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Plasticity of Lh cells caused by cell proliferation and recruitment of existing cells

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Abstract

Luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) control reproduction in vertebrates. Using a transgenic line of medaka, in which green fluorescent protein expression is controlled by the endogenous *lhb* promotor, we studied development and plasticity of Lh cells, comparing juveniles and adults of both genders. Confocal imaging and 3D reconstruction revealed hypertrophy and hyperplasia of Lh cells in both genders from juvenile to adult stages. We show that Lh cell hyperplasia may be caused by recruitment of existing pituitary cells that start to produce *lhb*, as evidenced by time lapse recordings of primary pituitary cell cultures, and/or through Lh cell proliferation, demonstrated through a combination of 5-bromo-2'-deoxyuridine incubation experiments and proliferating cell nuclear antigen staining. Proliferating Lh cells do not belong to the classical type of multipotent stem cells, as they do not stain with anti-sox2. Estradiol exposure *in vivo* increased pituitary cell proliferation, particularly Lh cells, whereas pituitary *lhb* and *gpa* expression levels decreased. RNA-seq and *in situ* hybridization showed that Lh cells express two estrogen receptors, *esr1* and *esr2b*, and the aromatase gene *cyp19a1b*, suggesting a direct effect of estradiol, and possibly androgens, on Lh cell proliferation. In conclusion, our study reveals a high degree of plasticity in the medaka Lh cell population, resulting from a combination of recruitment and cell proliferation.

Key Words

- ▶ gonadotropins
- ▶ luteinizing hormone
- ▶ pituitary
- ▶ estradiol
- ▶ cell proliferation

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Introduction

The gonadotropins luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) are key players in the vertebrate brain–pituitary–gonad (BPG) axis controlling sexual maturation. Whereas the equivalent hormones in mammals and birds are synthesized and secreted from the same cell in the pituitary (Pope *et al.* 2006), they are produced by distinct cells in most teleosts (Kanda *et al.* 2011, Weltzien *et al.* 2014). Teleosts are therefore ideal models for investigating gonadotropin development and regulation.

Although described in only a few species, rainbow trout (Nozaki *et al.* 1990), African catfish (Cavaco *et al.*

2001) and zebrafish (Golan *et al.* 2014), the pituitary grows during the entire lifespan in teleosts, with a particular increase in the gonadotrope cell population during puberty. However, whether gonadotrope hyperplasia is a result of recruitment of existing pituitary cells and/or proliferation of progenitor cells is unknown. Controlling mechanisms are also poorly understood.

Gonadotropin synthesis and secretion are mainly stimulated by gonadotropin-releasing hormone, GnRH. In addition, a variety of stimulatory and inhibitory factors from the brain participate in control of Lh and Fsh, with variable importance according to species and

life stage (Yaron *et al.* 2003, Zohar *et al.* 2010). Gonadal steroids also participate in control, through direct and indirect feedback mechanisms, allowing the continuous communication within the BPG axis that is necessary for synchronized and coordinated activity.

Diverging effects of steroids on gonadotropes have been reported, depending on species, physiological status or gonadal steroid examined. Both positive and negative feedback effects have been observed on the synthesis and release of Lh and Fsh in teleosts (Yaron *et al.* 2003, Zohar *et al.* 2010, Von Krogh *et al.* 2017). The precise effects of sex steroids at the pituitary level are far from fully deciphered, and the mechanisms involved are likely to be complex as steroids can also act at the brain level.

Medaka (*Oryzias latipes*) provides a useful model system, having genetic gender determination (Matsuda *et al.* 2002, 2007, Nanda *et al.* 2002) and being accessible to a range of molecular and genetic tools (Wittbrodt *et al.* 2002, Shima & Mitani 2004). In this study, we used the tg(*lhb*-hrGfpII) line (Hildahl *et al.* 2012) to study developmental changes in pituitary morphology and Lh cell population in both genders. We investigated whether LH cell plasticity may be due to both recruitment of existing pituitary cells and cell proliferation.

Materials and methods

Animal maintenance and sexing

Wild-type (WT, d-rR strain) and tg (*lhb*-hrGfpII) (Hildahl *et al.* 2012) medaka (*Oryzias latipes*) were maintained at 28°C on a 14/10 h light/darkness cycle in a re-circulating water system and three daily feedings. Experiments were performed according to the recommendations on the care and welfare of research animals at the Norwegian University of Life Sciences. Experiments using 5-bromo-2'-deoxyuridine (BrdU) were approved by the Norwegian Food Safety Authority (FOTS ID 8596). Juvenile (2-month-old) and adult (6-month-old) fish were sexed based on secondary sexual characters (Egami 1975), confirmed by genotyping (Ager-Wick *et al.* 2014).

Estradiol treatment

To study effects of estradiol on cell proliferation, adult fish (six females and six males) were incubated for 2 or 6 days in system water containing 100 µg/L (3.7×10^{-7} M) estradiol (Sigma, diluted 1:10⁵ in either dimethyl sulfoxide or 96% ethanol). Control fish (six of each gender) were incubated for 6 days with diluent only. A similar set-up

was used to study the effects of estradiol on pituitary *lhb* and *gpa* synthesis, comparing adult fish (seven of each gender) incubated in system water containing estradiol with fish incubated with diluent only.

