EARLY LIFE HISTORY TRAITS OF YELLOWTAIL FLOUNDER, Pleuronectes ferrugineus (STORER), IN RELATION TO MATERNAL AND PATERNAL EFFECTS WITH EMPHASIS ON SCALE

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by

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Abstract

The early life history (ELH) traits of fish relate directly to critical aspects of survival, development and growth that are reflected in aquaculture recruitment and the quality of progeny. The ELH traits of the eggs and larvae of yellowtail flounder, *Pleuronectes ferrugineus* (Storer), were investigated to determine which ELH traits relate to commonly used egg and larval quality criteria and how these traits could be used to predict recruitment in mass rearing aquaculture.

Chapter 1 shows that inducing females to spawn with GnRHa produces higher mortality during embryogenesis compared with spontaneously ovulating females. However, the variability in mortality remains relatively constant. In addition, temperature affects time to hatch, but not egg mortality, which was determined through comparing the dry mass of filtrate to the dry mass of known volumes of eggs.

Chapter 2 shows that egg size was more variable than carbon and nitrogen content and therefore, less effective as an egg quality criterion. It is hypothesised that variation in egg size is greatly affected by hydration processes that are under environmental influence.

Chapter 3 shows that abnormal cleavage patterns during the 4–8 cell stage of embryogenesis significantly decreased hatching success, but did not produce any curved larvae. This suggests that hatching success may be underestimated and that visual egg quality determinations based on viability criteria may be less effective than previously thought.

Chapter 4 shows that multivariate analyses of five larval and three egg ELH traits had significantly higher predictive capabilities than any single trait. However, because of its relationship to other ELH traits, larval standard length was found to be the best single, quantitative metric of egg batch quality. Nonetheless, a trained assessment of larval quality, based on visual observations, is nearly as effective. Males were typically found to contribute less than 5% to the variability in ELH traits (range 0 – 15%) while females generally contributed over 30% (range 6% – 83%). However, relative contributions varied according to whether the analysis was performed on individual or mean levels of data and (or) at what time larval measurements were made. This result suggests that, with regard to ultimate larval success, the maternal contribution decreases and the paternal contribution may increase through ontogeny.

Comparative statistical analyses shows that multivariate treatment is superior to univariate treatment of data at the same level. Furthermore, the method of aggregating data, e.g. mean-level or population-level groupings, may significantly change results. This scale result is due in part to significant changes in sample sizes as data are aggregated. Model I and Model II (reduced major axis) regressions are compared and were found to be different when ordinate data are highly variable at a given abscissa value.

Keywords: egg quality, larval quality, scale, early life history, *Pleuronectes ferrugineus*, yellowtail flounder, flatfish, Pleuronectidae, maternal effects, paternal effects, ontogeny, abnormal cleavage

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Especially for Nicole.

For Gjura Kuka (1920 – 1995)

mentor, teacher, best friend

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List of Abbreviations and Symbols

AFDM	ash free dry mass (mg)
ANCOVA	analysis of covariance
ANOVA	analysis of variance
C	carbon
C/N	carbon to nitrogen ratio
CV	coefficient of variation
Ddf	denominator degrees of freedom
df	degrees of freedom
DM	dry mass (mg)
DPH	days – post – hatch
ED	egg diameter (mm)
ELH	early life-history
F	Fisher's F – statistic
FA – FE	female A – female E
FERNS	fertilisation success of non-symmetrical eggs (%)
FERS	fertilisation success of symmetrical eggs (%)
FERVIA	fertilisation success of viable eggs (%)
GLM	general linear model
GnRHa	gonadotropin releasing hormone analogue
IO	induced ovulated (manually stripped with hormone injection)
MA – MI	male A – male I
MANOVA	multivariate analysis of variance
MED	mean egg diameter (mm)
MFERNS	mean fertilisation success of non-symmetrical eggs (%)
MFERS	mean fertilisation success of symmetrical eggs (%)
MFERVIA	mean fertilisation success of viable eggs (%)
MNBD	mean notochord body depth at anus (mm)
MQ	mean quality
MTDB	mean total body depth at anus (mm)
MVIA	mean viability of eggs (%)
MYV	mean yolk volume (mL)
n	number in group, population, etc.
N	nitrogen
NBD	notochord body depth at anus (mm)
Ndf	numerator degrees of freedom
PCC	partial correlation coefficient
p	p – value, probability
Q	quality
SL	standard length (mm)
SO	spontaneously ovulated (manually stripped without hormone injections)
T3, T5 or T7	tests using 3, 5 or 7 traits
TBD	total body depth at anus (mm)
VC	variance component
VIA	viability of eggs (%)
YV	yolk volume (mL)

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Introduction and Overview

In the early 1990's, interest in alternative aquaculture species began to increase. Halibut (*Hippoglossus hippoglossus*) culture has been ongoing for many years particularly in Norway and more recently in North America. As well, research on Atlantic cod has been ongoing in many countries and commercial culture is beginning to occur. Yellowtail flounder should be ideally suited as an aquaculture species because of its rapid growth and good market presence. In 1994, the Ocean Sciences Centre viewed yellowtail flounder as one of several new local species to culture. Preliminary research had been conducted to determine spawning and rearing protocols (Larsson et al., 1997; Manning and Crim, 1998) and the first year of reasonable culture success was 1995.

Yellowtail flounder are a multiple egg batch spawning (or serial spawning), iteroparous flatfish and relatively little is known about their natural spawning behaviour. Manual stripping was required in order to obtain eggs in the laboratory and during five years of research the periodicity of spawning remained variable (see Manning and Crim, 1998). Initial observations of egg batch quality such as viability (percentage of eggs that were spherical, clear, and had a relatively small perivitelline space), and egg size (diameter) were highly variable within and among females, and temporally, suggesting that internal and (or) external factors were contributing to egg quality. Other egg quality indicators such as fertilisation and hatching success were less variable. Early studies focussed on aspects of fecundity, egg viability, fertilisation and hatching success,

and on spawning and rearing protocols. Spawning protocols included inducing females with gonadotropin releasing hormone analogue (GnRHa) to initiate and synchronise spawning (Larrson et al., 1997; Manning and Crim, 1998; Bettles and Crim, unpublished data). However, investigations into early life-history (ELH) traits related to maternal and paternal influence, induction, and environmental factors, (temperature) were not specifically addressed.

I planned my investigations to focus on the factors affecting ELH traits as they pertain to aquaculture. My overall hypothesis states that ELH traits would provide a well-recognised suite of characteristics to compare within and among females, egg batches, and external factors. I purposely designed my experiments to overlap previous work and to incorporate current knowledge. I was also interested in testing the underlying statistical assumptions of analyses. Specifically, I was interested in using multivariate techniques that combined many ELH traits and comparing those results to more common univariate analyses. Furthermore, I was interested in the effects of scale as it pertains to predictive relationships and conducted experiments and analyses to investigate the influence of different levels of aggregate data. That is, I started with the lowest possible level whether that was individual eggs, larvae, batches, males, or females and then combined lower levels of data to form aggregate or mean levels and re-analysed the data. These aggregate levels were extended to the population level where possible; generally the among female aggregate. In some cases, sample size would be affected by the addition or deletion of a factor or

trait at one or more level. The by-product was that sample size as it relates to statistical significance was also investigated.

The sum of all these investigations provides an account of the ELH traits of yellowtail flounder from embryogenesis to population within the confines of laboratory investigation. The problems of extrapolating laboratory investigations to wild populations are recognised, but with the lack of information on yellowtail flounder in wild populations, it may be the only source. I will only briefly outline each chapter here to provide the reader with the logical connection of investigations. Chapter introductions substantially outline each investigation and review the relevant literature.

Chapter 1 concentrates on determining relative levels of variability within egg traits and how these traits are affected by induction and embryogenesis. The quality measure used was egg mortality. I overlapped this investigation with previous work by Bettles and Crim at the Ocean Sciences Centre (unpublished) to determine the mortality effect of inducing females to ovulate. An untested assumption of Bettles and Crim was that induction would not increase mortality during embryogenesis. The variability in data from Chapter 1 served as a baseline for the design of further experiments. Sample sizes were determined for subsequent investigations using an iterative calculation based on the variance associated with egg diameters. A new technique was also developed to determine egg mortality using a non-invasive approach (Appendix 1).

Chapter 2 extended the experimental design of Chapter 1 to the female and egg batch level and focussed on whether egg diameter was a good egg

quality determinant because of high egg diameter variability found in Chapter 1. I hypothesised that the biochemical egg constituents of carbon and nitrogen would be less variable than egg diameters among egg batches within and among females. Moreover, the first aggregate data analysis was performed comparing results obtained at the individual level (females) to those obtained at the population level (all females' data combined).

Chapter 3 was designed to answer one question; does abnormal cell cleavage affect egg and (or) larval quality? Specifically, does abnormal cell cleavage increase mortality during embryogenesis, decrease hatching success, or produce abnormal larvae? Somewhat of a side study, this investigation quantified abnormality in eggs and larvae; something that has not been done in yellowtail flounder. It also introduced the larval connection to egg quality used in Chapter 4. One addition to the main question was the effect that temperature had on egg abnormality. Temperature was suspected of being a contributing factor to variability in spawning periodicity and possibly a factor in egg quality among batches. A by-product of the temperature investigation was that a general temperature dependent development time was established for yellowtail flounder.

The primary goal of Chapter 4 was to investigate the interactions and relationships between egg and larval quality at various levels of aggregate data with regard to maternal and paternal contributions. Chapter 4 built upon all of the previous investigations. I used the variance estimates from the first three chapters to determine more accurately the sample sizes required. The

comparisons between egg and larvae were also extended. This extension incorporated a multivariate approach to egg and larval quality based upon a suite of up to seven larval traits and through multivariate analyses between egg and larval traits. An analysis of scale was incorporated starting at the individual level (larvae) through nested general linear models and continuing through to population level (all females combined).

CHAPTER 1

MORTALITY OF INDUCED AND SPONTANEOUSLY OVULATED YELLOWTAIL FLOUNDER, *PLEURONECTES FERRUGINEUS* (STORER), EGGS DURING EMBRYOGENESIS

1.1. INTRODUCTION

Yellowtail flounder (*Pleuronectes ferrugineus*) has great potential for aquaculture because of its suitability for mass rearing and its good market presence. Past studies on yellowtail flounder ELH have centred on aspects of fecundity and batch variability (Zamarro, 1991; Manning and Crim, 1998), induced ovulation (Smigielski, 1979; Larsson et al., 1997), larval survival and growth (Rabe and Brown, 2000), and temperature and early development (Howell, 1980; Laurence and Howell, 1981). Few studies that focus on egg mortality exist despite Shelbourne (1956) suggesting that rearing experiments in the laboratory may provide insights into possible causes of natural death and that early mortality knowledge could be used to better understand the population dynamics of a particular species. In fact, Houde (1987) concluded that small changes in ELH mortality rates, that include mortality during the egg stage, could have a 10-fold or greater impact on future recruitment.

Many external factors can contribute to mortality during embryogenesis, such as mechanical stress (Shelbourne, 1956; Holmefjord and Bolla, 1988; Caberoy and Quinitio, 1998; Hilomen-Garcia, 1998) and bacterial contamination

(Kusuda et al., 1986; Pittman et al., 1990; Pavlov and Moksness, 1993). However, egg mortality could also be a function of differential egg development caused by factors inherent to the eggs themselves, such as availability of essential nutrients. These inherent factors may be affected by maternal effects (e.g., egg mass, female length or mass, or whether the female was induced to ovulate) and (or) genetic composition (e.g., physiological processes affecting developmental rates).

This study will concentrate on the effects of induced ovulation and temperature on the mortality of yellowtail flounder eggs. Do spontaneously ovulated (SO) females produce better quality eggs than induced ovulated (IO) females? Research into the direct effects of induced ovulation on egg mortality is lacking (Kjørsvik et al., 1990) but is needed if induced ovulation is to be used more frequently by aquaculturists to control the reproductive process. There are temporal, spatial, and financial benefits to aquaculturists in knowing as early as possible and under what condition batches of eggs will have lower mortality. In general, any decrease in mortality would reflect an increase in egg "quality" (Bromage et al. 1994). Assessing egg mortality during embryogenesis may also provide an earlier indicator of egg quality than hatching success.

Two null hypotheses will be tested: (1) IO eggs will not have significantly different mortality than SO eggs, and (2) incubation temperature will not significantly affect mortality.

