

THE REPRODUCTIVE PHYSIOLOGY OF YELLOWTAIL  
FLOUNDER, *Pleuronectes Ferrugineus*,  
WITH AN EMPHASIS ON SPERM PHYSIOLOGY

CENTRE FOR NEWFOUNDLAND STUDIES

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SUSAN JANE CLEARWATER



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THE REPRODUCTIVE PHYSIOLOGY OF YELLOWTAIL FLOUNDER.  
*PLEURONECTES FERRUGINEUS*, WITH AN EMPHASIS ON SPERM PHYSIOLOGY

By

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in partial fulfilment of the requirements for the degree of  
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## ABSTRACT

Male and female yellowtail flounder *Pleuronectes ferrugineus* were individually identified, monitored and maintained in captivity for two reproductive seasons during which time both sexes produced viable gametes. Female spawners had elevated plasma levels of  $17\beta$ -estradiol ( $E_2$ ) in November indicating vitellogenesis had commenced; plasma  $E_2$  continued to increase until the beginning of the spawning season in June. Plasma testosterone (T) increased just prior to the spawning season, peaked after plasma  $E_2$  and decreased by the end of the spawning season in August.

Despite the year-round presence of milt with motile sperm, male yellowtail flounder showed a distinct seasonal change in plasma 11-ketotestosterone (11KT). 11KT peaked at the beginning of the spawning season when milt volumes were highest but decreased while milt volumes were still elevated. Plasma T showed a less distinct seasonal cycle than 11KT and peaked later in the season .

Compared to control treated fish, gonadotropin releasing hormone-analogue (GnRH $\alpha$ ) treatment delivered either by microspheres or cholesterol pellets successfully increased sperm production and milt volume in mature male yellowtail flounder during the spawning season. Sperm production remained high for 4 weeks after implantation of GnRH $\alpha$  and then decreased in all treatments (including the controls), indicating milt volume increased due to an initial increase in sperm production and remained high due to milt hydration. Plasma levels of T, 11KT and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one showed no clear

pattern of response to GnRH $\alpha$  treatment. GnRH $\alpha$  treatment did not have a negative effect on sperm fertilizing ability, percentage hatch or larval appearance. Sperm motility and seminal plasma pH was increased by GnRH $\alpha$  treatment.

Several aspects of yellowtail flounder sperm physiology were examined. Sperm were activated in buffer with an osmolality greater than 367 mOsm (pH 8.2-8.8) and diluted seawater with an osmolality greater than 387 mOsm (pH 7.6-8.2). Percentage sperm activated and swim times increased as osmolality increased up to approximately 800 mOsm. Changing seawater pH between 4.8 and 9.0 had minimal effect on sperm motility and no optimal pH was observed. Spectrophotometry was developed as a method to rapidly measure the sperm concentration of yellowtail flounder milt ( $1.26 \times 10^{10} \pm 0.10 \times 10^{10}$  cells ml<sup>-1</sup>). Cool storage (4°C) of milt diluted 10 fold in buffer with or without antibiotic successfully maintained sperm fertility and motility for seven days and had no adverse affect on egg hatching rates or larval appearance. Rapid dilution of urine contaminated milt in buffer, mitigates the negative effects of urine contamination on sperm motility and fertility.

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## LIST OF ABBREVIATIONS AND SYMBOLS

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
E <sub>2</sub>	17β-estradiol
CF	condition factor index
GnRH	gonadotropin releasing hormone
GnRHa	gonadotropin releasing hormone analogue
GSI	gonadosomatic index
GtH	gonadotropin
T	testosterone
11KT	11-ketotestosterone
17α,20βP	17α,20β-dihydroxy-4-pregnen-3-one
17α,20β21-P	17α,20β,21-trihydroxy-4-pregnen-3-one

## LIST OF COMMON NAMES AND LATIN NAMES USED IN TEXT

amago salmon	<i>Oncorhynchus rhodurus</i> (Jordan and McGregor)
American plaice	<i>Hippoglossoides platessoides</i> (Fabricus)
Atlantic cod	<i>Gadus morhua</i> L.
Atlantic salmon	<i>Salmo salar</i> L.
Atlantic croaker	<i>Micropogonias undulatus</i> L.
bighead carp	<i>Aristichthys nobilis</i>
black seabream	<i>Spondylisoma cantharus</i>
brook trout	<i>Salvelinus fontinalis</i>
brown trout	<i>Salmo trutta</i> L.
capelin	<i>Mallotus villosus</i> Müller
carp	<i>Cyprinus carpio</i> L.
catfish	<i>Heteropneustes fossilis</i> (Bloch)
channel catfish	<i>Ictalurus punctatus</i>
chum salmon	<i>Oncorhynchus keta</i>
coho salmon	<i>Oncorhynchus kisutch</i> (Walbaum)
dab	<i>Pleuronectes limanda</i> L. (formerly <i>Limanda limanda</i> )
european catfish	<i>Silurus glanis</i> L.
flounder	<i>Platichthys flesus</i> L.
goldfish	<i>Carassius auratus</i> L.

grey mullet	=striped mullet <i>Mugil cephalus</i> L.
halibut	<i>Hippoglossus hippoglossus</i> L.
japanese eel	<i>Anguilla japonica</i> (Temminck and Schlegel)
japanese flounder	<i>Paralichthys olivaceus</i> (Temminck and Schlegel)
masu salmon	<i>Oncorhynchus masou</i> (Brevoort)
Pacific herring	<i>Clupea harengus pallasii</i>
perch	<i>Perca fluviatilis</i> L.
pike	<i>Esox lucius</i> L.
plaice	<i>Pleuronectes platessa</i> L.
puffer	<i>Fugu niphobles</i>
rabbitfish	<i>Siganus guttatus</i> (Bloch)
rainbow trout	<i>Oncorhynchus mykiss</i> (Walbaum)
red sea bream	<i>Pagrus major</i> (Temminck and Schlegel)
sea bream	<i>Sparus aurata</i> L.
seabass	<i>Dicentrarchus labrax</i> L.
sea bass	<i>Lates calarifer</i> (Bloch)
silver carp	<i>Hypophthalmichthys molitrix</i> (Val)
snapper	<i>Pagrus auratus</i> (Bloch and Schneider)
sole	<i>Solea solea</i> L.
striped bass	<i>Morone saxatilis</i> (Walbaum)
starry flounder	<i>Platichthys stellatus</i> (Pallas)

turbot . . . . .	<i>Scophthalmus maximus</i> L.
yellow perch . . . . .	<i>Perca flavescens</i> L.
yellowtail flounder . .	<i>Pleuronectes ferrugineus</i> (Storer) (formerly <i>Limanda ferruginea</i> )
whitefish . . . . .	<i>Coregonus clupeaformis</i> (Mitchill)
winter flounder . . . . .	<i>Pleuronectes americanus</i> (Walbaum) (formerly . . . . . <i>Pseudopleuronectes americanus</i> )
witch flounder . . . . .	<i>Glyptocephalus cynoglossus</i> L.



## CHAPTER 1.

### General Introduction.

Yellowtail flounder *Pleuronectes ferrugineus* (Storer) are found in the Northwest Atlantic, between the Strait of Belle Isle in the north, and Cheseapeake Bay (U.S.A.) in the south. Yellowtail flounder are believed to be most abundant on the Grand Banks off the southeast coast of Newfoundland, elsewhere their distribution is sparse (Smith et al., 1977; Pitt 1983).

There is interest in developing the skills and knowledge to farm flatfish commercially in Newfoundland and yellowtail flounder were selected for this research programme from among the other local small flatfish species for several reasons. Yellowtail flounder have a higher market value than winter flounder *Pleuronectes americanus* (Walbaum) and are distributed further offshore (Goff, 1993). This is an advantage because recently winter flounder have been associated with areas of high pollution (e.g. untreated sewage outfalls) and are avoided by fish processing plants. Witch flounder *Glyptocephalus cynoglossus* L. tend to be found in deeper waters (42-274 m) than yellowtail flounder (58-65 m)(Pitt, 1970; Walsh, 1992) and witch flounder larvae are pelagic for at least a year before settling (Yevseyenko and Nevinsky, 1981). It is anticipated that such a long larval phase would be difficult to maintain successfully in the laboratory, therefore yellowtail flounder are preferred with only a 58-63 degree day larval phase (Yevseyenko and Nevinsky, 1981). American plaice *Hippoglossoides platessoides* (Fabricus) are thought to be slow-growing and relatively long-lived, with females reaching maturity at eight years, whereas yellowtail

flounder are thought to be relatively fast growing and mature at four to five years (Pitt, 1983; Scott and Scott, 1988). American plaice are also distributed deeper than yellowtail flounder, especially in the early stages of their life history.

Research on yellowtail flounder is also expected to compliment the halibut *Hippoglossus hippoglossus* L. research programme at the Ocean Sciences Centre (O.S.C.), St. John's, Newfoundland. Halibut are a giant flatfish and an adult fish (approximately 20 kg, 130-145 cm) is worth \$6-\$8.kg<sup>-1</sup> as well as being difficult to obtain alive. These three facts prohibit maintaining large experimental populations at the O.S.C., and destructive sampling for seasonal information. Also halibut larvae are difficult to maintain in the laboratory since they do not float at ambient salinities (Haug, 1990), unlike yellowtail flounder larvae (Smith et al., 1977). However, both species are batch spawners (Zamarro, 1988; Methven et al., 1992), middepth benthic feeders and both species are thought to move offshore during winter (Scott and Scott, 1988). A major problem in the management of halibut broodstock has been the decline in availability of high quality sperm through the spawning season (Methven and Crim, 1991). Studies of sperm physiology, hormonal control of spermiation and sperm storage in yellowtail flounder will provide useful comparative information in this area.

Since yellowtail flounder are distributed over a large north-south range the following information will pertain to the Grand Banks population unless otherwise stated. Yellowtail flounder are considered to be a sedentary species with long distance migrations not expected. Distribution is thought to be mainly controlled by depth since they tolerate large

fluctuations in salinity and temperature (Walsh, 1992). Adults and juveniles are usually found at temperatures between -1.0 and 7.7°C, most frequently at 60 m at temperatures of 3.5°C (Pitt, 1970; Walsh, 1988).

The spawning season for yellowtail flounder occurs between May and July (Howell, 1983; Pitt, 1983), peaking in early May and occurring earlier in the year at more southerly latitudes (Smith et al., 1977; Howell, 1983; Pitt, 1983). Spawning is thought to occur in areas on the Grand Banks adjacent to the Labrador current in depths of less than 100 m and water temperatures higher than 2°C (Pitt, 1983; Walsh, 1992). Spawning congregations or migrations have never been documented (Walsh, 1992). Yellowtail flounder are thought to spawn at, or near the bottom and the positively buoyant eggs float to the surface layer where early development occurs (Pitt, 1983; Walsh, 1992). Pelagic eggs have hatching times varying from 4.5 days (14°C) to 14.5 days (4°C) depending on temperature (Yevseyenko and Nevinsky, 1981). Larvae make diel migrations to deeper waters, larger larvae moving 20 m in a single migration (Smith et al., 1977). Larval metamorphosis occurs at a length of 12-14 mm (Yevseyenko and Nevinsky, 1981; Walsh, 1992), and benthic settlement occurs at a length of 12-20 mm (Smith et al., 1977). Larvae are present on the Grand Banks from June until September (Walsh, 1992). Bottom trawl studies showed the presence of juvenile yellowtail flounder on, and adjacent to, the Southeast Shoal (southern Grand Banks) therefore this may be a nursery site for yellowtail flounder (Walsh, 1990).

Female fish mature at five years (body length = 25-30 cm), while males mature at four years (body length = 25-30 cm). The age of maturity is earlier in southern populations (Howell, 1983; Pitt, 1983) and is probably explained by more rapid growth rates in these areas (Pitt, 1970). Fecundity studies relate egg production to age and body length, suggesting a 30-35 cm female, will spawn 350,000-500,000 eggs in a spawning season while a 50-55 cm female will spawn up to 4 million eggs. However only a small percentage of these eggs will be fertilized and of those only a small percentage will survive to hatching (Pitt, 1983). Fecundity is also positively related to ovary weight by some workers (Pitt, 1971; Howell and Kesler, 1977).

Studies of oocyte development show yellowtail flounder have group synchronous ovaries (*sensu* Wallace and Selman, 1981) and are serial spawners (Howell, 1983; Zamarro, 1988), with approximately 200,000 oocytes released per spawn by wild yellowtail flounder (Zamarro, 1988). A recent study of captive yellowtail flounder suggests only ~39,000 eggs are spawned per batch (Manning and Crim, 1995). Zamarro (1988) found no significant difference of numbers of fish with ovulated eggs in the ovary, between three spawning times, 0600-1100 h, 1400-1500 h and 1900-2300 h, concluding that there was no synchronism in the spawning hour. This suggests either the species may spawn in pairs or small groups of locally synchronised individuals or spawning congregations if they occur, happen near the sea bottom (Scott and Scott, 1988) where they are difficult to observe (for example by ship radar).

Seasonal development of the ovaries and oocytes was examined in detail by Howell (1983) who sampled females from a Rhode Island population of yellowtail flounder. At Rhode Island the spawning season lasted from April to June and increases in gonadosomatic index (GSI) and changes in the histology of the ovaries suggested that vitellogenesis began in August and development of the ovaries continued until the beginning of the spawning season. This contrasts with the seasonal cycle of winter flounder in which vitellogenesis is complete four months before the spawning season (Harmin and Crim, 1992).

Little is known about the reproductive cycle in male yellowtail flounder. Males are believed to be spermiated well in advance of and after, the spawning season (when ovulated eggs are found in a female fish). Preliminary observations suggest laboratory confined males possess low volumes of milt containing motile sperm year-round (Clearwater *unpublished data*). The type of testes, the cycle of reproductive development and the variations in sperm quality throughout the year have not yet been studied.

So far, no information has been gathered on the hormonal control of reproduction in male or female yellowtail flounder. Hormonal cycles and control of final reproductive events has been studied in other pleuronectids, particularly the plaice *Pleuronectes platessa* L., dab *Pleuronectes limanda* L. and winter flounder. Some information is available on halibut, Japanese flounder *Paralichthys olivaceus* (Temminck and Schlegel) and American plaice. The left-eyed flatfish, turbot *Scophthalmus maximus* L., has also been studied extensively for aquaculture. Prior to this study yellowtail flounder had not

been held in captivity throughout their reproductive cycle, therefore there was no information on whether yellowtail flounder would successfully reproduce in captivity. Many teleost fish species undergo normal reproductive development in captivity but fail to spawn (Zohar, 1989; Barton and Iwama, 1991), however understanding of the reproductive physiology will allow successful intervention if necessary to stimulate breeding in captivity. Once the seasonal cycle of reproduction is understood then it may be possible to further investigate the control of reproduction and stimulate breeding outside the normal spawning season. For example winter flounder males and females can be successfully stimulated to spawn up to two and three months ahead of the normal spawning season respectively, because gonadal recrudescence is mostly complete four months prior to spawning (Harmin and Crim, 1992). The first investigation in this study documented the reproductive cycle of sex steroids and gonad development in individually identified captive male and female yellowtail flounder.

Preliminary investigations suggest that yellowtail flounder males produce low volumes of viscous milt which makes milt collection and management of broodstock difficult (Clearwater *unpublished data*). A similar problem has been experienced with captive male halibut which show decreased availability of good quality sperm as the spawning season progresses (Methven and Crim, 1991). Low sperm production is the most common form of reproductive dysfunction in male teleosts held in captivity (Zohar, 1989). Administration of gonadotropin or gonadotropin releasing hormone analogue (GnRH $\alpha$ ) has successfully been used to increase milt volume or advance milt production in a wide range

of male teleosts (Zohar, 1989; Pankhurst, 1994), however little is known about the effect of hormone treatment on the quality of sperm so produced. Changes in sperm quality after GnRHa treatment have only been examined in a few species but no negative effect was found in yellow perch *Perca flavescens* L. (Dabrowski et al., 1994) or carp *Cyprinus carpio* L. (Ngamvongchon et al., 1987). The second investigation in this study aimed to show the effect of GnRHa treatment on milt volume and milt quality of male yellowtail flounder. The ultimate measure of milt quality is fertilization ability, therefore this study examined fertilization rates and larval hatching rates of the progeny of males treated with three different GnRHa treatments compared to a control treatment.

Good management of captive male broodstock requires an understanding of the sperm physiology of the species to allow high sperm production, collection and dilution of high quality milt (Munkittrick and Moccia, 1987; Billard et al., 1992). In teleosts, high sperm fertility is thought to be related to good sperm motility. Sperm are thought to remain immotile in the genital tract of the male and be activated by a change in their physicochemical environment by dilution of the seminal plasma as they are spawned. Changes in osmotic pressure, pH and ionic composition are probably the most important factors triggering sperm motility (Billard et al., 1992). Short-term sperm storage is useful when production of male and female gametes is not synchronised or gametes must be transported to the hatchery facility for fertilization (Stoss, 1983), also good quality sperm or sperm from males with favoured characteristics can be stored for fertilization of eggs from good female broodstock. Yellowtail flounder males produce low volumes of highly

concentrated sperm (Clearwater *unpublished data*), therefore reliable methods to measure sperm concentration and to dilute milt will facilitate good broodstock management (Billard et al., 1992). Similarly an understanding of the concentrations of sperm necessary to achieve high fertilization rates using 'dry' fertilization methods will ameliorate the problems of low sperm availability (Suquet et al., 1995). 'Dry' fertilization is the practice of mixing eggs and milt prior to the addition of seawater. The third investigation in this study examined the effect of pH and osmolality on sperm motility, and compared three different methods of measuring sperm concentration. The effect of sperm concentration on fertilization rates was examined in order to determine the optimal sperm:egg ratio for good fertilization rates. The effect of short term storage on fertilization rates and motility was examined in diluted milt stored with and without antibiotics.

Urine contamination has been shown to decrease sperm quality in carp (Perchec et al., 1995a and 1995b) and Atlantic salmon *Salmo salar* L. (Rana, 1995) and is difficult to avoid during the collection of milt from yellowtail flounder. The fourth investigation in this study examined the affect of urine contamination on sperm fertility and motility in yellowtail flounder, then examined the rapid dilution of urine contaminated milt to determine if the negative effects of urine contamination could be avoided.

In summary, this study documented the reproductive cycle of sex steroids and gonad development in individually identified captive male and female yellowtail flounder. The effect of 3 different GnRHa treatments on milt volume and milt quality of male yellowtail flounder was examined during the spawning season. Sperm motility, sperm concentration,



short term milt storage and the effect of urine contamination on sperm quality were studied in order to determine methods for management of yellowtail flounder broodstock and to add to the current understanding of teleost sperm physiology.

## CHAPTER 2.

### **Some aspects of the reproductive physiology of yellowtail flounder (*Pleuronectes ferrugineus*) in captivity.**

#### **2.1. Introduction**

Recently captured female yellowtail flounder *Pleuronectes ferrugineus* (Storer) have been successfully used in spawning induction experiments, producing viable gametes after administration of pituitary extract (Smigielski, 1979). However, prior to our experiments, yellowtail flounder had not been held continuously in captivity throughout the annual cycle of reproduction, therefore there was no information on whether they would mature or produce viable gametes. Annual cycles of plasma  $17\beta$ -estradiol ( $E_2$ ) and plasma testosterone (T) have been shown in female winter flounder *Pleuronectes americanus* (Walbaum) (Campbell et al., 1976; Harmin et al., 1995b), plaice *Pleuronectes platessa* L. (Wingfield and Grimm, 1977), and halibut *Hippoglossus hippoglossus* L. (Methven et al., 1992). Generally plasma  $E_2$  increases gradually during gonadal recrudescence, peaking just prior to oocyte hydration, while changes in plasma T although similar to plasma  $E_2$ , usually occur slightly after and lower than plasma  $E_2$ . Both steroids decrease rapidly in the plasma throughout the spawning period and reach a minimum in spent fish. In halibut, female fish show fluctuations of  $E_2$  and T throughout the spawning season, that are probably related to the 79 h spawning period of successive batches of oocytes (Methven et al., 1992). In female teleosts  $E_2$  is produced by the follicle layers surrounding the

developing oocytes and stimulates the liver to produce vitellogenin, the yolk protein precursor, which is then transported to the ovary and taken up by the developing oocytes (Kagawa et al., 1982; Ng and Idler 1983; Tyler 1991; Tyler et al., 1991). In salmonids and cyprinids the presence of T in the plasma is believed to be related to its role as a precursor for  $E_2$ , hence peak plasma T occurs after peak plasma  $E_2$  when the aromatase enzyme reduces the rate of conversion of T to  $E_2$  (Kagawa et al., 1983; Kagawa et al., 1984). T may also play a role in final oocyte maturation by feedback effects on gonadotropin secretion by the pituitary (Kagawa et al., 1983; Young et al., 1983; Kobayashi et al., 1989). The aim of this investigation was to maintain yellowtail flounder in captivity under conditions that would allow successful reproduction, to document the annual cycle of plasma steroids in individually identified female yellowtail flounder and correlate these cycles with the onset of vitellogenesis and ovulation.

In male teleosts milt is often present in the sperm duct one to two months earlier than the beginning of the spawning season in females, for example in starry flounder *Platichthys stellatus* (Pallas), and plaice (Barr, 1963). Winter flounder males produce sperm five months earlier than the female spawning season (Burton and Idler, 1987). In dab *Pleuronectes limanda* L., sperm are present throughout the year, becoming more abundant prior to the spawning season, when cellular activity in the testes is high, however sperm present outside the spawning season are few and inactive (Htun-Han, 1978). Dab, like yellowtail flounder were formerly classified in the genus *Limanda* (Sakamoto, 1984). Preliminary data showed that yellowtail flounder males possess small

volumes of motile sperm year-round despite the occurrence of a well-defined female spawning season from May to July. Since no histological studies of yellowtail flounder testes have been done it is not yet known whether spermatogenesis and spermiation is occurring year round or if the milt present outside the female spawning season is residual from the previous cycle of spermatogenesis.

The androgens 11-ketotestosterone (11KT) and testosterone (T) and the progestin 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ P) show seasonal cycles in males of many different teleost species (Fostier et al., 1983), however the role of the different steroids in spermatogenesis, spermiogenesis and milt production is not yet entirely understood (Nagahama, 1987). Early work on salmonids and goldfish *Carassius auratus* L. suggested that plasma androgens peaked during the spawning season and therefore were probably involved in milt production as well as gonadal recrudescence (Billard et al., 1982, Fostier et al., 1983). However, injection of plasma androgens to induce milt production has had variable success depending on the species and the timing of the treatment (Yamazaki and Donaldson 1968 and 1969; Billard et al., 1982; Ueda et al., 1984; Weber and Lee 1985; Saad and Billard 1987; Miura et al., 1991). This investigation monitored the presence of milt and motility of sperm in individually identified male yellowtail flounder in order to investigate initial observations that, in captivity, this species produces sperm year-round. The seasonal changes in plasma steroid levels were documented in order to determine the role of the androgens T and 11KT and the progestin 17 $\alpha$ ,20 $\beta$ P in the reproductive cycle of captive male yellowtail flounder. The stress of captivity can have a negative effect on

reproduction causing decreased plasma steroids, for example, in the brown trout *Salmo trutta* L. (Pickering et al., 1987, Sumpter et al., 1987) and wild snapper *Pagrus auratus* (Bloch and Schneider) (Carragher and Pankhurst, 1991). Blood sampling, handling and emmersion can all have negative effects on reproduction, therefore to minimise the stress experienced by some of the broodstock some individuals were blood sampled monthly and others were sampled every second month (bimonthly).

