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Fundulus heteroclitus gonadotropins.5: Small scale chromatographic fractionation of pituitary extracts into components with different steroidogenic activities using homologous bioassays

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Abstract

Fractionation and characterization of gonadotropins (GtH) from *Fundulus heteroclitus* pituitary extracts were carried out using a biocompatible liquid chromatographic procedure (Pharmacia FPLC system). Chromatographic fractions were monitored for gonadotropic activities (induction of oocyte maturation and steroid production) using homologous follicle bioassays *in vitro*. Size-exclusion chromatography eluted gonadotropic activity in one major protein peak (M_r ~ 30,000). Anion-exchange and hydrophobic-interaction chromatography (HIC) yielded two distinct peaks of 17beta-estradiol (E_2)- and 17alpha-hydroxy,20beta-dihydroprogesterone (DHP)-promoting activity with associated oocyte maturation. Two-dimensional chromatography (chromatofocusing followed by HIC) resolved pituitary extracts into two active fractions; both induced E_2 synthesis, but one was relatively poor in eliciting DHP and testosterone production. Thus, using homologous bioassays, at least two quantitatively different gonadotropic (steroidogenic) activities: an E_2-promoting gonadotropin (GtH I-like) and a DHP-promoting gonadotropin (GtH II-like), which has a lower isoelectric point but greater hydrophobicity than the former, can be distinguished from *F. heteroclitus* pituitaries by a variety of chromatographic procedures. This study complements previous biochemical and molecular data in *F. heteroclitus* and substantiates the duality of GtH function in a multiple-spawning teleost.

Background

During a breeding season, many fish known as fractional spawners have been found to undergo periodic reproductive activity correlated with the lunar cycle [1], including *Fundulus heteroclitus* (killifish) [2,3]. Such activity involves a complicated interplay of gonadotropic hormones (GtHs) and ovarian steroidogenic events as clutches of oocytes are periodically recruited into vitellogenic, maturational, and ovulatory processes [3,4].

The presence of two distinct GtHs in teleosts has been demonstrated in several species including chum salmon [5,6], coho salmon [7], killifish [8-10], common carp [11], Atlantic croaker [12], tuna [13], bonito [14], red seabream [15], striped bass [16], yellowfin porgy [17], goldfish [18]and Japanese eel [19]. These GtHs, distinguished by their β-subunits and termed GtH I and GtH II, are similar (38–48% sequence identity) to tetrapod follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively [20,21]. The biological significance of the two GtHs has been intensively studied in salmonids and other

annual spawners. However, much less information is available from other commercially important species that are fractional spawners such as tuna, flounder, red drum, croaker, and halibut. Due to habitat destruction and/or overfishing, the populations of some of these species are declining. *Fundulus* is also a fractional spawner and can act as an inexpensive, easily manipulated model system.

In order to understand the operant mechanisms involved in cyclic teleost reproduction and to provide a broader basis for comparative study of the teleost GtHs, we have focused our studies on the *F. heteroclitus* GtHs and their role in controlling the cyclic reproductive activity characteristic of this species and common to a large class of commercially important species other than salmonids. These efforts have included the development of a homologous bioassay for gonadotropic hormones using oocyte maturation and steroid production by isolated ovarian follicles [22], the characterization of steroidogenic responses to an *F. heteroclitus* pituitary extract (FPE) by the ovarian follicle [23-26]; specifically, the identification of 17α-hydroxy,20β-dihydroprogesterone (DHP) as the maturation-inducing substance (MIS) for *F. heteroclitus* upon stimulation by gonadotropin [27], and the cloning and sequencing of the two β-subunits of the GtHs from a cDNA library [8]. The α-subunit, shared by the two GtHs and thyrotropin hormone (TSH), has also been sequenced from the same cDNA library [28]. More recently, we reported the preparation and use of specific antibodies to the two β-subunits of *F. heteroclitus* GtHs [9]. Each antibody recognizes a different subset of pituitary cells in the central (GtH I) and peripheral (GtH II) proximal pars distalis, respectively, regions that display the typical tinctorial properties of gonadotrops. Similarly, Shimizu and Yamashita [10] also reported the preparation of antibodies against the α-subunit and the two β-subunits, and the purification of *F. heteroclitus* GtHs using an immunochemical assay. Thus, the molecular and immunochemical data available for *F. heteroclitus* show the presence of two distinct GtHs; however, a detailed analysis on the duality of GtH function is still lacking in this and other fractional spawners.

To further investigate the possible differential biological activities of the two distinct GtHs in *F. heteroclitus* may require the use of specific homologous bioassays. The intention of the present study was to discern any discrete differences in terms of maturational and steroidogenic activities of the two gonadotropins (GtH I and GtH II) shown to be present in *F. heteroclitus*. To accomplish this objective, we carried out the purification and characterization of GtH activities from *Fundulus* pituitary by fast protein liquid chromatography (FPLC) as determined by homologous bioassays.

Materials and methods

Animals and chemicals

Animals were collected from salt marshes in the Matanzas River, St. Augustine, Florida. Routine husbandry procedures were used to maintain the fish in the laboratory: captured fish (average weight = 7 g) were kept in a temperature-regulated (25°C) running sea water aquarium with a controlled photoperiod (14L:10D) and were fed with an enriched diet (mixture of boiled chicken eggs and dry flake food). Under this regimen, the laboratory-maintained fish had responsive ovarian follicles and active pituitaries throughout the year [29]. In effect, the ovarian follicles retrieved from these fish were responsive to pituitary extract stimulation, and underwent oocyte maturation normally in vitro. In addition, the pituitary glands from these laboratory fish also retained high gonadotropic potencies even outside of the normal breeding season. The care and use of, as well as all procedures involving, animals have been approved by Barry University's Institutional Animal Care and Use Committee (IACUC), in accordance with the guidelines of the IACUC of the National Institutes of Health (NIH).

