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Roles of Sex Steroids in Reproduction of the Sea Scallop

by

Chunde Wang

Submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

at

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DALHOUSIE UNIVERSITY

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by Chunde Wang

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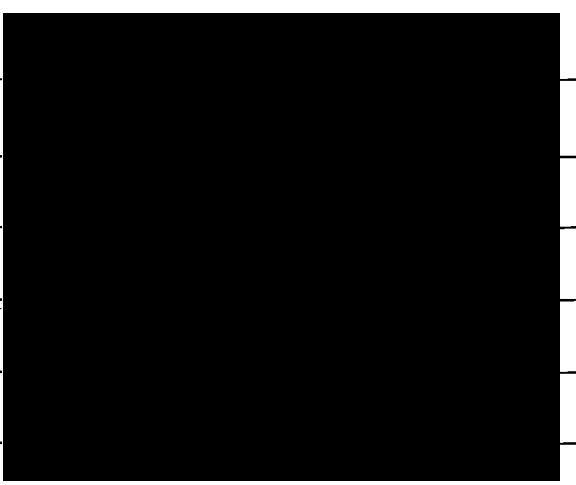

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Signature of Author

This thesis is dedicated to the memory of my mother,

and to the love of my family.

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Abstract:

Sex steroids, as well as their major metabolic enzymes and pathways, have been widely identified in molluscs. However, whether they are involved in molluscan reproductive control is generally not known. Thus, the aim of this study is to investigate the possible roles of sex steroids in the sea scallop.

In these studies, injections of estradiol, testosterone, progesterone and dehydroepiandrosterone (DHEA) significantly promoted sexual differentiation and shifted sex ratios, resulting in more males than females, as well as other morphological changes. *In vitro* experiments demonstrated that estradiol and progesterone potentiated 5-HT-induced gamete release in both sexes while testosterone was only effective in males. Examinations of the time scales of these effects and pharmacological studies utilizing antisteroids or RNA or protein synthesis inhibitors suggested that these effects may be mainly mediated by intracellular sex steroid receptors. Similar effects were also obtained by *in vivo* injections of these steroids. Radioligand binding studies demonstrated that specific estrogen binding sites exist in the cytosolic and nuclear fractions of both female and male scallop gonads. Furthermore, translocation of these estrogen binding sites may be involved in the sexual maturation of scallops. Specific progesterone binding sites were also characterized in the nuclear fraction of gonads of both sexes. Using degenerate primers based upon nucleotide sequences of vertebrate estrogen receptors, a cDNA fragment was amplified from the scallop liver by RT-PCR. Similarity comparison suggested that it might be a partial sequence of the scallop estrogen receptor gene. With this identified sequence as a probe, Northern blotting revealed the expression of a 3.1 Kb mRNA in the scallop gonad and liver.

The current studies suggested that sex steroids play important roles in the reproductive control of the scallops, possibly through the activation of sex steroid receptors. These findings may have potential applications in aquaculture and environmental issues.

Abbreviations:

3β–HSD: 3β–hydroxysteroid dehydrogenase

5-HT: serotonin

17β–HSD: 17β–hydroxysteroid dehydrogenase

AC-cAMP: adenylate cyclase-cyclic AMP

AMV reverse transcriptase: avian myeloblastosis virus reverse transcriptase

ANOVA: analysis of variance

ASO: accessory sex organ

ASW: artificial sea water

$B_{max:}$ maximum binding capacity

α-BCP-α-bag cell peptide

CBP: CREB binding protein

CDC: caudodorsal cell

CDCH: caudodorsal cell hormone

CDCP: caudodorsal cell peptide

cDNA: complementary DNA

CPM: count per minute

CREB: regulatory element binding protein

DB: dorsal body

DBH: dorsal body hormone

DCC: dextran-coated charcoal

DES: diethylstilbestrol

DHEA: dehydroepiandrosterone

E_2: estradiol

EDC: endocrine disruption chemicals

ELH: egg-laying hormone

G-6-PD: glucose-6-phosphate dehydrogenase

GSI: gonadosomatic index

HP: hydroxyprogesterone

HPLC: high performance liquid chromatography

HRE: hormone responsive element

HSP: heat shock protein

ITPG: isopropyl-β-D-thiogalactopyranoside

JO: juxtaganglionar organ

K_d: dissociation constant

LL: lateral lobe

PG E_2: prostaglandin E_2

RIA: radioimmunoassay

RT-PCR: reverse transcription polymerase chain reaction

SRA: steroid receptor RNA activator

SRC: steroid receptor co-activators

TBT: tributyltin

TISO: *Isochrysis galcilis* Clone Tahiti

TLC: thin layer chromatography

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Introduction

Reproductive activities, such as sex differentiation, sexual maturation, estrous cycles, and sexual behaviors, are well known to be regulated by sex steroids in vertebrates. Sex steroids have also been found in the invertebrates. For instance, almost all types of vertebrate sex steroids have been identified in gastropod, bivalve and cephalopod molluscs. Major metabolic enzymes and pathways for sex steroids have also been demonstrated in molluscs. In gastropods and bivalves, sex steroids have been suggested to be involved in the regulation of gametogenesis and gonadal maturation, as well as sex determination and sex reversal. In gastropods and cephalopods, the growth and activities of accessory sex organs may also be under the control of sex steroids. Finally, sex steroids may also be involved in detoxification of some heavy metals in molluscs.

Despite the enormous volume of studies on sex steroids in molluscs, surprisingly, no review is currently available on this topic. This chapter will therefore summarize the current status of the studies of sex steroids in molluscs, with foci on the identification, metabolism and actions of sex steroids.

As an introduction, this chapter will start with a brief review of sex steroids and their actions in vertebrates, as well as the major mechanisms of these actions. This will be followed by an overview of the reproductive endocrine system and hormones of molluscs. In the following sections, I will review evidence for the presence and metabolism of sex steroids in molluscs, their roles in the regulation of reproduction, and mechanisms of their actions. I will also discuss the possible involvement of sex steroids in molluscs in environmental issues as well as potential

applications in aquaculture.

I. Overview of Sex Steroids and Sex Steroid Receptors in Vertebrates

I-A. Sex Steroids and Their Actions

The estrogens, progestins, and androgens were first identified in mammals and found to participate mainly in reproduction and were thus collectively named "sex steroids". In vertebrates, these sex steroids are involved in various aspects of reproduction such as sex determination, sexual differentiation and maturation, and development of accessory sex tracts or organs (for reviews, see McEwen; 1978; McEwen, 1978; Gorski, 1979). In addition to reproductive regulation, they are also found to function in non-reproductive tissues such as those of the cardiovascular and digestive systems (Black, 1988; Radwanska, 1993; Song *et al.*, 1996; Wild, 1996).

I-B. Mechanisms of Sex Steroid Actions

Due to their lipophilic nature, sex steroids can readily diffuse through or incorporate into the phospholipid bilayer of the cell membranes. When steroids diffuse through the cell membrane and bind to steroid receptors, effects involving genomic actions will occur. Otherwise, if steroids bind to a membrane component, generally, a rapid and non-genomic membrane effect will be elicited. The former is referred to as the classical mechanism while the latter as the membrane mechanisms of steroid actions.

I-B-1. The classical mechanism: The classical mechanism of steroid actions has long been known and accepted in vertebrates. In this mechanism, steroids diffuse across the cell membrane and bind to specific intracellular receptors located in the nuclear

compartment. This binding causes conformational changes in the receptor, the dissociation of heat shock proteins (HSPs), the interactions with other proteins, and the phosphorylation and dimerization of the receptor. The receptor is then activated and becomes capable of binding the specific upstream promoter sequences, the steroid responsive elements (SREs), of target genes. The transcription and subsequent translation of target genes are thus induced, leading to physiological effects (Beato, 1989; Gronemeyer, 1991). These effects can be blocked by anti-steroid drugs that compete for steroid receptors, and by RNA and protein synthesis inhibitors, which block transcription and translation of the target genes. These phenomena can be utilized in effective strategies to distinguish the classical mechanism from the membrane mechanisms.

Intracellular sex steroid receptors have been identified, isolated, and characterized in various vertebrates and their genes were first cloned in the 1980s. The first such gene cloned was a human estrogen receptor (Walter *et al.*, 1985). In the following year, the progesterone receptor was cloned from the chicken (Conneely *et al.*, 1986; Jeltsch *et al.*, 1986) and the rabbit (Loosfelt *et al.*, 1986). Androgen receptors were subsequently cloned from humans and rats (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Trapman *et al.*, 1988). Following these pioneering studies, sex steroid receptors have now been cloned from all classes of vertebrates (Jensen, 1991). However, no sex steroid receptor has ever been cloned from invertebrates.

In recent years, three major break-throughs have been made with respects to sex steroid receptors. First, the sex steroid receptor isoforms have been widely found in vertebrates. The new isoforms usually have close binding affinities to the original

receptor but have different distribution in the tissues (Muramatsu and Inoue, 2000). Second, the discoveries of co-activators and co-repressors provide new insights in the mechanisms of the actions of sex steroids. It is now known that co-activators such as steroid receptor co-activators (SRCs) interact directly with liganded receptors to increase the transcriptional activity of the receptors (Muramatsu and Inoue, 2000; Tetel, 2000). Besides SRCs, other co-activators include steroid receptor RNA activator (SRA) which seems to function exclusively as an RNA transcript, and cAMP regulatory element binding protein (CREB) binding protein (CBP) which may work together with SRC-1 to increase the transcriptional activity of estrogen receptor. In contrast, co-repressors of steroid receptor decreased the transcriptional activity upon their interaction with the receptors (McKenna et al., 1999). Third, the three-dimensional structures of estrogen receptor ligand binding domains have been revealed by crystallographic analyses (Brzozowski et al., 1997; Pike et al., 1999; Kumar and Thompson, 1999). In both the α and β isoforms of estrogen receptor, the ligand binding domain consists of 11-12 α-helices which are arranged in 3 layers and 2 β-sheets. A binding cavity for the ligand is formed by some of the helices and other structures. Among these helices, the helix 12 (H12) is of particular importance because its position is dependent on the binding fashion of the ligand and the receptor. If an antagonist is bound, H12 will block the interaction of co-activator with the receptor and thus the transcriptional activity of the receptor.

I-B-2. Membrane mechanisms: Membrane effects of steroids were first identified in the late1920's (Cashin and Moravek, 1927) but had been largely neglected for about 60 years. At about the same time that the intracellular steroid receptors were

discovered, the membrane effects of sex steroids began to receive extensive attention and have now been thoroughly studied in various tissues. Now membrane effects of steroids have been found in neurons, oocytes, hepatocytes, and epithelial cells in vertebrates (Wehling, 1997; Christ *et al.*, 1999).

The membrane effects of steroids are elicited when steroids bind to a membrane component such as a specific membrane steroid receptor, a neurotransmitter receptor, an ion channel, or the phospholipid bilayer. The binding alters the cell excitability, triggers a second messenger system, or changes the membrane fluidity, resulting in a cellular effect (Olsen and Snowman, 1982; Olsen *et al.*, 1985; for reviews, see Robel and Baulieu, 1995; Robel and Baulieu, 1995; Ramirez and Zheng, 1996). One of the prominent characteristics of the membrane effect is the rapidity with which it is elicited and the fairly short time, from seconds to minutes, over which it is sustained. Another characteristic is that the membrane mechanism of steroids generally cannot be blocked by antisteroids, *i.e.*, the antagonists to steroid receptors, while the effects elicited by the classical mechanism can be blocked by antisteroids. These characteristics can be employed as strategies to distinguish the classical and membrane mechanisms of sex steroids.

Specific membrane receptors for estrogen and progesterone have been identified in vertebrates (Blondeau and Baulieu, 1984; Pappas *et al.*, 1995) and the membrane progesterone receptor gene has been cloned from porcine vascular smooth muscle cells (Falkenstein, 1996). The membrane estrogen receptor has been suggested to be similar to its intracellular counterpart in mammals by multiple antibody labeling and impeded-ligand binding (Pappas *et al.*, 1995). Razandi *et al.*

(1999) proposed that membrane and nuclear estrogen receptor may originate from the same transcript because transfection of the estrogen receptor cDNA into Chinese hamster ovary cells resulted in the expression of this protein in both the membrane and nuclear fractions. However, this hypothesis seems contradictory to the observation that the membrane effects generally cannot be blocked by antisteroids. It is possible that 1) membrane estrogen receptor may not account for most of the membrane effects; 2) there may be other membrane estrogen receptors that are different from the intracellular receptors in structure; 3) even though the membrane receptors are the same as its nuclear counterpart, they may have different binding affinity to antisteroids.

II. Overview of the Reproductive Endocrine System and Hormones in Molluscs

Reproductive responses in molluscs are triggered by interactions of exogenous factors (such as temperature, salinity, food, light, etc.) and endogenous factors (including neuronal and hormonal factors). Restated in physiological terms, neuronal or hormonal factors are released in response to exogenous stimuli, and these act upon various reproductive organs, directly or indirectly through the other endocrine centers. To help explain the possible actions of sex steroids in molluscs, the composition of endocrine system and hormones involved in the reproductive regulation are briefly reviewed here (for more complete reviews, refer to Joosse and Geraerts, 1983; Joosse, 1988; Smith and Croll, 1997). As a model, the reproductive endocrine system of gastropods will be described in detail.

An important point in this discussion, however, is that most opisthobranch and pulmonate gastropods and some prosobranch gastropods and bivalves are hermaphrodites (Runham *et al.*, 1973; Beninger and Pennec, 1991), i.e., both female and male gametes are present in the gonad of the same animal. Two types of hermaphrodites, simultaneous and sequential, are commonly seen. Simultaneous hermaphrodites possess female and male gametes in the gonad of the animal at the same time. In sequential hermaphrodites, female and male gametes develop sequentially during their life cycles, with usually a male phase preceding the female phase (protandric hermaphrodite). During the transition from one phase to another, the gametes and associated reproductive organs are resorbed followed by the generation of alternative sexual organs.

II-A. Gastropods

To some extent, the reproductive endocrine system in gastropods is similar to that of vertebrates in terms of its hierarchical organization and feedback mechanisms. In vertebrates, an axial endocrine system has been well established for the regulation of reproduction. This system includes the hypothalamus, pituitary gland, gonads and reproductive tracts and accessory organs (Rhoades and Pflanzer, 1992). In many orders of gastropods, a similar system exists including the central nervous system (CNS), endocrine centers associated with CNS such as the dorsal bodies (DBs, in pulmonates) or the juxtaganglionar organs (JOs, in opisthobranchs), the gonad, and the accessory sex organs (ASOs) and reproductive ducts. Cephalic sensory tentacles may also be involved in the reproductive regulation depending on species. A schematic representation of the endocrine system of a model gastropod is shown in

Figure 1.

II-A-1. CNS: In response to environmental changes (such as photoperiod) and behavioral and physiological stimuli (such as mating), the CNS of gastropods synthesizes and releases hormonal factors that regulate various aspects of reproduction. Among these, the most thoroughly studied are the hormones of the caudodorsal cells (CDCs) in pulmonates and bag cells (BCs) in opisthobranchs and their control of egg-laying (for recent reviews, see Ram and Ram, 1990; Smith and Croll, 1997).

CDCs are a cluster of neurosecretory cells in the cerebral ganglia of pulmonates. In opisthobranchs, BCs are located at the pleuroabdominal connectives. The CDCs and BCs are important endocrine centers in the CNS which exert their control over egg-laying and other associated behaviors in gastropods. This control is achieved through the secretion of CDC hormone (CDCH) and associated peptides (CDCPs) in pulmonates or egg laying hormone (ELH) and bag peptides (BPs) in opisthobranchs (Kupfermann, 1970; Geraerts, 1988; Ram and Ram, 1990). CDCH and ELH have high degrees of similarity in nucleotide coding organization and processing of the precursors. Both are encoded as parts of preprohormones which, after specific cleavage and packaging, produce the primary hormone and the associated peptides. A high degree of sequence similarity has been found between CDCH and ELH as well as among their associated peptides, such as α-CDCP and α-BCP (Kupfermann, 1970; Geraerts, 1988; Vreugdenhil *et al.*, 1988; Nagle *et al.*, 1990; Ram and Ram, 1990; Li *et al.*, 1994; Smith and Croll, 1997). It is presumed that the receptor for ELH may exist on the intercellular membranes in ovotestis

Figure 1. Schematic representation of the endocrine control of reproduction in a model gastropod. This model gastropod possesses features from both stylommatophoran and basommatophoran pulmonates. Lines represent controls from endocrine organs to target organs. Refer to text for details of the controls.
AG, albumen gland; BC, bursa copulatrix; CDC, caudodorsal cells; CG, cerebral ganglia; DB, dorsal bodies; LL, lateral lobes; MG, muciparous gland; OG, oothecal gland; OT, ovotestis; P, penis; PG, prostate gland; V, vagina.

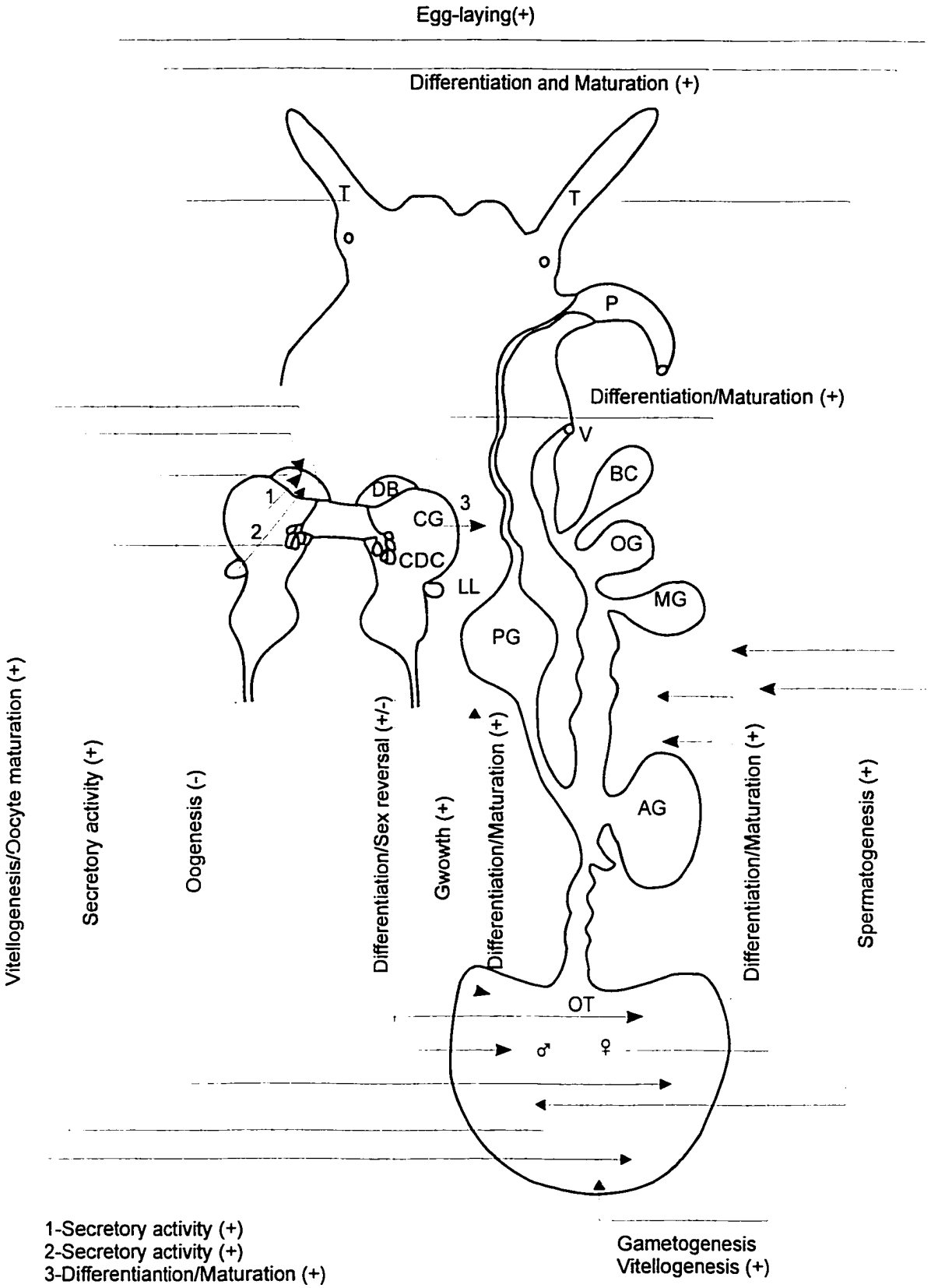

Egg-laying(+)
Differentiation and Maturation (+)
T
T
P
Differentiation/Maturation (+)
V
BC
OG
MG
AG
PG
DB
CG
CDC
LL
1
2
3
Vitellogenesis/Oocyte maturation (+)
Secretory activity (+)
Oogenesis (-)
Differentiation/Sex reversal (+/-)
Gwowth (+)
Differentiation/Maturation (+)
Differentiation/Maturation (+)
Spermatogenesis (+)
OT
♂
♀
1-Secretory activity (+)
2-Secretory activity (+)
3-Differentiantion/Maturation (+)
Gametogenesis
Vitellogenesis (+)

Figure 1

(Smith and Croll, 1997).

Roles of hormonal factors from the CNS on gonadal differentiation and growth, as well as differentiation and activities of ASOs have also been studied in gastropods. In protandric prosobranch snails, two hormonal factors released from the cerebral ganglia have been suggested to be essential for the development, differentiation, and maintenance of the gonad, as well as for the morphological changes in the gonad and ASOs during sex reversal (Choquet, 1971; Joosse, 1988). However, the natures of these factors have not been identified. The factor which stimulates proliferation of male and female cells is possibly a steroid since it is thermostable and pronase resistant (Lenngronne, 1986 in Joosse, 1988).

In pulmonates, factors secreted from the CNS control sex differentiation, gametogenesis, egg-laying behavior, and the activities of ASOs. An androgenic factor from the CNS is needed for male differentiation. This factor is secreted by the cerebral ganglia in response to long-day exposure and appears to be a protein. Implantation of long-day cerebral ganglia stimulated incorporation of [^{3}H] thymidine in the gonad (Sokolove *et al.*, 1984). Miksys and Saleuddin (1985) reported that the presence of the cerebral ganglia from mature snails induced the synthesis of polysaccharides in the albumin gland explants from immature animals in an *in vitro* organ culture experiment. A factor of peptide or protein nature from the cerebral ganglia may be partially responsible for this effect (Miksys and Saleuddin, 1987). A putative neurohormone, galactogenin, which stimulates the production of galactogen in the albumin gland, has also been found in the CNS of the land snail *Helix pomatia* (Goudsmit, 1975; Goudsmit, 1982).

Lateral lobes (LL), which are small lobes associated with the cerebral ganglia in basommatophoran pulmonates, play important roles in the onset of reproduction as well as the control of body growth. Hormones from the LLs promote maturation of the gonad and the ASOs while they delay the growth of the non-reproductive organs. LLs also stimulate the ovipository activity in the pond snail *Lymnaea stagnalis* (Geraerts, 1976a) These effects may be mediated by the stimulation of the activity of the DBs and inhibition of the secretion of a growth hormone from the light green cells in the cerebral ganglion (Geraerts, 1976b; Roubos *et al.*, 1980; Geraerts, 1981).

In gastropods with DBs or JOs such as the pulmonates and opisthobranchs, the reproductive control of the CNS may be achieved indirectly by its regulation on the activity of the DBs or JOs. This regulation can be neuronal in stylommatophoran pulmonates since their DBs are innervated by the CNS. In basommatophoran pulmonates, the regulation of the DBs by the CNS is more likely hormonal since the DBs are not innervated by the CNS.

II-A-2. DBs: Under the regulation of the CNS, the dorsal bodies (DBs), which are discrete endocrine organs associated with the CNS, also exert extensive direct control over various reproductive activities. The major actions of DBs include the stimulation of vitellogenesis, oocyte maturation in the gonads, and the development, differentiation and growth of the ASOs. These actions may be mediated by the production of a dorsal body hormone (DBH), the nature of which is uncertain at the present time (Krusch *et al.*, 1979; Wijdenes *et al.*, 1983b; Ebberink and Joosse, 1985; Nolte *et al.*, 1986; Joosse, 1988). This hormone functions like a female gonadotropic hormone (Geraerts, 1975). Removal of the DBs resulted in the cessation of oocyte

maturation and vitellogenesis, as well as egg-laying and ovipository activities (Geraerts, 1975; Geraerts, 1976b; Saleuddin *et al.*, 1980; Schollen and Saleuddin, 1986). These activities can be restored by re-implantation of the DBs (Geraerts, 1975). The DBs also control the growth, differentiation and secretion of the ASOs. The presence of DBs is essential for the development and cellular differentiation of the female, but not the male ASOs (Geraerts, 1975; Wijdenes and Runham, 1976). Synthesis of galactogen and other polysaccharides in ASOs had also been shown to be under the control of DBH (Veldhuijzen and Cuperus, 1976; Wijdenes *et al.*, 1983b; Miksys and Saleuddin, 1985; Miksys and Saleuddin, 1987).

II-A-3. Tentacles: In stylommatophoran pulmonates, tentacles appear to secrete one or more sex-specific hormones that affect the differentiation of germinal cells. The actions of the tentacles have mainly been shown in slugs by extirpation and injection experiments. In some species such as *Arion subfuscus*, *Arion ater* and *Helix asperea*, the tentacles exert a stimulatory effect on male germinal cells and an inhibitory effect on female germinal cells (Gomot, 1980). Takeda (1977, 1979, 1982) also showed that in *Limax flavus* and *Euhadra peliomphala*, a factor which originates from the collar cells near the optic ganglia of the tentacles, had similar effects. In other species such as *Ariolimax californicus*, tentacles have been reported to have the opposite effects (Gottfried and Dorfman, 1970c). In freshwater pulmonates, no endocrine role was found for tentacles.

II-A-4. Gonad: The gonads also exert important endocrine roles in gastropods. Under the direct regulation of the CNS, DBs and tentacles, the gonads secrete active

products to further affect the development and activities of the gonad itself as well as the ASOs.

Castration, implantation, and organ graft experiments provided useful information on the effects of gonadal hormones on the gonads. Sokolove *et al.* (1984) showed that in the slug *L. maximus*, implantation of the gonad from mature slugs induced rapid growth in the gonad, penis and albumen gland of immature animals. Gomot *et al.* (1980) also showed that in *H. aspersa*, culture of immature snail ovotestes in the presence of gonadal extracts from male dominant mature snails stimulated spermatogenesis. In *H. duryi*, castration led to a reduction in the circulating level of ferritin, a vitellogenic protein synthesized outside the gonad. Injection of extracts of gonad fragments or implantation of the active gonad fragments restored the ferritin level (Miksys, 1987). These results suggest that gonadal hormones control gametogenesis and vitellogenesis, important steps in gonadal maturation.

Other important functions of the gonad include its regulation of the differentiation, development, and activities of the ASOs. Effects of gonadal hormones on ASOs have long been recognized in stylommatophoran pulmonates. Abeloos (1943) found that removal of the ovary in *L. maximus* caused the female ASOs to regress. Castration also resulted in the cessation of the growth of penis in the slug *L. maximus* (McCrone and Sokolove, 1979). Consistent with these results, Laviolette (1954) observed that the reproductive tract from an immature slug showed significant enlargement if it was transplanted into a mature animal. Similarly, implantation of gonad fragments from mature animals induced rapid changes in immature slugs that had been exposed to short-day light cycles. These changes included rapid increases in

the ratios of the weight of penis and albumen gland to total body weight and an increase in the incorporation of [^{3}H] thymidine into the albumen gland (Sokolove *et al.*, 1984). Runham *et al.* (1973) showed that female and male reproductive tracts of the grey field slug *A. reticulatus* developed only when implanted in host animals that were in the female and male phase, respectively. Juvenile ASOs implanted into castrated slugs did not develop. Development of the head-wart, a specific ASO putatively responsible for sex pheromone release in some terrestrial snails, had been shown to be under the direct control of the gonad (Takeda *et al.*, 1987). These results indicated that the presence of gonadal hormones might be crucial in the regulation of reproduction in stylommatophoran pulmonates.

There has been considerable controversy about whether gonadal hormones have any effect on the ASOs in basommatophoran pulmonates. In *Biomphalaria glabrata* and *Bulinus truncatus*, ovarectomized mature animals were still able to deposit egg masses but without eggs and at a lower rate of production. These results indicated that at least the albumen gland and the oothecal gland were still functioning. The growth and secretion of the female ASOs, however, were not affected (Harry, 1965; Boer *et al.*, 1976; De Jong-Brink *et al.*, 1979; Geraerts, 1981). Joosse (1988) proposed that the gonadal hormones actually exert no effects on the ASOs in basommatophoran pulmonates. However, it is possible that the gonadal hormones exert partial control on the ASOs. In fact, Harry (1965) did observe retardation in the growth of the ASOs by castration in *B. glabrata*. Thus the gonadal hormones produced in the gonads of basommatophorans may still be able to cause changes in the ASOs under the continuous regulation of the CNS and DBs.

Despite these in-depth studies on the functions of the gonads, the chemical natures of the gonadal hormones are still not clear. However, as will be discussed later in this review, much evidence exists suggesting that the gonadal hormones are sex steroids.

As in vertebrates, feedback mechanisms may exist between the gonad and other endocrine control centers. Saleuddin *et al.* (1989) demonstrated that the synthetic activity of the DBs was affected by the presence or activity of the gonad. In virgin or castrated snails, the cells in the DBs seemed to be synthetically inactive. However, the DBs became synthetically active after mating. Runham *et al.* (1973) suggested that the maturation of the reproductive tract in the slug *A. reticulatus* induced by the hormones produced by the CNS were stimulated by factors secreted in the gonad. Thus, while the DBs regulate growth and activity of the gonads, the gonads possess a demonstrated ability to control the function of the DBs.

II-B. Bivalves

No discrete endocrine organs have yet been identified and characterized in bivalves. Most of the work on bivalves has been focused on the control of reproduction by the gonad and neurosecretory cells in the CNS.

Existing evidence indicates that the CNS exerts extensive control over the gonad. The secretory activities of the neurosecretory cells in the CNS are closely correlated with the gametogenic cycle (Illanes-Bucher and Lubet, 1980). A gonadotropic role of the cerebral ganglia has been demonstrated in the mussel by Lubet and Mathieu (1982) using organ culture techniques. They showed that a factor

from the cerebral ganglia stimulated the gonadal mitosis and the re-initiation of meiosis in males, and vitellogenesis in females. Mathieu (1987) showed that this factor stimulated the incorporation of [^{3}H]-thymidine into the germinal cells in the mussel. This factor has been partially characterized as an acid extractable, heat stable substance with a MW less than 5000 Da, indicating its peptide nature (Mathieu *et al.*, 1991). A similar factor with similar actions and physiochemical properties had also been found in the hemolymph (Mathieu *et al.*, 1988), suggesting that it may work as a hormone.

The CNS was also found to regulate reproduction-related glycogen synthesis and mobilization. Whittle *et al.* (1983) showed that the cerebral ganglia stimulate glycogen mobilization from storage tissues to the gonad while visceral ganglia promote glycogen synthesis. Robbins *et al.* (1990) had partially characterized a glycogen mobilization factor (GMF) from both the cerebral ganglia and haemolymph. This factor seemed to be of polypeptide/protein nature as it could be inactivated by trypsin digestion. A factor stimulating the synthesis of glycogen was also found in the CNS (Robbins *et al.*, 1991).

Monoamines from the CNS have also been studied for their effects in reproductive regulation. Serotonin (5-HT) has been studied extensively for its actions in the induction of spawning and final oocyte maturation in bivalves. In the gonads of both male and female bivalves, the gonadoducts, germinal epithelium, and acini are richly innervated by 5-HT-containing fibers (Matsutani and Nomura, 1986; Ram *et al.*, 1992; Paulet *et al.*, 1993; Croll *et al.*, 1995). Also, it has long been established that 5-HT is a potent spawning inducer in many bivalve species, and this phenomenon

has already been applied in aquaculture operations (Matsutani and Nomura, 1982; Gibbons, 1984; Citter, 1985). 5-HT has also been reported to induce final oocyte maturation *in vivo* and *in vitro* in bivalves (Wells and Wells, 1977; Matsutani and Nomura, 1982; Arnold, 1984; Matsutani and Nomura, 1987; Fong *et al.*, 1994b; Ram *et al.*, 1996). Such actions of 5-HT may be mediated by binding of 5-HT to specific receptors which have been characterized on the membranes of oocytes and sperm in various molluscan species (Bandivdekar *et al.*, 1991; Krantic *et al.*, 1993; Gobet *et al.*, 1994).

Besides 5-HT, catecholamines have also been suggested to be involved in the reproductive regulation in bivalves (Matsutani and Nomura, 1984; Matsutani and Nomura, 1986; Khotimchenko and Deridovich, 1991; Khotimchenko, 1991; Pani and Croll, 1995; Pani and Croll, 2000). Catecholamines have been identified in the CNS and gonads in the sea scallop (Smith *et al.*, 1998). Catecholamines display annual variations with the sexual maturation cycle (Osada and Nomura, 1989; Martinez and Rivera, 1994). Decrease of dopamine might be involved in spawning in scallops (Osada *et al.*, 1987; Pani and Croll, 2000). Furthermore, Fong *et al.* (1993) found that dopamine reduced 5-HT-induced spawning in both sexes of zebra mussel *Dreissena polymorpha.* However, further studies are still required to elucidate the mechanisms of these effects.

II-C. Cephalopods

In cephalopods, our knowledge about the reproductive endocrine system is mainly limited to the optic glands and the gonad (for reviews, see Wells & Wells,

1977; Wells and Wells, 1977; Arnold, 1984).

Optic glands are highly vascularized structures lying on both side of the optic tracts. These structures are essential for the development of the gonad (Richard, 1970; Wells and Wells, 1975), continued gametogenesis (Wells and Wells, 1975), vitellogenesis (O'Dor and Wells, 1973; O'Dor and Wells, 1975), and activities of the accessory sex organs (Wells, 1960; Froesch and Marthy, 1975). A hormone had been proposed to mediate the actions of the optic glands. This hormone is neither sex-specific nor species-specific (Wells and Wells, 1975; Wells, 1976; Wells and Wells, 1977). Froesch (1979) proposed that this hormone might be a steroid since the optic glands possess the cellular characteristics for steroid synthesis. However, Koueta and Boucaud-Camou (1992) had partially characterized a factor with low molecular weight, heat-liable, and sensitive to trypsin-digestion from the optic glands. This factor exhibited similar actions as those of the optic gland hormone. Thus the optic gland hormone could also be a peptide.

While a few reviews have been published on the endocrine control of reproduction by peptide and monoamine hormones in molluscs, none examined the presence, metabolism and possible involvement of sex steroid actions in molluscan reproduction (Joosse, 1988; Mathieu *et al.*, 1991; Koueta and Boucaud-Camou, 1992; Smith and Croll, 1997). However, sex steroids and their biosynthesis pathways have been widely identified in gastropod, bivalve and cephalopod molluscs (see below). Some investigators have also begun to examine the possible actions, such as gamete release, gametogenesis, vitellogenesis, and sex reversal, which might be controlled by

sex steroids in molluscs (Mori, 1967; Varaksina and Varaksin, 1988; Varaksina and Varaksin, 1991; Osada *et al.*, 1992a; Li *et al.*, 1998). These results suggest that sex steroids may be important endogenous factors in the regulation of molluscan reproduction. Therefore, it is the purpose of this review to examine the possible involvement of sex steroids in molluscan reproductive control.

III. Sex Steroids in Molluscs

III-A. Identification of Sex Steroids in Molluscs

Steidle (1930) first demonstrated the presence of estrogens in a gastropod (*Aplysia sp.*) and a cephalopod (*Octopus sp.*) by exploiting a mouse bioassay in which the weight gain of estrogen responsive tissue was used as an indicator. Hagerman (1956) later quantified the amount of estrogens in the ovary of *Spisula solidissima* by measuring the activity of diphosphopyridine nucleotide (DPN)-linked isocitric dehydrogenase, which is an estrogen driven enzyme in vertebrates (Karavolas, 1971). While these methods may be sensitive in detecting the presence or possible activities of sex steroids, their specificity is suspectable because the elicitation of these same effects by other substances could not be excluded. In addition, the exact identity of the steroids could not be determined.

Before the emergence of specific and sensitive techniques for detection of steroids, the demonstration of steroid-synthesizing enzymes was also employed as an effective and easiest way to suggest the existence of steroids. Enzymes such as 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD) involved in steroid synthesis in vertebrates have been identified

histochemically in molluscan species (Mori *et al.*, 1964; Mori *et al.*, 1965; Varaksina and Varaksin, 1988; Matsumoto *et al.*, 1997). *In vitro* studies showed that these enzymes were functional in molluscs (Gottfried and Dorfman, 1970c; Lehoux and Williams, 1971; Lupo di Prisco *et al.*, 1973; De Jong-Brink *et al.*, 1981; Le Guellec *et al.*, 1987), lending critical support to their value in predicting the existence of certain steroids. However, the presence of a steroidogenic enzyme does not necessarily indicate the existence of an assumed product or substrate, as the same enzyme may work on different or multiple substrates and thus generate different products.

Chromatography and radioimmunoassays (RIA) techniques provide necessary modern tools for direct measurement of steroids. Gas chromatography (Gottfried, 1967; Lupo di Prisco *et al.*, 1973) or gas chromatography combined with mass spectrometry (Reis-Henriques *et al.*, 1990), paper chromatography (Hathaway, 1965; De Longcamp *et al.*, 1974), thin layer chromatography (TLC) (De Longcamp, 1974; Krusch, 1979), and ion exchange chromatography (Reis-Henriques and Coimbra, 1990) have all been used to identify and quantify steroids in molluscan tissues. RIA is another reliable method for detection of sex steroids in which a radiolabeled standard steroid competes with endogenous steroids in crude cell extracts for the specific binding to antibodies against the hormones (Midgley *et al.*, 1971).

The application of these analytical techniques led to the direct identification of natural sex steroids or those synthesized from endogenous precursors in molluscs, as summarized in Table 1. Notably, significant levels of sex steroids have been identified in molluscs (Reis-Henriques *et al.*, 1990; Matsumoto *et al.*, 1997), suggesting that these sex steroids may have physiological effects in these animals.

Estrogens: The major forms of estrogens found in molluscs include 17β-estradiol, estrone, and estriol (Boticelli *et al.*, 1961; Lupo di Prisco *et al.*, 1973; Le Guellec *et al.*, 1987; Reis-Henriques *et al.*, 1990). Estrogens are most abundant in female animals, however, they are also found at much lower concentrations in males (Reis-Henriques *et al.*, 1990; Matsumoto *et al.*, 1997). Among these estrogens 17β-estradiol has been found in most of the molluscan species studied so far, and it is the most active form of the estrogens. Estrone may be metabolized from estradiol and may act as a storage of estrogen in the tissues as it is more stable than estradiol.(Matsumoto *et al.*, 1997).

Androgens: Androgens found in molluscs include testosterone, 11-keto-testosterone, 5α-dihydrotestosterone, 3α-androstanediol, androsterone, dehydroepiandrosterone (DHEA), and androstenedione (Boticelli *et al.*, 1961; Gottfried, 1967; Lupo di Prisco *et al.*, 1973; Le Guellec *et al.*, 1987; Reis-Henriques *et al.*, 1990). Unlike estrogens, androgens have mainly been found in male animals. Testosterone, as the major androgen, has been identified in gastropod, bivalve and cephalopod species. In some cases, it may be more potent when converted to 5α-dihydrotestosterone by 5α-reductase (Le Guellec *et al.*, 1987).

Progestins: Progestins found in molluscs include pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone (Lupo di Prisco *et al.*, 1973; Le Guellec *et al.*, 1987; Reis-Henriques and Combra, 1990; Reis-Henriques *et al.*, 1990). They may serve as precursors or intermediates in the synthesis of other sex steroids (De Longcamp *et al.*, 1974; Lupo di Prisco and Fulgheri, 1975).

Table 1. Identification of Endogenous Sex Steroids in Molluscs

Species	Tissues	Identity of Steroids	Methods	References
Arion ater	Bursa copulatrix Eggs	Estadiol, estrone 11-keto-testosterone testosterone, 17α-hydroxyprogesterone	Gas chromatography Gas chromatography	Gottfried *et al.*, 1967 Gottfriend and Lusis, 1967
Helix aspersa	Gonad	Estradiol, estrone, estriol; testosterone, 5α–DHT, DHEA, androsterone, androstenedione, 3α-androstanediol; progesterone	RIA	Le Guellec *et al.*, 1987
Aplysia depilas	Gonad Hepatic tissue	Estradiol, estrone; DHEA, testosterone; Progesterone, 17α-hydroxyprogesterone, pregnenolone Pregnenolone, 17α-hydroxypregnenolone	Gas chromatography	Lupo di Prisco *et al.*, 1973
Spisula solidissima	Ovary	"Estrogens"	Paper chromatography	Hagerman *et al.*, 1956
Pecten hericius	Ovary	"Estrogens", Progesterone	Column chromatography	Boticelli *et al.*, 1961
Mytilus edulis	Whole (females and males) Gonad	Estradiol, estrone; Testosterone, 5α–DHT, androsterone, androstenedione, androstanediol; Progesterone Progesterone	Gas chromatography -mass spectrometry RIA, Gas-liquid chromatography, ion exchange chromatography	Reis-Henriques *et al.*, 1990 Reis-Henriques and Coimbra, 1990
Crassostrea gigas *Patinopecten yessoensis*	Ovary Testis	Estradiol, estrone, estriol Estradiol	HPLC	Matsumoto *et al.*, 1997
Octopus vulgaris	Testis and male ASOs	Estradiol, testosterone, progesterone	RIA, HPLC	D'Aniello *et al.*, 1996

III-B. Biosynthesis of Sex Steroids in Molluscs

Biosynthesis of steroids had been examined by *in vitro* studies in which molluscan preparations were incubated with radiolabeled precursors and radioactive-labeled products were identified at the end of the experiments. One of the most commonly utilized precursors is cholesterol (Lupo di Prisco *et al.*, 1973; Sica, 1979; Blanchier *et al.*, 1986), which is the major sterol found in molluscs (Idler and Wiseman, 1972; Teshima and Kanazawa, 1973). In *in vitro* studies, it has been shown that cholesterol, in turn, can be synthesized directly from acetate (Lupo di Prisco *et al.*, 1973). It is also possible that the sterol precursors are not synthesized by molluscs, but are obtained from algal diets (De Longcamp *et al.*, 1974). Other substrates such as 24-methylene-cholesterol and 22-dehydrocholesterol in the sea scallop may also be used as precursors where cholesterol was found to be of minor importance as a precursor (Lehoux and Sandor, 1970).

Since sterol composition is very complex in molluscs (Idler and Wiseman, 1972; Idler, 1978; Joosse, 1978), it is possible that multiple sterols are utilized as precursors for the steroid synthesis in molluscs. This hypothesis is supported by the results from the *in vitro* experiments of Lupo di Prisco *et al.* (1973). Using radioactively-labeled acetate and cholesterol as precursors, Lupo di Prisco *et al.* (1973) found that not all endogenous steroids were synthesized from these precursors in the gonad and the hepatopancreas tissues. Therefore, it is possible that precursors other than cholesterol were used in the biosynthesis of these steroids.

III-C. Metabolism of Sex Steroids in Molluscs

Early studies used histochemical and biochemical techniques to indicate that molluscs possess many of the vertebrate-type enzymes, as well as the precursors, necessary for steroid biosynthesis and metabolism. Consequently, the presence of these enzymes in molluscs has been demonstrated by immunohistological studies and *in vitro* experiments. Two points must be kept in mind when interpreting the results from *in vitro* experiments: first, a specific conversion may not be catalyzed by the same enzyme nor follow the same pathway as in vertebrates; second, a specific enzyme may utilize a different precursor as substrate and produce a different product. Thus, it should be noted that a steroid identified in such *in vitro* experiments might not be of normal endogenous origin unless it is confirmed by *in vivo* studies.

Given these caveats, the major metabolic pathways and enzymes for sex steroids appear to be common to vertebrates and molluscs and are summarized in Figure 2 and Table 2.

III-D. Sites of Sex Steroid Synthesis

In mammals, sex steroids are synthesized mainly in the gonads and adrenal glands. However, molluscs do not have any organs homologous to the mammalian adrenal gland. Instead, most studies on the occurrence and biosynthesis of steroids in molluscs have been focused on the gonad, liver, and endocrine organs such as the dorsal bodies (DBs; see Section II of this chapter).

Steroid-producing cells are characterized by extensive smooth endoplasmic reticulum, a large number of mitochondria with tubular cristae, and lipid droplets (De Jong-Brink *et al.*, 1978; Krusch *et al.*, 1979) . Such cells have been localized in the

Figure 2. Major metabolism pathways for sex steroids in molluscs.
DHEA: dehydroepiandrosterone
3β-HSD: 3β–hydroxysteroid dehydrogenase
17β-HSD: 17β–hydroxysteroid dehydrogenase

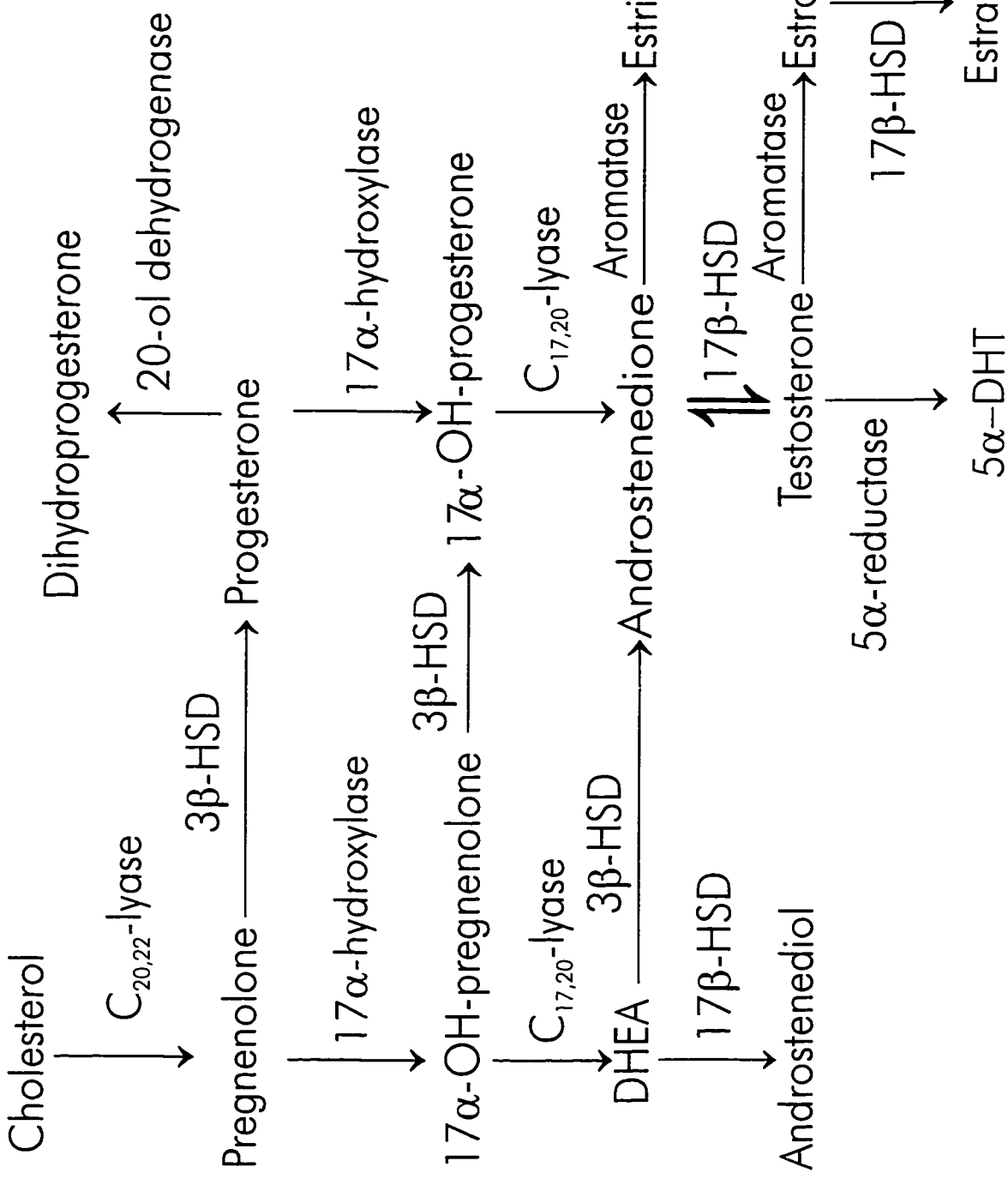

Cholesterol
$C_{20,22}$-lyase
Pregnenolone
3β-HSD
Progesterone
20-ol dehydrogenase
Dihydroprogesterone
17α-hydroxylase
17α-OH-progesterone
3β-HSD
$C_{17,20}$-lyase
DHEA
3β-HSD
Androstenedione
17β-HSD
Testosterone
Aromatase
Estrone
17β-HSD
Estradiol
17α-hydroxylase
17α-OH-pregnenolone
$C_{17,20}$-lyase
Androstenediol
17β-HSD
Aromatase
Estriol
5α-reductase
5α-DHT

Figure 2

Table 2. Major Enzymes for Steroid Synthesis in Molluscs

Enzymes	Species	Tissues	References
3α-hydroxysteroid dehydrogenase (3α-HSD)	*Crassostrea gigas*	gonad	Mori *et al.*, 1964, 1965
	Ariolimax californicus	ovotestis	Gottfried *et al.*, 1970
	Mytilus edulis	gonad	De LongCamp *et al.*, 1974
	Helix pomatia	dorsal body, ovotestis, and buccal ganglia	Krusch *et al.*, 1979
	Lymnaea stagnalis	gonad, liver	Jong-Brink *et al.*, 1981
	Octopus vulgaris	testis	D'Aniello *et al.*, 1996
	Patinopecten yessoensis	ovary	Matsumoto *et al.*, 1997
17β-hydroxysteroid dehydrogenase (17β-HSD)	*Crassostrea gigas*	kidney, liver	Mori *et al.*, 1965, 1966
		ovary	Matsumoto *et al.*, 1997
	Crassostrea virginica	sperm	Hathway, 1965
	Crepidula fornicata	gonad, liver	Bardon et a., 1971
	Mytilus edulis	gonad	De LongCamp *et al.*, 1974
	Patinopecten yessoensis	ovary	Matsumoto *et al.*, 1997
5α-reductase	*Ariolimax californicus*	ovotestis	Gottfried and Dorfman, 1970
	Mytilus edulis	gonad	De LongCamp *et al.*, 1974
17α-hydroxylase	*Ariolimax californicus*	ovotestis	Gottfried & Dorfman,1970
	Aplysia depilans	gonad, liver	Lupo di Prisco *et al.*, 1973
Aromatase	*Helix aspersa*	gonad, hemolymph	Le Guellec *et al.*, 1987
	Patinopecten yessoensis	gonad	Matsumoto *et al.*, 1997
C-20,22-lyase	*Ariolimax californicus*	ovotestis	Gottfried and Dorfman, 1970
C-17,20-lyase	*Placopecten magellanicus*	gonad	Idler *et al.*, 1969
	Ariolimax californicus	ovotestis	Gottfried and Dorfman, 1970
	Mytilus edulis	gonad	De LongCamp *et al.*, 1974
17α-20,21-desmolase	*Arion ater rufus*		Lehoux *et al.*, 1967
	Placopecten magellanicus	gonad	Idler *et al.*, 1969
	Ariolimax californicus	ovotestis	Gottfried *et al.*, 1970

gastropods, bivalves and cephalopods (see below). These cells must also possess both the precursors and enzymes for steroid synthesis. Thus the sites of steroid synthesis can also be inferred from the presence of steroid synthetic enzymes and the results from *in vitro* studies showing the occurrence of steroid synthesis.

III-D-1. Gastropods: Evidence exists for the possible synthesis of steroids in the gonad or hermaphroditic ovotestis, liver, CNS, DBs, and tentacle gland.

As in the vertebrates, the gonad may be the primary site for sex steroid synthesis in the gastropods. All the major sex steroids and steroid synthetic enzymes have been identified in the gonad or ovotestis, as shown in Table 1 and Table 2 (for review, see Lehoux and Sandor, 1970). The ability of the gonad for sex steroid biosynthesis has been demonstrated in various *in vitro* studies (Gottfried and Dorfman, 1970b; Lehoux and Williams, 1971; Lupo di Prisco *et al.*, 1973; De Jong-Brink *et al.*, 1981; Le Guellec *et al.*, 1987)

Despite the above studies, evidence for the presence of specific steroid-producing cells in the gonad of gastropods is sparse. In *B. glabrata*, extensive smooth endoplasmic reticulum, but not the characteristic numbers of mitochondria and lipid droplets, was found in the Sertoli cells of animals carrying spermatids and those after spermiation (De Jong-Brink *et al.*, 1977; De Jong-Brink *et al.*, 1981). This phenomenon was also found to be correlated with the diurnal activity of steroid-synthesizing enzymes. These results suggested that the Sertoli cells might be responsible for the synthesis of steroids in this species. Surprisingly, no putative steroid-producing cells have been identified in the female portion of the

hermaphroditic ovotestis. Thus, further studies are needed to determine if other putative steroid-producing cells exist in gastropod species.

The DBs have also received much attention regarding their possible production of steroid hormones. There have been disputes about the ability of DBs to synthesize steroids. Early studies showed that these organs possess the characteristic structures for steroid synthesis including numerous mitochondria, lipid droplets and extensive smooth endoplasmic reticulum (Boer *et al.*, 1968; Boer and Joosse, 1975). Saleuddin *et al.* (1989) also suggested that dorsal body hormone (DBH), the synthetic product of the DBs, could possibly be a steroid since the extract is heat and protease-resistant. In fact, the presence of ecdysteroids has been reported in the DBs of gastropods (Nolte *et al.*, 1986). However, Teunissen (1994) predicted that DBs are not able to synthesize steroids based on analyses of mRNA expressed in these organs. In her experiments, Teunissen (1994) used a differential screening procedure to clone genes that are unique to DBs from a CNS/DB cDNA library and found only one cytochrome P450 gene. Synthesis of steroids, however, requires activities of several cytochrome P450 enzymes. Although this is consistent with the results from her *in vitro* experiments in which no steroid was synthesized from radiolabeled precursors that are commonly used for steroid synthesis in insects, it is still premature to preclude the capability of DBs to synthesize steroids. A major concern regarding her experiments is that with the differential screening strategy, those cytochrome P450s which are common in both the CNS and DBs cannot be identified. In fact, as discussed below, the capability of CNS for steroid synthesis has been suggested in gastropods (Krusch *et al.*, 1979). Therefore, the possibility that DBs have all the

necessary steroid synthesis enzymes cannot be excluded by this study. Also, the cause for the failure to synthesize ecdysteroids in the *in vitro* study may be that the snails use different precursors for ecdysteroid synthesis than those used by insects. This possibility has also been proposed by Garcia (1995).

But is DBH one of the ecdysteroids? The major actions of DBH are sex-specific; it stimulates gametogenesis and activities of female ASOs but generally has no influence on male ASOs (Geraerts, 1975; Geraerts, 1976b; Miksys and Saleuddin, 1985; Schollen and Saleuddin, 1986). We know little about the actions of ecdysteroids in gastropods. However, while ecdysteroids may have sex-specific actions that have not yet been found, the major function of ecdysteroids, i.e., the stimulation of molting and metamorphosis, do not seem to be sex-specific. Thus DBH may not be an ecdysteroid. On the contrary, actions of sex steroids are usually sex-specific. Therefore, it is possible that DBH is a sex steroid.

In fact, DBs have been shown to be capable of synthesizing sex steroids. In their *in vitro* studies, Krusch *et al.* (1979) demonstrated that the DBs of the snail *H. pomatia* contained 3β-HSD and were able to convert pregnenolone to progesterone and dehydroepiandrosterone to androstenedione. The percentages of the conversions vary before and after ovoposition, suggesting that the variations in the concentrations of these sex steroids are correlated with reproductive activities, although results from *in vitro* experiments alone are not sufficient for a solid conclusion without corroborating results from *in vivo* studies.

In opisthobranch gastropods, the juxtaganglionar organs (JOs) have been suggested to be homologous to the DBs of pulmonates and also possess all the

characteristic features such as extensive smooth endoplasmic reticulum, a large number of mitochondria and lipid droplets that are necessary for steroid synthesis (Switzer-Dunlap, 1987). Therefore, JOs may also be a major site of steroidogenesis in these gastropod species.

The CNS may be another source of sex steroids. 3β-HSD and the conversions of sex steroids have been identified in the buccal ganglia of *H. pomatia* (Krusch *et al.*, 1979). In protandric snails in which the male phase proceeds the female phase during the reproductive cycle, factors secreted by the CNS are phase-specific. Factors secreted during the male phase stimulate spermatogenesis and activities in the male ASOs while inhibiting oogenesis and activities in the female ASOs, and these effects were reversed during the female phase (Choquet, 1971). Although it is possible that these actions are achieved by sex steroids from the CNS, evidence available now is not sufficient to confirm this hypothesis.

Other steroid-producing tissues may include the bursa copulatrix (or spermatheca gland), an accessory reproductive gland that stores sperm after copulation in gastropods such as the slug *A. ater rufus* (Gottfried *et al.*, 1967), or the hepatopancreas in *A. depilans* (Lupo Di Prisco *et al.*, 1973). The steroid-producing abilities of these tissues are indicated by the presence of steroids and associated steroid metabolic enzymes, as well as by *in vitro* synthesis studies.

III-D-2. Bivalves: In bivalves, the gonads and the hepatopancreas appear to be the main steroid-producing organs (De LongCamp *et al.*, 1974; Matsumoto *et al.*, 1997). Steroid-synthesizing enzymes such as 17β-HSD and 3β-HSD have been identified in the oyster *Crassostrea gigas* and the Japanese scallop *Patinopecten yessoensis* (Mori

et al., 1964; Mori *et al.*, 1965; Mori *et al.*, 1966; Varaksina and Varaksin, 1988; Mathieu *et al.*, 1991). Immunohistological studies demonstrated the presence of 3β-HSD, P450 aromatase and estradiol in the auxiliary cells, indicating that the synthesis of steroids in these cells. The digestive diverticulum (liver) may also synthesize steroids since it possesses both the enzymes for steroid synthesis and the main steroid precursors, such as cholesterol, pregnenolene and progesterone (Lupo di Prisco *et al.*, 1973).

III-D-3. Cephalopods: Sex steroids have been identified in the gonad (Carreau and Drosdowsky, 1977; Nikitina *et al.*, 1977) and the digestive gland (liver) in the cephalopod *Sepia officinalis* (Nikitina *et al.*, 1977). Henry (1994) identified progesterone in the follicles and hemolymph of females by RIA. In addition, D'Aniello *et al.* (1996) demonstrated the presence of testosterone, progesterone and 17β-estradiol in the testis, vas deferens, seminal vesicle, prostate, Needham's sac, and hemolymph, by RIA, HPLC, and immunohistological methods. Furthermore, tubular and interstitial cells in the testis also displayed strong 3β-HSD activity, suggesting that these cells are active in steroid synthesis.

III-E. Regulation of the Production of Sex Steroids in the Gonad

In gastropods, many reproductive activities, including those of the gonad, are under the direct or indirect control of the CNS, DBs, tentacles, and other endocrine centers, as discussed in Section II. It is reasonable to assume that the production of sex steroids, possibly an important function of the gonad, is regulated by these endocrine centers.

