PUBERTY IN AN EARLY-MATURING FLATFISH, THE YELLOWTAIL FLOUNDER, Limanda ferruginea STORER

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Puberty in an Early-Maturing Flatfish, the Yellowtail Flounder, *Limanda ferruginea* Storer.

by

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ABSTRACT

A description of puberty at the gonadal level, and investigations into its regulation and manipulation were undertaken in order to examine the maturation processes of the yellowtail flounder under culture conditions. Puberty in males occurred after one year of age. Male puberty was noted by the detection of plasma androgens during a novel testicular growth phase in which mitosis and meiosis were concurrent. Female pubertal age was plastic; maturation occurred in 1+, 2+ and rarely 3+ animals. Culture conditions promoting 0+ growth encouraged puberty in 1+ females. Immature ovaries in primary growth stages were steroidogenic and responsive to *in vitro* gonadotropic stimulation. The onset of puberty was detected by a plasma 17ß-estradiol peak in cortical alveolar stage females prior to vitellogenin incorporation. Mature gametes were produced within a year of the initiation of puberty in both sexes. Females became superior in body size when males underwent testicular maturation; an effect of elevated androgens on male growth was suggested.

Hormones with reputed dual roles in growth and reproduction were administered long-term in immature females and mature males. Treatment with a gonadotropin-releasing hormone analogue (GnRH-a) failed to advance puberty in females but synchronized the onset of puberty among treated subjects relative to controls. GnRH-a did not accelerate testicular recrudescence and had no effect on growth. High levels of testosterone, whether alone or in combination with GnRH-a, suppressed growth and early gametogenesis in both sexes. A recombinant bovine growth hormone (rbGH) formulation (Posilac®) stimulated growth in males and females, reducing sex differences in body size. A positive rbGH effect on testicular mass was attributed to enhanced body size. Growth stimulation by rbGH delayed

the onset of puberty in some females, presumably due to an increased utilization of energy reserves.

Inducing triploidy was examined as a maturation deterrent. A ten minute hydrostatic pressure treatment of 7 000 psi, initiated five minutes post-sperm activation (7-12°C), is recommended to induce high percentages (92-100%) of triploid larvae. Premetamorphic growth of triploid larvae was inferior to that of diploid larvae. Later, 2+ triploid females exceeded growth rates of maturing 1+ diploid females. Triploidy minimized gonadal development but did not prevent the production of gametes in either sex.

Yellowtail flounder demonstrated a propensity for early sexual maturation under culture conditions. Management of dietary fat levels, the therapeutic use of rbGH, and the production of triploids are proposed as measures which, alone or in combination, may help to suppress puberty in this pleuronectid flatfish.

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Reference

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List of Abbreviations and Symbols

2N. . . . . diploid

3N. . . . . triploid

ANOVA. . . . . analysis of variance

ANCOVA. . . . . analysis of covariance

BPG axis. . . . . Brain-Pituitary-Gonadal Axis

BSS. . . . . balanced salt solution

CA. . . . . cortical alveolar stage

CNR/cnr. . . . . circumnuclear ring

CPE. . . . . crude pituitary extract

E2. . . . . 17ß-estradiol

GABA. . . . . γ-aminobutyric acid

GH. . . . . growth hormone

GLM. . . . . general linear model

GnRH. . . . . gonadotropin-releasing hormone

GnRH-a. . . . . gonadotropin-releasing hormone analogue

GtH. . . . . gonadotropin

GSI. . . . . gonadosomatic index

HSD. . . . . honestly significant difference

HSI. . . . . hepatosomatic index

IBMX. 3-isobutyl, 1-methylxanthine

IGF. . . . . insulin-like growth factor

11-KT. 11-ketotestosterone

Mcs. . . . . microsphere

ND/nd. . . . . non-detectable

NPY. . . . . Neuropeptide Y

nsd. . . . . not significantly different

Oog. . . . . Oogonia

OL. . . . . ovarian length

OR. . . . . ovarian rank

pc. . . . . phagocyte

PG. . . . . primary growth

PG-Adv. . . . . advanced primary growth

p.h. . . . . post-hatch

PN/pn. . . . . perinucleolar

p.s.a. . . . . post-sperm activation

rbGH. . . . . recombinant bovine growth hormone

SAS. . . . . Statistical Analyses System

SC/sc. . . . . spermatocyte

SD. . . . . standard deviation

SE. . . . . standard error

SG/sg. . . . . spermatogonia

ST/st. . . . . spermatid

SZ/sz. . . . . spermatozoa

SZ-res. . . . . residual spermatozoa

T. . . . . testosterone

VG. . . . . vitellogenic

Wt. . . . . Weight

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CHAPTER 1

Thesis Introduction and Literature Review.

1.1. Introductory statement.

This thesis examines growth and sexual maturation in the yellowtail flounder, a teleost of recent evolutionary lineage (*Limanda ferruginea* Storer, Pleuronectidae). Specifically, this work describes puberty as well as the endocrine and histological correlates indicative of pubertal onset in both cultured males and females of this species. Furthermore, the thesis examines: growth patterns in relation to pubertal development and full maturity, effects of endocrine factors involved in both growth and reproduction, and the efficacy of the induction of triploidy on the suppression of gonadal maturation. In order to facilitate a greater understanding of the context of the different experiments, the present introduction reviews the endocrine control of reproduction and growth, gives an account of gametogenesis, provides an overview of the endocrine physiology of puberty in fish, and ends with an outline of the objectives for the thesis.

1.2. Endocrine control of reproduction.

The brain, pituitary and gonad are the three main endocrine centres regulating reproduction. These three centres form the gonadotropic or brain-pituitary-gonadal (BPG) axis. This axis represents the flow of hormonal signals from higher centres to the gonad, as well as the numerous feedback loops between the different regulatory centres. The initial physiological stimulus for reproduction is based on the processing of external environmental cues and internal metabolic signals by the brain. Neurons communicating external and internal cues to reproductive centres are distributed in different areas of the vertebrate brain.

They affect reproduction mainly by modulating the activity of neurons in the forebrain which produce gonadotropin-releasing hormone (GnRH). The stimulation of the BPG axis begins with an increase in the activity of GnRH neurons and with a responsiveness by the pituitary to GnRH. GnRH stimulates the synthesis and release of gonadotropins (GtH) from pituitary cells called gonadotropes. Gonadotropins in turn regulate gonadal activities such as sex steroid production and gametogenesis. Steroids produced by the gonads support gametogenesis and exert feedback effects to higher centres in the BPG axis. The three different classes of steroids include estrogens, androgens and progestins. In female fishes, an important estrogen-induced process includes vitellogenesis, that is the hepatic production of the yolk precursor protein, vitellogenin, which is then released into the blood and incorporated by developing oocytes.

1.2.1. Gonadotropin-Releasing Hormone (GnRH).

GnRH is a neuropeptide, composed of ten amino acids, which has been highly conserved throughout vertebrate evolution (Sherwood et al., 1994). The GnRH neurons regulating pituitary gonadotropin release are concentrated in the forebrain, specifically in the preoptic area and the hypothalamus. The forebrain GnRH neurons of most vertebrates have axons which terminate at the median eminence of the neurohypophysis, where GnRH is released into a hypothalamo-hypophysial portal blood system for transport to the pars distalis of the adenohypophysis. Teleosts are anatomically different in that the GnRH neurons directly innervate the pituitary gonadotropes (Sherwood et al., 1994).

The vertebrate brain usually contains at least two forms of GnRH. The names of the different GnRH forms found in vertebrates are associated with the animal or class in which

each particular variant was first isolated (Sherwood et al., 1994). A midbrain form, chicken GnRH-II (cGnRH-II), has been detected in all vertebrate classes and is suggested to function as a neuromodulator (Sherwood et al.,1993). In fish, one or two other GnRH molecules may be present in the forebrain. A single forebrain GnRH responsible for gonadotropin release is seen in: sturgeon and eel (mammalian GnRH), salmonids and cyprinids (salmon GnRH: sGnRH), and catfish (cfGnRH) (Sherwood et al., 1994). A second forebrain form, seabream GnRH (sbGnRH), has been detected in a number of species in addition to the phylogenetically common sGnRH (e.g. perciforms: gilthead seabream, *Sparus auratus*; cichlids, *Haplochromis burtoni*; striped bass, *Morone saxatilis*; scorpaeniform: grass rockfish, *Sebastes rastrelliger;* Powell et al., 1994, 1996; Gothilf et al., 1995; White et al., 1995; and other species reviewed in Holland et al., 1998a). Novel GnRH variants, present as the second forebrain form, are continuing to be discovered in a wider range of teleosts, as has been recently the case for Pacific herring, *Clupea harengus pallasi*, medaka, *Oryzias latipes*, and pejerrey, *Odontesthes bonariensis*, (Carolsfeld et al., 2000; Okubo et al., 2000; Stefano et al., 2000).

In species where both sbGnRH and sGnRH have been found in the forebrain, sbGnRH predominates in the forebrain centres regulating gonadotropin release (White et al., 1995; Ookura et al., 1999). Despite neuroanatomical associations of the different GnRH forms found in the brain, more than one form of brain GnRH may be seen in the pituitary as well (Sherwood et al., 1993; Kobayashi et al., 1997; Holland et al.,1998a, 1999). For instance, both sbGnRH and cGnRH-II were present in the pituitary of gilthead seabream, however, sbGnRH was determined to be the most physiologically relevant form to regulate

pituitary gonadotropic activity (Holland et al., 1998a). All three forms detected in the brain of striped bass were detected in the pituitary as well. Again sbGnRH was the most abundant and relevant form for reproduction, but pituitary levels of cGnRH-II also showed an association with gonadal development (Holland et al., 1999). A role for cGnRH-II should not be overlooked as it has been found to exert some stimulatory effects on gonadotropin regulation in goldfish, *Carassius auratus* (Khakoo et al., 1994).

In mammals, and potentially most vertebrates, GnRH release follows a pulsatile pattern produced by an endogenous pulse generator in the GnRH neuron (Dellovade et al., 1998; Terasawa, 1998). The basic pulsatile pattern is modulated by factors from adjacent neurons (Terasawa, 1998; see section 1.2.4.). Besides stimulating the release of gonadotropins, GnRH has been found to stimulate GtH synthesis in fish (Khakoo et al., 1994; Melamed et al., 1996, 1998; Hassin et al., 1998; Dickey & Swanson, 2000). Moreover, GnRH has been observed, in fish and other vertebrates, to upregulate its own receptors on gonadotropes as a self-priming mechanism to increase pituitary responsiveness to GnRH (Chieffi et al., 1991).

1.2.2. Gonadotropins.

Gonadal activity in tetrapods is regulated by two pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins are described as glycoprotein heterodimers, the two forms having a common alpha subunit and distinct beta subunits responsible for biological activity (Schulz et al., 1995b). In fishes, the presence of two pituitary gonadotropins (GtH-I and GtH-II) has been determined either by biochemical isolation or molecular cloning of the separate GtH ß-subunits. First isolated in chum salmon

Oncorhynchus keta, and coho salmon, *Oncorhynchus kisutch* (Suzuki et al., 1988a; Swanson et al., 1991), the list of species in which a dual gonadotropin system has been observed is still growing and includes not only teleosts (reviewed in Melamed et al., 1998), but early bony fish as well (white sturgeon, *Acipenser transmontanus*, Moberg et al., 1995).

While there is increasing evidence that most teleosts have a dual gonadotropin system, in two well-studied species, the European eel, *Anguilla anguilla*, and African catfish, *Clarias gariepinus*, only one gonadotropin molecule has been found in the pituitary (Koide et al., 1992; Quérat, 1995; Schulz & Goos, 1999). In the catfish, evidence from studies, both at the level of the pituitary and the gonad, support the proposal that the single, GtH-II-like gonadotropin regulates all gonadotropic function in males (Schultz et al., 1995a,b; Schulz & Goos, 1999).

In terms of biological function, GtH-I and GtH-II have been described as partially homologous to tetrapod FSH and LH. FSH-like GtH-I is associated with earlier stages of gametogenesis while LH-like GtH-II is connected with the stimulation of gamete maturation prior to spawning. In salmonids, GtH-I is the predominant circulating gonadotropin during spermatogenesis and vitellogenesis, while GtH-II remains low. A reversal of this pattern occurs during spermiation in males, and final oocyte maturation and ovulation in females (Suzuki et al., 1988b; Swanson, 1991; Breton et al., 1998). Both salmonid gonadotropins have similar steroidogenic potency on immature or developing male and female gonadal tissue, but GtH-II has been seen to have a greater stimulatory effect on progestin production in mature peri-ovulatory follicles (Suzuki et al., 1988c). An additional function of GtH-I in early gametogenesis is the stimulation of vitellogenin incorporation into oocytes. This was

demonstrated both *in vitro* and *in vivo* in rainbow trout, *Oncorhynchus mykiss*, while GTH-II had no effect (Tyler et al., 1991).

Gonadotropins isolated from the common carp have equal steroidogenic potency in both male and female goldfish gonadal tissue. In contrast with salmonid models, both carp gonadotropins stimulate final oocyte maturation and vitellogenin incorporation in goldfish oocytes *in vitro* (Van der Kraak et al., 1992; Schultz et al., 1995b). In red seabream, *Pagrus major*, GtH-II induces final oocyte maturation, but is more potent in stimulating estrogen production *in vitro* than GtH-I; both GtHs are equipotent in males (Tanaka et al., 1995; Kagawa et al., 1998). Even in salmonids the specific roles of each gonadotropin are not yet clear, as suggested by more recent data in rainbow trout where GtH-I has been shown to peak along with GtH-II at final maturation (Breton et al., 1998; Gomez et al., 1999).

With the development of molecular techniques, information on the transcription of GtH-Iß and GtH-IIß subunits is being reported for a wide variety of species. These studies report that mRNA of both beta subunits may be detectable in immature fish in addition to being present during all stages of gametogenesis (Gomez et al., 1999; Hassin et al., 1999, 2000; Kajimura et al., 2001). A molecular confirmation of the pattern where GtH-I is associated with early stages of gametogenesis, while GtH-II regulates final gamete maturation stages, has been reported in a number of species (Melamed et al., 1997; Gomez et al., 1999; Hassin et al., 1999, 2000). However, increases in GtH-Iß mRNA levels in rainbow trout during both spermiation and ovulation again point to a potential role for GtH-I in final maturation (Gomez et al., 1999). In some multiple spawning species with asynchronous oocyte development, the mRNA levels of GtH-Iß and GtH-IIß increase

synchronously from early through to late gametogenesis; a pattern which may characterize asynchronous developing species (goldfish, Sohn et al., 1999a; Japanese flounder, *Paralichthys olivaceus*, Kajimura et al., 2001; blue gourami, *Trichogaster trichopterus*, Jackson et al., 1999). Sexual dimorphism in gonadotropin subunit gene expression has been reported as well (Sohn et al., 1999a; Gen et al., 2000; Kajimura et al., 2001).

While studies use the measurement of GtH mRNA to assess pituitary activity, specific assays to measure intact hormones are required to determine the full meaning of observations based on gene expression. Gomez et al. (1999) compared pituitary levels of GtH subunit mRNA with levels of gonadotropins in both the pituitary and the plasma. The authors demonstrated that while patterns in gene expression of GtH-Iß matched well with changes in GtH-I, a delay was seen between GtH-IIß mRNA abundance and levels of GtH-II. This indicates that gene expression patterns do not always correlate with the presence of functional hormone.

1.2.3. Gonadal steroidogenesis and organization.

In addition to stimulating gametogenesis, the gonadal steroids, produced by the somatic cells of the testis and ovary, stimulate the gonadal ducts, induce physiological changes in behaviour, and promote any sexually dimorphic traits. Further, endogenous secretion of steroids at the time of sexual differentiation is important in sex determination (Nagahama, 1999). During spawning, gonadal steroids and their metabolites may act as pheromones (Zohar, 1989).

Fish gonads are organized into two zones: the germinal epithelium, composed of germ cells and somatic cells in intimate relationships with the germ cells, and interstitial

tissue. The germinal epithelium and interstitial tissue are separated by a basement membrane (Grier & Lo Nostro, 1999).

1.2.3.1. Testicular steroidogenesis and spermatogenesis.

The majority of male teleosts have lobular testes, where, within a lobule, spermatogenesis occurs in stationary cysts of germinal epithelium (Billard et al., 1982). Cysts are composed of a nest of germ cells surrounded by somatic Sertoli cells which synchronize spermatogenic development inside the cyst (Grier & Lo Nostro, 1999; Schulz et al., 1999). The cystic structure of the testis degenerates at the end of spermatogenic cycles, such that the remaining germinal epithelium consists only of spermatogonial stem cells and a reduced number of Sertoli cells. The cystic structure of the testis is rebuilt by the mitotic division of Sertoli cells and spermatogonia during recrudescence (Grier & Lo Nostro, 1999; Schulz et al., 1999).

Events in spermatogenesis begin with the mitotic proliferation of primary spermatogonia to produce secondary spermatogonia. The secondary spermatogonia become primary spermatocytes when they enter meiosis. Each primary spermatocyte during the first meiotic division produces two secondary spermatocytes, which proceed in the second meiotic division to produce a total of four haploid spermatids. In the latter part of spermatogenesis, the spermatids are transformed into spermatozoa by a process called spermiogenesis, which involves resorption of the spermatid cytoplasm and the formation of a flagellum. As spermiogenesis progresses, the cystic structure of the lobules degenerates and spermatids and/or spermatozoa from the numerous mature cysts are released into the lobular lumen. The spermiation phase in fish with lobular testes is initiated by the entry of spermatozoa into the

ducts (Billard & Cosson, 1992). Milt hydration and the acquisition of the capacity for sperm motility are major developments in the spermiation phase (Billard et al.,1990; Nagahama, 1994). The hydration of milt results in a marked increase in milt volume in preparation for spawning. Once this occurs a male is generally considered in full spermiating condition. In contrast to some species where sperm motility may not be acquired until certain changes occur within the sperm duct (Nagahama, 1994), spermatozoa stored in the testes of winter flounder, *Pseudopleuronectes americanus*, are motile and demonstrate high fertility, although motility can be enhanced by passage through the sperm ducts (Shangguan, 1998).

Testicular androgens are produced by the Leydig cells, which are located in the interstitial tissue of the testes. The predominant androgens in males include 11-ketotestosterone (11-KT), other 11-oxygenated androgens, androstenedione and testosterone (Borg, 1994). While androgens are produced by the testes, for some species androgen conversion may occur at other sites, such as the hepatic conversion of 11ß-hydroxyandrostenedione to 11-KT in the African catfish (Cavaco et al., 1997). In many teleosts 11-KT or another 11-oxygenated androgen is the main androgen stimulating spermatogenesis, while testosterone is more effective in feedback mechanisms to the BPG axis (Borg, 1994). This pattern is not universal as is the case in some hermaphroditic species where 11-KT may not be present (Borg , 1994). In the Japanese eel, *Anguilla japonica*, 11-KT treatment of testicular fragments *in vitro* induces spermatogonial mitosis, meiotic spermatogenesis and spermiogenesis via activation of Sertoli cells (Miura et al., 1991). Further study revealed that following gonadotropic stimulation, secreted 11-KT stimulated Sertoli cells to produce activin B which induced spermatogonial proliferation (Nagahama,

1994). This evidence links the observation of spermatogonial proliferation with the activation of the BPG axis in the male Japanese eel.

1.2.3.2. Ovarian steroidogenesis and oogenesis.

Oogonia represent the basic, proliferative, premeiotic cell population residing in the female germinal epithelium. A close association with somatic cells in the form of a follicle begins when oogonia become oocytes upon entering meiosis (Pudney, 1987; Grier & Lo Nostro, 1999). Folliculogenesis entails the surrounding of the oocyte by the follicle proper, an inner layer of granulosa cells, derived from somatic cells of the germinal epithelium, and an outer layer of basement membrane (Grier & Lo Nostro, 1999). The follicle complex is completed by an additional thecal cell layer, which is not part of the germinal epithelium and subsequently may be divided into the theca interna and externa layers. Ovarian steroidogenesis may follow a two-cell model, testosterone production by thecal cells and an aromatisation of testosterone in granulosa cells to the major estrogen in females, 17ß-estradiol (Nagahama, 1994). Not all teleosts follow a two cell model. There is evidence in two teleosts, mummichog, *Fundulus heteroclitus,* and medaka, *O. latipes*, that the thecal cells are not present and that the granulosa cells are responsible for ovarian steroidogenesis (Nagahama, 1994).

In oogenesis, oogonia proliferate mitotically forming small nests of cells. A proliferative stock of oogonia in the ovary is retained throughout the reproductive life in most teleosts, but in the remaining teleosts, and a number of other vertebrate classes, oogonial proliferation and entry of all oogonia into meiosis occurs early in ovarian development (Pudney, 1987; Scott, 1987). Once an oogonium becomes an oocyte, meiosis proceeds up

until the diplotene stage of prophase I, whereupon the oocyte enters a stage of meiotic arrest lasting until final oocyte maturation. The oocyte undergoes an initial primary growth phase during which folliculogenesis occurs, cytoplasmic organelles are synthesized, mRNA transcripts are produced for oocyte growth and future embryonic use, and the deposition of vitelline envelope proteins is begun to form the chorion - the outermost membrane of the egg (Tyler et al., 1999). A secondary growth phase, mainly characterized by exogenous vitellogenesis, is initiated when oocytes produce cortical alveoli (Scott, 1987). The contents of cortical alveoli are responsible for the formation of a peri-vitelline space upon fertilization and the water-hardening of the chorion, thus preventing polyspermy and providing protection for the zygote (Tyler et al.,1999). An accumulation of lipid vesicles may be seen prior to exogenous vitellogenesis; the source of the lipid is unclear (Tyler et al., 1999). During exogenous vitellogenesis, the oocyte increases substantially in size with the accumulation of yolk. Vitellogenin is a large glycolipophosphoprotein (300 to 640 kDa) which is incorporated into the oocyte by receptor-mediated endocytosis. Once inside the oocyte the vitellogenin is then enzymatically cleaved into yolk proteins such as lipovitellins and phosvitins (Tyler et al., 1999). The yolk is used to provide energy for embryo growth and survival in many species beyond hatching. In marine teleosts with pelagic eggs a proportion of the yolk proteins is hydrolysed forming a pool of free amino acids which drive oocyte hydration (Finn et al., 1999).

Major ovarian estrogens in female teleosts include 17ß-estradiol and estrone as is the case in other female vertebrates (Kime, 1987). During oogenesis, increases in levels of 17ß-estradiol are associated with the onset of vitellogenesis. Data in fish indicate that an increase

in the number of hepatic 17ß-estradiol receptors precedes the induction of hepatic vitellogenin synthesis by 17ß-estradiol (Le Dréan et al., 1994). Estradiol-17ß also stimulates the hepatic synthesis of vitelline envelope proteins in some species, while in other species the ovary may be the only, or an additional source, of vitelline proteins (Tyler et al., 1999).

Testosterone is the major female androgen in fishes and reaches high levels in the plasma, particularly during final oocyte maturation when conversion by aromatase into 17ß-estradiol decreases. As in males, an important role for testosterone in females includes providing feedback to the higher centres of the BPG axis. In addition to testosterone, 11-KT can be found in females of some species, similarly, estrogens may be found naturally in the plasma of males (Borg, 1994; Scott et al., 1999a).

1.2.3.3. Progestin and gamete maturation.

The production of progestins is stimulated by the rise in GtH-II levels during spermiation and final oocyte maturation. Certain progestins act as a Maturation-Inducing Steroid (MIS) in fish. In females, 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) and 17α,20ß,21-trihydroxy-4-pregnen-3-one (17,20ß,21-P) are two progestins which have been shown to be effective inducers of oocyte maturation (Kime, 1993). However, the actual MIS is not clear from plasma levels in many teleosts, particularly in batch-spawners where other progestins may be more abundant, such as potential precursors of the MIS (17,21-dihydroxy-4-pregnene-3,20-dione) or MIS metabolites (5ß-reduced, 3α-hydroxylated and sulphated forms) (Inbaraj et al., 1997; Mugnier et al., 1997; Scott et al. 1999b). In males, both 17,20ß-P and 17α,20α-dihydroxy-4-pregnen-3-one have been shown to be associated with spermiation (Kime, 1993). Besides progestins, corticosteroids such as 11-deoxycortisol and 11-

deoxycorticosterone have been implicated as maturation-inducing steroids (Zohar, 1989; Kime, 1993).

In females, the MIS has direct actions on the oocyte in terms of the resumption of meiosis from diplotene arrest, which is followed by germinal vesicle migration and breakdown in the oocyte (Nagahama, 1994). The MIS acts directly on the oocyte in a non-genomic manner through a plasma membrane receptor at the surface of the oocyte. Receptor binding of the MIS results in resumption of meiosis by activation of a cell-cycle regulator (maturational-promoting factor= complex of cdc2 kinase and cyclin B) (Nagahama, 1994). Oocyte hydration and/or ovulation in fish follow the initiation of final oocyte maturation but appear not to be controlled by the MIS (Zohar, 1989).

In males, GtH-II stimulates the Leydig cells to produce progestins, which, in some species, are converted to the MIS by the spermatozoa themselves (Nagahama, 1994). The role of the MIS in males is less clear, but an effect on the capacity for sperm motility through MIS actions on sperm duct pH has been reported (Nagahama 1994). In both males and females, progestins may serve as a source of pheromones which can be released to the environment through gonadal fluids and urine, as sulphates, as free unconjugated forms or as glucuronides (Kime, 1993).

1.2.4. Steroidal feedback to the brain and pituitary.

An important regulatory mechanism for the higher centres of the BPG axis comes in the form of gonadal feedback. Sex steroids predominantly supply negative feedback on GtH secretion in sexually mature fish. Surgical removal of the gonads results in increased GtH synthesis and secretion until exogenous androgen or estrogen treatment reinstates an

inhibitory tone (Zohar, 1989). Positive feedback effects of sex steroids in fish have been observed mainly in immature animals (Zohar, 1989- see section 1.4).

Steroid feedback may act, indirectly or directly, on the pituitary or hypothalamus to affect GnRH release, pituitary responsiveness to GnRH or basal GtH release. Aromatase activity, androgen receptors and estrogen receptors have been detected in the fish brain (Kah et al., 1993). This demonstrates the capacity of the brain to monitor steroid levels and thus reproductive status. Estrogen receptors appear to have a stronger relationship with the preoptic and hypothalamic areas associated with GnRH (Kah et al., 1993). While aromatizable androgens and estrogens have been reported to have stimulatory effects on GnRH synthesis, there has been no evidence that GnRH neurons express a fish estrogen receptor (Kah et al., 1999). Other neurons associated either directly or indirectly with sex steroid-concentrating areas of the brain are likely to mediate noted steroid effects on the GnRH system and GtH release.

A number of neuronal factors have been found to modulate GnRH and/or GtH release in fish, including: dopamine, norepinephrine, serotonin, glutamate, taurine, γ-aminobutyric acid (GABA), neuropeptide Y (NPY), and cholecystokinin (Trudeau & Peter, 1995). Most information available for fish refers to the stimulatory actions of GABA and NPY, and the inhibitory effects of dopamine, the neurons of which appear to be modulated by gonadal steroids.

An inhibitory action of dopamine on GtH-II release through effects on GnRH neurons and gonadotropes is clear in some species of teleosts (eel, catfish, goldfish, salmonids and tilapia; Dufour et al., 1988; Peter et al., 1991; Melamed et al., 1998), but is absent in Atlantic

croaker, *Micropogonias undulatus* (Copeland & Thomas, 1989). Recent evidence in female rainbow trout demonstrated that dopaminergic inhibition of GtH-II release was activated by high levels of 17ß-estradiol. Release of GtH-I, while under negative feedback by 17ß-estradiol, was not subject to inhibition by dopamine (Saligaut et al., 1998).

Stimulatory actions of both GABA and NPY on gonadotropin release have been shown to be dependent on reproductive stage and subject to steroid influence (Peter et al., 1991; Kah et al., 1999). GABA-ergic neurons, which express the estrogen receptor, are particularly good candidates for the mediation of steroid effects on GnRH gene expression (Kah et al., 1999). Studies indicate that stimulation of GtH release by GABA may occur, depending on the species, either by increasing GnRH release, decreasing dopaminergic inhibition, and/or affecting the gonadotropes directly (Trudeau & Peter, 1995; Kah et al., 1999).

Gonadal feedback on the brain and pituitary also may be supplied by non-steroidal factors such as inhibins and activins. In mammals, inhibins are known for selective inhibition of FSH synthesis and release, while activins stimulate these FSH parameters (Ge, 2000). Current evidence in fish has shown the expression of activin subunits in gonadal tissue, however, the production of inhibin has not as yet been detected (Ge, 2000). Recombinant homologous activin B has been found to stimulate GtH-Iß mRNA but inhibit GtH-IIß mRNA in goldfish (Yam et al., 1999). In female rainbow trout, human inhibin suppressed GtH-I release but increased the secretion of GtH-II in dispersed pituitary cells (Chyb & Breton, 1999). Desteroidized ovarian fluid induced a similar secretion pattern produced by heterologous inhibin, which suggests the presence of inhibins or inhibin-like compounds of

gonadal origin (Chyb & Breton, 1999; Chyb et al., 1999). The effects of activin or inhibin-like molecules on GtH-II is in sharp contrast to other vertebrates in which FSH is primarily affected (Ge, 2000). These non-steroidal gonadal factors potentially may be involved in the differential regulation of gonadotropins in fish.

1.3. Growth and reproduction.

A link between growth and reproduction has been observed in many fish species. Among these observations is the association between a faster growth rate and an earlier age of maturity (Alm, 1959; Thorpe, 1986; Le Bail, 1988). Studies examining growth and reproduction have discovered an interplay between the endocrine factors regulating these respective physiological processes.

1.3.1. Endocrine control of growth: the somatotropic axis.

As with reproduction, growth is regulated by an endocrine axis beginning with the brain and pituitary, but differs by ending with the liver as the main peripheral target organ. A variety of neuronal factors are implicated in the central control of growth hormone (GH) secretion from the pituitary. Stimulatory factors include: growth hormone-releasing hormone (GHRH or GRF), pituitary adenylate cyclase activating polypeptide (PACAP), dopamine, GnRH, thyrotropin-releasing hormone (TRH), NPY, glutamate (through the agonist NMA: N-methyl-D,L-aspartate), cholecystokinin and bombesin. Somatostatin (SRIF), serotonin, and norepinephrine have inhibitory actions on GH release in fish (reviewed in Peter & Marchant, 1995, Holloway and Leatherland, 1998).

While GHRH is a primary regulator of GH secretion in higher vertebrates, some studies report that GHRH has little to no effect on GH release in fish. Instead, PACAP,

which is encoded on the same gene as the GHRH-like peptide in fish, has a much stronger effect on GH release (Sherwood et al., 1994; Holloway & Leatherland, 1998; Montero et al., 2000). In terms of inhibition of GH release, somatostatin appears to be a major inhibitory influence on basal and stimulated GH secretion, and can block the stimulatory actions of dopamine, NPY, TRH and GnRH. Similarly, inhibitory effects of norepinephrine and serotonin are able to decrease basal and stimulated GH release (Peter & Marchant, 1995; Holloway & Leatherland, 1998).

Pituitary production of growth hormone occurs in cells called somatotropes. The main endocrine action of GH is to stimulate the hepatic synthesis and release of insulin-like growth factors I and II (IGF-I & -II), also referred to as somatomedins. Both IGF-I and GH have been found to provide negative feedback to GH secretion by pituitary somatotropes (Pérez-Sánchez et al., 1992; Björnsson, 1997).

Growth hormone is the primary regulator of somatic growth, however, linear growth promoting effects of GH are mainly mediated by IGF-I, which stimulates cartilage proteoglycan synthesis in skeletal tissue (McCormick et al., 1992). GH appears to be required for this effect of IGF-I to occur *in vivo,* but not *in vitro* (Peter & Marchant, 1995). Additional actions of IGF-I in fish include mitogenic and hypoglycemic effects, which have been noted in other vertebrates as well (Le Gac et al., 1993; McCormick et al., 1992).

Other physiological effects of GH include an anabolic induction of protein synthesis and catabolic actions namely, the breakdown of glycogen and the mobilization of lipid through lipolysis (O'Connor et al., 1993; Björnsson, 1997). Anabolic protein synthesis appears to occur in a variety of organs but not in the muscle; rather, muscle protein may

decrease in salmonids while water content increases (Björnsson, 1997). The muscle is proposed to supply the amino acids for GH-stimulated protein synthesis in the organs. Between decreases in muscle protein, lipolytic effects and increases in length, a leaning effect on the condition factor commonly is observed in fish treated with GH (Björnsson, 1997). Increased food conversion efficiency and a behaviourally evident increase in appetite are additional GH effects which may mitigate any loss of muscle protein over the long-term (Björnsson, 1997; Holloway & Leatherland, 1998).

1.3.2. Overlap of somatotropic and gonadotropic axes in the endocrine control of growth and reproduction.

The effects of gonadal steroids on the somatotropic axis represent one level of evidence of an interaction between hormonal factors regulating growth and reproduction in fish. Sexual stage, testosterone and/or 17ß-estradiol have been shown to affect basal GH release or somatotrope responsiveness to SRIF, TRH, dopamine, NPY and GnRH in a number of teleosts (Trudeau et al., 1992; Peter & Marchant, 1995; Holloway et al., 1997; Holloway & Leatherland, 1998; Lin et al., 1995; Melamed et al.,1995; Björnsson, 1997). A stimulatory effect of 17ß-estradiol on plasma GH levels in rainbow trout appears to involve decreasing SRIF levels and/or somatotrope responsiveness to SRIF (Holloway et al., 1997; Holloway & Leatherland, 1998). At the level of GH gene expression, a sex steroid action involving a stimulatory effect of testosterone has been noted only in goldfish; no effect has been found for 17ß-estradiol, despite its ability to increase GH content in the goldfish pituitary (Huggard & Habibi 1995; Zou et al., 1997; Holloway & Leatherland, 1998; Melamed et al., 1998).

Further evidence of overlap between the somatotropic and gonadotropic axes includes the GH-releasing action of gonadotropin-releasing hormone. This action has been observed in cyprinids, rainbow trout and a tilapia hybrid (*Oreochromis niloticus* x *Oreochromis auratus*) (Marchant et al. 1989; Lin et al., 1995; Peter & Marchant, 1995; Holloway & Leatherland, 1997; Melamed et al. 1995, 1996). In goldfish and grass carp, *Ctenopharyngodon idellus*, *in vivo* GnRH treatment caused sufficient GH release to stimulate growth (Marchant et al. 1989; Lin et al., 1995; Peter & Marchant, 1995). However, in other studies with rainbow trout pre-incubation with IGF-I was required for GnRH to have an effect on GH release *in vitro* (Blaise et al., 1997). A lack of an effect of GnRH on GH release has been reported for eel, *A. anguilla*, and European turbot, *Psetta maxima* (Rousseau et al., 1999).

Also at the neuroendocrine level, dopamine demonstrates a differential regulation of growth and reproduction by inhibiting GtH release while simultaneously stimulating GH release (Peter & Marchant, 1995). According to Wong et al. (1993), dopamine induction of GH release in goldfish was greatest in sexually regressed fish and diminished with increasing gonadal development.

Regarding crossover of the somatotropic axis into reproduction, GH has been shown to affect gonadal steroidogenesis. Moreover, there is evidence of gonadal GH receptors in fish (reviewed in Le Gac et al., 1993). In terms of IGFs, studies propose that IGF-I may act as a peripheral signal in the pubertal activation of gonadotropin release, and an intragonadal IGF system has been found in fish (Le Gac et al., 1993; Dufour et al., 1999; Perrot & Funkenstein, 1999).

1.4. Pubertal physiology of fish.

In immature fish, the BPG axis remains quiescent until signals induce its activation at the initiation of puberty. Low steroid production and low GtH levels may be detected in the plasma during the immature stage in certain species. However, even when these hormones are detectable in the plasma, there is little indication of a functional BPG axis wherein a flow of endocrine information between all the centres of the axis has been established. Depending on the species, the immature BPG axis may exhibit: a lack of GnRH synthesis or axonal transport of GnRH to the pituitary, an inhibition of GnRH release, an absence of pituitary responsiveness to GnRH, or low gonadotropic function in both synthesis and basal release (Dufour et al., 1988; Amano et al., 1997; Pavlick & Moberg, 1997; Gur et al., 2000). In immature black carp, a gonadal insensitivity to GtH, lasting up until four years of age, has been observed in addition to a lack of GnRH responsiveness by the pituitary (Gur et al., 2000). In contrast, immature individuals of other species have demonstrated steroidogenic and gametogenetic responses following *in vivo* gonadotropic treatment (Crim et al., 1982; Dufour et al., 1989; Sato et al., 1997). Thus, immature gonads generally demonstrate a sensitivity to gonadotropin, but the timing may be dependent on age.

A general definition describes puberty as a transitional period of reproductive development from immaturity to full sexual maturity, in which the potential for reproduction is newly acquired (Dufour et al., 1999; Schulz & Goos, 1999; Holland et al., 2000). The onset of puberty in fish can be recognized by the appearance of spermatocytes in males and by the presence of vitellogenic oocytes in females (Le Bail, 1988). These events are indicative of an activation of the BPG axis, which is noted by increases in the synthesis and

release of GnRH, gonadotropins and sex steroids. The end of the pubertal period occurs once first ovulation or first full spermiation is achieved (Dufour et al., 1999).

Effects of sex steroid administration in immature fish have established that gonadal steroids amplify or accelerate the pubertal development of the BPG axis. A lack of a strong negative feedback response to steroids in immature fish is in contrast to evidence for immature mammals. In rats a negative feedback response to gonadal steroids is more firmly established for immature than for mature animals (Ojeda & Urbanski, 1994). Aromatizable androgens, mainly testosterone, and 17ß-estradiol stimulate pituitary GtH content and levels of GnRH forms involved in GtH release in immature fish (Atlantic salmon, *Salmo salar*, Crim & Peter, 1978; rainbow trout, *O. mykiss*, Crim & Evans, 1979; 1983; Crim et al., 1981; Fåhræus-Van Ree et al., 1983; Gielen &Goos, 1983, 1984; masu salmon, *Oncorhynchus masou*, Amano et al., 1994, 1997; platyfish, *Xiphophorus maculatus*, Schreibman et al., 1986; European eel *Anguilla anguilla*, Dufour et al. 1983, 1988, 1989; Montero et al., 1995; Japanese eel, *Anguilla japonica*, Lin et al 1991; white sturgeon, *Acipenser transmontanus*, Pavlick & Moberg, 1997; black carp, *Mylopharyngodon piceus*, Yaron et al., 1995; Gur et al., 1995; striped bass, *Morone saxatilis*, Holland et al., 1998b; sea bass, *Dicentrarchus labrax*, Zanuy et al., 1999; African catfish, *Clarias gariepinus*, Cavaco et al. 1995; Dubois et al., 1998; Indian catfish, *Heteropneustes fossilis*, Tiwary et al., 2002). Non-aromatizable androgens have been found to be generally ineffective in stimulating higher reproductive centres, although exceptions have been seen with 11-ketotestosterone in platyfish and to a small degree in rainbow trout (Crim et al., 1981; Schreibman et al., 1986).

A few studies report that long-term treatment of testosterone is capable of stimulating pituitary GtH content and release in immature fish (Crim & Evans, 1982, 1983; Gielen & Goos, 1984; Tiwary et al., 2002). Frequently, testosterone treatment, while capable of providing other positive feedback actions, is unable to induce GtH release. In these cases GnRH treatment in combination with testosterone has been shown to stimulate the release of GtH and subsequent gonadal development (Crim & Evans, 1983; Fåhræus-van Ree et al., 1983; Gielen & Goos, 1984; Trudeau et al., 1993; Holland et al., 1998b). Due to an inhibitory action of dopamine on GtH release, only a combination of 17ß-estradiol, GnRH and treatments inhibiting dopamine action or synthesis was successful in stimulating GtH release and the initiation of puberty in prepubertal female eel, *A. anguilla* (Dufour et al., 1988).

The positive feedback actions of sex steroids on gonadotropin or GnRH in immature fish also involve actions at the gene expression level. In masu salmon, androgen stimulation of GnRH mRNA levels was age specific, occurring once females were two years of age (Amano et al., 1997). Stimulatory effects of steroids on GtH-IIß mRNA levels have been noted during the immature stage of a number of species (European eel, Quérat et al., 1991; rainbow trout, Xiong et al., 1994; coho salmon, Dickey & Swanson, 1998; goldfish, Huggard et al., 1996; Sohn et al., 1999b; black carp, Gur et al., 1995). Xiong et al. (1994) detected estrogen responsive elements in the GtH-IIß gene of rainbow trout with which estrogen receptors interact. The effects of sex steroids on the expression of the GtH-Iß gene during the immature phase are less clear. Steroids had no effect on GtH-Iß mRNA levels in immature/pubertal coho salmon or immature rainbow trout (Xiong et al., 1994; Dickey & Swanson, 1995,1998), but an inhibitory effect was detected in goldfish and pubertal male

coho salmon (Sohn et al., 1999b; Dickey & Swanson, 1998). In contrast to other species, low doses of testosterone stimulated GtH-Iß mRNA levels in immature male tilapia hybrid (*O. niloticus x O. aureus*), but no effect was seen on GtH-IIß mRNA levels at either low or high doses (Melamed et al., 1997).

Overall these studies indicate that sex steroids have a greater effect on increasing pituitary GtH-II content than that of GtH-I (Dufour et al., 1999). Other mechanisms leading to an increase in GtH-I levels in the pituitary have been examined. GtH-I gene expression is stimulated by GnRH in immature coho salmon, in which steroids had no effect (Dickey & Swanson, 2000). Acute GnRH treatment has been shown to increase the gene expression of all gonadotropin subunits in striped bass males already initiating puberty, while chronic treatment of GnRH with testosterone had a similar effect in immature males (Hassin et al., 1998, 2000).

While steroids accelerate the development of the BPG axis during puberty, the signals for the initiation of puberty are still unclear. As the age of maturity appears to be affected by growth rate, investigations have been undertaken to discover somatic cues which could communicate growth status to the BPG axis. Stimulatory effects of IGF-I, an important somatotropic hormone, have been found on both gonadotropin content and release in eel (*A. anguilla*) pituitary cells *in vitro* (Huang et al., 1998, 1999). In salmonids, IGF-I has been shown to increase the cellular content of GtH-I and the *in vitro* sensitivity of pituitary cells to GnRH (Baker et al.,1999; Weil et al., 1999). While the evidence for IGF-I as a potential growth related cue for puberty is encouraging, other factors relating to metabolic or nutritional status besides growth also need to be investigated.

1.5. Aims of the doctoral thesis.

The experimental fish for the present doctoral research is the yellowtail flounder, *Limanda ferruginea* Storer. It is a small flatfish species which has supported active commercial fisheries throughout its geographical range. Between 1992 and 2001, this fish has been investigated with regards to its potential development as a species for cold-water aquaculture. It has been found to adapt easily to captivity and is a convenient species for experimental work by virtue of its ability to withstand handling.

The yellowtail flounder is considered a cold-water species. It inhabits the Northwestern Atlantic with a geographical distribution which extends from Labrador and the Grand Banks of Newfoundland, southward to Chesapeake Bay (Walsh, 1992). Yellowtail flounder belong to the right-eyed flounder family, the Pleuronectidae. In 1984 this species was classified under the genus *Pleuronectes* (*Pleuronectes ferrugineus*). A number of studies have been generated while it was under this designation. It was returned under its historical genus *Limanda* in 1998 (Cooper & Chapleau, 1998).

The yellowtail flounder is a gonochoristic species, i.e. the sex does not change over time. Males have a pair of testes with sperm ducts on either side of the first haemal spine at the posterior margin of the abdominal cavity (Shangguan, 1998). The sperm ducts continue to the urogenital pore located just before the anterior end of the ventral fin and deviating slightly upwards from the midline towards the ocular surface. For females, the paired, conical-shaped ovaries are positioned on either side of the first haemal spine and extend posteriorly between the haemal spines and the body musculature (Howell, 1983). The female reproductive system is described as cystovarian, the ovarian wall of each hollow ovary is

continuous with a very short oviduct located at the ventral, anterior point of the ovary. Each oviduct joins with its bilateral counterpart to lead to the gonopore and a small cloaca at the midline of the animal (Howell, 1983; Bettles, 1997).

Previous work on the reproduction of yellowtail flounder has concentrated on the reproductive biology and physiology of mature individuals of this batch-spawning species. Adults show a seasonality in reproduction, with spawning lasting over the spring or summer period depending on the latitude (Royce et al.,1959; Colton et al., 1979; Manning & Crim, 1998). Ovarian development follows the group synchronous pattern, where a discrete population of developing vitellogenic oocytes arises from a previtellogenic stock in the fall to be spawned in the following spring or summer (Howell, 1983; Wallace & Selman, 1981). The batch-spawning or batch-ovulation strategy portions the vitellogenic oocyte population into a series of ovulatory events over time. Recent studies on the reproduction of captive adult yellowtail flounder have included: building seasonal steroid profiles in both males and females (Clearwater, 1996); the histological monitoring of spermatogenesis in mature males (Shangguan, 1998); examining natural ovulatory cycles and egg production in females (Manning & Crim, 1998); managing milt by dilution and storage (Clearwater, 1996; Clearwater & Crim, 1996); examining free and sulfated steroid profiles in males (Devereaux, 1998); developing a protocol for sperm cryopreservation (Richardson et al., 1999); as well as, assessing the effects of a GnRH-analogue ([D-Ala^{6},Pro^{9}-NHEt]LHRH) and/or photoperiod for the stimulation and advancement of spawning (Bettles, 1997; Linehan, 1996; Larsson et al., 1997; Clearwater & Crim, 1998; Lush & Crim, 1999). Results of GnRH use in adults indicated that sustained delivery of GnRH-analogue, alone, was an effective agent

for the synchronization and advancement of ovulation, both in naturally developing and photo-stimulated females (Bettles, 1997; Larsson et al., 1997; Lush & Crim, 1999). The use of GnRH-analogue in males indicated a stimulatory effect on spermiation and milt volume during spawning periods in captivity (Clearwater & Crim, 1998).

While an abundant amount of information has been accumulated on the reproduction of mature yellowtail flounder, no work had yet been done to describe the onset of puberty in immature yellowtail, nor had any studies linked endocrinology with gonadal histology in this species. Efforts in broodstock management and larviculture in 1997 and 1998 at the Ocean Sciences Centre in Logy Bay, Newfoundland, were successful in producing a large yield of cultured juveniles. This success provided an opportunity to examine, for the first time:

a) the process of puberty in male and female yellowtail flounder;

b) the effects of hormones with potential dual roles in reproduction and growth; and,

c) a method for the sterilization of the gonad.

These three themes are addressed in the following chapters.

Chapter 2 - This study examines the timing of puberty (age, size, season) in males and females from two year classes of cultured fish. In addition to histological methods to describe the onset of puberty and sexual maturation, sex steroids were measured in the plasma and in media from ovarian tissue incubations. Of interest was whether there is a plasticity in the timing of puberty in culture, and what role growth rate might play in the age and size that puberty is initiated. *In vivo* and *in vitro* steroid production was monitored beginning from an immature stage. This was done in order to detect signs of the activation of the BPG axis, and

to determine what role the gonad may have in the pubertal process.

Chapter 3 - This chapter investigates the effects of endocrine factors reputed to possess dual actions in growth and reproduction in fish. Immature females and maturing males were subject to long-term administration of recombinant bovine growth hormone, testosterone, GnRH-analogue, and a combination treatment of GnRH-analogue with testosterone. Each treatment was evaluated in terms of its effects on growth, the onset of puberty in females and spermatogenesis in males. The development of sex differences in growth was followed within each group.

Chapter 4 - The induction of triploidy in fish has been used as a method to sterilize fish in aquaculture. This practice was attempted for the first time for yellowtail flounder using a hydrostatic pressure treatment protocol on freshly fertilized eggs. The parameters for inducing triploidy and success rates in the production of triploids were determined in this study. Triploid larvae were compared to control groups in terms of larval growth performance. A group of triploid juveniles was reared to three years of age in order to examine the effectiveness of the treatment on gonadal development.

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CHAPTER 2

The Timing of Puberty in Cultured Male and Female Yellowtail Flounder, *Limanda ferruginea* Storer.

2.1. INTRODUCTION.

The onset of puberty requires the activation of endocrine pathways in areas of the brain and pituitary which regulate gonadal development. Once puberty is initiated, somatic energy reserves and energy from dietary intake are utilised for the support of gametogenesis and the development of accessory structures for reproduction. While fish are immature, the endocrine pathways controlling reproduction remain quiescent, and, frequently, high growth rates are observed. The duration of the immature phase prior to the initiation of puberty is variable both within and between species. At the individual level, the timing of puberty can be significantly affected by growth rate, a faster growth rate leading to an earlier age at first maturity (Alm, 1959; Le Bail, 1988).

The term puberty has been used in the fish literature to describe the physiological events and mechanisms involved in the first sexual maturation. Puberty is defined as the transitional period of reproductive development from a condition of immaturity to that of reproductive maturity, wherein the potential for reproduction is newly acquired (Dufour et al., 1999; Schultz & Goos, 1999; Holland et al., 2000). Evidence of the initiation of puberty in the fish gonad includes the observation of spermatocytes in males and the incorporation of yolk (vitellogenin) into oocytes in females (Le Bail, 1988). However, at an endocrine level, puberty is associated with an increase in basal gonadotropic secretion and steroidal output from the pituitary and gonads, respectively. These events occur prior to the hepatic

synthesis and release of vitellogenin in females, and may induce spermatogonial proliferation in addition to meiotic activity in males (Miura et al., 1991; Cavaco et al., 1998).

In order for pubertal development to culminate in the production of viable gametes, a full activation of the brain-pituitary-gonadal axis (BPG axis) must occur. This requires: an increase in the production of gonadotropin-releasing hormone (GnRH), the development of pituitary sensitivity to GnRH, increased synthesis and release of pituitary gonadotropin(s) (GtH), and gonadal responsiveness to GtH (Dufour et al., 1988; Gur et al., 2000). In fish, the full activation of the BPG axis during puberty is accelerated by positive feedback actions of sex steroids, particularly 17ß-estradiol or aromatizable androgens like testosterone (reviewed in Chapter 1. and Dufour et al., 1999). An insufficient activation of the BPG axis, at any level, may prolong pubertal development. For instance, in some male and female striped bass, *Morone saxatilis*, an incomplete cycle of pubertal gametogenesis preceded a second, successful, pubertal cycle the following year (Holland et al., 2000).

The present study examines the timing of puberty and the subsequent testicular and ovarian development to first maturity in a small pleuronectid species, the yellowtail flounder (*Limanda ferruginea*). Wild yellowtail flounder are reported to have a capacity for early sexual maturity; both sexes reach full maturity at two and three years of age in the southern limits of the species' geographic range (Royce et al., 1959). Two year classes of cultured fish were followed in the present study in order to determine if plasticity exists in the age and size at first maturity in culture.

Plasma levels of sex steroids were measured and gonadal histological analysis was performed in order to correlate endocrine factors with observed changes in gonadal size and

cytology. Seasonal changes in gonadal histology and hormone profiles have been reported separately for adult yellowtail flounder (Howell, 1983; Clearwater, 1996; Shangguan, 1998). However, no study has yet related histology with endocrinology, or examined puberty in this species. By examining these two aspects of gonadal development together in pubertal fish, this study attempted to discover: at what age and during what time window of the year the initiation of puberty was stimulated, whether the activation of the BPG axis could be detected by gonadal activity, and which steroids secreted at the onset of puberty could be candidates for supplying positive feedback to the brain and pituitary.

To study female puberty further, ovarian tissue was incubated *in vitro* and exposed to salmon crude pituitary extract, a source of heterologous gonadotropin, and to forskolin, an adenylate cyclase activator. These incubations were performed in order to determine whether levels of 17ß-estradiol in the plasma accurately reflected the steroidogenic competence of the ovary at different phases of its development. Crude pituitary extract was used to detect the presence of gonadotropin receptors in the ovary, as well as determine GtH sensitivity. Forskolin was used to test the intracellular capacity of steroidogenic cells to respond to a gonadotropin receptor signal.

Connections between body size, growth patterns and pubertal development were investigated in order to present a larger picture of the factors involved in puberty in this species. Body size data were examined for any evidence that growth performance during the immature phase affected the timing of the onset of puberty, as has been reported for other fish species. Male and female growth patterns were compared to determine whether sex differences in growth developed with puberty in cultured yellowtail flounder.

2.2. METHODS.

2.2.1. Source of fish, sampling times and husbandry.

This study examined two year classes of cultured fish which had been laboratory reared at the Ocean Sciences Centre of Logy Bay, Newfoundland. The two year classes were produced from the 1997 and 1998 spawning seasons of a captive adult broodstock. The spawning season of yellowtail flounder in captivity occurs from mid-May until late August /early September with peak spawning egg production between mid-July to early August (Manning & Crim, 1998). Since the juveniles of this experiment originated from a pool of different batches of larvae produced over a spawning season, a reference hatching date of August 15 was chosen for use in assigning an age to sampled fish. The period of peak spawning and the seven to ten day embryonic stage prior to hatching were used as guidelines in the choice of this reference point.

Sampling began after the fish from the 1997 and 1998 year classes had reached one year of age. Six fish per sex were sampled at each sampling date, which occurred generally every two to four months. The sex of an individual could be determined due to anatomical differences in gonadal growth, i.e. the testis grows anteriorly from the first haemal spine into the abdominal cavity, while the ovaries project posteriorly from the abdominal cavity. By holding individuals in front of a bright light source, the outline of the ovary could be perceived; in young, immature individuals the ovary itself would be illuminated.

Animals were sampled from two sites in this study, the Ocean Sciences Centre (OSC) and an experimental grow-out facility in Heart's Content, Newfoundland. This latter facility obtained 10 and 11 month old OSC animals from the 1997 and 1998 year classes in June,

1998 and July, 1999, respectively. Details on the timing and locations of sampling events and the relative health of the animals collected in either year class are listed in Table 2.1. After the first sample at 12.5 months of age, an outbreak of furunculosis prompted a cessation of sampling of 1997 year class animals from the OSC. Healthy 1997 year class animals were sampled from the experimental grow-out facility from 18 months until 34 months of age. In the sampling of 1998 year class fish, a less intense outbreak of disease was evident in the sample at 15.5 months from the experimental grow-out facility. Subsequent samples of 1998 females at 20 and 22 months of age were taken from a healthy, all-female OSC population. Sampling 1998 males from the experimental grow-out facility was continued, as no alternate male population was present at the OSC.

All fish sampled from the experimental grow-out facility were transported to the OSC before being processed, and were kept until individuals fed overtly. This behaviour was interpreted as an indicator of acclimation to new conditions, and was usually seen three weeks post-transport. At least five and often all six individuals of each sex survived this recovery period prior to data collection. However, in the sample of 1997 fish of 32 months of age (April), overt feeding had not taken place for most individuals. Fish from this sample had been transported two weeks after a water supply failure on site. The fish were killed after a total of six weeks following two female mortalities. Only two females and one male were feeding at this time. Due to these problems a supplemental blood sampling occurred on site at the experimental grow-out facility in May.

2.2.2. Environmental conditions and feeding.

Animals of both year classes experienced a temperature range of 8 to 13°C during their first 10-11 months of life at the OSC. Experimental fish of 12 months and older, whether at the OSC or the experimental grow-out facility, experienced temperature conditions which fluctuated seasonally. The fish kept at the experimental grow-out facility experienced larger fluctuations in temperature (~1 to 13-16°C) than the 1998 year class fish sampled at the OSC where temperature control permitted a range of 3-13°C (Figure 2.1). Holding tanks at both sites were flow-through designs receiving degassed, fresh sea water. Lighting, both natural and artificial, parallelled seasonal fluctuations in photoperiod.

Fish of both year classes were fed with a dry pellet salmonid diet containing 24% fat and 46-48% protein (Corey Feed Mills, Fredericton, NB and Moore-Clark, St. Andrew's NB). A marine fish diet containing 14% fat (Corey Feed Mills, Fredericton, NB) was used for OSC fish of the 1998 year class during most of their second year of life. One year old OSC animals were fed at least twice a day during the week and at least once a day during the weekend. Fish were fed multiple times per day at the experimental grow-out facility.

2.2.3. Data collection.

All fish were first anaesthetized using an overdose of 2-phenoxyethanol (Acros Organics, New Jersey, USA). Individuals were measured for both total length and standard length (tip of mouth to base of caudal fin) and were weighed to the nearest 0.1 g. Blood samples were collected from the haemal arch using prechilled, heparinized (i.e., rinsed in a 0.1% (w/v) heparin, 0.86% NaCl solution) syringes which were subsequently emptied into 1.5 ml heparinized Eppendorf tubes. Afterwards, the fish were killed by severing the spinal

cord just posterior to the cranium. The collected blood was kept on ice over the ensuing tissue processing period and later centrifuged for ten minutes at 8 325 x g (4˚C). The plasma was stored at -20˚C or -70˚C until hormone analysis could be undertaken at a later date.

Prior to dissection, female fish were held towards a bright light source and the development of the gonad was qualified according to an ovarian ranking (OR) scale developed for this study. This proportional scale relates the length of the ovary to the length of the ovarian cavity (Table 2.2). For males, an attempt was made to express milt from the urogenital pore by manual stripping before opening the abdominal cavity.

During dissection, the gonads, liver and remaining viscera were removed and weighed to the nearest 0.01 g. The length of each ovary was measured. Whole gonads or pieces of gonadal tissue were fixed in Bouin's fluid for one to three days, washed in 50% ethanol, then stored in 70% ethanol in preparation for histology.

2.2.4. *In vitro* incubations.

Ovarian tissue was sampled from the medial areas of both ovaries immediately after dissection. For each female, 750 mg of tissue were weighed and then immersed in sterile, ice-chilled balanced salt solution (BSS). This solution was a modified version of trout balanced salt solution (3.4 mM $CaCl_2 \cdot 2H_2O$, 3.1 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 133 mM NaCl, 40 mM Hepes, 1 g/L glucose Jalabert & Fostier, 1984). Additions of 1.0 M NaOH, and dissolving the salts to 94.5% of the prescribed final volume of solute, the BSS matched the pH and osmolarity measurements of yellowtail flounder blood plasma (pH 7.7; 331 mOsm).

The ovarian tissue was scissor-cut into small pieces and the fragments washed twice in BSS over a period of at least three hours prior to incubation. The fragments were randomly allocated among five sets of three replicate wells (~50 mg of tissue/ well) in a 24 well CoStar culture plate. Each set of wells represented a different treatment:

Set 1: control wells contained the BSS medium with 0.1 mM 3-isobutyl, 1-methylxanthine (IBMX). IBMX is a synthetic methylxanthine which delays the degradation of cyclic nucleotides (cAMP or cGMP) produced as intracellular second messengers following hormone receptor binding. Specifically, methylxanthines inhibit cyclic nucleotide phosphodiesterase which converts cyclic AMP to inactive 5'AMP.

Set 2: exposure to 10 μM forskolin (Sigma) in BSS-IBMX solution. Forskolin activates adenylate cyclase production of cyclic AMP in the steroidogenic cells.

Sets 3 - 5: exposure to heterologous gonadotropic stimulation in three different concentrations (5, 50 and 500 μg/ml) of crude salmon pituitary extract (CPE; Argent Chemical Laboratories, Redmond, WA, U.S.A.; lot# SP1211M) dissolved in BSS-IBMX medium.

