Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

XXVIII ciclo

Settore Concorsuale di afferenza: 07/H1 Medicina Veterinaria

Settore Scientifico disciplinare: VET/02 Anatomia e Fisiologia Veterinaria

Studies on the reproductive Physiology of two critically endangered species of the North Adriatic Sea: Adriatic sturgeon (*Acipenser naccarii*) and European eel (*Anguilla anguilla*)

Dottorando: Dott. Michaela Mandelli

Coordinatore Dottorato:

Chiar.mo Prof. Carlo Tamanini

Relatore: Chiar.ma Prof.

Albamaria Parmeggiani

Correlatore Chiar.mo

Dott.Oliviero Mordenti

Esame finale anno 2016

Table of contents

Abstracts

Reproductive physiology has many applications for a successful management of fish population in aquaculture. In particular the stock management of endangered species might take advantage of the knowledge concerning the reproductive cycle in order to improve the protocols and restore the population for restock activities.

The Adriatic sturgeon (*Acipenser naccarrii*) and the European eel (*Anguilla anguilla*) are two endemic species of the North Adriatic Sea and are both listed in the the IUCN Red List of threatened species as "critically endangered". Both the species nowadays rely on the experimental activities and new technologies that try to implement the farming conditions for an improvement of the population management for restocking purposes.

In the present study the onset of puberty was investigated through plasma Testosterone analysis in *A. naccarii.* With reference *to A. Anguilla* the effect of photoperiod was assessed on the endocrine profiles during hormonal induction. Furthermore a new tank design has lead to spontaneous spawning that was then compared to the manual stripping protocol.

La fisiologia della riproduzione presenta varie applicazioni per una gestione efficiente delle popolazioni ittiche in acquacoltura. In particolare la gestione degli stock nei casi di specie a rischio di estinzione potrebbe beneficiare degli studi sul ciclo riproduttivo al fine di migliorare i protocolli e di ripristinare la popolazione per progetti di ripopolamento.

Lo storione cobice (*Acipenser naccarrii*) e l'anguilla europea (*Anguilla anguilla*) sono due specie endemiche del nord Adriatico entrambe incluse nella Lista Rossa IUCN delle specie a rischio come "a rischio critico di estinzione". Ad oggi entrambe le specie devono affidarsi alle attività sperimentali ed alle nuove tecnologie che cercano di migliorare le condizioni di

allevamento per l'incremento della popolazione al fine di attività di ripopolamento. Nel presente lavoro è stato studiato il momento di inizio della pubertà in *A. naccarii* attraverso l'analisi del Testosterone plasmatico. Per quanto riguarda *A. anguilla* gli studi si sono focalizzati sugli effetti del fotoperiodo sui profili endocrini durante l'induzione ormonale. Inoltre, una nuova vasca è stata progettata e utilizzata portando le anguille ad una emissione spontanea dei gameti che è stata poi messa a confronto con il protocollo di spremitura manuale.

CHAPTER 1

Itroduction

Reproductive physiology of fish

The reproductive physiology of fish includes all the physiological processes essential for reproduction: fertilization, early development, gonad differentiation, puberty, gametogenesis, reproductive cycles, sexual behavior. All these processes are regulated by many different factors that refer to the brain pituitary-gonadal axis (Fig 1), and they interact with other important physiological functions such as growth, nutrition, osmoregulation, and response to stress factors. External factors such as temperature, pressure, photoperiod and water salinity can also influence the reproductive physiology. Environmental variables are hence important for an efficient management in aquaculture and need to be carefully monitored since fish exhibit adaptations in terms of physiological regulations for a huge variety of reproductive strategies and tactics (Oliveira 2006).

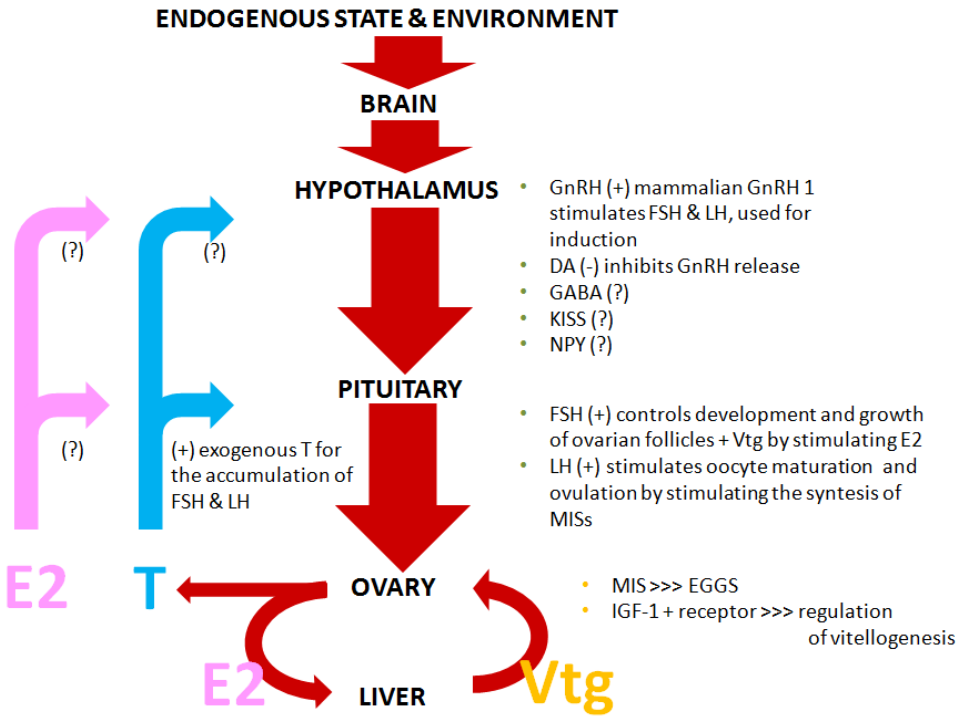


Fig.1: brain pituitary-gonadal axis in female sturgeon

It is now well known that reproductive physiology has many applications for a successful management of fish population in aquaculture, such as hormonal spawning induction, off-season spawning and age at puberty assessment. In particular the stock management of endangered species might take advantage of the knowledge concerning the reproductive cycle in order to improve the protocols and restore the population for restock activities.

Hormonal control sex differentiation: puberty

Puberty can be defined as the period and the physiological process related to the acquisition of the capacity to reproduce. In fish, the onset of puberty is generally recognized as the first production of spermatocytes in males, and the start of vitellogenesis in females (Devlin and Nagahama 2002). Different species exhibit a great variety of strategies with regards to the age of the onset of puberty (e.g. late puberty in sturgeons, Wootton 1984) which also depends on many environmental factors. Aquaculture is also known to affect puberty as continuous feeding on high energy diets, combined with temperature control induces early puberty in some species like the white sturgeon (Doroshov *et al*. 1997).

From the hormonal point of view the onset of puberty is due to the secretion of FSH (follicle stimulating hormone) and LH (luteinizing hormone), two pituitary gonadotropins that control the synthesis of gonadal sex steroids such as testosterone (T), 11-ketotestosterone (11KT) and estradiol-17β (E2). (Shulz *et al.* 2011). In particular Testosterone being a precursor both for androgens and estrogens (Fig.2), has been studied to be an effective parameter for the definition of sexual development stage on various species of sturgeon (Hurvitz *et* al. 2008, Falahatkar *et al.* 2013) and hybrids (Petochi et al. 2011).

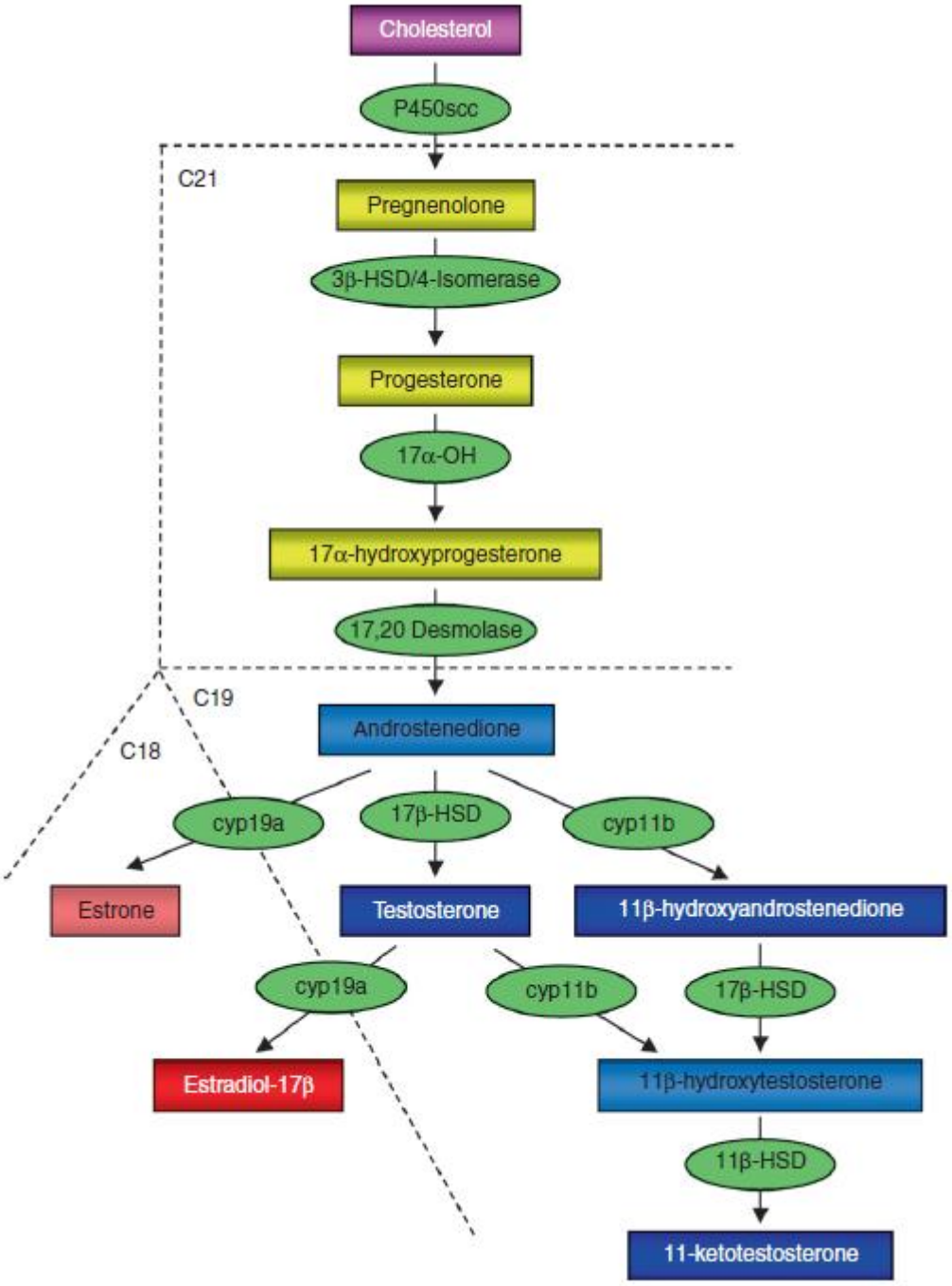


Fig.2 :Sex steroid production (Schulz et. Al. 2011)

Environment affecting physiology: photoperiod

Environmental parameters, such as water temperature, salinity, pressure and photoperiod are known to affect the fish physiology and, with reference to aquaculture, the reproduction performances. The manipulation of external factors have been studied for

economic purposes in order to control traits of interest. For instance photoperiod manipulation has been studied and applied to manage puberty (Okuzawa, 2002) and off-season spawning (Bromage *et al*., 2001), still protocols depend on the species and studies for particular populations are in progress. The farming environment design is therefore extremely important in order to properly manipulate each variable concerning the particular needs a species requires.

Photoperiod is one of the environmental factors that needs to be manipulated in aquaculture in order to improve the growth and reproduction performances since it affects the fish physiology and metabolism (Rodriguez *et a*l. 2009). Seasonal spawning species have been proved to vary their timing and migration by modifying the dark and light conditions. Photoperiod is therefore used in aquaculture in order to obtain a synchronization of the onset of the mating behavior, might it be spawning, migration or smolting (Bromage *et al*., 2001, Vøllestad *et al.* 1994, Durif *et al*. 2005). In the European eel for example the photoperiod was demonstrated to influence sexual maturation: in particular eels perform a greater egg production and spawning efficiency in dark conditions. (Mordenti et al. 2012).

Acipenser naccarii

The Adriatic sturgeon *Acipenser naccarii* (Bonaparte, 1836) is an endemic chondrostean of the North Adriatic Sea with anadromous habits. It once lived in the brackish waters of a wide area ranging from the Gulf of Venice to the coasts of Albania and Greece, returning upstream the major Adriatic tributaries for reproduction. Nowadays its distribution is limited only to landlocked ponds, and contested information on the presence of wild populations in Albania and Spain need to be verified. Since the massive alteration of its

habitat due to anthropization and the missing of natural connection between salt and fresh waters used by this species to reach the mating grounds, the Adriatic sturgeon seems to have lost its capacity to reproduce naturally, becoming highly threatened and it is now included within the IUCN Red list as critically endangered species. At present, this species has to rely on aquaculture procedures to reproduce in a controlled environment and to be hence released into the wild. Four principal captive stocks of *A. naccarii* are known and already characterized: the first one is located at Orzinuovi (Brescia, Italy) and consisted initially of 42 individuals which were collected directly from the Po river in 1977 and which represent reasonably the last living Adriatic sturgeons of certain wild origins (Congiu *et al.*, 2011). Other four stocks of potential breeders are located in aquaculture facilities in Orzinuovi, Parco del Ticino (Milan, Pavia and Varese), Treviso and Piacenza and consist each of a hundred of F1 fishes, all obtained by artificial reproduction of individuals caught in 1977. This means that, considering a reasonable estimate number of about 400 known Adriatic sturgeons all over Europe, more than the 90% are connected each other by kinship relations. The age and the number of surviving F0 individuals are inadequate to guarantee the future of the species, and the use of F1 is crucial for the broodstock management and we need to identify sex and maturity as earlier as

Fig.3: Adult female surgeon (*A. naccarii*)

possible in order to ease the selection of breeders and related crosses. Furthermore the onset of puberty is reached at 6-7 years of age (70 cm) in males and at 8-12 years (80-120 cm) in females, with a consequent difficulty in the management of the broodstock.

