GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia

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Developmental expression of GPR54 and rGnRH I

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ABSTRACT

The Kiss 1/GPR54 system has recently been shown to play a key role in the onset of puberty in mammals. Growing evidence suggests that this system is also conserved across vertebrates although very few studies so far have been performed in lower vertebrates. The aims of this study were firstly in the teleost Nile tilapia to screen tissues for GPR54 expression levels, secondly to measure the expression patterns of GPR54 and GnRH I receptor (rGnRH I) in whole brains during the onset of puberty and finally to determine the effects of continuous illumination (LL) on receptor expression levels. Results confirmed that GPR54 was predominantly expressed in the brain and pituitary of adult tilapia. Furthermore, a significant increase of GPR54 gene expression was found in tilapia brains at 11 weeks post hatch (wph) followed by rGnRH I at 13wph just prior to the histological observation of vitellogenic oocytes and active spermatogenesis in ova and testes at 17wph. These results suggest a correlation between the increase of GPR54 expression in the brain and the onset of puberty. Finally, a significant effect of LL was observed on GPR54 expression levels which were characterized by a delayed surge with significantly lower levels than those of control fish. The current study not only suggests a link between the Kiss 1/GPR54 system and the onset of puberty in a tropical batch spawning teleost that would be a highly conserved feature across vertebrates but also that the transcriptional mechanisms regulating GPR54 expression could be directly or indirectly influenced by light.

Keywords: GPR54; GnRH I; puberty; light; Nile tilapia.

INTRODUCTION

The mechanisms by which puberty is initiated in vertebrates has been a long sought question and the recent discovery of kisspeptin genes might help unravel this mystery. The brain-pituitary-gonadal (BPG) axis and the whole cascade of genes, receptors, enzymes and hormones involved in the control of puberty have been the object of many studies over the last ten years. Although such commitment clearly resulted in a better understanding of the GnRH and gonadotropin systems (for a detailed review see Gore, 2002), the way by which the BPG axis is initiated remains unclear in mammals and unknown in teleosts.

To date, more than 20 forms of GnRH have been found in vertebrates, eight of which are present in teleosts (Lethimonier *et al.* 2004; Pawson and McNeilly 2005). Of the three GnRH forms (I, II and III) found in brain of Nile tilapia and other perciforms, only GnRH I was found in the preoptic area of the hypothalamus and pituitary (hypophysiotropic) where it is also the most abundant (Parhar *et al.* 1996; Gothilf *et al.* 1996; Carolsfeld *et al.* 2000). GnRH I and its cognate receptor (rGnRH I) have thus been often used to study the mechanisms regulating gonadotropin release in many vertebrates.

The role played by environmental cues such as photoperiod are well reported across temperate vertebrate species and melatonin is known to be one of the key rhythmic signals entrained by light and used to synchronise physiological events such as reproduction in mammals (Malpaux *et al.* 2001; Simonneaux and Ribelayga 2003; Pevet 2003). On the other hand, in teleosts, convincing evidence of the cellular mechanisms and pathways are yet to be found despite the fact that melatonin also seems to play a key role in mediating photoperiod effects (Mayer *et al.* 1997; Falcon *et al.* 2007). Recently, kisspeptin (also called metastin) a product of the gene Kiss1 and its cognate G protein-coupled receptor 54 (GPR54) have been suggested to play an important role in the initiation of puberty in higher vertebrates. Preliminary evidence of such a role was reported in 2003 when two independent research

groups found that mutations of GPR54 caused hypogonadotropic hypogonadism in humans (de Roux *et al.* 2003) and mice (Funes *et al.* 2003). Since then, GPR54 has been cloned in other vertebrates including a few teleost species (Parhar *et al.* 2004; Nocillado *et al.* 2007; Mohamed *et al.* 2007) and have shown high homology of the amino acid sequence suggesting that the Kiss1/GPR54 system would be a conserved feature across vertebrates. Further studies have shown that kisspeptin could directly stimulate GnRH release via GPR54 in mouse (Messager *et al.* 2005). To date, this has not been shown in fish although GPR54 has been localized in GnRH neurons in tilapia (Parhar *et al.* 2004). In addition, kisspeptins also appear to coordinate the negative feedback loops of sex steroids in the hypothalamus of mammals (Rometo *et al.* 2007). Importantly, new evidence linking for the first time the Kiss1/GPR54 system and photoperiod has been reported in the hamster (Revel *et al.* 2006a). Many comprehensive reviews have recently been published (Colledge 2004; Popa *et al.* 2005; Seminara 2005; Aparicio 2005; Murphy 2005; Tena-Sempere 2006; Kuohung and Kaiser 2006; Smith *et al.* 2006a; Smith *et al.* 2006b; Roa and Tena-Sempere 2007; Roa *et al.* 2007). However, although the importance of this new system is without any doubt of prime importance, expression data in teleosts has been to date very scarce with only, to our knowledge, two published papers reporting studies performed in two seasonal marine species, the cobia (*Rachycentron canadum*) and grey mullet (*Mugil cephalus*). The latter have shown a positive correlation between the increase of GnRH expression and GPR54 during early development and puberty (Nocillado *et al.* 2007; Mohamed *et al.* 2007).

Further studies are clearly needed in a wider range of models to determine not only if the Kiss1/GPR54 system is highly conserved across vertebrates but also if such a system could be the missing link between perception of environmental cues and priming of the BPG axis. For this reason, teleosts could prove to be very good non-mammalian models due to the strong seasonality of their physiology, the range of reproductive strategies and the diversity of

environmental niches they inhabit. Indeed, photoperiod has been shown to be the main signal used by most of the fish species to entrain and synchronise reproduction (review by Bromage *et al.* 1995). Although this has been mainly reported in temperate teleost species, recent reports have also shown significant effects of photoperiod on reproductive physiology in Nile tilapia (Campos-Mendoza *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006).

