

Preparation and Use of Specific Antibodies to the β -I and β -II Subunits of Gonadotropic Hormone from *Fundulus heteroclitus* Pituitary

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Fundulus heteroclitus naturally spawns with a semilunar periodicity throughout most of the year in its southernmost habitat, an activity that can be maintained in the laboratory. The α and two β subunits comprising *F. heteroclitus* gonadotropic hormones (GtHs) I and II have been sequenced, and antibodies have been raised against unique peptides found in each of the two β subunits. On immunoblots of pituitary proteins, each antibody recognizes a single band with a molecular mass of 16–17 kDa, somewhat larger than the deduced sizes (11–13 kDa) of the unglycosylated subunits. Each antibody also recognizes a different subset of pituitary cells in the central (GtH I) and peripheral (GtH II) proximal pars distalis, regions that display the typical tinctorial properties of gonadotropins. The distribution and distinct separation of cells containing GtH β subunits I and II thus differ from those found for previously described teleost species, most of which are salmonids that engage in a single spawning episode during the year. The availability of these antibodies thus makes *F. heteroclitus* an inexpensive, easily manipulated model system for studies on the hormonal regulation of fractional spawning common to a large class of commercially important species other than salmonids. © 2001 Academic Press

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cytochemistry; killifish, mummichog (*Fundulus heteroclitus*); teleost.

Mammalian reproductive cycles are largely controlled by two gonadotropic hormones (GtHs) released from the pituitary gland: follicle-stimulating hormone (FSH) and leutenizing hormone (LH). FSH acts during the follicular phase of the reproductive cycle to stimulate the growth and development of the ovarian follicle(s), whereas LH acts to induce ovulation and to support the development of the corpus luteum during the luteal phase (Carr, 1993). The mammalian pituitary glycoprotein hormones LH, FSH, and thyroid-stimulating hormone (TSH) are heterodimers and share a common α subunit. Each hormone has a distinct β subunit that combines noncovalently with an α subunit to form the functional hormone.

The killifish or mummichog, *Fundulus heteroclitus*, is an intertidal teleost that has a semilunar reproductive cycle (Hsiao *et al.*, 1994; reviewed in Taylor, 1990). Recently, this cycle has been proposed as a model for the mammalian follicular phase since it shares many attributes with the mammalian follicular phase including rapid cycling, oocyte recruitment, oocyte maturation, and ovulation (Hsiao *et al.*, 1996).

The duality of the GtHs in teleosts has been demonstrated in several species including chum salmon (Suzuki *et al.*, 1988), coho salmon (Swanson *et al.*,

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1991), killifish (Lin *et al.*, 1992), common carp (Van Der Kraak *et al.*, 1992), Atlantic croaker (Copeland and Thomas, 1993), tuna (Okada *et al.*, 1994), bonito (Koide *et al.*, 1993), red seabream (Tanaka *et al.*, 1993), striped bass (Hassin *et al.*, 1995), yellowfin porgy (Tsai and Yang, 1995), goldfish (Yoshiura *et al.*, 1997), and Japanese eel (Yoshiura *et al.*, 1999).

The functional duality of the teleost GtHs is not yet fully understood. Many of the species in which GtHs have been intensively studied are salmonids and other annual spawners. Other species such as tuna and seabream are fractional spawners, but their large size and relatively long life render experimental work on their reproductive biology difficult and costly. Fractional spawners include many species of commercial importance such as flounder, red drum, croaker, seatrout, and halibut. Many of these species are now experiencing large population declines due to overfishing, habitat destruction, or both, and little is known about their reproductive biology. The killifish is also a fractional spawner and can act as an inexpensive, easily manipulated model system for this large class of commercially important species.

Under natural conditions, killifish spawn from early spring to late summer in the intertidal zone (Taylor, 1990). Spawning exhibits a semilunar rhythm with the spawning peaks roughly corresponding to the high tides at the full and new moons. In the laboratory, killifish exhibit the same pattern of semilunar spawning peaks and can be induced to spawn at any time of the year (Hsiao and Meier, 1989; Hsiao *et al.*, 1994).

