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GONADOTROPIN INVOLVEMENT IN THE CONTROL OF OOCYTE MATURATION AND OVULATION IN COHO SALMON (ONCORHYNCHUS KISUTCH): NEUROENDOCRINE CONTROL OF GONADOTROPIN SECRETION, EFFECTS ON STEROID PRODUCTION AND PROPERTIES OF OVARIAN GONADOTROPIN BINDING SITES.

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ABSTRACT

The involvement of gonadotropin in the regulation of reproductive development in coho salmon (Oncorhynchus kisutch) was studied by monitoring changes in plasma gonadotropin and steroid hormone levels and the induction of oocyte maturation and ovulation following the administration of gonadotropin releasing-hormones (Gn-RH). Further studies examined the properties of gonadotropin binding sites in ovaries from adult coho salmon and immature chum salmon (O. keta).

Intraperitoneal injections of luteinizing hormone-releasing hormone (LH-RH), its active analog des-Gly^{10}[D-Ala^{6}]LH-RH-ethylamide (LH-RHA DAla^{6}) and chum salmon Gn-RH caused an increase in plasma gonadotropin levels. LH-RHA DAla^{6}, which had a more prolonged effect on the maintenance of elevated plasma gonadotropin levels than LH-RH and chum salmon Gn-RH, induced oocyte maturation and ovulation. Intraperitoneal injections of pimozide, a dopamine receptor antagonist, also increased plasma gonadotropin levels suggesting that endogenous dopamine may inhibit gonadotropin secretion. Pimozide potentiated the effects of LH-RHA DAla^{6} on gonadotropin release. Studies comparing the effects of partially purified salmon gonadotropin (SG-G100) and LH-RHA DAla^{6} on the induction of ovulation demonstrated that the induction of ovulation was dependant on the duration rather than the magnitude of the initial increase in plasma gonadotropin levels.

Plasma testosterone and 17α,20βdihydroxy-4-pregnene-3-one (17α20βP) levels were increased and 17β-estradiol levels decreased following an elevation of plasma gonadotropin levels. High levels of 17α20βP were associated with both spontaneous and gonadotropin induced oocyte maturation. Declining 17β-estradiol production appears to determine the time of oocyte maturation and ovulation by having a permissive effect on 17α20βP production.

The presence of a gonadotropin receptor was demonstrated for the first time in the salmonid ovary. Gonadotropin binding to immature chum salmon ovaries was a saturable process as the uptake of ^{125}I-labeled salmon gonadotropin was reduced in a dose dependant manner by unlabeled gonadotropin. Gonadotropin binding was attributed to a single class of high affinity binding sites present in limited numbers. Similar sites were not present in liver, kidney or muscle. The ability of various teleost and mammalian gonadotropins to decrease the binding of ^{125}I-labeled salmon gonadotropin to ovarian tissue was in agreement with the ability of these hormone preparations to stimulate steroid production in vitro. Gonadotropin binding sites were demonstrated in thecal and granulosa cell layers from adult coho salmon, although the levels of binding were lower than those for immature chum salmon ovaries.

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CHAPTER 1 - INTRODUCTION

Reproduction in teleosts is controlled by the hypothalamic-pituitary-gonadal axis (see Peter, 1983a; Donaldson and Hunter, 1983; Idler and Ng, 1983; Fostier et al., 1983). The predominant pituitary hormone regulating gonadal function in teleosts is gonadotropin. Gonadotropin is a glycoprotein with a molecular weight of about 30,000 and consists of two subunits (Donaldson, 1973; Burzawa-Gerard, 1982; Idler, 1982; Idler and Ng, 1983) as do the gonadotropins in other vertebrate classes (Licht et al., 1977a). In female teleosts, gonadotropin stimulates cAMP production in the ovary, steroidogenesis, oocyte maturation and ovulation (see Idler, 1982; Idler and Ng, 1983). Idler and associates have identified a second pituitary hormone directly involved in gonadal development which is distinguishable from the glycoprotein gonadotropin on the basis of a lower carbohydrate content and in terms of its biological activity (see Idler, 1982; Idler and Ng, 1983). This hormone termed the low carbohydrate or vitellogenic gonadotropin participates in vitellogenesis by promoting the uptake of the yolk precursor vitellogenin into oocytes (Ng and Idler, 1983). Recent data suggests that the glycoprotein gonadotropin may also mediate vitellogenin uptake (Sundararaj et al., 1982; Breton and Derrien-Guimard, 1983). In the present context the term gonadotropin is taken to refer to the glycoprotein gonadotropin.

The diverse functions controlled by gonadotropin result from its stimulatory effects on the production of ovarian hormones which in turn mediate vitellogenin synthesis, oocyte maturation and ovulation. Estrogens, in particular 17β-estradiol, act in the liver to stimulate the production of vitellogenin (see Wallace and Selman, 1981; Wiegand, 1982; Ng and Idler, 1983). An increase in plasma 17β-estradiol levels during vitellogenesis has been correlated with the growth of vitellegenic oocytes (see Fostier et al., 1983). Furthermore,

gonadotropin stimulates the production of 17β-estradiol *in vivo* (Crim and Idler, 1978; Billard *et al.*, 1978) and *in vitro* (Yaron and Barton, 1980; Kagawa *et al.*, 1982a,b, 1983; Zohar *et al.*, 1982). Progestins, in particular 17α,20βdihydroxy-4-pregnene-3-one (17α20βP), have been identified as the most potent maturation inducing steroids in the majority of teleost species (see Jalabert, 1976; Goetz, 1983). High levels of 17α20βP were found in the incubation media when rainbow trout (Fostier *et al.*, 1981a) and amago salmon (Young *et al.*, 1982a) follicles were induced to mature in response to gonadotropin *in vitro*. Additionally, high levels of 17α20βP were found in the plasma of Atlantic salmon, coho salmon and rainbow trout induced to ovulate following injections of crude pituitary extracts (Wright and Hunt, 1982; Scott *et al.*, 1983). These findings coupled with the high levels of 17α20βP in the plasma of salmonids during the spawning period (Idler *et al.*, 1960; Campbell *et al.*, 1980; Fostier *et al.*, 1981b; Fostier and Jalabert, 1982; Scott *et al.*, 1982, 1983; Young *et al.*, 1983a) are consistent with the hypothesis that 17α20βP mediates oocyte maturation in salmonids. Less is known of the endocrine control of ovulation. It appears that ovulation is mediated by prostaglandins in that indomethacin (a prostaglandin synthesis inhibitor) blocks gonadotropin-induced ovulation and prostaglandins stimulate ovulation *in vivo* and *in vitro* (Stacey and Goetz, 1982; Goetz, 1983). However, the identity of the actual prostaglandin responsible for ovulation is not known (Goetz, 1983). Catecholamines may also mediate ovulation, however, it is not known whether catecholamines influence ovulation directly or act indirectly by stimulating prostaglandin synthesis (Jalabert, 1976; Stacey and Goetz, 1982; Goetz, 1983).

Extensive functional evidence indicates that gonadotropin secretion in teleosts is mediated by a gonadotropin releasing hormone (Gn-RH) (see Ball, 1981; Peter, 1982a,b, 1983a,b). Gn-RH activity has been demonstrated in crude

hypothalamic extracts of several teleost species (Breton and Weil, 1973; Crim et al., 1976; Crim and Evans, 1980). Additional studies in goldfish indicate that gonadotropin release is also regulated by a gonadotropin-release inhibitory factor (GRIF). The involvement of a GRIF in the control of gonadotropin secretion was suggested by the large increase in plasma gonadotropin levels in goldfish following lesions of the anterior ventral preoptic region or the pituitary stalk (Peter et al., 1978; Peter and Paulencu, 1980; Nagahama and Peter, 1982). Strong evidence has been presented suggesting that dopamine acts as a GRIF in goldfish (Chang and Peter, 1983a,b; Chang et al., 1983).

Reproductive failure, prespawning mortality and asynchrony of ovulation in captive broodstock have been major problems in fish culture operations (see Donaldson and Hunter, 1983). As a solution to these problems, attention has focused on the use of hormones to accelerate reproductive development. Crude and purified gonadotropin preparations have been used for many years to accelerate oocyte maturation and ovulation (see Harvey and Hoar, 1979; Lam, 1982; Donaldson and Hunter, 1983) but are costly owing to the collection and processing of pituitary glands. The search for a replacement to the use of gonadotropin has resulted in the testing of synthetic molecules which operate at different levels in the hypothalamic-pituitary-ovarian axis (Lam, 1982; Donaldson and Hunter, 1983). These compounds either operate at a higher level in the axis by stimulating the production and/or release of gonadotropin (e.g. antiestrogens and Gn-RH) or operate at a lower level in the axis by replacing hormones which would normally be produced in response to endogenous gonadotropin (e.g. 17α20βP and prostaglandins). The most promising results have been achieved using Gn-RH.

It is well established that synthetic luteinizing hormone-releasing hormone (LH-RH) and its "superactive" analogs stimulate gonadotropin secretion in salmonid

and cyprinid fishes (see Peter and Crim, 1979; Peter, 1982a,b, 1983a,b). However, attempts to stimulate reproductive development using Gn-RH have met with varying degrees of success. LH-RH induces ovulation in the ayu (Hirose and Ishida, 1974), goldfish (Lam et al., 1975, 1976) and plaice (Aida et al., 1978), although the need for excessively high doses or multiple injections negates the practical application of this technique. In other species, including coho salmon (Donaldson et al., 1981), common carp (Weil et al., 1980; Sokolowska, 1982; Breton et al., 1983), goldfish (Peter, 1982a,b; Chang and Peter, 1983a; Sokolowska et al., 1984) and pike (Billard and Marcel, 1980) attempts to induce ovulation using LH-RH and in some cases its active analogs have been unsuccessful. Researcher's in the People's Republic of China reported that the superactive analog des-Gly^{10}[D-ALa^{6}]LH-RH-ethylamide (LH-RHA DAla^{6}) induces ovulation in several Chinese carp species but the conditions for the successful induction of ovulation and the rate of ovulation in control fish are not clearly stated (Cooperative Team for Hormone Application in Pisciculture, 1977; Fukien-Kiangsu-Chekiang-Shanghai Cooperative Group, 1977; also see Peter, 1982a; Donaldson and Hunter, 1983). The inability to routinely use Gn-RH to accelerate reproductive development presumably relates to its transitory influence on gonadotropin secretion. However, few studies have attempted to evaluate the magnitude and duration of gonadotropin changes required to accelerate reproductive development. It is necessary to consider several factors when attempting to use Gn-RH to accelerate ovulation in teleosts. These include not only the dosage and type of releasing hormone, but factors which may influence the responsiveness of the pituitary to Gn-RH and the ability of the ovary to respond to gonadotropin.

Several factors must be considered when evaluating the pituitary response to Gn-RH. A major consideration is the effect of different types of Gn-RH on

gonadotropin secretion. In goldfish (Peter, 1980) and common carp (Breton et al., 1983), LH-RH analogs promote a longer duration increase in plasma gonadotropin levels than LH-RH. However, Crim et al. (1981a) reported no major difference in the potency of LH-RH and several of its active analogs on the stimulation of gonadotropin secretion in brown trout. Studies in the coho salmon suggest that LH-RHA DAla^6 has a greater potency than LH-RH. LH-RHA DAla^6 administered following an initial injection of partially purified salmon gonadotropin (SG-G100) was more effective in accelerating ovulation in coho salmon than SG-G100 alone or in combination with LH-RH (Donaldson et al., 1981). Recent evidence indicates that the structure of teleost Gn-RH differs from LH-RH (King and Miller, 1980; Barnett et al., 1982; Sherwood et al., 1983), raising the possibility that these molecules may have different effects on gonadotropin secretion. Sherwood et al. (1983) isolated a hypothalamic peptide from chum salmon which is believed to represent a Gn-RH. The chum salmon Gn-RH decapeptide differs from LH-RH with respect to two amino acids; the replacement of tryptophan for leucine in position 7 and leucine for arginine in position 8. Although little difference was found between the activity of chum salmon Gn-RH and LH-RH in the goldfish (R.E. Peter, C.S. Nahorniak and M. Sokolowska, unpublished data cited in Peter, 1983b), it is not known whether this is the case in other species. Differences in the affinity of teleost Gn-RH receptors for teleost Gn-RH and LH-RH may account for the relatively high doses of LH-RH required to stimulate gonadotropin secretion in teleosts. In mammals, multiple injections or long-term infusion of Gn-RH can result in self-suppression of gonadotropin release due to pituitary desensitization (Rivier et al., 1978; Clayton and Catt, 1981). In addition, high doses of Gn-RH disrupts reproductive function in mammals due to a direct inhibitory action on the ovary and testis (Catt et al., 1980; Hsueh and Jones, 1981). Suppression of gonadotropin release has been

reported in goldfish (Peter, 1980) and carp (Sokolowska, 1982) at high doses of mammalian Gn-RH. These observations may be of importance in studies with salmonids as they tend to be less sensitive to LH-RH than carp (Crim and Evans, 1980; Crim et al., 1981a; Breton et al., 1983) and therefore require relatively large amounts of releasing hormone. Pituitary responsiveness to Gn-RH varies on a seasonal basis, with the most responsive period found immediately prior to spawning (Crim and Cluett, 1974; Weil et al., 1975, 1978). Therefore, unlike the situation when using gonadotropin, the selection of fish with the most advanced maturity, may be an important consideration when attempting to use Gn-RH to accelerate ovulation. An additional consideration is that pituitary responsiveness to Gn-RH is diminished owing to the influence of a GRIF. In this regard, blocking the activity of dopamine by using the dopamine receptor antagonist pimozide, potentiates the pituitary responsiveness to Gn-RH in the goldfish (Chang and Peter, 1983a; Sokolowska et al., 1984).

To be effective in promoting oocyte maturation and ovulation, Gn-RH must evoke an increase in plasma gonadotropin levels which is of sufficient magnitude and duration to stimulate 17α20βP and prostaglandin synthesis. The necessary gonadotropin changes are not well defined and may vary depending on the species and the state of maturity. In fish which have a marked ovulatory surge of gonadotropin release such as the carp (Fish Reproductive Physiology and Peptide Hormone Research Group, 1978) and goldfish (Stacey et al., 1979a), it may be necessary to duplicate this surge to induce ovulation. Salmonids by comparison show only a slow and gradual increase in gonadotropin levels during the preovulatory period with little evidence for a surge associated with ovulation (Fostier et al., 1978; Jalabert et al., 1978a,b; Fostier and Jalabert, 1982; Scott et al., 1983). In this case, the duration rather than the magnitude of the increase in circulating gonadotropin

levels may be a more important consideration.

The sensitivity of the follicle to gonadotropin as determined by the amount of gonadotropin required to induce oocyte maturation in vitro varies inversely with the state of maturity (Fostier and Jalabert, 1982; Jalabert and Fostier, 1983). Larger amounts of gonadotropin were required to induce maturation of rainbow trout oocytes obtained 4-6 weeks prior to the expected time of ovulation than for oocytes obtained one week prior to the expected time of ovulation. The sensitivity of the follicle to gonadotropin may depend on the number of gonadotropin receptors. It is well established that the actions of gonadotropins in mammals are mediated by their binding to specific high affinity receptors located in the plasma membrane of target cells (see Catt and Dufau, 1976, 1977; Dufau and Catt, 1978; Catt et al., 1980). In mammals, changes in the number of gonadotropin receptors modify the sensitivity of gonadal tissue to gonadotropin (Richards, 1979; Catt et al., 1979, 1980). Little is known of the properties of gonadotropin receptors in teleosts or of changes in their number during ontogeny. In one study, Cook and Peter (1980a) reported that ovaries from goldfish having completed vitellogenesis or undergoing recrudesence bound more ^{125}I-labeled carp gonadotropin than ovaries from goldfish undergoing regression. Autoradiographic analysis indicated that the greatest accumulation of label within the ovary occurred in the special thecal cells (Cook and Peter, 1980a). These cells are considered to represent the major site of steroidogenesis in the ovary (Hoar and Nagahama, 1978; Nagahama, 1983). Recent work suggests that gonadotropin has two sites of action during 17α20βP biosynthesis. Both the thecal and granulosa cell layers contribute to the synthesis of 17α20βP in rainbow trout and amago salmon (Nagahama, 1983; Young et al., 1983b). Gonadotropin acting on the thecal cell layer stimulates the production of 17α-hydroxyprogesterone which is converted to 17α20βP in the

granulosa cell layer by a 20β-hydroxysteroid dehydrogenase (20β-HSD). Young et al. (1983b) reported that gonadotropin increases the activity of 20β-HSD in isolated granulosa cell layers from the amago salmon. These results suggest that gonadotropin receptors may be present in the thecal and granulosa cell layers.

The amount of gonadotropin required to induce oocyte maturation and ovulation may also depend on the biosynthetic capacity of the follicle at the time of injection. Although little is known of the prostaglandin changes in fish which ovulate spontaneously (see Goetz, 1983), 17α20βP levels in salmonids remain low until a few days prior to ovulation (Fostier et al., 1981b; Fostier and Jalabert, 1982; Scott et al., 1983; Young et al., 1983a). The possibility exists that attempts to accelerate ovulation may fail as the follicle has only a limited capacity to produce 17α20βP and prostaglandins. Two injections of crude pituitary extracts over a 72 hr period have been shown to increase 17α20βP production and induce ovulation in rainbow trout, Atlantic salmon and coho salmon (Scott et al., 1982; Wright and Hunt, 1982). In these cases, 17α20βP levels remain low for several days following injection, which presumably reflects the time lag to augment the activity of steroid converting enzymes in the follicle. Previous studies have shown that 17α20βP synthesis depends on mRNA and protein synthesis as actinomycin D and puromycin block the gonadotropin induced maturation of rainbow trout oocytes in vitro (Jalabert, 1976). This delay may be an important consideration when using Gn-RH in that injections of crude pituitary extracts or gonadotropin preparations which induce ovulation generally result in a much larger increase in plasma gonadotropin levels than injections of Gn-RH (see Bieniarz et al., 1980; Breton et al., 1983).

In addition to considering the effects of gonadotropin on 17α20βP production, attention must be given to other steroid hormones which may influence oocyte

maturation and ovulation. The preovulatory period in rainbow trout, amago salmon and coho salmon in characterised by declining plasma 17β-estradiol levels and high testosterone levels (Fostier and Jalabert, 1982; Sower and Schreck, 1982; Scott et al., 1983; Kagawa et al., 1983). Although 17β-estradiol is ineffective and testosterone has a limited effectiveness in promoting oocyte maturation (Jalabert, 1975; Young et al., 1982a), the maintenance of low 17β-estradiol and high testosterone levels may contribute to the proper steroid environment for 17α20βP synthesis and ovulation. It has been proposed that declining plasma 17β-estradiol levels may determine the time of ovulation by facilitating the release of gonadotropin necessary for 17α20βP production and ovulation (Fostier et al., 1978; Scott et al., 1983). 17β-estradiol may also directly influence the production of 17α20βP. High levels of 17β-estradiol reduce the effectiveness of gonadotropin on the promotion of oocyte maturation in vitro (Jalabert, 1975; Theofan, 1981 cited in Goetz, 1983; Jalabert and Fostier, 1983). The basis of this action is poorly understood, although in amphibians, 17β-estradiol reduces the conversion of pregnenelone to progesterone (Speigel et al., 1978). Little is known of the involvement of 17β-estradiol during induced ovulation. As gonadotropin stimulates 17β-estradiol production during vitellogenesis (see above) the control of estrogen biosynthesis during attempts to accelerate ovulation may be critical.

The major objective of this thesis has been to investigate the involvement of gonadotropin in the regulation of ovarian function in adult coho salmon. The possible use of mammalian and chum salmon Gn-RH as a means of accelerating reproductive development was investigated by studying their effects on plasma gonadotropin levels and ovulation (Chapter 3). Additionally, plasma gonadotropin levels in fish injected with LH-RHA $DAla^6$ and salmon gonadotropin were compared with the levels in fish which ovulate spontaneously to evaluate the importance of

the magnitude and duration of the increase in gonadotropin levels on ovulation. The significance of steroid hormone changes during the preovulatory period was investigated by studying the profiles of 17β-estradiol, testosterone and 17α20βP in the plasma of fish during spontaneous and induced ovulation (Chapter 4). Further studies were conducted to determine the sequence of changes in steroid biosynthesis during development by comparing the ability of ovarian follicles of various stages of maturity to produce these steroids in response to gonadotropin in vitro. Finally, Chapter 5 deals with an investigation of the properties of gonadotropin receptors in ovarian follicles from adult coho salmon and immature chum salmon.

CHAPTER 2 - MATERIALS AND METHODS

A. Experimental Animals

Chinook and chum salmon were obtained at ages 2-3 years from stocks raised at the West Vancouver Laboratory, Dept. of Fisheries and Oceans. These fish were held outdoors in 3 m-diameter fiberglas tanks supplied with aerated saltwater at 8-11°C. Fish were fed Oregon Moist Pellets (Moore-Clarke Co. Ltd., La Conner, Wash.) twice daily to satiation. Female chinook salmon used in experiments weighed about 1.5 kg and had an average oocyte diameter of 1.5-2.5 mm. Chum salmon weighed 400-600 g and contained oocytes of 0.6-1.0 mm in diameter.

Adult coho salmon were obtained from the Capilano Salmon Hatchery following their anadromous migration. Fish were transferred to the West Vancouver Laboratory and held outdoors in 3m-diameter fiberglas tanks supplied with aerated freshwater at 10°C. Adult coho salmon were starved as they do not feed during this stage of their lifecycle.

B. Hormone Injections, Tissue and Blood Sampling

Adult coho salmon were acclimated to the laboratory conditions for a minimum of four days prior to blood sampling or hormone injections. During all handling procedures, fish were anaesthetized by immersion in 0.02% 2-phenoxyethanol. Fish that were to receive hormone injections were screened on the day prior to injection to determine sex and maturity on the basis of the morphology of oocytes which were expelled following abdominal massage. Only those fish which had not undergone germinal vesicle breakdown (GVBD) were used for hormone injection. At this time, individual fish were weighed and identified by means of a coded numbered tag inserted into the musculature below the dorsal fin (Floy Tag and Manufacturing Inc., Seattle, Washington).

LH-RH and LH-RHA DAla^6 were purchased from Peninsula Laboratories Inc., San

Carlos, California or provided by Syndel Laboratories Ltd., Vancouver. Chum salmon Gn-RH was provided by Drs. J. Rivier and W. Vale, The Salk Institute, La Jolla, California and partially purified chinook salmon gonadotropin (SG-G100; Donaldson et al., 1972; Donaldson, 1973) was provided by Dr. E.M. Donaldson, West Vancouver, British Columbia. These hormones were dissolved in 0.65% saline immediately prior to injection. Pimozide (Janssen Pharmaceuticals Ltd., Beerse, Belgium) was obtained as a gift from Dr. R.E. Peter, University of Alberta, Edmonton, Alberta. Pimozide was suspended in saline containing 0.1% sodium metabisulphite. Hormones were administered intraperitoneally at the base of the pectoral fins using a 1 - ml tuberculin syringe (needle size; 22G). Injections volumes were adjusted to 0.4ml/kg bw. Details of the dosage and injection schedule are provided in the protocols to the experiments in Chap. 3.

Blood was removed from the caudal vessels using a heparinized 1 or 3 ml tuberculin syringe (needle size; 21G). Blood samples (generally 0.75 ml) were expelled into 1-ml polypropylene centrifuge tubes and held on ice prior to centrifugation at 4000 rpm for 10 min at 4°C (Sorval, Model RS-5). The plasma was then divided among several tubes and stored frozen at -40°C.

Oocyte maturity was determined according to the criteria established by Jalabert et al. (1978b). In live fish, oocyte maturity was assessed on the basis of oocytes expelled by abdominal massage. At terminal samplings, oocyte maturity was assessed by visual examination of oocytes within the ovary after the fish had been killed. Ovulation was indicated by the release of a stream of oocytes following the application of gentle pressure to the abdomen.

In most experiments, the gonosomatic index (GSI) was calculated, based on the ovary weight expressed as a percentage of the total body weight. The average oocyte diameter was calculated on the basis of 10-20 oocytes.

C. Stimulation of Steroid Production In Vitro

Chinook salmon at the mid-vitellogenic stage of development were killed by decapitation and the ovary quickly removed, weighed and placed in cold fish Ringer's (Donnen, 1976). Ten individual oocytes with intact follicle layers (follicles) were transferred to borosilicate glass culture tubes together with 200 µl of fish Ringer's. Hormones to be tested were dissolved in fish Ringer's and added to the incubation tubes in 200 µl aliquots. Incubations were routinely conducted for 24 hr in a water bath at 10°C in an atmosphere of 95% O_2:5% CO_2. Following incubation, 600 µl of Ringer's containing 0.1% gelatin was added, the media collected by aspiration and stored frozen at -40°C prior to 17β-estradiol measurement by RIA. Results were expressed as the amount of 17β-estradiol produced per tube.

A similar approach was used to examine the effects of gonadotropins on steroid production by follicles obtained from adult coho salmon. These studies were based on the incubation of 5 follicles per ml Ringer's, owing to their large size. Additionally, postovulatory follicles were dissected free from surrounding ovarian tissue and incubated in a similar fashion. In these studies, the amounts of 17β-estradiol, testosterone and 17α20βP released to the media were determined by RIA.

Studies were also conducted to evaluate the effects of gonadotropins on steroid production by testis pieces from adult coho salmon incubated in vitro. Testis were placed in cold fish Ringer's, minced with scissors into small pieces and divided into groups containing about 10 mg of tissue. The testis pieces were then rinsed with fresh Ringer's and incubated together with gonadotropin in 400 µl of Ringer's. After incubation for 20-24 hr at 10°C, tubes were placed on ice and 600 µl of cold Ringer's containing 0.1% gelatin added. Tubes were then centrifuged

at 4000 rpm for 10 min at 4°C to compact the testicular tissue. The media were collected and stored frozen prior to testosterone determination by RIA. The pellet of testicular tissue was dried, digested with 0.1 N NaOH and analysed for protein content by the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard. Results were expressed as the amount of testosterone produced per mg of protein.

In most studies, gonadal tissues were incubated with Ringer's alone or with graded amounts of SG-G100 using 3-4 replicates at each hormone concentration. Hormone specificity was examined by comparing the activity of fish and mammalian gonadotropin preparations. Additional chinook salmon gonadotropin preparations were obtained from Syndel Laboratories. These included acetone dried pituitary powder (ADP) and two preparations (SGA-2359 and 2360) prepared in a similar fashion to that described for G-75 II gonadotropin (Idler et al., 1975a). These latter fractions were identified during purification on the basis of their ability to augment cAMP production in immature rainbow trout ovaries and were estimated to have at least twice the potency of SG-G100 (Dr. T. Owen, pers. comm.). Ovine LH (NIH-LH-S-19) and ovine FSH (NIH-FSH-S-9) were obtained from the National Institutes of Health, Bethesda, Maryland) and hCG (3000 IU/MG) was obtained from Sigma, St. Louis, Missouri.

D. Gonadotropin Receptor Studies

Immature chum salmon were killed by decapitation and their ovaries removed and weighed. Ovaries from 2-4 chum salmon were chopped using scissors and homogenized with a loose fitting glass-teflon homogenizer in 10 volumes of buffer which consisted of 0.01 M phosphate, pH 7.5 containing 0.1 M NaCl, 2.5 mM $CaCl_2$, and 0.1% BSA. The homogenate was filtered through 3 layers of cheesecloth and the filtrate centrifuged at 3000 g for 10 min at 4°C. The pellet was resuspended in

buffer and the centrifugation step repeated. The second pellet was resuspended at a concentration of 50-150 mg original wet weight of tissue per 0.2 ml and used for binding studies. The same procedure was used to prepare tissue pellets from kidney, liver, muscle and testes for studies which examined the tissue distribution of gonadotropin binding sites. For a preliminary study to determine the distribution of gonadotropin binding sites in subcellular fractions of the ovary, tissue pellets were prepared by centrifugation of ovarian homogenates at 3000 g, 20,000 g and from the supernatant fluid obtained at 3000 g centrifuged at 20,000 g. In this case, the tissue pellets were weighed and resuspended in buffer at a concentration of 20 mg wet weight of pellet per 0.2 ml.

Adult coho salmon were killed by concussion and ovarian follicles separated from the connective tissue. Yolk was expressed by the application of pressure to rupture the oocyte and rinsed from the follicular tissue prior to homogenization. Conditions for homogenization and centrifugation were similar to that described for immature chum salmon ovaries. The 3000 g particulate fraction was weighed and resuspended in buffer at 20 mg wet weight of pellet per 0.2 ml. In other studies, intact ovarian follicles were used to study the binding of ^{125}I-labeled salmon gonadotropin in a manner similar to that described for the determination of steroid production *in vitro*. Additionally, thecal and granulosa cell layers were separated from preovulatory follicles as described by Kagawa *et al.* (1982b) to study binding to specific cell types in the ovarian follicle.

^{125}I-labeled salmon gonadotropin was prepared by the lactoperoxidase method as described in Section E. For receptor binding studies, 10 µg of SGA-2359 and 0.5-1.0 mCi ^{125}I were used for iodination. In most experiments, the ^{125}I-labeled salmon gonadotropin was further purified by group specific affinity chromatography on Con A Sepharose (Pharmacia) by modification of the procedure described by Dufau

et al. (1972). Columns containing Con A Sepharose to a height of about 1.2 cm were prepared in disposable 5-ml syringe barrels (inside diameter 1.1 cm) fitted with sintered glass discs. Gonadotropin was added to the column in 400 µl of elution buffer (0.05 M phosphate buffer, pH 7.5 containing 0.85% NaCl and 0.1% bovine gamma globulin or BSA). After equilibration for 10 min, damaged gonadotropin was eluted with 6 ml of buffer. Gonadotropin was displaced from the column by the addition of 0.1 M 1-o-methyl-α-D-glucopyranoside (Sigma) in elution buffer. Fractions which eluted from the column with 2 - 3 ml of buffer containing competing sugar were used for binding studies. In an additional study, ^{125}I-labeled salmon gonadotropin was subjected to filtration on a much larger column (1.3 x 32 cm) containing Sephacryl S-200 (Pharmacia).

Binding studies were conducted by combining 50 µl of unlabeled hormone solution or buffer alone, 50 µl of ^{125}I-labeled salmon gonadotropin (40,000-60,000 cpm) and 200 µl of tissue preparation in borosilicate glass tubes. Samples were routinely incubated for 18-24 hr at 20°C. Following incubation, 1 ml of cold assay buffer was added to each tube and the tubes centrifuged at 4000 rpm for 10 min at 4°C. The pellets were washed by resuspension in 1 ml of cold assay buffer and the centrifugation step repeated. The final supernatant was decanted and the radioactivity in the pellet counted. Groups of 10 intact follicles or thecal and granulosa cell layers from preovulatory follicles were incubated in borsilicate glass tubes for 18 hr at 20°C. In these studies, the incubation volume was adjusted to 1 ml by the addition of 900 µl of buffer together with 50 µl ^{125}I-labeled salmon gonadotropin and 50 µl of unlabeled competitor. Following incubation, the contents of the tubes were decanted on to filter paper and rinsed twice with 5 ml of incubation buffer. Ovarian tissue was removed from the filter paper and placed in new tubes for counting.

In all binding studies, determinations at each dose of unlabeled hormone were made in duplicate or triplicate. Reaction tubes containing ^{125}I-labeled salmon gonadotropin and ovarian tissue were incubated to determine total binding. Additional tubes containing 10 µg of SG-G100 in addition to the ^{125}I-labeled salmon gonadotropin and ovarian tissue were incubated to determine non-specific binding. The difference between total binding and non-specific binding was designated as specific binding.

E. Gonadotropin RIA

Plasma gonadotropin levels were measured by RIA using an antiserum directed against chinook salmon DEAE-1 gonadotropin (Pierce et al., 1976) provided by Dr. J. Pierce, University of California, Los Angeles. SGA-2360 gonadotropin was used for iodination and chinook salmon gonadotropin (Drs. R. Billard and B. Breton, Institut National de la Recherche Agronomique, Jouy en Josas, France) was used as the RIA standard. Validation of this system for the measurement of gonadotropin in coho salmon plasma is provided in Appendix 1.

^{125}I-labeled salmon gonadotropin was prepared for the RIA by either modification of the chloramine-T (Licht et al., 1977b) or lactoperoxidase (Thorell and Johannson, 1972) methods. Iodination by the chloramine-T method involved combining 40 µl 0.5 M phosphate buffer pH 7.5 (PB) with 500 µCi ^{125}I (5 µl, Amersham) and 4 µg SGA-2360 (20 µl). The reaction was initiated by addition of 1 µg chloramine-T in 0.05 M PB (10 µl). After 12 min, the reaction mixture was diluted with 200 µl of 0.25 % BSA in 0.05 M PB and immediately chromatographed (see below). For lactoperoxidase iodination, 40 µl of 0.5 M PB was mixed with 5 µl ^{125}I, 20 µl gonadotropin and 10 µl lactoperoxidase (100 IU/ml; Calbiochem, La Jolla, California). Iodination was achieved by the addition of 10 µl hydrogen peroxide (30% H_2O_2 diluted 1:30,000). After 2 min, the addition of H_2O_2

was repeated. Following a second 2 min incubation, the reaction mixture was diluted with 500 µl 0.05 M PB containing 0.85% NaCl (PBS) and chromatographed.

Separation of labeled gonadotropin from unreacted iodide involved chromatography through a 1 X 18 cm column containing G-50 (fine) Sephadex (Pharmacia). Prior to chromatography, the column was primed with 1 ml PBS containing 5% BSA to reduce adsorption of labeled hormone to the column and then rinsed with 20 ml PBS. The gonadotropin fractions were diluted in PBS containing 0.1% BSA and stored frozen for up to 3 weeks. The specific activity of labeled gonadotropin used in the RIA was 60-80 µCi/µg.

The gonadotropin content of plasma samples measured by RIA did not differ using ^{125}I-labeled gonadotropin prepared by the chloramine-T or lactoperoxidase methods. However, the incorporation of ^{125}I into gonadotropin was more consistent when the lactoperoxidase method was used and was the method of choice.

All steps in the gonadotropin RIA were conducted at 4°C using PBS containing 0.1% ovalbumin as the diluent buffer. For hormone measurement, 50 µl of standard or plasma was combined with 50 µl of diluent and 200 µl of dilute antibody containing 2.5% Normal Rabbit Serum (Calbiochem). At 24 hr, 6000 cpm of ^{125}I-labeled gonadotropin in 100 µl of diluent was added. At 48 hr, 100 µl of goat anti-rabbit gamma globulin (Calbiochem) was added. Following an additional 24 hr incubation, samples were centrifuged at 4000 rpm for 15 min. The resulting supernatants were then poured off and the pellets containing the antibody-bound hormone counted. Nonspecific binding was assessed by the same protocol except that the antibody was omitted. Non-specific binding generally represented about 5-8% of the added radioactivity. Using this format, approximately 85% of the added ^{125}I-labeled gonadotropin bound to excess antibody. The working dilution of antibody was adjusted so that 30-35% of the ^{125}I-labeled gonadotropin was bound

in the absence of competitor. Sensitivity of the assay was 0.05 to 0.1 ng/tube (1.0-2.0 ng/ml when using 50 µl plasma). Intraassay variability was 0.04-0.07 (N=6 determinations) and the interassay variability was 0.06 - 0.11 (N = 4 determinations).

F. Steroid Hormone RIA

17β-estradiol, testosterone and 17α20βP were measured by RIA in plasma samples obtained from adult female coho salmon and in the media from ovary and testis incubations in vitro. The steroid content of plasma samples was determined directly and did not depend on the extraction and chromatographic isolation of individual steroids. Plasma samples (50 µl) were combined with 1 - 3 ml of assay buffer (0.05 M phosphate, pH 7.6 containing 0.1% gelatin; Scott et al., 1982) and heated at 70°C for 1 hr as described by Scott et al. (1982, 1983). Aliquots of the media obtained from follicle incubations were extracted with eight volumes of diethyl ether and dried under nitrogen. The dried extracts were dissolved in assay buffer and measured directly by RIA. No correction was applied for losses incurred during the extraction as the recovery of radiolabeled steroids were greater than 95%. Media from testis incubations were assayed directly for testosterone content. In preliminary studies, media from the incubation of ovarian follicles and testicular pieces were assayed using 0.05 M tris-HCl at pH 8.0 containing 0.1 M NaCl, 0.1% NaN_3 and 0.1% gelatin as the RIA buffer. The validation of these protocols for steroid measurement is provided in Appendix 2.

17β-estradiol and testosterone were measured using rabbit anti-17β-estradiol-6(O-carboxymethyl)-oxime-BSA and antitestosterone-7αcarboxymethyl-thioether-BSA serum obtained from Miles Laboratories, Rexdale, Ontario. The anti-17β-estradiol serum cross reacts with 17β-estradiol, 17α-estradiol, estrone, estriol, testosterone and 17α20βP, at 100, 1.6, 1.4, 0.8, < 0.01, and < 0.01% levels,

respectively. The antitestosterone serum cross reacts with testosterone, 5αtestosterone, 11-oxotestosterone, 17β-estradiol and 17α20βP at 100, 17, 4.5, < 0.01 and < 0.01% levels, respectively. Radiolabeled (2,4,6,7-^{3}H) 17β-estradiol and (1,2,6,7-^{3}H) testosterone were obtained from Amersham. For hormone measurement, 200 µl of appropriate standard (Sigma) or diluted sample was combined with 200 µl of ^{3}H-steroid (4000 cpm) and 200 µl of diluted antiserum. Samples were incubated at room temperature for 16-20 hr. The samples were then cooled on ice for at least 15 min prior to the addition of 200 µl of a Dextran-coated charcoal suspension containing 0.5% Norit A charcoal and 0.05% Dextran T-70 in assay buffer. After a further 10 min incubation on ice, samples were centrifuged at 4000 cpm for 10 min at 4°C. The resulting supernatant fluid was poured directly into scintillation vials and combined with 6 ml of scintillation fluid (667 ml toluene, 333 ml Triton X-100, 4 g PPO and 0.2 g dimethyl POPOP) for counting.

17α20βP was measured by RIA using sheep anti 17α20β-3-carboxymethyl-oxime-BSA serum provided by Dr. A.P. Scott (Scott et al., 1982). This antiserum shows insignificant cross reaction with 17α-hydroxyprogesterone, progesterone, 17β-estradiol and testosterone (Scott et al., 1982). Radiolabeled 17α20βP was prepared from (1,2,6,7-^{3}H) 17α-hydroxyprogesterone (Amersham) by treatment with 3α,20β-hydroxysteroid dehydrogenase (Sigma) as described by Scott et al. (1982). The separation of radiolabeled 17α20βP from the parent compound was accomplished by chromatography on a precoated prewashed silica gel plate (LK5DF, 250 µm thickness, Whatman) developed with a dichloromethane (50 ml): diethyl ether (20 ml) mixture. Initially, standard 17α-hydroxyprogesterone and 17α20β (Steraloids, Wilton, New Hampshire) were chromatographed and their positions on the chromatogram identified in an iodide chamber. Chromatography of radiolabeled 17α-hydroxyprogesterone following treatment with the 3α,20β-hydroxysteroid dehydrogenase resulted in over

90% of the radioactivity on the plate migrating to the expected position of 17α20βP. This material was extracted from the chromatogram and stored in absolute ethanol for direct use in the RIA. For the RIA, 100 µl of standard of diluted sample was combined with 50 µl of tracer (1500 cpm) and 50 µl of antiserum at a final dilution of 1:25,000. The samples were incubated at room temperature for 16-20 hr. After incubation, the samples were cooled on ice for at least 15 min before the addition of 1 ml of cold Dextran-coated charcoal suspension. After standing for 10 min, the samples were centrifuged (4000 rpm for 10 min at 4°C), the supernatant poured into scintillation vials and combined with 8 ml of scintillation fluid.

The antibody concentrations used in each of the foregoing RIA procedures were adjusted so that 45-55% of the radiolabelled steroids were bound in the absence of competitor. All samples were analyzed in duplicate.

Intraassay and interassay variation were assessed using pooled plasma samples which corresponded to approximately 75 and 35% of the total binding observed in the absence of competitor. For all three assays, the intraassay and interassay variations were less than 7 and 14%, respectively.

G. Radiation Counting

Samples containing ^{125}I-labeled salmon gonadotropin were counted in a Picker Pace-1 automatic gamma counter and those containing ^{3}H-steroids were counted in a Packard Tri-Carb 460 C Liquid Scintillation Spectrometer. All samples were counted for 10 min or 10,000 counts.

H. Statistics

All data were expressed as the mean ± standard error. One way analysis of variance and Duncan's Multiple Range Test were used to determine differences between plasma gonadotropin and steroid values of the same experimental group at

different sampling times or differences between the values of experimental groups at the same sampling time. In these cases, $\log^{10}$ transformation was used to achieve homogeneity of variance. The same approach was used to calculate differences in the effectiveness of various gonadotropin doses on the stimulation steroid production in vitro. Comparisons based on two treatments were analyzed by Student's t-test. The effects of hormone treatments on oocyte maturation and ovulation were compared with the saline injected group using the Mann Whitney U test. Parallel line statistics were used to compute the potencies of different goadotropins on the stimulation of steroid production in vitro and from competitive inhibition studies based on RIA or receptor binding. When non-parallelism was apparent, potencies were calculated graphically from the dose giving a 50% response or were discussed separately in the text.

CHAPTER 3 - GONADOTROPIN CHANGES ASSOCIATED WITH SEXUAL MATURATION IN FEMALE COHO SALMON

A. Introduction

Considerable attention has focused on the development of techniques to accelerate reproductive development in Pacific salmon owing to the marked asynchrony of ovulation and high prespawning mortality of captive broodstock (Donaldson et al., 1982; Donaldson and Hunter, 1983). The present studies were undertaken to evaluate the possibility of using mammalian and teleost Gn-RH to replace fish pituitary extracts for the acceleration of ovulation in coho salmon. In Exp. I and II, plasma samples were taken at regular time intervals during the preovulatory period to determine the pattern of gonadotropin secretion in coho salmon at the time of oocyte maturation and ovulation. The effects of LH-RH, LH-RHA DAla^6 and chum salmon Gn-RH were investigated in Exp. III and IV by studying their short term effects on plasma gonadotropin levels and oocyte development. The effects of LH-RHA DAla^6 alone and in various combinations with SG-G100 on plasma gonadotropin levels and the acceleration of ovulation were examined in Exp. V. The possible involvement of dopamine in the regulation of gonadotropin secretion was investigated in Exp. VI by studying the effects of intraperitoneal injections of the dopamine receptor antagonist pimozide on plasma gonadotropin levels and ovulation.

B. Experimental Protocol

I. Gonadotropin and Ovarian Changes During Sexual Maturation.

A group of 120 adult coho salmon were held at the West Vancouver Laboratory during the 1979 spawning season. Subgroups of 10 females were bled at weekly or biweekly intervals during a seven week period which commenced on September 18. Plasma samples were analyzed for gonadotropin content. After blood sampling, fish

were killed and their ovaries removed for the determination of GSI and oocyte diameter.

II. Preovulatory Gonadotropin Changes

A group of 12 adult coho salmon were bled at 2-day intervals throughout the final stages of the preovulatory period. Four fish died prior to ovulation and were not considered for analysis. Gonadotropin was measured in plasma samples obtained from 8 fish which were sampled until ovulation. The time of ovulation was used as a reference for analysis, with data presented which covers the 12 day period preceding ovulation.

III. Effects of LH-RH and LH-RHA DAla^6 on Plasma Gonadotropin Levels and Oocyte Development

Adult coho salmon were divided into five groups after screening to select fish with oocytes containing a central germinal vesicle. Groups of 7-8 fish received single intraperitoneal injections of LH-RH (0.2 or 1.0 mg/kg) or LH-RHA DAla^6 (0.02 or 0.2 mg/kg). Control fish were injected with saline. Blood samples were taken at the time of injection (1100-1130 hr, October 31, 1980) and at increasing intervals to 96 hr for gonadotropin measurement. At 96 hr, the fish were killed and the GSI and oocyte diameter determined. The effects of the hormone treatments on oocyte development were assessed at 1.5 hr on the basis of oocytes expelled by abdominal massage and at 96 hr by examination of oocytes in the ovary. To facilitate comparisons between treatments, oocytes were classified numerically according to the stage of development: 1.0 for oocytes prior to maturation, in which the germinal vesicle occupied a central position; 2.0 for oocytes undergoing germinal vesicle migration, in which the germinal vesicle had migrated to a peripheral position; and 3.0 for oocytes which had completed GVBD, in which the germinal vesicle was no longer visible and the yolk had become transparent. The

arithmetic mean of these ratings was used to calculate an oocyte maturity index.

IV. Effects of Chum Salmon Gn-RH, LH-RH and LH-RHA DAla^6 on Plasma Gonadotropin Levels and Ovulation

Six groups consisting of 5-8 adult coho salmon were given single intraperitoneal injections of chum salmon Gn-RH, LH-RH and LH-RHA DAla^6 at 0.02 or 0.2 mg/kg. Control fish were injected with saline. Fish were too immature to assess oocyte development using abdominal massage at the time of injection. In preinjection samples from 8 fish, the average oocyte diameter was 5.1 ± 0.2 mm while the GSI was 14.7 ± 1.1%. The germinal vesicle was not visible in oocytes obtained from fish sampled at the time of injection. Gonadotropin levels were measured in the preinjection samples (10:00 hr, September 28, 1982) and in samples obtained 1.5, 24 and 48 hr following injection. Fish were checked for ovulation at 10 days post injection (October 8).

V. Effects of LH-RHA DAla^6 and SG-G100 on Plasma Gonadotropin Levels and Ovulation

Coho salmon were divided into nine experimental groups after an initial screening to eliminate fish in the final stages of oocyte maturation. Five groups of fish were given a single injection at time 0 (11:00-12:00 hr, October 15, 1981). Single treatment groups included a low and high dose of LH-RHA DAla^6 (0.02 and 0.2 mg/kg), SG-G100 (0.1 mg/kg) and combined injections of SG-G100 with the low and high dose of LH-RHA DAla^6. Three additional groups were given a second injection at 72 hr. Two of these groups were injected at time 0 and 72 hr with either the low or high LH-RHA DAla^6 dose while the third group was injected with SG-G100 followed at 72 hr with the high LH-RHA DAla^6 dose. A control group was injected with saline. Experimental groups consisted of 17-18 fish, eight of which were bled at various times after injection for gonadotropin determination. Blood samples were removed from 12 randomly selected fish at the time of injection. Fish

in groups receiving a single injection were bled on days 1, 2 and 3. Blood samples were taken from fish in all treatment groups on days 4, 5, 6, 8 and 10. All fish were checked for ovulation on a daily basis for 8 days and subsequently at 2-3 day intervals.

VI. Effects of Pimozide and LH-RHA DA1a^6 on Plasma Gonadotropin Levels and Ovulation

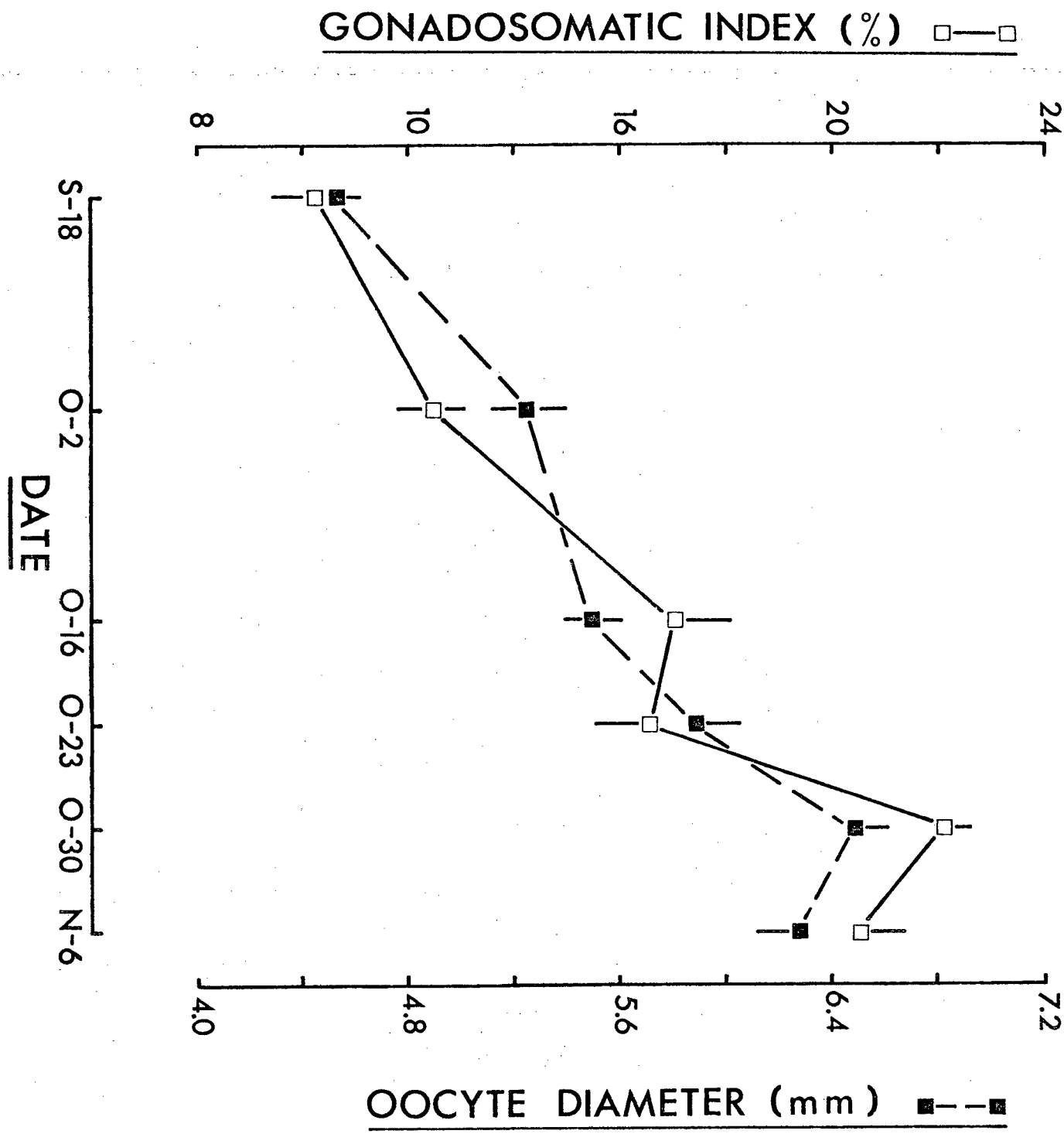
Four groups of 8 or 9 adult coho salmon were given single injections of saline, LH-RHA DA1a^6 (0.02 mg/kg), pimozide (10 mg/kg) or a combination of pimozide and LH-RHA DA1a^6. Blood samples were taken at the time of injection (10:00, October 30, 1982) and 1.5, 6, 24, 48 and 96 hr after injection for gonadotropin measurement. Fish were checked for ovulation on days 4, 6, and 8.