No external sexual dimorphism is found in this species: it is not possible to distinguish males from females by morphological characters at any stage of maturity. Currently the main technique that has been proved to be efficient for the determination of sex in sturgeons is surgical biopsy of mature gonads. Even if the survival rate is near to 100%, this procedure is invasive, and non invasive method for sexing sturgeons should be preferred in order not to cause unnecessary stress in the subjects. The assay based on blood plasma sex steroid levels could be used as alternative to gonad biopsy. This technique, which is non-invasive, less stressful and may lead to an earlier sex characterization, is broadly used to sex many species of Acipenseridae family. No studies are presently available on *Acipenser naccarii* maybe due to the fact that the world population can only count a few hundreds of fish, therefore an adequate number of samples might be difficult to be gathered. In this scenario, each single new entry can represent a valuable option to be tested for future crosses with F0/F1 surveyed individuals, in order to maintain the already endangered levels of genetic variability and differentiation. The study aimed to provide a method for a long term management of crosses of Adriatic sturgeons, based on the early sexing of younger individuals never coupled before, the identification of the gonadic activity of adults to select the females that are physiologically ready to reproduce. A further genetic analysis, carried out in collaboration with the Aquaculture Biotech Laboratory of the University of Bologna , provided a profile of all individuals aiming at the maximization of their residual genetic variability with a simple, not invasive blood collection.

For this reason, 18 Adriatic sturgeons (10 adults and 8 sub-adults of 7 years of age), found in a semi-natural land-locked pond at Abbiategrasso (Milan – Italy), were selected.

A further population was then available for sampling at Parco del Ticino near Milan, where a huge stock of more than 100 individuals is hosted. 54 animals of unknown age and sex were sampled in order to start a monitoring program in the area, during the sampling a stripping was performed to ascertain whether the animal was a mature male/female.

All the animals were blood sampled at constant intervals. Maximum total length (from the mouth to the extreme end of the caudal fin) and weight were assessed during blood sampling in order to limit the handling procedures on the animals. Weight was estimated thanks to a manual fish scale.

With reference to the main experiment involving the 18 selected animals one particular blood sample, collected at the beginning of April 2014, was analyzed being close enough to the spawning season to allow the detection of important differences in the plasma Testosterone levels.

The whole blood was collected into heparinized tubes from the caudal fin vasculature and separated by centrifugation for minutes at 3000g. Plasma was used for the dosage of steroid levels while the fraction

containing red blood cells was destined to genetic analyses.

The data were divided with reference to the two age groups of the animals. For the adults the sex was already known, with reference to the sub-adults a stripping was performed 2-3 month after sampling, and led to a distinction between adult males (AM), adult females (AF), young males (YM) and young females (YF).

Testosterone and Estradiol 17β concentrations were determined using a validated RIA (Gaiani *et al.*, 1984, Parmeggiani *et al.*, 2015), partly modified.

In the first experiment the biometric parameters were not found significantly different among the groups (length: p=0.6957; weight: p=0.7295). The plasma concentrations of Testosterone (T) in adults varied from 14.73 to 492.78 ng/mL (14.73-190.05 ng/mL in adult females and 173.23-492.78 ng/mL in adult males with an average value of 232.34 ng/mL (95.34 ng/mL in adult females and 371.37 ng/mL in adult males). With reference to the sub-adults the sampled animals can be divided into two groups: the first with a T level <100 ng/mL and the second with T >100 ng/mL. In the first group, T levels ranged from 2.17 to 9.48 ng/mL with an average of 5.42 ng/mL. The second group showed a T levels from 320.10 to 571.35 ng/mL with an average of 423.14 ng/mL. On the other hand the differences in T level were highly significant ($P<0.001$) among the 4 groups (AM, AF, YM, YF). In order to identify the significant combination of pairwise differences, the Kruskal-Wallis test was performed as a multiple comparison test within age and sex groups. The T concentration levels resulted significantly different ($P<0.05$) within the age and between sexes (AM>AF and YM>YF). In the same way the T concentration levels resulted significantly different ($P<0.05$) within sexes and between the age groups in females and not in males (YF<AF and YM not different from AM).

In order to define which animals should be chosen among the 18 for the current season the Testosterone levels confirmed that the female with the higher concentration of the hormone was the one that had spawned 24 months before, hence the one to be selected as a breeder for the incoming season. With reference to the adult males a higher concentration of T is commonly known to be an index of a higher gonadal activity, therefore the breeder stock should include in this case the males with T>400ng/mL.

With reference to the sub-adults, being 7 years old, the sexing was expected to be detected in males first. In fact among the sub-adults a high concentration in T was detected in three of them that after three months were confirmed 408 males after a

Gonadotropic Releasing Hormone (GN-RH) administration to induce spermiation (Mylonas *et al.*, 1995). It is therefore possible to say that early sex identification is achievable in *Acipenser naccarii* through their blood Testosterone levels. This study represents the first attempt to identify sex in Adriatic sturgeon by analyzing testosterone plasma levels: up to now, no studies were available on *Acipenser naccarii*.

With reference to the population at Parco del Ticino, the 54 animal presented a great variability due to the lack of historical data available, as they a had never been monitored before, 8 animals showed a high level of Estradiol and Testosterone (E2>0.50 ng/mL, T>40 ng/mL), all the others did not show a high E2 level (<050 ng/mL), instead T ranged from 0 to 132.50 ng/mL.

A further monitoring is needed, furthermore the previous experiment underline that the optimal period for blood sampling seems to be at the beginning of the steroid-gonadal activity, i.e. in this latitude, in late spring (April). A breeding and monitoring program has been defined. In the meantime, the animals that during sampling resulted being mature females were the ones with a high level of Estradiol providing confirmation of the effectiveness of the test.

The work on the *Acipenser naccarii* has also lead to the creation of a dedicate area at Cattolica Aquarium where a small stock is now hosted and reproduce to provide a new safe reservoir which is constant monitored.

Fig. 5: The tank dedicated to the Adriatic sturgeon at Cattolica Aquarium

Anguilla anguilla

European eels (*Anguilla anguilla*, Linnaeus, 1758) exhibit a very peculiar life cycle, a growth period in rivers is followed by morphological and physiological changes leading to the spawning migration to the sea. In the last century the population has shown a constant and sharp decline up to the 90% due to barriers to migration, mortality by hydropower turbines, loss and degradation of the natural habitat and overfishing. For this reasons the European eel is now included within the IUCN Red List of threatened species as "critically endangered". Artificial reproduction would then be desirable and crucial for the survival of the fish and even if technologies and experimental activities have recently lead to the spawning in farming conditions through hormonal induction treatments, a full life cycle in captivity has not been achieved yet. In order to optimize the induction protocols to produce a further step forward in the reproduction of the European eel, an in-depth analysis of the physiological mechanisms is needed.

Previous studies have demonstrated the photoperiod to influence sexual maturation (Mordenti et al. 2012): in particular eels perform a greater egg production and spawning efficiency in dark conditions. The effects of light and dark conditions were assessed on the levels of Estradiol 17β (E2), Testosterone (T) and thyroid hormones . For the experiment two groups were treated in two different tanks: the Light Group (14-hours light and 10 hours darkness) and the Dark Group (24 hours in dark conditions). All the animals were hormonal induced throughout the whole experiment and blood sampled at the fifth (P1), ninth (P2) and thirteenth (P3) week.

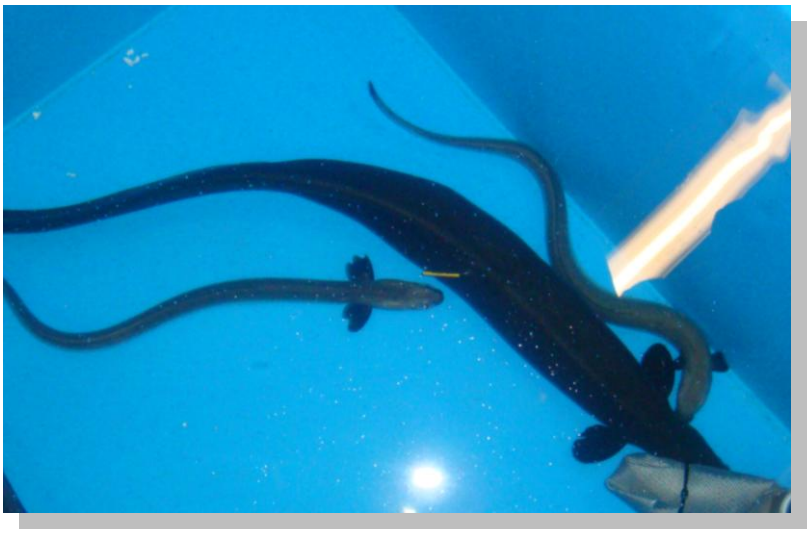


Fig.4: Adult female and males (*A. anguilla*)

Testosterone, Estradiol 17β, Total thyroxine (T4) and Total triiodothyronine (T3) concentrations were determined by RIA. E2 and T levels showed a statistically significant greater value in the Dark Group compared to the Light Group. On the contrary the photoperiod did not show any influence on the T3 and T4 plasma levels. At P2 the increase of E2 levels in conjunction with the decrease in T could suggest an enhance aromatase activity that converts T into E2 as it normally occurs in the biosynstesis of estrogens by the follicular cells.

Previous studies have pointed out that stress can affect reproduction by altering levels and patterns of reproductive hormones that influence maturation in fish (Barton and Iwama 1991). In order to reduce stress due to the handling procedures a new closed recirculating aquaculture system was designed leading to a natural fertilization and an increase in spontaneous spawning in a controlled environment. The system was provided with one

spawning and two incubation chambers. Twenty-four hours after the last hormonal induction through CPE (Carp Pituitary Extract) injection, each female was injected with a DHP solution to induce ovulation and immediately transferred in the new system with 4 spermiating males. A spontaneous reproduction occurred in more than 80% of the females and a complete transfer of the eggs to the incubation rooms was achieved. A subsequent study compared the fertility between eggs spawned spontaneously and those spawned by manual stripping. In the first case the number of eggs was significantly greater compared to the manual stripped group of females. Furthermore a higher fertilization rate was observed in the spontaneous-spawning group.

References

Barton BA, and Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases 1: 3-26.

Bromage NR, Porter M, and 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.

Congiu L, Pujolar JM, Forlani A, Cenadelli S, Dupanloup I, Barbisan F, Galli A, and FontanaF (2011) Managing Polyploidy in Ex Situ Conservation Genetics: The Case of the Critically Endangered Adriatic Sturgeon (*Acipenser naccarii*). *PlosONE*6 (3), e18249.

Devlin RH and Nagahama Y (2002) Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364.

Doroshov SI, Moberg GP, and Van Eenennaam (1997) Observations on the reproductive cycle of cultures white sturgeon, *Acipenser trasmontanus.*Environmental Biology of Fishes, 48(1): 265-278.

Durif C, Dufour S, and 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.

Falahatkar B, Akhavan SR, Tolouei Gilani MH and Abbasalizadeh A (2013) Sex identification and sexual maturity stages in farmed great sturgeon, *Huso huso L*. through biopsy. Iranian Journal of Veterinary Research, Shiraz University. 14(2): 133-139.

Gaiani R, Chiesa F, Mattioli M, Nannetti G, and GaleatiG (1984) Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. Journal of Reproduction Fertility,70: 55–59.

Hurviz A, Jackson K, Yom-Dim S, Degani Gand Levavi-Sivan B (2008) Sexual development in Russian sturgeon (Acipenser gueldenstaedtii) grown in aquaculture. Cybium 07/2008; 32(2):283-285.

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 64:1-8.

Okuzawa K (2002) Puberty in teleosts. Fish Physiology and Biochemistry, 26(1): 31-41.

Oliveira RF (2006) Neuroendocrine mechanisms of alternative reproductive tactics in fish. In: Sloman KA, Wilson RW, and Balshine S (eds.) Behaviour and Physiology of Fish, 1st edn., 24: 151–196.

Parmeggiani A, Govoni N, Zannoni A, Di Biase A, Sirri R, Forni M, Mandelli M, and Mordenti O (2015) Effect of photoperiod on endocrine profiles andvitellogenin expression in European eels Anguilla anguilla during artificially induced ovarian development. Theriogenology 83(4): 478-484.

Petochi T, Di Marco P, Donadelli V, Longobardi A, Corsalini I, Bertotto D, Finoia MG and Marino G (2011) Sex and reproductive stage identification of sturgeon hybrids (*Acipenser naccarii* × *Acipenser baerii*) using different tools: ultrasounds, histology and sex steroids Journal of Applied Ichthyology 27(2): 637–642.

Rodriguez A, Castello-Orvay F, and 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.

Schulz RW and No'brega RH (2011) Regulation of Spermatogenesis. Encyclopedia of Fish Physiology, 75: 627-634.

Vøllestad LA, Jonsson B, Hvidsten NA, and Naesje TF (1994) Experimental test of environmental factors influencing the seaward migration of European silver eels. Journal of Fish Biology, 45:641–651.

Wootton RJ (1984) Introduction: Strategies and 1: 83-113. tactics in fish reproduction Fishes. In: G.W. Potts and 15. Srijunngam, J. and K. Wattanasirmkit, 2001. R.J. Wootton, (ed.) Fish reproduction strategies and Histological structures of Nile Tilapia, Oreochromis tactics. Academic press, London, 1-12.

CHAPTER 2

Genotyping, pedigree reconstruction and endocrinological characterization of *Acipenser naccarii* (Bonaparte, 1836) through microsatellite markers and plasma steroid levels.

Co-authored by:

Ilaria Guarniero, Laura Stancampiano, Alessia Cariani, Nadia Govoni, Alba Parmeggiani, Damiano BarboniandOliviero Mordenti

Abstract

Acipenser naccarii is an endemic sturgeon of the north Adriatic Sea included in the IUCN Red List as critically endangered species and which survival depends currently on captive breeding programs. This study aims to provide a method for a long term management of crosses of Adriatic sturgeons, based on the early sexing of younger individuals never coupled before, the identification of the gonadic activity of adults to select the females physiologically ready to reproduce and the genetic profiling of all individuals in order to maximize their residual genetic variabilitywith a simple, not invasive blood collection. For this reason a population of 18 Adriatic sturgeons was characterized. The animals, ten adults with known sex and eight sub-adults not yet sexed, were of unknown origins and placed in a land-looked pond in Abbiategrasso (Milan, Italy). They were characterized by 10 specie-specific microsatellite loci and plasma Testosterone (T) levels. Analyses allowed to define the sex of the younger individuals (five females and three males), the gonadic phase of the adults and the different parental relationship which occurs between the sub-adults and four out of ten adults. Five adults did not generate any progeny. A very good breeding candidate was identified: an adult female collected in the Ticino river, called *Mostro*, which showed an interesting genetic profile, widely different from all other 17 specimens analyzed. On the base of these results, a list of possible future crosses was drawn.