Exposure to crude pituitary extract tested for tissue sensitivity to gonadotropin and for the production of gonadotropin receptors by the steroidogenic cells. The forskolin treatment tested whether the post-receptor pathways for the gonadotropic stimulation of steroidogenesis were functional. In the absence of a response to pituitary extract, the level of response to forskolin would indicate the intracellular status of the steroidogenic cells; that is whether the lack of a cell response to gonadotropin was due to an absence of gonadotropin receptors, or whether the whole cell was in a refractory state.

Sterile technique was observed during all steps of the procedure. Following tissue harvesting and cutting, all subsequent steps were performed in a laminar flow hood. The total volume per well was 1.2 ml. Tissue plates were placed in an incubator set at 9°C, a physiologically relevant temperature for cultured yellowtail flounder, and agitated continuously over a five day incubation period. At the end of the incubation, the fluid in the wells was recovered, transferred to two 0.5 ml Eppendorf tubes and stored at -20°C or -70°C.

Some modifications in the above procedure were required for some females with small ovaries (GSI<2%). A limited amount of tissue in these females required one or more of the following steps in order to maintain the same amount of tissue per well (~50mg/well): i) eliminating the lower 5 and 50 µg/ml of CPE treatments; ii) eliminating replication; or, iii) pooling tissue between females of similar GSI values. Eliminating replication was only necessary for 1998 females in the 13.5 and 14 month samples in October. In the 13.5 month sample only one female had enough tissue for a fully replicated incubation. For the remaining females, tissue was available for full replication (n=2 females) or no replication (n=3 females) of control, forskolin and the 500 µg/ml crude pituitary extract (CPE) treatments. Similarly, for females sampled at 14 months of age, tissue availability only permitted six non-replicated incubations, four of which included testing the two lower doses of CPE. One of the six incubations was based on pooled tissue from two 14A sample females, while another used pooled tissue from the 14B sample of five, small females (7-16.3 g; Table 2.1).

2.2.5. Hormone analysis.

Plasma was analysed for the presence of 17ß-estradiol and testosterone in females, or 11-ketotestosterone (11-KT) and testosterone in males. For the analysis of 17ß-estradiol

and testosterone, a no-extraction, solid phase ^{125}I radioimmunoassay was used (Diagnostic Products Corporation, Los Angeles, CA, USA). Two testosterone kits were available: one for free, unbound plasma testosterone and the other for total testosterone, which tests for the combination of free and protein bound testosterone. The total testosterone assay was used for females, but was not used initially for male samples as the assay had a 16% crossreactivity with 11-KT. Instead the free testosterone assay was used for early male samples but the restriction to free hormone did not reflect the actual testosterone load in the plasma. When available, additional plasma for these initial male samples was analysed for total testosterone; otherwise, total testosterone was estimated using the following regressions based on samples where both free and total testosterone levels had been measured. For values between 0.55 pg/ml and 4.5 pg/ml of free testosterone the following relationship was used for estimating total testosterone: log(total T)= 0.83723(log free T)+0.220 ($F(1,50)=20.27$, $P<0.0001$, $r^2=0.80$). To prevent over-estimation, another relationship was used for values of free testosterone below 0.55 pg/ml: total T=1.86(free T)+0.484 ($F(1,18)=61.1$, $P<0.0001$, $r^2=0.76$). Males with estimated levels of total T include: 6/6 1997 males sampled in June, 1999 (22 mo.); 1/6 1997 males in October, 1999 (25.5 mo.); 6/6 1998 males October, 1999 (13.5 mo.) and 5/6 1998 males December, 1999 (15.5 mo.).

The method used to analyse 11-KT levels was adapted from the protocol outlined in detail in Harmin & Crim (1993). The general sequence of the method employed in the present study is summarized. Samples of 100 µl of plasma were mixed and incubated for one hour with 10 µl of ethanol-dissolved ^{3}H-testosterone isotope (1 000 to 2 000 CPM). Each sample was extracted twice in 2 ml of diethyl ether. In each extraction, the organic soluble

layer was isolated by freezing the heavier aqueous layer on dry ice and decanting the organic layer into a fresh test tube. The organic solvent was evaporated overnight at room temperature and the residue dissolved in one ml of double distilled ethanol. The amount of steroid recovered by extraction was estimated by measuring the radioactivity due to ^{3}H-testosterone isotope in each sample extract. A volume of 100 µl of extract was sampled at least one hour after the addition of ethanol; 10 ml of liquid scintillation fluid were added to the sample, and the radioactivity measured in a beta counter (MINAXIß Tri-Carb® 4000 Series, Canberra Packard, Canada) .

Extracts were stored in a fridge at 4°C prior to further processing. In preparation for an assay, duplicate aliquots of 100 µl of each sample extract, and of each standard in the standard curve, were transferred to assay tubes and left to evaporate overnight at room temperature. The next day the residue was dissolved in assay buffer (phosphate buffered saline: 28 mM $NaH_2PO_4 \cdot H_2O$, 61 mM Na_2HPO_4, 154 mM NaCl, 0.1% (w/v) gelatin, pH 7.0) and kept at 4°C in a cold-room. Additions of 100 µl of ^{3}H-11-KT tracer, in the amount of 13 000 CPM/ tube, and 100 µl of 11-KT antiserum, using the dilution factors of 1:30 000 and 1:50 000, were made to each standard and sample assay tube. The mixture was left to incubate overnight in the cold room. The next morning 600 µl volumes of dextran-coated charcoal suspension, previously prepared and stored at 4°C, were added to the appropriate tubes and left for an hour. The assay tubes were then centrifuged at 2 200 x g for 15 minutes at 4°C creating a charcoal pellet. The supernatant was decanted into scintillation vials to which 10 ml of liquid scintillation fluid were added for subsequent radioactivity measurement in the beta counter.

The sensitivities and detectable ranges of all the assays are presented in Table 2.3. Inter-assay and intra-assay variation for the different assays are as follows: 5.7% and 4.25%, respectively, for 17ß-estradiol assays (n=16), 4.8% and 7%, respectively, for total testosterone (n=5), 18.4% and 6%, respectively, for free testosterone (n=4), and 29% and 3.9%, respectively, for 11-KT assays (n= 4). The average extraction efficiency for 11-KT assays was 88.9%. The high inter-assay variation for 11-KT was due to one assay in particular which appears to have underestimated levels for samples taken in October of 1999. Excluding this assay in calculating the inter-assay coefficient of variation reduced the value to 12%.

2.2.6. Histological analysis.

Preserved tissue samples, previously stored in 70% ethanol, were further processed in an ethanol dehydration series, a clearing step using xylene, and an infiltration step in molten Paraffin wax. The tissue was embedded in Paraffin wax (Paraplast Plus®, Oxford® Labware, St. Louis, MO, USA). Blocks of ovarian tissue with large vitellogenic oocytes were trimmed in order to expose the tissue and soaked overnight in a mixture of one part glycerin/nine parts 60% alcohol for tissue softening. Sections were cut at 7 µm and placed on albumin coated slides. All slides were stained with Ehrlich's haematoxylin and eosin.

Categories of ovarian oocyte stages were based on those characterized by Wallace & Selman (1981), Scott (1987), and Kjesbu & Kryvi (1989) and previous descriptions for yellowtail flounder females by Howell (1983). The different cell types are as follows:

Oogonia- Smallest germ cells with a single nucleolus and scant cytoplasm. Both the cytoplasm and the nucleoplasm are weakly basophilic.

Primary Growth Phase (PG)

Oocytes of the primary growth phase are divided here into three stages which coincide with the early perinucleolus, resting, and late perinucleolus stages defined by Howell (1983).

PG-PN- Perinucleolar PG Oocytes. Oocytes with usually deeply basophilic cytoplasm and a nucleus containing several (1-6) nucleoli.

PG- CNR- Circumnuclear Ring PG Oocytes - Oocytes exhibiting cytoplasmic zonation. The zones include a deeply basophilic circumnuclear ring (CNR) composed of Balbiani bodies, and an outer zone of less basophilic cytoplasm. The CNR is first seen in juxtaposition with the nuclear membrane. It moves concentrically outward away from the nucleus until it meets the oolemma, gradually becomes less distinct and dissipates.

PG-Adv- Advanced PG Oocytes - Large oocytes with a large pale staining cytoplasm of a granular appearance and a large nucleus containing many small nucleoli. These oocytes may appear with a peripheral chromophobic zone and/or a single Balbiani body (a small, circular, deeply basophilic body usually seen in the middle of the cytoplasm or adjacent to the nuclear membrane).

Secondary Growth Phase

Cortical Alveolar Stage - Cortical alveolar oocytes are similar in appearance to advanced PG oocytes but feature distinct chromophobic vacuoles, which may lie near the periphery or in the middle of the cytoplasm (Plate 2.1). The cortical alveoli seen in yellowtail flounder resemble those described by Kjesbu & Kryvi (1989) for cod, *Gadus morhua*, which, like yellowtail flounder, produce a pelagic egg without an oil droplet.

Exogenous Vitellogenesis- Vitellogenic oocytes are classified here in three stages wherein vitellogenin incorporation is initiated, is progressing but in an early stage, or is advanced (Plate 2.2).

VG-I- Oocytes where eosinophilic yolk globules appear at the periphery of the oocyte. Yolk globules may be seen within the peripheral chromophobic ring area, if present.

VG-II- An early vitellogenic oocyte where sufficient yolk uptake has filled the outer half of the cytoplasm with yolk globules. The oocyte has begun to increase in size at this time.

VG-III- Vitellogenic oocytes where the cytoplasm has been filled completely with yolk. This is a period where the oocyte shows a large growth in size. The zona radiata becomes particularly prominent later in this stage (Plate 2.2).

Hydrated Oocytes- Oocytes characterized by the coalescence of formerly distinct yolk globules and the absence of a nucleus. Due to tissue processing with alcohol, the periphery of these large oocytes may be highly indented producing an irregular shape.

Atretic Oocytes- These structures demonstrate yolk or cytoplasmic resorption and infolding of the follicle, which is enlarged in some cases. In early vitellogenic and advanced primary growth oocytes undergoing atresia, the cytoplasm is pale and a nucleus is absent.

For male yellowtail flounder, descriptions of cell types during testicular development agree with those characterized for winter flounder in Harmin et al. (1995). The testes of yellowtail flounder are the lobular-type seen in many teleosts (Billard et al., 1982), and lie on either side of the first haemal spine forming the posterior border of the abdominal cavity (Shangguan, 1998). In the lobular testis, spermatogenesis occurs within stationary cysts comprised of a nest of germ cells with somatic Sertoli cells at the periphery (Grier & Lo

Nostro, 1999; Schulz et al., 1999). The cyst structure deteriorates during advanced spermiogenesis and testicular sections show a lack of organization once the testis is ripe with mature spermatozoa.

Primary and Secondary Spermatogonia. Spermatogonial cells have large nuclei containing a single nucleolus. Primary spermatogonia typically have a pale cytoplasm and nucleoplasm and are the largest germ cells in the testis (Plate 2.3). Secondary spermatogonia were smaller and generally more basophilic than their progenitors.

Primary and Secondary Spermatocytes. Primary spermatocytes exhibit pale cytoplasmic staining, while the nuclear region is extremely basophilic due to chromatin condensation. Secondary spermatocytes are discerned as similar cells to primary spermatocytes but of lesser size with a stronger staining cytoplasm and a weaker staining nucleoplasm.

Spermatids have darker nuclear staining and a reduced cytoplasmic volume compared to spermatocytes. Cysts containing spermatids are characterized by a disaggregation of the cells within the cyst due to the onset of spermiogenesis. The differentiation of spermatids to spermatozoa (i.e. spermiogenesis) is evident by the elimination of cytoplasm and the production of eosinophilic flagella. A cyst containing mature spermatozoa is identifiable by tracts of flagella formed from similarly oriented sperm cells. As more cysts within a lobule produce mature spermatozoa, the structure of the cysts degenerates and the contents of adjacent cysts fill the lobular lumen, the testis soon appears dissociated as the whole testis fills with spermatozoa. The beginning of spermiation was detected in the present study as soon as milt could be expressed from the urogenital pore. Males were considered in full spermiating condition when milt volume increased to levels associated with spawning.

2.2.7. Statistical analysis.

Statistical analyses were performed using SAS (Statistical Analyses System, 1989). All body size and reproductive variables were expressed as means (±SD). Body size characteristics included standard length, body weight, and carcass weight (= body weight - viscera and gonad weights). Standard length was preferred over total length as cultured fish exhibit a high variability in caudal fin ray length due to conspecific aggression and some erosion of the fin rays. Reproductive variables included gonadal weight, ovarian length, hormone levels and the gonadosomatic index. Hepatosomatic index was calculated as a measure of energy storage, the liver being a major fat storage area in flatfish.

Gonadosomatic Index (GSI)= (total gonad weight / body weight) x 100%

Hepatosomatic Index (HSI)= (liver weight / body weight) x 100%

Due to differences in the degree of replication among different *in vitro* incubations, the data for replicate wells in individual incubations were reduced to means. Hence an individual incubation, representing tissue from a single female or pooled tissue from a set of females, became the replicated unit for statistical analyses. Mean tissue responsiveness data for any group of females is therefore presented in figures as mean 17ß-estradiol output (±SE). Two 1998 females, one in the 13.5 month sample and another in the 15.5 month sample, were excluded from analysis due to low or no steroidogenic output *in vitro* or in the plasma. Each female had a red sore, indicative of possible disease, the 15.5 month old female had no food in the digestive tract.

Changes in body size variables and reproductive variables for males and females over time were analysed by one-way ANOVA with the General Linear Models (GLM) procedure

of SAS (1989). One or two way ANOVA was used to compare year class differences within each sex. Incubation data were analysed by one-way ANOVA to examine treatment effects on tissue responsiveness, and two-way ANOVA for the added effect of sample/time differences. All ANOVAs were followed by pair-wise comparison tests including Tukey's HSD test and least square means test. Regression analyses between free and total testosterone were also performed with the GLM procedure.

Sex differences in growth were examined by heterogeneity of slopes tests. Only 1997 year class males and females sampled from 18 to 34 months were analysed for sex differences, since fish between these ages were sampled from one site without interruption.

Residuals were tested for homogeneity and normality in all analyses, $\log_{10}$ and arcsine transformations were used when required. The Kruskal-Wallis Test, Wilcoxon two sample test, and the Sheirer-Ray-Hare Extension of the Kruskal-Wallis Test (for two-way ANOVA situations) were employed as non-parametric alternatives when parametric assumptions in ANOVAs could not be met by $\log_{10}$ transformation. In the Sheirer-Ray-Hare test, data were ranked and then analysed by two-way ANOVA, the Chi-squares were calculated from effect Sum of Squares divided by total Mean Square (total Sums of Squares/ total degrees of freedom) (Sokal & Rohlf. 1995).

The F-statistic from a one-way ranked ANOVA may be used as an equivalent statistic to the Kruskal-Wallis Chi-square when n is high (a value of 25 or more was used in this study)(SAS, 1989). In these cases, pairwise comparisons were done by Tukey's HSD and least square means tests.

Table 2.1. Sampling information for yellowtail flounder of the 1997 and 1998 year classes.

Sampling Date	Age (mo.)	Source Population and Health Status of Sampled Individuals.
1997 year class		
Sept. 1998	12.5	OSC. : sample population infected with furunculosis.
Feb. 1999	18	Heart's Content : healthy sample population.
June 1999	22	Heart's Content : healthy sample population.
Oct. 1999	25.5	Heart's Content : healthy sample population.
Dec. 1999	28	Heart's Content : healthy sample population.
April 2000	32	Heart's Content : Fish stressed-water supply interruption 2 wks prior to transport. Only two females and one male were feeding overtly 6 weeks post-transport.
May 2000	32.5	Heart's Content : healthy fish. Supplemental blood sampling of six males and six females. Fish were not sacrificed and samples were taken on site.
June 2000	34	Heart's Content : healthy fish, blood sampled and sacrificed on site.
1998 year class		
Oct. 1999	13.5 14A 14B	Sept 30- Heart's Content: healthy sample population Oct 20- OSC: Six healthy females OSC group, and Oct 21- OSC: Five healthy males from recently infected tank Oct 20- OSC: Five healthy females from recently infected tank
Dec. 1999	15.5	Heart's Content: signs of furunculosis-like infection
April 2000	20	Females- OSC: healthy sample population. Males- Heart's Content: infected population.
May 2000	20.5	Heart's Content: Three supplemental males were sacrificed and blood sampled on site from an infected population.
June 2000	22	Females- OSC: Healthy mature and immature females. Males- Heart's Content: Males sacrificed on site from an infected population.

Table 2.2. Ovarian ranking scale used to describe ovarian development estimated by external examination.

Ovarian rank (OR)	**Proportion of ovarian length in relation to length of ovarian cavity.**	**Characteristics of ovaries observed from external examination.**
1	<1/2	Small, pink, translucent ovaries: very immature, triangular shape
2	1/2*	Small, elongating, translucent ovaries. Many yellow or pink; a reddish colour may indicate some change towards puberty.
3	1/2+	Majority still pale, translucent, immature. Reddish colour seen in the pubertal onset period. Few cases of orange opaque appearance or thickening of the ovary with pubertal vitellogenesis.
4	2/3	Orange colour prevalent, ovaries become opaque, and thicken with vitellogenesis. A few cases of ovaries that remain translucent and pale as in earlier ranks.
5	2/3+	Ovaries are orange, thickening or thick, opaque, and take an orange white hue when vitellogenesis is advanced. Some asymmetry in ovarian length has been observed in individuals, one ovary appearing longer than the other but both in the same stage of maturation. Sometimes the end of the ovary had reflexed 180° rather than continuing to grow posteriorly, which explained the asymmetry.
6	3/4	
7	3/4+	
8	full	

* Ovaries reaching the 1/2 proportion have their posterior tips aligned approximately with the widest part of the fish.

Table 2.3. Standard curve limits, and limits of detection for the different radioimmunoassays used in the study.

Assay	Lowest Standard Value	Maximum Standard Value	Limits of Detection
Coat-A-Count Estradiol	20 pg/ml	3 600 pg/ml	8 pg/ml
Coat-A-Count Total Testosterone	200 pg/ml	16 000 pg/ml	40 pg/ml
Coat-A-Count Free Testosterone	0.55 pg/ml	50 pg/ml	0.15 pg/ml
11-ketotestosterone	0.098 ng/ml	100 ng/ml	0.098 ng/ml

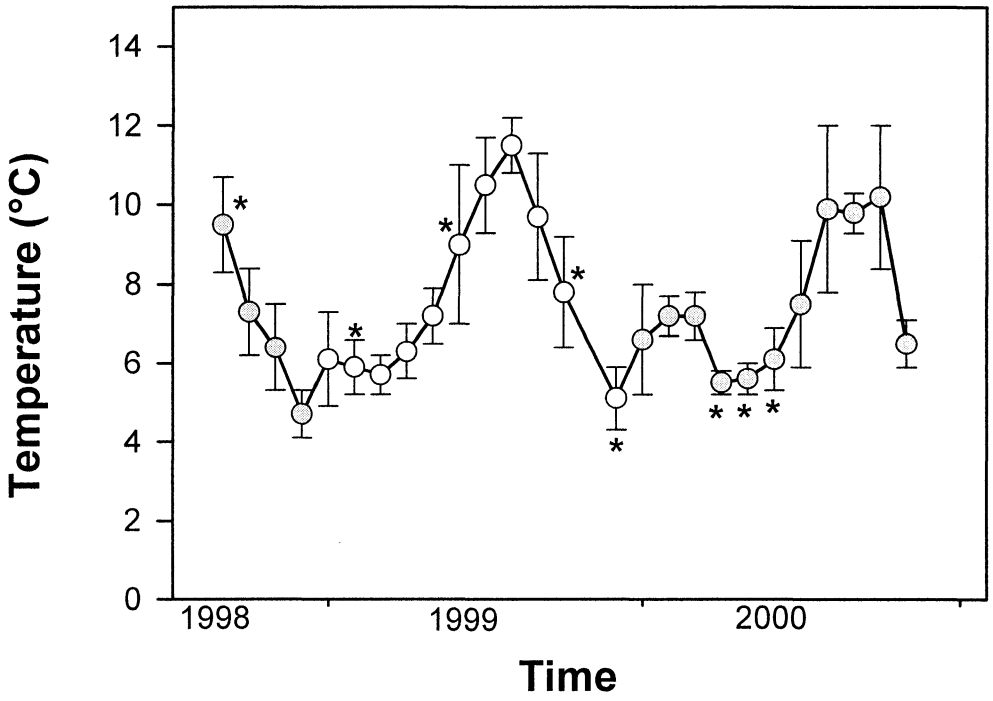


Figure 2.1. Water temperature profile from Sept. 1998 to December 2000 at the Ocean Sciences Centre facility. Monthly means (±SD) are plotted, shaded circles represent data for 1998 and 2000. ' * ' symbols indicate sample times.

2.3. RESULTS.

2.3.1. Female development of the 1997 year class.

Changes in body size, carcass weight, GSI, ovarian length (OL), HSI, and hormone levels were all highly significant ($P<0.0001$) over the sampling period (Sept. 1998 - June 2000) for 1997 females. Mean body weight increased linearly in 1997 females resulting in a ten fold difference in size over time (Figure 2.2). In contrast, increases in GSI followed a two phase pattern: an immature phase which demonstrated slow but statistically significant ovarian growth between 12.5 and 22 months; and, a pubertal phase, between 22 and 34 months, during which ovaries grew rapidly to peak values at full maturity (Figure 2.2). During immaturity, the ovaries of 1+ (i.e. one year old) 1997 females contained oocytes in primary growth stages (Figure 2.3). Immature ovaries progressed from having only oogonia and early perinucleolar oocytes (PG-PN) in September (12.5 mo.) (Plate 2.1C), to the circumnuclear ring stage (PG-CNR) predominant in February (18 mo.), then to the advanced primary growth stage (PG-Adv) in June (22 mo.) (Figure 2.3).

The initiation of puberty was detected in June at 22 months of age in two females. In these females, a low incidence of newly vitellogenic oocytes (VG-I) was seen among cortical alveolar oocytes (CA) and advanced primary growth oocytes with granular cytoplasm (Figure 2.3). Oocytes with granular cytoplasm dominated the ovaries of both immature and pubertal females in June. These oocytes often contained a single cytoplasmic Balbiani body and/or a peripheral chromophobic ring (not composed of cortical alveoli).

Puberty was well underway in October at 25.5 months when all females sampled had large yolky oocytes (VG-III), with the exception of one female. This individual had VG-I and

VG-II oocytes (GSI=2%) which indicated that vitellogenin incorporation had been initiated recently (Plate 2.2A). By December at 28 months, all females had evidence of VG-III oocytes and were still recruiting oocytes into vitellogenesis while PG-Adv oocytes dwindled (Figure 2.3; Plate 2.2D). Evidence of atresia was noted for the first time in December ovaries and was detected in all subsequent samples (Plate 2.2E). In April (32 months), females were in prespawning condition with ovaries dominated by VG-III oocytes with thick zona radiata follicles. Lesser vitellogenic oocytes were absent and cortical alveolar oocytes were rare which indicated that vitellogenic recruitment was complete (Figure 2.3). Advanced primary growth oocytes rarely seen in December, became prevalent once more in April. By June, females of 34 months of age were fully mature, ovaries had peaked in size, and two of the females had ovulated (Body Wt= 211-327g; Carcass Wt=153-239g; GSI 21-24%; OL=10.9-13.0 cm; OR= 8 ; Figures 2.2 & 2.3; Plate 2.2F,G).

Plasma levels of 17ß-estradiol followed the same biphasic pattern seen in GSI values and histology (Figure 2.2). In the first sample at 12.5 months, only one female had detectable 17ß-estradiol levels in the plasma (0.08 ng/ml: PG-PN). The next measurement in June at 22 months detected low amounts of 17ß-estradiol in the plasma of all females, with similar levels seen between immature females (0.12 to 0.21 ng/ml) and newly pubertal females (0.15 and 0.23 ng/ml). A comparison of immature and pubertal 1997 females in June is shown in Table 2.4. Levels of testosterone were not determined in these early samples.

Mean levels of 17ß-estradiol increased ten fold with vitellogenic activity following the initiation of puberty (Figure 2.2). Values were variable (0.44 -3.29 ng/ml) in October at 25.5 months of age; the female with the lowest levels was the recent pubertal initiate with

only VG-I &-II oocytes. Amounts of 17ß-estradiol were similar in December at 28 months of age (1.8 to 2.8 ng/ml), values above 2 ng/ml at this time were associated with obvious zona radiata follicles. For testosterone, very low levels were detectable in only three of five females in October ($\leq$0.13 ng/ml). By December all females had low amounts of testosterone in the plasma (0.2-0.48 ng/ml) (Figure 2.2).

Hormone levels at prespawning were best represented by the supplemental sample in May at 32.5 months. For most females, 17ß-estradiol levels were between 2.6 and 4.4 ng/ml while testosterone remained low, 0.29-0.5 ng/ml. However, one female had the highest levels yet seen, 10.5 ng/ml of 17ß-estradiol and 2.3 ng/ml of testosterone. A high variability in hormone levels was established at 34 months in June, during the early part of the spawning season, both in ovulating females (17ß-estradiol: 2.3 to 7.3 ng/ml; testosterone: 2-2.1 ng/ml) and preovulatory females (17ß-estradiol: 0.49 to 6.9 ng/ml; testosterone: 0.27 to 1.4 ng/ml).

A newly pubertal female was seen among the fully mature females at 34 months. The ovaries contained VG-I oocytes, on the threshold of entering the VG-II stage (Table 2.4). Levels of 17ß-estradiol and testosterone were 0.307 and 0.191 ng/ml, respectively. Besides the onset of vitellogenin incorporation, oogonial mitosis (prophase) was seen in this female.

2.3.2. Female development of the 1998 year class.

While females of the 1997 year class initiated puberty between 22 and 25.5 months of age, some 1998 year class females showed accelerated oogenesis at 13.5 months of age. Changes seen in 1998 females for whole body weight, standard length, carcass weight, and HSI were highly statistically significant over the period of October, 1999 to June, 2000 (P<0.0005). Patterns in GSI and sex steroids were divided between two groups of females,

those which matured after reaching one year of age and those which remained immature as 1+ fish (Figure 2.4). For maturing females, all reproductive variables increased significantly over time ($P<0.005$). For females which remained immature, reproductive variables showed small yet statistically significant changes from 13.5 to 22 months of age ($P \leq 0.02$).

Three sets of 1998 females were sampled in October, one at 13.5 months and two sets of small fish at 14 months of age (Table 2.4). A statistical comparison of these three groups of females indicated significant differences in body size ($P<0.0001$), GSI ($P=0.01$) and plasma 17ß-estradiol levels ($P=0.02$). A division between females with advanced ovarian development and those potentially remaining immature was seen in six females sampled at 13.5 months (Table 2.4). Three females over 40 g had GSI values equivalent to 18 and 22 month old females of the 1997 year class. The ovaries of these females featured cortical alveolar oocytes as well as PG-Adv oocytes with granular cytoplasm, and in some cases a single Balbiani body and/or peripheral chromophobic areas (Plate 2.1D,F). Plasma levels of 17ß-estradiol were elevated revealing pubertal activation of the ovaries. The three smaller females of the 13.5 month sample had clearly immature ovaries which had progressed to the PG-CNR stage, although one female had some PG-Adv oocytes with granular cytoplasm. This latter female had 17ß-estradiol levels similar to those of pubertal 22 month old 1997 females, while the other two females had barely detectable to low levels of 17ß-estradiol. Testosterone was non-detectable in all 13.5 month females, except the advanced female with the highest amounts of 17ß-estradiol (T=0.125 ng/ml).

The first group of 14 month old, October females (14A sample) overlapped in body size, ovarian size and 17ß-estradiol levels with values seen in slower developing females at

13.5 months (Table 2.4). No tissue was available for histology from this group due to use of tissue in an incubation trial. The second group of 14 month old fish (sample 14B) represented small females with immature ovaries weighing 0.05 to 0.15 g (Table 2.4). Ovaries contained PG-CNR oocytes, PG-PN oocytes, oogonial nests, as well as oocytes in transitional states between these three stages (Plate 2.1A,B). Oogonial mitosis also was observed in three females (Plate 2.1A). Very low but detectable plasma 17ß-estradiol levels were seen in the 14B sample females (mean 0.033 ng/ml, Table 2.4). Small plasma volumes precluded any analysis of testosterone levels in these females.

In subsequent samples in early December (15.5 mo.) and mid-April (20 mo.), four of six 1998 females in each sample demonstrated pubertal development, while the remaining females were still immature. The development of pubertal and immature females up to 22 months of age is described separately in the following sections.

2.3.2.1. Pubertal 1+ 1998 females.

In pubertal females, vitellogenesis was well underway by December at 15.5 months and recruitment of new vitellogenic oocytes was ongoing (Figure 2.3). By April (20 mo.), females had large ovaries containing large VG-III oocytes with thick zona radiata follicles. Only one female still had some VG-II oocytes among larger oocytes which suggests that recruitment into vitellogenesis was either still possible or had been recently completed. Three of the four pubertal females had advanced primary growth oocytes, but no cortical alveolar oocytes were seen. Atresia was detected in two females by April, and was seen again in June.

Rapid increases in GSI and plasma 17ß-estradiol levels accompanied vitellogenesis in pubertal females (Figure 2.4). In December, levels of 17ß-estradiol were still below 2

ng/ml, ranging between 0.51-1.63 ng/ml. Levels of 17ß-estradiol had increased to a range of 2 to 3.1 ng/ml by prespawning in April. Testosterone was low (<0.35 ng/ml) or non-detectable in December, but was detectable in all four females in April (0.18-0.58 ng/ml).

By June at 22 months of age, three of six mature females sampled had ovulated. Females at full maturity had high GSI values and were variable in body size, including small as well as large sized individuals (Body Wt= 62 -172 g; Carcass weight = 42-127 g; GSI= 22.7-28%; OL=6.7-12.0 cm; OR= 6-8). Ovaries had large yolky oocytes, and in ovulating females evidence of yolk coalescence and hydration (Figure 2.3). Hormone levels were variable in both preovulatory females (17ß-estradiol: 4.9-14.4 ng/ml; testosterone: 2.1- 7.1 ng/ml) and ovulating females (17ß-estradiol and testosterone levels were as high as 6.5 and 2.2 ng/ml, respectively, or, as low as 0.13 and 0.16 ng/ml, respectively).

A comparison of the degree of maturation between 1997 and 1998 pubertal females showed no year class differences in plasma hormone profiles, or in GSI values at full maturity. In contrast, mean ovarian weight for 1997 females at full maturity (60.9 ±10.2 g) was significantly greater (P<0.0001) than the mean for 1998 females (25.0 ±8.1 g). Comparing these means directly shows that females maturing as 1+ animals reached 41% of the ovarian investment of 2+ (i.e. two year old) maturing females. Further, fully mature ovaries for 1998 females were not significantly heavier than values for 1997 females in December and April, when GSI ranged between 9 and 16% of the whole body weight.

2.3.2.2. Immature 1+ 1998 females.

Ovaries of the four immature females sampled at 15.5 and 20 months of age remained small with mean GSI values equivalent to those seen in October (Table 2.4; Figure 2.4).

Oocytes remained in the PG-CNR stage of development throughout this period. Levels of 17ß-estradiol, detectable in the plasma of three of the four females, were within the range of values seen in females with PG-CNR oocytes in October (13.5 & 14 months). Plasma testosterone levels were non-detectable.

The initiation of puberty was detected in a sample of six females in June at 22 months of age. Mean GSI values and plasma 17ß-estradiol levels had increased significantly from levels seen between 14 and 20 months of age (Figure 2.4). When comparing these means to values seen at the initiation of puberty at 13.5 months, only GSI levels were significantly higher than those of 13.5 month old females (Table 2.4; Figure 2.4). Vitellogenic oocytes (VG-I) were seen in two females whose 17ß-estradiol levels were similar to those of 1997 females of the same age, while testosterone was not detected (Table 2.4). Three other females with cortical alveolar oocytes had higher 17ß-estradiol levels (Table 2.4; Plate 2.1E). Low amounts of testosterone were detectable in two of these females (0.083 & 0.22 ng/ml). The sixth female was still immature with a lower level of 17ß-estradiol than other females and no testosterone detectable in the plasma (Table 2.4). All females sampled had advanced primary growth oocytes of granular cytoplasm with single Balbiani bodies and/or peripheral chromophobic zones.

2.3.3. Ovarian tissue incubation *in vitro*.

In vitro incubation data for immature and newly pubertal females from the 1997 and 1998 year classes were sorted and analysed according to age and histological stage (Figure 2.5). Pooling the data from 1997 and 1998 year class females of 22 months of age was necessary for the advanced primary growth and VG-I stages.

For immature tissue (upper plot Figure 2.5), 17ß-estradiol production of 15.5 and 20 month old 1998 females was significantly lower ($P<0.0001$) than tissue sampled at any other time. Despite low output, tissue from these females showed a statistically significant steroidogenic response to both forskolin and crude pituitary extract (CPE), exceeding levels seen in control wells ($P<0.01$). Moreover, a dose response to CPE was demonstrated. Steroid output of 14 month old females in October also was significantly stimulated by forskolin and CPE ($P<0.005$), but no dose responsiveness to the latter was seen. Both sets of females were in the PG-CNR stage, although this could not be confirmed in some 14 month old females for which tissue was not available for histological analysis. For these females some PG-Adv oocytes may have been present, as was the case in one immature 13.5 month female. Immature 1997 and 1998 females (22 mo) with advanced primary growth oocytes, featuring chromophobic peripheries and/or a Balbiani body, demonstrated a steroidogenic response to stimulatory agents which was of borderline statistical significance ($P=0.05$). Steroid production from these females was not significantly different from 14 month old females ($P>0.05$).

Females initiating puberty demonstrated significantly higher levels of overall steroidogenic output than immature females ($P<0.0001$; Figure 2.5, lower plot). For advanced 13.5 month females with cortical alveolar oocytes, incubations revealed that the tissue was unequivocally responsive to both forskolin and the high dose of CPE ($P=0.0004$). This was not the case in a group of three older 22 month females with cortical alveolar oocytes, all from the 1998 year class ($P>0.05$). However, analyses of the data for these individuals revealed that two of the three females did respond significantly to both forskolin

and CPE stimulation ($0.05>P>0.0001$). Tissue from four 22 month females with vitellogenic oocytes showed a significant response to forskolin and a dose response to CPE ($P<0.05$), the highest CPE dose stimulating levels significantly higher than those of controls. Further comparison revealed that whether containing vitellogenic oocytes or only cortical alveolar oocytes, ovaries at the initiation of puberty did not differ in the overall level of *in vitro* steroid output ($P>0.05$).

A comparison of plasma levels of 17ß-estradiol between the different stages revealed an increasing trend during immature stages to peaks in the cortical alveolar stage and a subsequent decrease once vitellogenin began to be incorporated by oocytes. Pairwise comparison tests from statistical analysis on this data (stage effect, $P=0.0012$) showed three groupings in which mean plasma levels were not significantly different: i) early immature females; ii) vitellogenic and advanced primary growth females in June; and, iii) cortical alveolar oocyte females in October and June.

Tissue responses to stimulation by crude pituitary extract and forskolin became more statistically significant as puberty progressed (Figure 2.6). Changes in steroidal output patterns over puberty are represented by data from 1997 females in October at 25.5 months, December at 28 months and in maturing 1998 females in April at 20 months. Tissue output was similar between October and December ($P>0.05$) but tissue from April females, two months prior to spawning, became significantly more responsive ($P<0.0001$). A notable feature was a stronger dose sensitivity to crude pituitary extract in October and December which became less statistically distinct in April. Of particular interest is the reversal of the dose response in April as lower levels of crude pituitary extract induced the highest

steroidogenic response. Stimulation by forskolin remained similar over time although the response seemed depressed in December. Plasma 17ß-estradiol appeared to remain at stable levels during the main vitellogenic period after having increased since the initiation of puberty.

2.3.4. Relationships between ovarian rank estimates and gonadal stage.

Plotting GSI or ovarian length data against ovarian rankings determined by external examination showed that the ranking system can be used to estimate ovarian development in young yellowtail flounder (Figure 2.7). Immature ovaries had ranks of one to three, ovaries in early puberty show ranks of two to four, and maturing ovaries from three to eight (Figure 2.7). Ovarian thickness and colour, which is discernable in young fish, further helped to estimate ovarian development and the degree of vitellogenesis in maturing females (Table 2.2). Females with obvious, maturing, vitellogenic ovaries had ranks mainly of five and above. The lack of any pubertal females with an ovarian rank of one or an ovarian length of less than 2.5 cm suggests that the ovary must reach a certain size prior to the onset of puberty. However, the length of the ovary at the time of puberty is variable (2.75- 4.90 cm, 5.9 cm in a 34 month old). Ovarian weight at puberty has an even greater range in values, from 0.73 to 3.69 g. In contrast, the plot comparing GSI and ovarian rank data demonstrates that ovaries at the onset of puberty remain within a tight range of GSI values, mostly between 1.7-2.2% (the older 34 month old pubertal female with a GSI of 3.2% was an exception). Few immature or maturing ovaries in one or two year old fish overlapped with the 1.7-2.2% GSI range at the onset of puberty.

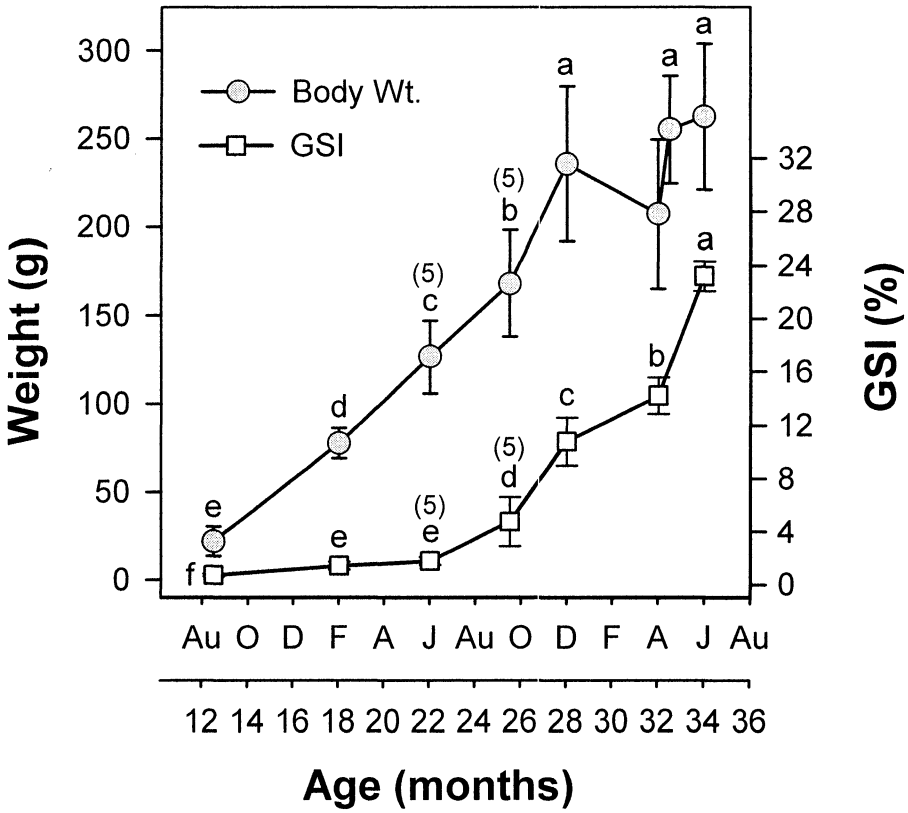
Table 2.4. Comparison of females at the onset of puberty (shaded rows) with immature females (unshaded rows) sampled coincidentally or at other periods.

Age (mo)		Wt (g)	GSI (%)	OL (cm)	OR	Stage	E2 (ng/ml)
1997: Onset of puberty in females sampled at 22 months of age (June, 1999).							
22.0	*n*=2	108 - 142	1.88 - 2.20	3.7 - 4.8	2, 4	VG-I	0.148 - 0.225
22.0	*n*=3	103 - 149	1.43 - 1.72	3.4 - 4.0	2	PG-Adv	0.119 - 0.213
1997: Onset of puberty in a female of 34 months (June, 2000).							
34.0	*n*=1	225	3.23	5.9	3	VG-I	0.307
1998: Onset of puberty in 1+ females at 13.5 months compared with immature females sampled at 14 months (Oct., 1999) and 15.5-20 months (Dec., 1999- April, 2000).							
13.5	*n*=3	42 - 67	1.72 - 1.79	2.8 - 3.4	2 - 3	CA	0.345 - 0.725
13.5	*n*=3	18 - 25	0.78 - 1.03	1.5 - 2.5	2 - 3	PG-CNR PG-Adv	0.009 - 0.065 0.194
14A	*n*=6	16 - 24	0.46 - 1.40	1.1 - 1.9	1 - 3	n/a	0.028 - 0.120
14B	*n*=5	7 - 16	0.46 - 0.96	0.9 - 1.1	1	PG-CNR	0.028 - 0.040
15-20	*n*=4	72 - 103	0.92 - 1.28	2.8 - 3.4	1 - 2	PG-CNR	0.021 - 0.065
1998: Onset of puberty in females sampled at 22 months of age (June, 2000).							
22.0	*n*=2	160 - 188	1.97 - 2.12	4.5 - 4.6	2- 3	VG-I	0.206 - 0.220
22.0	*n*=3	60 - 123	1.78 - 3.02	3.1 - 4.9	2- 4	CA	0.273 - 0.394
22.0	*n*=1	98	1.43	3.9	3	PG-Adv	0.152

Females of different year classes, ages and histological stages are shown.

Values in the table represent ranges for Wt= body weight; GSI= gonadosomatic index; OL= ovarian length; OR= ovarian rank; E2= plasma 17ß-estradiol levels; Stage= histological stage (most advanced oocyte stage present in the ovary).

Figure 2.2. Mean (±SD) body weight, GSI, 17ß-estradiol and testosterone changes over time for 1997 year class females. The main x-axis indicates month of the year starting from August, 1998 to August, 2000, the second axis indicates age in months. Means along the same plot which are labeled with the same letter are not significantly different ($P > 0.05$). The sample size (n) is indicated when the number of individuals in a sample is less than six.


Body Wt.
GSI
Weight (g)
GSI (%)
Age (months)
Au O D F A J Au O D F A J Au
12 14 16 18 20 22 24 26 28 30 32 34 36

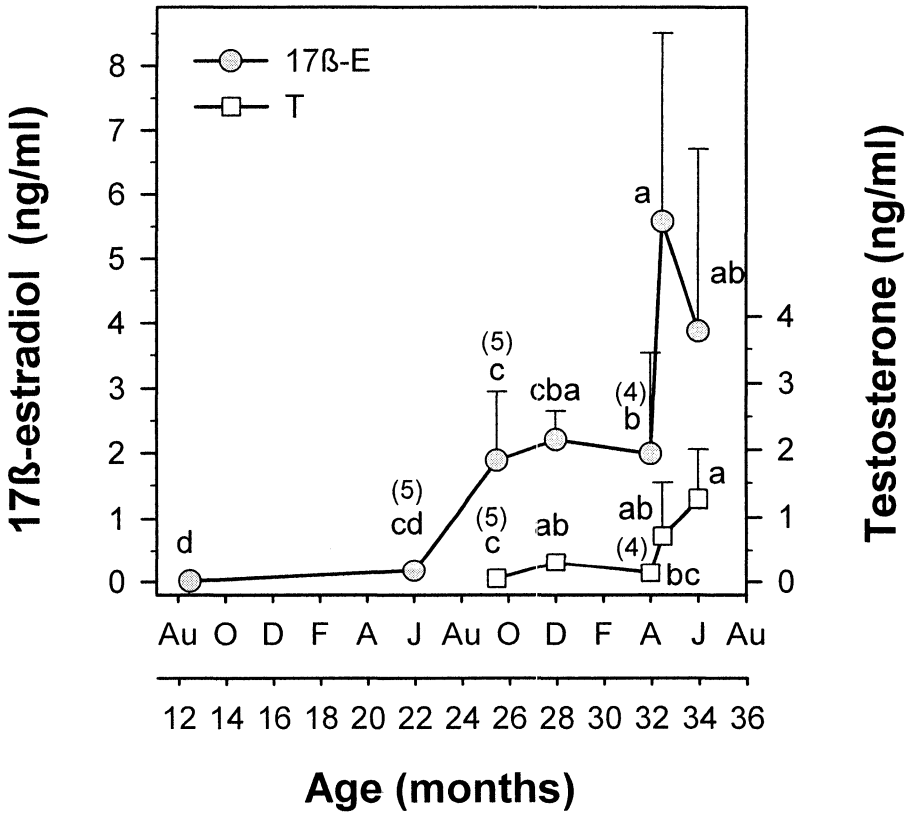
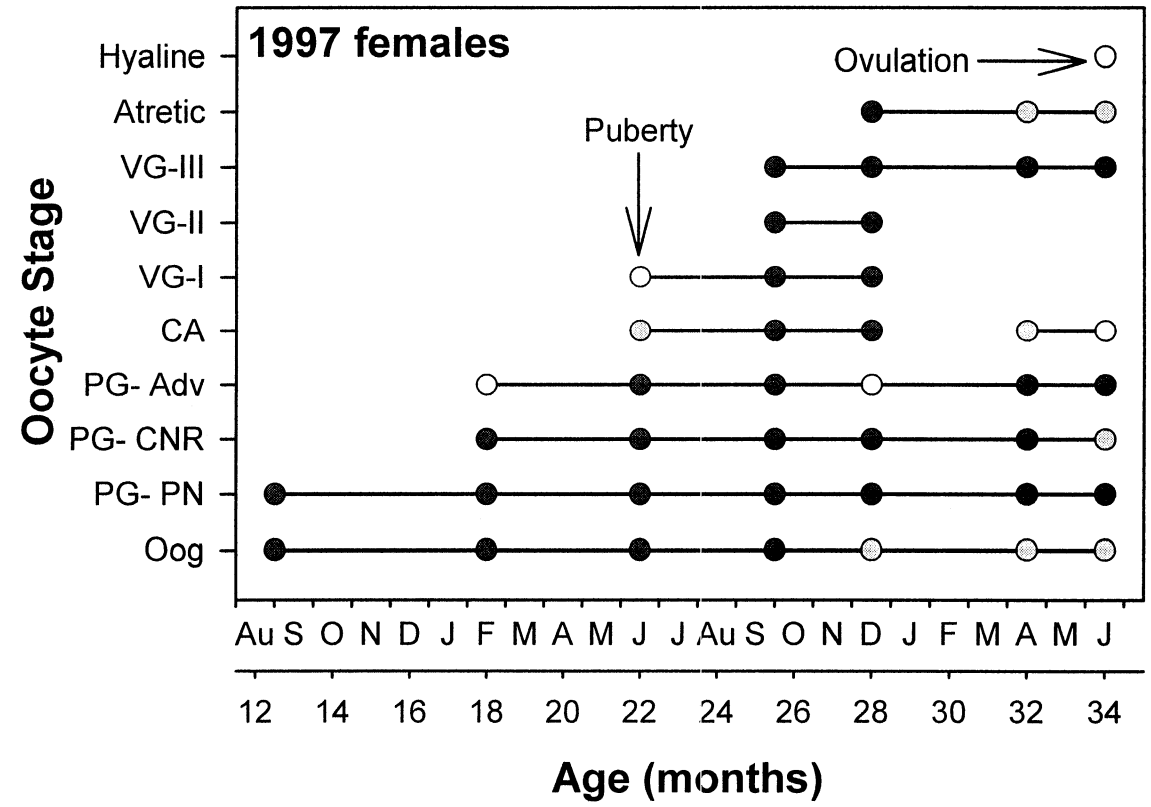

17ß-E
T
17ß-estradiol (ng/ml)
Testosterone (ng/ml)
Age (months)
Au O D F A J Au O D F A J Au
12 14 16 18 20 22 24 26 28 30 32 34 36

Figure 2.3. Histological changes in the ovaries of 1997 and 1998 females over time. The main x-axis indicates month of the year starting from August, 1998 to June, 2000 for 1997 females, and August, 1999 to June, 2000 for 1998 females; the second axis indicates age in months.

Different histological stages include: oogonial (Oog), Primary growth (PG) stage (substages - PN=perinucleolar, CNR= circumnuclear ring, Adv=advanced), Cortical Alveolar (CA), Vitellogenic (I, II, III), Atretic and Hyaline. The percentage of sampled females exhibiting each stage is indicated according to three classes: 0-33 %, 33-66 % and 66-100 % represented by clear, grey and dark grey symbols, respectively.


1997 females
Ovulation
Puberty
Hyaline
Atretic
VG-III
VG-II
VG-I
CA
PG- Adv
PG- CNR
PG- PN
Oog
Oocyte Stage
Au S O N D J F M A M J J Au S O N D J F M A M J
12 14 16 18 20 22 24 26 28 30 32 34
Age (months)

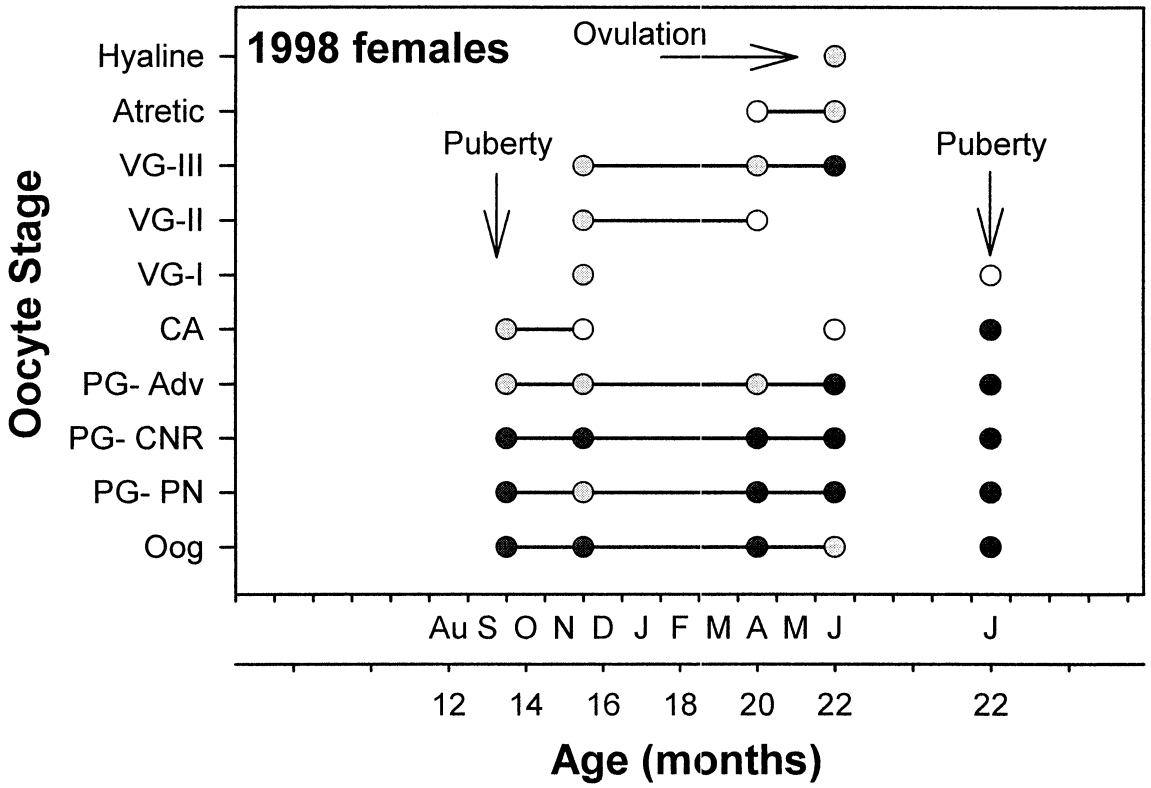
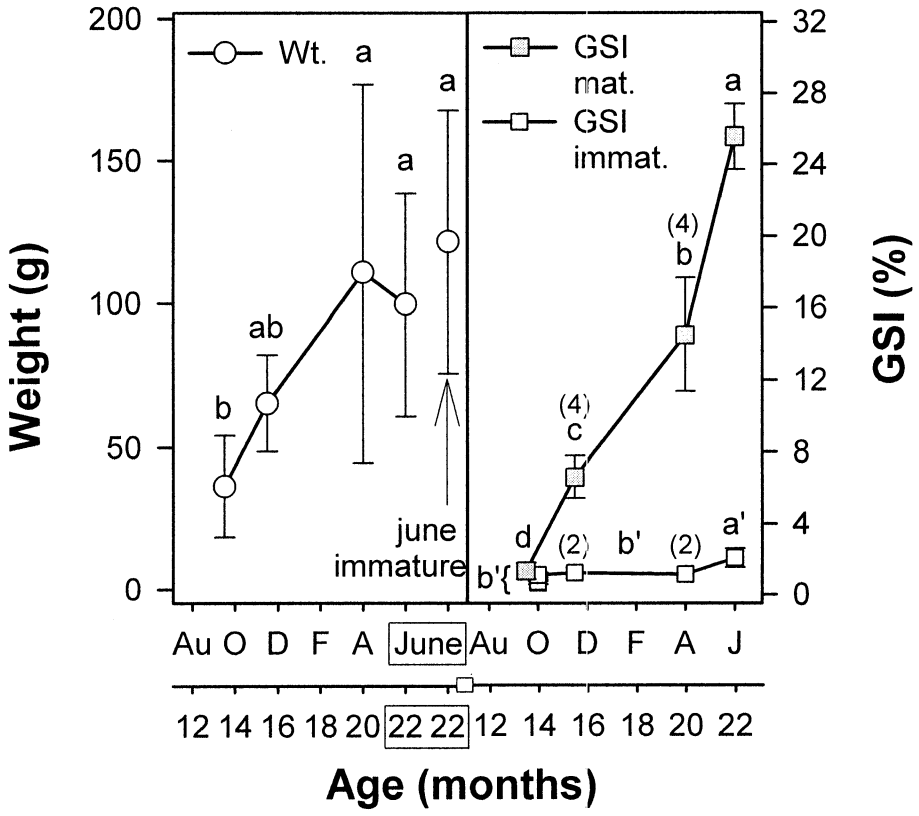

1998 females
Ovulation
Puberty
Puberty
Hyaline
Atretic
VG-III
VG-II
VG-I
CA
PG- Adv
PG- CNR
PG- PN
Oog
Oocyte Stage
Au S O N D J F M A M J J
12 14 16 18 20 22 22
Age (months)

Figure 2.4. Mean (±SD) body weight, GSI, 17ß-estradiol and testosterone changes over time for 1998 year class females. Data for maturing (mat.) and immature (immat.) females are plotted separately. The main x-axis indicates month of the year starting from August, 1999 to June, 2000, the second axis indicates age in months. Means which are labeled with the same letter are not significantly different ($P > 0.05$). Letters followed by an apostrophe refer to analyses on plotted data for immature females (GSI and 17ß-estradiol)- October females sampled at 13.5 months of age also are included in the analyses on immature females. The sample size (n) is indicated when the number of sampled individuals is less than six.


Weight (g)
GSI (%)
Wt.
GSI mat.
GSI immat.
june immature
Au O D F A June Au O D F A J
12 14 16 18 20 22 22 12 14 16 18 20 22
Age (months)

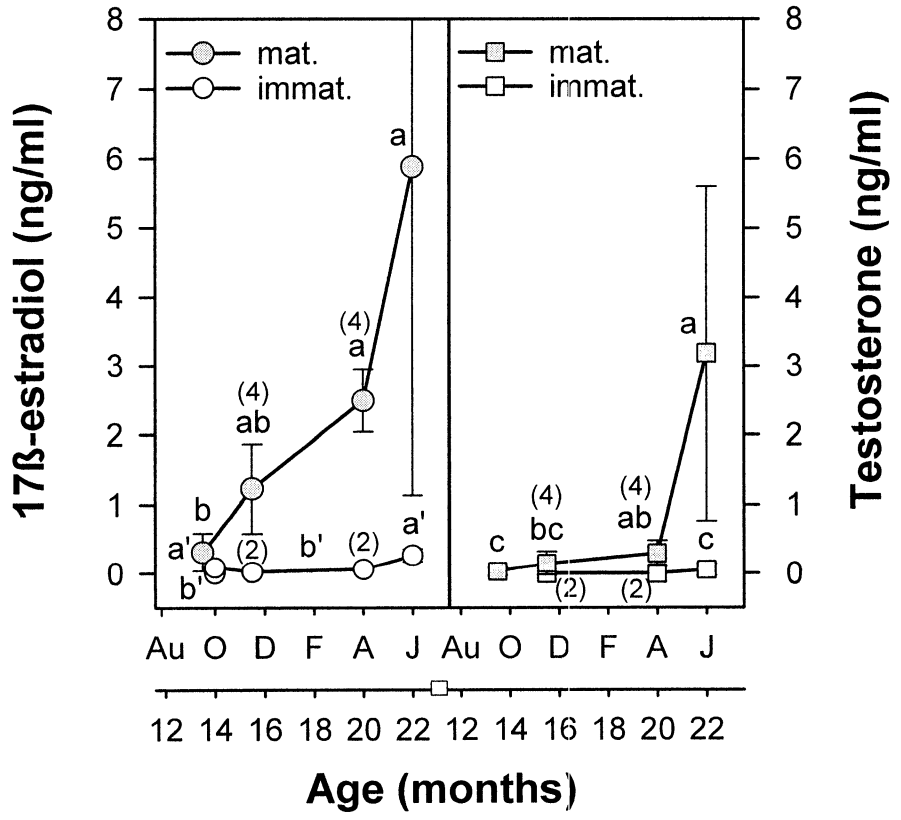
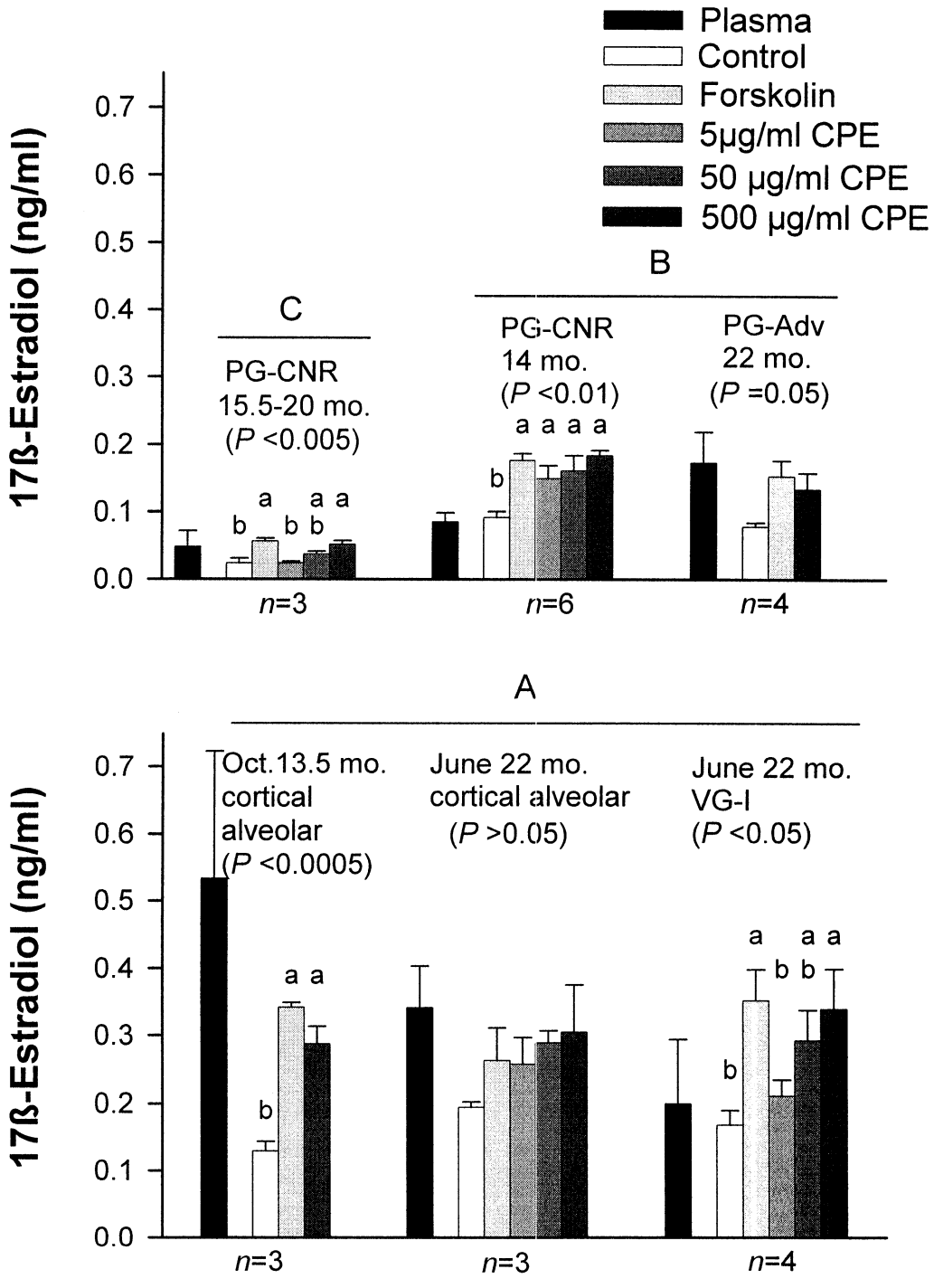

17ß-estradiol (ng/ml)
Testosterone (ng/ml)
mat.
immat.
Au O D F A J Au O D F A J
12 14 16 18 20 22 12 14 16 18 20 22
Age (months)

Figure 2.5. *In vitro* incubation results for immature (upper plot) and pubertal (lower plot) females of the 1997 and 1998 year classes. Plotted results for different groups of females include mean plasma 17ß-estradiol levels (±SD) and mean 17ß-estradiol output (±SE) of ovarian fragments in different incubation media (control, forskolin, and one to three doses of crude pituitary extract: CPE). Lower case letters indicate within group statistical comparisons of mean 17ß-estradiol output between incubation treatments. Upper case letters represent statistical comparisons of overall 17ß-estradiol output between different groups of females. Data labeled with the same letter are not significantly different ($P > 0.05$). The number of females (n) represented at each stage is noted below the corresponding plot.