The objectives of this study were thus to 1) screen tissue expression levels of GPR54 in order to confirm potential action sites, 2) measure the developmental expression patterns of GPR54 and GnRH I receptor (rGnRH I) during the onset of puberty and 3) determine whether continuous illumination (LL) could have an effect on GPR54 and GnRH I receptor expression levels.

MATERIAL AND METHODS

Animals

Mixed sex red Nile tilapias *(O. niloticus niloticus)* were obtained from the tropical aquarium facilities at the Institute of Aquaculture (University of Stirling, Stirling, UK). All fish used in these experiments were from the same stock and were produced in the facilities. Experimental fish were reared in a light proof closed water recirculation system (27 ± 1^{o}C) as previously described in Campos-Mendoza *et al.* (2004). Light intensity was 0.75W/m^{2} at the water surface (measured by a single channel light sensor, Skye instruments, Powys, UK). Nitrate, nitrite, ammonia and pH were monitored throughout the experiments with aquarium water quality kits (C-Test kits, New Aquarium Systems, Mentor, USA) and levels remained within safe limits. Fry were hand fed to satiation three times a day at 9am, 1pm and 6pm with a crumb mix of two feeds (Nutra Trout Fry 02 and Standard Expander 40, Skretting, Cheshire, UK). All trials were carried out in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986, UK.

Experiments and sampling

In all experiments, fish were killed by a lethal dose of anaesthetic (0.03M benzocaine solution, SIGMA, Poole, UK), and then decapitated. Tissues were sampled swiftly to avoid RNA degradation following fish death, frozen in liquid nitrogen and stored at -70^{0}C until total RNA extraction.

Experiment 1: Tissue screening

To determine the potential action sites of GPR54, samples from seven different tissues were sampled at midday from two-year old mature fish (three males and three females, mean weight of 135.8 ± 53.3g): brain, pituitary, heart, kidney, liver, gonad (mostly ovarian membrane in females) and muscle. Fish were previously reared in normal high density stocking conditions under a 12L:12D photoperiod.

Experiment 2: Developmental expression of GPR54 and rGnRH I during onset of puberty

Nile tilapia fry/fingerlings reared under standard conditions in the facilities (27 ± 1^{o}C, 12L:12D photoperiod, fed three times a day *ad libitum*) were sampled from 3wph (23 days post-fertilization) to 17wph. Initially, swimming larvae were kept in 7L incubators within the recirculating light proof system. Half way through the experiment, fish were transferred into 40L aquaria within the same rearing system. Sampling consisted of sacrificing fish at each time point (every 2 weeks) precisely at the same time of the day (12pm). The number of fish sampled and pooled (from 5 pools of 15 larvae heads at 3wph to 10 individual fish brains from 7 to 17wph) varied depending on the size of the fish (see Table 1). Whole bodies (larval stage) and/or gonads of sampled fish were fixed in Bouin's fixative and processed for histological examination in order to determine sex and gonadal staging.

Experiment 3: Effects of constant illumination on GPR54 and rGnRH I gene expression during the onset of puberty

A different batch of fish, from the same origin, was reared following experiment 2 under either 12L:12D photoperiod or constant illumination (LL) to test the effects of un-entrained photic conditions. LL regime was chosen as several authors reported the effects of such photoperiod or constant long-day regimes on growth and reproduction in Nile tilapia as well as other tropical and temperate species. Furthermore, Nile tilapia is a diurnal fish which feeds during the photophase. Sampling took place every 2 weeks starting at 7wph until 13wph (corresponding to the window where GPR54 and rGnRH I gene expression was shown to significantly increase in previous trial). Ten to twelve fish per treatment were randomly sampled at each time point in order to have a minimum of 5 males and 5 females however, in some cases only 4 individuals of one sex were sampled. Only eight individual fish brains (4 fish/sex) were analysed for gene expression. All gonad samples were fixed as previously mentioned and analysed by histology.

RNA extraction and cDNA synthesis

Frozen heads, brain samples and tissues were homogenized in 1ml TRI reagent (Sigma Aldrich, Saint Louis Missouri, USA) solution per 100mg of tissue. RNA pellets where reconstituted in 50µl of MilliQ water, quality checks and measurements were performed with a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK) and running 1µg of total RNA in a 1% agarose denaturating gel. cDNA was synthesized using 1µg of total RNA, 1ul of a blend (3:1) random hexamers (400ng/µl) - oligo dT (500ng/µl) (ABgene®, Epsom, UK) respectively, 1 µl of 10 mM dNTPs, 1 µl (200 units/ul) of reverse transcriptase III (Invitrogen®, Paisley, UK) with provided buffers in a final volume of 20µl. Following