The reproductive cycles of two related killifish species (*F. heteroclitus* and *F. grandis*) have been extensively studied both in the wild and in the laboratory (Hsiao and Meier, 1989; Taylor, 1990; Hsiao *et al.*, 1994, 1996; Cerdá *et al.*, 1996). The development and structure of the ovaries and the control of maturation of the oocytes have been described in detail (Wallace and Selman, 1978, 1980; Brummett *et al.*, 1982; Greeley *et al.*, 1986; Selman and Wallace, 1986, 1989; Lin *et al.*, 1987, 1989; Kanungo *et al.*, 1990). The two β subunits of the GtHs from *F. heteroclitus* have been cloned and sequenced from a cDNA library (Lin *et al.*, 1992). The α subunit, shared by the two GtHs and TSH, has also been sequenced from cDNA (Limesand *et al.*, 1995).

The purpose of the present investigation was to produce specific antibodies to the two β subunits of *F. heteroclitus* GtH. These antibodies are needed for sev-

eral reasons. Previously available antibodies (Farbridge *et al.*, 1990; Ikeuchi *et al.*, 1995; Suzuki *et al.*, 1988) were not made to closely related fish and are unsuitable for use with *F. heteroclitus*. Specific antibodies to killifish GtHs are needed to develop radioimmunoassays to measure the changes in plasma content of β -I and β -II over the semilunar spawning cycle. Little work has been done on the pituitary control of the reproductive cycle in *F. heteroclitus*, and available antibodies would definitively identify the pituitary gonadotrops and help determine whether the same or separate cells make β -I and β -II. One would also be able to determine how the pituitary content of GtH relates to the ovarian cycle and to investigate the ontogeny of the gonadotrops in relationship to the development of the gonads. Such studies have not been done on any fish with an asynchronous ovary.

METHODS

Animals

F. heteroclitus were collected from marshes in the vicinity of St. Augustine, Florida, maintained in the laboratory in a flow-through seawater system at a temperature of 22–28° under controlled lighting of L:D 12:12 h (Lin *et al.*, 1989), and fed tropical fish flake food (Wardley Laboratories, Secaucus, NJ) once daily until satiation. Fish were anesthetized in 100 ppm tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) before being killed.

Peptide Selection and Synthesis and Antibody Preparation

Unique peptide sequences were chosen from the conceptual translations of the *F. heteroclitus* GtH β subunit sequences (Lin *et al.*, 1992) representing amino acids 85–95 of GtH β -I (CTRLYAHIPSC) and amino acids 107–115 of GtH β -II (CMNDIPFYH). The peptides were chosen to be hormone specific and species specific. These peptides were synthesized and the antibody production carried out by Immuno-Dynamics, Inc., La Jolla, California. Briefly, the peptides were synthesized, purified, desalted over C4 columns, and then conjugated to keyhole limpet hemocyanin (KLH).

Rabbits were initially injected intradermally with 2.5 mg of the peptide-KLH conjugate in complete Freund's adjuvant and then boosted at 3 and 6 weeks with 2.5 mg of the peptide-KLH conjugate in incomplete Freund's adjuvant injected intramuscularly. Test bleeds were taken at 5, 7, and 9 weeks and tested in an enzyme-linked immunosorbent assay against the synthetic peptides. Sera from test bleeds were also used to stain immunoblots of *F. heteroclitus* pituitary proteins. Antibodies were purified from crude antisera on affinity columns constructed with the synthetic peptides conjugated to a thio-Sephadex matrix.

Immunoblots of *F. heteroclitus* Pituitary Proteins

Anesthetized fish were sacrificed by cervical transection and the pituitaries were rapidly dissected. Isolated pituitaries were homogenized within 2 min in a denaturing electrophoresis sample buffer consisting of 2% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, in 62 mM Tris-HCl buffer, pH 6.8. Samples were prepared for electrophoresis by sonication in a bath sonicator (Heat Systems Ultrasonics, Inc., Farmington, NY). Clarified pituitary homogenates were electrophoresed on 12% acrylamide SDS-PAGE gels by the method of Laemmli (1970). After electrophoresis, protein was transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore Corp., Bedford, MA) by the method of Pluskal *et al.* (1986).

