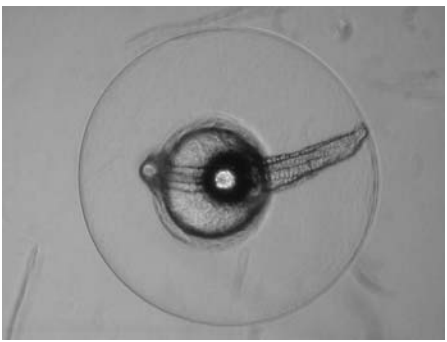

Bred stretched eel embryo 35 hours post fertilisation

Chapter 5

Artificial maturation and reproduction of European silver eel:

development of oocytes during final maturation

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CHAPTER 5

ABSTRACT

Attempts on artificial maturation of European eel (*Anguilla anguilla*) have largely been unsuccessful. The moment of stimulation of final maturation and ovulation is mainly based on weight increase related to the hydration response of the oocytes, which, in the European eel, is irregular. In contrast to Japanese eel, European eels show wide individual variability and much slower response to hormonal stimulation. In this study, the oocyte development of wild European silver eels was followed during final maturation. We describe 7 developmental stages based on 6 parameters: transparency, position and visibility of the nucleus, diameter of the oocyte, and diameter and number of oil droplets. Together, these parameters describe unidirectional changes from immature to over-ripe eggs. The developmental status of the gonads can thus be determined from biopsies. Of 23 female eels, 14 ovulated and were stripped, and 9 gave eggs that could be fertilised. Oocytes mature asynchronously, but this seems to be an artefact since fertility dropped with every new generation. As the timing of ovulation is crucial for fertility of the eggs, our developmental index of oocytes may result in more successful maturation protocols.

INTRODUCTION

Artificial reproduction of Japanese eel (*Anguilla japonica*) became successful with the application of 17, 20 β-dihydroxy-4-pregnen-3-one (DHP) for final maturation and ovulation resulting in fertility and hatching rates of 89.6 and 47.6% respectively (Ohta et al., 1996). DHP was found the most effective steroid for the induction of final maturation in at least eight different fish species (Goetz, 1983; Nagahama, 1987). DHP was also found to induce predictable *in vitro* ovulation of yellow perch (*Perca flavescens*) oocytes (Goetz & Theofan, 1979). This is probably mediated by an effect on prostaglandin synthesis (Goetz, 1983). The latter has been reported to stimulate *in vitro* ovulation of pike (*Esox lucius*) and European eel oocytes (Jalabert, 1976; Epler & Bieniarz, 1978; Epler, 1981). In Japanese eel, DHP was found to induce both final maturation (Yamauchi & Yamamoto, 1982) and ovulation of oocytes (Yamauchi, 1990).

Lokman & Young (2000) used Ohta's (et al., 1996) protocol on New Zealand freshwater eels (*A. dieffenbachii* and *A. australis*). They obtained larvae of *A. australis* and kept them alive for a few days. Recently, Pedersen (2003, 2004) applied variations of the same protocol on European eel and obtained a few larvae that stayed alive for 2 days. Those larvae showed, however, delayed hatching and abnormal morphology. In addition to body weight increase during final maturation Ohta et al. (1996) and Pedersen (2003) used changes in diameter and appearance of oocytes as additional parameters for initiating ovulation. Four oocyte stages were described (Pedersen, 2003): stage 1 (small, black non-transparent cells); stage 2 (larger eggs with a dark-grey cytoplasm containing numerous, small dark oil droplets); stage 3 (the greyish cytoplasm and the oil droplets are more transparent, oil droplets with increased diameter and decreased numbers); stage 4 (migratory nucleus with cytoplasm as well as oil droplets highly transparent). The different stages were, however, not described in detail nor quantified. Although both authors mentioned asynchronous oocyte development, this was not quantified. Time of priming and induction of ovulation may, however, determine fertility.

Low fertility and hatching rates are not restricted to European eel, but are also found with other commercially important fish species, notably marine fish such as Atlantic halibut *Hippoglossus hippoglossus* (Nordberg et al., 1991; Holmefjord et al., 1993; Bromage et al., 1994), sole *Solea solea* (Houghton et al., 1985), turbot *Scophthalmus maximus* (Bromley et al., 1986), gilthead seabream *Sparus auratus* (Carrillo et al., 1989) and some salmonids (Bromage et al., 1992). In this study, we artificially induced maturation of male and female European silver eel from Lake Grevelingen (the Netherlands). Cytological changes during oocyte maturation were studied and categorised. An identification key of oocyte maturation is presented and used to describe final stages of female eel maturation.

