North American Journal of Aquaculture

ISSN: 1522-2055 (Print) 1548-8454 (Online) Journal homepage: http://afs.tandfonline.com/loi/unaj20

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To cite this article: C. H. Slater , C. B. Schreck & S. A. Sower (1999) Rapid Clearance of d-Ala^6, Pro^{9}NEt Mammalian Gonadotropin Releasing Hormone from Chinook Salmon Plasma, North American Journal of Aquaculture, 61:4, 315-318, DOI: 10.1577/1548-8454(1999)061<0315:RCODAP>2.0.CO;2

To link to this article: http://dx.doi.org/10.1577/1548-8454(1999)061<0315:RCODAP>2.0.CO;2

North American Journal of Aquaculture 61:315–318, 1999

COMMUNICATIONS

Rapid Clearance of D-Ala^6,Pro^{9}NEt Mammalian Gonadotropin Releasing Hormone from Chinook Salmon Plasma

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Abstract.—Chinook salmon *Oncorhynchus tshawytscha* were injected with D-Ala^6,Pro^{9}NEt mammalian gonadotropin releasing hormone (GnRHa) at 5 μg/kg body weight, a typical dose administered to promote ovulation or spermiation. Following this injection, salmon were sampled serially, and plasma GnRHa levels were determined by chromatography and radioimmunoassay. Plasma levels of GnRHa were elevated following injection (>500 pg/mL), then levels declined rapidly and were nondetectable in all fish by 5 h postinjection. Using nonlinear regression, the data were fitted to an exponential decay model, which calculated a plasma half-life of 0.8 h, with a 95% confidence interval of 0.5–5.8 h. These studies provide further evidence of the rapid clearance of GnRHa, when used for the acceleration of final sexual maturation.

Maintenance of broodstock is a major concern in salmonid aquaculture because of high prespawning mortality and the large number of fish that do not ovulate or spermiate in a predictable manner. Accelerating final maturation can help avoid prespawning mortality by reducing the time broodstock are held in the hatchery. Accelerated final maturation also leads to an early hatch, thereby allowing fry to start growing sooner. Ultimately, this may lead to larger fish sizes at release, which is often a goal of aquaculture facilities. Efforts to accelerate and synchronize final maturation of salmonid broodstock have used injection of gonadotropin releasing hormones (GnRH) or superactive analogs (Donaldson et al. 1981; Sower et al.1982; Crim and Glebe 1984; Fitzpatrick et al. 1984; Slater et al. 1995). One such analog, D-Ala^6,Pro^{9}NEt mammalian GnRH (GnRHa), has proven effective in accelerating final maturation in a number of salmonid fish (Donaldson et al. 1981; Sower et al. 1982; Fitzpatrick et al. 1984; Crim and Glebe 1984; Breton et al. 1990; Slater et al. 1995).

Now that the efficacy of GnRHa use in aquaculture has been established, questions are emerging about the health and safety of animals (including humans) who may consume fish treated with GnRHa. In vitro studies have determined that GnRHa is resistant to degradation by cultured pituitary cells of rainbow trout *Oncorhynchus mykiss* and by cytosolic enzymes present in pituitary, kidney, and liver cells of gilthead seabream *Sparus aurata* (Goren et al. 1990; Zohar et al. 1990a; Weil et al. 1992). In this study, an attempt was made to address some of these concerns by establishing the half-life of GnRHa in the plasma of chinook salmon *O. tshawytscha.*

Methods

Experimental animals.—Chinook salmon were raised and maintained at the Fish Genetics and Performance Laboratory of Oregon State University in Corvallis, Oregon. Fish were maintained indoors under natural photoperiod and fed a commercial diet of semimoist pellets. They were held

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Received November 10, 1998; accepted April 5, 1999

in 2-m-diameter circular fiberglass tanks that were provided with flow-through well water at 12 ± 1°C. Fish used in this experiment were 2 years old.

