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# Controlled reproduction in *Anguilla anguilla* (L.): advanced studies on broodstock management, spawning techniques and system design for artificial seed production.

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**Esame finale anno 2015**

*In memory of prof. Massimo Trentini*

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## Sommario

Oggetto di studio è stata la specie *Anguilla anguilla* (L.) in quanto nel corso degli ultimi decenni, gli *stocks* naturali di anguille, in particolare le specie originarie delle zone temperate, quelle a maggior valore commerciale, anguilla europea (*Anguilla anguilla*), anguilla americana (*Anguilla rostrata*) e anguilla giapponese (*Anguilla japonica*), sono diminuiti sensibilmente (Casselman 2003; Dekker 2003; Tatsukawa 2003) a causa di fattori concomitanti quali la pesca eccessiva, la distruzione dell'ambiente, modifiche climatiche, oceanografiche e altre cause plausibilmente ancora incognite (EELREP 2006; van Ginneken & Maes, 2005). Ed in particolare è proprio l'anguilla europea ad essere inclusa nella Lista Rossa della IUCN, come specie in pericolo critico; conseguentemente sono state intraprese azioni per la sua salvaguardia: la cui applicazione, purtroppo, non ha ridotto il rischio di estinzione (Mordenti *et al*. 2012).

Lo studio delle caratteristiche morfo-fisiologiche di due popolazioni selvatiche di *Anguilla anguilla*, selezionate per la riproduzione artificiale, ha avuto lo scopo di descrivere la variabilità di risposta di esemplari femmina di anguille europee in termini di sviluppo delle gonadi e produzione di uova, in risposta ad un trattamento gonadotropico standardizzato (*Carp Pituitary Extract* - CPE), e di relazionare questa variabilità alle caratteristiche della popolazione. A tal fine, la maturazione sessuale, l'ovulazione e la fecondazione sono state indotte in due popolazioni di anguille provenienti da due differenti lagune del Nord-Adriatico (Comacchio - CM, Marano-Grado - MG). Su ogni esemplare sono stati misurati parametri esterni per la valutazione dello stato fisiologico (*Silver Index* - SI, *Eye Index* - EI, *Pectoral Fin Length Index* - PFLI, *Condition Factor* - K) e livelli plasmatici di ormoni steroidei (17β-estradiolo-E2, testosterone-T), alcuni soggetti/gruppo sono stati sacrificati per osservazioni istologiche, analisi del contenuto lipidico della frazione muscolare e per la determinazione dell'età tramite lettura dell'otolita. I parametri morfometrici mostrato il gruppo CM avere i più alti valori di peso corporeo (BW), lunghezza del corpo (BL) e K, mentre il gruppo MG ha presentato più alti valori di indice gonadosomatico (GSI), PFLI, EI, e SI. Per quanto riguarda le analisi ormonali, il gruppo CM ha mostrato livelli di T ed E2 significativamente più elevati rispetto al gruppo MG.

Tab. 1: parametri morfo-fisiologici di due popolazioni di *A.anguilla*

| | | ***Comachio (CM)*** | ***Marano-Grado (MG)*** |
|---|---|---|---|
| | | 8.6 (III) | - |
| **Silvering Index** | *%* | 77.1 (IV) | - |
| | | 14.3 (V) | 100 (V) |
| **Body Weight** | *g* | 1353±241***** | 358±94 |
| **Body Lenght** | *cm* | 85.62±4.98***** | 59.16±4.78 |
| **Condition Index** | | 2.13±0.22***** | 1.70±0.13 |
| **Eye Index** | | 10.12±1.35 | 10.80±2.19 |
| **Pectoral Fin Length Index** | | 4.68±0.40 | 5.55±0.37***** |
| **Gonadosomatic Index** | | 1.61±0.26 | 2.17±0.36***** |
| **Testosterone** | $ng.ml.^{-1}$ | 2.06±1.19 | 1.30±0.67 |
| **Estradiol** | $ng.ml.^{-1}$ | 2.26±1.52***** | 0.32±0.17 |
| **Lipid Content** | *%* | 32.85±1.20***** | 26.84±1.34 |
| | | 5 (40.0%) | 5 (54.3%) |
| **Age** | *years* | 6 (48.6%) | 6 (45.7%) |
| | | 7 (11.4%) | - |

Attraverso la valutazione istologica, per entrambe i gruppi sperimentali è stato riscontrato un trend positivo dello sviluppo gonadale; una maturazione più regolare è stata osservata nel gruppo MG, mentre il gruppo CM ha presentato uno sviluppo degli oociti ad andamento esponenziale. Il contenuto lipidico ha mostrato differenze significative fra CM e MG. Per quanto riguarda le prestazioni zootecniche, mentre le anguille MG hanno rilasciato le uova spontaneamente in acqua, per quelle del gruppo CM si è dovuto procedere allo *stripping* manuale. Le anguille MG sono risultate statisticamente più produttive con una quantità di uova rilasciate del 40,1 ± 6,33% del BW. Inoltre le femmine CM hanno ovulato principalmente tra la 19$^{a}$ e la 22$^{a}$ settimana invece in ovulazione della MG va dalla 24$^{a}$ alla 28$^{a}$ settimana. Questi risultati sembrano indicare che le dimensioni più grandi, maggiore K e il maggior contenuto di lipidi, riscontrati nel gruppo CM, non influenzano positivamente la capacità riproduttiva degli animali, sia in termini di quantità che di qualità delle uova prodotte. Le femmine più piccole con un più alto SI (gruppo GM) presentavano uno sviluppo gonadico più regolare, che ha portato gli animali a riproduzione spontanea.

Questo studio condotto su diverse popolazioni selvatiche di anguilla ha evidenziato come pur potendo esserci marcate differenze fra di loro, in termini di età, stadio di maturazione sessuale e contenuto lipidico della massa muscolare (per le anguille primaria fonte energetica durante la migrazione nelle zone di riproduzione naturale; percorrono 6000-7000 km in 5-6 mesi), il principale fattore discriminante nella selezione delle femmine

selvatiche da destinare alla riproduzione in cattività deve essere l'indice di argentinizzazione (*Silver Index*) che ne può determinare, a partire caratteri morfo-metrici e con un chiaro grado di precisione, lo stadio di sviluppo puberale. Inoltre si è riscontrato come maggiori dimensioni, migliori indici di condizione ($K_{fulton}$) o alti livelli di lipidi stoccati possano rendere il processo di induzione alla maturazione in cattività più veloce, senza per questo incidere positivamente sulle performance produttive.

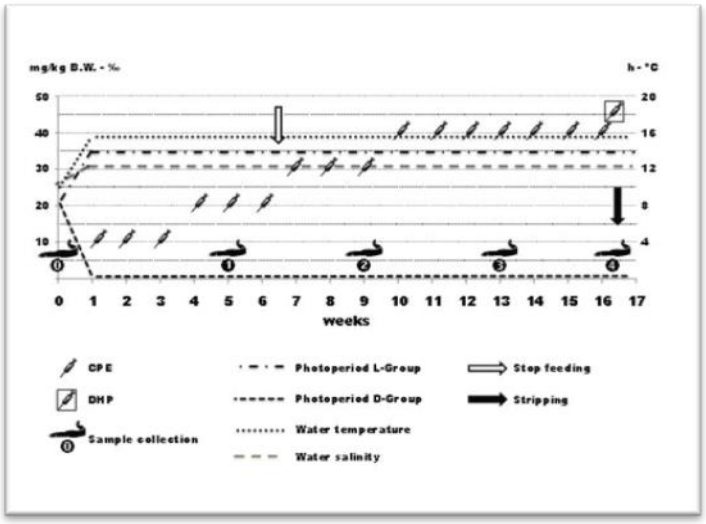



Fig. 1: rappresentazione grafica dello studio sperimentale sugli effetti del fotoperiodo sulla maturazione gonadica di femmine di *A.anguilla*

Infine, il protocollo di induzione ormonale adottato, con dosi crescenti di estratto ipofisario di carpa, si è dimostrato utile allo sviluppo ovarico, con un effetto di sincronizzazione che si è positivamente riflesso sulla produzione di uova (Di Biase *et al*. 2012; Mordenti *et al*. 2013).

Un secondo aspetto indagato è stato quello dell'influenza di diversi fotoperiodi sull'induzione alla maturazione sessuale di anguille selvatiche sottoposte ad un protocollo di induzione con estratti ipofisari (iniezioni settimanali (n. 16) di CPE con una dose sempre maggiore di 10, 20, 30 e 40 mg/kg di peso corporeo). Femmine c.d. argentine (alto valore di SI) sono state mantenute in condizioni di luce (L-Group) o buio (D-Group). In entrambi i gruppi, la maturazione finale e l'ovulazione è stata indotta mediante iniezione di 17-alfa,20-beta-diidrossi-4-pregnen-3-one (DHP) alla dose di 2 mg/Kg BW. Tutte le anguille esaminate sono risultate attivamente migranti (58 allo V e 2 allo stadio IV del *Silver Index*), mostravano un elevato EI (15,75 ± 1,40) ed erano distribuite in 3 classi di età (7-9 anni). In D-Group una delle 4 femmine in trattamento ha ovulato alla 13$^{a}$ iniezione di CPE, 12 ore dopo l'iniezione DHP; le rimanenti 3 femmine, che hanno ricevuto la 16$^{a}$ iniezione CPE, e ovulato tra 12 e 36 ore dopo l'iniezione DHP. Due delle 4 femmine L-Group che hanno ricevuto la 16$^{a}$ iniezione di CPE e ovulato tra le 12 e 36 ore dopo l'iniezione di DHP mentre le restanti 2 (L14 e L16) non hanno ovulato entro 36 ore dopo l'iniezione DHP. Il numero totale di uova rilasciato dalla D-Group (n. 1.485.600) era significativamente superiore a

quella del L-gruppo (n. 274.000). Non è stata registrata alcuna mortalità durante l'esperimento.

Questo studio effettuato sul fotoperiodo dimostra come la condizione di totale buio, nelle vasche di mantenimento dei riproduttori, condizioni positivamente le pratiche di riproduzioni in cattività. Infatti le performance zootecniche dei gruppi mantenuti in tale condizione sono state migliori (Mordenti *et al*. 2012).

Oltre all'influenza sulle performance zootecniche, si è proceduto all'osservazione dell'effetto del fotoperiodo sul profilo degli ormoni steroidei (17-β -estradiolo, E2; testosterone, T; ormoni tiroidei, T3 e T4), sull'espressione genica delle vitellogenine (vtg1, vtg2) nonché sull'espressione del recettore di membrana dell'estradiolo (esr1) durante lo sviluppo ovarico indotto da dosi crescenti di estratto ipofisario di carpa (CPE). Mentre gli andamenti del livello di ormoni steroidei sono risultati differenti, con una maggiore concentrazione nel plasma delle anguille tenute al buio, i livelli ti ormoni tiroidei, vitellogenine ed esr1 sono risultati simili fra i due gruppi. Inoltre le variazioni dell' indice gonado-somatico (GSI) dimostrano un effetto positivo della condizione di buio, di fatto suggerendo che lo stress inferiore possa condurre ad una migliore performance riproduttiva (Parmeggiani *et al*. 2014). Stabilito un criterio per la selezione del parco riproduttori e le migliori condizioni di stabulazione a fotoperiodo 0/24 di luce, si è proceduto nel chiarire quale fra la deposizione spontanea in vasca e la tecnica dello *stripping* potesse essere la più performante per la riproduzione in cattività dell'anguilla europea. Questo studio mirava quindi a confrontare il tasso di fecondazione delle uova e la vitalità degli embrioni ottenuti secondo le suddette tecniche. A tal fine, 18 anguille europee catturate in Val Bonello (Nord Adriatico) sono state indotte artificialmente (iniezione CPE) a maturazione sessuale e ovulazione; una iniezione finale di 17-alfa,20-beta-diidrossi-4-pregnen-3-one (DHP) è stato somministrata quando almeno il 30% degli oociti erano completamente trasparenti. Dopo l'iniezione di DHP, 9 anguille sono state trasferite in un innovativo sistema a ricircolo, dove erano presenti dei maschi fluenti (*sex ratio* 4/1) per consentire la deposizione spontanea

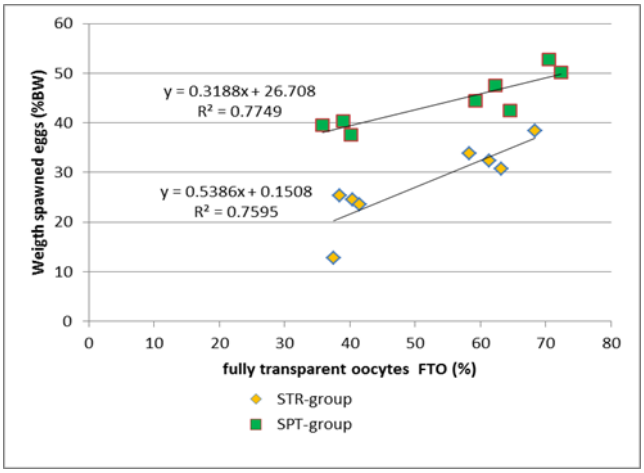



Fig. 2: rapporto tra la percentuale di oociti completamente trasparenti (% FTO) e il peso delle uova generate (% BW) confronto tra emissione spontaneamente (SPT) stripping (STR).

delle uova (gruppo SPT); le restanti 9 anguille sono state trasferite in una vasca da 250 L e l'ovulazione è stato controllata manualmente ad intervalli di quattro ore, fino al momento dello *stripping* (gruppo STR). Il numero di uova per femmina nel gruppo SPT sono stati significativamente maggiori rispetto a quello del gruppo STR. Inoltre, i tassi di fertilizzazione del gruppo SPT risultano notevolmente superiori a quelle osservate nel gruppo STR. Le migliori prestazioni sono state ottenute tra anguille in cui gli oociti completamente trasparenti raggiungevano, o superavano, un aliquota del 50% al momento in cui è stato somministrato il DHP (Di Biase *et al*. 2015).

Dalla comparazione fra deposizione spontanea e tecniche di inseminazione attraverso lo *stripping* si è dedotto come la prima porti ad una migliore resa qualitativa e quantitativa nella produzione di uova atte ad essere fecondate, inoltre il solo l'indice di accrescimento ponderale (BWI) non è adeguato alla determinazione del momento per l'induzione alla maturazione finale degli oociti (attraverso iniezioni di 17-α,20-β-dihydroxy-4-pregnen-3-one, DHP); piuttosto, la presenza di una percentuale di ovociti completamente trasparenti può essere utilizzato per temporizzare l' iniezione DHP e quindi ottenere le uova ad un buon tasso di fertilità.

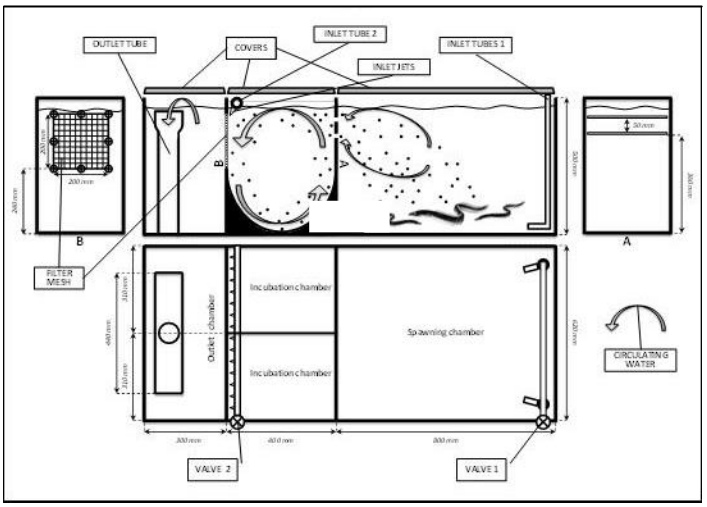


Fig. 3: Rappresentazione schematica del sistema a ricircolo ideato per la produzione artificiale di *A. anguilla*

Una volta attestato che la riproduzione in vasca è ottenibile senza l'intervento umano nella fase di fertilizzazione delle uova, si è reso necessario individuare una soluzione tecnica che potesse garantire le migliori condizioni di incubazione delle uova embrionate durante le loro fasi di sviluppo, evitando forme di stress da manipolazione e garantendo inoltre buone condizioni ambientali per la sopravvivenza delle forme larvali attese ala schiusa delle stesse uova. Per questo si proceduto all'ideazione (commissionando la realizzazione di un progetto originale), messa in opera e verifica della funzionalità di un sistema a ricircolo per la produzione in cattività di forme larvali di anguilla europea, sviluppando una tecnologia innovativa per la deposizione spontanea delle uova e loro incubazione. Il primo *step* è stato quello di ottenere una riproduzione spontanea in cattività della anguilla europea (*Anguilla anguilla*) utilizzando un sistema di ricircolo dotato di camere di

deposizione e di incubazione delle uova. Sono stati quindi verificati il tasso di deposizione delle uova, la fecondità e la qualità delle uova; quindi lo studio si è focalizzato sull'influenza di due livelli di flusso d'acqua all'interno delle camere (2,4 ± 0,05 L/sec a basso flusso; 0.8±0.05 L/sec ad alto flusso). A questo scopo 12 femmine di anguilla sono state indotte con dosi crescenti di estratto ipofisario di carpa (10, 20, 30 e 40 mg CPE/kg di peso corporeo). Ventiquattro ore dopo l'ultima iniezione CPE, ogni ovulazione femminile è stata indotta iniettando DHP, quindi trasferite nel sistema oggetto di studio, sono state mantenute per 16 ore con maschi fluenti (*sex ratio* 4/1) in modo da ottenere una riproduzione spontanea. La riproduzione è stata testata su 6 femmine in condizioni di tasso a basso flusso e 6 femmine in condizioni di tasso ad alta flusso.

I risultati hanno mostrato che il sistema a circuito chiuso, così come progettato, ha permesso di realizzare una riproduzione spontaneo per più dell'80% delle femmine, che hanno subito il trattamento gonadotropico, e favorito il trasferimento automatico e completo delle uova all'incubatoio.

I risultati indicano che il sistema a ricircolo ideato sembra soddisfare tutti i requisiti necessari per ottenere la riproduzione spontanea in anguille europee mantenute in cattività nonché a favorire il trasferimento delle uova ai sistemi di incubazione evitando ogni manipolazione delle stesse e quindi favorendone adeguato sviluppo embrionale.

Le condizioni idrodinamiche del sistema a circuito chiuso e la differente portata (variabile studiata nella sperimentazione) adottata nel processo non hanno ostacolato le attività riproduttive anguilla, ma il flusso più elevato nelle camere di incubazione si è dimostrato inadatto per sviluppo embrionale; conseguentemente si è constatata una perdita costante di vitalità delle uova che hanno raggiunto una mortalità del 100% alla più alta densità di incubazione. Per migliorare i risultati riproduttivi, sarebbe quindi preferibile adottare un basso tasso di flusso e una riduzione della densità incubazione (Mordenti *et al*. 2013).

## References


Casselman J.M. (2003) Dynamics of resources of American eel, Anguilla rostrata: declining abundance in 1990s. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer-Verlag, Tokyo, pp. 237–254.

Dekker W. (2003) Did lack of spawners cause the collapse of the European eel, (*Anguilla anguilla*) Fisheries Management and Ecology 10: 365-376.

Di Biase A., Casalini A., Emmanuele P., Mandelli M., Lokman P.M. & Mordenti O. (2015) Controlled reproduction in *Anguilla anguilla*: comparison between spontaneous spawning and stripping-insemination approaches. Aquaculture Research, accepted for publication on 12th March 2015

Di Biase A., Bastone G., Casalini A., Parmeggiani A., Costantini G. & Mordenti O. (2012) Study of the morpho-phisiological characteristics of two *Anguilla anguilla* populations selected for artificial reproduction. In: Word Aquaculture Society, Conference: "Global Aquaculture: Securing Our Future – AQUA 2012", 1–5 September 2012, Prague, Czech Republic, pp. 127–128.

EELREP (2006) Estimation of the Reproduction Capacity of European Eel. Final Report, EU Project, EELREP, Q5RS-2001-01836. Leiden University.

Mordenti O., Di Biase A., Bastone G., Sirri R., Zaccaroni A. & Parmeggiani A. (2013) Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. Aquaculture International, 21(5):1045-1063.

Mordenti O., Di Biase A., Sirri R., Modugno S. & Tasselli A. (2012) Induction of sexual maturation in wild female European eel (*Anguilla anguilla*) in darkness and light conditions. The Israeli Journal of Aquaculture – Bamidgeh, 1–9, doi:IJA 64.2012.726.

Parmeggiani A., Govoni N., Zannoni A., Di Biase A., Sirri R., Forni M., Mandelli M. & Mordenti O. (2014) Effect of photoperiod on endocrine profiles and vitellogenin expression in european eels *Anguilla anguilla* during artificially induced ovarian development. Theriogenology, Volume 83, Issue 4, Pages 478–484

Tatsukawa, K. (2003) Eel resources in East Asia. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer-Verlag, Tokyo, pp. 293–298.

van Ginneken V. & Maes G.E. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: a literature review. Rev. Fish Biol. Fish. 15, 367–398.

# Introduction

## Eel species of interest in aquaculture

Eels (Anguillidae*;* Infraclass Teleostei) are enigmatic and many details of their biology and life cycle and their evolutionary adaptability are still shrouded in mystery. The Anguillidae comprises 18 species, of which the four most readily identifiable species of commercial importance according to FAO (FishStatJ 2013) are *Anguilla japonica*, *A. anguilla*, *A. rostrata* and *A. australis*. Other anguillids (i.e. *A. mossambica*, *A. marmorata*, *A. bicolor, A. megastoma* and *A. obscura*) have been important food-fish, whether captured or farmed, and still retain economic importance in certain regions.

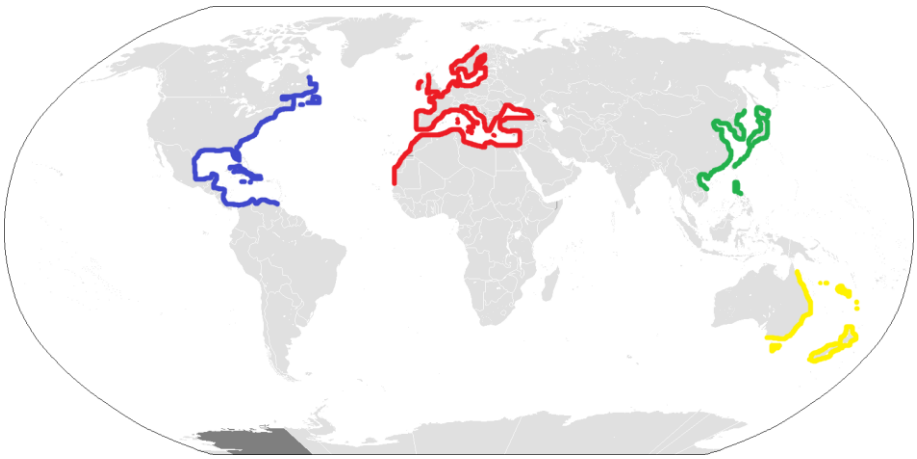


Fig.4: outline of the eels worldwide distribution, in blue *Anguilla rostrata,* in red A*nguilla anguilla,* in green *Anguilla japonica,* in yellow *Anguilla aistralis.*

The European eel has an area of distribution covering Europe, Iceland and North Africa (Schmidt 1909; Sorokin & Konstantinow 1960), the Japanese eel ranges from Vietnam, the Philippines, Taiwan, mainland China and the Korean Peninsula to the Japanese archipelago (Ege 1939; Matsui 1952), the American eel is found on the east coast of the United States of America (USA) and Canada (Schmidt1909; Jensen 1937) and the short-finned eel is native to Australia, New Zealand and the South Pacific (Ege 1939)(Fig.4).

Although little is known about their life cycles or migration routes, it is known that both the European and American eel spawn in the Sargasso Sea in the western Atlantic

Ocean (Schmidt 1922, 1925), the Japanese eel spawns off the Mariana Islands in the western Pacific (Tsukamoto 1992) and the Short-finned eel spawns in the Coral Sea, off New Caledonia(Castle 1963; Jellyman 1987).

Anguillids species life cycles (Fig. 5) can be divided into five main stages: eggs and larvae, carried to continents from marine spawning sites on currents; “glass eels”, of approximately 5-8 centimetres and < 0.5 g in body weight reaching the continental shelf and estuaries; “elvers”, with a darker skin color reaching freshwater habitats; “yellow eels”, over 10 cm long and living in freshwater habitats; and adult or “silver eels”, living in freshwater/estuaries before returning to the sea to spawn. All *anguillid* species are characterized as facultative catadromous fish (Tsukamoto *et al*. 1998; Tsukamoto & Arai 2001) that reproduce in the sea, where they die after spawning. Thus, the main growth phase occurs not necessarily in fresh water, but can also take place in brackish or marine habitats. Eels spawn in deep, nutrient-poor (oligotrophic) tropical and subtropical regions of the ocean and are capable of producing large numbers of small eggs (a typical European eel female can potentially produce 1 million eggs, the larger ones > 5 million) (Tesch 1977; Tsukamoto 2006). The resulting marine planktonic larvae, known as leptocephalus larvae, have a long-lasting dispersal stage that is unique among teleosts, the high fertility helping to compensate for potentially very high mortality during early development. Leptocephali grow from 5 mm to 60-70 mm in length and then change (metamorphose) as they cross the Continental Shelf into transparent ‘glass eels’ and migrate towards coasts (Grassi 1896; Jespersen 1942; Castle 1963; Tabeta & Takai 1975; Smith 1989; Mochioka 2003).

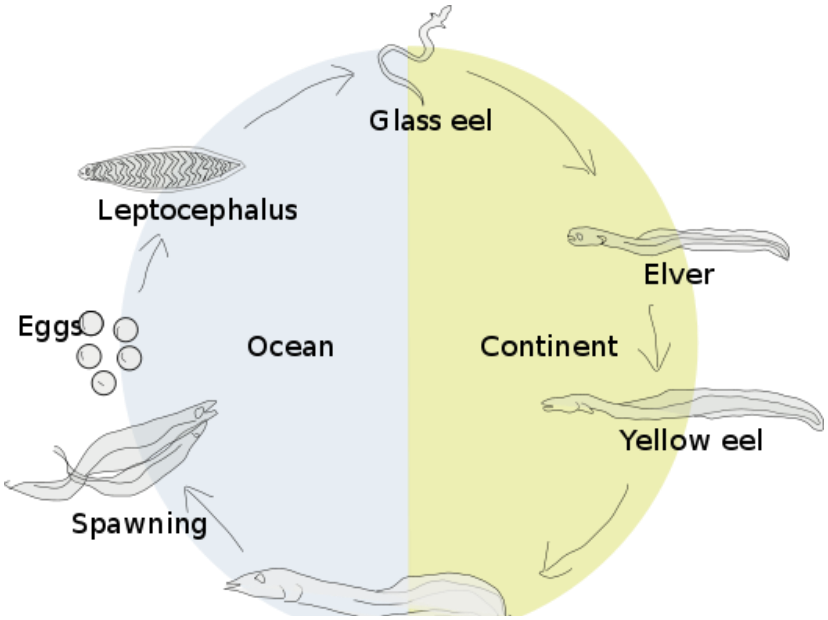



Fig.5: Eels life cycle. Licensed under CC BY-SA 2.5 via Wikimedia Commons
http://commons.wikimedia.org/wiki/File:Eel-life-circle1.svg#mediaviewer/File:Eel-life-circle1.svg

**Production and trade**

Anguillid species are harvested and traded on a global scale for consumption and are of considerable commercial interest - in fact, the European eel, much appreciated for its tasty meat, is offered in markets across Europe, where it demands a high wholesale price (15,00- € 16.80 / kg SO.GE.MI. Spa, 11/2012) for live, large-sized individuals. In retail markets, eels can be sold live, fresh, chilled, frozen, marinated, smoked and canned at prices that vary greatly with size and season, typically in the range of 22 - € 25.00 per kg (ISMEA, 12/2014).

Among the many popular eel dishes consumed around the world, *kabayaki* - marinated grilled eel - is a national dish in Japan, smoked and stewed eel (yellow and silver eels) is eaten in Europe, particularly in Scandinavia, Germany, Netherlands, and in North America; Spaniards favour eel larvae/glass eels (*Angulas*) as a traditional appetizer or tapas, particularly over Christmas, New Year's Day and on 7 January, and in parts of Italy, a dish of eel (*Capitone*) is also traditionally served on Christmas Eve. In UK coastal towns and London's East End, jellied eels (eels cut into chunks and served in a savoury jelly), sprinkled with vinegar, are sold from street stalls. Eel specialties in France include *Anguilles au vert* (eels cooked with spinach and sorrel leaves in white wine) and *Matelotte d'anguille* (eels braised in a red wine sauce). In Belgium, stewed eels in chervil sauce is eaten, and the central region of Portugal specializes in eel stew.

About 95% of eels in the food trade have been raised in captivity, but most of this production is based on catching and rearing wild-caught juvenile "glass eels" (Ringuet *et al*. 2002). For the European eel, all the continental life stages are therefore exploited by commercial fisheries for human consumption. Glass eels are also collected to provide the seed stock for eel farms. Prior to 1990, eel farming was almost exclusively carried out using species of local provenance: Japanese eel *Anguilla japonica* in Asia and European eel *Anguilla anguilla* in Europe. However, a decline in *A. japonica* stocks and recruitment, and the relatively abundant supplies and lower price of *A. anguilla* glass eels led to many Asian eel farms, in particular those in mainland China, switching to *A. anguilla* for their culture material at the end of the 1990s (Ringuet *et al*. 2002) declines in both *A. japonica* and *A. anguilla* stocks in recent years, the market for glass eels has continued to evolve, and new populations and Anguilla species such as American eel *Anguilla rostrata* from the Americas, African Longfin eel *Anguilla mossambica* from Africa and Giant Mottled eel *Anguilla marmorata* from Southeast Asia are now being exploited to supply East Asian farms with

glass eels (Crook & Nakamura 2013). It is at least clear that the involvement of Asian countries – particularly China - in the eel market has resulted in a trade that is more diffi-

Fig. 6: Global Aquaculture Production for species (tonnes)
Source: FAO FishStat

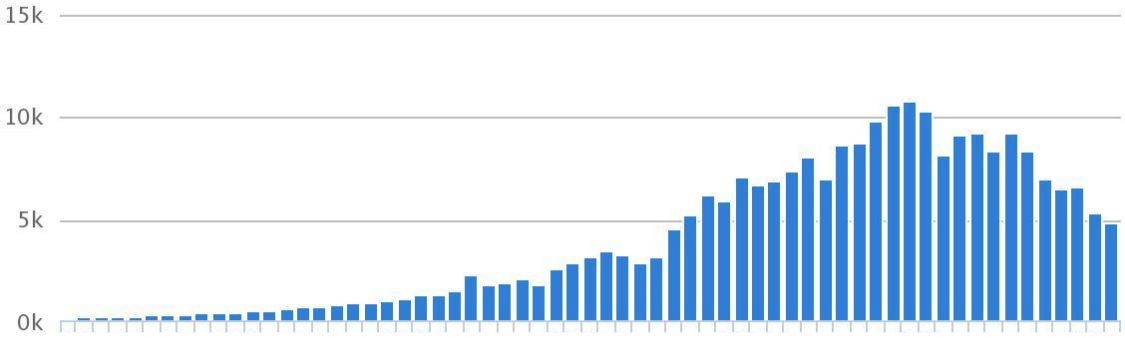
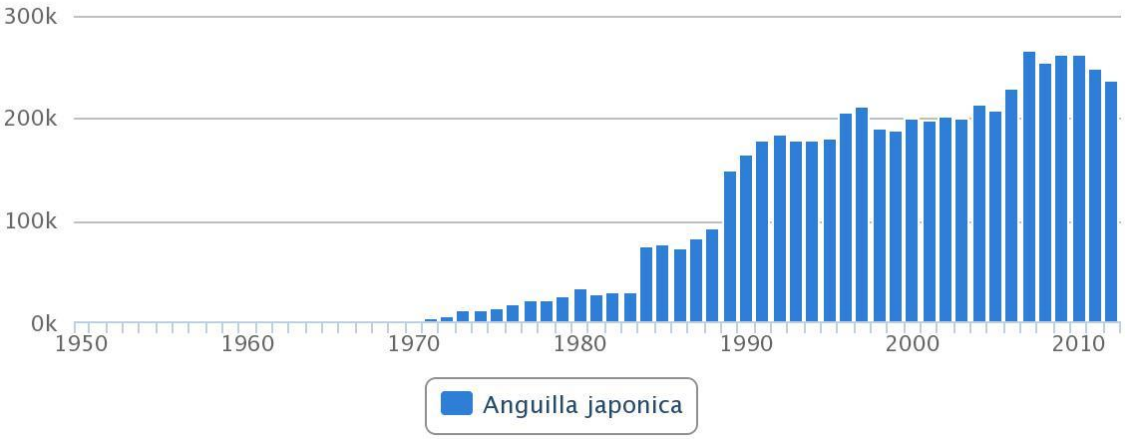



Fig. 7: Global Aquaculture Production for species (tonnes)
Source: FAO FishStat




cult to manage: live glass eels as well as semi-processed and processed eel products are now transported all over the world. Consequently the status of this resource (*Anguilla anguilla*) has grown from being a small European fishery to one of global significance (Dekker 2002).

What was once a European fishery feeding European farms and consumption, therefore became an industry of global significance.

In 2011, the top three producing countries of farmed European eels were the Netherlands, Denmark, and Italy; substantial quantities are also farmed in Spain, Greece, Sweden and Germany, together with smaller quantities in several other European and North African countries, including Algeria, Hungary, Macedonia, and Morocco (FAO 2004-2015).

As regard *Anguilla japonica*, China is by far the largest producer of farmed Japanese eels (e.g. 73 percent of global production in 2003) but Taiwan Province of China and Japan are also major producers. The other countries declaring farmed production to FAO of this species are the Republic of Korea and Malaysia (FAO 2005-2015).

Although only trade in *A. anguilla* is regulated through CITES, there are concerns over the impact that international trade may be having on other Anguilla species, in particular *A. japonica*, *A. rostrata* and the lesser-known tropical anguillids species. Furthermore, controlling trade in just one anguillids species through CITES and the stricter measures imposed by the EU (see below) is likely to have altered global eel trade dynamics and exploitation patterns, in particular for the high value commodity, glass eels (Crook & Nakamura 2013).

**Farming techniques**

Three rather different eel rearing techniques are primarily in use:

- *Extensive pond systems:* the traditional form of eel culture in Europe is in ponds of about 100-350 m². When eels reach marketable size they are transferred to larger ponds (1000-1500 m²). The ponds may be static or flow-through. The best temperature range in ponds is 18-25 °C.

- *Intensive culture in recirculation systems:* these systems consist of square or circular tanks from 25-100 m², usually built of cement or fibreglass. The eels are stocked at a size of 50 g. Densities reach up to 100-150 kg/m². Extruded dry feed (1.5-3 mm) is fed automatically several times a day. Individual growth rates are very different, and grading every 6 weeks is necessary in order to reach a high overall growth performance.

- *Valliculture:* eels are also extensively cultured in marine and brackish waters within a form of aquaculture known as valliculture. In these Mediterranean systems, mainly in Italy, in the north Adriatic, elvers of 15-35 g are stocked at the rate of 4-15 kg/ha. The elvers are mainly imported from France but also from Denmark, the Netherlands and Sweden.

