MANIPULATION OF OVULATION TIME IN THE YELLOWTAIL FLOUNDER, Limanda ferruginea STORER USING PHOTOTHERMAL CUES AND GONADOTROPIN HORMONE RELEASING HORMONE ANALOGUE (GnRH-a) ADMINISTRATION

PAULINE LYNN LUSH

Manipulation of ovulation time in the yellowtail flounder, *Limanda ferruginea* Storer using photothermal cues and gonadotropin hormone releasing hormone analogue (GnRH-a) administration

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Abstract

The yellowtail flounder (*Limanda ferruginea*) once considered a prime candidate for aquaculture, were photothermally manipulated in an attempt to advance the ovulation time of females of this species. The success of these techniques would be highly beneficial to the broodstock management of yellowtail flounder.

Two groups of female yellowtail flounder were held under compressed photothermal environmental laboratory conditions as an attempt to advance ovulation and spawning time. One group was administered a controlled release gonadotropin hormone-releasing hormone analogue (GnRH-a) in February, the other group was administered a sham pellet in an identical manner. A third group of fish was held under ambient photoperiod and temperature conditions. The experiment ran for two years using a different group of animals for each year. In year two, a sub-group (five females) of the ambient control fish was given a GnRH-a pellet in February. In year one a total of 6 females were used in each of the photothermally advanced group. In year two this number was increased to twelve in both photothermally treated groups.

Throughout annual reproductive development the degree of advancement of the females was monitored using both ovarian cannulation and plasma steroids (estradiol-17β and testosterone) analysis. No significant advancement was noted in either of the photothermal manipulated groups in the oocyte diameters obtained through the cannulation or in steroid levels.

Advanced spawning in the group photothermally manipulated and administered the GnRH-a pellet (PP-GnRH-a) was noted in March in both year one and two. The

photothermally manipulated group receiving the sham pellet (PP-Sham) did not spawn in advance of the ambient control group. Egg quality parameters, based on egg viability, fertilization and hatch rates, for the PP-GnRH-a group were excellent and were significantly higher than the PP-Sham group for viability and fertilization. Egg quality for the PP-GnRH-a group was not significantly different from the ambient group of females. The PP-Sham and control group both spawned in June, which is the expected ambient spawning time for yellowtail flounder in captive conditions. A single female from the ambient group administered the GnRH-a pellet in February spawned, however egg and larval quality was extremely poor, and larvae produced from this female did not survive for more than one day post hatch.

Further investigation is required to determine an appropriate way to advance ovulation in the yellowtail flounder using environmental cues, and to improve on general broodstock husbandry of this species.

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Chapter 1

Introduction

1.1 Overview of the life history and reproductive biology of the yellowtail flounder *(Limanda ferruginea)*

The yellowtail flounder (*Limanda ferruginea*) is a cold-ocean flatfish distributed from Chesapeake Bay in the south to its northerly limit in the Strait of Belle Isle in southern Labrador. It is most abundant on the Grand Banks, Banquereau, Sable Island and the St. Pierre Banks (Bigelow and Schroeder 1953; Pitt 1970; Scott and Scott 1988). Yellowtail flounder became commercially important in 1965 when landings increased, coinciding with a marked reduction in haddock landings (Pitt 1970). When groundfish abundance declined throughout the latter 20th century, focus changed to the captive farming of many of the species once fished off the east coast of Canada. Yellowtail flounder was thought to be an excellent candidate for cold-ocean aquaculture as it has a high quality flesh with established commercial markets, a fast growth rate, and is quite tolerant of captive conditions being a euryhaline and eurythermic species (Pitt 1970; Crim 1993; Bettles 1996; Manning and Crim 1998). As more species are being examined for their aquaculture potential, those that fetch high market prices are being focused on and researched to maximize productivity for any future aquaculture endeavor. One aspect vital to the culturing of a fish species is the management of a captive broodstock to achieve good quality gametes, which in turn will produce high quality offspring.

The reproductive biology of the yellowtail flounder has been detailed in several studies. Spawning in this species occurs in the spring and summer, starting in April and

continuing until September. Spawning shows a latitudinal cline with spawning commencing and concluding earlier in the more southern regions (Scott and Scott 1988). Pitt (1970) found that yellowtail from the Grand Banks reached a peak spawning during the latter half of June. Under captive conditions, spawning in yellowtail flounder have been observed to reach a peak between mid-July and August, but spawning was observed as early as May (Manning and Crim 1998).

Female yellowtail flounder are batch-spawners, meaning that one group of fully yolked and hydrated oocytes are released from the ovary while another group is still undergoing nuclear migration and hydration and will be spawned as the next batch (Zamarro 1991). Manning and Crim (1998) found that a one day interovulatory period is common in captive females, but no such data exists for wild stocks. Zamarro (1991) has estimated that the process of hydration lasts approximately 12 hours. Under captive conditions the yellowtail flounder does not spontaneously spawn, and both males and females must be manually stripped. This poses a problem for this easily stressed species, as it does not do well with the repeated handling required with batch spawners (Smigielski 1979).

In the wild, Grand Banks(off the eastern coast of Newfoundland) male yellowtail flounder are thought to become sexually mature at 5 years of age when length is approximately 31cm, and females at 6 years when length is 37cm (Pitt 1970). However, smaller, younger fish become mature in captivity, reaching maturity at less than 25 cm (Manning 2003). Females are considered to be group synchronous meaning that two distinct populations of oocytes are present in the ovary at one time (Wallace and Selman

1981; Howell 1983). Howell (1983) has described seven cytologically different developmental stages in the ovary of the yellowtail flounder and has classed them under two headings representing the two populations of cells present in the ovary. The previtellogenic cells, those that will not be released in the upcoming spawning season are subdivided into the oogonia (5-29 μm), early perinucleolus (10-78 μm), resting (23-140 μm) and late perinucleolus stages (39-174 μm). The second class, titled the vitellogenic oocytes, are those that are preparing to be spawned in the upcoming spawning season, and include the early (52-260 μm) and late (104-474 μm) maturing as well as the hyaline or hydrated oocytes (~400 μm). Howell's study also tracked the timing of these stages and noted that a previtellogenic stock of oocytes arises each year soon after the spawning season. These oocytes rapidly develop into the perinucleolus stage and reach the resting stage by fall. Oocytes will remain at this stage until the following spring when they develop into the late perinucleolus stage. Oocytes are in the previtellogenic stage for a full year before they begin to accumulate yolk in the form of a hepatically derived glycophospholipoprotein called vitellogenin. These oocytes are now considered a second class of oocytes, which are vitellogenic and will be spawned in the same year. Close to the spawning season the oocytes will undergo a hydration through the absorption of fluid and certain accompanying changes including the coalescing of yolk globules, and the migration of the nucleus. These changes prepare the oocyte for release and subsequent fertilization.

As stated previously, the yellowtail flounder is a batch spawner, a reproductive strategy employed by several species including the Atlantic cod (*Gadus morhua)*, the

Atlantic halibut (*Hippoglossus hippoglossus)*, and haddock (*Melanogrammus aeglefinus*). Zamarro (1991) noted in yellowtail flounder that as one batch is being spawned the next is ending the nuclear migration stage. This strategy allows the female to maximize chances of offspring survival by spawning egg batches at different times, and under a wider variety of environmental conditions which include food availability and temperature. This batch spawning strategy allows the opportunity for the offspring to hatch at the most conducive time to their survival. It also allows the potential for greater genetic variation by permitting several spawning events with a larger number of males, given that each batch is fertilized by a different male.

Variation between batch size has also been noted. Batches may differ in fecundity, and egg quality. Zamarro (1991) has noted through histological analysis that the number of oocytes maturing at the same time in a single batch was 200 000 ± 20 000. For captive females Manning and Crim (1998) noted that batch fecundity ranged from 10 000 to 60 000 oocytes.

Yellowtail flounder spawn in spring under long and increasing photoperiod and temperature conditions. It is the change in these photothermal cues that act on the neuroendocrine pathway to promote spawning. Bye (1987) has proposed that the cue most effective in promoting spawning in temperate species is photoperiod, provided that temperature is within range of tolerance for the species. Photoperiod acts at the highest level of the hypothalamus-pituitary-gonad (H-P-G) axis. Changes in light duration act directly on the eyes which in turn triggers the pineal gland to adjust the level of melatonin in the system of the animal. These changes affect the neurosecretions of gonadotropin

releasing hormone (GnRH) from the hypothalamus which promote the release of the gonadotropins (GTHI and GTHII) from the pituitary. These GTH's stimulate the production of steroids in the gonad, and resultant sexual maturation (Peter 1983; Fostier *et al.* 1983; Moyle and Cech 1988; Ekstrom and Meissl 1997). Vitellogenesis occurs under a different set of environmental conditions than final maturation, and it is these changes in the environment that promote the appropriate physiological responses. For the yellowtail flounder, vitellogenesis would occur under shorter daylength (i.e. fall), while hydration and spawning occur during longer daylength (i.e. spring). The opposite scenario would be the case in a fall spawning species such as the Atlantic salmon. This knowledge has sparked numerous studies in manipulating the timing of spawning in several species of fish, which is the major focus of this project.

1.2 Manipulation of the spawning cycle of fishes using photothermal and/or hormonal cues

Seasonal breeders, the majority of fish species outside of tropical regions, have distinct patterns of gonad maturation that culminate into a spawning event restricted to a specific time of the year. It is the information provided by the environment in the form of changing photoperiod, temperature, water level, spawning substrate or vegetation that promotes and regulates the spawning event. The precise timing of spawning under the correct environmental conditions ultimately promotes the best survival of offspring. The environmental conditions are those which are most suitable to the development of the young, factors which include optimum temperature for growth, and food availability such as plankton blooms which serve as a major food source for the larvae of many species. The development of the gonad most often begins under completely different conditions than those which promote the final maturation and release of gametes; in fact the external conditions controlling the advancement of gametogenesis may be different for each stage (Bye 1984).

Controlling the reproductive cycle of a species is imperative to broodstock management. Changing the timing of spawning so that specific groups of fish spawn at different times of the year may provide a year round productive broodstock to an aquaculture facility. Such production of gametes throughout the entire year will maximize production at the facility where certain months see very little or no production. Year round availability of gametes, and hence offspring will also be of a huge benefit to marketing fish of suitable size throughout a larger portion of the year.

Adjustment in photoperiod has been shown as an inexpensive and effective way to advance or delay spawning. Photoperiod control has been very effective in the spawning manipulation of temperate species, including the flatfishes (Bye 1984). Altering the photoperiod so that it is advanced or delayed compared to the natural photoperiod can cause noticeable changes in the timing of spawning for a species. Since light acts at the highest level of the H-P-G axis it seems likely that changes in such an important environmental factor would affect the maturation of the gonad. However, there is evidence that an endogenous rhythm or an internal clock exists which dictates the timing of reproduction in seasonal spawners regardless of the environmental conditions. Certain species kept under constant environmental conditions have been noted to exhibit the same rate of gonad maturation as the individuals kept under natural seasonal conditions (Bye 1984). This was best demonstrated in the catfish (*Heteropneustes fossilis*) where the usual seasonal change in ovarian weight persisted for three years under constant temperature and continual light or darkness. This indicates an endogenous cycle of reproductive development approximately equal to 12 months. It has been proposed that a similar cycling is common to most if not all species (Bye 1984). Even those species in deep sea vents devoid of temperature changes and in complete darkness show seasonal cycles of gonadal maturation (Bye 1984).

Regardless of the endogenous rhythm noted in some species, there are those species which respond well to environmental manipulation. The European turbot (*Scopthalmus maximus*) and the Atlantic halibut are both flatfish species which have been successfully induced to spawn out of season with photoperiod manipulations

(Devauchelle *et al.* 1988; Bjornsson *et al.* 1998). Various other species have also been induced including the rainbow trout (*Onchorhynchus mykiss*) and the Atlantic salmon (*Salmo salar*) (Randall *et al.* 1991; Oppendal *et al.* 2003). Early observations of the dab (*Limanda limanda*) showed that fish held under photoperiod regimes which included more than one period of daylight resulted in an advancement of spawning by six months (Bye 1987). Photoperiod trials done on the Atlantic halibut by Bjornsson *et al.* (1998) have shown that spawning can be altered if photoperiod cues are four months advanced or delayed from that observed by the wild population, however, large shifts in spawning time were not observed until three spawning seasons had passed. Other studies with certain salmonids have shown that exposure to long light induces out of season spawning. Rainbow trout submitted to continuous light spawned in advance to those fish kept under ambient light conditions (Randall *et al.* 1991).

Previously, photoperiod manipulation of yellowtail flounder has not been successful. Linehan (1996) held female yellowtail under compressed photoperiod regimes in November beginning at 7.25 hours of light and increasing light levels until February when it remained at a maximum of 17 hours of light per day. Advanced spawning was not observed in any females. In the study, temperature control was not attempted, and is thought to have been a contributing factor to the lack of success in the experiment. To advance the spawning cycle of this species a combination of appropriate photothermal cues may have to be used. It is also possible that yellowtail flounder will not spawn out of season without administering exogenous hormone, regardless of the photothermal regime at which the fish are held. The combination of the two treatments

was found effective in experiments with the Channel catfish (*Ictalurus punctatus*) where some fish held under certain photothermal manipulations required an injection of LHRH-a before spawning would occur (Kelly and Kohler 1996). This combined use of photothermal and hormonal manipulation has also shown considerable success in the summer flounder (*Paralichthys dentatus*). By using both of these techniques summer flounder have shown a capacity to spawn at various times throughout the year according to the photothermal regime to which they are exposed (Berlinsky *et al.* 1996; Watanabe *et al.* 1998; Bengston 1999).

While various forms of hormonal manipulation have proven successful in numerous species of fish, repeated administration of some of these hormones would not be suitable to a highly stressed batch spawning species such as the yellowtail flounder. The development of sustained release GnRH analogue cholesterol (GnRH-a) pellets has proven very successful in batch spawning animals especially those which are susceptible to repeated handling stress (Larsson *et al.* 1997). A single pellet implanted into an individual fish can release hormone over an extended period of time, thus reducing handling which is often needed with pituitary extract, steroids or repeated acute injections of GnRH-a. Previous work has involved the use of carp pituitary extract, however this preparation would be contaminated with other pituitary hormones in addition to those required for gonad maturation and spawning. However, these extracts are still being used and there is some argument that other pituitary hormones present in the preparations, such as prolactin and growth hormone, may play a role in annual sexual maturation (Bengston 1999). The use of the GnRH-a in a sustained release cholesterol pellet is

gaining widespread use, and is being successfully used in numerous species including the rainbow trout, *Oncorhynchus mykiss* (Breton *et al.* 1990), Atlantic salmon, *Salmon salar* (Crim and Glebe 1984), common carp, *Cyprinus carpio*
(Drori *et al.* 1994), gilthead seabream, *Sparus aurata* (Barbaro *et al.* 1997), striped bass, *Morone saxatilis* (Mylonas *et al.* 1998), winter flounder, *Pseudopleuronectes americanus* (Harmin and Crim 1992), southern flounder, *Paralichthys lethostigma* (Berlinsky *et al.* 1996) and the yellowtail flounder, *Limanda ferruginea* (Bettles 1997; Larsson *et al.* 1997).

GnRH-a has been used in a number of species to improve broodstock management in several ways, including initiating final maturation, synchronizing broodstock, and advancing spawning. Often females will fail to hydrate vitellogenic oocytes and GnRH-a can induce final maturation and spawning. For flatfish species, GnRH-a sustained release pellets have proven successful in advancing or inducing the spawning season of the winter, yellowtail and southern flounder without compromising egg quality (Harmin and Crim 1992; Berlinsky *et al.* 1996; Bettles 1997; Larsson *et al.* 1997). Oftentimes, in cyprinids, a dopamine antagonist is required to shift the balance of GnRH and dopamine to give the required surge of GnRH to promote release of the GTH from the pituitary (Peter *et al.* 1988). Fortunately, this is not the case with this flounder species, as a response is elicited with the use of the GnRH-a pellet alone. Yellowtail flounder and its response to GnRH-a pellets have been studied in recent years (Bettles 1997; Larsson *et al.* 1997).

Larsson *et al.* (1997) showed that GnRH-a treatment doubled egg production, synchronized spawning females, and shortened the interovulatory period. It also advanced spawning in females by 3-4 weeks over control fish. Bettles (1997) also showed that GnRH-a implants advanced spawning by a few months if administered when oocytes had reached a critical diameter, generally 400 μm. The number of fish responding to the implants improved as the natural spawning time neared, with the best results being observed when implants were given 2 weeks before the regular spawning season. Summer flounder show great success with advanced spawning when a combination of photothermal and hormonal manipulations are given (Watanabe *et al.* 1998), with select groups spawning out of season throughout most of the year. This is beneficial to broodstock management, allowing for maximum use of a facility. As yellowtail flounder were under investigation for aquaculture the combination of photothermal and GnRH-a implant manipulations may prove beneficial to the farming of this species by maximizing the use of the broodstock resources available.

1.3 Study Objective

The focus of this study is to determine the efficacy of photothermal manipulation of the yellowtail flounder both with and without administration of a GnRH-analogue, with the intention of improving the candidacy of the species for aquaculture by improved broodstock management. Effects of the manipulation on spawning performance, and egg and larval quality will be assessed.

Chapter 2

Monitoring maturation in photothermal manipulated female yellowtail flounder using ovarian cannulation and steroid profiles

2.1 Introduction

Yellowtail flounder spawn from June to August under captive conditions (Manning and Crim 1998). Numerous studies have shown that the spawning time of certain species including flatfish can be manipulated using environmental cues with or without exogenous hormonal manipulations. Initial attempts to advance the spawning time of yellowtail flounder with photomanipulation were unsuccessful (Linehan 1996). However, Bettles (1997) has shown that a certain degree of advancement can be accomplished with the use of a sustained release cholesterol gonadotropin releasing hormone analogue (D-Ala^6, Pro^9[GnRH-a]) implanted in the musculature of the yellowtail flounder held at ambient environmental conditions. Bettles (1997) also introduced a cannulation procedure useful in monitoring the maturational progress in the yellowtail flounder based on the procedures developed by Shehadeh *et al.* (1973) for grey mullet (*Mugil cephalus*). By inserting a small cannula into the ovipore and down into the ovarian lumen, a sample of the vitellogenic oocytes can be aspirated, subsequently measured and assessed for maturation. An increase in diameter would indicate an increase in the uptake of the hepatically derived yolk protein, vitellogenin. Maturing oocytes will continue the uptake of vitellogenin until hydration and subsequent release of the matured oocyte occurs. Maturational progress of females can be monitored

throughout a large portion of the annual reproductive cycle using this cannulation procedure.