Quantitative polymerase chain reaction (qPCR)

After estradiol treatment, total RNA from single pituitaries was extracted in TRIzol (Ambion) and cDNA prepared from 30 ng total RNA. qPCR was performed as previously described (Weltzien *et al.* 2005) with minor modifications, using a LightCycler96 with SYBR Green I (Roche) and specific primers (Table 1) designed with Primer3Plus software (Untergasser *et al.* 2007). PCR cycling parameters were 300 s at 95°C followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 6 s, followed by melting curve analysis to assess qPCR product specificity. Samples were run in duplicate on cDNA diluted 1:5. Relative expression levels were calculated as described (Weltzien *et al.* 2005), using the most stable combination of three selected reference genes (*rna18s*, *rpl7*, *gapdh*), according to RefFinder (Kim *et al.* 2010).

In situ hybridization (ISH)

ISH and fluorescent ISH (FISH) for *lhb*, *esr1* (Erα), *esr2b* (Erβ2) and *cyp19a1b* were performed as previously described (Fontaine *et al.* 2013). *esr1* and *esr2b* were cloned using primers from Zempo *et al.* (2013), while for *lhb* and *cyp19a1b*, we used previously described riboprobes (Okubo *et al.* 2011, Hildahl *et al.* 2012). Control experiments were performed with sense riboprobes. For confirmation of tg(*lhb*-hrGfpII), *in toto* pituitaries were hybridized with *lhb*-fluorescein (FITC) riboprobe for 18 h at 65°C. For *esr1*, *esr2b* and *cyp19a1b* ISH and FISH, free-floating 60 µm vibratome sections (Leica) were hybridized with digoxigenin-tagged (DIG) riboprobes for 18 h at 55°C. Color revelation was performed with sheep anti-DIG, and anti-FITC conjugated with peroxidase (1:250; Roche), together with custom-made TAMRA-conjugated and FITC-conjugated tyramides for FISH or with alkaline phosphatase (1:3000; Roche) and NBT/BCIP (Roche) for ISH.

Lh cell proliferation

(1) To count and locate newborn Lh cells, juvenile and adult tg(*lhb*-hrGfpII) fish were treated with 1 mM BrdU (Sigma) diluted in water with 15% DMSO for 4 h. They were then left for 4 days, without BrdU, under standard

Table 1 List of the primers used for qPCR.

	Accession number	Forward primer	Reverse primer	PCR efficiency
<i>lhb</i>	NM_001137653.2	CCACTGCCTTACCAAGGACC	AGGAAGCTCAAATGCTTGTAG	2
<i>gpa</i>	NM_001122906	CCAATCTGGCTTCTCAAAC	GCTCTGGAGAAGCAACATCC	1.96
<i>rna18s</i>	AB105163.1	CCTGCGGCTTAATTGACTC	AACTAAGAACGGCCATGCAC	2.02
<i>rpl7</i>	NM_001104870	TGCTTTGGTGGAGAAAGCTC	TGGCAGGCTTGAAGTTCTTT	2.03
<i>gapdh</i>	XM_004077972.3	CCTCCATCTTTGATGCTGGT	ACGGTTGCTGTAGCCAAACT	2

conditions to allow dividing cells to complete mitosis and express differentiated markers such as Gfp. (2) To determine the location of dividing cells, three groups of adult tg(*lhb*-hrGfpII) fish were treated with BrdU for 2, 1 h or 30 min, and then killed directly. (3) To investigate the effect of estradiol exposure on cell proliferation, fish were incubated with BrdU for 4 h immediately after estradiol treatment and then killed. Brain and pituitary were fixed in 4% paraformaldehyde overnight, and gradually dehydrated and stored in 100% MetOH until use.

Immunofluorescence

Immunofluorescence (IF) was performed on free-floating 60 µm sections as previously described (Fontaine *et al.* 2013). For BrdU IF and proliferating cell nuclear antigen (PCNA) IF, epitope retrieval was achieved using either 2 M HCl (in PBS with 0.1% Tween) for 1 h at 37°C or HistoVT One (Nacalai Tesque, Japan) for 20 min at 90°C. Then, a blocking step of 1 h was followed by primary antibody incubation: rat anti-BrdU (1:250; Abcam), mouse anti-ratPCNA (1:100; Santa Cruz Biotechnology) or custom-made polyclonal rabbit anti-medakaLhb (1:2000 (Burrow *et al.* 2018)). For goat anti-human Sox2 (1:500; Immune Systems, UK), blocking was prepared according to supplier recommendations. Amplification was performed using AlexaFluor 555 or 647 secondary antibodies (1:1000; Invitrogen). Nuclei in Lhb-IF and Sox2-labeled tissues were stained with DAPI (1:1000; 4',6-diamidino-2-phenylindole dihydrochloride; Sigma). Control experiments were performed using the same protocol without primary antibody.

Dispersed pituitary cell culture

Cell cultures ($n=5$ of each gender) were prepared by dissociating cells from 15 tg(*lhb*-hrGfpII) adult pituitaries, as described in detail in Ager-Wick *et al.* (2018). Cells were plated in a glass-bottomed dish (Mattek, USA) in modified L-15 medium (Life Technologies), adjusted to pH 7.75 and 290 mosmol/kg by incubating at 26°C with 1% CO₂.