169 synthesis, 180µl of MilliQ water was added to reach working dilution (1:10) and stored at -
170 20^0C.
171
172 *Primer design and Quantitative RT-PCR (qPCR)*
173 All primers were designed using using PrimerSelect Ver. 6.1 program (DNASTAR,
174 www.dnastar.com) based on GeneBank sequences of GPR54, rGnRH I (target genes) and β-
175 actin, accession numbers: AB162143, AB111356 and EF206801 respectively. β-actin has
176 been selected as a reference gene following a preliminary gene expression study in the brain
177 that showed remarkable stability under the experimental conditions tested. PCR products were
178 then cloned into a pCR 2.1 vector (Invitrogen, Paisley, UK) and sequenced using a CEQ-8800
179 Beckman sequencer (Coulter Inc., Fullerton, USA). The identity of the cloned PCR products
180 were then verified (100% overlapping) using BLAST (http://www.ncbi.nlm.nih.Gov
181 /BLAST/). Primers used for qPCR were designed on these sequences and optimized (Table
182 2). In all cases, qPCR reactions containing 1µl of each primer (7pmol/µl), 5µl cDNA (1:10
183 dilution), 10µl Syber Green master mix (ABgene®, Epsom, UK) and 3µl of MilliQ water in a
184 final volume of 20µl, were run in a thermocycler (Techne, Quantica, Cambridge, UK) using
185 the following program: 95°C for 15min (Taq activation) followed by 45 cycles of 95°C for 15
186 sec, 60°C for 15 sec and 72°C for 30 sec followed by a temperature ramp from 70°C to 90°C
187 for melting curve analysis. Gene copy number in each reaction was calculated by comparison
188 to a standard curve constructed from the results of a parallel set of reactions containing serial
189 dilutions of linearised plasmids containing Nile tilapia GPR54, rGnRH I and β-actin cloned
190 cDNA sequences. Concentrations of standards were determined spectrophotometrically and
191 converted to copy number by consideration of plasmid size and DNA absorption coefficient.
192 Samples were run in triplicate together with non template controls, standards and internal
193 controls to correct expression levels between plates. qPCR products were checked by

sequencing confirming 100% overlap against Genebank sequences. Expression levels for tissue expression profile are shown as absolute copy numbers per µg of total RNA, due to the variability of β-actin expression in different tissues, whereas expression values for experiments 2 and 3 were normalised against the reference gene (β-actin), multiplied by 100,000 (arbitrary number for graphs) and presented as relative expression.

Histological preparations and analysis

The fixed samples were trimmed and placed individually into cassettes and then dehydrated using methylated spirits, cleared in chloroform and impregnated with paraffin wax using an automated Tissue Processor (Thermo Fisher, Cheshire, UK). The samples were then embedded in molten paraffin wax using a histoembedder (Leica UK Ltd., Milton Keynes, UK). Once hardened the wax blocks were trimmed using a Rotarty microtome (Leica UK Ltd., Milton Keynes, UK). When the samples consisted of whole fish it was necessary to surface decalcify the blocks for 1 hour in Rapid Decalcifying solution (Cellpath, UK.) before cutting. Using a Rotary Microtome, the blocks were sliced into 5µm thick sections and transferred onto slides. These slides were stained according to a standard Haematoxylin and Eosin (H and E) protocol. The stage of oocyte development was visually determined and the leading oocytes were classified according to Coward and Bromage (1998) for oogenesis (chromatin nucleolar stage (1), early perinucleolar stage (2), late perinucleolar stage (3), cortical alveolar stage (4), vitellogenesis (5), maturation (6) and germinal vesicle migration (7)) and Babiker and Ibrahim (1978) for spermatogenesis (Immature (1), Maturing (2), Mature (3), Ripening (4), Ripe (5), Spawning (6) and Spent (7)) respectively.

219 **Statistical analysis**

220 Normality and homogeneity of variances were tested using Kolmogorov-Smirnov (with
221 Lilliefors' correction) and when appropriate, data was transformed using the natural
222 logarithm. Data from the experiment1 (tissue GPR54 gene expression) and comparison of
223 males *vs.* females for all trials were analysed by t-tests. In addition, temporal gene expression
224 data in experiment 2 were analysed by a) a general lineal model (GLM) from 3-11wph where
225 no sex differences were observed (mean values) and by b) a GLM per/sex from 7-17wph. For
226 purpose of clarity, temporal differences from 3 to 11wph are not presented on figure 2. Data
227 in experiment 3 were also analysed by a GLM including temporal and treatment factors (two
228 way ANOVA) followed by multiple comparison test (Tukey). For all tests, significance was
229 set at $p<0.05$. All analyses were performed with Sigmastat (V. 3.11) and Minitab (V. 14.13).
230 Values are presented as mean ± SEM.

231

232 **Results**

233 Raw gene expression data showed in all cases the same pattern of expression than the
234 normalized results presented in this study. Nonetheless, normalization decreased sample
235 variability showing that the reference gene used (β-actin) was appropriate in all cases.
236 In experiment 1, GPR54 expression was found to be highest in brain of male and female fish
237 with no significant differences between sexes (Fig. 1). Levels in the pituitary were shown to
238 be lower (~14 fold) than in the brain and significantly higher in females than in males (at least
239 2 fold) although sampling size was small (n=3). Expression levels were close to detection
240 limits in the other tissue samples (heart, kidney, liver, gonad and muscle) ranging between 7
241 to 78 absolute copies/µg total RNA.

242 In experiment 2, significant sex differences were found in GPR54 gene expression at 13
243 and 17wph (Fig. 2a) with higher levels in females. Developmental GPR54 expression levels

were generally low in the first 9wph but displayed a significant increase between 3 to 4 and 6 to 7wph which for purpose of clarity are not shown. Thereafter, GPR54 expression levels significantly increased in both sexes (~8.5 fold) from 9 to 11wph and were maintained so (plateau) until the end of the study (17wph) in females with males showing a significant reduction at 13wph. GnRH I type receptor also produced a similar pattern of expression as GPR54 with small but significant increases between 3-4, 6-7 and 7-9wph (Fig. 2b, not shown). Expression levels continued to increase significantly between 9 to 11wph (~3.5 fold) and 13wph (~2 fold) when peak expression levels were reached and maintained until 17wph. Significant sex difference of rGnRH I expression levels were only found at 13wph with higher levels in females. Fish had a final mean weight of 31.5 ± 4.4 (n=10) at 17wph. No significant correlations were found between GPR54/rGnRH I and growth (graphs not shown).

