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THE ENVIRONMENTAL CONTROL OF OOCYTE DEVELOPMENT IN THE

STRIPED MULLET, \textit{Mugil cephalus}

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

The environmental control of oocyte development in the striped mullet, *Mugil cephalus*, was investigated in 3 studies. The first examined individual variation in oocyte development under natural conditions. Data on oocyte stage, oocyte growth rates, and fecundity were collected during three reproductive seasons. Females initiated the cortical vesicle stage in September-October and vitellogenesis in October-December. The average clutch required 65 days to complete vitellogenesis and had an oocyte growth rate of 6.5 μm/day. Approximately 60% of the females initiated a single clutch while 40% initiated 2 or more clutches per season. Clutch fecundity ranged between 418,000-3,008,200 eggs and was related to female length and weight.

The second study examined the effects of four combinations of photoperiod and temperature (short/cold, short/warm, long/cold, and long/warm) on oocyte development. Trials were conducted with females during the refractory period, and with females that had primary growth stage, cortical vesicle stage, and vitellogenic stage oocytes. In general, the short/cold combination stimulated, while the long/warm combination inhibited oocyte development. Short photoperiod stimulated the onset of the cortical vesicle stage in cold or warm temperatures while cold temperature stimulated the onset of vitellogenesis under short or long photoperiod. Histological examination of oocytes found that the migration of the yolk nucleus of Balbiani's vitelline body occurred in the short/cold, but not in the long/warm combination. This is the earliest event during oocyte development in any animal shown to be influenced by environmental factors. A hypothesis is also presented to explain differences in *M. cephalus* reproductive seasons throughout the world.
The third study examined the effects of melatonin (MLT) on oocyte development. The first of two experiments tested the effects of MLT implants under either natural, inhibitory, or stimulatory photoperiod. The second experiment examined the effects of afternoon administration of MLT feed. While both of these techniques are used to advance the reproductive cycles of mammals, neither implants nor MLT feed affected oocyte development in *M. cephalus*.

The results of these studies have led to the development of hatchery techniques to spawn this species throughout the year.
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CHAPTER 1
INTRODUCTION

According to evolutionary theory, reproduction in a species should be timed to maximize the chances for survival and growth of the progeny. Environmental factors which influence the success of the offspring are considered the “ultimate factors” shaping reproductive strategies (Baker, 1938). In fish, Munro (1990) identified water quality, predation, and food availability as the three main ultimate factors affecting the survival of the fry. The optimal conditions with respect to each of these factors is dependent upon the location of the spawning grounds, the spawning season, and the timing of spawning. “Proximate factors”, on the other hand, include environmental conditions which directly influence the reproductive process such as photoperiod, temperature, salinity, lunar periodicity, and social interactions. These have been categorized according to the nature of their effect (i.e., primary, supplementary, or modifying) and the stage of reproduction which they influence (Munro, 1990). Proximate factors are also referred to as cues since they provide information on the optimal time and conditions for successful breeding and ultimately maximum survival of the offspring.

If the important proximate factors for a particular species exhibit little or no annual variation, then that species should exhibit little or no reproductive seasonality. If these factors exhibit distinct annual cycles, then the species should reproduce seasonally. Many tropical and subtropical species of animals reproduce throughout the year as a result of relatively constant environmental conditions. However, the majority of both plants and animals which inhabit latitudes greater than 20° reproduce on a seasonal basis (Sumpter,
1990). These regions experience distinct annual fluctuations in environmental factors such as photoperiod and temperature, both of which are primary cues for reproduction (de Vlaming, 1974). Furthermore, some species which are distributed throughout a wide range of latitudes have discrete reproductive seasons at higher latitudes, but much less distinct seasons at lower latitudes (see Bronson, 1985). However, given the number of factors which can influence the timing of reproduction, variation in seasonality between different populations of a species or between closely related species is often much more complicated (Sumpter, 1990). In these cases, the observed reproductive patterns cannot be understood by simply correlating environmental cycles to the reproductive cycle; experimental manipulation of suspected proximate factors is required.

The striped mullet, *Mugil cephalus*, is a widely distributed species whose pattern of reproductive seasonality from location to location has not been explained. This teleost fish is euryhaline and widely distributed between the latitudes of 0 and 42° (Thomson, 1963). Briggs (1960) described *M. cephalus* as 1 of only 14 species of fish which have a truly circumglobal distribution. Since it is found in a wide variety of habitats and is easily maintained in captivity, this species is particularly well suited for investigating the relationship between environmental factors and reproduction.

Brusle (1981) reviewed the extensive literature on the reproductive seasonality of *M. cephalus*, but was unable to draw any general conclusions. Conflicting reports on the reproductive season in the same locations as well as the manner in which data had been collected were cited as the major problems. However, there were clearly other problems as well. Many studies did not document specific stages of gametogenesis, while those that
did, used different terminology which made comparisons difficult. Furthermore, most of the studies examined the reproductive condition of fish captured at different times of the year. Very few attempts were made to examine the effects of manipulating environmental conditions. Consequently, there was very little experimental data to support correlations suggested by these studies.

This dissertation presents the results of research on the environmental control of *M. cephalus* oocyte development. The studies were conducted for the purpose of clarifying the pattern of reproductive seasonality observed in this species. Chapter 2 describes the individual to individual, and year to year variation in annual oocyte development observed in females maintained under similar conditions in captivity. Data on oocyte growth rates, the relationship between female size and fecundity, and the relationship between the size of the oocytes before and after final maturation and hydration are included. Chapter 3 presents the results of 5 experiments in which females in different stages of oocyte development were placed under 4 combinations of photoperiod and temperature. This work was conducted to increase the body of experimental data on the environmental control of oocyte development in this species. Chapter 4 provides the results of 2 experiments aimed at determining whether the hormone melatonin was involved in transducing environmental information in this species. Both experiments were designed to create a physiological short-day and thereby stimulate oocyte development with either chronic release melatonin implants or melatonin treated feed.
All of the findings are summarized in a brief conclusion (Chapter 5), which is then followed by four appendices. Appendix A presents annual water temperature data obtained from two natural habitats of *M. cephalus* (Pearl Harbor and Kaneohe Bay) on the island of Oahu, Hawaii, USA. These data were provided for comparison to the temperature data obtained in the studies described in Chapters 2-4. Appendix B describes two trials conducted at The Oceanic Institute in Hawaii to promote natural spawning in captivity. The results from these trials are provided to support the assertion that *M. cephalus* does not spawn in captivity. Appendix C describes an experiment to stimulate final maturation of *M. cephalus* oocytes *in vitro*. The results of this experiment are relevant to the discussion of oocyte competence provided in Chapter 2. Appendix D provides data obtained in preliminary MLT experiments in *M. cephalus*. These experiments were conducted to determine the appropriate dosage of hormone to use in the MLT implant and MLT feed experiments presented in Chapter 4. The final section is a list of references used in this dissertation.
CHAPTER 2

OOCYTE DEVELOPMENT IN Mugil cephalus

ABSTRACT

Oocyte development patterns were examined in individual striped mullet, Mugil cephalus, maintained in outdoor tanks in Hawaii. Data were collected during three reproductive seasons: 1986-87, 1987-88, and 1989-90, from 81 adult females. All of the fish were implanted with personal identification transponders (pittags), measured, and cannulated at two to four week intervals to obtain ovarian tissue samples for oocyte stage and diameter analysis. Those which completed vitellogenesis (stage III) were induced to undergo final maturation and spawning (stage IV) by hormone injection. Fecundity estimates were obtained from the number of eggs released into the spawning tanks.

The majority of the females initiated the cortical vesicle stage (stage II) in September-October and stage III in October-December. There was more year-to-year variation in the timing of stage III than in the timing of stage II. Females reached the critical oocyte diameter (COD), or point at which stage IV could be induced, between November-March, depending on when each female initiated vitellogenesis and her oocyte growth rate. The average oocyte growth rate from the initiation of stage III (175-225 \( \mu \)m) to the COD (600 \( \mu \)m) was 6.5 \( \mu \)m/day. Based on this rate, it therefore took an average of 65 days to complete vitellogenesis. Interval oocyte growth rates calculated from consecutive samples indicated that the growth rate decreases as the oocytes reach the COD. Fifty-seven percent of the females initiated a single clutch per season, of which
56% completed vitellogenesis (i.e., reached the COD) while 44% underwent atresia. Forty percent of the females initiated 2 or more clutches per season, of which 40% completed vitellogenesis in all of the clutches. Females completing 2 or more clutches per season initiated the first clutch earlier in the season and had a higher oocyte growth rate in comparison to females who only completed a single clutch. The size of spawned eggs was directly related to the size of the oocytes prior to final maturation being induced and was described by the expression: \( Y = 463 + 0.72X \) where \( Y \) is the spawned egg diameter and \( X \) is the oocyte diameter. Clutch fecundity estimates obtained from induced spawning trials ranged between 418,000-3,008,200 eggs and were directly related to female size.

These results provide the first estimates of the rate of oocyte growth in *M. cephalus* and the first direct evidence of multiple spawning in this species. The descriptions of individual variation in oocyte development will furthermore be useful in interpreting the data from females captured from the wild.

**INTRODUCTION**

The striped mullet, *Mugil cephalus*, has one of the widest habitat distributions of any teleost fish. This species is found between 42°N to 42°S (Thomson, 1963) and has been described as 1 of only 14 marine fish with worldwide distributions (Briggs, 1960). Gilbert (1994) re-examined the evidence for its geographic range and determined that it was more restricted in the Atlantic Ocean than was previously thought. However, neither the general range of latitudes described by Thomson (1963) nor the fact that *M. cephalus* is found in all major oceans, were affected by his findings.
M. cephalus and other globally distributed species which inhabit environments that seemingly limit their dispersal capabilities are enigmatic (Crosetti et al., 1993). Although M. cephalus migrates offshore during the reproductive season (see Brusle, 1981), this species is not pelagic. It is found during much of the year in lakes and rivers (Chubb et al., 1981; Wells, 1984), brackish water estuaries (Shanbhogue et al., 1987; Potter et al., 1983), and nearshore marine environments such as bays and lagoons (Moore, 1974; Silva and De Silva, 1981; Hur et al., 1984). One possibility is that fish identified as M. cephalus at different locations may be more than one species. However, recent studies examining mitochondrial DNA and allozyme frequencies between various populations around the world found considerable variation but not a level which would be indicative of distinct species (Crosetti et al., 1993; Gilbert, 1994).

The wide distribution and variety of habitats of M. cephalus present an opportunity to examine how the reproductive strategy of a species is adapted to different environmental conditions. For example, its annual photoperiod cycle varies from a constant year-round 12:07 hours in Ecuador (latitude 0°) to a minimum and maximum of 9:07 and 15:15 hours respectively in the Black Sea (latitude 42°N; List, 1984). Annual temperature cycles range from 5-27 °C in the Black Sea (Kulikova et al., 1986) and 9-27 °C during the reproductive season in the Sea of Azov (Apekin and Vilenskaya, 1979). In contrast, the temperature range in Southeast India is 26-31 °C (Manickasundaram et al., 1987). Finally, populations migrating between freshwater lakes and the sea experience salinity changes from 0 to 35 ppt (reviewed by Brusle, 1981). However, populations migrating from hypersaline lagoons to the sea experience changes from as high as 70 ppt.
to 35 ppt (Wallace, 1975). These varied conditions are associated with a wide range of maturation and spawning seasons even within the same geographic area (Brusle, 1981).

Rather than leading to a greater understanding of *M. cephalus* reproduction, however, studies from various regions of the world have generated confusion. Brusle (1981) described some of the problems which included the inability to capture “ripe” females from the wild, estimating spawning seasons on the basis of fry appearance, a lack of experiments to determine the number of clutches females could produce in one season, and the lack of behavioral data on both individuals and populations. Until recently, there had been only 1 study on the effects of photoperiod and temperature on *M. cephalus* reproduction (Kuo et al., 1974a).

While additional experiments have helped to clarify the environmental control of annual ovarian recrudescence (see Chapter 3), data on individual variation in reproductive performance are still lacking. A complete description of this variation would contribute toward a comprehensive understanding of *M. cephalus* oocyte development. While field studies obtain only one sample from each wild female captured, maintaining fish in culture facilities and utilizing nonlethal techniques such as tagging, anesthetization, and cannulation, allows repeated sampling of the same individuals during the course of the reproductive season. Data collected in this manner have already contributed to the understanding of the basic process of oocyte development in *M. cephalus* and its hormonal control (see Tamaru et al., 1991). Most of these latter studies, however, were conducted for the purpose of developing hatchery techniques and have focused on methods for inducing final maturation and spawning as well as other problems related to
commercial production (Shehadeh and Ellis 1970; Shehadeh et al., 1973; Kuo et al., 1973, 1974b; Kuo and Nash, 1975; Kuo, 1982; Kelley et al., 1987; Lee et al., 1987, 1988; Tamaru et al., 1989). The purpose of this chapter is to describe oocyte development in individual females maintained in captivity. The patterns observed in the study are then discussed in the context of determining oocyte development patterns in the wild.

MATERIALS AND METHODS

The data presented in this chapter were obtained from female M. cephalus held at The Oceanic Institute, Waimanalo, Hawaii, USA. The data were collected during 3 periods: August, 1986 - March, 1987, August 1987 - March, 1988, and August 1989 - March, 1990. All of the fish were maintained in rubber-lined dirt ponds (9.1 m x 5.7 m x 0.7 m) having an approximate volume of 30 m$^3$. Each pond was continuously aerated and a flowthrough seawater system provided a water exchange of 80-100% per day. Both morning (8-9 AM) and afternoon (3:30-4:30 PM) surface water temperature and salinity were measured 3 days a week using a mercury thermometer and an Aquafauna refractometer (Biomarine Inc.). The average weekly photoperiod in Hawaii was obtained from the National Oceanographic and Atmospheric Administration (NOAA) and was based on sea level sunrise and sunset data taken at Honolulu Harbor.

The fish fed primarily on algae, diatoms, and crustaceans which occurred naturally in the ponds. Purina trout chow was provided as a supplemental feed once a day at a rate of 2-5% of the body weight.

Data were collected from 81 females: 44 in 1986-87, 22 in 1987-88, and 15 in 1989-90. Fish in the last 2 periods were maintained in a single pond whereas fish in
period 1 were divided into 2 ponds (21 and 23 fish each). Each of the females was identified by a Personal Identification Transponder, or Pittag, purchased through ID Systems Incorporated. The fish were first anesthetized with 200 ppm 2-phenoxyethanol. Pittags were then implanted intramuscularly just below the dorsal fin with the use of a trochar. The alphanumeric code of each fish was subsequently read by passing a hand reader over the body of the fish. These tags allowed data to be collected from individual females over the entire reproductive season.

The lengths and weights of the fish were obtained at the beginning of the reproductive season using a 1 meter length board and a top loading scale. The condition factor index (CFI) of each fish was calculated using the formula: 

\[ \text{CFI} = \frac{\text{length (cm)}}{\text{weight (g)}} \times 100. \]

Samples of oocytes were obtained according to the method of Shehadeh et al. (1973). The females were first captured using a seine net and transferred to an anesthesia tank containing 200 ppm of 2-phenoxyethanol in seawater. Once anesthetized, a 30-50 cm long polyethylene tube having an outer diameter of 1.52 mm and an inner diameter of 0.86 mm was inserted into the gonoduct for a distance of 10-12 cm. This located the end of the tube inside the ovarian lumen from which a sample of oocytes was removed by suction. The sample was ejected onto a glass slide and examined under a microscope to determine the stage of the oocytes according to the descriptions of Kuo et al. (1974b). The oocytes were then placed into a test tube containing 10% buffered formalin for storage.
Oocyte size frequency distributions were obtained from all samples containing vitellogenic oocytes. A compound microscope was fitted with an ocular micrometer calibrated to 50 μm with the use of a stage micrometer. A portion of oocytes from each sample was placed onto a plastic grooved slide (see Liu and Kelley, 1995). The oocytes lined up in the grooves which facilitated both measuring and counting. The slide was placed onto the microscope stage and the horizontal diameters of 100 oocytes were measured to the nearest 50 μm interval. The size frequency distribution obtained from each sample was used to calculate the mean oocyte diameter and standard deviation.

Females were sampled repeatedly at 2-3 week intervals. Average oocyte growth rates during the entire process of vitellogenesis were obtained from females whose oocytes were sampled both at 175-225 μm and 600-650 μm. The rates were expressed as the increase in diameter per day by calculating the difference between the means of the two samples and dividing by the number of days. Interval oocyte growth rates were obtained by dividing the difference of the mean oocyte diameters of two consecutive samples by the number of days between the samples (i.e., the sampling interval).

Six hundred microns is considered to be the critical oocyte diameter (COD) for inducing final maturation and spawning (Kuo et al., 1974b). Females having oocytes with a mean diameter of 600 μm or greater were subjected to the following induced spawning protocol. After anesthetization, each fish was injected with 20 mg /kg body weight of Carp Pituitary Homogenate (CPH) obtained from Argent Chemical. The CPH was first reconstituted with 0.6-0.8 ml of 0.9% sterile saline solution using a homogenizer. The injection was administered intramuscularly just below the dorsal fin. The fish were then
transferred to 170 liter aquaria for spawning. The following morning, approximately 24 hours after the first injection, a second injection was administered which consisted of 50-100 \( \mu g/kg \) body weight of Des gly 6, D-Ala 10 Luteinizing Hormone Releasing Hormone analog (LHRH-a) purchased from Sigma Chemical. The LHRH-a was dissolved in 0.9% sterile saline using a vortex mixer. Prior to administering the injection, a second oocyte sample was obtained. Instead of anesthesia, a black hood was placed over the head of each fish, rendering them immobile. After sampling and injecting, the hood was removed and a screen was placed over the drainpipe of each aquarium to prevent spawned eggs from washing out of the tank. Two mature males were then placed into the aquarium with each female to fertilize the eggs after spawning.

Oocytes obtained from three females undergoing induced final maturation and spawning were examined histologically. These samples were fixed for a minimum of 24 hours in Bouin's solution (Humason, 1979), washed to remove the fixative, and dehydrated in punctilious ethanol. The oocytes were then embedded in methacrylate (Sorvall's embedding medium, Dupont). One micron sections were obtained using glass knives mounted on a MT-1 ultramicrotome (Sorvall, Inc.). The sections were affixed to glass slides by heating and then stained with either Mallory's triple stain (Pantin method) or hematoxylin and eosin. Sections were photographed using a Zeiss compound microscope fitted with a Nikon camera.

After each spawn, the fish were removed from the aquaria and returned to their respective ponds. Three random 50 ml samples of water were taken from each aquarium and poured onto a fine mesh screen. The eggs from each sample, which were retained on
the screen, were then counted. The counts from the three samples were then averaged to obtain an estimate of the density per 50 ml. The average was then extrapolated to the volume of the aquarium to obtain an estimate of the total number of eggs which were spawned.

The data were analyzed using the Minitab® Statistical Software Release 10 for Windows. Regression analyses were used to examine the relationship between fish size, oocyte growth rate, and fecundity. Analyses of variance (ANOVAs) were used for all other comparisons. Unless otherwise stated, numbers presented in the form of # ± # are the mean ± the standard error (SE).

RESULTS

Oocyte Stage Descriptions

Females in this study progressed naturally through the first three stages of oocyte development as described by Kuo et al. (1974b) for M. cephalus and Wallace et al. (1987) for other teleosts. Stage I or primary growth included both the chromatin-nucleolar and the peri-nucleolar oocytes. Peri-nucleolar oocytes, the larger of the two types, reached 150-175 μm in diameter and were characterized by a central germinal vesicle containing multiple nucleoli and clear cytoplasm (Fig. 2.1a). Stage II or cortical vesicle stage oocytes were generally larger than stage I oocytes (175-200 μm), and were characterized by oil droplets surrounding the germinal vesicle which appeared dark under a transmission microscope (Fig. 2.1a). Cortical vesicles could not be distinguished in fresh samples due to their small size but are known to appear coincidentally with the oil droplets (see
Stage III, or vitellogenic oocytes, were characterized by the formation of yolk globules throughout the cytoplasm which rendered the oocytes opaque under a transmission microscope. Stage III oocytes ranged in size from 175 µm to 760 µm, but rarely exceeded 700 µm. Those having a mean diameter less than 600 µm were designated as IIIa (Fig. 2.1a), those at or exceeding 600 µm were designated as IIIb (Fig. 2.1b). This subdivision was made to distinguish oocytes which had reached the COD, and therefore could be induced to undergo stage IV.