METHODS

Experimental animals and period of treatments

Silver eels (male and female) were caught in the fall of 2001 and 2002 during their seaward migration in the brackish Lake Grevelingen (Bout, Bruinisse, The Netherlands) at the North Sea sluice at 32 ppt. After arrival in the lab they were tagged with small passive transponders (TROVAN, EID Aalten BV, Aalten, The Netherlands).
Animals were treated from March 28 until August 5, 2002 (experiment 1) and from January 15 until July 2, 2003 (experiment 2). Experiment 1 was started with 51 males (100-150 g) and 32 females (83.1 ± 7.8 cm, 1160 ± 360 g). Experiment 2 was started with 100 males (100-150 g) and 30 females (72.7 ± 6.0 cm, 733 ± 180 g).

Animal housing & welfare

Males were kept in two 180-l tanks connected to a 2200-l recirculation system in artificial seawater (35 ppt, 18°C) under a 12/12-h light/dark regime. Females were kept in a 1500-l tank connected to a 2400-l recirculation system in artificial seawater (35 ppt, 18°C) under dark conditions. PVC pipes were added to serve as shelter. Both males and females were starved throughout the experiments. All fish received weekly treatments with antibiotics (Flumequin; Flumix, Eurovet, Bladel, The Netherlands, both of 50 mgl^{-1} for 1-2 h). Wounds were sealed with solutions of silver nitrate (1%) and potassium dichromate (1%).

Hormonal treatment protocol

Males were anaesthetised weekly (benzocain, 80 ppm) and injected IP with 125 IU Human Chorionic Gonadotropin (HCG; Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands). Males were checked for spermiation by hand stripping. A drop of sperm was collected in a syringe (1-ml) and mixed with artificial seawater from the holding tanks. Sperm motility was estimated using a microscope. The day before fertilisation, three to five males displaying high sperm motility were selected per female and were IP injected with a single booster dose of 1000 IU HCG (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands). Selected males were transferred to a 500-l tank with water of 20 °C.

Females were weekly anaesthetised (benzocain, 80 ppm) and injected IP with 20-mg carp pituitary extract (CPE; 'Catfish', Den Bosch, The Netherlands). From week 7

onwards, females were weighed two days after injection to determine the body weight index (BWI= body weight/initial body weight x 100). At the final stage, a female was primed by IP injecting a double dose of CPE (Lokman, personal comment). This is in contrast to the single dose primer that Ohta (et al., 1996) and Pedersen (2003) applied. Ovulation was induced by injecting a DHP-solution (2 mg DHP per kg female/175 µl 100% ethanol 1/1 diluted with buffered saline solution) at 8 locations in the ovary. DHP was injected at 21.00 h with the aim of ovulation occurring on the following day. After DHP injection, the female was transferred to a 1000 l tank in a 7000 l re-circulating system with water of 20 °C (2 °C increase; Ohta et al., 1996).

Oocyte development during final maturation and ovulation

Weekly biopsies of the ovary were taken when females started showing larger and softer abdomens. Additional biopsies were made at the time of priming, DHP injection and ovulation, respectively. Oocytes were sampled from a standardised location in the body (5 cm rostral to the genital pore) using an injection needle with an inner diameter of 1.2 mm. Freshly obtained oocytes were observed by phase contrast microscopy (NIKON Eclipse TS100) and photographed with a digital camera (NIKON Coolpix 4500). For measuring diameters of spherical oocytes and fat droplets, a 100x0.01=1 mm standard (Graticules LTD., Tonbridge, Kent, England) was photographed at same magnification. Diameters were measured after using UTHSCSA Image Tool 2.0 on photographs of fresh material. After microscopy and photography, oocytes were preserved in 4% buffered formalin.

Hand-stripping and fertilisation

Occurrence of ovulation was checked between 10 and 24 h after DHP injection. When eggs could be stripped easily, a sample was collected, observed, photographed and preserved as described. Then, males were stripped first. After collecting the sperm of 3 males (1-11 ml per male) motility was estimated by eye. Then the ripe female was anaesthetised and hand-stripped. The abdomen was kept dry and the released oocytes were collected in plastic Petri dishes with only a single layer of oocytes on the bottom. In the first experiment, females were stripped multiple times on the day of ovulation when possible. In the second experiment, females were stripped only once. Oocytes and sperm were mixed with a feather. Artificial seawater was added and the mixture was gently shaken for 30 s (Tanck, personal comment). Within 24 hours after ovulation, females were killed and the remaining gonad was weighed. The GSI was estimated calculating gonad weight/bodyweight x 100, corrected for stripping.

Statistics

Results were calculated and plotted as mean ± standard deviation. Significance in difference between oocyte diameter, fat droplet diameter and number of fat droplets was tested for each type of oocyte vs. the previous type and for each biopsy vs. the previous one with GLM (General Linear Model) repeated measures of SPSS 10.0 for Windows.