Compared to elvers or yellow eels, glass eels are the most commonly used stocking material for aquaculture purposes for several reasons:

- almost 100 percent of glass eels accept the initial food offered;
- they are easier to wean on artificial food;
- they have been collected for direct consumption for many decades, and the fishing industry was able to provide a good supply when aquaculture activity started;
- they are easy to transport;
- compared to elvers, they carry fewer pathogens, parasites, viruses or bacteria.

The intensive farming technique was developed to save on energy and wastewater costs and is mainly used in northern European countries. The eels are reared at very high densities (up to 120 kg eels/$m^3$ of water) in indoor tanks with a strong water flow to provide the necessary oxygen and removal of waste products. The effluent is recycled in a specially designed unit, and this highly sophisticated farming technique saves considerable water and energy, but it requires a highly trained and educated team of experts to run the facility. Furthermore, it requires a high investment and the overall farming risks are high as all the tanks are interconnected. Most operations are automatic (e.g. feeding, grading, water parameter controls, cleaning) to save manpower. In fact, only 1.5 employees are needed for an annual production of 100 tonnes (Corbari *et al*. 2001).

The semi-intensive farming is usually carried out in still water ponds at considerably lower densities or a maximum of 20 kg/$m^2$. Surface aerators provide the necessary oxygen and create a current which concentrates the sediment in the centre of the ponds. Water flushing, carried out twice daily, removes approximately 1/3 of the water volume and aids the removal of unwanted wastes and sediments. The waste water is usually discharged in a nearby stream. These farms occupy large areas and are located near freshwater streams as they require large volumes of water (approximately 4000 $m^3$ water/day/100 tonnes annual production) (Corbari *et al*. 2001).

The culture ponds have a simple design usually separated by the water discharge channels. Most farm operations are conducted manually (feeding, grading, cleaning, etc.) and approximately 20–30 persons are employed for each 100 tonnes produced. Heating of the water during the cold winter months is carried out using a coal boiler. These farms have a very low technical level, poor sanitary monitoring, and do not require highly educated staff to operate and manage the system (Corbari *et al*. 2001).

The *vallicoltura* is an ancient fish culture practice originated in the Mediterranean region namely the Adriatic and Tyrrhenian coasts. People exploit the seasonal migrations of some fish species from the sea into the lagoons by preventing the fish returning to the sea.

The term now applies to fish culture in coastal lagoons or brackish water bodies based on seasonal migrations of fish. This technique was developed by the upper Adriatic populations to exploit the seasonal migrations of some fish species from the sea into the lagoon and delta areas which were more suitable for their growth. The fish returned to the sea because of altered environmental conditions (temperature) of the sea or for reproduction. To exploit these periodic movements, large brackish areas were enclosed to prevent the fish returning to the sea and complex permanent capture systems, fish barriers, were developed consisting of barriers in the channels communicating with the sea to catch the adults. Later, from the simple ponding of fry freely entering the lagoon from the sea, came a man-made seeding of fry fished elsewhere and introduced into the basins to be reared for a few years (Ardizzone *et al*. 1988). This practice focuses on some fish species very tolerant of variations in salinity and temperature variations, such as seabream (*Sparus aurata*) , sea-ass (*Dicetrarchus labrax*), flatfish species (*Scophthalmus maximus, Solea solea*), mullet (*Mugilidae*), some mollusks (*Mytilus galloprovincialis*, *Tapes spp*.), and eels (*Anguilla anguilla*). The eel, in particular, is left permanently in pasture areas (Ardizzone *et al*. 1988; Ciccotti 2005; Ciccotti *et al*. 2000).

To date however juveniles (glass-eels) that could entry in the lagoons during the upstream migration is variable and often not sufficient to guarantee the exploitation of the full productivity of the valleys and the juveniles seeded in the valley are supplied by professional fishermen that catch the glass-eels in particular periods of the year. After about 5-7 years, the eels that have reached the adult stage begin their migration, heading towards the sea driven by a strong instinct of reproduction. This event typically occurs in the winter months. During this period, the sluices are opened, allowing communication between the sea and the valley; this, in turn assists the capture of eels of a commercial size, which are attracted by the salt water, in devices placed along the way and known as weirs. The knowledge of the migratory behavior has prompted fishermen to construct particular barriers in the channels of communication between the lagoons and the sea in order to capture adult fish at the time of their migration, at the same time not hindering the entry of juveniles into the lagoon.

## Status of recruitment

European, Japanese and American Eel populations have declined considerably over the last 30 years. This loss has been attributed to a number of factors, including changes in ocean currents, pollution, diseases, the loss of river habitat, the introduction of invasive species, local fishing and more recently, catches for international trade (Casselman 2003; Dekker 2003; Tatsukawa 2003; van Ginneken & Maes 2005; EELREP 2006).

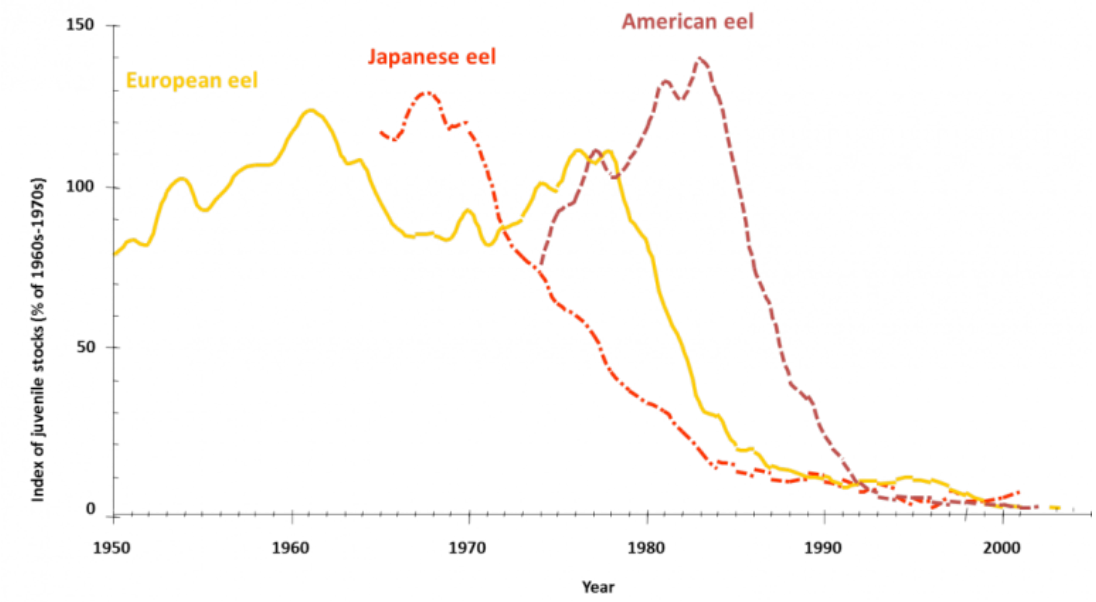



Fig. 8 : Development of European, American and Japanese eel population (*Anguilla anguilla, A. rostrata, A. japonica*) since 1950. Based on the average figures of glass eel stock between 1960-1979. From: Dekker W. (2003): Status of the European eel stock and fisheries. Pp. 237 – 254. In Aida, K., Tsukamoto, K. & Yamauchi, K. (Ed.), Eel Biology, Springer-Verlag, Japan, Tokyo, ISBN 4431004580, 497 p.

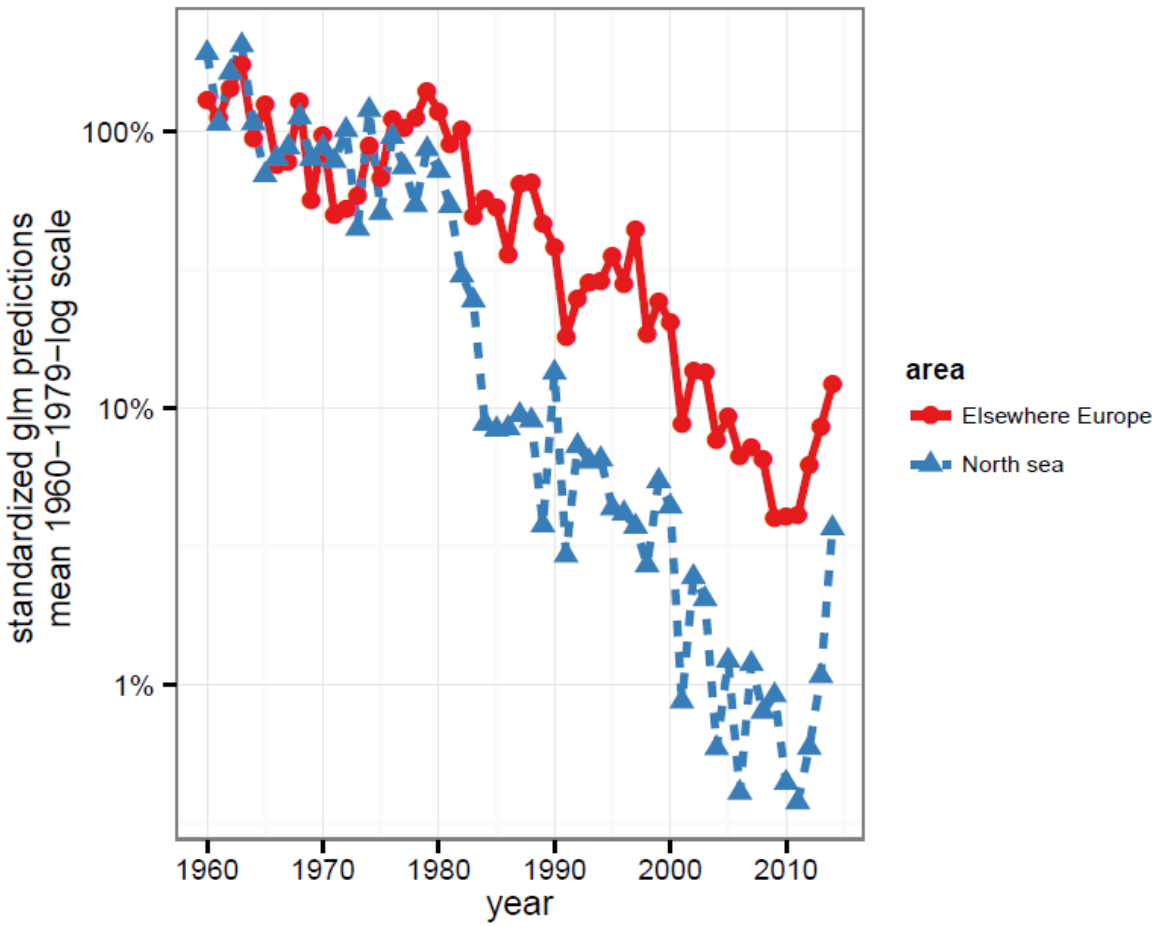



Fig. 9: WGEEL recruitment index: mean of estimated (GLM) glass eel recruitment for the continental North Sea and elsewhere in Europe, updated to 2014 (modified from: ICES Advice 2014, Book 9). The "North Sea" series are from Norway, Sweden, Germany, Denmark, the Netherlands, and Belgium. The "Elsewhere" series are from UK, Ireland, France, Spain, Portugal, and Italy.

In 2007, the European eel stocks were at an historical low and continued to decline, international trade known to be the driver of much of the harvesting of European glass eels. Subsequently, *A. anguilla* was proposed for listing in Appendix II of the Convention on International Trade in Endangered Species of Fauna and Flora (CITES). The listing came into force on 13 March 2009. *A. anguilla* is currently listed as Critically Endangered on the IUCN Red List. Arrivals of glass and yellow European, American and Japanese eels to recruit to continental stocks have fallen markedly since about 1980. The approximate synchrony of declines in recruitment is indicative of common factors (such as ocean warming) acting in the Northern Hemisphere. However, concerns have been expressed about possible overfishing of longfin eels (*A. dieffenbachii*) in New Zealand; indeed, there is tentative evidence for a decline in recruitment since the 1970s in the Waikato River in the North Island (Jellyman *et al*. 2009). The annual recruitment of glass eel to European waters has increased over the last three years, from less than 1% to 3.7% of the 1960–1979 level in the 'North Sea' series, and from 5% to 12.2% in the 'Elsewhere' series.

However, both recruitment indices are still below the 1960–1979 levels and there is therefore no change in the perception of the status of the stock. In September 2008 and again in 2014, European eel was listed in the IUCN Red List as a critically endangered species.

**Management plans**

In 1980, the European Committee for the Conservation of Nature and Natural Resources of the Council of Europe classified the eel as “vulnerable” (Lelek 1980). Matsuda (1999) considers the Japanese Eel to be “Critically Endangered” according to IUCN Red List criteria, and ICES (1999) considers the European Eel “outside safe biological limits” in the context of the Agreement for the implementation of the provisions of the United Nations Convention of the Law of the Sea of 10 December 1982 relating to the conservation and management of straddling fish stocks and highly migratory fish stocks (Anon 2001).

A management framework for eel within the EU was established in 2007 through an EU regulation (EC Regulation No. 1100/2007), but there is no internationally coordinated management plan for the whole stock area. The objective of the EU regulation is the protection, recovery, and sustainable use of the stock. To achieve the objective, EU Member States have developed Eel Management Plans (EMP) for their river basin districts, designed to allow at least 40% of the silver eel biomass to escape to the sea with high probability, relative to the best estimate of escapement that would have existed if no anthropogenic influences had impacted the stock. ICES has evaluated the conformity of the national management plans with EC Regulation No. 1100/2007 (ICES 2009, 2010) and progress in implementing EMP actions (ICES 2013). The EU Member States produced their first progress report in 2012. The next progress reporting is scheduled for 2015.

**Reproduction of eel species in captivity**

Due the decline of eel populations, researchers and professionals engaged in fishery works, including aquaculture, are facing an urgent need for success in artificial reproduction aimed to protect and conserve these species, as well as to obtain reliable supplies of glass eels for aquaculture (Kagawa 2005; Triscitta *et al*. 2013).

Eels have a complex migratory life cycle with the occurrence of two metamorphoses (for reviews: Sinha & Jones 1975, Tesch 1977, Haro 2003, Dufour & Rousseau 2007, Rousseau & Dufour 2008, Rousseau *et al*. 2009, 2012) and have spawn and die in the ocean;

since their sexual maturation has been completed during their reproductive migration towards the tropics, eels remain blocked at the silver prepubertal stage, as long as the reproductive migration is prevented (Dufour *et al*. 2003).

The pioneering work of Fontaine and his collaborators at the Muséum National d'Histoire Naturelle (Paris, France) proved in 1936 that injections of extract of urine from pregnant women (later proved to contain human chorionic gonadotropin, hCG) in males of European eel, or carp pituitary extract in females (Fontaine *et al*. 1964), respectively, induced spermatogenesis or full development of the ovary. These experiments led to the first observation of gonadal maturation in eels, suggesting that the arrest of the development of the gonads was caused by a deficiency in pituitary gonadotropin function. This inhibition at the pituitary level is the reason why eels do not breed naturally in captivity, however, the inhibition can be overruled through hormonal treatment that stimulates gametogenesis, leading to viable egg, sperm, and offspring production in European and Japanese eel (Ohta *et al*. 1997; Kagawa *et al*. 2005; Tomkiewicz 2012; Butts *et al*., 2014). Thus, Japanese scientists have succeeded in reproducing the Japanese eel and completed the lifecycle in culture (Ohta *et al*. 1997; Kagawa *et al*. 2005).

The ability to raise large numbers of juvenile eels in captivity will have a significant impact across the aquaculture industry and trade. Indeed, the eel farming sector could terminate the dependence on wild-caught fish (issues with seasonality and fluctuating prices for the purchase of wild juveniles, bureaucratic costs and restrictions, e.g., CITES). Moreover, it would be a huge advantage for the natural environment and ecosystem functioning if farming systems no longer depended on wild-caught juveniles, a scenario that should release pressure and help restore natural stocks. Furthermore, as required by regional and national plans on the recovery of eel stocks, it would be possible to plan for experimental restocking in lagoons or in restricted areas, through the release of juveniles (leptocephali, glass eels or elvers), a practice already in use for other freshwater species.

*Pituitary extract protocols for artificial maturation of eel*

Based on these pioneering experiments on the European eel, similar treatments (hCG in males and carp or salmon pituitary extract in females) were subsequently used to induce sexual maturation (gametogenesis and steroidogenesis) in various species of eel:

- *A*. *japonica*: Yamamoto & Yamauchi 1974, Yamauchi *et al*. 1976, Ohta *et al.* 1996, 1997, Tanaka *et al*. 2001;

- *A. rostrata*: Edel 1975, Sorensen & Winn 1984;
- *A. dieffenbachii and A*. *australis*: Todd 1979, Lokman & Young 2000.

Furthermore, the use of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), for induction of final maturation and ovulation, has led to increasing fertility and hatching rates (*A. japonica*: Ohta *et al*. 1996; *A. anguilla:* Di Biase *et al*. 2015) and is commonly used in current protocols. Thus, since the first attempts at artificial breeding of European eel by Fontaine in 1936, subsequent improvement of protocols and techniques have led to the production of fertilized eggs and larvae of Japanese eel in 1974 (Yamamoto & Yamauchi 1974), then to rearing of larvae (preleptocephalus), obtained in 1976 (Yamauchi *et al*. 1976).

In 2003, production of the first glass eels from *A. japonica* strengthened the hopes for eel aquaculture (Tanaka *et al*. 2003; Kagawa *et al*. 2005) which led, more recently, to the life cycle being closed was closed (Masuda *et al*. 2011). In other species of eel, the pre-pubertal block may be stronger, evidenced from the necessity for more injections of pituitary extract to induce full maturation. As regard European eels, Palstra *et al*. (2005) were able to fertilize eggs and produce embryos, and Tomkiewicz & Sorensen (2008) were able to prolong the larval life to 18 days until the resorption of the yolk sac. In *A. rostrata*, attempts to induce full maturation to achieve artificial reproduction have been few and have yielded limited results, larvae surviving for 6 days (Oliveira & Hable 2010).

All researchers, regardless of the species studied, have pointed out that the crucial factor for successful fertilization was the use of good quality oocytes at the right time for the induction of final maturation.

With reference to the European eel, research has focused on successful artificial propagation protocols in terms of hormone dose and timing and with regard to defining optimal environmental parameters (water temperature, water salinity, and photoperiod) (Durif *et al*. 2006; Mordenti *et al*. 2013) in order to obtain a high number of eggs for artificial fertilization. Nevertheless, one of the major problems of seed production remains the constant availability of high quality eggs. Egg quality, in terms of resulting fertilization, hatching and survival rates, is highly variable between batches from different individuals (Chai et al., 2010) or between different egg retrieval methods (Mordenti *et al*. 2014).

The reproductive success of eel reproduction using the 15-25 weekly injections of salmon or carp pituitary extract protocol could be very limited as it results in abnormal phenomena in oogenesis such a variations in yolk accumulation, egg membrane for-

mation, as well as difference in the process of oocyte maturation and plasma hormones levels (Adachi *et al*. 2003; Palstra & van den Thillard 2009).

In addition to this long and complex treatment, many difficulties are encountered in the females, such as the lack of standardized pituitary extracts, eel mortality during the treatment, large individual variations in the response to the hormonal treatment, with no, slow and fast responders, and general poor quality eggs (Rousseau *et al*. 2013).

European eel shows a highly individual response in timing and speed of maturation in contrast to Japanese eel (Pedersen 2003; Palstra *et al*. 2005). Therefore, the body weight index 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 (Palstra et al. 2005).

The difficulties in the induction of sexual maturation in female *A. anguilla* and thus in obtaining fertilised eggs may be due to several factors: it is possible that egg quality in the farmed, artificially matured females is low, either because of deficiencies in the maturational procedures which have all used gonadotropins from non-Anguillid fish or maybe because the nutritional status prior to maturation is in some ways suboptimal. In addition, unnatural rearing conditions may be detrimental to the development of good quality eggs in this species (Pedersen 2004).

In this context, the overall objective of the present dissertation is to describe a suitable source population that can provide a wild broodstock which shows a good response to pituitary extract protocols for artificial maturation, by comparing different eel populations coming from different locations in the Adriatic Sea; and after that, providing a close examination of the effect of light/dark condition, both in terms of zoo-technical performance and physiology of induced spawning female eels. Later an analysis will be presented that aims to compare matured female silver eels that spawned spontaneously and those that were spawned by manual stripping, with an emphasis on the effects of the two methods of spawning on ovulation and fertilization rate. Eventually a closed recirculating system for artificial seed production of the European eel will be illustrated: a technology, developed for spontaneous spawning and eggs incubation, that the designed closed-loop system made it possible to carry out successful spontaneous reproduction and favored the automatic and complete transfer of the eggs to the hatchery.

## References


Adachi S., Ijiri S., Kazeto Y. & Yamauchi K. (2003) Oogenesis in the Japanese eel, *Anguilla japonica*. In: K. Aida, K. Tsukamoto and K. Yamauchi [eds]. Eel Biology. Springer, Tokyo, Japan. pp. 502–518.

Anon 2001 CMFRI, Cochin. Annual report. 2000-2001.163 pp.

Ardizzone G.D., Cataudella S. & Rossi R. (1988) Management of coastal lagoon fisheries and aquaculture in Italy. FAO Fisheries Technical Paper, 293, 103 pp.

Butts I. A., Sørensen S. R., Politis S. N., Pitcher T.E., & Tomkiewicz J. (2014) Standardization of fertilization protocols for the European eel, *Anguilla anguilla*. Aquaculture, 426, 9-13

Casselman J.M. (2003) Dynamics of resources of American eel, *Anguilla rostrata*: declining abundance in 1990s. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer-Verlag, Tokyo, pp. 237–254

Castle P.H.J. (1963) Anguillid leptocephali in the Southwest Pacific. Zool.Publ. Victoria Univ. Wellington, 33: 1-14

Chai Y., Tosaka R., Abe T., Sago K., Sago Y., Hatanaka E., Ijiri S. & Adachi S. (2010) The relationship between the developmental stage of oocytes in various seasons and the quality of the egg obtained by artificial maturation in the feminized Japanese eel *Anguilla japonica*. Aquaculture Science 58, 269–278.

Ciccotti E., Busilacchi S. & Cataudella S. (2000) Eel, *Anguilla anguilla* (L.), in Italy: recruitment, fisheries and aquaculture. Dana, 12: 7-15

Ciccotti E. (2005) Interactions between capture fisheries and aquaculture: the case of the eel (*Anguilla anguilla* L., 1758). In: Cataudella S., Massa F., D. Crosetti (Eds.). Interactions Between Capture Fisheries and Aquaculture: A Methodological Perspective. Studies and Reviews, General Fisheries Commission for the Mediterranean, 78: 190-203.

Corbari L., Mezzani G. & Rossi R. (2001) Anguillicoltura. In:. Cataudella S & Bronzi P. (Eds.) Aquacoltura Responsabile, Unimar – Uniprom (in Italian).

COUNCIL REGULATION (EC) No 1100/2007 of 18 September 2007 establishing measures for the recovery of the stock of European eel. Official Journal of the European Union, 22/9/2007.

Crook V. & Nakamura M. (2013) Glass eels: Assessing supply chain and market impacts of a CITES listing on anguilla species. TRAFFIC Bulletin 25(1): 24-30.

Di Biase A., Casalini A., Emmanuele P., Mandelli M., Lokman P.M. & Mordenti O. (2015) Controlled reproduction in *Anguilla anguilla*: comparison between spontaneous spawning

and stripping-insemination approaches. Aquaculture Research, accepted for publication on 12th March 2015

Dekker W. (2002) Status of the European eel stock and fisheries. Proceedings of the International Symposium 'Advances in Eel Biology', Tokyo (Japan), 28-30 September 2001, 237-254.

Dekker W., Casselman J.M., Cairns D.K., Tsukamoto K., Jellyman D. & Lickers H. (2003) Worldwide decline of eel resources necessities immediate action: Quebec declaration of concern. Fisheries 28: 28–30.

Dufour S. & Rousseau K. (2007) Neuroendocrinology of fish metamorphosis and puberty: evolutionary and ecophysiological perspectives. J. Mar. Sci. Tech. Special Issue 55–68.

Dufour S., Burzawa-Gérard E., Le Belle N., Sbaihi M. & Vidal B. (2003) Reproductive endocrinology of the European eel, *Anguilla anguilla*. In: K. Aida, K. Tsukamoto and K. Yamauchi [eds]. Eel Biology. Springer, Tokyo, Japan. pp. 107–117.

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel. Mar. Ecol. Prog. Ser. 327: 171–181.

Edel R.K. (1975) The induction of maturation of female American eels through hormone injections. Helgol. Mar. Res. 27: 131–138.

EELREP, 2006. Estimation of the Reproduction Capacity of European Eel. Final Report, EU Project, EELREP, Q5RS-2001-01836. Leiden University.

Ege V. (1939) A revision of the genus *Anguilla* Shaw. A systematic, phylogenetic and geographical study. Dana-Rep., 16: 1-256

FAO 2004-2015. Cultured Aquatic Species Information Programme. Anguilla anguilla. Cultured Aquatic Species Information Programme . Text by The Danish Aquaculture Development Group (DANAQ). In: FAO Fisheries and Aquaculture Department [online]. http://www.fao.org/fishery/culturedspecies/Anguilla_anguilla/en

FAO 2005-2015. Cultured Aquatic Species Information Programme. Anguilla japonica. Cultured Aquatic Species Information Programme. Text by Xie, J. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 13 January 2005. [Cited 4 March 2015]. http://www.fao.org/fishery/culturedspecies/Anguilla_japonica/en

FAO (2015) Capture and Aquaculture Production (1950-2011) and Fisheries Commodities Production and Trade (1976-2009) datasets. FISHSTAT.

Fontaine M. (1936) Sur la maturation complète des organes génitaux de l'anguille mâle et l'émission spontanée des produits sexuels. C. R. Acad. Sci. Paris 202: 1312–1315. (in French).

Fontaine M., Lopez E., Callamand O. & Bertrand E. (1964). Sur la maturation des organes genitaux de l'anguille femelle (*Anguilla anguilla* L.) et l' emission spontanee des oeufs en aquarium. C. R. Hebdomadaires Seances Acad. Sci. 259, 2907–2910. (in French).

Grassi B. (1896) The reproduction and metamorphosis of the common eel, *Anguilla vulgaris*. Proc. Roy. Soc. 60: 260-271.

Haro A. (2003) Downstream migration of silver-phase anguillid eels. In: K. Aida, K. Tsukamoto and K. Yamauchi [eds]. Eel Biology. Springer, Tokyo, Japan. pp. 215–222.

ICES 1999. WGEEL. The report of the 2009 Session of the Joint EIFAC/ICES Working Group on Eels, September 2009; ICES CM 2009/ACOM:15. 117 pp. and country reports.

ICES 2009. SGAESAW. Report of the Study Group on Anguillid Eels in Saline waters. ICES CM/DFC:06. 189 p.

ICES. 2010. WGEEL. The report of the 2010 Session of the Joint EIFAC/ICES Working Group on Eels, September 2010; ICES CM 2009/ACOM:18. 198 pp. and country reports.

ICES. 2013. Report of the Joint EIFAAC/ICES Working Group on Eels (WGEEL), 18–22 March 2013 in Sukarietta, Spain, 4–10 September 2013 in Copenhagen, Denmark. ICES CM 2013/ACOM:18. 851 pp.

ISMEA, Istituto di servizi per il mercato agricolo alimentare. <http://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/2280/idSF/13/Merc/Milano/TD/mercato%2Bdi%2Bredistribuzione/G/16/M/12/A/2014>*12 January 2015*

Jellyman D.J. (1987) Review of the marine life history of Australian temperate species of *Anguilla*. Am. Fish. Soc. Symp. 1: 276–285.

Jellyman D.J., Booker D.J., & Watene E. (2009) Recruitment of *Anguilla* spp. glass eels in the Waikato River, New Zealand. Evidence of declining migrations?. Journal of Fish Biology, 74: 2014–2033. doi: 10.1111/j.1095-8649.2009.02241.x

Jensen A.S. (1937) Remarks on the Greenland eel, its occurrence and reference to *Anguilla rostrata*. Medd. om Grønland. 118(9).

Jespersen P. (1942) Indo-Pacific leptocephalids of the genus *Anguilla*: systematic and biological studies. Dana Rep. 22: 1–128.

Kagawa H., Tanaka H., Ohta H., Unuma T. & Nomura K. (2005) The first success of glass eel production in the world : basic biology on fish reproduction advances new applied technology in aquaculture. Fish Physiol. Biochem. 31: 193–199.

Lelek A. (1980) Threatened freshwater fishes of Europe. Council of Europe, Strasbourg, 148 pp.

Lokman P.M. & Young G. (2000) Induced spawning and early ontogeny of New Zealand freshwater eels (*Anguilla* spp.). NZJ Mar. Freshwater Res 34, 135–145.

Masuda Y., Imaizumi H., Oda K., Hashimoto H., Teruya K. & Usuki H., 2011. Japanese eel *Anguilla japonica* larvae can metamorphose into glass eel within 131 days after hatching in captivity. Nippon Suisan Gakk. 77: 416–418

Matsuda H. (1999) Resource management from point of view of biological resource conservation. In: Aida, K., Tsukamoto, K. & Yamauchi, K. (eds), Proceedings of symposium: Present Status and Conservation Measures of Eel Resources. Ocean Research Center of the University of Tokyo, Tokyo, Japan. Pp. 47-48.

Matsui I. (1952) Studies on the morphology, ecology and pond culture of the Japanese eel (*Anguilla japonica* Temminck and Schlegel). Journal of the Shimonoseki College of Fisheries, 2: 1–245.

Mochioka N. (2003) Leptocephali. In: Aida K, Tsukamoto K, Yamauchi K (eds). Eel Biology. Springer, Tokyo. 2003; pp. 51–60.

Mordenti O., Di Biase A., Bastone G., Sirri R., Zaccaroni A. & Parmeggiani A. (2013) Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. Aquaculture International, 21(5):1045-1063.

Mordenti O., Casalini A., Mandelli M. & Di Biase A. (2014) A closed recirculating aquaculture system for artificial seed production of the European eel (*Anguilla anguilla*): technology development for spontaneous spawning and eggs incubation Aquacultural Engineering 58, 88– 94.

Ohta H. & Tanaka H. (1997) Relationship between serum levels of human chorionic gonadotropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. Aquaculture 153: 123–134.

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Hirose K. (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17α, 20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. Aquaculture 139: 291–301.

Oliveira K. & Hable W.E. (2010) Artificial maturation, fertilization, and early development of the American eel (*Anguilla rostrata*). Can. J. Zool. 88, 1121–1128.

Okamura A., Horie N., Mikawa N., Yamada Y. & Tsukamoto K. (2014) Recent advances in artificial production of glass eels for conservation of anguillid eel populations. Ecol. Freshw. Fish 23, 95–110.

Palstra, A.P. & van den Thillart, G.E.E.J.M. (2009) Artificial maturation and reproduction of the European eel. In: Spawning migration of the European eel.(ed. By Thillart, G.E.E.J.M., Dufour, S., Rankin, C.), pp. 309–332. Springer, Netherlands.

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: Development of oocytes during final maturation. Aquaculture 249: 533–547.

Pedersen B.H. (2003) Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. Aquaculture 224: 323–338.

Pedersen B.H. (2004) Fertilization of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. Aquaculture, 237:461–473

Ringuet S., Muto F. & Raymakers C. (2002) Eels: Their harvest and trade in Europe and Asia. TRAFFIC Bulletin 19: 1-26.

Rousseau K. & Dufour S. (2008) Endocrinology of migratory fish life cycle in special environments: the role of metamorphoses. In: P. Sébert, D.W. Onyango and B.G. Kapoor [eds]. Fish Life in Special Environments. Science Publishers, Enfield (NH), USA. pp. 193–231.

Rousseau K., Aroua S., Schmitz M., Elie P. & Dufour S. (2009) Silvering: metamorphosis or puberty? In: G. Van den Thillart, S. Dufour and C. Rankin [eds]. Spawning Migration of the European Eel. Springer, Netherlands. pp. 39–64.

Rousseau K., Aroua S. & Dufour S. (2012) Eel secondary metamorphosis: silvering. *In*: S. Dufour, K. Rousseau and B.G. Kapoor [eds]. Fish Metamorphosis. Science Publishers, Enfield (NH), USA. pp. 216–249.

Rousseau K., Lafont A.G., Pasquier J., Maugars G., Jolly C., Sébert M.E., Aroua S., Pasqualini C. & Dofour S. (2013). Advances in eel reproductive physiology and endocrinology. In: Trischitta, F., Takei, Y., Sébert, P.,(Eds.) 2013. Eel Physiology. CRC Press. Downloaded by [Alma Mater Studiorum – Università di Bologna] at 01:02 10 december 2014

Schmidt J. (1922) The breeding places of the eel. Philosophical Transactions of the Royal Society. 211, 179–208.

Schmidt J. (1925) The breeding places of the eel. Ann. Rep. Smithsonian Inst., 1924: 279-316.

Schmidt J. (1909) On the distribution of the fresh-water eels (Anguilla) throughout the world. I. Atlantic Ocean and adjacent regions. Medd. Komm. Havunders., Ser. Fiskeri. 3(7).

Sinha V.P.R. & J.W. Jones (1975) The European Freshwater Eel. Liverpool University Press, Liverpool, UK.

Smith D.G. (1989) Introduction to leptocephali. In: Böhlke EB (ed). Fishes of the Western North Atlantic. Leptocephali. Part 9, Vol. 2. Sears Foundation for Marine Research, New Haven. pp. 657–668.

So.Ge.Mi. S.p.A. < http://www.mercatimilano.com/it/documenti/prezzi-e-statistiche/295-statistiche-quantita-prezzi> 12 Janaury 2015

Sorensen P.W. & Winn H.E. (1984) The induction of maturation and ovulation in American eels, *Anguilla rostrata* (Le Sueur), and the relevance of chemical and visual cues to male spawning behavior. J. Fish Biol. 25: 261–268.

Sorokinn V.P. & Konstantinov K.G. (1960) The eel *Anguilla anguilla* in the Kola Bay. Zoologischeskii Zhurnal 621-622.

Tabeta O. & Takai T. (1975) Leptocephali of *Anguilla japonica* found in the waters south of the Okinawa Islands. Bull. Japan. Sci. Fish., 41 (2): 137-145

Tanaka H., Kagawa H. & Ohta H. (2001) Production of leptocephali of Japanese eel (*Anguilla japonica*) in captivity. Aquaculture 201: 51–60.

Tanaka H., Kagawa H., Ohta H., Unuma T. & Nomura K. (2003) The first production of glass eel in captivity: fish reproductive physiology facilitates great progress in aquaculture. Fish Physiol. Biochem. 28, 493–497.

Tatsukawa K. (2003) Eel resources in East Asia. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer-Verlag, Tokyo, pp. 293–298.

Tesch F.W. (1977) The Eel: Biology and Management of Anguillid Eels. Chapman and Hall, London, UK.

Todd P.R. (1979) Hormone-induced maturation of New Zealand freshwater eels. Rapport et Procès-verbaux Réunions. Conseil Permanent International pour l'Exploration de la Mer 174: 91–97.