1.2. Materials and Methods

Suitable quantitative methods for determining egg mortality in some marine fish are required because of fundamental differences in egg properties. For example, eggs that float have to be cultured and sampled differently than eggs that are neutrally buoyant or those that sink. Although sub-sampling techniques that involve stirring are adequate for some species they are not appropriate for yellowtail flounder eggs whose eggs are positively buoyant and float near the surface of the water. Stirring causes mechanical stress, to which yellowtail flounder eggs are susceptible, and introduces bacterial contamination from dead eggs that are re-suspended to the surface water. The use of AFDM of dead eggs to estimate egg mortality during embryogenesis reduces the chance of bacterial contamination and mechanical stress to live eggs from stirring, and may provide an earlier indicator of egg quality than hatching success.

1.2.1. Broodstock

Adult yellowtail flounder were collected by SCUBA divers or by trawling from offshore fishing vessels in 1994 and held at the Ocean Sciences Centre for at least 1 year prior to spawning. Males and females were held in tanks and supplied with ambient seawater (mean temperature = 4.5°C, range = 2 – 6°C, during the study period). In the period leading up to study, all fish received the same commercial moist feed and they resided in the same tank. Induced ovulated females were implanted in the dorsal musculature with a pellet

containing 100 μg in pellet form gonadotropin-releasing hormone analogue (GnRHa) and spontaneously ovulated females were given a control implant without GnRHa and allowed to ripen naturally (Larsson et al., 1997).

Because yellowtail flounder do not release eggs in captivity (Larsson et al., 1997, S. Bettles pers. comm.), they must be manually stripped by applying light pressure on the gonads. In all experiments, eggs were manually stripped from individuals and care was taken not to contaminate the eggs with faecal matter or seawater.

1.2.2.Egg AFDM

From 17–23 June, 1995 eight SO and eight IO females were manually stripped of their eggs and 3 mL of each batch of eggs were fertilised with 100 μL of undiluted milt, which was combined from 3–5 males according to Larsson et al. (1997). The fertilised eggs were placed in separate 25-cm glass petri dishes and incubated at 6°C in a controlled temperature incubator. A sample of 20, 25, or 50 eggs from each batch of eggs was taken on various days during development. The samples were rinsed twice with 3% ammonium formate solution to remove any adhering salts, rinsed twice with distilled water, placed in pre-weighed foil vessels, and dried at 60°C until a constant mass was recorded at three consecutive times. These vessels were then heated at 400°C for 24 hr and re-weighed. Mean egg masses were determined by dividing the total mass or AFDM of each sample of eggs by the total number of eggs in that sample.

Pre-spawning (before gonads were mature i.e. no hydrated eggs) length and wet mass was taken for all females. The relationship of development time, and female length and mass, to AFDM for both SO and IO eggs was determined by linear regression through the mean AFDM determined on each day. Analysis of variance (ANOVA) was used to determine the overall AFDM differences between SO and IO females.

1.2.3.Comparisons between eggs from SO and IO females

1.2.3.1.Treatment

Seven females were induced to ovulate intramuscularly with GnRHa pellets and eight females were allowed to spontaneously ovulate. All batches of eggs had volumes of eggs greater than 10 mL, with viability >85%, and fertilisation counts >60% (Table 1.1). Viability was defined as the percentage of unfertilised eggs that were transparent, perfectly spherical, floating and that lacked a premature perivitelline space (Hirose et al., 1979; McEvoy, 1984; Larsson et al., 1997). Stripped eggs were kept on ice at about 2°C until fertilisation. Each batch of eggs was fertilised with the milt from at least two males (30 μL). The fertilisation techniques followed those of Larsson et al. (1997). Fertilisation counts were calculated as the percentage of eggs that developed to the four-cell stage of embryogenesis. These criteria were used to ensure an adequate amount of eggs for dry mass analysis. Although many batches of eggs were stripped, only five batches from two IO females and 10

batches from seven SO females had adequate survival to follow from fertilisation to hatch.

1.2.3.2.Incubation

Fertilised egg batches were incubated at ~8°C separately in round (70 cm diameter), 60-L incubators and maintained as static systems (no air flow or constant water flow). Temperature was regulated by suspending the incubators in a flow-through water bath maintained at 8 ± 1°C until hatching. The water in the incubators was filtered through a 1-μm glass wound filter, passed by ultraviolet light, and initially treated with 0.1 gL^{-1} streptomycin sulphate and 0.06 gL^{-1} penicillin G to inhibit bacterial growth.

1.2.3.3.Sampling and analyses

Incubators were cleaned daily by siphoning dead eggs from the bottom of each incubator then slowly refilled, resulting in a daily water exchange of about 25%. Each siphoned sample was filtered through a 20-μm mesh screen, rinsed with 3% ammonium formate and placed in a pre-weighed foil vessel for drying at 60°C. Drying times were determined by re-weighing samples until no change in dry mass was recorded at three consecutive times (about 48–72 h depending on the size of the sample). Sampling stopped when the first larva hatched.

Mortality was determined by subtracting the calculated volume of eggs from the total ovulated volume of eggs. The calculated volume was determined by extrapolating the AFDM of a single egg (mean value as determined in AFDM

section) to the total dry mass of the siphoned samples and comparing this to the corresponding dry masses of known volumes of eggs for each batch. The relationship of cumulative mortality with development time was fitted to an exponential rise to maximum non-linear regression for both SO and IO eggs.

1.2.4.Mortality of eggs reared at two temperatures

1.2.4.1.Treatment

This experiment simulated the incubation of eggs in a large-scale aquaculture operation. Only eggs from spontaneously ovulated females were used. Batches of eggs were manually stripped from 4–12 females and combined. Four combined batches (samples) were kept on ice at about 2°C until fertilisation with 30 μL of milt mixed from 4–6 males. Sperm motility was >50% to >90% motility in all cases as determined through microscopic inspection. Each sample was then split into two replicates, one for incubation at 10°C and one for incubation at 4.5°C. A total of 4 samples (8 replicates) were used. Temperatures were chosen to correspond to the upper and lower limits for successful incubation of yellowtail flounder eggs as shown in previous experiments (T. Avery, unpublished data) and by Laurence and Howell (1981). Replicates were thus identical in composition, fertilisation, and handling time (mechanical stress) at a given temperature.

1.2.4.2.Incubation

Eggs were incubated in round (45 cm), 15-L black plastic containers. Four replicates of each paired sample were placed in a water bath, and maintained at a mean temperature of 10.6 ± 1.0°C (range 9.5–12.7°C), the 4 sister replicates were placed in a temperature controlled room and maintained at a mean temperature of 4.3 ± 0.4°C (range 3.5–4.9°C). The water in all incubators was treated with 0.1 gL^{-1} streptomycin sulphate and 0.06 gL^{-1} penicillin G on a daily basis to inhibit bacterial growth and about 20% water exchange occurred when incubators were refilled after siphoning.

1.2.4.3.Sampling and analysis

Sampling followed the procedures given for the comparisons between eggs from SO and IO females with one exception. Siphoned sample volumes were calculated directly from a standard regression of the dry mass of three known volumes (0.5, 1, and 2 mL) of eggs taken directly from each sample before splitting and incubation.

1.3. Results

1.3.1.Relationship of egg dry mass to development

Linear regression analysis showed a significant relationship between IO eggs and development time (r^2 = 0.178, p = 0.023, n = 29). However this was not reflected in the regression of SO eggs and development time (r^2 = 0.149, p =

0.173, n = 14) (Fig. 1.1). This result was most likely a function of a smaller sample size rather than a goodness of fit. A comparison of the overall group means was performed using an ANOVA with development time as a covariate. It showed that the mean SO AFDM (24.84 ± 0.73 μg) was significantly heavier than the mean IO AFDM (20.09 ± 0.54 μg) (F = 20.49, p < 0.001, n = 43).

1.3.2.Mortality of spontaneous and induced ovulated eggs

Non-linear regression revealed a significant exponential rise to a maximum relationship for both SO (r^2 = 0.988, p < 0.001, n = 65) and IO (r^2 = 0.990, p < 0.001, n = 43) eggs, using the equation form $y = a \cdot (1 - B^t)$ (Fig 1.2). The maximum IO mortality as determined by the equation was higher (69.21) than the maximum SO mortality (36.01), but no significant differences were found between mortalities on each day (p > 0.126 for each day). The inflection point of SO eggs was reached at about 1.2 days and for IO eggs at about 3 days. Therefore, since both mortality rates decreased by day 3, post 3-day mortalities were chosen to represent an early determination of egg quality. An ANOVA showed higher post 3-day IO egg mortality (54.05 ± 24.36%), compared with 32.99 ± 18.43% for SO eggs (F = 10.09, p = 0.003, n = 42).

1.3.3.Mortality at two temperatures

The regression equations for each pair of replicate (split) batches were nearly identical (Table 1.2). These standards were used to calculate the total number of dead eggs in each sample.

Non-linear regression revealed a significant exponential rise to a maximum relationship for both IO ($p < 0.001$) and SO ($p < 0.001$) eggs, using the equation form $y = a \cdot (1 - e^{(b \cdot x)})$ (Fig 1.3). The maximum mortality at 10°C as determined by the equation was higher (69.09) than the maximum mortality reached at 4.5°C (36.01). Hatching times were longer in the 4.5°C batches (331.1 ± 17.8 h) than in the 10°C batches (96.9 ± 11.5 h).

1.4. Discussion

The first hypothesis was based on the premise that inducing females to spawn should not affect the egg quality, since it is assumed that the egg constituents in yellowtail flounder are laid down prior to induction. Moreover, the timing of the induction process was undertaken just prior to oocyte hydration, which was used more to synchronise rather than to initiate spawning. A more in-depth discussion is given in Harmin and Crim (1992).

Understanding the physiological processes associated with induced ovulation are important in aquaculture as it may impact on egg quality. Induced ovulation is not new to aquaculture but has shown varying effects on egg quality criteria, such as fecundity, fertilisation rates, hatching rates, or larval morphology (Larsson et al., 1997; Manning and Crim, 1998). Earlier studies by Smigielski (1979), Crim and Glebe (1984), Ramos (1986), and Lee et al. (1987) showed poorer quality of eggs from induced females compared with controls (spontaneous ovulation), whereas De Leeuw et al. (1985), Peter et al. (1988), and Manickam and Joy (1989) showed better egg quality. However, none of

these studies used mortality during embryogenesis as an egg quality criteria. Therefore, the use of egg mortality during embryogenesis as a measure of egg quality may not be directly comparable to past studies, but it does provide a general characteristic of egg batches that can be detected about 3 days after fertilisation.

Differences in IO and SO cumulative mortality should be viewed with caution. Only five batches of IO eggs passed the viability selection criteria, with three batches coming from the same female. These three batches had quite different individual mortalities. Therefore, batch variability seems to have an effect on mortality. Batch variability has also been shown in other egg quality criteria studies. For example, Kjesbu (1989) found that the middle batches of eggs for Atlantic cod (*Gadus morhua*) were of better "quality" (based on total fecundity) than early or late batches. A distinct temporal batch relationship was not found in the current study, but batch variability may be partly confounding the mortality results. On the one hand, the mean mortality of IO eggs was not statistically higher than SO eggs on each day, thus providing reasonable evidence to suggest that induced ovulation does not cause significantly higher mortality, and hence an acceptance of the first hypothesis. On the other hand, the trend of higher mortality in IO eggs is obvious, as indicated by the difference in maximum mortality as calculated from the exponential rise to maximum equations. This coupled with IO eggs having significantly lower AFDM than SO eggs and the acceptance of the first hypothesis becomes questionable. It is more probable that mortality was due to the physical or biochemical

characteristics of the eggs. Increased mortality could be a direct consequence of a lack of resources during development, i.e., not enough total nutrients to sustain the egg through development or a lack of some egg constituent caused by an interruption in oocyte maturation, which was brought about by induction.

A recent study by Ako et al. (1994) found significantly higher amounts of fatty acids and essential amino acids, larger egg diameters, and higher fertilisation rates in naturally (spontaneously) spawned females of milkfish (*Chanos chanos*) compared with induced ovulated females. No significant differences were found in nonessential amino acids. Conversely, in that study, induced females produced more eggs per spawning event than naturally spawned females. The significant increases in the amount of egg constituents were not simply a consequence of size, since quantities were expressed as mg per 100 mg egg dry mass. Therefore, the increased egg diameters in naturally spawned individuals were not directly connected to the biochemical content, suggesting that induction has a significant effect on the biochemical properties of the eggs. The increased number of eggs produced per spawning event also suggested that induction might cause a minor shift in reproductive strategy.