Blotted membranes were wet with methanol, washed 2×5 min in TBST (150 mM NaCl, 0.1% Tween 20, 0.1 M Tris-HCl, pH 7.6), blocked for 3 h in 5% bovine serum albumin (BSA) in TBST, and then incubated overnight at 4° in antiserum diluted 1:100 in 5% BSA in TBST. Blots were washed 3×10 min in TBST at room temperature and then incubated 60 min in alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000 in TBST. After being washed 3×10 min in TBST, the blots were rinsed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and then developed in BCIP/NBT substrate (0.0167% 5-bromo-4-chloro-3-indolyl phosphate, 0.0333% nitroblue tetrazolium) in alkaline phosphatase buffer. Control and experimental blot lanes were developed for the same length of time before development was stopped by being rinsed with distilled water.

Immunocytochemistry

Animals were anesthetized in 100 ppm MS-222 and then sacrificed by cervical transection. Brains were rapidly dissected and immersed in Zamboni's fixative (Stefanini *et al.*, 1961) overnight at 4°. Tissue was then transferred to 15% sucrose in phosphate buffer (PB; 0.1 M phosphate buffer, pH 7.4) and then to 30% sucrose in PB until sinking. Frozen sections were cut at a thickness of 14 μ m, collected on chrome-alum gelatin-coated slides, and air-dried. Sections were washed 3×10 min in TBS (50 mM Tris-HCl, pH 7.6, with 0.85% NaCl) and then in 0.4% Triton-X 100 in TBS for 15 min. Following 3×5 min washes in TBS, sections were blocked in 10% normal goat serum (NGS) in TBS for 60 min. Sections were incubated overnight in humid chambers at 4° with anti- β -I or anti- β -II diluted 1:100 in 10% NGS, 0.1% Triton X-100 in TBS. Sections were then washed 3×10 min in TBS and incubated with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:50 in 10% NGS, 0.1% Triton X-100 in TBS, for 60 min at room temperature. Following 3×10 min washes in TBS, slides were coverslipped with Gel/Mount (Biomedica Corp, Foster City, CA). Slides were observed and photographed on a Leitz DMRD microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) with epifluorescence optics.

Glenner-Lillie Stain for Pituitary

Tissue was fixed in Zamboni's fixative and processed, and frozen sections were cut as above (for immunocytochemistry) and air-dried. Sections were then cleared through an ethanol and xylene series, rehydrated, and stained for 60 min at 60° in a closed Coplin jar in Glenner-Lillie staining solution. The staining solution consisted of 0.2% Eosin B, 0.05% Aniline blue, 2.75 mM citric acid, and 4.5 mM disodium phosphate in aqueous solution. Sections were then dehydrated through an acetone and xylene series and coverslipped with PerMount (Fisher Scientific, Fair Lawn, NJ).

RESULTS

Immunoblot Analysis of Pituitary Proteins

On immunoblots of electrophoresed *F. heteroclitus* pituitary tissue proteins, each antibody recognized