Stage IV did not occur naturally in any of the fish in this study. In all cases, this stage of development had to be induced by hormone injections. Thirty-five hours after the first injection, a large oil drop was present in the cytoplasm. When viewed by the naked eye, these oocytes appeared to have a clear center. A histological series taken after a female received the first injection revealed that the oil drop formed from the coalescence of the smaller oil droplets observed in stage III (Fig 2.2a-d). When the drop was completely formed (Fig. 2.2d), the germinal vesicle was still present but displaced from the center, indicating that germinal vesicle breakdown (GVBD) had not taken place. Figure 2.2d also revealed that yolk globule fusion had been initiated but not hydration.

Final maturation and hydration were initiated and completed 12-24 hours after the second injection. Spawning took place shortly thereafter. Oocytes which had completed stage IV and were released into the water are referred to as eggs. Spawned eggs ranged in size from 839 to 1003 µm and were characterized by clear cytoplasm, a single oil drop, and the presence of cortical vesicles immediately under the oocyte membrane (Fig 2.1c).
Fig. 2.1: Microscopic appearance of different stages of *M. cephalus* oocytes: a) stages I, II, and IIIa, b) stage IIIb, and c) stage IV. Scale bars = 200 µm.
Fig. 2.2: Histological sections of oocytes undergoing oil droplet coalescence during stage IV on Jan. 14-15, 1986.

a) Jan. 14 at 0840 hrs, just prior to the first injection, b) Jan. 15 at 0831 hrs just prior to receiving the second injection, c) Jan. 15 at 1313 hrs, d) Jan. 15 at 1905 hrs. Scale bar = 100 μm.
Initiation of Oocyte Stages

Figure 2.3 provides the monthly percentage of females in stages I-IIIb during the 8 month period beginning in August and ending in March. Figure 2.3a summarizes the data for the 1986-87 season; the 1987-88 season is shown in Fig. 2.3b and the 1989-90 season is shown in Fig. 2.3c. In general, while a small percentage of females initiated stage II in August, most of the females initiated that stage in September and October. Likewise, a few females were found in stage IIIa in September, although most initiated vitellogenesis from October through December. Depending on the year, females first reached stage IIIb between November and January, with the peak months being January through March. During the 1986-87 and 1987-88 seasons, most females had regressed to stage I by the end of March. There was no significant difference in the initiation of stage II between the three seasons (P>0.05); however, females in 1986-87 initiated stages IIIa and IIIb significantly later than females in either 1987-88 or 1989-90 (P<0.05). There were no significant differences with respect to either the length, weight, or CFI of individual females and the initiation of stages II, and IIIa and IIIb (P>0.05).

Figure 2.4 shows the AM and PM water temperature data for each of the three seasons. On average, AM temperatures in the ponds were 2.9 ± 0.1 °C lower than PM temperatures. Water temperature decreased from September through December and remained relatively constant until March, at which point it began to increase. Table 2.1 shows the average monthly temperatures for each season. The 1986-87 season had the highest temperatures (25.2-29.0 °C) while the 1989-90 season had the lowest (24.0-27.8 °C). Since stage III was initiated latest during the 1986-87 season and earliest during the
Fig. 2.3: Initiation of stages I, II, IIIa and IIIb during each reproductive season.
Fig. 2.4: AM (lower line) and PM (upper line) water temperatures during each season.
1989-90 season, temperature may therefore have influenced the initiation of that stage, which is supported by the experimental data provided in Chapter 3. For comparison to natural *M. cephalus* habitats in Hawaii, water temperature data obtained from Kaneohe Bay and Pearl Harbor are provided in Appendix A. Photoperiod was not included on these graphs since it is constant year to year. However, the daylength in Hawaii ranges between 10.8 hrs in December to 13.4 hrs in June.

**Table 2.1**

*Average monthly water temperature (°C) in the study ponds for each season.*

<table>
<thead>
<tr>
<th>Season</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986-87</td>
<td>29.0 ±0.4</td>
<td>28.1±0.4</td>
<td>26.7±0.3</td>
<td>25.8±0.3</td>
<td>25.5±0.3</td>
<td>25.2±0.4</td>
<td>26.7±0.5</td>
</tr>
<tr>
<td>1987-88</td>
<td>28.4±0.4</td>
<td>27.4±0.4</td>
<td>25.7±0.4</td>
<td>24.9±0.3</td>
<td>24.5±0.4</td>
<td>25.3±0.4</td>
<td>26.3±0.4</td>
</tr>
<tr>
<td>1989-90</td>
<td>27.8±0.4</td>
<td>26.9±0.3</td>
<td>25.5±0.3</td>
<td>24.7±0.4</td>
<td>24.5±0.2</td>
<td>24.8±0.4</td>
<td>24.0±0.4</td>
</tr>
</tbody>
</table>

**Oocyte Size Frequency Distributions**

Figure 2.5 shows a series of oocyte size frequency distributions taken from a single female (#397C) at various points during the course of oocyte development. The example shown begins when the oocytes initiated stage IIIa (Nov. 17) and continues through the completion of stage IV (Feb. 2). Development from stage I to II took place between September 23 and October 21, and from stage II to stage IIIa between October 21 and November 17, when the series begins. The female had reached the COD (stage IIIb) when sampled on February 10 and was induced to undergo final maturation and spawning by injections of CPH (Feb. 10) and LHRH-a (Feb. 11) as previously described. Spawning took place the evening of February 11. Overall, the mean oocyte diameter of the clutch increased in size from 175-919 μm. On Nov. 17, all of the oocytes in the clutch were in the 175 μm size class. However, in subsequent samples, the oocytes were distributed in as
Fig. 2.5: Series of oocyte size frequency distributions from a single female (#397C, 1986-87) illustrating oocyte development in a typical clutch.
many as seven 50 μm size classes (Jan. 13, 458 ± 62 μm), which may have resulted from differences in when each oocyte initiated vitellogenesis as well as the rate of vitellogenin uptake. Spawned eggs exhibited a narrower distribution (Feb. 11, 919 ± 23 μm). This example was typical of the pattern of oocyte development from stage IIIa to IV observed in this study. In all samples taken, the size frequencies of oocytes were normally distributed, which is indicative of a single clutch. None of the 81 females showed the presence of 2 clutches of oocytes developing in the ovary at the same time.

Table 2.2 summarizes the standard deviations of oocyte size frequency distributions during development from stages IIIa to IV. The 370 samples are organized according to the size class of their means. The standard deviation of the clutches increased from 0 to 48 μm as the mean diameters increased from 175 to 325 μm. However, from that point on, the standard deviation remained relatively constant, averaging between 52-59 μm. Only 10 clutches were observed which had a mean diameter greater than 650 μm, because females with clutches at or greater than 600 μm were either induced to spawn or underwent atresia. The standard deviation in these 10 clutches was not significantly different than that of clutches in the 400-600 μm size range (P>0.05). This suggests that when atresia takes place, it occurs in an all or none fashion, rather than selectively removing only the largest oocytes in a clutch. Supporting evidence comes from the examination of samples taken while females were undergoing atresia. In these cases, stage III oocytes in all size classes were being resorbed simultaneously.

The average standard deviation of stage IV clutches was significantly smaller than either stage IIIa or IIIb (P<0.05). Remnant stage IIIb oocytes were observed in some
females checked immediately after spawning. These oocytes, which shortly underwent
atresia, either had not reached the COD, or were not responsive to the hormone injections
for some other reason. Unresponsiveness of the oocytes in one or both ends of the
distribution is a possible factor contributing to the narrowing of the distribution during
stage IV.

### Table 2.2
Standard deviations of oocyte size frequency distributions during stage IIIa and IV.

<table>
<thead>
<tr>
<th>Oocyte Stage</th>
<th>Oocyte Size Class (μm)</th>
<th>N</th>
<th>Standard Deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIa</td>
<td>175</td>
<td>21</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>33</td>
<td>17 ± 2</td>
</tr>
<tr>
<td></td>
<td>275</td>
<td>25</td>
<td>39 ± 1</td>
</tr>
<tr>
<td></td>
<td>325</td>
<td>40</td>
<td>48 ± 1</td>
</tr>
<tr>
<td></td>
<td>375</td>
<td>29</td>
<td>55 ± 2</td>
</tr>
<tr>
<td></td>
<td>425</td>
<td>27</td>
<td>59 ± 5</td>
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<tr>
<td></td>
<td>475</td>
<td>29</td>
<td>52 ± 2</td>
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<tr>
<td></td>
<td>525</td>
<td>22</td>
<td>55 ± 2</td>
</tr>
<tr>
<td></td>
<td>575</td>
<td>24</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>IIIb</td>
<td>625</td>
<td>58</td>
<td>53 ± 1</td>
</tr>
<tr>
<td></td>
<td>675</td>
<td>8</td>
<td>55 ± 3</td>
</tr>
<tr>
<td></td>
<td>725</td>
<td>1</td>
<td>56 ± 0</td>
</tr>
<tr>
<td></td>
<td>775</td>
<td>1</td>
<td>50 ± 0</td>
</tr>
<tr>
<td>IV</td>
<td>825</td>
<td>2</td>
<td>41 ± 7</td>
</tr>
<tr>
<td></td>
<td>875</td>
<td>18</td>
<td>26 ± 2</td>
</tr>
<tr>
<td></td>
<td>925</td>
<td>26</td>
<td>28 ± 1</td>
</tr>
<tr>
<td></td>
<td>975</td>
<td>5</td>
<td>27 ± 3</td>
</tr>
<tr>
<td></td>
<td>1025</td>
<td>1</td>
<td>38 ± 0</td>
</tr>
</tbody>
</table>

Figure 2.6a illustrates the range of frequency distributions observed in stage III
clutches. Female #1F48 had one of the narrowest distributions for a clutch with a mean
above 400 μm (mean ± SD = 470 ± 30 μm) while female #4E4B had one of the widest
(mean ± SD= 507 ± 86 μm). The coefficients of variation for these two examples are 6.4%
and 17% respectively. Figure 2.6b illustrates the range of size distributions observed in
Fig. 2.6: Examples of a) stage III (white bars = \#1F48 on Dec. 10, 1986, S.D. = 30 μm, black bars = \#4E4B on Jan. 13, 1987, 86 μm) and b) stage IV (white bars = \#416E on Jan. 7, 1988, 15 μm, black bars = \#4762 on Feb. 14, 1987, 48 μm) oocyte size frequency distributions illustrating the range observed in this study.
clutches which had completed stage IV. The coefficients of variation in these cases are 1.8% and 5.6% for the smallest (a) and widest (b) distribution, respectively.

**Oocyte Growth Rates**

Table 2.3 provides the average oocyte growth rates during stage III for clutches which a) reached the COD and b) those which did not. There were only 7 clutches which were initially sampled at exactly 175 μm as well as just after reaching 600 μm, and only 2 clutches sampled at exactly 175 μm and which later underwent atresia before reaching 600 μm. To increase the sample size, 23 clutches initially sampled when their mean diameters were in the 225 size class were also included (17 which reached the COD and 6 which did not). Table 2.3a shows that the average growth rate of clutches which completed stage III was 6.5 μm/day. The minimum and maximum growth rates observed were 4.1 and 12.3 μm/day, respectively. An estimate of 65 days to reach the COD after vitellogenesis was initiated was calculated from the expression: (600-175)/6.5. Similarly, the minimum and maximum number of days were estimated at 35 and 104 days based on 12.3 and 4.1 μm/day respectively.

Table 2.3b shows that the average growth rate of clutches which failed to reach the COD was 3.9 μm/day, which was significantly slower than clutches which completed stage IIIa (P<0.05). The minimum and maximum rates for these clutches which underwent atresia were 2.4 and 5.6 μm/day respectively.

Tables 2.4a and b provide data on the interval growth rates of clutches which reached the COD and those which did not. These data were grouped according to the 50 μm size class containing the mean oocyte diameter of the initial sample. Table 2.4a shows
Table 2.3
Average Oocyte Growth Rates During Stage III

a: Clutches Which Completed Stage III

<table>
<thead>
<tr>
<th>Oocyte Size Class (μm)</th>
<th>N</th>
<th>Initial Diameter (μm)</th>
<th>Final Diameter (μm)</th>
<th>Growth Period (Days)</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>7</td>
<td>175 ± 0</td>
<td>621 ± 23</td>
<td>71 ± 14</td>
<td>6.6 ± 1.5</td>
<td>5.1</td>
<td>8.7</td>
</tr>
<tr>
<td>225</td>
<td>17</td>
<td>233 ± 7</td>
<td>617 ± 17</td>
<td>62 ± 14</td>
<td>6.5 ± 1.8</td>
<td>4.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Combined</td>
<td>24</td>
<td>216 ± 28</td>
<td>618 ± 19</td>
<td>65 ± 14</td>
<td>6.5 ± 1.7</td>
<td>4.1</td>
<td>12.3</td>
</tr>
</tbody>
</table>

b: Clutches Which Did Not Complete Stage III

<table>
<thead>
<tr>
<th>Oocyte Size Class (μm)</th>
<th>N</th>
<th>Initial Diameter (μm)</th>
<th>Final Diameter (μm)</th>
<th>Growth Period (Days)</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
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<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>2</td>
<td>175 ± 0</td>
<td>481 ± 2</td>
<td>66 ± 15</td>
<td>4.8 ± 1.1</td>
<td>4.0</td>
<td>5.6</td>
</tr>
<tr>
<td>225</td>
<td>6</td>
<td>236 ± 6</td>
<td>476 ± 94</td>
<td>66 ± 16</td>
<td>3.8 ± 1.0</td>
<td>2.4</td>
<td>4.9</td>
</tr>
<tr>
<td>Combined</td>
<td>8</td>
<td>221 ± 29</td>
<td>477 ± 80</td>
<td>66 ± 15</td>
<td>3.9 ± 1.1</td>
<td>2.4</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Table 2.4
Interval Oocyte Growth Rates During Stage III

a: Clutches Which Completed Stage III

<table>
<thead>
<tr>
<th>Oocyte Size Class (µm)</th>
<th>N</th>
<th>Initial Diameter (µm)</th>
<th>Final Diameter (µm)</th>
<th>Interval Length (Days)</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>175</td>
<td>7</td>
<td>175 ± 0</td>
<td>357 ± 90</td>
<td>27 ± 2</td>
<td>6.7 ± 3.1</td>
<td>1.8</td>
<td>10.6</td>
</tr>
<tr>
<td>225</td>
<td>19</td>
<td>233 ± 7</td>
<td>384 ± 91</td>
<td>24 ± 6</td>
<td>6.0 ± 3.3</td>
<td>0.7</td>
<td>12.8</td>
</tr>
<tr>
<td>275</td>
<td>15</td>
<td>278 ± 15</td>
<td>430 ± 89</td>
<td>22 ± 6</td>
<td>6.8 ± 3.5</td>
<td>0.5</td>
<td>13.1</td>
</tr>
<tr>
<td>325</td>
<td>23</td>
<td>322 ± 16</td>
<td>467 ± 74</td>
<td>23 ± 7</td>
<td>6.1 ± 2.4</td>
<td>0.8</td>
<td>13.2</td>
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<td>375</td>
<td>13</td>
<td>373 ± 18</td>
<td>546 ± 52</td>
<td>24 ± 6</td>
<td>7.2 ± 1.4</td>
<td>4.2</td>
<td>9.6</td>
</tr>
<tr>
<td>425</td>
<td>15</td>
<td>426 ± 15</td>
<td>584 ± 46</td>
<td>24 ± 6</td>
<td>6.4 ± 1.3</td>
<td>4.5</td>
<td>8.8</td>
</tr>
<tr>
<td>475</td>
<td>20</td>
<td>470 ± 15</td>
<td>617 ± 32</td>
<td>26 ± 6</td>
<td>5.7 ± 1.5</td>
<td>2.5</td>
<td>8.4</td>
</tr>
<tr>
<td>525</td>
<td>18</td>
<td>522 ± 13</td>
<td>611 ± 28</td>
<td>26 ± 8</td>
<td>3.6 ± 1.4</td>
<td>1.4</td>
<td>6.2</td>
</tr>
<tr>
<td>575</td>
<td>8</td>
<td>573 ± 18</td>
<td>623 ± 22</td>
<td>25 ± 7</td>
<td>2.1 ± 0.6</td>
<td>1.2</td>
<td>2.8</td>
</tr>
<tr>
<td>625</td>
<td>11</td>
<td>616 ± 10</td>
<td>642 ± 17</td>
<td>24 ± 6</td>
<td>1.1 ± 0.5</td>
<td>0.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

b: Clutches Which Did Not Complete Stage III

<table>
<thead>
<tr>
<th>Oocyte Size Class (µm)</th>
<th>N</th>
<th>Initial Diameter (µm)</th>
<th>Final Diameter (µm)</th>
<th>Interval Length (Days)</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>175</td>
<td>4</td>
<td>175 ± 0</td>
<td>309 ± 74</td>
<td>19 ± 4</td>
<td>7.0 ± 3.2</td>
<td>4.2</td>
<td>11.1</td>
</tr>
<tr>
<td>225</td>
<td>8</td>
<td>233 ± 7</td>
<td>310 ± 41</td>
<td>24 ± 2</td>
<td>3.2 ± 1.5</td>
<td>1.1</td>
<td>5.5</td>
</tr>
<tr>
<td>275</td>
<td>8</td>
<td>269 ± 12</td>
<td>337 ± 41</td>
<td>21 ± 8</td>
<td>3.3 ± 1.6</td>
<td>0.8</td>
<td>5.5</td>
</tr>
<tr>
<td>325</td>
<td>13</td>
<td>320 ± 14</td>
<td>393 ± 43</td>
<td>26 ± 8</td>
<td>2.8 ± 1.2</td>
<td>1.0</td>
<td>4.7</td>
</tr>
<tr>
<td>375</td>
<td>8</td>
<td>369 ± 11</td>
<td>447 ± 36</td>
<td>26 ± 8</td>
<td>3.3 ± 2.1</td>
<td>0.0</td>
<td>6.7</td>
</tr>
<tr>
<td>425</td>
<td>4</td>
<td>413 ± 5</td>
<td>461 ± 31</td>
<td>25 ± 5</td>
<td>2.0 ± 1.2</td>
<td>0.9</td>
<td>3.0</td>
</tr>
<tr>
<td>475</td>
<td>3</td>
<td>470 ± 17</td>
<td>559 ± 13</td>
<td>23 ± 4</td>
<td>3.7 ± 0.5</td>
<td>3.4</td>
<td>4.3</td>
</tr>
</tbody>
</table>
that the rate decreased significantly as the mean diameter approached and passed the COD (P<0.01). Intervals starting at 175-475 μm averaged between 5.7-7.2 μm/day (range: 0.5-13.2 μm/day). Intervals starting at 525-625 μm averaged between 1.1-3.6 μm/day (range: 0.25-6.2 μm/day). Table 2.4b shows that the initial growth rate of clutches which underwent atresia was similar to clutches which completed vitellogenesis (175 μm interval, 7.0 μm/day vs 6.7 μm/day). However, the average growth rate of all subsequent intervals was significantly lower (P<0.05).

Unlike stage III, the increase in the mean diameter during stage IV was not due to the process of vitellogenesis, but rather the process of hydration, which takes place over a period of several hours. The average diameter of eggs produced from 50 induced final maturation and spawning trials ranged from 839-1003 μm. Figure 2.7 shows that the mean diameter at stage IV was dependent on the mean diameter at stage IIIb, prior to the first injection of CPH (P<0.01). The relationship was described by the equation: Y = 463 + 0.72X, where Y was the stage IV diameter and X was the stage IIIb diameter. One outlying data point (stage IIIb = 760 μm, stage IV = 940 μm) was omitted from this plot.

On average, the mean oocyte diameter increased 291 ± 4 μm as a result of hydration. A summary of oocyte and egg diameter analyses associated with induced spawning trials is provided in Table 2.5. Even though the COD was considered to be 600 μm, final

<table>
<thead>
<tr>
<th>Stage</th>
<th>N</th>
<th>Mean Diameter (μm)</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIb</td>
<td>52</td>
<td>621 ± 4</td>
<td>558</td>
<td>760</td>
</tr>
<tr>
<td>IV</td>
<td>52</td>
<td>912 ± 5</td>
<td>839</td>
<td>1003</td>
</tr>
</tbody>
</table>

Table 2.5
Stages IIIb and IV oocyte diameter analyses from induced spawning trials.
Stage IV Mean Diameter (μm)

Y = 463 + 0.72X
R-Squared = 0.4

Fig. 2.7: Comparison of the mean diameters of oocytes in stage IIIb and stage IV.
maturation and spawning was induced in females having a mean oocyte diameter as low as 558 µm. However, the majority of trials in which the mean diameter was below 600 µm produced either "non-viable" eggs or no response.

