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Ultrasonography to assist with timing of spawning in European eel

Pauline Jéhannet [a], Leon T.N. Heinsbroek [b], Arjan P. Palstra [a, *]

[a] Wageningen University & Research Animal Breeding and Genomics, Wageningen Livestock Research, PO Box 338, 6700 AH Wageningen, The Netherlands
[b] Aquaculture and Fisheries Group, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, The Netherlands

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ABSTRACT

Ovulation in the European eel can be induced by injection of DHP (17α,20β-dihydroxy-4-pregnen-3-one). The timing of injection is based on the developmental stage of the oocytes. The oocyte stage is determined by invasive biopsy or through external indicators of the oocyte hydration response: body weight index (BWI) and body girth index (BGI). However, in European eel, BWI and BGI are inaccurate indicators because the individual hydration response is highly variable. The aim of this study was to identify indicators of oocyte maturation non-invasively by applying ultrasonography. Ultrasound parameters that were determined were the body wall thickness; diameter of the cloaca opening; grey scale median (GSM) of the ovary, and diameters (vertical and horizontal) and surface area of ovary, liver and swim bladder. Data of farmed and wild eels were combined for correlation analyses and principal component analyses (PCA). Ovary and liver parameters were not correlated with the average oocyte stage. Body wall thickness was negatively correlated to the average oocyte stage but variability was high. The swim bladder vertical diameter (Sbv) was negatively correlated with the average oocyte stage and with the oocyte diameter. Swim bladder area (Sba) was also negatively correlated to the average oocyte stage but less accurate. Results showed that during the final stages of oocyte maturation, the swim bladder becomes smaller. This is most probably due to the pressure that is caused by the increasing ovaries as a result of the oocyte hydration response. Females with an average oocyte stage between 4.5 and 7.0 and an average oocyte diameter higher than 800 μm, had Sbv values decreasing from 4.3 to 2.4 mm and Sba values from 52 down to 11 mm^2. The correlation between Sbv and oocyte stage is more strict than for BWI or BGI which makes Sbv not only a parameter that can be determined non-invasively but also one that is more accurate in indicating the oocyte developmental stage. Therefore, ultrasonography, and in particular the ultrasound imaging of the swim bladder, represents a useful tool to assist with the timing of spawning as induced by injection of DHP.

1. Introduction

Ever since Fontaine (1964) [1] first matured female European eels with pituitary extracts, hypophysation has been routinely applied to induce the sexual maturation of eels. Repeated injections with either carp or salmon pituitary extracts do not only sexually mature female European eels *Anguilla anguilla*, but also Japanese eels *A. japonica* [2,3]; American eels *A. rostrata* [4]; long-finned eels *A. dieffenbacchii* and short-finned eels *A. australis* [5]. The injections with pituitary extracts immediately induce the hepatic synthesis of vitellogenins in European eels [6]. Once released into the blood, the vitellogenins are taken up by the oocytes where they contribute to the formation of yolk. Kagawa et al. [7] reported that yolk proteolysis is essential for water influx into the maturing oocytes of the Japanese eel.

Ohta et al. [8] discovered the maturation inducing steroid (MIS) in female Japanese eels and induced final oocyte maturation and ovulation by treating eels with 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Eels are treated with DHP when a body weight increase of 10% occurs as a result of oocyte hydration. However, in European eels, the hydration response is more variable in timing and speed [9] and therefore less accurate indicating oocyte development. Consequently, body weight increase, and also body girth increase [10], are not sufficiently accurate indicators of oocyte development and oocyte sensitivity to DHP. Therefore, oocyte biopsies are necessary taken either with a needle to perforate the body wall and sample the ovary, or by inserting a cannula into the cloaca to sample the oviduct. This invasive method is stressful, may

* Corresponding author.
E-mail address: arjan.palstra@wur.nl (A.P. Palstra).

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cause infection and, depending on the origin of the eels, anesthesis may be required. A non-invasive method that indicates oocyte development at high accuracy would avoid these negative impacts on the physiology of the mature eels.

Ultrasonography may present a solution. Ultrasound imaging is commonly used for sex determination and assessment of maturity in fish (reviewed by Ref. [11]), also in European eel [12–15]. These investigations focused on gonadal development to discriminate fast, slow and non-responding eels to hormonal treatment. The monitoring of ovulation by ultrasonography has been reported for striped bass *Morone saxalitis* [16] and halibut *Hypoglossus hypoglossus* [17].

Our study is the first, to our knowledge, that aims to find indicators of oocyte maturation to assist with timing of spawning in the European eel. Accurate staging could improve the timing of spawning induction by injection of DHP. DHP injection often represents a single chance after at least 12 weekly hormonal injections in the reproduction protocol for European eels to induce ovulation, final maturation and release of fertile eggs. Ultrasonography may therefore contribute significantly to higher reproductive success rates.

2. Material and methods

Experimental protocols complied with the current laws of the Netherlands and were approved by the animal experimental committee (DEC nr. 2014216).

