

## Social dominance in tilapia is associated with gonadotroph hyperplasia



Matan Golan, Berta Levavi-Sivan\*

The Robert H. Smith Faculty of Agriculture, Food and Environment, Department of Animal Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

### ARTICLE INFO

#### Article history:

Available online 6 May 2013

#### Keywords:

Tilapia  
Social status  
*Tol2*  
Gonadotroph  
FSH  
Hyperplasia

### ABSTRACT

Tilapias are emerging as one of the most important fish in worldwide aquaculture and are also widely used as model fish in the study of reproduction and behavior. During the reproductive season, male tilapia are highly territorial and form spawning pits in which the dominant males court and spawn with available females. Non-territorial males stand a much lower chance of reproducing. Using transgenic tilapia in which follicle stimulating hormone (FSH) gonadotrophs were fluorescently labeled with enhanced green fluorescent protein (EGFP), we studied the effect of social dominance on the hormonal profile and pituitary cell populations in dominant and non-dominant males. Immunofluorescence studies showed that FSH-EGFP-transgenic fish reliably express EGFP in FSH-secreting cells. EGFP expression pattern differed from that of luteinizing hormone. Dominant males had larger gonads as well as higher levels of androgens and gonadotropins in the plasma. Pituitaries of dominant males exhibited higher gonadotropin content and gene expression. Flow cytometry revealed pituitary hyperplasia as well as FSH cell hyperplasia and increased granulation. Taken together, these findings suggest that gonadotroph hyperplasia as well as increased production by individual cells underlie the increased reproductive activity of dominant tilapia males.

© 2013 Elsevier Inc. All rights reserved.

### 1. Introduction

In recent years, tilapias have become one of the most important fish in world aquaculture, second only to carp. Tilapias are highly suitable for aquaculture due to their high growth potential, short generation time, ease of spawning and good disease resistance, their adaptability to a wide range of salinities, oxygen levels, crowding and handling, and their high value as a food source (Coward and Little, 2001; Watanabe et al., 1985). These traits, along with the recently published genome, also make tilapia an increasingly popular model organism for evolutionary, behavioral and physiological studies. Despite their popularity as model species, transgenic tilapia are rarely produced due to technical constraints. When compared to other model fish species, such as zebrafish, tilapia has a relatively long generation time (6–7 months), unpredictable spawning, large and opaque eggs, tough chorion, and embryos that are not transparent (with no albino phenotype available) (Maclean et al., 2002). Due to these limitations, eggs have to be individually positioned and held in place under a microscope in order to insert the needle into the animal pole. As a result, even a skilled technician can only inject 50 to 60 embryos per day. Combined with the fact that classical transgenesis approaches produce very

low rates of transgenesis and even lower germline-integration rates, it is clear why transgenic tilapia are so rarely produced. Since the ability to induce transgenesis is an important attribute in model organisms we sought an effective and modular method to induce transgenesis in tilapia as a means of enhancing its value as a model fish. To date, the most widespread and efficient technique for the production of stable transgenic fish makes use of transposon systems. Transposon elements are mobile DNA sequences that can be cleaved from the genome and inserted in another location through homologous recombination by a transposase enzyme that performs the “cut-and-paste” activity (Miskey et al., 2005). Although transposons have been known for many years in plants and invertebrates, they have only recently been recognized and activated in vertebrates. The use of transposon systems for the induction of transgenesis in fish results in a very high efficiency of integration of the target DNA into the host genome when compared to traditional transgenesis methods. In conjunction with advanced cloning technologies (Kwan et al., 2007), transposon systems are an easy and effective method for the production of transgenic fish and functional investigations of genetic elements (Asakawa et al., 2008; Kawakami, 2007; Kawakami et al., 2004; Tafalla et al., 2006). We aimed to use transposon-assisted transgenesis to produce tilapia with labeled gonadotrophs and use them as a mean to study changes along the reproductive axis in males from different social status.

In all vertebrates, reproduction is dependent upon the coordinated actions of various hormones associated with the

\* Corresponding author. Address: Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, P.O. Box 12, Rehovot 76100, Israel. Fax: +972 8 9489307.

E-mail address: [berta.sivan@mail.huji.ac.il](mailto:berta.sivan@mail.huji.ac.il) (B. Levavi-Sivan).

brain–hypothalamus–pituitary–gonadal axis. Unlike mammals, fish lack hypophyseal portal vessels, and hypothalamic regulation of the pituitary is performed by hypothalamic fibers directly innervating the pituitary (Ball, 1981). The most important reproductive hypothalamic hormones are gonadotropin-releasing hormone (GnRH) that stimulates, and dopamine that inhibits the release of gonadotropins (GtHs) from the pituitary. The hypothalamic hormones released from these fibers bind to membrane receptors on the pituitary cells, triggering action potentials, a rise in cytosolic calcium, and exocytosis of gonadotropins into the circulation (Levavi-Sivan et al., 2010).

In mammals, the different cell types are dispersed throughout the anterior lobe; in teleosts, each cell type is concentrated in specific defined areas (Kasper et al., 2006; Peter et al., 1986). In tilapia, FSH-containing cells are localized adjacent to the hypothalamic nerve fibers ramifying in the proximal pars distalis (PPD), while luteinizing hormone (LH) cells are localized in a more peripheral region of the PPD (Aizen et al., 2007a). The action of these hormones requires binding to specific receptors anchored in the plasma membrane of target cells of the gonads, where they activate one or more second-messenger pathways, leading to the secretion of sex steroids [estradiol in females and 11-ketotestosterone (11-KT) in males] (reviewed by Levavi-Sivan et al. (2010)).

The regulation of the reproductive axis is highly dependent upon social information, especially in social species such as tilapia. In tilapiine cichlids, territorial behavior in the wild is confined to the breeding period. During this period males establish breeding territories and defend them for a few weeks (Turner and Robinson, 2000). Territorial behavior involves distinct aggression and fighting patterns and typical coloration (Giaquinto and Volpato, 1997), and is related to the probability of winning the match (Turner and Robinson, 2000). Larger fish are more likely to become dominant (Fernandes and Volpato, 1993) and in aquaria frequently reduce food intake by non-dominant individuals (Vera Cruz and Brown, 2007), although over time, non-dominant fish may outgrow a stable dominant male (Pfennig et al., 2011). As courtship in *Oreochromis* species is initiated only once a territory has been established (Turner and Robinson, 2000), dominant territorial males have a significantly higher chance of mating than non-territorial individuals.

In the cichlid *Astatotilapia burtoni*, territorial males exhibit distinct profiles in all major components of the brain–hypothalamus–pituitary–gonadal axis: dominant males have larger gonads as well as increased levels of the androgen 11-KT (Parikh et al., 2006). Brains of dominant *A. burtoni* males were found to express higher

levels of GnRH1 in larger GnRH neurons (Francis et al., 1993; White et al., 2002), while pituitaries of dominant males express elevated levels of the hypophysiotropic GnRH1 receptor. In addition, higher expression levels of LH and FSH were found in the pituitaries of territorial males, along with higher plasma gonadotropin levels (Maruska et al., 2011) and elevated levels of their cognate receptors in the gonad (Maruska and Fernald, 2011).

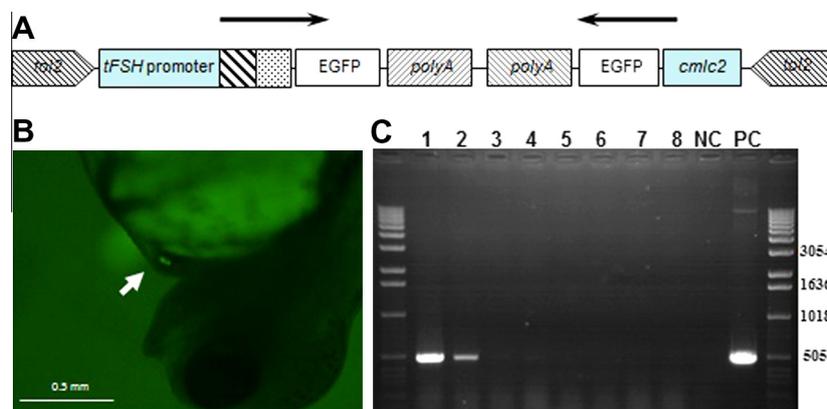
Here we report on the establishment of a stable transgenic line of tilapias in which FSH gonadotrophs are fluorescently labeled. We speculated that significant differences in gonadotropin levels exist between dominant and non-dominant tilapia males and that these differences will be manifested in changes in pituitary cell populations. We used the FSH-EGFP transgenic line to study gonadotroph cell dynamics in tilapia social dominance.