C. Results

I. Gonadotropin and Ovarian Changes During Sexual Maturation

Fig. 1 shows the average oocyte diameter and GSI in coho salmon sampled from mid-September to November. The average oocyte diameter increased significantly ($P < 0.01$) from 4.5 ± 0.1 mm in fish sampled in September to above 6.3 mm in fish sampled at the end of October. Similar changes were observed for the GSI, which doubled during the sampling period. The GSI increased from 10.2 ± 0.8% in September to above 21% at the end of October. The germinal vesicle was not visible in oocytes obtained on September 18 or October 2. By October 16, the germinal vesicle was visible in a central position approximately 1 mm from the oocyte surface. Fish sampled at subsequent times were at various stages of maturity, although oocyte development in individual fish was synchronous. These included oocytes in which the germinal vesicle had migrated towards the periphery, mature oocytes in which the germinal vesicle was not visible and the yolk had become translucent, and ovulated oocytes. No significant differences were

FIG. 1. Changes in the gonadosomatic index and average oocyte diameter during sexual maturation of coho salmon. Each value represents the mean ± standard error of measurements from 10 fish.


GONADOSOMATIC INDEX (%)
8
10
16
20
24
S-18
O-2
O-16
O-23
O-30
N-6
DATE
4.0
4.8
5.6
6.4
7.2
OOCYTE DIAMETER (mm)

distinguished with respect to the stage of maturity and the average oocyte diameter or GSI in samples taken on October 23, 30 and November 6.

Fig. 2 shows the gonadotropin levels in the plasma of female coho salmon sampled from September to November. Gonadotropin levels while similar in fish sampled on September 18 to October 16, increased ($P < 0.05$) at subsequent sampling times. The plasma gonadotropin concentration in one fish which had ovulated by the October 23 sampling was 45 ng/ml (data not shown in Fig. 2). Gonadotropin levels in fish which had not ovulated at this time were lower (mean 13.9 ± 4.1, range 4.9 - 29 ng/ml, N = 9). On October 30, the average gonadotropin concentration in the plasma of ovulated fish was higher than the levels in non-ovulated fish but the difference was not significant (Fig. 2). By November 6, plasma gonadotropin levels in ovulated fish were significantly higher ($P < 0.01$) than the levels in non-ovulated fish.

II. Preovulatory Gonadotropin Changes

The preovulatory changes in plasma gonadotropin levels are presented in Fig. 3. Plasma gonadotropin levels increased about 2-fold over the 12 day period preceding ovulation and peaked 2 days prior to ovulation. The gonadotropin levels 12 and 10 days prior to ovulation (6.0 ng/ml) were significantly lower ($P < 0.05$) than the levels at ovulation (12.2 ng/ml). Oocyte maturation, as defined by GVBD, occurred 2-4 days prior to ovulation based on the visual examination of oocytes expelled while checking for ovulation.

III. Effects of LH-RH and LH-RHA DAla^6 on Plasma Gonadotropin Levels and Oocyte Development

Gonadotropin levels in plasma samples taken immediately before injection were 4.5 ± 0.3 ng/ml (N = 38). Plasma gonadotropin levels in saline-injected fish did not change from this level during the 96 hr sampling period (Fig. 4). Gonadotropin

FIG. 2. Changes in plasma gonadotropin levels during sexual maturation of coho salmon. Each value represents the mean ± standard error of the indicated number of samples. Gonadotropin levels in fish which contained ovulated oocytes (■) are reported separately from the levels in fish which had not ovulated (□).

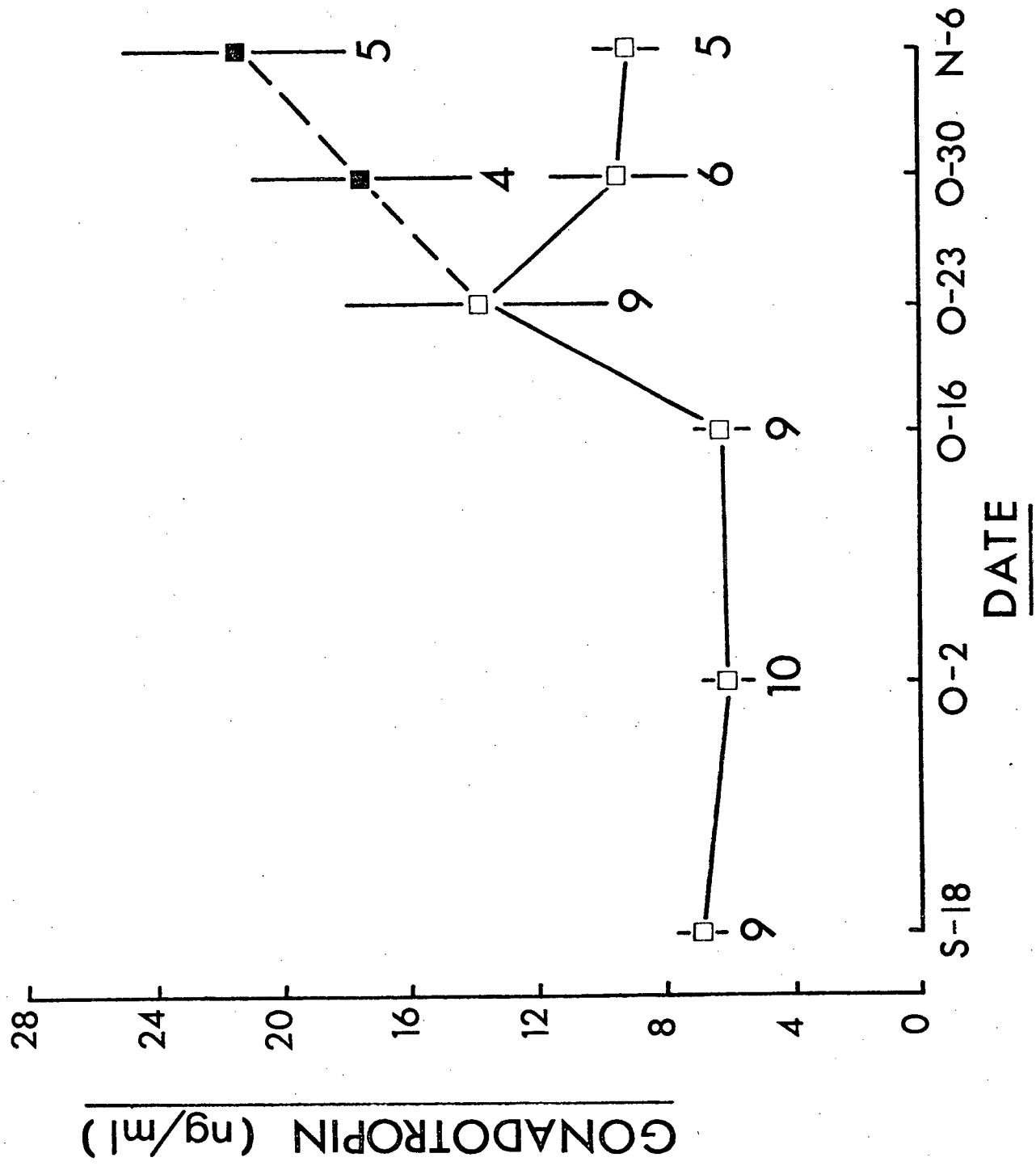
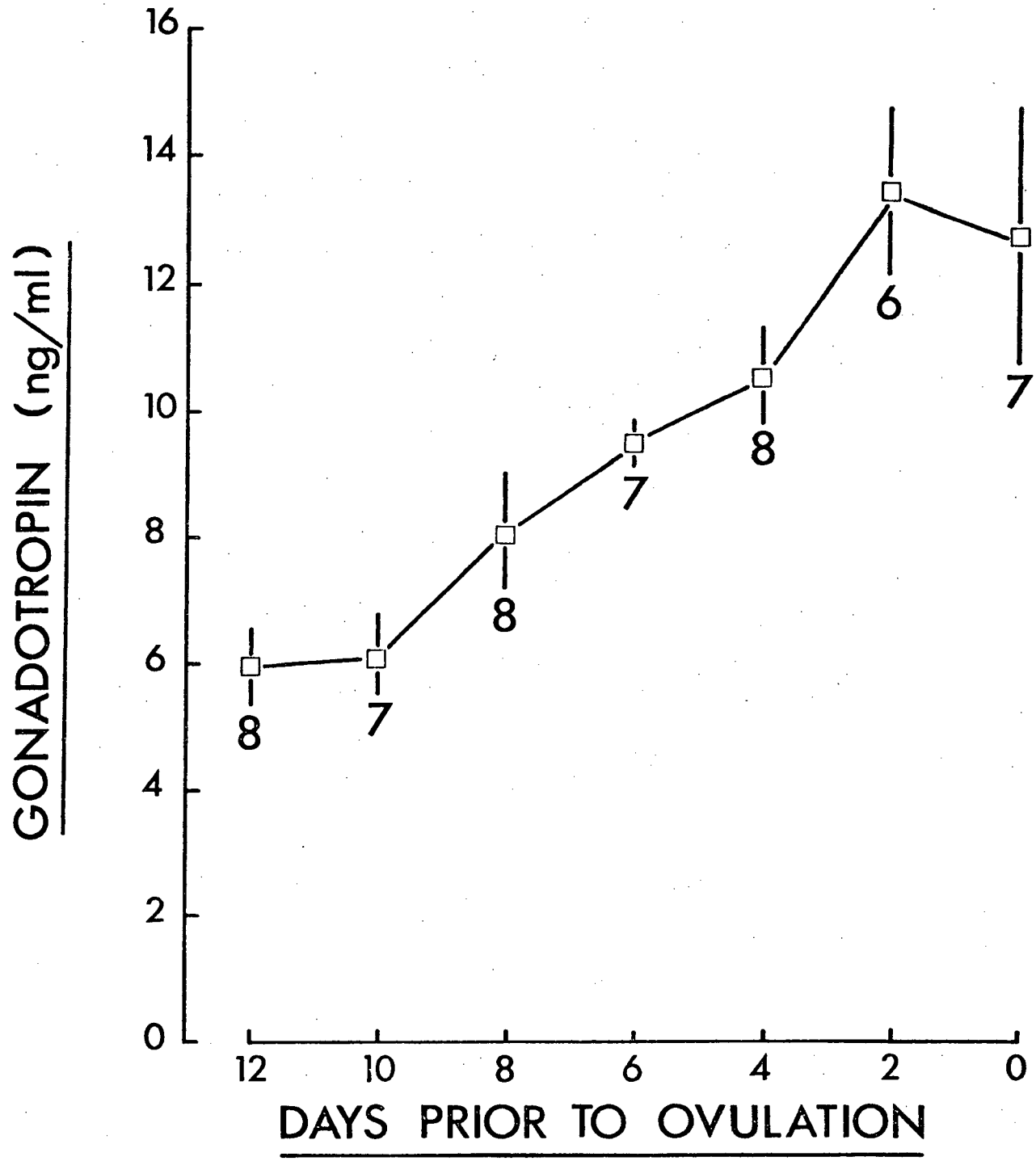

GONADOTROPIN (ng/ml)
28
24
20
16
12
8
4
0
S-18
O-2
O-16
O-23
O-30
N-6
DATE
5
4
9
9
10
9
5
6

FIG. 3. Preovulatory changes in plasma gonadotropin levels in coho salmon. Each value represents the mean ± standard error of the indicated number of samples.


GONADOTROPIN (ng/ml)
16
14
12
10
8
6
4
2
0
8
7
8
7
8
6
7
12
10
8
6
4
2
0
DAYS PRIOR TO OVULATION

levels in groups injected with LH-RH and LH-RHA DAla^6 were significantly higher ($P < 0.01$) than the levels in the saline-injected group at 1.5 and 3 hr. Gonadotropin levels had returned to control levels by 11 hr in fish injected with 0.2 mg/kg LH-RH and by 24 hr in fish injected with 1.0 mg/kg LH-RH. LH-RHA DAla^6-injected fish maintained higher ($P < 0.05$) gonadotropin levels than saline and LH-RH-injected fish from 11-96 hr.

No significant differences between saline and Gn-RH-injected groups were apparent in terms of body weight, GSI, oocyte diameter or oocyte maturity index at 1.5 hr post injection (Table 1). By 96 hr, 5 out of 7 fish in groups injected with 0.02 and 0.2 mg/kg/LH-RHA DAla^6 had undergone oocyte maturation. An accelerated rate of ovarian development was apparent in these groups by their having a higher oocyte maturity index ($P < 0.02$) than saline-injected fish. No fish ovulated during the 96 hr experimental period.

A comparison of LH-RHA DAla^6 - injected fish which failed to complete GVBD and those which completed GVBD revealed no significant difference with respect to plasma gonadotropin levels.

IV. Effects of Chum Salmon Gn-RH, LH-RH and LH-RHA DAla^6 on Plasma Gonadotropin Levels and Ovulation

Gonadotropin levels were 6.4 ± 1.9 ng/ml in plasma samples from 8 fish at the time of injection. Plasma gonadotropin levels in all hormone-treated groups were significantly higher ($P < 0.05$) than those in saline-injected fish at 1.5 hr (Table 2). Gonadotropin levels in groups injected with 0.02 and 0.2 mg/kg LH-RH were similar at 1.5 hr and declined to a level similar to saline-injected fish by 24 hr. Fish injected with 0.02 mg/kg chum salmon Gn-RH had lower gonadotropin levels at 1.5, 24 and 48 hr than fish receiving the high dose of chum salmon Gn-RH. Gonadotropin levels in fish injected with 0.02 mg/kg chum salmon Gn-RH were similar

FIG. 4. Plasma gonadotropin levels (mean ± standard error, N = 7-8) in coho salmon following single intraperitoneal injections of LH-RH (A) or LH-RHA DAla^{6} (B). At each sampling period, plasma gonadotropin levels which are similar ($P > 0.05$) as determined by Duncan's Multiple Range Test are identified by the same superscript.

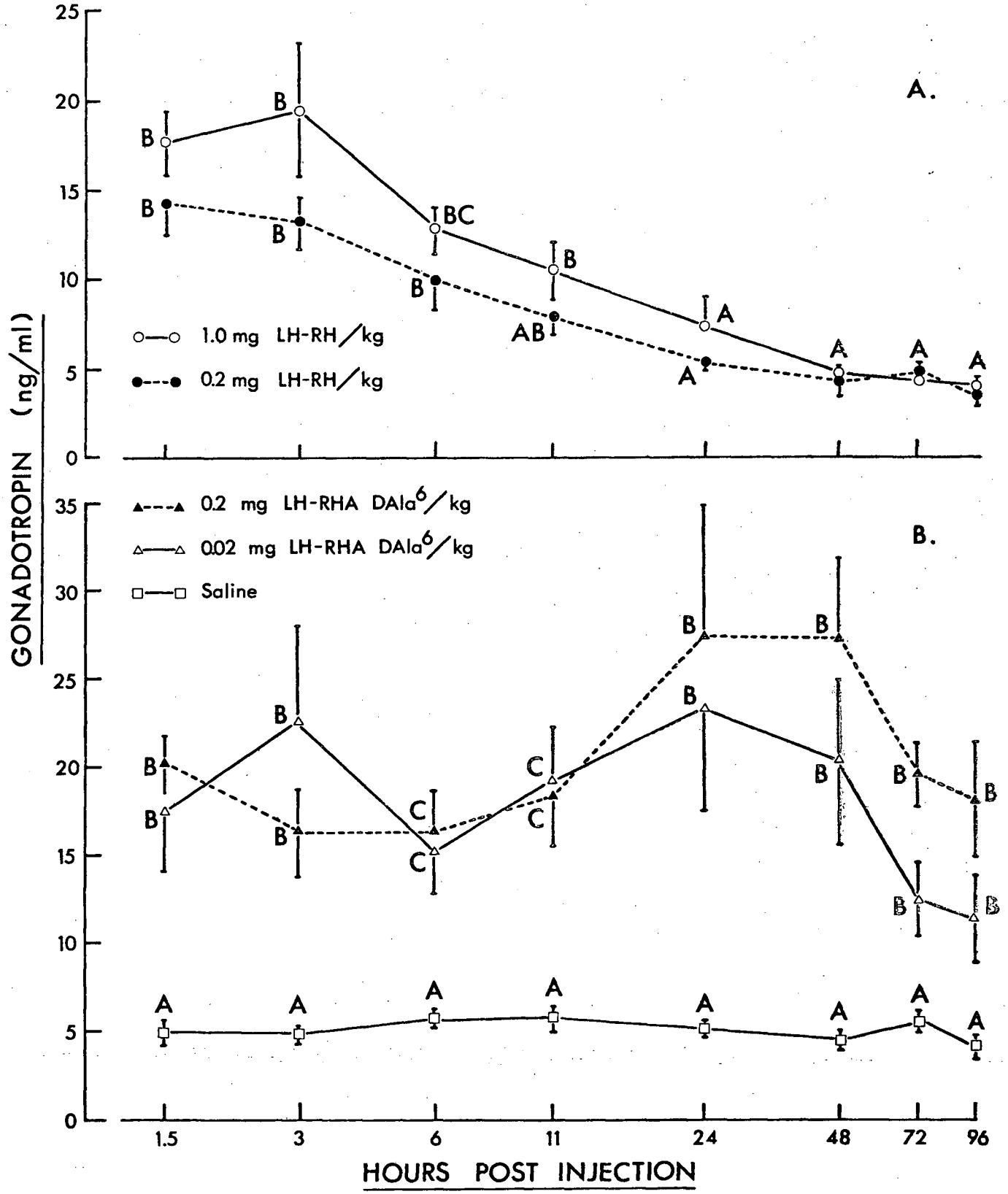

A.
B.
1.0 mg LH-RH/kg
0.2 mg LH-RH/kg
0.2 mg LH-RHA DAla6/kg
0.02 mg LH-RHA DAla6/kg
Saline
GONADOTROPIN (ng/ml)
HOURS POST INJECTION
0
5
10
15
20
25
30
35
1.5
3
6
11
24
48
72
96

TABLE 1 - Effects of LH-RH and LH-RHA DAla^6 on body weight, gonadosomatic index, oocyte diameter and oocyte maturity in coho salmon. Values represent the mean ± standard error.

Dosage	N	Body weight kg	Gonadosomatic index (%)[1]	Oocyte diameter (mm)	Oocyte maturity index 1.5 hr	Oocyte maturity index 96 hr
1.0 mg/kg LH-RH	8[2]	1.79 ± 0.13	18.49 ± 1.10	5.92 ± 0.09	1.25 ± 0.16	1.57 ± 0.20[2]
0.2 mg/kg LH-RH	8	2.08 ± 0.17	18.26 ± 0.83	5.87 ± 0.17	1.13 ± 0.13	1.38 ± 0.18
0.2 mg/kg LH-RHA DAla^6	7	2.31 ± 0.29	18.97 ± 0.94	5.95 ± 0.09	1.29 ± 0.19	2.57 ± 0.30*
0.02 mg/kg LH-RHA DAla^6	7	2.52 ± 0.32	20.47 ± 1.04	6.48 ± 0.12	1.29 ± 0.19	2.71 ± 0.18*
saline	8	2.32 ± 0.19	19.81 ± 0.99	5.93 ± 0.18	1.25 ± 0.16	1.50 ± 0.27

[1] ovary weight/body weight x 100

[2] N = 7 following 48 hr sample owing to a single mortality

* Significantly different from the saline-injected group as determined by the Mann-Whitney U test ($P < 0.02$).

to the levels in saline-injected fish by 24 hr. Fish injected with the high dose of chum salmon Gn-RH maintained higher plasma gonadotropin levels than saline and LH-RH injected fish at 24 hr but were similar to these groups at 48 hr. Groups injected with LH-RHA DA1a^6 had higher plasma gonadotropin levels ($P < 0.01$) than all other groups at 24 and 48 hr. All of the treatments were ineffective in accelerating the rate of ovulation (Table 2).

V. Effects of LH-RHA DA1a^6 and SG-G100 on Plasma Gonadotropin Levels and Ovulation

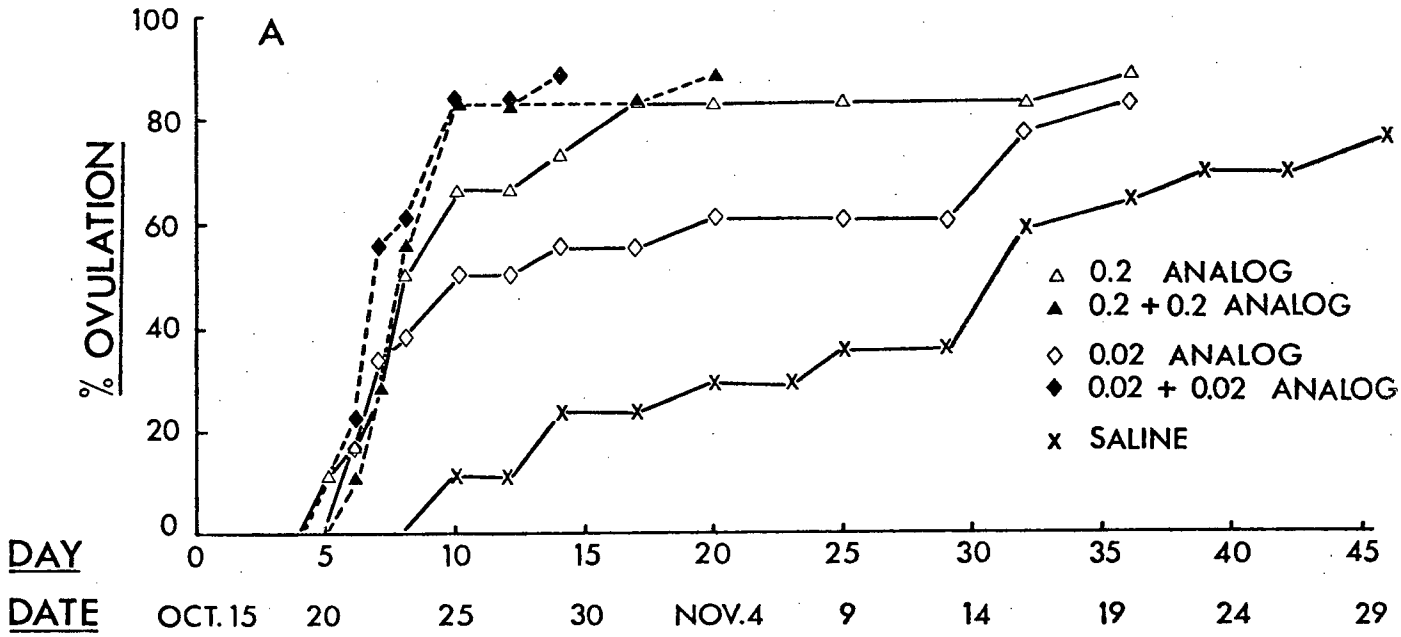
The effects of LH-RHA DA1a^6 alone and in various combinations with SG-G100 on ovulation are reported in Fig. 5. These results represent the combined data from fish which had blood samples removed for gonadotropin measurement and fish sampled only for ovulation; since blood sampling had no significant effect on ovulation. All hormone treatments accelerated the rate of ovulation by day 14 when compared to saline-injected fish ($P < 0.05$). In saline-injected fish, 50% ovulation occurred by day 32 whereas in the least effective of the hormone-treated groups (0.02 mg/kg LH-RHA DA1a^6) 50% ovulation occurred by day 14. In most hormone-treated groups, 50% ovulation occurred 8-10 days after injection. The effectiveness of treatments involving the injection of LH-RHA DA1a^6 alone on the acceleration of ovulation can be summarized as follows: double low = double high > single high > single low (Fig. 5A). The ovulatory response to a single injection of SG-G100 was similar to that observed following single injections of LH-RHA DA1a^6. Combined injections of LH-RHA DA1a^6 and SG-G100 were more effective in promoting ovulation than SG-G100 alone (Fig. 5B). The ovulatory response to two injections of LH-RHA DA1a^6 at either low or high doses was as effective as treatments involving the injection of SG-G100 and LH-RHA DA1a^6. No prespawning mortality occurred by day 14 in hormone-treated fish which had not ovulated. All hormone treated fish which subsequently died prior to ovulation had completed oocyte maturation. Furthermore,

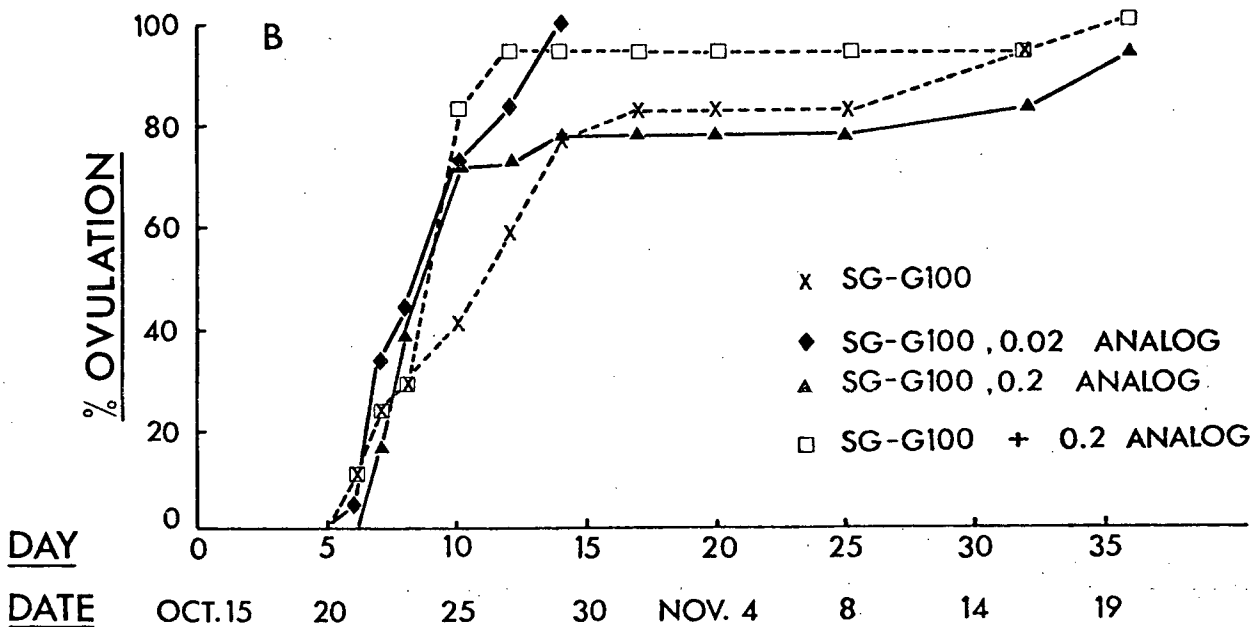
TABLE 2 - Effects of LH-RH, LH-RHA DAla^6 and chum salmon Gn-RH on plasma gonadotropin levels and ovulation in coho salmon. Gonadotropin levels (mean ± standard error) which are similar, at each sampling time, as determined by Duncan's Multiple Range Test ($P > 0.05$) are identified by a similar superscript. The number of fish which had ovulated was determined on day 10.

Treatment	N	Plasma gonadotropin (ng/ml) 1.5	24	48 hr	Number ovulated
Saline	8	6.9 ± 1.2^{a}	9.2 ± 0.5^{a}	8.6 ± 1.1^{ab}	0
LH-RH					
0.02*	8	16.5 ± 1.7^{bc}	7.0 ± 1.0^{a}	8.7 ± 1.0^{ab}	1
0.2	8	19.5 ± 2.4^{bcd}	7.2 ± 0.5^{a}	7.8 ± 0.7^{ab}	0
LH-RHA DAla^6					
0.02	8	21.3 ± 1.5^{cd}	26.7 ± 2.3^{c}	26.5 ± 3.4^{c}	2
0.2	7	26.0 ± 1.5^{d}	35.6 ± 6.4^{c}	35.5 ± 5.2^{c}	1
Chum salmon Gn-RH					
0.02	5	14.2 ± 0.9^{b}	8.0 ± 0.9^{a}	6.4 ± 0.7^{a}	0
0.2	7	24.8 ± 4.0^{cd}	14.2 ± 2.4^{b}	11.3 ± 1.6^{b}	1

* Injected dose (mg/kg)

FIG. 5. Effects of intraperitoneal injections of LH-RHA DAla^6 (A) and SG-G100 alone or in combination with LH-RHA DAla^6 (B) on ovulation in coho salmon. Values represent the cummulative percentage of the number of fish which ovulated in each of the hormone treated groups. N = 17 or 18.


A
% OVULATION
100
80
60
40
20
0
△ 0.2 ANALOG
▲ 0.2 + 0.2 ANALOG
◇ 0.02 ANALOG
◆ 0.02 + 0.02 ANALOG
x SALINE
DAY
0 5 10 15 20 25 30 35 40 45
DATE
OCT. 15 20 25 30 NOV. 4 9 14 19 24 29

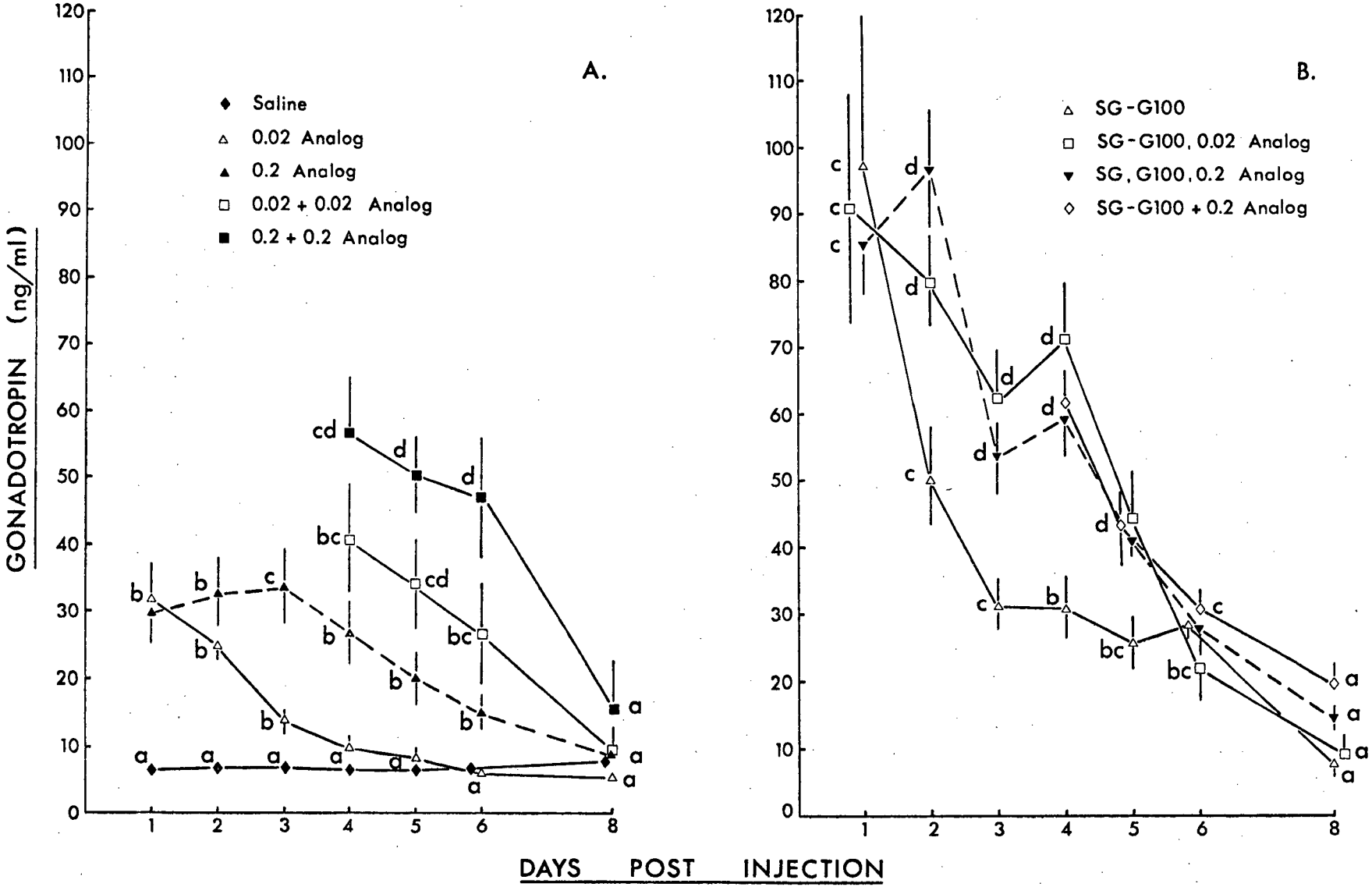

B
% OVULATION
100
80
60
40
20
0
x SG-G100
◆ SG-G100 , 0.02 ANALOG
▲ SG-G100 , 0.2 ANALOG
□ SG-G100 + 0.2 ANALOG
DAY
0 5 10 15 20 25 30 35
DATE
OCT. 15 20 25 30 NOV. 4 8 14 19

based on the morphology of oocytes released while checking for ovulation, all but one of the hormone-injected fish had completed oocyte maturation by day 10.

Plasma gonadotropin levels were 5.4 ± 0.8 ng/ml in 12 fish at the time of injection. Plasma gonadotropin levels in saline-injected fish showed little change with respect to this initial level during the 8 day sampling (Fig. 6a). All hormone treatments resulted in increased plasma gonadotropin levels when compared to saline-injected fish. Fish injected with 0.02 mg/kg LH-RHA DA1a^6 maintained elevated plasma gonadotropin levels for 3 days when compared to saline-injected fish. Gonadotropin levels in fish injected with 0.2 mg/kg LH-RHA DA1a^6 were similar to fish injected with 0.02 mg/kg LH-RHA DA1a^6 on days 1 and 2, but remained higher than saline-injected fish for 6 days. Fish receiving two separate injections of 0.02 or 0.2 mg/kg LH-RHA DA1a^6 at 0 and 72 hr had significantly higher plasma gonadotropin levels on days 4 to 6 than fish receiving a single injection of LH-RHA DA1a^6 at comparable doses. Plasma gonadotropin levels in these groups did not decrease to the levels in saline-injected fish until day 8. Injections of SG-G100 alone or in combination with LH-RHA DA1a^6 resulted in higher plasma gonadotropin levels at 1 and 2 days post injection than did injections of LH-RHA DA1a^6 alone (Fig. 6a and b). Although there was a rapid decrease in plasma gonadotropin levels in SG-G100 injected fish, the levels remained higher than those in saline-injected fish until day 8. Fish receiving combined injections of SG-G100 and 0.02 or 0.2 mg/kg LH-RHA DA1a^6 had higher plasma gonadotropin levels on days 2 to 5 than fish injected with SG-G100 alone. Fish receiving an initial injection of SG-G100 followed by 0.2 mg/kg LH-RHA DA1a^6 at 72 hr had higher plasma gonadotropin levels on days 4 and 5 than SG-G100-injected fish.

Gonadotropin titres in fish which ovulated by day 10 and fish which ovulated at later times were examined to determine if the acceleration of ovulation

FIG. 6. Effects of intraperitoneal injections of LH-RHA DAla^6 (A) and SG-G100 alone or in combination with LH-RHA DAla^6 (B) on plasma gonadotropin levels in coho salmon. Values represent the mean ± standard error of measurements from 8 fish. At each sampling period, plasma gonadotropin levels which are similar ($P > 0.05$) as determined by Duncan's Multiple Range Test are identified by the same superscript.


A.
B.
Saline
0.02 Analog
0.2 Analog
0.02 + 0.02 Analog
0.2 + 0.2 Analog
SG-G100
SG-G100, 0.02 Analog
SG, G100, 0.2 Analog
SG-G100 + 0.2 Analog
GONADOTROPIN (ng/ml)
DAYS POST INJECTION

was related to the magnitude or duration of the increase in plasma gonadotropin levels. Figure 7 illustrates these data for fish receiving single injections of LH-RHA DA1a^6 at 0.02 and 0.2 mg/kg. Fish which ovulated by day 10 in response to 0.02 mg/kg LH-RHA DA1a^6 had higher plasma gonadotropin levels on days 2-5 than fish which ovulated at later times (Fig. 7A). Fish which ovulated by day 10 following the injection of 0.2 mg/kg LH-RHA DA1a^6 had higher plasma gonadotropin levels on days 1-4 than fish which ovulated after 10 days (Fig. 7B). For other treatments, it was not possible to distinguish significant differences in plasma gonadotropin levels that related to the time of ovulation.

VI. Effects of Pimozide and LH-RHA DA1a^6 on Plasma Gonadotropin Levels and Ovulation

The effects of pimozide and LH-RHA DA1a^6 on plasma gonadotropin levels are shown in Fig. 8. Gonadotropin levels in saline-injected fish did not change during the sampling period. Gonadotropin levels in pimozide-injected fish were similar to saline-injected fish until 24 hr, but were higher at 48 and 96 hr. Groups injected with LH-RHA DA1a^6 alone or in combination with pimozide had higher gonadotropin levels than saline-injected fish at 1.5 to 96 hr. Fish receiving the combination of pimozide and LH-RHA DA1a^6 had higher gonadotropin levels than LH-RHA DA1a^6-injected fish at 6-96 hr.

In the pimozide-injected group, 3 out of 9 fish had ovulated by day 4 compared to 1 out of 8 or 9 fish in the other groups (Table 3A). By day 8, only one additional fish had ovulated in the pimozide and saline-injected groups. In contrast, 7 out of 8 fish receiving LH-RHA DA1a^6 and 8 out of 9 fish receiving pimozide and LH-RHA DA1a^6 had ovulated by day 8. The high variation in the rate of ovulation for fish in the pimozide-injected group suggested that this may have resulted from the differing stages of maturity of fish at the time of injection.

FIG. 7. Plasma gonadotropin levels in fish, which ovulated by day 10 (△) and after day 10 (▲) in response to single intraperitoneal injections of 0.02 (A) and 0.2 mg/kg/LH-RHA DAla^{6} (B). In each case, values represent the mean ± standard error of measurements from 4 fish. Gonadotropin levels in fish ovulating by day 10 and at later times were compared using the t-test ($P < 0.05$*, $P < 0.01$**).

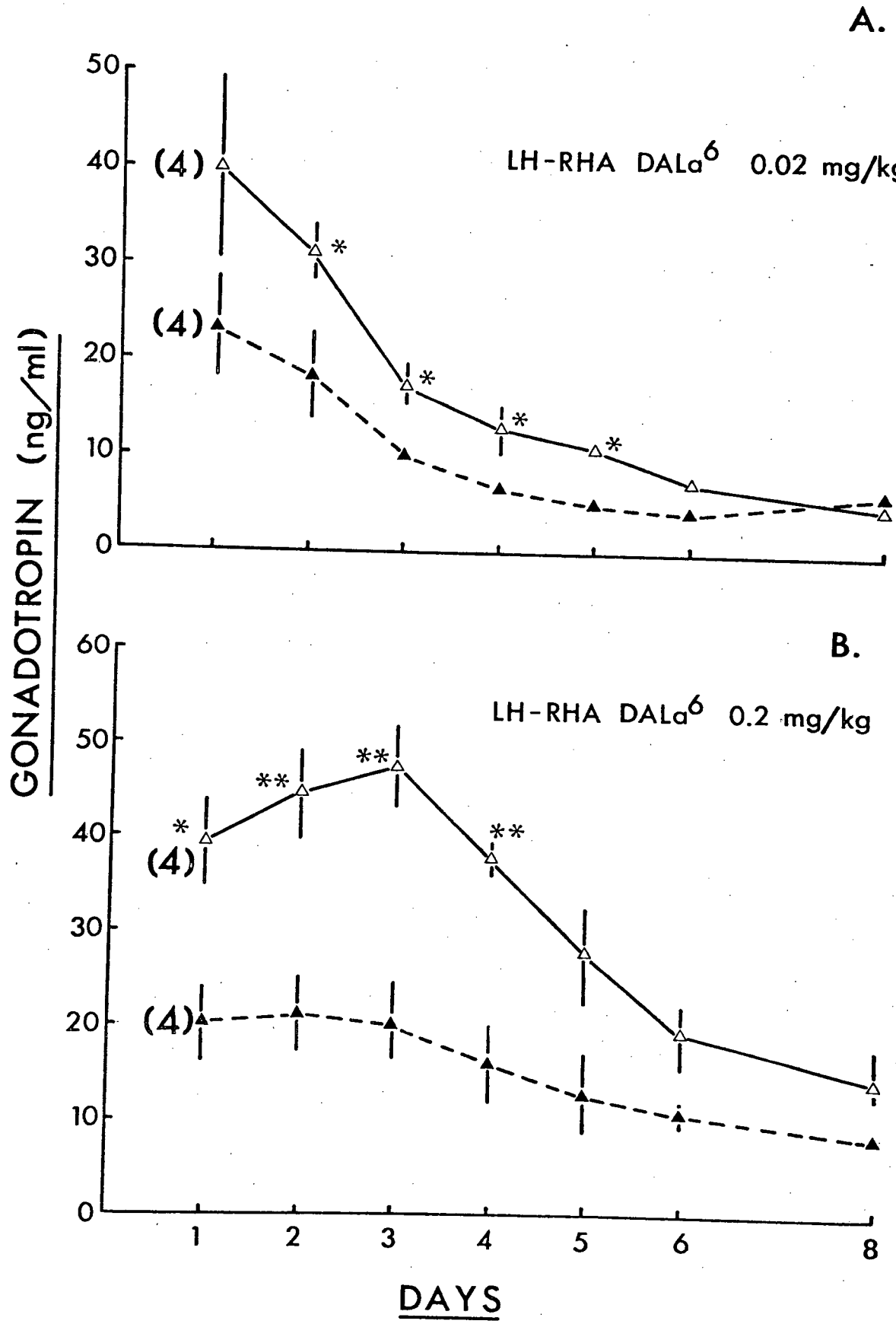
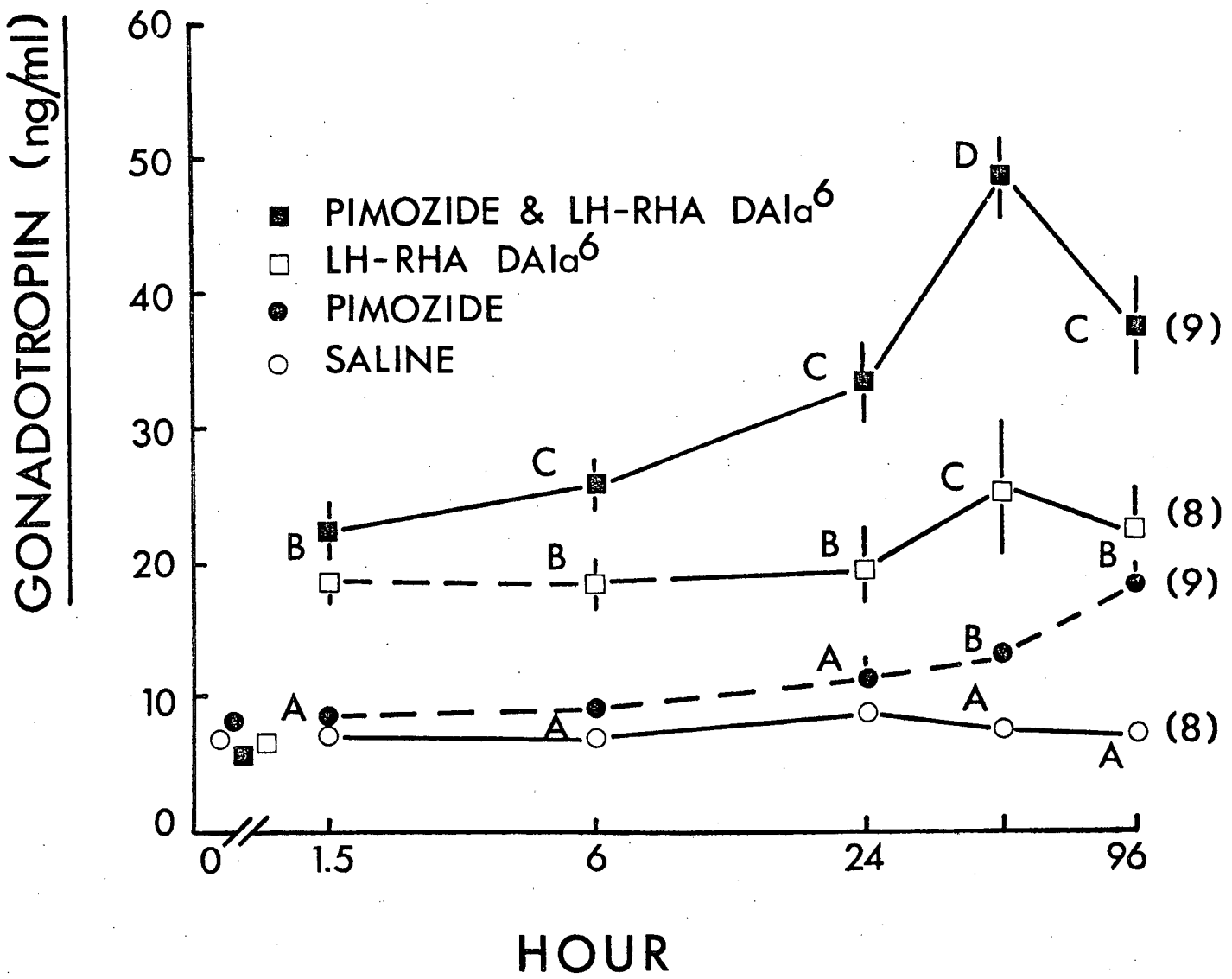

A.
LH-RHA DALa6 0.02 mg/kg
(4)
(4)
B.
LH-RHA DALa6 0.2 mg/kg
(4)
(4)
GONADOTROPIN (ng/ml)
DAYS
0
10
20
30
40
50
60
1
2
3
4
5
6
8

FIG. 8. Effects of pimozide and LH-RHA DAla^6 on plasma gonadotropin levels (mean ± standard error) in coho salmon. At each sampling period, plasma gonadotropin levels which are similar ($P > 0.05$) as determined by Duncan's Multiple Range Test are identified by the same superscript.


GONADOTROPIN (ng/ml)
60
50
40
30
20
10
0
PIMOZIDE & LH-RHA DAla6
LH-RHA DAla6
PIMOZIDE
SALINE
D
C
C
C
B
B
B
B
A
A
A
B
A
(9)
(8)
(9)
(8)
0
1.5
6
24
96
HOUR

To investigate this possibility, preinjection samples were analysed for 17α20βP. Initial 17α20βP concentrations were highly variable, with the levels in some fish approaching the levels seen in fish immediately prior to the completion of oocyte maturation (see Chap. 4, Exp. 1). To further examine the effects of these hormone treatments on ovulation, those fish having high 17α20βP levels (> 50 ng/ml)at the time of injection were eliminated from consideration (Table 3B). Having applied this correction, it was found that LH-RHA-DA1a^{6} injected alone or in combination with pimozide were more effective in accelerating ovulation than saline or pimozide injections. For example, 4 out of 6 fish receiving LH-RHA-DA1a^{6} and 6 out of 7 fish receiving pimozide and LH-RHA-DA1a^{6} had ovulated by day 6 whereas none of the pimozide or saline-injected fish had ovulated.

