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Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared

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Abstract This study aimed to describe the response variability of female silver eels in terms of gonad development and eggs production to a standardized gonadotropic treatment (Carp pituitary extracts—CPE), and to relate this variability to population characteristics. For this purpose, sexual maturation, ovulation, and fertilization were induced in two eel populations coming from different locations in Adriatic Sea (*Comacchio*—CM and *Marano-Grado*—MG lagoons), and after that, their reproductive capacity was valuated. External (Silver index—SI, Eye index—EI, Pectoral fin length index—PFLI, Condition factor—K) and hormonal (17β-estradiol—E2, testosterone—T) parameters were measured, and some subject/group were killed for histological and lipid analysis and age determination. Morphometric parameters showed the CM-Group to have highest values of Body weight (BW), Body length (BL), and K, while MG-Group presented highest PFLI and Gonadosomatic index (GSI) values. Regarding hormonal analysis, the CM-Group showed significantly higher T and E2 levels than the MG-Group, both groups showed considerably rapid increase at T5 (5th injection). A positive trend in gonadal development was found through histological evaluation; a more regular maturation was observed in the MG-Group, whereas the CM-Group presented an exponential oocytes development starting from T10 (10th week), which led to an anticipated spawning. Lipid content showed significant differences in T0 (start study), post-ovulation, and Control (30th week) between CM and MG eels. As to zootechnical performances, while MG eels released spontaneously into the water, the CMs were stripped in order to check ovulation. The MG eels were statistically the most productive with 40.1 ± 6.33 % BW of eggs released. Furthermore, CM females ovulated mainly between the 19th and 22nd week (77.8 % spawned eels) instead in the MG's ovulation goes from the 24th to the 28th week (100 % spawned eels). As fertilization is of concern, in both groups fertilized eggs were obtained with no difference in larvae production. These results seem to indicate that bigger dimensions, higher K, and larger lipid content (Comacchio eels) could fasten gonadic maturation without positively influencing reproductive performance of animals, both in term of quantity and quality of

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produced eggs. Smaller females with a highest SI (Marano-Grado eels) presented a more regular gonadic development, leading the animals to spontaneous spawning.

Keywords European eel (*Anguilla anguilla*) · Silver index · Artificial maturation · Gonad development · Eggs production

Abbreviations

BL	Body length
BW	Body weight
BWI	Body weight index
Control-Group	Control eels
CM-Group	Comacchio eels
CPE	Carp pituitary extracts
DHP	17α,20β-dihydroxy-4-pregnen-3-one
E2	17β-estradiol
EDh	Eye diameter horizontal
EDv	Eye diameter vertical
EI	Eye index
GSI	Gonadosomatic index
H&E	Hematoxylin and eosin
hCG	Human chorionic gonadotropin
IBW	Initial body weight
K	Condition factor
MG-Group	Marano-Grado eels
PFL	Pectoral fin length
PFLI	Pectoral fin length index
RIA	Radio-immuno assay
SI	Silver index
SPE	Salmon pituitary extracts
T	Testosterone

Introduction

European eels (*Anguilla anguilla*) have a complex life cycle. After a long growth period in continental waters (4–20 years), eels undertake a 5–6,000 km sea water migration from Europe to the Sargasso Sea (Aarestrup et al. 2009). Before migrating, eels go through a significant morphological and physiological change process known as "silvering" (van den Thillart and Dufour 2009): a pre-adaptive requirement for downstream migration and reproduction, it marks the end of the growth phase and the onset of sexual maturation (Durif et al. 2009).

A study by Palstra and van den Thillart (2010) pointed out that silvering is not a true metamorphosis but a mere initiation of maturation. The typical morphological modifications include a change in belly color from yellow to silver/bronze and in back and pectoral fins from white/gray to black and increased eye size (Fontaine 1994; Han et al. 2003a; EELREP 2006; Okamura et al. 2007). The physiological changes include degeneration of the digestive tract (Fontaine 1994; Han et al. 2003a, b), changes of visual pigments, more

developed swim bladder, and higher density of branchial chloride cells (Fontaine 1994). These modifications of silver eels have been proposed as a pre-adaptation for the oceanic migration back to Sargasso Sea (Han et al. 2003a; Durif et al. 2005).

Also silvering marks the start of lipid mobilization and sexual maturation (Palstra and van den Thillart 2010). Silvering is more flexible than generally presumed (Svedäng and Wickström 1997) and can be influenced by several trophic and environmental factors (Durif et al. 2005; Melia et al. 2006; van Ginneken and Maes 2005; van Ginneken et al. 2007; van den Thillart and Dufour 2009).

At present, the only way to obtain sexually mature eels is to artificially induce sexual maturation in migratory individuals caught in brackish and freshwater environments or cultured silver eels using repeated injection of carp (CPE) or salmon (SPE) pituitary extract and a final injection of 17, 20 β-dihydroxy-4-pregnen-3-one (DHP) (Ohta et al. 1996; Palstra et al. 2005; Burgerhout et al. 2011).

Since then, such experimental procedure has been used extensively, mainly to obtain viable larvae for aquaculture development of eels. Ovulated eggs and larvae were successfully obtained (Tanaka et al. 2001, 2003; Pedersen 2003, 2004; Okamura et al. 2009; Palstra and van den Thillart 2009; Oliveira and Hable 2010; Burgerhout et al. 2011); however, the fertility and hatchability of eggs remained very low.

Studies have focused especially on the successful protocols based on dose and timing of hormone injection and on the definition of environmental optimal parameters (water temperature, water salinity, photoperiod) (Durif et al. 2006).

However, eels receiving the same treatment showed high variability in their maturation response; thus, it is possible that these responses reflect not only individual reproductive capacities (Durif et al. 2006) but also the different habitats of each eel population.

The objective of this study was to describe the variability in the response of female silver eels to a standardized gonadotropic treatment (CPE) in terms of gonad development and egg production, and to relate this variability to population characteristics. For this purpose, sexual maturation and reproduction were induced in two population coming from different location in the Adriatic Sea. Their reproductive capacities were assessed throughout the experimental periods using morphological and physiological indicators and related to their initial characteristics.

