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Ex vivo medaka pituitary culture reveals differential CCK and GnRH regulation of Fsh and Lh cells and selective disruption by estradiol and bisphenol A

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Abstract

Ex vivo pituitary culture sits between *in vivo* exposure and dissociated cell culture, but its suitability for studying gonadotrope regulation and endocrine disruption is unestablished. In teleosts, luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) are produced by separate cells, allowing their regulation to be studied independently. We characterised intact cultured medaka (Oryzias latipes) pituitaries and applied the preparation to bisphenol A (BPA) and 17β-estradiol (E2). Pituitaries remained viable throughout a 10-day culture period, with metabolic activity peaking between days 2 and 6 before decreasing. Basal *lhb* transcript levels fell significantly by day 2 and *fshb* by day 4, consistent with loss of hypothalamic input, and both were restored by mdGnRH and CCK8 respectively, with responsiveness persisting until day 6 for *lhb* and day 8 for *fshb*. Using transgenic lines expressing a genetically encoded Ca^{2+} indicator in Lh or Fsh cells, agonist-evoked responses persisted to day 4 in Lh cells and throughout the 6-day period tested in Fsh cells. Unexpectedly, CCK8 also increased *lhb* transcript levels. Single-cell transcriptomic data showed that, among the CCK receptor paralogs, only *cck2rb* was detected in the pituitary, in 40.9% of $fshb^{+}/lhb^{-}$ and 15.3% of $lhb^{+}/fshb^{+}$ gonadotropes but only 1.6% of $lhb^{+}/fshb^{-}$ cells. Together with the known plasticity of medaka gonadotropes in culture, this suggests the *lhb* response to CCK8 reflects a small bi-hormonal population rather than a major regulatory pathway. E2 attenuated the CCK8-induced *fshb* increase, whereas BPA did not, and neither altered mdGnRH-induced *lhb* transcript levels. Agonist-evoked Ca^{2+} responses were not significantly affected by acute, 48-h *ex vivo* or 48-h *in vivo* exposure. *Ex vivo* culture is therefore a viable alternative to live-animal exposure for studying gonadotrope regulation and its disruption.

1. Introduction

The endocrine system is a highly dynamic network of hormonal signals that regulate a wide range of physiological functions, including energy metabolism, growth, stress response, immune responses, fluid balance, and cardiovascular regulation [1]. At its core, the pituitary serves as a key regulatory center, integrating stimuli from the brain to coordinate hormonal responses delivered via the bloodstream to the peripheral organs [2]. The pituitary gland produces and secretes various hormones, including growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), and the two gonadotropins: the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1]. The gonadotropins play a crucial role in reproductive function by promoting gametogenesis and steroidogenesis in the gonads [3].

Endocrine regulation is complex and highly plastic, involving stimulators, inhibitors, feedback loops and interactions across multiple organs. In the control of reproduction, gonadotropin-releasing hormone (GnRH), produced in the brain in response to environmental and internal signals, stimulates the pituitary gonadotropes, which in turn drive the gonads, while gonadal sex steroids feed back on both [4]. In addition to these main hormones, dopamine inhibits gonadotropin release in several teleost species [5],

highlighting the complexity of the regulatory network governing the brain–pituitary–gonadal (BPG) axis [6].

Endocrine-disrupting chemicals (EDCs) found in our environment can disrupt endocrine regulation by interfering with hormonal balance, posing significant risks to both human and wildlife health [7]. Frequently found in consumer products, cosmetics, pharmaceuticals, and chemical food additives [8], EDCs have been linked to a higher incidence of endocrine-related cancers, diabetes, obesity, and infertility in humans [9]. One of the well-known EDCs is BPA, which is widely used in reusable plastic containers, water bottles, food packaging, and storage containers [10]. In addition, 17β-estradiol (E2), although a natural endogenous estrogen, can act as an environmental estrogenic endocrine-disrupting compound when released from pharmaceutical waste, and industrial or household sources into the environment through sewage effluents. Estrogenic endocrine-disrupting chemicals (e-EDCs) have indeed been detected in soil, water, air, and food [11]. While these EDCs exhibit estrogenic activity, their effects on Lh and Fsh synthesis and secretion remain unclear. Thus, further investigation of their effects is important for understanding reproductive endocrine dysfunction.

The Japanese medaka (*Oryzias latipes*) is an established vertebrate model organism widely used for toxicology and physiological studies due to its high fecundity, relatively short life cycle, and well-characterized genetic background [12]. Interestingly, teleosts differ from mammals in the fact that Lh and Fsh are produced in distinct cells within the pituitary, which makes it easier to investigate their differential regulation. In addition, recent studies in teleosts demonstrated that hypothalamic cholecystokinin (CCK) primarily regulates Fsh hormone production, while GnRH mainly controls Lh [13, 14]. CCK receptor expression is enriched in Fsh cells in medaka, zebrafish and tilapia [13–15] and loss of the receptor disrupts gonadal development in zebrafish and medaka [14, 16], indicating that this arrangement is conserved across teleosts. This suggests that teleosts utilize two distinct gonadotropin regulators, while mammals depend on a single GnRH [17]. Consequently, teleosts provide a valuable model for studying the independent regulatory mechanisms governing Lh and Fsh.

Despite advances in endocrinology and toxicology research, traditional *in vivo* studies present ethical and practical challenges, including animal welfare concerns, regulatory restrictions, and variability in experimental outcomes [18]. To address these issues, *in vitro* studies, which utilize isolated tissues or cells rather than whole organisms, eliminate the risk of animal suffering from chemical exposures. As part of New Approach Methodologies (NAMs), *ex vivo* models complement *in vitro* dissociated cells by preserving tissue architecture and cellular interactions, allowing endocrine events to be studied at a level of organisation closer to the intact gland [18–20]. Such systems can deepen the mechanistic understanding of endocrine regulation and inform the development and interpretation of fully *in vitro* models. In this study, we first aimed to determine how long cultured medaka pituitaries remain viable and retain their capacity to respond to stimulation, at both the transcriptional and the Ca^{2+} level, in order to establish the window over which the preparation is suitable for studying Lh and Fsh cell regulation.

Secondly, we investigated the effects of the e-EDCs E2 and BPA in the *ex vivo* preparation and compared these findings with *in vivo* exposure.

2. Methods

2.1. Experimental Design

This study was conducted as a collaboration between the Norwegian University of Life Sciences (NMBU), Norway, and the Atmosphere and Ocean Research Institute, University of Tokyo, Japan. Phase 1 was performed at NMBU; phases 2 and 3 were performed at the University of Tokyo.

This study consisted of three phases summarised in Fig. 1. Phase 1 characterised *ex vivo* pituitary culture conditions: pituitaries were cultured for up to 10 days and assessed for metabolic activity, basal *lhb* and *fshb* expression, and transcriptional responsiveness to mdGnRH and CCK8 over time. Phase 2 assessed whether cultured pituitaries retained the capacity to couple stimulation to hormone release. Pituitaries cultured for 0, 2, 4 or 6 days were perfused with mdGnRH or CCK8, and intracellular Ca^{2+} responses were recorded in Lh and Fsh cells as a proxy for secretion. Phase 3 applied the model to two endocrine-disrupting chemicals: the effects of E2 and BPA on gonadotropin subunit expression were assessed by co-treatment with the corresponding agonist over 48 h, and their effects on agonist-evoked Ca^{2+} responses were assessed following acute perfusion, 48-h *ex vivo* exposure, and 48-h *in vivo* waterborne exposure.

2.2. Ethical consideration

The experiments conducted in Japan, comprising the Ca^{2+} imaging experiments and the ex vivo and in vivo exposures to 17β-estradiol and bisphenol A, were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with the Guiding Principles for the Care and Use of Experimental Animals of the University of Tokyo (permission numbers A2025AORI005 and A2025AORI010-01). The remaining experiments were conducted in Norway on pituitaries collected post mortem from euthanised fish, so that no procedures were performed on living animals; under Article 3 of EU Directive 2010/63/EU and the corresponding Norwegian regulations, killing animals solely for the use of their organs or tissues does not require a specific permission. Fish housed and euthanised at the Norwegian University of Life Sciences were handled in accordance with NMBU institutional guidelines, EU Directive 2010/63/EU and the regulations of the Norwegian Food Safety Authority. The study is reported in accordance with the ARRIVE guidelines.

2.3. Animals

The experiments were conducted using adult male and female Japanese medaka (> 3 months old, body weight 0.12–0.38 g; standard length 1.9–2.9 cm). Wild-type fish of the d-rR strain were used for the experiments performed in Norway and were obtained from the in-house breeding colony at the Norwegian University of Life Sciences, Ås, Norway. For Ca^{2+} imaging and the *ex vivo* and *in vivo* exposure

experiments, the transgenic lines Tg(*lhb*: inverse pericam) and Tg(*fshb*: inverse pericam), hereafter *lh*:IP and *fsh*:IP, were used; these lines express the genetically encoded Ca^{2+} indicator inverse pericam specifically in Lh and Fsh cells and were generated as described previously [20], and were maintained at the Atmosphere and Ocean Research Institute, The University of Tokyo, Japan, where the Ca^{2+} imaging and exposure experiments were performed. Sex was determined based on secondary sexual characteristics, including the size and shape of the dorsal and anal fins, as described by Kenji Murata and Tanaka [21]. The fish were maintained in a controlled laboratory environment within a recirculating aquaculture system, kept at 28°C with a 14-hour light / 10-hour dark cycle, a water pH of 7.3–7.5, and a dissolved oxygen concentration of 5.8 mg/L. They were fed a commercial zebrafish diet (Zebrafeed, Sparos, Portugal) three times daily. Additionally, from 25 days post-fertilization (dpf) onwards, they were also provided with live Artemia (Ocean Nutrition, Thailand) once a day.

2.4. Pituitary dissection and explant culture

Pituitary culture was performed as previously described by Royan, et al. [22]. A modified Leibovitz's L-15 medium (Gibco™, Thermo Fisher Scientific, Cat. No. 21083027) was prepared by supplementing the medium with 10 mM D-glucose (Sigma-Aldrich, Cat. No. G5400), 5% fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific, Cat. No. A5209401), and 1× penicillin–streptomycin (Gibco™, Thermo Fisher Scientific, Cat. No. 15140122). The medium was adjusted to pH 7.75 and 290 mOsm/kg and sterilized through a 0.2 µm filter (Fisherbrand™, Thermo Fisher Scientific, Cat. No. 15206869). Medaka were anesthetized in 0.02% tricaine methanesulfonate (MS-222; PHARMAQ Ltd., United Kingdom), and pituitary glands were dissected as previously described by Ager-Wick, et al. [23]. Individual pituitaries were then cultured in 500 µL of the prepared medium in Nunc™ non-treated 4-well multidishes (Thermo Scientific™, Thermo Fisher Scientific, Cat. No. 179820) at 27°C. Half of the culture medium was replaced every second day throughout the culture period.