D. Discussion

Although coho salmon undergo dramatic changes with respect to the GSI and average oocyte diameter following entry to freshwater (Fig. 1), plasma gonadotropin levels were unchanged until 1-2 weeks before ovulation with the highest levels seen at the time of final maturation and ovulation (Fig. 2 and 3). These results confirm and extend the observations of other workers (Crim et al., 1973, 1975; Billard et al., 1978; Fostier et al., 1978; Jalabert et al., 1978a,b; Jalabert and Breton, 1980; Stuart-Kregor et al., 1981; Bromage et al., 1982; Fostier and Jalabert, 1982; Breton et al., 1983a; Scott and Sumpter, 1983; Scott et al., 1983; Young et al., 1983a) that gonadotropin levels increase during the preovulatory period in salmonids. The increase in plasma gonadotropin levels to 10-20 ng/ml in ovulated coho salmon (Fig. 2 and 3) was consistent with measurements of gonadotropin levels in ovulated brown trout and brook trout (Crim et al., 1975), rainbow trout (Jalabert et al., 1978a; Fostier et al., 1978; Fostier and Jalabert, 1982; Scott et al., 1983) and coho salmon (Jalabert et al., 1978b). Since the

TABLE 3 - The effects of pimozide and LH-RHA DAla^6 on ovulation in coho salmon. The results represent the cummulative number of fish in each group which ovulated 4, 6 and 8 days after injection (A) and after correction to eliminate fish which were at an advanced stage of maturity at the time of injection (B).

		Cummulative number ovulated		
		Day		
Treatment	N	4	6	8
A. Uncorrected for maturity				
Saline	8	1	1	2
Pimozide	9	3	3	4
LH-RHA DAla^6	8*	1	6	7
LH-RHA DAla^6 and pimozide	9	1	8	8
B. Corrected for maturity				
Saline	7	0	0	1
Pimozide	6	0	0	1
LH-RHA DAla^6	6*	0	4	5
LH-RHA DAla^6 and pimozide	7	0	6	6

* Includes one non-ovulated fish dead on day 6.

present studies were based on a laboratory-held population of fish, the gonadotropin changes may not be representative of wild fish. For example, Crim et al. (1975) reported that gonadotropin levels in sockeye salmon sampled on the spawning grounds were about 300 ng/ml.

Although gonadotropin levels were generally higher in ovulated fish than fish undergoing maturation, this was only significant on the November 6 sampling (Fig. 2). Since these values were based on measurements from fish that were not serially sampled, the precise time of ovulation was not recorded. This may be of importance as gonadotropin levels in rainbow trout continue to increase following ovulation and do not reach maximal values until several weeks after ovulation (Jalabert and Breton, 1980; Scott et al., 1983). In coho salmon serially sampled during the preovulatory period, there was no difference in the gonadotropin levels in fish undergoing oocyte maturation and at ovulation (Fig. 3).

The demonstration that LH-RH, LH-RHA DAla^6 and chum salmon Gn-RH stimulate gonadotropin secretion in coho salmon (Table 2; Fig. 4, 6 and 8) confirm and extend previous studies which indicate that gonadotropin secretion in teleosts is mediated by a hypothalamic releasing hormone (Peter and Crim, 1979; Ball, 1981; Peter, 1982a,b, 1983a,b). Furthermore, the demonstration that pimozide stimulates gonadotropin secretion in coho salmon (Fig. 8) suggests that a dopaminergic inhibitory system shown to regulate gonadotropin secretion in cyprinids (Chang and Peter, 1983a,b) may also exist in salmonids.

Chum salmon Gn-RH, LH-RH and LH-RHA DAla^6 had similar effects on gonadotropin secretion at short-time intervals following injection (Table 2). The increase in plasma gonadotropin levels in coho salmon following injections of Gn-RH was similar to that observed in brown trout, rainbow trout and goldfish (Crim and Cluett, 1974; Weil et al., 1978; Peter, 1980; Crim et al., 1981a; Chang and Peter, 1983a). The

magnitude of the response in coho salmon was also similar to that in the common carp (Weil et al., 1975; Sokolowska, 1982; Breton et al., 1983) but considerably less than that described in an earlier report on this species (Breton and Weil, 1973) or in Chinese carps (Remlian et al., 1980). Peter (1980) also observed no difference in the magnitude of the initial increase in plasma gonadotropin levels in male goldfish following single intraperitoneal injections of LH-RH and LH-RHA DAla^6. Additionally, Crim et al. (1981a) found no differences in the magnitude of the increase in plasma gonadotropin levels in male brown trout at short-time intervals following injections of LH-RH, des-Gly^{10}LH-RH-ethylamide and des-Gly^{10}[D-Leu]^6-LH-RH- ethylamide. Despite differences in their structure, it appears that chum salmon Gn-RH, LH-RH and LH-RHA DAla^6 have a similar affinity for Gn-RH receptors in coho salmon. As a full range of doses was not tested, the possibility remains that differences in the activity of the releasing hormones may exist, particularly at lower doses. There is an indication that this may be the case in that injections of LH-RHA DAla^6 at 0.02 mg/kg resulted in higher plasma gonadotropin levels than injections of chum salmon Gn-RH at the same dose. As there was a dose dependance when the duration of the response was considered (Table 2; Fig. 4 and 6), the failure to detect differences in the potency of different Gn-RHs at short-time intervals following injection may result from only a small proportion of pituitary gonadotropin reserves being available for release.

Major differences in the activities of chum salmon Gn-RH, LH-RH and LH-RHA DAla^6 were apparent when the duration of the increase in plasma gonadotropin levels was considered (Table 2 and Fig. 4). The effects of chum salmon Gn-RH and LH-RH were transitory, although the effects of chum salmon Gn-RH appeared to last for a slightly longer duration than LH-RH (Table 2). This finding was consistent with data from the goldfish in which the potency of LH-RH and chum salmon Gn-RH were

reported to be similar (R.E. Peter, C.S. Nahorniak and M. Sokolowska, unpublished data cited in Peter, 1983b). The response to LH-RHA DAla^6 was of much longer duration than that to chum salmon Gn-RH or LH-RH (Table 2 and Fig. 4). The increased activity of LH-RHA DAla^6 was indicated by the maintenance of elevated plasma gonadotropin levels for at least 96 hr compared to only 11 hr following injections of LH-RH (Fig. 4). Studies in mammals (see Vale et al., 1977) and in other teleosts have also shown that LH-RH analogs have a more prolonged effect on gonadotropin secretion than LH-RH. For example, Peter (1980) reported that LH-RHA DAla^6 administered in two injections 12 hr apart or in three daily injections promoted a longer duration increase in plasma gonadotropin levels in male goldfish than injections of LH-RH. Breton et al. (1983) reported that single injections of des Gly^{10} [D-Ser^6]-LH-RH-ethylamide had a longer effect than LH-RH in the carp. Additional studies in coho salmon have shown that analogs of chum salmon Gn-RH containing [D-Arg] or [D-Ala] in position 6 and removal of position 10 glycine promote a longer duration increase in plasma gonadotropin levels than the native molecule (Donaldson et al., 1983). In this case, LH-RHA DAla^6 and analogs of chum salmon Gn-RH were shown to be equipotent (Donaldson et al., 1983). The basis for the prolonged effectiveness of LH-RHA DAla^6 compared to LH-RH and chum salmon Gn-RH in coho salmon is not known. Explanations include a possible longer metabolic clearance rate for LH-RHA DAla^6 or a slower degradation upon binding to Gn-RH receptors in the pituitary.

Although several factors preclude direct comparisons between species (state of maturity, site of injection, dosage and type of releasing hormone), the duration of response to LH-RHA DAla^6 in coho salmon was greater than that described for other teleost species. In the common carp, plasma gonadotropin levels were elevated for less that 24 hr following intracardiac injection of des-Gly^{10}[D-Ser^6]

LH-RH-ethylamide at 0.3 μg/kg (Breton et al., 1983). In adult female goldfish, plasma gonadotropin levels were elevated for 48 hr following intraperitoneal injections of LH-RHA DAla^6 at 0.1 mg/kg (Chang and Peter, 1983a). In coho salmon, plasma gonadotropin levels were elevated for up to 6 days following injections of LH-RHA DAla^6 at 0.2 mg/kg (Fig. 6). Additional evidence for the long-acting effects of LH-RHA DAla^6 in coho salmon was provided by the higher plasma gonadotropin levels on days 2-5 in fish receiving combined injections of LH-RHA DAla^6 and SG-G100 when compared to fish receiving SG-G100 alone (Fig. 5). To account for these species differences it may be necessary to consider the rate of uptake into the circulation following injection and the duration of binding to Gn-RH receptors.

In goldfish, the effects of LH-RHA DAla^6 on gonadotropin release was potentiated by two injections of LH-RHA DAla^6 over a 12 hr period (Peter, 1980; Chang and Peter, 1983a). There was no indication for the potentiation of gonadotropin release in coho salmon when LH-RHA DAla^6 was administered in two injections over a 72 hr period. The effect of a second LH-RHA DAla^6 injection was esentially additive to the effects of the first injection (Fig. 6). For example, the gonadotropin level measured on day 4 following two injections of LH-RHA DAla^6 was equivalent to the sum of the level measured on day 3 and the 25 ng/ml increase in gonadotropin level measured one day following a single injection of LH-RHA DAla^6.

Previous studies have shown that pituitary responsiveness to Gn-RH varies on a seasonal basis (Weil et al., 1975, 1978; Lin et al., 1984). Little evidence was found to indicate a major change in pituitary responsiveness to Gn-RH in coho salmon during the preovulatory period. However, a difference was found in that fish injected with 0.02 mg/kg LH-RHA DAla^6 about one month prior to the expected

time of ovulation maintained elevated plasma gonadotropin levels for only 3 days (Fig. 6) whereas fish of more advanced maturity maintained elevated plasma gonadotropin levels for at least 4 days (Fig. 4 and 8). Additionally fish which were induced to ovulate following single injections of LH-RHA DA1a^6 showed a larger increase in plasma gonadotropin levels which persisted for a longer duration than that in fish which failed to ovulate (Fig. 7). Whether these differences relate to changes in pituitary responsiveness or the subsequent effects of changing steroid feedback on gonadotropin secretion remains to be evaluated.

The dopamine receptor antagonist pimozide was shown to stimulate gonadotropin secretion in coho salmon (Fig. 8). Following a delay of 1.5 hr, combined injections of pimozide and LH-RHA DA1a^6 were more effective in promoting gonadotropin secretion than LH-RHA DA1a^6 alone (Fig. 8). Although the effects of pimozide alone were not marked, these results suggest that pimozide potentiates the response to LH-RHA DA1a^6 in coho salmon. Chang and Peter (1983a) reported that pimozide injected with the first or second of two LH-RHA DA1a^6 injections (12 hr injection interval), or prior to a single injection of LH-RHA DA1a^6 greatly potentiated the gonadotropin release-response to LH-RHA DA1a^6 in goldfish held at 12°C. However, the simulataneous injection of pimozide and LH-RHA DA1a^6 was no more effective in stimulating gonadotropin secretion than injection of LH-RHA DA1a^6 alone in goldfish at 12°C (Chang and Peter, 1983a). Subsequent studies based on goldfish held at 20°C have shown, like the data for coho salmon, that the simultaneous injection of pimozide and LH-RHA DA1a^6 was more effective than LH-RHA DA1a^6 alone (Sokolowska et al., 1984). Recent studies in rainbow trout (Billard et al., 1983a) provide confirmation that pimozide stimulates gonadotropin secretion in salmonids, although in this case, injections of pimozide were more effective than LH-RHA DA1a^6. Furthermore, there was no potentiation of the response in groups

receiving pimozide and LH-RHA DAla^6. The basis of the difference between the results in rainbow trout (Billard et al., 1983a) and coho salmon (Fig. 8) is not known, but may be a consequence of the lower doses of LH-RHA DAla^6 (1 µg/kg) used in studies with rainbow trout. Although it appears that dopamine inhibition may regulate gonadotropin secretion in salmonids and cyprinids, physiological evidence for a GRIF has only been described for cyprinids (see Peter, 1982a,b, 1983a,b). It has been suggested that the preovulatory gonadotropin surge in the goldfish is regulated by the removal of dopamine inhibition on gonadotropin release and the stimulation of gonadotropin secretion by Gn-RH. The significance of a GRIF such as dopamine in the regulation of gonadotropin secretion in salmonids remains to be investigated.

The results presented here (Fig. 5) confirm and extend previous studies on the use of LH-RHA DAla^6 to induce ovulation in salmonids. Consistent with recent studies (Donaldson et al., 1981; Sower et al., 1982), it was shown that LH-RHA DAla^6 injected 72 hr following an initial injection of SG-G100 accelerates ovulation in coho salmon. It was also shown that a combined single injection of 0.02 or 0.2 mg/kg LH-RHA DAla^6 together with SG-G100 accelerates ovulation in a higher percentage of fish than SG-G100 alone. In addition, the present results also demonstrate that single intraperitoneal injections of LH-RHA DAla^6 induce ovulation in coho salmon. Furthermore, by the administration of LH-RHA DAla^6 in two injections over a 72 hr period, an ovulatory response was obtained which was comparable to that observed following combined injections of SG-G100 and LH-RHA DAla^6. The effectiveness of two separate injections of LH-RHA DAla^6 on the promotion of ovulation in this study was comparable to recent studies in Atlantic salmon and rainbow trout in which LH-RH analogs were administered in cholesterol pellet implants which were designed for a long-term gradual release of hormone

(Crim et al., 1983a,b). LH-RHA DAla^6 alone and in combination with pimozide had similar effects on the acceleration of ovulation in coho salmon (Table 8). In contrast, Billard et al. (1983a) reported that the combination of LH-RHA DAla^6 and pimozide was more effective in accelerating ovulation than pimozide or LH-RHA DAla^6 alone in rainbow trout and brown trout. The inability to demonstrate an increased effectiveness of pimozide and LH-RHA DAla^6 in coho salmon may have resulted from the advanced state of maturity of fish at the time of injection.

LH-RH was ineffective in accelerating oocyte maturation in coho salmon (Table 1). In contrast, LH-RHA DAla^6 had at least 50 x the potency of LH-RH based on its stimulation of oocyte maturation. Since LH-RH and LH-RHA DAla^6 have a similar effect on plasma gonadotropin levels at 1.5 and 3 hr (Fig. 4), a potency estimate based on gonadotropin measurement at short-time intervals following injection would not agree with this observation. The failure of LH-RH injections to induce oocyte maturation suggests that short-lived changes in plasma gonadotropin levels are of insufficient duration to stimulate final maturation and ovulation.

Although single injections of LH-RHA DAla^6 and SG-G100 have very different effects on plasma gonadotropin levels (Fig. 6), these treatments have a similar effect on the acceleration of ovulation (Fig. 5). These treatments differ in that the injection of SG-G100 causes a much larger increase in plasma gonadotropin levels at 1 and 2 days post injection than LH-RHA DAla^6. It was previously shown that gonadotropin levels at 24 hr were not significantly different from the levels 1.5 hr following the injection of LH-RHA DAla^6 (Table 2 and Fig. 4). However, it is likely that measurements 24 hr after the injection of SG-G100 do not provide a measure of peak gonadotropin concentrations (see Cook and Peter, 1980b; Breton et al., 1983). These results suggest that it is not solely the magnitude of the initial increase in plasma gonadotropin level that determines the success of

hormone treatments on the stimulation of ovulation. The duration of the increase in plasma gonadotropin level appears to be more important.In the case of fish which ovulated following a single injection of LH-RHA DAla^6, it was possible to associate the duration of the increase in plasma gonadotropin levels with the acceleration of ovulation (Fig. 7).Additional evidence supporting this hypothesis was available from the higher plasma gonadotropin levels in fish receiving two separate injections of LH-RHA DAla^6 and the greater number of fish which ovulated in these groups compared to fish receiving single injections of LH-RHA DAla^6 (Fig. 5 and 6). Additionally, combined single injections of SG-G100 and LH-RHA DAla^6 resulted in higher plasma gonadotropin levels on days 2-5 when compared to SG-G100-injected fish and were more effective in accelerating ovulation. However, with the exception of fish receiving single injections of LH-RHA DAla^6, it was not possible to distinguish fish in other hormone treated groups which would ovulate based solely on gonadotropin measurements. This suggests, that although gonadotropin can be increased to a level seemingly appropriate for ovulation, factors in addition to gonadotropin are responsible for ovulation. Ultimately, the acceleration of ovulation may depend on the capacity of the ovary to respond to gonadotropin by the synthesis of appropriate steroids and prostaglandins (see Chapter 4).

The demonstration that single intraperitoneal injections of LH-RHA DAla^6 accelerate ovulation in coho salmon is in contrast with the majority of studies in other teleosts (see Introduction). The high rate of success using LH-RHA DAla^6 to accelerate ovulation in coho salmon compared to the carp and goldfish may relate to differences in the pattern of gonadotropin secretion at ovulation. In salmonids, there is a slow and gradual increase in plasma gonadotropin levels during the preovulatory period with no evidence for a gonadotropin surge immediately prior to

ovulation (Fig. 3; Fostier et al., 1978; Jalabert et al.,1978a,b; Fostier and Jalabert, 1982; Scott et al., 1983). In contrast, a dramatic ovulatory gonadotropin surge occurs in the goldfish (Stacey et al.,1979a) and carp (Fish Reproductive Physiology Research Group and Peptide Hormone Group, 1978). The ovulatory gonadotropin surge in the goldfish lasts at least 12 hr, during which gonadotropin levels increase about 10-fold above basal levels (Stacey et al., 1979a). The significance of differences in the pattern of gonadotropin secretion at ovulation is not fully understood, but presumably reflects changes in reproductive strategy. In the goldfish, ovulation and spawning are precisely coordinated by environmental factors while salmonids apparently lack this precise coordination (Peter, 1981). Goldfish generally complete gonadal development at cold temperatures and retain fully developed oocytes which ovulate 1-2 days after exposure to environmental conditions appropriate for spawning (warm temperature, suitable substrate) (Stacey et al., 1979a,b; Peter 1981). In salmonids, ovulation occurs at relatively cold temperatures, apparently as a consequence of completed ovarian development. Additionally once ovulated, oocytes can be held for several days prior to spawning (Bry, 1981; J. Stoss, personal communication).

The inability of injections of LH-RH analogs to accelerate ovulation in carp and goldfish presumably relates to a failure to duplicate the preovulatory gonadotropin surge. For example, the implantation of cholesterol pellets containing des-Gly^{10}[D-Trp]^{6}LH-RH-ethylamide in goldfish, results in a chronic elevation of plasma gonadotropin levels lasting at least 7 days, but fails to induce ovulation (Sokolowska et al., 1984). By comparison, a similar approach in landlocked Atlantic salmon accelerates ovulation at least two weeks in advance of control fish (Crim et al., 1983a). It was recently shown that ovulation could be induced in the goldfish (Chang and Peter, 1983a; Sokolowska et al., 1984) and the

common carp (Billard et al., 1983b) by using pimozide and LH-RHA DAla^6. It appears that the removal of dopamine inhibition by pimozide potentiates the effects of LH-RHA DAla^6 on gonadotropin release making it possible to mimic or exceed the normal ovulatory gonadotropin surge (Chang and Peter, 1983a). However, the rate at which plasma gonadotropin levels increase appears to be as important as the magnitude of the increase in the goldfish. This appears to be in direct contrast with the situation in coho salmon. The highest rate of ovulation in coho salmon occurred 6 - 10 days following hormone treatments and was associated with declining plasma gonadotropin levels (Fig. 6 and 7). Jalabert et al. (1978a,b) noted a similar relationship between plasma gonadotropin levels and ovulation in rainbow trout and coho salmon following the injection of gonadotropin preparations. In contrast to the situation in the goldfish, the duration of the increase in plasma gonadotropin level appears to be critical for the successful induction of ovulation in the coho salmon.

In conclusion, the present results show that LH-RHA DAla^6 provides a suitable alternative to the use of gonadotropin preparations for the acceleration of ovulation in coho salmon when administered about one month prior to the expected time of ovulation. Although combined injections of LH-RHA DAla^6 and SG-G100 may be of particular benefit when handling stress is to be minimized, two injections of a low dose of LH-RHA DAla^6 may be more economical with respect to hormone usage. Additional research is warranted to optimize the dosage and to investigate the effects of varying the interval between injections of LH-RHA DAla^6 and to further investigate the potential application of pimozide to accelerate ovulation.

CHAPTER 4 - 17β-ESTRADIOL, TESTOSTERONE, AND 17α20βP CHANGES ASSOCIATED WITH SEXUAL MATURATION IN FEMALE COHO SALMON

A. Introduction

In Chapter 3, it was shown that oocyte maturation and ovulation in coho salmon were associated with the elevation of plasma gonadotropin titres. It is generally accepted that gonadotropin does not directly promote oocyte maturation but acts on the follicular layers surrounding the oocyte to stimulate the production of steroidal mediators of maturation. Current evidence suggests that 17α20βP functions as the maturation inducing steroid in salmonids (see Introduction). The role of other steroids, such as 17β-estradiol and testosterone, is not completely understood. The present studies were conducted to assess the steroid changes associated with spontaneous and induced reproductive development, in particular, the steroid changes associated with the completion of vitellogenesis and the initiation of oocyte maturation. Experiments I, II, and III represent a continuation of the experiments described in Chapter 3 by examining the changes in 17β-estradiol, testosterone, and 17α20βP during spontaneous reproductive development (Exp. I) and following injections of LH-RH, LH-RHA DAla^6 and SG-G100 (Exp. II and III). Additionally, ovarian follicles obtained throughout the preovulatory period and following ovulation were examined to determine their capacity to produce these steroids in response to gonadotropin <u>in vitro</u> (Exp. IV). The possible involvement of 17β-estradiol in the regulation of 17α20βP synthesis was investigated in Exp.V by studying the effects of 17β-estradiol on the ability of SG-G100 to augment 17α20βP production by follicles incubated <u>in vitro</u>.

B. Experimental Protocol

I. Preovulatory Steroid Changes

17β-estradiol, testosterone and 17α20βP were measured in plasma samples

obtained by serial sampling of coho salmon up until the time of ovulation. A full description of the protocol is provided in Exp. II, Chapter 3.

II. Steroid Changes in Response to LH-RH and LH-RHA DAla^6

17β-estradiol, testosterone and 17α20βP were measured in plasma samples obtained from coho salmon following single intraperitoneal injections of LH-RH and LH-RHA DAla^6. Full details are provided in the protocol for Exp. III, Chapter 3.

III. Steroid Changes in Reponse to LH-RHA DAla^6 and SG-G100

17β-estradiol and 17α20βP were measured in plasma samples obtained at time 0, 1, 2, 4, 6, 8 and 10 days following injections of varied combinations of LH-RHA DAla^6 and SG-G100. Details are provided in the protocol for Exp. IV, Chapter 3.

IV. In Vitro Steroid Production by Ovarian Follicles During Sexual Maturation

Ovarian follicles obtained at various times during the preovulatory period and following ovulation were incubated in vitro with or without SG-G100 to evaluate their capacity to produce 17β-estradiol, testosterone and 17α20βP.

V. Effects of 17β-estradiol on 17α20βP Production In Vitro

Two experiments were conducted to examine the effects of 17β-estradiol on 17α20βP production in vitro. The first experiment utilized follicles from a fish prior to maturation which were characterized by a central germinal vesicle and the second experiment utilized follicles from a fish undergoing maturation which were characterized by a peripheral germinal vesicle. In each experiment, groups of five follicles were incubated with or without SG-G100 (0, 10, 100 and 1000 ng/ml) for 24 hr at 10°C. Additional groups of follicles were incubated in medium containing 17β-estradiol at 25 or 250 ng/ml in addition to gonadotropin. Three replicate incubations were made for each concentration of SG-G100 and 17β-estradiol. The amounts of 17α20βP released to the media were determined by RIA.

C. Results

I. Preovulatory Steroid Changes.

Preovulatory changes in plasma 17β-estradiol, testosterone and 17α20βP levels are shown in Fig. 9. 17β-estradiol levels decreased significantly from 16 ng/ml 12 days prior to ovulation to basal levels of 1-2 ng/ml 4 days before ovulation. Testosterone levels remained high (>125 ng/ml) during the preovulatory period reaching maximal levels about 6 days prior to ovulation and then gradually decreased until ovulation. 17α20βP increased from basal levels of less than 10 ng/ml 12 days prior to ovulation to 270 ng/ml 4 days prior to ovulation and then declined. Oocyte maturation occurred 2-4 days prior to ovulation based on the visual examination of oocytes expelled when checking for ovulation.

II. Steroid Changes in Response to LH-RH and LH-RHA DAla^6

No dose dependant differences were found when 17β-estradiol, testosterone and 17α20βP levels were compared in groups injected with 0.2 and 1.0 mg/kg LH-RH or in groups injected with 0.02 and 0.2 mg/kg LH-RHA DAla^6. Subsequent comparisons between the effects of LH-RH and LH-RHA DAla^6 on plasma steroid levels (Figs. 10, 11 and 12) were based on the pooled data for all fish injected with LH-RH and the pooled data for all fish injected with LH-RHA DAla^6. However, the steroid profiles in fish which completed GVBD by 96 hr were examined separately from those failing to complete GVBD (see Table 4). The one saline-injected fish which completed GVBD spontaneously showed similar steroid changes to those reported in Fig. 9 and was not included in subsequent comparisons. In this fish, 17β-estradiol was 4.9 ng/ml initially and decreased to 1.9 ng/ml at 96 hr. 17α20βP increased from 39 ng/ml to 325 ng/ml over the 96 hr sampling period. Testosterone levels were above 240 ng/ml at all sampling times.

Plasma 17β-estradiol levels following the injection of LH-RH and LH-RHA

FIG. 9. Preovulatory changes in plasma 17β-estradiol, testosterone and 17α20βP levels in coho salmon. Values represent the mean ± standard error of measurements from 8 fish.

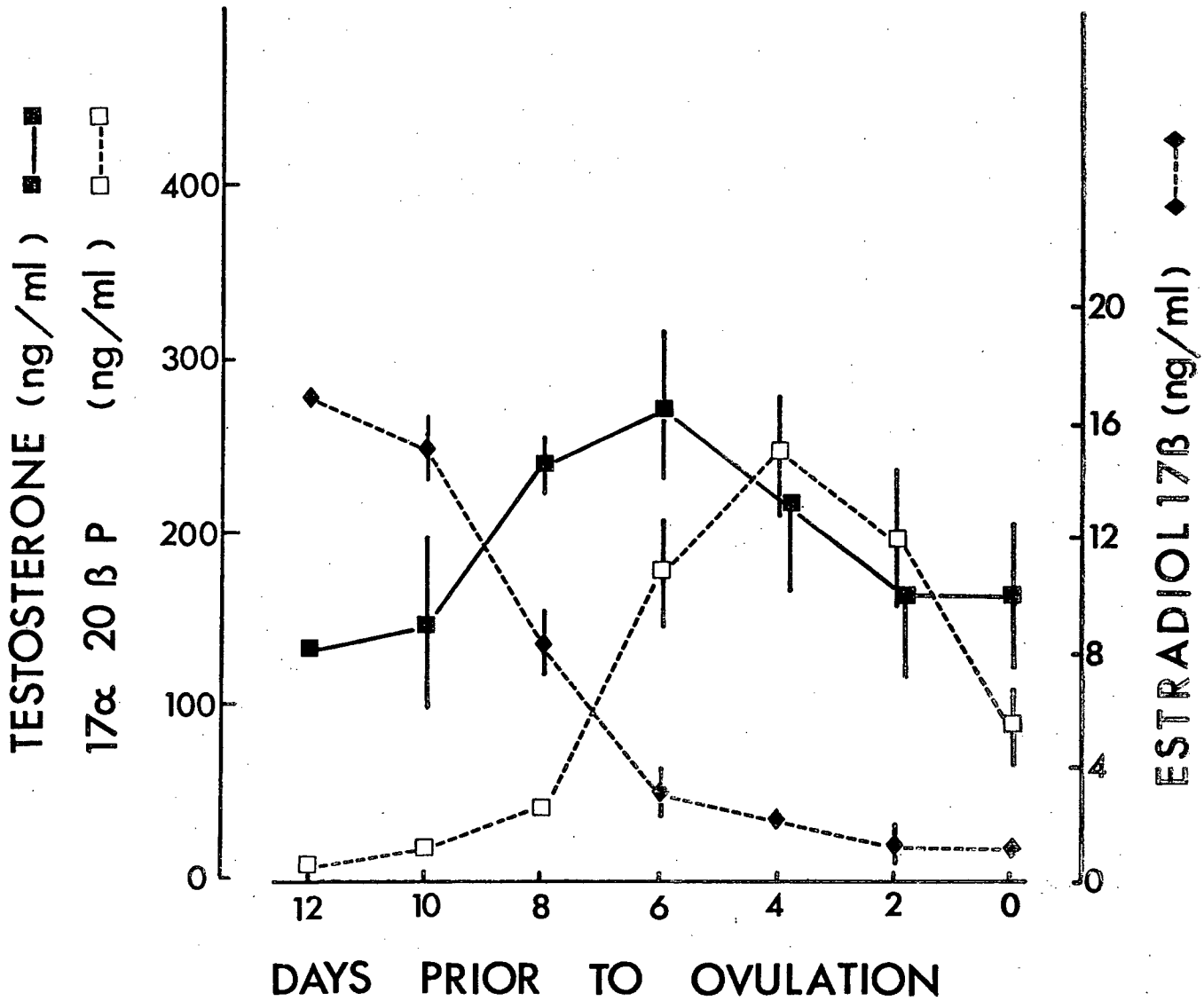

TESTOSTERONE (ng/ml)
17α 20 β P (ng/ml)
ESTRADIOL 17β (ng/ml)
400
300
200
100
0
20
16
12
8
4
0
12
10
8
6
4
2
0
DAYS PRIOR TO OVULATION

TABLE 4 - Oocyte development determined 96 hr following the injection of LH-RH, LH-RHA DAla^6 or saline. Fish were assigned to specific categories which correspond to the position of the germinal vesicle. A maturation index was calculated to numerically describe the average oocyte classification in each of the treatment groups.

		Oocyte classification			
Injected peptide	Dose (mg/kg)	Central Germinal vesicle 1	Peripheral Germinal vesicle 2	Germinal vesicle breakdown 3	Maturation index
LH-RH	1.0	3	4	0	1.57
LH-RH	0.2	5	3	0	1.38
LH-RHA DAla^6	0.2	1	1	5	2.57*
LH-RHA DAla^6	0.02	0	2	5	2.71*
Saline	0	5	2	1	1.50

* Significantly different from saline group as determined by the Mann Whitney U test ($P < 0.02$).

DAla^6 are shown in Fig. 10. Plasma 17β-estradiol levels in saline-injected fish were similar to preinjection levels (27 ng/ml) at 24 hr but then declined to 5.9 ng/ml by 96 hr. Plasma 17β-estradiol levels in LH-RH-injected fish followed the same trend as the saline-injected group. Plasma 17β-estradiol levels in LH-RHA DAla^6-injected fish which completed GVBD were reduced significantly compared to saline-injected fish at 48 to 96 hr. LH-RHA DAla^6-injected fish which failed to complete GVBD had higher plasma 17β-estradiol levels than saline-injected fish at 48 hr but then declined at 72 and 96 hr to levels similar to saline-injected fish.

Plasma 17α20βP levels in saline-injected fish were 9.1 ng/ml at the time of injection and increased significantly at 72 and 96 hr reaching 38 ng/ml (Fig. 11). Injections of LH-RH and LH-RHA DAla^6 resulted in elevated plasma 17α20βP levels by 3 hr when compared to saline-injected fish. The response to LH-RH was transitory, at 24 hr plasma 17α20βP levels had decreased to the levels in saline-injected fish and at 96 hr were significantly lower than the levels in saline-injected fish. LH-RHA DAla^6-injected fish which completed GVBD maintained high 17α20βP levels at 24 hr and then showed a further increase reaching 480 ng/ml at 72 and 96 hr. LH-RHA DAla^6-injected fish which failed to complete GVBD maintained elevated 17α20βP levels, but titres were only 55 ng/ml at the 96 hr sampling.

Testosterone levels in saline-injected fish were highly variable and showed little change during the sampling period (Fig. 12). No significant differences were found between the testosterone levels in LH-RH- and saline-injected groups, although testosterone levels in LH-RH-injected fish were decreased significantly at 72 and 96 hr when compared to preinjection levels. Testosterone levels in LH-RHA DAla^6-injected fish which completed GVBD were significantly higher than the levels in saline-injected fish at 24 to 96 hr. Testosterone levels in this group were

FIG. 10. Changes in plasma 17β-estradiol levels in response to single intraperitoneal injections of saline, LH-RH and LH-RHA DAla^6. LH-RHA DAla^6-injected fish have been separated into two groups based on whether the fish had completed GVBD at the 96 hr sampling. Values represent the mean ± standard error with the number of fish per group indicated. At each sampling time, plasma 17β-estradiol levels which are similar as determined by Duncan's Multiple Range Test ($P > 0.05$) are identified by the same superscript, i.e., A, B, and C.

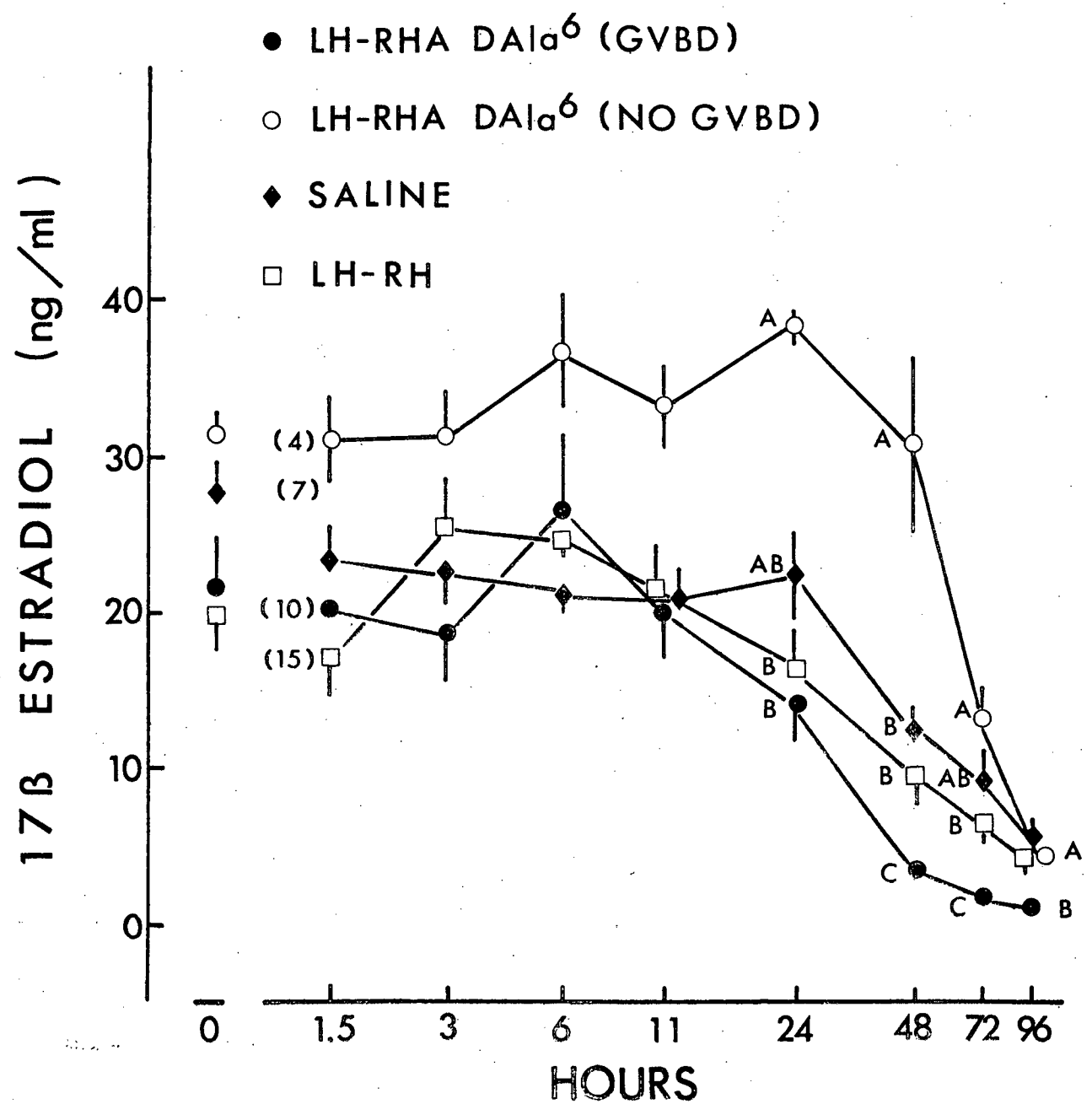

● LH-RHA DAla6 (GVBD)
○ LH-RHA DAla6 (NO GVBD)
◆ SALINE
□ LH-RH
17β ESTRADIOL (ng/ml)
40
30
20
10
0
(4)
(7)
(10)
(15)
A
A
AB
B
B
A
B
AB
B
A
C
C
B
0
1.5
3
6
11
24
48
72
96
HOURS

FIG. 11. Changes in plasma 17α20βP levels in response to single intraperitoneal injections of saline, LH-RH and LH-RHA DAla^6. Additional information is provided in the legend to Fig. 10.

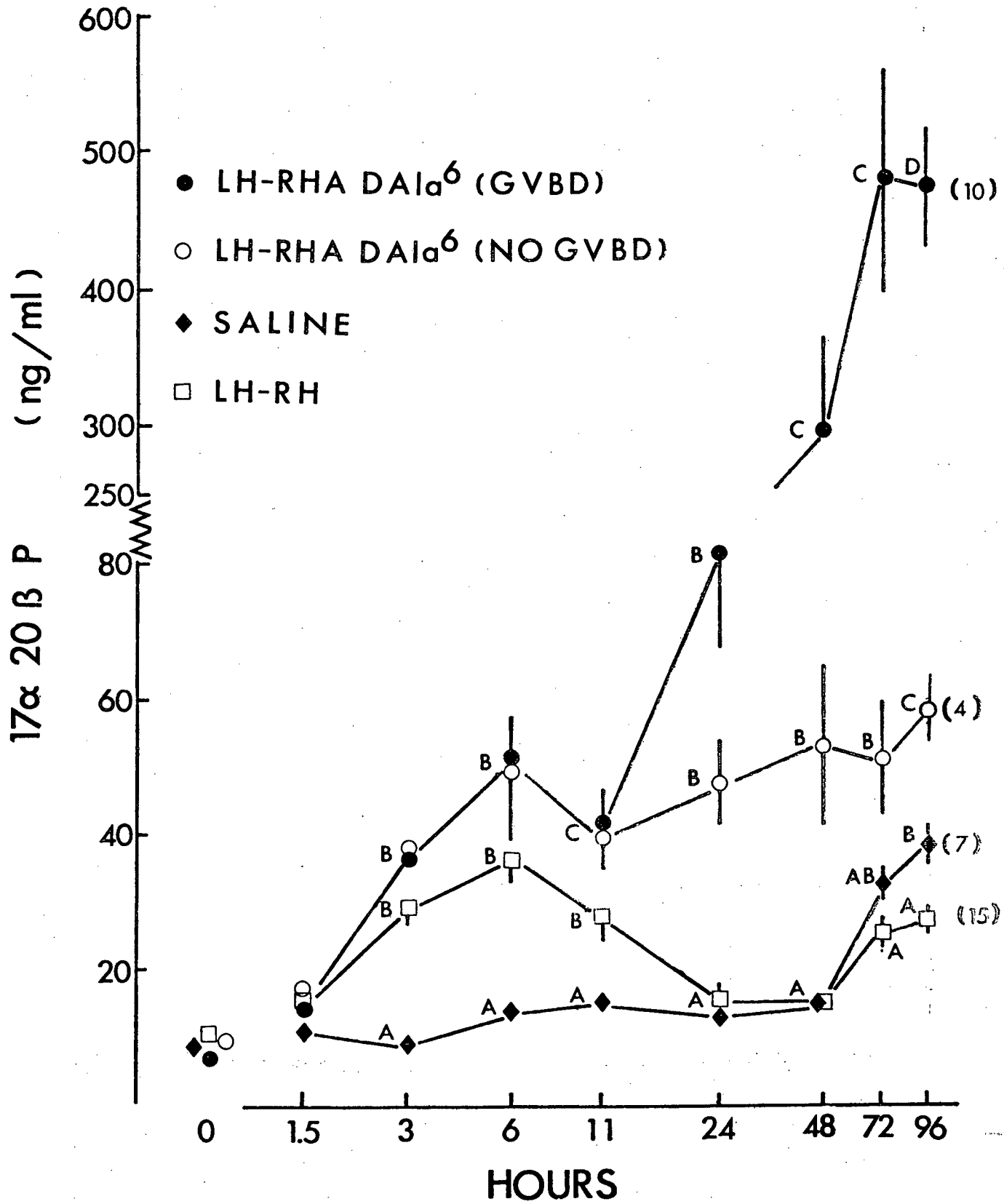
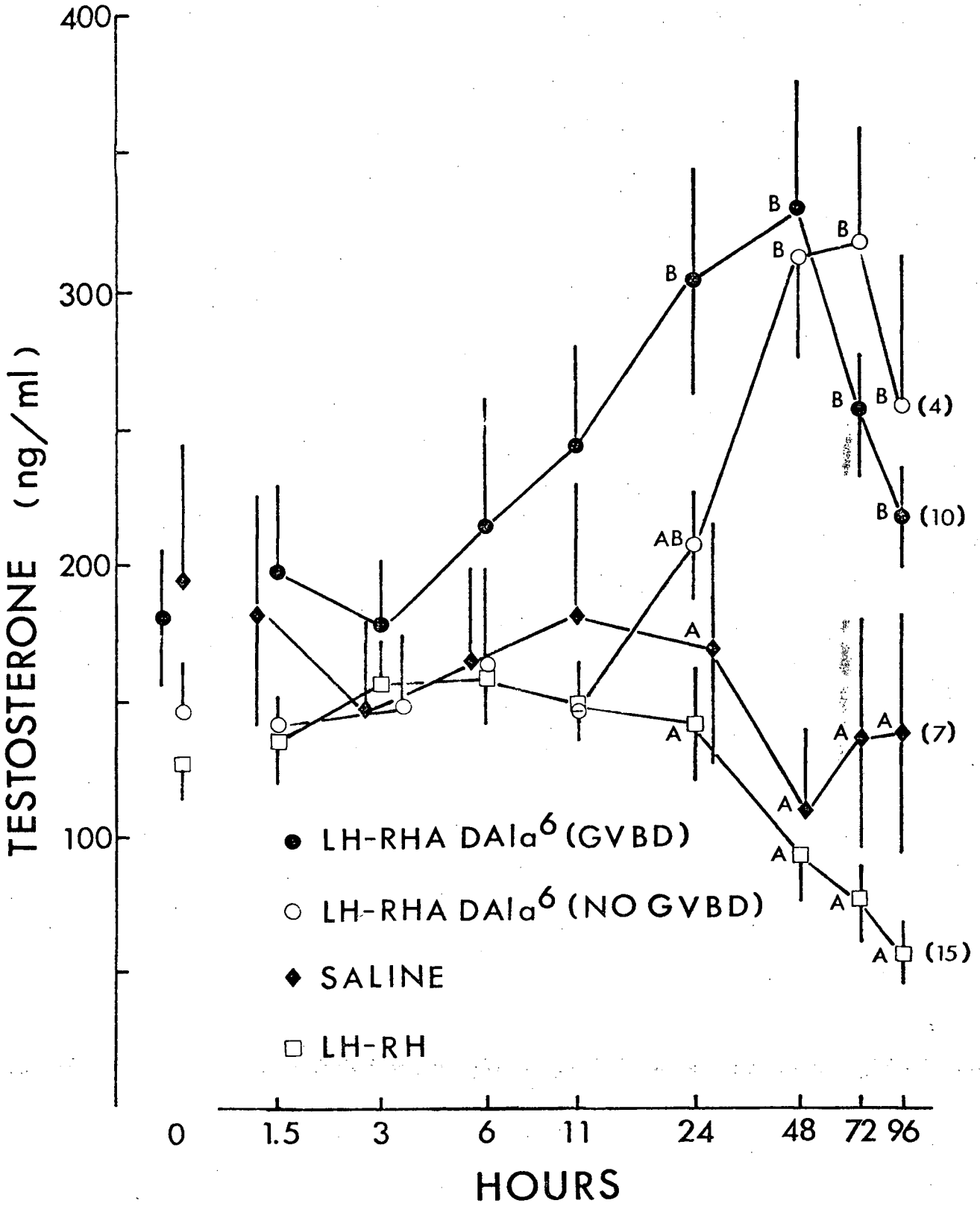

600
500
400
300
250
80
60
40
20
17α 20 β P (ng/ml)
● LH-RHA DAla6 (GVBD)
○ LH-RHA DAla6 (NO GVBD)
◆ SALINE
□ LH-RH
0
1.5
3
6
11
24
48
72
96
HOURS
(10)
(4)
(7)
(15)

FIG. 12. Changes in plasma testosterone levels in response to single intraperitoneal injections of saline, LH-RH and LH-RHA DAla^{6}. Additional information is provided in the legend to Fig. 10.


400
300
200
100
TESTOSTERONE (ng/ml)
B
B
B
B
B
B
B
(4)
B
(10)
AB
A
A
A
A
A
A
A
A
(7)
A
A
(15)
● LH-RHA DAla6 (GVBD)
○ LH-RHA DAla6 (NO GVBD)
◆ SALINE
□ LH-RH
0
1.5
3
6
11
24
48
72
96
HOURS

maximal at 48 hr and decreased significantly by 96 hr. Testosterone levels in LH-RHA DA1a^6-injected fish which failed to complete GVBD were elevated at 48 to 96 hr when compared to saline-injected fish.

III. Steroid Changes in Response to LH-RHA DA1a^6 and SG-G100

A. Temporal steroid changes following injections of LH-RHA DA1a^6 and SG-G100

Plasma 17β-estradiol and 17α20βP levels were 17.2 ± 1.2 ng/ml and 8.1 ± 0.9 ng/ml, respectively, in 12 fish sampled at the time of injection. The effects of various hormone treatments on plasma 17β-estradiol and 17α20βP levels are reported in Table 5 and 6.

No significant differences in plasma 17β-estradiol concentrations were observed between hormone-injected and saline-injected fish at 1 day post injection (Table 5). SG-G100 injected alone and in combination with LH-RHA DA1a^6 significantly reduced plasma 17β-estradiol levels by 2 days post injection when compared to saline-injected fish. Plasma 17β-estradiol levels in all hormone treated groups were lower than the levels in saline-injected fish by day 4 and remained lower until day 10. Owing to the high variation in 17β-estradiol levels between individual fish in each of the hormone-treated groups (see below), it was not possible to discriminate major differences in the effects of the various hormone treatments on days 4-10. However, groups receiving single injections of LH-RHA DA1a^6 at 0.02 mg/kg and SG-G100 tended to have higher 17β-estradiol levels than other groups.

All hormone treatments increased plasma 17α20βP levels compared to saline-injected fish by 1 day post injection and maintained higher 17α20βP levels than saline-injected fish until day 8 (Table 6). Plasma 17α20βP levels in the group injected with 0.02 mg/kg LH-RHA DA1a^6 had decreased by day 10 to a level similar to the saline-injected group. All other hormone-treated groups maintained higher

TABLE 5 - Effects of LH-RHA DAla^6 and SG-G100 on plasma 17β-estradiol levels. At each sampling period, plasma 17β-estradiol levels (mean ± standard error) which are similar as determined by Duncan's Multiple Range test ($P > 0.05$) are identified by a similar superscript.

	Plasma 17β-estradiol (ng/ml) at various days post injection					
Treatment	1	2	4	6	8	10
Saline	17.5 ± 1.1^a	20.9 ± 3.8^a	19.1 ± 2.7^a	17.9 ± 3.8^a	12.8 ± 3.9^a	11.8 ± 3.5^a
Single Injection						
0.02 LH-RHA*	15.0 ± 1.3^a	14.8 ± 2.4^{ab}	6.2 ± 1.6^b	3.2 ± 0.8^b	3.8 ± 1.0^b	3.7 ± 1.1^b
0.2 LH-RHA	18.4 ± 1.1^a	19.4 ± 2.7^a	8.3 ± 2.4^b	3.4 ± 1.0^b	2.0 ± 0.6^{bc}	1.4 ± 0.7^{cd}
SG-G100**	14.5 ± 2.5^a	9.22 ± 2.3^b	6.0 ± 2.1^b	3.5 ± 1.5^{bcd}	3.7 ± 1.9^b	2.1 ± 1.0^{bc}
SG-G100, 0.02 LH-RHA	17.8 ± 0.6^a	10.7 ± 1.1^b	2.7 ± 0.6^b	0.85 ± 0.17^d	1.6 ± 0.2^{bc}	0.53 ± 0.05^d
SG-G100, 0.2 LH-RHA	17.2 ± 2.4^a	11.0 ± 2.1^b	3.2 ± 1.0^b	1.5 ± 0.6^{cd}	1.9 ± 0.4^{bc}	0.84 ± 0.25^{cd}
Double Injection***						
0.02 + 0.02 LH-RHA			7.4 ± 2.9^b	3.5 ± 1.6^{bc}	2.0 ± 0.6^{bc}	0.67 ± 0.09^{cd}
0.2 + 0.2 LH-RHA			2.5 ± 0.5^b	1.4 ± 0.1^{bcd}	1.2 ± 0.1^c	0.69 ± 0.13^{cd}
SG-G100 + 0.2 LH-RHA			6.8 ± 2.3^b	1.4 ± 0.5^{cd}	1.5 ± 0.3^{bc}	0.56 ± 0.07^{cd}

* LH-RHA DAla^6 (mg/kg body wt)

** SG-G100 (0.1 mg/kg body wt)

*** First injection at time 0 + second injection at 72 hr.

TABLE 6 - Effects of LH-RHA DAla^6 and SG-G100 on plasma 17α20βP levels. At each sampling period, plasma 17α20βP values (mean ± standard error) which are similar (P > 0.05) as determined by Duncan's Multiple Range test are identified by a similar superscript.

