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Title: Sex Steroids, Gonadotropins, and Effects on the Immune Response in Maturing Spring Chinook Salmon (Oncorhynchus tshawytscha)

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Carl B. Schreck

Plasma concentrations of 17β-estradiol, 17α, 20β-dihydroxy-4-pregnen-3-one, androstenedione, testosterone, 11-ketotestosterone as well as gonadotropin I and II were measured in maturing adult female spring chinook salmon (*Oncorhynchus tshawytscha*) between April and September while migrating in the Willamette River and later while being held in hatcheries. Ovaries were also collected and their state of maturity determined. Steroid profiles were related to sample date and stage of egg maturity.

Plasma testosterone concentrations remained unchanged during the spring and early summer. In mid-July testosterone concentrations began to climb and reached maximum levels by the time spawning took place in September. 11-ketotestosterone was found in low concentrations throughout maturation, demonstrating a slight but significant rise just prior to spawning. Androstendione and 17β-

estradiol concentrations were generally high throughout maturation, dropping significantly at the time of spawning. 17α, 20β-dihydroxy-4-pregnen-3-one was detected at very low concentrations throughout maturation, demonstrating a rapid and significant rise to high levels at the time of spawning. In 1989 the gonadotropins were detected at low levels throughout maturation. Gonadotropin I increased only slightly at the time of spawning, whereas gonadotropin II demonstrated a dramatic and highly significant rise at the time of spawning. Gonadotropin I concentrations were much higher during the 1990 season, reaching maximum levels late in the summer then dropping significantly at the time of spawning. The profile of gonadotropin II levels during 1990 was very similar to those recorded in 1989.

From April until the end of June, all oocytes had central germinal vesicles. In July germinal vesicles were migrating, and by the end of August germinal vesicles were peripheral. In early September oocytes began to show germinal vesicles breakdown and ovulation occured in mid-September.

Male spring chinook were sampled in 1990. Circulating 11-ketotestosterone concentrations were stable throughout the spring and summer, rising significantly to maximum levels shortly before spawning. Plasma testosterone concentrations fluctuated during April and May, stabilized in June, then started a steady and significant increase to maximum levels at spawning. Androstenedione concentrations showed no significant differences in mean values over

time, but the maximum individual levels were measured just before spawning. 17β-estradiol and 17α, 20β-dihydroxy-4-pregnen-3-one concentrations were very low throughout maturation. Gonadotropin I concentrations remained unchanged through most of maturation, rose to maximum levels late in the summer, then dropped significantly just before spawning. Gonadotropin II was present at low levels throughout maturation, increasing only just prior to spawning.

Cortisol, a steroid hormone, is a known immunosuppressive agent in fish, and sex steroid hormones, specifically testosterone and 17β-estradiol, are known to affect the mammalian immune response. To determine if the high concentrations of sex steroids detected in the plasma of maturing spring chinook have any effect on the function of the immune system, leukocytes from the anterior kidney of juvenile spring chinook salmon were incubated in the presence of steroid and their ability to form specific antibody producing cells was used as a measure of immunocompetence. Testosterone and cortisol, but not 17β-estradiol or aldosterone, were found to significantly reduce the plaque forming response *in vitro*. Testosterone and cortisol administered together had a significantly greater effect than did either when administered alone. Testosterone did not produce any immunosuppressive effects *in vivo*.

Sex Steroids, Gonadotropins, and Effects on the Immune Response in
Maturing Spring Chinook Salmon (*Oncorhynchus tshawytscha*)

by

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I. General Introduction

The future of many salmon stocks in the Pacific Northwest and elsewhere is in jeopardy. Helping these stocks recover will require a coordinated effort on many fronts. One area will certainly be the modulation of reproduction to boost population sizes. A number of hormones are known to control reproduction in fish (Donaldson, 1973; Hunter and Donaldson, 1983). The gonadotropins and the gonadal steroids are most often studied. Secretion of gonadotropin is known to be controlled by the hypothalamus through a gonadotropin-releasing hormone (GnRH). The active molecular form of GnRH in salmonid fish was discovered by King and Millar (1980) and later isolated and sequenced by Sherwood *et al.,* (1983). Gonadotropin can be detected in the plasma of maturing salmonids (Crim *et al.,* 1975; Crim and Idler, 1978) and has been shown to stimulate female maturation (Jalbert *et al.,* 1978; Sower and Schreck, 1982a) and steroidogenesis (Nagahama and Kagawa, 1982; Kanamori *et al.,* 1988). Recent studies have demonstrated two distinct forms of gonadotropin in salmon pituitaries (Suzuki *et al.,* 1988; Kawauchi *et al.,* 1986,1989) which are unique in both structure (Itoh *et al.,* 1988) and biological activity (Suzuki *et al.,* 1987,1988).

Gonads of teleosts are known to produce steroids (Arai and Tamaoki, 1967) and many of these steroids can be detected in the plasma (Idler *et al.*, 1971; Campbell *et al.*, 1980). The dynamics of sex steroid levels in the plasma during the progress of sexual maturation have been investigated in a number of pacific salmon species other than the chinook(*Oncorhynchus tshawytscha*) such as chum salmon, *O. Keta*: (Ueda *et al.*, 1984), sockeye salmon, *O. nerka* (Schmidt and Idler, 1962; Truscott *et al.*, 1986), coho salmon, *O. kisutch* (Sower and Schreck, 1982b; Fitzpatrick *et al.*, 1986), pink salmon, *O. gorbuscha* (Dye *et al.*, 1986), masu salmon, *O. masou* (Yamauchi *et al.*, 1984), amago salmon, *O. rhodurus* (Young *et al.*, 1983).

An important aspect of any successful hatchery program is the rearing of healthy fish. Researchers and managers have tried to develop strategies to keep fish free of disease, although disease-related losses of juveniles and brood stock are still a major concern. Lowered disease resistance in maturing salmon of some species can lead to high prespawning mortality. The process of sexual maturation itself may be partly responsible for lowered resistance to disease. Maturing salmonids have high plasma levels of many hormones including cortisol and the sex steroids (Schmit and Idler, 1962). High concentrations of cortisol reduce the immune response of salmonid fish (Kaattari and Tripp, 1987; Tripp *et al.*, 1987; Maule *et al.*, 1987, 1989; Pickering, 1989; Pickering and Pottinger, 1989).

In mammals the connection between gonadal steroids and immune function has been well documented. The four main areas of

research which have provided this evidence include: 1) studies of the sexual dimorphism in the immune response of males and females; 2) research involving gonadectomy and sex hormone replacement to alter immune response; 3) studies of the altered immune response during pregnancy; 4) studies demonstrating that the organs responsible for the immune response contain receptors for gonadal steroids (see reviews by: Grossman, 1984, 1985). Possible connections between gonadal steroids and immune function in fish have not been investigated.

This thesis addresses some of the areas discussed above and is presented in the form of chapters. The second chapter describes the endocrine events associated with the upstream migration and sexual maturation of female spring chinook salmon. Plasma levels of 17β-estradiol, 17α, 20β-dihydroxy-4-pregnen-3-one, androstenedione, testosterone, 11-ketotestosterone as well as gonadotropin I and II were measured. Ovaries were also collected whenever possible and their state of maturity determined. Steroid profiles were related to sample date and stage of egg maturity.

The third chapter details my investigation of the possibility that sex steroids affect the function of the salmonid immune system. Pronephric leukocytes of juvenile spring chinook salmon were incubated in the presence of steroid and their ability to form specific antibody producing cells was used as a measure of immuno-competence.

The fifth chapter describes the endocrine events associated with the upstream migration and sexual maturation of male spring chinook salmon. Plasma levels of 17β-estradiol, 17α, 20β-dihydroxy-4-pregnen-3-one, androstenedione, testosterone, 11-ketotestosterone as well as gonadotropin I and II were measured. Steroid profiles were related to sample date.

Chapter 6 describes *in vivo* investigations of the effects of testosterone on the salmonid immune response.

II. Plasma profiles of the sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*).[1]

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II. Plasma profiles of the sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*)

INTRODUCTION

This study describes the endocrine events associated with the upstream migration and sexual maturation of female Willamette River spring chinook salmon (*O. tshawytscha*). The spring chinook is interesting because, unlike many species of salmon studied by other investigators, it enters freshwater 6 to 9 months before spawning in a state of relative sexual immaturity. This provides opportunity for observation of a large portion of the process of sexual maturation.

Oregon's Willamette River is fed by melting snow on the western slopes of the Cascade mountains and rains on the eastern slopes of the coast range. The Willamette joins the Columbia River some 165 kilometers from the Pacific ocean at the city of Portland, OR. Salmon returning to the Willamette to spawn enter the Columbia River estuary as early as February. Spring chinook mass in the lower Willamette and by mid-April begin to pass the Willamette Falls, a natural falls at river kilometer (RKM) 43 (208 km from ocean), now impassable except by fish ladder. The migration continues through mid-June with an average run size (1989-90) of 68,000 fish (Oregon Department of Fisheries and Wildlife, unpublished data). The upstream extent of the migration is now limited by the Dexter dam (RKM 329: 494 km from

ocean) where fish are removed from the river and held until spawning at Willamette Hatchery in Oakridge, OR. Spawning normally occurs in September.

A number of hormones are known to control reproduction in fish (Donaldson, 1973; Hunter and Donaldson, 1983). The gonadotropins and the gonadal steroids are most often studied. The dynamics of sex steroid levels in the plasma during the progress of sexual maturation have been investigated in a number of pacific salmon species other than the chinook salmon such as chum salmon, *O. Keta* (Ueda *et al.*, 1984), sockeye salmon, *O. nerka* (Schmidt and Idler, 1962; Truscott *et al.*, 1986), coho salmon, *O. kisutch* (Sower and Schreck, 1982b; Fitzpatrick *et al.*, 1986), pink salmon, *O. gorbuscha* (Dye *et al.*, 1986), masu salmon, *O. masou* (Yamauchi *et al.*, 1984), amago salmon, *O. rhodurus* (Young *et al.*, 1983).

In order to investigate the endocrine control of reproduction in female spring chinook we measured the plasma levels of 17β-estradiol, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), androstenedione, testosterone, 11-ketotestosterone as well as gonadotropin I and II. Samples were collected at various sites along the migratory route throughout maturation. Steroid profiles were related to sample date and stage of egg maturity. In 1990 samples were taken from actively migrating fish at the Willamette Falls during the start, middle, and end of the run to determine if sample date or sample site was a more important factor in determining plasma steroid concentrations.