Tomkiwicz J.(ed)(2012) Reproduction of European eel in aquaculture (REEL): Consolidation and new production methods. DTU Aqua rapport 249\2012.

Tomkiewicz J. & Soerensen R.S. (2008) Project Report: Artificial Reproduction of Eels III: Larval Culture (ROE III-LC). National Institute for Aquatic Resources, Technical University of Denmark (In Danish).

Trischitta F., Takei Y. & Sébert P. (Eds.) (2013) Eel Physiology. CRC Press. Downloaded by [Alma Mater Studiorum – Università di Bologna] at 01:02 10 december 2014

Tsukamoto K. (1992) Discovery of the spawning area for Japanese eel. Nature 356: 789–791.

Tsukamoto K. & Arai T. (2001) Facultative catadromy of the eel *Anguilla japonica* between freshwater and seawater habitats. Mar. Ecol. Progr. Ser. 220: 265-276.

Tsukamoto K., Nakai I. & Tesch W.V. (1998) Do all freshwater eels migrate? Nature 396:635–636

Tsukamoto K. (2006) Spawning of eels near a seamount. Nature 439: 929.

van Ginneken V.J.T. & Maes G.E. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecyle, evolution and reproduction: a literature review. Rev. Fish Biol. Fisheries 15: 367–398.

Yamamoto K. & Yamauchi K. (1974) Sexual maturation of Japanese eel and production of eel larvae in the aquarium. Nature 251, 220–222.

Yamauchi K., Nakamura M., Takahashi H. & Takano K. (1976). Cultivation of larvae of Japanese eel. Nature 263: 412

# CHAPTER 1

## STUDY OF THE MORPHO-PHISIOLOGICAL CHARACTERISTICS OF TWO *ANGUILLA ANGUILLA* POPULATIONS SELECTED FOR ARTIFICIAL REPRODUCTION.

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The European eel (*Anguilla anguilla*) has a rather complex life cycle: after a long period of growth in rivers (4-20 years), eels undertake a migration of 5-6000 km, from Europe to the Sargassi Sea, to perform the reproduction (Durif *et al*. 2005). At this stage the eel undergoes changes in its morphology (body color and fins, size of eyes, fins and head length) and physiological (gonadal development, degeneration of the intestinal tract, mobilization of lipids and changes in visual and gills), a process known as "silvering" (van den Thillart & Dufour 2009). This process represents the end of the growth phase and the beginning of sexual maturation and it is strongly influenced by numerous environmental and trophic factors (van den Thillart & Dufour 2009).

At present, the only way to achieve a complete sexual maturation of eels in captivity is to induce silver females captured in nature by using repeated injections of pituitary extracts under the complete control of the main environmental parameters (temperature and salinity, and photoperiod) (Mordenti *et al*. 2012).


Fig. 10 - geographical location of coastal lagoons under study, modified from http://www.demis.nl/mapserver

The aim of this study was to study the reproductive potential of two different populations of eel from the lagoon, using morphological and physiological markers. The characteristics of the 2 populations are subsequently placed in relation to reproductive results that will be available at the end of the artificial breeding program. For the trial two eel populations of the Adriatic Sea were used, coming respectively from Comacchio (Emilia-Romagna) (Group-CM) and the Marano-Grado lagoons (Friuli-Venezia-Giulia) (Group-MG) (fig.10).

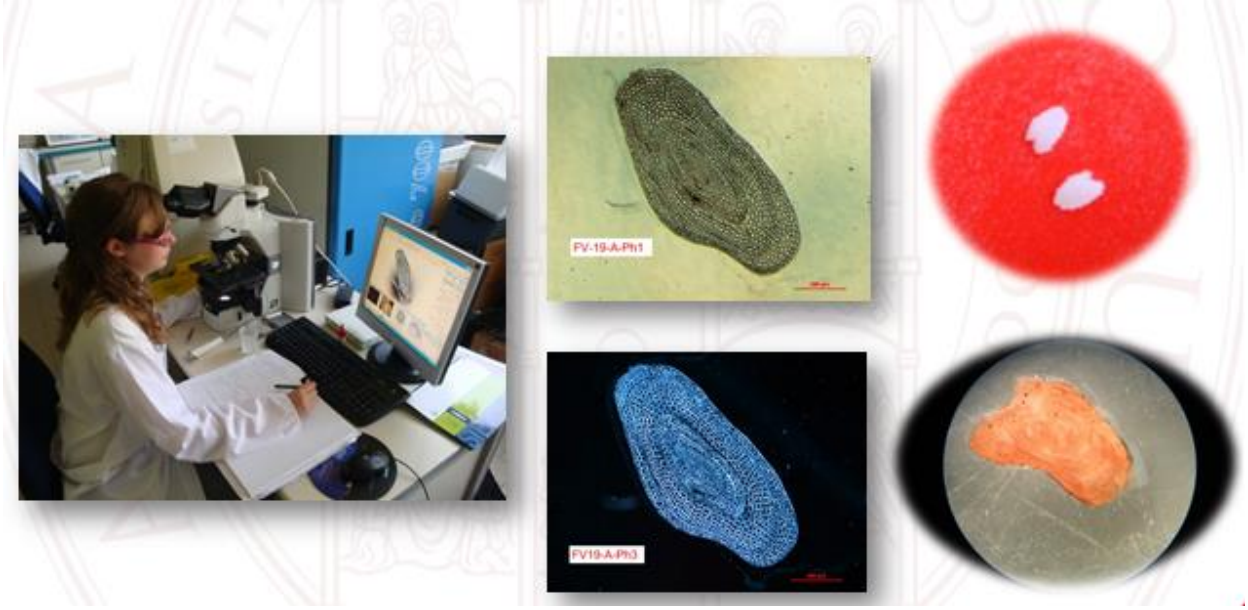

Fig. 11 - age evaluation of *Anguilla anguilla* using scalimetry and otolithometry, photos by Andrea Di Biase

Thirtyfive females for each group were selected and used for the determination of the maturation markers that allow to calculate the silvering index (SI), body weight (BW) and body length (BL) of the animal, condition index (K), eye index (EI), pectoral fin length index (PFLI) following Durif *et al*. (2005).

Later on, eels was performed a blood test that allowed the determination of plasmatic testosterone (T) and estradiol (E2). A sample of 8 eels/group was sacrificed after overdose with 2-phenoxyethanol, to calculate the gonadosomatic index (GSI) and for the determination of lipid content (L) of tissues. The age evaluation was made using scalimetriy in subjects used for artificial reproduction and otolithometry in fish sacrificed (Fig. 11).

The eels of both populations were all migrants and showed, despite similar age, significant differences both in morphological and physiological characteristics (Table 2).

Table 2– Characteristics of two populations

| | | *Comacchio (CM)* | *Marano-Grado (MG)* |
|---|---|---|---|
| **Silvering Index** | *%* | 8.6 (III) | - |
| | | 77.1 (IV) | - |
| | | 14.3 (V) | 100 (V) |
| **Body Weight** | *g* | 1353±241***** | 358±94 |
| **Body Lenght** | *cm* | 85.62±4.98***** | 59.16±4.78 |
| **Condition Index** | | 2.13±0.22***** | 1.70±0.13 |
| **Eye Index** | | 10.12±1.35 | 10.80±2.19 |
| **Pectoral Fin Length Index** | | 4.68±0.40 | 5.55±0.37***** |
| **Gonadosomatic Index** | | 1.61±0.26 | 2.17±0.36***** |
| **Testosterone** | $ng.ml.^{-1}$ | 2.06±1.19 | 1.30±0.67 |
| **Estradiol** | $ng.ml.^{-1}$ | 2.26±1.52***** | 0.32±0.17 |
| **Lipid Content** | *%* | 32.85±1.20***** | 26.84±1.34 |
| **Age** | *years* | 5 (40.0%) | 5 (54.3%) |
| | | 6 (48.6%) | 6 (45.7%) |
| | | 7 (11.4%) | - |

*: significant differences - Pvalue<0.05

The environmental characteristics of the two areas of origin seem to guarantee eel populations with a high Silvering Index, a fundamental aspect to achieve complete gonadal maturation in an artificial reproduction program.

MG eels were characterized by the best SI and a higher degree of gonadal maturation, while in CM females was found a high level of growth and an increased content of lipids and steroid hormones.

The GSI obtained in both populations (1.61±0.26 and 2.17±0.36 in CM ang MG group respectively) can be considered typical of a migrant eel (Durif *et al.* 2005) and confirm the high SI in Marano-Grado eels.

High BW and K of CM-group comes from high availability of food in the lagoon, mainly crustaceans and small fishes, lasting all year round.

Di Leo & 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 (Crangoncrangon and Palaemon sp.) and larger eels feeding preferentially on fish (in particular *Atherinaboyeri, Engraulisencrasicholus, Aphaniusfasciatus, Polatoschiustus* sp.).

The greater energy reserves for CM eels, could lead advantages in the long migration back to spawning areals. However, only the final results on the induced reproduction

may give further explanations on the aspects that most influence the gonadal maturation of eels kept in captivity.

Finally, it is clear how bigger size of CM-group are not due to older age of animals, as only 11.4% of females were one 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).

## References


De Leo G.A. & Gatto M. (1996) Trends in Vital rates of the European eel: evidence for density dependence?. *Ecological Applications,* **6**(4):1281-1294

Durif C., van Ginneken V., Dufour S., Muller T. & Elie P. (2009) Seasonal Evolution and Differences in Silvering Eels from Different Locations. In: van den Thillart G., Dufour S., Rankin JC (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 13-38

Durif C., Dufour S. & Elie P. (2005) The silvering process of Anguilla anguilla: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology*, **66**: 1025-1043

Melia P., Bevacqua D., Crivelli A., Panfili J., De Leo G.A. & Gatto M. (2006) Sex differentiation of the European eel in brackish and freshwater environments: a comparative analysis. *Journal of Fish Biology***69**:1228-1235

Mordenti O., Di Biase A., Sirri R., Modugno S. & Tasselli A. (2012) Induction of sexual maturation in wild female European eel (Anguilla anguilla) in darkness and light conditions. *The Israeli Journal of Aquaculture-Bamidgeh*, **IJA_64.2012.726**, 9 p

van den Thillart G. & Dufour S. (2009) How to estimate reproductive success of European silver eels. p.3-9 In: *Spawning Migration of the European Eel*. G. van den Thillart, S. Dufour, J.C. Rankin (eds), © Springer Science + Business Media B.V. 477 p

# CHAPTER 2

# CONTROLLED REPRODUCTION IN THE WILD EUROPEAN EEL: TWO POPULATIONS COMPARED.

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**Abstract**

This study aimed to describe the response variability of females silver eels in terms of gonad development and eggs production to 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 sacrificed 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 CM's 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 influence re-

productive performance of animals, both in term of quantity and quality of produced eggs. Smaller females with a highest SI (Marano-Grado eels) presented a more regular gonadic development, leading the animals to spontaneous spawning.

## Introduction

European eels (*Anguilla anguilla*) have a complex life cycle. After a long growth period in continental waters (4-20 years), eels undertake a 5-6000 kilometer 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 & 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 & 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; Han *et al*. 2003b), 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 & van den Thillart 2010). Silvering is more flexible than generally presumed (Svedang & Wickstrom 1997) and can be influenced by several trophic and environmental factors (Durif *et al*. 2005; Melia *et al*. 2006; van Ginneken & Maes 2005; van Ginneken *et al*. 2007; van den Thillart & 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 17α,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 have 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 & van den Thillart 2009; Oliveira & 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 females 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. 12). 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 & Gatto 1996; Holthaus *et al*. 2011).

The fish ponds (fishing *Valli*) system of *Marano* and *Grado* lagoon (Friuli Venezia Giulia Region – Italy) (Fig.12), covers a total surface of about 1720 hectares, 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 (*Dicentrarchuslabrax)*, Gilthead seabream (*Sparusaurata)*, Mullets Mugilidae and the European eel (*Anguilla anguilla) (*Cosolo *et al*. 2009; Gelli 2011*).*

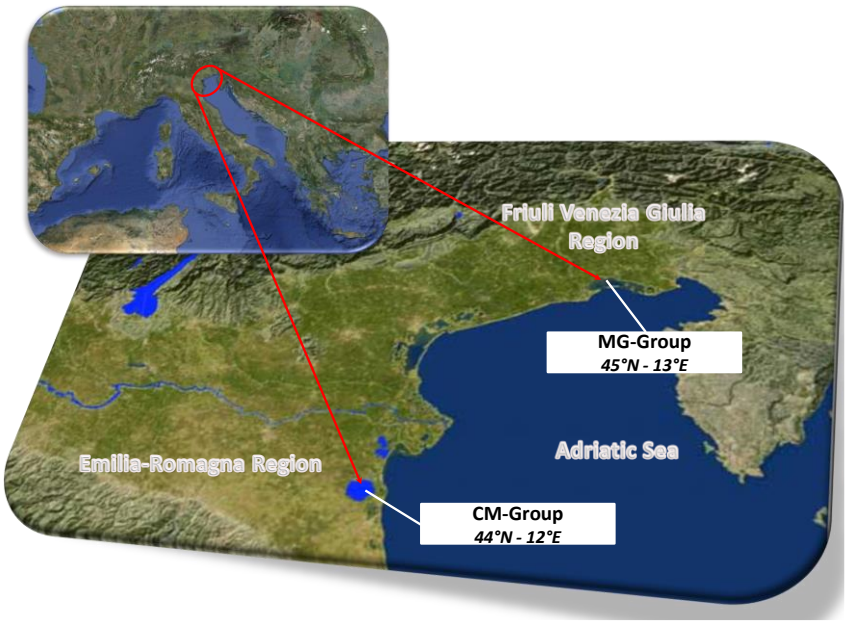



Fig. 12 - Map showing origin eels

Wild females 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, 94-203g in BW) reared in freshwater were purchased from a commercial eel supplier and they were gradually acclimated to sea water over 7 days.

35 female/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 subject/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).

Condition factor (K) = $(BW*BL^{-3})*10^{3}$

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

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

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

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

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 sacrificed (**T0**) with an overdose of anaesthetic (2-phenoxyethanol) and their gonads were carefully excised and weighed; the gonadosomatic index (GSI) was calculated according to the formula below:

Gonadosomatic index (GSI) = (GW $BW^{-1}$)*100

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

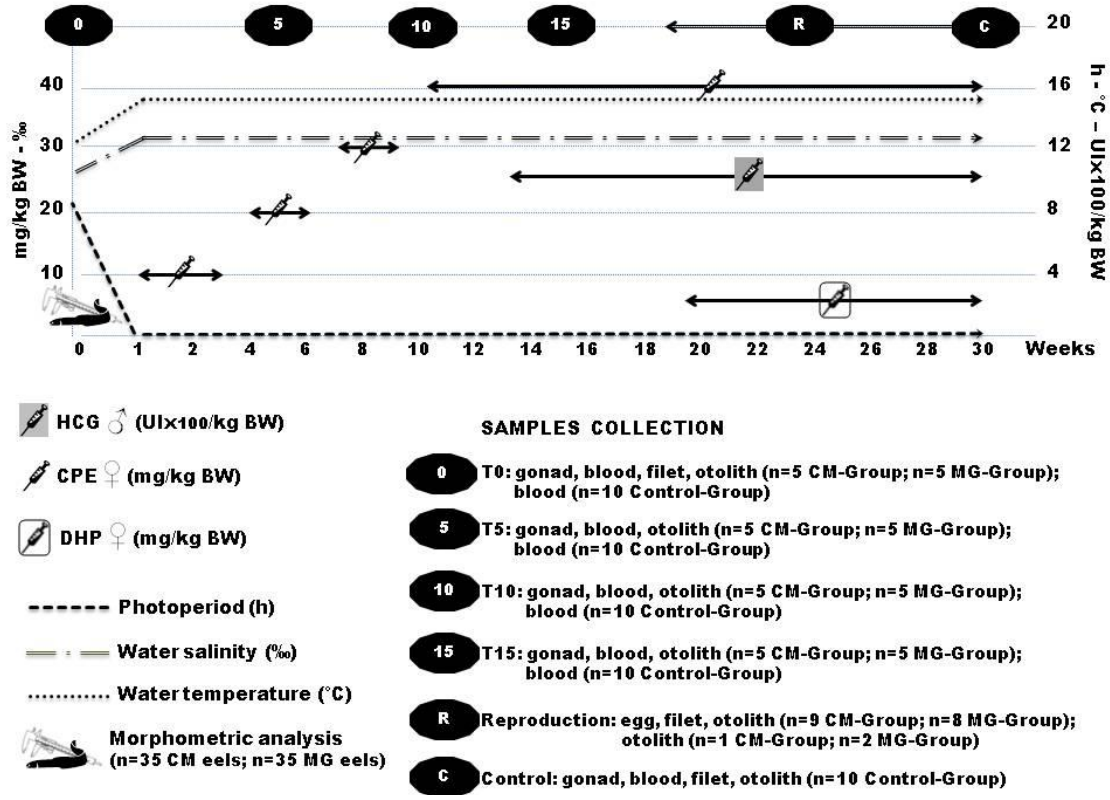



Fig. 13 - Schematic drawing of the experimental protocol adopted

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 $5^{th}$ (T5), $10^{th}$ (T10) and $15^{th}$ week (T15), 5 eels/group were randomly selected and sacrificed for GSI determination and gonad histo-

logical analysis (Fig. 14). GSI of Control-Group were calculated at the end of the trial (30$^{th}$ 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, i.e. 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).

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 (1$^{st}$-3$^{rd}$ week), 20 mg/kg BW (4$^{th}$-6$^{th}$ week), 30 mg/kg BW (7$^{th}$-9$^{th}$ week) and 40 mg/kg BW (Mordenti *et al*. 2012) (Fig. 13). 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 hours after 5$^{th}$ (T5), 10th (T10) and15$^{th}$ (T15) CPE injection, blood samples (1 mL) were collected from the caudal vein, transferred to heparinised tubes, centrifuged (4000xg, 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 12h 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 (12h, 18h and 24h) 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 (10mL) 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 minutes. 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 hours 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. 13) 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 sacrifice (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

Table 3 - 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* | 1353±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* |
| | | | 5 (40.0%) | 5 (54.3%) |
| ***Age*** | **(n=35)** | *years* | 6 (48.6%) | 6 (45.7%) |
| | | | 7 (11.4%) | - |

*: significance difference (P<0.05) between CM and MG eels

The external and internal measurements of the wild eels at the beginning of the experiment (T0) are reported in Table 3. 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 3).
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 3).

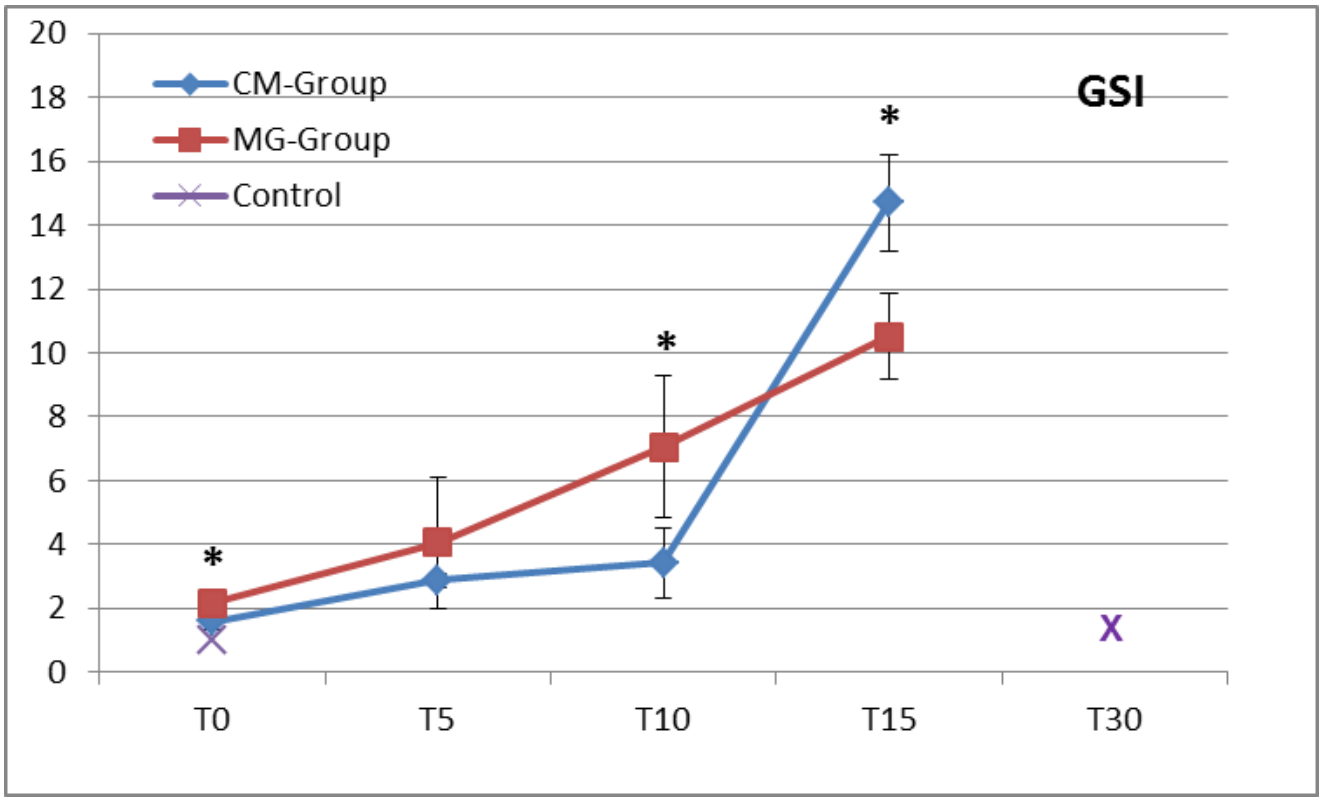



Fig. 14 - Evolution of gonado-somatic index (GSI) along the experiment

GSI data showed that raw gonad weight of all treated females followed a growth related to the increasing number of injections (Fig. 14). A slight increase in GSI up to the 10th injection was noticed in the CM-Group , after that a rapid increase up to the 15$^{th}$ injection while MG eels showed a regular increase the trial. GSI was found significantly higher in the MG-Group at T0 and T10, while at 15$^{th}$ 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 week was stable, ranging from 1.58 to 2.07, and comparable to values obtained at T0 (Fig. 14).

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 (5$^{th}$ CPE treatment) both groups showed larger previtellogenic oocytes (Fig. 14). At T10 (10$^{th}$ CPE treat

ment) 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. 14). At T15 (15$^{th}$ 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

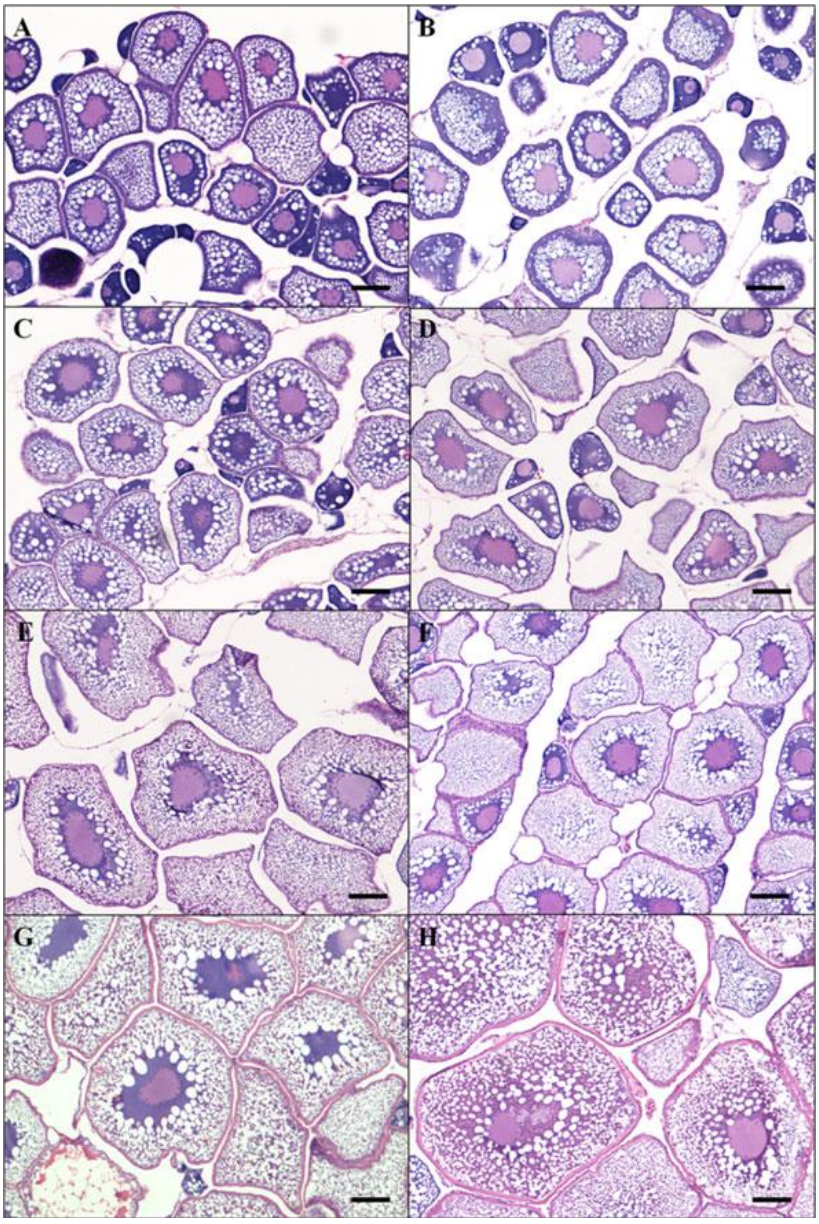
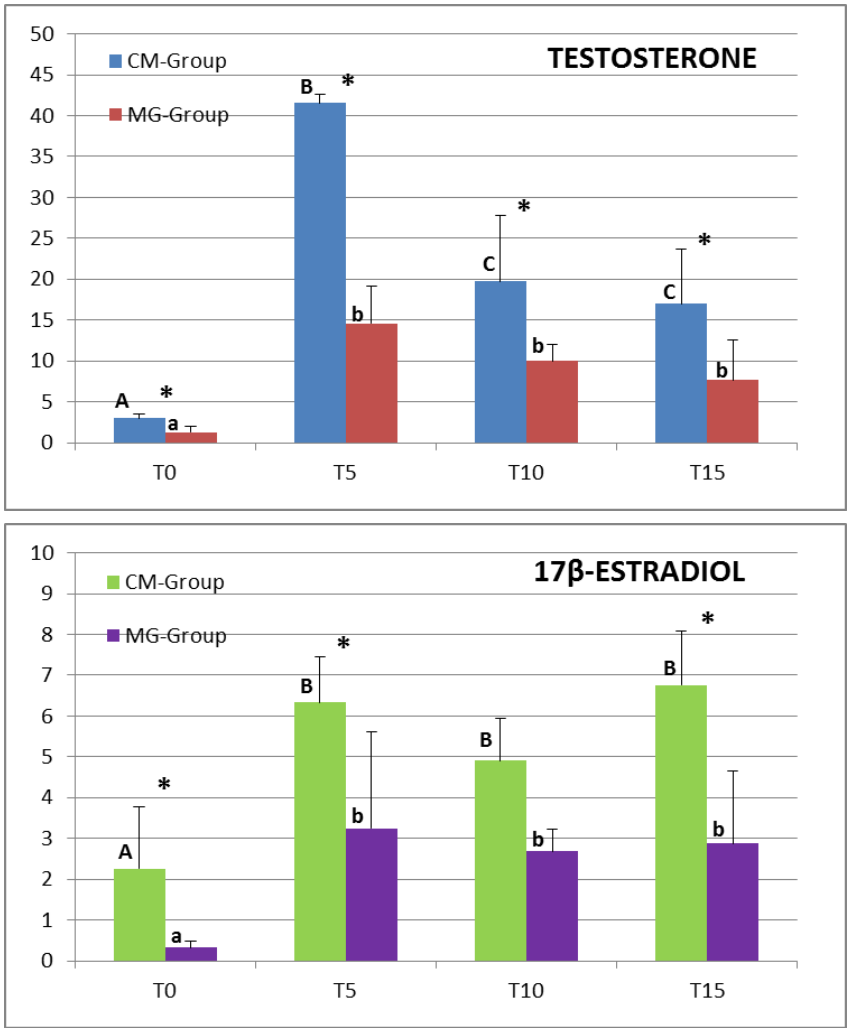



Fig. 15 - Histological sections of oocytes from the two populations (MG-Group: a, c, e, g; CM-Group: b, d, f, h) at different sampling points. Week 0 a, b Populations are at the same state of maturation (previtellogenic oocytes at oil drop st age). Week 5 c, d Populations are still at oil drop stage but with larger oocytes. Week 10e, f MG-Group (e) shows oocytes in early vitellogenesis with the first small yolk vesicles near the nucleusand 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 andsmallest oocytes and CM-Group (h) shows oocytes in late vitellogenesis with abundant yolk vesicles whichfill entirely the cytoplasm. (H&E staining; scale bars = 100 lm)

than yolk vesicles (Fig. 15). 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. 16-17).




Figs.16-17 - 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.

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. 16-17). 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). Fig. 18 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 not 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

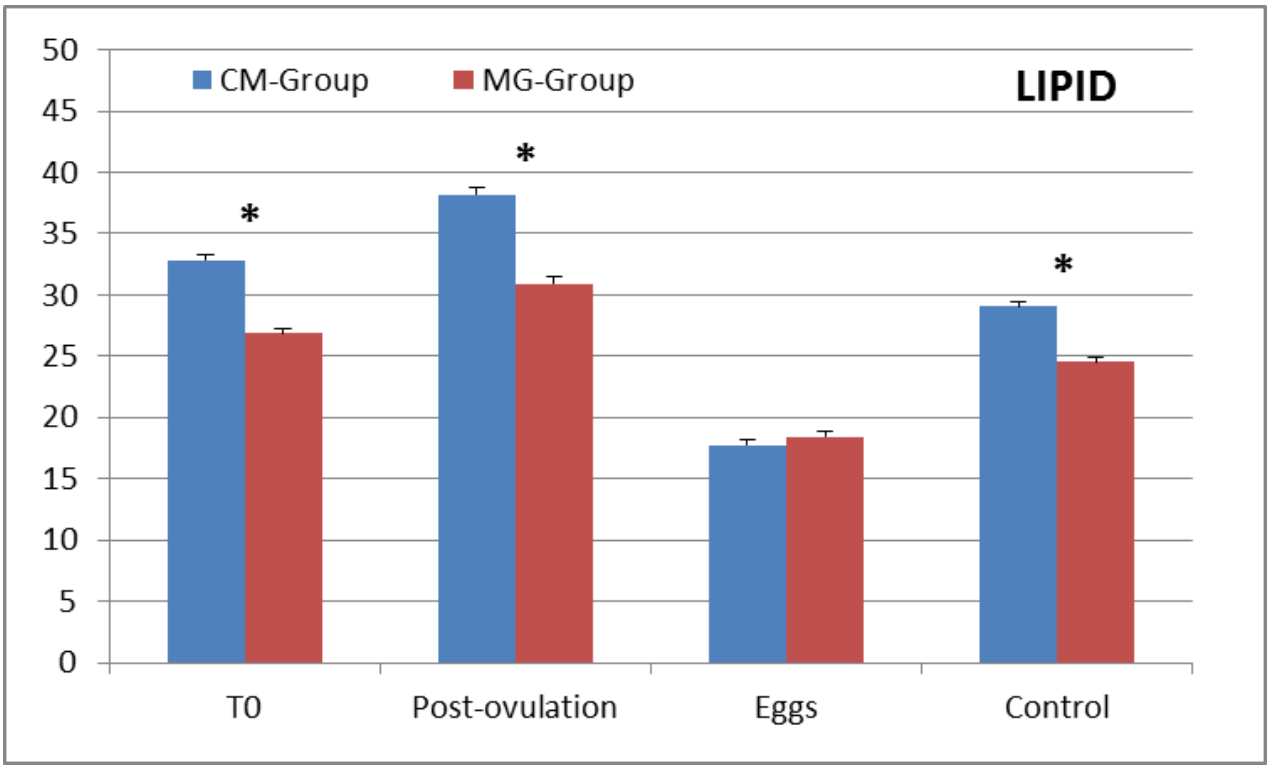



Fig. 18 - Total lipid content (% w.w.) in skin-on at T0, Post-ovulation, Control-Group (30 weeks) and unfertilized eggs.
*: significance difference (P<0.05) between CM and MG eels

after the last CPE injection, was significantly higher than that of CM-Group eels (Table 4). 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 frommanual 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. 19).

Table 4: Zootechnical performances obtained at the end of the experimental protocol

| | | **CM** | **MG** |
|---|---|---|---|
| ***BWI increase*** | *%* | 113.53±3.68 | 120.63±4.16* |
| ***Ovulatedeels*** | *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

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% to 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.