**Oocyte Development Patterns**

Individual females showed a number of different oocyte development patterns during the course of each reproductive season. Figures 2.8a-d show examples of four of the most common patterns which were coded as: # clutches which initiated stage III | # of clutches which completed stage III. In the first example (Fig. 2.8a), female #3F16 initiated a single clutch of oocytes in November, which underwent atresia after reaching 546 µm at the end of January. This type of pattern was therefore coded 1|0. Fig. 2.8b shows the second example (female #3853). This female initiated a single clutch of oocytes in November which reached the COD in February and was subsequently induced to spawn. The rapid increase of the mean oocyte diameter as a result of hydration during stage VI is shown as the spike in the pattern which took place on Feb. 26. Following expulsion of the eggs during the actual spawning event, the mean oocyte diameter dropped to 175µm. With the exception of a few remnant stage III oocytes which did not respond to the hormone injections, the only oocytes in the ovaries at that time were stage I and II. This pattern was coded as 1|1, signifying that 1 clutch was initiated and 1 was completed. Fig. 2.8c shows the third pattern which is from female #3E67. This female initiated a clutch in November which reached the COD in January. After spawning was induced several days later, a second clutch was initiated. By early March, this clutch had reached a mean diameter of 384 µm before undergoing atresia. On March 24, the only
Fig. 2.8: Examples of the four most common oocyte development patterns: a) 1|0, b) 1|1, c) 2|1, d) 2|2.
oocytes remaining in the ovaries were stages I and II. This third pattern was coded 2/1, signifying 2 clutches were initiated, of which 1 reached the COD and was spawned. The fourth commonly observed pattern is shown in Fig. 2.8d. Female #4479 initiated 2 clutches, both of which reached the COD and were spawned. This pattern was coded 2/2.

A specific maturation pattern was determined for 72 of the 81 females used in this study. Table 2.6 provides a summary of the possible types of patterns through three clutches and spawns, and the frequency in which they were observed. Each pattern type is coded as described above and ranked from most to least frequent. Thirty-two percent of the females produced a 1|1 pattern, 25% produced a 1|0, 21% produced a 2|1 and 15% produced a 2/2. Two females (3%) neither initiated nor completed a single clutch (0|0) while 1 female initiated 3 clutches, all of which reached the COD.

Fig. 2.9 shows the data from these 72 females regrouped according to the number of clutches initiated (a) and the number completed (b). Twenty-eight percent of the females initiated 1-2 clutches which failed to reach the COD and therefore were not

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<th>Oocyte Development Pattern</th>
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Fig. 2.9: Summary of the number of clutches initiated (a) and the number completed (b) by females in this study.
spawned during the season. Fifty-three percent of the females initiated and completed 1 clutch and were therefore spawned once during the season, while 16% were spawned 2-3 times during a single season.

Factors Influencing the Number of Clutches Completed

Figure 2.10a shows the relationship between the month in which the first clutch was initiated and the number of clutches completed during the season. In general, the earlier the female initiated the first clutch, the more clutches and spawns were produced per season. Females that were in stage III in September-October completed over 1.5 clutches per season in comparison to 0.7 for those which began stage III in December. A second influencing factor was the oocyte growth rate. Females that completed a higher number of clutches also had a higher average oocyte growth rate (Fig. 2.10b). Females that initiated a clutch, but failed to reach the COD, had the lowest oocyte growth rates.

Fecundity

Fecundity data were obtained from a total of 43 induced spawning trials involving 35 of the 50 females which reached the COD in this study. Data were not included from trials in which the egg screen overflowed or from those which had more than 20% bad or non-viable eggs. “Bad egg” spawns often resulted from an injection timing error in which underripe or overripe oocytes were induced to undergo final maturation. Since many oocytes did not respond to the hormone stimulus or only partially hydrated, these spawns usually produced lower than normal fecundity estimates. Twenty-six of the 35 females
Fig. 2.10: Relationship between the number of clutches completed and a) initiation of stage III and b) oocyte growth rate.
spawned once, while 9 spawned twice. The clutch fecundity from these trials ranged from 418,000-2,043,400 eggs and the total fecundity ranged from 418,000-3,008,200 eggs.

There was no significant difference between the fecundity of the first (1,018,075 ± 95,291 eggs) and second (1,167,514 ± 140,505 eggs) clutches of the 8 females that spawned twice (P<0.05). Furthermore, there was no significant difference between the average fecundity of these females (1,092,794 ± 100,919 eggs) and the fecundity of the females which spawned only once (972,073 ± 77,320 eggs, P<0.05). As a result, the total fecundity of females which spawned twice was significantly higher than females that spawned only once (2,185,589 ± 201,837 eggs, P<0.01).

Clutch fecundity increased significantly in relation to the length (Fig. 2.11a) and weight (Fig. 2.11b) of the female, but not to the condition factor index or CFI (P<0.01). As a result, larger females that produced 2 clutches had a total fecundity up to 7 times higher than smaller females that produced only a single clutch.

DISCUSSION

Bye (1990) pointed out that studies on the environmental control of fish reproduction are complicated by a high level of individual variation in responses to experimental conditions. Correctly interpreting the results of these studies requires an understanding of the range of individual variation in gonadal development under natural conditions. Furthermore, this information is essential to understanding the seasonal reproductive patterns in species with wide geographic distributions, such as *M. cephalus*. This chapter examined individual variation in *M. cephalus* reproduction by maintaining females in captivity and monitoring their oocyte development over time. The results
Fig. 2.11: Relationship between clutch fecundity and female length (a) and weight (b).
provide a foundation for the experimental study described in Chapter 3. The following
discussion relates the results presented here to those from other studies on this species and
makes recommendations for standardizing descriptions and techniques.

The numerous ways in which *M. cephalus* oocyte development has been described
has impeded the comparison of findings from different regions of the world. Some
authors focused on the general appearance, size, and development of the ovaries using
subjective staging terminology such as “glassy”, “bloodshot”, “pigmented yellow or
orange”, “running ripe”, “oozing”, “flaccid”, and “spent” as well as immature, maturing I,
maturing II, early maturing, late maturing, and developing (Kesteven, 1942; Rafail, 1968;
Moore, 1974; Brulhet, 1975; Stenger, 1959; Sulochanamma *et al.*, 1981). Other authors
focused on staging the oocytes using terms which included “transparent”, “opaque”,
“ripe”, “intense basophil”,”basophil confined to delicate granules”, “vacuolisation of the
ooplasm”, protoplasmic growth, trophoplasmic growth (which is further divided into
vacuolation, commencement of yolk accumulation, intensive yolk accumulation), atretic,
vitellogenic, preovulatory, primary yolk granule, secondary yolk granule, tertiary yolk
granule, chromatin-nucleolus, perinucleolus, early vitellogenic, mid-late vitellogenic,
mature, prematuration, yolk vesicle, cortical vesicle, primary growth, and yolk globule
Timoshek and Shilenkova, 1974; see also the review by Brusle, 1981). Ovarian and
oocyte stages have furthermore been designated in Roman numerals from I to VIII, Arabic
numbers from 0 to 6, as well as letters from A to F. In some studies using the same type
of designation, the numbers or letters correspond to the same stages; in others, they do not.

In this study, the description of *M. cephalus* oogenic stages conforms to the model proposed by Wallace *et al.* (1987) for teleost fishes in general. The numerical assignment of each stage follows the convention of Kuo *et al.* (1974b), with the exception that stage V (atresia) was not included. Atretic oocytes are degenerating oocytes and therefore were not considered to be a developmental stage *per se*. According to Wallace *et al.* (1987), teleost oocytes undergo 4 general stages of development: stage I - primary growth (which includes the chromatin-nucleolar and peri-nucleolar stage), stage II - cortical vesicle formation, stage III - vitellogenesis, and stage IV - maturation. Grouping chromatin-nucleolar and the peri-nucleolar oocytes stages into a single stage (i.e., primary growth) makes intuitive sense: both types of oocytes are present during the off season (May-August) and collectively define the immature state of the ovary. Oocytes undergoing the process of cortical vesicle formation should be considered a separate stage and not grouped with oocytes undergoing primary growth. Cortical vesicle formation rather than vitellogenesis is the first response to a stimulatory photoperiod (see Chapter 3) and signals the onset of the reproductive season. Recording the appearance of this stage of oocyte development is extremely important for understanding the environmental control of *M. cephalus* oogenesis in different regions of the world. Greeley *et al.* (1987) suggested that *M. cephalus* vitellogenesis should be subdivided to include a prematuration stage which defines when the oocytes acquire the capability to resume meiosis. The results of this study also support a subdivision of this stage. Prior to reaching a mean
oocyte diameter of 600 μm, oocytes generally show no response to exogenous GTH. Those with a mean diameter at or greater than 600 μm, however, undergo oil droplet coalescence after the hormone is administered. To recognize this differential response to GTH stimulation, stage III was subdivided into IIIa and IIIb. There seems to be no advantage to subdividing vitellogenesis further into early and middle “substages” on the basis of oocyte frequency distributions or the size of yolk globules.

Goetz (1983) used the term “final maturation” rather than “maturation” to describe the events associated with the resumption of meiosis I in stage IV. Since the term maturation is commonly misused in aquaculture to describe stage III, referring to stage IV as final maturation would alleviate confusion regarding stage descriptions. Final maturation would include oil drop coalescence, hydration, GVBD, and ovulation. In almost all cases in this study, as well as others, single oil drop oocytes responded to the second hormone injection by undergoing GVBD and hydration, and were subsequently ovulated and spawned. If the second injection was postponed for several days, the oocytes would lose their responsiveness and require a third injection (Moriwake and Moriwake, unpublished data). If the second injection were not administered at all, single oil drop oocytes would not undergo spontaneous maturation but would remain in that stage for several weeks or longer.

The course of natural final maturation is unknown, as is the serum profile of gonadotropin (GTH) during this stage. It is suspected that GTH stimulation and subsequently oil droplet coalescence, GVBD, and hydration occur in a continuous manner during natural final maturation since there seems to be no advantage to a pause which
would cause a loss of responsiveness. For this reason, the morphological differences following the first and second injections were not considered to be distinct substages, as they were in the classification scheme of Zhitenev et al. (1974).

Kuo et al. (1974b) provided an excellent series of photographs from cultured Hawaiian *M. cephalus* showing both the microscopic and histological appearance of stage I-III oocytes, as well as atretic oocytes. This chapter provides microscopic and histological appearance of oocytes undergoing stage IV. Sections of ovaries obtained from wild *M. cephalus* captured from the Black Sea and induced to undergo final maturation and spawning showed a similar histological appearance (Zhitenev et al., 1974). Typical of teleosts with pelagic eggs, yolk globule and oil droplet coalescence took place coincidentally with the resumption of meiosis (Wallace and Selman, 1981).

A mean diameter of 600 µm has been described previously as a marker for determining when a clutch of oocytes will initiate stage IV in response to GTH stimulation (Kuo et al., 1974b; Lee et al., 1987; Tamaru et al., 1989). While 600 µm appears to be the COD in Hawaiian, as well as perhaps Australian and Floridian *M. cephalus* (see Grant and Spain, 1975 and Greeley et al., 1987), the COD in *M. cephalus* found in other locations appears to be smaller. For example, in the Kerch Strait between the Black Sea and the Sea of Azov, final maturation and hydration could be induced at a mean diameter of 475 µm (Apekin and Vilenskaya, 1979). This resulted in spawned eggs of 650-750 µm in diameter from which viable larvae hatched. Similar results were obtained with female *M. cephalus* caught during their annual migration from Lake Fuad in Egypt, to the Mediterranean Sea (Kelley, et al., unpublished data). Final maturation and hydration were
induced in 10 females that had mean oocyte diameters ranging between 494-572 µm (535 ± 6 µm). The mean spawned egg diameter resulting from these trials ranged between 700-785 µm (764 ± 8 µm). Subsequent spawning trials with both wild and captive females indicated that clutches almost never reached a mean diameter of 600 µm.

As was shown in Fig. 2.8 and Table 2.5, the average size of spawned eggs ranged between 839 and 1003 µm, and was directly related to the size of the oocytes when final maturation was induced. According to Apekin and Vilenskaya (1979), naturally spawned eggs collected from the Sea of Azov have averaged between 720-780 µm in diameter, suggesting that final maturation was initiated when the oocytes were less than 550 µm. This estimate is in agreement with their report of the size of the vitellogenic oocytes obtained from migrating females. Finucane (1978) reported that naturally spawned eggs collected from plankton tows in the Gulf of Mexico ranged in size from 910-990 µm which suggests that final maturation was initiated when the oocytes ranged between 600-730 µm. This is also consistent with the oocyte diameters of migrating females in this region of the world (Greeley et al., 1987). Other reported diameters of naturally spawned eggs include 650-780 µm from Japan (Kawakami, 1917, in Brusle, 1981), 910-1080 µm from Japan (Nakano, 1918, in Brusle, 1981), 720 µm from Italy (Sanzo, 1936, in Brusle, 1981), 600-720 µm from the Black Sea (Vodyanitskii and Kazanova, 1954, in Brusle, 1981), and 650-850 µm from Japan (Hotta, 1955, in Brusle, 1981). Therefore, depending on the location, spawned egg diameters range between 600-1080 µm which suggests that the COD in wild populations ranges between 475-620 µm.
A mean diameter of 600 μm has also been described as the point at which the oocytes acquired the competence to resume meiosis (Greeley et al., 1987). In Hawaiian *M. cephalus*, however, competence has not been acquired in oocytes of this size. First, if no injections were administered, stage IIIb oocytes would continue vitellogenesis for several weeks. Secondly, stage IIIb oocytes would not respond to 17α-20β-dihydroprogesterone (17α-20β-P) *in vitro* until 24 hours after the female had received the first injection (Kelley and Goetz, unpublished data). Thirdly, it was shown that only oocytes obtained 3-6 hrs after the second injection would undergo *in vitro* final maturation when 17α-20β-P was absence from the media (see Appendix C). Finally, Susuki *et al.* (1991) showed that after reaching the COD, the follicles did not begin synthesizing maturation-inducing steroids until both injections had been administered.

Since most teleost species have a distinct annual reproductive season, the developmental stage of the oocytes will vary depending on the time of year (reviewed by Bye, 1990). On this basis, Wallace and Selman (1981) categorized adult teleost ovaries into 3 types: synchronous, asynchronous, and group synchronous. According to the data presented in this study as well as others (Kuo and Nash, 1975; Greeley *et al.*, 1987), the *M. cephalus* ovary is a group synchronous type, in which a distinct group of previtellogenic oocytes, derived from a everpresent pool, is stimulated to undergo vitellogenesis and eventually complete the process of development. Stage I oocytes were present in all samples examined regardless of the time of year, in both this study as well as others (see Kuo and Nash, 1975, and Chapter 3). In addition to these, stage II oocytes appeared in September and stage III oocytes appeared in October-November.
Recruitment can occur from oogonia, stage I, stage II, or even stage III depending on the species (Wallace and Selman, 1981). According to Wallace et al. (1987), the recruitment pool consists of stage I oocytes in species which spawn once per year, or later stages in species which spawn more than once. In this study on *M. cephalus*, two recruitment events appeared to take place during the annual reproductive cycle. At the beginning of the reproductive season in this study, a group of stage I oocytes was recruited into stage II. One to two months later, a subgroup from these oocytes was recruited into stage III. The study by Kuo and Nash (1975) also appears to show the same pattern. The recruitment pool consisted of both stage I and stage II oocytes during the reproductive season, but only stage I oocytes during the off-season.

Since oocytes undergoing primary growth can be found in the ovaries throughout the year, stage I is considered to be an immature stage in teleosts (Wallace et al., 1987). Stage II and stage III, on the other hand, are initiated in late summer/early fall and fall/winter, respectively, in many fishes including salmonids, carps, sticklebacks, gobies, surfperches, dabs, turbots, soles, and sea basses (Baggerman, 1990; Bye, 1990; Hontela and Stacey, 1990; Scott, 1990; Taylor, 1990). This study and that of Kuo and Nash (1975), showed that cultured *M. cephalus* in Hawaii have a similar pattern. Stage II was initiated at the same time each year, suggesting that it is controlled by a factor having a relatively constant annual cycle. In contrast, the initiation of stage III was more variable. Kuo et al. (1974a) showed that both photoperiod and temperature were important environmental factors in the mullet ovarian cycle, while the study presented in Chapter 3 showed that the initiation of stage II was primarily controlled by photoperiod and initiation
of stage III by temperature. These experimental results are consistent with the observation that in 1989, stage III was initiated 2 months earlier than in 1987, a year during which the temperature in the first part of the season was significantly higher than in 1989.

To date, there has been no documented case of *M. cephalus* spontaneously undergoing stage IV and spawning in captivity (Shehadeh and Ellis, 1970; Kuo *et al.*, 1973, and 1974b; Shehadeh *et al.*, 1973; Lee *et al.*, 1987). More recent attempts to promote natural spawning in ponds were unsuccessful (see Appendix 2). In the wild, *M. cephalus* usually migrate from nearshore environments such as bays, estuaries, and lakes to the open sea prior to initiating final maturation and spawning (reviewed by Brusle, 1981). Whatever cues are required to initiate this stage in the open sea are apparently lacking in culture environments. Since the females did not initiate final maturation unless induced to do so with hormone injections, no data were obtained on the factors which controlled this stage. This study could only show that there was year-to-year as well as individual to individual variation in when females reached the COD, the latter being related to when stage III was initiated and the oocyte growth rate of the clutches. Year to year and individual to individual variation also occur in the wild (see Fig. 1 and 3 of Greeley *et al.*, 1987) and may be responsible for some of the conflicting reports on the extent of the reproductive season of *M. cephalus* in the same region (reviewed by Brusle, 1981). Confusion could be reduced by sampling females from the same location for 2-3 years and correlating their stages of oocyte development to water temperature measurements.
Unlike that observed in Greeley et al. (1987), the standard deviation of stage III oocyte size frequency distributions in this study did not vary once the mean reached the 325 μm size class. The average standard deviation from this point on ranged between 48-59 μm. For the purpose of comparison, the frequencies of mid to late and prespawning vitellogenic oocytes shown in Figure 6 of Greeley et al. (1987) were estimated to be 25 μm or approximately half that observed in similar stage clutches of this study. Female mullet caught migrating from Lake Fuad, Egypt, had oocyte clutches with standard deviations ranging between 29-49 μm (41 ± 1 μm, Kelley et al., unpublished data).

Sulochanamma et al. (1981) also provided oocyte diameter frequencies for M. cephalus collected off the west coast of India. While the standard deviations were not provided, it was estimated from their Fig. 1 to be approximately 59 μm at the latter part of stage III.

It is not clear, however, whether the frequency distributions shown are single examples or combined data. Furthermore, while 200 oocytes were measured in that study, only 50 oocytes were measured per sample in Egypt while 100 oocytes were measured per sample in this study. Greeley et al. (1987) reported measuring samples that contained between 100-500 oocytes. The variation in the standard deviations in these studies may therefore lie in differences in sample size and oocyte measuring techniques. For purposes of comparison, a standardized procedure should be adopted for future studies. It is recommended that all oocyte samples be preserved in 10% buffered formalin and that 100 oocytes are measured per sample. While it would be most desirable to measure fresh oocytes, it is impractical when a relatively large number of samples are collected simultaneously.
This study provided a comprehensive analysis of *M. cephalus* oocyte growth rates. The average stage IIIa growth rate of 6.5 μm implies that the average length of time required to complete vitellogenesis was 65 days under the environmental conditions in the ponds. Vitellogenesis clearly slowed as the oocytes approached and passed the COD. Clutches which underwent atresia before reaching the COD had a growth rate which was approximately 50% slower than clutches which did reach the COD. Other studies have not provided comparable data on the oocyte growth rate in this species. However, the length of time required for the oocytes to complete stage III has been inferred from oocyte samples taken during different times of the season, and in general, by the length of the season. Apekin and Vilenskaya (1979) estimated that vitellogenesis took place over a period of 2-2.5 months in the Black Sea. Similarly, data provided by Greeley *et al.* (1987) suggest that vitellogenesis takes place over a period of 2-3 months in Florida. Both of these estimates are in agreement with the present study and indicate that, in general, the average oocyte growth rate in wild populations is similar to that observed under culture conditions.

However, rates from location to location are probably quite variable. First, the number of days required to complete vitellogenesis will depend on the point at which either the COD or competence to initiate final maturation is reached. Secondly, considerable individual variation in oocyte growth rates was observed. Thirdly, temperature has been shown to affect the rate of gonadal development in other species of teleosts (reviewed by Laevastu and Hayes, 1982). Although it has not been well studied in *M. cephalus*, females placed under a controlled photoperiod of 8 hours of light and a
temperature of 21 °C exhibited an average oocyte growth rate which, depending on how it was calculated, was 42-69% higher than that of the females maintained under ambient conditions in the present study (see Chapter 3).

