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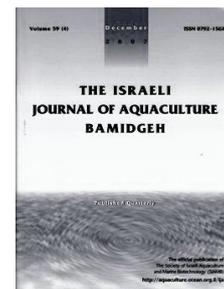
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Induction of Sexual Maturation in Wild Female European Eels (*Anguilla anguilla*) in Darkness and Light

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Abstract

Sexual maturity was induced in wild silver eel females of the European eel (*Anguilla anguilla*) using a protocol similar to that successfully used for *Anguilla japonica*. Eels were injected for sixteen weeks with increasing doses of carp pituitary extract (10, 20, 30, and 40 mg CPE/kg body wt) and kept in natural light or completely dark conditions. In both groups, final oocyte maturation and ovulation were induced by injection of 17,20 h-dihydroxy-4-pregnen-3-one (DHP) at 2 mg/g body wt. All sixty eels turned out to be silver and actively migrant (58 in stage V and two in stage IV), showing a particularly high eye index (15.75 ± 1.40), and distributed within three age classes (7-9 years). In the group kept in darkness, one of the four females ovulated 12 h after DHP injection in the thirteenth week. The remaining three females received the final (16th) CPE injection and ovulated 12-36 h after the DHP injection. In the group kept in light, two of the four females received the 16th CPE injection and ovulated 12-36 h after the DHP injection; the remaining two did not ovulate within 36 h post DHP injection. The total number of eggs released by the group kept in darkness (1,485,600) was significantly higher than that of the group kept in light (274,000). There was no mortality during the experiment.

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Introduction

Eels are amongst the organisms with the highest market potential for freshwater aquaculture in the world. To date, all seedlings for cultivation are wild glass eels or elvers collected in estuarine waters. However, in East Asia and Europe, the catch of glass eels differs greatly from year to year and, especially in the past thirty years, has decreased, resulting in a sharp rise in the price of seedlings. Natural stocks of eels, especially the commercially valuable temperate species, European eel (*Anguilla anguilla*), American eel (*A. rostrata*), and Japanese eel (*A. japonica*), have decreased markedly due to structural changes in water courses caused by new barrages and dams that prevent the comeback of juveniles, increasing pollution and contamination with toxic polychlorinated biphenyls (PCB), infection by the swim bladder parasite, *Anguillicola crassus*, viruses, oceanographic and climatic changes, overfishing, and other yet unknown factors (EELREP, 2006; van Ginneken and Maes, 2005).

The European eel is included on the Red List of the International Union for Conservation of Nature (IUCN) as a Critically Endangered Species. Consequently, it is protected by the imposition of a short fishing season, minimum size for capture, protection of larvae, and careful regulation of the fish trade. Unfortunately these measures are not enough to eliminate the danger of extinction for this mysterious fish. To prevent depletion of the natural glass eel stock and provide reliable supplies of seedlings for aquaculture, development of an artificially-induced breeding procedure is highly desirable.

Artificially induced breeding techniques for the three most common eel species have focused on hormonally-induced oocyte maturation and ovulation in females and spermatozoa maturation in males in sexually immature eels. The first successful ovarian development and ovulation in female silver eel *A. anguilla* were obtained using repeated injections of carp pituitary extract (CPE) and a final injection of deoxycorticosterone (Fontaine et al., 1964). Since then, such experimental procedures have been used extensively and good quality eggs were successfully obtained in a number of studies. However, the proportion of ovulating females and number of eggs produced was low, as were the fertility and hatchability of such eggs (EELREP, 2006; van Ginneken et al., 2005) - not high enough for commercial-type activity.

Subsequent studies focused on fine-tuning successful protocols: dose and rhythm of hormone injections, timing and type of ovulation primers, swimming and body indices to predict ovulation, and optimal environmental parameters such as water temperature, salinity, and pressure (Ohta et al., 1997; Kagawa et al., 1998; Pedersen, 2004; EELREP, 2006; Palstra et al., 2005, 2007; Dou et al., 2008).