Bioassay of blood plasma for reproductive steroids is also a good indicator of maturational progress as levels of estradiol-17β (E2) and testosterone (T) would increase at certain stages of annual sexual development. In females an increase in these steroids coincides with increased production of vitellogenin as plasma levels of E2 activate the liver to begin production of yolk (Moyle and Cech 1988). Clearwater (1997) found that E2 levels in female yellowtail flounder were elevated in November indicating the onset of vitellogenesis. These levels continued to increase until spawning commenced in June. Increased T in females occurred just before spawning, and peaked just after E2. It is expected that with manipulated fish these steroid profiles will be advanced compared to ambient environmental fish if spawning occurs earlier from the ambient. Hence, steroid profiles would be a good indicator of maturational progress, and a predictor of spawning time between ambient and photothermally manipulated species.

As the yellowtail flounder were being considered a potential cold-ocean aquaculture species, these non-lethal methods of assessing maturity would be preferred over the traditional methods of histologically sampling a population, thus retaining live broodstock. As steroid assays are costly, the more inexpensive route of cannulation may be more feasible. Results from cannulation data would be quicker to obtain and maturational progress of broodstock may be assessed immediately. However, data can be obtained to anticipate the advancement of the spawning cycle through both oocyte diameter and steroid profile. The objective of this study is to determine through ovarian

cannulation, and blood plasma steroid levels if advanced response to the photothermal manipulation is occurring, prior to the collection of ovulated eggs.

2.2 Materials and Methods

2.2a Environmental Experimental Conditions

Prior to the experiments, male and female yellowtail flounder were held together in 2000 L broodstock tanks at the Ocean Sciences Center, Logy Bay, Newfoundland and Labrador supplied with degassed and aerated ambient sea water under ambient photoperiod. Fish were collected by SCUBA divers from Bay Bulls and Witless Bay in summer of 1995 and 1998. Sources of light in holding tanks were natural lighting through an adjacent window as well as a timer controlled 60W incandescent light bulb suspended 80 cm above the water surface. Fish were fed a commercial moist pellet salmon diet (Connor's Brothers Ltd, St George, New Brunswick) at 2% body weight twice weekly, and occasionally during spawning. Individual fish were tagged using PIT (passive integrated transponder) tags injected in the right dorsal musculature or peritoneum.

Attempts to advance spawning in female yellowtail flounder were performed for two consecutive years on separate groups of females each year. The experimental population was increased and adjustments were made to photo and thermal cues in the second year. In September of both years, females were chosen from the broodstock and moved to 400 L experimental tanks supplied with degassed and aerated seawater. Females were inspected for sexual maturity before being placed in the experiment and

only fish not previously used for similar experiments were incorporated. Fish were considered sexually mature if the gonad was easily visible and extended two-thirds of the total distance in the ovarian cavity (Manning 2003). Condition factors as a function of length (L) and weight (W) were obtained (W/L^3 *100) for fish only in the second year of experimentation. In both years of experimentation, hormone or sham pellets were administered to fish in February (Chapter Three of this thesis).

Photomanipulated fish were held in tanks with the light source an incandescent 60W light bulb suspended 80 cm from the water surface, all extraneous light was blocked by black plastic shields encompassing the four sides of the tank. Water temperature was regulated using a Neslab™ unit to chill water during warmer ambient conditions, and by mixing ambient and heated seawater to achieve warmer temperatures during winter months. In addition to the PIT tags, fish were externally tagged in the right opercula using surgical thread and small coloured beads for easy visual recognition during the spawning season, and for cannulation in year 2. Upon completion of spawning, fish were returned to the 2000 L broodstock tanks.

Year 1 (1997-1998)

Fourteen female yellowtail were divided equally into two experimental tanks. All fish had been held in captivity for at least one year at the Ocean Sciences Centre (Table 2.1). Photoperiod and temperature was decreased from ambient to (11L: 13D) and 5°C beginning on September 24, 1997 with a continued daylength decrease by one hour for the next nine days, maintaining photoperiod at 8L:16D on day nine. Fish were

maintained under these environmental conditions for 125 days (January 27, 1998), after which photoperiod was increased by four hours (12L: 12D), and then again increased by 5 hours on day 132, making the final photoperiod (17L:7D). Temperature at this time was increased to 6°C (Fig 2.1). Fish were held under these final conditions until spawning was completed. In February, a GnRH-a hormone pellet was given to one photomanipulated group (PP-GnRH-a) with the other photomanipulated group being administered a sham pellet (PP-Sham). Males remained in the large 2000L broodstock tank, and were not placed in experimental tanks. The broodstock held at the Ocean Sciences Centre were used as ambient environmental controls and maintained under ambient photoperiod and temperature.

Year 2 (1998-1999)

Twenty-six female yellowtail flounder were weighed, measured and divided equally into the two experimental tanks. These females were selected from existing captive broodstock and from new fish brought from the wild (Table 2.2). In addition to the females, three males were added to each experimental tank. Fish were maintained under the same experimental conditions as the previous year, with the exceptions that photoperiod was decreased earlier following the regular spawning season and increased sooner in the reproductive cycle. Daylength began decreasing on September 10, 1998 (11L:13D) and was decreased over the next 5 days maintaining daylength at 8L:16D on day 5. The temperature remained at 5°C and photoperiod at 8L:16D for 81 days. Photoperiod and temperature was then increased (day 81- November 30, 1998) in the

same manner as year one, and maintained on this environmental regime until completion of spawning. Again, hormone or sham pellets were administered to the photomanipulated groups in February using the same methods as year one. Fish were given a one time injection of 10 µl of Tetraject LA- a long acting oxytetracycline antibiotic, and a daily addition of 3g of choloramine T to the tanks over a one week period, at the initiation of the experiment, as a parasite preventative. Environmental control group conditions were the same as year one.

2.2b Ovarian Cannulation and Sampling

All fish in year one and a randomly selected subset of six fish from each environmentally manipulated group in year two were selected for cannulation and blood sampling every month starting in January of year one and December of year two. In addition, 3-5 fish from the ambient broodstock were sampled at the same time. Cannulation prior to December has been proven ineffective, as oocytes are not easily removed from the ovary using this technique in early recrudescence (Linehan 1996; Bettles 1997). Bettles (1997) demonstrated cannulation to be an effective method to assess maturation in the yellowtail ovary, and that oocytes removed by this method were representative of the entire ovary. A polyethylene cannula (inner diam. =1.19 mm, outer diam. =1.70 mm) was inserted in the ovipore, guided through the oviduct and into the right ovary. Oocytes were then aspirated from the ovary by applying gentle suction. Placement of the cannula could be visualized in the ovary throughout the procedure; hence guiding the cannula in the ovary was not difficult. For the most part only

vitellogenic oocytes were removed, however on occasion clusters of previtellogenic oocytes appeared in the sample. After removal from the ovaries, oocytes were placed in an oocyte fixative solution of 1% formalin, 0.6% saline, and diameters were measured 24 hours later using a stereomicroscope (40X) fitted with an ocular micrometer.

Blood samples were obtained using a pre-heparinized 3 cc syringe fitted with a 23 G needle inserted just above the lateral line in or slightly anterior to the caudal peduncle and into the haemal canal; ½-1cc of blood was taken and kept on ice. Blood samples were transferred to microcentrifuge tubes and centrifuged at 10 000 rpm for 20 minutes at 10°C. Blood plasma was then pipetted in duplicate into small Eppendorf tubes and frozen at -20.0°C until steroid assay analysis.

2.2c Analysis of Plasma Steroids

Blood plasma was analyzed for levels of estradiol (E_2) and free testosterone (T) using I^{125}, following procedures validated by Harmin *et al.* 1995.

Blood samples were centrifuged and frozen until plasma was assayed with a no extraction, solid phase I^{125} Coat-a-Count® steroid assay kit (Diagnostic Products Corporation®, Los Angeles CA.). Both plasma estradiol (E2) and free testosterone were counted using Coat-a-Count® kits with specific antibodies for each of the two steroids. One hundred micro litres of plasma was placed in an antibody coated polypropylene tube, 1.0 ml of the radioactive iodine was added and the tube vortexed. Tubes were incubated for 3 hours at room temperature, and the decanted bound fraction was counted in a gamma counter (Packard Autogamma 5650 Series, United Technologies Packard) for one

minute. Calibration curves were determined and the amount of steroid in a sample was determined using these curves.

2.2d Statistical analysis

Data were analyzed using SAS statistical software package (SAS Institute Inc., Cary, NC) and Minitab statistical package. One-way analysis of variance (ANOVA) was performed to determine statistical difference between groups at various sampling dates for both oocyte diameters and plasma steroids. Tolerance for Type I error was set at a level of 0.05. For some parameters insufficient data were available for statistical analysis.

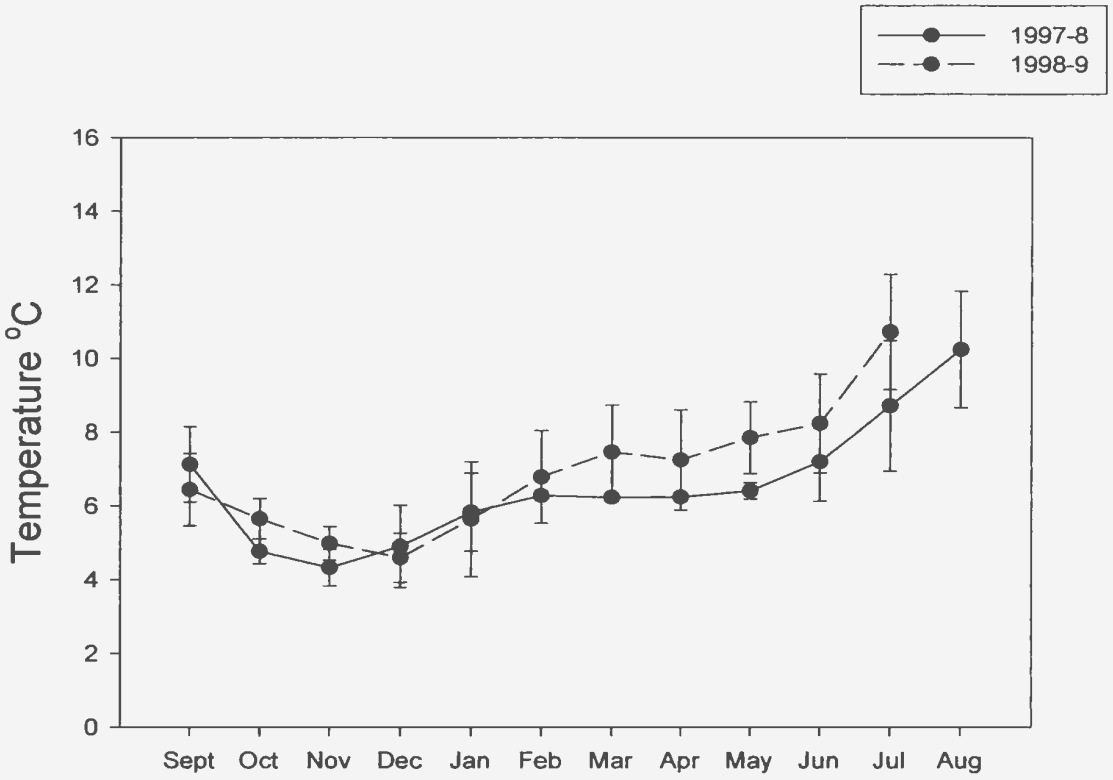


Figure 2.1: Temperature profile (°C) for yellowtail flounder (*Limanda ferruginea*) over the study period of 1997/8 and 1998/9 in the photothermally adjusted experimental tanks. Error bars denote standard deviation.

Table 2.1: Individual fish tag number and origin for photothermally manipulated females in 1997-8 (year one)

Treatment '98	Tag #	Captive/Wild
Photoadjusted-GnRH-a	OFO85C	c
	516BID	c
	715C28	c
	305872	c
	370643	c
	151F09	c
	782D7C	c
Photoadjusted -Sham	OE56OF	c
	154026	c
	164860	c
	340B2E	c
	3D670B	c
	OEO23E	c
	451A4A	c

Table 2.2: Individual fish tag number and origin for photothermally manipulated females in 1998-9 (year two)

Treatment '99	Tag #	Captive/Wild	Mass (g)	Length (cm)	Condition Factor
	313A38	c	194	27.5	0.93
	1F3232	c	280	32.5	0.82
	26266E	c	344	36.3	0.72
	138836	c	263	32.3	0.78
	39311F	c	298	39.8	1.13
	OA27OE	w	251	30.1	0.93
Photoadjusted - GnRH-a	18023E	w	292	31.5	0.93
	443338	w	229	30.3	0.83
	042D38	w	321	30.5	1.13
	451A4A	w	360	32.8	1.02
	1B2845	w	260	32.3	0.78
	166C11	w	280	31.5	0.91
	250327	w	214	27.5	1.03
	431COF	c	349	33.8	0.91
	370643	c	414	35.1	0.97
	163A23	c	566	40.1	0.88
	0F6375	c	327	32.5	0.95
	1C324E	c	260	32.5	0.76
	4A6303	w	308	32.1	0.94
Photoadjusted - sham	0F3900	w	215	30.1	0.81
	1D7972	w	240	32.3	0.72
	214045	w	375	35.1	0.87
	0B6005	w	217	31.3	0.71
	196149	w	341	35.1	0.81
	682F43	w	198	28.8	0.83
	3A6079	w	396	32.5	1.15

2.3 Results

2.3a Oocyte maturation

Monthly cannulation (Table 2.5) revealed that oocyte diameters for the most part increased for individual fish with each sampling (Tables 2.3, 2.4). In year one some animals did not show an increase with each month. One fish (OFO85C) from the photoadjusted – GnRH-a group and one fish (340B2E) from the photoadjusted-sham group showed a slight decrease between the January 1998 and February 1998 sampling. The fish from the GnRH-a group spawned in March and the fish from the sham group increased oocyte diameter over the subsequent sampling date. Statistical analysis of the means using a one-way analysis of variance revealed that no significant difference was found between the photoadjusted-GnRH-a and photoadjusted-sham at any cannulation sampling date for either experimental year (Table 2.6). Since no significant difference was noted, data for the two photoadjusted groups were combined and oocyte diameter frequency graphs were generated to compare the photothermal adjusted groups to the control ambient broodstock (Figs 2.2-2.10). Frequency graphs were used due to some sampling dates or individuals producing samples of fewer oocytes than others.

In the first two sampling months of year one (January 21, 1998 and February 11, 1998), there was a significant difference in oocyte diameter between the photothermal adjusted and control groups (Table 2.6). A noticeable shift in the oocytes diameters can be seen in Figs 2.2 and 2.3 where diameter frequency is toward the higher end on the oocyte diameter scale. Significant differences between the photothermally manipulated and ambient groups were not detected at any other sampling date either in year one or

year two. Most frequency graphs did not show a tendency to shift to the larger diameters for manipulated fish in comparison to the ambient; with the exception being April 1999 (Fig 2.10) where there was a slight shift, but no significant difference was detected between diameters for the control and photothermal adjusted groups.

Several individuals produced cannulation samples of only previtellogenic oocytes and/or ovarian fluid or debris from both experimental years. Ovarian debris generally consisted of over-ripened dark coloured misshapen oocytes, and connective tissue. The over-ripened oocytes may be a remnant from the previous spawning season, produced due to insufficient stripping or possibly atretic oocytes. In year one, no previtellogenic oocytes were observed from any cannulated females. Fourteen percent (two females) of photothermally manipulated females sampled produced fluid and debris only. One of these females died before the third sampling date. The other continued to produce debris until the termination of the experiment. A total of 29% (four females) of the animals died before spawning commenced. One control animal produced ovarian debris on the first sampling date, but on the three subsequent sampling dates produced vitellogenic oocytes (Table 2.3).

In year two, 46% (6 females) of the photothermally manipulated females cannulated produced previtellogenic oocytes, ovarian fluid or debris only, and at no sampling date were vitellogenic oocytes observed in the sample. Thirty-one percent (four females) of photothermally manipulated females cannulated died before the termination of the experiments. Sixty-six percent of these mortalities were not undergoing any obvious vitellogenesis (no vitellogenic oocytes). Of the fish not producing vitellogenic

oocytes, 67% were wild fish brought to the Ocean Sciences Centre in the summer of 1998 (beginning of year two of experiment) (Tables 2.2 and 2.4). One female in the photomanipulated group given the GnRH-a pellet (166C11) produced vitellogenic oocytes in each cannulation sample, the oocyte diameter continued to increase over the sampling period, but this fish failed to spawn, even though other individuals in this group spawned months prior to the last sampling. In the photomanipulated group not receiving the hormone pellet, two individual females (OB6005 and 196149) showed increasing oocyte diameters throughout the sampling dates, but in the end did not ovulate even at the ambient spawning time (Table 2.4).

2.3b Steroid Profiles

In both year one and two no statistically significant difference could be detected for either estradiol-17β or testosterone for any sampling date (Table 2.7 and 2.8). On occasion, some samples were not able to be analysed due to an insufficient amount of blood gathered during sampling, or not being able to collect a blood sample from certain individuals on a sampling date. As with cannulation, it was decided that if blood was not collected easily from a female, then further attempts to collect at this sampling date would not be attempted. Again, the focus was to maintain healthy viable animals to spawning time, and the stress of continued sampling and anaesthetic would likely cause high mortality.

A seasonal trend in steroid profiles is seen in Figs 2.11-2.14 for both testosterone and estradiol-17β. While no statistical significance can be determined, a large variation

on the sampling dates may have led to the lack of detectable significance. Data in year one clearly shows no difference between the three treatments in the first two sampling dates. Post implantation with the GnRH-a pellet in February, there is a difference in both estradiol-17β and testosterone with both being higher in the PP-Sham group, however no statistical significance could be detected. Testosterone continued to increase in the next sampling date for the PP-Sham group (May); however estradiol-17β decreased in the PP-Sham group on this date.

In year two of the experiment, while no significant difference could be detected at any of the sampling dates, testosterone showed a marked increase in March for the control (ambient conditions) animals. In the following sampling date, April, the PP-Sham showed an increase in testosterone, while the control group had a reduction in the amount of plasma testosterone.