Assay for GnRHa.—Plasma levels of GnRHa were determined by radioimmunoassay (RIA), using the procedure described by Stopa et al. (1988) as modified by Fahien and Sower (1990). Briefly, 200 μL of plasma were extracted with 400 μL ice-cold 2 M acetic acid during rapid mixing with a polytron (Brinkmann Instruments, Westbury, New York). The resulting mixture was centrifuged for 45 min at 10,000 × gravity. The supernatant was then loaded onto a September Pak Plus C18 cartridge (Waters Corp, Milford, Massachusetts) and washed with 10 mL 0.2% formic acid. The protein was eluted with 4 mL 80% acetonitrile. The acetonitrile fraction was collected, evaporated to dryness in a Speed Vac (Savant Instruments, Farmingdale, New York), and rehydrated with 400 μL of Milli-Q H_2O. One hundred microliters of this rehydrated sample was then assayed by RIA. The D-Ala^6,Pro^{9}NEt mammalian gonadotropin releasing hormone (number 7206, Peninsula Laboratories, Inc., Belmont, California) was iodinated following the chloramine-T method (Stopa et al. 1988). Antiserum of GnRHa was prepared at the University of New Hampshire by the method of Calvin et al. (1993). The GnRHa antiserum (number 35-19-5) was used at a final dilution of 1:5,000 for RIA. The GnRH antibody binding was 44%. The lower limit of detection for the assay was 39 pg/0.1 mL and the coefficient of variation was 13% between assays.

Injection of GnRHa.—Injection of GnRHa followed the protocol of Fitzpatrick et al. (1984). Five milligrams of GnRHa was dissolved in 500 mL of sterile 0.6% saline. The fish, six at a time, were first anesthetized in a buffered solution of tricanine methanesulfonate (MS-222). Then each fish (N = 18) received an injection equivalent to approximately 5 μg/kg body weight. Injections were administered intraperitoneally via the ventral surface anterior to the vent.

Experimental design.—The 18 injected fish were randomly assigned to one of three groups. Fish were serially sampled in an overlapping schedule. All fish in group 1 were sampled at 0.5, 5, 12.5, 48, and 96 h postinjection. All fish in group 2 were sampled at 1, 7.5, 15, 48, and 96 h postinjection. All fish in group 3 were sampled at 2.5, 10, 24, 72, and 96 h postinjection. Some mortality (two fish from groups 1 and 2) was observed after the sampling at 12.5 h postinjection.

Fish were anesthetized in a buffered solution of MS-222 before sampling. Between 1 and 3 mL of blood was drawn for each sample. Blood was drawn into a Vacutainer containing lithium–heparin by puncturing the caudal vein. Blood was centrifuged, and the plasma was drawn off, frozen, and stored at −20°C until assayed. For a control, three additional (noninjected) fish were sampled for plasma at the start of the experiment with the same method used for the experimental fish.

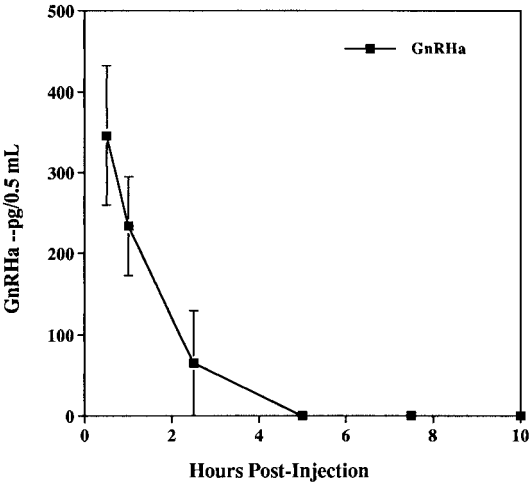

FIGURE 1.—Concentration of GnRHa remaining in the plasma of chinook salmon 0.5–10 h after a single intraperitoneal injection of GnRHa equivalent to approximately 5 μg/kg body weight; N = 6 at each time point.

Results and Discussion

Plasma levels of GnRHa were high following injection, but declined rapidly and were nondetectable in all fish by 5 h postinjection (Figure 1). Figure 1 only reflects the data obtained up to 10 h because GnRHa was nondetectable in all fish sampled beyond 10 h. Using nonlinear regression, the data were fitted to an exponential decay model, which determined the plasma half-life to be 0.8 h, with a 95% confidence interval of 0.5–5.8 h. Uninjected fish contained no detectable GnRHa in plasma. These data provide evidence for the rapid clearance of GnRHa. As stated earlier, GnRHa has proven effective in accelerating final maturation in a number of salmonid fish. Thus, our clearance data support the use of GnRHa as a viable and effective method for acceleration of final maturation.