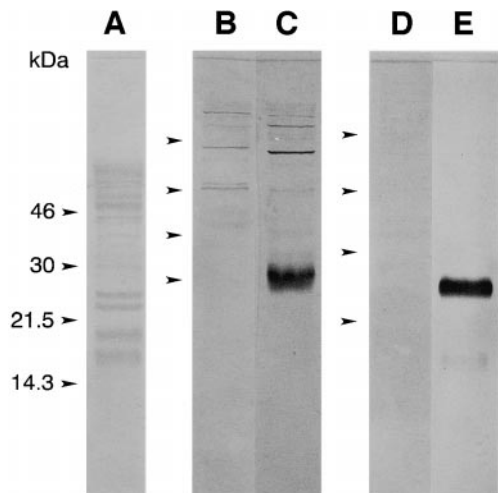


FIG. 1. Immunoblots of pituitary proteins. (A) Coomassie blue staining of pituitary proteins together with positions of markers for molecular mass (kDa) indicated by arrows on the left, as determined on a separate lane from the same blot. On separate immunoblots, lanes were stained without (B) or with (C) diluted GtH I antiserum and without (D) or with (E) diluted GtH II antiserum, followed by secondary antibody in each case. The corresponding positions of the respective molecular mass markers run simultaneously are indicated to the left of immunoblots B,C and D,E.

one band at a position somewhat above (ca. 16–17 kDa) that expected for the molecular masses of the GtH β -I or β -II subunits predicted from the conceptual translation of the nucleotide sequence (10.7 kDa for β -I and 12.9 kDa for β -II) (Fig. 1). The larger apparent sizes are presumably a consequence of subunit glycosylation. Immunoreactivity was eliminated by omission of the primary antibody or by substitution of preimmune serum from the same rabbit for the primary antibody.

Immunocytochemical Staining of Pituitary Tissue

In parasagittal sections of adult brains, the antibody to GtH β -I subunit recognized a subset of the cell bodies in the central (dorsal) part of the proximal pars distalis of the pituitary (Fig. 2A). In adjacent sections, the antibody to the β -II subunit recognized the cell bodies of the peripheral (ventral) proximal pars distalis of the pituitary (Fig. 2B). There was no apparent overlap of the regions containing cell bodies recognized by the two antibodies. Immunoreactivity was eliminated by omission of the primary antibody or by

substitution of the preimmune serum for the primary antibody.

Correlation with Tinctorial Properties of the Pituitary Cells

The antibodies to GtH β -I and β -II recognized subsets of the pituitary basophilic cells stained by the Glenner–Lillie stain (data not shown). The cell bodies stained blue by Glenner–Lillie are putative gonadotrops and thyrotrops and are distributed in the proximal pars distalis and along the rim of the pars intermedia that is made up of projections of tissue from the proximal pars distalis.

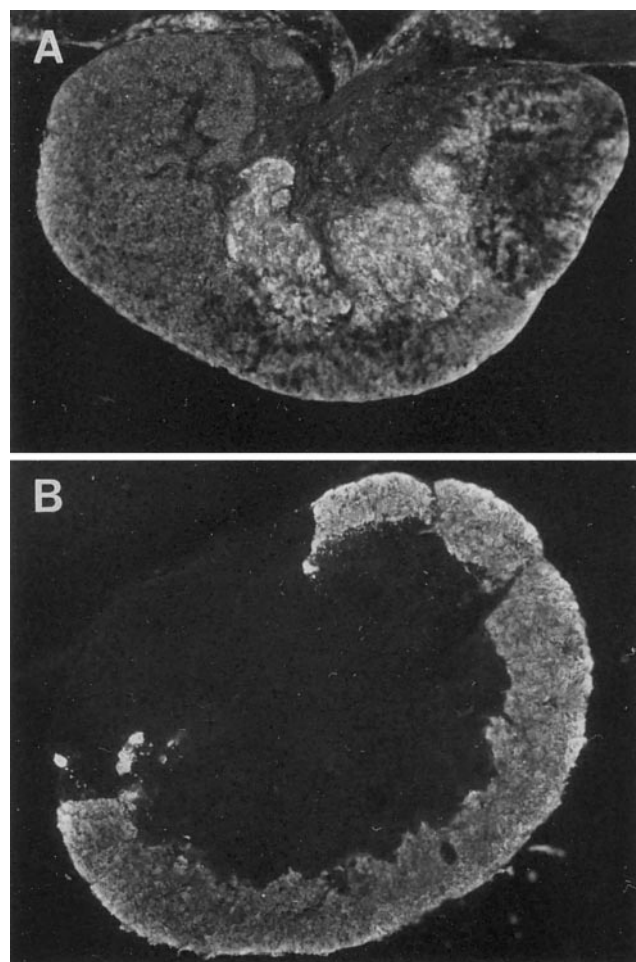


FIG. 2. Immunocytochemistry of adjacent parasagittal pituitary sections stained with diluted antiserum against GtH I (A) or GtH II (B), followed by secondary antibody conjugated with FITC. Approx. 100 \times .

