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Temporal expression of hepatic *estrogen receptor 1*, *vitellogenin1* and *vitellogenin2* in European silver eels

Arjan P. Palstra [a,b,1], Denhi Schnabel [a,c,1], Maaike C. Nieveen [a], Herman P. Spaink [a], Guido E.E.J.M. van den Thillart [a,*]

[a] Molecular Cell Biology, Institute of Biology, Leiden University (IBL), Einsteinweg 55, 2333 CC Leiden, The Netherlands
[b] Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain
[c] Departamento Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología/UNAM, Av. Universidad #2001, Col. Chamilpa C.P. 62210, Cuernavaca, Morelos, Mexico

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ABSTRACT

Because European silver eels have never been caught during or after their 6000-km reproductive migration to the Sargasso Sea, all existing knowledge on their sexual maturation comes from hormonal stimulation. Silver eels that start their oceanic migration are still immature with pre-vitellogenic oocytes. Hence we assumed that vitellogenesis should start with the expression of the estrogen receptor in the liver before the circulating 17β-estradiol (E2) can have any effect. In this study we followed the hepatic vitellogenesis upon 4 weekly injections with carp pituitary extracts (CPE). New molecular primers for the expression of the *estrogen receptor 1* (*esr1*), *vitellogenin1* (*vtg1*) and *vitellogenin2* (*vtg2*) in the liver were developed. Sequences of *vtg2* and *esr1* were not previously described in *Anguilla anguilla*. All eels showed weekly increase of the eye size and pectoral fin length, which are signs of early maturation. The same occurred with the gonadosomatic index, the oocyte stage and diameter, and number of deposited fat droplets. Early vitellogenesis appeared as a 3-step process (1) E2-levels and *esr1* expression were significantly increased already after one injection, (2) *vtg1* and *vtg2* expression were significantly increased after one and two injections, respectively, and (3) *vtg1* and *vtg2* expression increased further after three and four injections. Then also plasma calcium (corresponds with plasma vitellogenin) increased and yolk globuli appeared in the oocytes. These results show that *esr1* is the first of the three genes examined that is expressed during the onset of hepatic vitellogenesis. Furthermore, ovarian vitellogenesis (appearance of yolk globuli in oocytes) occurs 1–2 weeks later than the onset of hepatic vitellogenesis.

1. Introduction

The three most well-known eel species (European eel, *Anguilla anguilla*; American eel, *Anguilla rostrata* and Japanese eel, *Anguilla japonica)* are semelparous and migrate thousands of kilometers to their oceanic spawning grounds. The European eel swims 6000-km from the estuaries and freshwater rivers and lakes to the Sargasso Sea to reproduce. The females spend 8 to over 50 years (mean 12 years) as yellow eels on continental waters to grow and to build up the required fat reserves to fuel their trip and to deposit fats in the oocytes. The large and fatty females with sufficient fat stores (Larsson et al., 1990; Svedäng and Wickström, 1997) cease feeding and start their reproductive migration. They show numerous morphological and physiological changes in preparation of their oceanic journey ('silvering') and leave as silver eels. Knowledge on natural maturation and reproduction of European eels is absent. The silver eels venture into the ocean in autumn, still in a pre-pubertal condition, with less than 2% relative gonad mass. Laboratory experiments show that after artificial maturation female eels reach gonad masses of 40–60% of their body mass (Palstra et al., 2005). However, nobody ever observed maturing silver eels during oceanic migration nor caught them in the act of spawning.

Before leaving the continent as silver eels, the yellow eels are characterized by a severely depressed lipid mobilization (Palstra et al., 2009) and blockage of maturation. Pre-pubertal blockage of maturation is due to a deficient GnRH stimulation and a simultaneous inhibition of pituitary FSH and LH synthesis and release by dopamine (Dufour et al., 1988; Vidal et al., 2004). Oocytes of yellow and silver European eels are in a pre-vitellogenic stage (Versonnen et al., 2004) and have to be stimulated by gonadotropins. Like in salmonids, development until yolk incorporation is characterized by accumulation of cortical alveoli and fat droplets (Campbell et al., 2006). Information on vitellogenesis has increased substantially in recent years but little pro-

* Corresponding author. Fax: +31 71 5274900.
E-mail addresses: a.p.palstra@biology.leidenuniv.nl (A.P. Palstra), g.van.den.thillart@biology.leidenuniv.nl (G.E.E.J.M. van den Thillart).
[1] These authors contributed equally to this work.

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gress has been made in understanding pre-vitellogenic development (Pătino and Sullivan, 2002). Only recently, mechanistic studies on ovarian previtellogenesis have been published on salmonids (Campbell et al., 2006) and eel (*Anguilla australis*; Lokman et al., 2007).

The existing knowledge on eel maturation comes from the artificial induction of maturation by hormonal injections with (carp or salmon) pituitary extract in females. This approach has been used by many researchers in reproduction studies on Japanese eel (Yamamoto and Yamauchi, 1974; Yamauchi et al., 1976), American eel (Sorensen and Winn, 1984), New Zealand eels (*Anguilla dieffenbachii* and *A. australis*; Todd, 1981), and European eel (Boëtius and Boëtius, 1980). Current protocols are still based on the use of pituitary extracts although reproductive success is very limited. Larvae of *A. dieffenbachii*, *A. australis* (Lokman and Young, 2000) and *A. anguilla* (Bezdenezhnykh et al., 1983; Bezdenezhnykh and Prokhorcik, 1984; Pedersen, 2004; Palstra et al., 2005) were obtained by pituitary extract injections but they showed, however, obstructed or delayed hatching, abnormal morphology and died within a few days after hatching prior to exogenous feeding. In contrast, Japanese researchers succeeded in raising larvae up to exogenous feeding and even in producing glass eels from *A. japonica* treated with pituitary extracts (Kagawa, 2003; Kagawa et al., 2005) but they acknowledge that the yield was low and most larvae developed abnormally. Adachi et al. (2003) observed variations in yolk accumulation, egg membrane formation, oocyte maturation and plasma hormone levels. Sato et al. (2003) mentioned variable fertility and hatchability of eggs and also low survival of *A. japonica* larvae. These reproduction problems might be due to alterations in vitellogenesis induced by the weekly hormone injections.