III-E-1. Regulation by the CNS: From the previous discussion in Section II, it appears that in pulmonates and prosobranchs, androgenic and estrogenic factors, which promote gametogenesis and development of ASOs, are produced in the CNS. The importance of the regulation of the gonad by the CNS had been illustrated by the studies of Bailey (1973). In his experiments utilizing organ culture techniques, Bailey (1973) showed that the CNS is essential for the actions of gonads on differentiation of the juvenile ASO in the slug *A. reticulatus*. Culture of the reproductive tract with the gonad alone did not induce the ASO differentiation. Although Runham *et al.* (1973) argued that the maturation of the reproductive tract in the slug *A. reticulatus* was induced by the hormones produced by the CNS which in turn was stimulated by factors secreted in the gonad, it is also possible that the maturation of the ASOs is induced by the gonad hormone under the regulation of the CNS.

III-E-2. Regulation of hormone production by the DBs: Dorsal bodies or dorsal body hormones (DBHs) in pulmonates have been proposed to have female gonadotropic effects including promotion of oocyte growth and maturation, vitellogenesis, and development, maturation and secretive activities of female ASOs (Geraerts and Algera, 1972; Geraerts, 1975; Geraerts, 1976b; Wijdenes and Runham, 1976; Joosse and Geraerts, 1983; van Minnen *et al.*, 1983; Wijdenes *et al.*, 1983a; Miksys and Saleuddin, 1985; Schollen and Saleuddin, 1986; Miksys and Saleuddin, 1987; Miksys and Saleuddin, 1988). De Jong-Brink *et al.* (1986) determined that dorsal body hormone (DBH) works on the gonad by activating the adenylate cyclase-cyclic AMP (AC-cAMP) system. However, it has also been shown that the action of AC-cAMP system can only be induced in the follicle cells, the main sites for the

production of the sex steroids in the gonad. It is possible that the production of steroids in the gonad is the result of the activation of AC-cAMP system and is thus regulated by DBH. Therefore, DBH may at least have a tropic function on the production of gonadal hormones, especially those produced in the female gonads. Notably, many of the activities regulated by DBH are also controlled by the gonadal hormones. Therefore, these actions are probably indirectly achieved by control of gonadal hormone production. Although this hypothesis has been suggested by some authors (Geraerts, 1975), it still lacks solid evidence.

III-E-3. Regulation by the tentacles: A masculinizing factor from the tentacles that stimulates spermatogenesis while inhibiting oogenesis has been reported in various studies (Takeda, 1977, 1979, 1982; Gomot *et al.*, 1980). Co-culture of fragments of the ovotestis and the tentacles side by side in the medium stimulated spermatogenesis, but not oogenesis (Gomot *et al.*, 1980). Joosse (1988) suggested that this factor might control the production of androgenic steroids produced in the Sertoli cells. An *in vitro* study performed by Gottfried and Dorfman (1970a) had also demonstrated the action of the tentacles on the production of sex steroids in the gonad. When a fraction of the ovotestis homogenate was incubated with a tentacle homogenate fraction, the metabolism of steroids in the ovotestis was affected. Gottfried *et al.* (1970a) supposed that this effect may be caused by the extensive metabolism of the precursors and final products by the enzymes in the tentacles. While this may hold true in *in vitro* studies, it can not explain the effect of the tentacles on the ovotestis *in vivo*. Therefore further studies are needed to elicit the mechanism of the actions of the tentacles on the production of sex steroids by the gonad.

III-F. Do Sex Steroids Act as Hormones in Molluscs?

It is well known that in vertebrates, sex steroids act as hormones, i.e., they can be transported from the site of synthesis to the target tissues. A direct evidence for a factor to be a hormone would thus be its presence in the blood or haemolymph. Up to now, sex steroids have been reported in the haemolymph of cephalopods (Henry and Boucaud-Camou, 1994; D'Aniello *et al.*, 1996), suggesting that they might act as hormones in these species. It is possible that sex steroids also act as hormones in gastropods and bivalves. However, presence of sex steroids in haemolymph in gastropods or bivalves has not been proved.

IV. Regulation of Reproduction by Sex Steroids in Molluscs

Existing evidence indicates that sex steroids have wide actions in the regulation of reproductive activities such as gametogenesis, vitellogenesis, sex maturation and spawning or egg-laying in molluscs. To help explain the actions of sex steroids, some important reproductive events that are common in molluscs are briefly discussed here.

Gametogenesis, the production of gametes, is the central event in sexual maturation. It involves the accumulation and utilization of substrates and energy in the gonad during gonadal growth, differentiation and maturation. Vitellogenesis, the production of vitellin, is a crucial event in the gametogenesis. Vitellin, also known as yolk protein, is accumulated in the oocytes of oviparous animals during oocyte growth.

IV-A. Effects of Steroids in Gastropods

In the endocrine system described above, sex steroids have been found mainly in the gonad, although they have also been identified in the digestive gland and the accessory sex organs such as the albumin gland and the bursa copulatrix (Gottfried *et al.*, 1967). However, only the gonad has been found to possess steroid-producing cells such as the Sertoli cells in the testis (De Jong-Brink *et al.*, 1977; De Jong-Brink *et al.*, 1981). Presence of sex steroids in other tissues is not an indication that steroids are synthesized there if no steroid-producing cells are present. It may merely suggest that these tissues are targets for sex steroids. Furthermore, a substance that is capable of long term regulation should generally have a long lifetime and a low turnover rate in order for the efficiency of the control. Since sex steroids are the only substances found in the gonads that possess these characteristics, it is reasonable to suppose that some, if not most, of reproduction-related activities of the gonad are mediated by sex steroids.

IV-A-1. Effects of sex steroids on gametogenesis and sex maturation in gastropods: Direct evidence for the effects of sex steroids on gametogenesis has been obtained from *in vivo* experiments in which steroid hormones were injected intramuscularly into the snails (Aubry, 1962; Csaba and Bierbauer, 1979; Csaba and Bierbauer, 1981; Sakr *et al.*, 1992). Aubry (1962) showed that, in the gastropods *H. pomatia* and *L. stagnalis*, injections of estradiol stimulated oogenesis and inhibited spermatogenesis. Testosterone had inverse effects and progesterone stimulated gametogenesis in both sexes. But Csaba and Bierbauer (1979) showed that injection of testosterone stimulated oogenesis more conspicuously than spermatogenesis in the

same species. Testosterone seemed to have a stimulatory effect on spermatozoa maturation and release. 19-nortestosterone had even stronger effects than testosterone in stimulating spermatogenesis (Csaba, 1979; Csaba, 1981). Both estradiol and progesterone increased the number of secondary spermatocytes and primary oocytes in *H. pomatia* (Csaba, 1979; Csaba, 1981). Similarly, injection of testosterone in the land snail *Theba pisana* accelerated spermatogenesis, resulting in an increase in the number of spermatozoa and a decrease in that of primary spermatocytes. Treatment with testosterone also stimulated the growth of male germinal acini. In contrast to the results of Csaba *et al.* (1979), Sakr *et al.* (1992) found that testosterone seemed to inhibit oogenesis in this species, as the number of mature ova was decreased.

In conclusion, it seems that the actions of progesterone are not very sex-specific, with this steroid possibly serving merely as a precursor for the synthesis of other active sex steroids, as is frequently observed in vertebrates. The effects of estradiol and testosterone are generally sex-specific, but there are still controversies about their actions.

IV-A-2. Effects on accessory sex organs (ASOs): Direct evidence for the actions of sex steroids on ASOs is scarce. However, the effects of the gonad on the ASOs are possibly mediated by the actions of the sex steroids produced in the gonad. As discussed in Section II, gonadal hormones may regulate the differentiation, growth and activities of the ASOs in both basommatophoran and stylommatophoran pulmonates. Available evidence strongly suggests the secretion of two sex-specific gonadal hormones, possibly estrogens and androgens, which have been identified in the gonad. This hypothesis was re-enforced by evidence presented by Takeda (1985)

showing that exogenous androgens and estrogens stimulate the development of male and female ASOs, respectively, and these effects were blocked by their respective anti-steroids, antagonists to their specific receptors. Similar effects can also be induced by ovotestis homogenates, in which testosterone and estradiol, as well as 3β-HSD, were found. In another study, Takeda *et al.* (1987) showed that in the terrestrial snail, *Euhadra peliomphala,* testosterone has similar effects as ovotestis homogenate in inducing head-wart development when injected into castrated animals. Thus, it seems that testosterone secreted by the ovotestis may control the development of head-wart.

The effects of gonadal hormones on ASOs can also be inferred from imposex induced by tributyltin (TBT). TBT is widely used as an anti-fouling biocide and catalyst in industry and is known to have significant impacts on molluscs, especially gastropods (Deutsch, 1996; Morcillo *et al.*, 1998; Oberdorster *et al.*, 1998). The major impact of TBT on gastropods is imposex, the phenomenon in which distal male genitals develop in females and blockage of oviducts occurs (Deutsch and Fioroni, 1996). Available studies show that TBT induces imposex by altering the metabolism of steroids in gastropods as well as some bivalves. It has been found that TBT inhibits the activities of cytochrome P450 monooxygenases, which are important in the conversion of estrogen from testosterone and cholesterol to various other steroids (Morcillo *et al.*, 1998; Oberdorster *et al.*, 1998). TBT may also inhibit 17-sulphate conjugation of testosterone and its metabolites as well as their excretion. The consequence is the disturbance of steroid metabolism, especially that of estradiol and testosterone. Most commonly observed is a decrease of testosterone level and an

increase in the estradiol level (Spooner *et al.*, 1991). These changes probably directly induce the development of female characteristics and blockage of the female reproductive tract. Aromatase inhibitor, SH 489 and flavone, can also induce imposex (Bettin *et al.*, 1996). However, anti-androgen cyproterone acetate can repress the imposex induced by TBT, indicating that androgen receptors may also be involved in the induction of imposex (Bettin *et al.*, 1996). An alternative explanation could be that the changes in steroid metabolism affect the production of masculinization factors in the CNS, which then induces the imposex directly.

IV-A-3. Effects on sexual behaviors: The effects of steroids on egg-laying vary with species. In the slugs *Deroceras retculatus* and *L. flavus*, injection of estrogens stimulated egg-laying but with a lower rate of embryonic development while androgens increased the rate of embryo development without affecting egg-laying. If estradiol and dehydroepiandrosterone, an androgenic precursor, were administrated together, or if estrogen and androgen were administrated after removal of the optic tentacles, both egg-laying and development were enhanced. The author also reported that the same effects could be achieved by injection of a gonadotropin and were blocked by injections of metopirone (an inhibitor of steroid hydroxylation) (Takeda, 1977, 1979). It is thus of great interest to confirm that molluscs are able to respond to a vertebrate gonadotropin and the production of sex steroids is under the control of such a gonadotropin-like substance.

IV-B. Bivalves

IV-B-1. Effects of sex steroids on gametogenesis:

IV-B-1-a. Stimulation of gametogenesis by sex steroids: There are relatively few studies on the direct effects of sex steroids on gametogenesis in bivalve molluscs. Mori (1969a) reported the acceleration of sexual maturation by injection of 17β-estradiol in the Japanese oyster. In his subsequent studies, he showed that estradiol stimulated gametogenesis by accelerating glycogenolysis which provided the energy for the process of gametogenesis (more details later). Varaksina and Varaksin (1991) studied the effects of estradiol, progesterone and testosterone on oogenesis in the Yezo scallop *Mizuhopecten yessoensis*. They found that these steroids had similar effects in stimulating oogenesis: stimulation of mitotic growth of the gonad and increases in gonadal weight, gonadal/somatic index and sizes of acini, oocytes and oocyte nuclei. These steroids have also been found to have stimulatory effects in male Yezo scallops (Varaksina, 1992). In the coot clam *Mulinia lateralis*, administration of methyltestosterone promoted a 10-day earlier spawning and an increase in spawning frequency (Moss, 1989). Similar actions of sex steroids are common in vertebrate species (for reviews see Callard *et al.*, 1991; Nagahama, 1994) and may also be common in invertebrates. For instance, in the sea star, an invertebrate from Phylum Echinodermata, *in vivo* injection of estradiol induced an increase in gonad weight and a decrease in pyloric caeca. These results suggested that estradiol stimulated the transfer of energy reserves from the pyloric caeca to the gonad, as well as accelerating gametogenesis and vitellogenesis (Schoenmakers *et al.*, 1981). Thus, it is reasonable to speculate that the mobilization of energy from storage tissues to gonad is a long-term process probably under the regulation of steroids, especially estrogens in female bivalves.

As discussed earlier in Section I-B, four neuroendocrine factors of peptide or protein nature have been identified in *M. edulis* and seem to also have stimulatory effects on gametogenesis (Whittle *et al.*, 1983; Mathieu, 1987; Mathieu *et al.*, 1988; Mathieu *et al.*, 1991). Interactions might exist between these factors and sex steroids; on one hand, these neuroendocrine factors may act as gonadotropin-like factors that stimulate the synthesis and activities of sex steroids; on the other hand, the production of these factors may be under the control of sex steroids.

IV-B-1-b. Regulation of the metabolic processes within the gonad by sex steroids: The major metabolic processes in the gonad during sexual maturation are the breakdown of glycogen, and the synthesis of proteins and lipids. Levels of protein, glycogen, and lipids in the gonad increase with gametogenesis, accompanied by a net gain in gonadal dry weight which then drops dramatically after spawning (Thompson, 1977; Epp, 1988). This trend is in good agreement with the profile of estradiol, the level of which also increases with maturation and decreases after spawning (Matsumoto *et al.*, 1997). These lines of evidence suggest that metabolism of steroids may be closely correlated with metabolic processes in the gonad during the processes of gametogenesis. But it must be noted that this is a dynamic process including both anabolic and catabolic metabolism and the changes in contents are the net effects.

IV-B-1-b-i. Effects of sex steroids on glycogen metabolism: Glycogenolysis, the breakdown of glycogen, is the major metabolic event for glycogen in the gonad during gonadal maturation (Mori *et al.*, 1972a; Mori *et al.*, 1972b); synthesis of glycogen in the gonad during gametogenesis is relatively minor.

Glycogen accumulated in the gonads has three destinations during the process of gametogenesis. The first destination is the oocyte glycogen inclusions that will be utilized for embryonic development. The second destination of glycogen is its incorporation into the vitellin coat layer of the eggs which is also possibly utilized during embryo development (Dorange, 1989). The rest of glycogen is used as the major energy source for gametogenesis. During gametogenesis, glycogen stored in the gonad is broken down into glucose which is further broken down, resulting in the production of ATP, NADPH, and NADH which are necessary for the synthesis of other organic compounds including fatty acids and nucleic acids, etc.

Glycogen content has been observed to be inversely proportional to oocyte diameter throughout the gametogenesis while estradiol level is directly correlated (Barber and Blake, 1981; Matsumoto *et al.*, 1997). These observations suggested that glycogen may be responsible for oogenic development and this involvement is correlated with estradiol.

The interaction between glycogen utilization and estradiol has been studied extensively in the oyster. Injections of estradiol have been reported to accelerate glycogenolysis during sexual maturation in female oysters in both laboratory and field experiments (Mori *et al.*, 1972a; Mori *et al.*, 1972b). Although the exact mechanism underlying this action is not known, it is possible that estradiol exerts its action indirectly through glucose-6-phosphate dehydrogenase (G-6-PD). In mammals, G-6-PD can be induced by estrogen-treatment and is the major regulatory enzyme in the pentose phosphate pathway of glucose breakdown (Cummings and Baker, 1986; Rasmussen *et al.*, 1988; Vessal and Yazdanian, 1995; Murray et al., 1996). It is

known that estradiol affects the activity of G-6-PD at transcriptional, translational and also post-translational levels and these actions can be inhibited by antiestrogens and cycloheximide (Vessal and Yazdanian, 1995). In another invertebrate, the crustacean freshwater prawn, stimulation of the activity of G-6-PD by the injection of estradiol has also been reported. This stimulation can be inhibited by simultaneous injection of tamoxifen, which is an antagonist to estrogen receptor, or cycloheximide which blocks protein synthesis (Ghosh and Ray, 1992; Ghosh, 1994). The direct effects of steroids on G-6-PD have not been studied in molluscs. However, G-6-PD has been detected histochemically in the epithelia of the nephridium, digestive diverticulum, intestine, and other tissues in oysters. The activity of this enzyme was shown histochemically to increase with sexual maturation and decline after spawning in the oyster (Mori, 1967); this trend coincides with that of 17β-HSD and thus estradiol. It thus seems that estradiol exerts its effects through the classical mechanism to stimulate glycogenolysis in bivalves.

On the other hand, NADH produced during glycogen utilization is used in steroidogenesis. Steroidogenesis is triggered at the same time as glycogenolysis occurs. Mori *et al.* (1966) indicated that activity of 17β-HSD, which is responsible for the conversion of estradiol from estrone and testosterone from androstenedione through reduction (Ghosh, 1993), increased with sexual maturation. The consequence is a continuous increase in estradiol content which reaches a peak just before spawning (Matsumoto *et al.*, 1997). Both estradiol concentration and the activity of 17β-HSD reach peaks before spawning and drop thereafter. In areas with severe eutrophication, a state of excess organic nitrogen or phosphorus in the environment

and thus excess algae are present in the water, the scallops may become "over-maturated", as characterized by an abnormal accumulation of glycogen and fatty acids in the gonad. This physiological burden may lead to a decrease in the activity of G-6-PD. Because of the reciprocal relationship between the activity of G-6-PD and 17β-HSD, the activity of the latter may also be decreased, causing the disturbance of steroid metabolism (Mori *et al.*, 1966). Disturbance of steroid metabolism which intensifies the physiological burden before and during spawning may contribute to the seasonal mass mortality in areas with severe eutrophication (Mori, 1979).

IV-B-1-b-ii. Effects of sex steroids on protein synthesis during gametogenesis: As the major components of the oocyte, proteins are actively synthesized in the gonad. Variations of protein levels in the gonad also coincide with sexual maturation cycles (Barber and Blake, 1981; Epp *et al.*, 1988). It is thus possible that steroids are related to protein synthesis during gametogenesis. It has been known that steroids stimulate an increase in the content of total protein in the gonads of some invertebrate species such as the sea star *Sclerasterias mollis* (Barker and Xu, 1993). This may also hold true for molluscs, although no similar study is available yet. However, evidence exists suggesting that steroids may regulate gametogenesis by altering expression of some important gametogenesis-related proteins including 17β-HSD, G-6-PD, and vitellin.

Presence of 17β-HSD has been demonstrated in various molluscan species including *Patinopecten yessoensis* (Varaksina and Varaksin, 1988; Matsumoto *et al.*, 1997), *Crenomytilus grayanus* (Varaksina and Varaksin, 1988), *Mytilus edulis* (De Longcamp, 1974), and *Crassostrea gigas* (Mori *et al.*, 1965; Mori *et al.*, 1966; Matsumoto *et al.*, 1997). One of its functions may be to stabilize and retain steroids in

the gonad. Thus with maturation the accumulation of estrone in the ovaries of *Patinopecten yessoensis* and *Crassostrea gigas* may be the result of the activity of 17β-HSD (Matsumoto *et al.*, 1997).

It has been noted that the activity of 17β-HSD in bivalves varies with sexual maturation: it increases with gonad development and decreases after spawning. This trend coincides with that of estradiol and estrone. Unfortunately, no study has been carried out to determine whether 17β-HSD is induced directly by estradiol in molluscs. However, in other invertebrates such as the freshwater prawn, injection of estradiol induced an increase in the activity of 17β-HSD. This effect can be inhibited by concurrent injection of tamoxifen and cycloheximide, suggesting that estradiol increases 17β-HSD through the activation of its receptor (Ghosh *et al.*, 1993). Thus, it is possible that the increase in the activity of 17β-HSD with sexual maturation in molluscan species is also induced by estradiol. Production of NADH in the utilization of glycogen during sexual maturation may also indirectly potentiate the activities of 17β-HSD.

G-6-PD is an important enzyme involved in the utilization of glycogen. Activity of G-6-PD is essential for the production of NADH which is needed for the synthesis of steroids and lipids during sexual maturation. It is also a key enzyme in the hexose monophosphate shunt pathway in the synthesis of steroids (Ghosh *et al.*, 1994). As discussed in the preceding section, G-6-PD is an estrogen-driven enzyme in vertebrates and some invertebrates, probably also in molluscs. In the process of sexual maturation, G-6-PD plays a central role in the interaction of glycogenolysis, lipidogenesis and steroidogenesis.

In vertebrates, vitellogenesis can be induced by sex steroids such as estradiol (Elliott *et al.*, 1979; Maitre *et al.*, 1986). This phenomenon is also seen in some invertebrates including sea stars (Schoenmakers *et al.*, 1981; Takahashi and Kanatani, 1981) and crustacea (Ghosh and Ray, 1992).

Compared with other phyla, little work has been done in molluscs, although vitellin was identified in *Crassostrea gigas* (Suzuki *et al.*, 1992) and *Patinopecten yessoensis* (Osada *et al.*, 1992b). The latter authors also suggested that vitellogenesis in the scallops may be under the control of estradiol. This hypothesis was then supported by Li *et al.* (1998) who showed that the accumulation of vitellin in oyster ovaries is synchronized with the profile of estradiol, total protein synthesis (as indicated by RNA/DNA ratio), and oocyte growth during sexual maturation. Furthermore, vitellin content in the ovaries is increased by the application of estradiol *in vitro* and *in vivo*. It is notable that vitellin was not identified in the liver of oysters and scallops, but whether it is synthesized in the oocytes or the follicle cells is not known at the present time.

IV-B-1-b-iii. Actions of sex steroids on lipidogenesis: Lipid synthesis is essential for gametogenesis because lipids are needed not only for the cell membranes but also for the synthesis of lipoproteins including vitellin. An increase in *de novo* fatty acid synthesis has been observed in the female oysters in the late stages of gametogenesis (Kluytmans *et al.*, 1985).

Lipid synthesis in molluscs may be regulated by estrogens. In the oyster *C. gigas*, the biosynthesis of lipids during vitellogenesis is associated with glycogenolysis (Ruiz *et al.*, 1992). Conversion of glycogen to lipids during

vitellogenesis in bivalves has been suggested by Gabbott (1975). Utilization of glycogen provides substrates such as glucose and $NADPH_2$ for the biosynthesis of lipids. Enzymes such as G-6-PD in the pentose phosphate pathway and malate dehydrogenase in the Krebs cycle, both of which are estrogen-responsive in mammals, are closely related to lipidogenesis (Moulton and K. L. Barker, 1971; Murray et al., 1996). Ghosh *et al.* (1994) showed that injection of estradiol increased the activity of G-6-PD and malate dehydrogenase in the ovary of the freshwater prawn. It is possible that steroids also regulate biosynthesis of lipids through these enzymes in molluscs. Evidence from mammals and other invertebrates may lend support to this hypothesis. It is well known that estrogens induce lipid synthesis in mammals (Emersen *et al.*, 1979). In the sea star, *in vivo* injection of estradiol has also been reported to increase the lipid level in the pyloric caeca of females (Van der Plas *et al.*, 1982). However, no direct study has ever been done on the direct effects of steroids in lipid biosynthesis during gonad development in bivalves, or any other molluscs.

IV-B-1-c. Effects of sex steroids on mobilization of nutrients and energy to the gonad: When food is abundant, nutrients are stored in various tissues in forms of glycogen, lipid, and protein prior to periods of sex maturation. Glycogen is mainly stored in the adductor muscle of scallops, and in the mantle and connective tissues of other bivalves such as oysters and mussels. The major storage site for lipids is the digestive gland in most species and the adductor muscle in other species. Proteins are mainly stored in the adductor muscle or liver in bivalves. These nutrients are transferred to the gonad as sexual maturation proceeds due to high metabolic demands

at this specific period.

It has been observed that seasonal changes in energy reserve and body mass coincide with levels of steroids. As gametogenesis proceeds, increases in gonad weight are simultaneous with decreases in dry weight of storage tissues such as the adductor muscle (Ansell, 1974; Taylor and Venn, 1979; Barber and Blake, 1981; Epp, 1988). These changes are accompanied by decreases in the levels of glycogen (Gabbott, 1975; Martinez and Mettifogo, 1998), lipids and proteins (Barber and Blake, 1981) in storage tissues. Meanwhile, it is notable that the levels of estradiol increase with sexual maturation and decreases after spawning (Matsumoto *et al.*, 1997). The coincidence between the variations of steroids and the cycles of nutrients and energy transfer suggests that the metabolism of steroids may be closely related to nutrient mobilization. In *Mytilus edulis*, steroids induce lysomal activity in adiposgranular cells which are responsible for the transfer and utilization of energy reserves during gametogenesis (Moore *et al.*, 1978a; Moore *et al.*, 1978b). Peek *et al.* (1989) suggested that steroids might be involved in the hormonal control of the breakdown of these cells. Therefore, it is possible that sex steroids also regulate mobilization of nutrients and energy to the gonad during sex maturation.

IV-B-2. Effects of steroids on gamete release and spawning: Induction of gamete release by 5-HT in bivalves is a well-known phenomenon and has been widely applied in bivalve aquaculture (Matsutani and Nomura, 1982; Gibbons, 1984; Tanaka and Murakoshi, 1985). The induction of egg release can be blocked by 5-HT antagonists, such as methysergide and methiothepin, suggesting that the induction may be achieved via specific 5-HT receptors (Matsutani and Nomura, 1987; Fong *et*

al., 1994a; Ram *et al.*, 1996). The presence of 5-HT receptors has been demonstrated on the surface of egg and sperm membrane of different molluscan species by radioligand receptor binding studies and pharmacological studies. Receptor subtypes found in molluscan gametes include 5-HT_1 (Japanese scallop, Pacific oyster, zebra mussel and surf clam), 5-HT_2 (Japanese scallop and zebra mussel) and 5-HT_3 (surf clam) (Bandivdekar *et al.*, 1991; Fong *et al.*, 1993; Fong *et al.*, 1994a; Osada *et al.*, 1998).

Notably, 5-HT receptors on the oocyte surface can be induced by treatment with estradiol. Osada *et al.* (1998) found that during sexual maturation, increases in specific binding of 5-HT to oocyte membrane preparations are directly correlated with increases in oocyte diameters, which in turn correspond well with the variation of the level of estradiol in the gonad. They also demonstrated that estradiol can significantly increase both 5-HT binding and egg release induced by 5-HT. The potentiation can be abolished by actinomycin D, which is an RNA synthesis inhibitor. These results suggest that estradiol controls the expression or activity of 5-HT receptors on the surface of the oocyte and thus the sensitivity to 5-HT stimulation.

The induction of spawning by 5-HT can also be blocked by aspirin and indomethacin, inhibitors to prostaglandin synthesis (Matsutani, 1987). Also, neither aspirin nor indomethacin can block the 5-HT effect if prostaglandin E_2 (PG E_2) was also applied (Matsutani, 1987). These results suggested that prostaglandin is involved in the induction of spawning induced by 5-HT. Thus, it is possible that estradiol achieved its effects on 5-HT-induced spawning by increasing the production of either 5-HT receptors or prostaglandins.

In mammals, estradiol increases the synthesis of cyclooxygenase and phospholipase A, which are responsible for the synthesis of prostaglandins, and estradiol has been shown to regulate the level of prostaglandins in the ovary. Estradiol has also been reported to stimulate the synthesis of prostaglandin in scallops (Osada and Nomura, 1990), so the enhancing effect of estradiol might be achieved through the synthesis of prostaglandins. Catecholamines may also be involved in the regulation of spawning by steroids. The level of catecholamine has been shown to be affected by estradiol treatment (Osada and Nomura, 1989). In the scallop *Mizuhopecten yessoensis*, a possible dopamine receptor has been suggested and can increase the level of cAMP when stimulated. This effect can be blocked by galoperidol, a specific blocker of dopamine receptors (Khotimchenko *et al.*, 1989). Since there is conflicting evidence of the involvement of catecholamine in spawning (Mori *et al.*, 1972a; Mori *et al.*, 1972b; Matsutani and Nomura, 1987; Osada *et al.*, 1992a; Smith and Croll, 1997; Pani and Croll, 2000), it is not certain whether estradiol also functions by affecting the release of catecholamines.

IV-B-3. Effects of sex steroids on sex reversal: It is well known that sex steroids induce sex reversal in vertebrates such as fish, amphibians and reptiles (Vannini and Stagni, 1967; Yamamoto and Kajishima, 1968; Tang *et al.*, 1974; Dournon *et al.*, 1990; Chang *et al.*, 1995). In bivalves, there is evidence indicating that sex reversal can be induced by sex steroids, although the results were not statistically tested. Mori *et al.* (1966) studied the effects of estradiol on the sex ratio of the oyster *Crassostrea gigas*. They showed that injection of estradiol into the oysters at early stages of sex maturation induced reversal from male to female, resulting in a greater sex ratio

(female/male). But if the treatment was performed at later stages, the sex ratio was not affected. In the coot clam, *Mulinia lateralis*, methyltestosterone fed to spawned animals increased the ratio of male/female from 0.8 to 1.6 (Moss, 1989). Since sex control may have potential application in scallop aquaculture, well-designed and controlled studies are needed to determine the possible effects of sex steroids in sex determination.

IV-B-4. Effects of sex steroids on metal detoxification during reproductive cycles: The metal detoxification capability of animals is largely due to the binding of metals by metallothioneins, metal-binding proteins. As a result, the metals are accumulated in the animals during detoxification. Coimbra and Carraca (1990) showed that the levels of accumulation of Fe, Zn, Cu and Cd in the mussel *Mytilus edulis* vary with reproductive cycles, with maxima occurring at the stages of sexual maturity and spawning. Since their profiles correlated well with those of estrogens and progesterone, Coimbra and Carraca (1990) hypothesized that synthesis of metallothioneins may be driven by estrogens during sexual maturation. Induction of metallothioneins by sex steroids has been extensively studied in vertebrates (Bracken and Klaassen, 1987; Blazka and Shaikh, 1991; Kuo and Leavitt, 1999). In addition, Zn-binding metallothionein may provide a way to maintain the Zn level for zinc fingers of sex steroid receptors, as the exchange of Zn from metallothioneins to estrogen receptor has been observed (Cano-Gauci and Sarkar, 1996).

IV-C. Effects of Sex Steroids in Cephalopods

In cephalopods, the reproductive system is known to be under the control of

the optic gland, which in turn is controlled by the CNS, possibly through the actions of neuropeptide FMRFamide and gonadotropin-releasing hormone (D'Aniello *et al.*, 1996).

Until recently, few studies have been conducted on the functions of sex steroids in cephalopods. Available studies indicated that sex steroids may be involved in the formation of egg capsules. In *Sepia officinalis*, the egg capsule is composed of polysaccharides secreted by the oviducal gland and the nidamental gland. Henry and Boucaud-camou (1993) showed that synthesis of polysaccharides by the nidamental gland is controlled by a gonadal factor. Henry (1994) demonstrated that progesterone can stimulate the incorporation of [^{14}C]-glucose incorporation into polysaccharides secreted by the nidamental cells. Progesterone has been identified in the follicle cells of the gonad, as well as haemolymph, in females. Furthermore, the concentration of progesterone is directly correlated with the multiplication of follicle cells during vitellogenesis, suggesting that the follicle cells are the endogenous origin of progesterone or are responsive to progesterone. Thus it is possible that progesterone from the gonad regulates the formation of egg capsules during sexual maturation.

V. Sex Steroid Receptors in Molluscs

Despite the studies on the actions of sex steroids in molluscs discussed in previous sections, little research has investigated the mechanisms of the actions of sex steroids in molluscs. A few studies suggested that these actions might be achieved through the classical mechanism (Takeda, 1977, 1979, 1985). However, direct evidence of the presence of sex steroid receptors in molluscs is sparse.

Sex steroid receptors in vertebrates belong to the superfamily of nuclear receptors. A distinguishing character of a nuclear receptor is that it possesses two Zinc-fingers, which are Cys_4 domains stabilized by a Zn^{++}. This structure is essential for recognizing a specific DNA sequence called the Hormone Responsive Element (HRE). The presence of these two Cys_4 domains has been used as a strategy for identifying nuclear hormone receptor genes. For example, based upon this feature, Kostrouch *et al.* (1995) designed degenerate primers with which they amplified and cloned three steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. Steroid receptor genes with two Cys_4 domains were also cloned in the parasitic nematodes *Strongyloides stercoralis* (Siddiqui *et al.*, 2000) and *Onchocerca volvulus* (Yates *et al.*, 1995; Unnasch *et al.*, 1999). However, none of them seem to be a sex steroid receptor based on nucleotide sequence similarity comparisons.

The sequencing of the entire genome of some organisms including *C. elegans* and *Saccharomyces cerevisiae* provides us with further possibilities of looking for the existence of steroid hormone receptor genes in invertebrates. Based on the information of the presence or absence of the two Cys_4 domains, no genes with high sequence similarity to steroid receptors were found in *Methanococcus jannaschii*, *Escherichia coli*, or *S. cerevisiae*. However, up to 233 hormone receptor genes were predicted in the *C. elegans* genome (Clarke and Berg, 1998), although no strong similarity was demonstrated with known sex steroid receptors.

Receptors for steroids other than the sex steroids have also been found in higher invertebrates such as arthropods and echinoderms (Evans, 1988; Chan *et al.*, 1992). A well-studied example is the ecdysone receptor which is widely identified in

the arthropods and also nematodes (Barker *et al.*, 1991). The ecdysone receptor is one of the major steroid receptors functioning in the ecdysone-induced responses during the molting and metamorphosis of the larvae (Riddiford, 1993). Other steroid receptors involved include *usp, knirps, knirps-related, FTZ-F1, DHR3, embryonic gonad, tailless* and *seven-up* (for reviews, see Segraves, 1991; Oro *et al.*, 1992; Segraves, 1994; Buszczak & Segraves, 1998). However, none of them seems to be capable of binding specifically sex steroids. In fact, they generally do not have reproductive regulation activities.

Despite the presence of non-sex steroid receptors in these invertebrate organisms, no sex steroid receptor has been positively identified thus far in the invertebrates. However, sex steroid receptors have been predicted in invertebrates. Based on phylogenetic analysis of the known sequences of steroid receptors from vertebrates and invertebrates, it is supposed that sex steroid receptors in vertebrates may have originated from an ancestral sex steroid receptor in the invertebrates during evolution (Baker, 1997; Ohno, 1999). The number of gene loci of vertebrates (60,000) is about four times of that of invertebrates (15,000) due to duplications of the genome which were supposed to occur during the transition from invertebrates to vertebrates (Ohno, 1999). Thus, an ancestral gene in the invertebrates may be expected to evolve into four genes in the vertebrates. Based on the parsimony analysis of the steroid receptor genes, it is proposed that receptors for progesterone, androgen, mineralocorticoid and glucocorticoid in vertebrates originated from one common ancestral steroid receptor in the invertebrates while estrogen receptor and estrogen-like receptors evolved from another ancestral receptor (Baker, 1997; Ohno, 1999).

Therefore, Baker (1997) predicted that the ancestor for sex steroid receptors may exist in a tunicate or *Amphioxus*, and may be closest to estrogen receptor based on phylogenetic analyses. According to these predictions, there should be no sex steroid receptor in organisms lower than invertebrate chordata. However, Laudet (1997) proposed that the diversification of this ancestral receptor may have occurred much earlier, during the multicellularization event which led to the emergence of the metazoan phyla. If this were the case, sex steroids should exist at least in some of the invertebrates and may have reproductive regulation functions.

Some existing evidence supports the presence of sex steroid receptors in the invertebrates. In the sea star *Asterias rubens*, a high affinity site specific for estradiol was characterized by binding studies (De Waal *et al.*, 1982). In molluscs, binding sites for estrogen, progesterone and testosterone with dissociation constant values comparable to those of vertebrate have been characterized in *Octopus vulgaris* (D'Aniello *et al.*, 1996). In addition, as we have discussed earlier, some actions of sex steroids in molluscs can be blocked by antisteroids or RNA or protein synthesis inhibitors (Takeda, 1977; Takeda, 1979; Matsutani and Nomura, 1987).

In general, sex steroid receptors may exist in molluscs and mediate the actions of sex steroids in reproductive control.

VI. Potential Impacts of Endocrine Disruption Chemicals on Molluscs

Sufficient evidence exists indicating the presence of the endocrine disruption

chemicals (EDC) that mimic or affect the actions of sex steroids, especially estrogen and androgen, in the aquatic environment. These compounds include synthetic estrogens and androgens, industrial chemicals that have estrogenic or androgenic effects (such as nonylphenol and pesticides), and those substances such as tributyltin which interfere with the metabolism and dynamics of the steroid hormones in organisms.

The actions of many of the EDCs are thought to be achieved through their interference with steroid receptors. By binding to the steroid receptors, they either act as agonists by eliciting similar effects as endogenous hormones, or as antagonists by blocking the effects of endogenous steroids (DeRosa *et al.*, 1998). For example, it has been reported that vitellogenesis in fish can be induced by alkylphenol polyethoxylate surfactants (Jobling, 1996). Feminization of male fish exposed to estrogenic chemicals in effluent from sewage-treatment works and industry wastes has also been well documented (Harries, 1999). No work on EDCs other than TBTs has ever been carried out up to now in molluscs. However, as the importance of sex steroids in molluscan reproductive endocrine receives closer attention, steroidal EDCs may possibly be found to have profound impacts on molluscan reproduction. We may expect more investigations on this issue in the future.

Other non-steroid EDCs may act by altering the metabolism of steroids and causing imbalances in endocrine functions in the animals (DeRosa *et al.*, 1998), such as TBTs, as discussed earlier in Section IV-A-2.

VII. Conclusions and Objectives of the Thesis

Vertebrate type sex steroids have been found at physiologically significant levels in many molluscan species. Existing evidence indicated that these steroids are synthesized in the reproduction-related organs under the control of other higher regulatory centers such as the CNS.

The correlation between the fluctuation of sex steroids and the reproductive cycles suggested that these sex steroids might be involved in the regulation of reproduction in molluscs. The most direct evidence for this hypothesis comes from experiments employing injection of sex steroids into the animals which demonstrated that sex steroids influence gametogenesis and sexual maturation in molluscs. One of the most important actions of sex steroids in these processes may be their regulation of metabolism and mobilization of nutrients. This action may be achieved through the control of the activity of critical enzymes by sex steroids. Another well-studied phenomenon of sex steroids is their actions in gamete release. Estradiol has been found to stimulate egg release induced by 5-HT in scallops. Sex steroids have also been found to be involved in the induction of sex reversal, sex determination, sex behaviors and detoxification during reproductive cycles.

Based on the discussion above, we hypothesize that sex steroids may be extensively involved in reproductive regulation in molluscs. Their effects may be achieved through the same mechanisms as in vertebrates. Sex steroid receptors may exist in molluscan species. This thesis is thus aimed at testing these hypotheses.

I first examined the effects of sex steroids on sexual differentiation of juvenile scallops and on gamete release/spawning in mature adults. Then I tried to determine

the underlying mechanisms that are responsible for these actions. To ascertain the existence of sex steroid receptors in molluscs, estrogen receptor were characterized using radio-labeled ligand receptor binding assays and confirmed by molecular biology methods including RT-PCR and Northern blotting.

Materials and Methods

I. Animals and Chemicals

Juvenile and adult sea scallops, *Placopecten magellanicus*, were obtained from the Great Maritime Scallop Trading Co. (Chester, Nova Scotia) and collected from Mahone Bay, Nova Scotia. After their arrival in the laboratory, the scallops were cleaned by removing fouling and dirt from the shells and kept in the tanks with running natural seawater maintained at 14-16°C in the wet lab of the Aquatron Facility of Dalhousie University.

All the steroids used in these studies, including 17β-estradiol, testosterone, progesterone and dehydroepiandrosterone (DHEA) were purchased from Sigma Chemical Co. (Missasauga, Ontario). Isotopes [^{3}H] estradiol (143 Ci/mmol, 250 μCi), [^{3}H] progesterone (85 Ci/mmol, 250 μCi), and [α-^{32}P] dCTP (3000Ci/mmol, 250 μCi) were purchased from Amersham Pharmacia Biotech Inc (Piscataway, NJ). Cycloheximide and actinomycin D were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Formamide and formaldehyde were bought from Fisher Scientific Canada Ltd (Nepean, Ontario). The remaining chemicals were all bought from Sigma Chemical Co. if not otherwise specified.

II. Histological Procedures

The haematoxylin & eosin Y staining method modified from that of Wallington (1972) was employed to determine the sexes of animals in the juvenile scallop injection experiments. Briefly, the tissues were fixed in ethanolic Bouin's for

solution (see Appendix 1 for recipes) for 24-36 hours and stored in 70% ethanol. They were then dehydrated in an ascending series of ethanol solutions and embedded in paraffin. The entire gonad from each animal was sectioned saggitally at 10 μm thickness. Serial sections were mounted on glass slides and then cleared in xylene and rehydrated in descending series of ethanol solutions. After washing in tap water, they were stained in Mayer's Hematoxylin for 3 minutes. After soaking in Scott's tap water for 1 minute, the slides were stained in ethanolic Eosin Y solution for another 1 minute. After being dehydrated in serial ethanol solutions and cleared in xylene, the slides were mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

The sections were observed under a Leitz Aristoplan compound microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). Pictures of representative sections were taken with a Leitz Orthom at E automatic microscope camera (Wild Leitz, Wetzlar, Germany) using Kodak Select 200 color film (Kodak Canada Co., Toronto, Ont.). Negatives were digitally scanned using PhotoSmart scanner (Hewlett Packard Company, Palo Alto, CA), and the images were assembled and labeled with Photoshop 5.0 (Adobe Systems Incorporated, San Jose, CA).

III. Effects of Steroids on Gonadal Differentiation and Sex Determination in Juvenile Scallops

Juvenile scallops used in these experiments were 5.87 ± 0.68 gram (n=60, mean ± standard error (SE)) in average weight and 3.57 ± 0.11 cm (n=60, mean ± SE) in average shell height. Histological examination of representative specimens, using the procedures described above, indicated that no distinguishable gametes were

present in these juveniles (see Figure 3A).

The scallops were acclimated to laboratory conditions for at least 24 hours before injections were performed. The steroids used for injections, including 17β-estradiol, testosterone, progesterone, and dehydroepiandrosterone (DHEA), were first dissolved in 5 volumes of 100% ethanol and then mixed with 95 volumes of corn oil to make 1000 μg/ml stock solutions. Thirty micro-liters of one of the steroid solutions were injected into the adductor muscle of each animal. Another group of animals injected with 30 μl of the mixture of ethanol and corn oil (5:95) were used as vehicle controls. A final group of animals without any injections were used as blank controls. The injections were performed once every month for three continuous months. The experiments were repeated three times using different batches of animals.

All scallops were kept in the same tank with natural running seawater maintained at 14-16 °C and fed with the alga *Isochrysis galcilis* Clone Tahiti (TISO). The light cycle was maintained on a 16:8 (L:D) schedule. After three months, the scallops were dissected. The growth parameters including total body weights, soft body weights, gonad weights, and shell heights were measured. Histological examinations of the gonads were performed according to the procedures described in Section II. The scallops were determined to be a female if female gametes were present, a male if male gametes present, a hermaphroditic animal if both female and male gametes were present, or as undifferentiated if no gametes were detected.

Statistic analyses of the data were performed using the statistical package for Social Sciences (SPSS, SPSS Science, Chicago, IL). The growth parameters were statistically analyzed by two-tailed one-way analysis of variance (ANOVA) to

determine if there is a significant effect (Zar, 1996). Statistical differences in the differentiation rate and sex ratio were analyzed using Chi-square test. To measure any possible bias in determining the sexes of the animals, tests were performed by asking three persons to examine the same 20 unmarked slides. No significant inter-observer difference was found ($P>0.05$, Chi-square tests).

IV. Effects of Steroids on Gamete Release and Spawning

IV-A. *In Vitro* Effects of Sex Steroids on 5-HT-induced Egg or Sperm Release

Mature female and male scallops (100-120 mm in shell height) were kept in the laboratory under the same conditions as described above. The gonads were removed from the scallops and cut into small cubes of 2-3 mm per side length. The caps of 1.5 ml Eppendorf tubes were removed and pieces of 0.5 mm × 0.5 mm mesh were glued to the openings of the tubes. Portions of the tube bottoms were cut off to make openings and handles. The gonad pieces were put on the screens of the Eppendorf tubes which were placed in six-well plastic culture plates (15 ml/well). They were incubated with one of the steroid solutions (as described below) for 2 hours in the first set of experiments to determine the effects of different sex steroids. For experiments to examine the time scales of the effects, the gonad pieces were incubated with sex steroids for 15 or 30 minutes, instead of 2 hours. After the steroid treatments, the gonad pieces were washed and incubated with 10^{-5} M 5-HT for 90 minutes. The 5-HT concentration was chosen at 10^{-5} M based on results from preliminary experiments which showed that maximum effects of sex steroids could be observed at this concentration. All the incubations were performed at 15 ^{o}C.

Sex steroids, including testosterone, estradiol, progesterone, dehydro-epiandrosterone (DHEA) and 4α-androstrerone, were first dissolved in 100% ethanol and then diluted with artificial seawater (ASW, Marine Enterprises International, Baltimore, MD) to make 10^{-8}-10^{-4} M steroid solutions. The percentage of the volume of ethanol in the final solution was always less than 0.5%. Preliminary studies showed that presence of such a low level of ethanol had no effect on gamete release.

At the end of the experiments, the gonad pieces were removed, blotted on filter papers, and weighed. The eggs or sperm in each well were well mixed by bubbling a stream of air into the medium. In experiments with females, three separate 1 ml samples of the medium bathing each piece of gonad were placed into Petri dishes with 5 mm cross-grids marked on the bottom and the number of eggs were counted under a dissecting microscope. The average of these triplicate measures was used to calculate the total number of eggs released from each gonad piece. In experiments with males, a haemocytometer was used to measure the density of the sperm in the medium and the total number of sperm released from each gonad piece was calculated using the average of triplicate measures. The effects of steroids were assessed by comparing the numbers of eggs or sperm released per milligram (mg) wet weight of gonad. The number of released eggs or sperm per mg of gonad induced by 5-HT alone without pre-incubation with steroids was used as a control.

The data were standardized to percentages of the response in the control group. Statistic analyses of the data were performed using the SPSS statistical software package (SPSS Science, Chicago, IL). Briefly, the raw data were tested for normality of distribution using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and the

homogeneity of the variance using the Levene test. When normality or homogeneity assumption of the raw date was violated, the raw data were transformed as their natural logarithm and retested. If both the normality and homogeneity were met, two-tailed one-way ANOVA was performed to determine if the treatment had any significant overall effect. If a significant effect was detected, one-tailed Dunnett's tests were then performed to compare each experimental group with the control group with a prediction based on the statistical results of the overall ANOVA. In case that either the normality or homogeneity was not met with the transformed data, non-parametric analysis was performed using Kruskal-Wallis test (Zar, 1996).

IV-B. Morphological Observation of Parthenogenetic Larvae:

In some groups of the *in vitro* experiments described above, parthenogenetic embryos, i.e., embryos developed from unfertilized eggs were observed. The number of such embryos together with the number of the eggs from the same group was obtained to calculate the percentage of parthenogenetic embyos in this group. These embryos were transferred from the culture plates to Petri dishes containing clean seawater 24 hours after the 5-HT incubation and kept at 14-16 ^{o}C. Every 24 hours, 5-10 embryos were sampled and fixed in 4% PFA for 1 hour. The fixed embryos were then transferred on glass slides and observed under a compound microscope. Pictures were taken, assembled and labeled as described in Section II.

IV-C. Mechanisms of Actions of Sex Steroids on 5-HT-induced Gamete Release:

Pharmacological studies were designed to determine whether the actions of sex steroids on 5-HT-induced gamete release are mediated by the activation of

specific sex steroid receptors and subsequent transcriptional regulation. Tamoxifen (1.25 μM), flutamide (2.5 μM), and RU486 (1 μM) (antagonists to estrogen, androgen and progesterone receptors, respectively; Lazier, 1987; Maentausta *et al.*, 1993; Mcleod, 1993), as well as actinomycin D (8 μM) and cycloheximide (10μM) (inhibitors of RNA and protein synthesis, respectively; Lepran et al., 1982; Fahrbach, *et al.*, 1994), were tested to determine if they could block the actions of steroids in the *in vitro* assays.

In these experiments, the gonad pieces were incubated with one of the anti-steroids or RNA or protein inhibitors for 15 minutes before treatment with sex steroids and 5-HT as described above. The optimal concentrations of sex steroids obtained from the above *in vitro* experiments were utilized here. As in the *in vitro* experiments, the numbers of eggs or sperm released per milligram of gonad were used as indices for the effects of sex steroids. Gonad pieces pre-incubated with ASW alone, instead of anti-steroids or RNA or protein inhibitors, followed by sex steroids and 5-HT treatments, were used as controls for comparison of the effects of these antagonists. Other groups of gonad pieces were pre-treated with one of the antagonists or inhibitors for 135 minutes followed by a 90-minute 5-HT treatment, in order to determine whether the antagonists or RNA or protein inhibitors have any effects by themselves on 5-HT-induced gamete release.

Tests of the normality of the data and necessary transformation were performed for all the data as described in the Section IV-A. For all subsequent statistic tests, homogeneity of the data were first tested using Levene test. The data from the blank control group that was not treated with any drug were compared with those

from the group treated with 5-HT only by Student's t-test to ascertain that the scallop gonad pieces were responsive to 5-HT induction. A Student's t-test was also performed to compare the data from the 5-HT control group and that from the group exposed to both one of the steroids and 5-HT to determine whether sex steroid treatment had any effect. Then a two-tailed ANOVA was performed including the groups incubated with both sex steroids and 5-HT with or without pretreatment with one of the antisteroids or RNA or protein synthesis inhibitor. If a significant effect was detected, one-tailed Dunnett's tests were then performed to compare the antisteroid or inhibitor groups with the group that were exposed to the sex steroid and 5-HT only. Another two-tailed ANOVA was performed including the groups incubated with one of the antisteroids or inhibitors followed by 5-HT treatment alone and the 5-HT control group. Non-parametric analysis was employed using Kuskal-Wallis test when the normality or homogeneity of the data was violated.

IV-D. *In Vivo* Effects of Sex Steroids on Scallop Spawning:

IV-D-1. Direct induction of spawning by sex steroids: Mature scallops were acclimated to the laboratory conditions for at least 24 hours. Before the experiments, the scallops were matched according to their ripeness and size and numbered. The ripeness of the scallops was assessed using the standards of Naidu (1970). Only those at stage VII were considered to be fully ripe and used for the experiments. At this stage, the gonads were fully distended with no visible intestines.

The sex steroids 17β-estradiol, testosterone and progesterone were first dissolved in ethanol and then diluted with ASW to1000 μg/ml. The content of ethanol

in the vehicle was 1%, which had been proved to have no effect on spawning induction in preliminary experiments. Aliquots of 200 μl of steroid solution or the vehicle solution alone were injected into the gonads of scallops in the experimental or control groups. The scallops were then placed in separate 20 cm × 12 cm × 27 cm plastic trays filled with natural seawater and observed for 3 hours to record the response time of spawning after injection. This time delay was referred to as spawning latency. After 3 hours, the eggs or sperm in the trays were sampled and counted using the methods described above in section IV-A. After the experiments, the gonads were removed from the scallops and weighed. The numbers of eggs or sperm spawned per milligram of gonad and spawning latency were compared between the steroid-injected groups and the control group by t-test.

IV-D-2. *In vivo* effects of sex steroids on 5-HT-induced spawning: Scallops were matched, numbered, and injected with steroid solutions or the vehicle solution into the gonads using the procedures described above. Scallops which spawned after steroid or vehicle injections alone were discarded from further analysis. The remaining scallops were maintained in the tanks with running seawater for 24 hours. The scallops were then injected with 0.2 ml of 10^{-4} M 5-HT in ASW into the gonads. After the 5-HT injection, the scallops were housed in separate plastic trays and monitored to record the spawning latency. After 3 hours, the eggs or sperm in the trays were sampled and counted and the data were analyzed as described above.

IV-D-3. Statistical analyses: Statistic analyses of the data were performed using the SPSS statistical package (SPSS Science, Chicago, IL). After checking the normality of the distributions of the data with the Kolmogorov-Smirnov and Shapiro-Wilk tests,

Student's t-tests were performed to detect the difference between the experimental and the control group.

V. Radioligand Receptor Assays:

V- A. Preparation of Cell Extracts:

Scallops were opened by cutting the adductor muscle with a scalpel blade. The soft bodies were removed and dissected in cold ASW (4 °C) under a dissecting microscope. The gonads were removed and minced with razor blades in Petri dishes containing cold homogenization buffer (see Appendix 1 for recipes of this buffer and subsequent buffers or solutions). The protocol for the preparation of cell extracts were adapted from Smith and Thomas (1980) and Cuevas *et al.* (1992) with modifications. They were then washed twice by mixing with cold homogenization buffer followed by centrifugation to remove haemolymph and suspended in the same buffer. The minced tissues were homogenized using 3 strokes of an Ultra-Turrax-Antrieb T25 homogenizer (Staufen, Germany) at high speed with cooling on ice between pulses. The homogenate was then centrifuged at 1000 ×g at 4 °C for 30 minutes and the supernatant was set aside. In another tube, an equal volume of 0.5% dextran-coated charcoal (DCC) solution to that of the homogenate was centrifuged for 10 minutes at 4 °C. The supernatant of the homogenate was then mixed with the pellet of the DCC solution and incubated for 30 minutes at 4 °C. The mixture was again spun at 1000 ×g for 10 minutes to collect the supernatant. The supernatant was then ultracentrifuged at 100,000 g at 4 °C for 60 minutes. The derived supernatant was referred to as the cytosolic fraction.

The pellet from the first centrifugation of the homogenate above was rinsed with washing buffer for three times. After the last wash, the pellet was resuspended and incubated in extraction buffer for 60 minutes at 4 °C with shaking. The supernatant was then treated with DCC as described above and ultracentrifuged at 100,000 ×g for 1 hour at 4 °C. This derived supernatant was referred to as the nuclear fraction.

The protein contents of the cell extracts were measured according to the procedures of Bradford (1976). All the extracts were diluted to a protein content of 1 mg/ml using homogenization buffer for cytosolic fractions or extraction buffer for nuclear fractions.

V-B. Saturation Receptor Binding Assays:

Saturation analysis procedures were modified from those of Lazier and Haggarty (1979) and Smith and Thomas (1980). Varying amounts of radiolabeled ligand ([^{3}H] estradiol or [^{3}H] progesterone), alone or together with a 500-fold excess concentration of radio-inert ligand (DES or progesterone) in ethanol, were added to glass tubes so that after addition of cell extracts the concentrations of radiolabeled ligand were 1, 2, 5, 7.5, 10, 15 and 20 nM. Triple samples were set for each concentration. After steroids were air dried, 200 µl of cell extract was added to the tubes, resuspended with steroids, and incubated at 4 °C for 24 hours with agitation. After incubation, 200 µl of cold 0.5% DCC solution were added into each tube and incubated for 30 minutes on ice, followed by centrifugation at 1000 ×g for 15 minutes to remove free steroids. A 200 µl aliquot of supernatant was taken to a Mini Poly-Q

vial (Beckman, Somerset, NJ) containing 5 ml of Ecolite (+) liquid scintillation cocktail (ICN Biomedicals, Inc., Costa Mesa, CA) and counted on a 1215 RackBeta II liquid scintillation counter (LKB Wallac, Turku, Finland) for 2 minutes. Cytosolic and nuclear fractions from both males and females were assayed.

The counts of the sample tubes containing only radiolabeled steroids were measured as "total binding", whereas those containing both radiolabeled and an excess concentration of unlabeled steroids were referred to as "nonspecific binding". The specific binding was the difference between the two measures.

Analyses were performed according to Scatchard *et al.* (1949). The Scatchard plots were performed by plotting the ratios of bound to free radiolabeled ligand against the amounts of bound radioligand. Non-linear regression of the data was made using SigmaPlot 5.0 (Jandel Scientific Inc., Chicago, IL). Multiple binding sites were resolved manually according to the methods described by Rosenthal (1967). Briefly, two straight lines were found so that the vectorial sum of the points on the lines equaled to the vectorial length of the corresponding point on the Scatchard plot, as illustrated in Appendix 2. The dissociation constants (K_d) were the slopes of the lines and the maximum binding capacity (B_{max}) equaled the x-intercepts of the lines.

V-C. Competitive Binding Assays:

Procedures for competitive binding assays were modified from those of Weiss and Xu (1990) and Yamamoto *et al.* (1996). Aliquots of 200 µl of cell extracts were incubated with 10 nM [^{3}H] estradiol or [^{3}H] progesterone in the absence or presence of competitors at increasing concentrations at 4 ^{o}C for 24 hours with agitation.

Competitors included 10 nM-100 μM of unlabeled estradiol, diethylstilbestrol (DES), progesterone and testosterone for the estrogen binding sites, or progesterone, hydroxyprogesterone, estradiol and testosterone for the progesterone binding sites. After incubation, 200 μl of 0.5% DCC solution were added to each tube and incubated again for 30 minutes on ice and mixed every 5 minutes. The mixtures were then centrifuged at 1000 ×g for 30 minutes at 4 °C. A 200 μl aliquot of the resulted supernatant was carefully removed to a Mini Poly-Q vial containing 5 ml of scintillation liquid and counted for 2 minutes on a ReckBeta II Counter.

Binding of [^{3}H] estradiol (or [^{3}H] progesterone) in the absence of the competitors was referred to as total binding while that in the presence of DES (or progesterone) at the highest concentration (100 μM) was considered as non-specific baseline binding. Thus, specific binding in the presence of the competitor was expressed as percentage of total binding after the non-specific baseline was subtracted. That is:

$$\text{Specific binding (with competitor)\%} = \frac{\text{Binding (with competitor)} - \text{Non-specific binding}}{\text{Total binding} - \text{Non-specific binding}} \times 100\%$$

V-D. Variations of Binding Capacity with Sexual Maturation:

V-D-1. Comparison of total specific binding capacity between mature and spent scallops: Female mature (full of mature oocytes in the gonad, GSI = 16.9 ± 1.0, n = 6) and spent scallops (no mature oocytes in the gonad, GSI = 5.2 ± 0.9, n = 6) were sacrificed and their gonads were homogenized separately. Cytosolic and nuclear extracts were made following the procedures described in Section IV-A. Cell extracts

were incubated with 10 nM of [^{3}H] estradiol in the absence or presence a 500-fold excess concentration of DES for 24 hours at 4 °C. The total specific binding capacity is the difference between the number of counts per minute (CPM) per microgram of protein in the absence of unlabeled estradiol and that in the presence of unlabeled estradiol. Comparisons of total specific bindings of cytosol and nuclear fractions were made between mature and spent scallops.

V-D-2. Variation of relative binding capacity with sexual maturation: Scallops at different stages of sexual maturation were dissected. Their soft body weights and gonads weight were measured to obtain their gonadosomatic indices (GSI) (Barber and Blake, 1991), as follows:

$$GSI = \frac{\text{Gonad weight}}{\text{Soft body weight}} \times 100$$

The gonads were then minced, rinsed in homogenization buffer, and homogenized separately. Cytosolic and nuclear fractions were made and assayed for protein contents according to the procedure described by Bradford (1976). The cell extracts were diluted to a protein concentration of 1 μg protein/ml extract and then stored at -74 °C until they were assayed. Aliquots of 200 μl of cell extracts were incubated with 10 nM [^{3}H]-estradiol in the absence or presence of a 500× excess concentration of radio-inert DES at 4 °C for 24 hours. Then 200 μl of 0.5% DCC solution were added and incubated on ice for 30 minutes followed by centrifugation at 1000 ×g at 4 °C for 30 minutes. Two hundred micro-liters of supernatant, containing only bound radiolabeled estradiol, were transferred to a Mini Poly-Q vial containing 5 ml of Ecolite scintillation liquid and counted on a RackBeta II counter. Specific bindings

were calculated by subtracting the counts in the presence of excess concentration of radio-inert DES from those in the absence of radio-inert DES. The specific bindings of cytosolic and nuclear fractions were compared to obtain the relative binding capacity according to the following formula:

$$\text{Relative Binding Capacity} = \frac{\text{Specific binding of cytosolic fraction}}{\text{Specific binding of nuclear fraction}}$$

The relative binding capacity was then plotted against GSI to determine the variations in the distribution of estrogen binding sites between the cytosolic and nuclear fraction with sexual maturation. Both male and female scallops were examined.

