DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE

CORSO DI LAUREA IN BIOLOGIA MARINA

Gonadotropin plasmid gene therapy as an alternative method for oogenesis induction in European eel females (*Anguilla anguilla* Linnaeus, 1758)

Terapia genica con plasmidi per stimolare le gonadotropine come metodo alternativo per l'induzione dell'oogenesi nelle femmine di anguilla europea (Anguilla anguilla Linnaeus, 1758).

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Abstract

This study evaluates the feasibility of plasmid-based gene therapy as an alternative to induce oogenesis in European eel (Anguilla anguilla) females. The research evaluated the use of plasmids encoding the β subunits of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), administered via electroporation into skeletal muscle. The treatments lasted 12 weeks, with injections performed every three weeks. The results showed a significant increase in gonadosomatic and ocular indexes and plasma levels of testosterone, 11-ketotestosterone, and estradiol in plasmid-treated groups compared to the control one. Furthermore, advanced progression in oocyte development was observed, reaching mid- and late-vitellogenic stages in treated eels.

This approach offers significant advantages over traditional hormonal methods, including a more gradual and sustained response and reduced stress from repeated injections. Gene therapy, aligning with sustainable aquaculture practices, provides an innovative solution to overcome reproductive barriers in this critically endangered species, contributing to the sustainability of aquaculture and the conservation of European eel populations.

Riassunto

Questo studio esplora una nuova possibilità per stimolare la riproduzione nelle femmine di anguilla europea (*Anguilla anguilla*) attraverso l'uso della terapia genica con plasmidi. Invece di ricorrere ai metodi ormonali tradizionali, i ricercatori hanno testato plasmidi che codificano per le subunità β delle gonadotropine FSH (ormone follicolo-stimolante) e LH (ormone luteinizzante). Questi sono stati somministrati tramite elettroporazione nel muscolo scheletrico, con un ciclo di iniezioni ripetute ogni tre settimane per un totale di 12 settimane. Rispetto ai metodi ormonali convenzionali, questa tecnica offre diversi vantaggi: garantisce una stimolazione più graduale e prolungata, riducendo lo stress legato alle iniezioni frequenti. Inoltre, si inserisce perfettamente nelle pratiche di acquacoltura sostenibile, rappresentando una strategia innovativa per superare le difficoltà riproduttive di una specie in pericolo critico. Un passo avanti sia per la conservazione dell'anguilla europea che per la sostenibilità della sua produzione in acquacoltura. I risultati sono stati promettenti: le anguille trattate hanno mostrato un significativo aumento dell'indice gonadosomatico e oculare, oltre a livelli più elevati di testosterone, 11-chetotestosterone ed estradiolo rispetto al gruppo di controllo. Anche lo sviluppo ovocitario ha registrato un netto

avanzamento, con molte femmine che hanno raggiunto le fasi medie e avanzate della vitellogenesi.

1 Introduction

1.1 *Economic and Biological Importance of the European Eel*

The European eel (*Anguilla anguilla*) is a catadromous species of significant economic and ecological importance. This species undergoes a unique life cycle characterised by two long transoceanic migrations. After a growth phase in freshwater or brackish environments, adult individuals migrate thousands of kilometres to the Sargasso Sea, where spawning occurs. Juveniles, known as leptocephali, embark on a long journey back to European waters to complete their growth cycle (fig.1) (Asturiano, 2020; Mazzeo et al., 2014).

Economically, the European eel represents a high-value product, particularly in Asian and European markets, where it is valued both for direct consumption and its use in traditional dishes (Zhao & Wu, 2024).

Aquaculture depends on capturing wild juveniles (“glass eels”), which are then raised in farms to commercial size. However, this practice has exacerbated the pressure on natural stocks, which have declined dramatically in recent decades.

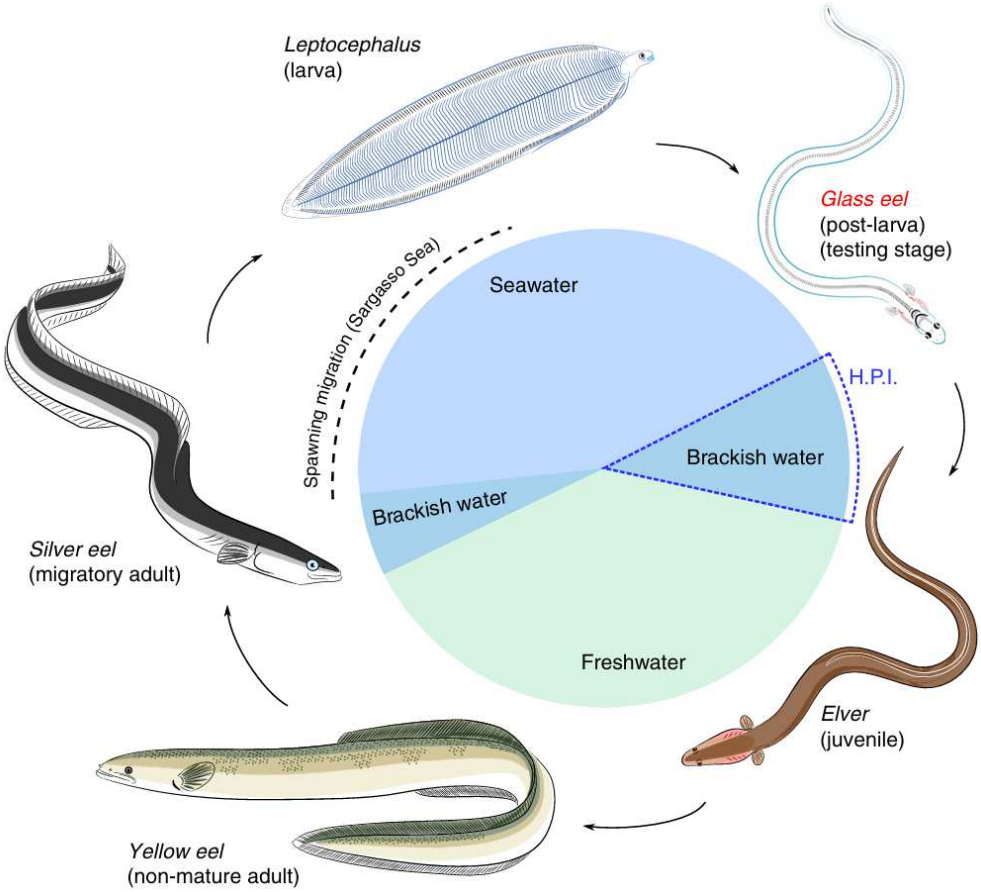

Figure 1 *The life cycle of the European eel (Anguilla anguilla). Eels are born as leptocephalus larvae in the Sargasso Sea. In their larval stage, they are carried by ocean currents across the Atlantic to the continental slope of Europe, where they transform into young, transparent eels known as glass eels. These glass eels migrate along the continental shelf and eventually reach the brackish waters of estuaries. Later, they undergo a metamorphosis into pigmented juveniles, called elvers, and begin their journey upstream into freshwater, where they grow into adult yellow eels. After several years, the yellow eels undergo another transformation into silver eels and embark on a long migration of thousands of kilometres back to the Sargasso Sea, where they spawn and complete their life cycle. A dashed blue polygon indicates the Hypothetical Period of Imprinting (H.P.I.). Artwork credit A. Cresci. (Cresci et al. 2019)*

The global population of the European eel has decreased by 95-99% compared to the 1960s-1980s, leading the International Union for Conservation of Nature (IUCN) to classify the species as “critically endangered” (Casalini et al., 2024). A similar decline has been observed in the Japanese eel (*Anguilla japonica*), where environmental factors such as temperature and photoperiod have been shown to influence reproductive cycles and physiological processes (Byun et al., 2025). Biologically, the European eel provides a unique model for studying reproductive physiology in teleosts. Its ability to complete such extensive migrations, combined with semelparous reproduction, offers insights into complex hormonal and physiological regulation (Asturiano, 2020; Muñoz-Cueto et al., 2020).

Recent studies on the Japanese eel have revealed a close interplay among melatonin, dopamine, and gonadotropin-releasing hormone (GnRH), which regulates ovarian development. Dopamine suppresses GnRH-induced luteinising hormone (LH) secretion, creating a major neuroendocrine bottleneck for reproduction in captivity (Vidal et al., 2004). Despite its biological significance, closing the reproductive cycle in captivity remains challenging.

Recent studies have suggested that eels captured during the silvering stage exhibit varying degrees of gonadal maturation readiness,

influenced heavily by environmental conditions such as brackish habitats (Casalini et al., 2024). Addressing these challenges is critical for reducing pressure on natural stocks and ensuring sustainable aquaculture.

