Co-treatment with androgens during artificial induction of maturation in female eel, Anguilla anguilla: effects on egg production and early development

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Co-treatment with androgens during artificial induction of maturation in female eel, *Anguilla anguilla*: effects on egg production and early development

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Running title: Effects of 17-MT on fertilization and hatching rates in *A. anguilla*

Abstract

Inclusion of sustained-release androgen implants into fertility treatment regimes of female freshwater eels has been proposed to benefit artificial maturation outcomes. This study sought to test whether co-administration of the androgen 17-methyltestosterone (17-MT) in combination with 'traditional' hypophysation of female European silver eels would affect egg quality and subsequent larval survival. Implants, containing 1 mg 17-methyltestosterone (17α-methyl-4-androsten-17β-ol-3-one in a matrix of cholesterol : cellulose = 95 : 5), were administered twice, at 1 and 5 weeks after onset of experimentation (MTA- and MTB-groups); control eels (Ctrl) were given a blank implant. Female eels received weekly injections with carp pituitary homogenates (CPH) from either Week 1 (MTA- and Ctrl groups) or Week 5 onwards (MTB-group). First 4 injections were made with 10 mg CPH / kg body weight (BW) in sterile physiological saline (0.9% NaCl); the subsequent 12 injections with 30 mg/kg CPH and thereafter, 40 mg/kg CPH was injected until fish were considered ready for induction of final maturation. Bi-weekly blood samples were collected (Day 0 onwards) and analyzed for levels of testosterone (T) and estradiol-17β (E2) by radioimmunassay. After reaching maturity, fertilized eggs were obtained upon spontaneous spawning of co-housed breeders and egg performance (fertilization, hatching, survival rates) was recorded. No differences were observed between eels from the MTA- and MTB-groups; however, egg performance was notably better in eels treated with 17-MT + CPH compared to eels receiving CPH only, with hatching rates reaching mean values as high as around 70% for both 17-MT and CPH co-treated groups, but only averaging 21.0 ± 8.4% for eggs from the Ctrl-group. Furthermore, 17-MT treated eels displayed a more distinct pre-spawning peak for levels of plasma T

and E2 and for body weight, the latter being indicative of increased batch fecundity. We conclude that androgen co-treatment holds promise for future development of eel captive breeding technologies.

Statement of relevance: Artificial propagation of the European eel is increasingly urgent to release the strain on natural populations. This study presents a novel approach to artificial maturation of female eels: centered around the incorporation of androgens during fertility treatment. Accordingly, increased performance was observed in terms of fertilization, hatching and survival rates, which contributes to completing the life cycle of this fish in captivity.

Keywords: *Anguilla anguilla*; induced maturation; 17-methyltestosterone; androgen; fertilization; egg quality

Highlights

- Large quantities of larvae from European eels were obtained after fertility treatment, spontaneous spawning by breeders and optimized husbandry
- Inclusion of 17-methyltestosterone (17-MT) in the fertility treatment regime enhanced batch fecundity, egg quality and larval survival up to 15 dph
- Inclusion of 17-MT in the fertility treatment regime reduced the time required to reach the pre-spawning time and greatly improved cost-efficacy

1. Introduction

Anguillid eels are a highly valued commodity in several regions on the planet. Their value is reflected in a global fishery for eels of a suite of sizes, used either for consumption or as stocking material in eel farming industries, chiefly in Europe and East Asia. Worldwide, eel stocks seem to be dwindling rapidly, due to several reasons that include the fishing industry, habitat destruction (e.g., the construction of dams that interfere with migration patterns), global warming, pollution or other factors (reviewed by Hanel et al., 2014). Thus, whilst early experiments aimed at artificially propagating the eel seem to have been prompted largely by scientific curiosity. Contemporary research partly reflects the urgent need to complete the life cycle to prevent these enigmatic animals from becoming extinct. Indeed, the need for protection of eels and a greater transparency of catch and trade volumes was tabled by the European Union at the 17th Meeting of the Washington Convention/CITES in September 2016, Johannesburg, South Africa (EU, 2016).

Current methods to induce maturation artificially are largely centered around the repeated – typically weekly – use of human chorionic gonadotropin in male eels and of pituitary homogenates in female eels. Accordingly, Japanese researchers first obtained eel larvae in 1974 (Yamamoto and Yamauchi, 1974) and completed the life cycle (acquisition of glass eels) in 2003 (Tanaka et al., 2003). Artificial propagation is now done routinely by several research groups in Japan, but survival of larvae until the glass eel stage remains exceedingly low, still prohibiting the commercial uptake of the approach.

Larval rearing is seen as a key bottleneck to mass production of glass eels (e.g., Okamura et al., 2014), and despite steadily improving outcomes from artificial

propagation studies by several groups in recent years (e.g., Ahn et al., 2012; Butts et al., 2014; Di Biase et al., 2015; Tanaka, 2015), compromised egg quality may well be at the root of this problem (e.g., Izumi et al., 2016; also see Rozenfeld et al., 2016 for association between maternal transcripts and hatching rates). Recently, some benefits of androgen treatment prior to administration of pituitary homogenates to female Japanese eels (*Anguilla japonica;* Matsubara et al., 2003a,b) and Australian shortfinned eels, *A. australis* (Lokman et al., 2015), were reported, but effects on egg quality (fertilization and hatching rates) were not investigated. Androgens have notable impacts on lipid physiology in eel (c.f., Divers et al., 2010; Damsteegt et al., 2016) and egg quality in several fish species is known to be affected by lipid content and/or composition. Therefore, we predicted that co-treatment of androgens and pituitary homogenates might affect the quality of eggs from artificially matured European eels, *A. anguilla*. To test this prediction, we administered slow-release implants containing 17-methyltestosterone (17-MT) in combination with traditional hypophysation to female European silver eels and determined fertilization and hatching rates of the eggs acquired thereafter.