RESULTS

I Artificial maturation and reproduction

Male maturation

In both experiments, some males began spermiating after 6 weekly injections. After 7-9 weekly injections, more than half of the males were spermiating lasting for the period of treatment of 25 weekly injections. Selected males for stripping showed sperm motility percentages between 30-50%. At the time of stripping (15-29 h after HCG booster injection) these percentages had increased up to 80-90%. After activation with seawater, sperm motility ceased within 1 minute.

Female maturation

In the first experiment twelve females died during maturation and three females did not show weight increase within 19 weeks. The remaining seventeen females (53.1% of total number of animals) fully matured within 19 weeks (Table 1). Three of these females died showing a decreasing BWI after peaking. During the second experiment twenty-four females died without fully maturing. These deaths were, however, probably due to a virus infection as they had red abdomens and ventral fins. The remaining six females fully matured (Table 1).

Table 1 Final maturation of 23 European female eels with exp.; experiment 1 or 2, inj.; the number of weekly CPE injections (exc. primer and DHP), tag; PIT-tag code, W_i; initial weight, $BWI_{1/2}$; BWI at moment 1 (priming) or 2 (DHP injection), $ECO_{1/2/3}$; the occurrence of an external cluster of extruded oocytes at the end of the oviduct at moment 1 (priming), 2 (DHP injection) or 3 (ovulation), t; the hours after DHP injection when ovulation started, GSI and fate of the females (for more explanation see text).

exp	inj	tag	Wi	BWI_1	BWI_2	ECO_1	ECO_2	ECO_3	t (h)	GSI	fate
2	13	EB3C	853	120.0	131.3	no	no	yes	14.5	37.6	stripped and fertilised
1	15	32A7	1262	112.8		yes	yes	yes	14	41.3	stripped and fertilised
2	15	6C26	784	109.4	109.9	yes	yes	yes	14	43.5	stripped and fertilised
1	16	A162	944	110.3		no	no	yes	24	38.7	stripped and fertilised
1	17	6DEC	1062	113.9	121.8	no	yes	yes	13	44.9	stripped and fertilised
1	18	ECC9	785	118.0	129	no	yes	yes	13		stripped and fertilised
1	19	514E	1136	111.0	119.8	no	no	yes	13		stripped and fertilised
2	23	F9FE	914	111.9	114.2	no	yes	yes	14.5	45.6	stripped and fertilised
2	25	8FDF	604	117.0	120.5	yes	yes	yes	13.5	48.9	stripped and fertilised
1	12	6673	1175	118.1	118.1	yes	yes	yes	<18	51	stripped, no fertilisation attempted
1	14	EA57	1204	111.6	114.1	no	no	yes	24	46.9	stripped, no fertilisation attempted
1	14	OC7O	968	119.1	121.2	no	no	yes	<18	43.3	stripped, no fertilisation attempted
1	16	FD1A	1002	124.9		no	yes	yes	10	36.3	stripped, no fertilisation
1	16	FCB8	1714	137.8		yes	yes	yes	10	60	stripped, no fertilisation
1	15	F184	1290	130.4		yes	yes	yes		41.6	no ovulation
1	15	FA0B	726	104.1		yes	yes	yes		28.2	no ovulation
1	17	O189	1005	106.9	117.5	no	no	no			no ovulation
1	18	E431	795	114.7	121.8	no	yes	yes			no ovulation
1	14	OD51	1112	103.7		no				35.8	died during final maturation
1	15	OAFD	889	102.4		yes					died during final maturation
1	16	E9C1	1202	96.3		yes					died during final maturation
2	18	1692	715	113.9	119.8	no	no			39.2	died during final maturation
2	20	3E61	966	111.3							died during final maturation

The final stage of female maturation and ovulation

During experiment 1, fourteen females were primed, and four did not ovulate (Table 1). Females F184 and FAOB showed dilation at priming. Dilation caused formation of an external cluster of extruded oocytes (ECO) and other gonadal material at the end of the oviduct. Female E431 had oocytes with single fat droplets or burst open at DHP injection and was considered over-ripe (Sugimoto et al., 1976). Ten females successfully ovulated (71.4% of surviving animals) and were stripped. During experiment 2, six females were primed. Female 3E61 died after priming (Table 1). Female 1692 died after DHP injection (Table 1). Four females successfully ovulated and were stripped. Thus, in total, fourteen females were stripped (Table 1). These eels matured between 12-25 injections (16.6 ± 3.7). They showed a BWI of 117 ± 8 (range 109-138) at priming and 120 ± 7 (range 110-131) at injection of DHP (Table 1). They had a GSI of 44.8 ± 6.5 (range 36.3-60.0). Females ovulated between 10 and 24 h after DHP injection in a quite narrow range of 13 to 14.5 h after DHP injection (14.8 ± 4.6). The weight increase between priming and DHP injection varied between 0 and 11.3% (Table 1). Of the 14 stripped females 9 were fertilised. BWIs of these females are depicted in Figure 1. From the other five females, FD1A and FCB8 were over-ripe (Sugimoto et al., 1976) and for three, fertilisation was not attempted because spermiating males were still lacking at that time.