## 2.2. Materials and methods

### 2.2.1. Holding conditions

Adult male and female yellowtail flounder were captured inshore in Conception Bay, Newfoundland by SCUBA divers during November 1992 and June 1993. The fish were held in 540 l tanks with flow-through seawater at either ambient temperatures or in a mixture of ambient and heated/chilled seawater. Water temperatures were recorded in each tank daily from January 1993-August 1994 using temperature monitors. Fish were exposed to a simulated natural photoperiod for St. John's, Newfoundland (47°20' N, 52°45' W) using a 60 W bulb (75-190 lux) 80 cm above each tank controlled by an on-off electronic timer (Bishop, 1988). From November 1992 to October 1993 the fish were fed weighed and chopped shrimp *Penaeus sp.* or capelin *Mallotus villosus* (Müller) three times a week at 0.6-2.1 % body weight day<sup>-1</sup>. From November 1993 the diet was changed to a commercial moist pellet feed (Connor Bros., Blacks Harbour, New Brunswick) and fed two to five times a week at 0.2-0.5 % body weight day<sup>-1</sup> until mid April 1994 then the rations were increased to 1.0-2.0 % body weight day<sup>-1</sup>.

Growth and reproduction was studied for two spawning seasons, 1993 (November 1992 to October 1993) and 1994 (November 1993 to August 1994). At the beginning of the 1993 study the fish were held together as they were captured, then on December 17, 1992 fish were individually tagged with passive injectible transponder (PIT) tags and sorted into three different tanks, termed groups I ("warm"), II and III (both "cold") (Table 1). The broodstock were initially separated into groups to compare the effect of different water

temperatures during winter and to reduce the risk of accidental loss of all broodstock. On June 8, 1993 group III was combined with group II in one tank to provide space for freshly caught broodstock. In September 1993 group I was combined with groups II & II, then in November all surviving broodstock (including "new" broodstock caught in June 1994) were placed in a single tank for the 1994 spawning season study. Space limitations at the laboratory dictated the broodstock groupings into tanks.

#### *2.2.2. Sampling*

Each month (except for February 1993), individual body length, weight and reproductive stage (Table 2) was recorded and fish were blood sampled, if scheduled. Measurements, milt collection and blood sampling was completed between 0900 to 1200 h. The blood sampling schedule was either monthly or every second month (bimonthly) for individual fish. A 0.6 ml blood sample was taken from the caudal sinus of unanesthetized fish using preheparinised 23 G needles and stored on ice. Approximately 3 h after sampling (after motility trials), blood was centrifuged in 1.5 ml Eppendorf tubes for 15 min,  $9170 \times g$  at  $4^{\circ}\text{C}$ . Blood plasma was removed in 140  $\mu\text{l}$  aliquots and stored in 0.5 ml Eppendorf tubes at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  for future analysis. Individual males were carefully dried and all available milt was manually stripped from the urogenital pore and collected directly at the pore in a 1 ml syringe then stored in an 0.5 ml Eppendorf tube on ice. Urine contamination was avoided by not collecting urine when it was expressed separately.

### 2.2.3. Gonad staging

Reproductive stage was assessed for each fish by the presence or absence of gametes and externally visible changes in the gonads (Table 2).

### 2.2.4. Milt analysis

As soon as possible after milt collection, usually after 2 h, motility was tested under 100x magnification in chilled filtered seawater (5°C). A 10 µl pipette tip was dipped into the milt sample and stirred quickly into a 100 µl sample of seawater already prepared on a prechilled slide on the microscope stage. Percentage sperm activated at the point of mixing and duration of forward motility of the majority of activated sperm (swim time in seconds) were noted in each motility trial. The volume of milt collected was measured in the collection syringe or in a marked Eppendorf vial. Spermatocrit (packed cell volume) was measured using haematocrit tubes after centrifuging at  $15,000 \times g$  for 30 min and where possible 3 replicates of spermatocrit were measured. The data for spermatocrit, percentage sperm cells activated and sperm swim times were grouped into arbitrarily defined classes (Table 3).

### 2.2.5. Radioimmunoassay

Steroids were extracted from 0.1 ml plasma samples and prepared for steroid assay according to Harmin and Crim (1993). Testosterone and 17β-estradiol ( $E_2$ ) concentration in the plasma was determined according to Harmin et al., (1995b) using iodinated steroids



as tracers (purchased from International Diagnostic Services, Scarborough, Ontario, Canada) with the exception that after the addition and incubation of ethylene glycol separating reagent the assay tubes were centrifuged at  $1650 \times g$  for 30 min rather than 15 min.

11KT and  $17\alpha,20\beta$ P were assayed using tritiated labels (prepared by C. Wilson, Nfld. Canada, after Truscott (1981)) according to Harmin and Crim (1993) 11KT procedure with the following exceptions. 11KT and  $17\alpha,20\beta$ P antibodies (courtesy of D.R. Idler, Nfld. Canada) were diluted at 1:40,000 and 1:2000 respectively, and the  $17\alpha,20\beta$ P label was added at ca. 5000 cpm per tube.

Extraction efficiencies were 84.88%, 87.6%, 90.94% and 86.88% for  $E_2$ , T,  $17\alpha,20\beta$ P and 11KT respectively and all steroid data were corrected accordingly. Interassay standards varied between 0.58-1.14 ng ml<sup>-1</sup> and 1.14-2.06 ng ml<sup>-1</sup> for  $E_2$  (n = 3) and T (n = 5) respectively.  $17\alpha,20\beta$ P (n = 5) interassay standards varied between 0.00-0.02 ng ml<sup>-1</sup>. 11KT interassay standards were 0.12 ng ml<sup>-1</sup> and 0.40 ng ml<sup>-1</sup>. All samples for individual fish over time were done within one assay, so that seasonal comparisons were made together. T antibody cross-reactivity was 0.0009% for estriol, <0.0007% for estrone, 0.0035% for estradiol, 0.96% for androstenedione, 0.19% for 11KT and 0.0034% for progesterone (Harmin et al., 1995b). 11KT antibody cross-reactivity with testosterone and 11 $\beta$ -hydroxy-testosterone was <0.1% (Ng and Idler, 1980).  $17\alpha,20\beta$ P antibody cross-reactivity was 14% for 5 $\alpha$ -pregnan-3 $\alpha,17\alpha,20\beta$ -triol, and there was no cross reaction for 17 $\alpha$ -hydroxyprogesterone, 17 $\alpha$ -hydroxy-20 $\alpha$ -dihydroprogesterone, progesterone, 20 $\beta$ -

dihydroprogesterone, cortisol, cortisone, 11-deoxycortisol, 20 $\beta$ -dihydrocortisone, 5 $\beta$ -pregnan-3 $\alpha$ , 17 $\alpha$ , 20 $\beta$ -triol (Peter et al., 1984).

#### 2.2.6. *Statistics*

Data were analyzed using one way or two way ANOVA and Duncan's multiple range means test ( $P < 0.05$ ) (Zar, 1984) on the Statistical Analysis System (SAS) computer package. Data were log, square root or arcsine transformed when necessary to fit the assumptions of ANOVA. Data are expressed as means  $\pm$  s.e. Gonad stage, spermatocrit, percent sperm activated and sperm swim time data were not analyzed statistically.

## 2.3. Results

### 2.3.1. *Water temperature*

In 1993 the effects of temperature on feeding, growth and reproductive development of adult yellowtail flounder was studied in three different groups of fish. From January to June group I was maintained in warmer water temperatures (2.5-6.4°C) compared with groups II and III (1.4-3.6°C and 1.8-4.2°C respectively, Fig. 1). Beginning in July, all three groups of fish were raised in similar water temperatures which rose significantly in the summer to 6.2°C in July, to 9.2°C in August, and reached 10.3°C by September. A similar thermocycle reoccurred again in 1994, when the fish were exposed to low water temperatures in the winter and much higher temperatures in the summer varying from 1.4°C in February to 12.2°C in August.

Preliminary analysis showed differences in growth of groups I, II and III were not consistent with different temperature regimes between the tanks or differences in food consumption. Also some spawning females occurred in all groups and spawning started at similar times between groups, therefore for further analysis of seasonal changes in growth and reproduction in 1993, groups I, II and III were lumped together into the 1993 data set. The increased sample sizes allowed for more meaningful comparison of seasonal changes in growth and plasma steroid concentrations between spawning and non-spawning individuals.

### 2.3.2. Male steroid profiles

In 1993 males showed no seasonal change in plasma T which varied between 0.6-3.0 ng ml<sup>-1</sup> from November 1992 to October 1993 (Fig. 2). Plasma 11KT was 0.6-15.0 ng ml<sup>-1</sup> in November. January and March then increased (compared to January) to 25.5 ng ml<sup>-1</sup> in April remaining high in May and June (28.0-41.3 ng ml<sup>-1</sup>) before decreasing to 19.2 ng ml<sup>-1</sup> in July and remaining low from August to October (1.9-7.0 ng ml<sup>-1</sup>).

In 1994 males showed seasonal changes in both plasma T and 11KT (Fig. 3). Plasma T ranged between 1.5-4.6 ng ml<sup>-1</sup> from December 1993 to April 1994, then increased (compared to March) to 6.4 ng ml<sup>-1</sup> in May and peaked in June at 8.0 ng ml<sup>-1</sup>. Plasma T then decreased significantly to 4.0 ng ml<sup>-1</sup> in July and remained low in August at 1.6 ng ml<sup>-1</sup>. Plasma 11KT ranged between 0.9-2.3 ng ml<sup>-1</sup> from December to March, increased to 7.7 ng ml<sup>-1</sup> in April, peaked in May and June at 10.7-11.0 ng ml<sup>-1</sup> then decreased to 1.4-3.4 ng ml<sup>-1</sup> in July and August.

Plasma 17 $\alpha$ ,20 $\beta$ P, was  $\leq 2.0$  ng ml<sup>-1</sup> in all samples and less than the detection limit of the assay in most samples therefore the data were not analysed further (data not shown).

### 2.3.3. Male gonad stages

Gonad stage data showed seasonal changes in the prevalence of males with expressible milt during the female spawning season. In 1993 only stage 4 males were present in July, both stage 3 and 4 males were present in August, then mainly stage 3 males were found from September to November (Fig. 2c). Stage 2 males were present in October after the

spawning season. In December 1993, 40% of the males had no milt (stage 2), then from January to March 1994 most males were in stage 3 and some were in stage 4. From June to August 1994 most males were in stage 4 (spermiated, Fig. 3c).

#### *2.3.4. Milt volumes and quality*

Milt volumes and sperm quality parameters (spermatocrit, % activated and swim times) also showed seasonal changes.

In 1993, milt volume ranged between 4.71-5.97 ml.kg<sup>-1</sup> (1.16-1.78 ml) in July and August, then decreased to 0.62 ml.kg<sup>-1</sup> (0.22 ml) in September and remained low in November (Fig. 2b). Numbers of milt samples with high spermatocrit (> 50%) increased from July to October when all spermatocrits were between 75-100% (n = 4) (Fig. 4). However, all spermatocrit classes were recorded in November (n = 4). The highest number of high motility (% activated and swim time) milt samples were present in July, then lower motility samples tended to dominate from August to November.

Prior to the 1994 spawning season, milt volume ranged between 0.29-0.52 ml.kg<sup>-1</sup> (0.11-0.20 ml) from December 1993 to May 1994, increased to 2.36 ml.kg<sup>-1</sup> (0.85 ml) in June then decreased to 1.43 ml.kg<sup>-1</sup> (0.52 ml) and 0.78 ml.kg<sup>-1</sup> (0.28ml) in July and August respectively (Fig. 3b). Low spermatocrit samples (< 50%) only, were present in June at the beginning of the spawning season and were dominant in July, however in August the majority of spermatocrits were 50-75% (Fig. 5). Immotile sperm were present throughout the sampling period except for July. During the spawning season percentage sperm

activated increased from June to July and decreased slightly in August. while swim times increased from June to July and remained high in August.

#### *2.3.5. Steroid profiles in female spawners and non-spawners in 1993*

In 1993 spawning females ( $n = 12$ ) showed seasonal changes in both plasma  $E_2$  and T, whereas non-spawning females ( $n = 11$ ) showed a seasonal increase in  $E_2$  after the spawning season but no seasonal change in T (Figs. 6a and 7a).  $17\alpha,20\beta P$  showed no significant seasonal change in either spawning ( $0.05$ - $0.74 \text{ ng ml}^{-1}$ ) or non-spawning ( $0.01$ - $0.52 \text{ ng ml}^{-1}$ ) females between November 1992 and October 1993.

#### *2.3.6. Steroid profiles in 'normal' and 'abnormal' female spawners in 1994*

Three out of eight spawning females in 1994 only released small quantities ( $\leq 5 \text{ ml}$ ) of low quality eggs (cloudy and/or non-spherical) in August. These three females were classed as 'abnormal' spawners (see Section 2.4.) and their steroid profiles were analysed separately from the 'normal' spawners. The abnormal spawners showed no seasonal change in plasma T which varied between  $0.5$ - $2.7 \text{ ng ml}^{-1}$  or  $E_2$  which varied between  $0.7$ - $4.3 \text{ ng ml}^{-1}$  (Fig. 8a). In the normal spawners plasma  $E_2$  was elevated in December at  $4.5 \text{ ng ml}^{-1}$  and steadily increased to  $11.3 \text{ ng ml}^{-1}$  in April and decreased gradually to  $0.9 \text{ ng ml}^{-1}$  in August (Fig. 9a). Plasma T varied between  $1.6$ - $6.3 \text{ ng ml}^{-1}$  in June, increased sharply to  $13.3 \text{ ng ml}^{-1}$  in July and decreased sharply to  $2.9 \text{ ng ml}^{-1}$  in August. There were

insufficient plasma samples taken from non-spawning females in 1994 ( $n = 1$ ) to analyse seasonal changes in plasma steroids statistically (data not shown).

### 2.3.7. *Spawning and non-spawning female gonad stages*

In 1993 spawners, only spent or resting (stage 2) females were observed in November 1992, then the majority of females were stage 3 until May. Spawning females were found in June, July and August and numbers of spent or resting females (stage 2) increased from July to September (Fig. 6b). Stage 3 females were present again in October and November 1993. Two stage 4 females were observed in April.

In 1993 non-spawners, stage 2 females were present throughout the year but were in highest numbers in August and September (Fig. 7b). Small numbers of stage 3 females were observed from December to July and then were the most numerous stage in October and November 1993. Immature females were observed in November, December, March and June.

The normal spawners in 1994 were predominately at stage 3 from January to May (Fig. 9b). Spawning females were found from June until August, while resting or spent females were first observed in July and then in August. Abnormal spawners in 1994 were classed as either stage 2 or 3 from December to July; in 1994 all three fish spawned  $\leq 5$  mls of low quality eggs.

Only one non-spawning female was recorded in 1994 and this fish remained at stage 3 from February until August (Data not shown).

#### *2.3.8. Rematuring fish*

Of nine fish that survived two spawning seasons, five spawned in both 1993 and 1994 (Table 4). Three did not spawn in 1993 then spawned in 1994 and one fish did not spawn in either year.

#### *2.3.9. Growth -Males*

Male body weight, body length and condition factor index (CF) ( $CF = (\text{body weight} / \text{body length}^3) \times 100$ ) showed no significant monthly changes in either 1993, or 1994 (Fig. 10).

#### *2.3.10. Growth-Females*

Female body weight, body length and CF increased between November 1992 and November 1993 (Fig. 11). In 1994 female body weight, body length and CF showed no significant monthly changes.

#### *2.3.11. Growth -Female spawners and nonspawners*

Female body weight, body length and CF were not significantly different between spawning and non-spawning individuals in 1993, but did vary seasonally (Figs. 12, 13 and 14). Body weight and CF showed a significant interaction between seasonal effect and spawning status, but body length did not. Body length showed no significant change between November 1992 and August 1993, then increased in September and remained



high until November. In 1994, eight out of nine fish spawned, therefore it was not possible to statistically compare changes in growth due to spawning status. Body weight, body length and CF, of spawning females only, showed no seasonal change.

Table 1. Groups of fish used in seasonal study and dates groups were moved between tanks.

<b>Month</b>	<b>Tank 100</b>	<b>Tank 105</b>	<b>Tank 106</b>
	(Warm)	(Cold)	(Cold)
January 1993	I	II	III
June 1993	I	II & III	-
September 1993	-	I, II & III	"New" broodstock
November 1993	-	I, II & III and "New" broodstock	-

Table 2. Male and female gonad stages (N.B. "sex unknown" can be determined retroactively for individually identified fish).

Sex	Characteristic	Stage	Name
Male	Sex unknown	1	Immature
	No sperm, sex known.	2	Resting
	Sperm present in small volumes, viscous.	3	Mature
	Sperm present in large volumes, dilute, non-viscous.	4	Spermiating
Female	Sex unknown.	1	Immature
	Sex known, ovary small, no swelling.	2	Resting or spent
	Female, ovary fat and firm, swelling of gonad obvious.	3	Vitellogenic
	Female ovary very fat, soft and swollen.	4	Hydrated (but not ovulated)
	Female spawning, eggs expressed from gonopore.	5	Spawning (hydrated and ovulated)

Table 3. Grouping of spermatocrit, percentage sperm cells activated and sperm swim time data into classes

<b>Parameter</b>	<b>Characteristics</b>	<b>Class</b>
Spermatocrit	<b>Percentage</b>	
	0-25%	1
	26-50%	2
	51-75%	3
	76-100%	4
Percentage Sperm Activated	<b>Percentage</b>	
	0%	1
	1-25%	2
	26-50%	3
	51-75%	4
	76-100%	5
Swim Times	<b>Seconds</b>	
	0	1
	1-5	2
	5-30	3
	>30	4

Table 4. Spawning activity in 1993 and 1994 of fish that survived both years in captivity.

Fish	Spawn (Yes/No)		Date spawning first detected.	
	1993	1994	1993	1994
53	Y	Y	13/7/93	11/8/94
55	Y	Y	22/7/93	22/7/94
58	Y	Y	8/6/93	29/6/94
57	Y	Y	12/8/93	18/8/94
59	Y	Y	13/7/93	22/7/94
52	N	Y	-	21/6/94
54	N	Y	-	18/8/94
60	N	Y	-	21/6/94
61	N	N	-	-

Fig. 1. Monthly average water temperatures for yellowtail flounder broodstock in 1993 and 1994. Group I (open circles), group II (squares) and group III (triangles). Group III joins group II in June, and group I joins groups II and III in September 1993. Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Lower and upper case letters indicate Duncan's groups for 1993 temperatures (July to September) and 1994 water temperatures respectively.

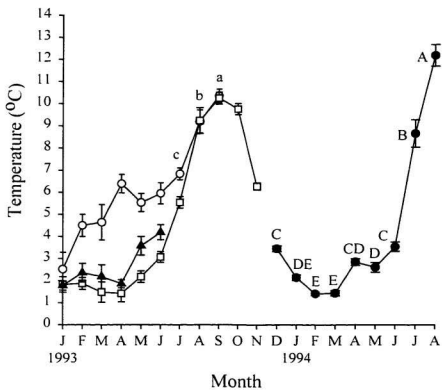


Fig. 2. Seasonal change in a) plasma steroids, b) milt volume and c) gonad stage in males in 1993. There was a seasonal change in plasma 11KT (squares) ( $F = 6.91$ ,  $P < 0.0001$ ) and milt volume ( $F = 7.51$ ,  $P < 0.0008$ ) but not in plasma T (circles) ( $F = 0.81$ ,  $P < 0.608$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Numbers indicate n value for each sample.

Gonad stages: 1 -immature; 2-resting; 3-mature (milt present); 4-spermiating (milt present).



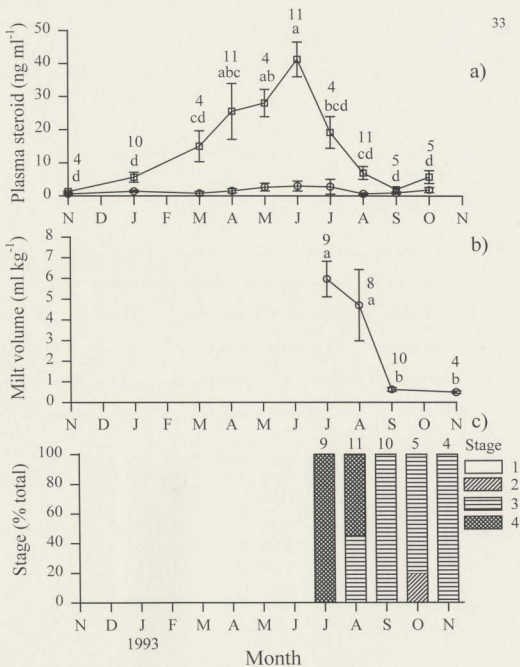
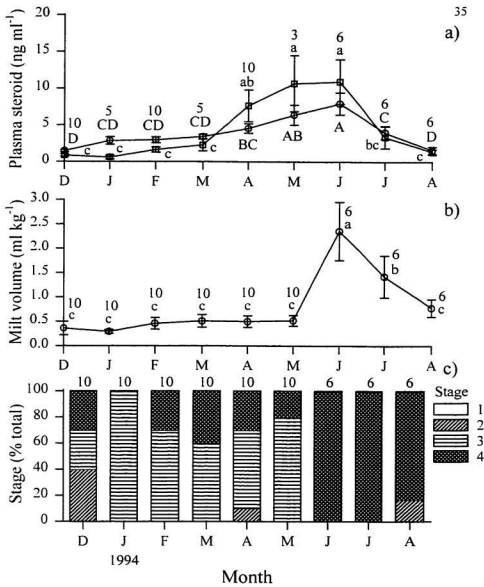
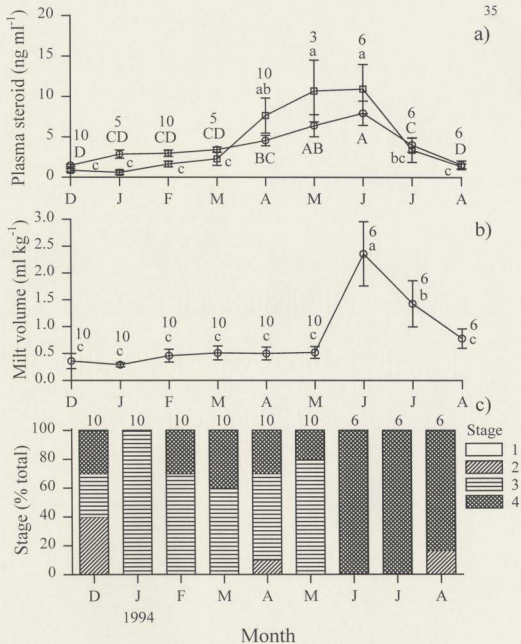


Fig. 3. Seasonal change in a) plasma steroids, b) milt volume and c) gonad stage in males in 1994. There was a seasonal change in plasma 11KT (squares) ( $F = 6.02$ ,  $P < 0.0001$ ), plasma T (circles) ( $F = 8.56$ ,  $P < 0.0001$ ) and milt volume ( $F = 8.51$ ,  $P < 0.0001$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Upper and lower case letters show Duncan's groupings of plasma T and 11KT means respectively. Numbers indicate n value for each sample.

Gonad stages: 1 -immature; 2-resting; 3-mature (milt present); 4-spermiating (milt present).





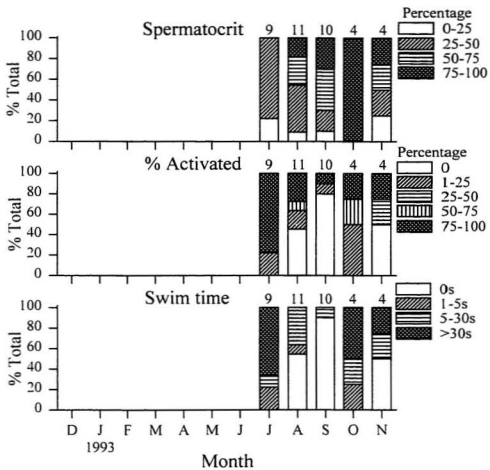


Fig. 5. 1994 males -Seasonal change in proportion of milt samples in different classes of spermatocrit (%), percentage activated sperm and swim times (s). Numbers show n value for each sample.