Photoperiod is one of the most important environmental factors that regulate fish physiology and metabolism. Consequently, photoperiod can be manipulated to improve performance, profitability, and sustainability of aquaculture activities, especially the daily endogenous rhythms of fish that advance or delay gonadal maturation, spawning period, and somatic growth (Nilsson et al., 1981; Rodriguez et al., 2009).

The aim of present study is to understand the impact of photoperiod regimen on gonad weight, ovarian development, and egg production in the silver eel. Sexual maturation was induced in two groups of eels (one kept in light and one in dark) by a modification of the standard gonadotropic treatment (carp or salmon pituitary extract followed by 17, 20 β -dihydroxy-4-pregnen-3-one injection). The process of maturation was evaluated using internal and external indicators, then compared to the initial physiological and morphological characteristics.

Materials and Methods

Wild eels (*Anguilla anguilla*) were caught in early March 2010 by traditional "lavoriero" (downstream trap system allowing capture of eels during migration to sea) in a brackish water lagoon (10°C; salinity 26‰) near sluices of the North Adriatic Sea in Val Noghera, Friuli Venezia Giulia, Italy. Sixty large females (>450 g) were selected and transported to the laboratory where they were measured and sampled, and maturation stage was determined by external indicators (Durif et al., 2006). Body length, body weight,

horizontal eye diameter, vertical eye diameter, and pectoral fin length were measured. Condition factor (K) was determined as $100(\text{body wt}/\text{body length}^3)$, eye index as $100(\text{EDh} + \text{EDv})(0.25^2)(\pi)10(\text{body length})$ where EDh = horizontal eye diameter and EDv = vertical eye diameter, and pectoral fin length index as $100(\text{pectoral fin length}/\text{body length})$. The stage of the eels relative to the silvering process was determined according to Durif et al. (2005).

Sixteen eels were randomly selected as controls. Twelve others were sacrificed with an overdose of anesthetic 2-phenoxyethanol, their gonads were carefully excised and weighed, and the gonadosomatic index (GSI) was calculated as $100(\text{gonad wt}/\text{body wt})$. Gonad samples were collected for histological analysis. Small pieces of gonads were taken from the caudal, central, and cranial regions and immediately fixed in 10% buffered formalin. Subsequently, they were dehydrated in a graded ethanol series and embedded in paraffin. Histological sections (4 μm) were cut, stained with hematoxylin and eosin (H&E), and evaluated under a light microscope to assess the maturation state according to Kagawa (2005). Twenty oocytes per sample were randomly chosen, four diameters per oocyte were measured, and the mean oocyte diameter was determined using Lucia software (Nikon UK, Kingston-upon-Thames, UK). Otoliths were collected during each sacrifice and samples were prepared according to Durif et al. (2006). Age was determined by considering the first ring as Year 1 of the eel's life.

Eels were kept in natural sea water in a 3,600-l tank connected to a recirculation system and maintained in outdoor conditions throughout the experiment. Salinity ranged 28-33‰, water temperature 11-24°C, and the photoperiod naturally lengthened from 12L:12D to 15L:9D. After a week of acclimation to local seawater conditions (salinity 32‰), 32 silver eels were randomly divided in two experimental groups (dark and light) and kept in two 700-l tanks. Unlike the temperature used in most maturation-inducing experiments using sexually immature eels (20-24°C; Ohta et al., 1996; Pedersen, 2003, 2004; Dou et al., 2007, 2008), we chose the lower temperature of $15.5 \pm 0.5^\circ\text{C}$ to approximate the temperature in Val Noghiera at the start of the reproductive oceanic migration. Also, low temperatures are more likely to reduce individual variations in sensitivity to gonadotropic treatments than high temperatures, which lead to more uniform responses to hormone treatment (EELREP, 2006).