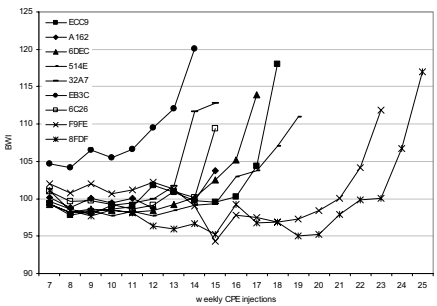

Figure 1 Body weight index (BWI) of maturing female eels with fertilised oocyte batches versus the number of weekly injections.

Fertilisation, cleavage and embryo formation

No attempt was made to fertilise those egg batches obtained by stripping first ovulating females in experiment 1. This is because the males were not spermiating yet at that time. Samples from females FD1A and FCB8 showed that all oocytes were over-ripe (Sugimoto et al., 1976), and fertilisation was not therefore attempted. Fertilisation was attempted and established for oocyte batches from the remaining nine females (Table 1). After transfer to rearing tanks, more than 90% of the eggs from all different batches floated. Sinking eggs soon turned white and were removed. During the first three hours after fertilisation (at 20˚C), eggs from 9 females showed early stages of development (Figure 2a). Most eggs showed meroblastic cleavage up to the eight-cell stage. Later cell divisions became difficult to observe since the percentage of surviving eggs was rather low. Egg batches of females F9FE and 6DEC, however, resulted in the development of about 1500 and 100 embryos respectively (Figure 2b). Embryonic development continued until 100 hours after fertilisation when last embryos died (Palstra et al., 2004b). Hatching was not observed.

a)

b)

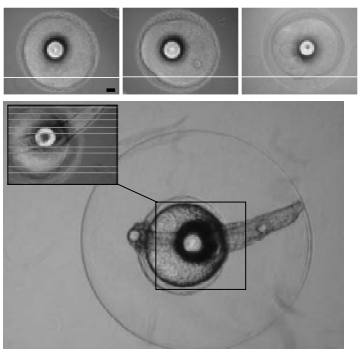

Figure 2 a) Activated eggs within 3 hours after fertilisation in 9 fertilised egg batches (scale bar = 100µm) with first stages of meroblastic cleavage. b) Stretched embryo with developed somites (see insert) at 32 hours after fertilisation reared at 20˚C. (Phase contrast microscopy)

II Development of oocytes during final maturation

Appearance of oocytes during final maturation

In the different biopsies 9 different types of oocytes could be distinguished:

a) Non-transparent, small oocytes.
b) Partially transparent oocytes with a visible central nucleus surrounded by numerous small fat droplets.
c) Larger, fully transparent oocytes with nucleus mostly not visible and with larger fat droplets in the centre.
d) Fully transparent oocytes with the nucleus between centre and periphery (GVM).
e) Fully transparent oocytes with the nucleus at the periphery and larger fat droplets starting to cluster opposite it.
f) Fully transparent oocytes with the nucleus still at the periphery and with even larger fat droplets now completely clustered opposite it.
g) Fully transparent oocytes with no visible nucleus and few large fat droplets.
h) Fully transparent oocytes with no visible nucleus and a single fat droplet.
i) Turbid oocytes with a single fat droplet.

Table 2 lists measurements of oocyte diameters, fat droplet diameters and number of fat droplets found in these types. Every type was significantly different from the previous one for at least one parameter except for type f vs. e, which however differed in number of fat droplets with 100%.

Final stages of oocyte development

Some oocyte characteristics from sequential biopsies of female F9FE (which egg batches showed embryonic development) during final maturation are illustrated in Figure 3 and 4. Figure 3a shows that BWI increased over time with 20%. The percentage transparency increased from 24 at s1 to 53% at s2 but decreased thereafter (Fig. 3b). Oocyte diameters increased only in the first samples s1 and s2 (Fig. 3c) and showed that transparency coincided with hydration. Fat droplet diameters increased while at the same time the number of fat droplets decreased from about 190 in the first biopsy to a few in the last (Fig. 3d). Fat fusion was observed and followed directly in time (Fig. 4). A fusion rate of 7.1 fat droplets per hour was found. Figure 5 shows that the first sample taken 2 weeks before priming (s1) contained mainly type b-c oocytes. Sequential samples taken 1 week before priming (s2), at priming (s3) and at DHP injection (s4) contained all types but less type b and more type g-h. The final sample at stripping (s5) mainly contained type g-h.