METHODS AND MATERIALS

Blood samples were collected from adult female spring chinook salmon at sites along their spawning migration in the Willamette River and during final maturation when fish were held at Smith Farm Experimental Hatchery, Oregon State University, Corvallis, Oregon. Blood was drawn by lithium heparinized vacutainer via puncture of the caudal vein. Samples were centrifuged, the plasma was drawn off and frozen on dry ice, then stored at -80° C until assayed. When possible, a sample of ovarian tissue was also collected and preserved in a clearing solution (Trant and Thomas, 1988). The tissue was later examined to determine maturity of oocytes. The stages of oocyte maturation used to describe these samples are the same used by Sower and Schreck (1982a) and Fitzpatrick *et al.*, (1986): stage one (I) premigratory or central germinal vesicle (CGV), stage two (II) migrating germinal vesicle (MGV), stage three (III) peripheral germinal vesicle (PGV), stage four (IV) germinal vesicle breakdown and coalescence of lipid drops (GVBD), and stage five (V) ovulation (O).

Sampling Sites. 1989 sampling began on 3 May when blood was collected from angler-captured fish at RKM 38 (203 km from ocean), below the Willamette Falls in Oregon City. By this date the fish may have already been in fresh water for as long as 3 months. Samples were next obtained from actively migrating fish at the fish passage facilities at the Willamette Falls (RKM 43) on 16 May. Fish were next

sampled at the upstream end of their spawning migration at the Dexter holding ponds (RKM 329) on 8 June and 11 July. On the latter date, fifteen fish (4 males and 11 females) were transported to Corvallis where the fish were held at constant temperature ($12 \pm 1^{0}C$) in a 10 m x 5 m above-ground tank (approximate depth 1.5 m). Blood samples were taken every other week through 23 August when all fish died due to a fungal infection of the gills. On 22 August, samples were taken from fish at Dexter in order to compare fish being held in Corvallis to fish which had spent the summer in the river. The death of all fish before spawning forced a return to the Dexter holding ponds on 7 September when samples were taken from two distinct populations of fish: those being strip-spawned on that date, and those judged to be several weeks from ovulation.

The 1990 sampling season began on 18 April with fish captured by gillnet as they began their spawning migration in the Columbia River estuary (RKM 40). This site is 163 km downstream of the first sample site used in 1989. Samples were again obtained at Oregon City from angler-captured fish in early May and from the Willamette Falls fish passage facility on 20 April, 23 May, and 25 June. These sample dates represented the start, peak, and end of the migration over the falls. Fish at the Dexter holding ponds were sampled on 15 and 29 June, 10 July, and 16 August. In 1990, the transfer of fish (7 males and 18 females) to Corvallis took place on 3 July. Fish were held in a 3.3 m x 1.3 m circular fiberglass tank on an outdoor concrete pad and supplied with water at a constant temp ($12 \pm 1^{0}C$). Fish were

then sampled every other week through 10 September. On 11 and 20 September samples were taken from another group of spring chinook being held at Smith Farm.

Salmon removed from the fish ladder at Willamette Falls and those being held in Corvallis were anesthetized using MS222 (50 mg/l) buffered with $NaCO_3$ (100 mg/l) before sampling. Fish bled at Dexter were given CO_2 before sampling.

Assays. Plasma levels of testosterone, 11-ketotestosterone, 17β-estradiol, and 17α,20β-dihydroxy-4-pregnen-3-one were determined by RIA following the procedure of Sower and Schreck (1982b), modified by Fitzpatrick et al. (1986). Androstenedione was assayed following the procedure of Schreck et al. (1989). Assays of gonadotropin I and II were performed using the method of Swanson et al. 1991.

Statistics. All plasma hormone data were first subjected to a nonparametric analysis of variance (Kruskill-Wallace) then comparisons between individual dates were completed using a nonparametric comparison of means test (Mann-Whitney U-test). A significance level of $P<0.05$ was used in all tests.

RESULTS

11-ketotestosterone was detected in the plasma of female spring chinook salmon at low levels (<10ng/ml) throughout the spring and summer, with the exception of a high initial measurement in 1989. Plasma levels of 11-ketotestosterone rose significantly in September (10-20 ng/ml) just prior to ovulation, then decreased, though not significantly, at ovulation (fig. 1a).

Testosterone was present in the plasma at 10-20 ng/ml during the spring and early summer. In mid-July, testosterone concentrations began to climb and reached maximum levels (100 ng/ml) prior to ovulation in September (fig. 1b).

Androstenedione concentrations fluctuated throughout maturation, but tended to follow the same pattern in each of the sample years (fig. 1c), although maximum levels in 1990 were nearly twice those in 1989. During April and May, levels varied greatly, but were generally high (20-40 ng/ml). In June and early July, the concentrations stabilized and then began to rise to maximum levels in late August, dropping significantly at ovulation in September.

Circulating levels of 17β-estradiol followed a pattern very similar to that of androstenedione, demonstrating highly variable values during spring and early summer, then sustaining high levels (20 ng/ml) until a few weeks before ovulation, starting a precipitous and

highly significant drop to only 0.5 ng/ml at the time of ovulation (fig. 2a).

Figure 1. Plasma concentrations (ng/ml) of 11-keto-testosterone, testosterone, and androstenedione during the upstream migration and sexual maturation of female spring chinook salmon. Each value represents the mean ($\pm$ standard error) of samples collected on that date. Plasma steroid levels are related to date and maturational stage (I central germinal vesicle; II migrating germinal vesicle; III peripheral germinal vesicle; IV germinal vesicle breakdown; V ovulation) of the oocytes.

Figure 1

Figure 2.   Plasma concentrations (ng/ml) of 17β-estradiol, 17α-
20β-dihydroxy-4-pregnen-3-one, gonadotropin I and
gonadotropin II during the upstream migration and
sexual maturation of female spring chinook salmon. Each
value represents the mean ($\pm$ standard error) of samples
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vesicle;   II migrating germinal vesicle;   III peripheral
germinal vesicle;   IV germinal vesicle breakdown;   V
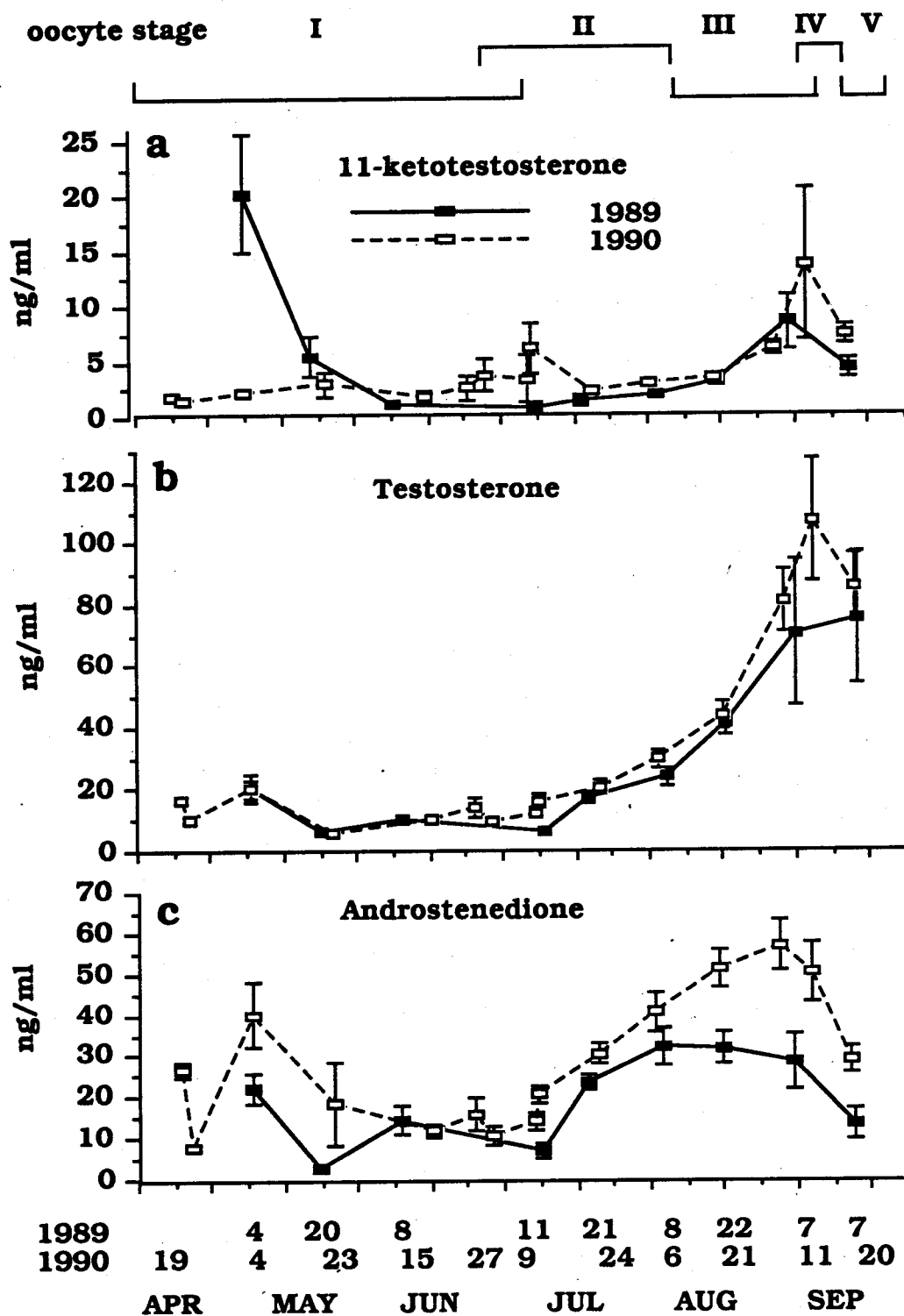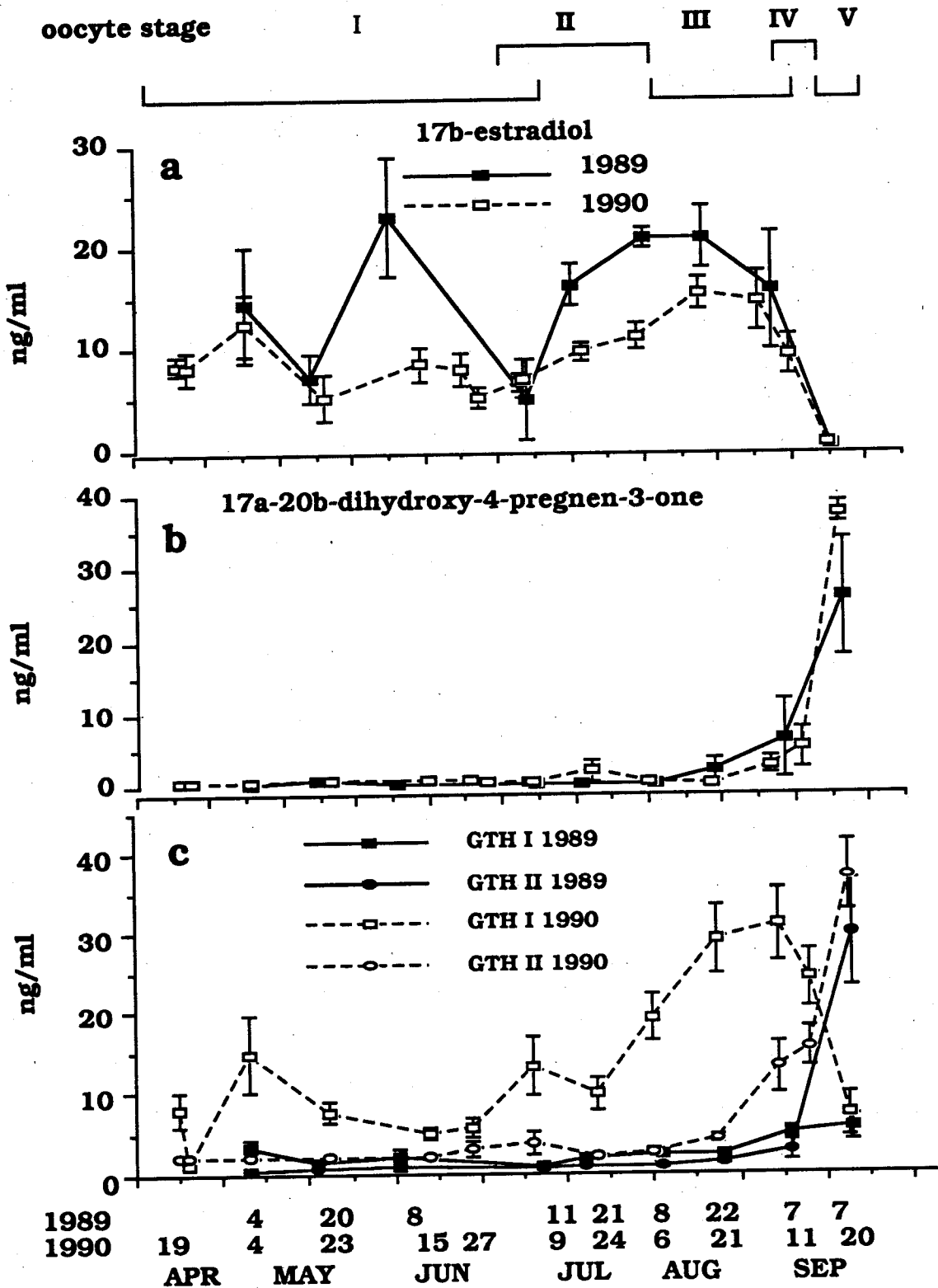ovulation) of the oocytes.