In this study we focused on the onset of liver vitellogenesis, which we assumed should start with the expression of the estrogen receptor in hepatocytes. As European yellow eels have not started vitellogenesis yet (Versonnen et al., 2004; van Ginneken et al., 2007a; Palstra et al., 2009) and cannot be used for artificial reproduction (Palstra and van den Thillart, 2009), it is likely that the estrogen receptor has not been adequately activated. Thus we hypothesized that the combination of circulating estradiol and the presence of the Esr1 in the liver of silver eels has to precede the production of vitellogenin. We studied the effects of 4 weekly injections with carp pituitary extract (CPE) on migratory female silver eels. The maturation response and especially the vitellogenic response of liver and oocytes, was determined by measuring morphometric and histological maturation parameters, blood plasma estradiol (E2) and calcium (Ca), and the expression of genes involved in vitellogenesis in the liver.

2. Methods

2.1. Silver eels

Wild migratory silver eels ($n = 47$) were caught in Lake Grevelingen (The Netherlands), near the sluices at the North Sea side (at 32‰, 12 °C), at the end of October of 2005. Larger eels (>70 cm in length and 800 g body weight) were selected from the catch. They were transported to the laboratory in a 1:1 eel:water ratio in plastic bags filled up with oxygen in 60 l tanks. Silver eels were randomly divided over the experimental groups; 10 eels were immediately measured and dissected as controls, another 10 eels were measured and dissected as rest group after 4 weeks at the end of the experiment. For hormonal stimulation, 27 experimental eels were tagged with small passive implantable glass encapsulated transponders (TROVAN, EID Aalten BV, Aalten, The Netherlands) that were subcutaneously injected at the base of the dorsal fin.

2.2. Treatment

The group of 27 eels received weekly intraperitoneal CPE injections at a dose of 20 mg/kg according to the method described before (Palstra et al., 2005), referred to as the CPE group. The CPE group was kept in a 1700-l recirculation system with artificial seawater of 18 °C. To prevent bacterial infections, eels were exposed weekly for 1.5 h to the antibiotic Flumiquin (Flumix, Eurovet, Bladel, The Netherlands, 50 mgl^{-1} for 1–2 h) in 200-l water in a separate tank.

2.3. Measurements and sampling

Each week, 5 or 6 eels were measured and sampled to analyze the treatment effects. Morphometric parameters included body length (BL), body weight (BW), eye diameter horizontal (EDh), eye diameter vertical (EDv) and pectoral fin length (PFL). The following indices were calculated according to the formulae below: condition factor (*K*), eye index (EI) and pectoral fin length index (PFLI).

1. Condition factor (K) = $100 * BW\ BL^{-3}$
   BW: body weight (g), BL: body length (cm).
2. Eye index (EI) = $100 * (((EDh + EDv) * 0.25)^2\ \pi * (10 * BL)^{-1})$
   EDh: eye diameter horizontal (mm), EDv: eye diameter vertical (mm).
3. Pectoral fin length index (PFLI) = $100 * PFL\ BL^{-1}$
   PFL: pectoral fin length (cm).
4. The silver index (SI) was calculated according to Durif et al. (2005).

Blood samples were taken from the caudal vein with heparin-flushed (10.000 IU ml^{-1}) 1 ml syringes, which were immediately placed on ice. The blood was centrifuged for 5 min at 14,000 rpm and blood plasma was stored at −80 °C. Liver and gonads were dissected and weighed. The following indices were calculated according to the formulae below: the gonadosomatic index (GSI) and the hepatosomatic index (HSI).

5. Gonadosomatic index (GSI) = $(GW\ BW^{-1}) * 100\%$
   GW: gonad weight (g), BW: body weight (g).
6. Hepatosomatic index (HSI) = $(LW\ BW^{-1}) * 100\%$
   LW: liver weight (g).

Liver tissue was stored in RNAlater (Ambion) at −20 °C for later analysis. Gonad tissue from a standardized rostral location was stored in Bouin solution at room temperature.

2.4. Blood measurements

As vitellogenic indicators in the blood plasma, 17β-estradiol (E2) and calcium (Ca) were chosen. E2 was measured by ELISA (human kit 55030, Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany). Levels of plasma calcium were measured by a colorimetric test (human kit 10011, Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany) and indicated circulating vitellogenin (Vtg). Ca was chosen as indirect measure of plasma Vtg in eels as previously reported by Versonnen et al. (2004) based on the significant positive correlation between Ca and Vtg that has been demonstrated for rainbow trout by Verslycke et al. (2002).

2.5. Histology

To remove the Bouin fixative, the gonads were washed in 0.1 M phosphate buffer and 70% ethanol until the solution became transparent. After dehydrating through an accumulating alcohol series the samples were embedded in air-free Technovit 7100 (Kulzer