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Chapter 7

A cellular implant system stimulates the early phase of sexual maturation in European eel

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Abstract

The European eel (*Anguilla anguilla*) is a critically endangered species, therefore there is an urgent need for reliable artificial reproduction protocols. Male eel spermiation can be efficiently induced via 6-8 weekly injections of purified human chorionic gonadotropin (hCG). Sexual maturation of female European eels can be induced via weekly injections of salmon or carp pituitary extract for up to 6 months. The weekly hormone injections result in handling stress and high transient hormone peaks in the blood plasma that negatively affect sexual maturation. In addition, fish pituitary extract is poorly defined and a potential source of pathogens. Most of these problems can be solved with a defined hormone preparation that slowly releases the required gonadotropins at the right dose into the circulation. However, the currently available slow release pumps require surgery of the fish and large amounts of purified hormone. We have now developed a slow release system consisting of live fish cells that constitutively secrete a specified gonadotropic hormone. In a proof-of-principle experiment, male European eels were administered a single injection with hCG-producing cellular implants. Using a recently developed bioassay, we could demonstrate that hCG was detectable in the eel blood plasma for up to 14 days after intraperitoneal injection of the cellular implant. The implant resulted in significantly increased blood testosterone levels and an increased eye index, both of which are strong indicators of sexual maturation. This proof-of-principle experiment shows that the cellular implant system functions as a slow release system and induces the early phase of sexual maturation in male eels.

Introduction

According to current knowledge, natural maturation of European eels (*Anguilla anguilla*) only occurs during and/or after the migration to the spawning area in the Sargasso Sea (Schmidt, 1923; Tesch, 2003). The influx of glass eels has dramatically declined in the past decades (Dekker, 2003) and the European eel is now even listed as critically endangered on the IUCN "Red List of Threatened Species" (Freyhof & Kottelat, 2008). Artificial reproduction may contribute to the restoration of the natural populations by reducing the pressure on the wild stock and allow sustainable eel aquaculture in the future. Artificial reproduction of eels, including European eels, has already been accomplished (Lokman & Young, 2000; Ohta et al., 1996, 1997; Oliveira & Hable, 2010; Palstra et al., 2005; Pedersen, 2003; Yamamoto & Yamauchi, 1972); however, until now full closure of the life cycle has only been achieved for Japanese eel (*Anguilla japonica*) (Ijiri et al., 2011). The main bottleneck toward closing the life cycle is lack of in depth knowledge on the maturation process and on the proper food for the larvae. Recently, the first insight into the feeding biology of eel larvae in their natural habitat was obtained, suggesting that specific plankton composition is essential for feeding and growth of these larvae (Riemann et al., 2010). To increase the chances of finding the correct food composition, frequent production of large numbers of high quality larvae are required, which are until now not available. As for artificial maturation of eels, the current standard protocols are based on treating males with human chorionic gonadotropin (hCG) (Fontaine, 1936; Miura et al., 1991; Ohta et al., 1996, 1997a), and females with pituitary extracts (PEs) from salmon or carp (SPE or CPE, respectively) (Fontaine et al., 1964; Kagawa, 2003; Kagawa et al., 2005; Ohta et al., 1997a).

Induction of maturation in male eels can be accomplished via 6-8 injections of hCG with success rates close to 100% (Miura et al., 1991; Ohta et al., 1997a; Pedersen, 2003). Induction of full maturation of female eels requires multiple weekly injections with PEs: Japanese eels (Yamamoto & Yamauchi, 1972; Ohta et al., 1996; 1997a) and New Zealand shortfinned eels (*Anguilla australis*) require 6-15 injections (Lokman & Young, 2000; Burgerhout et al., 2011), while European eels need 11-29 injections (Palstra et al., 2005; Pedersen, 2003, 2004). However, usually more than 50% of injected European female eels do not respond to the treatment (Burgerhout et al., unpublished data). In addition, egg quality and

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fertilization rates are very low. In teleosts, there is a clear negative correlation between exposure to stressors and the success rate of reproduction (Barton & Iwama, 1991; Foo & Lam, 1993; Pottinger et al., 1991; Schreck, 2010). The multiple PE injections cause stress as a result of handling and high transient hormone peaks in the blood plasma (Sato et al., 2000). Another disadvantage is that the composition of PEs (luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL) and other compounds) is variable and poorly defined. Furthermore, the crude extracts may contain pathogens (Oidtmann et al., 2003; Rivera-Milla et al., 2003).