2. Materials and methods

2.1 Animals

Wild female eels were caught using traditional "*lavoriero*" (downstream traps) in a brackish water lagoon near the sluices of the North Adriatic Sea (*Val Bonello*, Veneto, Italy). At this site, it was possible during the autumn-winter migration to catch *A. anguilla* specimens that were morphological identified as migrants (Silver Index IV and V; Di Biase et al., 2015; Durif et al., 2005). Eighteen females were selected from the catch and then transported to the laboratory (Department of Medical and Veterinary Science, University of Bologna, Cesenatico, Italy) where they were assigned randomly to one of three treatment groups, i.e., MTA, MTB or Control (6 fish/group), with mean body weights of 602 ± 65, 657 ± 85 and 649 ± 102 g, respectively. Cultivated male eels, 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 aquaculture system (RAS) consisting of two fish-rearing tanks (700 L each), 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; c.f., Mordenti et al., 2012; Parmeggiani et al., 2015) in seawater (salinity 32 $g.L^{-1}$) at a controlled temperature of 15.5 ± 0.5°C until gonadal maturation (see below) was complete. The animals were marked individually by placing fish tags (FLOY TAG Mod Floy T-Bar Anchor) in the dorsal muscle whilst under anesthesia with 400 ppm 2-phenoxyethanol, and maintained under starvation for the duration of the trial.

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

2.2 Manufacture of slow-release androgen implants

Slow-release androgen implants containing 1 mg 17-methyltestosterone (17α-methyl-4-androsten-17β-ol-3-one: Sigma Chemicals Co.; St Louis, MO, USA) in a matrix of cholesterol : cellulose = 95 : 5 were made in-house essentially as described previously (Lokman et al., 2015; Sherwood et al.,1988). Control implants (vehicle) contained matrix only.

2.3 Experimental design

2.3.1 Induction of oocyte growth

Female eels were assigned randomly to one of three treatment groups, i.e., MTA, MTB or Control (6 fish/group). MTA- and MTB-groups were administered 17-MT-containing implants, twice, at 1 and 5 weeks after onset of experimentation, whilst control eels (Ctrl) were given a blank implant. Furthermore eels received weekly injections with carp pituitary homogenates (CPH) from either Week 1 (MTA- and Ctrl groups) or Week 5 onwards (MTB-group). At the start (Day 0) of experimentation (Week 0 = W0), eels were anaesthetized with 400 ppm 2-phenoxyethanol and subjected to blood-sampling by syringe for measurement of plasma steroid levels (see Section 2.4). Additional blood samples were collected fortnightly from each fish until reaching the time for induction of spawning (Section 2.3.3). On the 7th day (W1), anaesthetized eels (400 ppm 2-phenoxyethanol) were injected intraperitoneally with sustained-release implants containing 1 mg of 17-MT (MTA- and MTB-groups) or matrix (Controls; Ctrl). On Day 35 (W5) of experimentation, the same procedure was repeated and a second implant was administered.

Treatment of eels with carp pituitary homogenate (CPH) commenced on Day 7, W1, in the MTA- and Ctrl-group. CPH was injected intramuscularly on a weekly basis, adopting a protocol in which doses increased with time (Mordenti et al., 2012), as follows: the first 4 injections were made with 10 mg CPH / kg body weight (BW) in sterile physiological saline (0.9% NaCl), the subsequent 12 injections with 30 mg/kg CPH and thereafter, 40 mg/kg CPH was injected until fish were considered ready for induction of final maturation (Section 2.3.2). Fish in the MTB-group were treated the same way as fish in the MTA-group, except that the 10 mg/kg injections during W1-W4 were withheld (fish were injected with 0.9% saline only); thereafter, these eels received the same CPH doses as the eels in the MTA-group.

2.3.2 Induction of final oocyte maturation and ovulation

Weekly injections with CPH continued until increases in body weight (weight gain of >10% over initial values; c.f., Dou et al., 2008; Oliveira and Hable, 2010; Ijiri et al., 2011; Burgerhout et al., 2011; Mordenti et al., 2013) and a gravid appearance indicated that oocyte hydration was likely to have started, a stage that we for convenience refer to as the *pre-spawning stage*. At this stage, ovarian biopsies from individual female eels were collected repeatedly, every 8 h, to optimize the timing for induction of final maturation. Biopsies were made by needle and syringe whilst eels were under anesthesia in 400 ppm 2-phenoxyethanol, and the developmental stage of oocytes was evaluated according to Palstra et al. (2005). Accordingly, guided by the presence of clearing oocytes (>30% of fully transparent oocytes; FTO), a peripheral nucleus and the presence of a few large fat droplets (diameter from 110 to 150 µm), ovulation was induced by intra-peritoneal injection with 2 mg/kg 17,20β-dihydroxy-4-pregnen-3-one (DHP; Di Biase et al., 2015).

If the desired stage of oocyte maturation (stage 5 according to Palstra et al., 2005) was not reached within 48 h from the last weekly CPH injection, an intramuscular booster with CPH (30 mg/kg BW) was given and ovarian biopsies were again taken as described above in order to properly time the injection with DHP. The relative change in body weight at time = j was captured by the Body Weight Index (BWI_j), as follows:

BWI_j = *(BW at time j / initial BW) x 100*

2.3.3 Induction of testicular maturation

Males were induced to mature by weekly injections with 1 IU/g BW hCG (Ohta et al., 1997; Pérez et al., 2000; Palstra et al., 2005) and started spermiating by 5 weeks after onset of treatment. Just before fertilization experiments, simultaneously with DHP injection of females, the males received a booster hCG injection (1 IU/g BW) to induce sperm maturation (Burgerhout et al., 2011).

2.3.4 Fertilization and egg incubation

All eighteen females matured and were allowed to spawn spontaneously (Di Biase et al., 2015). After DHP injection, each female was transferred to a new closed recirculating aquaculture system, in which the seawater temperature was raised to 20 ± 0.5°C (Dou et al., 2008; Mordenti et al., 2014) and maintained for 20 h in the company of spermiating males (*sex ratio* 4M : 1F) in order to facilitate spontaneous spawning.