DISCUSSION

The gross anatomy of the *F. heteroclitus* pituitary was first provided by Matthews (1936), and the general cytology was described by Matthews (1936), Sokol (1961), and Emmart *et al.* (1966). The morphology of the gland and the distribution of tinctorially defined cell types appear to agree with the general teleost pattern (Ball and Baker, 1969; Van Oordt and Peute, 1983; Yan and Thomas, 1991). Presumptive gonadotrops were first identified in the proximal pars distalis of the *F. heteroclitus* pituitary through their basophilic properties and by analogy to other species (Matthews, 1936; Sokol, 1961). The results of our Glenner–Lillie staining indicated the same general distribution of basophils, acidophils, and chromophobes as seen with the Gomori aldehyde–fuchsin stain used by Sokol (1961). The basophilic presumptive thyrotrops and gonadotrops were stained clear blue with the Glenner–Lillie stain, similar to aldehyde–fuchsin, and were distributed throughout the proximal pars distalis, including the fingers of the proximal pars distalis that project along the rim of the pars intermedia. Cords of acidophilic presumptive somatotrops were found in the ventral portion of the proximal pars distalis.

A few previous studies have used immunocytochemistry with heterologous antibodies to localize hormones in the *F. heteroclitus* pituitary. Emmart *et al.* (1966) employed an antibody to ovine prolactin and demonstrated immunoreactivity in the anterior (rostral pars distalis) of the pituitary. García-García *et al.* (1994) used antisera to human LH and human chorionic gonadotropin (hCG) on pituitary sections and found that immunoreactivity with both antisera was confined to the ventral rim of the proximal pars distalis, with the exception of some crossreactivity of the anti-hCG with apparent thyrotrops. This latter result may have been due to this polyclonal antibody recognizing the α subunit common to hCG, GtH, and TSH.

In this study we describe antibodies that recognize *F. heteroclitus* GtH β -I and β -II, both on immunoblots under denaturing conditions and in tissue sections, thus demonstrating the sensitivity and specificity of the antibodies. Each antibody to β -I and β -II recognizes a single 16- to 17-kDa protein (Fig. 1) and each stains distinctly different, nonoverlapping cell regions in the proximal pars distalis of the pituitary (Fig. 2).

Localization in separate groups of cells indicates that each of the two GtHs could be released selectively and supports the duality of GtH function in *F. heteroclitus*.

The distribution of gonadotrop cell bodies differs from that in salmonids, where the two gonadotropins are contained in separate groups of cells, but these cells are intermixed in the ventral region of the proximal pars distalis (Nozaki *et al.*, 1990; Naito *et al.*, 1991; Ikeuchi *et al.*, 1995). The distribution of gonadotrops in the platyfish pituitary also differs in important points: gonadotrops are found in the pars intermedia and in the proximal pars distalis. Many of the pars intermedia cells are predominantly β -I types, which in the *F. heteroclitus* pituitary are in the central (dorsal) portion of the proximal pars distalis. However, most of the gonadotrops in platyfish are immunoreactive to antibodies against both β -I and β -II gonadotropins, with only a small number exhibiting immunoreactivity to only one form, especially in the pars distalis (Schreibman *et al.*, 1990). This differs fundamentally from the distribution in *F. heteroclitus*, in which there is no apparent overlap in the immunoreactive cell groups.

