Available online at www.sciencedirect.com
ScienceDirect
General and Comparative Endocrinology 156 (2008) 312–322
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Appearances and chronological changes of mummichog *Fundulus heteroclitus* FSH cells and LH cells during ontogeny, sexual differentiation, and gonadal development

Akio Shimizu [a,*], Masami Hamaguchi [b], Haruka Ito [a], Makoto Ohkubo [a], Miho Udagawa [a], Kazunori Fujii [b], Tohru Kobayashi [c,1], Masaru Nakamura [d]

[a] *National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Kanazawa, Yokohama 236-8648, Japan*
[b] *National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan*
[c] *Inland Station, National Research Institute of Aquaculture, Fisheries Research Agency, Hiruta, Tamaki, Mie 519-0423, Japan*
[d] *Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Motobu, Okinawa 905-0227, Japan*

Received 29 October 2007; revised 20 January 2008; accepted 23 January 2008
Available online 10 February 2008

Abstract

To examine relationships between gonadal stages and the initial appearance and subsequent development of gonadotrophs, hatched larvae of the mummichog *Fundulus heteroclitus* were reared until first maturity under suitable conditions for maturation (20 °C–16L). Evident FSH cells generally appeared 1–2 weeks after hatching (wah), around or slightly before the morphological sex differentiation which occurred at 2 wah. During this period, 3β-hydroxysteroid dehydrogenase positive cells also appeared in the gonads. While FSH cells existed throughout the early phases of gonadal development such as cortical alveoli formation and basic spermatogenesis, LH cells appeared later (6–12 wah), after the commencement of the early gonadal development. Both FSH cells and LH cells were abundant at 36 wah when the fish had attained full maturity. These results indicate the possibility that FSH is responsible for gonadal differentiation by inducing steroidogenesis in the gonads, implying the importance of FSH on the early phases of gonadal development. These results also suggest cooperation of FSH and LH in later phases of gonadal development such as yolk globule accumulation and active spermatogenesis. The mode of changes in the abundances of the gonadotrophs according to the gonadal development was somewhat different from previously observed changes during the annual reproductive cycle in adult mummichog. Possible complementary roles of the two GTHs in vitellogenesis and spermatogenesis may be involved in the difference by providing flexibility to the controlling mechanism.

Keywords: Gonadotroph; FSH; LH; Pituitary; Ontogeny; Sexual differentiation; Gonadal development; Steroidogenic enzyme

1. Introduction

The mummichog *Fundulus heteroclitus* is a multiple spawning cyprinodont fish native to North America. This fish has been used as a convenient experimental model for studies in general physiology. *F. heteroclitus* is also a suitable experimental fish model for studying reproductive physiology because of its appropriate size and ease to reproduce in the laboratory (Shimizu, 2003). Especially, this fish has become important for research on gonadotropins (GTHs) since Lin et al. (1992) reported cDNA sequences encoding the follicle stimulating hormone (FSH; formerly GTH I) β subunit and the luteinizing hormone (LH; formerly GTH II) β subunit. Specific antisera for immunochemical and/or immunocytochemical identifications of both FSH and LH have been obtained (Calman et al., 2001; Shimizu et al., 2003b), and for the first time for

* Corresponding author. Fax: +81 45 788 5001.
E-mail address: aneko@affrc.go.jp (A. Shimizu).
[1] Present address: Laboratory of Reproductive Biology, Faculty of Agriculture, Ehime University, Ehime 790-8566, Japan.

0016-6480/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2008.01.022

small experimental fishes, the two distinct GTHs have been successfully purified from the pituitary (Shimizu and Yamashita, 2002).

Until now, the specific roles of the two GTHs are not well understood in multiple spawning fishes mainly because of the previous lack of appropriate experimental fish models. Especially, the functions and the expression profiles of FSH in non-salmonid fishes are important but as yet remain unclarified. This is because details of the FSH function have been mainly obtained from salmonid species which are single or annual spawners (i.e. spawn once in the lifetime or once a year, respectively). In non-salmonid fishes, biological activities of the two different GTHs have been examined almost only in the red seabream *Pagrus major* (Tanaka et al., 1993; Kagawa et al., 1998b, 2003) and the carp *Cyprinus carpio* (Van Der Kraak et al., 1992), and the immunoassay system for measuring plasma levels of FSH has not been developed yet. The timing of the initial appearance of the two GTH cells has been studied only in a small number of species (Magliulo-Cepriano et al., 1994; Miranda et al., 2001; García-Ayala et al., 2003; Pandolfi et al., 2006), and the roles of the two types of GTHs during sex differentiation and early gonadal development are still unclear. Until now a convenient experimental fish model that can be used for studying the multiple functions of GTHs throughout the life cycle (i.e. during ontogeny, gonadal differentiation, gonadal development and maturation, and annual and short reproductive cycles) has been lacking. We consider that the use of mummichog as an experimental model can overcome this problem.

This study aims to clarify the chronological changes of the two GTH cells, and to contribute to the basic data for understanding the functions of the two different GTHs in multiple spawning teleosts. We have previously reported (Shimizu et al., 2003b) the changes during the annual reproductive cycle in adult mummichog, and in this study we report the changes in early stages (i.e. from hatching to first maturity).