2.5. Viability assessment of culture pituitaries

Pituitary viability was assessed using the high-sensitivity PrestoBlue (hs-PB) assay (Invitrogen™, Thermo Fisher Scientific, Cat. No. A13261), which measures metabolic activity through the reduction of resazurin to the fluorescent product resorufin. The high-sensitivity formulation was selected for its improved signal-to-background ratio relative to standard resazurin-based reagents [24], and the assay was performed according to the manufacturer's instructions with incubation time and tissue-to-volume ratio optimised for whole pituitaries. Pituitaries were cultured in pools of four per well in 4-well culture plates containing 500 µL of modified L-15 medium. At each sampling time point, one pool of four pituitaries was washed and transferred to a well of a Nunc™ non-treated 24-well plate (Thermo Scientific™, Cat. No. 144530) containing 165 µL of modified L-15 medium supplemented with 10% hs-PB, and incubated for 4 h at 27°C. Wells containing medium and hs-PB without tissue served as blanks. To confirm that the fluorescence signal reflected the metabolic activity of viable cells, non-viable control pools were generated by exposing pituitaries to 1% Triton™ X-100 (BioXtra; Sigma-Aldrich, Cat. No. T9284) for 1 h prior to hs-PB incubation; untreated pools processed in parallel served as the corresponding viable control (Supplementary Figure S1). Following incubation, resorufin fluorescence was quantified using a

Spark® multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland) with excitation at 535 nm and emission at 595 nm, using an automatically optimised gain setting, 30 flashes per well and an integration time of 40 µs.

2.6. Chemical preparation

MdGnRH was dissolved in ultrapure water to prepare a 1 mM stock. CCK-8S peptide was dissolved in 10% DMSO (Sigma-Aldrich, Cat. No. D8418) in ultrapure water to prepare a 1 mM stock. E2 (Sigma-Aldrich, Cat. No. E8875) was dissolved in DMSO to prepare a 1 mg/mL stock, and BPA (Sigma-Aldrich, Cat. No. 239658) was dissolved in DMSO to prepare a 100 mM stock. Both were serially diluted in 100% DMSO to generate working solutions such that a constant volume was added to each culture. All stocks were aliquoted and stored at – 20°C. Agonist working solutions were prepared by diluting the stocks in L-15 medium immediately before use. Within each experiment, the final DMSO concentration was 0.1% (v/v) and was matched across all treatments.

2.7. *Lhb* and *fshb* stimulation

To assess gonadotropin subunit expression under culture conditions, individual pituitaries were cultured in 500 µL of modified L-15 medium and sampled on days 0, 2, 4, and 6 for measurement of basal *lhb* and *fshb* expression in the absence of stimulation.

The functionality of cultured pituitaries was then examined using medaka gonadotropin-releasing hormone (mdGnRH; GenScript, purity 96.6%) and cholecystokinin octapeptide (CCK-8; Sigma-Aldrich, Cat. No. C2175), prepared as described above. To optimise stimulation conditions, pituitaries were exposed to 100 nM mdGnRH for 2 h or 24 h, or to 200 nM mdGnRH for 2 h; 100 nM mdGnRH for 24 h produced the strongest induction of *lhb* and was used in all subsequent experiments (Supplementary Figure S2a). For *fshb*, pituitaries were treated with 100 nM or 200 nM CCK-8 for 48 h; 200 nM for 48 h gave the greatest induction and was used thereafter (Supplementary Figure S2b).

To determine how long cultured pituitaries remained responsive to stimulation, pituitaries were stimulated at days 2, 4, 6, 8 and 10 of culture using the optimised conditions described above (100 nM mdGnRH for 24 h; 200 nM CCK-8 for 48 h), and *lhb* and *fshb* expression was compared with unstimulated pituitaries sampled at day 2 of culture.

2.8. Quantitative RT-PCR of pituitary

For phase 1 studies, RT-qPCR was performed as previously described by Royan, et al. [22]. In brief, total RNA was extracted from individual pituitaries using TRIzol® Reagent (Invitrogen™, Thermo Fisher Scientific, Cat. No. 15596018) and chloroform-based phase separation, followed by cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers (Thermofisher Scientific). The resulting cDNA was diluted 1:5 and 3 µL was used per qPCR reaction. The amplification protocol consisted of an initial pre-incubation at 95°C for 10 min, followed by 42 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s. Gene expression of *lhb* and

fshb was quantified by SYBR Green-based qPCR using gene-specific primers on a LightCycler 96 system (Roche), and expression levels were normalized to β-actin (*actb*).

For phase 3 studies, RT-qPCR was performed as previously described by Uehara, et al. [13]. Briefly, total RNA was extracted from individual pituitaries using the FastGene RNA Basic Kit (Nippon Genetics, Japan) and reverse transcribed with the PrimeScript RT Kit (Takara, Kusatsu, Japan), both according to the manufacturers' instructions. Real-time PCR was performed using a KAPA SYBR Fast qPCR Kit (Nippon Genetics) on a LightCycler 480 II instrument (Roche). cDNA samples were amplified under the following conditions: an initial denaturation step at 95°C for 5 min, followed by 45 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. Amplification specificity was confirmed through melting curve analysis. Gene expression levels were normalized to β-actin (*actb*). Primer sequences are given in Supplementary Table S1.

2.9. Single-cell transcriptomic analysis of CCK receptor expression

Genome database annotations predicted the presence of three CCK receptor genes in medaka, namely *cck1r*, *cck2ra* and *cck2rb* (Supplementary Table S2). Gene names follow the convention proposed by Kayo, et al. [17], in which teleost CCK receptors are designated *Cck1r* and *Cck2r* rather than *Cckar* and *cckbr*, since both *Ccka* and *Cckb* activate both receptors. The orthologue referred to here as *cck2rb* is named *cckbrb* in Li, et al. [16] and *Cckrb* in Tanaka, et al. [15]. The expression patterns of these receptors were analysed using previously published single-cell RNA-seq (scRNA-seq) datasets from male and female adult medaka pituitaries [25], available at the NCBI Gene Expression Omnibus under accession GSE162787 and the NCBI Sequence Read Archive under SRP296792.

2.10. Ca^{2+} imaging

Two transgenic medaka lines, *fsh*:IP and *lh*:IP [20] were used. In these lines, Fsh and Lh cells express the genetically encoded Ca^{2+} indicator Inverse Pericam (IP), enabling visualisation of intracellular Ca^{2+} dynamics in living pituitary cells. Inverse Pericam displays an inverse relationship between fluorescence intensity and Ca^{2+} concentration: Ca^{2+} binding quenches the fluorescence of the protein, so that a rise in $[Ca^{2+}]_i$ is detected as a decrease in emitted fluorescence. An overview of the Ca^{2+} imaging approach, including a schematic of the perfusion setup and representative responses, is shown in Fig. 2.

Adult medaka were anaesthetised with 0.02% MS-222 and decapitated. Pituitaries were dissected and transferred to a recording chamber containing approximately 300 µL of artificial cerebrospinal fluid (ACSF; 134 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1.2 mM $MgCl_2$, 2.1 mM $CaCl_2$, 15 mM glucose, 0.1% BSA and 0.001% DMSO, pH 7.35–7.45). Solutions were delivered by continuous perfusion at room temperature using a peristaltic pump (Rainin Dynamax RP-1) at approximately 550 µL min^{-1}.

Imaging was performed on an upright fluorescence microscope (Eclipse E600FN; Nikon) with a scientific CMOS camera (Andor Zyla 4.2 PLUS, Oxford Instruments) and an LED excitation source (X-Cite 110,

Excelitas Technologies). Images were acquired every 5 s with 4 × 4 binning and a 50 ms exposure, using MicroManager v1.4.

To assess gonadotrope cell responses, pituitaries from *fshb* fish were perfused with 1 mL of 1 μM CCK8, whereas pituitaries from *lhb* fish were perfused with 1 mL of 1 μM mdGnRH. These concentrations were selected based on previous studies demonstrating agonist-evoked Ca^{2+} responses in Fsh cells following CCK8 [13] and in Lh cells following GnRH [20].

Fluorescence was quantified in Fiji/ImageJ v1.54p (National Institutes of Health). For each pituitary, a single region of interest was drawn to cover the labelled cell population, and mean intensity was extracted per frame. For each pituitary, fluorescence was normalised to the baseline value F_0 and expressed as $-\Delta F/F$, calculated as $-(F - F_0)/F_0$, so upward deflections correspond to increases in Ca^{2+}. Individual cells were not segmented, so the signal reflects the population-level response of labelled cells within that region. No preparations were excluded. Response amplitude was quantified as the peak $-\Delta F/F$ for Lh cells and as the mean of 10 consecutive frames (50 s) during agonist perfusion for Fsh cells.

2.11. E2 and BPA exposure

2.11.1. *Ex vivo* exposure for transcript analysis

Pituitaries were dissected and immediately transferred to individual wells containing one ml of modified L-15 medium supplemented with E2 (Sigma-Aldrich, Cat. No. E8875) at 0.005, 0.05 or 0.5 ng/mL, BPA (Sigma-Aldrich, Cat. No. 239658) at 50, 500 or 5000 ng/mL, or vehicle alone, and cultured for a total of 48 h at 27°C. In all groups, the medium was fully replaced after 24 h with fresh medium containing the same chemical concentrations. For *fshb* stimulation, 200 nM CCK-8 was present from the start of culture and maintained throughout the 48-h period. For *lhb* stimulation, pituitaries were exposed to the chemicals alone during the first 24 h, and 100 nM mdGnRH was added to the chemical-containing medium for the final 24 h. Control pituitaries received agonist alone or vehicle alone over the same time course. E2 and BPA stock solutions were prepared in DMSO and diluted in culture medium to the final concentrations indicated; the final DMSO concentration did not exceed 0.1% (v/v) and was consistent across all treatment and control groups. One pituitary was cultured per well (n = 5 per group).

2.11.2. Acute exposure during Ca^{2+} imaging

Pituitaries were dissected from Tg(*lh*:IP) or Tg(*fsh*:IP) medaka and mounted in the imaging chamber. Each pituitary was assigned to a single treatment and used once. Recordings consisted of four consecutive perfusion periods of 100 s: ACSF, followed by treatment (ACSF for controls, E2 at 0.5 ng/mL, or BPA at 5000 ng/mL), followed by agonist applied in the continued presence of the treatment (1 μM mdGnRH for Lh cells, 1 μM CCK-8 for Fsh cells), and finally ACSF washout.

2.11.3. *Ex vivo* exposure for Ca^{2+} imaging

Pituitaries were dissected and cultured for 48 h in medium containing E2 (0.5 ng/mL), BPA (5000 ng/mL) or vehicle, as described above, with the medium replaced after 24 h. No agonist was present during the

exposure period. After 48 h, pituitaries were transferred to the imaging chamber and Ca^{2+} responses recorded following perfusion with 1 μM mdGnRH (Lh cells) or 1 μM CCK-8 (Fsh cells).

2.11.4. *In vivo* exposure

Adult Tg(*lh*:IP) or Tg(*fsh*:IP) medaka were placed in 1 L tanks at four fish per tank (two males and two females) and exposed to E2 (0.5 ng/mL), BPA (5000 ng/mL) or vehicle for 48 h under static conditions, with the exposure water fully renewed after 24 h. These concentrations corresponded to the highest used in the *ex vivo* exposures. Fish were held at 27°C under a 14 h light / 10 h dark cycle and fed three times daily. After 48 h, fish were euthanised, and pituitaries were dissected as described above, then imaged directly without a culture period. Ca^{2+} responses were recorded in Lh cells following perfusion with 1 μM mdGnRH and in Fsh cells following perfusion with 1 μM CCK-8, as described above.