Absolute grade $(NH_4)_2SO_4$ was obtained from Research Plus, Inc. All other chemicals were obtained from Sigma unless specified otherwise.

Preparation of Fundulus *pituitary extract (FPE)*

Pituitaries from sexually mature fish {gonadosomatic index (GSI) = gonad weight ÷ eviscerated body weight × 100; GSI = ~10% for female and ~5% for male} were isolated immediately after decapitating the animals and stored frozen at -80°C (for up to one year) before the extraction procedure. The pituitary glands were collected from animals used in this and other studies in the laboratory over a period of three years. The pituitaries (0.2 mg wet wt per gland; 40 to 500 glands) were homogenized with a Teflon-coated pestle at 4°C in the starting buffer used for each chromatographic run. Homogenates were then centrifuged (13,000 *g*) for 1 h at 4°C and the supernatants were collected. The pellets were extracted once more with the starting buffer and recentrifuged. Supernatants from both extracts were combined to provide FPE and filtered through a 0.2-μm Nylon centrifugal microfilter (Centrex, Schleicher & Schuell) before injection onto chromatographic columns.

Pituitaries from both male and female fish were used. Due to a report of sexual differences in GtH [30], pituitaries from both sexes were not mixed. However, numerous experiments in our lab indicated that similar chromatograms are obtained from pituitary extracts of either sex (data not shown), so an indication of sex for the sample source is not provided.

Chromatography

Size-exclusion chromatography (SEC) was carried out using two columns (Pharmacia Superose 12 HR 10/30 and Superose 6 HR 10/30) connected in series on a Pharmacia FPLC system equipped with absorbance monitors at 214 and 280 nm (HR 10 flow cells). The columns were equilibrated with 50 mM NH_4HCO_3, pH 7.5, before the application of FPE. The same buffer was used to elute the sample isocratically with a flow rate of 0.5 ml/min. One-ml fractions were collected and evaporated to dryness by vacuum centrifugation (Savant SpeedVac Concentrator).

For anion-exchange chromatography (AEC), the column (Pharmacia Mono Q HR 5/5) was initially equilibrated with 50 mM Tris-HCl buffer (pH 7.5). FPE was applied to the column and unadsorbed proteins were allowed to percolate through the column using the same starting buffer. Elution of the adsorbed sample was then carried out with a linear, increasing gradient of NaCl (0 – 0.33 M) in 50 mM Tris-HCl, pH 7.5. The flow rate was 1.0 ml/min, the fraction size was 2.0 ml/tube, and eluted fractions were dialyzed overnight at 4°C against distilled water.

The starting buffer for hydrophobic-interaction chromatography (HIC) was 1.2 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate, pH 6.8. The hydrophobic-interaction column (Bio-Rad Bio-Gel TSK Phenyl-5-PW, 7.5 × 75 mm) was equilibrated with starting buffer before the application of FPE and a linear gradient of decreasing $(NH_4)_2SO_4$ concentration was begun after the unadsorbed protein eluted from the column. Eluted fractions of 1.0 ml were collected at a flow rate of 0.5 ml/min and dialyzed against distilled water.

For chromatofocusing, the column (Pharmacia Mono P HR 5/20) was initially equilibrated with 25 mM Bis-Tris-HCl, pH 6.3. After application of FPE, elution was carried out with 40 ml 10% Polybuffer 74 (Pharmacia), pH 4.0, and this was followed by a 1.0-M NaCl wash to elute the remaining adsorbed protein. Fractions of 2.0 ml were collected at a flow rate of 1.0 ml/min. Eluted fractions were pooled into five samples (I-V) and each sample was adjusted to 1.2 M $(NH_4)_2SO_4$ and rechromatographed on the hydrophobic-interaction column. Eluted fractions were dialyzed against distilled water. All dialyzed samples were evaporated to dryness by vacuum centrifugation, tightly sealed, and stored at 4°C for up to a month prior to reconstitution.

Bioassays

Dried fractions were reconstituted with 75% L-15 and measured for gonadotropic activities by RIA determinations of the amounts of 17α-hydroxy,20β-dihydroprogesterone (DHP), testosterone (T), and 17β-estradiol (E_2) generated after follicle culture at 25°C for 24 h and by scoring cultured follicles for oocyte maturation [germinal vesicle breakdown (GVBD)] after 48 hr as previously described [22]. For each bioassay, prematurational ovarian follicles (1.2 – 1.4 mm in diameter) were pooled from 10 to 20 animals.

Results

Each of the following chromatographic procedures has been performed at least ten times. Representative chromatographs are illustrated in the following results.

Size-exclusion chromatography (SEC)

SEC yielded several adsorbance peaks that were not well separated from each other (Fig. 1A). Oocyte maturation bioassays of the eluted fractions indicated a single peak with a maximum activity of 80% GVBD (similar to the maximum response of positive controls to unfractionated FPE) (Fig. 1B). Steroidogenic activities were also measured by RIA and were found to elute in a similar position (corresponding to an M_r of approx. 30,000) (Fig. 1C); maximum activities were also comparable to those stimulated by unfractionated FPE. The peak of T and DHP production coincided with the peak of oocyte maturation activity, although steroidogenic activity in earlier eluting fractions were indicated for both DHP- and E_2-production. None of the activities indicated in Figures 1B and 1C corresponded precisely with the peaks provided by the adsorbance trace (Fig. 1A).