## 2. Materials and methods

### 2.1. Isolation and cloning of FSH $\beta$ promoter region from Nile tilapia and construction of *tol2* expression vector

In the current work, we adopted the *tol2* transposon system (Kawakami, 2004) for the induction of transgenesis in tilapia as a means of increasing germline transmission efficiency in a species in which injection is complicated and transgenesis is relatively hard to achieve. The Tol2kit (Kwan et al., 2007) simplifies construction of *tol2* expression vectors and was used to produce *tol2*-compatible constructs.

Nile tilapia FSH (tFSH) promoter was sequenced using the published sequence for the *O. mossambicus* FSH gene (GenBank accession number AF289173). The primers used were tFSHp55F 5'-AACAAGCATAATGTAACCAAGGACAAC-3' and tFSHp2968R 5'-GCTGCCACAAGAGGGGATC-3'. BLAST alignment of the two sequences using discontinuous megablast comparison revealed 97% homology between the sequences of the two related species. The resulting ~3-kb fragment, including the first exon and intron (GenBank accession number JX887154), was used as the 5' element for the construction of the expression vector. attB4 and attB1R adaptors were added, respectively, to the 5' and 3' ends of the cloned tFSH promoter by PCR. All PCRs for insert amplification and addition of the Gateway adaptors were performed using Phusion polymerase (Finnzymes, Vantaa, Finland). The 5' element was inserted into the pDONRP4-P1R donor vector through a BP recombination reaction. The resulting plasmid was used as a 5' entry clone, along with pME-EGFP as the middle vector and p3E-polyA as the 3' entry clone, and introduced into the pDestTol2CG2 destination clone



**Fig. 1.** Tilapia transgenesis. A. Structure of the expression vector. FSH-regulating region consists of the gene's 5' flanking region, first exon (■), and first intron (□). EGFP acts as the reporter gene and is flanked by a polyadenylation signal. A second region is composed of EGFP driven by the *cmlc2* promoter inducing a signal in the heart, and its direction is opposite that of the FSH construct. The whole insert is flanked by the *tol2* sequences. B. Tilapia embryo 96 h post-fertilization. EGFP signal in heart (arrow). C. PCR analysis for the presence of transgene in genomic DNA from the caudal fins of eight (lanes 1–8) FSH-EGFP-injected tilapia fry. NC = negative (non-injected) control, PC = positive (injected plasmid) control. Ladder sizes (base pairs) are indicated.

through a LR Threeway Multisite Gateway reaction (Invitrogen, Carlsbad, CA). All Gateway methods and protocols were performed according to the Invitrogen Multisite Gateway Manual and as detailed in Kwan et al. (2007). The construct includes the tFSH promoter fused to an EGFP cassette and followed by a polyadenylation signal. In addition, a *cmlc*-promoter-driven EGFP cassette is present in the plasmid which produces a green signal in the embryo heart (Huang et al., 2003). This feature enables easy identification and screening of transgenes when expression of the target labeled genes is weak or occurs late in development. Expression vector composition and structure are illustrated in Fig. 1A.

## 2.2. Capped mRNA transcription

Transposase mRNA was produced using the mMessage mMachine SP6 kit (Ambion, Austin, TX) and pCS2FA-transposase (Kwan et al., 2007) as the template. For every reaction, 2 µg of NotI-digested template plasmid was used in a 2-h incubation at 37 °C. Transcribed mRNA was purified using Ambion MegaClear and further concentrated using ethanol/ammonium acetate precipitation.

## 2.3. Establishment of a protocol for tilapia egg injection using the *tol2* system

Fifteen adult tilapia females were kept in groups of three to five individuals in 240-l glass aquaria in a recirculating system at a temperature of  $27 \pm 2$  °C. Males were not housed with females but were kept in the same recirculating system so that pheromone signals would be available to the females. Females were monitored every morning to identify ripeness, as judged by extension and redness of the genital papilla. Ripe females were stripped of eggs 10 h after first light and eggs were fertilized using sperm that was freshly stripped from a male. Sperm was added to the eggs, activated with water and incubated for 5 min to allow fertilization to occur. After fertilization, eggs were washed of excess sperm and kept at 21 °C in water containing antibiotics (50,000 U/l penicillin, 50 mg/l streptomycin) and methylene blue. The low temperature slows first cell cleavage and allows up to 2 h for injection (Rahman and Maclean, 1992).

Eggs with regular shape, good yolk coloration and a visible animal pole were chosen and placed under a microscope to visualize animal pole and micropyle. Each egg was held in place by gentle suction with a glass pipette using a vacuum pump and injected with 2 µl of DNA/RNA mix in a dye buffer (0.1 M KCl, 10% phenol red) using a borosilicate pipette with an outer diameter of 3 µm.

Several DNA/RNA ratios were tested based on *tol2* injection protocols published for zebrafish (Kawakami, 2004) and *Xenopus* (Hamlet et al., 2006). After injection, eggs were incubated in downwelling Zuger jars at 27 °C.

## 2.4. Identification of possible founders

Hatched embryos (4–6 days after fertilization) were examined under a fluorescent microscope. Potential founders were identified by an EGFP signal in the heart (Fig. 1B) and positive fish were grown to sexual maturity. Genomic DNA from caudal fin clips of positive fish was extracted using DNAeasy kit (Qiagen, Hilden, Germany). The extracted genomic DNA was used as template for PCR analysis to identify possible founders. A forward primer from within the 5' flanking region of the FSH gene (tFSH2811F 5'-agcgcctcatttgataccatccc-3') and a reverse primer from the EGFP ORF (436R 5'-ttgtactcagctt-3') were used to amplify an amplicon of ~500 bp that exists only in cells that contain the transgene.

## 2.5. Southern blotting

DIG-labeled probes were produced using the PCR DIG probe synthesis kit (Roche, Mannheim, Germany). Genomic DNA from F1 EGFP-positive fish was extracted using the Qiagen DNAeasy kit and digested with EcoRV. This enzyme cuts once within the construct. Digested DNA (10 µg) was used for Southern blotting using the DIG-labeled probes according to the DIG Southern manual (Roche). Bands were visualized using NBT/BCIP.

## 2.6. Immunofluorescence

Brains were extracted with pituitaries attached from transgenic fish and fixed in 4% paraformaldehyde for 4 h at room temperature and then cryoprotected by immersion in PBS containing 20% (w/v) sucrose and 30% OCT (w/v) overnight at 4 °C. Tissue was frozen in OCT blocks and sectioned in a cryostat at a thickness of 12 µm. Slides were heated in 0.01 M citrate buffer to 90 °C for 5 min to eliminate EGFP fluorescence. Sections were blocked in 5% (v/v) normal goat serum with 0.3% (w/v) Triton X-100 for 1 h at room temperature and then incubated overnight at 4 °C with specific antibodies raised in rabbit against recombinant tilapia LHβ (Kasuto and Levavi-Sivan, 2005), recombinant tilapia FSHβ (Aizen et al., 2007a) or anti-GFP antibodies raised in mouse (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in antibody dilution buffer (1% (w/v) BSA, 0.3% Triton X-100 in PBS). Samples were stained using secondary anti-rabbit antibodies conjugated to Alexa fluorescent dyes (Invitrogen) diluted 1:300 and incubated for 2 h at room temperature. Following staining, slides were washed and mounted using anti-fade solution (2% propyl-gallate and 75% glycerol in PBS).

## 2.7. Dominant experiment setup

To test for the effect of dominance on the reproductive status of males, adult (14 months old), size-matched transgenic male tilapia (~80 g) were housed in harems consisting of 4 males and 9 transgenic females in 240-l glass aquaria. No significant size difference was allowed between the males (maximum 5% difference). Size matching and harem structure were used as a means of alleviating aggression and diffusing it among several individuals, thus reducing stress in non-dominant individuals. Water quality was maintained by a central filtration unit and temperature was maintained at  $27 \pm 2$  °C. Male dominance was established within 1 day, as evidenced by nuptial coloration and aggression of the dominant male (Giaquinto and Volpato, 1997) (see Supplementary Movie 1) and allowed to proceed for 7 days. At the end of the trial period, a dominant and non-dominant male were collected from each tank ( $n = 8$ ). As tilapia kept in tanks tend to exhibit aggressive behavior, dominant individuals may cause injury and stress to other tank mates. In order to avoid sampling stressed subdominant individuals, fish that exhibited dark coloration (indicative of stress in tilapia) or frayed fins were not used for the analysis. Blood was quickly drawn from the caudal vasculature of the fish and they were then euthanized using tricaine (250 mg/l). Gonads were weighed for GSI determination (gonadal weight percentage of body weight) and pituitaries were collected individually and processed for fluorescence-activated cell sorting (FACS), gonadotropin content measurement and gene expression. All experimental procedures were in compliance with the Animal Care and Use Guidelines of the Hebrew University and were approved by the local Administrative Panel on Laboratory Animal Care.