2.1. Broodstock

Experimental farmed female eels (N = 11) were part of a group of 71 females and 100 males that originated from the commercial farm Peeters BV (St. Anthonis, The Netherlands). They were transferred to the Wageningen Research facilities in Yerseke (The Netherlands) where they were subjected to a simulated fresh water and seawater migration of 1447 km in a swim-gutter over a period of seven weeks. Thirty-six females and fifty males were swimming at 0.57 m s^{-1} while thirty-five other females and fifty males did not swim. Experimental wild female eels (N = 5) were part of a group of 35 female and 48 male wild migratory eels which were caught early December in the Veerse Meer in the Netherlands and were subjected to a simulated seawater migration of 2164 km over a period of six weeks in a similar way (Mes et al. [18] for detailed description of photothermal regimes). After simulated migration, farmed and wild eels were transported to the Wageningen University and Research animal experimental facilities (CARUS, Wageningen, The Netherlands) for reproduction experiments.

2.2. Animal housing and welfare

Farmed and wild silver eels were housed under dark conditions, in two separate recirculating aquaculture systems (RAS), each consisting of four 350-L tanks. Farmed eels were kept at 15.6 ± 1.6 °C in artificial sea water (Red Sea Salt Reef Crystal, Aquarium system, Sarrebourg, France) at 34.5 ± 2.1 ppt, wild eels at 15.8 ± 2.0 °C and 34.5 ± 1.0 ppt. For each RAS, the whole water volume was pumped over a settling filter, a poligazer filter and a biological moving bed biofilm reactor. Water was continuously pumped through a UV-filter and a heat exchanger with cooler. PVC pipes were added to the tanks to serve as refuge. Eels were starved during the whole experiment. Wounds were treated with betadine or sealed with 1% silver nitrate solution.

2.3. Hormonal treatment

Females were weekly intramuscular injected with 20 mg carp pituitary extract per kg (CPE; Catvis, Den Bosch, The Netherlands) which was dissolved in 0.2 ml physiological salt solution, without applying anaesthetics. From week 10 onwards, the females were weighed two days after receiving their weekly injection. The BWI was calculated as: (BW/BW at the moment of first CPE injection) x 100%. When BWI exceeded 110%, body girth (BG) was measured to determine the BGI. The BGI was calculated as (BG/BL) x 100%. When BGI exceeded 0.24, females received an extra single CPE dose to booster the progress of maturation. The following day, selected females were weighed again. When BWI exceeded 120%, BG was measured again and females were subjected to ultrasonography (as described below). Subsequently, ovary biopsies were either sampled by using a 1.2 mm diameter needle on a 1 ml syringe or by inserting a cannula through the cloaca in the oviduct. Freshly obtained oocytes were observed and photographed under a microscope connected to a camera (Moticam 580) at 2× magnification. Oocyte diameter was determined using the free-software Image J (version 2.0). Oocyte development was characterised according to the staging as described by Palstra et al. [9]. In short, these authors consider seven developmental stages of oocytes during their increase in size due to a hydration response: from an opaque oocyte (stage 0) to an opaque oocyte with a centred nucleus becoming visible (stage 1); to a fully transparent oocyte with the fat droplets clustered (stage 2); then showing germinal vesicle migration (stage 3); with the nucleus at the periphery (stage 4); with the nucleus at the periphery and only with few large fat droplets (stage 5); then showing germinal vesicle breakdown (stage 6), and ultimately with germinal vesicle breakdown and a single fat droplet. Females having fully transparent oocytes with few large fat droplets (<40) and with a nucleus at the periphery (i.e. stage 5) were treated with DHP (Sigma-Aldrich, Zwijndrecht, The Netherlands) at a dose of 2 mg DHP per kg, dissolved in 175 μl 100% ethanol and diluted with 175 μl buffered saline solution, at 6 locations in the ovarium to induce final oocyte maturation and ovulation. This solution was injected at 06:00 h pm to induce egg release on the following morning.

2.4. Ultrasonography

2.4.1. Equipment and image analysis

Ultrasonography was performed using a compact and portable system (MyLabFive™Vet, Esaote, Genoa, Italy). The used probe was a linear array transducer and was 40 mm long with a frequency ranging from 6 to 18 MHz (LA435, Esaote). Cross-sectional images with B-mode were performed at the highest resolution (18 MHz). The penetration mostly used was 4 cm depth since it suited best the size of the females [14]. The gain was set at 100% to be homogeneous within females for the grey scale median (GSM) measurements.

Cross-sectional images were recorded on the internal storage and analysed later with the tools provided by the ultrasound unit. Vertical and horizontal diameters of organs were measured by means of the “distance” tool. For determination of the surface area of cross-sectional images, organ contours were drawn with the tool “area” and the corresponding value was directly provided. Diameter and surface area of liver, ovary and swim bladder were measured as well as the body wall thickness.

For GSM measurements, images recorded on the internal memory of the ultrasound unit were exported to a computer. GSM analysis was reported by several studies on human carotid plaques [19–22] using Adobe Photoshop (CS6 extended). Organs contours were drawn with the “lasso” tool and GSM, mean and standard