The ultimate goal of artificial maturation is that the protocols result in similar physiological conditions as those occurring in nature. Under natural circumstances, plasma hormone levels show gradual changes (Suetake et al., 2003), rather than the high transient peaks that occur after each injection with hormones (Sato et al., 2000). Therefore, slow release systems with defined hormone preparations would better mimic the natural situation. Currently available slow release pumps that last up to six weeks require surgery on the fish (Kagawa et al., 2009, 2013). Other slow release methods, like the lipophilized gelatin emulsion (Sato et al., 1995, 1997) only dampen the peak and still have to be injected weekly.

Here, we present a newly developed slow release system consisting of cellular implants from fish cells that secrete constitutive levels of a gonadotropic hormone. These implants have a defined hormone production, are pathogen free, and can be directly injected into the peritoneal cavity.

Methods

Recombinant DNA constructs

The building blocks for the hCG expressing plasmid were obtained from the following sources. The full length cDNA clone IRAUp969C05103D, which contains the code for the hCG β chain, was purchased from RZPD. The plasmid pBC-hCGsyn α that contains a synthetic DNA fragment encoding the human glycoprotein hormone alpha chain, codon-optimized for expression in teleost cells (hCGsyn α) and flanked by a 5' *Bst*XI site and a 3' *Sna*BI site, was purchased from

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BaseClear BV (Leiden, The Netherlands). The Gateway-based Tol2kit plasmids p5E-bactin2, p3E-IRES-nlsEGFPpA, pME-MCS and pDestTol2pA2 were described elsewhere (Kwan et al., 2007).

The hCG expressing plasmid was constructed from the building blocks, according to the following cloning scheme. The *Bst*XI-*Sna*BI fragment of pBC-hCGsyn α was cloned into the *Bst*XI and *Sna*BI sites of p3E-IRES-nlsEGFPpA, thereby replacing the nlsEGFP code with the hCGsyn α code and resulting in p3E-IRES-hCGsyn α . The hCG β code was cloned into the pME-MCS vector, yielding pME-hCG β . The plasmid pBactin-hCG β -IRES-hCGsyn α -polyA, hereafter referred to as pZFS010 (Fig. 1), was made via a recombination event between p5E-bactin2, pME-hCG β , p3E-IRES-hCGsyn α and pDestTol2pA2 using the Gateway LR Clonase kit according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands).

The neomycin selection plasmid was constructed according to the following cloning scheme. The lacZ gene was PCR amplified from pMP2838 (a kind gift from Dr. Jeroen Bakker) using forward primer (5'-AGC TAA GCT TGA ATT CAC CAT GGA AGA TCC CGT CGT TTT ACA ACG TCG-3') and reverse primer (5'-AGC TGG ATC CCC TGA CAC CAG ACC AAC TGG TAA TG-3') and digested with *Hind*III and *Bam*HI and subsequently cloned into the *Hind*III and *Bam*HI sites of pcDNA5/FRT (Invitrogen), yielding pcDNA5/FRT-lacZ. The neomycin resistance gene was also PCR amplified from pMP2838 using forward primer (5'- AGC TGG ATC CCC CGG GCT GCA GCC AAT ATG GGA TCG GCC ATT GAA CAA GAT GGA TTG CAC GCA G-3') and reverse primer (5'-AGC TCT CGA GTC AGA AGA ACT CGT CAA GAA GGC G-3'), digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI and *Xho*I sites of pcDNA5/FRT-lacZ, yielding pZFS013 (Figure 1). The neomycin resistance gene in pZFS013 also confers resistance against geneticin and was used to select for stably transfected clones.

Large-scale plasmid purification was performed using the EndoFree Plasmid Maxi Kit (Qiagen, Venlo, The Netherlands). All plasmids were sequence verified (BaseClear, Leiden, The Netherlands). The pZFS10 and pZFS013 plasmids were linearized with *Afe*I and *Psi*I, respectively, and the linearized plasmids were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Diegem, Belgium).

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Generation of hormone-producing cellular implant

Fat head minnow cells (FHM, *Pimephales promelas*), a kind gift from the Hubrecht Laboratory (Utrecht, The Netherlands), were cultured at 25°C in 67% L15 medium supplemented with 10% fetal bovine calf serum 100 IU/mL penicillin and 100 µg/mL streptomycin. The FHM cells were transfected with a 5:1 volume ratio of the linearized pZFS010 (hCG code) and pZFS013 (neomycin code) plasmids, respectively, using FuGENE6 Transfection Reagent in serum-free medium according to the manufacturer's protocol (Roche, Basel, Switzerland). At 8 hours after transfection, the serum-free medium was replaced with serum-containing medium. At two days after transfection, cells were transferred to 12-well microplates and stably transfected cells were selected for by culturing in the presence of 500 µg/mL geneticin (G418; Duchefa, Haarlem, The Netherlands). After approximately one month of selection, four individual clones of G418-resistant cells were obtained, named FHM-hCG clones 2.1 to 2.4.