## 2.8. Fluorescence-activated cell sorting

The pituitary from each male was processed and analyzed individually. Pituitaries were generally processed according to Levavi-Sivan et al. (2004). Briefly, they were removed from adult fish, washed, minced and digested with trypsin (Biological Industries, Beit Ha'emek, Israel). Fragments were incubated in 1 ml trypsin solution (2.5 g/l trypsin, 2.25% w/v glucose, 0.5 mM EDTA and 10 units/ml DNaseI) on an orbital shaker for 40 min at room temperature with occasional pipetting to disintegrate cell clumps. The reaction was stopped by the addition of 1/5 volume of charcoal-stripped fetal calf serum (FCS). Suspended cells were strained through a 40- $\mu$ m nylon mesh and centrifuged for 5 min at 150 g. The pellet was resuspended in PBS enriched with 2% (v/v) FCS and analyzed in a BD FACSibur analyzer (Becton and Dickinson, Franklin Lakes, NJ). At least 15,000 cells were analyzed for every sample. Parameters analyzed included forward scatter (proportional to cell size), side scatter (proportional to cell granulation) and cell number. Acquisition parameters were adjusted by comparing wild-type and transgenic fish and a threshold for specific fluorescence was defined (Supplementary Fig. 1). For cell sorting pituitaries from eight fish were dissociated and sorted in a BD FACSAriaII cell sorter. Specific gates were determined for collecting EGFP-positive and negative cells. For each group 20,000 cells were collected, pelleted and processed for RNA extraction.

## 2.9. Measurement of 11-KT and gonadotropin concentration

For 11-KT measurement, steroids were extracted from the plasma by mixing with 5 volumes of diethyl ether (100  $\mu$ l plasma and 500  $\mu$ l ether). Subsequently, the plasma was frozen and the steroid-containing ether transferred to a different tube for evaporation. After evaporation of the solvent, steroids were redissolved in 100  $\mu$ l phosphate buffer (0.1 M phosphate, 0.15 M NaCl, 1 mM EDTA, 0.1% w/v BSA, 0.01% w/v sodium azide pH 7.4). Levels of 11-KT were measured by ELISA using acetylcholinesterase as a label as described in Aizen et al. (2007a). Sensitivity of the assay was 1.56 pg/ml and intra- and interassay covariances were less than 7% and less than 11%, respectively.

Levels of gonadotropins in the plasma and pituitaries were measured by a specific ELISA developed for tilapia (Aizen et al., 2007b). Sensitivity for plasma measurements was 15.84 pg/ml for LH and 0.24 pg/ml for FSH. For pituitary content measurements, sensitivity was 2.43 ng/ml and 1.52 ng/ml for LH and FSH, respectively. Interassay covariance was 14.8% and 12.5% and intraassay CV was 7.2% and 8% for LH and FSH, respectively. To determine the hormonal content of pituitaries, gonadotropins from 4500 cells from each pituitary were extracted by freezing the cells in distilled water. Total pituitary content was calculated by multiplying the value obtained for a single cell by the total number of cells in the pituitary as determined by FACS.

## 2.10. Determining gene expression levels

Gene expression levels were determined according to Biran et al. (2008) and Levavi-Sivan et al. (2006). Gene-specific primers for the tilapia 18S and prolactin genes, as well as for LH $\beta$  and FSH $\beta$  subunits, were designed with the aid of Primer Express 2.0 software (Table 1). Prolactin expression was measured since its levels are reported to be higher in non-reproducing males and hence it served as an additional marker for decreased reproductive activity. Total RNA from 50,000 cells from each pituitary was extracted using Trizol (Invitrogen). RNA was reverse-transcribed using the Verso cDNA kit (Thermo Fisher Scientific, Surrey, UK). For real-time PCR, a 3- $\mu$ l sample of diluted cDNA was used as a template with 200 nM of each primer and 10  $\mu$ l of Platinum SYBR Green qPCR

**Table 1**  
Primers used for quantitative Real-time PCR.

Primer	Position	5' to 3' sequence	Slope	R <sup>2</sup>
18S F	660	GCACCACCACCCACAGAATC	-3.555	0.998
18S R	897	CGACCATAAACGATGCCAACTAG		
$\beta$ LH F	44	TGCTCCTTGCTCTGATGTGA	-3.641	0.999
$\beta$ LH R	226	CCTTGGTGATGCAGTGTCCAC		
$\beta$ FSH F	174	CTGTGCCCAAGAACATCA	-3.543	0.998
$\beta$ FSH R	424	AGGTCCCGCAGTCTGTGTT		
tPRL F	228F	TGCCACACTCTCTACTACA	-3.285	0.998
tPRL R	412R	GATCGTTTTGGATAAGGTGCTT		

SuperMix-UDG (Invitrogen) in a final volume of 20  $\mu$ l. All samples were run in duplicate. An individual standard curve was run for every gene using graded dilutions of cDNA (0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625). The R<sup>2</sup> value and slope, calculated by linear regression for all of the genes tested, are detailed in Table 1. A dissociation-curve analysis was run after each real-time experiment, to confirm the presence of a single product. To control for false positives, a reverse-transcriptase negative control was run for each template and primer pair. Gene expression levels were normalized to the amount of an endogenous reference, the rRNA subunit 18S, by the comparative threshold (C<sub>T</sub>) cycle method using the formula  $2^{-\Delta C_T}$  where  $\Delta C_T$  corresponds to the difference between the C<sub>T</sub> measured for prolactin or gonadotropin subunits and that measured for 18S rRNA. The ribosomal subunit 18S was used as an internal reference gene, as its expression was found to be stable and uninfluenced by reproductive status (C<sub>T</sub> = 16.99  $\pm$  0.55 and 16.65  $\pm$  0.43 for dominant and non-dominant males, respectively;  $p$  = 0.2262,  $n$  = 8). A detailed description of the method is described elsewhere (Levavi-Sivan et al., 2004).

## 2.11. Statistics

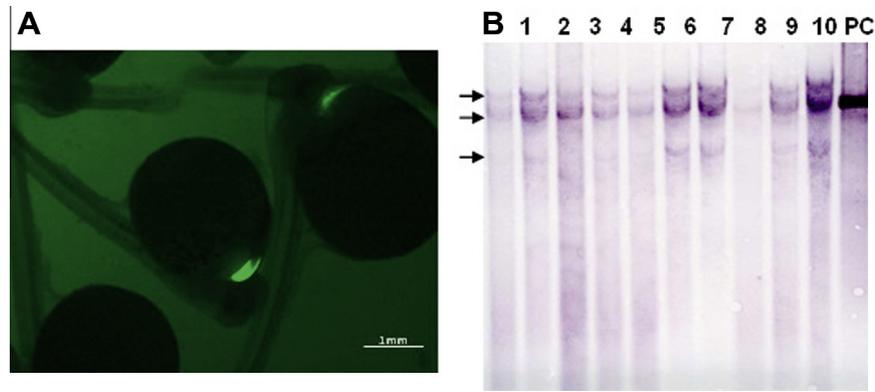
D'Agostino and Pearson omnibus normality test was used to check that values were normally distributed. Significance of differences between group means was determined by Student *t*-test analysis of paired values using GraphPad Prism 4.03 software (San Diego, CA). Paired analysis was used to reduce effects of variances arising from comparing males from different tanks or males of different sizes (as size matching was performed for each tank). Differences were determined as significant at  $p$  < 0.05.

## 3. Results

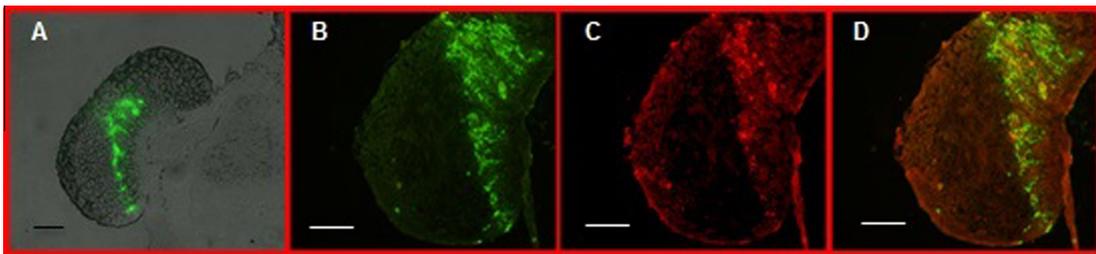
### 3.1. Establishment of an egg-injection protocol

In our experiments, the best results for both transgenesis and survival were obtained using equal concentrations (25  $\mu$ g) of mRNA and DNA per embryo (Supplementary Table 1). Under these conditions, survival rates were high (80%) and transient expression was observed in over 50% of surviving embryos. Using higher concentrations of plasmid DNA and transposase mRNA increased mortality without increasing transgenesis efficiency. Because of the small number of eggs that can be injected in each session, we did not test a wider range of concentrations on a higher number of eggs; nevertheless, even in these limited experiments, results were highly satisfactory and in accordance with those in zebrafish (Kawakami, 2004) and with a recent report in tilapia (Fujimura and Kocher, 2011).

