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Ontogeny of Gonadotropin-Inhibitory Hormone (GnIH) in the cichlid fish *Cichlasoma dimerus*.

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Short title: Ontogeny of GnIH in C*ichlasoma. dimerus.*

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Abstract

RFamide peptides are expressed in the early stages of development in most vertebrates. Gonadotropin-inhibitory hormone (GnIH) belongs to the RFamide family, and its role in reproduction has been widely studied in adult vertebrates, ranging from fish to mammals. As only three reports evaluated GnIH during development, the aim of this study was to characterise the ontogeny of GnIH in a fish model, *Cichlasoma dimerus.* We detected the presence of two GnIH-immunoreactive (GnIH-ir) cell clusters with spatial and temporal differences. One cluster was observed by 3 days post-hatching (dph) in the *nucleus olfacto-retinalis* (NOR) and the other in the *nucleus posterioris periventricularis* by 14 dph. The number of GnIH-ir neurons increased in both nuclei, whereas their size increased only in the NOR from hatchling to juvenile stages. These changes occurred from the

moment larvae started feeding exogenously and during development and differentiation of gonadal primordia. We showed by double-label immunofluorescence that only GnIH-ir neurons in the NOR co-expressed GnRH3 associated peptide. In addition, GnIH-ir fibre density increased in all brain regions from 5 dph. GnIH-ir fibres were also detected in the retina, optic tract and optic tectum, suggesting that GnIH acts as a neuromodulator of photoreception and the integration of different sensory modalities. Also, there were GnIH-ir fibres in the pituitary from 14 dph, which were in close association with somatotropes. Moreover, GnIH-ir fibres were observed in the *saccus vasculosus* from 30 dph, suggesting a potential role of GnIH in the modulation of its function. Finally, we found that *gnih* was expressed from 1 dph, and that the pattern of variation of its transcript levels was in accordance with that of cell number. Present results are the starting point for the study of new GnIH roles during development.

Introduction

The role of neuropeptides in adult life has been studied in depth but their function in ontogeny is not well established (1). Among peptides involved in reproduction, the importance of GnIH is increasingly recognised. This neuropeptide is a member of the RFamide peptide family, which includes kisspeptin and neuropeptide FF (NPFF), among others. Contrary to GnRH, GnIH has an inhibitory effect on gonadotropin synthesis and release in birds and mammals (for reviews, see 2-4), while its role in fish remains unclear (for reviews, see 5,6). Although GnIH was first characterised in this group in 2002 (7), knowledge of its role increased only recently. Most studies focused on the reproductive function of GnIH and reported both stimulatory and inhibitory effects of the hormone (7-12, for reviews, see 6). This dual effect has been proposed to depend on the action of sex-steroids and neuroestrogens and on the abundance of GnIH, GnRH and oestrogen receptors in the plasmatic membrane (13).

Little is known about spatial-temporal expression patterns and roles of GnIH during development (for reviews, see 1,14), since most published work focused on GnIH anatomical distribution and function in adult vertebrates (for reviews, see 2-6). Even though some studies have evaluated GnIH (gene: *gnih*) expression during embryonic stages in birds and mammals (15-17), the GnIH system has been examined to a greater extent throughout sexual development and the pre-pubertal period (18-25). To our knowledge, only three reports have examined GnIH during fish development. They demonstrated that GnIH was expressed from early developmental stages in the preoptic area and in different brain regions. Two of these studies reported the expression of GnIH mRNA during the first months of development in *Danio rerio* (12) and *Dicentrarchus labrax* (26), while the other analysed GnIH spatial-temporal expression pattern in *Labeo rohita* by immunohistochemistry (27).

The social cichlid fish *Cichlasoma dimerus* is an excellent model for studies in neuroendocrinology, the endocrinology of somatic growth, and behaviour (28-30). We have recently sequenced in our laboratory the GnIH precursor polypeptide, allowing us to identify three putative GnIH peptides. Also, we analysed the distribution of GnIH-immunoreactive somata and fibres in brain and pituitary gland of adult *C. dimerus*. Moreover, one of the putative GnIH peptides (cdLPQRFa-1) was shown to stimulate growth hormone (GH) and inhibite β-luteinizing hormone (β-LH) release *in vitro*, while the another (cdLPQRFa-2) was shown to stimulate only the release of β-follicle-stimulating hormone (β-FSH) into the culture medium (31).

The early development of *Cichlasoma dimerus* has been well characterised from embryonic to juvenile stages (32-33). Larvae hatch at 54-56 hours post-fertilization, but yolk-sac larvae initially remain attached to the substrate by adhesive glands. At 5 days post-hatching (dph), their mouth opens and larvae begin to swim freely and feed exogenously. The yolk-sac is completely reabsorbed around 11 dph. Paired gonadal primordia develop at 12 dph but remain undifferentiated until 38 dph (32). Ovarian histological differentiation occurs by 42 dph in females, as evidenced by the presence of primary oocytes, but spermatogonial proliferation is not observed in males until 72 dph

(33). Juvenile stage is reached by 45 dph, when body shape and pigmentation patterns become similar to those of the adult (32). The ontogeny of different neuropeptides and pituitary hormones has been also analised in *C. dimerus* (34-37).

Taking into account the importance of GnIH in endocrinology and the limited knowledge of its function during development, the aim of the current study was to evaluate the ontogeny of GnIH in *C. dimerus.*

Materials and methods

1. Animals

Reproductive adult *C. dimerus* of both sexes were captured in *Esteros del Riachuelo*, Corrientes, Argentina, and transferred to the laboratory, where they were acclimated to a constant temperature (25 ± 2°C) and photoperiod (14 L: 10 D) in 400 l freshwater aquaria prior to study. Fish were fed daily with commercial pellets (Kilomax Iniciador, #310; *Mixes del Sur, Provincia de Buenos Aires, Argentina*). Four females and 2 males were placed in 130 l community aquaria, and eggs were obtained from 8 spawnings of different fish pairs. Two days after fertilization, each offspring was isolated in an individual 15 l aquarium and maintained at the same temperature and photoperiod conditions as described above. Larvae of *C. dimerus* hatch at 54-56 h after fertilization (32). From that moment, larvae were sampled at different days post-hatching: day 0 (newly hatched), and 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 15, 17, 20, 23, 27, 30, 33, 37, 42, 52, 62, 75 and 85 dph. Five specimens were randomly sampled for each stage. Larvae were fed with newly hatched nauplii of *Artemia* sp when reaching the *free-swimming* stage (5-6 dph). After 12 dph, they were fed with ground commercial pellets Kilomax Iniciador (#703) (Mixes del sur) of increasing size and proportion until 18 dph, when commercial food completely replaced *Artemia* sp. Commercial pellets were processed with a mortar

and 3 different sieves in order to obtain 3 grain sizes. Larvae were fed with grains matching the size of their mouths (Fig. 1); hence, the size of grain fed was progressively increased as the animals grew. In all cases, larvae were fed 4 times a day throughout the study.