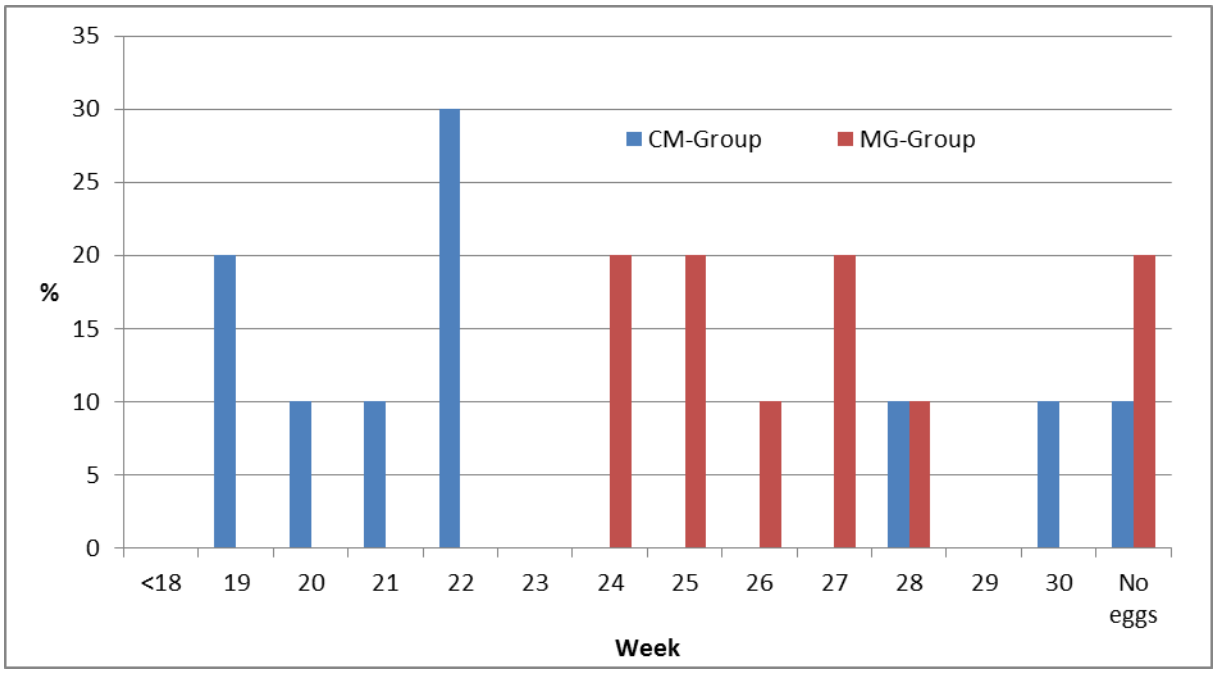



Fig. 29 - Females ovulated during experimental protocol (n=10 for both groups)

## 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. 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 & Van de 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 pre-requisite for the female eel sensitivity to gonadotropic stimulation (EELREP 2006; Dufour & Van de 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 towards 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 & van den Thillar 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 is, 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 (1353±241g and 358±94g in CM-Group and MG-Group respectively) and had a higher BL of about 26 cm (85.62±4.98cm 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 & 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 (*Crangoncrangon* and *Palaemon* sp.) and larger eels feeding preferentially on fish (in particular *Atherinaboyeri*, *Engraulisencrasicholus*, *Aphaniusfasciatus, 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

are 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 are not due to older age of animals, as only 11.4% of females were one 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, Svedang *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 & 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 (Messaoury *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 have a stimulatory effect on LH synthesis (Huang *et al*. 2001). This apparent relationship between the two steroids confirm that T act 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 pre-vitellogenic stage, contrary to what observed by other authors (Burzawa-Gerard & 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 where 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 $10^{th}$ until $15^{th}$ 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 & Boetius, 1985) in order to sustain normal gonadal development and to swim to the spawning grounds in the Sargasso Sea (Svedang & Wickstrom 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 & 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 if lipid content do triggers silvering in eels. Larssoon *et al.* (1990) suggested that there is a “critical fat mass” for triggering silvering while Svedang & Wickstrom (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 & van de 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 & van de Thillart 2010), while in captivity it seems to have no advantageous aspect. Anyway, highest lipid storage, associated to 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 to 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 contra-

ry, *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, i.e. protein, were also exhausted in addition to lipid. Concerning BWI, in eels, the increase in female BW (close to 10% in one 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 a*l. (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 ($19^{th}$-$22^{nd}$ week) and of all MG females ($24^{th}$-$27^{th}$ week) associated to a good level of homogeneity of oocytes obtained in the two populationsis 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 suggests 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 females 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 seems 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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## References


Aarestrup K., Okland F., Hansen M.M., Righton D., Gargan P., Castonguay M., Benrnatchez L., Howey P., Sparholt H., Pedersen M.I. & McKinley R.S. (2009) Oceanic Spawning Migration of the European Eel (*Anguilla anguilla*). *Science* 325, 1660

Aroua S., Schmitz M., Baloche S., Vidal B., Rousseau K. & Dufour S. (2005) Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. *Neuroendocrinology,* 82:221-232

Boetius I. & Boetius J. (1985) Lipid and protein content in *Anguilla anguilla* during growth and starvation. *Dana,* 4:1-17

Bono G., Cairoli F., Tamanini C. & Abrate L. (1983) Progesterone, estrogen, LH, FSH and PRL concentrations in plasma during estrous cycle in goat. *Reproduction Nutrition Development,* 23:217-222

Burgerhout E., Brittijn S.A., Kurwie T., Decker P., Dirks R.P., Palstra A.P., Spaink H.P. & van den Thillart G.E.E.J.M. (2011) First artificial hybrid of the eel species *Anguilla australis* and *Anguilla Anguilla*. *BMC Developmental Biology,* 11:16

Burzawa-Gerard E. & Dumas-Vidal A. (1991) Effects of 17β-estradiol and carp gonadotropin on vitellogenesis in normal hypophysectomized European silver female eel (*Anguilla anguilla* L.) employing a homologous radioimmunoassay for vitellogenin. *General and Comparative Endocrinology,* 84:264-276

Chiba H., Iwatsuki K., Hayami K., Hara A. & Yamuchi K. (1994) Changes in serum steroid hormones and vitellogenin levels in cultured female European eel *Anguilla anguilla* during artificially induced ovarian development. *Journal World Aquaculture Society,* 25(4):553-560

Cosolo M., Utmar P., Roppa F. & Sponza S. (2009) Interactions between fish resources and cormorants *Phalacrocoraxcarbo* in the Grado and Marano Lagoon (NE-Italy). *Acrocephalus,* 30(149):17-23

Cottril R.A., McKinley R.S., Van der Kraak G., Dutil J.D., Reid K.B. & McGrath K.J. (2001) Plasma non-esterified fatty acid profiles and 17β-oestradiol levels of juvenile immature and maturing adult America eels in the St Lawrence River. *Journal of Fish Biology,* 50:364-379

De Leo G.A. & Gatto M. (1996) Trends in Vital rates of the European eel: evidence for density dependence?. *Ecological Applications,* 6(4):1281-1294

Dufour S. & van den Thillart G. (2009) Reproduction Capacity of European Eels. In: van den Thillart G, Dufour S, Rankin JC (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 465-473

Durif C.M.F., van Ginneken V., Dufour S., Muller T. & Elie P. (2009) Seasonal Evolution and Differences in Silvering Eels from Different Locations. In: van den Thillart G., Dufour S., Rankin J.C. (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 13-38

Durif C., Gosset C., Rives J., Travade F. & Elie P. (2003) Behavioral study of downstream migrating eels by radio-telemetry at a small hydroelectric power plant. In: Dixon D A (eds) *Biology, Management and Protection of Catadromous Eels*. Bethesda, MD: American Fisheries Society, Symposium pp 343–356

Durif C., Dufour S. & Elie P. (2005) The silvering process of the *Anguilla anguilla*: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology,* 66:1025–1043

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel. *Marine Ecology Progress Series,* 327:171–181

EELREP (2006) Estimation of the reproduction capacity of European eel. Final report, EU Project, EELREP, Q5RS-2001-01836. Leiden University

Folch B.J., Lees M. & Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry,* 226:497-509

Fontaine Y.A. (1994) L'argenture de l'anguille: metamorphose, anticipation, adaptation. *BulletinFrancais De La Peche Et De La Pisciculture,* 335:171-185

Gaiani R. Chiesa F., Mattioli M., Nannetti G. & Galeati G. (1984) Androsteneidione and T testosterone concentrations in plasma and milk of the cow throughout pregnancy. *Journal of reproduction and fertility,* 70:55

Gelli F. (2011) Per uno studio di fattibilità della produzione di anguilla marinata delle valli del Friuli Venezia Giulia. In: *Prime ricerche svolte su anguilla europea (Anguilla anguilla)*, Centro Stampa Regione Emilia-Romagna, Bologna (Italy) pp 50-75

van Ginneken V. & van den Thillart G. (2000) Eel fat stores are enough to reach the Sargasso. *Nature*, 403:156-157

van Ginneken V. & Maes G.E. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: a literature review. *Reviews in Fish Biology and Fisheries,*15:367–398

van Ginneken V., Durif C., Balm S.P., Boot R., Verstegen M.W.A., Antonissen E. & van den Thillart G. (2007) Silvering of European eel (*Anguilla anguilla* L): seasonal changes of morphological and metabolic parameters. *Animal Biology,* 57(1):63–77

Han Y.S., Liao I.C., Tzeng W.N., Huang Y.S. & Yu J.Y.L. (2003a) Serum estradiol-17β and testosterone levels during silvering in wild Japanese eel *Anguilla japonica*. *Com Biochem Physiology,* 136:913-920

Han Y.S., Liao I.C., Huang Y.S., He J.T., Chang C.W. & Tzeng W.N. (2003b) Synchronous changes of morphology and gonadal development of silvering Japanese eel *Anguilla japonica*. *Aquaculture* 219:783-796

Holthaus K.B., Savorelli F., Gelli F., Modugno S., Trentini P.L. & Tasselli A. (2011) Il comprensorio vallivo di Comacchio e l'indice di maturità sessuale su campioni di anguille. In: *Prime ricerche svolte su anguilla europea* (*Anguilla anguilla*)., Centro Stampa Regione Emilia-Romagna, Bologna (Italy), pp 19-28

Huang Y.S., Sbaihi M., Le Belle N. & Dufour S. (2001) Combination of testosterone with cortisol stimulates the silvering of female European eel (*Anguilla anguilla.* L). In: Yu JYT (ed) *Proceedings of 4th Congress of the Asian and Oceania Society for Comparative Endocrinology*. Institute of Zoology. Academia Sinica, Taipei, Taiwan, pp 215-222

Ijiri S., Kazeto Y., Takeda N., Chiba H., Adachi S. & Yamauchi K. (1995) Changes in serum steroid hormones and steroidogenic ability of ovarian follicles during artificial maturation of cultivated Japanese eel, *Anguilla japonica*. *Aquaculture,* 135: 3-16

Ijiri S., Kayaba T., Takeda N., Tachiki H., Adachi S. & Yamauchi K. (1998) Pretreatment reproductive stage and oocyte development induced by salmon pituitary homogenate in the Japanese eel *Anguilla japonica*. *Fisheries Science*64: 531-537

Ijiri S., Tsukamoto K., Chow S., Kurogi H., Adachi S. & Tanaka H. (2011) Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. *Aquaculture Europe,*36(2):13-17

Kagawa H. (2005) Estimation of gonadal status of Japanese female eel *Anguilla japonica* by a single injection of salmon pituitary extract. *Fisheries Science,* 71:1084–1090

Kamei H., Kaneko T. & Aida K. (2006) Steroidogenic activities of follicle-stimulating hormone in the ovary of Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology,*146:83-90

Larsson P., Hamrin S. & Okla A. (1990) Fat content as a factor inducing migratory behavior in the eel (*Anguilla anguilla* L.) to the Sargasso Sea. *Naturwissenschaften,* 77:488-490

Matsubara H., Lokman P.M., Kazeto Y., Adachi S. & Yamauchi K. (2005) Serum steroid profiles in artificially maturing female Japanese eel, *Anguilla japonica*. *Aquaculture,* 243:393-402

Melia P., Bevacqua D., Crivelli A.J., Panfili J., De Leo G.A. & Gatto M. (2006) Sex differentiation of the European eel in brackish and freshwater environments: a comparative analysis. *Journal of Fish Biology,* 69:1228-1235

Messaouri H., Baloche S., Leloup-Hatey J. & Burzawa-Gerard E. (1991) Hepatic 17β-estradiol receptors and vitellogenesis in *Anguilla anguilla* L. after treatment by 17β-estradiol alone associated with bovine growth hormone. *General and Comparative Endocrinology,* 82: 238

Mordenti O., Di Biase A., Sirri R., Modugno S. & Tasselli A. (2012) Induction of sexual maturation in wild female European eel (*Anguilla anguilla*) in darkness and light conditions. *The Israeli Journal of Aquaculture – Bamidgeh,* IJA_64.2012.726, 9 p

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Hirose K. (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4- pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture,* 139: 291–301

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Iinuma N. (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiologyand Biochemistry,* 17:163–169

Okamura A., Yamada Y., Yokouchi K., Horie N.,Mikawa N., Utoh T., Tanaka S. & Tsukamoto K. (2007). A silver index for the Japaese eel (*Anguilla japonica*). *Environmental Biology of Fishes*80:77-89

Okamura A., Yamada Y., Horie N., Utoh T., Mikawa N., Tanaka S. & Tsukamoto K. (2008) Effects of silvering state on induced maturation and spawning in wild female Japanese eel *Anguilla japonica*. *Fisheries Science*74:642-648

Okamura A., Yamada Y., Mikawa N., Horie N., Utoh T., Kaneko T., Tanaka S. & Tsukamoto K. (2009) Growth and survival of leptocephali (*Anguilla japonica*) in low-salinity water. *Aquaculture,* 296:367–372

Oliveira K. & Hable W.E. (2010) Artificial maturation, fertilization, and early development of the American eel (*Anguilla rostrata*). *Canadian Journal of Zoology,* 88:1121-1128

Ozaki Y., Koga H., Takahashi T., Adachi S. & Yamauchi K. (2008) Lipid content and fatty acid composition of muscle, liver, ovary and eggs of captive-reared and wild silver Japanese eel *Anguilla japonica* during artificial maturation. *Fisheries Sciences,* 74:362-371

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. *Aquaculture,* 249:533–547

Palstra A.P. & van den Thillart G.E.E.J.M. (2009) Artificial maturation and reproduction of the European eel. In: van den Thillart G., Dufour S., Rankin J.C. (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 309-331

Palstra A.P., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2009) Effect of swimming on silvering and maturation of the European eel, *Anguilla anguilla* L. In: van den Thillart G., Dufour S., Rankin J.C. (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 229-251

Palstra A.P. & van den Thillart G.E.E.J.M. (2010) Swimming physiology of European eels (*Anguilla anguilla* L.): energetic costs and effects on sexual maturation and reproduction. *Fish Physiology and Biochemistry,* 36:297-322

Pedersen B.H. (2003) Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. *Aquaculture,* 224:323–338

Pedersen B.H. (2004) Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture,* 237:461–473

Perez L., Penaranda D.S., Dufour S., Baloche S., Palstra A.P., van den Thillart G.E.E.J.M. & Asturiano J.F. (2011) Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. *General and Comparative Endocrinology,* 174:51-59

Peyon P., Baloche S. & Burzawa-Gerard E. (1996) Potentiating effect of growth hormone on vitellogenin synthesis induced by 17β-estradiol in primary culture of female eel (*Anguilla anguilla* L.) hepatocytes. *General and Comparative Endocrinology,*102:263-273

Roncarati A., Gelli F., Brunelli F., Cavallini G., Forlini L., Pignata S. & Melotti P. (2008) Quality traits of ComacchioValli eels before, during and after the pickling process. In: 1st International Congress of Seafood Technology, 18-21 May 2008. (ed) S Cakli, U Celik, C Altinelataman, Izmir Turkey, pp 295-299

Stearns S.C. & Koella J.C. (1986) The evolution of phenotypic plasticity in life-history traits: predictions of reactionnorms for age and size at maturity. *Evolution,* 40(5):893-913

Svedäng H., Neuman E. & Wickström H. (1996) Maturation patterns in female European eels: age and size at the silver stage. *Journal of Fish Biology,* 48:342–351

Svedäng H. & Wickström H. (1997) Low fat contents in female silver eels: indications of insufficient energetic stores for migration and gonadal development. *Journal of Fish Biology,* 50:475–486

Tanaka H., Kagawa H. & Ohta H. (2001) Production of leptocephali of Japanese eel (*Anguilla japonica*) in captivity. *Aquaculture,* 201:51-60

Tanaka H., Kagawa H.,Ohta H., Unuma K. & Nomura N. (2003) The first production of glass eel in captivity: fish reproductive physiology facilitates great progress in aquaculture. *Fish Physiology and Biochemistry,* 28:493–497

van den Thillart G. & Dufour S. (2009) How to Estimate the Reproductive Success of European Silver Eels. In: van den Thillart G., Dufour S., Rankin J.C. (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 1-9

van den Thillart G., Palstra A. & van Ginneken V. (2009) Energy requirements of European eel for trans-Atlantic spawning migration. In: van den Thillart G., Dufour S., Rankin J.C. (eds) *Spawning Migration of the European Eel*. Reproduction index, a useful tool for conservation management, vol 30. Springer, New York, USA, pp 179-199

# CHAPTER 3

## INDUCTION OF SEXUAL MATURATION IN WILD FEMALE EUROPEAN EELS IN DARKNESS AND LIGHT CONDITION

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**Abstract**

The aim of present study was to induce sexual maturity in wild silver eels females of the European eel using a protocol similar to the one already successfully used for *Anguilla japonica*. Weekly injections (n. 16) of carp pituitary extracts (CPE) with an increasing dose of 10, 20, 30 and 40 mg/kg BW were given to silver females kept in light (L-Group) or dark (D-Group) conditions. In both groups, the final oocyte maturation and ovulation was induced by injection of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) at 2 mg/g BW. All the eels turned out to be silver and actively migrant (n 58 in stage V and n 2 in stage IV), showing a particularly high EI (15.75±1.40) and they were distributed within 3 age classes (7-9 years). In D-Group one of the 4 females at 13th CPE treatment ovulated 12h after the DHP injection. The remaining 3 females, which received the final (16th) CPE injection, ovulated between 12 and 36 h after the DHP injection. Two out of the 4 L-Group females which received the 16th CPE injection ovulated between 12 and 36 h after the DHP injection and the remaining 2 (L14 and L16) did not ovulate within 36 h post DHP injection. The total number of eggs released by the D-Group (n. 1,485,600) was significantly higher than that of the L-Group (n. 274,000). No mortality was observed during the experiment.

## 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 thesemeasures 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 use dextensively 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 17α, 20β-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 $100*(\text{body weight}/\text{body length}^3)$, eye index as $100*(((EDh + EDv) *0.25)^2\pi *(10* BL)^{-1})$ where EDh = horizontal eye diameter and EDv = vertical eye diameter, and pectoral fin length index as 100*(pectoral fin length/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 gonado-somatic index (GSI) was calculated as 100*(gonad weight/body weight). 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 *at al*. (2006). Agewas 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±0.5°C to approximate the temperature in Val Noghera at the start of the reproductive oceanic migration. Also, low temperatures are more likely to reduce individual variations insensitivity 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; $-0.04 \times 10^3$ lux at the bottom of the aquarium without water). The second group was gradually brought over a period of seven days to light conditions($0.40 \times 10^3$ 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.

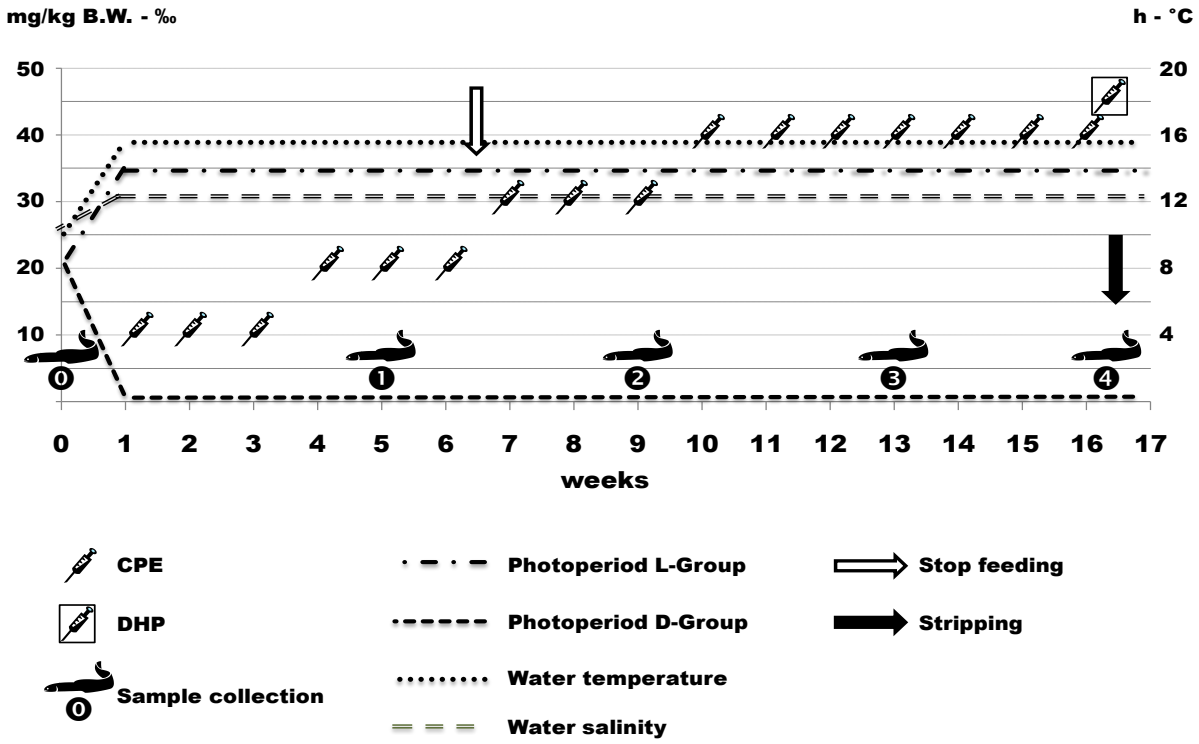


Fig. 20 - Schematic drawing of the experimental protocol adopted in European silver eel

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 BW during weeks 1-3, to 20 mg/kg during weeks 4-6, 30 mg/kg during weeks 7-9, and 40mg/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*(body weight/initial body weight).

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 17α,20β-dihydroxy-4-pregnen-3-one (DHP)/kg body weight 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 weight 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

No mortality was observed during the experiment and no eels were removed because of diseases. The external and internal measurements of the wild eels at the start of the experiment (T0) are reported in Tab. 5.

Table 5. Characteristics of silver eel adopted for the experimental procedure

| **Parameters** | | |
|---|---|---|
| ***External*** | | |
| **Eels** | *n* | 60 |
| **Body Weight (BW)** | *g* | 572.4±87.5 |
| **Body Length (BL)** | *cm* | 68.2±4.6 |
| **Condition factor (K)** | | 1.82±0.38 |
| **Eye index (EI)** | | 15.75±1.40 |
| **Pectoral fin length index (PFLI)** | | 5.5±0.4 |
| **Silver index (SI)** | | IV-V |
| ***Internal*** | | |
| **Eels** | *n* | 12 |
| **Gonadosomatic index (GSI)** | | 1.51±0.21 |
| **Age** | *years* | 7-9 |

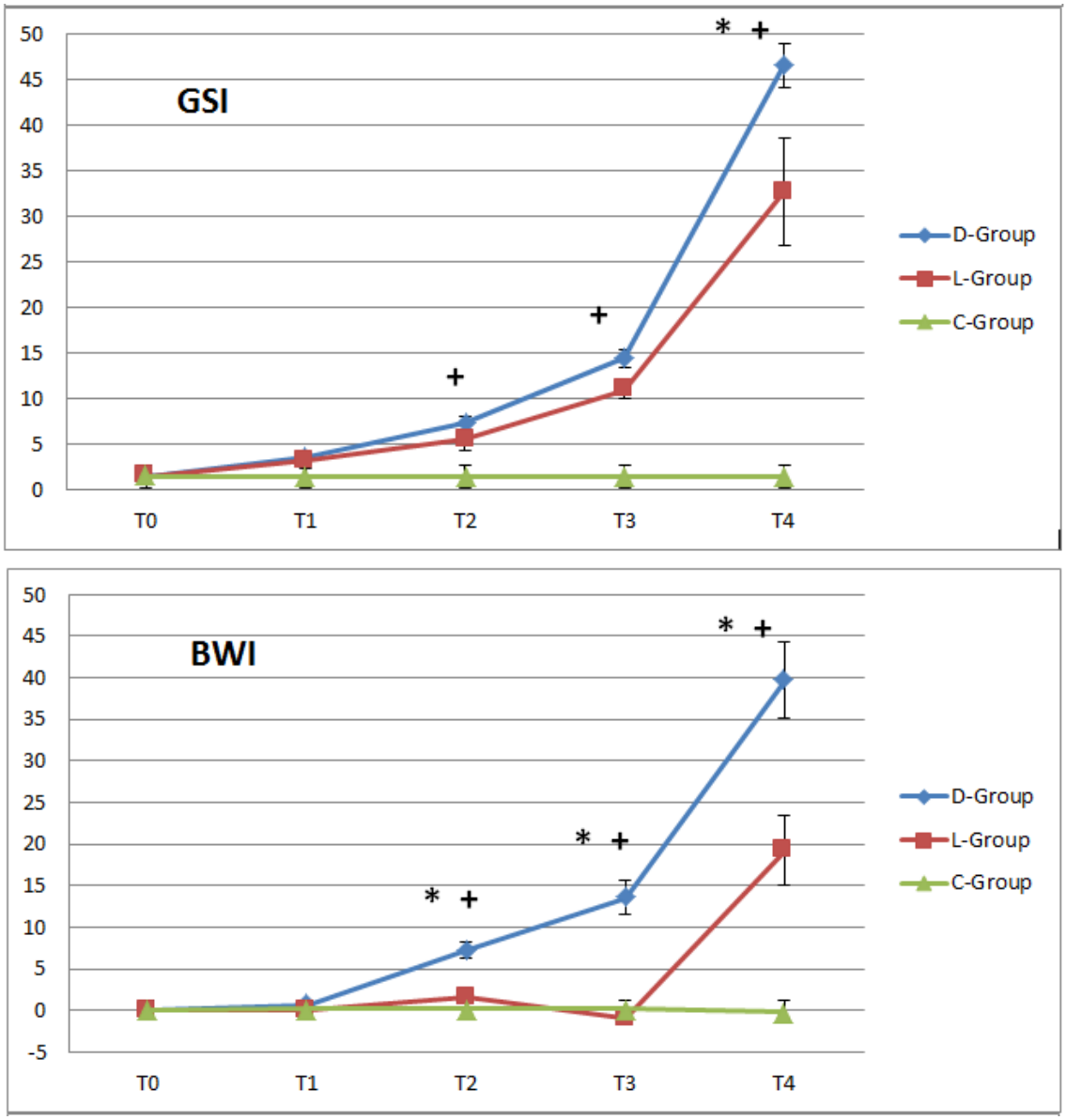


Fig. 21 e 22. Changes in body weight index (BWI) and gonadosomatic index (GSI) during the whole experiment

*: significant difference (P<0.05) between D-Group and L-Group eels;
+: significant difference (P<0.05) between treated and control eels.

All eels were silver and actively migrant: 58 eels were in stage V and only 2 were in stage IV. Morphometric parameters showed a particularly high EI (15.75±1.40). Eels sacrificed for internal parameters (T0) showed low variability in GSI (±0.21) and age classes that were not directly related to the BW eels (Table 5). Overall, results showed that raw gonad weight followed an exponential increase with the increasing number of injections while the GSI of control eels decreased regularly: after 16week, mean GSI of control eels was reduced by 15.2% (Fig. 21). Gonads of all treated eels underwent an increase of as much as 46.5% and 32.7% of final GSI (T4) in D-Group and L-Group respectively. At T4

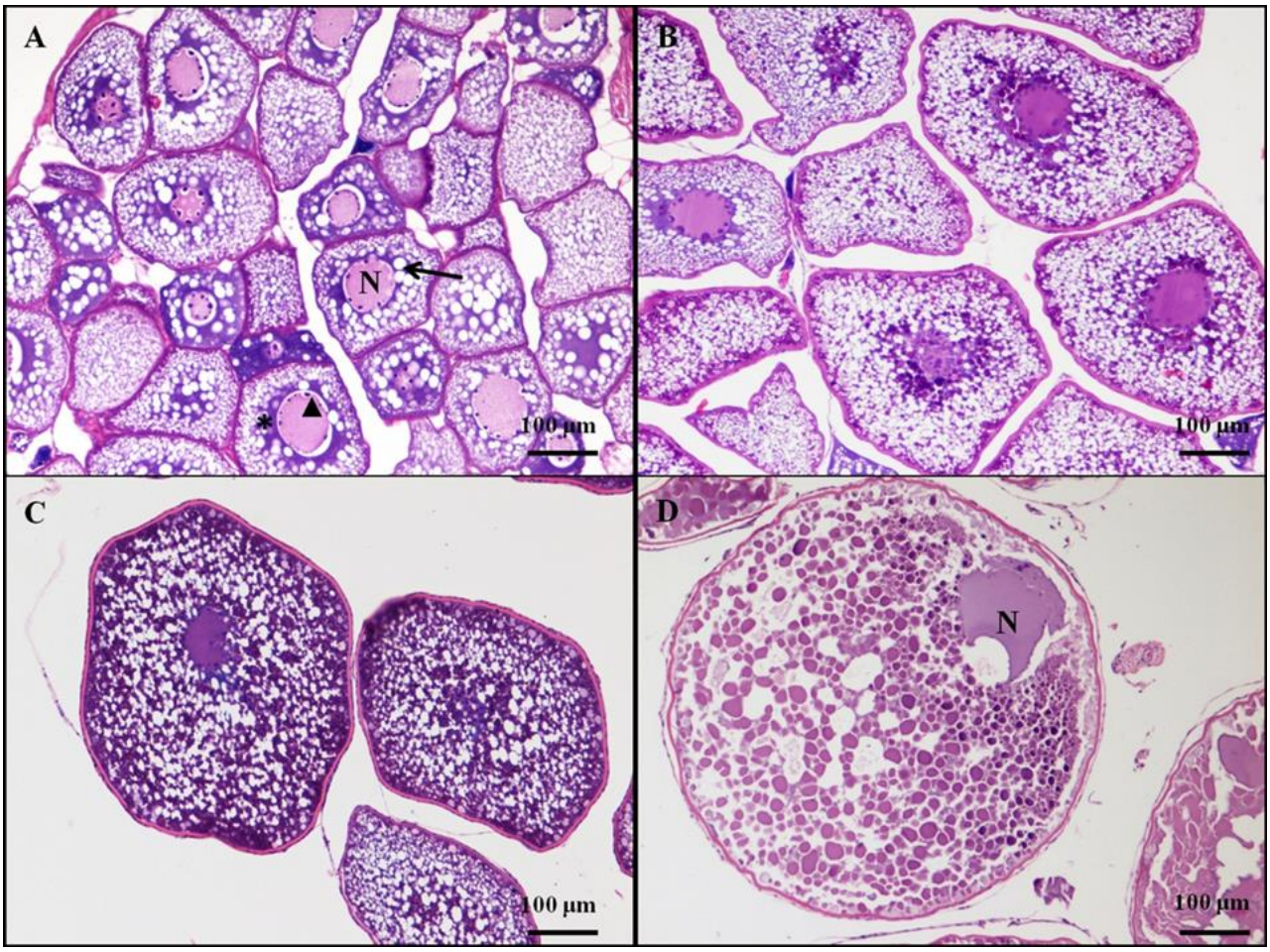


Fig. 23. Histological analysis of the oocytes. (a) T1: oocytes are in early vitellogenic. The nucleus (N) is central and multiple nucleoli (arrowhead) are peripherally located. (b) T2): oocytes are still in early vitellogenic stage. The nucleus is still centrally located. (c) T3: oocytes are in mid-vitellogenesis, cortical alveoli start to fuse together and the most part of the cytoplasm is filled with yolk globules. (d) T4: the eggs after stripping show the cytoplasm entirely occupied by large yolk globules and cortical alveoli fused together to form large lipid droplets. The nucleus (N) is migrated to the cell periphery. (H&E staining; scale bar = 100 µm).

the D-Group showed significantly higher GSI than the L-Group. Furthermore, individual response to light conditions was highly variable among L-Group eels: data observed at the end of the trial for each organ (gonad) ranged between 24.3 and 41.0%. Differences in GSI between treated and control eels became significant after the 9th injection (T2) (Fig. 21).

GSI increase was confirmed by BWI data, which revealed a constant growth in D-Group eels (Fig. 21) while in L-Group the increase was recorded starting from T3. At T4 the mean increase was 139.7% and 119.2% in D-Group and L-Group respectively. Beginning with the 9th injection (T2) (Fig. 21)

BWI of D-Group eels was significantly higher than that of L-Group eels. The BWI of control eels did not change significantly. The mean increase in BWI recorded after the 12h treatment with DHP (DHP injection – first stripping) was 10.4% (from 129.3 to 139.7) in D-

Group eels; 9.8% (from 123.2 to 133.0) in ovulated females L-Group eels (L13 and L15) and 1.5% (from 115.1 to 116.6) in unovulated females (L14 and L16) L-Group eels. Histological data showed a gonadic tissue organized in lamellae, supported by a stroma rich in adipose tissue (Palstra *et al*. 2007).

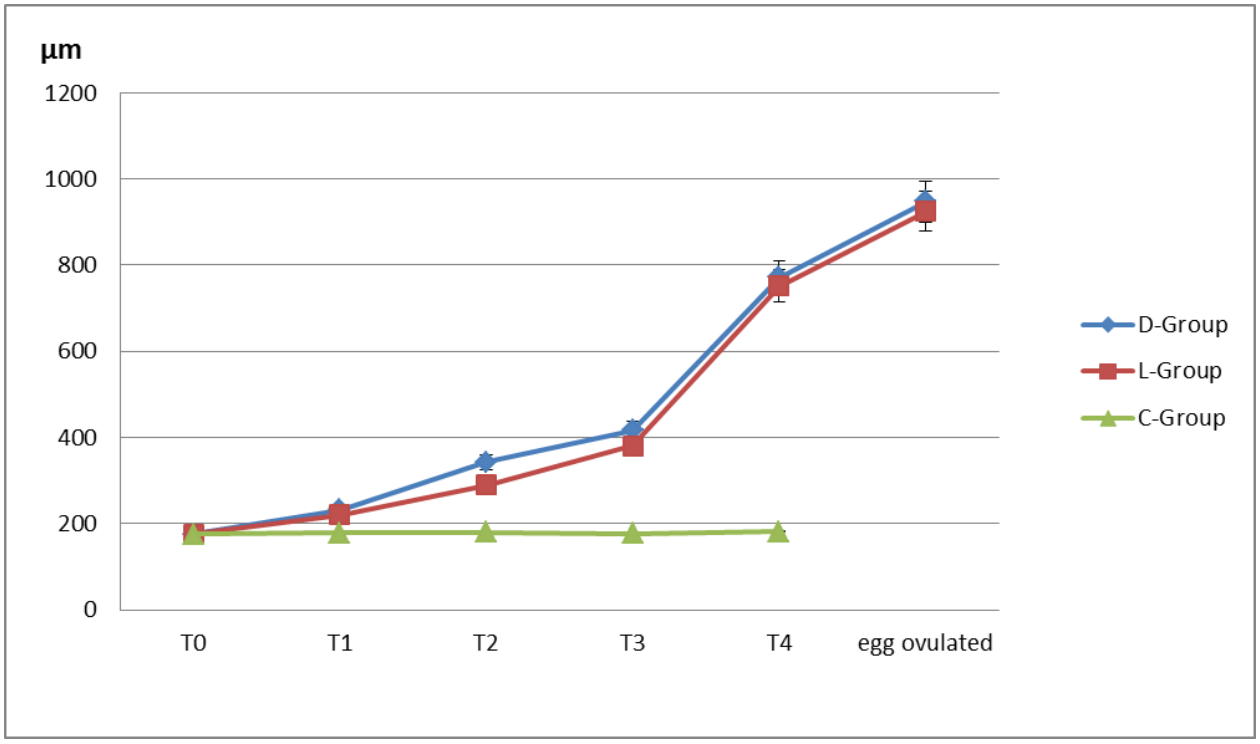


Fig. 24. Increase in the oocytes diameter during experiment

For the duration of the trial the eels of the C-Group showed oocytes in previtellogenic stage (oil drop stage): oocytes had a central round large nucleus (or germinal vesicle), multiple nucleoli and abundant cortical alveoli completely filling the cytoplasm. T1 eels showed oocytes in early vitellogenic stage (primary yolk globule stage) both in L- and D-Groups: oocytes showed abundant cytoplasm, still prominent cortical alveoli and the appearance of the first yolk globules around the germinal vesicle (Fig. 23). T2 eels from L- and D-Groups showed oocytes still in early vitellogenic stage: cortical alveoli filled entirely the cytoplasm together with more abundant densely-staining yolk globules. The nucleus was still centrally located. T3 eels from L- and D-Groups showed oocytes in mid-vitellogenesis: cortical alveoli fused together forming large lipid droplets; most of the cytoplasm was filled with yolk globules (Fig. 23).