Gonadal histological observations showed that first sign of active oocyte development (cortical alveolar stage) appeared from 11wph although all females sampled at 9wph possessed oocytes at stage 2 (early perinucleolar)(Table 3). First vitellogenic oocytes were only observed at 17wph. All males sampled up to 13wph were at an immature stage with testes predominantly containing spermatogonia. First sign of active spermatogenesis was observed at 15wph with testes possessing a few spermatids (stage 2). At 17wph, 80% of the male fish sampled (4 fish) were at the ripening stage 4 characterised as active spermatogenesis and containing different stages with abundant spermatozoa.

In experiment 3, GPR54 gene expression levels measured in the brain of fish exposed to 12L:12D (control) photoperiod and constant illumination (LL) were not significantly different at 7wph (Fig. 3a). Thereafter, GPR54 expression levels increased significantly in fish from both treatments and reached significantly higher levels (2.2 fold) in control as compared to LL at 9wph. Under the control photoperiod treatment, GPR54 expression levels remained elevated for the remainder of the study. However, under the LL treatment, GPR54 expression

continued to increase between 9 and 11wph, thereafter no differences were then observed. Expression levels between treatments at both 11 and 13wph were not statistically different.

rGnRH I expression levels in the brain was similar in fish under both photoperiodic treatments throughout the study (from 7 to 13wph) (Fig. 3b). Levels of both treatments significantly increased between 7 and 9wph (2.5 fold) and then again between 9 and 11wph (2.5 fold). Levels remained steady at 13wph. No statistical differences were observed between treatments at both latter sampling points although mean rGnRH I expression levels appeared to be slightly higher in control fish. No significant sex differences were found in expression levels of both target genes throughout experiment 3. No significant growth differences were observed throughout the duration of the experiment between photoperiodic treatments with mean end weight at 15wph of 27.11 ± 1.33g (control) and 28.97 ± 1.17g (LL). Weight of the fish in both experiment 2 and 3 were similar at 17wph (31.6 ± 14.08g, n=10) and no significant correlations were found between GPR54/rGnRH I and growth (graphs not shown). Gonadal staging in this experiment showed no major differences between treatments although no statistical analyses could be performed due to the format of the data. Males in control group initiated spermatogenesis (stage 2) between 7 and 9wph and reached advanced ripening stage in both treatments by 13wph. Females started earlier at 7wph reaching the early perinuclear stage (3) in control fish and the cortical alveolar stage (4) in the LL treatment. Females from the control group seemed to catch up with LL by 9wph and the stages remained similar (late perinuclear to cortical alveolar) in both groups until 13wph when the first females of both groups reached vitellogenesis (Table 4).

Discussion

The kisspeptin receptor GPR54 has been shown to stimulate GnRH secretion in mammals (Messager *et al.* 2005) and has been specifically localized in Nile tilapia GnRH neurons (Parhar *et al.* 2004) although to our knowledge, no studies have looked at its expression in tissues other than brain and gonads in fish. In the current study, the Nile tilapia kisspeptin receptor (GPR54) showed high tissue specificity (expressed in brain and pituitary) in accordance with its suggested role in the BPG axis (Seminara 2005; Smith *et al.* 2006a). Although no significant differences between males and females were shown in brain tissues in experiment 1, higher expression levels of GPR54 were observed in females in the pituitary. Such sexual dimorphism in expression levels have already been reported for rGnRH I in the same species (Levavi-Sivan *et al.* 2004). Interestingly, male brain expression levels showed more variation than females. This could be explained either by the small sample size (n=3) or more likely by the social nature/behavior of this species (i.e. dominant males could potentially have higher GPR54 levels than their submissive counterparts). Such social interactions have been shown to exist in another cichlid (*Astatotilapia burtoni*) where territorial (dominant) males were shown to have significantly higher rGnRH I expression levels than non territorial males (Au *et al.* 2006; Hofmann 2006). Also, studies have reported that GnRH mRNA and its receptors are differentially regulated and sexually dimorphic during development (Gore 2002; Levavi-Sivan *et al.* 2004). GPR54 levels in all other tissues were very low (especially taking into account the amount of cells present in ~100mg tissue) and suggests that GPR54 might not play a functional role in these tissues, although it should be noted that in mammals, it has been reported that low occupancy (20%) of GnRH receptors (not shown for GPR54) is enough for a strong (80%) biological response (Naor *et al.* 1980). The low GPR54 transcripts found in the ovary in this study compared to those found in grey mullet (Nocillado *et al.* 2007) could be explained by the fact that in the present study, most of the oocytes were

315 removed from the gonad to prevent the high fatty acid and glycoprotein content of tilapia eggs
316 from potentially affecting RNA extraction, cDNA synthesis and qPCR sensitivity.
317 Importantly when comparing expression levels between different studies, methodology
318 aspects such as type (i.e. random hexamers, oligo dT), concentration of primers used during
319 cDNA synthesis, final template dilution used and normalization strategy should be considered
320 as they could all account for target copy variability (Ginzinger 2002).

321 Furthermore, results in the current study clearly showed a GPR54/rGnRH I surge in
322 whole brains of Nile tilapia, which correlated with the onset of puberty as shown through
323 histological observations (experiment 2 and 3). Indeed, in experiment 2 male fish were at an
324 immature stage (pre-gametogenesis) up to 13wph while females appeared to initiate oogenesis
325 soon after the first significant increases of GPR54/rGnRH I expression occurring between 7
326 and 9wph. The highest surge of expression occurred by 11wph and 13-15wph for GPR54 and
327 rGnRH I respectively.