The eels were individually marked by fish-tags (Floy Tag Mod Floy T-Bar Anchor). One group was gradually brought over a period of seven days to completely dark conditions (24 h/day without light; -0.04×10^3 lux at the bottom of the aquarium without water). The second group was gradually brought over a period of seven days to light conditions (0.40×10^3 lux at the bottom of the aquarium without water during 06:00-20:00) using three 36W/950 halogen lamps placed 30 cm above the water surface. The eels were fed daily *ad libitum* a mixture of Gadidae fresh ovary (Grandi et al., 2000). Eels were disinfected weekly with peracetic acid (oxygen, 0.01/ml for 10 min). Light and dark groups stopped eating after 45 days while the control group continued eating for the entire duration of the trial.

The light and dark groups received weekly intramuscular injections of carp pituitary extract (CPE; Palstra et al., 2005). The injection dose was increased from 10 mg/kg body wt during weeks 1-3, to 20 mg/kg during weeks 4-6, 30 mg/kg during weeks 7-9, and 40 mg/kg during weeks 10-16. At weeks 5, 9, and 13, four eels from each group were randomly selected and sacrificed for gonad histological analysis and to determine GSI. At the same time the animals were weighed and the body weight index (BWI) was calculated as $100(\text{body wt}/\text{initial body wt})$.

Twenty-four hours after the last CPE injection, the eels were weighed and ovulation was induced by injecting 10 locations in the ovary with 2 mg of 17,20 h-dihydroxy-4-pregnen-3-one (DHP)/kg body wt dissolved in 95% ethanol and diluted with buffered saline solution (Palstra et al., 2005). Ovulation was checked 12, 24, and 36 h after the DHP injection by applying gentle pressure on the abdomen in an anterior to posterior direction (Ohta et al., 1996). Immediately after stripping, eggs were weighed, four samples of 500 mg eggs/female were transferred to a petri dish with a glass pipette and counted under a stereoscopic microscope, and diameters were measured.

At the end of the experiment, the last four eels in each group were weighed and sacrificed to determine final BWI and GSI. Residual gonads (eggs not released but retained in the abdominal cavity) were removed and weighed, and parts of the gonads were fixed for histological analysis.

Body wt index, GSI, histological data, and reproductive performance were statistically analyzed using Student's *t* test where differences between treatments were considered statistically significant when $p \leq 0.05$.

Results

There was no mortality during the experiment and no eels were removed because of disease. Initial measurements are given in Table 1. The eye index was particularly high.

Table 1. Initial characteristics of silver eels, *Anguilla anguilla* ($n = 60$) used in the experiment.

External ($n = 60$)	
Body wt (g)	572.4±87.5
Body length (cm)	68.2±4.6
Condition factor (K)	1.82±0.38
Eye index	15.75±1.40
Pectoral fin length index	5.5±0.4
Silver index	IV-V
Internal ($n = 12$)	
Gonadosomatic index	1.51±0.21
Age (year)	7-9

All eels were silver and actively migrant: 58 eels were in stage V and only 2 in stage IV. GSI varied little and age was not directly related to body weight.

BWI rose in eels kept in darkness from week 5 onwards and in eels kept in light from week 13; by week 19, the mean BWI in the dark group increased 139.7% and in the light group 119.2% (Fig. 1). The mean BWI increase after the DHP treatment in week 12 (first stripping) was 10.4% (from 129.3 to 139.7) in the dark group, 9.8% (from 123.2 to 133.0) in the two ovulated females in the light group, and 1.5% (from 115.1 to 116.6) in the two females of

the light group that did not ovulate. Oocyte diameters increased with maturation.

The gonad tissue was organized in lamellae, supported by stroma rich in adipose tissue (Palstra et al., 2007). Control eels had oocytes in the previtellogenic stage (oil drop stage) throughout the trial where they had a large central round nucleus (or germinal vesicle), multiple nucleoli, and abundant cortical alveoli that completely filled the cytoplasm. At 5 weeks, eels in the light and dark groups had oocytes in the early vitellogenic stage (primary yolk globule stage) where they had abundant cytoplasm, still-prominent cortical alveoli, and the first yolk globules appeared around the germinal vesicle (Fig. 2). By week 9, eels in both groups had oocytes still in the early vitellogenic stage; cortical alveoli entirely filled the cytoplasm, densely-staining yolk globules were more abundant, and the nucleus was still centrally located. In week 13, eels from the light and dark groups had oocytes in mid-vitellogenesis; cortical alveoli fused together forming large lipid droplets and most of the cytoplasm was filled with yolk globules. The three gonadal segments (caudal, central, cranial) of all animals displayed the same developmental stage.