RNA-seq analysis

Read counts for genes of interest were determined using data previously published in Ager-Wick *et al.* (2014), and summarized at the level of Ensembl annotated genes. Raw read counts were normalized using conditional quantile normalization with the R-package cqn (version 1.6.0) (Hansen *et al.* 2012).

Imaging

Vibratome slices were mounted between slide and coverslip with Vectashield (H-1000 Vector, UK) mounting medium, and spacers added between the slice and the coverslip when mounting whole pituitaries. Time lapse recordings of dissociated pituitary cells were performed in a humid chamber at 26°C with 1% CO₂, conditions optimized for medaka (Ager-Wick *et al.* 2018). Confocal images were acquired using a LSM710 microscope (Zeiss) with 10× (N.A. 0.3), 25× (N.A. 0.8) or 40× (N.A. 1.2) objectives. Channels were acquired sequentially to avoid signal crossover between filters. Images were taken with a zoom stereomicroscope (Nikon) and a sCMOS camera (Andor Zyla, UK). Images were processed using ZEN software (v2009, Zeiss). Z-projections from confocal image stacks were obtained using Fiji (v2.0.0 (Schindelin *et al.* 2012)). 3D reconstruction was built using 3D viewer plugin (Schmid *et al.* 2010).

Cell counting and measurements

Counting and measurements were performed blindly, including body weight, standard length and brain and pituitary sizes measured on fixed tissue ($n=10-12$). Pituitaries were measured in three dimensions, and volume was estimated by height×width×length. Gfp cells were measured in 60 µm parasagittal fixed brain-pituitary slices ($n=5$). For each fish, the 15 largest Gfp-positive cells from three different slices were manually measured in two orthogonal axes using ZEN software. Cells were counted in whole fixed and DAPI-treated (5 h) pituitaries ($n=7-9$). Selected z-sections were

analyzed using Cell Profiler software (v2.1.0 (Carpenter *et al.* 2006)) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). BrdU-labeled and double-labeled cells (BrdU and Gfp) were manually counted using Fiji and cell-counter plugin. The fluorescence intensity in the mean region of interest (ROI) was measured with Fiji on a cell culture using 10× objective ($n=11$ cells), and ROI of exactly the same area. Mean ROI fluorescence intensity was adjusted based on background levels from two ROI.

Statistics

Data were analyzed using GraphPad Prism (v6.0c) with significance set at $P<0.05$. Nonparametric analyses were used when data did not meet the Shapiro–Wilk normality test. Potential pituitary or brain size differences were tested by two-way ANOVA, followed by Tukey's multiple comparison test. Potential differences in body weight, standard length, pituitary cell number or proportion and effects of estradiol on cell proliferation were tested by one-way ANOVA and Tukey's. Correlations between pituitary length or volume and body weight or standard

length were tested using Pearson correlation coefficients (R^2), separating genders and stages. R^2 of 0.75, 0.50, and 0.25 were described as substantial, moderate, or weak, respectively, according to (Henseler *et al.* 2009). Kruskal–Wallis followed by Dunn's multiple comparisons test (nonparametric) was used to test for differences in the number of BrdU–Gfp-labeled cells. After removing outliers using the ROUT method, qPCR data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Due to lack of normal distribution, *lhb* in females were tested by Kruskal–Wallis followed by Dunn's multiple comparisons test.

Results

Verification of *lhb*-transgenic line

The high specificity of the tg(*lhb*-hrGfpII) line (Hildahl *et al.* 2012) in both adult and juvenile fish was confirmed by the clear overlap between Gfp-labeled cells and *lhb*-expressing cells labeled by FISH as well as Lh β -producing cells labeled by IF (Fig. 1).

Figure 1

Confocal images of parasagittal pituitary sections from tg(*lhb*-hrGfpII) juvenile (A, B, C, D, E and F) and adult (G, H, I, J, K and L) fish. Tissues sections are labeled for *lhb* mRNA (orange, A, G) by FISH or Lh β proteins (cyan, B, H) by IF. Endogenous hrGfpII is shown in magenta (C, I). Merged images for *lhb* mRNA and endogenous hrGfpII are shown in D and J. Merged images for Lh β protein and endogenous hrGfpII are shown in E and K. Merges of all labeling, together with DAPI counterstaining (gray), are shown in F and L. A, anterior; D, dorsal; P, posterior; V, ventral; scale bar: 50 μ m.

Morphological analysis of the pituitary and the Lh cell population

A significant difference between genders was observed for brain size in adults, but not for bodyweight, standard length (Supplementary Fig. 2A and B) or pituitary size or volume (Fig. 2C and D). Significant differences between juvenile and adult fish were observed for pituitary size (all three axes being almost doubled) and volume (up to 7-fold increase). Regarding pituitary size relative to brain or body size, a substantial correlation was found between pituitary length and brain length in juvenile males and females ($R^2=0.82$ and $R^2=0.89$, respectively; Supplementary Fig. 2E, G, H, I, J, K and L), whereas no apparent correlations were found in adults.

Regarding location of Lh cells, 3D rendering of pituitaries from the *tg(lhb-hrGfpII)* line did not reveal any apparent differences between genders (data on males not shown) or between juveniles (Fig. 3A, B, C, D, E and Supplementary Videos 1, 3) and adults (Fig. 3F, G, H, I and J and Supplementary Videos 2, 4). Most cells were localized on the ventral and lateral surfaces of the median part of the pituitary, with a few cells also in the posterior part.