328 Although significant sex differences of GPR54 gene expression were observed at 13 and
329 17wph with higher levels found in females, peak levels of expression were already reached
330 for both sexes by 11wph. If the role of GPR54 on the initiation of the BPG axis in tilapia is
331 confirmed, these results could suggest that the timing of such stimulation would have
332 occurred simultaneously in both sexes. Theses results contrast with those found by Mohamed
333 *et al.* (2007) where male cobia displayed significantly higher GPR54 expression levels.
334 Interestingly, rGnRH I expression levels significantly peaked at 13wph for females and two
335 weeks later in males (15wph). Such sexual dimorphism, already reported in a tilapia hybrid,
336 *O. niloticus x O. aureus* (Levavi-Sivan *et al.* 2004), correlate well with the later gonadal
337 development observed in males as compared to females. However, we acknowledge that
338 sample size might have been too low to depict further significant differences between sexes.
339 Nonetheless, although females appeared to initiate gametogenesis earlier, both sexes only

reached advanced stages of gonadal development (early to late vitellogenic stages in females and ripening testes in males) by the end of the experiment (15-17wph). This delay in male gonadal development most probably reflects the difference in time and energy required to accomplish full development in comparison to females.

Thus, in agreement with what has already been reported in other vertebrates, the onset of puberty in Nile tilapia would be correlated to increases in GPR54 and rGnRH I. Although these findings do not demonstrate a direct link between these two receptors and puberty, it suggests that such a connection could be at work as both receptors consecutively switch on prior to the onset of active gametogenesis. These results bring further evidences to recent data obtained in two marine teleost species (with longer life cycles and different reproductive strategies) in which a similar pattern of GPR54 expression at the respective gonadal stages has been shown (Mohamed *et al.* 2007; Nocillado *et al.* 2007). To date and to our knowledge, the present study is the first one to study the ontogeny of expression prior and throughout puberty in a batch spawner fish. In cobia, GPR54 expression was shown to peak when male fish were at an early stage of puberty followed by a rise in all three GnRH subtypes (Mohamed *et al.* 2007). Similarly, grey mullet showed higher GPR54 expression in brain during early gonadal development stages (Nocillado *et al.* 2007). In the present study (experiment 2), the fact that oocytes in females were already at a late perinucleolar and cortical alveolar stages when GPR54 expression levels peaked suggests a) that the intense surge in GPR54 and rGnRH I expression (observed at 11 and 13wph respectively in the current work) might not be needed for the initial stages of oocyte development and/or b) that kisspeptin, gonadotropin and sex steroid content previously released are enough to trigger onset of gametogenesis.

After the surge of gene expression in both GPR54 and rGnRH I, high levels were then maintained throughout the remaining of the experiments. This is in agreement with previous

365 studies performed in higher vertebrates which have shown that puberty is associated with an
366 increase in expression of GnRH mRNA and pulsatile releases of GnRH and LH which peak
367 when reproductive function is attained and tends to remain steady (plateau) (Gore 2002;
368 Clarke and Pompolo 2005). However, present results are in conflict with previous findings
369 obtained in the grey mullet where expression levels were shown to decrease at intermediate
370 and advanced gonadal stages (Nocillado *et al.* 2007). Differences in the profile of expression
371 and sexual dimorphism could be due to the different reproductive strategies between species
372 (continuous batch spawner in Nile tilapia vs. iteropare for grey mullet and cobia) and other
373 factors such as the timing of sampling and interspecies differences.

374 Photoperiod is without any doubt one of the most powerful and noise free signal along
375 with temperature that fish and other vertebrates can rely on to synchronise their reproductive
376 physiology. However, there has not been any clear definition of the pathway through which
377 photoperiod exerts its effects in fish physiology (Mayer *et al.* 1997; Falcon *et al.* 2007). The
378 aim of experiment 3 was firstly, to confirm findings of experiment 2 and determine whether
379 photoperiod could have an effect on the GPR54/rGnRH I expression patterns as well as
380 gonadal development. Indeed, the pattern of GPR54 and rGnRH I expression observed in
381 experiment 3 reproduced very well what was shown in the experiment 2 although there was a
382 shift in the timing of the expression surge which occurred earlier (GPR54 peaking at 9wph in
383 control fish compared to 11wph in experiment 2) with a higher amplitude (almost 2 fold).
384 Histology results in this experiment also confirmed those shown in the previous one with
385 control males and females starting to develop after the initial surges in gene expression of
386 both target genes (7-9wph) and reaching more advanced stages by 11-13wph. No evident
387 differences could be observed in developmental stages between both control and LL
388 treatments. However, males under the control photoperiodic regime matured earlier than
389 males in experiment 2 (stage 3 reached at 15 and 11wph, respectively for experiments 2 and

390 3). Natural stock variability in addition to possible sex ratio interactions could explain these
391 differences (Lorenzen *et al.* 2000).