1.2 *Challenges of captive reproduction*

Reproducing the European eel in captivity is hindered by biological and environmental barriers. Adult eels fail to complete gonadal maturation under artificial conditions, remaining arrested in the pre-pubertal stage.

This inhibition is attributed primarily to dopamine's suppression of gonadotropin-releasing hormone (GnRH), the neuropeptide that activates the hypothalamus-pituitary-gonad (HPG) axis (Vidal et al., 2004).

Under normal conditions, GnRH stimulates the secretion of follicle-stimulating hormone (FSH) and luteinising hormone (LH), which are essential for gametogenesis and oocyte maturation (Muñoz-Cueto et al., 2020; Casalini et al., 2024). (Fig.2)

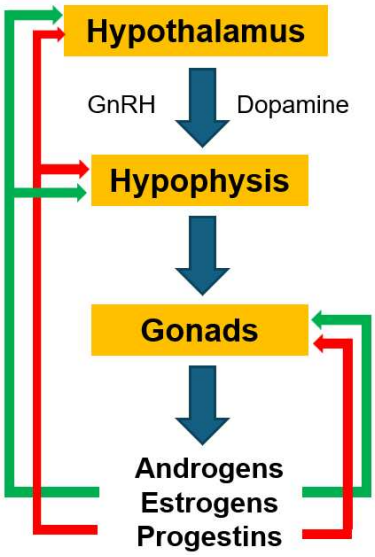

Figure 2 *Hypothalamus-pituitary-gonad (HPG) axis. Sex hormones regulate the endocrine system through two feedback mechanisms. In the case of negative feedback, indicated in red, these hormones inhibit the release of GnRH from the hypothalamus and the secretion of gonadotropins from the pituitary gland, thus contributing to maintaining hormonal homeostasis. In contrast, positive feedback, represented in green, occurs when estrogens stimulate the release of GnRH and gonadotropins.*

Studies on the silvering process in Adriatic eels highlighted the critical role of brackish lagoons in accelerating gonadal development, where favourable trophic conditions promote rapid growth and maturation readiness (Casalini et al., 2024). Captive environments without such stimuli often lead to incomplete oocyte development and poor gamete quality. Additionally, variations in melatonin secretion, induced by photoperiod and lunar phases, have been observed in Japanese eels, underscoring the hormone's role in synchronising oogenesis and

reproductive activity. Temperature fluctuations also play a significant role in eel reproduction. Artificial environments with constant temperatures often fail to mimic natural migration conditions, which are critical for triggering hormonal cascades essential for maturation (Byun et al., 2025). Therefore, replicating temperature cycles observed during natural migrations may enhance gonadal development and gamete quality (Peñaranda et al., 2016; Rozenfeld et al., 2019; Ferrão et al., 2024).

1.3 *Limitations of traditional hormonal treatments*

Traditional hormonal treatments, such as the use of carp (cPE) or salmon pituitary extracts (sPE) in females or human chorionic gonadotropin (hCG) in males, remain the most common methods to induce sexual maturation in eels. However, these approaches have significant limitations. Pituitary extracts lack precise control over the FSH-to-LH ratio, essential for vitellogenesis and oocyte maturation. This hormonal imbalance often leads to poor egg quality and incomplete maturation (Mazzeo et al., 2014; Casalini et al., 2024).

The response to hormonal treatments also varies with ovarian developmental stages: FSH predominates during early vitellogenesis, while LH is crucial for final maturation and ovulation. Not considering these temporal dynamics often results in ineffective treatments and suboptimal outcomes (Mazzeo et al., 2014; Peñaranda et al., 2018).

Although cPE and sPE are effective, they are not species-specific and may elicit adverse immune responses in females, necessitating repeated administrations due to their short half-life (Pérez et al., 2011). Frequent injections can also cause stress in captive eels, further compromising gamete quality and overall reproductive success.

Environmental factors such as temperature fluctuations further complicate treatment efficacy. Studies have shown that variable thermal regimes, which mimic natural migration conditions, significantly enhance ovarian development and gamete quality compared to static temperatures (Mazzeo et al., 2014; Byun et al., 2025; Peñaranda et al., 2016).

In the last years, recombinant gonadotropins (rGths) have been tested as alternative treatments (reviewed by Molés et al., 2020). These species-specific molecules, produced in eukaryotic systems, provide improved stability and biological activity, but their high production costs limit large-scale applications (Asturiano, 2020). Furthermore, Peñaranda et al. (2018) reported the first application in European eels, showing that a combination of rFSH and rLH induces full maturation and spermiogenesis. However, challenges remain regarding sperm quality variability and the economic feasibility of these treatments.

1.4 *Gene therapy as a new frontier*

A significant advantage of gene therapy lies in its species-specificity and prolonged effect. Using muscle-specific promoters ensures localised and sustained gonadotropin expression, minimising off-target effects while improving treatment efficiency. Unlike traditional methods, plasmid-based approaches align with sustainable aquaculture practices, as they are cost-effective and non-integrative, reducing risks of mutagenesis (Molés et al., 2020). Combined with advances in genomic research, these strategies offer a potential solution to overcoming the challenges of European eels reproduction in captivity.

Recent studies have highlighted that plasmid-based therapies could represent a promising alternative for European eel reproduction, but they still need testing. Plasmids encoding the β subunits of FSH (fshb) and LH (lhb) can be introduced into muscle cells via electroporation, stimulating endogenous gonadotropin production. This method circumvents the need for repeated hormone administration, reducing stress and treatment costs (Molés et al., 2020).

Moreover, these therapies have significantly increased plasma estradiol levels and upregulated key genes involved in vitellogenesis, such as *cyp19a1* and estrogen receptors (*esr1*) in *Epinephelus coioides* (Lv et al., 2018). This upregulation aligns with findings in Japanese eels, where

increased *cyp19a1* expression correlates with heightened estradiol levels, supporting mid-to-late vitellogenesis (Byun et al., 2025).

First applications of plasmid-based therapies have been tested in other teleost species, such as European sea bass (*Dicentrarchus labrax*), where plasmids encoding single-chain gonadotropins (scFSH and scLH) were successfully employed. These studies demonstrated that electroporation-facilitated gene transfer can lead to prolonged gonadotropin expression in muscle, increased steroid hormone production, and enhanced spermatogenesis in males (Mazón et al., 2013, 2014). Similarly, in European eels, experimental treatments with scFSH and scLH plasmids triggered steroidogenesis and spermatogenesis in males, with the combined administration of both plasmids producing more consistent results (Zapater et al., 2023).

Preliminary data suggest that these therapies could be further adapted to stimulate ovarian development in female European eels, offering a promising strategy for the future of sustainable aquaculture and aligning with the research goals highlighted in this study (Zapater et al., 2024).

2 Objectives of the study

The primary objective of this study is to test the applicability of plasmid-based gene therapy to improve European eel oogenesis in captivity, using plasmids containing the genes for the β-subunits of follicle-stimulating hormone (FSH) or luteinizing hormone (LH), administered via electroporation into the skeletal muscle of female European eels (*Anguilla anguilla*).

The study determines if they can stimulate the endogenous production of gonadotropins and promote gonadal maturation without using exogenous hormones, becoming an alternative method to induce oogenesis in females of this species. This approach can potentially represent an innovative solution for improving the captive reproduction of European eels, thereby contributing to the conservation of the species and the sustainability of aquaculture.

3 Materials and methods

This study was carried out in strict accordance with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Experimental Animal Ethics Committee of the Universitat Politècnica de València (UPV), and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2023-VSC-PEA-0039).

3.1 Animal and maintenance conditions

Immature female specimens of *Anguilla anguilla* were obtained from the Albufera of Valencia, Spain. The fishermen of the Albufera lagoon caught the fish. Subsequently, they transported them to the aquaculture facilities of the Universitat Politècnica de València (UPV, Spain), where they were acclimated and maintained, according to Peñaranda et al. (2018). Forty-two specimens were used, with an average weight of 756.95 ± 12.74 g.

3.2 Plasmid construction and purification

For the plasmids of Fsh and Lh from European eel, these were constructed from the available sequences in GenBank for the β and α

subunits (GenBank accession number: CAA43373.1, (common α subunit), βfsh (AAN73407.1), and βlh (CAA43374.1)) to obtain the single-stranded Gths. At the 5' end, the cDNA encoding the Fsh-β or Lh-β subunits was placed, followed by a sequence in-frame to code for six histidines, the C-terminal peptide (CTP) of hCG and the common α subunit. These constructs were cloned into the pcDNA3 expression vector (Invitrogen) by a commercial company, Gencript.