The current study shows that inducing ovulation decreases the mean AFDM of eggs. Egg diameters have been previously positively correlated to egg dry mass in yellowtail flounder (Manning and Crim, 1998), therefore, AFDM measures and studies concentrating on egg diameters should be comparable. However, Manning and Crim (1998) used mean dry mass and mean egg diameter measures from all females combined, and their results therefore, do not

consider inter- or intra-individual variability in either variable: a caveat against which Chambers et al. (1989) warns. The problem is one of scale and how data are aggregated together at different levels. The present experiment also does not compare individual egg size to mortality but uses batches of eggs and the mean egg AFDM. Therefore, there is inconclusive evidence to suggest that egg size alone is the main factor contributing to mortality.

Maternal effects of length and mass within both the SO and IO groups were not considered since the range in female length and mass was not adequate to perform rigorous statistical analyses. However, female effects may have contributed to some of the IO and SO differences although Manning and Crim (1998) showed that yellowtail flounder fecundity and egg quality was highly variable and independent of female size. In contrast, Hislop (1988) showed that female length is more important than female age, based on the fecundity of haddock (*Melanogrammus aeglefinus*).. This suggests that the current range of female lengths and masses was not great enough to elucidate any underlying relationship, either because all females were older (suggesting an age-specific component) or because there is no correlation between egg mortality and female size in yellowtail flounder.

Clearly, the induction mechanism in yellowtail flounder is not fully understood and although it may produce reasonable results with respect to egg size, viability, fertilisation rates, and hatching success (Larsson et al., 1997; Manning and Crim, 1998), this is not the case with egg mortality during embryogenesis. The overlying, high inter- and intra-individual variability and the

analysis caveats mentioned by Chambers et al. (1989) obscure the true relationship of mortality, induced ovulation, and "egg quality". In fact, the presence of such high variability suggests that particular attention should be paid to differences in females and batch effects, especially when selecting broodstock.

The second hypothesis is based on the extent to which temperature will affect physiological processes (such as the metabolic rate) and their effect on the corresponding mortality. Temperature acts on internal physiological processes by affecting the rate at which biochemical reactions occur and are catalysed (Laurence and Howell, 1981). Secondary effects of temperature include increased bacterial contamination (Pittman et al., 1990) that would have to be controlled in order to obtain a reasonable assessment of temperature effects on mortality. All else being equal, the expected result of an increase in temperature should be an increase in developmental rate only.

An increase in temperature had little effect on the cumulative mortality of yellowtail flounder eggs, but did increase the mortality rate (linear portion of the cumulative mortality curve) slightly in eggs raised at 10°C. In contrast, Pittman et al. (1990) showed significantly different cumulative mortality in egg incubation and larval-rearing of halibut (*Hippoglossus hippoglossus*) raised at three temperatures (3, 6, and 9°C). Eggs incubated at 3°C and 9°C had higher cumulative mortalities at hatch than those raised at 6°C. In comparison, Dwyer (1987) found no significant differences between the mortality of lake trout (*Salvelinus namaycush*) eggs incubated at three temperatures (1.8, 6.4, and

9.8°C). In both of these studies, the methodologies did not adequately indicate the degree of filtration, whether the tank systems were flow-through or static, or whether antibiotics were used; therefore, it is impossible to determine the degree to which bacterial contamination was a factor. The use of antibiotics in the current study did not eliminate bacterial growth, but it seemed to be successful in reducing or inhibiting bacteria to a level that did not adversely affect the eggs.

The second hypothesis is accepted. The only obvious factor affected by temperature was the development rate of the eggs, which was three times longer for the eggs reared at 4.5°C than those raised at 10°C. This developmental rate difference was expected and was similar to that found by Laurence and Howell (1981). They found an almost twofold increase in time to 50% hatch for yellowtail flounder of 132 h at 10°C and 224 h at 4.5°C, based on the equation 50% hatch = 501.86 · temperature$^{-0.58}$. However, their results were in concert with various salinity measures that may account for the differences between their values and those herein. Devauchelle et al. (1988) showed a similar twofold decrease in incubation time (135–75 h) with a 6°C change in temperature (13–19°C) for the turbot (*Scophthalmus maximus*), and Wanzenböck and Wanzenböck (1993) had similar results with white fin gudgeon (*Gobio albipinnatus*).

1.5. Summary

Variability in serially batch spawning fish is common and must be factored into any culture situation. Variability is seen in batch volumes and fertilisation counts for the Atlantic halibut (*H. hippoglossus*) (Norberg et al., 1991) and for

yellowtail flounder (Manning and Crim, 1998), which suggests that batch production in serial-spawning flatfish has a different role in the reproductive strategies of these fish. Induced ovulation may reduce the variability in some ontogenetic or reproductive event, for example, the co-ordination of spawning, but from the present data it seems that inducing ovulation may not be effective if IO mortality is consistently higher and eggs from IO females consistently weigh less. From an egg quality standpoint, based solely on mortality, it seems to be more beneficial to allow females to spontaneously ovulate.

Mortality during embryogenesis seems to be a reasonable indicator of egg quality. Bromage et al. (1994) state that parameters of quality should provide reliable predictions of performance, should be easy to perform, and should be able to be carried out as soon as possible after stripping to avoid unnecessary hatchery costs associated with rearing unproductive batches of eggs. The dry-mass technique of the current study reflects all of the proposed criteria of a good quality parameter and adds the benefit of being non-invasive (no mechanical stress to the eggs). The technique would be especially useful if egg batches were incubated separately, as individual batch quality would be able to be assessed.

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Table 1.1. Batch characteristics of females for comparison of induced ovulated and spontaneously ovulated females.

Female	Date	Batch # (of total #)	Batch Volume (mL)	Viability (%)	Fertilisation Success (%)
Spontaneous Ovulated					
3P	29-Jun	16 (41)	20	88.7	64
3P	02-Jul	19 (41)	17	89.3	83
3B	21-Jul	12 (17)	12	97 7	84
3W	13-Jul	8 (11)	31	96 7	62
ACH	22-Jun	5 (10)	55	92 3	81
Annab	13-Jul	3 (25)	41	94 0	76
Annab	21-Jul	10 (25)	55	72.0	32
Cass	27-Jul	11 (30)	31	94 3	44
Isis	02-Jul	8 (22)	39	84 7	-
Isis	12-Jul	17 (22)	38	94 7	92
Induced Ovulated					
4P	25-Jun	11 (33)	32	86 3	72
4P	29-Jun	15 (33)	41	95 3	80
4V	27-Jun	8 (33)	20	92 3	63
4V	30-Jun	11 (33)	27	97 7	63
4V	19-Jul	30 (33)	21	96.7	81

Table 1.2. Standard regression equations calculated from the dry mass (mg) of known volumes (mL) of eggs from spontaneously ovulated yellowtail flounder.

Egg batch number	Standard regression equation	r^2
1	Volume = 54.9 · dry mass + 0	0.856
2	Volume = 54.4 · dry mass + 0	0.960
3	Volume = 58.5 · dry mass + 0	0.999
4	Volume = 57.0 · dry mass + 0	0.996

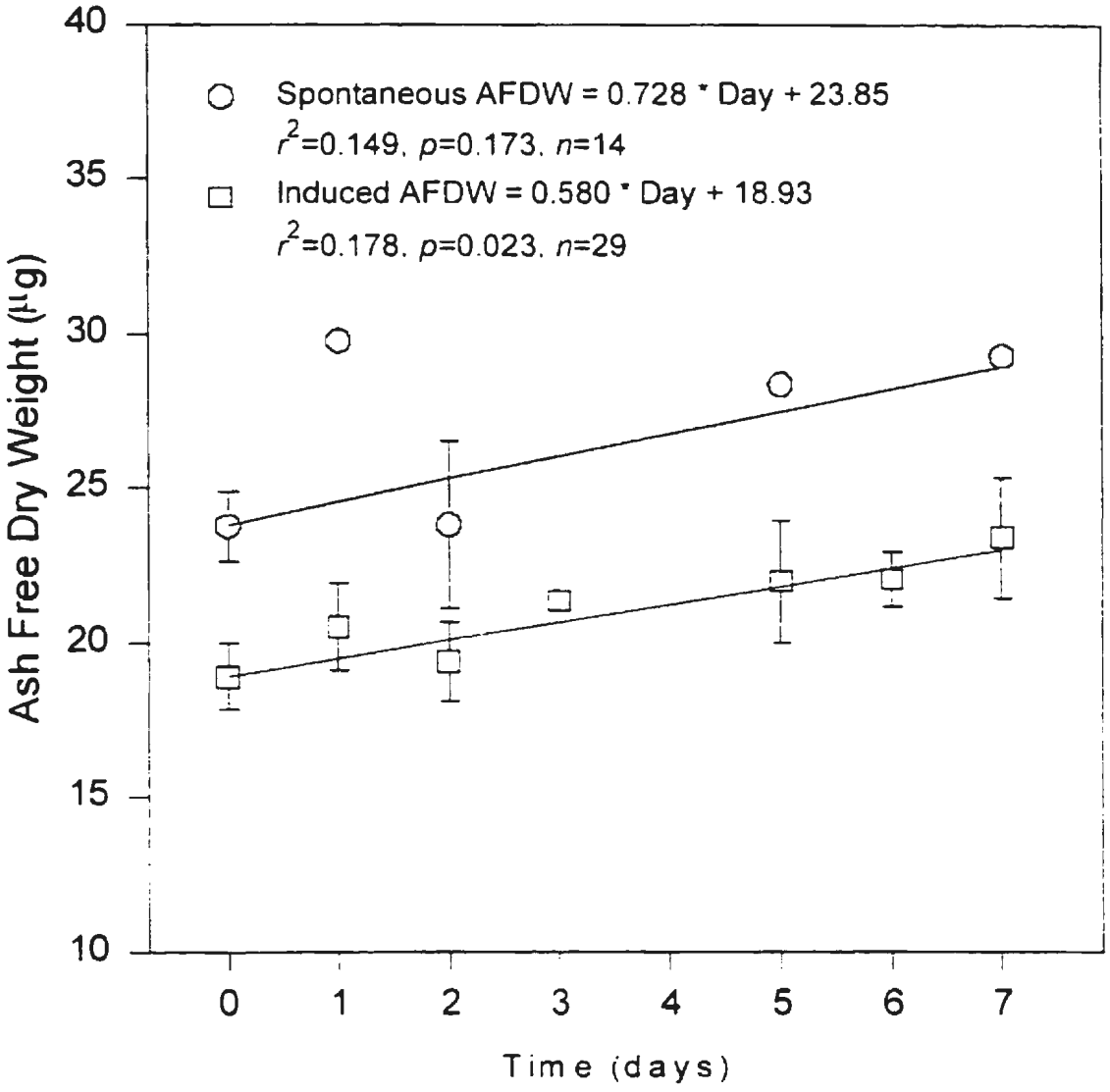

Figure 1.1. Ash free dry mass (AFDM) of eggs during embryogenesis for both hormonally induced ovulated (squares) and spontaneously ovulated (circles) yellowtail flounder. Data points represent the mean (±SD) AFDM.