392 Secondly, our results suggested an effect of photoperiod on GPR54 gene expression
393 levels which were shown to be significantly reduced at 9wph in fish exposed to LL compared
394 to fish under 12L:12D control photoperiod. Although rGnRH I expression levels appeared to
395 be lower in fish exposed to LL between 11 and 13wph, no significant differences between
396 treatments were observed. One possible explanation is that the GPR54 surge in the LL
397 treatment would have been enough to trigger (gate) the GnRH cascade. These results would
398 therefore suggest that the mechanism involved in the transcription of GPR54 and possibly its
399 ligand Kiss-1 (not tested in this study) could be affected by environmental cues as recently
400 proposed in mammals (Revel, *et al.* 2006b; Roa *et al*. 2007). These findings will obviously
401 have to be confirmed. To our knowledge, data linking photoperiod effects in the Kiss/GPR54
402 system has only been recently reported in hamsters and sheep (Revel *et al.* 2006a; Greives *et*
403 *al.* 2007; Wagner *et al.* 2007). Kiss1 mRNA (not GPR54) was shown to be expressed in
404 higher quantities in long days (summer reproductive phase) rather than short days (winter
405 inhibition) (Revel *et al.* 2006a). The reverse is true in sheep (Wagner *et al.* 2007). Most
406 importantly, it was shown by Revel *et al.* (2006a) that pineal ablation did not reduce Kiss1
407 mRNA levels during short days as opposed to their control and sham counterparts. This
408 suggests that melatonin would mediate the short day down regulation of Kiss-1 expression in
409 hamsters. Furthermore, photoperiod and the reproductive state of hamsters were shown to
410 significantly affect the number and size of Kisspeptin-immunoreactive neurons (Greives *et al.*
411 2007). Both these studies confirmed the importance of the Kiss-1/GPR54 system in the
412 interpretation of environmental stimuli and subsequent regulation of the reproductive axis in
413 hamster. No such data is yet available in fish. The present study brings preliminary evidences
414 suggesting for the first time in fish that light could act on the Kiss-1/GPR54 system. Although

Nile tilapia is not a seasonal species as such, light has been shown to impact on its growth and reproductive physiology (Campos-Mendoza *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006) and mechanisms at work in higher vertebrates are likely to be conserved in Nile tilapia considered as one of the most evolved teleost species (perciforms). It could also be hypothesized that melatonin could have a direct or indirect role in regulating GPR54 expression levels as has been shown in higher mammals (Revel *et al.* 2006a; Greives *et al.* 2007; Wagner *et al.* 2007). As such, further co localization and regulatory studies in fish and vertebrates in general are clearly needed to help us better understand these regulatory mechanisms controlling puberty and reproduction in species with different reproductive strategies.

Overall, GPR54 and rGnRH I patterns of gene expression in experiment 2 and control fish from experiment 3 were comparable although in the latter, higher levels (circa 2 fold) were found throughout which could explain the more advanced stages of gametogenesis observed. The lack of strong correlation between expression levels and growth suggest that gene expression increased irrespectively of the size of the fish sampled and that growth (size) would not be a requirement for the onset of pubertal gene expression.

In conclusion, results showed a correlation between the GPR54, rGnRH I and the onset of puberty in a tropical batch spawning teleost. These findings are in accordance with results obtained in seasonal marine teleosts which further suggest a conserved role of this system across vertebrates. Moreover, the recent discovery of a functional Kiss1/GPR54 receptor in zebrafish (*Danio rerio*) provides a new tool to further study the Kiss system in teleosts (van Aerle *et al*. 2007). Furthermore, current results showed an effect of photoperiod on GPR54 expression with continuous illumination resulting in a reduced GPR54 expression compared to the control treatment. These preliminary findings suggest a potential link between

environmental stimuli and the kisspeptin/GnRH systems. Further investigations are clearly required to test and confirm these results and demonstrate how such mechanisms would work.

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570

571 Table 1. Experimental sampling structure of Nile tilapia.

572

Stage (wph)	**No. fish pooled**	**n=**	
		Female	**Male**
3	15 (heads)	5	
4	5 (heads)	5	
5	3 (heads)	5	
6	2 (heads)	5	
7	1 (brain)	5	5
9	1 (brain)	7	3
11	1 (brain)	7	3
13	1 (brain)	6	4
15	1 (brain)	5	5
17	1 (brain)	5	5

573

574 Table 2. Sequences of primers used for qPCR gene expression of developmental Nile tilapia.

575

Primer name	Sequence	Product size	Gene Bank Accession No.
β-actin F β-actin R	5′-TCTCTTCCAGCCTTCCTTCC-3′ 5′-GGTACCTCCAGACAGCACAGT-3′	130 bp	EF206801
rGnRH-I F rGnRH-I R	5′-GTGGCTTGCCGGAGACTTTG-3′ 5′-AGAGGGTTGAGGATGGCTGACT-3′	123 bp	AB111356
GPR54 F GPR54 R	5′-ATGCCTGGCTGGTCCCTCTGTTCT-3′ 5′-GGCGGCCAGGTTTGCTATGTA-3′	136 bp	AB162143

576 Table 3. Histological staging of male (M) and female (F) Nile tilapia sampled in experiment 2

577 every two weeks from 7 to 17wph.

WPH	**7**		**9**		**11**		**13**		**15**		**17**	
Stages	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
1	4	6	3		4		4					
2				7					4			
3						5		2	1	2	1	1
4						1		4		3	4	3
5												1
6												
Total	4	6	3	7	4	6	4	6	5	5	5	5

578

579 Table 4. Histological staging of male (M) and female (F) Nile tilapia exposed to either
580 12L:12D (control) or constant illumination (LL) photoperiods from first feeding (experiment
581 3). Sampling took place at 7, 9, 11 and 13wph.