For the extraction and purification of plasmids containing recombinant Lh and Fsh gonadotropins, the QIAGEN® Plasmid Mega kit (QIAGEN Inc.) was used.

3.3 Experimental design

The experiment was conducted on female *A. anguilla* with three experimental treatments:

1. Animals injected with 200 μg of the plasmid encoding the gonadotropin Fsh.
2. Animals injected with 150 μg of the plasmid encoding Fsh plus 150 μg of the plasmid encoding the gonadotropin Lh.
3. Control animals injected with the empty plasmid (pcDNA3).

The animals were anaesthetised prior to injection into the dorsal muscle. The injections were followed by *in vivo* electroporation using needle

array electrodes and the ECM830 electroporator (BTX), according to the protocol previously applied to European sea bass by the receiving group (Mazón et al., 2014). (fig.3)

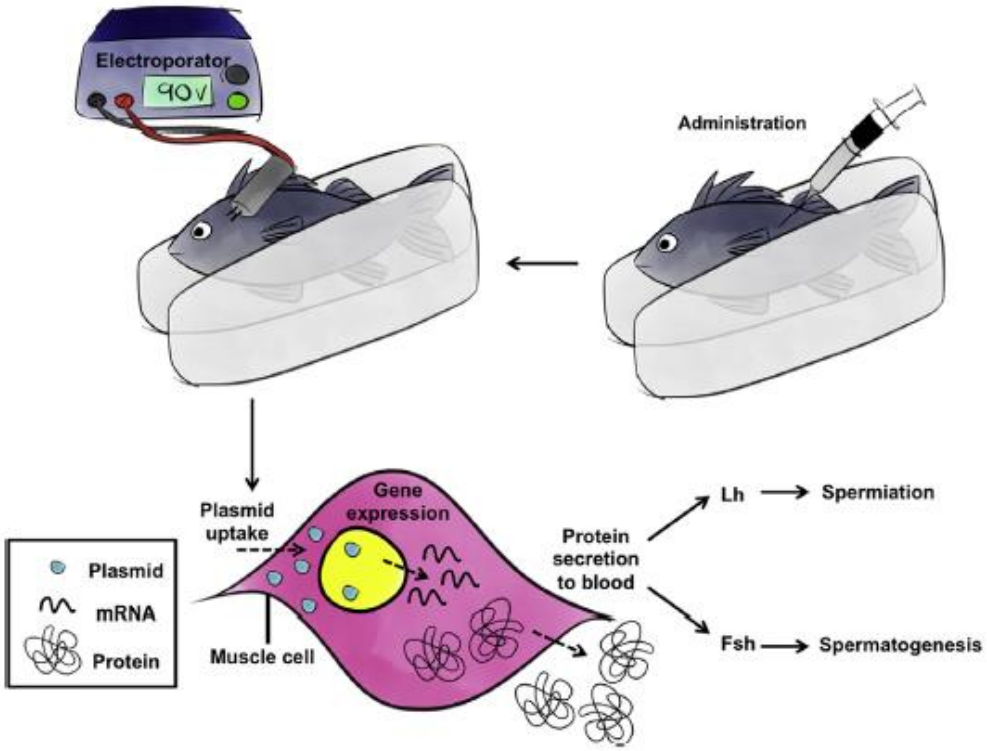

Figure 3 *Schematic representation of the somatic gene transfer technique applied in systemic treatments of Lh and Fsh to male sea bass, a method used by Mazon et al. (2015); this same system was also applied to female eels in this study.*

The experiment lasted 12 weeks, with plasmids injections administered on days 0 and 3 every three weeks, from March 22, 2024, to June 17, 2024.

Biometric measurements were conducted every three weeks throughout the experimental period. These assessments included body weight, total body length, horizontal and vertical eye diameters, and skin and fin

colouration observations across all experimental groups.

To evaluate the progression of maturation, blood samples were collected at the time of injection for steroid level analysis (Peñaranda et al., 2010).

Animals were sacrificed at the end of the experiment to determine the GSI and HSI and to perform macroscopic and histological analyses of the gonads. All efforts were made to minimise suffering. Fish were sacrificed with benzocaine overdose, followed by decapitation.

3.4 Biometric parameters

Before the biometric parameter assessment, the animals were anaesthetised using benzocaine at a concentration of 60 ppm to ensure their welfare and minimise stress during the procedures.

Body weight

A precision electronic balance measured individual body weight (BW) in grams (± 0.01 g).

Hepatosomatic index and gonadosomatic index

The HSI and GSI were calculated using the following formulas:

- HSI (%) = (Liver weight / Body weight) × 100
- GSI (%) = (Gonad weight / Body weight) × 100

Liver and gonad weights were determined to the nearest 0.01 g through dissection under controlled laboratory conditions.

Eye index

The eye index (EI) was calculated following the method described by Pankhurst (1982):

$$EI = \left[\frac{\pi \left(\frac{D_h + D_v}{4} \right)^2}{L_t} \right] \times 100$$

Where:

- D_h = Horizontal eye diameter (mm)
- D_v = Vertical eye diameter (mm)
- L_t = Total body length of the eel (mm)

Measurements were performed using precision callipers, ensuring proper positioning of the eels to minimise errors.

Skin and fin colouration

Skin and fin colouration (SC and FC) were assessed visually and categorised into distinct stages for consistency:

- 0: Transparent
- 1: Light grey

- 2: Dark grey
- 3: Black

Skin colouration was evaluated along the dorsum, while fin colouration was focused on the pectoral fins. Observations were conducted under standardised lighting conditions to reduce variability.

3.5 Hormone concentration in blood plasma

Blood samples were collected using heparinised syringes and centrifuged at 3500 rpm for 15 min at 4 °C to obtain blood plasma stored at -80 °C until further steroid analysis.

Testosterone

The plasma levels of testosterone (T) were determined by a specific immunoassay (EIA) developed by Rodríguez et al. (2000) for sea bass. First, plasma samples were extracted with methanol, the organic solvent was evaporated, and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA) by vortexing. The assay was performed in a total volume of 150 µl, composed of 50 µl of standard testosterone or plasma extract, 50 µl of diluted enzymatic tracer (0.083 IU/ml; E-AChE, Cayman Chemical, MI, USA) and 50 µl of specific antiserum (1/3.000.000). All the dilutions were made in EIA buffer. The competition was left overnight at 4 ºC. The plates were washed, and the

addition of Ellman's reagent (200 µl per well) revealed the bound enzymatic activity. The enzymatic reaction was left to develop for 4 h in the dark at room temperature under slight agitation; the absorbance at 414 nm was then measured. The assay sensitivity of testosterone was 0.010 ng/ml.

11-Ketotestosterone

The plasma levels of 11-ketotestosterone (11-KT) were determined by an EIA developed for the Siberian sturgeon (Cuisset et al., 1994) and modified for its use in sea bass (Rodríguez et al., 2005). First, plasma samples were extracted with methanol, the organic solvent was evaporated, and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA) by vortexing. The assay was performed in a total volume of 150 µl, composed of 50 µl of standard 11-KT or plasma extract, 50 ul of diluted enzymatic tracer (0.1 IU/ml; E-AChE, Cayman Chemical, MI, USA) and 50 µl of specific antiserum (1/320,000). All the dilutions were made in EIA buffer. The competition was left overnight at 4 ºC. The plates were washed, and the addition of Ellman's reagent (200 µl per well) revealed the bound enzymatic activity. The enzymatic reaction was left to develop for 4 h in the dark at room temperature under slight agitation; the absorbance at 414 nm was then measured. The assay sensitivity of 11-KT was 0.0012 ng/ml.

Estradiol

The plasma levels of estradiol (E2) were measured by a conventional enzyme immunoassay (EIA), and it was validated for its use on sea bass in the Institute of Aquaculture of Torre la Sal (CSIC), Spain. First, plasma samples were extracted with methanol, the organic solvent was evaporated, and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA) by vortexing. The assay was performed in a final volume of 150 µl in 96-well microtiter plates coated with mouse anti-rabbit IgG monoclonal antibodies (Clone RG-16, Sigma-Aldrich, Inc). Each component, i) the E2 acetylcholinesterase conjugate (E-AChE, Cayman Chemical, MI, USA) used as tracer (0.083 IU/ml), ii) a specific anti-E2 rabbit antiserum (diluted to 1:2,500,000), iii) E2 standards (ranging from 80 ng/ml to 0.039 ng/ml) or samples, were added in a volume of 50 µl. Plates were incubated overnight at 37 ºC, rinsed, and colour development was performed by adding 200 µl/ well of Ellmans's reagent and incubation under gentle agitation for two h at 20 ºC in the dark. Optical density was read at 405 nm in a microplate reader (Bio-Rad microplate reader model 3550). The assay's sensitivity was around 0.156 ng/ml (Bi/B0 = 90%), and half-displacement (Bi/B0 = 50%) occurred around 1.90 ng/ml.