After 20 h, all breeders were removed from the spawning chamber and the relative weight of spawned eggs (%BW) was calculated as

$$\%BW = (BW_{DHP\ injection} - BW_{post\text{-}spawning}) \times 100 / BW_{DHP\ injection}$$

Spawned eggs were directed to the incubation chamber by water flow (please refer to Mordenti et al. (2014) for details on husbandry system) and a sample was carefully collected with a fine-meshed net and transferred to a measuring cylinder containing seawater. The floating rate (%) was determined on buoyant eggs, obtained after maintaining an egg sample for 30 minutes in a 500 ml beaker:

floating rate (%) = (buoyant eggs / total number of eggs) x 100

Each batch of spawned eggs was monitored at 2 hpf in order to determine the % of fertilized eggs, i.e., eggs that reached the 8- or 16-cell stage, in the floating fraction, collected from the incubation chamber as described above. Three subsamples of 1,000 eggs were scored using a dual counter clicker and estimates of numbers of fertilized eggs averaged for each batch. A check on fertilization success was also made on the sunken eggs (Di Biase et al., 2015). Accordingly,

fertilization rate (%) = number of fertilized eggs / total number of eggs × 100

Each batch of eggs was weighed after interrupting the flow after which floating eggs were gently collected by overflow into a beaker. The density of eggs was so high that only a limited amount of water, which was carefully eliminated as much as possible prior to weighing, was collected along with the eggs. From these floating eggs, we subsequently transferred 21.5 g of eggs (about 30,000 eggs) at 3 hpf into a rearing system composed of 6 tanks at 20 ± 0.5 °C. Eggs were divided equally between these 6 tanks at a density of 250 eggs.L^{-1}, which could guarantee self-cleaning of the filter mesh into the rearing system (see below); successful self-cleaning was confirmed

regularly by visual inspection. The rearing system, which had a design similar to a kreisel (Greve 1968; Okamura et al., 2009), was equipped with a pump, a dual stage temperature controller, sump filter, UV-C sterilizer and a proto-skimmer (ACQUAPROGET SRL, Rimini – Italy) for the recirculation of the water. It set up with a circular revolving water current with inlet jets on the top of the incubation chamber to guarantee the constant cleaning of the filter mesh. During the whole period of experimentation, i.e., throughout larval development and reabsorption of the yolk-sac, the larvae were maintained at a starvation regime. Some time from 52 to 60 hours of incubation, the hatching rate was calculated by sampling the number of embryos and larvae in 1 liter of incubation water according to equation:

hatching rate (%) = (number of hatched larvae / 250) x 100

where 250 is the number of eggs/L incubated;

Survival rate was estimated both at 5 dph (yolk sac stage) and 15 dph (feeding stage), by sampling 1 L of incubation water and expressing the number of vital larvae as a percentage of the number of hatched larvae in 1 L, as follows:

survival rate (%) = (number of live larvae / number of hatched eggs) x 100

2.4 Quantification of plasma levels of testosterone (T) and 17β-estradiol (E2)

Blood samples (1 mL) were collected in heparinised tubes, centrifuged (4000 *g*, 20 min) and stored at –80°C until analysis for plasma hormone levels. Steroids were extracted by mixing 0.2 mL of plasma with 5 mL of diethyl ether for 30 min on a rotary mixer. The tubes were centrifuged at 2000 *g* for 15 min and the supernatants evaporated to dryness at 37 °C in a fume hood. The dry extracts were stored at -20 °C until reconstitution in assay buffer for measurement of T (10% extract, equivalent

to 20 µl plasma) or E2 (10 µl plasma equivalent) by radioimmunoassay that was validated for use with eel plasma (RIA; see Parmeggiani et al., 2015); tritiated T (30 pg; 83.4 Ci/mmol Perkin Elmer Inc. Boston, MA) or E2 (15 pg; 81 Ci/mmol Perkin Elmer Inc, Boston, MA) were added, followed by rabbit anti-testosterone serum (0.1 mL, 1:50,000; antiserum produced in our laboratory) or rabbit anti-E2 serum (0.1 mL, 1:40,000; antiserum produced in our laboratory), respectively. After incubation and separation of antibody-bound and –unbound steroid by charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer), tubes were centrifuged (15 min, 3000*g)*, the supernatant was decanted and radioactivity immediately measured using a β-scintillation counter (Packard C1600, Perkin Elmer, USA).

The sensitivity of the T assay was 1.70 pg/tube, and the intra- and inter-assay coefficients of variation were 5.7% and 10.2% respectively. The sensitivity of the E2 assay was 0.85 pg/tube and the intra- and inter-assay coefficients of variation were 4.9% and 9.7% respectively. Cross reactivity of various steroids with the antisera were as reported by Parmeggiani et al. (2015). The assay results for both hormones were expressed as ng/mL.

2.5 Statistical analyses

Data on zootechnical performance (BW, egg weights and egg performance scores) were analyzed by one way repeated measures ANOVA, followed by Bonferroni-corrected post-hoc *t*-tests. Changes in levels of steroid hormones over time within each treatment group and differences between the control group and treated groups at each sampling time were evaluated by one way repeated measures ANOVA

followed by Tukey's multiple range test. Data are presented as means ± SE and a difference of $P < 0.05$ was considered significant.

3. Results

3.1 Zootechnical performance

3.1.1 BWI and time required to reach the pre-spawning stage

Administration of fertility drugs resulted in changes in relative body weight over time, as showed in figure 1. Thus, treatment with CPH-only saw average body weights being maintained close to starting weights (BWI ~ 100%) for much of the first 24 weeks of experimentation; notable deviations from BWI (=100%) were evident by a transient peak at Week 13 (mean BWI = 107.8%) and a transient trough at Week 23 (mean BWI = 96.5%)(P<0.05). Among the CPH-only group, increases in body weight prior to final maturation appeared from W25 onwards; in contrast, BWI in eels assigned to MTA and MTB treatment regimes started to increase soon after co-treatment with 17-MT and CPH. The BWI of eels in the MTB-group, not treated with low doses of CPH during the first 4 weeks, lagged that of eels in the MTA-group initially, but eventually caught up. A dramatic increase in BWI ensued in both 17-MT and CPH co-treated groups from Week 12 onwards, peaking at around BWI_{W14} = 130% (P<0.05), just prior to spawning (Fig. 1). The time required to reach this stage was significantly shorter for both 17-MT + CPH co-treated groups than for the Ctrl-group (P<0.05). Accordingly, significantly less CPH was needed to reach the pre-spawning stage in eels that received both 17-MT and CPH (P<0.05) (Fig.2).