Materials and methods

The *Valli di Comacchio* (10,400 ha) are three shallow, closed lagoons (Valle Campo, Magnavacca, and Fossa di Porto) located near Ferrara (Emilia-Romagna Region—Italy) (Fig. 1). The fishery in Comacchio has been operating for centuries taking advantage of the autumn–winter migration of European eels to the ocean. Several species thrive in the *Valli*, but the fishery has always been dominated by *Anguilla anguilla*, which comprises up to 90 % of fishery yield in mass (De Leo and Gatto 1996; Holthaus et al. 2011).

The fish ponds (fishing *Valli*) system of *Marano* and *Grado* lagoon (Friuli Venezia Giulia Region—Italy) (Fig. 1) covers a total surface of about 1,720 ha, out of a total area of 20,000 ha of coastal wetlands. Within Grado lagoon, there are 38 fishing *Valli* (1,400 ha), and in Marano, there are 17 (320 ha) small and intensively managed ones. Grado fishing *Valli* are larger and extensively managed with an average 80 % of the total surface occupied by water. The most important fish species are European seabass (*Dicentrarchus labrax)*, Gilthead seabream (*Sparus aurata)*, Mullets Mugilidae and the European eel (*Anguilla anguilla) (*Cosolo et al. 2009; Gelli 2011*).*

Fig. 1 Map showing origin eels

Wild female eels were caught early in December 2010 using traditional "*lavoriero*" (downstream trap) in brackish water lagoon: one population came from *Marano-Grado* lagoon (*MG-Group*) and the other from *Comacchio* lagoon (*CM-Group*). At the same time, cultivated male eels ($n = 50$ fish, 94–203 g in BW) reared in freshwater were purchased from a commercial eel supplier and they were gradually acclimated to sea water over 7 days.

35 females/group were randomly selected at the catch and then transported to the laboratory where they were measured to obtain an external indicator of their maturation state (Durif et al. 2006); 5 subjects/group were maintained as control animal (untreated *Control-Group*).

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).

$$\text{Condition factor (K)} = \left(\text{BW} \times \text{BL}^{-3}\right) \times 10^3$$

BW: body weight (g), BL: body length (cm).

$$\text{Eye index (EI)} = 100 \times \left(\left((\text{EDh} + \text{EDv}) \times 0.25\right)^2 \pi \times (10 \times \text{BL})^{-1}\right)$$

EDh: eye diameter horizontal (mm), EDv: eye diameter vertical (mm).

$$\text{Pectoral fin length index (PFLI)} = 100 \times \text{PFL BL}^{-1}$$

PFL: pectoral fin length (cm).

The initial stage of eels relative to the silvering process (silver index—SI) was determined according to the classification system described by Durif et al. (2005).

Ten eels (5 CM eels and 5 MG eels) were randomly selected and immediately killed (T0) with an overdose of anesthetic (2-phenoxyethanol) and their gonads were carefully

excised and weighed; the gonadosomatic index (GSI) was calculated according to the formula below:

$$\text{Gonadosomatic index (GSI)} = \left(\text{GW} \cdot \text{BW}^{-1}\right) \times 100$$

GW: gonad weight (g), BW: body weight (g).

Samples of gonads were collected for histological analysis. Small pieces of gonads were taken and immediately fixed in 10 % buffered formalin. Subsequently, they were dehydrated in a graded ethanol series and embedded in paraffin. Section series of 4 μm were then cut and stained with hematoxylin and eosin (H&E). Histological sections were evaluated under light microscope to assess the state of maturation according to Kagawa (2005) and Perez et al. (2011).

Moreover, at 5th (T5), 10th (T10), and 15th week (T15), 5 eels/group were randomly selected and killed for GSI determination and gonad histological analysis (Fig. 2). GSI of Control-Group were calculated at the end of the trial (30th week).

After a week of acclimation to local seawater condition (salinity 32 ‰), all the subjects were kept in five 700 L tanks (four with females and one with males) connected to a recirculation system and maintained in indoor conditions for the duration of the experiment. A seawater controlled temperature system was set at 15.5 ± 0.5 °C.

The animals were marked individually (CM-Group: CM 1–25; MG-Group: MG 1–25; Control-Group: 1–5 (CM) and 6–10 (MG) by inserting fish tags (FLOY TAG Mod Floy T-Bar Anchor) and were gradually brought under "completely dark conditions", during a period of 7 days, that is, 24 h/day dark (-0.04×10^3 lux at the bottom of the aquarium without water) (Mordenti et al. 2012). The eels did not eat for the entire duration of the trial (30 weeks).