All injections were carried out using plasmids that also induced EGFP expression in the heart to simplify the embryo-screening process for hard-to-visualize gene products. The image of an injected embryo is shown in Fig. 1B.



**Fig. 2.** A. Positive F1 embryos from the FSH-EGFP line showing a strong signal in the heart. B. Southern blot of genomic DNA from 10 F1-transgenic FSH-EGFP fish using a transgene-specific probe. At least three integrations can be seen (arrows). PC = positive control (plasmid containing the transgene).



**Fig. 3.** Transgene expression in the pituitary of a F1 FSH-EGFP female. A. Non-stained EGFP fluorescence. B. Anti-GFP immunofluorescence (green). C. Anti-FSH $\beta$  immunofluorescence (red). D. Merged image of anti-GFP (green) and anti-FSH $\beta$  (red) immunofluorescence. Scale bar = 100  $\mu$ m.

### 3.2. Analysis of transgene genome integration in injected founders

Genome integration of the transgene was determined by PCR using primers specific for the insert. An example of the results for FSH-EGFP-injected fish is shown in Fig. 1C. Strong presence of the transgene was identified in fish #1, and a weaker band was obtained in fish #2. The six other fish that were tested did not show presence of the transgene in the caudal fin despite testing positive for EGFP expression in the heart at the age of 5 days. This might have been due to either transient expression during the early developmental stages or to the chimeric nature of the integration (i.e. the fact that the transgene did not integrate into all of the founder cells).

All fish exhibiting a signal in the heart during embryonic development were raised to adulthood and tested for their ability to transfer the transgene by mating them to a wild-type female. Only those individuals showing transgene presence in the caudal fin also transferred the transgene to their offspring. Positive founders transferred the transgene to 13–19% of their offspring (Fig. 2A), suggesting that the genome integration was chimeric.

To identify the number of genome integrations, genomic DNA from 10 F1 transgenic fish from the FSH-EGFP line was Southern blotted. At least three integrations could be identified in the DNA (Fig. 2B). The fact that one band corresponded to the same size as the plasmid used as a positive control suggests that in addition to transposition events, integration of the whole plasmid, also occurred.

F1 offspring mated to wild-type fish produced 50% positive F2 offspring. This Mendelian pattern of inheritance provides further evidence for genomic transgene integration. To date, the trait and its inheritance pattern have been stable for three generations.

### 3.3. Transgene expression in the pituitary

Transgene expression in the pituitary was studied on 12  $\mu$ m cryostat sections. Without staining, strong EGFP expression ap-

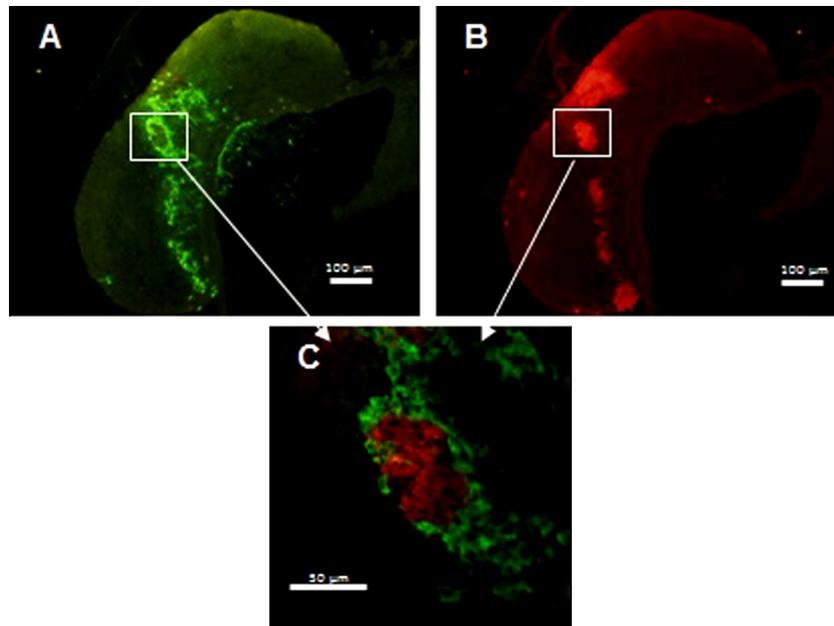
peared in the PPD in the dorsal part close to the neurohypophysis (Fig. 3A). To verify that the labeled cells also express FSH, we performed double-labeling immunofluorescence against GFP and against tilapia FSH $\beta$ . The labeling pattern of both antibodies was similar (Fig. 3B–D), establishing that the EGFP-labeled cells are, in fact, FSH-secreting gonadotrophs. Gene expression analysis on FACS sorted cells found no FSH expression in the EGFP-negative fraction (Supplementary Fig. 2).

We also used specific antibodies against tilapia LH $\beta$  to visualize LH dispersion pattern relative to the location of the FSH (labeled with anti-GFP antibodies). We found LH and FSH gonadotrophs to be situated in close proximity within the pituitary but their staining does not overlap. LH cells are situated in dense clusters that are more peripherally located compared to FSH cells, and these areas are often surrounded by FSH cells (Fig. 4).

### 3.4. Effect of dominance on gonadotropins and androgens

The gonadotropin plasma levels of transgenic ( $n = 9$ ) and non-transgenic ( $n = 7$ ) male tilapia of the same reproductive status were found to be similar (Supplementary Fig. 3; Student t-test,  $p = 0.7$  and  $p = 0.98$  for LH and FSH, respectively).

Using the FSH-EGFP-labeled F1 adult tilapia, we investigated the role of gonadotropins in the reproductive changes induced by male dominance in this highly social species. After 7 days of interaction, the testis size of non-dominant males ( $n = 8$ ) was significantly reduced compared to their dominant counterparts ( $n = 8$ ) as evidenced by the large difference in GSI (GSI =  $0.86\% \pm 0.14$  and  $0.2\% \pm 0.05$  for the dominant and non-dominant males, respectively;  $p = 0.0098$ ). Testes of dominant males were at least three times larger than those of non-dominant males of the same size (Fig. 5A). Cell counts revealed that the pituitaries of dominant fish contain more cells than those of non-dominant males (Fig. 5B;  $p = 0.0009$ ). By using FSH-EGFP-transgenic tilapia for these experiments, we were able to test for the percentage of FSH cells in



**Fig. 4.** Transgene expression in the pituitary of a F1 FSH-EGFP female. A. Anti-GFP immunofluorescence (green). B. Anti-LH $\beta$  immunofluorescence (red). C. Detail of the region marked in A and B showing cells with both EGFP (green) and LH (red) staining.

the pituitary of males from different social statuses. All dominant males had a significantly higher percentage of labeled cells in their pituitaries compared to non-dominant males (Fig. 5C;  $p < 0.0001$ ). When taking into account total pituitary cell number, dominant male pituitaries exhibited a 90% increase in the number of FSH cells per pituitary, compared to non-dominant males (Fig. 5D;  $84,367 \pm 4185$  vs.  $44,253 \pm 4863$  labeled cells per pituitary for dominant and non-dominant fish, respectively;  $p < 0.0001$ ).

While the size of FSH cells, determined from forward-scatter values, did not differ between the different social statuses (Fig. 5E;  $p = 0.6035$ ), cell complexity and granulation, as reflected from side-scatter values, were significantly higher in FSH cells of dominant males (Fig. 5F;  $p < 0.0001$ ).

The increased ability of the pituitaries from dominant males to produce FSH was also evident in the presence of higher levels of FSH in the plasma and pituitary of dominant fish (Fig. 6A and C;  $p = 0.027$  and  $p = 0.0088$ , respectively).

Since the fish used in this study were labeled only for FSH, we did not measure the difference in numbers of LH cells between dominant and non-dominant fish. However, LH content in the plasma and pituitaries displayed a trend similar to that of FSH, being higher in the plasma and pituitaries of dominant males (Fig. 6B and D;  $p = 0.0475$  and  $p = 0.0055$ , respectively). The high pituitary gonadotropin content in dominant males corresponded to high expression levels of both LH $\beta$  and FSH $\beta$  subunits as revealed by real-time PCR analysis (Fig. 7A and B;  $p = 0.0078$  and  $p = 0.0225$ , respectively). In contrast, prolactin levels were found to be significantly higher in non-dominant males (Fig. 7C;  $p = 0.0156$ ).