Introduction

The Adriatic sturgeon*Acipenser naccarii*(Bonaparte, 1836) is an endemic chondrostean of the North Adriatic Seawith anadromous habits. It once lived in the brackish waters of a

wide area ranging from the Gulf of Venice to the coasts of Albania and Greece, returning upstream the major Adriatic tributaries for reproduction. Nowadays its distribution is limited only to landlocked ponds,and contested information on the presence of wild populations in Albania (Ludwig *et al.*, 2003) and Spain (Doukakis*et al.*, 2000; Garrido-Ramos *et al.*, 2009,) need to be verified. Since the massive alteration of its habitat due to anthropization and the missing of natural connection between salt and fresh waters used by this species to reach the mating grounds, the Adriatic sturgeon seems to have lost its capacity to reproduce naturally, becoming highly threatened andit is now included within the IUCN Red list as critically endangered species.

At present, this species has to rely on aquaculture procedures to reproduce in a controlled environment and to be hencereleased into the wild. Four principal captive stocks of *A. naccarii* are known and already characterized: the first one is located at Orzinuovi (Brescia, Italy) and consisted initially of 42 individuals -most of which deceased during the years- which were collected directly from the Po river in 1977 and which represent reasonably the last living Adriatic sturgeons of certain wild origins (Congiu*et al.*, 2011). Other three stocks of potential breeders are located in aquaculture facilities in Orzinuovi itself, Treviso and Piacenza (both Italy) and consist each of a hundredof F1 fishes, all obtained by artificial reproduction of the F0 individuals caught in 1977. This means that, considering a reasonable estimate number of about 400known Adriatic sturgeons all over Europe, more than the 90% are connected each other by kinship relations. In the perspective of the speciesconservation and from the genetic point of view, the first group of few surviving F0 individuals represents doubtless the higher value stock of breeders.Nevertheless, the age and the number of surviving F0 individuals is inadequate to guarantee the future of this species, and the use of F1 individuals as breeders become mandatory. Since the high

relatedness between F1 individuals, it is absolutely crucial to prevent a further loss of genetic variability and differentiation. Both microsatellite and mitochondrial DNA markers in fact have already detected in these individuals lower levels of genetic variability and different distribution of haplotype and allele classes in comparison to F0 individuals, clear signs of the genetic bottleneck that occurred in F1 individuals of*A. naccarii*(Ludwig *et al.*, 2003; Congiu*et al.*, 2011).

From the genetic point of view, *A. naccarii* belongs to the group of 240-chromosome sturgeon speciesand is considered to be tetraploid or functionally tetraploid. This condition seems to be due to autopolyploidization and the chromosome segregation into gametes ismainly tetrasomic, in which the four homologous chromosome copies (a, b, c, d) can generate six equiprobable diploid gamete variants by random pairing (ab, ac, ad, bc, bd, cd), even if the coexistence of tetrasomic and disomic inheritance model cannot be totally excluded (Boscari*et al.*, 2011). Genotyping of individuals and family/pedigree reconstruction by neutral microsatellite DNA markers is widely used in fish species belonging to different taxonomical groups, with both diploid and tetraploid chromosome set. Several unlinked microsatellite DNA markers are available for *Acipenser* species (McQuown*et al.*, 2000; Henderson-Arzapalo and King, 2002; Zane *et al.*, 2002; Forlani*et al.*, 2008; Chassaing*et al.*, 2011) both species-specific for *A. naccarii* and/or optimized for other species of the genus, which cross amplify also in Adriatic sturgeon because of the slow evolutionary rate tipical of the Acipenseridae family (Robles *et al.*, 2004; Chassaing*et al.*, 2011).

Sexing is crucial for the broodstock management and we need to identify sex and maturity as earlier as possible in order to ease the selection of breeders and related crosses. Furthermore the onset of puberty is reached at 6-7 years of age (70 cm) in males and at

8-12 years (80-120 cm) in females (Bronzi*et al.*, 2005, Grandi and Chicca, 2008), with a consequent difficulty in the management of the broodstock itself.

No external sexual dimorphism is found in this species: it is not possible to distinguish males from females by morphological characters at any stage of maturity, larval, juvenile and adult. Even if the mechanism of sex determination in sturgeons is poorly understood, the presence of a sex ratio 1:1 in wild adult populations suggests that these species have a genetically determined gender. Despite the wide efforts spent and the different approaches tried, up to now no sex-specific molecular markers were detected (Keyvanshokooh and Gharaei, 2010).

Currently the main technique that has been proved to be efficient for the determination of sex in sturgeons is surgical biopsy of mature gonads (Doroshov*et al.*, 1997). Even if the survival rate is near to 100% (Feist*et al.*, 2004), this procedure is invasive, and non-invasive method for sexing sturgeons should be preferred in order not to cause unnecessary stress in the subjects (Di Marco *et al.*, 1999). The assay based on blood plasma sex steroid levels could be used as alternative to gonad's biopsy. This technique, which is non-invasive, less stressful and may lead to an earlier sex characterization, is broadly used to sex many species of Acipenseridae family (Webb *et al.*, 2002, Barannikova*et al.*, 2004, Feist*et al.*, 2004). No studies are presently available on *Acipenser naccarii* maybe due to the fact that the world population can only count a few hundredsof fish, therefore an adequate number of samples might be difficult to be gathered.

Based on bibliographic data, the conservation of the Adriatic sturgeon seemed to rely up today on the management of the F0 and F1 individuals genotyped by Congiu*et al.*(2011) and Boscari*et al.* (2014). In this scenario, each single new entry can represent a valuable

option to be tested for future crosses with F0/F1 surveyed individuals, in order to maintain the already endangered levels of genetic variability and differentiation.

This study aims to provide a method for a long term management of crosses of Adriatic sturgeons, based on the early sexing of younger individuals never coupled before, the identification of the gonadic activity of adults to select the females that are physiologically ready to reproduce and the genetic profiling of all individuals aimed at the maximization of their residual genetic variability with a simple, not invasive blood collection.

For this reason, 18 Adriatic sturgeons,found in a semi-natural land-locked pond at Abbiategrasso (Milan – Italy), were typified. These individualshave not been surveyed to date and can represent, after appropriate genetic characterization by microsatellite markers and plasmasexual steroid analysis, a newbroodstock which members may contribute to the conservation of the species and the managing of residual genetic variability, increasing the number of possible crosses with the F0/F1 individuals already typed.

Materials and Methods

Ethics. All experiments were performed according to European guidelines on animal experimentation and care.

Fish specimens,blood sampling and biometric measurements

All the animals included in the present study, with the exception of one (individual nr 18), were part of a single stock that was born in 1996, conceivably deriving from the F0

breeders kept in Orzinuovi. Once they reached sexual maturity artificial reproductions were performed between randomly chosen individuals leading to the generation of an offspring in 2007 referred in the present work as sub-adults.

All these animals have been kept in a semi-natural environment: a small and closed cove, with no possible escaping or infiltration of other fish.

The individual nr 18, alsocalled "*Mostro*",was caught in the Ticino River in 2008, when the Panperduto dam was dried to create a climbing ladder for sturgeons.

One particular blood sample, collected at the beginning of April 2014, was analyzed beeingclose enough to the spawning season to allow the detection of important differences in the plasma Testosterone levels. The whole blood was collected into heparinized tubes from the caudal fin vasculature and separated by centrifugation for 15 minutes at 3000g. Plasma was used for the dosage of steroid levels while the fraction containing red blood cells was destined to genetic analyses.

The data were divided with reference to the two age groups of the animals. For the adults the sex was already known, with reference to the sub-adults a stripping was performed 2-3 month after sampling, and led to a distinction between adult males (AM), adult females (AF), young males (YM) and young females (YF). Identification data of the ten adults and eight sub-adults sampled such asmicrochip ID number, sex (where known at sampling time) and maturity stage are reported in Table 1.

Progressive nr.	Sex*	Maturity stage
1 (Albina)	F	A
2	ND	SA
3	M	A
4	ND	SA
5	ND	SA
6	ND	SA
7	M	A
8	M	A
9	M	A
10	ND	SA
11	ND	SA
12	M	A
13	M	A
14	ND	SA
15	ND	SA
16†	F	A
17	M	A
18 (Mostro)	U	A

Table 1. ID number and name (in brackets), sex (F: female, M: male, ND: not determined, U: uncertain) and maturity stage (A: adult, SA: sub-adult). Individual nr. 1 was named *Albina* for her unusual pale livery and individual nr. 18 was named *Mostro* for its length (more than 2m). † Dead in 2014.

Maximum total length (from the mouth to the extreme end of the caudal fin) and weight were assessed during blood sampling in order to limit the handling procedures on the animals. Weight was estimated thanks to a manual fish scale.Condition factor *k* was calculated according to the formula $k = (BW*BL^{-3}) \times 10^3$ where BW: body weight (g), BL: body length (cm) (Roncarati*et al.*, 2014; Falahatkarand Imanpour, 2014). Biometric data were statistically analyzed by STATA 11.2 (StataCorp. 2009).

Testosterone radioimmunoassay

Testosterone concentration was determined using a validated RIA (Gaiani*et al.*, 1984, Parmeggiani*et al.*, 2014), partly modified. The hormone was extracted from plasma samples (0.2 mL) with 5 ml of diethyl ether by stirring on "rotor" for 30 minutes and centrifugation at2000gfor 4 min. At a rate of 0.1 mL, was added tritiated testosterone (30 pg/tube) and 0.1mL of a solution of 1: 50,000of antibody anti-testosterone.

The dried extracts were dissolved in 1 mL of an RIA-phosphate buffer (Na2HPO4 74.26 mmol/L, EDTA Na 12.49 mmol/L, NaN3 7.69 mmol/L) containing 0.1% bovine serum albumin, pH 7.5, and were shaken for 10 min. The sample (0.1 mL), 1,2,6,7-3H testosterone (T) (0.1 mL, 30 pg/tube) and rabbit anti-testosterone serum (0.1 mL, 1:50,000) were incubated overnight at 4°C; 1 mL of charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer) was then added to the tubes. After 15 min at 4°C, the tubes were centrifuged for 15 min at 3000g, the supernatant was decanted and radioactivity was immediately measured using a β scintillation counter (Packard C1600, PerkinElmer, USA). In parallel to the ether extracts of plasma, the assay was conducted on known quantities of the hormone, in order to set up a curve reading. The average recovery, evaluated in preliminary tests, was 80%. The cross reactions of various steroids with the rabbit anti-testosterone serum were as follows: testosterone 100%, DHT 25.4%, dione 0.43%, cortisol <0.001%, progesterone <0.001%. Testosterone level determined by RIA was validated by verifying that serial dilutions were parallel to standard curves.Since data were not normally distributed a Kruskal-Wallis one-wayanalysis of variance by ranks non-parametric test was used for statistical analysis (STATA 11.2, StataCorp.2009).

Genotyping and parental allocation

Genomic DNA was extracted from blood samples starting from 25μL of blood using the QiagenDNeasy Blood and Tissue kit and quantified by Qubitfluorometer (Invitrogen) according to manufacturers' protocols. The genetic profiling was assessedbyten polymorphic microsatellite loci selected for their good level of polymorphism: AnacA6, AnacB7, AnacB10, AnacE4, AnacB11(Forlani*et al.*, 2008), An16(Zane *et al.*, 2002), Spl120 (McQuown*et al.*, 2000), AoxD234 (Henderson-Arzapalo and King, 2002), LS54 and Aox23 (Chassaing*et al.*, 2011). Forward primers were fluorescently labeledand 50ng of each sample DNA was amplified using the PCR conditions described by authors. Genetic profiles were obtained by capillary electrophoresis in an ABI3100 fragment analyzerusing LIZ500 as internal size standard. Alleles were scored by Peak Scanner Software v1.0 (Life-Technology).

Given the tetraploid condition of the Adriatic sturgeon and the impossibility to assess allelic frequencies, the genetic variability was measured by estimating the number of alleles per locus, the maximum number of bands per individuals and the intra-locus band sharing parameter (IBS), which estimates for each single locus the number of allelic classes shared overall the samples (Forlani*et al.,* 2008).

Poisson regression was used to compare the levels of homozygosity (reordered as number of loci showing a single band per individual) between sub-adults and adult animals. In order to avoid bias in the results, loci with one or more missing data were not included in the analysis.

The unweighted pair-group method using arithmetic mean (UPGMA) clustering algorithm illustrating the pattern of differentiation among samples was constructed by

BioNumericssoftware (Applied Maths, Sint-Martens-Latem, Belgium) using the matrix of dominant records obtained converting microsatellite codominant data according to Rodzen*et al*. (2004).

Parental allocation was performed by the Windows-based software wHDP by Galli*et al*. (2011) based on an exclusion method and a likelihood approach in case of multiple allocations and which is specifically developed for species carrying a quadruple set of chromosomes.

Themultilocus genetic distances between sampleswere evaluated as band-sharing dissimilarity index, i.e. one minus the similarity index.In particular, two different index were evaluated: the first is the similarity index described in Equation 1 of Lynch (1990), specific for autopolyploid species, implemented in Polysat package for R software (Clark and Jasieniuk, 2011). The second is based on the equationofWetton *et al*. (1987) and is not implemented in any software up to now.In order to automate and speed up the evaluation of multilocus pairwise band sharing (MBS) according to Wetton *et al*. (1987) a dedicated Phyton script has been developed. This programanalyze a .csvinput file containing the acquired data (Locus, Alleles, Gender) and provides as output a report that lists, for each female/male couple, the computed band sharing parameters and related genetic distance, with details of matching loci (contact the corresponding author for the script free download procedure).

Genetic distances were then plotted by MDS implemented in STATA 11.2 (StataCorp. 2009).

To avoid bias due to missing data, when necessary loci with an incomplete genotyping were excluded by calculation of genetic distances.

Results

Biometric analysis

Fish average weight and length were 23.5±8.5kg, 21.60±2.4kg for adults and sub-adults respectively, and 139.4±19cm and 132.9±4.7cm respectively (ranges in Table 2 and 3). The biometric parameters were not found significantly different among the groups (length: p=0.6957; weight: p=0.7295).

With reference to the Condition Factor *k* all the animals showed values in favor of the weight parameter with the exception of animal nr 18 (Mostro) that registered a *k* in favor to the length, therefore a not yet satisfactory nutritional status(Table 2 and 3).

Testosterone analysis

The plasma concentrations of Testosterone (T) in adults varied from 14.73 to 492.78ng/mL (14.73-190.05 ng/mL in adult females and 173.23-492.78 ng/mL in adult males (Table 2) with an average value of 232.34 ng/mL (95.34 ng/mL in adult females and 371.37 ng/mL in adult males). With reference to the sub-adults (Table 3) the sampled animals can be divided into two groups: the first with a T level <100 ng/mL and the second with T >100 ng/mL. In the first group (animals 11, 10, 15, 6, 2), T levels ranged from 2.17 to 9.48 ng/mL with an average of 5.42 ng/mL. The second group (animals 5, 4, 14) showed a T levels from 320.10 to 571.35 ng/mL with an average of 423.14 ng/mL.

