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Effect of aromatable androgen (17-methyltestosterone) on induced maturation of silver European eels (*Anguilla Anguilla*): Oocyte performance and synchronization

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Abstract

The reproductive performances of silver European eel in term of gonad development and egg production, employing slow-release implants with the androgen 17-MT (1 mg) in combination with traditional weekly injection of carp pituitary extract (CPE) was evaluated. Wild female European eels (*Anguilla anguilla*) underwent a standard induction protocol with CPE and were randomly divided into three groups (N-group, no implant; Y-group, with implant; and control, C-group, no treatment). The results showed that 17-MT-treated females (Y-group) reproduced spontaneously about 6 weeks earlier than the N-group females with a saving of almost 40% in CPE and time of induction. Concerning artificial induction of maturation in female silver eels, our study demonstrated that they positively respond to androgen exposure also in terms of eggs productivity. Indeed, Y-group was more productive than N-group: in Y-group, 11 eels ensured an eggs production that exceeded 50% of initial body weight (BW), whereas in N-group only three eels have exceeded this value. The results suggest that 17-MT should be considered in future protocols for the improvement of the artificial reproduction of female silver European eels.

KEYWORDS
17-methyltestosterone, *Anguilla anguilla*, eggs production, gonad development, ovarian maturation

1 | INTRODUCTION

The European eel is included on the Red List of the International Union for Conservation of Nature (IUCN) as a Critically Endangered Species. Consequently, it is protected by the imposition of a short fishing season, minimum size for capture, protection of larvae and careful regulation of the fish trade. Unfortunately, these measures are not enough to reduce the danger of extinction for this species. To prevent depletion of the natural glass eel stock and provide reliable supplies of seedlings for aquaculture, development of an artificially induced breeding procedure is highly desirable (Mordenti, Di Biase, Sirri, Modugno & Tasselli, 2012).

At present, the only way to obtain sexually mature eels is to artificially induce sexual maturation in migratory individuals caught in brackish and freshwater environments or cultured silver eels using repeated injection of carp (CPE) or salmon (SPE) pituitary extract and a final injection of 17,20b-dihydroxy-4-pregnen-3-one (DHP) (Ijiri et al., 2011; Oliveira & Hable, 2010; Parmeggiani et al., 2015). Nevertheless, this method is not yet standardized and presents high variability between batches from different individuals (Chai et al., 2010; Okamura, Horie, Mikawa, Yamada & Tsukamoto, 2014). Thus, it is necessary to improve techniques for the production of high-quality eggs to make mass production of larvae possible (Di Biase et al., 2016). Indeed, if this target could be reached,

research could focus and concentrate on larval weaning techniques, as first successful early feeding of European eel larvae has already been obtained (Butts, Sørensen, Politis & Tomkiewicz, 2016; Di Biase et al., 2016).

More recently, alternative induced spawning protocols have also been developed, and arguably, the most eye-catching outcomes have been achieved in response to treatment with androgens (11-K testosterone [11-K]) of female silver eels (Lokman, Wylie, Downes, Di Biase & Damsteegt, 2015; Matsubara et al., 2003). For several years, the effect of androgen on eel reproductive activity was investigated. Sudo and Tsukamoto (2015), in *A. japonica*, report that androgen is involved in silvering and also in migratory behaviour at the onset of the spawning migration (higher locomotor activity and increasing behavioural drive in the spawning migration). Furthermore, it has been suggested that 11-KT synchronously accelerates early development of the ovaries and morphological changes in the Japanese eel (Sudo et al., 2012). Androgen treatment of eels resulted in morphological and physiological changes (silvering), such as increases in eye diameter and degeneration of the digestive tract (Rohr, Lokman, Davie & Young, 2001; Sudo et al., 2012). It has been shown that 17-α methyltestosterone (17-MT) provides a potential usefulness to participate the process of vitellogenesis when it combines with SPE treatments in Japanese eel (Wang & Lou, 2007); furthermore, the synergistic effect actually enhances the growth and synchronous development of ovarian follicle throughout the reproductive process. Lokman et al. (2015) demonstrated that incorporation of androgen in female silver short finned eels accelerates the gonadal maturation, increase lipid accumulation and oocyte size without any apparent detrimental effect on eggs quality.

The aim of this study was to verify the reproductive performances of European eel silver in term of gonad development and egg production employing slow-release implants with the androgen 17-MT in combination with traditional weekly injection of carp pituitary extract (CPE).