There was no difference in Lh cell size between juvenile males and females, but cells were longer in adults than juveniles ($P<0.0001$) (Fig. 4B). Cell width was significantly greater ($P<0.0001$) in adult females than juveniles, and cells were longer and wider in adult females than adult males ($P<0.05$). We observed that adult Lh cells increased more in length than width from juvenile to adult stages, resulting in more elongated cells in adults, often with an extension at their extremity (Fig. 4C and D).

There was no difference in the number of Lh cells or total number of pituitary cells (nuclei labeled by DAPI) in juveniles, in contrast to the clear difference seen between juveniles and adults ($P<0.001$) and between adult males and females ($P<0.05$; Fig. 5). Although the proportion of Lh cells did not differ by gender, in adult females, there was a significant increase from juveniles ($P<0.01$; Fig. 5C). This was not observed in males, presumably because of high individual (bimodal) variation in adult males.

Recruitment of new Lh cells

Time lapse recordings of dissociated pituitary cells from *tg(lhb-hrGfpII)* adult males (Fig. 6A, B, C, D and Supplementary Video 5) and females (data not shown)

Figure 2

Measurements of the pituitary in three axes by confocal imaging. Illustrations of the lateral view (A) and ventral view (B) of the brain and pituitary of medaka showing the measurements (L: Length; W: width; H: height). Anterior to the left. Histograms representing (C) mean ($\pm$ s.d.) of the size of the three axes (in μm) and (D) mean ($\pm$ s.d.) of the estimated volume ($L \times W \times H$) of the pituitary from juvenile males ($n = 5$), juvenile females ($n = 4$), adult males ($n = 5$), and adult females ($n = 5$). The sizes among the four groups were significantly different ($P < 0.05$) according to a two-way ANOVA and Tukey's multiple comparison test when letters are different (a and b).

Figure 3

Snapshots of different perspectives from 3D reconstruction of whole pituitary from *tg(lhb-hrGfpII)* juvenile female medaka (A, B, C, D and E) and adult female medaka (F, G, H, I and J). Snapshots were taken from different viewpoints: ventral (A, D, F, I), lateral (C, E, H, J), dorsal (B, G). Anterior to the top. Ventral to the left in C and H, and to the right in E and J. Lh cells (Gfp) are in green and nuclei stained with DAPI are in gray. Scale bar in red is expressed in μm .

showed some Gfp cells moving and clustering. A few Gfp-negative cells were also revealed to start expressing Gfp after 24–48 h in culture. The proportion of new Gfp-expressing cells varied between cultures, always with a time-dependent increase in fluorescence intensity. Using a 10 $\times$ objective, simultaneous recording of ROI mean intensity for numerous cells (Fig. 6E) showed that several cells started to express Gfp between 35 and 45 h after plating. Some cells obtained fluorescence intensities similar to the other Gfp-expressing cells around 55 h after plating, only 10 h after starting to express Gfp. The ROI mean intensity increased linearly, but with different slopes between cells. In some cells, ROI mean intensity fluctuated with time, but never apparently disappeared. A few cells died in culture, as shown by time lapse recordings and ROI mean intensity measurements (Fig. 6B and C, and cell number 6 in Fig. 6E).

Evidence for newborn Lh cells in the medaka pituitary

After 4 h of BrdU treatment followed by 4 days of recovery to allow time for cell division and differentiation, we observed several BrdU-labeled cells in the brain and pituitary in both juveniles and adults (Fig. 7A, B, C and D). Some BrdU-labeled cells in the pituitary were also labeled by Gfp. The number of BrdU-Gfp cells (Fig. 7E) did not differ significantly between genders or stages. Interestingly, the number of double-labeled cells varied tremendously, with none found in some fish and others with up to 80 BrdU-Gfp-labeled cells.

Location of dividing cells in the medaka pituitary

BrdU incorporation experiments using incubation times down to 30 min consistently revealed BrdU-labeled cells

Figure 4

Morphological analysis of the Lh cells. Confocal planes of parasagittal section from juvenile (A, C) and adult (D) pituitary from *tg(lhb-hrGfpII)* fish. (A) High magnification showing the two axes used to measure the cell size (L: length, the longest size; W: width). (B) Histogram representing mean ($\pm$ s.d.) of two cell size parameters as shown in A, of the 15 largest cells from 5 *tg(lhb-hrGfpII)* fish, from juvenile and adult fish of both genders. The sizes among four groups were significantly different ($P < 0.05$) using two-way ANOVA with Tukey's multiple comparison test when letters are different (a, b and c). (D) Arrows indicate elongated cells observed in adult. Scale bars: 10 μ m.

in all parts of the pituitary (Fig. 8A and B). A number of doublets (i.e., two closely situated cells labeled with BrdU), suggested recently divided cells. The presence of cells throughout the pituitary labeled by PCNA, an essential protein for DNA replication during the cell cycle, confirmed active cell division (Supplementary Fig. 3).

Mitosis of Lh cells in medaka pituitary

BrdU exposure always resulted in double-labeled cells (Gfp and BrdU), regardless of incubation time. Occasional observation of double-labeled cells in anaphase (Fig. 8C, D and E) or PCNA-labeled Gfp cells (Fig. 8F, G and H)

clearly demonstrated that Gfp cells might undergo mitosis.