Anion-exchange chromatography (AEC)

AEC of FPE provided a complex elution profile (Fig. 2A). Bioassays for oocyte maturation also indicated that activity was distributed throughout the eluted fractions (Fig. 2B), as was also the case for the various steroidogenic activities (Fig. 2C). Nevertheless, well defined peaks of E_2- and DHP-promoting activity were eluted at NaCl concentrations of about 0.15 and 0.23 M, respectively (Fig. 2C). No steroidogenic activities were detected before the start of the NaCl gradient. Similarly, a 1.0-M NaCl wash eluted little additional activity from the anion-exchange column.

Hydrophobic-interaction chromatography (HIC)

HIC of FPE also yielded numerous UV-adsorbing components (Fig. 3A). Bioassays for oocyte maturation indicated that activity was restricted to fractions eluting near the end of the gradient (Fig. 3B). No steroidogenic activities were detected in unabsorbed material (before the start of the gradient) or during the early part of the chromatographic process, but were also confined to the second half of the gradient [$(NH_4)_2SO_4$ concentration ≤ 0.6 M] (Fig. 3C). Two distinct peaks of E_2-producing activities were detected at an $(NH_4)_2SO_4$ concentration of about 0.31 M and 0.17 M (peaks a and b, respectively) and heterogeneity was not particularly evident. T-promoting activity was broadly associated with both peaks, while DHP-promot-

Table 1: Maturation-inducing and steroidogenic activities in active fractions derived from a combination of chromatofocusing followed by HIC[a]

Sample	GVBD (%)	pg per well			X 100	
		E_2	T	DHP	T/E_2	DHP/E_2
FPE[b]	38 ± 7	5,850 ± 2,000	264 ± 48	332 ± 68	4.5	5.7
FPE[c]	40 ± 5	4,450 ± 2,000	240 ± 56	312 ± 48	5.4	7.0
I-1[d]	12 ± 12	3,150 ± 1,204	147 ± 53	126 ± 18	4.7	4.0
II-8[d]	29 ± 8	3,300 ± 450	145 ± 14	139 ± 49	4.4	4.2
III-10[d]	4 ± 3	2,120 ± 701	60 ± 60	43 ± 15	2.8	2.0
V-19[d]	26 ± 15	4,483 ± 393	271 ± 23	339 ± 91	6.0	7.6

[a] Results presented as means ± SEM derived from three chromatographic series. In the absence of added gonadotropin, no GVBD took place and T- and DHP-production were undetectable, while E_2-production averaged 1,150 pg per well. The latter baseline value was subtracted from the induced E_2 data to provide the indicated values for E_2. [b] Thawed, immediately added to L-15 medium, and tested for activity at a concentration of 0.5 pit. equiv./well (0.25 pit. equiv./ml). [c] Thawed, stored for up to a week at 4°C, dialyzed overnight against distilled water, dried by vacuum centrifugation, reconstituted with L-15 medium, and tested for activity at a concentration of 0.5 pit. equiv./well. [d] Roman numerals refer to portions of the chromatofocusing runs (Fig. 4) while Arabic numerals refer to active fractions found after subsequent HIC (Fig. 5).

ing activity co-eluted primarily with peak b. It is important to note that this DHP-promoting activity also coincided with the peak of oocyte maturation (Fig. 3B).

Chromatofocusing

Chromatofocusing of FPE with a decreasing pH gradient gave a complex but reproducible elution profile of 280-nm adsorbing material followed by a major protein peak eluted with the high salt wash (Fig. 4). The presence of Polybuffer in the eluant precluded adsorptivity measurements at 214 nm; it also proved to be toxic to cultured follicles so that bioassays could not be performed directly on eluant fractions. Polybuffer was therefore removed by rechromatographing five major portions (indicated as I to V in Fig. 4) on a Phenyl-5-PW column (Fig. 5 I-V). The Polybuffer, which was not adsorbed on the Phenyl-5-PW column, eluted at the beginning of each chromatogram. Various fractions from each hydrophobic-interaction run (indicated as 1 to 19 in Fig. 5) were then tested for steroidogenic activity and ability to induce maturation, and positive results were obtained for four fractions (indicated by shaded bars in Fig. 5). This exercise was performed three times and the pooled results for the active fractions together with appropriate controls are provided in Table 1. These latter data indicate that the GtHs in FPE survive the manipulative procedures associated with chromatographic fractionation, dialysis, vacuum centrifugation and reconstitution for up to a week at 4°C (perhaps a slight loss in E_2-promoting activity is suggested). Among the four active fractions (I-1, II-8, III-10, V-19) eluted chromatographically (Fig. 5, Table 1), fraction III-10 had relatively poor DHP-promoting and maturation-inducing activities but had E_2-promoting activity, while fraction V-19 appeared enriched in DHP-promoting activity. In general, T-promoting activity tended to elute with DHP-promoting activity (Table 1).

Discussion

GtH bioassay

Fundulus pituitary extract (FPE) was fractionated by various biocompatible liquid chromatographic procedures in an attempt to purify the GtHs and characterize their biological activities. Chromatographic fractions were tested for their gonadotropic activities by using a homologous bioassay system which utilized intact *F. heteroclitus* ovarian follicles *in vitro* [22]. Two indicators of gonadotropic activity were employed, one being the ability of the fractions to stimulate prematurational oocytes (1.2–1.4 mm in diameter), which are arrested at prophase I of meiosis, to resume the meiotic process by undergoing GVBD. The other indication of gonadotropic activity was the ability of the eluted fractions to stimulate the ovarian follicles to produce three reproductively important steroid hormones (DHP, T, and E_2).