3.1.2 Pre-spawning oocyte stage

As shown in Fig. 3A, co-treatment with 17-MT and CPH resulted in a larger percentage ($F(2,14)=51.3;P<0.05$) of FTO at Stage 5 than what was seen after treatment with CPH only.

3.1.3 Egg quality

3.1.3.1 Floating eggs. Recovered eggs from spontaneous spawning (rate of spawned eggs is showed in fig.3B) events largely floated for all treatment regimes ($F(2,14)=4.75;P<0.05$), rates averaging 72.7 ± 1.8 %, 79.3 ± 1.6 % and 80.5 ± 2.2 % in Ctrl-, MTA- and MTB- groups, respectively (Fig. 4A).

3.1.3.2 Fertilization rates. The fertilization rate of eggs from control females (71.2 ± 7.5 %) was lower ($F(2,14)=10.9;P<0.05$) than that of eggs from slow-release androgen implanted eels (MTA-group, 89.5 ± 1.4 %; MTB-group, 91.3 ± 1.3 %); no difference was apparent among rates of fertilization between both co-treated groups (Fig. 4B). Unlike floating eggs, sunken eggs were not fertilized for any of the females under study.

3.1.3.3 Hatching rates. Eggs hatched after 40-50 hours and remained suspended in the water column through the flow in the *kreisel*. Rates of hatching were notably different between treatment groups ($F(2,14)=10.2;P<0.05$), reaching mean values as high as around 70% for both 17-MT and CPH co-treated groups (Fig. 4C), but only averaging 21.0 ± 8.4%) for eggs from the Ctrl-group.

3.1.3.4 Larval survival. Larval survival by 5 dph (Fig. 5A) was quite variable among individuals within treatment groups, ranging between 54-68%. Accordingly, treatment

effects were not found (F(2,16)=2.9;P=0.08), although survival tended to be higher in the groups that received 17-MT implants. These trends persisted, such that by 15 dph, mean survival was significantly lower (F(2,16)=31.2;P<0.05) among offspring from the Ctrl-group (< 10%) than among those from 17-MT-treated fish (45-50%; see Fig. 5B).

3.2 Plasma steroid levels during artificially induced oogenesis

3.2.1 Testosterone

Plasma levels of T fluctuated in a band between 0.8 and 2.3 ng/mL for samples across much of the experimental period, regardless of treatment regime (Fig. 6A). A small transient peak (2.2 - 2.3 ng/mL) was seen for levels of T in 17-MT treated fish between W2 – W4, being different from values at corresponding times in controls (0.9 – 1.0 ng/mL). However, on W14, plasma T levels increased dramatically in eels treated with 17-MT, averaging 4.8 ± 1.84 and 5.8 ± 2.35 ng/mL in the MTA- and MTB-groups, respectively(P<0.05). Thereafter, T plasma levels returned to pre-peak values (around 1.7 ng/mL) until spawning in eels in the MTA- and MTB- groups had completed (W18). There was no evidence for any strong increase in plasma T levels among control eels at any time during the experimental period (Fig. 6A).

3.2.2 Estradiol

Notable variability was observed in plasma E2 levels during the experimental period, values typically averaging 0.3 – 1.0 ng/mL (P<0.05) (Fig. 6B). Trends were limited to a general falling in concentrations of E2 during the first 4 weeks of the study in all treatment groups, and a distinct, transient peak in E2 levels at W14 in the MTB-group (1.7 ± 0.60 ng/mL); a smaller peak in levels of E2 (1.1 ± 0.18 ng/mL), significantly

different from concentrations in control eels (0.4 ± 0.60 ng/mL), was evident for eels in the MTA-group ($P<0.05$) (Fig. 6B). No pre-spawning E2 peak was seen in plasma from control eels.

4. Discussion

Artificial propagation may prove crucial to the survival, or alternatively, the rate of recovery, of European eel stocks, in future. Many years of research have seen Japanese scientists succeed in completing the life cycle of the Japanese eel, but problems with egg quality and larval feed have been cited as factors that limit mass production of seedlings for aquaculture or stock enhancement purposes (e.g., Okamura et al., 2014; Izumi et al., 2016). We posed that androgen treatment, previously seen to shorten the duration of fertility treatment in shortfinned eels (Lokman et al., 2015), might be effective in modulating egg quality, given that androgens have wide-ranging effects on ovarian lipid physiology (Divers et al., 2010; Endo et al., 2011; Damsteegt et al., 2016) and on expression of the Fsh receptor (Setiawan et al., 2012) in anguillids. The present study provides support for this prediction, as reflected in increased fertilization, hatching and survival rates of eel larvae produced from androgen-treated maternal parents.

Research on *A. australis* revealed that high doses (3-30 mg/fish) of 17-MT result in unusual oocyte cytology (Lokman et al., 2015), prompting the use of a lower-dose implant of 1 mg/eel in our study. Androgen or vehicle administration was combined with hypophysation, commencing either at the same time, or 4 weeks after initiation of 17-MT treatment. Accordingly, eels in pre-spawning condition were obtained from all

treatment groups, remarkably with 100% efficacy. Using our previously described approach to enable fully matured females to spawn spontaneously when co-housed with mature males (Di Biase et al., 2015), spawned eggs were obtained from all crosses. The floating rates from different egg batches were not affected by androgen administration, however, subsequent rates of fertilization, hatching and survival were notably higher in androgen co-treated eels than those subjected to 'traditional' hypophysation, reinforcing the previously proposed value of androgens in induced spawning protocols. Some previous works have speculated that making shorter the female maturation process could jeopardize the right development of vitellogenesis or final egg quality (Levavi-Sivan et al., 2004). In the present work, hormonal treatment lead to a complete synchronization of females, thus obtaining opposite results. We think that this should be ascribed at first to non-species specificity of steroidal hormones (17-MT), not leading to an immunological reaction against the hormone itself. Reduced treatment time, induced by 17-MT, also reduces the CPE exposure time, again minimizing the immune reaction against the heterologous extract. Additionally, 17-MT could be converted by aromatase to 17-ME2, binding to hepatic estradiol receptor (Hornung et al., 2004; Lockman et al., 2015).