Thus, five processes hydration, transparency, fat fusion, GVM and GVBD were found to proceed in time. Hydration occurs in the first stage of final maturation, while fat fusion could be observed to develop over several weeks. As GVM and particularly GVBD are characteristic for the last phase, the 5 processes should describe the development of the oocyte. Using these keys we categorised the distinguished types of oocytes into seven developmental stages of final oocyte maturation (Fig. 6).

Table 2 Parameters of different types of mature oocytes (a-h). Panel A: oocyte diameters. Panel B: fat droplet diameters. Panel C: number of fat droplets per oocyte. Mean, standard deviation (stdev) and range are given and the number of measurements on fat droplets or oocytes from particular samples of particular eels. Significance levels are given for each type vs. the previous type. *** Denotes a significance of P<0.001, ** of P<0.01, * of P<0.05, 0 of P>0.05 and – indicates too few data to test. (For more explanation see text)

a)

type	oocyte diameter (µm) mean	stdev	range	n oocytes	n samples	eels	sign.
a	451	117	316-644	35	3	F9FE, 8FDF	
b	653	70	532-776	29	2	F9FE, 8FDF	***
c	790	38	723-864	29	4	F9FE, 8FDF	***
d	797	29	757-847	11	3	F9FE, 8FDF	0
e	826	39	784-897	11	4	F9FE, 8FDF	**
f	827	45	767-890	6	4	F9FE, 8FDF	-
g	831	38	716-887	25	4	F9FE, 8FDF	0
h	800	61	675-922	32	4	F9FE, 8FDF	0

b)

type	fat droplet diameter (µm) mean	stdev	range	n fat droplets	n oocytes	n samples	eels	
a								
b	32.5	15.9	10.0-61.1	40	2	1	8FDF	
c	42.9	14.5	15.0-81.0	64	4	2	F9FE	**
d	39.9	18.7	11.6-140.3	92	5	3	F9FE, 8FDF	*
e	60.3	25.5	27.6-162.1	83	8	2	F9FE	***
f	80.9	44.1	16.4-202.1	115	7	3	F9FE, 8FDF	0
g	97.5	61.7	18.4-311.7	78	6	2	F9FE, 8FDF	*
h	343	12.4	326.2-354.6	4	4	2	F9FE, 8FDF	***

c)

type	number of fat droplets mean	stdev	range	n oocytes	n samples	eels	
a	>200						
b	>200						
c	215	22	183-233	4	2	F9FE, 6C26	
d	94	22	90-98	4	2	F9FE, 6C26	-
e	115	33	100-130	6	2	F9FE, 6C26	-
f	52	22	34-39	6	2	F9FE, 6C26	-
g	19	2	11-25	10	4	F9FE, 6C26	***
h	1	0		10	4	F9FE, 6C26, EB3C	***

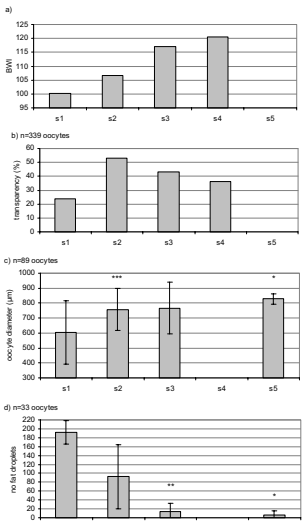

Figure 3 Developmental characteristics of oocytes at final maturation from a single female (F9FE). a) BWI, b) percentage of transparent oocytes, c) oocyte diameter, and d) number of fat droplets. Sample moments are given on the x-axis: s1= 2 weeks before priming, s2= 1 weeks before priming, s3= at priming, s4= at DHP injection and s5= at stripping. Changes in oocyte diameter and number of fat droplets were statistically tested. Significance levels are given for each biopsy vs. the previous one. *** Denotes a significance of P<0.001, ** of P<0.01, * of P<0.05.

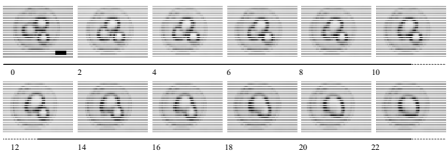

Figure 4 Fat droplet fusion followed in time within a single water activated oocyte (scale bar = 250µm). The axis gives the 2-minute time lapse between each picture.

Developmental stages of eel oocytes during final maturation

Stage 0 opaque oocytes (Table 2: type a).
Stage 1 opaque oocytes with a centred nucleus becoming visible (Table 2: type b).
Stage 2 fully transparent oocyte; fat droplets clustered (Table 2: type c).
Stage 3 fully transparent oocyte with GVM (Table 2: type d).
Stage 4 fully transparent oocyte with nucleus at periphery (Table 2: type e).
Stage 5 fully transparent oocyte with nucleus at periphery with few large fat droplets (Table 2: type f).
Stage 6 fully transparent oocyte with GVBD; few fat droplets (Table 2: type g).
Stage 7 fully transparent oocyte with GVBD; single fat droplet (Table 2: type h).