On the other hand the differences in T level were highly significant (P<0.001) among the 4 groups (AM, AF, YM, YF). In order to identify the significant combination of pairwise differences, the Kruskal-Wallis test was performed as a multiple comparison test within

age and sex groups. The T concentration levels resulted significantly different ($P<0.05$) within the age and between sexes (AM>AF and YM>YF). In the same way the T concentration levels resulted significantly different ($P<0.05$) within sexes and between the age groups in females and not in males (YF<AF and YM not different from AM).

Animal Ref. Nr	1	16	18	3	12	7	13	8	17	9
Length	145	120	185	144	117	140	130	129	138	146
Weight	28	14	43	28	14	20	20	20	21	27
Condition factor (k)	0.92	0.81	0.68	0.94	0.87	0.73	0.91	0.93	0.80	0.87
Sex	F	F	F	M	M	M	M	M	M	M
Testosterone	81,24	190,05	14,73	173,23	406,15	372,24	438,74	457,38	492,78	259,06

Table 2. Biometrics, sex and Testosterone plasma concentration in adults. Body length is expressed in cm, weight in Kg and Testosterone level in ng/mL. F: female; M: male.

Animal Ref. Nr	11	10	2	6	15	4	5	14
Length	135	130	131	135	139	125	130	138
Weight	25	20	21	23	22	17	23	22
Condition factor (k)	1.01	0.91	0.93	0.93	0.82	0.87	1.01	0.84
Sex Group	1	1	1	1	1	2	2	2
Testosterone	2,17	3,49	7,2	9,48	4,75	320,1	571,35	377,96

Table 3. Biometrics and plasma concentration of Testosterone in sub-adults. Body length is expressed in cm, weight in Kg and Testosterone level in ng/mL. The animals were divided into two sex groups with reference to the concentration of Testosterone (Sex Group 1, T<100ng/mL and Sex Group 2, T>100ng/mL).

Genotyping and parental allocation

The observed allelic ranges overlap the expected ones and the total number of alleles per locus is similar to those reported in bibliography (Table 4). Since the maximum number of

alleles per locus per individual ranged from three to four, all the ten loci appear to be part of a tetraploid system, even locus An16 that in Zane *et al*. (2002) showed a diploid behavior.

Locus	Nr individuals analysed	Allelic range	Allele number	Max nr bands per individual	IBS coefficient
AnacA6(§)	18	291-305	7	4	0.37
AnacB7(§)	18	151-173	7	4	0.40
AnacB10(§)	18	207-258	11	4	0.40
AnacE4(§)	18	333-355	9	4	0.31
AnacB11(§)	18	131-163	10	4	0.33
An16(§)	18	176-204	6	3	0.29
Spl120(§)	18	264-304	6	4	0.51
AoxD234(§)	18	214-274	9	4	0.46
LS54(§)	18	130-190	9	4	0.35
Aox23(§)	18	139-175	7	4	0.29
AnacA6(*)	42	289-313	9	4	0.44
AnacB7(*)	42	152-176	11	4	0.46
AnacB10(*)	42	212-258	11	4	0.39
AnacE4(*)	42	326-354	10	4	0.50
AnacB11(*)	42	132-162	8	4	0.42
An16(*)	20	171-221	12	2	0.19
Spl120(*)	42	263-303	9	3	0.35
AoxD234(*)	30	215-275	14	4	0.32
LS54(*)	30	140-190	2	NA	0.45
Aox23(*)	30	140-174	6	NA	0.44

Table 4. (§) Samples analyzed in this study; (*) Data from referring bibliography: loci AnacA6, -B7, -B10, -E4, -B11, Spl120, Aoxd234 in Forlani*et al*., 2008; locus An16 in Zane *et al*., 2002; locus LS54 in Chassaing*et al*., 2011.

IBS= intra-locus band sharing coefficient, i.e. percentage of bands shared by all individuals for the same locus. NA=data not available.

A total of 75 different alleleswere observed, one of which was fixed (allele 222 locus AnacB10). Alleles have a different distribution between adults and sub-adults: the latter show only 53 out of 75 variants, which means a decrease of 30,48% of the variability between the two generations. With the exception of locus AnacB7 that appear conserved into the two groups analyzed, all other loci have lost from one to four allelic variants.

Despite the overall loss of genetic variability the level of heterozygosity (defined as the presence of 2 or more bands per locus per individual) appears similar in both age groups (p=0.68).

From a strictly genetic point of view, the individual nr. 18 (Mostro) showed a peculiar genotype: four private alleles were identified (allele 143 locus AnacB11, 284/Spl120, 150/LS54 and 169/Aox23), while among the remaining 17 examined sturgeons only one showed a single private allele (individual nr. 1, "Albina", allele 301 locus AnacA6).

Even assuming a tetrasomic pattern of chromosome segregation, it was not possible to reconstruct the effective genotype of partially heterozygotes (i.e. individuals that showed two or three bands per locus) and related "true" allelic frequencies. For this reason the intra-locus band-sharing coefficient was reported instead of heterozigosity and ranged from 0.29 of loci AN16 and Aox23, and 0.51 of locus Spl120.

The UPGMA tree (Fig.1) constructed to illustrate the pattern of genetic divergence between sturgeon samples using the matrix of dominant data showed the separation of two main clusters and a third one separated by other two (individuals 3 and 18).

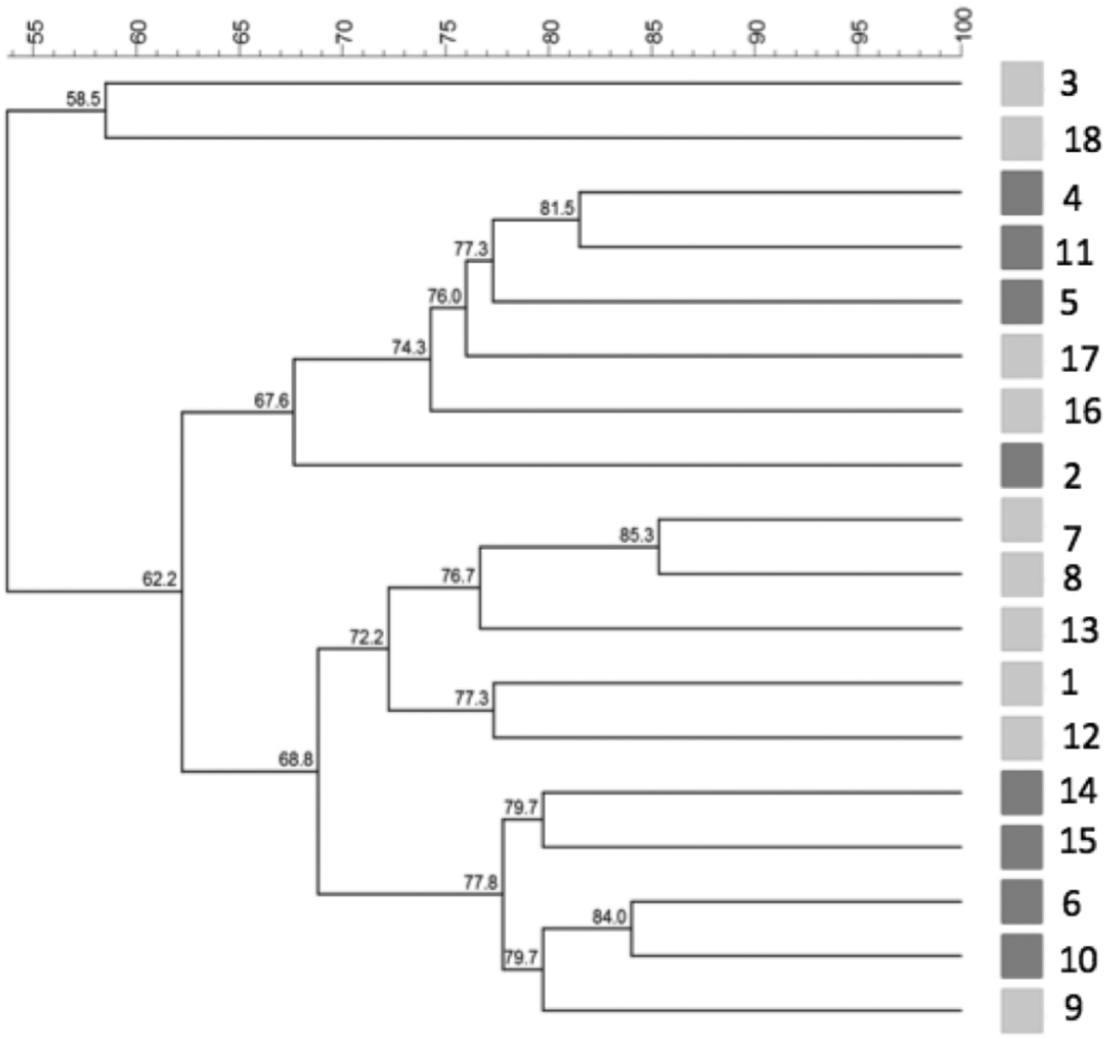


Fig.1 Cluster analysis of the 18 sturgeon samples by UPGMAmethod. Light grey: adults; dark grey: sub-adults.

For the parental allocation procedure, locus AnacB10 was excluded for technical reasons and parentage test was performed with nine loci.

Complete allocation was possible for all 8 sub-adults (Fig. 2). In particular two main families were identified: Albina (female nr 1) X male nr 9, which formed the first one with 3 full sib (animals nr 6, 10 and 15), and female nr 16 X male 17 with other 3 sons (2, 4 and 5). Same adults but with a different combination produced the remaining two sub-adults (nr 11, son of Albina X 17; and nr 14, son of 16 X 9).

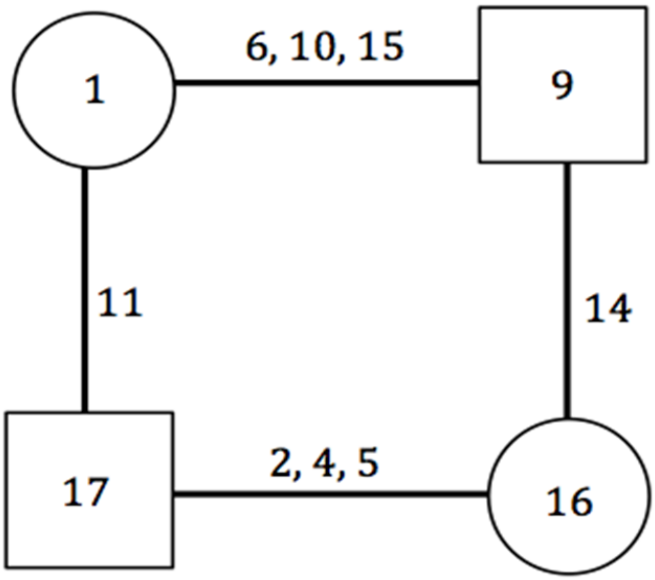


Fig. 2. Parental allocation scheme according to results of parentage analysis software for tetraploid individuals (wHDP by Galli*et al*., 2010). In squares: putative fathers; in rounds: putative mothers.

Adults n. 7, 8, 12, 13 do not seem to be strictly related to the eight sub-adults and therefore to participate in the reproductive event. Even if the Mostro was added to the broodstock only in a second moment and it was surely not used as a breeder, it was included in the parentage assessment as potential parent, to test the power of the algorithm implemented in wHDP software. This individual was correctly rejected as parent.

As regard horizontal kinship, individual nr 6, 10 and 15 from one hand, and 2, 4 and 5 from the other, form two separate groups of full sib (family 1 and family 2). While individual nr 11 and 14 share one of the parents with both sub-adults belonging to family nr.1 and to family nr.2.

The two multilocus pairwise genetic distances based on Wetton *et al*. (1987) and Lynch 1990 equations are reported in Table 5,above and below the diagonal respectively. Results are mostly overlapping, even if method based on Wetton's equation and implemented in

the script here developed appears to be more conservative than method based on Lynch distances. In fact crossing genetic distances with pedigree/kinship reconstruction the threshold value, which separates related from unrelated individuals,is slightly higher according to Wetton distance (0.600, 14 X 2 pairwise comparison) rather than Lynch distance (0.575, same comparison).

	1 [f]	3 [m]	4 [m]	5 [m]	6 [f]	7 [m]	8 [m]	9 [m]	10 [f]	11 [f]	12 [m]	13 [m]	14 [m]	15 [f]	16 [f]	17 [m]	18 [f]
1 [f]	-	0.778	0.543	0.462	0.347	0.480	0.447	0.551	0.469	0.400	0.333	0.490	0.545	0.478	0.451	0.577	0.741
2 [f]	0.536	0.667	0.429	0.434	0.600	0.451	0.542	0.640	0.680	0.412	0.462	0.538	0.600	0.617	0.462	0.434	0.600
3 [m]	0.814	-	0.625	0.842	0.556	0.722	0.697	0.471	0.529	0.771	0.778	0.622	0.484	0.563	0.611	0.737	0.550
4 [m]	0.538	0.635	-	0.351	0.600	0.515	0.548	0.667	0.697	0.294	0.647	0.600	0.563	0.576	0.282	0.351	0.526
5 [m]	0.523	0.823	0.316	-	0.569	0.577	0.551	0.647	0.569	0.308	0.547	0.623	0.609	0.500	0.321	0.333	0.536
6 [f]	0.338	*0.582*	0.609	0.603	-	0.469	0.348	0.347	0.250	0.469	0.440	0.440	0.349	0.289	0.440	0.686	0.774
7 [m]	0.493	0.688	0.500	0.580	0.457	-	0.234	0.574	0.429	0.520	0.373	0.255	0.476	0.455	0.429	0.577	0.741
8 [m]	0.480	0.690	0.552	0.594	0.361	0.213	-	0.511	0.435	0.447	0.375	0.458	0.450	0.429	0.447	0.551	0.647
9 [m]	0.545	0.436	0.654	0.640	0.281	0.553	0.511	-	0.293	0.551	0.633	0.592	0.333	0.404	0.577	0.608	0.686
10 [f]	0.436	0.532	0.633	0.575	0.221	0.420	0.423	0.271	-	0.469	0.480	0.360	0.302	0.244	0.600	0.686	0.774
11 [f]	0.456	0.804	0.290	0.283	0.527	0.549	0.520	0.564	0.548	-	0.490	0.490	0.409	0.435	0.412	0.434	0.519
12 [m]	0.340	0.830	0.624	0.576	0.433	0.356	0.356	0.654	0.500	0.510	-	0.346	0.591	0.478	0.490	0.737	0.782
13 [m]	0.493	0.646	0.590	0.633	0.430	0.264	0.447	0.604	0.391	0.540	0.330	-	0.409	0.391	0.529	0.351	0.745
14 [m]	0.533	0.465	0.519	0.566	0.348	0.470	0.437	0.326	0.290	0.449	0.583	0.411	-	0.333	0.574	0.333	0.609
15 [f]	0.459	*0.599*	0.543	0.502	0.286	0.451	0.437	0.392	0.271	0.463	0.435	0.374	0.337	-	0.469	0.667	0.708
16 [f]	0.451	0.603	0.260	0.312	0.426	0.415	0.455	0.574	0.557	0.411	0.499	0.533	0.537	0.455	-	0.358	0.660
17 [m]	0.626	0.770	0.352	0.328	0.718	0.596	0.571	0.614	0.710	0.321	0.695	0.776	0.633	0.692	0.369	-	0.500
18 [f]	0.768	0.491	0.537	0.555	0.783	0.725	0.656	0.669	0.755	0.553	0.796	0.741	0.583	0.718	0.669	0.533	-

Table 5. Genetic distances. Above the diagonal: distances calculated with the Wetton's formula implemented in the Phyton script here developed; behind the diagonal: dissimilarity index based on Equation 1 of Lynch (1990), specific for autopolyploid species, implemented in Polysat package for R software (Clark and Jasieniuk, 2011). Threshold values which separate consanguineous from non consanguineous: 0.600 (Wetton's formula) and 0.575 (Lynch distance).