Plasma
Control
Forskolin
5µg/ml CPE
50 µg/ml CPE
500 µg/ml CPE
17ß-Estradiol (ng/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C
PG-CNR
15.5-20 mo.
(P <0.005)
b a b a b a
n=3
B
PG-CNR
14 mo.
(P <0.01)
b a a a a
PG-Adv
22 mo.
(P =0.05)
n=6
n=4
A
Oct.13.5 mo.
cortical
alveolar
(P <0.0005)
b a a
June 22 mo.
cortical alveolar
(P >0.05)
June 22 mo.
VG-I
(P <0.05)
b a b a b
n=3
n=3
n=4

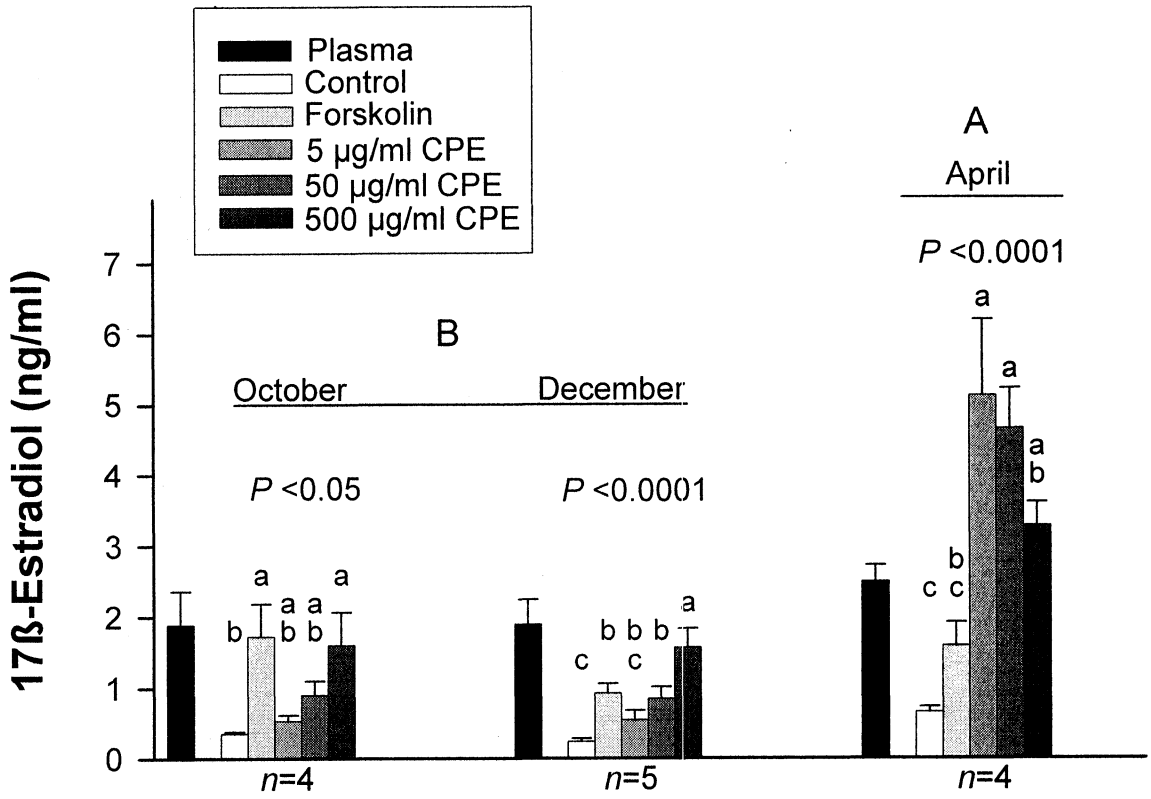


Figure 2.6. *In vitro* incubation results for females during the progression of puberty. Females from the 1997 (October, 1999- 25.5 mo.; and December, 1999- 28 mo.) and 1998 (April, 2000; 20 mo.) year classes are represented. Plotted results for different groups of females include mean plasma 17ß-estradiol levels (±SD) and mean 17ß-estradiol output (±SE) of ovarian fragments in different incubation media (control, forskolin, and three doses of crude pituitary extract: CPE). Lower case letters indicate within group statistical comparisons of mean 17ß-estradiol output between incubation treatments. Upper case letters represent statistical comparisons of overall 17ß-estradiol output between different groups of females. Data labeled with the same letter are not significantly different ($P > 0.05$). The number of females (n) represented at each stage is noted below corresponding plot.

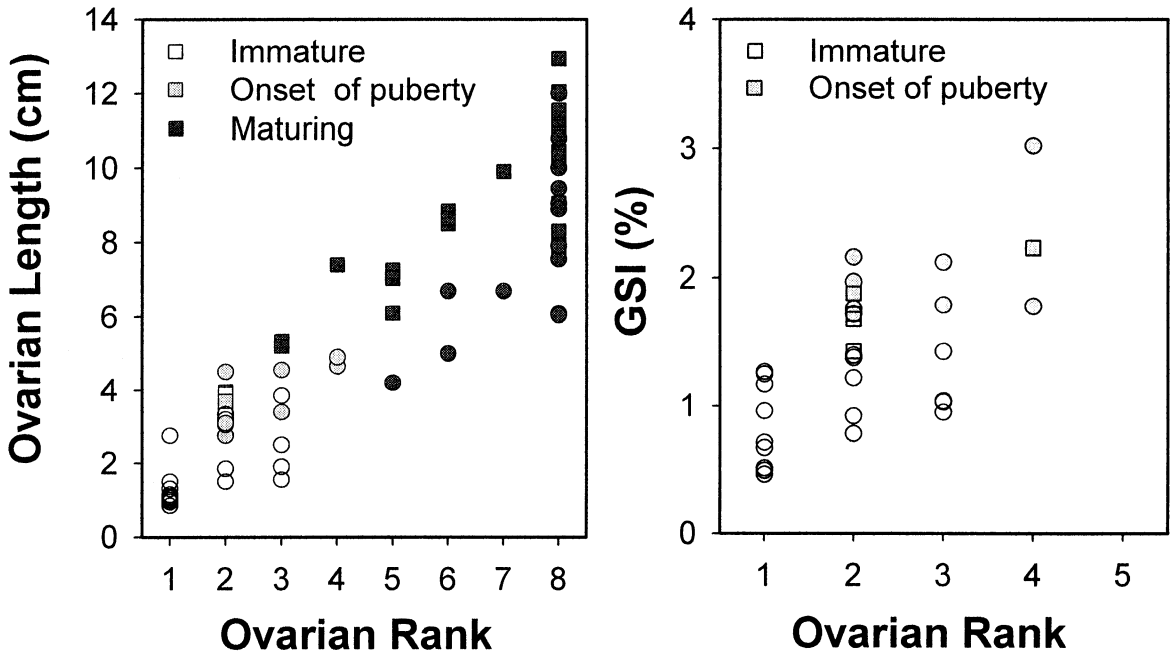


Figure 2.7. Scatter plots of ovarian length and GSI values determined post-dissection in relation to ovarian ranks estimated for individual females using an external examination technique prior to dissection. The scatter plot for the relationship with GSI illustrates only data for immature and newly pubertal females. Data for 1997 and 1998 females are represented by squares and circles, respectively. Dark filled symbols represent maturing females, pale filled symbols females at the onset of puberty, and open symbols immature females.

Plate 2.1. Ovarian histology for 1997 and 1998 females prior to exogenous vitellogenesis.

A- Immature 1998 female (14 mo.; October, 1999) with perinucleolar oocytes (**pn**), oogonial nest (**on**) and evidence of oogonial mitosis. Insert- mitotic figure in anaphase.

B- Immature 1998 female (14 mo.; October, 1999) with perinucleolar oocytes (**pn**), oogonial nest (**on**), evidence of oogonial mitosis (metaphase) and early circumnuclear ring stage oocytes (**cnr**) as well as oocytes in transitional points between these stages.

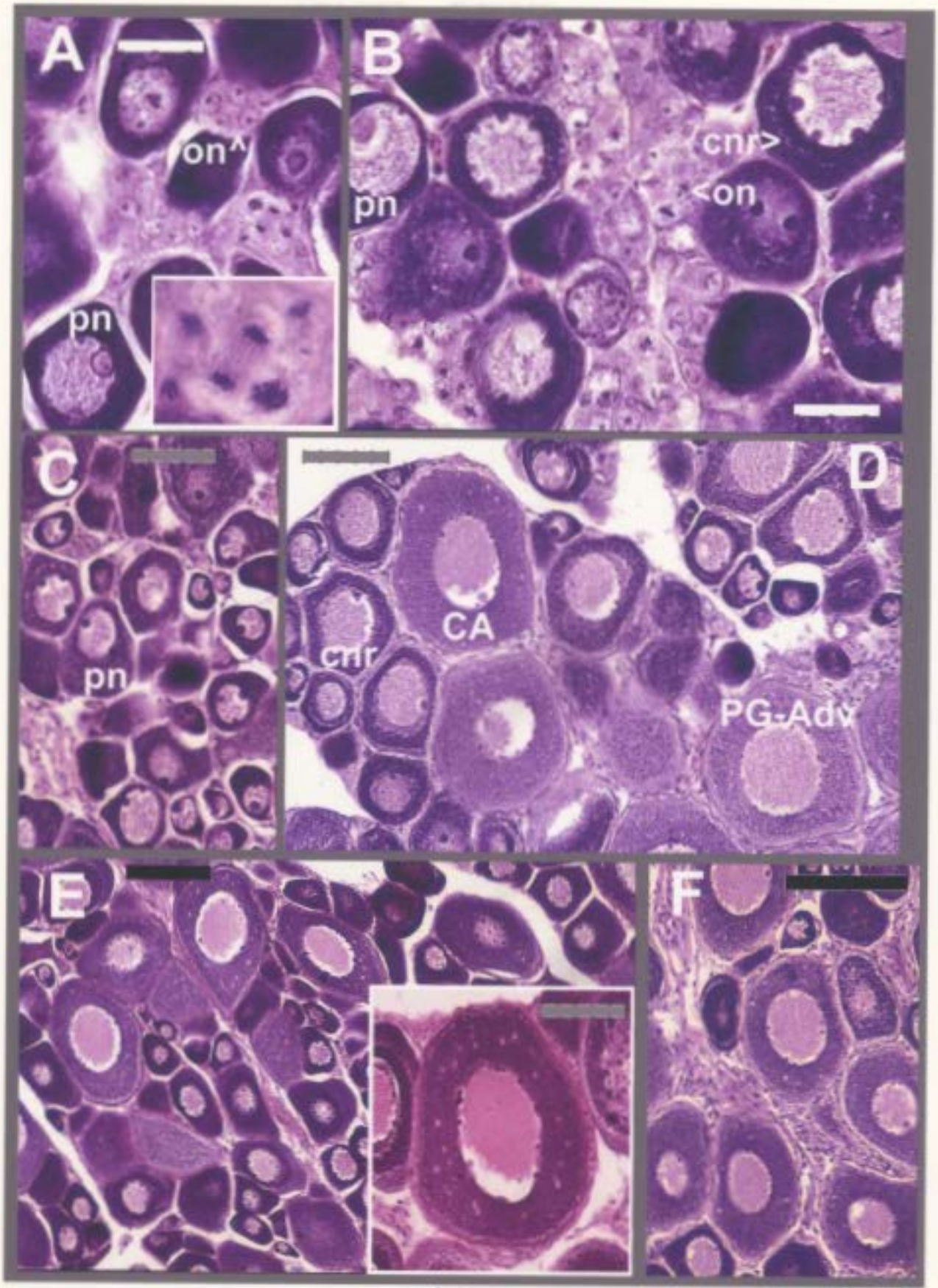
C- Immature 1997 female (12.5 mo.; September, 1998) with early perinucleolar stage oocytes (**pn**) and very few oogonia.

D- Pubertal 1998 female (13.5 mo.; October, 1999) with cortical alveolar stage oocytes (**CA**) as well as advanced primary growth (**PG-Adv**) and primary growth circumnuclear ring stage oocytes (**cnr**).

E- Pubertal 1998 female (22 mo.; June, 2000) in the cortical alveolar stage. Insert - close-up view of a cortical alveolar oocyte- note in addition to the chromophobic alveoli, a chromophobic area at the periphery of the oocyte which also may be seen in similar stage oocytes in the main view (some advanced primary growth oocytes may also show this feature; not shown).

F- A pubertal 1998 female of 13.5 months of age (October, 1999) also with cortical alveolar stage oocytes.

White scale bars=25 μm; grey scale bars= 50 μm; black scale bars = 100 μm.



2.44

Plate 2.2. Ovarian histology for 1997 and 1998 females during pubertal vitellogenesis.

A-C- Early vitellogenesis in 1997 females sampled in October (25.5 mo.) oocytes with peripheral yolk globules (**VG-I** in A) and oocytes (**VG-II**) where yolk globules are beginning to fill the ooplasm. **bb**= Balbiani body

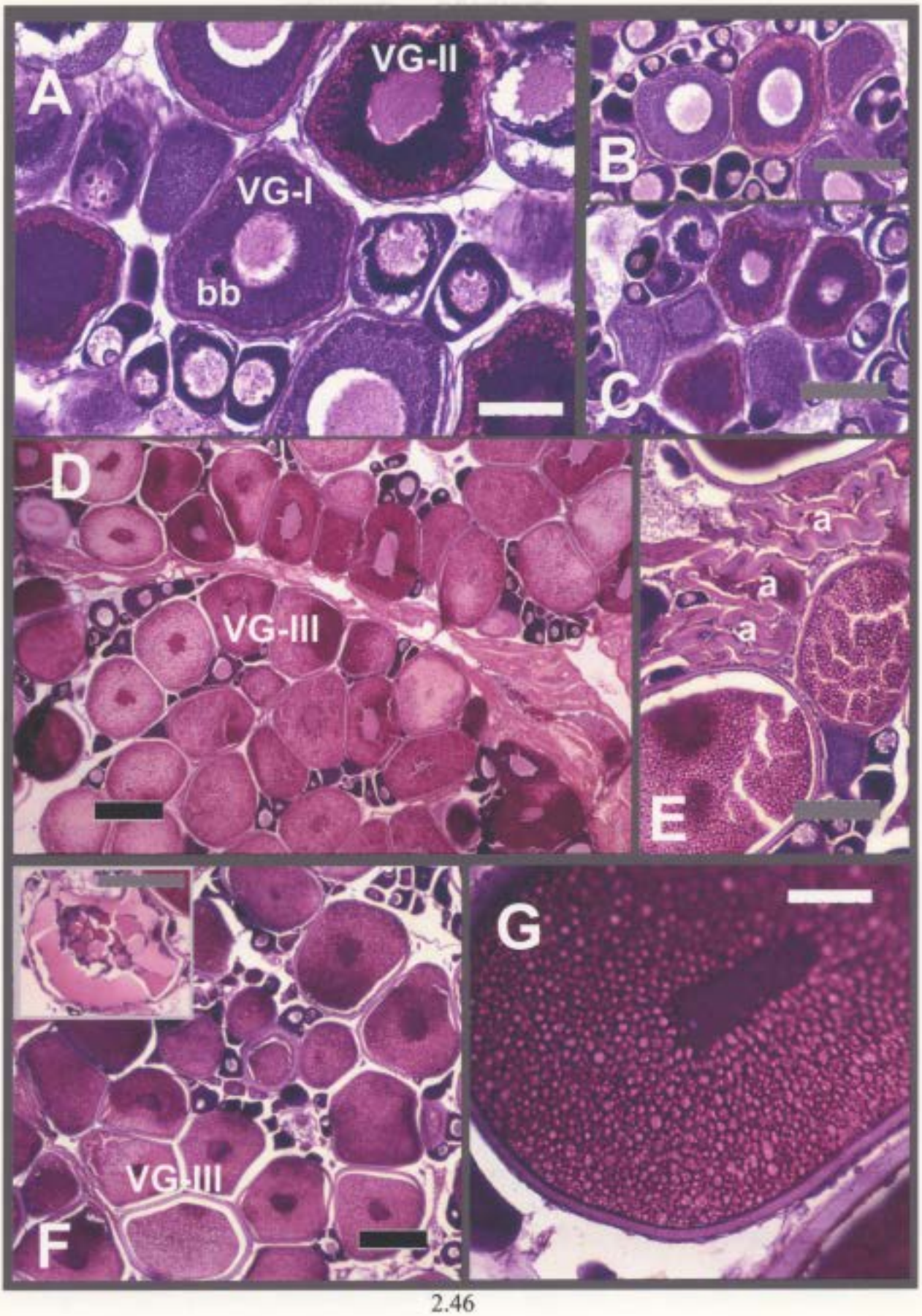
D- Vitellogenic 1997 female sampled in December, 1999 (28 mo.) showing more advanced vitellogenic oocytes (**VG-III**).

E- Atretic follicles (**a**) in a vitellogenic 1997 female sampled in December, 1999 (28 mo.).

F- Prespawning female in June, 2000 (34 mo.) oocytes are large with thick zona radiata follicles. Insert- a hyaline oocyte seen in another June sampled female which had ovulated.

G- Close up of a mature oocyte with a thick zona radiata follicle.

White scale bars=50 μm; grey scale bars= 100 μm; black scale bars = 200 μm.



2.46

2.3.5. Male development of the 1997 and 1998 year classes.

For 1997 males sampled over 21.5 months (Sept.1998 - June, 2000), highly significant differences ($P<0.0001$) were seen over time for changes in weight, length, carcass weight, GSI and 11-ketotestosterone (11-KT) (Figure 2.8). Changes in testosterone levels were less evident but were significant ($P=0.043$; Figure 2.8). Similarly for 1998 males sampled over a nine month period (October, 1999 - June, 2000), body size and GSI changes were highly significant ($P<0.0005$), but variations in 11-KT and testosterone were not statistically significant ($P>0.05$) (Figure 2.8).

2.3.5.1. Onset of puberty in 1997 and 1998 year class males.

A mixture of immature males and males initiating puberty was seen in the first samples of 1997 and 1998 year class fish (Table 2.5). Half the 1997 males sampled in early September at 12.5 months of age, and some 1998 males sampled at 14 months, had immature testes weighing less than 0.01 g; no 11-ketotestosterone (11-KT) was detected in the plasma of these males (Table 2.5). Immature testes were translucent and were densely populated by primary spermatogonia, although some secondary spermatogonia and mitotic activity may be detected as well (Plate 2.3A,B).

Clear evidence of the initiation of puberty in male yellowtail flounder was indicated by: testicular growth (the tissue taking on a cloudy appearance), an increased presence of secondary spermatogonia and mitotic activity, the presence of spermatocytes, and detectable levels of 11-KT in the plasma (Table 2.5; Plate 2.3B,C). However, in some instances, endocrine puberty lagged behind histological evidence of the initiation of puberty, namely, the appearance of primary spermatocytes. For 1997 males at 12.5 months, two males were

found with low numbers of spermatocytes, but 11-KT was detectable only in one male with the largest GSI (Table 2.5). Similarly for 1998 males sampled at 13.5 months of age in October, two males were found in the very initial stages of puberty, with few spermatocytes amid mitotically dividing spermatogonia. Again, only one of these two males had detectable plasma 11-KT (Table 2.5). Surprisingly, the male showing both endocrine and histological evidence of the onset of puberty had a lower total testicular weight (0.03 g, ~0.015 g/ testis), than the male lacking detectable 11-KT (0.06 g total testicular weight).

A weak relationship between body size and the onset of puberty was seen in male yellowtail flounder. In 1997 fish of 12.5 months of age, it was the longest and heaviest individuals which were pubertal. Among 1998 males sampled at 13.5 months of age, the heaviest male had the highest GSI, highest hormone levels, and the most advanced testes with secondary spermatocytes and spermatids (Table 2.5; Figure 2.9; Plate 2.3D). Other males of the sample were smaller and less advanced, although they included males in which early puberty was well established with elevated levels of 11-KT (Table 2.5). Sampling males later at 14 months demonstrated a division in testicular status. Males weighing 16 to 19 g were pubertal with mean GSI and 11-KT values equivalent to those of 13.5 month males ($P>0.05$; Table 2.5). Males of lesser size were immature, nevertheless, further investigation showed that evidence of testicular growth could be detected even in males as small as 7.8 and 9.9 g (total testis weight=0.01 and 0.07 g; GSI=0.13 and 0.71%). Overall, these results demonstrate that all males of one year of age undergo puberty regardless of size, but that faster growing males are the first to initiate testicular development.

It was clear that plasma 11-KT increases rapidly to levels of 2 to 4 ng/ml while males are in an early stage of puberty and total testicular weights are still low (0.08-0.38 g; Table 2.5). Regarding testosterone, estimated total testosterone levels may reach as high as 0.8 to 1.5 ng/ml for 13.5 month old 1998 males in which early puberty was well established. Measurement of testosterone was not possible for males sampled at other times due to insufficient amounts of plasma associated with small body size.

2.3.5.2. Pubertal development in 1998 one year old males.

Subsequent pubertal development in 1998 males showed a significant peak in mean GSI of 4.03% (2.47 - 6.56%) in December at 15.5 months (Figure 2.8). Spermatogenic activity was high and every cell type including spermatozoa was seen in all males at this time (Figure 2.9; Plate 2.3E, F). Spermiogenesis was advanced in three males where new spermatozoa dominated the germ cell distribution. A small amount of viscous milt could be expressed from two of these males. Very few secondary spermatogonia were found in the sampled testes, and mitosis was no longer observed. In most males the testis tissue was dissociated as mature cysts degenerated and released spermatozoa into the lobular lumen.

Following December, mean GSI decreased significantly and remained between 1.9 and 2.4% in April (20 mo.), May (20.5 mo.) and June (22 mo.); an overall range in individual GSI values of 0.96-3.13% was seen during this interval (Figure 2.8). Testes sampled in April and June showed lobules full of mature spermatozoa (Plate 2.3G). Nests of primary spermatogonia were mainly the only other germ cells seen during this period. However, two to three males in each sample had a few secondary spermatogonia; primary spermatocytes were infrequently detected as well in these April sampled males but were absent in June

sampled males (Figure 2.9). In April, only small amounts of dilute milt could be expressed from males. By June, at 22 months, males were in full spermiating condition where spawning levels of milt could be expressed.

Although changes in mean hormone levels were not statistically significant over time in 1998 males, increasing trends in plasma levels of both androgens were noted (Figure 2.8). Levels of 11-KT during established early puberty in October (13.5 mo.) and peak spermatogenesis in December (15.5 mo.) were 4 ng/ml or less. By April (20 mo.) levels ranged up to 5.7 ng/ml and peaked to 25.9 ng/ml in one male in May (20.5 mo.). Testosterone showed a similar pattern to 11-KT but with lower maximal levels at 1.1, 2.5 and 4 ng/ml for December, April and May samples, respectively. In June (22 mo.), males with higher GSI (2.7 - 3.0%) had high levels of 11-KT (4.8 - 11.1 ng/ml) and testosterone (2.1 - 4.5 ng/ml). In contrast, two males with lower GSI (<2%) had lower amounts of 11-KT (0.7 &1.4 ng/ml) and testosterone (0.5 & 0.76 ng/ml).

2.3.5.3. Pubertal development in 1997 one year old males.

Samples describing the first cycle of spermatogenesis in 1997 males, after the initiation of puberty in September (12.5 mo.), were available only for February at 18 months and June at 22 months of age. By February, testes had grown significantly larger since the initiation of puberty. Mean GSI seen in February (1.38%) was not significantly different from mean values in June for 1997 males (2.0%) or 1998 males sampled between 20 and 22 months of age (Figure 2.8). Spermatozoa dominated the testes in both samples but areas of spermatogenic tissue were still present even in June. While secondary spermatogonia were declining in February and were usually rare or absent in June, all males at both times had

every type of meiotic cell (Figure 2.9). Plasma hormone levels, only available from June males, were variable. Levels of 11-KT ranged between 0.5 to 7 ng/ml, while estimated total testosterone ranged from 0.5 to 3.2 ng/ml. An association with GSI was less clear than in 1998 males in June, but higher hormone levels were usually seen with higher GSI. Elevated testosterone (>2 ng/ml) was associated with higher 11-KT (>2 ng/ml).

2.3.5.4. Testicular recrudescence in 1997 two year old males.

Sampling of 1997 males in their second cycle of spermatogenesis began in October at 25.5 months of age. The sampling period for recrudescing 1997 males was concurrent with pubertal development in younger 1998 males. Testes of two year old 1997 males in October were beige to white in colour, and generally appeared regressed, although sperm was still present in the ducts. A slight decrease in mean GSI (1.4%) was seen compared to the spawning period (Figure 2.8). The testes of all males in October were comprised of dense recrudescent tissue. Mitotically-dividing spermatogonia and primary spermatocytes were seen in four males; spermatocytes were still few in number in most of these individuals. The remaining two males of the sample were more advanced with germ cells ranging from spermatogonia to new spermatozoa (Figure 2.9). New spermatozoa were seen in isolated areas and were associated with spermatids amid recrudescent tissue. These new cells were distinguished from residual spermatozoa since the latter remained mainly in the sperm ducts. Mitotic activity was absent in these advanced males. Some phagocytes were seen in at least one male among the residual spermatozoa. Levels of 11-KT (1.8 to 4.6 ng/ml) and testosterone (1.1 to 2.0 ng/ml) were similar to values in June, and did not differ between males with new spermatozoa and those in early recrudescence (Figure 2.8).

As recrudescence continued in December at 28 months, mean GSI levels reached a peak of 6.1% (3.2-7.7%) (Figure 2.8). Spermatogenic activity was also at its peak, and new sperm production was seen in all males (Figure 2.9). Testes were reverting to a dissociated state, and, in half the males, few or no secondary spermatogonia or primary spermatocytes remained. Levels of 11-KT and testosterone increased from previous samples ranging between 4.1 to 9.4 ng/ml and 1.9 to 3.3 ng/ml, respectively (Figure 2.8). Milt was expressed in reduced amounts compared to October and could be watery or viscous in character.

By April (32 months of age), mean GSI had decreased to a level (mean=3.26%; 1.8-4.4%) equivalent to that seen in June at 34 months of age (mean=3.33%; 2.6-4.8%). In both April and June, testes appeared completely dissociated and contained mature spermatozoa and primary spermatogonial nests (Figure 2.9). Secondary spermatogonia were observed along the lobule walls in a few males in June. Copious amounts of milt were expressed from males at both sample times. Plasma 11-KT values increased sharply in April and May (range 5.6 to 24.7 ng/ml). Similar increases in testosterone levels were seen during this period (range 1.5 - 6.8 ng/ml) (Figure 2.8). Androgen levels in April and May were significantly higher than levels seen during early recrudescence in October (Figure 2.8). By June, 11-KT was highly variable, the observed range spanning from 0.2 to 57.1 ng/ml. Levels did not relate to GSI values. Testosterone levels paralleled the individual variability in 11-KT (0.09 - 10 ng/ml).

Comparing the results of recrudescent 1997 males with pubertal males of 1998, overall recrudescent males had significantly higher GSI ($P<0.015$), 11-KT ($P<0.001$) and testosterone ($P<0.001$) levels than pubertal males of 1998. Both recrudescent males and

pubertal 1998 males displayed peak GSI means in December. Despite a larger peak in 1997 males, it was not significantly different from the mean of pubertal males ($P>0.05$). In June samples, highly variable levels of 11-KT were seen for recrudescent 1997 males at 34 months, and newly mature males at 22 months for both 1997 and 1998 year classes. June levels of 11-KT and testosterone were not significantly different between these three samples. For GSI, mean values were not significantly different between year classes in newly mature 22 month old males, but values of 22 month old males were significantly smaller than those for 34 month old testes which suggests an increase in sperm production with age.

Table 2.5. Comparison of males at the onset of puberty with immature males sampled coincidentally or at other periods.

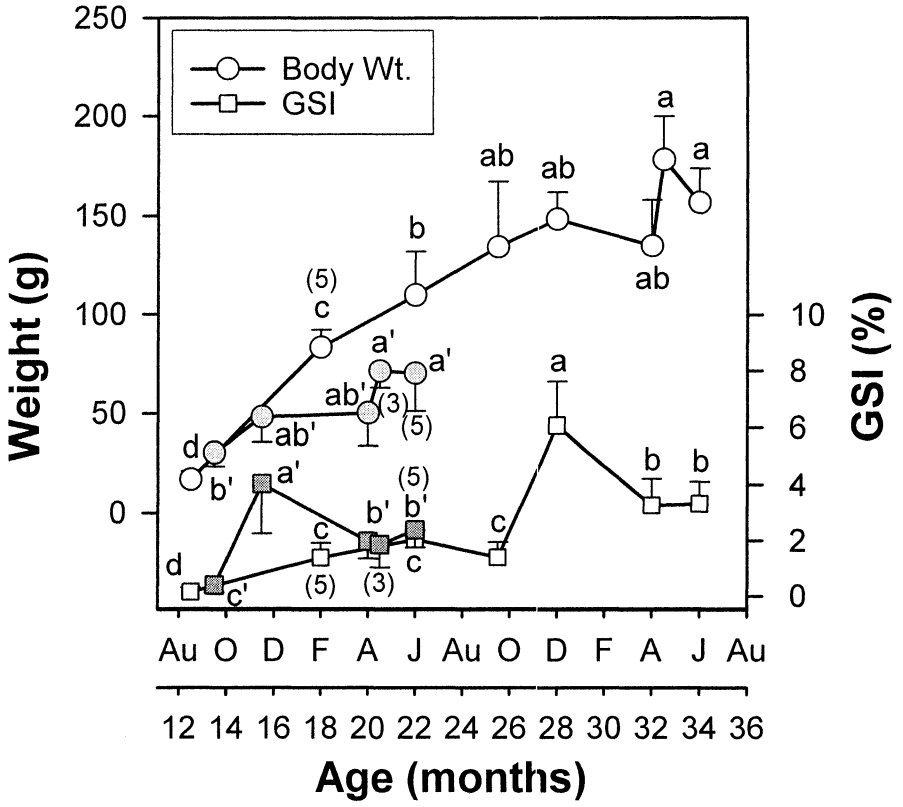
Age (mo.)	Wt (g)	Gonad Wt (g)	GSI (%)	Histological Stage	11-KT (ng/ml)
1997: Onset of puberty in males at 12.5 months of age (early September, 1998)					
12.5 *n*=2	18	0.07	0.39	SC-1, Mitosis	0.88
	24	0.04	0.17	SC-1, Mitosis	ND
12.5 *n*=4	12 - 19	<0.01/0.04	0, 0.28	SG-1/ SG-2	ND
1998: Onset of puberty in males sampled at 13.5 and 14 months of age (October,1999).					
13.5 *n*=1	43	0.38	0.88	ST, Mitosis	3.4
13.5 *n*=3	27 - 32	0.08 - 0.13	0.25 - 0.43	SC-1, Mitosis	2.5 - 3.1
13.5 *n*=2	28	0.03	0.11	SC-1, Mitosis	0.17
	23	0.06	0.26	SC-1, Mitosis	ND
14A *n*=3	16 - 19	0.15 - 0.25	0.81 - 1.57	SC-1, Mitosis	2.0 - 4.0
14A *n*=2	7 - 14	<0.01	0	SG-1/SG-2	ND

Males of different year classes, ages and histological stages are shown.

Values in the table represent ranges for Wt= body weight; Gonad Wt= total testicular weight; GSI= gonadosomatic index; and 11-KT= plasma 11-ketotestosterone levels (ND=non-detectable); Histological Stage = most advanced stage present in the testes and whether mitosis is detected.

Unshaded, pale shaded and dark shaded rows highlight data for immature males, males at the very initiation of puberty, and males where early puberty is well established, respectively.

Figure 2.8. Mean (±SD) body weight and GSI changes (upper plot), and 11-ketotestosterone (11-KT) and testosterone fluctuations (lower plot), over time for 1997 and 1998 year class males. Clear and shaded plots represent data for 1997 (sample period: September, 1998 to June, 2000) and 1998 males (sample period: October, 1999 - June, 2000), respectively. The main x-axis indicates month of the year, the second axis indicates age in months. Means along the same plot which are labeled with the same letter are not significantly different (P >0.05). Letters followed by an apostrophe refer to analyses on weight and GSI data for 1998 males and testosterone data for 1997 males. Plots without letters showed no significant differences in mean values over time (P >0.05). The sample size (n) is indicated when the number of sampled individuals is less than six.


Body Wt.
GSI
Weight (g)
GSI (%)
250
200
150
100
50
0
10
8
6
4
2
0
Au O D F A J Au O D F A J Au
12 14 16 18 20 22 24 26 28 30 32 34 36
Age (months)

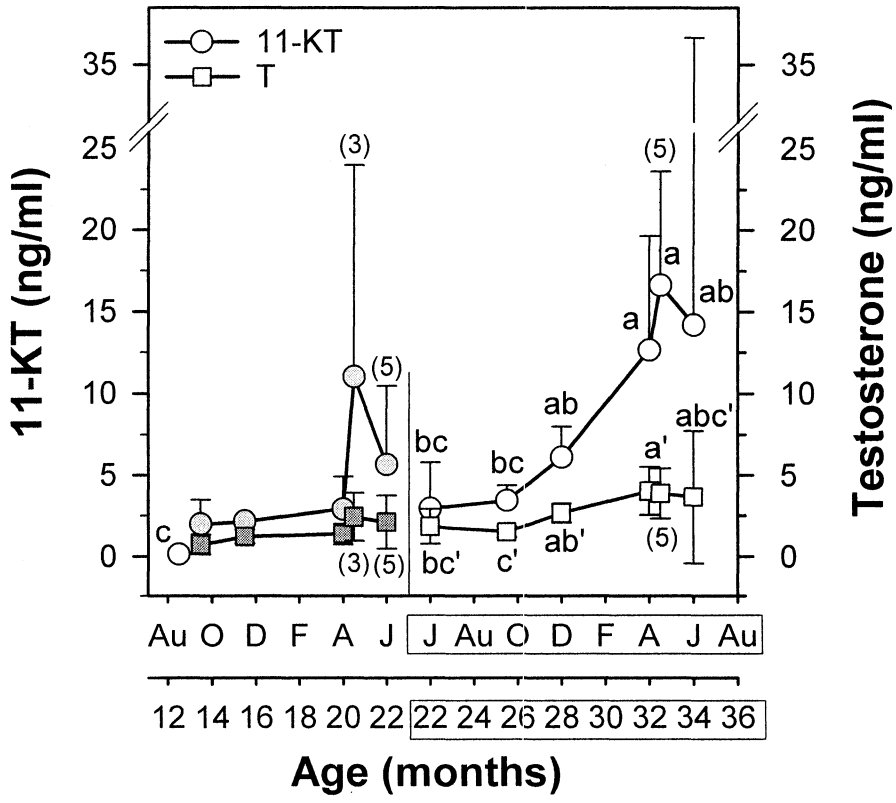
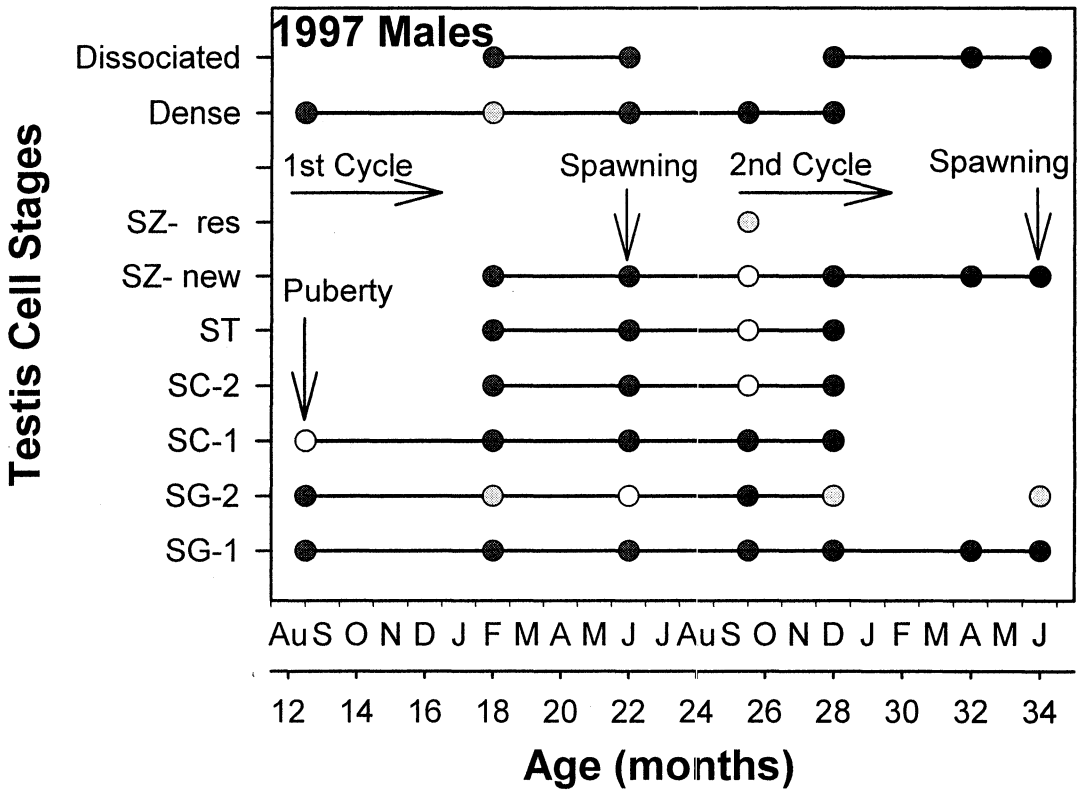

11-KT
T
11-KT (ng/ml)
Testosterone (ng/ml)
35
25
20
15
10
5
0
Au O D F A J J Au O D F A J Au
12 14 16 18 20 22 22 24 26 28 30 32 34 36
Age (months)

Figure 2.9. Histological changes in the testes of 1997 and 1998 males over time. The main x-axis indicates month of the year starting from August, 1998 to June, 2000 for 1997 males, and August, 1999 to June, 2000 for 1998 males; the second axis indicates age in months. Different cell types include: primary spermatogonia (SG-1), secondary spermatogonia (SG-2), primary spermatocytes (SC-1), secondary spermatocytes (SC-2), spermatids (ST), new spermatozoa (SZ- new) and residual spermatozoa (SZ-res). State of the testis is also indicated, i.e. whether the testis was comprised of dense areas of tissue and/or dissociated areas of tissue. The percentage of sampled males exhibiting each cell type or tissue state is indicated according to three classes: 0-33 %, 33-66 % and 66-100 % represented by clear, grey and dark grey symbols, respectively. The number of males per sample was six with the exceptions of n=5 for 1997 males sampled at 18 months of age and 1998 males sampled at 22 months of age.


1997 Males
Testis Cell Stages
Dissociated
Dense
SZ- res
SZ- new
ST
SC-2
SC-1
SG-2
SG-1
1st Cycle
Spawning
2nd Cycle
Spawning
Puberty
AuS O N D J F M A M J JAuS O N D J F M A M J
12 14 16 18 20 22 24 26 28 30 32 34
Age (months)

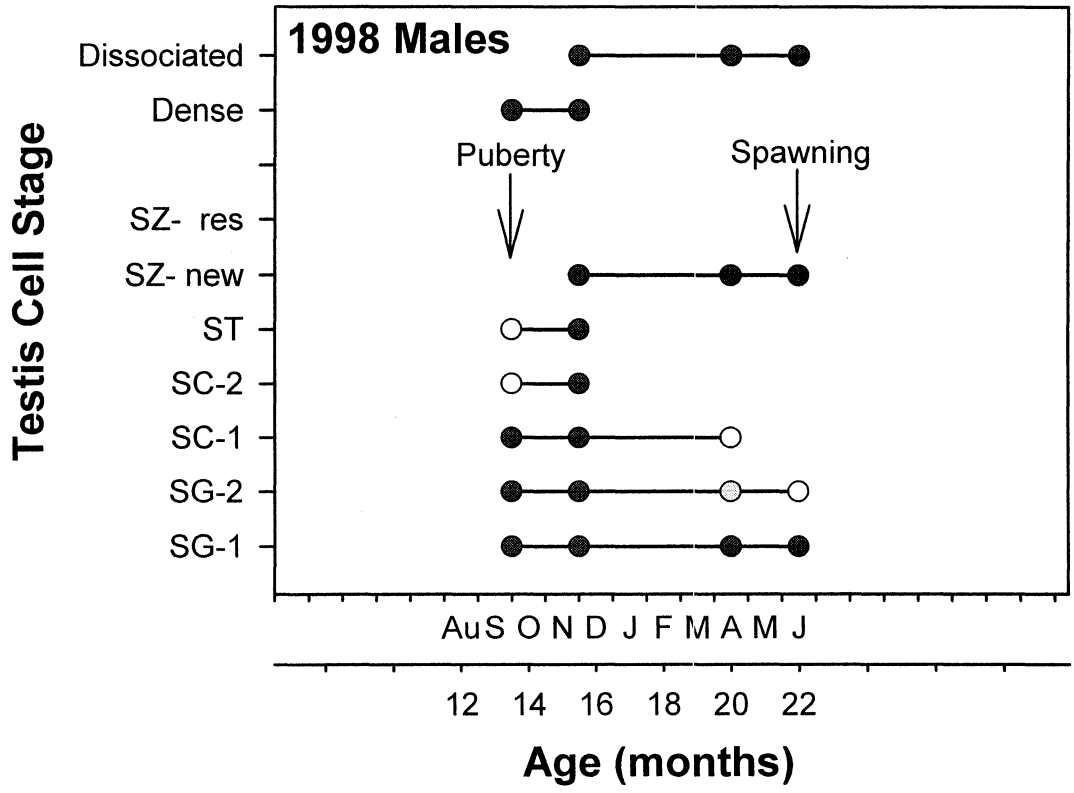

1998 Males
Testis Cell Stage
Dissociated
Dense
SZ- res
SZ- new
ST
SC-2
SC-1
SG-2
SG-1
Puberty
Spawning
AuS O N D J F M A M J
12 14 16 18 20 22
Age (months)

Plate 2.3. Testicular histology for 1997 and 1998 males.

A- Immature male with only nests of primary spermatogonia (sg-1).

B- Spermatogonial proliferation in a 1997 pubertal male (September, 1998) with both primary (sg-1) and secondary spermatogonia (sg-2).

Insert- a cyst of mitotic spermatogonia in metaphase.

C- Meiotic activity in 1998 males (14 mo.; October, 1999) with mainly primary spermatocytes (sc-1).

D- Meiotic activity in a recrudescing 1997 male (25.5 mo.; October, 1999). Spermatogonia, primary and secondary (sc-2) spermatocytes are seen as well as spermatids (st).

E- Ongoing meiosis and spermiogenesis and the beginning of the dissociation of the testicular cyst structure in a pubertal 1998 male (15.5 mo.; December, 1999).

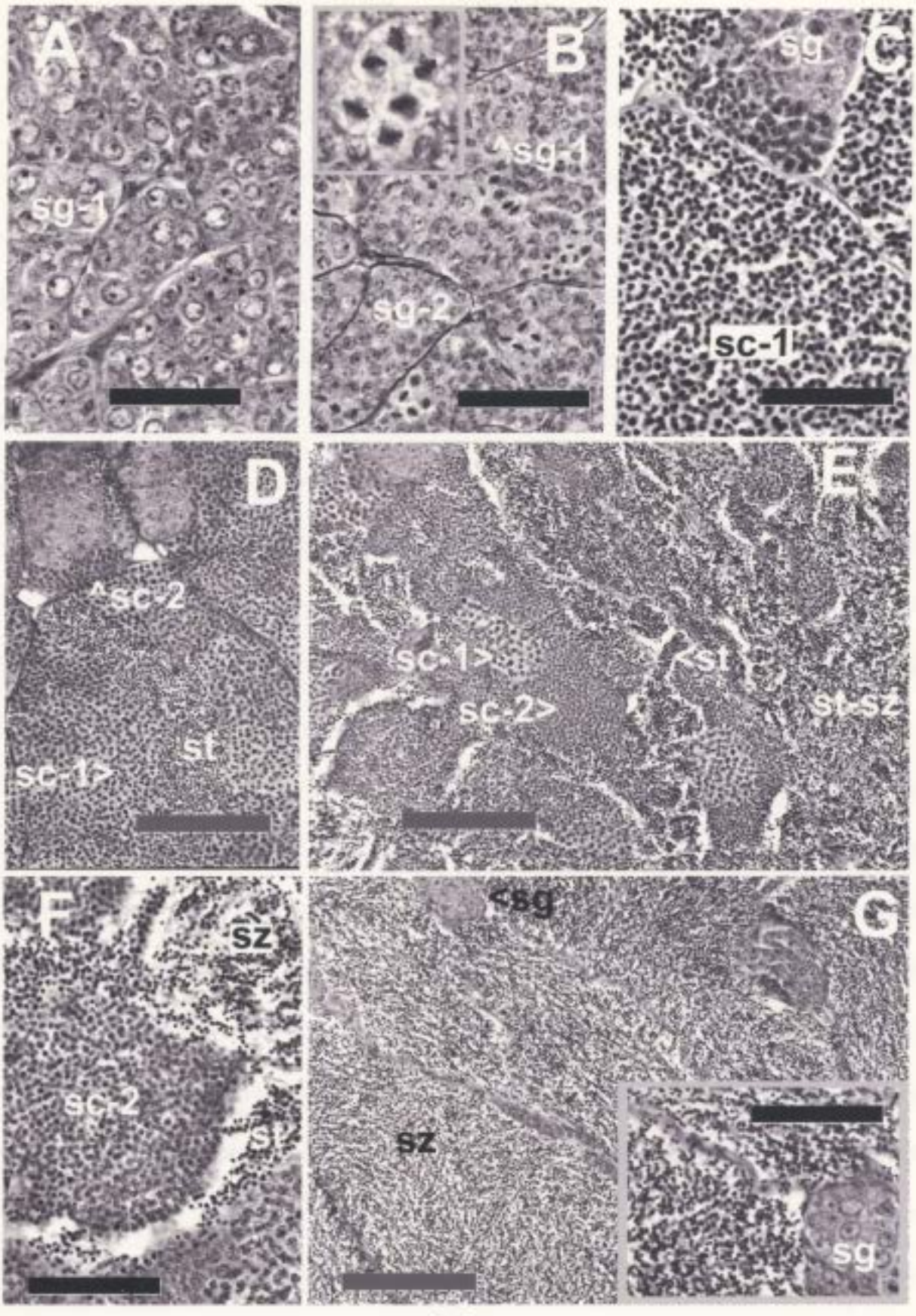
F- Secondary spermatocytes, spermatids and spermatozoa (sz) in an 1998 15.5 month pubertal male (December, 1999).

G- 1998 male at 22 months (June, 2000) at full maturity during spermiation.

Insert- close-up of spermatozoa.

sg= spermatogonia (**sg-1** primary; **sg-2** secondary); **sc**= spermatocytes (**sc-1** primary; **sc-2** secondary); **st**= spermatids; **sz**= spermatozoa.

Black scale bars = 50 μm; grey scale bars= 100 μm.


A
sg-1
B
^sg-1
sg-2
C
sg
sc-1
D
^sc-2
sc-1>
st
E
sc-1>
sc-2>
<st
st-sz
F
sz
sc-2
st
<sg
G
sz
sg

2.3.6. Changes in hepatosomatic index.

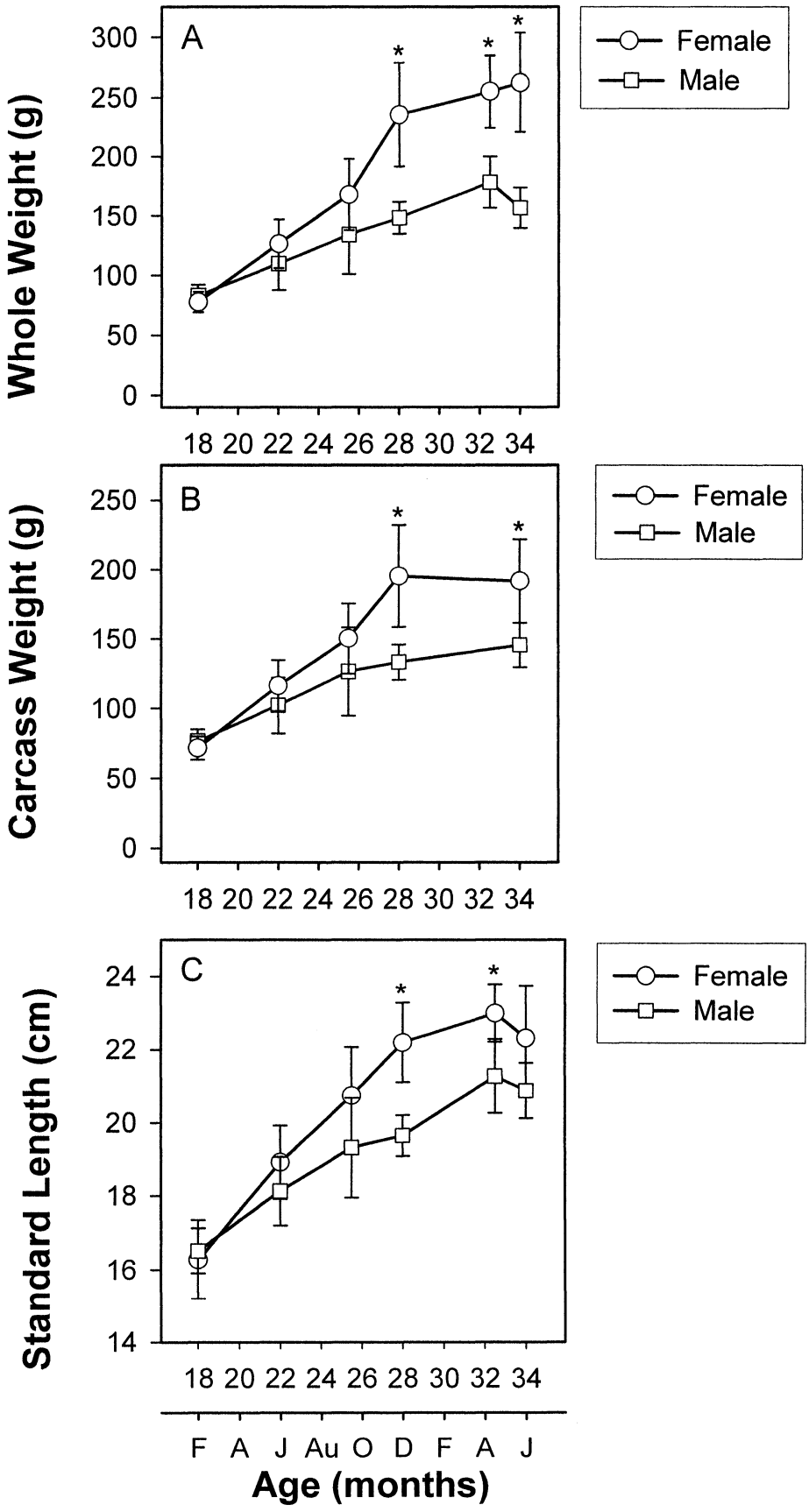
Hepatosomatic index profiles for 1997 and 1998 females showed an increasing trend in autumn, peaking in winter and declining in spring. This pattern was seen in immature and maturing females alike, although minimum values in June were lower in fully mature females (data not shown). These trends may better reflect seasonal changes in temperature conditions and metabolism, rather than gonadal activity. Changes in hepatosomatic index in males were similar to patterns in females.

2.3.7. Sex differences in growth.

Comparing the growth curves of males and females of the 1997 year class showed that males had a slower growth rate in whole body weight, carcass weight and standard length (Figure 2.10). Females eventually showed a decrease in growth during vitellogenesis from December (28 mo.) to June (34 mo.).

Sex differences in body size were significant in whole body weight ($P<0.001$) and carcass weight ($P<0.01$) by 28 months of age in December. For standard length, appreciable differences were noted by October (25.5 mo.), were significant at 28 months ($P=0.0005$) and 32.5 months (May, $P<0.01$), yet were not significant in June (34 mo.) ($P=0.056$). Divergent patterns in body size parameters appeared to become established as early as June (22 mo.) and October (25.5 mo.). Tests for heterogeneity of slopes indicated that growth rates in males and females differed significantly ($P<0.005$). For whole body weight, differences between regression slopes, determined by sex-time interaction terms, were highly significant ($P=0.0001$). Differences between regression relationships for males and females in carcass weight and standard length showed a lower order of statistical significance ($0.02<P<0.05$).

Figure 2.10. Sex differences in whole body weight (A), carcass weight (B) and standard length (C) over time in 1997 year class males and females (February, 1999 to June, 2000). A female mean (±SD) denoted with an asterisk '*' is significantly higher (P <0.05) than the male mean from the same sample event.


A
B
C
Female
Male
Whole Weight (g)
Carcass Weight (g)
Standard Length (cm)
300
250
200
150
100
50
0
24
22
20
18
16
14
18 20 22 24 26 28 30 32 34
F A J Au O D F A J
Age (months)
*

2.4. DISCUSSION.

Yellowtail flounder experience early sexual maturation in culture. In both year classes males initiated puberty between 12.5 to 14 months of age. In contrast, females initiated puberty as early as 13.5 or 22 months in the 1998 population, and 22 or 34 months in the 1997 population. Therefore, a plasticity in the timing of the onset of puberty was present, both within and between year classes, in female yellowtail in culture, but was not evident among males. Following the onset of puberty, sexual maturation in both sexes continued to full maturity in one uninterrupted progression. Ovulation and spermiation in newly mature yellowtail was synchronized with the captive, adult spawning season extending from mid-May to late August (Manning & Crim, 1998). In some teleosts, incomplete pubertal gametogenesis may occur prior to a complete cycle leading to full maturity (female grouper, *Epinephelus aeneus*, Hassin et al., 1997; male and female striped bass, *Morone saxatilis*, Holland et al., 2000). Moreover, first maturing striped bass females may not reach the same level of gonadal development as adults (Holland et al., 2000). This was not the case in the present group of cultured yellowtail flounder where GSI levels exceeded the maximal mean value of 18.5% reported for wild females sampled in southern New England (Howell, 1983).

2.4.1. The onset of puberty in females.

Immature females had low amounts of 17ß-estradiol in the plasma which could be detected as early as the perinucleolar stage (PG-PN). *In vitro* incubations demonstrated that immature tissue was steroidogenically competent, and was responsive to both forskolin and crude pituitary extract. Responsiveness to both these agents revealed the presence of GtH receptors in primary growth phase tissue (PG-CNR), and that intracellular mechanisms

involving adenylate cyclase were functional and could mediate a gonadotropic signal resulting in steroidogenesis.

Plasma levels of 17ß-estradiol increased during the immature phase, peaking in tissue with advanced primary growth oocytes (PG-Adv). Immature tissue showed statistically greater steroidal output *in vitro* when sampled at periods when the onset of puberty may occur (October, 13.5-14 mo. & June, 22 mo.). In contrast, immature females sampled in December and April had lower steroidal output *in vitro* despite being older than, and in a similar histological stage (PG-CNR) as, 14 month old females. A greater steroidal output by immature females in June may be associated with the presence of PG-Adv oocytes. However, as PG-Adv oocytes were few or absent in immature females sampled at 14 months of age, an alternate explanation is required regarding higher *in vitro* steroidal output at this time. It seems plausible that factors (environmental/endogenous) which stimulate the initiation of puberty in advanced females could partially up-regulate the brain-pituitary-gonadal (BPG) axis in immature females. In striped bass, annual peaks in GtH-Iß mRNA, which coincided with the period of vitellogenesis in adults, were seen in immature animals and older pubertal females alike (Hassin et al., 1999). Partial up-regulation of the immature BPG axis could include increases in basal GtH release. In the present results such a phenomenon could explain increased plasma 17ß-estradiol levels seen in immature females sampled in June. Temporal increases in basal GtH release in immature fish during periods of pubertal onset could facilitate earlier sexual maturation, particularly in energetically permissive culture environments.