Histological analysis of the eggs of stripped animals showed cytoplasm entirely occupied by large yolk globules and a nucleus that had completed migration to the cell periphery.

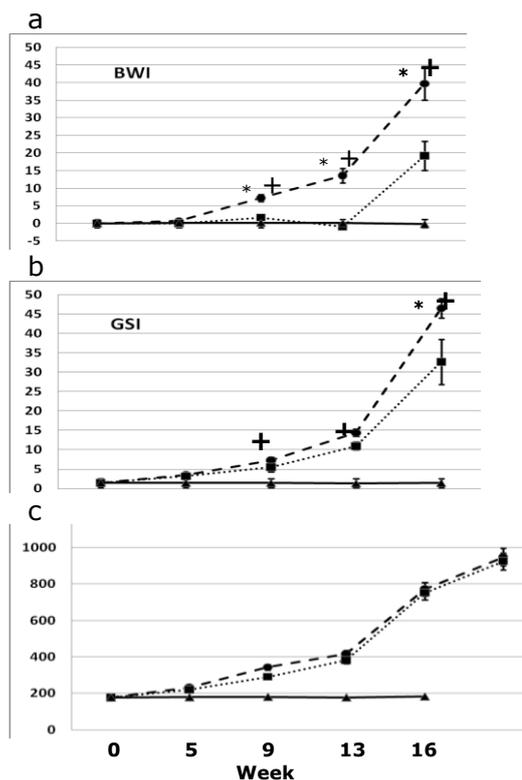


Fig. 1. (a) Body weight index (BWI), (b) gonadosomatic index (GSI), and (c) oocyte diameter (μm) of hormonally-treated European eels (*Anguilla anguilla*) kept in total darkness (dashed line) or natural photoperiod (dotted line), and of control eels that were not hormonally treated. * significant difference ($p < 0.05$) between dark and light groups; + significant difference ($p < 0.05$) between treated and control eels.

Histological analysis of residual gonads showed oocytes with cytoplasm entirely occupied by large yolk globules but a nucleus that had not completed migration to the cell periphery.