V-D-3. Demonstration of estrogen binding sites in the liver of the scallops: Cytosolic and nuclear fractions were made from the scallop livers, as well as the fractions from the gonads of the same animals. The cell extracts were incubated with 10 nM of [^{3}H] estradiol in the absence or presence a 500-fold excess concentration of DES for 24 hours at 4 ^{o}C. The total specific binding capacity is the difference between the number of counts per minute (CPM) per microgram of protein in the absence of unlabeled estradiol and that in the presence of unlabeled estradiol.

VI. Cloning of Estrogen Receptor in the Sea Scallop

VI-A. Cloning of an Estrogen Receptor cDNA Fragment by Reverse Transcription Polymerase Chain Reaction (RT-PCR):

VI-A-1. Preparation of poly A$^+$-mRNA: Preparation of mRNA for reverse transcription was performed using a SOLIDscript ^{TM} Solid Phase cDNA Synthesis Kit (CPG, New Jersey). Briefly, 100 mg of liver or gonad were removed from ripe female

scallops and put into 1 ml of ice-cold hybridization buffer from the kit and homogenized at high speed. The homogenate was then centrifuged at 14,000 ×g at room temperature for 1 minute. The derived supernatant was transferred to another tube and incubated with freshly prepared oligo $(dT)_{25}$-bound MPG streptavidin particles at room temperature for 5 minutes. Oligo $(dT)_{25}$-bound MPG streptavidin particles were made by incubating oligo $(dT)_{25}$ probe with MPG streptavidin magnetic particles at room temperature for 5 minutes in the probe binding buffer from the kit. After incubation with tissue homogenates, the particles were separated magnetically and washed with hybridization buffer before proceeding to reverse transcription steps.

VI-A-2. Preparation of solid-phase cDNA library: The mRNA-bound MPG streptavidin particles were incubated with avian myeloblastosis virus (AMV) reverse transcriptase in the presence of RNase inhibitor and dNTP (all these reagents were from the SOLIDscript ™ Solid Phase cDNA Synthesis Kit) in the reverse transcription buffer at 42 °C for 1 hour (O'Driscoll *et al.*,1993). At the end of the reaction, the particles were separated magnetically, washed and resuspended in TE buffer.

VI-A-3. Designing of degenerate primers for PCR: Amino acid sequences of estrogen receptors from various vertebrates including human, rat, chicken, alligator, turtle, frog and fish were compared to find conserved regions (blocks) using Block Maker, an on-line program from Fred Hutchinson Cancer Research Center (http://www.blocks.fhcrc.org/blockmkr). From these blocks, degenerate primers were designed using another on-line program, CODEHOP (http://blocks.fhcrc.org/

blocks/codehop.html). Three upstream primers corresponding to conserved regions of the DNA-binding domain and three downstream primer corresponding to conserved regions of the steroid-binding domain of estrogen receptor were chosen. The strategies for selection of the primers included low degeneracy (less than 8), appropriate length (20-26 oligonucleotides), and melting temperature (55-65 °C). Primers were synthesized by Dalton Laboratory Ltd. (North York, Ontario).

VI-A-4. PCR: PCR (Oste, 1988) was performed using the following conditions: 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM primers, and 2 unit/100 µl Taq DNA polymerase (MBI Fermentas, Amherst, New York). Cycles for reactions with degenerate primers were: 94 °C for 3 minutes for the initial denaturation step, 30 cycles of 94 °C for 30 seconds for the denaturation steps, 40 °C for 30 seconds and 54 °C for 10 seconds for the annealing steps, and 68 °C for 45 seconds for the extension steps, and 68 °C for 10 minutes for the final extension step. For reactions with non-degenerate primers, temperatures that were 3-5 °C lower than the melting temperatures of the primers were chosen as annealing temperatures. After PCR, the samples were run on a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide and transilluminated under UV to visualize the bands. The desired bands were then excised and purified by GeneClean (Bio 101, Carlsbad, CA) and saved for sub-cloning and sequencing.

VI-A-5. Cloning and sequencing of the PCR product: The PCR product was ligated into pCR2.1 TA vectors (Invitrogen, Carlsbad, CA) at a rate of 3:1 catalyzed by T_4 ligase for 4 hours at 16 °C. After ligation, the vectors were transformed into TOP10F' One-Shot *E. coli* cells (Invitrogen, Carlsbad, CA) and plated on LB agar

plates containing kanamycin and coated with X-Gal and IPTG (Stratagene, La Jolla, CA). Since multiple cloning sites of the pCR2.1 vector are located within the *lacZ*α fragment which encodes part of the β-galactosidase, bacteria transformed with a vector with an insert will form a white colony while those without an insert will form a blue colony. After an overnight incubation at 37 °C, the white colonies were selected and grown in LB broth containing 50 μg/ml kanamycin overnight. The cells were collected to isolate plasmid DNA using a MiniPrep kit from Qiagen (Missassauga, Ontario). To confirm the presence of an insert of the appropriate size, a fraction of the purified plasmid DNA was digested with restriction enzyme EcoR I, and separated and visualized on a 0.7% agarose gel. If confirmed, the rest of the plasmid DNA was used for sequencing by ABI using Universal M13 Reverse primer or M13 forward-20 primer in the Institute for Marine Biology, National Research Council (Halifax, Nova Scotia).

VI-B. Northern Blotting:

VI-B-1. Preparation of total RNA: Total RNA preparations were made using TRI REAGENT kit (MRC, Cincinnati, OH) based upon the methods described by Chomczynski (1993). In general, the tissues were homogenized in the TRI REAGENT buffer and the homogenate was incubated at room temperature for 5 minutes. Then 0.2 ml chloroform per ml of TRI REAGENT buffer were added to the homogenate, mixed vigorously, and incubated at room temperature for 10 minutes. The mixture was then centrifuged at 12,000 ×g for 15 minutes at 4 °C. The upper aqueous phase containing total RNA was recovered and transferred to a new tube.

Half volume of isopropanol to that of the RNA aqueous phase was added to the tube and incubated for 10 minutes at room temperature. The mixture was centrifuged at 12,000 ×g for 8 minutes at room temperature. The RNA pellet was washed with 75% ethanol, air dried for 5 minutes, and dissolved in DEPC-treated water.

VI-B-2: Blotting transfer: Twenty μg of total RNA were denatured in a buffer containing 50% formamide 17.5% formaldehyde, and 0.5× gel running buffer (see Appendix 1 for recipes for this buffer and the subsequent buffers or solutions) at 65 °C for 15 minutes and chilled on ice for 10 minutes. Then the RNA sample was mixed with 2 μl of formaldehyde gel loading buffer and loaded into 18% formaldehyde and 1% agarose gel in 1× formaldehyde gel running buffer. Before electrophoresis, the surface of the buffer tank, plate, and dams of the electrophoresis apparatus were cleaned with 30% hydrogen peroxide and soaked in 0.1% DEPC-treated water. Gel running buffer was added to submerge half the thickness of the gel. No running buffer was added to the wells before the samples. The gel was pre-run for 5 minutes at 60V before the RNA sample was loaded. After the samples were loaded, they were run for several minutes until they had progressed into the gel. Then more running buffer was added to submerge the gels and the electrophoresis was run at 40V for 4 hours. After electrophoresis, the gel was soaked in distilled water for 30 minutes with water changes every 5 minutes to eliminate the formaldehyde. The gel was then stained with 0.5 μg/ml ethidium bromide in 0.1 M ammonium acetate for 30 minutes, destained with 0.1 M ammonium acetate for another 30 minutes, and briefly rinsed with distilled water. After the gel was photographed, it was denatured in 0.05 M NaOH/0.15 NaCl for 30 minutes, neutralized with 0.1 M Tris (pH 8.0)/0.15 M NaCl for 30 minutes, and

soaked in 10× SSC for 15 minutes. S & S NC™ nitrocellulose membrane (0.1 μm, Schleicher & Schuell, Keene, New Hampshire) pre-wetted for 10 minutes in water and treated with 10× SSC for 10 minutes was used for blotting transfer. RNA was transferred to the membrane with 10× SSC as the transfer solution overnight by capillary blot procedures (Southern, 1975). After a brief blotting with filter paper, the membrane was UV-crosslinked in a Strategene Crosslinker and wrapped with the Saran wrap.

VI-B-3. Probe labeling: The DNA used as probes was prepared from either the plasmid digestion or PCR amplification using the cloned cDNA fragment as a template. A DecaLabel™ DNA Labeling Kit (MBI Fermentas, Amherst, New York) was used to label the probe DNA by random labeling techniques (Feinberg and Vogelstein, 1983). In brief, the template DNA and the deca-nucleotides were denatured by boiling for 5 minutes and chilling on ice. Mixtures of dATP, dTTP and dGTP, [α-^{32}P]-dCTP (Amersham, Piscataway, New Jersey) and the Klenow fragment (exo$^-$, 5 U/μl) were added and incubated at 37 °C for 5 minutes. Then the dNTP mixture was added and incubated at 37°C for another 5 minutes. Finally, 0.5M EDTA (pH 8.0) was added to stop the reaction.

VI-B-4: Hybridization: The nitrocellulose membrane blotted with RNA was wetted in 2× SSPE buffer before being immersed in the pre-hybridization buffer. The membrane was incubated in the hybridization tube containing pre-hybridization buffer at 65 °C for 30 minutes. Then the pre-hybridization solution was replaced with hybridization buffer. Radiolabeled probe and 100 μg herring sperm DNA per ml of hybridization buffer were denatured by boiling for 5 minutes and chilled on ice before

they were added to the hybridization tube. The hybridization was performed at 65 °C for 24 hours. After hybridization, the membrane was first washed with 0.5% SDS in 2× SSPE at room temperature for 5 minutes, then washed twice with 0.5% SDS in 2× SSPE for 30 minutes at 65 °C. Finally, it was washed with 0.2× SSPE, 0.5% SDS for 15 minutes at 65 °C. After a brief rinse with 2× SSPE at room temperature, the membrane was blotted on filter papers to remove excess solution and placed between two sheets of Saran wrap. The membrane was then exposed to Kodak Bio Max X-ray film (Kodak Canada Co., Toronto, Ont) with intensifying screen at -74 °C for 12-72 hours depending on its radioactivity. The film was developed on a Kodak X-OMAT P20 Processor (Kodak Canada Co., Toronto, Ont).

Results

I. Effects of Sex Steroids on Gonadal Differentiation and Sex Determination in Juvenile Scallops

No differences were found in the average soft body weight, average gonad weight or average gonadosomatic index (GSI) between the experimental and the control groups (Table 3; $P>0.1$, $n=19$, ANOVA for all data). These results indicate that sex steroids had no detectable effects on the growth of the gonads or the soft bodies over 3 months in the present studies. However, differences in differentiation rate (the percentage of sex-differentiated animals in total) and sex ratio (the ratio between the number of male animals to that of females) were observed between sex steroid-injected groups and the control groups. Other morphological changes were also observed in sex steroid-injected females.

I-A. Morphological Observations of Normal Gonadal Differentiation in Scallops

I-A-1. Undifferentiated: Undifferentiated animals were observed in all the groups. In undifferentiated animals, the development of the gonads varied from no visible acini (Figure 3A) to developing acini (Figure 3B), but with no distinguishable oocytes or spermatids present.

I-A-2. Male gonads: Sections of a typical male gonad from the non-injection group is shown in Figure 4. As can be seen from this figure, germinal cells which were about 5-6 μm in diameter, were generally found along the walls of acini. Primary spermatogonia, which were about 3-4 μm in diameter, were seen close to the walls of

Table 3. Measurements of Juvenile Scallops in the Sex Steroids Injection Experiments

Treatments	Soft body Weight (g)	Gonad Weight (g)	GSI	Number
Estradiol	3.61±0.76	0.062±0.031	1.89±0.96	51
Testosterone	3.53±0.64	0.071±0.033	1.81±0.48	48
Progesterone	3.23±0.66	0.057±0.022	1.64±0.46	53
DHEA	3.43±0.62	0.058±0.021	1.63±0.43	48
Vehicle	3.39±0.82	0.055±0.025	1.59±0.37	42
No Injection	3.96±0.92	0.071±0.024	1.79±0.36	48

Figure 3. Morphology of undifferentiated scallops.

A. Animals before injection were sexually undifferentiated. No acinus was present in the gonad. Scale bar = 50 μm.
B. Undifferentiated animals after experiments. Developing acini (A) were present in the gonad. Scale bar = 15 μm.

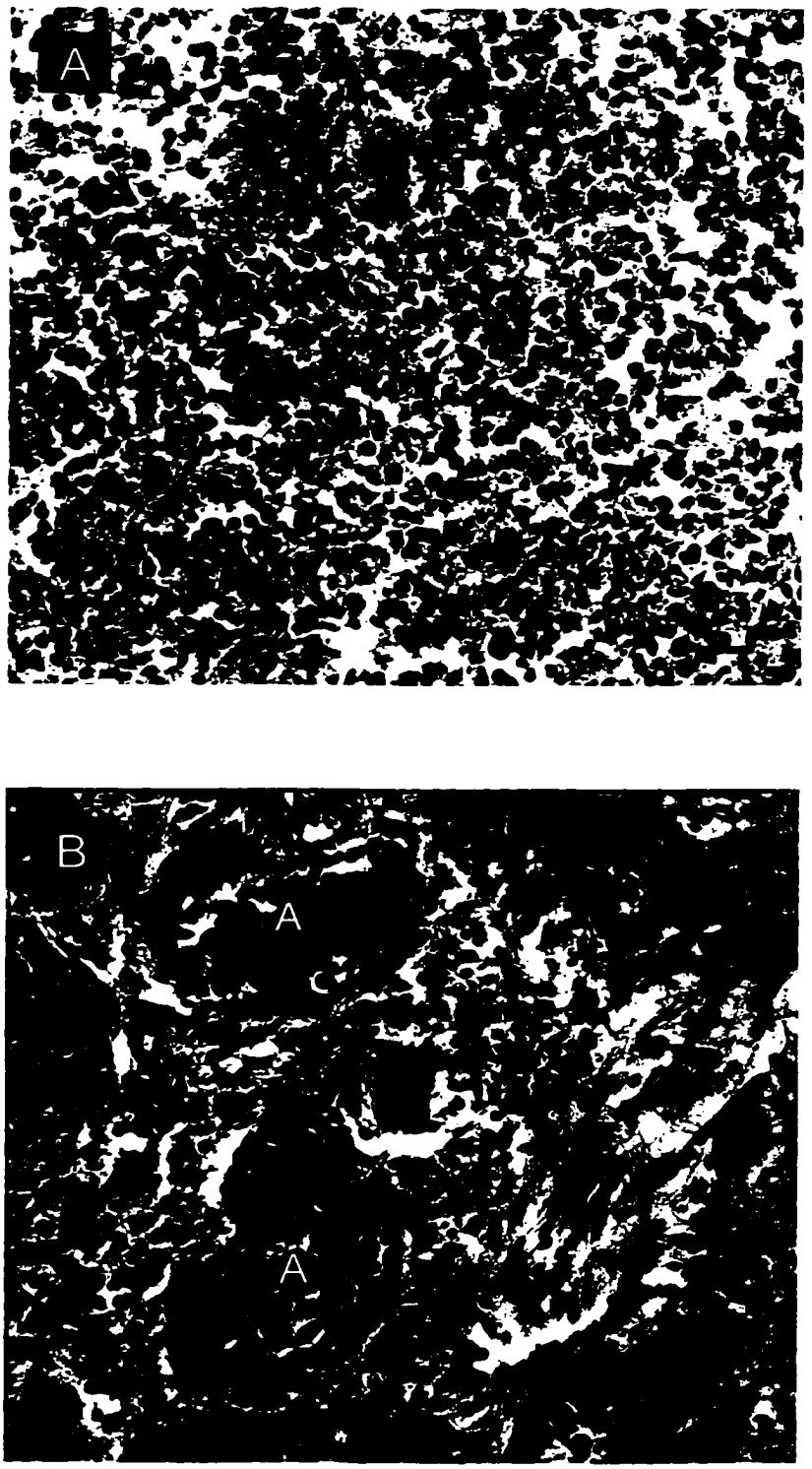


Figure 3

Figure 4. Morphology of the normal male juvenile scallop gonad.
GC: germinal cell; PS: primary spermatogonia; SS: secondary spermatogonia; SP: spermatid; GD: gonadoduct
A. Low magnification, scale bar = 50 μm.
B. High magnification, scale bar = 10 μm.

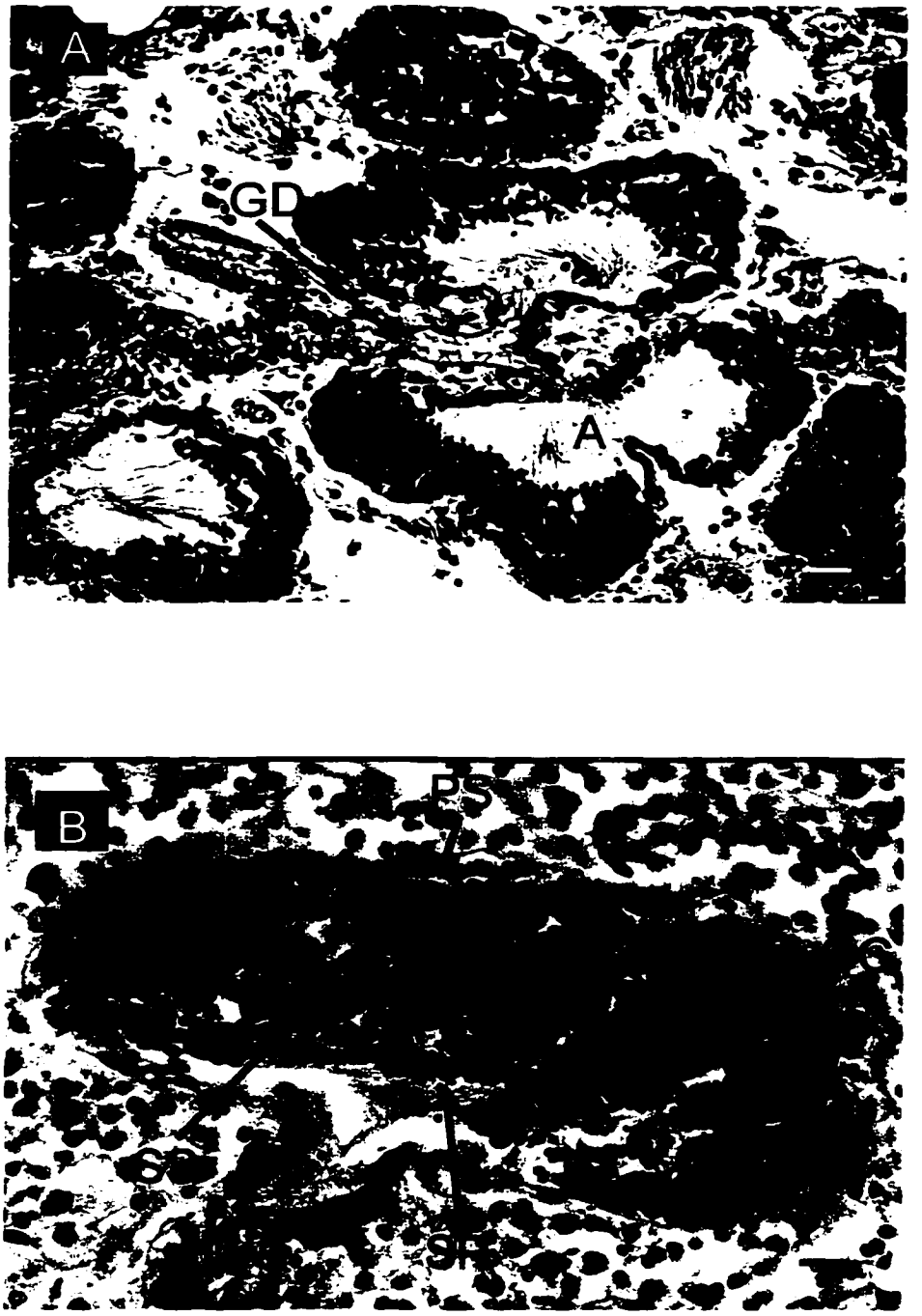


Figure 4

acini. Closer to the center of the acini were secondary spermatogonia which measured about 3-4 μm. Spermatids were the smallest cells which were about 1-2 μm in diameter in the male acini and were usually found close to the ciliated gonadoducts.

I-A-3. Female gonads: Figure 5 shows sections of a typical female gonad from the blank control group. Oocytes of varying stages of maturity were readily seen near the walls of the acini. Each oocyte was characterized by a large germinal vesicle that accounted for approximately 2/3 of its volume, with a strongly Eosin-stained nucleus in the middle. In close contact with the developing oocytes were small auxiliary cells that were around 3-5 μm in diameter. In most of the sections, the lumens of the acini were relatively empty. Ciliated gonadoducts were seen associated with the acini.

I-B. Effects of Sex Steroids on Gonadal Differentiation:

Undifferentiated animals were observed in all the sex steroid-injected groups and control groups. The differentiation rates in the sex steroid-injected groups and the control groups are shown in Table 4. Higher differentiation rates were observed in sex steroid-injected groups (60.78% for the estradiol injection group, $P<0.001$; 58.33% for testosterone group, $P<0.005$; 56.60 % for progesterone group, $P<0.01$; and 56.25 % for DHEA group, $P<0.01$; Chi-square test for all data) compared to the non-injection control group (37.50%). Injection of vehicle also led to a higher differentiation rate (47.62%), but it was not significantly different from the non-injection control group ($P>0.1$, Chi-square test).

I-C. Changes in Sex Ratio Induced by Sex Steroid Injections:

Figure 5. Morphology of the normal female juvenile scallop gonad.
OG: oogonia; OC: developing oocyte; AC: auxillary cell; GD: gonadoduct
A. Low magnification, scale bar = 50 μm.
B. High magnification, scale bar = 10 μm.

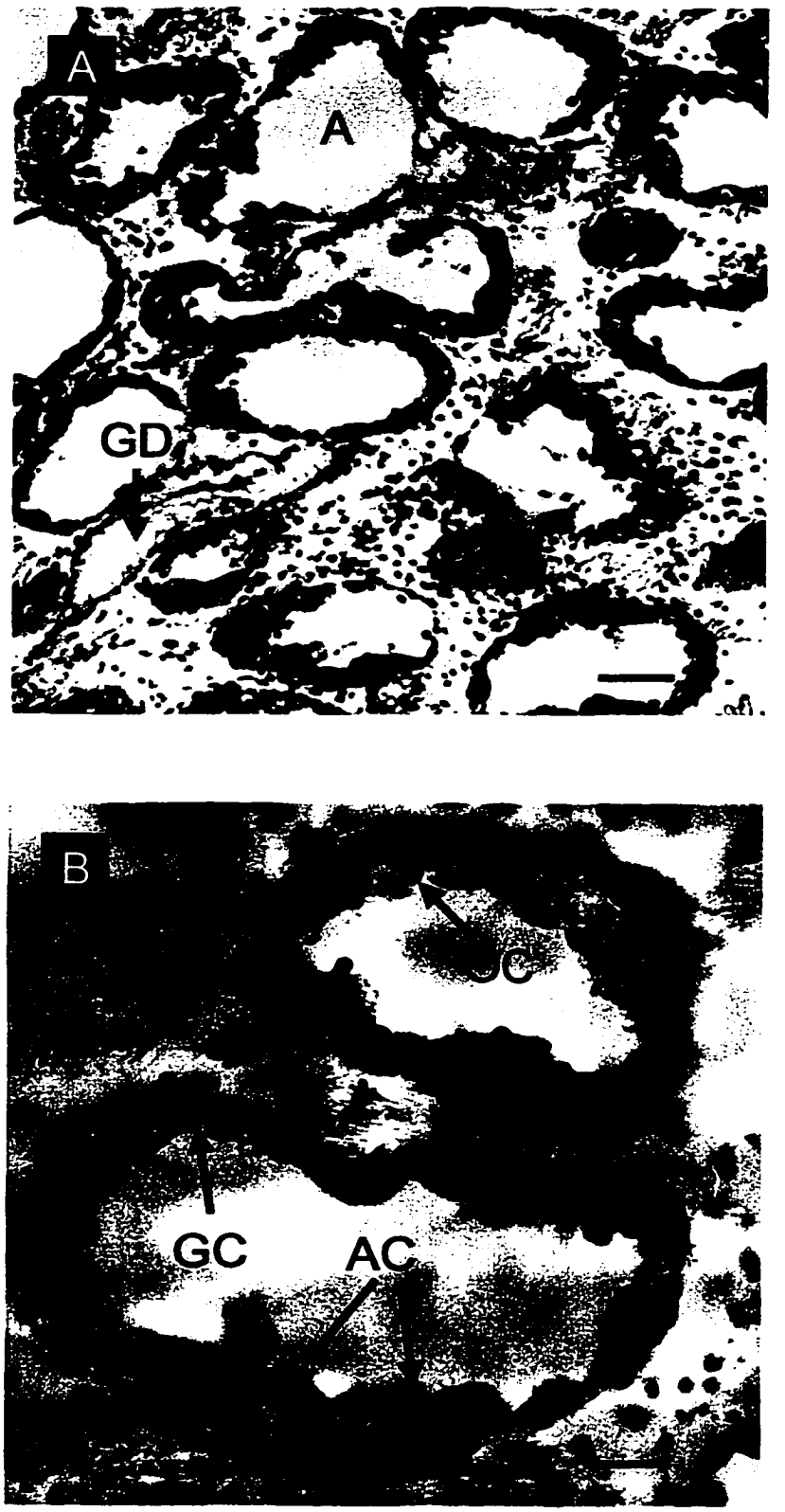


Figure 5

Table 4. Effects of Sex Steroid Injections on Differentiation Rate and Sex Ratio

(U: undifferentiated animal; H: hermaphroditic animal)

Treatments and Trials		Male	Female	Other		Differentiation Rate (%)	Sex Ratio (M/F)
				U	H		
Estradiol Injection	#1	14	3	3		60.78	6.75
	#2	8	1	4			
	#3	5	0	13			
	Total	27	4	20			
Testosterone Injection	#1	13	4	3		58.33	6.00
	#2	6	0	6			
	#3	5	0	12			
	Total	24	4	20			
Progesterone Injection	#1	10	2	7	1	56.60	8.67
	#2	10	1	3			
	#3	3	0	13			
	Total	26	3	23	1		
DHEA Injection	#1	13	4	1	2	56.25	5.25
	#2	8	0	5			
	#3	0	0	15			
	Total	21	4	21	2		
Vehicle Injection	#1	10	8	1		47.62	1.25
	#2	1	1	10			
	#3	0	0	11			
	Total	11	9	22			
No Injection	#1	8	8	3		37.50	1.22
	#2	2	0	13			
	#3	0	0	14			
	Total	10	8	30			

The sex ratios (male/female) of the sex steroid-injected groups and the control groups are given in Table 4.

Injections of sex steroids caused significant changes in the sex ratio ($P<0.005$, Chi-square test). Sex ratios were significantly shifted to 6.75 by injection of estradiol ($P<0.001$), to 6.00 by testosterone ($P<0.005$), to 8.67 by progesterone ($P<0.001$), and to 5.25 by DHEA ($P<0.005$), compared to 1.22 in the vehicle-injection group and 1.25 in the non-injection control group (Chi-square test for all comparisons). However, the sex ratio in the vehicle control group was not different from that of the blank control group ($P>0.5$, Chi-square test).

I-D. Other Morphological Changes in Sex Steroid-injected Groups:

Morphologically, the undifferentiated and male animals from the sex steroid-injected groups were not different from those from the control groups. No difference was found between the females from the vehicle control group and those from the non-injection control group. However, morphological changes were observed in females from the sex steroid-injected groups compared to those from the control groups. These include the eutrophy of oocytes in the estradiol- and DHEA-treated groups, breakdown of oocytes in the testosterone-treated group, and hermaphrodites in the progesterone- and DHEA-injected groups.

I-D-1. Eutrophy of oocytes in estradiol- and DHEA-treated juveniles: Extraordinary large oocytes were seen in the females from the estradiol-injected and the DHEA-injected groups. In these animals, usually only one or two large and well-developed oocytes were present in the acini while the rest were still small and at early differentiation stages (Figure 6). To compare the sizes of these large oocytes with those

Figure 6. Eutrophy of oocytes in estradiol- and DHEA-treated juveniles.
OC: oocyte; GD: gonadoduct
A. Low magnification, scale bar = 50 μm.
B. High magnification, scale bar = 35 μm.

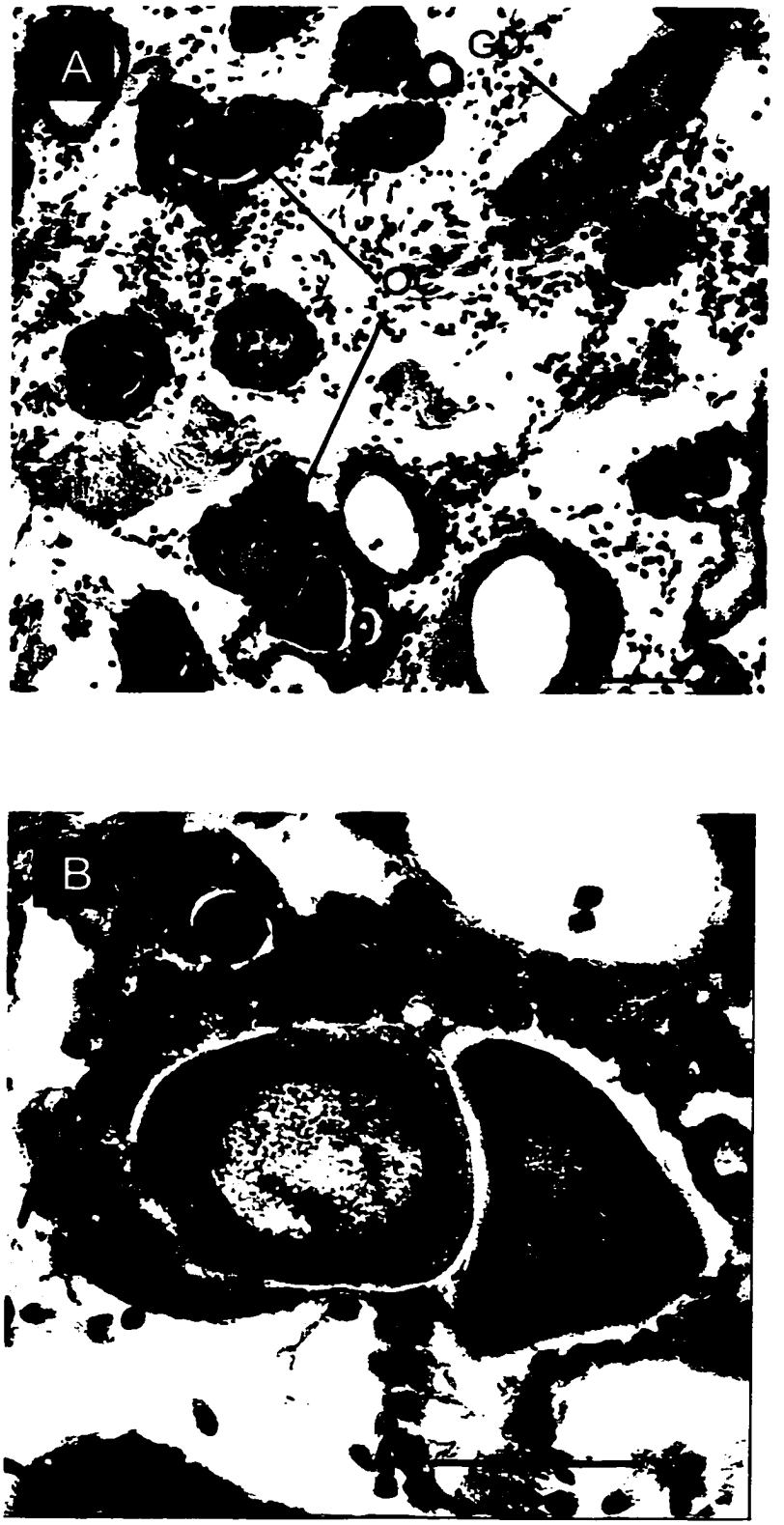


Figure 6

from the blank control group, 5 largest oocytes from the gonad of each female in these groups were measured. The diameter of large oocytes in the estradiol-injected group was 48.57 μm ± 2.94 μm in the long axis and 40.10 μm ± 2.44 μm in the short axis (n=15). In the DHEA-injected group, the diameter of the large oocytes was 46.83 μm ± 2.15 μm in the long axis and 32.86 μm ± 1.72 μm in the short axis (n=20). These diameters were significantly larger than the diameter of the largest oocytes in the control groups (22.60 μm ± 1.22 μm in long axis and 21.01 μm ± 1.11 μm in short axis, n=15, $P<0.001$, t-test).

In the gonads of the females from the estradiol-injected group, 36.67% of the acini contained large oocytes (n=60) while in the DHEA-injected group, 35.00% of the acini had large oocytes (n=80). In other sex steroid-injected groups or the control groups, less than 1% of the acini were found to have large oocytes.

I-D-2. Breakdown of oocytes in female gonads of testosterone-injected scallops: In the testosterone-injected group, breakdown of oocytes was observed in all the female scallops. The acini containing such oocytes represented 17.50% of the acini in the sections (n=80). In these acini, the cytoplasmic and nuclear membranes of the oocytes were often ruptured, thus no boundary was visible between the nuclear and cytoplasmic contents. These cytoplasm and nuclei mixtures were often irregular in appearance (Figure 7). No similar phenomenon was seen in other steroid-injected or control groups.

I-D-3. Hermaphrodites in the progesterone- and DHEA-treated groups:

Three hermaphroditic animals were observed in these studies: one from the progesterone-injected group and two from the DHEA-injected group, representing 1.89% and 4.17% of the animals in each group. At the same time, no hermaphroditic animals were found in other steroid-injected groups or any of the control groups. The occurrence

Figure 7. Breakdown of oocytes in female juvenile scallops in the testosterone-injected group (as indicated by arrowheads).
A. Low magnification, scale bar = 20 μm.
B. High magnification, scale bar = 10 μm.

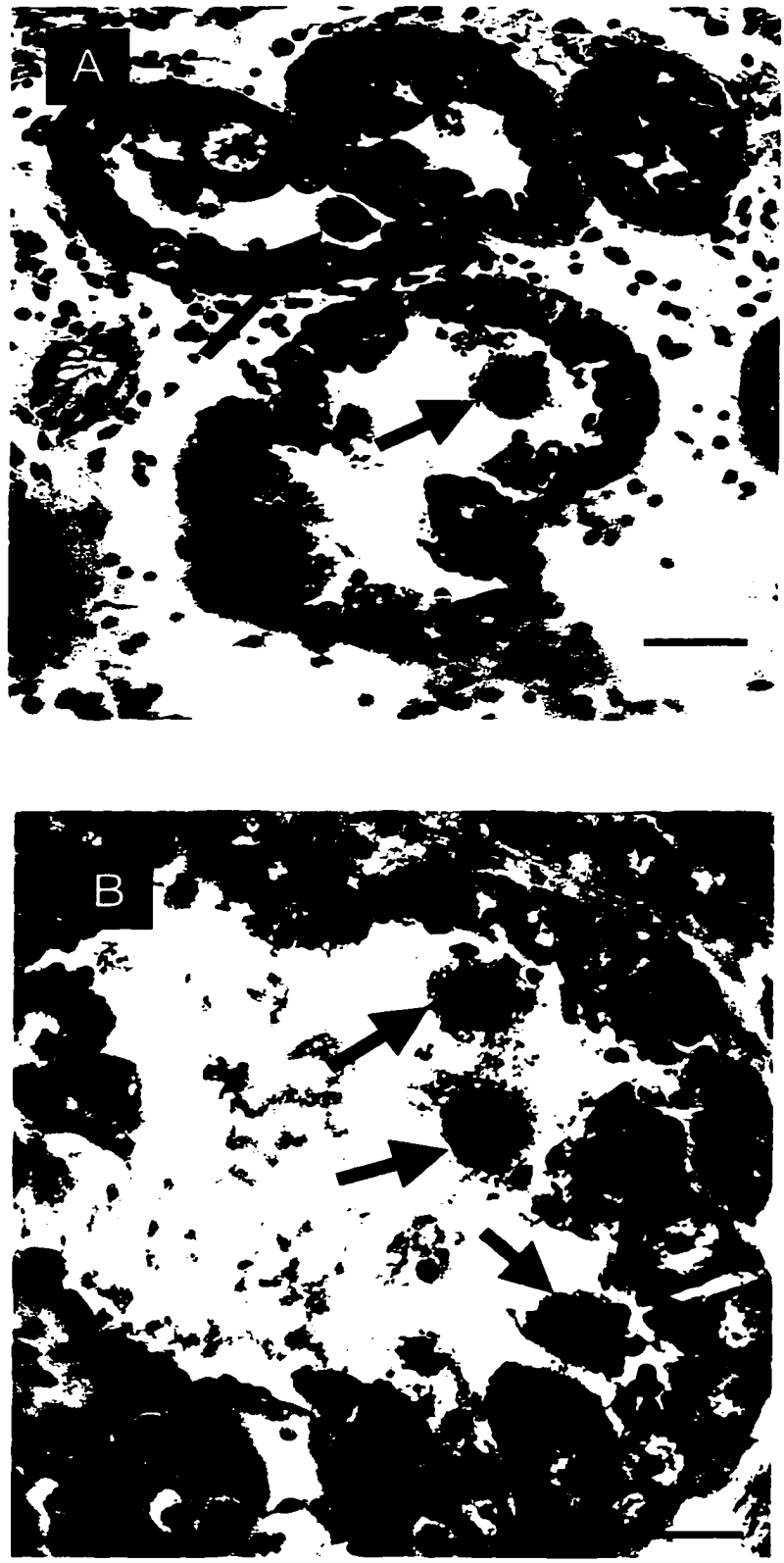


Figure 7

of hermaphrodites in the progesterone- or DHEA-injected groups was significantly higher than that of either of the control groups ($P<0.001$ for both groups, Chi-square test).

In the hermaphroditic specimen from the progesterone-treated group, female and male acini occupied different portions of the gonad (Figure 8). In the two hermaphroditic specimens from the DHEA treatment group, only one or two male acini were found among many female acini. Figure 9 shows a section of a hermaphroditic gonad from a DHEA-injected juvenile. As can be seen from this figure, an acinus containing spermatids appeared adjacent to acini with well-developed oocytes.

II. Effects of Sex Steroids on Gamete Release and Spawning:

II-A. *In Vitro* Effects of Sex Steroids on 5-HT-induced Gamete Release (120 minutes):

The procedure of Osada *et al.* (1992) was adapted to screen the actions of sex steroids on 5-HT-induced gamete release. Results from preliminary experiments showed that estradiol, progesterone and testosterone had stimulatory effects in females and/or males while dehydroepiandrosterone (DHEA) and 4-androstene-3,17-dione had no significant effects on 5-HT-induced gamete release. Therefore, efforts were focused on the actions of the first three sex steroids in both sexes.

II-A-1. Estradiol: Estradiol had stimulatory effects on 5-HT-induced egg release in the sea scallops (Figure 10A). As homogeneity of the raw data was violated, natural logarithm transformation of the data was performed. Statistical analyses of the transformed data showed that treatments with 10^{-5}-10^{-8} M estradiol significantly

Figure 8. Hermaphroditism from the progesterone-injected group.
Male and female acini occupied different portions in the gonad.

A. Overview of the hermaphrodite gonad, male part is at the left side and female part is at the right side. M: male portion; F: female portion. Scale bar = 50 μm.
B. The male portion of the gonad. Scale bar = 20 μm.
C. The female portion of the gonad. OC, oocyte. Scale bar = 20 μm.

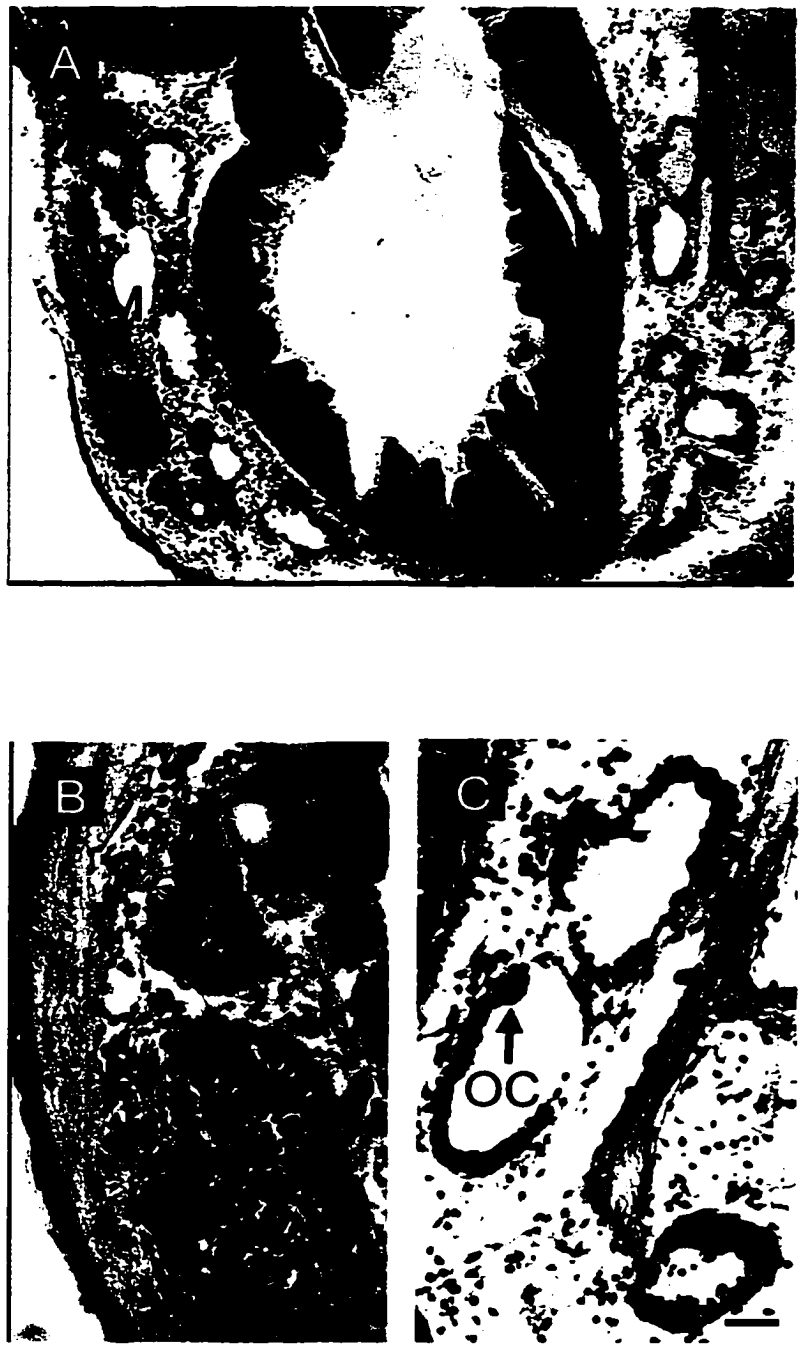


Figure 8

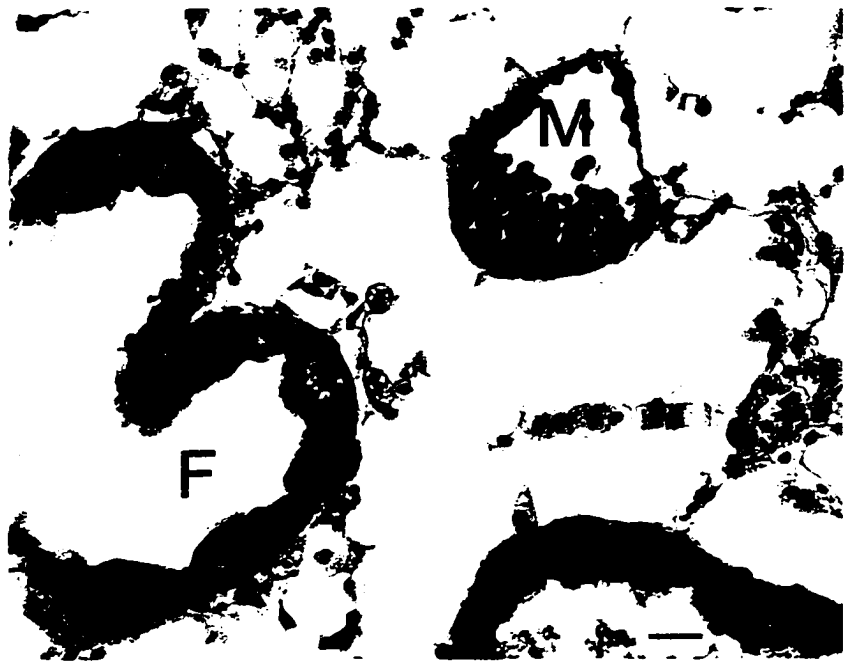


Figure 9. Hermaphroditism from the DHEA-injected group.
Only one male acinus (M) was present among many female acini (F).
Scale bar = 10 μm

increased the number of eggs released per unit weight of gonad (P=0.04, n=5, ANOVA). A maximum response, as high as 375.9% of that of the control group, was observed in the group treated with 10^{-6} M estradiol (P<0.005, n=5, Dunnett's test). Treatment with estradiol at lower concentrations induced smaller yet significant effects, which were around 180% of the effect of the control group (P<0.05, n=5, Dunnett's test). Treatment with 10^{-4} M estradiol had no significant effect on 5-HT-induced egg release (P>0.05, n=5, Dunnett's test).

Estradiol at concentrations of 10^{-5}-10^{-7} M also had stimulatory effects on sperm release induced by 5-HT (P=0.002, n=7, ANOVA; Figure 10B). Statistical analyses were performed using natural logarithm-transformed data because this transformation increased the normality of the data. A maximum response which was 132.2% higher than that of the control group, was observed in the 10^{-6} M estradiol group (P<0.005, n=7, Dunnett's test). Treatment with estradiol at 10^{-5} M or 10^{-7} M resulted in about an 80% increase in the responses, compared to the control group (P<0.01 for both concentrations, n=7, Dunnett's test). Estradiol at 10^{-4} or 10^{-8} had no significant potentiating effects (P>0.05, n=7, Dunnett's test).

II-A-2. Testosterone: Testosterone had no significant effects on 5-HT-induced egg release (P>0.05, n=6, ANOVA; Figure 11A). In contrast, testosterone had stimulatory effects on sperm release induced by 5-HT (P=0.002, n=5, ANOVA). Statistical analyses were performed using natural logarithm-transformed data because the homogeneity of the raw date was not met. As shown in Figure 11B, pretreatment with 10^{-6}-10^{-8} M testosterone significantly increased the sperm release induced by 5-HT (P<0.005 for all, n=5,

Figure 10. *In vitro* effects of estradiol (120 minutes) on 5-HT-induced gamete release. Gonad pieces were incubated with 10^{-4}-10^{-8} M estradiol in the experimental groups or with artificial seawater in the control group for 2 hours, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group.
* $P<0.05$, ** $P<0.01$, *** $P<0.005$, Dunnett's test.
A. Female, each value represents the mean±SE of 5 experiments
B. Male, each value represents the mean±SE of 7 experiments

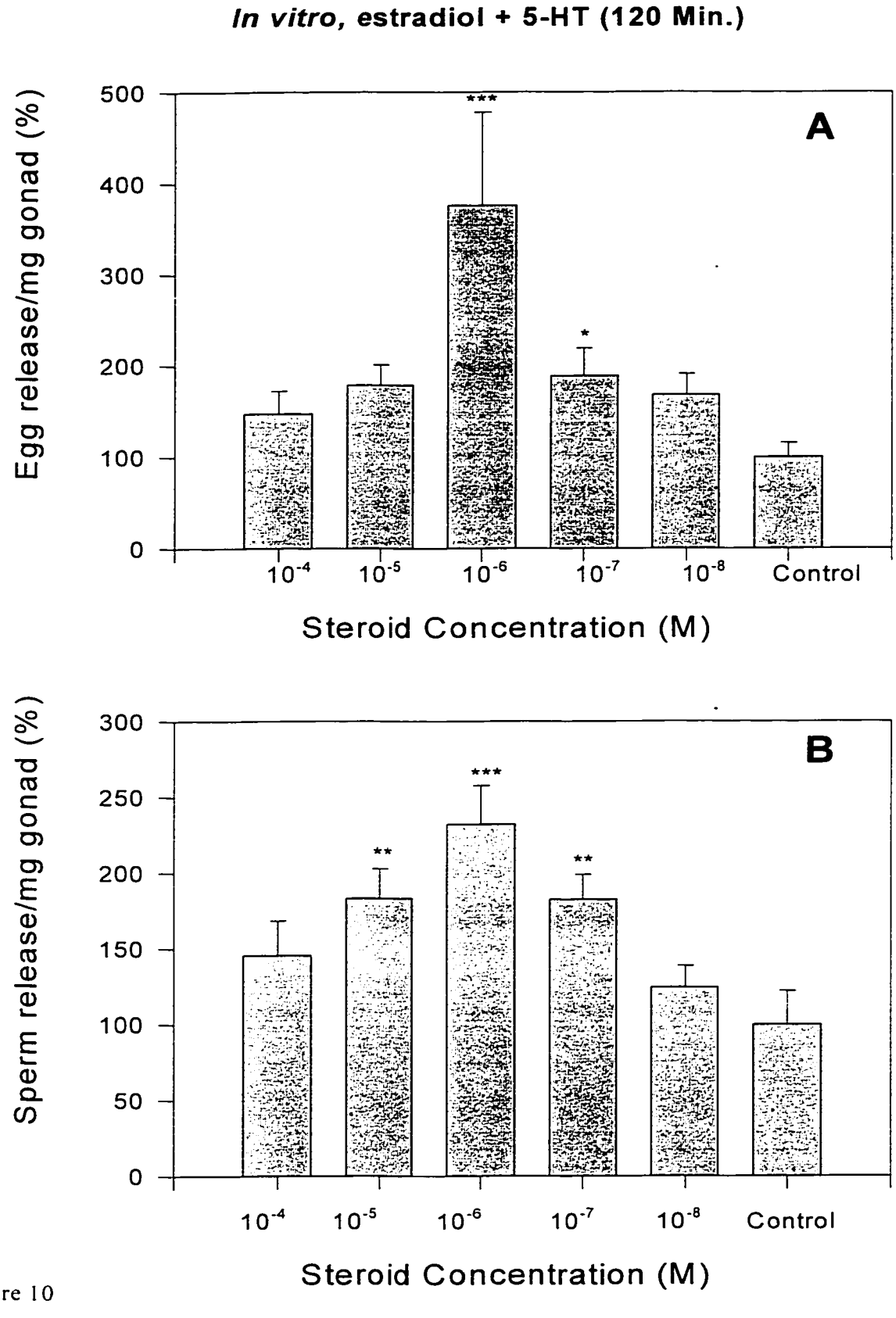

In vitro, estradiol + 5-HT (120 Min.)
A
Egg release/mg gonad (%)
500
400
300
200
100
0
***
*
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Control
Steroid Concentration (M)
B
Sperm release/mg gonad (%)
300
250
200
150
100
50
0
**
***
**
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Control
Steroid Concentration (M)

Figure 10

Figure 11. *In vitro* effects of testosterone (120 minutes) on 5-HT-induced gamete release. Gonad pieces were incubated with 10^{-4}-10^{-8} M testosterone in the experimental groups or with artificial seawater in the control group for 2 hours, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. ***P<0.005, Dunnett's test.

A. Female, each value represents the mean±SE of 6 experiments.

B. Male, each value represents the mean±SE of 5 experiments.

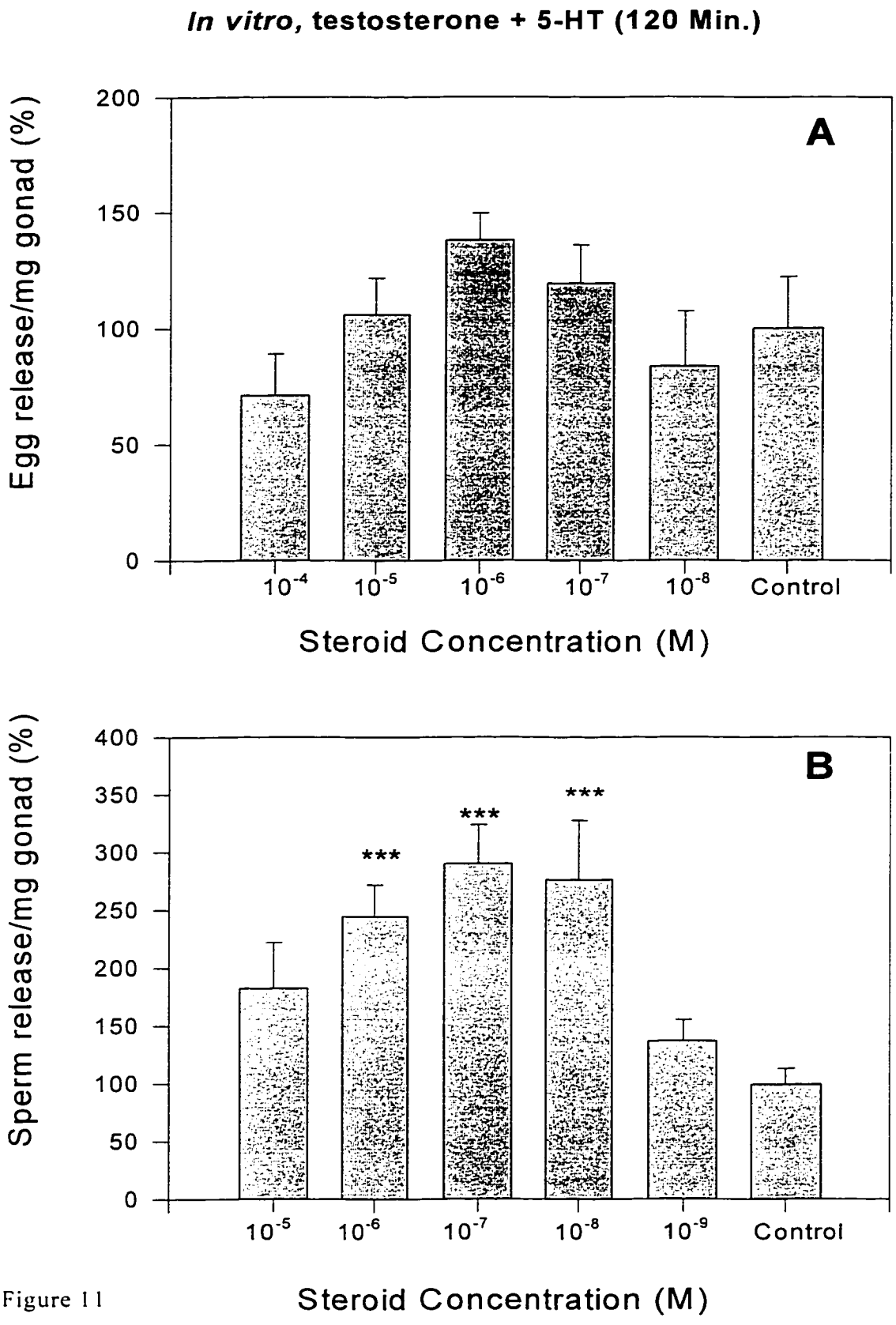

In vitro, testosterone + 5-HT (120 Min.)
Egg release/mg gonad (%)
200
150
100
50
0
A
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Control
Steroid Concentration (M)
Sperm release/mg gonad (%)
400
350
300
250
200
150
100
50
0
B
***
***
***
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
Control
Steroid Concentration (M)

Figure 11

Figure 12. *In vitro* effects of progesterone (120 minutes) on 5-HT-induced gamete release. Gonad pieces were incubated with 10^{-4}-10^{-8} M progesterone in the experimental groups or with artificial seawater in the control group for 2 hours, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. * $P<0.05$, ***$P<0.005$, Dunnett's test.

A. Female, each value represents the mean±SE of 5 experiments

B. Male, each value represents the mean±SE of 6 experiments

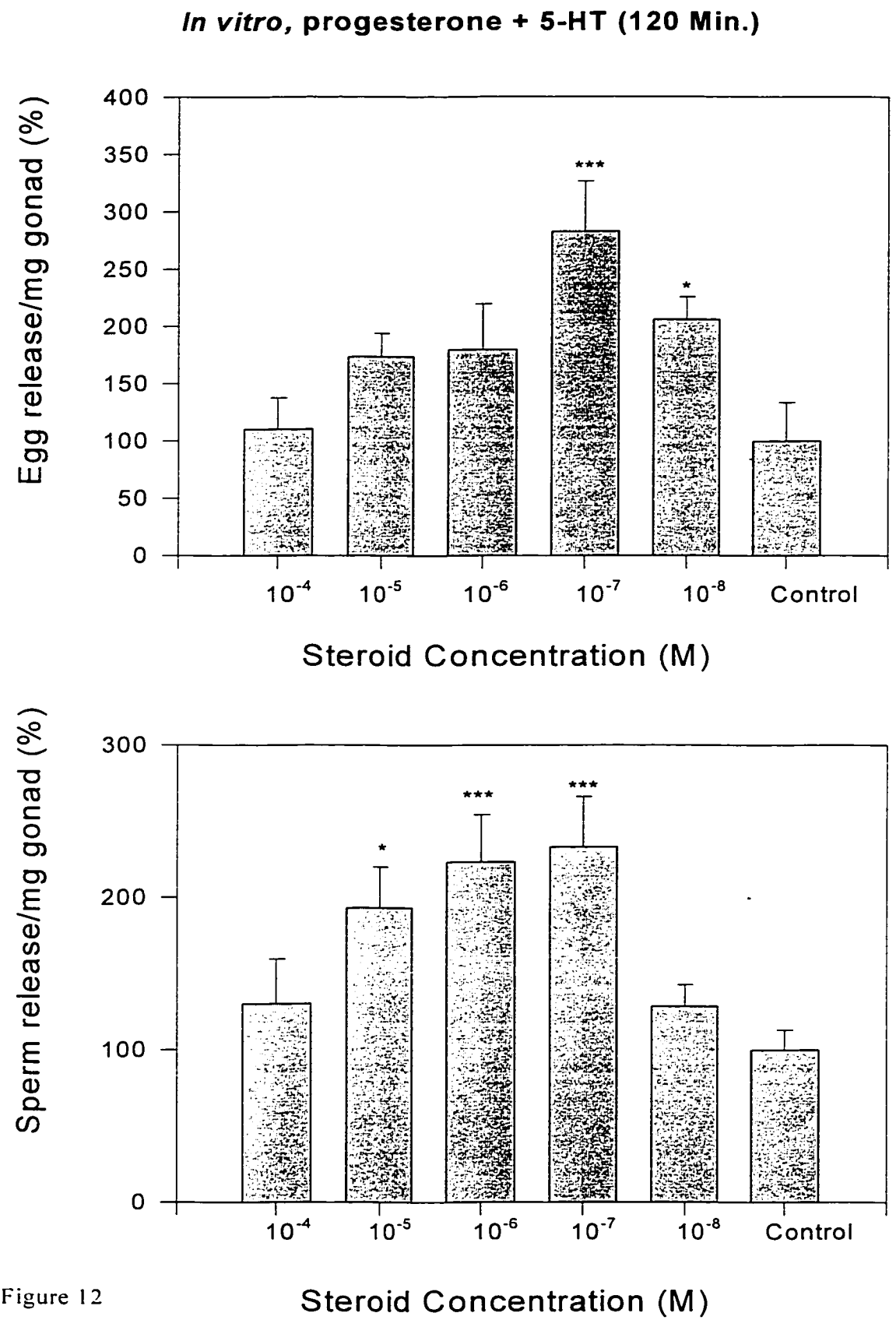

In vitro, progesterone + 5-HT (120 Min.)
Egg release/mg gonad (%)
400
350
300
250
200
150
100
50
0
***
*
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Control
Steroid Concentration (M)
Sperm release/mg gonad (%)
300
200
100
0
*
***
***
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Control
Steroid Concentration (M)

Figure 12

Dunnett's test). The maximum response of 298.4% of that of the control group was observed in the 10^{-7} M testosterone treatment group. The minimum significant effect, which was 244.7% of the response of the control group, was obtained at 10^{-6} M. Testosterone at concentrations 10^{-5} M or 10^{-9} M had no significant effects on 5-HT-induced sperm release ($P>0.05$, n=5, Dunnett's test).