Plasma levels of the androgen 11-KT were found to be  $\sim 10$  times higher in dominant vs. non-dominant males (Fig. 8;  $p = 0.0342$ ).

#### 4. Discussion

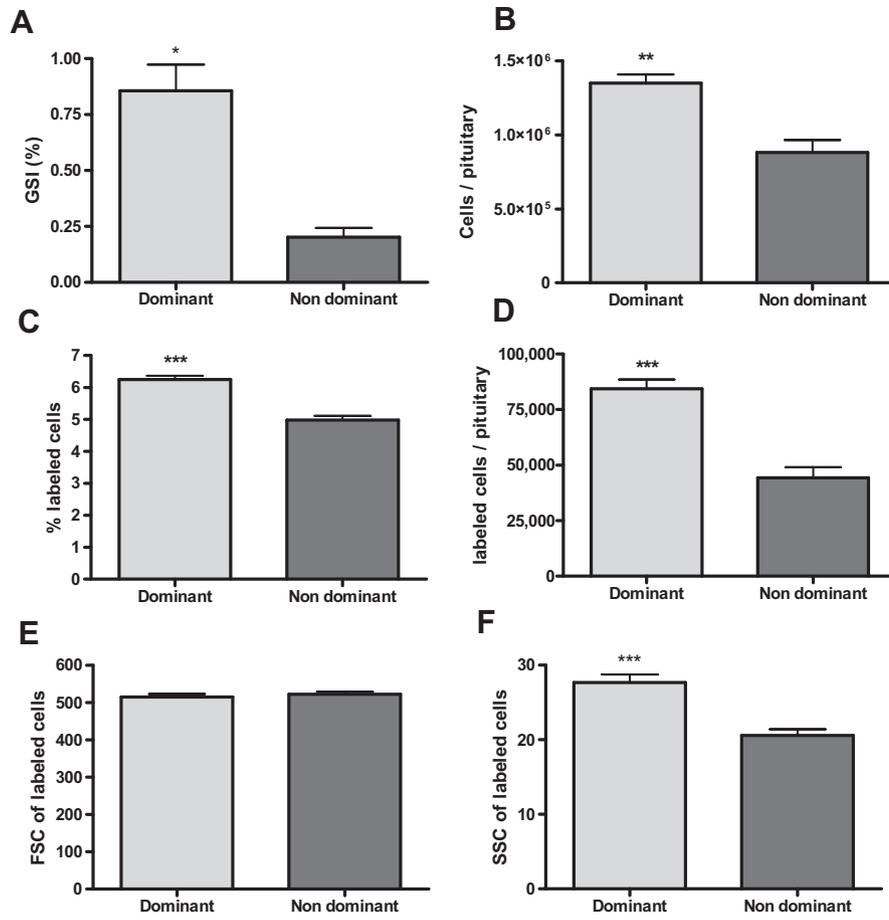
The ability to induce transgenesis is an important attribute in model organisms as transgenic animals offer a wide array of research possibilities which are difficult to achieve by other means. Using linearized plasmid-injection methods, the germline trans-

mission rate of the transgene is usually less than 2.5% of surviving injected embryos (Rahman et al., 1997). In zebrafish, the use of transposons increases germline transmission from  $\sim 5$ –10% in traditional DNA-injection strategies to  $\sim 50$ % using the *tol2* system (Kawakami et al., 2004) and therefore we chose to use this technique to enhance transgenesis efficiency in tilapia. At the time, no published data existed on transgenesis in tilapia using a transposon system; however, a recent article (Fujimura and Kocher, 2011) has reported the use of the *tol2* system in tilapia. Our results are comparable to those of Fujimura and Kocher (2011) and demonstrate the significant increase in transgenesis efficiency when applying the *tol2* system for tilapia. Utilization of the Tol2kit for construction of the expression plasmids, as demonstrated in the current study, further facilitates the use of *tol2*-assisted transgenesis by providing a reliable and modular method in which different reporter or target genes can be driven by various regulating factors. Using the methods described here, one can reliably produce transgenic tilapia by injection of only 50 to 100 eggs from just one or two spawns.

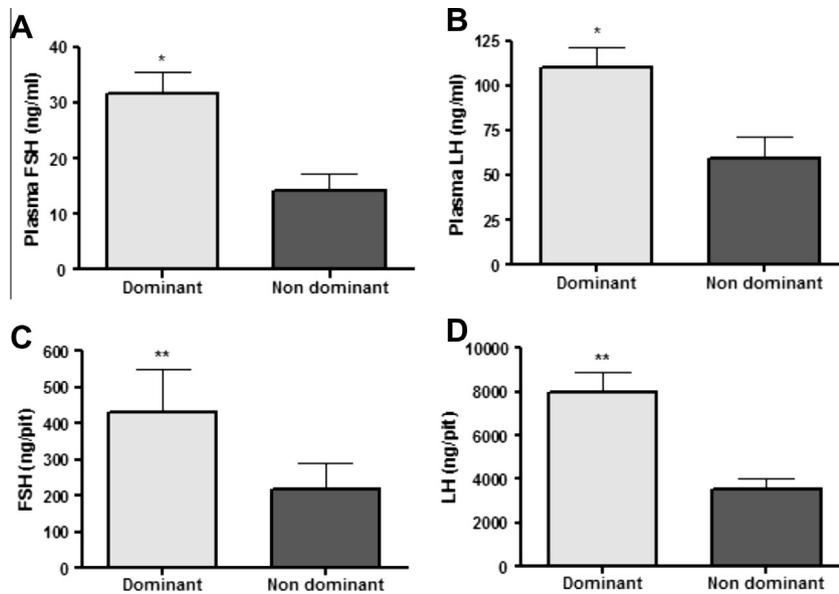
The protocols developed in the current study were used to produce transgenic tilapia in which pituitary FSH cells are labeled. Due to the efficiency of genomic integration using the *tol2* system, multiple insertions often occur, as seen in the case of the FSH-EGFP fish described here. The high insertion number ensures strong expression of the transgene and thus easy identification of labeled cells.

Using specific antibodies we confirmed that the labeled cells were indeed FSH-secreting cells. This distribution pattern is in line with previous reports using species-specific (Aizen et al., 2007a) and non-species-specific antibodies (Kasper et al., 2006); it was also seen in in-situ hybridization studies (Yaron et al., 2003) demonstrating a distinct expression pattern in which LH and FSH gonadotrophs are located in different areas but in close proximity to each other. This discrepancy between the two gonadotroph types is unique to fish and provides a valuable tool for the study of their distinct roles.

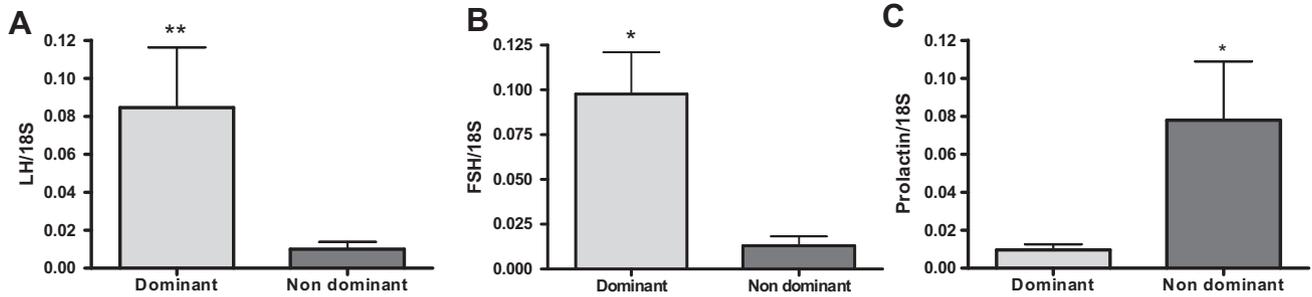
The FSH-EGFP-transgenic line was used to study the physiological differences underlying dominance in tilapia. We found the GSI of dominant males to be significantly higher than that of their non-dominant counterparts. The large difference in gonadal size be-



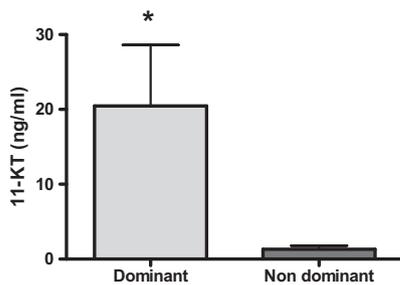
**Fig. 5.** Effect of social status on reproductive parameters. GSI (A), cell number (B), % FSH cells (C), number of labeled cells per pituitary (D), forward-scatter (FSC) values (E) and side-scatter (SSC) values (F) of dominant and non-dominant fish. Asterisks indicate means that are significantly different (\* $p < 0.01$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ ). The results are expressed as mean  $\pm$  s.e.m ( $n = 8$ ).