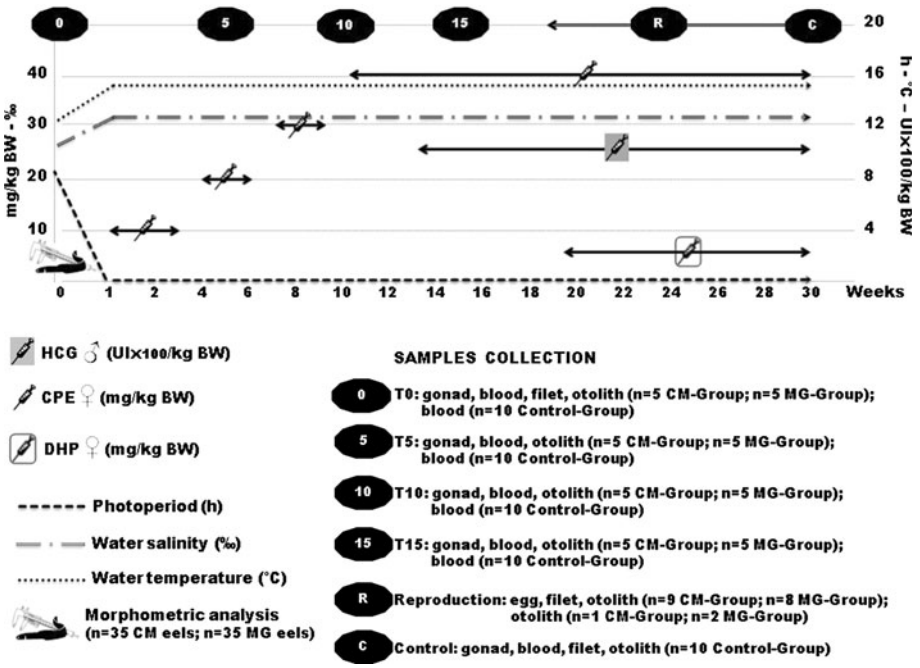

Fig. 2 Schematic drawing of the experimental protocol adopted

The 2 groups of 25 females received intramuscular injections once a week with carp pituitary extracts (CPE) (Palstra et al. 2005) at a dosage of 10 mg/kg BW (1st–3rd week), 20 mg/kg BW (4th–6th week), 30 mg/kg BW (7th–9th week), and 40 mg/kg BW (Mordenti et al. 2012) (Fig. 2). CPE administration was carried on until BW exceeded 110 % of initial body weight (IBW) (*Body weight index* (BWI) = (BW IBW^{-1}) × 100—BW: body weight (g), IBW: initial body weight (g)), which means the beginning of oocyte hydration, and additional CPE injection was administered to enhance maturation competency (Ijiri et al. 2011).

Males were induced following standard protocols (Ohta et al. 1997; Palstra et al. 2005) and started spermiation after 5 weeks treatment. Just before fertilization, the males received a booster hCG injection to reactivate spermiation (Burgerhout et al. 2011).

At start (T0), and 48 h after 5th (T5), 10th (T10), and 15th (T15) CPE injection, blood samples (1 mL) were collected from the caudal vein, transferred to heparinised tubes, centrifuged (4000×*g*, 10 min), and stored at −80 °C until analysis for plasma hormone levels. Plasma was extracted with diethyl ether (approximately 1:10 v/v) and processed for measurement of 17β-estradiol (E2) and testosterone (T). Plasma E2 and T were determined using a validated radio-immuno assay (RIA) as described by Bono et al. (1983) and Gaiani et al. (1984), respectively.

In order to determine the parallelism between hormone standards and endogenous hormone in plasma eel, a pooled sample of eel plasma, containing high concentration of E2, T was serially diluted (1:1–1:8) with RIA buffer and determined by RIA.

Twenty-four hours after the last CPE injection, the females were weighed (*BW-Last-CPE*) and ovulation was induced by injecting a DHP-solution (2 mg/kg BW dissolved in 95 % ethanol and diluted with buffered saline solution) (Palstra et al. 2005) in 10 different locations in the ovary.

After the DHP injection, each eel was transferred to a 150-L tank (salinity 32 ‰, water temperature 20 ± 0.5 °C), connected to a recirculation system and maintained for 12 h with spermiating males (*sex ratio* 4/1) in order to obtain natural reproduction. Eggs were collected by a net (mesh size: 300 μm) and moved to an incubation tank.

The artificial fertilization program started in case DHP injection did not lead to spontaneous spawn within 12 h. The ovulation was checked at hourly intervals (12, 18 and 24 h) by applying gentle pressure on the abdomen in cranial to caudal direction (Ohta et al. 1996) and the eggs were collected into a 3-L plastic sterilized bowl. The first flow of eggs (about 50 g) was not used for fertilization (Burgerhout et al. 2011). Three males per female were hand stripped and milt was collected in a syringe (10 mL) and kept in the refrigerator for a maximum of 12 h. The collected sperm was added to dry eggs in bowls and mixed. Fresh seawater was added, and after approximately 3–4 min, the eggs were placed into buckets with fresh sterile seawater (∼20 L) for 15 min. Sperm motility was checked prior to fertilization under a microscope, after mixing a drop of sperm with a drop of seawater. Only sperm with at least 50 % motility (continuous activity of >50 % of spermatozoa) was used for fertilization (Burgerhout et al. 2011).

Each inseminated egg batch was kept in a 150-L polyethylene tank and maintained at the same temperature used to induce ovulation (20 ± 0.5 °C), for 8 h up to the morula stage. We considered only eggs that reached the morula stage to be confirmed as fertilized.

In order to examine the final eggs production, the eels were weighted at the end of the natural emission or stripping (egg production: [BW-LastCPE] − [BW-PostOvulated]).

Filet (skin-on) samples were collected from the central body (dorsal fin attachment-anus) in T0, post-ovulation, and Control-Group (30th week) eels (Fig. 2) and stored at −80 °C until lipid extraction. Moreover, samples of unfertilized eggs were collected by

ovulated females. Total lipids were extracted with chloroform–methanol 2:1 (v/v) as described by Folch et al. (1957). Lipid content of each tissue was expressed as weight of total lipids per weight of each tissue (% wet wt).

Otoliths were collected during each killing (T0, T5, T10, T15, and post-ovulation), and samples were prepared according to Durif et al. (2006). Age was determined by taking the first ring as Year 1 of the eel's life.

Characteristics of the silver eels, hormonal and lipid data, and reproductive performances were statistically analyzed. Statistics were performed using analysis of Variance on SSP (Smith's statistical Package); $P \leq 0.05$ was considered statistically significant.

All the fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Approval for this study was obtained by Ethics Committee of Bologna University.

Results

The external and internal measurements of the wild eels at the beginning of the experiment (T0) are reported in Table 1.

The whole MG-Group was silver and actively migrant at maximum silvering degree (V), while in CM-Group, the most represented silver index was IV, with 3 fish at pre-migrant stage (III) and only five eels at stage V (Table 1).

Morphometric parameters showed statistically significant differences for most of considered parameters despite similar age classes. CM-Group eels showed highest BW, BL, and Condition factor values, while in the MG-Group, the highest PFLI and GSI values were observed (Table 1).