Treatments	**Control**								**LL**							
WPH	**7**		**9**		**11**		**13**		**7**		**9**		**11**		**13**	
Stages	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
1	4	4	5		1				5		3					
2		3	2		5		2				2		8		1	
3				1	1	1	2			2		2		1	2	1
4				3		3	1	4		3		3		3	2	3
5								1								1
6																
Total	4	7	7	4	7	4	5	5	5	5	5	5	8	4	5	5

582

583 **Figure legends**

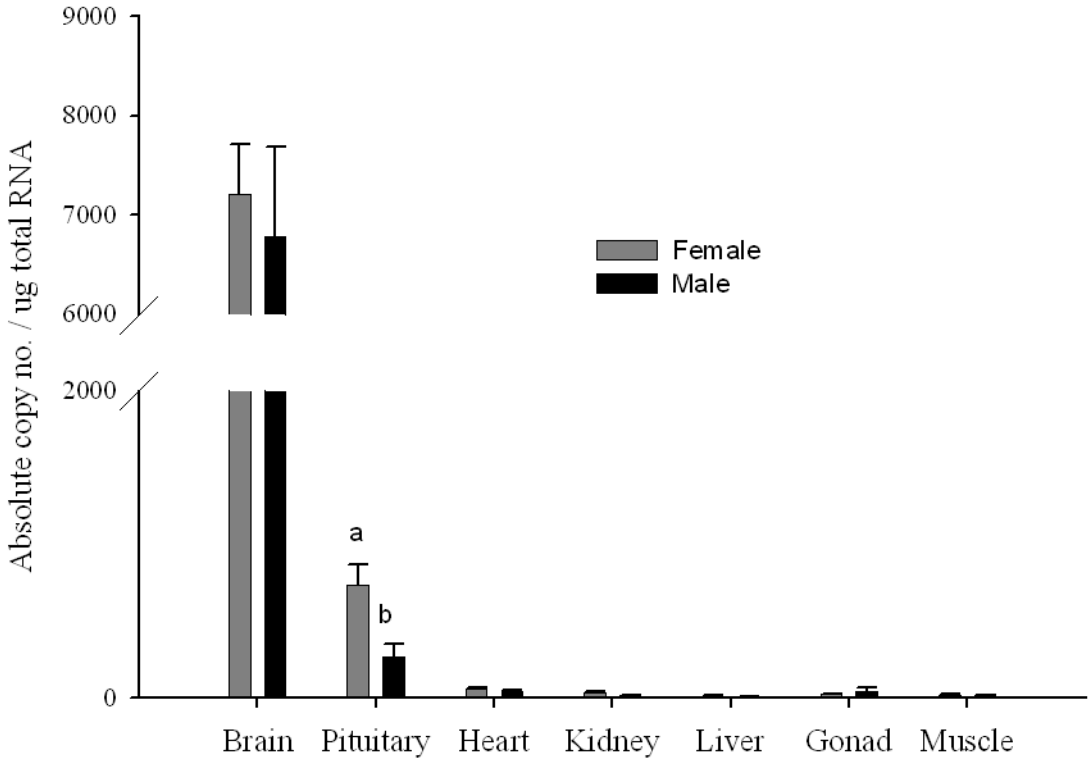
584 Fig. 1. Differential tissue expression of GPR54 (absolute copy numbers) in male and female
585 Nile tilapia. Values expressed as mean ± SEM (n=3). Superscripts denote significant
586 difference between sexes in each tissue.

587 Fig. 2. Relative gene expression of GPR54 (a) and rGnRH I (b) during onset of puberty in
588 Nile tilapia. Values expressed as mean ± SEM (n=3-7, see table 1). Superscripts denote
589 significant temporal differences for a given sex from 7 to 17wph (capital and lower case
590 letters for females and males, respectively) while asterisks (*) denote significant differences
591 between sexes at given time points.

592 Fig. 3. Relative gene expression of GPR54 (a) and rGnRH I (b) during onset of puberty in
593 Nile tilapia exposed to either 12L:12D (control) or constant illumination (LL) photoperiods
594 from first feeding. Values expressed as mean ± SEM (n=8). Superscripts denote significant
595 differences between sampling points and treatments.