**Fig. 6.** Effect of social status on gonadotropin levels. A. FSH plasma levels of dominant and non-dominant fish. B. LH plasma levels of dominant and non-dominant fish. C. Pituitary content of FSH in dominant and non-dominant fish. D. Pituitary content of LH in dominant and non-dominant fish. Asterisks indicate means that are significantly different (\* $p < 0.05$ , \*\* $p < 0.01$ ). The results are expressed as mean  $\pm$  s.e.m ( $n = 8$ ).



**Fig. 7.** Effect of social status on gene expression levels. A. Expression levels of LH $\beta$  (A), FSH $\beta$  (B) and prolactin (C) in pituitaries of dominant and non-dominant fish. Asterisks indicate means that are significantly different (\* $p < 0.05$ , \*\* $p < 0.01$ ). The results are expressed as mean  $\pm$  s.e.m ( $n = 8$ ).



**Fig. 8.** Effect of social status on 11-ketotestosterone levels. 11-KT levels in dominant and non-dominant fish plasma. Asterisks indicate means that are significantly different (\* $p < 0.05$ ). The results are expressed as mean  $\pm$  s.e.m ( $n = 8$ ).

tween fish from different social statuses has been previously reported in tilapia (Pfennig et al., 2011) and *A. burtoni* (White et al., 2002).

To support this enhanced reproductive state, physiological differences between dominant and non-dominant males are evident in the pituitary. Gonadotropins play a major role in the regulation of reproduction (Levavi-Sivan et al., 2010), and we found significantly higher levels of gonadotropins in both the pituitaries and plasma of dominant males. Higher gonadotropin content of dominant male pituitaries was accompanied by higher expression levels of both LH and FSH mRNA, as in the closely related *A. burtoni* (Maruska et al., 2011). In tilapia, both LH and FSH are potent inducers of androgen production by the testes (Aizen et al., 2007a; Kasuto and Levavi-Sivan, 2005), whereas in salmonids and eel, FSH-mediated steroidogenesis is involved in the stimulation of early stages of spermatogenesis while LH levels increase only during the last phases of the process (Schulz et al., 2010). Since in tilapia these processes occur continuously throughout the long breeding period, both gonadotropins are constantly in demand, accounting for the simultaneous rise in the levels of both gonadotropins in reproductively active individuals.

The ability to identify FSH cells allowed us to compare their number, relative abundance, size and granularity in dominant versus non-dominant males. Using flow cytometry, we found that pituitaries of dominant males contain more cells than pituitaries of their size-matched counterparts. Normal, non-pathological pituitary volume enlargement and hyperplasia has been reported in mammals in response to pregnancy (Dinç et al., 1998; Elster et al., 1991; Gonzalez et al., 1988; Horvath et al., 1999) and is attributed mainly to lactotrophs. Hyperplasia of the pituitary is also highly affected by castration and is responsive to steroids (Inoue et al., 1985; Nolan and Levy, 2006). The transgenic labeling of FSH cells allowed us to identify a 90% increase in their number in the pituitaries of dominant males. The observed FSH cell hyperplasia in dominant males is likely one of the mechanisms responsible for the elevated levels of FSH found in their plasma and pituitaries.

The increase in FSH cell number accounts for  $\sim 30\%$  of the total pituitary hyperplasia observed in these males. Since only FSH cells were labeled in this research, we cannot tell at this point exactly which of the other cell types account for the increase in total cell number detected in dominant fish. However, the observed rise in LH levels in the pituitary and plasma of dominant males suggests that at least part of the total hyperplasia may be attributed to LH gonadotroph hyperplasia.

Hyperplasia of gonadotrophs during the reproductive cycle is well documented in mammals (Childs, 1995; Childs and Unabia, 1997; Childs et al., 1992; Inoue et al., 1985; Oishi et al., 1993). In fish, gonadotroph hyperplasia is reported in several species during puberty, including rainbow trout, African catfish, mummichog and zebrafish (Chen and Ge, 2011; Nozaki et al., 1990; Schulz et al., 1997; Shimizu et al., 2008) and during the reproductive cycle in the molly (Young and Ball, 1982), and is related to elevated gonadotropin levels in the plasma.

In mammals, GnRH and activin are suggested to play a significant role in the proliferation of gonadotrophs (Childs and Unabia, 1997; Childs and Unabia, 2001) while activin A and B were found to increase FSH expression levels in pituitary cells of goldfish (Yuen and Ge, 2004) and tilapia (Yaron et al., 2001). In territorial *A. burtoni*, high levels of hypophysiotropic GnRH (GnRH1) expression have been documented in the hypothalamus (White et al., 2002) along with a rise in its cognate receptor in the pituitary (Au et al., 2006; Maruska et al., 2011). These reports suggest activin and GnRH as possible inducers of gonadotroph proliferation in fish but further investigation is required to confirm this.

Side-scatter analysis showed a change in gonadotroph granularity in fish of different social statuses. The granular structure of gonadotrophs is well-established in mammals (Batten and Hopkins, 1978; Crawford and McNeilly, 2002; Norman and Litwack, 1997; Slater, 1991). In fish, most former studies were conducted on gonadotrophs of unidentified content in which a granular structure is evident (Abraham, 1974; Ekengren et al., 1978; García-Ayala et al., 1998; Kaneko et al., 1986; Leatherland et al., 1974; Peute et al., 1976; Batten, 1986; Young and Ball, 1982). Ultrastructure of identified FSH gonadotrophs has only been studied in salmonids (Naito et al., 1993) and Mediterranean yellowtail (Pilar García Hernández et al., 2002) and in both species, FSH is retained in specific granular and globular structures within the cells. Despite the fact that no ultrastructural study of tilapia FSH gonadotrophs has been conducted to date, it seems reasonable to assume that a granular structure of FSH cells is also present in this species. Our findings revealed that FSH cells are not only more numerous in dominant males, but also exhibit a higher side-scatter value, implying that in dominant males, these cells have a more complex structure, as result of increased granularity, than those of non-dominant males. Changes in cell granulation can be detected throughout the reproduction cycle (Kaneko et al., 1986; Young and Ball, 1982) and are correlated to gonadotropin production (Childs et al., 1987). The fact

that FSH-secreting gonadotrophs of dominant males are more granular than those of non-dominant males implies that these cells are more active in FSH synthesis and thus contain higher levels of gonadotropins, as reflected by the elevated FSH levels found in the pituitaries and plasma.

The higher gene expression levels of FSH found in the pituitary cells of dominant fish further supports the concept of enhanced FSH production. However, whereas expression levels are determined within a mixed cell population and can be attributed to a higher number of FSH-expressing cells within that population (Fig. 5C), FACS side-scatter measurements are performed on individual cells and therefore provide a more detailed picture of the changes underlying dominance within a specific pituitary cell population.

The hyperplasia of FSH cells in dominant males was not accompanied by hypertrophy of these cells, as forward-scatter values, indicative of cell volume, did not differ between fish of different social status. This implies that the observed hypertrophy and increased granulation are sufficient for the production of higher levels of FSH. Taken together, these findings suggest that increased mRNA expression, cell granularity and higher cell numbers are the factors underlying the higher levels of FSH found in dominant male tilapia.

The higher levels of gonadotropins produced by the pituitary of dominant males significantly elevated androgen levels in the dominant individuals. The ability of gonadotropins to induce androgen synthesis is well documented in tilapia (Aizen et al., 2007a; Kasuto and Levavi-Sivan, 2005; Levavi-Sivan et al., 2010). In turn, increased levels of androgen induce territorial behavior (Fernald, 1976), as well as enhanced testis growth and higher sperm production (Maruska and Fernald, 2011). These changes account for the increased reproductive activity of dominant males, enhancing their chances of successful mating.

Whereas all of the reproductive markers that we tested were found to be elevated in dominant males, prolactin expression was significantly higher in the non-dominant males. High levels of prolactin in non-reproductive males is reported in other species of fish in which prolactin is related to parental care and decreased courtship, territorial and spawning behavior (Páll et al., 2004; Kindler et al., 1991). In mammals, high levels of prolactin are also associated with parental care (Schradin, 2008) and with a decrease in circulating androgen levels (Schradin and Yuen, 2011; Ziegler et al., 2009). The means by which prolactin inhibits the reproductive axis in non-dominant individuals remains to be investigated.