Table 2.3: Average oocyte diameters or presence of fluid/residual oocytes (debris) for individual yellowtail flounder females from the photoadjusted and control treatment groups - year 1 – 1997-1998

Treatment	Tag #	Average Oocyte Diameter (µm)			
		Jan-98	Feb-98	Mar-98	May-98
Photoadjusted - GnRH-a	OFO85C	376.74	375.44	Spawned	ns*
	516BID	Debris	Debris	Dead	
	715C28	371.81	388.71	Spawned	ns
	305872	Dead			
	151F09	Debris	Debris	Debris	Fluid/Blood
	370643	355.42	355.94	Spawned	ns
	782D7C	329.16	332.54	Spawned	ns
Photoadjusted - Sham	OE560F	333.06	389.22	495.56	547.04
	154026	404.86	416.11	523.12	545.22
	164860	Dead			
	340B2E	376.22	369.52	469.82	490.61
	3D670B	339.56	370.24	454.74	375.71
	OEO23E	333.06	379.86	452.41	498.94
	451A4A	326.56	336.44	Dead	Dead
Control	164A13	Debris	293.02	472.68	357.76
	176056	231.14	245.18	422.24	501.28
	392D5E	307.71	ns	ns	528.06

*ns- not sampled

Table 2.4: Average oocyte diameters, presence of previtellogenic (previts) or fluid/residual oocytes (debris) for individual yellowtail flounder females from the photoadjusted and control treatment groups - year 2 – 1998-1999

Treatment	Tag #	Average Oocyte Diam (µm)				
		Dec-98	Jan-99	Feb-99	Mar-99	Apr-99
Photoadjusted-GnRH-a	1B2845	Previts	Previts	Previts	ns*	ns
	OA270E	Previts	Dead			
	18023E	Previts	Previts	Previts	Dead	
	1F3232	336.18	401.96	420.94	Spawned	
	451A4A	322.41	Dead			
	138836	374.41	412.36	Dead		
	166C11	ns	356.2	370.56	520.11	513.26
Photoadjusted - Sham	370643	351.26	416.11	424.58	469.82	512.72
	OB6005	336.7	366.08	375.44	451.62	523.91
	196149	340.86	350.74	322.66	338.11	360.62
	214045	Previts	Previts	Previts	Previts	Previts
	1D7972	Previts	Previts	Debris	Debris	Debris
	163A23	Previts	Debris	Debris	Debris	Fluid
Control	Ctrl 1	365.31	422.76	401.71	Spawned	
	Ctrl 2	347.88	Dead			
	Ctrl 3	332.28	356.72	369.21	Spawned	
	Ctrl 4	260.11	Previts	Debris	Spawned	
	Ctrl 5	ns	382.21	401.71	381.42	429.78
	Ctrl 6	ns	ns	ns	472.16	555.85

*ns – not sampled

Table 2.5: Cannulation sampling dates for year one and year two of study for photothermally manipulated and control female yellowtail flounder

1997-98	1998-99
January 21	December 1
February 11*	January 19
March 30	February 9*
May 20	March 23
	April 26

* hormone/sham pellet implant date

Table 2.6: Statistical analysis of average oocyte diameters between environmental treatments for female yellowtail flounder

Date		Between Treatments				Between Treatments and Controls	
	p-value	F-value	Significant		p-value	F-Value	Significant
Jan-98	0.928	0.01	No		0.015	7	Yes
Feb-98	0.701	0.16	No		0.002	12.65	Yes
Mar-98	*	*	*		0.959	0	No
May-98	*	*	*		0.626	0.26	No
Dec-98	0.74	0.32	No		0.74	0.32	No
Jan-99	0.89	0.12	No		0.887	0.12	No
Feb-99	*	*	*		0.798	0.24	No
Mar-99	*	*	*		0.919	0.01	No
May-99	*	*	*		0.891	0.02	No

* both photothermal treatments not cannulated post GnRH-a implantation.

Table 2.7: Statistical analysis of testosterone analysis between environmental treatments for female yellowtail flounder

Date		Between Treatments				Between Treatments and Controls	
	p-value	F-value	Significant		p-value	F-Value	Significant
Jan-98	0802	0.068	No		0.802	0.068	No
Feb-98	0.125	2.93	No		0.120	2.70	No
Mar-98	*	*	*		0.471	0.606	No
May-98	*	*	*		*	*	*
Dec-98	0.288	1.26	No		0.442	0.869	No
Jan-99	0.295	1.23	No		0.447	0.862	No
Feb-99	0.471	0.574	No		0.586	0.562	No
Mar-99	*	*	*		0.182	2.20	No
Apr-99	*	*	*		0.384	0.880	No

*both photothermal treatments not cannulated post GnRH-a implantation

Table 2.8: Statistical analysis of estradiol-17β analysis between environmental treatments for female yellowtail flounder

Date		Between Treatments				Between Treatments and Controls	
	p-value	F-value	Significant		p-value	F-Value	Significant
Jan-98	0.861	0.032	No		0.452	0.855	No
Feb-98	0.144	2.52	No		0.344	1.18	No
Mar-98	*	*	*		0.391	0.881	No
May-98	*	*	*		*	*	*
Dec-98	0.721	0.135	No		0.649	0.446	No
Jan-99	0.842	0.0420	No		0.920	0.0838	No
Feb-99	0.531	0.429	No		0.588	0.557	No
Mar-99	*	*	*		0.928	0.00870	No
Apr-99	*	*	*		0.948	0.00469	No

*both photothermal treatments not cannulated post GnRH-a implantation

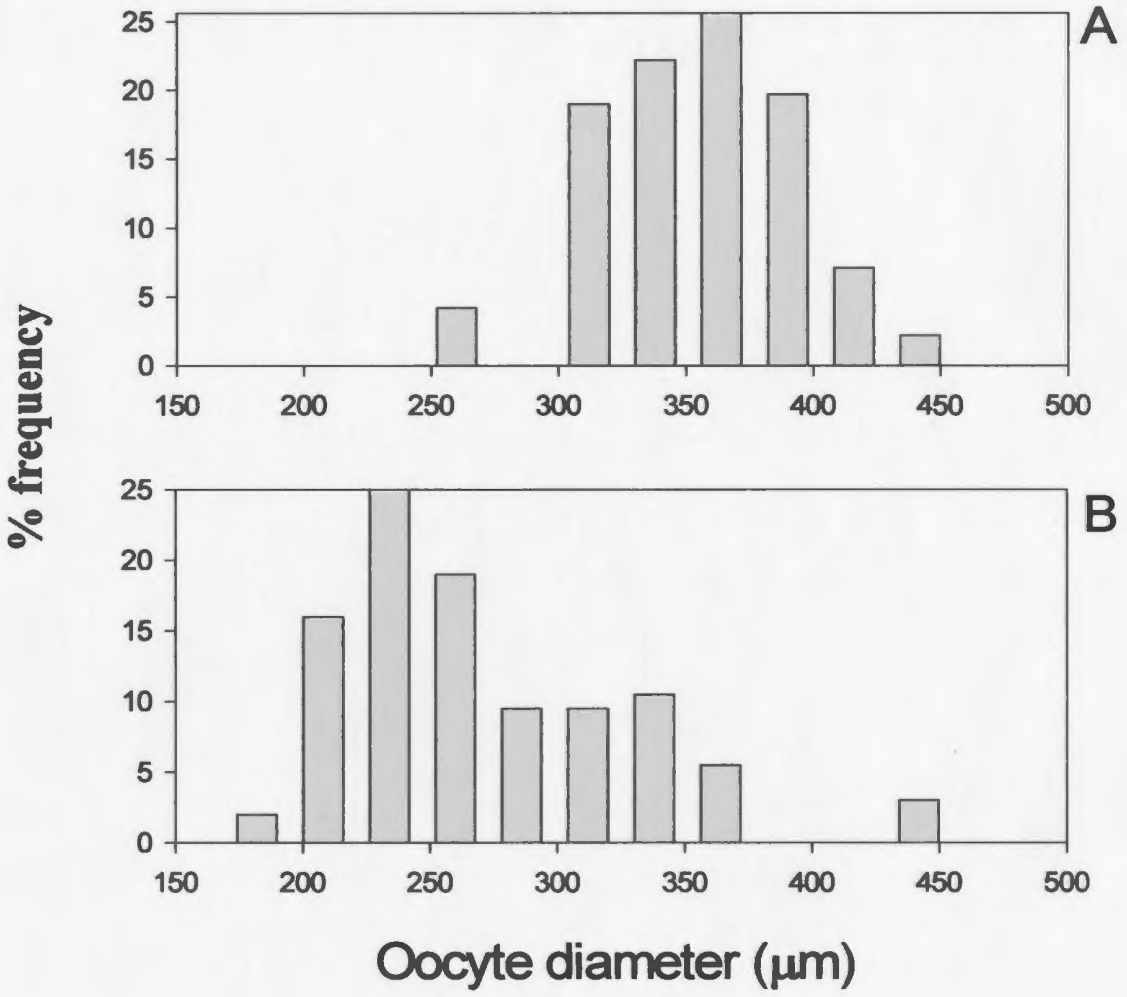


Figure 2.2: Percent frequency (%) of oocyte diameters (µm) for January 1998 (year one) for photothermally adjusted (A) N=10 and ambient control (B) N=2 female yellowtail flounder (*Limanda ferruginea*)

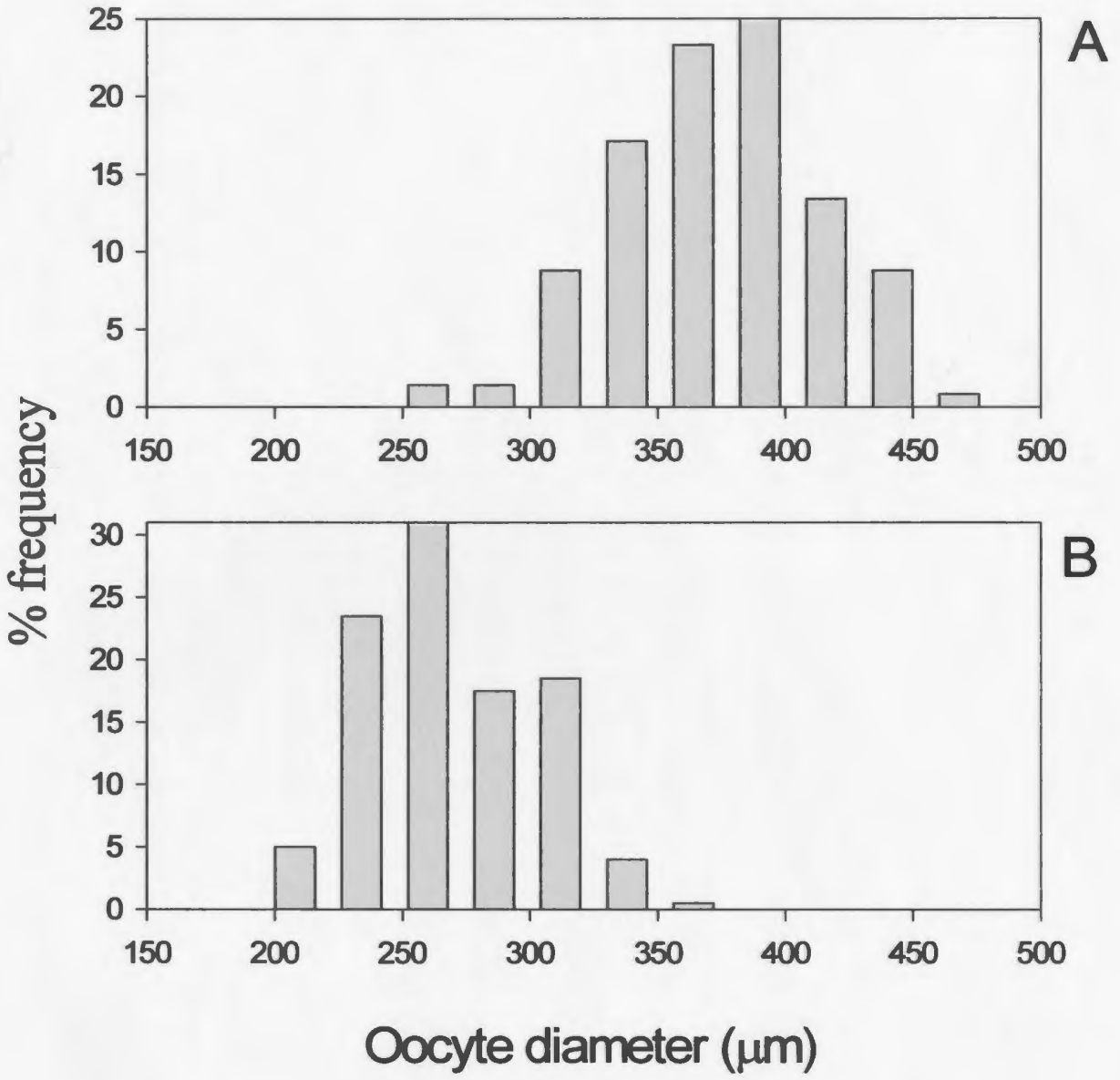


Figure 2.3: Percent frequency (%) of oocyte diameters (μm) for February 1998 (year one) for photothermally adjusted (A) N=10 and ambient control (B) N=2 female yellowtail flounder (*Limanda ferruginea*)

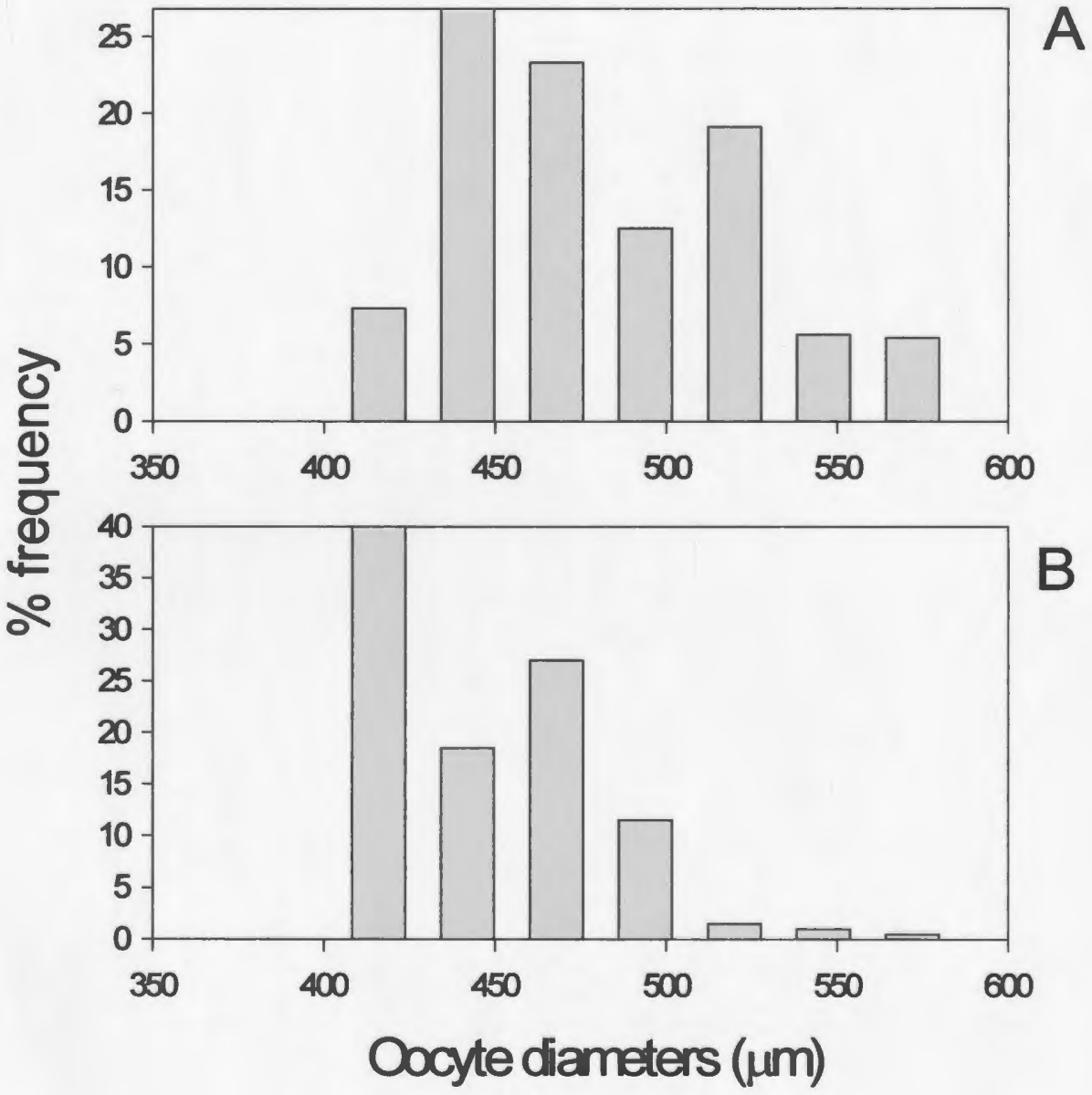


Figure 2.4: Percent frequency (%) of oocyte diameters (µm) for March 1998 (year one) for photothermally adjusted (A) N=5 and ambient control (B) N=2 female yellowtail flounder (*Limanda ferruginea*)

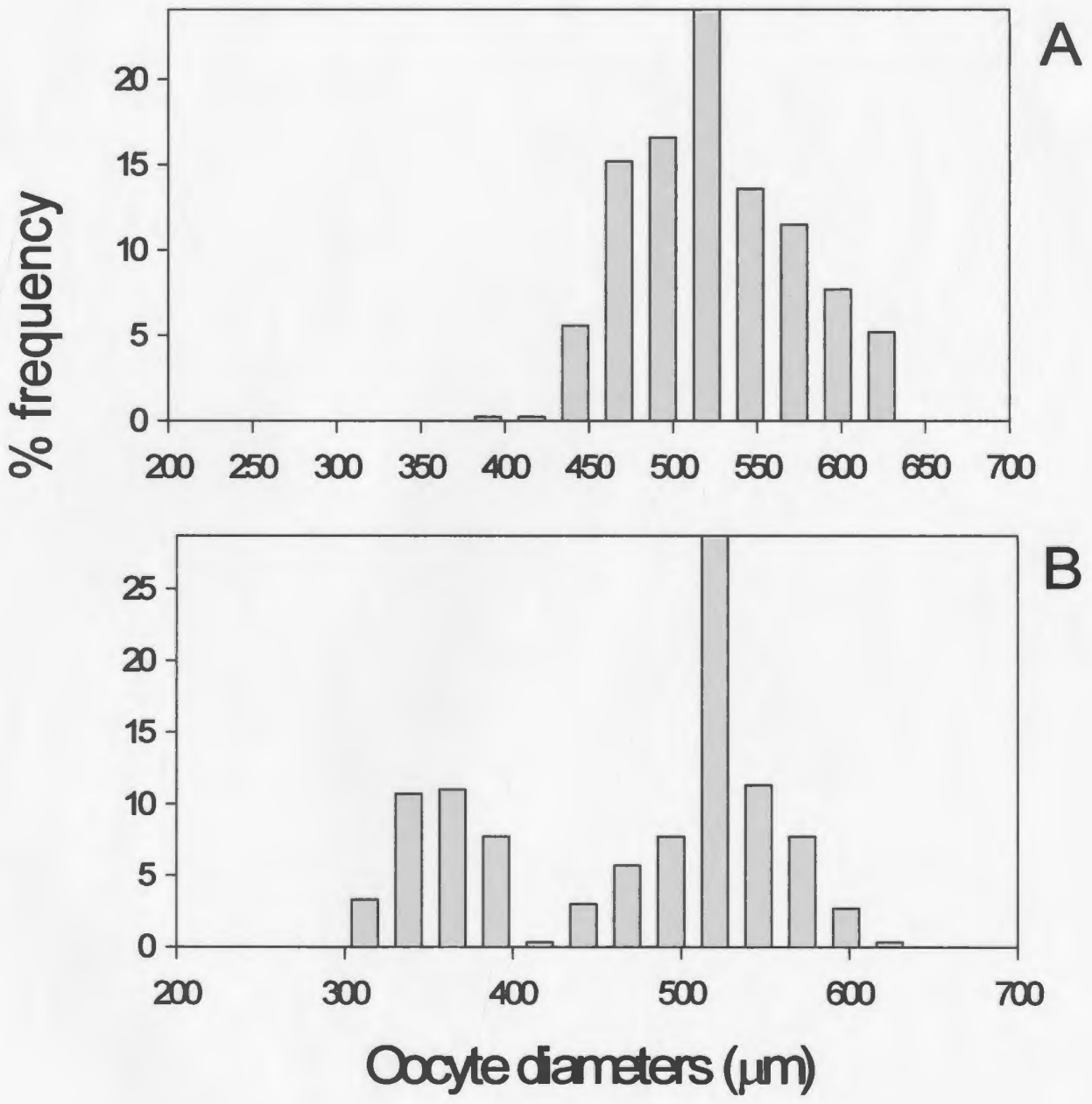


Figure 2.5: Percent frequency (%) of oocyte diameters (µm) for May 1998 (year one) for photothermally adjusted (A) N= 5 and ambient control (B) N= 3 female yellowtail flounder (*Limanda ferruginea*)