Lh cells are not multipotent stem cells

We found several cells in brain and pituitary that were positive for Sox2, a marker for multipotent stem cells (cells with the ability to self-renew for long periods of time and differentiate into various cell types) in mammals (Mollard *et al.* 2012) and medaka (Alunni *et al.* 2010, Lust & Wittbrodt 2018) (Fig. 8I, J, K, L and M). However, Sox2-labeled cells were mostly located in the dorsal pituitary, close to the *pars nervosa*, with only a few cells scattered

Figure 5

Cell counting for the four different groups of fish: juvenile males ($n = 8$) and females ($n = 9$), and adult males ($n = 9$) and females ($n = 7$). Cells were counted using the process described in Supplementary Fig. 1, from confocal images of *tg(lhb-hrGfpII)* whole pituitaries. (A) Mean ($\pm$ s.d.) of the total number of cells in the pituitary. (B) Mean ($\pm$ s.d.) of the number of Gfp cells in the pituitary. (C) Mean ($\pm$ s.d.) of the percentage of Gfp cells related to the total number of cells in the pituitary. For each graph, one-way ANOVA with Tukey's multiple comparison test revealed significant differences ($P < 0.05$) when letters are different (a, b and c).

Figure 6

(A, B, C and D) Time lapse of confocal images of primary pituitary cell culture from *tg(lhb-hrGfpII)* adult male pituitaries (snapshots extracted from Supplementary Video 5) imaged with 25 $\times$ objective. Arrows show the location of the new cells expressing Gfp. The star shows two cells clustering and the arrow head shows a cell disappearing. Scale bars: 50 μ m. (E) Background corrected mean ROI intensity for 11 different cells over time, from another cell culture imaged with a 10 $\times$ objective.

throughout the pituitary. We never observed Gfp in Sox2-labeled cells (Fig. 8N, O and P).

Effects of estradiol on pituitary and Lh-specific cell proliferation

Two days of estradiol treatment (diluted in EtOH) had no significant effect on pituitary cell proliferation, in either males or females (Fig. 9A and B). However, after 6 days of estradiol treatment, the total number of BrdU-labeled cells ($P < 0.0001$), the number of double-labeled

Figure 7

Cell proliferation in the pituitary. (A) Confocal plane of brain and pituitary from *tg(lhb-hrGfpII)* adult female medaka with BrdU IF labeling in magenta and Lh cells (Gfp labeling) in cyan, superimposed to a transmitted light microscope image in order to view the anatomy. (B, C and D) Confocal magnification of the selected region shown in A. Arrows show cells that are double labeled with Gfp and BrdU IF. Anterior to the left. Scale bars: 50 μ m. (E) Plotted graph presenting the number of double-labeled cells: Gfp and BrdU IF in the pituitary. One dot represents one individual and horizontal bars represent the mean (largest one in the middle) and $\pm$ s.e.m. No significant differences between gender or stages according to one-way ANOVA followed by Kruskal-Wallis multiple comparison test.

Figure 8

Identification of some Lh cell progenitors. (A and B) Projection of 15 confocal Z-plans (1 µm step between each picture) of a parasagittal section of medaka brain-pituitary from *tg(lhb-hrGfpII)* adult male (cyan) incubated with 1 mM BrdU for 1 h, fixed immediately after treatment and then labeled with BrdU (magenta) for IF. (B) High magnification of the selected area shown in A, with arrows showing doublets: two cells close to each other that could represent sister cells. Anterior to the left. (C, D and E) Confocal plan of parasagittal section of medaka brain-pituitary from *tg(lhb-hrGfpII)* adult male (green). Fish have been incubated with BrdU 1 mM for 4 h, fixed immediately after treatment and then labeled with BrdU (magenta) for IF, showing an Lh cell in the anaphase of mitosis. (F, G and H) Confocal plan of parasagittal section of medaka brain-pituitary from *tg(lhb-hrGfpII)* adult medaka male (green) labeled with PCNA (yellow) for IF, with arrows showing a cell labeled with both Gfp and PCNA. Scale bars: 20 µm. (I, J, K, L, M, N, O and P) confocal images of parasagittal section of medaka brain-pituitary from *tg(lhb-hrGfpII)* adult male (cyan) labeled with Sox2 (magenta) for IF. Scale bars: 100 µm. (J, K, L and M) Magnification of the pituitary shown in I. (N, O and P) Magnification of the dashed square zone shown in P from another confocal plan, showing the Lh cell population and a peripheral Sox2-positive cell. Scale bar: 20 µm.

cells (BrdU and Gfp) ($P<0.001$) and the proportion of double-labeled cells ($P<0.0001$) increased in males. Double-labeled cells represented about 50% of the total number of BrdU-positive cells in the pituitary of males. In contrast, only the total number of BrdU-labeled cells significantly increased ($P<0.01$) after 6 days of treatment in females. Interestingly, by diluting estradiol in DMSO instead of EtOH, similar results were obtained for males,

but no significant change was observed for females (Supplementary Fig. 4). Our results indicate that EtOH could be a better solvent for estradiol than DMSO.