2.12. Data analysis

All statistical analyses were performed using GraphPad Prism v11.0.1 (GraphPad Software, San Diego, CA, USA). Unless otherwise stated, n denotes the number of individual fish, one pituitary being obtained per fish; for the PrestoBlue viability assay, n denotes the number of pools of four pituitaries. Normality was assessed using the Shapiro–Wilk test, and parametric or equivalent non-parametric tests applied accordingly. Comparisons across more than two groups used ordinary one-way ANOVA followed by Dunnett's multiple comparisons test against the designated control, or the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Two related groups were compared using a paired two-tailed Student's t-test; two independent groups using an unpaired t-test with Welch's correction or the Mann–Whitney U test, as appropriate. PrestoBlue fluorescence was background-corrected against blank wells containing medium only. Relative gene expression was calculated using the efficiency-corrected method of Pfaffl [26] and normalised to *actb*. Ca^{2+} responses were quantified as described above. Data are presented as mean ± s.e.m. Significance was set at $P < 0.05$ and indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

3. Results

3.1. Metabolic activity of cultured pituitaries maintained over 10 days of culture

Metabolic activity was maintained throughout the 10-day culture period. Corrected fluorescence intensity rose above Day 0 levels by Day 2 and remained elevated through Days 4 and 6, before declining towards baseline at Days 8 and 10, which did not differ significantly from Day 0 (Fig. 3a).

3.2. Pituitary *lhb* and *fshb* mRNA levels decrease in culture

lhb expression was significantly reduced in cultured pituitaries at days 2, 4 and 6 compared with day 0 (Fig. 3b). A similar decline was observed for *fshb* expression which dropped roughly ten-fold by day 2,

although this difference did not reach statistical significance, and was significantly reduced at days 4 and 6 (Fig. 3c).

3.3. MdGnRH and CCK8 stimulate *lhb* and *fshb* mRNA levels in cultured pituitaries

When exposing the pituitaries for 24 h to 100 nM mdGnRH, *lhb* mRNA levels were significantly higher compared with untreated pituitaries, at day 2, 4 and 6 of culture (Fig. 3d). However, while mRNA levels were also higher at day 8 and 10, those were not statistically significantly different from the control group. In contrast, treatment with 200 nM CCK8 for 48 h significantly increased *fshb* mRNA levels, with statistically significant responsiveness maintained up to 8 days in culture (Fig. 3e). To evaluate possible sex-dependent differences in responsiveness to the stimulators, male medaka pituitaries were also examined. The expression profiles of *lhb* and *fshb* in mdGnRH- and CCK8-treated samples were similar to those observed in female medaka (Supplementary Figure S3). During optimization of *lhb* stimulation conditions, pituitaries were additionally exposed to 200 nM CCK8 for 48 h to evaluate its potential stimulatory effect on *lhb* expression. Surprisingly, treatment with 200 nM CCK8 resulted in a statistically significant increase in *lhb* gene expression compared with the control group (Supplementary Figure S4). In contrast, 100 nM mdGnRH for 24 h did not increase *fshb* transcript levels at any point during culture (Supplementary Figure S5).

3.4. *Cck2rb* is restricted to fshb-expressing and bi-hormonal gonadotropes

To further investigate the underlying mechanism, Fsh- and Lh-cell-specific single-cell RNA sequencing analyses were performed to identify the relevant CCK receptors involved. Of the three CCK receptor paralogs examined, *cck1r* and *cck2ra* transcripts were essentially undetectable across all annotated pituitary cell types, including gonadotropes, lactotropes, folliculo-stellate cells and the cga-expressing population. In contrast, *cck2rb* transcripts were detected in a restricted subpopulation of cells located within the gonadotrope cluster, identified by *fshb* and *lhb* transcript distribution (Fig. 4a).

cck2rb transcripts were detected across the gonadotrope population but were markedly enriched in *fshb*-dominant cells, which showed both the highest per-cell counts and the highest proportion of positive cells. A smaller fraction of *lhb*-dominant cells also carried detectable *cck2rb* transcripts, at levels above those observed in lactotropes and red blood cells (in which detection reflects ambient RNA background levels), indicating low-level receptor expression in a very few Lh cells rather than complete absence (Fig. 4a).

To determine which gonadotrope populations express the receptor, we restricted the analysis to the three gonadotrope clusters (n = 2739 cells) and classified cells by *lhb* and *fshb* expression using a threshold of 4 for both genes (Fig. 4b). Across all gonadotropes, 8.9% of cells were *cck2rb*-positive. *cck2rb*-positive cells were strongly overrepresented among cells expressing *fshb* alone (173 of 423 cells, 40.9%) and among bi-hormonal cells co-expressing *lhb* and *fshb* (19 of 124, 15.3%), whereas cells

expressing *lhb* alone were largely *cck2rb*-negative (24 of 1508, 1.6%), a frequency well below that of the gonadotrope population as a whole (Fig. 4c). Cells falling below the threshold for both subunits showed an intermediate frequency (27 of 684, 4.0%). Hypergeometric testing confirmed significant enrichment in *fshb*-expressing cells ($P = 2.2 \times 10^{-100}$) and in bi-hormonal cells ($P = 0.005$).

3.5 MdGnRH and CCK8 induce $[Ca^{2+}]_i$ response in Lh and Fsh cells in ex vivo culture

Using transgenic medaka lines expressing fluorescent markers in Lh and Fsh cells, pituitaries from *lh*:IP and *fsh*:IP fish were dissected to evaluate the effects of mdGnRH and CCK8 on intracellular calcium signaling. No statistically significant sex-dependent differences were detected at day 0 (Supplementary Figure S6). Pituitaries from both sexes were therefore included in the imaging experiments performed during the 6-day culture period. To assess the effect of mdGnRH on calcium signaling in Lh cells, pituitaries were examined at days 0, 2, 4, and 6 of culture. Perfusion with 1 µM mdGnRH significantly stimulated intracellular Ca^{2+} responses in Lh cells immediately after dissection (day 0), as well as in pituitaries cultured for 2 and 4 days (Fig. 5a-f). However, the Ca^{2+} response to mdGnRH was no longer statistically significant in pituitaries maintained in culture for 6 days (Fig. 5g, h). Fsh cell responsiveness to 1 µM CCK8 was assessed at Days 0, 2, 4, and 6 of culture (Fig. 5i–p). CCK8 significantly increased intracellular Ca^{2+} levels at all time points, indicating that Fsh cells remained functionally responsive throughout the 6-day culture period, unlike Lh cells.

The viability and functional windows of the *ex vivo* model are summarised in Table 1.

Table 1
Viability and functional windows of *ex vivo* cultured medaka pituitaries.

Endpoint	Target	Stimulus	Readout	Maintained until	Figure
Tissue viability	Whole pituitary	—	Metabolic activity	Day 10	3a
Transcription	*fshb*	CCK8	qPCR	Day 8	3e
Transcription	*lhb*	mdGnRH	qPCR	Day 6	3d
Transcription	*lhb*	CCK8	qPCR	Day 6*	S4b
Functional response	Lh cells	mdGnRH	Agonist-evoked Ca^{2+} response	Day 4	5f
Functional response	Fsh cells	CCK8	Agonist-evoked Ca^{2+} response	Day 6	5p

* Later time points not tested.

3.6. E2, but not BPA, attenuates the CCK8-induced increase in *fshb*

To test whether estrogenic compounds interfere with gonadotropin regulation, pituitaries were exposed to E2 or BPA for 48 h and stimulated with 100 nM mdGnRH (final 24 h) or 200 nM CCK8 (48 h); transcript levels were compared with agonist-alone and vehicle controls. Both agonists significantly increased their respective transcripts relative to vehicle (Fig. 6a, b, d).

Co-treatment with E2 did not significantly alter mdGnRH-induced *lhb* transcript levels at any concentration tested (0.005, 0.05 and 0.5 ng/mL; Fig. 6a). In contrast, E2 attenuated the CCK8-induced increase in *fshb* at all three concentrations relative to CCK8 alone (Fig. 6b). BPA did not significantly alter mdGnRH-induced *lhb* levels (Fig. 6c) or the CCK8-induced increase in *fshb* (Fig. 6d), although the *fshb* response across BPA concentrations was non-monotonic. In the BPA experiment, mdGnRH increased *lhb* expression approximately 50-fold relative to vehicle (Fig. 6c), although this difference did not reach significance following Kruskal–Wallis analysis with Dunn's correction.

3.7 Acute, 48 h ex vivo and in vivo exposure to E2 and BPA did not detectably alter agonist-evoked Ca^{2+} responses

Having established that E2 modulates gonadotropin subunit transcript levels, we next asked whether these chemicals also affect acute agonist-evoked signalling in gonadotropes. Intracellular Ca^{2+} responses were recorded in identified Lh and Fsh cells as an indicator of cellular activation.

Freshly dissected pituitaries were perfused with ACSF, E2 (0.5 ng/mL) or BPA (5000 ng/mL) for 100 s prior to agonist stimulation (Fig. 7a-d). In Lh cells, mdGnRH evoked a transient increase in Ca^{2+} that did not differ significantly between groups (Fig. 7a and b). In Fsh cells, CCK8 evoked a sustained response, and neither treatment differed significantly from control (Fig. 7c, d).

To test whether prolonged exposure was required, pituitaries were cultured with E2 (0.5 ng/mL) or BPA (5000 ng/mL) for 48 h prior to imaging. Neither treatment significantly altered responses in either cell type (Fig. 7e-h).

Finally, pituitaries from medaka exposed to E2 or BPA *in vivo* for 48 h were imaged. Consistent with the *ex vivo* results, neither compound significantly altered mdGnRH-induced responses in Lh cells or CCK8-induced responses in Fsh cells (Fig. 7i–l).

Across the three exposure routes, mean response amplitudes in Fsh cells were lower than control following E2, consistent in direction with the reduction in CCK8-induced *fshb* expression described above. None of these differences reached statistical significance, however, with four pituitaries per group these experiments had limited power to detect effects of the size observed.

4. Discussion

Ex vivo pituitary culture provides a controlled system for investigating pituitary regulation and its disruption by environmental factors. Compared with whole-organism studies, *ex vivo* cultures reduce systemic complexity, while still offering a more physiologically relevant model than dissociated cell

cultures, as they preserve the intact tissue architecture and cellular interactions. However, the viability and functionality of the pituitary in *ex vivo* culture conditions had not been thoroughly investigated, and it remained unclear whether this model performs comparably to *in vivo* or *in vitro* systems. In this study, the viability of cultured pituitaries was assessed over a 10-day period using the hs-PB assay to measure metabolic activity (Fig. 3a). Metabolic activity was maintained throughout the 10-day culture period, rising significantly above Day 0 levels by 48 h and remaining elevated until day 6 before declining. The initial increase may reflect recovery from dissection and adaptation to culture conditions, although an injury-associated increase in metabolic activity or proliferation of resident cells cannot be excluded. The later decline is consistent with the limited viability reported for pituitary explant cultures, attributed to inadequate perfusion and oxygenation of the tissue core [27]. Regardless of the underlying cause, the changing metabolic state over the first 48 h should be taken into account when designing experiments in this model.