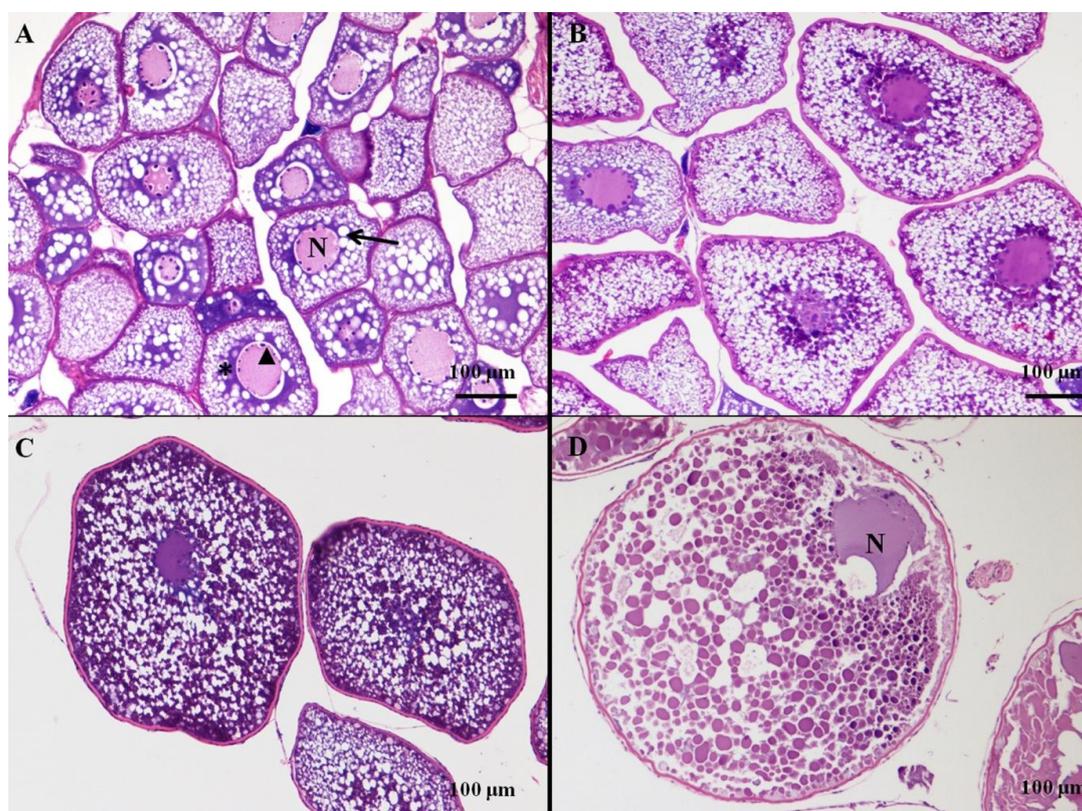


Fig. 2. Histological analysis of the oocytes: (A) week 5, oocytes in early vitellogenetic stage, nucleus (N) is central, and multiple nucleoli (arrowhead) are peripherally located, (B) week 9, oocytes still in early vitellogenetic stage and nucleus still centrally located, (C) week 13, oocytes in mid-vitellogenesis, cortical alveoli beginning to fuse together, and yolk globules fill most of the cytoplasm, (D) week 19, eggs after stripping show cytoplasm entirely occupied by large yolk globules, cortical alveoli fused together to form large lipid droplets, and nucleus (N) migrated to cell periphery (H&E staining).

Table 2. Zootechnical performance of female eels after sixteen weekly injections of carp pituitary extract and one injection of 17,20 h-dihydroxy-4-pregnen-3-one (DHP).

Eel no.	Gonad wt (g)		GSI	Spawned eggs after DHP injection (g)			Stripped eggs (%)	Eggs from ovulated females			
	Total	Residual		12 h	24 h	36 h		Total	per g	per eel	per kg body wt
Dark group											
D13	482.6	275.2	44.7	15.5	145.5	46.5	207.5	43.0	1,644±27	348,600	323,100
D14	537.0	282.2	49.2	-	206.9	47.9	254.8	47.5	1,682±30	428,100	392,400
D15	400.4	97.2	45.9	223.0	64.0	16.2	303.2	75.7	1,720±28	509,000	583,700
D16	250.1	131.5	46.2	37.5	66.0	15.5	119.0	47.5	1,670±29	199,900	369,500
Total spawned eggs dark grp (g)				276.0	482.4	126.1	884.5				
Light group											
L13	283.8	169.5	41.0	-	82.5	31.8	114.3	40.3	1,717±26	192,000	277,500
L14	175.1	175.1	28.0	-	-	-	-	-	-	-	-
L15	247.6	198.8	37.5	-	-	48.8	48.8	19.7	1,647±21	82,000	124,100
L16	134.2	134.2	24.3	-	-	-	-	-	-	-	-
Total spawned eggs light grp (g)				-	82.5	80.6	163.1				
Mean no. eggs per kg body wt dark group										417,200±99,300*	
Mean no. eggs per kg body wt light group										200,800±76,700	
Total no. eggs dark group									1,485,600*		
Total no. eggs light group									274,000		

* significant difference between groups ($p < 0.05$)

Ovulated eggs were not released into the water but were retained in the abdominal cavity. There were small losses of eggs during handling. One of the four eels of the dark group had a BWI of over 120, 24 h after the 13th CPE injection. On that day, it was given a DHP injection that prompted ovulation 12 h later. The remaining three females received the 16th CPE injection and ovulated 12-36 h after the DHP injection (Table 2). Of the four females in the light group, two ovulated 24-36 h after DHP induction following the 16th CPE injection and two did not ovulate within 36 h after DHP injection.