Animals were handled according to the Principles of Laboratory Animal Care (guidelines on the care and the use of fish in research, teaching and testing, Canadian Council on Animal Care, 2005), which were approved by the *Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina* (Protocol #95).

2. Immunohistochemical localisation of GnIH during ontogeny of *C. dimerus*

Larvae of *C. dimerus* were euthanised by hypothermic anaesthesia in ice-cold water and decapitation from 42 dph. All larvae were fixed in Bouin's solution for 18 h, as previously described by Pandolfi *et al*. (34-35). They were then embedded in paraplast (Fisherbrand, Fisher, Wash, USA) and cut in coronal or parasagittal sections at 7 or 10 µm intervals. Sections were mounted on gelatin-coated slides, deparaffinised in xylene and rehydrated through a graded ethanol gradient to phosphate-buffered saline (PBS, pH 7.4). Immunohistochemical procedures were similar to those previously described by Di Yorio *et al*. (31). Briefly, sections were incubated for 5 min in 0.3 % (v/v) hydrogen peroxidase to block endogenous peroxidase activity, then washed in PBS and incubated for 1 h with PBS containing 5 % (w/v) non-fat dry milk (blocking solution) at room temperature (RT). After washing with PBS, slides were incubated overnight with rabbit anti-GnIH antiserum (1:3000 in PBS, raised against bullfrog fGRP, in Prof. Tsutsui's laboratory) at 4 °C. Later, sections were washed in PBS and incubated for 1 h with biotinylated anti-rabbit IgG (1:500 in PBS, Sigma-Aldrich) at RT. Subsequently, they were incubated for 1 h with peroxidase-conjugated streptavidin (1:500 in PBS, Invitrogen, Carlsbad, CA, USA) at RT. The final reactive products were visualised with DAB Substrate Kit (Cell Marque™, CA, USA). Sections were gently counterstained with haematoxylin, mounted with

DPX, examined under a Zeiss Primo Star microscope coupled to a digital camera (Canon PowerShot A640) and photographed. The number of GnIH cells was counted and their diameter measured in coronal sections with an objective micrometre to evaluate changes in GnIH cell populations through development. As brains were sectioned at 7 µm intervals, only cells with a clearly visible nucleus were counted (39).

Specificity controls were performed by preadsorption of primary antiserum with an excess of either synthetic cd-LPQRFa-1 or cd-LPQRFa-2 (5 µg/500 µl) (Supplementary Figure S1), as previously described by Di Yorio *et al*. (31). An additional control was performed by omitting the primary antiserum (data not shown).

3. mRNA expression of *gnih* precursor polypeptide during ontogeny

Larval samples were taken from 0 dph to 27 dph in order to analyse *gnih* expression levels. Five larvae of *C. dimerus* were euthanised by hypothermic anaesthesia in ice-cold water (from 0 to 5 dph) or anesthetised in cold water and euthanised by decapitation (from 8 dph). Total larvae (up to 5 dph) or heads (in larvae from 8 to 27 dph) were homogenised in 400 µl of TRI-Reagent (MRC, Inc., Cincinnati, USA), and total RNA was obtained following the manufacturer's instructions. RNA quantification and purity were determined by NanoDrop ND-2000 (Thermo Scientific, Massachusetts, USA). Subsequently, 1 µg of total RNA was treated with DNAse I (Promega, Madison, Wi, USA) to avoid possible genomic DNA contamination. First-strand cDNA was synthesised using random hexamers (Invitrogen) and MMLV enzyme (Promega), as previously described by Delgadin *et al*. (30). GnIH and β-actin RNA transcript levels were quantified by fluorescent-based RT-qPCR using cDNA products diluted 1:10 in autoclaved distilled water. RT-qPCR was performed using FastStart Universal SyBR green Master (5 µl, ROCHE) with a mixture of forward and reverse specific primers designed from sequences previously obtained (1.5 µl; primers concentrations: 1 µM for GnIH and 0.5 µM for β-actin; Table 1), 2.5 µl of cDNA template and 1 µl of autoclaved distilled water per tube (29). The RT-qPCR protocol was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of

95 °C for 15 s, and 60 °C for 1 min. Each sample was run in duplicate and template omission served as negative controls. For each gene amplification, a single melting peak was obtained and efficiencies were close to 100%. *β-actin* gene was used as the reference gene, since its expression was stable among replication measures and showed minimum variation between days (data not shown). The starting concentration (N0) per sample was calculated by LinRegPCR as initial fluorescence in arbitrary units, which takes into account PCR efficiencies per sample and amplicon. N0 was used as the response variable (40-41).

4. Double-label immunofluorescence

Larvae at 27-30 dph were anesthetised and euthanised in ice cold water, and fixed in Bouin's solution as described above. Samples were then embedded in paraplast (Fisherbrand) and cut at the level of the pituitary, in parasagittal or coronal sections at 10 µm intervals. Sections were mounted on charged slides, deparaffinised in xylene, and rehydrated through a graded ethanol gradient to PBS.

4.1. GnIH-GnRH in the *nucleus olfacto retinalis* (NOR) and hypothalamus

After rehydration, sections were incubated for 1 h with PBS containing 5% non-fat dry milk (blocking solution) at RT. Next, they were incubated overnight with monoclonal LRH13 antiserum (1:1000 in PBS) or guinea pig anti-sea bass GnRH3 associated peptide (sGAP) antiserum (1:500 in PBS) at 4 °C. Subsequently, sections were washed in PBS and incubated with biotinylated anti-mouse IgG (1:200 in PBS, Invitrogen) or anti-guinea pig IgG (1:1000 in PBS; Vector laboratory, Burlingame, California, USA), respectively, for 1 h at RT. They were then incubated with streptavidin-Alexa 647-conjugated (1:150 in PBS, Invitrogen) (infrared) for 1 h at RT. Afterwards, sections were incubated overnight

with rabbit anti-GnIH antiserum (1:300 in PBS) at 4 °C. After washing slides in PBS, they were incubated for 1 h with an anti-rabbit IgG- Alexa 594-conjugated (1:150 in PBS, Invitrogen) (red) at RT.