In all the animals the three gonadal segments (caudal, central and cranial) displayed the same developmental stage. Data concerning oocytes diameters, reported in Fig. 24, showed an average increase in size with increasing maturation of the eel.

Table 6: Zootechnical performances obtained by females eels ad the end experimental protocol

| | ***Eels*** | ***Gonad*** | | | ***Spawned eggs*** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Weight* | | *GSI* | *12h* | *24h* | *36h* | *Total* | *Stripped* |
| | | *Total (g)* | *Residual(g)* | | | *(g)* | | *(g)* | *(%)* |
| D-Group | **D13** | 482.6 | 275.2 | 44.7 | 15.5 | 145.5 | 46.5 | 207.5 | 43.0 |
| | **D14** | 537.0 | 282.2 | 49.2 | - | 206.9 | 47.9 | 254.8 | 47.5 |
| | **D15** | 400.4 | 97.2 | 45.9 | 223.0 | 64.0 | 16.2 | 303.2 | 75.7 |
| | **D16** | 250.1 | 131.5 | 46.2 | 37.5 | 66.0 | 15.5 | 119.0 | 47.5 |
| | ***Total*** | | | | *276.0* | *482.4* | *126.1* | *884.5* | |
| L-Group | **L13** | 283.8 | 169.5 | 41.0 | - | 82.5 | 31.8 | 114.3 | 40.3 |
| | **L14** | 175.1 | 175.1 | 28.0 | - | - | - | - | - |
| | **L15** | 247.6 | 198.8 | 37.5 | - | - | 48.8 | 48.8 | 19.7 |
| | **L16** | 134.2 | 134.2 | 24.3 | - | - | - | - | - |
| | ***Total*** | | | | - | *82.5* | *80.6* | *163.1* | |

Regarding ovulation, eggs were not spontaneously released into the water but retained in the abdominal cavity. There were only small losses of eggs during handling. One of the four eels of D-Group (D16) showed a BWI of over 120 24 h after the 13th CPE injection. On the same day it was given a DHP injection which prompted the ovulation, 12 hours later. The remaining three females, received the 16th CPE injection and ovulated between 12 and 36 h after the DHP injection. Out of the four L-Group females, 2 (L13 and L15) after the 16th CPE injection ovulated between 24 and 36 h after the DHP induction and the remaining 2 (L14 and L16) did not ovulate within 36 h post DHP injection (Table 5). Three of the 4 D-Group females which ovulated released from 43% to 47.5% of the total weight of the gonad, while the remaining one (D15) exceeded 75%. The 2 eels of the L-Group which ovulated released 40.3% and 19.7% of the total weight of the gonad (L13 and L15 respectively).

Regarding spawned eggs at fixed intervals (12h, 24h and 36h) after the DHP injection, the eggs/female obtained in each group from manual stripping is shown in Tab. 6. At the 12th hour the female D15 released a large number of eggs (73.5% of its total ovulation). This eel was the most productive with 300.3g of eggs released and only 97.2g of oocytes retained in the abdominal cavity, thus exceeding the 500,000 eggs. L-Group females did not ovulate within 12 h after injection. The peak in production of eggs was obtained 24 h after the DHP induction with 54.5% and 82.5% of the eggs released in D-Group and L-Group respectively: eels D13 and D14 were the most productive with 70.1 and 81.2% of the total weight of the gonad. Thirty-six h after the DHP injection, the D-Group eels did not

exceed 15% of the weight of the gonad whereas in L-Group the L15 eel released 100% of its eggs (Table 6).

Histological analysis of the eggs of stripped animals showed a cytoplasm entirely occupied by large yolk globules and a nucleus that had completed migration (Fig. 23) to the cell periphery while histological analysis of residual gonad showed oocytes with cytoplasm entirely occupied by large yolk globules but the nucleus had not completed the migration to the cell periphery.

Table 7: Number of eggs/gram, number of eggs/female and total number of eggs/Group obtained by ovulated females

| **Eels** | **Eggs** | | | |
|---|---|---|---|---|
| | *n°/g* | *n°/eel* | *n° kg b.w.* | |
| **D13** | 1,644±27 | 348,600 | 323,100 | - |
| **D14** | 1,682±30 | 428,100 | 392,400 | - |
| **D15** | 1,720±28 | 509,000 | 583,700 | - |
| **D16** | 1,670±29 | 199,900 | 369,500 | - |
| **L13** | 1,717±26 | 192,000 | - | 277,500 |
| **L15** | 1,647±21 | 82,000 | - | 124,100 |
| ***Mean*** | *1,680±38* | | *417,200±99,300** | *200,800±76700* |
| ***Total D-Group (n°)*** | *1,485,600** | | | |
| ***Total L-Group (n°)*** | *274,000* | | | |

*: significant difference (P<0.05)

The number of eggs/gram (Table 7), the eggs' diameter (Fig. 24) and weight (0.59±7.9*103mg) of ovulated females were very homogeneous and showed no statistical differences both within and between the two experimental groups (L-Group and D-Group). The total number of eggs released by the D-Group eels was significantly higher than those released by the L-Group (eels maintained in "light conditions").

## Discussion

The Val Noghera lagoon, in the northern Adriatic Sea, is an environment with outstanding characteristics where it is still possible to find female eels with high SI to be used (in captivity) to start a program of artificial reproduction and where it is possible to restore the conditions for hosting and breeding eels. As noted by Durif *et al*. (2006), good reproductive responses came from high initial stage of female maturation. Indeed, in our study all the eels were at migrating stage IV and V (high SI) which is a pre-requisite for the use of fe-

male eel sensitivity to gonadotropic stimulation (EELREP 2005). Also our T0 female eels displayed GSI which can be considered typical of a migrant eel (Durif 2003). The EI recorded in our eels was higher (about 50% higher) than that observed by Durif *et al*. (2005) in Stage V eels. Regarding the EI, even EELREP (2005) showed a good correlation between initial ocular index and the maturation sensitivity. The high silvering value of Val Noghera's eels, presumably stems from the fact that the eels were caught in March, a few months after the transition from III (pre-migrating) to IV (migrating) stage which, according to Durif (2003), probably occurs at the end of the summer to early autumn (September-November). On the other hand, van Ginneken *et al*. (2005, 2007) noted that eels captured in the lake Grevelingen, the Netherlands, showed the highest stage of gonads' development in the fall and this is why they collected their specimens for the hormonal treatment at that time, just prior to their seaward migration. Therefore, maturation rate may not only be species-dependent, but may also be environmental-dependent.

Durif *et al*. (2005) say that at this silvering stage eels stop feeding, while in our study treated eels stopped feeding after the 6th hormonal injection (45th day) and C-Group eels continued feeding for the entire duration of experiment. This can be explained by the fact that our eels were collected in a natural and enclosed lagoon where they were unable to undertake their migration. According to Durif *et al*. (2003, 2005) downstream migration itself is flexible and if environmental conditions are not favorable, e.g. in the presence of barriers or dams, eels stop their migration for long periods of time, even months at a time, so eels that missed a favorable 'environmental window' would probably stop silvering and revert back to the growth phase. Svedäng & Wickstrom (1997) conclude that silvering is much more flexible than assumed, and that eels can stop metamorphosing and resume feeding if the chances of successfully migrating are compromised. Probably in our case the hormonal induction and the favorable environmental conditions inside the tanks restarted the silvering process. Moreover, the eels were fed with a mixture of Gadidae fresh ovary, high in n-3 fatty acids, effective in the feed of eels broodstocks (Furuita *et al*., 2007).

GSI and BWI analysis show a positive trend and the progressive maturation of the gonads of the hormonally treated eels, especially among those which were kept in dark conditions. Concerning BWI, in *A.japonica* the increase in female BW (close to 10% in one week) is used as a reliable indicator of the last phase of ovarian maturation (Ohta *et al*. 1996). In our study however this weight gain does not appear to be enough to initiate ovulation with DHP. Actually our results show that the increased production of eggs was ob-

tained from eels with BWI around 140%, while eels with BWI below 120% were unovulated females. Moreover, GSI obtained in our study is in line with that recorded by Palstra *et al*. (2005) with ovulated *A.anguilla* (GSI 44.8±6.5). Our histological analysis show a positive trend and a progressive maturation of the oocytes of treated eels in the three gonadal segments, typical of a synchronous species (Murua *et a*l. 2006) while Palstra *et al*. (2005) show asynchronous ovarian development in eels kept in captivity, thus supporting the idea that asynchronous oocyte development has an artificial rather than a natural origin. The good level of homogeneity of oocytes obtained in our study is probably the result of the low-dosage of hormone administered initially to obtain a synchronization in the maturation of eels. Subsequently the dosage was increased, thus allowing the oocytes plenty of time to mature during the entire duration of the study. At the end of the hormonal treatment we had some small differences in the vitellogenic stage, probably related to the sudden acceleration of the maturation process. Synchronizing of female maturation associated to male maturation, taking 4-5 weeks by intramuscular injection with HCG (Pedersen 2003; Kagawa *et al*. 2009) is a desirable step in artificial reproduction, because the time window for high-quality eggs is very narrow (Ijiri *et al*. 2011).

The ovulated females (a total of 6) were stripped after 13 (n 1) and 16 (n 5) weekly injections, a timing which is comparable to that reported by Palstra *et al*. (2005) (71% eels matured and stripped between 12 and 17 injections). Pedersen (2003) on the other hand reported maturation after 24-25 weekly injections with wild European eels in spring and 14-22 weekly injections with farmed European eels in winter using 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. Therefore 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. 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).

Regarding ovulation our results lead to interesting considerations: while the percentage of ovulated females in similar studies is fairly low (Ohta *et al*. 1996; Pedersen 2003; EELREP 2005; van Ginneken *et al*. 2005; Kagawa *et al*. 2005), we had a 100% success rate (every single eel in the D-Group ovulated). On top of that, our protocol, based on increasing doses of CPE, was also able to produce a significant amount of eggs with females kept in dark condition: all the D-Group eels reached high levels of eggs production

while the L-Group eels had a lower response to the hormonal stimuli, thus highlighting the positive effect on eggs production of the darkness factor.

The absolute fertility we obtained is lower than van Ginneken's *et al.* (2005) (772,000-3,945,000 eggs/female). However, their values are probably overestimated because they are calculated on the weight of oocyte and GSI and not on the real weight of ovulated eggs.

Regarding eggs, the size and the transparency achieved along with the complete migration of the nucleus suggest good quality. The diameter of the oocytes at the migratory nucleus stage and that of the ovulated eggs are in line with those observed by other researchers 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).

The ovulation stretched up to 36 hours after DHP injection, a value higher than that recorded in Japanese eel (15-23h) (Tanaka *et al*. 2003; Ohta *et al*. 1996) and European eel (Pedersen 2003, 2004; Palstra *et al*. 2005), probably due to the lower water temperature which caused a delay in the response to hormonal treatment. Such a delay did not affect the quality of the eggs and we obtained oocytes without cytoplasmatic degeneration, unlike Ohta *et al*. (1997).

Finally, the absence of mortality during the experiment is another very positive result considering that several studies with European eel reported high mortality rates (EELREP, 2005; van Ginneken et al., 2005; Pedersen, 2003, 2004; Palstra et al., 2005, 2010). The lack of mortality can be explained in many ways, but the hardiness of the eels used and the lower doses of hormone during the first 6 weeks certainly helped reducing the phenomena of stress.

## Conclusions

The high silvering value of Val Noghera eels contributed to the success of the artificial reproduction trial.

The great gonadal maturation and the high egg production of the D-Group eels show that darkness is conducive to a good reproductive performance.

Finally, the adopted protocol, which includes increasing doses of CPE, appears to have contributed to the synchronization of the maturation of the eels, the absence of mortality and the high production of good quality eggs.

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## References


Dou S.Z., Yamada Y., Okamura A., Tanaka S., Shinoda A. & Tsukamoto K. (2007) Observation on the spawning behavior of artificially matured Japanese eel *Anguilla japonica* in captivity. *Aquaculture*, 266:117–129.

Dou S.Z., Yamada Y., Okamura A., Tanaka S., Shinoda A. & Tsukamoto K. (2008) Temperature influence on the spawning performance of artificially-matured Japanese eel, *Anguilla japonica*, in captivity. *Environmental Biology of Fishes*, 82:151–164.

Durif C., Gosset C., Rives J., Travade F. & Elie P. (2003) Behavioral study of downstream migrating eels by radio-telemetry at a small hydroelectric power plant. In:*Biology, Management, and Protection of Catadromous Eels* (Dixon, D. A., eds.). Bethesda, MD: American Fisheries Society, Symposium, 343–356pp.

Durif C., Dufour S. & Elie P. (2005) The silvering process of the eel: a new classification from the yellow resident stage to the silver migrating stage. *Journal of Fish Biology*, 66:1–19.

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel.*Marine Ecology Progress Series*, 327:171–181.

Durif C. (2003) La migration d'avalaison de l'anguilleeuropéenne Anguilla anguilla: Caractérisation des fractions dévalantes, phénomène de migration et franchissementd'obstacles. These de doctorat, Université Paul Sabatier, Toulouse.

EELREP (2005) Estimation of the reproduction capacity of European eel. Final report. http://www.fishbiology.net/eelrepsum.html

Fontaine M., Bertrand E., Lopez E. & Callamand O. (1964) Sur la maturationdesorganesgénitaux de l'anguillefemelle (*Anguilla anguilla* L.) et l'émissionspontanéedesoeufs en aquarium. *Comptesrendushebdomadaires des séances de l'Académie des sciences*, 259:2907–2910.

Furuita H., Hori K., Suzuki N., Sugita T. & Yamamoto T. (2007) Effect of n-3 and n-6 fatty acids in broodstock diet on reproduction and fatty acid composition of broodstock and eggs in the Japanese eel, *Anguilla japonica*. *Aquaculture*, 267:55-61.

van Ginneken V., Vianen G., Muusze B., Palstra A., Verschoor L., Lugten O., Onderwater M., van Schie, S. Niemantsverdriet, P. van Heeswijk R., Eding E. & van den Thillart G. (2005) Gonad development and spawning behaviour of artificially-matured European eel (*Anguilla anguilla*). *Animal Biology*, 55:203–218.

van Ginneken V. & MaesG.E. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: a literature review. *Reviews in Fish Biology and Fisheries*, 15:367–398.

van Ginneken V., Durif C., Balm S.P., Boot R., Verstegen M.W.A., Antonissen E. & van den Thillart G. (2007) Silvering of European eel (*Anguilla anguilla* L): seasonal changes of morphological and metabolic parameters. *Animal Biology*, 57(1):63–77.

Grandi G., Poerio F., Colombo G. & Chicca M. (2000) Effects of diet supplementation with carp ovary on gonad differentiation and growth of the European eel. *Journal of Fish Biology*, 57:1505-1525.

Ijiri S., Tsukamoto K., Chow S., Kurogi H., Adachi S. & Tanaka H. (2011) Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. *Aquaculture Europe*, 36(2): 13-17.

Kagawa H. (2005) Estimation of gonadal status of Japanese female eel *Anguilla japonica* by a single injection of salmon pituitary extract. *Fisheries Science*, 71:1084–1090.

Kagawa H., Iinuma N., Tanaka H., Ohta H. & Okuzawa K. (1998) Effects of rearing period in seawater on induced maturation in female Japanese eel (*Anguilla japonica*). *Fisheries Science*, 64:77–82.

Kagawa H., Tanaka H., Ohta H., Unuma K. & Nomura N. (2005) The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. *Fish Physiology and Biochemistry*, 31:193–199.

Kagawa H., Kasuga Y., Adachi J., Nishi A., Hashimoto H., Imaizumi H. & Kaji S. (2009) Effects of continuous administration of human chorionic gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in male Japanese eel, *Anguilla japonica*. *Aquaculture*, 296:117–122.

Murua H., Saborido-Rey F., Tomkiewicz J., King P. & Rideout R. (2006) Female Reproductive Strategies of Marine Fish Species of the North Atlantic. *Abstract of 3rd Workshop of Gonadal Histology of Fishes*, 11-12 July, New Orleans.

Nilsson L., Nyman L., Westin L. & Ornhagen H. (1981) Simulation of the reproductive migration of European eels (*Anguilla anguilla* (L.)) through manipulation of some environmental factors under hydrostatic compression. *Speculations in Science and Technology,* 4:475–484.

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Hirose K. (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4- pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture*, 139:291–301.

Ohta H., Kagawa H., Tanaka H., Okuzawa, K. & Linuma N. (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiology and Biochemistry*, 17:163–169.

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. *Aquaculture*, 249:533–547.

Palstra A.P., Curiel D., Fekkes M., de Bakker M., Székely C., van Ginneken V. & van den Thillart G.E.E.J.M. (2007) Swimming stimulates oocyte development in European eel (*Anguilla anguilla* L.). *Aquaculture*, 270:321–332.

Palstra A.P., Schnabel D., Nieveen M.C., Spaink H.P. & van den Thillart G.E.E.J.M. (2010) Temporal expression of hepatic estrogen rtecepror 1, vitellogenin1 and vitellogenin2 in European silver eels. *General and Comparative Endocrinology*, 166:1-11.

Pedersen B.H. (2003) Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. *Aquaculture*, 224:323–338.

Pedersen B.H. (2004) Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture*, 237:461-473.

Rodriguez A., Castellò-Orvay F. & Gisbert E. (2009) Somatic growth, survival, feed utilization and starvation in European elver*Anguilla anguilla* (Linneus) under two different photoperiods. *AquacultureResearch*, 40:551-557.

Svedäng H. & Wickström H. (1997) Low fat contents in female silver eels: indications of insufficient energetic stores for migration and gonadal development. *Journal of Fish Biology*, 50:475–486.

Tanaka H., Kagawa H., Ohta H., Unuma K. & Nomura N. (2003) The first production of glass eel in captivity: fish reproductive physiology facilitates great progress in aquaculture. *Fish Physiology and Biochemistry*, 28:493–497.

# CHAPTER 4

# EFFECT OF PHOTOPERIOD ON ENDOCRINE PROFILES AND VITELLOGENIN EXPRESSION IN EUROPEAN EELS DURING ARTIFICIALLY INDUCED OVARIAN DEVELOPMENT

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**Abstract**

The aim of this work was to determine the effects of dark and light conditions on the E2, testosterone and thyroid hormones levels and on the gene expression levels (vitellogenin 1, vitellogenin 2 and estradiol receptor 1) in European eels (*Anguilla anguilla*) during ovarian development induced by increasing doses of carp pituitary extracts (CPE). The subjects were divided into two groups: 14-h-light:10-h-dark (Light Group) and 24h darkness (Dark Group). All the eels received intramuscular injections with carp pituitary extract (CPE) at a dosage of 10 mg/Kg BW once a week for the first three weeks, 20 mg/kg BW $4^{th}$-$6^{th}$ week, 30 mg/kg BW $7^{th}$-$9^{th}$ week and 40 mg/kg up to the end of the experiment ($13^{th}$ week). Vitellogenin and estradiol receptor expression levels did not show significant differences between the two housing conditions while in both groups vitellogenin mRNA increased starting from first CPE injection. Testosterone (T) and 17β-Estradiol (17β-E2) plasma levels were significantly higher in the Dark Group compared to the Light one starting from the $9^{th}$ and the $13^{th}$ week respectively. These results suggest that darkness could be a useful variable for standardizing gonadal maturation in eels kept in captivity.

## Introduction

Eels exhibit a very peculiar life cycle, after a long growth period in rivers, they show significant morphological and physiological changes from yellow to silver stage (process known as 'silvering') before the seawater spawning migration (Fontaine *et al*. 1995; Han *et al*. 2003). The morphological modifications include a change in belly and fin colour and an increased eye size (Han *et al*. 2003; Rohr *et al*. 2001). The physiological changes include degeneration of the digestive tract (Han *et al.* 2003), changes in visual pigments (Pankhurst 1982), a more developed swim bladder (Kleckner 1980; Yamada *et al.* 2001), higher branchialchloride cells density (Fontaine *et al*. 1995), higher muscle fat contents (Han *et al*. 2001), and more developed gonads (Han *et al*. 2003). The artificial reproduction of the European eel has become a critical issue in the context of the dramatic decline of the population. Most of the efforts are based on the aim to reproduce the species in captivity, which leads aquaculture to be the main field of study and one of the few opportunities for the species to survive. To artificially induce gamete maturation studies have focused on hormone injection dosage and frequency, timing and type of ovulation primers, body indices, swimming performances and environmental parameters such as water temperature, salinity and pressure (Ohta *et al.* 1997; Dou *et al*. 2008). Photoperiod is one of the most important environmental factors for the regulation of fish physiology and metabolism and it has been manipulated in order to improve the performance and profitability of the aquaculture activities (Rodriguez e*t al.* 2009). Modified photoperiods have been shown to alter the spawning timing in a wide range of seasonal spawning species (Bromage *et al*. 2001). Vøllestad *et al.* (1994) demostrated that photoperiod affects downstream migration using tagged silver eels and observing they migrate faster as the daylight decreased, suggesting that photoperiod could have an effect on silvering. Durif *et al*. (2005) also hypothesized that photoperiod (or the decrease in temperature) could activate the last stages of the silvering process as well as they affect smolting in Salmonids. The obvious advantage is the synchronization of the onset of the migration via homeostatic and hormonal priming, so that to get the future spawers physiologically ready for migration at the same time.

Our previous study (Mordenti *et al*. 2012) has demonstrated that the photoperiod influences sexual maturation of eels showing that dark conditions lead to a better reproduc-

tive performance in terms of a higher egg production and spawning record. As from bibliography, no information is yet available with respect to the influence of photoperiod on the sexual steroid production and on the gene expression levels of wild silver eels kept in captivity. Therefore the effects of light and dark conditions were investigated on the E2, testosterone and thyroid hormones levels. In addition we investigated hepatic gene expression of vitellogenin 1 and 2 (vtg1 and vtg2) and estradiol receptor 1 (esr1) of wild female European silver eels during pituitary extracttreatment.

## Materials and methods

### *Animals*

European silver eels (*Anguilla anguilla*) were caught early in spring using traditional *lavoriero* in brackish water lagoon near the sluices of the North Adriatic Sea (salinity 26‰, water temperature 10°C) (Val Noghera, Friuli Venezia-Giulia, Italy). Larger eels (n=60) were selected and then transported to the laboratory where they were measured to obtain external indicators of their maturation state (mean body weight of 572.4±87.5 g, mean body length of 68.2±4.6 cm and silver index stage of IV-V) (Mordenti *et al*. 2012; Dourif *et al*. 2006).

All procedures for the sampling of fish and sacrifice were approved by the Ethical Committee of the Bologna University (n° 19/69/12).

The eels were housed in a 3,600 L tank connected to a recirculation system in natural seawater (salinity ranged between 28‰ to 32‰, water temperature 14±2°C, photoperiod 12L:12D) and fed daily *ad libitum* with a mixture of Gadidae fresh ovary (Grandi *et al*. 2000).

After one week twelve female eels were sacrificed with an overdose of anesthetic (2-phenoxyethanol), blood and liver were sampled , and gonad tissue collected for histology purposes (P0). Twenty-four eels were randomly divided into 2 experimental groups (Dark group n=12 and Light group n=12) and kept in two 700 L tanks (water temperature 14±2°C and salinity 32‰); Dark Group eels were maintained under dark conditions for 24-h (0L:24D) ($0.04* 10^3$ lux at the bottom of the aquarium without water) and Light Group under 14h light conditions (14L:10D) ($0.40*10^3$ lux at the bottom of the aquarium without water) for the whole period of the experiment. Light was provided by three 36W/950 halogen

lamps 30 cm placed above the water surface. At this point the animals were starved throughout the duration of the experimental period.

After 7 days the hormonal induction started and all the eels received intramuscular injections with carp pituitary extract (CPE) at a dosage of 10 mg/Kg BW once a week for the first three weeks, 20 mg/kg BW $4^{th}$-$6^{th}$ week, 30 mg/kg BW $7^{th}$-$9^{th}$ week and 40 mg/kg up to the end of the experiment ($13^{th}$ week). This hormonal induction protocol has been developed by our research group and published in a previous work (Mordenti *et al.* 2012). At the $5^{th}$ (P1), $9^{th}$ (P2) and $13^{th}$ (P3) week, 4 animals from the Dark and Light Groups were anesthetized, weighed in order to calculate body weight index (BWI) and blood samples were collected from the caudal vasculature. Animals were then sacrificed and their gonads weighted to calculate the gonado-somatic index (GSI), samples of liver were collected, frozen in liquid nitrogen, and stored at -80°C until analysis. Gonad tissue samples were also collected and formalin-fixed for histology.

BWI and GSI were calculated according to the formulae below:

BWI= (body weight/initial body weight)*100

GSI= (gonad weight/body weight)*100

*Hormonal analysis:17-β-estradiol, testosterone and thyroid hormones*

Blood samples (0.5-1 mL) were collected in heparinised tubes, centrifuged (4000xg, 20 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 17-β-estradiol and testosterone. The plasma 17-β-estradiol (E2) was determined using a RIA method as previously described (Bono *et al*. 1983). The sensitivity was 0.96 pg/tube, and the intra-coefficient of variation was 5.8%. Cross reactions of various steroids with antiserum raised against 17-β-E2 were: 17-β-estradiol (100%), 17-α-estradiol (0.62%), estrone (1.5%), estrone-3-sulphate (< 0.3%), estriol (0,8%), estriol-3-sulphate (0.03%). The results were expressed as ng/mL.

Testosterone (T) hormone concentration was measured by RIA (Gaiani *et al.* 1984). The sensitivity of the assay was 1.68 pg/tube, and the intra-assay coefficient of variation was 6.7%. Cross reactions of various steroids with antiserum raised against testosterone were: testosterone (100%), dihydrotestosterone (25.44%), androstenedione (0.6%), progesterone and cortisol (<0.0001%). The results were expressed as ng/mL. In order to determine the parallelism between hormone standards and endogenous hormone in eel

plasma, a pool sample containing high concentrations in $E_2$ and testosterone was serially diluted (1:1-1:8) with RIA buffer. A regression analysis was used to determine parallelism between the two hormone levels in the same assay. Total thyroxine (T4) and Total triiodothyronine (T3) were determined using an commercial RIA kit (Institute of Isotopes, Budapest, Hungary); the sensitivity of the assay were 6.8 nmol/L and 0.3 nmol/L and the intraassay coefficient of variations were 5.4% and 4.8% respectively. In order to determine the parallelism between T3 and T4 standards and endogenous hormones in plasma eels, a pool samples containing high concentration of thyroid hormones was serially diluted (1:1-1:8) with RIA buffer. A high degree of parallelism was confirmed by regression test ($r^2$ =0.98, $p<0.01$), demonstrating the specificity of procedure for determining thyroid hormones concentration in eels.

*RNA extraction and qPCR*

Total RNA was extracted from liver collected at P0, P1, P2, and P3 point by using Tri-Pure reagent (Roche Diagnostic GmBH, Mannheim, Germany) according to the manufacturer's instructions. Purified RNA was spectrophotometrically quantified (A 260 nm); the quality was checked by gel electrophoresis on 1% agarose gel. Five ug of RNA was treated with DNAse free RNA kit (Zymoresearch, Orange, CA, USA) according to manufacturer's instructions then 1 ug of RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA), in a final volume of 20 ul. Quantitative real-time PCR (qPCR) performed using iCycler Thermal Cycler (Bio-RAD). Primers for vitellogenin (vtg1 and vtg2), estradiol receptor (esr1) and the reference gene β-actin were designed using the Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto Ca, USA).

Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Tab. 7.

A master-mix of the following reaction components was prepared to the indicated end-concentrations: 0.5 ul forward primer (0.2 µM), 0.5 ul reverse primer (0.2 µM), 9.5 ul water and 12.5 ul IQ SYBR Green BioRadSupermix (Bio-RAD). Two ul of cDNA were added to 23 ul of the master mix. All samples were analyzed in duplicate. The real-time PCR protocol employed was: initial denaturation for 3 min at 95°C, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, followed by a melting step with a slow heating from 55°C to 95°C with a rate of 0.5°C/second. Real-time efficiencies were acquired by amplifi-

cation of a standardised dilution series and corresponding slopes and PCR efficiencies were calculated using iCycleriQ Real Time PCR Detection System (Bio-Rad Laboratories Inc.). The expression level of interest genes in Light or Dark Group was calculated as fold of increase in relation to the P0 group using $2^{\Delta\Delta CT}$ method (Livakand & Schmittgen 2001).

Table 7: Forward and reverse primer sequences, PCR product length and accession number (AN) in the NCBI database.

| Gene | Primer Sequence (5'-3') | Product Length (bp) | Acc. No. |
|---|---|---|---|
| vtg1 | For.: GACAGTGTAGTGCAGATGAAG<br>Rev.: ATAGAGAGACAGCCCATCAC | 117 | EU073127 |
| vtg2 | For.: GATGCTCCCCTAAAGTTTGTG<br>Rev.: AGCGTCCAGAATCCAATGTC | 123 | EU073128.1 |
| esr1 | For.: TCGCTTGGGCTAAGAAAGTAC<br>Rev.: CCGATTATCAGCACCTCCAG | 97 | EU073125 |
| B-actin | For.:AGCCTTCCTTCCTGGGTATG<br>Rev.: GTTGGCGTACAGGTCCTTAC | 101 | DQ286836 |

*Histological procedures*

Formalin-fixed gonad samples were processed for routine histology. Sections were cut at 3 µm and stained with hematoxylin and eosin. Histological sections were then evaluated under light microscope to assess the state of maturation according to Palstra *et al*. (2007) and Pérez *et al*. (2011).

*Statistics*

All mRNA and hormone data were first analyzed by a two-way ANOVA (photoperiod and time) followed by one-way ANOVA with Tukey post-hoc comparison to evaluate differences within Light or Dark Group over time. Data were analyzed through t-student test comparing dark *vs* light conditions at every time point. The data are presented as means ± SEM and a difference of at least $p<0.05$ was considered significant. Statistical analysis was carried out using R software (http://www.R-project.org).

## Results

Table 8 shows the BWI and GSI changes during the experiment. Significant differences in BWI were not observed between the Light or Dark Group and within the two groups respectively. The GSI presented a significant difference (P<0.05) during all experimental period in Light and Dark Group, in addition a statistically significant increase was observed in the Dark Group compared to the Light Group at P3 sampling time (P<0.05).

Table 8: Changes in body weight and gonado-somatic index throughout the treatments.

| Sampling time | BWI% | | GSI% | |
|---|---|---|---|---|
| | L | D | L | D |
| **P0** | 100 | | 1.5±0.2$^{aA}$ | |
| **P1** | 100.5±6.9 | 104.3±11.2 | 3.2±0.4$^{b}$ | 3.5±0.2$^{B}$ |
| **P2** | 104.7±8.7 | 104.5±6.4 | 5.5±0.6$^{c}$ | 7.2±0.4$^{C}$ |
| **P3** | 91.5±3.9 | 92.7±7.7 | 10.9±0.5$^{d}$ | 14.4±0.2*$^{D}$ |

Asterisk indicates statistically significant difference between two groups (Light and Dark) within each experimental time. Different lowercase or capital letters indicate statistically significant differences within Light and Dark group respectively. Means are given ± SEM.

*Plasma hormones*

Temporal changes in plasma hormones are reported in figures 25,26 and 27. E2 plasma levels (Fig. 25) showed a statistically significant higher value in the Dark Group compared to the Light Group (2.71±0.55 *vs* 1.29±0.35 ng/mL; P<0.05) at the 13$^{th}$ week of the experimental period (P3). In the Dark Group the CPE injection induced a slight increase in E2 concentration getting to statistically significant value only between P3 and P0-P1. In the Light Group a constant trend during the testing period was observed, without showing any statistically significant fluctuation.

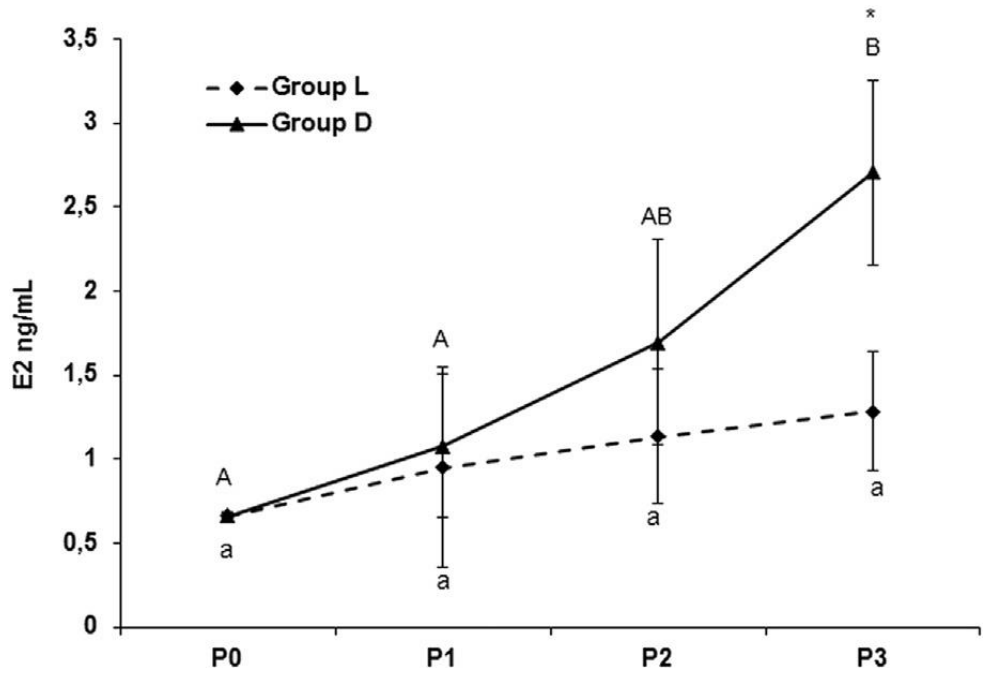


Figure 25: Plasmatic concentration of 17-βestradiol (E2) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capital letters indicate statistically significant differences (P<0.05, ANOVA post hoc Tukey test) within Light or Dark group respectively. *indicates statistically significant difference (P<0.05, Student's test) between two groups.

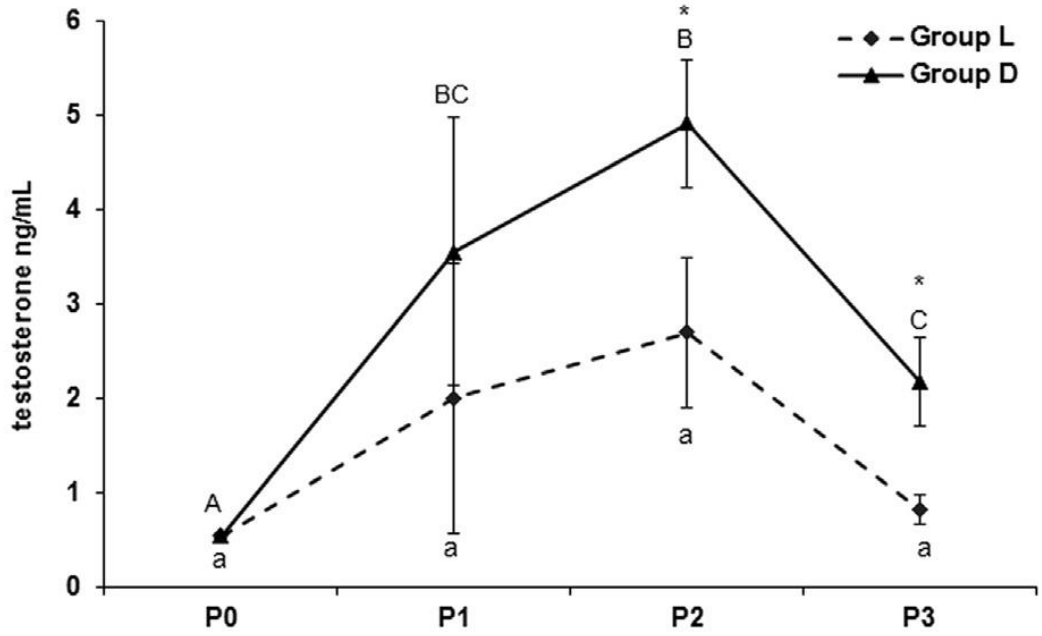


Figure 26: Plasmatic concentration of testosterone (T) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capital letters indicate statistically significant differences (P<0.05, ANOVA post hoc Tukey test) within Light or Dark group respectively. *indicates statistically significant difference (P<0.05, Student's test) between two groups.

