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Development of Improved GnRH Aanalogs for Controlled Reproduction of *Sparus Aurata* in Farming Conditions

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C. Abstract

The Sparus aurata is a seabream of high commercial value but it does not ovulate and spawn in captivity. The ultimate aim of our study was to provide the tools for induction of spawning. In our studies we have demonstrated that GnRH of salmon and mammalian origin are rapidly degraded, mainly at the Tyr⁵-Gly⁶ and the Pro⁹-Gly¹⁰ positions, by enzymes present in the pituitary, liver and kidney of the Sparus. Other, minor degradation sites were: 3-4, 6-7, and 7-8 bonds of the neurohormone. Analogs of mammalian and salmon GnRH were more resistant to degradation, and one of the salmon GnRH analogs that we have synthesized was almost completely resistant to degradation. These analogs were found to be superactive in inducing gonadotropin secretion in vivo. However, surprisingly and in contrast to their action in mammals, they were not more active than the native hormones when tested in a dynamic superfusion system of pituitary gland fragments. The in vivo vs in vitro results suggest that resistance to degradation of the analog is the major factor in determining its superactivity in this species. The results obtained by these studies were essential in the establishment of a protocol for the administration of GnRH analogs which resulted in successful daily spawning for a period of 30-90 days in over 80% of the treated females.

D. Objectives of the original research proposal.

The main objectives of this work were to study the hormonal profiles associated with the onset of oocyte atresia in the female *Sparus aurata* raised in captivity and to establish the background and tools for an improved methodology, based on fish gonadotropin-releasing hormone and its analogs, for the induction of oocyte maturation, ovulation and spawning.

All these objectives were fulfilled.

1. Degradation of Gonadotropin Releasing Hormones

As discussed in the second year end report, the relative susceptibility of GnRH and its analogs to degradation by enzymes present in different tissues is considered an important factor in determining the bioactivity of the neuropeptides. In both mammals and fish, pituitary bound peptidases were suggested to terminate the action of GnRH, by cleaving it to non-active fragments. In our previous report (see second annual BARD report) it was indicated that native sGnRH and LHRH are rapidly degraded by two pituitary cytosolic peptidases, a Tyr⁵-Gly⁶ endopeptidase and a Pro⁹-Gly¹⁰NH₂ peptidase. When considering relative potencies of exogenously administered forms of GnRH, as is the case in spawning induction treatments in fish, the involvement of peripheral metabolic tissues in the cleavage and inactivation of the neuropeptides should be evaluated. In the present work we studied the pattern and kinetics of degradation of native sGnRH and LHRH by kidney and liver, and the effect of structural modifications in the native peptides, as found in their analogs, on their susceptibility to degradation by the kidney and liver.

1.1 Materials and Methods

Animals

Two to three year old female Sparus aurata weighing 500 - 600 gm were used. All females used for experimentation were sexually mature, having oocytes undergoing final stages of vitellogenesis.

Preparation of subcellular fractions

Immediately after sacrifice, kidney, or liver were removed into homogenization buffer; 10 mM Tris-HCl, 0.25 M sucrose, 1 mM dithiothreitol (DDT), 0.5 mM CaCl₂. One gram of tissue from four fish was homogenized in 5 ml buffer. The homogenization of the tissues and preparation of cytosolic and membrane fractions was done as described in the second end year report. Our previous studies showed that both in the kidney and liver at least 90% of GnRH degrading activity resides in the cytosolic fraction.

Testing for GnRH degrading activity

10 nmol of the tested GnRH preparation were incubated with 100 µg protein of the cytosolic fraction of kidney or liver. Final volume was 500 µl of 10 mM Tris-HCl, 10 mM sucrose, 1 mM DTT, and 0.5 mM CaCl₂, at pH 7.2. Incubations were carried out for 15, 45, 90, and 180 min in a shaking water bath at 20±0.5°C. Control reactions included both the use of preheated homogenates (10 min at 100°C) and incubations without addition of GnRH. The incubations were terminated by heating in a boiling water bath for 6 min. The 0 time incubations were terminated immediately after adding the tested peptide to the tissue cytosolic fraction. The incubation mixture was centrifuged (15,000 x g for 5 min), the supernatant filtered through a 0.45 µm filter (Millipore), adjusted to the initial conditions of the HPLC gradient (10% CH₃CN 0.1% trifluoroacetic acid (TFA)) and refrigerated in siliconized 1.5 ml plastic tubes until analysis. This mixture was applied to a reverse phase high pressure liquid

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chromatography (HPLC, Waters, Milford) to separate degradation products. A plastic cartridge column (Radial Pak, Waters, 100 x 8 mm, 10 μ m particle size) was used. Elution started with isocratic flow for 15 min at 10% CH₃CN (with 0.1% TFA) followed by a linear gradient of 24 min ranging from 10% to 40.6% CH₃CN (0.1% TFA). Flow rate was 3 ml/min and UV absorbance at 280 nm was monitored. The main degradation products were collected and identified by comparing their retention times with those of synthesized fragments and by analyzing their amino acid composition.

Statistical analysis

Means and standard errors were calculated for the amounts of the whole peptides (GnRH and analogs) or their fragments measured at different incubation times. These values were compared using students' t test. For each tested hormone a decay curve (amount of peptide vs. incubation time) was established. These exponential decay curves were linearized by a logarithmic transformation (log amount of peptide vs. incubation time). The linearity of the obtained curves was tested by linear regression analysis. The rate of degradation of sGnRH, LHRH, and their analogs were compared by analyzing the linearized decay curves using an F test. A half life time was calculated for each tested hormone, on the basis of the slope of its linearized decay curve.