We next examined the temporal expression patterns of *lhb* and *fshb* in *ex vivo* cultured pituitaries and found that transcript levels declined significantly after 2 and 4 days in culture, respectively (Fig. 3b and c). The reduction in *fshb* expression is consistent with previous findings in medaka, where *fshb* was significantly reduced after 24 h of culture relative to levels measured 1 h post-dissection [28]. Comparable reductions have been reported following disruption of hypothalamic input: targeted chemogenetic ablation of Gnrh3 neurons decreased the numbers of $fshb^{+}$, lhb^{+} and $fshb^{+}/lhb^{+}$ cells in zebrafish [15] and both *fshb* and *lhb* transcript levels are reduced in zebrafish $cck1^{-/-};cck2^{-/-}$ and $cck2rb^{-/-}$ mutants [16]. Together, these findings suggest that the decline in *lhb* and *fshb* expression observed here reflects the absence of hypothalamic stimulatory input, although loss of gonadal steroid feedback and adaptation to culture conditions may also contribute. These factors may not contribute equally to the two cell types, since Lh cells in teleosts express multiple estrogen receptors and are subject to estrogenic feedback [29] so removal of gonadal estrogen may contribute more to the decline in *lhb* than in *fshb*.

In medaka, mdGnRH stimulates Lh cells [20] and CCK8 stimulates *fshb* expression [13], with a comparable CCK effect on *fshb* reported in primary cultured zebrafish pituitary cells [16]. We therefore tested a range of concentrations and incubation periods for both agonists and found that 24-h exposure to mdGnRH and 48-h exposure to CCK8 increased *lhb* and *fshb* transcript levels, respectively, in pituitaries of both sexes (Supplementary Figures S2, S3). Restoring *lhb* expression with mdGnRH required a shorter incubation period than restoring *fshb* with CCK8. The basis for this difference is not clear from the present data. One possibility lies in the signalling pathways engaged by the two receptors: *Cck2rb* activates cAMP, Ca^{2+} and MAPK pathways in medaka [13], whereas the receptor subtype mediating the mdGnRH response in Lh cells, and whether it couples to Gq, Gs or both, has not been established. Identifying that receptor and its coupling may help explain the different time courses.

We next determined how long each transcript remained responsive during culture. Fsh cells continued to respond to CCK8 until day 8, whereas a statistically significant response of Lh cells to mdGnRH was no longer detected after day 6 (Fig. 3d and e). This difference is consistent with reports that Lh and Fsh

cells in the teleost pituitary differ in receptor profiles, signaling pathways and cellular organization [30], although the basis for their differing persistence in culture remains unclear. We next used intracellular Ca^{2+} responses as an indicator of cellular activation in Lh and Fsh cells. No statistically significant difference between sexes was detected in the Ca^{2+} response to mdGnRH or CCK8 in either cell type (Supplementary Figure S6), so we examined how long both cell types remained responsive during *ex vivo* culture. As previously reported in dispersed pituitary cell culture [31], the two cell types displayed distinct response profiles. In Lh cells, intracellular Ca^{2+} returned rapidly to baseline following mdGnRH stimulation, whereas in Fsh cells the response to CCK8 remained elevated after the agonist was washed out. This may reflect differences in Ca^{2+} influx and intracellular store dynamics, and in ion channel expression between *lhb*- and *fshb*-expressing cells, as described in Atlantic cod [31]. Whereas previous studies examined Lh and Fsh Ca^{2+} responses in freshly dissected pituitaries in medaka [13, 20] and zebrafish [14], we show that Ca^{2+} responses can still be evoked after culture until day 4 in Lh cells and day 6 in Fsh cells (Fig. 5) and the two cell types cannot be assumed to remain informative over the same period.

Together, our findings show that this *ex vivo* pituitary model remains metabolically active for up to 10 days, and that transcriptional and Ca^{2+} responses to stimulation are retained for at least 4 days, with *fshb* responsiveness persisting to day 8 and Fsh cell Ca^{2+} responses to day 6 (Table 1). This extends the usable window beyond the 24–48 h typically employed in previous pituitary culture studies [13, 16, 28], and this is consistent with observations in murine anterior pituitary explants, which remained viable for up to 96 h in culture while sustaining T3 production over a 48-h incubation [32]. However, the loss of hypothalamic input has consequences on Lh and Fsh cells activity in *ex vivo* culture, and therefore GnRH and CCK8 should be provided to compensate for this loss of inputs to provide more physiological conditions.

Reproductive regulation in vertebrates was traditionally thought to be conserved, with GnRH acting as the sole hypothalamic factor controlling both LH and FSH production and release. Work in teleosts has revealed a different arrangement, in which CCK functions as an Fsh-releasing hormone while GnRH primarily stimulates Lh synthesis [13, 14]. In the present study we confirmed that mdGnRH stimulates both *lhb* transcription and Ca^{2+} activity in Lh cells. However, CCK did not act exclusively on *fshb* in *ex vivo*. CCK-8 also significantly increased *lhb* transcript levels in cultured medaka pituitaries. Uehara, et al. [13] reported an apparent increase in *lhb* following CCK-8 treatment of *ex vivo* medaka pituitaries that did not reach statistical significance, wherease no Ca^{2+} response to CCK-8 was detected in Lh cells in the same study. Our transcript result is therefore consistent in direction with theirs, and we consider the apparent divergence between transcript and Ca^{2+} readout further below. Comparable observations have been reported in zebrafish. Li, et al. [16] found CCK-induced *lhb* expression both *in vivo* and in primary pituitary culture, detected *cck2rb* transcript in Lh-protein-positive cells by combined *in situ* hybridisation and immunostaining, and showed that a GnRH3-neutralising antibody only partially attenuated the *lhb* response in hypothalamic–pituitary co-culture, although the authors note this experiment lacked a non-immune IgG control. Hollander-Cohen, et al. [14] reported that calcium levels in Lh cells were increased

weakly and inconsistently: all Fsh cells strongly responded in every fish examined, whereas the proportion of responding Lh cells varied between 20 and 100% and their responses were poorly synchronised. This variability is compatible with CCK acting on a responsive subpopulation of Lh cells rather than on Lh cells uniformly, as proposed by Li, et al. [16]. Whether such a subpopulation accounts for the *lhb* response we observed, and what weight it carries *in vivo*, is considered below.

We next examined the distribution of CCK receptors in the medaka pituitary. Transcripts for *cck1r* and *cck2ra* were essentially absent, whereas *cck2rb* was largely restricted to gonadotropes. Within this population, *cck2rb* was expressed predominantly in *fshb*-expressing cells and additionally in a subset of cells co-expressing *lhb* and *fshb*, while cells expressing *lhb* alone were largely receptor-negative. The Fsh-dominant expression agrees with Uehara, et al. [13], who localised *cck2rb* to Fsh cells by *in situ* hybridisation; our data extend this by resolving a *cck2rb*-positive bi-hormonal subset that is not distinguished when *fshb*- and *lhb*-expressing cells are scored as mutually exclusive populations. In zebrafish, CCK-associated increase in *lhb* has likewise been attributed to a bi-hormonal *lhb*$^+$/*fshb*$^+$ gonadotrope subpopulation expressing *cck2rb* [16], and such bi-hormonal gonadotropes have independently been identified by pituitary single-cell transcriptomics [15]. Cell-type-specific transcriptomes of tilapia gonadotropes similarly show CCK receptor as the most enriched GPCR in Fsh cells, with lower-level expression also detectable in Lh cells [30], although that approach cannot resolve individual cells. In our study, mdGnRH did not increase *fshb* at any point during culture. This should be interpreted cautiously, since GnRH has been shown to evoke Ca^{2+} responses in medaka Fsh cells, albeit weaker than those evoked by CCK [13, 20] and since mdGnRH was applied for 24 h whereas CCK8 was applied for 48 h.

We have previously demonstrated bi-hormonal gonadotropes *in vivo* in juvenile and adult medaka pituitaries, using both the double-transgenic Tg(*lhb*/*fshb*) reporter line and dual-fluorescence in situ hybridisation for *lhb* and *fshb* mRNA in wild-type fish [28], with further support from single-cell RNA sequencing of freshly dissected pituitaries [33]. Importantly, we also showed that medaka gonadotropes are plastic: when paracrine and endocrine input is removed in dispersed pituitary cell culture, *fshb*-expressing cells begin to produce *lhb* over 72 h, expanding the bi-hormonal population, and this conversion is enhanced by Gnrh1 [28]. Because some of these bi-hormonal cells express *cck2rb* in our scRNA-seq data, the *ex vivo* conditions used here (in which brain input is removed and Gnrh is supplied) could increase the number of CCK-responsive bi-hormonal cells and thereby contribute to the rise in *lhb* mRNA we observed following CCK stimulation. The present preparation preserves tissue architecture and the gonadotrope networks through which much of this signalling occurs and is therefore less prone to this effect than dissociated culture; nonetheless, a contribution from culture-induced plasticity cannot be excluded. Functional evidence in medaka points the same way: disruption of *cck2rb* impairs folliculogenesis, an Fsh-dependent process, whereas gnrh1 knockout disrupts final oocyte maturation and ovulation, and no other signalling pathway compensates for the loss of Gnrh1 [34]. Additionally, the small number of *cck2rb*-expressing cells among *lhb*-expressing gonadotropes *in vivo*, together with the absence of a CCK-evoked Ca^{2+} response in medaka Lh cells reported by Uehara, et al. [13], suggests that

any direct stimulatory action of CCK on Lh cells is limited and of minor physiological importance relative to the other factors regulating these cells *in vivo*. It may also explain why Ca^{2+} responses of zebrafish in Lh cells to CCK were weak and varied widely between individuals [14], whereas significant increases in *lhb* transcript levels following CCK treatment have been reported in cultured pituitary preparations from multiple species, including dissociated zebrafish pituitary cells [16] and, in the present study, intact medaka pituitaries.

We next investigated the effects of two well-characterised EDCs, E2 and BPA, using *ex vivo* cultured pituitaries (Fig. 6). E2 has previously been shown to suppress *fshb* in isolated medaka pituitary [35]; however, our findings extend this to agonist-stimulated conditions and to lower and environmentally relevant concentrations. In our study, E2 exposure did not significantly alter mdGnRH-induced *lhb* expression at any concentration tested, whereas the CCK8-induced increase in *fshb* was significantly attenuated. The attenuation of the CCK8-induced *fshb* response is consistent with previous work in medaka showing that gonadal sex steroids reduce both *fshb* transcript levels and Fsh cell number in both sexes [35–37]. The lack of a statistically significant *lhb* response contrasts with our previous report that *in vivo* E2 exposure reduces *lhb* expression in medaka [29]. Notably, medaka Lh cells express both *esr1* and *esr2b*, so the absence of a detectable effect here is unlikely to reflect an absence of estrogen receptors. This suggests instead that indirect actions, whether via the hypothalamus or via gonadal feedback, contribute substantially to the *in vivo* response. Consistent with this, differences in *lhb* expression apparent between genotypes in intact female medaka are lost following ovariectomy, indicating that ovarian estrogen contributes to *lhb* expression in the intact animal [13]. Our *ex vivo* preparation, which lacks both hypothalamic input and gonadal feedback, isolates direct pituitary effects over a shorter exposure window. A similar pattern was reported in Atlantic cod, where von Krogh, et al. [38] found that 72 h E2 exposure of dispersed Atlantic cod pituitary cells did not affect *lhb* expression, while *fshb* was upregulated. Beyond possible species differences, their cultures were not stimulated with mdGnRH or CCK8, which may further account for the divergent gonadotropin responses.