4.2. GnIH-immunoreactive fibers and FSH- and GH-immunoreactive cells in the pituitary

For β-FSH immunodetection, sections were pretreated for epitope unmasking with citrate buffer 10 mM, pH 6, for 10 min at 90 °C, cooled at RT and washed in PBS (37). All sections were incubated with blocking solution for 1 h at RT. Next, they were incubated overnight with rabbit anti-GnIH antiserum (1:3000 in PBS) at 4 °C. Sections were then washed in PBS and incubated with biotinylated anti-rabbit IgG (1:500 in PBS, Sigma-Aldrich) for 1 h at RT. Later, they were incubated with streptavidin-Alexa 647-conjugated (1:150 in PBS, Invitrogen) (infrared) for 1 h at RT. Subsequently, sections were incubated overnight with rabbit anti-chum salmon GH antiserum (1:1000 in PBS, lot 8502) or anti-*Fundulus heteroclitus* β-FSH antiserum (1:500 in PBS) at RT. After washing slides in PBS, they were incubated with an anti-rabbit IgG- Alexa 594-conjugated (1:300 in PBS, Invitrogen) (red) for 1 h at RT.

Double-label immunofluorescence system with two primary antibodies raised in rabbit was performed because GnIH-ir fibres and pituitary immunoreactive-cells are clearly identifiable from each other. Specificity controls were previously carried out in our laboratory with two different approaches: preadsorption of primary antibody with an excess of its antigen (31,37,38,39) and western blot (38,39). It is important to mention that sGAP antiserum recognise *C. dimerus* sGAP and co-localise with GnRH3 expressing neurons in larvae and adults of this species (39). Finally, samples were mounted in PBS-glycerin (1:1) and examined under a confocal laser microscopy (Olympus FV-30 attached to an Olympus Bx61 microscope).

5. Statistical analysis

Variation in average cell number during ontogeny was evaluated using the non-parametric Friedman test, followed by Dunn's multiple comparison tests against 3 dph for the NOR or 14 dph for the *nucleus posterioris periventricularis* (NPP). Variation in cell diameter was analysed with repeated measures ANOVA, followed by Dunnett's multiple comparison tests against 3 dph for the NOR or 14 dph for the NPP. Normality, homoscedasticity and sphericity assumptions were tested prior to analysis. Statistical significance was established at $p<0.05$. Data are presented as mean ± standard error of the mean (SEM).

Results

GnIH distribution during ontogeny of C. dimerus

Immunohistochemistry showed the presence of GnIH-ir nuclei rostrally in the *nucleus olfacto-retinalis* (NOR) (Fig. 2.1) and in the *nucleus posterioris periventricularis* (NPP) of the hypothalamus at either side of the third ventricle (Fig. 2.2). No GnIH-ir fibres or GnIH-ir cells were detected when the primary antiserum was incubated with synthetic cd-LPQRFa-1 (Fig. S1). However, some cells and fibres stained weakly when the antibody was preadsorbed with synthetic cd-LPQRFa-2 (data not shown).

Nucleus olfacto-retinalis

GnIH-ir somata were detectable in this nucleus by 3 dph (Fig. 3 B). Their number progressively increased during ontogeny ($\chi^2_{(10)}= 29.60$; $p=0.001$) and was significantly different in larvae at 33 dph ($p=0.02$) and 85 dph ($p=0.006$), as compared to larvae at 3 dph (Fig. 3 B-E and F). There was a

significant increase in the average diameter of these cells from 14 dph, as compared to larvae at 3 dph ($F_{(10,20)}$= 13.51; p<0.0001) (Fig. 3 F).

Nucleus posterioris periventricularis

Weakly stained GnIH-ir somata were detected in this nucleus by 14 dph (Fig. 4 B). A gradual increase in the number of these neurons was observed as larvae developed ($\chi^2_{(7)}$= 19.11; p=0.008), with larvae at 42 dph (p=0.014) and 85 dph (p=0.006) showing significant differences compared to larvae at 14 dph (Fig. 4 B-E and F). The average diameter of GnIH-ir somata was unaltered through development (6.7 ± 0.1µm) ($F_{(7,14)}$=1.79; p=0.168; Fig. 4 F).

Fibres in the brain

Few GnIH-ir fibres were detected in the brain of larvae up to5 dph. Their density progressively increased along the anterior-posterior axis as larvae developed from that day (Fig. 3,4,5,6). A high density of fibres was observed at both sides of the third ventricle and in the *nucleus lateralis tuberis* (NLT) by 14 dph (Fig. 5). Subsequently, these regions contained the highest density of fibres in the brain. It is important to point out that mid- and posterior- brain regions of larvae at 85 dph contained higher number of fibres than the same regions in adult brains (data not shown).

Pituitary

Even though GnIH-ir fibres were detected from 14 dph to 85 dph (Fig. 5 B-D), they appeared to diminish in number from 37 dph. It is important to mention that fibre immunostaining in the pituitary was not as intense as that observed in the hypothalamus and other brain regions. No GnIH-ir cells were observed at the pituitary level.

Saccus vasculosus

This structure became morphologically distinguishable by 8 dph. GnIH-ir fibres were detected in the tractus of *sacci vasculosi* from 23 dph and in the *saccus vasculosus* itself from 30 dph (Fig. 5 E-G).

Retina-Optic Tract-Optic Tectum

Emerging GnIH-ir fibres were observed in the inner plexiform layer of the retina by 5 dph (Fig. 6 B), whereas an increase in fibre density was detected from 8 dph (Fig. 6 C-D). GnIH-ir fibres were also observed in the optic chiasm, optic nerve and optic tract from 8 dph (Fig. 6 E-G). Few GnIH-ir fibres were detected in the optic tectum at that day, but their density increased subsequently (Fig. 6 H-J). Taking into account a previous description of the optic tectum of *C. dimerus* (40), GnIH-ir fibres were located in the intermediate layers, between the *stratum marginale* and the *stratum periventriculare* (SPV), closer to the latter.

GnIH expression levels in ontogeny

GnIH mRNA was first detected at 1 dph, its expression increasing from 12 dph and reaching a peak at 20 dph (Fig. 7).

Double label- immunofluorescence

GnIH-GnRH in the NOR and the NPP

The pattern of expression of GnRH variants was examined during ontogeny in a previous study on *C. dimerus* (35). Based on the results reported in that study and GnIH localisation described in the present work, we decided to evaluate whether there was co-localisation of any GnRH variants with

GnIH in the NOR and NPP. We first used an antibody that recognises all GnRH variants (LRH-13), and found that only neurons in the NOR co-expressed both peptides (data not shown). Considering that GnRH3 is expressed in the NOR of *C. dimerus* (35,39), we next investigated potential co-localisation of GnRH3 and GnIH using an antibody that recognises the GnRH3 associated peptide (sGAP). We observed that sGAP and GnIH immunostaining co-localised in neurons of the NOR (Fig. 8 A-C) and in some fibres. Other fibres in the NOR and hypothalamus were labelled with only one of these peptides (Fig. 8 D-F).