Superactive analogs of GnRH (like GnRHa) are assumed to gain much of their additional biological activity through resistance to degradation. However, tissues differ in their ability to degrade GnRH (Bi-

enert et al. 1983; Zohar et al. 1990b). Studies of rat tissue have determined that the hypothalamus and pituitary degrade analogs, D-Phe^6,Pro^{9}NEt mammalian GnRH and D-Ser(Bu^t)^6,Pro^{9}NEt mammalian GnRH, at only 10% the rate of unaltered mammalian GnRH (analog half life: 12.5–24 h), while peripheral tissues such as liver, kidney, and lungs still degrade the analogs at a rate of 35–50% of the native peptide (analog half-life: 2.5–4 h) (Bienert et al. 1983). This situation allows GnRH analogs to have heightened biological effect at the target tissue (pituitary) while still exhibiting rapid clearance from the body.

The short plasma half-life of GnRHa is confirmed by experiments with goldfish *Carassius auratus* that found the plasma half-life of salmon GnRH and D-Arg^6,Pro^{9}NEt salmon GnRH to be only 0.54 h and 1.2 h, respectively (Huang et al. 1991). In vitro experiments with tissue homogenates of the gilthead seabream determined a slightly longer half-life for GnRHa: 5 h when incubated with kidney cell cytosol and 13 h in liver cell cytosol (Zohar et al. 1990a). These results may be due to the fact that GnRH degradation has been found to be primarily initiated by membrane-bound endopeptidases (Molineaux et al. 1988), which would not be present in the cytosolic fractions studied. Injection of striped bass *Morone saxatilis* with 20 μg/kg GnRHa resulted in elevated plasma GnRHa levels for 24–48 h (Mylonas et al. 1998); however, 20 μg/kg is four times the dose used in the present experiment. Our data support these studies on the rapid clearance of GnRHa from the plasma.

Degradation of mammalian GnRH by intact pituitary cells results in the accumulation of pGlu-His-Trp (GnRH 1–3) with rapid degradation of the C-terminal portion of the peptide (Molineaux et al. 1988). In vitro studies with gilthead seabream pituitary cell cytosol by Goren et al. (1990) determined part of the salmon and mammalian GnRH degradation pathway. These authors showed that degradation first results in GnRH 1–5, 6–10, and 1–9 fragments, with rapid secondary degradation of the 6–10 and 1–9 fragments and only the 1–5 fragment accumulating. It was found that GnRHa was resistant to this degradation pathway of salmon and mammalian GnRH (Zohar et al. 1990a). It is probable that membrane-bound endopeptidases described by Molineaux et al. (1988), which would not be present in the cytosolic fractions, may initiate the degradation of GnRHa. Because GnRH receptor binding and activation requires both ends of the GnRH peptide to be held in the proper configuration (Sealfon et al. 1997), it is unlikely that any GnRHa metabolites (short sections of the peptide, such as GnRHa 1–3 or 1–5) would remain biologically active. In fact, it is much more likely that these short peptides could remain detectable by RIA even though they are biologically inactive.

Given the rapid clearance demonstrated in the present study, it is unlikely that any GnRHa would be available to affect humans or other animals that might consume treated fish as soon as 24 h after treatment. Some biologically active GnRHa may still be present in body compartments other than the plasma (we did not assay any other tissues) after 24 h. However, this is a remote possibility given the results of experiments on goldfish where only trace amounts of radioactivity were detected in the muscle 2 h after an intra-arterial injection with ^{125}I D-Arg^6,Pro^{9}NEt salmon GnRH (Huang et al. 1991).

Current aquaculture techniques that use sustained release of GnRHa analogs via biodegradable implants maintain high levels (in the microgram range) of GnRHa in the body for 3–6 weeks (Goren et al. 1995; Mylonas et al. 1998). These protocols are very effective stimulators of spermiation and ovulation, but the fact that bioactive peptides remain in the body for weeks makes marketing of treated fish unwise without thorough knowledge of the peptide release profile and a significant waiting period between treatment and consumption. Injection of GnRHa in saline is also effective at stimulating spermiation and ovulation (Fitzpatrick et al. 1984; Breton et al. 1990; Slater et al. 1995; Mylonas et al. 1998). This protocol may require multiple injections, but it does not require the complex and expensive procedure of biodegradable implant production and allows rapid clearance of the peptide from treated individuals, a desirable characteristic if sale of treated individuals for consumption by humans or other animals (i.e., pet food) is an option.

Acknowledgments

We thank Yueh-Chen Chiang and Kim Perry for technical assistance. This work was supported by National Oceanic and Atmospheric Administration, New Hampshire/Marine Sea Grant (project R/ICD-96) and National Science Foundation (IBN-9004332). This is scientific contribution 1981.

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