This homologous bioassay system has been extensively verified and shown to be sensitive and specific for *F. heteroclitus* GtHs [22]. It thus avoided pitfalls that may have arisen using a heterologous bioassay system [31]. Although a report has appeared that prolactin and growth hormone stimulate ovarian steroidogenesis when injected into *F. heteroclitus* [32], no biological activity was detected for either hormone using our *in vitro* bioassay system (data not shown). Another advantage of our homologous bioassay system was that many fractions could be assayed simultaneously with a large number of appropriate-sized follicles that can be pooled from several fish and randomized, thus eliminating most of the between-animal

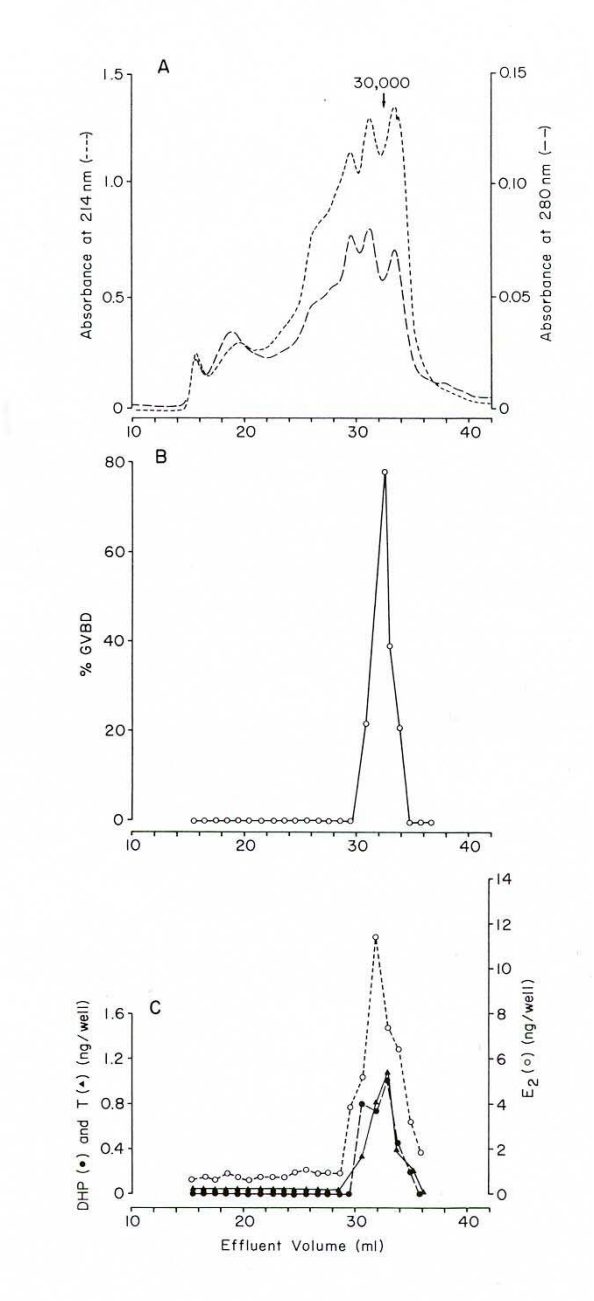

Figure 1
Size-exclusion chromatography (SEC) of FPE. Approximately 78 pituitary equivalents in a volume of 200 μl were applied to the Superose columns and the effluent was monitored for UV-absorbance (A). One-ml fractions were collected and tested for (B) maturational and (C) steroidogenic activity. For this and subsequent figures, negative controls (no gonadotropic extracts or fractions added) had 0–5% GVBD, DHP and T secretion were not detectable, and E_2 secretion averaged less than 0.5 ng/2-ml well. For this experiment only, positive controls (FPE added at a concentration of 0.5 pit. equiv./well) were 65% GVBD with DHP-, T-, and E_2-secretion of 1.25, 0.75, and 7.4 ng/well, respectively.

variation in the procedure. To further minimize the inherent variation in the responsiveness of ovarian follicles to gonadotropin and to assure the availability of large numbers of sensitive follicles and active pituitary glands throughout the year, we also developed a routine husbandry procedure to maintain a large population of reproductively healthy *F. heteroclitus* in the laboratory [29]. With this husbandry procedure, we were able to proceed with the FPE fractionation and to carry out homologous bioassays throughout the year.

Chromatographic resolution of gonadotropic activity

Size-exclusion chromatography (SEC) produced several UV-absorbing peaks, none of which correlated precisely with biological activity found around 30 kDa (Fig. 1). Active fractions induced oocyte maturation in high frequency and stimulated the production of all three steroids tested (DHP, T, and E_2), with estradiol being predominant (as was the case for unfractionated FPE). The size of *F. heteroclitus* GtHs thus approximates previous lower estimates (ranging in molecular weight from 25 to 62 kDa) made by SEC for fully glycosylated, nondenatured teleost GtHs [5,7,30,33-36]. Recently, a molecular weight of about 40 kDa (gel filtration) was reported for *F. heteroclitus* (Arasaki strain, Japan) GtH I and II [10]. The large variation in these molecular weights may be population-specific or due to the presence of aggregates or to differences in the methods used for their estimation.

After anion-exchange chromatography (AEC) (Fig. 2A), maturational activity was found to be spread throughout the chromatogram (Fig. 2B). Similarly, steroidogenic activities for all three steroids tested were distributed among the various eluted fractions, although very distinct peaks of E_2- and DHP-activity were discerned at NaCl concentrations of 0.15 and 0.24 M, respectively (Fig. 2C). Hence at least two different GtHs, one with predominantly E_2-producing activity and the other with relatively high DHP-producing activity, could be distinguished by the steroidogenic bioassay data. However, the appearance of all types of gonadotropic activity throughout the AEC profile implies the presence of charge-heterogeneity in the *F. heteroclitus* GtHs. Such heterogeneity has previously been well documented for a variety of teleost species [37-39] as well as other vertebrates [40-45]. Charge-heterogeneity in GtHs has been previously explained by differences in sialic acid content [46] or by post-translational modifications of amino acid residues [43], but the precise differences between various isohormones are not well understood. In most cases, however, electrostatically distinct GtHs of the same type (i.e., FSH-like or LH-like) have been considered to be qualitatively identical in biological action.