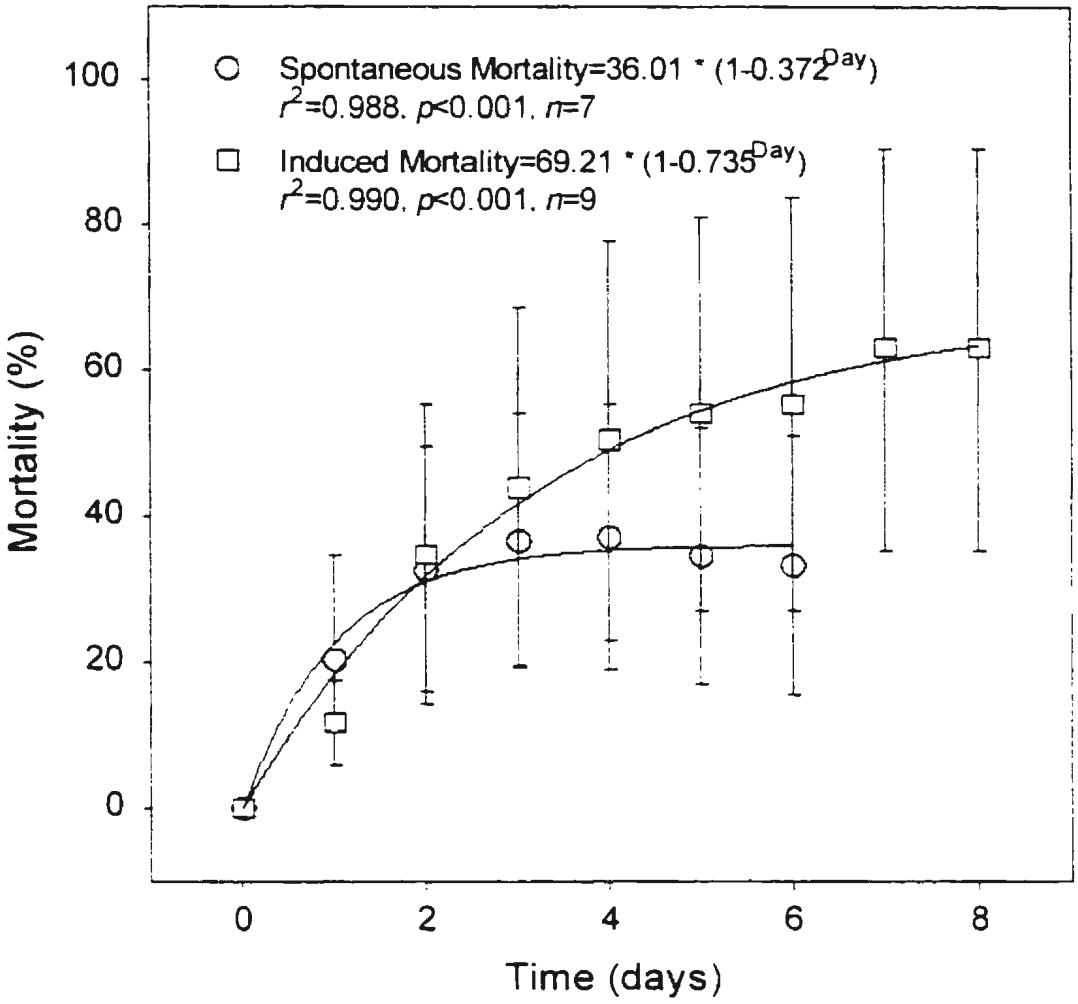

Figure 1.2. Mortality relationship between hormonally induced ovulated (squares) and spontaneously ovulated (circles) yellowtail flounder eggs. Data points represent the mean (±SD) mortalities.

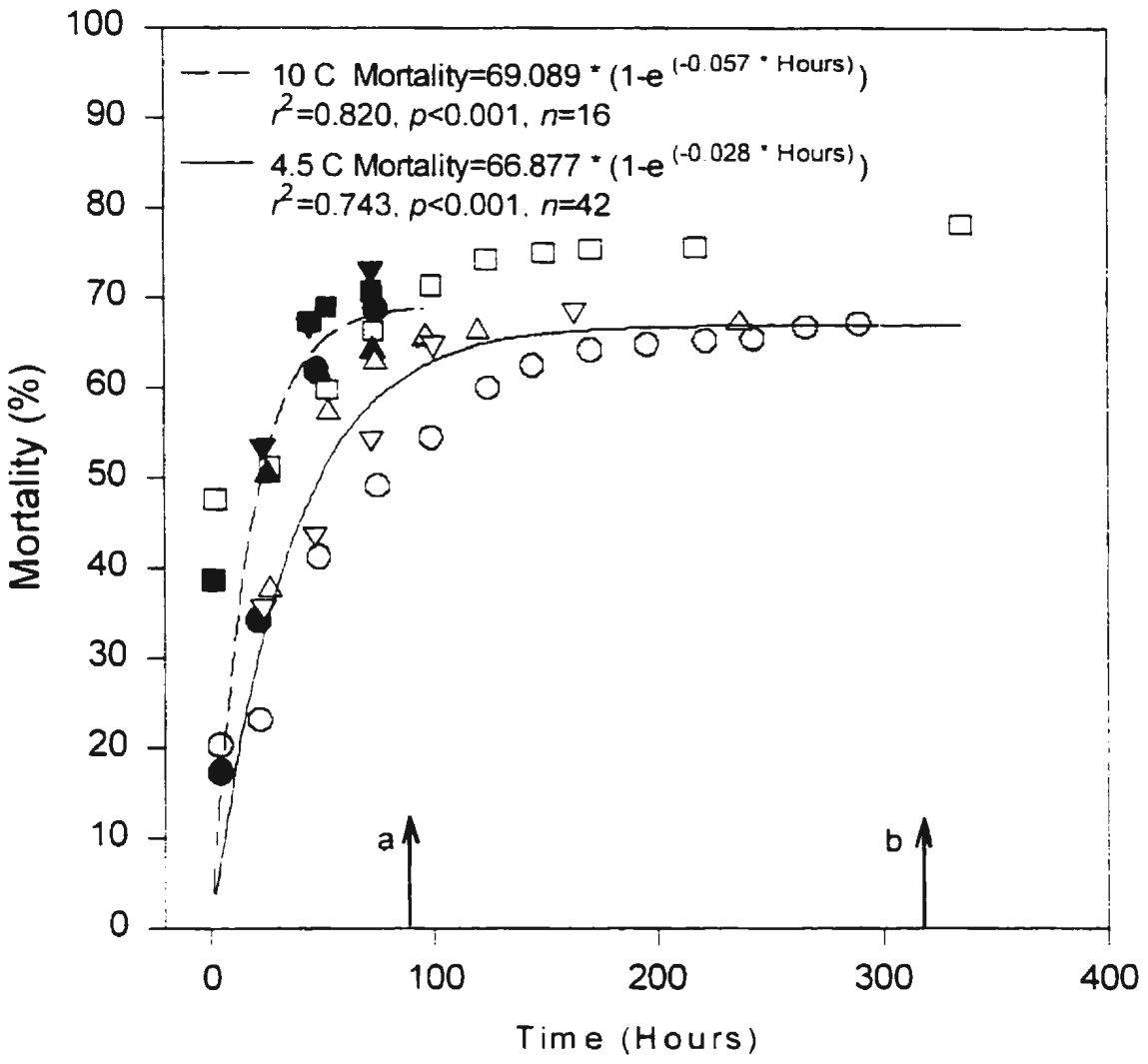

Figure 1.3. Comparison of mortality and development time of batches of eggs from spontaneously ovulated yellowtail flounder raised at two temperatures. Open symbols (4.5 C) and solid symbols (10 C) of the same type correspond to split batches of eggs. Data points represent individual batch mortalities. Arrows represent mean hatching times: *a* = eggs raised at 10°C and *b* = eggs raised at 4.5°C.

Chapter 2

Inter- and intra-female variability of biochemical and morphological measures in the eggs of yellowtail flounder, *Pleuronectes ferrugineus* (Storer)

2.1. Introduction

Chambers et al. (1989) summarised that eggs grouped together (e.g., small versus large), simple correlations of traits, or the confounding of egg size with other factors such as female age, length, or mass are three reasons why the often used egg quality generality that larger eggs produce larger larvae that live longer without food (Blaxter and Hempel, 1963; Kjørsvik et al., 1990; Litvak and Leggett, 1992) is not axiomatic. No evidence was found to support the claim that larger eggs produce larger larvae when data were analysed at the individual larval level where eggs were raised separately (Chambers et al., 1989). Therefore, variability within and among individuals should be incorporated to any analyses that involve intra- and inter-individual variability. Proper exploration of data requires that tests for significance among factors on an individual basis be done prior to grouping individual data; something that is often not reported or not done.

In addition, using mean values is not appropriate in regression analyses when a functional relationship or a central trend is sought between two factors and both factors have measurement variability (Ricker, 1973, 1975). The correct analysis to use is a geometric mean or Model II regression procedure (Ricker, 1973, 1975; Jolicoeur, 1975), but a common analysis approach to use is the

mean values of one trait as "groups" for the abscissa. This tactic excludes variability and the data are often pooled at a level higher than the level at which conclusions are drawn.

Yellowtail flounder (*Pleuronectes ferrugineus*) is an important commercial groundfish stock and has shown recent success as an aquaculture species. As such, aspects of recruitment variability, egg quality, and broodstock management should be explored to better understand the dynamics of this species in the North Atlantic and its suitability for captive breeding and rearing. Larsson et al. (1997) and Manning and Crim (1998) have shown inter- and intra-individual variability in batch fecundity, egg viability, and fertilisation and hatching rates. Manning and Crim (1998) showed a significant positive correlation between egg dry mass and egg diameter and a significant negative correlation between egg diameter and time in the spawning season using data at the population level, which was obtained by pooling individual-level data. Similar relationships have been shown for other marine fish species (Bagenal, 1971; Ware, 1975). However, individual variability is obscured in many of these analyses by using mean values or by pooling individual-level data (Pepin and Miller, 1993; Chambers and Leggett, 1996), thereby concealing the individual-level variability upon which natural selection acts (Sober 1984). Consequently, the use of population-level data in recruitment processes has been questioned (Bertram et al., 1997).

The present study will determine the inter- and intra-individual variability in and among females of yellowtail flounder using two common egg quality criteria,

dry mass and egg diameter, and two biochemical measures, carbon and nitrogen content. Carbon and nitrogen content were used instead of lipid content as CHN analysis is less expensive and easier to conduct than lipid analysis. Moreover, CHN analysis provides an estimate of carbohydrate and lipid content. In addition, I was interested in establishing that egg dry mass has some underlying relationship to biochemical content of carbon and nitrogen. I postulate that the biochemical measures will be a better measure of egg quality because of the increased precision afforded by measuring individual components of the eggs rather than all components combined as represented by egg dry mass. In addition to providing general physical and biochemical characteristics of yellowtail flounder eggs and exploring the relationships between these traits both within and among different aggregate levels of data, the impact of using Model I regression analyses rather than Model II regression analyses will be discussed.

2.2. Materials and Methods

2.2.1. Broodstock and batches of eggs

Adult yellowtail flounder were collected by SCUBA divers or by trawling from offshore fishing vessels and held at the Ocean Sciences Centre for at least 1 year prior to spawning. Females were held in a common 1 m square, 400-L tank supplied with degassed, filtered seawater and additional aeration. A natural photoperiod was maintained through natural and artificial lighting. Some temperature regulation was available through the addition of cooled seawater. The mean temperature was 8.7 ± 1.4°C (range 5.8–10.3°C) during egg collection

with only the first day (5.8°C) being below 8.8°C. This temperature agrees with the minimum temperature (~6°C) observed for the onset of spawning in captive yellowtail flounder (Larsson et al., 1997; Manning and Crim, 1998). All fish received the same commercial moist feed in the period leading up to the commencement of the study. Fish were not fed during manual stripping to prevent faecal contamination of the eggs.

Four to six batches of eggs were collected from each of five females ranging in length from 29 to 36 cm and in mass from 242 to 414 g from 9 July, 1996 to 22 July, 1996. Females were checked and (or) stripped every second day between 09:00–11:00 h. Batch volumes were all greater than 18 mL, except for one batch (5 mL). Egg viabilities were all greater than 43% as defined by the percentage of unfertilised eggs that are transparent, spherical, floating, and that lacked a premature perivitelline space (Hirose et al. 1979; McEvoy, 1984; Larsson et al., 1997). These batches represented a reasonable cross-section of yellowtail flounder egg batches as presented in Larsson et al. (1997) and Manning and Crim (1998) and they did not deviate noticeably from previously reported values for volumes or viability (referred to as gamete potential in Manning and Crim (1998)).

Manning and Crim (1998) suggested that captive yellowtail flounder have a spawning periodicity of 1–2 d with 1 d being the most common interval. Zamarro (1991) hypothesised that yellowtail flounder have a shorter periodicity of only 12–24 h based on observed batch frequencies from similar species. However, Zamarro (1991) also suggests that the periodicity may be linked to

temperature and that yellowtail flounder raised in higher temperatures would probably have longer inter-ovulatory periods. Selecting a 2-d stripping schedule limits the handling of the females and provides a better chance of obtaining a true ovulation. On the other hand, a lengthy interval between stripping batches may produce more overripe eggs. Judging by the initial measure of egg quality (viability), the size of the batches and the regularity in batch production, a 2-d schedule was used.

2.2.2.Carbon, nitrogen, dry mass, and egg diameters

Four egg quality measures were employed in the study: carbon content (C), nitrogen content (N), dry mass, and egg size (diameter). Carbon and nitrogen measurements were obtained because they are relatively easy to obtain and provide an estimate of lipids and proteins. Each batch of eggs was fertilised according to Harmin and Crim (1992), with modifications as in Manning and Crim (1998). The same male was used to fertilise all batches of eggs except for 1 d. Fertilised eggs were used for all measurements and biochemical determinations because of the difficulties in handling unfertilised eggs and their susceptibility to leakage (Manning and Crim, 1998).