1.2 Results

Degradation of GnRH and analogs by kidney cytosolic enzymes

The native peptides sGnRH and LHRH are rapidly degraded by kidney cytosolic enzymes (Figs. 1, 2, Table 1) with the main initial cleavage sites at the Tyr⁵-Gly⁶ and Pro⁹-Gly¹⁰NH₂ bonds. LHRH is cleaved faster than the sGnRH (Fig. 2, Table 1). The early appearance of the fragments 1-5, 6-10, and 1-9 of sGnRH and LHRH (Fig. 1) indicates the activity of a Tyr⁵-Gly⁶ endopeptidase and a Pro⁹-Gly¹⁰NH₂ peptidase in the kidney. The rate of degradation of both sGnRH and LHRH is significantly higher in the kidney than in the pituitary (Table 1). The half life times of sGnRH and LHRH in the kidney are 62 min and 42 min respectively.

The 1-5 fragment of sGnRH and LHRH is further cleaved to the 1-4 and mainly to the 1-3 fragments (Fig. 1). The initial increase and later decrease in the fragment 6-10 and the accumulation of tryptophan indicates the secondary degradation of the fragment 6-10 by an exopeptidase. The 1-7 fragment is probably a product of secondary degradation of the fragment 1-9.

Substitution of the position 6 glycine by a D amino acid in the [D-Trp⁶]-LHRH blocks the cleavage at the 5-6 bond. However, in a striking contrast to the situation in the pituitary, this substitution does not result in a substantial increase in the cleavage at the Pro⁹-Gly¹⁰NH₂ bond. This indicates that the Pro⁹-Gly¹⁰NH₂ peptidase in the kidney is considerably less active than in the pituitary, at least in regard to the mammalian peptides. As a consequence, the analog [D-Trp⁶]-LHRH is much more resistant to cleavage in the kidney than it is in the pituitary. The half life time of [D-Trp⁶]-LHRH in the kidney is 232 min with 21.4% of the peptide degraded at 45 min and 41.8% degraded at 180 min as compared to a half life time of this peptide in the pituitary of 120 min with 30.7% degraded at 45 min and 59% degraded at 180 min.

The two GnRH analogs with both a D amino-acid substitution at position 6 and a Pro⁹-NET carboxy terminus (the [D-Ala⁶Pro⁹NET]-LHRH and the [D-Arg⁶Pro⁹NET]-

sGnRH) were relatively resistant to degradation by kidney cytosolic enzymes (Fig. 1, 2, Table 1). As in the pituitary, the piscine analog was the most resistant in the kidney. However, both of the latter analogs were degraded by the kidney somewhat faster than by the pituitary.

Degradation of GnRH and analogs by liver cytosolic enzymes

As is the case in the pituitary and the kidney, the fragments which appear first throughout liver degradation of the native sGnRH and LHRH are the 1-5, 6-10, and 1-9 (Fig. 3 for sGnRH). This indicates that initial cleavage in the liver is carried out also by a Tyr⁵-Gly⁶ endopeptidase and by a Pro⁹-Gly¹⁰NH₂ peptidase. The kinetics of degradation of the native peptides in the liver is very similar to that observed in the pituitary (Fig. 3, 4, and Table 1). There are no significant differences in the half life times of the native peptides or in their degraded quantities after 45 and 180 min of incubation between the liver and the pituitary ($P > 0.01$). Also, secondary degradation of the native peptides by liver cytosol is similar to that observed only in the pituitary. The 1-5 fragment is relatively stable as indicated by its progressive accumulation and by the absence of the 1-3 fragment (Fig. 3). Secondary degradation is limited to the 1-9 and 6-10 fragments.

Modification of the carboxy terminus to Pro⁹NET as in the [Pro⁹NET]-LHRH results in only a slight reduction in the degradation rate (Fig. 4). This might indicate a dominant activity of the Tyr⁵-Gly⁶ endopeptidase in cleaving the whole peptide. In agreement, and as is the case in the kidney and in contrast to the situation in the pituitary, analogs with a D amino-acid substitution at position 6 (the [D-Ala⁶]-LHRH and the [D-Trp⁶]-LHRH, are cleaved relatively slowly, indicating a low activity of the Pro⁹-Gly¹⁰NH₂ peptidase (Fig. 4, Table 1). The half life time of [D-Trp⁶]-LHRH in the liver is 294 min with only 11.6% degraded after 45 min of incubation as compared to 120 min with 30.7% degraded after 45 min of incubation in the pituitary.

The GnRH analogs with both a D amino-acid substitution at position 6 and a Pro⁹NET carboxy terminus are highly resistant to degradation in the liver (Fig. 4, Table 1). These analogs are degraded at a slower rate in the liver than in the kidney or the pituitary (Table 1). As is the case in the kidney and in the pituitary, the piscine GnRH analog, [D-Arg⁶Pro⁹NET]-sGnRH is the most cleavage-resistant analog in the liver. Only 1.8% and 2.2% of this peptide were degraded, respectively, after 45 and 180 min of incubation with liver cytosolic enzymes.

1.3 Discussion

The present and previous studies (BARD second year end report) are the first to describe in detail the pathways and kinetics of degradation of native and modified forms of GnRH in fish. Both central (pituitary) and peripheral (kidney and liver) degradation were studied. The pattern of initial cleavage of the native peptides, sGnRH and LHRH, was found to be similar in the pituitary, kidney, and liver. The major sites of initial cleavage were the Tyr⁵-Gly⁶ and the Pro⁹-Gly¹⁰NH₂ bonds. This indicates that the initial cleavage of native forms of GnRH in the tissues that were studied is carried out by two major peptidases, a Tyr⁵-Gly⁶ endopeptidase and a Pro⁹-Gly¹⁰NH₂ peptidase. The presence of the same degradative activities in all three tissues suggests that the enzymes which degrade GnRH are not specific to the neuropeptide, but are rather general proteolytic enzymes. This suggestion is supported by our findings that the cleavage at the 5-6 bond is inhibited by peptides which differ structurally from GnRH,

such as substance P and Tyr⁸-Substance P.