2 | MATERIALS AND METHODS

2.1 | Animals

Wild female European eels (*Anguilla anguilla*) were caught during one single day using traditional "*lavoriero*" (downstream trap) in a brackish water lagoon near the sluices of the North Adriatic Sea (*Val Bonello*, Veneto, Italy) (Mordenti et al., 2016), during their downstream migration (autumn–winter season) to the sea for their long migration to spawning area. Larger female eels (>500 g body weight [BW]) were selected at the catch and then transported to the laboratory

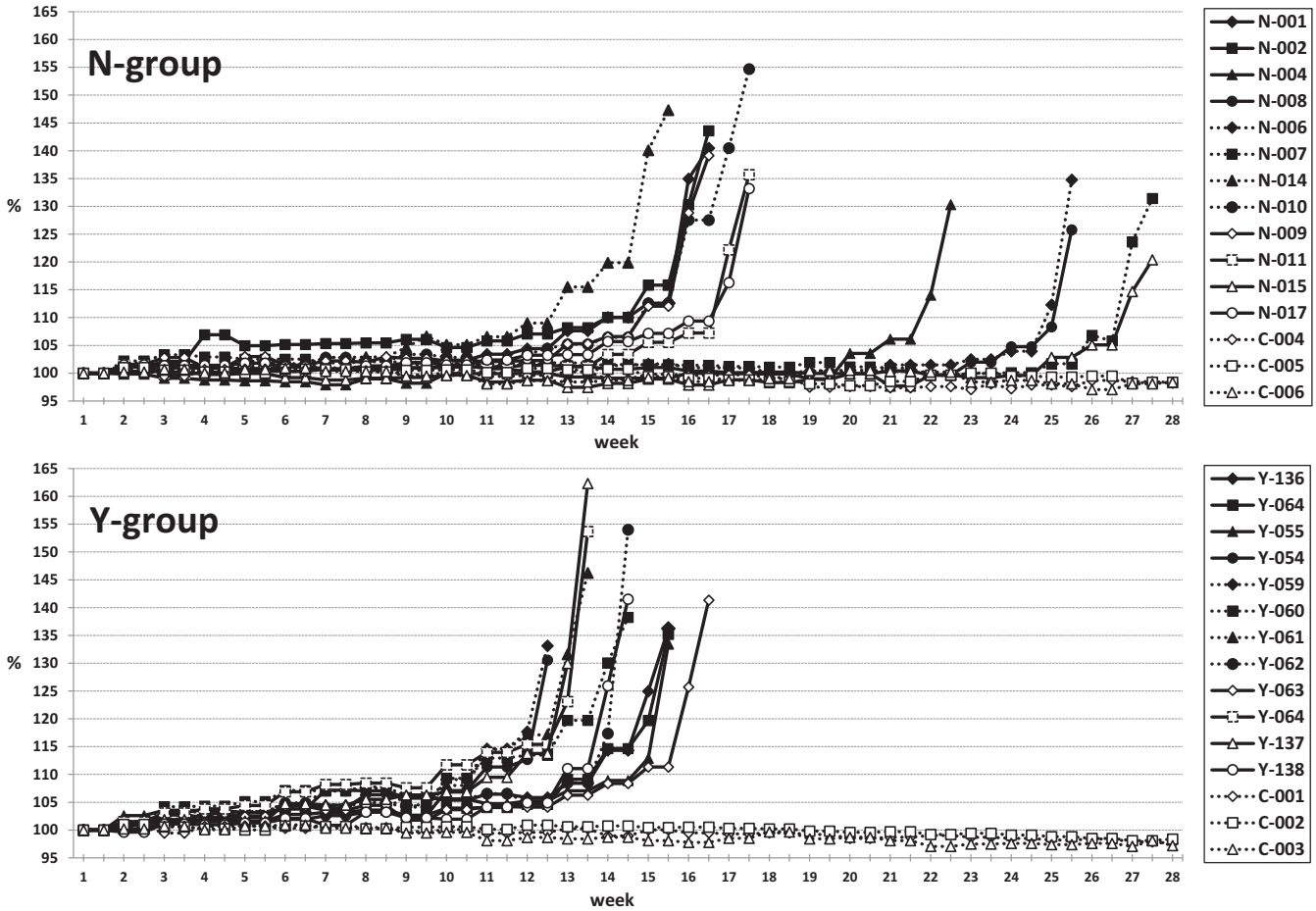

FIGURE 1 Body weight index (BWI) trend recorded in female eel during reproductive cycle