GSI data showed that raw gonad weight of all treated females followed a growth related to the increasing number of injections (Fig. 3). A slight increase in GSI up to the 10th injection was noticed in the CM-Group, after that a rapid increase up to the 15th injection, while MG eels showed a regular increase in the trial. GSI was found significantly higher in

Table 1 Initial characteristics of silver eel adopted for the experimental procedure

	CM	MG
Silver index (SI) ($n = 35$) (%)		
III	8.6	–
IV	77.1	–
V	14.3	100
Body weight (BW) ($n = 35$) (g)	1,353 ± 241*	358 ± 94
Body length (BL) ($n = 35$) (cm)	85.62 ± 4.98*	59.16 ± 4.78
Condition factor (K) ($n = 35$)	2.13 ± 0.22*	1.70 ± 0.13
Eye index (EI) ($n = 35$)	10.12 ± 1.35	10.80 ± 2.19
Pectoral fin length index (PFLI) ($n = 35$)	4.68 ± 0.40	5.55 ± 0.37*
Gonadosomatic index (GSI) ($n = 5$)	1.61 ± 0.26	2.17 ± 0.36*
Age ($n = 35$) (years)	5 (40.0 %)	5 (54.3 %)
	6 (48.6 %)	6 (45.7 %)
	7 (11.4 %)	–

* Significance difference ($P < 0.05$) between CM and MG eels

the MG-Group at T0 and T10, while at 15th week (T15), higher GSI in CM eels was observed (from 3.43 ± 1.10 in T10 to 14.70 ± 1.51 in T15). The GSI of Control-Group after 30 weeks was stable, ranging from 1.58 to 2.07, and comparable to values obtained at T0 (Fig. 3).

Histological observation showed gonads arranged in lamellae, supported by a stroma rich in adipose tissue. At T0 (no CPE treatment), the two populations, despite different GSI, were at the same degree of maturation showing oocytes in pre-vitellogenic stage (oil drop stage): oocytes had a central round large nucleus (or germinal vesicle), multiple nucleoli, and abundant cortical alveoli filling completely the cytoplasm. At T5 (5th CPE treatment), both groups showed larger previtellogenic oocytes (Fig. 4). At T10 (10th CPE treatment), eels showed oocytes in early vitellogenic stage (primary yolk globule stage): the MG-Group showed the first small yolk vesicles near the nucleus, while the CM-Group (F) was at oil drop stage (Fig. 4). At T15 (15th CPE treatment), CM-Group showed oocytes in late vitellogenesis with abundant yolk vesicles that entirely filled the cytoplasm; the nucleus of some oocytes started to move to the periphery of the cell. On the contrary, MG-Group showed smallest oocytes in mid-vitellogenesis with still more abundant lipid droplets than yolk vesicles (Fig. 4). At the end of the trial (30 weeks), all Control-Group animals showed oocytes in previtellogenic stage.

With regard to hormonal analysis, changes in serum T and E2 levels are shown in Figs. 5, 6. The serum T levels in both groups exhibit significantly heavy increase in T5. In the CM-Group, it drastically increase from an initial 2.06 ± 1.19 ng/mL to a peak of 41.56 ± 1.02 ng/mL after the 5th injection, followed by a sharp drop in T10 (19.76 ± 8.08) (10th injection).

An important increase in T5 of the MG-Group (from 1.30 ± 0.67 to 14.58 ± 4.61 ng/mL) was followed by gradual decreases in T10 (9.99 ± 2.04) and T15 (7.65 ± 4.89) even though not significant. The CM-Group showed considerable higher T levels than the MG-Group from T0 to T15. (Fig. 5).

The E2 levels in both groups increased significantly up to the 5th injection (from 2.26 ± 1.52 to 6.34 ± 1.10 ng/mL and from 0.32 ± 0.17 to 3.25 ± 2.37 ng/mL in CM and MG-Group respectively) and then fluctuated at same levels until the 15th injection. The CM-Group showed relevant higher E2 levels than the CM-Group in T0, T5, and T15 (Fig. 6).

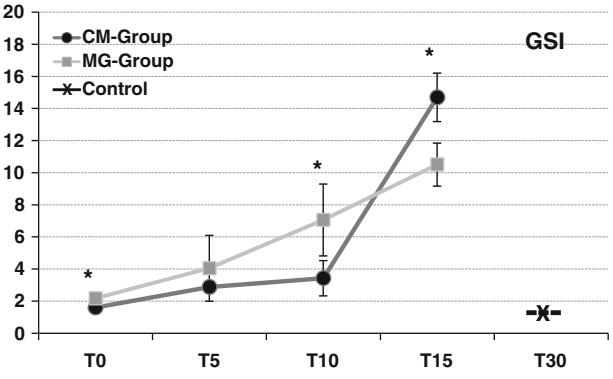

Fig. 3 Evolution of gonado-somatic index (GSI) along the experiment. * Significance difference ($P < 0.05$) between CM and MG eels