GnIH-ir fibres and FSH-ir and GH-ir cells at pituitary level

Considering that GnIH-ir fibres were detected at the pituitary level and that GnIH regulates GH and gonadotropin release in adult *C. dimerus* (31), double-label immunofluorescence was performed to identify potential contacts between GnIH-ir fibres and GH or FSH cells. Double-label immunofluorescence between GnIH-ir fibres and ir-LH cells was not performed because these cells develop by 60 dph (37), by which time GnIH-ir fibres had diminished considerably. We detected few contacts between GnIH-ir fibres and GH-ir cells (Fig. 8 G-I) and no contacts between GnIH-ir fibres and FSH-ir cells (Fig. 8 J-L).

Discussion

Even though studies of RFamide peptides during vertebrate development are scarce, they show interesting results that reflect their differential functions in ontogeny and in adults (1). GnIH belongs to the RFamide family and its role in the regulation of reproduction, feeding and growth in adult vertebrates has been widely discussed, with similar and opposing results in birds, mammals and fish (2-6).

The antiserum used in the present study was raised against a bullfrog peptide, closely related to GnIH, and demonstrated to be specific in affinity purifications (7) and in immunoassay for diverse fish species, including *C. dimerus* (7, 9, 27, 31). We have previously observed in our laboratory that preadsorption of this antibody with an excess of cd-LPQRF-1, the putative *C. dimerus* GnIH, resulted in complete loss of GnIH staining, whereas preadsorption with cd-LPQRFa-2 led to the loss of most, but not all, GnIH immunostaining. These results indicate that the antibody can be used in *C. dimerus* to recognise putative GnIH. However, cross-reactions with an unknown peptide from the RF-amide family cannot be ruled out.

We identified two GnIH-ir cell clusters during early life stages, with different spatial and temporal expression patterns: GnIH-ir neurons in the anterior region of the brain were detected in the NOR by 3 dph, while those at the hypothalamic level were found in the NPP by 14 dph. The location of GnIH-ir neurons in the NOR was similar to that previously described in adults of *C. dimerus* (31) and other fish species (7, 8, 43, 44). However, Biswas *et al*. (27) reported the presence of GnIH-ir cells in the olfactory epithelium and bulb, which varied in immunostaining intensity during development, and observed no immunoreaction in adult *Labeo rohita*. Considering that the present study and that by Biswas *et al*. (27) used the same antiserum, it is possible that species-specific differences exist in the expression of GnIH in the NOR. In line with this, previous studies have shown great intra- and inter-specific variability in this brain region (45). On the other hand, we observed an increase in the number of GnIH-ir neurons in the NOR from 5 dph, reaching a maximum by 85 dph. This gradual increase began when larvae started to feed exogenously and continued during the development and differentiation of gonadal primordia. The diameter of GnIH-ir neurons underwent a remarkable change between 5 dph and 14 dph, just before the development of gonadal primordia.

Based on the localisation of GnIH-ir neurons, their morphology, and on previous studies of GnRH expressing neurons in our model species, we decided to examine whether GnIH-ir somata co-localised with any GnRH variant. To explore this possibility, we first used an antibody that recognises all GnRH variants and found that only the labelled-GnIH neuronal population in the NOR co-localised with immunoreactive-GnRH. As GnRH3 somata occur in this brain region (35,39), we performed double-label immunofluorescence with an antibody against sGAP, and found the co-expression of GnRH3 and GnIH in the NOR and in fibres localised in this nuclei and at the hypothalamic level. These results are supported by previous studies showing the presence of RFamide peptides in the NOR or *nervus terminalis*, where they co-localise with GnRH (45-46), although not completely in some species (45,47). Recently, Spicer *et al.* (11) demonstrated the lack of co-localisation of GnIH and GnRH3, and the absence of a GnIH-ir cell cluster in the *nervus teminalis* of *Danio rerio*, after using a specific antiserum raised against zebrafish-LPXRFa (11). On the contrary, another study on the same fish species reported the presence of a cluster of GnIH-ir cells in that region, after performing *in situ* hybridization (44). The opposing results of both studies may reflect the different methodologies employed. Taking into account that GnIH and GnRH3 co-localise in neurons of the NOR in *C. dimerus*, that GnRH3-ir was first detected at hatching while GnIH-ir was observed by 3 dph, and that GnRH3 migrates to the NOR after originating in the olfactory placode (35), we could speculate that GnIH is expressed once GnRH3-ir cluster settles in the NOR.

With respect to the NPP neuron cluster, it was first detected by 14 dph and was smaller than the NOR population. These neurons have been found in all fish species studied so far, including adult *C. dimerus* (for review, see 6). However, some species-specific differences may exist in their temporal detection, since Biswas *et al*. (27) showed that they occur from hatching in *L. rohita*. On the other hand, we observed a gradual increase in the number of cells from 14 dph, reaching a maximum by 85 dph, with no changes in their size. As in the NOR, the increment in cell number took place during the development and differentiation of gonadal primordia, suggesting a possible role of GnIH in the

modulation of those processes. Future studies of possible sexual differences in the number of cells in NOR and NPP during gonadal differentiation are planned.

Present results showed that NPP neurons did not co-express any of the GnRH variants, whereas some of the GnIH-ir fibres in the hypothalamus co-expressed GnRH3. This may indicate that hypothalamic fibres expressing only GnIH come from the NPP cluster, while those co-expressing GnIH and GnRH3 peptides belong to neurons in the NOR. Finally, the presence of GnIH-ir neurons in the periventricular region of the third ventricle of the hypothalamus may reflect a different developmental origin of this cell cluster as compared to that of the NOR cluster. This is in agreement with some studies suggesting the existence of two populations of RFamide neurons with different developmental origins, one population arising from the olfactory placode and the other originating from the third ventricle (1).

The presence of immunoreactive fibres at the pituitary level in larvae from 14 dph to 85 dph was an interesting finding, considering that no pituitary GnIH-ir fibres have been detected in adult *C. dimerus* (31). This result is in accordance with those obtained during the development of *Labeo rohita* (27) and in adults of *Carassius auratus* (7), *Oncorhynchus nerka* (9), *Oreochromis niloticus* (10,48), *Dicentrarchus labrax* (43) and *Solea senegalensis* (8). By contrast, Biswas *et al*. (27) found GnIH-ir cells at the pituitary level only during early developmental stages. The presence of GnIH-ir fibres in larvae of *C. dimerus* and their absence in adults indicate that the mode of action of GnIH on pituitary cells may differ between larvae/juveniles and adults of this species.