Effects of estradiol on *lhb* expression in the pituitary

Using a combination of *rna18s* and *rpl7* as reference genes, a significant decrease in the level of *lhb* ($P<0.05$) and

Figure 9

(A) Effect of estradiol on cell proliferation in *tg(lhb-hrGfpII)* adult medaka. Histograms represent the mean ($\pm$ s.d.) of the number of double-labeled cells (Gfp and BrdU IF), the total number of BrdU-labeled cells in the pituitary on the left axis, and the percentage of double-labeled cells in relation to the total number of BrdU-labeled cells on the right axis, according to different estradiol treatment in males and females. Fish were treated for either 2 (5 females and six males) or 6 (6 females and six males) days with estradiol or with EtOH only for 6 days (control; six females and six males). The number of double-labeled cells among the four groups were significantly different ($P < 0.05$) using two-way ANOVA with Tukey's multiple comparison test when letters are different (a and b). (B) Effect of estradiol on *lhb* and *gpa* expression levels in adult medaka. Fish were treated for either 2 or 6 days with estradiol or with EtOH only (7 fish of each gender in each group). Means ($\pm$ s.e.m.) of the expression levels of *lhb* and *gpa* have been normalized with the expression level of the combination of *rna18s* and *rpl7*. Significant differences between the four treatment groups ($P < 0.05$) are shown when letters are different (a and b) using one-way ANOVA with Tukey's multiple comparison test.

gpa ($P < 0.05$) mRNA was found in both male and female pituitaries after both 2 and 6 days of estradiol treatment (Fig. 9C).

Lh cells express steroid receptors

RNA-seq data from FACS-sorted Gfp cells from juvenile (data not shown) and adult females showed expression of the three Ers known in medaka (Supplementary Fig. 5A); *esr1* (Er α ; ENSORLG00000014514), *esr2a* (Er β 1; ENSORLG00000017721), *esr2b* (Er β 2; ENSORLG00000018012), with relatively high levels for *esr1* and *esr2b*. ISH revealed similar localization of *esr1* and *esr2b* in the brains of adult males and females (Supplementary Fig. 6). Both receptors were also expressed

in the pituitary, especially the region where Lh cells are located. Double FISH confirmed the expression of these two receptors in some of the Lh cells in both genders, although they were also expressed in other cells (Fig. 10).

Lh cells express aromatase

RNA-seq data revealed expression in Lh cells of the brain form of aromatase, *cyp19a1b* (ENSORLG00000005548), but not *cyp19a1a* (ENSORLG00000002949) (Supplementary Fig. 5B). FISH for *cyp19a1b* confirmed the presence of aromatase in different brain regions (Fig. 11), as well as the pituitary. Double FISH for *lhb* and *cyp19a1b* confirmed the RNA-seq observations, showing that most Lh cells also express *cyp19a1b*.

Figure 10

Expression of the estrogen receptors *esr1* ($\text{Er}\alpha$) and *esr2b* ($\text{Er}\beta 2$) in the Lh cells. Confocal planes of parasagittal sections from brain and pituitary from adult males (A, B, C, D, E, F, G and H) and females (I, J, K, L, M, N, O and P) wild-type medaka. Tissues have been labeled with double-color FISH for *lhb* (yellow) and either *esr1* (blue, A, B, C, D and I, J, K, L) or *esr2b* (blue, E, F, G, H and M, N, O, P). Anterior to the left. Scale bars: 50 μm .

Discussion

Lh is a key player in the BPG axis, controlling reproductive function. However, little is known about Lh cells themselves and the population they form in the pituitary. In this study, we used 2-month-old juveniles

and 6-month-old adults of the *tg(lhb-hrGfpII)* line of medaka (Hildahl *et al.* 2012) to investigate developmental changes in Lh-producing gonadotropes during puberty. We demonstrate that Gfp is a reliable reporter for both *lhb* mRNA and Lh β protein expression, in juveniles and adults, thus showing the usefulness of this line to visualize

Figure 11

Expression of the aromatase *cyp19a1b* in the Lh cells. Confocal planes of parasagittal sections from brain and pituitary from adult males (A, B, C, D and E) and females (F, G, H, I and J) tg(*lhb-hrGfpII*) medaka. Endogenous Gfp is shown in yellow and *cyp19a1b* expression revealed by FISH is shown in magenta, and nuclear DAPI staining is shown in gray. (B, C, D and E) show higher magnifications of the pituitary shown in A and G, H, I, J show higher magnifications of the pituitary shown in F. Anterior to the left. Scale bars: 50 μ m.

and localize all Lh-producing (Gfp) cells, referred to as Lh cells, following the definition of endocrine cells used by Pogoda & Hammerschmidt (2009).

We then studied the morphology of the whole pituitary and showed that it grows from juvenile to adult stage, without any gender difference. This is not surprising, as the brains of fish have previously been shown to keep growing, even during adulthood (Lindsey & Tropepe 2006). Moreover, several studies have already reported allometric growth of different organs in relation to body size during development (Osse & Boogaart 1995, Van Snik *et al.* 1997, Huysentruyt *et al.* 2009). In medaka, we observed a correlation between pituitary length and brain size in juveniles, but this was not the case in adults.

No correlation was found between size or volume of the pituitary and fish length or weight in adults, suggesting that factors other than those for normal fish growth participate in control of pituitary growth.