3.6 Analysis of ovarian tissues

Macroscopic analysis

Macroscopic analysis of the gonads was performed during the final sampling after 12 weeks of treatment to evaluate the external and structural characteristics of the ovaries. Dissections were conducted on all females from the control and experimental groups using sterilised instruments to ensure optimal hygienic conditions.

After the gonads were removed, they were visually examined to document differences in size, shape, surface structure, and pigmentation. Macroscopic observations included the assessment of ovarian volume, surface appearance (degree of convolutions and compactness), pigmentation (ranging from dark to lighter shades such as whitish or pinkish tones), and tissue texture. Each sample was photographed using a high-resolution digital camera to document the observed macroscopic characteristics, allowing direct comparison between the treatment and control groups. Images were taken under standardised lighting conditions to minimise visual variability. The recorded parameters were used to identify distinctive signs of ovarian maturation, such as increased volume, greater surface convolutions, and pigmentation changes, which were later correlated with histological observations.

Histological analysis

A gonadal fragment from each specimen was preserved in 1% glutaraldehyde fixative following the method described by McDowell and Trump (1976). The samples were then subjected to a graded ethanol dehydration series (75–90%) before being embedded in Technovit resin (Kulzer). Semi-thin sections (2 µm) were obtained using a Reichert-Jung microtome and subsequently stained with the Cleveland-Wolfe method (Herlant, 1960). Microscopic examination and imaging were performed at 20x magnification using a Nikon Eclipse E600 microscope (Nikon Instruments Europe).

Vitellogenesis stage: classification of oocytes according to their development

The staging system used in this study was adopted from the classification proposed by Pérez et al. (2011). The previtellogenic (PV) stage is characterised by oocytes at the perinucleolar stage, with few or no lipid droplets, or by oocytes in the lipid droplet stage but lacking yolk vesicles (Fig. 1). The subsequent early vitellogenic (EV) stage is defined by small yolk vesicles confined to the periphery of the oocyte. The mid-vitellogenic stage is characterised by abundant yolk vesicles, clearly visible and distributed throughout the cytoplasm from the membrane to the nucleus, with lipid droplets more abundant than yolk vesicles. Finally,

in the late vitellogenic stage, the yolk vesicles are enlarged and more numerous than in the previous stages.

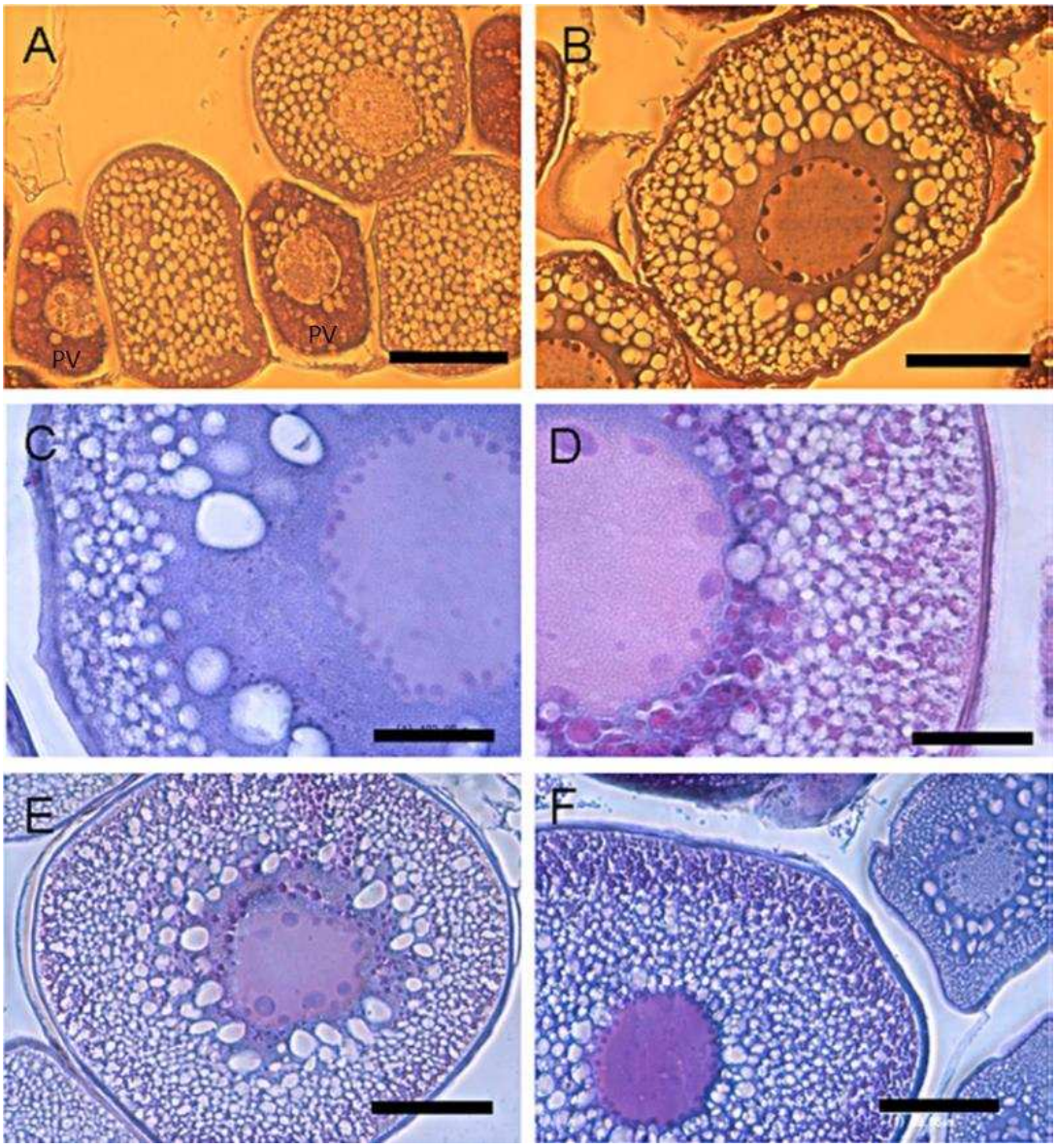

Figure 4 *Different oocyte development phases in European eel females. (A) Previtellogenic stage. (B) Lipid droplet stage. (C) Early vitellogenic oocyte. (D) Mid-vitellogenic oocyte. (E) Mid-vitellogenic oocyte. (F) Late-vitellogenic oocyte. Scale bar: 100 µm. (Source: Pérez et al., 2011).*

Oocyte diameter

The diameters of the oocytes were measured using ImageJ software, version 1.54. All images of ovarian tissue sections were calibrated to a fixed scale, where 488 pixels corresponded to 100 μm.

At least 50 measurements of the largest diameter oocytes were made for each eel with the Camera Control Unit software (Pérez et al., 2011).

3.7 Statistics

Data is presented as SEM ± mean. Normality was verified with the Shapiro-Wilk test.

Before performing statistical analysis on the percentage values, a base-10 logarithm transformation was applied to all values.

The size of oocytes showed a non-normal distribution in all groups, with p-values lower than 0.05 in the normality test.

For the Ocular Index, GSI, body weight, T, 11KT, and E2 tests confirmed normal distribution with p-values above 0.05.

For HSI, non-normal distributions in the FSH and FSH + LH groups were observed, with p-values below the significance threshold (0.05) in the normality test.

Kruskal-Wallis test with Dunn’s posthoc test

The Kruskal-Wallis test was used to analyse variables when the data did not follow a normal distribution. This non-parametric test compares the means among groups and is particularly useful for asymmetric or skewed distributions. It was applied to non-normally distributed data, such as oocyte size and HSI, to determine whether significant differences existed between group means.

Mixed-effects model

For variables measured repeatedly over time, such as the Eye Index and body weight, a mixed-effects model (REML) was applied, which accounts for the correlation between repeated observations within the same animal. The REML allows separate estimation of fixed effects (e.g., time and treatment) and random effects (e.g., inter-subject variability).

One-way ANOVA

A one-way ANOVA test was applied for variables with a normal distribution, such as GSI and plasma concentrations of E2, 11-KT, and T. This parametric test allows for the comparison of means across groups, assuming that the data are normally distributed and that variance between groups is homogeneous.