Three to six sub-samples of 20, 25, or 50 eggs from each batch of eggs were taken for dry mass and carbon-hydrogen-nitrogen (CHN) analysis. Mean values were calculated from the CHN sub-samples and were used in the analyses. The samples were rinsed twice with 3% ammonium formate solution to remove any adhering salts, placed in pre-weighed and HPLC-grade acetone

rinsed silver vessels, and dried at 60°C until the mass was constant. The silver vessels were then analysed in a CHN analyser (Perkin Elmer, Elemental Analyzer 2400CHN, Connecticut, USA). Silver vessels have background noise levels of zero for both N and H and a signal to noise ratio of 10:1 for C (Europa Scientific Inc., D2001, Cincinnati, Ohio), making them a superior choice for the small quantities of eggs used in this study. Corresponding egg diameters from each batch were calculated as the mean of 30–62 eggs. Egg diameters were determined using an Olympus microscope at 40x magnification.

Relationships between C, N, dry mass, and egg diameter were determined using both Model I and Model II linear regression analyses. Residual analysis was performed on all regressions.

2.2.3.Comparisons within and among females: individual versus population

The C, N, dry mass, and egg diameters were compared on a batch (within) and female (among) basis. The results of a Model I regression analysis were compared to the results of a Model II regression analysis where appropriate. A generalised linear model (GLM) was used to determine if significant differences existed between batches within females and among females, and to determine the relative proportion of variability of each. Coefficients of variation (CV) were calculated using the mean and standard deviation of all egg diameters, dry mass, C, N, and C/N values. All dry mass, C, N, and C/N values were standardised for egg volume before CVs were calculated. Residual analysis was performed on all regressions and GLMs.

2.3. Results

2.3.1. Carbon, nitrogen, dry mass and egg diameters

Since egg dry mass was found to be close to significantly related to egg diameter (r^2 = 0.133, F = 4.53, p = 0.045) the use of egg diameter as a covariate was tested by the homogeneity of slopes procedure. The interaction term tested for each model was egg diameter · independent variable. Homogeneity of slopes was shown for dry mass compared with C (p = 0.869) and for C compared to N content (p = 0.297), therefore egg diameter was used as a covariate for both models. The C content and dry mass were significantly related (r^2 = 0.861, F = 116.24, n = 24 p < 0.001) and correlated (Fig. 2.1). A significant relationship was also shown for C and N (r^2 = 0.962, F = 486.62, n = 24, p < 0.001) (Fig. 2.2). Model II regression parameters were not noticeably different from Model I parameters in either case (Table 2.1).

Linear regression across batches and females (population level) showed that C and N content were not related to egg batch number (p = 0.266 and 0.118, respectively) (solid lines in Fig. 2.3). Carbon and N content per batch was relatively constant within individuals, with no significant relationships (p > 0.05 for each female), except for batch 6 for female B. The dry mass of an individual egg for batch 6 from female B was the lowest of all batches (20.04 ± 0.904 g), but the egg diameter of 0.924 ± 0.025 mm was not noticeably different than the grand mean of 0.934 ± 0.019 mm.

Coefficients of variation for C and N were slightly higher than for dry mass with all samples standardised by egg volume (Table 2.2). Egg diameter showed a relatively low CV compared with dry mass, C, and N. The mean C/N ratio for all samples was 4.08 ± 0.054, with a corresponding CV of 1.31% showing a high protein content that is relatively constant in proportion across the eggs.

2.3.2. Variability within and among females

Inter- and intra-individual (female) variability was determined using dry mass only because of the high correlation between dry mass, C, and N. Dry mass is significantly affected by both individual females and batches within females, with variation among females about three times that of batches within females (Table 2.3). A Tukey-Kramer pairwise comparison performed to minimise the Type I experiment-wise error rate at an α level of 0.05 showed that females C and E had significantly higher dry mass than females A, B, and D. Female labels are ordered according to female mass from A (lightest) to E (heaviest). Female C (267 g) produced significantly heavier eggs than female D (337 g). Linear regressions of dry mass with egg diameter performed on individual females were all not significant, however, the overall linear regression was significant (r^2 = 0.171, F = 4.53, p = 0.045) (Table 2.4, Fig. 2.4), although marginally. The corresponding Model II regression on all mean data combined (population level) was noticeably different from the Model I regression (Table 2.4, Fig. 2.4).

Excluding batches 10 and 11 as they had only 1 sample, the CVs for egg diameter for each batch were all less than the corresponding CVs for dry mass and were considerably less variable within and among batches (Figs. 2.5a and 2.5b). Batch 7 and 8 are of particular interest as they show extreme variation in dry mass with relatively little variation in egg diameter. The CVs of egg diameter and C/N ratio remained relatively constant among females, but the CVs for dry mass, C, and N were highly variable (Fig. 2.5c). Furthermore, CVs generally increased as females increased in mass.

2.4. Discussion

2.4.1. Assessment of early life-history traits

Bromage et al. (1994) state that "mean values, even with appropriate confidence intervals, can never truly represent the full range of egg quality in a stock of broodfish." This statement should be obvious, but many studies neglect individual variability when assessing egg quality criteria. Broader relationships between factors at aggregate levels of organisation above those upon which conclusions are drawn may mask the underlying relationships that are of greater importance. For example, consideration of the variability within and among females is important for broodstock management (Bromage et al., 1994) and the information obtained by observations at the broodstock or larval level may provide important insights into population dynamics (e.g., Pepin and Meyers, 1991; Miller et al., 1995; Chambers and Waiwood, 1996). Conversely, population-level dynamics are generally not reflected at the individual level (see

Pepin and Miller, 1993; Rice et al., 1993) possibly because of differences in individuals (Chambers, 1993).

Development, survival and growth rate are critical parameters in population dynamics (Sissenwine, 1984; Chambers et al., 1988), and these can be assessed through studying early life-history traits at both the population and individual levels. The current study shows high variability in egg quality traits for yellowtail flounder and significant differences among individuals that appear not to be linked to female size. At the individual female level, increasing egg size does not produce heavier eggs nor does an increase in female mass produce consistently better eggs. In fact, as female mass increases, the variation in egg traits also increases. However, the range of females may not be enough to produce a well defined relationship. Furthermore, the variability associated with egg size is smaller than egg dry mass for corresponding batches suggesting that dry mass may better reflect the actual amount of available nutrients than does egg diameter. In addition, dry mass is more variable than egg diameter across batches and within 60% of the individuals. This suggests that egg size is not a clear representative of nutritional content for yellowtail flounder.

Selman and Wallace (1989) provide a review of egg growth in teleosts and summarise that >80–90% of dry mass is composed of vitellogenin, whereas Kjesbu et al. (1996) found that the water content of Atlantic cod (*Gadus morhua*) eggs is 92–94%. This indicates that dry mass would be a better indicator of nutritional content than egg diameter, and thus, is a better egg quality criterion; a view implicitly held by Kjesbu et al. (1996) where they focussed on dry mass

rather than egg diameter. Therefore, how did egg size (diameter) become so firmly entrenched as a good egg quality criterion when the often referenced review by Kjørsvik et al. (1990) summarised that under controlled conditions (laboratory) egg size has no direct implications on overall egg quality in rainbow trout (*Salmo gairdneri*), Atlantic salmon (*Salmo salar*), catfish (*Claria macrocephalus*) and carp (*Cyprinus carpio*). In marine fish species, the relationship between egg size and egg quality is less clear. The relationship is most probably the result of the rapid water uptake that occurs concomitant with egg maturation, which accounts for three to four-fold increases in egg volume (Selman and Wallace, 1989 and references therein). The processes of hydration, vitellogenesis, and egg maturation are under some environmental, physiological, and genetic influence that seemingly would contribute to variations observed in early life-history trait distributions. These effects are much less understood in multiple batch spawning teleosts.

Miller et al. (1995) have shown that temperature exerted a significant effect on egg diameter and larval size in Atlantic cod. Similarly, Benoît and Pepin (1999) found yellowtail flounder larval length to be temperature dependent. Chambers and Waiwood (1996) found similar environmental results, including an apparent adaptive response that results in larger Atlantic cod eggs being produced to increase egg buoyancy implying that salinity may affect egg size. They also found significant and varied differences in egg sizes among females, but no apparent relationship between egg size and female length or mass. Similar results were found for yellowtail flounder (Manning and Crim, 1998).

Although similarities exist between Atlantic cod and yellowtail flounder, these results cannot be extended to all species. The paradigm status of the "bigger is better" hypothesis is one example of how specific results become axiomatic even when only tested on a small number of species.

There was no relationship found between egg diameter and dry mass at the individual female level. This finding coupled with significant batch variability at about a third of the variability associated with individuals suggests that particular attention should be paid to individuals compared with batches or egg size. This is consistent with Bromage et al. (1994) and the results for Atlantic cod (Chambers and Waiwood, 1996). In contrast, there was a significant relationship at the population level (all females combined) illustrating that higher level aggregates of data do not necessarily represent what is occurring at lower levels. For example, Chambers et al. (1989) concluded that there was no relationship between egg size and other early life history traits in the capelin (*Mallotus villosus*) when the analyses were performed on an individual larval basis. More importantly, they state that relationships based on average trait values do not appropriately compare the traits that are generally in question. The generalisation that larger eggs produce larger offspring that survive longer (e.g., Blaxter, 1969; Hempel, 1979; Hunter, 1981; Rothschild, 1986) may be a function of experimental design and (or) the neglect of confounding factors that are not fully considered in the analyses (Chambers et al., 1989).

2.4.2.Regression models

The overall regression analyses show significant Model I and Model II relationships between egg size and dry mass. What analysis is appropriate depends on what information is wanted. Model I regression analysis is beneficial for predictive models (Ricker, 1973) where mean dry mass can be obtained from a known egg diameter. However, population dynamics rely on functional relationships that take into account variability in all factors, thereby making a Model II regression analysis more appropriate. Sokal and Rohlf (1995) state that under conditions where both x and y are variable, the Model I regression coefficient is expected to be lower in absolute value than the true slope of the functional relationship. This was shown to be the case for the population-level regression comparison in the current study. Model I should not be used in these types of analyses if only to satisfy the assumption of the independent variable being controlled by the investigator (Laws and Archie, 1981). Violation of this assumption should be investigated to determine its sensitivity to regression analyses when using mean level values and how this affects the functional and predictive relationships. This point is particularly important when using population-level relationships to infer results at the individual level (Pepin and Miller, 1993; Chamber and Leggett, 1996).

So why do so many investigations use a Model I regression analysis when Model II regression analysis is required? The mathematics needed to obtain the results from Model II regressions are rather more complicated than the

calculations required for a Model I regression although confidence limits are readily calculable. Current statistical packages generally do not incorporate a simple means to calculate Model II coefficients or confidence limits. There is also an overwhelming breadth of knowledge and literature on how to use Model I regression analysis in biological data most likely because Model I regression analysis are easier to understand, calculate, and compare with one another. Furthermore, simple textbook cases of regression analysis are generally more concerned with the predictive model rather than the functional case. These are strong reasons why studies generally do not use the Model II procedure, but that does not make the Model I analysis correct. The grouped values or arbitrary creation of x values based on some qualitative trait mentioned by Chambers et al. (1989) is reflective of the problem. Misleading results can be obtained when data are pooled in such a manner (Pepin and Miller, 1993; Bromage et al., 1994; Chambers, 1993; Chambers and Leggett, 1996; Miller et al., 1995; Bertram et al., 1997).

2.5. Summary

Eggs generally contain a high proportion of water that may affect egg size to a greater degree than previously thought. The assumption that larger eggs have more nutritional material must be shown *prior* to egg size being used as an egg quality measure. In addition, one cannot assume that an egg size and nutritional content relationship is transferable between species as egg quality measures are generally species specific (e.g., Bromage et al., 1994).

Consequently, dry mass, specifically C and N content, may be considerably more precise as an egg quality criterion, especially if egg size via hydration is affected by environmental factors.

No significant relationships were shown between females and early life-history traits at the individual level, but significance was obtained at the population level. In itself, this result is not a problem as population trends are useful information. Problems occur when the population trends are applied to the individuals. In recent years many more studies have focussed on the variability in the individuals because the application of population-level trends to individuals assumes an underlying randomness in the trait in question, which may not be the case as individuals are not all the same.

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Table 2.1. Comparison of Model I and Model II linear regression parameters for the relationship of carbon (C) with dry mass (DM), C with nitrogen (N), and egg diameter (ED) with dry mass for 25 batches of yellowtail flounder eggs from five females.