Figure 2
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Figure 2

DHP was detected at <1 ng/ml throughout the spring and summer (fig. 2b). Plasma levels started to increase in late August, ending with a very dramatic and significant spike (25-35 ng/ml) at the time of ovulation.

During 1989 circulating levels of gonadotropin I were low (<5 ng/ml) on all sampling dates, and demonstrated only a slight increase in ovulated fish on the last sample date (fig. 2c). During the 1990 season gonadotropin I was detected at much higher concentrations which reached maximum levels late in the summer and then declined significantly at spawning. Its plasma profile mirrors almost exactly those of androstenedione and 17β-estradiol. gonadotropin II was detected at very low levels (1 ng/ml) during most maturation in both years, demonstrating a dramatic and highly significant increase in ovulating fish (fig. 2c).

Fish sampled on 22 August 1989 in Corvallis, and on 23 August 1989 at Dexter in order to compare the development of fish held in Corvallis under constant water temperature with fish which spent the summer in the river (temperature 10-25° C) did not differ in their plasma levels of all hormones (p< 0.05 ANOVA).

From April until the end of June, all oocytes had central germinal vesicles. In July germinal vesicles were migrating, and by the end of August germinal vesicles were peripheral. In early September oocytes began to show germinal vesicles breakdown and ovulation occured in mid-September (table 1).

Table 1

MATURIONAL STAGE OF OOCYTES SAMPLED DURING 1990

date	n (number of fish)	oocyte maturation stage
18 April	4	CGV (I)
15 June	4	CGV (I)
29 June	5	CGV (I)/MGV (II)
7 July	4	MGV (II)/CGV (I)
25 July	1	MGV (II)
2 August	2	MGV (II)
9 August	1	PGV (III)
27 August	1	PGV (III)
6 September	2	GVBD (IV)
10 September	6	GVBD (IV)/PGV (III)
20 September	6	OV (V)

DISCUSSION

The unique life history of the spring chinook allowed us to document the endocrine events associated with sexual maturation for 6 months, over a 494 km spawning migration. The data present a more complete profile of the process of sexual maturation in an anadromous salmonid than do similar studies which investigated salmon returning to fresh water in the fall, only 1 or 2 months before spawning. This study documented the dynamics of all of the steroid hormones known to be important in the process of sexual maturation and for the first time in the chinook, the dynamics of the recently distinguished gonadotropin I and II were investigated.

Willamette River spring chinook entering fresh water in early April have elevated 17β-estradiol and oocytes at stage I. This suggests that these fish are already in the period of exogenous vitellogenesis (Sower and Schreck, 1982a; Fitzpatrick et al., 1986). During the period of exogenous vitellogenesis, high levels of 17β-estradiol stimulate the liver to produce vitellogenin, the yolk protein which is absorbed from the blood by the developing oocytes (Crim and Idler, 1978; van Bohemen and Lambert, 1981; van Bohemen et al., 1981). Plasma levels of 17b-estradiol have been correlated with vitellogenin production in a number of salmonid species (Bromage et al. 1982; Whitehead et al. 1983; Udea et al., 1984). Results of the present study show that production of 17β-estradiol, and presumably vitellogenin,

reach a maximum about a month before ovulation. This agrees with the findings of other studies of salmon (Udea *et al.*, 1984; Truscott *et al.*, 1986; Dye *et al.*, 1986; Fitzpatrick *et al.*, 1986), although none of these studies sampled fish more than 2 months prior to ovulation, and consequentially most only detected the decline in 17β-estradiol concentrations detected during final maturation.

Chinook appear to conform to the presently held model of salmonid final maturation in which the surge of DHP detected at ovulation plays a pivotal role. As exogenous vitellogenesis ends, oocytes move into stage IV, and both 17α-progesterone and DHP are present in the plasma and attain high levels by the time of ovulation (stage V). 17α-progesterone rises more gradually than the sharp increase seen in DHP (Sower and Schreck, 1982b; Young *et al.*, 1983; Ueda *et al.*, 1984; Dye *et al.*, 1986; Truscott *et al.*, 1986; Fitzpatrick *et al.*, 1986). DHP has been proven to trigger final maturation *in vitro* in a large number of teleosts (see review by Scott and Canario, 1987) and has been determined to be the maturation-inducing substance in salmonids, which acts through a major cytoplasmic mediator, or maturation-promoting factor, to induce final meiotic maturation (Nagahama and Yamashita, 1989).

The profiles of gonadotropin I and II obtained in our chinook salmon help to confirm recent theories regarding their role in development and sexual maturation. The two gonadotropins are prominent at different points in the life cycle: gonadotropin I is the only gonadotropin detectable in juvenile coho salmon (Swanson *et al.*

1989; Nozaki *et al.* 1990), but in mature adults gonadotropin II is predominate (Swanson, unpublished). The biological activity of the two gonadotropins also differs. gonadotropin I and II have equal activity in stimulating 17β-estradiol production, however gonadotropin II is more potent in stimulating maturational steroid synthesis (DHP) and at high doses significantly inhibits 17β-estradiol production in post-vitellogenic follicles (Suzuki *et al.*, 1987,1988). In light of this new evidence it seems that the high levels of 17β-estradiol detected during exogenous vitellogenesis are stimulated by gonadotropin I (and perhaps low levels of gonadotropin II) and the ovulatory surge of gonadotropin detected at the end of exogenous vitellogenesis is gonadotropin II, which stimulates the surge of maturational steroid synthesis (DHP) while also causing the detected drop in 17β-estradiol.

Both testosterone and 11-ketotestosterone are present in the plasma of female chinook, but testosterone predominates. It rises 10 times higher than 11-ketotestosterone, and to more than twice the levels seen in males. 11-ketotestosterone remains at low levels but generally increases as maturation continues. These data conform with other studies of maturing salmon (Ueda *et al.*, 1984; Dye *et al.*, 1986; Truscott *et al.*, 1986; Fitzpatrick *et al.*, 1986). Androgens may play many roles in maturing salmonids. Schmidt and Idler (1962) suggested an osmoregulatory role when they reported changes in the ratios of testosterone to 11-ketotestosterone when sockeye salmon migrated from sea water to fresh water. Idler *et al.* (1961) demonstrated that 11-ketotestosterone increased skin coloration and

thickness in sockeye salmon, and Sower *et al.* (1983) found that 17α-methyl-testosterone increased skin thickness in rainbow trout. testosterone may affect final maturation. It has been shown to stimulate germinal vesicle breakdown *in vitro* in amago salmon (Young *et al.*, 1982). Androgens may also serve as a reservoir of material for conversion to other steroids. An *in vitro* study by Kagawa *et al.* (1982) reported that testosterone and/or androstenedione produced in the thecal cell layer of the oocyte is then converted to 17β-estradiol by the aromatase enzyme system of the granulosa cell layer. The results of the present study suggest that androstenedione could be a precursor to 17β-estradiol in chinook. During both 1989 and 1990, the profile of plasma androstenedione mirrored that of 17β-estradiol almost exactly. Androstenedione was generally found in higher concentration, though not significantly higher until late July.

In general, time (sample date) was a more important variable than was distance upstream or holding temperature in determining plasma hormone concentrations. Some interesting site specific trends are apparent. In 1989, the 20 May and 11 July levels of androstenedione, testosterone and 17β-estradiol were significantly lower than sample dates before and after, and in 1990 the 20 April, 29 June, and 10 July testosterone levels were all significantly lower than surrounding dates. All of these samples are from fish which were removed from fish traps; 20 April 1990, and 20 May 1989 represent fish removed from the fish ladder at Willamette Falls; and 29 June and 10 July 1990, and 11 July 1989 represent fish removed from the fish

ladder at Dexter. Pickering *et al.* (1987) found that mature male brown trout subjected to a 1 hour handling and confinement stress had a significant decrease in plasma testosterone and 11-ketotestosterone levels. Pickering (1989) showed that a cortisol implant significantly reduced plasma 17β-estradiol, testosterone and gonadotropin in brown trout. The low plasma levels of steroids in fish removed from fish ladders in this study could be stress induced. The fish sampled at Dexter do experience some handling stress as they are confined to one end of a raceway and then given CO_2 before sampling. The fish sampled at Willamette Falls however, are not subjected to any stress other than that presented by passage through the fishway itself. This fish ladder effect is not apparent later in maturation (August-September) when all fish start exhibiting similar hormone profiles.