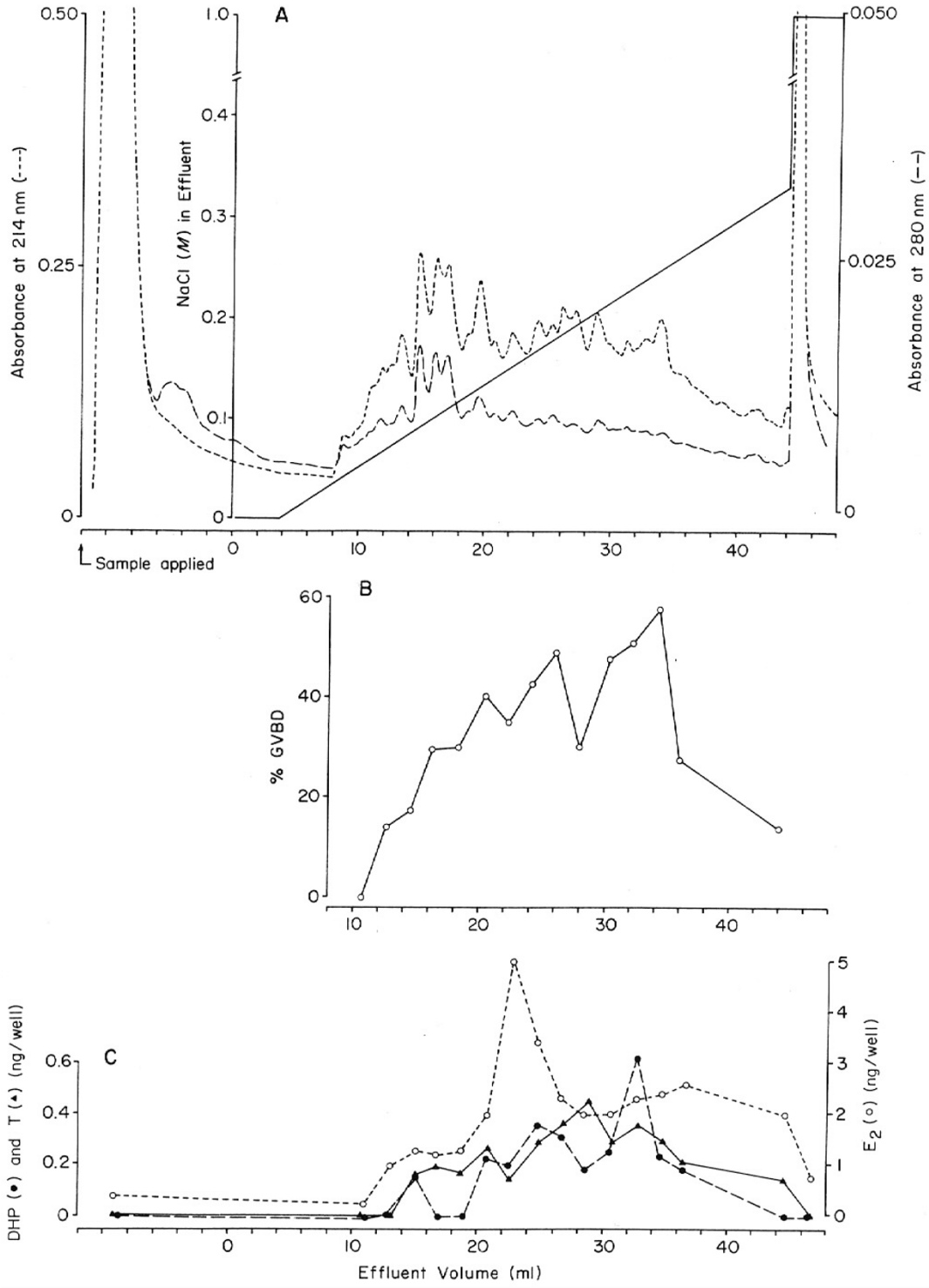

Figure 2
Anion-exchange chromatography (AEC) of FPE. Approximately 40 pituitaries were extracted twice with 0.5 ml 50 mM Tris-HCl (pH 7.5) and the extracts were combined, centrifugally filtered, and applied to a Mono Q column via the 10-ml Superloop. The effluent was monitored for UV-absorbance (A) and a gradient of NaCl was applied after non-adsorbing material percolated through the column. Two-ml fractions were collected and tested for (B) maturational and (C) steroidogenic activity. For this experiment only, positive controls (FPE added at a concentration of 0.5 pit. equiv./well) were 43% GVBD with DHP-, T-, and E_2-secretion of 0.68, 0.54, and 3.4 ng/well, respectively.