Treatment	Plasma 17α20βP (ng/ml) at various days post injection					
	1	2	4	6	8	10
Saline	6.0 ± 0.7^{a}	7.3 ± 2.6^{a}	20.7 ± 5.5^{a}	48.3 ± 20.2^{a}	79.0 ± 32.6^{a}	74.3 ± 26.3^{a}
Single Injection						
0.02 LH-RHA*	46.8 ± 5.4^{c}	90.9 ± 34.7^{bc}	228 ± 79^{bc}	222 ± 71^{bc}	136 ± 26^{b}	95.6 ± 21.6^{ab}
0.2 LH-RHA	30.7 ± 4.2^{bc}	47.0 ± 6.2^{b}	197 ± 67^{bc}	386 ± 97^{bcd}	418 ± 83^{cd}	146 ± 28^{bc}
SG-G100**	28.1 ± 3.9^{b}	76.3 ± 15.1^{bc}	112 ± 54^{b}	187 ± 76^{b}	298 ± 86^{bc}	380 ± 97^{d}
SG-G100, 0.02 LH-RHA	40.8 ± 6.9^{bc}	84.6 ± 14.0^{bc}	245 ± 79^{bc}	379 ± 72^{bcd}	487 ± 56^{d}	379 ± 54^{d}
SG-G100, 0.2 LH-RHA	41.5 ± 6.9^{bc}	95.9 ± 19.2^{c}	358 ±105^{c}	410 ± 102^{bcd}	445 ± 79^{cd}	357 ± 52^{d}
Double Injection***						
0.02 + 0.02 LH-RHA			253 ± 80^{bc}	454 ± 85^{cd}	311 ± 54^{c}	223 ± 53^{cd}
0.2 + 0.2 LH-RHA			380 ± 93^{c}	649 ± 73^{d}	557 ± 61^{d}	386 ± 67^{d}
SG-G100 + 0.2 LH-RHA			162 ± 48^{bc}	455 ± 87^{cd}	649 ± 83^{d}	417 ± 45^{d}

* LH-RHA DAla^6 (mg/kg body wt)

** SG-G100 (0.1 mg/kg body wt)

*** First injection at time 0 + second injection at 72 hr.

17α20βP levels than saline-injected fish on day 10. The extreme variation in 17α20βP levels between fish in each of the hormone treated groups made generalizations regarding the effectiveness of the hormone treatments difficult. Differences in 17α20βP levels between treatment groups appear to be unrelated to the maximal levels of 17α20βP but rather to the number of fish which had high levels of 17α20βP and subsequently ovulated (see below and Table 7).

B. Steroid changes in relation to the time of ovulation

To determine if ovulation was associated with specific steroid changes, plasma 17β-estradiol and 17α20βP concentrations have been examined in relation to the time of ovulation (Fig. 13, 14, 15 and 16). Furthermore, to evaluate the effects of administering hormones in a single injection or in two injections over a 72 hr period, the steroid changes in the single and dual injected groups have been examined separately. Table 7 shows the number of fish which ovulated during the periods of 6-7, 8-10, 12-14 or greater than 14 days post injection in each of the treatment groups.

Plasma 17β-estradiol levels in saline-injected fish which ovulated on day 10 and day 14 declined to basal levels of about 2 ng/ml by day 4 and day 6 respectively (Fig. 13A). Saline-injected fish which did not ovulate by day 14 showed a slight increase in plasma 17β-estradiol levels followed by a decline to a level (15 ng/ml) slightly below the initial concentration. Saline-injected fish which ovulated on day 10 and day 14 showed large increases in plasma 17α20βP levels by day 6 and day 8 respectively (Fig 13B). In contrast, saline-injected fish which did not ovulate by day 14 showed a gradual increase in plasma 17α20βP levels to 30 ng/ml by day 10.

The steroid changes, when related to the time of ovulation, were similar in fish receiving single injections of LH-RHA DA1a[6], SG-G100 and SG-G100 in

TABLE 7 - The number of fish which ovulated during different time periods in response to various combinations of LH-RHA DAla^6 and SG-G100 administered in a single injection or two separate injections 72 hr apart. Each treatment group contained 8 fish.

Treatment	Number of fish ovulated at various days 6-7	8-10	12-14	>14
Saline	0	1	1	6
Single injection				
0.02 LH-RHA*	2	2	0	4
0.2 LH-RHA	0	4	1	3
SG-G100**	2	0	4	2
SG-G100, 0.002 LH-RHA	1	5	2	0
SG-G100, 0.02 LH-RHA	3	2	1	2
Double Injection***				
0.02 + 0.02 LH-RHA	3	3	1	1
0.2 + 0.2 LH-RHA	2	5	0	1
SG-G100 + 0.2 LH-RHA	1	4	3	0

* LH-RHA DAla^6 (mg/kg body wt)

** SG-G100 (0.1 mg/kg body wt)

*** First injection at time 0 + second injection at 72 hr.

FIG. 13. Changes in plasma 17β-estradiol (A) and 17α20βP (B) levels in relation to the time of ovulation for saline- injected fish. Steroid levels in individual fish have been grouped according to the time of ovulation (○ 8-10, ◆ 12-14, ◇ > 14 days post injection). Values represent the mean ± standard error, where applicable, with the number of fish indicated in parenthesis.

17β - ESTRADIOL (ng/ml)
A.
0
4
8
12
16
20
24
28
2
4
6
8
10
DAYS POST INJECTION
17α 20β P (ng/ml)
B.
0
40
80
120
160
200
240
280
2
4
6
8
10
(6)
(1)
(1)

combination with LH-RHA DAla^6. Analysis of variance indicated no significant differences between the minimal 17β-estradiol and maximal 17α20βP values in those fish induced to ovulate by day 14. The data from these treatment groups have been pooled according to the time of ovulation (Table 7) and are presented in Fig. 14. 17β-estradiol levels decreased from 18.4 ng/ml to 4.6 ng/ml in fish which did not ovulate by day 14, but these levels were higher on days 2-10 when compared to fish which ovulated (Fig. 14A). Plasma 17β-estradiol levels in fish which ovulated by day 7 were lower on day 2 when compared to fish which ovulated 8-10 days after injection and were also lower on days 2 and 4 when compared to fish which ovulated 12-14 days after injection. Fish which ovulated 8-10 days after injection had lower 17β-estradiol levels on day 4 than fish which ovulated 12-14 days after injection. Fish which did not ovulate by day 14 had lower 17α20βP levels on days 2-10 than fish which ovulated (Fig. 14B). Fish which ovulated by day 7 had higher plasma 17α20βP levels until day 6 when compared to fish which ovulated 8-10 days post injection and to day 8 when compared to fish which ovulated 12-14 days post injection. Fish which ovulated 8-10 days after injection had higher 17α20βP levels on days 4 and 6 than fish which ovulated 12-14 days after injection.

The combined data for plasma 17β-estradiol and 17α20βP levels in fish receiving double hormone injections (Table 7) are illustrated in relation to the time of ovulation in Fig. 15 and 16. No significant differences in plasma 17β-estradiol levels were observed for fish which ovulated 6-7 and 8-10 days after injection (Fig. 15A). Both of these groups had lower plasma 17β-estradiol levels on days 4, 6, and 8 than fish which ovulated 12-14 days after injection.Plasma 17α20βP levels in fish which ovulated by day 7 were higher on day 4 when compared to fish which ovulated 8-10 days after injection and were also higher on days 4 and 6 when compared to fish which ovulated 12-14 days after injection (Fig. 15B). Fish

FIG. 14. Changes in plasma 17β-estradiol (A) and 17α20βP (B) levels in relation to the time of ovulation for coho salmon receiving a single injection of LH-RHA DA1a^{6}, SG-G100 or combined injections of LH-RHA DA1a^{6} and SG-G100 (see Table 4). Steroid levels in individual fish have been grouped according to the time of ovulation (● 6-7, ○ 8-10, ◆ 12-14, ◇ > 14 days post injection). At each sampling period, plasma steroid levels (mean ± standard error, N) which are similar as determined by Duncan's Multiple Range Test ($P > 0.05$) are identified by the same superscript.

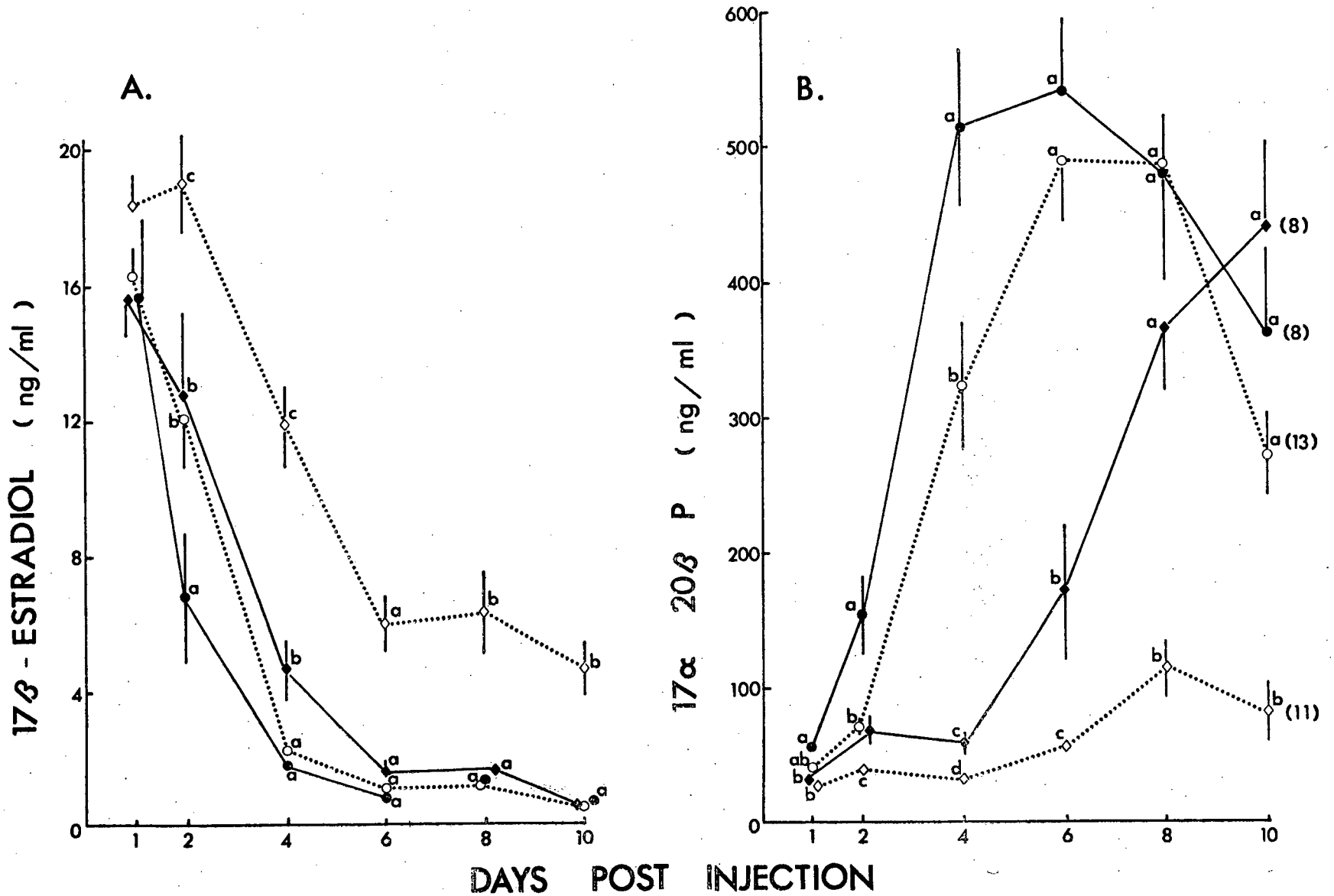

A.
B.
17β - ESTRADIOL (ng/ml)
17α 20β P (ng/ml)
DAYS POST INJECTION
(8)
(8)
(13)
(11)

FIG. 15. Changes in plasma 17β-estradiol (A) and 17α20βP (B) levels in relation to the time of ovulation for coho salmon receiving two separate injections of LH-RHA DAla^6 or SG-G100 followed by LH-RHA DAla^6 (see Table 4). For additional information see the legend to Fig. 14.

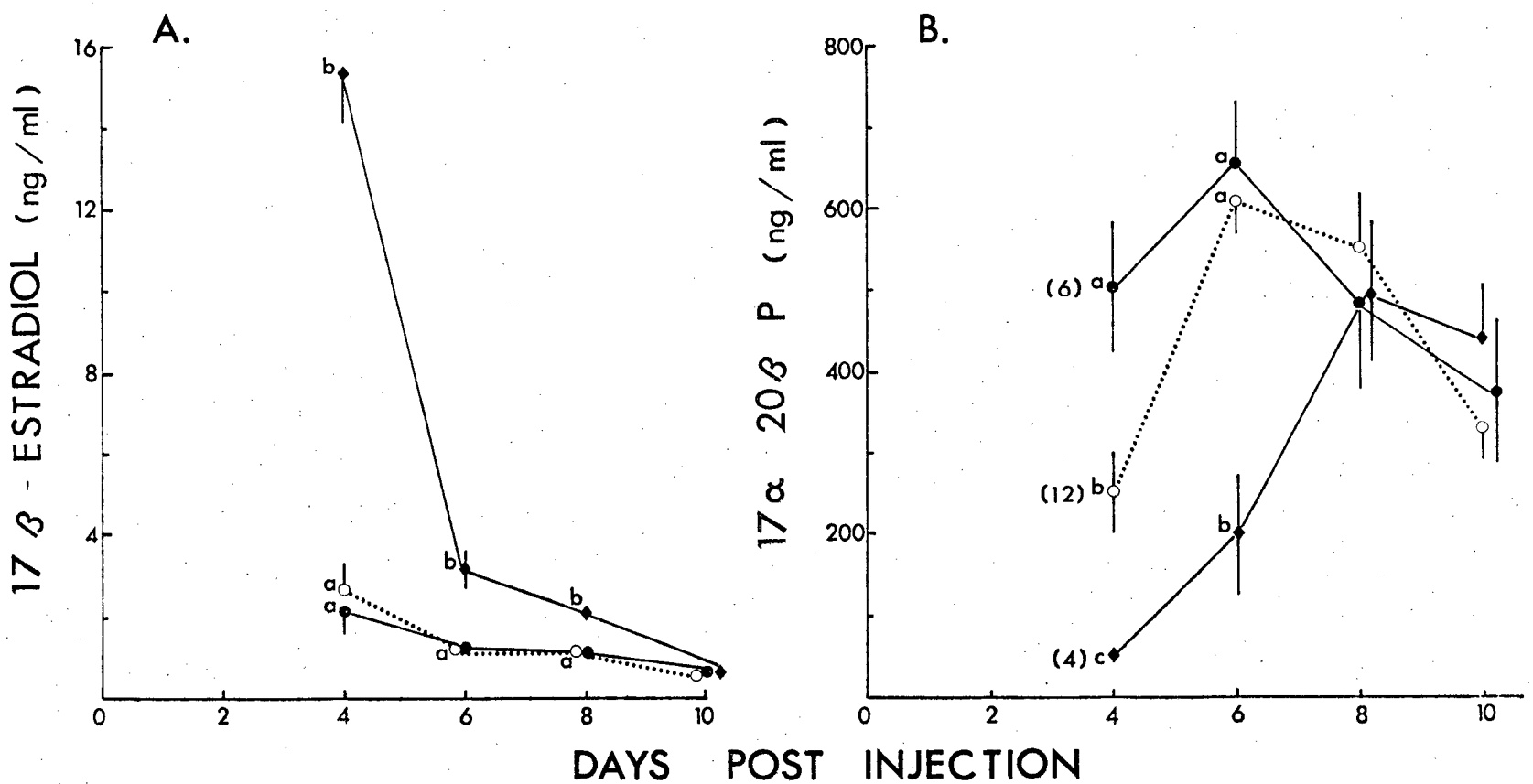
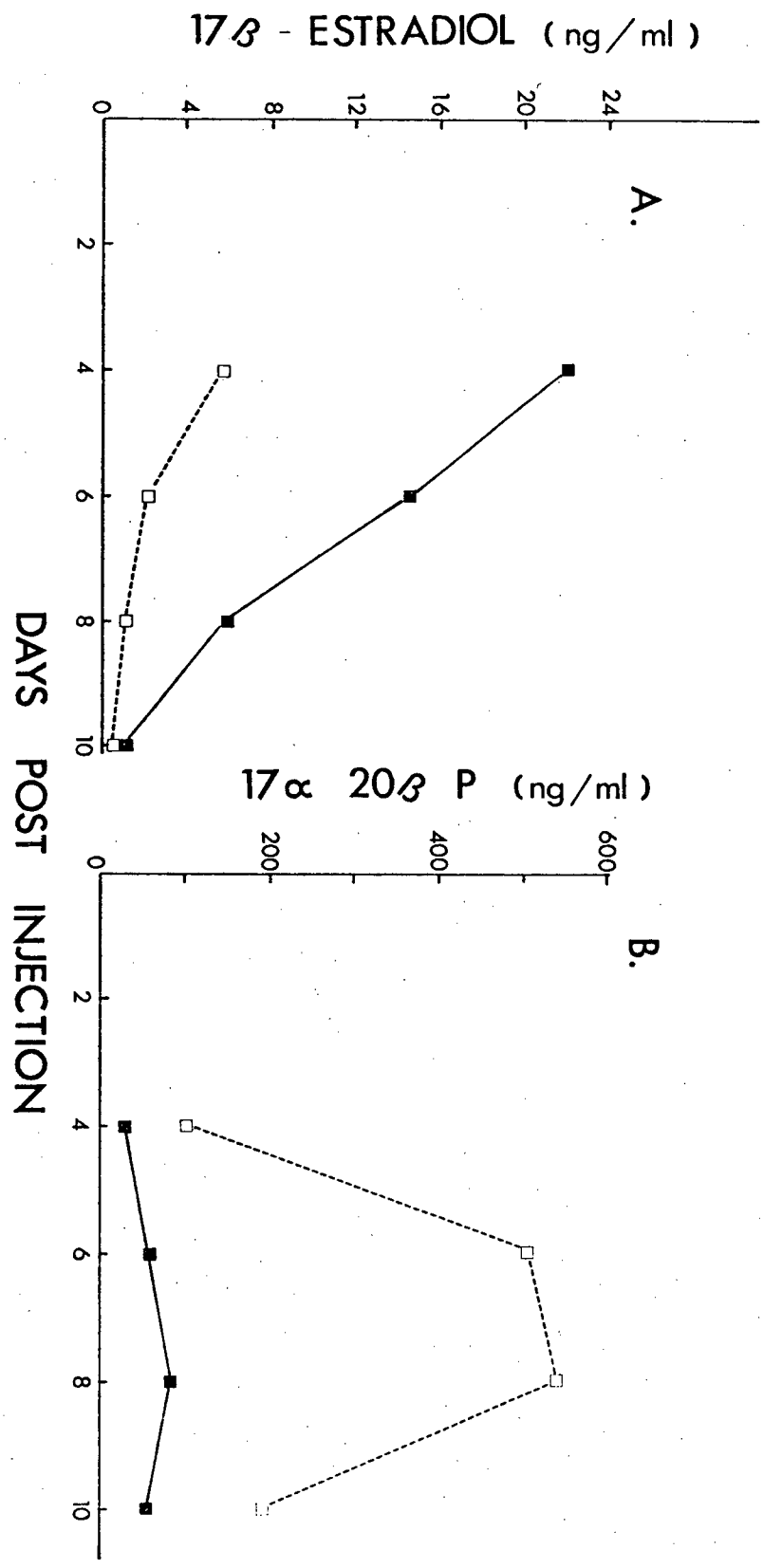

A.
B.
17 β - ESTRADIOL (ng/ml)
17α 20β P (ng/ml)
DAYS POST INJECTION
(6) a
(12) b
(4) c

FIG. 16. Temporal changes in plasma 17β-estradiol (A) and 17α20βP (B) levels in two fish which failed to ovulate by day 14 in response to two injections of LH-RHA DAla^6 over a 72 hr period. Values are based on measurements from one fish receiving 0.02 mg LH-RHA DAla^6/kg (□) and a second fish receiving 0.2 mg LH-RHA DAla^6/kg (■).


17β - ESTRADIOL (ng/ml)
0
4
8
12
16
20
24
A.
2
4
6
8
10
DAYS POST INJECTION
17α 20β P (ng/ml)
0
200
400
600
B.
2
4
6
8
10

which ovulated on days 8-10 had higher plasma 17α20βP levels on days 4 and 6 than fish which ovulated on days 12-14. The plasma 17β-estradiol level in one fish which failed to ovulate was 22 ng/ml on day 4 and declined to 1.1 ng/ml by day 10 (Fig. 16). In this case, plasma 17α20βP remained low (< 100 ng/ml) during the sampling. Plasma 17β-estradiol levels in a second fish which did not ovulate, decreased to below 2 ng/ml by day 6 and showed a large surge in 17α20βP to over 500 ng/ml at this time (Fig. 16). This fish had completed oocyte maturation by day 7 based on the visual examination of oocytes released while checking for ovulation, although ovulation did not occur until day 20.

Fish which ovulated 6-7 and 8-10 days following a single (Fig. 14) or double injection (Fig. 15) had similar plasma 17β-estradiol and 17α20βP concentrations on days 4 to 10. Fish which ovulated on days 12-14 following two injections had higher ($P < 0.01$) plasma 17β-estradiol levels on days 4 and 6 than fish ovulating at this time but given only a single injection. No temporal differences in plasma 17α20βP levels were apparent for fish ovulating 12-14 days following single or dual hormone injections.

IV. In Vitro Steroid Production by Ovarian Follicles During Sexual Maturation

Figures 17, 18 and 19 show the levels of 17β-estradiol, testosterone and 17α20βP in the media following the incubation of ovarian follicles at various stages of maturity with or without SG-G100. The gonadal characteristics and plasma steroid levels in donor fish are presented in Table 8.

A. 17β-Estradiol

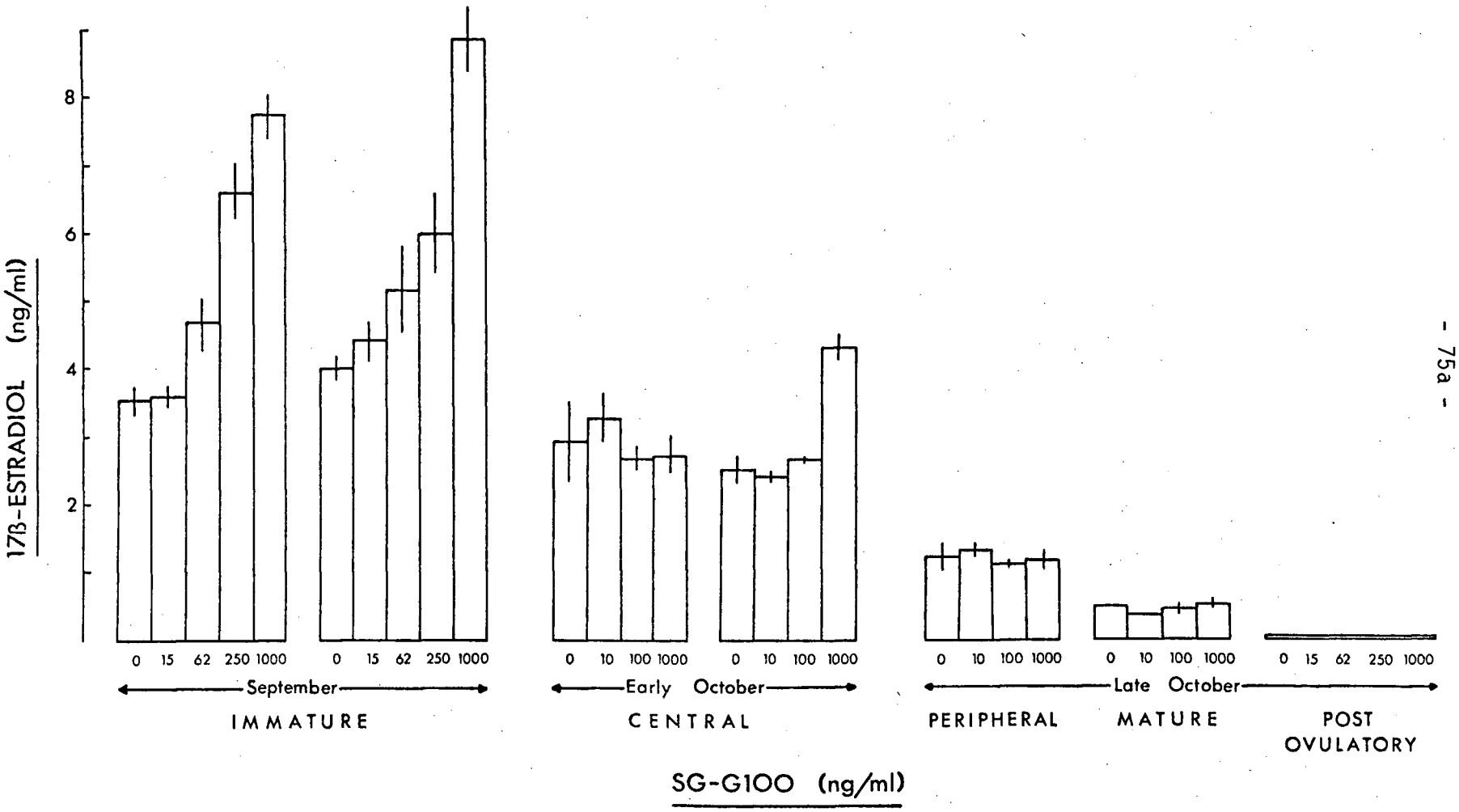
SG-G100 stimulated a dose-related increase in 17β-estradiol production by ovarian follicles obtained in September (Fig. 17). 17β-estradiol production by follicles from one of these fish was 3.5 ± 0.2 ng/ml in the absence of gonadotropin and increased significantly ($P < 0.05$) to 4.6 ± 0.5 ng/ml in response to 62.5 ng/ml

TABLE 8 - Gonadal characteristics and plasma sex steroid levels in coho salmon utilized for *in vitro* steroid production studies.

Date	Maturity status	Oocyte diameter (MM)	Plasma steroid concentration (ng/ml) 17β-estradiol	testosterone	17α20βP
September 16	Immature	4.1	12.0	7.6	< 1.0
September 16	Immature	4.3	15.4	9.4	< 1.0
October 15	Central GV*	5.1	22	108	5.6
October 15	Central GV	5.4	18	140	3.8
October 25	Migratory GV	5.6	9.4	275	18
October 25	Mature	5.5	2.0	185	220
October 27	Postovulatory	-	0.8	110	240

* GV = germinal vesicle

FIG. 17. *In vitro* 17β-estradiol production by ovarian follicles of coho salmon at different stages of sexual maturity. Follicles were incubated with Ringer's alone (0) or with various doses of SG-G100 for 24 hr at 10°C. Values represent the levels of hormone in the media (mean ± standard error) based on three replicate incubations.

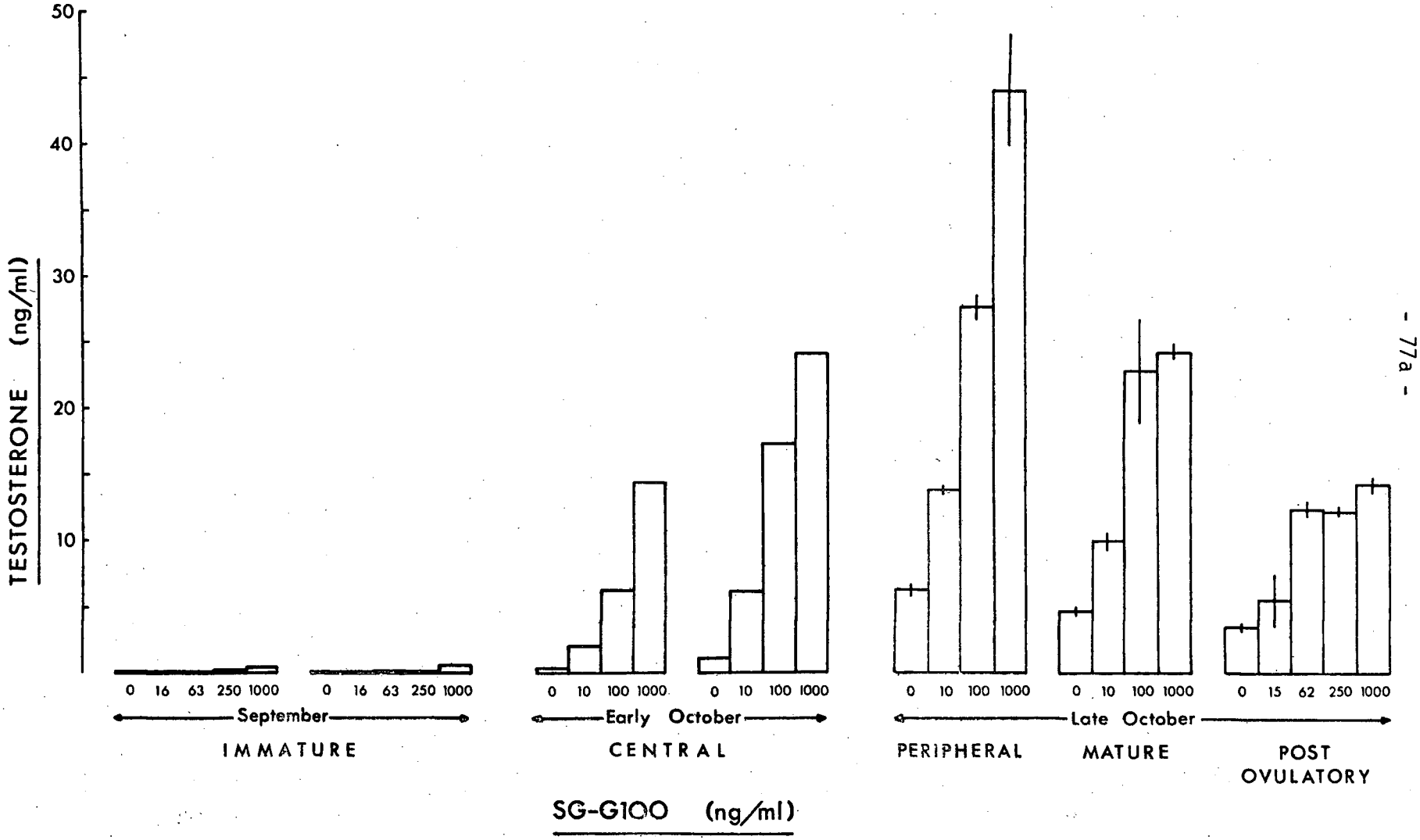

17β-ESTRADIOL (ng/ml)
8
6
4
2
0 15 62 250 1000
0 15 62 250 1000
September
IMMATURE
0 10 100 1000
0 10 100 1000
Early October
CENTRAL
0 10 100 1000
0 10 100 1000
Late October
PERIPHERAL
MATURE
0 15 62 250 1000
POST OVULATORY
SG-G100 (ng/ml)

SG-G100. Further increases occurred in response to 250 and 1000 ng/ml SG-G100 reaching 7.7 ± 0.3 ng/ml at the higher concentration. The pattern of 17β-estradiol production by follicles from a second fish obtained in September was similar. Basal 17β-estradiol production was 4.0 ± 0.2 ng/ml in the absence of gonadotropin. SG-G100 at 250 ng/ml increased 17β-estradiol production significantly ($P < 0.05$) with a further increase ($P < 0.05$) seen in response to 1000 ng/ml SG-G100 reaching 8.9 ± 0.5 ng/ml. Ovarian follicles characterized by a central germinal vesicle showed a diminished response to SG-G100 in terms of 17β-estradiol production. Basal 17β-estradiol production was 2.9 ± 0.6 ng/ml by follicles from one of these fish and did not change in response to SG-G100 at doses up to 1000 ng/ml. 17β-estradiol production by follicles from a second fish was unchanged from basal levels of 2.5 ± 0.2 ng/ml when incubated with SG-G100 at doses up to 100 ng/ml. However, SG-G100 at 1000 ng/ml promoted a significant increase ($P < 0.01$) in 17β-estradiol levels to 4.3 ± 0.2 ng/ml. Follicles obtained from coho salmon in late October were insensitive to SG-G100 at doses up to 1000 ng/ml in terms of 17β-estradiol production. There was a progressive reduction in basal 17β-estradiol production which appeared to relate to oocyte maturity. Follicles characterized by a peripheral germinal vesicle produced 1.2 ± 0.2 ng/ml 17β-estradiol compared to 0.5 ± 0.1 ng/ml 17β-estradiol by follicles obtained after maturation in vivo. 17β-estradiol production by postovulatory follicles was 0.1 ng/ml when incubated with Ringer's alone.

B. Testosterone

Ovarian follicles obtained from coho salmon in September produced relatively small amounts of testosterone in the absence of gonadotropin (0.07 ± 0.01 and 0.08 ± 0.01 ng/ml; Fig 18). Testosterone production by follicles from one of these fish was elevated ($P < 0.05$) in response to 62.5 ng/ml SG-G100 and showed

FIG. 18. *In vitro* testosterone production by ovarian follicles of coho salmon at different stages of sexual maturity. Follicles were incubated with Ringer's alone (0) or with various doses of SG-G100 for 24 hr at 10°C. Values represent the levels of hormone in the media (mean ± standard error) based on three replicate incubations.

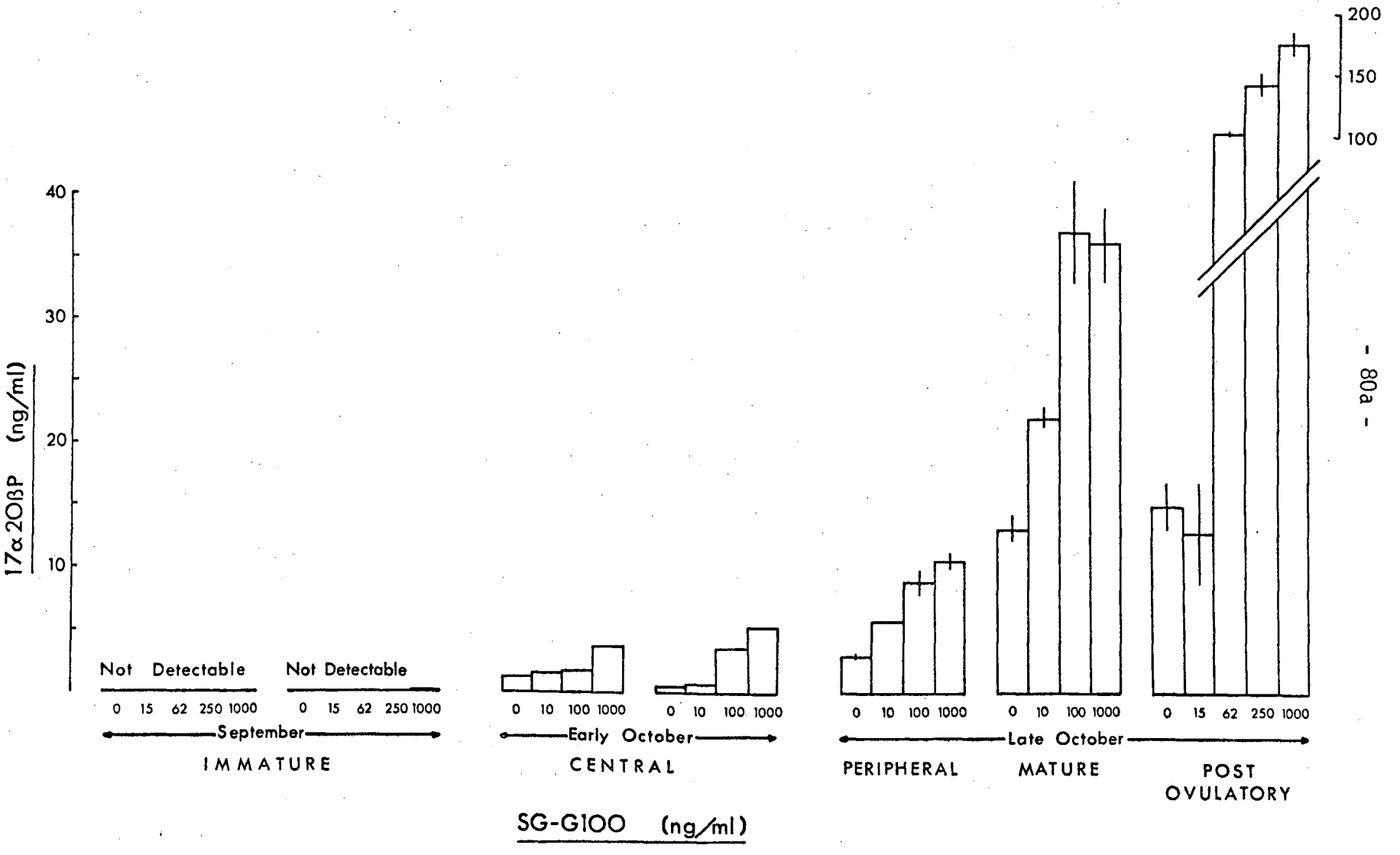

TESTOSTERONE (ng/ml)
50
40
30
20
10
0 16 63 250 1000
0 16 63 250 1000
September
IMMATURE
0 10 100 1000
0 10 100 1000
Early October
CENTRAL
0 10 100 1000
0 10 100 1000
Late October
PERIPHERAL
MATURE
0 15 62 250 1000
POST OVULATORY
SG-G100 (ng/ml)

a further increase ($P < 0.01$) to 0.43 ± 0.02 ng/ml in response to SG-G100 at 1000 ng/ml. Testosterone production by follicles from a second fish increased in response to 1000 ng/ml SG-G100 ($P < 0.01$) reaching 0.59 ± 0.02 ng/ml. Ovarian follicles obtained from coho salmon in mid-October showed an increased capacity to produce testosterone. Although basal testosterone production by follicles from one of these fish was low (0.36 ± 0.03 ng/ml), SG-G100 at 10, 100 and 1000 ng/ml stimulated a dose related increase ($P < 0.01$) in testosterone production. The 14.5 ± 2.7 ng/ml of testosterone produced in response to 1000 ng/ml of SG-G100 was about 40-fold higher than the levels produced in the absence of SG-G100. Testosterone production by ovarian follicles obtained from a second fish at this stage, showed a similar pattern of response. Testosterone production increased from 1.2 ± 0.1 ng/ml in the absence of SG-G100 to 24.4 ± 0.6 ng/ml in response to SG-G100 at 1000 ng/ml. Follicles characterized by a peripheral germinal vesicle produced greater amounts of testosterone than follicles of less advanced maturity. Testosterone production was increased in a dose related manner ($P < 0.01$) by 10, 100 and 1000 ng/ml SG-G100. In this case, testosterone production was increased from basal levels of 6.4 ± 0.4 ng/ml in the absence of SG-G100 to 44.4 ± 4.2 ng/ml in response to 1000 ng/ml SG-G100. Ovarian follicles obtained following maturation in vivo produced 4.8 ± 0.2 ng/ml of testosterone when incubated with Ringer's alone. Testosterone production was increased significantly ($P < 0.05$) in response to 10 ng/ml of SG-G100 and further increased ($P < 0.05$) to maximal levels in response to 100 and 1000 ng/ml SG-G100 reaching 24.4 ± 1.8 ng/ml at the higher concentration. Testosterone production by postovulatory follicles was 3.6 ± 0.2 ng/ml when incubated with Ringer's alone and increased significantly ($P < 0.01$) when incubated with SG-G100 at 62 ng/ml. No further increases in testosterone production occurred when incubated with SG-G100 at 250 or 1000 ng/ml.

C. 17α20βP

Ovarian follicles obtained from one fish in September produced nondetectable levels of 17α20βP (less than 50 pg/ml) when incubated with Ringer's alone or SG-G100 at doses up to 1000 ng/ml (Fig. 19). Similar results were obtained for follicles obtained from a second fish, although detectable levels of 17α20βP were measured in 2 out of the 3 replicates for follicles incubated with 1000 ng/ml of SG-G100. In these cases, 17α20βP levels were 125 and 220 pg/ml respectively. Ovarian follicles obtained in mid-October showed increased 17α20βP production. Follicles from one of these fish produced 1.2 ± 0.2 ng/ml of 17α20βP in the absence of gonadotropin and did not change following incubation with 10 or 100 ng/ml of SG-G100. However, 17α20βP production in response to 1000 ng/ml of SG-G100 was significantly higher ($P < 0.01$) than the other groups reaching 3.8 ± 0.5 ng/ml. Follicles from a second fish at this stage produced low levels of 17α20βP (0.6 ± 0.02 ng/ml) when incubated with Ringer's alone but increased significantly ($P < 0.01$) when incubated with SG-G100 at 100 or 1000 ng/ml. In this case, 17α20βP levels in response to 1000 ng/ml SG-G100 were higher ($P < 0.05$) than the levels produced in response to 100 ng/ml SG-G100 reaching 5.3 ± 0.7 ng/ml at the higher dose. Ovarian follicles obtained in late-October in which the germinal vesicle had migrated towards the periphery produced greater amounts of 17α20βP than did previous stages. 17α20βP production by follicles increased significantly ($P < 0.05$) in response to 10 ng/ml SG-G100 from basal levels of 3.0 ± 0.2 ng/ml to 5.8 ± 0.1 ng/ml. A further increase ($P < 0.05$) was observed in response to 100 and 1000 ng/ml SG-G100 to 10.6 ± 0.8 ng/ml at the higher concentration. Follicles obtained following maturation <u>in vivo</u> produced 13.2 ± 1.1 ng/ml of 17α20βP in the absence of gonadotropin and showed a significant elevation ($P < 0.05$) in response to 10 ng/ml of SG-G100. 17α20βP production further increased in response to 100 or 1000 ng/ml

FIG. 19. *In vitro* 17α20βP production by ovarian follicles of coho salmon at different stages of sexual maturity. Follicles were incubated with Ringer's alone (0) or with various doses of SG-G100 for 24 hr at 10°C. Values represent the levels of hormone in the media (mean ± standard error) based on three replicate incubations. 17α20βP levels which were less than 50 pg/ml were considered to be non-detectable.


17α 20ßP (ng/ml)
40
30
20
10
200
150
100
Not Detectable
Not Detectable
0 15 62 250 1000
0 15 62 250 1000
September
IMMATURE
0 10 100 1000
0 10 100 1000
Early October
CENTRAL
0 10 100 1000
0 10 100 1000
Late October
PERIPHERAL
MATURE
0 15 62 250 1000
POST OVULATORY
SG-G100 (ng/ml)

of SG-G100 reaching maximal levels of 37.3 ± 4.3 ng/ml in response to 100 ng/ml of SG-G100.Postovulatory follicles in comparison with other stages produced very large amounts of 17α20βP. In this case, media 17α20βP levels were 14.6 ± 6.2 ng/ml in the absence of SG-G100 and increased to 199 ± 21 ng/ml in response to 1000 ng/ml of SG-G100.

V. Effects of 17β-estradiol on 17α20βP Production In Vitro

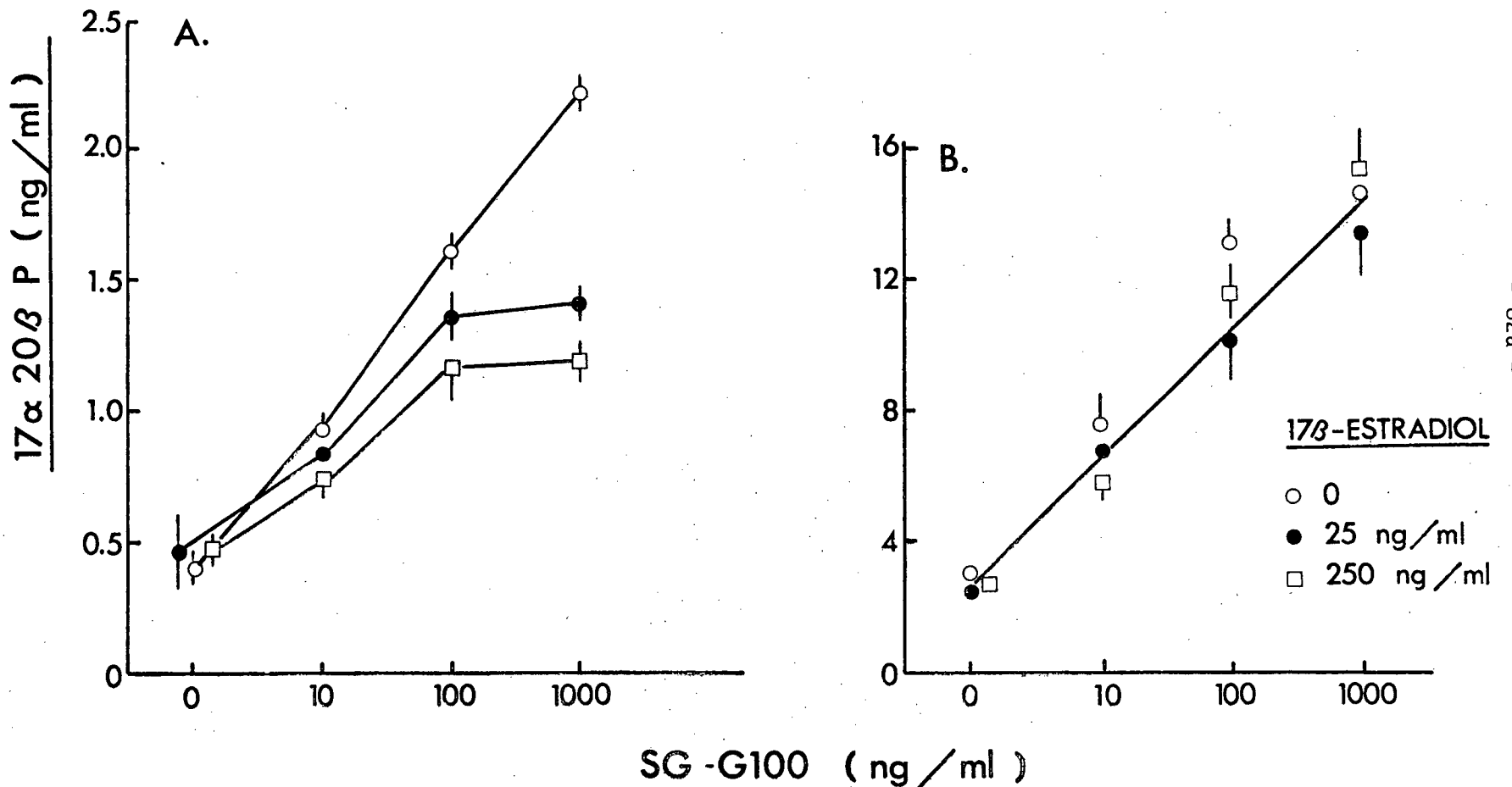
Fig. 20A shows the levels of 17α20βP released to the media by follicles characterized by a central germinal vesicle following gonadotropin stimulation in the presence or absence of 17β-estradiol SG-G100 stimulated a dose related increase in 17α20βP production from 0.4 ± 0.1 ng/ml in the absence of SG-G100 to 2.2 ± 0.2 ng/ml in the presence of 1000 ng/ml SG-G100 The addition of 17β-estradiol at 25 and 250 ng/ml did not influence basal or SG-G100 at 10 and 100 ng/ml stimulated 17α20βP production. The amounts of 17α20βp produced by follicles incubated with 1000 ng/ml SG-G100 and 17β-estradiol was lower ($P < 0.01$) than the levels produced by follicles incubated with 1000 ng/ml SG-G100 alone. Fig. 20B show the results of a similar experiment conducted on follicles characterized by a peripheral germinal vesicle. In this case, 17α20βP production increased from basal levels of 4.5 ± 0.2 ng/ml to 20.6 ± 0.6 ng/ml in response to SG-G100 at 1000 ng/ml. The addition of 17β-estradiol at 25 or 250 ng/ml did not influence basal or gonadotropin stimulated 17α20βP production.

D. Discussion

Preovulatory Steroid Changes

The preovulatory period in coho salmon was characterized by declining 17β-estradiol levels 10 days prior to ovulation and high testosterone levels with peak levels evident 6 days prior to ovulation (Fig. 9). The preovulatory period was also characterized by a large increase in plasma 17α20βP levels coincident to

FIG. 20. Effects 17β-estradiol on the production of 17α20βP *in vitro* in response to graded amounts of SG-G100. Values represent the mean ± standard error based on three determinations using follicles characterized by a central germinal vesicle (A) and by a peripheral germinal vesicle (B).