II-A-3. Progesterone: Like estradiol, progesterone had stimulatory effects in both females and males.

Since normality assumption of the raw data was violated, natural logarithm-transformation was performed on the data for the action of progesterone on 5-HT-induced egg release prior to statistical analyses. In females, as shown in Figure 12A, progesterone at 10^{-7} M and 10^{-8} M significantly potentiated egg release induced by 10^{-5} M 5-HT ($P<0.01$, n=5, ANOVA). The responses for progesterone at 10^{-7} M and 10^{-8} M were 283.0% and 206.0%, respectively ($P<0.005$ for the 10^{-7} M group and $P<0.05$ for the 10^{-8} M group, n=5, Dunnett's test), of the response in the control group. At concentrations lower than 10^{-7} M, progesterone did not induce significantly augmented egg release ($P>0.05$, n=5, Dunnett's test).

Similar results were also obtained with males (Figure 12B). Progesterone at concentrations between 10^{-7} M and 10^{-5} M significantly increased sperm release induced by 5-HT ($P=0.003$, n=6, ANOVA). The maximal response, which was 233.4% of that of the control group, was observed at 10^{-7} M progesterone ($P<0.005$, n=6, Dunnett's test). A minimal response of 193.4 of that of the control group was obtained at 10^{-5} M progesterone ($P<0.05$, n=6, Dunnett's test). Treatments at 10^{-4} or 10^{-8} M progesterone had no statistically significant effects ($P>0.05$, n=6, Dunnett's test).

II-B. Parthenogenesis:

Swimming embryos were observed about 40 hours after 5-HT incubation and survived for about 72 hours. By then, the embryos had developed into the blastula stage with a diameter of about 65 μM (Figure 13). The cilia were readily visible under a light microscope.

Parthenogenetic embryos were observed in the groups pretreated with estradiol (22 embryos from 186300 eggs) or progesterone (39 embryos from 237400 eggs) and then with 5-HT. They had also been found in the control groups that were incubated with 5-HT only (18 embryos from 152300 eggs). The occurrence was not different among these groups ($P>0.1$, Chi-square test). No parthenogenetic embryos were obtained from eggs not treated with 5-HT. However, it should also be noted that not as many eggs were obtained when the gonad pieces were not incubated with 5-HT.

II-C. Time Scales of the Actions of Sex Steroids:

To determine the mechanisms of the actions of sex steroids, the time scales of these effects were examined in an attempt to distinguish the classical mechanism from membrane mechanisms. In these experiments, the time of pre-treatment with sex steroids were shortened to 30 or 15 minutes, instead of 2 hours. Again, the number of eggs or sperm released per milligram of gonad after 5-HT treatment was used as an index of steroid action, as used in Section II-A.

II-C-1. 30 minutes trials:

II-C-1-a. Estradiol: As shown in Figure 14, treatment with estradiol at a range of 10^{-6} - 10^{-8} M for 30 minutes potentiated the effect of 5-HT on egg release ($P=0.01$, $n=6$, ANOVA using natural logarithm-transformed data since the homogeneity assumption of

Figure 13. Parthenogenetic embryos.
A: 48 hours after 5-HT incubation. Scale bar = 10 μm
PB: polar body
B: 72 hours after 5-HT incubation. Scale bar = 10 μm

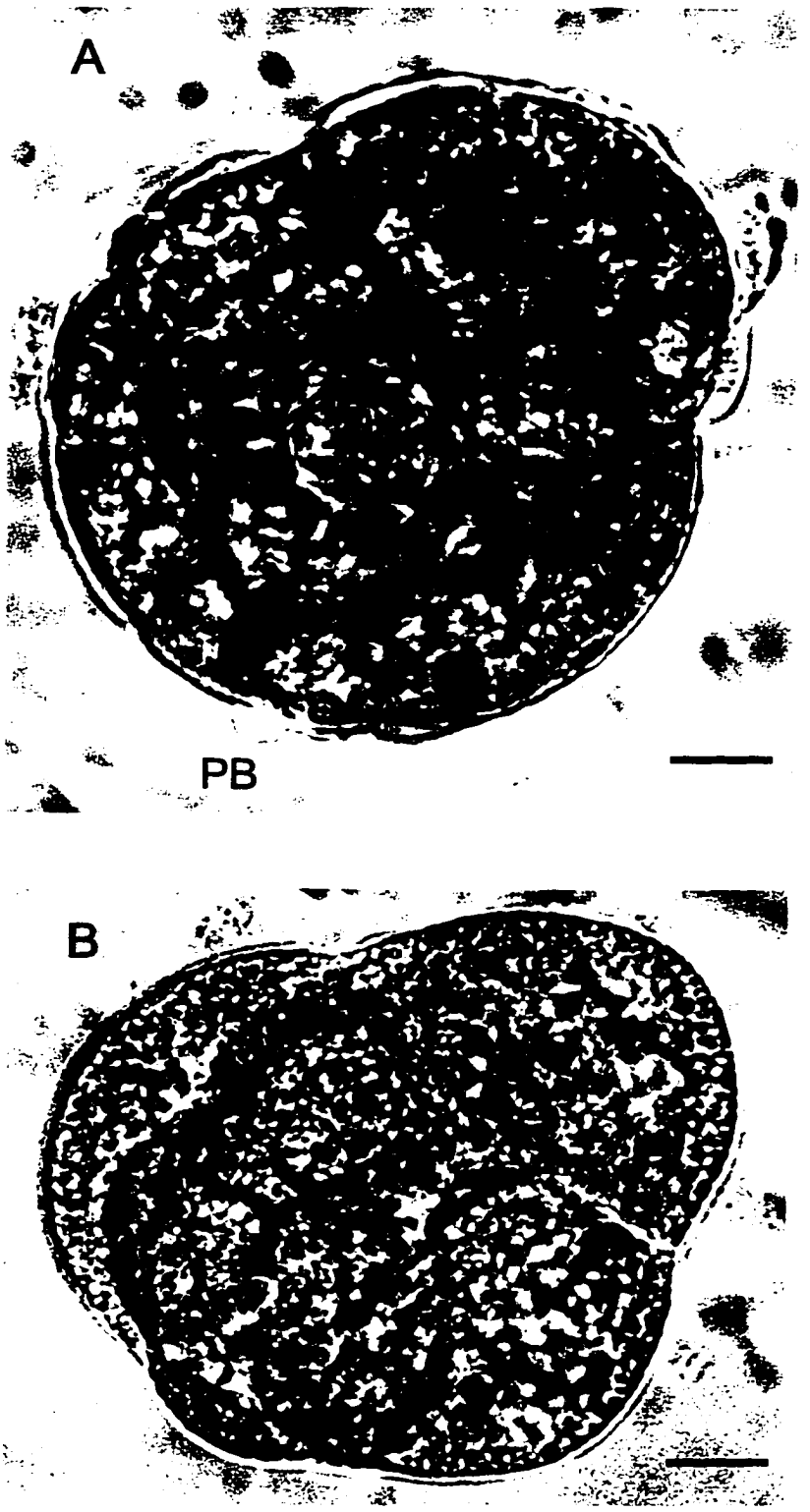


Figure 13

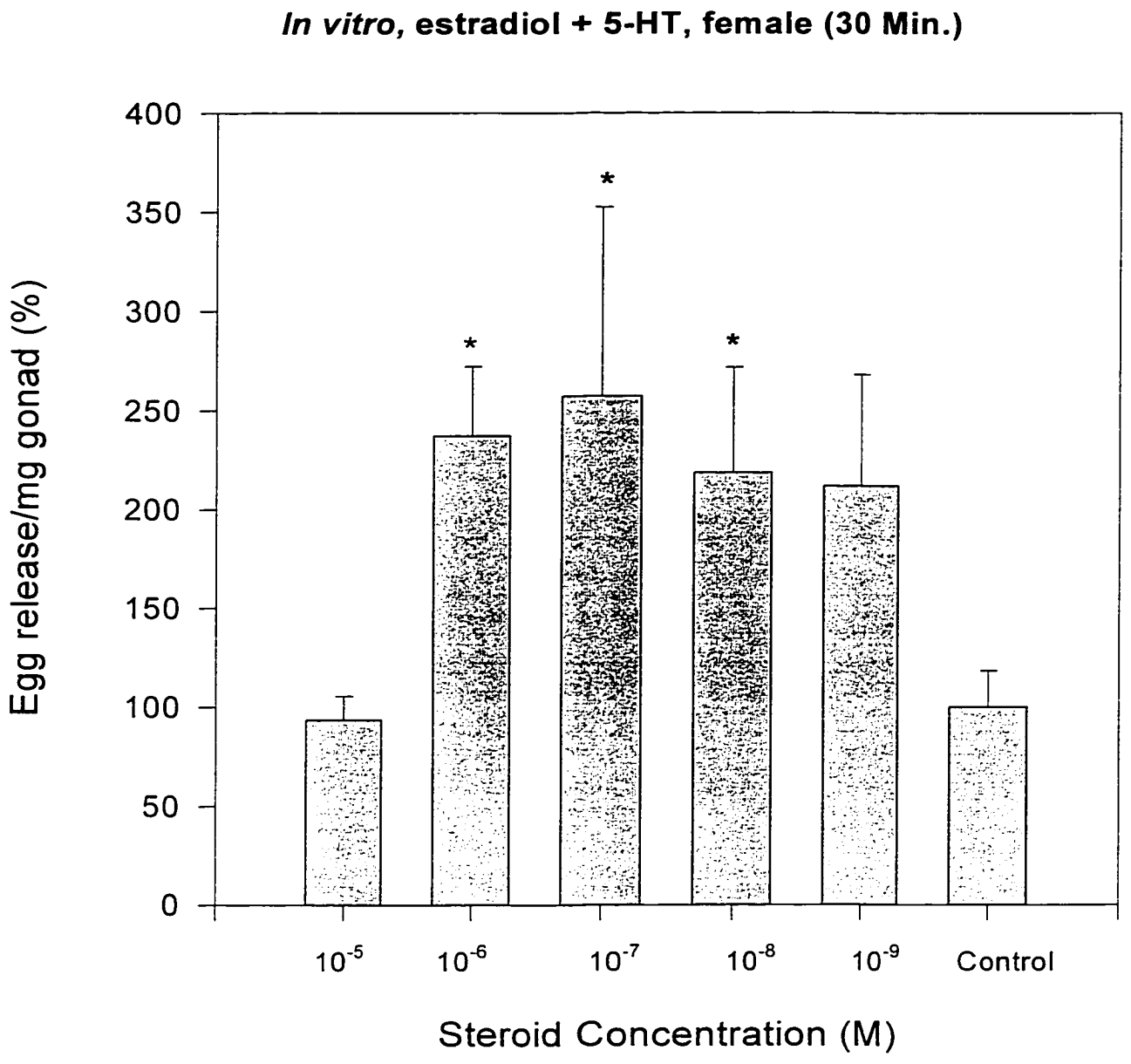


Figure 14. *In vitro* effects of estradiol (30 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M estradiol in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. *P<0.05, Dunnett's test. Each value represents the mean±SE of 6 experiments.

the raw data was violated). A maximum stimulatory effect of 237.4% of that of the control group was observed at 10^{-6} M ($P<0.05$, n=6, Dunnett's test) and a minimum significant effect of 218.7% of the control group. A maximum was obtained at 10^{-8} M ($P<0.05$, n=6, Dunnett's test). No significant effects were found for treatments with estradiol at 10^{-5} M or 10^{-9} M ($P>0.05$, n=6, Dunnett's test).

Treatment of male gonad preparations with estradiol for 30 minutes did not have significant effects on 5-HT-induced gamete release ($P=0.440$, n=6, ANOVA; Figure 15).

II-C-1-b. Testosterone: Treatment with testosterone for 30 minutes had inhibitory effects on 5-HT-induced egg release ($P<0.001$, n=7, ANOVA using natural logarithm-transformed data because homogeneity of the raw data was violated). As shown in Figure 16, treatments with 10^{-5} –10^{-8} M testosterone significantly inhibited egg release ($P<0.005$ for 10^{-5} –10^{-7} M, $P<0.05$ for 110^{-8} M, n=7, Dunnett's test). The maximum response, which was 74.6% inhibition of that of the control group, was observed at 10^{-5} M. The minimal significant inhibition (33.8%) was observed at 10^{-8} M.

In males, treatment of gonad pieces with testosterone significantly increased 5-HT-induced sperm release. Testosterone within a concentration range of 10^{-6}-10^{-9} M significantly elevated sperm release induced by 5-HT ($P<0.001$, n=5, ANOVA; Figure 17). A peak response of 270.7% of that in the control group was observed at 10^{-7} M ($P<0.005$, n=5, Dunnett's test). A minimum significant effect of 201.3% of that of the control group occurred at 10^{-9} M. No significant elevation was obtained at 10^{-5} M ($P>0.05$, n=5, Dunnett's test).

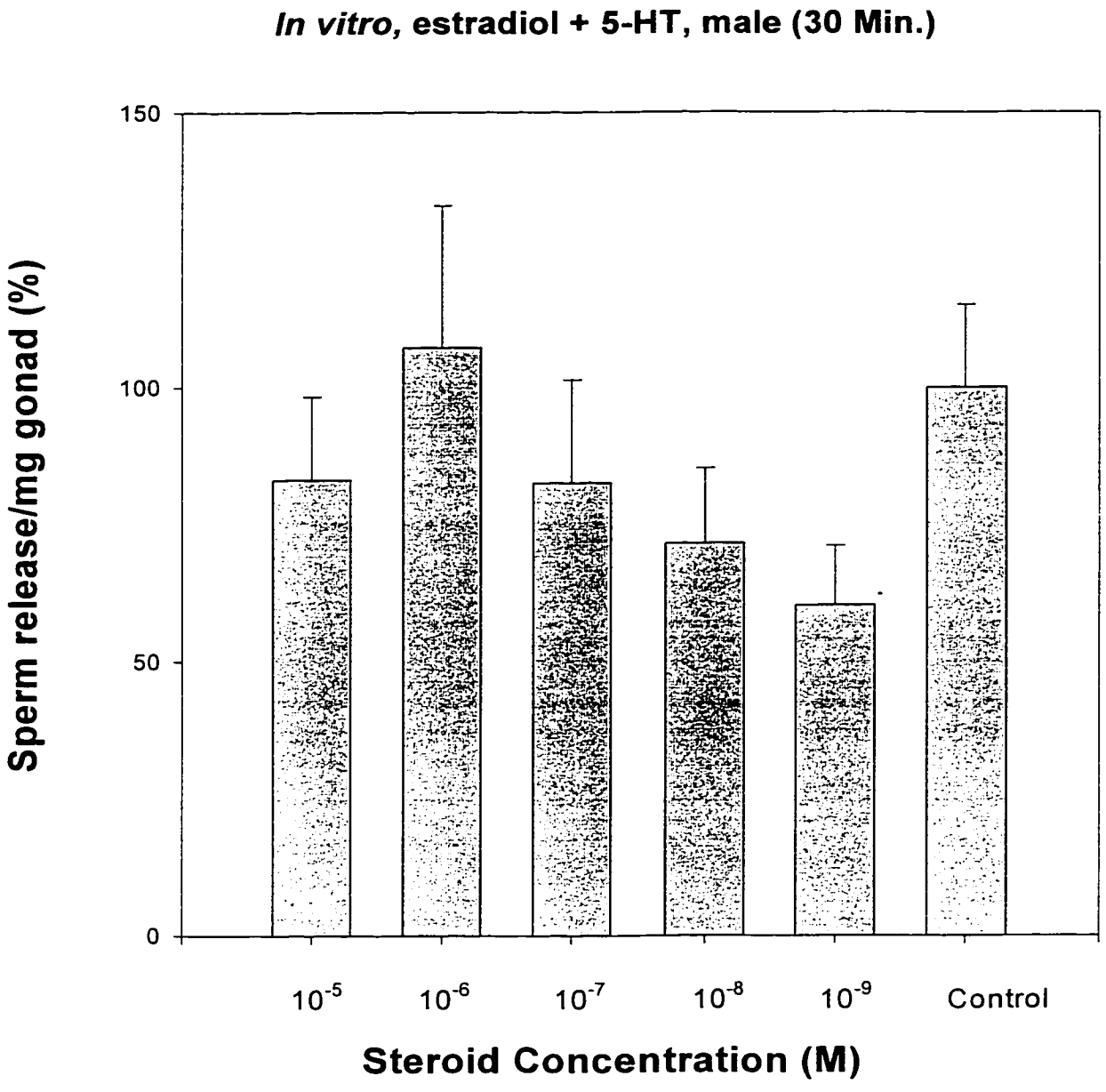


Figure 15. *In vitro* effects of estradiol (30 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-5}-10^{-9} M estradiol in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

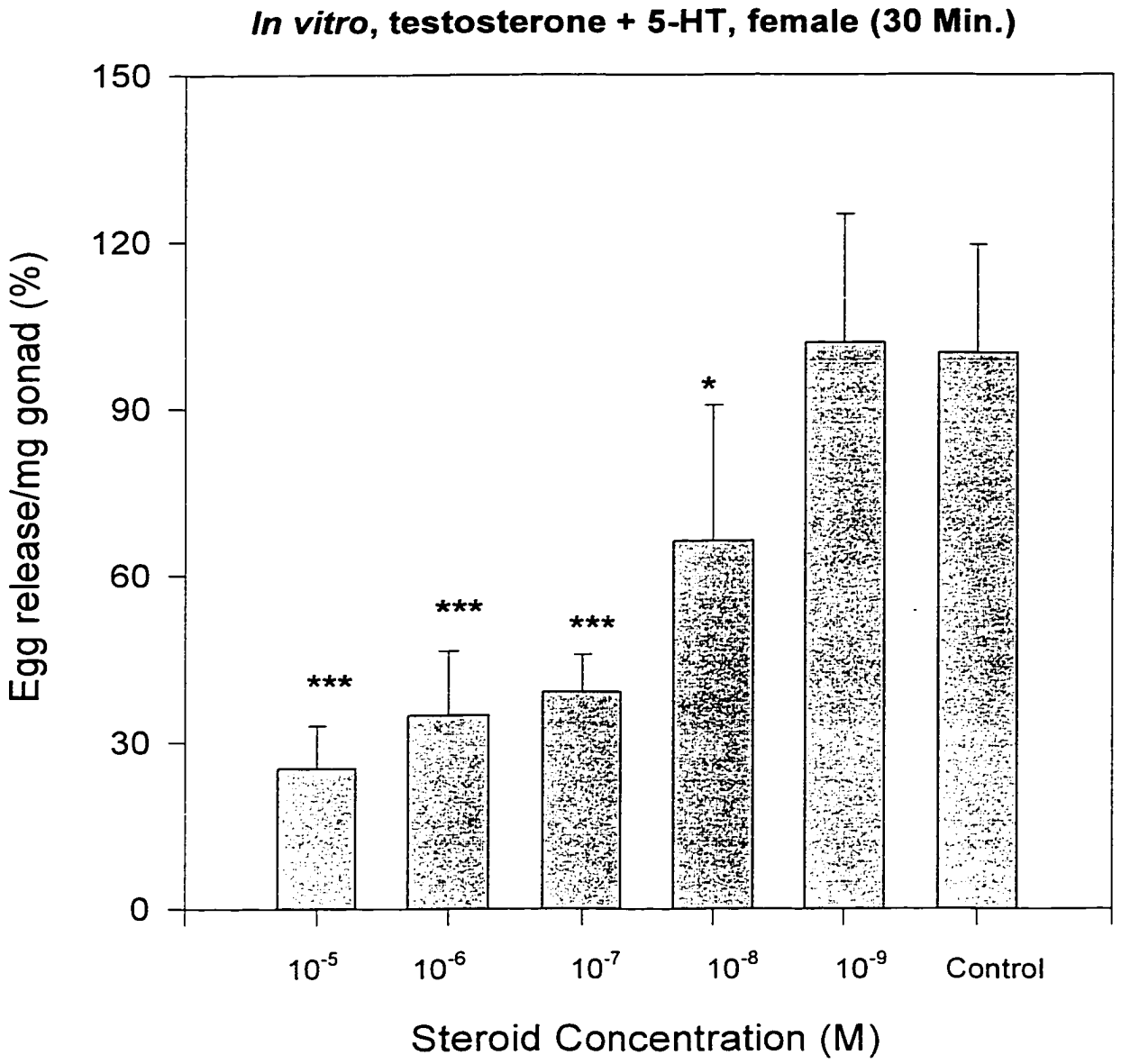


Figure 16. *In vitro* effects of testosterone (30 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M testosterone in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. *P<0.05, ***P<0.005 Dunnett's test. Each value represents the mean±SE of 7 experiments.

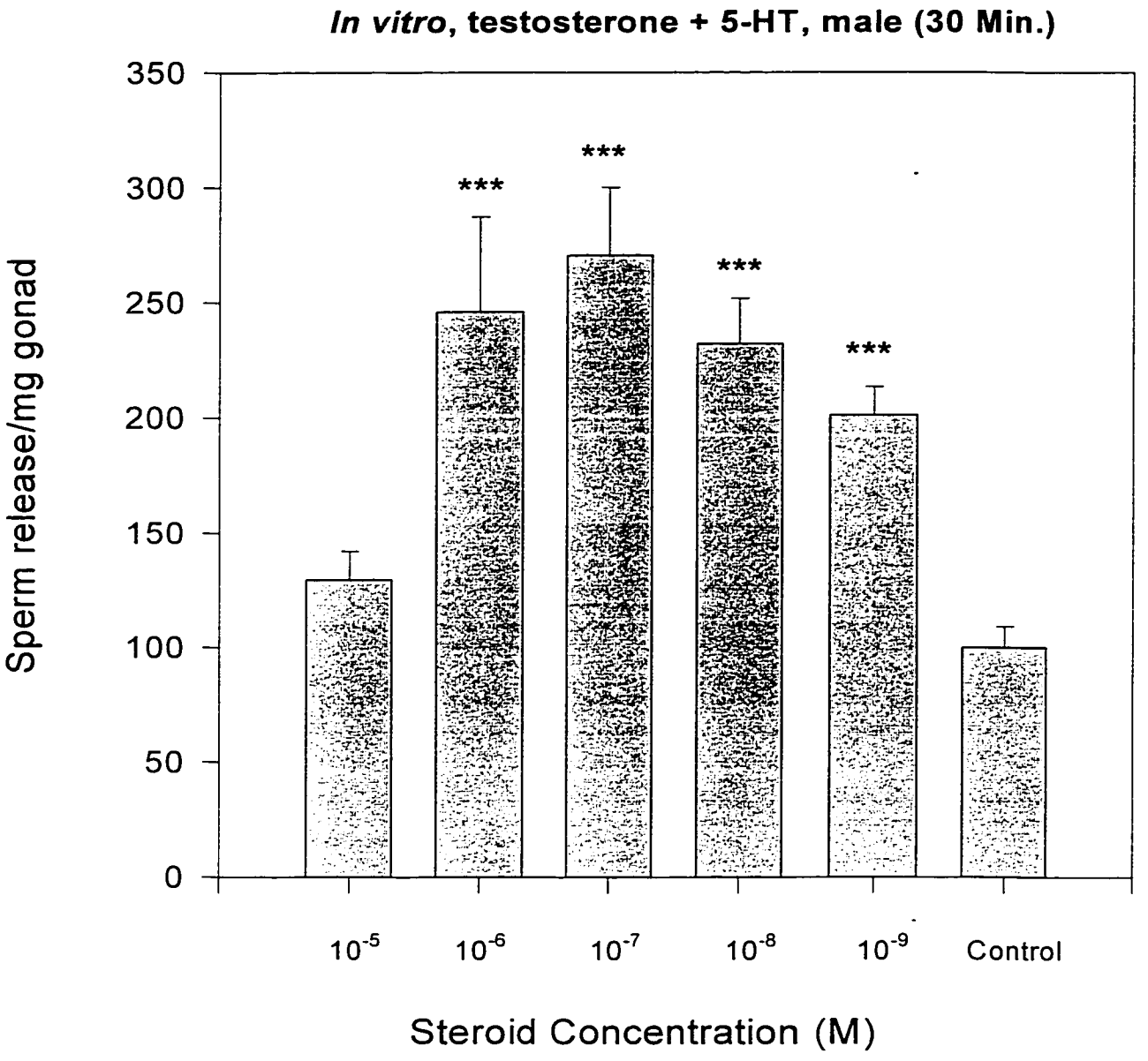


Figure 17. *In vitro* effects of testosterone (30 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-5}-10^{-9} M testosterone in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. **P<0.01, ***P<0.005, Dunnett's test. Each value represents the mean±SE of 5 experiments.

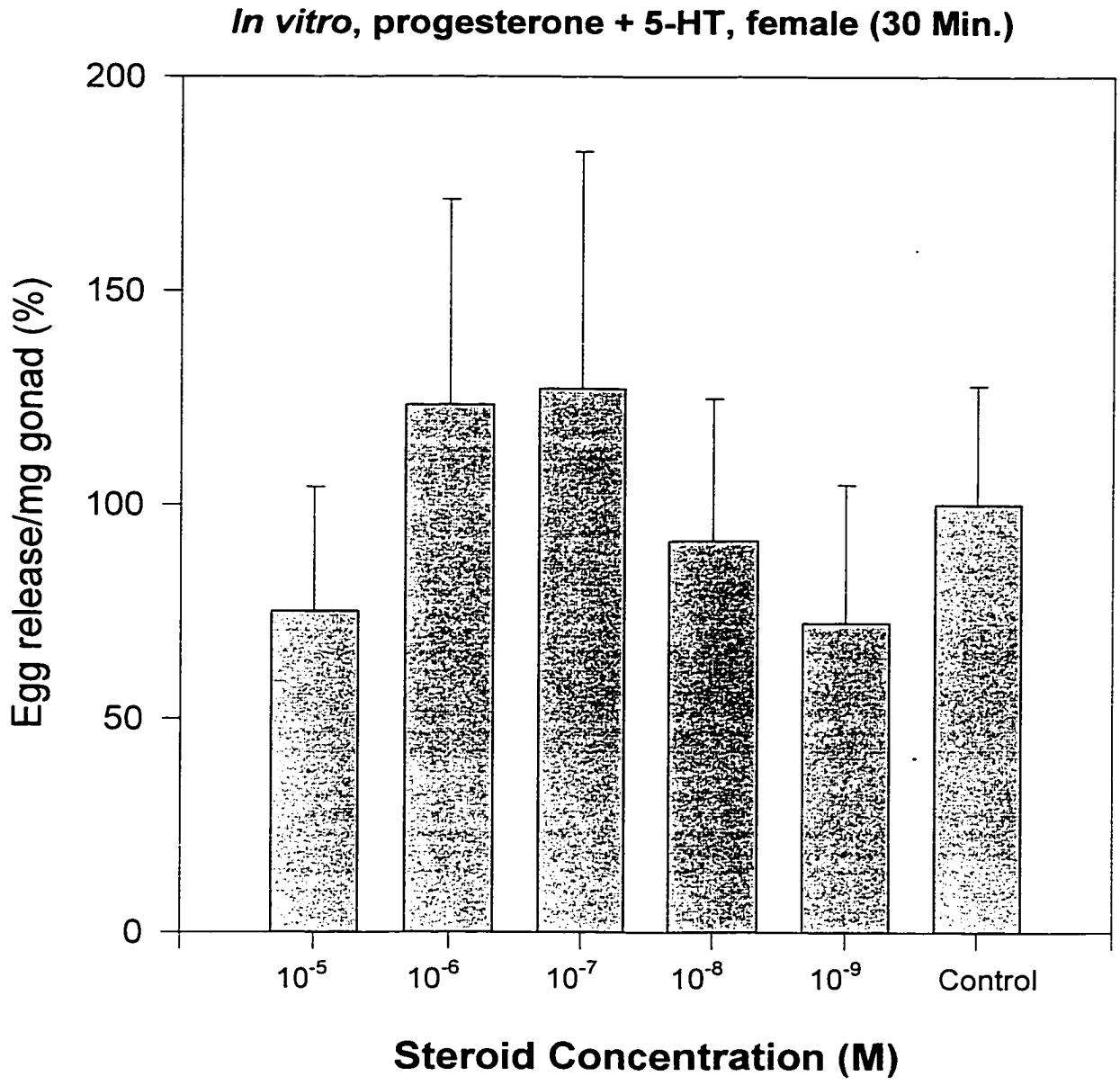


Figure 18. *In vitro* effects of progesterone (30 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M progesterone in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 4 experiments.

II-C-1-c. Progesterone: Progesterone treatment for 30 minutes did not show any significant effects on 5-HT-induced egg release (P=0.097, n=4, ANOVA; Figure 18).

Treatment with progesterone for 30 minutes had stimulatory effect on 5-HT-induced sperm release (P=0.006, n=5, ANOVA; Figure 19). Progesterone at 10^{-6} M and 10^{-7} M significantly potentiated the effects of 5-HT ($P<0.05$ for the 10^{-6} M group, $P<0.01$ for the 10^{-7} M group, n=5, Dunnett's test for all groups). Beyond this concentration range, no significant effect was found ($P>0.05$, n=5, Dunnett's test). A maximal effect of 303.0% of the control group was obtained at 10^{-7} M while the minimal significant effect (264.9% of the control group) was observed at 10^{-6} M.

II-C-2. 15 minutes trials: When the treatment time was further decreased to 15 minutes, no effect was observed in any of the experimental groups (estradiol in females, Figure 20; estradiol in males, Figure 21; testosterone in females, Figure 22; testosterone in males, Figure 23; progesterone in females, 24; progesterone in males, 25; $P>0.05$, n=6, ANOVA, for all experiments).

The effects of sex steroids on 5-HT-induced egg or sperm release are summarized in Table 5.

Table 5. Summary of the effects of sex steroids on 5-HT-induced gamete release (+: stimulatory, -: inhibitory, 0: no effect, na: not applicable)

Steroids	Female			Male		
	120 Min.	30 Min.	15 Min.	120 Min.	30 Min.	15 Min.
Estradiol	+	+	0	+	0	0
Testosterone	0	–	0	+	+	0
Progesterone	+	0	0	+	+	0

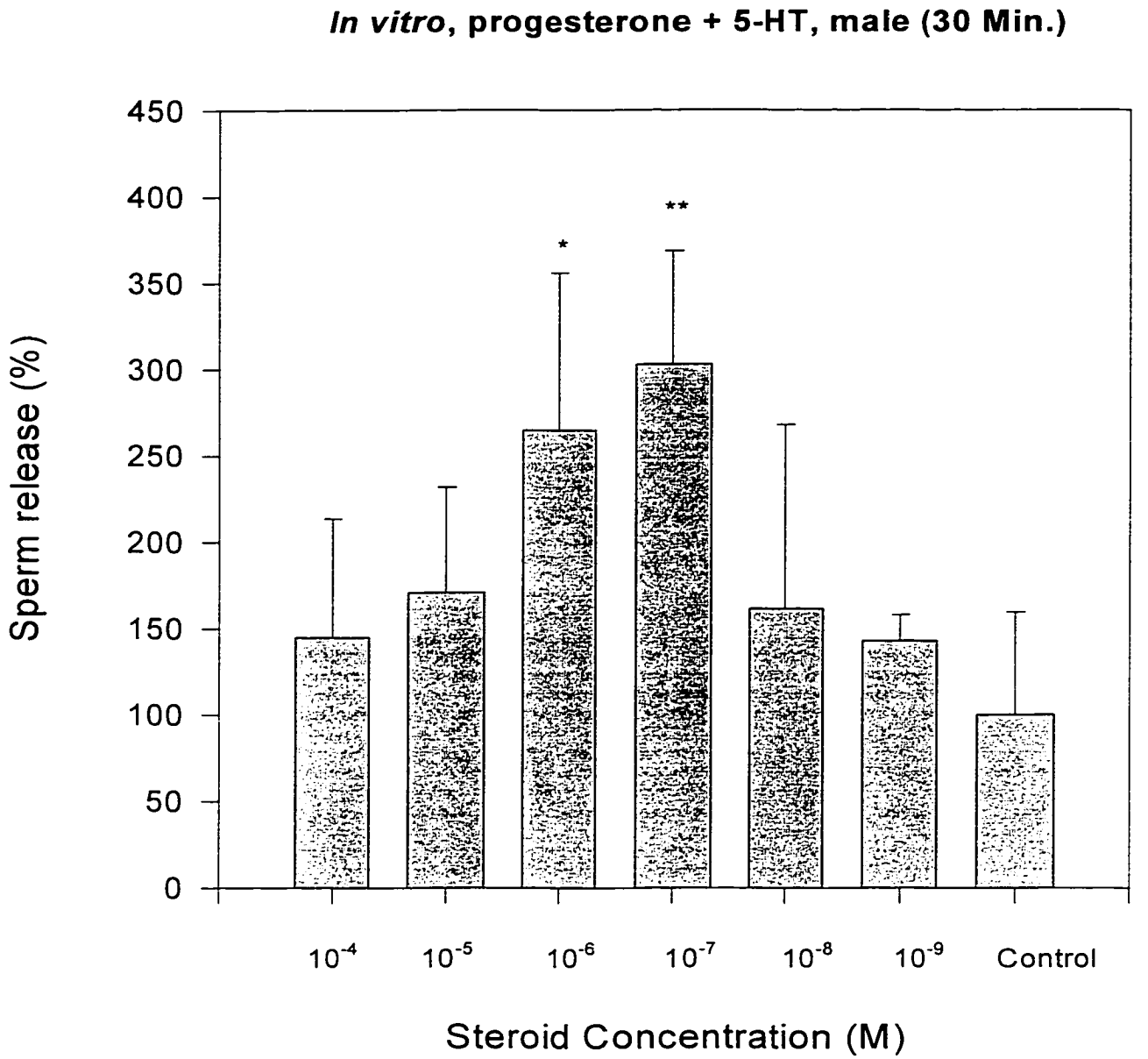


Figure 19. *In vitro* effects of progesterone (30 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-4}-10^{-9} M progesterone in the experimental groups or with artificial seawater in the control group for 30 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. *P<0.05, **P<0.01, ***P<0.005, Dunnett's test. Each value represents the mean±SE of 5 experiments.

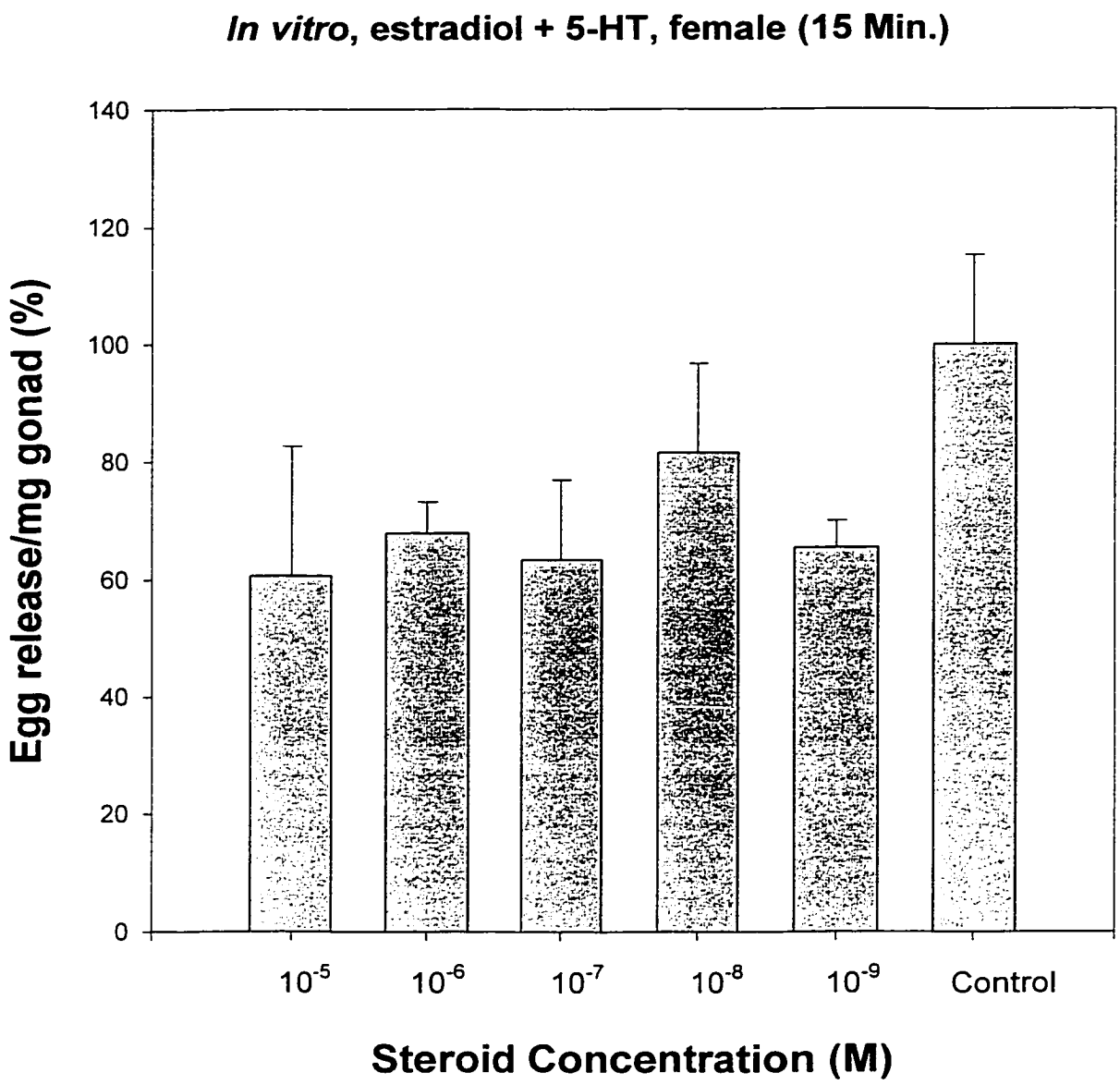


Figure 20. *In vitro* effects of estradiol (15 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M estradiol in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

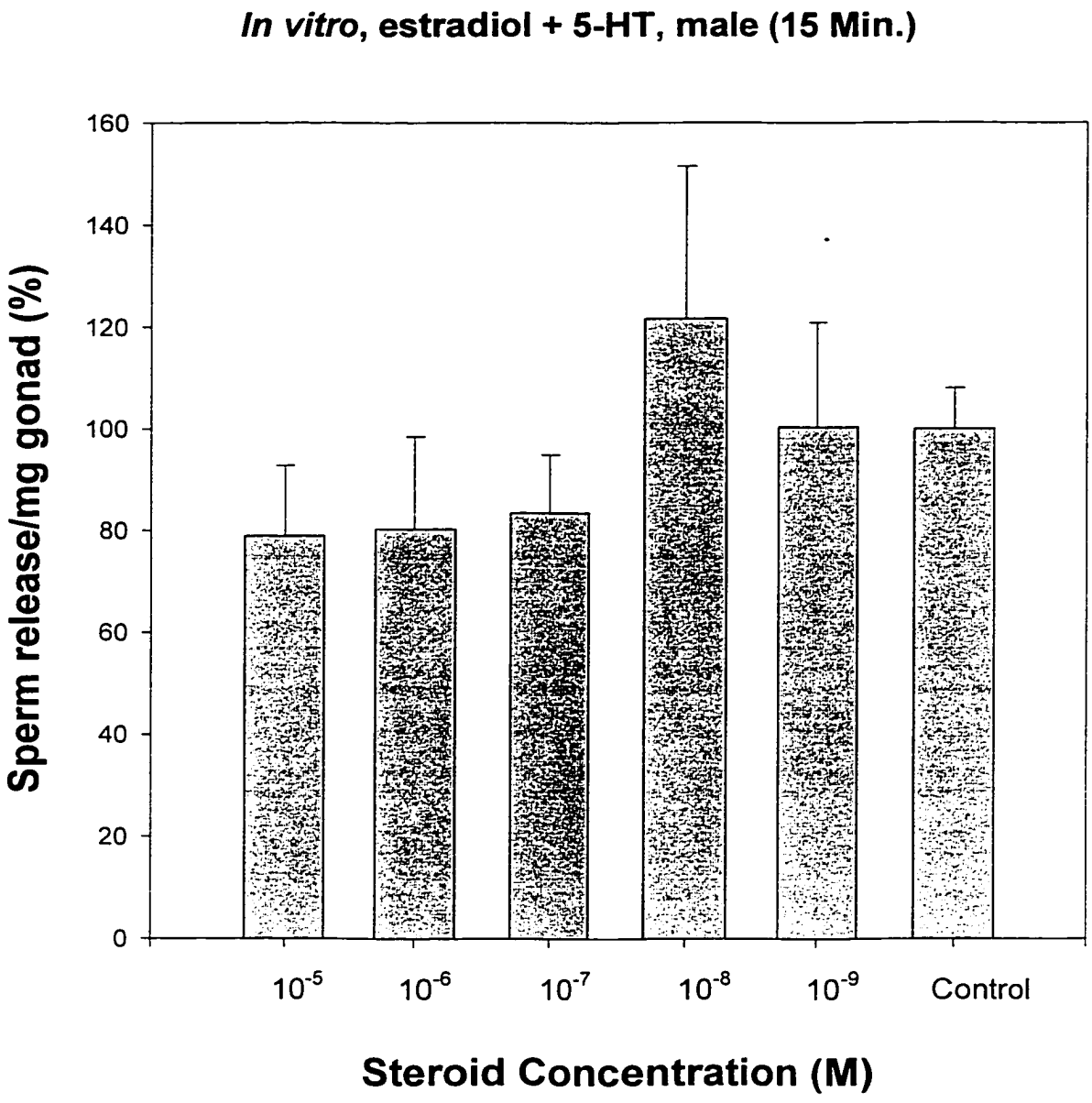


Figure 21. *In vitro* effects of estradiol (15 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-5}-10^{-9} M estradiol in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

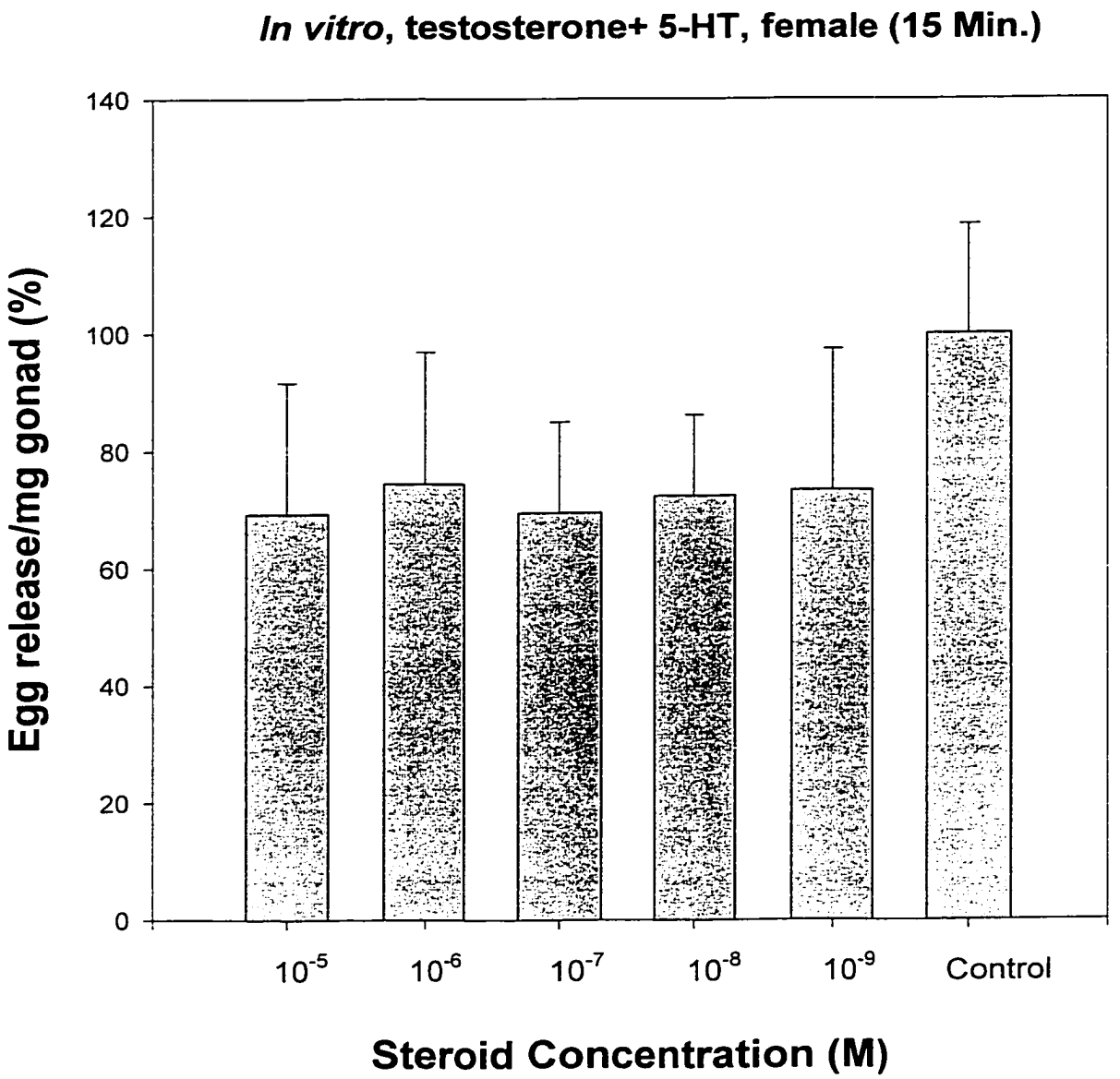


Figure 22. *In vitro* effects of testosterone (15 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M testosterone in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

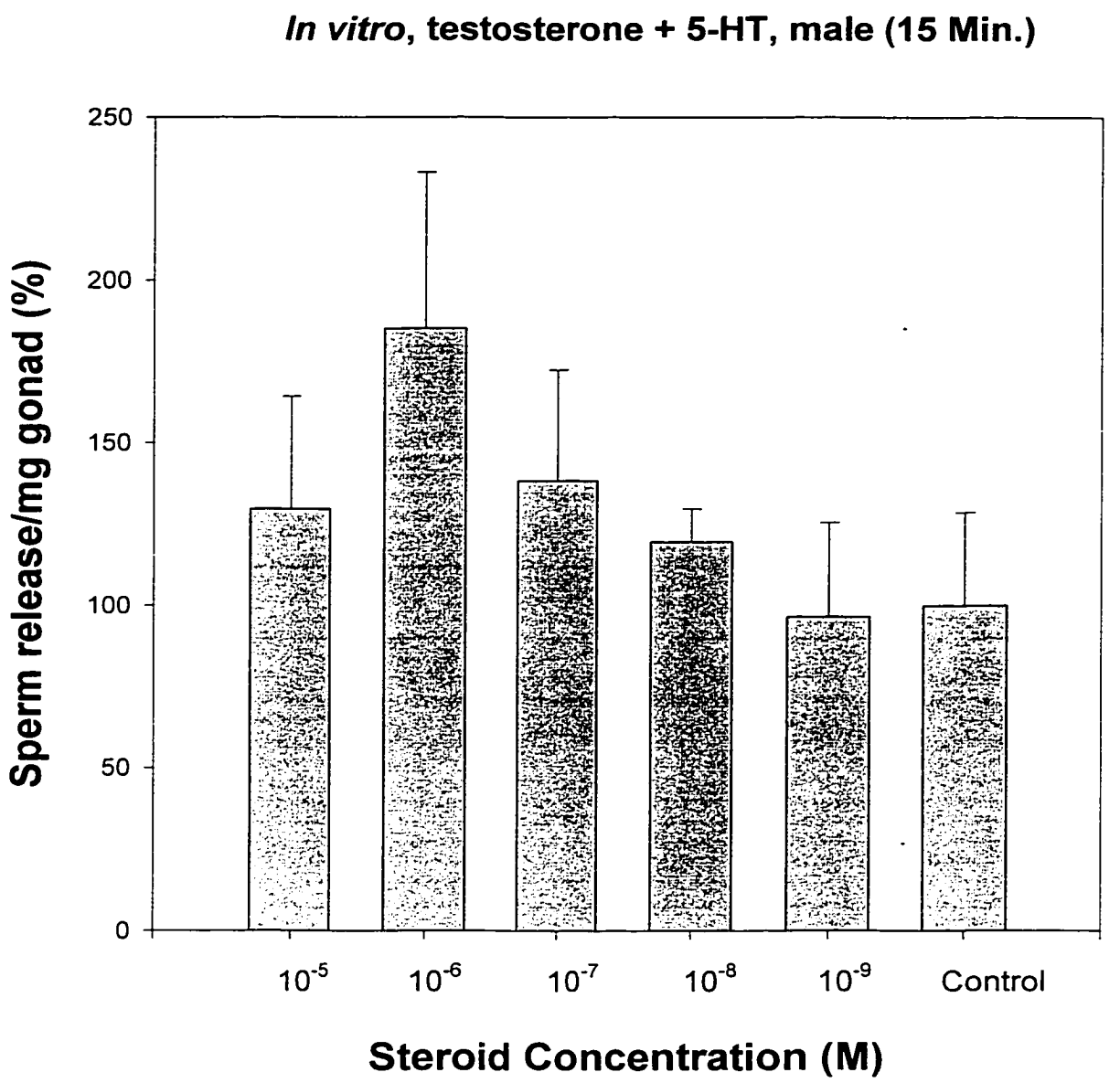


Figure 23. *In vitro* effects of testosterone (15 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-5}-10^{-9} M testosterone in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

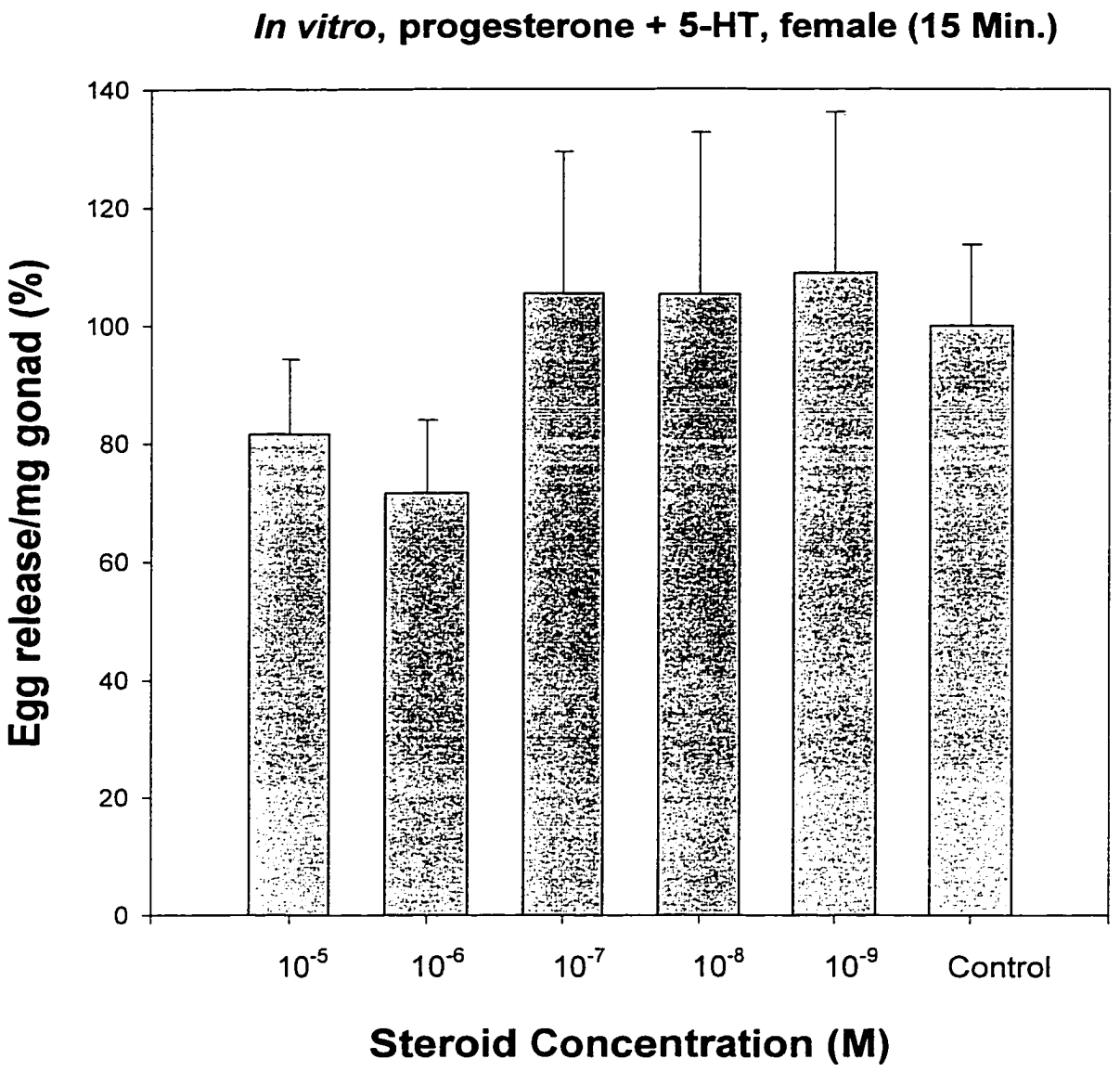


Figure 24. *In vitro* effects of progesterone (15 minutes) on 5-HT-induced egg release. Gonad pieces were incubated with 10^{-5}-10^{-9} M progesterone in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

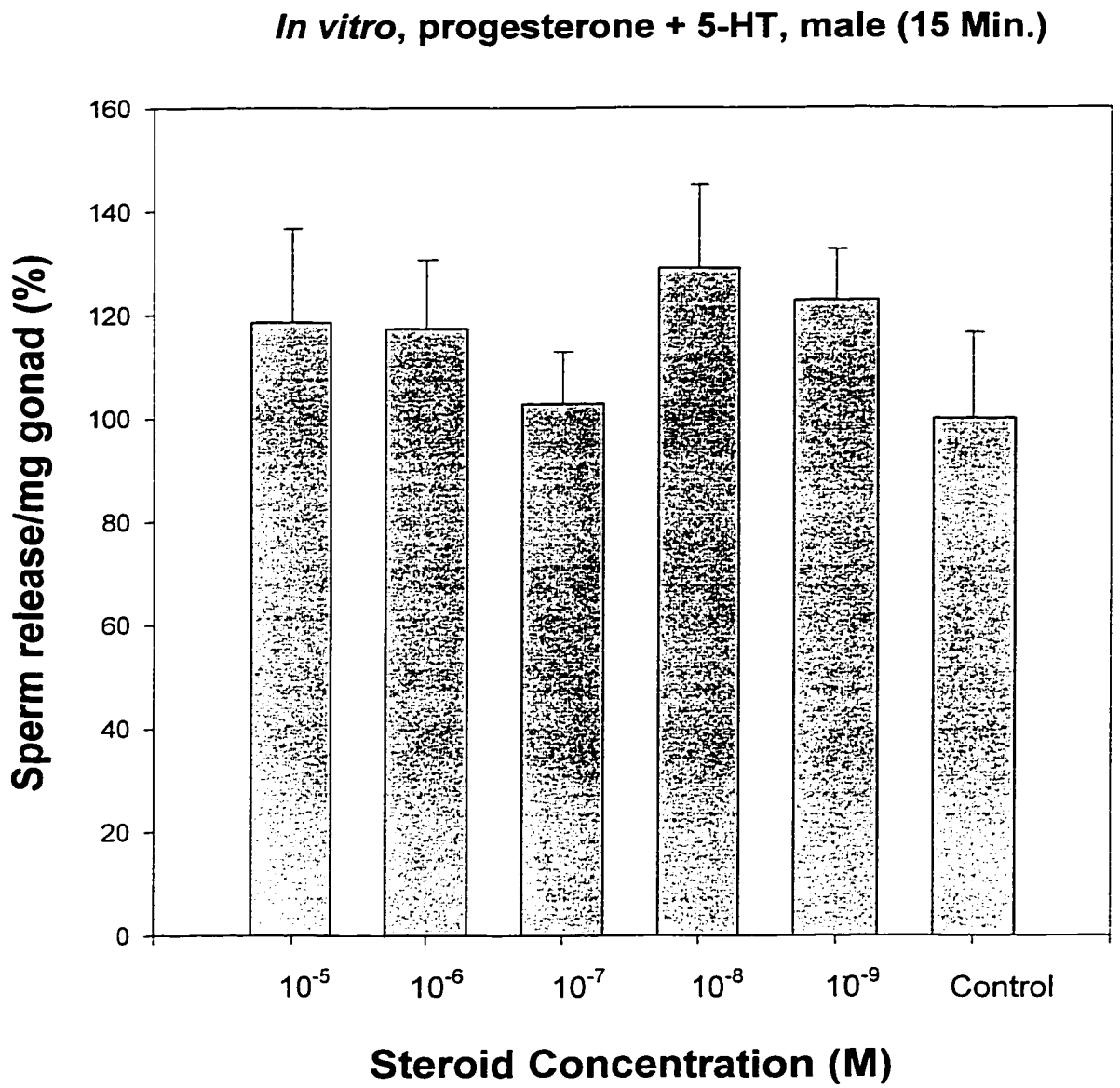


Figure 25. *In vitro* effects of progesterone (15 minutes) on 5-HT-induced sperm release. Gonad pieces were incubated with 10^{-5}-10^{-9} M progesterone in the experimental groups or with artificial seawater in the control group for 15 minutes, and then with 10^{-5} M 5-HT for 90 minutes. Data were standardized to percentages of the response in the control group. Each value represents the mean±SE of 6 experiments.

II-D. Possible Involvement of Intracellular Sex Steroid Receptors in the Actions of Sex Steroids:

To investigate the possible involvement of specific receptors in the actions of sex steroids, the effects of anti-steroids or RNA or protein synthesis inhibitors on 5-HT-induced gamete release were examined.

In the experimental groups, the gonad pieces were pre-treated with one of the anti-steroids or an RNA or protein synthesis inhibitor before incubation with sex steroids and 5-HT. The numbers of gametes released in the experimental groups were compared with those of the control group in which the gonad pieces were incubated with sex steroids and 5-HT only. In other control groups, the effect of 5-HT on spawning and those of anti-steroids or RNA or protein synthesis inhibitors alone on 5-HT-induced spawning were examined.