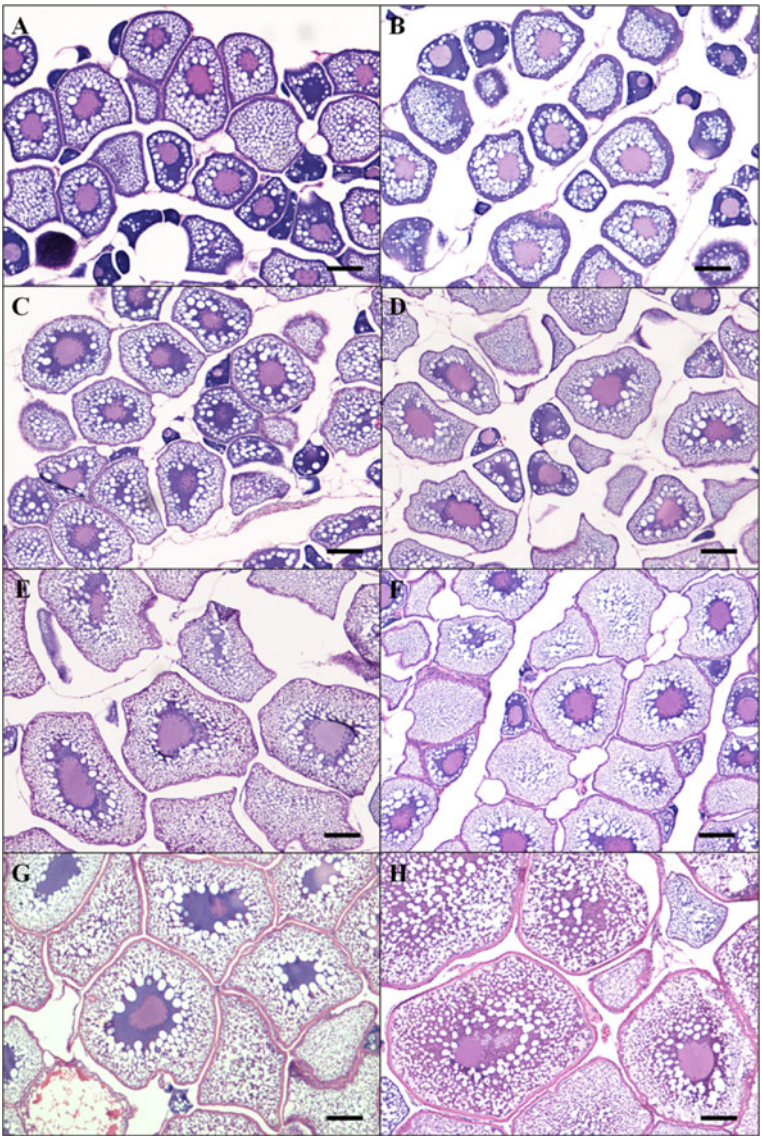

Fig. 4 Histological sections of oocytes from the two populations (*MG-Group*: **a**, **c**, **e**, **g**; *CM-Group*: **b**, **d**, **f**, **h**) at different sampling points. *Week 0* **a**, **b** Populations are at the same state of maturation (previtellogenic oocytes at oil drop st age). *Week 5* **c**, **d** Populations are still at oil drop stage but with larger oocytes. *Week 10* **e**, **f** MG-Group (**e**) shows oocytes in early vitellogenesis with the first small yolk vesicles near the nucleus and CM-Group (**f**) is still in oil drop stage even if oocytes became larger. *Weeks 15* **g**, **h** MG-Group (**g**) shows oocytes in mid-vitellogenesis with lipid droplets still more abundant than yolk vesicles and smallest oocytes and CM-Group (**h**) shows oocytes in late vitellogenesis with abundant yolk vesicles which fill entirely the cytoplasm. (H&E staining; *scale bars* = 100 μm)

In the Control-Group, T and E2 levels ranged around basal level during the whole experiment and were not much different from initial values (T0) (data not shown).

Figure 5

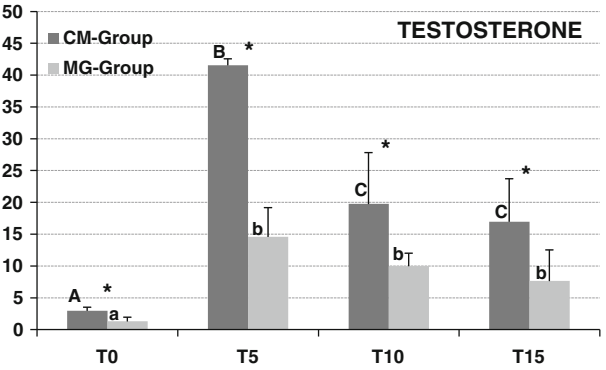

Figure 6

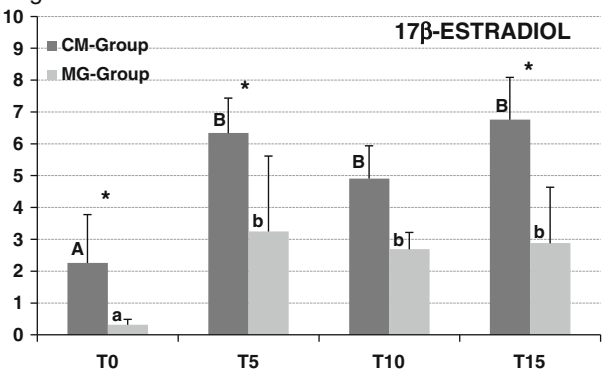

Fig. 5, 6 Testosterone and 17β-estradiol levels (ng/mL) along the experiment. *Asterisks* show significant differences ($P < 0.05$) between CM and MG eels. *Capital letters* show significant differences ($P < 0.05$) within CM-Group. *Small letters* show significant differences ($P < 0.05$) within MG-Group

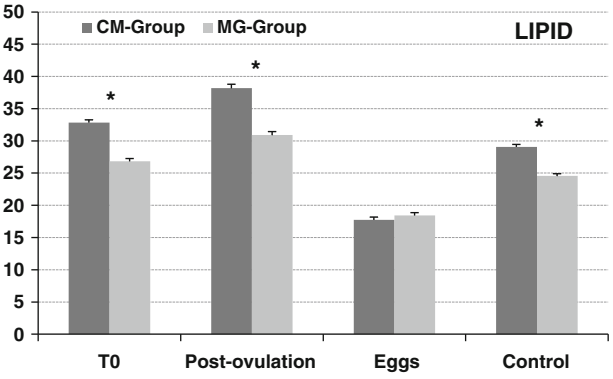

Fig. 7 Total lipid content (% w.w.) in skin-on at T0, post-ovulation, Control-Group (30 weeks) and unfertilized eggs. * Significance difference ($P < 0.05$) between CM and MG eels

Figure 7 shows total lipid content of skin-on filet samples collected from CM, MG, and Control eels.