The earlier initiation of puberty in advanced 1998 females was a striking difference between 1997 and 1998 year classes. Earlier maturation in one year old yellowtail flounder clearly demonstrated that a 22 month period of immaturity, or primary growth, is not physiologically required prior to the full activation of the female BPG axis. In fish, a relationship has been shown between growth rate and the age at which puberty is initiated (Alm, 1959; Le Bail, 1988). Factors such as environmental conditions (i.e. photoperiod and temperature) and nutritional status may affect the age of pubertal onset, either by affecting growth rate, or potentially through growth-independent effects. In the present study, the initiation of puberty was clearly associated with faster growth rates in 1998 females aged 13.5 months, where the three largest females, exceeding 40 g, were pubertal. Elevated temperatures during the first year of life and feeds with a high energy content were two factors associated with culture conditions which likely contributed to reductions in pubertal age in female yellowtail. Temperature effects on gonadal development or the BPG axis have been observed in fish (Lam, 1983; Blázquez et al., 1998). Regarding nutritional status, body condition and energy reserves have been recognized for their importance in the initiation of fish puberty (Rowe et al., 1991). Internal signals reflecting growth performance or condition, such as insulin-like growth factor-I (IGF-I), insulin, and possibly leptin (if present in fish), are of current interest in terms of their effects on the activation of the fish BPG axis (Dufour et al., 1999).

Endocrine puberty in yellowtail flounder was detected in females with cortical alveolar oocytes. Females in the cortical alveolar stage had significantly higher plasma 17ß-estradiol levels than females initiating vitellogenin incorporation, whose levels were notably

equivalent to those seen in females with advanced primary growth oocytes. Significant increases in steroid production *in vitro* also were noted for females with cortical alveolar oocytes. Once elevated, the level of steroidal output *in vitro* remained unchanged with the appearance of early vitellogenic oocytes (VG-I).

Females with cortical alveolar oocytes sampled at 13.5 months of age in October had particularly high levels of 17ß-estradiol in the plasma. These high levels may have been a result of a strong gonadotropic pulse. Such a pulse may have been more prominent in these females as the window of opportunity for the initiation of puberty (June into November, in this study) was beginning to close, resulting in an acceleration of events. Alternatively, strong pulses at the onset of puberty may be ephemeral events which reflect the fresh activation of the BPG axis. A strong gonadotropic pulse may be required to produce an effective 17ß-estradiol signal for the initiation of hepatic vitellogenin synthesis. Significantly lower plasma 17ß-estradiol levels seen in females which had initiated vitellogenin uptake may reflect new baseline levels of plasma GtH following an acute stimulation of the pituitary at the initiation of puberty. The lack of a significant difference between cortical alveolar females and newly vitellogenic females in tissue responsiveness *in vitro* appears to support this suggestion.

Increases in plasma steroid levels have been connected with stages preceding exogenous vitellogenesis in other species. In female black carp, the onset of puberty at four years of age occurs in the cortical alveolar stage (Gur et al., 2000). For pubertal striped bass, plasma steroid levels increase with the appearance of early secondary growth oocytes accumulating lipid vesicles (Holland et al., 2000). In adult English sole (*Parophrys vetulus*) increased 17ß-estradiol levels accompany the cortical alveolar stage and the appearance of

cytoplasmic lipid droplets (Johnson et al., 1991).

Plasma testosterone appears to have a limited presence during the initiation of puberty in yellowtail flounder. Pulses of testosterone were seen in some pubertal females with cortical alveolar oocytes and in a female initiating vitellogenesis (34 month old). In contrast, levels in immature females were non-detectable, as they were in half of the early vitellogenic females sampled following the initiation of puberty. The detection of testosterone at the onset of puberty may indicate that the enzyme aromatase, which converts testosterone into 17ß-estradiol, had been recently up-regulated and was not at optimal capacity.

Studies indicate that 17ß-estradiol and aromatizable androgens, such as testosterone, accelerate or amplify the pubertal activation of the BPG axis through positive feedback to the brain and pituitary (Dufour et al., 1999). The present results for female yellowtail flounder show little opportunity for testosterone to stimulate early female puberty, although pulses seen in some females at the initiation of puberty may serve briefly to enhance the development of the BPG axis. A potential positive feedback stimulation of the BPG axis in female yellowtail flounder would be more likely supplied by the consistent presence of 17ß-estradiol. Testosterone could be important in a feedback role later when levels become increasingly detectable with vitellogenesis.

Additional examination of the results for females showed that the onset of puberty was dependent on ovarian size. Only females with an estimated ovarian rank of two, where the ovary had grown half-way down the ovarian cavity, could become pubertal. However, rather than an absolute ovarian length or weight, it was GSI, the proportion of ovarian weight to body weight, that had a greater association with the onset of puberty. Presumably, ovaries

which had not reached a certain GSI during a time window for the onset of puberty remained immature and continued to exhibit slow gonadal growth until the next seasonally directed period for pubertal development. Although only one 34 month old immature female was seen, the larger GSI of this female at the onset of puberty may indicate that the GSI threshold required for puberty may change with age or body size. A relationship between GSI and puberty has been implied by data for black carp; a three fold increase in GSI occurred prior to pubertal onset, the result of a coincident two fold increase in oocyte diameter, as well as oogonial proliferation (Gur et al., 2000). Annual increases in ovarian GSI prior to puberty also have been noted in carp and striped bass (Horváth, 1986; Holland et al., 2000).

The time window for the initiation of female puberty spanned a six month period, from June into November. For 1997 and 1998 females which matured as 2+ animals a June to October period was seen in this study. For 1998 females maturing as 1+ animals the window was shortened to a period extending from early October into November. The timing of the onset of puberty coincided with moderate to high water temperature conditions (from ~6 °C to mean values of 11-12°C in culture), and changes in daylength from long summer (16 hrs) to decreasing autumn (10-11 hrs) photoperiod conditions. Some indication of the timing of puberty was seen in Howell's (1983) results for wild adults where early maturing oocytes, including cortical alveolar stage and/or early vitellogenic oocytes, were detected in April and peaked in September. Females in which these oocytes were found were described as "developing virgin" or recovering spent adults. Since spent adults first appeared in May in an April-June spawning season, any early maturing oocytes detected in April were probably from "developing virgin" or pubertal females. This supports evidence in the present

study that the initiation of puberty can occur early in the adult spawning season.

2.4.2. Ovarian development.

Vitellogenic development continued for up to a year for 2+ maturing females (June-June), but in 1+ maturing females vitellogenesis occurred within an eight or nine month period. The results presented for 1998 maturing 1+ females showed that ovarian differentiation and full oogenesis was accomplished within 22 months. Howell (1983) proposed that a two year period was required for an oogonium to develop into a mature oocyte ready for ovulation in wild females. The results for advanced 1998 females showed that oogenesis alone required less than two years. In addition, ovarian development in cultured pubertal animals followed the group synchronous pattern (Wallace & Selman, 1981) previously noted in wild females by Howell (1983). This pattern was clear in cultured females in April and June when late vitellogenic oocytes, alone, formed a distinct population from a previtellogenic stock.

The duration of vitellogenesis in other flatfish species is variable. In winter flounder, *Pseudopleuronectes americanus*, ovarian GSI increases rapidly from August to December, progressing more slowly during the winter months before the final increases at prespawning (Burton & Idler, 1984; Harmin et al., 1995). European plaice, *Pleuronectes platessa*, have an approximate six month period of vitellogenesis (Barr, 1963a; Wingfield & Grimm, 1977). Contrary to a prolonged period of vitellogenesis, the dab (*Limanda limanda*) shows only a duration of three to four months prior to the spawning period (Htun-Han, 1978a,c).

Atretic vitellogenic oocytes could be observed as early as December in 1997 females. Detecting atresia six months earlier than spawning indicates that potential fecundity is

adjusted over most of the vitellogenic period in yellowtail. The presence of atretic oocytes well in advance of spawning also has been reported for adult yellowtail flounder, dab, European plaice, English sole and striped bass (Barr 1963a; Htun-Han, 1978a; Howell, 1983; Johnson et al., 1991; Holland et al., 2000). In cod, *Gadus morhua*, atresia was noted mainly in spawning animals, or prespawning animals of poor condition, moreover, the severity of atretic activity was linked to nutritional status (Kjesbu et al., 1991).

Plasma levels of 17ß-estradiol following the initiation of puberty remained at moderate levels throughout vitellogenesis (<4 ng/ml). It is only in May and June, at the beginning of the spawning season, that higher mean levels of 17ß-estradiol (~ 4 to 6 ng/ml) were seen. Steroid profiles for adult females in captivity reported by Clearwater (1996) illustrated higher mean levels of 17ß-estradiol during vitellogenesis (~ 4-8 ng/ml), with peak levels occurring in April (10-11 ng/ml).

In vitro incubation results in the present study revealed that vitellogenic ovarian tissue, sampled at different times following the initiation of puberty, exhibited a growing sensitivity to gonadotropic stimulation. By prespawning, levels of 17ß-estradiol produced *in vitro* greatly exceeded levels in the plasma, and a reversal in dose response to crude pituitary extract was seen. These findings indicated an up-regulation in tissue responsiveness to gonadotropin, but not particularly for forskolin. An increase in gonadotropin receptors at prespawning would explain why the highest levels of 17ß-estradiol were produced by the lower doses of crude pituitary extract. Likewise, the down-regulation of GtH receptors in response to high gonadotropic stimulation should explain the lower tissue response to the high dose of crude pituitary extract.

Plasma testosterone levels were detectable in all pubertal females only by mid- to late vitellogenesis (December-April), and remained very low (<0.6 ng/ml) until increases were seen in May. Peak mean levels were reached in June at full maturity. Similarly in captive adult females, mean levels of testosterone were less prominent in the plasma than 17ß-estradiol (Clearwater, 1996). However, mean testosterone values were much higher than in pubertal females during vitellogenesis (~1.5 to 8 ng/ml), as well as during the spawning season (~9-13 ng/ml) (Clearwater, 1996). A pattern of lower plasma testosterone production during vitellogenesis and a peak during spawning is seen in other flatfish as well (European plaice, Wingfield & Grimm, 1977; Atlantic halibut, *Hippoglossus hippoglossus*, Methven et al., 1992; winter flounder, Harmin et al., 1995). Testosterone levels in yellowtail flounder, and other flatfish, match generally declining 17ß-estradiol levels only during the late-prespawning and spawning periods.

Studies have shown that increased testosterone levels during spawning are promoted by decreases in aromatase activity resulting from steroidogenic shift towards the production of C21 steroids or progestins by mature follicles (Nagahama, 1994). A Maturation-Inducing Steroid (MIS) is produced to initiate final oocyte maturation, which features the resumption of meiosis as well as the migration and breakdown of the germinal vesicle (Nagahama, 1994). A steroidogenic shift to produce the MIS may explain the high inter-individual variability in hormones of preovulatory and ovulatory females noted in the present study. Increased testosterone and decreased 17ß-estradiol in some females, as well as decreases in both hormones to values less than 0.5 ng/ml observed for a preovulating and an ovulating female, are potential evidence of this change. Methven et al. (1992) observed a drop in both

17ß-estradiol and testosterone prior to the first ovulation in Atlantic halibut, another batch-spawning species. Following the first ovulation, peaks in vitellogenin and both steroids occurred during the spawning period before finally decreasing after spawning. Repeated peaks of testosterone and fluctuations in 17ß-estradiol also were seen during the spawning season for batch-spawning European turbot, *Psetta maxima* (Howell & Scott, 1989). Repeated pulses of 17ß-estradiol and vitellogenin may reflect ongoing vitellogenesis in late vitellogenic oocytes or their maintenance during prolonged ovulatory activity in batch-spawners.

2.4.3. Onset of puberty in males.

In males, endocrine puberty was associated with novel testicular growth and the presence of primary spermatocytes amid proliferating spermatogonia. Both 11-KT and testosterone were detectable in males where early puberty was well established, with 11-KT being the dominant hormone at this time. However, a delay in the detection of androgens was seen in some males in very early puberty when primary spermatocytes were few and testes still very small. The presence of testosterone during early puberty could have a role in accelerating the maturation of the male BPG axis. Unlike observations for pubertal females, testosterone was detected consistently throughout pubertal development. While only aromatizable androgens are usually noted to stimulate the BPG axis, some stimulatory effects of 11-KT have been reported in platyfish, *Xiphophorus maculatus* (Schreibman et al., 1986). In addition a recent study for African catfish, *Clarias gariepinus,* reported a stimulatory effect of 11-KT on gonadotropin subunit gene expression, but not for GtH release (Rebers et al., 1997).

Evidence that androgens are associated with spermatogonial proliferation has been shown in the Japanese eel, *Anguilla japonica*, and pubertal African catfish (Miura et al., 1991; Cavaco et al., 1998). In Japanese eel, 11-KT stimulates Sertoli cell production of activin B which promotes spermatogonial mitosis (Nagahama, 1999). No clear connection between plasma androgens and pubertal mitosis could be made for male yellowtail flounder. Measuring intra-testicular levels may be required in order to make such a link, as detectability in the plasma may lag behind initial production. Nevertheless, as mitosis, early gonadal growth and meiosis appear to be coinciding activities during early puberty in yellowtail, it is possible that 11-KT and/or testosterone could be stimulating both mitotic and meiotic activity. Some support for this hypothesis can be seen in recrudescing males in which plasma 11-KT and testosterone were elevated, testes were dominated by proliferating spermatogonia, and spermatocytes, although present, were few in number. This observation was permitted as adult males have a more discernable period of mitosis prior to entry into meiosis, unlike the situation in pubertal males.

While androgens have been reported to stimulate spermatogonial proliferation, results in rainbow trout indicate that mitotic activity can be induced by IGF-I (Loir & Le Gac, 1994). An action of IGF-I suggests an endocrine route whereby somatic growth may affect testicular development. A relationship between body growth and male puberty was seen in yellowtail flounder where the largest males in the 1997 and 1998 year classes were the most advanced. While the data suggest that faster growth rate promotes the earlier initiation of puberty, growth performance was not a determinant factor for male puberty as even very small males initiated puberty.

2.4.4. Testicular development.

The spermatogenic cycle in pubertal and recrudescing yellowtail flounder in this study may be summarized as follows: In September and October, immature or previously mature testes develop rapidly. Mitosis was observed in males sampled at this time, as well as the initiation of meiosis with the presence of primary spermatocytes. However, a minority of males showed advanced spermatogenesis, including spermiogenesis (in recrudescent males), in October. By December, peaks in GSI values may be seen, spermatogenesis is advanced, and new milt production may be expressed in small amounts. Mitosis ceases to be observed in December, or even October in advanced recrudescent males. In April, most males had completed or were completing meiosis and spermiogenesis. The structure of the mature testis was mainly dissociated, containing mostly spermatozoa and isolated cysts of primary spermatogonia. This condition persisted in June, although small amounts of secondary spermatogonia may be seen along lobule walls, as well as an increase in the number of primary spermatogonia, suggesting a small amount of mitotic activity. In contrast, testes of pubertal males of the 1997 year class, sampled in June, still demonstrated some active spermatogenesis including spermiogenesis even though the tissue was dominated by mature spermatozoa.

Previous work on mature male yellowtail flounder has shown that milt with motile spermatozoa could be expressed year-round, although milt volume fluctuated seasonally (Clearwater, 1996). Since yellowtail in captivity do not spawn spontaneously, substantial amounts of milt in the testes and ducts may remain into the recrudescent period. This was noted in the present study in October, but reduced milt expressibility and changes in viscosity

in early December suggested that the residual spermatozoa had been resorbed. The lingering of residual spermatozoa and the rapid production of new spermatozoa entering the ducts by late autumn (December), explain the year-round presence of motile milt reported by Clearwater (1996).

Shangguan (1998), in a histological study, showed that while adult male yellowtail have a seasonality in spermatogenesis, some males demonstrated limited spermatogenic activity in July. Later samples in September and December demonstrated testes in a variety of states, postspawning, spermatogenic, as well as testes dominated by spermatozoa. Testicular development in the present study, whether for males in their first or second cycles, was highly synchronized, although during early recrudescence males showed varying degrees of advancement. The detection of spermatogenic activity during spawning in both the present group of 1997 pubertal males and in Shangguan (1998), may be due to slower rates of testicular development extending activity into the spawning period. Slower rates of development could be caused by cooler water conditions. In older animals, prolonged spermatogenesis may be a factor of age, either due to larger amounts of spermatogenic tissue, or a delay in the reinitiation of meiosis after spawning due to energetic constraints. Shangguan (1998) reported males with testes containing only spermatogonia as late as December. This was not observed for young recrudescing animals in the present study.

In both pubertal and recrudescent male yellowtail flounder, peaks in GSI were associated with peaks in spermatogenic activity in late autumn; increases in testicular mass resulted from progressive meiotic divisions. Subsequent decreases in GSI with advanced spermiogenesis reflected the resorption of cytoplasm in spermatids as they became

spermatozoa. Coinciding peaks in GSI and spermatogenic activity in the autumn also were reported for winter flounder. Rapid recrudescence and the completion of spermatogenesis was followed by a winter fasting period in which spermatozoa were maintained until spawning in spring (Burton & Idler. 1984; Harmin et al., 1995). Conversely, in plaice and dab, peak GSI was associated with both meiotic activity and spawning, as spermatogenic development preceded spawning without a sperm maintenance period in these species (Barr, 1963b; Htun-Han, 1978b,c). Observations of continued spermatogenesis into spawning in yellowtail flounder suggest that a slower rate of testicular development could be an alternate strategy for males subject to adverse conditions compared to the pattern where spermatogenesis is completed early and spermatozoa are maintained for several months. An autumn recrudescence pattern has been reported for wild yellowtail flounder (Pitt, 1970).

The evidence for rapid recrudescence in males sampled in October raises the question as to whether a true regressed phase is present; this issue was similarly raised in Shangguan (1998). In winter flounder, a regressed phase lasts at least one to two months: the testes are small (GSI< 1%) and bloody, and spermatogonia, undergoing mitosis, populate the testis (Harmin et al., 1995). Similarly, male dab and male plaice have a distinguishable regressed phase with minimal GSI values (< 0.5%) (Barr, 1963b; Wingfield & Grimm, 1977; Htun-Han, 1978c). In contrast, yellowtail males in captivity did not have the same degree of testicular regression, as the testes themselves did not become flaccid or bloody. Some males sampled in August-September for Manning et al. (chap. 3) had GSI values less than 1% and/or low androgen levels below 1 ng/ml. In the present study meiosis was reinitiated in October, or September given that two recrudescent males had spermiogenic cysts by early

October. It is therefore proposed that a regressed phase in cultured males is very brief, possibly a few weeks in which testicular reconstitution by spermatogonial mitosis begins and residual sperm is pushed from the testis proper to the duct system. It is unknown whether males in the wild display a more prominent regressed phase than those in culture. A reduced period of testicular regression may be advantageous for this species. Firstly, males which delay regression, and maintain sperm over the prolonged female spawning season, may be conferred with an advantage in fitness. Secondly, rapid recrudescence shortly after spawning and completion of spermatogenesis prior to winter temperature lows may be advantageous for cold water species like yellowtail or winter flounder.

Whether pubertal or recrudescing males were examined, plasma androgen levels remained similar between the different phases of spermatogenesis and spermiogenesis. For yellowtail flounder, levels of 11-KT were always at least slightly higher than testosterone during spermatogenesis, a pattern that was more prominent in recrudescing males. The highest androgen levels were associated with prespawning in April and May, and with spawning in some males in June. In these samples, 11-KT was clearly the dominant hormone, and was up to six fold higher than testosterone levels in some individuals. This pattern of peak levels near spawning was observed in pubertal and recrudescent males alike, but was statistically evident in the latter. Quantitative dominance and greater spermatogenic activity over testosterone have been attributed to 11-KT and other 11-oxygenated androgens in male teleosts (Fostier et al., 1983; Borg, 1994).

Androgen profiles for the young males of the present study showed similar patterns to those of seasonal profiles reported for older captive males by Clearwater (1996). Adult

peak levels were seen in May and/or June, with mean 11-KT reaching as high as ~41 ng/ml. These elevated levels declined with time. Clearwater (1996) reported seasonal profiles for two consecutive years. In one year, 11-KT was the dominant androgen through most of the cycle, while testosterone, which showed no seasonal differences, equaled 11-KT levels only during early spermatogenesis. In the second year, testosterone was dominant or similar to 11-KT until prespawning and spawning levels. The data from these two years indicate that variability in relative androgen profiles is possible. Milt volume expressed per kg body weight showed that the peak in mean milt output was much lower in the second year than in the first year (Clearwater, 1996). An effect of poor growth seen in both years may be a factor in decreases in milt output in the second year, possibly through an effect on androgen levels during spermatogenesis.

Seasonal androgen profiles showing stable or slowly increasing levels during spermatogenesis, and peaks around the spawning season, have been reported in various teleosts (reviewed in Fostier et al., 1983; Borg, 1994). Reviewing the information available for other flatfish, a dominance of 11-KT over testosterone was seen in Atlantic halibut during peak production in the spawning period, although testosterone at other stages of the cycle may equal or exceed 11-KT concentrations (Methven et al., 1992). In winter flounder, 11-KT levels were much higher than testosterone levels throughout the cycle, with both reaching maximal levels around spawning (Harmin et al., 1995). In contrast, levels of 11-KT were low and showed no seasonal change in greenback flounder, *Rhombosolea tapirina*, while testosterone was elevated and dominant throughout the cycle (Barnett & Pankhurst, 1999).

As in other teleosts, peaks in 11-KT in yellowtail during prespawning and early

spawning periods were associated with full spermiation, which is characterized by increases in milt volume due to the dilution of milt within the ducts (Billard et al., 1990). A high variability in hormone levels was seen in yellowtail males in the present study, particularly at early spawning in June when both very high and very low levels were observed. According to Clearwater (1996), adult male yellowtail demonstrated decreases in androgen levels while milt volumes remained at high levels in the middle of the spawning season in July. As in females, a steroidogenic shift promoting progestin synthesis occurs with final gamete maturation (Yaron, 1995). Both androgens and progestins have been associated with milt hydration in teleosts, where roles for both steroid classes may involve actions on the sperm duct, while progestins mediate the acquisition of sperm motility in some species (Billard et al., 1990; Yaron, 1995). In contrast, spermatozoa residing in the testicular lobules of winter flounder already have the capacity to become motile, although the passage of spermatozoa through the sperm ducts enhanced motility performance (Shangguan, 1998).

2.4.5. Age and size at maturity: cultured versus wild fish.

Ages at maturity seen in culture equaled those seen in wild yellowtail in the southern range of its distribution in New England and on the Scotian Shelf (Table 2.6; Royce et al., 1959; Beacham, 1983). As in culture, a large number of males and females alike could be fully mature at two years of age in New England (Royce et al., 1959). Ages at maturity tended to vary as much as two years in the wild (Table 2.6), which was seen in cultured females (2 - 4 yrs) but not in cultured males.

Cultured fish matured at lengths which overlapped with the lower range values reported by Beacham (1983) for Scotian Shelf fish and by Durán et al. (1998) for fish on the

Grand Banks. Larger sizes were reported in other, generally older, studies (Table 2.6). Using age-length and age-weight relationships reported by Walsh et al. (1998) for wild yellowtail on the Grand Banks, the lowest sizes of maturity observed by Durán et al. (1998) correspond to weight and age ranges of 3 to 4 years (60-140 g) in males and 4 to 5 years (140-260 g) in females. These weight estimates for wild fish are similar to the present results for cultured males and females of both year classes. However, the 1998 females reaching full maturity at 22 months of age included females weighing between 60 to 100 g, much smaller than estimated weight ranges for wild females. The presence of mature females in this size range is undoubtedly the result of precocious initiation of puberty promoted by culture conditions.

2.4.6. Development of sexual differences in growth.

Sex differences in growth seen in yellowtail flounder have been reported in other flatfish. Faster growth has been attributed to females in both European turbot and Atlantic halibut in culture (Björnsson, 1995; Imsland et al., 1997). In addition, superior growth rates in females have been previously reported for yellowtail flounder in the wild (Pitt, 1974; Walsh et al., 1998). In the present study, slower growth in 1997 year class males began with full maturity, which occurred earlier than in female conspecifics. However, differences from females in body size were significant only in the second spermatogenic cycle, which may imply an effect of repeated reproductive cycles. Cultured female yellowtail developed a slower growth pattern with approaching maturity during the latter part of pubertal vitellogenesis. Seasonal growth decreases associated with reproduction may be temporary. Björnsson (1995) reported that seasonal changes in growth could be tied with reproduction but indicated that long-term growth for males and females was linear in halibut.

Table 2.6. Age and size at full maturity and at the onset of puberty, a comparison of cultured with wild populations of yellowtail flounder.

Cultured Yellowtail Flounder

	Full Maturity		**(means ±SD)**	**Onset of Puberty**		**(means ±SD)**
Yr/Sex	Age (mo.)	Mean Total Length (cm)	Mean Weight (g)	Age (mo.)	Mean Total Length (cm)	Mean Body Weight (g)
1997 ♀	34	24.3 ±1.4	262.3 ±41.5	22	20.5 ±1.1	126.4 ±20.6
1998 ♀	22	18.3 ±2.1	99.6 ±38.8	22 13.5	19.6 ±2.5 14.9 ±1.1	121.6 ±46.0 50.3 ±14.1
1997 ♂	22	20.1 ±1.1	109.7 ±22.1	12.5	11.6 ±0.6	18.7 ±4.8
1998 ♂	22	17.3 ±1.4	70 ±19.0	13.5	12.2 ±1.4	26.0 ±8.7

Wild Yellowtail Flounder

Sex	Age (yrs)	Total Length (cm)	Comments
Royce et al. (1959) - New England			
♀	2 - 4	32 50%	For both sexes, 50% were mature by two years of age, 100% were mature a four years of age.
♂	2 - 3 (4)	<26	
Beacham (1983) - Scotian Shelf			
♀	2.9 - 4.3	23.4 - 29.4	Values indicate range in median size and age at maturity among different areas of the Shelf.
♂	2 - 4	20.1 - 24.4	
Pitt (1970) - Grand Banks of Newfoundland			
♀	65	37.4 ±1.3	Earliest sign of maturation detected in 4 yr old males, and five year old females of ~33 cm.
♂		31.0 ±1.4	
Durán et al. (1998)- Grand Banks of Newfoundland			
♀	-	24 - 35	Smallest sizes where mature individuals were seen (<50%) were 16 & 19 cm for ♂ and 21-22 cm for ♀.
♂	-	21 - 27	
Morgan & Walsh (1997)- Grand Banks of Newfoundland			
♀	6.1 - 6.8	~34	Changes in 50 % maturity stats from 1988 to 1993/95: reductions in age and size occurred in males, a slight reduction in age was seen in females.
♂	4.4 - 5.5	25 - 30	

2.5. SUMMARY.

Early sexual maturity characterized both male and female cultured yellowtail flounder. Males matured as 1+ animals in both 1997 and 1998 year classes. In contrast, females demonstrated a plasticity in the age at which first maturity was attained, both within and between year classes: 1998 females maturing as 1+ or 2+ individuals and 1997 females maturing as 2+ or 3+ individuals in culture. Similar ages at maturity are found in wild yellowtail from southern areas of the geographic range, where higher temperature conditions permit high rates of growth.

The onset of puberty in males occurred in September and October at 12.5 to 14 months of age, near the time of the autumnal equinox and when mean water temperatures were still elevated between 7 and 10°C. For females the initiation of puberty was seen during a broader time window, as early as June to as late as October or November. This period coincided with the spawning, post-spawning and early recrudescence periods in captive adults. Environmental conditions ranged between long and shortening photoperiodic cues, and moderate to elevated water temperatures. Only older 1997 and 1998 females approaching two or three years of age initiated puberty as early as June. The number of pubertal females increased in late summer and early fall into October. Younger 1998 females initiated puberty in the latter part of the time window for puberty in October and November. In both males and females the onset of puberty is hypothesized to be seasonally directed.

In females, immature ovaries were steroidogenically competent and showed a responsiveness to GtH. During the immature phase the ovary grew to a prerequisite ovarian size (GSI ~2%) and became dominated by advanced primary growth oocytes before the onset

of puberty could take place. Endocrine puberty was detected in females with cortical alveolar oocytes prior to evidence of vitellogenin incorporation. Increases in steroidal output were seen both in the plasma and *in vitro* at this time. Higher plasma levels in the cortical alveolar stage were indicative of an activation of the brain-pituitary-gonadal axis. Testosterone was undetectable in immature and in many early pubertal females (CA, VG-I and VG-II stages) in the present study. Detectable levels of testosterone were seen in some cortical alveolar and early vitellogenic females at the onset of puberty. Pulses of testosterone at this time may help further activate the BPG axis, but 17ß-estradiol is hypothesized to provide positive feedback during much of the early pubertal period in females.

In males, endocrine puberty was detected with the observation of primary spermatocytes, but evidence for a link with spermatogonial proliferation was less clear. However, as spermatogonial mitosis and meiosis appeared to be concurrent activities in pubertal males, androgens, possibly at the intratesticular level, may be associated with mitotic cycles in this species. Once endocrine puberty was detected, both 11-KT and testosterone were present during the rest of testicular development. Therefore, testicular androgens have ample opportunity, from an early stage, whereby they could serve in a presumed positive feedback role in the maturation of the male BPG axis.

In both males and females, full maturity was reached in one reproductive cycle. Ovarian development from immaturity to full maturity was easily tracked by an external examination technique in young females. Full gonadal differentiation and sexual development could be completed within a two year period in both sexes of yellowtail flounder. In males, meiotic activity could be rapid, followed by a prolonged period of sperm

maintenance prior to spawning. However, an alternate strategy may be followed where meiotic activity is prolonged throughout the winter and spring in some males. In females, vitellogenesis could occur over an eight to twelve month period. Atresia could be seen through most of vitellogenesis, from December to spawning even during pubertal development.

Estradiol-17ß and 11-KT were the dominant plasma hormones during most of gametogenesis in females and males, respectively. During female pubertal development, plasma and *in vitro* levels were closely associated. However, at prespawning an increased gonadal sensitivity to gonadotropic stimulation was seen *in vitro* which exceeded *in vivo* plasma levels.

Size at puberty was variable in females and generally small in males. Faster growing females in the 1998 year class initiated puberty as one year old fish while smaller females remained immature. However, some 1998 1+ females of small size did become sexually mature. Comparison with data in the wild suggested that these small females exhibited a precocious sexual maturation in association with culture conditions. In males, larger individuals initiated puberty ahead of smaller conspecifics, but all males matured at the same age regardless of size. Sex differences in growth were present, with females growing at a faster rate than males.

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CHAPTER 3

Hormones with Potential Duality in Growth and Reproduction: Effects of Long-Term Administration of Gonadotropin-Releasing Hormone Analogue, Testosterone and Recombinant Bovine Growth Hormone on 1+ Yellowtail Flounder, *Limanda ferruginea* Storer.

3.1. INTRODUCTION.

Growth and reproduction often are observed as competitive processes, with reproduction sequestering energy which could be used for somatic growth. Conversely, this competition appears to be reduced in the decision for puberty, as is indicated by reports that faster growth rates in immature fish promote a reduction in the age at first maturity (Alm, 1959; Le Bail, 1988). Endocrine factors which could be connected with the growth-reproduction inter-relationship are receiving greater attention in fish. There is increasing evidence that certain hormones demonstrate a duality in function, i.e. reproductive hormones having somatotropic effects and *vice versa.*

Classical models of the regulation of growth and reproduction involve two separate endocrine systems represented by the somatotropic and gonadotropic axes, respectively. In the gonadotropic axis, hypothalamic GnRH (gonadotropin-releasing hormone) stimulates the pituitary to secrete gonadotropins (GtH) which regulate gametogenesis and the production of gonadal steroids. The gonadal steroids, in addition to stimulating gametogenesis and other physiological changes required for reproduction, provide negative feedback to higher centres of the gonadotropic axis in mature animals (Goos, 1987). In the fish somatotropic axis, central neuronal factors establish inhibitory (somatostatin) and stimulatory (growth hormone-releasing hormone, pituitary adenylate cyclase activating polypeptide) tones on pituitary

growth hormone secretion (Holloway & Leatherland, 1998; Montero et al., 2000). Growth hormone (GH) in turn stimulates the liver to produce insulin-like growth factors (e.g. IGF-I and IGF-II), which regulate GH secretion through negative feedback (Peter & Marchant, 1995; Duval et al., 2002). The actions of IGF-I in fish include the promotion of skeletal growth by stimulating cartilage proteoglycan synthesis. Further evidence suggests that GH modulates IGF-I action in skeletal growth (Peter & Marchant, 1995).

Three hormones for which dual functions in fish growth and reproduction have been observed include gonadotropin-releasing hormone (GnRH), growth hormone and testosterone. Evidence of duality for GnRH was demonstrated in cyprinids, where in addition to its primary hypophyseal role in gonadotropin release, GnRH stimulated the release of GH both *in vitro* and *in vivo* (Marchant et al., 1989; Trudeau et al., 1992; Lin et al., 1993). In grass carp, *Ctenopharyngodon idellus*, and goldfish, *Carassius auratus*, *in vivo* GnRH stimulation of GH release led to somatic growth (Marchant et al. 1989; Lin et al., 1995). Similar reports of a GnRH effect on GH secretion in tilapia hybrid (*Oreochromis niloticus* x *O. aureus*) indicate that this somatotropic action of GnRH is present in higher as well as lower orders of teleosts (Melamed et al., 1995).

Apart from regulating growth, GH has been shown to stimulate ovarian steroid production (Singh et al., 1988), or have potentiating (Van Der Kraak et al., 1990) to synergistic effects (Le Gac et al., 1993) on GtH stimulated steroid secretion *in vitro*. An additional GH action in female reproduction includes an enhancement of the stimulation of vitellogenin synthesis by 17ß-estradiol in European eel, *Anguilla anguilla* (Peyon et al., 1996). In males, GH was shown to promote the secretion of progestins *in vitro* at the time

of spermiation (Le Gac et al., 1991). Additional, although indirect, gonadotropic actions of GH may be imparted via its regulation of IGF-I, which itself has been implicated as a potentially important peripheral signal for reproduction. Evidence has shown that IGF-I promotes GtH release in European eel while still exerting negative feedback effects on GH secretion (Huang et al., 1998, 1999). At the gonadal level, a stimulatory effect of IGF-I on spermatogonial proliferation in males has been demonstrated (Loir & Le Gac, 1994). More direct evidence of an overlap between somatotropic and gonadotropic axes includes the discovery of GH receptors and the presence of IGF systems in fish gonads (Le Gac et al., 1992, 1993; Perrot et al., 2000).

Testosterone is a major plasma androgen in both male and female fish. In addition to androgenic effects in males, testosterone may be a potent provider of negative feedback to higher centres of the gonadotropic axis in both sexes (Borg, 1994). Furthermore, testosterone has a non-classical positive feedback role in immature and pubertal fish. Experimental evidence has established that testosterone, either directly or through aromatization to 17ß-estradiol, accelerates the full activation of the gonadotropic axis during puberty by stimulating pituitary GtH content and levels of GnRH involved in GtH release (Atlantic salmon, *Salmo salar*, Crim & Peter, 1978; rainbow trout, *Oncorhynchus mykiss*, Crim & Evans, 1979, 1983; Crim et al., 1981; Fåhræus-van Ree et al., 1983; masu salmon *Oncorhynchus masou*, Amano et al., 1994, 1997; platyfish, *Xiphophorus maculatus*, Schreibman et al., 1986; eel, *A. anguilla*, Dufour et al.1983, 1988; Montero et al., 1995). In terms of somatotropic function, growth promoting effects resulting from the administration of androgens, including testosterone, have been well documented in fish (Donaldson et al.,

1979; Higgs et al., 1982). More direct effects on the somatotropic axis have been suggested for goldfish, where testosterone increased pituitary GH gene expression (Huggard & Habibi, 1995). In terms of GH release, it has been shown in a number of species that testosterone and/or 17ß-estradiol treatment increases GH plasma levels, which may be the result of either direct or indirect actions on the somatotropic axis (reviewed Holloway & Leatherland, 1998).

The present study examines the interactions between growth and reproduction in male and female yellowtail flounder, an early maturing pleuronectid. Given the evidence for dual functions of GnRH, growth hormone and testosterone in fish, both sexes of yellowtail flounder were treated long-term with slow release formulations of these hormones in order to determine if such dual effects could be observed in this species. Recently, in Holland et al. (1998), the use of microsphere technology permitted the prolonged release of testosterone which significantly increased pituitary GtH content in pubertal female striped bass, *Morone saxatilis*. Three of the same microsphere preparations used in Holland et al. (1998), containing testosterone, GnRH analogue (GnRH-a), and a combination treatment of GnRH-a and testosterone, were obtained for use in the present study. A combination of GnRH-a and testosterone frequently is used to promote the release of pituitary GtH which accumulates with testosterone exposure (Crim & Evans, 1983; Holland et al., 1998). For growth hormone treatment, Posilac®, a slow release formulation of recombinant bovine GH (rbGH), was used which previously has been shown to be effective in salmonids and tilapia, *O. mossambicus* (McLean et al., 1997; Leedom et al., 2002). A responsiveness to bovine growth hormone already has been demonstrated in yellowtail flounder by Taylor (1997).

Treatments were initiated at 18 months of age, which permitted the comparison of

treatment effects in immature females approaching the onset of puberty and maturing males during their first and, later, second cycles of spermatogenesis. In terms of growth, an additional point of comparison was the evaluation of hormonal treatment effects on the development of sex differences in growth, which according to Manning et al. (chap. 2) occur between 22 and 28 months of age. Regarding reproduction, important issues included determining what effects these treatments might have on: the immature gonadotropic axis and pubertal development in females, spermiation in first-time maturing males, or later recrudescence in adult males with a fully mature gonadotropic axis.

Growth enhancing effects, regardless of sex, were anticipated for all treatments, with males demonstrating slower increases than females due to their earlier maturity. In females, treatment with testosterone alone was expected to accelerate ovarian development during the normal time window for the onset of puberty. GnRH-a treatment, either alone or in combination with testosterone, was proposed to advance the onset of puberty earlier than the normal pubertal period; the combination of GnRH-a with testosterone was expected to have the strongest advancing effect. An earlier pubertal onset was similarly hypothesized for rbGH treated females, rbGH treatment either acting directly or through increased levels of IGF-I. In males, androgenic influences of testosterone on spermatogenesis and GnRH-a action on GtH release were expected to have stimulatory effects on testicular recrudescence, with possibly an additive effect seen in males in the combination GnRH-a and testosterone treatment. Larger gonadosomatic index levels were hypothesized in Posilac® treated males, as rbGH may, through IGF-I action, have an effect on spermatogonial proliferation. The reproductive effects of treatments on both sexes were assessed at the level of the gonad.

3.2. METHODS.

3.2.1. Experimental animal history and pre-experimental handling.

Experimental fish were obtained from the 1997 year class of animals reared at the Ocean Sciences Centre (OSC) in Logy Bay, Newfoundland. Prior to the experiment the subjects were kept for five months (June to December, 1998) at an experimental grow-out facility in Heart's Content, Newfoundland. While at this facility the fish were graded according to growth performance and vaccinated against furunculosis (*Aeromonas salmonicida)* and *Vibrio sp.* One hundred and fifty fish from medium to fast growing graded animals were selected on site (72.2 ±7.9 g, range 56 to 96 g). Equal sex representation (n=75 per sex) was accomplished by an external examination technique described in Manning et al. (chap. 2). The fish were transported back to the OSC the day after selection on December 1, 1998. The animals were 15.5 months old at this time. Individual fish were tagged with PIT tags (passive integrated transponders, Biomarck, Boise, Idaho USA) in two events: seventy fish were tagged on Dec. 15-18, 1998 and the remaining fish on Jan 11-12, 1999. Fish were starved for a period of three days before tagging. The tag was inserted intraperitoneally through a small, two to three mm incision made at the dorsal edge of the abdominal cavity. Insertion in the muscle was not possible due to the small size of the fish. No sutures were required after tag insertion as the incision was small and closed without external intervention.

The experimental fish were divided evenly among two adjacent 2 000 litre tanks each equipped with a bottom drain, an aeration source and supplies of fresh degassed sea water. Ambient fluctuations in photoperiod were experienced prior to and during the experimental periods. Water temperatures ranged from 4°C in the winter to ~13.5°C in the summer (Figure

3.1). A supply of ambient sea water was mixed with, or replaced with, an alternate water supply of either chilled or heated sea water as the season required.

3.2.2. Hormonal treatments.

Two types of slow release systems were used for hormone delivery in this study. The choice of slow release systems permitted long-term administration of treatments with infrequent handling. The first delivery system used biodegradable microspheres to deliver treatments of mammalian GnRH analogue (GnRH-a: [D-Ala^6, Pro^{9}NEt]-GnRH), testosterone, and a combination treatment of GnRH-a and testosterone, herein referred to as GnRH-a & T. Information on the preparation of microsphere treatments and their release profiles are detailed in Holland et al. (1998) and Mylonas et al. (1995). The second delivery system, Posilac® (Monsanto, St. Louis, Missouri, USA), contained 500 mg of recombinant bovine growth hormone (rbGH) suspended in a 1.4 ml sesame oil formulation. Control groups for each delivery system consisted of microspheres or oil formulation devoid of hormones.

Lyophilized microsphere treatments, prepared by M.C.H. Holland, were received at the Ocean Sciences Centre in separate, ready-for-use test tubes, which were stored at -20°C until scheduled injection dates. The microsphere vehicle, composed of 1% sodium-carboxymethyl-cellulose, 0.2% Tween 80, 0.14% methyl p-hydrobenzoate, 0.014% propyl p-hydroxy-benzoate and 5% sorbitol (Holland et al., 1998), was kept at 4°C. Prior to injection, the microspheres were brought to room temperature in a dessicator and the vehicle added to each tube in order to provide a mixture of 20 mg microspheres/ml of vehicle. Animals receiving microsphere treatments, including the control group, were injected

intramuscularly at a dose of 10 mg of microspheres/kg body weight. For the testosterone and GnRH-a treatments this corresponded to doses of 4 mg T/kg, and 300 μg GnRH-a/kg (Holland et al., 1998). The same individual levels of hormone were maintained in the GnRH-a & T combination treatment, however, because two types of microspheres were present the dose of microspheres was 20 mg (10 mg T + 10 mg GnRH-a)/kg body weight. The same injection volume 0.5 μl/g of fish was used in all microsphere delivery treatments.

According to results in McLean et al. (1997) for coho salmon, *Oncorhynchus kisutch*, a single injection of a high dose of Posilac® (~4 mg/g) released rbGH for 20 weeks at ~10°C. Given that temperature conditions over the greater part of the experimental period would be lower than 10°C, a six month interval between Posilac® injections was planned for the present study. Fish were injected intraperitoneally with a dose of 2 mg rbGH/g body weight which corresponded to injection volumes of 5.6 μl/g for both rbGH (Posilac®) treated and oil control fish. Posilac® (lot # 97C15/20) and the oil control (lot # 97M12) formulations were stored at 4°C prior to use.

3.2.3. Hormonal injections and fish handling.

Hormonal injections began in mid-February, 1999 (T1) when the fish were 18 months of age; all fish belonging to the different treatments were chosen at random. Injections of microsphere treatments took place at an average of 7.3 ±1.1 week intervals over the experimental duration (Table 3.1). A total of six injections of the microsphere treatments occurred for females and seven for males; the extra injection given for males permitted males to reach a more developed testicular stage for assessment of hormonal effects on recrudescence. rbGH and oil treatments were renewed only once in August, 1999 (T5). All

individuals experienced a fasting period of two to three days before any handling. Each group was composed of 12 females (13 in the testosterone and oil groups) and 12 males (11 males in the oil group). Intraperitoneal injections of rbGH and oil control treatments entered through the dorsal-posterior corner of the abdominal cavity. Intramuscular injections of microsphere treatments were placed in the caudal region, just dorsal to the vertebral column into a sinus between the epaxial musculatures. An application of direct pressure for a few seconds was necessary after injection with microspheres. A 1:1 mixture of antibiotic powder (Cicatrin, Burroughs Wellcome Inc.) and denture fixative (Orahesive, ConvaTec, Bristol-Myers Squibb) was applied to the wound to deter leakage of microspheres and bleeding (Mair, 1989). No anaesthetic was used during injections which could be performed quickly.

Some post-injection mortalities occurred after initial oil (n=8 mortalities) and rbGH (n=3 mortalities) treatments. Four replacement tagged fish were injected on Feb 18, 1999 but more individuals were needed to replace the mortalities. Seven fish from the original fish population in Heart's Content were treated with formalin for ectoparasites, then tagged intraperitoneally on March 16, 1999, the wound was given ten days to heal prior to injections with the appropriate treatment on March 26.

At each injection date all subjects were measured for total and standard lengths, and then weighed. Although fish in the rbGH and oil groups were only re-injected once, at T5 after six months, they were measured and handled at the same time as microsphere groups. After handling, the experimental fish were randomly and evenly allocated back to the two experimental tanks. Since injections and reallocations occurred six times in the experiment, with an equal chance of an individual subject experiencing either of the tanks at any one

time, tank effects were unlikely to be present in the results.

The reproductive status of individuals was assessed each time the fish were measured. Milt production stage in males was checked by applying gentle pressure at the urogenital pore. The gonadal status of females was estimated using the ovarian rank system previously assessed in Manning et al. (chap. 2). This method tracked gonadal development by relating ovarian length to the length of the ovarian cavity. This was determined simply by holding the fish in front of a bright light source. Ranks were given from a scale of 1 to 8: ranks of 1-2 are seen in immature females, with ovaries either half way down the ovarian cavity or smaller; ranks of 2-4 can be seen in females initiating puberty; values of 3 or more may be seen in females with varying stages of vitellogenic growth, with fully mature females generally showing ranks of 6 (3/4 of the ovarian cavity filled) to 8 (cavity full).

3.2.4. Feeding protocol.

The experimental fish were switched from a dry pellet, salmonid commercial feed (Nutra Fry, Moore Clarke) to a hand-made, shrimp-based, moist pellet diet after six weeks into the experiment. The salmonid feed, which had been used throughout the juvenile period prior to the experiment, had an excessive fat content (20-24%). The shrimp-based diet with a lower fat content of 8% was more suitable for yellowtail flounder, and was accepted readily by the fish soon after introduction. Daily food ration was maintained at a level of 1.5% body weight per day and portioned in one to two feedings.

3.2.5. Sampling of experimental animals and data collection.

Post-injection mortalities at T1 (February 10, 1999) were used to evaluate the gonadal stage of males (n=5) and females (n=6) at the beginning of the experiment. After six months

of treatment in August, 1999 (T5), preliminary samples of four males and four females were sacrificed from each group. The final samples of fish occurred in November-December, 1999 (T7) for females and in January, 2000 (T8) for males (Table 3.1). All sampled fish were anaesthetised with an overdose of 2-phenoxyethanol (Acros Organics, New Jersey, USA). They were then weighed, measured for total and standard lengths, and blood sampled. Blood was sampled using ice-chilled, heparinized, one cc syringes with 23 gauge needles. The blood collection was then emptied into 1.5 ml heparinized Eppendorf tubes. All blood samples were kept on ice until they were centrifuged (8 325 x g for 10 minutes at 4°C). The plasma was divided into 0.5 ml aliquots and stored between -20°C and -70°C.

Females had their ovarian development ranked before dissection and males were checked for milt. After severing the spinal cord, the gonads, liver and remaining viscera were dissected and weighed. Pieces of gonadal tissue were fixed in Bouin's fluid for one to three days, and were serially transferred to 50% then 70% ethanol in preparation for histology.

3.2.6. *In vitro* incubations of ovarian tissue.

Ovarian tissue was sampled for tissue incubation trials at the T5 and T7 sample events. The number of females processed for incubations in the preliminary and final sample events were four and six females per group, respectively. A total of 750 mg of ovarian tissue, taken from the middle of the ovary, was cut into fragments and washed twice in incubation medium over a period of at least three hours. Incubation medium consisted of a Balanced Salt Solution (BSS) modified from that used by Jalabert & Fostier (1984) for trout (3.4 mM $CaCl_2 \cdot 2H_2O$, 3.1 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 133 mM NaCl, 40 mM Hepes, 1 g/L glucose). By adjusting the pH with additions of 1.0 M NaOH and

dissolving the salts to 94.5 % of the prescribed final volume of solute, the BSS had the same pH and osmolarity values as the blood plasma of yellowtail flounder (pH 7.7; 331 mOsm). Ovarian fragments were randomly allocated among fifteen wells (~50 mg tissue/well) of a 24 well Costar incubation plate. Three replicate wells were allotted to each of five exposure treatments: i) a control treatment of BSS containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX: a cyclic nucleotide phopho-diesterase inhibitor); ii) a treatment of 10 μM forskolin (Sigma), an adenylate cyclase activator, in BSS-IBMX solution; and, iii-v) three doses (5, 50 and 500 μg/ml) of crude salmon pituitary extract (CPE: Argent Chemical Redmond, WA, USA; lot# SP1211M) in BSS-IBMX solution. All plates were placed in an incubator set at 9°C and agitated continuously over a five day period. At the end of the incubation, the fluid in the wells was recovered and stored in two 0.5 ml Eppendorf tubes at -20°C to -70°C.

3.2.7. Hormone analysis.

Plasma levels of 17ß-estradiol and testosterone in females, and 11-ketotestosterone and testosterone in males, were determined by radioimmunoassay (RIA). Tissue incubation medium was similarly analysed for 17ß-estradiol production. Estradiol-17ß and total testosterone were assayed using a no-extraction, solid phase ^{125}I radioimmunoassay kit (Coat-a Count, Diagnostic Products Corporation, Los Angeles, CA USA). The total testosterone assay used had a 16% crossreactivity with 11-ketotestosterone. For the measurement of 11-ketotestosterone, diethyl ether extraction of the plasma samples (100 μl) was required prior to performing the assay. The steroid extraction and RIA protocols followed the methods of Harmin & Crim (1993), previously outlined in Manning et al. (chap. 2). Inter- and intra-assay variabilities were 5.7% and 6.0% for 17ß-estradiol (n=13), and 9.0% and 7.1% (n=4) for total

testosterone, respectively. For 11-ketotestosterone, intra-assay variation was estimated at 3.9%, only two assays were performed one for blood samples collected at the preliminary sample (August, 1999 T5) and the other for the final blood collection (January, 2000, T8). The inter-assay variation was high, an average of 36% for three different internal standards used in each of the two assays. However, as only group differences in 11-KT within each sample event (or assay) were examined, the high inter-assay variability was not a concern.

3.2.8. Histological analysis.

Gonadal tissue, previously stored in 70% ethanol, was processed through an ethanol dehydration series, a clearing step with xylene, and an embedding step in Paraffin wax (Paraplast Plus®). Sections were cut at 7 μm, mounted on albumin treated slides, and stained using Ehrlich's haematoxylin and eosin. Ovarian and testicular development was categorized by the histological stages described fully in Manning et al. (chap. 2). Certain abbreviations have been used in the text to describe three classes of vitellogenic oocytes seen in females: VG-I, oocytes with initial yolk globules at the periphery of the oocyte cytoplasm; VG-II, oocytes with active yolk incorporation partially filling the cytoplasm; VG-III, large oocytes with cytoplasm filled with yolk granules.

3.2.9. Statistical analysis.

Statistical analysis was performed using the Statistical Analyses System (SAS, 1989). Gonadosomatic and hepatosomatic indices were calculated using the following formulae:

Gonadosomatic Index (GSI)= 100 x total gonad weight/intact body weight

Hepatosomatic Index (HSI)= 100 x liver weight/intact body weight

All growth and reproductive variables were expressed as means (± SD). *In vitro* incubation

data for a given group were reported as mean (± SE) female 17ß-estradiol output for each incubation level. Cumulative specific growth rates were calculated at T5 (August) and T7 (December) sample times (Table 3.1) for data in both length and weight. Likewise, inter-measurement specific growth rates were calculated for each sex to describe short-term growth performance during intervals between consecutive measurements.

Specific growth rates = $100 \times (\ln M(t_i + t_{i+n}) - \ln M(t_i)) / (t_{i+n} - t_i)$; M=weight or length

Changes in length, weight or condition factor over time for males, females, and both sexes combined were analysed by repeated measures analysis (general linear models (GLM) procedure). Two-way analysis of variance (ANOVA: GLM procedure) and one-way ANOVA were used to test the statistical significance of group, sex and *in vitro* exposure effects in reproductive data, specific growth rate data, body size data and *in vitro* incubation data. All ANOVAs were followed by Tukey's HSD and least square means tests. In all analyses residuals were examined for homogeneity and normality, and $\log_{10}$ or arcsine transformations used when required. The Kruskal-Wallis Test, Wilcoxon two sample test, and the Sheirer-Ray-Hare Extension of the Kruskal-Wallis Test (for two-way ANOVA situations) were employed as non-parametric alternatives when parametric assumptions in ANOVAs could not be met by $\log_{10}$ transformation. The F-test from a ranked ANOVA, which approximates a Kruskal-Wallis Test when n is high (SAS, 1989), was used with Tukey's HSD and least square means tests for large data sets ($n \geq 30$).

Condition factor (k) = weight x 100/ (total length)^3

Table 3.1. Handling and sampling schedule in relation to the time course of the experiment and the age of the experimental fish.

Measurement time (T): Groups injected during handling.	**Age (mo.)**	**Time between measure-ments (weeks)**	**Weeks since the first injections at T1**	**Dates of handling**
T1: All groups	18.0	0	0	February 9,16 & 18, 1999
T2: Mcs groups only	19.5	7.3 wks	7.3 wks	April 1-2, 1999
T3: Mcs groups only	21.0	7.0 wks	14.3 wks,	May 19-20, 1999
T4: Mcs groups only	22.5	5.7 wks	20 wks	June 29, 1999
T5: All groups	24.0	7.0 wks	27 wks	August 16 & 18, 1999
T6: Mcs groups only	26.0	8.0 wks	35 wks	October 13-14, 1999
T7: Mcs groups ♂ only	28.0	9.0 wks	44 wks	December 14, 1999
Sample events				
T1 Initial sample	18.0	0	0	February 10, 1999
T5 Preliminary sample ♂ and ♀	24.5	8.0 wks	28-29 wks	August 24 to September 2, 1999 n=4 per group
T7 Final sample ♀	27.5	7.0 wks	42 wks	November 24 to December 8, 1999 n=6-9 per group
T8 Final sample ♂	29.0	5.0 wks	49 wks	January 17 to 24, 2000 n=6-8 per group

Mcs= microsphere preparation.

All groups= refers to microsphere preparations, as well as Posilac® (rbGH) and oil control treatments.

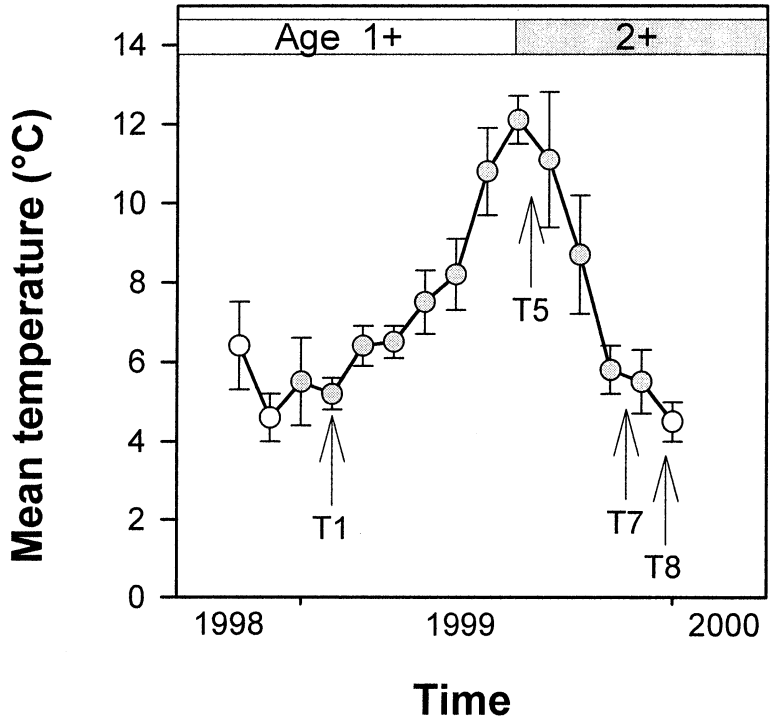


Figure 3.1. Mean (±SD) water temperature profile over the duration of the experiment. Shaded circles represent data for 1999. Sample dates T1, T5, T7 and T8 are indicated.

3.3. RESULTS.

3.3.1. Growth.

3.3.1.1. Effects of hormonal administration.

Male and females exhibited similar growth responses to the different hormonal treatments (Figure 3.2A-D). Superior increases in growth in both length and weight were evident for rbGH treated individuals, while the poorest growth performance was seen for fish injected with testosterone containing treatments (Figure 3.2A-D; Tables 3.2, 3.3; Plate 3.1). Plots for females in Figure 3.2(A,C) reflect the trends observed when the data for both sexes are combined (combined male and female data not shown).

Repeated measures analysis showed that hormone treatments significantly affected body size patterns, whether sexes were analysed individually or together ($0.0001 \leq P \leq 0.001$ T1-T7). The temporal development of group effects in body size was examined by one-way ANOVA (from T1 to T7). At the beginning of the experiment (T1), no group differences in mean body size could be detected in either males or females ($P \geq 0.10$). For females, significant group effects were present by T2 in weight ($P=0.01$) and in length ($P<0.0001$). Females treated with rbGH became statistically longer and heavier than all other groups by T2 and T3, respectively. A superior mean body size for rbGH treated females was maintained for the remainder of the experiment (Figure 3.2A,C). Females of the GnRH-a & T combination group were significantly smaller in length and weight than all other groups except testosterone treated females by T4 and again at T5. By T7, females of both testosterone containing groups were significantly smaller than all other female groups in weight, but were not significantly different from microsphere control females in length

(Figure 3.2A,C). For males, group effects in mean body size did not become statistically significant until T3 ($P<0.01$). Males receiving rbGH became significantly larger than all other groups from T4 onwards in both length and weight (Figure 3.2B,D). Males from both testosterone containing groups were significantly smaller than microsphere and GnRH-a treated males by T6, but were never significantly smaller than oil control males in either length or weight (Figure 3.2B,D). GnRH-a injected fish of both sexes showed no statistical differences in body size from either of the two control groups at any time during the experiment (Figure 3.2A-D).

Mean cumulative specific growth rates, calculated for length and weight at both T5 and T7 sample events, showed highly significant group effects ($P<0.0001$) whether data for males and females were analysed separately or in combination (Tables 3.2 & 3.3). Examining females, rbGH treated fish had a significantly higher mean growth performance in length than was seen in other groups (T5 & T7; Tables 3.2 & 3.3). A similar effect was seen by T5 for mean growth rate in weight, but by T7 the mean for rbGH treated females was not significantly different from the means of oil control or GnRH-a treated females. Females from groups containing testosterone demonstrated significantly lower mean cumulative growth rates in both body size parameters (T5 & T7; Tables 3.2 & 3.3). For males, rbGH treatment significantly increased cumulative specific growth rates in both length and weight above those of all other groups (T5 & T7; Tables 3.2 & 3.3). Mean rates of GnRH-a & T treated males were significantly lower than those of controls by T5, but the means for testosterone treated males were not (Table 3.2). By T7 both these groups showed significantly lower mean growth rates in both length and weight (Table 3.3). Mean

cumulative specific growth rates observed in males and females receiving the individual GnRH-a treatment were not significantly different from those of control groups (Tables 3.2 & 3.3).