Since the gonadotropins are members of a pituitary glycoprotein family that includes thyrotropin, the possible cross-reactivity of the antibodies is a valid concern. The sequences of the peptides used as immunogens in this study have little similarity to the sequence of thyrotropin. The GtH β -I subunit is more similar to the TSH β subunit than to the β -II subunit (Lin *et al.*, 1992). Thyrotropin has not been sequenced from *F. heteroclitus*, but the peptide selected from the GtH β -I of *Fundulus* differs in 10 of 11 amino acids when aligned with the TSH β subunit from rainbow trout (Ito *et al.*, 1993) and differs in 9 of 11 amino acids when aligned with the TSH β subunit from European eel (Salmon *et al.*, 1993). Somatotrops also occur in the same region of the pituitary that stains with the anti β -I serum. However, the peptides used as immunogens have no sequence similarity to known sequences of somatotropin, so crossreactivity appears unlikely.

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REFERENCES

- Ball, J. N., and Baker, B. I. (1969). The pituitary gland: Anatomy and histophysiology. In "Fish Physiology" (W. S. Hoar and D. J. Randall, Eds.), Vol. II, pp. 1–110. Academic Press, New York.
- Batten, T. F. C. (1986). Immunocytochemical demonstration of pituitary cell types in the teleost *Poecilia latipinna* by light and electron microscopy. *Gen. Comp. Endocrinol.* **63**, 139–154.
- Brummett, A. R., Dumont, J. N., and Larkin, J. R. (1982). The ovary of *Fundulus heteroclitus*. *J. Morphol.* **173**, 1–16.
- Carr, B. R. (1993). The normal menstrual cycle. In "Textbook of Reproductive Medicine" (B. R. Carr and R. E. Blackwel, Eds.), pp. 209–219. Appleton & Lange, New York.
- Cerdà, J., Calman, B. G., LaFleur, G. J., Jr., and Limesand, S. (1996). Pattern of vitellogenesis and follicle maturational competence during the ovarian follicular cycle of *Fundulus heteroclitus*. *Gen. Comp. Endocrinol.* **103**, 24–35.
- Copeland, P. A., and Thomas, P. (1993). Isolation of gonadotropin subunits and evidence for two distinct gonadotropins in Atlantic croaker (*Micropogonias undulatus*). *Gen. Comp. Endocrinol.* **91**, 115–125.
- Emmart, E. W., Pickford, G. E., and Wilhelmi, A. W. (1966). Localization of prolactin within the pituitary of a cyprinodont fish, *Fundulus heteroclitus* (Linnaeus), by specific fluorescent antiovine prolactin globulin. *Gen. Comp. Endocrinol.* **7**, 571–583.
- Farbridge, K. J., McDonald-Jones, G., McLean, C. L., Lowry, P. J., Etches, R. J., and Leatherland, J. F. (1990). The development of monoclonal antibodies against salmon (*Oncorhynchus kisutch* and *O. keta*) pituitary hormones and their immunohistochemical identification. *Gen. Comp. Endocrinol.* **79**, 361–374.
- García-García, A., Muñoz-Cueto, A., Rodríguez, R. B., and Sarasquete, C. (1994). Protein G-horseradish peroxidase based method for light-microscope immunocytochemistry: Application to the pituitary gland of the killifish, *Fundulus heteroclitus*. *Eur. J. Histochem.* **38**, 229–236.
- Greeley, M. S., Jr., Calder, D. R., Taylor, M. H., Hols, H., and Wallace, R. A. (1986). Oocyte maturation in the mummichog (*Fundulus heteroclitus*): Effects of steroids on germinal vesicle breakdown in intact follicles *in vitro*. *Gen. Comp. Endocrinol.* **62**, 281–289.
- Hassin, S., Elizur, A., and Zohar, Y. (1995). Molecular cloning and sequence analysis of striped bass (*Morone saxatilis*) gonadotropin-I and gonadotropin-II subunits. *J. Mol. Endocrinol.* **15**, 23–35.