The production of biologically active hCG by the FHM-hCG clones was measured using a recently developed bioassay (Minegishi et al., 2012). The FHM-hCG clones were cultured for 16 hours in serum-free medium, since the serum could interfere with the bioassay. The conditioned medium was added to HEK293 cells that stably express the European eel's LH receptor and contain a stably integrated luciferase reporter gene driven by a cAMP responsive-element. After incubation at 37°C for five hours for cell stimulation, a luciferase assay was performed using Steadylite plus Reporter Gene Assay System (Perkin Elmer, Waltham, MA, USA) The luminescence signal was measured on a multilabel plate reader (Victor, PerkinElmer). A serial dilution of pure hCG (Sigma-Aldrich) in DPBS (Invitrogen, Paisly, UK) was used as a reference.

Stable FHM-hCG clone 2.1, hereafter referred to as "2.1", and untransfected wild type FHM cells, hereafter referred to as "WT", were used for the trial. Three and a half weeks before the trial, 4 flasks of 75cm² were seeded to upscale the number of cells. One and a half week later, two Hyperflasks (Corning, Tewksbury, USA) of WT and 2.1 cells were seeded according to manufacturer's protocol to obtain sufficient cells for the *in vivo* trial.

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Animals

Male European eels (n=28; 110.1 ± 3.6 g body weight (BW) \pm standard error (SE); 38.8 ± 0.4 cm body length (BL) \pm SE) were obtained from a commercial eel farm (Nijvis-Holding B.V., Nijmegen, The Netherlands). All eels used during this trial were silver eels according and the silver index that was calculated as indicated by Durif et al. (2005). After transport, the eels were immediately transferred from fresh water to natural sea water (32 ± 1 ppt) in a ca. 6000L tank connected to a recirculation system. Animals were kept at $21 \pm 1^\circ\text{C}$ in the dark and acclimated to these new conditions for 1 month prior to the trial. As silver eels cease feeding during maturation they were not fed during the experiment. All animal experiments complied with the current laws of the Netherlands and were approved by the animal experimentation committee of Leiden University (DEC #11004).

Implant injection experiment

In this study male eels were chosen, because (1) they respond relatively fast to a single hCG injection, (2) there are only few non-responders compared to the female eels, and (3) testosterone levels are a reliable indication of maturation in male eels (Ohta et al., 1997b; Peñaranda et al., 2010). An outline of the experimental design is shown in Figure 1. At the start of the trial, all eels were tagged with a small passive transponder with a unique identification code (Trovan, EID Aalten B.V., Aalten, The Netherlands). In addition, a blood sample was taken and the following morphometric parameters were measured: total body length (BL), body weight (BW), eye diameter horizontal and vertical (EDh and EDv), and pectoral fin length (PFL). The blood was centrifuged at $13,000 \times g$ for 5 minutes at 4°C to obtain blood plasma that was stored at -80°C until measurement. The eye index (EI) was calculated using the formula: $\text{EI} = 100 * (((\text{EDh} + \text{EDv})/4)^2 \pi / (10 * \text{BL}))$ (Pankhurst, 1982).

For each of WT and 2.1 cellular implants, a total of $\sim 10^9$ cells were harvested from Hyperflasks using trypsin, after washed with PBS and resuspended in culture medium to $\sim 70 * 10^6$ cells/mL. The 28 male eels were randomly divided into 4 groups of 7 individuals and intraperitoneally injected according to the following scheme: the first negative control group (saline group) where each individual was injected with 2 mL 0.9% saline and the positive control group (hCG group) where

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each individual was injected with 1000 IU hCG dissolved in 2 mL 0.9% saline (Sigma-Aldrich, Zwijndrecht, The Netherlands). Eels of the second negative control group (WT group) were injected with 2 mL resuspended WT cells and eels of the experimental group (2.1 group) were injected with 2 mL of the 2.1 cells.