Mean carcass weight showed no statistically significant group differences at the preliminary sample (T5) of males and females in August, 1999 (Table 3.2). For females sampled in December, 1999 (T7) mean carcass weights for individuals of testosterone containing groups were significantly lower than means in other groups, as was the case in body weight (Table 3.3). For males sampled in January, 2000 (T8), carcass weight means of testosterone containing groups were significantly lower than the means for the microsphere control and rbGH groups, but were not lower than the means for oil control or GnRH-a treated males (Table 3.3). In both males and females, rbGH treated fish had superior carcass weight means at final sample dates (Table 3.3).

Mean specific growth rates were calculated for each measurement interval for both sexes in order to determine the variability in growth rates between groups over time and changes in temperature. Males and females were analysed separately by one-way ANOVA, significant group effects were found for both sexes in length and weight-based rates at almost all intervals ($<0.0001 < P < 0.05$). Exceptions included growth rates during: i) T4-T5 for both sexes, when temperature values were high and the length of time since a fresh rbGH injection was the longest; and, ii) T6-T7 for females when mean length growth rates were not significantly different between any of the groups. The mean inter-measurement specific growth rate data are tabulated in Appendix 3A (Table 3A-1 females, 3A-2 males). The main trends are as follows:

- For control groups, mean inter-measurement specific growth rates varied over time. Both males and females showed their lowest mean growth rates in weight between T4-T5 when water temperatures were high. In males, a progressive decrease to these minimal levels was seen over T1-T4, a pattern not seen in females. Mean growth rates in weight recovered for both sexes over T5-T7, despite elevated temperature conditions during T5-T6. Trends for mean growth rates in length were less clear in control males and females.

- Mean inter-measurement specific growth rates for both sexes of GnRH-a treated fish were statistically similar to those of either one or both control groups at all times.

- Males and females of the testosterone and GnRH-a & T groups demonstrated equivalent, subdued, or on occasion significantly decreased growth rates in length and weight relative to control groups. Sharp drops in mean growth rates followed by an interval of subdued growth were seen in females between T2 and T4, particularly for body weight. Female growth rates in weight increased when temperatures were higher (T4-T6) but decreased during T6-T7 with a fall in temperature. Mean weight growth rates for males of testosterone containing groups showed the same sharp decreases as was seen for females, but growth in following intervals was more firmly suppressed (T: T2-T5; GnRH-a & T: T1-T4). This was in contrast to the progressive decreases over time noted for weight growth rates of control males. A recovery in weight growth rates was seen at the same time as controls in testosterone treated males during T5-T7, but began earlier in GnRH-a & T treated males (T4-T7).

- Only during intervals following fresh injections (T1-T2 and T5-T6) were mean growth rates in length for rbGH treated males and females significantly higher than those of both control

groups. At other intervals rbGH males always had the highest mean growth rates in length, while females did not. For growth rates in weight, means of rbGH treated females were never significantly higher than those of both control groups. While values were statistically greater than those of oil controls after fresh injections, they remained similar to those of microsphere control females at these times. A clearer effect on weight was seen for rbGH treated males which had statistically greater mean growth rates than all other male groups from T2 to T3 and T5 to T7. Mean growth rates of rbGH fish progressively decreased with the passage of time after a fresh injection. The lowest growth rates in this group were seen between T4 and T5.

Repeated measures analysis showed no group differences in condition factor (k) over the experimental period, whether males and females were analysed together or separately. One way ANOVA analysis at each measurement time did reveal group differences in condition factor in males, but only following T5 ($P<0.05$). By T8, rbGH males had the highest mean k, significantly above those of testosterone containing groups, but not the means of GnRH-a and control treated males. For females, group difference in mean k were seen at T4 and T7. Females from testosterone containing groups and the rbGH treatment group had significantly lower conditions factors than one or both control groups by T7.

3.3.1.2. Sex effects.

In addition to group effects, analyses on data sets with both sexes combined showed highly significant sexual differences in changes in body size (repeated measures analysis on weight and length; $P<0.0001$, T1-T7) and in cumulative specific growth rates (two-way ANOVA, $P<0.0001$, T5 & T7). In all groups females grew faster than males. Sex effects in

body size were not apparent in most of the groups at T1, but were seen in length and weight for the rbGH group ($P<0.05$) and in weight only for the testosterone group ($P<0.05$). However, in the remaining groups statistically discernable sex effects arose later between April and June ($P<0.05$). Sex differences became more pronounced ($P<0.01$) between May (T3) and August (T5) in all groups, and persisted up to T7 in length, weight, carcass weight and cumulative specific growth rates ($0.05>P\geq 0.0001$). Only in rbGH treated fish were sex differences, previously significant at T5 ($P\leq 0.01$), reduced at T7 in the case of length ($P=0.025$), or absent in weight ($P=0.07$), carcass weight ($P=0.09$) and cumulative specific growth rates based on both body size parameters ($P>0.2$).

In order to determine whether differences between males and females of a given treatment remained similar with time, inter-measurement specific growth rates were analysed for sex effects. A variable significance of sex effects was seen in most groups over time, but demonstrated no discernible pattern. In contrast, the rbGH treatment group showed no significant sex effects in weight rates, or only marginally significant effects in length based rates ($P\sim 0.05$) between T1 and T3 which disappeared at later intervals ($P\geq 0.70$).

Table 3.2. Group comparison of mean (±SD) cumulative specific growth rates (SPG), body size, and post-dissected carcass weight for males and females at the T5 sample time.

Group	*n*	Cumulative SPG Rate length %/d	Cumulative SPG Rate weight %/d	Standard Length (cm)	Body Weight (g)	Carcass Weight (g) *n*=4/grp
Females (T5): August, 1999. Age: 24 months.						
Mcs	12	0.09 ±0.03 ^{b}	0.29 ±0.11 ^{b}	20.1 ±1.6 ^{bc}	154 ±35 ^{bc}	146 ±48 ^{a}
T	13	0.06 ±0.02 ^{c}	0.15 ±0.05 ^{c}	19.2 ±1.0 ^{cd}	130 ±26 ^{cd}	119 ±20 ^{a}
GnRH-a	11	0.09 ±0.02 ^{b}	0.29 ±0.08 ^{b}	20.2 ±0.7 ^{b}	153 ±21 ^{b}	139 ±21 ^{a}
G&T	12	0.05 ±0.02 ^{c}	0.14 ±0.05 ^{c}	18.7 ±1.0 ^{d}	120 ±23 ^{d}	120 ±16 ^{a}
Oil	13	0.09 ±0.03 ^{b}	0.30 ±0.08 ^{b}	19.9 ±1.3 ^{bc}	151 ±36 ^{bc}	123 ±46 ^{a}
rbGH	12	0.12 ±0.03 ^{a}	0.37 ±0.11 ^{a}	21.9 ±1.3 ^{a}	196 ±41 ^{a}	176 ±43 ^{a}
Males (T5): August, 1999, Age: 24 months.						
Mcs	12	0.05 ±0.02 ^{b}	0.16 ±0.08 ^{b}	18.5 ±0.9 ^{b}	109 ±14 ^{b}	97 ±10 ^{a}
T	12	0.04 ±0.02 ^{bc}	0.07 ±0.05 ^{cd}	17.9 ±0.8 ^{bc}	98 ±13 ^{bc}	101 ±18 ^{a}
GnRH-a	11	0.05 ±0.03 ^{b}	0.10 ±0.10 ^{c}	18.1 ±1.2 ^{bc}	103 ±18 ^{bc}	88 ±22 ^{a}
G&T	11	0.03 ±0.01 ^{c}	0.04 ±0.05 ^{d}	17.5 ±0.7 ^{c}	94 ±11 ^{c}	90 ± 9 ^{a}
Oil	11	0.05 ±0.02 ^{b}	0.13 ±0.05 ^{bc}	17.8 ±1.4 ^{bc}	102 ±20 ^{bc}	107 ±26 ^{a}
rbGH	12	0.10 ±0.03 ^{a}	0.28 ±0.12 ^{a}	19.6 ±1.4 ^{a}	135 ±29 ^{a}	125 ± 9 ^{a}

Mcs= microsphere control; G&T= GnRH-a & T.

Means (±SD) within a column of data which are noted by the same superscript character are not significantly different (P>0.05). Males and females were analysed separately. Cumulative specific growth rates and body size means were calculated for all individuals in each group. Mean carcass weight was determined only on the sampled individuals (n=4 per sex).

Table 3.3. Group comparison of mean (±SD) cumulative specific growth rates (SPG), body size, and post-dissected carcass weight for males and females at the T7 sample time.

Group	*n*	Cumulative SPG Rate length %/d	Cumulative SPG Rate weight %/d	Standard Length (cm)	Body Weight (g)	Carcass Weight (g)
Females- Final sample (T7): November-December, 1999. Age: 27.5 months.						
Mcs	7	0.08 ±0.02 ^{b}	0.30 ±0.07 ^{b}	21.2 ±1.4 ^{bc}	207 ±49 ^{b}	183 ±40 ^{b}
T	9	0.06 ±0.02 ^{c}	0.16 ±0.04 ^{c}	20.1 ±1.6 ^{c}	156 ±34 ^{c}	141 ±29 ^{c}
GnRH-a	6	0.09 ±0.02 ^{b}	0.34 ±0.05 ^{ab}	21.8 ±1.3 ^{b}	216 ±40 ^{b}	185 ±34 ^{b}
G&T	6	0.06 ±0.02 ^{c}	0.18 ±0.03 ^{c}	19.7 ±0.8 ^{c}	144 ±18 ^{c}	134 ±17 ^{c}
Oil	9	0.09 ±0.02 ^{b}	0.33 ±0.05 ^{ab}	21.6 ±1.2 ^{b}	219 ±48 ^{b}	186 ±38 ^{b}
rbGH	8	0.12 ±0.02 ^{a}	0.38 ±0.09 ^{a}	24.9 ±2.1 ^{a}	305 ±84 ^{a}	274 ±74 ^{a}
Males- (T7): December, 1999. Age: 28 months.						
Mcs	7	0.05 ±0.02 ^{b}	0.18 ±0.05 ^{b}	19.4 ±0.8 ^{b}	133 ±17 ^{b}	124 ±17 ^{b}
T	8	0.03 ±0.02 ^{c}	0.09 ±0.04 ^{c}	18.1 ±1.0 ^{c}	107 ±14 ^{c}	101 ±12 ^{c}
GnRH-a	6	0.05 ±0.01 ^{b}	0.14 ±0.05 ^{b}	19.4 ±0.8 ^{b}	132 ±20 ^{b}	122 ±16 ^{bc}
G&T	7	0.03 ±0.01 ^{c}	0.07 ±0.05 ^{c}	18.1 ±1.0 ^{c}	107 ±18 ^{c}	101 ±16 ^{c}
Oil	7	0.05 ±0.01 ^{b}	0.16 ±0.03 ^{b}	18.5 ±1.0 ^{bc}	124 ±16 ^{bc}	111 ±15 ^{bc}
rbGH	7	0.11 ±0.03 ^{a}	0.35 ±0.01 ^{a}	22.1 ±2.2 ^{a}	224 ±73 ^{a}	209 ±68 ^{a}

Mcs= microsphere control; G&T= GnRH-a & T.

Means (±SD) within a column of data which are noted by the same superscript character are not significantly different (P>0.05). Males and females were analysed separately.

Mean carcass weight in males was determined at the T8 final sample time for males (January, 2000; Age=29 months) , but all other values for males represent values at T7 for comparison with females.

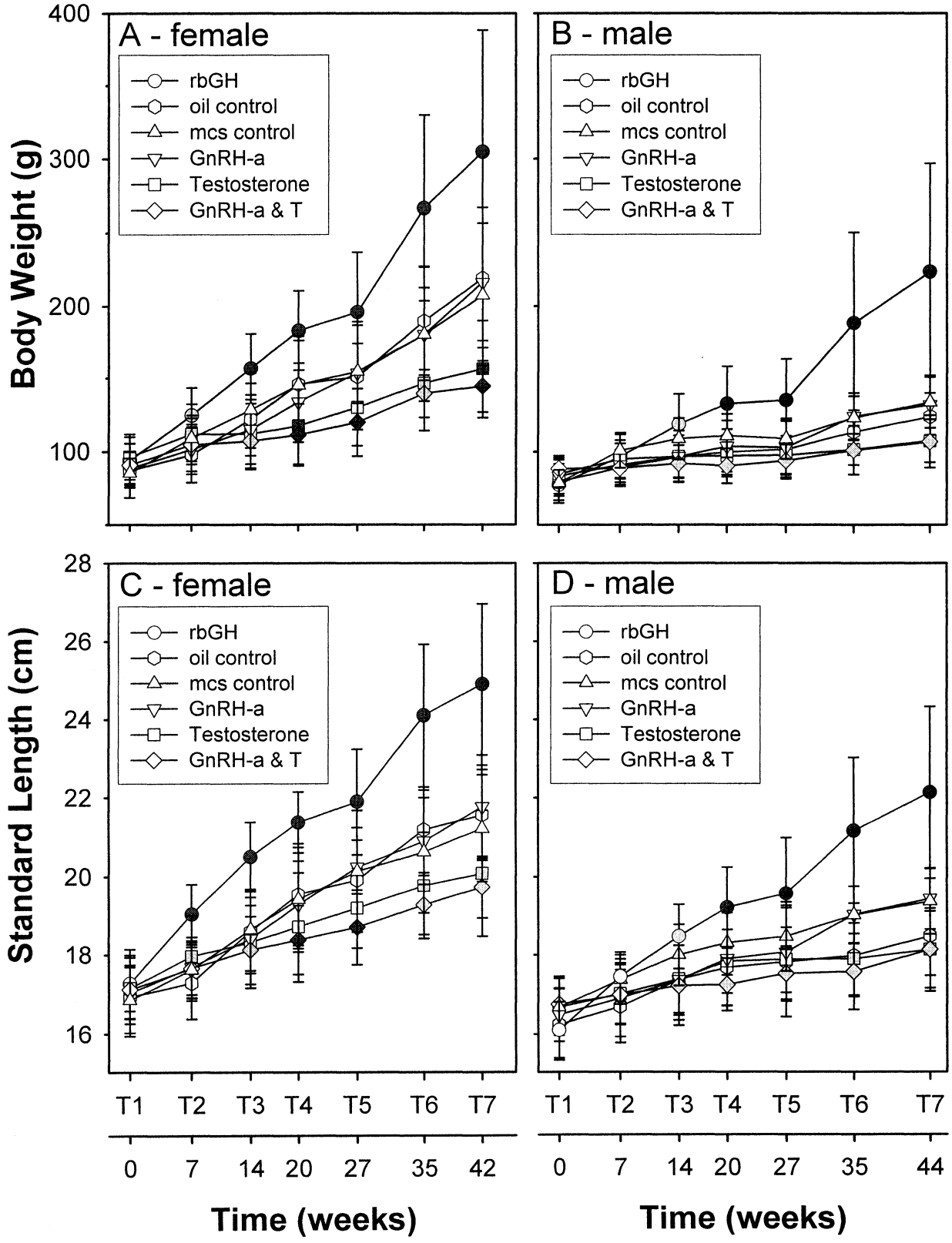

A - female
B - male
C - female
D - male
rbGH
oil control
mcs control
GnRH-a
Testosterone
GnRH-a & T
Body Weight (g)
Standard Length (cm)
400
300
200
100
28
26
24
22
20
18
16
T1
T2
T3
T4
T5
T6
T7
0
7
14
20
27
35
42
44
Time (weeks)
Time (weeks)

Plate 3.1. Males from four different groups sampled at the end of the experiment. Leftmost=rbGH, middlelower left= GnRH-a , middle upper right = microsphere control, rightmost =GnRH-a & Testosterone.

3.3.2. Male reproduction.

Males sampled at the beginning of the experiment (February, 1999: T1; Age 18 mo.) were in an advanced state of maturation. Histological analysis revealed that testes were dominated by spermatozoa although active spermatogenesis and spermiogenesis were ongoing at this time (mean GSI= 1.38 ±0.53%; mean total testis weight=1.14 ±0.36 g). From T1, milt could be expressed from males of all groups. Males receiving GnRH-a in their treatments had noticeably enhanced amounts of milt as early as the beginning of April (T2), while other groups were not in full spermiating condition until mid-May (T3).

The preliminary sample event of four males per group in August-September, 1999 (T5; Age 24.5 mo.) coincided with the end of the normal spawning period of captive yellowtail flounder. All males in the experimental groups were finishing, or had finished, their first reproductive cycle. No treatment differences were seen in GSI or total testicular weight. Low values were seen in some males of all groups, but particularly for males of GnRH-a containing groups (Table 3.4). Testicular histology of preliminary sampled males showed that the testes and ducts of most subjects were still densely packed with spermatozoa. In all groups, signs of regeneration of spermatogonial populations could be detected along the edges of the lobular walls (Plate 3.2A). In certain individuals, the regeneration had progressed such that areas of continuous spermatogonial tissue were formed. Spermatogonial tissue was most evident in two GnRH-a treated males (covering 45% & 90% of testis section area), and to a lesser extent (3- 40%) in one or two males of other groups. No areas of continuous spermatogonial tissue were seen in microsphere control males. Additional testicular features at this time included phagocytes, actively resorbing sperm in the ducts and

testes. Phagocytes usually were detected in low GSI males. This activity was more pronounced in some males of GnRH-a containing groups. One GnRH-a & T treated male in particular, which had essentially a regressed testis (GSI 0.6%) with few spermatozoa, displayed a high number of active phagocytes (Plate 3.2B).

Androgen levels in males of the preliminary sample (T5) demonstrated significant group differences (11-KT, $P<0.05$; T, $P<0.0001$). Mean plasma levels of testosterone were significantly elevated in groups where testosterone was administered (Table 3.4). Mean levels of 11-KT were lowest in males of the testosterone group; only in one male was 11-KT detectable (0.125 ng/ml). Males of other treatments had more variable 11-KT levels. Higher levels of 11-KT were observed more consistently in males of the GnRH-a, rbGH and oil control groups (Table 3.4).

The final sample of males occurred in January, 2000 (T8; Age 29 mo.). Between samples in August, 1999 (T5) and January, 2000 (T8), changes in testicular development were suggested by milt expressibility in October, 1999 (T6) and December, 1999 (T7). In October, during early male recrudescence, collectable amounts ($\geq$0.1 ml) of homogeneous milt still could be expressed from most males. Milt of a heterogeneous nature (i.e. white viscous fluid amid thin clear fluid) was expressed in many males in December, a time of peak spermatogenesis. Heterogeneous milt at this time may represent remnants of residual spermatozoa or the release of new sperm production to the ducts. An absence of milt was noted for a few individuals in the GnRH-a (1), GnRH-a & T (3) or oil (2) groups in December. Among the remaining males examined at this time, small amounts of homogeneous milt were expressed, particularly in microsphere controls and testosterone

treated males. By January (T8), thick homogenous milt could be expressed, once again, in collectable amounts for most (86-88%) males of control, testosterone and rbGH groups. Likewise, four of six GnRH-a treated males had collectable homogeneous milt, but the remaining two males of this group, and most GnRH-a & T group males, had dilute heterogeneous milt. Two GnRH-a & T treated males still had no milt in the ducts in January.

In contrast to previous results in August (T5) significant group differences were seen in mean GSI and mean testicular weight in January (T8; $P<0.0001$). High mean GSI values were seen for oil control, rbGH and microsphere control males. An intermediate GSI mean, which was still statistically similar to some high GSI groups, was noted for GnRH-a treated males. Significantly lower GSI means described testosterone and GnRH-a & T treated males (Table 3.4). Similar trends were seen in mean total testicular weight with the exception that rbGH treated males had significantly heavier testes than all other groups (Table 3.4).

Histological results on January (T8) samples confirmed that all males of the rbGH and both control groups were well into spermiogenesis, and that some had completed spermatogenesis. Lobules were densely packed with spermatozoa, and contained either dissociated tissue with most cysts having degenerated, or only primary spermatogonial nests amid the spermatozoa. Males which still exhibited active spermatogenesis had primary and secondary spermatocytes and spermatids in limited areas, but no secondary spermatogonia or mitotic cells were seen (Plate 3.3A). GnRH-a treated males were similar to controls except that two of the six males demonstrated slower spermatogenic activity. In these two males, the amount of spermatozoa produced was limited and secondary spermatogonia were still present amid meiotic cells. In addition, mitosis was observed in one male.

In contrast to the above groups in January, males of the testosterone group had testes with generally no intermediate meiotic cells between spermatogonial tissue and newly produced spermatozoa. Among the eight males sampled, generally half the testis was comprised of spermatogonial tissue (~10-90% of the testis section area). Spermatogonial mitosis was observed in five of the males. When spermatogenic activity was detected, it was reduced to rare sightings of primary spermatocytes in only two males and some spermatids in one other male. Despite a lack of observable meiotic activity, new sperm production was evident in six males, varying from low amounts of spermatozoa to densely filled lobules (Plate 3.3B,C). In the remaining two males it was not clear whether the sparse sperm cells in the testes and sperm ducts were new or residual (i.e. from the previous cycle).

The response of males to GnRH-a & T treatment was divided; two males exhibited meiotic activity while another four to five males had testes in a spermatogonial stage with generally no meiotic activity. In these latter individuals, 10 to 98 % of the testis section area was composed of tissue populated mostly or exclusively by spermatogonia. Mitotic activity was detected in three of these spermatogonial stage males. Some limited evidence of spermatogenesis was seen in two individuals: a few spermatocytes were found in one male, and two isolated cysts of new spermatozoa were noted in the other male (Plate 3.3F insert). In the male with the most spermatogonial tissue the testes appeared extremely regressed (GSI=0.4%), with no spermatozoa present in the sperm ducts or the testis despite being fully mature in the previous season. For the other spermatogonial stage males it was questionable whether spermatozoa observed in the testes were residual from the previous cycle or produced from a new cycle (Plate 3.3F). In these individuals GnRH-a & T treatment may

have induced a higher degree of inhibition than that seen in testosterone treated males. However, in contrast to spermatogonial stage males, new sperm production was indicated clearly in the two meiotic males of the GnRH-a & T group. Spermatocytes (primary dominant) and spermatids were seen in one male with low sperm production, and in the other mostly spermatids were detected amid significant amounts of spermatozoa (~50% the estimated density in control males) (Plate 3.3D,E). Besides the most regressed GnRH-a & T male, the only other male to have no milt in the ducts was the most advanced male (only clear fluid was found).

Phagocytes were detected in males of all treatments, however, a higher frequency of phagocytes could be seen in the two slower developing GnRH-a group males, as well as most testosterone and GnRH-a & T treated males. Among these males phagocytes included a varying number of active cells with sperm heads in the cytoplasm. These active cells were not isolated to individuals where new sperm production was inconclusive, but also were seen in males which clearly had produced new spermatozoa.

At the endocrine level, significant group differences were seen in both 11-KT and testosterone ($P<0.0001$; Table 3.4). For mean plasma 11-KT, clearly defined statistical groupings were seen which followed the trends observed with mean GSI: testosterone containing treatment males had the most depressed levels, GnRH-a treated males had intermediate levels, while high levels were seen in control and rbGH group males (Table 3.4). Testosterone treatment significantly elevated plasma levels of testosterone above those of all other groups, while the GnRH-a group had the lowest mean levels of this androgen (Table 3.4).

Table 3.4. Group comparison of mean (±SD) gonadosomatic index (GSI), total gonad weight, and levels of 11-ketotestosterone (11-KT) and testosterone for males sampled at T5 and T8.

Group	*n*	GSI (%)	Total Gonad Weight (g)	11-KT (ng/ml)	Testosterone (ng/ml)
Preliminary male sample (T5), August-September, 1999. Age: 24.5 months.					
Mcs	4	1.4 ±0.8 [a]	1.4 ±0.7 [a]	0.71 ±0.78 [ab]	0.75 ±0.32 [b]
T	4	1.0 ±0.3 [a]	1.1 ±0.3 [a]	0.03 ±0.06 [b]	10.74 ±5.15 [a]
GnRH-a	4	0.9 ±0.2 [a]	0.8 ±0.1 [a]	1.24 ±0.83 [a]	0.45 ±0.30 [b]
G&T	4	0.8 ±0.2 [a]	0.7 ±0.3 [a]	0.85 ±0.79 [ab]	4.45 ±1.22 [a]
Oil	4	1.3 ±0.5 [a]	1.3 ±0.3 [a]	1.73 ±1.10 [a]	0.95 ±0.44 [b]
rbGH	4	1.2 ±0.5 [a]	1.5 ±0.6 [a]	1.74 ±0.43 [a]	0.85 ±0.24 [b]
Final male sample (T8), January, 2000. Age 29 months.					
Mcs	7	3.6 ±0.6 [ab]	4.8 ±0.8 [b]	5.02 ±2.28 [a]	2.57 ±0.98 [b]
T	8	0.5 ±0.1 [c]	0.6 ±0.1 [c]	0.32 ±0.01 [c]	11.80 ±5.24 [a]
GnRH-a	6	2.0 ±1.3 [b]	2.8 ±2.1 [b]	1.68 ±1.07 [b]	1.20 ±0.52 [c]
G&T	7	0.6 ±0.2 [c]	0.6 ±0.2 [c]	0.44 ±0.20 [c]	12.32 ±8.35 [a]
Oil	7	3.9 ±0.4 [a]	4.7 ±0.6 [b]	5.48 ±0.76 [a]	1.99 ±0.06 [b]
rbGH	7	3.3 ±0.7 [ab]	7.2 ±2.0 [a]	5.85 ±2.32 [a]	2.67 ±0.71 [b]

Mcs= microsphere control; G&T= GnRH-a & T.

Means (±SD) within a column of data which are noted by the same superscript character are not significantly different (P>0.05). Data at each sample time were analysed separately.

Plate 3.2. Gonadal features of males and females of the preview sample at T5 (August-September, 1999; Age 24.5 mo.).

Males:

A- microsphere control male with spermatozoa and evidence of early spermatogonial proliferation;

B- GnRH-a & T male with regressed testis and phagocytic activity.

lbw= lobule wall; **pc**=phagocytes; **sg**=spermatogonia; **sz**= spermatozoa.

Females:

C- microsphere female with early pubertal ovary,

D- GnRH-a female with an early pubertal ovary,

E- GnRH-a & T female with primary growth oocytes,

F & G- abnormally large vacuolated oocyte (F) and atretic oocyte (G) in a GnRH-a & T female.

At= atretic oocyte; **VG-I**= newly vitellogenic oocyte with peripheral yolk globules; **VG-II**= early vitellogenic oocyte.

Black scale bars= 50 μm; white scale bars=100 μm

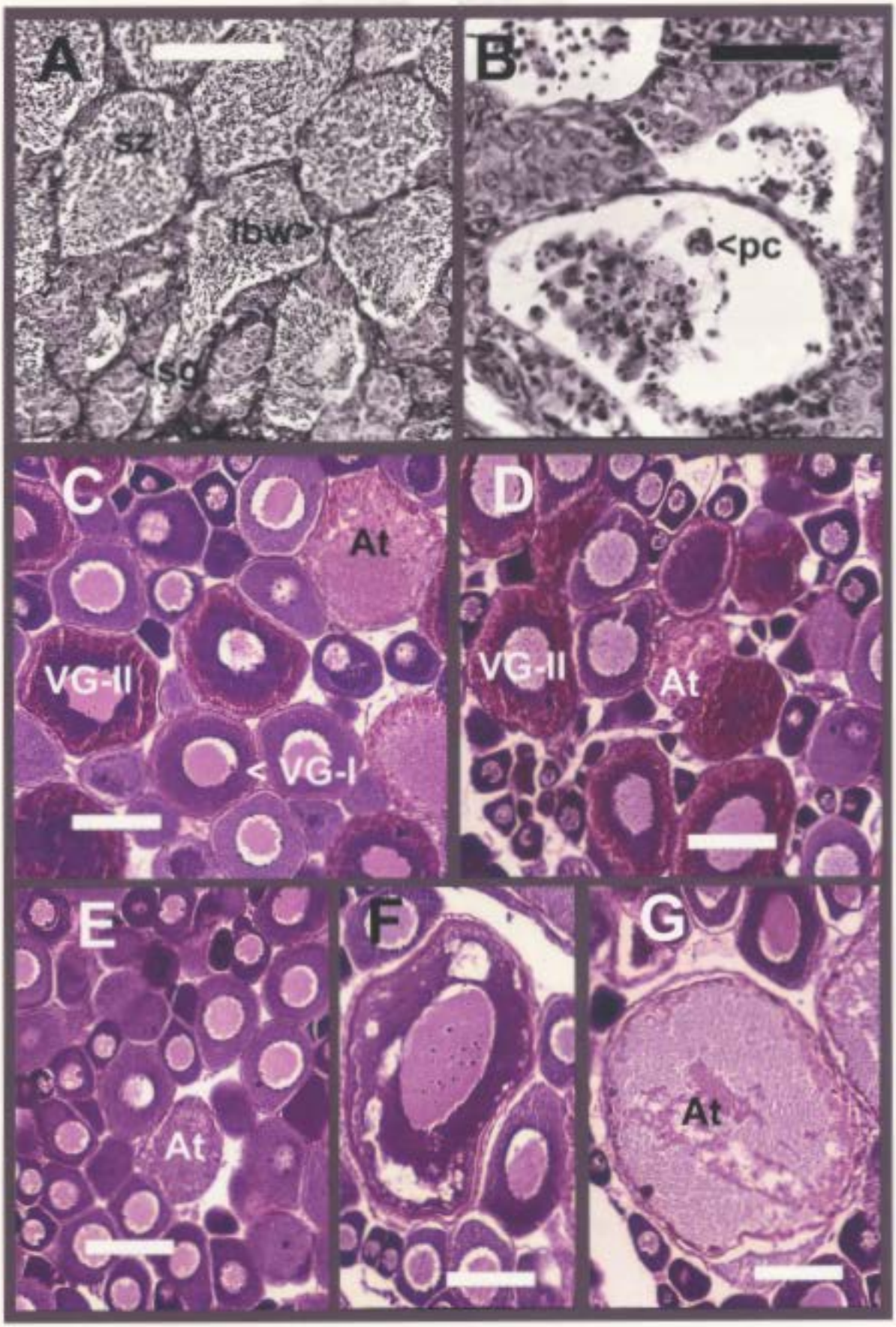

A
sz
tbw
sg
B
pc
C
At
VG-II
VG-I
D
VG-II
At
E
At
F
G
At

Plate 3.3. Testicular features at the final sample for males in January, 2000 (T8; Age 29 mo.).

A- late spermatogenic microsphere control male;

B- testosterone male with high levels of new sperm production;

C- testosterone male with limited amounts of new sperm production;

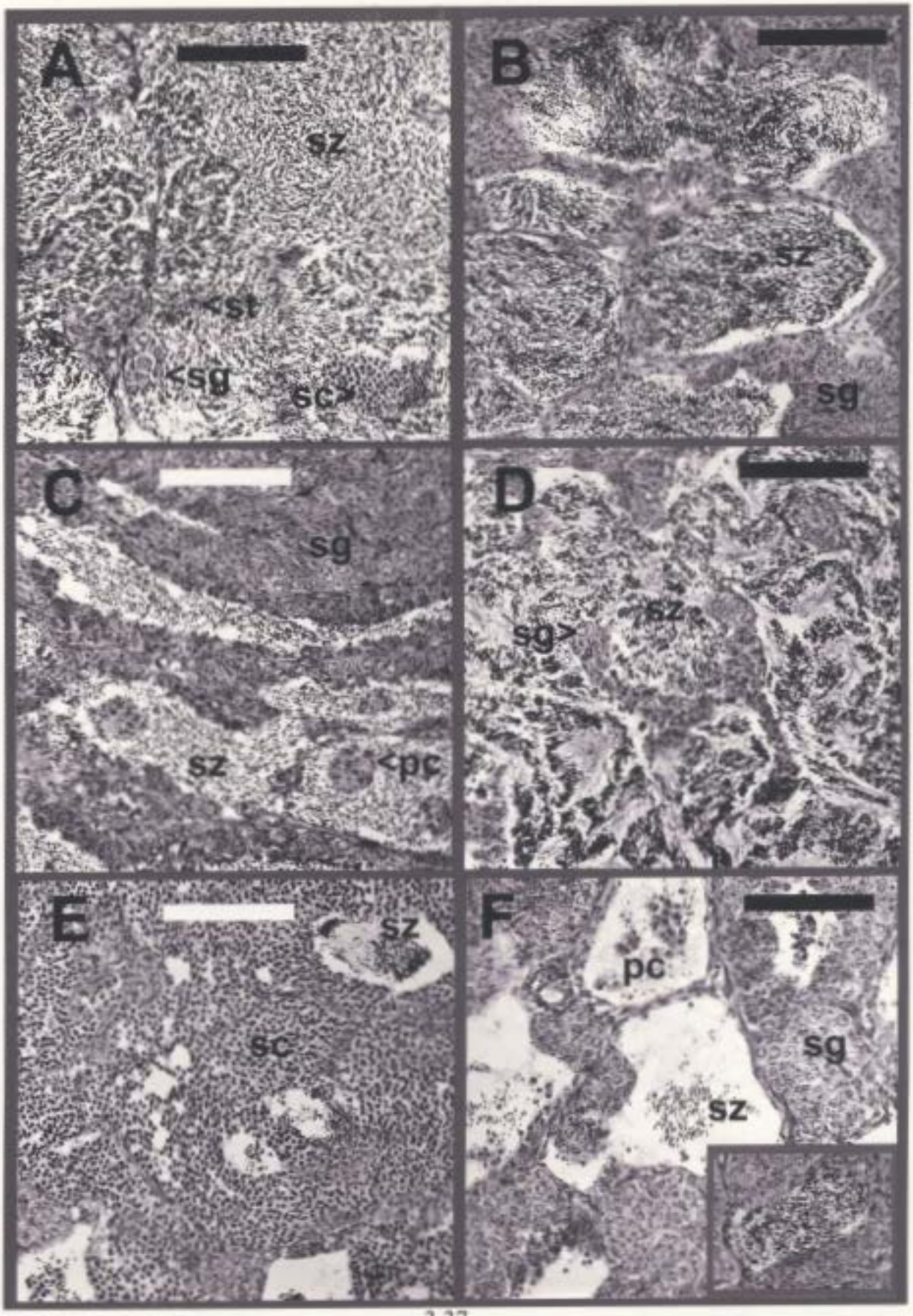
D- GnRH-a & T male with clear new sperm production;

E- GnRH-a & T male with ongoing spermatogenesis;

F- GnRH-a & T male where new sperm production is inconclusive, although the insert shows a cyst of new spermatozoa.

pc=phagocytes; **sg**=spermatogonia; **sc**=spermatocytes: **st**=spermatids; **sz**= spermatozoa.

All scale bars= 100 μm


A
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3.3.3. Female reproduction.

At the beginning of the experiment in February, 1999 (T1; Age 18 mo.), females were immature with small ovaries full of primary growth oocytes, most were in the circumnuclear ring stage (mean GSI=1.42 ±0.31%, ovarian rank=1-2). However, a total of seven females maturing as 1+ fish were distributed among the 1+ immature females in the microsphere treatment groups (2 microsphere control, 2 GnRH-a, 1 testosterone, 2 GnRH-a & T). All seven females ovulated between May (T3) and August, 1999 (T5), females in GnRH-a containing groups demonstrating earlier ovulatory activity. All 1+ maturing females were excluded from analyses of reproductive parameters.

Between February (T1) and the preliminary sample in August-September, 1999 (T5; Age 24.5 mo.), ovarian development of immature females was followed by changes in ovarian ranks (Figure 3.3). No clear indication was seen by ovarian ranks that any treatments accelerated ovarian growth or the onset of puberty in females prior to August (T5).

Sampling three to four immature females per group in August revealed no significant group differences in mean GSI, total gonad weight or 17ß-estradiol levels (Table 3.5). It is clear, however, that GnRH-a treated females had the highest means for all three variables (Table 3.5). Significantly higher testosterone levels were seen in those groups where exogenous testosterone was administered ($P<0.0001$; Table 3.5). In other groups, the highest mean levels of testosterone were seen in GnRH-a group females, but levels were not significantly greater than those of the control or rbGH groups (Table 3.5).

Results of *in vitro* incubations showed that ovarian tissue from females in testosterone containing treatments had produced minimal (one female in the GnRH-a &T

group) or no quantities of 17ß-estradiol. Moreover, the tissue from these females was not responsive to forskolin or gonadotropic stimulation via crude pituitary extract (data not shown). Statistical analysis comparing the *in vitro* performance of the six different groups showed a significant group effect (two-way ANOVA, $P<0.0001$) which disappeared when only non-testosterone groups were considered (two-way ANOVA, $P=0.31$).

The 17ß-estradiol production data for each individual group was analysed separately. Only the GnRH-a group showed a statistically significant response to *in vitro* stimulatory agents (Kruskal-Wallis test $P=0.016$; ranked ANOVA $F(4,10)=17.3$, $P=0.002$; Figure 3.4, upper plot). For these females, tissue exposed to either forskolin or the 500 µg/ml dose of crude pituitary extract produced the highest 17ß-estradiol levels. While Wilcoxon two sample tests did not show that these increases were significantly higher than the control response ($P=0.08$), pairwise comparisons following a ranked ANOVA did support the overall treatment effect. Control and rbGH groups showed a similar pattern in 17ß-estradiol production, but a lack of statistical significance in these groups was due to a high female variability in tissue responsiveness (Figure 3.4, upper plot).

Histologically, evidence of vitellogenesis, and thus the initiation of puberty, was seen in all groups in August (T5). In control and rbGH groups, the most advanced oocytes in females included early vitellogenic oocytes with peripheral yolk globules (VG-I) or more developed early vitellogenic oocytes (VG-II : 3 oil females and 1 microsphere control; Plate 3.2C). In contrast, in one oil control and one rbGH treated female, oocytes remained in an advanced primary growth stage characterized by granular cytoplasm. A higher synchronicity in ovarian development, which reflected endocrine patterns, was seen in the GnRH-a group

where all females had VG-II oocytes (Plate 3.2D).

Minimal vitellogenic development was seen in testosterone containing groups. Most GnRH-a & T group females were previtellogenic, and had either cortical alveolar or advanced primary growth oocytes (Plate 3.2E). However, one GnRH-a & T treated female had VG-I oocytes. In addition, some extremely large abnormal oocytes with prominent vacuoles, and atretic oocytes of similar size, were seen in this vitellogenic female (Plate 3.2F,G). As in the GnRH-a & T group, testosterone treated females had ovaries with advanced primary growth oocytes, and in one female some additional cortical alveolar stage oocytes. Yet, evidence of vitellogenesis was seen in atretic oocytes containing yolk globules in two females. Similar examples of atretic resorption of early vitellogenic oocytes were detected in all other groups, including oocytes which had progressed to the VG-II stage. In addition, atretic primary growth oocytes were detected in females from both testosterone containing groups, as well as in the single immature female sampled from the rbGH group.

From August, 1999 (T5: Age 24 mo.) to December, 1999 (T7: Age 27.5 mo.), ovarian ranks indicated that pubertal development was clearly underway in oil control and GnRH-a treated females, while, on average, slower development was seen in microsphere control and rbGH treated females (Figure 3.3). In contrast, ovarian ranks of females from testosterone containing groups indicated that ovarian development was inhibited (Figure 3.3).

At the final sample for females in November-December (T7; Age 27.5 mo.), highly significant group effects were seen in GSI, total gonad weight and plasma hormone levels which confirmed the observations seen in external ovarian ranks ($P<0.0001$; Table 3.5). The general pattern for reproductive variables was as follows: females from GnRH-a and both

control groups had high mean levels, testosterone and GnRH-a & T groups had the lowest levels, while means for the rbGH group had intermediate values. Statistically, GnRH-a, microsphere control and oil control treated females had similar mean GSI, ovarian weight, and plasma hormone levels. Notably, GnRH-a group females had consistently high values and less variability than other groups in all reproductive variables except testosterone levels (Table 3.5). Females from the rbGH group were in the same statistical grouping as control and GnRH-a treated females when comparing ovarian weight and testosterone means, but were similar only to microsphere controls in 17ß-estradiol levels. In mean GSI, rbGH injected females were grouped with testosterone and GnRH-a & T group females. These testosterone containing groups had distinctly lower means in GSI, ovarian weight and 17ß-estradiol than most or all other groups. Predictably, higher testosterone levels in the plasma were associated with testosterone administration (Table 3.5).

In vitro incubation data demonstrated that tissue from testosterone and GnRH-a & T treated females did not produce 17ß-estradiol, even in the presence of forskolin or crude pituitary extract. Examining only the data from the non-testosterone containing groups revealed no significant group differences (two-way ANOVA, P=0.058; Figure 3.4, lower plot); group differences were present in an analysis with all six groups (P<0.0001).

Within individual groups, tissue from GnRH-a and both control groups responded significantly to forskolin, as well as low and high doses of crude pituitary extract (Figure 3.4, lower plot). Conversely in the rbGH group, mean steroidal output in response to stimulatory agents was not significantly elevated from mean production levels of control wells. A high inter-individual variability in tissue responsiveness was seen in the rbGH group, which was

similarly evident in the microsphere control group but not other groups (Figure 3.4, lower plot). Further investigation indicated a division in the rbGH group incubation results; two subgroups of individuals were seen which were significantly different from each other in overall 17ß-estradiol output *in vitro* ($P<0.0001$). The mean *in vitro* steroidogenic output of tissue from three females demonstrated a high sensitivity to tissue stimulation (one-way ANOVA, $P<0.0005$; Figure 3.4, lower plot-rbGH mat). In contrast, the mean output of three other females showed no response to stimulatory agents (one-way ANOVA, $P=0.59$; Figure 3.4, lower plot-rbGH delay). Examining these latter three females individually showed that while one female had non-responsive tissue (one-way ANOVA, $P=0.6$), the tissue of the other two females were responsive to *in vitro* stimulation in a limited but statistically significant manner (one-way ANOVA, $P<0.002$).

Histological features supported the trends seen in the above results. Large vitellogenic oocytes (VG-III) were seen in microsphere control, oil control and all GnRH-a treated females indicating that pubertal development was advanced (Plate 3.4A). One female in each control group, however, lagged behind the development seen in other females by virtue of the fact that they only had either VG-I (oil) or VG-II (microsphere control) yolky oocytes. The presence of these early vitellogenic oocytes was indicative of the recent initiation of puberty. Atretic activity was not detected in oil control or GnRH-a treated females, and was rare in the microsphere control group (one female).

The inhibition of vitellogenesis was evident in testosterone and GnRH-a & T treated females, where, generally, cortical alveolar oocytes or primary growth oocytes were the most advanced cells observed (Plate 3.4B,E). Only one testosterone treated female sampled in

December had atretic oocytes in which yolk globules were seen (VG-I). In one GnRH-a & T treated female, a very few cells had escaped inhibition to become vitellogenic oocytes, two of which were VG-III oocytes (Plate 3.4F). Atretic primary growth oocytes were seen in only two females in each testosterone containing group.

Of the eight rbGH treated females sampled, three had maturing ovaries (GSI 6.6-7.6%) populated by large vitellogenic oocytes (VG-III). These females were the same individuals whose tissue exhibited a high steroidogenic output *in vitro* (Plate 3.4C). Of the remaining rbGH injected females: one had primary growth oocytes in the circumnuclear ring stage, two had very few VG-I oocytes (Plate 3.4D), and two more had VG-I and VG-II oocytes. These latter five females demonstrated delayed and potentially suppressed pubertal development (GSI <2%). Three of these females were those whose tissue produced low amounts of steroid *in vitro*, despite having some VG-I or VG-II oocytes. Atretic vitellogenic oocytes (VG-I & II) were noted only in the female with the non-responsive tissue. A similar division in plasma 17ß-estradiol was seen among rbGH females. Levels of 17ß-estradiol were: between 2.9 and 3.3 ng/ml for developing females, between 0.27 and 0.43 ng/ml for delayed females with some vitellogenesis, and at a low value of 0.16 ng/ml for the single immature female with only primary growth oocytes. Females demonstrating these different degrees of ovarian development could not be separated on the basis of size, weight or condition factor with the exception of three of the five delayed females. These individuals had low condition factors (<1.30 based on carcass weight). However, some fish in other groups with condition factors in this range were maturing. It should be noted that the immature female was the smallest rbGH female, and appeared not to respond to rbGH.

Table 3.5. Group comparison of mean (±SD) gonadosomatic index (GSI), total gonad weight, and plasma levels of 17ß-estradiol and testosterone for females sampled at T5 and T7.

Group	*n*	GSI (%)	Total Gonad Weight (g)	17ß-estradiol (ng/ml)	Testosterone (ng/ml)
Preliminary female sample (T5), August -September, 1999. Age: 24.5 months.					
Mcs	3	2.2 ±0.5 [a]	3.4 ±1.4 [a]	0.46 ±0.42 [a]	0.06 ±0.10 [b]
T	4	2.2 ±0.4 [a]	2.8 ±0.4 [a]	0.24 ±0.11 [a]	6.11 ±3.75 [a]
GnRH-a	3	3.2 ±0.1 [a]	4.8 ±0.8 [a]	1.08 ±0.37 [a]	0.28 ±0.12 [b]
G&T	3	2.8 ±0.9 [a]	3.7 ±1.5 [a]	0.22 ±0.11 [a]	4.69 ±1.33 [a]
Oil	4	2.4 ±0.4 [a]	3.3 ±1.7 [a]	0.64 ±0.53 [a]	0.08 ±0.10 [b]
rbGH	4	2.3 ±1.0 [a]	4.6 ±3.2 [a]	0.29 ±0.19 [a]	0.05 ±0.11 [b]
Final female sample (T7), November-December, 1999. Age: 28 months.					
Mcs	6	7.4 ±2.9 [a]	16.6 ±8.5 [a]	1.76 ±1.28 [ab]	0.13 ±0.09 [b]
T	8	2.1 ±0.3 [b]	3.2 ±0.6 [b]	0.30 ±0.08 [c]	9.48 ±3.73 [a]
GnRH-a	6	8.4 ±1.1 [a]	18.3 ±4.3 [a]	2.78 ±0.79 [a]	0.23 ±0.16 [b]
G&T	6	2.3 ±0.5 [b]	3.3 ±0.6 [b]	0.26 ±0.08 [c]	8.08 ±3.57 [a]
Oil	9	8.5 ±2.7 [a]	19.2 ±8.1 [a]	2.03 ±0.87 [a]	0.17 ±0.11 [b]
rbGH	8	3.8 ±2.9 [b]	12.0 ±10.3 [a]	1.39 ±1.45 [b]	0.14 ±0.15 [b]

Mcs= microsphere control; G&T= GnRH-a & T.

Means (±SD) within a column of data which are noted by the same superscript character are not significantly different ($P>0.05$). Data at each sample time were analysed separately.

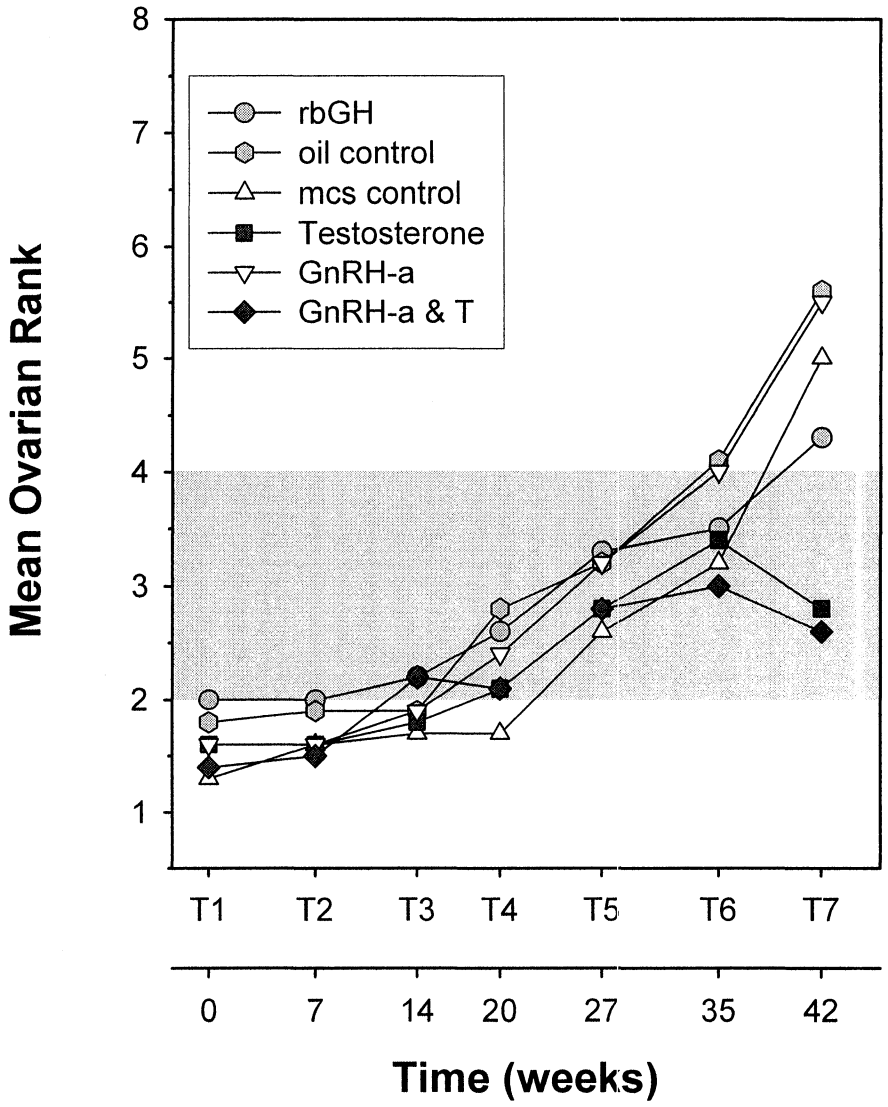


Figure 3.3. Tracking ovarian development in females of different groups through the use of ovarian ranks (based on the external evaluation of ovarian growth in length). The shaded area represents rank values in which the onset of puberty may occur. Mean ranks for each group are shown for measurement times T1 to T7.

Figure 3.4. Ovarian tissue incubation results for different groups of females sampled in August, 1999 (T5; Age 24.5 mo.) (upper) and December, 1999 (T7; Age 27.5 mo.) (lower). Mean (±SE) 17ß-estradiol production among females (n=3-4/group in August, 6/group in December) from microsphere control, GnRH-a, oil control and rbGH groups are shown. Ovarian steroid production is represented for tissue in control, forskolin, 5 µg/ml, 50 µg/ml and 500 µg/ml crude pituitary extract (CPE) exposure treatments. Results for rbGH females in December (lower plot) are divided between three maturing females (rbGH mat) and three females which appear to be delayed (rbGH delay). Upper case letters represent comparisons between hormone treatment groups based on overall steroid production performance. Lower case letters indicate comparisons between incubation exposure treatments within hormone treatment groups. Means (±SD) which are noted by the same superscript character are not significantly different (P>0.05).

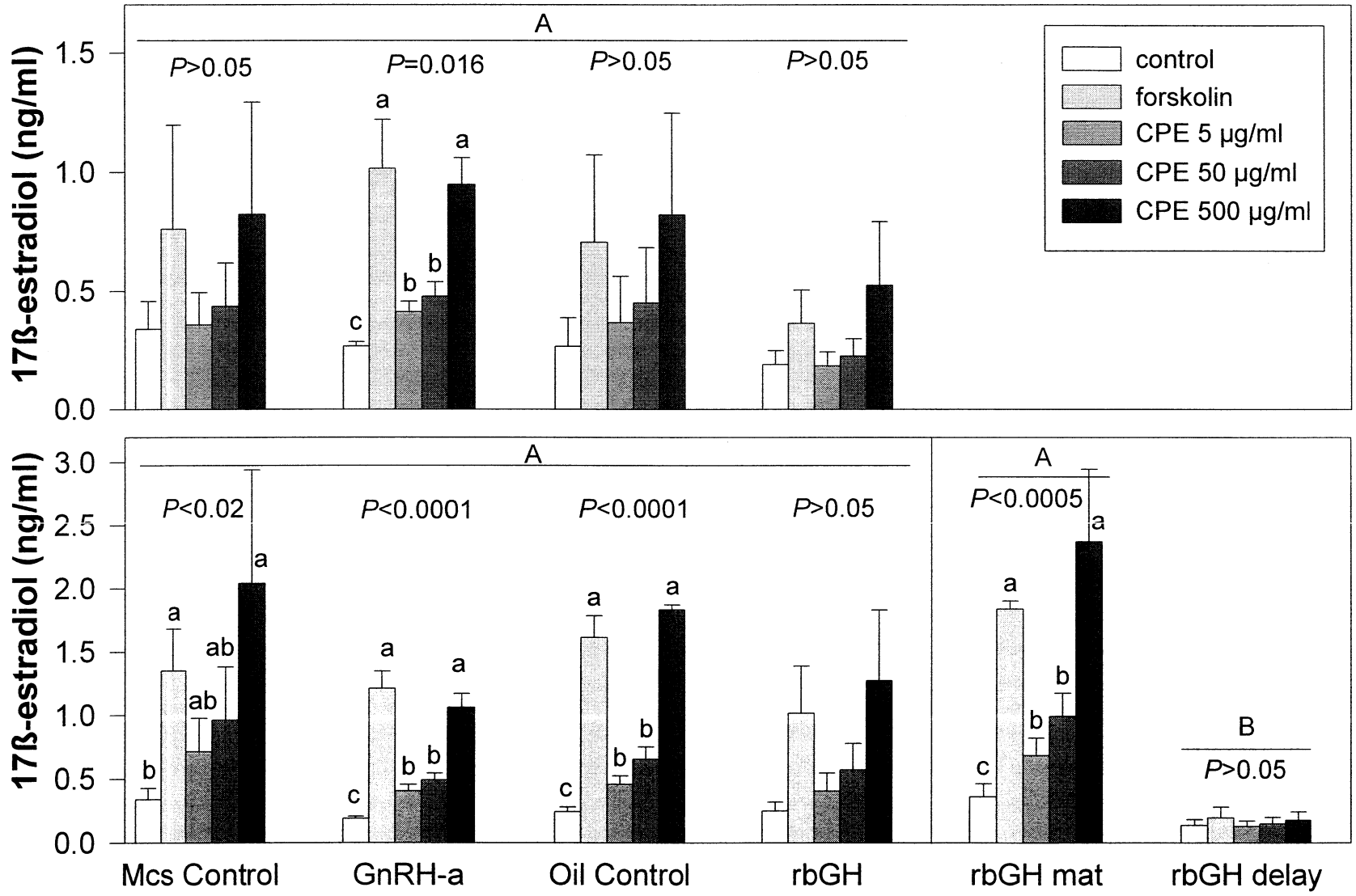

17ß-estradiol (ng/ml)
1.5
1.0
0.5
0.0
A
P>0.05
P=0.016
P>0.05
P>0.05
control
forskolin
CPE 5 µg/ml
CPE 50 µg/ml
CPE 500 µg/ml
17ß-estradiol (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
P<0.02
P<0.0001
P<0.0001
P>0.05
A
P<0.0005
B
P>0.05
Mcs Control
GnRH-a
Oil Control
rbGH
rbGH mat
rbGH delay

Plate 3.4. Ovarian development at the final sample for females in December, 1999 (T7; Age 27.5 mo.).

A- oil control female with well-developed vitellogenic oocytes;

B- Testosterone female with primary growth oocytes and a cortical alveolar stage oocyte;

C- Vitellogenic rbGH female;

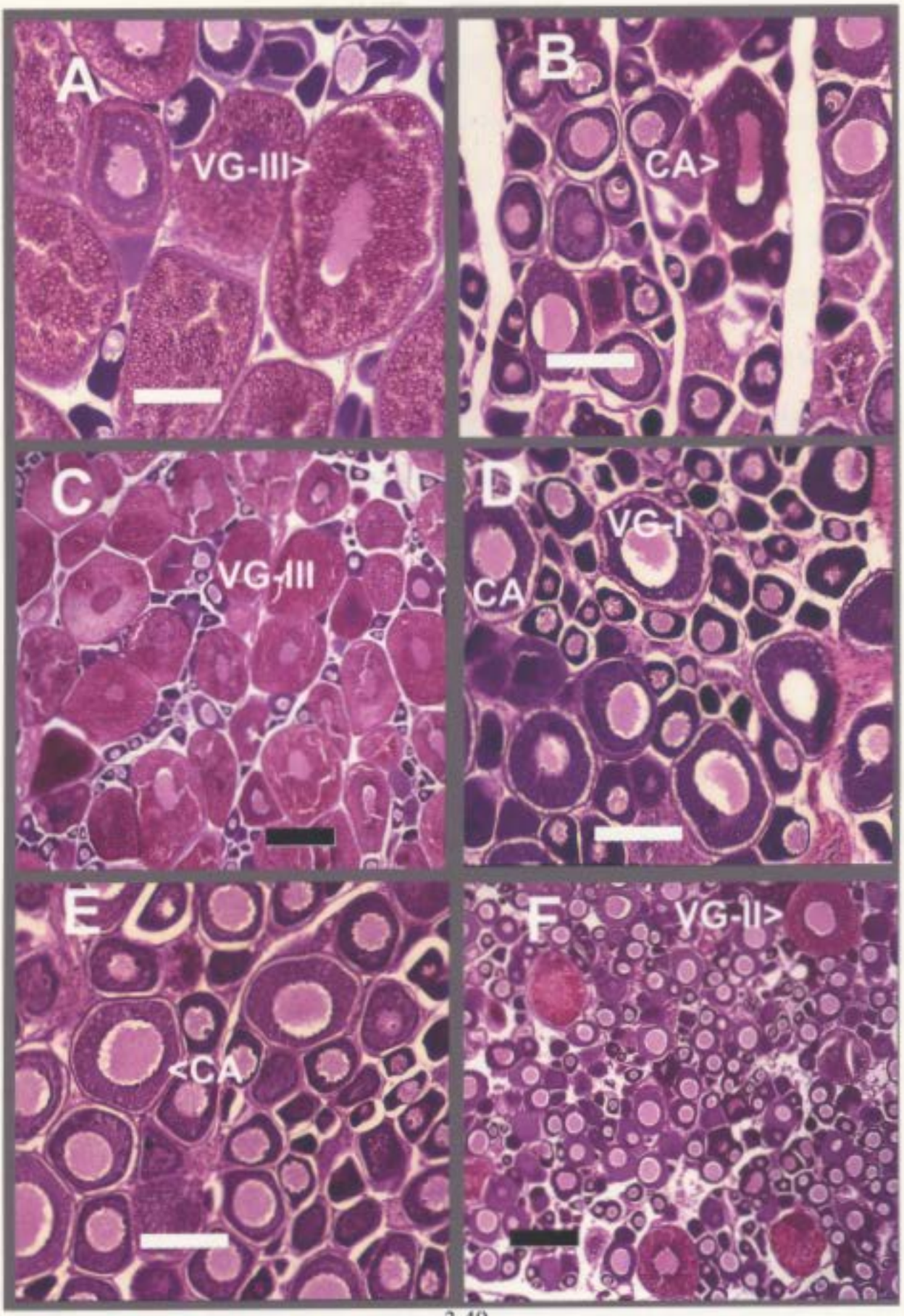
D- delayed rbGH female with rare evidence of very small amounts of yolk;

E- GnRH-a & T female with only cortical alveolar and primary growth oocytes;

F- GnRH-a & T female with some vitellogenic oocytes amid mostly previtellogenic stage cells.