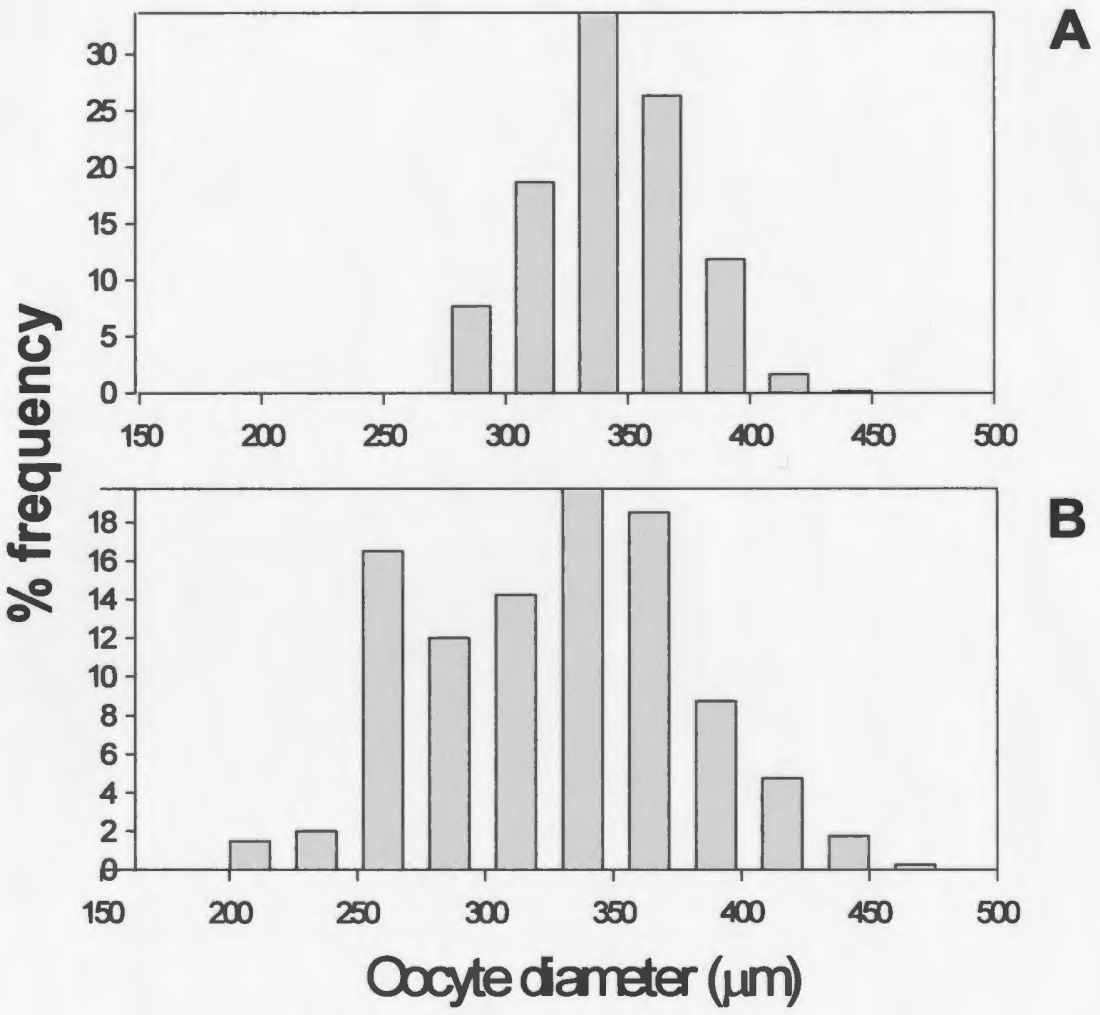


Figure 2.6: Percent frequency (%) of oocyte diameters (µm) for December 1998 (year two) for photothermally adjusted (A) N= 6 and ambient control (B) N= 4 female yellowtail flounder (*Limanda ferruginea*)

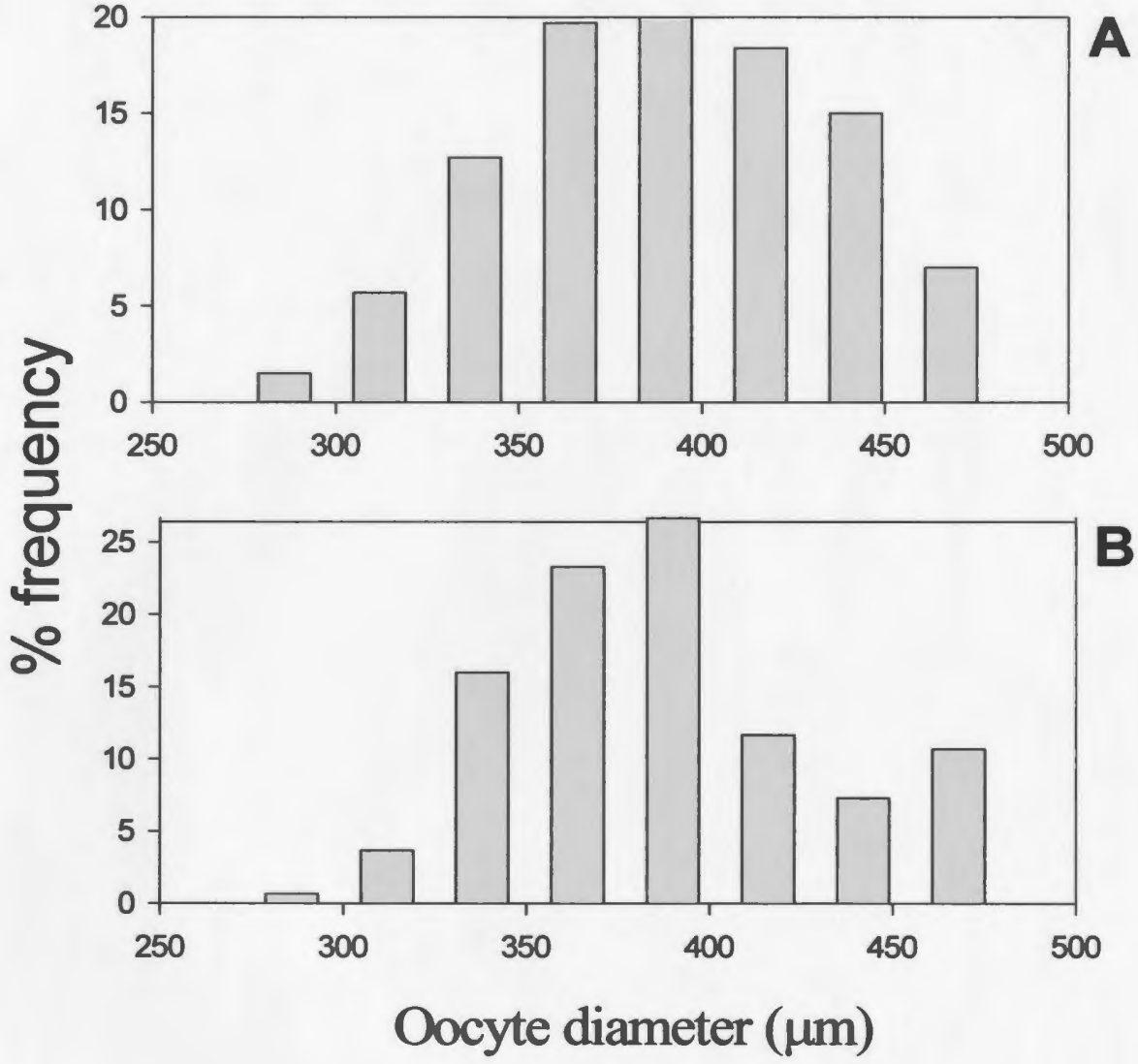


Figure 2.7: Percent frequency (%) of oocyte diameters (µm) for January 1999 (year two) for photothermally adjusted (A) N= 6 and ambient control (B) N= 3 female yellowtail flounder (*Limanda ferruginea*)

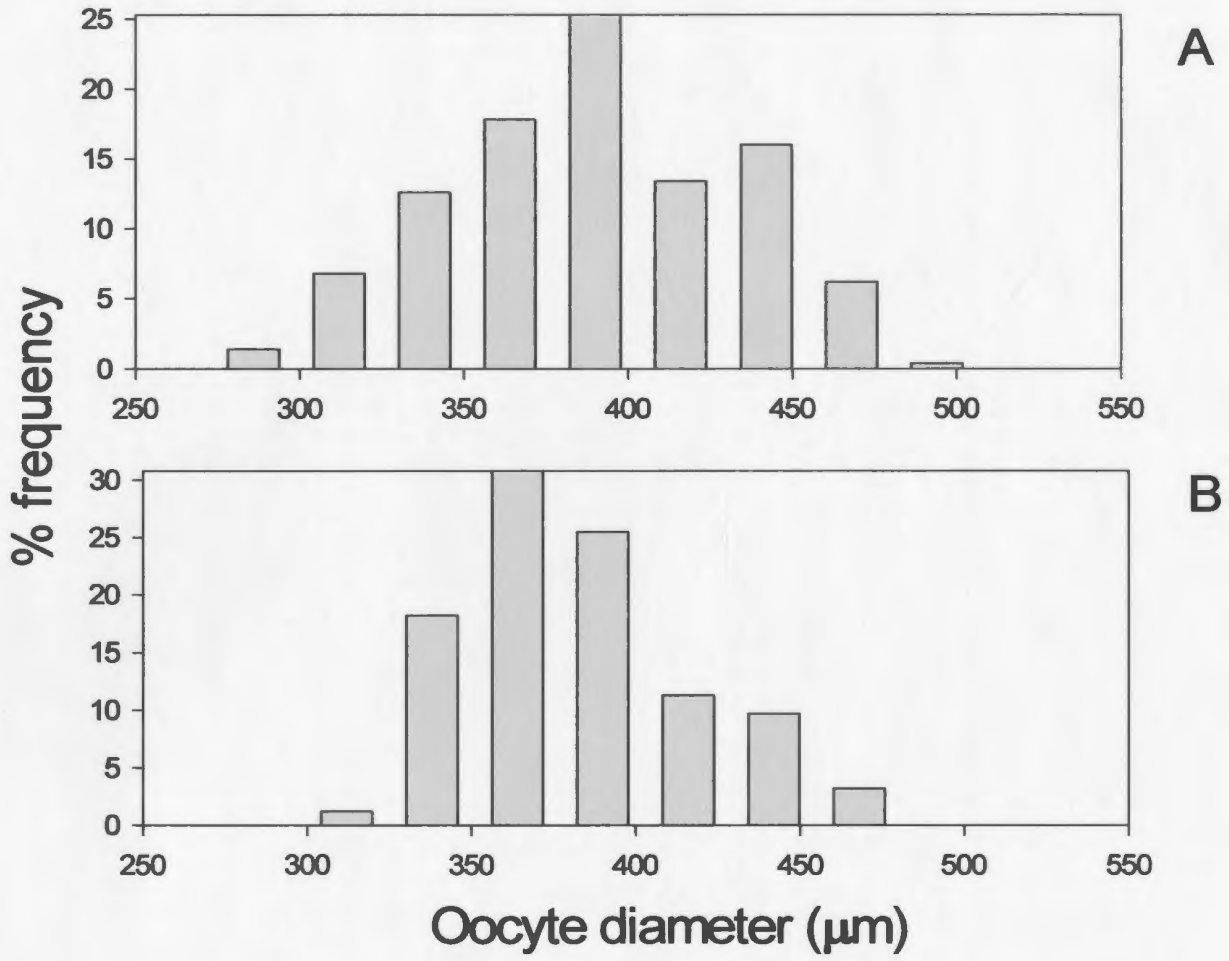


Figure 2.8: Percent frequency (%) of oocyte diameters (µm) for February 1999 (year two) for photothermally adjusted (A) N= 5 and ambient control (B) N= 3 female yellowtail flounder (*Limanda ferruginea*)

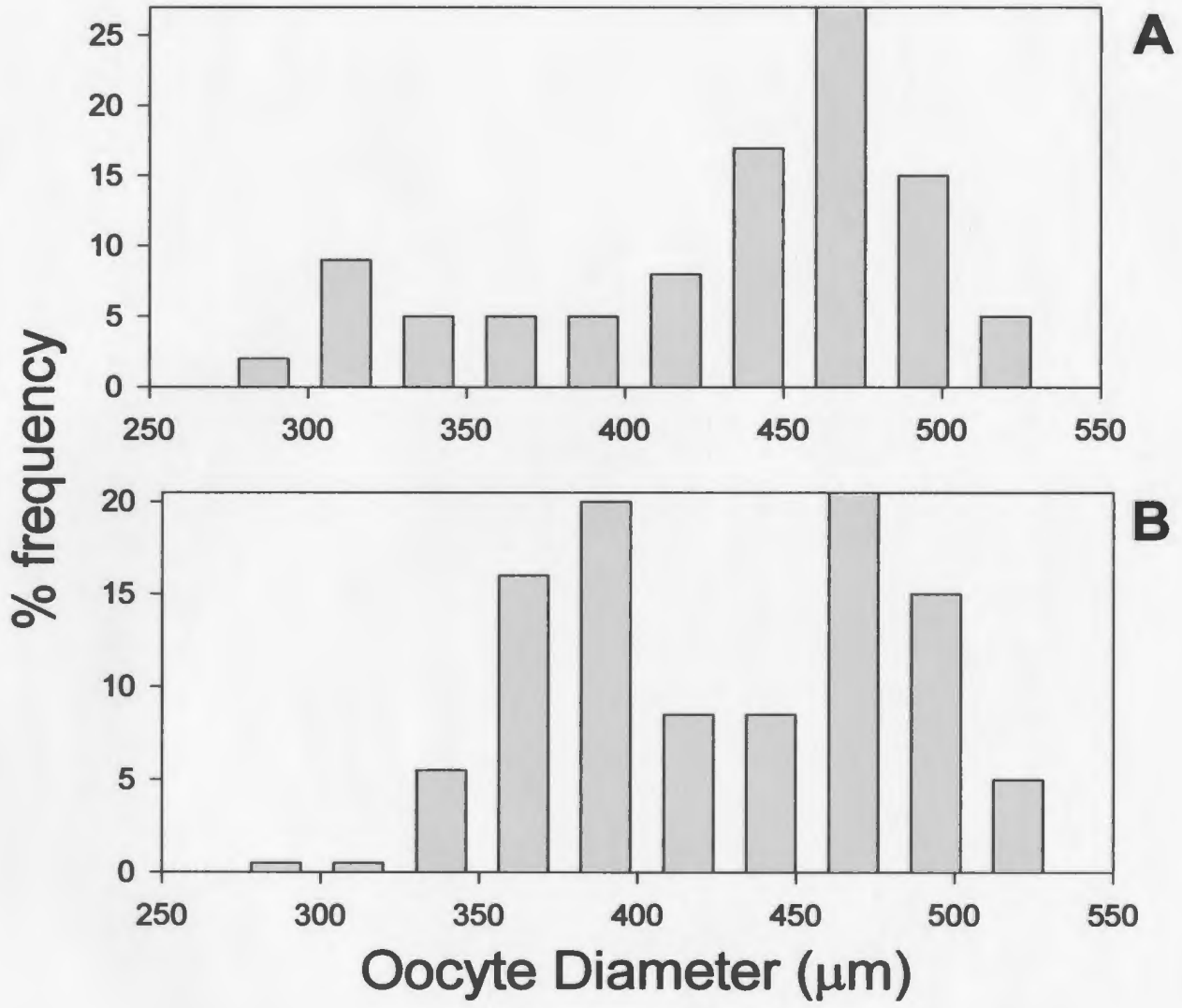


Figure 2.9: Percent frequency (%) of oocyte diameters (μm) for March 1999 (year two) for photothermally adjusted (A) N= 4 and ambient control (B) N= 2 female yellowtail flounder (*Limanda ferruginea*)