*M. cephalus* females are believed to spawn only once per year (Kesteven, 1942; Timoshek and Shilenkova, 1974; Grant and Spain, 1975; Greeley *et al.*, 1987). The basis for this conclusion is the presence of only a single clutch of oocytes in the ovaries of females collected during the reproductive season. The present study, however, showed that in captivity, some females can produce 2-3 clutches per year. The second and third clutches were not the result of partial spawning as observed by Moore (1974) since they were only initiated after the first clutch had been cleared from the ovaries. In fact, there were no instances in which 2 clutches were observed simultaneously in the same sample. Multiple clutches were only detected in the present study by repeated sampling of the same individual female. For this reason, some wild *M. cephalus* females may also be producing more than one clutch per season.

Two other observations also support this possibility. First, the occurrence of multiple clutches in the present study was correlated to a faster oocyte growth rate as well as initiating the first clutch earlier in the season. This is not surprising; both of these factors would lead to multiple spawning females completing their first clutch in time to initiate and complete a second clutch before the end of the season. Sampling during the first couple of months in the season found females in all different stages of vitellogenesis since the first clutch of multiple spawners was often at or near the COD when the first clutch of single spawners was initiating vitellogenesis. This pattern is also seen in the
capture studies of Apekin and Vilenskaya (1979) and Greeley et al. (1987). Secondly, multiple spawning took place despite frequent handling and sampling of the females in this study. Handling stress has been shown to have an inhibitory effect on oocyte development in fish (Campbell et al., 1991). Since wild females are not subjected to handling, it is even more likely that they would be producing multiple clutches than females maintained in captivity.

Multiple spawning in cultured females could have been the result of supplemental feeding which provided the additional energy requirements necessary to generate a second clutch. To compare the condition of the fish in this study to the condition of wild fish, the fork lengths of the females used in the study were converted to standard lengths using the equation provided by Greeley et al. (1987). The length-weight relationship was then obtained which yielded a regression coefficient of 3.127, which is essentially the same as the 3.126 value obtained from wild females by Greeley et al. (1987). Secondly, this study found no significant relationship between CFI and the number of clutches initiated or completed. It therefore seems unlikely that multiple spawning in captive females was a result of a difference in the condition of captive and wild fish.

Brusle (1981) summarized the absolute and relative fecundities from 13 studies carried out on *M. cephalus* in different regions of the world. The values ranged from 340,000 to 7,206,000 eggs and 648-1,369 eggs/gram body weight, respectively. Absolute fecundity should actually be referred to as potential fecundity since these estimates were obtained prior to final maturation and spawning. Greeley et al. (1987) reported potential fecundities ranging between 250,000-2,500,000 eggs. In light of the present study, these
estimates should be more conservatively described as potential clutch fecundities until the question of multiple spawning in wild populations is resolved. Clutch fecundity estimates from the present study are in good agreement with the estimates from these other studies. There is also complete agreement that clutch fecundity was positively correlated to body size. Finally, results from the present study indicate that the fecundity of second and third clutches is not significantly different than that of the first clutch. As a result, females that spawned more than once per season literally doubled or tripled their total annual fecundity in comparison to females that spawned only once.
CHAPTER 3

THE ENVIRONMENTAL CONTROL OF OOCYTE DEVELOPMENT IN
*MUGIL CEPHALUS*

ABSTRACT

This study examined the effects of four different combinations of photoperiod (L, hrs of light) and temperature (°C): short/cold (8L/20.9-21.9 °C), short/warm (8L/28.0-29.4 °C), long/cold (16L/20.6-21.7 °C), and long/warm (16L/29.4-30.6 °C) on the annual cycle of oocyte development in the striped mullet, *Mugil cephalus*. Five 8-10 week trials involving 205 sexually mature adult females were conducted between April, 1988, and June, 1989. Trial 1 was initiated immediately after the reproductive season when females had primary growth (stage I) and leftover cortical vesicle (stage II) stage oocytes. Trial 2 was initiated during the middle of the offseason when females had only stage I oocytes. Trial 3 was initiated at the beginning of the reproductive season when females had both stage I and stage II oocytes. Trial 4 was initiated during the middle of the reproductive season when the females had stages I, II, and III (vitellogenic) oocytes. Finally, trial 5 replicated trial 2 and was initiated during the middle of the following offseason when females had only stage I oocytes. In general, the short/cold treatment had the most stimulatory effect while the long/warm treatment had the most inhibitory effect on oocyte development. Short photoperiod stimulated the onset of stage II from stage I in either cold or warm temperatures. Cold temperature stimulated the onset of stage III from stage II under either short or long photoperiod. The combination of long photoperiod and warm temperature caused regression from either stage II or stage III to stage I. Histological
examination of stage I oocyte samples obtained during trial 5 indicated that the migration of the yolk nucleus of Balbiani’s vitelline body took place under the short/cold treatment, but did not take place under the long/warm treatment. This is the earliest event during oocyte development in any animal shown to be influenced by environmental factors.

The stage III oocyte growth rate between sampling intervals was significantly higher under the short/cold and long/cold treatments in comparison to the short/warm treatment, suggesting that water temperature was the major influencing factor. Based on these results, a hypothesis is presented to explain the differences in reproductive seasons reported for various locations throughout the world.

**INTRODUCTION**

Species which have a wide geographic distribution provide an opportunity to examine how reproductive cycles are adapted to different environmental conditions. For example, a pattern observed between populations of animals inhabiting higher and lower latitudes is shown in Bronson (1985). In this study, the breeding season of the rodent genus *Peromyscus* expands from 3 months at 60°N to 12 months at 20°N as a result of differences in photoperiod and temperature cycles between those latitudes. The spawning season of the milkfish, *Chanos chanos*, also expands from high to low latitudes (Kumagai, in Lee, 1985) and, furthermore, reverses from the Northern to Southern hemisphere, as one would expect if it was regulated by the same environmental factors. Sumpter (1990) points out, however, that only a very small percentage of animals, particularly fish, have been investigated with respect to the factors controlling their reproductive cycles and cautions against making generalizations until additional species have been studied.
With respect to fish, the striped mullet, *Mugil cephalus*, is a candidate species for this type of study since it is one of only a few truly circumglobal teleosts (Briggs, 1960). Depending on the time of year and the location, *M. cephalus* inhabits (both freshwater and hypersaline), rivers, estuaries, lagoons, bays, and the open ocean (see Brusle, 1981 for a review). *M. cephalus* is found in latitudes ranging from 0-42°F (Thomson, 1963), and therefore, has adapted to photoperiods ranging from a constant 12:07 hours year round in Ecuador to a 9:07-15:15 hour annual cycle in the Black Sea. Some reported annual temperature cycles include 5-27°C in the Black Sea (Kulikova et al., 1986), 6-33°C in the Gulf of Mexico (Dindo and MacGregor, 1981) and 26-31°C in India (Manickasundaram et al., 1987). Furthermore, since it typically migrates from inland or coastal waters to the open sea for spawning, *M. cephalus* experiences salinity changes from 0-35 ppt in freshwater lake-ocean migrations to 70-35 ppt in hypersaline lake-ocean migrations (Wallace, 1975).

Brusle (1981) reviewed the extensive literature regarding its spawning periodicity in different locations in the world. Since there were many conflicting reports from the same regions, however, no real generalizations on the environmental regulation could be synthesized. He cited a number of problems in obtaining data, including estimating seasonality on the basis of fry occurrence. However, Sumpter’s (1990) comment also seems to apply, that merely correlating observed reproductive cycles to environmental factors without conducting experiments is essentially useless for understanding the control of the cycle. For *M. cephalus*, correlational studies abound while experimental studies are almost non-existent.
On the basis of results obtained by Abraham et al. (1966), and Blanc-Livni and Abraham (1969) from fish raised in freshwater ponds (0 ppt), and Kuo et al. (1973) from fish maintained in seawater (32 ppt), Brusle (1981) concluded that salinity was probably not an environmental cue for *M. cephalus*. More recent support for this conclusion comes from Tamaru et al. (1994), in which adult females transferred from 32-35 ppt to either 0 ppt or 13-20 ppt prior to the reproductive season initiated both stages II and III and completed vitellogenesis to the point at which stage IV could be induced (Tamaru et al., 1994). However, none of these studies tested hypersaline conditions, nor did they simulate changes which might occur during natural migrations, and therefore, dismissing salinity as a possible factor influencing reproduction in *M. cephalus* may be premature.

Kuo et al. (1974a), examined the effects of a short photoperiod (6 hours of light) and temperatures ranging between 17-26°C and provided the first evidence that oocyte development was regulated by both photoperiod and temperature. However, the treatment conditions were all stimulatory since the primary goal of the study was to develop methodology for attaining year-round spawning. No other study on oocyte development using either the same or inhibitory photoperiod and temperature combinations has been reported.

Additional experimentation is required to understand how environmental factors influence oocyte development in this species and ultimately its patterns of reproductive seasonality of in different locations in the world. Therefore, a series of trials was initiated in which females were exposed to 4 combinations of photoperiod and temperature at different points in their annual reproductive cycle. The objective of the
experiment was to determine the effects of these two environmental factors on different stages of oocyte development.

MATERIALS AND METHODS

This study was conducted at The Oceanic Institute in Hawaii, USA. The experiment consisted of five 8-10 week trials using a total of 205 adult females. For each trial, 32-48 females were divided into four groups and stocked into separate 8 m³ round fiberglass tanks (4 m in diameter by 0.6 m deep). The tanks were located indoors and were supplied with continuous aeration. A flowthrough seawater system allowed a water exchange of 50-100% per day. Water temperature was controlled by 2 water cooled titanium chillers (Universal Marine Industries). The units were attached to 2 of the tanks to lower their temperature approximately 5 °C from ambient (26 °C). The hot water outflow from the units was supplied to the other 2 tanks to raise their temperature approximately 5°C above ambient. Each tank was fitted with a separate bank of fluorescent lights (345 lux) controlled by a 24 hour timer. Black plastic covers installed over a frame mounted on the top of the tanks prevented extraneous light from entering. The timers were set to 8 hours of light (8L) on one cold and one warm tank, and 16 hours of light (16L) on the other cold and warm tanks. This resulted in each tank having a different combination of photoperiod and temperature: short/cold, short/warm, long/cold, and long/warm.

The timers and water temperature were checked twice daily, once in the morning and once in the afternoon, and adjusted as necessary. The salinity was monitored 3 times a week using an Aquafauna refractometer (Biomarine Inc). During each trial, the fish
were fed Purina trout chow once a day at an approximate rate of 2-5% of their body weight. Each female was identified by a Personal Identification Transponder, or pit tag, which had been implanted in the musculature below the dorsal fin. The fork lengths and weights of the fish were obtained using a 1 meter length board and a top loading scale. The condition factor index (CFI) of each fish was calculated using the formula: length (cm)/weight (g)\(^3\) x 100.

Samples of oocytes were obtained on 2-4 week intervals by cannulation and staged as described in Chapter 2. Vitellogenic oocytes (stage III) were preserved in 10% buffered formalin and later measured as described in Chapter 2 to obtain the size frequency distribution for each sample. Estimates of oocyte growth rates during vitellogenesis were obtained using two different methods. The first method was based on samples obtained from the same female at the beginning of vitellogenesis (i.e., 175-250 µm) and when the oocytes had reached the COD (i.e., 600 µm). The difference in the mean oocyte diameters was divided by the number of days between the two samples to yield the increase in oocyte diameter per day. This method provided the average oocyte growth rates during the entire process of vitellogenesis. The second method also obtained the increase in oocyte diameter per day, but between two consecutive samples (i.e., during a single sampling interval). The data from intervals with starting diameters ranging between 175-475 µm were pooled since it was shown that the oocyte growth rate during this phase of vitellogenesis was not significantly different (see Chapter 2). These data provided an estimate of the oocyte growth rate of females not sampled at exactly the beginning and end of vitellogenesis.
A portion of each oocyte sample obtained during trial 5 was placed into Bouin's solution (Humason, 1979) for histological preparation. The samples were fixed for a minimum of 24 hours, washed, dehydrated in punctilious ethanol, cleared in toluene, and embedded in methacrylate (Sorvall's embedding medium, Dupont). Glass knives mounted on a MT-1 ultramicrotome (Sorvall, Inc.) were used to obtain 1-3 um sections, which were stained with either Mallory's triple stain (Pantin Method) or hematoxylin and eosin.

For statistical comparisons, the data were subjected to Analysis of Variance (ANOVA) using the Minitab® Statistical Software Release 10 for Windows. Unless otherwise stated, numbers presented in the form of \( \# \pm \# \) are the mean ± the standard error (SE).

RESULTS

Trial 1

The first trial was initiated on April 26, 1988, which was shortly after the completion of the natural spawning season in Hawaii. The trial ended 10 weeks later on July 5, 1988. The environmental conditions and female size data are summarized in Table 3.1. A total of 48 females were used in this trial (12 per tank). By chance, the females in

<table>
<thead>
<tr>
<th>Treatment (photo/tem)</th>
<th>Photo (hrs)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>N</th>
<th>Length (cm)</th>
<th>Weight* (grams)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>short/cold</td>
<td>8L</td>
<td>20.9 ± 0.1</td>
<td>34.3 ± 0.2</td>
<td>12</td>
<td>44.0 ± 0.6</td>
<td>1281 ± 72</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>short/warm</td>
<td>8L</td>
<td>28.8 ± 0.2</td>
<td>34.0 ± 0.2</td>
<td>12</td>
<td>42.8 ± 0.9</td>
<td>1128 ± 70</td>
<td>1.42 ± 0.03</td>
</tr>
<tr>
<td>long/cold</td>
<td>16L</td>
<td>20.6 ± 0.1</td>
<td>34.3 ± 0.2</td>
<td>12</td>
<td>46.0 ± 0.8</td>
<td>1431 ± 81</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>long/warm</td>
<td>16L</td>
<td>30.5 ± 0.2</td>
<td>33.9 ± 0.2</td>
<td>12</td>
<td>43.7 ± 0.9</td>
<td>1176 ± 71</td>
<td>1.39 ± 0.02</td>
</tr>
</tbody>
</table>

* Female weight of the long/cold group was significantly higher than the other groups (P>0.05).
the long/cold treatment had a significantly higher weight than the females in the other 3 treatments (Table 3.1, \(P<0.05\)); however, since all of the females were sexually mature, this difference is not believed to have affected the outcome of the trial.

Oocyte samples were obtained on weeks 0, 4, 8, and 10. Figure 3.1 summarizes the results of the oocyte stage analysis. At the beginning of the trial, there was no significant difference in oocyte stage between treatments \((P>0.05)\). Approximately 50% of the females in each tank had stage I oocytes, while the other 50% had both stage I and stage II oocytes. At the end of the 10 weeks, all of the females in the long/warm treatment had only stage I oocytes, indicating that regression from stage II to stage I had taken place. In the other three treatments, there was no significant difference in oocyte stage between the week 0 and week 10. Two of the 12 females in the short/cold treatment had initiated stage III and had mean oocyte diameters of 175 \(\mu m\) and 544 \(\mu m\), respectively. One female in the short/warm treatment also initiated stage III and had a mean oocyte diameter of 175 \(\mu m\). All other females had either stage I or stage II oocytes. The results of this trial indicate that neither short photoperiod nor cold temperature had a significant stimulatory effect on oocyte development immediately after the end of the natural spawning season.

**Trial 2**

Trial 2 was initiated on July 5, 1988, during the middle of the off-season, and was completed 10 weeks later on September 15. At the start of this trial, all of the females had only stage I oocytes. Forty-eight females were used (12 per tank) and oocyte samples
Fig 3.1: Percentage of females having oocyte stages I - III during trial 1.
were obtained on weeks 0, 4, 8, and 10. There was no significant difference (P<0.05) in either length, weight or CFI between treatments (Table 3.2).

Figure 3.2 summarizes the oocyte stage analysis for this trial. None of the females in the long/warm treatment initiated either stage II or stage III during the 10 week trial. All remained in an immature state, having only stage I oocytes throughout the entire 10 weeks. In contrast, 25% of the females in the short/cold treatment had completed the cortical vesicle stage and had initiated vitellogenesis within the first 4 weeks. Four weeks later the number had increased to 75% and, by the end of the trial, 92% of the females had stage III oocytes in their samples. None of the females reached the COD by week 10; the mean diameters of stage III oocyte samples ranged from 246-535 μm. Females that had stage III oocytes by week 4 were the most advanced (451-535 μm). Only 1 stage II sample (female #420E on week 8) was obtained in this group. Under these conditions, females both initiated and completed stage II and initiated stage III in less than 4 weeks.

In the short/warm treatment, 67% of the females had progressed to stage II by week 8 while only 1 (8%) had progressed to stage III. By the end of the trial, 75% of the females were in stage II while only 17% (2) had initiated stage III. All but 1 of the females that had initiated stage II by week 8 were still in that stage 2 weeks later. In

Table 3.2

Environmental conditions and female size in trial 2.

<table>
<thead>
<tr>
<th>Treatment (photo/temp)</th>
<th>Photo (hrs)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>N</th>
<th>Length (cm)</th>
<th>Weight (grams)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>short/cold</td>
<td>8L</td>
<td>21.2 ± 0.1</td>
<td>32.6 ± 0.2</td>
<td>12</td>
<td>44.3 ± 0.9</td>
<td>1234 ± 95</td>
<td>1.39 ± 0.04</td>
</tr>
<tr>
<td>short/warm</td>
<td>8L</td>
<td>29.2 ± 0.2</td>
<td>32.3 ± 0.2</td>
<td>12</td>
<td>42.9 ± 1.1</td>
<td>1172 ± 73</td>
<td>1.47 ± 0.04</td>
</tr>
<tr>
<td>long/cold</td>
<td>16L</td>
<td>21.2 ± 0.2</td>
<td>32.5 ± 0.1</td>
<td>12</td>
<td>43.2 ± 0.8</td>
<td>1179 ± 61</td>
<td>1.45 ± 0.03</td>
</tr>
<tr>
<td>long/warm</td>
<td>16L</td>
<td>30.3 ± 1.2</td>
<td>32.1 ± 0.2</td>
<td>12</td>
<td>43.3 ± 0.7</td>
<td>1191 ± 56</td>
<td>1.46 ± 0.02</td>
</tr>
</tbody>
</table>
Fig 3.2: Percentage of females having oocyte stages I - III during trial 2.
comparison to the short/cold treatment, the rate of oocyte development was considerably slower under short/warm treatment. The majority of the females in the long/cold treatment remained in stage I throughout the duration of the trial. Three females (25%) progressed to stage II while only 1 female initiated stage III and reached a mean diameter of 524 μm. The results of this trial indicate that photoperiod rather than temperature is the main factor influencing the progression from stage I to stage II. Temperature, on the other hand, had a significant influence on the rate of oocyte development and, therefore, determined the duration of stage II and onset of stage III.

Trial 3

Trial 3 began on September 30, 1988, and was completed 10 weeks later on December 13. A total of 37 females were used (10 females in long/cold treatment, and 9 females in the other 3 treatments), all of which began the trial in stage II. Due to a lack in the availability of stage II females, 25 were stocked on September 30, while the remaining 12 were stocked 2 weeks later on October 14. As a result of this staggered start, samples were collected from each female at 2 week intervals for 8 rather than 10 weeks (i.e., the first fish stocked were sampled from September 30 to November 29, while the remainder were sampled from October 14 to December 13). All tanks had females stocked on both dates to insure uniformity in the sampling. There was no significant difference in the size or CFI of the females among treatments (P>0.05, Table 3.3).

Figure 3.3 summarizes the results of this trial. Sixty-seven percent of the females in the short/cold treatment progressed from stage II to stage III during the first two weeks of the trial, whereas all had initiated stage III by week 6. By week 8, thirty-three percent
Fig 3.3: Percentage of females having oocyte stages I - III during trial 3.
of the females had reached the COD. At this point, the mean oocyte diameter for all of the females in this treatment ranged from 243-686 μm (490 ± 49 μm). As in trial 2, the females with the largest mean oocyte diameters were those that had initiated stage III by week 2.

Table 3.3
Environmental conditions and female size in trial 3.