At= atretic oocyte; **CA**= cortical alveolar stage oocyte; **VG-I**= newly vitellogenic oocyte with peripheral yolk globules; **VG-II**= early vitellogenic oocyte; **VG-III**= large vitellogenic oocyte.

White scale bars = 100 μm; black scale bars= 200 μm.


A
VG-III>
B
CA>
C
VG-III
D
VG-I
CA
E
<CA
F
VG-II>

3.4. DISCUSSION.

Treatment with recombinant bovine growth hormone resulted in increased body size and higher cumulative specific growth rates in both immature females and mature males. However, while cumulative growth rates of rbGH group females were significantly higher than other groups for length at the end of the experiment (December, 1999: T7; Age 27.5 mo.), they were not so for weight. This was not due to a decreased response to rbGH, but due, in part, to the gonadal weight increases in females of oil control and GnRH-a groups, which were greater than in rbGH treated females.

Enhancing effects of rbGH over controls were mostly due to higher growth rates which occurred in intervals following the administration of fresh injections. Thus, it appears rbGH release from the Posilac® formulation was highest immediately after injection; and that high levels of rbGH were required for significantly higher growth rates over controls, as well as overcoming individual variability in rbGH responsiveness. A recent study has shown that juvenile tilapia (*Oreochromis mossambicus*) were refractory in growth response to low doses of rbGH, and that higher levels were needed possibly due to a low binding affinity of tilapia GH receptors for a heterologous GH (Leedom et al., 2002). Therefore, in the present study, observations of significantly higher growth rates after fresh injections, particularly in length, may indicate that high levels of circulating rbGH are required to overcome a high specificity of the yellowtail flounder GH receptor for its own growth hormone.

Growth hormone has been reported to be more effective in growth in length than in weight, a difference which can lead to a decrease in condition factor (k) (Higgs et al., 1977; Björnsson, 1997; McLean et al., 1997; Leedom et al., 2002). In McLean et al. (1997),

Posilac® treatment had a decreasing or leaning effect on condition factor which was greatest during peak periods of growth in length. However, the condition factors of injected fish returned to control levels with time. In yellowtail flounder, no significant leaning effect following fresh injections was observed. This may indicate that rbGH had equivalent effects on length and weight. While growth rates in length were significantly higher than controls after fresh injections in both sexes, growth rates in weight for females never significantly exceeded rates of microsphere controls after fresh injections. In contrast to females, males had prolonged significant increases in weight specific growth rates, with the exception of the interval following the first injection of rbGH. The prolonged effect in males in terms of weight was probably due to the development of slower growth rates in maturing males seen in other groups. That rbGH males and females did not always exceed the weight growth rates seen in microsphere controls may be related to the invasive nature of intraperitoneal compared to intramuscular injections, or that energy demands for increases in length did not permit a full stimulation of weight gain after fresh injections.

An important result in the present study was the demonstration that rbGH treatment was highly effective in males despite their maturity. In addition, rbGH removed or reduced sex differences in growth parameters over time. While this may, in part, be due to a high individual variability among males and females in rbGH responsiveness, it is in sharp contrast to the increasing sex differences in growth and body size in all other groups through the experimental period. In Manning et al. (chap. 2) sex differences in body size were statistically established by December at 28 months of age. These differences developed during pubertal development in females and the second spermatogenic cycle in males. In the

present results, where fish were tagged and followed individually, sex differences were well established in both weight and length much earlier, between mid-May, 1999 (T3; Age 21 mo.) to late August, 1999 (T5; Age 24.5 mo.). This period coincided with full spermiation, which occurred for the first time in the present males, and with the normal spawning period in captivity. Sex differences in growth and decreases in growth rates associated with reproductive maturity are well established phenomena in fish. Faster female growth patterns have been observed previously in wild populations of yellowtail flounder (Pitt, 1974; Walsh et al., 1998) and other flatfish in culture (Atlantic halibut, *Hippoglossus hippoglossus*, Björnsson, 1995; European turbot, *Psetta maxima*, Imsland et al., 1997).

Regarding the effects of rbGH on male reproduction, males from rbGH and both control groups showed similar GSI, 11-KT and testosterone levels, as well as histological features of spermatogenic development. One difference from controls was the significantly higher mean testicular weight in rbGH treated males in January, 2000 (T8; Age 29 mo.). However, this increase in gonadal weight did not translate into higher GSI; instead gonadal growth remained in proportion to stimulated increases in body size. This suggests a strong link between gonadal size and body size, a relationship which may be regulated by GH or IGFs. Evidence to support a somatotropic involvement in testicular activities has been shown in a number of studies. This evidence includes the detection of testicular GH and IGF receptors, as well as the demonstration of IGF gene expression in the fish testis (Le Gac et al., 1992, 1993, 1996; Perrot & Funkenstein, 1999). In addition, IGF-I has been reported to stimulate spermatogonial mitosis in rainbow trout, *Oncorhynchus mykiss* (Loir & Le Gac, 1994). Although the fish testis produces IGFs, circulating IGF-I of hepatic origin bound by

testicular IGF receptors, has been proposed to be physiologically relevant during spermatogonial proliferation and early meiotic stages in trout (Le Gac et al., 1999). Together these studies show a link between growth and male reproduction whereby somatotropic agents could affect testicular size and productivity through actions on spermatogonial proliferation. Of particular resonance with the present study are reports that long-term treatment, with homologous GH or heterologous bovine GH, stimulates testicular IGF gene expression in immature trout (Le Gac et al., 1996; Perrot & Funkenstein, 1999). Thus, it may be possible that rbGH treatment had a stimulatory effect on testicular recrudescence in the present study which may have facilitated the proportional increase in testicular mass with increases in body size.

It is clear that rbGH treatment had no adverse effects on spermatogenesis despite the stimulation of growth (length T5-T6; weight T5-T7) during periods of gonadal regression and early recrudescence (T5-T6: August-October), as well as peak spermatogenesis (T6-T7: October-December; the present study and Manning et al., chap. 2). However, for immature females treated with rbGH, the onset of puberty was either delayed, or it occurred at the same time and progressed at the same rate as in females from control groups. Bovine growth hormone treatment in immature female coho salmon promoted the development of previtellogenic oocytes, a result which was surmised to have arisen from increases in body size associated with bGH treatment (Higgs et al., 1976, 1977). It was hypothesized that rbGH treatment in yellowtail flounder, either by direct rbGH effects, indirect effects through elevated IGF-I, or growth acceleration, would encourage the development of immature ovaries towards an earlier onset of female puberty. Although increases in mean body size

were seen following rbGH treatment, the onset of puberty was not advanced in females of the present study.

As the degree of pubertal development in rbGH treated females was variable, no direct effect of rbGH on the initiation of puberty is proposed to have taken place. Delays in the onset of puberty seen in some females is believed to be due to a rbGH-induced utilization of energy reserves for somatic growth, at the expense of reproduction. The second injection of rbGH in August stimulated growth rates at a time when the onset of puberty was detected in rbGH and other groups. This promotion of growth which occurred during a window of opportunity for maturation, and during a period of high water temperature conditions, may have reduced lipid levels below physiological thresholds for maturation. In fact, GH has been shown to have lipolytic actions in rainbow trout liver tissue leading to the mobilization of fatty acids and glycerol (O'Connor et al., 1993). Thorpe (1986) proposed for salmon that fish physiologically assess the rate of accumulation of excess energy stores in relation to a genetically determined threshold during a certain time of the year. This proposal was supported by studies which indicate a connection between fat accumulation and maturation in male salmonid parr (Rowe et al., 1991; Shearer & Swanson, 2000). In yellowtail, growth promotion and fat catabolism by rbGH treatment may have altered a physiological assessment for maturation such that the onset of puberty was delayed. A high proportion of rbGH females delayed the initiation of puberty until the latter part of November to early December, or in one female to the following year. According to Manning et al. (chap. 2) the end of November was the latest period for the onset of puberty in female yellowtail flounder.

It is questionable whether or not all delayed females which had initiated puberty

would have continued to full maturity. From an endocrine perspective, these delayed females had similar plasma levels of 17ß-estradiol to those seen at the onset of puberty in Manning et al. (chap. 2). Nevertheless, in some delayed rbGH females an interruption of pubertal development may have been a likely outcome as atretic activity and/or a scarcity of vitellogenic oocytes was observed. In two of three delayed, early vitellogenic females, whose tissue was incubated *in vitro*, mean levels of stimulated *in vitro* output were less than 0.14 ng/ml. This was lower than mean levels of stimulated output in the third female tested, which reached 0.36 ng/ml, within the range of values for females at the onset of puberty as determined by Manning et al. (chap. 2). As *in vitro* performance was suppressed for the former two females, the tissue of one being unresponsive to *in vitro* stimulatory agents, an interruption of pubertal development seemed probable for these individuals.

GnRH-a treatment had no significant effect on the growth of yellowtail flounder, unlike the reports for cyprinids in which *in vivo* GnRH treatment promoted growth (Marchant et al. 1989; Lin et al., 1995). A recent study has shown that GnRH was unable to stimulate *in vitro* GH release from eel (*A. anguilla*) or European turbot pituitary cells (Rousseau et al., 1999). Initial reports also were negative for rainbow trout, but further investigation revealed that salmon GnRH could stimulate GH release *in vitro* when pituitary cells were pre-incubated with IGF-I (Blaise et al. 1995,1997). The permissive effect of IGF-I was dependent on sexual stage, occurring in immature to early pubertal fish, but not in mature fish (Blaise et al., 1997). Therefore, although no growth promoting effect of GnRH-a was evident in yellowtail flounder, a role for GnRH on GH release could be possible under specific physiological conditions.

Regarding reproductive effects, GnRH-a treatment ultimately did not accelerate female pubertal development or male recrudescence. For males, histological analysis revealed that similar rates of spermatogenic development during recrudescence were seen between GnRH-a and control males, although two GnRH-a males did show slower development. Despite an overall histological similarity to controls, GnRH-a males had lower GSI, total gonad weight and androgen levels in January, 2000 (T8) than males of control and rbGH groups, indicating some degree of gonadal suppression. In goldfish, *Carassius auratus*, continuous GnRH treatment *in vitro* desensitized pituitary gonadotropes to further GnRH stimulation and reduced pituitary GnRH receptor content (Habibi, 1991a,b). Therefore, continuous exposure to GnRH-a in previously mature male yellowtail may have adversely affected gonadotropin (GtH) levels, and, in turn, decreased levels of both 11-KT and testosterone during recrudescence. Depressed androgen and/or GtH levels are likely responsible for the smaller GSI values seen in most GnRH-a treated males. Since meiotic division appeared to be proceeding at similar or slightly slower rates than controls, low GSI values may be a result of reduced spermatogonial proliferation. Such an effect may arise from reduced levels of 11-KT, which has been shown to stimulate spermatogonial division in Japanese eel (Miura et al., 1991).

According to Manning et al. (chap. 2), the initiation of puberty in female yellowtail flounder could take place as early as June, or as late as autumn in October or November. Neither GnRH-a treated females nor control group females of the present experiment initiated puberty outside of this period. The inability of GnRH-a to advance puberty earlier than controls may indicate that the onset of puberty is tightly controlled by environmental

factors or growth and metabolic cues peripheral to the gonadotropic axis. *In vitro* evidence from Manning et al. (chap. 2) indicated that immature ovaries are responsive to GtH from an early stage. Thus, the absence of pubertal development in GnRH-a treated females prior to the normal seasonal period for pubertal onset may be based on a lack of GnRH sensitivity or low GtH synthesis by the pituitary.

While GnRH-a did not advance female puberty, the present results did demonstrate that GnRH-a treatment synchronized pubertal development among the females in the group. This is supported by evidence from both samples, but particularly in the preliminary sample (August-September, 1999: T5; Age 24.5 months) during early puberty. In contrast, control groups demonstrated larger individual variability in: a) the timing of the onset of puberty; b) most reproductive parameters; and, c) 17ß-estradiol production *in vitro*. A synchronizing effect in puberty agrees well with the now common practice of using GnRH-a to synchronize ovulation in broodstock. This practice has been shown to be effective in enhancing the reproductive performance of captive adult female and male yellowtail flounder (Larsson et al., 1997; Clearwater & Crim, 1998). In adult winter flounder, *Pseudopleuronectes americanus*, GnRH-a administered during early gonadal recrudescence increased GSI and plasma sex steroid levels in both males and females (Harmin et al., 1995). These findings are similar to the present results for females, but are contrary to the results for males. Additional findings by Harmin et al. (1995) demonstrated a lack of a GnRH-a effect in regressed winter flounder, this resembles the inability of GnRH-a treatment to advance puberty in immature females of the present study.

Testosterone treatment, whether alone or in combination with GnRH-a, resulted in

depressed growth rates, suppressed to inhibited spermatogenic development and prevented pubertal development in females. These effects are consistent with reports of the use of high doses of testosterone in other species (Donaldson et al., 1979; Berglund et al., 1995). In striped bass, testosterone microsphere treatment produced mean plasma levels of 2 to 3.6 ng/ml over a five week period, after which levels decreased to 0 ng/ml by 9-10 weeks at a temperature of 15°C (Holland et al., 1998). In contrast, the same microsphere treatments used in striped bass produced higher mean testosterone levels in yellowtail flounder of the present study. Even after five to eight week intervals since an injection, mean plasma levels of testosterone in treated subjects were found to be between 4.5 and 12.3 ng/ml. A tendency for higher levels was seen when temperatures were colder. These testosterone levels were within the upper physiological range of values found in adult yellowtail flounder, in which mean levels remain below 5 ng/ml during most of gametogenesis but peak during spawning to 8 and 13 ng/ml for males and females, respectively (Clearwater, 1996). Conversely, testosterone levels from microsphere treatments clearly exceeded the levels recently reported for pubertal females and young mature males in which peak mean levels at final gamete maturation were below 4 ng/ml (Manning et al., chap. 2). The lack of agreement between profiles reported in Holland et al. (1998) and levels seen in the present study may be the result of slower clearance rates due to lower water temperatures, as well as inter-specific differences in androgen metabolism; such a delay in clearance could have caused a build-up of testosterone with repeated injections.

In fish, growth enhancing effects of testosterone have been documented generally when low doses of the androgen were used, while higher doses have been determined to be

ineffective or suppressive on growth (Donaldson et al., 1979). Testosterone treatment in yellowtail flounder resulted in significantly lower cumulative specific growth rates compared to rates seen in controls. Despite slower growth rates, males and females of testosterone containing groups remained similar in mean body size to either their oil or microsphere control counterparts at the end of the experiment. The only exception where testosterone treatment clearly led to a significantly smaller body size relative to all other groups was seen in mean female body weight. This result was mainly due to weight gains accompanying ovarian maturation in the other groups. Nevertheless, the statistical significance of testosterone depression of female body weight was not explained solely by maturity in other females; for it was found that females receiving testosterone treatment were significantly lower than other groups in mean carcass weight as well.

In Berglund et al. (1992), treatments of 11-ketoandrostenedione and testosterone demonstrated both positive and negative effects on the growth of immature salmon parr (*Salmo salar*). Whether the effects of androgen treatments were positive or negative was dependent on the time of year (Berglund et al., 1992, 1995). In the current study, the examination of inter-measurement specific growth rates revealed that testosterone treatment had mainly negative effects on growth. Positive growth effects were not seen, although mean growth rates of testosterone containing groups did increase to levels equivalent to those seen in control groups. These increases, noted particularly in weight growth rates, coincided with high temperature conditions occurring mainly between July and October (T4-T6). Therefore, a decrease in testosterone suppression of growth at these times may be due to a greater metabolic clearance of testosterone.

Sharp decreases in inter-measurement specific growth rates, followed by a suppression of weight gain, were observed in males and females of testosterone containing groups prior to July (T4). In contrast, maturing males of both control groups had progressively decreasing growth rates in weight over the February (T1; Age 18 mo.)- July (T4; Age 22.5 mo.) period, which was not seen in immature control females. Given that sharp growth decreases were seen accompanying testosterone treatment in both sexes, the progressive decreases seen in control males may have been due to an increasing testosterone or total androgen presence which occurs during the early full spermiation phase between April and June (Clearwater, 1996; Manning et al., chap. 2). Concurrently, it is during this period that statistically significant differences in body size were observed to develop between males and females in all groups of the present study. Overall, these results suggest that high levels of testosterone may have a physiological role in suppressing the somatotropic axis during periods of final gametogenesis. In Berglund et al. (1992), the period of growth suppression by testosterone in salmon parr coincided with the normal period of pubertal development. The authors suggested that increasing androgen levels seen with maturation were responsible for slower growth rather than energetic concerns for 1+ maturing fish (Berglund et al. 1992). Berglund et al. (1992) additionally noted that androgens had a stronger effect on growth in males than in females. The results of the present study similarly showed that females of both testosterone containing groups, as in other groups, grew significantly faster than their male counterparts. A sex difference may be present in testosterone metabolism or tolerance to mediate this difference between female and male growth responses.

High levels of testosterone produced mainly negative effects on the reproduction of yellowtail flounder. In pubertal female striped bass, testosterone microsphere treatment was combined with low or high levels of GnRH-a. Only in the combination with high levels of GnRH-a was GtH release observed in addition to the accumulation effect of testosterone on pituitary GtH content. While the combination of high levels of GnRH-a with testosterone was able to enhance the development of the gonadotropic axis, it was still not able to increase rates of vitellogenic development in pubertal females (Holland et al., 1998). In view of the fact that GnRH-a alone is effective in adult yellowtail flounder, and that this species is characterized by early maturity, high levels of GnRH-a were not used in the present study. Instead, prolonged treatment with the lower dose GnRH-a & T microsphere preparation used in Holland et al. (1998) had been hypothesized to: i) provide sufficient stimulation for early GtH release; ii) advance the onset of puberty in immature females; and, iii) potentially accelerate recrudescence in males.

In males, high levels of testosterone during recrudescence distinctly disrupted meiotic activity, depressed 11-KT levels and resulted in low GSI means in both testosterone containing groups. Many studies report a stimulatory effect of testosterone on spermatogenesis (Borg, 1994; Berglund et al., 1995). However, there are reports of inhibitory effects from high doses on GSI, spermatogenesis and androgen levels (Donaldson et al., 1979; Higgs et al., 1982; Berglund et al., 1995). The last injection for males in the present study occurred in December (T7) which coincided with the normal timing of peak GSI and spermatogenesis reported for yellowtail flounder (Manning et al., chap. 2). High testosterone at this time may have caused sufficient negative feedback on GtH release to cause an

interruption in spermatogenesis. According to Billard et al. (1982), inadequate GtH and androgen levels following hypophysectomy result in a degeneration of meiotic cells. Meiotic cells were clearly lacking in most males of testosterone containing groups; therefore, reduced GtH levels may explain the low GSI means, reduced amounts of new spermatozoa, and the presence of significant amounts of spermatogonia in males of these groups.

By reducing levels of GtH and/or 11-KT, high levels of testosterone may have artificially induced a regressed testicular condition. This seems plausible given that half the males in testosterone containing groups demonstrated mitotic activity in January (T8) when mitosis in cultured males is usually complete by December (Manning et al., chap. 2). Moreover, phagocytic activity was elevated in these males. The resorption of spermatozoa by active phagocytes may have contributed to reductions in GSI, as well as the inability to determine conclusively whether new sperm production had occurred in certain males of both testosterone containing groups. Berglund et al. (1995) similarly observed phagocytic activity in the testes of maturing salmon parr given high levels of testosterone.

For GnRH-a & T treated males, the observation of two males with ongoing spermatogenesis suggested that GnRH-a was able to induce GtH release despite high plasma testosterone. In contrast, other GnRH-a & T treated males appeared to be more inhibited than most males injected with testosterone alone. This may indicate that long-term GnRH-a treatment could have an additional inhibitory effect on pituitary GtH secretion, as was postulated to explain reduced GSI values in males of the GnRH-a treatment group. That new sperm production occurred in males of both testosterone containing groups is indicative that, despite interrupted spermatogenesis, adult males had a greater gametogenetic tolerance to

high testosterone than immature/pubertal females.

The detection of a few vitellogenic oocytes or atretic oocytes containing yolk globules in females of testosterone containing groups indicated that some females were physiologically pubertal, while in others the onset of puberty may have been inhibited. It could be argued that pubertal female yellowtail flounder are testosterone sensitive. Manning et al. (chap. 2) recently reported that plasma testosterone levels remained below 0.6 ng/ml for the greater part of pubertal vitellogenesis. The release of testosterone from microsphere preparations clearly exceeded the low levels of this androgen in pubertal females.

In vitro incubation results for females from testosterone containing treatments demonstrated that ovaries were neither responsive to gonadotropic stimulation, nor capable of mediating a gonadotropic signal intracellularly via adenylate cyclase. Therefore, testosterone treatment had induced a down-regulation of the post-receptor pathways for ovarian steroidogenesis. Despite the absence of 17ß-estradiol production *in vitro*, levels in the plasma were similar to values at the onset of puberty reported in Manning et al. (chap. 2). This difference may be due to a low aromatisation of the high plasma levels of testosterone by intact ovaries, although an extra-gonadal site of conversion may be possible.

A similar inhibition of ovarian steroidogenesis *in vitro* was seen following *in vivo* testosterone treatment in pubertal female striped bass, whether with or without additional treatment of a low dose of GnRH-a (Holland et al., 1998). It should be noted, however, that *in vitro* 17ß-estradiol levels produced by control striped bass females were already very low (<50 pg/ml) (Holland et al., 1998). A stimulation of *in vitro* steroidogenesis in striped bass was seen with a testosterone and high dose GnRH-a combination, which successfully

increased levels of GtH in the plasma (Holland et al., 1998). These results appear to indicate that inhibitory effects of testosterone on the ovary are dependent on circulating levels of GtH.

Inhibitory effects of testosterone on gonadal steroidogenesis in both pubertal females and recrudescing males in the present study could be the result of low gonadotropic output over a prolonged period of time and/or direct effects of testosterone on the steroidogenic cells themselves. At the level of the gonad, testosterone and other naturally occurring androgens have been found to suppress basal and GtH-stimulated steroidogenesis of testicular tissue of immature African catfish *in vitro* (Cavaco et al., 1999). The authors determined that androgen treatment had impeded steroidogenesis prior to 11ß-hydroxylation, and had direct effects on the size and ultrastructure of the Leydig cells - halving the number of mitochondria which are important in steroid biosynthesis.

At the level of the pituitary, exposure of immature females to high testosterone levels may have promoted a premature negative feedback response to testosterone which affected gonadotropin release even in the presence of GnRH-a. In males, a negative feedback response and some readjustment of testosterone sensitivity would have been established during the first cycle of spermatogenesis. High testosterone levels, which occur naturally in the prespawning and early spawning periods, may serve as a physiological signal to prevent the stimulation of new cycles of reproductive activity during spawning periods. Some support for this idea stems from the fact that testosterone microsphere treatment did not appear to affect full spermiation in males between April (T2; Age 19.5 mo.) and August (T5; Age 24 mo.). Furthermore, observations from the few testosterone treated females which matured as 1+ animals (two GnRH-a & T treated females and another in the testosterone group)

showed that ovulation proceeded while these females were still receiving treatments. Therefore, testosterone treatment appeared not to compromise pituitary function during the final stages of gametogenesis, while early gametogenesis was clearly affected.

Positive actions of sex steroids on pituitary GtH content have been mainly reported to affect GtH-II. In salmonid models, GtH-II is associated with the final stages of gametogenesis, while GtH-I is associated with early stages such as spermatogenesis and vitellogenesis (Suzuki et al., 1988; Swanson et al., 1991; Breton et al., 1998).Variable results have been reported by the few studies in which the response of pituitary GtH-I to sex steroids has been observed. In immature male tilapia only low doses of testosterone increased GtH-Iß mRNA in dispersed pituitary cells *in vitro* (Melamed et al., 1997). In other studies effects of testosterone on GtH-Iß were negative or absent (Dufour et al., 1999). Although it has not been determined, a dual gonadotropin system seems likely to be present in yellowtail flounder, as has been recently discovered in two other pleuronectiforms, Atlantic halibut and Japanese flounder, *Paralichthys olivaceus* (Weltzien et al., 1999; Kajimura et al., 2001). The observations of high testosterone inhibition of pubertal development and interruptions of spermatogenesis, but not spermiation or ovulation, in yellowtail flounder would be consistent with an effect on the GtH-I regulation of the gonad.

3.5. SUMMARY.

This study demonstrates several interesting features, some contrary to the initial hypotheses, of long-term treatment with potentially "dual-effect" hormones, all of which were initially predicted to be growth-enhancing, regardless of sex. Initial predictions were also of reproductive stimulation such as pubertal advancement and increased reproductive development. Long-term treatment with recombinant bovine growth hormone did improve growth for both males and females, reducing sex effects in growth. However, while it may have increased reproductive output (high testicular weight) in males, it delayed puberty in some females. Further, long-term treatment with GnRH-a by itself had no significant effects on growth, and, overall, accelerated neither female pubertal development, nor male recrudescence. However, this hormone did synchronize female puberty within the normal period for its initiation. Finally, long-term treatment with testosterone (singly or in combination with GnRH-a) suppressed both growth and reproduction in males and females. While the effects of these hormones were not as initially hypothesized, and, with the exception of testosterone, did not show clear direct dual effects in hormone function, the results of the study point to several interesting questions regarding the relationship between growth and reproduction. These questions include: whether growth hormone treatment could be used as a deterrent for early female sexual maturation; do high testosterone levels act as a physiological cue to deter new cycles of gametogenesis and growth during spawning periods; and, to what degree is the activation of the gonadotropic axis and its GnRH responsiveness at puberty regulated by environmental cues and/or energetic cues? Yellowtail flounder as an early maturing flatfish is an excellent model for investigating these questions.

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Appendix 3A

Inter-measurement specific growth rate tables.

Table 3A-1. Group comparison of mean inter-measurement specific growth rates (%/day) in weight and standard length for females receiving different hormonal treatments.

Table 3A-2. Group comparison of mean inter-measurement specific growth rates (%/day) in weight and standard length for males receiving different hormonal treatments.

Table 3A-1. Group comparison of mean inter-measurement specific growth rates (%/day) in weight and standard length for females receiving different hormonal treatments.

Weight	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**	**T5-T6**	**T6-T7**
Mcs	0.47 ±0.22 ^{ab}	0.30 ±0.21 ^{bc}	0.29 ±0.14 ^{bc}	0.14 ±0.15 ^{a}	0.35 ±0.13 ^{ab}	0.30 ±0.15 ^{a}
T	0.32 ±0.20 ^{b}	0.00 ±0.23 ^{d}	0.11 ±0.29 ^{c}	0.22 ±0.14 ^{a}	0.20 ±0.10 ^{c}	0.14 ±0.06 ^{b}
GnRH-a	0.30 ±0.19 ^{b}	0.24 ±0.25 ^{c}	0.37 ±0.31 ^{ab}	0.24 ±0.24 ^{a}	0.25 ±0.21 ^{bc}	0.27 ±0.10 ^{a}
GnRH-a &T	0.32 ±0.15 ^{b}	0.04 ±0.10 ^{d}	0.09 ±0.25 ^{c}	0.15 ±0.22 ^{a}	0.31 ±0.07 ^{bc}	0.07 ±0.05 ^{b}
Oil	0.38 ±0.22 ^{b}	0.42 ±0.12 ^{ab}	0.44 ±0.15 ^{a}	0.06 ±0.15 ^{a}	0.29 ±0.11 ^{bc}	0.29 ±0.10 ^{a}
rbGH	0.60 ±0.32 ^{a}	0.47 ±0.10 ^{a}	0.39 ±0.23 ^{ab}	0.12 ±0.21 ^{a}	0.50 ±0.14 ^{a}	0.25 ±0.14 ^{a}
Temp (°C)	6.0 ± 0.7	6.9 ± 0.8	8.0 ± 0.9	11.3 ± 1.2	10.9 ± 1.5	6.4 ± 1.1
Length	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**	**T5-T6**	**T6-T7**
Mcs	0.09 ±0.06 ^{b}	0.11 ±0.06 ^{ab}	0.10 ±0.08 ^{ab}	0.07 ±0.04 ^{a}	0.08 ±0.05 ^{b}	0.06 ±0.04 ^{a}
T	0.10 ±0.09 ^{b}	0.04 ±0.07 ^{c}	0.05 ±0.09 ^{bc}	0.06 ±0.06 ^{a}	0.06 ±0.04 ^{b}	0.03 ±0.04 ^{a}
GnRH-a	0.08 ±0.06 ^{b}	0.08 ±0.06 ^{bc}	0.11 ±0.07 ^{a}	0.08 ±0.07 ^{a}	0.05 ±0.04 ^{b}	0.07 ±0.02 ^{a}
GnRH-a &T	0.07 ±0.06 ^{b}	0.05 ±0.04 ^{c}	0.04 ±0.06 ^{c}	0.04 ±0.03 ^{a}	0.07 ±0.03 ^{b}	0.05 ±0.02 ^{a}
Oil	0.07 ±0.03 ^{b}	0.14 ±0.04 ^{a}	0.12 ±0.06 ^{a}	0.04 ±0.04 ^{a}	0.08 ±0.03 ^{b}	0.04 ±0.03 ^{a}
rbGH	0.21 ±0.06 ^{a}	0.15 ±0.04 ^{a}	0.10 ±0.05 ^{ab}	0.05 ±0.07 ^{a}	0.16 ±0.04 ^{a}	0.06 ±0.04 ^{a}

Mcs= microsphere control; Temp= mean (±SD)water temperature during a given interval between consecutive measurements. Means (±SD) within a column of data which are noted by the same superscript character are not significantly different (P>0.05).

Table 3A-2. Group comparison of mean inter-measurement specific growth rates (%/day) in weight and standard length for males receiving different hormonal treatments.

Weight	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**	**T5-T6**	**T6-T7**
Mcs	0.49 ±0.26 ^{a}	0.15 ±0.10 ^{b}	0.04 ±0.16 ^{bc}	-0.04 ±0.12 ^{a}	0.12 ±0.06 ^{c}	0.13 ±0.04 ^{b}
T	0.25 ±0.10 ^{b}	0.04 ±0.16 ^{b}	0.01 ±0.08 ^{bc}	0.01 ±0.12 ^{a}	0.15 ±0.12 ^{c}	0.08 ±0.08 ^{b}
GnRH-a	0.19 ±0.15 ^{b}	0.12 ±0.17 ^{b}	0.13 ±0.20 ^{ab}	-0.02 ±0.16 ^{a}	0.23 ±0.11 ^{bc}	0.10 ±0.07 ^{b}
GnRH-a &T	0.01 ±0.13 ^{c}	0.06 ±0.13 ^{b}	-0.01 ±0.10 ^{c}	0.08 ±0.09 ^{a}	0.13 ±0.13 ^{c}	0.09 ±0.06 ^{b}
Oil	0.27 ±0.14 ^{b}	0.15 ±0.10 ^{b}	0.08 ±0.12 ^{bc}	0.02 ±0.13 ^{a}	0.31 ±0.07 ^{b}	0.14 ±0.06 ^{b}
rbGH	0.45 ±0.23 ^{a}	0.41 ±0.17 ^{a}	0.26 ±0.18 ^{a}	0.03 ±0.12 ^{a}	0.52 ±0.19 ^{a}	0.28 ±0.04 ^{a}
Temp (°C)	6.0 ± 0.7	6.9 ± 0.8	8.0 ± 0.9	11.3 ± 1.2	10.9 ± 1.5	6.4 ± 1.1
Length	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**	**T5-T6**	**T6-T7**
Mcs	0.08 ±0.05 ^{b}	0.07 ±0.04 ^{b}	0.04 ±0.05 ^{bc}	0.02 ±0.03 ^{a}	0.03 ±0.03 ^{bc}	0.03 ±0.03 ^{bc}
T	0.04 ±0.08 ^{b}	0.04 ±0.05 ^{c}	0.07 ±0.05 ^{ab}	0.00 ±0.03 ^{a}	0.03 ±0.04 ^{bc}	0.02 ±0.02 ^{c}
GnRH-a	0.06 ±0.08 ^{b}	0.05 ±0.04 ^{bc}	0.06 ±0.05 ^{ab}	0.02 ±0.05 ^{a}	0.05 ±0.03 ^{b}	0.03 ±0.02 ^{bc}
GnRH-a &T	0.03 ±0.05 ^{b}	0.03 ±0.03 ^{c}	0.02 ±0.03 ^{c}	0.03 ±0.05 ^{a}	0.00 ±0.05 ^{c}	0.05 ±0.02 ^{ab}
Oil	0.06 ±0.04 ^{b}	0.08 ±0.03 ^{ab}	0.04 ±0.04 ^{bc}	0.01 ±0.05 ^{a}	0.05 ±0.03 ^{b}	0.04±0.02 ^{abc}
rbGH	0.16 ±0.04 ^{a}	0.12 ±0.05 ^{a}	0.10 ±0.05 ^{a}	0.04 ±0.07 ^{a}	0.15 ±0.05 ^{a}	0.07 ±0.03 ^{a}

Mcs= microsphere control; Temp= mean (±SD)water temperature during a given interval between consecutive measurements. Means (±SD) within a column of data which are noted by the same superscript character are not significantly different (P>0.05).

CHAPTER 4

The Production and Preliminary Evaluation of Triploid Yellowtail Flounder, (*Limanda ferruginea*, Storer).

4.1. INTRODUCTION.

Sexual maturation in fish frequently occurs at the expense of somatic growth. In culture situations, high food quality and fast growth rates often reduce the age at which sexual maturation is initiated. For early maturing species such a reduction is particularly undesirable as it may produce large numbers of precociously mature individuals of small size and poor growth potential. Problems associated with maturation may be circumvented with the production of sterile fish. The induction of triploidy is one of a number of strategies which may be used to suppress gonadal maturation. Triploidy can be induced by treatments which cause the retention of the second polar body after fertilization. Reproductive dysfunction is imposed by the presence of three sets of homologous chromosomes; the odd number of chromosomes creates difficulties during the formation of homologous pairs in meiosis I.

It is often reported that male and female triploids exhibit different degrees of reproductive dysfunction. Triploid females demonstrate a significant reduction in gonadal development, their ovaries generally containing oogonia and few oocytes (Purdom, 1972; Benfey & Sutterlin, 1984; Malison et al., 1993; Hussain et al., 1995). Moreover, levels of plasma steroids in triploid females remain low or even undetectable while those of maturing diploids increase (Lincoln & Scott, 1984; Benfey et al., 1989b; Hussain et al., 1995; Amano et al., 1998; Kobayashi et al., 1998). In contrast to females, the development and the

steroidogenicity of gonadal tissue in triploid males remain similar to levels seen in diploids (Lincoln & Scott, 1984; Benfey et al., 1989b; Malison et al., 1993; Hussain et al., 1995). This is likely due to the fact that spermatogonial mitosis, cyst formation and division of steroidogenic cells are premeiotic events in males. Additionally it has been reported that meiotic activity occurs in triploid males and that small amounts of functional, but aneuploid, spermatozoa may be produced (Benfey et al., 1986; Benfey, 1999; Zhang & Arai, 1999). While triploid males may not be physiologically sterile, the aneuploid condition of their spermatozoa renders them functionally sterile with regard to the production of viable embryos.

The yellowtail flounder is a small pleuronectid flatfish which has been investigated as a candidate species for cold-water aquaculture since 1992. Evidence from wild populations in New England (in the species' southern geographic range), and recent data obtained in culture, indicate that males and females can become fully mature as early as two years of age (Royce et al., 1959; Manning et al., chap. 2). Considering the propensity for early maturation in this species, the production of triploids was investigated as a strategy to deter gonadal development.

The present study determined the appropriate conditions for inducing triploidy when using hydrostatic pressure to retain the second polar body post-fertilization. Larvae and juveniles were reared in preliminary experiments which assessed the growth performance of triploid yellowtail flounder. The efficacy of triploidy in suppressing gonadal development was evaluated for both males and females up to three years of age.

4.2. METHODS.

4.2.1. Gamete collection.

Eggs and milt were collected, for experimental use, from a population of wild-caught, adult yellowtail flounder held in captivity at the Ocean Sciences Centre, Logy Bay, Newfoundland. These adults were kept in 2 000 litre tanks, each equipped with a bottom drain, an aeration source and a continuous supply of fresh degassed ambient sea water. An alternate water supply providing heated or chilled sea water could be used as the season required. Natural light cycles were provided by ambient daylight and artificial lights set to a natural photoperiod. Broodstock were conditioned with a moist pellet commercial feed throughout the year (Connors Brothers Ltd., St. George, New Brunswick). Since females of this batch-spawning species frequently ovulate on a daily basis (Manning & Crim, 1998), females were checked for eggs daily during experimental periods. Eggs and milt were stripped by hand from females and males as described in Manning & Crim (1998).

4.2.2. Experiment 1: Inducing triploidy by hydrostatic pressure treatment.

4.2.2.1. Hydrostatic pressure treatment methodology.

Pressure shock treatments for triploid induction experiments were performed during the spawning seasons of 1997 and 1998. The viability of egg collections from individual females was assessed visually on the basis of morphological characteristics previously correlated to fertility in Manning & Crim (1998). Batches with 75% or higher estimated viability were preferred for use in pressure shock treatments. Egg collections of suitable quality from a minimum of two females were mixed and kept on ice until fertilization. Only motile milt collections from at least two males were pooled for use in egg fertilization.

Motility was checked microscopically (100x magnification) after activating a small amount of milt (~1 µl) with sea water (8°C) on a microscope slide. Once the milt collections were combined, the pooled milt was diluted 10x in sperm extender (1.7 mM $CaCl_2 \cdot 2H_2O$, 7 mM $MgSO_4 \cdot 7H_2O$, 86 mM glycine, 150 mM sucrose, 30 mM Tris-HCl, pH 8.0; Billard et al., 1993). Since milt collections from males frequently were small in volume (0.1-0.3 ml/male) and quite viscous, sperm extender was used to promote the even spread of spermatozoa among the eggs during fertilization. The milt "solution" was mixed to homogeneity and checked for motility prior to use in fertilization steps.

The hydrostatic pressure apparatus (TRC Hydraulics Inc, Dieppe NB) used in the present study has been described previously in Pepper et al. (1996). The apparatus consisted of a stainless steel, cylindrical chamber which accommodated a 1.8 litre, plastic receptacle for freshly fertilized eggs. The chamber was sealed with a steel head equipped with a pressure gauge and release valve. Hydrostatic pressure was applied through the use of a manual hydraulic pump. Prior to any treatment session, the pressure chamber was cooled by filling it with ice and letting the ice turn into a slurry. This kept the chamber from increasing in temperature over the course of treatment. The ice was removed and the chamber was filled with fresh sea water shortly before fertilization steps were initiated. The sea water used in the present experiment, whether to fill the pressure chamber, the 1.8 litre egg receptacle, or to activate the gametes during fertilization, was always taken from the same source.

The volume of eggs used in a pressure shock treatment varied according to availability, but was generally a minimum of five ml, which represented approximately 7 500 to 10 000 eggs. Fertilization steps were performed in a petri dish set on ice. Volumes of

diluted milt, eggs and activating sea water were mixed according to the proportion of 0.1 : 1 : 1, respectively. The addition of the sperm activating sea water demarked the time zero reference point for the application of pressure treatment. A two minute period was allowed for fertilization to occur in the petri dish. During this interval the 1.8 litre egg receptacle was filled with fresh sea water and a record was made of the water temperature. After two minutes the egg-sperm mixture was transferred to the egg receptacle which was then topped up with sea water and capped with a plastic ring equipped with an overlay of fine mesh. As yellowtail flounder eggs are pelagic, the receptacle and meshed ring were necessary to restrict the movement of the eggs within the chamber. The egg receptacle was inserted into the water-filled chamber of the apparatus, displacing most of the water therein. The chamber head was quickly screwed on and tightened. Any trapped air was expelled by a few pumps, at which point the release valve was closed to seal the pressure chamber.

Hydrostatic pressure within the apparatus was increased to the desired treatment level by use of the manual hydraulic pump. Treatment levels were attained within 30 to 55 seconds of pumping and were maintained over the duration of the treatment. Pressure treatments were performed at levels of 5 000 to 10 000 psi and were initiated at 5, 10, 15 and 30 minutes post-sperm activation. Sham control treatments followed the same procedure except no pressure was applied. Treatment durations included 5, 10, 15, 20 and 30 minute intervals. Following treatments, the pressure was released slowly, and the egg receptacle was removed. The water temperature within the receptacle was recorded; generally little change ($\leq 1^{\circ}C$) in temperature occurred over the duration of pressure treatment. The eggs were transferred to one litre beakers, which were placed in an incubator set at 8°C. Water changes were done every two

days over the egg incubation period using 1 μm filtered, UV sterilized sea water with antibiotic (0.1 g/l streptomycin/0.06 g/l penicillin). Any dead eggs or larvae were removed during daily maintenance checks.

4.2.2.2. Ploidy assessment.

Larvae were incubated until yolk-sac absorption, which occurred approximately 18 to 20 days post-fertilization at 8°C. For larvae from any given treatment, cell suspensions were prepared from individual larvae or groups of larvae (n= 5-18) using a method modified from that of Blacklidge & Bidwell (1993a) (see Appendix 4A). Ploidy assessment was performed by flow-cytometric measurement of the DNA content of cells stained with propidium iodide (Sigma). Cell suspensions were analysed on a FacStar^{plus} flow cytometer (Becton Dickinson, Mississauga, ON). Blood from captive adults was sampled and blood cell suspensions prepared for use as diploid reference samples in all analyses. These suspensions were prepared according to Blacklidge & Bidwell (1993b) (see Appendix 4A).

4.2.3. Experiment 2: The effect of triploidy on larval growth and survival.

In this experiment, larvae from two pressure treatments were compared to those from a sham control treatment (no pressure added) in order to discern the effects of triploidy on larval growth. Eggs collected during the 1998 spawning season were subject to the same quality criteria as in experiment 1. On any given day, egg collections of satisfactory quality were pooled and then divided into three equal portions. Each portion was subjected to one of three ten minute pressure treatments (7 000 psi, 5 000 psi or sham control 0 psi, initiated five minutes post-sperm activation at 11.1 to 13.4°C). Previous results from experiment 1 indicated that 7 000, 5 000, and 0 psi treatments yielded high, low and zero percentages of

triploid larvae, respectively. A 5 000 psi treatment was used to discern whether exposure to hydrostatic pressure levels at the threshold for inducing triploidy had an effect on larval performance. Diploid larvae from this group, therefore, would serve as a control for the triploid condition. Sham control larvae acted as control subjects for both triploidy and exposure to hydrostatic pressure.

Due to a limited egg supply from broodstock, the pressure treatments were performed on egg collections from non-consecutive dates (July 26, 29 and 31). Eggs treated on July 26 were incubated for a few days in a 4-5°C cold room, while later treatments were incubated at 9°C. This difference in conditions was imposed in an attempt to synchronize hatching between treatments performed two to five days apart. Maintenance of eggs during the embryonic incubation period was as previously described in experiment 1.

On August 7 (day 0) the newly hatched larvae from the different treatments were transferred to three 250 litre, cylindro-conical, upwelling incubators supplied with fresh ambient sea water (tank1: 7 000 psi treatments; tank 2: 5 000 psi treatments; tank 3: 0 psi control treatments). An air stone positioned centrally at the bottom provided aeration and promoted water circulation. As tank availability was limited, no replicate tanks could be obtained for this study. Egg collections from the three different dates (July 26, 29, 31) were equally represented among the three tanks, with the unfortunate exception that sham control embryos from July 31 were accidentally spilled during egg maintenance. Samples of larvae from each treatment on each different date were kept in a 4-5°C cold room until yolk-sac absorption and processing for ploidy analysis (individual analysis, n=15 preparations of individual larvae; group analysis, n=3 preparations of groups of 15 larvae).

Starting from day two post-hatch of larval rearing, an algal suspension (*Isochrysis sp.* and/or *Nannochloropsis sp.*, depending on availability) was added in two allotments of seven and six litres per tank in the morning and evening periods, respectively. Additions of rotifers (*Brachionus plicatilis*) enriched with Culture Selco (INVE, Dendermond, Belgium) began on day 3. Each tank received three feedings of one million rotifers (density 4 000/l) at 1100h, 1800h and 2300h daily. Morning feedings had been enriched overnight in an *Isochrysis* algal suspension. The proportion of small-sized rotifers was increased by selective filtration of rotifer stock cultures between days eight and fourteen. Newly hatched *Artemia* nauplii (*Artemia franciscana*) were introduced on day 34 in one feeding per day at a prey density of 800/l. On day 38 post-hatch, *Artemia* nauplii enriched with DHA Selco (INVE, Dendermond, Belgium) or Algamac (Bio-Marine, Hawthorne, CA, USA) were added at all three feeding times. The prey density at each feeding was 1 300 *Artemia* per litre, this density was doubled to 2 600 per litre on day 44. Additions of rotifers were decreased over time until day 62 when they were eliminated from the diet. The experiment was terminated on day 76 post-hatch (October 22, 1998).

Larvae experienced 24 hours of light and ambient temperature fluctuations during the experiment. Mean temperatures over the rearing period were 11.8 ± 0.2°C. High temperature conditions of 15.6°C were seen at the beginning of the experiment, levels decreased to 8.3°C by the end of the experiment. Temporary drops to 3°C were seen on four occasions which were associated with upwelling events in Logy Bay.

Measurements of ten larvae per tank occurred on days 6, 21, 36 and 51 post-hatch. Notochord/standard length, myotome height and eye diameter were recorded for each larva.

Total length was measured as of day 51. On day 36, only the 5 000 psi treatment larvae in tank 2 were measured as the other tanks had low numbers of larvae at that time. During all measurements, notes were made on the developmental stages of the larvae from first-feeding to metamorphosis. Notable characteristics of metamorphosis included skin pigmentation, pigmentation of the blood, eye migration and settling behaviour. At the end of the experiment on day 76, fifteen individuals from each tank were sampled, anaesthetized with an overdose of 2-phenoxyethanol (Acros Organics, New Jersey, USA), and measured as described previously. Ploidy analysis was done on these individuals to determine whether the percentage of triploids had changed since yolk-sac absorption. As the fish were larger, but still too small to blood sample, they were dissected and the liver homogenized to obtain cell suspensions for ploidy analysis. After homogenization, all subsequent steps were identical to those for cell suspension preparations of larvae at yolk-sac absorption.

4.2.4. Experiment 3. Effects of triploidy on sexual maturation in yellowtail flounder.

4.2.4.1. Treatment and rearing.

Ploidy analysis on larvae from initial pressure treatments in 1997 indicated that experiments using 9 000 to 10 000 psi had been successful in producing a high rate of triploid larvae. With this initial success, a preliminary effort was made to rear triploid larvae. Four batches of eggs were collected from the last ovulating female of the 1997 spawning season (August 27 to September 5, 1997). Each batch of eggs was treated for ten minutes with a pressure of 9 000 psi starting at five minutes post-sperm activation (9.2-9.7°C). A lower pressure was not used at the time due to the lack of confirmation regarding the effectiveness of lower pressure treatments.

Treated eggs were added to three 250 litre cylindro-conical upwelling incubators where the eggs subsequently hatched (Sept 6-15, 1997) and were raised until December, 1997. Rearing techniques were similar to those outlined in experiment 2. In late-December the fish were moved from the upwelling incubators to a fibreglass tank with four 40 litre compartments. Each compartment had a degassed heated water supply and was equipped with a water surface drain covered with nylon mesh. The fish were weaned from *Artemia* on to a salmon starter diet (Moore-Clarke, St. Andrew's, NB) in January, 1998. In the spring of 1998 the fish were transferred to a 400 litre tank equipped the same features described for adult broodstock tanks (Section 4.2.1). The fish were fed salmonid diets, which had a high lipid content (~20 %), until 18.5 months of age when they were switched in 1999 to a moist pellet, shrimp-based diet with a low fat content (8%) more suitable for yellowtail flounder. The fish seemed to be having difficulty eating the dry pellet and readily accepted the moist pellet, which yielded good growth results in a concurrent experiment (Manning et al., chap.3). A marine fish, dry pellet diet (14% lipid; Corey Feed Mills, Fredericton, NB) was introduced in March, 2000, and was used until the end of the experiment. No adverse reaction was seen to dry pellet feed at this time. A food ration of 1.5% body weight/day was maintained throughout the experiment.

Juvenile fish produced from this experiment were reared for three years until full maturity was seen in both sexes of untreated diploid fish of the same age and year class. Measurements of total and standard lengths on individual animals began in February, 1998 (five months of age). Prior to this time, size measurements were done only on mortalities. The pooled weight of all the animals in a water-filled container served as an estimate of the

mean weight per fish between February and July, 1998 (5-10 months of age). An individual record of both weight and length was constructed starting from November, 1998 (14 months of age). Measurements occurred every three to four months until reproductive evaluation (see Section 4.2.4.2) of males in July, 2000 (34 months) and females in October, 2000 (37 months) and January, 2001 (40 months).

Due to a limited amount of eggs at the beginning of the experiment, no diploid control group was available for growth comparison. Instead, the growth of 2+ (two-year-old) experimental females was compared to groups of 1+(one-year-old) diploid females of similar size assigned to another experiment. An 11 month age difference between females in this growth comparison was due to a poor growth performance by experimental fish in their first year of life. One year old diploid females from the 1998 year class were collected and separated according to weight attained after one year under general culture conditions. Two groups of diploid females, 28 females demonstrating a fast growth rate and 32 females exhibiting slower growth as 0+(Age <12 mo.) animals, were followed for fourteen months from October, 1999 (14.5 months of age) to December, 2000 (28 months of age). Each group was reared during this period in separate 400 litre tanks identical to the tank containing the experimental fish reared from pressure treated eggs. These tanks were equipped with the same water supplies and experienced the same temperature and photoperiod conditions as those of experimental fish. Feeding ration for these diploid groups also was the same at 1.5% body weight per day. All diploid individuals were measured every two to three months, these measurements generally coincided with measurements of experimental fish. A number of early maturing diploid females reaching full maturity by 22 months of age was seen in both

tanks. Mature and immature diploid females were discerned based on their ovarian ranks (described section 4.2.4.2).

4.2.4.2. Reproductive evaluation.

During routine measurements, males were checked for milt production by stripping attempts, and female ovarian development was estimated by external examination and the use of an ovarian ranking system. Ranks were given by relating the length of the ovary to the length of the ovarian cavity (Manning et al., chap. 2). Ovarian ranks (OR) of 1-3, 2-4, and 5-8 in diploids corresponded to three stages: immature ovaries, ovaries initiating puberty and ovaries with advanced vitellogenic growth proceeding to full maturity.

Fish sampled for reproductive evaluation were anaesthetized with an overdose of 2-phenoxyethanol. The fish were then measured for total and standard lengths, weighed, and blood sampled with heparinized syringes prior to decapitation. During dissection, the gonads, the liver and the remaining viscera were removed and their respective weights were recorded. Blood was centrifuged at 8 325 x g for 10 minutes at 4°C, and the plasma stored at -20°C for later hormone analysis. Blood cells were prepared for ploidy analysis which had not been performed since the larval stage. Blood smears were prepared and stained with Giemsa stain.

For males, a preliminary examination of sperm motility was done on stripped milt. The next day males were sacrificed and milt was collected directly from the sperm ducts. These post-mortem collections were supplemented by sampling the exudate from dissected testes. The milt collection of each male was tested for motility, fertilization success and hatching success. Milt from experimental males was compared to a pool of milt stripped from two diploid adult males. This pool was diluted 25x in sperm extender as the milt was highly

viscous. Eggs collected from two females were combined for use in fertilization trials. The pooled egg collection had an initial mean viability of 72%. Three replicates of 100 µl of eggs were placed in petri dishes set on ice. A ten microlitre volume of milt was mixed with each replicate volume of eggs. The gamete mixture was activated with 100 µl of 1 µm filtered, UV sterilized sea water containing antibiotics (0.1 g/l streptomycin / 0.06 g/l penicillin). After a fertilization period of two minutes, one ml of sea water was added followed by 20 ml after five minutes. The eggs were incubated at 8°C with water changes occurring every two days. Fertilization success, percentage of irregular cleavage of blastomeres, hatching success and larval production percentages were calculated as follows:

Fertilization success (FS)= 100% x (number of fertilized eggs / total number of eggs);

Irregular cleavage (IR)= 100% x (number of fertilized eggs with irregular cleavage of blastomeres / number of fertilized eggs);

Hatching success (HS)= 100% x (number of hatched larvae / the number of fertilized eggs);

Larval production (LP)= 100% x (number of larvae / total number of eggs).

In vitro incubations were performed using tissue sampled from the ovaries of the first six females sacrificed in October, 2000 (Age 37 mo.). These incubations tested the tissue's steroidogenic capacity and ability to respond to heterologous gonadotropic stimulation. For each female, a sample of 450 mg of ovarian tissue was cut into small pieces which were randomly allocated to nine wells (~50 mg tissue/well) in a 24 well Costar incubation plate. The incubation medium contained 0.1 mM 3-isobutyl, 1-methylxanthine (IBMX) in a balanced salt solution-BSS (i.e. trout BSS: 3.4 mM $CaCl_2 \cdot 2H_2O$, 3.1 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 133 mM NaCl, 40 mM Hepes, 1 g/L glucose; Jalabert

& Fostier, 1984), which through dilution and pH adjustment was made to match the osmolarity and pH of yellowtail plasma (331 mOsm, pH 7.7). The first set of three replicate control wells contained BSS-0.1 mM IBMX medium alone. In the second set of wells the BSS-0.1 mM IBMX medium was supplemented with 10 μM forskolin (Sigma), an adenylate cyclase activator. For the third set of wells, the BSS-0.1 mM IBMX medium contained 500 μg/ml of crude salmon pituitary extract (CPE; Argent Chemical Redmond, WA, USA; lot# SP1211M). The volume of medium in each well was 1.2 ml. All plates were placed in an incubator set at 9°C and agitated continuously over a five day period. After this period, the fluid in the wells was recovered and stored in two 0.5 ml Eppendorf tubes at -20°C.

Gonadal tissue from both males and females was sampled for histological analysis. Tissue was fixed in Bouin's fluid for two days and transferred to 50%, then 70% ethanol in preparation for histological processing. At a later date the tissue was sent through an alcohol dehydration series, cleared in xylene, and embedded in Paraffin wax (Paraplast Plus®). Sections were cut at 5 μm and 6 μm for males and females, respectively. The sections were placed on slides coated with albumin and stained with Ehrlich's haematoxylin and eosin. Cytological staging followed previous descriptions of yellowtail flounder gametogenesis in Manning et al. (chap. 2). Abbreviations for vitellogenic oocyte stages used in the text include: VG-I - oocytes with initial signs of peripheral yolk globules; VG-II - early stage, growing, vitellogenic oocytes where the cytoplasm has been partially filled with yolk globules; VG-III - growing vitellogenic oocytes with a cytoplasm which has been filled with yolk globules.

Hormone levels in the plasma and in incubation medium were determined by radioimmunoassay. Levels of 11-ketotestosterone in males were measured according to

protocols outlined in Harmin & Crim (1993) and Manning et al. (chap. 2), which involved a diethyl ether extraction of plasma prior to performing the radioimmunoassay. For 17ß-estradiol and total-testosterone levels, no-extraction, solid phase ^{125}I radioimmunoassay kits were used (Coat-a Count, Diagnostic Products Corporation, Los Angeles, CA USA). The total testosterone assay had a 16% crossreactivity with 11-ketotestosterone.

4.2.5. Statistical analysis.

All statistical analyses was performed using the GLM procedure in SAS (Statistical Analyses System, 1989). Gonadosomatic indices and specific growth rates were calculated:

Gonadosomatic index (GSI)= (total gonad weight / body weight) x 100%

Specific growth rates (SPGR) = $100*[\ln M(t_f)- \ln M(t_0)] / (t_f-t_0)$;

M=weight or length measurement, t_f= final date, t_0 = start date.

Heterogeneity of slopes analyses were used in growth comparisons to determine whether regression coefficients for body size versus time relationships were similar among different experimental groups. Analysis of covariance (ANCOVA) followed in cases when heterogeneity of slopes analysis indicated that regressions were essentially parallel. Egg quality, *in vitro* incubation, and body size comparisons were analysed by one or two-way ANOVA. Analyses were followed by Tukey's HSD and least square means tests. Residuals were tested for homogeneity and normality, and $\log_{10}$ transformations were used when required. The Kruskal-Wallis test, Wilcoxon two sample test and the Sheirer-Ray-Hare extension of the Kruskal-Wallis test (for two-way ANOVA situations) were used as non-parametric alternatives when ANOVA assumptions could not be met by $\log_{10}$ transformation.

4.3. RESULTS.

4.3.1. Experiment 1: Inducing triploidy by hydrostatic pressure treatment.

High proportions of triploid larvae were produced from ten minute hydrostatic pressure treatments of 7 000 to 10 000 psi initiated at five minutes post-sperm activation (p.s.a.) (Table 4.1). These treatments were executed under a wide range of temperature conditions: 7-11.3°C for treatments in 1997 (mean= 9.5 ±0.9°C) and 7.4-13.4°C for treatments in 1998 (mean= 9.8 ±1.6°C). Even at high temperature conditions the initiation of pressure treatments at five minutes p.s.a. was capable of inducing high rates of triploidy (up to 100%). Between 8 000 to 10 000 psi, shorter five minute treatments initiated five minutes p.s.a. (7-11.2°C) were similarly effective in inducing high proportions of triploids, but more variable results were seen when using 7 000 psi (Table 4.1). Pressure treatments below 7 000 psi revealed a decreasing proportion of triploids, between 60 and 100% at 6 000 psi, and 0 to 40% at 5 000 psi, despite using ten minute treatment durations. There appeared to be good agreement between mean proportions of triploids estimated by analyses of individual larvae and analyses of groups of larvae (Table 4.1).

Pressure treatments of 15 to 30 minutes initiated at five minutes p.s.a. were as effective as shorter treatments, but in some cases had a negative impact on survival with no added advantage in triploidization. Initial poor egg quality appeared to be linked with these observations. Trials where pressure shocks were initiated at 10 to 30 minutes post-sperm activation showed deleterious effects on survival. Blastomeres showed an increased incidence of irregular cleavage, and hatching success was lowered considerably in some treatments. Ploidy analysis was not performed on larvae from these latter treatments.

Table 4.1. Mean proportions of triploids (3N) obtained from a range of hydrostatic pressure treatments initiated at five minutes post-sperm activation.

Pressure level (psi)	Duration of treatment (min.)	N_R	Individual larvae Mean (±SD) proportion of 3N (%)	N_{RI}	Groups of larvae Mean (±SE) proportion of 3N (%)	N_{RG}
10 000	10	1	-	-	100	1
	5	1	-	-	100	1
9 000	10	1	-	-	100	1
	5	2	93	1	100	2
8 000	10	2	100	1	100	1
	5	1	100	1	-	-
7 000	10	6	98.8 ± 2.9	6	100	5
	5	2	37 (7 - 67*)	2	50*	1
6 500	10	1	86	1	-	-
6 000	10	3	82.3 ±20.4	3	83.3 ±16.7	3
5 500	10	1	80	1	-	-
5000	10	4	21.7 ±18.4	4	20.0 ±10.4	3

'*' values corresponding to the same treatment; '-' ploidy analysis not obtained

N_R = number of replicate treatments for which ploidy analysis was performed

N_{RI} = number of replicate treatments represented by analyses on individual larvae (n=15 preparations per replicate)

N_{RG} = number of replicate treatments (N_R) represented by analyses on groups of larvae (n=1 - 3 preparations of groups of 10 to 18 larvae per replicate treatment).