A.
B.
17α 20β P (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
16
12
8
4
0
10
100
1000
SG -G100 (ng/ml)
17β-ESTRADIOL
0
25 ng/ml
250 ng/ml

the completion of oocyte final maturation. A similar temporal pattern of change in these hormones has recently been described during the preovulatory period in rainbow trout (Fostier and Jalabert, 1982; Scott et al., 1983). This suggests that the pattern of steroid secretion associated with oocyte maturation and ovulation is similar in coho salmon and rainbow trout.

The high levels of 17α20βP in the plasma of coho salmon at the time of oocyte maturation (Fig. 9) was consistent with the concept that 17α20βP functions as the maturation inducing steroid in salmonids (Jalabert, 1976; Young et al., 1982a; Goetz, 1983; Nagahama et al., 1983). However, the functional significance of changes in 17β-estradiol and testosterone during the preovulatory period remains unclear. Although 17β-estradiol is ineffective and testosterone has only a limited effectiveness in promoting maturation in salmonids (Jalabert, 1975; Young et al., 1982a), the maintenance of low 17β-estradiol and high testosterone levels may contribute to the proper steroid environment for 17α20βP synthesis and oocyte maturation. Testosterone has been shown to enhance the effectiveness of gonadotropin and 17α20βP on the induction of oocyte maturation in vitro (Jalabert, 1976; Young et al., 1982a) and therefore may be directly associated with oocyte maturation. 17β-estradiol and testosterone may also have important regulatory effects on the control of gonadotropin synthesis and release. Antiestrogens have been shown to stimulate gonadotropin secretion in teleosts (see Peter, 1982a,b) suggesting that declining 17β-estradiol levels may permit increased gonadotropin secretion. The decline in 17β-estradiol levels (Fig. 9) and the concomitant rise in gonadotropin levels (Fig. 3; Chap. 3) are suggestive of a cause and effect relationship, although proof that this represents release from a negative feedback is lacking. Aromatizable androgens and estrogens have been shown to increase pituitary gonadotropin levels in sexually immature salmonids (Crim and

Evans, 1979; Crim et al., 1981b; Geilen et al., 1982). The high levels of testosterone during the preovulatory period in coho salmon may function in a similar fashion to increase pituitary gonadotropin levels in preparation for increased gonadotropin secretion associated with oocyte maturation and ovulation and may also have a role in spawning behavior.

Based on short-term studies which examined the pattern of steroid secretion by coho salmon ovarian follices in vitro (Fig. 17, 18 and 19), it appears that the preovulatory changes in 17β-estradiol, testosterone and 17α20βP (Fig. 9) result primarily from changes in the activity of steroid converting enzymes located in the follicle. Although results of the in vitro studies were based on determinations from only one or two fish at each stage of maturity, these findings have been confirmed by recent reports detailing the pattern of steroid secretion by amago salmon follicles (Kagawa et al., 1983; Young et al., 1983a).

Follicles from adult coho salmon upon entry to freshwater produce high levels of 17β-estradiol but then show a progressive loss of sensitivity to SG-G100 and by the postovulatory stage produce negligible amounts of hormone (Fig. 17). Previous studies have also shown that gonadotropin stimulates 17β-estradiol production during vitellogenesis (Billard et al., 1978; Yaron and Barton, 1980; Kagawa et al., 1982a,b; Zohar et al., 1982) although recent evidence indicates that this action is a consequence of providing a suitable substrate for aromatization (Kagawa et al., 1982b; Young et al., 1982b,c, 1983c). For example, the conversion of exogenous testosterone to 17β-estradiol by isolated granulosa cell layers from the amago salmon was not enhanced by the addition of gonadotropin (Kagawa et al., 1982b; Young et al., 1982b, 1983c). It is doubtful that the preovulatory decline in 17β-estradiol is related to a lack of substrate since coho salmon have high levels of testosterone throughout the preovulatory period (Fig. 9 and 12) and produce

large quantities of testosterone in response to SG-G100 *in vitro* (Fig. 18). It is more likely that the preovulatory decline in 17β-estradiol levels results from declining aromatase activity. A reduction of ovarian aromatase activity during the preovulatory period in rainbow trout has been suggested on the basis of the reduced incorporation of ^{3}H-labeled androstendione into estrogens (van Boehman and Lambert, 1981). In addition, Young *et al.* (1983c) reported that isolated granulosa cell layers from preovulatory follicles and postovulatory follicles from the amago salmon fail to convert exogenous testosterone to 17β-estradiol.

Follicles obtained from coho salmon in September produce only low amounts of testosterone (Fig. 18) which may reflect its high rate of conversion to 17β-estradiol. The high levels of testosterone in the plasma of maturing coho salmon (Table 5, Fig. 1 and 4) were consistent with the enhanced capacity of preovulatory follicles to produce testosterone in response to SG-G100 *in vitro* (Fig. 18). The increase in testosterone production by follicles in October presumbly relates to its decreasing rate of conversion to 17β-estradiol but may also reflect an increase in the activity of enzymes associated with testosterone production.

It is apparent from measurements of 17α20βP production by follicles incubated *in vitro* that the capacity to produce this steroid develops 1-2 months prior to ovulation (Fig. 19). The low levels of 17α20βP in the plasma of coho salmon prior to maturation (Table 8; Fig. 9, 11 and 14) were consistent with the limited capacity to produce this steroid *in vitro*. Additonally, basal 17α20βP production by follicles from fish which matured *in vivo* was higher than the levels of 17α20βP produced by follicles prior to maturation even when incubated with SG-G100. This observation suggests that oocyte maturation is associated with an increased capacity of the follicle to produce 17α20βP which presumably reflects increasing

amounts of 20β-HSD. Postovulatory follicles produced far greater amounts of 17α20βP in response to SG-G100 than preovulatory follicles which had undergone maturation. This was somewhat suprising in view of the comparable plasma 17α20βP levels in these fish (Table 8) and the tendency for 17α20βP levels to decline at ovulation (Fig. 9 and Fig. 13). Young et al. (1983a) also found that postovulatory follicles from the amago salmon produced far greater amounts of 17α20βP in response to SG-G100 than preovulatory follicles. The basis of the increased capacity of postovulatory follicles to produce 17α20βP may relate to hypertrophy of the granulosa cell layer which is the likely source of the hormone (Young et al., 1983a,b).

Testosterone production by follicles following maturation in vivo and by postovulatory follicles was decreasing while 17α20βP production was highest (Fig. 18 and 19). This finding was consistent with the tendency for plasma testosterone levels to decline at maturation (Fig. 9) and the decreasing plasma testosterone levels following LH-RHA DAla^6 injection in fish which completed GVBD (Fig. 12). The basis for the diminished capacity of the follicle to produce testosterone is not known, however, both testosterone and 17α20βP are produced from 17αhydroxyprogesterone. Whether decreasing amounts of testosterone result from the predominance of 20β-HSD which mediates the conversion of 17αhydroxyprogesterone to 17α20βP or a concomitant reduction in the activities of C19-21 desmolase and 17β-HSD which mediate the conversion of 17αhydroxyprogesterone to testosterone remains to be investigated. In mammals, 17α20βP binds irreversibily to desmolase blocking testosterone production (Inano et al., 1967). A similar mechanism in coho salmon could contribute the decreasing production of testosterone when 17α20βP production is high.

Steroid Changes in Response to Elevated Plasma Gonadotropin Levels

The changes in plasma 17α20βP and testosterone levels following injections of LH-RH and LH-RHA DAla^6 (Fig. 11 and 12) were consistent with the differential effects of these treatments on plasma gonadotropin levels (Fig. 4, Chapt. 3). Injections of LH-RH promote a transient increase in plasma 17α20βP levels which persists for less than 24 hr (Fig. 11). Although 17α20βP levels in LH-RHA DAla^6-injected fish were highly variable, these levels were higher than those in LH-RH-injected fish at 11-96 hr (Fig. 11). Injections of LH-RH and LH-RHA DAla^6 also had very different effects on plasma testosterone levels (Fig. 12). LH-RH-injected fish showed a significant decrease in plasma testosterone levels at 48-96 hr when compared to preinjection values. In contrast, testosterone levels in LH-RHA DAla^6-injected fish were increased at 24 hr and 48 hr in fish which completed and failed to complete GVBD, respectively (Fig. 12). Furthermore, the testosterone levels in LH-RHA DAla^6-injected fish were higher than the levels in saline and LH-RH-injected fish at 48-96 hr. These differences were consistent with the short duration increase in plasma gonadotropin levels in LH-RH-injected fish and the longer duration increase in LH-RHA DAla^6-injected fish (Fig. 4, Chapt. 3). Why testosterone levels decrease in LH-RH-injected fish is not understood. LH-RH-injected fish also had lower 17α20βP levels than saline-injected fish at 96 hr. Additional research is warranted to investigate the possibility that a transitory elevation of plasma gonadotropin levels depletes the supply of steroid precursors and a long-term increase is necessary to provide additional substrate for steroid synthesis.

The effects of gonadotropin on plasma 17β-estradiol levels were more difficult to interpret. Injections of saline and LH-RH had similar effects on plasma 17β-estradiol levels (Fig. 10). In this case, plasma 17β-estradiol levels

were decreased significantly relative to preinjection values by 48 hr. Furthermore, this decrease occurred in the apparent absence of changes in plasma gonadotropin levels (Fig. 4, Chapt. 3). The effects of LH-RHA DAla^6 on plasma 17β-estradiol levels were highly variable. 17β-estradiol levels in LH-RHA DAla^6-injected fish which were induced to mature decreased at 48-96 hr to levels significantly lower than those in saline or LH-RH-injected fish (Fig. 10). In contrast, LH-RHA DAla^6-injected fish which failed to mature had higher 17β-estradiol levels than saline-injected fish at 24 and 48 hr but then showed a sharp decrease to levels similar to those in saline-injected fish by 72 and 96 hr. It is possible that these disparate results are a consequence of differences in the maturity of fish at the time of injection. A comparison of the steroid profiles in saline-injected fish (Fig. 10, 11 and 12) and those of spontaneously ovulating fish (Fig. 9) suggest that hormone injections were administered about 8-10 days prior to the expected time of ovulation. A similar conclusion was reached on the basis of the time of ovulation in laboratory held coho salmon. Owing to this advanced state of maturity, the decline in 17β-estradiol levels in saline-injected fish would be expected in the absence of gonadotropin stimulation. Although the difference was not significant, LH-RHA DAla^6-injected fish which failed to mature tended to have higher 17β-estradiol levels than other groups at the time of injection which could account for the delay in reducing 17β-estradiol levels.

Results of a second experiment using fish of less advanced maturity provide further evidence for a gonadotropin induced decrease in plasma 17β-estradiol levels (Table 5). The effects of gonadotropin on plasma 17β-estradiol levels were dose- and time-dependent. The injection of SG-G100 alone or in combination with LH-RHA DAla^6 results in higher plasma gonadotropin levels on days 1 and 2 than injections of LH-RHA DAla^6 alone (Fig. 6, Chapt. 3) and promotes a significant reduction in

plasma 17β-estradiol levels by day 2 compared to day 4 following LH-RHA DAla^6 injection (Table 5). Owing to the high variation in 17β-estradiol levels between individual fish in each of the hormone treated groups (Fig. 14, 15 and 16) it was not possible to discriminate major differences in the effects of the various hormone treatments on days 4 to 10. In general, fish which ovulated in response to the various hormone treatments showed a more pronounced reduction in plasma 17β-estradiol levels than fish which failed to ovulate. Studies based on the production of 17β-estradiol *in vitro* by ovarian follicles from coho salmon (Fig. 17) and from other salmonids (van Boehman and Lambert, 1981; Nagahama and Kagawa, 1982; Kagawa *et al.*, 1983; Young *et al.*, 1983c) suggest that aromatase activity declines from maximal levels during vitellogenesis to very low levels at ovulation. In part, the high variation in plasma 17β-estradiol levels in response to elevated plasma gonadotropin levels may reflect differences in the maturity of fish at the time of injection. Sower *et al.* (1983) recently provided confirmation of these results in that injections of salmon gonadotropin and LH-RHA DAla^6 were shown to decrease plasma 17β-estradiol levels in adult female coho salmon and steelhead trout. Furthermore, Zohar *et al.* (1982) demonstrated a gonadotropin induced inhibition of 17β-estradiol secretion by rainbow trout oocytes incubated in an open-perifusion system. It is likely that the gonadotropin-induced decrease in plasma 17β-estradiol levels results from an inhibition of aromatase activity although the precise mechanism of this action is poorly understood.

There are several aspects of the regulation of aromatase activity that remain unclear. First, it appears that there is a gradual loss of aromatase activity throughout the preovulatory period (Fig. 9). However, elevated gonadotropin levels were only evident during the 1-2 week period preceding ovulation (Fig. 2 and 3, Chap. 3). Zohar *et al.* (1982) suggest during the preovulatory period in rainbow

trout that gonadotropin levels show a gradual increase but more importantly show a marked daily fluctuation to very high levels which directly contribute to the decline in ovarian aromatase activity. However in coho salmon,there was no evidence to indicate a significant daily cycle in plasma gonadotropin levels (see Fig. 4, Chapt. 3). It is possible that the increase in plasma gonadotropin levels at the end of the preovulatory period (Fig. 2 and 3; Chapt 3) is secondary to an earlier increase from the very low and often undetectable levels present in immature fish. In addition to gonadotropin, the possible involvement of other hormones in regulating aromatase activity can not be excluded. For example, FSH is required to maintain aromatase activity in mammals (Leung and Armstrong, 1980). The possible involvement of the "vitellogenic gonadotropin" (Idler, 1982) in controlling aromatase activity merits further investigation. A third aspect of the regulation of aromatase activity which is poorly understood is the mechanism whereby gonadotropin switches from having a stimulatory to an inhibitory effect on 17β-estradiol secretion. During vitellogenesis in the amago salmon, gonadotropin acting on the thecal cell layer stimulates the production of aromatizable androgens which are converted to 17β-estradiol in the granulosa cell layer which contains aromatase (Kagawa et al., 1982b; Young et al., 1982b, 1983c). Young et al. (1983b) have shown that gonadotropin acting directly on the granulosa cells induces 20βHSO activity. The loss of aromatase activity (Fig. 17) appears to coincide with the ability to produce 17α20βP in vitro (Fig. 19). It is possible that gonadotropin acting via a receptor which appears in the granulosa cells in the latter stages of the preovulatory period provides a second site of action which in addition to having a stimulatory effect of 20βHSD activity is inhibitory to 17β-estradiol production.

The gonadotropin-induced decrease in plasma 17β-estradiol levels in coho

salmon contrasts with data for carp (Weil et al., 1980) and goldfish (Stacey et al., 1984). In the adult carp, plasma 17β-estradiol levels increase following injections of LH-RH or crude pituitary extracts (Weil et al., 1980). In goldfish, plasma 17β-estradiol levels increase during spontaneous and brain-lesion induced ovulation (Stacey et al., 1983). It is possible that this may relate to the synchronous pattern of oocyte development in coho salmon and the asynchronous pattern of oocyte development in the carp and goldfish. For example, when oocytes from maturing goldfish were subdivided according to size, small follicles respond to gonadotropin by increased 17β-estradiol production whereas large follicles showed a reduced response (W. Garcia and R.E. Peter, personal communication).

Plasma 17α20βP levels were low (< 10 ng/ml) at the time of injection in Exp. II and III, but increase rapidly following hormone treatment. In Exp. II, plasma 17α20βP levels were elevated 3 hr following injections of LH-RH and LH-RHA DA1a^6 (Fig. 11). In Exp. III, plasma 17α20βP levels were increased at the first sampling (24 hr) in all hormone-treated groups (Table 6, Fig. 14, and 15). These results suggest that the enzymes required for the synthesis of 17α20βP were present at least one month prior to the expected time of ovulation. A similar conclusionwas reached on the basis of 17α20βP production by ovarian follicles in vitro (Fig. 19). 17α20βP levels in LH-RHA DA1a^6 injected fish were increased to an apparent plateau at 6-11 hr, with the large increase observed in fish which completed GVBD evident by 48 hr (Fig. 11). In Exp. IV, the increase in plasma 17α20βP levels to greater than 150 ng/ml was delayed up to 6 days in fish which ovulated by day 14 (Fig. 14 and 15). Recent studies in rainbow trout, coho salmon and Atlantic salmon have also shown a delay prior to the large increase in 17α20βP levels following injections of crude pituitary extracts (Scott et al., 1982; Wright and Hunt, 1982). It is likely that the amounts or activities of enzymes associated with

17α20βP synthesis are limiting and directly contribute to this delay. For example, Jalabert (1976) reported that gonadotropin-induced maturation of rainbow trout oocytes *in vitro* was sensitive to actinomycin D and puromycin. 17α20βP-induced maturation was not sensitive to these inhibitors, suggesting that the actions of gonadotropin are mediated by mRNA and protein synthesis required for the production of enzymes necessary for 17α20βP synthesis (Jalabert, 1976). Suzuki *et al.* (1981) reported that gonadotropin stimulated the activity or induced the formation of 20β hydroxysteroid dehydrogenase (20βHSD) in the ayu. It is likely that the the delay associated with the surge in 17α20βP levels in coho salmon is a consequence of this action.

Steroid Changes Associated with Induced Oocyte Maturation and Ovulation

The induction of oocyte maturation was related to the duration and magnitude of the increase in plasma 17α20βP levels. A transient elevation of 17α20βP levels as seen following injections of LH-RH or a short-term increase at low titres (55 ng/ml) as seen in some LH-RHA DA1a^{6}-injected fish failed to promote GVBD (Fig. 11). LH-RHA DA1a^{6}-injected fish which completed GVBD showed a long-term increase in 17α20βP reaching 480 ng/ml by 72 hr (Fig. 11). GVBD was induced in rainbow trout oocytes incubated *in vitro* within 60 hr of treatment with 1 μg/ml of 17α20βP for 30 sec to 15 min (Jalabert, 1976). Although this dose was higher than the levels reported here, continuous exposure of rainbow trout oocytes to 30 ng/ml of 17α20βP (Jalabert, 1976) or amago salmon oocytes to 5-30 ng/ml of 17α20βP (Young *et al.*, 1982a; Nagahama *et al.*, 1983) was sufficient to promote maturation in 50% of the oocytes during a 3 day incubation. The apparent difference between the higher levels of 17α20βP necessary to promote GVBD *in vivo* and that required *in vitro* may relate to the binding of 17α20βP to plasma proteins *in vivo*. The binding of 17α20βP to plasma proteins would presumably reduce the concentration of free

hormone available for binding to 17α20βP receptors on the outer surface of the oocyte. Fostier and Breton (1975) reported that the addition of carbon treated plasma to rainbow trout oocytes incubated *in vitro* increased the median effective dose of 17α20βP required to stimulate GVBD by about 10-fold necessitating the use of 100-200 ng/ml.

Recent data on 17α20βP changes during induced ovulation in goldfish (Stacey *et al.*, 1983) were very different from the data reported here for coho salmon. Oocytes from the goldfish matured within 5 hr of hypothalamic lesions which stimulate a massive increase in plasma gonadotropin levels but show only a transient increase in 17α20βP levels to 20 ng/ml at 5 hr postlesioning (Stacey *et al.*, 1983). Unlike coho salmon which require a long-term elevation of 17α20βP at high levels to induce oocyte maturation, goldfish require only a short-term increase in 17α20βP at relatively low levels to promote oocyte maturation. However, Nagahama *et al.* (1983) failed to detect a major difference in the activity of 17α20βP on the stimulation of oocyte maturation when oocytes from rainbow trout, amago salmon, ayu and goldfish were incubated *in vitro*. As 17α20βP was not detected in the plasma of goldfish which ovulate spontaneously, it is possible that 17α20βP could exert its effect locally before the rate of synthesis was sufficient to increase plasma levels.

Oocyte maturation is a preliminary step to ovulation in teleosts. These events are normally closely linked, with oocyte maturation occurring 2-4 days prior to ovulation in coho salmon. These events can be dissociated when attempting to induce ovulation in that fish can be induced to mature but fail to ovulate at the expected time. An examination of oocytes expelled when checking for ovulation in Exp. III indicated that all but one of the hormone-treated fish had completed oocyte maturation by day 10. However, many of these fish did not ovulate by day 14

and in certain cases did not ovulate until after day 20. The basis of the dissociation between oocyte maturation and ovulation is poorly understood. It was possible to distinguish fish which were induced to mature from fish which ovulate on the basis of 17α20βP levels. With one exception (see below), there was a marked difference in the maximal 17α20βP levels in fish which ovulated by day 14 (450 ng/ml) and fish which ovulated at later times (100 ng/ml) (Fig. 14, 15, and 16). These results suggest that a long-term elevation of plasma 17α20βP at relatively low concentrations was sufficient to promote oocyte maturation. Since 17α20βP levels in the former group were lower than the levels in spontaneously ovulating fish (Fig. 9 and 13) and fish induced to ovulate (Fig. 14 and 15) it appears that high levels of 17α20βP may have a role in ovulation. Ovulation occurs following oocyte maturation and after the detachment of granulosa cells from the oocyte (Jalabert and Szolloski, 1975; Jalabert, 1978; Fostier and Jalabert, 1982). In rainbow trout, low levels of 17α20βP were required to promote oocyte maturation whereas higher levels of 17α20βP were required to promote detachment of the granulosa cells from the oocyte *in vitro* (Jalabert, 1978). A similar requirement for high levels of 17α20βP *in vivo* may in part contribute to the failure of some fish to ovulate. As ovulation appears to be mediated by prostaglandins (Jalabert, 1976; Stacey and Goetz, 1982; Goetz, 1983), it is likely that low levels of 17α20βP are not the sole reason for the failure of fish to ovulate. Furthermore, high levels of 17α20βP do not necessarily ensure ovulation. For example, the 17α20βP profile in one fish which did not ovulate until day 20 (Fig. 16) was comparable to that observed in fish which ovulated by days 8-10 (Fig. 14 and 15). Scott *et al.* (1982) also found that injections of crude pituitary extracts which result in a large increase in plasma 17α20βP levels did not always result in ovulation in rainbow trout. Previous studies have shown that the injection of

17α20βP promotes oocyte maturation in rainbow trout and coho salmon but has a variable effect on ovulation (Jalabert et al., 1976, 1978a,b). In fish which were close to maturity and have high levels of endogenous gonadotropin, injection of 17α20βP is sufficient to induce oocyte maturation and ovulation (Jalabert et al., 1976). In less mature fish, characterized by lower endogenous gonadotropin levels, it was necessary to supplement 17α20βP with gonadotropin to ensure that both final maturation and ovulation occur (Jalabert et al., 1978a,b). As the dissociation between maturation and ovulation was most pronounced in groups receiving a single hormone injection (see Table 7) it is likely that a transitory elevation of gonadotropin contributes to both the low levels of 17α20βP and the failure to ovulate. Furthermore, 17α20βP has been shown to decrease gonadotropin secretion in salmonids (Jalabert et al., 1978b; Jalabert and Breton, 1980) which could compound the effects of a gonadotropin deficiency required for ovulation.

The preovulatory period in salmonids involves a switch in steroidogenesis from 17β-estradiol to 17α20βP (Fig. 9; Fostier and Jalabert, 1982; Scott et al., 1983). Declining 17β-estradiol levels appear to be the primary signal which determines the time of oocyte maturation and ovulation during both spontaneous and induced development. For fish which ovulate spontaneously, the decline in 17β-estradiol levels in the plasma precedes the large increase in 17α20βP required for oocyte maturation (Fig. 9 and 13). Similarly, changes in 17β-estradiol production appear to determine the time of oocyte maturation and ovulation following hormone treatments which elevate plasma gonadotropin levels. In short-term studies, fish which were induced to mature following injections of LH-RHA DAla^6 could be distinguished from fish which fail to mature on the basis of 17β-estradiol levels (Fig. 10). In this case, fish which failed to mature maintained higher 17β-estradiol levels and did not show a large surge in 17α20βP

levels (Fig. 10 and 12). For fish which were induced to ovulate, the time of ovulation was related to the rate of decline in plasma 17β-estradiol levels (Fig. 14 and 15). In this case, the drop in 17β-estradiol to less than 2 ng/ml preceded or was concomitant to an increase in 17α20βP levels to 450-500 ng/ml. Furthermore, an increase in 17α20βP to high levels (> 200 ng/ml) did not occur in the absence of a decline in 17β-estradiol levels to less than 2 ng/ml (Fig. 13 and 16). These results raise the possibility that 17β-estradiol may have a regulatory influence on 17α20βP synthesis. High levels of 17β-estradiol in vitro have been shown to reduce the effectiveness of gonadotropin on the stimulation of oocyte maturation in rainbow trout (Jalabert, 1975) and brook trout (Theofan, 1981 cited in Goetz, 1983). As 17β-estradiol had no effect on 17α20βP induced maturation in these species (Jalabert, 1975; Goetz, 1983) it appears that 17β-estradiol blocks the actions of gonadotropin on 17α20βP synthesis. However, Young et al. (1982a) reported that high levels of 17β-estradiol had no influence on gonadotropin induced maturation of amago salmon oocytes in vitro. Interpretation of these results are complicated by the possibility that the sensitivity of the follicle to 17β-estradiol may change during development. The possible involvement of 17β-estradiol on 17α20βP production in coho salmon was investigated by studying the effects of 17β-estradiol on the gonadotropin induced stimulation of 17α20βP production in vitro (Fig. 20). For follicles characterized by a central germinal vesicle, 17β-estradiol reduced the effectiveness of gonadotropin on the stimulation of 17α20βP synthesis. However, 17β-estradiol was without effect in follicles with a peripheral germinal vesicle. These results provide further evidence that 17β-estradiol can modify 17α20βP production, but further studies will be necessary to determine the mechanism of this action.

Traditional methods used for the induction of ovulation in teleosts generally

rely on the administration of pituitary extracts in two successive injections (see Harvey and Hoar, 1979; Lam, 1982). Priming by the injection of a low dose of gonadotropin has been shown to increase the effectiveness of subsequent hormone injections on the induction of ovulation in coho salmon (Jalabert et al., 1978b; Donaldson et al., 1981; Hunter et al., 1981; Sower et al., 1982). However, the basis of this priming action in largely unknown. In the present study, 72.5 and 91.7% of the fish ovulated by day 14 in groups receiving one and two injections respectively (Table 7). The high rate of response to a single injection and the variation in the time of ovulation make it difficult to establish whether fish ovulate as a consequence of the first or second injection. Fish which failed to ovulate in groups receiving a single injection could be distinguished from fish which ovulated on the basis of higher 17β-estradiol levels on days 2-10 (Fig. 14A). Fish which ovulated on days 12-14 in groups receiving a second hormone injection at 72 hr had similar plamsa 17β-estradiol levels on day 4 to those fish which failed to ovulate in response to a single hormone injection (Fig. 14A and 15A). On subsequent sampling days, fish in the former group showed a marked decrease in plasma 17β-estradiol level and a surge in plasma 17α20βP level (Fig. 15). These results suggest that fish which ovulated on days 12-14 in groups receiving two injections did so as a consequence of the second injection. Since steroid changes associated with induced ovulation appear to follow a progression, the first injection may initiate this sequence by an inhibition of aromatase activity. The second injection would then be able to reduce aromatase activity to basal levels and stimulate the preovulatory 17α20βP surge. However, a detailed examination of this hypothesis would necessitate the sampling of fish earlier in the season when there may be a greater dissociation between fish ovulating in response to one or two injections.

CHAPTER 5 - THE FUNCTIONAL PROPERTIES OF GONADOTROPIN RECEPTORS IN ADULT COHO SALMON AND IMMATURE CHUM SALMON

A. Introduction

The actions of gonadotropins in mammals are mediated by their binding to specific high affinity receptors located in the plasma membrane of target cells (see Dufau and Catt, 1978). Information on the nature of gonadotropin receptors has been derived from physiological studies which compare the effects of various types of gonadotropins on the stimulation of biological responses and from the direct binding of radiolabeled gonadotropins to gonadal tissue in an analogous fashion to RIA (see Licht, 1980). These approaches were used to determine the properties of gonadotropin receptors in Pacific salmon. Experiments in section 1 examine the specificity of gonadotropin receptors by determining the effects of mammalian and teleost gonadotropins on steroid production in vitro. The validity of using ^{125}I-labeled salmon gonadotropin as a probe to study gonadotropin receptors was evaluated by determining the effects of iodination on the biological activity of salmon gonadotropin (section II). The properties of gonadotropin receptors were determined by the direct binding of ^{125}I-labeled salmon gonadotropin to ovarian tissue from immature chum salmon (section III) and adult coho salmon (section IV).

B. Experimental Protocol.

I. Effects of Teleost and Mammalian Gonadotropins on the Stimulation of Steroid Production In Vitro.

The effects of chinook salmon and mammalian gonadotropins on the stimulation of steroid production by mid-vitellogenic ovarian follicles from chinook salmon and postovulatory follicles from coho salmon were investigated. A preliminary study investigated the time course of the effects of SG-G100 on 17β-estradiol production

by chinook salmon ovarian follicles incubated at in vitro 10°C and 20°C. Routinely gonadotropins were tested over a wide range of doses in triplicate. Incubations were conducted for 20-24 hr at 10°C. The steroidogenic response was quantified in terms of the amounts of 17β-estradiol produced by chinook salmon follicles and the amounts of 17α20βP produced by coho salmon postovulatory follicles.

II. Effects of Iodination on the Biological Activity of Gonadotropin.

The effects of iodination on the biological activity of salmon gonadotropin were determined by comparing the effects of untreated and ^{125}I-labeled salmon gonadotropin on testosterone production by minced testicular tissue and ovarian follicles from coho salmon incubated in vitro. Iodination was performed by the lactoperoxidase method as described in Chapter 2 except that 0.6 nmoles of KI supplemented with 100,000 cpm ^{125}I was used in place of 1 mCi ^{125}I. After iodination, labeled gonadotropin and untreated gonadotropin were serially diluted with Ringer's and incubated with gonadal tissue for 24 hr at 10°C. In one of these experiments, the activities of gonadotropin subjected to the iodination conditions but with the iodide or H_2O_2 omitted were also evaluated. In order to establish whether the iodination conditions used in these studies resulted in the labeling of gonadotropin, trichloroacetic acid (TCA) precipitation was used to determine the incorporation of ^{125}I to gonadotropin. Aliquots of the iodination mixture (25 µl) and 200 µl of saline containing 1.0% BSA were combined with 1 ml of 10% TCA. After 10 min at 4°C, the mixture was centrifuged at 3000 g for 10 min. The incorporation of ^{125}I to gonadotropin was estimated from the percentage of radioactivity in the TCA pellet.

III. Properties of Gonadotropin Binding Sites in Immature Chum Salmon Ovaries

A series of competitive binding studies based on the ability of ^{125}I-labeled salmon gonadotropin to bind to ovarian tissue and for unlabeled gonadotropin to

compete for these binding sites were conducted to determine the properties of gonadotropin receptors in immature chum salmon ovaries. Initial studies were conducted to localize the site of gonadotropin binding by studying the uptake of ^{125}I-labeled salmon gonadotropin to various subcellular fractions obtained by centrifugation of ovarian homogenates. Further studies examined the effects of time, temperature and tissue concentration on gonadotropin binding. The binding of ^{125}I-labeled salmon gonadotropin to the ovary and testes and the liver, kidney and muscle from female chum salmon was examined to determine the tissue distribution of gonadotropin binding sites.

The affinity and number of gonadotropin binding sites in the chum salmon ovary were determined by two methods. For the first method, a constant amount of ^{125}I-labeled salmon gonadotropin (40,000 cpm) and increasing amounts of SGA-2359 were incubated with the 3000 g particulate ovarian fraction for 20 hr at 20°C. For the second method, increasing amounts of ^{125}I-labeled salmon gonadotropin (0.26 to 9.82 x 10^5 cpm) were incubated alone or in combination with 10 µg SG-G100 and the 3000 g particulate fraction from 50 mg of ovarian tissue. The binding inhibition curves obtained from these experiments were converted to Scatchard plots to determine the affinity and number of gonadotropin binding sites (Scatchard, 1949). The specific activity of ^{125}I-labeled salmon gonadotropin was determined by the self-displacement of increasing amounts of ^{125}I-labeled salmon gonadotropin in the gonadotropin RIA. The proportion of ^{125}I-labeled salmon gonadotropin that reacted specifically with ovarian tissue was identified by incubation with an excess of ovarian tissue. As only radiolabeled gonadotropin that is capable of specific binding is believed to represent biologically active hormone (Dufau and Catt, 1978), specific activity was corrected for the maximal bindability of the tracer preparation.

The specificity of gonadotropin binding sites was evaluated by determining the capacity of various teleost and mammalian gonadotropin preparations to compete with the binding of ^{125}I-labeled salmon gonadotropin to ovarian tissue.

IV. Properties of Gonadotropin Binding Sites in Adult Coho Salmon Ovaries

The properties of gonadotropin binding sites in adult coho salmon ovaries were determined by studying the binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from coho salmon ovaries at different stages of development. Additional studies examined the binding of ^{125}I-labeled salmon gonadotropin to intact ovarian follicles and isolated thecal and granulosa cell layers from preovulatory follicles at different stages of development.

C. Results

I. Effects of Teleost and Mammalian Gonadotropins on the Stimulation of Steroid Production In Vitro.

Fig. 21 shows the time course of the effects of SG-G100 on 17β-estradiol production by chinook salmon follicles incubated in vitro at 10 and 20°C. SG-G100 increased ($P < 0.05$) media 17β-estradiol levels by 4 hr for follicles incubated at 10°C (Fig. 21a). The levels of 17β-estradiol produced in response to SG-G100 continued to increase as a function of time and by 24 hr were about 6 fold-higher than the levels produced by follicles incubated with Ringer's alone. SG-G100 increased ($P < 0.05$) media 17β-estradiol levels by 2 hr for follicles incubated at 20°C (Fig. 21b). Media 17β-estradiol levels were increased to a maximum by 8 hr in response to SG-G100 and remained unchanged at 12 and 24 hr. The amounts of 17β-estradiol produced by follicles in response to SG-G100 at 20°C were lower than the levels produced by follicles incubated at 10°C and represented only about a 3-fold increase in 17β-estradiol production compared to follicles incubated with Ringer's alone.

FIG. 21. Time course of the effects of SG-G100 on 17β-estradiol production by chinook salmon ovarian follicles incubated *in vitro* at 10° (A) and 20°C (B). Values represent the mean ± standard error of the amounts of 17β-estradiol released to the media based on three replicates when incubated with Ringer's alone (○) or 1 μg/ml SG-G100 (■).

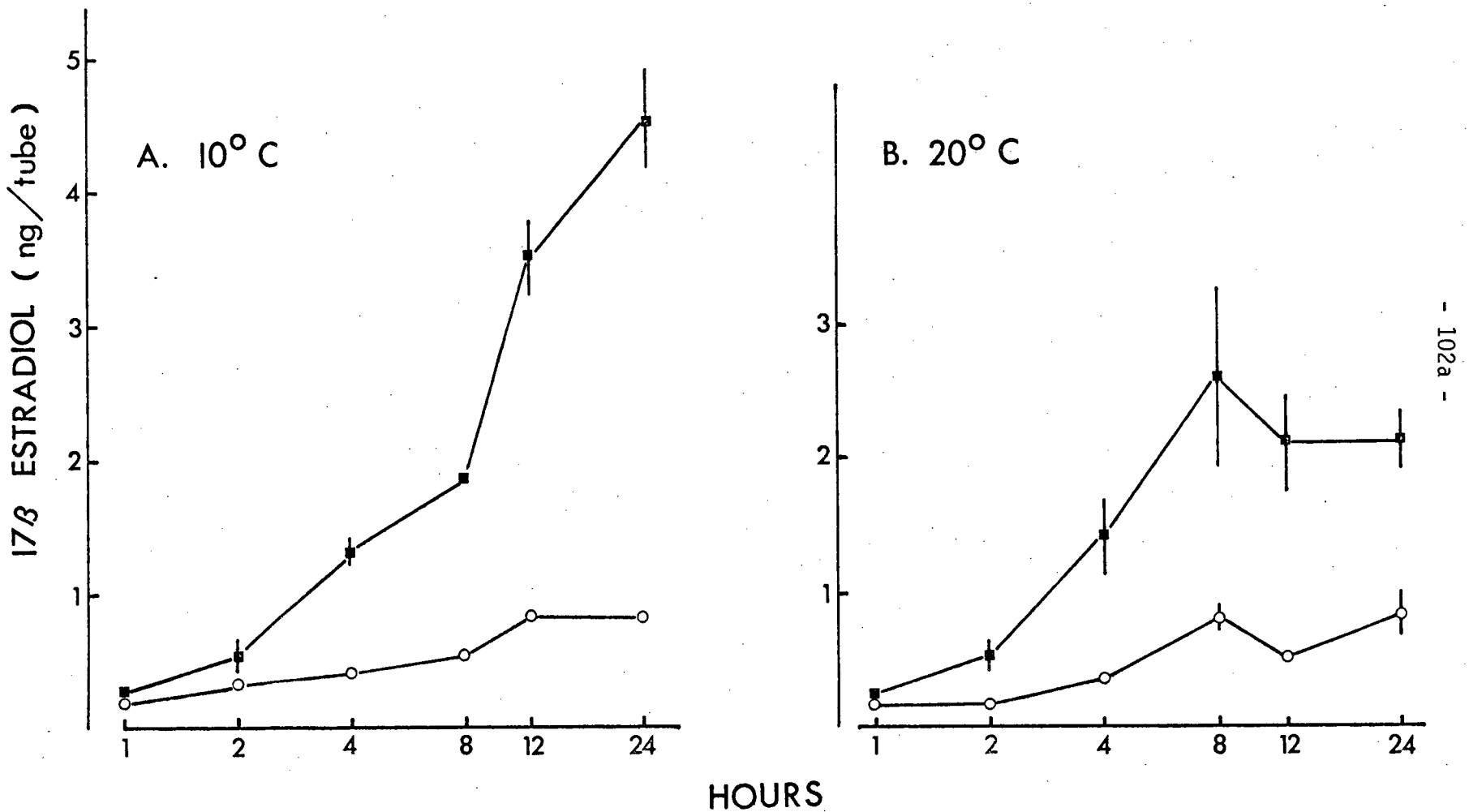

17β ESTRADIOL (ng/tube)
A. 10° C
B. 20° C
1
2
3
4
5
1
2
4
8
12
24
HOURS

Fig. 22 shows the effects of various salmon gonadotropin preparations on 17β-estradiol production by chinook salmon ovarian follicles. Each of the four gonadotropin preparations tested stimulated a dose related increase in 17β-estradiol production. No difference was found with respect to the maximal response ellicited by the different gonadotropin preparations. Upon comparing the ascending portion of the dose response curves, it was found that SGA-2359 was about 20 times and SGA-2360 about 15.1 times as active as the acetone dried pituitary powder. SG-G100 was 7.7 times as active as the acetone dried pituitary powder.

Fig. 23 shows the effects of SG-G100, ovine LH and ovine FSH on 17β-estradiol production by chinook salmon ovarian follicles. SG-G100 at 50 ng/ml stimulated a significant increase ($P<0.05$) in 17β-estradiol production with the maximal response evident at 500 ng/ml. Ovine LH and FSH were inactive at 5 and 20 μg/ml as the amounts of 17β-estradiol produced by these hormones did not differ ($P>0.05$) from the levels produced by follicles incubated with Ringer's alone.

Fig. 24 shows the effects of SG-G100, acetone dried pituitary powder, ovine FSH, ovine LH and hCG on 17α20βP production by adult coho salmon postovulatory follicles. SG-G100 and acetone dried pituitary powder stimulated a dose related increase in 17α20βP production. SG-G100 was about 9.5 times as active as the acetone dried pituitary powder. Mammalian gonadotropins were inactive at doses up to 25 μg/ml.

II. Effects of Iodination on the Biological Activity of Gonadotropin.

Untreated and ^{125}I-labeled salmon gonadotropin had similar effects on testosterone production by coho salmon testicular tissue incubated in vitro (Fig. 25). Additionally, the activity of gonadotropin subjected to the iodination conditions but with the iodide or H_2O_2 omitted did not differ from untreated gonadotropin. In a separate experiment, iodinated gonadotropin was shown to be

FIG. 22. Effects of various salmon gonadotropin preparations on the stimulation of 17β-estradiol production by chinook salmon ovarian follicles. Values are expressed as the mean ± standard error of the amounts of 17β-estradiol released to the media based on three replicates except for controls which were based on nine replicates.

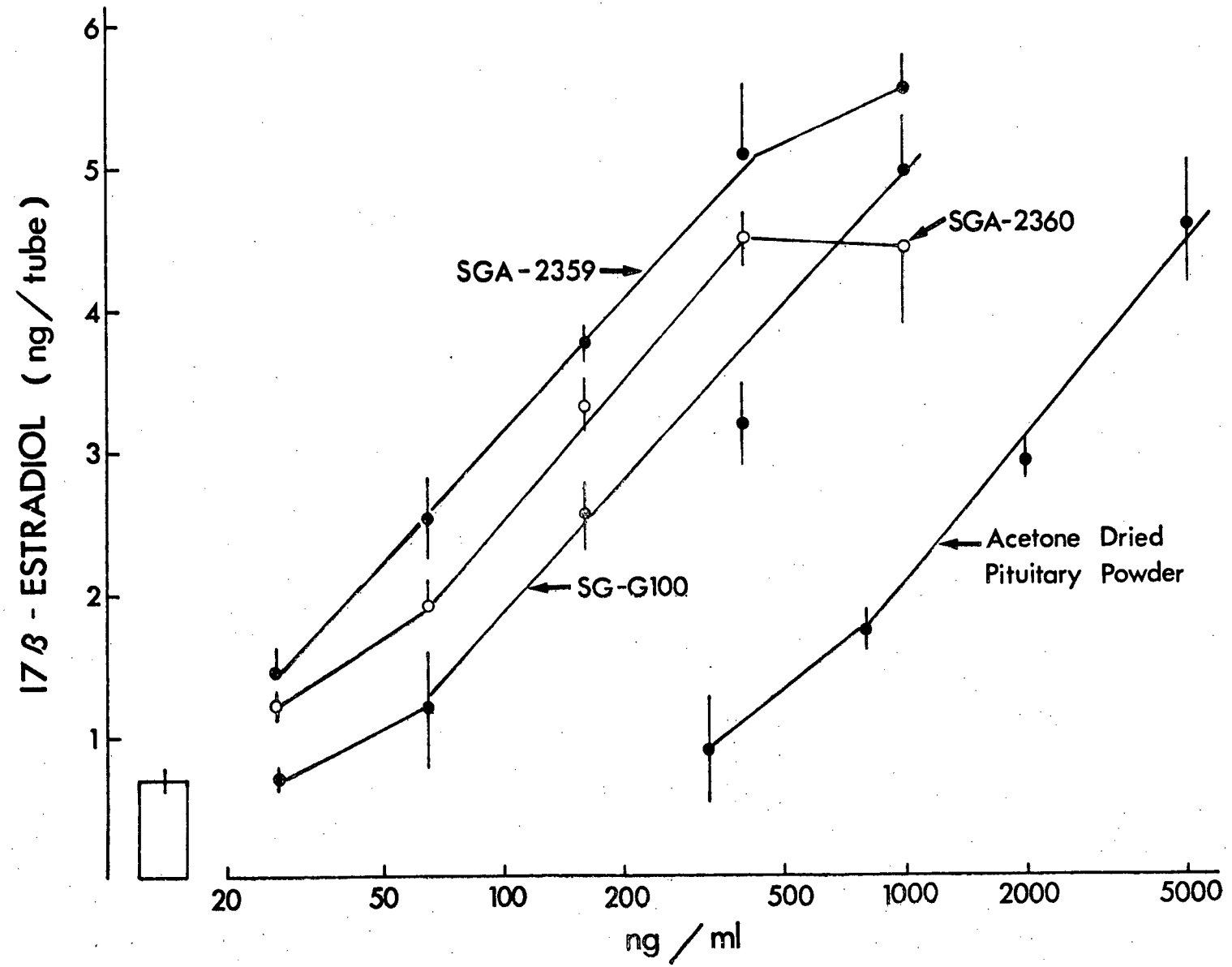

17β - ESTRADIOL (ng / tube)
6
5
4
3
2
1
SGA-2359
SGA-2360
SG-G100
Acetone Dried
Pituitary Powder
20
50
100
200
500
1000
2000
5000
ng / ml

FIG. 23. Effects of SG-G100, ovine LH and ovine FSH on the stimulation of 17β-estradiol production by chinook salmon ovarian follicles incubated in vitro. Values represent the mean ± standard error of the amounts of 17β-estradiol released to the media based on three replicate incubations.

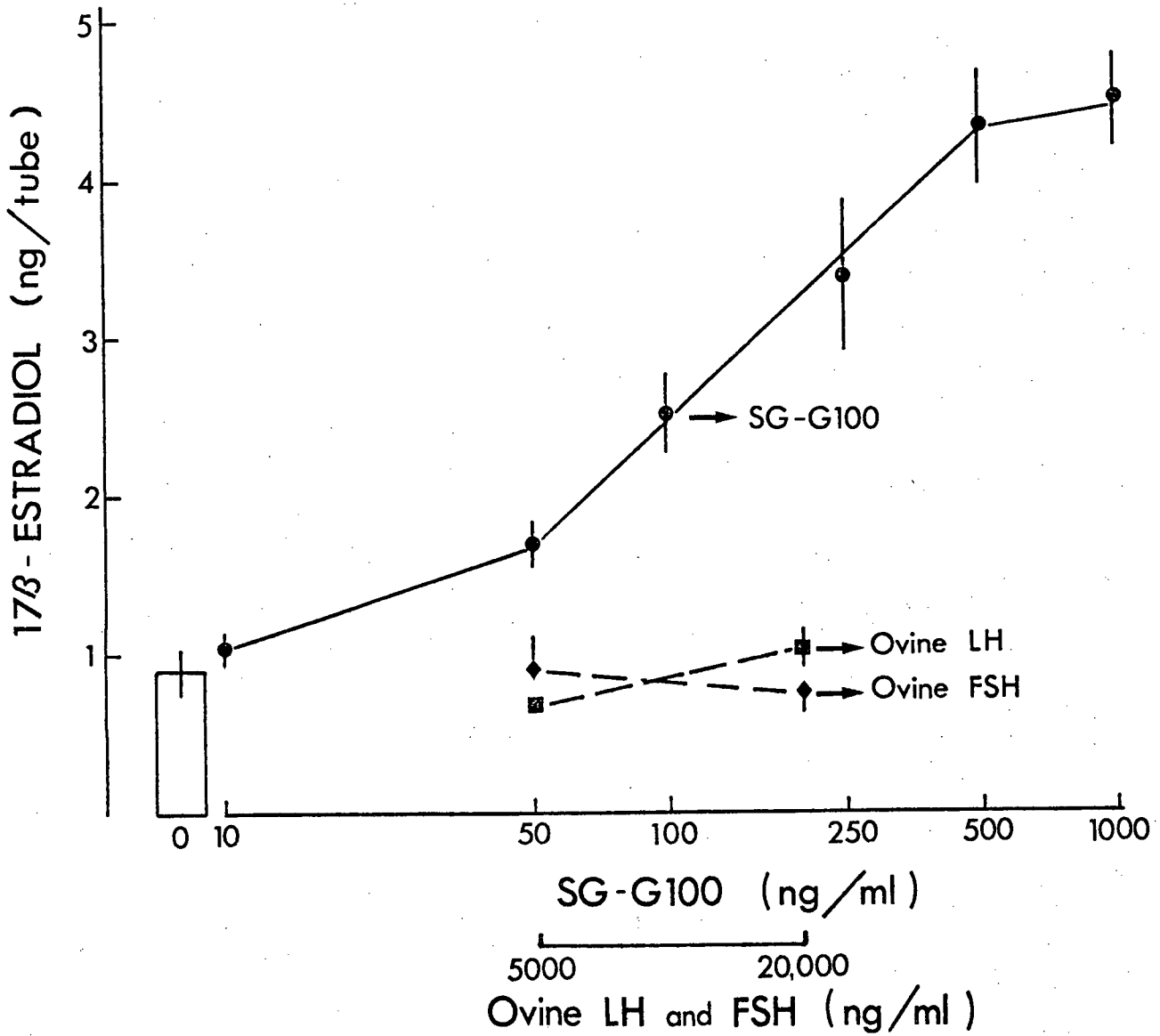

17β - ESTRADIOL (ng/tube)
5
4
3
2
1
SG-G100
Ovine LH
Ovine FSH
0
10
50
100
250
500
1000
SG-G100 (ng/ml)
5000
20,000
Ovine LH and FSH (ng/ml)

FIG. 24. Effects of SG-G100, acetone dried pituitary powder, ovine LH, ovine FSH and hCG on 17α20βP production by coho salmon postovulatory follicles incubated in vitro. Values represent the mean ± standard error of the amounts of 17α20βP released to the media based on three replicates.