In the current study, high rates of fertilization and hatching were observed, leading to consistent and large yields of larvae, typically in the tens of thousands. Anecdotally, larvae obtained from androgen co-treated eels in this study seemed more numerous, but actual estimates on larval yield were not collected. In any case, the consistent high yield of larvae, resulting from improved husbandry (Okamura et al., 2009; Mordenti et al., 2014), from spontaneous spawning (Horie et al., 2008; Di Biase et al., 2015) and from inclusion of androgens in the induced spawning protocol (this study; Lokman et al., 2015), is a very exciting outcome – this contrasts markedly with outcomes from a

range of other, mostly older, studies (e.g., Pedersen, 2003), and hopefully, heralds a new phase in captive breeding of European eel; future larval rearing experiments will undoubtedly further assess the value of inclusion of androgens in the induced maturation protocol in more detail.

Egg quality has remained an exceedingly difficult trait to define other than in terms of hatching and/or fertilization rates. Moreover, this trait is unstable, perhaps nowhere more so than in the eel, in which mis-timing (delay) of *in vitro* fertilization is known to have dramatic impacts on fertility (e.g., over-ripening; e.g., Ohta et al., 1996; review by Tanaka, 2015). The issue of mis-timing may be a confounding factor when interpreting egg performance data from eels subjected to strip-spawning (S. Adachi, Hokkaido University, pers. comm); however, the increasing use in recent years of spontaneous spawning of breeders is likely to ensure that spawning occurs soon after ovulation, leading to improved fertilization outcomes, as reported by Horie et al. (2008) and Di Biase et al. (2015).

Findings from previous decades have further associated the maternal diet, especially the lipid content and composition, with egg quality and fertilization outcomes in marine fishes. Likewise, several biochemical indicators have been correlated with egg quality in salmonids. Recent studies have employed proteomics (hapuku, *Polyprion oxygeneios*; Kohn et al., 2015) or massive parallel sequencing approaches, such as RNA-sequencing (striped bass, *Morone saxatilis*; Chapman et al., 2014), in order to get insights into proteins or (maternal) transcripts that could describe or predict egg quality. In addition, the screening of large numbers of candidate genes by qPCR has been trialed, also for eel; accordingly, expression of the genes encoding LSM7 homolog U6 small nuclear RNA and mRNA degradation associated (Izumi et al., 2016)

and of β-Tubulin, Carnitine O-palmitoyltransferase liver isoform-like 1, prohibitin-2 and phosphatidylinositol glycan biosynthesis class F protein 5 (Rozenfeld et al., 2016) correlated with hatching rate and it would be worthwhile to investigate whether the abundance of any of the identified molecular players in the eel egg can be regulated by androgens.

Of notable interest is the difference in body weight profile between androgen-treated and control fish. The latter group maintain a BWI close to 100% until the pre-spawning increase. However, in androgen co-treated eels, BWIs remain well about 100% throughout the experimental period, a trend that resounds the findings on androgen effects on body weight reported previously (Lokman et al., 2015). Indeed, BWI values of around 110% were the norm, increasing to 130% during the pre-spawning stage. This high BWI was associated with a significantly greater yield of eggs from the androgen-treated groups (~45% of BW) than that from control eels (~ 35% of BW, data not shown). Together with the increased egg performance data, these observations demonstrate a substantially greater larval yield from female eels treated with androgen, and hence, a much improved economy of scale. Moreover, in keeping with a previous report (Lokman et al., 2015), the time required until the pre-spawning stage, together with the associated savings in costs of fertility drugs, add further impetus to considering androgen co-treatment in induced spawning protocols.

Interestingly, treatment with androgens did not seem to have much of an effect on plasma levels of steroid hormones for most of the experimental period – a dramatic difference was only evident at the pre-spawning stage, when a clear, distinct peak was observed for both T and E2 in androgen-, but not vehicle-treated, eels. Increased steroid biosynthesis, and, in turn, levels of plasma steroids, is a likely reflection of increased expression of steroidogenic protein genes in the ovary at that time (e.g.,

steroidogenic acute regulatory protein: Reid et al., 2013; P450 side-chain cleavage: Kazeto et al., 2006; P450 17α-hydroxylase: Kazeto et al., 2000; P450 aromatase: Ijiri et al., 2003). Minor differences in plasma steroid levels between treatment groups at other times (especially W4) are more likely to reflect random scatter in the data. Aside from scatter, cross-reactivity with 17-MT in the T assay seems plausible, especially in the first few weeks after implantation when levels of 17-MT are likely to be most elevated.

The release rates of 17-MT from the implants are not known and the interval during which androgens levels are increased, therefore, is open to speculation; Lokman et al. (2015) observed significant decreases in plasma levels of 11-KT by 3 weeks after implantation with 17-MT, even at doses lower than those described in the present study, suggesting that 17-MT interferes with steroidogenesis, or at least, 11-KT synthesis. Co-treatment with CPH could presumably override any inhibiting effects of 17-MT on steroidogenesis, as essentially no differences in plasma steroid levels were evident between experimental groups other than during the pre-spawning period. Similarly, the males are able to produce steroidogenic enzymes and peaks of androgens under a weekly treatment with hCG (Peñaranda et al., 2016). Moreover, Peñaranda et al. (2014) studied the regulation of aromatase and androgen receptor expression during testis and ovary development in European eel. These results could be related to what found in this study (pre-spawning E2 peak), partially explaining the effect of 17-MT on synchronization and maturation of eggs.