Sampling of blood (200 μ L) and morphometric measurements occurred at 1, 3, 5, 7, 14, 21, 35 and 42 days after injection. In addition, at each time point, the eels were checked for production of milt. At every sampling point, the animals were anesthetized with clove oil (1 mL of a 10% solution in 96% ethanol added to 1 L of sea water). At the end of the trial (42 days after injection) the animals were sacrificed using an overdose of clove oil (5 mL of a 10% solution added to 1 L sea water), followed by decapitation. After sacrificing the eels, the gonad and liver were weighed to calculate the gonadosomatic index (GSI; $100 * (\text{weight gonads} / \text{BW})$) and the hepatosomatic index (HSI; $100 * (\text{weight liver} / \text{BW})$). The gonads were preserved overnight in 4% paraformaldehyde (PFA, buffered in PBS), and transferred to 70% ethanol for histological analysis.

Plasma hormone measurements

The hCG concentration in the blood plasma samples was measured using the bioassay as described above (Section Generation of hormone-producing cellular implant. Pure hCG (Sigma-Aldrich) that was serially diluted in blood plasma obtained from a negative control eel was used as a reference. To inactivate the eel toxins that affect the HEK293 cells of the bioassay the plasma samples were heated overnight at 37°C prior to measurements as described elsewhere (Minegishi et al., 2012). In the first week after injection, the hCG levels in undiluted plasma samples obtained from the hCG group were too high for the linear range of the standard curve. Therefore, we also measured 10-fold and 50-fold dilutions of these samples in the bioassay. The levels of testosterone in the plasma samples were measured using a Testosterone ELISA kit (HUMAN Diagnostics Worldwide GmbH). The absorbance of the ELISA plates and luminescence of the bioassay plates were measured on a multilabel plate reader (Victor, PerkinElmer).

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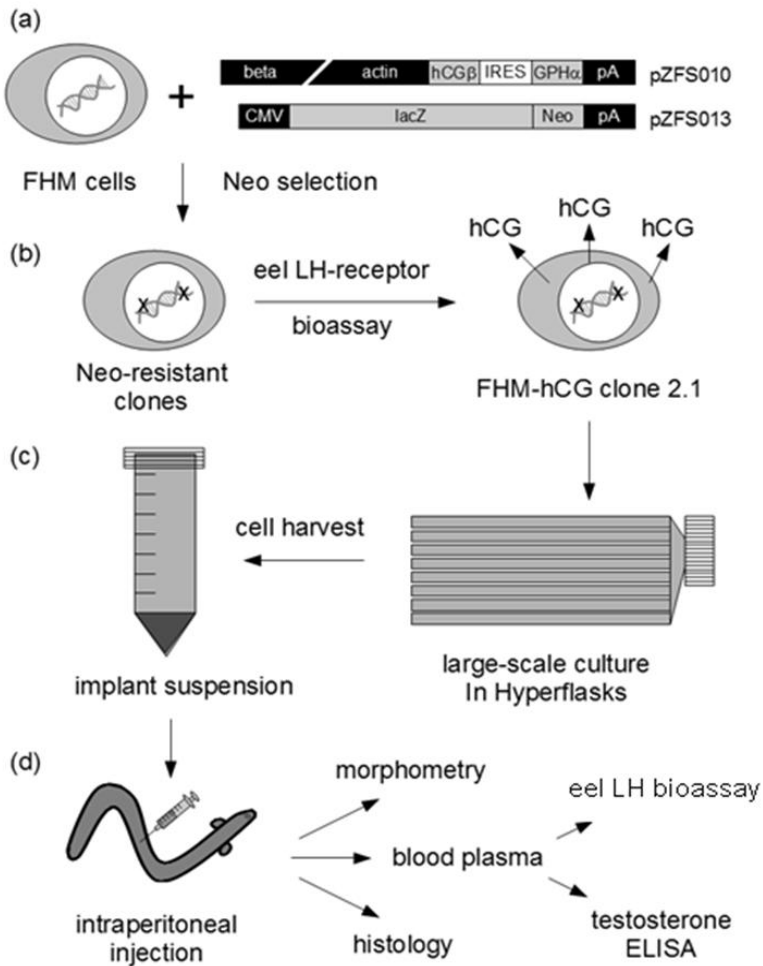


Figure 1. Schematic overview of the workflow. (a) FHM cells were stably transfected with a mixture of a bicistronic expression construct encoding both peptide chains of hCG (pZFS010) and a neomycin resistance selection construct (pZFS013). (b) The amount of hCG in conditioned medium of neomycin-resistant clones was analysed using a bioassay based on the eel LH receptor. (c) The clone that produced the highest amount of hCG (clone 2.1) was expanded in Hyperflasks and concentrated into a small volume. (d) Male eels were intraperitoneally injected with 2.1 cells or with a control substance (wild type cells, pure hCG, or saline). Immediately before injection and at eight time points after injection, the eye index was measured, blood samples were obtained, and eels were checked for production of milt. The hCG and testosterone levels in the blood plasma

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Figure 1 (continued). were determined using the eel LH bioassay and an ELISA, respectively. After 42 days, all eels were sacrificed, their hepatosomatic and gonadosomatic indices were determined, and their testes were microscopically analysed.