This study represents a comprehensive investigation of the sexual maturation of an anadromous salmonid. The unique life history of the spring chinook allowed us to document the endocrine events associated with sexual maturation for 6 months, over a 494 km spawning migration. This large timescale illustrated that the much studied period of final maturation is the final outcome of a continum of endocrine events which begins many months before ovulation and spawning.

III. Testosterone Alters the Immune Response of Chinook Salmon
(*Oncorhynchus tshawytscha*)[1]

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III. Testosterone Alters the Immune Response of Chinook Salmon

(*Oncorhynchus tshawytscha*).

INTRODUCTION

Sexually mature salmon demonstrate immune deficiencies, such as mature sockeye which are unable to produce isohemaggultinins, antibodies readily produced in immature fish (Ridgway, 1960, 1962). Disease related mortality of maturing salmon broodstock is a serious problem. Sexually maturing salmonids have high plasma levels of many hormones including cortisol and the sex steroids (Schmit and Idler, 1962). Cortisol is known to reduce the production of specific antibodies and disease resistance of salmonid fish (Kaattari and Tripp, 1987; Tripp *et al.*, 1987; Maule *et al.*, 1987,1989; Pickering, 1989; Pickering and Pottinger, 1989), but the possibility that other hormones associated with maturation in salmon, specifically the sex steroids, may also be affecting immune function has not been studied.

In mammals, the connection between gonadal steroids and immune function has been well documented. The four main areas of research which have provided this evidence include: 1) studies of the sexual dimorphism in the immune response; 2) research involving gonadectomy and sex hormone replacement to alter immune response; 3) studies of the altered immune response during pregnancy; 4) studies demonstrating that the organs responsible for

the immune response contain receptors for gonadal steroids (see reviews by: Grossman 1984, 1985).

Estrogen enhances the action of splenic macrophages in the removal of antibody-coated cells (Schreiber *et al.,* 1988) but has been shown to inhibit other cellular responses, specifically, inhibiting natural killer cells (Seaman *et al.,* 1978; Hanna and Schneider, 1983; Pung and Luster, 1986). Conversely, estrogen also stimulates the mammalian humoral, or antibody response (Paavonen *et al.,* 1981; Trawick and Bahr, 1986; Erbach and Bahr, 1988; Sthoeger *et al.,*1988). Estrogen also increases the mammalian immune response by enhancing interleukin 1 synthesis (Hu *et al.,* 1988; Polan *et al.,* 1988). Progesterone has been shown to inhibit splenic macrophage activity and lymphocyte transformation (Schreiber *et al.,* 1988; Wyle and Kent, 1977) while testosterone inhibits the mammalian antibody response (Fujii *et al.,* 1975; Sthoeger *et al.,* 1988), but its effect is dependent on the antigen used (Rife *et al.,* 1990). Testosterone also interferes with lymphocyte transformation (Wyle and Kent, 1977). Possible connections between gonadal steroids and immune function in fish have not been investigated.

Earlier work in our laboratory has demonstrated that adult spring chinook salmon (*Oncorhynchus tshawytscha*) elaborate high concentrations of sex steroid hormones in the plasma during sexual maturation (Slater *et al.,* in press). Prespawning mortality can be a serious problem when working with spring chinook salmon. Spring chinook enter fresh water 6 to 9 months before spawning and spend

the summer months in very warm river water or confined in hatchery raceways where disease can spread easily. This study set out to determine if the concentrations of sex steroids detected in the plasma of maturing spring chinook have any effect on the function of the immune system. In order to avoid the confounding effects of already high concentrations of cortisol and sex steroid hormones found in sexually maturing adults, and to isolate experimentally the effects of different hormones, juvenile chinook salmon, known to have low concentrations of sex steroids in their plasma (Patino and Schreck, 1986), were investigated. Testosterone and 17β-estradiol were chosen for testing because they both are present in the plasma of maturing salmon at high concentrations during the summer months and because of their known roles as modulators of the mammalian immune response.

The antibody production by salmonid leukocytes is known to change seasonally, particullary in the spring during the process of smoltification (Maule *et al.* 1987). We also had some evidence that antibody production changed in the winter as well (Schreck and Malue unpublished). These changes are documented in this study as experiments started in the fall of 1990 and continued until the spring of 1991.

GENERAL METHODS

Juvenile spring chinook salmon (60 ± 5g) were obtained from Anadromous, Inc., Corvallis, Oregon (Rogue River stock, 1989 brood year) and held at Smith Farm Experimental Hatchery, Oregon State University, Corvallis, Oregon. Fish were maintained in 2 m circular, flow through tanks, at 12 ± 1°C, under natural photoperiod, and fed a commercial diet of semi-moist pellet (Bio-moist Co. Warington, OR). Fish were held for at least 30 days before any experiments were started.

The ability of leukocytes to form specific antibody producing cells was used as a measure of immunocompetence. The antigen used was TNP-LPS. The procedure followed was the hemolytic plaque assay described by Tripp *et al.* (1987) with the following modifications: 50 µl of leukocyte cell suspension and 50 µl tissue culture media (TCM) were added to wells of 96-well, flat bottom microculture plates. Cells were incubated at 18°C for 7 days in the presence of antigen or antigen and steroid. Cell cultures were not fed during incubation. On day 7 the tissue culture plates were centrifuged to remove cells from suspension. TCM was drawn off and 50 µl fresh TCM (no antigen or steroid) was added to wells. 10 µl of dilute steelhead complement and 10 µl of sheep red blood cells haptenated with TNP were then added to all wells. Using a Pasteur pipet, the contents of each well were gently mixed and transfered to one half of a Cunningham chamber.

The chambers were sealed with wax and incubated at 18°C for 2 hours. Chambers were then examined under a stereo dissecting microscope and number of plaques per culture recorded. All experiments were performed on leukocytes removed from the anterior kidney.

Aliquots of leukocyte suspension from individual fish were incubated in TCM containing antigen and steroid (treatment) or antigen and no steroid (control). Each control or treatment was replicated. After a seven day incubation, the number of plaque forming cells (PFCs) per treatment culture was compared to the number of PFCs in control cultures and expressed as percent of control, as a measure of the effect of the steroid on immunocompetence. All experiments included cultures containing no antigen, as negative controls, and cultures incubated with antigen and cortisol, a known immunosuppressive steroid, as a positive control. Concentrations of sex steroids which are within physiological limits of adult salmon were tested, testosterone at 10 and 100 ng/ml and 17β-estradiol at 5 and 50 ng/ml. Aldosterone, a steroid similar to cortisol, but not naturally present in teleosts and shown not to be immunosuppressive (Tripp *et al.*, 1987), was also employed as a negative control at concentrations of 10 and 100 ng/ml in several experiments.

Statistics: All data were subjected to the Freidman's nonparametric analysis of variance and, where significant between-group differences were found, pairwise comparisons were conducted using Dunnett's test as described in Zar (1984) at the $P<0.05$ level.

Data is presented as percent of control, while the statistical analyses were performed on the raw data.

EXPERIMENTAL DESIGN AND RESULTS

The first experiment was run on 10-21 September 1990. Sixteen fish were used (n=16). Aliquots of pronepheric leukocyte suspension from each fish were incubated separately with testosterone, 17β-estradiol, and cortisol. Testosterone and cortisol at 100 ng/ml significantly lowered the number of plaque forming cells to 42% and 31% of control, respectively, while all other treatments had no significant effect (fig. 3).

The second experiment was started on 24 September and involved 5 fish (n=5). Aliquots of pronepheric leukocyte suspension from each fish were exposed to combinations of testosterone and cortisol. The combined dose of 100 ng/ml of each testosterone and cortisol was much more effective than either alone, suppressing plaque formation by 82% relative to controls (fig. 4a).

On 1 and 8 November, aliquots of pronepheric leukocyte suspension from 10 fish (n=10), five fish on each date, were incubated with a number of different doses of testosterone in order to gauge the range of the immunosuppressive response. Cortisol at 100 ng/ml and testosterone at 500 and 1000 ng/ml all had significant, but equal, effects on the production of PFCs, reducing PFCs to between 35% and 55% of control (fig. 4b).

Figure 3.   Numbers of plaque forming cells produced by leukocytes of chinook salmon incubated with steroid (T=testosterone; E2=17β-estradiol; C=cortisol). Bars represent means (± standard error) of plaque-forming cells expressed as percent of controls. Bars marked "*" are significantly different from controls (p<0.05).

Figure 3
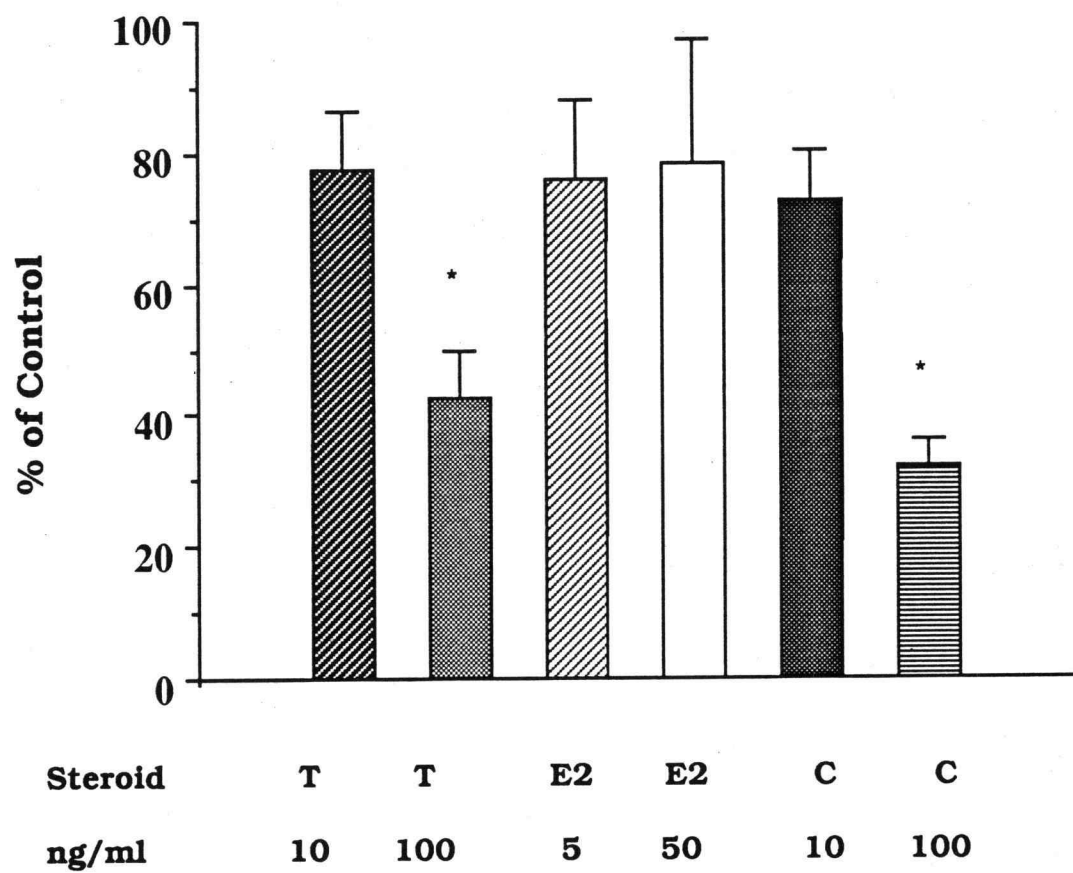
Figure 4.   Numbers of plaque forming cells produced by leukocytes of chinook salmon incubated with steroid (T=testosterone; C=cortisol). Bars represent means ($\pm$ standard errors) of plaque-forming cells expressed as percent of controls. Bars marked "*" are significantly different from controls ($p<0.05$). a. Plaque forming response of leukocytes incubated with combinations of T and C. b. Plaque forming response of leukocytes incubated with increasing doses of T.