In conclusion, we demonstrate here that female European silver eels respond to co-treatment with 17-MT and CPH by accelerated oogenesis, larger batch size, improved fertility and enhanced larval survival. We thus contend that inclusion of androgens in

treatments aimed at inducing artificial maturation of anguillid eels may be a worthwhile pursuit.

Conflict of interest

We declare that we have no conflicts of interest in the authorship or publication of this paper.

Author Contributions

ADB, PML and OM conceptualized the experiments. ADB, AC, PE conducted the in vivo experiments. AP and NG carried out the RIA**.** ADB, NG and PML drafted the ms for subsequent critiquing by co-authors.

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Fig.1: changes in relative body weight over time, in artificially matured female silver eels, *Anguilla anguilla* .

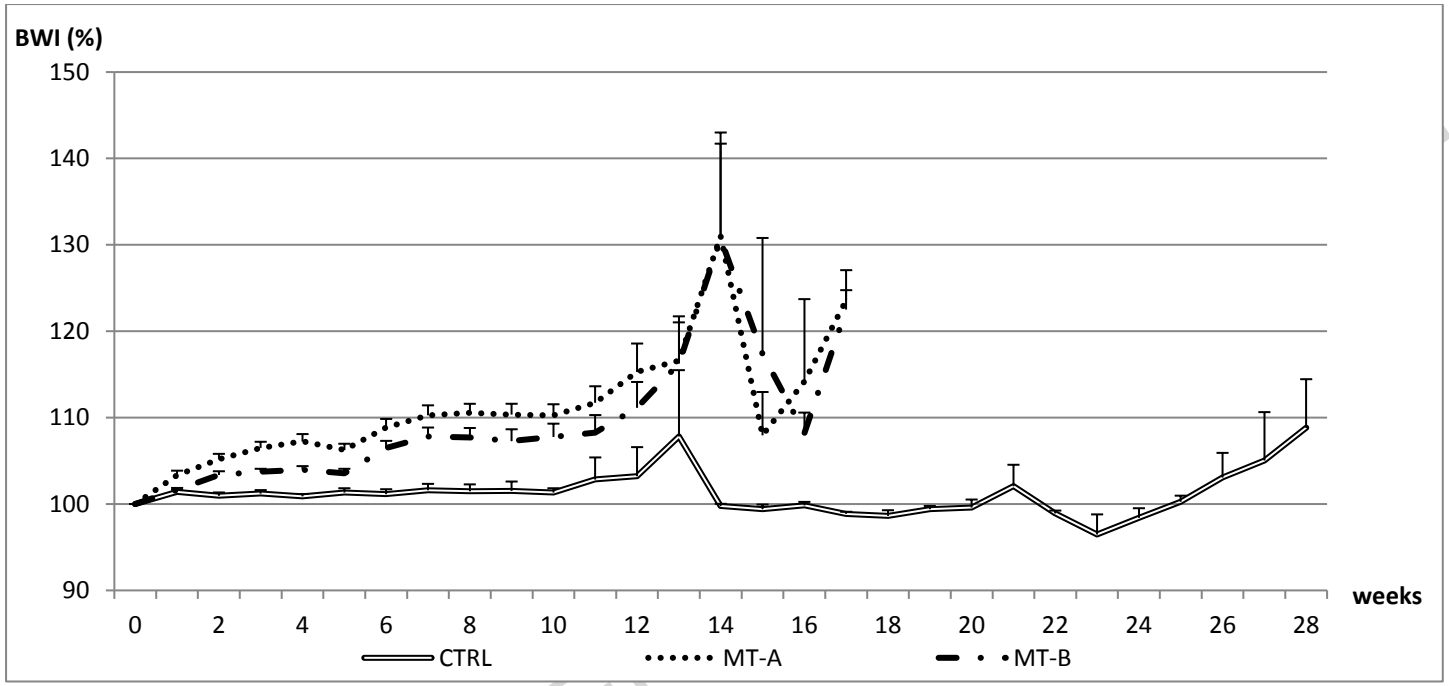

Fig.2: Total amount of CPE (mg/kg) and the time-to-spawn in artificially matured female silver eels, *Anguilla anguilla.* The means of total amount of CPE (CTRL=787±100 mg/kg, MTA=415±31 mg/kg and MTB=357±24 mg/kg) are significantly different between CTRL and MTA (*; $p<0.05$) and between CTRL and MTB (§; $p<0.05$), the R value are respectively $R^2 = 0,999$, $R^2 = 0,993$ and $R^2 = 0,995$ for Ctrl-, MTA- and MTB- groups.