Female D15 was the most productive. It released 73.5% of its ovulation only 12 h after DHP induction and produced 303.2 g of released eggs with only 97.2 g oocytes remaining in the abdominal cavity. Females in the light group did not ovulate within 12 h after injection. Production peaks were obtained 24 h after DHP induction with 54.5% and 82.5% of the eggs released in the dark and light groups, respectively. Eels D13 and D14 released 70.1% and 81.2% of the total weight of the gonad. By 36 h after DHP injection, eels in the dark group did not exceed 15% of the weight of the gonad whereas eel L15 released 100% of its eggs. The number of eggs/g and weight ($0.59 \pm 7.9 \times 10^3$ mg) of ovulated females were homogeneous did not statistically differ within or between the light and dark groups although the total number of eggs released by eels kept in the dark was significantly higher than the number released by those kept in the light.

Discussion

The Val Noghera lagoon, in the northern Adriatic Sea, has outstanding environmental characteristics in which it is still possible to find female eels with a high silver index that can be used in captivity to start a program of artificial reproduction and to restore conditions for hosting and breeding eels. Good reproductive responses can be obtained from females in advanced initial stages of maturation (Durif et al., 2006). Indeed, in our study all the eels were in migrating stages IV and V (high silver index) which is a required for female eel sensitivity to gonadotropic stimulation (EELREP, 2006). The captured female eels had a GSI typical of migrant eels (Durif, 2003). The eye index in our eels was about 50% higher than in other in Stage V eels (Durif et al., 2005). There was good correlation between initial ocular index and maturation sensitivity, as in past studies (EELREP, 2006). The high silvering value of the Val Noghera eels presumably stems from the fact that the eels were caught in March, a few months after the transition from stage III (pre-migrating) to IV (migrating) that probably occurs at the end of the summer/early autumn, i.e., September-November (Durif, 2003). In contrast, eels captured in Lake Grevelingen (Netherlands) reached the highest stage of gonad development in the fall and this is why specimens are collected for hormonal treatment at that time, just prior to seaward migration (van Ginneken et al., 2005, 2007). Therefore, the rate of maturation may not be only species-dependent, but may also be environmental-dependent.

In some cases, eels stop feeding at this silvering stage (Durif et al., 2005). However, in our study, while treated eels stopped feeding after the 6th hormonal injection (day 45), control eels continued feeding throughout the experiment. This may be because our eels were collected in a natural enclosed lagoon where they were unable to undertake migration. Downstream migration is flexible and, if environmental conditions are unfavorable, e.g., there are barriers or dams, eels stop migrating for long periods of time, even months; eels that miss a favorable 'environmental window' probably stop silvering and revert to the growth phase (Durif et al., 2003, 2005). Silvering is much more flexible than assumed, and eels can stop metamorphosing and resume feeding if the chance of successfully migrating is compromised (Svedäng and Wickström (1997). Probably in our case, hormonal induction and favorable environmental conditions in the tanks restarted the silvering process. Moreover, the eels were fed a mixture of Gadidae fresh ovary which is high in n-3 fatty acids and effective in feeding eel broodstocks (Furuita et al., 2007).

The GSI and BWI showed positive trends and progressive gonad maturation in the hormonally-treated eels, especially among those kept in dark conditions. In *A. japonica*

the increase in female BW (close to 10% per week) is a reliable indicator of the last phase of ovarian maturation (Ohta et al., 1996). In our study, however, weight gain did not appear to be enough to initiate ovulation with DHP. Our results show that the production of eggs was increased in eels with a BWI around 140%, while eels with a BWI below 120% were un-ovulated females. The GSI obtained in our study agrees with that of ovulated *A. anguilla* (44.8 ± 6.5 ; Palstra et al., 2005).