Appearance of stripped oocytes

Most DHP injected females could be stripped easily resulting in large quantities of transparent oocytes (1007 ± 55 µm, n=7 oocytes from female F9FE). These contained few large fat droplets (137 ± 84 µm, n=538 fat droplets from female F9FE) and the nucleus was not visible (GVBD). Some females (32A7, 6C26, A162) however, still contained large numbers of oocytes (resp. 37.5, 93 and 62%) in which the nucleus was still visible. Of two females (EA57, OC7O) only a small number of oocytes could be stripped containing many small fat droplets and GVM.

DISCUSSION

Male maturation

In this study, spermiation of European eels started after 5 weekly injections. Already one week later high motility sperm was obtained. Pedersen (2003) found spermiation to start after 4 weekly HCG injections and Müller et al. (2002) also after 5 weekly injections. Ohta & Unuma (2003) found the first spermiation in Japanese eel after 5-6 weekly injections. High motility sperm was obtained early in comparison with results of Perez (et al., 2000) who obtained this only after 10 injections. In our study males showed

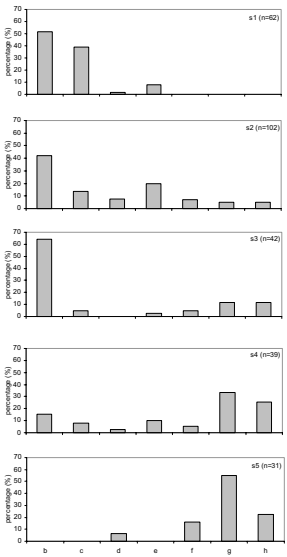

Figure 5 Percentage of oocyte types at final maturation in sequential biopsies (s1-5) from a single female (F9FE) with s1= 2 weeks before priming, s2= 1 weeks before priming, s3= at priming, s4= at DHP injection and s5= at stripping. A gradual shift is displayed from type b-c at s1 to type g at s5.

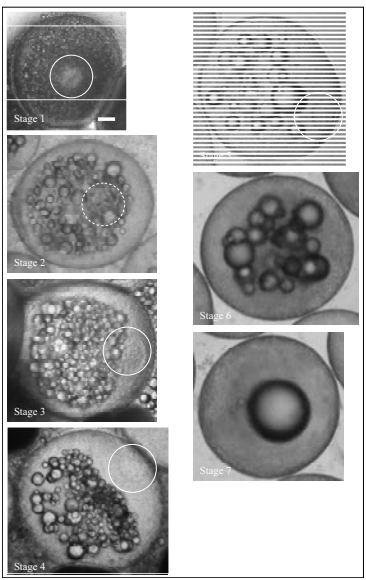

Figure 6 Seven developmental stages in oocyte maturation (scale bar = 100µm). Encircled are the positions of the migrating GV. (Stage 1-5 phase contrast microscopy, stage 6-7 light microscopy). For more explanation see text)

moderate motility (30-50%) and high motility sperm (80-90%) only after a booster dose of 1000 IU HCG. Using farmed eels, Pedersen (2003) obtained sperm motility close to 100% without a booster injection. During the whole experimental period of 6-25 weekly injections, males could be selected with high motility sperm. Sperm was successfully applied for artificial fertilisation within 5 minutes of stripping. The motility of eel sperm after activation was observed to continue for 30-60 s under the microscope, which is comparable to that recorded for most other teleosts (Coward et al., 2002).

Female maturation

Mortality among experimental females was high in the first and second experiment at 37.5% and 80%, respectively. Similar or higher mortalities were found by other research groups but were not reported (personal comments Durif, Pedersen, van Ginneken). Fourteen females were stripped between 12-25 weekly injections. This timing is comparable to that reported by Pedersen (2003) who found maturation after 24-25 weekly injections with wild European eels (n=3: 623-837 g) and 14-22 weekly injections with farmed European eels (n=9: 571-820 g) using a comparable dose of salmon pituitary extract (SPE). Ohta (et al., 1996) reported a range of 9-12 weekly injections with farmed Japanese eels in a weight range of 701-980 g with SPE. The maturation response of European and Japanese eels is depicted in Figure 7. European eel thus shows both a delayed as well as a more extended response in comparison to Japanese eel. These differences seem to be species specific and not a matter of wild vs. farmed eels, weight or the source of the pituitary extract (CPE or SPE). In addition to a highly variable response time we also observed that the body weight increase of European eels is highly variable. From Figure 1 is evident that the slopes of BWI vs. time can be both low and steep. Japanese eel respond also in this matter in a more uniform way. The BWI of Japanese eels increases from 100 to above 110 in one week (Ohta et al., 1996). Thus the increase in female bodyweight is used as a reliable indicator of the last phase of ovarian maturation of Japanese eel (Yamamato et al., 1974; Sugimoto et al., 1976; Oka 1979; Wang et al., 1980; Yamauchi and Yamamoto, 1982; Satoh et al., 1992; Tachiki and Nakagwa, 1993). It appears that a similar procedure is not applicable for European eel (this study, Pedersen 2003). The other approach to predict the right time for final maturation can be the evaluation of the developmental stages of the oocytes in the ovary.