596 Figure 1

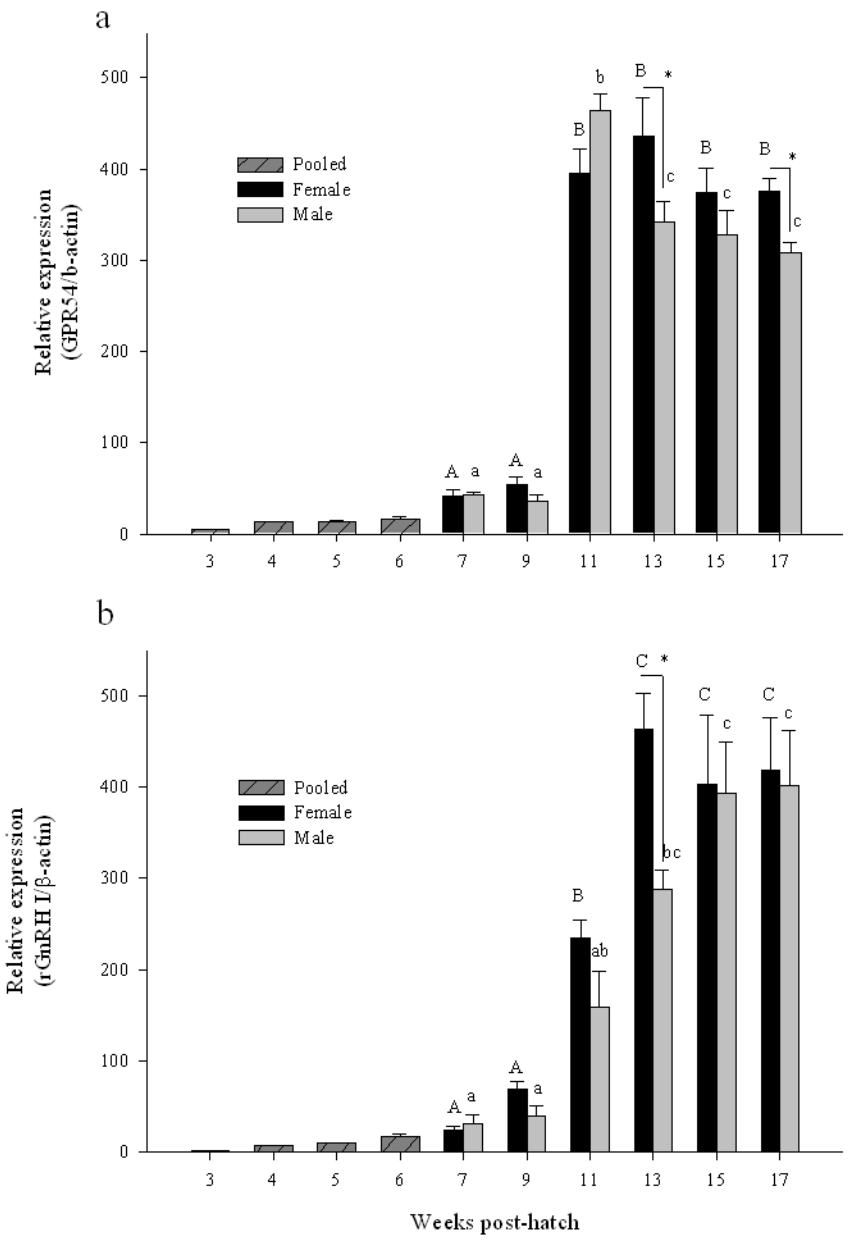
597



598

599 Figure 2

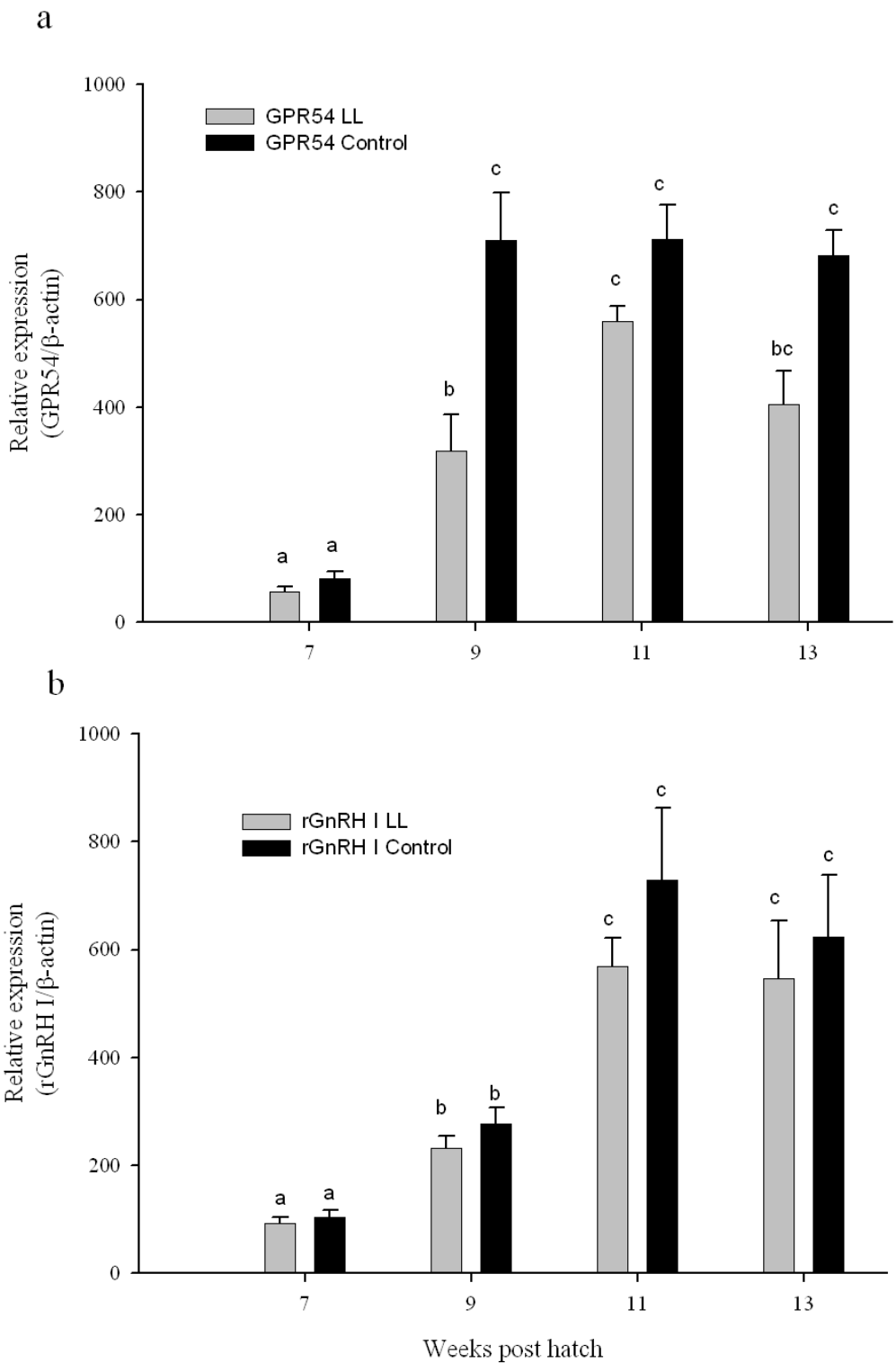
600



601

602 Figure 3

603



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