Figure 4
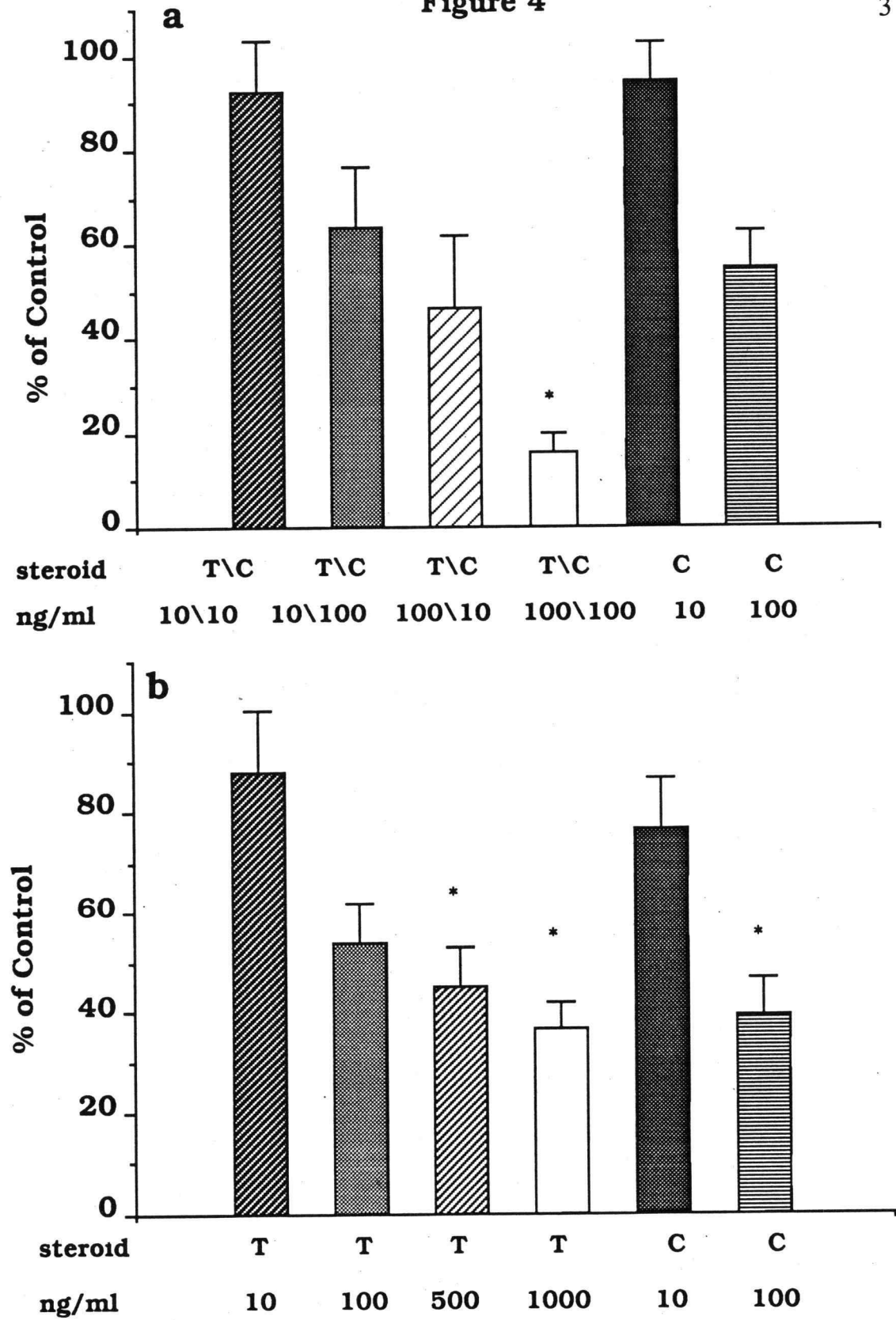
On four successive days between 14 and 17 November aliquots of pronepheric leukocyte suspension from 4 fish (new fish on each day) (n=16) were incubated in the presence of testosterone, aldosterone, and cortisol (separately). Only cortisol at 100 ng/ml significantly affected the plaque forming response, and it only reduced numbers of PFCs by 30%. (fig. 5a). This experiment was repeated in January and February 1991 with similar results (not shown). In March 1991, the experiment was repeated again and aliquots of pronepheric leukocyte suspension from 10 fish (n=10) were incubated in the presence of testosterone, aldosterone, and cortisol (separately). This time both testosterone and cortisol at 100 ng/ml again demonstrated significant immunosuppressive effects, reducing the plaque forming response by 45% and 60% (fig. 5b). Aldosterone showed no significant effects.

Figure 5.   Numbers of plaque forming cells produced by leukocytes of
            chinook salmon incubated with steroid (T=testosterone;
            A=aldosterone; C=cortisol). Bars represent means ($\pm$
            standard errors) of plaque-forming cells expressed as
            percent of controls. Bars marked "*" are significantly
            different from controls (p<0.05).

Figure 5
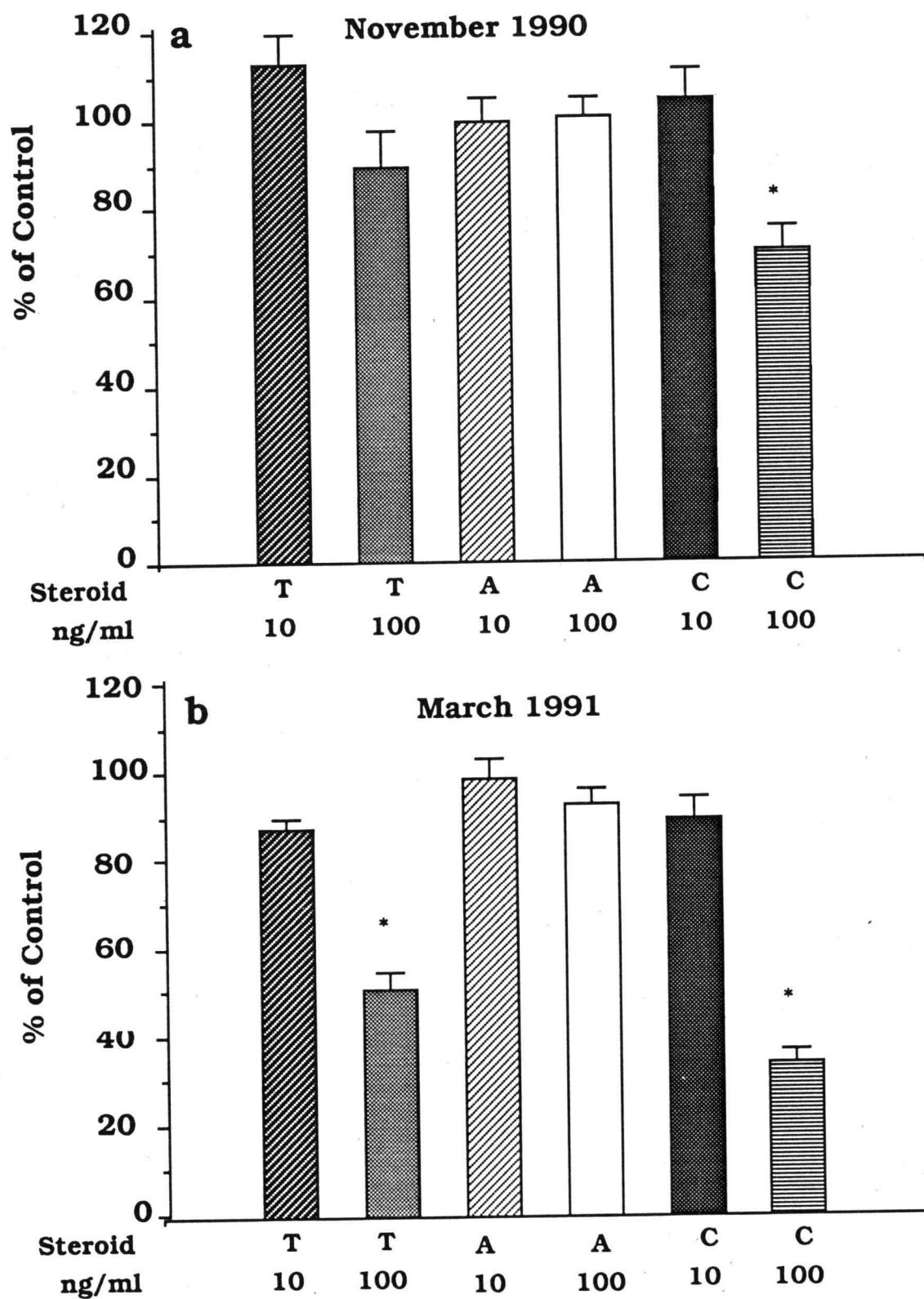
DISCUSSION

The results of this study demonstrate a significant immuno-suppressive effect of testosterone *in vitro*. The immunosuppressive effect was comparable to that of an equal dose of cortisol, but the effect of testosterone and cortisol together was greater than that of either alone. Pearce *et al.* (1981) found that androgen receptors are confined to thymocytes which are relatively resistant to glucocorticoids in mouse and rat thymus glands, suggesting that androgens and glucocorticoids may affect separate cell subpopulations. This could account for the additive immunosuppressive effect of testosterone and cortisol. While we were unable to demonstrate an effect of 17β–estradiol on the production of plaque forming cells, this estrogen is known to increase antibody production in mammals (Paavonen *et al.*, 1981). Gonadal steroids affect interleukin synthesis in mammals (Hu *et al.*, 1988; Polan *et al.*, 1988) while in fish cortisol suppresses the number of antibody producing cells in salmon by inhibiting the release of an interleukin-like immune-factor (Kaattari and Tripp 1987; Tripp *et al.* 1987). In higher vertebrates testosterone is thought to be immunosuppressive by increasing the activity of suppressor T cells. This theory of testosterone action on T cells has been strengthened by recent studies; Rife *et al.* (1990) demonstrated immunosuppressive effects of testosterone when a T cell-dependent antigen was used, but not with a T cell-independent

antigen. The present study indicates that this is not the case in fish. TNP-LPS is a T cell-independent antigen, but immunosuppression was still demonstrated.