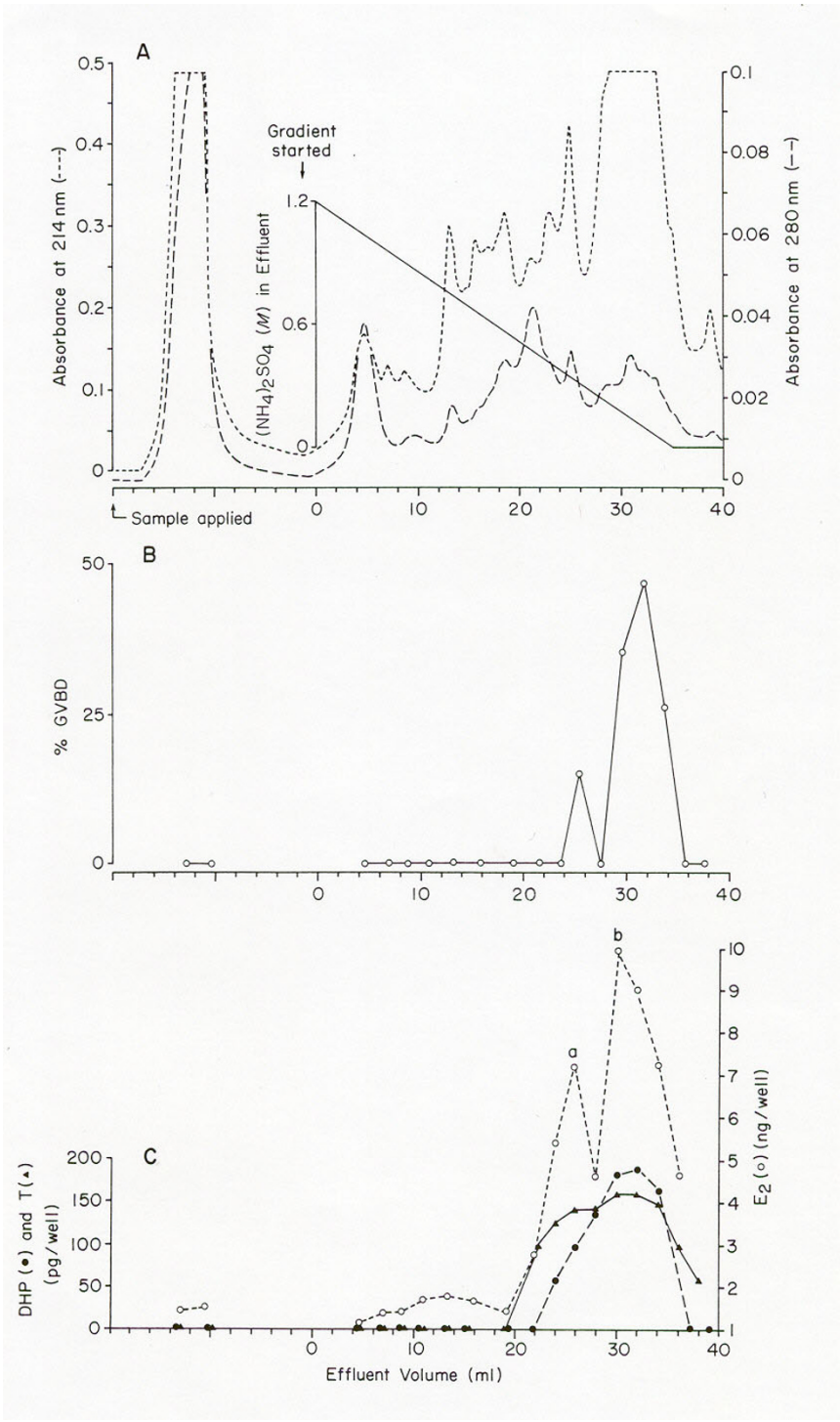

Figure 3
Hydrophobic-interaction chromatography (HIC) of FPE. Approximately 56 pituitaries were extracted twice with 0.5 ml 1.2 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate buffer (pH 6.8) and the extracts were combined, centrifugally filtered, and applied to a Phenyl-5-PW column via the 10-ml Superloop. The effluent was monitored for UV-absorbance (A) and a linear gradient of decreasing $(NH_4)_2SO_4$ concentration was started after the unadsorbed protein eluted from the column. Fractions of 1.0 ml were collected and tested for (B) maturational and (C) steroidogenic activity. Two peaks of E_2-generating activity are indicated (a, b). For this experiment only, positive controls (FPE added at a concentration of 0.5 pit. equiv./well) were 50% GVBD with DHP-, T-, and E_2-secretion of 1.3, 0.8, and 7.4 ng/well, respectively.