Lh cells are located in the ventral and lateral surfaces of the median part (*proximal pars distalis*, PPD), and a few cells in the posterior part (*pars intermedia*, PI) of the pituitary in both juvenile and adult medaka, concurs with reports from zebrafish (So *et al.* 2005), tilapia (*Oreochromis niloticus*) (Golan *et al.* 2014) and Chinese sturgeon (*Acipenser sinensis*) (Cao *et al.* 2009). However, this distribution differs from that observed in European hake (*Merluccius merluccius*) (Candelma *et al.* 2017) and European eel (Schmitz *et al.* 2005), where Lh cells are

distributed throughout the PPD. Although we did not observe any differences between genders, there was a higher absolute number and a higher proportion of Lh cells, in combination with larger Lh cells, in adult females than juveniles. The increased size of Lh cells in adults could be explained by the fact that, at least in females, Lh cells are known to store a considerable quantity of hormone-containing vesicles until the Lh surge around ovulation (Zohar 1988, Karigo *et al.* 2012). The increased number of cells could be explained by the necessity to increase hormone production in order to maintain a high concentration in the growing target tissues. Indeed, the pituitary gland must adapt the proportion of all endocrine cell types to meet different hormonal demands throughout the lifecycle of the animal.

We then looked for the origin of the new Lh cells and demonstrated that in dispersed pituitary cell cultures, some cells of unknown phenotype are able to start producing *lhb*. This clearly indicates recruitment of existing pituitary cells for production of *lhb*. Unfortunately, we could not identify the cell type before it started to produce Gfp. Further investigations are necessary to understand whether the new *lhb*-producing cells come from phenotypic conversion of differentiated cells (transdifferentiation as shown for mammotropes and somatotropes in the mammalian pituitary (Porter *et al.* 1991)), differentiation of progenitor cells in the pituitary or activation of quiescent Lh cells. Interestingly, the number of cells starting to express Gfp varied between cell cultures, and several of the new Gfp-expressing cells appeared simultaneously. We assume that cell density, the presence of paracrine factors in culture or the physiological status of the donor fish could influence the recruitment of existing cells for the expression of *lhb*. So far, we have not been able to determine exactly what induces this phenomenon, but GnRH and sex steroids may be good candidates for further studies.

In the mammalian brain, it has been demonstrated that, even with high concentrations and direct cardiac perfusion, BrdU incorporation during DNA repair is insufficient to label any damaged or apoptotic cells (Bauer & Patterson 2005). BrdU is therefore a useful marker for revealing the location and number of recently divided and currently dividing cells. Our experiments, using 4 h of incubation with BrdU followed by 4 days of recovery in both juveniles and adults, revealed some cells had been double-labeled with both Gfp and BrdU. The G1 and the S phases are the longest of the cell cycle (Borrell & Calegari 2014) and last only a few hours (Cameron & Greulich 1963, Hahn *et al.* 2009, Turrero Garcia *et al.* 2016, Akle *et al.* 2017). Therefore, by waiting 4 days after

the integration of BrdU by the cells in S phase, these cells should have completed mitosis. Consequently, our results suggest that new Lh cells arise from mitotic activity, both in juveniles and adults.

Such a sustained cell proliferation could be achieved either by maintenance of undifferentiated progenitors (Chapouton & Godinho 2010) or by mitosis of existing gonadotrope cells. It has previously been demonstrated that anterior pituitary cells of mammals retain the capacity to divide (Taniguchi *et al.* 2002). Mitosis has been reported in prolactin and LH cells, contributing to their proliferation in rats (Sakuma *et al.* 1984, Takahashi 1995). Here, fish killed immediately after short pulses of BrdU always revealed some double-labeled (BrdU-Gfp) cells suggesting that Lh cells were dividing. We also observed some Lh cells in anaphase, as well as some double-labeled cells with Gfp and PCNA, confirming that Lh cells are able to divide in the medaka pituitary.

The existence of specific niches of multipotent progenitor cells has been reported in the adult mammalian pituitary. Indeed, Sox2, an important multipotent progenitor cell marker (Fauquier *et al.* 2008b, Kelberman *et al.* 2009), has been used to localize stem cells in a concentrated layer lining the mammalian pituitary cleft, the intraglandular structure at the border of the neurohypophysis and adenohypophysis, with a few additional cells in the pituitary (Fauquier *et al.* 2008a,b). In medaka, Sox2 has also been shown to be a multipotent progenitor cell marker in the brain (Alunni *et al.* 2010) and retina (Lust & Wittbrodt 2018). Here, we observed Sox2-positive cells in brain regions previously shown to express Sox2 in zebrafish (Germana *et al.* 2011) and in the dorsal part of the medaka pituitary. In the pituitary, Sox2-positive cells are located in close proximity to the *pars nervosa*, where the neuronal projections from the hypothalamus enter the pituitary, with very few extra cells scattered throughout the pituitary. Therefore, this study reveals the location of potentially multipotent stem cells in the adult fish pituitary, in a region equivalent to that identified for mammals. Although a significant number of dividing (PCNA-positive) cells were also Sox2 positive in zebrafish and medaka brain (Alunni *et al.* 2010, Diotel *et al.* 2013) and also differentiated Lh cells retain the capacity to divide in medaka pituitary, and none of the Lh cells expressed Sox2, suggesting that Lh cells are probably not multipotent progenitor cells.