In the plot of values of MDS 1st and 2nd principal component (Fig. 3), animals cluster according to UPGMA tree, with two main groups composed by individuals belonging to family 1 (1 X 9= 6, 10, 15) plus genetically similar sturgeons (7, 8, 12, 13 and the half-sib 14) and by family 2 (16 X 17= 2, 4, 5) plus the half-sib 11. Individuals 18 and 3, which in UPGMA tree behave as an out-group, are at the margin of distribution.

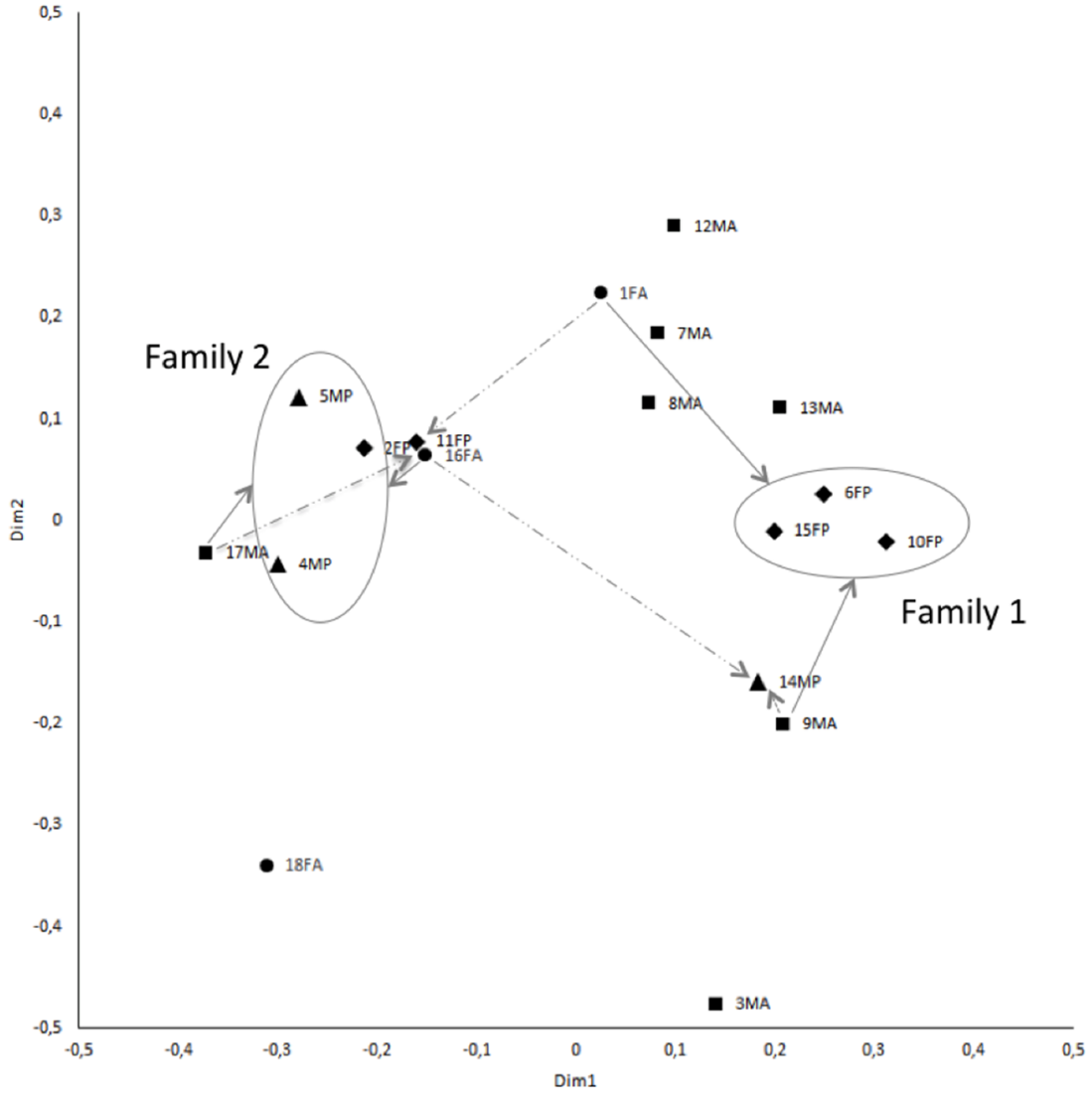


Figure 3. Plot of MDS first and second principal coordinates obtained from pairwise genetic distances. FA: female adult, FP: female pre-pubertal (i.e. sub-adults), MA: male adult, MP: male pre-pubertal (i.e. sub-adults). In the circles: the two main triplets of full sib (Family 1 and 2). Arrows connect mothers/fathers to their offspring.

Discussion

The Adriatic sturgeon *Acipenser naccarii* is classified as Critically Endangered by IUCN red list of threatened species. There are no evidences of natural reproduction since 1980 in the Italian side of its geographical distribution, and since 1990 in Albania (Bronzi*et al.*, 2013).Therefore, the reproduction and survival of the species depends almost on aquaculture and restocking practices. However, none or few of the individuals released

during the multiple restocking events that occurred in the past were then recaptured, suggesting that the Adriatic sturgeon is no longer able to reproduce in the wild and its complex life cycle has been irretrievably compromised mainly by human activity. Indeed, the only animal recaptured analyzed in this study (nr 18, Mostro), did not show good physical conditions, with a weight/size ratio to the limits of survival, index of a possible difficulty in feeding (Falahatkar*et al.*, 2014; Vaz*et al.*, 2015).

During the first attempts of artificial reproduction in the 80s, few breeders were randomly chosen paying no attention to the genetic aspects and risks of inbreeding. Therefore all the three F1 reared in the facilities of Treviso, Piacenza and Orzinuovi, showed clear signs of founder effect: a significant loss in the number of alleles per locus, an increase in average band sharing rates and a shift in the allele class and frequency distribution as regards nuclear loci analyzed, and the loss of the 50% of haplotype diversity with respect to mitochondrial markers (Ludwig *et al.*, 2003, Congiu*et al.*, 2011).

In order to allow the survival of the species and maintain the already endangered genetic variability and differentiation, it would be desirable to incorporate an adequate number of new founders as soon as possible to guarantee a proper management of residual genetic variability in the next years. In this scenario, each single new entry in the breeder stock can represent a valuable option to be tested for future crosses.

All the 18 animals involved in this study were tested for Testosterone level and genetic profiles in order to establish a multi-year crosses plan which takes into account their sex, gonadic phase and the genetic compatibility of possible partners.

Sturgeons reach sexual maturity after many years. No sexual dimorphism is found in the species and the nearly mature juveniles can reach or even overtake the length and weight

of a mature animal (sub-adults analyzed showed biometric parameters and k index overlapping those of adults). Once the sexual maturity is reached, females are ready for reproduction only every 24-36 months (Bronzi*et al.*, 2005). For this reason, it is necessary to carefully plan crosses in order to use the best animals from the genetic point of view, at their best physical and physiological conditions. A complete study by Doroshov*et al.* (1997), in *A. transmontanus* describes the trend of the reference plasma sex steroid levels in sturgeons: in males Testosterone (T) and 11-ketotestosterone (11-KT) concentrations increase following the onset of meiosis, in females androgens (T) raise at oocyte growth and estrogens (17β-estradiol, E2) during vitellogenesis, reliably assuming the different levels in T in sub-adults are connected to early sex differentiation, where males mature substantially earlier than females.

Still the results are linked to many variables such as species, captivity, handling, temperature and diet (Cataldi*et al.*, 1998; Di Marco *et al.*, 1999).

In order to preserve the overall fitness of such a rare population, a non-invasive sampling method should be used avoiding continuous handlings that would induce further stress and therefore a modification in the physiological parameters. In this study, a single blood sampling resulted to be the best solution allowing the collection both for physiological and genetic purposes.

In order to define which animals should be chosen for the current season the Testosterone levels confirmed that the female with the higher concentration of the hormone was the one that had spawned 24 months before, hence the one to be selected as a breeder for the incoming season. With reference to the adult malesa higher concentration of T is commonly known to be an index of a higher gonadal activity, therefore the breeder stock should include in this case the males with T>400ng/mL.

This fact underline that the optimal period for blood sampling seems to be at the beginning of the steroid-gonadal activity, i.e. in this latitude, in late spring (April).

With reference to the sub-adults, being 7 years old, the sexing was expected to be detected in males first. In fact among the sub-adults a high concentration in T was detected in three of them that after three months were confirmed males after a Gonadotropic Releasing Hormone (GN-RH) administrationto induce spermiation (Mylonas*et al.*, 1995). It is therefore possible to say that early sex identification is achievable in *Acipenser naccarii* through their blood Testosterone levels.

Once determined the Testosterone level of adults and sex of younger animals, the genetic profile of the 18 Adriatic sturgeons was evaluated in order to determine the best crosses possible for the maximization of the available genetic variability. Except for the year of birth and acquisition by the Province of Milannothing was known about adults and almost nothing about the sub-adults.

The 18 samples analyzed in this study were morphologically evaluated and specifically amplified at loci *LS54* and *Aox23* in order to verify if allele lengths were those of *A. naccarii* according to Chassaing*et al.*, 2011, which asserts that the Adriatic sturgeon can be distinguished from other species genotyping these loci. The observed allele classes and distribution actually fall into the specific range of *A. naccarii* and do not overlap ranges belonging to other species (data not showed).

The integrated evaluation of cluster analysis by UPGMA, paternity/kinship test and distance values, revealed that the use of unweighted pair-group method by arithmetic mean can successful identify not only full-sib groups, as already observed by Rodzen*et al.*(2004) in sturgeons, but also half-sibs. The two main clusters observed in fact are

representative of the two main families observed: the first one consisting of female 1 (Albina, mother), male 9 (father), sub-adults 6, 10, 15 (full-sibs) and 14 (half-sib); and the second one made by female 16 (mother), male 17 (father), sub-adults 2, 4, 5 (full-sibs) and 11 (half-sib). Applying this method *a posteriori* to understand kinship relations between unknown individuals, animal nr. 7/8/13 and 1/12 form groups which behave as full-sibs, since they cluster in a way very close to that sowed by the three full-sib 6/10/15, with branches and knots supported by similar values. On the basisoftree topology therefore, all the crosses, which involve pair of mates chosen inside the same cluster,are to be avoided. This result is supported by the analysis of the genetic distances (see table 5). Unfortunately the couplesrandomly chosen in 2007 for the artificial reproduction that produced the majority of newborn, belonged to the same cluster, exacerbating the already inevitable loss of variability due to the use of too few breeders. For example couple 16 X 17, which clusters together in the UPGMA tree, has a genetic distance valuewhich fall into the range observed for full-sib (0.369 by Lynch method and 0.358 by Wetton method), while other couples showed pairwise genetic distance values below or very close to threshold which separate consanguineous from non-consanguineous.

For this reason, the unplanned selectionand the random choice of breeders must be avoided, and long-term plans of crosses have to be carefully programmed by analyzing UPGMA tree topology and genetic distance values.

As regard the two distance methods proposed,it is preferable to use the Wetton's formula here implemented. Analyzing the table of male X female crosses in fact,Wetton distance appears to be more restrictive in the setting of the threshold which separates consanguineous from non-consanguineous (0.575 according to Lynch and 0.6 according to

Wetton), allowing 15 possible crosses above threshold over the 18 foundwith Lynch's method (table 6).

	ID NR	3	4	5	7	8	9	12	13	14	17
WETTON	1	**0.778**	0.543	0.462	0.480	0.447	0.551	0.333	0.490	0.545	0.577
	2	**0.667**	0.429	0.434	0.451	0.542	**0.640**	0.462	0.538	0.600*	0.434
	6	0.556	***0.600***	0.569	0.469	0.348	0.347	0.440	0.440	0.349	**0.686**
	10	0.529	**0.697**	0.569	0.429	0.435	0.293	0.480	0.360	0.302	**0.686**
	11	**0.771**	0.294	0.308	0.520	0.447	0.551	0.490	0.490	0.409	0.434
	15	0.563	0.576	0.500	0.455	0.429	0.404	0.478	0.391	0.333	**0.667**
	18	0.550	0.526	0.536	**0.741**	**0.647**	**0.686**	**0.782**	**0.745**	**0.609**	0.500
LYNCH	1	**0.814**	0.538	0.523	0.493	0.480	0.545	0.340	0.493	0.533	**0.626**
	2	**0.699**	0.371	0.381	0.465	0.548	**0.627**	0.481	0.558	0.575*	0.411
	6	*0.582*	**0.609**	**0.603**	0.457	0.361	0.281	0.433	0.430	0.348	**0.718**
	10	0.532	**0.633**	***0.575***	0.420	0.423	0.271	0.500	0.391	0.290	**0.710**
	11	**0.804**	0.290	0.283	0.549	0.520	0.564	0.510	0.540	0.449	0.321
	15	***0.599***	0.543	0.502	0.451	0.437	0.392	0.435	0.374	0.337	**0.692**
	18	0.491	0.537	0.555	**0.725**	**0.656**	**0.669**	**0.796**	**0.741**	**0.583**	0.533

Table 6. Plan of future possible crosses, males in columns and females in rows. In bold: best possible crosses (maximum genetic distance between the two possible parents, value above the threshold value indicated by the star).