4 Results

4.1 Biometric parameters

Body weight

No statistically significant differences were observed among the three experimental groups at any of the sampling points or throughout the experiment, as no significant interaction was detected between the time factor and the experimental groups.

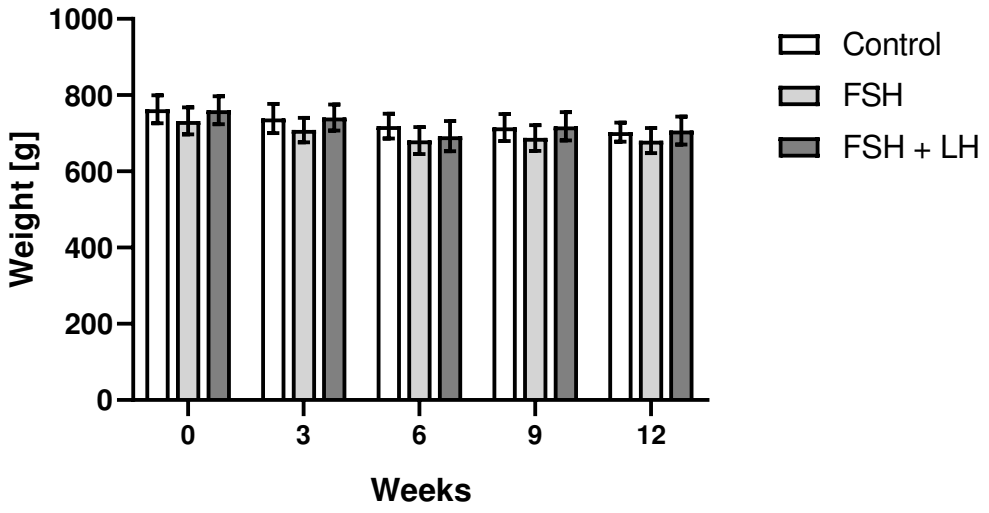

Figure 5 *Body weight of the European eel females (n = 7 fish/control/week; n = 13 fish/FSH/week; n = 11 fish/FSH+LH/week) measured at the different sampling points (weeks 0 to 12). Data represent the mean ± SEM of each group and sampling point.*

Hepatosomatic index and gonadosomatic index

The experimental treatments FSH and FSH + LH did not produce statistically significant effects on HSI values compared to the control group after 12 weeks of experiment (Fig. 3.A).

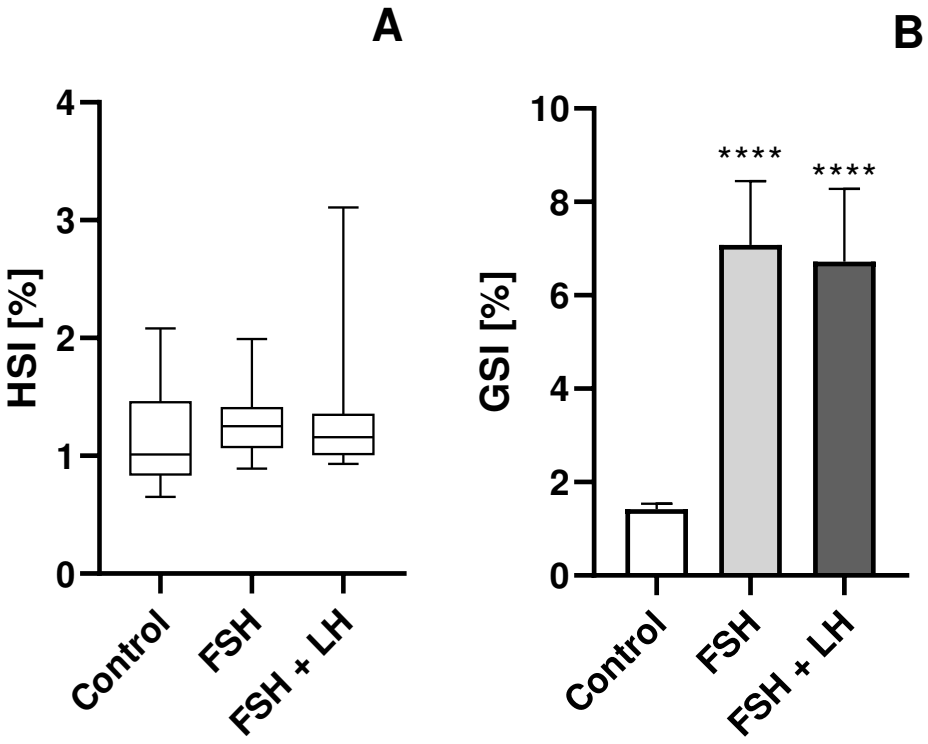

Figure 6 Hepatosomatic (A) and gonadosomatic (B) index of European eel females (n = 7 fish/control; n = 13 fish/FSH; n = 11 fish/FSH+LH) at the end of the experiment (12 weeks). Graph (A) results are boxplots, including the median and the first and third quartiles. The central line represents the median of the values. Results for graph (B) are represented with a bar chart. Asterisks indicate statistical differences compared to control group values ($P < 0.05$; Tukey's test). A more significant number of asterisks corresponds to a higher level of statistical significance.

The mean HSI values of the three groups were similar, with substantial variability within each group, as indicated by the error bars.

The GSI results showed a statistically significant difference among the experimental groups, with increased values in the groups treated with FSH and FSH + LH compared to the control group ($p < 0.001$) (Fig. 3.B)

Conversely, no significant difference was observed between the FSH-treated and FSH + LH-treated groups.

Eye index

The progression of the eye index in experimental groups treated with different hormonal combinations (FSH and FSH+LH) and in the control group over four weeks is shown in Fig. 4. The mixed-effects model revealed a significant effect of time ($P < 0.0001$), indicating that the eye index varied significantly over the weeks.

Furthermore, a significant effect of treatment ($P < 0.0001$) was observed, highlighting differences between the treated groups and the control one.

A significant interaction between time and treatment ($P = 0.0002$) was also detected, demonstrating that the effects of the treatment were influenced by the progression of time.

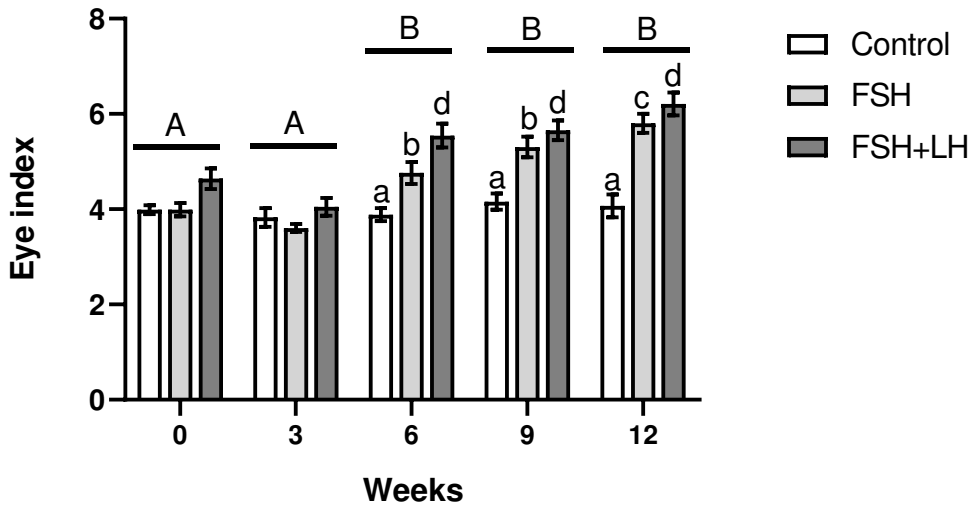

Figure 7 *Mean eye index values (n = 7 fish/control/week; n = 13 fish/FSH/week; n = 11 fish/FSH+LH/week) of experimental groups of European eel females measured at five sampling points throughout the experiment (weeks 0 to 12). Uppercase letters indicate statistical differences between weeks, while lowercase letters indicate statistical differences between treatments every week (P < 0.05; Tukey's test) between means. Data represent the mean ± SEM of each group and sampling point.*

At week 0, all groups exhibited comparable eye index values with no statistically significant differences. Similarly, in week 3, the eye index remained similar across groups.

However, starting from week 6, significant differences emerged: the group treated with FSH showed a considerable increase in the eye index compared to the control group (P = 0.0115), and the FSH+LH group exhibited a highly significant increase compared to the control (P <

0.0001). That week, no significant differences were detected between the FSH and FSH+LH groups (P = 0.0753).