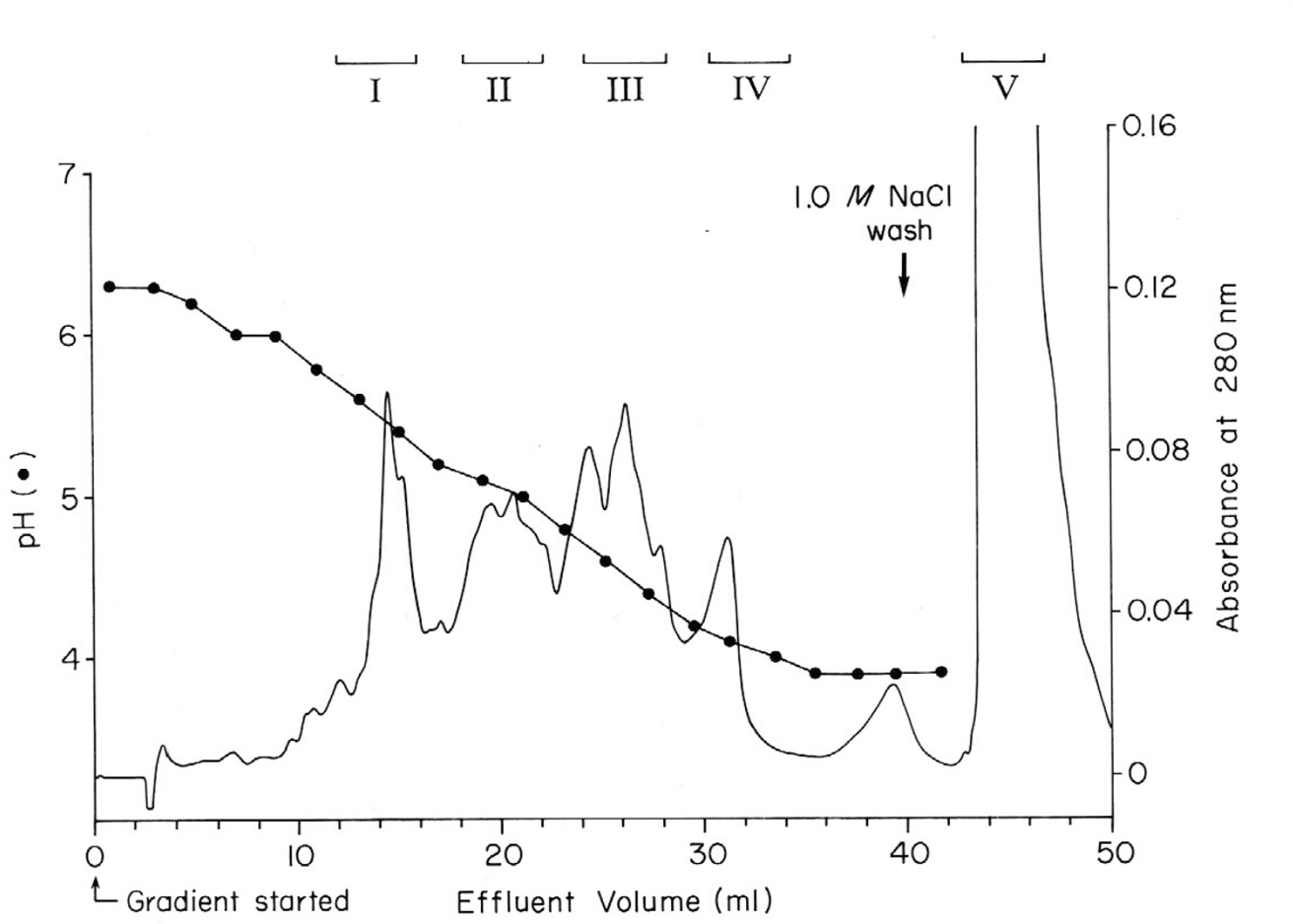

Figure 4
Chromatofocusing of FPE. Approximately 500 pituitaries were extracted twice with 0.5 ml 25 mM Bis-Tris-Cl (pH 6.3) and the extracts were combined, centrifugally filtered, and applied to a Mono P column via the 10ml Superloop. After the absorbance recording returned to the baseline (tracing not shown; no gonadotropic activity was present in collected fractions), 10% Polybuffer 74 was pumped onto the column via the 50ml Superloop and this was followed by a salt wash. The effluent was monitored for absorbance at 280 nm. Two-ml fractions were collected and measured for pH. Several fractions (indicated as I-V) were also pooled and subjected to further hydrophobic-interaction chromatography.

Gonadotropic fractions relatively rich in either E_2- or DHP-promoting activity were also discerned either by hydrophobic-interaction (HIC) (eluting at -0.31 and -0.17 M $(NH_4)_2SO_4$, respectively; Fig. 3C) or chromatofocusing followed by HIC (in order to remove Polybuffer). Fractions relatively enriched in E_2- and DHP-promoting activity were found to elute from the chromatofocusing column at pH 4.5 and after the salt wash, respectively, and these activities subsequently eluted from the Phenyl-5-PW column at $(NH_4)_2SO_4$ concentrations of -0.34 M and -0.12 M, respectively (Figs. 4, 5; Table 1). Thus a consistent elution pattern by HIC is indicated. The need to elute DHP-promoting activity from the chromatofocusing column with a salt wash is also consistent with its relatively late elution during AEC (Fig. 2C).

Based on previous reports that employed similar procedures, it would appear that the gonadotropic activity purified by Swanson *et al.* [33] from coho salmon pituitaries primarily corresponds to those fractions described here that lack preferential steroidogenic activity (i.e., Table 1, fractions I-1 and II-8), while the gonadotropin described by Copeland and Thomas [34] for Atlantic croaker is similar to our DHP-promoting gonadotropin (Table 1, fraction V-19). None of the fractions we have analyzed from any single chromatographic run have yielded single,