Analyzing the UPGMA tree topology and MDS plot,a third group formed by individual nr. 3 and 18 was observed. The genetic profile of the latter was very interesting: it certainly belongs to *A. naccarii* species (genotyping of loci LS54, Aox23 and D-Loop data analysis, not showed), but it showed a particular genetic profile with four private alleles. Although the evidence of a genotype quite different from all other sturgeon analyzed in this paper, at present it is not possible to understand if this animal represent a wild one or a restocked F1 and further analyses are requested. However, it represents a very interesting

new resource for the breeder stock: this female can be coupled with six out of ten possible males of the breeders stock to maximize residual genetic variability. On the contrary,the differences showed by animal nr. 3 (its position both in UPGMA tree and MDS plot) are probably to be ascribed to the incomplete genotyping of this animal due to technical problems.

With the present study a comprehensive and integrated method based on physiological and genetic data, allows a long term breeding plan, avoiding a less effective plan based only on genetic data with a year-by-year management of the available breeders.

Conclusion

(i) This study represents the first attempt to identify sex in Adriatic sturgeon by analyzingtestosterone plasma levels: up to now, no studies were available on *Acipenser naccarii*.

(ii)We have identified and genotyped 18 new potential breeders never analyzed before, that can be added to Adriatic sturgeon broodstock, which species survival depends at the moment on aquaculture practices and ex situ management of few known individuals.

(iii) A new valuable animalof unknown origin was found, with a genetic profile quite different from all other individual here sampled. This female can be used in six out of ten crosses with males of the breeder park, avoiding risk of consanguinity.

(iv) Based on theanalysis of genetic profile, at least 15 new couples were identified to be paired during next yearsmaximizing residual genetic variability

(v) The integrated analysis of genetic and physiological data could be the best solution possible in order to maximize the residual genetic variability, avoiding stress for the animals. Many results in fact can be obtained by a single blood sampling providing an effective and cost-effective method for aquaculture management.

Acknowledgements

The authors are very grateful to Dr Alessandro Grossi, responsible of Centro IttiogenicoCittàMetropolitana di Milano (Milan, Italy) for his help and for supplying the sturgeons, to Dr Alex Lucchi for his help with BioNumerics software, to Dr Giulia Pastore and Chiara Lattanzi for their help in sample preparation and finally to EngDamianoBarboni who developed the Phyton script here proposed.Supporting Grant: *Provincia di Ravenna (Italy) - BENATUR Project.*

References

Barannikova, I.A., BayunovaL.V. &SemenkovaT.B. (2004) Serum levels of testosterone, 11-ketotestosterone and oestradiol-17β in three species of sturgeon during gonadal development and final maturation induced by hormonal treatment. *Journal of Fish Biology***64**, 1330–1338.

Boscari E., Barbisan F. &CongiuL. (2011) Inheritance pattern of microsatellite loci in thepolyploid Adriatic sturgeon (*Acipensernaccarii*). *Aquaculture***321**, 223-229.

Boscari E., Pujolar J.M., DupanloupI., Corradin R. &CongiuL. (2014) Captive Breeding Programs Based on Family Groups in Polyploid Sturgeons. *PLoSONE***9(10)**, e110951.doi:10.1371/journal.pone.0110951.

Bronzi P., Vecsei P. &ArlatiG. (2005) Threatened fishes of the world: *Acipensernaccarii* Bonaparte, 1836 (Acipenseridae). *Environmental Biology of Fishes***72(1)**, 66-66.

Bronzi P., CongiuL., RossiR., Zerunian S. &ArlatiG. (2013)*Acipensernaccarii*. The IUCN Red List of Threatened Species 2013: e.T224A13037056. (http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T224A13037056.en.)

Cataldi E., Di MarcoP., Mandich A.&CataudellaS. (1998) Serum parameters of Adriatic sturgeon *Acipensernaccarii* (Pisces: Acipenseriformes): effects of temperature and stress. *Comparative Biochemistry and Physiology part A***121**, 351–354.

Chassaing O., Hanni C. &BerrebiP. (2011)Distinguishing species of European sturgeons *Acipenser*spp. using microsatellite allele sequences. *Journal of Fish Biology***78**: 208–226.

Clark L.V. &JasieniukM. (2011)Polysat: an R package for polyploid microsatellite analysis. *Molecular Ecolology Resources***11(3)**, 562-566.

Congiu L., PujolarJ.M., ForlaniA., CenadelliS., DupanloupI., BarbisanF., Galli A.& FontanaF. (2011) Managing Polyploidy in Ex Situ Conservation Genetics: The Case of the Critically Endangered Adriatic Sturgeon (*Acipensernaccarii*). *PlosONE***6 (3)**, e18249.

Di Marco P., McKenzieD.J., MandichA., BronziP., Cataldi E.&CataudellaS. (1999) Influence of sampling conditions on blood chemistry values of Adriatic sturgeon *Acipensernaccarii* (Bonaparte, 1836). Journal of Applied Ichthyology**15(4-5)**, 73–77.

Doroshov S.I., MobergG.P.&Van EenennaamJ.P. (1997) Observations on the reproductive cycle of cultured white sturgeon (*Acipensertransmontanus* Richardson). *Environmental Biology of Fishes***48**, 265-278.

Doukakis P., BirsteinV.J., DeSalleR., LudwigA.N., LudwigA., MachordomA., Almodóvar A.& ElviraB. (2000) Failure to confirm previous identification of two putative museum specimens of the Atlantic sturgeon, *Acipensersturio*, as the Adriatic sturgeon, *A. naccarii. Marine Biology***136(2)**, 373-377.

Falahatkar B. &ImanpourJ. (2014) The relationship between morphological and sexual indices in suitable brood Russian sturgeon *Acipensergueldenstaedtii* (Brandt andRatzeburg, 1833). *International Journal of Aquatic Biology***2(4)**, 193-200.

Feist G., Van EenennaamJ.P., DoroshovS.I., SchreckC.B., Schneider R.P.&FitzpatrickM.S. (2004) Early identification of sex in cultured white sturgeon, *Acipensertransmontanus*, using plasma steroid levels. *Aquaculture***232**, 581-590.

Forlani A., Fontana F. &CongiuL. (2008) Isolation of microsatellite loci from the endemic and endangered Adriatic sturgeon (*Acipensernaccarii*). *Conservation Genetics***9**, 461–463.

Gaiani R., ChiesaF., MattioliM., Nannetti G.&GaleatiG. (1984)Androstenedioneand testosterone concentrations in plasma and milk of the cow throughout pregnancy. *Journal of Reproduction Fertility***70**, 55–59.

Galli A., GreppiG., Boscari E. &BonacinaC. (2011) Parental allocation in *Acipensernaccarii* – statistical aspects and software development. *Journal of Applied Ichthyology***27**, 651–654.

Garrido-Ramos M.A., RoblesF., de la HerránR., Martínez-EspínE., LorenteJ.A., Ruiz-Rejón C.& Ruiz-RejónM. (2009) Analysis of Mitochondrial and Nuclear DNA Markers in Old Museum Sturgeons Yield Insights About the Species Existing in Western Europe: *A. sturio*, *A. naccarii*and*A. oxyrinchus* in Biology, Conservation and Sustainable Development of Sturgeons. *Fish and Fishery***29**, 25-49.

Grandi G. &Chicca M. (2008) Histological andultrastructural investigation of early gonad development and sex differentiation in Adriatic sturgeon (*Acipensernaccarii*, Acipenseriformes, Chondrostei). *Journal of Morphology***269(10)**, 1238-62.

Henderson-Arzapalo A. & KingT.L. (2002)Novel microsatellite markers for Atlantic sturgeon (*Acipenseroxyrinchus*) population delineation andbroodstock management. *Molecular Ecology Notes***2**, 437–439.

Keyvanshokooh S. &GharaeiA. (2010) A review of sex determination and searches for sex-specific markers in sturgeon. *Aquaculture Research***41**, e1-e7.

Ludwig A., CongiuL., PitraC., FickelJ., GessnerJ., FontanaF., Patarnello T. & ZaneL. (2003)Nonconcordant evolutionary history of maternal and paternal lineages in Adriatic sturgeon. *Molecular Ecology***12**, 3253–3264.

Lynch M.(1990)The Similarity Index and DNA Fingerprinting. *Molecular Biology and Evolution***7(5)**, 478-484.

McQuown E.C., SlossB.L., SheehanR.J., RodzenJ., Tranah G. & MayB. (2000)Microsatellite Analysis of Genetic Variation in Sturgeon: New Primer Sequences for *Scaphirhynchus*and*Acipenser. Transactions of the American Fisheries Society***129**, 1380–1388.

Mylonas C.C., TabataY., Langer R. & ZoharY. (1995) Preparation and evaluation of polyanhydride microspheres containing gonadotropin-releasing hormone (GnRH), for inducing ovulation andspermiation in fish. *Journal of Controlled Release*, 35: 23-34.

Parmeggiani A., GovoniN., ZannoniA., Di BiaseA., SirriR., ForniM., Mandelli M.&MordentiO. (2015) Effect of photoperiod on endocrine profiles andvitellogenin expression in European eels *Anguilla anguilla* during artificially induced ovarian development. *Theriogenology***83(4)**, 478-484.

Robles F., de la HerranR., LudwigA., RejonC.R., RejonM.R. &Garrido-RamosM.A.

(2004) Evolution of ancient satellite DNAs in sturgeon genomes. *Gene***338,**133–142.

Rodzen J.A., FamulaT.R. &MayB. (2004) Estimation of parentage andrelatedness in the polyploid white sturgeon (*Acipensertransmontanus*) using a dominant marker approach for duplicated microsatellite loci. *Aquaculture***232**, 165–182.

Roncarati A., MordentiO., Stocchi L. &MelottiP. (2014) Comparison of growth performance of common catfish *Ameiurusmelas*, Rafinesque1820, reared in pond and in recirculating aquaculture system. *Journal of Aquaculture Researh& Development***5**, 218 doi:10.4172/2155-9546.1000218.

StataCorp(2009)Stata Statistical Software: Release 11. College Station, TX: StataCorp LP.

Vaz P.G., KebreabE., HungS.S.O., FadelJ.G., Lee S. &FangueN.A. (2015) Impact of Nutrition and Salinity Changes on Biological Performances of Green and White Sturgeon. Fine ML, ed. *PLoS ONE***10(4)**,e0122029. doi:10.1371/journal.pone.0122029.

Wetton J.H., CarterR.E., ParkinD.T. & WaltersD. (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature***327**, 147-152.

Zane L., PatarnelloT., LudwigA., Fontana F. &CongiuL. (2002)Isolation and characterization of microsatellites in the Adriatic sturgeon (*Acipensernaccarii*). *Molecular Ecology Notes***2**, 586–588.

CHAPTER 3

Effect of photoperiod on endocrine profiles and vitellogenin expression in European eels during artificially induced ovarian development

Co-authored by:

Parmeggiani Albamaria, Govoni Nadia, Zannoni Annalisa, Sirri Rubina, Forni Monica, Andrea Di Biase and Mordenti Oliviero.

Printed in:

Theriogenology, Volume 83, Issue 4, Pages 478–484

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 (vitello- genin 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 dark- ness (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 differ- ences between the two housing conditions while in both groups vitellogenin mRNA in- creased 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 start- ing 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 pop- ulation. 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 indi- ces, 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 aquacul- ture 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 sil- vering 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 reproductive 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 cap- tivity. Therefore the effects of light and dark conditions were investigated on the E2, testos- terone 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 Euro- pean 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 natu- ral seawater (salinity ranged between 28‰ to 32‰, water temperature 14±2°C, photoperi- od 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 histol- ogy 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 un- der 14h light conditions (14L:10D) (0.40*10^3 lux at the bottom of the aquarium without wa- ter) 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 sam- ples 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 col- lected, 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 ex-tracted 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 antise- rum 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%), pro-gesterone and cortisol (<0.0001%). The results were expressed as ng/mL. In order to de- termine 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 triiodo- thyronine (T3) were determined using an commercial RIA kit (Institute of Isotopes, Buda- pest, Hungary); the sensitivity of the assay were 6.8 nmol/L and 0.3 nmol/L and the in- traassay 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 hor- mones 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 manu- facturer'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 treat- ed with DNAse free RNA kit (Zymoresearch, Orange, CA, USA) according to manufactur- er'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 Interna- tional, 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 amplification 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 $2^{\cdot\cdot CT}$ 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 Rev.: ATAGAGAGACAGCCCATCAC	117	EU073127
vtg2	For.: GATGCTCCCCTAAAGTTTGTG Rev.: AGCGTCCAGAATCCAATGTC	123	EU073128.1
esr1	For.: TCGCTTGGGCTAAGAAAGTAC Rev.: CCGATTATCAGCACCTCCAG	97	EU073125
B-actin	For.:AGCCTTCCTTCCTGGGTATG 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 eval- uated 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 differ- ences 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}	$14.4\pm0.2^{*D}$

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 in- crease 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.