Although we could have expected a higher degree of Lh cell proliferation in juvenile fish that are in the fastest growth phase, we did not observe any significant differences in the capacity of Lh cells to proliferate in the

pituitary, either between juveniles and adults, or between genders. However, in all groups, we found individuals without newborn cells and others with numerous newborn Lh cells, suggesting Lh cell proliferation is controlled by one or several biological factors. Estrogens have been shown to affect brain development and to modulate embryonic and adult neurogenesis (Martinez-Cerdeno *et al.* 2006, Mouriec *et al.* 2008, Barha *et al.* 2009, Kah *et al.* 2009, Diotel *et al.* 2013, Coumailleau *et al.* 2015). To the best of our knowledge, nothing is known about the effect of estradiol on cell proliferation in fish pituitary. In mammals, however, several studies have investigated the role of sex steroids on LH cell proliferation. Indeed, mitotic LH cells drastically increase after castration in male rats (Sakuma *et al.* 1984), and ovariectomy in female rats (Smith & Keefer 1982), suggesting a negative effect of steroids on LH cell proliferation. In contrast, our results revealed a positive effect of estradiol on cell proliferation in medaka. Interestingly, estradiol had a stronger effect in males than in females. This is possibly due to higher background level of estradiol in females (Bhatta *et al.* 2012), and therefore, the estradiol treatment is less obvious in females.

Although it has mostly been reported that estradiol treatment increases *lhb* and *gpa* synthesis in fish (Yaron *et al.* 2003, Li *et al.* 2018), our results show a clear decrease of both *lhb* and *gpa* mRNA levels following estradiol treatment, in both genders. To the best of our knowledge, the only study where a negative effect of estradiol on *lhb* and *gpa* synthesis in fish has been reported was also from medaka (Zhang *et al.* 2008). The authors showed a decrease in mRNA levels for *lhb* and *gpa*, using even lower estradiol concentrations. Several studies have shown that estradiol could decrease *lhb* synthesis in mammals (Liu & Jackson 1977, Di Gregorio & Nett 1995). Indeed, it has been found that estradiol has a bimodal effect on *lhb* and *gpa* synthesis, mostly depending on the reproductive stage of the animal (Lindzey *et al.* 2006). The decrease in *lhb* and *gpa* expression levels after estradiol treatment shown in our studies can be explained by the fact that estradiol increases Lh cell proliferation, as transcription of many genes is thought to be silenced during mitosis (Taylor 1960, Prescott & Bender 1962, Parsons & Spencer 1997).

In addition to demonstrating an effect of estradiol on Lh cell proliferation and that Lh cells have the capacity to divide, we also showed that Lh cells express estrogen receptors, suggesting a direct effect of estradiol. Lh cells have been shown to express *esr2b* in zebrafish females (Li *et al.* 2018). Our results show, in agreement with those of

Zempo *et al.* (2013), that several brain areas express *Esr* in medaka. Whereas RNA-seq has been done on whole Lh cell populations in the pituitary, thus masking heterogeneity in the cell population, FISH revealed that some, but not all, Lh cells express *esr1* and *esr2b*. Additionally, our results showed that Lh cells are not the only pituitary cells to possess *Ers*; this fits with the fact that proliferation of cell types in the pituitary, other than Lh cells, is activated by estradiol.

Androgens can be converted into estrogens (Simpson & Davis 2001) by aromatase. Fish are unique among vertebrates as aromatase B (*cyp19a1b*) is strongly expressed in the adult brain, especially in some progenitor cells (Noctor *et al.* 2001, Pellegrini *et al.* 2007, Lam *et al.* 2009, Marz *et al.* 2010, Rothenaigner *et al.* 2011). Our results show substantial aromatase expression in medaka brain and pituitary, in agreement with a previous publication (Okubo *et al.* 2011). The high aromatase expression suggests that estradiol can be produced in the pituitary from androgens. The positive effect of estradiol on *lhb* expression has been shown across different species and also for testosterone after its aromatization to estradiol (Yaron *et al.* 2003). Thus, control of pituitary Lh cell proliferation could be via estrogen produced by the gonads or locally in the pituitary.

In conclusion, our study provides information on the Lh cell population and its high degree of plasticity in the medaka pituitary. We demonstrate that the pituitary and Lh cell population evolve between juvenile and adult stages, with new Lh-producing cells arising due to both mitosis of Lh cells and recruitment of existing cells for the production of *lhb*. Our findings offer insights into the possible role of estradiol in facilitating pituitary cell proliferation, including Lh cells. In addition, Lh cells that have the capacity to divide also express *esr1*, *esr2b* and *cyp19a1b*, suggesting a direct effect of estrogens and androgens on Lh cell proliferation. Although this study has focused on Lh cells, a similar study conducted on the Fsh cell population could further elucidate how plasticity of gonadotrope cells is controlled.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0412>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval

Animal experiments were performed according to recommendations on the care and welfare of research animals at the Norwegian University of Life Sciences, with specific approval from the Norwegian Food Safety Authority (FOTS ID 8596).

Authors' contribution statement

R F and E A W conducted the experiments. R F, E A W, F A W and K H conceived the research and analyzed the data. R F, E A W and F A W wrote the manuscript, with input from the other authors.

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