Results showed variations both between and within the samples of eels. Relevant differences in lipid content were found in T0 (32.85 ± 0.43 in CM compared to 26.84 ± 0.44

in MG), post-ovulation (38.18 ± 0.55 in CM compared to 30.91 ± 0.55 in MG), and Control (30th week) (29.08 ± 0.39 in CM compared to 24.56 ± 0.36 in MG) between CM-Group and MG-Group, while no statistically important differences were found between CM and MG eggs. Finally, the lipid contents in CM and MG eggs (17.74 ± 0.46 and 18.43 ± 0.46 in CM and MG, respectively) were statistically lower than T0, post-ovulation, and Control.

With reference to zootechnical performance, the BWI of MG-Group eels, obtained after the last CPE injection, was significantly higher than that of CM-Group eels (Table 2). Also, at the 30th week, two individuals in MG-Group and one in CM-Group did not completely respond to CPE and therefore were not treated with DHP.

Concerning ovulation, 8 CM eels did not spontaneously spawn but retained eggs in the abdominal cavity. There were only small losses of eggs during handling; therefore, ovulation was checked by stripping. In MG-Group, all eels ovulated spontaneously.

Regarding spawned eggs after the DHP injection, the eggs/female obtained in each group from manual stripping or natural emission is shown in Table 2. MG-Group eels were statistically more productive with 40.1 ± 6.33 % BW of eggs released.

However, eels from CM-Group showed an earlier response to hormonal treatment: CM-Group females ovulated especially between the 19th and the 22nd week (77.8 % spawned eels), while in MG ells, the ovulation started at the 24th week and finished at the 28th week (100 % spawned eels)(Fig. 8).

As fertilization is of concern, in both treated groups, fertilized eggs were obtained, even if they remained at low levels: in CM eggs, obtained by stripping, fertilization ratio was 1.5–8.6 %, while in spontaneously laid eggs (MG females), it was not possible to precisely assess the percentage of success. Also, fertilization was successful for 8 out of 9 CM-Group females with 6 fertilization resulting in hatched larvae. Concerning MG-Group, fertilization was successful for all the females (8 eels), and all fertilization resulted in hatched larvae. Incubation time was 38.3 ± 2.5 degrees day in both two groups and maximum survival time of larvae was 12 days on starvation.

Discussion

The results of this work suggest that morpho-physiological features in European eel affect ovarian development, being an important parameter in artificial reproduction protocols.

Table 2 Zootechnical performances obtained by female eels at the end of the experimental protocol

	CM	MG
BWI increase (%)	113.53 ± 3.68	120.63 ± 4.16*
Ovulated eels (*n*/total)	9/10	8/10
By Stripping (*n*)	8	–
Natural (*n*)	1	8
Ovulated eels (% BW)	25.16 ± 7.88	40.10 ± 6.33*
By Stripping	27.13 ± 5.59	–
Natural	9.4	40.10 ± 6.33

* Significance difference ($P < 0.05$) between CM and MG eels

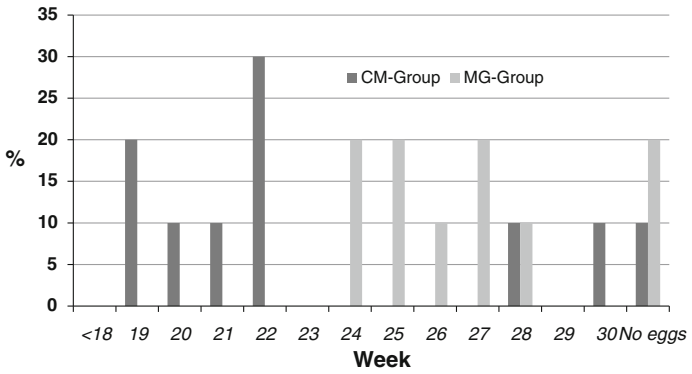

Fig. 8 Females ovulated during experimental protocol ($n = 10$ for both groups)

The Silver index is based on many morphological and physiological parameters of wild female silver eels and has therefore a better predictive value with respect to real silvering stage (Dufour and van den Thillart 2009).

The *Marano-Grado* and *Comacchio* lagoons, in the northern Adriatic Sea, are environments with outstanding characteristics where it is still possible to find female eels with high SI to be used to start a program of artificial reproduction and where it is possible to restore the conditions to host and breed eels. In our study, EI and PFLI in both populations were around 10 and 5, respectively, which can be considered typical of silver migratory stage (Durif et al. 2005, 2009). As noted by Durif et al. (2006), good reproductive responses came from females of high SI and this seems a prerequisite for the female eel sensitivity to gonadotropic stimulation (EELREP 2006; Dufour and van den Thillart 2009).

However, all *Marano-Grado* eels were at maximum migrating stage (V), while *Comacchio* eels, even though showing good SI level, included females in pre-migrating stage (III). Moreover, the percentage of stage V eels that were caught by downstream trap were higher in MG-Group (100 % stage V) and lower in CM-Group (only 14.3 %) than that observed by Durif et al. (2009) (75 % stage V) in different types of hydro-systems. Finally, the better maturation degree of MG eels was confirmed also by the highest PFLI at capture. Hypothetically ,the highest SI of *Marano-Grado* lagoon eels, located far north of the Adriatic Sea, could presumably derive from a drastic lowering of water temperature, which usually occurs in this area at the beginning of winter, due to cold winds from northeast. This can anticipate eels migration toward the sea, while mildest environmental conditions in *Comacchio* lagoon in December might be less favorable for downstream migration. This is in agreement with Durif et al. (2003, 2005) which state that downstream migration is flexible and its intensity is dependent from environmental conditions (so called "environmental window").

In females, the gonad weight increases during silvering (Durif et al. 2005; van Ginneken et al. 2007; Palstra and van den Thillart 2010); nevertheless, the GSI rarely goes above 1.5 % and usually is constant as long as eel remains in fresh- or coastal water (Durif et al. 2009).

The GSI obtained at T0 in both populations (1.61 ± 0.26 and 2.17 ± 0.36 in CM and MG–Group, respectively) can be considered typical of a migrant eel (EELREP 2006; Durif et al. 2005) and confirms the high SI in *Marano-Grado* eels.