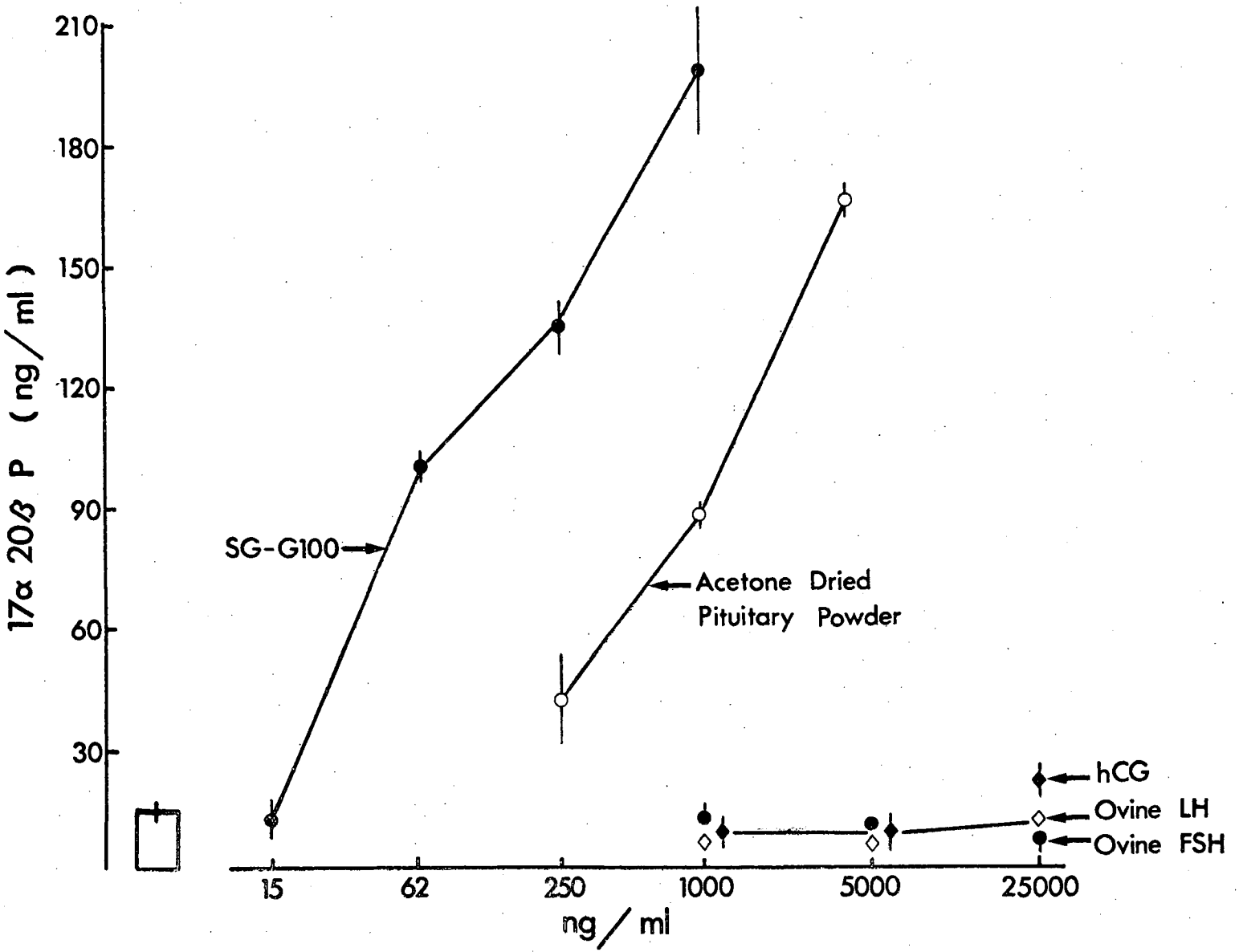
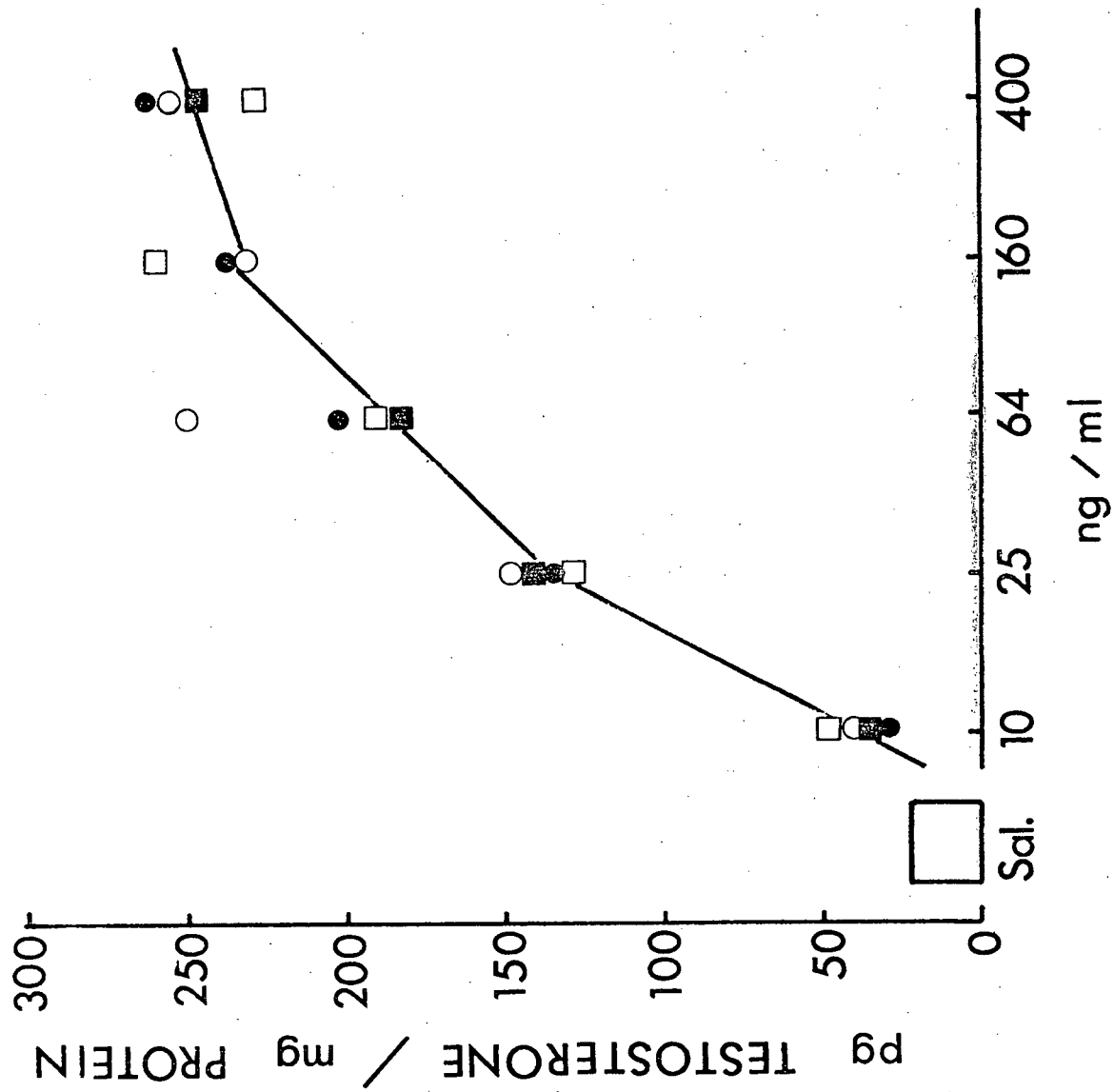

17α 20β P (ng/ml)
210
180
150
120
90
60
30
SG-G100
Acetone Dried
Pituitary Powder
hCG
Ovine LH
Ovine FSH
15
62
250
1000
5000
25000
ng/ml

FIG. 25. Testosterone production by coho salmon testicular tissue *in vitro* in response to untreated and various forms of iodinated salmon gonadotropin. Values represent the mean levels of testosterone released to the media based on three replicate incubations using untreated (●) or ^{125}I-labeled (□) gonadotropin and gonadotropin subjected to the iodination conditions but with the iodide (○) or H_2O_2 (■) omitted. The average coefficient of variation was 0.19.


pg TESTOSTERONE / mg PROTEIN
300
250
200
150
100
50
0
Sal.
10
25
64
160
400
ng / ml

equipotent to untreated hormone in terms of its effect on testosterone production by coho salmon ovarian follicles incubated in vitro (Fig. 26). In these experiments, the iodinated gonadotropin was estimated to contain 0.89 (Fig. 25) and 1.09 (Fig. 26) molecules of iodide per molecule of gonadotropin.

III. Properties of Gonadotropin Binding Sites in Immature Chum Salmon Ovaries

The binding of ^{125}I-labeled salmon gonadotropin to various subcellular fractions prepared by centrifugation of immature chum salmon ovarian homogenates is shown in Fig. 27. Saturable binding was demonstrated in the 3000 g, 20,000 g and 3000-20,000 g fractions by a decrease in the percentage of radioactivity bound to ovarian tissue when coincubated with 10 µg of SG-G100. Saturable binding was highest in the 3000 g pellet accounting for 2.2% of the added radioactivity. In this case, SG-G100 displaced approximately 40% of the total radioactivity bound to the 3000 g pellet.

As the level of saturable binding was low in the initial experiments, additional chromatographic purification of ^{125}I-labeled salmon gonadotropin was attempted in an effort to selectively isolate gonadotropin fractions with higher binding activity. Chromatography of ^{125}I-labeled salmon gonadotropin on either ConA Sepharose or Sephacryl S-200 increased the proportion of added radioactivity that bound specifically to the 3000 g particulate fraction from immature chum salmon ovarian homogenates (Fig. 28). This increase was attributed primarily to a reduction in the amount of hormone that bound non-specifically to ovarian tissue. Whereas 2.6% of the original ^{125}I-labeled salmon gonadotropin preparation showed specific binding, the level of specific binding increased to 4.4% following chromatography on ConA Sepharose and 3.5% following chromatography on Sepharcyl S-200. Further, chromatographic purification of the label on Con A Sepharose and Sephacryl S-200 increased the proportion of radioactivity displaced by SG-G100 from

FIG. 26. Testosterone production by coho salmon ovarian follicles *in vitro* in response to intact and iodinated salmon gonadotropin. Values represent the levels of testosterone released to the media (mean ± standard error) based on three replicate incubations.

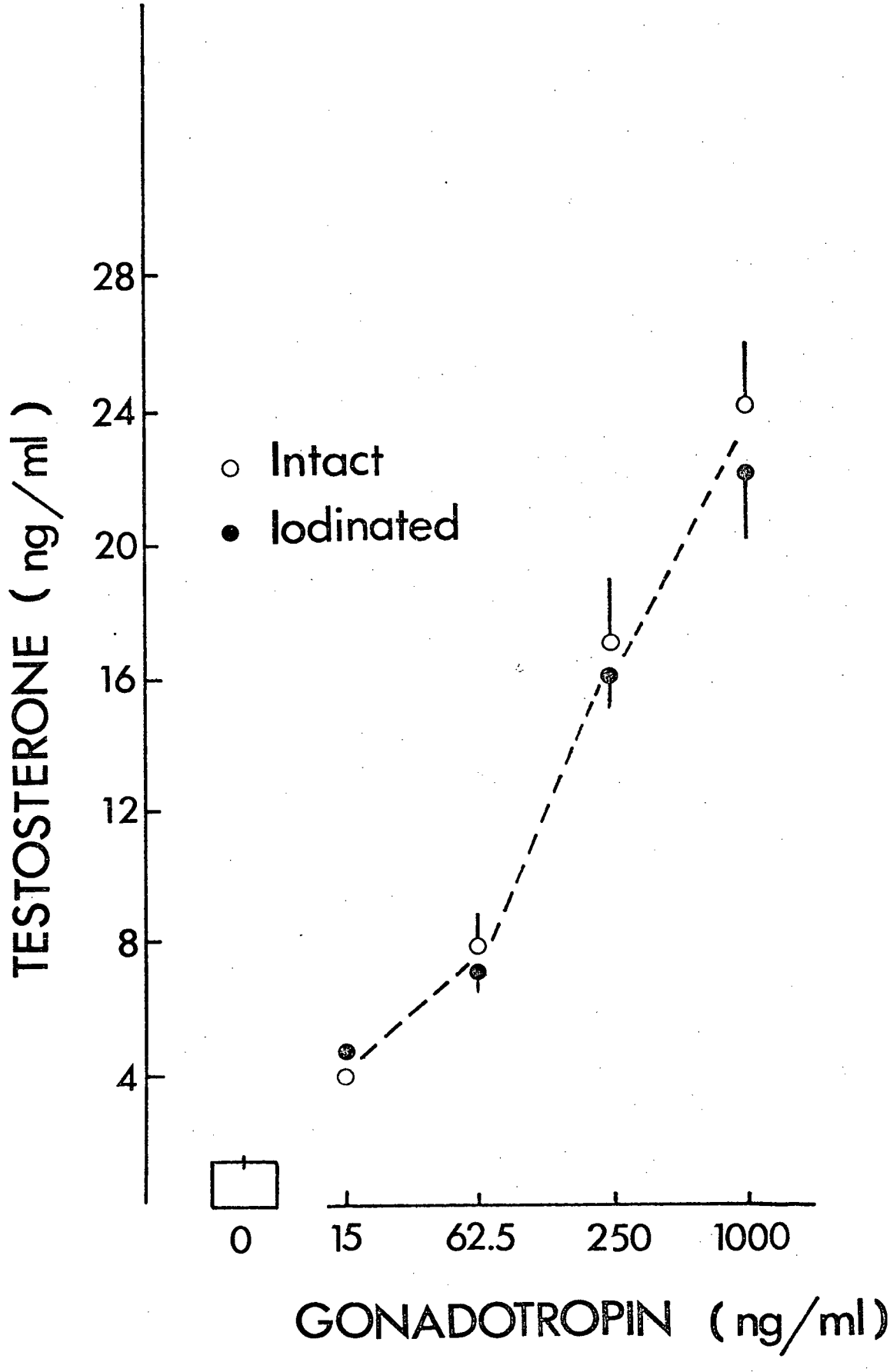

TESTOSTERONE (ng/ml)
28
24
20
16
12
8
4
Intact
Iodinated
0
15
62.5
250
1000
GONADOTROPIN (ng/ml)

FIG. 27. Total binding (open-bar) and nonspecific binding (hatched-bar) of ^{125}I-labeled salmon gonadotropin to immature chum salmon ovary fractions equivalent to 20 mg wet weight of tissue pellet. Tissue pellets were prepared by centrifugation of ovarian homogenates at 3000 g, 20,000 g and the supernatant fluid from the 3000 g pellet recentrifuged at 20,000 g. Values represent the percentage of added radioactivity (mean ± standard error) bound to the ovarian tissue based on three replicate determinations.

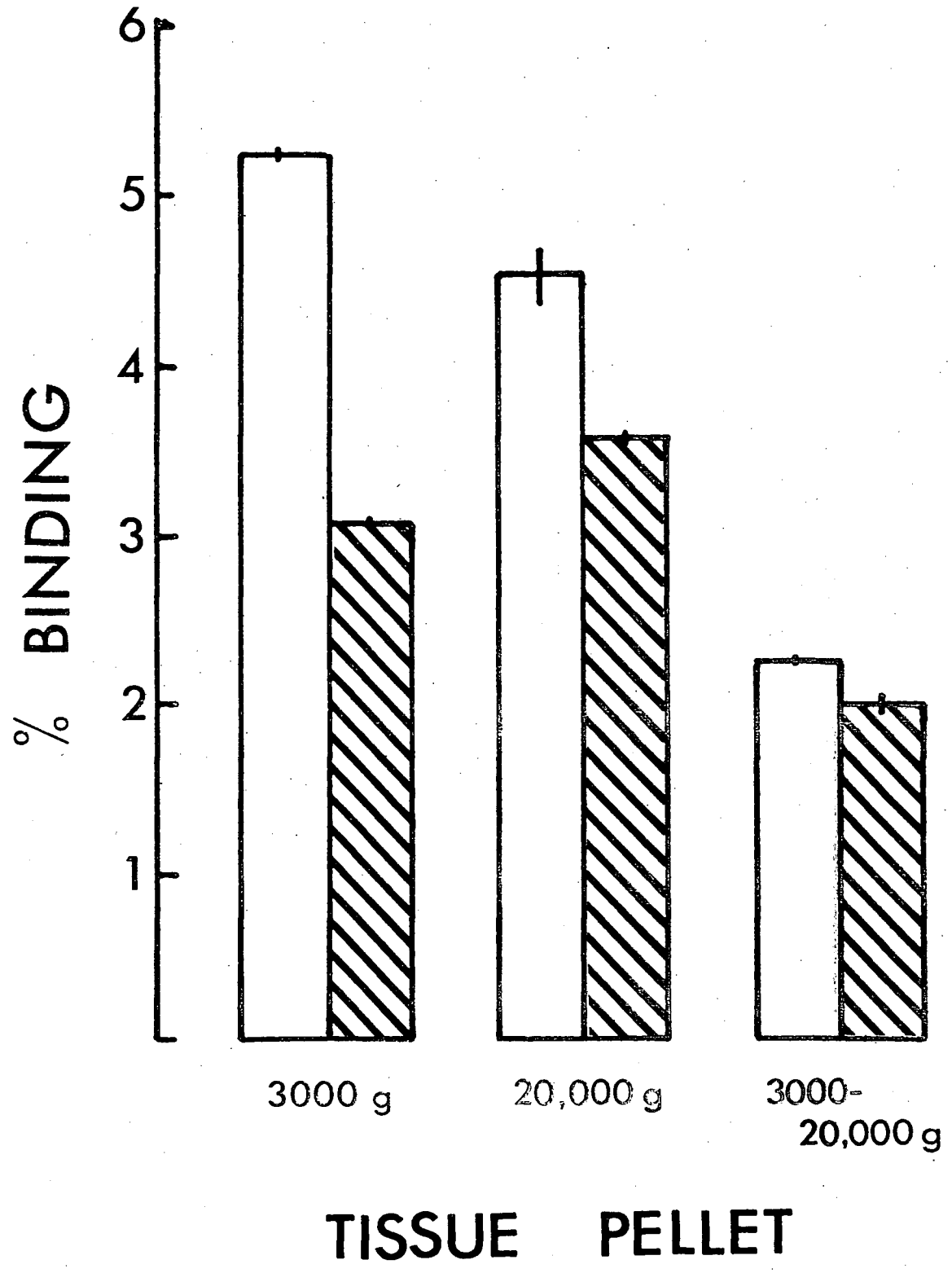
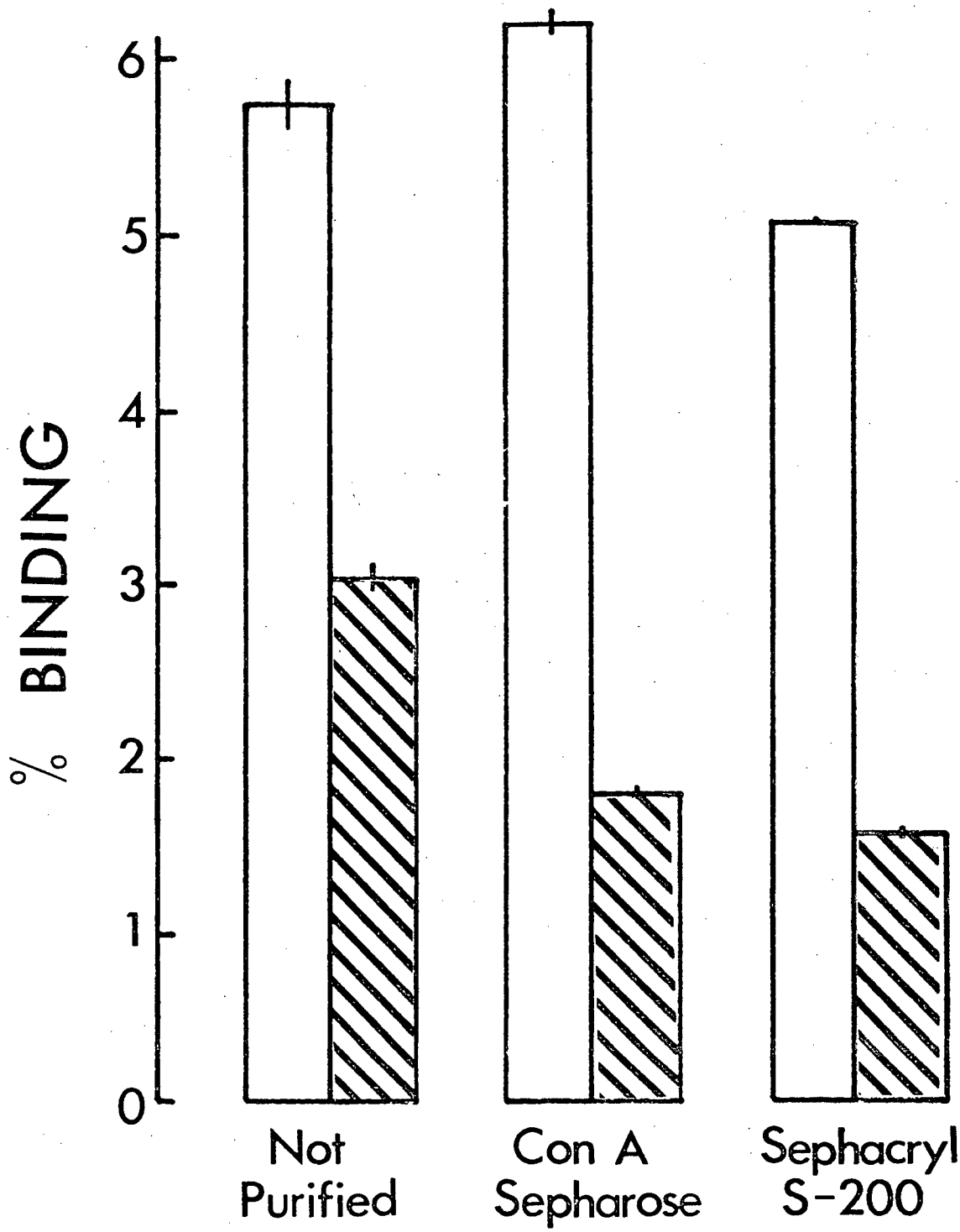

6
5
4
3
2
1
% BINDING
3000 g
20,000 g
3000-
20,000 g
TISSUE PELLET

FIG. 28. Total binding (open bar) and nonspecific binding (hatched bar) of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction of immature chum salmon ovarian homogenates following chromatography of the label on Con A Sepharose and Sephacryl S-200. Values represent the percentage of added radioactivity (mean ± standard error) bound to the ovarian tissue based on three determinations.


% BINDING
0
1
2
3
4
5
6
Not Purified
Con A Sepharose
Sephacryl S-200

45 to 70% of the total radioactivity bound in the absence of competitor.

The effects of time and temperature on the specific binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from 100 mg of ovarian tissue are shown in Fig. 29. Temperature influenced both the rate and proportion of ^{125}I-labeled salmon gonadotropin that bound specifically to ovarian tissue. Specific binding increased more rapidly at 20°C than 10°C. Specific binding was maximal by 20 hr for ovarian tissue incubated at 20°C whereas specific binding showed a slight increase at 36 hr relative to the level at 20 hr for ovarian tissue incubated at 10°C. The amount of ^{125}I-labeled salmon gonadotropin that bound specifically to ovarian tissue at 20°C (4.0%) was about 25% higher than the amount specifically bound to ovarian tissue at 10°C. Specific binding at 4°C was low (0.5%) when compared to ovarian tissue incubated at 10° or 20°C.

Figure 30 shows the specific binding of ^{125}I-labeled salmon gonadotropin as a function of increasing amounts of ovarian tissue. Specific binding increased as a linear function of tissue concentration when incubated with the 3000 g particulate fraction prepared from 25-300 mg of ovarian tissue. Specific binding accounted for 6.1 ± 0.1% of the added radioactivity incubated with the particulate fraction derived from 300 mg of ovary but declined at higher tissue concentrations.

SG-G100 at 0.1 and 10 µg reduced the binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from immature chum salmon ovarian homogenates but had no effect on the binding of ^{125}I-labeled salmon gonadotropin to similar fractions prepared from liver, kidney or muscle (Fig. 31). The binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from immature chum salmon testes homogenates was also reduced in a dose dependent fashion by SG-G100 (Fig. 31).

The affinity and number of gonadotropin binding sites in the 3000 g

FIG. 29. Time course of specific binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction of immature chum salmon ovary homogenates at 4°C (□), 10°C (●) and 20°C (○). Each point is the mean of duplicate determinations.

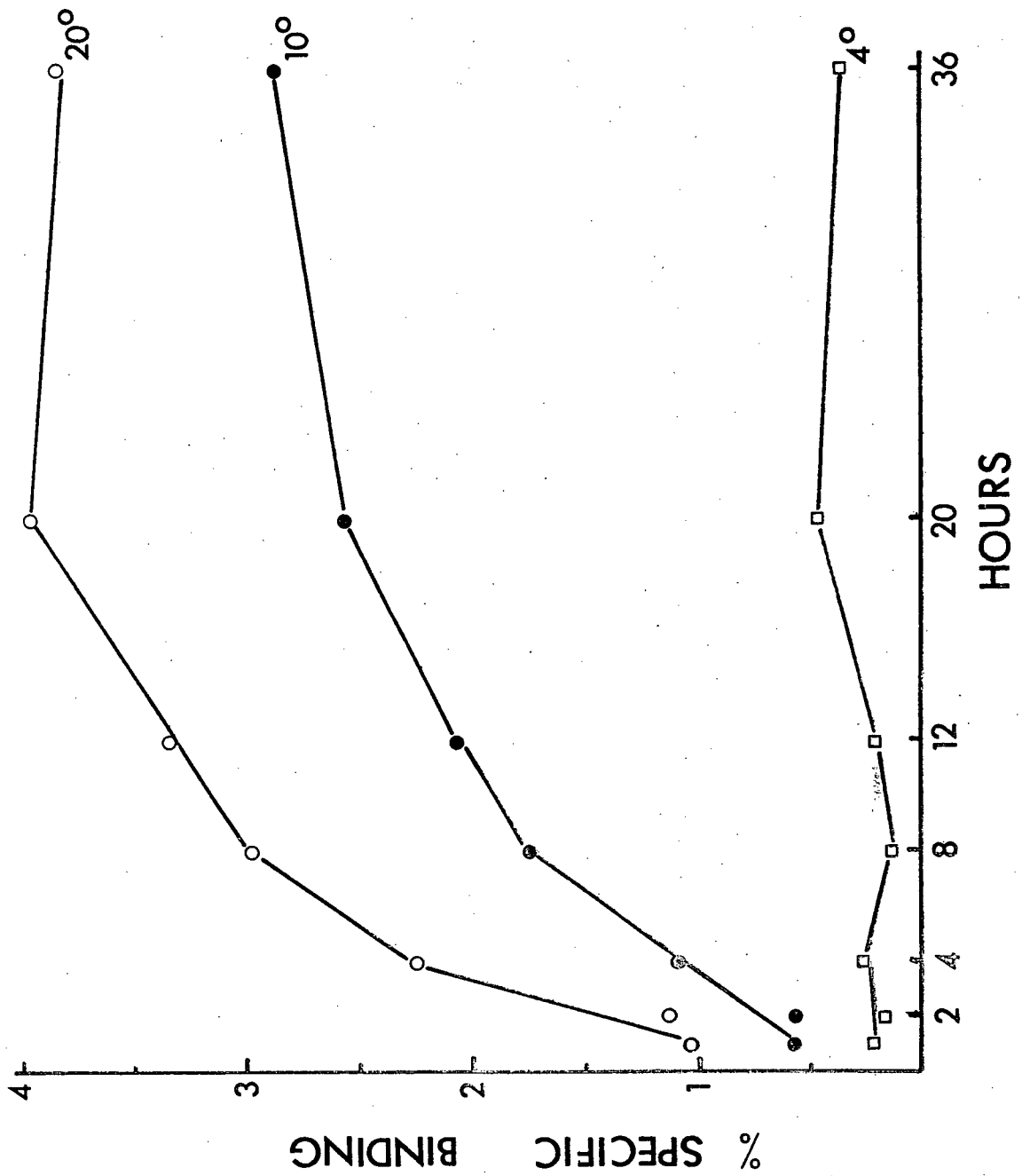

20°
10°
4°
% SPECIFIC BINDING
HOURS
1
2
3
4
2
4
8
12
20
36

FIG. 30. The specific binding of ^{125}I-labeled salmon gonadotropin to increasing quantities of the 3000 g particulate fraction prepared from immature chum salmon ovary homogenates. Each point is the mean of duplicate determinations.

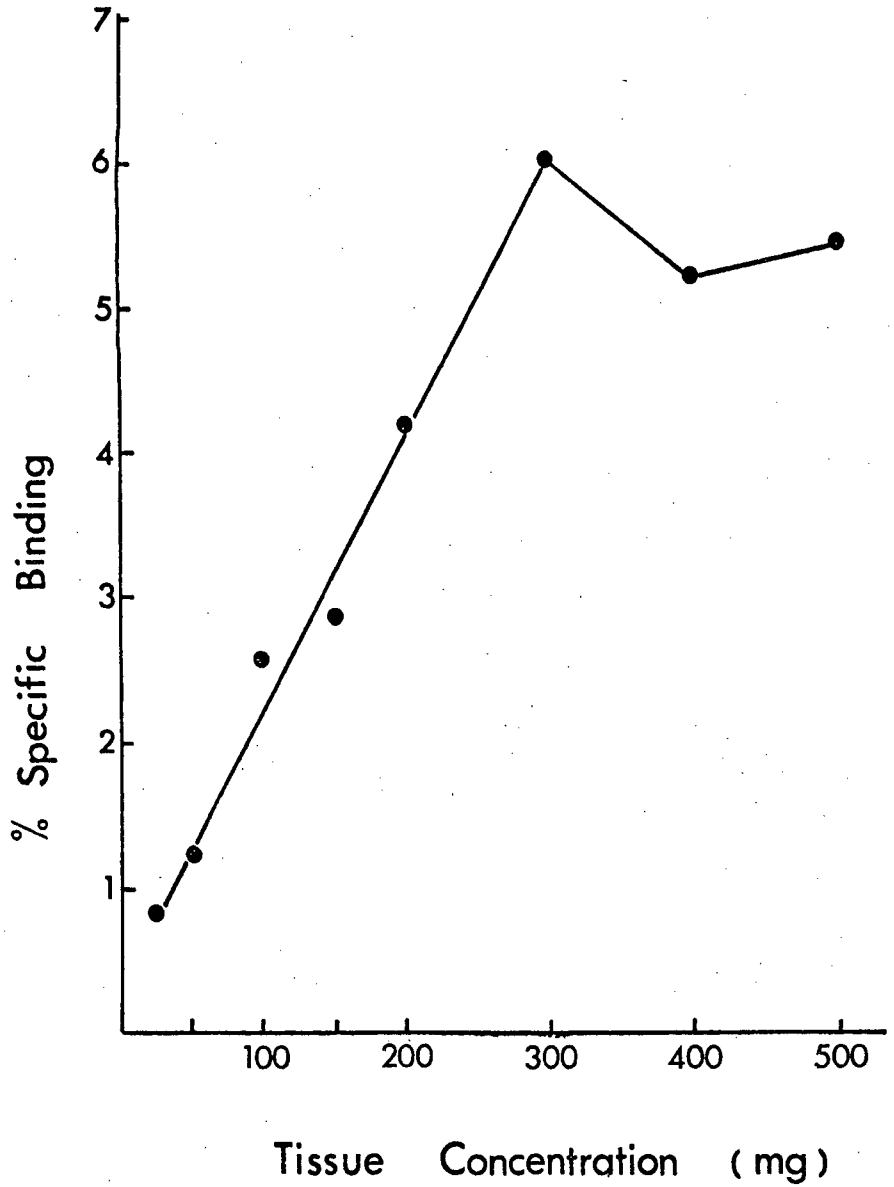
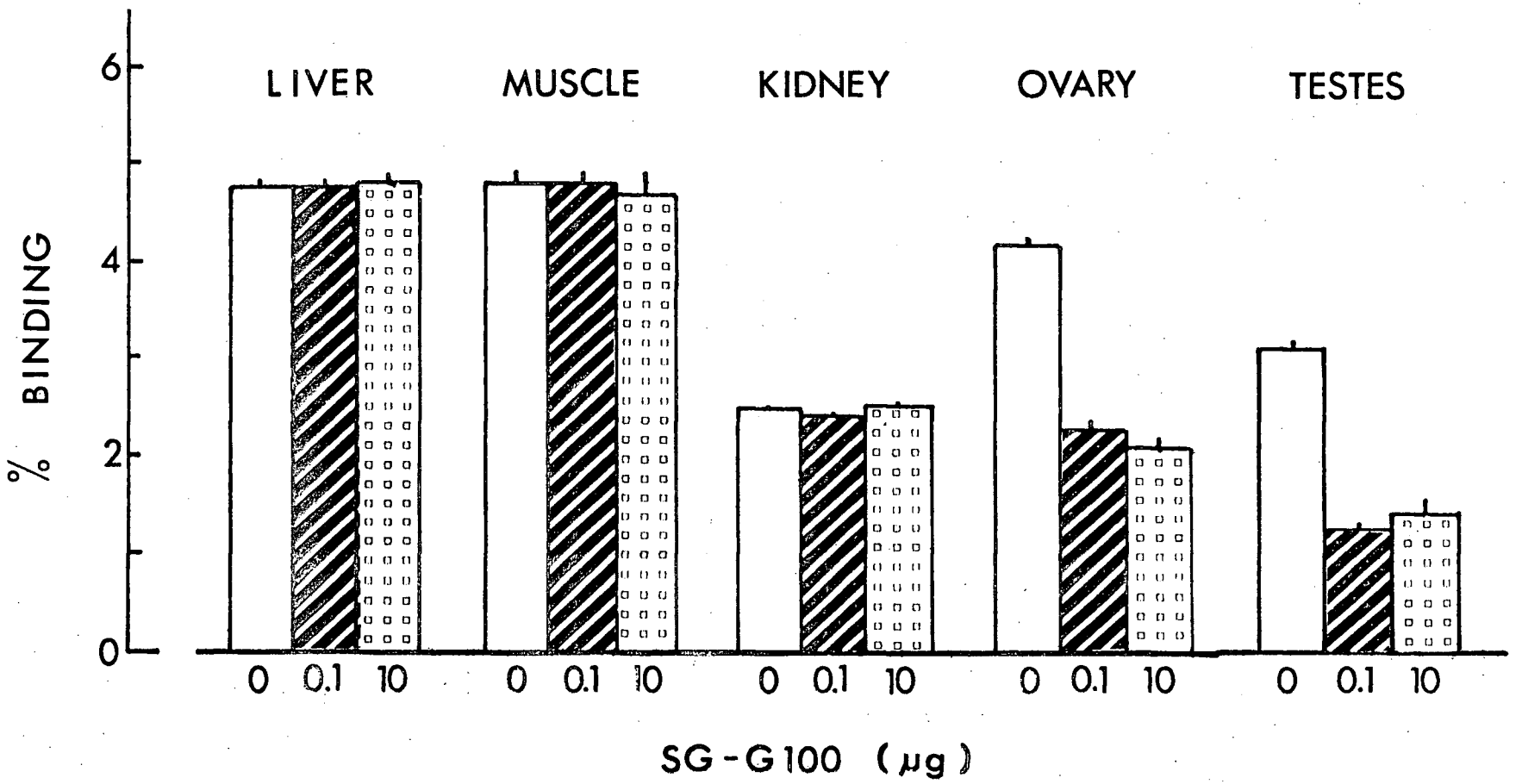

% Specific Binding
7
6
5
4
3
2
1
100
200
300
400
500
Tissue Concentration (mg)

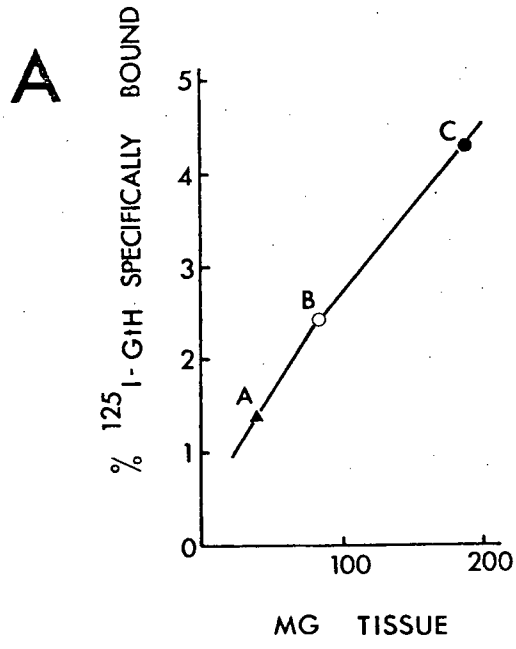
FIG. 31. The binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from 50 mg original wet weight of ovary, liver, kidney, muscle and testes from immature chum salmon. Values represent the percentage of added radioactivity bound to the tissue (mean ± standard error) when incubated with 0, 0.1 and 10 µg SG-G100. Results were based on three replicates.

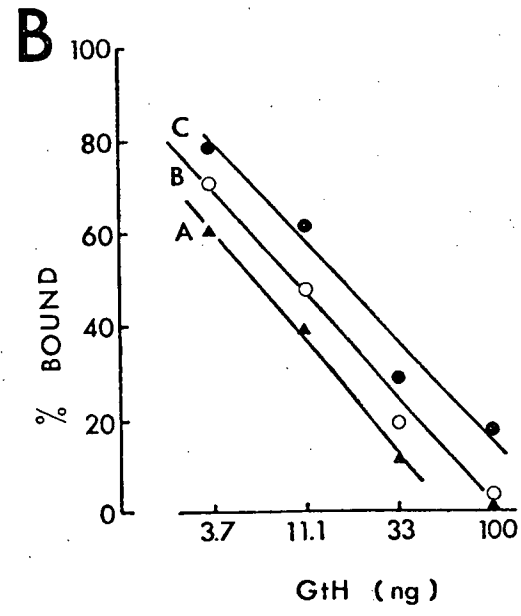

LIVER
MUSCLE
KIDNEY
OVARY
TESTES
% BINDING
6
4
2
0
0 0.1 10
0 0.1 10
0 0.1 10
0 0.1 10
0 0.1 10
SG-G100 (µg)

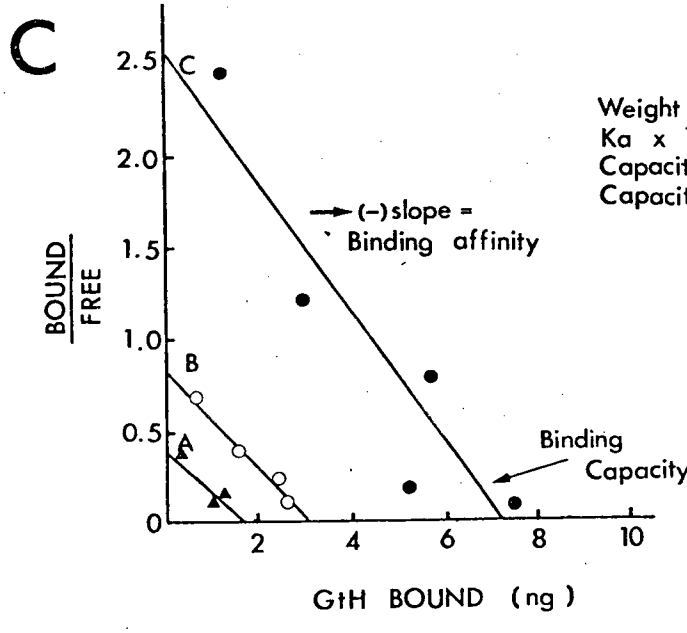
particulate fraction of the ovary determined from competition studies using a constant amount of ^{125}I-labeled salmon gonadotropin and increasing amounts of unlabeled SGA-2359 are shown in Fig. 32. To evaluate whether the amount of ovarian tissue influenced the estimation of the binding parameters, competition studies were performed at three levels of ovarian tissue. Specific binding increased from 1.3 to 4.2% of ^{125}I-labeled salmon gonadotropin incubated with the 3000 g particulate fraction prepared from 38-188 mg of ovarian tissue (Fig. 32a). SGA-2359 effected a dose related reduction of the specific binding of ^{125}I-labeled salmon gonadotropin (Fig. 32b). The ability of SGA-2359 to displace labeled gonadotropin was influenced by the amount of ovarian tissue, as the sensitivity to SGA-2359 increased at low tissue levels. The affinity and number of gonadotropin binding sites were estimated by Scatchard analysis (Fig. 32c) from the competition data shown in Fig. 32b. Calculation of the binding parameters was done following correction to account for the proportion of ^{125}I-labeled salmon gonadotropin capable of specific binding. This level (6.0%) was determined from incubations containing 300 and 450 mg of ovarian tissue. The estimation of the binding capacity was not influenced by the amount of ovarian tissue. The binding capacity of the 3000 g particulate fraction was 37-44 pg gonadotropin per mg of tissue. The estimation of the affinity of the gonadotropin binding sites was influenced by the amount of tissue as the affinity constant ranged from 1.4 - 3.5 x $10^{9}M^{-1}$ when incubated with the 3000 g particulate fraction prepared from 38-188 mg of ovarian tissue.

Figure 33a shows the results of a saturation experiment in which increasing amounts of ^{125}I-labeled salmon gonadotropin were incubated with the 3000 g particulate fraction prepared from 50 mg of ovarian tissue. Specific binding increased a linear function of ^{125}I-labeled salmon gonadotropin concentration over

FIG. 32. Effect of tissue concentration on the determination of the affinity constant and number of gonadotropin binding sites in the immature chum salmon ovary. A. Specific binding of ^{125}I-labeled salmon gonadotropin as a function of tissue concentration. B. Competition curves for the specific binding of ^{125}I-labeled salmon gonadotropin as a function of increasing amounts of unlabled SGA-2359. C. Scatchard plot for the competition data shown in B.

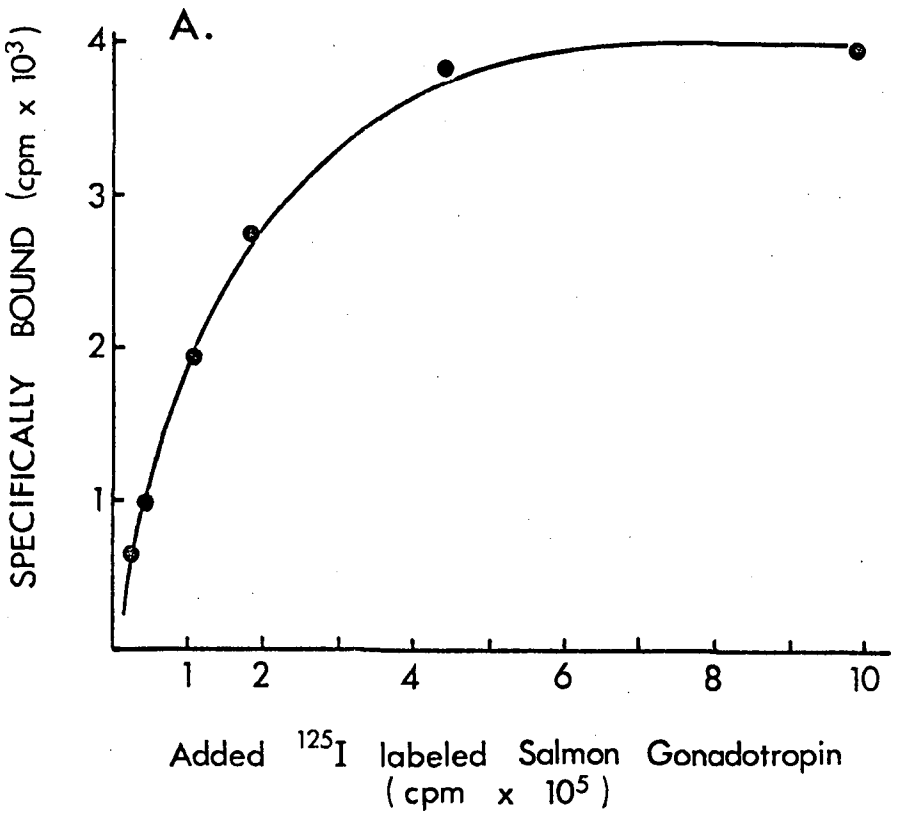


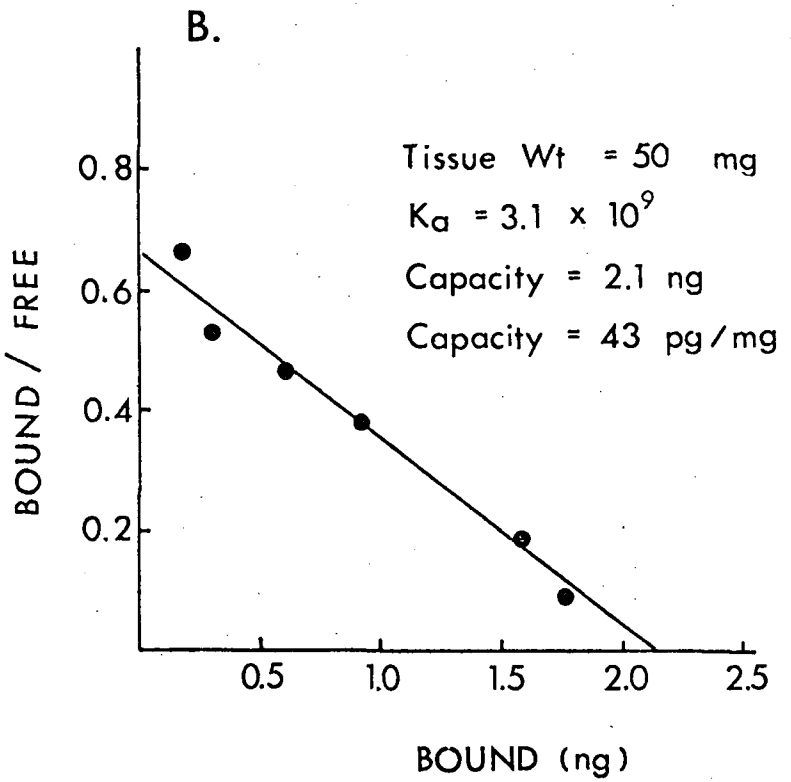




	A	B	C
Weight (mg)	38	83	188
Ka x 10^9 M	1.5	2.8	3.5
Capacity (ng)	1.7	3.1	7.1
Capacity (pg/mg)	44	37	38

FIG. 33. The specific binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from immature chum salmon ovarian homogenates as function of increasing amounts of ^{125}I-labeled salmon gonadotropin (A) and Scatchard analysis of the binding data (B). Values represent the mean of duplicate determinations.


A.
SPECIFICALLY BOUND (cpm x 10^3)
4
3
2
1
1
2
4
6
8
10
Added ^{125}I labeled Salmon Gonadotropin
(cpm x 10^5)


B.
BOUND / FREE
0.8
0.6
0.4
0.2
0.5
1.0
1.5
2.0
2.5
BOUND (ng)
Tissue Wt = 50 mg
K_a = 3.1 x 10^9
Capacity = 2.1 ng
Capacity = 43 pg / mg

the range of 0.26 - 1.83 x 10^5 cpm. Binding was saturated at 5.44 and 9.82 x 10^5 cpm of ^{125}I-labeled salmon gonadotropin. Scatchard analysis of the binding data indicated a single class of high affinity binding sites (Ka: 3.1 x $10^9 M^{-1}$) with a binding capacity of 43 pg/mg tissue (Fig. 33b).

Figure 34 shows the effects of various teleost and mammalian gonadotropin preparations on the binding of ^{125}I-labeled salmon gonadotropin binding to the 3000 g particulate fraction prepared from 200 mg of ovarian tissue. SGA-2359, SG-G100 and acetone dried pituitary powder caused a dose related decrease in the binding of ^{125}I-labeled salmon gonadotropin. SGA-2359 was approximately 29 times and SG-G100 8.2 times more active than the acetone dried pituitary powder. The Con A_1 fraction was less effective than acetone dried pituitary powder. Based on the amounts required to cause a 50% reduction in the binding of ^{125}I-labeled salmon gonadotropin, the Con A, gonadotropin fraction had 0.5 times the activity of acetone dried pituitary powder. Mammalian gonadotropins (ovine LH, ovine FSH and hCG) reduced the binding of ^{125}I-labeled salmon gonadotropin but only at high concentrations. No major differences in the potencies of the mammalian gonadotropins were evident.

IV. Properties of Gonadotropin Binding Sites in Adult Coho Salmon Ovaries

Figure 35 shows the binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction prepared from adult coho salmon ovaries. Specific binding was not detectable in preovulatory follicles characterized by central or peripheral germinal vesicle. Preovulatory follicles which had undergone GVBD showed specific binding of ^{125}I-labeled salmon gonadotropin when using 1.25 and 20 mg of tissue but not when using 5 mg of tissue. Postovulatory follicles showed specific binding of ^{125}I-labeled salmon gonadotropin. However, the level of specific binding by ovarian tissue from adult coho salmon was low accounting for at most 3.0% of the

FIG. 34. Effects of teleost and mammalian gonadotropin preparations on the specific binding of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction of immature chum salmon ovarian homogenates. Values were expressed as a percentage of the radioactivity specifically bound to ovarian tissue in the absence of competitor. Result are the mean of duplicate determinations.