Neither acute nor 48-h *ex vivo* E2 exposure significantly altered agonist-evoked Ca^{2+} responses in Lh or Fsh cells. To our knowledge, the effect of E2 on gonadotrope Ca^{2+} signalling has not previously been examined in fish, so no direct comparison is available. In mammals, by contrast, E2 has been reported to sensitise rat pituitary cells to GnRH through protein kinase C- and calcium-dependent pathways [39], to trigger rapid non-genomic release of intracellular Ca^{2+} [40], and to increase low voltage-activated calcium current density in GH_3 pituitary cells over 24–72 h [41]. The absence of a detectable effect here may reflect the shorter exposure durations used and the concentration tested. More broadly, the dissociation between our two readouts with a significant reduction in CCK8-induced *fshb* transcription with no detectable change in agonist-evoked Ca^{2+} responses, is consistent with E2 acting predominantly through nuclear receptors to modulate transcription rather than through rapid membrane-initiated effects on Ca^{2+} signalling in this preparation. Moreover, a genuine difference between teleost and mammalian gonadotropes cannot be excluded.

As with E2, BPA exposure did not significantly alter mdGnRH-induced *lhb* transcript levels, and no significant effect on *fshb* was detected at any concentration tested. The group means nonetheless did not show linear dose-dependency: *fshb* levels were lower at 50 and 5000 ng/mL than at 500 ng/mL. Such non-monotonic concentration–response relationships are well documented for BPA, occurring in over 20% of experiments surveyed [42]. Comparable patterns have been reported in fish, including an inverted U-shaped response with maximal effect at an intermediate concentration across multiple reproductive endpoints in female rare minnow exposed to three BPA concentrations [43], and in pituitary cells, where xenoestrogens elicit non-monotonic estrogen receptor-mediated calcium and prolactin responses [44]. Such patterns have been attributed to activation of multiple receptors with opposing downstream effects, receptor desensitization at higher concentrations, or negative feedback regulation, although direct experimental support remains limited [45]. Given that only three concentrations were tested with high inter-individual variability (n = 5), the apparent non-monotonicity should be interpreted with caution and would require a dose-response characterization to confirm.

As with E2, we did not detect an effect of BPA on agonist-evoked Ca^{2+} responses in Lh or Fsh cells, and the same was true following *in vivo* exposure. This is in contrast to a study conducted in rats in which the authors reported altered serum LH and FSH levels following 28 days of *in vivo* BPA exposure, suggesting an effect on secretion activity [46]. The discrepancy between our and this study may be explained by the substantially longer BPA exposure period they used. No detectable change in Ca^{2+} responses followed 48 h BPA exposure in either *ex vivo* or *in vivo* conditions, consistent with the transcript data. For E2, by contrast, an effect on CCK8-induced *fshb* expression was detected without a corresponding change in Ca^{2+} responses. The two readouts report different processes, so this is not unexpected: agonist-evoked Ca^{2+} signalling reflects receptor engagement and acute intracellular Ca^{2+} mobilisation, whereas *fshb* transcription is a slower, downstream process on which E2 may act at the pituitary level. One technical consideration should be noted, in that inverse pericam is expressed under the control of the *lhb* and *fshb* promoters, so treatments altering subunit transcription could in principle also alter indicator expression during the longer exposures, although this does not apply to the acute experiments. Overall, transcriptional readouts appear to reveal effects that are not apparent at the level of acute Ca^{2+} signalling, and gene expression may therefore represent an informative endpoint for investigating endocrine-disrupting effects in this model.

Conclusion

In conclusion, this study shows that *ex vivo* cultured medaka pituitaries remain viable for up to 10 days and functionally responsive for between 4 and 8 days depending on the endpoint examined (metabolic activity, *lhb* and *fshb* transcript levels, or agonist-evoked Ca^{2+} responses in Lh and Fsh cells; Table 1). Basal gonadotropin transcript levels nonetheless declined rapidly in culture, consistent with the loss of hypothalamic input, indicating that restoring these inputs would be necessary to more closely approximate physiological conditions. In the course of this characterisation, we found that CCK8 increased *lhb* as well as *fshb* transcript levels in cultured pituitaries, and that *cck2rb* is mainly expressed

in *fshb*-expressing cells but also in a bi-hormonal gonadotrope population co-expressing *lhb* and *fshb*, while cells expressing *lhb* alone are largely *cck2rb*-negative. Of the two endocrine disruptors tested, only E2 significantly affected gonadotrope function, reducing CCK8-induced *fshb* expression without a detectable change in agonist-evoked Ca^{2+} responses. By defining the functional window over which transcriptional and secretory readouts remain informative, this work provides a characterised *ex vivo* platform for investigating the regulation of Lh and Fsh cells and its disruption by exogenous compounds and for deepening the mechanistic understanding of gonadotrope regulation in a preparation that retains tissue context. It also offers a New Approach Methodology for assessing chemical effects on gonadotrope function without exposing live fish to test compounds.

Declarations

Additional Information

Competing interests

The author(s) declare no competing interests.

Use of AI and AI-assisted technologies

During the preparation of this work, the authors used Claude (Anthropic; Claude Opus 5) to assist with language editing and checking the manuscript against journal requirements. The authors reviewed and edited all content and take full responsibility for the content of the published article.

Author Contribution

S.V., R.F., S.Ka., M.H.B.M., E.R and M.R.R. designed the study. S.V. performed the viability assays, RT-qPCR, Ca^{2+} imaging, and chemical exposure experiments. S.K.U. and M.R.R. provided technical assistance with the Ca^{2+} imaging. C.H. performed the single-cell RNA-seq analysis. S.V. wrote the first draft. S.V. and R.F. wrote the manuscript with input from all other authors. S.V., M.R.R., S.Ka. and M.H.B.M. acquired funding. All authors read and approved the final manuscript.

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Data Availability

Processed single-cell RNA-seq data analysed in this study were retrieved from the Gene Expression Omnibus, accession GSE162787 (https://identifiers.org/geo:GSE162787). All other data generated or analysed during this study are available from the corresponding author on reasonable request.

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Figures

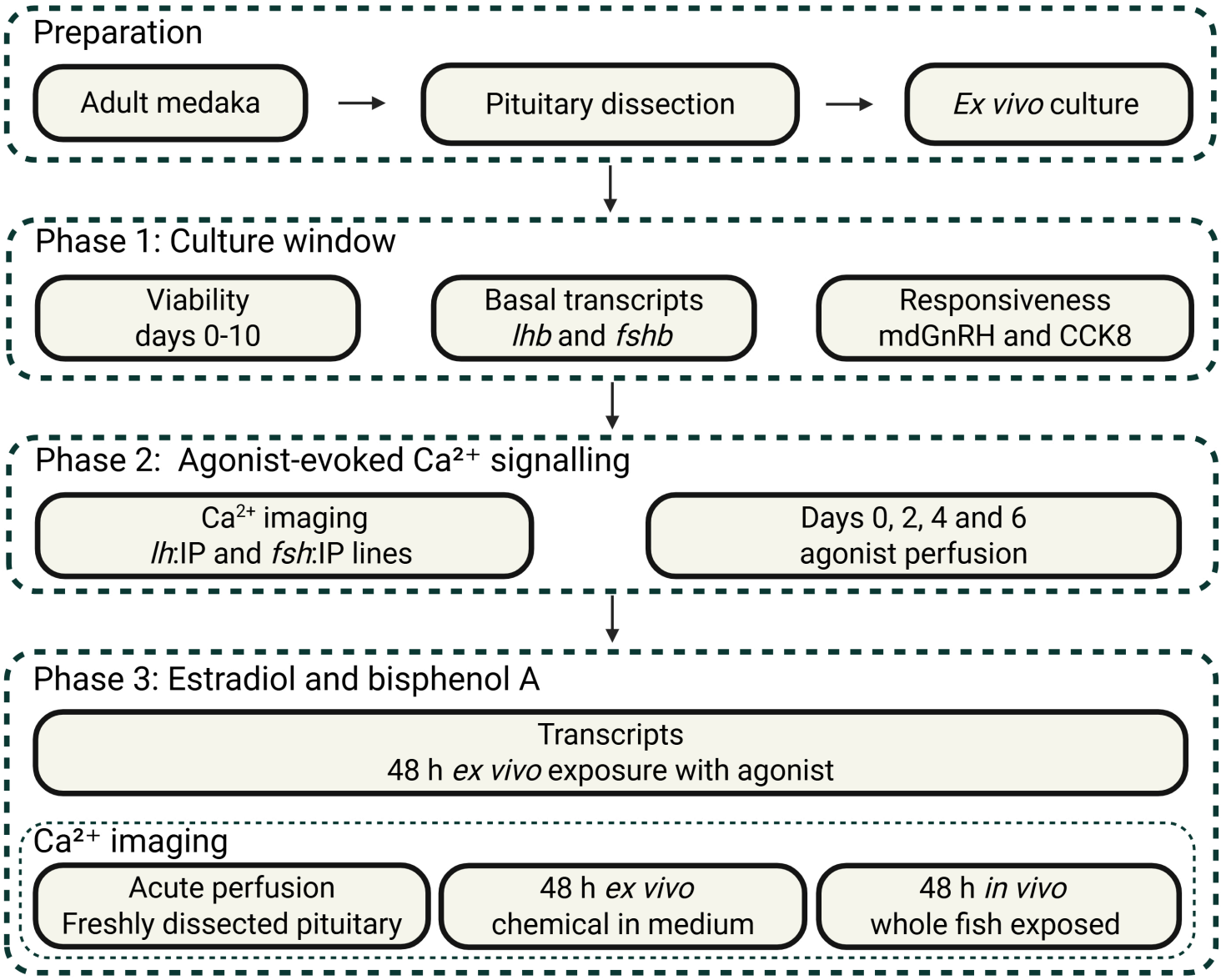


Figure 1

Experimental design. Pituitaries were dissected from adult medaka and maintained in ex vivo culture. Phase 1. Metabolic activity was assessed over 10 days, and basal lhb and fshbtranscript levels and their responsiveness to mdGnRH and CCK8 were measured over time. Phase 2. Pituitaries from lh:IP and fsh:IP transgenic lines were cultured for 0, 2, 4 or 6 days, perfused with mdGnRH or CCK8, and intracellular Ca^{2+}responses were recorded in Lh and Fsh cells. Phase 3. Effects on gonadotropin subunit expression were assessed after 48 h co-treatment with the corresponding agonist, and effects on agonist-evoked Ca^{2+} responses were assessed after acute perfusion, 48 h ex vivoexposure, and 48 h in vivo waterborne exposure. For in vivo exposure, whole fish were exposed for 48 h; pituitaries were then dissected and imaged without a culture period.