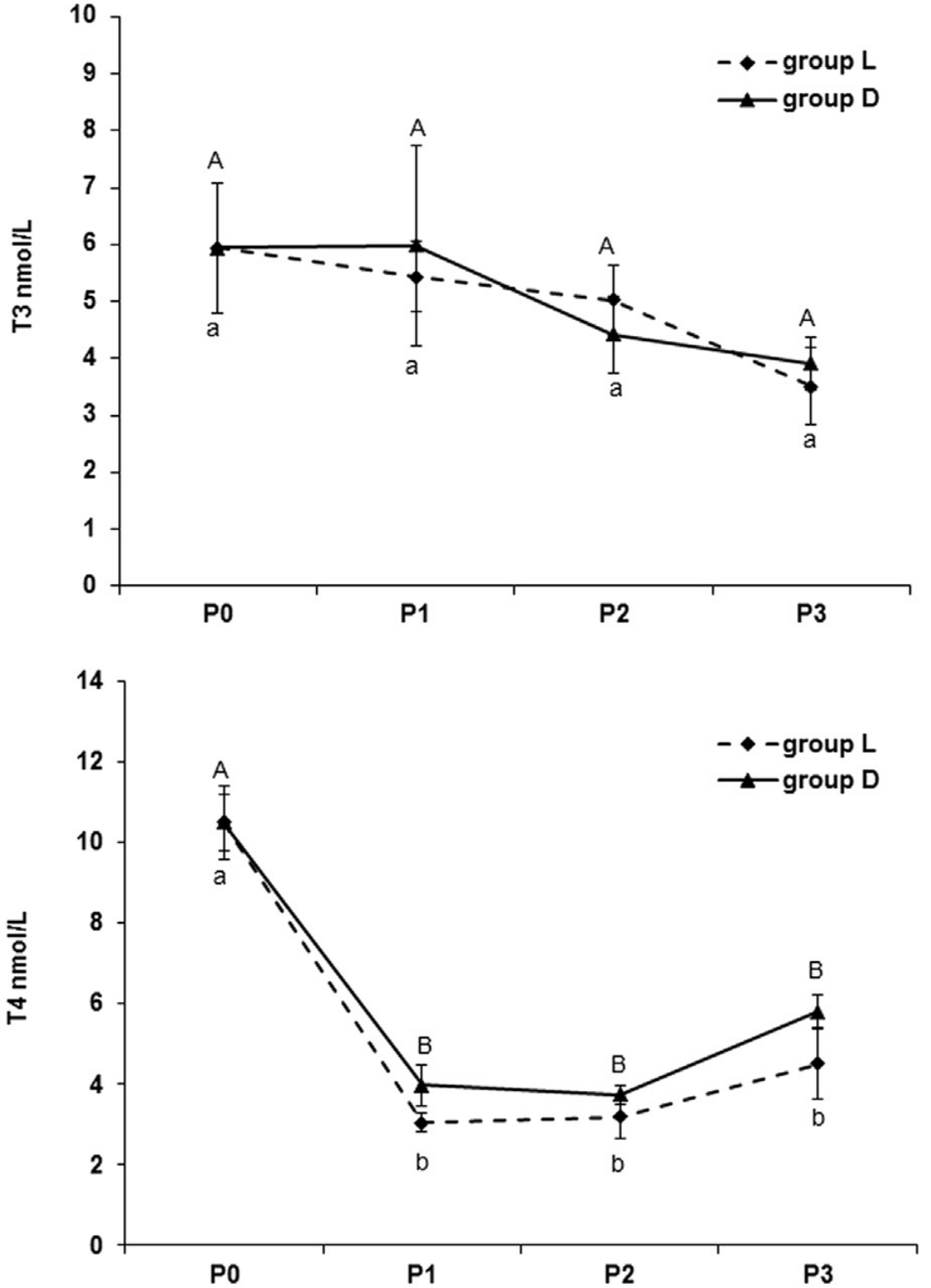


Figure 27:Plasmatic concentration of total Triiodothyronine(T3) and Thyroxine (T4) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capital letters indicate statistically significant differences (P<0.05, ANOVA post hoc Tukey test) within Light or Dark group respectively. *indicates statistically significant difference (P<0.05, Student's test) between two groups.

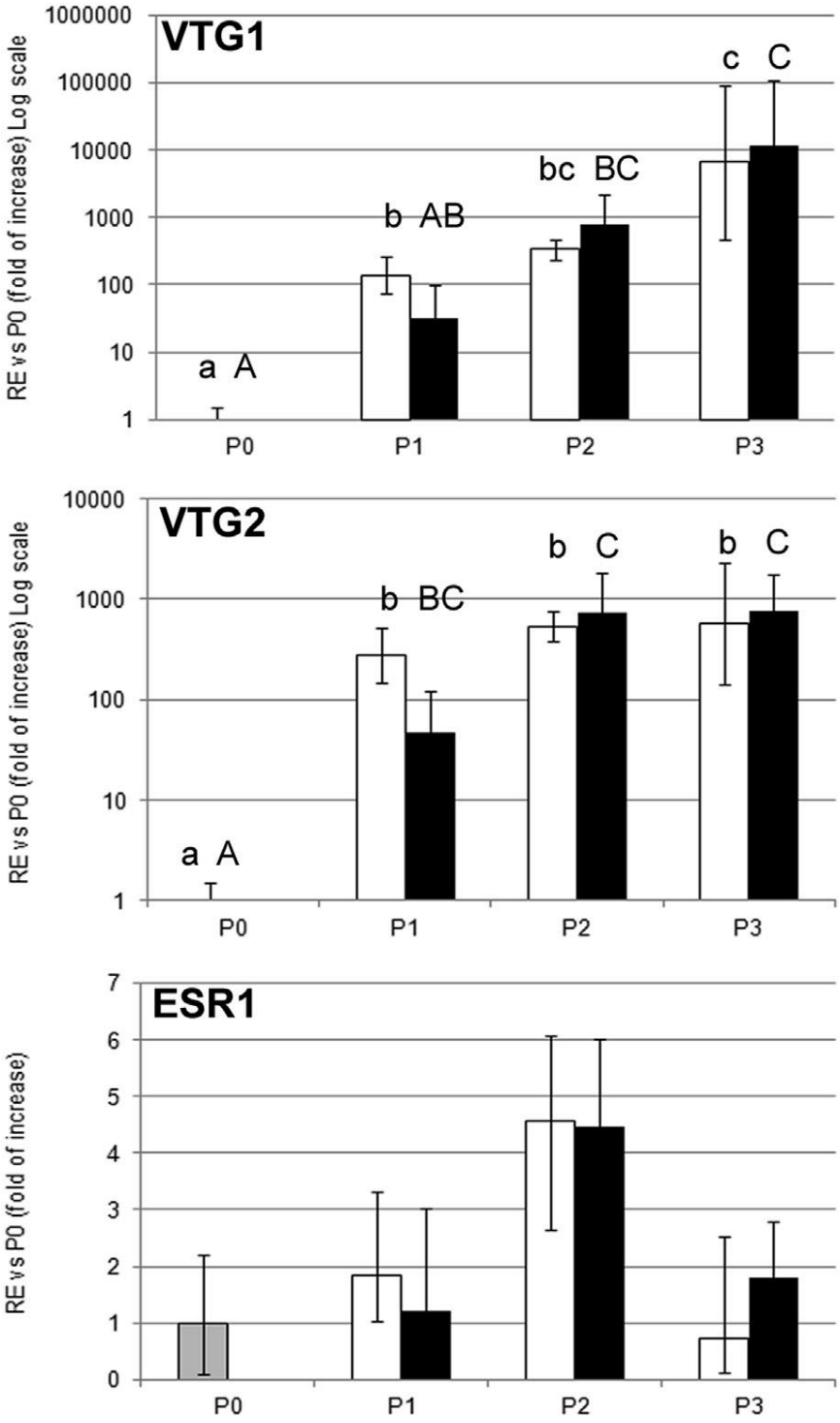


Figure 28: Relative gene expression (RE) of vitellogenin 1 (vtg1), vitellogenin 2 (vtg2) and estrogen receptor 1 (esr1) in liver samples after CPE treatments (P1, P2, P3) in different light condition (L and D groups) and in control group (P0). mRNA data are presented as fold of increase ($2\Delta\Delta CT$ method) in respect to the P0 group. Error bars represent the range of expression. Different lowercase or capital letters indicate statistically significant differences ($P<0.05$, ANOVA post hoc Tukey test) within Light (open bars) or Dark (shaded bars) group respectively.

Dark conditions induced a statistically significant higher value in testosterone (T) plasma levels compared to the Light Group at P2 (4.92±0.68 *vs* 2.70±0.80 ng/mL; P<0.05) and P3 sampling points (2.17±0.47 *vs* 0.82±0.16; P<0.05) (Fig. 16). Hormonal CPE injection has not induced a significant variation in T plasma levels in the Light Group during the whole experiment, while in the Dark Group a significant increase (P<0.05) was ascertained starting from the 5$^{th}$ week (P1), followed by a decrease at the 13$^{th}$ week (P3) keeping higher concentration levels compared to P0. The T3 and T4 plasma levels (Fig. 27) did not show any significant difference between D- and L-Groups up to the end of the treatment. In both groups T4 plasma levels showed a significant decrease after the first five CPE injections (P1) compared to P0. The interaction of two factors (photoperiod x time) did not significantly influenced the plasma levels of the hormones analyzed (Table 9).

*Vtg1, vtg2 and esr1 gene expression levels in liver*

The relative gene expression of the vtg1, vtg2 and esr1 genes was reported in Fig 28. All studied genes have not showed any significant difference between Dark and Light Groups up to the end of the treatment. CPE injection induced a progressive increase on both vtg1 and vtg2 expression levels. Vtg1 showed a significant increase in the Light Group starting from the 5$^{th}$ week (P1) while in the Dark Group a statistically significant increase was observed later (9$^{th}$ week, P2), reaching the highest value at P3 sampling point in both groups. Vtg2 gene expression data showed a statistically significant increase starting from the 5$^{th}$ week (P1), afterwards the expression level remained constant. The esr1 gene expression data showed a similar value in all the samples. The interaction of two factors (photoperiod x time) did not significantly influenced the mRNA gene expression levels (Table 9).

Table 9: Two-way ANOVA analysis of the photoperiod effect and CPE treatment (time) on hormones plasma levels and on estradiol receptor1 mRNA, vitellogenin1 and 2 mRNA expressions.

| | Photoperiod | Time | Interaction |
|---|---|---|---|
| E2 (ng/mL) | p=0.043 | n.s. | n.s. |
| T (ng/mL) | p=0.0087 | p=0.0043 | n.s. |
| T3 (nmol/L) | n.s. | n.s. | n.s. |
| T4 (nmol/L) | n.s. | p=0.0027 | n.s. |
| vtg1 mRNA | n.s. | p=0.0000 | n.s. |
| vtg2 mRNA | n.s. | p=0.0000 | n.s. |
| esr1 mRNA | n.s. | n.s. | n.s. |

n.s. indicates non-significant differences

*Histology*

The gonad tissue was organized in lamellae, supported by stroma rich in adipose tissue which permit the typical lamellar fan-shaped arrangement (a peculiarity of eel if compared to other fish species). This lipidic content (triglycerides) is normally dissolved by xylene during routine processing: the result on histological sections was the presence of optically empty vacuoles between an oocyte and another (corresponding to adipocytes or part of their content if ruptured) and subsequent separation of oocytes cords. Control eels (P0) before the hormonal treatment had oocytes in the previtellogenic stage (oil drop stage) showing a large central round nucleus (or germinal vesicle), multiple nucleoli and abundant cortical alveoli that completely filled the cytoplasm (data not shown). At the 5$^{th}$ week (P1), eels from Light Group and Dark Grouphad oocytes in the early vitellogenic stage (primary yolk globule stage) with abundant cytoplasm, still prominent cortical alveoli and the first yolk globules appeared around the germinal vesicle (Fig.29 A,D). By week 9 (P2), eels in both groups had oocytes still in the early vitellogenic stage; cortical alveoli entirely filled the cytoplasm, densely stained yolk globules were more abundant and the nucleus was still centrally located (Fig.29 B,E). In week 13 (P3), eels from Light Group and Dark Grouphad oocytes in mid vitellogenesis; cortical alveoli fused together forming large lipid droplets and most of the cytoplasm was filled with yolk globules (Fig.29 C,F). There were no significant morphological differences between Light Group and Dark Group in term of gonadal maturation.

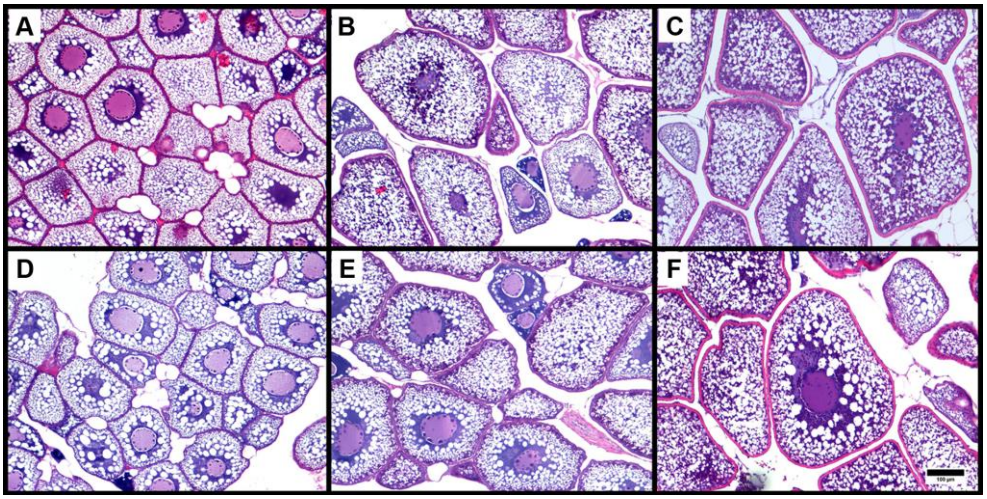


Figure 29: Representative histological images of gonad samples.
(A,D) 5th week (P1), eels from D-Group(A) and L-Group (D) show oocytes in the early vitellogenic stage. (B,E) 9th week (P2), eels from D-Group (B) and L-Group (E) have larger oocytes still in the early vitellogenic stage. (C,F) 13th week (P3), eels from D-Group (C) and L-Group (F) show oocytes in mid vitellogenesis stage (H&E staining, bar=100µm).

## Discussion

In the present study the effects of light and dark conditions on gonadal steroidogenic activity, thyroid hormones and vitellogenin gene expression were observed in female European silver eels (*Anguilla anguilla*) treated with increasing doses of carp pituitary extracts. At the start of the experiment the animals showed a stage IV and V in silvering as a fundamental prerequisite to permit artificial reproduction in a controlled environment for this particular endangered animal. The Silver Index is based on many morphological and physiological parameters of wild female eels and has therefore a better value with respect to real silvering stage (Han *et al*. 2003; Rohr *et al*. 2001; Dufour *et al*. 2009). Moreover in our previous work (Mordenti *et al.* 2012) we observed that silver eels do not undergo to CPE induction did not show any variation related to the photoperiod regimen on gonadal weight, ovarian development and egg production. It was here demonstrated that CPE stimulation induces a significant increase of the GSI value after the 5$^{th}$ injection (P1 sampling point) in agreement with what reported by Palstra *et al*. (2005), moreover at the 13$^{th}$ week (P3) the GSI was found higher in dark conditions compared to the Light Group supporting the hypothesis that the constant darkness positively influences the gonadal growth, even if no differences on gonadal maturation were observed. From the endocrine point of view, testosterone significantly increased after the first 5 weeks of the CPE treatments in the Dark group, while in the light condition the differences of the hormone levels are not significant during the whole trial. We can also record a significant difference in the 17-β-estradiol plasma levels in the Dark Group, while in light condition no evident variations were observed. In the Dark Group the rise of E2 levels in conjunction with the decrease in T could suggest an enhanced aromatase activity which converts T into E2 as it normally occurs in the biosynthesis of estrogens by the follicular cells. Both Chiba *et al.* (1994) in *Anguilla anguilla* and Lokman *et al.* (2001) in *Anguilla dieffenbachii* have reported an increase in E2 and testosterone plasma levels in eels stimulated with pituitary extracts at different timings and dosage without specifying the photoperiod conditions. In the present experimental model a response of testosterone and E2 levels was exclusively observed in animals kept in the dark, this seems to disagree with what reported by the above mentioned authors. Such different experimental situations might be difficult to compare, still the data of the present experiment seems to demonstrate eels to prefer dark conditions for gonadal growing and for the onset of the gonadal steroidogenic activity.

It is well known that the hepatic vitellogenin gene expression is regulated by the binding of estrogen receptors-estradiol 17β; the vitellogenin protein is then incorporated into the growing oocytes and proteolytically cleaved into smaller yolk proteins (Pérez *et al*. 2011; Specker *et al*. 1994). Two types of estrogen receptors are described in fish liver: estrogen receptor 1 (esr1) and estrogen receptor 2 (esr2). The esr1 plays an important role in the onset of hepatic vitellogenesis in European eel while the role of the esr2 at this stage remains unclear (Palstra *et al.* 2010). The present study shows that CPE administration in European silver eels induces a significant increase in both vitellogenin (vtg1 and vtg2) gene expression levels according to Palstra *et al.* (2010). After four hormonal injections the levels of vtg1 and vtg2 increase compared to the control group (P0), remaining high for the whole experiment. No differences were observed on vtg1 and vtg2 expression levels between light and dark groups. The resulting data are in agreement with the gonadal histological observations where both groups (Dark and Light Group) showed oocytes at the same development stage (mid-vitellogenesis) with cortical alveoli fused together and cytoplasm filled with yolk globules at the 13$^{th}$ week of the hormonal treatment. The experimental model have showed that CPE did not influence the esr1 mRNA expression. It might be due to the fact that the first sampling (5$^{th}$ week) was not appropriate, in fact as reported by Palstra *et al*. (2010) the esr1 expression increases very early (after one CPE injection) and then returns to the basal level at the 4$^{th}$ week. Therefore it is possible to assume that the first sampling was too late to detect the increase in the esr1 mRNA expression as well as the first increase in the E2 plasma level.

In both groups a higher concentration of the T4 compared to T3 was found, as already reported in other fish species by different authors (Raine & Leatherland, 2000; Raine *et al*. 2001). In this experimental model the photoperiod does not seem to have influenced the pattern of the TH, as observed in previous studies on trout by Cyr *et al.* (1998), and on rainbow trout by Pavlidis *et al*. (1991). In the present study the TH levels were observed to be higher during early ovarian development and then a decrease occurred in conjunction with the increasing levels of E2. All the above mentioned trends were in accordance to the current studies on European eels. The experimental model involved animals at the last stage of migration where the somatic body growth is at the maximum level, therefore the inhibition of the THs during the development progression of the ovarian steroidogenesis could reflect the shift in energy sharing where there is no more somatic growth and all the energy is devoted to the gonadal growth as observed in the overview by Cyr & Eales (1996). It is well-known that the neuroendocrine transductor of darkness in many classes

of vertebrates, including mammals and fish, is melatonin. Sebert *et al*. (2008) evaluated a long-term effect of this indoleamine on the GnRHs neurons and on the dopaminergic system in European eels and found an inhibitory impact on the reproductive activity, through the administration of melatonin the dopaminergic system was activated so that to inhibit the release of gonadotropin. The present experimental model bypassed the hypothalamic–pituitary system by directly administering CPE, this lead to better steroidogenesis performances in animals kept in dark conditions. In fact, as previously referred by Mordenti et al. (2012), the only animals that reached a full maturation at stage 6 (fully transparent oocyte with germinal vesicle breakdown, few fat droplets were the ones reared in dark conditions.

The animals kept in dark conditions showed a significant increase both on T and E2 plasma levels compared to pre-CPE administration, while in light conditions no effects of CPE injection on steroid levels were detected. Moreover the variations of the GSI demonstrate a positive effect of the dark condition housing. This fact suggests, as yet reported by Rodriguez *et al*. (2009) for growth performance and feed utilization, that these animals keep in the dark show a lower stress and a better reproductive performance.

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## References


Bono G., Cairoli F., Tamanini C. & Abrate L. (1983) Progesterone, oestrogen, LH, FSH, and prolactin concentrations in plasma during the oestrus cycle in goat. *Reproduction Nutrition Development*, 23:217-222.

Bromage N.R., Porter M. & Randall C. (2001) The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture*, 197:63-98.

Chiba H., Iwatsuki K., Hayami K., Hara A. & Yamuchi K. (1994) Changes in serum steroid hormones and vitellogenin levels in cultured female European eel *Anguilla anguilla* during artificially induced ovarian development. *Journal World Aquaculture Society*, 25(4):553-560.

Cyr D.G. & Eales J.G. (1996) Interrelationships between thyroidal and reproductive endocrine systems in fish. *Reviews in Fish Biology and Fisheries*, 6:165-200.

Cyr D.G., MacLatchy D.L. & Eales J.G. (1988) The influence of short-term 17 betaestradiol treatment on plasma T3 levels and in in vitro hepatic T4 5'-monodeiodinase activity in immature rainbow trout, *Salmogairdneri*. *General and Comparative Endocrinology*, 69:431-438.

Dou S.Z., Yamada Y., Okamura A., Shinoda A., Tanaka S. & Tsukamoto K. (2008) Temperature influence on the spawning performance of artificially-matured Japanese eel *Anguilla japonica* in captivity. *Environmental Biology and Fisheries*, 82:151-164.

Dufour S. & van den Thillart G.E.E.J.M. (2009) Reproduction capacity of European eels. In: Van den Thillart G, Dufour S, Rankin C editors. Spawning migration of the European eel. Springer, *Fish and Fisheries Series*, 30:465-473.

Durif C., Dufour S. & Elie P. (2005) The silvering process of *Anguilla anguilla*: A new classification from the yellow resident to the silver migrating stage. Journal of *Fish Biology*, 66:1025–1043.

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel. *Marine Ecology Progress Series*, 327:171-181.

Fontaine Y.A., Pisam M., Moal C.L. & Rambourg A. (1995) Silvering and gill 'mitochondria-rich' cells in the eel, *Anguilla anguilla*. *Cellular Tissue Research*, 281:465-471.

Gaiani R., Chiesa F. & Mattioli M. (1984) Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. *Journal Reproduction Fertility*, 70(1):55-59.

Grandi G., Poerio F., Colombo G. & Chicca M. (2000) Effects of diet supplementation with carp ovary on gonad differentiation and growth of the European eel. *Journal of Fish Biology*, 57(6):1505-1525.

Han Y.S., Liao I.C., Huang Y.S., He J.T., Chang C.W. & Tzeng W.N. (2003) Synchronous changes of morphology and gonadal development of silvering Japanese eel *Anguilla japonica*. *Aquaculture*, 219:783-796.

Han Y.S., Tzeng W.N., Huang Y.S. & Liao I.C. (2001) Silvering in the eel: changes in morphology, body fat content, and gonadal development. Journal Taiwan *Fisheries Research*, 9:119-127.

Kagawa H., Iinuma N., Tanaka H., Ohta H. & Okuzawa K. (1998) Effects of rearing period in seawater on induced maturation in female Japanese eel *Anguilla japonica*. *Fisheries Science*, 64:77-82.

Kleckner W.B. (1980) Swim bladder volume maintenance related to initial oceanic migratory depth in silver-phase *Anguilla rostrata*. *Science*, 208:1481-1482.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 25(4):402-8.

Lokman P.M. & Young G. (2000) Induced spawning and early ontogeny of New Zealand freshwater eels (*Anguilla dieffenbachia* and *A. australis*). *New Zealand Journal of Marine and Freshwater Research*, 34:135-145.

Lokman P.M., Wass R.T., Suter H.C., Scott S.G., Judge K.F. & Young G. (2001) Changes in steroid hormone profiles and ovarian histology during salmon pituitary-induced vitellogenesis and ovulation in female New Zealand Longfinned eels, *Anguilla dieffenbachia* gray. *Journal of Experimental Zoology*, 289:119-129.

Mordenti O., Di Biase A., Sirri R., Modugno S. & Tasselli A. (2012) Induction of sexual maturation in wild female European eel (*Anguilla anguilla*) in darkness and light conditions. *The Israeli Journal of Aquaculture-Bamidgeh,* IJA_64.2012.726, 9 pages

Ohta H., Kagawa H., Tanaka H., Okuzawa K., Iinuma N. & Hirose K. (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiology Biochemistry*, 17:163-169.

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. *Aquaculture*, 249:533–547.

Palstra A.P., Curiel D., Fekkes M., Bakker M., Székely C., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2007) Swimming stimulates oocyte development in European eel (*Anguilla anguilla L*.). *Aquaculture*, 270:321–332.

Palstra A.P., Schnabel D., Nieveen M.C., Spaink H.P. & van den Thillart G.E.E.J.M. (2010) Temporal expression of hepatic estrogen receptor 1, vitellogenin 1 and vitellogenin 2 in European silver eels. *General and Comparative Endocrinology*, 166:1-11.

Pankhurst N.W. (1982) Relation of visual changes to the onset of sexual maturation in the European eel *Anguilla anguilla*. *Journal Fish Biology*, 21:127-140.

Pedersen B.H. (2004) Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture*, 37:461-473.

Pérez L., Peñaranda D.S., Dufour S., Baloche S., Palstra A.P., van den Thillart G.E.E.J.M. & Asturiano J.F. (2011) Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females.*General and Comparative Endocrinology*, 174:51-59.

Raine J.C. & Leatherland J.F. (2000) Morphological and functional development of the thyroid tissue in rainbow trout (*Oncorhynchusmykiss*) embryos. *Cellular Tissue Research*, 301:235-243.

Raine J.C., Takemura A. & Leatherland J.F. (2001) Assessment of thyroid function in adult medaka (*Oryziaslatipes*) and juvenile rainbow trout (*Oncorhynchusmykiss*) using immunostaining methods. *Journal Experimental Zoology*, 290:366-378.

Rodriguez A., Castello-Orvay F. & Gisbert E. (2009) Somatic growth, survival, feed utilization and starvation in European elver *Anguilla anguilla* (Linnaeus) under two different photoperiods. *Aquaculture Research*, 40(5):551-557.

Rohr D.H., Lokman P.M., Davie P.S. & Young G. (2001) 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis.Comparative Biochemistry Physiology*, 130a:701-714.

Sebert M.E., Weltzien F.A., Moisan C., Pasqualini C. & Dufour S. (2008) Dopaminergic systems in the European eel: characterization, brain distribution and potential role in migration and reproduction. *Hydrobiology*, 602:27-46.

Specker J.L. & Sullivan C.V. (1994) Vitellogenesis in fishes: status and perspectives. In: Davey KG, Peter RG, Tobe SS. (eds). *Perspectives in Comparative Endocrinology*, National Research Council of Canada, Ottawa; p. 304-315.

Vøllestad L.A., Jonsson B., Hvidsten N.A. & Naesje T.F. (1994) Experimental test of environmental factors influencing the seaward migration of European silver eels. *Journal of Fish Biology*, 45:641–651.

Yamada Y., Zhang H., Okamura A., Tanaka S., Horie N., Nikawa N., Utoh T. & Oka H.P. (2001) Morphological and histological changes in the swim bladder during maturation of the Japanese eel. *Journal Fish Biology,* 58:804-814.

PavlidisM., DessyprisA. & Christofidis I. (1991) Seasonal fluctuations in plasma thyroid hormones, in two strains of rainbow trout (*Oncorhynchusmykiss*), during the first and second reproductive cycle; relation with their photoperiodically altered spawning time. *Aquaculture*, 99:365-385.

# CHAPTER 5

# CONTROLLED REPRODUCTION IN *ANGUILLA ANGUILLA*: COMPARISON BETWEEN SPONTANEOUS SPAWNING AND STRIPPING-INSEMINATION APPROACHES.

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**Abstract**

This study aimed to compare the fertility of eggs between artificially matured female silver eels that spawned spontaneously and those that were spawned by manual stripping. The effects of the two methods of spawning on ovulation and fertilization rate were also investigated. For this purpose, 18 wild female European eels captured in *Bonello* lagoon (North Adriatic Sea) were carp pituitary extract-injected to undergo sexual maturation and ovulation; a final injection of 17,20-β-dihydroxy-4-pregnen-3-one (DHP) was administered when at least 30% of the oocytes were fully transparent. After the DHP-injection, 9 eels were transferred to a new closed recirculating aquaculture system, where they were housed with spermiating males (sex ratio 4/1) to allow spontaneous spawning (SPT-group); the remaining 9 eels were transferred to a 250 L tank and ovulation was checked at four-hourly intervals by manual stripping (STR-group). The number of eggs per female in the SPT-group was significantly greater than that in the STR-group. Furthermore, fertilization rates in the SPT-group were notably higher than those observed in the STR-group. Significantly, the best performances were obtained among eels in which at least 50% of oocytes were fully transparent at the time DHP was administered. We conclude that the fertility of eggs from spontaneously spawning eels is superior to that of eggs acquired by strip-spawning and artificial fertilization.

## Introduction

The eel is amongst the highest-value species produced from freshwater aquaculture in the world. To date, all seedlings for cultivation originate from wild glass eels or elvers collected in estuarine waters. However, over the last several decades, natural stocks of eels, especially those representing the commercially valuable temperate species such as European eel (*Anguilla anguilla*), American eel (*Anguilla rostrata*) and Japanese eel (*Anguilla japonica*), have decreased markedly (Casselman 2003; Dekker 2003; Tatsukawa 2003) due to overfishing, environmental destruction, oceanographic/climatic changes and other as yet unknown factors (EELREP 2006; van Ginneken & Maes, 2005). Indeed, *Anguilla anguilla* is now included in the IUCN Red List of critically endangered species (Freyhof & Kottelat, 2010). Unfortunately, this measure has not reduced the risk of extinction (Mordenti, Di Biase, Sirri, Modugno & Tasselli 2012). One effective way to preserve these species is through the establishment of techniques for mass production of glass eels to reduce the fishing pressure on wild glass eel stocks (Okamura, Horie, Mikawa, Yamada & Tsukamoto 2013).

With reference to the European eel, research has focused on successful artificial propagation protocols in terms of hormone dose and timing and with regard to defining optimal environmental parameters (water temperature, water salinity, and photoperiod) (Durif, Dufour & Elie 2006; Mordenti, Di Biase, Bastone, Sirri, Zaccaroni & Parmeggiani 2013) in order to obtain a high number of eggs for artificial fertilization. Nevertheless, one of the major problems of seed production remains the constant availability of high quality eggs. Egg quality, in terms of resulting fertilization, hatching and survival rates, is highly variable between batches from different individuals (Chai, Tosaka, Abe, Sago K., Sago Y., Hatanaka, Ijiri & Adachi 2010) or between different egg retrieval methods (Mordenti, Casalini, Mandelli & Di Biase 2014). Thus, it is necessary to improve techniques for the production of high quality eggs in order to make mass production of eel larvae possible. The use of 15-25 weekly injections of salmon or carp pituitary extract can advance oocytes to the migratory nucleus stage after which final oocyte maturation and ovulation are typically induced by 17,20β-dihydroxy-4-pregnen-3-one (DHP)(Kagawa, Tanaka, Ohta, Okuzawa & Hirose 1995; Ohta, Kagawa, Tanaka, Okuzawa & Hirose 1996; Ohta, Kagawa, Tanaka, Okuzawa & Iinuma1997; Palstra. Cohen, Niemantsverdriet, van Ginneken & van den Thil-

lart 2005; Mordenti *et al*. 2012; Mordenti *et al*. 2013). However, for this routine method, the relationship between timing of the DHP-induced final stage and the resulting egg quality remains to be explored. The objective of the present study was to investigate the quality and quantity of eggs produced by artificially matured European eels (*Anguilla anguilla*) that were allowed to spawn spontaneously or that were subjected to stripping-insemination, and to relate these fertility parameters to the timing of DHP administration.

## Materials and methods

### *Animal source and maintenance*

Wild female eels were caught using traditional "*lavoriero*" (downstream trap) in a brackish water lagoon near the sluices of the North Adriatic Sea (*Val Bonello*, Veneto - Italy). The *Val Bonello* (50 ha) is a closed lagoon located in the Po Delta, where a fishery has been operating for centuries, taking advantage of the autumn-winter migration of euryhaline fishes to the sea: several species thrive in the *Valli*, but the fishery has always been dominated by *Anguilla anguilla*. Large female eels were selected from the catch and then transported to the laboratory. Cultivated male eels (n=50 fish, 118-237g in BW), reared in freshwater, were purchased from a commercial supplier at the same time, and upon transfer to the laboratory, gradually acclimated to seawater over 7 days. All eels were kept in a recirculating system consisting of two fish-rearing tanks (700 L), one with females and one with males; fish were maintained in complete darkness (-0.04 $x10^3$ lux at the bottom of the tank without water) (Mordenti et al., 2012) in seawater (salinity 32 $gL^{-1}$) at a controlled temperature of 15.5±0.5°C until gonadal maturation (sections b and c, below) was complete. This system was equipped with a foam separation tank (protein skimmer) and a biological filter containing plastic porous balls. The tank was also provided with a thermal regulation system, a UV-sterilizer lamp, an ozonizer and an aerator (electromagnetic air compressor) to adjust the rearing water conditions.