In week 9, the FSH group continued to show a significantly higher eye index compared to the control group (P = 0.0016), and the FSH+LH group again demonstrated a highly significant increase compared to the control (P < 0.0001). Once more, no statistically significant differences were found between the FSH and FSH+LH groups (P = 0.4839).

In the last week, this trend persisted: the FSH group exhibited a significant increase in the eye index compared to the control group (P = 0.0002), and the FSH+LH group showed a highly significant increase compared to the control (P < 0.0001).

No significant differences were observed between the two hormonally treated groups (P = 0.4145).

Skin and fins colouration

Regarding skin and fins colouration at the end of the experiment, eel females in the control group exhibited a light and more uniform tone, with natural pigmentation and no visible changes (Fig. 5), and the pectoral fins remained grey and semi-transparent.

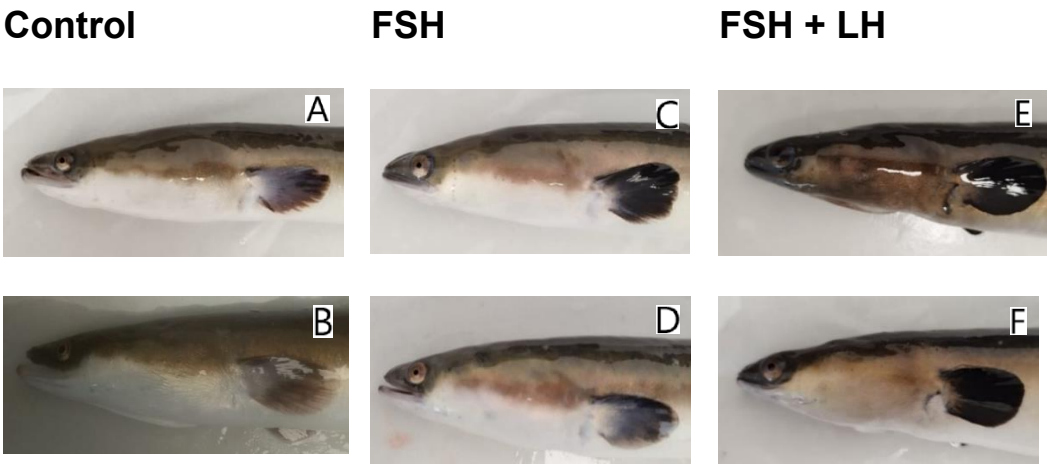

Figure 8 *Photographs taken to observe the differences in skin and pectoral fin colouration between the experimental groups of European eel females. The photographs are categorised according to the experimental groups: Control (A–B), FSH (C–D), and FSH+LH (E–F). Observations were made during the final sampling (week 12).*

A noticeable skin darkening was observed in FSH-treated eels, with more intense pigmentation than in the control group. Pigmentation was mainly concentrated in dorsal areas and along the fin edges.

The pectoral fins in this group showed a significant increase in pigmentation, with darker regions tending toward black, although it did not reach a completely black tone in some females.

In contrast, the effect was even more pronounced in FSH + LH-treated eels. Their skin was considerably darker, with intense and uniform pigmentation covering the entire body in many cases. The pectoral fins, in particular, were entirely blackened, displaying a uniform black.

4.2 Hormone concentration in blood plasma

Testosterone plasma levels

The analysis revealed that the Time factor significantly affects T plasma levels ($P < 0.0001$), indicating a significant increase in T levels over time within each treatment. Additionally, a significant effect was observed among the experimental groups ($P = 0.0009$), highlighting differences in T levels between groups at each sampling point. A significant interaction between time and experimental groups ($P < 0.0001$) demonstrates that the effect of treatments changes progressively over the weeks. (Fig.6)

At week 0, no significant differences were observed among the treatment groups. However, starting from week 3, significant differences emerged between groups. Specifically, T levels in the FSH-treated group were significantly higher compared to the control ($P = 0.0145$), while FSH + LH-treated fish showed intermediate values. This pattern became more pronounced in subsequent weeks: at week 6, T levels were significantly higher in the FSH group compared to the control ($P = 0.0040$) and in the FSH + LH group compared to the control ($P = 0.0046$).

At week 9, differences were even more pronounced, with T levels significantly elevated in the FSH group ($P = 0.0002$) and the FSH + LH group ($P = 0.0003$) compared to the control.

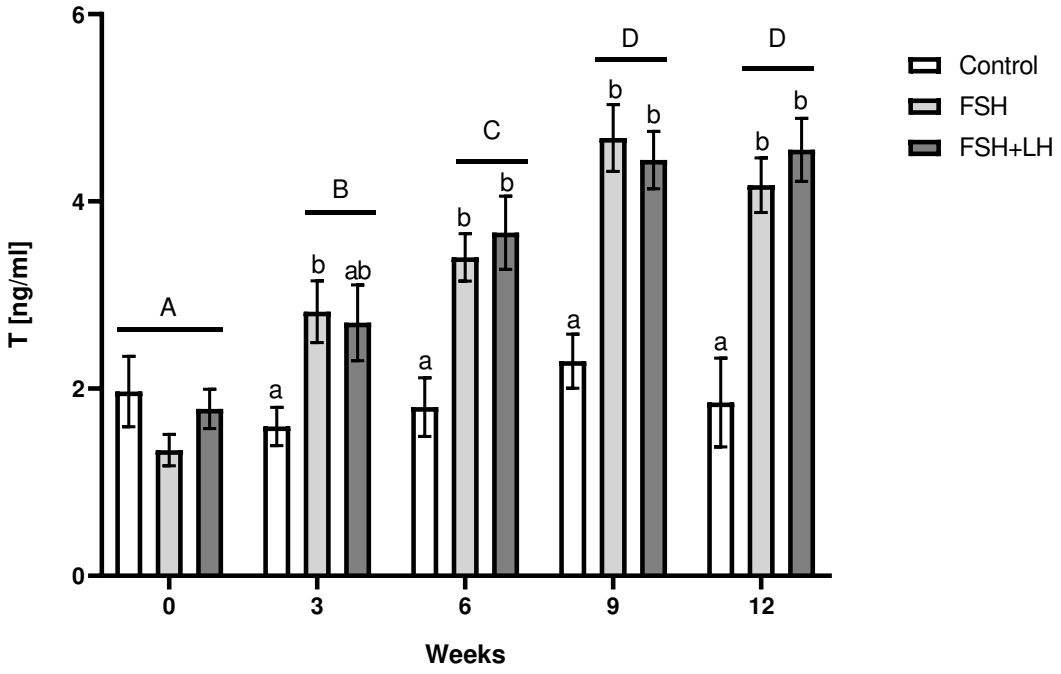

Figure 9 *Blood plasma testosterone level of eels (n = 7 fish/control/week; n = 13 fish/FSH/week; n = 11 fish/FSH+LH/week) during 12 weeks of treatment (weeks 0 to 12). Uppercase letters indicate statistical differences over time between the different samplings of the same group, while lowercase letters indicate statistical differences between treatments (P < 0.05; Tukey's test) each week. Data represent the mean ± SEM of each group and sampling point.*

At week 12, T levels remained significantly higher in the FSH (P = 0.0043) and FSH + LH groups (P = 0.0015) relative to the control. In parallel, between the groups treated within each treatment, a progressive and significant increase in testosterone levels was observed over the weeks.

11-Ketotestosterone plasma levels

Analysis revealed that the Time factor significantly affects 11-KT plasma levels (P = 0.0001). There is a significant effect between the two groups treated and the control (P = 0.0031).