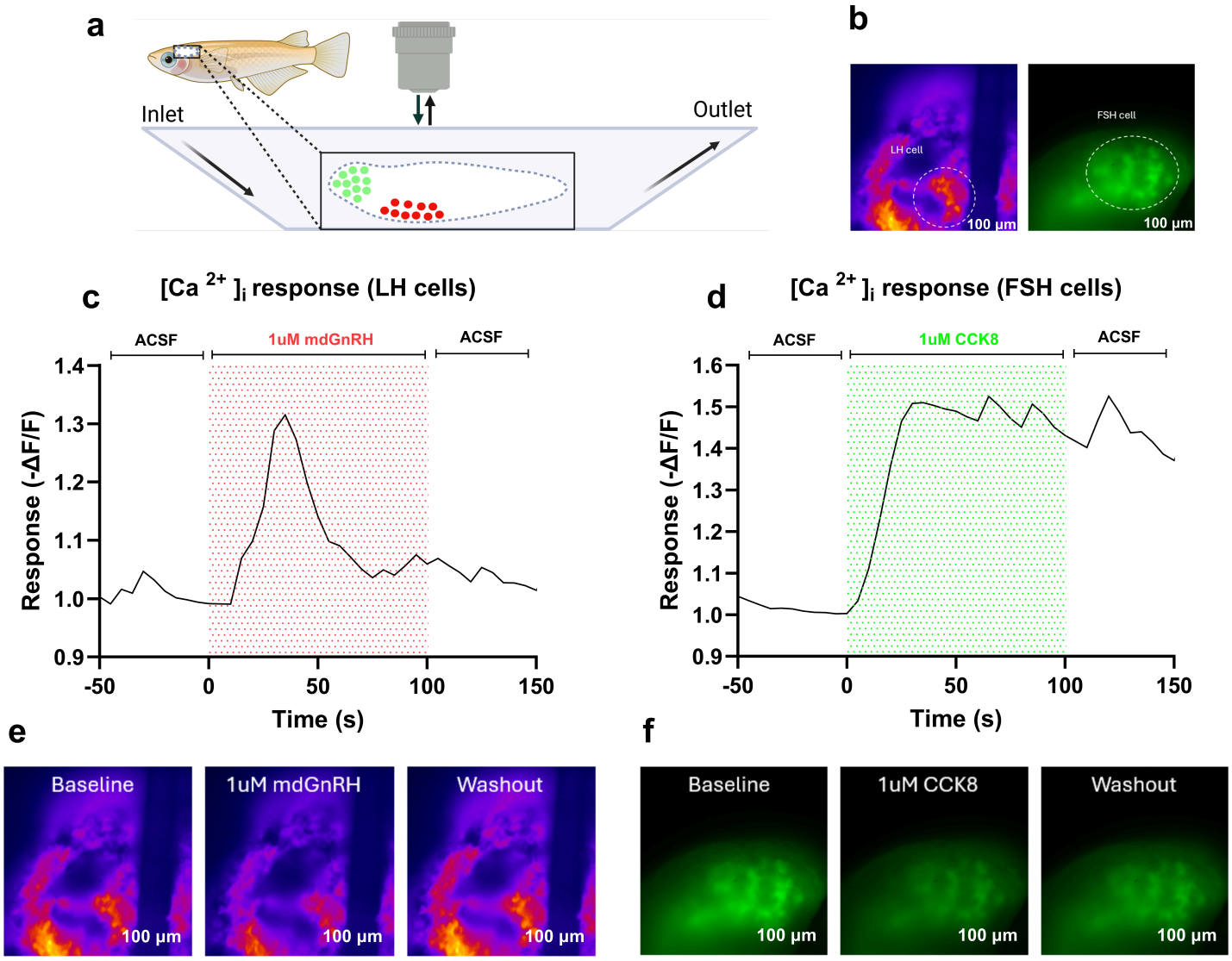


Figure 2

Ca²⁺ imaging in Lh and Fsh cells of lh:IP and fsh:IP transgenic medaka. (a) Schematic of the Ca²⁺ imaging setup: the pituitary is held in a perfusion chamber and imaged from above while solutions flow from inlet to outlet. (b) Epifluorescence images of Lh cells in lh:IP (left, pseudocoloured) and Fsh cells in fsh:IP (right); dashed outlines indicate the regions of interest. Inverse Pericam fluorescence decreases when Ca²⁺ binds, so a drop in fluorescence intensity reflects a rise in [Ca²⁺]. Traces are therefore plotted as −ΔF/F, such that upward deflections correspond to increases in intracellular Ca²⁺. (c) Representative trace of [Ca²⁺] dynamics in Lh cells of one pituitary during perfusion with 1 µM mdGnRH (medaka gonadotropin-releasing hormone), shown to illustrate the response profile. The trace is the mean signal from a single region of interest encompassing the labelled cell population. Shading indicates the 100 s perfusion period; the preparation was perfused with ACSF before and after stimulation. (d) Representative trace of $[Ca^{2+}]_i$ dynamics in Fsh cells of one pituitary during perfusion with 1 µM CCK8 (cholecystokinin octapeptide); same conventions as in (c). (e) Representative images of Lh cells at

baseline, during perfusion with 1 µM mdGnRH, and after washout. The reduction in fluorescence during stimulation reflects cell activation. (f) As in (e), for Fsh cells perfused with 1 µM CCK8.

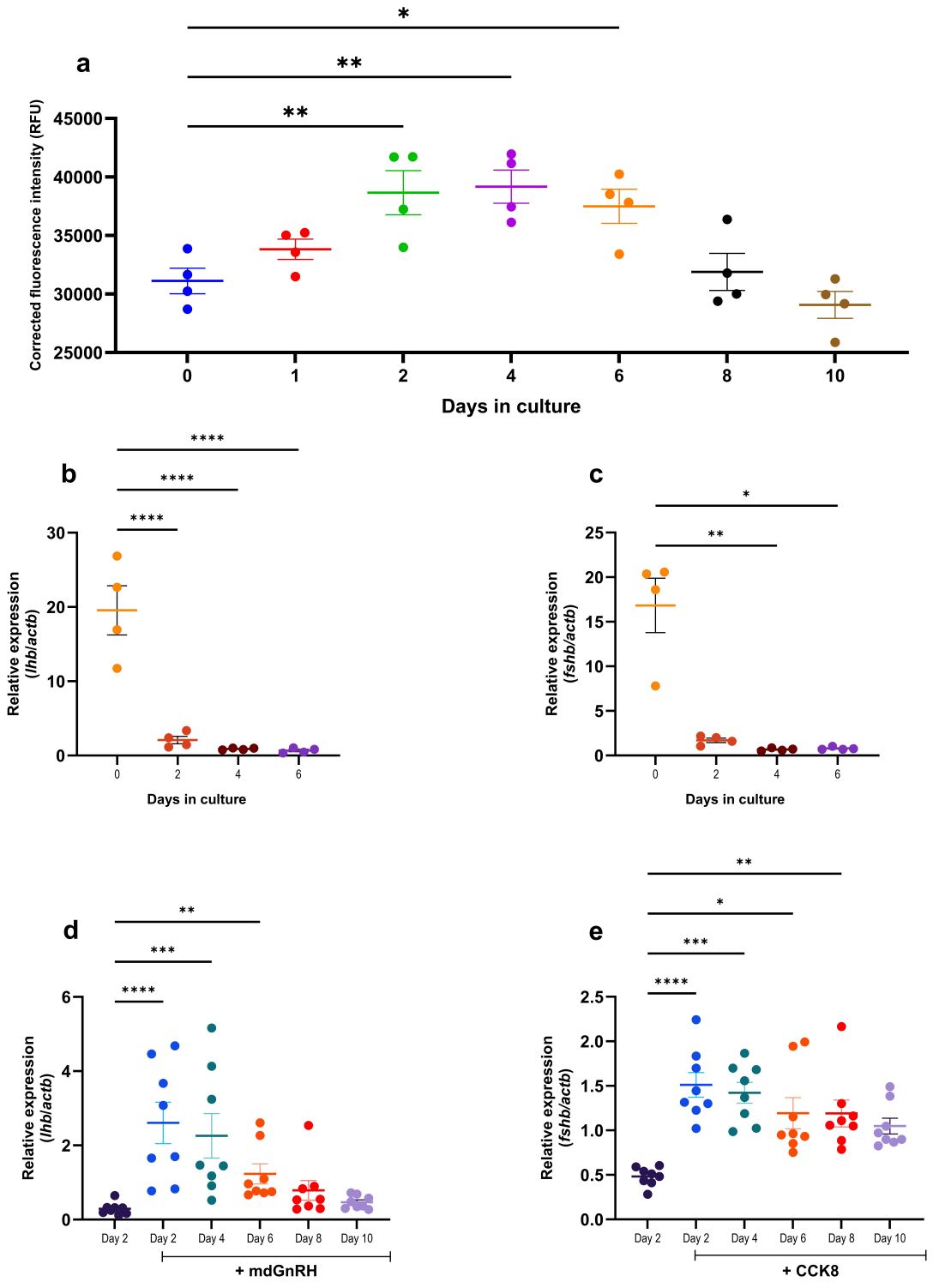


Figure 3

Cultured medaka pituitaries remain metabolically active and transcriptionally responsive for several days, despite a rapid decline in basal gonadotropin transcript levels. **a,** Metabolic activity of cultured

pituitaries measured using the high-sensitivity PrestoBlue (hs-PB) assay over 10 days, expressed as blank-corrected fluorescence intensity (RFU). Activity rose above day 0 levels by 48 h, remained elevated until day 6, and declined thereafter.**b, c,** Basal lhb (b) and fshb (c) transcript levels after 0, 2, 4 and 6 days in culture, measured by RT-qPCR and normalised to actb. Both fell sharply within the first two days and remained low thereafter. d, e, Transcript levels following stimulation at different times in culture. Pituitaries were stimulated at days 2, 4, 6, 8 and 10 of culture with 100 nM mdGnRH for 24 h (d) or 200 nM CCK8 for 48 h (e), and compared with unstimulated pituitaries sampled at day 2. Significant induction of lhb was detected up to day 6 and of fshb up to day 8. Transcript levels remained above the unstimulated reference at later time points, but the differences were not statistically significant. In a, each point represents one pool of four pituitaries (n = 4 pools per time point); in b–e, each point represents one pituitary from an individual fish (n = 4 in b, c; n = 8 in d, e). Lines and error bars indicate mean ± s.e.m. Normality was assessed using the Shapiro–Wilk test. Normally distributed data were analysed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; data that did not meet the normality assumption were analysed by the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Comparisons were made against day 0 (a–c) or against the unstimulated day-2 group (d, e). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; comparisons without an asterisk were not significant.