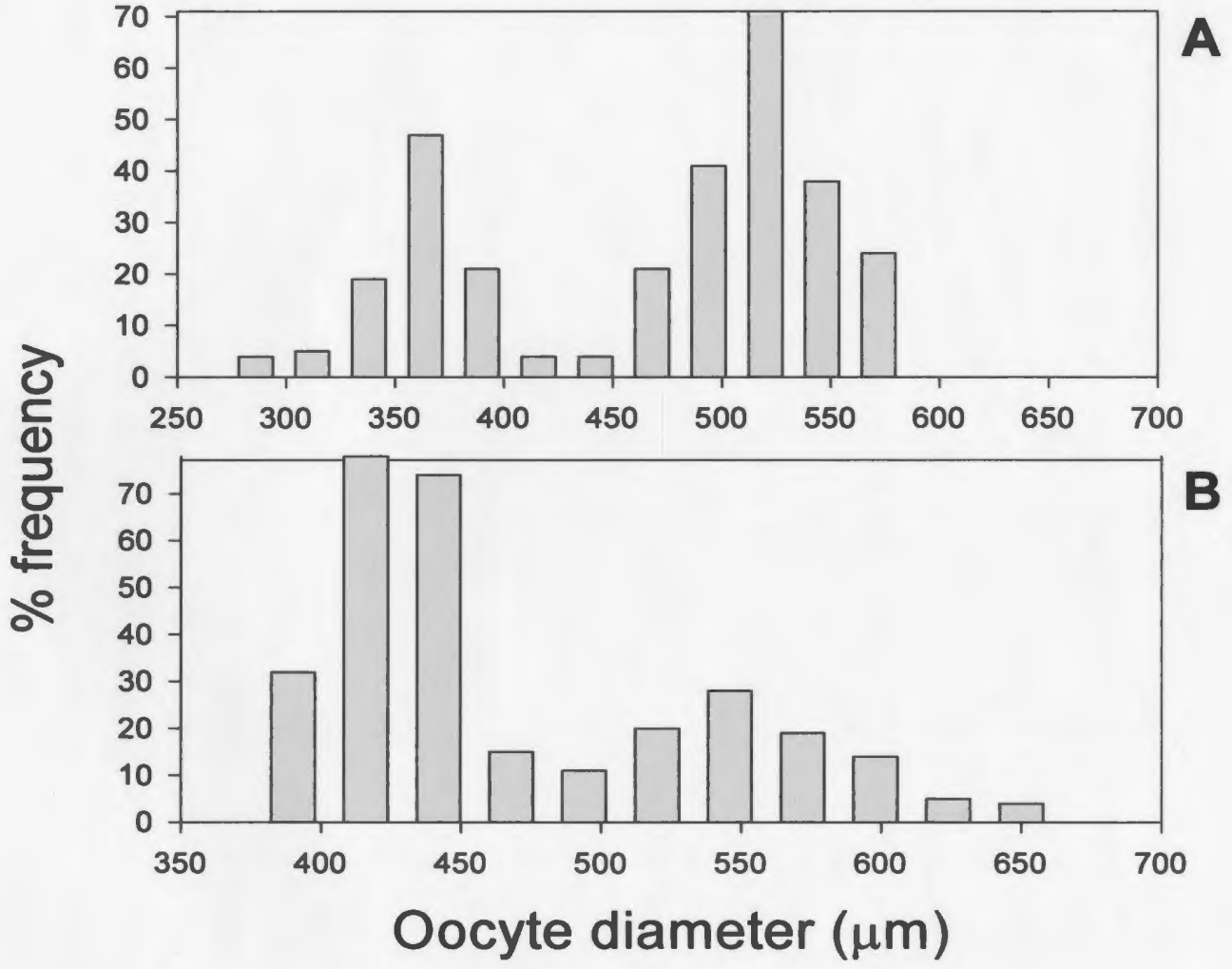


Figure 2.10: Percent frequency (%) of oocyte diameters (μm) for April 1999 (year two) for photothermally adjusted (A) N= 4 and ambient control (B) N= 2 female yellowtail flounder (*Limanda ferruginea*)

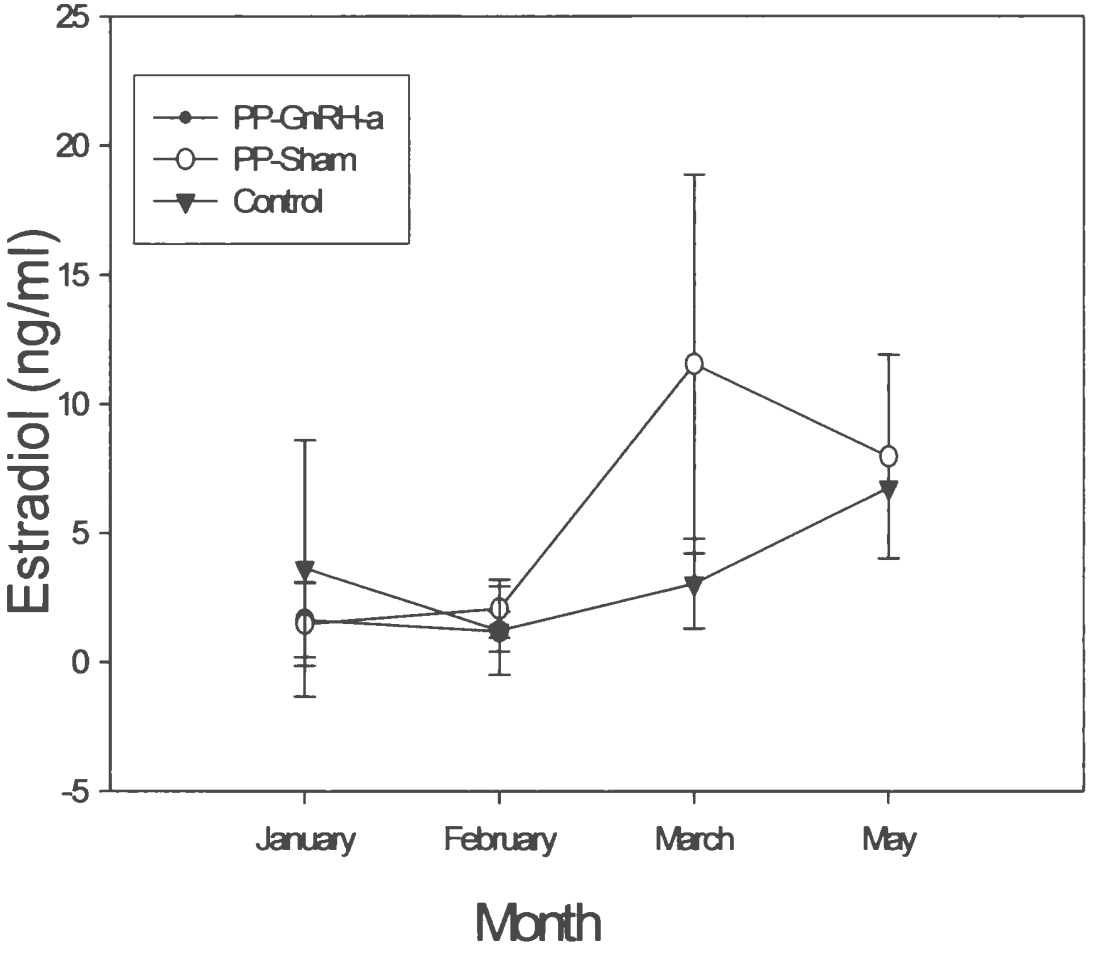


Fig 2.11: Estradiol profile for year one female yellowtail flounder (*Limanda ferruginea*) in the three treatment groups: photoadjusted with GnRH-a administration (PP-GnRH-a), photoadjusted with sham pellet (PP-Sham), and ambient females (control). Error bars denote standard deviation.

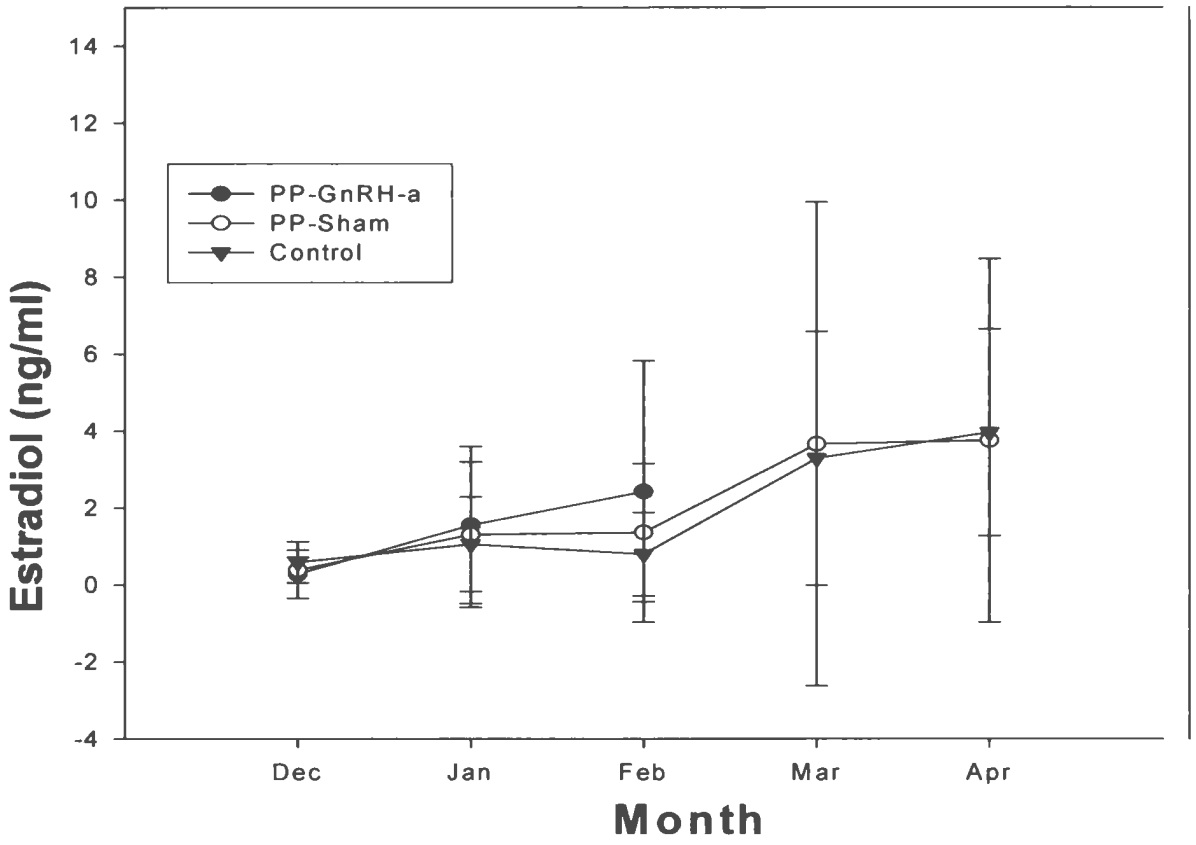


Fig 2.12: Estradiol profile for year two female yellowtail flounder (*Limanda ferruginea*) in the three treatment groups: photoadjusted with GnRH-a administration (PP-GnRH-a), photoadjusted with sham pellet (PP-Sham), and ambient females (control). Error bars denote standard deviation.

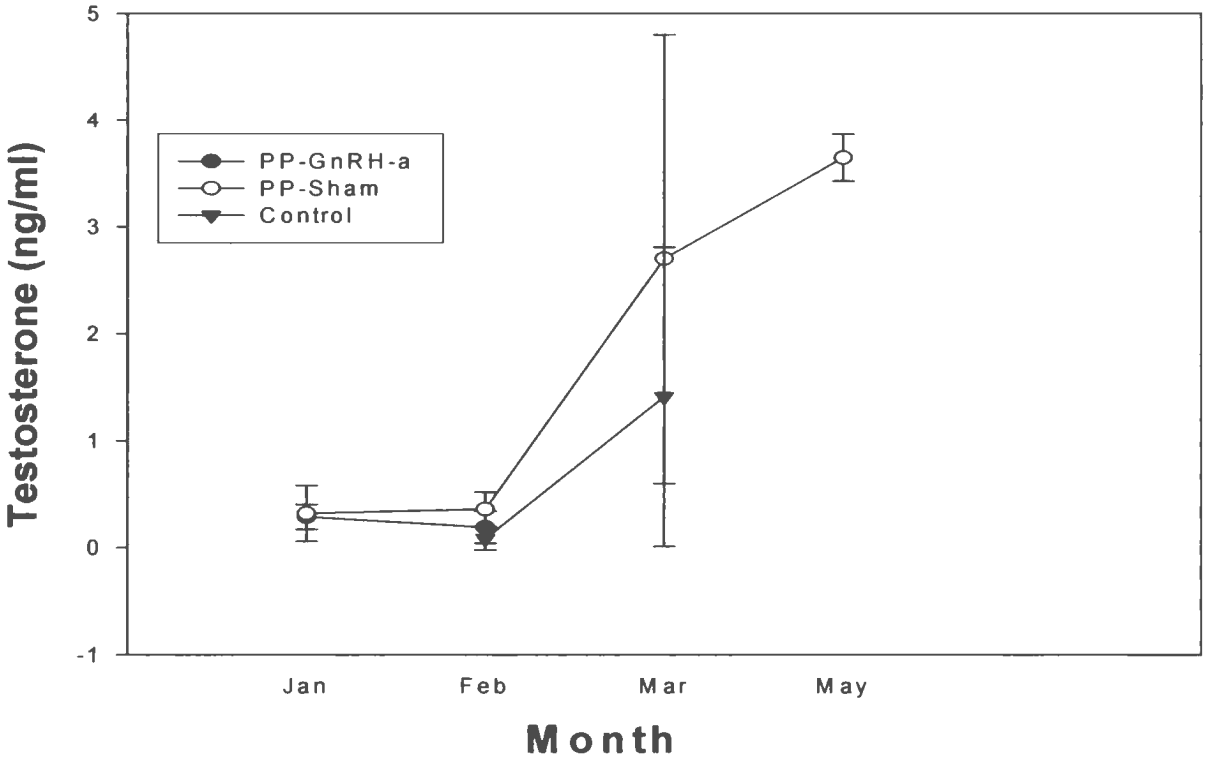


Fig 2.13: Testosterone profile for year one female yellowtail flounder (*Limanda ferruginea*) in the three treatment groups: photoadjusted with GnRH-a administration (PP-GnRH-a), photoadjusted with sham pellet (PP-Sham), and ambient females (control). Error bars denote standard deviation.

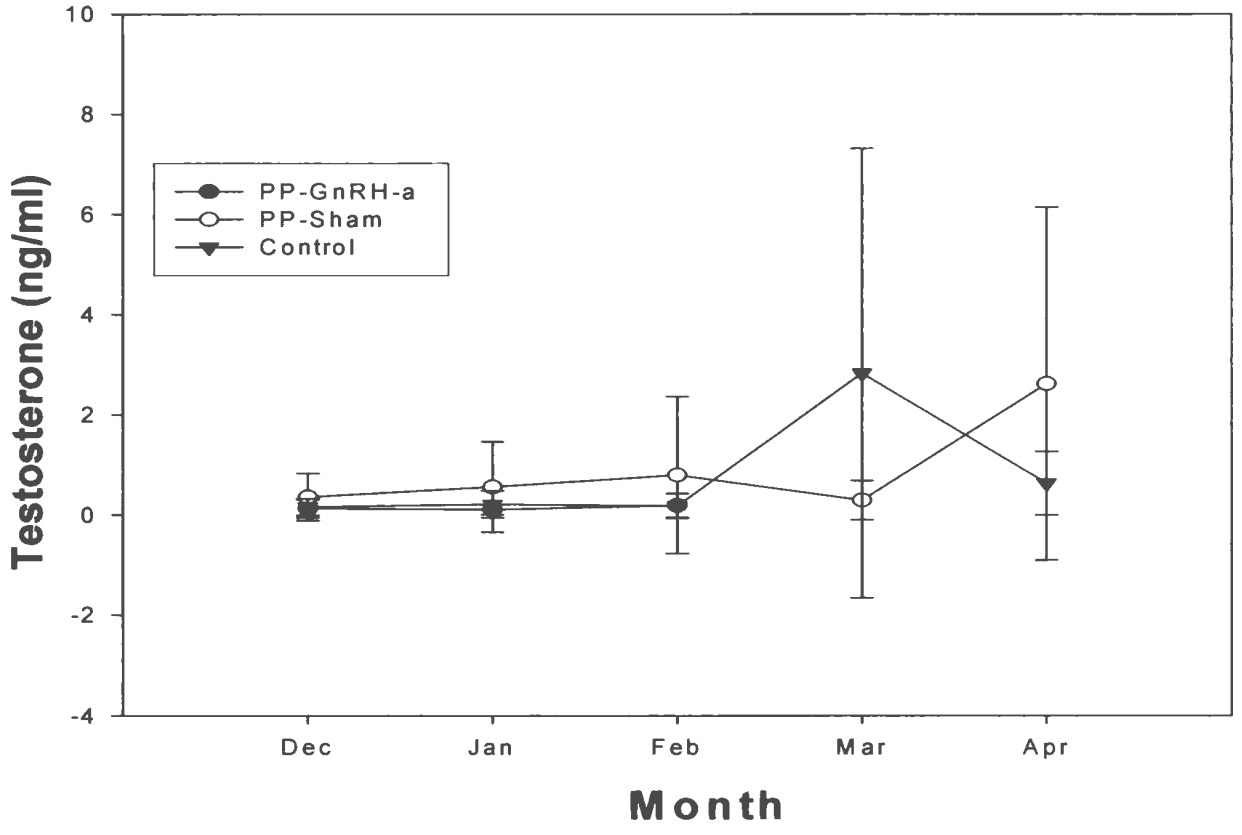


Fig 2.14: Testosterone profile for year two female yellowtail flounder (*Limanda ferruginea*) in the three treatment groups: photoadjusted with GnRH-a administration (PP-GnRH-a), photoadjusted with sham pellet (PP-Sham), and ambient females (control). Error bars denote standard deviation.

2.4 Discussion

Bettles (1997) established the oocyte cannulation method initially introduced for the grey mullet (*Mugil cephalus*) by Shehadeh *et al.* (1973) as a legitimate method of staging oocyte development in yellowtail flounder, and its use was continued for the purpose of this study. Bettles' study confirmed that ovarian cannulation of the yellowtail flounder met the criteria of Harvey and Hoar (1979) in that, the yellowtail flounder ovary permitted a direct passage to the lumen, oocytes were uniformly distributed throughout the ovary, and oocyte diameter was known for all stages of development, thereby allowing a determination of maturational stage. Bettles also determined that the oocytes aspirated from the anterior 1/3 of the right ovary were representative of those throughout the remainder of the ovary. Shehadeh *et al.* (1973) also mentions that oocytes sampled from the mid portion of the ovary are the most representative, and error can be avoided by not sampling the extreme top and bottom of the ovary. In this study, it seemed that removal of maturing vitellogenic oocytes was readily accomplished on ripening females and vitellogenic oocytes were easily aspirated using the cannula. Placement of the cannula in the ovary could be observed externally, so samples were consistently taken from the mid-ovarian region.