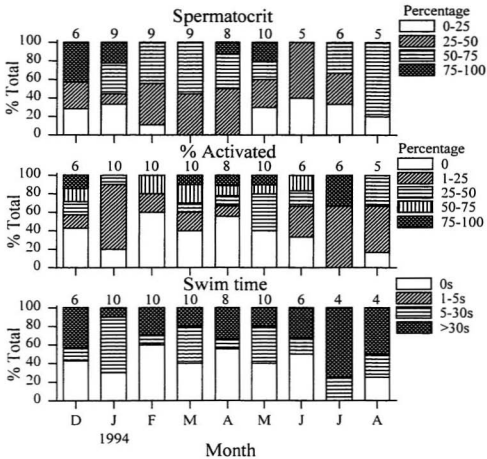


Fig. 6. Seasonal change in a) plasma steroids and b) gonad stage in female spawners in 1993. Plasma T (circles) ( $F = 6.80$ ,  $P < 0.0001$ ) and  $E_2$  (squares) ( $F = 6.51$ ,  $P < 0.0001$ ) changed seasonally but plasma  $17\alpha,20\beta P$  (triangles) did not ( $F = 0.61$ ,  $P < 0.785$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Lower and upper case letters show Duncan's groupings of means for plasma  $E_2$  and T respectively. Numbers show n values for each sample.

Gonad stages: 1-immature; 2-resting or spent; 3-vitellogenic; 4-hydrated (but not ovulated); 5-spawning (hydrated and ovulated).



a) 41

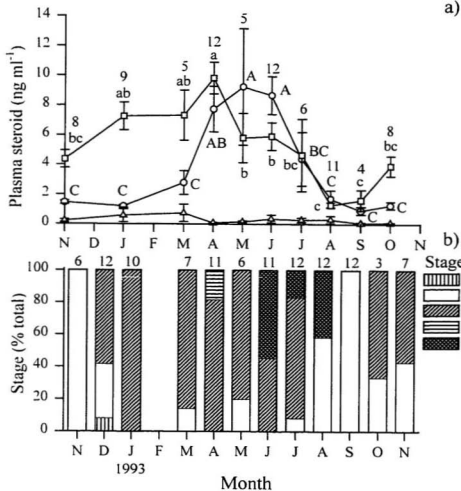


Fig. 7. Seasonal change in a) plasma steroids and b) gonad stage in non-spawning females in 1993. Plasma  $E_2$  (squares) ( $F = 7.61$   $P < 0.0001$ ) changed seasonally but plasma T (circles) ( $F = 1.48$ ,  $P < 0.188$ ) and  $17\alpha,20\beta P$  (triangles) did not ( $F = 0.48$ ,  $P < 0.862$ ). Plasma  $E_2$  means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Numbers show n values for each sample.

Gonad stages: 1-immature; 2-resting or spent; 3-vitellogenic; 4-hydrated (but not ovulated); 5-spawning (hydrated and ovulated).

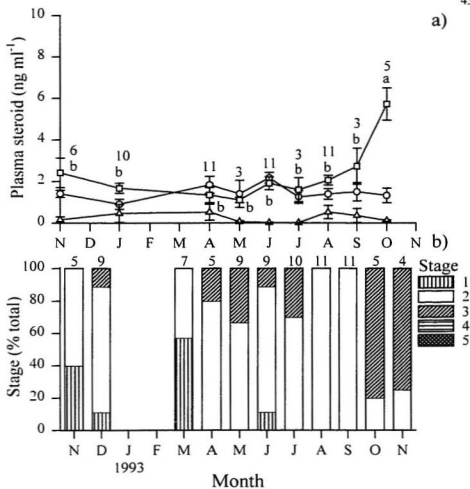


Fig. 8. Seasonal change in a) plasma steroids and b) gonad stage in abnormal female spawners in 1994. There was no seasonal change in either plasma T (circles) ( $F = 0.50$ ,  $P < 0.819$ ), plasma  $E_2$  (squares) ( $F = 0.49$ ,  $P < 0.831$ ) or plasma  $17\alpha,20\beta P$  (triangles) ( $F = 1.15$ ,  $P < 0.384$ ).  $n = 3$  for plasma steroid measurements and gonad stages, except for gonad stage measurements in June when  $n = 2$ .  
Gonad stages: 1-immature; 2-resting or spent; 3-vitellogenic; 4-hydrated (but not ovulated); 5-spawning (hydrated and ovulated).

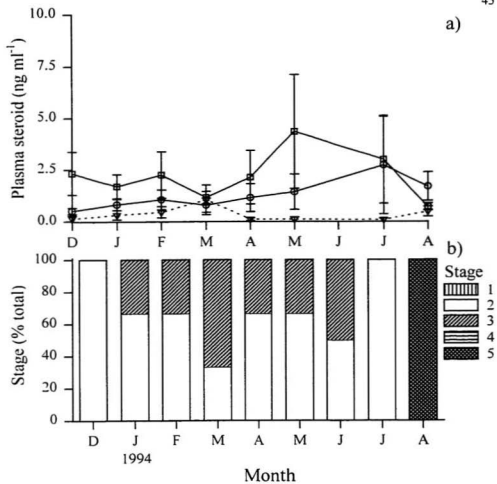


Fig. 9. Seasonal change in a) plasma steroids and b) gonad stage in normal female spawners in 1994. Plasma T (circles) ( $F = 4.87$ ,  $P < 0.0041$ ) and  $E_2$  (squares) ( $F = 3.21$ ,  $P < 0.024$ ) changed seasonally but plasma  $17\alpha,20\beta P$  (triangles) did not ( $F = 1.26$ ,  $P < 0.317$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Lower and upper case letters show Duncan's groupings of means for plasma  $E_2$  and T respectively. Numbers show  $n$  values for each sample.

Gonad stages: 1-immature; 2-resting or spent; 3-vitellogenic; 4-hydrated (but not ovulated); 5-spawning (hydrated and ovulated).

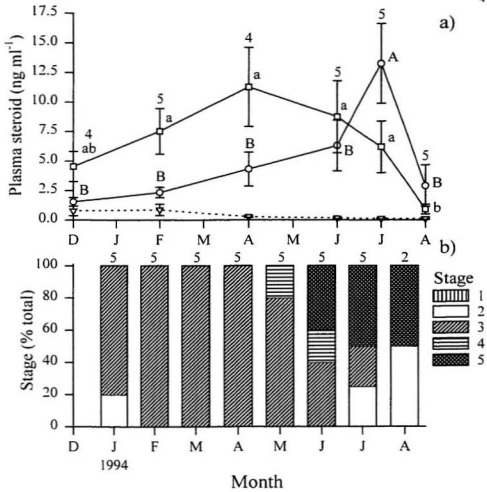


Fig. 10. Male body weight, body length and CF, showed no seasonal change in either 1993 (open circles) ( $P < 0.782$ ,  $P < 0.625$ ,  $P < 0.118$  respectively) or 1994 (filled circles) ( $P < 0.999$ ,  $P < 0.987$ ,  $P < 0.883$  respectively). Numbers show n values for each sample.



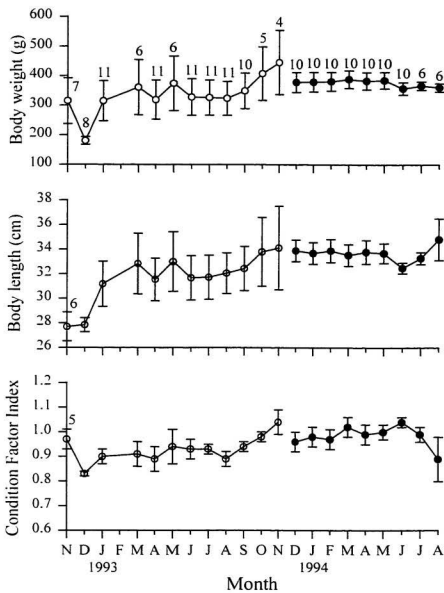


Fig. 11. Seasonal change in female body weight, body length and CF for 1993 (open circles) and 1994 (filled circles). Body weight ( $F = 7.74$ ,  $P < 0.001$ ), body length ( $F = 4.49$ ,  $P < 0.0001$ ) and CF ( $F = 10.11$ ,  $P < 0.0001$ ) increased in 1993 but showed no change in 1994 ( $P < 0.970$ ,  $P < 1.000$ ,  $P < 0.824$  respectively). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). Numbers show n values for each sample.

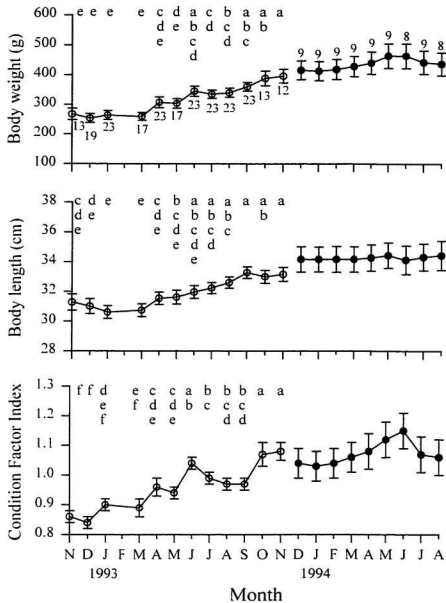


Fig. 12. Comparing body weight of spawning (filled circles) and non-spawning females (open circles) from 1993 and 1994. Body weight was not different between spawners and non-spawners in 1993 ( $F = 0.36$ ,  $P < 0.548$ ) and there was only one non-spawner in 1994 (data not shown). Body weight changed seasonally in 1993 ( $F = 8.81$ ,  $P < 0.0001$ ) but not in 1994 ( $F = 0.17$ ,  $P < 0.994$ ). There was a significant interaction between seasonal change and spawning status in 1993 females ( $F = 2.08$ ,  $P < 0.0243$ ). Numbers show  $n$  values for each sample.

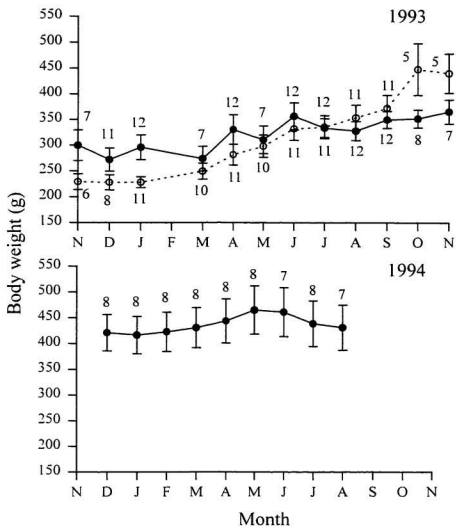


Fig. 13. Comparing body length of spawning (filled circles) and non-spawning females (open circles) from 1993 and 1994. Body length was not different between spawners and non-spawners in 1993 ( $F = 0.63$ ,  $P < 0.430$ ) and there was only one non-spawner in 1994 (data not shown). Body length changed seasonally in 1993 ( $F = 4.44$ ,  $P < 0.0001$ ) but not in 1994 ( $F = 0.02$ ,  $P < 1.000$ ). There was no interaction between seasonal change and spawning status in 1993 females ( $F = 0.80$ ,  $P < 0.643$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ). n values were the same as in Fig 12.

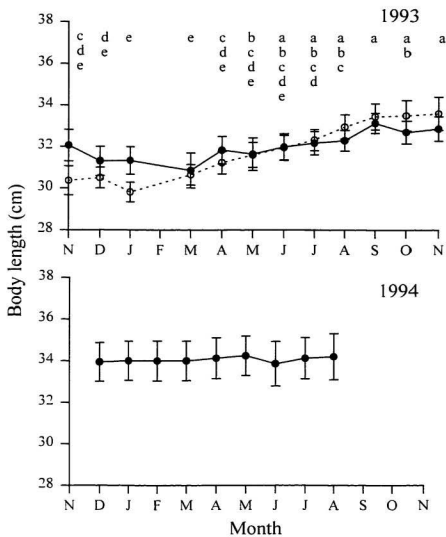
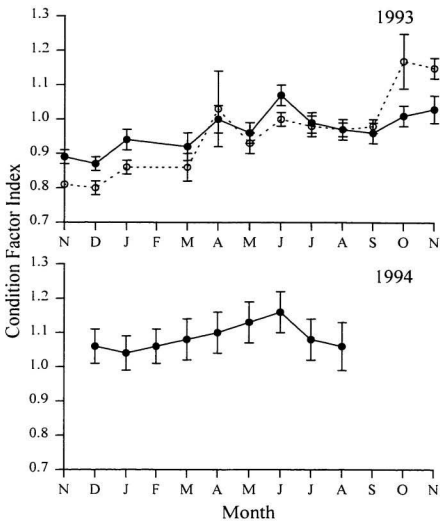


Fig. 14. Comparing condition factor (CF) of spawning (filled circles) and non-spawning females (open circles) from 1993 and 1994. CF was not different between spawners and non-spawners in 1993 ( $F = 1.65$ ,  $P < 0.201$ ) and there was only one non-spawner in 1994 (data not shown). CF changed seasonally in 1993 ( $F = 11.84$ ,  $P < 0.0001$ ) but not in 1994 ( $F = 0.40$ ,  $P < 0.917$ ). There was an interaction between seasonal change and spawning status in 1993 females ( $F = 2.58$ ,  $P < 0.004$ ).  $n$  values were the same as in Fig 12.





#### 2.4. Discussion

Male and female yellowtail flounder were successfully maintained in captivity for two reproductive seasons during which time both males and females produced viable gametes that were successfully used to produce larvae for other studies at the laboratory.

The cycle of plasma androgens in male yellowtail flounder showed a distinct seasonal peak similar to that observed in other male teleosts and other flatfish species, despite the year-round presence of motile sperm. In both 1993 and 1994 plasma 11KT remained relatively low from November to March, increased prior to the spawning season (June to August), peaked in June during the spawning season then decreased in the second half of the spawning season reaching minimum plasma concentrations between August and September (Figs. 2 and 3). Plasma T did not change significantly in 1993, but in 1994 showed a seasonal cycle similar to, but of smaller amplitude than 11KT, peaking in June and decreasing before the end of the spawning season. Milt volume also changed seasonally, in 1993 milt volumes were high in July and August while plasma androgens were decreasing rapidly. In 1994 plasma 11KT and T increased two months before milt volume increased in June, and in July milt volumes had decreased but were still elevated compared to pre-June levels, however both plasma 11KT and T had decreased. Yellowtail flounder males therefore show a seasonal cycle of plasma androgens that increase prior to the increased milt volume during the spawning season and decrease while milt volumes are still above pre-June levels. While milt volumes also show an obvious seasonal cycle, small amounts of milt containing motile sperm are present all year round (Figs. 4 and 5).

The milt may be residual from the preceding spawning season or spermatogenesis may be occurring all year round, however histological studies of the testes are necessary to determine this. Male dabs also have sperm present all year and milt volume is highest in the first two months of the three month spawning season (Htun-Han, 1978).

Since milt volumes are still elevated while plasma androgens are decreasing these data suggest that in male yellowtail flounder, similar to other male teleosts, a change from the production of C19 (e.g. androgens) to C21 (e.g. progestins) steroids may be expected to take place around the time of spawning. Plasma  $17\alpha,20\beta\text{P}$  was measured in both male and female yellowtail and was always  $\leq 2.0 \text{ ng ml}^{-1}$  and  $\leq 4.3 \text{ ng ml}^{-1}$  respectively, however this may not indicate the importance of its physiological role in yellowtail flounder. For example, in plaice  $17\alpha,20\beta\text{P}$  and  $17\alpha,20\beta21\text{-trihydroxy-4-pregnen-3-one}$  ( $17\alpha,20\beta21\text{-P}$ ) are the most effective progestins for inducing final oocyte maturation *in vitro*, however only low concentrations are ever found in the plasma (Canario and Scott, 1990). There may be other reasons for low plasma  $17\alpha,20\beta\text{P}$  in yellowtail flounder. Japanese flounder *Platichthys olivaceus* (Temminck and Schlegel) males show a daily cycle of milt production that is synchronised with female ovulation and plasma  $17\alpha,20\beta\text{P}$  shows a short, low amplitude surge at 2200 h (Matsuyama et al., 1995b). If yellowtail flounder undergo a similar daily cycle, the sampling schedule used in this study (monthly samples 0900 to 1200 h) would not detect these changes. Alternatively  $17\alpha,20\beta\text{P}$  may not be the important progestin in yellowtail flounder; so far  $17\alpha,20\beta21\text{-P}$  has been shown to be the maturation inducing steroid in female turbot *Scophthalmus maximus* L. (Mugnier et al.,

1995). Atlantic croaker *Micropogonias undulatus* L. (Trant et al., 1986; Trant and Thomas 1988) and striped bass *Morone saxatilis* (Walbaum) (King et al., 1995).

In yellowtail flounder spermatocrit tends to decrease during June to August, when milt volumes were highest, which indicates that some hydration of the milt may have occurred, but not in all individuals simultaneously (Figs. 4 and 5). In rainbow trout *Oncorhynchus mykiss* (Walbaum) (Büyükhapıoglu and Holtz, 1984; Munkittrick and Moccia, 1987), Atlantic salmon *Salmo salar* L. (Aas et al., 1991) and turbot (Suquet et al., 1992b) sperm concentration decreases as the spawning season progresses but in captive halibut sperm concentration increases (Methven and Crim, 1991). When the effect of increasing the frequency of stripping milt from males was examined it was shown to be correlated with a decrease in sperm concentration (Büyükhapıoglu and Holtz, 1984; Munkittrick and Moccia, 1987; Aas et al., 1991; Suquet et al., 1992b). The average milt volumes present in yellowtail flounder during the spawning season (0.2-1.8 ml) are similar to other teleost species for example extreme values in pike *Esox lucius* L. (0.1-1.5 ml), perch *Perca fluviatilis* L. (0.5-1.5 ml), whitefish *Coregonus* sp. L. (0.02-2.5 ml) and turbot (0.2-2.2 ml) (Suquet et al., 1994). Sperm motility also appeared to increase during the spawning season but this parameter was highly variable.

In the Newfoundland population of wild yellowtail flounder spawning occurs from May to July, peaking in June (Pitt, 1970). The beginning of egg release in captive females was similar to the peak spawning time found in wild populations of yellowtail flounder around Newfoundland, starting by the beginning of June in 1993 and by mid June in 1994 (Figs.

6 and 9). In 1994, plasma  $E_2$  was highly variable from December 1993 to August 1994. Eight out of nine females in this group spawned, however three of the spawning females only released small quantities ( $\leq 5$  ml) of low quality eggs in August (Fig. 8). Since recent studies have shown that captive female yellowtail flounder are capable of spawning 14-22 batches of eggs with an average batch volume of  $\sim 22$  ml in one season (Manning, 1996) these three females were classed as 'abnormal' spawners and their steroid profiles were analyzed separately from the 'normal' spawners. Under the same criteria all female spawners in 1993 were normal.

In the 1993 spawners plasma T remained low until March, increased rapidly to a peak at the beginning of the spawning season and then decreased until the end of the spawning season in August. In the 1994 'normal' spawners, plasma T peaked rapidly in July in the middle of the spawning season and decreased by August. In the 1993 spawners and in the 1994 'normal' spawners plasma  $E_2$  was elevated in November and increased steadily until April when it peaked and then began to decrease throughout the spawning season. In 1993 plasma  $E_2$  increased in October however the change was not statistically significant (Fig. 6a). In 1993 the non-spawning females showed no seasonal change in plasma T or  $E_2$  until after the spawning season when plasma  $E_2$  increased in October to  $5.7 \text{ ng ml}^{-1}$ , indicating the start of vitellogenesis for the following season (Fig. 7a). The increase in plasma  $E_2$  correlates well with the seasonal changes in gonadosomatic index (GSI) and histology recorded for female yellowtail flounder by Howell (1983) which also suggests that vitellogenesis commences in October.

Howell (1983) studied a Rhode Island population of yellowtail flounder that spawned from April to June, which is approximately two months ahead of the captive yellowtail flounder in this study. The start of vitellogenesis is defined histologically as when yolk globules first appear in the oocyte and the oocytes increase in diameter (Wallace and Selman 1981; Guraya 1994). Howell (1983) does not explicitly state when yolk globules first appeared in oocytes during the reproductive cycle but the definition provided for 'early maturing' oocytes states that yolk globules begin to appear in the final stages of their development. Transformation of 'early maturing' to 'late maturing' oocytes occurred from September to October, therefore yolk globules probably first appeared in August. GSI also remained relatively low from 2.5% in June to 3.2% until September when it steadily increased to 11% in February, sharply increased in early April at the beginning of the spawning season when it peaked at 19%, then decreased throughout the spawning season (Howell, 1983). If vitellogenesis begins in August or two months after the spawning season in the Rhode Island population of yellowtail flounder, vitellogenesis may begin in October in the captive yellowtail flounder in this study which supports the conclusions drawn from the seasonal changes in plasma  $E_2$  concentrations. Vitellogenesis may continue during the beginning of the spawning season as the youngest batches of oocytes continue to mature even while plasma  $E_2$  is decreasing. Red sea bream *Pagrus major* (Temminck and Schlegel) are also batch spawners and show continued vitellogenesis throughout the spawning season (Matsuyama et al., 1988). The physiological role of T in female teleosts is not well understood, this pattern of seasonal change could

be related to the role of T as a precursor to  $E_2$  (Kagawa et al., 1983; Kagawa et al., 1984) or to its possible role in stimulating the pituitary to release GtH in the pre-spawning GtH surge (Kagawa et al., 1983; Young et al., 1983; Kobayashi et al., 1989). The later peak in plasma T in 1994 may be related to the possible later start to spawning in 1994 compared to 1993.

The abnormal spawners showed no seasonal change in plasma T ( $0.5\text{--}2.7\text{ ng ml}^{-1}$ ) or  $E_2$  ( $0.7\text{--}4.3\text{ ng ml}^{-1}$ ), but plasma  $E_2$  concentrations were greater than observed in the non-spawners in 1993 ( $1.1\text{--}2.7\text{ ng ml}^{-1}$ ). Two of the abnormal spawners were classed as stage 3 while one remained at stage 2 throughout the spawning season. Together these observations indicate that the abnormal spawners began to develop vitellogenic oocytes prior to the spawning season and then failed to completely undergo vitellogenesis and oocyte maturation. Several factors may have contributed to this. All three of the abnormal spawners were blood sampled monthly while 4 out of the 5 normal spawners were sampled bimonthly. The stress of excess handling and an invasive procedure such as blood sampling may have had a negative effect on reproduction as is observed in many other teleost species (Barton and Iwama, 1991). However, in 1993 the numbers of bimonthly and monthly sampled fish in the spawning and non-spawning groups were almost equal. Alternatively the high water temperatures experienced in 1994 in approximately mid June may have inhibited spawning (Fig. 1). Pitt (1970) found wild yellowtail flounder around Newfoundland at temperatures ranging from  $-1.0\text{--}7.7^\circ\text{C}$ . From January to June in 1993 the average temperatures in captivity were within the range reported for wild fish, however

between August to October 1993 and in July and August 1994 average water temperatures were  $>7.7^{\circ}\text{C}$ . Captive turbot, sole *Solea solea* L. and seabass *Dicentrarchus labrax* L. do not release viable eggs above a certain water temperature (Bye, 1990). In turbot gametogenesis is retarded at  $<8^{\circ}\text{C}$  while egg quality deteriorates above  $16^{\circ}\text{C}$ , and in sole, egg release only occurs between temperatures of  $8\text{--}12^{\circ}\text{C}$  (Bye, 1990). High temperatures during the latter half of the spawning season may be inhibitory to ovulation in female yellowtail flounder.