II-D-1. Inhibition of estradiol actions by anti-steroids, actinomycin D and cycloheximide: Since the normality of the raw data was not met, statistical analyses were performed using natural logarithm-transformed data. As shown in Figure 26, egg release was effectively induced by 10^{-5} M 5-HT, compared to the blank control group ($P<0.005$, $n=9$, t-test). This effect was facilitated by pretreatment with 10^{-6} M estradiol ($P<0.05$, $n=9$, t-test, compared to the group incubated with only 5-HT). However, pre-treatments of the gonad pieces with antisteroids or RNA or protein synthesis inhibitors had significant effects on the action of estradiol ($P<0.005$, $n=9$, ANOVA). Based on these results, one-tailed Dunnett's tests were performed with the prediction that one or more of the antisteroids or inhibitors blocked the action of estradiol. The results showed that pretreatment with 1.25×10^{-6} M tamoxifen, which is an antagonist to estrogen receptors,

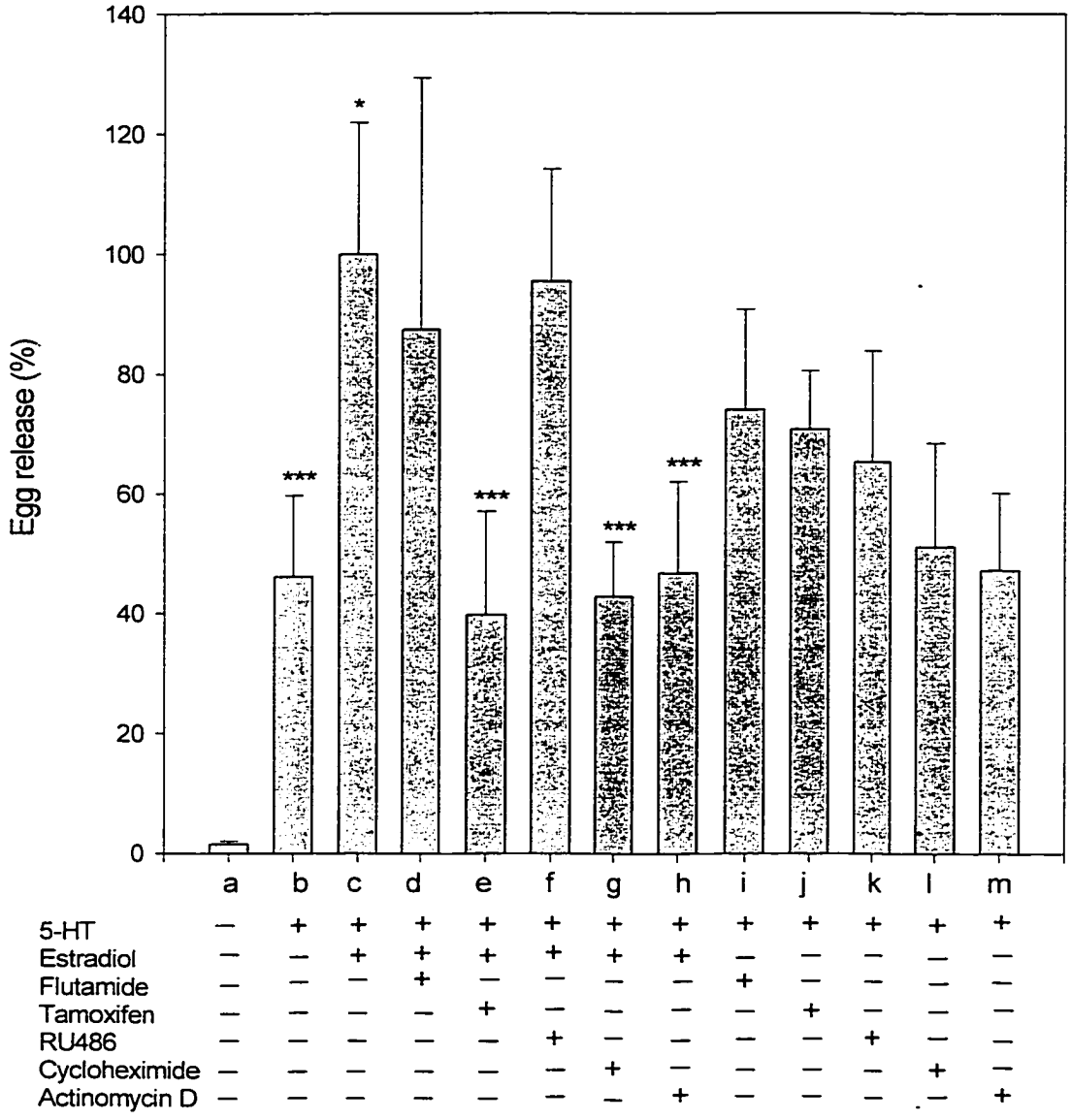


Figure 26. Inhibition of the effects of estradiol on egg release by antisteroids or RNA or protein synthesis inhibitors. Gonad pieces were pre-incubated with one of the antisteroids (flutamide, tamoxifen or RU486), cycloheximide or actinomycin D for 15 minutes, followed by incubation with estradiol for 2 hours and 5-HT for 90 minutes. The data were standardized to the percentage of the response induced by estradiol and 5-HT and expressed as mean±SE (n=9). b is significantly different from a, c is significantly different from b, and e, g and h are significantly different from c. *P<0.05, ***P<0.005, Dunnett's test for all comparisons.

significantly decreased egg release compared to the group which was incubated with only estradiol and 5-HT, ($P<0.005$, n=9, Dunnett's test). Similar results were also obtained in groups pretreated with 0.8 μM actinomycin D ($P<0.005$, n=9, Dunnett's test), which is an RNA synthesis inhibitor, and 10 μM cycloheximide ($P<0.005$, n=9, Dunnett's test), which is a protein synthesis inhibitor. Flutamide, an antagonist to androgen receptor, and RU486, an antagonist to progesterone receptor, had no inhibitory effects on the potentiation of 5-HT-induced egg release by estradiol ($P>0.05$, n=9, Dunnett's test). In the groups treated with only flutamide, tamoxifen, RU486, actinomycin D or cycloheximide, no significant effect was observed on 5-HT-induced egg release ($P>0.05$, n=9, ANOVA, compared to the group incubated with 5-HT only).

Similar results were also obtained with males (Figure 27). Pretreatment with antisteroids or RNA or protein synthesis inhibitors had a significant effect on estradiol actions ($P<0.005$, n= 9, ANOVA). ANOVA and all subsequent statistical analyses were performed using natural logarithm-transformed data since normality of the raw data was not met. Based on these results, one-tailed Dunnett's tests indicated that the potentiation of 5-HT-induced sperm release by 10^{-6} M estradiol were abolished by pretreatment with tamoxifen ($P<0.005$), actinomycin D ($P<0.005$) and cycloheximide ($P<0.01$) (n=9, Dunnett's test, compared to the group incubated with estradiol and 5-HT). Notably, RU486 also abolished a part of the effects ($P<0.05$) while flutamide did not have any significant effect ($P>0.05$) (n=9, Dunnett's test, compared to the group incubated with estradiol and 5-HT). Treatments with the anti-steroids or RNA or protein synthesis inhibitors did not alter the effect of 5-HT significantly ($P>0.05$, n=9, ANOVA, compared to the group incubated with 5-HT alone).

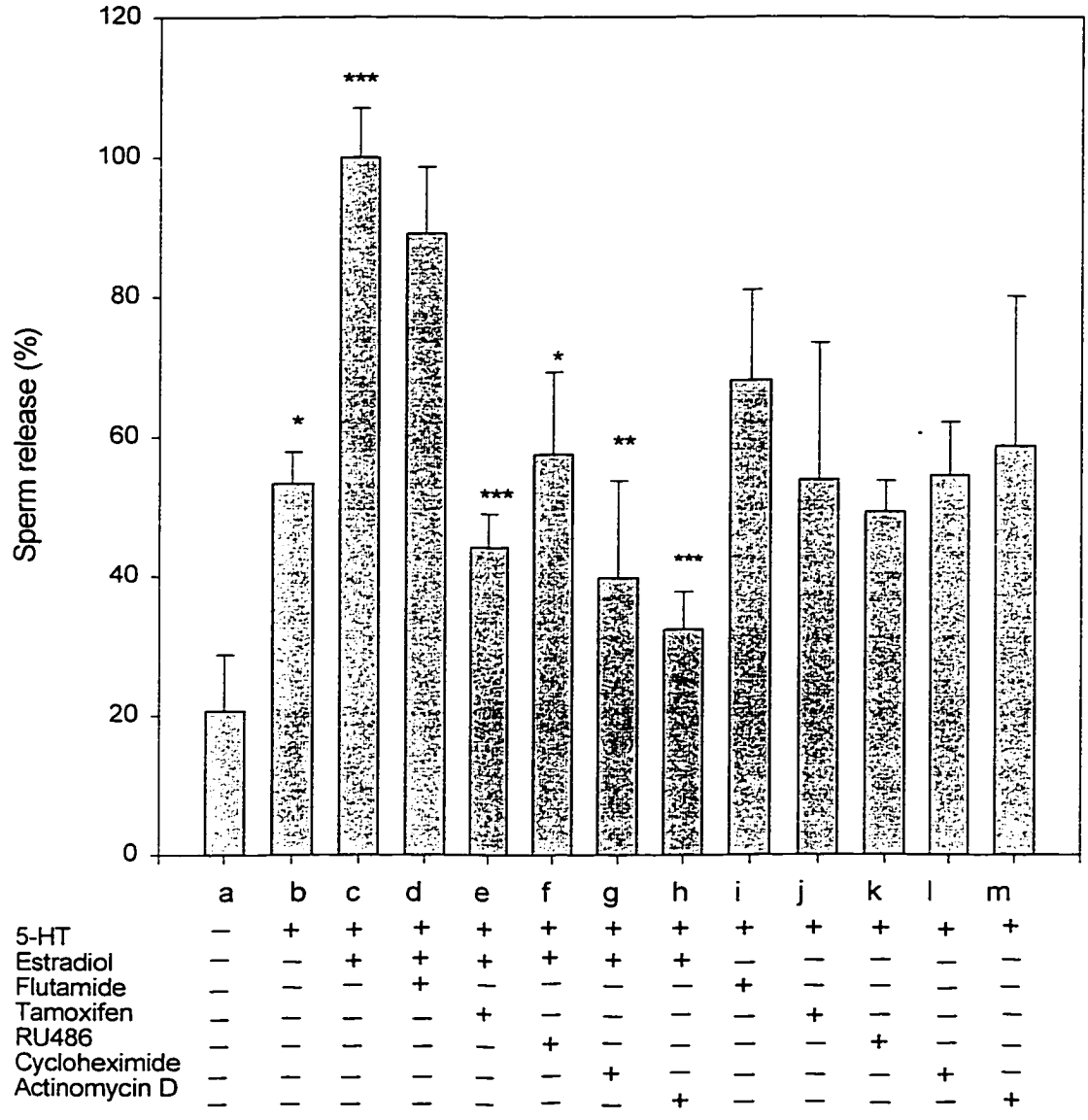


Figure 27. Inhibition of the effects of estradiol on sperm release by antisteroids or RNA or protein synthesis inhibitors. Gonad pieces were pre-incubated with one of the antisteroids (flutamide, tamoxifen or RU486), cycloheximide or actinomycin D for 15 minutes, followed by incubation with estradiol for 2 hours and 5-HT for 90 minutes. The data were standardized to the percentage of the response induced by estradiol and 5-HT and expressed as mean±SE (n=9). b is significantly different from a, c is significantly different from b, and e, f, g and h are significantly different from c, *P<0.05, **P<0.01, ***P<0.005, Dunnett's test for all comparisons.

II-D-2. Inhibition of the action of testosterone on sperm release: Since normality of the raw data was violated, natural logarithm transformation was performed prior to the statistical analyses. Pretreatment with 10^{-6} M testosterone significantly increased the sperm release induced by 10^{-5} M 5-HT ($P<0.005$, n=6, t-test, compared to the 5-HT control group). Pretreatment with antisteroids or RNA or protein synthesis inhibitors had a significant effect on the action of testosterone ($P=0.001$, n= 6, ANOVA; Figure 28). The promotion of sperm release by testosterone was abolished by flutamide ($P<0.05$), cycloheximide ($P<0.005$), and actinomycin D ($P<0.01$) (n=6, Dunnett's test for all groups, compared to the group incubated with testosterone and 5-HT). However, tamoxifen and RU486 also blocked the effects of testosterone ($P<0.005$, n=6, Dunnett's test, compared to the group incubated with testosterone and 5-HT). Treatment with one of the antisteroids, cycloheximide, or actinomycin D followed by 5-HT incubation had no significant effects compared to the group incubated with 5-HT alone ($P>0.05$, n=6, ANOVA).

II-D-3. Inhibition of the actions of progesterone on egg and sperm release: Since normality of the raw data was not met, natural logarith-transformation was performed before all the statistical tests. In these experiments, egg release induced by 10^{-5} M 5-HT was significantly promoted by progesterone at 10^{-7} M ($P<0.05$, n=9, t-test, compared to the 5-HT control group). Pretreatments with antisteroids or RNA or protein synthesis inhibitors had a significant inhibitory effect on actions of progesterone ($P<0.005$, n=9, ANOVA). With thess results as a prediction, the results of one-tailed Dunnett's tests showed that pre-treatments with RU486 ($P<0.005$), flutamide ($P<0.05$), actinomycin D ($P<0.01$) and cycloheximide ($P<0.005$) significantly blocked the effects of progesterone

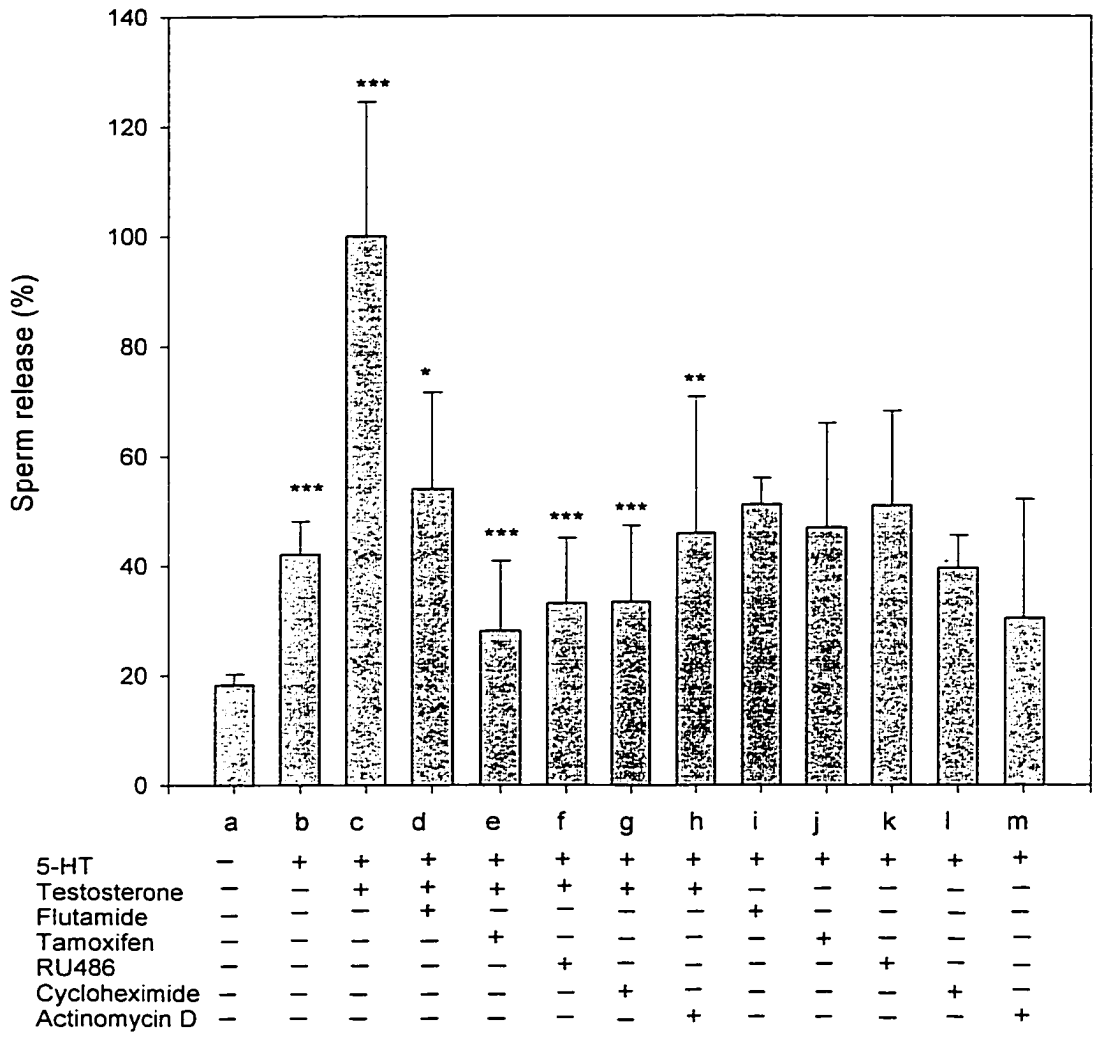


Figure 28. Inhibition of the effects of testosterone on sperm release by antisteroids or RNA or protein synthesis inhibitors. Gonad pieces were pre-incubated with one of the antisteroids (flutamide, tamoxifen or RU486), cycloheximide or actinomycin D for 15 minutes, followed by incubation with testosterone for 2 hours and 5-HT for 90 minutes. The data were standardized to the percentage of the response induced by testosterone and 5-HT and expressed as mean±SE (n=6). b is significantly different from a, c is significantly different from b, and d, e, f, g and h are significantly different from c. **P<0.01, ***P<0.005, Dunnett's test for all comparisons.

(n=9, Dunnett's test, compared to the group incubated with progesterone and 5-HT; Figure 29). Treatments with anti-steroids, actinomycin D and cycloheximide alone had no significant effects on 5-HT-induced egg release (P>0.05, n=9, ANOVA).

In experiments with male gonad preparations, pre-treatments with antisteroids or the inhibitors showed a significant inhibitory effect on progesterone effect (P=0.024, n= 6, ANOVA). Since normality of the raw data was not met, natural logarithm transformation was performed. Treatments with RU486, flutamide, actinomycin D and cycloheximide all blocked the potentiating effect of progesterone on 5-HT-induced sperm release (P<0.05, n=6, Dunnett's test, compared to the group incubated with progesterone and 5-HT; Figure 30). In the control groups, treatments with anti-steroids and RNA or protein synthesis inhibitor did not significantly affect sperm release induced by 5-HT (P>0.05, n=6, ANOVA).

The results of the inhibition of the actions of sex steroids by antisteroids and RNA or protein synthesis inhibitors are summarized in Table 6.

Table 6. Summary of inhibitions of actions of sex steroids by antisteroids and RNA or protein synthesis inhibitors
(+: stimulatory, -: inhibitory, 0: no effect, na: not applicable)

Steroids		Tamoxifen	Flutamide	RU486	Actinomycin D	Cycloheximide
Estradiol	**Female**	–	0	0	–	–
	Male	–	0	–	–	–
Testosterone	**Female**	na	na	na	na	na
	Male	–	–	–	–	–
Progesterone	**Female**	–	0	–	–	–
	Male	–	0	–	–	–

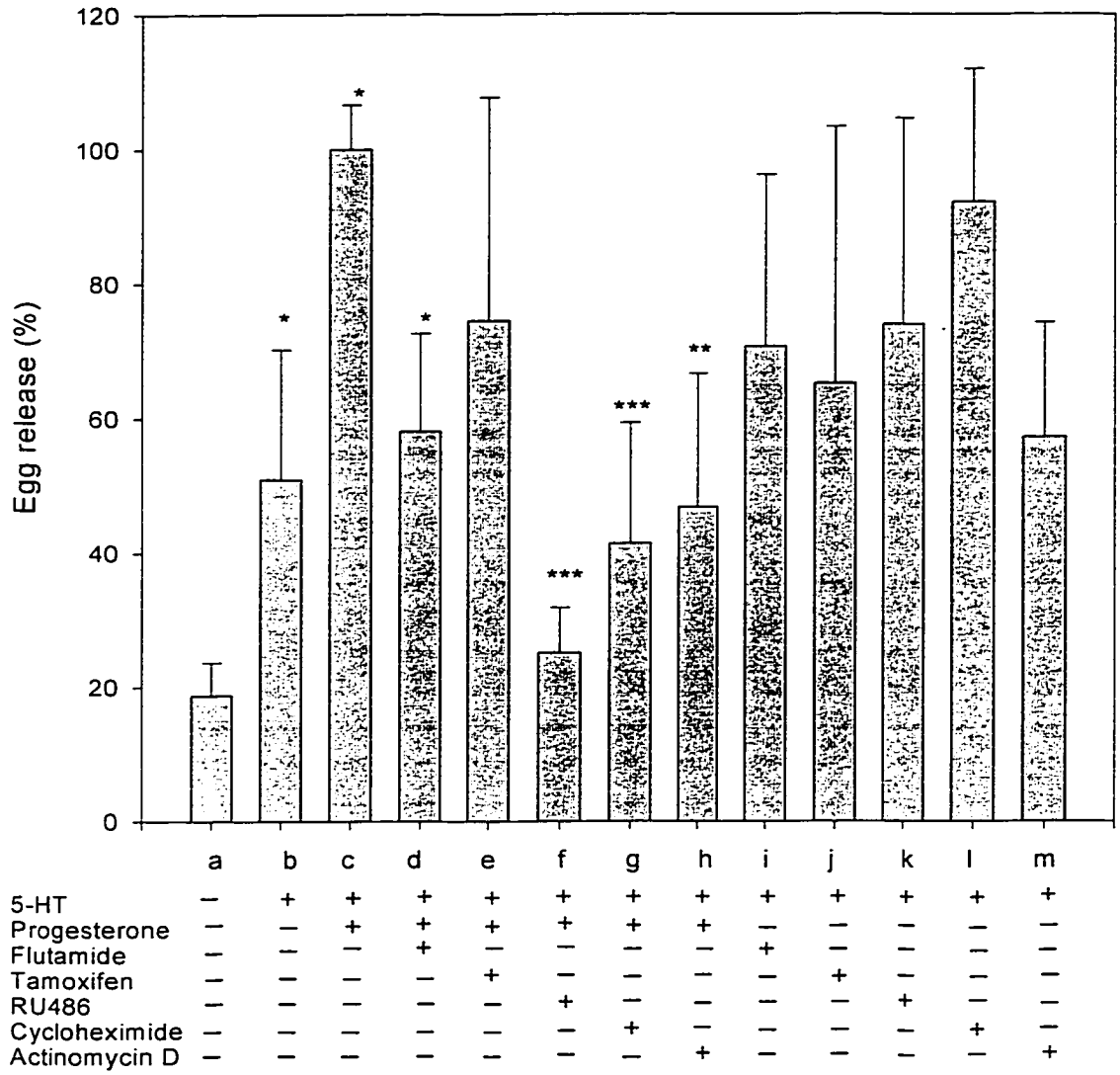


Figure 29. Inhibition of the effects of progesterone on egg release by antisteroids or RNA or protein synthesis inhibitors. Gonad pieces were pre-incubated with one of the antisteroids (flutamide, tamoxifen or RU486), cycloheximide or actinomycin D for 15 minutes, followed by incubation with progesterone for 2 hours and 5-HT for 90 minutes. The data were standardized to the percentage of the response induced by estradiol and 5-HT and expressed as mean±SE (n=9). b is significantly different from a, c is significantly different from b, and d, f, g and h are significantly different from c, *P<0.05, **P<0.01, ***P<0.005, Dunnett's test for all comparisons.

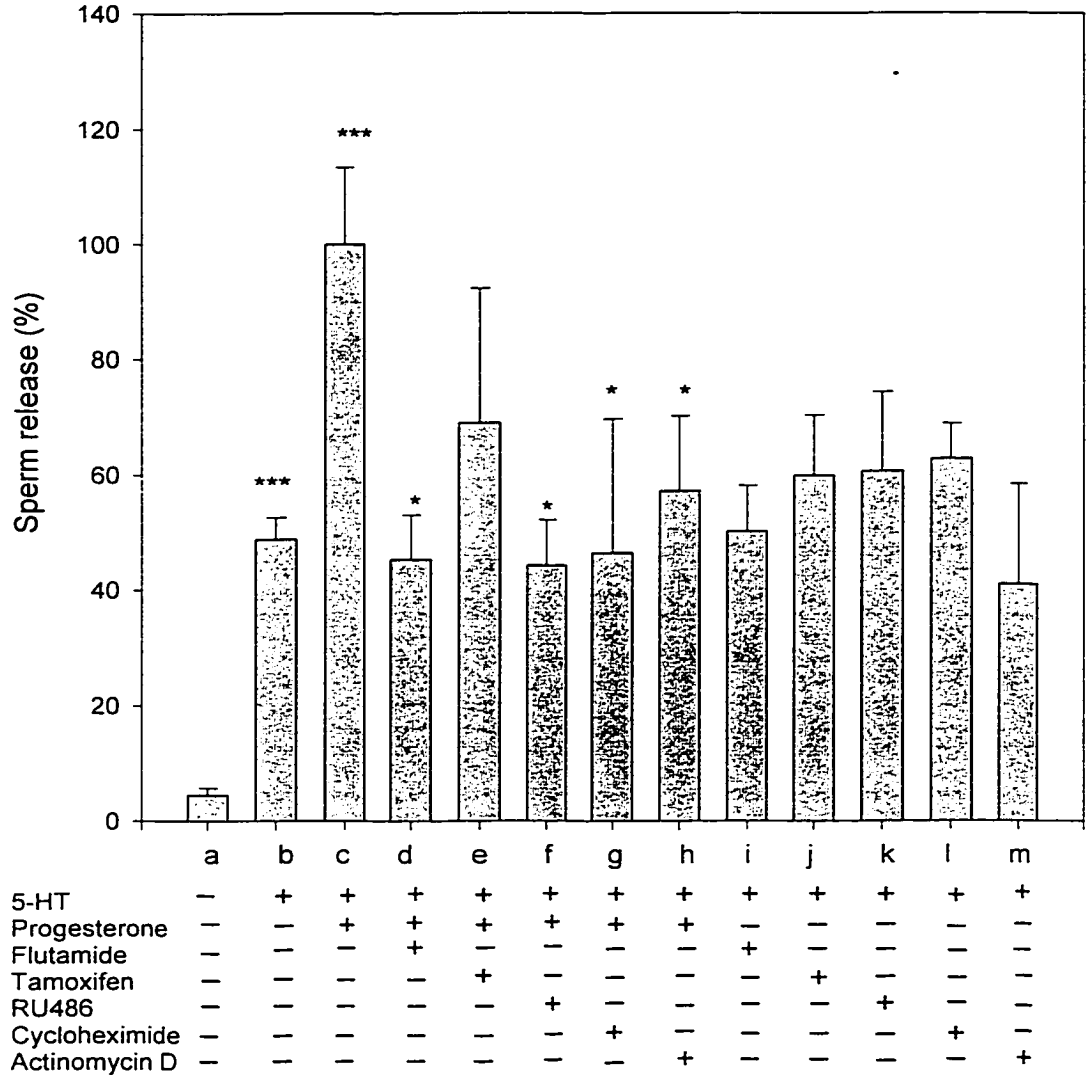


Figure 30. Inhibition of the effects of progesterone on sperm release by antisteroids or RNA or protein synthesis inhibitors. Gonad pieces were pre-incubated with one of the antisteroids (flutamide, tamoxifen or RU486), cycloheximide or actinomycin D for 15 minutes, followed by incubation with progesterone for 2 hours and 5-HT for 90 minutes. The data were standardized to the percentage of the response induced by progesterone and 5-HT and expressed as mean±SE (n=6). b is significantly different from a, c is significantly different from b, and d, f g and h are significantly different from c, *P<0.05, ***P<0.005, Dunnett's test for all comparisons.

II-E. *In vivo* Effects of Sex Steroids on Spawning:

The results of the *in vitro* studies described in the preceding sections suggested that sex steroids might be useful in the induction of spawning in the practice of scallop aquaculture. In order to examine the applicability of these discoveries in scallop culture,we tested the *in vivo* effects of sex steroids on scallop spawning.

In the experimental group, estradiol dissolved in ethanol and diluted in ASW was injected into the gonads of both male and female ripe scallops. Scallops in the control group were injected with the vehicle only. After injection, the scallops were closely observed to record their response time and the eggs or sperm released in the first 3 hours were measured.

II-E-1.Effects of estradiol on scallop spawning:

II-E-1-a. Direct induction: In females, injection of estradiol induced all individuals to spawn (n=14) while only 78.6% in the control group (n=14) injected with vehicle spawned. Figure 31A showed that injection of estradiol resulted in a 300.7% increase in the number of gametes released compared with the control group ($P<0.005$, n=14, t-test). The spawning latency, the time between injection and spawning, of the estradiol-injected group was 53.9% shorter than that of the control group ($P<0.005$, n=14, t-test) (Figure 31B).

Estradiol slightly potentiated spawning in male scallops induced by 5-HT. Figure 32A showed that estradiol induced a 49.8% increase in the number of gametes released in the estradiol-injected group compared with that of the control group ($P<0.05$, n=14, t-test). But the average response time of the experimental group was not different from that of the control group (Figure 32B).

Figure 31. Direct induction of spawning by injection of estradiol in females.
Mature scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of ASW) or estradiol solution (1000 μg/ml vehicle) into gonads. The numbers of eggs spawned per milligram of gonad in the first 3 hours were used as indices of gamete release (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=14). ***P<0.005, t-test.

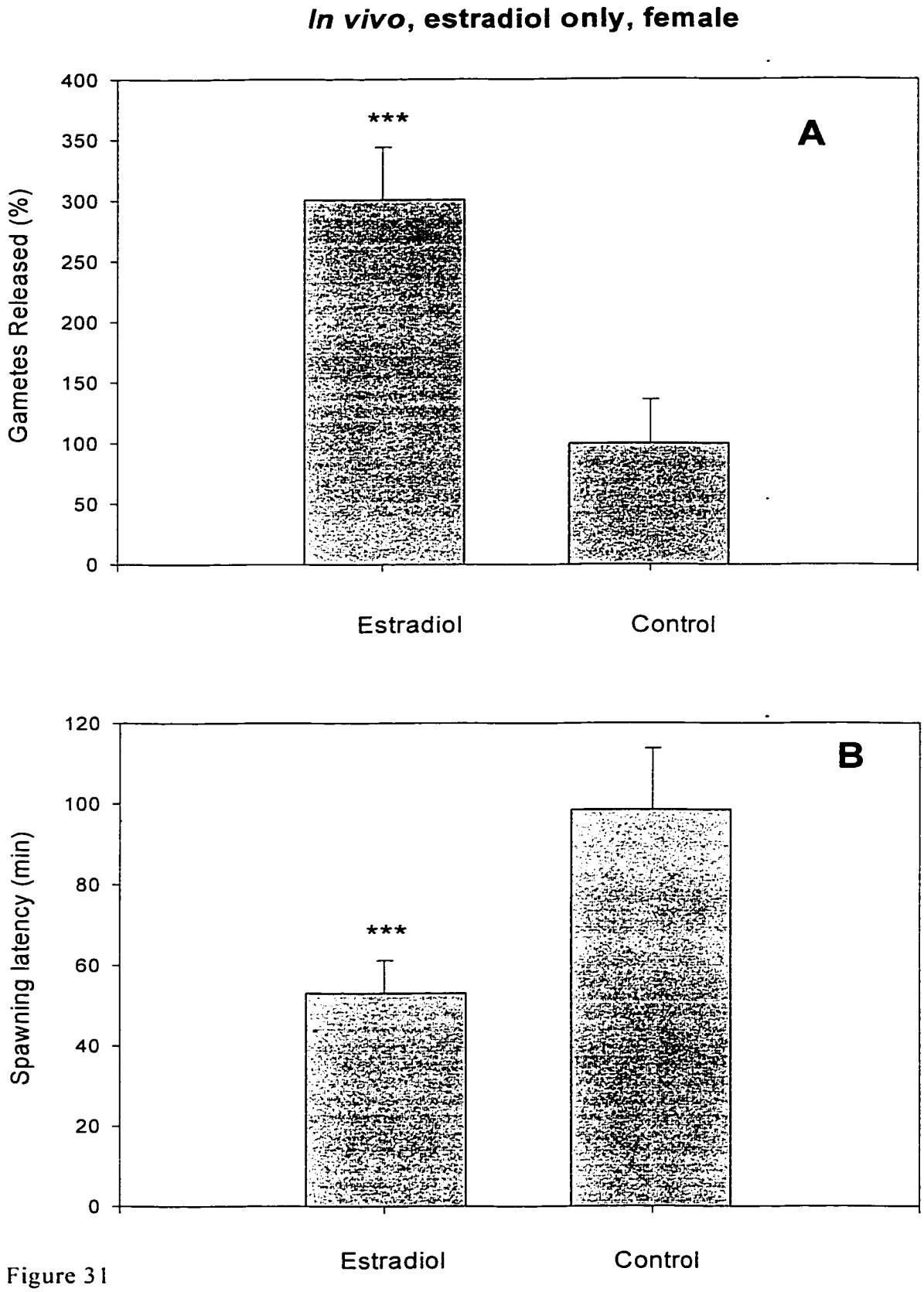

In vivo, estradiol only, female
Gametes Released (%)
400
350
300
250
200
150
100
50
0
***
A
Estradiol
Control
Spawning latency (min)
120
100
80
60
40
20
0
***
B
Estradiol
Control

Figure 31

Figure 32. Injection of estradiol directly induced spawning in male scallops.
Mature male scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of ASW), or estradiol solution (1000 μg/ml vehicle) into gonads. The numbers of sperm spawned per milligram of gonad in the first 3 hours were used as indices of gamete release (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=14). *P<0.05, t-test.

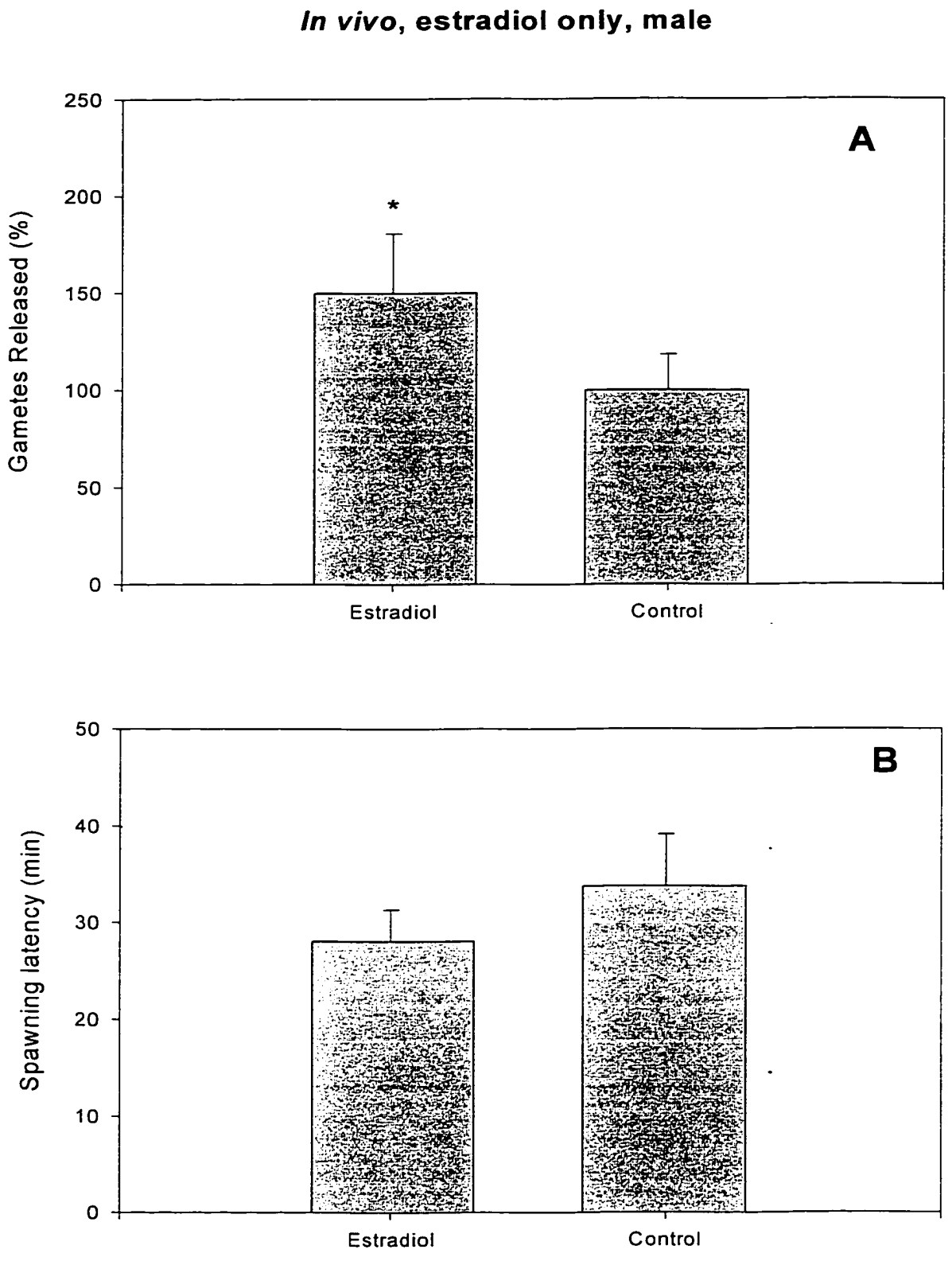

In vivo, estradiol only, male
A
Gametes Released (%)
250
200
150
100
50
0
*
Estradiol
Control
B
Spawning latency (min)
50
40
30
20
10
0
Estradiol
Control

Figure 32

II-E-1-b. Potentiation of 5-HT-induced spawning: Estradiol also potentiated 5-HT-induced spawning *in vivo* in both females and males. The results represented in Figure 33A indicated that injection of estradiol in females caused a 97.8% increase in the number of gametes released compared with the control group ($P<0.05$, n=6, t-test). The spawning latency in the estradiol-injected group was also 31.6% shorter than that of the control group ($P<0.05$, n=6, t-test; Figure 33B).

In males, injections induced all scallops to spawn in both the experimental and the control group. However, the number of gametes released in the estradiol-injected group was 73.8% higher than that of the control group ($P<0.05$, n=12, t-test; Figure 34A) while the average spawning latency in the estradiol-injected group was not different from that of the control group ($P>0.05$, n=12, t-test; Figure 34B).

II-E-2. Effects of testosterone on spawning: Effects of testosterone on direct spawning induction and on 5-HT-induced gamete release were also studied in both sexes.

II-E-2-a. Direct induction: In females, injection of testosterone did not have significant effect on gamete release (Figure 35A) or spawning latency (Figure 35B) ($P>0.05$, n=14, t-test for both gametes released and spawning latency).

In males, scallops spawned after injections in both the experimental and the control group. However, the gametes released in the experimental group was 133.1% higher than that in the control group ($P<0.005$, n=14, t-test; Figure 36A) while the spawning latency of the experimental group was 36.1% shorter than the control group ($P<0.01$, n=14, t-test) (Figure 36B).

II-E-2b. Potentiation of 5-HT-induced spawning: Testosterone had no significant effect on 5-HT-induced spawning in female ($P>0.05$, n=12, t-test for both gamete

Figure 33. *In vivo* effects of estradiol on 5-HT-induced spawning in female scallops. Mature female scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of s ASW), or estradiol solution (1000 μg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 μl 10^{-4} 5-HT. The numbers of eggs spawned per milligram of gonad in the first 3 hours were used as indices of gamete release (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=6). *P<0.05, t-test.

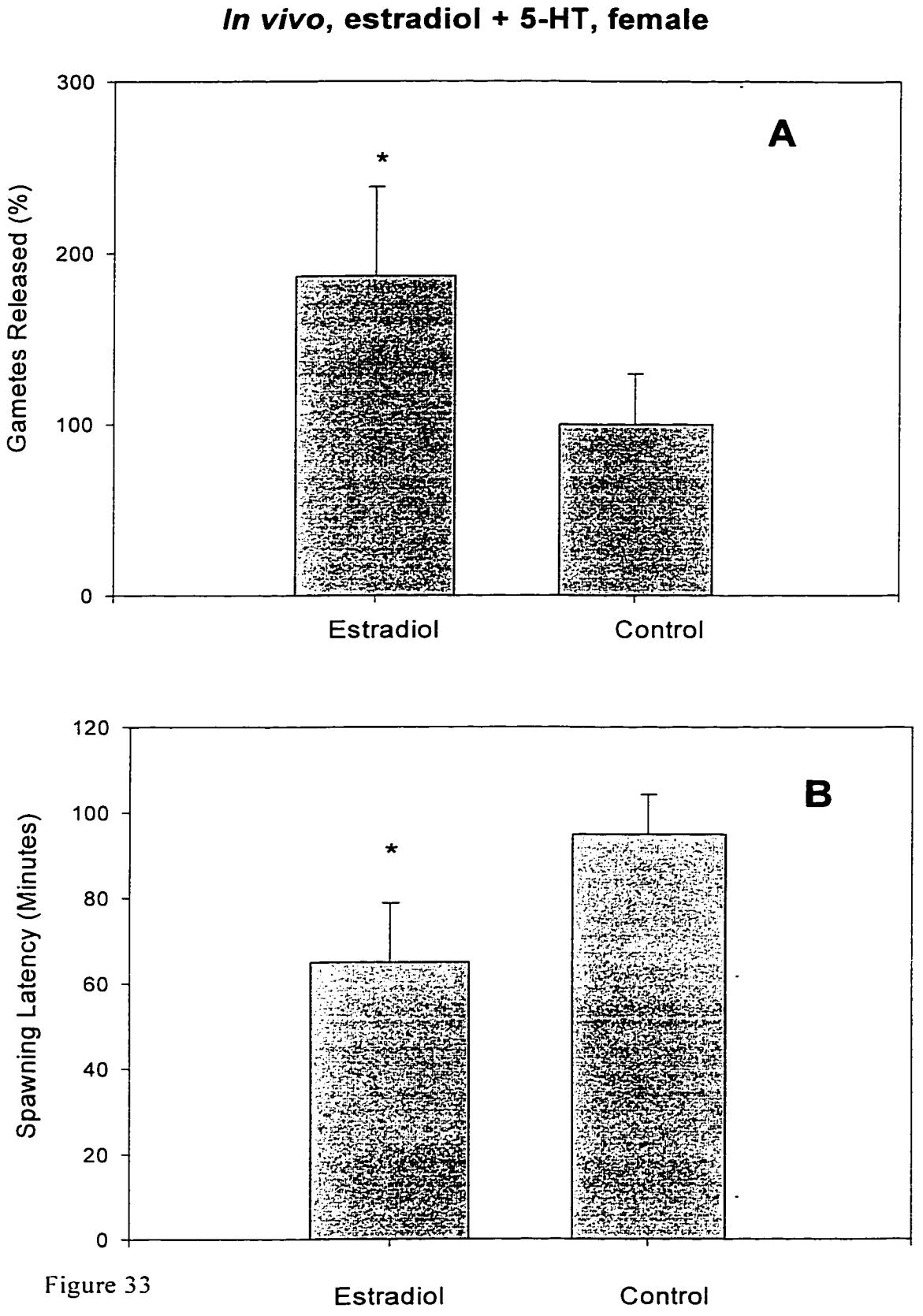

In vivo, estradiol + 5-HT, female
Gametes Released (%)
300
200
100
0
*
A
Estradiol
Control
Spawning Latency (Minutes)
120
100
80
60
40
20
0
*
B
Estradiol
Control

Figure 33

Figure 34. *In vivo* effects of estradiol on 5-HT-induced spawning in male scallops. Mature male scallops were injected with 200 µl vehicle (1 part of ethanol and 99 parts of ASW), or estradiol solution (1000 µg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 µl 10^{-4} 5-HT. The numbers of sperm spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=12). *P<0.05, t-test.

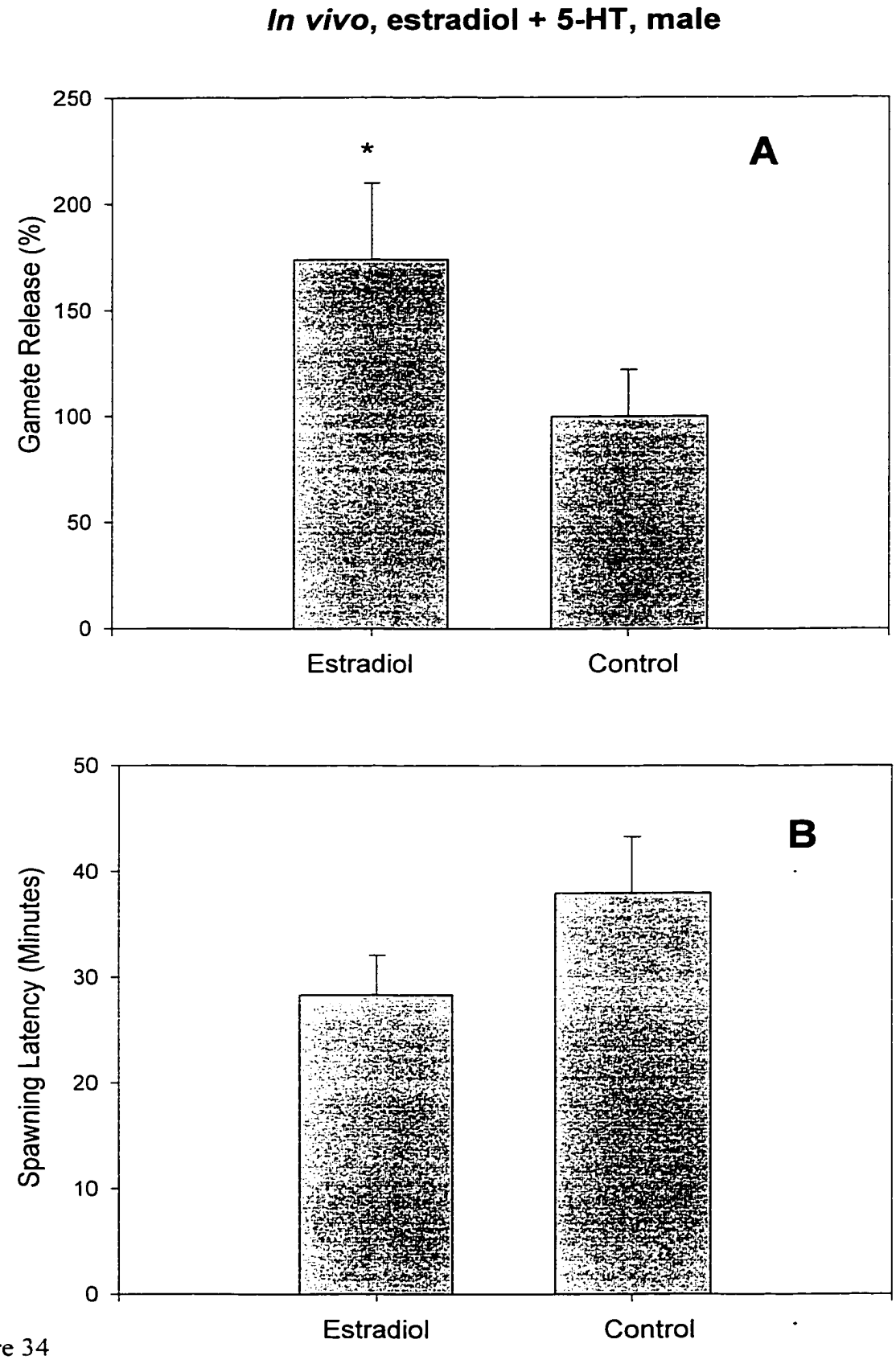

In vivo, estradiol + 5-HT, male
Gamete Release (%)
250
200
150
100
50
0
*
A
Estradiol
Control
Spawning Latency (Minutes)
50
40
30
20
10
0
B
Estradiol
Control

Figure 34

Figure 35. Direct effects of testosterone injection on spawning in female scallops. Mature scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of ASW), testosterone solution (1000 μg/ml vehicle) into gonads. The numbers of eggs spawned per milligram of gonad in the first 3 hours were used as gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=14).

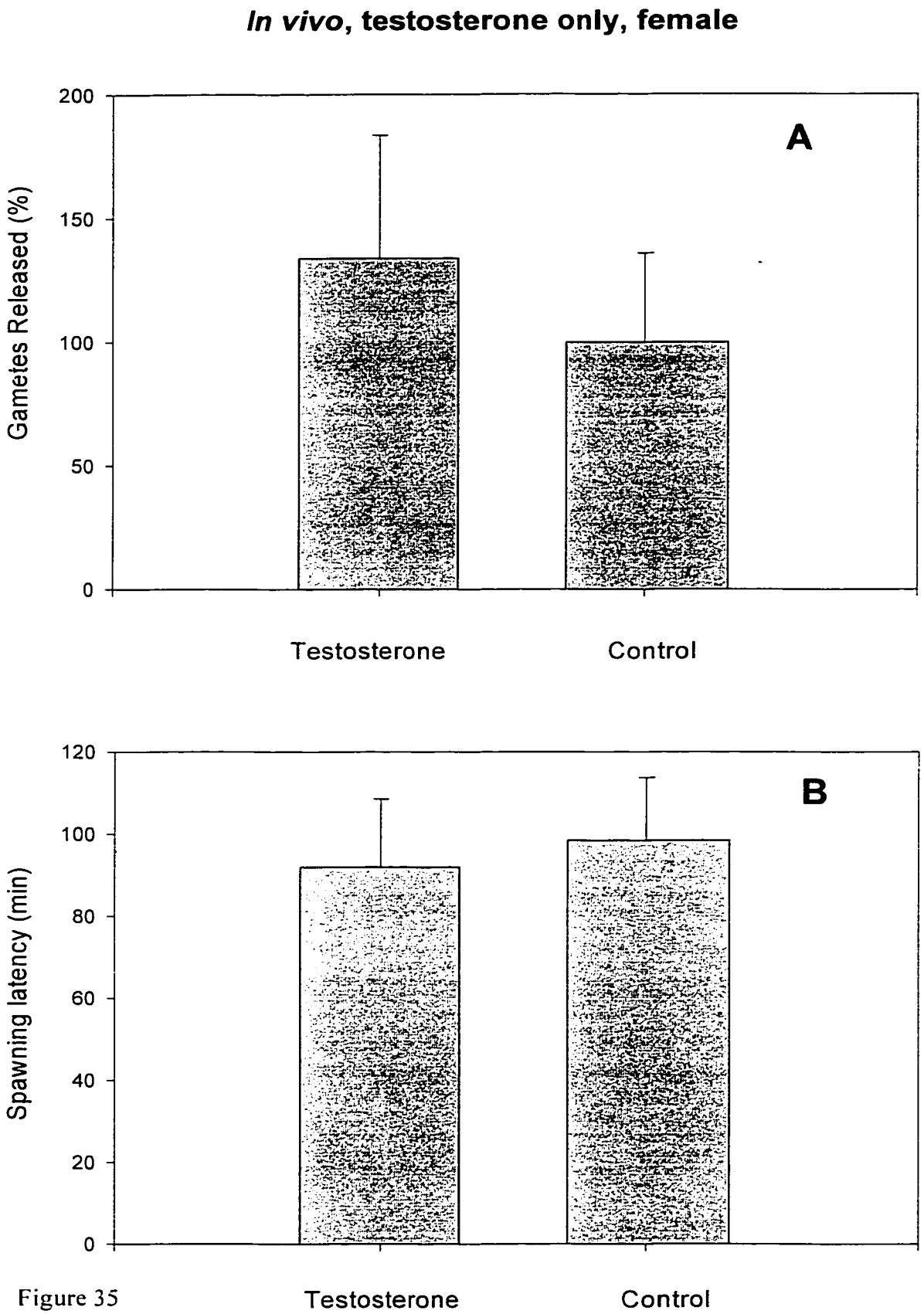

In vivo, testosterone only, female
Gametes Released (%)
200
150
100
50
0
A
Testosterone
Control
Spawning latency (min)
120
100
80
60
40
20
0
B
Testosterone
Control

Figure 35

Figure 36. Injection of testosterone directly induced spawning in male scallops.
Mature male scallops were injected with 200 μl of vehicle (1 part of ethanol and 99 parts of ASW), or testosterone solution (1000 μg/ml vehicle) into gonads. The numbers of sperm spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=14). **<0.01, ***P<0.005, t-test.

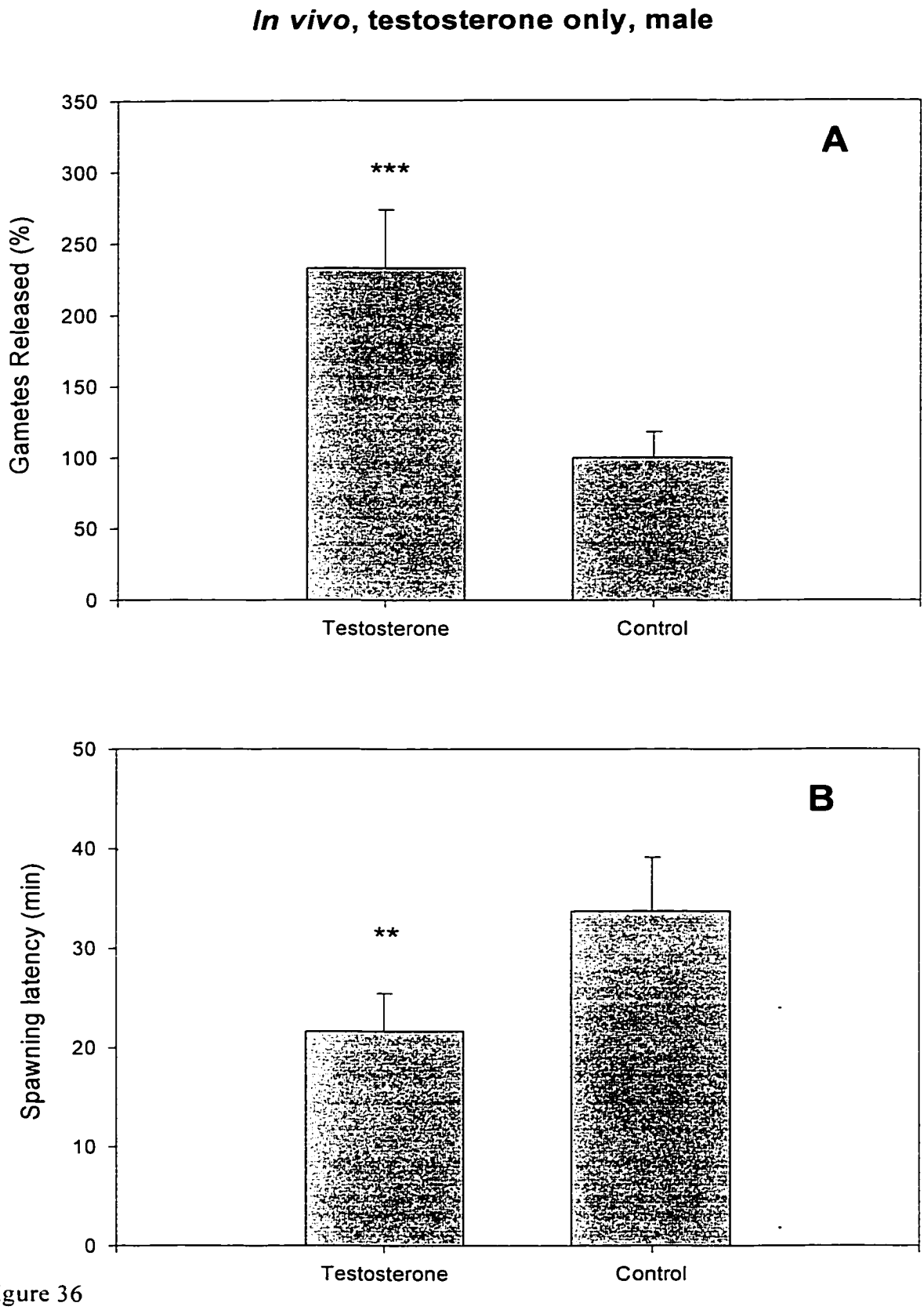

In vivo, testosterone only, male
A
***
Gametes Released (%)
350
300
250
200
150
100
50
0
Testosterone
Control
B
**
Spawning latency (min)
50
40
30
20
10
0
Testosterone
Control

Figure 36

released, Figure 37A, and spawning latency, Figure 37B).

Testosterone also promoted the spawning induced by 5-HT in male scallops. The gametes released was 152.6% higher in the experimental group pre-injected with testosterone than in the control group pre-injected with the vehicle ($P<0.01$, $n=12$, t-test; Figure 38A). Injection of testosterone shortened the spawning latency in the testosterone-injected group by 38.2% compared to that of the control group ($P<0.05$, $n=12$, t-test) (Figure 38B).

II-E-3. Effects of 5-HT-induced spawning by progesterone: Because progesterone had stimulatory effects on *in vitro* gamete release in both sexes, its effects on *in vivo* spawning in both sexes were also examined. Injection of progesterone into the gonads of ripe scallops did not induce spawning directly in either sex. However, pre-injection of progesterone inhibited 5-HT-induced spawning in females while promoting 5-HT-induced spawning in males.

In female scallops, pre-injection of progesterone inhibited spawning induced by 5-HT. Only 55.6% of the scallops spawned in the progesterone-injected group ($n=9$) while all the scallops in the control group spawned. The gametes released in the experimental group was 89.2% lower than that of the control group ($P<0.05$, $n=9$, t-test; Figure 39A). The spawning latency of the progesterone-injected group was 102.7% longer than that of the control group ($P<0.05$, $n=9$, t-test; Figure 39B).

In male scallops, scallops in both the experimental and the control groups spawned after the injections. However, the gametes released was increased by 46.2% in the experimental group compared with that of the control group ($P<0.05$, $n=12$, t-test; Figure 40A). The spawning latency of the progesterone-injected group was 50.2%

Figure 37. *In vivo* effects of testosterone on 5-HT-induced spawning in female scallops. Mature female scallops were injected with 200 µl vehicle (1 part of ethanol and 99 parts of ASW), or testosterone solution (1000 µg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 µl 10^{-4} 5-HT. The numbers of eggs spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=14).