On the other hand, double-label immunofluorescence results showed for the first time the presence of GnIH-ir fibres in contact with somatotropes, an association already reported in adult *D. labrax* (43). In addition, we have shown here that GnIH fibres decreased in number at the pituitary level from 37 dph. It is important to mention that fibres immunoreactive for seabream-GnRH (the main

hypophysiotropic variant of GnRH in *C. dimerus*) were observed entering the posterior infundibular stalk and the neurohypophysis from 30 dph (35). These results suggest that a change in pituitary regulation could occur at the beginning of gonadal differentiation in *C. dimerus*, although further studies are required.

Interestingly, GnIH-ir fibres were detected in the tractus of *sacci vasculosi* from 23 dph and also in the *saccus vasculosus* (SV) from 30 dph. The presence of GnIH-ir fibres in the SV has been reported only in adult *D. labrax* (43); hence, this study shows for the first time the occurrence of GnIH-ir fibres during larval stages both in the tractus and the SV. Although the function of the SV remains unknown, recent findings suggest that it might be involved in the regulation of seasonal reproduction of *Oncorhynchus masou*, since it expressed *rodopsin* and *β-thyroid-stimulating hormone* and was also able to respond to changes in photoperiod (49). Taking into account these findings and those obtained in the present study, we propose a new function for GnIH in the regulation of a structure that could be involved in the control of reproduction. However, more extensive studies are needed to elucidate this point.

An increase in GnIH-ir fibre density was observed in the inner plexiform layer of the retina, optic tract and optic tectum from 5 dph. A high number of fibres in these brain regions was also detected during the ontogeny of *L. rohita* (27) and in adults of other fish species such as *D. labrax* (43), *O. niloticus* (48), *S. senegalensis* (8) and *D. rerio* (44). These results provide anatomical evidence supporting a potential role of GnIH as a neuromodulator of photoreception and integration of different sensory modalities, not only in adults but also throughout development. In line with this, GnIH expression levels have been shown to be modulated by photoperiod and melatonin in adults of *D. rerio* (50) and *Amphiprion melanopus* (51). Moreover, Paullada-Salmeron *et al*. (26) demonstrated that expression levels of GnIH and its receptor were altered by photoperiod during *D. labrax* development. The presence of GnIH-ir fibres in anterior brain regions, such as the olfactory bulb, and

in the visual system suggests that GnIH might integrate and modulate the olfactory-retinal system, as previously proposed for neurons in the NOR (52). This is an interesting issue to evaluate in future studies.

Finally, to complement immunohistochemical studies, we evaluated *gnih* mRNA expression during the stages of development prior to gonadal differentiation. *gnih* mRNA was first detected at 1 dph and its levels increased by 20 dph. This rise may be related to the increase in number of GnIH immunoreactive somata during development of gonadal primordia. In a previous study on *D. rerio*, Zhang *et al.* (12) first detected mRNA encoding *gnih* before hatching (prim-5 stage). Unfortunately, we were not able to establish whether *gnih* was expressed before hatching or whether it began to express at 1 dph, since we evaluated the expression of *gnih* precursor from hatching.

In conclusion, we report the first description of the GnIH system ontogeny in a cichlid fish, *C. dimerus*, showing spatial and temporal differences in the two brain nuclei previously described in adults. Moreover, both the number and size of neurons in both clusters was shown to change from hatchling to juvenile stages. These changes began when larvae started feeding exogenously and continued during development of gonadal primordia. They were in accordance with variations in mRNA levels, at least in the first stages of development. On the other hand, it was shown for the first time that GnIH-ir neurons in the NOR co-expressed the GnRH3 variant and that GnIH-ir fibres could directly modulate somatotrope function. In addition, GnIH-ir fibres were observed along the optic tract, opic tectum and retina, suggesting a possible role of GnIH as a neuromodulator of photoreception and the integration of different sensory modalities. Finally, the detection of GnIH innervation in the *saccus vasculosus* indicates that GnIH may modulate the function of this organ. Present results are the starting point for future experiments during sexual differentiation and the study of new functional roles of GnIH during development.

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References

1. Sandvik GK, Hodne K, Haug TM, Okubo K, Weltzien FA. Review: RFamide Peptides in Early Vertebrate Development. Front Endocrinol 2014; **5**: 203.

2. Tsutsui K. Review: How to contribute to the progress of neuroendocrinology: New insights from discovering novel neuropeptides and neurosteroids regulating pituitary and brain functions. Gen Comp Endocrinol 2016; **227**: 3-15.

3. Tsutsui K, Bentley GE, Bedecarrats GT, Osugi T, Ubuka T, Kriegsfeld LJ. Review: Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. Front Neuroendocrinol 2010; **31**: 284-295.

4. Tsutsui K, Ubuka T. Review: GnIH Control of Feeding and Reproductive Behaviors. Front Endocrinol (Lausanne) 2017; **7**: 170.

5. Ogawa S, Parhar IS. Review: Structural and functional divergence of gonadotropin-inhibitory hormone from jawless fish to mammals. Front Endocrinol (Lausanne) 2014; **5**: 177.

6. Muñoz-Cueto JA, Paullada-Salmerón JA, Aliaga-Guerrero M, Cowan ME, Parhar IS, Ubuka T. Review: A Journey through the Gonadotropin-Inhibitory Hormone System of Fish. Front Endocrinol (Lausanne). 2017; **8**: 285.

7. Sawada K, Ukena K, Satake H, Iwakoshi E, Minakata H, Tsutsui K. Novel fish hypothalamic neuropeptide. Eur J Biochem 2002; **269**(24): 6000-6008.

8. Aliaga-Guerrero M, Paullada-Salmerón JA, Piquer V, Mañanós EL, Muñoz-Cueto JA. Gonadotropin-inhibitory hormone in the flatfish, *Solea senegalensis*: Molecular cloning, brain localization and physiological effects. J Comp Neurol 2018; **526**(2): 349-370.

9. Amano M, Moriyama S, Iigo M, Kitamura S, Amiya N, Yamamori K, Ukena K, Tsutsui K. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. J Endocrinol 2006; **188**(3): 417-423.

10. Biran J, Golan M, Mizrahi N, Ogawa S, Parhar IS, Levavi-Sivan B. LPXRFa, the piscine ortholog of GnIH, and LPXRF receptor positively regulate gonadotropin secretion in Tilapia (*Oreochromis niloticus*). Endocrinology 2014; **155**(11): 4391-4401.