The male yellowtail flounder showed no increases in body weight, length or CF in either 1993 or 1994 (Fig. 10). Female growth was different between the two years that fish were maintained in captivity (Fig. 11). Female body weight, body length and CF increased between November 1992 and November 1993, indicating that the fish grew in captivity. Body weight and body length appeared to increase steadily throughout the year but CF showed a slight decrease from June to September that may be related to the spawning period. In contrast during 1994 there was no significant change in body weight, length or CF, however CF did show a non significant peak in June followed by a non significant decrease in July and August that was similar to that observed in 1993. The main difference in captivity conditions between the two years was diet. From mid November 1993 the diet was changed from shrimp to a lower ration of moist commercial pellets. In mid April 1994 the pellet rations were increased, however this produced no growth in the females by August. There was a significant interaction between spawning status and seasonal change in the body weight and CF of females in 1993 which shows



that the pattern of seasonal change in body weight and condition in spawners is different to that in non-spawners (Figs. 12 and 14). In 1993 spawners both body weight and CF tended to be higher (not statistically significant) than in non-spawners before the spawning season and then lower (not statistically significant) than in non-spawners after the spawning season. These data tend to suggest that in female spawners a decrease in body weight occurs towards the end of the spawning season due to a decrease in ovarian weight. Howell (1983) showed that in the Rhode Island yellowtail flounder female GSI peaked (19%) at the beginning of the spawning season then decreased over the next four months to a post-spawning low (2.5%).

In conclusion both male and female yellowtail flounder were successfully maintained and fed in captivity throughout the reproductive cycle and produced viable gametes during the spawning season observed in wild yellowtail flounder.

## CHAPTER 3.

### **Gonadotropin releasing hormone-analogue (GnRHa) treatment increases sperm motility, seminal plasma pH and sperm production in yellowtail flounder (*Pleuronectes ferrugineus*).**

#### **3.1. Introduction**

This experiment investigated several aspects of the use of gonadotropin releasing hormone-analog (GnRHa) in male teleosts. Unlike most male teleosts investigated so far, captive yellowtail flounder *Pleuronectes ferrugineus* (Storer) possess motile sperm throughout the year ( $0.55 \text{ ml kg}^{-1}$ ), and show an increase in milt volume ( $0.52\text{-}5.97 \text{ ml kg}^{-1}$ ) at the time of spawning (May-August) (see Chapter 2). Female yellowtail flounder produce multiple batches of eggs, usually one batch per day over several weeks and male yellowtail flounder are thought to be involved in several spawning events in a season. Production of small volumes of milt is a common problem in fish farming (Zohar, 1989) and makes sperm collection difficult. Gonadotropin releasing hormones (GnRH) and their analogues (GnRHa) have been used very successfully in a wide range of freshwater and marine teleosts to either advance spermatogenesis and/or enhance milt production (Zohar, 1989; Pankhurst, 1994). Stimulation of milt volume is required over an extended period of time for the broodstock management of yellowtail flounder and for experimental purposes. Therefore, this experiment investigated the effect of prolonged administration

of gonadotropin releasing hormone analogue (GnRHa) on milt volumes in a repeat spawning male teleost.

The effect of GnRHa on reproduction in males is mediated by stimulation of the pituitary to release gonadotropin (GtH), which then stimulates the testes to produce steroids (Crim et al., 1983a; Weil and Crim, 1983; Takashima et al., 1984; Rosenblum and Callard, 1987). The different roles of the androgens testosterone (T), 11-ketotestosterone (11KT) and the progestin 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\beta$ P) in spermatogenesis, spermiation and milt hydration are not yet completely clear, and may vary between species (Loir and Billard, 1990). Recent evidence suggests that 17 $\alpha$ 20 $\beta$ P may be important in the final stages of milt hydration and the acquisition of sperm motility in teleosts (Fostier et al., 1987; Ueda et al., 1985; Miura et al., 1992), and may have its effects via alterations in sperm duct pH (Miura et al., 1992; Miura et al., 1995). Since yellowtail flounder produce small volumes of highly concentrated milt, the process of milt hydration may not be occurring in our captive broodstock. Therefore, we measured the response of the plasma steroids T, 11KT and 17 $\alpha$ 20 $\beta$ P, sperm motility and seminal plasma pH to GnRHa treatment, even though seasonal trends in 17 $\alpha$ 20 $\beta$ P have not been observed.

The effect of GnRHa treatment on sperm quality has only been examined in a few species, but there has been no indication of reduced sperm quality in carp *Cyprinus carpio* L. (Ngamvongchon et al., 1987) and yellow perch *Perca flavescens* L. (Dabrowski et al., 1994). The ultimate measure of sperm quality is fertilization ability, therefore this

experiment investigated the effect of GnRH $\alpha$  administration on egg fertilization rates, larval hatch rates and larval appearance.

### **3.2. Materials and methods**

#### *3.2.1. Experimental design*

On 12 and 13 July 1994, 29 male fish (average weight 570 g -range 200-877 g) were divided into four groups (Table 5) in four identical, covered 250 l tanks provided with a continuous supply of fresh ambient seawater which ranged from 6-16°C over the duration of the experiment. One of four treatments were randomly assigned to each tank, either control pellet implant, low dose GnRH $\alpha$  pellet implant, high dose GnRH $\alpha$  pellet implant or 30  $\mu\text{g kg}^{-1}$  GnRH $\alpha$  microsphere injections. Because counts of fertilized eggs in the fertilization trials could only be completed for 18 fish a day (see Section 3.2.2.) the entire experiment was further divided into two groups (across treatments) so that the fish could be treated and sampled over two consecutive days and data could be collected from the fertilization trials. Two days before hormone treatment all fish were manually stripped of all available sperm before being anesthetized with 2-phenoxyethanol (0.05% dosage), blood sampled and weighed and this sampling day was designated day -2. This procedure was repeated at 4, 12, 29, 48 and 88 days after hormone treatment. Sperm motility, pH, spermatocrit and milt volume collected were measured in all milt samples. Sperm collected on days -2 (before hormone treatment), 4 and 12 were also tested for fertility; after this time no viable eggs were available from our broodstock females.

### 3.2.2. Fertility Trial

Sperm fertility was tested by mixing 5  $\mu$ l undiluted milt to a 60  $\mu$ l aliquot (~125 eggs) of oocytes (egg diameter, 0.75-1.1 mm) in a petri dish and then adding an equal volume of seawater (with antibiotics added concentrations, 30 mg l<sup>-1</sup> penicillin G, 50 mg l<sup>-1</sup> streptomycin sulphate). This preparation was mixed again and left for 2 min, after which 25 ml of seawater was added to the petri dish and the dish was placed in an incubator set at 5°C. Three replicates of each sperm sample were tested. Fertilization trials usually started 2 h after milt collection and all replicates of a fertilization trial were fertilized within 1.0-1.5 h, including one set of 'blank replicates' with no milt added to test for parthenogenetic egg development. Approximately 6 hours after fertilization, numbers of fertilized eggs were counted by observing the 2-32 cell stage of development under 20x magnification (it is difficult to discriminate between fertilized and unfertilized eggs after the 32 cell stage). Eggs were counted as fertilized (2-32 cell stage, -floating or not), unfertilized (clear, floating and showing no cell division) or unviable (uneven, opaque and/or not floating). Since counting began while some eggs were still at the 2-cell stage, (in order to complete counts before the eggs developed past 32 cells), initial counts were checked at the end of the trial. Fertilization rates were calculated from numbers of fertilized eggs out of the total number of eggs (since later work showed that "clear eggs that did not float" were incorrectly identified as nonviable at the time of data collection). Every two days after fertilization, dead eggs (opaque eggs showing no embryo development) and hatched larvae were counted and removed from the petri dish and the

seawater was changed. Larvae were also classified as 'bent' if notable deformities or unusual swimming patterns were present. This procedure was repeated every two days until all eggs had either hatched or died.

### 3.2.3. Motility, spermatocrit and milt pH determinations

As soon as possible after sperm collection and completion of the fertilization trial, (usually four hours) motility was tested under 100x magnification in chilled filtered seawater (5°C). A 10 µl pipette tip was dipped into the sperm sample and stirred quickly into a 100 µl sample of seawater already prepared on a prechilled slide on the microscope stage. Percentage sperm activated at the point of mixing and duration of forward motility of the majority of activated sperm (swim time in seconds) were noted in each motility trial. Milt pH was measured on indicator paper (ColourpHast pH sticks, EM Science, New Jersey, U.S.A., pH range 6.5-10.0 and 5.0-10.0), and milt volume was measured in marked Eppendorf vials. Spermatocrit was measured using sealed haematocrit tubes after centrifuging at  $15,000 \times g$  for 30 min. and where possible 3 replicates of spermatocrit were measured. Cell concentration was calculated according to the relationship between spermatocrit and sperm concentration (cells ml<sup>-1</sup>) ( $R^2 = 0.83$ ) (see Chapter 4):

$$10 \frac{\text{spermatocrit} - 1137.63}{119.53} = \text{concentration}$$

Cell concentration (cells ml<sup>-1</sup>) data was combined with milt volume (ml kg<sup>-1</sup> body weight) data to give sperm production (cells kg<sup>-1</sup> body weight).

### 3.2.4. Blood samples

0.6 ml blood samples were taken from the caudal sinus of anesthetized fish using preheparinised 23 G needles, and stored on ice. Approximately two hours after sampling blood samples were centrifuged in 1.5 ml Eppendorf tubes for 15 min at  $9170 \times g$  at  $4^{\circ}\text{C}$ . Blood plasma was removed in 140  $\mu\text{l}$  aliquots and stored in 0.5 ml Eppendorf tubes at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  for future analysis.

### 3.2.5. GnRH $\alpha$ pellet preparation

100% cholesterol pellets were prepared (Crim et al., 1983b; Sherwood et al., 1988) using the GnRH analogue (D-Ala<sup>6</sup>.Pro<sup>9</sup>-NHet)LHRH (Syndel Lab.s, Vancouver, B.C.), to make either 0  $\mu\text{g}$ , 20  $\mu\text{g}$  or 100  $\mu\text{g}$  dosage pellets, giving 0  $\mu\text{g kg}^{-1}$  (control pellet), 44  $\mu\text{g kg}^{-1}$  (low dose pellet) and 197  $\mu\text{g kg}^{-1}$  (high dose pellet) dosages respectively. The analogue was weighed and dissolved in 50% ethanol for washing and transfer. The solution was then mixed into cholesterol powder and dried in a  $30^{\circ}\text{C}$  oven. Molten cocoa butter was mixed with the dry, pulverised paste and then 27 mg portions were compressed into pellets with a mold.

### 3.2.6. Microsphere preparation

Biodegradable microspheres made of a copolymer of a fatty acid dimer and sebacic acid (FAD:SA 25:75 molar ratio), containing GnRH $\alpha$  were suspended in vehicle (1% sodium-carboxymethylcellulose, 0.2% Tween 80, 0.14% methyl p-hydroxybenzoate,

0.014% propyl p-hydroxybenzoate and 5% Sorbitol (Courtesy Dr. Y. Zohar & C.C. Mylonas, Maryland, U.S.A.) (Mylonas et al., 1995) and injected in the dorsal musculature of the fish using an 18 G needle. The average GnRH $\alpha$  dosage was 30  $\mu\text{g kg}^{-1}$  for each fish.

### 3.2.7. Radioimmunoassay

Steroids were extracted from 0.1 ml plasma samples and prepared for steroid assay according to Harmin and Crim (1993). Testosterone concentration in the plasma was determined according to Harmin et al., (1995b) using iodinated testosterone as a tracer (purchased from International Diagnostic Services, Scarborough, Ontario, Canada) with the exception that after the addition and incubation of ethylene glycol separating reagent the assay tubes were centrifuged at  $1650 \times g$  for 30 min rather than 15 min.

11KT and 17 $\alpha$ .20 $\beta$ P were assayed using tritiated labels (prepared by C. Wilson, Nfld. Canada, Truscott (1981)) according to the 11KT procedure used by Harmin and Crim (1993) with the following exceptions. 11KT and 17 $\alpha$ .20 $\beta$ P antibodies (courtesy of D.R. Idler, Nfld. Canada) were diluted at 1:40,000 and 1:2000 respectively, and the 17 $\alpha$ .20 $\beta$ P label was added at ca. 5000 cpm per tube.

Extraction efficiencies were 88.06%, 84.59% and 85.83% for 17 $\alpha$ .20 $\beta$ P, T and 11KT respectively and all steroid data were corrected accordingly. 17 $\alpha$ .20 $\beta$ P, T and 11KT interassay standards varied between 0.02-0.03 ng ml $^{-1}$ , 0.87-1.38 ng ml $^{-1}$  and 0.40-0.50 ng ml $^{-1}$  respectively (n = 2 for each steroid). All samples for individual fish over time were



done within one assay, so that seasonal comparisons were made together. T antibody cross-reactivity was 0.0009% for estriol, <0.0007% for estrone, 0.0035% for estradiol, 0.96% for androstenedione, 0.19% for 11KT and 0.0034% for progesterone (Harmin et al., 1995b). 11KT antibody cross-reactivity with testosterone and 11 $\beta$ -hydroxy-testosterone was <0.1% (Ng and Idler, 1980). 17 $\alpha$ ,20 $\beta$ P antibody cross-reactivity was 14% for 5 $\alpha$ -pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol, and there was no cross reaction for 17 $\alpha$ -hydroxyprogesterone, 17 $\alpha$ -hydroxy-20 $\alpha$ -dihydroprogesterone, progesterone, 20 $\beta$ -dihydroprogesterone, cortisol, cortisone, 11-deoxycortisol, 20 $\beta$ -dihydrocortisone, 5 $\beta$ -pregnan-3 $\alpha$ , 17 $\alpha$ , 20 $\beta$ -triol (Peter et al., 1984).

### 3.2.8. *Statistics*

One way ANOVA, two way ANOVA and Duncan's Multiple Range Test (Zar, 1984) were used to analyse sperm and steroid data. Fertilization rates, larval hatch rates and larval appearance data were analysed using a two way ANOVA with three replicates for each fish nested with each treatment and group, therefore the effect of treatment, group, replicates and the interaction of treatment and group effects was analysed in this model. Square root, arcsine and log transformations were used to fulfill the assumptions of ANOVA. The level of statistical significance was set at  $P < 0.05$ . Kruskal Wallis non-parametric one way ANOVA was used to analyze day -2 swim time data.

### 3.3. Results

#### 3.3.1. Sperm production, milt volume and spermatocrit

On day -2, prior to hormone treatment sperm production (cells  $\text{kg}^{-1}$ ), milt volume ( $\text{ml kg}^{-1}$ ) and spermatocrit were similar for the four different groups of males (Figs. 15, 16 and 17). After hormone treatment sperm production and milt volume were increased for up to 4 weeks in all groups of males exposed to GnRH $\alpha$  ( $P < 0.010$ ,  $P < 0.0001$  respectively). However, spermatocrit fell significantly ( $P < 0.0001$ ) for both control and hormone-treated males between days 12 and 29 remaining low for the remainder of the experimental period.

By the last day of this experiment, the number of males in the GnRH $\alpha$  microsphere group and the high dose pellet groups were reduced to three due to mortalities from handling and perhaps due to high water temperatures (Table 5). Despite the loss of individuals in all groups, by day 88 the total sperm production (Fig. 18, low dose v. control,  $P < 0.04$ ) and total milt volume (Fig. 19, all hormone treated males v. control,  $P < 0.001$ ) remained significantly elevated.

#### 3.3.2. Percent sperm activated, sperm swim times and pH

Prior to hormone treatment (day -2) percent sperm activated and sperm swim times were not different between the four groups of males (Fig. 20). Milt pH was not measured prior to hormone treatment. On day 88 insufficient milt was available from the microsphere treated fish to collect data on milt pH or sperm motility.

Following hormone treatment, percent sperm activated, sperm swim times and milt pH were higher in all hormone treated groups compared to the control group ( $P < 0.0001$ ,  $P < 0.0001$  and  $P < 0.03$  respectively), however no pattern of change across time was detected. On average, percentage sperm activated was higher in the high dose pellet treatment ( $83.9 \pm 7.1\%$ ) than in all the other treatments except the microsphere treated fish. The low dose pellet fish and microsphere treated fish both had higher percent sperm activated ( $49.2 \pm 8.4\%$  and  $52.3 \pm 8.8\%$ , respectively) than the control treated fish ( $19.2 \pm 8.4\%$ ). On average, swim times were  $19.2 \pm 6.8$  s in the control group, while they ranged between  $47.2$ - $57.2 \pm 5$  s in the treatment groups. Across time, milt pH averaged  $6.8 \pm 0.1$  in the control fish and  $7.2$ - $7.3 \pm 0.1$  in the hormone treated fish.

### *3.3.3. Egg fertilization rates, larval hatch rates and larval appearance*

Before hormone treatment there was no difference in fertilization rates or larval appearance associated with sperm from the four treatment groups of males, however hatch rates were low in the low dose pellet group compared to the controls ( $P < 0.006$ ) (Figs. 21 and 22).

Four days after hormone treatment there was no affect of hormone treatment on fertilization rates, hatch rates or larval appearance ( $P < 0.060$ ,  $P < 0.371$ ,  $P < 0.544$  respectively).

Twelve days after hormone treatment there was a significant interaction between treatment effects and group effects on fertilization rates, hatch rates and larval appearance

( $P < 0.014$ ,  $P < 0.0003$ ,  $P < 0.039$  respectively). The interaction effect on hatch rates and larval appearance was probably driven by the unexplained failure of group 2 eggs in the control treatment to hatch (unlike all other fertilization trials in this experiment); there was no explanation for this failure.

There was a significant maternal (group) effect on fertilization rates and hatch rates on days -2 and 12 (fertilization rates-day -2  $P < 0.0001$ , day 12  $P < 0.016$ ; hatch rates -day -2  $P < 0.0001$ , day 12  $P < 0.007$ ) and on larval appearance on day -2 only ( $P < 0.0003$ ). There was high variation in the replicates for fertilization rates on days -2 and 4, for hatch rates on day 12 and larval appearance on day -2 only.

#### 3.3.4. Steroids

T, 11KT and  $17\alpha,20\beta\text{P}$  were detectable in the plasma but not significantly different between treatments on day -2 (Fig. 23). Plasma testosterone levels decreased significantly from  $1.44 \pm 0.28 \text{ ng ml}^{-1}$  after day 4 and thereafter remained low ( $1.04\text{--}0.33 \text{ ng ml}^{-1}$ ) ( $P < 0.020$ ). there was no significant effect of treatment and no significant interaction between these two effects. Plasma 11KT and  $17\alpha,20\beta\text{P}$  levels showed no significant effect of treatment, day or any significant interaction between day and treatment.

Table 5. Sample sizes for each treatment on each day of the experiment after hormone treatment.

DAY	TREATMENT			
	Control	GnRHa Microspheres	Low GnRHa dose pellet	High GnRHa dose pellet
-2	8	7	7	5
4	6	7	7	5
12	5	6	7	4
29	4	6	5	4
48	4	3	5	3
88	4	3	5	3

Fig. 15. The effect of hormone treatment on sperm production (cells  $\text{kg}^{-1}$ , mean  $\pm$  s.e.) over time. Circles = control pellets, squares = GnRH $\alpha$  microspheres, triangles = low dose GnRH $\alpha$  pellets and diamonds = high dose GnRH $\alpha$  pellets.

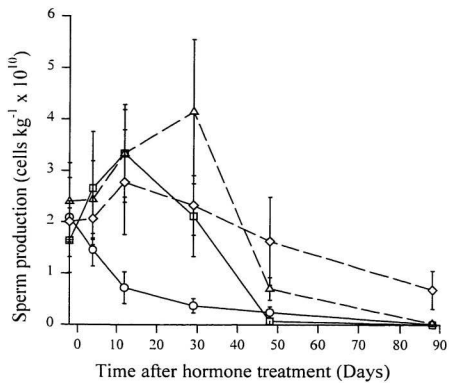


Fig. 16. The effect of hormone treatment on milt volume ( $\text{ml kg}^{-1}$ , mean  $\pm$  s.e.) over time.

Circles = control pellets, squares = GnRH $\alpha$  microspheres, triangles = low dose

GnRH $\alpha$  pellets and diamonds = high dose GnRH $\alpha$  pellets.



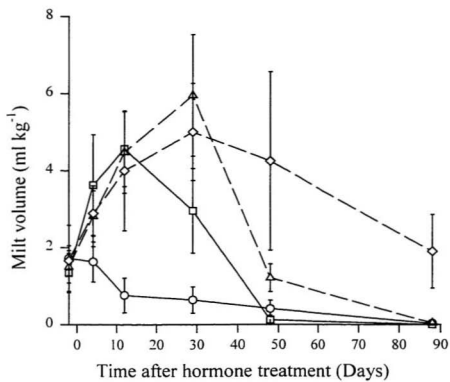


Fig. 17. The effect of hormone treatment on spermatocrit over time. Circles = control pellets, squares = GnRHa microspheres, triangles = low dose GnRHa pellets and diamonds = high dose GnRHa pellets. Letters show Duncan's grouping according to days after treatment.

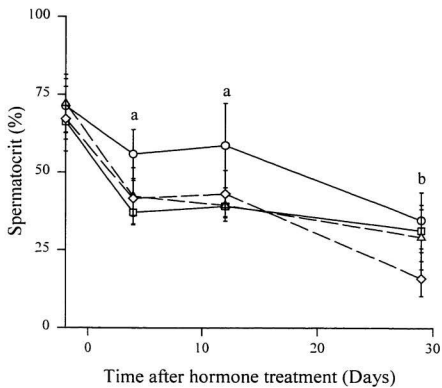


Fig. 18. Total sperm production (cells  $\text{kg}^{-1}$ , mean  $\pm$  s.e.) collected from survivors 88 days after implantation ( $F = 3.92$ ,  $P < 0.0398$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).

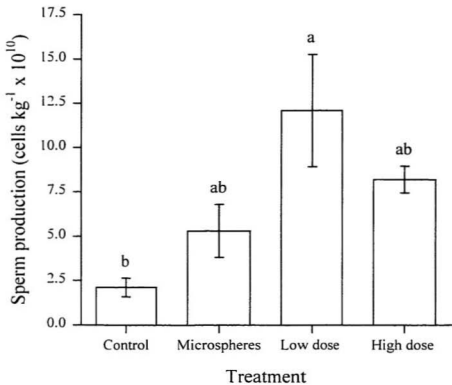


Fig. 19. Total milt volume ( $\text{ml kg}^{-1}$ , mean  $\pm$  s.e.) collected from survivors 88 days after implantation ( $F = 11.10$ ,  $P < 0.001$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).

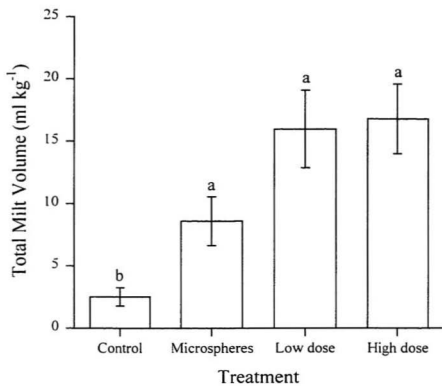


Fig. 20. Changes in milt pH, percent sperm activated and sperm swim time with time after hormone treatments. Circles = control pellets, squares = GnRHa microspheres, triangles = low dose GnRHa pellets and diamonds = high dose GnRHa pellets.



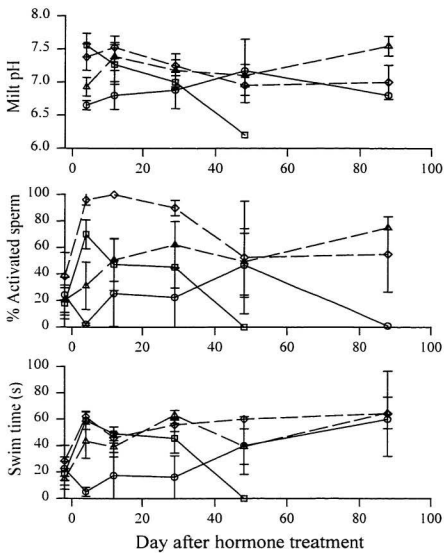


Fig. 21. Comparison of % Fertilized Eggs/Total Eggs from each fertilization trial on days -2 (before hormone treatment), 4 and 12 after different hormone treatments. Open symbols are group 1 and filled symbols are group 2 fertilization trials.