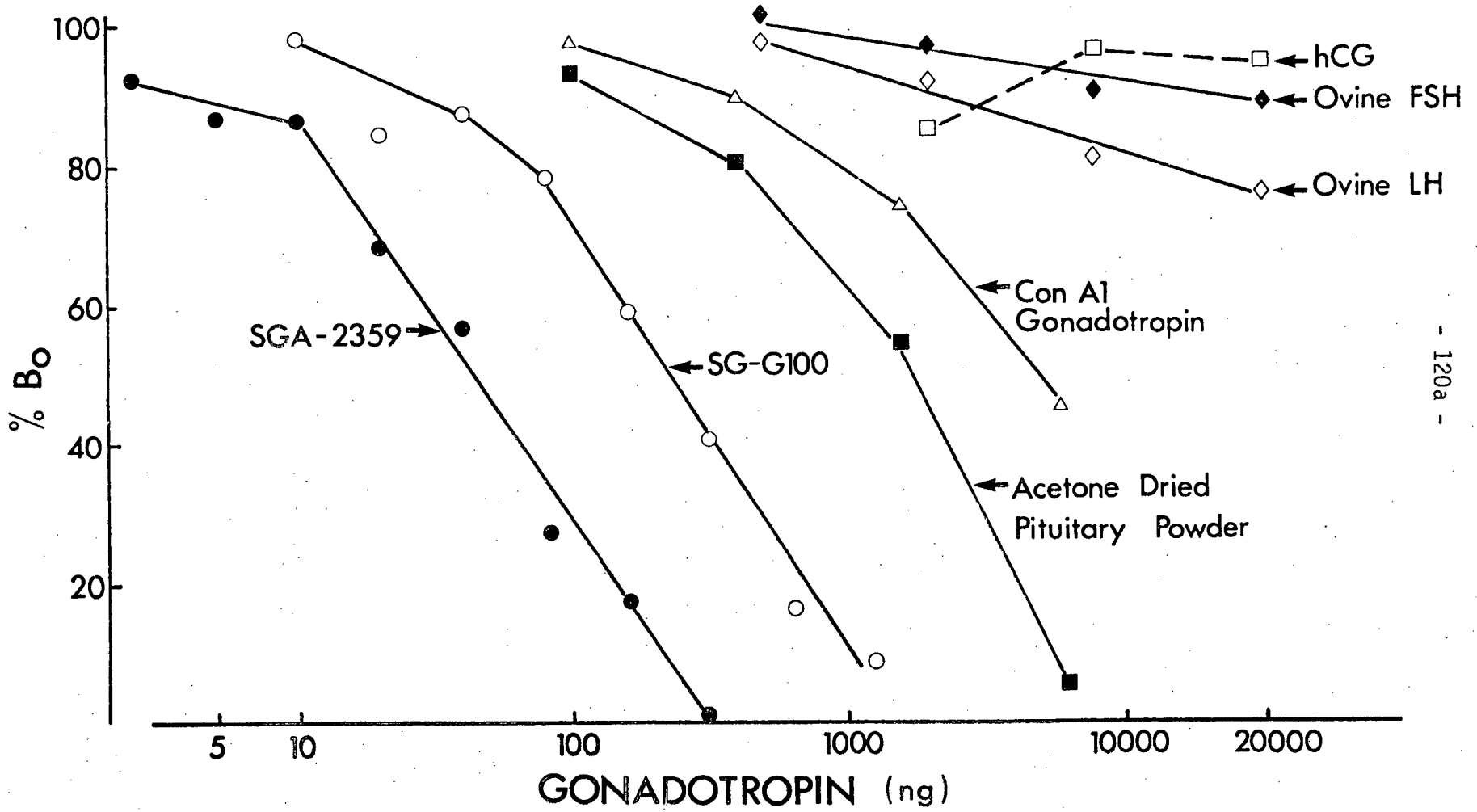
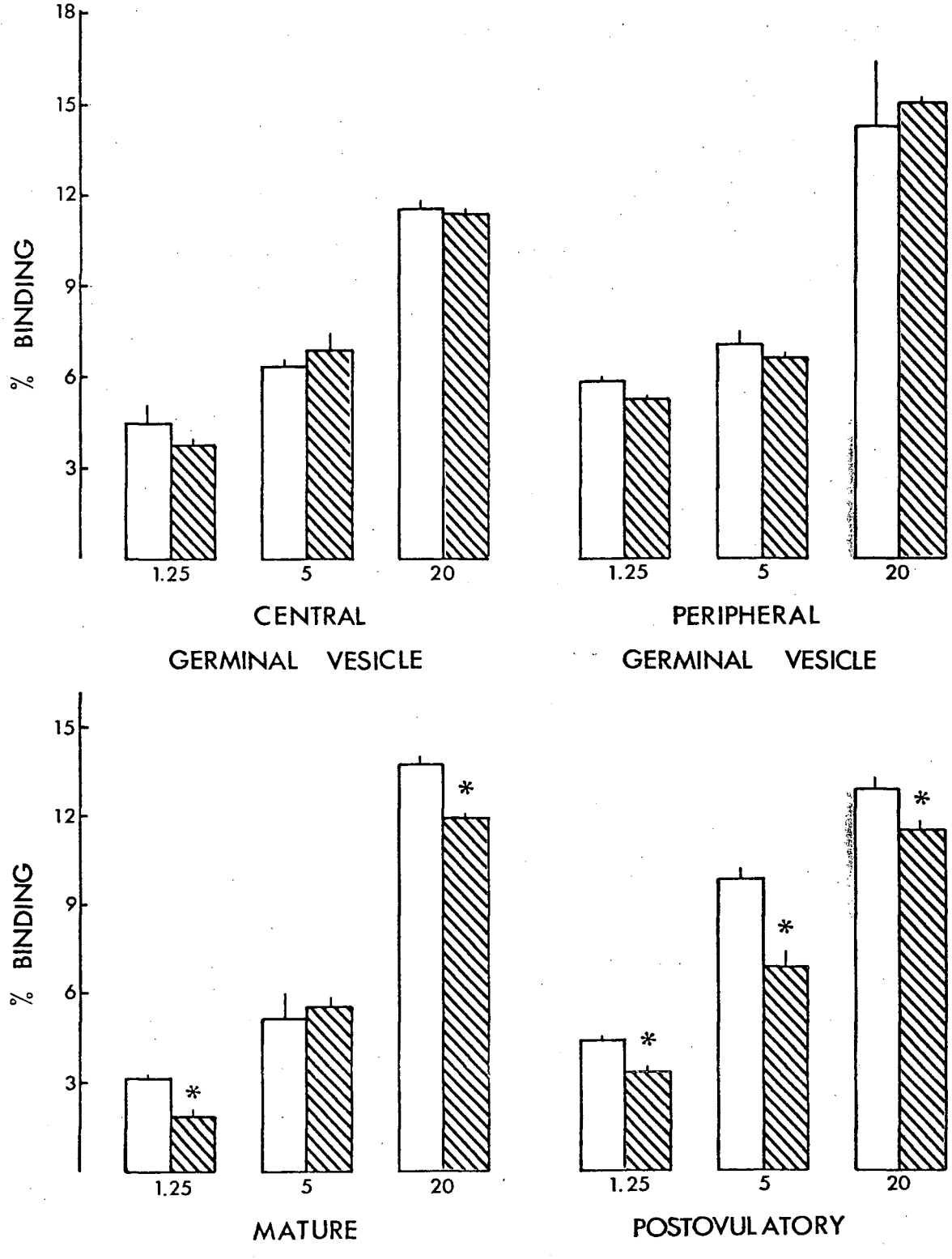

100
80
60
40
20
% Bo
SGA-2359
SG-G100
Con Al
Gonadotropin
Acetone Dried
Pituitary Powder
hCG
Ovine FSH
Ovine LH
5
10
100
1000
10000
20000
GONADOTROPIN (ng)

FIG. 35. Total binding (open bars) and nonspecific binding (hatched bars) of ^{125}I-labeled salmon gonadotropin to the 3000 g particulate fraction of adult coho salmon ovaries. Binding studies were conducted at three levels of ovarian tissue (1.25, 5 and 20 mg/tube) obtained from fish at differing stages of maturity. Values represent the percentage of added radioactivity (mean ± standard error) bound to the ovarian tissue based on three determinations.


% BINDING
18
15
12
9
6
3
1.25
5
20
CENTRAL
GERMINAL VESICLE
PERIPHERAL
GERMINAL VESICLE
MATURE
POSTOVULATORY
*

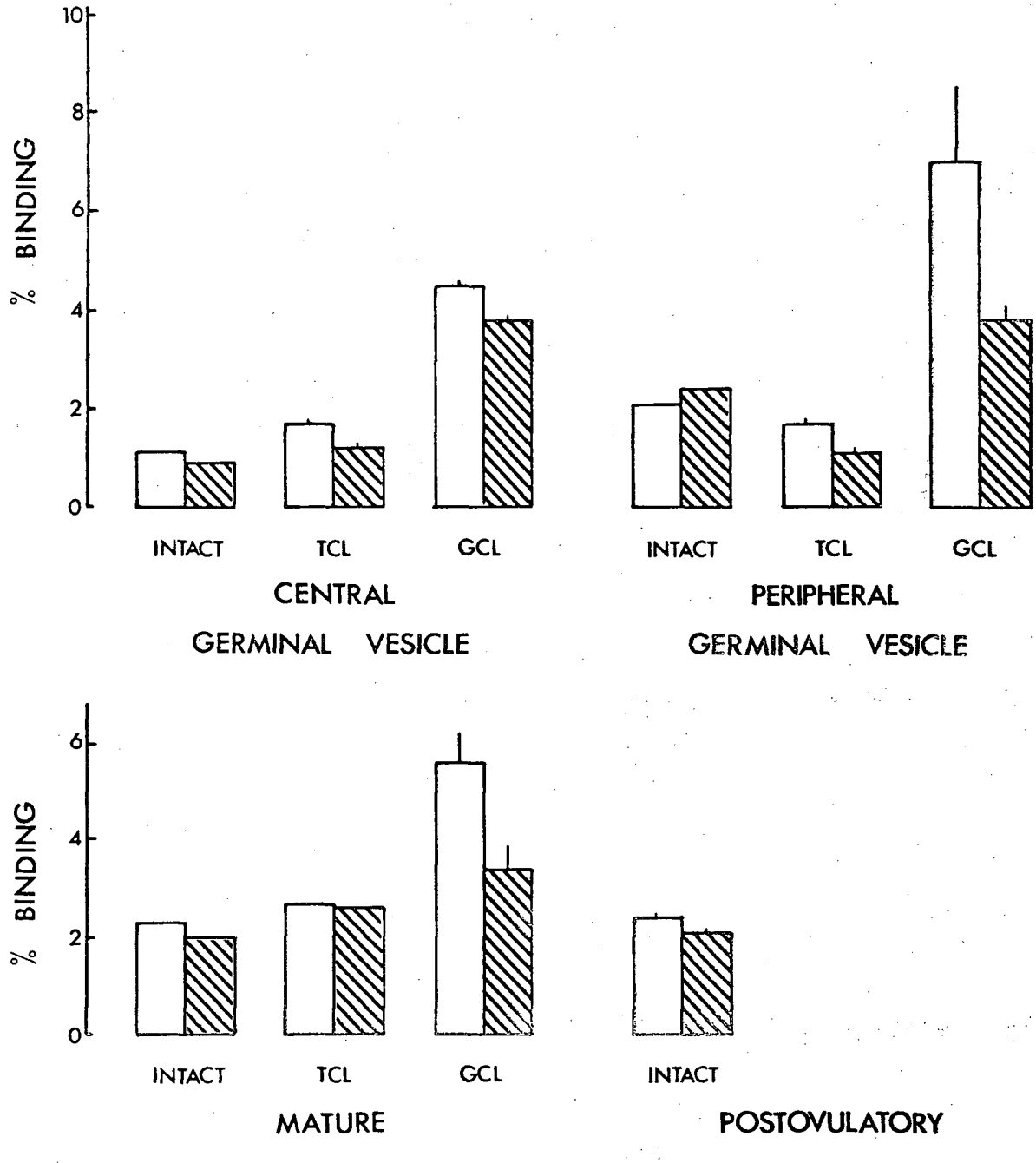
added radioactivity with the majority of the radioactivity associated with the ovarian tissue bound nonspecifically. A series of experiments which examined gonadotropin binding to various subcellular fractions prepared from preovulatory follicles and the influence of time, temperature and incubation buffer composition failed to demonstrate significant specific binding of gonadotropin to preovulatory follicles (data not reported). Additional tests done in conjunction with some of these studies showed that 5 - 8% of the ^{125}I-labeled salmon gonadotropin bound specifically to ovarian tissue from immature chum salmon.

Figure 36 shows the binding of ^{125}I-labeled salmon gonadotropin to intact ovarian follicles and isolated thecal and granulosa cell layers from preovulatory follicles. Intact follicles showed negligible specific binding of ^{125}I-labeled salmon gonadotropin. The uptake of ^{125}I-labeled salmon gonadotropin by isolated thecal cell layers and particularly isolated granulosa cell layers from preovulatory follicles was greater than that for intact follicles. Specific binding to thecal cell layers obtained from oocytes characterized by a central or peripheral germinal vesicle represented 0.6% of the added radioactivity. Specific-binding was not detected in isolated thecal cell layers from preovulatory follicles which had matured in vivo. Granulosa cell layers generally bound greater amounts of ^{125}I-labeled salmon gonadotropin specifically than thecal cell layers. Granulosa cell layers obtained from follicles characterized by a peripheral germinal vesicle showed 3.2% specific binding of ^{125}I-labeled salmon gonadotropin. Granulosa cell layers from oocytes characterized by a central germinal vesicle or following maturation bound lesser amounts of gonadotropin specifically. Thecal and granulosa cell layers could not be separated from postovulatory follicles.

D. Discussion

These studies have demonstrated gonadotropin binding sites in chum and coho

FIG. 36. Total binding (open bars) and nonspecific binding (hatched bars) of ^{125}I-labeled salmon gonadotropin to intact ovarian follicles and isolated thecal cell layer (TCL) and granulosa cell layers (GCL) from adult coho salmon at different stages of maturity. Values represent the percentage of added radioactivity (mean ± standard error) bound to the ovarian tissue based on three determinations.


% BINDING
10
8
6
4
2
0
INTACT
TCL
GCL
CENTRAL
GERMINAL VESICLE
INTACT
TCL
GCL
PERIPHERAL
GERMINAL VESICLE
% BINDING
6
4
2
0
INTACT
TCL
GCL
MATURE
INTACT
POSTOVULATORY

salmon ovaries which share several of the properties that characterize physiologically relevant gonadotropin receptors in other vertebrates. Gonadotropin binding to ovarian tissue was a saturable process as the uptake of ^{125}I-labeled salmon gonadotropin to ovarian tissue was reduced in a dose dependant manner by unlabeled gonadotropin. The ability of different gonadotropin preparations to compete with the binding of ^{125}I-labeled salmon gonadotropin to ovarian tissue was in agreement with the effects of these gonadotropins on the stimulation of steroid production in vitro. These results suggest that gonadotropin binding sites identified by direct binding studies show a similar hormone specificity as seen for physiological gonadotropin receptors which mediate steroid producton. Saturable gonadotropin binding was demonstrated in the ovary and testis but not in the liver, kidney or muscle. These results suggest that saturable gonadotropin binding sites were restricted to the expected gonadotropin target tissues.

Studies using immature chum salmon ovarian tissue have shown that saturable gonadotropin binding sites were localized in the 3000 g particulate fraction (Fig. 27). Mammalian gonadotropin receptors are found in the plasma membrane (see Saxena, 1976; Dufau and Catt, 1978) and as with the studies in chum salmon are localized in low speed pellets following homogenization (Catt et al., 1974, 1976; Dufau and Catt, 1978).

The binding of ^{125}I-labeled salmon gonadotropin to chum salmon ovarian tissue was dependent on time, temperature and tissue concentration (Fig. 29 and 30). From 5 to 9% of the added ^{125}I-labeled salmon gonadotropin bound specifically to ovarian tissue. This level of specific binding while low in comparison with the proportion of radiolabeled mammalian gonadotropins which bind specifically to gonadal tissue of mammalian origin compares favorably with the binding activity of non-mammalian tetrapod gonadotropins to gonadal tissue from a variety of tetrapod species. For

example, up to 60% of ^{125}I-labeled hCG and 20-40% of ^{125}I-labeled mammalian LH preparations will bind specifically to mammalian gonadal tissue (see Catt et al., 1974, 1976). Labeled mammalian FSH preparations commonly exhibit less than 12% specific binding to mammalian gonadal tissue (Catt et al., 1976; Nimrod et al., 1976; Sairam, 1979) although in certain cases up to 40% specific binding has been reported (Darga and Reichert, 1978; Chiauzzi et al., 1982). Mammalian FSH preparations exhibit high binding activity (up to 40% specific binding) to gonadal tissue from birds, reptiles and amphibians (Licht and Midgley, 1976; Ishii and Farner, 1976; Adachi et al., 1979; Bona Gallo and Licht, 1979). Previous attempts to demonstrate specific binding of mammalian FSH preparations to gonadal tissue from teleosts have been unsuccessful (Adachi et al., 1979; Adachi and Ishii, 1980). Studies using radiolabeled ostrich FSH, turkey FSH, sea turtle FSH and cobra gonadotropin have shown 5-16% specific binding when incubated with gonadal tissue from a variety of tetrapod species (Licht et al., 1977a,b, 1979; Bona Gallo and Licht, 1979; Bona Gallo et al., 1983). Unlike studies using FSH, attempts to demonstrate specific binding of LH type gonadotropins in non-mammalian vertebrates have met with limited success. Despite high biological activity, hCG and mammalian LH preparations fail to bind specifically to gonadal tissue from birds, reptiles and amphibians (Licht et al., 1977a; Licht, 1980; Etches and Cheng, 1981). Similarily, studies using^{125}I-labeled sea turtle LH and ostrich LH did not show appreciable specific binding to gonadal tissues from a variety of tetrapod species (Licht, 1980; Bona Gallo et al., 1983). In other studies, ^{125}I-labeled turkey LH was shown to bind specifically to gonadal tissue from birds and reptiles but this was represented by less than 10% of the added radioactivity (Bona Gallo and Licht, 1979; Bona Gallo et al., 1983). Recently, Schlaghecke (1983) reported that ^{125}I-labeled hCG binds specifically to testicular tissue from rainbow trout. In

these studies specific binding accounted for about 1% of the added radioactivity when incubated with testicular tissue at 37°C.

Low levels of specific binding have been attributed, in certain cases, to a decrease in the biological activity of gonadotropins following radiolabelling (see Catt et al., 1976; Birnbaumer, 1978). In the present studies, ^{125}I-labeled salmon gonadotropin prepared by the lactoperoxidase method and untreated gonadotropin had similar effects on the stimulation of steroid production (Fig. 25 and 26) suggesting that iodination does not reduce the activity of salmon gonadotropin.

In the present study, further chromatographic purification of ^{125}I-labeled salmon gonadotropin on ConA Sepharose or Sephacryl S-200 increased the proportion of label that bound specifically to ovarian tissue (Fig. 28). These approaches have been applied to gonadotropin receptor studies in other vertebrate classes with similar results (Dufau et al., 1972; Catt et al., 1976; Licht and Bona Gallo, 1978; Dufau and Catt, 1978). Perhaps the most effective technique used to selectively increase the proportion of radiolabeled gonadotropin capable of receptor binding is receptor purification. In this procedure, radiolabeled gonadotropin bound to gonadal tissue is first dissociated at low pH or high temperature and then utilized in a receptor binding assay. When applied to studies with ^{125}I-labeled human FSH, receptor purification results in a several fold increase in the proportion of radiolabeled hormone capable of receptor binding and increased biological activity relative to the starting material (see Catt et al., 1976). Attempts to utilize receptor purification to increase the proportion of ^{125}I-labeled salmon gonadotropin capable of specific binding have been unsuccessful, however the procedure merits further investigation.

Gonadotropin binding to ovarian tissue from immature chum salmon was temperature dependant. Specific binding increased more rapidly and was of higher

magnitude at 20°C than 10°C (Fig. 29). Ovarian tissue incubated at 4°C showed only low levels of specific uptake. Kubokawa and Ishii (1980) reported that testicular tissue from reptiles and amphibians but not birds or mammals incubated at 0°C showed high specific binding of ^{125}I-labeled rat FSH. The present results suggest that the ability of gonadal tissues to bind gonadotropins specifically at low temperatures may not be a feature common to all poikilotherms. The effects of temperature on binding differed from that reported for steroid production (Fig. 21). 17β-estradiol production by chinook salmon ovarian follicles was higher at 10°C than at 20°C. The differing effects of temperature on binding and steroid production need not imply that binding sites identified using ^{125}I-labeled salmon gonadotropin are distinct from physiological receptors which mediate steroid production. As temperature directly influences the activity of steroidogenic enzymes (see Kime, 1982; Fostier et al., 1983), differences between the temperature optima for steroid production and receptor binding may occur.

Saturable gonadotropin binding sites appear to be restricted to the expected gonadotropin target tissues. Saturable gonadotropin uptake was found in particulate fractions prepared from ovaries and testes of immature chum salmon but not in liver, kidney or muscle (Fig. 31). Sundararaj and Goswami (1977) suggest that gonadotropin mediates steroidogenesis in interrenal tissue from the Indian catfish. Whether gonadotropin has a similar effect in salmonids is not known, although the kidney which would also contain interrenal tissue failed to show saturable binding of ^{125}I-labeled salmon gonadotropin.

Scatchard analysis indicated that gonadotropin binding to immature chum salmon ovaries was due to a single class of high affinity binding sites. The binding affinity for these sites (1.7-3.5 X $10^{9}M^{-1}$) was similar to that described

for LH and FSH receptor interactions in other vertebrate classes (Saxena, 1976; Catt et al., 1976; Bona Gallo and Licht, 1979). The binding capacity was also comparable to that reported for LH and FSH binding sites in other vertebrates.

In the present studies, attempts were made to evaluate the specificity of gonadotropin receptors by comparing the effects of different gonadotropins on the binding of ^{125}I-labeled salmon gonadotropin to ovarian tissue and their effect on the stimulation of steroid production (Fig. 22, 23, 24 and 34). However, it was not possible to evaluate the specificity of gonadotropin receptors in the immature chum salmon ovary by both of these approaches owing to the low steroidogenic capacity of the ovarian tissue. For example, in preliminary studies basal 17β-estradiol production was variable, and also SG-G100 at doses up to 5 μg/ml caused less than a 2-fold increase in 17β-estradiol production. However, separate studies which examined the pattern of steroid production by chinook salmon and coho salmon ovarian follicles in response to different gonadotropins were in agreement with the ability of these hormones to compete with the binding of ^{125}I-labeled salmon gonadotropin to immature chum salmon ovarian tissue. The limited ability of mammalian gonadotropins to stimulate steroid production in vitro and to compete for ^{125}I-labeled salmon gonadotropin binding sites was in general agreement with other studies which examined the actions of these hormones in salmonids. Idler et al. (1975b) reported that hCG, ovine LH and ovine FSH were ineffective in promoting cAMP production in immature rainbow trout gonads. Also, ovine LH and hCG were ineffective in accelerating the rate of oocyte maturation in rainbow trout and amago salmon (Nagahama et al., 1980). Kagawa et al., (1982) reported that very high doses of ovine LH stimulates 17β-estradiol production in amago salmon follicles while FSH and hCG had limited effectiveness.

Ng et al. (1980) suggested that separate receptors for glycoprotein and

vitellogenic gonadotropins were present in the winter flounder ovary on the basis of immunofluorescence localization studies. It was not possible to directly address the question of whether gonadotropin binding sites identified in the present studies were specific for glycoprotein gonadotropin as vitellogenic gonadotropin was not available for comparison. However, a crude fraction not retained on ConA Sepharose ($ConA_1$) which would be expected to contain vitellogenic gonadotropin had only a limited effectiveness in competing for ^{125}I-labeled salmon gonadotropin binding sites in chum salmon ovaries (Fig. 34). In preliminary studies, the ability of the $ConA_1$ fraction to compete for gonadotropin binding sites was consistent with its relative activity as determined by RIA, which suggests that the activity present in this fraction may represent contamination by the glycoprotein gonadotropin. Further studies using purified vitellogenic gonadotropin would be necessary to provide a definitive statement on hormone specificity.

Unlike the studies with immature chum salmon, ovarian tissue from adult coho salmon either failed to show specific binding of ^{125}I-labeled salmon gonadotropin or showed only very low levels of specific binding (Fig. 35 and 36). These data contrast with data from mammals in that the number of LH receptors in the ovary increase as a function of follicular size (Channing and Kammerman, 1973; Lee, 1976; Ryan and Lee, 1976) and development (Nimrod et al., 1977; Diekman et al., 1978; Richards, 1979; McNeilly et al., 1980). Also, Cook and Peter (1980a) reported that ovaries from goldfish having completed vitellogenesis or undergoing recrudesence bound more ^{125}I-labeled carp gonadotropin than ovaries from goldfish undergoing regression. There are several possible explanations which could account for the low levels of specific gonadotropin binding to ovarian tissue from adult coho salmon (and for the low specific binding of radiolabeled LH preparations in

nonmammalian vertebrates). It is possible that the proportion of ovarian tissue which contains gonadotropin receptors is low. A vast excess of gonadal tissue containing only non-specific gonadotropin binding sites could mask the presence of a limited number of specific gonadotropin binding sites.

However, attempts to selectively increase the proportion of specific cell types by studying gonadotropin binding to isolated thecal and granulosa cell layers did not result in increased levels of specific binding (Fig. 36). Low levels of specific binding could result from degradation of either labeled gonadotropin or receptor during incubation. To test these possibilities, short-term binding studies with a proteolytic enzyme inhibitor (phenyl methyl sulfonylflouride) included in the incubation buffer were conducted. These conditions did not influence the specific binding of ^{125}I-labeled salmon gonadotropin (unpublished results). An additional consideration is that separate gonadotropin receptors exist in immature and adult salmonids. A change in the specificity of these receptors could account for differences in the amount of specific ^{125}I-labeled salmon gonadotropin binding. In this regard, Young et al. (1983d) reported a change in the activity of mammalian gonadotropins on the stimulation of 17β-estradiol production in vitellogenic amago salmon and 17α20βP production in preovulatory animals. In these studies ovine LH was less effective in stimulating 17α20βP production than 17β-estradiol. In the present studies, the relative activities of SG-G100 and acetone dried pituitary powder were similar in chinook and coho salmon ovarian follicles at different stages of development (Fig. 22 and 24). These results suggest that the specificity of gonadotropin receptor interactions in Pacific salmon did not change during development.

In the present studies, saturable gonadotropin binding sites were demonstrated in both thecal and granulosa cell layers from preovulatory coho salmon

ovarian follicles (Fig. 36). Recently, H. Kagawa and Y. Nagahama (pers. comm.) found a similar distribution of saturable gonadotropin binding sites in amago salmon ovarian follicles. The presence of gonadotropin binding sites in thecal and granulosa cells is consistent with the recently proposed two-cell model for 17α20βP synthesis in salmonids (Nagahama, 1983; Young et al., 1983b). In this model, gonadotropin binding to a receptor located in the thecal cell layer would result in the activation of steroidogenesis leading to the production of 17α-hydroxyprogesterone. Gonadotropin binding to a separate receptor in the granulosa cell layer would increase the production of 20βHSD responsible for the conversion of 17αhydroxyprogesterone to 17α20βP. It would be predicted based on the pattern of 17α20βP production in vivo (Fig. 9 and 13; Chapt. 4) and in vitro (Fig. 19; Chapt. 4) that the number of gonadotropin receptors in granulosa cells would increase during development and reach their highest levels at the time of oocyte maturation and ovulation. The present results provide an indication that this may indeed be the case as follicles characterized by a peripheral germinal vesicle and follicles having undergone maturation showed higher levels of saturable gonadotropin binding than follicles characterised by a central germinal vesicle. However, the low levels of saturable binding and the high variation in uptake preclude a direct evaluation of gonadotropin receptor number by Scatchard analysis. A second question which remains unanswered is whether the specificity of gonadotropin binding sites in thecal and granulosa cell layers differ. However, an accurate assessment of these questions will await further developments to selectively increase the binding activity of ^{125}I-labeled salmon gonadotropin or to increase the proportion of ovarian tissue showing saturable gonadotropin binding.

CHAPTER 6 - SUMMARY AND CONCLUSIONS

The preovulatory period in coho salmon was characterized by a gradual increase in plasma gonadotropin levels with no evidence for a defined surge associated with ovulation. 17β-estradiol levels declined ten days prior to ovulation, reaching basal levels four days prior to ovulation. Testosterone levels were high during the preovulatory period but decreased at ovulation. 17α20βP increased from very low levels ten days prior to ovulation to maximal levels four days prior to ovulation. The preovulatory increase in 17α20βP coincides with germinal vesicle breakdown which is consistent with the view that 17α20βP functions as the maturation inducing steroid in salmonids.

A dual system controlling gonadotropin secretion was demonstrated in adult female coho salmon. Intraperitoneal injections of mammalian and piscine Gn-RH were shown to increase plasma gonadotropin levels. LH-RH, LH-RHA DAla^6 and chum salmon Gn-RH had similar effects on plasma gonadotropin levels initially, although the response to LH-RHA DAla^6 was of longer duration. LH-RH and chum salmon Gn-RH maintained elevated plasma gonadotropin levels for up to 24 hr whereas the effects of LH-RHA DAla^6 persisted for up to six days. Intraperitoneal injections of pimozide, a dopamine receptor antagonist, also increased plasma gonadotropin levels suggesting that dopamine may function to inhibit gonadotropin release. Although pimozide was less effective than LH-RHA DAla^6 on the stimulation of gonadotropin secretion, pimozide potentiated the actions of LH-RHA DAla^6.

The acceleration of oocyte maturation and ovulation was related to the magnitude and duration of the increase in plasma gonadotropin levels. LH-RH having a transitory influence on plasma gonadotropin levels was ineffective whereas LH-RHA DAla^6 having a prolonged action accelerated oocyte maturation and ovulation. Two injections of LH-RHA DAla^6 over a 72 hr period were additive to the effects of a

single injection with respect to plasma gonadotropin levels and were a more effective means of accelerating ovulation. A single injection of SG-G100 which resulted in higher plasma gonadotropin levels than LH-RHA DA1a^6 for 48 hr had similar effects on the acceleration of ovulation. These results suggest that the induction of ovulation was dependent on the duration rather than the magnitude of the initial increase in plasma gonadotropin levels. Combined injections of SG-G100 and LH-RHA DA1a^6 resulted in higher plasma gonadotropin levels than injections of SG-G100 alone and were more effective in accelerating ovulation. Two injections of LH-RHA DA1a^6 alone or injections of LH-RHA DA1a^6 in combination with SG-G100 represent effective means of inducing ovulation in coho salmon. The suitability of LH-RHA DA1a^6 for the induction of ovulation relates to its prolonged effect on the maintenance of elevated plasma gonadotropin levels.

Treatments which elevate plasma gonadotropin levels increased the production of testosterone and 17α20βP. LH-RH caused a transient increase in 17α20βP levels whereas LH-RHA DA1a^6 and SG-G100 resulted in a long-term elevation of 17α20βP levels. The induction of oocyte maturation and ovulation was related to the duration and magnitude of the increase in plasma 17α20βP levels. A short-term increase in 17α20βP at low levels (55 ng/ml) failed to induce oocyte maturation whereas a long-term increase in 17α20βP at high titres (100 ng/ml) resulted in oocyte maturation but not ovulation. 17α20βP levels in coho salmon which were induced to ovulate were increased to 450-500 ng/ml.

Gonadotropin decreased plasma 17β-estradiol levels in a dose and time dependent manner. Declining 17β-estradiol levels determined the time of oocyte maturation and ovulation as low levels of 17β-estradiol were a requirement for maximal 17α20βP production. An increase in 17α20βP levels to above 200 ng/ml did not occur in the absence of a decline in 17β-estradiol to less than 2 ng/ml. The

production of large amounts of 17α20βP in response to SG-G100 in vitro occurred when 17β-estradiol production was reduced. Furthermore, exogenous 17β-estradiol reduced the production of 17α20βP in response to SG-G100 in vitro when 20β HSD activity was low.

Gonadotropin binding sites were identified in chum and coho salmon ovaries which share several of the properties wich characterize physiologically relevant gonadotropin receptors in other vertebrates. The binding of ^{125}I-labeled salmon gonadotropin to immature chum salmon ovarian tissue incubated in vitro was a saturable process. The addition of unlabeled SG-G100 reduced the uptake of ^{125}I-labeled salmon gonadotropin by ovarian tissue. Saturable gonadotropin binding sites were localized in the 3000 g particulate fraction. Gonadotropin binding sites with similar properties were identified in particulate fractions prepared from chum salmon testes but not in liver, kidney or muscle. The saturable binding component was due to a single class of high affinity binding sites present in limited numbers. These binding sites were characterised by a high affinity (Ka: 1.3 - 3.5 x 10^9 M^{-1}) and a binding capacity of about 40 pg/mg of tissue. Gonadotropin binding sites exhibited a high degree of hormone specificity. Salmon gonadotropin preparations inhibit the binding of ^{125}I-labeled salmon gonadotropin to immature chum salmon ovarian tissue whereas mammalian gonadotropins including ovine LH, ovine FSH and hCG were ineffective. The ability of different salmon gonadotropin preparations to compete for ^{125}I-labeled salmon gonadotropin binding sites were consistent with the relative effects of these preparations on steroid production. These results suggest that gonadotropin binding sites identified by the binding of ^{125}I-labeled salmon gonadotropin share properties similar to physiologically relevant receptors which mediate steroidogenesis. A crude pituitary fraction expected to contain vitellogenic gonadotropin had limited

ability to compete for gonadotropin binding sites suggesting that these sites were directed to the glycoprotein gonadotropin. Relative to immature chum salmon, ovarian tissue from adult coho salmon showed only low levels of saturable gonadotropin binding. The gonadotropin binding sites in adult coho salmon ovary were present in both thecal and granulosa cell layers surrounding the follicle. However, the low binding activity precludes a direct evaluation of the properties of these sites.

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APPENDIX 1

Gonadotropin RIA: Assay Validation

The suitability of the gonadotropin RIA for the measurement of coho salmon plasma and pituitary gonadotropin was investigated by comparing the slopes of RIA displacement curves using gonadotropin standard and serial dilutions of plasma and pituitary extracts (Fig. 1). The slopes of the displacement curves for plasma and pituitary extracts were similar to the standard suggesting a high degree of immunorelatedness between standard and endogenous gonadotropin.

The accuracy of the gonadotropin RIA was evaluated by comparing known amounts of gonadotropin added to aliquots of a female coho salmon plasma pool and the measured excess of hormone determined by RIA (Fig. 2). The slope of the resulting regression line between the added and measured amounts of gonadotropin ($b = 1.05$) does not differ from 1 and the intercept (-0.46) does not differ from 0 ($P > 0.05$). Thus the amounts of gonadotropin measured by RIA were correlated with the amount of added gonadotropin.

To evaluate whether the gonadotropin antisera reacts with biologically active hormone, studies were conducted to investigate the effects of pretreatment with gonadotropin antisera on the ability of gonadotropin to stimulate steroid production. In one of these studies, 5 µg of SG-G100 was combined with either gonadotropin antiserum or normal rabbit serum in 100 µl of PBS. Following incubation for 24 hr at 10°C, aliquots of the pretreated gonadotropin preparations were incubated with minced testicular tissue from adult coho salmon. The amounts of testosterone released into the media were determined by RIA. Gonadotropin preincubated with 2 or 20 µl of normal rabbit serum induced a dose related increase in testosterone production whereas pretreatment with 20 µl of gonadotropin antiserum resulted in lower ($P < 0.01$) testosterone production (Fig. 3). In a

separate experiment, 5 µg of SGA-2360 in 100 µl of PBS was combined with 25 µl of either normal rabbit serum or the gonadotropin antiserum. After incubation for 24 hours at 10°C, 200 µl of goat anti-rabbit gamma globulin was added and the incubation continued for an additional 8 hours. Following centrifugation for 10 min at 3000 g, aliquots of the supernatant fluid were incubated with ovarian follicles obtained from adult coho salmon. SGA-2360 pretreated with normal rabbit serum stimulated a dose related increase in testosterone production during a 24 hr incubation (Fig. 4). SGA-2360 pretreated with gonadotropin antisera produced signficantly less testosterone ($P < 0.01$) than did hormone pretreated with normal rabbit serum. These data suggest that the gonadotropin antisera is capable of neutralizing the activity of gonadotropin in male and female coho salmon.

Since salmon pituitary hormones other than gonadotropin were not available for comparison, it was not possible to directly evaluate the specificity of the gonadotropin RIA. However, tests were conducted to investigate the relationship between the amounts of gonadotropin present in various salmon pituitary preparations as determined by RIA and bioassay. Fig. 5 shows the RIA displacement curves for four gonadotropin preparations using ^{125}I labeled SGA-2360 as the radioligand. The relative potencies of these pituitary extracts as determined by RIA and bioassay are shown in Table 1. These data indicate a close agreement between the gonadotropin content determined by either RIA or bioassay. This suggests that pituitary factors other than gonadotropin which are present in the acetone dried pituitary powder but are removed during purification do not influence the measurement of gonadotropin by RIA.

FIG. 1. RIA (logit-log) dose response curves for gonadotropin standard and serial dilutions of coho salmon plasma samples and pituitary extracts. Each point represents the mean of three determinations.

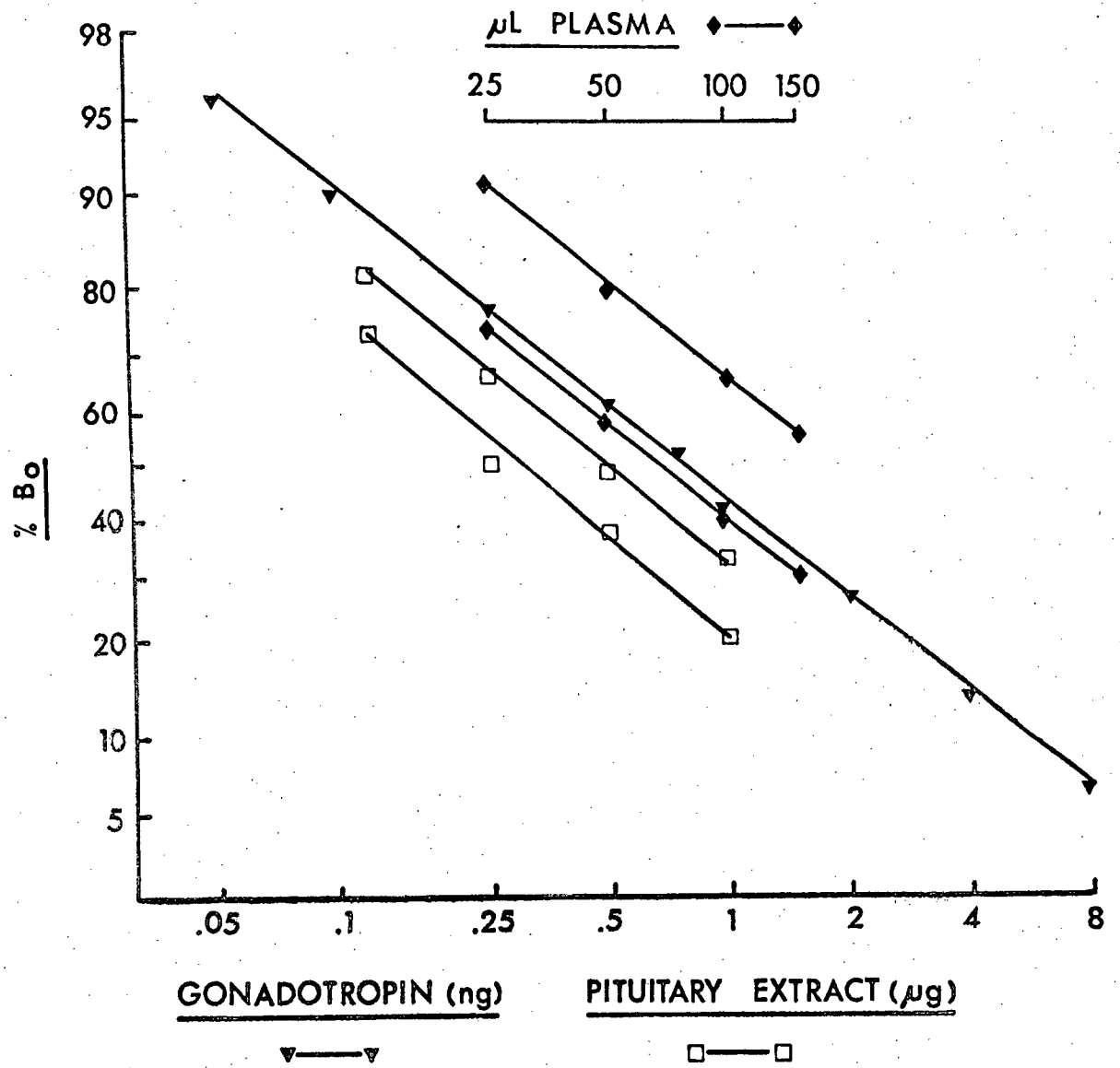
µL PLASMA
25 50 100 150
98
95
90
80
60
40
20
10
5
% Bo
.05 .1 .25 .5 1 2 4 8
GONADOTROPIN (ng)
PITUITARY EXTRACT (µg)

FIG. 2. Recovery of known amounts of gonadotropin added to plasma samples as determined by RIA. Values represent the mean ± standard error of 4 determinations.

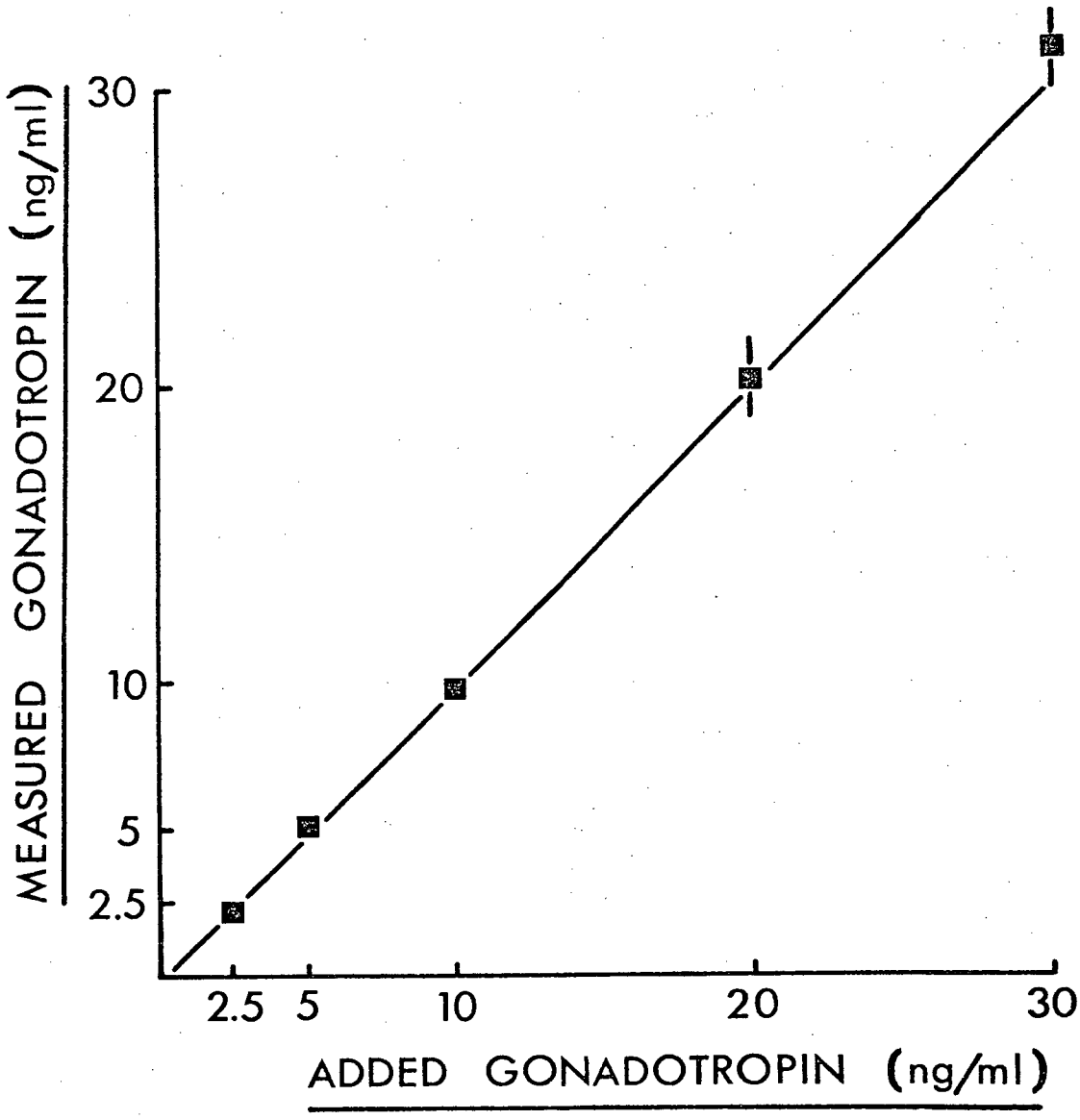
MEASURED GONADOTROPIN (ng/ml)
30
20
10
5
2.5
2.5
5
10
20
30
ADDED GONADOTROPIN (ng/ml)

FIG. 3. Testosterone production by minced testicular tissue from adult coho salmon in response to SG-G100 pretreated with normal rabbit serum (NRS) or gonadotropin antiserum (SG-RS). Values represent the mean ± standard error of 3 determinations.

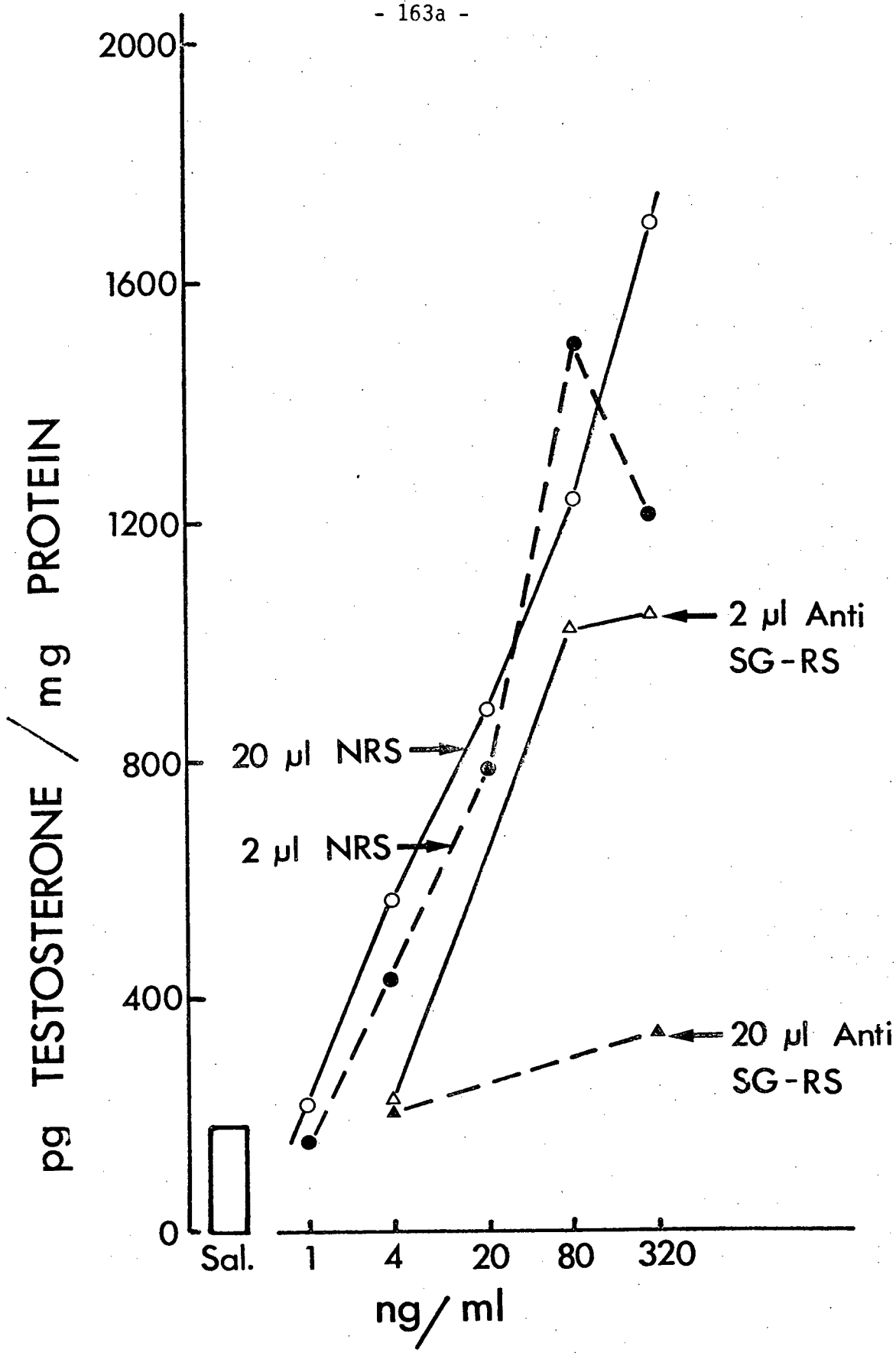
2000
1600
1200
800
400
0
pg TESTOSTERONE / mg PROTEIN
20 µl NRS
2 µl NRS
2 µl Anti SG-RS
20 µl Anti SG-RS
Sal.
1
4
20
80
320
ng/ ml

FIG. 4. Testosterone production by ovarian follicles from adult coho salmon in response to SGA-2360 pretreated with normal rabbit serum (○) or antiserum to salmon gonadotropin (●). Values represent the mean ± standard error of 3 determinations.