Degradation of both native forms, sGnRH and mammalian LHRH by the pituitary, kidney, and liver of *Sparus aurata* is rapid, 33 to 55% of the peptide being cleaved as soon as 45 min of incubation with the tissues' cytosolic fraction. In all studied tissues, LHRH was found to be degraded faster than sGnRH (Table 1).

Although in all studied seabream tissues initial cleavage of GnRH involves the Tyr⁵-Gly⁶ endopeptidase and the Pro⁹-Gly¹⁰ peptidase, some differences exist in GnRH degradation pattern amongst the tissues. The cleavage pattern of GnRH analogs with either a position 6 or a position 10 modification suggests that the Pro⁹-Gly¹⁰NH₂ activity is more intensive in the pituitary than in the kidney or liver (data on degradation in the pituitary presented in BARD second year end report). [D-Trp⁹]-LHRH, which is resistant to cleavage at the 5-6 bond is cleaved intensively at the 9-10 bond in the pituitary, but only very moderately in the kidney and liver. Also, the degradation rate in the liver of Pro⁹NET-LHRH, which is resistant to cleavage by the Pro⁹-Gly¹⁰NH₂ peptidase, is very similar to that of the native LHRH. This suggests that at least in the liver the Tyr⁵-Gly⁶ endopeptidase is the major contributor to LHRH degradation and that the contribution of the Pro⁹-Gly¹⁰NH₂ peptidase is limited.

Another important difference in GnRH degradation patterns between the studied tissues is that the secondary cleavage of the native forms of GnRH is more intensive in the kidney as compared to the pituitary and liver. Whereas enzymatic degradation of sGnRH in the pituitary and liver results in the dominant production of the fragments 1-5, 6-10, and 1-9, in the kidney the appearance of these fragments is followed by the more abundant production of shorter fragments, namely the 1-3, 1-6, and 1-7. Also, whereas the 1-5 fragment of sGnRH and LHRH is stable in both the pituitary and liver, which results in its accumulation, in the kidney this fragment undergoes secondary degradation.

Substitution of the position 6 amino acid of the native peptides (glycine) by a D amino-acid renders the 5-6 bond of the peptide resistant to degradation. No or only a little 1-5 fragment was observed when these analogs were incubated for increasing durations with cytosolic fraction of pituitary, kidney, or liver. However, the 9-10 bond of such analogs is still susceptible to cleavage by the Pro⁹-Gly¹⁰NH₂ peptidase. When, in addition to the position 6 modification, the carboxy-terminus of the native peptides, glycine-amide, is modified to Pro⁹NET, the second site of initial cleavage, the 9-10 bond, also becomes resistant to cleavage, resulting in analogs highly resistant to degradation.

2. Studies on receptors of GnRH

2.1 Introduction

The aim of this study was to develop a method for demonstrating GnRH-binding sites in *Sparus aurata* pituitary membranes. Such a system is a prerequisite for investigations regarding the role of GnRH receptors in the physiological control of pituitary gonadotropin secretion. In the research describe above, it was found that analogs of GnRH had different rates of degradation. Investigations with receptors give us the possibility of comparing affinities of different analogs to their hypophyseal receptors.

2.2 Materials and Methods

Fish were killed by decapitation and pituitaries were collected and homogenized in ice-cold 0.25M sucrose in 10mM Tris-HCl, pH 7.4 (100 μ l/pituitary) with a glass homogenizer. The supernatant fraction of a 5 min, 250 x g (4°C) centrifugation was centrifuged at 10,000 x g for 30 min at 4°C. The resulting pellet (membrane fraction) was resuspended in 10 mM Tris-HCl (pH 7.4). In all radioreceptor assays fresh membrane preparations were used.

The binding assays were conducted in polypropylene microfuge tubes precoated overnight with 1% BSA in 10 mM Tris-HCl, pH 7.4. The assay buffer consisted of 10 mM Tris-HCl, pH 7.4, containing 0.1% BSA. Pituitary membrane fraction (prepared from the equivalent of 1/8-2 pituitaries as indicated) was added to 125 I-GnRHa (20,000 cpm or as indicated), with or without 10^{-6} M unlabeled GnRHa (peptide concentration at which maximum displacement was obtained) at a final volume of 250 μ l assay buffer. Following a 1 hour incubation on ice, bound and free hormone were separated by centrifugation for 5 min at 15,000 x g (Eppendorf microfuge), through 100 μ l of 10% (w/v) sucrose in 10 mM Tris-HCl, pH 7.4).

After aspiration of the supernatant, the tips of the tubes were cut off and radioactivity was determined. Specific binding was determined by subtracting non-specific binding (in the presence of 10^{-6} M unlabeled GnRHa) from total binding (no unlabeled GnRHa added).

2.3 Results

Specific binding as a function of tissue concentration

Increasing membrane concentrations (1/64 to 2 pituitary equivalents per tube) were incubated with constant amounts of labeled GnRHa. After incubation the specific binding was measured. Specific binding was found to be a function of tissue concentration with a positive linear correlation over the range of 1/16-3/4 pituitary per tube (Figure 5). The specific binding reaches saturation at about 1-2 pituitary equivalents. At saturation there is about 40% binding indicating that only about this proportion of the labeled peptide is biologically active possessing binding ability.