- Hsiao, S.-M., and Meier, A. H. (1989). Comparison of semilunar cycles of spawning activity in *Fundulus grandis* and *F. heteroclitus* held under constant laboratory conditions. *J. Exp. Zool.* **252**, 213–218.
- Hsiao, S.-M., Greeley, M. S., Jr., and Wallace, R. A. (1994). Reproductive cycling in female *Fundulus heteroclitus*. *Biol. Bull.* **186**, 271–284.
- Hsiao, S.-M., Limesand, S. W., and Wallace, R. A. (1996). Semilunar follicular cycle of an intertidal fish: The *Fundulus* model. *Biol. Reprod.* **54**, 809–818.
- Ikeuchi, T., Mochida, K., Ura, K., Adachi, S., and Yamauchi, K. (1995). Specific antibodies against synthetic peptides of salmonid GTH I and II β subunits. *Zool. Sci.* **12**, 317–323.
- Ito, M., Koide, Y., Takamatsu, N., Kawauchi, H., and Shiba, T. (1993). cDNA cloning of the beta subunit of teleost thyrotropin. *Proc. Natl. Acad. Sci. USA* **90**, 6052–6055.
- Kanungo, J., Petrino, T. R., and Wallace, R. A. (1990). Oogenesis in *Fundulus heteroclitus*. VI. Establishment and verification of conditions for vitellogenin incorporation by oocytes *in vitro*. *J. Exp. Zool.* **254**, 313–321.
- Koide, Y., Itoh, H., and Kawauchi, H. (1993). Isolation and characterization of two distinct gonadotropins, GtHI and GtHII, from bonito (*Katsuwonus plelamis*) pituitary glands. *Int. J. Peptide Protein Res.* **41**, 52–65.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.
- Limesand, S. W., Lin, Y.-W. P., Price, D. A., and Wallace, R. A. (1995). *Fundulus heteroclitus* gonadotropins. 4. Cloning and sequencing of gonadotropin hormone (GTH) α -subunit. In "Proceedings of the Fifth International Symposium on the Reproductive Biology of Fish" (F. W. Goetz and P. Thomas, Eds.), p. 34. Fish Symposium 95, University of Texas, Austin, TX.
- Lin, Y.-W. P., LaMarca, M. J., and Wallace, R. A. (1987). *Fundulus heteroclitus* gonadotropin(s) I. Homologous bioassay using oocyte maturation and steroid production by isolated ovarian follicles. *Gen. Comp. Endocrinol.* **67**, 126–141.
- Lin, Y.-W. P., Greeley, M. S., Jr., and Wallace, R. A. (1989). *Fundulus heteroclitus* gonadotropin(s) 2. Year-round husbandry of animals with active pituitaries and active follicles. *Fish Physiol. Biochem.* **6**, 139–148.
- Lin, Y.-W. P., Rupnow, B. A., Price, D. A., Greenberg, R. M., and Wallace, R. A. (1992). *Fundulus heteroclitus* gonadotropins. 3. Cloning and sequencing of gonadotropin hormone (GTH) I and II β -subunits using the polymerase chain reaction. *Mol. Cell. Endocrinol.* **85**, 127–139.
- Matthews, S. A. (1936). The pituitary gland of *Fundulus*. *Anat. Rec.* **65**, 357–367.
- Naito, N., Hyodo, S., Okumoto, N., and Nakai, Y. (1991). Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. *Cell Tissue Res.* **266**, 457–467.
- Nozaki, M., Naito, N., Swanson, P., Miyata, K., Nakai, Y., Oota, Y., Suzuki, K., and Kawauchi, H. (1990). Salmon pituitary gonadotropins. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. *Gen. Comp. Endocrinol.* **77**, 348–357.
- Okada, T., Kawazoe, I., Kimura, S., Sasamoto, Y., Aida, K., and Kawauchi, H. (1994). Purification and characterization of gonadotropin I and II from pituitary glands of tuna (*Thunnus obesus*). *Int. J. Peptide Protein Res.* **43**, 69–80.
- Pluskal, M. G., Przekop, M. B., Kavonian, M. R., Vecoli, C., and Hicks, D. A. (1986). Immobilon PVDF transfer membrane: A new membrane substrate for Western blotting of proteins. *BioTechniques* **4**, 272–282.
- Salmon, C., Marchelidon, J., Fontaine, Y. A., Huet, J. C., and Querat, B. (1993). Cloning and sequence of thyrotropin β -subunit of a teleost fish the eel (*Anguilla anguilla*). *C. R. Acad. Sci. D Sci. Nat.* **316**, 749–753.