Testosterone does not seem to affect mature T cells directly. Rife *et al.* (1990) found no testosterone receptors in lymphocytes from mouse spleen, and Cohen *et al.* (1983) could find no testosterone receptors in human peripheral T cells. Testosterone must then affect T cells indirectly, through a second messenger or during development through the known androgen receptors in the thymus.

The loss of the immunosuppressive effects of cortisol and testosterone during the winter (late November through February) is not understood. Similar results have been obtained with head kidney, splenic, and peripheral leukocytes from coho salmon (Schreck, unpublished). Maule *et al.* (1987) found changes in the immune response of coho salmon during the time of smoltification. This change was attributed to a natural increase in plasma cortisol usually associated with the process of smoltification itself, and as such were seen as a developmental, rather than seasonal effects. Results of the present study point to a seasonal effect on immune response. In a single population of fish, immunosuppressive effects of steroids which were lost during the winter, returned in the spring. The antibody response of teleosts is known to be temperature dependent (Avtalion, 1969), but in the present study all fish were held at constant (12 ± 1° C) temperature, and the antibody response itself was not affected, just the immunosuppressive effects of testosterone and cortisol. This

seasonal variation in the salmonid immune response must be controlled by some factor(s) other than temperature, such as photoperiod.

The results of this study demonstrate a significant immuno-suppressive effect of testosterone *in vitro*, in the chinook salmon. Although more investigation will be required to determine the mechanism for testosterone's action, it is evident that modulation of the immune system by sex steroids in adult salmon may play a role in the reduced disease resistance seen in many maturing populations during the spawning season.

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APPENDICES

V. Appendix I:

Profiles of the Sex Steroids and Gonadotropins in the Plasma of Male

Spring Chinook Salmon (*Oncorhynchus tshawytscha*).

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V. Appendix I:

Profiles of the Sex Steroids and Gonadotropins in the Plasma of Male Spring Chinook Salmon (*Oncorhynchus tshawytscha*).

INTRODUCTION

Plasma concentrations of 17β-estradiol, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), androstenedione, testosterone, 11-ketotestosterone as well as gonadotropin I and II were measured in maturing adult male spring chinook salmon (*Oncorhynchus tshawytscha*) during 1990. Samples were taken during the study of female salmon described in chapter II. Results from male fish were not included in chapter II because males were only sampled during one season and sample sizes were small.

METHODS

Male salmon were sampled along with the females used in chapter II, therefore all methods, sample sites, and dates are the same as those detailed in chapter II under the 1990 sampling season.

RESULTS

Circulating 11-ketotestosterone concentrations were fairly stable (20-30 ng/ml) throughout the spring and summer rising significantly to maximum levels (70 ng/ml) late in August (fig. 6a). Plasma testosterone concentrations fluctuated between 10 and 30 ng/ml during April and May. Testosterone concentrations stabilized at around 15 ng/ml in June, then started a steady and significant increase to maximum levels of 60 ng/ml just prior to spawning in September (fig. 6b). Androstenedione concentrations were highly variable during the maturational process. There were no significant differences in mean values over time, but the maximum individual levels were measured just before spawning on 11 September.

17β-estradiol and DHP remained at very low concentrations (3 ng/ml) throughout maturation (fig. 7a).

Gonadotropin I concentrations remained unchanged through most of maturation, rose to maximum levels (25 ng/ml) late in the summer, then dropped significantly just before spawning. Gonadotropin II was present at low levels throughout maturation, increasing only just prior to spawning (fig. 7c).

Figure 6.   Plasma concentrations (ng/ml) of 11-keto-
testosterone, testosterone, and androstenedione
during the upstream migration and sexual maturation of
male spring chinook salmon. Each value represents the
mean and standard error of samples collected on that
date.

Figure 6

a 11-ketotestosterone

b Testosterone

c Androstenedione

1990   19   4   23   27   9 24   6   21   11   20

APR   MAY   JUN   JUL   AUG   SEP

Figure 7.   Plasma concentrations (ng/ml) of 17β-estradiol, 17α-20β-
             dihydroxy-4-pregnen-3-one, gonadotropin I and
             gonadotropin II during the upstream migration and
             sexual maturation of female spring chinook salmon. Each
             value represents the mean and standard error of samples
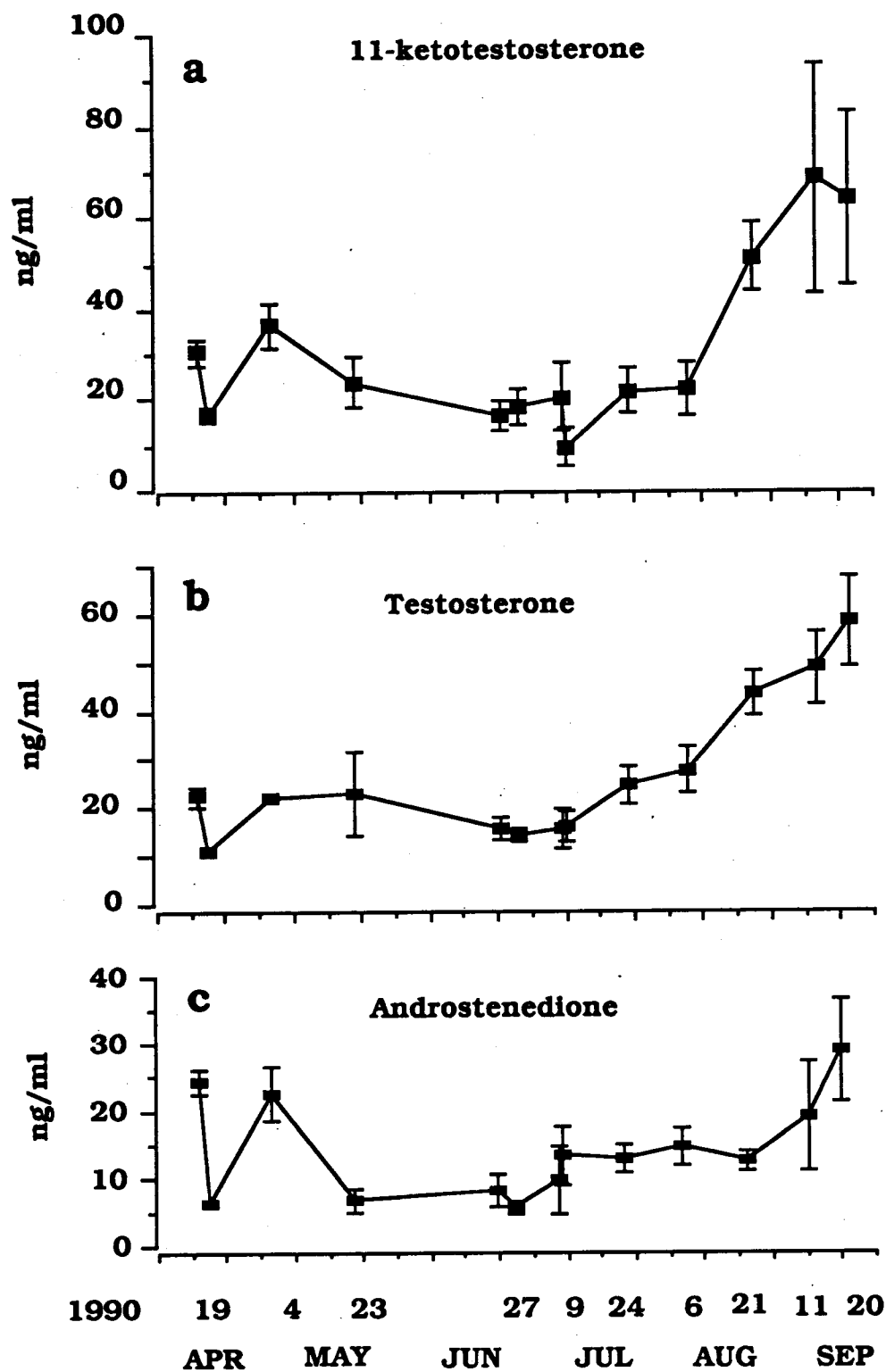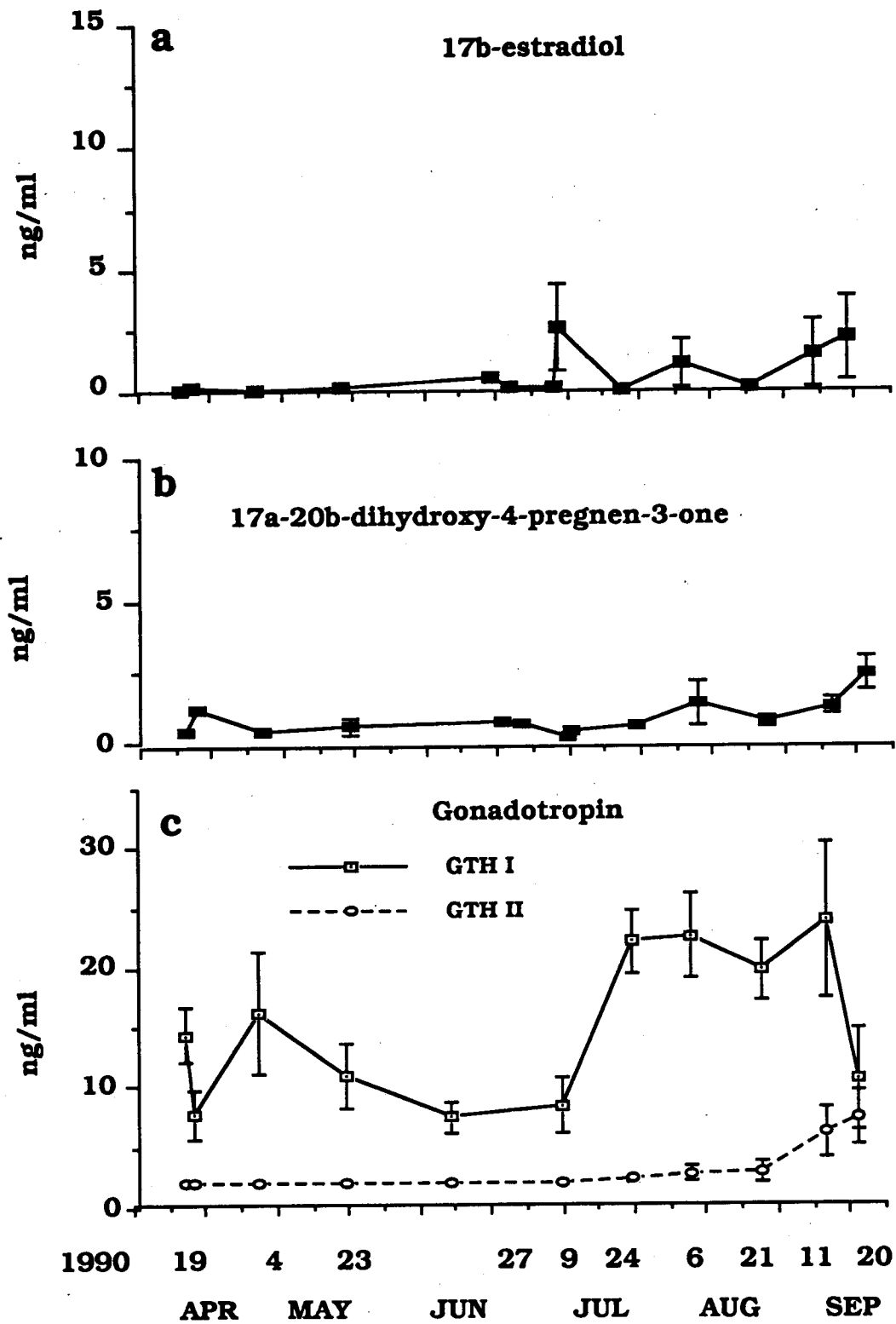   collected on that date.