Histological analysis

The gonad samples were dehydrated in a series of ethanol (70%-80%-90%-100%), followed by incubation in 100% Histo-Clear (National Diagnostics) and a 1:2 mixture of Histo-Clear and paraffin, respectively. The tissue samples were then embedded in paraffin. Sections (7 μ m thick) were obtained using a Leica microtome (Leica RM2165) and, after rehydration, nuclei and cytoplasm were stained using Mayer's haematoxylin and eosin (H-E) staining. Testis stages were determined following Peñaranda et al. (2010).

Statistics

A Kolmogorov-Smirnov test was used to test for normality of all data. As all data were not normally distributed (Kolmogorov-Smirnoff; $p > 0.05$) a Mann-Whitney U non-parametric test was used to analyze the results. Differences in eye index, plasma levels of testosterone and hCG were tested between the groups and within the groups over time. Statistical difference was considered significant at $p < 0.05$. In all cases values are expressed as average \pm standard error.

Results and Discussion

Hormone-producing implants

Current maturation protocols with European eels are performed at temperatures between 18°C and 20°C (Palstra et al., 2005; Pedersen, 2003, 2004). Since Fathead Minnow cells (FHM) have optimal growth characteristics at this temperature (unpublished data), these cells were chosen as starting material for the construction of hormone producing cellular implants. The standard protocol for the induction of sexual maturation of male eels is based on injections with purified hCG (Ohta et al., 1996, 1997a) which is a heterodimer of an alpha and a beta chain. In order to get high constitutive expression of both polypeptide chains in FHM cells, we designed the plasmid pZFS010 that is based on the zebrafish

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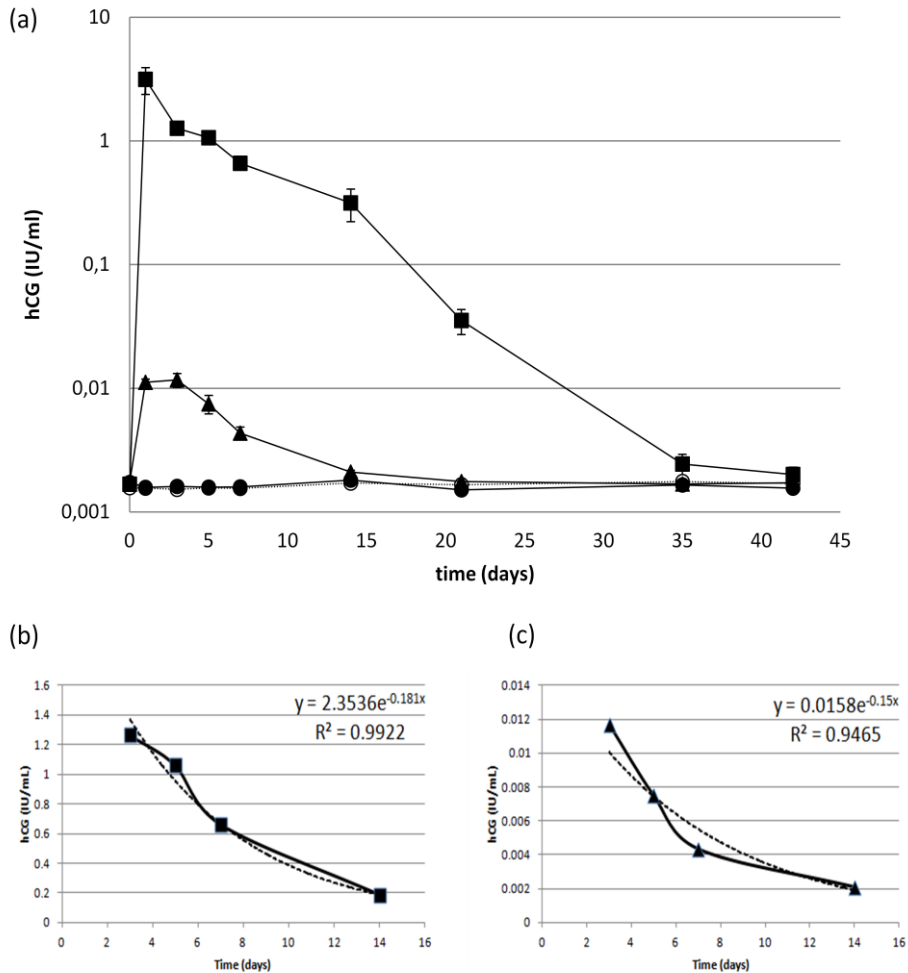