Abscissa	Ordinate	Coefficient	Model I	Model II
C	DM	a	5.91 (-4.49, 16.3)	8.45 (0.513, 16.4)
		b	1.90 (1.65, 2.16)	1.84 (1.65, 2.04)
C	N	a	-0.294 (-1.04, .452)	-0.139 (-0.798, 0.520)
		b	0.252 (0.234, 0.271)	0.249 (0.233, 0.265)
ED	DM	a	-3.29 (-32.4, 25.9)	-45.6 (-71.6, -19.7)
		b	32.2 (1.01, 63.3)	77.5 (49.3, 105.6)

Note: Values in parentheses are the 95% confidence limits.

Table 2.2. Summary of sample values (mean ± SD), and coefficients of variation (CV) for egg diameter (ED), dry mass (DM), carbon (C), and nitrogen (N) content of yellowtail flounder eggs from four to six replicate batches from five females.

Female	ED (mm)	DM (µg)	C (µg)	N (µg)	C/N
A	0.927±0.00812 (0.876)	25.4±0.668 (2.63)	12.4±0.334 (2.70)	3.06±0.102 (3.32)	4.04±0.0557 (1.38)
B	0.935±0.0135 (1.45)	25.7±0.370 (1.44)	12.6±0.311 (2.47)	3.06±0.108 (3.54)	4.12±0.0462 (1.12)
C	0.933±0.00875 (0.938)	28.1±1.29 (4.59)	13.8±0.522 (3.78)	3.37±0.137 (4.06)	4.09±0.0501 (1.23)
D	0.913±0.00707 (0.775)	25.9±0.202 (0.778)	12.6±0.436 (3.45)	3.07±0.124 (4.05)	4.11±0.0334 (0.812)
E	0.964±0.0101 (1.04)	28.1±1.08 (3.84)	13.6±0.786 (5.80)	3.35±0.228 (6.81)	4.05±0.0470 (1.16)

Note: Values in parentheses are the coefficients of variation (CV%).

Table 2.3. (*a*) GLM of batches nested within individuals (females) for the dry mass of eggs from yellowtail flounder.

Source	*df*	Corrected *df* *	SS	MS	*F*	*p*-value
Model	23	23	271	11 8	21 1	<0 001
Female	4	4	186	46 6	13 0	<0.001
Batch	19	19 18	69.7	3 57	6.56	<0 001
Error	109	109	60 9	0 559		
Total	132	132	332			

(*b*) Results of a Tukey–Kramer pairwise comparison at $\alpha = 0.05$, with a line clustering groups that are not significantly different.

Female	A	B	D	C	E
Mass (g)	242	249	337	267	414

* The corrected degrees of freedom (*df*) for the proper denominator since both female and batch were random factors in the model.

Table 2.4. Comparison of linear regression equations for dry mass (DM) and egg diameter (ED) for five female yellowtail flounder.

Female	Regression equation	r^2	*p*-value
A	DM = – 4.55 ED + 29.7	0.0031	0.930
B	DM = –11.5 ED + 36.5	0.177	0.579
C	DM = –17.2 ED + 44.2	0.0136	0.826
D	DM = – 4.01 ED + 39.6	0.0197	0.860
E	DM = – 37.4 ED + 64.1	0.122	0.564
Overall (Model I)	DM = + 32.2 ED + 3.29	0.172	0.044*
Overall (Model II)	DM = + 77.4 ED – 45.6	n/a	n/a

Note: Results of the overall Model I and Model II linear regressions are provided to show the change in slope and to compare the predictive (Model I) and functional (Model II) regression models. The Model I analysis is generally used when a Model II analysis is required.

* significant at α=0.05

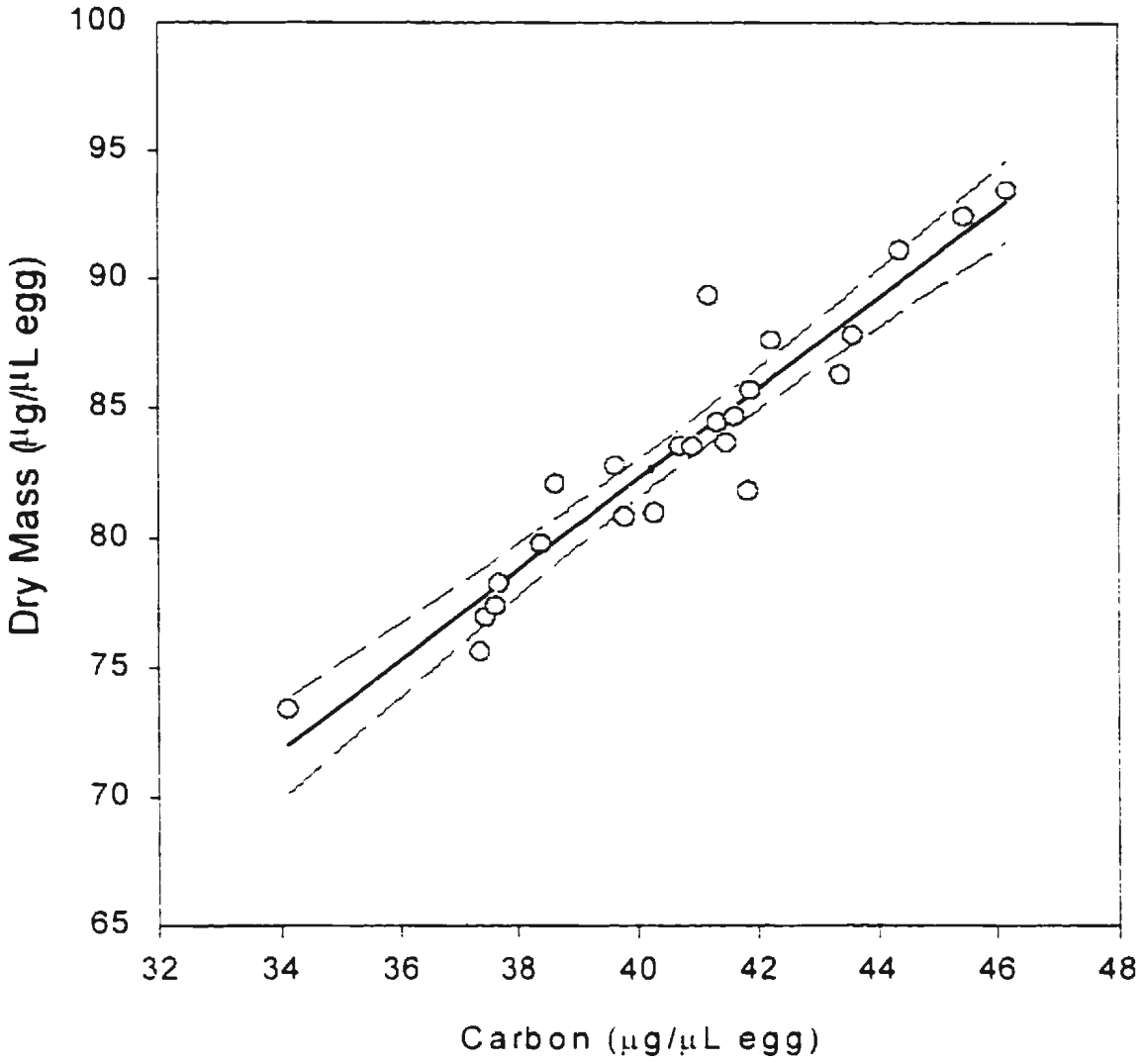

Figure 2.1. Relationship of carbon content (µg/µL/egg) to dry mass (µg/µL/egg) of yellowtail flounder eggs from five females. The solid line represents the Model I linear regression with 95% confidence intervals (short dash lines) and the dotted line represents a Model II regression.

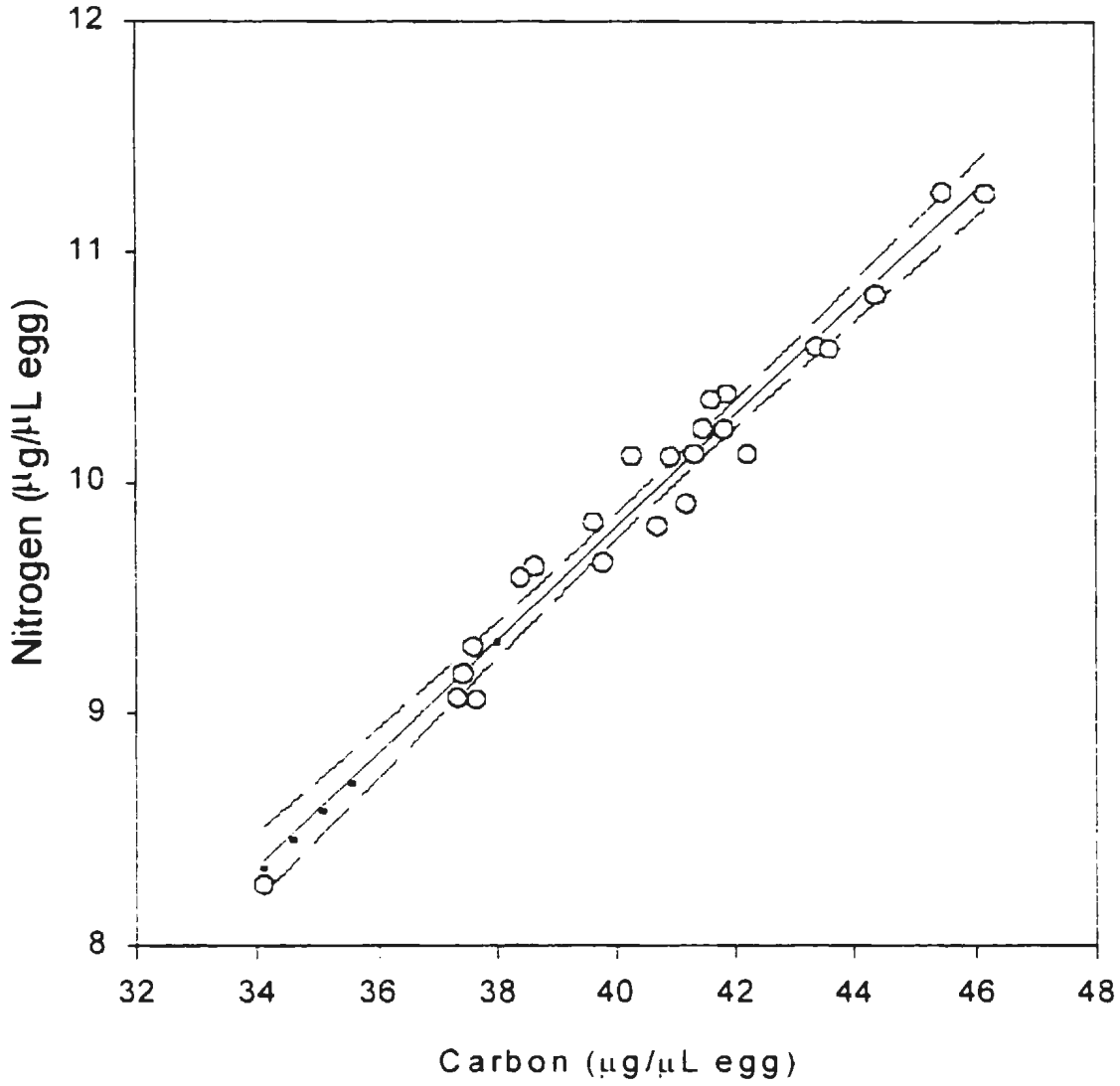

Figure 2.2. Relationship of carbon content (µg/µL/egg) to nitrogen content (µg/µL/egg) of yellowtail flounder eggs from five females. The solid line represents the Model I linear regression with 95% confidence intervals (short dash lines) and the dotted line represents a Model II regression.