where they were measured and sampled to obtain an external indicator of their maturation state (silver index, SI) (Durif, Dufour & Elie, 2005; Mordenti et al., 2012, 2013). Out of 64 caught eels, 30 (47%) were at SI level V, 20 (31%) at SI IV and 14 (22%) at SI III. Only eels with SI V were selected and used for reproduction. At the same time, captive male eels (n. 50), reared in freshwater, were purchased from a commercial supplier. Upon transfer to the laboratory, all animals were gradually acclimated to natural seawater over 10 days. All eels were kept in a Recirculating Aquaculture System (RAS) consisting of two fish-rearing tanks (1200 L each), one with females and one with males. Fish were maintained in complete darkness (-0.04×10^3 lux at the bottom of the tank without water) in seawater (salinity 31 ± 1 g/L) at a controlled temperature of 15.5 ± 0.5°C until gonadal maturation was complete (Mordenti et al., 2012, 2013). The animals were marked individually by inserting fish-tags (FLOY TAG Mod Floy T-Bar Anchor) in the dorsal muscle whilst under anaesthesia 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.

TABLE 1 CPE consumption and number of injections needed to induce spawning

	CPE Consumption		
	mg/female	mg/kg BW	CPE induction *n*
N-group			
N-001	285.6	343.9	16
N-002	279.9	350.0	16
N-004	415.6	504.4	22
N-008	707.2	597.6	25
N-006	420.5	601.7	25
N-007	482.1	666.9	27
N-014	291.5	321.9	15
N-010	270.9	382.6	17
N-009	213.3	340.2	16
N-011	215.8	363.3	17
N-015	535.5	650.6	27
N-017	244.3	366.3	17
Mean	363.5 ± 151.7	457.4 ± 135.9*	20.0 ± 4.8*
Y-group			
Y-136	209.3	314.3	15
Y-074	197.8	309.5	15
Y-055	261.2	308.7	15
Y-054	272.1	211.8	12
Y-059	208.3	216.6	12
Y-060	203.7	291.5	14
Y-061	192.9	255.5	13
Y-062	158.9	278.5	14
Y-063	206.2	342.1	16
Y-064	136.5	256.2	13
Y-137	160.0	252.6	13
Y-138	142.7	276.3	14
Mean	195.8 ± 42.2	276.1 ± 39.7	13.8 ± 1.3

*Significance difference ($p < .01$) between Y-group and N-group eels.
BW, body weight; CPE, carp pituitary extract.

2.2 | Induction of maturation

To perform the fertilization of eggs, males gonad maturation was induced by weekly injection with 1 IU/g body weight (BW) human chorionic gonadotropin (hCG); spermiation started after 10 weeks (Mordenti, Casalini, Mandelli & Di Biase, 2014; Ohta et al., 1997; Palstra, Cohen, Niemantsverdriet, Van Ginneken & Van den Thillart, 2005). Just before mating, the males (BW 134.5 ± 27.5 g) received an hCG booster injection (1 IU/g BW) to further induce spermiation (Burgerhout et al., 2011).

Females were randomly divided into three groups, two consisting of 12 fish (N-group and Y-group) and one consisting of six fish (control, C-group).

Eels from N-group and Y-group were intramuscularly injected (i.m.) with 5 mg/kg BW (1^{st}–4^{th} week), 15 mg/kg BW (5^{th}–8^{th} week) and 30 mg/kg BW (9^{th} week–final maturation) CPE following the originally developed protocol by Mordenti et al. (2012). Injections were repeated every 7 days until increases in body weight and a gravidic appearance indicated that oocyte hydration was likely to have started.

At the first CPE injection, Y-group eels were implanted intraperitoneally (i.p.) with a slow-release implants containing 1 mg of 17α-methyl-4-androsten-17β-ol-3-one (17-MT) (Sigma Chemicals Co.; St Louis, MO, USA), and at the fifth CPE injection they received the 2^{nd} and last implant.

N-group was treated with CPE but received no implant, while C-group did not receive any CPE injection and any androgen implant until final experiment.

Slow-release androgen implants (2 mm in diameter and 8 mm in length, and weighed 28–30 mg) 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, Crim, Carolsfeld & Walters, 1988).