Oocyte maturation

Non-transparent oocytes that are found until final maturation are small and fully filled with fat droplets, which are products of the secondary yolk / midvitellogenic stage (Adachi et al., 2003). Fast growth and increase in transparency occurs in the tertiary yolk stage / late vitellogenic stage (Adachi et al., 2003). The latter is considered as a result of fusion of yolk globules (reviewed in Wallace and Selman, 1981). Oocytes now undergo their final maturation during which the chromosomes resume meiosis and proceed to the second meiotic metaphase with the concomitant formation of the first polar body (Goetz, 1983). The increase in transparency coincides with swelling of the oocytes due to hydration (up to 800-900 µm in this study). Pronounced hydration up to 30% between opaque oocytes of maximum diameter and fully transparent oocytes was observed in this study which corresponds with other marine teleosts spawning pelagic eggs (Wallace and Selman, 1981).

European eel spawns up to 4 million eggs which are not sticky and which rise to the water surface with a speed of over 2 meters per hour (van Ginneken et al., 2005a). Simultaneous with hydration we observed also fusion of fat droplets (Figs. 3 and 4), which in all cases caused a reduction from >200 to a few droplets (10-1).

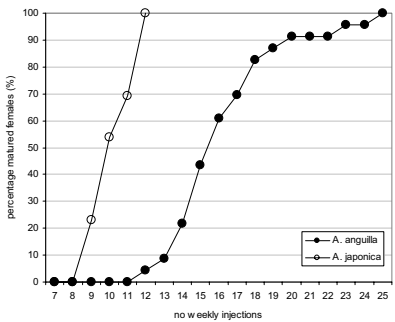

Figure 7 Frequency of occurrence of matured females of *A. anguilla* (this study) vs. *A. japonica* (Ohta et al., 1996). *A. anguilla* shows a much slower response to injection of pituitary extract.

Some discrepancy exists between different authors with respect to the diameter of the European eel egg (Boetius & Boetius, 1980). In literature a wide range of diameters is reported. In most cases the stage of the oocyte was not clear and therefore comparison is restricted to results of Pedersen (2003) and Japanese eel. Ohta (et al., 1997) and Adachi (et al., 2003) mention diameters of oocytes at GVM of 700-800 µm in Japanese eel. Oocytes at GVM in European eel have diameters of 800 µm (this study). Pedersen (2003) reports diameters of 700-850 µm. Adachi (et al., 2003) mentions diameters of oocytes where the germinal vescicle reaches the periphery of 850-900 µm. Oocytes from European eel in this stage are 750-950 µm (this study). Pedersen reports diameters of 750-860 µm. No significant differences seem to exist between the two species. In this study, oocyte hydration occurred only up to stage 2. Oocyte diameter did not continue to change significantly indicating that further hydration stopped. Therefore, we can conclude that the oocyte diameter does not suffice as discrimination tool for oocyte development of European

eel. Until the time of spawning, the GV moves forwards to the periphery as the lipid droplets coalesce. Finally, prior to ovulation, the GV migrates a short distance to the surface of the oocyte after which it breaks down (GVBD). The time and rate at which the GV migrates to a peripheral position varies between species. In this study, oocytes showed GVM for about 48 hours although individual differences were high.

Fat droplets were measured and counted per individual oocyte stage (Table 2). Counting fat droplets in oocyte stage 1 was not possible because of limited transparency and high numbers (>200). Fat droplet numbers did not differ significantly between stages 3, 4 and 5 (GVM to periphery). Concerning lipid coalescence in higher teleosts, Goetz (1983) states that the degree of lipid coalescence follows a phylogenetic pattern. Lipid coalescence in ovulated oocytes of higher teleosts results in the formation of one major fat droplet (reviewed by Goetz, 1983). In contrast, ovulated oocytes in lower teleosts still contain a large number of lipid droplets (reviewed by Goetz, 1983). Goetz (1983) states that European eel, like Japanese eel, is a major exception to this trend in which one to several large lipid droplets are present in oocytes following GVBD (Epler and Bieniearz, 1978; Yamauchi and Yamamoto, 1974). Although this is true for most observed oocytes in this study, we also found oocytes with single fat droplets still containing a peripheral nucleus. Oocytes with single fat droplets soon turned over-ripe. Females peaking in BWI (females FD1A, FCB8) possessed large quantities of over-ripe oocytes and were not fertile. DHP sensitivity dropped since most over-ripe females could not be induced to ovulate (females F184, FAOB, E431). Soon after a peak in BWI females developed an ECO and ovulated spontaneously.