## 5. Conclusion

Our findings provide insights into the mechanisms driving increased reproductive activity in dominant males. High plasma gonadotropin levels in dominant individuals result from gonadotroph cell hyperplasia as well as increased production by individual cells, inducing increased androgen production. In turn, the increase in androgen levels enhances testicular development and sperm production, as well as aggression and reproductive behavior.

The methods developed in this study provide an effective and modular protocol to induce transgenesis in tilapia. The use of transposon-assisted transgenesis dramatically improves the efficiency of transgenic fish production. These developments, coupled with the recent publication of the tilapia genome, mark another step toward establishing this economically important fish species as a valuable model organism.

## Acknowledgments

The authors would like to acknowledge Prof. Koichi Kawakami for the permission to use the *tol2* system and Dr. Chi-Bin Chien

for kindly providing the *tol2kit* plasmids. This research was supported by Grant No. 1350/06 from the Israel Science Foundation (ISF). Matan Golan's research was generously supported by the Hoffman Leadership and Responsibility fund, at the Hebrew University.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2013.04.032>.

## References

- Abraham, M., 1974. Ultrastructure of the cell types and of the neurosecretory innervation in the pituitary of *Mugil cephalus* L. from freshwater, the sea, and a hypersaline lagoon II. The proximal pars distalis. *Gen. Comp. Endocrinol.* 24, 121–132.
- Aizen, J., Kasuto, H., Golan, M., Zakay, H., Levavi-Sivan, B., 2007a. Tilapia follicle-stimulating hormone (FSH): immunocytochemistry, stimulation by gonadotropin-releasing hormone, and effect of biologically active recombinant FSH on steroid secretion. *Biol. Reprod.* 76, 692–700.
- Aizen, J., Kasuto, H., Levavi-Sivan, B., 2007b. Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins. *Gen. Comp. Endocrinol.* 153, 323–332.
- Asakawa, K., Suster, M.L., Mizusawa, K., Nagayoshi, S., Kotani, T., Urasaki, A., et al., 2008. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc. Natl. Acad. Sci. USA* 105, 1255–1260.
- Au, T.M., Greenwood, A.K., Fernald, R.D., 2006. Differential social regulation of two pituitary gonadotropin-releasing hormone receptors. *Behav. Brain Res.* 170, 342–346.
- Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. *Gen. Comp. Endocrinol.* 44, 135–170.
- Batten, T.F.C., 1986. Immunocytochemical demonstration of pituitary cell types in the teleost *Poecilia latipinna*, by light and electron microscopy. *Gen. Comp. Endocrinol.* 63, 139–154.
- Batten, T.F.C., Hopkins, C.R., 1978. Discrimination of LH, FSH, TSH and ACTH in dissociated porcine anterior pituitary cells by light and electron microscope immunocytochemistry. *Cell Tissue Res.* 192, 107–120.
- Biran, J., Ben-Dor, S., Levavi-Sivan, B., 2008. Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. *Biol. Reprod.* 79, 776–786.
- Chen, W., Ge, W., 2011. Ontogenic expression profiles of gonadotropins (fshb and lhb) and growth hormone (gh) during sexual differentiation and puberty onset in female zebrafish. *Biol. Reprod.* 86, 1–11.
- Childs, G.V., 1995. (Division of labor among gonadotropes). In: Gerald, L. (Ed.), *Vitamins and Hormones*. Academic Press, pp. 215–286.
- Childs, G.V., Unabia, G., 1997. Cytochemical studies of the effects of activin on gonadotropin-releasing hormone (GnRH) binding by pituitary gonadotropes and growth hormone cells. *J. Histochem. Cytochem.* 45, 1603–1610.
- Childs, G.V., Unabia, G., 2001. Epidermal growth factor and gonadotropin-releasing hormone stimulate proliferation of enriched population of gonadotropes. *Endocrinology* 142, 847–853.
- Childs, G.V., Unabia, G., Lee, B.L., Lloyd, J., 1992. Maturation of follicle-stimulating hormone gonadotropes during the rat estrous cycle. *Endocrinology* 131, 29–36.
- Childs, G.V., Unabia, G., Tibolt, R., Lloyd, J.M., 1987. Cytological factors that support nonparallel secretion of luteinizing hormone and follicle-stimulating hormone during the estrous cycle. *Endocrinology* 121, 1801–1813.
- Coward, K., Little, D.C., 2001. Culture of the 'aquatic chicken': present concerns and future prospects. *Biologist (London)* 48, 12–16.
- Crawford, J., McNeilly, A., 2002. Co-localisation of gonadotrophins and granins in gonadotrophs at different stages of the oestrous cycle in sheep. *J. Endocrinol.* 174, 179–194.
- Diñç, H., Esen, F., Demirci, A., Sari, A., Gümele, H.R., 1998. Pituitary dimensions and volume measurements in pregnancy and post partum. *Acta Radiol.* 39, 64–69.
- Ekengren, B., Peute, J., Fridberg, G., 1978. Gonadotropic cells in the Atlantic salmon, *Salmo salar*. *Cell Tissue Res.* 191, 187–203.
- Elster, A.D., Sanders, T.G., Vines, F.S., Chen, M.Y., 1991. Size and shape of the pituitary gland during pregnancy and post partum: measurement with MR imaging. *Radiology* 181, 531–535.
- Fernald, R.D., 1976. Effect of testosterone on behavior and coloration of adult male cichlid fish (*Haplochromis burtoni*, Gunther). *Horm. Res.* 7, 172–178.
- Fernandes, M.d.O., Volpato, G.L., 1993. Heterogeneous growth in the Nile tilapia: social stress and carbohydrate metabolism. *Physiol. Behav.* 54, 319–323.
- Francis, R.C., Soma, K., Fernald, R.D., 1993. Social regulation of the brain–pituitary–gonadal axis. *Proc. Natl. Acad. Sci. USA* 90, 7794–7798.
- Fujimura, K., Kocher, T.D., 2011. Tol2-mediated transgenesis in tilapia (*Oreochromis niloticus*). *Aquaculture* 319, 342–346.
- García-Ayala, A., García-Hernández, M.P., Quesada, J.A., Agulleiro, B., 1998. Gonadotropic and thyrotropic cells from the Mediterranean Yellowtail (*Seriola*