The results demonstrate that oocyte maturation of photothermal manipulated fish proceeded at a rate statistically similar to that of unmanipulated females. Females, which produced vitellogenic oocytes, showed increased oocyte size over the sampling period. Ripening oocytes will continue to increase in size throughout maturation reaching an average pre-spawning diameter of 433 μm in May (Bettles 1997). In my study, average

oocyte diameters for some individuals in both experimental years were above 500μm at the last sampling date (May 1998/April 1999). This value exceeds the average pre-spawning diameter noted by Bettles. Both photothermally manipulated groups showed a prevalence of previtellogenic oocytes and/or debris in the cannulation samples, with the presence of previtellogenic oocytes being prominent in year two. The presence of these previtellogenic oocytes are noted in the group synchronous ovary as described in Wallace and Selman (1981). In the group synchronous ovary, such as that found in batch spawners, previtellogenic oocytes and vitellogenic oocytes are present concurrently. The previtellogenic oocytes are those that will be used in future spawning seasons. The presence of previtellogenic oocytes in early sampling dates (December 1998/January 1999) are not bothersome, as Bettles (1997) and Linehan (1996) determined that first successful cannulation with the presence of vitellogenic oocytes occurs in December. It is the lack of vitellogenic oocytes in the later sampling dates that may indicate the regular annual maturation cycle was not occurring in these individuals. Linehan (1996) determined that vitellogenesis begins to occur in the yellowtail flounder in November; hence vitellogenic oocytes should be present in the ovary of a developing female at the time of first cannulation in this study. The year two cannulation samples in this study, representing the experimental population, do not indicate that advanced spawning, or any spawning event for that matter will likely occur for many individuals in the population, as a high percentage produced previtellogenic oocytes only. Also in year two of experimentation, certain individuals in both photothermally manipulated groups showed an increase in diameter of vitellogenic oocytes over the sampling dates, but in the end

failed to ovulate (tag numbers 166C11, OB6005, 196149) It is important to note that these individuals were all new fish brought to the facility just prior to incorporation in the experiment. This may have been a major factor in the lack of spawning performance by these individuals, as capture and acclimation stress may have played a role.

This lack of maturation is also prevalent in year one as only ovarian debris was extracted from a number of females. For the most part the animals did not produce any vitellogenic oocytes, once debris was cannulated, with the exception being a single control female. This ovarian debris, which resembled discoloured misshapen oocytes, may be oocytes held in the ovary from the previous spawning season, or possibly atretic oocytes. The possibility of this debris being atretic oocytes is lessened by the fact that the debris was aspirated from the ovary from the first sampling date. It would be expected that a certain degree of maturation would occur before the gonad becomes atretic, often due to some external stressor. Atresia, the process whereby vitellogenic oocytes undergo degeneration and resorption prior to spawning, has been described in numerous species, including the Atlantic cod (*Gadus morhua*) (Kjesbu *et al.* 1991), and the European turbot (*Scophthalmus maximus*) (Bromley *et al.* 2000). These studies both demonstrated that nutritional stress often results in atresia. Kjesbu *et al.* (1991) stated that under favourable conditions, which include sufficient body reserves and ample food supply, the energy costly process of vitellogenesis can be regulated to the maximum reproductive advantage. If ample reserves were not present in the experimental yellowtail flounder, then vitellogenesis may be halted for the year. Burton (1991) found this to be the case with winter flounder (*Pseudopleuronectes americanus*). However,

condition factors measured in year two do not indicate the fish, either wild or captive, to be in poor condition to reach reproductive potential.

Steroid analysis did not lend much evidence to the fact that the photothermal manipulated females were showing an advancement in the seasonal steroid profile. Clearwater (1997) stated that female yellowtail spawners have a peak in plasma estradiol just prior to the spawning season. Plasma testosterone was shown to increase just before spawning, but peaking after estradiol. Early sampling dates with my experiment, prior to March, showed no obvious or statistically significant difference between the treatments. The photothermally treated animals were not advanced in comparison to the ambient controls. A peak in estradiol in March for the photothermally manipulated group not administered hormone (PP-Sham) in year one showed some promise that advancement in spawning time due to the environmental adjustments may occur. However, this was not the case. In year two, no such peak occurred in either group. Estradiol levels continued to increase over the sampling period but did not peak to the levels that occurred in year one. Plasma estradiol levels were three times higher in March of year one than in March of year two. This lends further evidence that animals used for experimentation in year two were not preparing to spawn, be it from capture stress, size, lack of acclimation to captivity, or some other unknown factor.

Testosterone showed a similar trend as that of estradiol. A peak of testosterone was noted in May in sampling year one for the PP-Sham group. As stated in Clearwater (1997), a peak in testosterone will occur just prior to spawning cycle. Again, as with the estradiol data, this would lend one to believe that spawning would occur earlier as the

peak was reached in May. No advanced spawning for this group was noted. For testosterone in year two, levels did not reach those obtained in year one and the peak of testosterone from the sampling dates tested was not as noticeable as it was for the year one fish. Again, this data indicates a lack of physiological preparation for spawning by these animals. Overall, no difference in plasma steroid levels between the ambient control fish and the photothermally manipulated fish was noted, which lends further evidence as to the lack of response to the environmentally advanced conditions.

Several factors may have influenced the fact that reproductive advancement did not occur in the experiment using photothermal manipulation alone. Numerous studies have noted advanced spawning in species of fish manipulated with photoperiod and/or temperature cues. It would be expected that at some point in the annual reproductive maturation that a significant difference would be detected between the photothermal advanced fish and the ambient controls. This was not the case in this study and possibly some other influence must be causing this lack of advanced response. It has been noted that often more than one year of photomanipulation is required to elicit a response from fish, and that an endogenous annual rhythm is so entrained in an animal that it is difficult to elicit a different response (Bye 1987). A second year using the same females would be required to determine if this is a possibility. As females were replaced in year two of the experiment, due to a high mortality in year one, this was not a possibility. However, if broodstock survival could be improved, a second year in captive spawning under the advanced environmental cues could determine whether two annual cycles would be required before an advance in spawning would be seen. Both Manning and Crim (1998)

and Clearwater (1997) held spawning broodstock over a two year period, therefore it is possible to have some females survive for two spawning cycles, however, these studies experienced a high mortality rate. Nonetheless, the yellowtail flounder has been observed to be a highly stressed species, and the repeated handling during spawning may have attributed to the high mortality. Linehan (1996) determined that photomanipulation alone did not supply a sufficient amount of cues to advance the spawning time of yellowtail flounder, and suggested that some influence of temperature may be required. As temperature was held similarly in both photothermal adjusted tanks, and high temperatures as those in Linehan's study were not experienced during the short-day phase of the experiment, this would not likely be the cause of the lack of advancement. Temperatures were held between 5-6 ^{o}C during the short light phase of the experiment and temperature increased to a maximum of 11^{o}C during the long light phase (spawning time). Interruptions of reproduction in yellowtail flounder do not generally occur until temperatures reach greater than 12^{o}C (Manning and Crim 1998).

Some species of teleosts have been noted to skip a year of spawning. Burton (1991) observed that winter flounder could be induced into a non-reproductive state due to inadequate nutrition. It is possible that some animals, which did not spawn at all, were responding to nutritional inadequacies. New fish brought in from the wild in year two, which constituted a large portion of those that did not mature, may have been stressed due to the change in diet. When the animals were brought from the wild they were placed on a formulated salmon feed. Manning and Crim (1998) noted reproductive improvements in female yellowtail flounder when placed on the salmon ration as opposed

to the previous diet of shrimp. However, it is unlikely that a salmon diet, which is extremely high in fat content, would be nutritionally sound for a cold-water marine flatfish. Most broodstock programs now recognize the need to develop a diet specifically aimed at a particular species. Some animals require a higher protein content in the diet than others, others require higher lipid with the particular lipids in the correct balance to promote and improve spawning performance (Izquierdo *et al.* 2001). It has been noted by Lie *et al.* (1993) that with the exception of salmonids and European turbot, muscle lipid reserves are utilized during the maturation of the ovaries; hence a sound understanding of the nutritional requirements of a species is paramount to allow gonad maturation to progress. The correct balance of vitamins must also be available to broodstock fish, while Vitamin C is not important in an Atlantic cod broodstock diet, it is vital in the diet of the gilthead seabream (Izquierdo *et al.* 2001). It is imperative that broodstock nutrition becomes the cornerstone of developing a high quality broodstock of any species.

Handling stress may have been a factor in the lack of advanced maturation for some fish in both years. Watanabe *et al.* (1998) noted that excessive handling can reduce spawning success and induce mortality in summer flounder. Hislop *et al.* (1978) found when wild haddock were kept in captivity for short times in small tanks they experienced a high level of stress. Manning and Crim (1998) found that manually checking yellowtail flounder females for gonad swelling twice daily, with an occasional blood sample resulted in mortalities and interruption of ovulatory activity. It has also been widely observed that capture and confinement stress of certain fish species results in

reduced levels of plasma sex steroids (testosterone and estradiol-17β) (Clearwater and Pankhurst 1997; Haddy and Pankhurst 1999). As many experimental animals died during the course of this study it would not be surprising that the high mortality and lack of maturation were elicited due to a stress response brought on by the repeated handling for the monthly cannulation, and transfer from the wild to small experimental tanks. The cannulation procedure itself may cause unknown damage to the ovary and oocytes and this may cause a halt in maturation. It was observed that on one occasion blood was present in the cannulated sample of a single fish; this may indicate that cannulation is extremely invasive, and damage to the ovary can occur during the procedure.

Individuals from the photothermally adjusted group given the sham pellet (PP-Sham) spawned at the same time as the ambient controls with no advancement in spawning time (data presented in Chapter 3). Further information about the requirements to photomanipulate these animals will have to be gathered in order to successfully improve broodstock practices with this species. As in this experiment, the use of compressed photoperiod did not prove successful; however, these animals may prove responsive to a phase shifted photoperiod regime, whereby an entire 12 month annual light and temperature cycle is experienced by the broodstock animals before spawning. Phase shifting has proven successful with other species such as striped bass (*Morone saxatilis*), Atlantic halibut (*Hippoglossus hippoglossus*), and haddock (*Melanogrammus aeglefinus*) (Bjornsson *et al.* 1998; Vuthiphandchai *et al.* 2002; Martin-Robichaud and Berlinsky 2004). In phase shifted photo-regimes the animal experiences a full photothermal cycle, just at an advanced stage of a selected amount of weeks or months.

This allows adequate time for vitellogenesis and final maturation to occur. Strangely, for those individual yellowtail flounder in my study that produced vitellogenic oocytes, the average pre-spawning average diameter described by Bettles (1997) was attained, and surpassed. Again, possibility of success after a second year held on a photoperiod regime should also be investigated for the yellowtail flounder, as Bye (1987) suggested that a single year of photomanipulation may not be adequate to elicit the desired response. However, the combination of photomanipulation and GnRH-a implant was successful in spite of being given to fish before they reached the 'critical' oocyte size of 400μm set by Bettles (1997) for environmentally ambient yellowtail flounder. This data will be further discussed in chapter three with relation to egg quality and larval viability.

Full yellowtail flounder broodstock husbandry should also be investigated further before commercial application of photomanipulation techniques. It seems apparent that high mortality, and stress play a negative role in the reproductive biology of this species, and poor quality eggs and interrupted spawning have been noted for yellowtail flounder (Manning and Crim 1998) in response to stress. Further investigations to minimize handling stress and to improve nutritional and captive conditions must be investigated along with reproductive manipulations to produce top quality broodstock.

Chapter 3

Manipulation of ovulation in yellowtail flounder using photothermal cues with and without gonadotropin hormone-releasing hormone (GnRH-a)

3.1 Introduction

Yellowtail flounder were being investigated as a valued aquaculture species. The high quality flesh as well as a large tolerance to temperature and salinity changes made it very desirable for culture. Yellowtail flounder follow the determinate group synchronous sexual development pattern described by Wallace and Selman (1981) and are serial or batch spawners which produce an average of 14-22 batches in a spawning season in captivity. Yellowtail flounder females also have a tendency towards disrupted ovulation patterns and variable fertilization and survival rates (Manning and Crim 1998). Manning and Crim (1998) also observed that 64% of the female population spawned in year one of a study detailing reproduction of female yellowtail flounder in captive conditions, and 84% in year two. This species has been known to be quite susceptible to stress, and due to the repeated handling required during captive spawning it was suggested in the above mentioned study, that this may play a large role in disruptions to ovulation and poor spawning performance.

When considering a species for mariculture good broodstock management is extremely important. To promote a maximum producing species and industry, broodstock should be manipulated to spawn beyond the natural spawning season. Many species respond to photoperiod cues which may, or may not be in conjunction with thermal cues; such species as the Atlantic halibut (*Hippoglossus hippoglossus*) have been shown to successfully both advance and delay spawning with appropriate photoperiod

changes (Bjornsson *et al.* 1998). Temperate species, such as the yellowtail flounder, are most responsive to the more reliable calendar of photoperiod as a prominent cue promoting the timing of gonadal development and spawning. Previous work done by Linehan (1996) has shown that yellowtail flounder do not advance the spawning time with compressed photoperiod cues alone. It is possible that temperature cues may play a part in promoting advanced spawning. Linehan also proposed that photomanipulation for this species start sooner than November, when females in the reported experiment began the photomanipulation regime. It was suggested in the 1996 study that vitellogenesis might begin sooner than initially anticipated. Linehan suggested that while vitellogenic oocytes are first cannulated from the ovary in December, environmental cues triggering the onset of vitellogenesis are experienced well before this time. It has been well established that environmental cues are the stimuli that trigger reproductive processes in fish. For species most tuned to annual photoperiod changes, light duration triggers the pineal gland to adjust the production of melatonin in relation to daylength, which in turn stimulates the hypothalamus to produce gonadotropin releasing hormone (GnRH). The GnRH acts on the pituitary to produce gonadotropin (GTH). The GTH in turn stimulates the gonad to produce the appropriate steroids which cause the gonad to mature. In female fish, GTHI stimulates the gonad to begin producing estradiol which promotes the liver to begin the production of vitellogenin; this glycolipophosphoprotein is carried by the blood and taken up by the oocytes, beginning the process of exogenous vitellogenesis. Oocytes continue to take up the vitellogenin until a critical size, after which other hormones, such as progesterone, are produced to accomplish final maturation and ovulation (Peter 1983;

Fostier *et al.* 1983; Moyle and Cech 1988; Ekstrom and Meissl 1997).

Photomanipulation is often accompanied by an appropriate temperature regime for the species in question, even those species most responsive to photomanipulation. This was found in southern flounder, *Paralichthys lethostigma* (Smith *et al.* 1999), summer flounder, *Paralichthys dentatus* (Watanabe *et al.* 1998), and haddock, *Melanogrammus aeglefinus* (Martin-Robichaud and Berlinsky 2004). Temperature should be within the reasonable limit of tolerance, and should be balanced against the photoperiod regime in use with lower temperatures occurring during short day cycles, and higher temperatures occurring during long light cycles.

Another method gaining large favour in manipulating spawning time and enhancing performance is the use of gonadotropin releasing hormone analogue (GnRH-a). GnRHs are highly conserved brain decapeptides that play a vital role in reproduction, with several specific types of GnRH reported in various animals being described (Sherwood *et al.* 1993). Numerous studies have proven the effectiveness of GnRH analogues on promoting spawning in those species which will not spawn in captivity, advancing ovulation, and improving egg and sperm quality. Such studies have been performed on sea bass, *Lates calcarifer* (Garcia, 1990), southern flounder, *Paralichthys lethostigma* (Berlinsky *et al.* 1996), winter flounder, *Pseudopleuronectes americanus* (Harmin and Crim 1992), rainbow trout, *Oncorhynchus mykiss* (Breton *et al.* 1990), Atlantic salmon, *Salmo salar* (Crim *et al.*1986), and the grey mullet, *Mugil cephalus* (Lee *et al.* 1987). Of the several forms of GnRH described, mammalian GnRH-a has proven successful in advancing and improving spawning in the yellowtail flounder (Bettles 1997;

Larsson *et al.* 1997). When GnRH-a is combined in pellet form with cholesterol, release of GnRH-a occurs over a longer period of time than when GnRH-a is used alone or with cellulose. Cholesterol content and release duration are positively correlated (Crim *et al.* 1988). This slower, yet longer release of hormone works well when used with batch spawning animals, as spawning can occur over a period of weeks.

The yellowtail flounder's response to long acting GnRH-a cholesterol pellet has proven favourable. Bettles (1997) proved yellowtail flounder ovulation advancement with the administration of this hormone analogue prior to the regular spawning season. Most consistent results, with regard to number of ovulating females, were noted when GnRH-a was administered closest to the natural spawning season of June. When hormone was administered in February, four months prior to the natural spawning period for yellowtail flounder, only 25% of females implanted spawned early. Of the groups implanted in April and June 100% of females spawned. April implanted fish spawned 20 days post-implantation, while June implanted fish spawned 14 days post-implantation. In February implanted fish, up to 32 days elapsed before first spawning. Bettles concluded from the study, that in order to observe any marked consistent advancement to ovulation, the administration of the GnRH-a pellet should be given in conjunction with environmental manipulation, such as advanced photothermal regimes. Other species such as the southern flounder (Smith *et al.* 1999) and summer flounder (Watanabe *et al.* 1998) have shown advanced spawning when both of these cues (environmental and hormonal) have been used together.

The effect of GnRH-a administration has proven beneficial in also enhancing

spawning performance, aside from advancing ovulation. In a study conducted by Larsson *et al.* (1997) on yellowtail flounder it was concluded that GnRH-a not only advanced ovulation by two to three weeks, but also significantly increased fertilization and hatching rates, doubled egg production, synchronized females, and shortened the interovulatory period. These have all been improvements based on the difficulties associated with the captive spawning of the yellowtail flounder as described by Manning and Crim (1998).

The synchronous use of advanced photothermal conditions and the administration of GnRH-a on female yellowtail flounder broodstock to promote advanced ovulation and its effects on egg and larval quality is the main focus of the current study.

3.2 Materials and Methods

3.2a Environmental Experimental Conditions

Environmental conditions and experimental animals are identical to those described in **Chapter Two** of this thesis.

3.2b Hormone Administration

Fish, in one of the environmentally manipulated groups, received a sustained release cholesterol pellet containing gonadotropin hormone-releasing hormone analogue at a concentration of 100 μg/pellet. Bettles (1997) observed advanced ovulation when he injected yellowtail females with GnRH-a pellets only after oocyte diameters reached a size of 400 μm. However, in order to increase the chances of obtaining a significant advancement in spawning the hormone pellet was given prior to oocytes reaching this diameter, administering the hormone in February for both years. The pellet was embedded in the right dorsal musculature of fish in one of the environmentally manipulated groups. A sham pellet containing all ingredients but the GnRH-a was embedded in the same manner in the other environmentally manipulated group, hereafter referred to as PP-GnRH and PP-sham groups respectively. In year two, a GnRH-a pellet was administered to five ambient control broodstock in the same manner.

3.2c Egg Quality Analysis

Fish were checked weekly after the GnRH-a pellet was implanted for ovulation and to assess degree of swelling in the gonad. The PP–sham group was checked weekly beginning in April in the same manner. Once ovulation occurred, oocytes were stripped using gentle manual pressure on the gonad. Oocytes were collected in chilled graduated plastic specimen jars daily at approximately 10:00 am. Sperm from at least two males was also collected at this time in 1cc syringes. Urine contamination was carefully avoided. This daily stripping of gametes continued until spawning ceased. Batch volume was recorded and batches less than 5 ml were considered to be residual and sometimes not assessed for quality beyond the viability stage.