Figure 2: (a) Concentration of blood plasma hCG after a single intraperitoneal injection. Male eels (n=7 per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲). Blood samples were taken immediately before and at 1, 3, 5, 7, 14, 21, 35, and 42 days after injection. The blood plasma hCG concentration was determined using a bioassay based on the eel LH receptor. (b) Trend line (dotted line) of hCG plasma levels between 3 and 14 days after injection of 1000 IU pure hCG (■). (c) Trend line (dotted line) of hCG plasma levels between 3 and 14 days after injection of hCG-producing FHM 2.1 cells (▲).

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beta-actin promoter and an expression cassette in which an IRES element is flanked by the codes for the alpha and beta chains of hCG (Figure 1). Antibiotic selection of FHM cells transfected with pZFS010 and the selection plasmid pZFS013 resulted in four stably transfected clones. To determine the production level of biologically active hCG by the FHM clones, we used a recently developed bioassay based on the eel LH receptor. This bioassay shows a linear response to a more than 100,000-fold concentration range of hCG diluted in saline (Minegishi et al., 2012). The two clones with highest activity, clones 2.1 and 2.4, produced similar amounts of hCG (91 and 94 mIU hCG/day/ 10^6 cells, respectively). Since clone 2.1 showed better growth rates, this clone was selected for the trial in male European eels.

The hormone production of implants in male European eels

To determine the *in vivo* effects of hCG-producing cellular implants on maturation of male eels we performed the following trial (see also Figure 1 and experimental design in Methods). Four groups of male silver eels received a single injection of 1000 IU hCG (hCG group), 0.9% saline (saline group), untransfected wild type FHM cells (WT group) or FHM 2.1 cells (2.1 group). The injected amount of 2.1 cells was equivalent to an *in vitro* production of ~13 IU hCG per day, which corresponds with a theoretical daily *in vivo* maximum dose of ~120 mIU/g BW (~13 IU/110 g/day). Before and after administration of the injections, morphometric measurements and blood samples were obtained at nine consecutive time points covering a period of 42 days. The blood plasma hCG levels were measured using the bioassay as described above. At 1 and 3 days after injection of the cellular implant, the hCG concentration in the eel plasma was approximately 10 mIU/mL (Figure 2a, 2.1 group). This was the highest plasma hCG concentration measured in this group, although the actual peak level may have been higher between 1 and 3 days after injection. After 3 days, the plasma hCG concentration gradually decreased, but remained significantly higher ($p < 0.05$) than the background signal of the saline and WT groups for up to 14 days after injection (Fig. 2a). A single dose of 1000 IU pure hCG (~9 IU/g BW) resulted in a plasma hCG concentration of approximately 5 IU/mL at 1 day post injection (Figure 2a, hCG group), although the actual peak hCG plasma level could again have been reached before or after this time point. After 3 days, the plasma hCG

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level gradually decreased and the hormone level was still significantly above background ($p < 0.05$) at 42 days after injection.

To determine the *in vivo* half-life of hCG in eel, trend lines were generated based on the plasma hCG levels at 3, 5, 7, and 14 days post injection (Figure 2b,c). According to the trend line formula, pure hCG had an *in vivo* plasma half-life of 3.83 days (Figure 2b). Injection of the FHM 2.1 cells resulted in a plasma hCG half-life equivalent to 4.62 days, which is longer than both values found for pure hCG and which suggests that hCG was synthesized *de novo* by the injected cells. At 1 day after injection of FHM 2.1 cells, a plasma hCG concentration of 10 mIU/mL was observed. This is less than one-tenth of the calculated theoretical daily maximum dose of ~120 mIU/g BW. Since the hCG spreads throughout the eel's entire body, it would not be reliable to calculate the total amount of implant-derived hCG that is present in the eel from the concentration of hCG that was measured in the blood plasma. Instead, we estimated the amount of pure hCG that would result in similar plasma levels as the amount of hCG produced by the implant. The single injection of pure hCG (~9 IU/g BW) resulted in a plasma hCG concentration of 5 IU/mL after 1 day. From these results we could estimate that the actual daily *in vivo* production level of the FHM cells was equivalent to a single injection of ~18 mIU hCG/g BW ($((10 \text{ mIU/mL}) / (5 \text{ IU/mL})) * (9 \text{ IU/g BW}))$). This is almost seven times lower than the theoretical daily maximum *in vivo* dose of ~120 mIU hCG/g BW based on a daily *in vitro* production of 13 IU hCG. The lower *in vivo* production might be the result of a combination of factors, such as (1) damage of the cells during the injection, (2) limited *in vivo* access to nutrients and growth factors, (3) inefficient release of hormone to the circular system, and (4) attack of the cellular implant by the immune system of the eel.