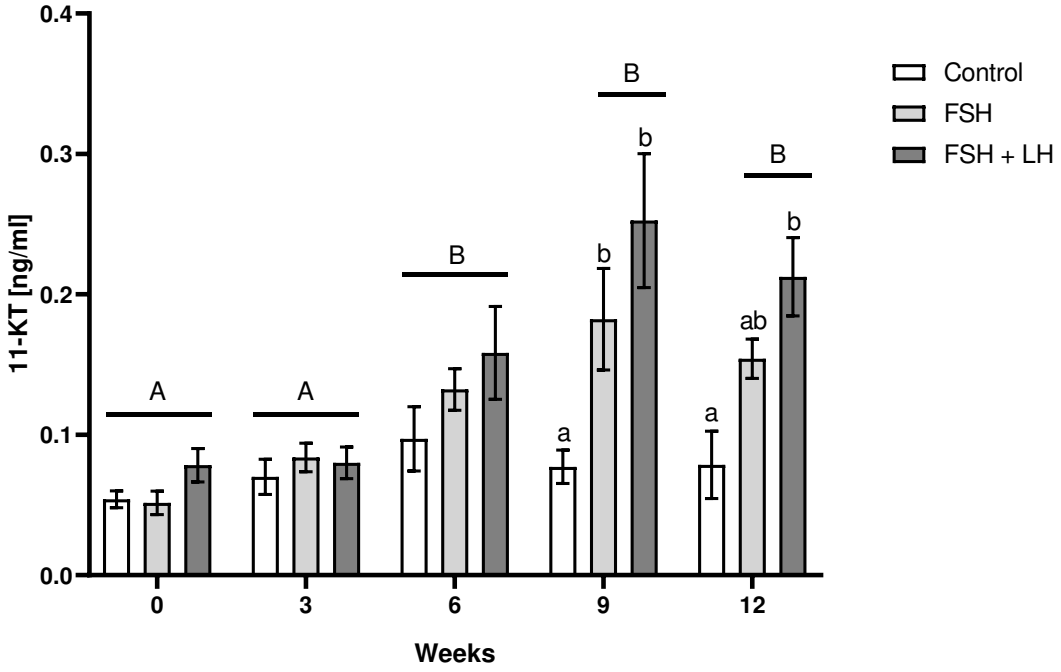

Figure 10 *Blood plasma 11-ketotestosterone level of eels (n = 7 fish/control/week; n = 13 fish/FSH/week; n = 11 fish/FSH+LH/week) during 12 weeks of treatment (weeks 0 to 12). Uppercase letters indicate statistical differences over time between the different samplings of the same group, while lowercase letters indicate statistical differences between treatments (P < 0.05; Tukey's test) each week. Data represent the mean ± SEM of each group and sampling point.*

The comparison between groups on week 9 showed significant differences between control and FSH (P = 0.0376) and between control

and FSH + LH (P = 0.0097). In week 12, there were substantial differences between the control and FSH + LH groups (P = 0.0056).

Estradiol plasma levels

Statistical analysis of estradiol plasma levels revealed significant differences among the different time points and across the groups, while the interaction between time and group factors was not significant.

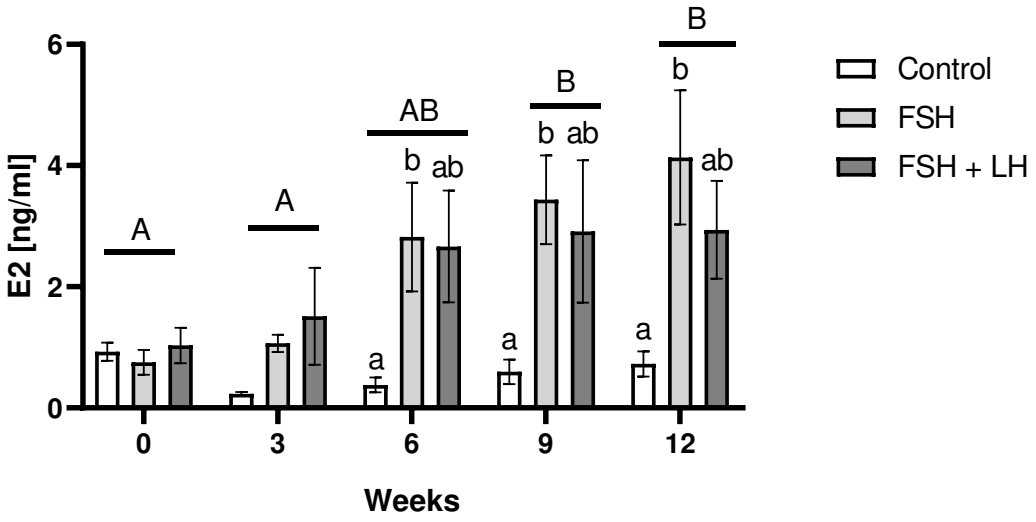

Figure 11 *Blood plasma estradiol level of eels (n = 7 fish/control/week; n = 13 fish/FSH/week; n = 11 fish/FSH+LH/week) during 12 weeks of treatment (weeks 0 to 12). Uppercase letters indicate statistical differences over time between the different samplings of the same group, while lowercase letters indicate statistical differences between treatments (P < 0.05; Tukey's test) each week. Data represent the mean ± SEM of each group and sampling point.*

Specifically, a progressive increase in blood plasma estradiol levels was observed in the FSH and FSH + LH groups compared to the control group, particularly at later time points (weeks 9 and 12).

Results from Tukey's multiple comparisons tests highlighted significant differences between the initial time points (weeks 3 and 6) and subsequent dates, with notable increases in estradiol levels on week 9 ($p = 0.0281$) and week 12 ($p = 0.0128$) compared to the first measurement (week 3). When comparing groups, estradiol levels were higher in the treated groups (FSH and FSH + LH), with no significant differences observed between the two treatments.

4.3 Analysis of ovarian tissues

Macroscopic analysis

Macroscopic analysis revealed substantial differences concerning ovaries appearance between the control and groups treated with plasmids (Fig. 9).

In the control eels, the ovaries were smaller, with a thin, linear, and underdeveloped structure. The surface exhibited darker pigmentation and limited convolutions, indicating low maturation. In contrast, an apparent increase in ovarian volume was observed in the groups treated with plasmids.

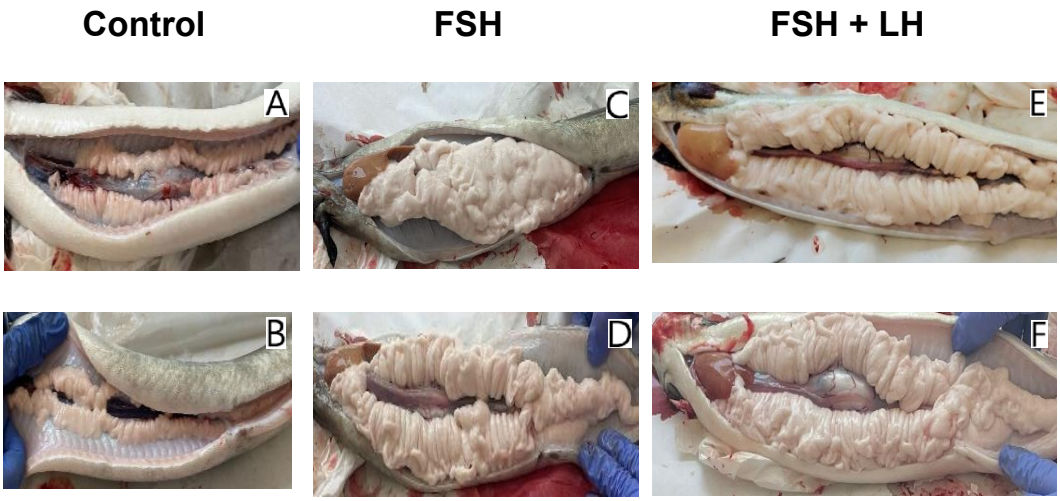

Figure 12 *Photographs were taken to analyse the macroscopic differences in ovarian development between the European eel experimental groups. The photographs are categorised according to the experimental groups: Control (A–B), FSH (C–D), and FSH+LH (E–F). Observations were performed during the final sampling (12 weeks).*

The ovaries aspect was turgid and enlarged, with a more organised structure and a highly developed, convoluted surface typical of advanced maturation. The tissue exhibited a lighter pigmentation, ranging from whitish to pinkish tones, and displayed a compact and uniform texture. The ovaries were broader and elongated, with prominent convolutions suggesting active oocyte proliferation.

Histological analysis

All females in the Control group remained in the previtellogenic stage (PV) at the end of the experiment (week 12), showing no progression in the absence of hormonal stimulation.

In the FSH group, oocyte development advanced significantly, with 53.85% of the oocytes reaching early vitellogenesis (EV), 30.77% progressing to mid vitellogenesis (MV), and 15.38% achieving late vitellogenesis (LV). Regarding the FSH + LH group, the PV stage was observed in 8.33% of the oocytes, 50% progressed to EV, 25% reached MV, and 16.67% were in LV.