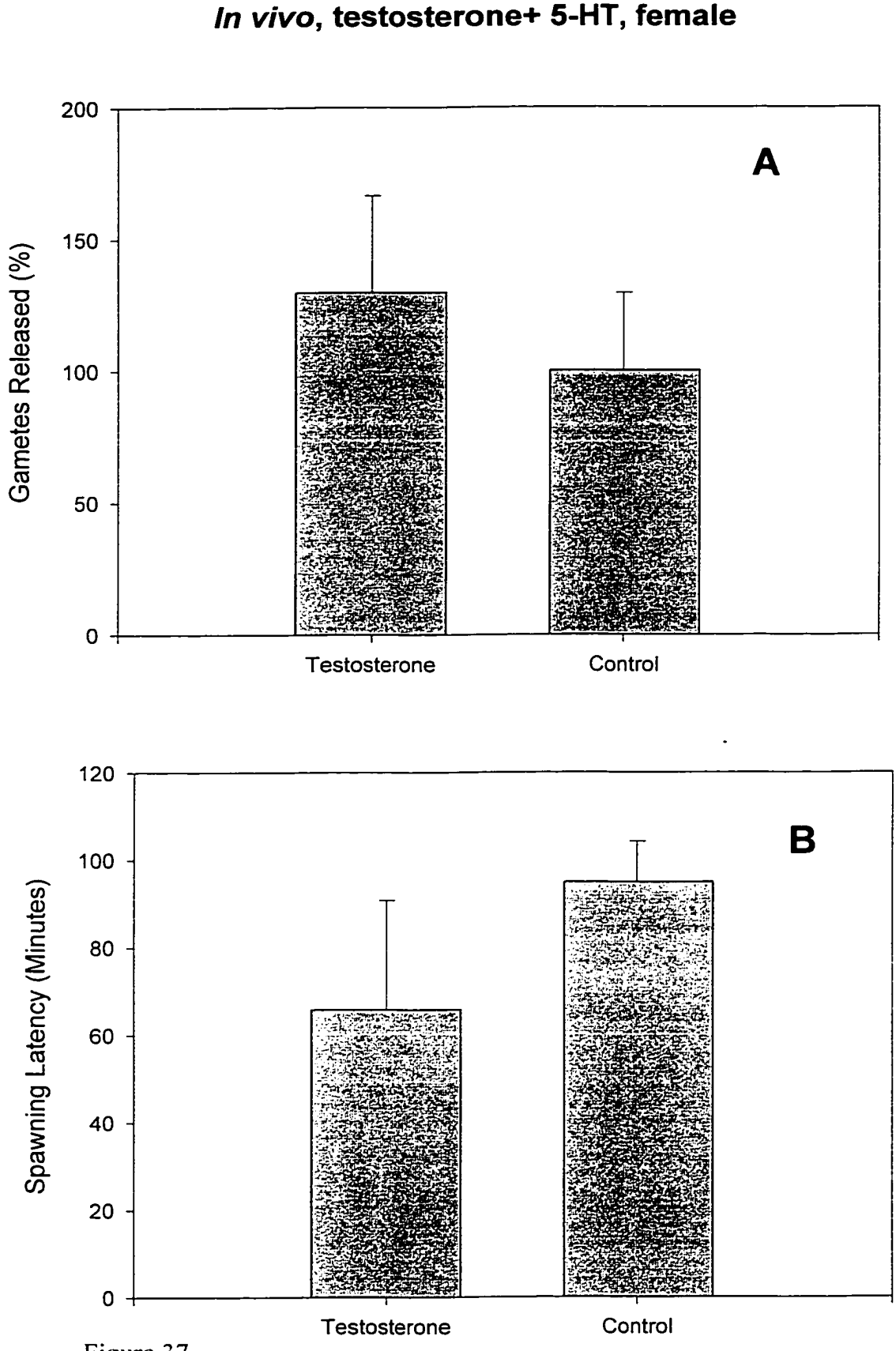

In vivo, testosterone+ 5-HT, female
A
Gametes Released (%)
200
150
100
50
0
Testosterone
Control
B
Spawning Latency (Minutes)
120
100
80
60
40
20
0
Testosterone
Control

Figure 37

Figure 38. *In vivo* effects of testosterone on 5-HT-induced spawning in male scallops. Mature male scallops were injected with 200 µl vehicle (1 part of ethanol and 99 parts of ASW), or testosterone solution (1000 µg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 µl 10^{-4} 5-HT. The numbers of sperm spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=12). *P<0.05, **<0.01, t-test.

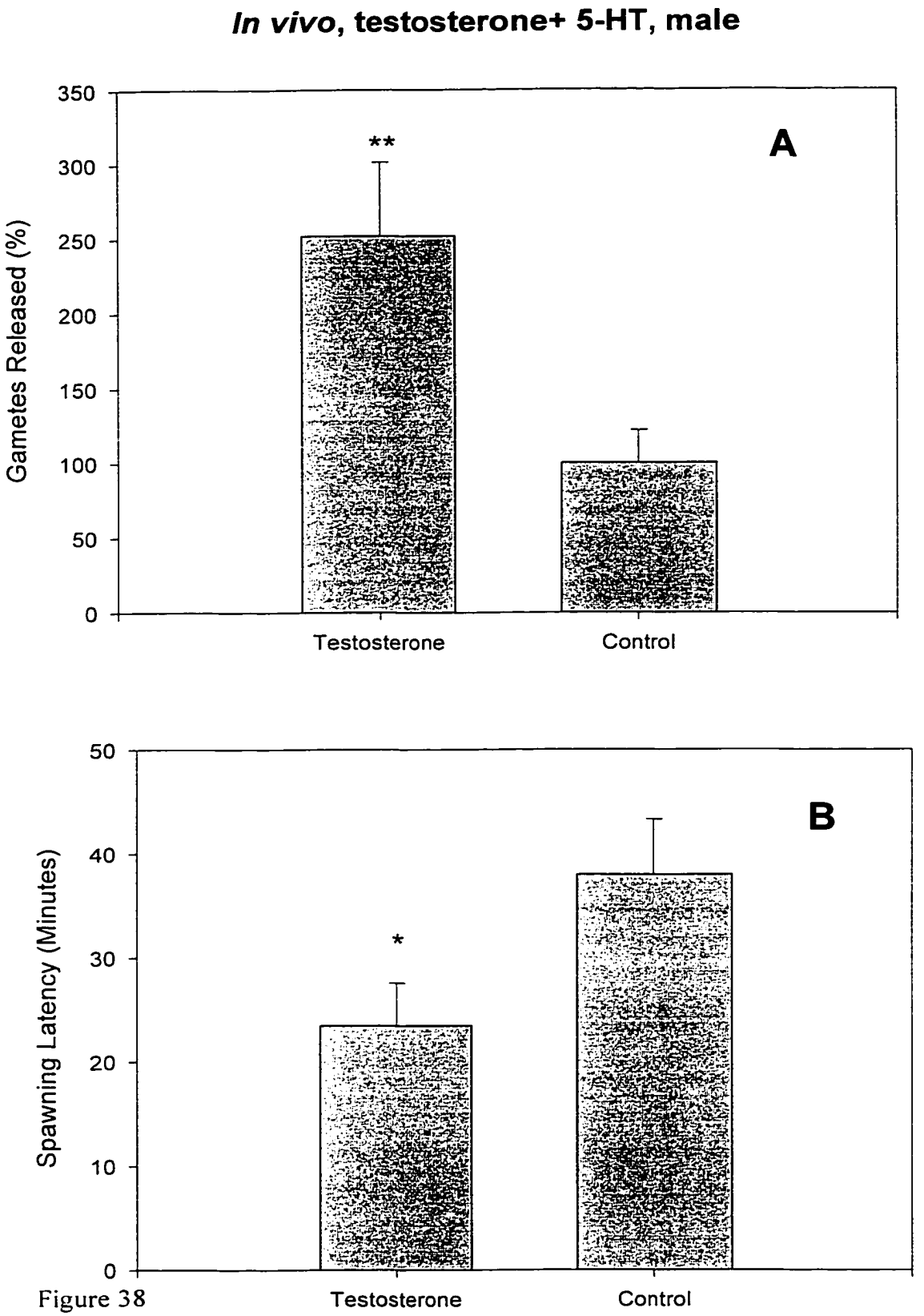

In vivo, testosterone+ 5-HT, male
Gametes Released (%)
350
300
250
200
150
100
50
0
**
A
Testosterone
Control
Spawning Latency (Minutes)
50
40
30
20
10
0
*
B
Testosterone
Control

Figure 38

Figure 39. *In vivo* effects of progesterone on 5-HT-induced spawning in female scallops. Mature female scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of ASW), or progesterone solution (1000 μg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 μl 10^{-4} 5-HT. The numbers of eggs spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=9). *P<0.05, t-test.

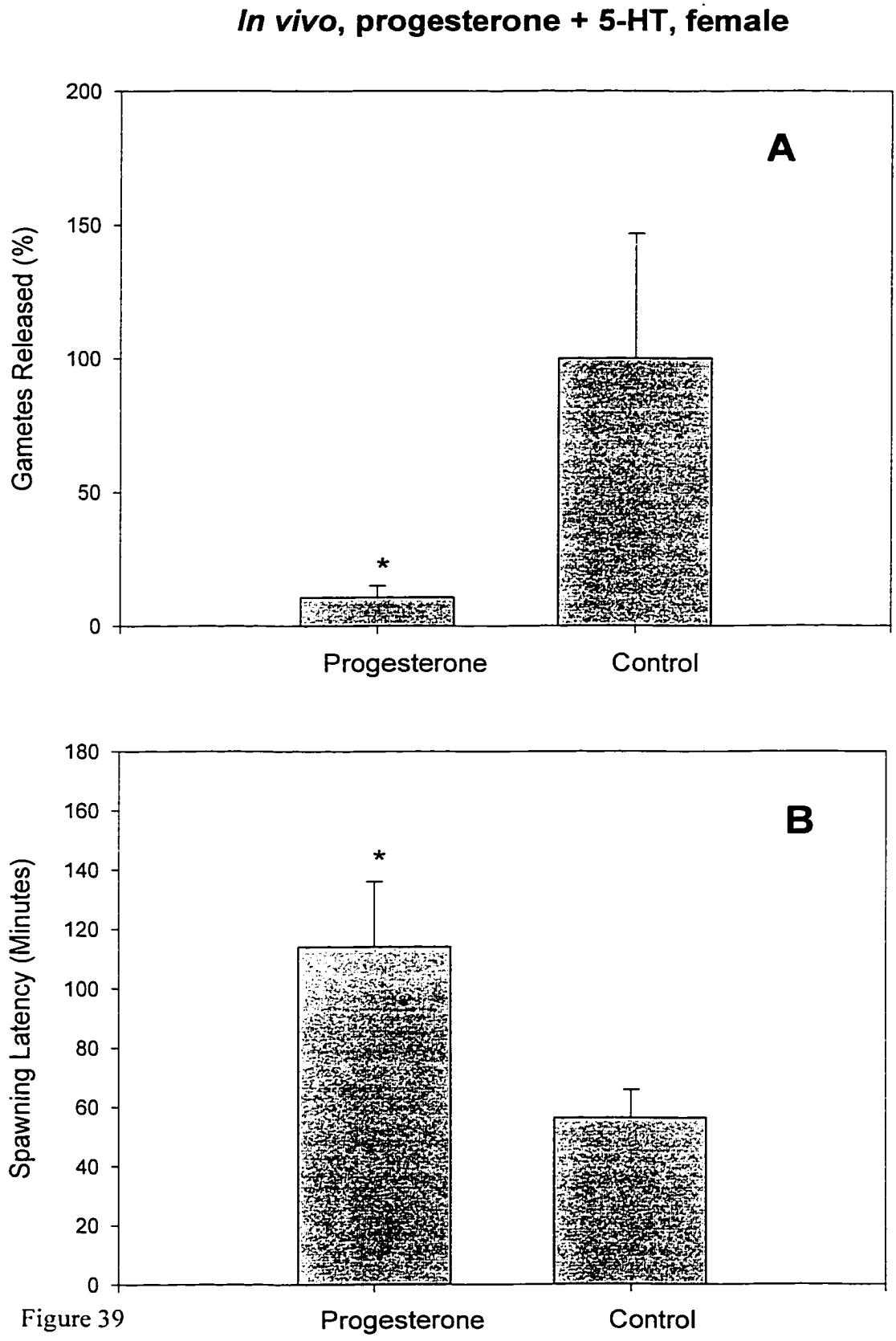

In vivo, progesterone + 5-HT, female
A
Gametes Released (%)
200
150
100
50
0
*
Progesterone
Control
B
Spawning Latency (Minutes)
180
160
140
120
100
80
60
40
20
0
*
Progesterone
Control

Figure 39

Figure 40. *In vivo* effects of progesterone on 5-HT-induced spawning in male scallops. Mature male scallops were injected with 200 μl vehicle (1 part of ethanol and 99 parts of ASW), or progesterone solution (1000 μg/ml vehicle) into gonads. After 24 hours, the scallops were injected with 200 μl 10^{-4} 5-HT. The numbers of sperm spawned per milligram of gonad in the first 3 hours were used as indices of gametes released (A). The response time of spawning after injection was referred to as spawning latency (minutes) (B). Each value represents mean ± SE (n=12). *$P<0.05$, t-test.

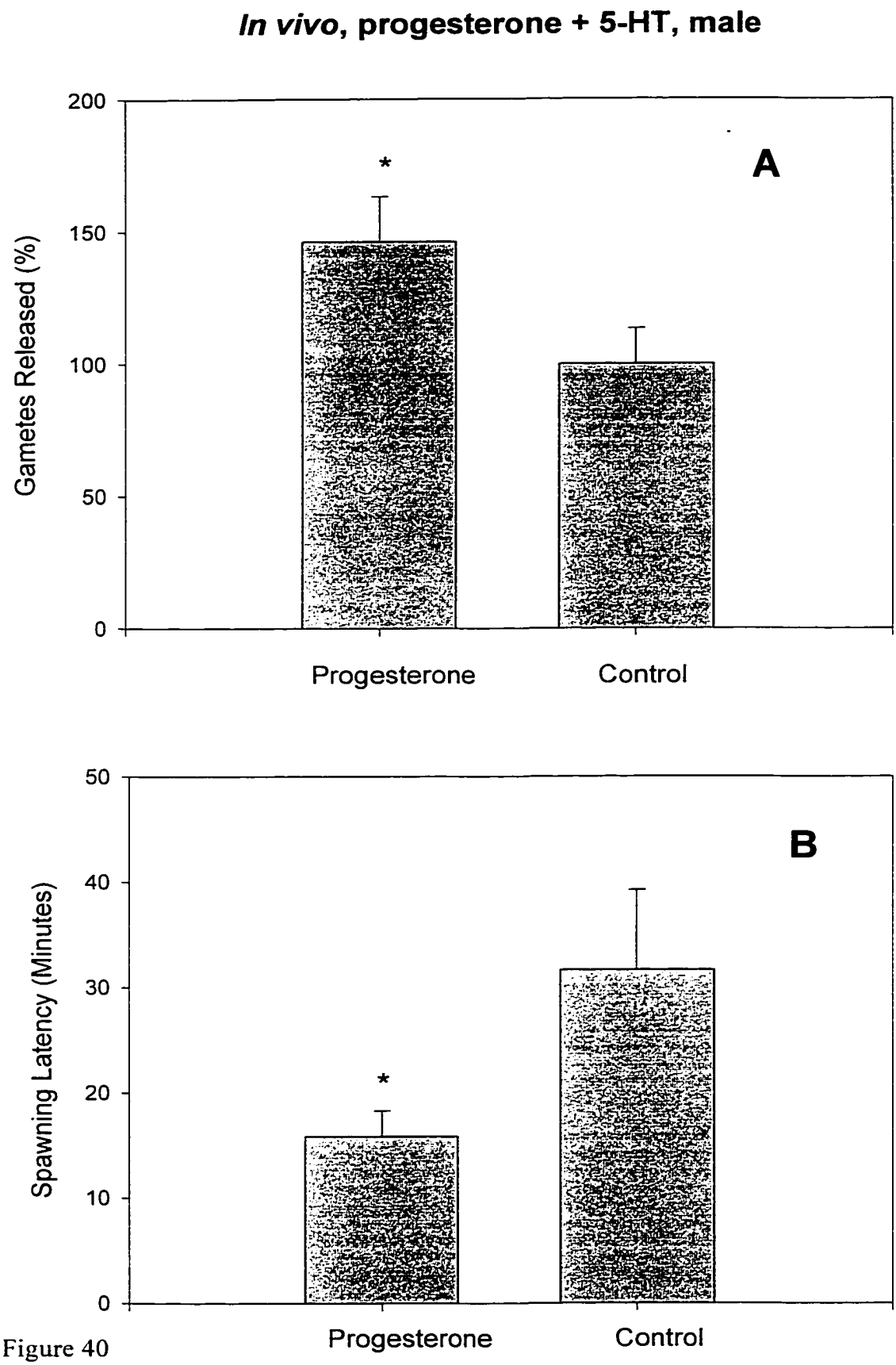

In vivo, progesterone + 5-HT, male
Gametes Released (%)
200
150
100
50
0
*
A
Progesterone
Control
Spawning Latency (Minutes)
50
40
30
20
10
0
*
B
Progesterone
Control

Figure 40

shorter than that of the control group ($P<0.05$, n=12, t-test; Figure 40B).

The results of the *in vivo* effects of sex steroids on scallop spawning are summarized in Table 7.

Table 7. Summary of the *in vivo* effects of sex steroids on scallop spawning (+: stimulatory, -: inhibitory, 0: no effect, na: not applicable)

Steroids		Direct (Steroid only)	Indirect (Steroid + 5-HT)
Estradiol	**Female**	+	+
	Male	+	+
Testosterone	**Female**	na	na
	Male	+	+
Progesterone	**Female**	0	−
	Male	0	+

III. Radioligand Receptor Binding Assays of Estrogen Binding Sites in the Scallop:

Results from the *in vitro* and *in vivo* experiments suggested the existence of specific sex steroid receptors in the scallop gonads. To provide further evidence, characterization of possible estrogen receptors in the scallop gonad preparations by saturation and competitive radioligand receptor binding analyses was attempted. As estrogens have been implicated in the process of sexual maturation, the distributions of the estrogen binding sites during various stages of sexual maturation were also studied.

III-A. Saturation Analyses:

III-A-1. Female gonad: The existence of both cytosolic and nuclear estrogen binding sites was demonstrated in female gonad preparations by saturation analysis. As seen from

Figure 41A, the binding curve for the cytosolic fraction seemed to consist of two components-- one saturated between 5.0-7.5 nM and another not saturated below 20 nM. Scatchard analysis apparently revealed two sites: a high-affinity site with limited capacity and a low-affinity site with high capacity. The dissociation constant (K_d) for the high affinity site was 0.52 nM and its maximum binding capacity (B_{max}) was 62.57 fmol/mg protein (Figure 41B). The low affinity site was not characterized because higher concentrations of radiolabeled estradiol were needed in order to do so.

Figure 42 represents the data for saturation analysis of the nuclear fraction of female gonad. The binding curve indicated the existence of two sites: one was saturated at around 2 nM and another was not saturated below 20 nM, as shown in Figure 42A. Scatchard analysis of the data revealed a high affinity binding site with a K_d of 1.71 nM and a B_{max} of 174.3 fmol/mg protein, and a low affinity binding site with a K_d of 31.84 nM and a B_{max} of 1.12 pmol/mg protein (Figure 42B).

III-A-2. Male gonad: Saturation analysis of the cytosolic fraction of male gonad showed a similar pattern to that of the cytosolic fraction of female gonad (Figure 43A).

As in the female gonad cytosol, the binding curve for the male gonadal cytosolic fraction appeared to include two components: the first was saturated at around 5 nM and the second between 15 nM and 20 nM. Scatchard analysis indicated two binding sites: a high affinity one with a K_d of 0.57 nM and a B_{max} of 38.3 fmol/mg protein, and a low affinity high capacity site, the accurate characterization of which required higher concentrations of radiolabeled estradiol (Figure 43B).

A similar binding curve to that for female gonad nuclear fraction was also obtained for the nuclear fraction of the male gonad. As shown in Figure 44A, the first site

Figure 41. Saturation analysis of binding of [^{3}H] estradiol by cytosolic fraction of female scallop gonads.

A. Saturation curve of the binding. 200 µl of cytosolic fraction were incubated with 0.5-20 nM [^{3}H] estradiol in the presence or absence of a 500 fold excess concentration of DES at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment.
B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.

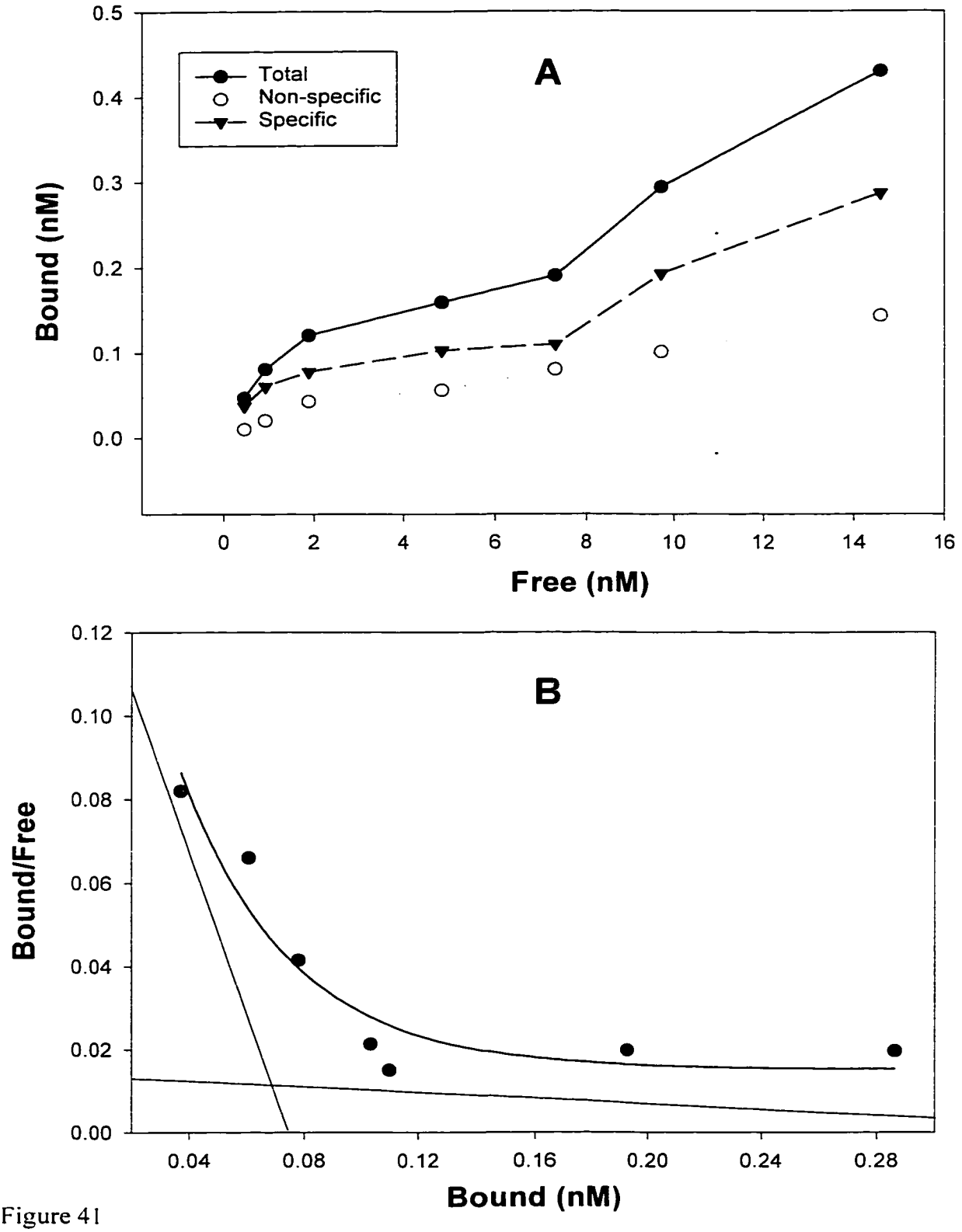
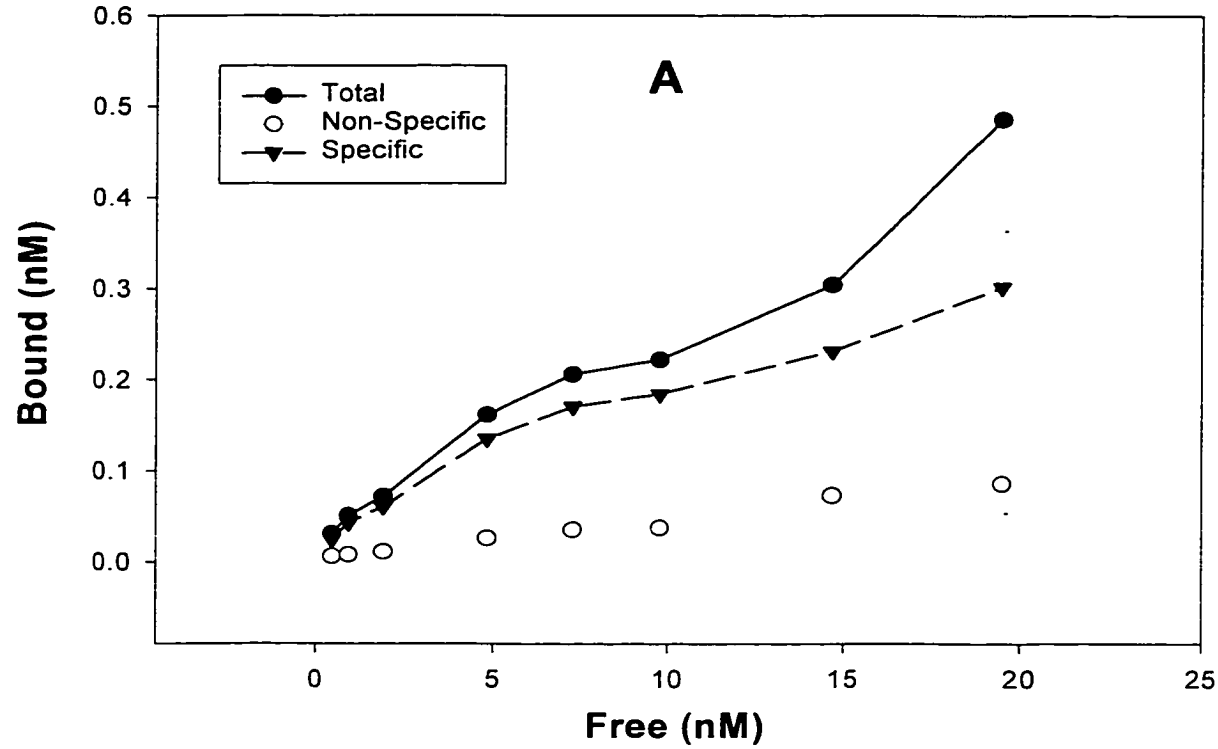

A
Total
Non-specific
Specific
Bound (nM)
Free (nM)
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
16
B
Bound/Free
Bound (nM)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.04
0.08
0.12
0.16
0.20
0.24
0.28

Figure 41

Figure 42. Saturation analysis of binding of [^{3}H] estradiol by nuclear fraction of female scallop gonads.

A. Saturation curve of the binding. 200 µl of nuclear fraction were incubated with 0.5-20 nM [^{3}H] estradiol in the presence or absence of a 500 fold excess concentration of DES at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment.
B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.


A
Total
Non-Specific
Specific
Bound (nM)
Free (nM)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25

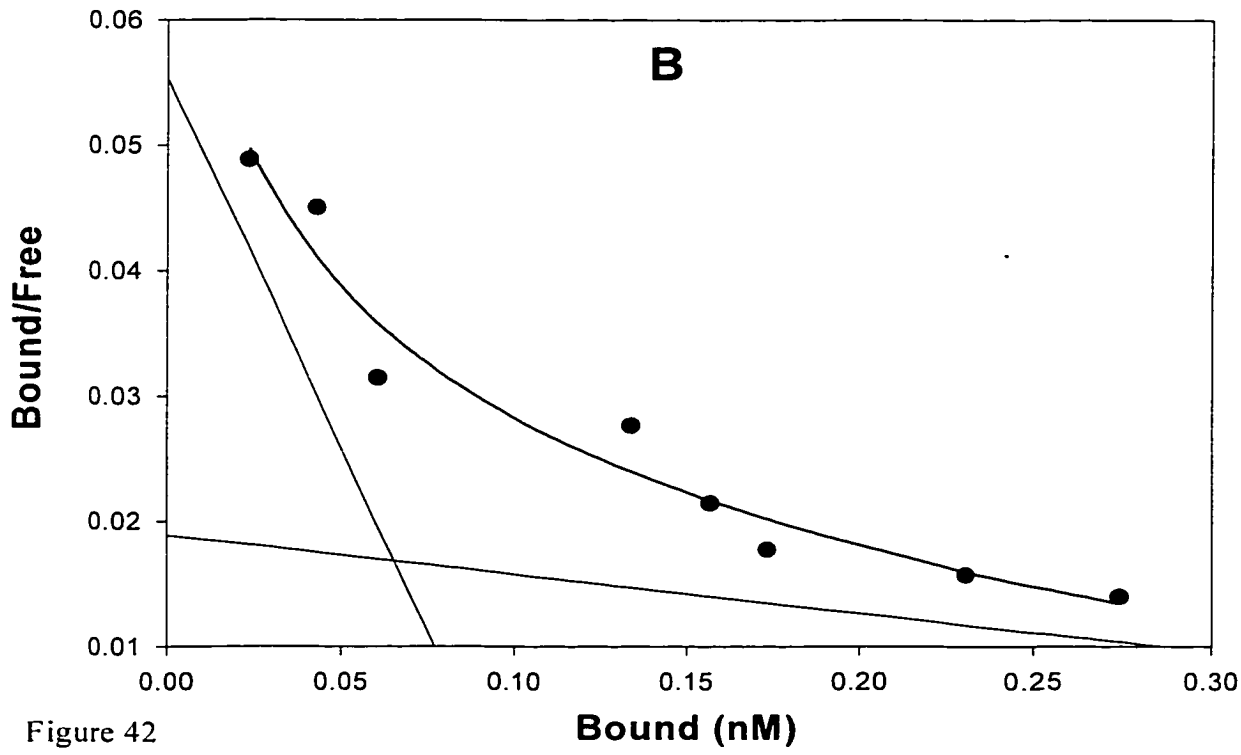
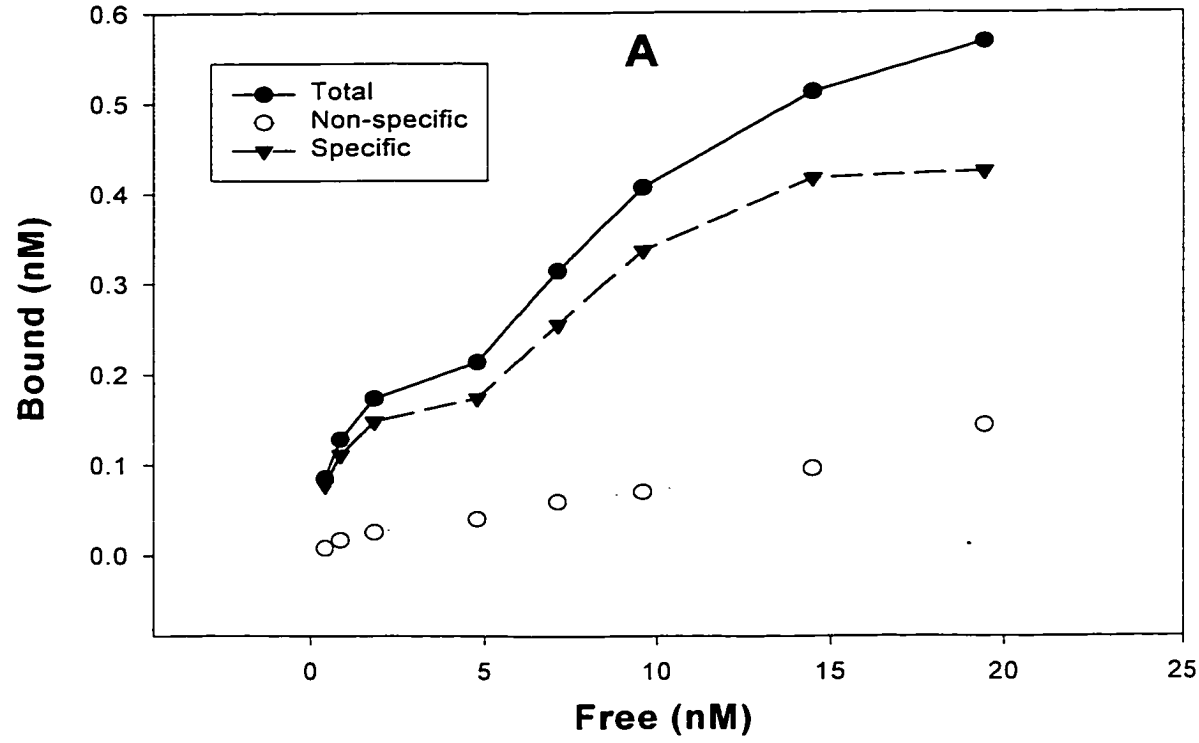

B
Bound/Free
Bound (nM)
0.01
0.02
0.03
0.04
0.05
0.06
0.00
0.05
0.10
0.15
0.20
0.25
0.30

Figure 42

Figure 43. Saturation analysis of binding of [^{3}H] estradiol by cytosolic fraction of male scallop gonads.

A. Saturation curve of the binding. 200 µl of cytosolic fraction were incubated with 0.5-20 nM [^{3}H] estradiol in the presence or absence of a 500 fold excess concentration of DES at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment.
B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.


A
Total
Non-specific
Specific
Bound (nM)
Free (nM)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25

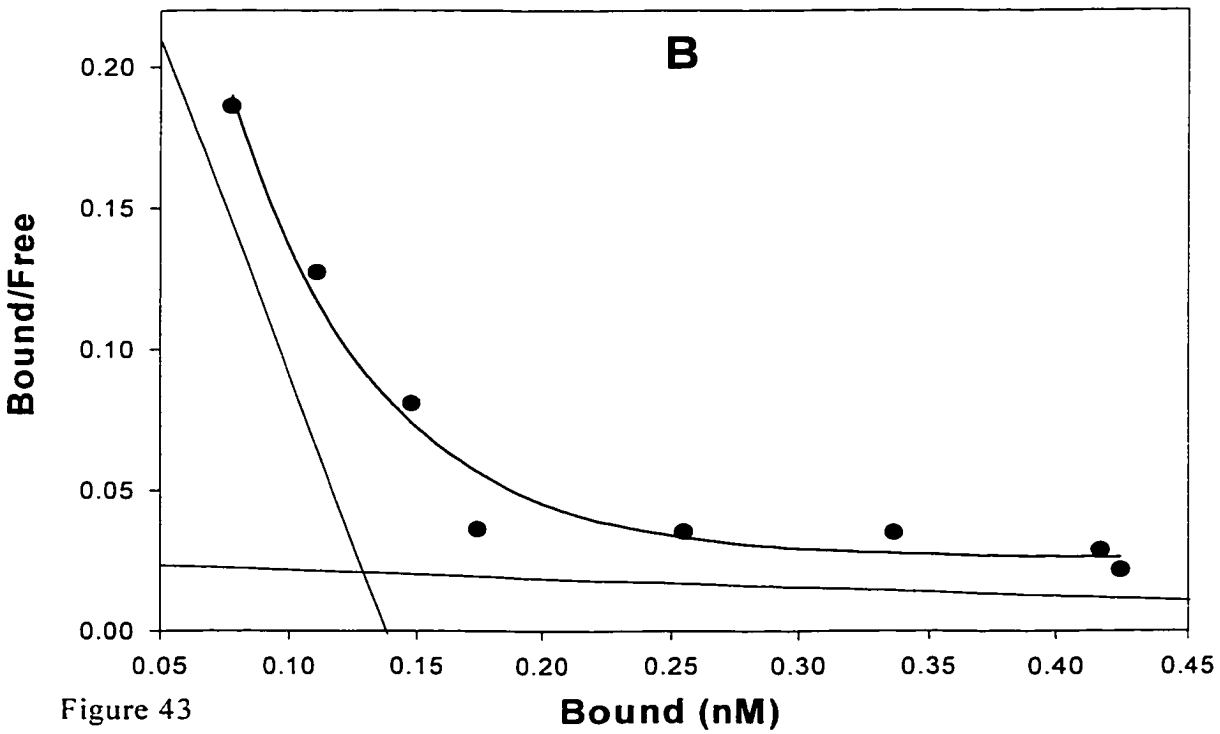

B
Bound/Free
Bound (nM)
0.00
0.05
0.10
0.15
0.20
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45

Figure 43

was saturated at around 7.5 nM and the second not saturated up to 20 nM. Scatchard analysis also demonstrated the presence of two binding sites. The dissociation constant for the high affinity site was 1.82 nM and its B_{max} is 63.97 fmol/mg protein. The low affinity site had a K_d of 25.51 nM and a B_{max} of 0.293 pmol/mg protein (Figure 44B).

In summary, comparison of the saturation analysis data between female and male cell extracts showed that, in preparations from ripe scallops:

(1) Two sites in the cytosolic fraction were identified in both sexes and the K_d values for the high affinity site were similar (0.52 nM vs 0.57 nM).

(2) Two sites were identified in the nuclear fractions of the gonads of both sexes. Both populations of sites have similar K_d values, *i.e.*, 1.71nM vs 1.82 nM for the high affinity sites, and 31.84 nM vs 25.51nM for the low affinity sites.

(3) In both the cytosolic and nuclear fractions, the maximum binding capacity of estrogen binding sites is higher in females than in males.

(4) In both sexes, the maximum binding capacity is higher in the nuclear fraction than in the cytosolic fraction.

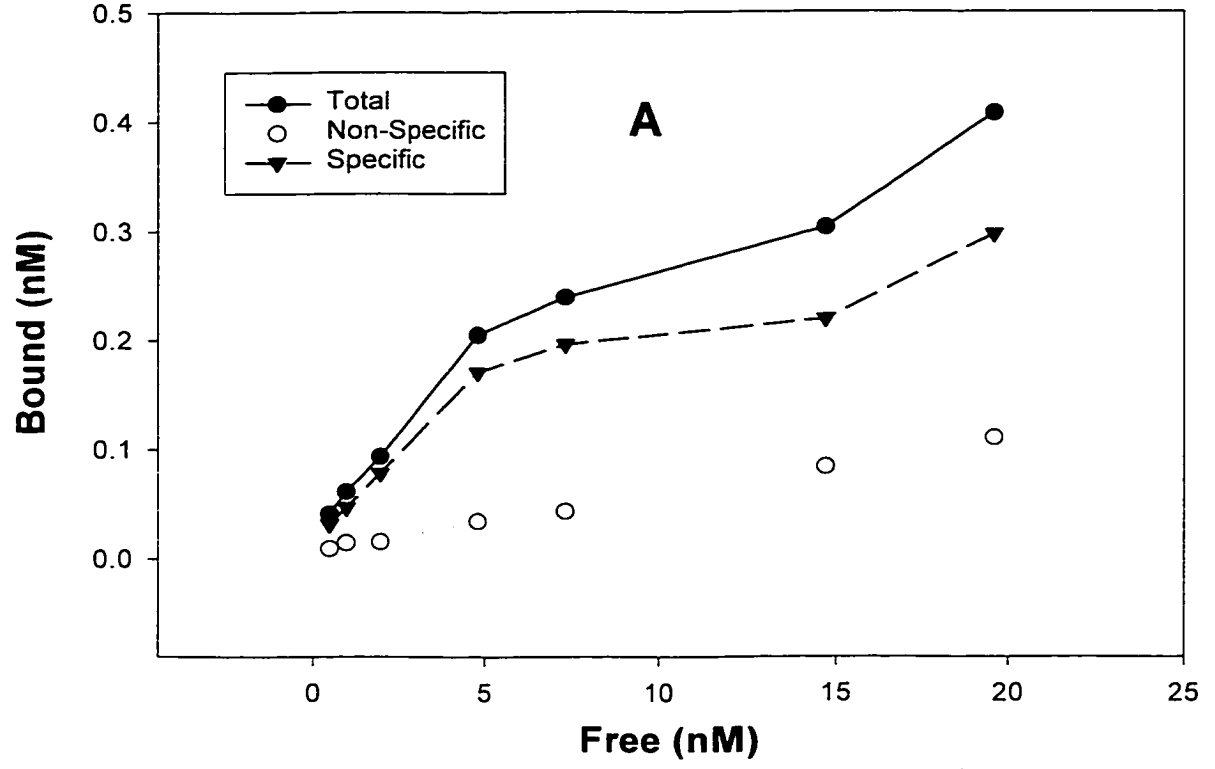
III-B. Competitive Analyses

To determine the specificity of the estrogen binding sites in scallop gonad fractions, competitive analyses were carried out in the cytosolic and nuclear fractions of both male and female scallop gonads. The capacities of DES, estradiol, progesterone and testosterone to compete for the estrogen binding sites against [^{3}H] estradiol were investigated.

Figure 44. Saturation analysis of binding of [^{3}H] estradiol by nuclear fraction of male scallop gonads.

A. Saturation curve of the binding. 200 µl of nuclear fraction were incubated with 0.5-20 nM [^{3}H] estradiol in the presence or absence of a 500 fold excess concentration of DES at 4 ^{o}C for 24 hours. Bound steroids were separated from free by DCC treatment.

B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.


A
Total
Non-Specific
Specific
Bound (nM)
Free (nM)
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25

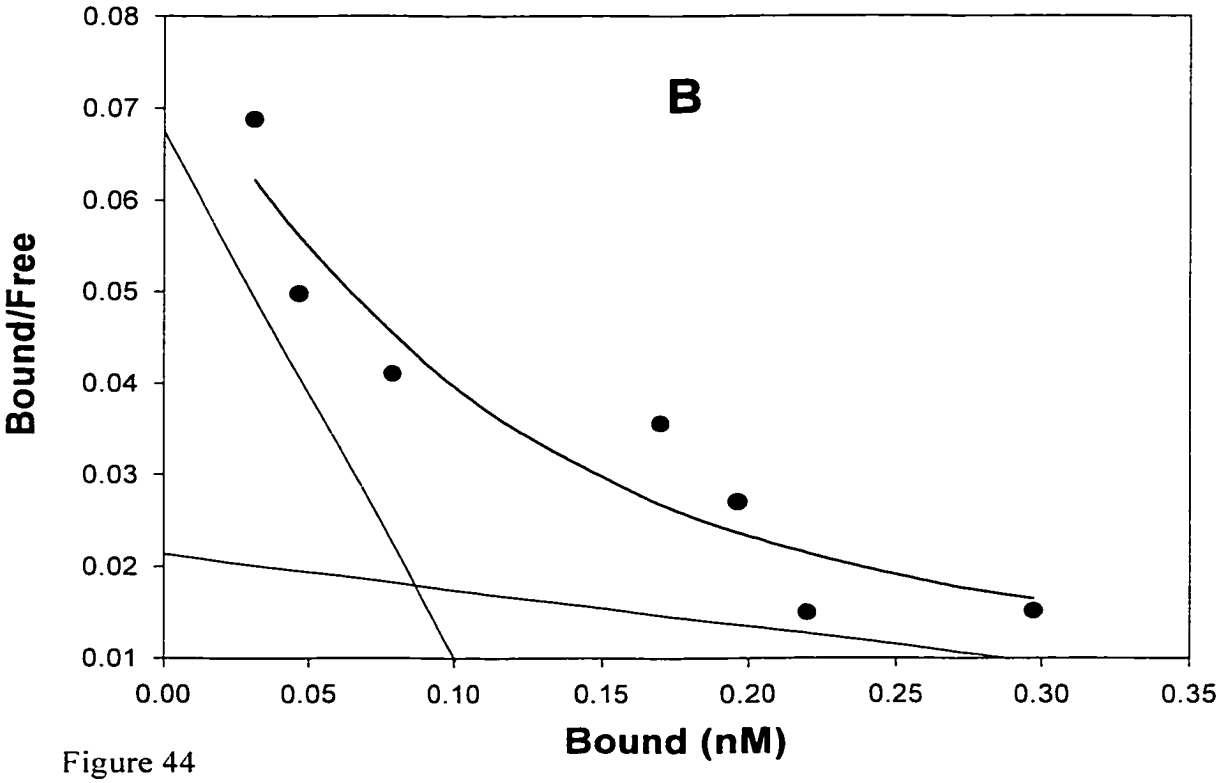

B
Bound/Free
Bound (nM)
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35

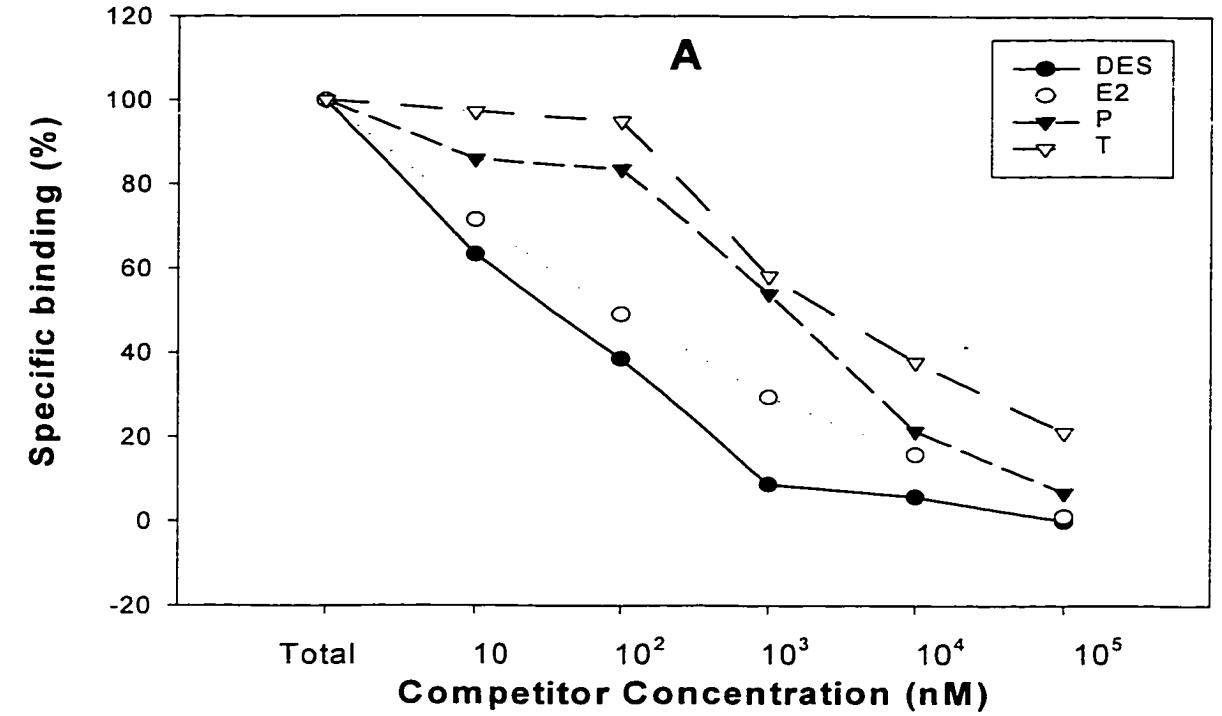
Figure 44

Incubation of the female gonad cytosol fractions with 10 nM of [^{3}H] estradiol alone or in the presence of increasing concentrations of competitors showed that at low concentrations (10^{-8}-10^{-6} M), DES and estradiol, but not progesterone and testosterone, effectively diminished the binding of [^{3}H] estradiol (Figure 45A). Based on their ability to inhibit 50% of total binding, DES had an about 3-fold higher affinity while progesterone and testosterone had 15- and 30-fold lower affinities than estradiol in competing for the binding sites. The concentrations of competitors needed to inhibit 50% of total binding were 33.5 nM for DES, 86.1 nM for estradiol, 1.28μM for progesterone and 2.45 μM for testosterone. Surprisingly, progesterone and testosterone were able to inhibit the [^{3}H] estradiol binding to a considerable extent at very high concentrations 10^4-10^5 nM). The percentages of displacement of [^{3}H]estradiol binding in the presence of 100 × concentration of competitors (1μM) are given in Table 8, showing the same tendency as obtained from 50% displacement.

Competition analyses in female gonad nuclear fractions showed a similar tendency as in the female gonad cytosolic fraction (Figure 45B). DES and estradiol were good competitors for the binding sites while progesterone and testosterone were weak in competition for the binding sites at the concentration range of 10-10^5 nM. DES is 10-fold more competent than estradiol to compete for the binding sites. Progesterone and testosterone were 80- and 110-fold less competitive than estradiol. At the highest concentration (10^5 nM), the four steroids all reduced the [^{3}H] estradiol binding drastically.

The competitive curves of the male gonad cytosolic fraction are quite similar to those of the female preparations (Figure 46A). With increasing concentrations, DES and

Figure 45. Competitive analysis of binding of [^{3}H] estradiol to the cytosolic and nuclear fractions of female scallop gonads.
Increasing concentrations of competitors were incubated with 200 µl of cell extracts and 10 nM [^{3}H] estradiol at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment. Data were expressed as percentages of specific binding in the absence of competitor (denoted as Total). DES, diethylstilbestrol; E2, estradiol; P, progesterone; T, testosterone
A. Cytosolic fraction
B. Nuclear fraction


A
DES
E2
P
T
Specific binding (%)
120
100
80
60
40
20
0
-20
Total
10
10^2
10^3
10^4
10^5
Competitor Concentration (nM)

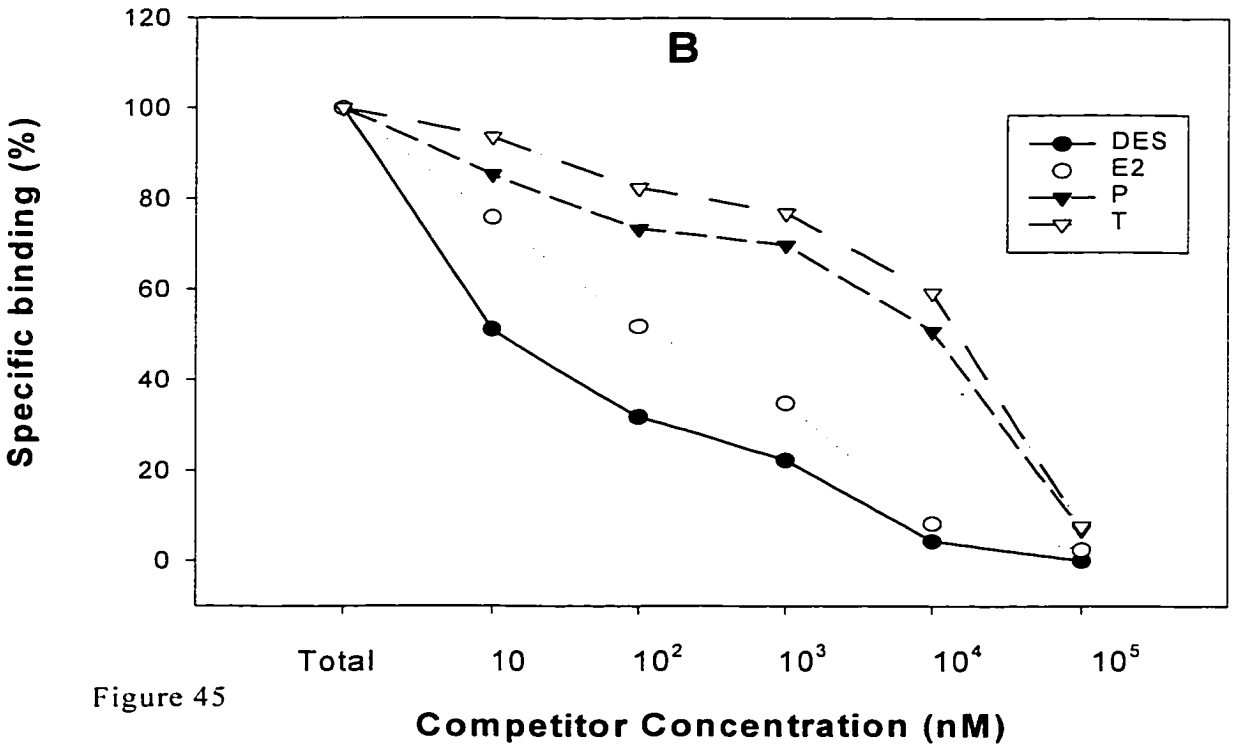

B
DES
E2
P
T
Specific binding (%)
120
100
80
60
40
20
0
Total
10
10^2
10^3
10^4
10^5
Competitor Concentration (nM)

Figure 45

estradiol effectively decreased [^{3}H] estradiol binding. Progesterone and testosterone, on the contrary, did not compete well for the binding sites at low concentrations (1-10^3 nM). At higher concentrations (10^4-10^6 nM), progesterone and testosterone also drastically blocked the total binding. Based on the concentration of the competitors at which 50% total binding was blocked, DES is 5-fold more competitive than estradiol, and progesterone and testosterone were 30- and 44-fold less competent than estradiol.

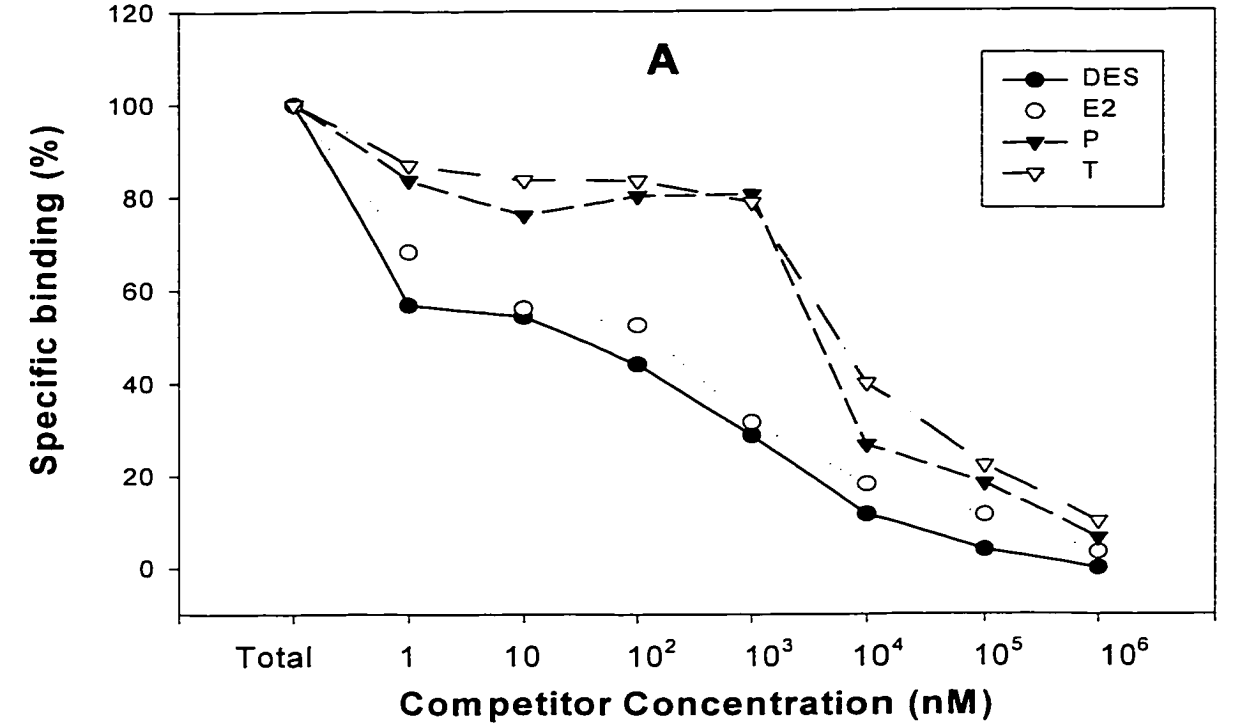
Similarly, in the nuclear fraction of the male gonad, DES and estradiol competed well with [^{3}H] estradiol for the binding sites (Figure 46B). Progesterone and testosterone were less effective in blocking the total binding. DES had a 2-fold higher affinity for the estradiol binding sites than estradiol itself. Progesterone and testosterone were 18- and 45-fold less competent than estradiol in competing for the estradiol binding sites.

In general, DES is about 2-10 times more competitive than estradiol in binding to estrogen binding sites, progesterone showed 20-80 fold less affinity to estrogen binding sites, and testosterone is 50-130 fold less competent than estradiol. Estrogen binding sites in cytosol and nuclear fraction have same ligand preferences.

Table 8. Relative Binding Affinity of Sex Steroids to Estrogen Binding Sites

	Female Cytosolic Fraction	Female Nuclear Fraction	Male Cytosolic Fraction	Male Nuclear Fraction
DES	129.2	119.1	104.1	123.9
Estradiol	100.0	100.0	100.0	100.0
Progesterone	65.2	46.3	31.1	89.3
Testosterone	59.2	35.3	28.6	73.3

Figure 46. Competitive analysis of binding of [^{3}H] estradiol to the cytosolic and nuclear fractions of male scallop gonads.
Increasing concentrations of competitors were incubated with 200 µl of cell extracts and 10 nM [^{3}H] estradiol at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment. Data were expressed as percentages of specific binding in the absence of competitor (denoted as Total). DES, diethylstilbestrol; E2, estradiol; P, progesterone; T, testosterone
A. Cytosolic fraction
B. Nuclear fraction


A
DES
E2
P
T
Specific binding (%)
120
100
80
60
40
20
0
Total
1
10
10^2
10^3
10^4
10^5
10^6
Competitor Concentration (nM)

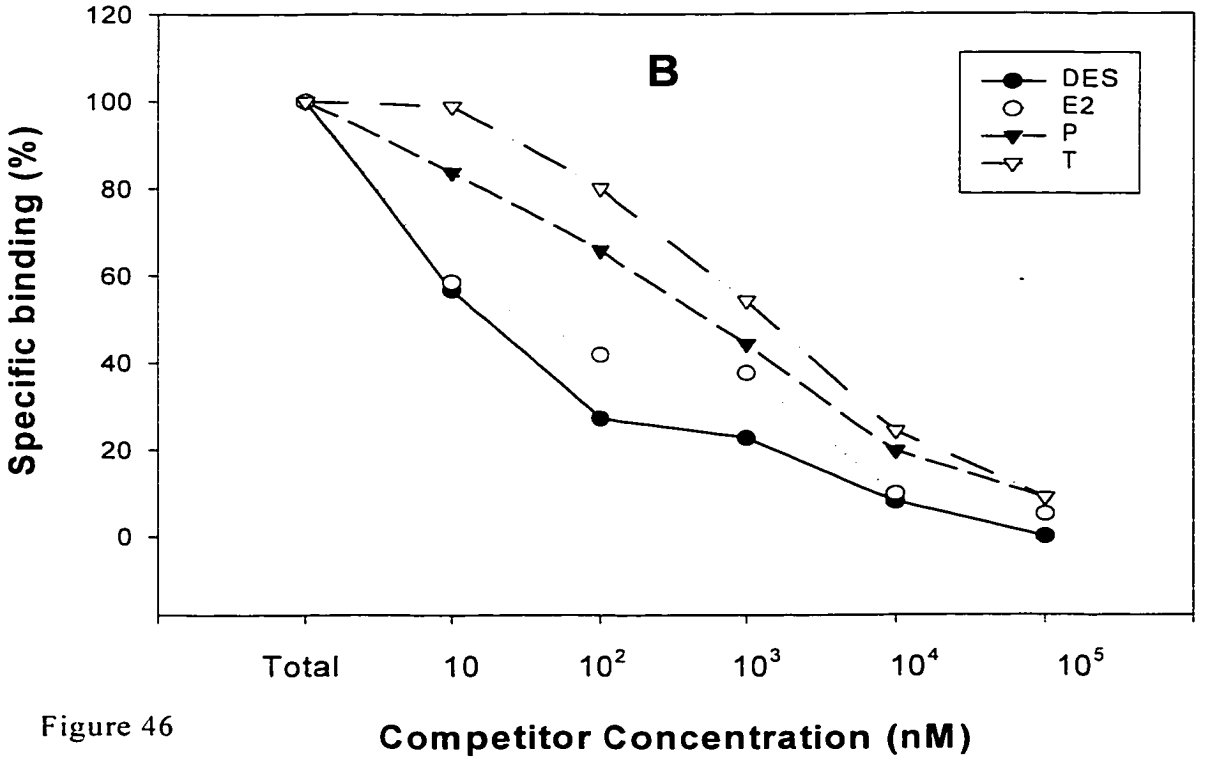

B
DES
E2
P
T
Specific binding (%)
120
100
80
60
40
20
0
Total
10
10^2
10^3
10^4
10^5
Competitor Concentration (nM)

Figure 46

III-C. Distribution of Estrogen Binding sites during Sexual Maturation

Estradiol has been implicated in the regulation of sexual maturation. Thus, it is possible that the concentrations of the estrogen binding sites vary with sexual maturation. To test this hypothesis, we examined the involvement of activation of estrogen receptors by investigating the changes in estrogen binding in female and male gonads with sexual maturation.