Effects of the cellular implant on sexual maturation of the European male eels

An increasing eye index (EI) is an external marker of sexual maturation in eels and associated with increased production of male sex steroids (e.g. Pankhurst, 1982; Durif et al., 2005; Peñaranda et al., 2010). The EI of the 2.1 group continuously increased after injection of the cellular implant and reached a maximum level at 21 days post injection ($\Delta EI = 2.22 \pm 0.27$), whereas the EI in the WT and saline group did not significantly change (Figure 3). After 21 days, the EI of the 2.1 group showed some regression; however, it remained at a significantly higher level ($p <$

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0.05) compared with the WT and saline groups until the end of the trial. The hCG group (positive control) showed a stronger increase in EI, which reached a plateau level ($\Delta EI = 3.94 \pm 0.40$) at 28 days after injection and stayed at that high level until the end of the trial.

Testosterone (T) and its oxidized form 11-keto testosterone (11-KT) are important steroids for sexual maturation in eel (Nagahama, 1994; Peñaranda et al., 2010). Therefore, the plasma T values at the different time points were measured using ELISA. At one day after injection of the cellular implant, the plasma T concentration was already significantly increased as compared to the saline and WT negative control groups ($p < 0.05$, Figure 4). The plasma T level reached a maximum value of ~ 13 ng/mL at 7 days after injection, thereafter it gradually decreased. The plasma T concentrations in the positive control group (hCG)

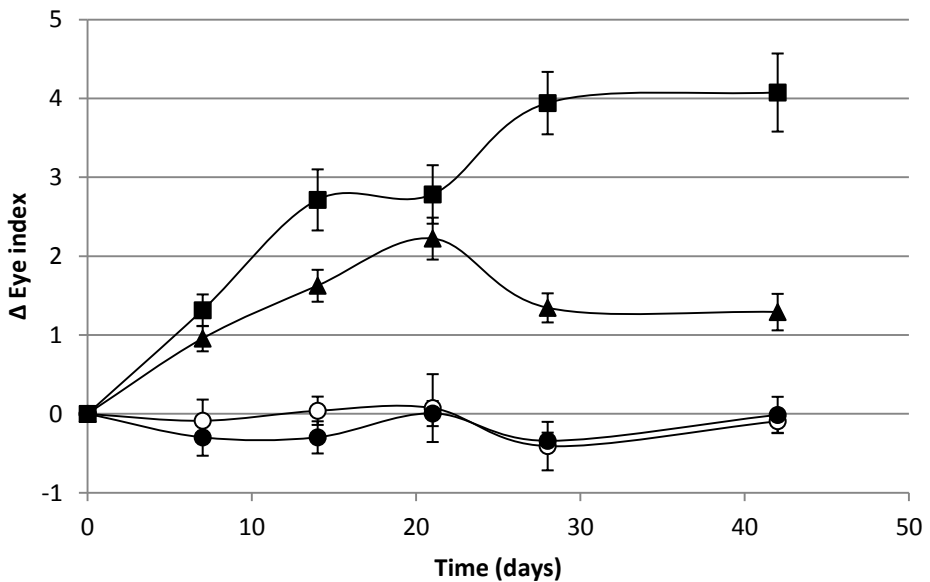


Figure 3: Change of the eye index (ΔEI). Male eels ($n=7$ per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲). The EI was determined at 7, 14, 21, 28, and 42 days after injection. The ΔEI was calculated by subtracting the average EI measured immediately before injection from the average EI measured at the indicated time points.