11. Spicer OS, Zmora N, Wong TT, Golan M, Levavi-Sivan B, Gothilf Y, Zohar Y. The gonadotropin-inhibitory hormone (Lpxrfa) system's regulation of reproduction in the brain-pituitary axis of the zebrafish (*Danio rerio*). Biol Reprod 2017; **96**(5): 1031-1042.

12. Zhang Y, Li S, Liu Y, Lu D, Chen H, Huang X, Liu X, Meng Z, Lin H, Cheng CH. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. Peptides 2010; **31**(6): 1034-1043.

13. Ubuka T, Parhar I. Dual Actions of Mammalian and Piscine Gonadotropin-Inhibitory Hormones, RFamide-Related Peptides and LPXRFamide Peptides, in the Hypothalamic-Pituitary-Gonadal Axis. Front Endocrinol (Lausanne) 2018; **8**: 377.

14. Poling MC, Kauffman AS. Review: Regulation and Function of RFRP-3 (GnIH) Neurons during Postnatal Development. Front Endocrinol (Lausanne) 2015; **6**: 150.

15. Ubuka T, Ueno M, Ukena K, Tsutsui K. Developmental changes in gonadotropin-inhibitory hormone in the Japanese quail (Coturnix japonica) hypothalamo-hypophysial system. J Endocrinol 2003; **178**(2): 311-318.

16. Yano T, Iijima N, Hinuma S, Tanaka M, Ibata Y. Developmental expression of RFamide-related peptides in the rat central nervous system. Brain Res Dev Brain Res 2004; **152**(2): 109-120.

17. Legagneux K, Bernard-Franchi G, Poncet F, La Roche A, Colard C, Fellmann D, Pralong F, Risold PY. Distribution and genesis of the RFRP-producing neurons in the rat brain: comparison with melanin-concentrating hormone- and hypocretin-containing neurons. Neuropeptides 2009; **43**(1): 13-19.

18. Ubuka T, Ukena K, Sharp PJ, Bentley GE, Tsutsui K. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. Endocrinol 2006; **147**(3): 1187-1194.

19. Johnson MA, Fraley GS. Rat RFRP-3 alters hypothalamic GHRH expression and growth hormone secretion but does not affect KiSS-1 gene expression or the onset of puberty in male rats. Neuroendocrinol 2008; **88**: 305-315.

20. Quennell JH, Rizwan MZ, Relf HL, Anderson GM. Developmental and steroidogenic effects on the gene expression of RFamide related peptides and their receptor in the rat brain and pituitary gland. J Neuroendocrinol 2010; **22**(4): 309-316.

21. Iwasa T, Matsuzakia T, Murakamia M, Kinouchia R, Osugi T, Gereltsetseg G, Yoshida S, Irahara M, Tsutsui K. Developmental changes in the mammalian gonadotropin-inhibitory hormone (GnIH) ortholog RFamide-related peptide (RFRP) and its cognate receptor GPR147 in the rat hypothalamus. International J Dev Neurosci 2012; **30**(1): 31-37.

22. Poling MC, Kim J, Dhamija S, Kauffman AS. Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene expression and RFamide receptors in the mouse hypothalamus. Endocrinol 2012; **153**(4): 1827-1840.

23. Zhao L, Zhong M, Xue HL, Ding JS, Wang S, Xu JH, Chen L, Xu LX. Effect of RFRP-3 on reproduction is sex- and developmental status-dependent in the striped hámster (Cricetulus barabensis). Gene 2014; **547**(2): 273-279.

24. Glanowska KM, Burger LL, Moenter SM. Development of gonadotropin-releasing hormone secretion and pituitary response. J Neurosci 2014; **34**(45): 15060-15069.

25. Kiyohara M, Son YL, Tsutsui K. Involvement of gonadotropin-inhibitory hormone in pubertal disorders induced by thyroid status. Sci Rep 2017; **7**(1): 1042.

26. Paullada-Salmerón JA, Loentgen GH, Cowan M, Aliaga-Guerrero M, Rendón-UncetaMD, Muñoz-Cueto JA. Developmental changes and day-night expression of the gonadotropin-inhibitory hormone system in the European Sea Bass: Effects of rearing temperature. Comp Biochem and Physiol A Mol Integr Physiol 2017; **206**: 54-62.

27. Biswas S, Jadhao AG, Pinelli C, Palande NV, Tsutsui K. GnIH and GnRH expressions in the central nervous system and pituitary of Indian major carp, *Labeo rohita* during ontogeny: An immunocytochemical study. Gen Comp Endocrinol 2015; **220**: 88-92.

28. Alonso F, Cánepa MM, Guimarães Moreira R, Pandolfi M. Social and reproductive physiology and behavior of the Neotropical cichlid fish *Cichlasoma dimerus* under laboratory conditions. Neotrop Ichthyol 2011; **9**(3): 559-570.

29. Pérez Sirkin DI, Suzuki H, Cánepa MM, Vissio PG. Orexin and neuropeptide Y: tissue specific expression and immunoreactivity in the hypothalamus and preoptic area of the cichlid fish *Cichlasoma dimerus*. Tissue Cell 2013; **45**(6): 452-459.

30. Delgadin TH, Pérez Sirkin DI, Di Yorio MP, Arranz SE, Vissio PG. GH, IGF-I and GH receptors mRNA expression in response to growth impairment following a food deprivation period in individually housed cichlid fish *Cichlasoma dimerus*. Fish Physiol Biochem 2015; **41**(1): 51-60.

31. Di Yorio MP, Perez Sirkin DI, Delgadin TH, Shimizu A, Tsutsui K, Somoza GM, Vissio PG. Gonadotrophin-Inhibitory Hormone in the Cichlid Fish *Cichlasoma dimerus*: Structure, Brain Distribution and Differential Effects on the Secretion of Gonadotrophins and Growth Hormone. J Neuroendocrinol 2016; **28**(5).

32. Meijide FJ, Guerrero GA. Embryonic and larval development of a substrate-brooding cichlid *Cichlasoma dimerus* (Heckel, 1840) under laboratory conditions. J Zool 2000; **252**(4): 481-493.

33. Meijide FJ, Lo Nostro FL, Guerrero GA. Gonadal Development and Sex Differentiation in the Cichlid Fish *Cichlasoma dimerus* (Teleostei, Perciformes): A Light- and Electron-Microscopic Study. J Morphol 2005; **264**(2): 191-210.