Our histological analysis showed a positive trend and progressive oocyte maturation in the three gonadal segments in treated eels, typical of synchronous species (Murua et al., 2006). In contrast, ovarian development is asynchronous in eels kept in captivity, supporting the idea that asynchronous oocyte development has an artificial rather than natural origin (Palstra et al., 2005). The homogeneity of oocytes in our study probably results from the low-dosage of hormone initially administered to obtain synchronization of maturation in the eels. The dosage was subsequently increased, allowing the oocytes time to mature throughout the study. At the end of the hormonal treatment, there were small differences in the vitellogenic stage, probably related to the sudden acceleration of the maturation process. Synchronizing female maturation with male maturation within 4-5 weeks by intramuscular injection of human chorionic gonadotropin (HCG; Pedersen, 2003; Kagawa et al., 2009) is desirable in artificial reproduction because the time window for high-quality eggs is very narrow (Ijiri et al., 2011).

One ovulated female was stripped after 13 weekly injections and five were stripped after 16 weekly injections. Similarly, 71% eels matured and were stripped after 12-17 injections (Palstra et al., 2005). On the other hand, using salmon pituitary extract (SPE), wild European eels matured after 24-25 weekly injections in spring and farmed European eels matured after 14-22 weekly injections in winter (Pedersen, 2003). A range of 9-12 weekly injections of SPE were required for farmed Japanese eels ranging 701-980 g (Ohta et al., 1996). Thus, the European eel shows both a delay in response to treatment and a longer maturation period than the Japanese eel. These differences seem to be species specific and not a matter of wild vs. farmed eels, weight, source of the pituitary extract (CPE or SPE), or season (Palstra et al., 2005).

While the percentage of ovulated females in similar studies is fairly low (Ohta et al., 1996; Pedersen, 2003; EELREP, 2006; Kagawa et al., 2005; van Ginneken et al., 2005), we had a 100% success; every female in the dark group ovulated. Further, our protocol, based on increasing doses of CPE, resulted in a significant amount of eggs in females kept in dark conditions; eels kept in the light had a lower response to the hormonal stimuli, highlighting the positive effect of darkness on egg production. Our absolute fertility was lower than the 772,000-3,945,000 eggs/female reported by van Ginneken et al. (2005). However, their values may be overestimated because they were based on oocyte weight and GSI rather than the actual weight of the ovulated eggs.

The egg size and transparency, and the complete migration of the nucleus suggest good egg quality. The oocyte diameter at the migratory nucleus stage and that of the ovulated eggs are similar in *A. japonica* (Ohta et al., 1996; Tanaka et al., 2003; Kagawa et al., 2005) and *A. anguilla* (Pedersen, 2003, 2004; Palstra et al., 2005, 2010). Ovulation required up to 36 h after DHP injection, longer than the 15-23 h recorded in Japanese eel (Ohta et al., 1996; Tanaka et al., 2003) and European eel (Pedersen, 2003, 2004; Palstra et al., 2005), probably because our lower water temperature caused a delay in response to hormonal treatment. Such a delay did not affect the egg quality and we obtained oocytes without cytoplasmic degeneration, unlike Ohta et al. (1997).

The absence of mortality during the experiment is another very positive result considering that several studies with European eels reported high mortality rates (Pedersen, 2003, 2004; EELREP, 2006; Palstra et al., 2005, 2010; van Ginneken et al., 2005). The lack of mortality can be explained in many ways, but the hardiness of the eels and the lower doses of hormone used during the first six weeks certainly helped reduce stress.

In conclusion, the high silvering value of the Val Noghera eels contributed to the success of the artificial reproduction trial. The great gonadal maturation and high egg production of the eels kept in darkness show that darkness is conducive to good

reproductive performance. Finally, the adopted protocol, i.e., increasing doses of CPE, appears to have contributed to synchronization of maturation, absence of mortality, and high production of good quality eggs.

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