### *Induction of maturation in female eels*

At the experimental premises, the female eels were measured and sampled to obtain an external indicator of their maturation state (silver index - SI) (Durif, Dufour & Elie

2005; Di Biase, Bastone, Casalini, Parmeggiani, Costantini & Mordenti 2012) and their condition factor (K) was calculated according to the formula below:

$$K = (BW*BL^{-3})*10^{6}$$

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

Nine females were selected for artificial maturation (see below) and subsequent spontaneous spawning (*SPT-group*) and another nine for egg retrieval by strip-spawning (*STR-group*); only eels with comparable body weights (600±100gr) were used for artificial reproduction. The animals were marked individually by inserting fish-tags (FLOY TAG Mod Floy T-Bar Anchor) in the dorsal muscle whilst under anesthesia with 400ppm 2-phenoxyethanol, and maintained under starvation for the duration of the trial. Once a week, females received an intramuscular injection with carp pituitary extracts (CPE) 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) or 40 mg/kg BW ($10^{th}$ week-final maturation) (Mordenti *et al*. 2012). Weekly administrations of CPE continued until the beginning of oocyte hydration, i.e., until the BW exceeded 110% of initial body weight (IBW), similar to what has been done by many other researchers (Dou, Yamada, Okamura, Shinoda, Tanaka & Tsukamoto 2008; Oliveira & Hable 2010; Ijiri, Tsukamoto, Chow, Kurogi, Adachi & Tanaka 2011; Burgerhout, Brittijn, Kurwie, Decker, Dirks, Palstra, Spaink & van den Thillart 2011; Mordenti *et al*. 2013). However, the timing of the subsequent DHP injection was notably changed – thus, rather than administering DHP at a fixed time after the last CPE injection, the timing was optimized for individual eels. To this end, females were repeatedly ovary-biopsied (~ 0.3 mL, equating to ca. 500 follicles) every 8 h by needle and syringe whilst under anesthesia in 400 ppm 2-phenoxyethanol. Thereafter, once at least 30% of the oocytes were fully transparent, displaying their nucleus at the periphery and containing few large fat droplets (diameter from 110 to 150 µm)(i.e., Fully Transparent Oocytes, FTO), ovulation was induced by intra-peritoneal DHP injection (2 mg/kg). The developmental stage of the FTO corresponded to stage 5 of gamete development in *A. anguilla* according to Palstra *et al.* (2005) and to stage 7 in *A. japonica* according to Unuma, Hasegawa, Sawaguchi, Tanaka, Matsubara, Nomura & Tanaka (2011). In case the desired stage of oocyte maturation (stage 5 according to Palstra *et al.* 2005) was not reached within 48 h from the last routine weekly CPE injection, a booster with CPE (i.m. 40 mg/kg BW) was administered and ovarian biopsies were again taken as described above in order to properly time the injection

with DHP. The body weight at the final CPE administration, whether a routine weekly or a follow-up booster injection, was used to calculate the Body Weight Index (BWI), as follows:

Body weight Index (BWI) = (BW $IBW^{-1}$)*100

BW: body weight at DHP (g), IBW: Initial body weight (g).

*Induction of maturation in male eels*

Males were induced to mature following standard protocols (Ohta *et al*. 1997; Palstra *et al*. 2005); briefly, they were injected with 1 IU/g BW hCG and started spermiation after a 5-week treatment. Just before fertilization experiments, the males received a booster hCG injection (1 IU/g BW) to induce sperm maturation (Burgerhout *et al*. 2011). Sperm motility was monitored and only males with at least 50% sperm motility (continuous activity of >50% of spermatozoa) were used for experimentation (Burgerhout *et al*. 2011).

*Experimental design: effects of insemination method on reproductive parameters*

Eighteen females were matured and either allowed to spawn spontaneously (*SPT-group*; n=9) or used for egg retrieval by strip-spawning (*STR-group*; n=9); after DHP injection, each female of the *SPT-group* was transferred to a new closed recirculating aquaculture system, in which the seawater temperature was raised to 20±0.5°C (Dou *et al*. 2008; Mordenti *et al*. 2014) and maintained for 20 h in the company of spermiating males (*sex ratio* 4M/1F) in order to facilitate spontaneous spawning. The system, composed of one spawning chamber, two incubation chambers and one outlet chamber, has been described in more detail in Mordenti *et al*. (2014). After 20 h, all breeders were removed from the spawning chamber. In contrast, DHP injection of each eel in the *STR-group* was followed by transfer to a 250 L tank supplied with recirculating seawater at a temperature of 20±0.5°C. The artificial fertilization program started 8 h post DHP-injection when females were assessed for ovulation at 4-hourly intervals (8h, 12h, 16h and 20h) by applying gentle pressure on the abdomen in a cranial-to-caudal direction (Ohta *et al*. 1996); eggs were collected into a 3-L plastic sterilized bowl. Four males per female were hand stripped and milt was collected in a syringe (10mL) and kept in the refrigerator for a maximum of 12 h. The collected sperm was mixed with 100ml fresh seawater and then added to the dry eggs in the bowl. After approximately 3-4 min, the eggs were placed into buckets with fresh sterile seawater (~20 L) for 15 minutes. Each inseminated batch was kept in a 150 L polyeth-

ylene tank and maintained at the same temperature used to induce ovulation (20±0.5°C).

*Analyses: reproductive performance*

For each spawning event, the relative weight of spawned eggs (%BW) was calculated as the difference in body weight post-spawning and that at the time of DHP injection. The total fertilization rate (%) for each batch of spawned eggs was observed at 2 hpf and determined by calculating the % of eggs that reached the 8-cell stage; for this purpose, 3 subsamples of 1000 eggs were scored and averaged for each batch. Furthermore the fertilized, floating rate (%) was assessed on buoyant eggs, obtained after maintaining an egg sample for 30 minutes in a 500 ml beaker; again, only embryos in the 8-cell or 16-cell states were considered as fertilized. A check on fertilization success was also made on the sunken eggs.

All 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 the Ethics Committee of Bologna University.

*Statistical analysis*

Reproductive performances were statistically analyzed: statistics were performed using a t-test following a Student's t distribution; $P \leq 0.01$ was considered statistically significant.

## Results

The internal and external indicators of maturation state of the wild eels at the beginning of the experiment (T0) are reported in Table 10. All eels were silver, actively migrant and had a maximum silvering score (V) (only one eel was at stage IV).

*Reproductive performance*

SPT-group females ovulated between the 15th and the 28th week while in the STR-group, ovulation started at the 17th week and finished at the 29th week. One female/group (STR-8 and SPT-5) did not spawn but retained eggs in the abdominal cavity (Table 11).

Table 10. Characteristics of silver female European eels adopted for artificial maturation experiments.

| | | STR-group | SPT-group |
|---|---|---|---|
| Eels | *n* | 9 | 9 |
| Body Weight (BW) | *g* | 598.42±54.99 | 566.71±63.35 |
| Body Length (BL) | *cm* | 69.60±2.17 | 67.69±2.98 |
| Condition factor (K) | | 1.78±0.12 | 1.83±0.17 |
| Silver index (SI) | | V | IV-V |

There were no differences (df=16; $t_{Student}$=0.54; P=0.60) between both experimental groups with regard to the % of oocytes at stage 5 (FTO) prior to DHP administration, mean values hovering around 50%. Similarly, the BWI of SPT- (121.35±7.44%) and STR-groups (119.88±7.36%), obtained after DHP injection showed no evident differences from a statistical point of view (df=16; $t_{Student}$=0.42; P=0.68).

Table 11. Reproductive performance of artificially matured female European eels that were strip-spawned (STR-group) or that spawned spontaneously (SPT-group) after hormone treatment (see text for details).

| | **BWI** *(% BW)* | **Oocytes** *(% FTO)* | **Ovulation** *(Yes/No)* | **Spawned eggs** *(% BW)* |
|---|---|---|---|---|
| **STR-group** | | | | |
| ***1*** | 122.2 | 61.4 | Yes | 32.40 |
| ***2*** | 108.3 | 40.4 | Yes | 24.50 |
| ***3*** | 115.5 | 37.5 | Yes | 12.70 |
| ***4*** | 128.5 | 68.4 | Yes | 38.40 |
| ***5*** | 112.8 | 63.2 | Yes | 30.80 |
| ***6*** | 118.1 | 58.3 | Yes | 33.80 |
| ***7*** | 131.3 | 38.5 | Yes | 25.40 |
| ***8*** | 123.7 | 33.5 | No | - |
| ***9*** | 118.5 | 41.5 | Yes | 23.60 |
| ***Mean*** | ***119.88±7.36*** | ***49.90±13.37*** | | ***27.70±7.93*** |
| **SPT-group** | | | | |
| ***1*** | 117.7 | 64.5 | Yes | 42.50 |
| ***2*** | 116.7 | 62.3 | Yes | 47.50 |
| ***3*** | 118.5 | 35.8 | Yes | 39.60 |
| ***4*** | 124.9 | 70.5 | Yes | 52.80 |
| ***5*** | 134.1 | 32.4 | No | - |
| ***6*** | 107.9 | 59.2 | Yes | 44.50 |
| ***7*** | 125.8 | 38.9 | Yes | 40.40 |
| ***8*** | 126.2 | 40.2 | Yes | 37.60 |
| ***9*** | 120.4 | 72.3 | Yes | 50.20 |
| ***Mean*** | ***121.35±7.44*** | ***52.90±15.88*** | | ***44.39±5.38**** |

* Significant difference (P<0.01) between eels in the STR-group and the SPT-group. BWI: body weight index (increase in relative body weight after injection of fish with 17α,20β-dihydroxy-4-pregnen-3-one); FTO: fully transparent oocytes at the time of treatment with 17α,20β-dihydroxy-4-pregnen-3-one.

Table 12. Fertilization rates of eggs from artificially matured female European eels that were strip-spawned (STR-Group) or that spawned spontaneously (SPT-group) after hormone treatment (see text for details).

| | | Fertilization | |
|---|---|---|---|
| | ***(Yes/No)*** | ***Total (%)*** | ***Floating (%)*** |
| **STR-Group** | | | |
| ***1*** | Yes | 8.20 | 21.50 |
| ***2*** | Yes | 3.90 | 10.80 |
| ***3*** | Yes | 2.90 | 18.70 |
| ***4*** | Yes | 9.70 | 13.50 |
| ***5*** | Yes | 11.10 | 27.80 |
| ***6*** | Yes | 8.60 | 16.70 |
| ***7*** | Yes | 7.40 | 20.20 |
| ***8*** | - | - | - |
| ***9*** | Yes | 9.80 | 15.40 |
| ***Mean*** | | ***7.70±2.89*** | ***18.07±5.27*** |
| | | | |
| **SPT-Group** | | | |
| ***1*** | Yes | 64.20 | 93.20 |
| ***2*** | Yes | 52.30 | 74.50 |
| ***3*** | No | 0.00 | 0.00 |
| ***4*** | Yes | 57.30 | 78.60 |
| ***5*** | - | - | - |
| ***6*** | Yes | 44.70 | 81.20 |
| ***7*** | No | 0.00 | 0.00 |
| ***8*** | Yes | 38.90 | 68.40 |
| ***9*** | Yes | 48.60 | 70.70 |
| ***Mean*** | | ***38.25±24.81**** | ***58.32±36.78**** |

* Significance difference (P<0.01) between eels in the STR-group and the SPT-group

The females that spawned spontaneously (SPT-group) were statistically more productive (df=14; $t_{Student}$=0.92; P<0.01) than those stripped manually (Table 11). In both groups, a positive correlation (STR-group: $R^2$=0.7595; STP-group: $R^2$=0.7749)(Fig.30) was observed between the FTO and the relative weight of spawned eggs: in fact, the two females that retained the eggs (STR-8 and SPT-5) displayed the lowest percentage of FTO at the time of DHP injection within their respective groups (33.5% in STR-8 and 32.4% in SPT-5). Likewise, the most productive females had the highest percentage of FTO (Table 11). In both experimental groups, fertilized eggs were obtained: in the STR-group, fertilization was successful for all females (8 eels; i.e., excluding STR-8 and SPT-5), while it was successful for 6 out of 8 SPT-group females. The eggs from the SPT-3 and SPT-7 females, although released spontaneously, were not fertilized as the males did not emit milt whilst being co-housed with the females in the spawning chamber (Table 12); moreover, courtship behavior was not observed.

The total fertilization rate of the eggs of SPT females was higher (38.25%) than that from eels that were spawned by stripping-insemination (7.70%) (df=14; $t_{Student}$=3.46; P<0.01). Similarly, the rate of fertilized, floating eggs (Table 3) was significantly different between the two experimental groups (df=14; $t_{Student}$=3.06; P<0.01), edging higher in the SPT-group. Unlike floating eggs, sunken eggs were not fertilized for any of the females under study. When excluding the two spawning events with null outcome (SPT-3 and SPT-7), the total fertilization rate and that for the floating eggs in the SPT-group were 51% and 78%, respectively, notably higher than the corresponding values of 8% and 18% in the STR-group (Table 12).

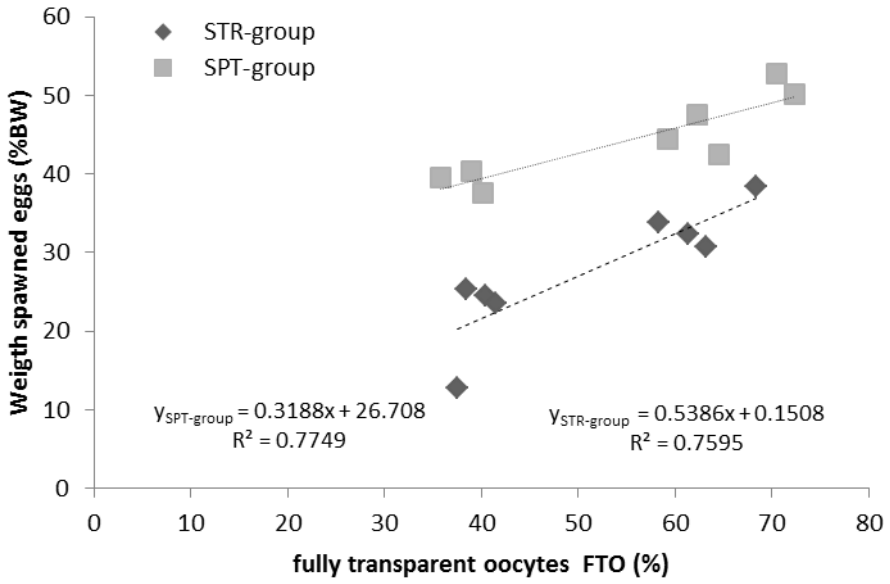


Fig. 30: relationship between the percentage of fully transparent oocytes (% FTO) and the weight of spawned eggs (%BW) from female European eels that spawned spontaneously (SPT) or that were spawned by stripping (STR).

## Discussion

Fertilization, hatching and survival rates can be highly variable between eggs from different individuals (Chai *et al*. 2010) or between different methods of egg retrieval (Mordenti *et al*. 2014). Although pituitary extract-induced resumption of oogenesis in captive silver eels can advance oocyte development to the migratory nucleus stage, ovulation and

final maturation are routinely induced by DHP. However, the relationship between the timing of the induction of the final stages of development by DHP and the resulting quality of the eggs needs to be further explored. Accordingly, we administered DHP to artificially matured European eels (*Anguilla anguilla*) whose ovarian follicles had varying % of fully hydrated oocytes and evaluated the resulting fertility after spontaneous spawning or in response to stripping-insemination.

Silver eels captured in the wild have been mostly used as broodstock for induced maturation trials because under conditions of captivity in aquaculture ponds, almost all eels differentiate into males, making it difficult to obtain females from farmed eel stocks (Okamura *et al*. 2013). However, even while working with eels from wild populations from the same area, a high degree of variability is evident with respect to the gonadal response to hormone treatment (in the present work, ovulation occurred between the 15th and the 29th week). Wild female eels originating from other brackish areas of the North Adriatic Sea (Mordenti *et al*. 2012) ovulated between the 19$^{th}$ and the 30$^{th}$ week. Other studies (Pedersen 2003, Pedersen 2004; Palstra *et al*. 2005; Palstra & van den Thillart 2009) that employed wild eels in their reproduction programs have also been characterized by large variation in time-to-maturity. Okamura *et al*. (2013) proposed that these differences in time-to-maturation could reflect specific differences in the maturity state of female eels just before the first hormone injection; it is plausible that especially gonadotropin receptor abundance may be important in this context.

We observed that among zootechnical performance indicators, the body weight increase (BWI) at DHP injection was highly variable (from 108% to 134%) and it did not appear to be an adequate predictor of the final phase of ovarian maturation, as also reported by Palstra *et al*. (2005) and Mordenti *et al*. (2012), and for *A. japonica* by many authors (Seoka, Yamada, Iwata, Yanagisawa, Nakagawa & Kumai 2003; Dou *et al*. 2008; Chai *et al*. 2010; Ijiri *et al*. 2011; Unuma *et al*. 2011, Unuma, Sawaguchi, Hasegawa, Tsuda, Tanaka T., Nomura & Tanaka H. 2012). The lack of a correlation between the BWI and the percentage of FTOs in the gonads in our study further reinforces the unsuitability of the BWI as a predictor of the right time for induction of final maturation in the European eel. Having said that, our findings do not concur with those from Palstra *et al*. (2005), who showed a relationship between the number of oocytes with single fat droplets (over-ripe and not fertilizable oocytes) and higher BWIs.

Ovarian biopsies obtained after the booster injection (the additional CPE administration following regular weekly injections) showed good synchronous development of oo-

cytes, evidenced by 50% of oocytes being fully transparent. The stage synchrony of oocytes probably results from the low dosage of hormone initially administered to the eels (Mordenti *et al*. 2013). Synchronous maturation in the final phase is very important as it enhances the quantity of spawned eggs on the one hand and prevents the obstruction of the vent by immature ovarian tissue on the other; it is not coincidental that in this study the animals that provided the best yields (in terms of spawned eggs per %BW) had the highest percentage of FTOs. Furthermore, the use of high-dose CPE injections (40mg/kg BW) from the 10$^{th}$ week onwards led to an acceleration in the final maturation phase, as observed by Chai *et al*. (2010) in *A. japonica,* and probably to a better quality of the eggs in terms of fertilization rate. However, there is a risk that migratory nucleus-stage oocytes develop too fast after the booster injection with CPE, and that a high proportion of oocytes contains a single fat droplet (over-ripe) prior to treatment with DHP (Chai *et al*. 2010). Unfortunately, over-ripening in eels progresses faster than in other teleosts (Ohta *et al*. 1996; Unuma, Kondo, Tanaka, Kagawa, Nomura & Ohta 2005).

Inappropriate timing of hormone administrations to pre-spawning eel broodstock may be a cause of poor egg quality. Unuma *et al*. (2011) suggested that proper timing of ovulation induction is essential, but that additional factors are also important for the acquisition of good quality eggs. To predict the best time of ovulation induction, the developmental stages of the oocytes in the ovary during final maturation should be evaluated. The present study highlights that the best results in terms of spawned egg quantity and fertilization rate can be obtained when the proportion of FTO is at least 50% at the time of DHP injection. Indeed, it seems likely that the recent promising findings on spontaneous spawning of eels (Mordenti *et al*. 2014) carried out by our team may be due to fine-tuning of the timing of DHP administration.

The present study convincingly illustrates that in our hands, and with the maturity criteria that we have employed, the spontaneous spawning method produces higher quality eggs than what can be obtained by the stripping-insemination method, probably because the timing of spawning and fertilization is optimized by parent eels themselves (Okamura *et al*. 2013). In addition, the high incidence of spontaneous spawning in European eels in captivity was striking. The presence of good quality males in the tank is a decisive factor for spawning success: only a few minutes after a female was placed in the tank, typical mating behavior was observed in males (approaching the head and urogenital region of the female) (van Ginneken, Vianen, Muusze, Palstra, Verschoor, Lugten, Onderwater, van Schie, Niemantsverdriet, van Heeswijk, Eding & van den Thillart 2005). In sharp

contrast, the only two females with unfertilized eggs (SPT-3 and SPT-7) were not approached by males displaying courtship behavior. The better performance of the SPT-group compared to the STR-group is also reflected in the higher fertilization rate in the former group: for example, among the floating eggs in the SPT-eels, 5 out of 6 females had a fertilization rate exceeding 70%. Retrieving floating eggs from all spawned females contrasts notably with the report on *A. japonica* by Seoka *et al*. (2003), in which floating eggs were obtained from 4 out of 31 females. The buoyancy of eggs, important for oceanic survival and dispersal as well as for the initiation of early embryogenesis (Kagawa, Kishi, Gen, Kazeto, Tosaka, Matsubara, Matsubara & Sawaguchi 2011), has often been used as an indicator in the assessment of egg quality (Unuma *et al*. 2005). Whilst buoyancy is not universally indicative of egg quality in teleost fish (e.g., Kohn & Symonds 2012), the ratio of floating eggs to total spawned eggs at least correlates positively with egg hatchability in eels (Unuma *et al*. 2005).

## Conclusion

The present work shows that in *Anguilla anguilla* the spontaneous-spawning method can yield better egg production, both in terms of quality and quantity, than the stripping-insemination method. Furthermore, BWI is not itself a proper predictor to guide the timing of DHP injection; rather, the presence of a high proportion of fully transparent oocytes can be used to time DHP injection in order to obtain eggs of good fertility.

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## References


Burgerhout E., Brittijn S.A., Kurwie T., Decker P., Dirks R.P., Palstra A.P., Spaink H.P. & van den Thillart G.E.E.J.M. (2011) First artificial hybrid of the eel species *Anguilla australis* and *Anguilla anguilla*. *BMC Developmental Biology* 11:16 DOI 1471-213X/11/16, 1-9.

Casselman J.M. (2003) Dynamics of resources of American eel, *Anguilla rostrata*: declining abundance in 1990s. In: *Eel Biology* (Ed. by Aida K., Tsukamoto K. &Yamauchi K.), pp.255-274. Springer-Verlag, Tokyo.

Chai Y., Tosaka R., Abem T., Sago K., Sago Y., Hatanaka E., Ijiri S. & Adachi S. (2010) The relationship between the developmental stage of oocytes in various season and the quality of the egg obtained by artificial maturation in the feminized Japanese eel *Anguilla japonica. Aquaculture Science* 58, 269-278.

Dekker W. (2003) Status of the European eel stock and fisheries. In: *Eel Biology* (Ed. by Aida K., Tsukamoto K. &Yamauchi K.) pp.237-254. Springer-Verlag, Tokyo.

Di Biase A., Bastone G., Casalini A., Parmeggiani A., Costantini G. & Mordenti O. (2012) Study of the morpho-physiological characteristics of two *Anguilla anguilla* populations selected for artificial reproduction. Word Aquaculture Society, Conference: "Global aquaculture: securing our future - AQUA 2012" 1-5 September 2012, Prague, Czech Republic, 127-128.

Dou S.Z., Yamada Y., Okamura A., Shinoda A., Tanaka S. & Tsukamoto K. (2008) Temperature influence on the spawning performance of artificially-matured Japanese eel, *Anguilla japonica*, in captivity. *Environmental Biology of Fishes* 82, 151–164. doi:10.1007/s10641-007-9268-8.

Durif C.M.F., Dufour S. & Elie P. (2005) The silvering process of *Anguilla anguilla*: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology* 66, 1025–1043.

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel. *Marine Ecology Progress Series* 327, 171–181.

EELREP (2006) Estimation of the reproduction capacity of European eel. Final report, EU Project, EELREP, Q5RS-2001-01836. Leiden University.

Freyhof J. & Kottelat M. (2010) *Anguilla anguilla.* IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. Available at: www.iucnredlist.org (downloaded 8 January 2014).

Ijiri S., Tsukamoto K., Chow, S., Kurogi H., Adachi S. & Tanaka H. (2011) Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. *Aquaculture Europe* 36, 13-17.

Kagawa H., Tanaka H., Ohta H., Okuzawa K. & Hirose K. (1995) In vitro effects of 17 alpha -hydroxyprogesterone and 17 alpha , 20 beta -dihydroxy-4-pregnen-3-one on final maturation of oocytes at various developmental stages in artificially matured Japanese eel *Anguilla japonica*. *Fisheries Science* 61, 1012-1015.

Kagawa H., Kishi T., Gen K., Kazeto Y., Tosaka R., Matsubara H., Matsubara T. & Sawaguchi S. (2011) Expression and localization of aquaporin 1b during oocyte development in the Japanese eel (*Anguilla japonica*). *Reproductive Biology and Endocrinology* 9, 71.

Kohn Y.Y. & Symonds J.E. (2012) Evaluation of egg quality parameters as predictors of hatching success and early larval survival in hapuku (*Polyprion oxygeneios*) *Aquaculture* 342–343: 42–47.

Mordenti O., Di Biase A., Sirri R., Modugno S., Tasselli A. (2012) Induction of sexual maturation in wild female European eel (*Anguilla anguilla*) in darkness and light conditions. *The Israeli Journal of Aquaculture - Bamidgeh, IJA_64.2012.726, 9 pages.*

Mordenti O., Di Biase A., Bastone G., Sirri R., Zaccaroni A. & Parmeggiani A. (2013) Controlled reproduction in the wild European eel (*Anguilla anguilla*): two populations compared. *Aquaculture International* 21, 1045-1063.

Mordenti O., Casalini A., Mandelli M. & Di Biase A. (2014) A closed recirculating aquaculture system for artificial seed production of the European eel (*Anguilla anguilla*): Technology development for spontaneous spawning and egg incubation. *Aquacultural Engineering* 58, 88– 94.

Ohta H., Kagawa H., Tanaka,H., Okuzawa K. & Hirose K. (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4- pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture* 139, 291–301.

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Iinuma N. (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiology and Biochemistry* 17, 163–169.

Okamura A., Horie N., Mikawa N., Yamada Y. & Tsukamoto K. (2013) Recent advances in artificial production of glass eels for conservation of anguillid eel populations. *Ecology of Freshwater Fish* 23, 95–110.

Oliveira K. & Hable W.E. (2010) Artificial maturation, fertilization and early development of the American eel, *Anguilla rostrata*. *Canadian Journal of Zoology* 88, 1121–1128.

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: Development of oocytes during final maturation. *Aquaculture* 249, 533–547.

Palstra A.P. & van den Thillart G.E.E.J.M. (2009) Artificial maturation and reproduction of the European eel. In: *Spawning migration of the European eel.* (ed. by van den Thillart G.E.E.J.M., Dufour S. & Rankin C.), pp. 309–332. Springer, Netherlands.

Pedersen B.H. (2003) Induced sexual maturation of the European eel *Anguilla anguilla* and fertilization of the eggs. *Aquaculture* 224, 323–338.

Pedersen B.H. (2004) Fertilization of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla. Aquaculture* 237, 461–473.

Seoka M., Yamada S., Iwata Y., Yanagisawa T., Nakagawa T. & Kumai H. (2003) Differences in the biochemical content of buoyant and non-buoyant eggs of the Japanese eel, *Anguilla japonica*. *Aquaculture* 216, 355-362.

Tatsukawa K. (2003) Eel resources in East Asia. In: *Eel Biology* (Ed. by Aida K., Tsukamoto K. & Yamauchi K.), pp. 293-298. Springer-Verlag, Tokyo.

Unuma T., Kondo S., Tanaka H., Kagawa H., Nomura K. & Ohta H. (2005) Relationship between egg specific gravity and egg quality in the Japanese eel, *Anguilla japonica*. *Aquaculture* 246, 493-500.

Unuma T., Hasegawa N., Sawaguchi S., Tanaka T., Matsubara T., Nomura K. & Tanaka H. (2011) Fusion of lipid droplets in Japanese eel oocyte: stage classification and its use as a biomarker for induction of final oocyte maturation and ovulation. *Aquaculture* 322-323, 142-148.

Unuma T., Sawaguchi S., Hasegawa N., Tsuda N., Tanaka T., Nomura K. & Tanaka H. (2012) Optimum temperature of rearing water during artificial induction of ovulation in Japanese eel. *Aquaculture* 358-359, 216-223.

van Ginneken, V.J.T. & Maes G.E. (2005) The European eel (*Anguilla anguilla*), its lifecycle, evolution and reproduction: A literature review. *Reviews in Fish Biology and Fisheries* 15, 367–398. doi:10.1007/s11160-006-0005-8.

van Ginneken V.J.T., Vianen G., Muusze B., Palstra A.P., Verschoor L., Lugten O., Onderwater M., van Schie S., Niemantsverdriet P., van Heeswijk R., Eding E.P. & van den Thillart G.E.E.J.M. (2005) Gonad development and spawning behaviour of artificially-matured European eel (*Anguilla anguilla*). *Animal Biology* 55, 203–218.

# CHAPTER 6

## *A CLOSED RECIRCULATING SYSTEM FOR ARTIFICIAL SEED PRODUCTION OF THE EUROPEAN EEL: TECHNOLOGY DEVELOPMENT FOR SPONTANEOUS SPAWNING AND EGGS INCUBATION.*

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**Abstract**

The objective of the present study was to obtain spontaneous reproduction in captivity of the European eel (*Anguilla anguilla*) by using a new closed recirculating aquaculture system provided with spawning and incubation chambers. The influence of two levels of water-flow rates (Low-Flow: 0.8±0.05 L/sec and High-Flow: 2.4±0.05 L/sec) on the spawning, fecundity and egg quality was also investigated. For this purpose 12 silver eel females were induced with increasing doses of carp pituitary extract (10, 20, 30 and 40 mg CPE/kg BW). Twenty-four hours after the last CPE injection, each female ovulation was induced by injecting a DHP-solution and then transferring them to a new closed recirculating aquaculture system, where they were maintained for 16 hours with spermiating males (*sex ratio* 4/1) in order to obtain spontaneous reproduction. The reproduction was tested with 6 females in Low-Flow rate conditions and 6 females in High-Flow rate conditions.

The results showed that the designed closed-loop system made it possible to carry out a more spontaneous reproduction for more than 80% of the females that underwent standardized gonadotropic treatment and favored the automatic and complete transfer of the eggs to the hatchery. The results also point out that high or low water current conditions in the tank do not hinder the mating and the emission of gametes by the breeders, but the high flow rate in the two incubation chambers showed unsuitable hydrodynamic conditions for embryonic development resulting in a constant loss of viable eggs which reached a mortality of 100% among females with the highest incubation density.

## Introduction

The eel is one of the species with the highest market for freshwater aquaculture in the world. To date, all the seedlings for cultivation are wild glass eels or elvers collected in the estuarine waters. However, over the last several decades, natural stocks of eels, especially the commercially valuable temperate species, European eel (*Anguilla anguilla*), American eel (*Anguilla rostrata*) and Japanese eel (*Anguilla japonica*), have decreased markedly (Casselman 2003; Dekker 2003; Tatsukawa 2003) due to overfishing, environmental destruction, oceanographic/climatic changes and other as yet unknown factors (EELREP 2006; van Ginneken & Maes 2005).

The European eel was recently included in the Red List of the IUCN, as a Critically Endangered Species; as a consequence a short fishing season, a minimum capture size, larvae protection and an implemented trade regulation have been imposed to protect this species. Unfortunately the application of the measures has not reduced the risk of extinction (Mordenti *et al*. 2012).

One effective solution to the issue would be to set artificial reproduction techniques for the production of seedlings for aquaculture so that to reduce the demand of wild glass eels.

At present, the only way to obtain sexually mature eels is to artificially induce sexual maturation in silver eel females using repeated injection of carp (CPE) or salmon (SPE) pituitary extract and a final injection of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) while the males are induced following standard protocol with hCG injection (Ohta *et al*. 1996; Palstra *et al*. 2005; Burgerhout *et al*. 2011). Males and females are then hand stripped and milt and eggs are separately collected (Burgerhout *et al*. 2011).

Concerning European eels, studies have focused on the successful protocols based on hormone injection dose and timing and on the definition of optimal environmental parameters (water temperature, water salinity, and photoperiod) (Durif *et al*. 2006; Mordenti *et al*. 2012) in order to obtain gametes by stripping for artificial fertilization; while laboratory experiments that have shown spontaneous spawning of artificially matured European eels in captivity remain elusive.

The objective of the present study was to obtain spontaneous reproduction in captivity of the European eel (*Anguilla anguilla*) by using wild female silver eels. For this purpose a new closed recirculating aquaculture system provided with spawning and incubation chambers was tested.

In this study the influence of two levels of water-flow rates on the spawning, fecundity and egg quality was also investigated.

## Materials and methods

### *Animal*

Wild female eels were caught using traditional "*lavoriero*" (downstream trap) in the brackish water lagoon near the sluices of the North Adriatic Sea (*Valli di Comacchio*, Emilia-Romagna, Italy).

Larger female eels (>500 gr body weight - BW) were selected (n 23) at the catch and then transported to the laboratory where they were measured and sampled to obtain an external indicator of their maturation sta-te (silver index - SI) (Durif *et al*. 2005; Di Biase *et al*. 2012). Only 12 eels with a maximum level in SI (V) were selected and used for reproduction. The animals were marked individually by inserting fish-tags (FLOY TAG Mod Floy T-Bar Anchor) and maintained in starvation for the entire duration of the trial.

At the same time, cultivated male eels (n= 25 fish, 104-212g in BW) reared in freshwater were purchased from a commercial eel supplier and they were gradually acclimated to sea water over 7 days.

All the subjects were kept in two 700 Litre tanks (one with females and one with males) connected to a recirculation system and maintained completely in dark conditions (-0.04 $x10^3$ lux at the bottom of the tank without water) (Mordenti *et al*. 2012) and seawater condition (salinity 32‰), up to a complete gonadal maturation. A seawater controlled temperature system was set at 15.5±0.5°C.

The females received intramuscular injections once a week with carp pituitary extracts (CPE) 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 (10th-final maturation)(Mordenti *et al*. 2012).

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

*Induction of maturation*

Twenty-four hours after the last CPE injection (increase in female BW around 120%) (Mordenti et al., 2013), 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 areas of the ovary.

After the DHP injection, each female was weighted then transferred to a new closed recirculating aquaculture system, where the seawater temperature was raised to 20±0.5°C (Dou et al., 2008), and maintained for 16 hours with spermiating males (*sex ratio* 4/1) in order to obtain spontaneous reproduction. Sperm motility was checked and only males with at least 50% sperm motility (continuous activity of >50% of spermatozoa) were used for the reproduction (Burgerhout *et al*. 2011).

*System description*

A new closed recirculating system is shown in Figs. 31-32. This system consisted in two fish-rearing tanks (0.47$m^3$/tank; water volume 0.43$m^3$/tank; water surface area 0.93$m^2$/tank), a foam separation tank (0.05$m^3$) (protein skimmer) and an biological filter (0.21$m^3$) contained plastic porous balls (0.15$m^3$) (Fig. 31). The total amount of water in this system was 1.12$m^3$ and was transported by a circulating pump (1.1 kW; max delivery 16000 L/hr). The rearing water reached the biological filter and the protein skimmer with an up-flow style (natural falling water) and the treated rearing water was returned to the rearing tank by a circulating pump. The system was also provided with a thermal regulation system (compact cooling equipment, 1.4 kW) to adjust the rearing water conditions (20±0.5°C), a UV-sterilizer lamp (max delivery 500L/hr, 36W), an ozonizer (250mg $O_3$/h) and an aerator (electromagnetic air compressor; delivery 170L/min, pressure 34 kPa, 150W) (Fig.31).

The core of the system was the reproduction tank, which was made of four components: one spawning chamber (240 L), two incubation chambers (52 L/cad) and one outlet chamber (90 L).