<table>
<thead>
<tr>
<th>Treatment (photo/temp)</th>
<th>Photo (hrs)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>N</th>
<th>Length (cm)</th>
<th>Weight (grams)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>short/cold</td>
<td>8L</td>
<td>21.2 ± 0.2</td>
<td>33.4 ± 0.3</td>
<td>9</td>
<td>41.8 ± 1.6</td>
<td>1096 ± 135</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>short/warm</td>
<td>8L</td>
<td>29.4 ± 0.1</td>
<td>33.3 ± 0.3</td>
<td>9</td>
<td>39.4 ± 1.4</td>
<td>911 ± 102</td>
<td>1.45 ± 0.02</td>
</tr>
<tr>
<td>long/cold</td>
<td>16L</td>
<td>21.2 ± 0.2</td>
<td>33.4 ± 0.3</td>
<td>10</td>
<td>43.7 ± 1.2</td>
<td>1294 ± 103</td>
<td>1.52 ± 0.03</td>
</tr>
<tr>
<td>long/warm</td>
<td>16L</td>
<td>30.6 ± 0.1</td>
<td>33.2 ± 0.3</td>
<td>9</td>
<td>42.6 ± 1.3</td>
<td>1211 ± 116</td>
<td>1.52 ± 0.03</td>
</tr>
</tbody>
</table>

None of the females in the long/warm treatment progressed to stage III during this trial. By week 8, 78% had only stage I oocytes in their samples indicating that these conditions either caused atresia of the stage II oocytes or resorption of the cortical vesicles in the stage II oocytes. In the other warm temperature treatment (short/warm), 44% of the females initiated stage III. Unlike the long/warm treatment group, females that did not initiate vitellogenesis did not regress to stage I but remained in stage II throughout the duration of the trial. Those that progressed to stage III had mean oocyte diameters ranging between 231-412 μm by week 8 (319 ± 37 μm), which was significantly smaller than the diameters of the short/cold group (P<0.05). In the other cold temperature treatment (long/cold), 90% of the females progressed from stage II to stage III. This response was similar to that of the short/cold treatment group and indicated that temperature had a more pronounced influence on the transition from stage II to stage III. Twenty percent of the females reached the COD by week 8 while the overall range in
mean oocyte diameters was 268-653 μm (526 ± 40 μm). Females with the largest oocyte
diameters had initiated vitellogenesis by week 2. The results from trial 3 were consistent
with those from trial 2 and indicated that temperature influenced the rate of oocyte
development and, as a result, the duration of stage II and the onset of stage III. The
results further confirmed that stage II oocytes regress to stage I under long photoperiod
and warm temperature conditions.

**Trial 4**

Trial 4 was conducted during the 10 week period between February 22, 1989, and
May 3, 1989. Thirty-two females were used (8 per tank). There was no significant
difference between treatments in the length, weight, or CFI of the females (P>0.05, Table
3.4).

<table>
<thead>
<tr>
<th>Treatment (photo/temp)</th>
<th>Photo (hrs)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>N</th>
<th>Length (cm)</th>
<th>Weight (grams)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>short/cold</td>
<td>8L</td>
<td>21.8 ± 0.3</td>
<td>31.6 ± 0.2</td>
<td>8</td>
<td>45.0 ± 1.4</td>
<td>1331 ± 128</td>
<td>1.43 ± 0.05</td>
</tr>
<tr>
<td>short/warm</td>
<td>8L</td>
<td>28.0 ± 0.2</td>
<td>31.3 ± 0.2</td>
<td>8</td>
<td>43.7 ± 1.5</td>
<td>1241 ± 108</td>
<td>1.50 ± 0.06</td>
</tr>
<tr>
<td>long/cold</td>
<td>16L</td>
<td>21.7 ± 0.1</td>
<td>30.4 ± 1.0</td>
<td>8</td>
<td>43.5 ± 0.9</td>
<td>1279 ± 71</td>
<td>1.55 ± 0.04</td>
</tr>
<tr>
<td>long/warm</td>
<td>16L</td>
<td>29.4 ± 0.1</td>
<td>31.1 ± 0.3</td>
<td>8</td>
<td>43.6 ± 0.7</td>
<td>1201 ± 65</td>
<td>1.44 ± 0.02</td>
</tr>
</tbody>
</table>

At the start of this trial, all of the females had stage III oocytes. There was no
significant difference among treatments with regard to oocyte size (P>0.05, 418 ± 57,
417 ± 61, 472 ± 30, and 387 ± 53 μm for the short/cold, short/warm, long/cold, and
long/warm groups, respectively). Sampling during the trial was conducted on 2 week
intervals.
The results are summarized in Fig. 3.4. By week 10, 75% of the females in the short/cold treatment still had stage III oocytes, which ranged in size from 521-678 µm. One of these females underwent atresia after reaching 644 µm on week 4, initiated a second clutch of oocytes between weeks 6-8, and reached 589 µm by week 10. Both females which did not have stage III oocytes at week 10 were in stage II. One of these had reached a mean diameter of 662 µm on week 8 before undergoing atresia during the last 2 weeks of the trial. The other female underwent atresia between weeks 6-8 after reaching 508 µm. All of the females in the long/warm treatment group underwent atresia within the first 4 weeks: 75% during the first 2 weeks, and the remaining 25% during the second 2 weeks. Fifty percent of the females had both stage I and II oocytes at week 2; however, by week 6, all of the females had only stage I oocytes. This suggests that stage III oocytes were resorbed first, followed later by stage II oocytes.

All of the females in the other warm temperature treatment (short/warm) also underwent atresia; 87% doing so by week 4. Unlike the long/warm treatment group, the majority remained in stage II through the end of the 10 weeks, which is also consistent with the results of trial 3. The fourth treatment group (long/cold) showed a response which was intermediate between that of the short/cold and short/warm treatment groups. By week 6, 75% of the females still had stage III oocytes, however, by week 8, this number had dropped to 25%. The 2 females that did not undergo atresia had mean oocyte diameters of 671 and 715 µm. Atresia took place both in females with large mean oocyte diameters (#7A3E, 640 µm on week 4) as well as in females with smaller mean diameters (#1F2C, 286 µm on week 6). Furthermore, all of the females that underwent
Fig 3.4: Percentage of females having oocyte stages I - III during trial 4.
atresia had both stage I and stage II oocytes at the end of the trial. These results of this trial suggest that both warm temperature and long photoperiod bring about atresia in stage III oocytes, the former in 4 weeks while the latter in 8 weeks. Warm temperature and long photoperiod combined brought about atresia within 2 weeks. The results also suggest that both cold temperature and short photoperiod delayed or prevented the resorption of stage II oocytes.

Trial 5

Trial 5 started on June 22, 1989, and ended after 10 weeks on August 31, 1989. Similar to trial 4, 32 females were used (8 per tank) who ranged in length from 37.1 cm to 48.3 cm and weight from 699 grams to 1,402 grams. There was no significant difference between treatments in either length, weight, or CFI (P>0.05, Table 3.5).

<table>
<thead>
<tr>
<th>Treatment (photo/temp)</th>
<th>Photo (hrs)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>N</th>
<th>Length (cm)</th>
<th>Weight (grams)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>short/cold</td>
<td>8L</td>
<td>21.9 ± 0.2</td>
<td>32.3 ± 0.1</td>
<td>8</td>
<td>43.7 ± 0.7</td>
<td>1096 ± 50</td>
<td>1.31 ± 0.05</td>
</tr>
<tr>
<td>short/warm</td>
<td>8L</td>
<td>28.9 ± 0.1</td>
<td>32.3 ± 0.1</td>
<td>8</td>
<td>42.8 ± 1.5</td>
<td>1073 ± 83</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>long/cold</td>
<td>16L</td>
<td>21.0 ± 0.2</td>
<td>32.4 ± 0.1</td>
<td>8</td>
<td>44.2 ± 0.7</td>
<td>1140 ± 38</td>
<td>1.33 ± 0.05</td>
</tr>
<tr>
<td>long/warm</td>
<td>16L</td>
<td>30.5 ± 0.1</td>
<td>32.3 ± 0.2</td>
<td>8</td>
<td>42.2 ± 0.9</td>
<td>984 ± 46</td>
<td>1.31 ± 0.04</td>
</tr>
</tbody>
</table>

This trial was essentially a replicate of trial 2, but was started 2 weeks earlier in the year. All of the females had only stage I oocytes when stocked into the tanks and sampling was conducted at 2 week intervals. Figure 3.5 shows the results which are similar to those of trial 2. Seventy-five percent of the females in the short/cold treatment had initiated stage III by week 10 while none of the females in either the long/warm or the long/cold treatment groups had initiated stage II or III. Fifty percent of the females in the
Fig 3.5: Percentage of females having oocyte stages I - III during trial 5.
short/warm treatment group initiated stage II; however, none had progressed to stage III. Females in the short/cold treatment group progressed from stage I to III in less than 4 weeks, and by week 10 had mean oocyte diameters ranging between 175 μm to 580 μm. None of the females had reached the COD by the end of the trial. In general, the response to the treatments in this trial was slower than the response in trial 1. In both the short/cold and the short/warm treatment groups, a smaller percentage of females had progressed beyond stage I by week 8. Furthermore, none of the females in the long/cold treatment progressed beyond stage I in this trial. Since trial 5 was started earlier than trial 1, some of the females may still have been in a refractory state when stocked into the tanks.

Histological examination of samples taken from the short/cold and long/warm groups provided additional detail on the effects of photoperiod and temperature on oocytes in stage I. In general, the process of cannulation removed portions of intact ovarian lamellae containing various sizes of stage I oocytes. The smallest of these were typically located along the outer edge of the lamellae while the larger were located toward the interior of the lamellae (Fig. 3.6). Many smaller oocytes could not be distinguished as chromatin-nucleolar or peri-nucleolar stages due to the amount of distortion which resulted from histological preparation. The larger oocytes were much less distorted and could be identified clearly as being in the peri-nucleolar stage.

In the short/cold group, a consistent sequence of intracellular changes was observed as the oocytes progressed to stage II (Fig. 3.7). At the start of the trial, 6 of the 8 females were at the stage shown in Fig 3.7a. The larger stage I oocytes were characterized by relatively uniform cytoplasmic staining, a smooth nuclear membrane, and
Fig. 3.6: Histological section of stage I oocytes showing their typical arrangement within ovarian lamellae. Scale bar = 200 μm.
Fig. 3.7: Histological sections of oocyte samples taken from females in the short/cold group. a) week 0, b) week 2, c) week 4, d) week 6. yn = yolk nucleus, gv = germinal vesicle, od = oil droplet, cv = cortical vesicle. Scale bar = 50 μm.
the yolk nucleus of Balbiani’s vitelline body located adjacent to the nucleus (i.e., juxtanuclear). As in a previous report (Abraham et al., 1968), the yolk nucleus was present in only a relatively small number of oocytes per section. When sampled 2 weeks later, the oocytes in all six females still showed a juxtanuclear yolk nucleus and smooth nuclear membrane; however, the cytoplasm had assumed a heterogeneous appearance (Fig. 3.7b). Many of these oocytes sectioned poorly and showed distortions similar to the smaller stage I oocytes. Two of the 8 females had oocytes with heterogeneous cytoplasm when initially sampled on June 22. Two weeks later at week 4 of the trial, the oocytes in 1 of these females had the same appearance whereas the other had oocytes which had uniformly staining cytoplasm. Therefore, in 75% of the females, the first visible effect of short photoperiod and cold temperature was the appearance of some type of material in the cytoplasm which gave the oocytes a heterogeneous appearance by week two.

On week 4, 3 females still had oocytes with heterogeneous cytoplasm while 5 had oocytes with a uniform appearance. The yolk nucleus had begun to migrate in four of the latter females (Fig. 3.7c). The process of migration lasted from less than 2 weeks to more than 4 weeks and was accompanied by a noticeable increase the size of the oocyte. In addition, the nuclear membrane became more irregular in appearance. The final transition from stage I to stage II was characterized by the appearance of oil droplets surrounding the nucleus, which now had a distinctly irregular membrane, cortical vesicles which appeared to be forming near the periphery of the cytoplasm, and the disappearance of the yolk nucleus (Fig. 3.7d).
In contrast to the short/cold group, none of these changes were observed in the long/warm group. Seven of the 8 females had oocytes with uniform cytoplasm when the trial was initiated on June 22. There was no noticeable change in the appearance of these oocytes throughout the trial (Figs. 3.8a-d). In August, the oocytes were the same size, the cytoplasm was uniform, the nuclear membrane was smooth, and the yolk nucleus was still in a juxtanuclear position. The eighth female had oocytes with heterogeneous cytoplasm at the start of the trial. After 2 weeks, the cytoplasm had become uniform; however, the yolk nucleus remained in a juxtanuclear position. The oocytes in this female continued in that state throughout the remainder of the trial.

Progression from stage I to stage II in the short/warm group followed the same pattern as that observed in the short/cold group. Two of the four females in this group that did not reach stage II by the end of the trial progressed to the point where the yolk nucleus was migrating. None of the females in the long/cold group progressed to stage II by the end of the trial. However, by August 31, all but 1 of the females had oocytes with migrating yolk nuclei suggesting that oocyte development was slowed but not arrested under an inhibitory photoperiod coupled with a stimulatory temperature.

Tables 3.6 and 3.7 summarize the average and interval oocyte growth rate data obtained during this study, respectively. Since the rates were calculated from stage III oocyte samples, and since the environmental conditions in each treatment were not significantly different among trials (P<0.05), the data from individual trials were pooled. Average oocyte growth rate data were obtained from only 7 females, all of which were in either the short/cold or the long/cold treatments in trials 3 and 4. There was no significant
Fig. 3.8: Histological sections of oocyte samples taken from females in the long/warm group. a) week 0, b) week 2, c) week 4, d) week 6. yn = yolk nucleus, gv = germinal vesicle. Scale bar = 50 μm.
difference in the rates between these 2 treatments, which averaged 10.6 µm/day and 11.0 µm/day (P > 0.05). The short/cold treatment had the higher interval oocyte growth rates (9.2 µm/day), followed by the long/cold treatment (8.0 µm/day), and the short/warm treatment (4.4 µm/day). As with the average oocyte growth rate data, there was no significant difference between the cold temperature treatments (P > 0.05). However, the interval oocyte growth rate under the short/warm treatment was significantly lower than that observed under both the short/cold and long/cold treatments (P < 0.05). Minimum and maximum values indicate that there was a considerable range of growth rates within treatments. These data suggest that temperature had a greater influence than photoperiod on the rate of oocyte growth during vitellogenesis.

**Table 3.6**

Average oocyte growth rates under controlled environmental conditions.
(Combined data from all five trials)

<table>
<thead>
<tr>
<th>Treatment (photo/temp)</th>
<th>N</th>
<th>Initial Diameter (µm)</th>
<th>Final Diameter (µm)</th>
<th>Growth Period (days)</th>
<th>Oocyte Growth Rate (µm/day) Mean ± SE</th>
<th>Min</th>
<th>Max</th>
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<tbody>
<tr>
<td>short/cold</td>
<td>5</td>
<td>195 ± 12</td>
<td>638 ± 14</td>
<td>39 ± 2</td>
<td>11.0 ± 0.7</td>
<td>9.6</td>
<td>13.5</td>
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<td>long/cold</td>
<td>2</td>
<td>175 ± 0</td>
<td>646 ± 7</td>
<td>40 ± 1</td>
<td>10.6 ± 0.2</td>
<td>10.4</td>
<td>10.8</td>
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**Table 3.7**

Interval oocyte growth rates under controlled environmental conditions.
(Combined data from all five trials)

<table>
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<tr>
<th>Treatment (photo/temp)</th>
<th>N</th>
<th>Initial Diameter (µm)</th>
<th>Final Diameter (µm)</th>
<th>Growth Period (days)</th>
<th>Oocyte Growth Rate (µm/day) Mean ± SE</th>
<th>Min</th>
<th>Max</th>
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</thead>
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<tr>
<td>short/cold</td>
<td>55</td>
<td>294 ± 14</td>
<td>430 ± 15</td>
<td>15 ± 0.5</td>
<td>9.2 ± 0.5</td>
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<td>short/warm</td>
<td>14</td>
<td>304 ± 33</td>
<td>566 ± 29</td>
<td>14 ± 0.2</td>
<td>4.4 ± 0.7</td>
<td>0.9</td>
<td>8.6</td>
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<tr>
<td>long/cold</td>
<td>31</td>
<td>319 ± 20</td>
<td>435 ± 19</td>
<td>15 ± 0.2</td>
<td>8.0 ± 0.6</td>
<td>0.3</td>
<td>13.9</td>
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</table>
DISCUSSION

Bye (1990) said that considerable individual variation in responses has complicated studies on the environmental regulation of fish reproduction. However, while individual responses did vary to some degree in the present study, the effects of the four different combinations of photoperiod and temperature on different stages of oocyte development were unambiguous. An eight hour daylength coupled with a temperature averaging between 21.2-21.9 °C was clearly stimulatory, while a 16 hour daylength and temperatures averaging 28.0-30.6 °C was clearly inhibitory. An eight hour daylength stimulated the progression from stage I (primary growth) to stage II (cortical vesicle stage) in both cold and warm temperatures, indicating that photoperiod was the principle environmental cue for that stage of oocyte development. Cold temperatures combined with either a short or long photoperiod stimulated the progression of stage II to stage III (vitellogenesis). It therefore seems that once the oocytes reach stage II, temperature becomes the more influential factor. Finally, once the oocytes had reached stage III, cold temperature was clearly more effective than short photoperiod in sustaining vitellogenesis while warm temperature was more effective than long photoperiod in causing atresia. In short, the annual ovarian cycle of M. cephalus is regulated by both photoperiod and temperature, with each factor exerting its effect at different stages of oocyte development.

After reviewing the extensive bibliographies presented in Pillay (1972) and Alvarez-Lajonchere (1974) in addition to conducting a search of more recent literature, only 1 other experimental study could be found which examined the effects of controlled photoperiod and temperature on M. cephalus oocyte development. Kuo et al., (1974a)
conducted a series of experiments using a short photoperiod (6L) in combinations with temperatures ranging between 17-26°C. Because the intent was to develop year round egg production techniques, the photoperiod and temperatures used were all within a stimulatory range and, therefore, the females in all of the treatments matured. While the experimental design was not conducive for determining the respective roles of photoperiod and temperature, these authors inferred from their results that both factors had a regulatory function, with the former initiating oocyte development and the latter “regulating vitellogenesis toward functional maturity”. The results of this study offer the necessary proof for that general conclusion.

Previtellogenic stages in salmonids, sticklebacks, and minnows appear to be independent of environmental control (reviewed by Lam and Munro, 1987). This does not appear to be the case in M. cephalus. The histological analysis of samples collected during trial 5 showed that depending on the conditions, the progression from primary growth to the cortical vesicle stage (i.e., stage I to II) can be slowed or even completely arrested. The earliest recognizable event influenced by photoperiod and temperature was the migration of the yolk nucleus of Balbiani’s vitelline body. This is the earliest event during oocyte development in any animal shown to be influenced by environmental factors.

The yolk nucleus is composed of RNA and protein and is believed to be derived from one or more nucleoli in the germinal vesicle (Guraya, 1979). The material making up this structure is apparently extruded into the cytoplasm where it complexes with mitochondria, dictyosomes, and lipid bodies to form the entire Balbiani vitelline body.
The yolk nucleus and associated components subsequently migrate away from the germinal vesicle and disperse once they reach the cortical ooplasm (Guraya, 1979). Dispersal generally takes place prior to, or at the beginning of vitellogenesis; however, it does not appear to be involved in yolk formation (Riehl, 1976, in Guraya, 1979). While its function is still unknown, the identification of elongation factor 1α (EF-1α) in Balbiani’s vitelline body of *Xenopus laevis* oocytes indicates that it is a site of protein synthesis (Viel *et al.*, 1990).

Guraya (1963) observed the formation of “basophilic masses” composed of RNA derived from the yolk nucleus in the teleost, *Channa marulius*. These masses appeared, then subsequently disappeared, during yolk nucleus migration as the material became uniformly distributed throughout the cytoplasm. Abraham *et al.* (1968) also described the formation of a basophilic reticulum in the cytoplasm during yolk nucleus migration of *M. cephalus* oocytes. In the present study, migration of the yolk nucleus was preceded by an alteration in the staining patterns of the cytoplasm, which seems to be the same phenomenon. As with the actual movement of the yolk nucleus, the formation of this material, which gave the cytoplasm a heterogenous appearance, was stimulated by the short/cold treatment and inhibited by the long/warm treatment.

This study also provided the growth rates of oocytes undergoing vitellogenesis in the various treatments. Bye’s (1990) statement mentioned at the beginning of this discussion certainly applies to this aspect of *M. cephalus* oocyte development: oocyte growth rates within the same treatment showed considerable individual variation. However, by comparing the average and interval and interval values for the various
treatments, it is clear that temperature has a greater effect than photoperiod on the rate of vitellogenesis. Cold temperatures produced significantly faster oocyte growth rates in comparison to warm temperatures. Chapter 2 provided oocyte growth rate data for *M. cephalus* females held under natural photoperiod and temperature conditions. For females which completed vitellogenesis, the average rate was 6.5 μm/day (photoperiod = 10.9–11.3 hrs, temperature = 24.7-26.4 °C) while rates for intervals starting from 175-475 μm ranged between 5.7-7.2 μm/day (photoperiod = 10.9-12.1 hrs, temperature = 23.7-27.0 °C). These rates, as well as the temperatures under which they occurred, were intermediate between those of the cold and warm temperature groups in this study. Chapter 2 also showed that females that underwent atresia before completing vitellogenesis had lower average and interval rates of 3.9 μm/day and 2.0-7.0 μm/day, respectively. Atresia of stage III oocytes was common in the warm temperature treatments in this study. Whether caused by temperature or some other factor, atresia is likely to occur when the oocyte growth rate of a female drops below 4 μm/day.