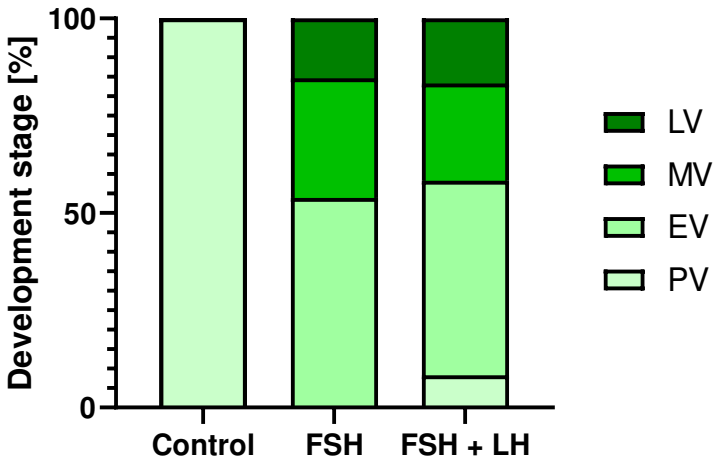

Figure 13 *Percentage of evaluated oocyte development stages (n = 2449 oocytes; n = 7 fish/control; n = 13 fish/FSH; n = 11 fish/FSH+LH) in the three experimental groups at week 12 of treatment. PV previtellogenic stage; EV, early vitellogenic stage; MV, mid-vitellogenic stage; LV, late vitellogenic stage.*

Oocyte diameter

The measurements revealed a highly significant increase ($P < 0.0001$) in the mean oocyte diameter in the FSH and FSH+LH groups compared to the Control group (Fig. 11). However, no significant differences were

observed between the FSH and FSH+LH groups (P = 0.1806). The average diameter, calculated by including all oocytes at different developmental stages within each experimental group, was 119 ± 32.53 µm in the Control group. This value increased to 231 ± 69.76 µm in the FSH group and 229 ± 83.18 µm in the FSH+LH group.

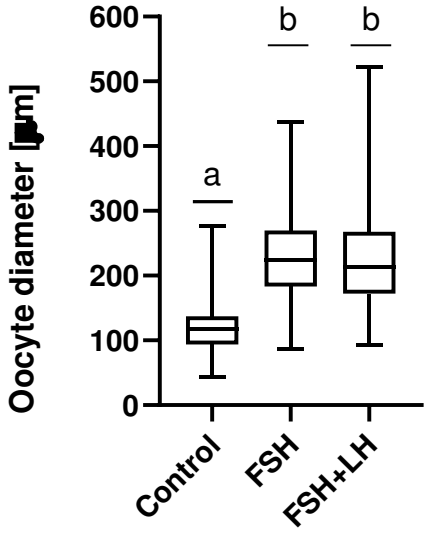

Figure 14 *Diameter of oocytes (n = 7 fish/control; n = 13 fish/FSH; n = 11 fish/FSH+LH; n = 2449 oocytes) of European eels divided into three treatments; the diameter measurement dates back to the last week of treatment (week 12). The central line represents the median of the values. Results are shown as boxplots, including the median and the first and third quartiles. Different letters indicate statistical differences between treatments (P < 0.05; Dunn's test) between medians.*

5 Discussion

The results of this study confirm that plasmid-based gene therapy is a promising technique to stimulate reproductive maturation in female European eels, offering a valid alternative to traditional methods. The absence of adverse effects on body weight and hepatosomatic index suggests that this technique is well-tolerated and does not induce significant metabolic stress.

The observed increase in gonadosomatic index (GSI), accompanied by changes in oocyte diameter and the macroscopic and histological appearance of the gonads, demonstrates effective activation of the hypothalamic-pituitary-gonadal axis and sustained advancement of vitellogenesis. These changes are supported by increased plasma levels of testosterone, 11-ketotestosterone, and estradiol, reflecting hormonal regulation consistent with reproductive maturation. Comparing the concentrations of these steroids with those obtained with other induction methods, there is consistency with the results previously obtained by Mazzeo et al. (2014) and Pérez et al. (2011).

The results of this study align with findings from juvenile female orange-spotted groupers, where an increase in plasma E2 levels and induced ovarian development was observed. However, unlike the current study,

previous research exclusively utilised gene therapy with Lh (Lv et al., 2018).

Skin pigmentation and the ocular index offer indirect evidence of advanced reproductive states in treated groups, indicating that external biometric parameters can serve as valuable complementary indicators in future monitoring protocols. Moreover, gene therapy promotes a reproductive maturation process closer to natural physiological conditions than traditional methods, reducing reliance on repeated exogenous hormonal treatments and lowering animal stress levels (Cuisset et al., 1994; Peñaranda et al., 2018).

The timing of maturation observed with gene therapy treatments is characterised by a more gradual progression than conventional methods. Plasmid injection provoked longer-lasting and higher plasma Lh levels. However, the plasma gonadotropin level reaches its maximum two weeks after the first injection, as observed in previous work on European sea bass (Mazon et al., 2013). In groups treated with plasmids encoding FSH and FSH + LH, early signs of reproductive maturation, such as increased ocular index and testosterone levels, were detected as early as the sixth week. By the twelfth week, the effects were consolidated, significantly increasing GSI, larger oocyte diameters, and advanced oocyte development (mid and late vitellogenesis stages).

Conversely, conventional hormonal treatments, including repeated injections of pituitary extracts or hCG, often result in a faster but less sustained response, but this occurs at the cost of significant stress caused by frequent injections and side effects, such as disrupted metabolism and decreased egg quality (Mazzeo et al., 2014; Pérez et al., 2011). In comparison, plasmid treatments induce a more gradual progression, with noticeable effects observed around the sixth week and full maturation reached by the twelfth week. Additionally, traditional methods often fail to maintain an optimal balance between FSH and LH during the different stages of maturation, negatively affecting egg quality (Mazzeo et al., 2014; Casalini et al., 2024).

By leveraging the prolonged expression of endogenous hormones, gene therapy reduces the need for handling interventions and minimises associated risks.

Plasmid-based gene therapy represents an innovative approach that could transform aquaculture, providing a sustainable solution to overcome reproductive barriers in species with reproductive limitations, such as the European eel. This method reduces dependence on traditional, often invasive and expensive hormone treatments, improving animal welfare by reducing workers' time handling breeding animals.

From an ethical and sustainability perspective, gene therapy could reduce pressure on wild populations by ensuring a sustainable supply.

Limitations and future developments

Despite the promising results, this study has some limitations that require further investigation to ensure the full applicability of gene therapy for the reproduction of European eels. Firstly, the study was conducted on a limited sample. It is therefore necessary to verify the reproducibility of these results in large-scale aquaculture environments, where environmental, management, and genetic factors could significantly influence treatment efficacy. Large-scale applications should involve different facilities, specialised personnel, and defined protocols, affecting the results.

Another critical limitation is the variability in individual responses to the treatments. While plasmids successfully stimulated vitellogenesis in most individuals, some exhibited weaker or delayed responses, likely due to the indirect nature of the method. Unlike conventional hormonal treatments that deliver precise hormone doses, plasmid therapy involves injecting genetic material that may or may not lead to hormone synthesis in muscle cells, resulting in varied physiological responses across specimens (Peñaranda et al., 2016).

From a technological standpoint, a critical development will be optimising the protocols for plasmid production to reduce costs and improve the treatment's stability and efficiency. Additionally, it will be necessary to evaluate less invasive methods for plasmid administration to maintain high gene transfer efficiency while minimising animal stress (Zapater et al., 2023).

Exploring the long-term impact of gene therapy on treated animals will be essential in the future. Follow-up studies could focus on aspects such as egg quality and fertility, the success of subsequent generations, and the potential transmission of undesirable effects.

To broaden the applicability of gene therapy, other teleost species with similar reproductive challenges should also be explored. Combining this technology with other innovations could further enhance its effectiveness.

6 Conclusion

The results of this study demonstrate that plasmid-based gene therapy effectively stimulates vitellogenesis in female European eels, offering a promising alternative to traditional methods. This technique has shown physiological effects, reducing the need for repeated administration of exogenous hormones and minimising animal stress. The observed changes in gonadosomatic index (GSI), oocyte diameter, and hormonal levels suggest the efficacy of this method in promoting reproductive maturation.

While conventional hormonal treatments may induce quicker changes, gene therapy provides a more sustainable and less invasive option, with comparable or even superior results in the short term, particularly regarding oocyte quality and overall sustainability. The potential of gene therapy to revolutionise European eel captive breeding is significant, offering an innovative and sustainable solution to current reproductive challenges while contributing to species conservation and the global sustainability of aquaculture.

However, it is important to note that this experiment was limited to twelve weeks. Longer-term studies are necessary to evaluate the medium-term results and to standardise protocols for future applications. Further research and practical experimentation are essential to fully realise gene

therapy's potential in aquaculture, ensuring its effectiveness and sustainability.

In conclusion, despite the remaining challenges, the prospects for using gene therapy in aquaculture are promising. With continued investment in research and development, this technology could become a turning point for the sustainability of aquaculture and the conservation of endangered fish species. Integrating biotechnological innovations in aquatic resource management is crucial for promoting the adoption of gene therapy and ensuring a positive impact on biodiversity conservation.

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