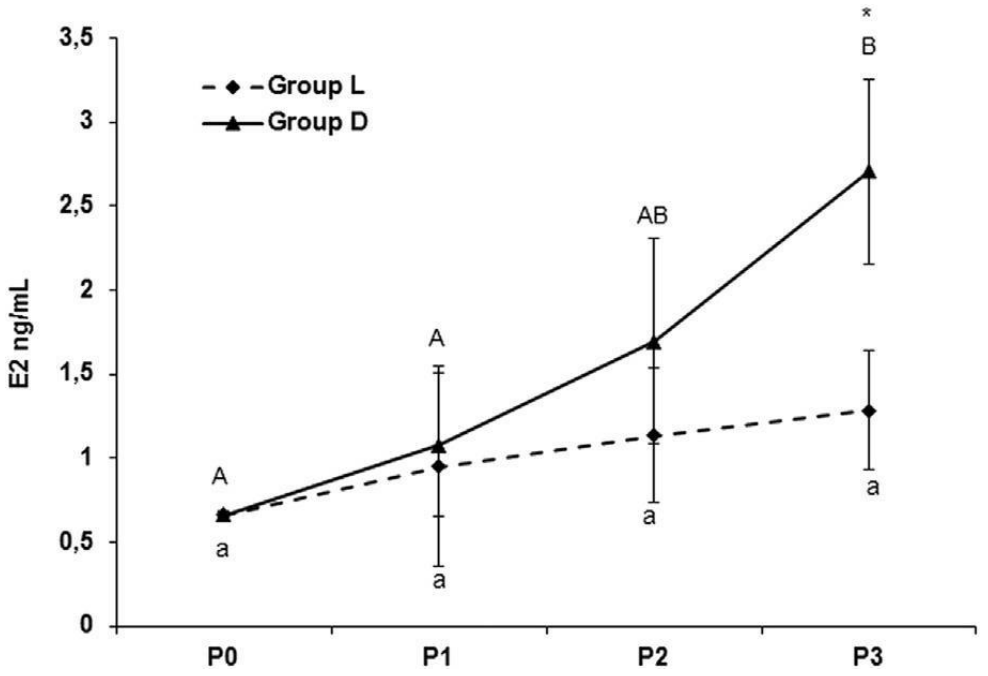


Figure 25: Plasmatic concentration of 17-βestradiol (E2) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capi- tal letters indicate statistically significant differences (P<0.05, ANOVA post hoc Tukey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

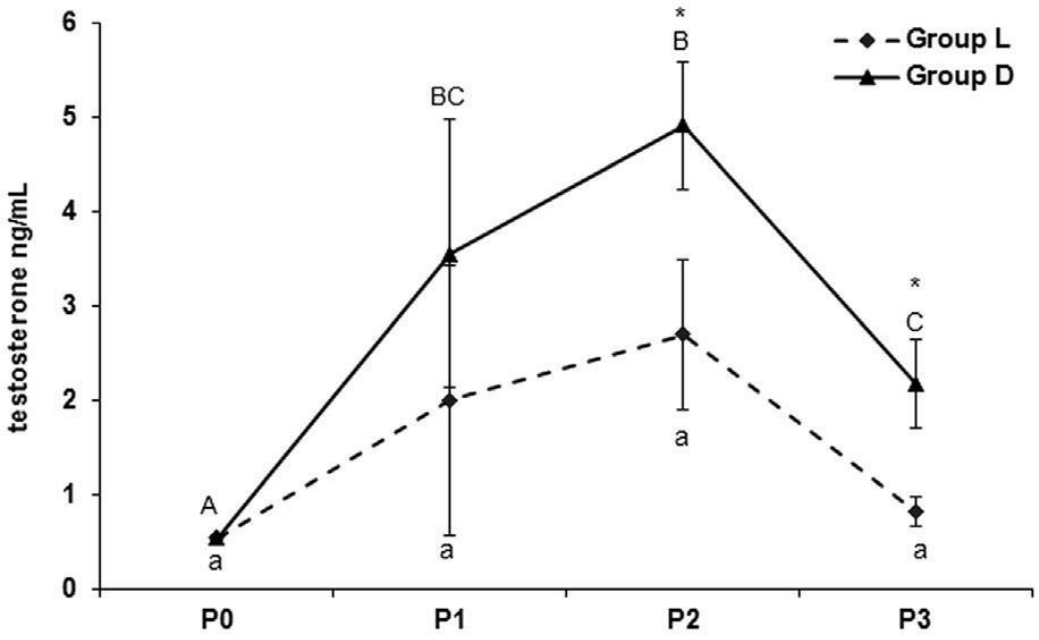


Figure 26: Plasmatic concentration of testosterone (T) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capital letters indicate statistically significant differences (P<0.05, ANOVA post hoc Tukey test)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

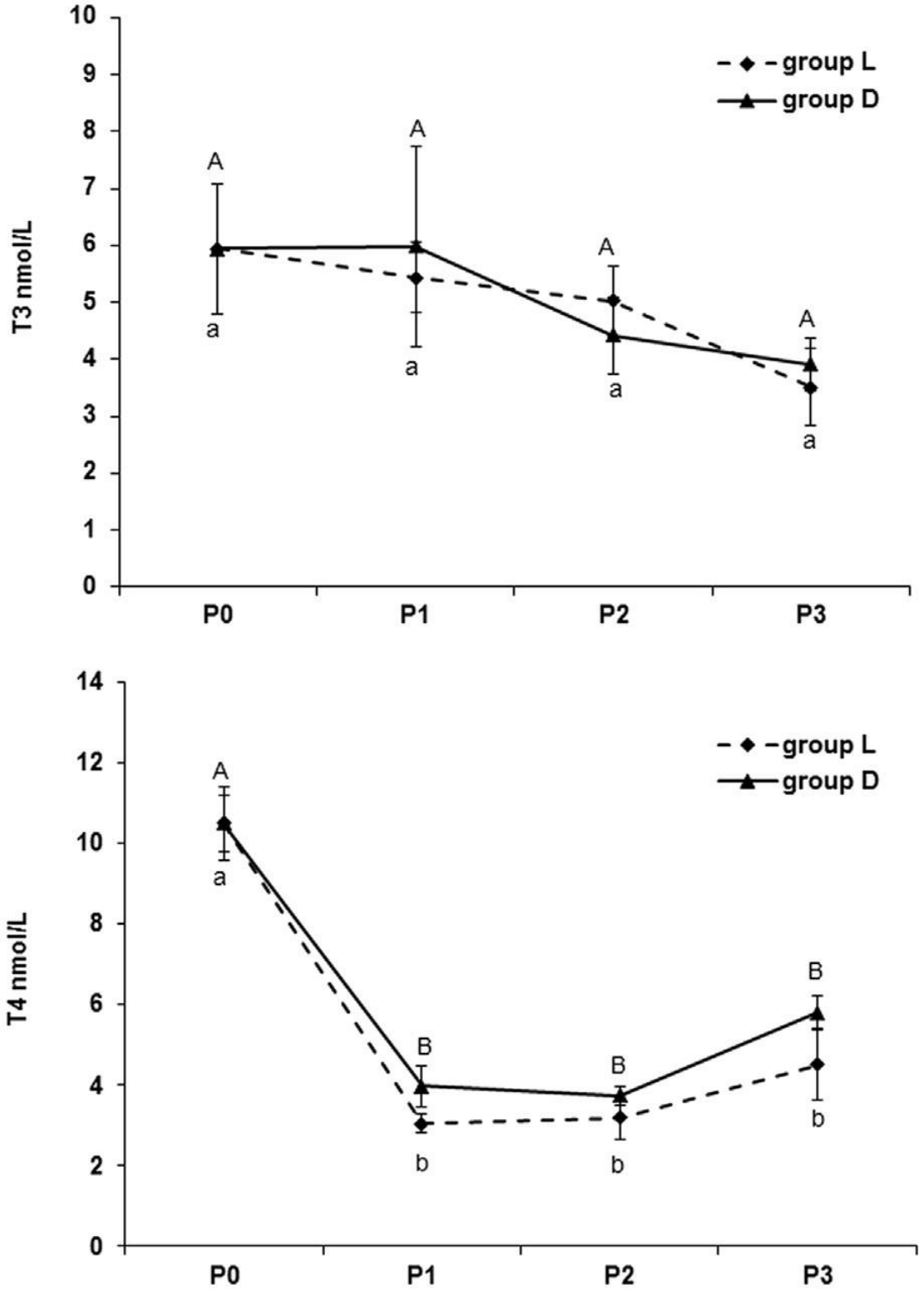


Figure 27:Plasmatic concentration of total Triiodothyronine(T3) and Thyroxine (T4) in European eels before (P0) and after (P1,P2,P3) CPE treatments in different light conditions. Data are presented as mean±SEM. Different lowercase or capital letters indicate statistically signif- icant differences ($P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

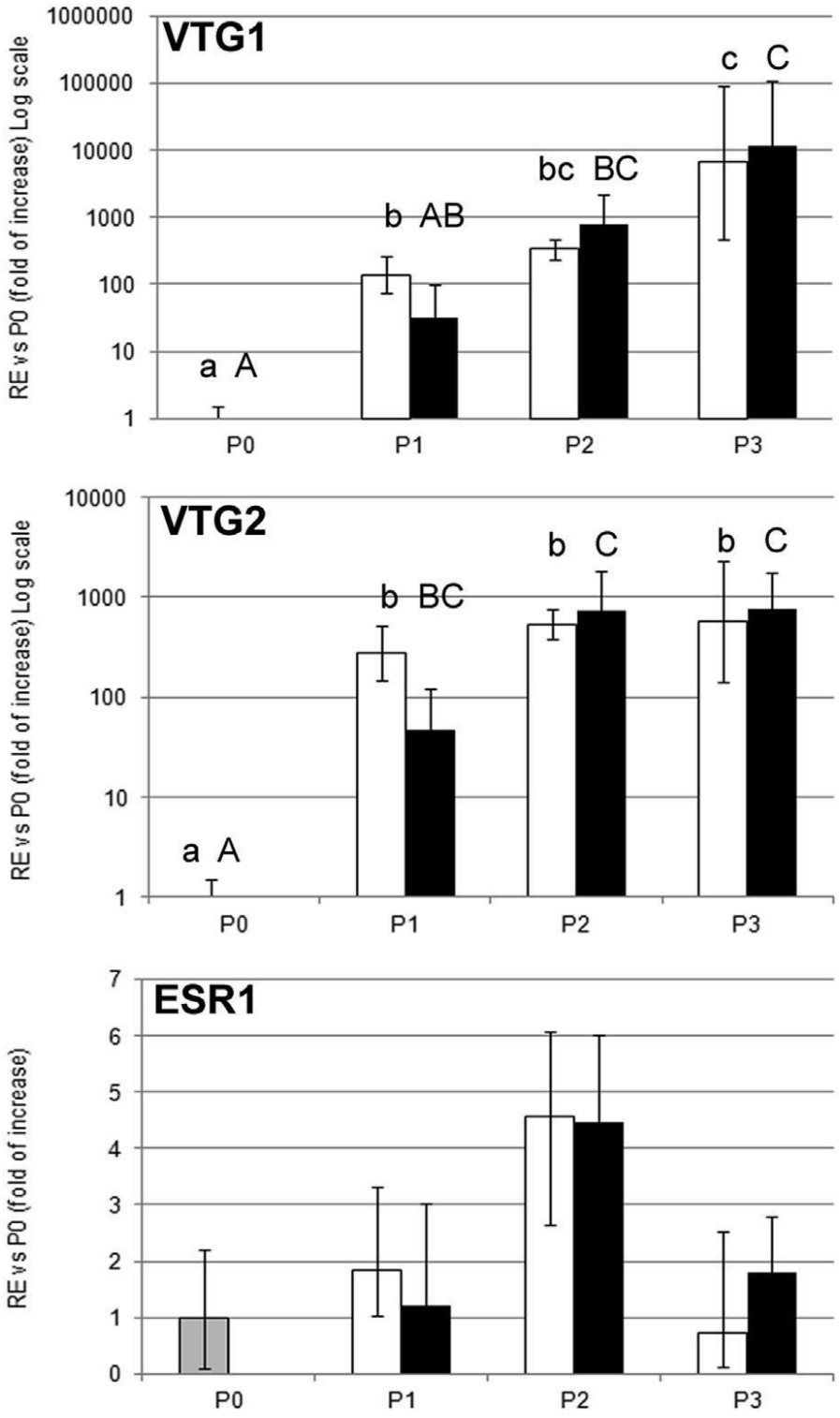


Figure 28: Relative gene expression (RE) of vitellogenin 1 (vtg1), vitellogenin 2 (vtg2) and estrogen receptor 1 (esr1) in liver samples after CPE treatments (P1, P2, P3) in different light condition (L and D groups) and in control group (P0). mRNA data are presented as fold of increase (2‥CT method) 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 injec- tion 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 in- jections (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 5thweek (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 en- tirely filled the cytoplasm, densely stained yolk globules were more abundant and the nu- cleus 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.

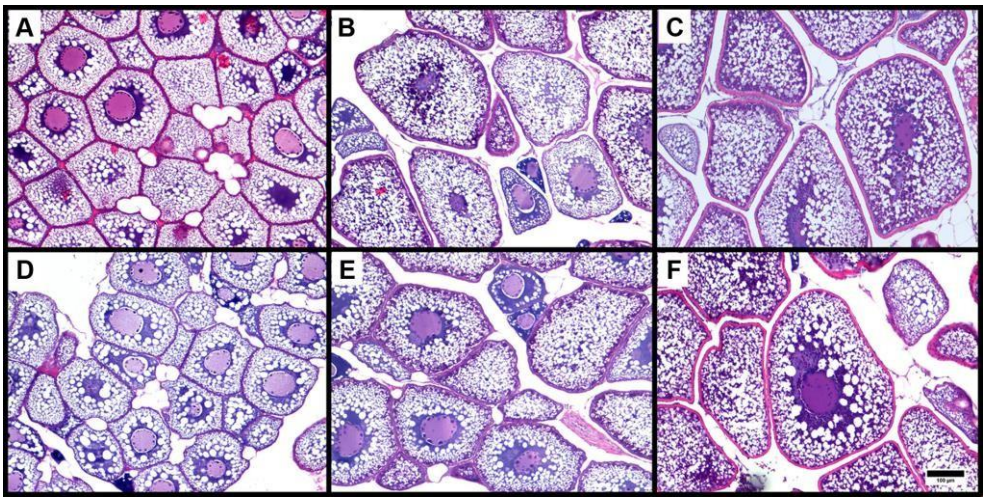


Figure 29: Representative histological of gonad samples

(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 steroidogen- ic activity, thyroid hormones and vitellogenin gene expression were observed in female European silver eels (*Anguilla anguilla*) treated with increasing doses of carp pituitary ex- tracts. 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 sup- porting 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 in- crease in E2 and testosterone plasma levels in eels stimulated with pituitary extracts at dif- ferent 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 forgonadal 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 in- to 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: es- trogen 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 administra- tion 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 injec- tions 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 gonad- al 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 13th 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 (5th 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 4th 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 al- ready 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 sys- tem 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 perfor- mances 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 demon- strate 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.

Acknowledgements

This study was supported by the Emilia Romagna Region and by RFO grant University of Bologna.

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 Nu- trition 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. *Aquacul- ture*, 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 endo- crine 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 im- mature rainbow trout, *Salmogairdneri*. *General and Comparative Endocrinology*, 69:431-438.

Dou S.Z., Yamada Y., Okamura A., Shinoda A., Tanaka S. & Tsukamoto K. (2008) Tem-perature influence on the spawning performance of artificially-matured Japanese eel *An-guilla 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 clas- sification 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 con- dition 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 Biolo- gy*, 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 mor- phology, 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 migrato- ry 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 vitello-genesis 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 induc- tion 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: de- velopment 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 (*An- guilla 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 vi- tellogenesis during experimental maturation of European eel (*Anguilla anguilla*) fe- males.*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 im- munostaining methods. *Journal Experimental Zoology*, 290:366-378.

Rodriguez A., Castello-Orvay F. & Gisbert E. (2009) Somatic growth, survival, feed utiliza- tion and starvation in European elver *Anguilla anguilla* (Linnaeus) under two different pho- toperiods. *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 sec- ond reproductive cycle; relation with their photoperiodically altered spawning time. *Aquaculture*, 99:365-385.

CHAPTER 4

A closed recirculating system for artificiaò seed production of the European eel: Technology development for spontaneous spaening and egg incubation

Co-authored by:

Mordenti Oliviero, Casalini Antonio and Andrea Di Biase

Printed in:

Aquacultural Engineering (2014) vol. 58, pp. 88-94

Abstract

The objective of the present study was to obtain spontaneous reproduction in cap- tivity of the European eel (*Anguilla anguilla*) by using a new closed recirculating aquacul- ture 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 recirculat- ing 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 stand- ardized 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 cap- tivity of the European eel (*Anguilla anguilla*) by using wild female silver eels. For this pur- pose a new closed recirculating aquaculture system provided with spawning and incuba- tion chambers was tested.