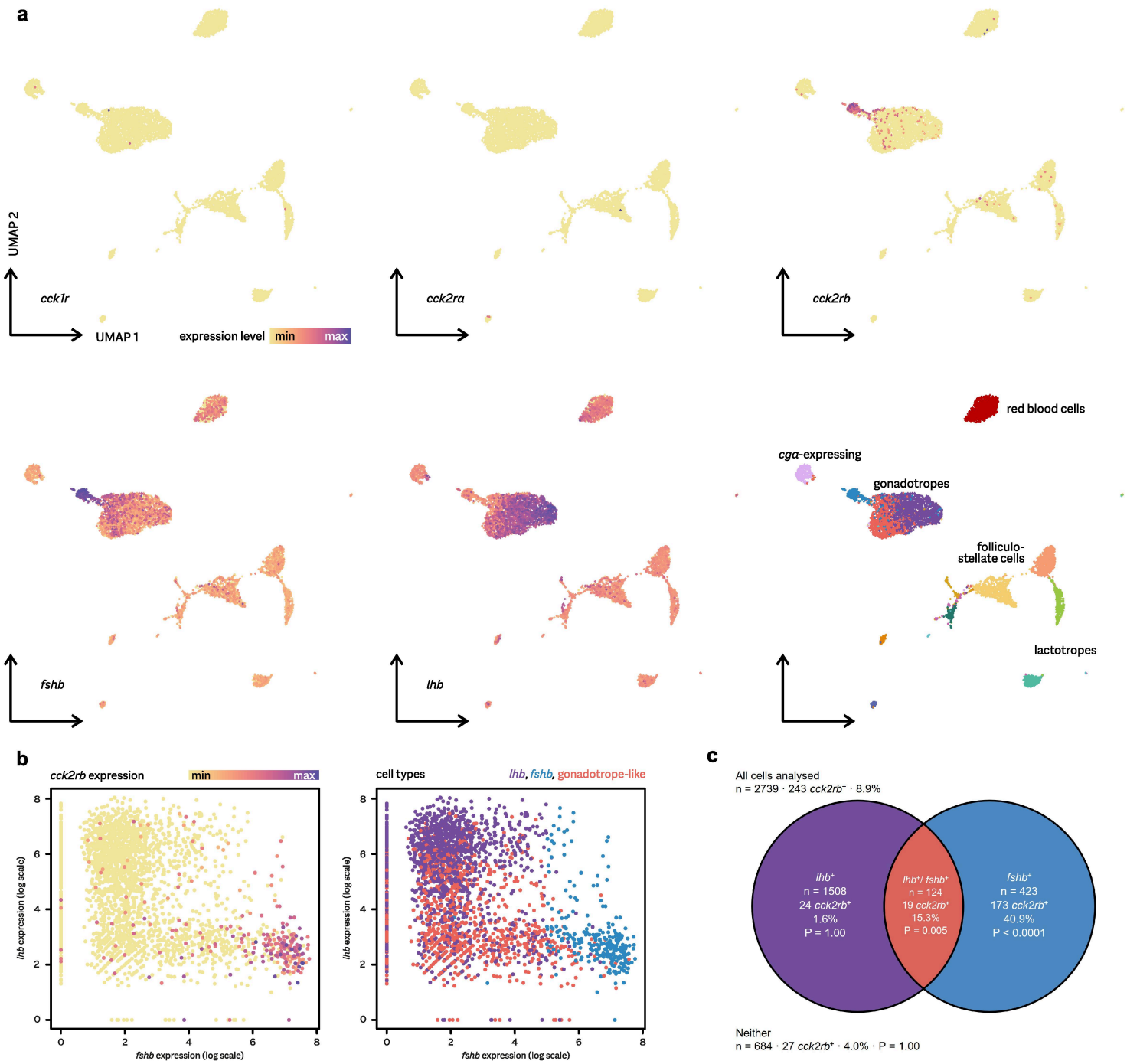


Figure 4

cck2rb is expressed predominantly in fshb-expressing gonadotropes. a, UMAP projections of single-cell RNA-seq data from adult medaka pituitaries (dataset GSE162787), coloured by expression of the three cholecystokinin receptor genes cck1r, cck2ra and cck2rb (top row) and of fshb and lhb(bottom row). Colour scale indicates normalised expression from low (yellow) to high (purple). The bottom-right panel shows the annotated cell clusters for reference; cga-expressing cells, gonadotropes, folliculo-stellate cells, lactotropes and red blood cells are indicated. cck1r and cck2ra were essentially undetectable across all cell types, whereas cck2rb was detected within the gonadotrope cluster. b, Expression of

lhbplotted against fshb(normalised, log scale) in individual cells of the three gonadotrope-related clusters. Left, points coloured by cck2rb expression; right, the same cells coloured by cluster identity (lhb-expressing gonadotropes, fshb-expressing gonadotropes, and gonadotrope-like cells co-expressing both). cck2rbexpression is concentrated in cells with the highest fshb and lowest lhblevels. c, cck2rbexpression across gonadotrope subtypes. Analysis was restricted to the three gonadotrope clusters (n = 2,739 cells), and cells were classified by lhb and fshb using a threshold of normalised expression 4 for both transcripts. Across all gonadotropes, 243 cells (8.9%) were cck2rb-positive. cck2rb was detected in 173 of 423 $fshb^{+}/lhb^{-}$ cells (40.9%) and 19 of 124 $lhb^{+}/fshb^{+}$ cells (15.3%), but in only 24 of 1,508 $lhb^{+}/fshb^{-}$ cells (1.6%) and 27 of 684 cells below threshold for both transcripts (4.0%). Over-representation of $cck2rb^{+}$ cells within each subset relative to the overall rate was tested using a one-sided hypergeometric test; exact P values are shown. The test assesses enrichment only, so P = 1.00 indicates absence of enrichment rather than absence of a difference.

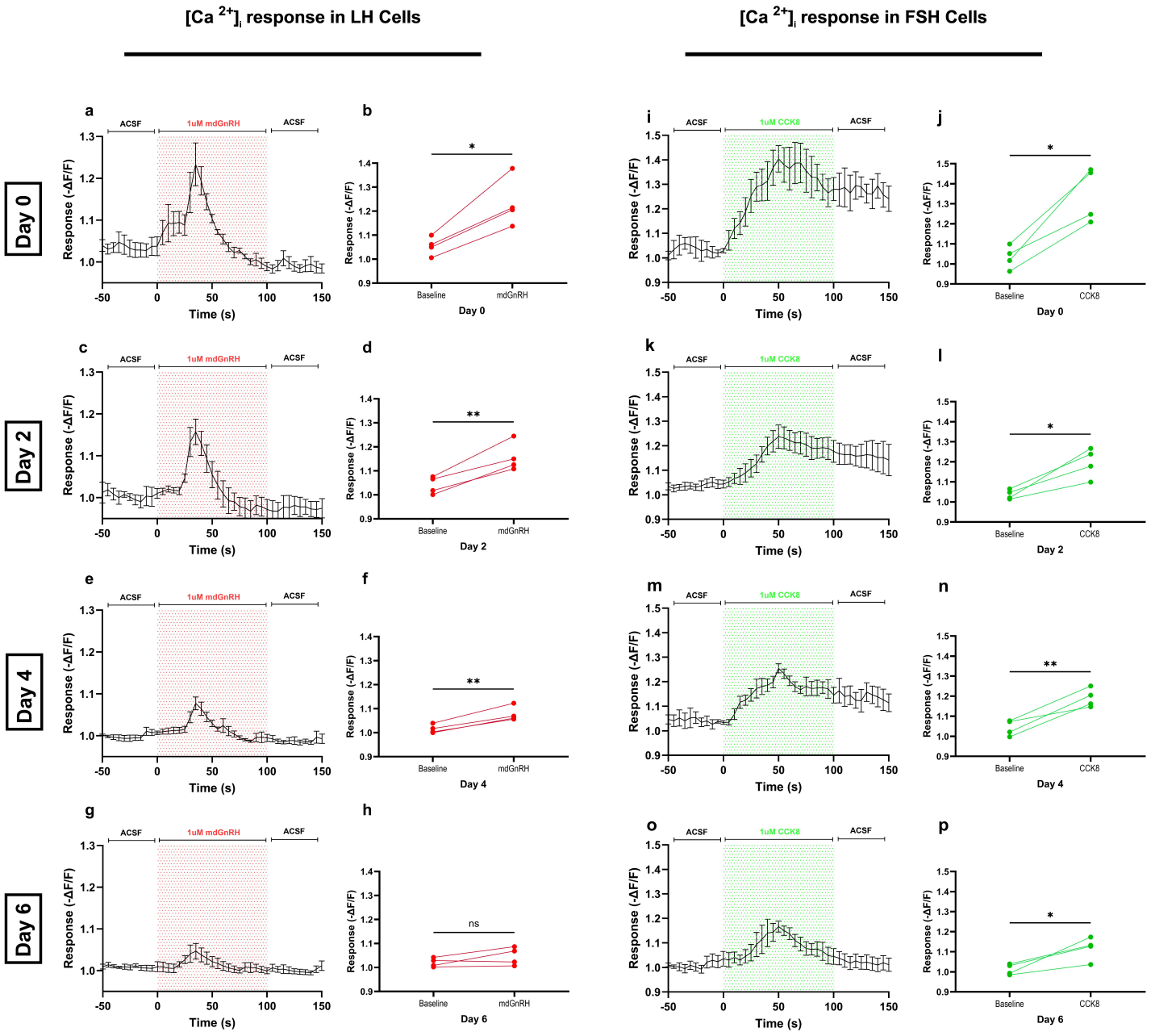


Figure 5

Intracellular Ca²⁺responses of Lh and Fsh cells during pituitary culture. a–h, Lh cells perfused with 1 μM mdGnRH. i–p, Fsh cells perfused with 1 μM CCK8. Pituitaries were examined at day 0 (a, b, i, j), day 2 (c, d, k, l), day 4 (e, f, m, n) and day 6 (g, h, o, p) of culture. a, c, e, g, i, k, m, o, averaged traces of intracellular Ca²⁺responses (−ΔF/F, mean ± s.e.m.). Shading indicates the 100 s agonist perfusion period; preparations were perfused with ACSF before and after stimulation. b, d, f, h, j, l, n, p, corresponding paired comparisons of baseline and stimulated fluorescence. For Lh cells, the peak mdGnRH-induced value was used; for Fsh cells, the mean stimulated response was calculated from 10 consecutive image frames (50 s) acquired during CCK8 perfusion and compared with the corresponding baseline fluorescence. Lh cells responded significantly to mdGnRH at days 0, 2 and 4, but no longer at day 6. Fsh

cells responded significantly to CCK8 at all four time points. Each point represents one pituitary preparation, quantified from a single region of interest encompassing the labelled cell population (n = 4 fish per time point). Baseline and stimulated values were compared using a paired two-tailed Student's t-test. *P < 0.05, **P < 0.01; ns, not significant.