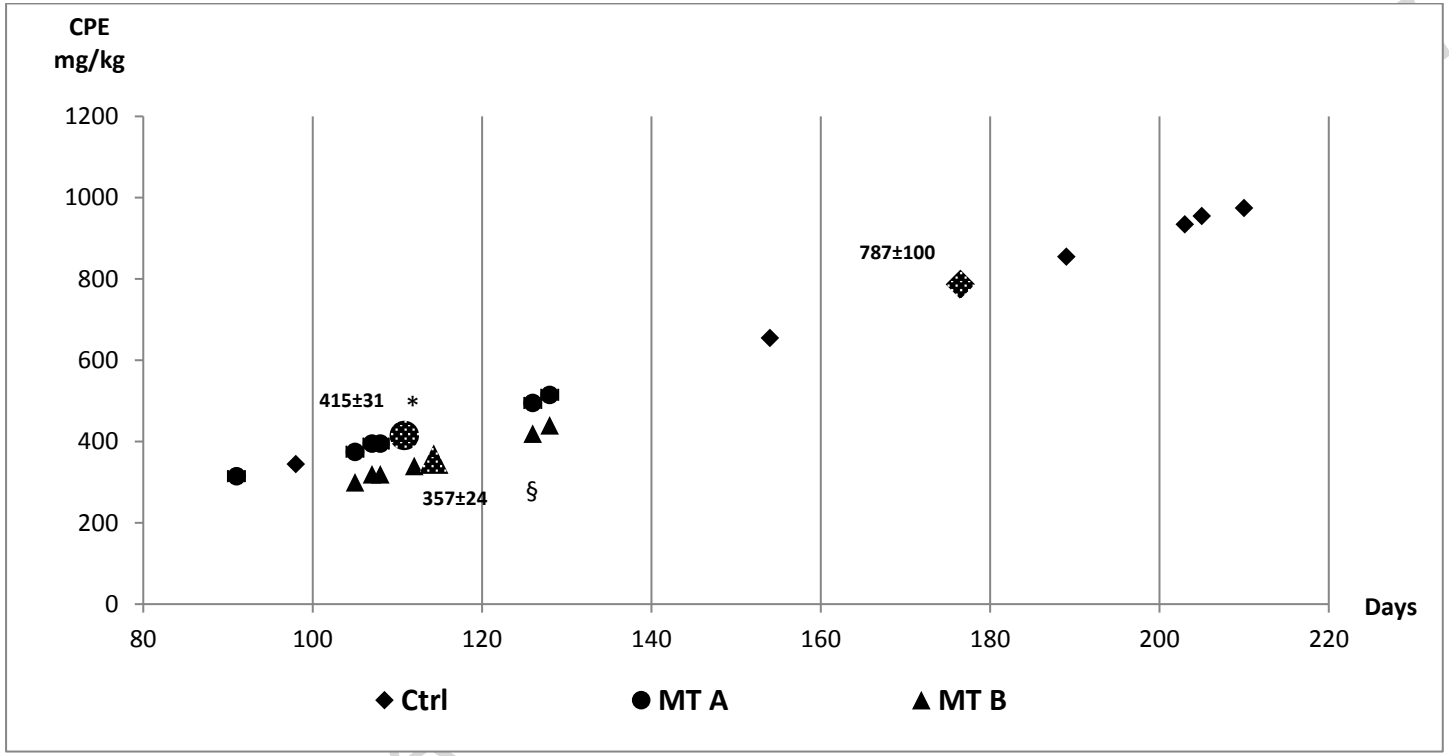

Fig.3A and 3B: Rate of Fully Transparent Oocytes (A) and Spawned egg (B) in artificially matured female silver eels, *Anguilla Anguilla,* expressed as percentage of BW at DHP-injection - Significance difference ($p<0.05$)

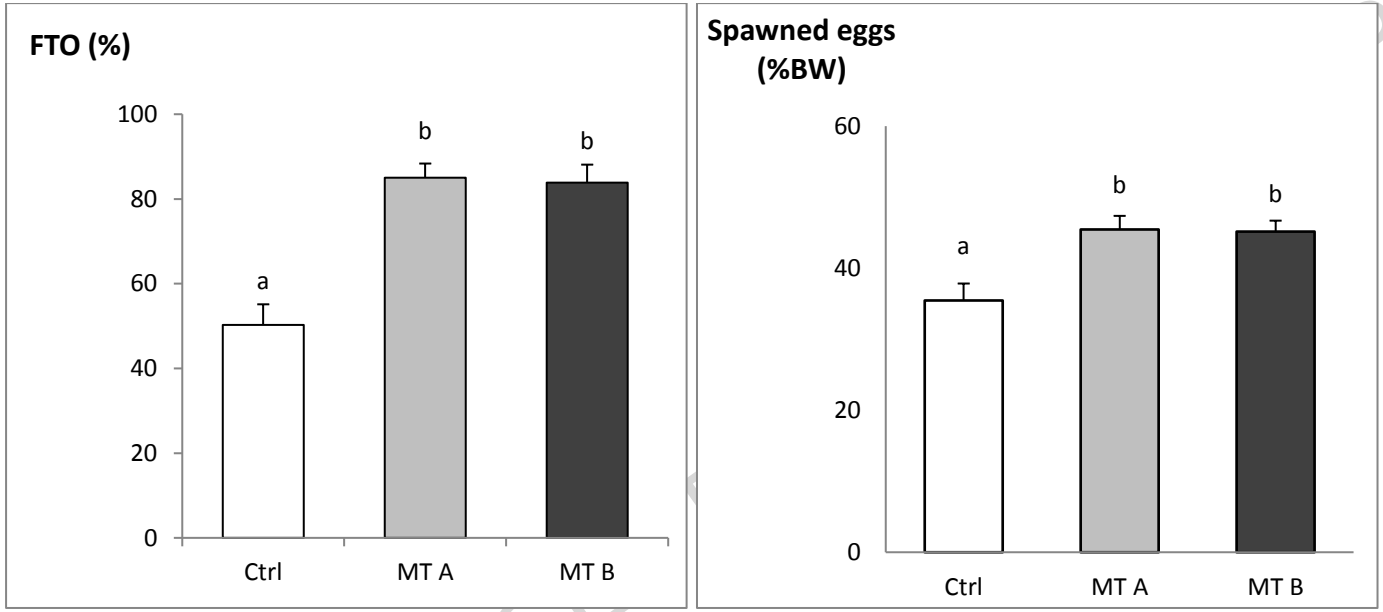

Fig.4A, 4B and 4C: Floating eggs rate, fertilization rate and hatching rate in artificially matured female silver eels, *Anguilla anguilla* - Significance difference ($p<0.05$)