4.3.2. Experiment 2: The effect of triploidy on larval growth and survival.

Ploidy analysis performed on larvae after yolk-sac absorption indicated that high proportions of triploids were produced by pressure treatments of 7 000 psi (ten minute treatments initiated at five minutes p.s.a. (11.1-13.4°C); Table 4.2). Similar treatments at the 5 000 psi level produced low proportions of triploid larvae, with none being produced in one replicate treatment (Table 4.2). No triploid larvae were detected in sham control treatments of 0 psi (Table 4.2).

Statistical analysis on initial egg quality, assessed six to eight hours following treatments, showed that fertilization success values and proportions of fertilized eggs with irregular blastomere cleavage (4-8 cell stage) were similar among the different treatment groups ($P>0.05$, Table 4.2). However, it should be noted that the highest incidence of irregular blastomere cleavage was seen in the 7 000 psi treatment group.

Developmental changes in the experimental larvae were recorded at each measurement event over the course of the study. At six days post-hatch (p.h.), larvae in all treatment groups were in the first-feeding stage and had simple tubular intestines. During this sensitive stage, mortality rates were high in all treatments. Mortalities abated when feeding activity appeared to be well established following day nine post-hatch. By day 21 p.h. the larvae were still in an early developmental stage. A coiling of the intestine, forming a single loop, was noted in all ten larvae sampled from the 7 000 psi group. Fewer larvae exhibited this feature in other groups (5/10 in the 0 psi group; 7/10 in the 5 000 psi group).

Observations made on day 36 p.h. for larvae from the 5 000 psi treatment group revealed significant changes. These changes included a more developed trunk musculature

and gills with prominent filaments. Eight of the ten larvae sampled were in a preflexion stage where the caudal end of the notochord was straight with hypural rays. Development was more advanced in the remaining two individuals of the sample: in one subject the end of the notochord was flexing upwards, in the other individual the notochord flexion stage was complete and fully developed fins with rays were seen. By day 51 p.h. larvae sampled from all treatments were in a postflexion stage with the exception of one individual in the 7 000 psi group. Evidence of metamorphosis, indicated by eye migration, was seen in two large individuals, one in the 5 000 psi and another in the 0 psi group.

Proportions of metamorphic individuals at the end of the experiment (day 76 p.h.) were similar among all treatment groups, as were the number of individuals showing pigmentation or settling behaviour (Table 4.3). A higher number of individuals in which metamorphosis was advanced was noted in 0 psi and 5 000 psi treatments. Ploidy analysis indicated that a high percentage of triploids was preserved at the end of the experiment in the 7 000 psi pressure treatment group (Table 4.3). In the 5 000 psi treatment group, however, no triploids were found in the sample.

Increases in notochord/standard length were similar between treatments up until day 36 (Figure 4.1). After this time, larvae from 5 000 and 0 psi treatments showed identical growth patterns, faster than those of 7 000 psi treatment larvae. Heterogeneity of slopes analysis indicated that the length-time regression coefficient (or growth rate) for the 7 000 psi treatment group was significantly lower than those of the other groups ($P<0.0001$). No difference was seen between the regression coefficients of the 5 000 psi and 0 psi treatment groups. The timing of this growth divergence occurred after larvae had begun to feed on

enriched *Artemia* added on day 38. As noted above, significant developmental events occurring during this period included notochord flexion and fin ray development, followed by metamorphosis.

Survival rates were low in all treatment groups. The highest larval yield was noted in the 5 000 psi group while mortalities occurred most heavily in the 7 000 psi and the sham control 0 psi treatments. These latter two groups produced similar numbers of individuals (Table 4.3). Considering the lower initial stocking numbers of the sham control group, the 7 000 psi group would appear to have had the lowest survival. An increase in the number of mortalities in the 7 000 psi group during the last week of the experiment was a contributing factor to the lower larval yield upon sampling.

Table 4.2. Summary of initial egg volume, egg quality and ploidy analysis results for the three pressure treatment groups in experiment 2.

Pressure (psi)	N_R	Egg Volume (ml)	Mean FS (%)	Mean IR (%)	Mean & range in proportion of 3N (%)	Dates of egg collection
7 000 Tank 1	3	46.4	37 ±12 [a]	32 ±18 [a]	98 ± 4 (93-100)	07/26, 29, 31
5 000 Tank 2	3	45.5	41 ± 9 [a]	22 ±10 [a]	24 ±21 (0 - 40)	07/26, 29, 31
SHAM 0 Tank 3	2	31.4 (44.3)*	39 ± 4 [a]*	19 ±19 [a]*	0	07/26, 29

All mean values are expressed as mean (±SD); means noted by the same superscript letter are not significantly different (P >0.05).

Values denoted by an asterisk '*' include data from the third sham control 0 psi treatment performed on 07/31, accidentally spilled during egg maintenance.

N_R = number of replicate pressure treatments per group

Egg volume= total volume of eggs contributing to each treatment group

FS= fertilization success

IR= percentage of fertilized eggs with irregular cleavage of blastomeres.

The mean and range in the proportion of triploids (3N) produced from the different replicate treatments in each group is noted. Ploidy analyses performed at yolk-sac absorption were determined on cell suspensions of individual larvae (n=15 larvae per replicate treatment).

Table 4.3. Final sample information for the three pressure treatment groups in experiment 2: proportion of triploids, developmental characteristics and survival performance at 76 days post-hatch.

Characteristics	**Tank 1 7 000 psi**	**Tank 2 5 000 psi**	**Tank 3 0 psi**
Proportion of triploid individuals (%)	92	0	0
Pre-metamorphic individuals	3 / 15	4 / 15	1 / 15
Evidence of metamorphosis	12 / 15	11 / 15	14 / 15
Advanced metamorphosis	2 / 12	5 / 11	5 / 14
Pigmented individuals	2 / 15	3 / 15	4 / 15
Proportion of settled individuals of the total number of fish surviving to the end of the experiment	23 / 32 (72 %)	112 / 142 (79 %)	28 / 41 (68 %)

The percentage of triploids in treatments was determined on a sample of 15 individuals for which the developmental characteristics were recorded. Although 15 individuals were sampled from the sham control 0 psi group, only the first five were tested for ploidy analysis as no triploid larvae were found during the analysis at yolk-sac absorption.

Evidence of metamorphosis: eye migration and/or pigmentation of the blood.

Advanced metamorphosis: expressed as number of metamorphic individuals demonstrating full eye migration and in some individuals body pigmentation as well.

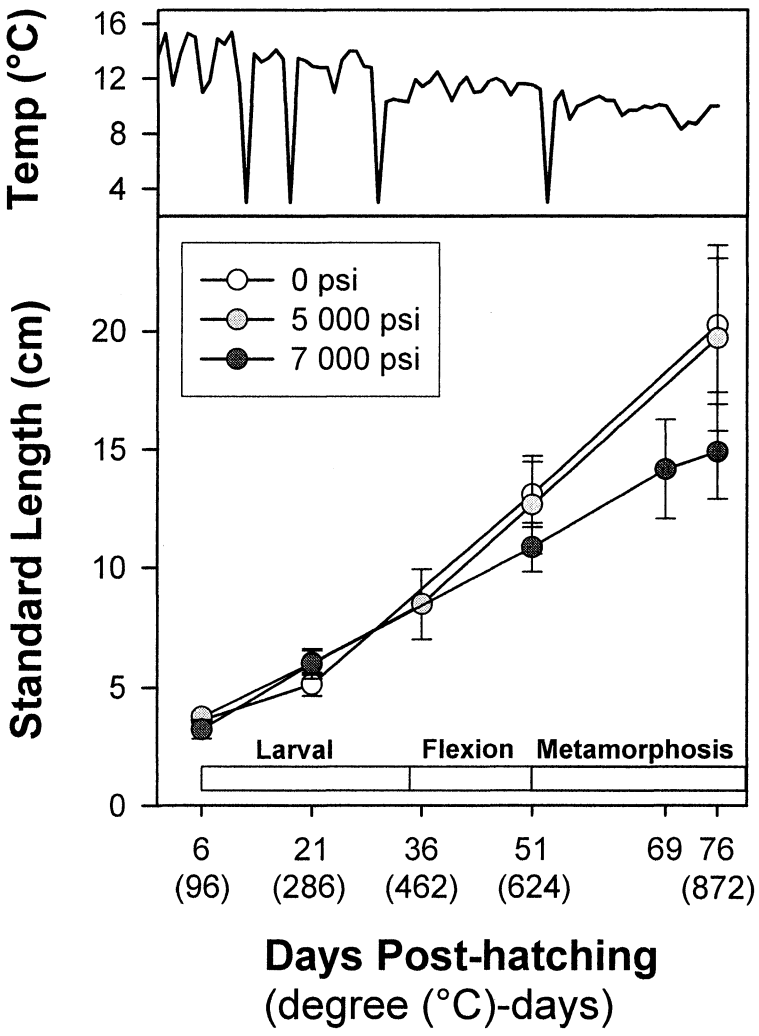


Figure 4.1. Comparison of growth patterns of larvae from pressure treatments in experiment 2. The three pressure treatments include: a 7 000 psi treatment inducing a high percentage of triploids, a 5 000 psi treatment inducing a low percentage of triploids, and a 0 psi-sham control treatment representing only diploids. The water temperature profile and the main developmental phases observed over the course of the experiment are also shown. Developmental time in degree-days = sum of temperatures recorded for each day_i of development post-hatching; values are shown in parentheses.

4.3.3. Experiment 3: Effects of triploidy on sexual maturation in yellowtail flounder.

Preliminary larval rearing efforts in 1997 produced a small population of 38 juveniles. Twenty-four fish survived to final sampling after three years in culture. Ploidy analysis on blood cells collected at final sampling times confirmed that all 24 individuals, 17 females and seven males, were triploid. These fish had the same range of morphological variations observed in cultured diploids (i.e. varying degrees of albinism and incomplete eye migration). One exception was an individual with jaw and head deformities: the lower jaw was bent to the side and the abocular surface of the head had a concave depression. This individual was still able to feed and showed no disadvantage in terms of body size.

4.3.3.1. Juvenile growth.

Growth records for triploids began at five months of age and continued to 40 months of age. The first evidence of ovarian differentiation was detected macroscopically when juveniles were nine months old in June of 1998. At ten and 14 months of age, 15 of 31 individuals could be identified as females simply from external examination. By the next measurement at 18.5 months of age in March, 1999, the number of females had increased to 18 of 25 fish. Apart from an additional mortality, the sex representation (7 males; 17 females) remained unchanged until final sampling, whereupon the accuracy of sexing triploid individuals by external examination was confirmed.

Poor growth performance was seen during the first five to eight months of life resulting from a prolonged larval growth period and slow early juvenile growth (Figure 4.2). An improvement in growth in length was seen following eight months of age. Increases in length were relatively linear with time, although a slight decrease in growth rate was seen

with age (Figure 4.2). In weight gain patterns, growth rates increased markedly following 18.5 months of age, particularly in females (Figure 4.2). This increase coincided with a change to a moist pellet diet. Some preference for a moist pellet rather than the dry pellet feed was seen in triploids at this time. Behaviourally, triploids were more covert in their feeding activity than diploids.

Divergent growth patterns for male and female triploids began at 24.5 months of age (Figure 4.2). The difference between male and female growth curves was greater for body weight than for length (Figure 4.2). According to heterogeneity of slopes analysis, regression coefficients for male and female size relationships with time were not significantly different for length or for weight ($P>0.05$). Subsequent ANCOVA results did show that, overall, males were smaller than females in both length and weight ($P<0.01$). Comparing males and females at each measurement event showed that significant sex differences in body size were present only in June at 33 months of age (one-way ANOVA, $P<0.02$). This was the last time males and females were measured together prior to the sampling of males.

The growth of 2+ triploid females was compared to the growth data of three groups of 1+ diploid females (Table 4.4; Figure 4.3). Two groups consisted of either maturing or immature diploid females, both of which had exhibited fast growth rates as 0+ individuals. The third diploid group was comprised of immature females which had demonstrated slow growth as 0+ animals. In late-October, 1999 triploid females of 24.5 months of age (September, 1999) were nearly the same size as 14.5 month old (October, 1999) diploid females which had exhibited fast underyearling growth (Figure 4.3). Growth curves for triploids lay between those of immature diploid females with either slow or fast growth

histories, and followed similar upward trends in body size (Figure 4.3). In contrast, maturing, formerly fast growing females showed the slowest growth patterns, intersecting those of triploid females. Calculations of specific growth rates for the different groups clearly showed that immature females with a previous slow growth history had the highest growth rates in both length and weight (Table 4.4). Triploid growth rates approached only those of immature fast-growth females, while exceeding those of maturing females (Table 4.4). For both length-time and weight-time relationships, heterogeneity of slopes analyses showed that the regression coefficients (growth rates) for triploid females were statistically similar to those for immature diploids with a fast growth history (weight P=0.29; length P=0.078), but differed significantly from those of other diploid curves (P<0.0001).

Table 4.4. Comparison of specific growth rates (SPGR) calculated for 2+ triploid (3N) females with those calculated for immature and maturing 1+ diploid (2N) females of different growth histories (fast or slow underyearling 0+ growth).

Groups	SPGR-Standard Length (%/day)	Size range in Standard Length (cm)	SPGR-Weight (%/day)	Size range in Weight (g)	Age and time span for growth
3N♀ *n*= 17	0.070	16.1 ±1.7 20.9 ±1.6	0.236	86 ±32 208 ±51	24.5 - 37 09/99-10/00
2N ♀ fast mature *n*= 13	0.045	16.9 ±0.8 20.3 ±1.1	0.154	92 ±14 173 ±25	14.5 - 28 10/99-12/00
2N ♀ fast immature *n*= 7	0.083	17.2 ±0.6 24.1 ±1.5	0.276	99 ±12 303 ±65	14.5 - 28 10/99-12/00
2N ♀ slow immature *n*= 18	0.113	12.7 ±0.9 20.1 ±1.5	0.375	36 ±7 162 ±34	14.5 - 28 10/99-12/00

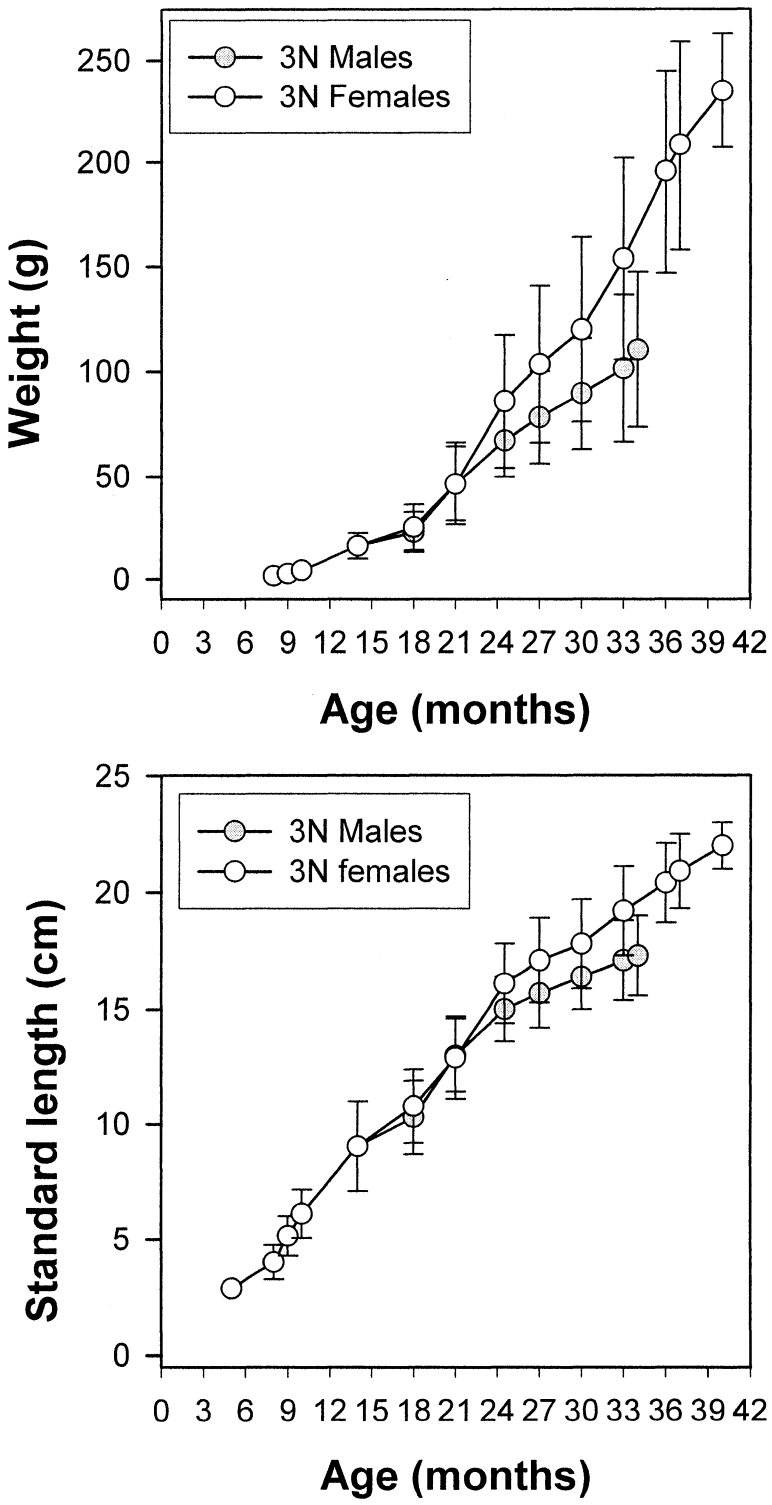
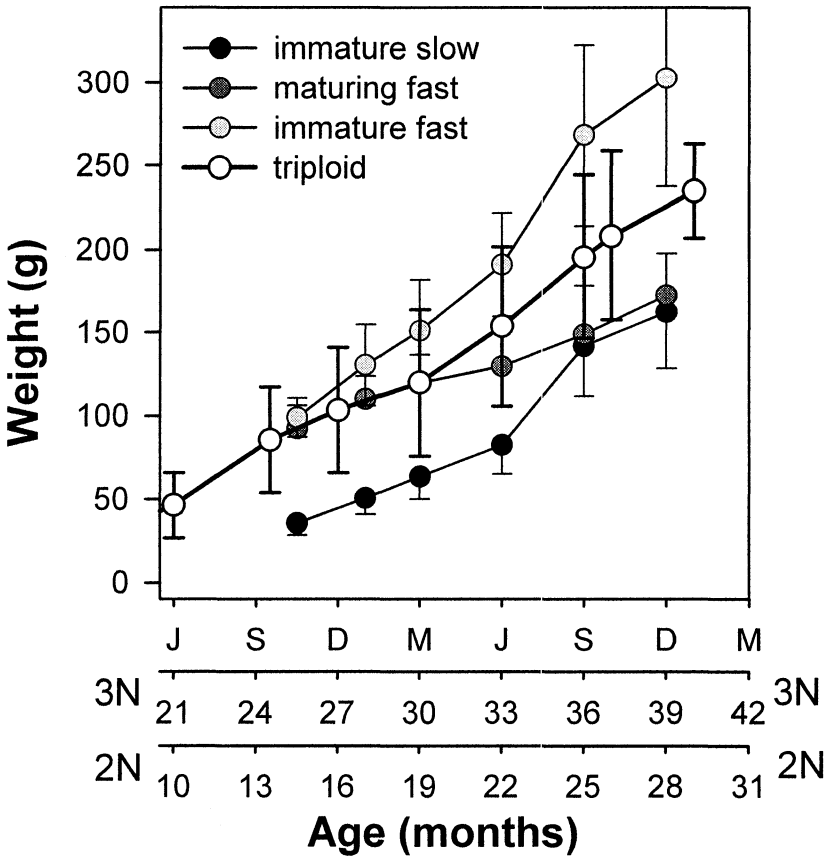
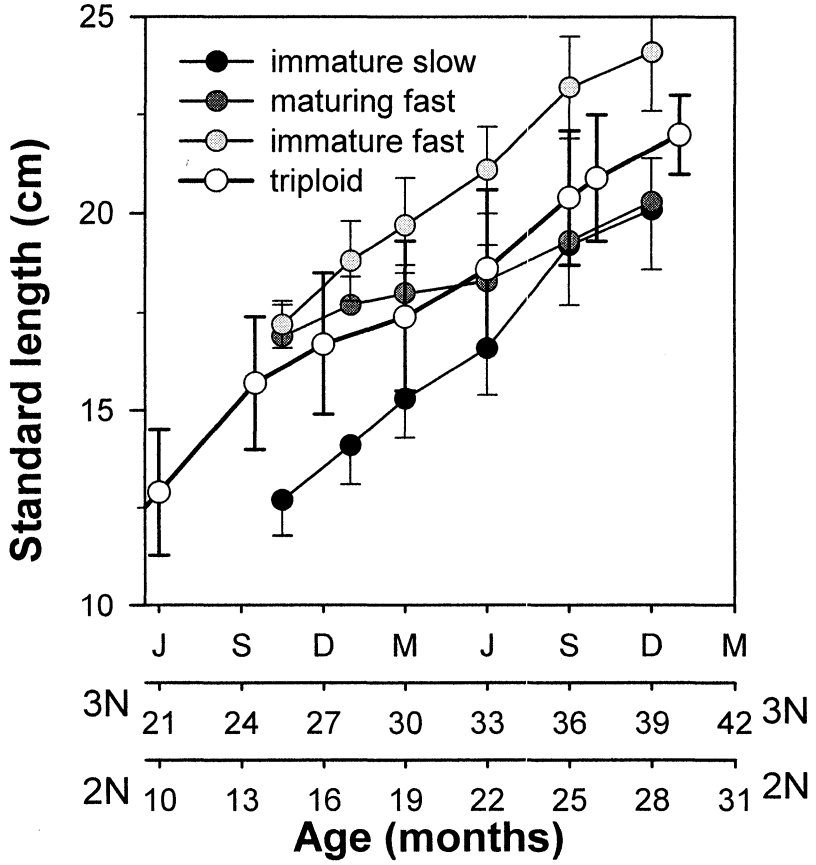


Figure 4.2. Mean (±SD) weight and standard length growth curves for male (n=7) and female (n=17-18) triploid yellowtail flounder from experiment 3.

Figure 4.3. Comparison of growth curves over time (June, 1999 - January, 2001) in mean (±SD) weight and standard length between 2+ triploid females (n=17) and three sets of 1+ diploid females: immature (n=7) and early maturing (n=13) females which demonstrated fast underyearling growth rates and immature females (n=18) which demonstrated a slow underyearling growth rate. The main x-axis indicates months and years during which the growth comparison occurred, the second axis represents the ages of the triploid females during the comparison, and the third axis indicates the ages of the diploid females during the comparison.


immature slow
maturing fast
immature fast
triploid
Weight (g)
300
250
200
150
100
50
0
J S D M J S D M
3N 21 24 27 30 33 36 39 42 3N
2N 10 13 16 19 22 25 28 31 2N
Age (months)


immature slow
maturing fast
immature fast
triploid
Standard length (cm)
25
20
15
10
J S D M J S D M
3N 21 24 27 30 33 36 39 42 3N
2N 10 13 16 19 22 25 28 31 2N
Age (months)

4.3.3.2. Male gonadal development.

Triploid males were recorded as immature until limited amounts of milt could be expressed from four of the seven males at 30 months of age in March, 2000. By June, when diploids were in full spermiating condition, the amount of milt that could be expressed from triploids was still limited in four of the males, and was only just detectable at the urogenital pore in the remaining three males. All seven males were killed at 34 months of age in July, during the peak of the spawning period for diploids. Due to the small amounts of milt produced, milt sampled directly from the sperm ducts was supplemented by milt exuding from the dissected testes.

Motility assessment demonstrated that milt from triploid males had either few or no motile spermatozoa (Table 4.5). Heterogeneous populations of cells were seen in the milt collections of most males. These observations characterized samples of milt collected from stripping attempts as well as collections made directly from the sperm ducts and testes. In fertilization trials, milt from triploid males demonstrated poor fertility (Table 4.5). Very few larvae were produced from these trials. Most larvae were abnormal, frequently exhibiting curvature of the notochord. Only four triploid males produced one or two larvae of normal appearance (Table 4.5). In contrast to triploids, milt pooled from two adult diploid males showed higher fertilization and hatching success values, and far higher numbers of normal larvae (Table 4.5). Comparing mean fertilization rates of triploids and diploids showed that milt from triploid males exhibited 7 to 45% (mean=~ 20%) of the fertility performance of milt pooled from diploid males. For larval production rates, triploids exhibited values which were 0 to 20% (mean ~7%) of the value obtained when using milt pooled from diploids.

After 15 days post-fertilization at 9°C a total of three normal larvae and five abnormal larvae survived from original pooled totals of six normal and 61 abnormal larvae. Thus, a high degree of mortality was seen among the progeny of triploid males prior to losses associated with yolk-sac absorption. Conversely, minimal larval mortality was seen among the progeny of adult diploid males where six mortalities occurred among 165 larvae.

The testes of triploid males were small and the amount of milt in the sperm ducts was minimal. Total testicular weights (<1 g) and GSI values were very low in all seven males (Table 4.5). Testes for males with GSI values between 0.18 and 0.36 % had a translucent character typically seen only at the initiation of puberty in diploids. Histologically, the testes appeared regressed in most males, containing mainly primary spermatogonia and low amounts of spermatozoa within the testicular lobules (Plate 4.1A). In contrast, diploid testes were full of spermatozoa during the spawning period with only a few nests of primary spermatogonia (Plate 4.1B). Some mitotic activity was seen in two triploid males, and an increased amount of spermatogonial tissue was noted in another three males in which mitotic activity was not confirmed. Spermatozoa in triploids were observed to have larger sperm heads than those seen in diploids (Plate 4.1 D & E). Besides spermatozoa, two other cell types were seen within the lobular spaces of all triploid males. One cell type, as densely basophilic as sperm heads, but unflagellated and larger in size, resembled spermatids. Phagocytes were the other cell type present. These cells were actively ingesting spermatozoa and potential spermatids, and could appear very basophilic as a result of this activity. A high number of phagocytes was seen in the male with the most regressed testes (male 6 Table 4.5; Plate 4.1F&G).

Some meiotic activity was noted in two triploid males (males 2&3 Table 4.5); spermatocytes and cells tentatively identified as spermatids were seen surrounded by spermatogonial tissue which included secondary spermatogonia (Table 4.5; Plate 4.1C). Meiotic areas within testicular tissue appeared to be in a process of dissociation. In addition, cells similar in appearance to both spermatocytes and spermatids were found amid material, formerly in the ducts and testicular lumen, which coated the outer surface of the testis in histological sections (Plate 4.1C). This outer material was a result of fluid leaking from the testis during dissection and fixation; spermatozoa in mature diploid testes frequently is seen in this manner. The detection of meiotic cells in this material suggests that a degenerative process may have occurred involving a premature release of meiotic cells into the lobular lumen.

At an endocrine level the two meiotic males (males 2& 3) had the highest and lowest androgen levels while the other, essentially regressed males had intermediate levels of androgens (Table 4.5). Notably, the male with the highest androgen levels had the highest values in fertilization trials for triploids (male 2, Table 4.5). A reference diploid male had very low androgen levels, which is not atypical given the high variability in androgen levels of diploid males sampled during spawning (Manning et al., chap. 2; Clearwater, 1996).

Table 4.5. Fertility trial, testicular development and androgen level summary for seven 34 month old triploid males sampled during the regular spawning period for diploids (July): fertility data for individual triploids are compared to the results for milt pooled from two adult, diploid males.

Male	Motility	Mean FS (%)	Mean HS (%)	Mean LP (%)	Normal larvae of total	GSI (%)	11-KT (ng/ml)	T (ng/ml)
1	minimal	7.0	24.2	1.7	0 / 10	0.48	0.88	0.70
2	minimal	19.0	31.3	5.9	2 / 27	1.10	6.14	1.4
3	minimal	4.0	13.2	0.6	0 / 3	0.35	0.16	0.14
4	0	8.0	22.3	2.0	1 / 9	0.27	1.41	0.61
5	0	5.6	44.4	2.5	2 / 12	0.36	1.18	0.37
6	0	3.0	0	0	0 / 0	0.18	1.26	0.36
7	minimal	8.7	14.4	1.2	1 / 6	0.41	2.01	0.69
Avg SE/SD		7.9 $\pm 2.0_{SE}$	21.4 $\pm 5.4_{SE}$	2.0 $\pm 0.7_{SE}$		0.46 $\pm 0.30_{SD}$	1.9 $\pm 2.0_{SD}$	0.60 $\pm 0.40_{SD}$
2N milt	high	42.5	68.4	29.0	143/165	n/a	0.36*	0.09*

'*' androgen values of one of the diploid males in the fertility trial

Motility= individual sperm motility assessment

FS=fertilization success; HS= mean hatching success; LP= larval production

Number of normal larvae among the total number of larvae produced from the three replicates in each fertilization trial.

11-KT= plasma levels of 11- ketotestosterone; T= plasma levels of testosterone

Plate 4.1. Testicular histology for 34 month old triploid male yellowtail flounder sampled in July, 2000.

A- Limited sperm production of a mature triploid male in July (**sz**=spermatozoa).

B- Spermiating diploid male in June.

C- Delayed spermatogenesis in a triploid male. Spermatogonial tissue as well as meiotic cells (spermatocytes (**sc**) and spermatids (**st**) seemingly in a state of degeneration) are seen. Spermatozoa and cells similar to meiotic cells are seen in milt coating outer surface of testis. Insert shows a close-up of a cyst of meiotic cells in a triploid male.

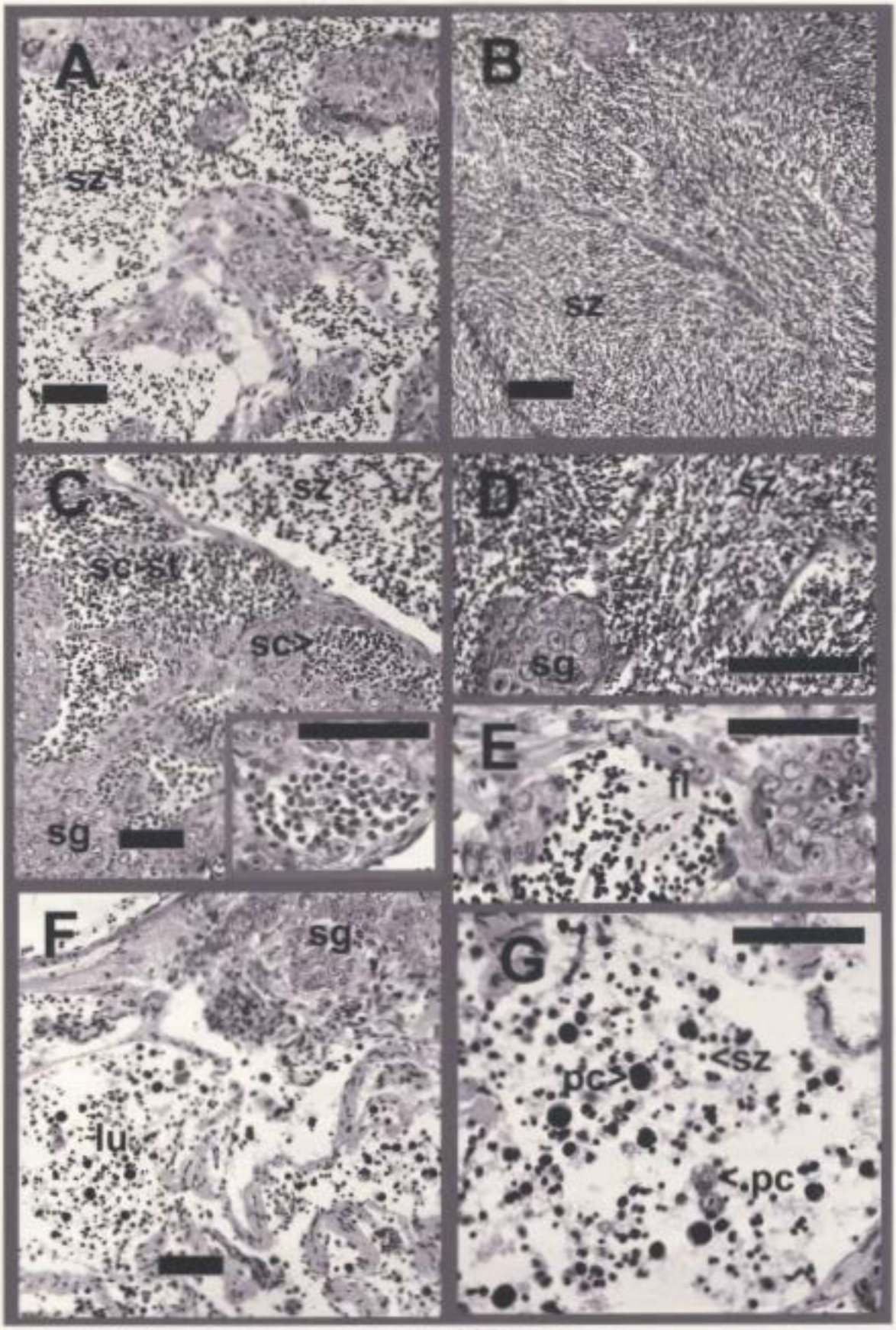
D- Close -up of spermatozoa in a diploid male testis. **sg**= spermatogonia

E- Close-up of spermatozoa in a triploid male. Note larger heads of spermatozoa and size of spermatogonia relative to cells in the diploid male (D). **fl**= flagella.

F- Regressed triploid male (6) with phagocytes in the lobular lumen (**lu**). **sg**=spermatogonia.

G- Close-up of phagocytes (**pc**) actively taking in sperm heads (**sz**), an inactive phagocyte is present as well.

Scale bars= 50 μm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4.3.3.3. Female gonadal development.

Observations based on ovarian ranks (OR) showed that the ovaries of triploid females remained very small (OR=1) until two years of age in September, when evidence of ovarian growth was seen in two females (OR=2-3). One of these females was of average size while the other was the largest of the group. As more females displayed ovarian growth between 24.5 months and 33 months of age (June), the hypothesis was tested that ovarian growth was associated with body size. Five females demonstrating ovarian growth (OR=2-3) in December at 27 months of age were larger in weight and length than females with ovarian ranks of one ($P \leq 0.003$). However, a connection with body size disappeared ($P<0.10$) when the number of females displaying ovarian growth increased in March at 30 months of age. By June at 33 months of age, six females had ovarian ranks of two to four, in the range for the initiation of puberty seen in diploids. Two additional females had ovarian ranks of six, previously having values of three in March. The ovaries of these females appeared non-reproductive, but were uncharacteristically swollen. When these females were stripped, a clear fluid was collected, but no ovulated eggs were detected.

Twelve females aged 37 months were sacrificed in October, 2000. Externally, seven of the twelve females appeared to be immature (OR=1). Of the five remaining females, three showed signs of initial ovarian growth (OR=2-3), while two females had larger ovaries (OR=5-6). These latter two individuals were the same females which had been checked for eggs four months earlier. Gonadosomatic index values and ovarian weights were low for all twelve females (GSI range= 0.44 - 2.11%; ovarian weight range 0.7 - 5.1 g; Table 4.6). Despite the small ovarian size, histological analysis showed that vitellogenesis was underway

in ten of the twelve females. Five of these females were in the VG-II stage while the other five were in the VG-III stage. Vitellogenic oocytes were interspersed amid tracts of oogonia, as were other earlier stage oocytes (i.e. perinucleolar stage to VG-I/II oocytes) (Plate 4.2A,B,C). The amount of oocytes occupying the tissue was estimated as a percentage of the tissue section area. In the ten vitellogenic females, 5 to 80% of the tissue area was comprised of oocytes in previtellogenic and vitellogenic stages (Plate 4.2C,F). A few atretic oocytes were detected in only four of the ten vitellogenic females.

The two females with the highest degree of ovarian growth (OR=5-6) were among the ten vitellogenic females. Both visual evidence upon dissection and histological evidence indicated the presence of a small number of residual eggs within the ovaries of these individuals (Plate 4.2A). While other vitellogenic females appeared to be in a pubertal state of vitellogenesis, these two females clearly had ovulated and were in a recrudescing state in October, 2000.

Cortical alveolar oocytes were the most advanced cells in the two non-vitellogenic females sacrificed in October, 2000 (Plate 4.2D,E). The number of oocytes in the ovaries was low, representing five and ten percent of the tissue area. The ovaries of these two females were very small (GSI=0.44 & 0.59%). Only in these two females did ovarian ranks (OR=1) accurately reflect that the ovaries were immature at the time of sampling.

Plasma levels of 17ß-estradiol and testosterone were low but detectable in all twelve females. Levels in the ten vitellogenic females ranged from 0.29 to 0.80 ng/ml for 17ß-estradiol (mean 0.60 ±0.16) and from 0.05 to 0.32 ng/ml for testosterone (mean 0.19 ±0.08). For the two females with cortical alveolar oocytes, levels of 17ß-estradiol (0.24 & 0.38

ng/ml) overlapped with the lower values seen in vitellogenic females. Levels of testosterone (0.20 & 0.23 ng/ml) resembled average values seen in vitellogenic females.

In vitro incubation results from the first six females sacrificed in October, 2000 were organized according to reproductive stage (Figure 4.4). A cortical alveolar stage female, a recrudescing vitellogenic female, one of those found with residual eggs, and four females in a state of pubertal vitellogenesis were examined (Figure 4.4). All five vitellogenic females demonstrated a higher steroidogenic capacity in response to forskolin and crude pituitary extract (CPE) than was indicated by *in vivo* plasma levels (Figure 4.4). In contrast, for the cortical alveolar stage female, 17ß-estradiol levels produced *in vitro* were comparable to plasma levels (Figure 4.4). In four of the six individuals, the tissue response to both forskolin and crude pituitary extract was clear and statistically significant ($0.0001<P<0.005$). For the two remaining females (one pubertal and the recrudescing female) the tissue response to these agents was of borderline statistical significance ($P=0.051$ & 0.061) despite a visually apparent response. Mean levels of 17ß-estradiol production for the group of four pubertal females were much higher than levels produced by the ovarian tissue of the other two females (Figure 4.4). This included levels in control wells, which were strikingly higher for pubertal females. In one pubertal female in particular, mean 17ß-estradiol output reached very high levels (forskolin wells= 3.5 ng/ml; CPE wells= 5.5 ng/ml) even though the ovaries were still in an early stage of vitellogenesis (VG-II oocytes).

In addition to the October sample, five triploid females were sacrificed at 40 months of age (January, 2001). Ovarian ranks of these five females in October, 2000 ranged from one to four. The same range was seen when these individuals were sampled in January, 2001.

Upon dissection it was clear that four females had ovaries where vitellogenic oocytes could be seen macroscopically in the tissue. Ovaries of these females were small, but reached sizes exceeding those of ovaries sampled in October (GSI range=1.0 - 4.3%; ovarian weight range=2.2 - 10.7 g). The fifth female was presumably immature with a GSI of 0.38% and an ovarian weight of 0.98 g, values similar to those seen in cortical alveolar stage females in October.

Table 4.6 compares the mean results for triploid females with data from Manning et al. (chap. 2) for two-year-old pubertal diploids sampled at similar times of the year. Mean ovarian weight, GSI and 17ß-estradiol were higher in diploid females in October. Diploids had ovaries with VG-II or VG-III oocytes at this time, as was the case in triploid females. A greater difference between diploid and triploid females was seen when comparing December (2N)/January(3N) samples (Table 4.6). Plasma testosterone levels had a stronger presence in triploids in October compared to diploids at the same time of year (Table 4.6). Only three of five diploids in October had detectable levels of testosterone. Conversely, all triploid females in the October sample had detectable levels of the androgen. This was not the case in diploids until December.

Abnormalities were detected in the ovaries of some triploid females (Plate 4.3). Oocytes with two nuclei were seen in six of the twelve females sacrificed in October. More than one example was seen within certain individuals, which suggested that binucleate oocytes may be a common feature in the ovaries of some triploid yellowtail flounder (Plate 4.3A,B,E). In one binucleate oocyte, the cytoplasm showed marked swirling elements (Plate 4.4B). In another oocyte, a nucleoplasmic bridge appears to be connecting the two nuclei

(Plate 4.3F). Other abnormalities detected in the oocytes of triploids included the presence of pale patches in the cytoplasm (Plate 4.3C). In one oocyte, an evagination of nucleoplasm was observed (Plate 4.3D). Two small cytoplasmic patches with the same level of eosinophilic staining as the nucleoplasm were seen below this evagination. This may be indicative of preceding periods of ejection of nucleoplasm (Plate 4.3D).

Giemsa stained blood smears, prepared from triploid and diploid females, showed that triploids had predictably larger erythrocytes with larger sized nuclei than diploids (Plate 4.4). Some erythrocytes of triploids had irregular shapes, this was not seen in the blood smears of diploids (Plate 4.4).

Table 4.6. Reproductive parameter comparison between three year old triploid females and two year old pubertal diploids sampled at similar times of the year.

Females Age and Time of sample	**Ovarian Weight (g)**	**GSI (%)**	**17ß-estradiol (ng/ml)**	**Testosterone (ng/ml)**	**Range in Ovarian Ranks**
Triploids					
3N (37 mo.) October, 2000 *n*=12	**2.4** ±1.6	**1.1** ±0.6	**0.55** ±0.19	**0.19** ±0.08	**1-3, 6***
3N (40 mo.) January, 2001 *n*=5	**4.8** ±4.0	**2.0** ±1.5	n/a	n/a	**1-4**
Diploids					
2N (25.5 mo.) October, 1999 *n*=5	**8.2** ±4.2	**4.8** ±1.9	**1.89** ±1.10	**0.09** ±0.04	**3-6**
2N (28 mo.) December, 1999 *n*=6	**25.6** ±2.7	**10.8** ±1.8	**2.20** ±0.40	**0.31** ±0.10	**5-8**

Data are presented as mean (±SD)

n/a=not available

'*'values reached by the two ovulating females.

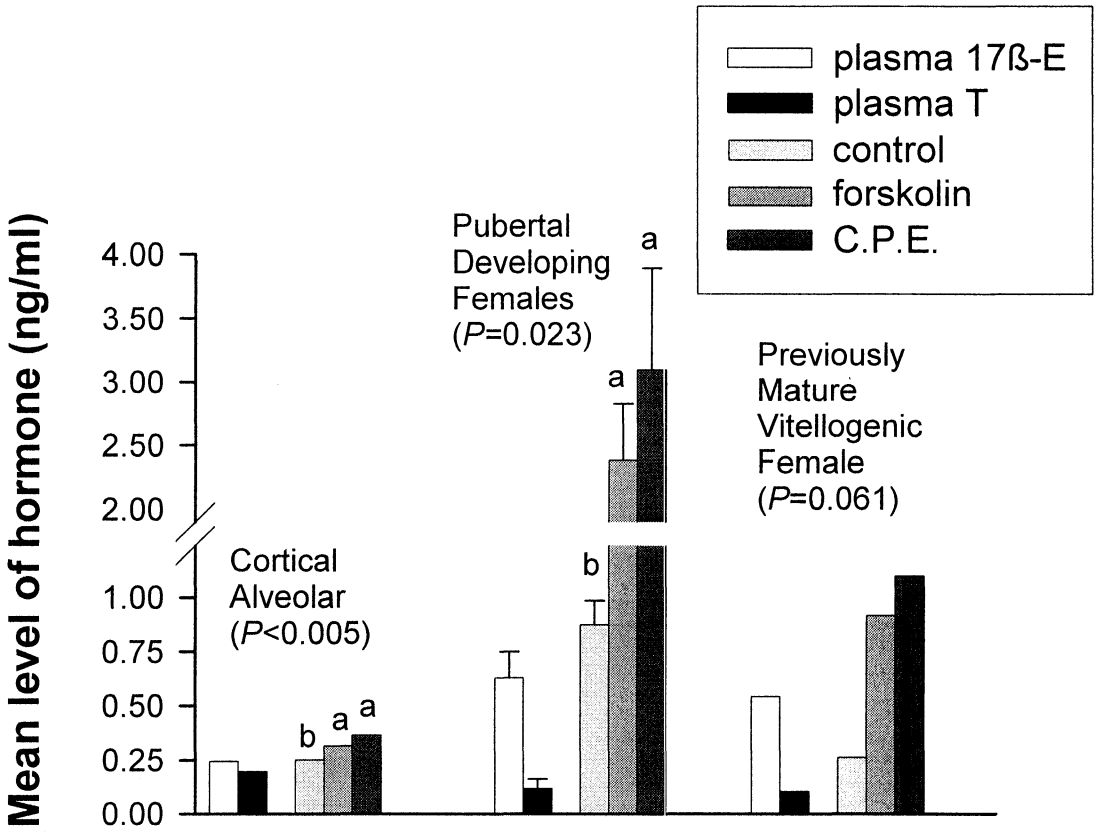


Figure 4.4. Mean (±SD) plasma levels of 17ß-estradiol and testosterone (T), and mean (±SE) *in vitro* 17ß-estradiol production by ovarian tissue of triploid females. Steroid production from tissue incubated in control, forskolin and crude pituitary extract (CPE) media are shown for females of different stages: a cortical alveolar stage female (n=1), pubertal vitellogenic females (n=4) and a recrudescing female (n=1). Means noted by the same letter are not significantly different (P>0.05).

Plate 4.2. Ovarian histology for 37 month old triploid females sampled in October, 2000. Oocytes of different stages are seen amid significant amounts of oogonial tissue.

A- Recrudescing female with large vitellogenic oocytes (**VG-III**) demonstrating a residual egg (**RE**) in the lumen as evidence of prior ovulatory activity.

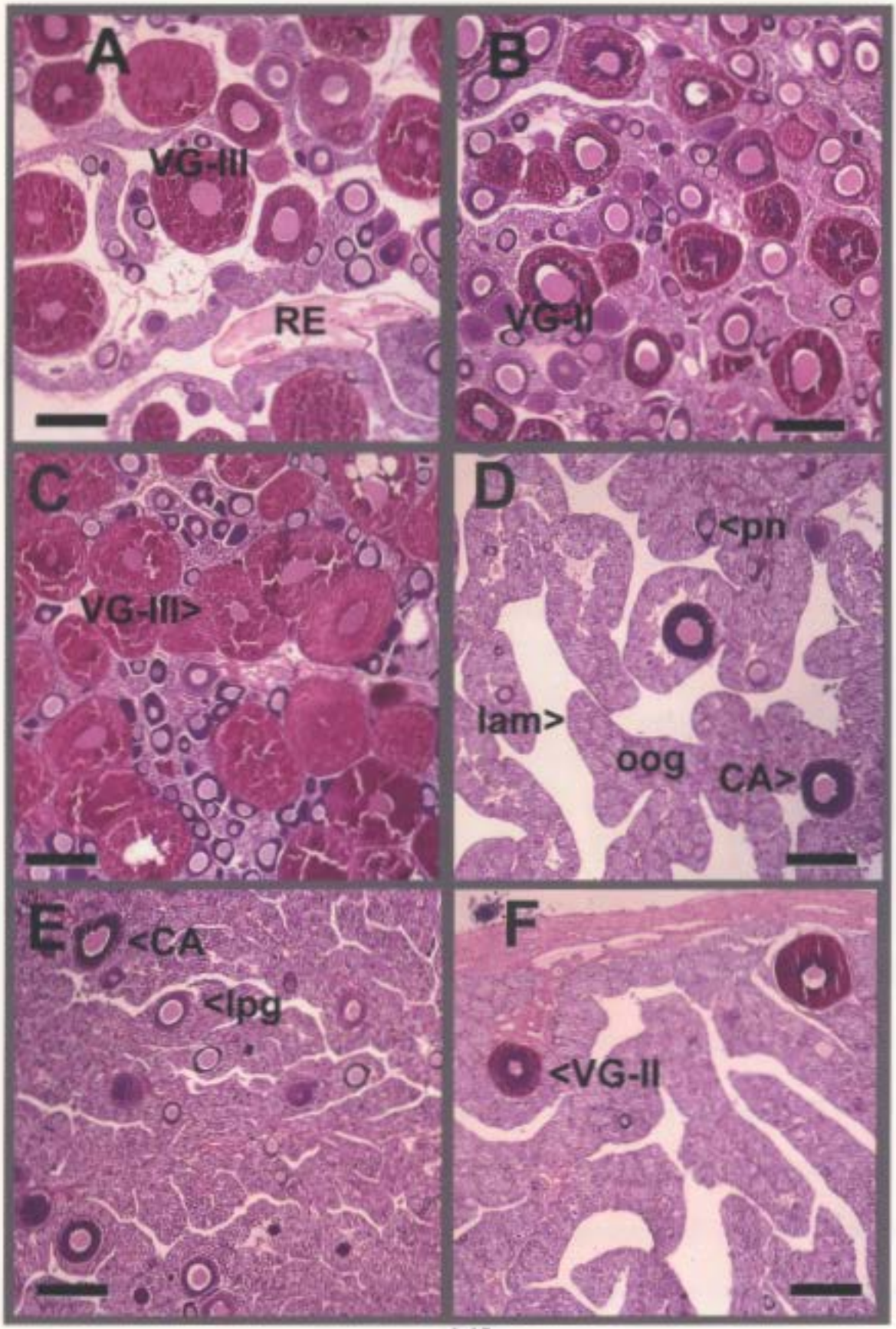
B- Vitellogenic pubertal female in early vitellogenesis with **VG-II** and lesser oocytes

C- A more advanced female with large vitellogenic **VG-III** oocytes. This female had the highest percentage of oocytes seen among the females sampled at this time (~80 of tissue area) and the least amount of oogonial tissue..

D & **E**- Two females with few cortical alveolar (**CA**) and primary growth oocytes (perinucleolar (**pn**) , late stage primary growth (**lpg**) oocytes) and large areas of oogonial tissue (**oog**). Individual lamellae (**lam**) are in clear (D).

F- Female with very few oocytes yet isolated vitellogenic oocytes are present.

Scale bars=200 μm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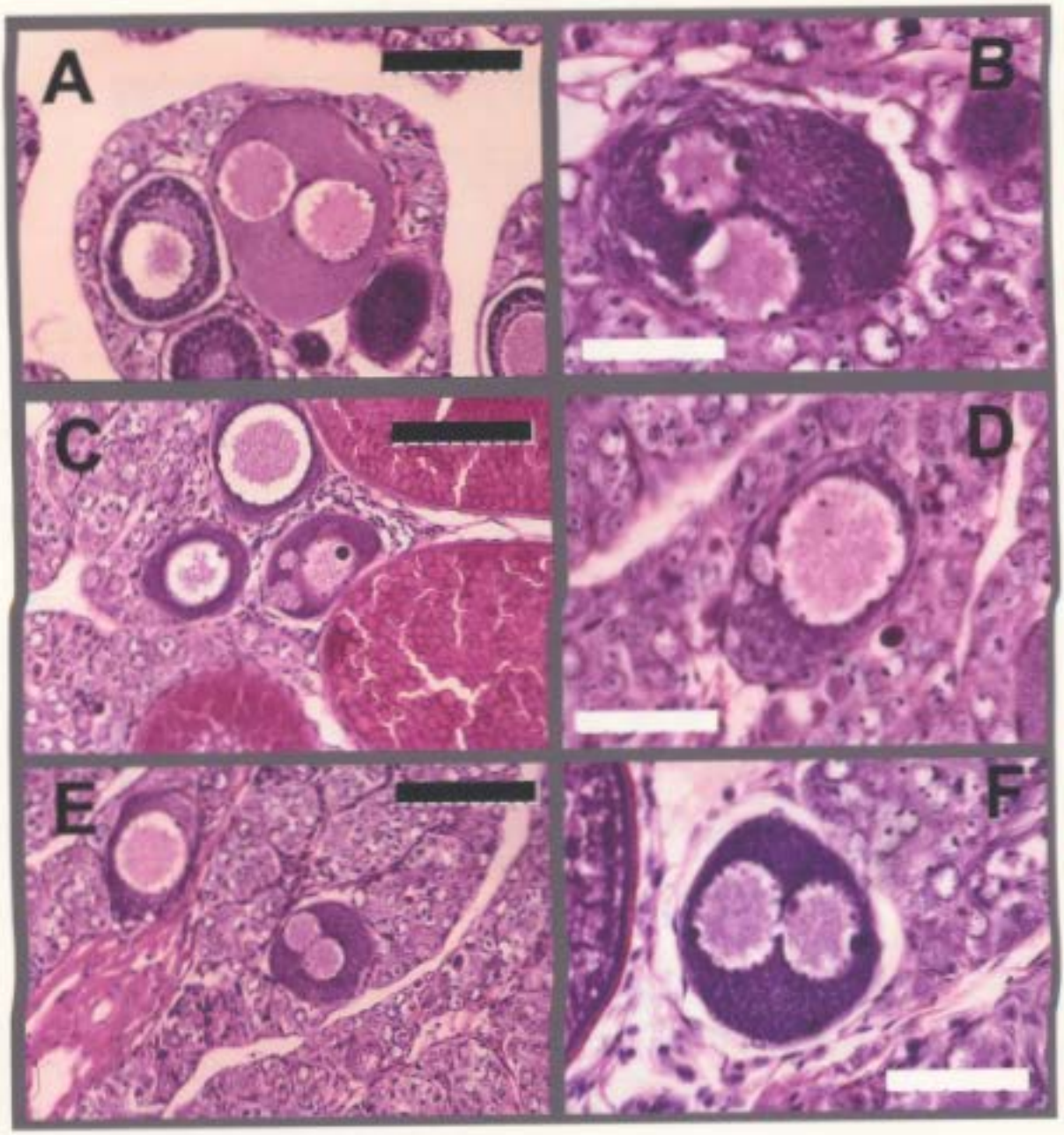

Plate 4.3. Cytological abnormalities in the ovaries of triploid females.

A- binucleate primary growth oocyte; **B**- binucleate oocyte with heterogeneous cytoplasm; **C**- oocyte with pale cytoplasmic patches; **D**- oocyte with evaginating nucleoplasm and pale cytoplasmic patches; **E**- binucleate oocyte; **F**- binucleate oocyte with a nucleoplasmic bridge. Black scale bars= 100 µm ; white scale bars = 50 µm

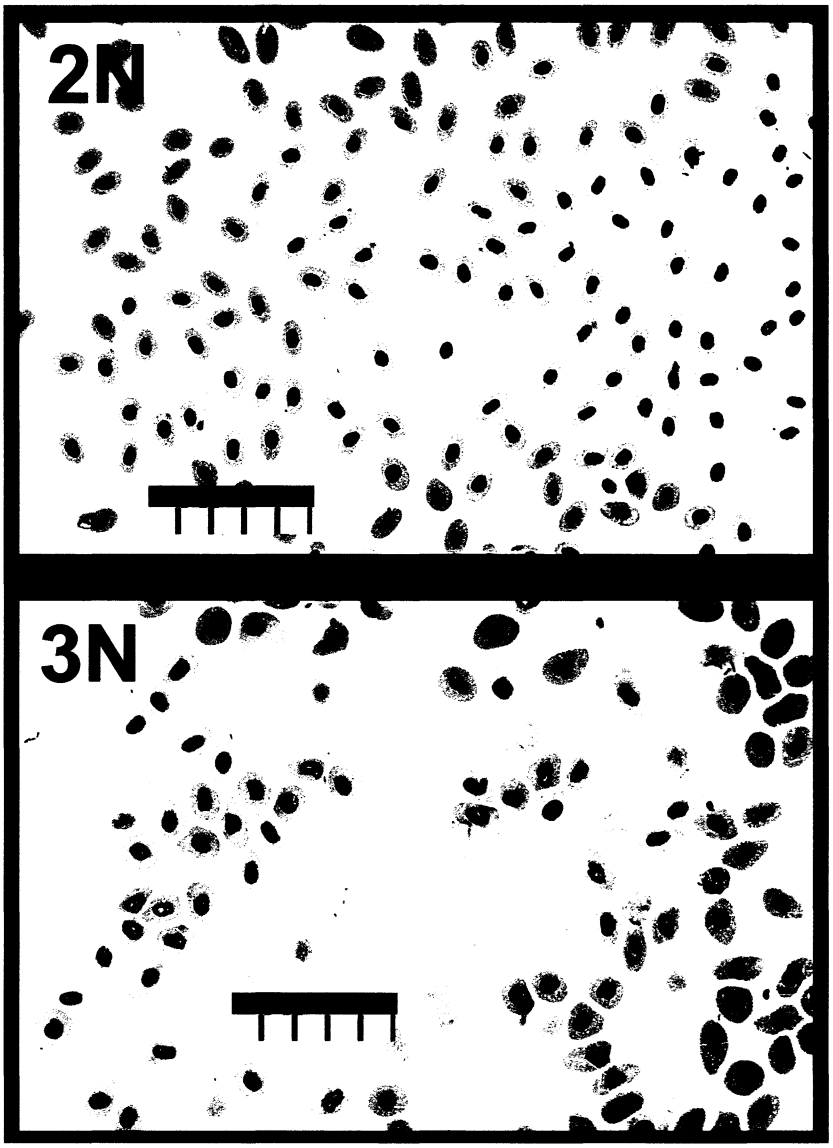

Plate 4.4. Comparison of erythrocytes from diploid and triploid yellowtail flounder.

Scale bars= 50 μm

4.4. DISCUSSION.

4.4.1. Experiment 1: Inducing triploidy by hydrostatic pressure treatment.

A high percentage (92 - 100%) of triploid yellowtail flounder was produced from ten minute hydrostatic pressure shocks of at least 7 000 psi when they were initiated at five minutes post-sperm activation (7-13.4°C). Similar success in inducing triploidy was obtained with five minute treatments but using higher pressure levels (≥8 000 psi; 7-11.2°C). Consistent results were obtained under a wide range of temperature conditions, which indicates that the protocol may be practical under non-laboratory situations. The results of the present study demonstrate that hydrostatic pressure treatment in yellowtail flounder was as effective as cold-thermal shock treatment which was used for other flatfish species (plaice, *Pleuronectes platessa*, plaice x flounder hybrids, *P. platessa x Platichthys flesus*, Purdom, 1972; European turbot, *Psetta maxima*, Piferrer et al., 2000).

A five minute duration of pressure treatment was the shortest tested in the present study. Peruzzi & Chatain (2000) reported that two minute pressure treatments (12-13°C) were effective in the production of triploid sea bass, *Dicentrarchus labrax*. A treatment duration shorter than five minutes may be effective in inducing triploidy in yellowtail flounder eggs at higher pressures (≥8 000 psi). However, using a shorter duration may require a greater degree of temperature control in order to obtain the same level of success in triploidization.

According to Chourrout (1984), pressure shocks below the optimal levels for inducing triploidy in rainbow trout (*Oncorhynchus mykiss*) led to the production of non-viable aneuploid embryos. The aneuploid condition was a result of an incomplete retention of the second polar body. In the present study, suboptimal pressure levels or treatment durations

merely increased the number of diploids relative to triploids with no apparent effects on survival. However, embryo survival was deleteriously affected by delaying the initiation of treatment until ten to thirty minutes post-sperm activation. It seems probable that pressure shocks at these times disrupted the extrusion process of the second polar body, and perhaps other events. Incomplete retention of the polar body promoting aneuploidy seems a plausible explanation for the increased embryo mortality in these cases.