**Fig.31**

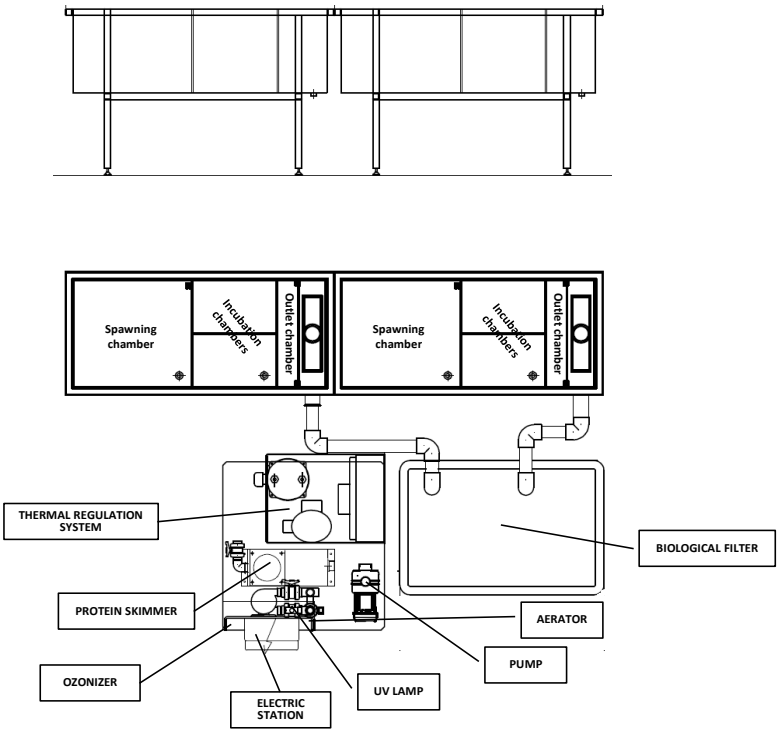
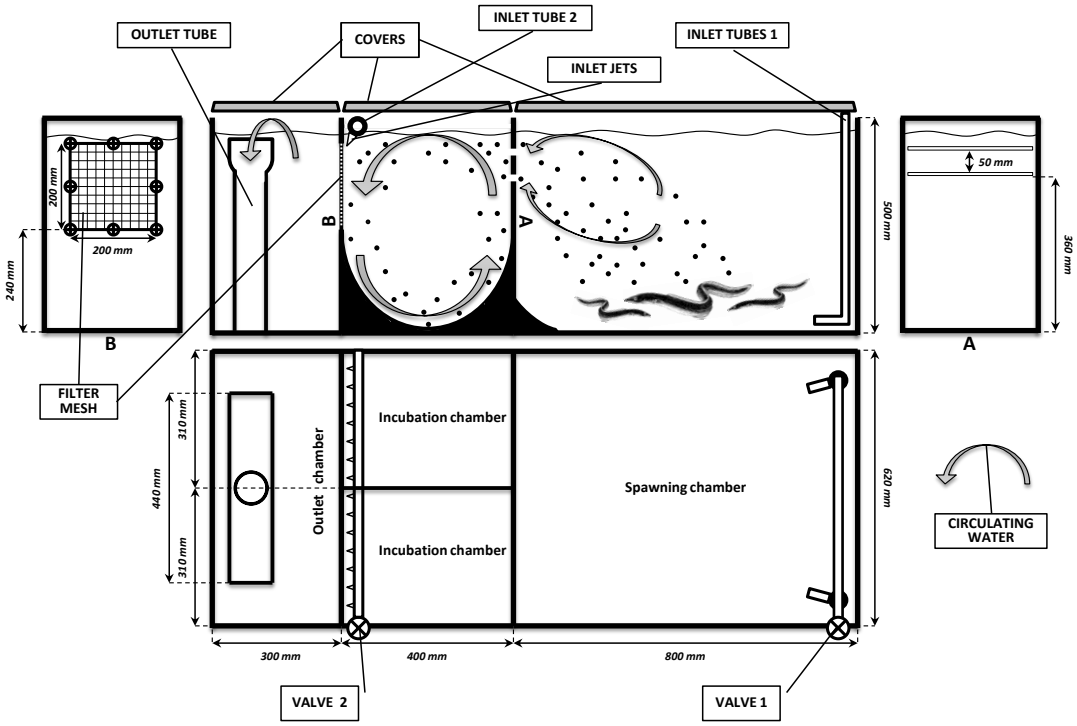



**Fig.32**




Figs 31-32. Schematic illustration of the closed recirculating aquaculture system for artificial seed production of *A.anguilla*

The spawning chamber is connected to the incubation chambers through two 5-mm lengthwise splits located on the top side of the dividing panel. Two pipes allow the water to enter from the base of the spawning chamber (inlet tubes 1) in order to guarantee the water exchange and promote, once the gametes are released, the entrance of the eggs into the incubation chambers (Fig. 32). The water exchange rate is regulated by a valve (valve 1) (Fig. 32).

The incubation chamber has a cylindrical base and a tube on the top (inlet tube 2) and it is provided with inlet jets that produce a circular revolving current: an outlet mesh screen (200mmx200mm; 300 micron diameter and exchangeable) is located on the dividing panel between the incubation and the outlet chambers; inlet jets push water across this mesh screen (Fig. 32). The water flow and the current speed in the incubation chamber are calibrated by the inlet tube valve of the spawning chamber (valve 1) and by the hatchery water pipe valve (valve 2) (Fig. 32).

The system described has a vertical configuration and is inspired by the one originally developed by Greve in 1975 (called “planktonkreisel”) for the maintenance of planktonic animals, later modified by various researchers for larval *Palinurus japonicas* culture (Matsuda & Takenouchi, 2007) and for rearing eel leptocephali (Okamura *et al*. 2009).
The overall tank water level is determined in the outlet chamber by adjusting the outlet tube height; at this point the water is discharged into the filtration system therefore adjusting the environmental parameters (Fig. 32). The water outflow takes place by overflowing, thanks to a collecting tray (440mm x 100mm) positioned at the top of the outlet tube, whirls in the outlet chamber were so avoided.

Finally, 3 covers positioned on the tank had the purpose of maintaining the conditions of darkness and trapping the breeders inside the tank.

*Reproduction*

During the reproduction trial, two levels of fixed water-flow rate, 0.8±0.05 L/sec (Low-Flow) and 2.4±0.05 L/sec (High-Flow), were achieved in incubation chamber by adjusting the valve 1 (0.5±0.05 L/sec in Low-Flow and 1.5±0.05 L/sec in High-Flow) and valve 2 (0.3±0.05 L/sec in Low-Flow and 0.90±0.05 L/sec in High-Flow). The reproduction was tested with 6 females in Low-Flow rate conditions and 6 females in High-Flow rate conditions.

The water-flow upper limit rate (2.4±0.05 L/sec) is the common rate practiced in two 700-Litre tanks used during the hormonal treatment and the lower one (0.8±0.05 L/sec) is an estimated minimum flow rate to minimize the current while maintaining system minimal functionality. After 16 hours all the breeders were removed from the spawning chamber.

*Analytical methods*

For all reproductive cycles the amount of spawned eggs (% BW) and the egg stocking density during the incubation period (n ° egg / L) were determined.

The spawned egg rate was obtained by the difference between the BW at the time of the DHP treatment (BW-DHP) and the BW in post-ovulation (BW-Post-ovulation) when females were removed from the spawning chamber (16h).

The stocking density was determined by dividing the ovarian biomass obtained from each female by the total volume of both the incubation chambers (104 L). For the numerical calculation of the eggs a quantity of 1680 eggs/gr of ovarian biomass was considered (Mordenti *et al*. 2012).

In addition the embryonated egg rate (%) was determined for each female at the beginning (6hr), at 12 hr and 24hr as intermediate times of the total incubation period by calculating the average of 3 samples of 1000 eggs. The spawning starting point was calculated by monitoring the animals from the $8^{th}$ hour after the induction every 15 minutes up until the release of the eggs. Finally, we calculated the embryo survival rate (%) at 12 and 24 hr, taken as 100 the embryonated egg rate at 6hr. Hatching was not assessed due to the fact that maternal factors contribute to the individual variability in wild stocks (natural feeding, endocrine asset, lipid and fatty acid content, age) (Okamoto et al., 2009; Mordenti et al., 2013) indeed they can lead to abnormal development of embryos at embryogenesis completion.

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

Throughout the experimental period operational anomalies were not recorded in the designed recirculating system both in high and in low flow rate conditions.

The system water flow permitted the complete passage of the eggs from the spawning to the incubation chamber both in Low Flow and in High Flow rate conditions. In fact, once the spontaneous breeding was over, no eggs were found within the spawning chamber, with the exception of very small amounts that might be due to the leakage of the female genital orifice during removal. Egg return from the incubation to the spawning chamber was not registered during incubation.

A constant circular water movement (circular revolving current) was recorded in the incubation chamber during all the reproduction cycles both in Low and High flow rate conditions; egg deposit was not recorded on the tank bottom.

Eggs were not flowing into the outlet chamber thanks to a 300µ mesh mounted on the panels that was large enough to enable a proper water flow in both Low and High flow rate conditions.

Besides promoting the water circular revolving current, the inlet jets on the top of the incubation chamber guaranteed the constant cleaning of the filter mesh (self-cleaning) so that the panels were not replaced during the incubation.

Reproduction data showed that the eels responding positively to hormonal treatment were able to reproduce spontaneously in the reproduction chamber and led to the production of fertile eggs. The results also showed that the different flows adopted in the system did not produce a negative impact on spontaneous reproduction inside the tank: only one female/group did not spontaneously ovulated but retained the eggs in the abdominal cavity (Table 13).

Regarding spawned eggs after the DHP injection (Table 13), the eggs/female in each group obtained from spontaneous emission were not statistically different while some variability was observed in the 2 experimental groups (27-42% BW Low-Flow BW group and 28-45% in high-flow group).

Similarly, with regard to egg stocking density, data shown in Table 13, no statistically significant differences were found between the two experimental groups. The highest densities of incubation were recorded with larger eels (2-HF and 5-LF eel eel) and with females that have exceeded the 40% BW of spawned eggs (Table 13).

Tab. 13 – Body weight (DHP and post-ovulation), ovulation, spawned eggs and stocking density eggs exhibit during spontaneous reproduction

| | Eels | | | Eggs | |
|---|---|---|---|---|---|
| | **Body Weight** *DHP-time* | **Ovulation** | **Body Weight** *postovulation* | **Spawned eggs** | **Stocking density** |
| | ***(g)*** | ***(Yes/No)*** | ***(g)*** | ***(%BW)*** | ***(n°eggs/L)*** |
| | | | ***LOW-FLOW*** | | |
| ***1-LF*** | 652 | Yes | 476 | 27 | 2844 |
| ***2-LF*** | 640 | Yes | 378 | 41 | 4349 |
| ***3-LF*** | 604 | Yes | 423 | 30 | 2927 |
| ***4-LF*** | 774 | No | - | - | - |
| ***5-LF*** | 886 | Yes | 523 | 41 | 5868 |
| ***6-LF*** | 618 | Yes | 358 | 42 | 4193 |
| ***Mean*** | ***695.7±111.2*** | | ***431.6±68.3*** | ***36.2±7.1*** | ***4036.2±1238*** |
| | | | ***HIGH-FLOW*** | | |
| ***1-HF*** | 608 | No | - | - | - |
| ***2-HF*** | 831 | Yes | 507 | 39 | 5235 |
| ***3-HF*** | 632 | Yes | 348 | 45 | 4594 |
| ***4-HF*** | 648 | Yes | 467 | 28 | 2931 |
| ***5-HF*** | 636 | Yes | 363 | 43 | 4418 |
| ***6-HF*** | 611 | Yes | 409 | 33 | 3257 |
| ***Mean*** | ***661±84.7*** | | ***418.8±67.7*** | ***37.6±7.0*** | ***4087±963*** |

*Significance difference (P<0.05) between Low–Flow and High-Flow eels

With reference to the embryonated egg rate, Table 14 shows no significant differences between the two experimental groups at the initial stage of embryonic development (6hr), while at 12hr and at the end of the incubation (24hr), eels maintained in the low flow rate system, showed statistically higher embryo survival levels than the ones kept in a high-flow rate. A significantly worse survival rate was found in eggs maintained in high-flow rate conditions after 12 hours of incubation and only in two cases (4-HF eel and 6-HF eel) quantities of fertilized eggs at 24hr were observed (Table 14). Eggs kept in a low flow rate condition showed a significant worsening in the survival rate only at the end of the incubation (24 hr) (Table 14).

Similarly, the survival rate recorded in Low-Flow eels at 12hr and 24hr (76.5% and 62.8% respectively) was statistically higher than that of High-Flow eels (38.3% and 5.6% respectively). In addition, the survival rate at 24hr was significantly higher within the respective groups in eels characterized by a low percentage of released eggs. In fact, eels 1-LF (27% BW) and 3-LF (30% BW) that reproduced spontaneously under low flow rate conditions showed an 80% embryonic survival as the hatching approached (77.9% and

81.9% respectively), at the same time in the high-flow group, the only eels that showed fertile eggs at 24 hours were those with lower % of spawned eggs (4-HF and 6-HF eels). On the contrary the 2 larger females, 5-LF eel and 2-HF eel, which released a high number of eggs, were found to be those with the lowest value of survival rate at 24hr (with 38.7% and 0.0% respectively) in their groups.

Tab. 14 – Embryo survival rate and survival rate obtained in the experiment

| | **Embryo survival rate (%)** | | | | | |
|---|---|---|---|---|---|---|
| | $\frac{N.Embryo\ hr}{Tot\ Embryo}100$ | | | $\frac{N.Embryo\ 6hr}{N.Embryo\ 6hr}100$ | $\frac{N.Embryo\ 12hr}{N.Embryo\ 6hr}100$ | $\frac{N.Embryo\ 24hr}{N.Embryo\ 6hr}100$ |
| | ***6hr*** | ***12hr*** | ***24hr*** | ***6hr*** | ***12hr*** | ***24hr*** |
| | | | | ***LOW-FLOW*** | | |
| ***1-LF*** | 13.1 | 11.4 | 10.2 | 100 | 87.0 | 77.9 |
| ***2-LF*** | 9.8 | 7.1 | 5.8 | 100 | 72.4 | 59.2 |
| ***3-LF*** | 12.7 | 11.2 | 10.4 | 100 | 88.2 | 81.9 |
| ***4-LF*** | - | - | - | - | - | - |
| ***5-LF*** | 10.6 | 6.2 | 4.1 | 100 | 58.5 | 38.7 |
| ***6-LF*** | 11.4 | 8.7 | 6.4 | 100 | 76.3 | 56.1 |
| ***Mean*** | ***11.5±1.4+*** | ***8.9±2.3**** | ***7.4±2.8**** | ***100*** | ***76.5±12.1**** | ***62.8±17.5**** |
| | | | | ***HIGH-FLOW*** | | |
| ***1-HF*** | - | - | - | - | - | - |
| ***2-HF*** | 11.3 | 4.2 | 0.0 | 100 | 37.2 | 0 |
| ***3-HF*** | 9.3 | 3.8 | 0.0 | 100 | 40.1 | 0 |
| ***4-HF*** | 10.7 | 5.1 | 2.2 | 100 | 47.7 | 20.6 |
| ***5-HF*** | 12.1 | 3.9 | 0.0 | 100 | 32.2 | 0 |
| ***6-HF*** | 11.1 | 3.6 | 0.8 | 100 | 34.4 | 7.2 |
| ***Mean*** | ***10.9±1.0§+*** | ***4.1±0.6#*** | ***0.6±0.9*** | ***100*** | ***38.3±6.0*** | ***5.6±9.0*** |

*: Significance difference (P<0.05) between Low–Flow and High-Flow eels;
§: Significance difference (P<0.05) between (6h) and (12h);
+: Significance difference (P<0.05) between (6h) and (24h);
#Significance difference (P<0.05) between (12h) and (24h)

## Discussion

The plant was designed with the aim to obtain spontaneous spawning in *Anguilla anguilla* in captivity as it has been done for many years in the most important European marine farming fish (*Dicentrarchus labrax*, *Sparus aurata*, *Scophthalmus maximus*, *Solea solea*, *Gadus morhua* and *Hippoglossus hippoglossus*) (Brown *et al*. 2006). Currently the reproduction of fish in captivity is mainly controlled by environmental manipulations such as photoperiod, water temperature and salinity or spawning substrate (Kelley *et al*. 1991;

Bromage *et al*. 1993; Carrillo *et al*. 1995; Brooks *et al*. 1997; Anguis & Canavate 2005; Mylonas *et al*. 2010). However, other factors as tank size, water volume and/or depth, hydrodynamic conditions, water flow rate and stocking density were shown to influence reproductive success in some cultured fishes (Tsadik & Bart, 2007; Mylonas *et al*. 2010; Oca & Masalo 2013). Furthermore the spontaneous reproduction of fish in captivity reduces all the issues related to the stripping of ovulating females and spermiating males for gamete collection and to the egg manipulation during the artificial fertilization and their transfer to the hatcheries. It has been widely demonstrated that the stress induced by frequent manipulation to obtain gamete emission can negatively affect the breeder performances and lead them to death (Campbell *et al*. 1992; Bromage 1995; Brooks *et al*. 1997; Schreck *et al.* 2001). In particular in aquaculture the broodstock and egg handling may determine deterioration in quality of the eggs (Hilomen-Garcia 1998; Kjorsvik *et al*. 1990; Brooks *et al*. 1997; Mylonas *et al*. 2004) and, in the case of species that do not spawn spontaneously, it is difficult to determine the optimal latency period for the collection of the gametes. It was demonstrated, in fact, that an early or delayed stripping is directly related to a loss in egg quality (Bromage *et al*. 1994; Brooks *et al*. 1997). Moreover, the spontaneous spawning is preferable since it has the advantage of eliminating the time lapse for the artificial fertilization. In eels, in particular, insemination soon after ovulation is a prerequisite of success, for egg quality degenerates relatively soon after ovulation (Abe *et al*. 2010; Ijiri *et al*. 2011).

The hydrodynamic conditions and the water inlet and outlet characteristics of the system did not hinder the eel reproduction in the tank. The designed closed-loop system made it possible to carry out a more spontaneous reproduction for more than 80% of the females that underwent standardized gonadotropic treatment and favored the automatic and complete transfer of the eggs to the hatchery. The results of this work suggest that it is possible to obtain a spontaneous reproduction in captivity with European eels as already recorded in some work on Japanese eels (Dou *et al*. 2007, 2008; Horie *et al*. 2008; Okamura *et al*. 2009; Ahn *et al*. 2012). It is also important to highlight that spontaneous reproduction was possible with wild silver eels and not with farmed females or feminised eels (Ijiri *et al*. 2011) and therefore already adapted to life in captivity. The results also point out that high or low water current conditions in the tank do not hinder the mating and the emission of gametes by the breeders. The designed system, in short, seems to meet all the requirements for a spontaneous reproduction of the European eel, thus eliminating all the issues related to the "stripping" and the egg handling. The absence of mortality during the experiment is another extremely positive result considering that several studies with Euro-

pean eels reported high mortality rates (EELREP 2006; van Ginneken *et al*. 2005; Pedersen 2003, 2004; Palstra *et al*. 2005, 2010).

With reference to the egg production, similar fertilization percentages at 6hr in the two experimental groups show that the different flow levels did not affect the ovarian fertilization phase during mating. Interestingly, the quantity of eggs spontaneously emitted (36.2±7.12 %BW in LF eels and 37.6±7.0 %BW in HF eels) was higher than that obtained by Mordenti *et al.* (2013) (27.13±5.59% BW) on the same eel population that underwent an identical environmental/hormonal protocol where instead stripping was performed for artificial egg fertilization. The fertilization rate above 10% can be seen as a positive result, in fact it is calculated on the total production of each wild female, instead most of the current experiments assess it on small amount of eggs (from 100-200 to 1000-2000) that are incubated in Petri dishes or plastic bowls (Pedersen 2004; Okamoto *et al*. 2009; Oliveira & Hable 2010; Ahn *et al*. 2012). Furthermore, the results are unlikely to be compared to other freshwater eel species (e.g. *Anguilla japonica* and *Anguilla australis*), as the major difficulties in captive breeding the European eel are well-known (Burgerhout *et al*. 2011; EELREP 2006). The results, however, showed that a high flow rate in the incubation chamber causes a steady loss of viable eggs as time goes by, with a result of a 100% mortality at 24hr for 3 on 5 females (HF group). Probably a high flow rate causes mechanical shocks within the incubation chamber that can lead to embryo death linked to the high fragility of eel eggs. On the other hand mechanical shock sensitivity of fertilized eggs has been reported to vary among species (Hilomen-Garcia 1998; Alderdice *et al*. 1988; Jensen & Alderdice 1989; Thorsen *et al*. 2003).

Regarding the survival rate at the end of incubation (24 hr), a reduced number of fertile eggs was shown in both groups with increasing stocking density. No coincidence that the best results were obtained with those eels characterized by lower body weight and reduced egg emission rate which showed a stocking density of less than 3000 eggs/L while the worst was recorded with eels that were close to 6,000 eggs/L.

It is clear, at this point, that the recirculation system must be reformulated by increasing the volume of the two incubation chambers without however altering the circular revolving water current. A second solution would be to remove the breeder once it reaches a determined incubation density that should not exceed 2000 eggs/L according to observations led in this study. At the moment, in fact, the bibliography does not provide precise information about stocking density of incubated eel eggs and then it would be better to remain at prudent levels compared to those recorded in the experiment.

The plant, which has been designed to avoid any egg manipulation from the release to the hatching, does not allow the separation of viable eggs from the dead ones as it happens for some farmed species and leading to a health risk. After fertilization, in fact, dying or dead eggs are colonized with bacteria/fungus, and in case they are not quickly removed from the incubation trays, viable eggs may also be colonized (Brooks *el al*. 1997). During our experiments, however, no health issues were reported probably due to the rather short time of incubation, for an amount of 38±2.5 degrees day (Mordenti *et al*. 2013) and to the fact that once the hatching was obtained, it was enough to block the water flow for a few minutes to get the separation of the eggs from the dead larvae: eggs, which were positioned on the bottom were easily removed by siphoning while larvae remained motionless on the water column. It is clear, finally, that the designed plant can be used for larvae storage up to the yolk sac resorption and it has all the characteristics to perform a first larval weaning phase without altering the conditions of rearing water.

## Conclusions

The system seems to meet all the necessary requirements in order to obtain a spontaneous reproduction in European eels in captivity and to foster a spontaneous egg transfer to the incubation systems where a proper embryonic development takes place.
The hydrodynamic conditions of the closed-circuit system and the different flow rate adopted in the trial have not hampered the eel reproductive activities, but the high flow rate in the two incubation chambers showed unsuitable hydrodynamic conditions for embryonic development resulting in a constant loss of viable eggs which reached a mortality of 100% among females with the highest incubation density.

In order to improve the reproductive results, it would be then preferable to adopt a low flow rate and a reduction in incubation density.

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## References


Abe T., Ijiri S., Adachi S. & Yamauchi K. (2010) Development of an in vitro culture system for producing eel larvae from immature ovarian follicles in Japanese eel *Anguilla japonica*. *Fisheries Science*, 76, 257-265.

Ahn H., Yamada Y., Okamura A., Horie N. & Mikawa N. (2012) Effect of water temperature on embryonic development and hatching time of the Japanese eel *Anguilla japonica*. *Aquaculture,* 330-333:100-105.

Alderice D.F., Jensen J.O.T. & Velsen F.P.J. (1998) Incubation of sablefish (*Anoplopama fimbria*) in a system designed for culture of fragile marine teleost eggs. *Aquaculture*, 71(3):271-283.

Angius V. & Canavate J.P. (2005) Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture*, 243:133-145.

Bromage N., Bruce M., Basavaraja M., Rana K., Shields R., Young C., Dye J., Smith P., Gillespie M. & Gamble J. (1994) Egg quality determinants in finfish: the role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglossus hipoglossus*. *Journal World Aquaculture Society,* 25:13-21.

Bromage N., Randall C., Duston J., Thrush M. & Jones J. (1993) Environmental control of reproduction in salmonids. In: Muir J., Roberts R. (Eds.), *Recent Advances in Aquaculture*, vol. IV. Blackwell Sciences, Oxford, pp. 55-66.

Bromage N.R. (1995). Broodstock management and seed quality-general consideration. In: Bromage N.R., Roberts R.J., (Eds.), *Broodstock Management and Egg and Larval Quality*. Blackwell Science, Oxford, pp. 1-24.

Brooks S., Tyler C.R. & Sumpter J.P. (1997) Egg quality in fish: what makes a good egg?. *Reviews in Fish Biology and Fisheries,* 7:387-416.

Brown N.P., Shields R.J. & Bromage N.R. (2006) The influence of water temperature on spawning patterns and egg quality in the Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture,* 261:993-1002.

Burgerhout E., Brittijn S.A., Kurwie T., Decker P., Dirks R.P., Palstra A.P., Spaink H.P. & van den Thillart G.E.E.J.M. (2011) First artificial hybrid of the eel species *Anguilla australis* and *Anguilla anguilla*. *BMC Developmental Biology*, 11:16.

Campbell P.M., Pottinger T.G. & Sumpter J.P.l. (1992) Stress reduces the quality of gametes produced by rainbow trout. *Biology of Reproduction*, 47:1140-1150.

Carrillo M., Zanui S., Prat F., Cerda J., Ramos J., Mananos E. & Bromage N. (1995) Sea bass (*Dicentrarchus labrax*). In: Bromage N.R., Roberts R.J. (Eds.), *Broodstock Management and Egg and Larval Quality*. Blackwell Science, Oxford, pp. 138-168.

Casselman J.M. (2003) Dynamics of resources of American ee, *Anguilla rostrata*: declining abundance in 1990s. In: Aida, K., Tsukamoto, K., Yamauchi, K., (Eds.), *Eel Biology*. Springer-Verlag, Tokyo, pp.237-254.

Dekker W. (2003) Status of the European eel stock and fisheries. In: Aida, K., Tsukamoto, K., Yamauchi, K., (Eds.), *Eel Biology*. Springer-Verlag, Tokyo, pp.237-254.

Di Biase A., Bastone G., Casalini A., Parmeggiani A., Costantini G. & Mordenti O. (2012) Study of the morpho-phisiological characteristics of two *Anguilla anguilla* populations selected for artificial reproduction. Word Aquaculture Society, Conference: "Global aquaculture: securing our future - AQUA 2012" 1-5 September 2012, Prague, Czech Republic, 127-128.

Dou S.Z., Yamada Y., Okamura A., Tanaka S., Shinoda A. & Tsukamoto K. (2007) Observation on the spawning behavior of artificially matured Japanese eel *Anguilla japonica* in captivity. *Aquaculture,* 266:117–129.

Dou S.Z., Yamada Y., Okamura A., Tanaka S., Shinoda A. & Tsukamoto K. (2008) Temperature influence on the spawning performance of artificially-matured Japanese eel, *Anguillajaponica*, in captivity. *Environmental Biology of Fishes,* 82:151–164.

Durif C., Dufour S. & Elie P. (2005) The silvering process of the *Anguilla anguilla*: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology*, 66:1025–1043.

Durif C.M.F., Dufour S. & Elie P. (2006) Impact of silvering stage, age, body size and condition on the reproductive potential of the European eel. *Marine Ecology Progress Series*, 327:171–181.

EELREP (2006) Estimation of the reproduction capacity of European eel. Final report, EU Project, EELREP, Q5RS-2001-01836. Leiden University.

Greve W. (1975) The "Meteor Planktonkuvette": a device for the maintenance of macrozooplankton aboard ships. *Aquaculture,* 6:77-82.

Hilomen-Garcia G.V. (1998) Sensitivity of fertilized milkfish (*Chanoschanos forsskal*) eggs to mechanical shock and simulated transport. *Aquaculture*, 159:239-247.

Horie N., Utoh T., Mikawa N., Yamada Y., Okamura A., Tanaka S. & Tsukamoto K. (2008) Influence of artificial fertilization methods of the hormone-treated Japanese eel *Anguilla japonica* upon the quality of eggs and larvae (Comparison between stripping insemination and spontaneous spawning methods). *Nippon Suisian Gakkaishi,* 74(2):26-35 (in Japanese, with English abstract, tables and figures).

Ijiri S., Tsukamoto K., Chow S., Kurogi H., Adachi S. & Tanaka H. (2011) Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. *Aquaculture Europe*, 36(2):13-17.

Jensen J.O.T. & Alderdice D.F. (1989) Comparison of mechanical shock sensitivity of eggs of five Pacific salmon (*Oncorhyncus*) species and steelhead trout (*Salmo gairdneri*). *Aquaculture,* 78:163-181.

Kelley C.D., Tamaru C.C., Lee C.S., Moriwake A. & Miyamoto G. (1991) Effects of photoperiod and temperature on the ovarian cycle of the striped mullet, *Mugilcephalus*. In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S. (Eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of fish*. Fish Symp '91, Sheffield, pp. 142-144.

Kjorsvik E., Mangor-Jensen A. & Holmefjord I. (1990) Egg quality in fishes. *Advances in Marine Biology,* 26:71-113.

Matsuda H. & Takenouchi T. (2007) Development of technology for larval Panulirus japonicas culture in Japan: a rewiew. *Bulletin of Fisheries Research Agency*, 20:77-84.

Mordenti O., Di Biase A., Sirri R., Modugno S. & Tasselli A. (2012) Induction of sexual maturation in wild female European eel (*Anguilla anguilla*) in darkness and light conditions. *The Israeli Journal of Aquaculture – Bamidgeh*, IJA_64.2012.726, pp 9.

Mordenti O., Di Biase A., Bastone G., Sirri R., Zaccaroni A. & Parmeggiani A. (2013) Controlled reproduction in the wild European eel (*Anguilla anguilla*): two population compared. *Aquaculture International,* 21:1045-1063

Mylonas C., Fostier A. & Zanuy S. (2010) Broodstock management and hormonal manipulation of fish reproduction. *General and Comparative Endocrinology*, 165:516-534.

Mylonas C., Papadaki M., Pavlidis M. & Divanach P. (2004) Evaluation of egg production and quality in the Mediterranean red porgy (*Pagrus pagrus*) during two consecutive spawning seasons. *Aquaculture,* 232:637-649.

Oca J. & Masalo I. (2013) Flow pattern in aquaculture circular tanks: influence of flow rate, water depth and water inlet & outlet features. *Aquacultural Engineering,* 52:65-72.

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Hirose K. (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17, 20β-dihydroxy-4- pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture*, 139:291–301.

Ohta H., Kagawa H., Tanaka H., Okuzawa K. & Iinuma N. (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiology and Biochemistry,* 17:163–169.

Oliveira K. & Hable W.E. (2010) Artificial maturation, fertilization, and early development of the American eel (*Anguilla rostrata*). *Canadian Journal of Zoology*, 88:1121-1128.

Okamoto T., Kurokawa T., Gen K., Murashita K., Nomura K., Kim S.K., Matsubara H., Ohta H. & Tanaka H. (2009) Influence of salinity on morphological deformities in cultured larvae of Japanese eel, *Anguilla japonica*, at completion of yolk resorption. *Aquaculture,* 293:113-118.

Okamura A., Yamada Y., Mikawa N., Horie N., Utoh T., Kaneko T., Tanaka S. & Tsukamoto K. (2009) Growth and survival of leptocephali (*Anguilla japonica*) in low-salinity water. *Aquaculture,* 296:367–372.

Palstra A.P., Schnabel D., Nieveen M.C., Spaink H.P. & van den Thillart G.E.E.J.M. (2010) Temporal expression of hepatic estrogen receptor 1, vitellogenin1 and vitellogenin2 in European silver eels. *General and Comparative Endocrinology*, 166:1-11.

Palstra A.P., Cohen E.G.H., Niemantsverdriet P.R.W., van Ginneken V.J.T. & van den Thillart G.E.E.J.M. (2005) Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. *Aquaculture,* 249:533–547.

Pedersen B.H. (2003) Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. Aquaculture 224, 323–338.

Pedersen B.H. (2004) Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture,* 237:461–473.

Schreck C.B., Contreraz-Sanchez W. & Fitzpatrick M.S. (2001) Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture,* 197:3-24.

Tatsukawa K. (2003) Eel resources in East Asia. In: Aida K., Tsukamoto K., Yamauchi K. (Eds.), *Eel Biology*. Springer-Verlag, Tokyo, pp.293-298.

Thorsen A., Trippel E.A. & Lambert Y. (2003) Experimental methods to monitor the production and quality of eggs of captive Marine Fish. *Journal Northwest Atlantic Fishery Science,* 33:55-70.

Tsadik G.G. & Bart A.N. (2007) Effects of feeding, stocking density and water-flow rate on fecundity, spawning frequency and egg quality of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture*, 272:380-388.

van Ginneken V. & Maes G.E. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: a literature review. *Reviews in Fish Biology and Fisheries*, 15:367–398.

van Ginneken V., Vianen G., Muusze B., Palstra A., Verschoor L., Lugten O., Onderwater M., van Schie S., Niemantsverdriet P., van Heeswijk R., Eding E. & van den Thillart G. (2005) Gonad development and spawning behaviour of artificially-matured European eel (*Anguilla anguilla*). *Animal Biology*, 55(3):203–218.

## *Conclusion*

The *Marano-Grado* and *Comacchio* lagoons, in the northern Adriatic Sea, are environments with outstanding characteristics where it is still possible to find female European eels, with high Silver Index, to be used to start a program of artificial reproduction, nevertheless the lagoons produce wild females eels with different morphometric parameters, hormonal and lipid levels. Their high silvering values contributed to the success of the artificial reproduction trial; on the other hand bigger dimensions, highest Condition factor and higher lipid storage seems to fasten gonad maturation of *Comacchio* eels without positively increasing reproductive performances. In fact *Marano-Grado* females, with a smaller size but a higher silver index, showed a more regular gonad development, leading eels to spontaneous spawning.

The adopted protocol, which includes increasing doses of carp pituitary extract (CPE), appears to have contributed to the gamete recruitment and synchronization during gonad development with high eggs production.

Besides the intrinsic characteristics of a wild population, also a particular photoperiod can positively influence the gonadal maturation and an high egg production, resulting in a good reproductive performance. In fact the study on the effects of light and dark conditions on gonadal steroidogenic activity, thyroid hormones and vitellogenin gene expression shows how bypassing the hypothalamic–pituitary system by directly administering CPE, it possible to lead to better steroidogenesis performances especially in animals kept in dark conditions. Moreover, the animals kept in dark conditions showed a significant increase both on T and E2 plasma levels compared to pre-CPE administration, while in light conditions no effects of CPE injection on steroid levels were detected, and the variations of the GSI demonstrate a positive effect of the dark condition housing.

In order to obtain good quality zygotes, a further step has been accomplished by the comparison between two fertilization techniques: spontaneous spawning and stripping-insemination approaches. This shows that in *Anguilla anguilla* the spontaneous-spawning method can yield better egg production, both in terms of quality and quantity, than the stripping-insemination method. Furthermore, the body weight index is not itself a proper predictor to guide the timing of DHP injection; rather, the presence of a high proportion of fully transparent oocytes can be used to time DHP injection in order to obtain eggs of good fertility.

Establishing as a new objective the spontaneous reproduction in captivity of the European eel it was necessary to develop a new closed recirculating aquaculture system that could guarantee conditions of hydrodynamic different from than do traditional tanks, provided with spawning and incubation chambers. It was tested the influence of two levels of water-flow rates on the spawning, fecundity and egg quality. The system developed seems to meet all the necessary requirements in order to obtain a spontaneous reproduction in European eels in captivity and to foster a spontaneous egg transfer to the incubation systems where a proper embryonic development takes place. The hydrodynamic conditions of the closed-circuit system and the different flow rate adopted in the trial have not hampered the eel reproductive activities, but the high flow rate in the two incubation chambers showed unsuitable hydrodynamic conditions for embryonic development resulting in a constant loss of viable eggs which reached a mortality of 100% among females with the highest incubation density. In order to improve the reproductive results, it would be then preferable to adopt a low flow rate and a reduction in incubation density.