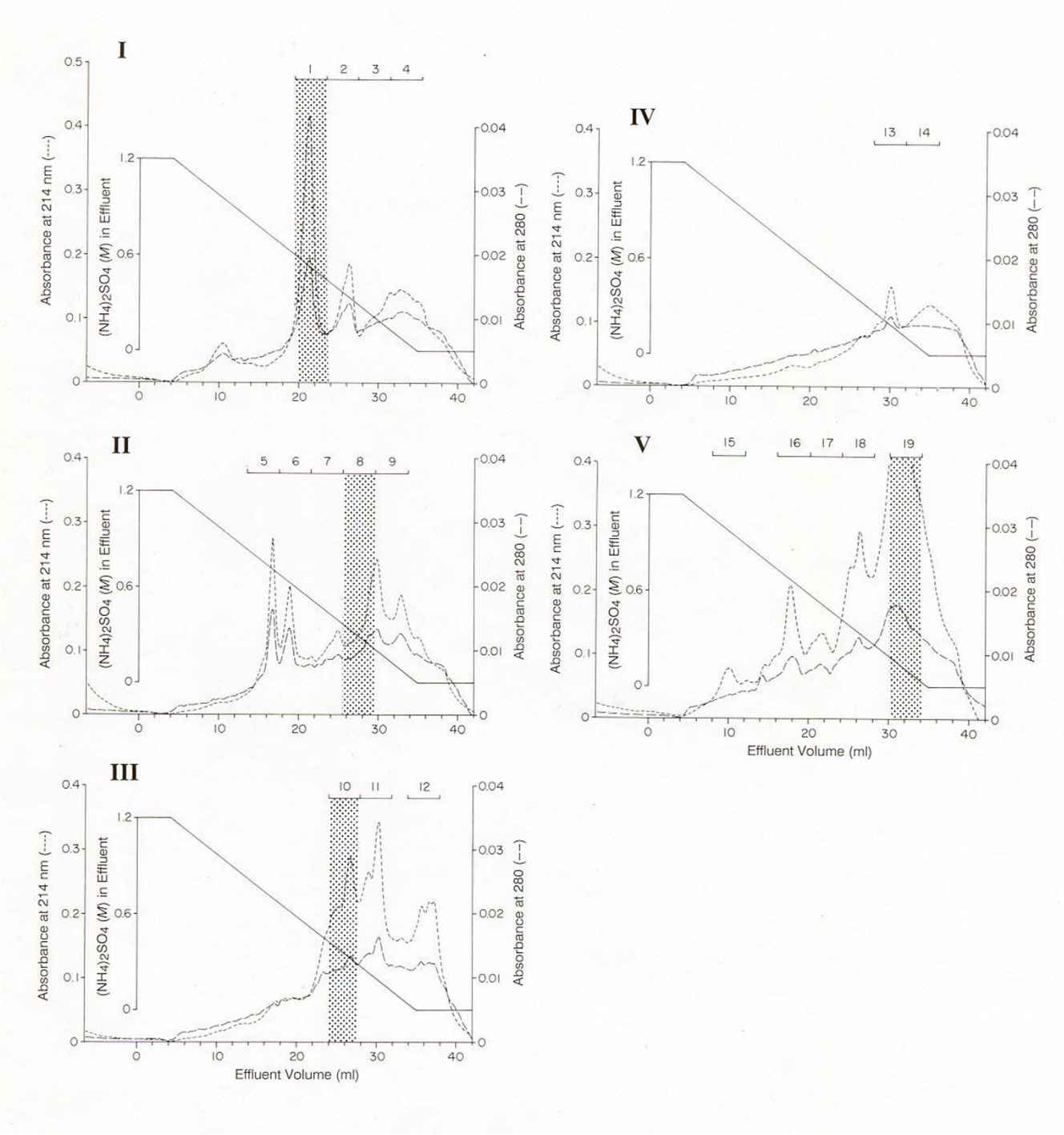

Figure 5
Mono-P fractions #I-V (containing Polybuffer) were readjusted with start buffer [1.2 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate, pH 6.8]. Each individual Mono-P fraction was rechromatographed on a Phenyl-5-PW column. A linear gradient of decreasing salt concentration was started after the Polybuffer eluted. Shaded areas indicate fractions containing gonadotropic activities (see Table 1).

silver-stained bands at the expected size on electrophoretic gels (data not shown). This may indicate that the amount of GtH protein present in the chromatographic fractions was below the detection level (<5 ng) of silver staining [47]. On the other hand, the homologous oocyte maturation and steroid production assays used in this study are far more sensitive in discerning biological activities.

A series of articles have attested to the duality of the GtHs in teleosts [7-10,12,19,48]. These studies provided ample biochemical and immunological evidence that there are two chemically distinct GtHs that reside in separate pituitary gonadotrops and have different ontogenies. Unfortunately, attempts to distinguish different steroidogenic activities between GtH I and GtH II have been less definitive because biological activities of GtHs overlap considerably. Although GtH II seems to be more potent than GtH I in stimulating DHP-production by salmon ovarian follicles, no significant difference in E_2-steroidogenic activity has been found between GtH I and GtH II [7,48-50]. As a corollary, therefore, GtH I appear to preferentially promote E_2-production in salmonids. Since E_2 and DHP are primarily involved in vitellogenic processes and the resumption of meiotic maturation, respectively, GtH I and GtH II would seem to correspond to the vitellogenesis-promoting "carbohydrate-poor" and maturation-promoting "carbohydrate-rich" GtHs described by Idler and his colleagues for salmon and flounder [51-54]. Based on this, our own results for *F. heteroclitus* appear to indicate that GtH I-like and GtH II-like activities reside in fractions III-10 and V-19, respectively, derived from chromatofocusing of FPE (Table 1). Similar activities were found in fractions obtained by AEC (Fig. 2C) and HIC (Fig. 3C).

The chromatographic separation of the GtHs evaluated by bioassay cannot directly discern which fraction is GtH I and GtH II, and even though there is an overlap in the steroidogenic activity, the homologous bioassay data clearly distinguish two well defined peaks of different biological activities. Additionally, our HIC profiles as determined by the specific biological activities (oocyte maturation and steroidogenesis) are in agreement with those reported by Shimizu and Yamashita [10] who identified the *F. heteroclitus* GtH I and GtH II using an immunochemical analysis. We conclude, therefore, that at least two gonadotropic activities are present in *F. heteroclitus* pituitaries: an E_2-promoting gonadotropin (GtH I-like) and a DHP-promoting gonadotropin (GtH II-like) which has a lower isoelectric point but greater hydrophobicity than the former. Consistent with our previous findings that DHP is the maturation-inducing steroid in *F. heteroclitus* [27] the DHP-promoting gonadotropin is also associated with high maturation-inducing activity (Fig. 3). Taken together, the homologous bioassay data presented here, and our previous immunochemical study indicating that each of the two GtHs could be released selectively [9], help to substantiate the duality of GtH function in a teleost which is a multiple spawner.

Conclusions

Using homologous bioassay, at least two quantitatively different gonadotropic (steroidogenic) activities: an E_2-promoting gonadotropin (GtH I-like) and a DHP-promoting gonadotropin (GtH II-like), which has a lower isoelectric point but greater hydrophobicity than the former, can be distinguished from *F. heteroclitus* pituitaries by a variety of chromatographic procedures. This study complements previous biochemical and molecular data in *F. heteroclitus* and substantiates the duality of GtH function in a multiple spawner.

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