High percentages of triploid yellowtail flounder were still observed over time following larval rearing (experiment 2) and long-term juvenile grow-out (experiment 3). These results indicate that proportions of triploids determined on larvae after yolk-sac absorption adequately reflected the percentage of triploid fish surviving prolonged rearing stages.

4.4.2. Experiment 2: The effect of triploidy on larval growth and survival..

Initial efforts in 1997 (experiment 3) suggested that the larval rearing phase of triploids may be prolonged. This prompted the following question for triploid yellowtail flounder: are larval rates of growth and development affected by the triploid condition itself, or by the method of triploidization, namely, the exposure of eggs to hydrostatic pressure? The results of experiment 2 in 1998 showed that rates of development were similar between larvae from 7 000 psi (high % of 3N), 5 000 psi (low initial % of 3N) and sham control 0 psi (all 2N) treatments. All three groups showed the same proportion of metamorphic individuals and degree of settling behaviour. In contrast, the growth rate of 7 000 psi triploid larvae became significantly slower than the rates seen for mainly diploid larvae of the 5 000 and 0 psi treatment groups between day 36 and 76. These results for triploid larval growth should

be viewed as preliminary until future experimentation with replication can be performed in order to remove potential tank effects. Nevertheless, the overlapping curves of all three groups prior to day 36, and those of 5 000 and 0 psi groups after day 36, suggest that tank effects were minimal on growth performance. That 5 000 psi and 0 psi larvae had similar growth patterns demonstrates that pressure treatment at the threshold for inducing triploidy did not affect larval growth; therefore, growth decreases in 7 000 psi larvae appear to be due to the triploid condition.

Slower growth in triploid larvae following day 36 was clearly associated with periods of significant developmental change involving notochord flexion and metamorphosis. Triploid yellowtail larvae may be challenged in their capacity to attain the same growth rates demonstrated by diploids during these periods. The complexity of eye migration peculiar to flatfish metamorphosis may pose an additional difficulty for triploids. A significant environmental change also occurred following day 36 with the introduction of enriched *Artemia*. More information on the larval physiology of this species is required in order to determine what factors (respiratory, haematological, endocrine) may be contributing to growth decreases in triploids, particularly leading up to metamorphosis.

Some negative effects of triploidy on early growth or development have been noted in salmonids. Johnstone et al. (1991) reported that triploid Atlantic salmon (*Salmo salar*) required a longer period for development to first-feeding than diploids. Conversely, most studies, including the present study, report similar developmental rates for diploids and triploids (Benfey, 1999). Regarding early growth, Jungalwalla (1991) indicated that triploid Atlantic salmon fry demonstrate reduced growth and feed acceptance over the first two to

three months of feeding. Similarly, O'Flynn et al. (1997) found that triploid Atlantic salmon fry were repeatedly smaller than diploids among different year classes. Although slower growth rates were observed during the fry stage, compensatory growth at a later age was reported for triploid Atlantic salmon by both Jungalwalla (1991) and O'Flynn et al. (1997).

4.4.3. Experiment 3: Effects of triploidy on sexual maturation in yellowtail flounder.

4.4.3.1. Juvenile growth.

While reports vary, most studies indicate that the growth performance of juvenile triploids is comparable or inferior to the performance of immature diploids (Purdom, 1972; Benfey & Sutterlin, 1984; Lincoln & Scott, 1984; Benfey et al., 1989b; Johnstone et al.,1991; Galbreath et al., 1994; Hussain et al., 1995; Felip et al., 1997; O'Keefe & Benfey, 1999). However, a higher growth performance for triploids relative to diploids has been reported to occur when diploids undergo sexual maturation and spawning. Constant growth rates exhibited by triploids during periods of reproductive activity, when diploids show slower growth, give triploids a growth advantage.

In the present study, triploid juveniles exhibited particularly poor growth as 0+ animals. Results from experiment 2 would suggest that poor early growth was related to the triploid condition. However, the small body size exhibited by triploids at the end of their first year may have been influenced by other factors. These factors include a late hatch-date in September and decreasing water temperatures which prolonged the larval rearing period. Normally, larvae reared under general culture conditions have an earlier hatch-date during July or August, and experience high temperature conditions which promote larval growth and earlier weaning onto formulated feeds. The small tanks used to rear the present group of

triploids during the post-metamorphic stage may have had an additional negative impact on early growth patterns compared to those of diploids reared in large tanks under general culture conditions.

Evaluating the growth performance of triploid yellowtail flounder at a later age showed that two year old triploid females had growth rates statistically similar to those of a group of immature 1+ diploid females which previously had exhibited fast underyearling growth. However, growth for triploid females fell short of the high growth rates exhibited by a group of immature 1+ diploids compensating for a prior slow growth record. Notably, triploid growth in both length and weight clearly surpassed growth rates seen in early maturing 1+ diploid females with a previous fast growth history. Thus, triploidy in older yellowtail flounder permitted a growth performance which was better than that of early maturing fish, and approached growth rates seen in immature diploids. According to Lincoln (1981a), mature diploid plaice x flounder hybrids showed compensatory growth following ovulatory activity, such that, ultimately, there was little difference between diploids and triploids at the end of the experiment. In the present study, compensatory increases in mature diploids in autumn were insufficient to reach the same body size as triploids. Triploid females outgrew mature diploid individuals during the protracted period of ovulatory activity demonstrated by this batch-spawning species.

For diploid yellowtail flounder, significant differences in body size were established between males and females by 20 - 22 months of age, with females showing superior growth (Manning et al., chap. 3). This period coincided with full maturity in diploid males. For triploids of the present study, a divergence between male and female growth patterns

although visually evident was less distinct statistically. Only for the final measurement at 33 months of age was a statistical difference in body size detected, with females again being the larger sex. As reported for diploids, the tendency for triploid male growth patterns to diverge from those of triploid females was associated with testicular maturation. Milt was detected for the first time at 30 to 33 months of age, a year later than in diploid males which would be in the spermiation phase of their second reproductive cycle at this time (Manning et al., chap. 2). The delay in testicular development seen in the present group of triploids could be due to either one of two factors: a poor growth rate during the first year of life, or an effect of triploidy. Further efforts in rearing triploids should be performed in order to determine if maturity in males is in fact delayed by triploidy.

4.4.3.2. Male gonadal development.

According to studies in other species, triploidy in males does not prevent the physiological maturation of the testes. Triploids frequently produce spermatozoa and have testicular androgen levels similar to those detected in diploids (Lincoln & Scott, 1984; Benfey et al., 1989b; Malison et al., 1993; Hussain et al., 1995). However, the production of spermatozoa relative to diploid males is limited, which results in a dilute milt. Additionally, triploids may exhibit reduced testicular size and delays in spermatogenic cycles (Benfey & Sutterlin, 1984; Lincoln & Scott, 1984; Benfey et al., 1986; Benfey et al., 1989b; Malison et al., 1993; Hussain et al., 1995; Benfey, 1999). The results for triploid male yellowtail flounder in the present study were no exception to the general pattern seen among other species: testes displayed steroidogenic competence, yet very limited testicular growth and spermatogenic development.

As a result of limited sperm production, the testes of triploid yellowtail appeared regressed even though males were sacrificed in July, during the main spawning period in captivity. A degeneration of meiotic tissue and/or a blockade on the advancement of meiotic cells into spermiogenesis have been either proposed, or shown to explain low sperm production in triploids (Swarup, 1957 cited in Lincoln & Scott, 1984; Lincoln, 1981b; Benfey & Sutterlin, 1984). In the present group of males, two individuals demonstrated evidence of meiotic activity and a degeneration of meiotic tissue. Other males in which meiotic tissue was not detected had abnormal cells resembling spermatids in the lobular spaces of the testes. These observations suggest that low sperm yield in triploid male yellowtail flounder was a result of reproductive dysfunction involving the premature degeneration of spermatogenic cysts and release of meiotic cells. Areas of intact spermatogonial tissue, seen in some males, may be further evidence of reproductive dysfunction in triploid yellowtail. These areas may represent tissue in which meiotic division was not initiated during the previous cycle. Alternatively, the prevalence of spermatogonial tissue in certain males may indicate that spermatogonial proliferation had been renewed early, perhaps as a result of low amounts of spermatozoa within the testes. The detection of mitotic cells in two males of the present group supports the latter proposal.

Milt from triploid yellowtail flounder contained few or no motile spermatozoa, and included a variety of other cells. These cells were likely the degenerative meiotic cells and phagocytes noted in the testes and sperm ducts. Milt of similar description has been reported for triploid plaice x flounder hybrids (Lincoln, 1981b). Spermatozoa seen in triploid yellowtail flounder had larger heads than sperm cells from diploid males. This has been

observed and quantified in triploid rainbow trout and European plaice (*P. platessa*), and is probably linked to a higher DNA content associated with the aneuploid condition of the spermatozoa (Lincoln, 1981b; Lincoln & Scott, 1984).

Collections of milt from triploid yellowtail flounder demonstrated a low fertilization success. Most larvae produced from fertilization trials with triploid milt were abnormal and non-viable. This would be consistent with reports that spermatozoa from triploids are functionally sterile, that is they are capable of fertilization but yield non-viable aneuploid progeny (Lincoln 1981b; Lincoln & Scott, 1984; Benfey et al., 1986). Although a high mortality was seen among larvae in the present study, three larvae of normal appearance were seen to survive to the final stages of yolk-sac absorption. These normal larvae may have represented viable individuals (since only three larvae were found, survival through larval development was not tested). Studies have shown that triploids of some species are capable of producing euploid spermatozoa or even viable euploid offspring (Van Eenennaam et al., 1990; Kawamura et al. 1995). The potential for the production of euploid spermatozoa in triploids, together with the present findings of normal appearing larvae in yellowtail, caution that functional sterility may not be guaranteed in triploid males of this species. In aquaculture, functional sterility in triploids is important if there are concerns that captive populations may genetically contaminate local native populations, particularly in the context of transgenic animals. For yellowtail flounder, poor embryo survival compounded with low fertility and low sperm production dramatically decreases the likelihood of the production of viable larvae from crosses between triploid males and diploid females. Should milt production and quality improve in triploid yellowtail flounder with age, the probability of the production of viable

larvae could increase. Increased milt yield and fertility could be possible in triploid yellowtail given that triploid European plaice, another pleuronectid species, produced highly motile milt of moderate fertility, and showed no evidence of meiotic cell degeneration within the testes (Lincoln, 1981b).

As triploidy is generally less effective in suppressing gonadal maturation in males than it is in females, the production of all-female triploids has been proposed to be more useful for aquaculture situations. In yellowtail flounder, the decreased growth which accompanies male maturity supports this view. Given the low level of testicular development in triploid yellowtail flounder, growth decreases seen during male maturation were more likely associated with endocrine maturity rather than an energetic investment into reproduction. This effect of maturation on male growth patterns seems sufficient cause to focus future efforts on triploid females of this species.

4.4.3.3. Female gonadal development.

Although most three year old triploid females had vitellogenic oocytes, and two females had actually ovulated, triploidy was successful in suppressing ovarian growth by limiting the number of oocytes in the ovary. Oocytes were interspersed within oogonial tissue, which agrees with the general description for triploids of other species (Purdom, 1972; Benfey & Sutterlin, 1984; Malison et al., 1993; Hussain et al., 1995). The suppressive effect of the triploid condition on the amount of oocytes present in yellowtail flounder ovaries was individually variable. Some studies indicate that the number of oocytes in the ovaries of triploids increases with time, even in cases where entry into meiosis had been completely suppressed for a period of years (Lincoln, 1981c; Lincoln & Scott, 1984; Hussain et al.,

1995).

According to the ovarian rank data, ovarian growth in triploid yellowtail flounder became evident only at two years of age, which suggests that the presence of oocytes was limited until that age. Using ovarian ranks to follow triploid ovarian development permitted the detection of ovarian growth, and helped discern the fore-running females which ovulated from the rest of the group. However, unlike diploids, vitellogenic development was not obvious in triploid yellowtail flounder. Vitellogenic ovaries, sampled in October, 2000 at 37 months and in January, 2001 at 40 months of age, frequently appeared immature or had ranks equivalent to those of diploid ovaries at the initiation of puberty. Even the ovaries of ovulating females appeared relatively undeveloped (OR=3) until June, 2000 (33 months) when a sudden increase in length was seen - this was probably due to distension following the production of ovarian fluid. Given these observations it seems possible that any ovarian growth detected in triploids may represent vitellogenic activity. Ovarian growth which was detected in more than two females following 24.5 months of age could have represented an increase in the numbers of previtellogenic oocytes, or it may have indicated pubertal development. In the two ovulating females, it was clearly the latter. That no evidence of residual eggs was seen for the remaining females does not preclude the possibility that an abortive or anovulatory pubertal cycle may have occurred in some females prior to three years of age. Among the triploid literature, abortive vitellogenic oocytes were seen in five year old plaice x flounder hybrids (Lincoln, 1981c). In triploid rainbow trout, vitellogenic development occurred at three years of age, but did not result in ovulation (Kobayashi et al., 1998).

The capacity for triploids to mature and reach ovulation has been previously reported by other investigators (Johnstone et al., 1991; Benfey, 1995; Brämick et al., 1995; also reviewed in Benfey, 1999). Studies in which eggs ovulated from triploids were collected and fertilized with normal haploid spermatozoa have shown that the resulting embryos were non-viable (Johnstone et al., 1991; Benfey, 1995; Brämick et al., 1995). Benfey (1995) observed that vitellogenic oocytes in the ovaries of a triploid brook trout female, *Salvelinus fontinalis*, demonstrated abnormal asynchronous development, and that ovulated eggs in the lumen were variable in size. Similarly, Johnstone et al. (1991) noted a high variability in the size of ovulated eggs produced by triploid Atlantic salmon. Although the October, 2000 sample for females in the present study was during early vitellogenesis, and not near ovulation, oocyte development in more developed (VG-III stage) vitellogenic females resembled the group synchronous pattern typical for yellowtail flounder.

Despite clear evidence of ovarian maturation in most females, mean GSI and plasma hormone levels for triploids in the present study were less than values in pubertal diploids at similar times of the year. Moreover, observations for ovulating triploids indicated that even at full maturity the ovaries of triploid yellowtail attained a fraction of the development observed in diploids. According to Manning et al. (chap. 2), ovarian development in diploids may account for up to 28% of the body weight at full maturity. Full maturity in diploid yellowtail may occur at 22 or 34 months of age, with a very few individuals initiating puberty later as three year old fish (Manning et al., chap. 2). In contrast, only two triploid females successfully reached full maturity (i.e. final oocyte maturation and ovulation) by 34 months of age. The majority of the remaining females seemed likely to reach full maturity by 45 to

48 months of age, but at a much lower cost than diploids in terms of gonadal growth.

Plasma 17ß-estradiol levels in triploids were clearly sufficient to stimulate vitellogenesis, even in cases where the number of oocytes present in the ovary was very low. All females had 17ß-estradiol levels similar to those reported by Manning et al. (chap. 2) for diploid females at the initiation of puberty and early vitellogenesis (up to VG-II oocytes). Regarding testosterone, which was detectable in all females, a higher presence was noted in triploids than is usually seen in diploids during early vitellogenesis (Manning et al., chap. 2). In triploid females still in the cortical alveolar oocyte stage, levels of 17ß-estradiol in the pubertal range may indicate that these individuals soon would have become vitellogenic. According to other reports for triploid females, sex steroid levels remain low or undetectable, but increase as oocytes appear or become more numerous (Lincoln & Scott, 1984; Benfey et al., 1989b; Hussain et al., 1995). It has been suggested that a critical number of oocytes is required in order to produce a threshold level of 17ß-estradiol for the induction of hepatic vitellogenesis (Benfey et al., 1989a; Hussain et al., 1995). As vitellogenesis was underway in triploid female yellowtail, including some with very few oocytes, the 17ß-estradiol threshold for stimulating vitellogenesis in this species may be low.

Beyond vitellogenesis, the detection of ovulation in two females was indicative of a full activation of the gonadotropin-releasing hormone (GnRH) system in the brain and an adequate production of gonadotropin(s) by the pituitary. In sockeye salmon, *Oncorhynchus nerka*, triploid females with ovaries containing only oogonia had lower pituitary and hypothalamic levels of GnRH compared to immature diploids with previtellogenic oocytes (Amano et al., 1998). The appearance of sufficient numbers of oocytes with steroidogenically

competent follicles is likely a requirement for the activation of the GnRH system and the promotion of gonadotropin release in triploids; positive feedback action by gonadal steroids being the main mediating mechanism for the activation (Breton & Sambroni, 1996).

In vitro incubation results demonstrated that the ovarian tissue of triploids was clearly responsive to both adenylate cyclase activation by forskolin and heterologous gonadotropin stimulation with crude salmon pituitary extract. A trend for *in vitro* 17ß-estradiol production to exceed *in vivo* plasma levels was seen in vitellogenic triploid females. For diploids in the autumnal period of vitellogenesis (October, December), levels of *in vitro* steroid production in response to stimulatory agents were similar to *in vivo* plasma levels (approximately 2 ng/ml) (Manning et al., chap. 2). In contrast, triploids, similarly sampled in October, had equivalent to higher steroidal output *in vitro,* despite a reduced number of vitellogenic oocytes in the ovaries. This was noted particularly in the four pubertal females whose tissue was sufficiently upregulated that even levels in control wells exceeded levels in the plasma. Peak *in vitro* levels reached 5.5 ng/ml in one pubertal triploid, which matched *in vitro* values of prespawning diploids in April (Manning et al., chap. 2). The lack of agreement between plasma levels and *in vitro* steroid production in pubertal vitellogenic triploids suggests that either gonadotropin levels are low or there is a factor suppressing 17ß-estradiol output *in vivo*. While GtH levels may in fact be low, the removal of an *in vivo* inhibitory factor on steroidogenesis could explain the high *in vitro* steroidal output from pubertal tissue in control wells.

4.5. SUMMARY.

A high proportion of triploid yellowtail flounder was obtained using hydrostatic pressure shocks to induce the retention of the second polar body post-fertilization. A ten minute treatment of 7 000 psi, initiated at five minutes post-sperm activation (7-12°C) , is recommended for inducing triploidy in this species. Preliminary evidence suggests that larval triploids exhibit growth disadvantages during metamorphosis but develop at the same rate as diploids. Comparisons at a later age showed that two-year-old triploid females had growth rates approaching those of immature diploid females while exceeding those of maturing diploid females. Triploidy was effective in minimizing gonadal development in yellowtail flounder, but permitted physiological maturation in both males and females. Males produced limited amounts of spermatozoa of poor motility and low fertility. However, a few larvae of normal appearance hatched from fertilized eggs which may have been viable over the long-term. Two females ovulated at 34 months of age, and a high proportion of three year old pubertal females with vitellogenic oocytes were likely to become fully mature at 45 months of age. Although ovarian development in triploids was low in comparison to diploids, tissue of pubertal individuals showed a high steroidogenic competence *in vitro*.

Decreased growth rates during metamorphosis are a disadvantage for the culture of triploid yellowtail. It should be noted that rearing conditions available for the present study were not optimal for long-term larval rearing; thus, growth rates of triploid larvae presented here may not represent the growth potential of larvae grown in general larval rearing conditions. Further experimentation is needed to examine this aspect of triploid biology in this species. Developmental rates were similar between triploids and diploids; hence larval

rearing of triploids should not take longer than the time required for diploids. The value of triploidy in yellowtail flounder was shown in its minimizing effect on ovarian development in females. Since triploid males developed slower growth even with the limited testicular maturation seen in the present study, females should be the focus of further investigation into the development of triploid yellowtail flounder for aquaculture. Higher growth rates of triploid females compared to maturing diploids make triploid females even more attractive given that the yellowtail flounder is prone to early sexual maturation in culture.

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Appendix 4A.

Flow cytometry methodology.

Protocol based on Blacklidge & Bidwell (1993a).

Larvae were anaesthetized in a 1- 2.5% (v/v) 2-phenoxyethanol / ACD (acid citrate dextrose, see below) solution kept on ice. Individual larvae were transferred to a 1.5 ml Eppendorf tube and the volume of ACD solution reduced to ~50 µl by aspirating excess solution with a syringe. For pooled samples the larvae were added to the Eppendorf tube and then centrifuged to force the larvae into a clump in the bottom of the tube. The ACD solution was then reduced to a small volume (~50 µl). A hand homogenizer designed for Eppendorf tubes was then used with gentle pressure to make the larval mash, enough pressure to cause a smear of pigment from the eyes on the side of the tube being sufficient. A volume of 600 µl of ACD was added to the tube and the volume slowly aspirated twice through a 23 gauge hypodermic needle and then once through a 26 hypodermic gauge needle to help liberate cells. The sample was passed through a 32 µm nylon mesh filter draining into another 1.5 ml Eppendorf tube, and the filter rinsed with an additional 200 µl of ACD solution. All solutions and samples were kept on ice. A volume of 750 µl of the sample was transferred to a polyethylene test tube and an equal volume of Vindelov's propidium iodide solution (see below) added to the test tube and left overnight in a refrigerator (4°C).

Protocol based on Blacklidge & Bidwell (1993b).

A fresh 250 µl blood sample was taken with an ACD primed, heparinized syringe and added to one ml ACD and mixed. The blood cells were concentrated either by leaving the blood sample to settle overnight in a refrigerator or by centrifuging the sample. A 25 µl

sample of concentrated, red blood cells was added to two ml of ACD and agitated with a vortex blender. A volume from this suspension was diluted 10x and a cell count done on a haemacytometer in order to determine the volume required to make a suspension of 1 x 10^6 cells / ml of phosphate buffered solution. Subsequently, 500 µl of the final suspension was pipetted to a polyethylene tube followed by 500 µl of Vindelov's propidium iodide solution and kept on ice in the dark, usually overnight.

Acid Citrate Dextrose (ACD)

480 mg citric acid, 1.32 g sodium citrate, 1.47 g glucose (dextrose) in 100 ml H_20

Vindelov's propidium iodide

121 mg Tris base, 1 mg RNAse, 5 mg propidium iodide, 0.1 ml Triton X100 in 100 ml H_20

A pH of 8.0 was obtained with additions of HCl

The solution was passed through a 0.45 µm filter after preparation and prior to use.

CHAPTER 5

Overview and Discussion.

5.1. The timing of puberty: the roles of season and growth.

Phenotypic plasticity in age and size at the onset of puberty was seen in female yellowtail flounder in culture. In contrast, cultured males invariably initiated puberty at the earliest opportunity after one year of age. An earlier age at full maturity in females in the present study was accompanied by a reduction in size, in some cases to the point where maturity could be described as precocious. The pattern illustrated by females agrees with the position of Stearns & Crandall (1984) that changes in age and size at maturity follow a plastic trajectory, in which neither age nor size is the focus of natural selection over a species' evolution. Plasticity in both traits would be an evolutionary advantage, by virtue of the fact that it would permit the individual to mature when environmental conditions favour both reproduction and survival.

It has been well established that growth rate and the age of maturation are correlated in fish, with faster growing individuals maturing earlier than slower growing conspecific individuals (Alm, 1959; Thorpe, 1986). Studies also have shown a genetic basis for the timing of maturation, including evidence that the trait for early maturity is heritable (Thorpe et al., 1983; Schreibman et al., 1986). However, according to studies by Thorpe and colleagues, the heritable characteristic is a faster overall rate of development, where growth performance and early maturation are linked (Thorpe et al., 1983; Thorpe, 1991). While genetic factors establish potential performance, environmental factors (e.g. food quality, food abundance, temperature, day length, etc..) determine whether a genetically programmed

growth capacity can be realized.

The link between growth and maturation raises a pressing question: by what mechanism does growth rate affect the physiological decision for maturation?

Thorpe (1986) presented a proposal for salmon that fish are "physiologically aware of their growth rate through their rate of acquisition of surplus energy, and hormone kinetics associated with its storage". Further, if the rate of acquisition of surplus energy exceeds a genetically predetermined level, during a certain period of the year in which environmental cues are stimulatory, then the brain-pituitary-gonadal axis (BPG axis) will be activated (Thorpe, 1986). Rowe et al. (1991) demonstrated that maturing male Atlantic salmon parr (*Salmo salar*) had accumulated a greater amount of mesenteric fat earlier in the spring than males which remained immature. In addition, they found that fasting during the spring suppressed maturation by reducing the accumulation of mesenteric fat stores. Other studies in salmonids similarly have found a link between fat accumulation and the percentage of early maturing individuals (Silverstein et al., 1998; Shearer & Swanson, 2000). Two important aspects of Thorpe's (1986) model and Rowe et al.'s (1991) results include: firstly, that there is a seasonality to when energy reserves are evaluated regarding an individual's capacity for reproduction, and, secondly, that hormonal signals reflecting growth and nutritional status affect the onset of puberty.

Regarding the question of seasonality in the present work, male yellowtail flounder initiated puberty in late summer (early September) to early autumn (October) (Chapter 2). Females showed a wider time window for the onset of puberty from June into November (Chapters 2 & 3). However, apparent differences between male and female yellowtail

flounder in the timing of pubertal onset are more likely linked to age. One-year-old fish were seen to initiate puberty in late summer (males) and in the autumn (females) implying that growth during the summer months may be particularly important for maturation at this age. Only older females approaching two years (22 months) or three years of age (34 months) initiated puberty in June or were vitellogenic in the summer months (Chapters 2 & 3). This suggests that only females of a larger size and thus greater energy storage are able to activate puberty in the spring and summer.

The June to November time window for the onset of puberty seen for cultured yellowtail flounder coincided with spawning (May to Aug/Sept) and early recrudescence in captive adults (Clearwater, 1996; Manning & Crim, 1998; and the present study, Chapter 2). Additional evidence of a seasonality in the onset of female puberty was indicated by results in chapter 3; namely, that GnRH-a treatment was able only to synchronize female puberty rather than advance it earlier than the normal period of the year. These observations suggest that the initiation of puberty in yellowtail flounder is regulated by environmental cues, specifically the same cues which stimulate spawning and recrudescence in mature individuals.

While environmental factors have long been known to regulate reproduction (review Lam, 1983), the mechanism whereby these factors affect the BPG axis has not always been clear. In both masu salmon, *Oncorhynchus masou*, and sockeye salmon, *Oncorhynchus nerka*, a short photoperiodic cue has been shown to increase GnRH levels in the brain and pituitary, as well as pituitary levels of one or both gonadotropin ß-subunits (Amano et al., 1994, 1995, 1997). Further, seasonal increases in gene expression of GtH subunits have been

noted in immature striped bass females, *Morone saxatilis*, one and/or two years prior to puberty (Hassin et al., 1999). These reports reveal evidence that the immature BPG axis can be upregulated by stimulatory environmental cues. It is during these seasonal periods that a peripheral signal communicating somatic status could provide sufficient additional upregulation to stimulate the initiation of puberty.

Hormones reflecting growth rate or energy storage, which could affect the BPG axis directly or indirectly, may include: Insulin-like growth factor-I (IGF-I), steroids produced by certain types of adipose tissue, or the hormone leptin. IGF-I is the most promising peripheral cue linking growth with reproduction in fish. As previously reviewed in chapters 1 & 3, IGF-I has been shown to exert positive effects on pituitary gonadotropin content and gonadotrope sensitivity to GnRH in fish (Huang et al., 1998, 1999; Baker et al., 1999; Weil et al., 1999a). IGF-I could be a physiologically relevant link between growth rate and the onset of puberty in yellowtail flounder. However, observations in chapter 3 suggest that energy-reserve status may be a predominant influence in the decision for puberty. Treatment of yellowtail with rbGH (Posilac®) produced significantly faster growth rates, and thus was probably effective in elevating IGF-I levels in the circulation of treated fish. Yet a significant proportion of rbGH treated females showed delays in the onset of puberty relative to control groups. This delaying effect was hypothesized to be due to a decrease in energy reserves resulting from the stimulation of somatic growth, and a potential lipolytic action by GH on lipid reserves. These findings imply that energy-reserve status is a major determining factor for the initiation of puberty, regardless of an elevated level of somatotropic hormones (rbGH and/or IGF-I).

As previously mentioned, a relationship between maturation and accumulation of fat was found for male Atlantic salmon by Rowe et al. (1991). These authors proposed that the estrogenicity of adipose tissue could establish a link between energy stores and the BPG axis, where estrogen could accelerate the development of the BPG axis via a positive feedback action. Clear evidence of an estrogenic capacity of adipose tissue has been shown recently for black carp, *Mylopharyngodon piceus* (Gur et al., 2000). In immature female black carp the gonadal fat pad was capable of producing estrogen, as well as responding to gonadotropin at puberty, but visceral fat was not estrogenic (Gur et al., 2000). A role for estrogen secretion by the gonadal fat pad in puberty was suggested for this species. With regard to yellowtail flounder, adipose tissue is present neither in the viscera nor is it associated with the gonad. Therefore, steroid production from adipose tissue may not be a physiologically-relevant mechanism for communicating energy-reserve status to the BPG axis in this species. The main lipid storage area in yellowtail flounder appears to be the liver, although lipid accumulation in hypodermal areas has been noted in winter flounder, *Pseudopleuronectes americanus*, and in pterygiophorial areas of the unpaired fins in yellowtail flounder and other flatfish (Maddock & Burton, 1994; Begg et al., 2000). While the steroidogenic capacity of adipose tissue in the carcass is unknown, evidence for female yellowtail flounder in the present study (Chapter 2) seems to indicate that ovarian tissue alone is responsible for 17ß-estradiol levels in the plasma.

Another hormone associated with adiposity, at least in mammals, is leptin (Johnson et al., 2000). The secretion of leptin by mammalian adipocytes occurs during periods of fat deposition, and shows a positive correlation with body fat levels (Cunningham et al., 1999;

Johnson et al., 2000). Leptin acts on hypothalamic regions regulating appetite to decrease food intake when adiposity is elevated (reviewed Johnson et al., 2000). Effects of leptin on puberty and the reproductive axis have been shown in a number of mammalian models, wherein leptin's role in communicating metabolic status to the brain appears to explain correlations between reproduction and critical body fat levels (Cunningham et al., 1999). The presence of a leptin-like molecule, recognized by antibodies from mammalian leptin, recently has been detected in the liver, brain, blood and heart, but not the muscle or visceral fat, of several species of fish (Johnson et al., 2000). In green sunfish, *Lepomis cyanellus*, blood levels of recognized leptin were significantly reduced in starved compared to fed individuals (Johnson et al., 2000). This information suggests that a leptin-like hormone may be present and linked with energy-reserve status in fish as is the case in mammals. Regarding a connection with reproduction, Weil et al. (1999b) have reported that high levels of recombinant human leptin increased the basal release of GtH-I and GtH-II from dispersed pituitary cells of male and female rainbow trout, *Oncorhynchus mykiss*. This activity was observed in individuals at certain, mainly reproductive, stages of development. In contrast, *in vivo* treatment with recombinant human leptin has shown no effect on reproduction in immature coho salmon, *Oncorhynchus kisutch* (Baker et al., 2000). Thus far investigation into a leptin-like molecule in fish is in a preliminary or early stage. Further study will be required to confirm the presence of leptin in fish, as well as determine its role in communicating energy-reserve/metabolic status to the brain and potentially the BPG axis.

From the present study, a high accumulation of fat is hypothesized to be a significant contributing factor to early maturation in yearling yellowtail flounder. Young yellowtail

flounder of one year of age were noted to have abnormally white livers presumably due to the use of high fat diets which were designed for salmonids. The detection of a leptin-like molecule in the liver of fish may be physiologically relevant for yellowtail flounder, flatfish and other fishes in which the liver represents a primary storage area for lipid.

Apart from an obvious endocrine model in the signaling of somatic condition, or a model involving fat reserves, a signaling system based on protein could be possible. Amino acid levels in the plasma have been shown to demonstrate changes with feeding status in winter flounder, and thus may serve as indicators of nutritional status to the brain (Burton, 1995). In this case, protein reserves and protein metabolism could be more closely monitored in relation to the decision for puberty than fat levels. A protein-based model may be very relevant for a lean-bodied flatfish like yellowtail flounder.

Whatever the mechanism may be for relaying somatic condition to the BPG axis, there are likely to be well defined physiological criteria for the onset of puberty in immature yellowtail, or recrudescence in adults. The "all-or-nothing" nature of yellowtail flounder maturation noted in the present study indicates a complete activation of the BPG axis. A partial activation of the gonadotropic axis producing incomplete pubertal gametogenetic cycles has been reported in some species (grouper, *Epinephelus aeneus*, Hassin et al., 1997; striped bass, Holland et al., 2000).

5.2. Gonadal physiology at the onset of puberty in yellowtail flounder.

In male yellowtail flounder, testes at the onset of puberty were characterized by a novel growth phase in which mitotic proliferation of spermatogonia and entry into meiosis were concurrent activities. Endocrine puberty clearly was associated with testes of this

description which suggests that androgens are linked with both mitosis and meiosis at the onset of puberty. However, at very early stages of puberty, when testes were small and primary spermatocytes few in number, androgens were either low or undetectable in the plasma. While androgens appeared to be correlated with meiotic activity, a lack of a discrete mitotic phase, and a low testicular mass at the onset of puberty, make it unclear whether androgens also regulate mitotic division in pubertal male yellowtail. In order to determine conclusively whether mitotic activity is linked with endocrine puberty in this species, the measurement of intratesticular androgen levels seems necessary. Examining the data obtained from older mature male yellowtail revealed that androgen levels were elevated during early recrudescence. Spermatogonial proliferation was the primary testicular activity in these early recrudescent males, although a low number of spermatocytes indicated the reinitiation of meiosis (Chapter 2). Furthermore, in mature triploid males androgen levels between 0.9-2 ng/ml were seen in individuals exhibiting regressed testes in which mitosis for some cases was detected as well (Chapter 4). As mature males appear to have a discrete period of spermatogonial mitosis that is lacking in pubertal males, clearer links between androgens and mitotic activity may be observed in post-pubertal males.

In female yellowtail flounder, an activation of the BPG axis was detected by an increase in 17ß-estradiol output during the cortical alveolar oocyte stage (Chapter 2). Since the number of females in certain ovarian histological stages (early vitellogenic VG-I and VG-II) was low in chapter 2, data from females in chapters 2 and 3 were pooled and analysed to verify certain trends. Only females from control and rbGH treatments were included from chapter 3 while females receiving reproductive hormones were excluded. Including rbGH

treated females was justified as these females showed steroid levels equivalent to those of control females in similar ovarian histological stages. The endocrine data for immature females, cortical alveolar stage females and early vitellogenic females are shown with data from October sampled vitellogenic triploid females (Figure 5.1). Statistical analysis of the data confirmed results in chapter 2 showing that:

a) Cortical alveolar females had higher mean plasma levels of 17ß-estradiol than mean levels seen in newly vitellogenic females (VG-I stage: peripheral yolk globules) ($P<0.05$).

b) Levels of 17ß-estradiol production *in vitro* were not statistically different between cortical alveolar stage females and females with VG-I oocytes ($P=0.75$).

As *in vitro* levels of steroid output did not differ between the two stages, higher plasma levels detected in cortical alveolar stage females were likely due to higher gonadotropin levels in the circulation. Thus, it seems cortical alveolar stage females were sampled at a time when a pulse of gonadotropin was secreted with the pubertal activation of the BPG axis. Plasma levels were lower during the VG-I stage as new basal levels of GtH secretion would be established with time. A gonadotropin surge at the onset of puberty, in addition to reflecting a release of stored gonadotropin from the pituitary, may be necessary to stimulate hepatic vitellogenin synthesis. It is interesting that cortical alveolar stage females had statistically similar plasma 17ß-estradiol levels to females in a later stage of early vitellogenesis (VG-II: Figure 5.1) when the ovary demonstrated a greater state of upregulation in response to gonadotropic stimulation *in vitro*.

Estradiol-17ß was the dominant hormone compared to testosterone in females during puberty (Figure 5.1). Testosterone was not detected in females during the primary growth

phase, but was detectable at low levels in 50% of the females in the cortical alveolar stage and 33% of VG-I stage females at the beginning of vitellogenesis (Figure 5.1). Low levels of testosterone were detected in the majority of females with subsequent vitellogenesis. A dominance of 17ß-estradiol similarly was seen in triploid yellowtail, however, triploids had a higher mean testosterone to mean 17ß-estradiol ratio than diploids.

Triploid females demonstrated some notable differences from diploids during puberty. In contrast to diploids, triploid females in pubertal vitellogenesis (VG-II & VG-III stages) exhibited a high steroidogenic capacity *in vitro* which was not reflected *in vivo*. Plasma levels of 17ß-estradiol even in females with more advanced VG-III oocytes remained much lower (<0.8 ng/ml) than similarly staged diploids (Chapter 4; see Figure 5.1). This difference between *in vivo* and *in vitro* steroid performance suggests that plasma GtH levels may be subdued in triploids, possibly due to a lesser activation of the BPG axis during early puberty. However, what was most striking regarding triploid females was that their ovaries were sufficiently steroidogenic to induce and support vitellogenesis despite the low numbers of oocytes found in the tissue. These observations lead to the hypothesis that the tendency for early maturity in female yellowtail flounder may be related to the fact that high levels of 17ß-estradiol or large amounts of ovarian tissue are not required for vitellogenesis. Another factor supporting the idea that females have a low threshold for puberty may include the early age at which immature ovaries were steroidogenically competent and able to respond to gonadotropic stimulation (diploids 13.5-14 months, Chapter 2).

Figure 5.1. Mean plasma steroid levels and mean *in vitro* 17ß-estradiol production levels from ovarian tissue of diploid females in different oocyte stages prior to and during early puberty. Comparison plots for vitellogenic triploid females are shown.

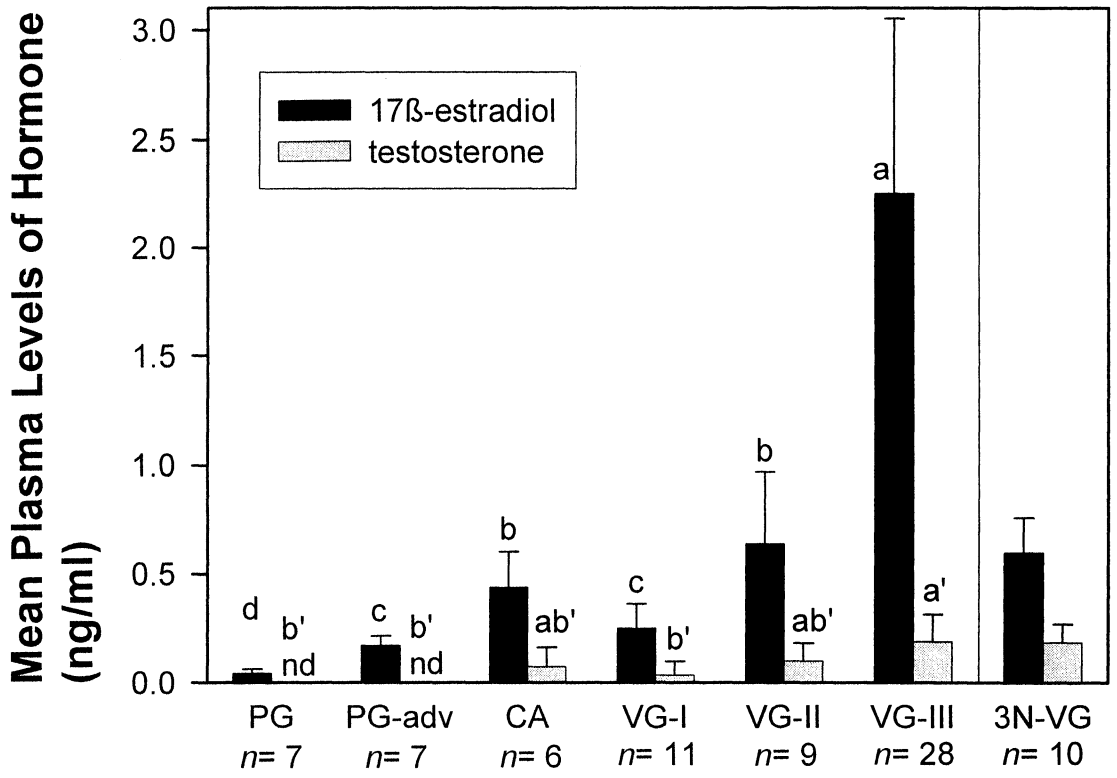
Upper plot- Mean (±SD) plasma levels of 17ß-estradiol and testosterone are plotted for diploid and for vitellogenic triploid females. Vitellogenic diploids were sampled June-December (22 to 28 mo.) while vitellogenic triploids were sampled in October (37 mo.). Statistically significant stage differences were found for each hormone ($P<0.0001$). Means which are labeled with the same letter are not significantly different ($P>0.05$). Letters followed by an apostrophe refer to statistical analysis for testosterone.

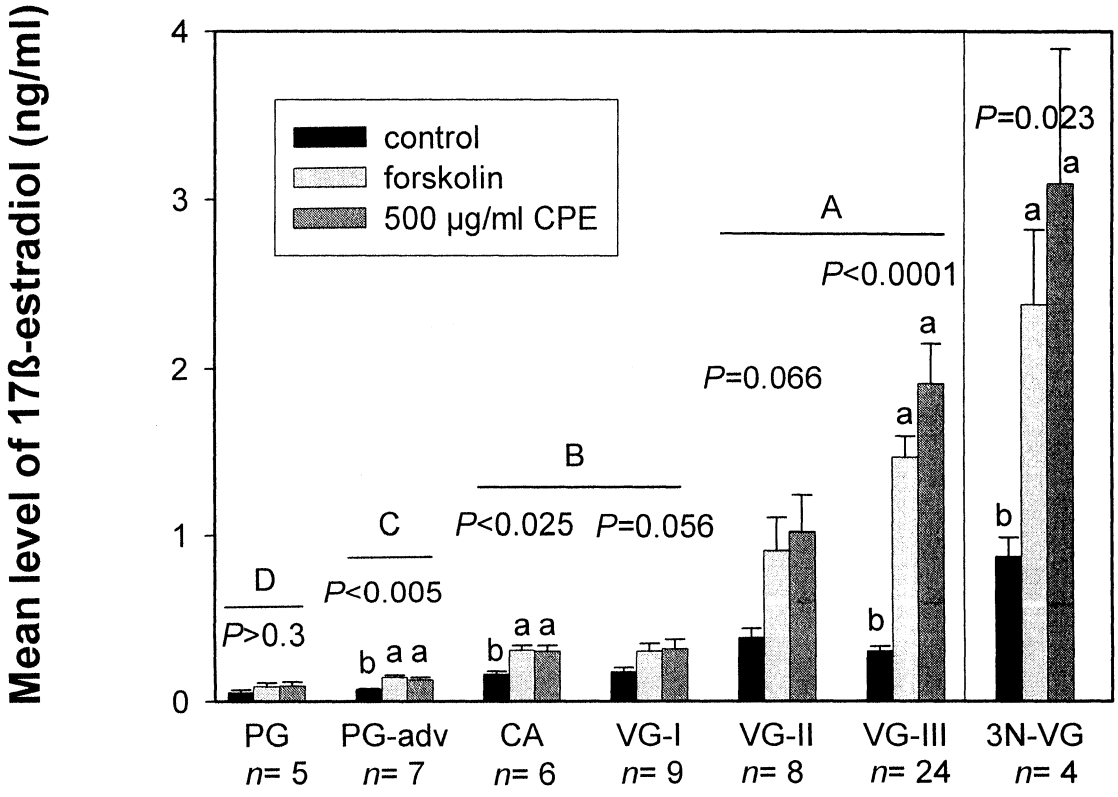
Lower plot- Mean (±SE) *in vitro* 17ß-estradiol production levels for ovarian tissue incubated in control, forskolin, and crude pituitary extract (CPE: 500 μg/ml) media are plotted for groups of females in different oocyte stages. Triploid females demonstrating pubertal vitellogenesis are shown for comparison.

Lower case letters indicate within group statistical comparisons of mean 17ß-estradiol output among incubation treatments. Upper case letters represent statistical comparisons of overall 17ß-estradiol output among different groups of diploid females (overall stage effect: $P<0.0001$). Data labeled with the same letter are not significantly different ($P>0.05$).

n= number of females represented; **nd**= non-detectable

PG= primary growth stage; **PG-Adv**= advanced primary growth; **CA**= Cortical alveolar stage; **VG**= vitellogenic stages I, II, III ; **3N-VG**= vitellogenic triploids.


Mean Plasma Levels of Hormone (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
17ß-estradiol
testosterone
d
b'
nd
c
b'
nd
b
ab'
c
b'
b
ab'
a
a'
PG
n= 7
PG-adv
n= 7
CA
n= 6
VG-I
n= 11
VG-II
n= 9
VG-III
n= 28
3N-VG
n= 10


Mean level of 17ß-estradiol (ng/ml)
4
3
2
1
0
control
forskolin
500 µg/ml CPE
D
P>0.3
C
P<0.005
B
P<0.025
P=0.056
A
P<0.0001
P=0.066
P=0.023
b
a a
b
a a
b
a
a
b
a
a
PG
n= 5
PG-adv
n= 7
CA
n= 6
VG-I
n= 9
VG-II
n= 8
VG-III
n= 24
3N-VG
n= 4

5.3. Steroids and positive feedback?

Positive feedback effects of testosterone on the immature BPG axis have been reported frequently in teleosts (Dufour et al., 1999; reviewed in Chapters 1 and 3). No positive feedback effects were noted in the present study, instead testosterone administration, with or without GnRH-a, disrupted the onset of puberty in females, recrudescence in males and suppressed growth in both sexes (Chapter 3). These negative effects were proposed to be due to high levels of testosterone. Early gametogenesis in both sexes appeared to be particularly affected while spermiation in pubertal developing males and ovulation in a few females was unimpeded. Physiologically, high levels of testosterone, which are detected in final stages of gametogenesis in this species (Chapter 2), may prevent the initiation of new gametogenetic cycles during spawning when gonadotropin levels are high and seasonal environmental factors stimulatory.

High testosterone levels additionally may act as a signal to deter somatic growth during periods of final gamete maturation. Besides the observed effects of testosterone treatment, sex differences in body size became statistically significant when high androgen levels were detected during prespawning and early spawning periods in full spermiating males (Chapter 3). The fact that triploid male yellowtail flounder also developed slower growth with maturation was suggestive that endocrine maturity, rather than energetics involved in gonadal growth, was responsible for the growth divergence from females. This suggestion was based on the findings that while testes were capable of producing significant amounts of androgens, their testicular development was particularly low (Chapter 4).

In order to demonstrate a positive feedback role for testosterone in females one might have to mimic levels seen at puberty. An increased proportion of females with detectable testosterone at the cortical alveolar oocyte stage may indicate a potential positive feedback signal during the initiation of puberty. On the other hand, positive feedback signals in immature females may be provided by 17ß-estradiol as this was the dominant hormone at puberty in yellowtail. A selective response to estrogen was seen in immature female European eel where exogenous 17ß-estradiol treatment increased mGnRH and pituitary GtH-II levels *in vivo*, while testosterone was ineffective (Dufour et al., 1983; Montero et al., 1995). Conversely, immature male eel showed strong increases in pituitary GtH-II levels in response to both testosterone and 17ß-estradiol (Dufour et al., 1983).

Regarding the present results in male yellowtail flounder, testosterone was again the less dominant hormone, but generally was present with 11-KT during its rise at puberty and in recrudescence. As noted above, high levels of exogenous testosterone were seen to disrupt meiotic activity in recrudescing males in their second spermatogenic cycle (Chapter 3). Interestingly in Atlantic salmon, testosterone exerted negative feedback on GtH-I levels of castrated males during periods of early recrudescence, but had a stimulatory effect during the regular spawning period (Borg et al., 1998). Whether testosterone has positive actions on the immature BPG axis in male yellowtail flounder remains unknown. In some species, 11-ketoandrogens have been reported to have positive feedback effects on male reproduction. In mature male Atlantic salmon parr which had been castrated following their first spawning, 11-ketoandrostenedione increased pituitary and plasma levels of GtH-I (Borg et al., 1998). In addition, a stimulatory effect of 11-ketotestosterone on GtH-α and GtH-IIß gene

expression has been reported in African catfish (Rebers et al., 1997). A positive feedback action by 11-ketotestosterone could be possible in yellowtail flounder given its dominance in the plasma during pubertal and post-pubertal spermatogenesis.

5.4. Practical aspects for aquaculture.

The yellowtail flounder has shown promise as a candidate species for cold-water, marine aquaculture. Efforts at the Ocean Sciences Centre have shown that rearing is not complicated and high numbers of larvae and juveniles may be produced. While early maturity is a concern, with appropriate management the timing of puberty could be manipulated. Early maturity is a frequent problem for many species in culture and different approaches may be taken to reduce its prevalence. One approach for yellowtail flounder could be selective breeding. Evidence in the present work has shown that there are some female phenotypes which delay the onset of puberty until three years of age in culture. The development of selected strains of later maturing females with good growth has yet to be attempted. Focusing on all-female populations, diploid or triploid, is suggested as females showed faster growth rates than maturing males, and only cultured females demonstrated a plasticity in the timing of puberty (Chapters 2, 3 & 4).

All-female populations could be produced either by 17ß-estradiol treatment at sex differentiation or by gynogenesis. Inducing gynogenesis could be accomplished by combining pressure shock treatments (as per the treatment protocols already determined in the present work for inducing triploidy (Chapter 4)) with a UV irradiation protocol for the destruction of sperm DNA. The spermatozoa remain capable of egg activation although UV irradiation negates the contribution of paternal DNA to the zygote genome. A pressure shock retains the

second polar body, thus producing a viable diploid condition. However, this method of gynogen production is successful only when genetic sex determination for females is homogametic (XX female homogametic sex, XY male heterogametic sex). In species where females are the heterogametic sex, the use of estrogen exposure during sex differentiation would be an effective alternative to obtain all-female diploid populations with the option of producing all-female triploid populations.

The value of inducing triploidy in yellowtail flounder was seen in its clear minimizing effect on gonadal development. However, triploidy did not prevent maturation from proceeding to final stages of gametogenesis in either sex by three or four years of age (Chapter 4). The fact that triploid yellowtail flounder of both sexes completed gametogenesis indicates that further study is needed to verify the functional sterility of their gametes. Other studies have reported not only the production of gametes in triploids, but that in certain species these gametes (spermatozoa or eggs) can be euploid and capable of yielding viable progeny (reviewed in Benfey, 1999). In some situations, triploidization is used as a sterilization measure to protect the genetic integrity of wild fish populations should culture variants of the same species escape and intermingle with their wild counterparts. Cultured variants which could be of concern would include genotypes arising from selective breeding or transgenic manipulation. Triploidization similarly has been employed to sterilize fish, of non-endemic species, which are introduced into ecosystems as biological control agents and whose reproduction must be regulated. The fact that triploidy does not always guarantee functional sterility should serve as a warning to managers who depend on the sterilization aspect of triploidy, particularly if the long-term effectiveness of triploidy has not been tested

in long-lived iteroparous species.

Thus far for yellowtail flounder, milt collected from triploid males showed poor motility and low fertility *in vitro*, but the production of a few larvae of normal appearance which survived to yolk-sac absorption suggests that functional sterility may not be assured (Chapter 4). In future studies, the ploidy and survival of larvae from crosses between diploid females and triploid males should be verified. The potential of triploid males to produce higher numbers of spermatozoa with age, and the ploidy of these spermatozoa should be examined as well. At some point an effort to determine the ploidy of vitellogenic oocytes and ovulated eggs will be required. Currently, the functional sterility of triploids is not a pressing issue regarding the culture potential of yellowtail flounder. Culture systems for this species have remained land-based given that the health of yellowtail flounder depends on water temperatures below 13˚C and buffering against decreases in water temperature associated with upwelling events. The current focus regarding the application of triploidy in cultured yellowtail flounder is its use as a maturation deterrent. In this regard triploidy was effective in minimizing gonadal growth which is particularly beneficial as it decreases the amount of energy diverted to reproduction.

Whether rearing diploids or triploids, early maturity may be controlled best by managing adiposity in combination with selected breeding. In addition to fatty livers found in cultured flounder of one year of age, signs of an accumulation of fat along the dorsal and ventral fin margins (pterygiophores) has been detected in newly metamorphosed juveniles. Hence, adiposity should be regulated long-term, starting with the introduction of low fat diets when weaning fish off live feed. Further measures to reduce excess energy stores, such as

food restriction or rbGH treatment, can be imposed prior to periods of pubertal activation. Growth hormone treatment is probably impractical on the long-term, but could be used on the short-term to induce lipolysis of fat reserves and to direct energy stores toward somatic growth rather than reproduction. In fish, poor food quality, food deprivation and over-feeding of high energy diets promote a condition in which the liver becomes refractory to GH stimulation; this leads to low IGF-I levels and high GH levels in the plasma (Perez-Sanchez & Le Bail, 1999; Perez-Sanchez, 2000). In the case of overfeeding, these changes represent an endocrine mechanism to respond to adiposity at the expense of growth performance. These findings stress the importance of optimizing diets for the requirements of cultured species.

5.5. Summary.

In summary, yellowtail flounder is an excellent model species for examining the physiology of growth and reproduction in fish. That yellowtail flounder can be handled easily makes them attractive for research. A substantial amount of information on the biology of this species has been gathered over the past ten years of research into its potential for aquaculture (reviewed in Chapter 1). The present thesis has been able to add to this existing information by describing puberty in both sexes of this fish. Regarding the importance of this study to fish reproduction in general, this thesis may represent the first detailed account of puberty in a flatfish (Pleuronectiformes). It also may be the first study in which the steroid performance of ovarian tissue from triploid fish was examined *in vitro*. In this respect pubertal triploid females demonstrated a considerable difference from diploid females.

The major findings of the different chapters and potential future directions for research are outlined below.

In the first study of the thesis (Chapter 2), gonadal endocrinology was related to histological changes in the gonads for the first time in both male and female yellowtail flounder. Examining two year classes of young fish demonstrated that culture conditions reduce the age of sexual maturity, but that females still maintain a phenotypic plasticity in this trait. Evidence was found that growth rate influenced whether females entered puberty as one year old fish. For males, which all matured as one year old fish, a tendency was seen for larger individuals to initiate puberty earlier than smaller individuals of the same year class. Immature ovaries were steroidogenic and capable of responding to gonadotropic stimulation. Endocrine puberty in females was detected by a peak in 17ß-estradiol as early as the cortical alveolar oocyte stage. In males endocrine puberty was associated with both mitosis and meiosis as concurrent activities, although at very early stages of puberty (when few spermatocytes were present) androgens could be non-detectable. In both males and females, puberty, once initiated, proceeded to full maturity.

The second study of the thesis (Chapter 3) examined the effects of hormones with reputed dual roles in growth and reproduction, namely, gonadotropin-releasing hormone analogue (GnRH-a), testosterone and recombinant bovine growth hormone (rbGH). The major findings suggested that high levels of testosterone, whether alone or in combination with GnRH-a, suppress early stages of gametogenesis in immature females and recrudescing males. Additional negative effects of testosterone treatment on growth may explain a connection between elevated testosterone and decreases in growth associated with sexual maturity. GnRH-a in immature females was unable to advance the timing of puberty relative to controls

but did synchronize puberty as in ovulation. As the immature ovary is able to respond to gonadotropic stimulation, the inability of GnRH-a to advance puberty suggests that female puberty is dependent on the capacity of the pituitary to produce sufficient GtH and/or respond to GnRH. No effect of GnRH-a was seen on growth. The use of rbGH stimulated growth in males and females. It additionally decreased sex differences in growth usually resulting from slower growth rates in maturing males, as was noted in control and other experimental groups. In reproduction, rbGH treatment caused delays in the timing of puberty in some females, presumably by indirect actions involving energy storage. For rbGH treated males a stimulatory effect may be present during recrudescence.

In the third study (Chapter 4), the use of hydrostatic pressure treatments to induce triploidy was successful in producing up to 100% triploid individuals. Initial growth performance between hatching and metamorphosis indicates that growth of triploid larvae was inferior to growth of larvae from other groups. This included larvae from hydrostatic pressure treatments at the threshold for inducing triploidy, as well as larvae from sham control treatments. In contrast, growth of two year old triploid females approached growth rates for immature, yearling, diploid females and exceeded those of maturing, yearling, diploid females. Inducing triploidy in yellowtail flounder reduced gonadal development, but did not prevent maturation in either sex. By nearly three years of age, males produced very small amounts of milt with few motile spermatozoa, which in artificial fertilization trials demonstrated a reduced fertility. The majority of larvae that hatched were abnormal and non-viable although a minimal number of larvae of normal appearance were seen to survive to yolk-sac absorption.

Ovulation was detected in two females at 33-34 months of age, the majority of the remaining females were in a pubertal vitellogenic state preparing for full maturity by four years of age. Maturation by five years of age was possible for one to three other females. Ovaries had reduced numbers of oocytes amid tracts of oogonia. Nevertheless, there was sufficient production of 17ß-estradiol to induce vitellogenesis.

The research described in this thesis lays a foundation for further studies on flatfish pubertal physiology, particularly regarding early maturation, the inter-relationship of growth and reproduction, and the effect of induced triploidy. In terms of puberty in yellowtail flounder, future research should examine the roles of 17ß-estradiol, 11-ketotestosterone and very low levels of testosterone in positive feedback to the BPG axis at the onset of puberty. Examining the effects of steroids on the BPG axis more thoroughly will require: cloning of gonadotropin subunit genes; isolation of GnRH forms and intact gonadotropin(s); and, the development of assays for these substances. Assaying yellowtail flounder vitellogenin or its mRNA also will be needed to verify when, and at what levels of 17ß-estradiol, the synthesis of vitellogenin is stimulated during puberty. A comparison between diploids and triploids may help to determine to what degree pubertal vitellogenesis is regulated by ovarian size, steroid production or the number of follicles in the tissue.

For males, intragonadal production of androgens should be measured during the immature and early pubertal periods in an attempt to correlate androgen production with spermatogonial proliferation. *In vitro* incubations used in the present study were useful for assessing the steroidogenic responsiveness of the ovaries, and could be used to test the effects of IGF-I, 11-KT or activins on mitotic and meiotic activity in testicular tissue.

A natural extension from the present work in the investigation of yellowtail flounder puberty would include the injection of immature fish with a heterologous gonadotropin. The *in vivo* gonadal response could be observed to determine whether effects of exogenous gonadotropin treatment are dependent on season or histological stage of the gonad. An important question would be whether exogenous GtH treatment could produce sufficient steroid levels to activate the BPG axis of immature fish and thus demonstrate a positive feedback action by sex steroids *in vivo*.

Duality of action for hormones primarily associated with growth or reproduction could be investigated further. Potential links, such as IGF-I, between reproduction and somatic growth/condition could be studied intensively. Assays for fish IGF-I currently available may help elucidate the role of the somatotropic axis and growth rate in the onset of puberty. Investigations into somatic energetics should be prioritized as well in order to understand the role of body stores, growth and dietary lipid levels on the timing of the onset of puberty, particularly with regard to precocious maturation which was seen in a few small one-year-old females in the present study.

Regarding triploidy, pubertal vitellogenic ovaries from triploids showed a highly up-regulated steroidogenic output *in vitro* which was particularly interesting. An absence of an intra-ovarian inhibitory factor was suggested to explain an elevated steroid production by pubertal tissue in control incubation medium. The detection of binucleate oocytes in ovaries of triploids was similarly intriguing and may hint at mechanisms whereby oocytes attempt to deal with their triploid condition. What these findings may represent in terms of the gonadal physiology of flatfish, and triploid females in general, warrants further study.

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