Lower SI and GSI of CM eels are, anyway, compensated by a higher BW, BL, and K with respect to MG eels. Indeed, CM eels BW is almost 4 times higher than MG-Group

(1,353 ± 241 g and 358 ± 94 g in CM-Group and MG-Group, respectively) and had a higher BL of about 26 cm (85.62 ± 4.98 cm and 59.16 ± 4.78 in CM- and MG-Group, respectively).

High BW and K of CM-Group come from high availability of food in the lagoon, mainly crustaceans and small fish, lasting all year round and from reduced food competition (eel represents 90 % of fishery yield mass). De Leo and Gatto (1996) showed that in the *Valli di Comacchio,* prey-size spectrum is incredibly large and abundant: smallest eels eating mainly insect larvae, intermediate eels eating snails, mussels and crustaceans (*Crangon crangon* and *Palaemon* sp.) and larger eels feeding preferentially on fish (in particular *Atherina boyeri*, *Engraulis encrasicholus*, *Aphanius fasciatus, Polatoschiustus* sp.). On the contrary, smaller dimensions of the MG eels can partially be due to higher food competition with other estuarine fish species (sea bream, sea bass, mullets, etc.) living in Marano-Grado lagoon (Gelli 2011).

Regarding BL, female eels start to silver when they reach 50 cm length (Durif et al. 2005, 2009); however, it is clearly more advantageous to attain a bigger size: energy store is greater and fecundity is increased (Durif et al. 2009). Indeed, the same authors showed that when BL of eels of the same age class were compared, migrant eels were always longer than resident ones indicating that migrating eels had benefited of a higher overall growth rate suggesting that a high growth period precedes migration and therefore silvering. On the contrary, our data show a higher silvering in the population with a lower BL (*Marano-Grado* eels).

Finally, it is clear how bigger size of CM-Group is not due to older age of animals, as only 11.4 % of females were 1 year older than eels from *Marano-Grado*. This aspect confirms that age classes were not directly related to the BW and that length and age at migration are extremely variable in female silver eels, probably as a reflection of the variability in habitats and growth conditions (Melia et al. 2006; Durif et al. 2009).

The data recorded in our subjects do not appear to be in line with those observed by Durif et al. (2009) which showed that when body length of eels of same age class were compared, migrating eels benefit of higher overall growth rate suggesting that a high growth period precedes migration and silvering. Also, Svedäng et al. (1996) showed that age at the onset of maturity (silvering) in female European eels were inversely related to growth rate. Probably, age at silvering differs between habitats, thereby excluding the possibility of a common reaction norm in age at silvering relative to growth rate (Stearns and Koella 1986).

It is clear then that silvering level may not only be related to somatic growth, but may also be related to diversity of habitat or under the influence of certain, yet unknown, environmental factors. In this respect, Durif et al. (2009) showed that high productivity habitats will rapidly yield less fecund females, while poorer environments will more slowly yield large fecund females: eels appear to adopt both strategies.

With reference to experimental treatment, GSI and histological analysis showed a positive trend and the progressive maturation of the gonads of hormonally treated eels in the two experimental groups. Anyway, while *Marano-Grado* females showed a constant and regular time of maturation, *Comacchio* eels presented an exponential development of oocytes starting from T10, leading to an earlier egg emission with respect to MG-Group.

This answer could originate from higher basal plasma T and E2 level observed in CM-Group starting from T0.

It is indeed known that vitellogenin protein is synthesized by the liver under effect of E2 and incorporated in the oocyte under the control of GTH (Messaouri et al. 1991; Peyon et al. 1996; Durif et al. 2009): T and E2 then amplify the activity of the gonadotropic axis

by stimulating the production of LH (Durif et al. 2009), and injection of T with cortisol in European eel has a stimulatory effect on LH synthesis (Huang et al. 2001). This apparent relationship between the two steroids confirm that T acts as a precursor for E2 synthesis during the vitellogenic phase (Durif et al. 2009).

The E2 profiles observed in both groups were similar to that observed by Chiba et al. (1994) in European eel during artificially induced ovarian development, showing an increase at the beginning of treatment with pituitary extract, than fluctuating at same levels during vitellogenesis. This increase in E2 is a peculiar feature of artificially matured eels and may be a consequence of artificial maturation. Ijiri et al. (1995), Matsubara et al. (2005), and Perez et al. (2011) too showed that, during artificial maturation in Japanese eel, E2 levels in vitellogenesis phase were low, while in European eel were higher.

Also Ijiri et al. (1995) and Matsubara et al. (2005) state that in European eel the serum levels of E2 increased dramatically at the end of vitellogenesis and during the migratory nucleus stage. However, the final increase in E2 did not appear in our studies, especially in *Comacchio* eels where migratory nucleus stage started (T3).

Finally, our data are in agreement with Aroua et al. (2005) and Perez et al. (2011), showing an increase in E2 in hormonally treated eels at the previtellogenic stage, contrary to what observed by other authors (Burzawa-Gerard and Dumas-Vidal 1991; Durif et al. 2009) that noted this peak only when gonads enter vitellogenesis.

Confirming data of Chiba et al. (1994), in this study, serum concentrations of T were much higher than those of E2 during experiment, especially in *Comacchio* females. Our hypothesis is that the initial high T level in CM-Group led to a more responsive performance to hormonal treatment inducing an acceleration in mid- and last stages of vitellogenesis. This seems to coincide with an initial ovarian maturation from 10th until 15th week.

With regard to lipid content, this research underlined a highest energetic reserve in *Comacchio* eels, persisting for the whole study length.

Lipid storage is fundamental for final phase of biological cycle of eel as it represents major energy reserve (about 80 % of total Energy reserve is based on lipid according to Boetius and Boetius 1985) in order to sustain normal gonadal development and to swim to the spawning grounds in the Sargasso Sea (Svedäng and Wickström 1997).

In vertebrates, sexual maturation occurs when individuals have reached a certain age and size and accumulated enough energy to ensure the success of reproduction (Durif et al. 2009).