- Schreibman, M. P., Holtzman, S., and Cepriano, L. (1990). The life cycle of the brain-pituitary-gonad-axis in teleosts. In "Progress in Comparative Endocrinology" (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.), pp. 399–408. Wiley-Liss, New York.
- Selman, K., and Wallace, R. A. (1986). Gametogenesis in *Fundulus heteroclitus*. *Am. Zool.* **26**, 173–192.
- Selman, K., and Wallace, R. A. (1989). Cellular aspects of oocyte growth in teleosts. *Zool. Sci.* **6**, 211–231.
- Sokol, H. W. (1961). Cytological changes in the teleost pituitary gland associated with the reproductive cycle. *J. Morphol.* **109**, 219–235.
- Stefanini, M., De Martino, C., and Zamboni, L. (1961). Fixation of ejaculated spermatozoa for electron microscopy. *Nature* **216**, 173–174.
- Suzuki, K., Kanamori, A., Nagahama, Y., and Kawauchi, H. (1988). Development of salmon GTH I and GTH II radioimmunoassays. *Gen. Comp. Endocrinol.* **71**, 459–467.
- Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol. Reprod.* **44**, 29–38.
- Tanaka, H., Kagawa, H., Okuzawa, K., and Hirose, K. (1993). Purification of gonadotropins (PmGTH I and II) from red seabream (*Pagrus major*) and development of a homologous radioimmunoassay for PmGTH II. *Fish Physiol. Biochem.* **10**, 409–418.
- Taylor, M. (1990). Estuarine and intertidal teleosts. In "Reproductive Seasonality in Teleosts: Environmental Influences" (A. D. Munro, A. P. Scott, and T. J. Lam, Eds.), pp. 109–124. CRC Press, Boca Raton, FL.
- Tsai, H.-J., and Yang, L.-T. (1995). Cloning and sequencing of the cDNA encoding the pituitary gonadotropin II β -subunit of yellowfin porgy (*Acanthopagrus latus*). *J. Fish Biol.* **46**, 501–508.
- Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. *Gen. Comp. Endocrinol.* **85**, 217–229.
- Van Oordt, P. G. W. J., and Peute, J. (1983). The cellular origin of pituitary gonadotropins in teleosts. In "Fish Physiology" (W. S. Hoar, D. J. Randall, and E. M. Donaldson, Eds.), Vol. IX, Part A, pp. 137–186. Academic Press, New York.
- Wallace, R. A., and Selman, K. (1978). Oogenesis in *Fundulus heteroclitus* I. Preliminary observations on oocyte maturation in vivo and in vitro. *Dev. Biol.* **62**, 354–369.
- Wallace, R. A., and Selman, K. (1980). Oogenesis in *Fundulus heteroclitus* II. The transition from vitellogenesis into maturation. *Gen. Comp. Endocrinol.* **42**, 345–354.
- Yan, H. Y., and Thomas, P. (1991). Histochemical and immunocytochemical identification of the pituitary cell types in three sciaenid fishes: Atlantic croaker (*Micropogonias undulatus*), spotted seatrout, (*Cynoscion nebulosus*), and red drum (*Sciaenops ocellatus*). *Gen. Comp. Endocrinol.* **84**, 389–400.
- Yoshiura, Y., Kobayashi, M., Kato, Y., and Aida, K. (1997). Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH-I β and -II β) from the goldfish. *Gen. Comp. Endocrinol.* **105**, 379–389.
- Yoshiura, Y., Suetake, H., and Aida, K. (1999). Duality of gonadotropin in a primitive teleost, Japanese eel (*Anguilla japonica*). *Gen. Comp. Endocrinol.* **114**, 121–131.