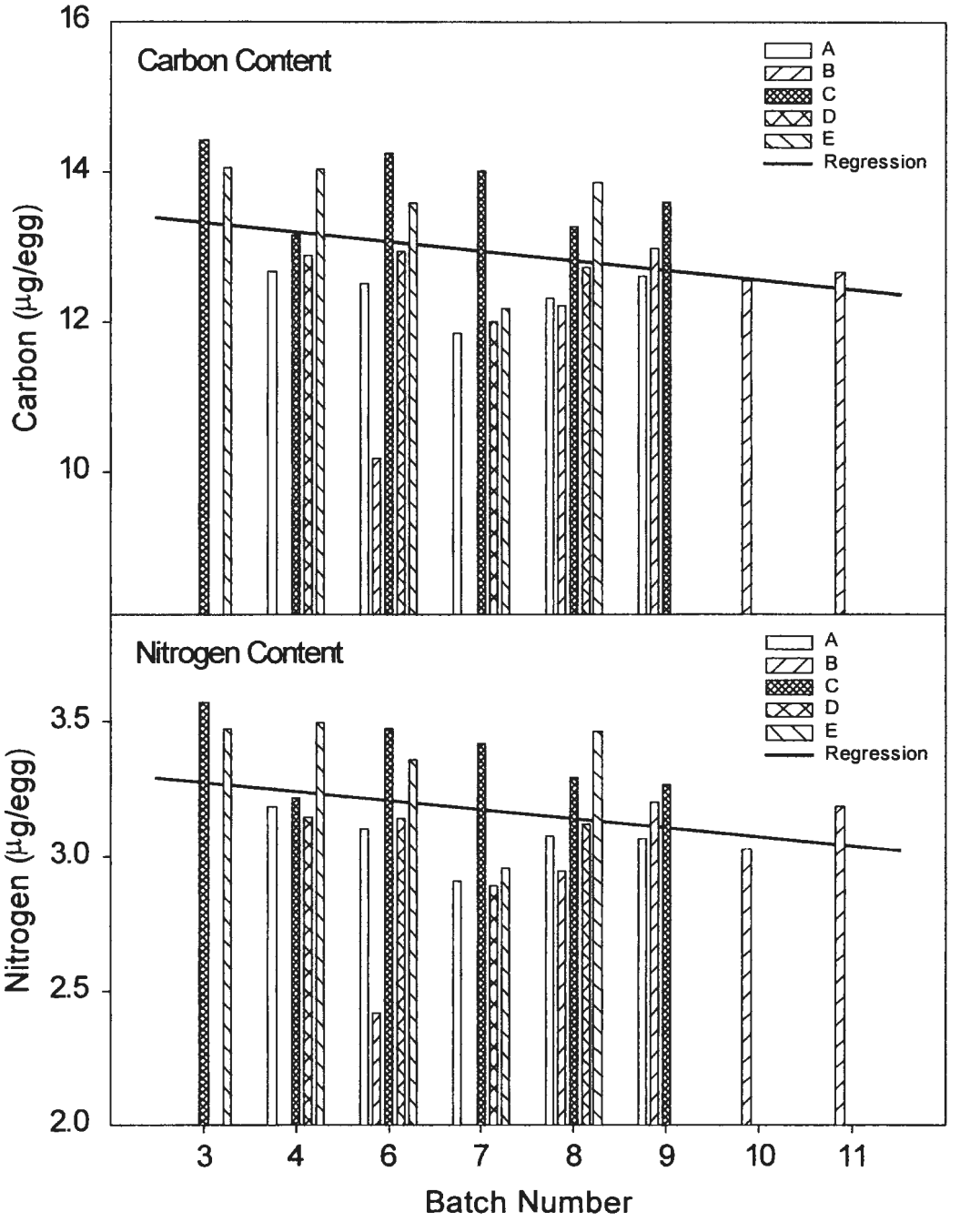

Figure 2.3. Carbon and nitrogen content per batch of yellowtail flounder eggs. Bars represent the mean carbon and nitrogen content per egg determined from 4–6 analytical replicate samples from each of five females (A–E). The solid lines represent the overall Model I linear regression and are not significant ($p>0.05$).

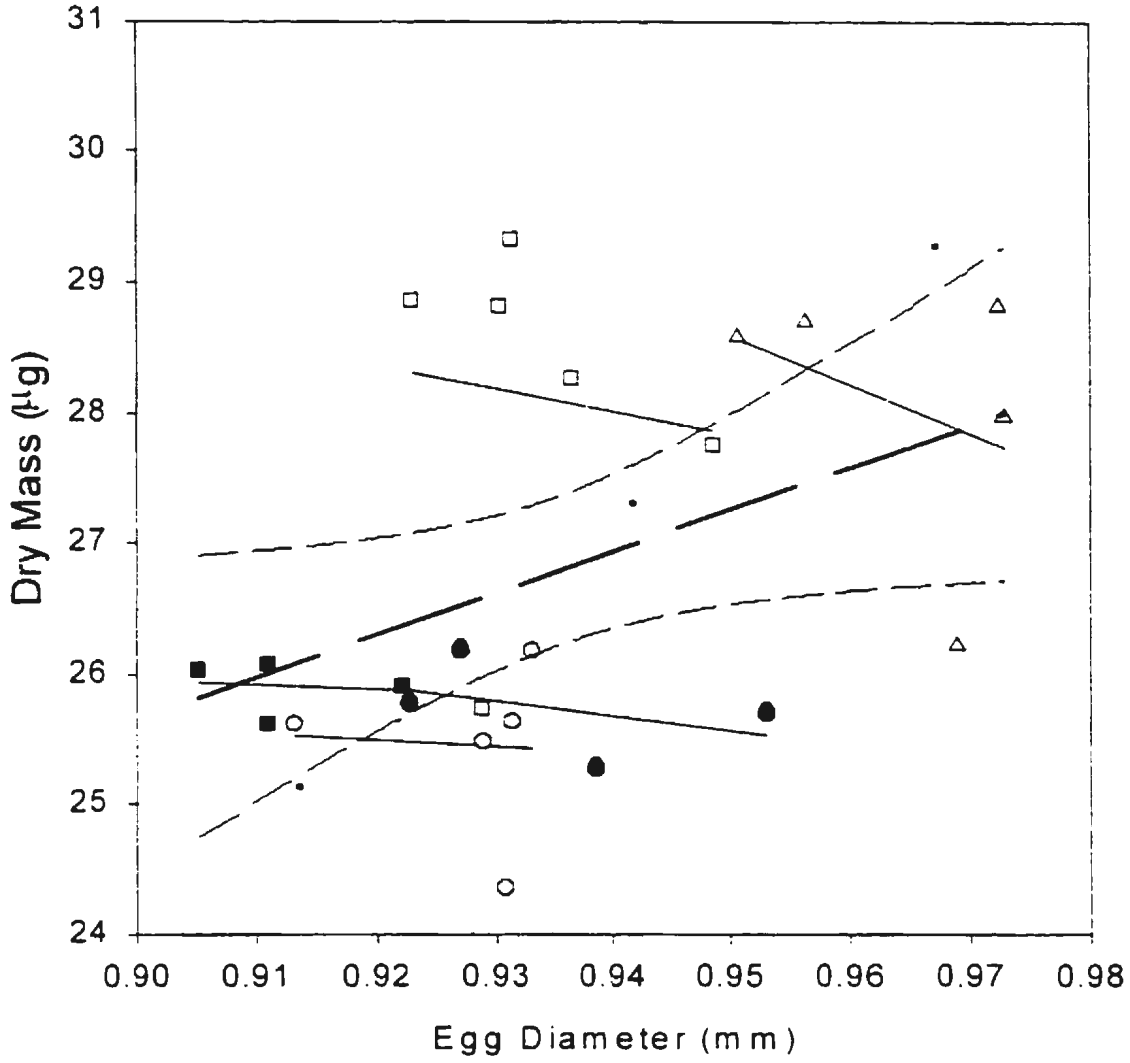

Figure 2.4. Variability in batch egg diameter and dry mass for each of five females (symbols). Data points represent the mean dry mass from 4–6 analytical replicate samples. The solid lines represent the overall Model I linear regressions for each female and are not significantly different from zero ($p>0.05$). The long dashed line represents the overall Model I regression with 95% confidence intervals (short dash line), and the dotted line represents the overall Model II regression.

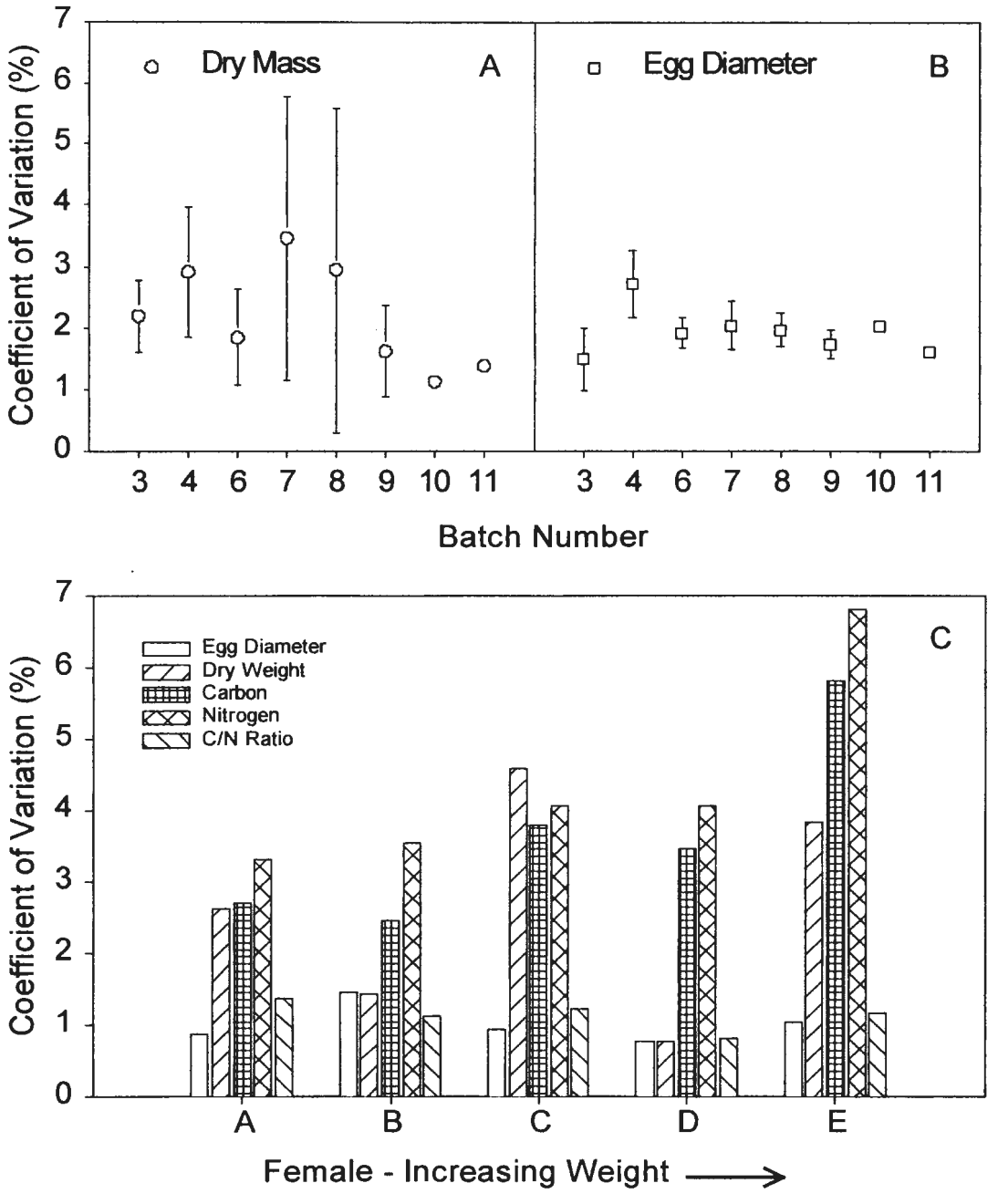

Figure 2.5. Coefficient of variation (CV) for dry mass (A) and egg diameter (B). Data points represent mean (±SD) values from 2–5 samples from individual females except for batches 10 and 11 which are single samples. CV for 5 egg quality measures from individual females (C) ordered by increasing female mass (242–414 g).

Chapter 3

Effects of abnormal cell cleavages on eggs and larvae of yellowtail flounder *Pleuronectes ferrugineus* (Storer)

3.1. Introduction

"Egg quality" is a widely used term, but it is difficult to define. Many studies have used a suite of determinants to provide a framework for egg quality based on one or several determinants which seem to correlate well with larval condition (Kjørsvik et al., 1990). However, many of the suggested egg quality criteria such as: broodstock nutrition and genotype, and the size, bacterial colonisation, biochemical composition and over-ripening of eggs (Kjørsvik et al., 1990; Bromage et al., 1994; Brooks et al., 1997) have been shown to hold true only in individual or small groups of related species, especially in marine fish. For example, the frequent use of egg size as a key indicator of egg quality is frequently questioned because 1) the definition of egg quality is often different for each study, 2) relationships between egg size and egg quality are often confounded by other factors or suffer from multicollinearity, or 3) the results are generally limited to the species under study. Consequently, egg size is used ubiquitously as an egg quality criterion despite a lack of supporting empirical evidence. Recent research has shown that abnormal cell cleavage during embryogenesis has some merit as an early egg quality criterion. However, abnormal cell cleavage studies suffer from the same shortcomings as egg size studies.