Figure 7

DISCUSSION

The major steroid in the plasma of male chinook, as in other male salmon, is 11-ketotestosterone (Idler *et al.*, 1971; Truscott *et al.*, 1986; Dye *et al.*, 1986; Fitzpatrick *et al.*, 1986). It was present at higher concentrations than testosterone, as much as twice as high in some individuals, but due to great individual variability these differences were often not significant. The plasma profiles of testosterone and 11-ketotestosterone in the males were generally the same as in the females except that concentrations of 11-ketotestosterone were as much as five times as high, and testosterone only half, those recorded in females. Idler *et al.* (1961) found that 11-ketotestosterone affected secondary sexual characteristics: snout length, skin coloration, and skin thickness in sockeye salmon. Both testosterone and 11-ketotestosterone have been indicated as necessary to the process of spermatogenesis but not spermiation. Baynes and Scott (1985) and Fitzpatrick *et al.* (1986) found no link between 11-ketotestosterone and milt production, and plasma levels of both androgens have been demonstrated to fall in milt-producing individuals (Ueda *et al.*, 1986). Androstenedione was also present in the plasma of males in moderate concentrations. Its maximum concentrations were only half those of the other androgens and its role in the sexual maturation of male salmonids is unknown.

In the present study DHP concentrations were highest in the last samples obtained, but because no milt-producing individuals were

sampled, the results do not confirm or refute the claims that DHP is linked to milt production in salmonids (Scott *et al.,*, 1983; Ueda *et al.,* 1986; Fitzpatrick *et al.,* 1986). DHP has been shown to affect the ionic composition of rainbow trout seminal fluid (Baynes and Scott, 1985).

17β-estradiol was present at low concentrations throughout maturation. Other studies have found 17β-estradiol higher in coho salmon in the later stages of spermatogenesis and lowest in milt-producing individuals (Sower and Schreck, 1982b; Fitzpatrick *et al.* 1986), but because no milt producing individuals were sampled in the present study, we do not know if this is also the case in chinook. 17β-estradiol concentrations in male salmonids are known to be low and remain relatively unchanged when compared with females (Sower and Schreck, 1982b; Truscott *et al.,* 1986; Fitzpatrick *et al.,* 1986).

Gonadotropin I levels generally followed the same plasma profile as the androgens. Plasma levels of gonadotropin II began to rise at the same time as levels of 17β-estradiol and DHP. It is likely that gonadotropin I is responsible for the levels of androgens detected throughout the sampling season and, as in the females, gonadotropin II is responsible for the increase in DHP detected late in maturation.

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VI.   Appendix II:

Testosterone Alters the Immune Response of Chinook Salmon

(*Oncorhynchus tshawytscha*): *In vivo* studies.

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VI. Appendix II:

Testosterone Alters the Immune Response of Chinook Salmon

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INTRODUCTION

Steroid hormones such as cortisol and testosterone have been shown to modulate the antibody response of the salmonid immune system (Tripp *et al.*, 1987; Maule *et al.*, 1989; Slater and Schreck, in press). This study set out to determine if the high concentrations of testosterone detected in the plasma of maturing spring chinook (Slater *et al.*, in press) have any effect on the function of the immune system. In order to avoid the confounding effects of already high concentrations of cortisol and sex steroid hormones found in sexually maturing adults, and to isolate experimentally the effects of different hormones, juvenile chinook salmon, known to have low concentrations of sex steroids in their plasma (Patino and Schreck, 1986), were studied.

METHODS

Juvenile spring chinook salmon were obtained from Anadromous, Inc., Corvallis, Oregon (Rogue River stock, 1989 brood year) and Dexter holding ponds, Lowell, Oregon (Willamette River stock, 1990 brood year) and held at Smith Farm Experimental Hatchery, Oregon State University, Corvallis, Oregon. Fish were maintained in 2m circular, flow through tanks, at $12 \pm 1^{\circ}C$, under natural photoperiod, and fed a commercial diet of semi-moist pellet (Bio-moist). Fish were held for at least 30 days before any experiments were started.

The ability of leukocytes to form specific antibody producing cells was used as a measure of immunocompetence. The procedure followed was the hemolytic plaque assay exactly as described in chapter III.

Fish from Anadromous, Inc. (wt: $60 \pm 5g$), were used in experiments in the fall of 1990. Dexter fish (wt: $40 \pm 5g$) were used for experiments in the spring of 1991.

Testosterone was administered to juvenile chinook salmon via an interperitoneal injection of microencapsulated steroid, following the procedure of Fitzpatrick (1990). The microcapsules were made by dissolving testosterone in 1.6 g of menhaden oil, and adding to this 0.65 g of kaomel and 0.95 g of tripalmitin. These ingredients were mixed at $40-60^{\circ}C$, and then 5.4 ml of 2% (w/v) polyvinyl alcohol (at

the same temperature) was added. This mixture was immediately sonicated until frothy and well mixed (about 20 sec), at which time 8 ml of ice cold water was added. A sample of microcapsules was examined under the microscope (100x) to ensure that the steroid was dissolved and that the microcapsules were uniform in shape. In the fall 1990 experiment, 115 mg of testosterone was incorporated into 16 ml of microcapsules. Each fish received a 0.5 ml injection of the microcapsule preparation. Control fish (sham) received microcapsules to which no testosterone was added. Injected fish were given a right or left pelvic fin clip to distinguish treatment group. For the spring 1991 experiment, the testosterone dose was raised to 144 mg testosterone in 16 ml microcapsules and another control (no injection or fin clip) group was added. Five fish from each treatment group were placed in each of three 1 m circular tanks. On day 2, 5, and 7 (1990), or day 1, 4, and 7 (1991) after injection, all fish in one tank were quickly netted and killed by overdose of anesthetic (MS-222 200g/L). Blood was collected in heparinized capillary tubes after severing the caudal peduncle. Samples were then centrifuged and the plasma was removed and frozen at -20° C until assayed. Plasma testosterone concentrations were determined by RIA following the procedure of Sower and Schreck (1982), modified by Fitzpatrick et al. (1986).

Statistics: All data were subjected to the Freidman's nonparametric analysis of variance and, where significant between-group differences were found, pairwise comparisons were conducted

using the Dunnett's test as described in Zar (1984) at the P<0.05 level.

RESULTS

The first experiment was carried out between 14 and 21 November 1990. Fish treated with testosterone had plasma testosterone concentrations which were significantly higher than sham injected fish on all sampling days (fig 8a). The number of plaque forming cells produced by testosterone injected and sham injected fish did not differ significantly on any sample date. However, the plaque forming response of both the testosterone and sham groups was significantly lower on day 7 than on day 2 or 5 (fig. 8b)

The experiment was repeated in March of 1991. Plasma testosterone concentrations were significantly higher in the testosterone-injected group than either the control or sham-injected groups on day 1 and 4 and had returned to near control levels by day 7 (fig. 9a). There were no significant differences in plaque forming response in leukocytes from the three treatment groups on day 1 (fig. 9b). On day 4, both the sham and testosterone groups had significantly reduced plaque forming response as compared to the control group. On day 7, the number of plaque forming cells in the sham group was still significantly lower than the control group, but did not differ significantly from the testosterone group.
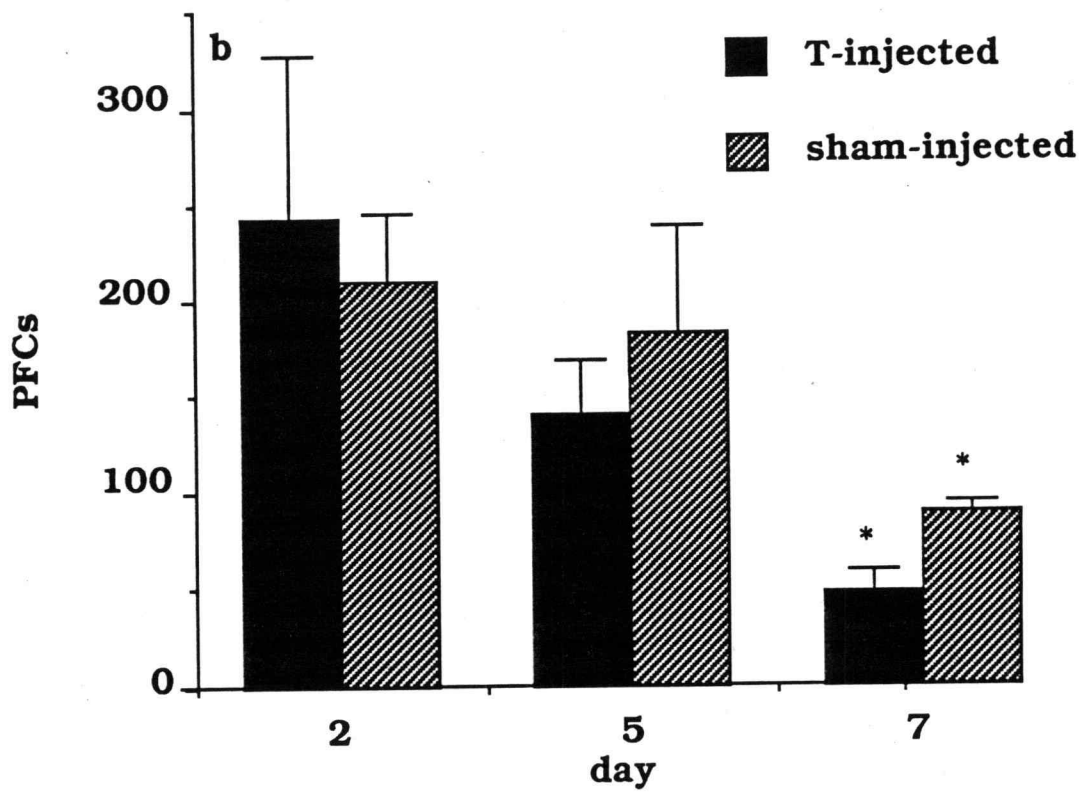
Figure 8.   Plasma testosterone concentrations and numbers
of plaque forming cells (PFC) produced by juvenile spring
chinook salmon injected with testosterone filled and
empty microcapsules. Bars represent means ($\pm$ standard
errors) of: a. plasma testosterone concentration (ng/ml).
b. plaque-forming cells per culture. Bars marked "*" are
significantly different from controls ($p < 0.05$).