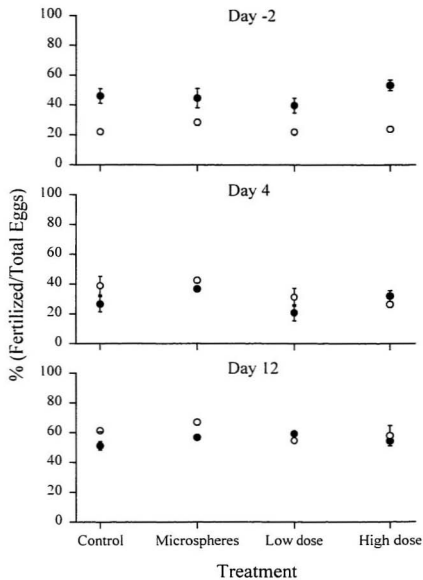


Fig. 22. Comparison of % Hatch/Total Fertilized Eggs (triangles) and % Bent Larvae/Hatched Eggs (circles) from each fertilization trial on days -2 (before hormone treatment), 4 and 12 after different hormone treatments. Open symbols are group 1 and filled symbols are group 2 fertilization trials.

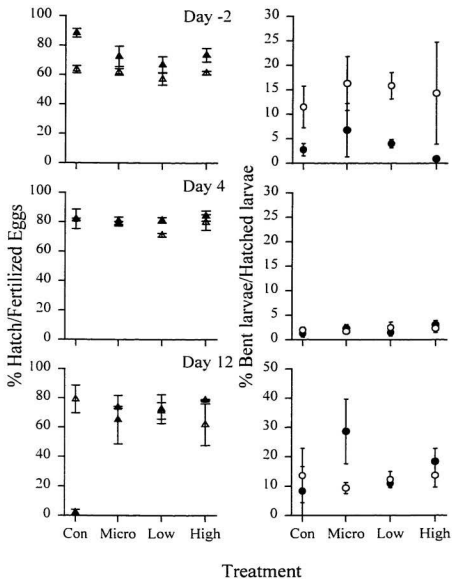
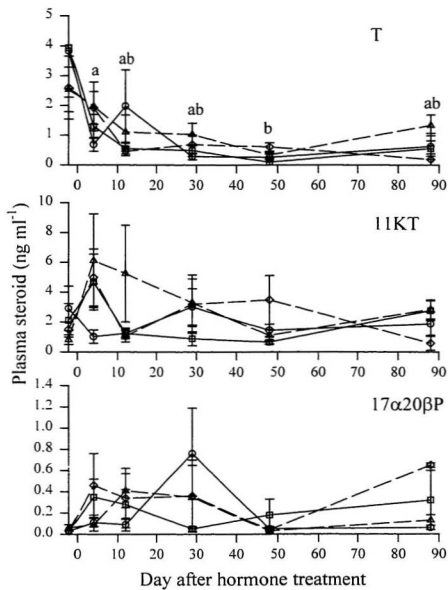


Fig. 23. Change in plasma steroids with time after implantation with either control pellets (circles), or GnRHa microspheres (squares), low dose GnRHa pellets (triangles) or high dose GnRHa pellets (diamonds). Means with different letters indicate significantly different days across treatments (Duncan's multiple range means test,  $P < 0.05$ ).



### 3.4. Discussion

GnRHa delivered by either microspheres or pellets successfully increased sperm production in yellowtail flounder males compared to control treated males (Fig. 15). Sperm production remained high for up to 29 days after implantation then decreased in all treatments (including control pellets) 48 days after implantation and remained low for the remainder of the experiment. The increase in milt volume was more variable than sperm production and depended on the hormone treatment (Fig. 16). The GnRHa containing microspheres appeared to cause a peak in milt volume at 12 days, compared to the low dose pellets which appeared to stimulate a later peak at 29 days. Milt volumes in the high dose pellet group also peaked at 29 days but appeared to remain elevated until at least 48 days after implantation and possibly at 88 days after treatment. Because a two way ANOVA showed that there was a significant interaction between treatment and time, no further analysis was conducted to determine which means were significantly different.

*In vitro* studies indicate that fish implanted with cellulose:cholesterol pellets containing GnRHa, receive an initial 'surge' of hormone over 24 hours, followed by a constantly decreasing release of hormone (Sherwood et al., 1988). 100% cholesterol pellets containing GnRHa caused a 4 to 8 week response of the pituitary and/or gonads in salmonids (Crim et al., 1983a; Crim et al., 1983b; Crim et al., 1988) and in winter flounder *Pleuronectes americanus* (Walbaum) (Harmin et al., 1995a). In yellowtail flounder the two different GnRHa dose pellets appeared to have a similar effect on milt volume until 29 days post implantation. After that time the high dose GnRHa pellets



seemed to have a longer stimulatory effect on milt volume, although total milt volume collected from survivors was not different between the 3 hormone treatments. Similar plasma androgen responses occurred in prespermiating winter flounder males regardless of the single dose of GnRHa ranging from 2-200  $\mu\text{g kg}^{-1}$  for each fish (Harmin and Crim, 1993). Harmin and Crim (1993) suggested that even 2  $\mu\text{g kg}^{-1}$  represented an overdose of GnRHa in winter flounder, which is why dose-dependent effects were not observed, however in the 200  $\mu\text{g kg}^{-1}$  group, plasma GnRHa was elevated for several days longer than the other treatments and spermiation was stimulated in 3 out of the 5 fish treated compared to lower numbers in the other treatments.

Hormone release from the GnRHa containing microspheres is temperature dependent (Tabata and Langer, 1993; Mylonas et al., 1995). Mature male striped bass *Morone saxatilis* (Walbaum) held at 18°C showed a 4-10 fold increase in sperm production for at least 14 days after microsphere injection, with a peak in sperm production two days after injection (Mylonas et al., 1995). In Atlantic salmon *Salmo salar* L. held at 11°C, milt volumes increased 2.5-fold 10 days after microsphere injection, however milt was only collected once (Mylonas et al., 1995). In this experiment water temperatures varied between approximately 10-15°C, and the sperm production and milt volume response to microspheres appeared to peak at 12 days post injection.

It remains to be seen if yellowtail flounder males will respond to GnRHa treatment at other stages of the reproductive cycle. Winter flounder males show a positive response to hormone stimulation (increased GSI and plasma androgens only) at all times of the year

except for a brief period directly after the spawning season when the gonads are thought to be sexually regressed (Harmin et al., 1995a). GnRHa treatment significantly advanced the onset of milt production by 2 months, however winter flounder have already undergone gonadal recrudescence at this point in their seasonal cycle. In contrast captive yellowtail flounder males show less distinct seasonal cycles of milt production, which may be an artefact of captivity (see Chapter 1). It is likely that it will be possible to stimulate milt production by GnRHa treatment for a larger proportion of the annual reproductive cycle of yellowtail flounder than in winter flounder.

Spermatoctrit decreased significantly between 12 and 29 days after implantation in all treatments including the control, therefore the decrease in sperm concentration may have been due to seasonal changes in the milt (Fig. 17). Total sperm production of day 88 survivors was significantly higher than controls in the low dose pellet group only, whereas total milt volumes produced by survivors were higher than the controls in all hormone treated groups (Figs. 18 and 19). When changes in sperm production are analysed over time, sperm production was significantly higher in all treated groups compared to the control group, but decreased in all treatments including the controls between days 29 and 48 (Fig. 15). Together these data indicate that hormone treatment initially increased both sperm production and milt volume, however after 29 days sperm production decreased in all treatments while milt volume remained high. GnRHa treated rabbitfish *Siganus guttatus* (Bloch) (Garcia, 1993), landlocked Atlantic salmon (Weil and Crim, 1983), winter flounder (Harmin et al., 1995a) and carp (Takashima et al., 1984) all showed both

increased milt volume and increased sperm concentration for at least part of their seasonal cycle.

Even if GnRH $\alpha$  treatment of yellowtail flounder males only stimulated milt hydration rather than sperm production, the effect on milt collection is still useful. Small milt volumes (0.1-0.3 ml) are difficult to collect and handle, particularly if the milt is highly concentrated and viscous. In addition, urine contamination, which can have a negative effect on sperm quality, is difficult to avoid (Clearwater and Crim, 1996). Finally, the sperm:egg ratios required for high fertilization rates in the laboratory using 'dry' fertilization methods is easily met by the most dilute sperm produced by hormone treated yellowtail flounder (see Chapter 4).

Male yellowtail flounder showed no clear pattern in the response of plasma steroids, either between GnRH $\alpha$  treatments or across days (Fig. 23). This experiment was conducted during the spawning season, and captive yellowtail flounder males kept under similar conditions for measurement of seasonal changes in plasma steroids, showed a peak in plasma 11KT (10.95 ng ml<sup>-1</sup>) and T (7.95 ng ml<sup>-1</sup>) in May and June and then both plasma steroids levels decreased to around 1.5 ng ml<sup>-1</sup> in August (see Chapter 2). Initial plasma T and 11KT of fish in this experiment were 3.27 ng ml<sup>-1</sup> and 1.90 ng ml<sup>-1</sup> which is lower than that measured in seasonal groups at the same time. Plasma steroid levels may have been negatively affected by the stress of captivity as has been shown in other teleost species (Pickering et al., 1987; Sumpter et al., 1987).

Plasma  $17\alpha 20\beta\text{P}$  was  $< 1.2 \text{ ng ml}^{-1}$  throughout this experiment, even though it has been shown to be involved in milt hydration in other species of teleost (Miura et al., 1991; Ohta et al., 1996). There are several possible reasons for this. In both male and female plaice *Pleuronectes platessa* L. it has been shown that  $17\alpha 20\beta\text{P}$  is rapidly metabolized in the gonads before it is released into the bloodstream, but nonetheless it is produced in large amounts in the testis of spermiating males, and the ovaries of spawning females (Scott and Canario 1992; Inbaraj et al., 1995; Scott et al., 1995). Therefore in some species, particularly batch spawners, it may be impossible to observe an increase in plasma concentrations of progestins or the sampling schedules used may not have been sensitive enough to record a rapid peak of progestins just prior to spermiation. Alternatively,  $17\alpha 20\beta\text{P}$  may not be the important progestin in all teleosts.  $17\alpha 20\alpha\text{P}$ , the isomer of  $17\alpha 20\beta\text{P}$ , is produced by carp, goldfish *Carassius auratus* L. and flounder *Paralichthys flesus* L. testicular fragments *in vitro* (Asahina et al., 1990; Barry et al., 1990; Asahina et al., 1993; Asahina et al., 1994) and is also found in the plasma, urine and milt of plaice (Scott et al., 1991).

In yellowtail flounder, sperm quality as measured by fertilization rates showed no significant difference between treatments (Fig. 21). Only very extreme differences in sperm viability would have been detected by this method since highly concentrated sperm samples were used to fertilize the eggs. However, hatch rates and percentage bent larvae also showed no significant difference between treatments, indicating that GnRHa treatment of mature male yellowtail flounder has no negative effect on sperm fertilizing ability and

subsequent embryo quality up to hatch (Fig. 22). The differences observed between fertilization groups reflects a maternal effect since different batches of eggs were used for the two groups of fertilization trials. The significant interaction terms observed on day 12 were probably driven by an unexplained failure of the group 2 eggs in the control treatment to hatch. Treatment of pike *Esox lucius* L. with salmon GtH or pituitary extracts produced sperm with good fertilizing ability, whereas oocytes in the same experiment showed decreased quality (Billard and Marcel, 1980). Treatment of mature female yellowtail flounder with GnRHa during the spawning season resulted in higher quality eggs than control fish that spawned later in the season (Larsson et al., 1997). Sperm fertilization rates were not different between male grey mullet *Mugil cephalus* L. injected with 17 $\alpha$ -methyltestosterone and controls (Lee et al., 1992).

Carp and yellow perch treated with GnRHa show good sperm motility (Ngamvongchon et al., 1987; Dabrowski et al., 1994). For yellowtail flounder, motility trials showed higher percent activation and longer swim time for sperm of the hormone treated groups compared to the control group; milt pH was also higher in the hormone treated groups (Fig. 20). Interestingly 17 $\alpha$ 20 $\beta$ P has recently been shown to have a positive effect on the acquisition of sperm motility in masu salmon *Oncorhynchus masou* (Brevoort) and Japanese eel *Anguilla japonica* (Temminck and Schlegel) via alterations in sperm duct pH (Morisawa and Morisawa, 1988; Miura et al., 1992; Miura et al., 1995). Baynes and Scott (1985) have found correlations between 17 $\alpha$ 20 $\beta$ P and changes in the ionic composition of the seminal plasma of rainbow trout *Oncorhynchus mykiss* (Walbaum). As has already

been discussed, milt hydration in many species of male teleosts appears to be stimulated by  $17\alpha,20\beta\text{P}$  and plasma levels of this hormone may not always indicate the physiological importance of the steroid (Matsuyama et al., 1995a). Yellowtail flounder showed increased milt production, volume, motility and pH suggesting that GnRHa treatment may have affected these parameters and possibly exerted its influence by changes in progesterin metabolism in the testis. However, in yellowtail flounder, milt pH can be influenced strongly by urine contamination which is difficult to avoid in this species, and may have occurred in the control fish causing the pH to decrease in comparison to the treated fish. The role of  $17\alpha,20\beta\text{P}$  in milt hydration and the acquisition of motility in yellowtail flounder should be further examined by injecting fish with gonadal steroids and observing their effects on milt parameters.

## CHAPTER 4.

### **Milt storage, sperm concentration and the effects of pH and osmolality on sperm motility, in yellowtail flounder (*Pleuronectes ferrugineus*).**

#### **4.1. Introduction**

Like most other externally fertilizing marine teleosts (Morisawa and Suzuki, 1980), yellowtail flounder *Pleuronectes ferrugineus* (Storer) sperm is immotile when collected from the urogenital pore and is activated upon dilution in seawater. Preliminary data showed that mass forward motility tends to last from 20-60 s (Clearwater and Crim, unpublished data). Changes in osmotic pressure, pH and ionic composition of the diluent compared to the seminal plasma are thought to be the most important factors in triggering sperm activation in teleost fish (Billard et al., 1992), although changes in other factors such as CO<sub>2</sub> concentration may also be important in certain species, for example turbot *Scophthalmus maximus* L. (Dreanno et al., 1995). In marine fish an increase in osmotic pressure relative to the seminal plasma is the most commonly known factor causing sperm activation, while sperm are less sensitive to changes in pH (Billard et al., 1992). In contrast in salmonids sperm activation is inhibited by high concentrations of K<sup>+</sup> in the seminal plasma (Morisawa et al., 1983). Some progress has been made recently on elucidating the mechanisms of sperm motility in salmonids. The current model suggests that the dilution of K<sup>+</sup> ions in the seminal plasma affects the sperm membrane, possibly through the actions of Ca<sup>2+</sup> and calmodulin, and causes the intracellular synthesis of cyclic

AMP. The cyclic AMP causes the activation of a protein kinase resulting in the phosphorylation of a unidentified protein which in turn possibly has an affect on the sperm flagella that eventually results in motility (Morisawa, 1985). In contrast, very little is known about sperm activation in other teleost species (Billard et al., 1992).

Yellowtail flounder males produce relatively low volumes of highly concentrated sperm (0.2-1.8 ml), therefore in order to manage male yellowtail flounder broodstock effectively it is important to be able to determine the sperm concentration of milt and dilute it to an optimal concentration for fertilization without activating the sperm. This investigation examined the motility response of yellowtail flounder sperm to diluents of different osmotic pressure and pH and examined three different methods of measuring sperm concentration, counting in a haemocytometer, spermatocrit, and spectrophotometry. Sperm storage is also useful when broodstock spawning times are not synchronised, when gametes must be transported to the incubation facility or to allow selective use of gametes from desirable spawners (Stoss, 1983). Many sperm storage experiments investigate changes in motility after storage and neglect changes in fertility. Except for Saad et al. (1988) the investigations that do examine fertilizing ability of stored sperm do not examine fertility at a range of sperm concentrations which has the advantage of demonstrating subtle changes in sperm quality. The effect of short term cool storage on yellowtail flounder sperm motility and fertility was examined at a range of milt dilutions and compared to the fertility of freshly collected sperm.



## 4.2. Materials and methods

### 4.2.1. Broodstock

From May to September 1995, three groups of adult yellowtail flounder (160-814 g body weight) were held in 250 l tanks (12 fish per tank, including at least 2 spawning females) with flow-through seawater at either ambient temperatures or in a mixture of ambient and heated/chilled seawater (ranging 1.0-11.0 °C seasonally). Fish were exposed to a simulated natural photoperiod for St. John's, Newfoundland (47°20' N, 52°45' W) using a 60 W bulb (75-190 lux) 80 cm above each tank controlled by an on-off electronic timer (Bishop, 1988). Fish were fed 2-4 times per week on a commercial moist pellet feed (Connor Bros., Blacks Harbour, New Brunswick) at rations of ~1.0-2.0 % body weight day<sup>-1</sup>.

### 4.2.2. Milt collection

Milt samples were collected from the urogenital pore of male yellowtail flounder using 1 ml syringes while taking care to avoid urine contamination, then stored in either syringes or test tubes on ice.

### 4.2.3. Measurement of pH and osmolality

Milt and urine pH were measured with indicator paper (ColourpHast pH sticks, EM Science, New Jersey, U.S.A., pH range 6.5-10.0 and 5.0-10.0), buffer pH and seawater pH were measured using a Corning Model 12 Research pH Meter. Sperm samples were

centrifuged in 1.5 ml Eppendorf vials for 5 min at  $9170 \times g$  at  $4^{\circ}\text{C}$  to obtain seminal plasma. Osmolality was measured using a Fiske 110 osmometer.

#### *4.2.4. Effect of pH and osmolality on sperm motility*

Buffer (DCSB4 -adapted from Chambeyron and Zohar (1990). 0.001 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.083 M glycine, 0.030 M Trizma basic and 0.150 M sucrose, pH 8.0) and seawater were used as diluents. Osmolality was altered by diluting buffer with NaCl (100-500 mM) or diluting seawater with distilled water. pH was altered by diluting either buffer or seawater with 0.1 M HCl or 0.1 M NaOH. These methods were adapted from Chambeyron and Zohar (1990). The effects of changes in pH or osmolality of buffer or seawater on percentage sperm activated and sperm swim times of pooled milt samples were tested in motility trials. A 10  $\mu\text{l}$  pipette tip was dipped in the undiluted milt and then rapidly stirred in 100  $\mu\text{l}$  of diluent on a microscope slide under 100x magnification. Percentage sperm activated at the point of mixing and duration of forward motility of the majority of activated sperm (swim time in seconds) were noted in each motility trial.

#### *4.2.5. Measurement of sperm cell concentration by spectrophotometry*

Urine samples were collected from anaesthetized fish using silastic tubing (PE 90 - internal diameter = 0.86 mm, external diameter = 1.22 mm) inserted through the urogenital pore into the bladder. Gentle pressure on the bladder expressed urine through the tubing into an Eppendorf tube which was then stored on ice. Seminal plasma was

pipetted from milt samples that had been centrifuged for  $9170 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Three replicates of each milt sample were diluted in buffer to 1000 fold or 5000 fold for counting in a haemocytometer and 100 fold for spectrophotometric measurements. Diluted milt, diluted seminal plasma and diluted urine samples were measured at 13 different wavelengths in the spectrophotometer to select a wavelength appropriate for routine measurement based on the relationship between these three variables. Buffer absorbance was also measured at all wavelengths compared to a diluted water blank. Once the wavelength (420 nm) was selected the absorbance, concentration and spermatocrit of 28 milt samples was measured to examine relationships between these three variables. This method was adapted from Suquet et al., 1992a and Ciereszko and Dabrowski, 1993.

#### *4.2.6. Spermatocrit and haemocytometer counts*

A  $10 \mu\text{l}$  sample of milt diluted 1000 fold or 5000 fold in buffer was counted in each side of the haemocytometer after a 15 min settling time. Spermatocrit was measured in a  $10 \mu\text{l}$  capillary tube: preliminary data established that centrifuging for 15 min at  $15,000 \times g$  was suitable for the measurement of spermatocrit. Three replicates (or more where possible) of undiluted milt in haematocrit tubes were measured for each sample.

#### *4.2.7. Short term storage of milt*

Freshly collected milt samples that showed good sperm motility ( $\geq 90\%$  activation,  $\geq 20$  s swim time) were pooled and diluted for storage to a final volume of 1.8-2.0 ml at 10.

100, 1000 and 10,000 fold and to a final volume of 0.5 ml at 50,000 fold, using buffer either with antibiotic (30 mg l<sup>-1</sup> penicillin G, 50 mg l<sup>-1</sup> streptomycin sulphate) or without antibiotic. A fertilization trial was conducted on the day the milt was collected and then each following day for seven days after collection, except for day six. Each day fertilization trials were run using all the dilutions of the two stored treatments (with and without antibiotic) and compared to a fresh milt sample with high motility collected on the day of the fertilization trial. The fresh milt samples were used undiluted and diluted 100, 1000, 10,000 and 50,000 fold in buffer (without antibiotic added), in the fertilization trials. A 5 µl or 10 µl sample (depending on the sperm concentration) of the 10 fold and 100 fold diluted milt and a sample of milt on a 10 µl pipette dipped in the undiluted milt were tested in motility trials conducted immediately after the fertilization trial was completed. Four of the fertilization trials were maintained until the eggs hatched and the numbers of hatched larvae and their morphological appearance (normal or 'bent') were recorded. Sperm concentrations were measured using the haemocytometer method.

#### *4.2.8. Fertilization trials*

Seawater, sperm and oocytes were kept on ice throughout the fertilization process. all seawater used in the experiment had antibiotics added (30 mg l<sup>-1</sup> penicillin G, 50 mg l<sup>-1</sup> streptomycin sulphate). Prelabelled petri dishes were placed on ice in groups of six and 60 µl aliquots of oocytes (approximately 125 eggs) were added to each dish. A 5 µl sperm sample was added to each dish and stirred. next a 60 µl aliquot of seawater was added to

each dish and stirred with a pipette tip. Exactly two minutes after the first addition of seawater each sample was diluted with 25 ml of seawater, and placed in an incubator set at 5°C. Three replicates of each sperm sample were tested. Fertilization trials usually started 2.5 h after milt collection and all replicates of a fertilization trial were fertilized within 1 h, including one set of 'blank replicates' with no milt added to test for parthenogenetic egg development. Approximately 6 h hours after fertilization, numbers of fertilized eggs were counted by observing the 2-32 cell stage of development under 20x magnification (it is difficult to discriminate between fertilized and unfertilized eggs after the 32 cell stage). Eggs were counted as fertilized (2-32 cell stage, -floating or not), unfertilized (clear, floating and showing no cell division) or unviable (uneven, opaque and/or not floating). Since counting began while some eggs were still at the 2-cell stage, (in order to complete counts before the eggs developed past 32 cells), initial counts were checked at the end of the trial. Fertilization rates were calculated from numbers of fertilized eggs out of the total number of eggs (since later work showed that "clear eggs that did not float" were incorrectly identified as nonviable at the time of data collection).

#### *4.2.9. Statistics*

Data were analysed using one way ANOVA and Duncan's multiple range test (Zar, 1984) to group significantly different treatments.  $\alpha = 0.05$ . Data were transformed to meet the assumptions of ANOVA or where stated the F value was tested against a F distribution generated by 500 randomizations of the data set.

### 4.3. Results

#### 4.3.1. *Effect of pH and Osmolality on sperm motility*

Buffer osmolality varied between 232-282 mOsm and pH 8.2-8.8. seawater osmolality varied between 821-901 mOsm and pH was 7.6-8.2. Four trials were completed, trial I had no replicates within the trial.