In the initial experiments, cytosolic and nuclear gonadal extracts were made from ripe scallops which were full of mature gametes (GSI = 16.9 ± 1.0, n = 6) in the gonads and recently spawned or spent scallops that were not bearing mature gametes (GSI = 5.2 ± 0.9, n = 6). After being diluted to 1 mg protein/ml, both the cytosolic and nuclear fraction were incubated with 10 nM [^{3}H] estradiol at 4 °C for 24 hours to reach equilibrium. Parallel test tubes contained 10 nM [^{3}H] estradiol plus a 500-fold excess concentration of DES to measure the non-specific binding. Specific binding capacities were obtained by subtracting non-specific binding from total binding in the absence of excessive DES. As can be seen from Figure 47, the binding capacity of the cytosolic fraction in ripe scallops was not significantly different from that of spent ones, but the binding capacity of nuclear fraction in the ripe scallops was 53.8% higher than in the spent ones ($P<0.01$, n=6, t-test).

In the second set of experiments, scallops of different stages during the reproductive cycle (females with GSI ranging 3.0-22.2, n=15; males with GSI ranging 3.4-21.8, n=15) were collected and cell extracts were made from their gonads separately. After being diluted to a protein concentration of 1 mg/ml, the cell extracts were incubated with 10 nM [^{3}H] estradiol at 4 °C for 24 hours. The specific bindings of cytosolic and

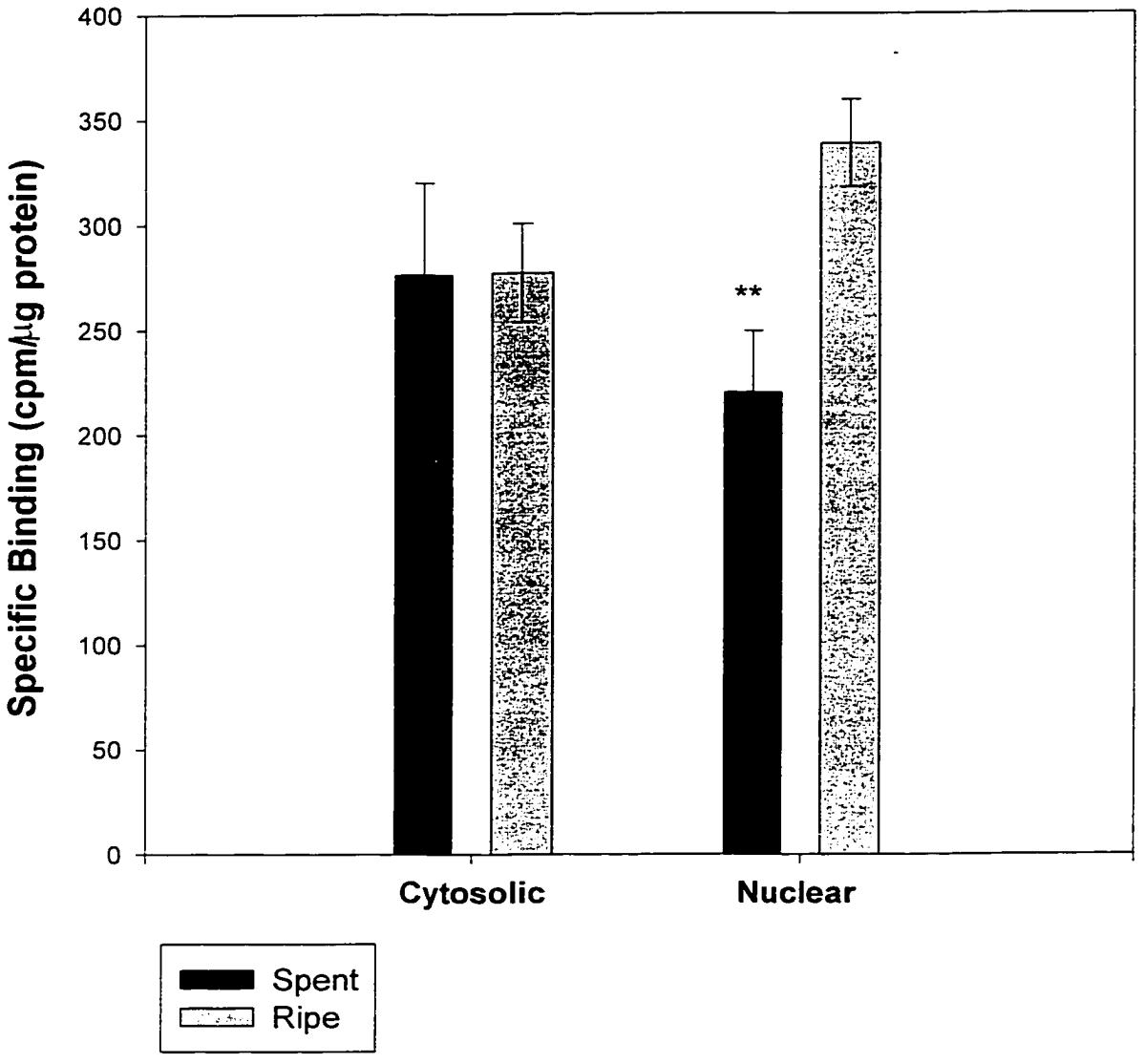


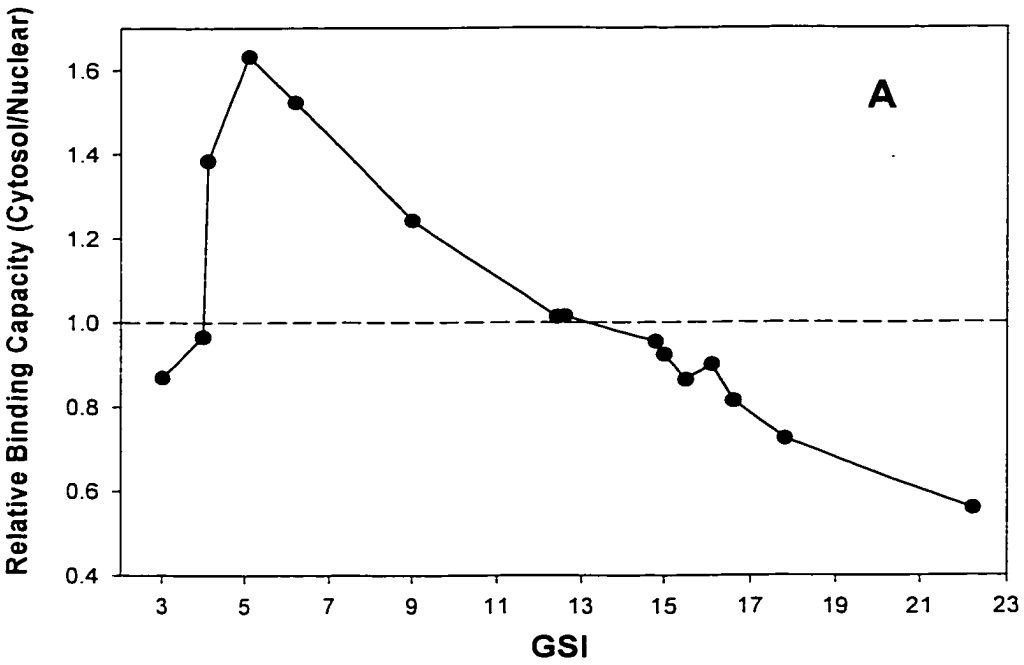
Figure 47. Comparison of binding capacities of cytosolic and nuclear fractions in spent and ripe females.
Cytosolic and nuclear fractions made from mature or spent female scallops were incubated with 10 nM of [^{3}H] estradiol in the absence or presence of 500-fold excess concentration of DES for 24 hours at 4 °C. Bound steroids were separated from free by DCC treatment. Values represent mean±SE (n=6). **P<0.01, n=6, Student's t-test.

nuclear fractions were measured as above. Relative binding capacity ratios between cytosolic and nuclear fraction (C/N ratios) were calculated by dividing the specific binding of the cytosolic fraction by that of the nuclear fraction. The results for females and males are shown in Figure 48A and 48B, respectively.

In the females, when GSI was low, the binding capacity of the cytosolic fraction was lower than that of the nuclear fraction, resulting in a low C/N ratio. At this point, the gonads of the scallops were almost transparent and with very few mature eggs. With the initiation of gametogenesis, the ratio of binding capacity between the cytosol and nuclei increased to a peak of 1.63 when GSI was about 5. This was the stage when the gonads of the scallops began to gain rapid growth. Then the ratio of the concentrations of estrogen binding sites in the cytosol and nuclei decreased indicating the accumulation of estrogen binding sites in the nuclei. When the GSI was about 12, the binding capacity of the cytosol was observed to be equivalent to that of the nuclear fraction. At this stage, the scallop gonad was half full, but the intestine was still visible. Immediately before spawning, when the scallop gonads were fully distended and the intestine was totally obscured, the ratio of binding capacity was lowest, 0.56, in the entire cycle (Figure 48A).

Similar results were also obtained in males, as shown in Figure 48B. At the spent stage when the gonads were generally transparent, the binding capacity of the cytosol was much lower than that of the nuclear fraction with a binding capacity ratio of 0.59. With the gonadal development, the ratio of binding capacity between the cytosolic and nuclear fractions reached a peak of 2.35 at the stage when the GSI was 6. This peak may be correlated with the initiation of the rapid growth of the gonads. The ratio decreased dramatically afterwards with the ratio of binding capacity reaching 1 when the GSI was

Figure 48. Variation of relative specific binding capacity (cytosol/nuclear) with sexual maturation.
Cytosolic and nuclear fractions made from female and male scallop gonads of varying stages of sexual maturation were incubated with 10 nM of [^{3}H] estradiol in the absence or presence of 500-fold excess concentration of DES for 24 hours at 4 °C. Bound steroids were separated from free by DCC treatment.
A. Female
B. Male


A
Relative Binding Capacity (Cytosol/Nuclear)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
3
5
7
9
11
13
15
17
19
21
23
GSI

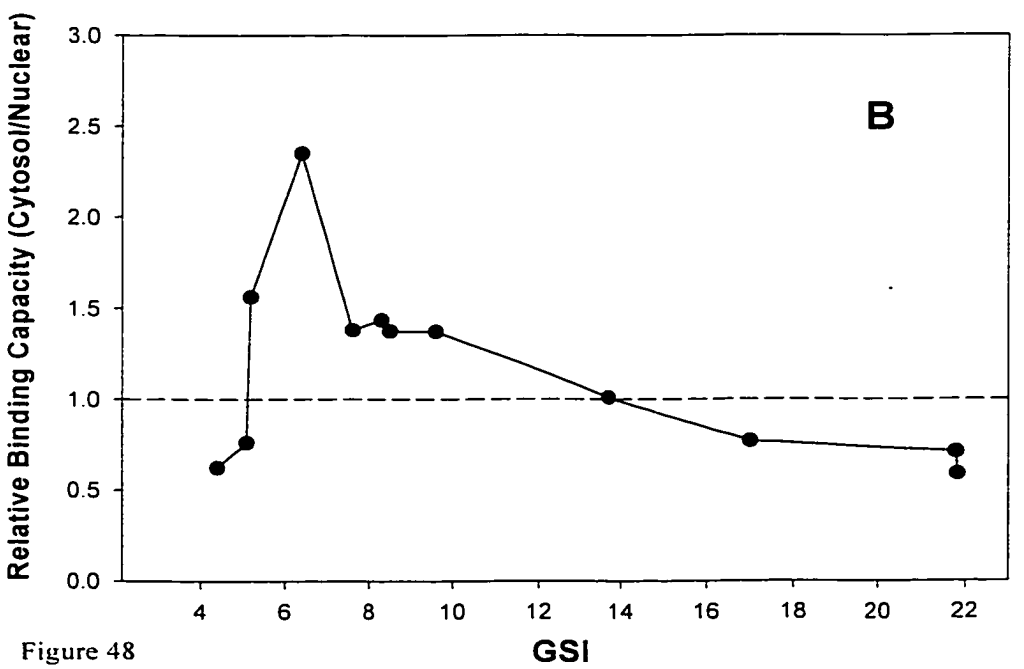

B
Relative Binding Capacity (Cytosol/Nuclear)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
6
8
10
12
14
16
18
20
22
GSI

Figure 48

about 14. In male scallops, the gonads at this stage were about 3/4 full and the intestines were barely visible. The ratio continued to decrease and the lowest value (0.62) was observed when the gonads were full and no intestines were visible.

III-D. Existence of Estrogen Binding Sites in the Liver of Scallops

Estrogen binding sites were also demonstrated in the liver of scallops by examining the specific binding of the liver fractions after incubation with 10 nM [^{3}H] estradiol for 24 hours at 4 °C. The specific binding of the gonad fractions of the same scallops were also obtained for comparison. The GSI of the scallops was 4.29±0.44 (n=3). The results were shown in Figure 49.

It can be seen from Figure 49 that considerable, but lower specific binding for estrogens existed in the liver than in the gonad preparations. In particular, the specific binding in the nuclear fraction was close in the gonad and the liver extracts, but that in the cytosolic fraction was lower in the liver than in the gonad.

IV. Radioligand Receptor Binding Assays of Progesterone Binding Sites in the Scallop

IV-A. Saturation Analysis:

IV-A-1. Female gonad: Progesterone binding sites were also characterized in female gonad nuclear fractions. Two binding sites can be observed in the curves shown in Figure 50A. The first site was located within 0.5-5 nM while the second appeared saturated

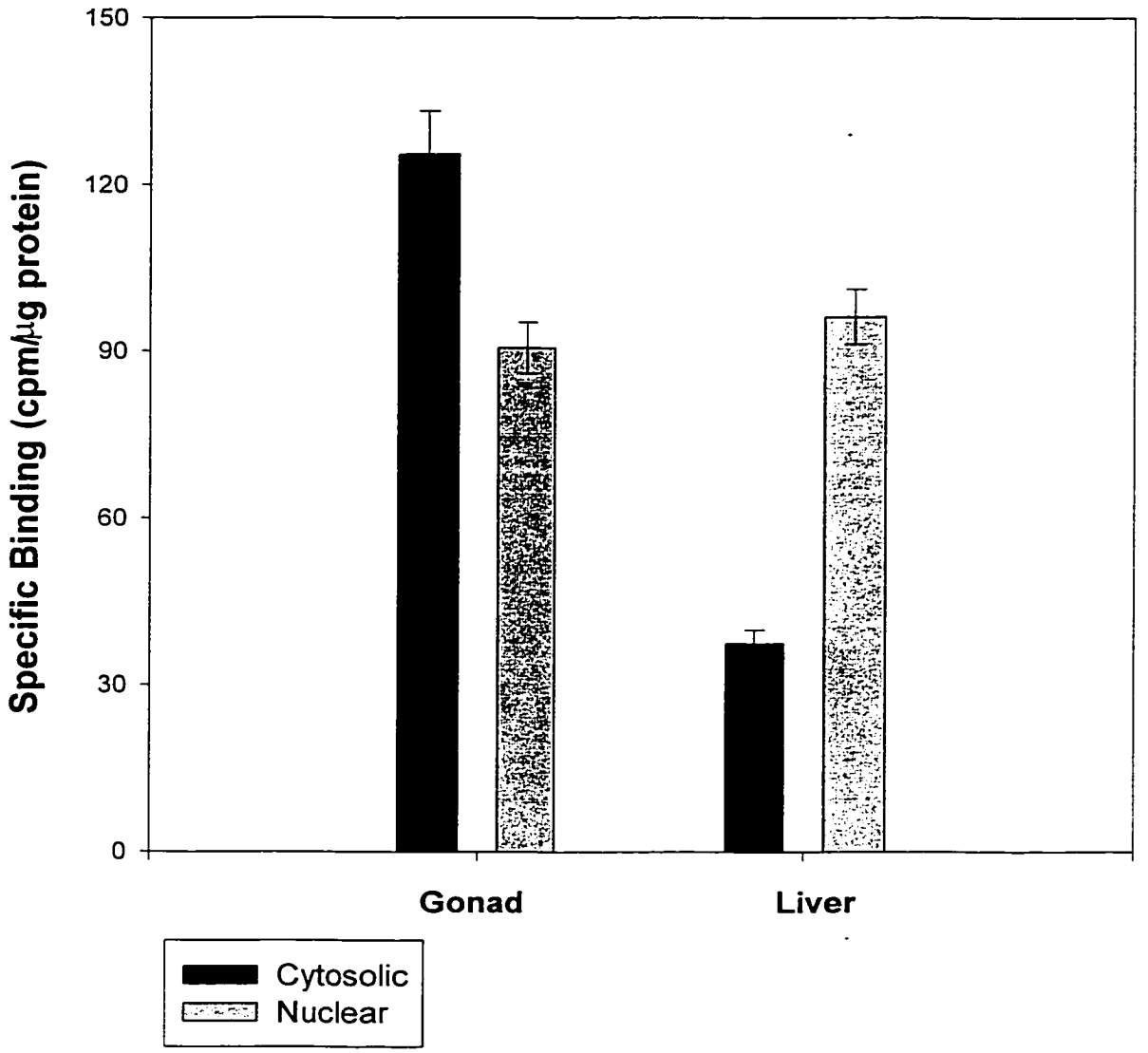


Figure 49. Comparison of specific estrogen bindings in the liver and in the gonad fractions.
Cytosolic and nuclear fractions made from the livers and the gonads were incubated with 10 nM of [^{3}H] estradiol in the absence or presence of 500-fold excess concentration of DES for 24 hours at 4 °C. Bound steroids were separated from free by DCC treatment. Values represent mean±SE (n=3).

between 5-20 nM. Scatchard analysis clearly indicated the presence of two binding sites: one with a high affinity (K_d=1.94 nM) and low maximum binding capacity (B_{max}=17.2 fmol/mg protein) and the other with low affinity (K_d=52.63 nM) and high capacity (B_{max}=0.25 pmol/mg protein) (Figure 50B).

IV-A-2. Male gonad: The binding curves for saturation analysis of progesterone binding sites in the nuclear fraction of male scallops are shown in Figure 51A. It appeared that the specific binding curve was saturated between 15-20 nM. However, two binding sites were revealed from the Scatchard analysis based upon the same data. The high affinity site had a K_d of 2.69 nM and a maximum binding capacity of 23.6 fmol/mg protein while the K_d value for the low affinity was 43.48 nM and its maximum binding capacity was 0.38 pmol/mg protein (Figure 51B).

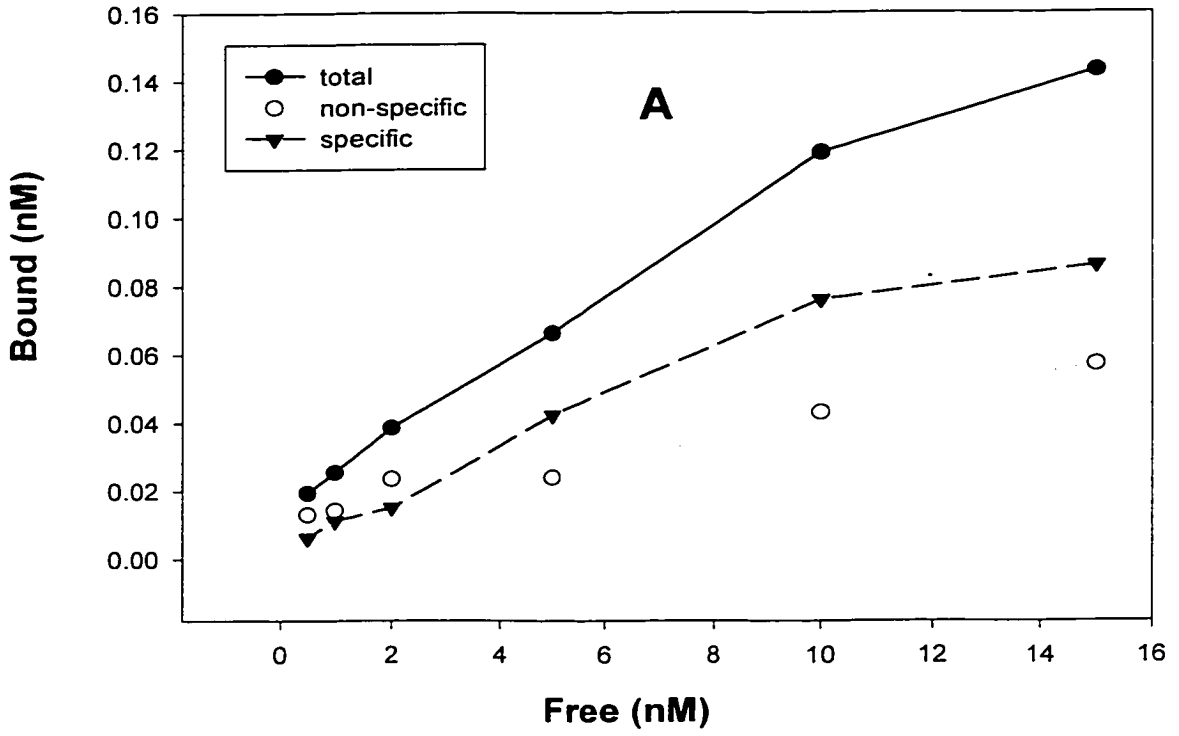
IV-B. Competitive Analyses:

IV-B-1. Female gonad: Competitive analysis showed that unlabeled progesterone competed well with [^{3}H] progesterone. Hydroxyprogesterone and testosterone also competed for progesterone binding sites to some extent. Estradiol did not block the [^{3}H] progesterone binding effectively. Inferred from the results of 50% displacement, hydroxyprogesterone and testosterone were 200-fold and 1000-fold, respectively, less competent than progesterone in competing for the binding sites. Estradiol at 10^{-4} M only diminished the [^{3}H] progesterone binding by 20% (Figure 52A).

IV-B-2. Male gonad: Competitive analysis showed a tendency similar to that for the female nuclear fraction (Figure 52B). Progesterone effectively diminished the [^{3}H] progesterone binding. Hydroxyprogesterone and testosterone also competed for the

Figure 50. Saturation analysis of binding of [^{3}H] progesterone by nuclear fraction of female scallops gonads.

A. Saturation curve of the binding. 200 µl of nuclear fraction were incubated with 0.5-20 nM [^{3}H] progesterone in the presence or absence of a 500 fold excess concentration of progesterone at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment.
B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.


A
total
non-specific
specific
Bound (nM)
Free (nM)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
12
14
16

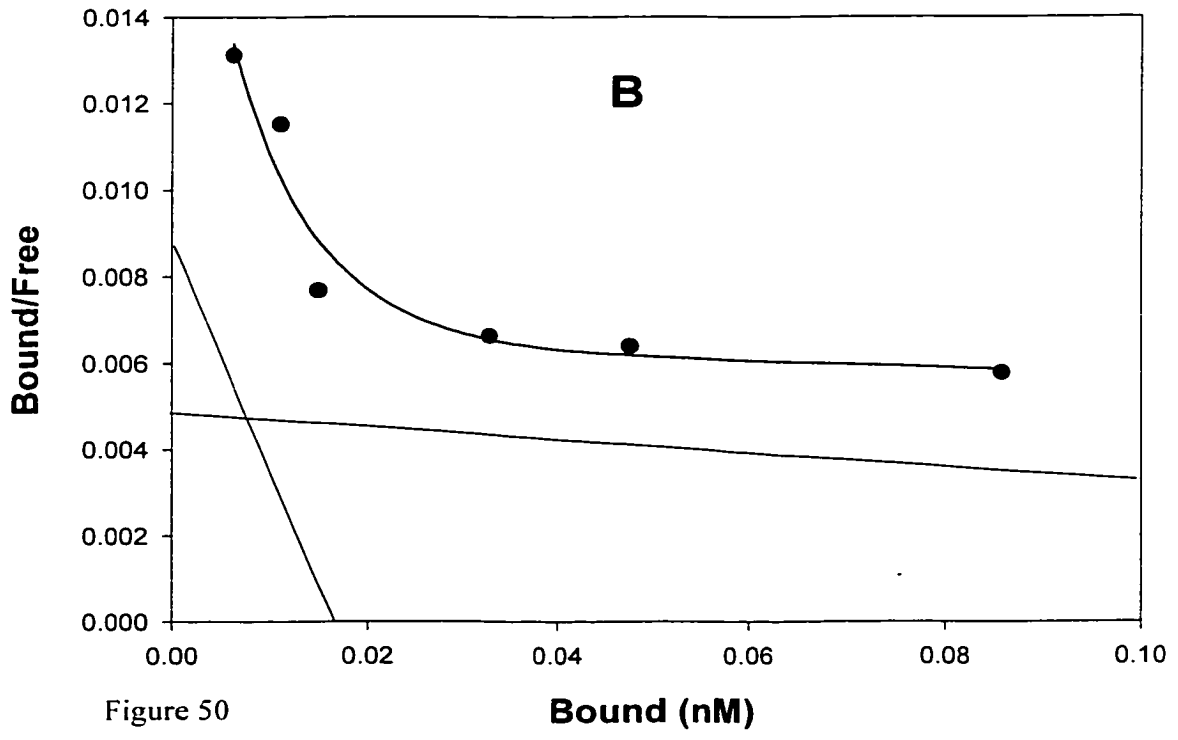

B
Bound/Free
Bound (nM)
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.00
0.02
0.04
0.06
0.08
0.10

Figure 50

progesterone binding sites, but were 80- and 100-fold less competent in competing for the binding sites. Estradiol, at a concentration up to 10^5 M, only blocked 20% of the total binding.

V. Cloning of Estrogen Receptor Gene in the Sea Scallop

V-A. Cloning of an ER cDNA Fragment from the Sea Scallop:

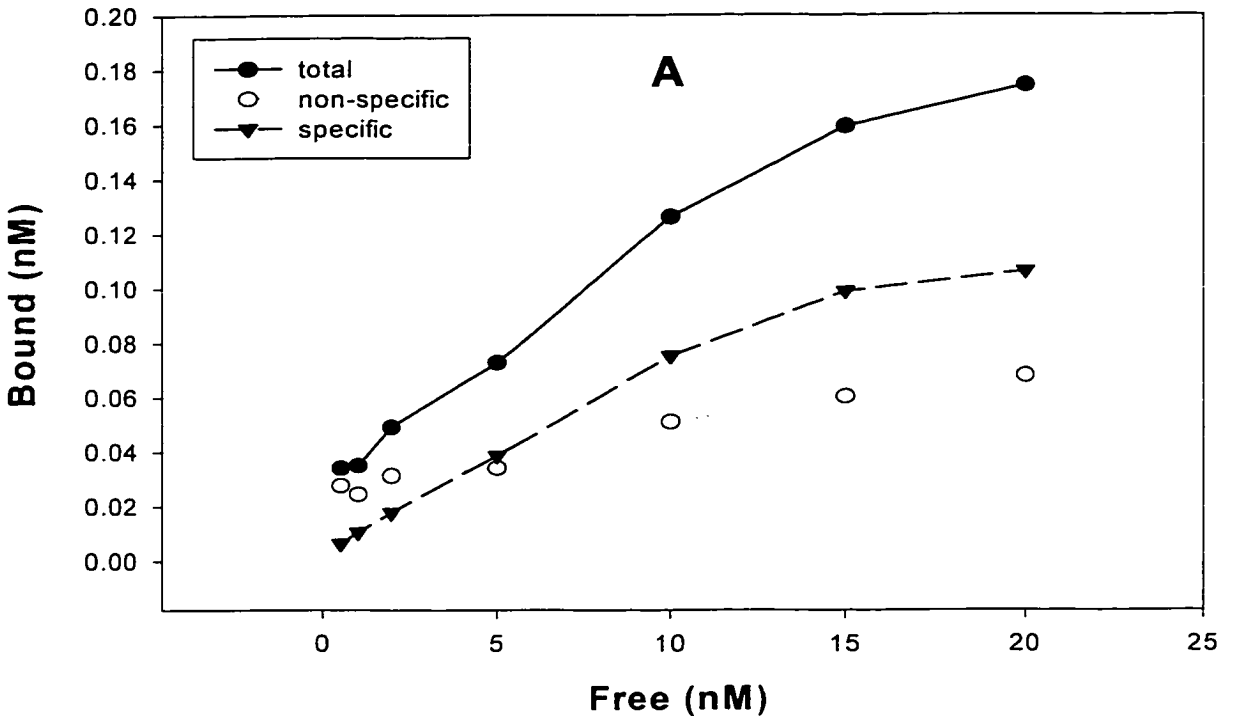
V-A-1: Designing of degenerate primers for RT-PCR: Sequences of estrogen receptors from 14 vertebrate species were selected in a file in the FASTA format which was submitted to Block Maker on-line to find blocks containing conserved regions. Seven blocks were returned, three of which corresponded to the highly conserved DNA binding domain and four to the estrogen binding domain. Degenerate primers were then produced by the program CODEHOP at the same web site. Sixty upstream primers were suggested based on the conserved sequences of DNA-binding domain, and 64 downstream primers for the estrogen-binding domain. Following consideration of low degeneracy and appropriate length and melting temperature, three upstream primers were selected from sixty primers corresponding to DNA-binding domain and three downstream primers from sixty-four primers derived from the estrogen-binding domain.The upstream primers were named pecten-up-1, pecten-up-2 and pecten-up-3, while the downstream primers were named pecten-down-1, pecten-down-2 and pecten-down-3 (Table 9). Non-degenerate primers for actin from the sea scallop were also designed according to the published sequence (Patwary *et al.*, 1996) (Table 9).

V-A-2. Amplification of estrogen receptor cDNA fragment from the sea scallop: Poly-A+-mRNA was captured from the gonad homogenate using biotinylated poly-dT

Figure 51. Saturation analysis of binding of [^{3}H] progesterone by nuclear fraction of male scallop gonads.

A. Saturation curve of the binding. 200 µl of nuclear fraction were incubated with 0.5-20 nM [^{3}H] progesterone in the presence or absence of a 500 fold excess concentration of progesterone at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment.

B. Scatchard analysis of data from A. Graphic analysis of multiple binding sites was performed according to Rosenthal (1967) as shown in Appendix 2.


A
total
non-specific
specific
Bound (nM)
Free (nM)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25

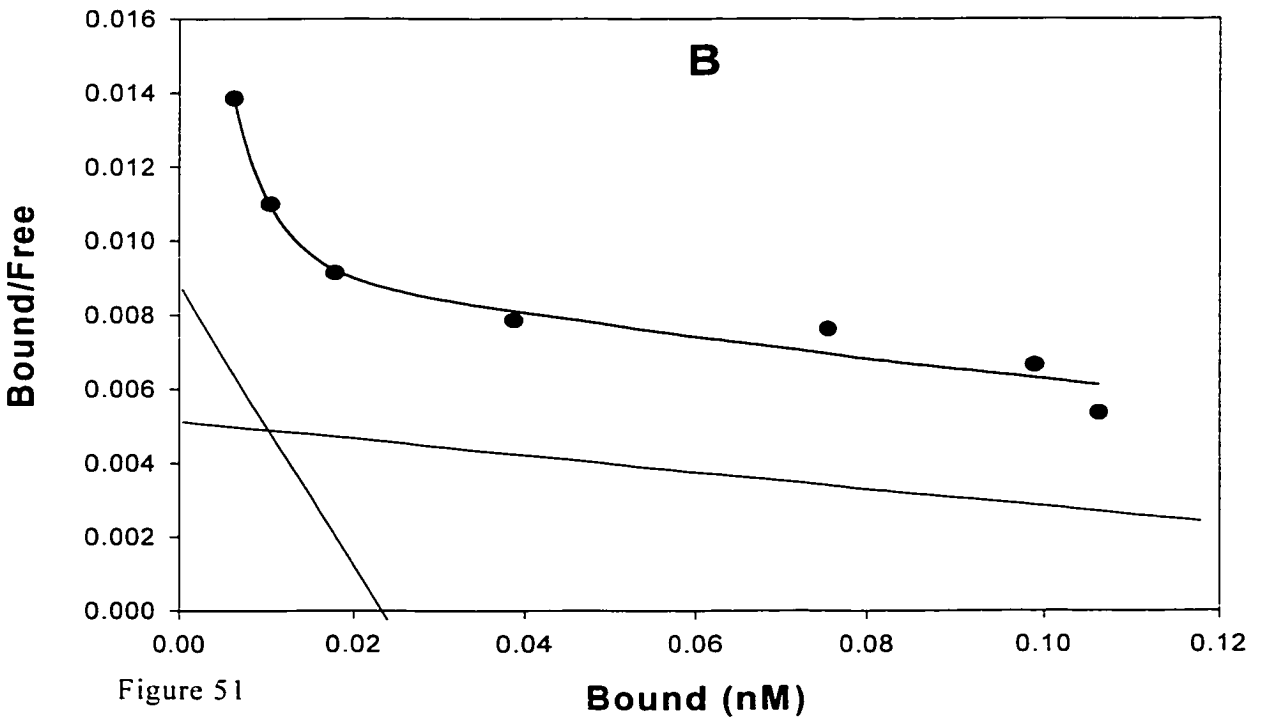
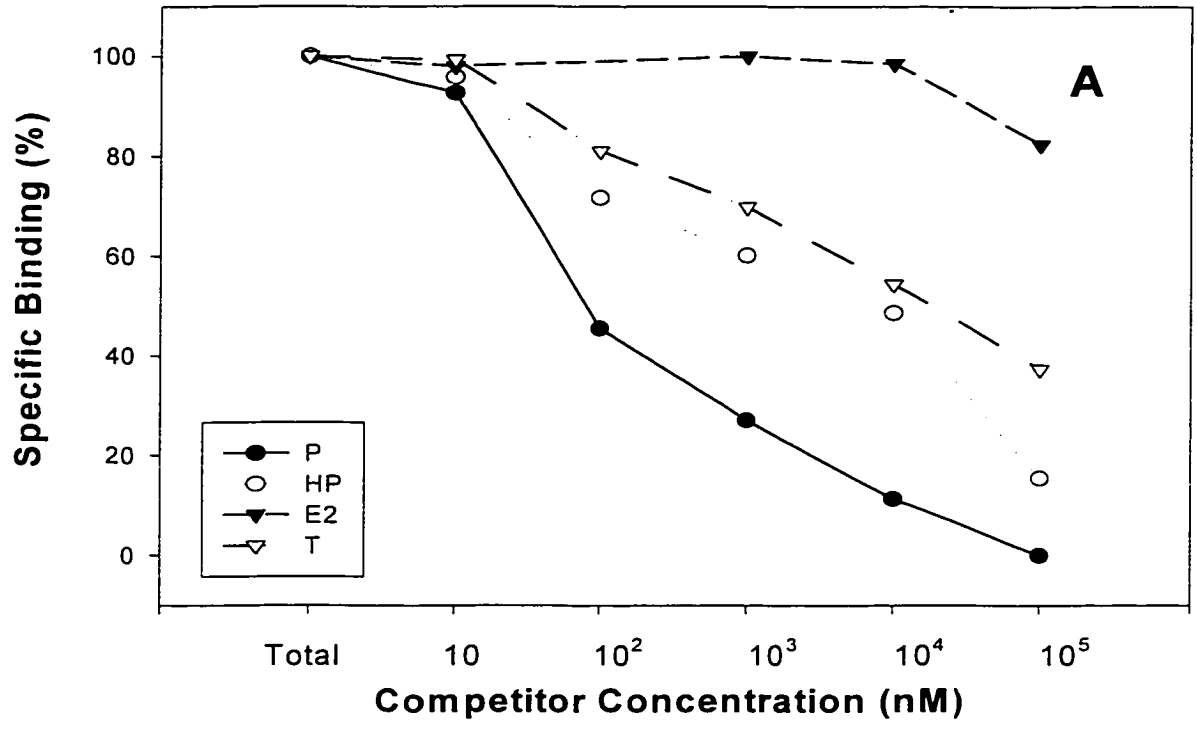

B
Bound/Free
Bound (nM)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.00
0.02
0.04
0.06
0.08
0.10
0.12

Figure 51

Figure 52. Competitive analysis of binding of [^{3}H] progesterone to nuclear fractions of male and female scallop gonads.
Increasing concentrations of competitors were incubated with 200 µl of nuclear fraction and 10 nM [^{3}H] progesterone at 4 °C for 24 hours. Bound steroids were separated from free by DCC treatment. Data were expressed as a percentage of specific binding in the absence of competitor (denoted as Total). P, progesterone; HP, hydroxyprogesterone; E2, estradiol; T, testosterone
A. Female nuclear fraction
B. Male nuclear fraction


A
Specific Binding (%)
100
80
60
40
20
0
P
HP
E2
T
Total
10
10^2
10^3
10^4
10^5
Competitor Concentration (nM)

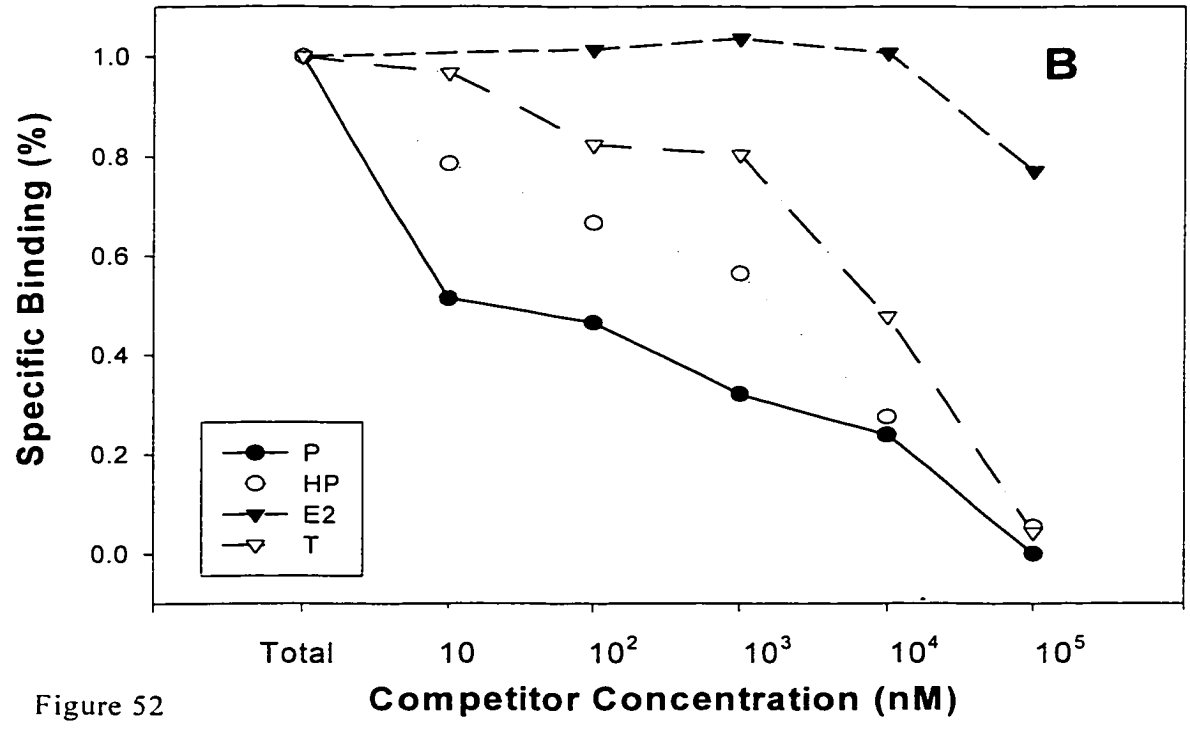

B
Specific Binding (%)
1.0
0.8
0.6
0.4
0.2
0.0
P
HP
E2
T
Total
10
10^2
10^3
10^4
10^5
Competitor Concentration (nM)

Figure 52

probes attached to streptavidin-coated MPG magnetic particles. They were reverse transcribed into first-strand cDNA by AMV reverse transcriptase using poly-dT as 5'-end primers. The quality and integrity of the cDNA pool was tested with actin primers at an annealing temperature of 60 °C. A strong and clear band of expected length (900 bp) and a smaller band (800 bp) were observed on the agarose gel after electrophoresis and staining (Figure 53), suggesting that the cDNA pool was not degraded.

Different combinations of upstream and downstream primers were tried at different annealing temperature with 2.5 mM [Mg^{++}]. A 730 bp band and two small bands (400bp and 450 bp) were amplified with the primers of pecten-up-2 and pecten-down-3. In another reaction, a 710 bp band was amplified with primers pecten-up-3 and pecten-down-3 (Figure 53). No band was amplified from the control reactions in which no template DNA was included or with just one of the two primers. As predicted from the sequences of estrogen receptor from vertebrate species, the 730 bp and the 710 bp fragment were expected with the corresponding primers. Since the 710 bp band was stronger on the agarose gel, only it was excised from the gel and purified by GeneClean.

V-A-3. Cloning and sequencing of the PCR products:

Purified PCR products were ligated with 50 μg of pCR2.1 vector at a ratio of 3:1. The ligate was chemically transformed into the provided host cells and plated on LB plates. Seventy-two positive colonies with a positive rate of 64.5% were obtained. Positive colonies were cultured in liquid medium and plasmid DNA was made from them. After confirmation of the insert size by EcoR I digestion, the clones were then sequenced with the M13 Reverse primer and M13 Forward primer.

The nucleotide sequence for the 710 bp band and the amino acid sequence which it is predicted to encode are given in Figure 54. Alignment of the cloned sequence with corresponding region of estrogen receptor genes from vertebrate species is shown in Figure 55. It can be seen that the cloned partial sequence flanks the region from the conserved DNA-binding domain to the conserved estrogen-binding domain, as expected. It has very high homology at the DNA- and estrogen-binding domain and a low homology in between.

Table 9. PCR Primers for Estrogen Receptor and Actin
(Codes for degenerate base: r=A, G; y=C, T; s=G, C; w=A, T; h=A, T, C)

Primers	Sequences
pecten-up-1	CAr GGn CAy AAy Gry TAy ATs TGy CC
pecten-up-2	TGy GAr GGh TGy AAr GCy TTy TTy AA
pecten-up-3	TGT CCC GCy ACC AAy CAr TGy AC
pecten-down-1	AGr TGy TCC ATr CCy TTr AA
pecten-down-2	AGr TGC TCC ATr CCT TTr TT
pecten-down-3	ATG ATG GCC TTG AGG CAG ACr wAy TCy TC
PMER-1	GCC GCC TCA GAA AGT GTT ATG
PMER-2	GCA GAC GTA CTC CTC AGG CT
actin-up	ATG TGT GAC GAC GAG GTA GCA
actin-down	GGC GTA CAG ATC CTT ACG GAT

V-B. Northern Blotting Analysis

To confirm the existence of the cloned gene in the sea scallop, Northern blotting analysis was carried out with total RNA from scallop gonads, livers and adductor muscles utilizing the cloned cDNA as a probe.

The probe DNA was prepared by PCR from the EcoR I-digested plasmid DNA

Figure 53. Electrophoresis of PCR products of a test gene (actin) and putative estrogen receptor gene amplified from the scallop gonad cDNA pool.

M: 100 bp ladder

1, 2: pecten-up-3 + pecten-down-3

3: pecten-up-2 + pecten-down-3

4, 5: actin-up + actin-down

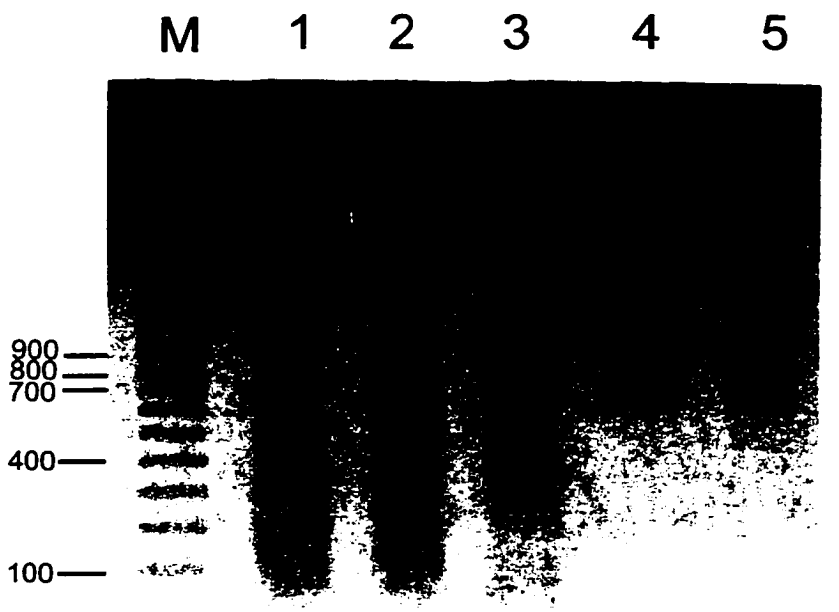


Figure 53

```
ATGTGTCCCGCCACTTATCAGTGTACAATGGACAGGAATCGTAGGAAG  48
 M  C  P  A  T  Y  Q  C  T  M  D  R  N  R  R  K

AGCTGCCAGGCATGCCGCCTCAGAAAGTGTTATGAAGTGGGGATGGTG  96
 S  C  Q  A  C  R  L  R  K  C  Y  E  V  P  M  V

AAAGGAGGCTTGCGTAAGGTTCCNGGGGGGCGGGTTCTCAGGAAGGAT 144
 K  G  G  L  R  K  V  X  G  G  R  V  L  R  K  D

AAGCGGTATTGTGGCCCTGCTGGTGACAGAGAGAAAACCTACGGTGAC 192
 K  R  Y  C  G  P  A  G  D  R  E  K  P  Y  G  D

CTGGAGCACAGGACAGCGCCCCCTCAGGACGGGGGTAGGAACAGCAGC 240
 L  E  H  R  T  A  P  P  Q  D  G  G  R  N  S  S

AGCAGTCTCAATGGTGGTGGAGGATGGCGTGGGCCCAGAATCACCATG 288
 S  S  L  N  G  G  G  G  W  R  G  P  R  I  T  M

CCTCCTGAACAGGTGCTGTTCCTGCTGCAGGGGGCAGAGCCTCCGGCC 332
 P  P  E  Q  V  L  F  L  L  Q  G  A  E  P  P  A

CTGTGTTCTCGTCAGAAGGTGGCCCGCCCCTACACAGAGGTCACCATG 380
 L  C  S  R  Q  K  V  A  R  P  Y  T  E  V  T  M

ATGACCCTGCTCACCAGCATGGCTGACAAGGAGCTGGTGCACATGATC 428
 M  T  L  L  T  S  M  A  D  K  E  L  V  H  M  I

GCTTGGGCTAAGAAAATACCAGGTTTCCAGGAGCTGTCTCTCCATGAC 476
 A  W  A  K  K  V  P  G  F  Q  E  L  S  L  H  D

CAGGTGCAGCTGCTGGAGAGTTCCTGGCTGGAGGTGCTGATGATCGGA 524
 Q  V  Q  L  L  E  S  S  W  L  E  V  L  M  I  G

CTCATATGGCGGTCCATCCACTGCCCTGGGAAACTCATCTTCGCCCAG 572
 L  I  W  R  S  I  H  C  P  G  K  L  I  F  A  Q

GACCTCATCCTGGACAGGAGTGAAGGGGACTGTATAGAGGGTATGGCT 620
 D  L  I  L  D  R  S  E  G  D  C  V  E  G  M  A

GAGATCTACGACATGCTCCTGGCCACTGTGTCTCNCTTCCGCATGCTT 668

 E  I  F  D  M  L  L  A  T  V  S  X  F  R  M  L

AAACTGAAGCCTGAGGAGTACGTCTGCCTCAAGGCC 704

 K  L  K  P  E  E  Y  V  C  L  K  A
```

Figure 54. Nucleotide and amino acid sequence of the cloned partial sequence.

Figure 55. Similarity comparison of amino sequence of the putative scallop estrogen receptor gene with those of some vertebrate species.

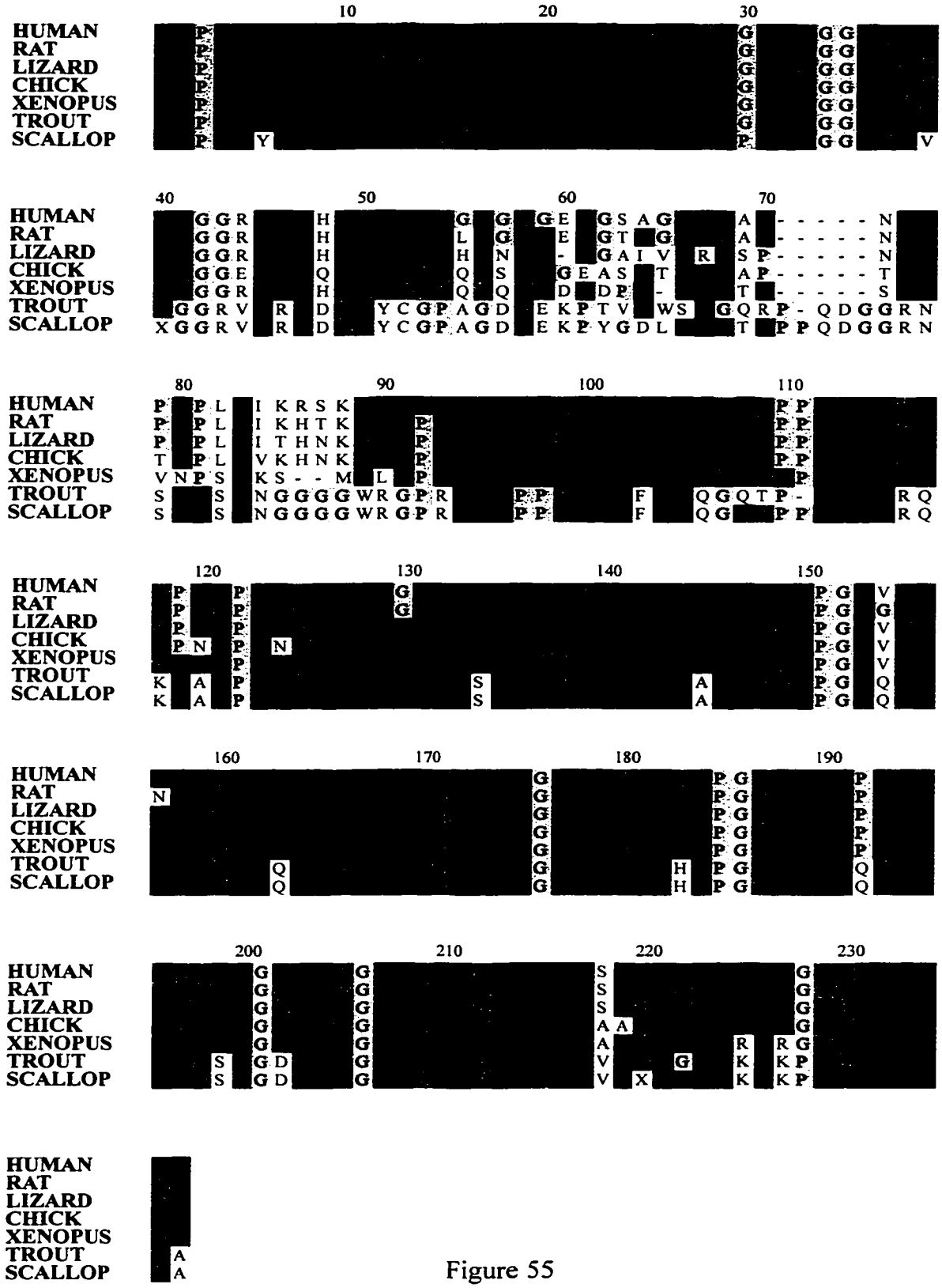


Figure 55

using non-degenerate primers based on the determined sequence (PMER-1 and PMER-2, see Table 9 for their sequences) and running at high annealing temperature. The PCR product displayed a strong and clear band on the gel and was thus used in probe labeling without purification. The probe DNA was also prepared from the EcoR I digest of the plasmid DNA containing the 710 bp band by GeneClean. One hundred nanogram of probe DNA and 50 μCi of [α-^{32}P] dCTP were used in a labeling reaction.

With this probe, a band of 3.1 kb was displayed from the total RNA preparations of the liver and gonad but not from the adductor muscle (Figure 56).

Figure 56. Northern blotting of total RNA preparations from scallop with the cloned gene as a probe.
L: liver
G: gonad
M: adductor muscle

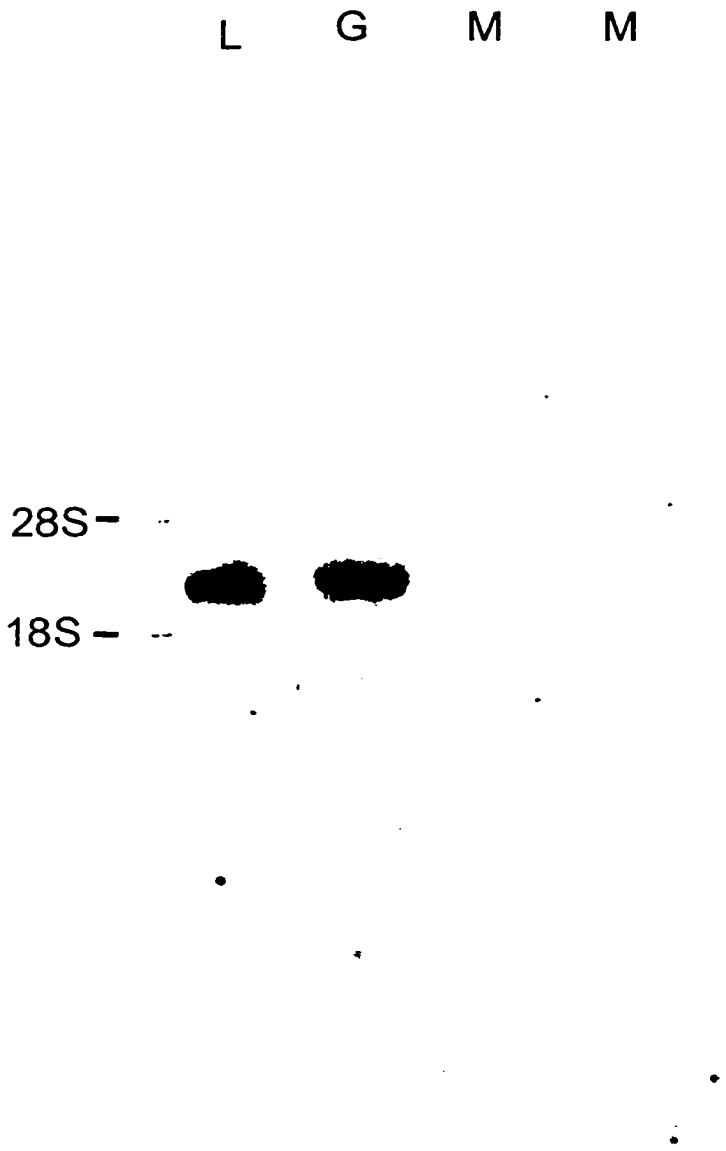


Figure 56

Discussion

Vertebrate type sex steroids have long been identified in molluscs. However, there have been disputes about their functions in invertebrates including molluscs. The results from the current studies suggest that sex steroids have important functions in the reproductive control in the sea scallops and their actions may be achieved through sex steroid receptors.

One of the major effects I found is that injections of sex steroids into undifferentiated juveniles significantly promote gonadal differentiation and shift the sex ratios, as well as inducing morphological changes. Another major action of sex steroids appears to be their regulation of gamete release and spawning in scallops. It appears that estradiol has stimulatory effects in both sexes; testosterone has stimulatory effects in males, and has inhibitory or no effects in females; progesterone has stimulatory effects in both sexes except for *in vivo* spawning in females.

I then demonstrated that the classical mechanism of steroids may be involved in these actions, but membrane effects may have also contributed to the overall actions. Using radioligand binding assays, estrogen and progesterone binding sites that are similar to vertebrate receptors in binding affinity, capacity and specificity were characterized in the sea scallop. I also showed that synthesis and activation of the estrogen receptor might be involved in the process of gonadal maturation. Furthermore, a partial sequence of a probable estrogen receptor gene was cloned from the sea scallop and its expression in the sea scallop was demonstrated by Northern blotting.

In general, my results suggest that sex steroids may have extensive control of

the reproduction of the sea scallop, and these regulations are possibly achieved through the activation of specific sex steroid receptors.

I. Effects of Sex Steroids on Gonadal Differentiation and Sex Determination in Juvenile Scallops

I-A. Effects of Sex Steroids on Gonadal Differentiation in Juvenile Scallops

We have shown in these studies that sex steroids significantly promoted gonadal differentiation. Higher differentiation rates were observed in the sex steroid-injected group than the non-injection control group.

As discussed in the Introduction, previous evidence exists for the stimulation of gonadal differentiation by sex steroids in bivalves. In Yezo scallop *Mizuhopecten yessoensis*, injections of estradiol, progesterone and testosterone had stimulatory effects on both oogenesis and spermatogenesis (Varaksina and Varaksin, 1991; Varaksina *et al.*, 1992). Their results were in agreement with the results from current studies in that sex steroids directly stimulated morphological differentiation in bivalves. However, the mechanisms that are responsible for the cellular differentiation during gametogenesis in molluscs are not clear.

My results also agree with those of Varaksina and Varaksin (1991) and Varaksina *et al.* (1992) in that effects of sex steroids on gametogenesis do not seem to be steroid-specific. In their studies, estradiol, progesterone and testosterone had similar effects on gametogenesis in both males and females; the results from the current studies showed that all the steroids injected stimulated gonadal differentiation. Together, these results imply that, besides their direct effects, sex steroids may also

indirectly stimulate sex differentiation, possibly by regulating the metabolism and mobilization of nutrients and energy.

In the Introduction, I have discussed the possible involvement of sex steroids in the regulation of the metabolism of glycogen, protein and lipids. For example, estradiol may stimulate glycogenolysis and lipidogenesis by regulating the activities of some important enzymes such as glucose-6-phosphate dehydrogenase (G-6-PD) and malate dehydrogenase in molluscs (Mori, 1969a; Mori *et al.*, 1972a; Mori *et al.*, 1972b). Vitellogenesis, which is an essential event for the development of gametes, was also suggested to be controlled by estradiol (Li *et al.*, 1998). In addition to these specific proteins, synthesis of total proteins in molluscs may also be under the regulation of steroids as was seen in the sea star (Barker and Xu, 1993). Therefore, administration of sex steroids in the scallops may accelerate the metabolic rate in the gonad, providing more materials and thus more energy for the gonadal differentiation.

Before gonadal differentiation, the reserves in the storage tissues such as the adductor muscle and liver are relatively small. However, since the process of gonadal differentiation resembles that of gametogenesis in adults in terms of development of gametes, transfers of nutrients and energy from the storage tissues to the gonad during sex differentiation may still exist. As discussed in the Introduction, these transfers may be facilitated by sex steroids (see Section IV-B-1-c of the Introduction).

It is interesting to note that the significant stimulatory effects of sex steroid injections on gonadal differentiation become insignificant when compared to the vehicle control group. The difference in significance was caused by an insignificant stimulatory effect of vehicle. It is thus possible that the presence of corn oil (mainly

fatty acids, of which oleic acid is the major component) potentiated the effects of sex steroids. It is also possible that in these studies, sex steroids promoted gonadal differentiation by stimulating metabolism of this fatty acid. In either case, the interactions between sex steroids and the vehicle may be necessary for these effects.

Fatty acids may potentiate the effects of sex steroids in two ways. First, it is possible that fatty acids combine with sex steroids to form esters. Estradiol- and DHEA-fatty acid esters have been reported in mammals and these steroidal esters are more potent than non-esterized steroids (Hochberg, 1991; Larner *et al.*, 1993). No fatty acid esters for testosterone and progesterone have been reported. However, the possibilities that testosterone and progesterone are converted to estradiol or pregnenolone or other steroids and then form esters with fatty acids cannot be excluded. Second, fatty acids could affect the binding of sex steroids to their receptors and thus modulate their efficacies. For example, C20:4 unsaturated fatty acid can stimulate estradiol binding while inhibiting progesterone binding (Benassayag *et al.*, 1999).