- dumerilii*; Risso, 1810): immunocytochemical and ultrastructural characterization. *Anat. Rec.* 250, 448–458.
- Giaquinto, P.C., Volpato, G.L., 1997. Chemical communication, aggression, and conspecific recognition in the fish Nile tilapia. *Physiol. Behav.* 62, 1333–1338.
- Gonzalez, J.G., Elizondo, G., Saldivar, D., Nanez, H., Todd, L.E., Villarreal, J.Z., 1988. Pituitary gland growth during normal pregnancy: an in vivo study using magnetic resonance imaging. *Am. J. Med.* 85, 217–220.
- Hamlet, M.R.J., Yergeau, D.A., Kuliyeve, E., Takeda, M., Taira, M., Kawakami, K., et al., 2006. Tol2 transposon-mediated transgenesis in *Xenopus tropicalis*. *Genesis* 44, 438–445.
- Horvath, E., Kovacs, K., Scheithauer, B., 1999. Pituitary hyperplasia. *Pituitary* 1, 169–179.
- Huang, C.J., Tu, C.T., Hsiao, C.D., Hsieh, F.J., Tsai, H.J., 2003. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev. Dyn.* 228, 30–40.
- Inoue, K., Tanaka, S., Kurosumi, K., 1985. Mitotic activity of gonadotropes in the anterior pituitary of the castrated male rat. *Cell Tissue Res.* 240, 271–276.
- Kaneko, T., Aida, K., Hanyu, I., 1986. Ultrastructural changes in the pituitary gonadotropes during the annual reproductive cycle of the female chichibu-goby *Tridentiger obscurus*. *Cell Tissue Res.* 246, 137–144.
- Kasper, R.S., Shved, N., Takahashi, A., Reinecke, M., Eppler, E., 2006. A systematic immunohistochemical survey of the distribution patterns of GH, prolactin, somatolactin, beta-TSH, beta-FSH, beta-LH, ACTH, and alpha-MSH in the adenohypophysis of *Oreochromis niloticus*, the Nile tilapia. *Cell Tissue Res.* 325, 303–313.
- Kasuto, H., Levavi-Sivan, B., 2005. Production of biologically active tethered tilapia LH $\beta$  by the methylotrophic yeast *Pichia pastoris*. *Gen. Comp. Endocrinol.* 140, 222–232.
- Kawakami, K., 2004. Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. *Zebrafish: Genetic. Genom. Informa. Methods Cell Biol.* 77, 201–222.
- Kawakami, K., 2007. Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol.* 8, S7.1–S7.10.
- Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N., Mishina, M., 2004. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev. Cell.* 7, 133–144.
- Kindler, P.M., Bahr, J.M., Gross, M.R., Philipp, D.P., 1991. Hormonal regulation of parental care behavior in nesting male bluegills: do the effects of bromocriptine suggest a role for prolactin? *Physiol. Zool.* 64, 310–322.
- Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., et al., 2007. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* 236, 3088–3099.
- Leatherland, J.F., Ball, J.N., Hyder, M., 1974. Structure and fine structure of the hypophyseal pars distalis in endogenous African species of the genus Tilapia. *Cell Tissue Res.* 149, 245–266.
- Levavi-Sivan, B., Biran, J., Fierman, E., 2006. Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine D2 receptors in female tilapia pituitary. *Biol. Reprod.* 75, 642–650.
- Levavi-Sivan, B., Bogerd, J., Mananos, E.L., Gomez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.* 165, 412–437.
- Levavi-Sivan, B., Safarian, H., Rosenfeld, H., Elizur, A., Avitan, A., 2004. Regulation of gonadotropin-releasing hormone (GnRH) receptor gene expression in Tilapia: effect of GnRH and dopamine. *Biol. Reprod.* 70, 1545–1551.
- Maclean, N., Rahman, M.A., Sohm, F., Hwang, G., Iyengar, A., Ayad, H., et al., 2002. Transgenic tilapia and the tilapia genome. *Genesis* 295, 265–277.
- Maruska, K.P., Fernald, R.D., 2011. Plasticity of the reproductive axis caused by social status change in an African cichlid fish: II. Testicular gene expression and spermatogenesis. *Endocrinology* 152, 291–302.
- Maruska, K.P., Levavi-Sivan, B., Biran, J., Fernald, R.D., 2011. Plasticity of the reproductive axis caused by social status change in an African cichlid fish: I. Pituitary gonadotropins. *Endocrinology* 152, 281–290.
- Miskey, C., Izsvak, Z., Kawakami, K., Ivics, Z., 2005. DNA transposons in vertebrate functional genomics. *Cell. Mol. Life Sci.* 62, 629–641.
- Naito, N., Suzuki, K., Nozaki, M., Swanson, P., Kawachi, H., Nakai, Y., 1993. Ultrastructural characteristics of two distinct gonadotropes (GTH I- and GTH II-cells) in the pituitary of rainbow trout *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* 11, 241–246.
- Nolan, L.A., Levy, A., 2006. The effects of testosterone and oestrogen on gonadectomised and intact male rat anterior pituitary mitotic and apoptotic activity. *J. Endocrinol.* 188, 387–396.
- Norman, W.A., Litwack, G., 1997 (Hormones), second ed. Academic Press.
- Nozaki, M., Naito, N., Swanson, P., Dickhoff, W.W., Nakai, Y., Suzuki, K., et al., 1990. Salmonid pituitary gonadotropins II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). *Gen. Comp. Endocrinol.* 77, 358–367.
- Oishi, Y., Okuda, M., Takahashi, H., Fujii, T., Morii, S., 1993. Cellular proliferation in the anterior pituitary gland of normal adult rats: influences of sex, estrous cycle, and circadian change. *Anat. Rec.* 235, 111–120.
- Páll, M.K., Liljander, M., Borg, B., 2004. Prolactin diminishes courtship behaviour and stimulates fanning in nesting male three-spined sticklebacks. *Gasterosteus aculeatus*. *Behaviour.* 141, 1511–1519.
- Parikh, V.N., Clement, T.S., Fernald, R.D., 2006. Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. *Behav. Brain Res.* 166, 291–295.
- Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., et al., 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Prog. Horm. Res.* 42, 513–548.
- Peute, J., Bruyn, M.G.A., Seldenrijk, R., Oordt, P.G.W.J., 1976. Cytophysiology and innervation of gonadotrophic cells in the pituitary of the Black Molly (*Poecilia latipinna*). *Cell Tissue Res.* 174, 35–54.
- Pfennig, F., Kurth, T., Meißner, S., Standke, A., Hoppe, M., Zieschang, F., et al., 2011. The social status of the male Nile tilapia (*Oreochromis niloticus*) influences testis structure and gene expression. *Reproduction* 143, 71–84.
- Pilar García Hernández, M., García Ayala, A., Zandbergen, M.A., Agulleiro, B., 2002. Investigation into the duality of gonadotrophic cells of Mediterranean yellowtail (*Seriola dumerilii*, Risso 1810): immunocytochemical and ultrastructural studies. *Gen. Comp. Endocrinol.* 128, 25–35.
- Rahman, A., Maclean, N., 1992. Production of transgenic tilapia (*Oreochromis niloticus*) by one-cell-stage microinjection. *Aquaculture* 105, 219–232.
- Rahman, M.A., Iyengar, A., Maclean, N., 1997. Co-injection strategy improves integration efficiency of a growth hormone gene construct, resulting in lines of transgenic tilapia (*Oreochromis niloticus*) expressing an exogenous growth hormone gene. *Transgenic Res.* 6, 369–378.
- Schradin, C., 2008. Differences in prolactin levels between three alternative male reproductive tactics in striped mice (*Rhabdomys pumilio*). *Proc. R. Soc. B Biol. Sci.* 275, 1047–1052.
- Schradin, C., Yuen, C.-H., 2011. Hormone levels of male African striped mice change as they switch between alternative reproductive tactics. *Horm. Behav.* 60, 676–680.
- Schulz, R.W., de França, L.R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., et al., 2010. Spermatogenesis in fish. *Gen. Comp. Endocrinol.* 165, 390–411.
- Schulz, R.W., Zandbergen, M.A., Peute, J., Bogerd, J., van Dijk, W., Goos, H.J., 1997. Pituitary gonadotrophs are strongly activated at the beginning of spermatogenesis in African catfish, *Clarias gariepinus*. *Biol. Reprod.* 57, 139–147.
- Shimizu, A., Hamaguchi, M., Ito, H., Ohkubo, M., Udagawa, M., Fujii, K., et al., 2008. Appearances and chronological changes of mummichog *Fundulus heteroclitus* FSH cells and LH cells during ontogeny, sexual differentiation, and gonadal development. *Gen. Comp. Endocrinol.* 156, 312–322.
- Slater, M., 1991. Plurihormonality in the secretory granules of the normal human pituitary. An immunoelectron microscopic study. *Cell. Mol. Life Sci.* 47, 267–270.
- Tafalla, C., Estepa, A., Coll, J.M., 2006. Fish transposons and their potential use in aquaculture. *J. Biotechnol.* 123, 397–412.
- Turner, G.F., Robinson, R.L., 2000 (Reproductive biology, mating systems and parental care). In: Beveridge, M.C.M., McAndrew, B.J. (Eds.), *Tilapias: Biology and Exploitation*. Kluwer Academic Publishers, pp. 33–58.
- Vera Cruz, E.M., Brown, C.L., 2007. The influence of social status on the rate of growth, eye color pattern and Insulin-like growth factor-I gene expression in Nile tilapia, *Oreochromis niloticus*. *Horm. Behav.* 51, 611–619.
- Watanabe, W.O., Kuo, C.M., Huang, M.C., 1985. Salinity tolerance of Nile tilapia fry (*Oreochromis niloticus*), spawned and hatched at various salinities. *Aquaculture* 48, 159–176 (Amsterdam, Netherlands).
- White, S.A., Nguyen, T., Fernald, R.D., 2002. Social regulation of gonadotropin-releasing hormone. *J. Exp. Biol.* 205, 2567–2581.
- Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. *Int. Rev. Cytol.* 225, 131–185.
- Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Levavi-Sivan, B., Elizur, A., 2001. Regulation of gonadotropin subunit genes in tilapia. *Comp. Biochem. Phys. B* 129, 489–502.
- Young, G., Ball, J.N., 1982. Ultrastructural changes in the adenohypophysis during the ovarian cycle of the viviparous teleost *Poecilia latipinna*: I. The gonadotrophic cells. *Gen. Comp. Endocrinol.* 48, 39–59.
- Yuen, C.W., Ge, W., 2004. Follistatin suppresses FSH beta but increases LH beta expression in the goldfish – evidence for an activin-mediated autocrine/paracrine system in fish pituitary. *Gen. Comp. Endocrinol.* 135, 108–115.
- Ziegler, T.E., Prudom, S.L., Zahed, S.R., Parlow, A.F., Wegner, F., 2009. Prolactin's mediative role in male parenting in parentally experienced marmosets (*Callithrix jacchus*). *Horm. Behav.* 56, 436–443.