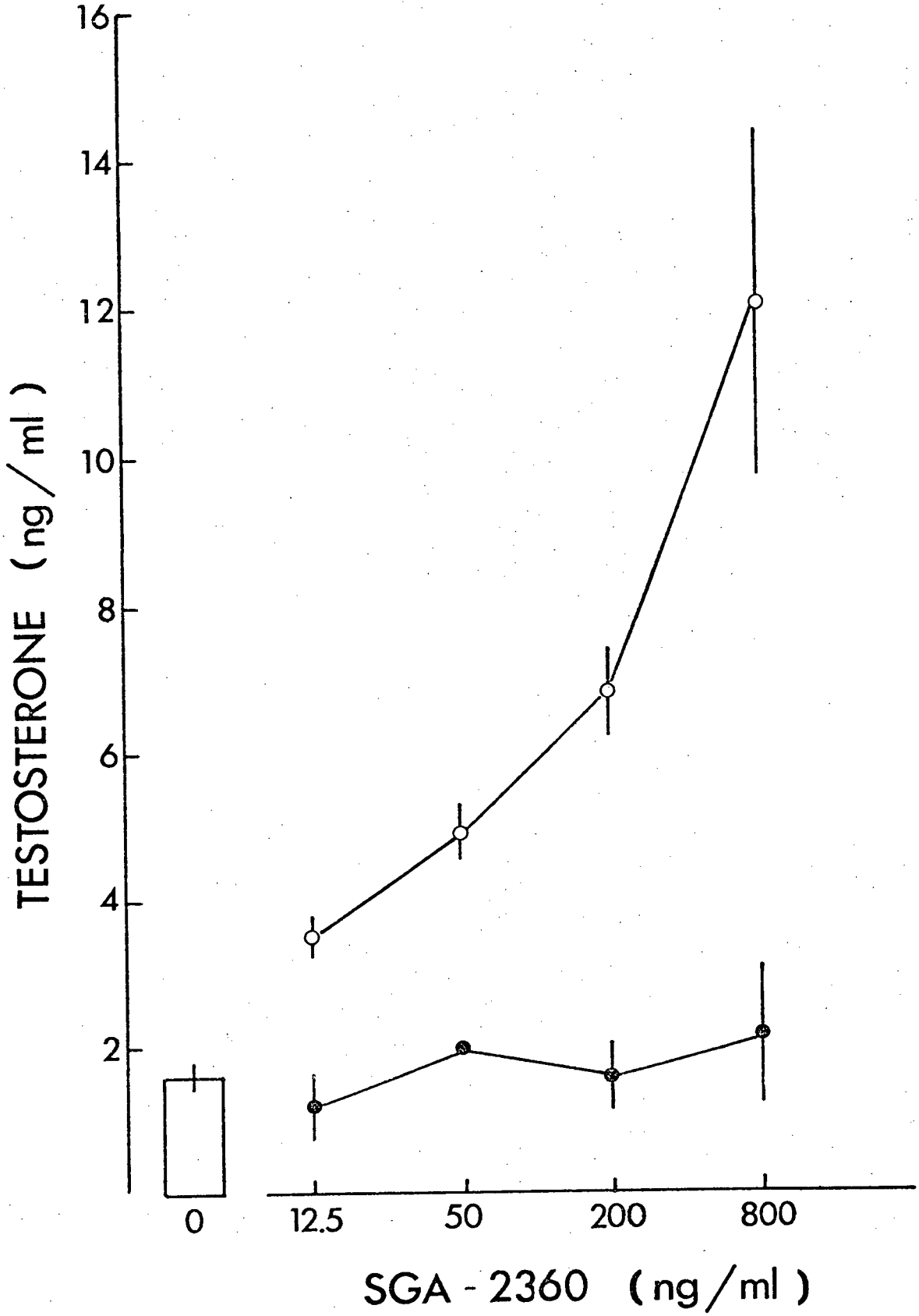
16
14
12
10
8
6
4
2
TESTOSTERONE (ng/ml)
0
12.5
50
200
800
SGA - 2360 (ng/ml)

Fig. 5 - RIA (logit/log) displacement curves for acetone dried pituitary powder, SG-G100, SGA-2360 and SGA-2359. Each point represents the mean of two determinations.

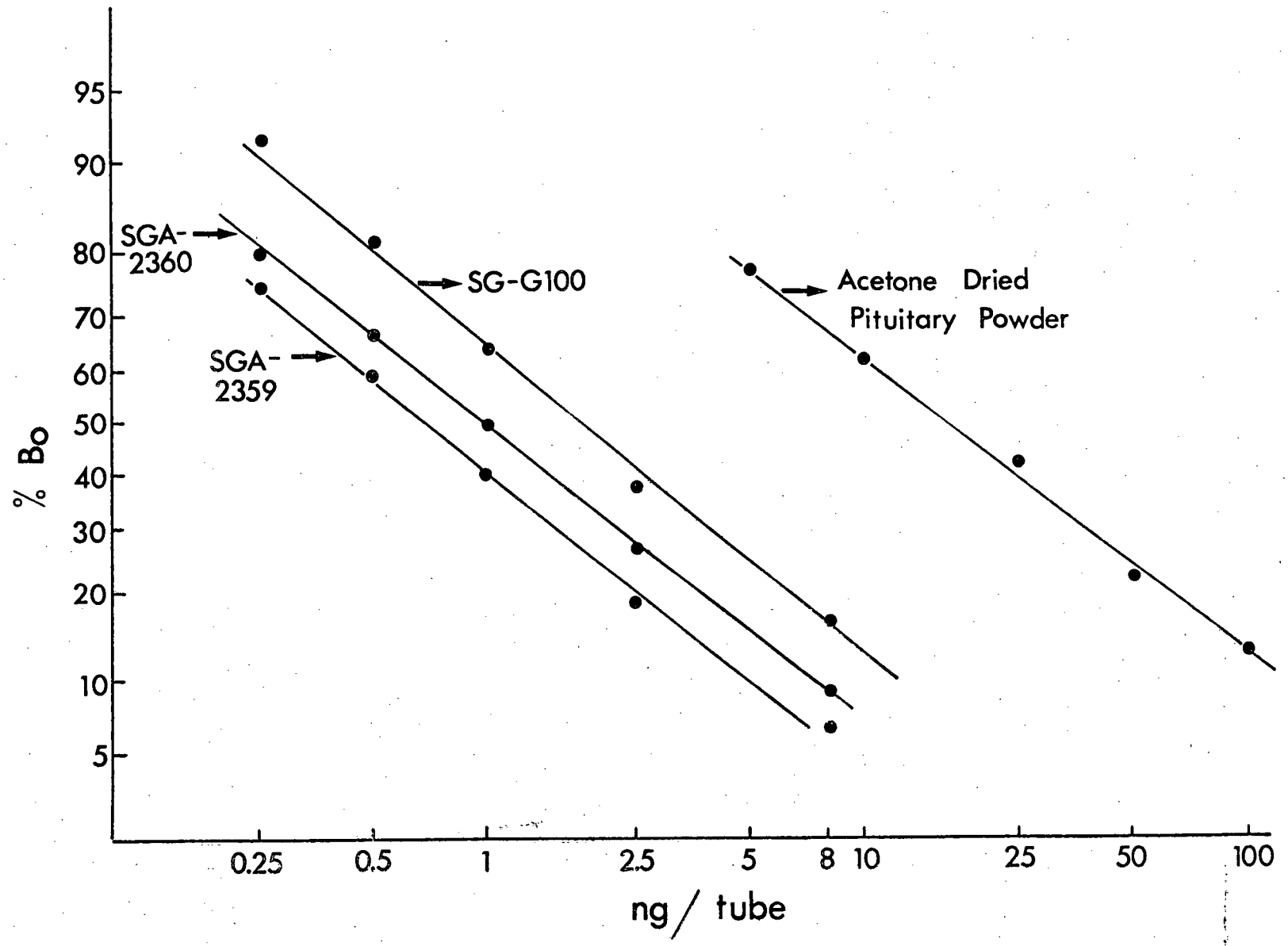
95
90
80
70
60
50
40
30
20
10
5
% Bo
SGA-
2360
SGA-
2359
SG-G100
Acetone Dried
Pituitary Powder
0.25
0.5
1
2.5
5
8
10
25
50
100
ng / tube

TABLE 1 - Relative potency of various pituitary fractions as determined by RIA and bioassay[1].

Pituitary preparation	RIA	Bioassay 1[2]	Bioassay 2[3]
Acetone dried pituitary powder	1.0	1.0	1.0
SG-G100	9.1	7.7	7.1
SGA-2360	16.8	15.1	17.8
SGA-2359	23.4	20.0	25.0

1 Relative potencies calculated on the basis of acetone dried pituitary powder which is assigned the value of 1.0

2 Stimulation of 17β-estradiol production in chinook salmon ovaries in vitro.

3 Stimulation of testosterone production in coho salmon testis pieces in vitro.

APPENDIX 2

17β-Estradiol, Testosterone and 17α20βP RIAs: Assay Validation

The applicability of the 17β-estradiol, testosterone and 17α20βP RIAs to coho salmon plasma samples were evaluated by comparing the dose response curves for the steroid standards and serial dilutions of heated plasma (Fig. 1, 2 and 3). The slopes of the displacement curves for the steroid standards and coho salmon plasma were similar suggesting that plasma constituents did not effect the immunological properties of the endogenous steroids.

As measurements of plasma steroid levels by RIA were based on direct measurements from heated plasma samples, it was considered essential to evaluate the accuracy of these values by comparing the levels obtained following diethyl ether extraction and chromatographic purification of steroids. Plasma samples used for assay validation were obtained from maturing and ovulated coho salmon. For the 17β-estradiol RIA, 10 plasma samples were measured following heating and after diethyl ether extraction and chromatography on LH-20 Sephadex as described by Haning et al. (1979). 17β-estradiol levels in plasma samples measured directly and after chromatography were highly correlated (correlation coefficient (r)= 0.98, slope (b)=1.01, intercept (a) 0.56; Fig 4). For the testosterone assay, comparisons were made between 8 plasma samples measured directly and following diethyl ether extraction and chromatography on Celite columns as described by Abraham et al. (1972). The correlation coefficient between testosterone levels measured by the direct assay and after chromatography was 0.97 (b = 0.98, a = 1.48; Fig. 5). No difference was found with respect to 17α20βP in plasma samples measured directly and following diethyl ether extraction (r = 0.99, b = 0.96, a = 3.8, n = 6; Fig. 6).

The accuracy of the direct methods for the measurement of plasma steroid

levels by RIA was evaluated by comparing the recovery of known amounts of steroid added to plasma before heating. Three separate plasma pools were obtained by combining plasma samples of known titre so that a 200 µl aliquot measured in the RIA would contain approximately 50 pg of either 17β-estradiol, testosterone or 17α20βP. Known amounts of steroid were added to aliquots of the plasma pools so that a 200 µl sample would contain an additional 12.5, 25, 50, 100 or 150 pg of steroid. The relationship between the amounts of steroid added and the recovery of added steroid as determined by RIA are shown in Fig. 7, 8 and 9. In each of the steroid assays, the regression line slope between expected and measured quantities does not differ from 1 and the intercept does not differ from 0 ($P>0.05$). Thus the amounts of steroid measured by RIA were closely related with the amount of added steroid.

The suitability of the 17β-estradiol, testosterone and 17α20βP RIA procedures for the measurement of steroids produced by ovarian follicles incubated in vitro was evaluated in a similar fashion to that described for coho salmon plasma samples. It was found necessary to extract the incubation media from ovarian follicle incubations with diethyl ether in order to obtain parallelism between dilutions of the incubation medium and the RIA steroid standards. Known amounts of steroid added to the incubation medium were highly correlated with the amounts of hormone determined by RIA as were the levels of 17β-estradiol and testosterone measured with or without chromatography (data not shown). Since the recovery of ^{3}H labeled steroids added to the incubation medium prior to extraction was greater than 95%, no correction was used to account for losses incurred during extraction.

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FIG. 1. RIA (logit-log) dose response curves for 17β-estradiol standard and dilutions of heated coho salmon plasma. Each point represents the mean of two determinations.

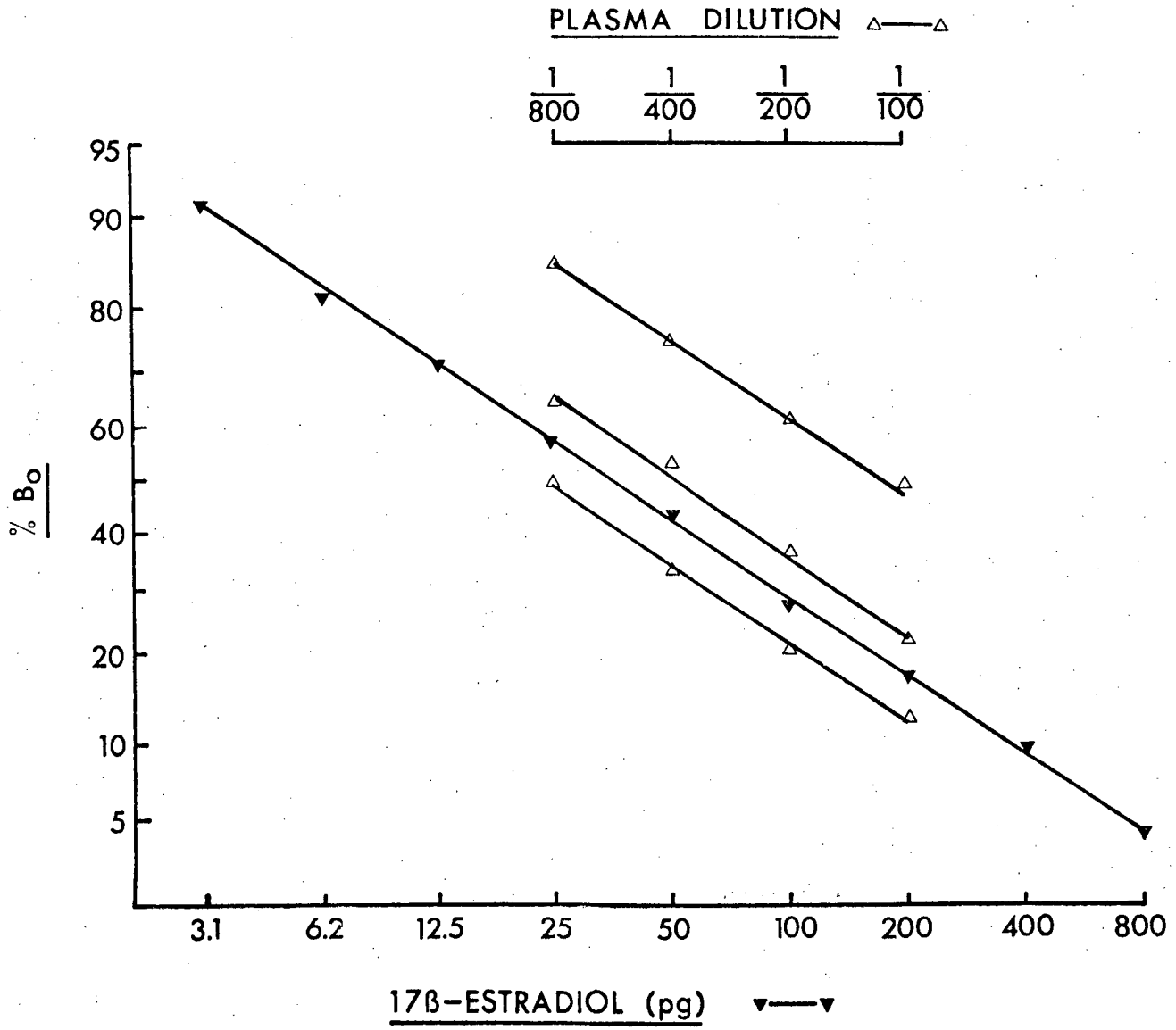

PLASMA DILUTION
1/800
1/400
1/200
1/100
95
90
80
60
40
20
10
5
% Bo
3.1
6.2
12.5
25
50
100
200
400
800
17ß-ESTRADIOL (pg)

FIG. 2. RIA (logit-log) dose response curves for testosterone standard and dilutions of heated coho salmon plasma. Each point represents the mean of two determinations.

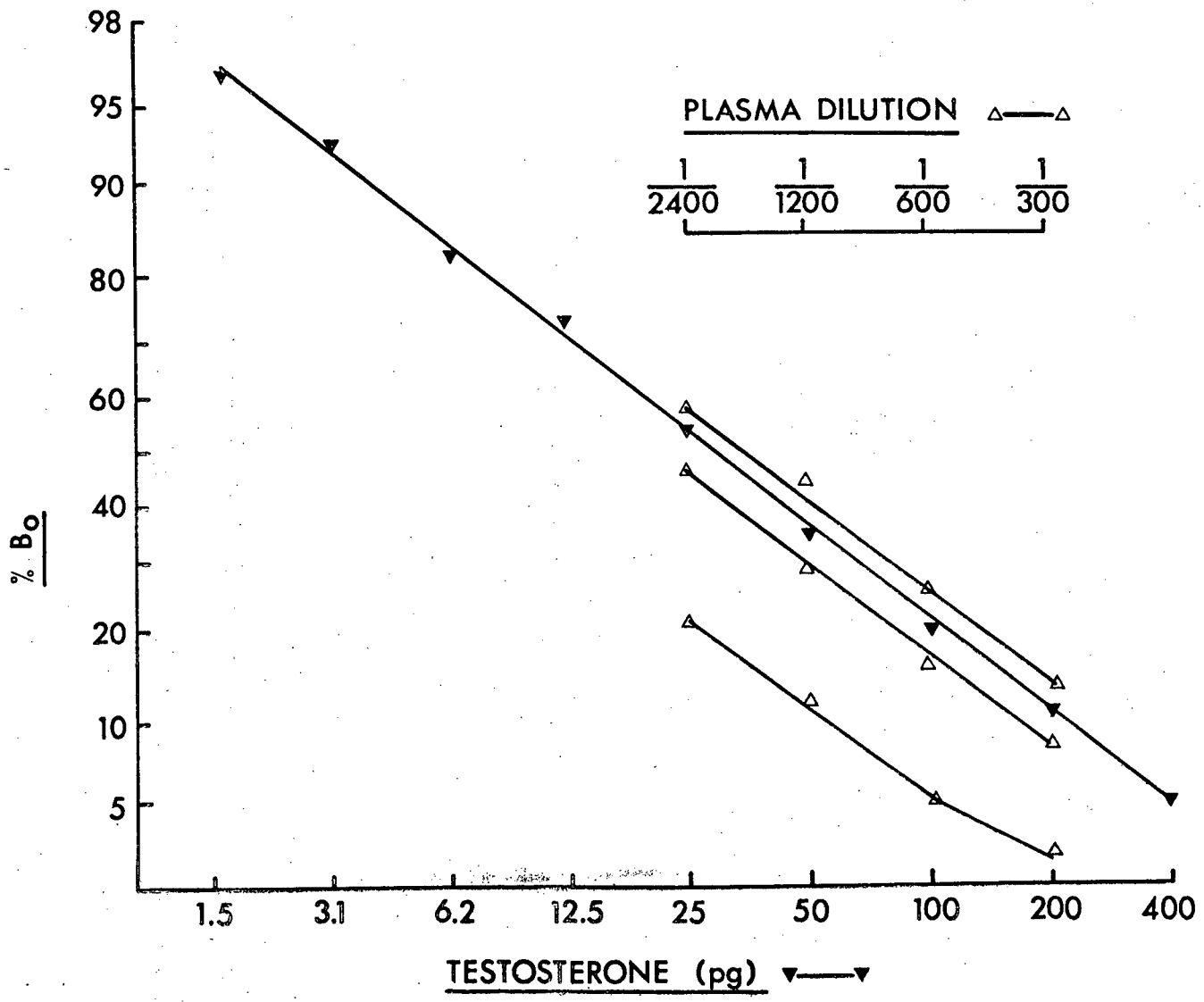

98
95
90
80
60
40
20
10
5
% B_o
PLASMA DILUTION
1/2400
1/1200
1/600
1/300
1.5
3.1
6.2
12.5
25
50
100
200
400
TESTOSTERONE (pg)

FIG. 3. RIA (logit-log) dose response curves for 17α20βP standard and dilutions of heated coho salmon plasma. Each point represents the mean of two determinations.

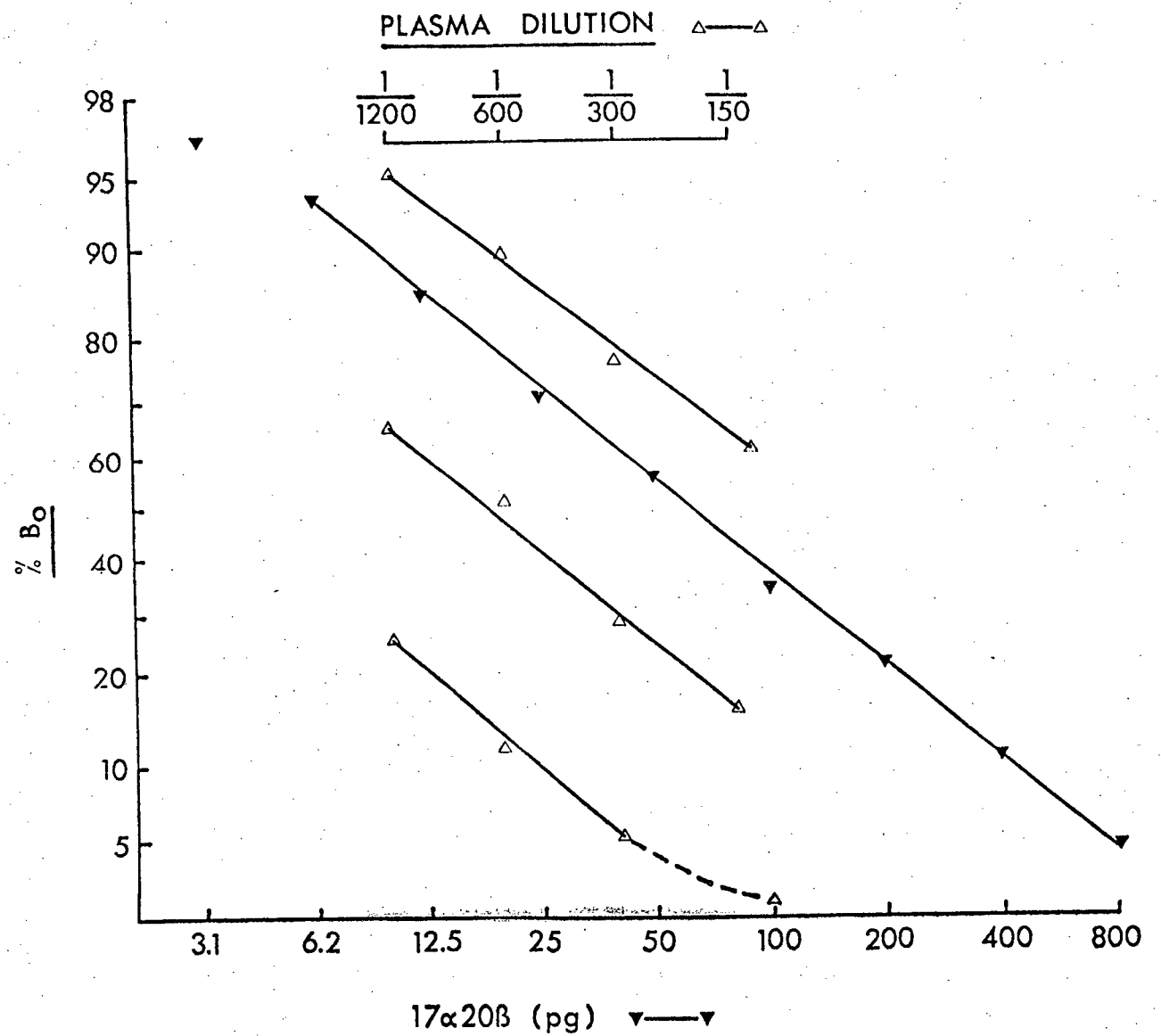

PLASMA DILUTION
1/1200
1/600
1/300
1/150
98
95
90
80
60
40
20
10
5
% Bo
3.1
6.2
12.5
25
50
100
200
400
800
17α20ß (pg)

FIG. 4. Relationship between the levels of 17β-estradiol in plasma samples determined using the direct method and following diethyl ether extraction and chromatography on LH-20 Sephadex. The line represents the relationship Y = X.

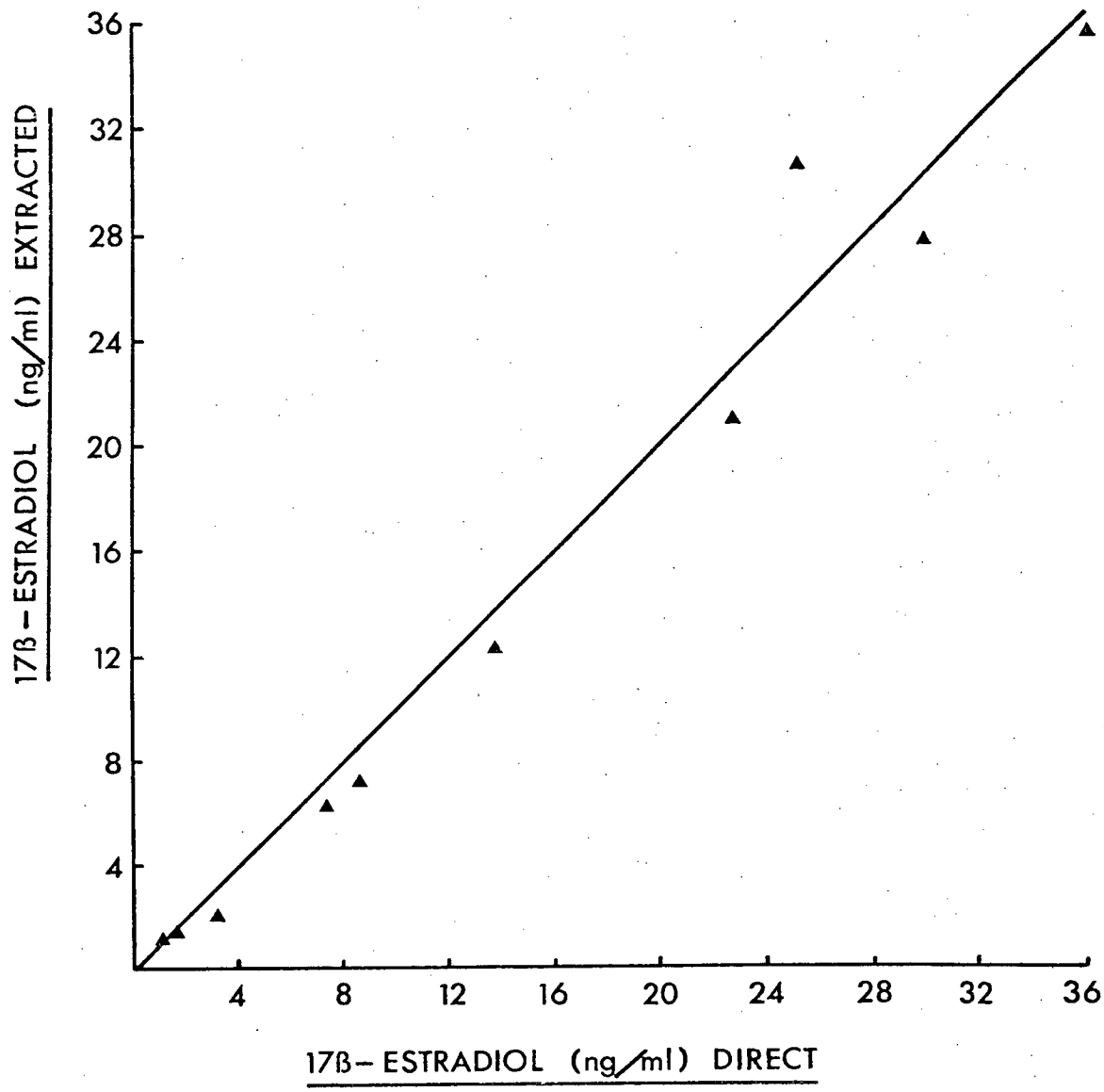

17ß – ESTRADIOL (ng/ml) EXTRACTED
36
32
28
24
20
16
12
8
4
4
8
12
16
20
24
28
32
36
17ß – ESTRADIOL (ng/ml) DIRECT

FIG. 5. Relationship between the levels of testosterone in plasma samples determined using the direct method and following diethyl ether extraction and chromatography on Celite columns. The line represents the relationship Y = X.

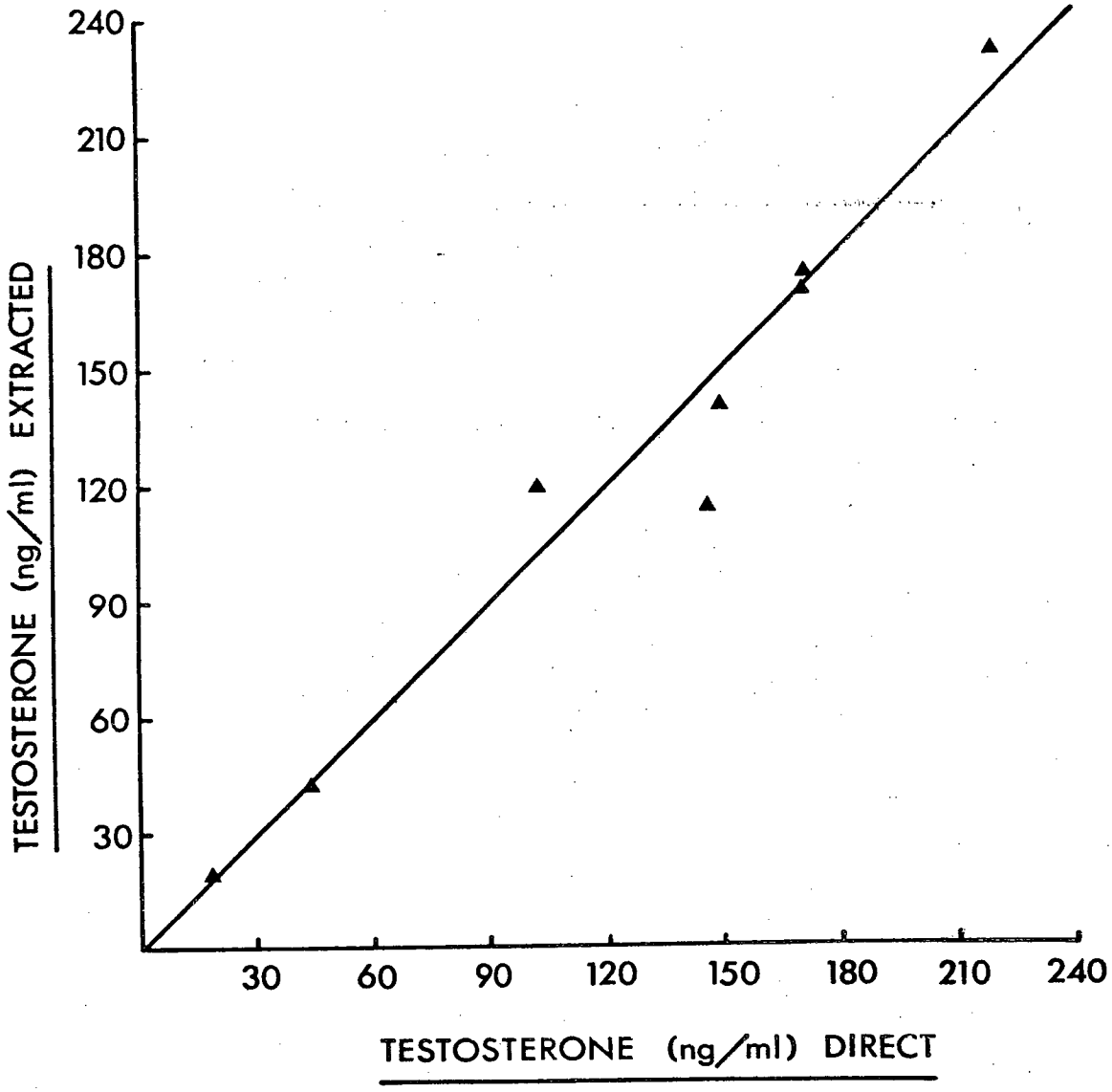

240
210
180
150
120
90
60
30
TESTOSTERONE (ng/ml) EXTRACTED
30
60
90
120
150
180
210
240
TESTOSTERONE (ng/ml) DIRECT

FIG. 6. Relationship between the levels of 17α20βP in plasma samples determined using the direct method and following diethyl ether extraction. The line represents the relationship Y = X.

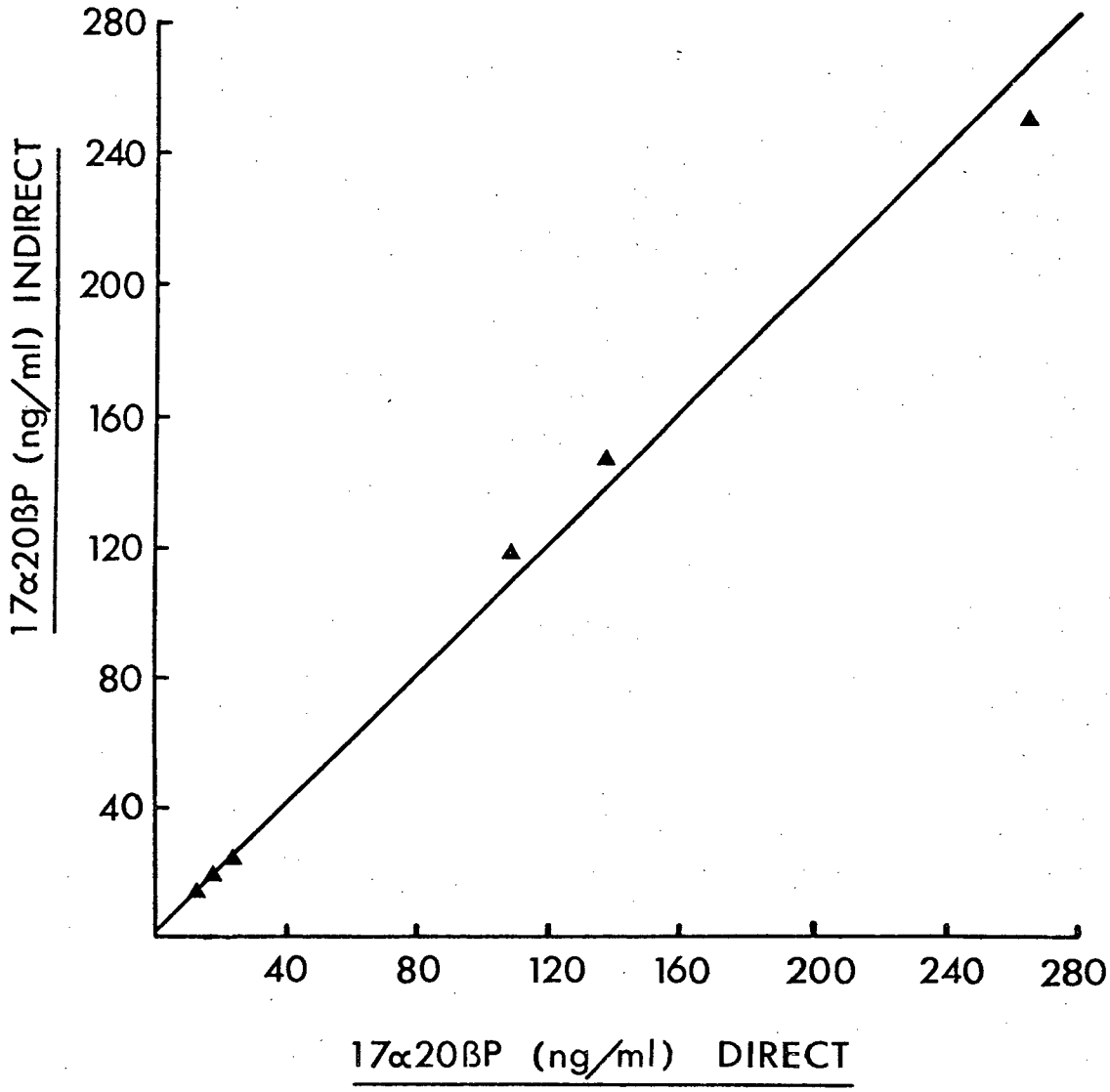

280
240
200
160
120
80
40
17α20βP (ng/ml) INDIRECT
40
80
120
160
200
240
280
17α20βP (ng/ml) DIRECT

FIG. 7. Recovery of known amounts of 17β-estradiol added to plasma samples prior to heating as determined by RIA. Each point represents the mean ± standard error of three determinations.

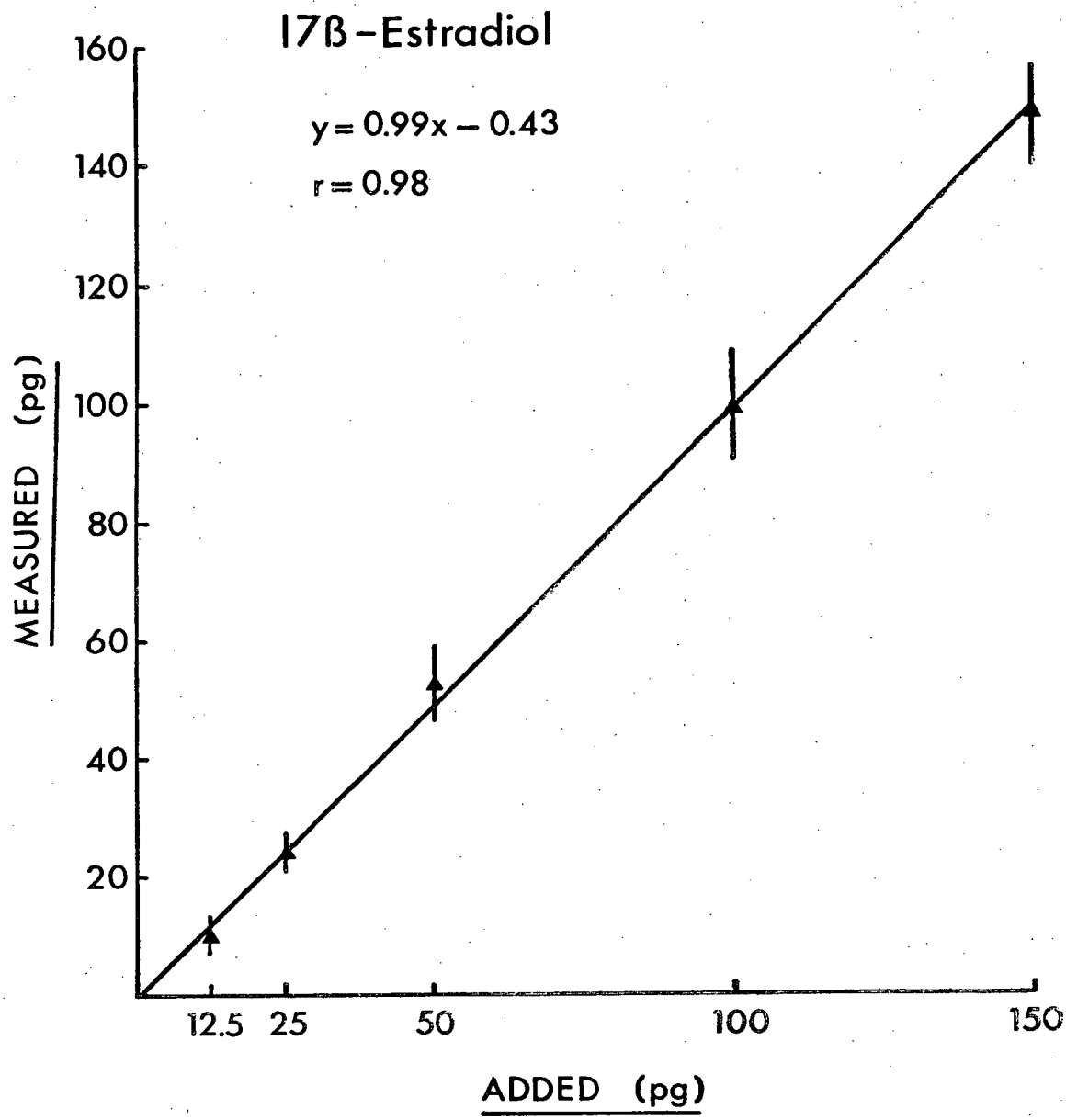

17ß-Estradiol
y = 0.99x – 0.43
r = 0.98
160
140
120
100
80
60
40
20
MEASURED (pg)
12.5
25
50
100
150
ADDED (pg)

FIG. 8. Recovery of known amounts of testosterone added to plasma samples prior to heating as determined by RIA. Each point represents the mean ± standard error of three determinations.

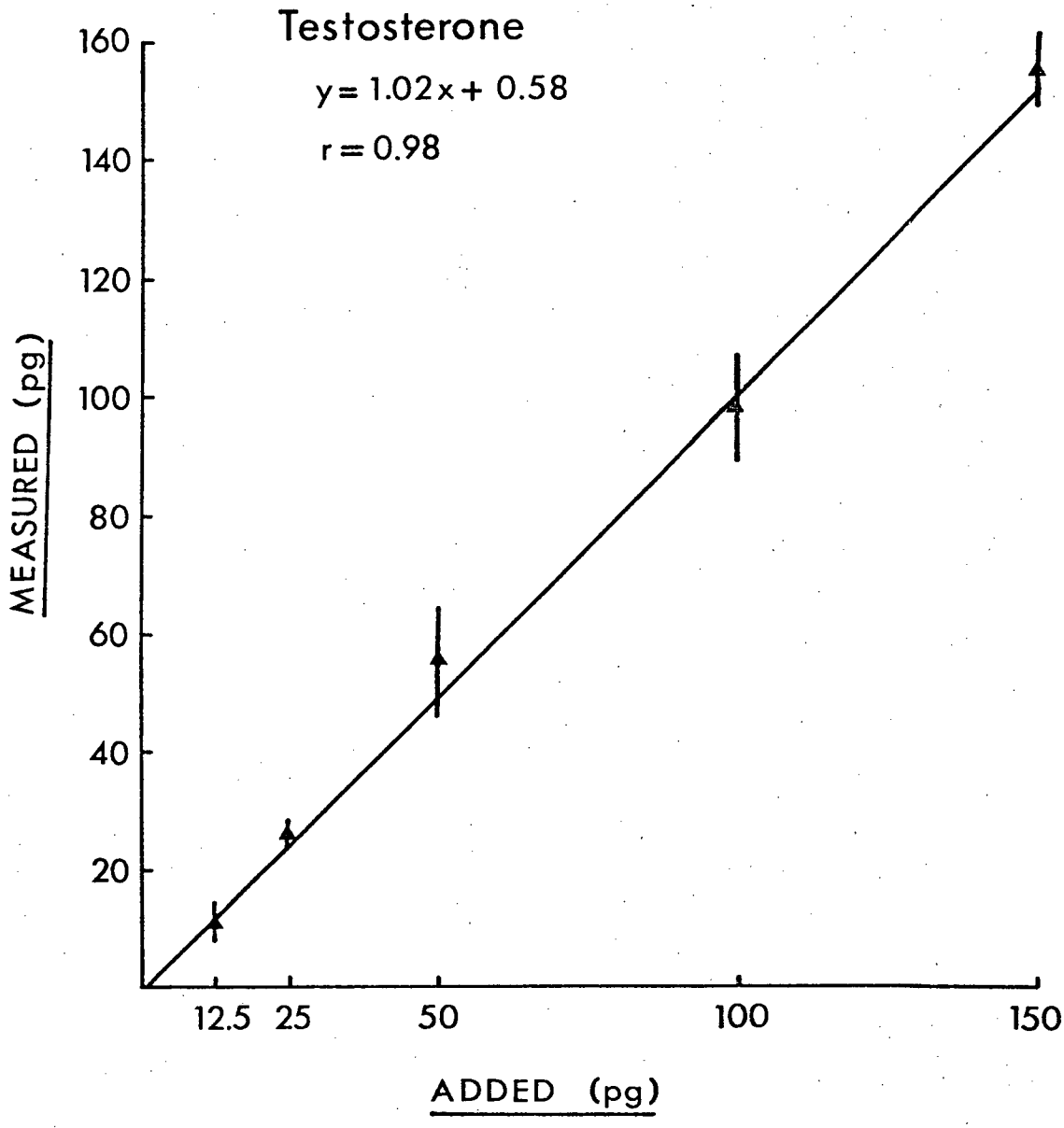
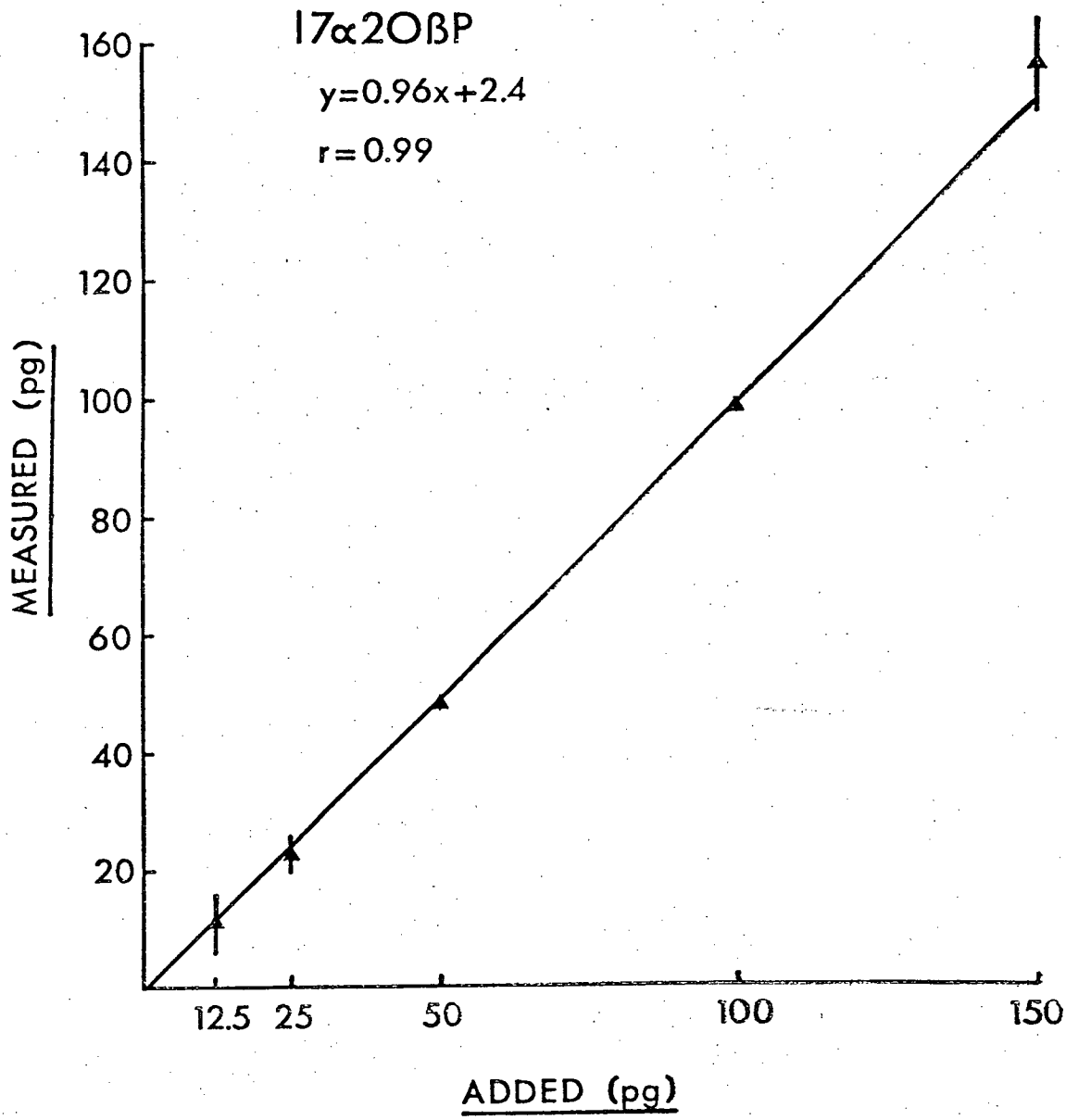

Testosterone
y = 1.02x + 0.58
r = 0.98
160
140
120
100
80
60
40
20
MEASURED (pg)
12.5
25
50
100
150
ADDED (pg)

FIG. 9. Recovery of known amounts of 17α20βP added to plasma samples prior to heating as determined by RIA. Each point represents the mean ± standard error of three determinations.


17α20ßP
y=0.96x+2.4
r=0.99
160
140
120
100
80
60
40
20
MEASURED (pg)
12.5
25
50
100
150
ADDED (pg)