Figure 8
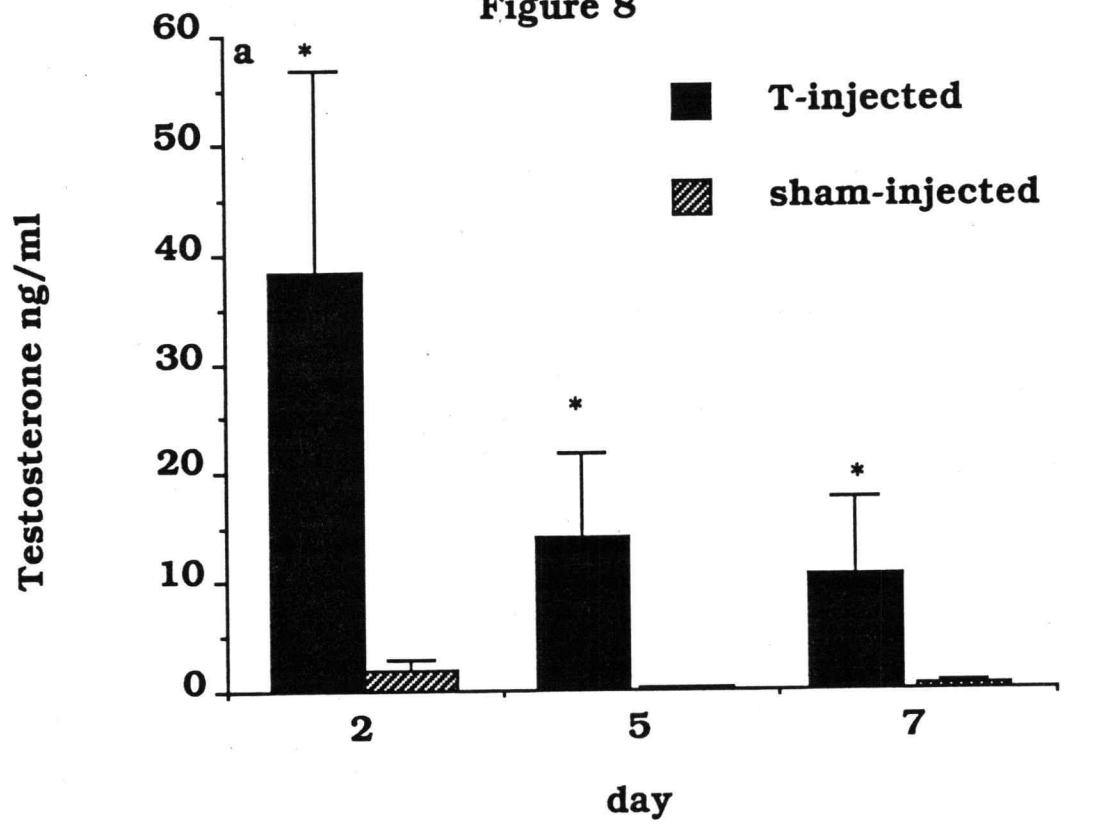
Figure 9.   Plasma testosterone concentrations and numbers
             of plaque forming cells (PFC) produced by juvenile spring
             chinook salmon injected with testosterone filled and
             empty microcapsules. Bars represent means ($\pm$ standard
             errors) of: a. plasma testosterone concentration (ng/ml).
             b. plaque-forming cells per culture. Bars marked "*"
             are significantly different from controls (p<0.05).

Figure 9
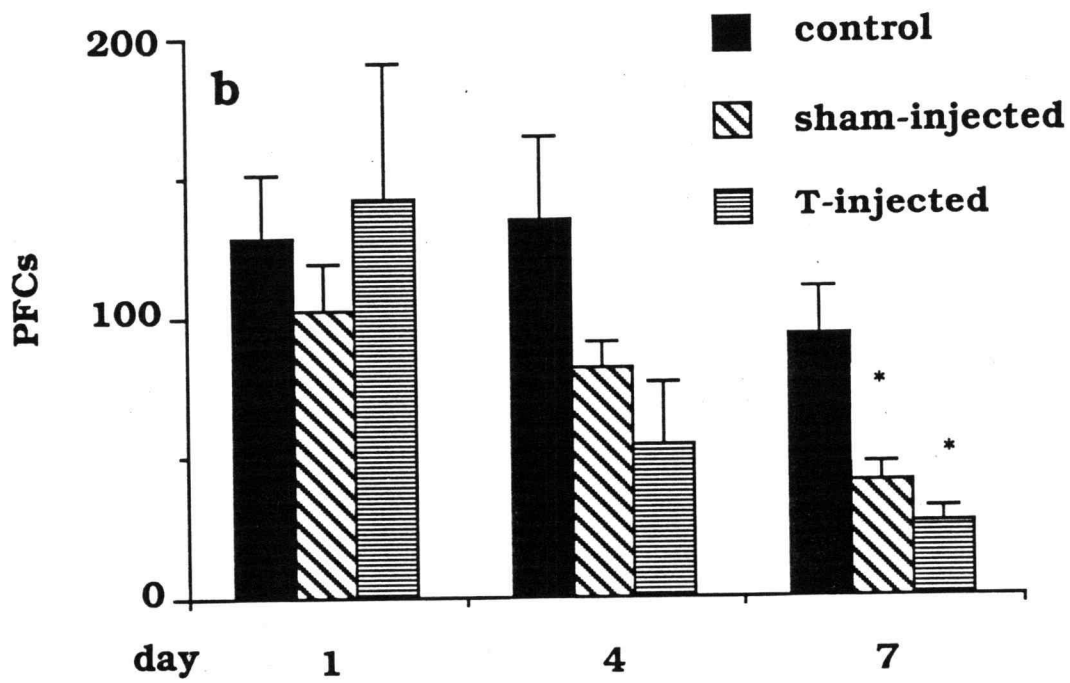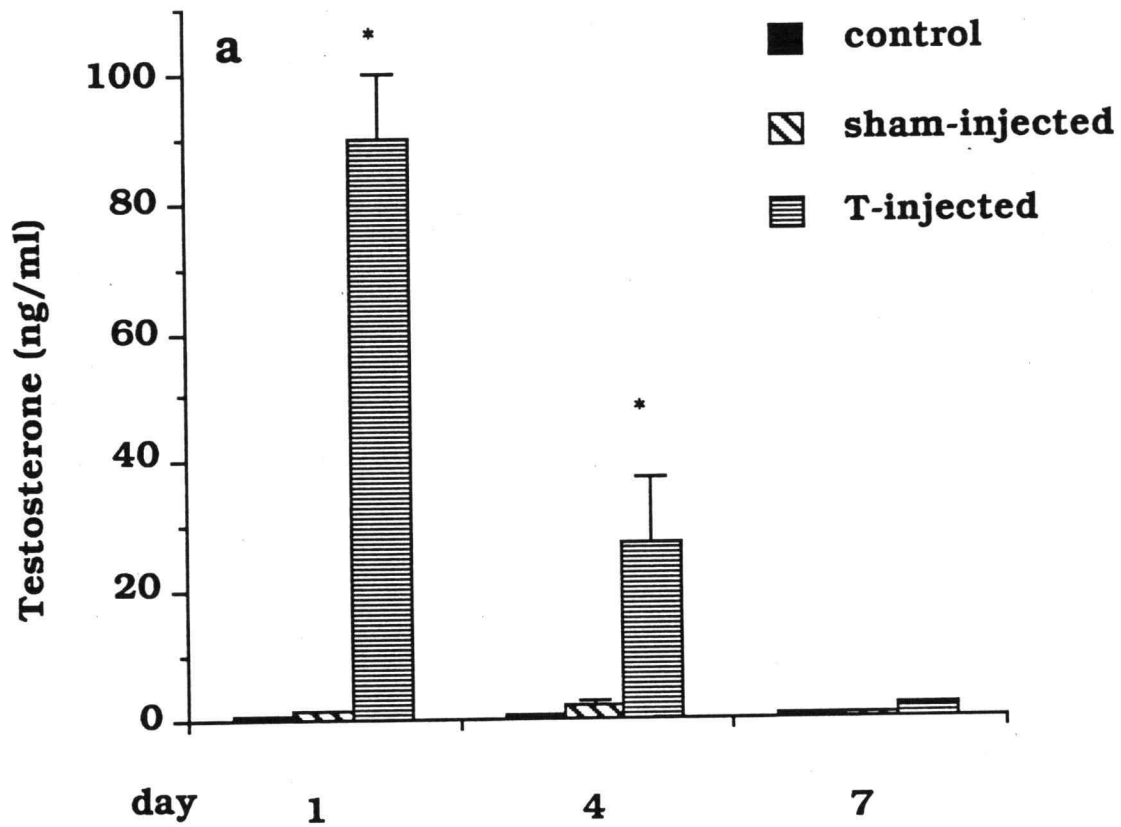
DISCUSSION

The fact that testosterone did not demonstrate immuno-suppression *in vivo* does not necessarily negate any biological significance of the findings. Maule *et al.* (1989) found differences between results of the plaque forming assay and actual disease resistance in response to cortisol in coho salmon. The fact that sham-injected fish demonstrated immunosuppression on days 4 and 7 is consistent with the findings of Maule *et al.* (1989) who showed that the numbers of plaque forming cells dropped significantly seven days after coho salmon were subjected to acute stress. The injection and fin clip procedure used on experimental animals constituted such an acute stress. Stress also alters the number and location of leukocytes. Maule *et al.* (1987) reported that circulating white blood cells and the relative number of splenic leukocytes both declined when cortisol levels were elevated. Therefore, it is possible that different subpopulations of leukocytes were sampled on different days. The confounding effects of stress and cortisol on experimental animals makes demonstration of immunosuppression by injection of sex steroids very difficult *in vivo*.

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