Sperm remained nonmotile until buffer reached 463 mOsm except for Trial IV when sperm were not activated until >554 mOsm (Fig. 24). Percentage of sperm cells activated and length of swim time increased as buffer osmolality increased and reached a maximum in buffer of 774-796 mOsm in all four trials. Percentage sperm activated and sperm swim times were greater in buffer than that observed in undiluted seawater.

Similar effects of osmolality on percentage sperm activation and duration of sperm swimming were obtained in dilutions of seawater (Fig. 25).

No sperm cells were activated in buffer (232-282 mOsm) at pH ranging from 5.0-9.1 (data not shown).

Sperm cells were always activated in seawater from pH 4.8-9.0. seawater osmolality ranged between 821-901 mOsm (Fig. 26). The percentage of sperm cells activated at different pH showed little variation except in replicate I when only 30% of the sperm cells were activated at pH 5.5 compared to 70-90% in all other samples. Swim times showed little variation in seawater of different pH.

#### 4.3.2. *Measurement of sperm concentration by spectrophotometry*

There was a negative linear relationship between diluted milt absorbance and increasing wavelength (Fig. 27). Pooled seminal plasma diluted 100 fold showed minimal absorbance at all wavelengths decreasing from 0.010 to 0.000 at 400 nm and above (Fig. 28). Three seminal plasma samples taken from different males and diluted 100 fold showed the same absorbances as the 100 fold pooled sample. Pooled seminal plasma diluted 10 fold was 0.07 at 350 nm, 0.03 at 420 nm and less than 0.01 at 550 nm and above. Note that the maximum absorbance is less than 0.1. Urine samples showed a negative relationship between absorbance and increasing wavelength (Fig. 29). Pooled urine diluted 100 fold showed an absorbance of 0.24 at 350 nm while pooled urine diluted only 10 fold showed an absorbance of 1.34 at 350 nm decreasing to 0.77 at 420 nm. Two urine samples from individual males diluted 100 fold showed a higher absorbance than the pooled sample but three samples had lower absorbances. Buffer absorbance was found to be negligible in comparison to diluted water at all wavelengths measured (data not shown). A positive linear relationship was found between sperm absorbance at 420 nm and log sperm cell concentration (Fig. 30). Regression analysis gave the following equation with an  $R^2 = 0.9221$ :

$$\text{Absorbance} = 1.0538 (\log (\text{sperm cell concentration})) - 9.6548.$$

which gives (for sperm cells  $\text{ml}^{-1}$ ):

$$10^{\frac{(\text{absorbance} - 9.6548)}{(-1.2538)}} = \text{concentration}$$

There was a positive linear relationship between spermatocrit and log sperm cell concentration.  $R^2 = 0.8313$ :

$$\text{Spermatocrit} = 119.53(\log(\text{sperm cell concentration})) - 1137.63.$$

which gives (for sperm cells  $\text{ml}^{-1}$ ):

$$10^{\frac{(\text{spermatocrit} + 1137.63)}{119.53}} = \text{concentration}$$

Absorbance at 420 nm and spermatocrit also showed a positive linear relationship,  $R^2 = 0.8778$ :

$$\text{Absorbance} = 0.0078(\text{spermatocrit}) + 0.4361.$$

Sperm concentrations ranged from  $0.28 \times 10^{10}$  to  $2.61 \times 10^{10}$  cells  $\text{ml}^{-1}$  and the average sperm concentration was  $1.26 \times 10^{10} \pm 0.10 \times 10^{10}$  cells  $\text{ml}^{-1}$ .

#### 4.3.3. Sperm storage fertilization rates

On the day milt was collected for storage fertilization rates between milt diluted in buffer with or without antibiotic were not different except for milt diluted 10 fold without antibiotic and milt diluted 10,000 fold with antibiotic (Fig. 31). After storage for one day the fertilization rates of the stored milt were comparable to, if not slightly higher than, freshly collected milt at similar sperm concentrations. After two days storage decreased fertility of the stored milt (compared to milt collected on the day of the fertilization trial)



was evident in the lower sperm concentrations and this trend continued until day seven when the 100 fold stored milt dilutions were not fertile but the fertility of the 10 fold stored milt dilutions were still comparable to freshly collected milt (Fig. 32)(day seven data not shown). The two exceptions were anomalously low fertilization rates in the 100 fold dilution of freshly collected milt on day 4 and undiluted freshly collected milt on day 5. Addition of antibiotics to the storage medium improved sperm fertility in the 100 fold and 1000 fold dilutions after four and five days storage but did not improve fertility compared to storage without antibiotics after seven days storage. Sperm motility and fertility were both high in the stored milt diluted 10 fold (with and without antibiotic) throughout the experiment, but in the milt diluted 100 fold, both with and without antibiotic, percentage sperm activated decreased to 1% after four days storage while sperm fertility remained high (Figs. 33 and 34).

#### *4.3.4. Hatch rates and larval appearance after storage*

Hatch rates were not different between sperm treatments and between different dilutions on the day of milt collection or after two or five days storage (Fig. 35). After four days storage hatch rates were not different between the three milt treatments except for low hatch rates in the undiluted freshly collected milt and in the 10,000 fold dilution of freshly collected milt. On the day milt was collected larvae showing deformities and/or unusual swimming patterns ("bent") were observed from eggs fertilized both by sperm diluted with antibiotic and sperm diluted without antibiotic (Fig. 36). Rates of bent larvae remained

relatively low from eggs fertilized on days two, four and five of the storage experiment (and were not significantly different between treatments or dilutions).

Fig. 24. The effect of increasing buffer osmolality on sperm motility, in trials a) I, b) II, c) III and d) IV. Open symbols show percentage sperm activated and filled symbols show swim times in seconds (s)(N.B. symbols overlaid). Circles show milt diluted in buffer and squares show milt diluted in full strength seawater for comparison. Buffer pH varied between 8.2-8.8.

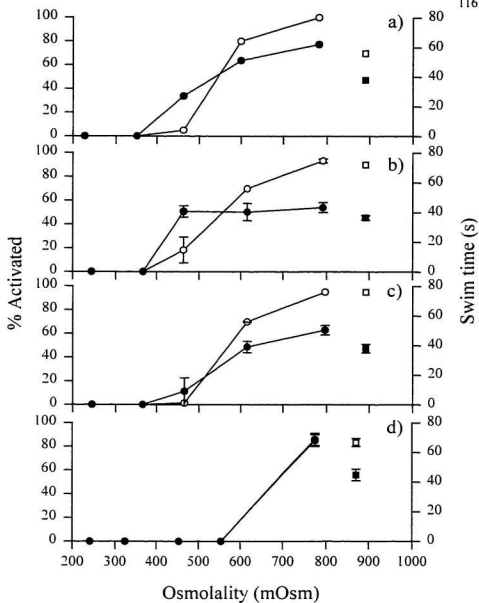


Fig. 25. The effect of increasing seawater osmolality on sperm motility, in trials a) I, b) II, c) III and d) IV. Open symbols show percentage sperm activated and filled symbols show swim times (s). Circles show milt in diluted seawater and squares show milt in undiluted seawater for comparison. Buffer pH varied between 7.6-8.2.

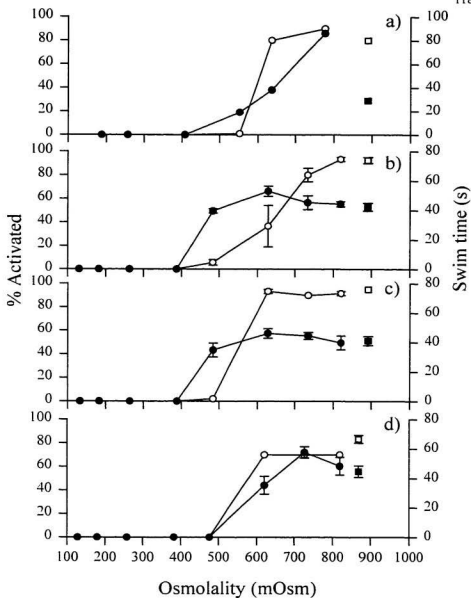


Fig. 26. The effect of changing seawater pH on sperm motility, in trials a) I, b) II, c) III and d) IV. Open symbols show percentage sperm activated and filled symbols show swim times (s). Circles show milt in pH altered seawater and squares show milt in unaltered seawater for comparison. Seawater osmolality varied between 821-901 mOsm.

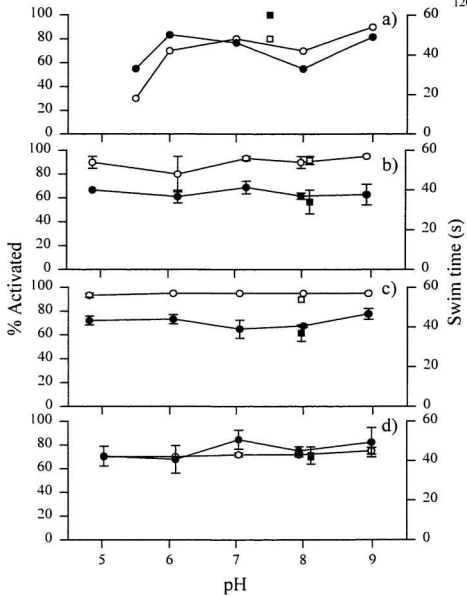




Fig. 27. Absorbance of nine milt samples diluted 100 fold in buffer at 13 different wavelengths.

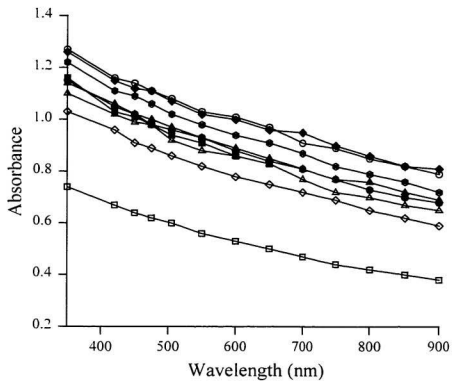


Fig. 28. Absorbance of pooled seminal plasma diluted 100 fold (circles) and 10 fold (squares) at different wavelengths. Note that the maximum absorbance is less than 0.1. Three seminal plasma samples taken from different males and diluted 100 fold showed the same absorbances as the 100 fold pooled sample.

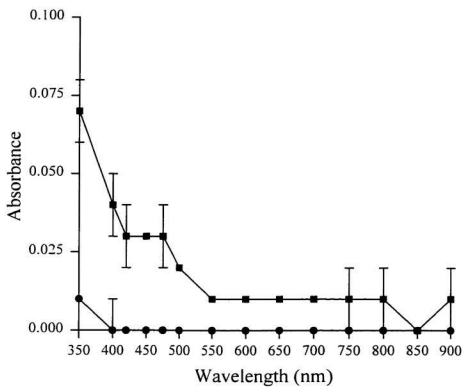


Fig. 29. Absorbance of urine at 14 different wavelengths in 5 different individual samples (diluted 100 fold) and 2 pooled urine samples (diluted 100 fold or 10 fold). Filled symbols show individual samples, open circles show pooled urine diluted 100 fold and open squares show pooled urine diluted 10 fold.

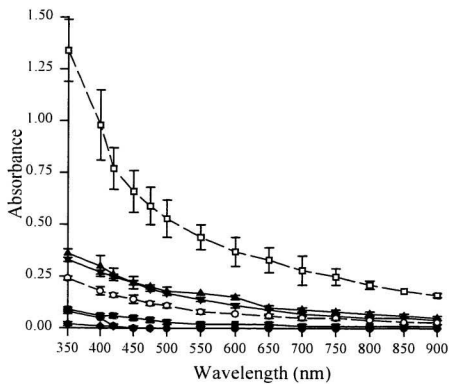


Fig. 30. Relationship between log sperm cell concentration and a) absorbance of diluted milt (100 fold) at 420nm ( $R^2 = 0.922$ ) and b) spermatocrit ( $R^2 = 0.831$ ) and c) between spermatocrit and absorbance at 420nm ( $R^2 = 0.878$ ).

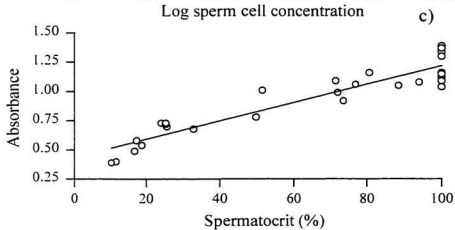
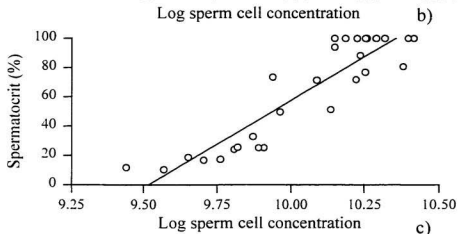
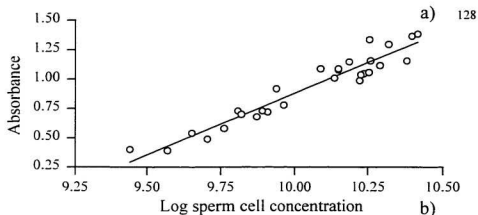




Fig. 31. Change in fertilization rates at different sperm concentrations in fresh milt (open squares), and milt stored with (filled circles) and without (open circles) antibiotic a) on day of collection ( $F = 93.06$ ,  $P < 0.0001$ ) b) one day after collection ( $F = 58.73$ ,  $P < 0.0001$ ) and c) two days after collection ( $F = 138.00$ ,  $P < 0.0001$ ). Letters indicate Duncan's groupings of means.

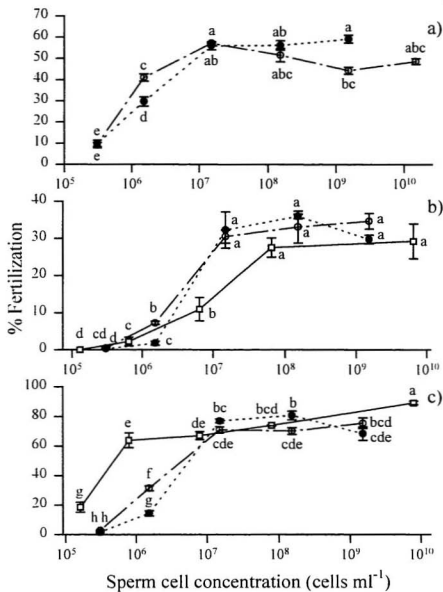


Fig. 32. Change in fertilization rates at different sperm concentrations in fresh milt (open squares), and milt stored with (filled circles) or without (open circles) antibiotic a) three days after collection ( $F = 145.41$ ,  $P < 0.0001$ ) b) four days after collection ( $F = 103.85$ ,  $P < 0.0001$ ) and c) five days after collection ( $F = 86.03$ ,  $P < 0.0001$ ). Letters indicate Duncan's groupings of means.

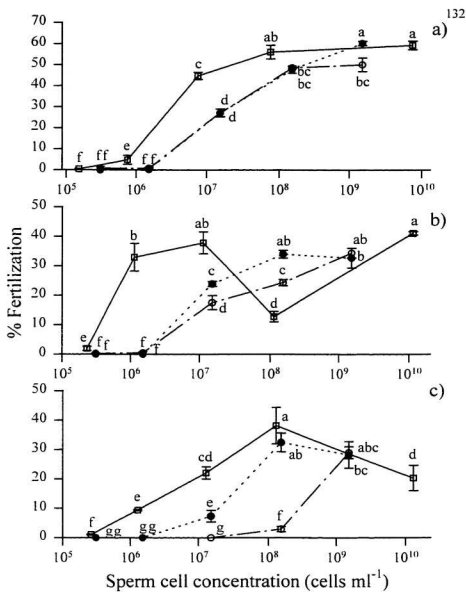


Fig. 33. Change in percentage sperm cells activated with storage (day 0 is day milt collected) in a) stored milt diluted with antibiotic and b) stored milt diluted without antibiotic. Diagonal striped bars show milt diluted 10 fold and cross hatch bars show milt diluted 100 fold.

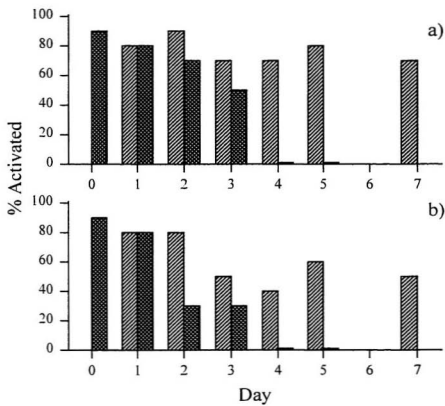


Fig. 34. Change in duration of mass forward motility of sperm (swim time) with storage (day 0 is day milt collected) in a) stored milt diluted with antibiotic and b) stored milt diluted without antibiotic. Diagonal striped bars show milt diluted 10 fold and cross hatch bars show milt diluted 100 fold.

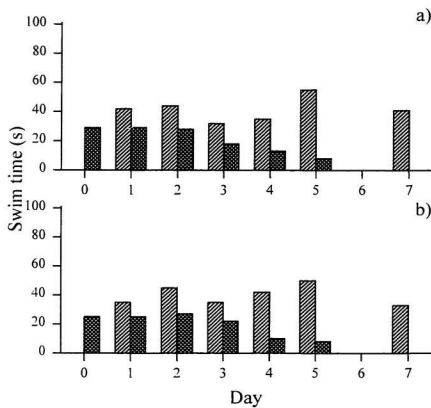




Fig. 35. Percentage hatched larvae from fertilized eggs in fertilization trials with fresh milt (open squares), stored milt diluted with (filled circles) and without (open circles) antibiotic on a) the day milt was collected for storage ( $F = 2.10$ ,  $P < 0.071$ ) and b) two days ( $F = 1.76$ ,  $P < 0.095$ ), c) four days ( $F = 8.57$ ,  $P < 0.0001$ ) and d) five days ( $F = 0.29$ ,  $P < 0.950$ ) after milt was collected for storage. Asterisk indicates data not included in the statistical analysis because  $<3$  eggs were fertilized in at least one of the replicates. Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).

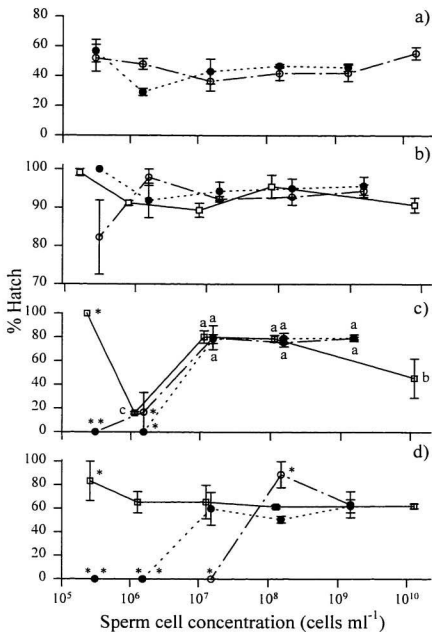
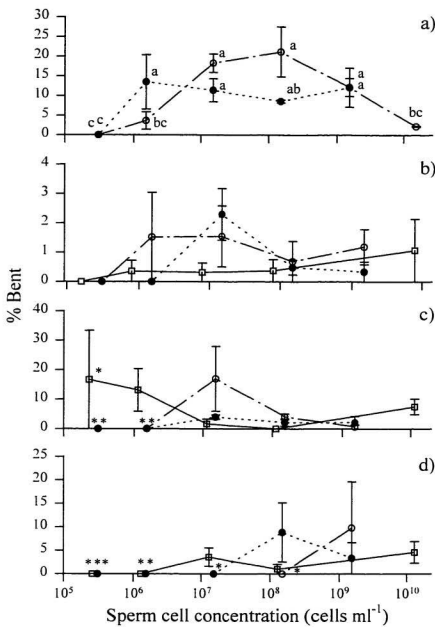


Fig. 36. Percentage 'bent' larvae from hatched eggs in fertilization trials with fresh milt (open squares), stored milt diluted with (filled circles) and without (open circles) antibiotic on a) the day milt was collected for storage ( $F = 8.12$ ,  $P < 0.0001$ ) and b) two days ( $F = 1.18$ ,  $P < 0.336$ ). c) four days ( $F = 1.65$ ,  $P < 0.168$ ) and d) five days ( $F = 0.76$ ,  $P < 0.628$ ) after milt was collected and stored. An asterisk indicates data not included in the statistical analysis because  $<3$  eggs were fertilized in at least one of the replicates. Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).



#### 4.4. Discussion

Yellowtail flounder sperm is activated in buffer of  $>367$  mOsm and diluted seawater of  $>387$  mOsm (Figs. 24 and 25). Percentage sperm activated were highest and swim times were longest in buffer of 774-796 mOsm and seawater of approximately 630 mOsm: both of these characteristics were slightly lower in undiluted seawater (870-890 mOsm). Maximum motility at a lower osmotic pressure than undiluted seawater has been observed in other marine teleosts, for example the puffer *Fugu niphobles* (Morisawa and Suzuki, 1980; Stoss, 1983). Morisawa and Suzuki (1980) suggest that in the wild eggs and sperm are probably spawned into a mixture of ovarian fluid, seminal plasma and sea water and therefore at a slightly lower osmolality than seawater. No sperm were activated in buffer of  $\leq 351$  mOsm or diluted seawater of  $\leq 387$  mOsm. Yellowtail flounder seminal plasma osmolality is approximately 318 mOsm which suggests that sperm remain immotile in the genital tract of male yellowtail flounder (see Chapter 5). Halibut *Hippoglossus hippoglossus* L. sperm were motile in a range of osmotic pressures from 380-1150 mOsm and motility tended to increase from 400-1100 mOsm depending on the diluents used (Billard et al., 1993). Changing seawater pH had very little effect on yellowtail flounder sperm motility when osmolality was  $\geq 821$  mOsm and no optimum pH was observed (Fig. 26). Sperm remained immotile in buffer of pH 4.99-9.07 when the osmotic pressure was  $\leq 282$  mOsm. In other teleosts sperm are motile in a wide pH range and some species show an optimum pH (Scott and Baynes, 1980; Stoss, 1983; Billard et al., 1992). For example halibut sperm show highest percentage activation at pH 8.0-8.5, and longest swim

times at pH 7.5-8.0. but were motile in solutions of pH 6.5-10.0 (approx. 800 mOsm) (Billard et al., 1993). pH from 6.9-8.9 also had little affect on motility of sea bream *Sparus aurata* L. sperm but activation was inhibited in diluents of <480 mOsm and increased at higher osmotic pressures (Chambeyron and Zohar, 1990).

Visual counts of sperm, considered to be the most reliable method of assessing sperm concentration, are usually made using a haemocytometer however this method is time consuming (1 h per sample). Spectrophotometry was shown to be a rapid method (10 min per sample) to measure the concentration of yellowtail flounder sperm. In addition only 20  $\mu$ l of milt is required for measurement of sperm concentration since a sample of  $\geq 2$  ml of a 100 fold dilution is required for the spectrophotometer. Since absorbance measurement will not affect the sperm cells, the 100 fold dilution can be used for fertilization of eggs if required. A wavelength of 420 nm was selected to measure sperm absorbance since seminal plasma absorbance was zero at this wavelength and it was also the wavelength used to measure turbot sperm absorbance (Suquet et al., 1992a). However, it is important to note that the turbot milt was diluted with a solution of the detergent Triton X-100 to avoid sperm aggregation. Triton X-100 has been shown to cause sperm demembranation (Redondo-Muller et al., 1991), and therefore may have caused the release of light absorbing substances (Ciereszko and Dabrowski, 1993). Spectrophotometry was also successfully used to measure sperm concentration in the milt of carp *Cyprinus carpio* L. at 410 nm (Takashima et al., 1984) and the milt of rainbow trout *Oncorhynchus mykiss* (Walbaum), whitefish *Coregonus clupeaformis* (Mitchill) and yellow perch *Perca*

*flavescens* L., at 505 nm (Ciereszko and Dabrowski, 1993). In this study two dilutions of seminal plasma and urine (10 fold and 100 fold) were used to determine the absorbance of these substances at both the concentration expected in a normal sperm sample (100 fold) and in a concentration (10 fold) much higher than that expected in routine measurement. Concentrated seminal plasma (10 fold) did not have a significant absorbance, however urine at both 100 fold and 10 fold showed absorbance that would be significant in comparison to sperm absorbance (Figs. 28 and 29). Urine contamination must therefore be avoided when collecting sperm, both to allow accurate measurement of sperm concentration and to improve fertility since it has been shown that urine contamination can negatively affect sperm fertility (Clearwater and Crim, 1996). The variation in absorbance between replicates of the same milt sample was negligible while the variation in counts obtained from the haemocytometer showed a coefficient of variation ranging from 3-30% which is another drawback to this time consuming method.