Time course for equilibration of binding

The time course for equilibration of the binding of the labeled GnRHa was studied by incubating 1/4 pituitary equivalent with the labeled hormone and ending the incubation after 10, 20, 30, 60, 90, 120, 180, 240 minutes. Equilibration was reached by 30 minutes (Figure 6) and both total and specific binding appeared to be stable for at least the following hour. As standard experimental condition for the subsequent studies, an incubation time of one hour was chosen.

Specificity of receptor binding

The specificity of the binding was demonstrated by displacement experiments in which pituitary membrane fractions (1/4 pituitary equivalent) were incubated with labeled [D-Ala⁶-Pro⁹-NET]-LHRH and increasing concentrations of [D-Ala⁶-Pro⁹-NET]-LHRH, [D-Arg⁶-Pro⁹-NET]-sGnRH, [D-Ala⁶-Pro⁹-NET]-sGnRH, [D-Trp⁶-Pro⁹-NET]-LHRH, [D-Arg⁶]-sGnRH, [D-Trp⁶]-LHRH, [D-Ala⁶]-LHRH, [D-Trp⁶-Pro⁹-NET]-sGnRH, sGnRH,

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Chicken II, mLHRH, Substance P, Lys-Vasopressin, and NPY. The competition curves obtained were mutually parallel (Figure 7, 8, 9, 10). The relative affinities, in relation to sGnRH which was used as a standard in all trials is presented in Table 2. Lys-Vasopressin, α -MSH, and Substance P did not cross-react with the receptor. The analogs with the highest affinities were those that had substitutions in position 6 and 10. Analogs with these substitutions had higher affinities than native hormones or analogs that had substitutions only in position 6. Analogs of the salmon native hormone had affinities as high or higher than analogs of the mammalian native hormone.

Saturation of receptor binding

To study the saturation of binding, pituitary membrane fractions (1/4 pituitary equivalent per tube) were incubated with increasing concentrations of labeled GnRH α . Specific binding was found to be saturable (Figure 11).

2.4 Discussion

We successfully developed a method to measure receptors to GnRH. The receptors were shown to be specific for GnRH and saturable. Using this methodology we were able to compare relative affinities of different analogs to the receptor. The bioactivity of a particular GnRH is probably due to its affinity to the receptor as well as to its degradation rate. In this regard, we found that analogs with substitutions at position 6 and position 10 in their amino acid sequence have higher affinity to the receptor than native forms of the hormone.

3. Structure activity relationships of gonadotropin-releasing hormones and their analogs

3.1 Introduction

In order to have a better evaluation of relative *in vivo* bioactivities of GnRH and its analogs, we tested, for native GnRH and some of its analogs, the relationship between the applied dose and the elicited response, in terms of GtH release. A dose-response study has applied implications as it points out the superactive GnRH analogs to be selected for spawning induction therapies and their minimal doses needed to induce a maximal biological response.

3.2 Methods

Experiment 1 - February 1988

Fish and stocking: Adult 3-4 year old female *Sparus aurata*, 500-800 gram body weight, were used throughout the study. They were held in outdoor tanks of 20 m³ at densities of 10 kg/m². Fish undergoing vitellogenesis were selected for experimentation. Oocyte developmental stages were determined by microscopic examination of an ovarian biopsy.

Hormones: [D-Ala⁶Pro⁹NET]-LHRH was purchased from Sigma Chemicals. [D-Trp⁶]-LHRH was purchased from Ferring, [D-Arg⁶Pro⁹NET]-sGnRH from

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Syndel Laboratories and [D-Ala⁶Pro⁹NET]-sGnRH and native salmon GnRH (sGnRH) from Bachem.

Treatments: Groups of seven females were injected intramuscularly with different doses (1.0-20 ug/kg body weight) of either [D-Ala⁶Pro⁹NET]-LHRH, [D-Trp⁶]-LHRH, [D-Arg⁶Pro⁹NET]-sGnRH, [D-Ala⁶Pro⁹NET]-sGnRH, or sGnRH. A control group was injected with 0.9% saline. Injected volume was 500 ul per kilogram of body weight (BW). Blood (600 ul) was sampled from the caudal vessels of all fish immediately before and at 1.5, 4, and 8 hours after the injection. Samples were centrifuged (2000 x g) for 30 minutes at 4°C and the plasma fraction stored at -70°C until analyzed for GtH content by homologous RIA. Analysis of variance on log-transformed data and Student-Newman-Keuls multiple range test were used to test for significant differences in plasma GtH levels between the experimental groups.

Experiment 2 - November 1988

Fish: Female *Sparus aurata*, 600-1000 gram body weight, that had been maintained in outdoor tanks (20 cubic meters) were used for the study. Fish undergoing late stages of vitellogenesis were selected. Oocyte developmental stages were determined by microscopic examination of an ovarian biopsy.

Hormones: [D-Ala⁶Pro⁹-NET]-LHRH was purchased from Sigma Chemicals. LHRH-13 was provided by the Weizman Institute.

Treatments: Groups of seven females were injected intramuscularly with different doses (0.2, 1, or 5 ug/kg body weight) of either LHRH-13 or [D-Ala⁶Pro⁹NET]-LHRH. A control group was injected with 0.9% saline. A blood sample (600 ul) was taken from each female before as well as 1.5 and 4 hours after the injection. Blood samples were centrifuged (2000 x g, 4°C) for 30 minutes. The plasma fraction was stored at -70°C until assayed for GtH by homologous RIA.

Experiment 3 - April 1989

Fish: The age and source of fish was the same as described in experiment 1. However, since the experiment took place after the breeding season, the ovaries of the selected females were in a previtellogenic state.