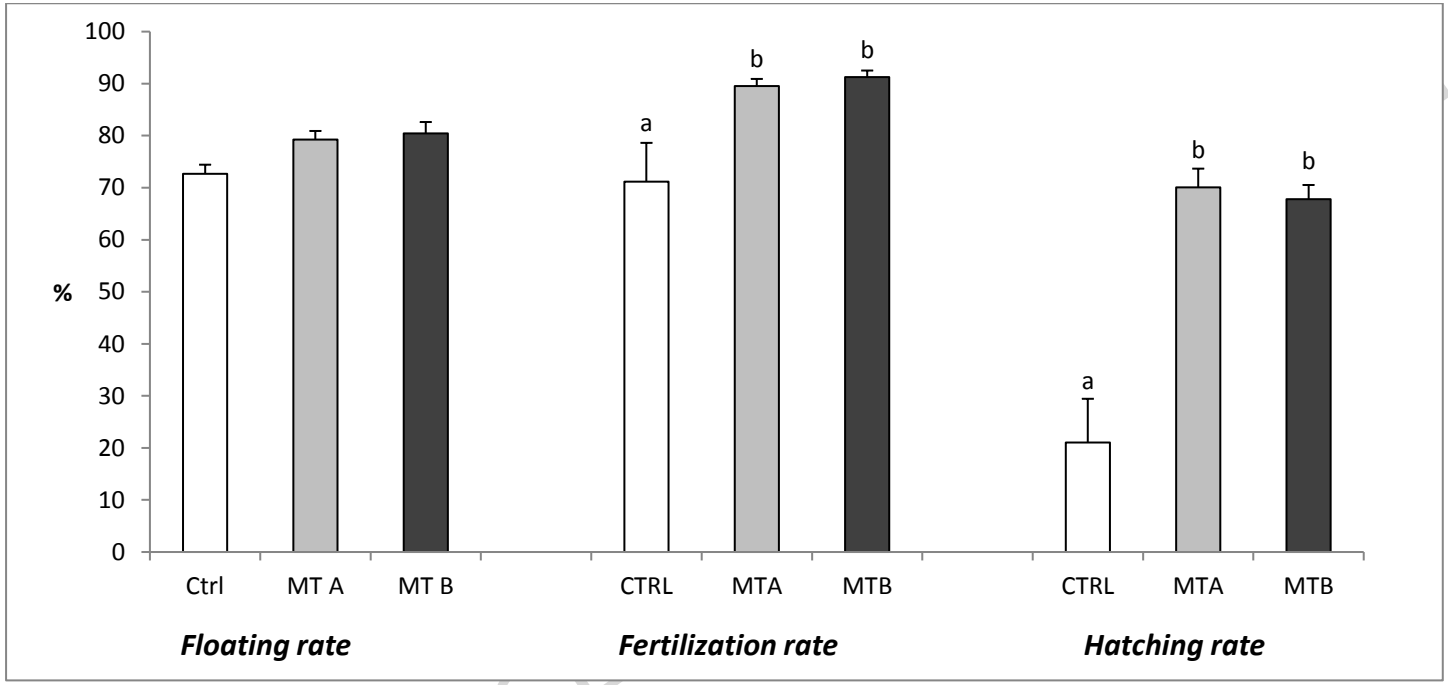

Fig.5A and 5B: Survival rate at 5 dph (A) and Survival rate at 15 dph (B), in artificially matured female silver eels, *Anguilla anguilla* - Significance difference ($p<0.05$)

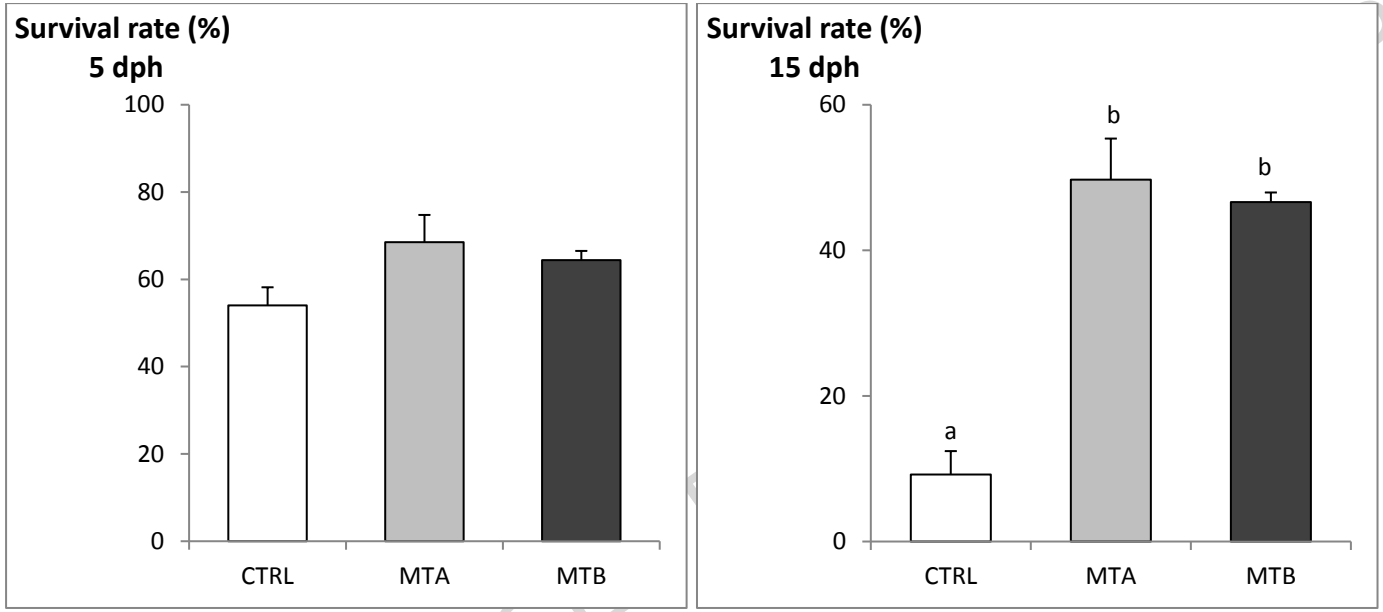

Fig.6A and 6B: Changes in plasma testosterone (A) and 17-β-E2 (B) levels of female European eels (*Anguilla Anguilla*) implanted with slow-release implants containing 1 mg of 17-MT and carp pituitary extracts (CPE) at various doses. An asterisk (*) indicates values significantly different from control at each sampling time (P<0.05).