At the beginning of oocyte hydration, that is, when the BW exceeded 110% of initial body weight (IBW), each female was repeatedly ovary-biopsied (every 8 hr) and the developmental stage of oocytes was evaluated. This procedure was necessary to perform the best individual final induction timing. Ovulation was induced by intraperitoneal DHP (17,20b-dihydroxy-4-pregnen-3-one) injection (2 mg/kg) only when at least 50% of the oocytes were fully transparent (Fully Transparent Oocytes [FTO]), displayed their nucleus at the periphery and contained few large fat droplets (Di Biase et al., 2016; Palstra et al., 2005). The developmental stage of the FTO corresponded to stage five of gamete development in European eel,

TABLE 2 Morphometric parameters and eggs yields of treated eels groups (N-group and Y-group)

	Body weight (BW)		BWI			Spawned eggs	
	Initial	At last CPE	At last CPE	At DHP	CPE=>DHP		
	g	*g*	%	%	%	*g*	% BW
N-group							
N-001	830.7	1121.0	134.9	140.5	5.6	150.0	18.1
N-002	799.6	1042.0	130.3	143.6	13.3	391.7	49.0
N-004	824.0	939.7	114.0	130.3	16.3	289.0	35.1
N-008	1183.4	1282.0	108.3	125.8	17.5	403.6	34.1
N-006	698.9	784.8	112.3	134.8	22.5	374.9	53.6
N-007	722.9	893.5	123.6	131.4	7.8	299.2	41.4
N-014	905.8	1269.2	140.1	147.3	7.2	392.0	43.3
N-010	708.1	995.1	140.5	154.7	14.2	513.0	72.5
N-009	627.0	807.8	128.8	139.1	10.3	303.1	48.3
N-011	594.0	726.0	122.2	135.7	13.5	210.3	35.4
N-015	823.2	944.3	114.7	120.4	5.7	145.0	17.6
N-017	666.9	775.6	116.3	133.2	16.9	367.8	55.2
Mean	782.0 ± 157.0	965.1 ± 186.0	123.8 ± 11.1	136.4 ± 9.4	12.6 ± 5.3	320.0 ± 110.0	40.9 ± 15.6
Y-group							
Y-136	665.7	832.0	125.0	136.2	11.2	377.8	56.8
Y-074	639.1	764.8	119.7	135.2	15.5	360.0	56.33
Y-055	846.1	955.3	112.9	133.4	20.5	344.0	40.7
Y-054	1284.8	1448.0	112.7	130.6	17.9	625.0	48.7
Y-059	961.8	1132.0	117.7	133.1	15.4	593.0	61.7
Y-060	698.9	909.1	130.1	138.2	8.2	416.0	59.5
Y-061	755.0	993.6	131.6	146.2	14.6	478.0	63.3
Y-062	570.6	669.6	117.4	154.0	36.7	456.8	80.1
Y-063	602.7	757.7	125.7	141.4	15.6	426.8	70.8
Y-064	533.0	656.2	123.1	153.7	30.6	389.1	73.0
Y-137	633.3	823.0	123.0	162.3	32.4	374.5	59.1
Y-138	516.4	650.3	125.9	141.5	15.6	374.2	72.5
Mean	725.6 ± 218.3	882.6 ± 230.9	122.6 ± 6.5	142.2 ± 10.0	19.5 ± 8.9*	434.6 ± 90.7	61.9 ± 11.1*

*Significance difference ($p < .01$) between Y-group and N-group eels.
BW, body weight; BWI, body weight index; CPE, carp pituitary extract.

following Palstra et al. (2005), and to stage seven in A. japonica following Unuma et al. (2011).

After DHP injection, each female was transferred to a new closed RAS described by (Mordenti et al., 2014) and maintained for 20 hr with spermiating males (*sex ratio* 4M/1F) in seawater at a temperature of 20 ± 0.5°C to produce a thermic shock (Dou et al., 2008; Mordenti et al., 2014), to facilitate spontaneous spawning.

2.3 | Analyses: reproductive performance

For all reproductive cycles, the amount of CPE consumption (mg/female and mg/kg BW) and number of CPE injection to final maturation were registered.

The principal external indicators of ovary maturation were observed during the experiment: the body weight at each CPE and at final DHP injection was measured and used to calculate the body weight index (BWI), as follows:

$$\text{body weight Index (BWI)} = \frac{\text{body weight at } n.\text{injection}}{\text{initial body weight}} \times 100 \quad (1)$$

The difference between the BWI at last CPE and BWI at DHP was also calculated.

For each spawning event, the amount of spawned eggs (g) was calculated as the difference in female BW between post-spawning and DHP injection; the relative weight of spawned eggs (%BW) was calculated as a percentage of the initial BW.

2.4 | Statistical analyses

Reproductive performances of female were statistically analysed to identify differences in hormones consumption, in BWI and in eggs

production by group. Statistics were performed using one-way analysis of Variance (ANOVA) followed by Tukey HDS post-hoc test, on SSP (Smith's statistical Package); $p < .05$ was considered statistically significant.