Application of the oocyte maturation key

The seven developmental oocyte stages were categorised in an identification key. This key was used to determine the average maturation stage of oocyte samples. Figure 8 shows average stages of individual females of which batches were fertilised. Individual variation in developmental speed is clear. Administration of a CPE booster causes a change of –0.3 up to 3.4 stages a day later. In most cases less developed batches showed greatest response. After DHP administration development in most cases continued either induced still by CPE or by DHP. Individual maturation in these females converges towards the moment of ovulation. On average oocyte maturation stage was 4.0 ± 1.2 at CPE injection, 5.1 ± 1.2 at DHP injection and females ovulated at 5.9 ± 0.5 ($P<0.01$ vs. stage at CPE injection). On average oocyte batches developed with speeds of 1.1 stage after CPE injection and 0.7 stage after DHP injection. Females 6DEC and F9FE of which eggs showed embryological development ovulated at average oocyte stage 5.9 reflecting fully transparent oocytes with GVBD and only few fat droplets.

Synchronous or asynchronous ovarian development?

In literature, eels are considered having synchronous ovaries typical for teleosts spawning once and then die (Wallace & Selman, 1981). In this study, ovaries showed asynchronous development of oocytes maturing over several generations (Figure 5). Most eels ovulated more than once over periods up to several days. These findings are supported by other authors concerning European eel (Bezdenezhnykh & Prokhorcik, 1984; Pedersen, 2003), New Zealand eel (Lokman & Young, 2000) and also Japanese eel (Pedersen, 2003).

However, as discussed before, we were not able to fertilise other than first stripped batches. Also, in some cases females were stripped almost completely empty. These females showed a large first generation oocytes. In the case of small early oocyte generations we attempted to induce ovulation of later ones although fertility dropped. These observations support the idea that asynchronous oocyte development has an artificial rather than a natural origin.

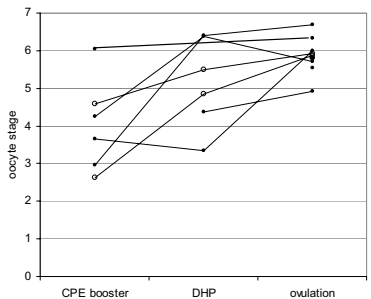

Figure 8 Average oocyte developmental stages in biopsies of individual eels of which eggs were fertilised (lines) at CPE booster injection, DHP injection and ovulation. Lines connect samples from the same female. Open circles reflect batches that showed embryological formation.

Oocyte stage and ovulation time

In this study ovulation was induced between 10 and 24 h after DHP injection with most females (8 out of 14) in a quite narrow range of 13-14.5 h after DHP injection. Pedersen found comparable ovulation times between 13.5 and 17.5 h after DHP injection. Ohta (et al., 1996) and Kagawa (et al., 1997) found ovulating females of Japanese eel between 15 and 21 h after DHP injection, independent on circadian rhythm (Kagawa, 2003). It might be that oocytes of European eel are more sensitive to DHP than those of Japanese eel. Differences in oocyte appearance clearly exist between both species (Pedersen, personal comment). Differences in timing of ovulation are, however, at least partly, determined by the developmental stage and diameter of the oocytes. In this study we found indications of a negative correlation between timing of ovulation and development of

oocytes. Goetz & Theofan (1979) and Goetz (1983) confirm this, although the level of synchrony between DHP as inducer of final maturation and ovulation at the used dose is uncertain. Correlation between timing of ovulation and oocyte diameter was found by Ohta (et al., 1997). *In vitro* experiments on Japanese eel showed that oocytes between 700-800 µm were sensitive to DHP (Ohta et al., 1997). Oocytes over 800 µm in diameter became more sensitive to the steroid (Ohta et al., 1997). For DHP induced ovulation of Japanese eel, a minimum oocyte diameter of 750 µm is used as a criterion (Pedersen, personal comment). Oocytes of European eel in this and Pedersen's (2003) study were, on average, larger in comparison with Japanese eel (Kagawa et al., 1995; Ohta et al., 1997) at the time of DHP injection. Oocytes at the desired developmental stage need to be induced to ovulate within 17 hours after DHP injection since Ohta (et al., 1996) found fertility and also hatching rates decreasing rapidly after.
European eel shows a highly individual response in timing and speed of maturation in contrast to Japanese eel. Therefore, BWI is an unreliable indicator of the last phase of ovarian maturation of European eel. Hence other tools are necessary to quantify the maturation stage of oocyte samples. In this study, seven oocyte maturation stages were categorised in an identification key. We used this key to determine the average maturation stage of oocyte samples. The average stage, level of transparency and oocyte diameters proved to be useful complementary characteristics in quantifying the individual maturation status.

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