34. Pandolfi M, Paz DA, Maggese C, Ravaglia M, Vissio P. Ontogeny of immunoreactive somatolactin, prolactin and growth hormone secretory cells in the developing pituitary gland of *Cichlasoma dimerus* (Teleostei, Perciformes). Anat Embryol 2001; **203**(6): 461-468.

35. Pandolfi M, Parhar IS, Ravaglia MA, Meijide FJ, Maggese MC, Paz DA. Ontogeny and distribution of gonadotropin-releasing hormone (GnRH) neuronal systems in the brain of the cichlid fish *Cichlasoma dimerus*. Anato Embryol 2002; **205**(4): 271-281.

36. Pandolfi M, Cánepa MM, Ravaglia MA, Maggese MC, Paz DA, Vissio PG. Melanin-concentrating hormone system in the brain and skin of the cichlid fish *Cichlasoma dimerus*: anatomical

localization, ontogeny and distribution in comparison to alpha-melanocyte-stimulating hormone-expressing cells. Cell Tissue Res 2003; **311**(1): 61-69.

37. Pandolfi M, Lo Nostro FL, Shimizu A, Pozzi AG, Meijide FJ, Rey Vazquez G, Maggese MC. Identification of immunoreactive FSH and LH cells in the cichlid fish *Cichlasoma dimerus* during the ontogeny and sexual differentiation. Anato Embryol 2006; **211**(5): 355-365.

38. Pandolfi M, Paz DA, Maggese C, Meijide FJ, Vissio PG. Immunocytochemical localization of different cell types in the adenohypophysis of the cichlid fish Cichlasoma dimerus (Heckel, 1840). Biocell 2001; **25**(1): 35-42.

39. Pandolfi M, Muñoz Cueto JA, Lo Nostro FL, Downs JL, Paz DA, Maggese MC, Urbanski HF. GnRH systems of *Cichlasoma dimerus* (Perciformes, Cichlidae) revisited: a localization study with antibodies and riboprobes to GnRH-associated peptides. Cell Tissue Res 2005; **321**(2): 219-232.

40. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative realtime polymerase chain reaction (PCR) data. Neurosci Lett 2003; **339**(1): 62-66.

41. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research 2009; **37**(6): e45.

42. Vissio PG, Cánepa MM, Maggese MC. Brain-derived neurotrophic factor (BDNF)-like immunoreactivity localization in the retina and brain of Cichlasoma dimerus (Teleostei, Perciformes). Tissue Cell 2008; **40**(4): 261-270.

43. Paullada-Salmerón JA, Cowan M, Aliaga-Guerrero M, Gómez A, Zanuy S, Mañanos E, Muñoz-Cueto JA. LPXRFa peptide system in the European sea bass: A molecular and immunohistochemical approach. J Comp Neurol 2016a; **524**(1): 176-198.

44. Corchuelo S, Martinez ERM, Butzge AJ, Doretto LB, Ricci JMB, Valentin FN, Nakaghi LSO, Somoza GM, Nóbrega RH. Characterization of Gnrh/Gnih elements in the olfacto-retinal system and ovary during zebrafish ovarian maturation. Mol Cell Endocrinol 2017; **450**: 1-13.

45. D'Aniello B, Polese G, Luongo L, Scandurra A, Magliozzi L, Aria M, Pinelli C. Neuroanatomical relationships between FMRFamide-immunoreactive components of the nervus terminalis and the topology of olfactory bulbs in teleost fish. Cell Tissue Res 2016; **364**(1): 43-57.

46. Stell WK, Walker SE, Chohan KS, Ball AK. The goldfish nervus terminalis: a luteinizing hormonereleasing hormone and molluscan cardioexcitatory peptide immunoreactive olfacto retinal pathway. Proc Natl Acad of Sci 1984; **81**(3): 940-944.

47. Chiba A. Co-localization of gonadotropin-releasing hormone (GnRH)-, neuropeptide Y (NPY)-, and molluscan cardioexcitatory tetrapeptide (FMRFamide)-like immunoreactivities in the ganglion cells of the terminal nerve of the masu salmon. Fish Sci 1997; **63**(1): 153-154.

48. Ogawa S, Sivalingam M, Biran J, Golan M, Anthonysamy RS, Levavi-Sivan B, Parhar IS. Distribution of LPXRFa, a gonadotropin-inhibitory hormone ortholog peptide, and LPXRFa receptor in the brain and pituitary of the tilapia. J Comp Neurendocrinol 2016; **524**(14): 2753-2775.

49. Nakane Y, Ikegami K, Iigo M, Ono H, Takeda K, Takahashi D, Uesaka M, Kimijima M, Hashimoto R, Arai N, Suga T, Katsuya K, Abe T, Maeda R, Senga T, Amiya N, Azuma T, Amano M, Abe H, Yamamoto N, Yoshimura T. The saccus vasculosus of fish is a sensor of seasonal changes in day length. Nat Commun 2013; **4**: 2108.

50. Yumnamcha T, Khan ZA, Rajiv C, Devi SD, Mondal G, Sanjita Devi H, Bharali R,Chattoraj A. Interaction of melatonin and gonadotropin-inhibitory hormone on the zebrafish brain-pituitary reproductive axis. Mol Reprod Dev 2017; **84**(5): 389-400.

51. Choi YJ, Habibi HR, Kil GS, Jung MM, Choi CY. Effect of cortisol on gonadotropin inhibitory hormone (GnIH) in the cinnamon clownfish, Amphiprion melanopus. Biochem Biophys Res Commun 2017; **485**(2): 342-348.

52. Kawai T, Oka Y, Eisthen H. The role of the terminal nerve and GnRH in olfactory system neuromodulation. Zoolog Sci. 2009; **26**(10): 669-680.

Figure legends

Figure 1 Timeline representing the days in which larvae were sampled and important events related to feeding and sexual differentiation during ontogeny of *C. dimerus*. Coloured lines indicate periods of feeding on different diets. Overlapping lines correspond to adaptation periods to a change in feeding.

Figure 2 Localisation of GnIH-immunoreactive somata detected by immunohistochemistry in brain parasagittal sections of a 42 days post-hatching larva of *C. dimerus*. (A) Schematic parasagittal section of a larva brain. Scale bar: 200 µm. Boxed areas indicate the microphotograph parasagittal sections showing GnIH-immunoreactive somata (arrow) in the *nucleus olfacto-retinalis* (1) and in the *nucleus posterioris periventricularis* (2). GnIH-immunoreactive fibres are indicated by arrowheads. Scale bar: 20 µm.

