tissues were then sectioned with a microtome into 5–7 μm slices, which were then stained in hematoxylin and eosin (H + E). Gonads were analysed using a light microscope (Axio Scope A1, Zeiss, Germany) with axiovs40 v 4.8.2.0 software (Carl Zeiss MicroImaging GmbH, Germany).

2.5. Data analysis

Data are presented as mean values with standard deviation (mean ± SD). Percentage data (GSI) were subjected to *arcsin* transformation before they were statistically analysed. Statistically significant differences ($P < 0.05$) were determined using a one–way ANOVA variance analysis, followed by use of a t–test (length, weight, GSI) or Chi-square Pearson (sex identification). The statistical analysis was conducted using Microsoft Excel and Statistica v. 13.1 (StatSoft Inc., USA).

3. Results

A preliminary verification (W_1; Fig. 1) of lamprey sex ($n = 100$), based on their appearance, indicated there were a larger number of males than females (73:27 individuals) in the population that was used in this study. Subsequently ultrasonic examinations (Fig. 2A, 2B), however, indicated the male/female ratio was about 1:1 (49:51), which was confirmed by the post–mortem and histological examinations ($P < 0.05$; test Chi-square Pearson). A secondary sex verification (W_2), conducted after 5 months (April), when external secondary sexual characteristics were obvious, confirmed the effectiveness of use of ultrasonic assessments for sex verification in immature lampreys in each group (pool). All previous sex determinations (conducted in November) using ultrasonography (Fig. 2A, 2B) were conducted correctly and the results were consistent with the findings with assessments of secondary sexual characteristics such as: a sexual papilla, a skinfold (females; Fig. 3A) and an ovipositor (males; Fig. 3B).

4. Discussion

Sexual dimorphism in most fish species is poorly visible. For example, females of certain species are much larger than males of the same age (European eel *Anguilla anguilla* (Kucharczyk et al., 2016), barbel *Barbus barbus* (Nowosad et al., 2016)). Only during the

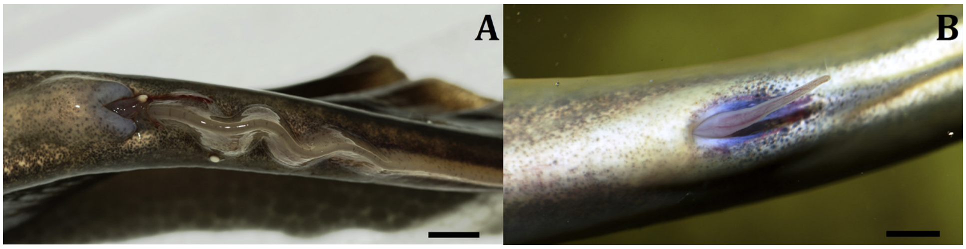

Fig. 3. Genital warts of female (A) and male (B) river lamprey *Lampetra fluviatilis* during spawning (April); Scale bars: 5 mm.

reproduction period, however, do the secondary sexual characteristics become obvious which allow for sex identification, such as spawn rash, significant enlargement of the abdomen in females or manifestation of the sexual papilla (Nowosad et al., 2014, 2016). In most fish species, it is, however, impossible to distinguish the sexes other than during the reproduction period with macroscopic indicators. This is particularly important when the specimens are acquired from natural populations, because fish of both sexes swim to the spawning ground separately and at different times. In such cases, it is possible to acquire brooders of only one sex or in an incorrect proportion of specimens for reproduction. One example is a case in the Łyna River (north of Poland), where 63 ide brooders were caught, 60 of which were females, one was infertile, one was an ide and asp hybrid, and only one proved to be male (unpublished data).

Ultrasonic imaging is used in breeding, disease diagnostics and in scientific research (Sande and Poppe, 1995; Poppe et al., 1998; Poppe and Nygaard, 1999; Næve et al., 2018). Ultrasonic examination in fish breeding can provide invaluable information for distinguishing the sex of individual animals (Du et al., 2017) and for determination of the extent of fish infestation with parasites (Kucharczyk et al., 2016). An ultrasonic examination can also allow for an assessment of the sexual maturity of individuals (Petochi et al., 2011; Du et al., 2017). It is used to predict the stage of brooder development for reproduction and to determine the amount of spawn contents present in a female. The technique is widely applied in caviar-producing aquacultural farms (Masoudifard et al., 2011). Ultrasonic examination is also the most effective method of sex identification in sexually immature fish. Biopsy and endoscopy can be used in sexually mature fish. The presence (+) or absence (–) of gonads is determined in sexually immature fish. Up to 2000 fish per day can be examined using this method. The use of ultrasonic examinations in distinguishing sex has been confirmed in such species as: rainbow trout (*Oncorhynchus mykiss*; Hliwa et al., 2014), Atlantic salmon (*Salmo salar*; Reimers et al., 1987; Mattson, 1991; Poppe et al., 1998; Næve et al., 2018), cod (*Gadus morhua;* Karlsen and Holm, 1994), Atlantic halibut (*Hippoglossus hippoglossus*; Shields et al., 1993), striped bass (*Morone saxatilis*; Blythe et al., 1994), Pacific Herring (*Clupea harengus pallasi*; Bonar et al., 1989), flounder (*Pleuronectes americanus* and *Pleuronectes ferruginea*; Martin-Robichaud et al., 1998; Martin-Robichaud and Rommens, 2001), haddock (*Melanogrammus aeglefinus*; Martin-Robichaud and Rommens, 2001), Neosho madtom (*Noturus placidus*; Pallid sturgeon *Scaphirhynchus albus*; Albers et al., 2013), shovelnose sturgeon (*Scaphirhynchus platorynchus*; Colombo et al., 2004) and European eel (Bureau du Colombier et al., 2015; Kucharczyk et al., 2016).

Ultrasonic examinations in river lampreys have confirmed the effectiveness of use of this technique for sex verification. It must be emphasised that the recommendations provided as a result of findings from these previous studies based on the size and colour of river lampreys (Witkowski, 2000) are not reliable and there is significant error in sex determinations, which was confirmed in the present study. Sex identification using an ultrasonograph allows for rapid determination of the ratio of males and females caught for reproduction under controlled conditions, as well as for determination of the sex ratios in monitoring catches without having to kill the animals (Novelo and Tiersch, 2012). Lampreys are an essential component of the ecosystem and contribute to aquatic animal and plant biodiversity. Only lamprey larvae were protected in Poland before 2004. Currently, adult individuals are also under full species protection. This species has been entered in the Habitat Directive and are protected in multiple Nature 2000 sites in Poland. The unique advantage of using ultrasonography is the possibility of rapid sex determination of animals, both in the field and in a laboratory setting (Evans et al., 2004). Using ultrasonography for sex identification also allows for minimising animal acquisition from the natural habitat, as it is not necessary to catch more animals in situations of a sexual imbalance in populations. This is parituclarly important with lampreys because of the reproduction processes occurring only once in their lifetime before they die shortly after spawning.

5. Conclusions

The results of the present study confirmed that the sex of European river lamprey can be identified during the time period when migration of these animals is occurring to the spawning site, long before the secondary sexual characteristics appear. The use of ultrasonography is an intravital, non–invasive method which enables rapid sex verification in lampreys and can be used in the field and during monitoring or for collecting breeding stocks. This technique enables early determination of the reproductive success of river lamprey, which is further aided by determination of the male/female ratio.

Conflict of interest

The authors declare no conflict of interest.

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