3 | RESULTS

Final maturation and spontaneous spawning were obtained in 24 females treated hormonally.

Trend of BWI until the spawned eggs is reported in Figure 1. Generally speaking, an increase in mean BWI higher than 5% (105.1 ± 1.3) was observed in Y-group 2 weeks after the second 17-MT treatment. On the contrary, N-group did not exceeded a BWI gain of 2% (101.8 ± 1.1), but in one eel. Additionally, in Y-group, reproduction was limited to a 4-week period (between 12–13th and 16–17th week), whereas in N-group it was spread over 12 weeks (15–16th to 27–28th) (Figure 1).

Regarding CPE consumption (Table 1), the results showed that CPE amount (mg/Kg BW) in N-group females was statistically highest ($p < .01$) with respect to Y-group females. Likewise, the number of CPE induction resulted statistically highest ($p < .01$) in N-group with respect to Y-group (Table 1). In fact, 17-MT-treated females (Y-group) reproduced spontaneously about 6 weeks before the N-group females, saving almost 40% of CPE consumption.

With reference to the BWI, Table 2 shows no significant differences between the two experimental groups at the time of the last CPE induction and at DHP induction while the increase in BWI since the last CPE to DHP was statistically highest ($p < .05$) in the Y-group compared to N-group.

The Y-group females were more productive than N-group females. With regard to spawned eggs, data (Table 2) show statistical differences ($p < .01$) between CPE + 17-MT-treated eels and CPE-only-treated eels. In Y-group, 11 eels (91.7%) ensured an eggs production that exceeded 50% of initial BW, whereas in N-group only three eels (25%) have exceeded this value. Females ensuring the highest eggs yield were Y-054 in absolute terms (625 g) and Y-062 in relative terms (80.06% BW), while the less productive female was N-001 (150 g and 18.06% BW respectively).

4 | DISCUSSION

This study examined the effects of androgen 17-methyltestosterone on zootechnical performances of female silver European eels in the artificial reproduction.

Concerning artificial induction of maturation in female silver eels, our study demonstrated that females respond to androgen exposure in terms of hormonal response time at CPE treatments and in terms of eggs productivity. The results suggest that 17-MT may be directly involved in the improvement of the artificial reproduction of female silver European eels reducing the time to complete gonadic maturation by several weeks. Indeed, in similar trials (Di Biase et al., 2016; Mordenti et al., 2013, 2014), wild eels from the same area (Northern Adriatic Sea) subjected to hormonal treatment with CPE only, rarely reached full gonadal maturation before 20th week.

Lin, Zhang, Zhang, Van Der Kraak and Peter (1991) and Lin, Zhang, Wang and Chen (1998) demonstrated in *A. japonica* that multiple injections and implantations of 17-MT were effective in stimulating an increase in ovarian development. Furthermore, Wang and Lou (2006) demonstrated that in Japanese eel gonadal development was significantly induced by co-treatment with salmon pituitary homogenate and 17-MT and Wang and Lou (2007) suggested that the androgen was not only converted to oestrogens by aromatase, but also had a potential usefulness to the process of vitellogenesis and oogenesis. More recently, Lokman et al. (2015) in *A. australis* and Di Biase et al. (2017) in *A. anguilla* demonstrated that female silver eels strongly respond to androgen exposure during artificial induction of maturation with CPE. In addition, a sustained-release androgen implant during artificial maturation by CPE injection requires less time in induction, without any negative effect on quality of obtained eggs. Additionally, the data demonstrated that it is possible to obtain the same results without hypophysation with CPE during the first 4–5 weeks (Di Biase et al., 2017). The same authors underline that the amount of time, and consequently, of handling, hormone and money, needed to reach the pre-ovulatory stage is notably decreased in female eels pre-treated with 17-MT. Finally, in *A. japonica*, it has been underlined that a reduction in times of gonadic maturation and of post-ovulatory aging lead to a better quality of ovulated eggs and to an increase in larval survival (Nomura et al., 2013; Okamura et al., 2014). Indeed, the success rates of induced maturation and production of viable eggs in *A. anguilla* were generally low compared with other "Anguillan" species, caused