Spermatoctrit was also linearly related to the log of sperm concentration (Fig. 30) however the spermatoctrit of yellowtail flounder milt is sometimes difficult to measure. This is due to the lack of a clear interface between sediment and supernatant and layers of opaque material forming on top of the clear supernatant. Ciereszko and Dabrowski (1993) described similar problems with the measurement of yellow perch spermatoctrit but successfully established a relationship between sperm concentration and spermatoctrit by a 2-5 fold dilution of the milt samples prior to measurement of spermatoctrit. The sperm concentration of yellowtail flounder milt is also relatively high ( $1.26 \times 10^{10}$  cells ml<sup>-1</sup>)

therefore dilution of milt samples may improve measurements of spermatocrit. Spermatocrit and sperm concentration are related in a number of species, for example rainbow trout (Bouck and Jacobsen, 1976; Munkittrick and Moccia, 1987; Ciereszko and Dabrowski, 1993), pike *Esox lucius* L. (DeMontalembert et al., 1980, cited in Suquet et al., 1992a) carp (Takashima et al., 1984) and whitefish (Ciereszko and Dabrowski, 1993). However, no significant relationship between sperm concentration and spermatocrit could be established for turbot (Suquet et al., 1992a) and individual sperm volume is thought to change during the spawning season in seabass *Dicentrarchus labrax* L. and halibut (Billard et al., 1992).

The milt storage experiment demonstrated that yellowtail flounder milt can be diluted 10 fold or 100 fold in buffer with antibiotic and stored for five days or diluted 10 fold for seven days and achieve fertilization rates comparable to undiluted fresh milt (Figs. 31 and 32). Short term storage of undiluted and diluted milt has been used successfully in a range of teleost species with highly variable results. The important factors are temperature, gaseous exchange, preventing bacterial growth (i.e. addition of antibiotics) and prevention of dessication (Stoss, 1983). Storage of undiluted milt has been most successful in salmonids, for example rainbow trout sperm was stored successfully under oxygen at 0°C for 34 d (Stoss and Holtz, 1983). Storage of diluted milt has not been as successful as undiluted milt in salmonids but has succeeded in other teleost species. Fifty percent of European catfish *Silurus glanis* L. sperm retained motility after 25 d storage in a diluent that included antibiotics (Redondo-Muller et al., 1992). Japanese eel *Anguilla japonica*



(Temminck and Schlegel) milt diluted in artificial seminal plasma retained 40% motility after storage for 28 d at 3°C (Ohta and Izawa, 1995). Pieces of macerated channel catfish *Ictalurus punctatus* testes stored in a diluent retained motility after 9 weeks (Guest et al., 1976) and diluted carp sperm retained motility after 30 days storage (Sneed and Clemens, 1956). Diluted common carp, silver carp *Hypophthalmichthys molitrix* (Val) and bighead carp *Aristichthys nobilis* milt was stored for 10 d, 7 d and 7 d respectively (Chen et al., 1992).

Diluting milt before storage allows better control of the physiochemical environment the sperm is stored in. A good diluent is isotonic, has a good buffering capacity, contains nutrients, stabilizing colloids and antioxidants and is antibacterial (Stoss, 1983). Milt dilution for storage may reduce the negative effects of urine contamination of the milt (Clearwater and Crim, 1996), reduce the risk of dessication when only small volumes of milt are available and allow easier mixing of the stored preparation to improve gaseous exchange and respiration of the stored sperm. The storage method, dilution and storage in a fridge in open test tubes loosely covered with parafilm was chosen for ease and practicality, with a view to this method being used at a fish farm with minimal specialized equipment and also in experimentation in the laboratory. In order to achieve longer storage times many improvements may be possible, for example storage in an oxygen saturated atmosphere, identifying the essential ingredients of the storage diluent and increasing the concentration of antibiotics added to the buffer. In this experiment, addition of antibiotics to the dilution medium improved sperm survival in storage in the 100 fold

and 1000 fold dilutions four and five days after dilution but this effect was not seen in the 10 fold dilution after seven days of storage. Undiluted carp milt stored with  $50\text{ }\mu\text{g ml}^{-1}$  streptomycin + 50IU bipenicillin  $\text{ml}^{-1}$  markedly improved sperm survival *in vitro* (Saad et al., 1988), in comparison with  $30\text{ }\mu\text{g ml}^{-1}$  ( $48.9\text{ IU ml}^{-1}$ ) penicillin G and  $50\text{ }\mu\text{g ml}^{-1}$  ( $87.5\text{ IU ml}^{-1}$ ) streptomycin sulphate in this investigation. In rainbow trout 9 mg streptomycin + 9000IU penicillin  $\text{ml}^{-1}$  had a negative effect on stored trout sperm however lower doses improved sperm survival *in vitro* (Stoss et al., 1978). The hatch rates and larval appearance from fertilization trials by stored yellowtail flounder sperm were not lower than fresh sperm (Figs. 35 and 36) indicating that storage does not negatively affect the progeny produced from fertile sperm. Similar results were found with undiluted carp sperm stored for 15 d with antibiotic (Saad et al., 1988).

There are two possible explanations for lower fertilization rates in stored sperm at high dilutions (>100 fold, i.e. lower concentrations), either these results reflect a decrease in sperm quality due to storage that is not revealed in fertilization trials with high concentrations of sperm or high dilution rates are harmful for the sperm cells. Saad et al., (1988) showed a decrease in the quality of undiluted carp sperm due to storage by testing the fertility of stored sperm at a range of sperm concentrations. Low sperm concentrations resulted in low fertilization rates indicating that the decrease in quality of carp sperm was due to storage *per se* rather than high dilution rates. Alternatively Chambeyron and Zohar (1990) showed that high dilution rates were detrimental to sea bream sperm motility, however these results may be biased by the low numbers of sperm observed in motility

trials of more dilute sperm. Similarly Billard (1983) claimed that increasing milt dilution decreased both sperm survival and fertilization rates but the methods did not account for decreased numbers of sperm in the higher dilutions. However, it has been shown that increased protein concentration in milt diluents will increase sperm survival in both rainbow trout (Billard, 1983) and turbot (Suquet et al., 1994). Therefore, increasing milt dilution may reduce sperm survival by decreasing the concentration of seminal plasma proteins. Increased protein concentration also increases the survival of rainbow trout sperm survival during cryopreservation (Legendre and Billard, 1980) possibly by protecting sperm membranes during freezing. The storage of non-diluted sperm with antibiotics added was not investigated but would be an interesting extension of this work, since it has been successful in a number of other species, especially salmonids (Stoss, 1983; Saad et al., 1988). Ultimately this storage experiment demonstrates that short term storage of yellowtail flounder milt is possible and if sperm concentrations are high fertilization rates comparable to fresh sperm can be achieved 7 days after storage with no adverse affect on larval hatch rates or larval appearance.

Examination of the fertilization trials shows that generally fertilization rates of the fresh milt did not begin to decrease until sperm cell concentrations fell below  $1 \times 10^4$  cells  $\text{ml}^{-1}$ , which is approximately equivalent to 5000 sperm cells per egg. These sperm:egg ratios are likely to be particular to the fertilization method used in this investigation which allows a high degree of mixing of eggs and sperm prior to sperm activation with a small volume of seawater. Undiluted milt is viscous and does not mix easily with eggs, therefore

taking these observations together, it is recommended that milt diluted 10-100 fold in the buffer DCSB4 be used in fertilization of yellowtail flounder eggs, particularly when high volumes of eggs are being fertilized. In turbot 6000 sperm per egg is the minimum ratio to achieve maximum fertilization rates, which is lower than that observed in other species (Suquet et al., 1995). Suquet et al. (1995) also noted that when egg quality was low higher numbers of sperm were required to achieve maximum fertilization success.

## CHAPTER 5.

### **Rapid milt dilution mitigates the negative effects of urine contamination on sperm quality in yellowtail flounder (*Pleuronectes ferrugineus*).**

#### **5.1. Introduction**

Urine contamination of milt as it is collected from the male is detrimental to sperm quality in Atlantic salmon *Salmo salar* L. (Rana, 1995) and carp *Cyprinus carpio* L. (Perchee et al., 1995a and b). The mechanism by which urine has its negative effect on sperm quality is not well understood and may be different in different species. In carp, urine contaminated milt samples show low motility due to prior activation of some of the sperm by the urine, which results in hydrolysis of ATP in the sperm cells (Perchee et al., 1995b). Carp sperm motility can be restored by incubation of the sperm in 200 mM KCL and 30 mM Tris-HCl (pH 8.0) (Redondo-Muller et al., 1991; Perchee et al., 1995a). In salmonids sperm motility is inhibited by high K<sup>+</sup> ion concentrations in the seminal plasma: sperm are activated when they are spawned into freshwater and the K<sup>+</sup> ion concentration decreases (Morisawa, 1985). Urine contamination of Atlantic salmon milt prematurely diluted K<sup>+</sup> ion concentrations in the milt and reduced subsequent sperm motility (Rana, 1995). Rana (1995) suggested that the intra-male variation in protein and osmolality of urine and the variation in amount of urine contamination that may occur at the point of milt collection may account for much of the random and unpredictable variation in milt

quality between and within males. In yellowtail flounder *Pleuronectes ferrugineus* (Storer) preliminary work showed that similar random variation in sperm fertility and motility was associated with urine contamination of milt as it was collected. Furthermore it was found that samples of yellowtail flounder urine from different individuals or from an individual at different times could either decrease sperm motility or have no effect on sperm motility. Therefore, the effect of urine contamination on sperm motility and fertility was investigated in a series of fertilization trials. In order to focus this investigation milt was diluted 10 fold in urine for 15 min, and only urine that decreased sperm motility was used in fertilization trials. Urine contamination is difficult to avoid in yellowtail flounder because of the low volumes of viscous milt available for collection and the small diameter of the urogenital pore. Scott et al. (1991) also reported that it was difficult to obtain uncontaminated milt by hand-stripping goldfish *Carassius auratus* L., Pacific herring *Clupea harengus pallasi*, plaice *Pleuronectes platessa* L., flounder *Platichthys flesus* L. and dab *Pleuronectes limanda* L.. Rapid dilution of urine contaminated milt in buffer was investigated to examine its effect on sperm motility and fertility. If this method is successful it will be an easy and practical solution to the problem of urine contamination.

## 5.2. Materials and methods

### 5.2.1. Milt collection

During July and August 1995, milt samples were collected from the urogenital pore of captive male yellowtail flounder broodstock (for confinement conditions see Section 4.2.1.) using 1 ml syringes while taking care to avoid urine contamination, then stored in either syringes or test tubes on ice.

### 5.2.2. Sperm motility

A 10  $\mu$ l pipette tip was dipped in the undiluted milt and then rapidly stirred in 100  $\mu$ l of diluent on a microscope slide under 100x magnification. Percentage sperm activated at the point of mixing and duration of forward motility of the majority of activated sperm (swim time in seconds) were noted in each motility trial. If milt had been diluted 10 fold, 100 fold or 1000 fold in either buffer (DCSB4 -adapted from Chambeyron and Zohar (1990), 0.001 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.083 M glycine, 0.030 M Trizma basic and 0.150 M sucrose, pH 8.0) or urine, a 5  $\mu$ l or 10  $\mu$ l sample (depending on dilution), was tested under 100x magnification in 100  $\mu$ l chilled filtered seawater (5°C), rather than dipping a 10  $\mu$ l pipette in the sample. Pre-fertilization motility trials were started within 1.5 h of urine and milt collection and post-fertilization motility trials were started 10 min after fertilization trials were completed.

### *5.2.3. Collection of urine and selection of milt and urine for experiments*

For replicate one of Experiment I (see Section 5.2.4.), urine samples were taken directly from the bladder, after anaesthetizing the fish and cutting open the gut wall. Urine samples for replicate two of Experiment I and all of Experiment II (see Section 5.2.5.) were obtained using a catheter on an anaesthetized fish. Motility trials were used to test milt samples prior to use in urine dilutions. Milt with good sperm motility ( $\geq 90\%$  activation,  $\geq 20$  s swim time) was pooled and then samples were diluted in urine (90% urine) for 15 min to test for the effect of urine on sperm motility. Urine that negatively affected sperm motility was then pooled and used in the fertilization trials. Milt diluted in urine was compared to milt diluted in buffer.

### *5.2.4. Experiment I -the effects of urine contamination on sperm motility and fertility*

Fertilization rates and post-fertilization motility was compared between non-diluted milt, milt diluted in urine for  $\geq 15$  min and milt diluted in buffer at a range of sperm concentrations.

### *5.2.5. Experiment II -the effects of rapid dilution of contaminated milt on sperm motility and fertility*

Fertilization rates and post-fertilization motility of milt diluted for 1 min in urine prior to dilution in buffer (rapid dilution) was compared with milt diluted in urine for 15 min prior to dilution in buffer (urine contamination) and milt diluted only in buffer (buffer).



Milt not diluted prior to use in fertilization trials (non-diluted) was used in fertilization trials for comparison of super- and sub-saturation sperm concentrations relative to egg numbers. Initial dilutions in urine or buffer were 10 fold (90% urine or buffer).

#### *5.2.6. Fertilization trials*

Seawater, milt and oocytes were kept on ice throughout the fertilization process, all seawater used in the experiment had antibiotics added (30 mg l<sup>-1</sup> penicillin G, 50 mg l<sup>-1</sup> streptomycin sulphate). Prelabelled petri dishes were placed on ice in groups of six and 60 µl aliquots (~125 eggs) of oocytes were added to each dish. A 5 µl milt sample was added to each dish and stirred, next a 60 µl aliquot of seawater was added to each dish and stirred with a pipette tip. Exactly 2 min after the first addition of seawater each sample was diluted with 25 ml of seawater, and placed in an incubator set at 5°C. Three replicates of each milt sample were tested. Fertilization trials usually started 2.5 h after milt collection and all replicates of a fertilization trial were fertilized within 1 h, including one set of 'blank replicates' with no milt added to test for parthenogenetic egg development. The sequence of treatments was random in each fertilization trial. Approximately 6 h hours after fertilization, numbers of fertilized eggs were counted by observing the 2-32 cell stage of development under 20x magnification (it is difficult to discriminate between fertilized and unfertilized eggs after the 32 cell stage). Eggs were counted as fertilized (2-32 cell stage, -floating or not), unfertilized (clear, floating and showing no cell division) or unviable (uneven, opaque and/or not floating). Since counting

began while some eggs were still at the 2-cell stage. (in order to complete counts before the eggs developed past 32 cells), initial counts were checked at the end of the trial. Fertilization rates were calculated from numbers of fertilized eggs out of the total number of eggs (since later work showed that "clear eggs that did not float" were incorrectly identified as nonviable at the time of data collection).

#### *5.2.7. Measurement of pH and osmolality*

Milt and urine pH was measured on indicator paper (ColourpHast pH sticks, EM Science, New Jersey, U.S.A., pH range 6.5-10.0 and 5.0-10.0), buffer pH and seawater pH was measured using a Corning Model 12 Research pH Meter. Milt samples were centrifuged in 1.5 ml Eppendorf vials for 5 min at  $9170 \times g$  at 4°C to obtain seminal plasma. Osmolality was measured using a Fiske 110 osmometer.

#### *5.2.8. pH and osmolality and ionic composition of seminal plasma and urine*

Urine and seminal plasma samples were selected for the measurement of pH, osmolality and ionic composition based on sperm motility. Highly motile sperm were diluted 10 fold in urine and sperm motility was tested in seawater  $\geq 15$  min after dilution. Urine samples that had a positive or no effect on sperm motility were grouped together as were urine samples that negatively affected sperm motility. The ionic composition of freshly thawed seminal plasma and urine samples was analyzed using the SYNCHRON CX3 system (a multi-analyte discrete analyzer, courtesy of the Health Sciences Centre, Memorial

University of Newfoundland). Sodium, potassium and chloride were measured by indirect potentiometry using ion sensitive electrodes housed in a flow cell. Carbon dioxide was measured using a silicone rubber covered pH electrode. Calcium, magnesium and phosphorus were measured spectrophotometrically in a colorimetric reaction.

### 5.2.9. Statistics

Data were analysed using one way ANOVA and Duncan's multiple range test (Zar, 1984) to group significantly different treatments.  $\alpha = 0.05$ . Data were transformed to meet the assumptions of ANOVA. Where stated sperm motility data in the urine contamination experiment were analysed using a Kruskal-Wallis non parametric one way ANOVA.

## 5.3. Results

### 5.3.1. Experiment I -the effects of urine contamination on sperm motility and fertility

Egg fertilization rates of milt diluted 1000 fold were significantly lower in the urine dilution compared to the buffer dilution in both replicates one and two of Experiment I (Fig. 37 and 38). Egg fertilization rates of milt diluted in buffer were not significantly different to non-diluted milt in replicate one but were significantly lower than non-diluted milt in replicate two. Post fertilization motility of urine-diluted milt was zero in both replicates while milt diluted in buffer showed 50-90% sperm activation and 29-44 s sperm swim times and non-diluted milt had 50-100% sperm activated and sperm swim times of 20-45 s.

*5.3.2. Experiment II -the effects of rapid dilution of contaminated milt on sperm motility and fertility*

Egg fertilization rates were compared statistically between milt treatments diluted 10,000 fold for all replicates. This dilution was chosen for comparison because sub-saturation concentrations of sperm were present (non-diluted milt was included in the analysis for statistical confirmation of this assumption). Fertilization rates of rapidly diluted milt were significantly higher than urine contaminated milt in replicates one and two of Experiment II while milt diluted in buffer showed higher fertilization rates than the two other treatments (Fig. 39 and 40). Non-diluted milt gave higher fertilization rates than milt diluted 10,000 fold regardless of treatment. Post-fertilization motility was compared between milt samples diluted 100 fold because milt diluted 1000 fold was too dilute for motility trials. Percent sperm activated in milt diluted in buffer was significantly higher than in rapidly diluted milt and lowest in urine contaminated milt in replicate one. In replicate two percent sperm activated was significantly different between the three treatments but non-parametric analysis could not group the treatments. In replicate one swim times were not significantly different between the treatments while in replicate two milt diluted in buffer had a significantly longer swim time than rapidly diluted milt and urine contaminated milt. Rapidly diluted milt had a longer sperm swim time than urine contaminated milt.

In replicate three of Experiment II, fertilization rates of rapidly diluted milt were not significantly different to fertilization rates of urine contaminated milt (Fig. 41). Both

rapidly diluted and urine contaminated milt samples had significantly lower fertilization rates than milt diluted in buffer. Non-diluted milt had significantly higher fertilization rates than milt diluted 10,000 fold regardless of treatment. In post-fertilization motility tests of 100 fold diluted milt, both percent sperm activated and swim times were not significantly different between milt diluted in buffer, rapidly diluted milt and urine contaminated milt.

#### *5.3.3. The pH, osmolality and ionic composition of urine and seminal plasma*

The pH of urine is lower than seminal plasma whereas the osmolality of seminal plasma and urine are very similar (Table 6). The ionic composition of urine and seminal plasma are compared in Table 7.

Table 6. Comparison of pH and osmolality (mOsm) of samples used in sperm dilutions and fertilization trials.

Sample	Effect on sperm motility	pH $\pm$ s.e.	osmolality $\pm$ s.e.	n
Urine	Negative	6.2 $\pm$ 0.1	312.5 $\pm$ 1.3	18
Urine	None or positive	6.7 $\pm$ 0.1	313.1 $\pm$ 2.4	13
Seminal plasma	High motility	7.5 $\pm$ 0.1	318.0 $\pm$ 1.9	14
Buffer	Does not activate	8.0 $\pm$ 0.01	287.0 $\pm$ 4.7	3
Seawater	Activates motility	7.4 $\pm$ 0.1	855.7 $\pm$ 11.8	3

Table 7. pH, osmolality (mOsm) and ionic composition of seminal plasma and urine (mmol l<sup>-1</sup>)

Sample	Effect on sperm motility	Na	K	Cl	CO <sub>2</sub>	PO <sub>4</sub>	Ca	Mg	pH	osmo
Urine	Negative	118.7	4.9	119.0	5.0	48.9	3.3	65.7	6.3	302.4
(n = 7)		±5.8	±0.6	±14.1	±0	±19.9	±0.4	±14.5	±0.1	±1.8
Urine	Positive or none	120.3	2.5	157.3	5.0	4.7	7.6	72.1	6.7	309.2
(n = 3)		±20.3	±0.5	±6.2	±0	±1.6	±3.0	±37.1	±0.1	±2.2
Seminal Plasma	High motility	153.7	2.6	150.1	5.6	4.43	1.6	10.0	-	-
(n = 9)		±7.0	±0.37	±2.1	±0.4	±1.5	±0.2	±5.1	-	-





Fig. 37. Experiment I -Replicate 1. Effect of dilution of milt in buffer (filled circles) or urine (open circles) compared to non-diluted milt (filled squares) on a) fertilization rates, b) percentage sperm activated and c) swim time (s). Percentage fertilization was compared between undiluted milt (1 fold) and milt diluted 1000 fold ( $F = 227.24$ ,  $P < 0.0001$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).

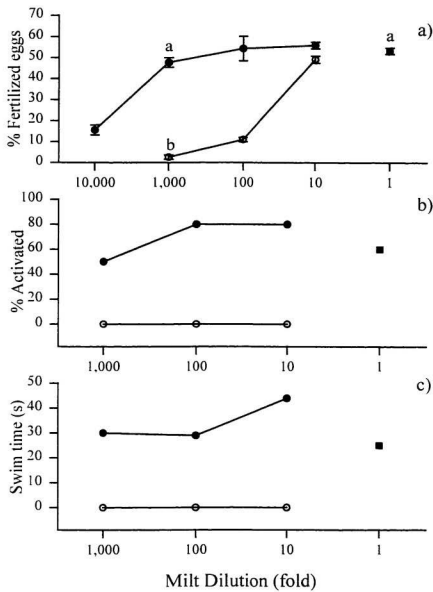


Fig. 38. Experiment I -Replicate 2. Effect of dilution of milt in buffer (filled circles) or urine (open circles) compared to non-diluted milt (filled squares) on a) fertilization rates, b) percentage sperm activated and c) swim time (s). Percentage fertilization was compared between undiluted milt (1 fold) and milt diluted 1000 fold ( $F = 36.30$ ,  $P < 0.0001$ ). Means with similar letters were not significantly different (Duncan's multiple range means test,  $P < 0.05$ ).