Eggs (for convenience, oocyte will be referred to as an egg once collected) were checked in triplicate for viability under a stereoscope at 150X magnification. Yellowtail eggs are considered to be viable if they are round, clear, positively buoyant and lack a perivitelline space (Larsson *et al.* 1997). Triplicate aliquots of 100 µl from each batch were then 'dry' fertilized in plastic petri dishes. Five µl of pooled sperm samples checked for motility from at least two males was added to the eggs. One hundred µl of seawater was added to activate the sperm. After 2 minutes, 2 ml of sea water was added, an additional 5 minutes was given for any sperm that may still be motile, after which 20 ml of sea water was added to the petri dish. Egg fertilization rates were counted at the 4-8 cell cleavage stage, generally after 6 hours of incubation at 6-7°C.

In order to obtain hatch rates, development was permitted to continue in these fertilized plates. Seawater was replaced every 2-3 days until hatching occurred 8-12 days

later and larvae were counted. Hatch rates were calculated as a function of the number of viable fertilized eggs which hatched. All seawater used in fertilization and hatch experiments was 1 μm filtered, UV sterilized and treated with 0.006% penicillin G and 0.1% streptomycin sulfate (Sigma Chemicals, Illinois) to inhibit bacterial and fungal growth in the plates.

3.2d Larval Rearing

Batches of eggs were fertilized in large containers and transferred to 300 liter incubators supplied with 3L/min of 6-7°C aerated seawater. At first hatch, 10L of *Isochrysis* (Tahitian Strain) (T-*Iso*) algae was slowly dripped into the incubator water through thin plastic tubing. At 24 degree days (dd) of larval development rotifers (*Branchionus plicatilis* American Strain) were introduced. Rotifers were T-*Iso* enriched by feeding *Isochrysis* algae over the day. Larval feeding schedule consisted of 10L slow drip *Isochrysis* algae and 3 million rotifers added in the morning. Another 3 million rotifers remained in 10L of algae (enrichment) for 8 hours, and were subsequently fed to the larvae in the afternoon. Larvae were monitored every 3-4 days for signs of food in the gut by microscopic analysis of 200 larvae at each sampling.

3.2e Statistical Analysis

Data were analyzed using SAS statistical software package (SAS Institute Inc., Cary, NC). Hierarchical analysis of variance (ANOVA) was employed to test for differences in egg parameters including egg viability, fertilization, and hatch between both experimental years. The effects of year were not significant ($p>0.05$), therefore, data from 1998 and 1999 were combined. All data represented as a percentage (egg viability, fertilization, and hatch) were arcsine square root transformed before performing analysis. The effect of treatment on the various parameters was investigated using a nested analysis of variance (ANOVA). Comparison of batch volume produced per female was tested using a one-factor ANOVA. Tolerance for Type I error was set at a level of 0.05. For some parameters (hatch) insufficient data were available for statistical analysis.

3.3 Results

No advanced spawning was noted in the photothermally manipulated (PP-sham) group in comparison to the ambient broodstock group. Spawning commenced three months in advance for the PP-GnRH-a group when compared to spawning of the PP-Sham and the ambient broodstock group. In year one spawning commenced on March 9 for the PP-GnRH-a and on June 4 for the PP-sham, with spawning for the ambient broodstock beginning six days after the PP-sham. Year two gave similar results with fish from the PP-GnRH-a group commencing spawning on March 1, the PP-sham group beginning on June 22, and the ambient broodstock group on June 23. In year two, in the

ambient group administered the GnRH-a pellet (GnRH-a control group), a single female produced eggs on March 12. Two other females from the GnRH-a control group produced fluid which did not contain any eggs. One female from the PP-GNRH-a group in year one produced only fluid, as did two females from the PP-sham group. No eggs were detected in any fluid samples. Females which ovulated in the other three groups in year two, produced eggs with no individual producing only fluid (Tables 3.1 and 3.2).

For the PP-GnRH-a group in year one, 26 days elapsed between implantation and ovulation, in year two the time was reduced by six days. In year one, two females (29%) in the PP-sham group did not ovulate any eggs, only producing fluid. The same occurred with one female in the PP-GnRH-a group. In year two, more than 77% of the surviving females in the PP-GnRH-a group did not ovulate, and greater than 90% of survivors did not ovulate in the PP-sham group.

Egg quality parameters were generally significantly different between groups, however no detectable significant difference for each treatment between years was found ($p<0.05$), hence egg quality data from both years were combined to compare treatment effect on egg quality. Data for the combined years are shown in Fig 3.1. Viability and fertilization rates were significantly different between treatments ($F= 3.41$; $p=0.0338$: $F=10.80$; $p=0.0008$ respectively), (Fig 3.1). However, treatment had no effect on hatch rates ($F=2.21$; $p=0.1491$) (Table 3.1, 3.2 Fig 3.1). The experimental animals given the cholesterol pellet, without photomanipulation in year two of experimentation had a significantly lower viability and fertilization rate from the other three. Eggs from this group frequently had a dimpled appearance and were dark in colour compared to the

other treatment groups (Fig 3.2a, 3.2b). Larvae that hatched from eggs from the GnRH-a control group were curved and the yolk sac had a similar dimpled appearance to the eggs. None of the hatched larvae exhibited swimming activity, and were rated as moribund. These larvae were very different when compared to the straight, clear yolk sac, active larvae produced by the other treatments (Fig 3.3a, 3.3b). The PP-GnRH-a group showed higher viability than the PP-sham and GnRH-a control group. However there was no significant difference between the PP-GnRH-a and the ambient controls. Viability and fertilization rate was significantly lower in the ambient GnRH-a group over all other groups. The PP-GnRH-a group showed the largest average batch volume, being significantly larger than the other treatments ($F=4.14$, $p=0.0173$). One female in year one in the PP-GnRH-a group produced almost 650 ml of eggs, out producing all other animals in that year. In year two a single female produced greater than 700 ml in the ambient control group. Both these values were significantly higher than others within the treatments ($F=1.96$; $p=0.0194$).

Hatch rates were not determined for the ambient broodstock group in year one. In year two, hatch data were not collected for the PP-sham group as incubation equipment was not capable of keeping a consistent controlled temperature required to raise the larvae at the time the individual fish from this group were spawning. Later batches for the ambient control were tested for hatch success as these animals continued to spawn once incubation problems were remedied.

Eighty per cent of larvae raised from the PP-GnRHa group had food in the gut seven days after first feeding (Fig 3.4). Another batch of eggs from the PP-GnRH-a

group was incubated but due to low water supply in the hatchery the eggs suffered total mortality and no larval data were collected.

Table 3.1: Spawning summary and egg quality parameters for manipulated (PP-GnRH-a, PP-sham) and control female yellowtail flounder broodstock Year One – 1997-8

Treatment	Fish #	First ovulation	# batches	Total vol(ml)	Avg Viab(%)	Avg fert(%)	Avg hatch(%)
PP-GnRH-a	OFO85C	9-Mar	23	647	92	76	63
	516BID	Dead					
	715C28	11-Mar	3	30	60	80	72
	305872	Dead					
	370643	26-Mar	8	160	81	67	34
	151F09	Dead					
	782D7C	18-Mar	1	Fluid	na	na	na
PP-Sham	OE56OF	4-Jun	10	275	71	82	80
	164860	Dead					
	154026	9-Jun	3	135	55	72	na
	340B2E	14-Jul	1	Fluid	na	na	na
	3D670B	7-Jul	10	405	58	75	na
	OEO23E	16-Aug	2	fluid	na		
	451A4A	Dead					
Control	1F4534	15-Jun	2	30	64	na	na
	6E5A09	12-Aug	11	327	47	na	na
	3E0849	26-Jun	5	102	55	na	na
	1E7552	23-Jun	7	162	55	na	na
	1E6D65	26-Jun	2	52	62	na	na

na – data not available

Table 3.2: Spawning summary and egg quality parameters for manipulated (PP-GnRH-a, PP-Sham), and control female yellowtail flounder broodstock Year Two - 1998-9

Treatment	Fish #	First ovulation	# batches	Total vol(ml)	Avg Viab(%)	Avg fert(%)	Avg hatch(%)
PP-GnRH-a	166C11	DNO					
	1B2845	DNO					
	443338	DNO					
	18023E	Dead					
	250327	DNO					
	OA27OE	Dead					
	451A4A	Dead					
	042D38	DNO					
	313A38	DNO					
	26266E	DNO					
	138836	Dead					
	39311F	1-Mar	18	245	89	65	56
	1F3232	4-Mar	6	60	76	56	32
PP-Sham	1D7372	Dead					
	682F43	DNO					
	196149	DNO					
	163A23	DNO					
	3A6079	DNO					
	0B6005	DNO					
	4A6303	DNO					
	0F3900	Dead					
	0F6375	DNO					
	214045	DNO					
	431C0F	DNO					
	1C324E	DNO					
	370643	22-Jun	5	170	55	60	69
GnRH-a control	C2	DNO					
	C3	22-Feb*	1	fluid			
	C4	22-Feb*	1	fluid			
	C5	DNO					
	C6	12-Mar	15	190	36	29	12
Control	Ctrl A	23-Jun	21	710	87	74	55
	Ctrl B	23-Jun	8	90	61	79	75
	Ctrl C	23-Jun	5	125	56	72	87
	Ctrl D	28-Jun	4	125	65	65	55
	Ctrl E	6-Jul	4	55	76	52	39
	Ctrl F	22-Jul	1	10	72	94	77

DNO- did not ovulate *ovulation not complete – no oocytes in fluid

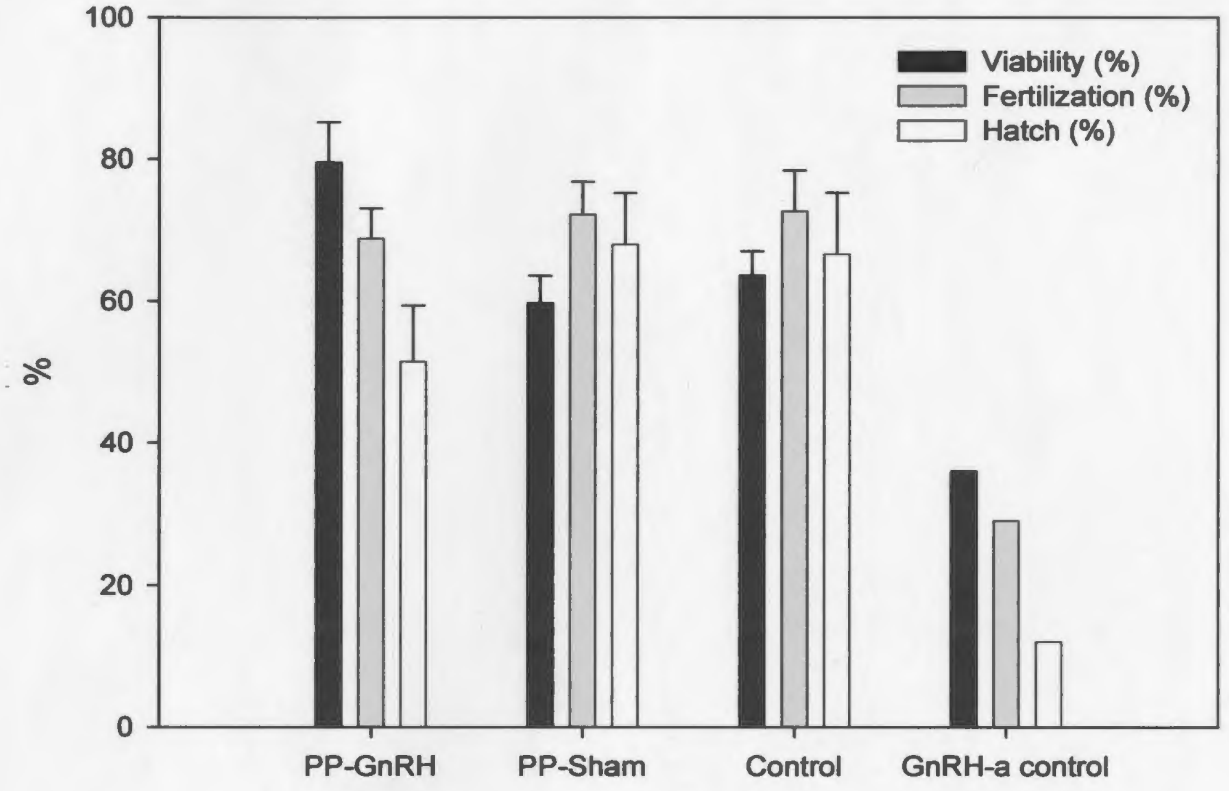


Fig 3.1 Egg quality parameters based on viability, fertilization and hatch rates (%) for the four treatment groups of female yellowtail flounder broodstock. (PP-GnRH, PP-Sham, Control and GnRH-a control). Error bars denote standard deviation

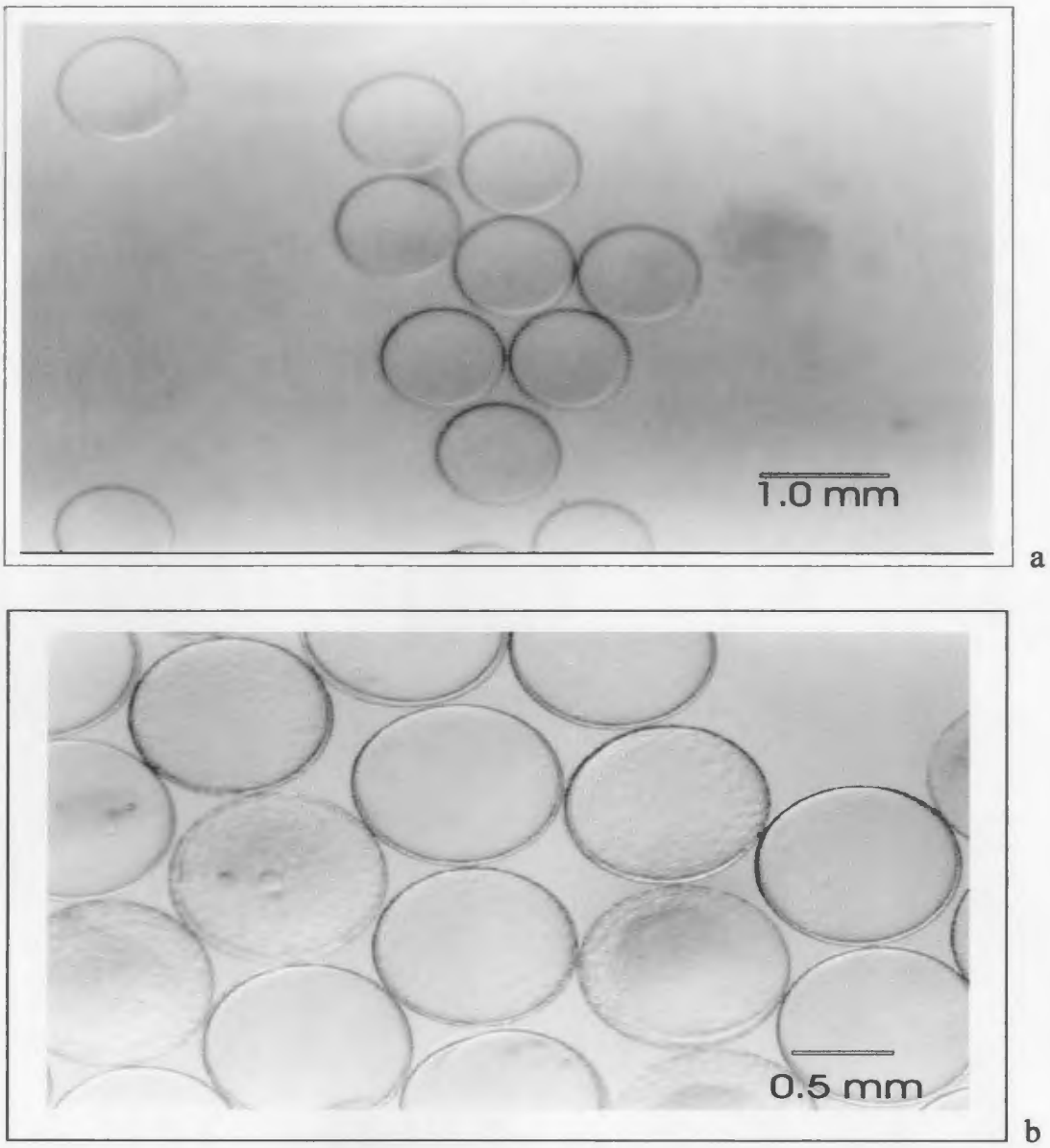
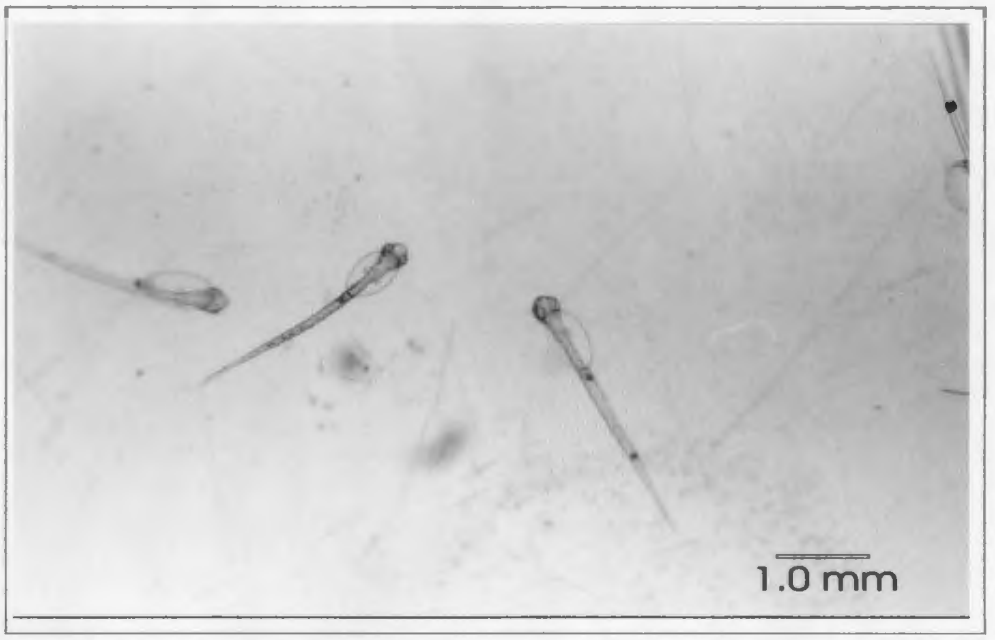
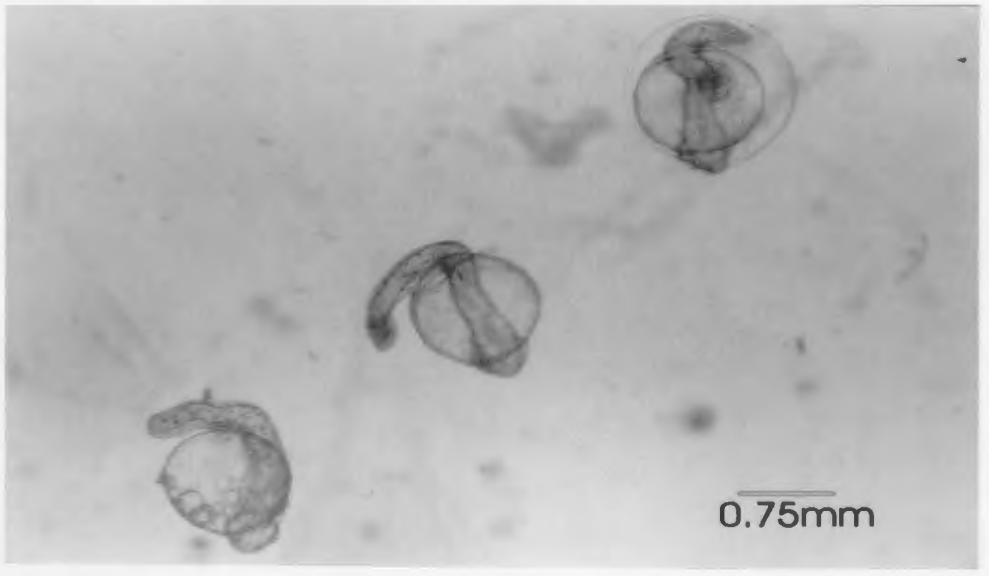


Fig 3.2a: Viable eggs from advanced female yellowtail flounder photothermally manipulated and administered GnRH-a in February

Fig 3.2b: Viable and non-viable dimpled eggs from female yellowtail flounder administered GnRH-a in February without prior photothermal manipulation



a



b

Fig 3.3a One day post hatch straight normal larvae spawned from female yellowtail flounder photothermally manipulated and administered GnRH-a in February

Fig 3.3b One day post hatch curved abnormal larvae spawned from female yellowtail flounder administered GnRH-a in February without prior photothermal manipulation.