Hormones: [D-Arg⁶Pro⁹NET]-sGnRH and Chicken II were purchased from Syndel Laboratories. Native LHRH (mLHRH), [D-Ala⁶Pro⁹NET]-LHRH, and [D-Ala⁶]-LHRH were purchased from Sigma. Native salmon GnRH (sGnRH) was purchased from Bachem Chemicals and [D-Arg⁶]-sGnRH and [D-Trp⁶Pro⁹NET]-sGnRH were provided by INRA Laboratories, France.

Treatments: This experiment was carried using the same protocol and methodology as experiment 2. Fish were administered the above mentioned hormones or 0.9% saline. Analysis of variance on log-transformed data and Student-Newman-Keuls multiple range test were used to test for significant differences in plasma GtH levels between the experimental groups.

3.3 Results and Discussion

Experiment 1

The *in vivo* results are shown in Fig. 12. At all sampling times and at all tested doses all the GnRH analogs were significantly more potent than the native sGnRH in inducing GtH release. At the highest tested doses 20 µg/kg BW - all GnRH analogs were equipotent, at all sampling times. Differences in biopotencies between GnRH analogs appeared when their injected dose was reduced. These differences were mainly apparent at 1.5 hours post-injection and disappeared at later sampling times. At 5 and 7.5 µg/kg BW (1.5 hours) the analog of the piscine GnRH, [D-Ala⁶Pro⁹NEt]-sGnRH, was less potent than all the other three analogs which were equipotent to each other. At the lowest dose tested, 1 µg/kg BW, the two analogs of fish GnRH were significantly less potent than the two analogs of mammalian GnRH. These data demonstrate for the first time in fish that doses of GnRH analogs as low as 1 µg/kg BW induce GtH secretion. They suggest that such low doses can be used for the induction of ovulation and spawning in *Sparus aurata*. Moreover, the *in vivo* bioactivity data suggest that at present there is no need to use fish GnRH analogs in spawning induction treatments, at least in *Sparus aurata*. The classic analogs of LHRH, D-Trp⁶-LHRH and [D-Ala⁶Pro⁹NEt]-LHRH are more potent than the fish GnRH analogs. These mammalian GnRH analogs are commercially available at relatively low prices.

It is clear from our above results that differences in *in vivo* potencies of GnRH analogs might be overcome by a moderate increase in their injected dose (from 1 to 2.5 or 5 µg/kg BW). This should be taken into consideration when developing a spawning induction therapy in fish in which the most potent analogs are more expensive. Such is the case, for example, in cyprinids, in which the [D-Arg⁶Pro⁹NEt]-sGnRH is the most potent analog, but also about 5 times more expensive than the LHRH analogs.

Experiment 2

Before the administration of the hormones (0 hour) all the groups showed low levels of plasma GtH. One and a half hours after the injection, the hormone treated fish showed a dose dependent increase in plasma GtH (Figure 13). At all doses the GtH levels in the fish treated with [D-Ala⁶Pro⁹NEt]-LHRH was higher than the fish treated with LHRH-13. This was also found to be the case four hours post-treatment, with the exception of the 5 µg/kg BW dose where GtH levels were similar for the two hormones (data not shown).

LHRH-13 has been found to be a highly superactive analog in mammals. On the basis of this finding, its bioactivity was tested in fish. From the results of this experiment it is clear that LHRH-13 is less active and has no advantage over the commercially available [D-Ala⁶Pro⁹NEt]-LHRH.

Experiment 3

The results of this experiment are presented in Figure 14. At all the sampling times and at all doses, four of the analogs ([D-Ala⁶Pro⁹NEt]-LHRH, [D-Ala⁶]-LHRH, [D-Arg⁶Pro⁹NEt]-sGnRH, [D-Arg⁶]-sGnRH) are superactive as compared to native hormones. Among these four analogs there were not significant differences for most of the doses and sampling times and they seem to be essentially equipotent.

[D-Trp⁶Pro⁹NEt]-sGnRH, however, is clearly less active than the other analogs and is similar in activity to the native avian Chicken II form of the peptide. Chicken II induced significantly higher GtH levels than the other native forms of the peptide up until 4 hours after injection when administered at a dose of 1 or 5 µg/kg BW. The mammalian and salmon native forms of the peptide were equipotent to each other. sGnRH injected at a dose of 0.2 µg/kg BW did not induce GtH release above control levels whereas at the higher doses it did. Administration of any of the other GnRH forms did result in an elevation of plasma GtH levels for at least 1.5 hours after treatment.

4. Conclusions and Perspectives

A close correlation exists in Sparus aurata between the bioactivity in vivo of different forms of GnRH, in terms of inducing gonadotropin release, and their affinities to receptors as well as their resistance to degradation in peripheral tissues. The native peptides (sGnRH, LHRH, Chicken II) have a relatively low in vivo bioactivity. These same peptides also have a relatively lower affinity to pituitary receptors. Furthermore, sGnRH and LHRH were shown to be degraded relatively quickly by all the studied tissues.

All the GnRH analogs which were shown to have a higher biological activity in vivo (peptides with modifications at position 6 or at positions 6 and 10) resulted in analogs that were also highly resistant to degradation by kidney and liver enzymes. These GnRH analogs were also found to have greater affinities to pituitary receptors.