2. Materials and methods

2.1. Fish

Fertilized eggs of the Arasaki strain (Shimizu, 1997) mummichog were kept under running sea water. Temperature and photoperiod were controlled at 20 °C and 16L–8D, respectively, (suitable conditions for maturation; Shimizu, 2003). Just hatched larvae were reared under the same conditions by feeding *Artemia* naupli (for the first 2 weeks) and then commercial food (fine pellets) for juvenile marine fishes (C-400 and C-700, Kyowa Hakko Kogyo). Samplings were undertaken just after and 1, 2, 3, 4, 6, 8, 12, 16, 24, and 36 weeks after hatching (wah). Fish were killed by deep anesthesia with tricaine methanesulfonate, and the whole body (cranial and abdominal cavities opened in individuals >2 wah) was fixed with Bouin's solution for 3 days. The samples were preserved in 70% ethanol until the examination.

2.2. General histological procedure

Pituitaries (with the head region in individuals <8 wah) and gonads (with the body in individuals <8 wah) were dehydrated with ethanol series and embedded with Paraplast Plus (Sigma Chemical). Sagittal sections of 5 μm thickness were obtained from the pituitary sample and used for immunocytochemistry. Transverse sections of 5 or 10 (for matured ovaries) μm thickness were obtained from the gonad samples. These were deparaffined and stained with Mayer's hematoxylin combined with periodic acid-Schiff (PAS) or eosin staining, and were used for microscopic examinations. Some of the gonadal sections (0–2 wah) were used for evaluating steroidogenic activity by immunocytochemical identification of a steroidogenesis related enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD). Some of the ovarian sections (8–36 wah) were used for immunocytochemical identification of vitellogenin and its processing product, lipovitellin.

2.3. Polyclonal antibodies

Rabbit antiserum (identification number 616; Shimizu and Yamashita, 2002) against the synthetic peptide (corresponding to mummichog (Fh) GTHα 65–85; Limesand et al., 1995) and rabbit antiserum (identification number 299; Shimizu and Yamashita, 2002) against the synthetic peptide (corresponding to Fh LHβ 91–105; Lin et al., 1992) were used as the primary antibodies for immunocytochemical identification of GTHα and LHβ, respectively. The specificities of these antisera have been previously confirmed (Shimizu and Yamashita, 2002; Shimizu et al., 2003b).

Rabbit antiserum against C-terminal sequences of the rainbow trout *Oncorhynchus mykiss* 3β-HSD (Kobayashi et al., 1996, 1998) was used for immunocytochemical identification of steroidogenic cells in the gonads. The specificity of the anti-3β-HSD was confirmed by using adsorbed antisera by means of adding an excessive amount of the antigen (the synthetic peptide). The immunoreactivities almost disappeared by such antigen additions.

Rabbit antiserum against purified mummichog vitellogenin (Kakuno et al., 2001) was used for immunocytochemical identification of vitellogenin and lipovitellin. The specificity of the anti-vitellogenin has been confirmed previously (Kakuno et al., 2001).

The sera were diluted with EDTA-gel-PBS (10 mM phosphate buffered saline containing 0.1% gelatin and 50 mM EDTA, pH 7.5) and were used for preparing the working solutions of the primary antibody.

2.4. Monoclonal antibody

To minimize the interaction between the staining systems in the double or the triple staining, mouse monoclonal antibody was used for FSH staining. GTHs mixture (mixture of FSH and LH) was obtained from mummichog pituitary glycoprotein by a combination method of gel-filtration and anion exchange chromatography (Shimizu and Yamashita, 2002) and used for immunization. All the following procedures adhered to the guidelines of animal experiments set by the Fisheries Research Agency. BALB/c mice of 4 weeks age were intraperitoneally immunized with the GTHs mixture in the presence of Freund's complete (first injection) or incomplete (second injection) adjuvant (Sigma Chemical). Two days before the fusion, the mice were intravenously boosted with the GTHs mixture. The mice were anesthetized and killed by cervical dislocation, and their spleens were removed. Cell suspension of the splenocytes were prepared in Dulbecco's modified Eagle's media (MEM: Gibco), and the cells were fused with mouse myeloma cells (SP-2) in the presence of polyethylene glycol MW1500 (Boehringer-Mannheim). The fused cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 15% fetal calf serum (FCS), 1 U/ml of interleukin-6, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and HAT supplement (Gibco), and were cultured at 37 °C in a humid atmosphere, and 7% v/v CO_2 air. After 10 days, the supernatant was obtained from each well, and FSH positive LH negative clones were selected via enzyme-linked immunosorbent assays using purified mummichog FSH and LH (Shimizu and Yamashita, 2002) as antigens. Hybridomas from FSH positive LH negative wells were individually picked up and plated out in individual wells in IMDM containing HT supplement (Gibco). Finally, individual hybridomas were cloned twice by limiting dilution, and a specific clone to FSH was obtained and cultured in the FCS-IMDM for 2 weeks. The superna-