Van Ginneken and van den Thillart (2000) demonstrated that for their swimming effort of 6,000 km, 40 % of European eels' energy reserves are needed while the remaining 60 % of their energy stores can be used for gonad development.

Anyway it is not yet clear whether the lipid content do triggers silvering in eels. Larsson et al. (1990) suggested that there is a "critical fat mass" for triggering silvering, while Svedäng and Wickström (1997) did not find any link between fat content and silvering. The same authors underline how silvering of female eels and energetic stores for migration and spawning may not always coincide. Present results seems to confirm this last hypothesis, as eels with higher lipid content (*Comacchio*) showed a lower SI with respect to *Marano-Grado* eels.

Highest lipid storage in *Comacchio* eels did not lead to a real advantage in terms of reproduction: no higher lipid mobilization from muscle to gonads (eggs) was indeed observed and egg production was lower with respect to *Marano-Grado* eels. Our hypothesis is that highest energetic storage was not used by eels for reproduction, as triggering of lipid mobilization and sexual maturation requires swimming, activity absent

in our study (recirculation system did not create any flow). Indeed, no change in lipid mobilization was found between yellow and silver eels from same location without swimming (EELREP 2006). Also Palstra et al. (2009) and Palstra and van den Thillart (2010) showed that lipid mobilization and early maturation are linked to migration and that swimming itself may be the natural trigger for these processes hypothesizing that lipolysis becomes activated during and due to sustained swimming. Finally, Palstra et al. (2009) showed that during simulated migration in captivity (endurance swimming), lipid mobilization is activated, which allows the transport to and the deposition of lipid in the oocytes. Concluding, main lipid storage lead to clear advantages in the wild, as only eels with sufficient lipid stores will start their reproductive migration to the spawning grounds in the Sargasso (Palstra and van den Thillart 2010), while in captivity, it seems to have no advantageous aspect.

Anyway, highest lipid storage, associated with a better condition factor and a bigger size of CM eels, seems to have led to a highest production of steroid hormones (T and E2) that can have fastened reproductive process and can partially explain earlier egg laying with respect to MG eels (Cottril et al. 2001; Han et al. 2003a). This is in contrast with observations in *A. japonica* (Okamura et al. 2008), suggesting that time required to reach the final maturation phase in oocytes relates to the developmental stage of gonad and the silvering stage before hormonal treatments.

Moreover, Roncarati et al. (2008) observed that high lipid content in *Comacchio* eels is associated with a high cholesterol content. This lipid molecule is first converted into T through a series of enzymatic reactions and then T is aromatized in E2 (in the granulose cell) (Chiba et al. 1994; Kamei et al. 2006). Also lipid mobilization for energy and deposition in the oocytes occurs most probably through the action of cortisol that is well-known as activator of lipid mobilization and has numerous positive effects on maturation parameters.

On the contrary, *Marano-Grado* eels, characterized by a smaller size but a higher SI, showed a more regular gonadic development leading, although after a longer time, to a spontaneous egg emission.

Moreover, the increase in lipid content observed in post-ovulation females with respect to the beginning of the trial could be explained by a reduction in body mass, which occurred during gonadic maturation.

Indeed in the wild, during downstream migration, eels lost weight, which can be due to diminished energy stores, but also to water loss; moreover, weight loss cannot be based on fat alone, but needs to be compensated by protein oxidation as well (van den Thillart et al. 2009).

Finally, Ozaki et al. (2008) showed that lipid content of muscle in *A. japonica* did not vary during artificial maturation, suggesting that in muscle, other components, for example, protein, were also exhausted in addition to lipid.

Concerning BWI, in eels, the increase in female BW (close to 10 % in 1 week) is used as a reliable indicator of the last phase of ovarian maturation and is necessary to start ovulation with DHP (Ohta et al. 1996). In our study, the higher weight gain in MG eels (120.3 ± 4.16) than CM eels (113.53 ± 3.68) favored increased egg production (%BW) of MG females. Indeed, Mordenti et al. (2012) showed that increased production of eggs was obtained from European eels with BWI more than 120 %.

MG females, who ovulated naturally, showed a timing which is comparable to that reported by Pedersen (2003) (maturation after 24–25 weekly injections) in wild European eels, while CM eels seem to have a timing similar to farmed eels (maturation after 14–22 weekly injections) (Pedersen 2003). However our data confirmed that the European eel

shows both a delay in the response to the treatment and a longer period to reach maturation compared to the Japanese eel. Ohta et al. (1996), Ijiri et al. (1998), and Okamura et al. (2008) reported, respectively, a range of 9–12, 8–10, 5–9 weekly injections with SPE in Japanese eels. 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; Mordenti et al. 2012).

Also, ovulation in a limited time range of 80 % of CM females (19th–22nd week) and of all MG females (24th–27th week) associated with a good level of homogeneity of oocytes obtained in the two populations is probably the result of low-dosage of hormone administered initially to obtain a synchronization in the maturation of eels (Mordenti et al. 2012).

Moreover, fertilization and hatching rates of eggs obtained from CM and MG eels suggest that silvering state of females, as noted by Okamura et al. (2008), does not affect egg quality when maturation is induced by hormonal treatment.

Conclusions

Present work aimed at comparing reproductive performance in two different European eel populations after controlled reproduction procedure.

Marano-Grado and *Comacchio* lagoons produce wild female eels with different morphometric parameters, hormonal and lipid levels; nevertheless, their high silvering values contributed to the success of the artificial reproduction trial.

Bigger dimensions, highest Condition factor and higher lipid storage seem to fasten gonad maturation of *Comacchio* eels without positively increasing reproductive performances.

Marano-Grado females, with a smaller size but a higher silver index, showed a more regular gonad development, leading eels to spontaneous spawning.

Eels with a higher energy storage in terms of critical fatty mass might have more chance to complete seawater spawning migration, but in captivity conditions, without swimming, high lipid content does not increase quality and quantity of produced eggs, nor increases fertilization rate.

Finally, the adopted protocol, which includes increasing doses of CPE, appears to have contributed to the gamete recruitment and synchronization during gonad development with high eggs production.

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