Bye (1990) stated that once in progress, gametogenesis is relatively insensitive to environmental manipulation. In this study, however, females responded to either stimulatory or inhibitory treatments regardless of whether their oocytes were in stage I, II, or III. Since stage II is stimulated by photoperiod which has a relatively constant cycle year after year, the initiation of this stage is also relatively constant year after year (see Chapter 2). The onset and duration of stage III, however, is significantly influenced by temperature which has a more variable cycle and, as a consequence, is also more variable.
(see Chapter 2). Year to year variation in ovarian development is undoubtedly the result of continuous sensitivity rather than insensitivity to environmental conditions.

Grant and Spain (1975) studied the spawning season for *M. cephalus* in North Queensland, Australia. At this location (19°S latitude), the photoperiod (11-13.25 hrs, estimated from List, 1984) and temperature (21-33 °C) cycles are similar to those in Hawaii but have opposite phases since it is located in the southern hemisphere. These authors found that the reproductive season occurred from May through August which is exactly reverse that of Hawaiian *M. cephalus*. This is as expected if oocyte development in this population was regulated in a manner consistent with the experimental results obtained here.

A comparison of the reproductive seasons of populations in the same hemisphere, however, produces a more complicated picture. Figure 3.9 summarizes reports of the spawning seasons for wild *M. cephalus* populations in the northern hemisphere, and was derived primarily from the review by Brusle (1981). The spawning season from Sri Lanka was obtained from Silva and DeSilva (1981). Arranging the seasons according to latitude shows that, in general, *M. cephalus* spawns during the summer in higher latitudes (40-45 °N) but during the winter in lower latitudes (15-30 °N). This change occurs even though a) the photoperiod cycles at these locations are all in the same phase, b) the photoperiod cycles have the same inflection points and therefore the same direction throughout the year, and c) any threshold value, such as 12.5 hours of light, is reached earlier in lower latitudes than in higher latitudes (calculated from List, 1984).
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Fig. 3.9: Summary of *M. cephalus* spawing seasons in the northern hemisphere. Data obtained from Brusle (1981) and Silva and DeSilva (1981).
Photoperiod alone could account for this change only if there was a complete reversal in responsiveness (i.e., long photoperiod stimulates the initiation of stage II in higher latitudes, while short photoperiod stimulates the initiation of stage II is lower latitudes). Baggerman (1980) reported that male and female sticklebacks, *G. aculeatus*, matured in response to 16 hrs of light from September to November, but responded to both 8 and 16 hrs of light from January to April. These observed changes in responsiveness are the basis of her photoreactivity threshold hypothesis. However, sensitivity did not reverse in that study since 16 hrs of light remained stimulatory in the spring. Furthermore, similar changes in responsiveness were not observed in the present study, nor have they been observed in any other studies of which this author is aware.

At first glance, it also seems difficult to explain the change on the basis of temperature cycles since the reproductive season coincides with increasing temperatures in higher latitudes but decreasing temperatures in lower latitudes. The change makes sense, however, if oocyte development in *M. cephalus* can only occur within an optimal range, a possibility which is supported by a number of studies. First, this study clearly showed that temperatures above 28 °C were inhibitory. Secondly, while experimental evidence is lacking, it is also likely that oocyte development is either slowed or arrested at temperatures below 15 °C. Kuo *et al.* (1974a) found that at 17 °C, yolk deposition decreased in comparison with 21 or 26 °C, which negatively affected egg quality. Thirdly, reports which correlated water temperature data with the reproductive season indicate that oocyte development in the wild is occurring at a range of 15-27 °C (Apekin and Vilenskaya, 1979; Grant and Spain, 1975; Moore, 1974).
Throughout the year, temperature measurements from natural *M. cephalus* habitats are known to range from 5-35 °C whereas lethal tolerance estimates have been placed at 4 °C and 37 °C (Kulikova *et al.*, 1986; Moore, 1974). In the Black Sea, the temperature ranges between 5-27 °C (Apekin and Vilenskaya, 1979, Kulikova *et al.*, 1986). At this location, the reproductive season begins in May when the temperature increases to 15.9-19.5 °C, continues through the summer as the temperature peaks at 27 °C, and ends in September-October as the temperature drops to 16 °C. In lower latitude locations such as Hawaii, the reproductive season begins in the Fall when the temperature drops below 27 °C, continues through the winter when the temperature reaches a low of 21 °C, and ends in March-April when the temperature increases again to 27 °C. It is therefore hypothesized, that temperature, rather than photoperiod, is the main factor responsible for the variation in *M. cephalus* spawning seasons within the northern hemisphere. The offseason in higher latitudes results from temperatures dropping below the lower limit of the optimal range for vitellogenesis while the offseason in lower latitudes results from temperatures rising above the upper limit of the range.

While this hypothesis explains the observed change in spawning seasonality within the northern hemisphere, it has not addressed the onset and duration of stage II at these locations. The annual appearance of this stage outside of Hawaii has not been well documented. In Israel, Abraham (1963) and Abraham *et al.* (1966) found vacuolated oocytes (which were interpreted to be stage II) in August and September through December, respectively.
For the purpose of stimulating studies on this aspect, it is hypothesized that stage II is initiated in response to decreasing photoperiod (i.e., late summer/early fall) at all latitudes in the northern hemisphere. In lower latitudes, stage III follows shortly after stage II, with the oocytes reaching a spawnable size within 1-4 months, depending on the temperature. At higher latitudes, the oocytes either proceed to early stage III or remain in stage II before being arrested as a result of decreasing temperatures. The oocytes remain in that stage until the temperature increases again in the spring, and reach a spawnable size during the summer. This latter strategy which involves an initiation phase, a dormant phase, and a continuation and completion phase is commonly found in other species of temperate water fish including sticklebacks, salmonids, carps, gobies, surfperches, dabs, turbots, soles, and sea basses (Baggerman, 1990; Bye, 1990; Hontela and Stacey, 1990; Scott, 1990; and Taylor, 1990).

An alternative hypothesis to explain the reproductive seasonality observed in higher latitudes is suggested by the results of trial 2 and the histological analysis of trial 5: that temperature alone is stimulating the onset of stage II in late spring/early summer. In both trials, oocyte development from stage I to stage II seemed to be taking place in the long/cold group, but at a slower rate than either the short/cold or the short/warm groups. It appears that the majority of females in this group would have been in stage II if the trials had been extended for an additional 2-4 weeks. In higher latitudes, the onset of stage II would have to be stimulated by the increase in temperature above 15 °C in Spring. Further experimentation should be conducted to investigate this possibility.
This study also suggests that the duration of stage II is dependent on the relationship between the photoperiod and temperature cycles. Stage II had a shorter duration in females in the short/cold group in comparison to females in the short/warm group. Under natural conditions, stage II also appeared to have a longer duration during the warmest of three years and a shorter duration during the coldest (see Chapter 2).

It has been suggested that photoperiod is a more important environmental regulator than temperature, with the latter acting only as a rate modifier (reviewed by Bye, 1987). In Hawaiian *M. cephalus*, however, temperature plays a prominent role in defining the annual reproductive cycle in females and may be the key to understanding the variation in *M. cephalus* reproductive seasons throughout the world. Furthermore, temperature tolerance ranges for reproduction may be an important factor determining the geographic distribution of fish species. For example, species with tolerance ranges of 10-20 °C would be primarily restricted to temperate latitudes whereas species with tolerance ranges of 25-35 °C would be restricted to tropical latitudes. However, species such as *M. cephalus*, which have an intermediate range of 15-27 °C, could succeed in both temperate and tropical latitudes by spawning in the summer in higher latitudes and winter in lower latitudes. Therefore, determining the temperature tolerance range for reproduction in a species may provide important clues to understanding its ecology.

Finally, the results of this study have already been used to develop a technique for inducing year-round spawning in Hawaiian *M. cephalus* (unpublished data). In general, understanding the environmental control of reproduction can lead to the achievement of
continuous egg production, an important step toward realizing the aquaculture potential of a species.
CHAPTER 4

THE EFFECTS OF MELATONIN ON OOCYTE DEVELOPMENT IN
MUGIL CEPHALUS

ABSTRACT

This chapter describes the results of two experiments designed to test the effects of chronic melatonin (MLT) treatment on oocyte development in the striped mullet, Mugil cephalus. The first experiment examined the effects of continuous administration of MLT. Females were implanted with silastic capsules containing 60 mg of MLT and placed under either natural, inhibitory (16 hrs of light), or stimulatory (8 hrs of light) photoperiod conditions. In the second experiment, females were placed under inhibitory photoperiod (14 hrs of light) and were fed a pelleted diet which contained 30 mg of MLT/kg of feed. The feed was provided after 10 hours of light each day (i.e., 3 pm) in an effort to simulate the endogenous MLT profile of a stimulatory 10 hour day.

The results of the first experiment indicated that MLT capsules had neither an inhibitory nor stimulatory effect on oocyte development. Implanted females showed the same response as control females to all 3 photoperiod conditions. The MLT feed provided in the second experiment also failed to stimulate oocyte development under an inhibitory photoperiod. Both MLT feed and implants have been used in mammals to advance the reproductive cycle of short-day breeders. M. cephalus is a short-day teleost fish and preliminary experiments have shown that both MLT implants and feed significantly increased the serum level of MLT in this species. Potential reasons for the failure of MLT treatments to advance the reproductive cycle in M. cephalus are discussed.
INTRODUCTION

Most animals living in temperate or higher latitudes reproduce on a seasonal basis (Sumpter, 1990) and depend upon environmental factors to time the production of eggs and sperm. Photoperiod and temperature are the two most common cues, particularly the former due to the year after year consistency in its cycle at any given location. Recent studies have provided evidence that melatonin (MLT) may transduce photic information and thereby serves as an internal zeitgeber in vertebrates (reviewed by Cassone, 1990). MLT is synthesized at night primarily by the pineal gland (reviewed by Arendt, 1995). The duration of darkness determines the duration of MLT synthesis each day, which appears to be the most important aspect of this signal with regard to its effect on reproduction and other physiological processes (Williams and Helliwell, 1993). In mammals, when the daily exposure is increased by administration of exogenous MLT, reproduction is generally inhibited in long-day breeders and stimulated in short-day breeders (Lincoln, 1983).

Due to the impracticality of maintaining large farm animals under controlled environmental conditions, a variety of techniques have been developed to administer exogenous MLT for the purpose of simulating a physiological short day (Staples et al., 1992). These include oral introduction through MLT treated feed (Kennaway and Seamark, 1980; Kennaway et al., 1982), daily injections (Bittman, 1984), and continuous release implants (Staples et al., 1992). Feed and injections were timed to extend the duration of the MLT signal whereas implants were originally intended to mask the signal.
Recent studies, however, have suggested that implants appear instead to produce a short-day signal (Lincoln and Ebling, 1985; O’Callaghan et al., 1991).

In fish, it has been shown that MLT is synthesized from both the pineal gland and the retina (Gern et al., 1978; Falcon et al., 1987, Kezuka et al.; 1991, Kezuka et al., 1992) with the duration of synthesis dependent on the duration of the scotophase (Kezuka et al., 1988). However, the relationship between MLT and reproduction in fish has not been investigated to the extent it has in mammals. Most studies have examined the effects of pinealectomy on either gonad size, secondary sex characteristics, oviposition, pituitary gonadotrophs, or serum gonadotropin levels (see review by Ralph, 1978, also, Hontela and Peter, 1980, Day and Taylor, 1983). Less emphasis has been placed on testing the effects of exogenous MLT. Injections of MLT have reportedly inhibited the seasonal increase in gonadal size or oocyte development in the goldfish, Carassius auratus (Fenwick, 1970), the Japanese killifish, Oryzias latipes (Urasaki, 1972), the killifish, Fundulus similis (deVlaming et al., 1974), the catfishes, Heteropneustes fossilis (Sundararaj and Keshavanath, 1976), and Mystus tengara (Saxena and Anand, 1977), and the three spined stickleback, Gasterostens aculeatus (Borg and Ekstrom, 1981). All of these species could be considered long-day breeders since they generally spawn in the spring or summer in the northern hemisphere and respond positively to long photoperiod regimes.

The objective of the present study was to investigate the effects of exogenous MLT on oocyte development in a short-day fish. Two experiments were conducted on the striped mullet, Mugil cephalus. The first experiment examined the effects of implanting
chronic release MLT capsules. The second experiment examined the effects of afternoon administration of MLT feed.

MATERIALS AND METHODS

Both experiments were conducted at The Oceanic Institute, Waimanalo, Hawaii, USA. The first took place between June 3 and October 7, 1991, in two outdoor 35 m$^3$ cement rectangular tanks and two indoor 8 m$^3$ liter fiberglass round tanks. One outdoor tank was exposed to natural photoperiod while a moveable black plastic hood was fitted onto the other to reduce the length of the photoperiod to 8 hrs. Both indoor tanks were fitted with black plastic covers, fluorescent lighting (345 lux), and 24 hr timers. The photoperiod on one of the tanks was maintained at 16 hrs of light (i.e., long photoperiod) while the other was maintained at 8 hrs of light (i.e., short photoperiod). All four tanks were continuously aerated and a flowthrough seawater system provided a water exchange of approximately 80-100% per day. Both morning and afternoon water temperature and salinity were measured 3 days per week using a mercury thermometer and an Aquafauna refractometer (Biomarine Inc.).

The tanks were stocked with 107 adult female *M. cephalus* with each tank having an approximate stocking density of 0.7-0.8 fish/m$^3$. Purina trout chow was provided as a feed once a day at a rate of 2-5% of the body weight. Individual females were identified by a Personal Identification Transponder, or Pitttag (ID Systems Incorporated) located in the musculature just below the dorsal fin. The length and weight of each fish were obtained at the start of the experiment using a 1 meter length board and a top loading
balance. The condition factor index (CFI) was calculated using the formula: length (cm)/weight (g)\(^3\) x 100.

Table 4.1 provides the design of the experiment which was intended to detect either inhibitory or stimulatory responses to the treatments.

<table>
<thead>
<tr>
<th>Location</th>
<th>Tank (lit)</th>
<th>Photoperiod</th>
<th>Implants</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outdoors</td>
<td>35,000</td>
<td>Natural</td>
<td>60 mg MLT</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Short (8 hrs)</td>
<td>60 mg MLT</td>
<td>5</td>
</tr>
<tr>
<td>Indoors</td>
<td>8,000</td>
<td>Long (16 hrs)</td>
<td>60 mg MLT</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Short (8 hrs)</td>
<td>60 mg MLT</td>
<td>5</td>
</tr>
</tbody>
</table>

Five females from each of the four tanks were implanted with 60 mg of melatonin (MLT) in the form of four 15 mg silastic capsules. Preliminary experiments to determine the \textit{in vitro} and \textit{in vivo} release rates of these capsules are presented in Appendix IV. To make the capsules, 2.4 cm lengths of silastic tubing (0.058 in. ID, 0.077 in. OD, Dow Corning) were capped on one end with medical grade elastomer (Dow Corning). Using a pipettor, 15 mg of melatonin purchased from Sigma Chemical were loaded into each piece of tubing. The open end of the tubing was then capped with elastomer to complete each capsule.

To minimize stress, each treatment fish was implanted with two of the four capsules on June 3, while the remaining two capsules were implanted 1 week later. The females were first captured from the tanks with the use of a seine net and transferred to a
small anesthesia tank containing 200 ppm of 2-phenoxyethanol in seawater. Once
anesthetized, the capsules were implanted into the fish by first removing a single scale on
the dorsal surface of the body and making a small incision in the underlying dermis. A
trochar was then inserted into the incision and the capsules were expelled in the
musculature. At the same time, a sample of oocytes was obtained from each female
according to the method of Shehadeh et al. (1973). A 30-50 cm long polyethylene tube
having an outer diameter of 1.52 mm and an inner diameter of 0.86 mm was inserting into
the gonoduct for a distance of approximately 10-12 cm. A sample of oocytes was then
removed by suction and examined under a microscope to determine the stage of
development according to the descriptions of Kuo et al. (1974b). After June 3, all
females were sampled at two week intervals until the end of the experiment.

The second experiment was initiated on June 18, 1992, and was completed 12
weeks later on September 10. The experimental design is shown in Table 4.2. Twenty-six
females and 30 males were divided into two equal groups of 13 females and 15 males.

Table 4.2
Design of the melatonin feed experiment.

<table>
<thead>
<tr>
<th>Photoperiod</th>
<th>Treatment</th>
<th>Replicate</th>
<th># Females</th>
<th># Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long (14 hrs)</td>
<td>MLT Feed</td>
<td>1</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Control Feed</td>
<td>1</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Since the topic of this dissertation is oocyte development, only the data obtained from
females will be presented in this chapter. One group served as the treatment group and
the other as the control. For the purpose of replication, each group was stocked into 2
indoor tanks of the same type as described above. The photoperiod timers on all tanks were set to 14 hrs (0600-2000 hrs) which is approximately 30 minutes longer than the peak offseason daylength under natural conditions in Hawaii.

The fish were fed Purina trout chow (#5 pellet) at a rate of 1% of their body weight per day. The control tanks received untreated feed while the MLT feed tanks received feed containing a concentration of 30 mg MLT/kg of pellets. The results of preliminary experiments carried out to determine the appropriate dosage of MLT are presented in Appendix D. To prepare the MLT feed, 30 mg of MLT were dissolved in 30 ml of punctilious ethanol and sprayed onto 1 kg of chow with an atomizer. The chow was then dried in a fume hood and frozen until use. Feeding was carried out once a day at 3 pm, at which time a screen was lowered over the central standpipe in each tank to prevent the pellets from flowing out the drain. The screens were left in place for 1 hour then removed at 4 pm.

All females were measured, sampled, and staged on June 18 as described for the first experiment. Oocyte samples were subsequently taken and staged at 3 week intervals.

Blood samples were taken during both experiments in order to evaluate whether the implants and feed were releasing MLT into the circulatory system. The fish were captured and anesthetized as described above. A 3 cc syringe with a 20 gauge needle was inserted into the caudal vein of each fish and 2 ml. of blood were removed. The blood was placed in a glass test tube and allowed to clot for 1-2 hours. The samples were then centrifuged at 3000 rpm for 20 minutes and the serum removed and stored in an ultracold freezer (-85 °C) for later analysis by MLT radioimmunoassay (RIA). In the first
experiment, blood samples were obtained between 10 am and 2 pm on July 15, 1991 (6 weeks after implantation), from all of the fish in both indoor tanks and from 10 fish (5 control and 5 implanted) from each outdoor tank. On the last day of the second experiment (September 10), blood samples were obtained from 10 fish (5 controls and 5 MLT feed) at each of the following times: 1600 hrs, 2000 hrs, 2400 hrs, 0400 hrs, 0800 hrs. The 2400 and 0400 hr samples were obtained in the dark without exposing the fish to light.

The MLT RIAs were performed in Dr. K. Aida’s laboratory in Tokyo University, Japan. The procedures have been previously reported in Maeda et al. (1984), and Kezuka et al. (1988), who validated the RIA for use in M. cephalus. Additional experiments to validate the RIA for use in this species are described in Appendix D. Melatonin was extracted from the serum and dried with the use of a Sep-Pak C18 cartridge (Waters Associates) and nitrogen gas. The extract was then redissolved in 500 μl of 20 mM gel-PBS prior to performing the RIA.

For statistical comparison of the treatments in both experiments, the data were subjected to analysis of variance (ANOVA) using Minitab® Statistical Software Release 10 for Windows. Unless otherwise stated, numbers presented in the form of # ± # are the mean ± the standard error (SE).

RESULTS

Experiment 1: Melatonin Implants

Table 4.3 summarizes the data on water quality and female size in experiment 1. There were no significant differences among treatments with respect to any of these
factors (P>0.05). Since lowering salinity is a common treatment for external parasites of marine fishes, the salinity in both outdoor tanks was maintained at 22 ppt. For consistency, the salinity was also reduced in the indoor tanks.