Our data do not demonstrate a strict colinearity between biological activity of GnRH analogs and their affinities and degradation rates. The [D-Arg⁶Pro⁹NEt]-sGnRH was found to be the most resistant to degradation by pituitary, kidney, and liver of Sparus aurata. It was also found to have the highest affinity to the GnRH receptor. However, this analog was not found to be more potent than [D-Ala⁶Pro⁹NEt]-LHRH or [D-Trp⁶]-LHRH in inducing GtH release in this species. This indicates that factors other than increased resistance to degradation are also involved in determining the degree of superactivity of GnRH analogs. In this regard, a circulating binding protein may be a contributor to the increased biopotency of these peptides.

The in vivo bioactivity data suggest that there is no need to use fish GnRH analogs in spawning induction treatments. The mammalian analogs of LHRH with modifications at position 6 or at positions 6 and 10 are at least as potent as the fish analogs. Considering that the mammalian analogs are more readily available at a lower cost, they are the prime candidates for use in commercial level spawning induction therapies. Moreover, it is clear from our results that even if there are slight differences in biological activity among the modified GnRH forms in a particular species, these can be overcome by a moderate increase in dose. At 5 µg/kg body weight many of the modified GnRH forms tested are equipotent.

On the basis of the research presented in this report, the analogs which have been shown to be highly bioactive, and which are commercially available, will be used to study the most efficient spawning treatment in terms of mode of administration. It is planned to introduce these analogs into polymer matrices. These polymer matrices will then be implanted into the fish such that the GnRH analog is released over a prolonged period of time. Such slow release delivery systems will provide a reproducible and efficient spawning therapy that can be used in commercial aquaculture facilities.

PEPTIDE	PITUITARY			KIDNEY			LIVER		
	T 1/2 (min)	% Degradation at: 45 min	180 min	T 1/2 (min)	% Degradation at: 45 min	180 min	T 1/2 (min)	% Degradation at: 45 min	180 min
sgNRH	77 ^a	35.4 ¹	78.3 ¹	62	36.6 ¹	88	81 ^a	33.2 ¹	77.0 ¹
LHRH	48 ^b	50.2 ²	87.7 ¹¹	42	50.1 ²	93.1 ¹¹	48 ^b	48.8 ²	91.0 ¹¹
[D-Trp ⁶]-LHRH	120	30.7	59.0	232	21.4	41.8	294	11.6	27.4
[D-Ala ⁶ -Pro ⁹ NET]-LHRH	454	12.6	24.2	299	15.3	36.1	771	6.5	15.7
[D-Arg ⁶ -Pro ⁹ NET]-sgNRH	2,186	3.9 ³	7.0	867	4.1 ³	10.9	>2500	1.8	2.2

Table 1. The rate of degradation of sgNRH, LHRH, and their analogs by cytosolic enzymes of the pituitary, kidney, and liver of *Sparus aurata*. Given are half life times of the peptides decay curves and percentages out of the initial amount of peptide (10 nmol) which were degraded after 45 and 180 min of incubation with the tissue cytosolic fraction. Values bearing the same symbols are not significantly different.

P E P T I D E

RELATIVE ED₅₀

sGnRH	1.000
mLHRH	1.500
cLHRH-II	0.811
D-Arg ⁶ -sGnRH	0.076
[D-Arg ⁶ ,Pro ⁹ NET]-sGnRH	0.035
[D-Ala ⁶ ,Pro ⁹ NET]-sGnRH	0.054
[D-Trp ⁶ ,Pro ⁹ NET]-sGnRH	0.306
D-Trp ⁶ -LHRH	0.124
[D-Trp ⁶ ,Pro ⁹ NET]-LHRH	0.043
D-Ala ⁶ -LHRH	0.226
[D-Ala ⁶ ,Pro ⁹ NET]-LHRH	0.139
Substance P	315.5

Table 2. Relative binding affinities of GnRH and analogs to Sparus aurata hypophyseal receptors.

Fig. 1: Degradation of sGnRH, LHRH, [D-Trp⁶]-LHRH and [D-Ala⁶-Pro⁹NET]-LHRH by kidney cytosolic enzymes of *Sparus aurata*. Shown are HPLC chromatographs of degradation products obtained after 0, 15, 45, 90, and 180 min of incubation of 10 nmoles of the tested peptides with 100µg protein of kidney cytosolic fraction.

Fig. 2: Rates of degradation of native and modified forms of GnRH by kidney cytosolic enzymes. Shown are the quantities of the peptides which remained intact after increasing periods of incubation with kidney cytosolic fraction.

Fig. 3: Degradation of sGnRH by liver cytosolic enzymes in Sparus aurata. Shown is an HPLC chromatograph of degradation products obtained after 0, 15, 45, 90, and 180 min of incubation of 10 nmoles of sGnRH with 100 μ g protein of liver cytosolic fraction.

Fig. 4: Rates of degradation of native and modified forms of GnRH by liver cytosolic enzymes. Shown are the quantities of peptides which remained intact after increasing periods of incubation with liver cytosol

Figure 5 Specific binding of labeled GnRH analog as a function of increasing pituitary membrane concentration

Figure 6

Time course for equilibration of GnRH analog binding to
pituitary membrane

Figure 7

Displacement of labeled GnRH analog specifically bound to pituitary membrane by unlabeled peptides

Figure 8

Displacement of labeled GnRH analog specifically bound to pituitary membrane by unlabeled peptides

Figure 9

Displacement of labeled GnRH analog specifically bound to pituitary membrane by unlabeled peptides

Figure 10

Displacement of labeled GnRH analog specifically bound to pituitary membrane by unlabeled peptides

Fig 11 Saturation of GnRH binding sites

Fig 12 IN VIVO BIOACTIVITY OF sGnRH AND ANALOGS

Figure 13 GtH secretion in response to different doses of GnRH analogs

GtH secretion in response to different doses of GnRH analogs.

Bars with same letters are not significantly different.