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

Materials and methods

Animals

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 re-production. 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 x103 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 tem- perature system was set at 15.5±0.5°C.

The females received intramuscular injections once a week with carp pituitary ex- tracts (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 re-ceived 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 (0.47m3/tank; water volume 0.43m3/tank; water surface area 0.93m2/tank), a foam separation tank (0.05m3) (protein skimmer) and an biological filter (0.21m3) contained plastic porous balls (0.15m3) (Fig. 31). The total amount of water in this system was 1.12m3 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 rear- ing 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 O3/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 compo- nents: one spawning chamber (240 L), two incubation chambers (52 L/cad) and one outlet chamber (90 L).

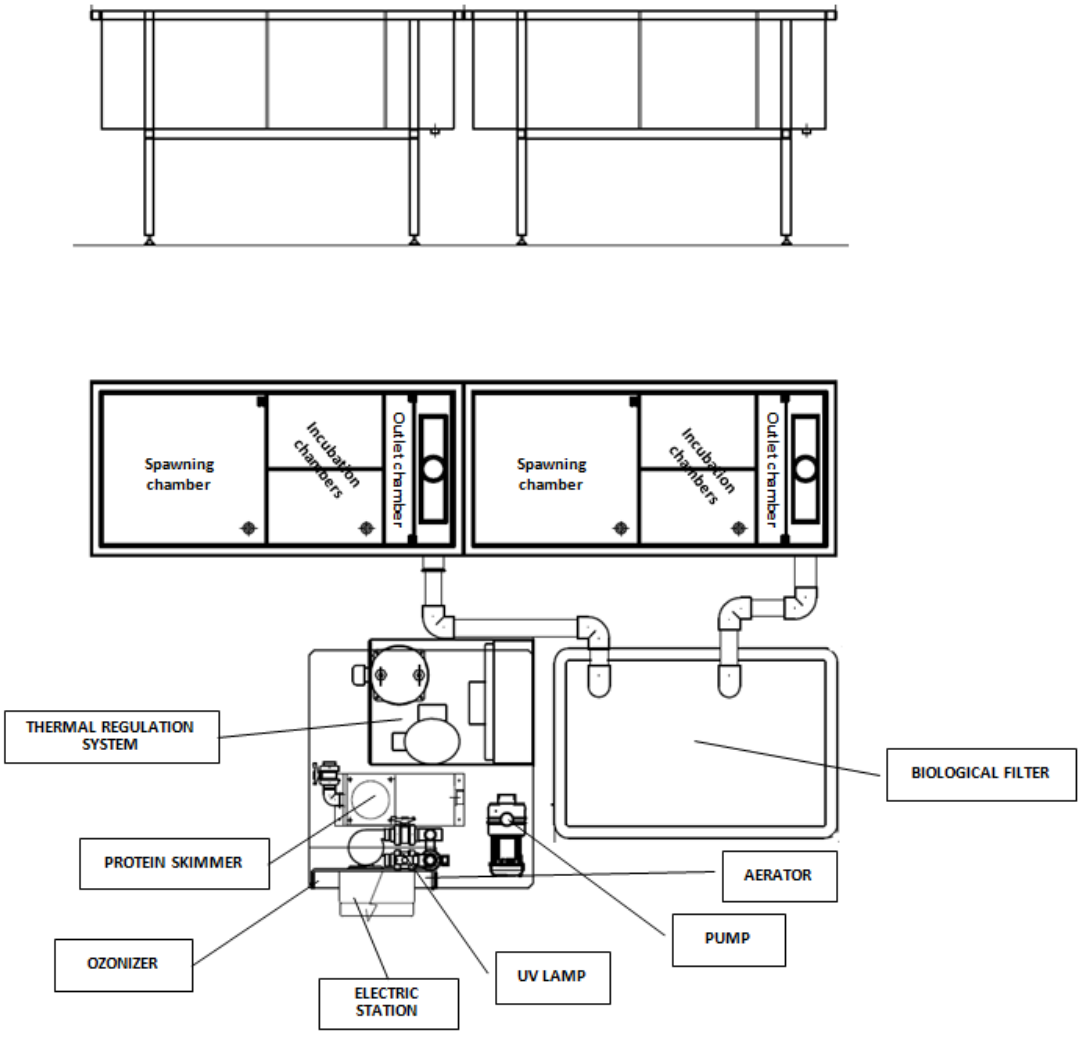
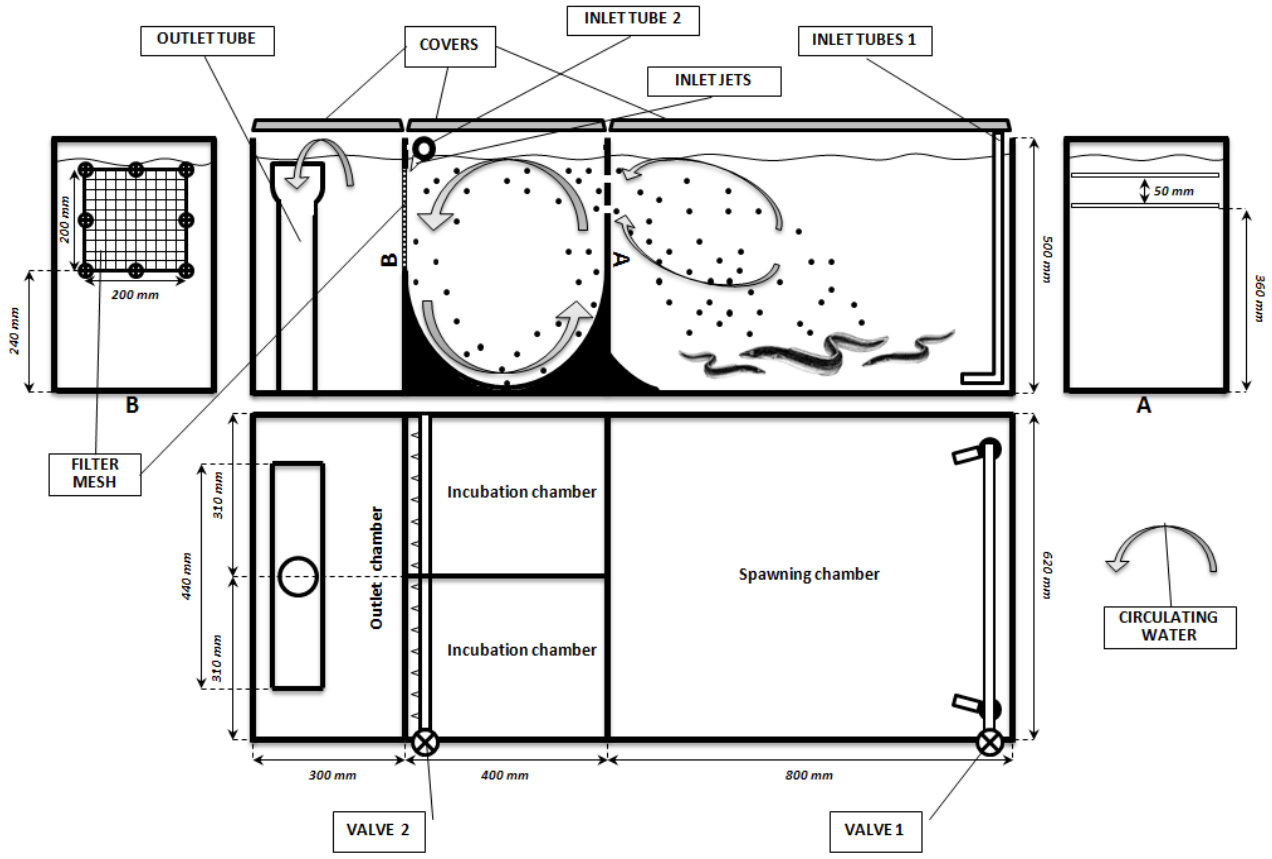


Fig.31



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 wa- ter 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 divid- ing 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 hatch- ery water pipe valve (valve 2) (Fig. 32).

The system described has a vertical configuration and is inspired by the one origi- nally developed by Greve in 1975 (called "planktonkreisel") for the maintenance of plank- tonic 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 adjust- ing 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 condi- tions 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 ad- justing 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 stock- ing 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 cal- culation of the eggs a quantity of 1680 eggs/gr of ovarian biomass was considered (Mor- denti *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 calcu-

lated by monitoring the animals from the 8th 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 con- cerning 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 spawn- ing 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 cham- ber, with the exception of very small amounts that might be due to the leakage of the fe- male

genital orifice during removal. Egg return from the incubation to the spawning cham- ber 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 statistical- ly 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 differ- ences 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 fer- tile 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**
				OW-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; My- lonas *et al*. 2010). However, other factors as tank size, water volume and/or depth, hydro- dynamic conditions, water flow rate and stocking density were shown to

influence repro- ductive 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 ma- nipulation 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 de- terioration 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 fertiliza- tion. 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 repro- duction 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 emis- sion of gametes by the breeders. The designed system, in short, seems to meet all the re- quirements for a spontaneous reproduction of the European eel, thus eliminating all the is- sues 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; Peder- sen 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 artifi- cial 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 in- cubated 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 oth- er freshwater eel species (e.g. *Anguilla japonica* and *Anguilla australis*),

as the major diffi- culties 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 mechani- cal 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 in- creasing 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 observa- tions led in this study. At the moment, in fact, the bibliography does not provide precise in- formation about stocking density of incubated eel eggs and then it would be better to re- main 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 hap- pens

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 posi- tioned 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 stor- age 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.

Acknowledgements

This study was supported by Emilia-Romagna Region, Italy. Authors would like to thank INNOVAQUA srl (Reggio Emilia, Italy) for technical support.

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 ja- ponica* upon the quality of eggs and larvae (Comparison between stripping insemination and spontaneous spawning methods). *Nippon Suisian Gakkaishi,* 74(2):26-35 (in Japa- nese, 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.

CHAPTER 5

Controlled reproduction in *Anguilla anguilla*: comparison between spontaneous spawning and strippinginsemination approaches

Co-authored by:

Di Biase Andrea, Casalini Antonio, Emmanuele Pietro, Lokman P. Mark and Mordenti Oliviero

Printed in:

Aquaculture Research (2015) 1-9

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 col- lected in estuarine waters. However, over the last several decades, natural stocks of eels, especially those representing the commercially valuable temperate species such as Euro- pean 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 (Mor- denti, Di Biase, Sirri, Modugno & Tasselli 2012). One effective way to preserve these spe- cies 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 & Tsuka- moto 2013).

With reference to the European eel, research has focused on successful artificial prop- agation 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., Hat- anaka, 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 Thillart 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 - Ita- ly). 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 com- plete. This system was equipped with a foam separation tank (protein skimmer) and a bio- logical filter containing plastic porous balls. The tank was also provided with a thermal reg- ulation system, a UV-sterilizer lamp, an ozonizer and an aerator (electromagnetic air com- pressor) to adjust the rearing water conditions.

Induction of maturation in female eels

At the experimental premises, the female eels were measured and sampled to ob- tain 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 opti- mized 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 trans- parent, displaying their nucleus at the periphery and containing few large fat droplets (di- ameter 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 corre- sponded 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:

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

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; Pal- stra *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 injec- tion, each female of the *SPT-group* was transferred to a new closed recirculating aquacul- ture

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 col- lected 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 polyethylene 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 calcu- lated 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 ferti- lized, 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 concern- ing 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 signif- icant.

Results

The internal and external indicators of maturation state of the wild eels at the begin- ning 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); moreo- ver, 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).

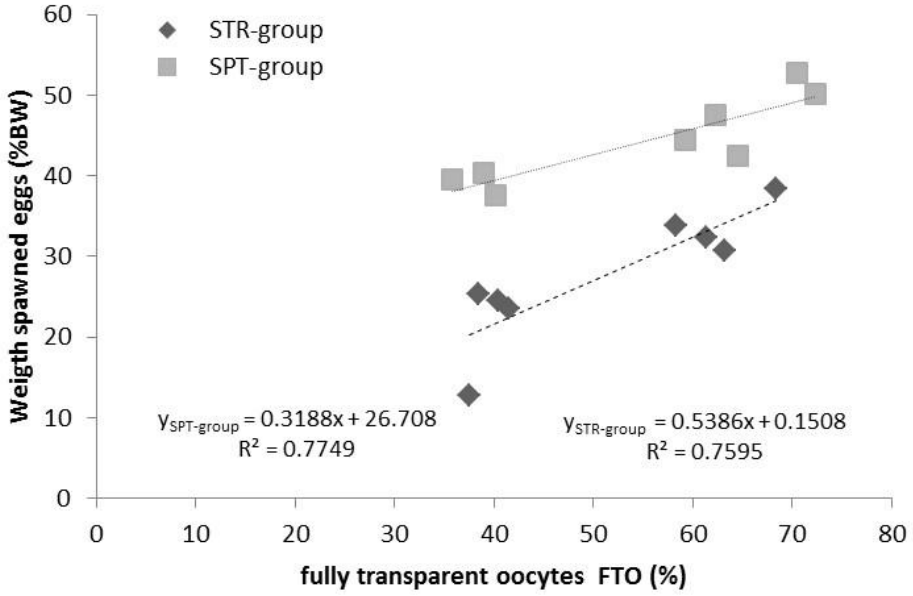


Fig. 30: relationship between the percentage of fully transparent oocytes (% FTO) and the weight of spawned eggs (%BW) from female European eels that spawned spontaneously (SPT) or that were spawned by stripping (STR).

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 (Mor- denti *et al*. 2014). Although pituitary extract-induced resumption of oogenesis in captive silver eels can advance oocyte development to the migratory nucleus stage, ovulation andfinal maturation are routinely induced by DHP. However, the relationship between the tim- ing 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 ma- tured European eels (*Anguilla anguilla*) whose ovarian follicles had varying % of fully hy- drated oocytes and evaluated the resulting fertility after spontaneous spawning or in re- sponse 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 in- crease (BWI) at DHP injection was highly variable (from 108% to 134%) and it did not ap- pear 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 administra- tion following regular weekly injections) showed good synchronous development of oocytes, evidenced by 50% of oocytes being fully transparent. The stage synchrony of oo- cytes 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 10th 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 quali- ty eggs than what can be obtained by the stripping-insemination method, probably be- cause 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 Euro- pean eels in captivity was striking. The presence of good quality males in the tank is a de- cisive 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, Onder- water, 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 ap- proached 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 meth- od 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.

Acknowledgements

This study was supported by Emilia-Romagna Region, Italy. Authors would like to thank INNOVAQUA srl (Reggio Emilia, Italy) for technical support. No conflict of interest has been identified.

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, *An-*

guilla 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 develop ment 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.