Injection of fatty acids into juvenile scallops may provide both materials and energy for the morphological changes in the gonad during sex differentiation. Fatty acids are essential components of the membrane phospholipids and oxidation of fatty acids produces ATP for the activities of cells. Furthermore, sex steroids may accelerate the metabolism of corn oil so that the fatty acid can be converted into phospholipids or ATP more quickly and efficiently and thus stimulating gonadal differentiation. Breakdown of fatty acids has been reported to be stimulated by DHEA and pregnenolone in guinea pig and rat (Belanger *et al.*, 1992; Goto, 1998).

The stimulatory effect of DHEA may be due to its induction of peroxisome proliferation which is essential for fatty acid breakdown (Goto, 1998).

In conclusion, sex steroids may promote gonadal differentiation directly by their effects on cellular differentiation or indirectly by affecting the metabolism and mobilization of nutrients, especially metabolism of fatty acids. In addition, effects of sex steroids may be enhanced by their interactions with fatty acids.

I-B. Effects of Sex Steroids on Sex Ratios:

In these studies, I have shown that injections of sex steroids into undifferentiated juvenile scallops induced changes in sex ratio and gonadal morphology. Significantly more males than females were found following injections of testosterone, estradiol, progesterone, and DHEA while the sex ratios in the control groups were not different from 1:1. My results therefore supported the hypothesis that sex steroids are involved in sex determination in molluscs, as discussed below.

The mechanisms underlying sexual determination vary among animal groups. In most vertebrates and insects, gender is determined by the presence of the sex chromosomes, XY (such as in mammals), ZW (birds) or X (insects). In some insects such as bees and ants, sex depends on the ploidy, i.e., haploid animals become males and diploid animals are females (Campbell *et al.*, 1999). Exogenous factors such as hatching temperature for reptiles may also affect sex determination presumably by affecting metabolism and thus the titers of sex steroids (Pieau *et al.*, 1999).

Given the complexity of sex determination mechanisms in the animal kingdom, a general model that can account for all the mechanisms and the exceptions,

sex reversal, and hermaphrodites has been sought. A hypothesis based on the ratio of androgens to estrogens was proposed by Bogart (1987). In this model, the sex of an animal is eventually determined by the balance between androgens and estrogens during sexual differentiation. This balance may be controlled by the activity of the aromatase which may be regulated by different factors in different animals. For example, there might be an aromatase inhibitor factor existing on the Y chromosome in mammals or aromatase activator on the W chromosome in birds. Hatching temperature in reptiles may affect the metabolism of sex steroids so that the balance between estrogens and androgens shifts. Thus, although the balance between androgens and estrogens is achieved in different ways, the gender of the animal is determined by this balance. In theory, this hypothesis can explain all of the major sex determination mechanisms in vertebrates and insects.

Currently, we know very little about sex determination mechanisms in molluscs. However, the hypothesis of Bogart (1987) may apply to molluscs for the following reasons. 1) Sex determination in some bivalves have been suggested to be achieved through XY-system, *i.e.*, XY animals are males and XX animals are females (Guo and Allen, 1994; Guo *et al.*, 1998a, b). According to Bogart (1987), the balance between estrogens and androgens may be affected by the presence of the Y chromosome which bears an aromatase inhibitor. However, this hypothesis cannot explain the sex ratios observed in triploid oysters. Guo *et al.* (1998) reported that triploid oysters produced by inhibition of polar body II had 46% females while those obtained by crossing tetraploid males with diploid females had 91% females. They have also observed 36% hermaphrodites in a triploid group produced by inhibiting

polar body II. It is possible that more than one factor regulating the conversion of androgens to estrogens exist on the sex chromosomes. In fact, Haley *et* al. (1979) had supposed that sex determination in American oyster might be controlled by 3 or more loci. 2) Vertebrate types of sex steroids such as estradiol and testosterone have been identified in molluscs, as discussed in the Introduction, although their roles as hormones have not been definitely established in molluscs. 3) The balance between androgens and estrogens has been shown to affect the secondary sex characteristics in tributyltin-induced imposex, i.e., development of male characteristics in females exposed to tributyltin. 4) Injections of sex steroids have been reported to induce sex reversal in bivalves (Mori *et al.*, 1969b; Moss, 1989).

If the hypothesis of Bogart (1987) applies to the sea scallops, it is thus not surprising that injection of testosterone and DHEA, both androgens, resulted in more males than females in the experimental groups of the present study. This is in agreement with the results from previous experiments which showed that testosterone had androgenic effect in molluscs (Csaba and Bierbauer, 1979; Csaba and Bierbauer, 1981; Varaksina and Varaksin, 1991; Sakr *et al.*, 1992; Varaksina *et al.*, 1992). It is also consistent with my results from the gamete release and spawning experiments which demonstrated the androgenic effect of testosterone in the scallops. These results suggested that testosterone and DHEA might have similar actions in molluscs as in vertebrates. However, if we assume that estradiol and progesterone have similar actions in molluscs as in vertebrates, the results from the estradiol and progesterone-injected groups seem to contradict the hypothesis of Bogart (1987). Nonetheless, it is premature to make such an assumption. In fact, in addition to their actions as female

hormones, estradiol and progesterone have also exhibited androgenic actions in bivalves and gastropods. It has been reported that both substances stimulated spermatogenesis in molluscs (Csaba and Bierbauer, 1979; Csaba and Bierbauer, 1981; Varaksina and Varaksin, 1991; Sakr *et al.*, 1992; Varaksina *et al.*, 1992). In my *in vitro* and *in vivo* studies I also showed that estradiol and progesterone potentiated sperm release and spawning in male scallops. In other invertebrates, estradiol has also been found to stimulate male reproductive activities. For example, injection of estradiol in juvenile red sea urchins favored male sex differentiation (Unuma *et al.*, 1999).

The androgen-like actions of estradiol and progesterone may be caused by the following mechanisms. 1) Injection of estradiol and progesterone may promote the synthesis of active androgens in the animal and thus favor the development of male gonad. In vertebrates, estradiol and progesterone have been reported to stimulate the conversion of testosterone or 4-androstene-3,17-dione to 5α-dihydrotestosterone (DHT) which was supposed to be a more potent androgen (Tilakaratne and Soory, 1999). Progesterone may also serve as a precursor for the synthesis of active androgens. It has been well established that molluscs are able to synthesize testosterone from progesterone (Lehoux and Sandor, 1970; De Longcamp *et al.*, 1974; Lupo di Prisco and Fulgheri, 1975), as summarized in Figure 2. 2) Estradiol or progesterone may bind directly to an androgen receptor and elicit androgenic effects. Binding of estradiol and progesterone to androgen receptors has been demonstrated in vertebrates. For example, binding of estradiol and progesterone to androgen receptors with high affinity were shown in the human prostate tumor cells (Veldscholte *et al.*,

1990). Mitogenic action of estradiol in androgen-sensitive Shionogi mammary carcinoma cells can be blocked by the antiandrogen, hydroxyflutamide, indicating that the action of estradiol was achieved by its binding to androgen receptors (Luthy *et al.*, 1988). This possibility was also shown in my *in vitro* studies in which I showed that potentiation of 5-HT-induced sperm release by progesterone can be partially inhibited by flutamide, an antiandrogen.

In conclusion, sex steroids may be involved in sex determination in molluscs, as in vertebrates. However, some sex steroids may have different actions in molluscs than in vertebrates.

I-C. Other Morphological Changes Induced by Sex Steroids:

I-C-1. Eutrophy of oocytes: My results showed that estradiol and DHEA stimulated the growth of extraordinarily large oocytes. No similar phenomenon has been previously reported in bivalves. However, estradiol has been suggested to be involved in growth of molluscan oocytes in other studies. In mussels and scallops, the levels of estradiol are correlated with the development of gonads (Reis-Henriques and Coimbra, 1990; Matsumoto *et al.*, 1997). Injections of estradiol have been shown to stimulate oogenesis in *Patinopecten yessoensis* (Varaksina and Varaksin, 1991). From these actions, it appears that estradiol has female hormone actions in molluscs, although it has not been proved to act as a hormone in such animals (see section III-F of the Introduction). The actions of estradiol in molluscs are also in agreement with their actions promoting cell proliferation and growth that have been widely reported

in vertebrate tissues (for reviews, see Sutherland *et al.*, 1983; Ernst *et al.*, 1989; Robker and Richards, 1998).

I-C-2. Breakdown of oocytes caused by testosterone in female juveniles: In this study, I observed that injection of testosterone resulted in breakdown of oocytes in the gonads of female juveniles. In morphology, this phenomenon is similar to oocyte atresia (necrosis) which is commonly observed in bivalves. Oocyte atresia usually occurs at the end of vitellogenesis before spawning. When the animals are very ripe and no spawning occur in a certain period, lysis of oocytes begins (Paulet *et al.*, 1986). During oocyte atresia, usually the stainings of nuclei and cytoplasm become pale, the oocytes deform in shape, and then the cell membranes rupture and the contents of the nuclei and cytoplasm are released, leaving cell debris in the acini (Dorange, 1989; Beninger and Pennec, 1991). However, while these morphological characteristics are similar to the breakdown of oocytes observed in this study, oocyte atresia is different in that it usually happens at the end of vitellogenesis while breakdown of acini was observed here early during gametogenesis before vitellogenesis. These results suggested that testosterone may have inhibitory effects on oogenesis.

I-C-3. Hermaphrodites in the progesterone- and DHEA-injected groups: Hermaphrodites represented 1.89% of the animals in the progesteronė-treated group and 4.17% in the DHEA-treated group, while no hermaphrodites were seen in other sex steroid-injected groups or the control groups. Naidu (1970) reported that 1.3% of the animals in the natural sea scallop population in Port au Port Bay, Newfoundland were hermaphrodites. Merrill and Burch (1960) observed only 2 hermaphrodites from

about 3000 sea scallops in the natural population of George Bank. In a *Mytilus edulis* population in the Gulf of Finland, 0.11% of the animals were hermaphrodites (Sunila, 1981). Although my results in the estradiol and progesterone groups are not different from the finding of Naidu (1970), they are significantly higher than those of the control groups, the results of Merrill and Burch (1960), and those observed in mussels ($P<0.001$, post-hoc Chi-square tests). These results suggested progesterone and DHEA may stimulate the induction of hermaphrodites. A similar hypothesis has also been proposed by Kat (1983). Notably, this hypothesis has recently received support from the induction of hermaphrodites in the reedfrogs by treatment with estradiol (Hayes, 1999).

II. Effects of Sex Steroids on Gamete Release and Spawning

II-A. *In Vitro* Effects of Sex Steroids on Gamete Release

It is well known that gamete release, the detachment and discharge of gametes from germinal epithelium of the gonad, can be induced by 5-HT (Matsutani *et al.*, 1987; Osada *et al.*, 1992). This phenomenon may be a direct consequence of gamete maturation induced by 5-HT, as eggs obtained by 5-HT treatment, but not by mechanical stimuli, have been observed to undergo germinal vesicle breakdown (GVBD) (Matsutani and Nomura, 1987; Osada *et al.*, 1992a). It is also possible that 5-HT causes breakdown of the acini walls and thus induces release of gametes from the gonad. However, the exact mechanisms for gamete release are not clear at the present time.

II-A-1. Enhancement of gamete release by activation of sex steroid receptors: It has been well established that estradiol potentiates 5-HT-induced egg release in the Japanese scallop *Patinopecten yessoensis* (Osada *et al.*, 1992). Here I demonstrated that estradiol had similar actions in the sea scallop. Furthermore, I showed that estradiol also had stimulatory effects on 5-HT-induced sperm release, progesterone had similar effects in both sexes, but testosterone was effective in males only. These results indicate that these sex steroids have sex-specific effects in molluscs, as in vertebrates.

To investigate the underlying mechanisms of the actions of sex steroids, pharmacological studies employing antisteroids or RNA or protein synthesis inhibitors were performed. My results indicated that the actions of estradiol and progesterone in both sexes and testosterone in males were blocked by anti-steroids, actinomycin D and cycloheximide. These results suggested that the actions of the sex steroids were achieved via the classical mechanism which involves the activation of intracellular steroid receptors. Both transcription and translation appear to be involved as the actions of sex steroids were inhibited by both actinomycin D and cycloheximide. Similar results were also obtained by Osada *et al.* (1992) who showed that actinomycin D blocked the promotion of 5-HT-induced egg release by estradiol.

Two mechanisms may be responsible for the actions of estradiol in 5-HT-induced gamete release. One mechanism may be that estradiol regulates the actions of 5-HT by stimulating synthesis of prostaglandin E_2 (PGE_2). Support for this mechanism came from pretreatment of gonad pieces with aspirin (a prostaglandin synthesis inhibitor) which significantly blocked the action of estradiol (Osada *et al.*,

1992). The involvement of prostaglandins on regulation of 5-HT actions in spawning induction has also been investigated in *Patinopecten yessoensis* (Matsutani and Nomura, 1986; Matsutani and Nomura, 1987). Estradiol has been shown to regulate the synthesis of prostaglandins in *Patinopecten yessoensis*, and this action can be blocked by epitiostanol, an antiestrogen (Osada and Nomura, 1990).

Another possible mechanism may be that estradiol promotes the synthesis of 5-HT receptors on the membrane of gametes so that they are more sensitive to 5-HT stimulation. Presence of 5-HT receptors on the oocyte cell membranes has been demonstrated in bivalve species such as the Atlantic surf clam *Spisula solidissima*, the Japanese scallop *Patinopecten yessoensis* and the Pacific oyster *Crassostrea gigas* (Kadam, 1991; Osada *et al.*, 1998). It has been shown that estradiol stimulated the synthesis of 5-HT receptors on the surface of oocyte membrane (Osada *et al.*, 1998).

In both mechanisms, it appears that synthesis of proteins, either the enzymes involved in prostaglandin synthesis or 5-HT receptors, is involved, since the effects can be abolished by pretreatment with actinomycin D. It is thus possible that estradiol exerts its actions through the classical mechanism in which activation of a specific estrogen receptor is involved. In agreement with this hypothesis, the presence of the estrogen receptor in female and male gonad preparations was demonstrated in my radiolabeled ligand receptor binding studies.

II-A-2. Specificity of actions of sex steroids in gamete release: It has also been observed that the actions of a sex steroid can be reduced by an anti-steroid to another sex steroid receptor. For example, the action of estradiol in males was partially blocked by RU486; action of testosterone in males was blocked by tamoxifen and

RU486; and actions of progesterone in both sexes blocked by flutamide. These results are in agreement with the results from the radiolabeled ligand receptor binding studies which showed that at high concentrations, a sex steroid can compete for the receptor for another sex steroid to some extent. However, the exact mechanisms for each case may not be the same.

In male scallops, the inhibition of the actions of estradiol by RU486 may be explained by two possible mechanisms. First, it is possible that RU486 blocked the activation of estrogen receptor. RU486 has been demonstrated to have antiestrogenic activities in vertebrates (McDonnell and Goldman, 1994). Bakker *et al.* (1987) showed that estradiol-induced growth of human mammary cancer MCF-7 cells was abolished by RU486. Another possibility is that in males, estradiol also bound to progesterone receptor and elicited an additive effect on the top of that initiated by estrogen receptor. This agrees with the fact that activation of progesterone receptor by progesterone stimulated 5-HT-induced sperm release. In this case, RU486 could compete with estradiol for the same binding sites.

There are several mechanisms that may be responsible for the inhibition of the actions of testosterone in males by tamoxifen and RU486. First, testosterone may be converted into estradiol by aromatase in the tissue and a stimulatory effect could thus be elicited by binding of converted estradiol to estrogen receptor and can be blocked by tamoxifen. This conversion exists widely in vertebrates and also in some molluscs (De Jong-Brink *et al.*, 1981). Second, testosterone may elicit a stimulatory effect on sperm release by binding to the estrogen receptor or progesterone receptor. Tamoxifen and RU486 might inhibit binding of testosterone to the estrogen and

progesterone receptors and thus block the effects of testosterone. This possibility has been widely reported in vertebrates. For example, testosterone and 5α-dihydrotestosterone (DHT) were shown to induce estrogenic and progestagenic effects which can be blocked by antiestrogens and antiprogesterones, respectively, in human endometrial adenocarcinoma cells of the Ishikawa Var-1 line (Markiewicz and Gurpide, 1997). In human endometrial stromal cells, DHT induced the expression of plasminogen activator inhibitor I gene. This effect can be partially inhibited by an antiprogesterone and partially by an antiandrogen, indicating that this androgen exerts its action partially by the activation of progesterone receptor (Casslen *et al.*, 1992). In fish, the induced accumulation of vitellin in the liver by DHT can be inhibited by tamoxifen (Le Menn *et al.*, 1980). Third, RU486 may bind directly to androgen receptor and block the actions of testosterone. Similar binding has been shown in vertebrate cells (Kemppainen *et al.*, 1992; Hackenberg *et al.*, 1996).

Similarly, the blockade of actions of progesterone in males by flutamide can be explained by its binding to the androgen receptor. But this may not be the case with females, since activation of the androgen receptor did not have any effect on egg release. However, it is possible that flutamide acted as an antagonist to the progesterone receptor in both sexes. A similar action was demonstrated by Brann *et al.* (1989). They showed that inhibition of estradiol-induced prolactin release in rats by progesterone could be in turn blocked by flutamide.

Thus it is possible that the actions of estradiol, testosterone and progesterone are all achieved through the activation of sex steroid receptors. Cross-reaction may

also exist between steroids and their receptors, possibley because common co-activators are used by different steroid receptors (Tetel, 2000).

II-A-3. Possible contribution of membrane effects: As discussed in the Introduction, sex steroids may bind to a membrane component and elicit rapid membrane effects. Thus, membrane effects may have contributed to the overall effects noted in this thesis.

To examine this possibility, the time interval for steroid treatment was shortened to 30 minutes and 15 minutes. The results showed that the effects of estradiol in female and testosterone and progesterone in males persisted when the treatment time was shortened to 30 minutes. When the treatment time was further shortened to 15 minutes, the effects of all the steroids were abolished. This time scale should be enough for membrane effects to occur if there are any. However, the time may not be enough for sufficient diffusion of steroids into the tissue and thus no effects were elicited.

As discussed earlier in the Introduction, the time scale for the classical mechanism of steroids is usually hours while that for the membrane effects is from seconds to minutes. In these studies, those effects that were only observed when the gonad pieces were incubated with steroids for 2 hours are likely caused by the activation of intracellular receptors which involves protein synthesis. In other words, the classical mechanism may be dominant in the actions of estradiol in males and progesterone in females in the 2 hour treatment experiments. In contrast, membrane effects cannot be ruled out in the actions of estradiol in females and testosterone and progesterone in the males.

Membrane effects of sex steroids have been widely reported in vertebrate tissues such as neurons, oocytes, sperm, and epithelial cells (Frye *et al.*, 1992; Baulieu and Robel, 1995; Farhat *et al.*, 1996). These rapid actions of sex steroids have also been found in gastropod molluscs. Kavaliers (1999) demonstrated that 3α-hydroxy-5α-pregnan-20-one and progesterone had antinociceptive effects in the land snail, *Cepaea nemoralis*. Although no report is currently available on the membrane effects of sex steroids in bivalves, this possibility cannot be excluded. Thus, it is possible that sex steroids bind to neurotransmitter receptors or other membrane components and cause changes in the membrane properties, thus triggering gamete release.

II-A-4. Effective concentrations of the sex steroids in the *in vitro* actions: The effective concentration ranges (at which significant effects were observed) together with the optimal concentrations (at which maximum effects were observed) for the *in vitro* actions of sex steroids are summarized in Table 10. Generally, the effective concentration range is 10^{-5}-10^{-8} M for estradiol, 10^{-5}-10^{-9} M for testosterone, and 10^{-5}-10^{-8} M for progesterone. The optimal concentrations are either 10^{-6} M or 10^{-7} M. Similar effective concentrations were found in *in vitro* studies of other molluscs. For example, in the Japanese scallop, 10^{-5}-10^{-6} M estradiol significantly potentiated 5-HT-induced egg release (Matsumoto *et al.*, 1997); treatment of Japanese scallop ovary pieces with 10^{-6} M estradiol stimulated vitellogenesis (Li *et al.*, 1998). In *Sepia officinalis*, 10^{-7}-10^{-9} M progesterone significantly promoted polysaccharides synthesis in the main nidamental glands (Henry and Boucaud-Camou, 1994).

Table 10. Effective concentrations ranges of *in vitro* actions of sex steroids (Optimal concentrations are denoted in the parentheses)

Steroids	Female		Male	
	120 Min.	30 Min.	120 Min.	30 Min.
Estradiol	10^{-6}-10^{-7} (10^{-6})	10^{-6}-10^{-8} (10^{-7})	10^{-5}-10^{-7} (10^{-6})	na
Testosterone	na	10^{-5}-10^{-8} (10^{-5})	10^{-6}-10^{-8} (10^{-7})	10^{-6}-10^{-9} (10^{-7})
Progesterone	10^{-7}-10^{-8} (10^{-7})	na	10^{-5}-10^{-7} (10^{-7})	10^{-6}-10^{-7} (10^{-7})

These effective concentrations are much higher than the actual sex steroids levels observed in molluscs. The contents of estradiol in mature female gonad are 0.6-1.1 ng/g wet weight (estimated to be 2.4-4.4 × 10^{-12} M assuming a 90% water content in the wet body weight) in the Japanese scallops and 1.1-1.5 ng/g wet weight (4.4-6.1 × 10^{-12} M) in the Pacific oysters (Matsumoto *et al.*, 1997), and 0.95 ng/g wet weight (3.8 × 10^{-12} M) in the prostate of male *Octopus vulgaris* (D'Aneillo *et al.*, 1996). The contents of progesterone have been reported to be 4.1-36.6 ng/g dry weight (1.3-11.6 × 10^{-12} M assuming a 90% water content in the body) in the mussel *Mytilus edulis* (Reis-Henriques and Coimbra, 1990) and 4.8 ng/g wet weight (16.8 × 10^{-12} M) in the seminal vesicle of *Octopus vulgaris* (D'Aneillo *et al.*, 1996). Testosterone at a content of 5.2 ng/g wet weight (19.8 × 10^{-12} M) has been detected in the testis of *Octopus vulgaris* (D'Aneillo *et al.*, 1996). Because of partitioning of water across the cell membrane, the actual plasma concentrations of sex steroids may be a little higher than the above figures. The dissociation constants from the radioligand receptor binding studies may provide some clues for the estimation of physiological concentrations in these animals. The current study demonstrated that the dissociation constants for the estrogen binding sites are 0.52-1.82 nM in the sea scallop; D'Aneillo *et al.* (1996)

reported that the dissociation constant for estrogen binding sites was 1.84 nM, testosterone binding sites 0.5 nM and progesterone binding sites 1.6 nM in *Octopus vulgaris*. Together, these facts suggest that the physiological concentrations (the concentration for a physiological effect to occur *in vivo*) for sex steroids may be between 0.01-1 nM in animals, although there may be variations among steroids and with the sex and physiological status of the animals.

In the intact animals, transportation of sex steroids may be facilitated by proteins such as steroid hormone binding proteins. In contrast, in the *in vitro* experiments, sex steroids diffused into the tissue passively and thus a higher concentration may be required for this diffusion. This may partially explain why the effective concentrations observed in the current studies are higher than the concentrations of sex steroids detected in molluscs.

II-A-5. Parthenogenetic embryos: In these studies, parthenogenetic embryos were observed in the groups that were treated with 5-HT alone or in combination with estradiol or progesterone. Sex steroids do not seem to contribute to the activation of eggs because a similar percentage of parthenogenesis also occurred in the group that were treated with 5-HT alone. It is possible that 5-HT is contributing to the stimulation of parthenogenesis, although not enough eggs were obtained in the non-treatment control group to allow for a statistically meaningful comparison.

Artificial induction of parthenogenesis has been reported in bivalves by treatments with heat, hypertonic seawater, KCl, $CaCl_2$ or other polar body inhibition agents such as cytochalasin B and caffeine (Guo *et al.*, 1991; Scarpa *et al.*, 1992). However, the activated eggs either did not develop or developed into abnormal

ciliated non-cellular masses (Scarpa *et al.*, 1992). The embryos obtained in the current studies seemed to have developed normally, although their occurrence was low.

Serotonin has been known to induce maturation of bivalve oocytes by re-initiation of meiosis (Ram *et al.*, 1992; Deguchi and Osanai, 1995; Moreau *et al.*, 1996). The most readily seen phenomena are germinal vesicle breakdown (GVBD) in some species and polar body emission in others. My results are consistent with these actions of 5-HT. Thus, it is possible that parthenogenesis in the current studies was induced by 5-HT. This finding may have potential application in bivalve aquaculture in efforts to produce lines through uniparental inheritance or mono-sex animals.

II-B. *In Vivo* Induction of Spawning by Sex Steroids

In my *in vivo* experiments in which sex steroids were injected into the gonads of the scallops, I found that administration of estradiol to both sexes and testosterone to males directly induced spawning. Injection of these steroids not only increased the spawning intensity, but also shortened the response time (spawning latency) of the scallops.

The mechanisms underlying these actions are currently not known. But from the time scales of the response, especially those for the males, it is possible that the membrane mechanism was involved. In these experiments, some males started spawning almost immediately after steroid injection (2 minutes). This time interval was obviously too short for a genomic effect but enough for membrane effects to occur. Thus, it is possible that sex steroids stimulated membrane effects in the nerves of the gonad, causing contractions of perigonadal muscles and resulting in spawning.

It is also possible that the sex steroids elicited membrane effects in the muscle cells and directly stimulated spawning.

In agreement with the results from my *in vitro* experiments, I have also shown that sex steroids had effects on 5-HT-induced spawning in my *in vivo* experiments. Injection of estradiol promoted 5-HT-induced spawning in both sexes; testosterone potentiated spawning in males only; and progesterone had different effects on 5-HT-induced spawning: it inhibited spawning in females while potentiating spawning in males.

In mature scallops, spawning can be triggered by many factors such as injection operation, water flow, or a temperature change. We have observed that injection of vehicle only induced male scallops to spawn, and this induction may have contributed to the overall effects. However, my results showed that injections of most steroids further increased spawning intensity and shortened spawning latency while injection of progesterone in females had opposite effects. These results suggested that sex steroids have specific effects on spawning induction.

Although some effects are similar in both *in vitro* and *in vivo* experiments, the mechanisms responsible for the *in vitro* and *in vivo* effects may be different. As discussed before, in the *in vitro* effects the classical mechanism may be involved while the membrane mechanisms may have contributions. In the *in vivo* experiments, since sex steroids were injected 24 hours before 5-HT was injected, the membrane mechanisms do not seem to be contributing to the overall actions. Therefore it seems that the classical mechanism may be the main cause for these actions. However, since we know very little about the actions of sex steroids in invertebrates and our

knowledge about membrane effects are mainly from vertebrates, the possible contribution of long-lasting membrane effects can not be excluded.

Spawning is a different process from gamete release. As discussed earlier, gamete release may involve breakdown of acini and the discharge of gametes from the gonad pieces. In contrast, spawning involves the acini, the nerves, and the spawning-related muscles in the gonad. Besides the mechanisms for *in vitro* gamete release, stimulation of nerves, contraction of muscles and discharge of gametes occur during spawning. Steroids may act on any of the events to affect spawning.

The findings that estradiol could further increase the spawning induced by 5-HT in females and that testosterone and progesterone could potentiate the spawning in males may be readily applied in the production of bivalve seed. Serotonin has been widely used to induce spawning in bivalves (Matsutani and Nomura, 1982; Gibbons, 1984; Braley, 1985; Tanaka and Murakoshi, 1985; Matsutani and Nomura, 1987; Unnasch *et al.*, 1999). In the practice of bivalve aquaculture, availability of brood stocks may be a limiting factor so that it is often important to obtain as many gametes as possible from individuals. This is even more critical for a genetic breeding program in which enough offspring have to be obtained from a pair of brood stocks. But serotonin does not induce spawning in all the bivalve species tested and very often it only induces male animals to spawn (Citter, 1985; Tanaka and Murakoshi, 1985; Martinez *et al.*, 1996; Martinez and Olivares, 1999). Sex steroids provide alternative spawning inducers, especially when combined with serotonin. These strategies have been tested in a commercial hatchery and resulted in a close to 2-fold

increase in spawning of female scallops in a genetic breeding project (Mallet, personal communication).

III. Characterization of Estrogen Receptor in the Sea Scallop:

I have characterized estrogen binding sites in the cytosolic and nuclear fractions of both female and male scallop gonads. Two populations of estrogen binding sites, one with high and another with low affinities, were observed in all these preparations. These sites resemble their vertebrate counterparts in terms of binding affinities, specificity, and their presence in both the cytosolic and nuclear fractions.

III-A. Existence of Classical Estrogen Binding Sites:

High affinity binding sites have been characterized in the cytosolic and nuclear fractions of both sexes of mature sea scallops. The dissociation constants (K_d) of the high affinity sites in the cytosolic fractions in females and males were very close, 0.52 nM in the female cytosol and 0.57 nM in the male cytosol, respectively. Similarly, the dissociation constants of the high affinity sites in the nuclear fraction were also close in females and males, i.e., 1.71 nM in the female nuclear fraction and 1.82 nM in the male nuclear fraction, respectively. These values are generally within the range of the parameters for vertebrate estrogen receptors (Table 11).

It is generally believed that the estrogen binding sites in what are referred to as the cytosolic and nuclear fractions both originated from the nucleus but they represent unbound and bound receptors, respectively (Yamashita, 1998). During the preparation of the cell fractions, the unbound receptor proteins are extracted with the

Table 11. Comparison of Parameters of Estrogen Receptors

Species	Tissues	Fraction	Kd (nM)	Bmax (fmol/mg protein)	References
Sea scallop (*Placopecten magellanicus)*	Ovary Testis	Cytosol Nuclei Cytosol Nuclei	0.52 1.71 0.57 1.82	62.6 174.3 38.3 64.0	Present studies
Octopus vulgaris	Testis	Cytosol	1.84	0.003±0.001	D'Aniello *et al.*, 1996
Sea Star (*Asterias rubens*)	Female pyloric caeca	Cytosol	0.23±0.03	17.8±0.9 (fmol/ml cytosol)	De Waal *et al.*, 1982
Human	Prostate	Cytosol Nuclei	0.1 0.1		Ekman *et al.*, 1983
Rat	Ovary	Whole	0.6		Kuiper *et al.*, 1996
Chick	Oviduct	Nuclear	0.1	35.0	Kon *et al.*, 1980
Japanese Quail	Liver	Nuclei	0.2		Turner, 1984
African claw frog (*Xenopus laevis)*	Male liver Female liver	Cytosol Nuclei	22.4±6.0 15.0±2.8	89.0 136.0	Lutz & Kloas. 1999
Frog (*Rana esculenta)*	Testis	Cytosol Nuclei	1.94±0.43 2.72±1.20	8.0 9.0	Fa sano *et al.*, 1989
Turtle (*Chrysemys picta)*	Testis	Whole	0.8 0.7	20.0 1.0-4.0	Dufaure *et al.*, 1983 Mark *et al.*, 1983
American alligator	Oviduct	Whole	0.5		Vonier *et al.*, 1997
Largemouth Bass (*Micropterus salmoides)*	Female liver (ripe)	Cytosol Nuclei	1.0 1.0	23.4 37.0	Garcia *et al.*, 1997
Atlantic croaker (*Micropogonias undulatus)*	Testis	Cytosol Nuclei	0.4 0.33		Loomis & Thomas (1999)
Sea Lamprey (*Petromyzon marinus)*	Testis	Cytosol Nuclei	0.52 0.39	56.0 68.0	Ho *et al.*, 1987

low ionic strength homogenization buffer, however, the bound receptors can only be extracted with the high ionic strength extraction buffer (homogenization buffer plus 0.6 M KCl). The reason why the binding affinities in the nuclear fractions are lower than those of cytosolic factions may be because of the existence of a co-factor in the cytoplasm but absent in the nuclear fraction (Clark *et al.*, 1988).

The binding capacities for these estrogen binding sites have also been characterized. The maximum binding capacities (B_{max}) for the cytosolic fractions were 62.57 fmol/mg protein in females and 38.30 fmol/mg protein in males, while those of the nuclear fractions were 174.3 fmol/mg protein in females and 63.97 fmol/mg protein in the males. The facts that these sites have high affinity and limited binding capacity are in agreement with the characteristics of the classical steroid receptors in vertebrates.

It is of interest to note that the binding capacity, i.e., the number of the binding sites, is higher in females than in males in both the cytosolic and nuclear fractions. This is in agreement with the fact that the concentration of estrogens in gonads is generally higher in females than that in males (Matsumoto *et al.*, 1997). It is well known that the estrogen receptor in vertebrates is itself an estrogen-responsive protein, *i.e.*, the synthesis of the estrogen receptor can be induced by estrogens (Clark and Peck, 1979). Thus, it is not surprising to see a low concentration of the estrogen receptor in a tissue in which the estrogen content is low.

Another interesting phenomenon is that, in both sexes, the binding capacity was higher in the nuclear fraction than in the cytosolic fraction. The animals used for the saturation analyses were very ripe and estrogen levels are known to be highest at

this point during the sexual maturation cycle (Matsumoto *et al.*, 1997). It is known that estradiol binding causes retention of the estrogen receptors in the nuclear fraction during the preparation of cell extracts. Thus this phenomenon suggests that in ripe scallops, there are more bound receptors than unbound ones. The changes in the binding capacity of estrogen binding sites between the cytosolic and nuclear fraction may reflect the physiological status elicited by estradiol during the maturation cycle.

Competitive studies showed that these sites are very specific for natural and synthetic estrogens, although progesterone and testosterone, at very high concentrations, can also compete for these sites. The relative affinities for sex steroids in all the fractions are in the same order, that is DES > estradiol >> progesterone ≥ testosterone. Similar binding affinity order has also been observed in other vertebrate estrogen receptors (Notides, 1970; Colburn and Buonassisi, 1978).

These estrogen binding proteins could not have been the steroid hormone binding globulin (SHBGs) from the haematocytes, if they are present in molluscs, because SHBGs usually bind equally to estradiol and testosterone (Rosener *et al.*, 1991). In fact, the rinsing of the minced tissue with the homogenizing buffer before homogenization should essentially remove most of the haematocytes in the preparation. Based on the binding affinity and specificity characteristics, it is obvious that these sites belong to the classic, or Type I estrogen receptor (Clark and Peck, 1979).

III-B. Are the Low Affinity Binding Sites the Type II Estrogen Binding Sites?

In these studies, I have also observed secondary low affinity binding sites in the cytosolic and nuclear fractions of both sexes. The secondary binding sites were characterized in the nuclear fractions of both sexes. The dissociation constants for the low affinity sites were 31.84 nM for female nuclei and 25.51 nM for male nuclei. The maximum binding capacity for the in female nuclei was 1.12 pmol/mg protein and that for male nuclei was 0.29 pmol/mg protein. Since the concentration range of [^{3}H] estradiol utilized in the saturation analyses was limited (0.5 nM to 20 nM), it was not sufficient to accurately characterize the second sites in the cytosolic fractions of both sexes based on these data. In order to characterize the secondary sites in the cytosolic fractions, the highest concentration should be at least doubled.

It is possible that the secondary binding sites characterized in these studies are counterparts of type II estrogen receptors in vertebrates because they all have medium affinity to estrogens, i.e., a K_d within the range of 30-50 nM, and a high binding capacity. Existence of a Type II estrogen receptors has been reported in many vertebrates (Lopes *et al.*, 1987; Kornyei *et al.*, 1993; Yamamoto *et al.*, 1996). Type II cytosolic sites, unlike Type I sites, do not translocate into the nucleus upon binding estrogens and thus may not be the precursors of Type II nuclear sites (Clark and Peck, 1979). Their primary function may be to concentrate estrogens and maintain high availability in the cytoplasm. The functions of type II nuclear sites are not known.

III-C. Possible Involvement of Estrogen Receptors in Sexual Maturation

As discussed in section III-A, the changes in the binding capacity between the cytosolic and nuclear fractions may reveal the effects of estrogens during sexual

maturation. To investigate this hypothesis, I compared the binding capacity of the cytosol and the nuclear fractions between spent and ripe female scallops. No significant difference in the binding capacity of the cytosol was found between spent scallops and ripe scallops. However, the binding capacity of the nuclear fraction in ripe scallop gonads was much higher than in spent ones. This may be the direct consequence of activation and retention of the estrogen receptors in the nuclear fraction. These results suggested that estrogens and estrogen receptors might be involved in sexual maturation in scallops.

We then examined the distribution of estrogen bindings in the cytosolic and nuclear fractions in scallops at different stages of sexual maturation cycle. I have tried to compare the total binding in the cytosolic and nuclear fractions, but because of the large variations in the total binding among individuals, the relative distribution of estrogen binding sites between the cellular fractions were compared. The results showed that, in both sexes, the ratio of binding capacity of the cytosol to that of the nuclei (C/N ratio) was low when the GSI was low. A rapid increase in the C/N ratio was observed with the increase of GSI when gametogenesis was initiated. This was followed by a continuous decrease in the C/N ratio with sexual maturation. The ratio reached its lowest value when the scallops were ripe.

Changes in the C/N ratio presumably reflect the dynamics of synthesis and activation of estrogen receptors in the animals. Therefore, these results may indicate that, at the beginning of gametogenesis, synthesis of estrogen receptor was very active. Since the estrogen level at this stage was relatively low, most of the synthesized estrogen receptor remained unbound in the animal, thus leading to a high

C/N ratio. Due to the increases in the estrogen concentration with the progress of sexual maturation, more and more estrogen receptor molecules were activated, resulting in a high estrogen binding in the nuclear fraction. Thus, although estrogen receptors may have been continuously synthesized in the cytoplasm, the C/N ratio decreased. The C/N ratio reached its lowest value before spawning when the cytosolic estrogen level was the highest in the cycle. These results suggest that the synthesis and activation of estrogen receptor could have been involved in the process of sexual maturation, especially in the initiation of gametogenesis, in both sexes.

In general, it appears that the profiles of the synthesis and activation of estrogen receptors, as well as the levels of estrogens, correlate well with the process of sexual maturation in scallops. Levels of progesterone have also been reported to be correlated with reproductive cycle in the mussel *Mytilus edulis* (Reis-Henriques *et al.*, 1990), thus it is possible that progesterone receptors are also involved in the reproductive control in molluscs.

III-D. Estrogen Receptors Might Function in Male Scallops

So far I have shown that a considerable quantity of estrogen binding sites exist in the male scallop gonad. These results suggest that they may play roles in the reproduction of males. Although the concentrations of both estrogen receptors and estrogens are low in males, it is not safe to state that estrogen and estrogen receptor play lesser important roles in males than in females of molluscs. In mammals, activation of estrogen receptor is necessary for the production of sperm in males. It has been reported that the interaction of estrogen receptors and estrogens is essential for the initiation of spermatogenesis and absorption of epididymis fluid in mammals

(Hess, 1997; Hess, 2000). A similar effect has also been reported in molluscs. Varaksina *et al.* (1992) showed estradiol stimulated spermatogenesis in the scallop *Mizuhopecten yessoensis.* Administration of estradiol also accelerated spermatogenesis in other molluscan species such as *Helix pomatia* (Csaba *et al.*, 1979, 1981). It is possible that these actions were achieved through the activation of estrogen receptors in these molluscan species.

In these studies, I have demonstrated that estradiol has effects in male scallops. I have shown that injection of estradiol into undifferentiated juvenile scallops resulted in more males than females. In the *in vitro* experiments, I demonstrated that estradiol promoted sperm release induced by 5-HT treatment and this effect can be inhibited by tamoxifen, an antiestrogen, suggesting the involvement of estrogen receptors. I further showed that estradiol directly stimulated spawning or indirectly potentiated 5-HT-induced spawning in male scallops. These results are consistent with the presence of estrogen receptors in male gonad preparations. Moreover, in studies of the distribution of estrogen bindings between the cytosolic and nuclear fractions, my results suggested that synthesis and activation of estrogen receptors might be involved in sexual maturation of males. Together, the results from the current studies indicate that estrogen may regulate male reproductive activities through the activation of estrogen receptors.

IV. Estrogen Receptor Gene in the Sea Scallop

A partial sequence of 710 bp was cloned by RT-PCR from an RNA preparation of sea scallop gonads. Amino acid sequence similarity comparisons

showed that this cloned fragment is 57.6% similar to the corresponding region of human estrogen receptor, 59.3% to that of the chick, 53.8% to that of the African clawed frog, and 92.3% to that of the rainbow trout (see Figure 54). As can also be seen from the same figure, this sequence has particularly high similarity with estrogen receptors from vertebrates in the functional domains, the DNA binding domain and the steroid binding domain. These results suggested that the protein encoded by the entire gene might be able to bind both estrogens and the estrogen responsive element (ERE). Since only a part of the ligand binding domain is cloned, not enough date is available to predict the binding specificity at the present time. Thus, it is possible that the cloned sequence is a partial sequence of the estrogen receptor.

This possibility is re-enforced by the recently finished fruit fly genome (http://www.ncbi.nih.gov/). A sequence was retrieved using the online BLAST similarity search tool (http://www.ncbi.nih.gov/) with the human estrogen receptor gene as a probe. Over its full length, the derived estrogen receptor protein sequence from the fruit fly has 34% identical amino acids with human estrogen receptor and most of the similarities were found in the DNA and estrogen binding domains (Figure 57), suggesting that this sequence may be similar to the putative ancestor gene for vertebrate estrogen receptors. The cloned putative scallop estrogen receptor gene has 29.9% identity in the amino acid sequence compared with the corresponding region of the fruit fly sequence. Although more studies are still needed to prove that this sequence encodes a functional estrogen receptor in the fruit fly, this finding lends support to my results.

Together, these findings provide useful clues to an dispute related to the evolution of the estrogen receptor. As discussed in the Introduction, an estrogen-like ancestor sex steroid receptor has been hypothesized to emerge in some invertebrates. However, there have been disputes over when this receptor occurred. Baker (1997) proposed that the receptor could not have evolved in invertebrates earlier than tunicates or *Amphioxus*, that is, before the second major duplication of the genome occurred. But Laudet (1997) argued that the diversification of nuclear receptors may have occurred early, when metazoan phyla emerged. The results from my study and from the studies of the fruit fly appear to support the latter hypothesis.

To demonstrate that the presumed estrogen receptor gene was expressed in the scallops, Northern blotting was performed using the identified sequence as a probe. Hybridization of total RNA preparations from both the gonad and liver of female sea scallops with such a probe revealed a 3.1 kb band. However, no band appeared in the RNA preparation from adductor muscle.

The finding that the putative estrogen receptor seemed to be expressed in the gonad agrees with the evidence that the gonad is responsive to estradiol treatments. In the current and previous studies, estradiol has been suggested to be involved in gonadal activities, such as sex determination, sexual differentiation and maturation, gamete release and spawning, 5-HT synthesis and vitellogenesis (Osada *et al.*, 1992; Matsumoto *et al.*, 1997; Li *et al.*, 1998).

The results also suggest that the liver may be a target organ for estrogens. This result is in agreement with the presence of estrogen binding sites in the liver as demonstrated in the current study. Metabolism of glycogen, lipids and proteins in the

```
          1          11         21         31         41         51
Scallop   MCPATYQCTMDRNRRKSCQACRLRKCYEVGMVKGGLR--KVPGGRVLRKDKRYCGPAGDR
           CPA  +C +++ RRK+CQACR +KC  +GM+K G+R  +V GGR   +
Fly       TCPANNECEINKRRRKACQACRFQKCLLMGMLKEGVRLDRVRGGRQKYRRNPVSNSYQTM
           CPA N+C I+K RRK+CQACR +KC  +GM+K G+R DR RGGR K++R
Human     MCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDR-RGGRMLKHKRQRDDGE---

          61         71         81         91        101        111
Scallop   EKTYGD---------------------LEHRTAPPQ---DGGRNSSSSLNGGGGWRGPRI
          +  Y                      L  +T PPQ       N  +            +
Fly       QLLYQSNTTSLCDVKILEVLNSYEPDALSVQTPPPQVHTTSITNDEAXXXXXXXXXXXXV
                    S  D++   +     P  L ++          S+T D+
Human     ---GRGEVGSAGDMRAANLW----PSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILY

          121        131        141        151        161        171
Scallop   TMPPEQVLFLLQPAEPPALCSRQKVARPYTEVTMMTLLTSMADKELVHMIAWAKKVPGFQ
            P    +F       P                +++++L+ + DKELV +I WAK++PGF
Fly       VTPNGTCIFQNNNNNDPNE--------------ILSVLSDIYDKELVSVIGWAKQIPGFI
             + T  F   +                     ++  L+++ D+ELV +I WAK++PGF+
Human     SEYDPTRPFSEAS--------------------MMGLLTNLADRELVHMINWAKRVPGFV

          181        191        201        211        221        231
Scallop   ELSLHDQVQLLESSWLEVLMIGLIWRSIHCPGKLIFAQDLILDRS-EGDCVEGMAEIFDM
          +L L+DQ++LL+ SW E+L + L +RS+   GKL FA D+ +D     +C  G  E +
Fly       DLPLNDQMKLLQVSWAEILTLQLTFRSLPFNGKLCFATDVWMDEHLAKEC--GYTEFYYH
          DL L+DQ+ LL+ +W EIL + L +RS+   GKL FA ++ +D +  K C  G  E +
Human     DLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGK-CVEGMVEIFDM
          241        250
Scallop   LLATVSXFRMLKLKPEEY   258
           +         +  + EEY
Fly       CVQIAQRMERISPRREEY   258
           +  + R   ++ + EE+
Human     LLATSSRFRMMNLQGEEF   258
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Note: Amino acids 1-32 in the human estrogen receptor is a part of the DNA-binding domain; amino acids 33-102 is the hinge of the human estrogen receptor; and amino acids 103-158 is a part of the estrogen-binding domain of the human estrogen receptor.

Figure 57. Similarity comparison of estrogen receptor proteins from the human and the sea scallop with the putative estrogen receptor protein from the fruit fly.

liver may be under the control of estradiol. As discussed in the Introduction, the activity of glucose-6-phosphate dehydrogenase (G-6-DP), the critical enzyme for utilization of glycogen, may be under the control of estradiol in the liver of oysters (Mori *et al.*, 1972a, 1972b). Another possible activity that may be controlled by estrogens in the liver is vitellogenesis. It is well known that in fish vitellogenesis in the liver is induced by estradiol injection (Callard *et al.*, 1991; Macky and Lazier, 1993; Nagahama, 1994; Macky *et al.*, 1996). However, no study has ever been performed to investigate the similar actions of estradiol in molluscs. Li *et al.* (1998) showed that estradiol stimulated vitellogenesis in the gonad, but they did not examine the levels of vitellin-like proteins in the liver. Thus the possibility that estrogens control vitellogenesis in the liver can not be excluded.

Estrogen receptor mRNA was not expressed in the adductor muscle, suggesting that the adductor muscle may not be a target tissue for estrogens. This is also in agreement with the fact that the adductor muscle mainly serves as a site for nutrients and energy storage for reproduction.

The size of the estrogen receptor mRNA is smaller than most estrogen receptors found in vertebrates. For example, the sizes of the major form of estrogen receptors are 7.6 kb in the zebra finch (Jacobs *et al.*, 1996), 6.5 kb in the mouse (Hillier *et al.*, 1989; Ikegami *et al.*, 1993), 6.3 kb in humans (Piva *et al.*, 1993), and 3.5 kb in the rainbow trout (Pakdel *et al.*, 1989). Other less expressed forms of estrogen receptors may have larger or smaller size due to alternative splicing, breakdown of the message, or variations in the lengths of 3′ or 5′ end untranslated

sequences. We are not clear whether the variation in the size of this gene has any evolution implication.

The above results indicated that an estrogen receptor gene might exist in the sea scallop, *Placopecten magellanicus*. This finding agrees well with other results of the current studies. I have shown that estradiol stimulated gonadal differentiation, affected sex determination, and caused morphological changes in juvenile scallops. Estradiol potentiated gamete release in both sexes and these actions were possibly mediated by the estrogen receptors because they can be inhibited by pre-treatment with an antiestrogen as well as RNA and protein synthesis inhibitors. Estradiol also potentiated 5-HT-induced spawning *in vivo* and as discussed earlier, these effects could have been achieved through the classical mechanism in which activation of estrogen receptors is involved. Furthermore, my results from radioligand binding assays provided strong evidence for the existence of such an estrogen receptor in the sea scallop. This is also the first sex steroid receptor gene ever cloned from an invertebrate species so far.

Because the cDNA sequence I cloned is very similar to the estrogen receptor gene from rainbow trout, there could be concerns that it may be cloned due to a contamination in RT-PCR. However, the result from Northern blotting indicated that the cloned gene exists in the gonad and liver of the sea scallop. Even if the sequence were cloned due to contamination, the result from Northern blotting still indicated that a gene similar to vertebrate estrogen receptor gene exists in the sea scallop, since the hybridization of Northern blotting was performed at quite high stringency (65 ^{o}C).

V. Future Directions

Results from the current and previous studies suggest that sex steroids may play important roles in molluscan reproductive control and some mechanisms of sex steroids common in vertebrates may also function in molluscs. However, compared to our knowledge in vertebrates, we know very little of the actions of sex steroids in molluscs.

I have indicated in this study that estrogen receptor gene may exist in molluscs. Thus cloning of its full-length sequence is necessary to prove its presence with certainty. Cloning of this gene will also provide us more information for examination of the evolution and origin of estrogen receptor throughout the animal kingdom. Expression of the gene and functional studies of the protein will help better characterize this estrogen receptor. Upon expression of the protein, radioligand binding studies of this protein will provide us with useful information to determine whether it is a functional estrogen receptor that binds estrogens specifically. It will also be very useful to raise an antibody against this protein. This antibody can then be used for Western blotting or immunohistological studies to determine the distribution and patterns of the estrogen receptor in different tissues or organs. These studies will further our understanding of the mechanisms of sex steroids in invertebrates.

Efforts should also be devoted to the actions of sex steroids in molluscs. Due to the apparent differences between vertebrates and invertebrates, distinct effects of sex steroids are expected in invertebrates. I have provided some primary results on this topic, but more studies are required to help us fully understand the actions of sex steroids in invertebrates. Some major questions to be answered regarding sex steroids

and their receptors in invertebrates may include 1) What are the major roles of sex steroids in invertebrates? Do they have actions in somatic growth or CNS plasticity? Do they have effects in organogenesis and early development? 2) Are there specific membrane sex steroid receptors in molluscs? 3) If their major functions are sex-related, what are the mechanisms of their reproductive regulation? Are these functions achieved through the interactions between sex steroids and the other endocrine factors? Or in other words, are these primary or secondary actions? Answers to these questions might provide us more insights into the evolution of sex steroid receptors throughout the animal kingdom. They may also imply new potential application of sex steroids in aquaculture and the environmental impacts of endocrine-disruptive substances which induce similar actions as sex steroids. For example, since testosterone seems to have very specific effects in males at nearly physiological concentrations, it is possible that testosterone also has other physiological functions such as regulation of sexual maturation cycles in males. It might also affect vitellogenesis in molluscs as was observed in fish (Lazier et al., 1996). It will also be interesting to examine if sex steroid treatment can control sex of animals at larval stages or stimulate sexual maturation in adults. Study of estrogen receptors in molluscs may also provide a way to assess the impact of endocrine disruption chemicals in these animals.

Appendix 1: Recipes for Buffers and Solutions

A. Solutions for Histological Staining:

1. Ethanolic Bouin's solution:

150 ml 80% ethanol
60 ml 37% formaldehyde
15 ml glacial acetic acid
1 ml picric acid

2. Mayer's Hematoxylin:

1 g hematoxylin
1000 ml distilled water
0.2 g sodium iodate
50 g ammonium alum
1 g citric acid
50 g chloral hydrate

3. Scott's tap water:

2 g sodium bicarbonate
20 g magnesium sulfate
1000 ml distilled water

4. Ethanolic Eosin Y solution:

Stock solution:

1 g water soluble Eosin Y
20 ml distilled water
80 ml 95% ethanol

Working solution:

1 part Eosin stock solution
3 parts 80% ethanol
add 0.5 ml glacial acetic acid to per 100 ml working solution before use

B. Solutions for Receptor Binding Assays

1. Homogenization buffer:

40 mM Tris-HCl, pH 7.4
1 mM EDTA
1mM mercaptoethanol
30% glycerol

2. Extraction buffer:

0.6 M KCl in homogenization buffer

3. Washing buffer:

10 mM Tris-HCl
3 mM $MgCl_2$
0.25 mM sucrose

4. 0.5% Dextran-coated charcoal (DCC) solution:

0.5% Norit-A Charcoal
0.05% dextran T-70

C. Solutions for Molecular Cloning

1. 10×PCR buffer:

0.75 M Tris-HCl, pH 8.8
0.20 M $(NH_4)_2SO_4$
0.1% Tween 20
25 mM $MgCl_2$

2. TE buffer:

1 M Tris-HCl
0.5 M EDTA, pH 8.0

3. LB Agar:

10 g NaCl
10 g tryptone
5 g yeast extract
20 g agar
add distilled water to 1 liter, pH 7.0

4. LB broth:

10 g NaCl
10 g tryptone
5 g yeast extract
add distilled water to 1 liter, pH 7.0

D. Buffers for Northern Blotting

1. Formaldehyde gel loading buffer:

50% glycerol
1 mM EDTA, pH 8.0
0.5% bromophenol blue

2. Gel running buffer:

0.1 M MOPS

0.04 M sodium acetate
5 mM EDTA
adjusted to pH 7.0 with NaOH, sterize by filtration with a 0.22 μm filter

3. Pre-hybridization buffer:

5× Denhardt's
5 × SSPE
0.2% SDS
50 μg/ml denatured herring sperm DNA

4. Hybridization buffer:

50% formamide
5 × Denhardt's
5 × SSPE
0.2% SDS

5. 50× Denhardt's:

10 g Ficoll
10 g polyvinylpyrrolidone
10 g bovine serum albumin
add distilled water to 1 liter

6. 20 × SSC:

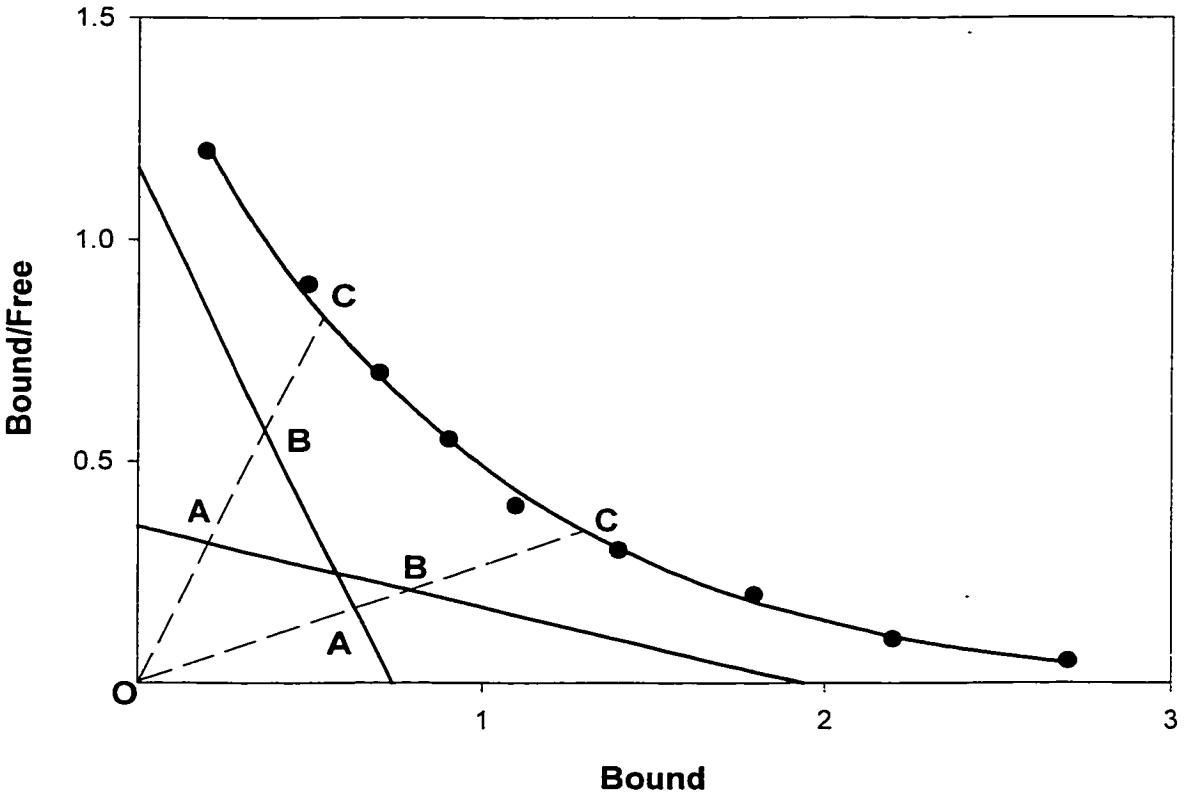
175.3 g NaCl
88.2 g sodium citrate
800 ml distilled water
adjust pH to 7.0 with 10.0 N NaOH
adjust to 1 liter with distilled water

7. 20 × SSPE buffer:

3 M sodium chloride
20 mM EDTA
1 M Tris HCl, pH 8.00

Appendix 2: Graphical Resolution of Multiple Binding Sites

Resolution of multiple binding sites by graphical analysis of Scatchard plot. Two straight lines are found so that for any vector from the origin, OA + OB = OC. The slope of the line equals to the dissociation constant (K_d) and its x-intercept equals to the maximum binding capability (B_{max}).



Appendix 3. Taxonomy of Animal Kingdom

Taxonomy of animals (fossil groups not included) used in Zoological Record, from website: http://www.biosis.org

Protozoa (Phylum)
Porifera (Phylum)
Archaeocyatha (Phylum)
Coelenterata (Phylum)
Ctenophora (Phylum)
Echinodermata (Phylum)
Platyhelminthes (Phylum)
Nematoda (Phylum)
Acanthocephala (Phylum)
Mesozoa (Phylum)
Nematomorpha (Phylum)
Nemertinea (=Rhynchocoela) (Phylum)
Annelida (Phylum)
Rotifera (=Rotatoria) (Phylum)
Cephalorhyncha (Phylum)
Chaetognatha (Phylum)
Cycliophora (Phylum)
Echiura (Phylum)
Gastrotricha (Phylum)
Gnathostomulida (Phylum)
Kinorhyncha (Phylum)
Lobopodia (Phylum)
Loricifera (Phylum)
Placozoa (Phylum)
Pogonophora (Phylum)
Priapulida (Phylum)
Sipuncula (Phylum)
Vestimentifera (Phylum)
Brachiopoda (Phylum)
Bryozoa (=Ectoprocta; Polyzoa) (Phylum)
Entoprocta (=Kamptozoa) (Phylum)
Mollusca (Phylum)
Aplacophora (Class)
Polyplacophora (Class)
Monoplacophora (Class)
Gastropoda (Class)

Prosobranchia (Subclass)
Heterobranchia (Subclass)
Opisthobranchia (Subclass)
Gymnomorpha (Subclass)
Pulmonata (Subclass)
Archaeopulmonata (Order)
Basommatophora (Order)
Stylommatophora (Order)
Cephalopoda (Class)
Nautiloidea (Subclass)
Coleoidea (Subclass)
Bivalvia (Class)
Protobranchia (Subclass)
Pteriomorphia (Subclass)
Palaeoheterodonta (Subclass)
Heterodonta (Subclass)
Anomalodesmata (Subclass)
Scaphopoda (Class)

Onychophora (Phylum)
Pentastomida (Phylum)
Arthropoda (Phylum)
Hemichordata (Phylum)
Phoronida (Phylum)
Chordata (Phylum)
Urochordata (Subphylum)(=Tunicata)
Cephalochordata (Subphylum)(=Acrania)
Vertebrata (Subphylum)
Pisces ("Group")
Amphibia (Class)
Reptilia (Class)
Aves (Class)
Mammalia (Class)

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