Table 4.3

<table>
<thead>
<tr>
<th>Location/Photo</th>
<th>Treatment</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>Length (cm)</th>
<th>Weight (g)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outdoor/natural</td>
<td>-</td>
<td>25.6 ± 0.17</td>
<td>22.2 ± 1.2</td>
<td>46.7 ± 0.3</td>
<td>1571 ± 50</td>
<td>1.53 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>MLT</td>
<td>25.6 ± 0.17</td>
<td>22.2 ± 1.2</td>
<td>47.4 ± 1.1</td>
<td>1765 ± 206</td>
<td>1.63 ± 0.08</td>
</tr>
<tr>
<td>Outdoor/short</td>
<td>-</td>
<td>25.8 ± 0.17</td>
<td>22.3 ± 1.2</td>
<td>46.2 ± 0.4</td>
<td>1505 ± 51</td>
<td>1.52 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>MLT</td>
<td>25.8 ± 0.17</td>
<td>22.3 ± 1.2</td>
<td>46.0 ± 1.7</td>
<td>1572 ± 175</td>
<td>1.59 ± 0.04</td>
</tr>
<tr>
<td>Indoor/long</td>
<td>-</td>
<td>25.2 ± 0.19</td>
<td>22.1 ± 1.2</td>
<td>44.3 ± 2.0</td>
<td>1391 ± 178</td>
<td>1.53 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MLT</td>
<td>25.2 ± 0.19</td>
<td>22.1 ± 1.2</td>
<td>46.8 ± 0.9</td>
<td>1510 ± 96</td>
<td>1.47 ± 0.04</td>
</tr>
<tr>
<td>Indoor/short</td>
<td>-</td>
<td>25.2 ± 0.18</td>
<td>21.9 ± 1.2</td>
<td>45.5 ± 0.6</td>
<td>1458 ± 67</td>
<td>1.54 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MLT</td>
<td>25.2 ± 0.18</td>
<td>21.9 ± 1.2</td>
<td>46.3 ± 0.8</td>
<td>1443 ± 69</td>
<td>1.45 ± 0.03</td>
</tr>
</tbody>
</table>

Fig. 4.1 summarizes the oocyte stage data obtained from the 2 outdoor tanks. Statistical comparisons were based on the number of weeks required for the females to progress from stage I to stage II, or stage II to stage III. Oocyte development in the short photoperiod tank was significantly advanced in comparison to the natural photoperiod tank (P<0.05). However, there was no significant difference between control and implanted females in either tank (P>0.05). Fig. 4.2 summarizes the results from the indoor tanks. Long photoperiod significantly retarded, while short photoperiod significantly advanced oocyte development in comparison to the outdoor natural photoperiod tank (P<0.05). As in the outdoor tanks, there was no significant difference between control and implanted females in either indoor tank (P>0.05).

These results indicate that melatonin capsules had no observable effect on oocyte development under either natural, inhibitory, or stimulatory photoperiod conditions.
Fig. 4.1: Oocyte stage data from the outdoor tanks in the melatonin implant experiment.
Fig. 4.2: Oocyte stage data from the indoor tanks in the melatonin implant experiment.
Table 4.4 provides a summary of the MLT RIA data. With the exception of the indoor/short-day tank, implanted females had a higher average level of MLT in comparison to the controls; however, the difference was not significant (P>0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Outdoor/natural (pg/ml)</th>
<th>Outdoor/short (pg/ml)</th>
<th>Indoor/long (pg/ml)</th>
<th>Indoor/short (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53 ± 4</td>
<td>74 ± 19</td>
<td>114 ± 32</td>
<td>253 ± 179</td>
</tr>
<tr>
<td>MLT Implants</td>
<td>609 ± 455</td>
<td>557 ± 408</td>
<td>181 ± 38</td>
<td>167 ± 29</td>
</tr>
</tbody>
</table>

Two implanted females, one in the outdoor/natural tank and one in the outdoor/short-day tank had unusually high values (2188 and 2426 pg/ml), which is reflected in the standard errors for those tanks. Elimination of those data points from the analysis resulted in average values for those two groups of 155 ± 26.5 and 149 ± 28.9 pg/ml, respectively. Similarly, the average values of the implanted females in the indoor/short-day tank was lower than controls because of one unusually high control value (1327 pg/ml). Elimination of this data point yielded an average and standard error of 74 ± 14.7 pg/ml for the controls in that group. Overall, the average level of MLT in the control fish was 134 ± 260 pg/ml in comparison to 378 ± 663 pg/ml in the implanted fish. Elimination of the three data points yielded overall averages and standard errors of 82 ± 11.7 and 164 ± 14.8 pg/ml, respectively. Regardless of whether the data points are left in or taken out of the data set, the average midday serum MLT level of females which were implanted 6 weeks earlier was at least double that of the controls.
Experiment 2: Melatonin Feed

Table 4.5 summarizes the data on water quality and female size in experiment 2. As in experiment 1, there were no significant differences among treatments or replicates with regard to these factors (P>0.05). Since all of these tanks were located indoors, the salinity during the experiment was maintained at ambient seawater. The temperature averaged 26.7 °C in all tanks.

Table 4.5  
Environmental conditions and female size in the melatonin feed experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replicate</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>Length (cm)</th>
<th>Weight (g)</th>
<th>CFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLT Feed</td>
<td>1</td>
<td>26.7 ± 0.1</td>
<td>32.0 ± 0.03</td>
<td>45.6 ± 1.4</td>
<td>1407 ± 136</td>
<td>1.47 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.7 ± 0.1</td>
<td>32.0 ± 0.03</td>
<td>45.7 ± 1.1</td>
<td>1353 ± 121</td>
<td>1.41 ± 0.07</td>
</tr>
<tr>
<td>Control Feed</td>
<td>1</td>
<td>26.7 ± 0.1</td>
<td>32.0 ± 0.03</td>
<td>44.7 ± 1.9</td>
<td>1326 ± 146</td>
<td>1.44 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.7 ± 0.1</td>
<td>32.1 ± 0.04</td>
<td>44.9 ± 1.3</td>
<td>1388 ± 141</td>
<td>1.52 ± 0.09</td>
</tr>
</tbody>
</table>

The results of this experiment are summarized in Fig. 4.3. None of the females in either the control tanks or MLT feed tanks initiated stage III during the course of the experiment. Furthermore, the majority of the controls and all of the MLT-feed females had only stage I oocytes on September 10. Therefore, under an inhibitory photoperiod, MLT feed was ineffective in stimulating oocyte development.

A summary of the MLT RIA data is presented in Table 4.6. There were no significant differences between the fish which received control feed and those which received MLT feed (P>0.05). As in the first experiment, there was considerable individual variation in MLT levels in both groups. Five of the fifty samples (3 control feed and 2 MLT feed) had values over 1000 pg/ml. Eliminating these data points yielded control
Fig. 4.3: Oocyte stage data from the melatonin feed experiment.
values for 2000 hrs and 0400 hrs of $350 \pm 110$ and $317 \pm 83$, respectively, and MLT feed values for 1600 and 2000 hrs of $239 \pm 44$ and $275 \pm 75$, respectively. These changes, however, did not affect the conclusions drawn from these data: there were still no significant differences among controls and MLT feed groups at any of the five sample times.

**Table 4.6**

**RIA data for the melatonin feed experiment.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1600 hrs (pg/ml)</th>
<th>2000 hrs (pg/ml)</th>
<th>2400 hrs (pg/ml)</th>
<th>0400 hrs (pg/ml)</th>
<th>0800 hrs (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control feed</td>
<td>185 ± 51</td>
<td>1770 ± 1067</td>
<td>208 ± 41</td>
<td>746 ± 434</td>
<td>178 ± 56</td>
</tr>
<tr>
<td>MLT feed</td>
<td>570 ± 334</td>
<td>519 ± 251</td>
<td>243 ± 90</td>
<td>113 ± 24</td>
<td>251 ± 145</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The effects of MLT on fish reproduction have been investigated by two separate approaches: a) removal of the endogenous source of MLT by pinealectomy and/or blinding, and b) administration of exogenous MLT (reviewed by de Vlaming, 1974, and Ralph, 1978). Pinealectomy has been progonadal, antigonadal, or had no effect depending on the study, the time of year, and the environmental conditions under which the experiments were conducted. In contrast, exogenous MLT has been either antigonadal or produced no effect. A more recent study using exogenous MLT reported a progonadal effect in addition to either no effect or an antigonadal effect (Borg and Ekstrom, 1981). This conclusion was based on a larger average GSI in the treated fish in comparison to the controls during one of their 7 experiments. However, the average oocyte stage of the treated fish in that experiment was identical, if not less developed than the controls, which does not support their conclusion.
The present study also investigated the effects of exogenous MLT on fish reproduction, but differed from previous studies in several aspects. First, the species used, *M. cephalus*, is a short-day rather than a long-day teleost fish. It was therefore hypothesized that MLT treatment would have a stimulatory rather than inhibitory effect on oocyte development, as it does in short-day mammals (Lincoln, 1983). Secondly, this study represents the first time that MLT-chronic-release implants and afternoon oral administration of MLT have been used in an attempt to stimulate fish reproduction. These techniques, particularly implants, are commonly used to advance the breeding cycles of domesticated mammals to the extent that an MLT implant is now commercially available (Staples *et al.*, 1992). Thirdly, the dosages of MLT used in each experiment were selected on the basis of preliminary trials involving the use of a MLT RIA to measure blood serum levels (see Appendix D). The strategy of these experiments was to mimic physiological levels observed at night in this species rather than to generate a pharmacological level. Finally, many of the previous experiments used the gonadosomatic index (GSI) rather than oocyte stage to assay the gonadal response to the hormone (see review by Ralph, 1978). The validity of this index in evaluating the results of administering exogenous MLT has been questioned (Vodicnik *et al.*, 1979). Short photoperiod was clearly shown to stimulate the progression of oocytes from stage I to II in this species (see Chapter 3). Assuming that MLT was transducing photoperiod information, then monitoring this progression provided a very specific assay for determining the effects of the treatments.
The design of the implant experiment was similar to the pinealectomy experiment conducted by Urasaki (1973) in the sense that both treated and control fish were placed under short, long, and natural photoperiods. In this way, the photoperiod conditions during the experiment could be minimized as a factor complicating the interpretation of the results. The feed experiment, however, required a long photoperiod regime since it was designed to mimic, physiologically, a short day.

Despite these considerations, neither chronic release MLT implants nor MLT feed had a significant effect on oocyte development in *M. cephalus*. There are several possible explanations for these results. First, the treatments could have been administered at the wrong time of year, which has been suggested as the reason why some pinealectomy studies in fish have failed to elicit a response (see de Vlaming, 1974). Both experiments, however, were conducted in June because previous experiments had shown that females placed under a short photoperiod during that month initiated oocyte development (see Chapter 3). If either MLT capsules or MLT feed produced a physiological short day, as these techniques apparently do in mammals (Kennaway et al., 1982; O'Callaghan et al., 1991), then a progonadal response would be expected. Secondly, the dosages may have been too low to elicit a response. As mentioned above, the dosages were chosen on the basis of preliminary experiments to duplicate serum levels present at night. Many hormones which do not appear to be primary regulators of fish reproduction have been shown to influence gonadotropin release and, therefore, have the potential to influence the course of gametogenesis (Peter et al., 1991). In this sense, this study was not designed to determine whether MLT could affect reproduction in *M. cephalus, per se*, but was
designed to determine whether ML T was the primary transducer for the effects of photoperiod on reproduction.

However, the RIA data obtained to evaluate the performance of the capsules and feed suggest that the dosage may have been a problem in the second experiment. The ML T feed at the end of the experiment did not appear to be having a detectable effect on the serum levels of ML T in the treated fish. Why this occurred is not clear since the preliminary experiments indicated that the dosage used should significantly increase the serum level of ML T for at least four hours after feeding (see Appendix D). It’s possible the clearance of ML T increased during the course of the experiment. However, there presently is no data available on chronic clearance rates of this hormone in fish. While orally administering hormones can be both unreliable and inefficient, this technique was selected because administering daily injections in this species would either have been fatal or would have had an inhibitory effect on reproduction. In an effort to improve the reliability of the technique, the fish were placed indoors where the feed would be their sole source of food and the feed was provided once a day for 1 hour only.

A number of previous experiments involving pinealectomy or daily injections suggest that ML T can influence reproduction in fish (see de Vlaming, 1974, and Ralph, 1978), while other studies have failed to demonstrate an effect (Schonherr, in de Vlaming, 1974; Rasquin, 1958; Pang, 1967; and Peter, 1968). de Vlaming (1974) suggested that the time of year and photoperiod conditions during the experiments, which were not reported in these latter studies, may have been responsible for their results. However, a more recent study did take these factors into consideration, yet also failed to demonstrate
an effect of either the eyes or the pineal gland on the reproductive cycle (Day and Taylor, 1983).

While it has been demonstrated that MLT influences mammalian reproduction, pinealectomy, injections, and constant-release capsules have failed to affect gonadal development in birds, and consequently, there is little or no evidence that MLT is involved in either bird photoperiodism or reproduction (reviewed by Cassone, 1990). In general, “a unique or consistent involvement of pineal organs with the reproductive process has not been demonstrated unequivocally in any nonmammalian class” (Ralph, 1978). Ralph (1978) also suggested that “considerable bias has encouraged the finding of an antigenadal role for the pineal in the various classes of vertebrates”. This may also be the case in fish since the results of many experiments have been complicated and difficult to interpret. For example, Hontela and Peter (1980) conducted 2 experiments on goldfish during the months of March and April respectively. Pinealectomy and blinding appeared to cause a reduction in gonadal size when conducted in March. In contrast, fish which were either pinealectomized, blinded, or both pinealectomized and blinded in April, did not have significantly smaller gonads than controls. Even so, these authors concluded that pinealectomy and blinding in the Spring had an inhibitory effect on reproduction.

The present study does not provide unequivocal evidence against MLT synchronizing annual oocyte development in M. cephalus. Its main contribution lies in the fact it approached the problem in a different manner than previous experiments on fish. One can only conclude from the results that proving this hormone is or is not the internal zeitgeber for annual reproductive cycles in fish will not be an easy task.
CHAPTER 5
CONCLUSION

This dissertation focused on the environmental control of oocyte development in the striped mullet, *Mugil cephalus*. This research increased the understanding of *M. cephalus* reproduction and, specifically, its pattern of seasonality observed throughout the world. The first study obtained data on the variation in oocyte development and growth rates between individual females. The second study provided experimental data on the effects of different photoperiod and temperature combinations on oocyte development. The third study examined the effects of chronic administration of melatonin on oocyte development to determine whether this hormone is involved in the physiological transduction of environmental information in this species.

The first study monitored the initiation of oocyte stages and oocyte growth rates in fish maintained in captivity under natural photoperiod and temperature conditions. During all three seasons, females initiated the cortical vesicle stage (i.e., stage II) between August and October, with the majority having oocytes in this stage in September. The initiation of vitellogenesis (i.e., stage III) took place between September and December and showed greater year to year variation than stage II. This type of variation may account for some of the confusion concerning the results of previous studies in the same geographic region. Only one clutch of vitellogenic oocytes was produced at a time; however, females produced 1-3 clutches per season, with the number depending on when vitellogenesis was initiated and the oocyte growth rate. Many authors have concluded that *M. cephalus* spawns only once a year on the basis of finding only a single clutch in the
ovaries which they examined. The present study provides the first conclusive evidence that this species can spawn more than once per year and may be doing so in their natural habitat.

Oocyte growth rates during vitellogenesis varied from individual to individual as well as from early to late vitellogenesis. The average rate was 6.5 μm/day, which indicates that vitellogenesis requires an average of 65 days for completion. Clutches which underwent atresia before completing vitellogenesis had significantly lower growth rates than those which completed vitellogenesis. Individual females reached the COD between November and March, depending on their oocyte growth rate. Final maturation and spawning were induced by 2 hormone injections. The diameter of the spawned eggs was directly related to the diameter of the vitellogenic oocytes prior to the first injection, while the number of eggs produced was directly related to the size of the female.

In the second study, 5 experiments were conducted in which females in different stages of oocyte development were placed under 4 different combinations of photoperiod and temperature: short/cold, short/warm, long/cold, and long/warm. The results of the first experiment suggest that once females have regressed to either stage I or II immediately after the natural season, they become refractory to stimulatory photoperiod and temperature conditions. However, the second experiment showed that two to three months later, short photoperiod will stimulate the progression from stage I to stage II while cold temperature stimulated the progression from stage II to stage III. This same pattern in which photoperiod was the primary factor influencing stage II while temperature was the primary factor influencing stage III was similarly observed in experiments 3, 4 and
5. Long photoperiod and warm temperature caused complete regression to stage I in all five experiments. Histological analysis of the oocyte samples obtained during experiment 5 indicated that the migration of the yolk nucleus of Balbiani’s vitelline body was dependent on environmental conditions. Yolk nucleus migration was stimulated in short photoperiod and cold temperature but inhibited in long photoperiod and warm temperature. To the author’s knowledge, this the earliest stage of oocyte development in any animal reported to be influenced by environmental factors. Based on these findings, temperature, rather than photoperiod is hypothesized as the predominant factor determining the pattern of reproductive seasonality observed in this species at different latitudes. Populations of *M. cephalus* in higher latitudes initiate stage III when the temperature rises above 15 °C, which appears to be the lower limit for reproduction. Populations in lower latitudes initiate stage III when the temperature drops below 27 °C, which appears to be the upper limit for reproduction. As a result, *M. cephalus* does not show the pattern of discrete seasons at high latitudes to continuous seasons at low latitudes observed in species in which reproduction is influenced primarily by photoperiod.

Two experiments were conducted in the third study. The first tested the effects of melatonin (MLT) silastic capsules on oocyte development in females placed under either stimulatory, inhibitory, or natural photoperiods. The second experiment tested the effects of orally administering MLT through the feed. Both techniques have been used extensively in mammals and birds to simulate a physiological short day. Both techniques also have been shown to affect the reproductive cycles of these animals as well as other activities which exhibit diurnal or seasonal cycles. In *M. cephalus*, however, neither MLT
implants nor MLT feed had either a stimulatory or inhibitory effect on oocyte development.
APPENDIX A

TEMPERATURE DATA FROM NATURAL HABITATS OF
MUGIL CEPHALUS

Chapters 2, 3, and 4 provide water temperature data for the experimental ponds and tanks used during the studies presented in this dissertation. For the purpose of comparing these data to annual temperature cycles in natural M. cephalus habitats, this appendix provides water temperature data from Kaneohe Bay, Oahu, Hawaii during 1987-89 and Pearl Harbor during 1987-88. The Kaneohe Bay data were obtained at 8 am and 4 pm by Lloyd Watarai of the Hawaii Institute of Marine Biology (HIMB) and were taken with the use of a water/soil type temperature probe (Campbell Scientific). The probe was placed approximately 2.5 m deep off Coconut Island where HIMB is located. Data from Pearl Harbor were provided by the Honolulu branch of the National Marine Fisheries Service (NMFS). NMFS monitored, on a weekly basis, a total of 39 stations in the harbor, 5 of which were used here for comparison to the ponds. The five stations, #28 (West Loch), #13 (Middle Loch), #39 (East Loch), #1 (East Loch), and #32 (Harbor mouth) were averaged since the time that the measurements were taken was different for each station. Furthermore, mullet are observed at all of these locations during the year. Temperatures were obtained with a thermometer which was lowered to approximately 1 meter below the surface.

Figure A.1a summarizes the data from Kaneohe Bay. During the 2 years monitored, the lowest temperature reached was 21.7 °C, which occurred in April of 1987. The highest temperature was 28.6 °C in September of 1987. These data also show a year to year fluctuation similar to that seen in the experimental ponds (see Chapter 2). The
lowest temperature reached in April of 1988 was 24.4 °C, which is approximately 3 degrees higher than the previous year.

Figure A.1b summarizes the data from Pearl Harbor. The lowest average temperature was 22.68 °C, which occurred in April, 1987. The highest average temperature was 29.1 °C, which occurred in September, 1987. The lowest and highest temperatures recorded at specific stations were 21.6 °C and 29.6 °C, which occurred in April and September, respectively.