Figure 3 Localisation of GnIH-immunoreactive somata detected by immunohistochemistry in coronal sections of the *nucleus olfacto-retinalis* (NOR) during development of *C. dimerus*. (A) Schematic parasagittal section of a larva brain. Line shows the position of the NOR coronal sections. Scale bar: 200 µm. Microphotograph section showing the GnIH-immunoreactive somata (arrow) and fibres (arrowheads) in larvae at 3 days post-hatching (dph) (B), 8 dph (C), 17 dph (D) and 85 dph (E). Scale bar: 20 µm. (F) Analysis of the variation in cell number (solid line) and cell diameter (dashed line) in the NOR during ontogeny. Results are presented as means ± SEM (n=3). * p=0.02; ** p=0.006, for

the comparison with cell number at 3 dph; $^{+++}$ p<0.0008; $^{++++}$ p<0.0001, for the comparison with cell diameter at 3 dph.

Figure 4 Localisation of GnIH-immunoreactive somata and fibres detected by immunohistochemistry in coronal sections of the *nucleus posterioris periventricularis* (NPP) during development of *C. dimerus*. (A) Schematic parasagittal section of a larva brain. Line shows the position of the NPP coronal sections. Scale bar: 200 µm. Microphotograph section showing the GnIH-immunoreactive somata (arrow) and some representative fibres (arrowheads) in larvae at 14 days post-hatching (dph) (B), 20 dph (C), 42 dph (D) and 85 dph (E). Third ventricle (3V). Scale bar: 20 µm. (F) Analysis of the variation in cell number (solid line) and cell diameter (striped line) in the NPP during ontogeny. Results are presented as means ± SEM (n=3). * p=0.014; ** p=0.006, for the comparison with cell number at 14 dph.

Figure 5 GnIH-immunoreactive fibres detected by immunohistochemistry in coronal sections of the pituitary and *saccus vasculosus* during development of *C. dimerus*. (A) Schematic parasagittal section of a larva brain. Lines show the position of the coronal sections of the pituitary (1) and *saccus vasculosus* (2). Scale bar: 200 µm. Microphotograph section showing the GnIH-immunoreactive fibres (asterisks) at the pituitary level in larvae at 14 days post-hatching (dph) (B), 33 dph (C) and 85 dph (D). Scale bar: 20 µm. GnIH-immunoreactive fibres at the hypothalamic level are indicated by arrowheads. Microphotograph section showing the GnIH-immunoreactive fibres (arrowheads) in the tract and the *saccus vasculosus* of larvae at 33 dph (E), 75 dph (F) and 85 dph (G). Scale bar: 20 µm. *Saccus vasculosus*: SV.

Figure 6 GnIH-immunoreactive (GnIH-ir) fibres detected by immunohistochemistry in coronal sections of the retina, optic chiasm-optic tract and optic tectum during development of *C. dimerus*. (A) Schematic parasagittal section of a larva. Lines show the position of the coronal sections of the retina (1), optic chiasm-optic tract- (2) and optic tectum (3). Scale bar: 200 µm. (1) *Camera lucida* drawing of a coronal section of a larva, and a boxed area indicating the microphotograph section showing GnIH-ir fibres (arrowheads) in the *retina* of larvae at 5 days post-hatching (dph) (B), 8 dph (C) and 14 dph (D). Scale bar: 100 µm and 20 µm, respectively. (2) *Camera lucida* drawing of a coronal section of a larva, and a boxed area indicating the microphotograph section showing GnIH-ir fibres (arrowheads) in the optic chiasm-optic tract of larvae at 8 dph (E), 12 dph (F) and 42 dph (G). Scale bar: 100 µm and 20 µm, respectively. (3) *Camera lucida* drawing of a coronal section of a larva, and a boxed area indicating the microphotograph section showing GnIH-ir fibres (arrowheads) in the optic tectum of larvae at 8 dph (H), 17 dph (I) and 37 dph (J). Scale bar: 100 µm and 20 µm, respectively.

Figure 7 Pattern of variation in mRNA expression of *gnih* precursor during the first 23 days post-hatching of larvae of *C. dimerus*. β-Actin was used as the reference gene. Values are expressed in arbitrary units (a.u.) as means ± SEM (n=3).

Figure 8 Double-label immunofluorescence in the *nucleus olfacto-retinalis* (NOR) (A-C), *nucleus posterioris periventricularis* (NPP) (D-F), and pituitary (G-L), in brain sections of 27-30 days post-hatching larvae. (A-C) Parasagittal sections of the NOR showing GnIH-immunoreactive (GnIH-ir) somata (green) (A), sGAP-ir somata (red) (B) and overlay of A and B (C). Scale bar: 10.0 µm. (D-F). Parasagittal sections of the NPP showing GnIH-ir somata and some representative fibres (green) (D), sGAP-ir somata (red) (E), and overlay of D and E (F). Scale bar: 20.0 µm. (G-I). (G-L) Coronal sections

at the pituitary level showing GnIH-ir fibres (green) (G and J), GH-ir cells (red) (H) and β-FSH-ir cells (red) (K). Overlay of G and H (I) and of J and K (L). Scale bar: 20.0 µm. Pit: pituitary. Immunoreactive somata or cells are indicated by arrows and fibres by arrowheads. Co-localisation is indicated by asterisks and contacts by open circles.

Supplementary Figure S1 Preadsorption test of anti-GnIH antisera with an excess of synthetic cd-LPQRFa-1 in immediately serial parasagittal sections of the brain of 27 days post-hatching larvae of *C. dimerus*. (A) Schematic parasagittal section of a larva brain. Scale bar: 200 µm. Boxed areas indicate the microphotograph parasagittal sections showing GnIH-immunoreactive (GnIH-ir) somata in the *nucleus olfacto-retinalis* (NOR) (1) and in the *nucleus posterioris periventricularis* (NPP) (2). (B) Microphotograph section showing GnIH-ir somata (arrow) in the NOR and the corresponding preadsorption test of anti-GnIH antisera with an excess of synthetic cd-LPQRFa-1 (C). (D) Microphotograph section showing GnIH-ir somata (arrow) and fibres (arrowhead) in the NPP and the corresponding preadsorption test of anti-GnIH antisera with an excess of synthetic cd-LPQRFa-1 (E). Scale bar: 20 µm.

Table 1

Primers used for mRNA expression by real time PCR.

Name	forward primer (5'→3')	reverse primer (5'→3')	GeneBank Accesion N°
GnIH	AGCCACCCAACCATTATCAG	AGATGAGTTCGGTGTCCTTTC	Di Yorio *et al*. 2016 (30)
β-actin	GCTGTCCCTGTACGCCTCTGG	GCTCGGCTGTGGTGGTGAAGC	EU158257