IN VIVO BIOACTIVITY OF ANALOGS

FINAL REPORT OF THE AMERICAN INVESTIGATOR

TITLE: "DEVELOPMENT OF IMPROVED GnRH ANALOGUES FOR CONTROLLED REPRODUCTION OF SPARUS AURATA IN FARMING CONDITIONS."

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The OBJECTIVES of our participation in this project was to contribute to the study of the fundamental reproductive physiology of the gilthead seabream (Sparus aurata) so that its successful culture can be implemented and facilitated.

In the three and a half years that this project has been in effect (date of initiation was September 1, 1985.) we have been able to achieve almost all of our specific aims. They are:

1. To provide information on the histology and cytology of the brain, pituitary gland, and gonads in Sparus at specific developmental stages from fry to adulthood.

2. To provide information in Sparus using primarily immunocytochemical (ICC) technology the normal distribution in ontogeny of endocrine regulating brain hormones, neurotransmitters, pituitary hormones, and gonadal sex steroids.

3. To determine in Sparus by cytological and immunocytochemical methods the response of the brain, pituitary, gonadal (BPG) axis to the administration of specific hormones and of specific experimental manipulations.

4. To use the platyfish (Xiphophorus maculatus), a small, freshwater teleost to assay the immunological specificity, and effectiveness of action of newly synthesized GnRH's and specific antisera.

Over 100 untreated fish which represented a variety of stages of sexual maturation were selected for sacrifice from four age groups: four to five months, six to eight months, one year, and two to three years old. Histological sections of brain and pituitary gland were stained with either Masson's Trichrome or periodic acid-Schiff (PAS) methods. Adjacent sections were subjected to immunocytochemical (ICC) procedures following the peroxidase-anti-peroxidase (PAP) method of Sternberger, et al.

The antisera used in this study included anti-gonadotropin (GtH), -growth hormone (GH), -gonadotropin releasing GnRH, -arginine vasotocin (AVT), -serotonin (5HT), -somatostatin (SRIF), -dopamine (DA), -adrenocorticotropin hormone (ACTH), -prolactin (PRL), -thyrotropin stimulating hormone (TSH). All antisera used on Sparus were also exposed to specimens of platyfish brain and pituitary where the specific responses of pituitary cells to various immunoagents have been previously detailed.

The pituitary gland of Sparus aurata is ovoid-shaped and is suspended from the diencephalon by a short, broad, infundibular stalk, immediately posterior to the optic chiasma and anterior to the saccus vasculosus. The gland is composed of the adenohypophysis and the neurohypophysis. A saccus vasculosus sits on the base of the brain immediately posterior to the pituitary gland and at the fissure formed by the lateral halves of the diencephalon.

The adenohypophysis can be divided into three regions, based on morphological, tinctorial, and immunological characteristics of the cells that occupy each region.

The pituitary gland of Sparus aurata is anatomically and cytologically similar, but not identical, to that of other teleosts. The restriction of distinct cell types to specific regions clearly divides the gland into three well-defined regions. One notable difference found in Sparus aurata is the distribution of thyrotropes. In most other teleost species, thyrotropes are found intermingled with somatotropes in close proximity to neurohypophysial projections into the CPD.

Ir-TSH containing cells are located in one or two small clusters in the interior of the CPD. Frequently thyrotropes are in proximity to, or surrounded by, gonadotropes. The reduced area of prolactin cells is characteristic of saltwater fish and reflects the demonstrated role of prolactin as a freshwater survival factor. There were significant changes in the pituitary gland between young and mature fish in the population gonadotropes, size and cell population of the pars intermedia, relative amounts of neuro- and adenohypophysial tissue and in the shape of the gland attending these differences. In mature fish, the gonadotropes appear to have proliferated throughout the ventral portion of the CPD and to have extended posteriorly towards, and even trailed into, the PI. This proliferation no doubt occurs at, and reflects, the time of sexual maturation. The examination of fish, at shorter intervals, between 8 and 12 mos. of age, is certain to reveal the times of gonadotrope proliferation and sexual maturation. As a result of this proliferation of adenohypophysial elements, the gland takes on an oval shape that is already obvious in seven-to-eight month old fish. The intensity of ir-GH is patently greater in 6 - 8 mos old animals than in the older fish. This has prompted an analysis of mechanisms that control synthesis and release of growth hormone in the different age groups and to determine the physiological/practical significance of these observations. This

study is now nearing completion.

The description of the distribution of neurohormones and neurotransmitters in the pituitary glands of 6 - 8 mos old fish will serve as a basis for future studies. These investigations, which must be broadened to include an analysis of these substances in the brain, need to be extended to other age groups under normal and experimental conditions.

In another study we determined the effects of GnRH administration on seabream pituitary gland gonadotrope activity (synthesis and release) as assessed by the simultaneous use of immunocytochemistry (ICC) and radioimmunoassay (RIA).

Thirty, two to three year old, female (300-650 gm) S. aurata were used in this study. Fifteen received a single intramuscular injection of GnRHa (7.5 ug/kg body weight). Fifteen control animals received saline. At time zero (n=5), 0.5 (n=5), 1.5 (n=10) and 24 (n=10) hrs after injection, fish were bled for RIA and pituitaries removed and fixed in Bouin's solution for ICC and histological analysis. Blood GTH levels were determined by a homologous S. aurata RIA (Zohar and Breton, unpublished). Fixed material was dehydrated in a combination of ethanol and butanol. Five micron thick paraffin sections were placed on subbed slides. Alternating groups of sections were stained with Masson's trichrome, periodic acid-Schiff (PAS), or with the indirect immunoperoxidase (PAP) method using antiserum to the beta subunit of carp gonadotropin (GTH; E. Burzawa-Gerard) at 1:1800. Control procedures included the substitution of normal rabbit serum for anti-cGTHb and the absorption of anti-serum with carp GTH. Comparisons of ICC responses were made on material processed simultaneously. Cell (CI) and nuclear (NI) indices [the averages of (length + width)/2] were determined.