In general, the average temperatures observed in the experimental ponds were several degrees higher than either of these 2 natural habitats, which was mostly likely due to the depth of the water, the depth at which the temperature was measured, and the fact that the ponds had a black liner. The temperatures in the indoor tanks during the 5 experiments presented in Chapter 3 are very similar to these natural maxima and minima.
Fig. A1: Water temperature data from Kaneohe Bay and Pearl Harbor, Hawaii, USA.
Chapter 2 contained references to the fact that natural final maturation and spawning has not been observed in captive *M. cephalus*. The author also made 2 attempts to obtain natural spawning in a pond at The Oceanic Institute, Hawaii, USA, both of which were unsuccessful. The first attempt took place February 7-22, 1989, and was designed to simulate a spawning migration by transferring females with stage IIIb oocytes from a smaller to larger body of water. This strategy had been successful in the milkfish, *Chanos chanos* (Kelley, unpublished data) and was routinely used to establish a spawning broodstock in commercial milkfish hatcheries in Indonesia. Three females having average oocyte diameters ranging between 592-631 μm were transferred from a 30 m$^3$ pond described in Chapter 1 to a dirt pond having an estimated volume of 173 m$^3$. Six fully mature males (i.e., milt could be easily expressed from the urogenital pore by applying pressure to the abdomen) were placed into the pond with the females. The fork lengths of the fish ranged from 35-46.5 cm (39.1 ± 3.9) and the weights ranged from 613 to 1130 g (849 ± 181). Purina trout chow was provided daily, but the fish fed primarily on the natural benthos in the pond. An egg collector, which had been installed on the drainpipe, was checked and cleaned daily. The average daily water temperature and salinity during the trial ranged between 25.5-27.3 °C and 34-35.5 ppt, respectively. After no eggs were observed for 2 weeks, the fish were returned to their original ponds.

The second trial was conducted between July 24, 1989, and March 21, 1990. Similar to the first trial, fish were transferred from 30 m$^3$ ponds to the 173 m$^3$ pond. In
this case, however, 9 females and 10 males were transferred during the offseason (May-August) and were left in the pond for a period of 8 months. When transferred, all of the females had only stage I oocytes while 8 of the 10 males were immature (i.e., no milt could be expressed from the urogenital pore or obtained by cannulation). Two of the males were fully mature. Fork lengths ranged from 29.5 to 46 cm (38.0 ± 3.9) and weights ranged from 340 to 1325 g. (829.2 ± 213.5). The average daily water temperature and salinity during the trial ranged between 21.5-29.3 °C and 32-35.5 ppt respectively. No eggs were observed in the collector on any day during the trial. On March 21, 1990, all of the fish were removed and sampled in the manner described in Chapter 2. Two females were found to have atretic oocytes, 3 were found to have stage III oocytes, 3 had stage II oocytes and 1 had only stage I oocytes. Eight of the 10 males were found to be mature. These data suggest that the majority of the fish matured during the trial, but the females failed either to complete vitellogenesis or initiate final maturation and spawning.
APPENDIX C

IN VITRO STIMULATION OF FINAL OOCYTE MATURATION IN MUGIL CEPHALUS

Similar to a number of other teleost fishes, M. cephalus females held in captivity do not undergo final maturation and spawning in captivity unless induced to do so by administration of exogenous hormones. The induced spawning protocol described in Chapter 2 of this dissertation involves a first injection of 20 mg/kg body wt. Carp Pituitary Homogenate (CPH) followed 24 hours later by a second injection of 50-100 μg/kg body wt. of Luteinizing Hormone Releasing Hormone analog (LHRH-a). Spawning generally takes place 12-16 hours after the second injection. In an effort to understand why M. cephalus requires two injections rather than one, an experiment was carried out in 1988 in which oocytes obtained during induced spawning trials were stimulated to undergo final maturation in vitro.

The experiment was conducted on January 19-20, 1988. Two females (#4E37 and #4458), with mean oocyte diameters exceeding the COD (see chapter 2), were each injected with CPH on January 18 and placed into a 170 liter spawning tank. Twenty-four hours later, the fish were captured and given a second injection of LHRH-a. Immediately prior to the injection and 6 hours after the injection, the females were cannulated in order to obtain a samples of oocytes. The samples were gently expelled into a petri dish containing a modified version of Cortland's medium developed by Dr. Frederick Goetz of the University of Notre Dame. The medium was made by adding the following ingredients to 1 liter of double distilled water:

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8.5 g of sodium chloride (NaCl)
0.38 g of potassium chloride (KCl)
0.23 g of calcium chloride dihydrate (CaCl\textsubscript{2} \cdot 2H\textsubscript{2}O)
0.1 g of sodium bicarbonate (NaHCO\textsubscript{3})
0.41 g of sodium phosphate monobasic (NaH\textsubscript{2}PO\textsubscript{4})
0.23 g of magnesium sulphate (MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O)
1 g of α-D glucose
4.76 g of Hepe’s buffer

The pH of this solution was adjusted to 7.8 with 1N NaOH, after which the following antibiotics were added:

100 mg of streptomycin
100,000 IU of penicillin

Prior to obtaining the oocyte samples, 17α-20β dihydroprogesterone (17α-20β-P) was added to the medium in the following dosages (μg/ml): 0, 0.06, 0.125, 0.3, 0.6, 1.25, 2.5, 5, 10. Two culture wells were loaded with each dosage of 17α-20β-P medium.

Aliquots of approximately 30-60 oocytes were teased apart and placed separately in each of the culture wells. The tissue culture plates were then placed on a rotating platform mixer. Twenty-four hours later, the number of hydrated and nonhydrated oocytes was counted in each well and the percentage of hydrated oocytes was then calculated.

Figure C.1 summarizes the results of this experiment for a) female #4E37 and b) female #4458. The samples from both females showed a similar response. Vitellogenic oocytes obtained immediately prior to the second injection (0 hrs) were stimulated to undergo hydration in dosages of 17α-20β-P above 0.6 μg/ml. None of the oocytes hydrated in the control (0 μg/ml), 0.06 and 0.125 μg/ml wells. Vitellogenic oocytes
Fig. C1: *In vitro* stimulation of final maturation in oocytes obtained from female #4E37 (a) and #4458 (b). Black bars are oocytes obtained just prior to the second hormone injection while the white bars are oocytes obtained 6 hrs after the second injection.
obtained 6 hours after the second injection hydrated in all dosages of 17α-20β-P, including the controls. These data indicate that the oocytes became competent to undergo final maturation 3-6 hours after the second injection was administered.
Chapter 4 provides the results of two experiments testing the effects of melatonin (MLT) implants and MLT feed on oocyte development in *Mugil cephalus*. However, that chapter did not provide the preliminary work for those experiments which included validation of the melatonin RIA in *M. cephalus*, an experiment to determine the MLT diurnal cycle in this species, experiments to determine the *in vitro* and *in vivo* release rates of the implants, and an experiment to determine the appropriate dosage of MLT for the MLT feed experiment. While the author reviewed the design of the experiments and assisted in the collection of blood samples, this work was primarily carried out by Virginia Moriwake who was also assisted by Dr. Clyde Tamaru, Aaron Moriwake, and Garret Miyamoto. With the permission of both V. Moriwake and the author’s graduate committee chairperson, the author obtained the data from the experiments and prepared the following summary solely for the purpose of providing the background for the experiments presented in Chapter 4.

**Validation of the MLT RIA**

As mentioned in Chapter 4, the MLT RIA was provided by Dr. K. Aida and was performed in his laboratory in Tokyo University. It had previously been validated for use in *M. cephalus* based on parallelism between inhibition curves of an MLT standard and serial two-fold dilutions of MLT plasma fractions (see Kezuka *et al.*, 1988). To further validate the RIA for this species, two other tests were conducted. Intra- and inter-assay
coefficients of variation (CV’s) were obtained by pooling *M. cephalus* plasma into three samples containing relatively low, medium, and high percent bound (B/Bo). Six aliquots from each sample were obtained and simultaneously subjected to the MLT extraction and RIA process described in Chapter 4 to determine the intra-assay CV. Four additional aliquots from each sample were extracted and run through separate assays to obtained the inter-assay CV. Table D.1 shows the results of this first test, which were reviewed by Dr. Aida. While these values were higher than the “desirable” level of 10%, they were still considered to be acceptable.

Table D.1
*Intra- and inter-assay coefficients of variation (CV’s) for the melatonin RIA.*

<table>
<thead>
<tr>
<th>Percent Bound (B/Bo)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.2</td>
<td>18.3</td>
<td>13.1</td>
</tr>
<tr>
<td>50.4</td>
<td>11.0</td>
<td>20.9</td>
</tr>
<tr>
<td>59.6</td>
<td>6.3</td>
<td>11.4</td>
</tr>
</tbody>
</table>

The second validation experiment tested the recovery efficiency of the RIA. Six known standards were mixed with pooled *M. cephalus* plasma and then subjected to the RIA procedure. The percent recovery of the added MLT was then calculated and is provided in Table D.2. The percentage of MLT recovered was lower than 70% when

Table D.2
*Recovery efficiency of the melatonin RIA.*

<table>
<thead>
<tr>
<th>Added Standard (pg/ml)</th>
<th>N</th>
<th>Expected (pg/ml)</th>
<th>Actual (pg/ml)</th>
<th>% Recovery (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>-</td>
<td>130 ± 23</td>
<td>-</td>
</tr>
<tr>
<td>62.5</td>
<td>5</td>
<td>192.5 ± 23</td>
<td>224 ± 35</td>
<td>116 ± 18</td>
</tr>
<tr>
<td>125</td>
<td>5</td>
<td>255 ± 23</td>
<td>226 ± 57</td>
<td>89 ± 22</td>
</tr>
<tr>
<td>250</td>
<td>5</td>
<td>380 ± 23</td>
<td>313 ± 58</td>
<td>82 ± 15</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>630 ± 23</td>
<td>476 ± 36</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>1000</td>
<td>5</td>
<td>1130 ± 23</td>
<td>734 ± 148</td>
<td>65 ± 13</td>
</tr>
<tr>
<td>2000</td>
<td>5</td>
<td>2130 ± 23</td>
<td>1128 ± 104</td>
<td>53 ± 5</td>
</tr>
</tbody>
</table>
1000 and 2000 pg standards were added. Standards having 500 pg/ml or less had recoveries above 70%. Since the physiological levels found in *M. cephalus* plasma are generally less than 500 pg/ml, the RIA was considered to be acceptable for use in this species.

**MLT Diurnal Cycle**

MLT is synthesized at night and therefore shows a very distinct diurnal cycle in all animals examined to date. This experiment was conducted to determine whether the same pattern exists in *M. cephalus*. Thirty adult fish (15 males and 15 females) were placed into three 8 m³ indoor tanks (5 males and 5 females per tank). The fish were acclimated to the tanks for a period of 6 days. On the seventh day, blood samples were obtained from five fish during each of the following sample times: 1200, 1600, 2000, 2400, 0400, and 0800. The photoperiod in the tanks was set at 12 hours of light which occurred between 0700-1900. The samples were subjected to the MLT RIA as described in Chapter 4. Figure D.1 summarizes the results which are consistent with other animals. Average MLT levels ranged between 48-471 pg/ml. The peak occurred at midnight (2400) while the lowest level occurred at 0800.

*In vitro* release rates of MLT silastic capsules

As was mentioned in Chapter 4, a number of studies had previously tested the effects of MLT silastic implants in higher vertebrates. The release rates from these implants depended on the length and shape. None of these studies had determined the release rate of MLT from the specific size of silastic tubing used in the present study (i.e., 0.058 in. ID, 0.077 in. OD). Therefore, two preliminary experiments were carried out to
determine their performance. The design of the first experiment is presented in Table D.3.

Four different dosages of 1.5 cm MLT silastic capsules (0 mg, 5 mg, 7.5 mg, and 15 mg) were made as described in Chapter 4. These capsules were then placed into a twenty 12mm x 75mm glass test tubes (5 replicates per dosage) containing 2 ml of 20 mM phosphate buffer.

Table D.3
Design of the melatonin implant *in vitro* release rate experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>MLT/capsule (mg)</th>
<th>Capsules per testtube</th>
<th>MLT/testtube (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>7.5</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>15</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

One capsule each of the 0 and 15 mg dosages was placed into a separate test tube, while two of the 7.5 mg capsules and 3 of the 5 mg capsules were placed in separate test tubes. Therefore, with the exception of the controls, each test tube had a total of 15 mg of MLT in the form of either 1, 2, or 3 capsules. All of the test tubes were placed on a rotating platform mixer which was set to run continuously at 50 rpm. The temperature was recorded daily from 1 of the tubes. The buffer solution was removed for MLT RIA analysis each day and replaced. The 24 hour release rate was calculated by dividing the concentration of MLT in the tubes by the number of minutes between the addition and removal of the media and multiplying by 1440 minutes.

Similar to steroid containing silastic capsules (see Tamaru *et al.*, 1990), the release rate patterns of MLT capsules showed a sharp spike on day 1, which immediately dropped
by day 2. This phenomenon may be the result of a rapid release of hormone that was already embedded in the capsule membrane at implantation. From day 2 on, the release rates was relatively constant. Fig. D.2a summarizes the data beginning on day 2. Since days 2-7 were not significant different within treatments, the data from days 3-6 are not shown. MLT was undetectable in the control capsules (treatment 1). The highest levels of MLT were measured in treatment 2 (three 5 mg capsules) while the lowest levels were measured in treatment 4 (one 15 mg capsule). Fig. D.2b shows the relationship between the total release rate and the packing density. Each capsule had a volume of 25.46 mm$^3$; therefore, the packing densities of the 5, 7.5, and 15 mg capsules were approximately 0.2, 0.3, and 0.6 mg/mm$^3$, respectively. As the packing density increased from 0.2 to 0.6 mg/mm$^3$, the average release rate decreased from 9.2 to 2.6 μg/day. Fig. D.2c shows that the release rate per capsule was not significantly different for each of the packing densities and averaged between 2.6-3.06 μg/day, or approximately 1 mg per year. These data suggest that the release rate of a 15 mg capsule will not significantly change as the concentration of MLT in the capsule decreases.

**In vivo release rates of MLT silastic capsules**

This experiment was designed to determine the effects of MLT silastic capsules on the serum level of MLT in *M. cephalus*. Twenty adult fish (12 females and 8 males) were divided into 4 groups (3 females and 2 males per group). Group 1 (0 mg) served as the controls and were each implanted with an empty silastic capsule. Group 2 (15 mg) were each implanted with a single 15 mg MLT capsule. Group 3 (30 mg) were each implanted with two 15 mg MLT capsules and group 4 (45 mg) were each implanted with three 15
Fig. D2: Melatonin implant in vitro release rate experiment.  a) average release rates per sample day, b) relationship between total release rate and packing density, and c) estimated release rate per capsule for each packing density.
mg MLT capsules. The experiment was started on February 27, 1991 (Day 0) and was terminated on April 24, 1991 (Day 56). All of the fish were stocked into a single outdoor 35 m³ rectangular tank. Blood samples were obtained at midnight and at noon from each fish on days 0, 1, 7, 14, 21, 28, 42, and 56. The serum obtained from these samples was subjected to the MLT RIA.

Table D.4 provides the range of average MLT levels obtained at midnight and noon for each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Midnight MLT serum level (pg/ml)</th>
<th>Noon MLT serum level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg</td>
<td>5</td>
<td>105 ± 53.2</td>
<td>51 ± 51.1</td>
</tr>
<tr>
<td>15 mg</td>
<td>5</td>
<td>199 ± 80.0</td>
<td>116 ± 56.9</td>
</tr>
<tr>
<td>30 mg</td>
<td>5</td>
<td>301 ± 271.4</td>
<td>237 ± 260.1</td>
</tr>
<tr>
<td>45 mg</td>
<td>5</td>
<td>340 ± 230.6</td>
<td>259 ± 179.8</td>
</tr>
</tbody>
</table>

In comparison to the previous experiment summarized in Fig. D.1, both the midnight and noon MLT levels in the control group were significantly lower in this experiment, the reasons for which are unclear. However, the average serum MLT levels in the 30 and 45 mg groups were significantly higher than the controls (0 mg) at both midnight and noon (ANOVA, P<0.05).

Fig. D.3 provides the midnight and noon serum MLT levels for each sample day during the experiment. One fish in the 30 mg group had unusually high levels on day 14 (1602 and 1544 pg/ml at midnight and noon respectively) which accounts for the high average level on that day. Serum MLT levels in the 45 mg group decreased from 353-445
Fig. D3: Melatonin implant *in vivo* release rate experiment. Midnight (a) and noon (b) serum melatonin levels for each treatment.
pg/ml on day 7 to 170-187 pg/ml by day 56. Since the release rate of the implants should not have varied during the experiment, this change may represent a physiological response to continuous high levels of the hormone.

On the basis of these results and the results of the previous experiment, two decisions were made regarding the protocol for the implant experiment presented in Chapter 4. The length of the 15 mg capsules was increased from 15 mm to 24 mm which, would theoretically increase the daily release rate of each capsule to 4 μg. Secondly, the fish would be implanted with four 15 mg capsules rather than three in an effort to sustain serum MLT levels above 400 μg/ml.

Preliminary experiments using MLT feed.

Two experiments were conducted to determine whether MLT feed could affect the serum levels of MLT in *M. cephalus* and, if so, what the appropriate dosage would be to simulate a physiological short day. In the first experiment, 30 subadult fish (weights = 186-408 g) were stocked into two indoor 4.1 m³ fiberglass tanks fitted with black plastic covers (15 fish per tank). Since these tanks were not fitted with 24 hour photoperiod timers, the hoods were placed over the tanks at 1630 and removed at 0430 on the day of the experiment to produce a photoperiod of 12 hours. Both tanks of fish were fed Purina trout chow at a rate of 1% of their body weight per day. In the control tank, the fish received untreated trout chow, while the treatment tank received MLT chow. This feed was made as described in Chapter 4 and contained a dosage of MLT calculated to be 12.5 mg/kg of fish. The experiment was conducted on March 1, 1991. The fish in both tanks were fed at at 0900. Five fish were removed from each tank at 1100, 1300, and 1500 and
anesthetized as described previously in this dissertation. A blood sample was taken from each fish and subsequently subjected to the MLT RIA. The results of this experiment are provided in Table D.5. While 2 of the controls had unusually high MLT levels when sampled at 1500, this experiment showed conclusively that oral administration of MLT is effective in increasing the serum levels of that hormone in this species.

Table D.5
Results of the preliminary melatonin feed experiment.

<table>
<thead>
<tr>
<th>Tank</th>
<th>N</th>
<th>1100</th>
<th>1300</th>
<th>1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>61 ± 19</td>
<td>103 ± 93</td>
<td>1,348 ± 1,649</td>
</tr>
<tr>
<td>MLT Feed</td>
<td>15</td>
<td>14,173 ± 14,821</td>
<td>28,988 ± 15,114</td>
<td>27,291 ± 12,798</td>
</tr>
</tbody>
</table>

The second experiment focused on defining the appropriate dosage to use for the MLT feed experiment described in Chapter 4 since 12.5 mg per kg of fish was clearly an excessive level. On February 20, 1992, sixty subadult *M. cephalus* (210-516 g) were divided into 4 groups of 15 fish and stocked into 4 separate 3 m³ indoor fiberglass tanks. The fish were acclimated to the tanks for 4 days during which time they were not provided food in order to insure they would eat when the experiment started. On February 24, the fish in each tank were fed Purina trout chow containing one of the following dosages of MLT: 0 mg/kg body wt (control), 0.1 mg/kg body wt., 1.0 mg/kg body wt., and 10.0 mg/kg body wt. The feed was made in the same manner as previously described. Feeding took place at 1500 hrs. Blood samples were obtained from five fish in each tank at 1600, 1800, and 2000 hrs. After sampling, each fish was placed in one of four additional 3 m³ tanks so that by 2000 hrs, all of the fish in each group had been transferred out of their
original tanks. At 0600 hrs on February 25, a final blood sample was obtained from all sixty fish. The serum was extracted from the samples and subsequently subjected to the MLT RIA. The results of this experiment are shown in Table D.6.

### Table D.6
Results of the melatonin feed dose response experiment.

<table>
<thead>
<tr>
<th>Treatment (mg/kg body wt)</th>
<th>N</th>
<th>1600 hrs</th>
<th>1800 hrs</th>
<th>2000 hrs</th>
<th>0600 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>15</td>
<td>43 ± 30</td>
<td>29 ± 6</td>
<td>63 ± 10</td>
<td>45 ± 33</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
<td>1783 ± 972</td>
<td>865 ± 443</td>
<td>219 ± 90</td>
<td>34 ± 23</td>
</tr>
<tr>
<td>1.0</td>
<td>15</td>
<td>8971 ± 8790</td>
<td>4837 ± 2907</td>
<td>3383 ± 1745</td>
<td>66 ± 36</td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td>118857 ± 40579</td>
<td>81556 ± 89319</td>
<td>16511 ± 18449</td>
<td>284 ± 148</td>
</tr>
</tbody>
</table>

The strategy of the MLT feed experiment described in Chapter 4 was to elevate serum MLT levels above 500 pg/ml from the time of feeding to 2000 hrs, at which point natural production of the hormone would have begun. Based on the results shown here, a dosage of 0.3 mg/kg body wt was selected for the experiment.
REFERENCES


Susuki, K., K. Asahina, C.S. Tamaru, C.S. Lee, and H. Inano. 1991. Biosynthesis of 17α, 20β-dihydroxy-4-pregnen-3-one in the ovaries of grey mullet (*Mugil cephalus*) during...