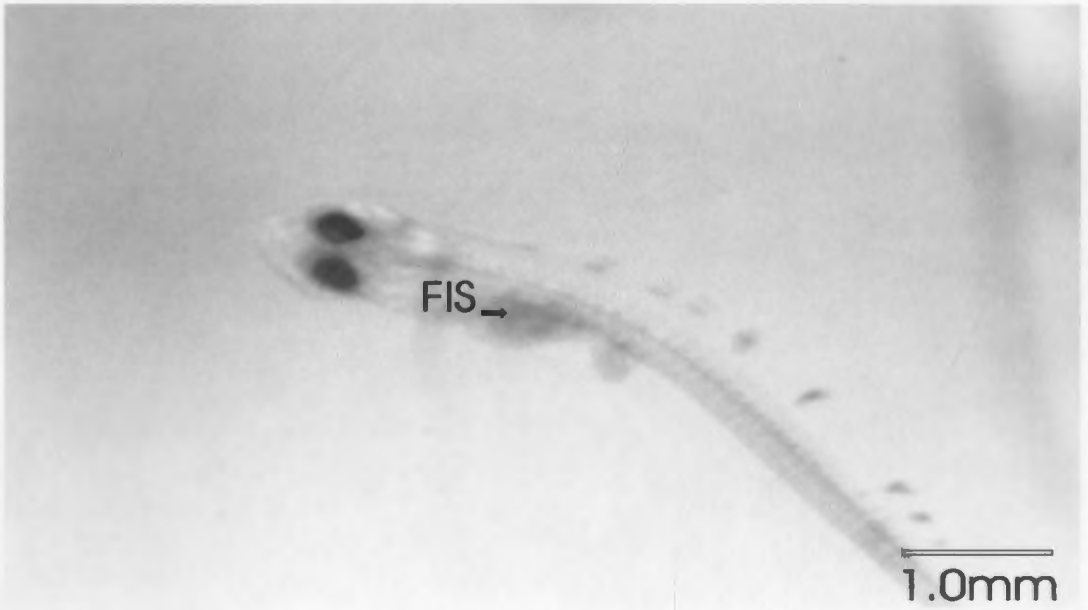


Fig 3.4 Forty-two degree day larvae with food in stomach (FIS) hatched from eggs incubated from photothermally manipulated females implanted with GnRH-a in February

3.4 Discussion

Successful advanced ovulation using a combination of photothermal and exogenous hormonal cues have been proven successful in several species. Berlinsky *et al.* (1996) induced final maturation in eleven of twelve southern flounder (*Paralichthys lethostigma*) using GnRH-a implants in photothermally conditioned captive females. Previous experiments attempting to manipulate natural spawning of the southern flounder with photothermal manipulation alone had proven unsuccessful (Arnold *et al.* 1977). This has also been the case with yellowtail flounder. Previous photoperiod advancement attempts have proven unsuccessful, and suggestions as to initiating photoadvancement earlier during recrudescence and incorporating temperature control have been discussed as possible solutions for improving response to advancing ovulation through photoperiod in female yellowtail flounder (Linehan 1996). In my study, these two aspects were addressed, without success. Short day length cycle was initiated two months prior to the Linehan study, setting photoperiod to eight hours of light in September, much earlier during recrudescence than the previous study. Temperature was controlled at approximately 5°C during this phase, so high temperature experienced in the Linehan study during the short daylength, providing a set of mixed environmental cues, and during the initiation of vitellogenesis was likely not a problem. In year two of my study, because of the lack of successful advanced ovulation in year one, the long light cycle began three months earlier than in year one, 17 hours of light was set in November as opposed to February. In summer flounder (*Paralichthys dentatus*), a fall spawner, thermal manipulations (without photoperiod) were shown to play a dominant role in the

rate of vitellogenesis, however this study also showed that photoperiod tended to be the trigger for the commencement of vitellogenesis in the species (Watanabe *et al* 1998). Hence, at least for the summer flounder, both temperature and photoperiod play a vital role in commencement and continuation of vitellogenesis. This fact was taken into account in the current study on yellowtail flounder, however no advancement in ovulation was observed using photothermal manipulations alone.

When environmental manipulations were combined with hormone administration in the form of a GnRH-a cholesterol pellet, advanced spawning occurred. Bettles (1997) was only successful in advancing spawning in yellowtail flounder with a minimum vitellogenic oocyte diameter of 400 μm, but he did not use photothermal manipulation concurrently. Watanabe *et al.* (1998) also determined a minimal oocyte size for the success of LHRH-a pellet in summer flounder. They found that a mean oocyte diameter of 258 μm was required to elicit a response from the implant.

Average oocyte diameter for the females in this study was 375 μm when GnRH-a was administered. Bettles (1997) found that when GnRH-a pellets were implanted in females in February only 25% of the animals spawned. In year one of my experiment 100% of females surviving to spawning ovulated when photothermal cues were combined with February GnRH-a administration. This suggests that the 400 μm oocyte size is not as critical as initially proposed by Bettles (1997). However, in year two only two individuals (7%) under these conditions spawned. It is possible, since these fish were new to the facility, the stress of capture and captivity was a factor in the lack of spawning response. It has been noted by Smith *et al.* (1999) that conditioning and age of

wild broodstock may be factors in spawning success, and suggested that fish be held in captive conditions for a minimum of 1.5 years before incorporation into a spawning manipulation experiment. Devauchelle *et al.* (1988) found that with turbot brought from the wild, a minimum of two years in captivity was required before efficient spawning occurred. Manning and Crim (1998) also noted that 20% more female yellowtail flounder spawned the second year in captivity. In year one of the current study, fish had been in captivity for greater than one year, however in year two more than 61% of the experimental population were wild fish. It should be noted that broodstock animals are extremely valuable and in the case of yellowtail flounder, often difficult to obtain. Therefore, one must often use animals as they become available, even if they are not the desired quality. The yellowtail flounder females used in this experiment were assessed for gonad size prior to incorporation into the experiment in both years. Only those females that exhibited gonads extending 2/3 of the body length were used in the environmental manipulation experiments. This gonad size has been determined to be an appropriate indicator for post pubescent yellowtail flounder (Manning 2003). Condition factors (CF) were calculated in year two and the average CF was 0.90. This supports the contention that these individuals were not in poor reproductive potential condition when brought into captivity. Pitt (1970) observed that 50% of wild female yellowtail flounder mature at 37cm. Manning (2003) determined that yellowtail flounder reared in captivity become mature before reaching a length of 25 cm. While most animals were below Pitt's average, there were still spawning individuals in the experiment that were less than 37 cm in length, however some spawners in year two were larger than Pitt's stated size of

mature females. No females were smaller then the maturity size noted by Manning (2003). Pitt (1970) also noted in his study that female yellowtail flounder reach first sexual maturity at approximately 33cm in length. This reference approximates the length of most animals in my experiment; in fact one of the spawning females in the PP-GnRH-a group in year two was 32.5cm in length. If larger animals were available for my experiment they would have been selected, however, the value and limited access to broodstock was a determining factor as to which animals were available for use in this experiment.

Females implanted in February without photothermal manipulation did not respond well. Most females did not ovulate, or produced only fluid. The only female which did produce eggs produced very poor quality eggs and significantly poorer than all other experimental groups. Interestingly, these eggs were significantly poorer than eggs produced from the PP-GnRH-a group. Eggs from the control GnRH-a broodstock in this study appeared discoloured and dimpled and showed a low fertilization and hatch rate. Mylonas *et al.* (1992) concluded from a study on the effects of GnRH-a on ovulation in brown trout (*Salmo trutta*) that induced ovulation by GnRH-a early in the season caused poor egg fertilization due to insufficient time to resume meiotic maturation before ovulation. While no advancement using photothermal manipulations alone could be induced, the lack of advanced environmental cues adversely affected response to the hormone implant. Some physiological preparation may in fact be occurring in response to the environmental cues which could not be detected with oocyte diameters or steroid analysis (Chapter 2), or confirmed with advanced ovulation. It is possible that some cue

from the advanced photothermal regime progressed females in the PP-GnRHa group so that meiosis could be completed and ovulated oocytes were able to be fertilized. This is something that requires further investigation. Egg quality for females implanted with GnRH-a in February by Bettles'(1997) study exceeded or was similar to ambient control fish, however only a quarter of all females implanted ovulated.

The use of the sustained release cholesterol pellet has proven very successful in batch spawning fishes susceptible to handling stress, such as the yellowtail flounder (Larsson *et al.* 1997). Yellowtail flounder have been noted as an easily stressed animal, being described as delicate and excitable by Smigielski (1979), so the use of a sustained release pellet reduces the amount of handling required for repeat injections, thus reducing stress for the animal. Early spawning of the experimental animals administered the hormone pellet in conjunction with photothermal cues in this experiment agreed with the results of Bettles (1997) and Larsson *et al.* (1997), that eggs produced were of a high quality and met or exceeded the quality of eggs produced in the other treatment groups based on egg viability, fertilization, and hatch rates.

Oddly, the photothermally manipulated group not administered the GnRH-a pellet did not commence spawning in advance of the control broodstock group. No significant difference was noted in the oocyte maturation or steroid progress (Chapter 2), and advanced spawning did not occur without the use of hormone in conjunction with the photothermal regime. It was hoped that after the successful spawning and good egg quality produced in the PP-GnRH-a group in year one that advanced spawning would occur in the PP-sham group, although no advanced oocyte growth could be detected

earlier in the year through cannulation. This did not prove to be the case, and the advancement for the PP-sham group was not observed. It has been discussed that many fish species have an entrained internal 'zeitgeber' that controls the time of spawning regardless of the environmental cues to which the animals are subjected (Bye 1984). As noted earlier, a longer time in captivity may be required to acclimate to captive conditions and a longer time exposed to the adjusted photothermal regime may be required to advance spawning time to any degree; this has been found to be the case with European turbot (Devauchelle *et al.* 1988).

The use of compressed photoperiod, such as that used in this experiment, may not be the best choice for advancing spawning in the yellowtail flounder. While this method of advancing spawning time has been successful in numerous other species, the yellowtail flounder may require more than a few months to advance the reproductive cycle. Many studies using environmental cues to advance spawning have focused on using a phase-shifted photothermal regime, whereby an entire 12 month cycle is experienced by the animal just at an advanced time, and daylength increases or decreases at the same daily rate as under ambient conditions. Lush *et al.* (2002) have had success with phase shifting the spawning cycle of captive Atlantic cod (*Gadus morhua*). Successful three-month advanced ovulation has been noted by instantly advancing the natural photoperiod by three months early during recrudescence, whereby the animals experience the amount of daylength occurring in December, but in September. The animals spawned for a second time in nine months using this type of photoperiod control. However, more than nine months may be required by other species, and for fish newly

brought in from the wild. Martin-Robichaud and Berlinsky (2004) found the same success using phase-shifted photothermal cues on haddock (*Melanogrammus aeglefinus*). Like the yellowtail flounder, both the Atlantic cod and haddock are batch spawners, so this type of photoadvancement may be something to consider for future attempts at photoadvancing yellowtail flounder.

In year one of experimentation, females were held in experimental tanks without males. The lack of males in the experimental tanks was considered a possible cause for the unsuccessful photoadvancement of the PP-sham group. It was thought that some pheromonal or behavioural cue may be missing when one sex is held without the presence of the other. In year two, three males were added to each photothermally advanced group, in hope that if pheromones or particular behaviours were required to induce development or ovulation they would be present. However, this did not prove to be the case, and as stated above, no advance spawning was noted using advanced photothermal cues alone. Pheromones have been proven essential in inducing final maturation and spawning in such species as the catfish (Van Weerd and Richter 1991).

Broodstock nutrition would be vital to explore in the improvement of yellowtail flounder culture. In a report submitted to the Aquaculture Association of Canada, Crim and Brown (1998) stated that development of an appropriate broodstock diet for the yellowtail flounder would be of utmost importance. The on-growing salmon diet currently fed, while an improvement over the previous raw shrimp diet (Manning and Crim 1998), does not seem adequate for a cold water marine species such as the yellowtail flounder. Broodstock diets require the correct balance of fat, protein and

micronutrients, such as vitamins C and E, specific to parental fish and particular species (Izquierdo *et al.* 2001). It has been found in other species that adequate nutrition is vital to allow reproductive processes to occur (Burton 1991). Future consideration as to optimal captive conditions such as stocking density and appropriate broodstock diet need to be investigated for the yellowtail flounder.

Overall, while successful photothermal advancement was not achieved in this study, three month advanced ovulation did occur when an advanced photothermal regime was combined with the administration of GnRH-a. This advancement was not observed in this study when GnRH-a was administered at the same time without prior advanced photothermal conditioning. However, advanced spawning was noted in Bettles' 1997 study with February GnRH-a administration on ambient females. It is important to note in my study that GnRH-a successfully advanced ovulation in implanted females with oocytes less than the prescribed size of 400 μm by Bettles (1997), when used in conjunction with advanced photothermal cues.

Certain adjustments in future attempts to advance the spawning time of yellowtail flounder can be made by attempting a phase shifted photothermal regime as opposed to the compressed schedule used in this study, and to ensure that animals have sufficient time to acclimate to captive conditions before beginning to advance environmental conditions. General improved broodstock management, aiming for optimum husbandry practices, in conjunction with these spawning advancement techniques may result in a longer production of higher quality gametes and a healthier broodstock which may reach a higher reproductive potential.

Chapter 4

Conclusions

Successful aquaculture of any species relies heavily on sound broodstock management strategies. One such strategy is the manipulation of the spawning cycle so that individuals or groups spawn beyond the natural spawning period. This is most often accomplished with photoperiod, temperature, hormone administration or some combination of these factors. It is the environmental factors which influence the production of hormones that initiate and sustain the reproductive processes in teleosts. The administration of exogenous hormone has proven beneficial with promoting, advancing and enhancing spawning in numerous culture candidate species. With the use of these cues animals can be induced to spawn beyond the regular spawning season, thereby reducing amount of time that sees little or no production in an aquaculture facility due to lack of gamete supply.

Yellowtail flounder were being investigated as a potential species for mariculture. Its high quality flesh, tolerance to a wide variety of conditions, and established markets make it a good candidate for culture. Yet, studies to manipulate the spawning time have seen limited results. It was the purpose of this study to determine if photothermal manipulation with and without the administration of GnRH-a would advance ovulation in female yellowtail flounder. Previous studies have seen little success in advancing ovulation of yellowtail flounder well before its ambient spawning season. Linehan (1996) had no success in advancing ovulation using a compressed photoperiod regime commencing in November. Bettles (1997) had some success and induced

advanced ovulation in 25% of yellowtail flounder females implanted with a sustained-release GnRH-a cholesterol pellet in February, and when oocyte diameters were at least 400 μm. First ovulation post-implant was reported in March. Better results in April and June were observed as implants were administered closer to the natural spawning time. It was proposed by Bettles, that improved advanced ovulation could be observed if GnRH-a administration was used in conjunction with photothermal advancement.

In this study, advanced spawning occurred in early March when an advanced photothermal regime was used in conjunction with GnRH-a implants (PP-GnRH-a group). Successful spawning with the PP-GnRH-a group occurred even though pre-hormone administration oocyte diameters were less than those suggested by Bettles (1997). However, no advancement in ovulation was noted in females under the advanced photothermal regime alone. Of those individuals implanted with GnRH-a in February, but not subjected to an advanced photothermal regime (GnRH-a control), 20% spawned (1 female of 5) but produced poor quality eggs that would not be of any value in an industrial setting. Egg quality parameters, measured as egg viability, fertilization and hatch rates for the PP-GnRH-a group met or exceeded the other treatment groups. The GnRH-a control group was significantly poorer in egg quality parameters than all other groups except with regards to hatch rate, where no significant difference was detected. However, hatched larvae from this group were inactive and rated as moribund.

Monitoring oocyte development throughout the duration of the experiment showed no significant advanced vitellogenesis in the photothermally treated groups over ambient broodstock. Estradiol and testosterone profiles also did not indicate any

significant advancement in steroid production. However, with the advanced spawning of the PP-GnRH-a group in March, successful environmentally induced advanced ovulation was anticipated, but this did not occur.

The first year of this experiment was more successful than the second. In year one, 75% of PP-GnRH-a group ovulated, however in year two only 22% spawned. In year two over 61% of fish were brought from the wild only weeks before incorporation in the experiments, in year one 100% were captive animals. Limited time to acclimate to captive conditions may have prevented the year two females from responding to photo-thermal advancement without the GnRH-a hormone pellet. Females were also smaller than the reproductive size observed by Pitt (1970) for wild fish. However, condition factor did not indicate these animals were in poor condition, nor did sexually mature animal size disagree with that reported by Manning (2003) for yellowtail flounder.

Several other aspects of yellowtail flounder broodstock husbandry, such as diet, stocking density and holding tank dimensions should be addressed to optimize husbandry and in turn broodstock management.

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