Results

1. Zero hrs (uninjected controls)

Gonadotropes stain with aniline blue and PAS and contain immunoreactive (ir-) GTH. Their cellular index (CI) is 8.6u and their nuclear index (NI) is 4.5u [CI and NI are averages of (length + width)/2]. Blood GTH levels of controls remained low (2.4 +/- 0.22 ng/ml; mean +/- S.E.) and constant over the experimental period.

2. 0.5 hours After Injection:

Thirty minutes after the administration of a GnRH analog (GnRHa), circulating levels of GTH increased sharply from 2.4 +/- 0.22 (0 time) to 160 +/- 41 ng/ml. The size of the gonadotropes and of their nuclei were somewhat enlarged (CI= 9.3u +/- S.E., NI= 4.9u +/- S.E.) and the intensity of their immunoreactivity to anti-GTH was only slightly decreased.

3. 1.5 hrs after Injection

Ninety minutes after the administration of GnRHa, GTH levels increased from 2.22 ± 0.16 to 200 ± 33 ng/ml. Gonadotropes were significantly enlarged (CI= 10.0u, NI= 5.1u) and the immunoresponse to anti-GTH was most intense.

4. 24 hrs after injection:

Twenty four hrs after GnRHa treatment, GTH levels decreased to 23.3 ± 4.6 ng/ml. Ir-GTH intensity and cell dimensions were reduced (CI= 7.7u, NI= 4.3u).

This is the first report to correlate circulating levels of GTH with the cytological profile of the cells that produce them. Our observations are in agreement. They suggest that there is intense release, and the initiation of synthesis, of GTH 0.5 hrs after a single injection of GnRHa. Ninety minutes after treatment cytology and immunoreactivity indicate that both release and synthesis are at their maxima. The role of GnRH in its ability to stimulate the release of GTH is well known. However, its ability to activate synthesis of the hormone is less well documented. In summary, our morphological and physiological observations indicate that GnRHa produces immediate and intense effects on both release and synthesis of pituitary GTH in Sparus.

In other ongoing projects that stem from these studies we have:

1. developed in vitro methods to determine pituitary gland responses to humoral stimulants in dispersed cell cultures and,
2. begun to develop methods of evaluating ICC responses so that we will eventually be able to quantify secretory/synthesis activity in cells and relate this to their morphology.

Finally, this project has been highly successful as judged by the wealth of information and by the application of these data to a ever growing, successful, farming of Sparus. Additionally, much of this technology is also being utilized by laboratories and fish farms around the world. We believe that our contribution to the structure function relationship of the brain pituitary gonad axis in Sparus aurata, the gilthead seabream, has helped to foster this success.

F. Description of cooperation.

The research was carried out in three locations: The Weizmann Institute, IOLR in Eilat and at Brooklyn College. At the Weizmann Institute the synthesis, purification and characterization of GnRH analogs was carried out. Antisera were generated and studies on the biodegradation and bioactivity of these analogs were established. These studies also involved two students who carried out their studies, alternatively, at the Weizmann Institute and in Eilat. The determination of hormonal profiles, as well as studies aimed at the induction of ovulation and spawning, were carried out in Eilat. Specimens were sent frozen to Brooklyn College for histology, cytology and immunohistochemistry. In addition, the American collaborator has arrived here twice, each time for a period of over a month, to work at the Weizmann Institute and in Eilat.

G. Evaluation of the research achievements.

In a large proportion of marine fish, oogenesis is not completed under intensive culture conditions. Oocytes develop to the last stages of vitellogenesis and then undergo rapid atresia. Maturation, ovulation and spawning, do not occur. The commercial culture of *Sparus aurata* is dependent on the completion of its life cycle in captivity. In our studies we have synthesized salmon GnRH analogs; determined the degradation pathways of GnRH and its analogs by enzymes located in the pituitary gland, kidney and liver of the Sparus; determined the biological activity of these analogs in vivo as well as in vitro. The successful accomplishment of the research goals of this project were essential for the development of a protocol for the induction of spawning in the Sparus by GnRH analogs. To date, spawning is induced routinely in Eilat, and enough eggs are supplied all year round. A commercial fish farm is already functioning in Eilat and several months ago, Sparus eggs were even exported to Europe.

H. List of publications:

The following publications resulted from this project:

1. Zohar, Y., Goren, A., Tosky, M., Pagelson, G., Leibovitz, D. and Koch, Y. The bioactivity of gonadotropin-releasing hormones and its regulation in the gilthead seabream *Sparus aurata*: in vivo and in vitro studies. *Fish Physiol. Biochem.* 7: 59 (1989).
2. Goren, A., Zohar, Y., Fridkin, M., Elhanati, E. and Koch, Y. Degradation of gonadotropin-releasing hormone in the gilthead seabream *Sparus aurata*: I. Cleavage of native salmon GnRH and mammalian LHRH in the pituitary. *Gen. Comp. Endocrinol.* (in press).
3. Zohar, Y., Goren, A., Fridkin, M., Elhanati, E. and Koch, Y. Degradation of gonadotropin-releasing hormone in the gilthead seabream *Sparus aurata*: II. Cleavage of native salmon GnRH, mammalian LHRH and their analogs in the pituitary, kidney and liver. *Gen. Comp. Endocrinol.* (in press).