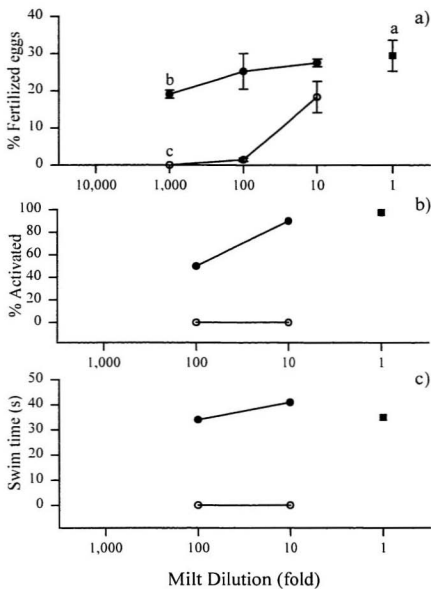


Fig. 39. Experiment II -Replicate 1. Effect of 3 different milt treatments compared to non-diluted milt on a) fertilization at four different dilutions. Diluted in buffer (filled circles), rapidly diluted milt (triangles), urine contaminated milt (open circles) and non-diluted milt (filled squares). Different letters represent significantly different fertilization rates of milt diluted 10,000 fold compared to non-diluted milt ( $F = 439.06$ ,  $P < 0.001$ ). Effect of 3 milt treatments on b) percentage activated sperm and swim time of 100 fold dilutions. Different letters show Duncan's grouping of significantly different percentage activation means ( $F = 26.05$ ,  $P < 0.001$ ). Swim times were not significantly different between treatments ( $F = 1.34$ ,  $P < 0.329$ ).

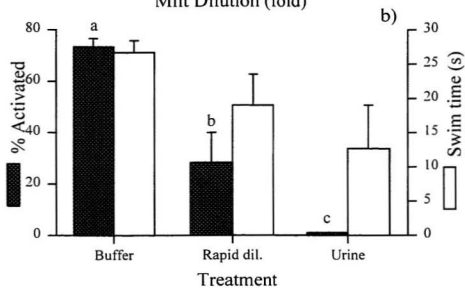
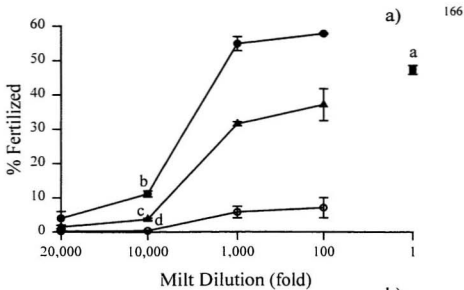


Fig. 40. Experiment II -Replicate 2. Effect of 3 different milt treatments compared to non-diluted milt on a) fertilization at five different dilutions. Diluted in buffer (filled circles), rapidly diluted milt (triangles), urine contaminated milt (open circles) and non-diluted milt (filled squares). Different letters represent significantly different fertilization rates of milt diluted 10.000 fold compared to non-diluted milt ( $F = 288.17$ ,  $P < 0.0001$ ). Effect of 3 milt treatments on b) percentage activated sperm and swim time of 100 fold dilutions. Different letters show Duncan's grouping of significantly different swim times between treatments ( $F = 51.77$ ,  $P < 0.0002$ ). Percentage activation was significantly different between treatments ( $\chi^2 = 7.78$ ,  $P < 0.0204$ ), but Kruskal-Wallis cannot group the treatments.

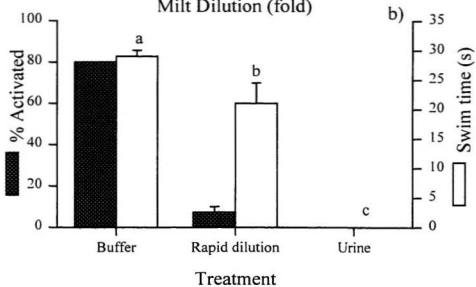
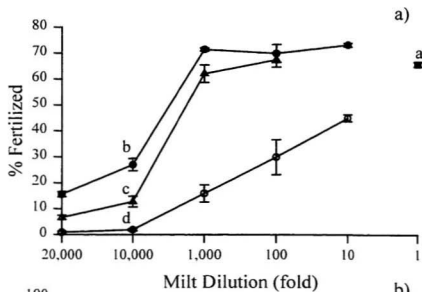
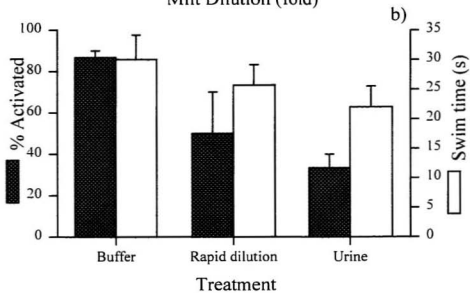
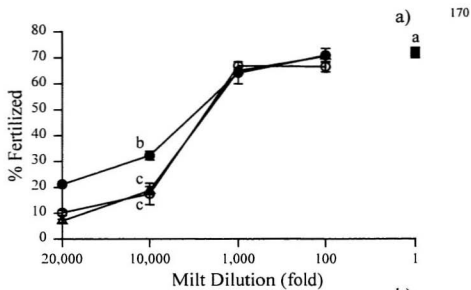




Fig. 41. Experiment II -Replicate 3. Effect of 3 different milt treatments compared to non-diluted milt on a) fertilization at four different dilutions. Diluted in buffer (filled circles), rapidly diluted milt (triangles), urine contaminated milt (open circles) and non-diluted milt (filled squares). Different letters represent significantly different fertilization rates of milt diluted 10,000 fold compared to non-diluted milt ( $F = 64.89$ ,  $P < 0.0001$ ). Effect of 3 milt treatments on b) percentage activated sperm and swim time of 100 fold dilutions. Percentage activated sperm ( $\chi^2 = 5.74$ ,  $P < 0.057$ ) and swim times ( $\chi^2 = 1.51$ ,  $P < 0.469$ ) were not significantly different between treatments.



#### 5.4. Discussion

Contamination of yellowtail flounder milt with urine that decreased sperm motility also decreased sperm fertility (Figs. 37 and 38). However, rapid dilution of contaminated yellowtail flounder milt reduced the negative effects of urine on sperm fertility (Figs. 39, 40 and 41). Urine contamination is difficult to avoid during the collection of yellowtail flounder milt since males produce low volumes of viscous milt when in captivity and the bladder is located directly between the dorsal ends of the two testes. A catheter can be inserted in the urogenital pore to drain the bladder, however because of the small diameter of the pore and the excitable nature of yellowtail flounder, the fish must be anaesthetized to avoid damaging the internal organs. This process is time consuming and repeatedly anaesthetizing a fish is likely to negatively affect its health and production of gametes and therefore is not practical for routine maintenance of captive broodstock. The viscous milt cannot be removed using this method because of the small diameter of the catheter required (PE 90 -internal diameter = 0.86 mm, external diameter = 1.22 mm).

Carp sperm is prematurely activated by urine contamination and ATP concentrations decrease in inverse proportions to urine concentration (Perchec et al., 1995b). In Atlantic salmon urine contamination decreased the  $K^+$  ion concentration in the milt which decreased subsequent sperm motility (Rana, 1995). Unlike salmon or carp, urine contamination of yellowtail flounder milt does not appear to prematurely activate sperm (data not shown); the mechanism whereby it decreases subsequent motility in seawater is unknown.

In order to understand the possible negative effects of urine contamination on sperm fertility, this investigation only examined urine that decreased sperm motility. The fertility effects of urine contamination that does not decrease sperm motility remains unknown.

Experiment I clearly showed that urine that decreased sperm motility also decreased sperm fertility, and this became more evident at decreasing sperm concentrations relative to egg numbers in the fertilization trials. Replicates one and two of Experiment II showed that rapid dilution (at one minute post contamination) of urine contaminated milt reduced the negative effects of urine on sperm fertility and sperm motility. Replicate three showed no difference in fertilization rates of the three treatments, however urine contamination did not appear to decrease sperm motility. These experiments were designed to examine the 'worst case scenario' of urine contamination with 10  $\mu$ l of milt diluted in 90  $\mu$ l of urine for either 1 min ('rapid dilution') or  $\geq 15$  min ('urine contaminated'). In practice milt could be diluted within 30 s of the time of collection, this would probably reduce the negative effects of urine contamination on sperm even more so than dilution at 1 min post contamination. Also, since rapid dilution is effective for milt diluted 10 fold in urine (90% urine) this data shows that even highly contaminated milt can be used for fertilization. The results of these experiments also indicate that poor sperm motility indicates low fertility and demonstrate the importance of testing sperm fertility at a range of dilutions.

The pH and osmolality of yellowtail flounder seminal plasma (7.5 and 318 mOsm respectively) is similar to that found in other marine teleosts, for example turbot *Scophthalmus maximus* L. (7.3 and 306 mOsm respectively) and sea bream *Sparus aurata*

L. (7.8 and 365 mOsm respectively). However, the pH is lower and the osmolality is higher compared to that found in most amphihaline and freshwater teleosts that have been investigated (Suquet et al., 1994, Table 8). The  $K^+$  concentration in the seminal plasma of yellowtail flounder is similar to other marine species and lower than in carp, goldfish and salmonid seminal plasma.  $K^+$  ions inhibit sperm motility in the seminal plasma of cyprinids and salmonids and the  $K^+$  ion concentration is elevated compared to the blood plasma concentrations, however,  $K^+$  ions have little effect on the sperm motility of marine species (Morisawa, 1985). Seminal plasma  $Na^+$  concentration in yellowtail flounder is similar to seminal plasma concentrations in other marine species and salmonids, and is higher than in cyprinids. The seminal plasma  $Na^+$  concentration in cyprinids is lower than in the blood plasma whereas in salmonids the  $Na^+$  concentration is very similar in the seminal plasma and blood plasma (Morisawa, 1985). The  $Mg^{2+}$  ion content is higher in yellowtail flounder seminal plasma than in other marine or freshwater species investigated so far, however the  $Cl^-$  ion and  $Ca^{2+}$  ion contents are roughly similar.

Comparison of the composition of yellowtail flounder urine samples and seminal plasma of motile sperm shows that urine that decreased sperm motility had a very high concentration of phosphate, although this was highly variable (Table 7). Both urine that decreased sperm motility and urine that had no affect on sperm motility showed a high concentration of  $Mg^{2+}$  in comparison to seminal plasma. Urine pH was  $6.2 \pm 0.1$ , and  $6.7 \pm 0.1$  (negative and no affect on motility respectively) compared to  $7.5 \pm 0.1$  in seminal plasma (Table 6), however pH appears to have little affect on the motility of yellowtail

flounder sperm (see Chapter 4). The osmolality of seminal plasma was  $318.0 \pm 1.9$  compared to  $312.5 \pm 1.3$  and  $313.1 \pm 2.4$  in urine that decreased sperm motility and urine that had no effect on sperm motility respectively, therefore osmotic shock does not appear to be the cause of a decrease in sperm motility after urine contamination. Rana et al., (1992) showed that collection of Atlantic salmon milt via a catheter had avoided urine contamination and resulted in higher sperm motility, spermatocrit and sperm concentration than non-catheterised milt. Urine contamination diluted the milt by up to 80% and resulted in a large variation in the osmolality and ionic composition of the non-catheterised milt. Since using a catheter to collect milt from captive yellowtail flounder is not a practical solution to the problem of urine contamination, rapid dilution of the freshly collected milt is a simple solution to improve the quality of milt collected.

## CHAPTER 6.

### General Discussion.

Captive male and female yellowtail flounder produced viable gametes during the spawning season of wild yellowtail flounder *Pleuronectes ferrugineus* (Storer) in Newfoundland (May to July) (Chapter 2). Both males and females showed a distinct annual cycle of plasma sex steroid levels that were similar to those observed in other teleost species (Pankhurst and Carragher, 1991) (Figs. 2, 3, 6 and 9). Individual fish were blood sampled either monthly or only once every two months (bimonthly) and although there was some indication that spawning may be inhibited by monthly blood sampling (abnormal spawners in 1994, Fig. 8) both males and females produced viable gametes.

Yellowtail flounder females appear to not spawn spontaneously under the confinement conditions provided in this study since large volumes of low quality eggs could be stripped from females that were not being examined regularly during the spawning season. Many teleost species in captivity show normal gonad development but fail to spawn spontaneously (Zohar, 1989; Barton and Iwama, 1991; Crim, 1991). This is thought to be caused by the lack of appropriate environmental cues for spawning in the captive environment (Crim, 1991). For example, goldfish *Carassius auratus* L. are induced to ovulate and spawn spontaneously in captivity by the addition of vegetation to their environment (Stacey et al., 1979). Yellowtail flounder may require higher water volumes

and/or higher water pressure than provided in this study since they are thought to spawn pelagic eggs at depths of <100 m (Pitt, 1970; Walsh, 1992).

Water borne pheromones stimulate reproductive behaviour and spawning in salmonids and cyprinids and are important in synchronising ovulation, spermiation and spawning between the males and females (Stacey and Cardwell, 1995).  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate, a potent pheromone in goldfish, has been found in the urine of plaice *Pleuronectes platessa* L. (Scott and Turner, 1991). Therefore it is likely that pheromones are important in the spawning of yellowtail flounder. Yellowtail flounder females are batch spawners and it is likely that males participate in several spawning events, therefore spermiation would need to be tightly synchronised with ovulation and spawning in females (Defraipont and Sorenson, 1993). However, if yellowtail flounder females are not spawning spontaneously in captivity the appropriate pheromonal cues may not be released to stimulate spermiation and/or spawning in males in captivity. Alternatively male yellowtail flounder may not be undergoing normal spermiation, since only relatively low milt volumes were collected during the spawning season, and therefore the males may not be stimulating the females to spawn. Both female seabass *Dicentrarchus labrax* L. and female goldfish will fail to spawn unless in the presence of spermiated males (Stacey et al., 1979; Zohar, 1989). Future research on yellowtail flounder reproduction in captivity should investigate the effect of known pheromones on ovulated females and mature males.



Failure to spawn spontaneously in captivity may be advantageous for management of broodstock since it allows collection of eggs and performance of 'dry' fertilization with selected milt samples. 'Dry' fertilization will result in higher fertilization rates than spontaneous spawning in the tank (Scott and Baynes, 1980) and also allows the selection of combinations of 'mates' from male and female broodstock. However, the extra handling and stress associated with collection of successive batches from a daily spawner may negatively affect the quality of eggs produced as the season progresses as has been shown in other teleost species (Campbell et al., 1992; Campbell et al., 1994; Wilson et al., 1995).

The seasonal cycle of plasma  $17\beta$ -estradiol in female yellowtail flounder suggests that vitellogenesis begins in October and continues until the spawning season. This information has implications for the potential success of manipulating the seasonal cycle of yellowtail flounder in captivity. In winter flounder *Pleuronectes americanus* (Walbaum) ovulation and spawning in females could be advanced by up to three months since vitellogenesis was complete four months before the beginning of the spawning season (Harmin and Crim, 1992). A recent study of female yellowtail flounder investigated the effect of advancing the photoperiod in November, but no difference occurred in the oocyte profiles of the treated fish compared to the controls (Linehan, 1996). Since vitellogenesis commences in October, advancing the photoperiod in September or October may be more effective at influencing the onset of vitellogenesis and ovarian maturation.

Although yellowtail flounder males showed a distinct annual cycle of plasma steroids and an increase in milt volume during the spawning season, small volumes of milt

containing motile sperm can be obtained from males throughout the year (Figs. 2, 3, 4 and 5). Since it was not possible to collect samples for histological examination of the testes in this study it is not known if spermatogenesis continues throughout the year or shows a seasonal cycle of production in yellowtail flounder males. The milt collected from males outside the May to July spawning season may be residual sperm from the previous spawning season. However unlike dab *Pleuronectes limanda* L. which also produce small amounts of milt year-round (Htun-Han, 1978), motile sperm were observed throughout the year in yellowtail flounder milt. Alternatively the final stages of spermiation may not be occurring in captivity, resulting in the relatively low volumes of high density milt sampled during the spawning season and residual sperm outside the spawning season, or spontaneous milt release may be occurring, thereby reducing the volumes measured in monthly sampling. Future work on freshly caught wild yellowtail flounder males during the spawning season might provide information on the milt volumes produced by wild fish and indicate if larger volumes than those measured in this study are produced by wild fish during the spawning season. Since at least some of the sperm collected from yellowtail flounder males throughout the year are motile this implies they may be fertile. Therefore if fertile eggs can be produced, the year-round production of yellowtail flounder may be possible.

GnRHa treatment delivered either by microspheres or by pellets successfully increased sperm production and milt volumes produced by mature male yellowtail flounder during the spawning season for 4 to 6 weeks post implantation (Chapter 3, Figs. 15 and 16).

Female yellowtail flounder spawn an average of 14 batches during a spawning season; each batch is ovulated one or two days apart (Manning and Crim, 1995). Therefore sufficient milt can be obtained from male broodstock to fertilize all eggs produced by using GnRHa treatment if necessary. GnRHa treatment of yellowtail flounder males did not have a negative effect on fertilization rates (when high concentrations of sperm were used), hatch rates or larval appearance (Figs. 21 and 22). This is consistent with results from other species that show that although GnRHa treatment may negatively affect egg quality, sperm quality is not affected (Billard and Marcel, 1980). Sperm motility and milt pH was higher in milt produced by all the hormone treated yellowtail flounder groups compared to the control treatment (Fig. 20). GnRHa treatment may have stimulated the acquisition of sperm motility in yellowtail flounder males via changes in sperm duct pH since increases in sperm duct pH are also associated with increased sperm motility in rainbow trout *Oncorhynchus mykiss* (Walbaum) and chum salmon *Oncorhynchus keta* (Morisawa and Morisawa, 1986). The progestin  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha,20\beta$ P) is also associated with the acquisition of motility via alterations in sperm duct pH. in masu salmon *Oncorhynchus masou* (Brevoort) and the Japanese eel *Anguilla japonica* (Temminck and Schlegel) (Miura et al., 1992; Miura et al., 1995). Japanese flounder *Paralichthys olivaceus* (Temminck and Schlegel) males have a diurnal cycle of spermatogenesis and milt production during the spawning season and this is associated with a short low amplitude surge in plasma  $17\alpha,20\beta$ P simultaneous with milt production (Matsuyama et al., 1995b). In landlocked Atlantic salmon, catfish and carp, GnRHa

treatment has been shown to have an effect on milt volume by stimulating the pituitary to produce gonadotropin and thereby stimulating the testes to produce steroids (Crim et al., 1983a; Weil and Crim, 1983; Takashima et al., 1984; Rosenblum and Callard, 1987). No clear pattern of changes in plasma steroids was observed in yellowtail flounder after GnRHa treatment, but this may have been because the sampling schedule did not synchronise with increases in plasma steroid levels. Therefore, although  $17\alpha,20\beta\text{P}$  also showed no seasonal changes that might be associated with reproduction in yellowtail flounder (see Chapter 2), future work should focus on injection of mature males with  $17\alpha,20\beta\text{P}$  and rapid diurnal changes in plasma or testicular levels of this steroid associated with successive spawning events.

When held under ambient photoperiod and seasonal changes in water temperature both male and female yellowtail flounder underwent an annual cycle of reproduction similar to that observed in wild fish (Chapter 2). These conditions were sufficient to stimulate the production of viable gametes in season in the majority of captive fish however high water temperatures ( $>7.7^{\circ}\text{C}$ ) may have inhibited ovulation in some females in the latter half of the spawning season in 1994 (Fig. 8), since these abnormal spawners spawned low volumes ( $<5$  ml) of low quality eggs but their ovaries remained distended and firm which is characteristic of prespawning (vitellogenic) females. Comparison of the spawning success of females held at  $<7.7^{\circ}\text{C}$  throughout the spawning season and females held at higher water temperatures should determine the role of temperature in spawning.

In 1993 two different water temperatures were provided for the captive yellowtail flounder during winter (2.5-6.4°C, 1.4-4.2°C respectively, Fig. 1), however this had no detectable affect on growth or reproduction, therefore providing average water temperatures between 1.4-4.2°C appears sufficient to maintain yellowtail flounder broodstock in the laboratory throughout winter. Although average water temperatures remained above zero, subzero temperatures did occur during the winter but were not associated with mortalities.

Several methods were developed that will further the study of yellowtail flounder sperm and improve the management of male yellowtail flounder broodstock (Chapters 4 and 5). The buffer, DCSB4, is suitable for dilution of yellowtail flounder milt since it keeps the sperm immotile and is suitable for oocyte fertilization. If the sperm concentration is unknown milt can be diluted at least 100 fold and achieve fertilization rates equivalent to or possibly higher than undiluted milt. Spectrophotometry is a rapid and reliable method to measure the sperm concentration of yellowtail flounder milt and a ratio of approximately 5,000 sperm:egg will be needed to achieve high fertilization rates in yellowtail flounder using the 'dry' fertilization method. Using spectrophotometry to measure sperm concentration will also be a useful method for future studies of seasonal changes in sperm production. Rapid measurement of sperm concentration will aid fertilization trials so that similar concentrations of sperm can be tested for viability at a sperm:egg ratio that will distinguish sperm quality. Short-term storage of high quality yellowtail flounder milt is possible in a 10 fold dilution in buffer in a refrigerator. This

is a simple method which will be useful if the spawning of females is not synchronised with male milt production or when high milt volumes are not available. GnRHa treatment of males during the spawning season will also relieve the problems of low sperm availability and will not adversely affect the quality of progeny produced by treated males (at least up to larval hatch). If urine contamination of milt is a concern when milt is being collected rapid dilution of the contaminated milt in buffer will improve its viability and motility (Figs. 39, 40 and 41).

This study focussed on problems associated with the production of sperm and the collection of high quality milt from male yellowtail flounder, however future work on yellowtail flounder sperm should attempt to define other parameters that relate to sperm quality apart from fertilization rates. No parameter has yet been defined that can reliably predict sperm viability. Currently, motility is the most commonly used parameter to predict sperm quality (Billard et al., 1992). So far motility has not yet been correlated with sperm fertilization rates, for example in Atlantic salmon *Salmo salar* L. (Aas et al., 1991) and in Atlantic cod *Gadus morhua* L. (Trippel and Nielson, 1992). Nonetheless, the relationship between high motility and high sperm fertility is often claimed (Aas et al., 1991) for example the studies by Morisawa et al., (1983) Stoss (1983), Cosson et al., (1985). Many studies of sperm fertility and motility suffer from one of two faults in their methodology. Firstly high concentrations of sperm are used in fertilization trials. As previously discussed (see Chapter 4) if high numbers of sperm are present relative to egg numbers, high fertilization rates will be achieved even when sperm quality is low.

Secondly sperm motility is a difficult parameter to measure accurately and many motility trials examine sperm at high concentrations and depend on subjective estimates from microscopic examination of numbers of sperm cells activated and duration of mass forward motility (Billard and Cosson, 1992). These measurements are useful for a rough indication of sperm motility, however Cosson et al., (1985) have developed a method of measuring rainbow trout sperm motility with stroboscopy after a two-step dilution. Motile sperm can be videoed and later these records can be analysed objectively for sperm velocity (Billard et al., 1992). This method of measuring sperm motility may be more suitable for establishing correlations/relationships between motility and fertilization rates than more subjective motility estimates. The data in this investigation indicated that motility and fertilization rates at low sperm concentrations may be related. For example in the sperm storage trials (see Chapter 4), decreases in sperm motility were noted in the 100 fold milt dilutions after four days of storage (Figs. 33 and 34) and were reflected in lower fertilization rates (Fig. 32). Similarly in the investigation of the effect of urine contamination on sperm motility and fertility decreases in fertilization rates of sperm with low motility became more obvious when lower sperm concentrations (100 fold) were used in fertilization trials (Figs. 37 to 41). Therefore future work on the sperm physiology of yellowtail flounder should focus on establishing an accurate method of measuring sperm motility using the methods established for rainbow trout as an example (Cosson et al., 1985). The relationship between sperm motility and fertilization rates could then be established at low sperm concentrations using spectrophotometry to measure sperm

concentration. If no relationship between sperm motility in seawater and fertilization rates can be established, then an investigation of the relationship between sperm motility in ovarian fluid and fertilization rates would be interesting since sperm motility has been shown to increase in ovarian fluid in several teleost species (Stoss, 1983). Recently correlations between motility and different compounds in the seminal plasma have been documented in the cyprinid *Alburnus alburnus*. However, the authors explicitly stated that the relationships between either motility and fertility or between seminal plasma parameters and fertility have yet to be established (Lahnsteiner et al., 1996).



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