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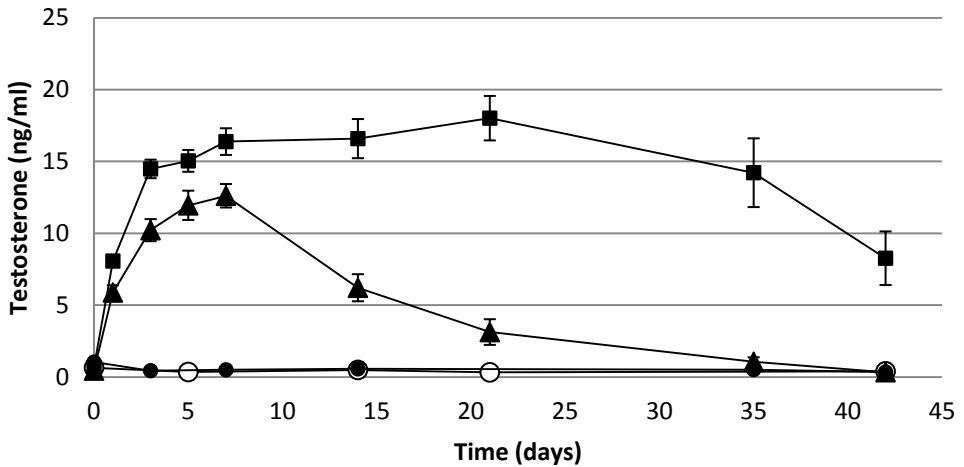


Figure 4: Temporal change of plasma testosterone (T) levels. Male eels (n=7 per group) were injected with 1000 IU pure hCG (■), 0.9% saline (○), untransfected wild type FHM cells (●), or hCG-producing FHM cells (▲). Blood samples were taken immediately before and at 1, 3, 5, 7, 14, 21, 35, and 42 days after injection. The blood plasma T levels were measured using ELISA.

were always significantly higher than those of the cellular implant group ($p < 0.05$). The highest concentration (~ 18 ng/mL) was measured at 21 days after hCG injection and even at 42 days post injection the plasma T concentration was still ~ 8 ng/mL. Although 11-KT, an oxidized form of T, also plays a major role in fish reproduction, the plasma 11-KT was not measured due to limited plasma sample amounts. Repeated sampling from 100g animals didn't allow sufficient sample size for additional assays.

Throughout the trial, all eels were checked for the production of milt. At 28 days after hCG injection, the first eels of the positive control group started the production of milt and at 42 days after injection, five out of six (83%) of the positive control eels produced milt (data not shown). In contrast, none of the eels from the other groups produced milt at any time point. At the end of the trial ($t=42$ days), gonads and livers from all males were weighed in order to calculate the GSI and HSI (data not shown). In addition, the histology of the gonads was studied in more detail using microscopy. The HSI did not significantly differ

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between the positive control (hCG), negative control (WT and saline groups) and the cellular implant (2.1) groups. The GSI of the positive control group (hCG) was more than fifty times higher as compared to that of the negative control groups, which is not unexpected based on the highly efficient milt production. Advanced maturation of the positive control group was further confirmed by histological analysis, which showed mature spermatozoa of stage 4-6 in the lumen of the testis (Peñaranda et al., 2010). In contrast, the GSI of the cellular implant group was not increased as compared to the negative control groups and the testes were immature (stage 1-2) in all these three groups (data not shown).

In the current experiment FHM cells were used, whereas species-specific cells would probably be more suited. Eel cells are more likely to survive longer inside the eel and might be better adapted to the extracellular environment in the coelomic lumen of the eel. Although it is not known how the European eel's immune system responds to the cellular implant, our results show significantly increased plasma hCG levels in the implant group for up to 14 days after injection. It is possible that the FHM cells were broken down by the host's immune response or died as a result of lack of nutrition.

Conclusions and future prospects

In this study we demonstrated a new approach to induce maturation in eels by hormone producing cellular implants. The male eels showed a clear biological response to the implants. However, the hormone production of the cells within the host was too low and needs to be optimized.

There are several ways to significantly increase the production of hCG by the cells. Firstly, by using more powerful transcriptional promoters. In the cell line described in the current study the expression of hCG is driven by the zebrafish beta-actin promoter. Recently we sequenced the transcriptomes of multiple eel tissues (unpublished data), which revealed many genes that are expressed at a much higher abundance than the actin gene. We expect that using the promoters of those genes will result in much higher hormone production levels in the near future. Secondly, optimization of the intracellular ratio of the hCG alpha versus the beta polypeptide chains may increase the hormone production by the cell (Dr. Yves Combarnous, pers. comm.).

Acknowledgements

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