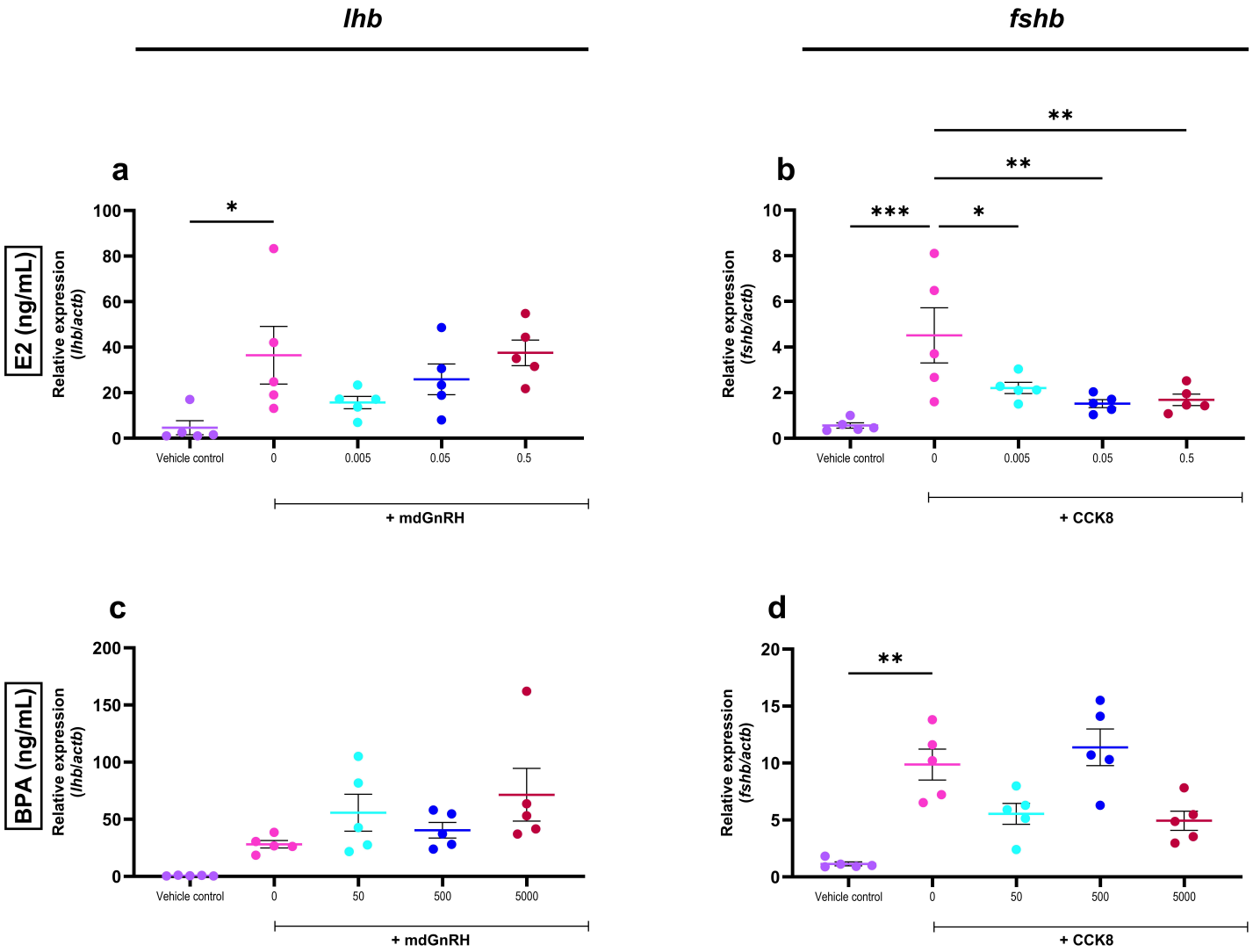


Figure 6

Effects of E2 and BPA on agonist-stimulated lhb and fshbexpression in cultured medaka pituitaries. Pituitaries were cultured for 48 h in medium containing 17β-estradiol (E2; a, b), bisphenol A (BPA; c, d) or vehicle, with the medium replaced after 24 h. For fshb (b, d), 200 nM CCK8 was present throughout the 48-h period. For lhb (a, c), pituitaries were exposed to the chemicals alone during the first 24 h, and 100 nM mdGnRH was added to the chemical-containing medium for the final 24 h. Relative expression was measured by RT-qPCR and normalised to actb. Horizontal bars beneath the x-axis indicate the groups that received agonist; 0 denotes agonist alone without E2 or BPA, and the vehicle control received neither. Concentrations are nominal. a, mdGnRH increased lhbtranscript levels relative to the vehicle control. Co-exposure to E2 at 0.005, 0.05 or 0.5 ng/mL did not significantly alter the mdGnRH-induced

response at any concentration. b, CCK8 increased fshb transcript levels relative to the vehicle control, and co-exposure to E2 significantly attenuated this increase at all three concentrations tested. c, mdGnRH increased lhb transcript levels approximately 50-fold relative to the vehicle control, although this did not reach statistical significance. Co-exposure to BPA at 50, 500 or 5000 ng/mL did not significantly alter lhbexpression. d, CCK8 increased fshbtranscript levels relative to the vehicle control; co-exposure to BPA did not significantly alter this increase at any concentration, although mean levels varied non-monotonically across the concentration range. Each point represents one pituitary from an individual fish; horizontal lines and error bars indicate mean ± s.e.m. (n = 5 per group). Normality was assessed using the Shapiro–Wilk test; normally distributed data were analysed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, and data that did not meet the normality assumption by the Kruskal–Wallis test followed by Dunn's multiple comparisons test, α = 0.05. Agonist-alone groups were compared with the vehicle control, and co-treatment groups with the corresponding agonist-alone group. *P < 0.05, **P < 0.01, ***P < 0.001; comparisons not marked were not statistically significant.

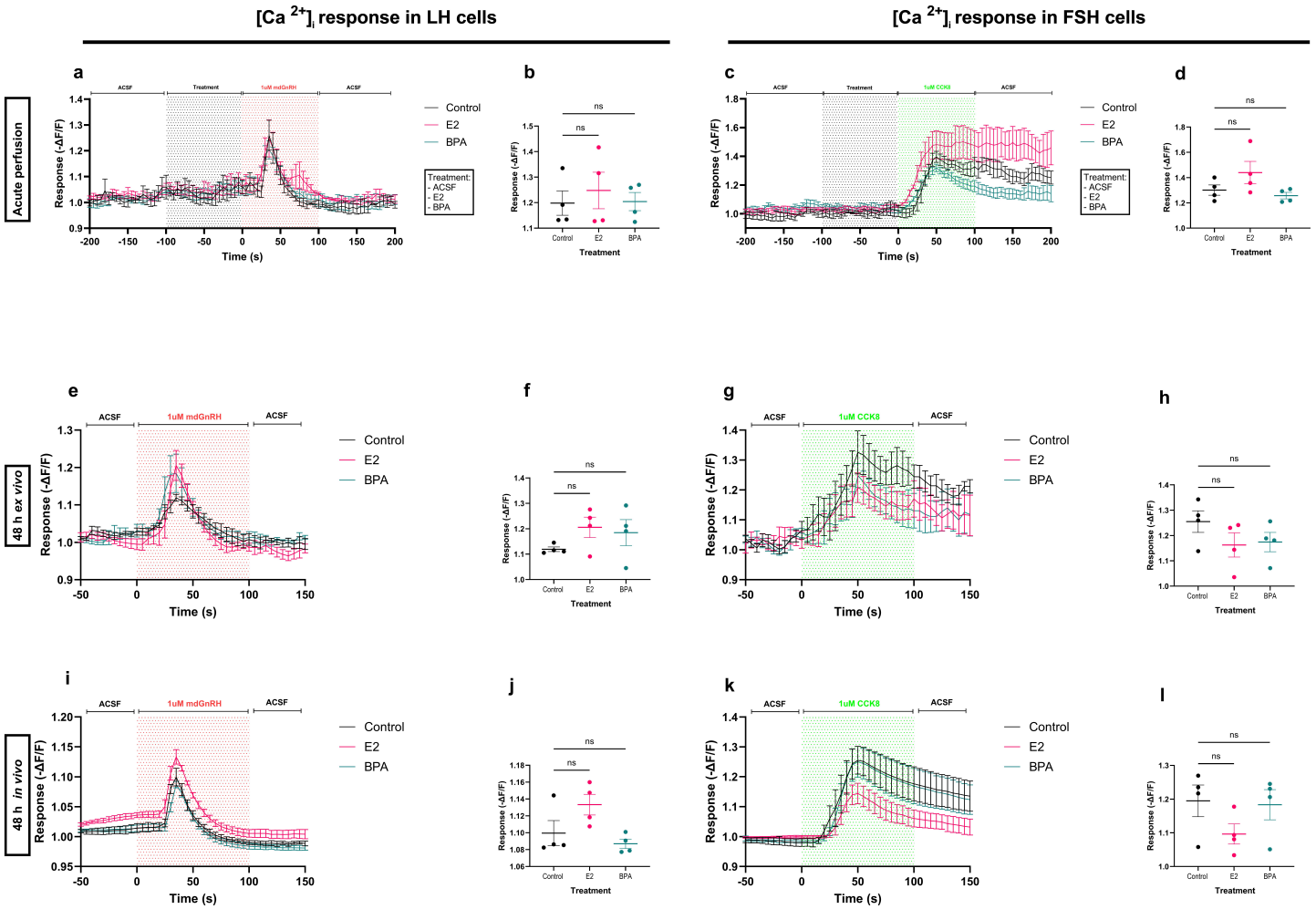


Figure 7

E2 and BPA did not detectably alter agonist-evoked Ca^{2+} responses in Lh or Fsh cells after acute, 48-h ex vivo or 48-h in vivoexposure. Intracellular Ca^{2+}responses were recorded in Lh cells of Tg(lh:IP) medaka (a, b, e, f, i, j) and Fsh cells of Tg(fsh:IP) medaka (c, d, g, h, k, l) following perfusion with 1 μM mdGnRH or 1 μM CCK8, respectively. a–d, acute exposure: freshly dissected pituitaries were perfused with ACSF (control), E2 (0.5 ng/mL) or BPA (5000 ng/mL) for 100 s, after which the agonist was applied in the continued presence of the treatment. e–h, 48-h ex vivo exposure: pituitaries were cultured in control medium, E2 (0.5 ng/mL) or BPA (5000 ng/mL) for 48 h before imaging, with no agonist present during the exposure period. i–l, 48-h in vivo exposure: adult medaka were exposed via water to control conditions, E2 (0.5 ng/mL) or BPA (5000 ng/mL) for 48 h; pituitaries were dissected immediately afterwards and imaged without culture. Concentrations are nominal. a, c, e, g, i, k, averaged Ca^{2+} responses ($-\Delta F/F$, mean ± s.e.m.). In a and c, grey shading indicates the acute treatment period; red and green shading indicate the mdGnRH and CCK8 stimulation periods, respectively. b, d, f, h, j, l, quantification: peak $-\Delta F/F$ for Lh cells, and the mean of 10 consecutive image frames (50 s) during agonist perfusion for Fsh cells. Each point represents one pituitary from an individual fish, quantified from a single region of interest encompassing the labelled cell population (n = 4 per group). Normality was assessed using the Shapiro–Wilk test. Groups were compared by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test against the control, $\alpha = 0.05$; ns, not significant. Mean Fsh cell response amplitudes were lower than control following E2 across all three exposure routes, but none of these differences reached statistical significance; with four pituitaries per group, these experiments had limited power to detect effects of the size observed.

Supplementary Files

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