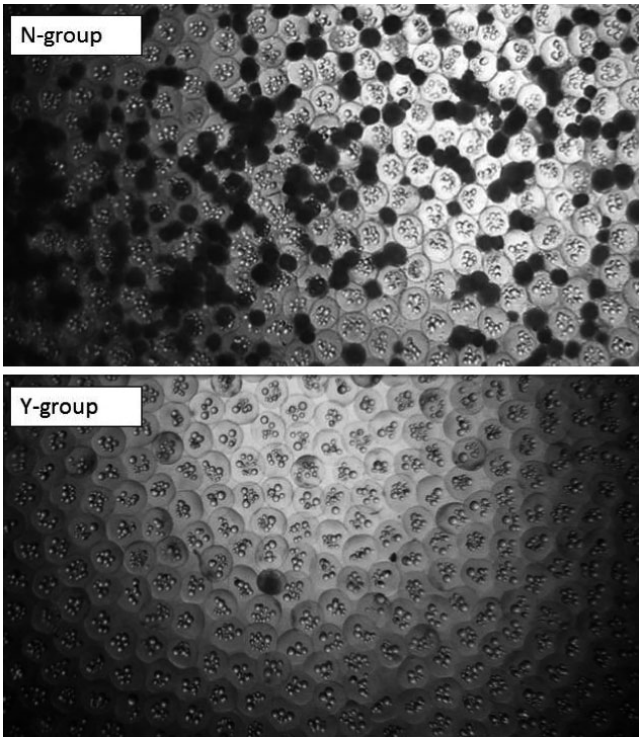

FIGURE 2 Fully transparent oocytes (FTO) in pre-ovulatory phase

by low responsiveness of their gonads to exogenous hormones (Okamura et al., 2014). In conclusion, the use of 17-MT in artificial reproduction of eel seems advisable not only for reduction of time to maturation and of CPE injections number, but also for the production of good-quality eggs in terms of hatching and/or fertilization rates.

An exciting finding from present study is the fact that the use of androgen implants in artificial reproduction of European eel seems to lead to an acceleration in female maturation as well as to a synchronization of gonadal maturation. This is a very important aspect, as the hormonal answer of wild eel is often heterogeneous (Mordenti et al., 2012, 2013) and usually 11–29 weeks are required before reaching the final maturation phase of oocytes by weekly injections of SPE or CPE (Di Biase et al., 2016; Okamura et al., 2014; Palstra et al., 2005; Pérez et al., 2011). Furthermore, this synchronization process agrees with what observed by Wang and Lou (2007) in Japanese eel, where the methyltestosterone causes the phenomenon of synchronous development when it combines with SPH treatments. It should be also noticed that the synchronization in female maturation is desirable in artificial reproduction because the time window for high-quality eggs is very narrow (Ijiri et al., 2011). Finally, having defined times in gametes emission by breeders is a mandatory and successful prerequisite for embryonated eggs management in aquaculture. The mechanism beneath this synchronization could be linked to the increased amount and/or the activation of androgen receptors at the teca and granulosa level, as shown in Japanese eel (Tosaka et al., 2010). Another important effect of 17-MT could be the increase in vitellogenin (VTG) synthesis. Indeed, 17-MT proved to be converted in fathead minnow to 17-methylestradiol, which activates the hepatic oestrogen receptors, leading to VTG production (Hornung et al., 2004). Although these two parameters have not been quantified in present study, a combination of androgen receptors activation and an increase in vitellogenin synthesis cannot be excluded as basic mechanism leading to synchronization of oocyte maturation and the improved quality of eggs.

One last aspect derived from this study is a better response to treatment with DHP of females given a sustained-release androgen implant, and a higher relative fertility of these same females.

Probably, the treatment with 17-MT lead to a higher percentage of FTO in pre-ovulatory phase (Di Biase et al., 2016; Palstra et al., 2005) and consequently to a higher production of spawned eggs (Figure 2). Indeed, Di Biase et al. (2016) observed a positive correlation in *A. anguilla* between the FTO and the relative weight of spawned eggs. Furthermore, sustained-release androgen implants resulted in fully matured oocytes of Japanese eel (Lin et al., 1998). Lokman et al. (2015) demonstrated that 17-MT implants in silver female shortfins eel induced a number of readily identifiable effects on body weight (increased), on GSI (increased), on oocyte diameters (increased). They are also likely to reflect their importance for a suite of physiological events, including oogenesis.

In summary in our study, silver European eels strongly respond to androgen exposure in terms of time of hormonal response to CPE treatments, in terms of synchronization of gonadal maturation and in terms of eggs production. The data suggest that incorporation of androgen into the induced spawning protocol is an improvement that may help us get a step closer to completing the breeding of European eel in captivity.

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