Dushkina (1975) recommended using post-hatching larval condition measures to qualify and quantify pre-hatching egg characteristics as the best method to determine egg quality. The recommendation was based on studies of the eggs of herring (*Clupea harengus*) which were found able to be fertilised long after the eggs were capable of producing good quality larvae. The goal in egg quality studies is that some pre-hatching measures will provide an early predictive capability as to the quality of larvae. In fact, it is this early predictive capability that is required in aquaculture in order to minimise rearing time and costs (Bromage et al., 1994, Shields et al., 1997).

A recent early predictor approach is to relate embryonic egg quality determinants to egg survivorship, fertilisation success, and hatching success (Kjørsvik et al., 1990; Brooks et al., 1997). For example, Kjørsvik (1994) reports on how Atlantic cod (*Gadus morhua*) egg viability (quality) based on malformed eggs can be used to assess eggs for aquaculture. However, she states that "further quantitative analyses need to be done, but defects visible in the early blastula stage will affect the subsequent survival potential, even though many embryos survive through hatching." This statement is further supported by detailed work on the zebrafish (*Danio rerio*) where blastomeres have been shown to correspond to embryonic tissues depending on their cleavage planes (Strehlow et al., 1994)

Abnormal cell cleavages early in embryogenesis are widely thought to be detrimental to the success of eggs (Kjørsvik et al., 1990; Kjørsvik, 1994; Shields et al., 1997). However, the majority of the supporting evidence is primarily based

indirectly on egg abnormalities in conjunction with egg over-ripening (McEvoy, 1984; Bromage et al., 1994), the increased susceptibility of abnormal eggs to bacterial contamination (Barker et al., 1989; Hansen and Olafsen, 1989; Pavlov and Moksness, 1993), pollution (Longwell, 1988; Westernhagen, 1988; Longwell et al., 1992), or a suite of determinants (McEvoy, 1984; Bromage et al. 1994).

Manning and Crim (1998) have shown that up to 27% of yellowtail flounder (*Pleuronectes ferrugineus*) eggs have abnormal cell cleavages in any one batch. Despite the acceptance afforded abnormal cell cleavage as an egg quality determinant, the effect of cell symmetry on hatching success or larval morphology outside of concomitant factors has yet to be determined. This study sets out to determine the effect of cell cleavage symmetry on mortality, hatching success, hatching rate, and larval condition in yellowtail flounder.

3.2. Materials and Methods

Adult yellowtail flounder were collected by SCUBA divers or by trawling from offshore fishing vessels and held at the Ocean Sciences Centre for at least two years prior to being used in this study. Females were held in a common 400-L tank supplied with degassed, filtered seawater with additional aeration. A natural photoperiod was maintained. The mean water temperature was 8.7 ± 1.4°C (range 5.8–10.3°C) during egg collection. This temperature range is in accord with the minimum temperature observed for the onset of spawning (about 6°C) and the maximum temperature for reliable egg collection in captive yellowtail flounder (Larsson et al., 1997; Manning and Crim, 1998). All fish

received the same commercial moist feed in the period leading up to the commencement of the study. Fish were not fed during manual stripping to prevent faecal contamination of the eggs.

Egg batches were stripped from three females from 16 July to 01 August, 1997 ranging in length from 35–39 cm and mass from 523–1086 g. Milt from several males was mixed together and used in excess to fertilise each batch of eggs separately. Each egg batch was then incubated at 4.5°C in 15-cm glass petri dishes filled with seawater that contained 0.1 gL^{-1} streptomycin sulphate and 0.06 gL^{-1} penicillin G to reduce bacterial contamination. Incubation was static. Four batches of eggs from three females were used to determine hatching success and hatching rate, but only three batches were available for larval condition measurements (each from a separate female). All eggs were assessed for initial egg quality using commonly cited methods (spherical, clear, and with no perivitelline space before fertilisation (McEvoy, 1984; Kjørsvik et al., 1990)).

"Abnormal embryo", "abnormal cell cleavage", "abnormal cleavage", "abnormal egg", "irregular cell cleavage", "irregular cleavage", "malformed egg", and other similar terms have all been used to describe the abnormal state of the blastula of fish eggs usually during the 4-cell to 8-cell stage of embryogenesis. Shields et al. (1997) provide a detailed description of abnormal cell cleavages most of which are highly correlated with each other. Therefore, this study adopts cell symmetry as an indicator of abnormality in eggs and the use of the terms abnormal and normal respectively refer to the asymmetrical and symmetrical pattern of blastomeres within a developing embryo. Egg abnormality was

determined at the 4-cell stage of development because Kjørsvik et al. (1990) reasoned that defects in cells at earlier stages of embryogenesis would influence the further development of the embryos more strongly than cell defects occurring later in development. Any eggs that were not divided in a normal, boxed formation were considered abnormal (Fig. 3.1). The distinction is made because previous work on abnormal embryos rarely defined what made an egg abnormal (Shields et al., 1997).

Because of the variability in the number of abnormal embryos within a batch of eggs on the day of sampling, it was impossible to sample equal numbers of eggs from each female. Therefore, 24–62 eggs were selected from egg batches from each of the normal and abnormal groups. Eggs were removed individually from initial petri dishes into new petri dishes using a pipette. All eggs underwent the same handling. Sampling provided 4 pairs of replicate batches; pairs being 1 petri dish of normal and one petri dish of abnormal eggs from each female.

Mortality during embryogenesis was determined by recording the number of dead eggs in each replicate once per day until all larvae hatched. A deviation from the commonly used term hatching rate is employed in this study as any "rate measure", in the context of scientific use, includes an element of time. Therefore, hatching success is defined as the number of eggs that hatched as a percentage of fertilised eggs and hatching rate will more appropriately be used to represent the relationship of hatching success over time.

The difference in hatching success between abnormal and normal eggs was determined using analysis of variance (ANOVA) and Tukey's Studentized Range Test with an experiment-wise error rate of α=0.05. Hatching rate was estimated by fitting a linear regression to the last three points of the hatching success data for normal eggs and compared to the slope of the middle two data points for the abnormal eggs. Extrapolation of the linear regression to the abscissa provides an estimation of the onset of hatch. Larval condition was determined morphologically and was primarily determined through measurements of larval length. Larval length measurements were done three days after hatch and differences were determined using an general linear model (GLM) that included date as a blocking effect and nested egg batches within females.

3.3. Results

Mortality was lower for normal embryos each day compared to abnormal embryos (Fig. 3.2). Overall, about 60% of the abnormal embryos died compared with only about 5% of the normal embryos. The pattern of mortality indicated that by day 3 the majority of eggs that would eventually die had done so. Eggs began hatching on day 7 and all eggs hatched within the following 48 h (Fig. 3.3).

The normal hatching rate was significant (r^2 = 0.999, p = 0.021, n = 8) (Fig. 3.3). Most hatching seemed to occur over a short period, probably within 48 h. There was a significant overall difference in hatching success (p < 0.001), however, no significant difference in hatching success between normal and

abnormal eggs on either day 7.8 (p = 0.292) or 8.3 (p = 0.851) was found suggesting that the rate of development is similar between the groups. This was confirmed by the slope of the linear regression through day 7.8 and day 8.3 of the abnormal eggs compared to the slope of the normal regression. The two slopes were not significantly different (p = 0.768).

Larval morphology was consistent between the two groups. Only one larva from the normal embryos and only two larvae from the abnormal embryos were curved upon hatching. All other larvae were straight and otherwise morphologically normal. Larval lengths were not significantly different between groups (p = 0.227) (Fig. 3.4).

3.4. Discussion

The mortality of abnormal embryos was higher than normal embryos, but 40% survival of abnormal embryos was good considering that abnormal cell cleavage is not necessarily a precursor for egg mortality in yellowtail flounder. In comparison, Kjørsvik (1994), in a study on Atlantic cod (*Gadus morhua*) comparing abnormal embryos and hatching success, showed a high positive correlation between the observed rate of abnormal embryos and hatching success. Westernhagen (1988) showed a similar result for Atlantic cod from the Baltic Sea, but that study was done in conjunction with polluted water and is probably not directly comparable due to differences in water quality (pollution) and differences between reared and wild fish (Blaxter, 1975). Nevertheless, the current study reflects the general findings of Kjørsvik (1994) and Westernhagen

(1988); abnormal embryos of yellowtail flounder show a significantly lower hatching success than normal embryos.

Pollution invariably adds to the incidence of curved larvae (Westernhagen, 1988) and may be useful as an indicator of pollution in wild populations (Kjørsvik, 1994). The observation of curved larvae as an indicator of pollution could be extended to water quality in aquaculture as well. There was no difference in the observed number of straight larvae hatching from abnormal and normal embryos in this study suggesting, at least, that water quality was not a factor. No correlation between straight larvae and hatching success could be determined because there were only 3 curved larvae. In contrast, Kjørsvik (1994) found that the number of straight larvae was significantly higher in normal embryos compared to abnormal embryos and that hatching success correlated positively with the number of straight larvae. This result may be somewhat species specific.

Cell symmetry has been cited as the "most reliable" egg quality indicator (e.g., Brooks et al., 1997), but is typically a component of a suite of characters (e.g., Kjørsvik and Lonning, 1983; McEvoy, 1984). The current study suggests that if confounding factors associated with cell cleavage are kept constant or minimised then a proportion of abnormal eggs are capable of producing viable larvae. Therefore, cell symmetry may not be as reliable an indicator as some suggest especially when considered on its own (Shields et al., 1997). Furthermore, extrapolating specific results made on individual species, under laboratory conditions, or in conjunction with other factors as general statements

is not always justified. Generalities based on species specific data can be especially problematic in marine teleosts within which life histories are not as well known as in salmonids, for example. The following observations provide some insights.

Brooks et al. (1997), citing Bromage et al. (1994), contend that the assessment of cell cleavage at the early stages of development is the "only reliable indicator of egg quality for halibut". However, Bromage et al. (1994) state that the proportion of eggs that are spherical and show normal cleavages after fertilisation is *thought* to provide the best index of egg quality for halibut (*Hippoglossus hippoglossus*) stating that the assessments are "qualitative" and, in concert with fertilisation rates, "reasonable" and conclude that the relationship "needs to be tested thoroughly" - which was subsequently done by Shields et al. (1997). In brief, Bromage et al. (1994) showed a constant rate (about 15%) of abnormal eggs during the over-ripening process probably because the abnormal eggs are dying and being replaced at the same rate by normal eggs that become abnormal. In contrast, Dushkina (1975) showed that abnormal cell cleavage was more pronounced the longer unfertilised herring (*Clupea harengus*) eggs were left in water before fertilisation. It is unclear whether it is over-ripening, cell symmetry, or some other factor that is causing the decrease in egg quality, although abnormal cell cleavage may correlate well with over-ripening. Over-ripening may be a precursor for abnormality in eggs, but abnormality is not necessarily an indicator of over-ripening. This example highlights the need for

studies into the effects of cell symmetry *per se*, preferably on individual eggs and their larva.

Bacterial contamination may also confound results. Bacteria have been shown to inundate what is considered to be good quality eggs (as defined by McEvoy, 1984), but the inundation of those eggs probably occurs indirectly once poor quality eggs have been infected and the bacteria have proliferated (Barker et al. 1989; Hansen and Olafsen, 1989; Pavlov and Moksness, 1993). Pavlov and Moksness (1993) show that abnormal eggs, which make up between about 2–32% of a brood of wolffish (*Anarhichas lupus*) eggs, are more susceptible to bacterial contamination. The present study used streptomycin and penicillin together to control bacteria, and separated the abnormal embryos from the normal embryos. Visual observation confirmed that this was an adequate approach.

Many studies refer to the review by Kjørsvik et al. (1990) citing that there is clear evidence that abnormal cleavage is a reliable indicator of egg quality. However, a reappraisal of the review and the literature cited therein does not provide a clear correlation or, more importantly, a clear causation between abnormal early blastomere cleavage and decreased egg quality criteria such as fertilisation or hatching success. Furthermore, Kjørsvik et al. (1990) cautions that fertilisation success does not always correlate well with survival or development in later embryonic stages. If the ultimate measures of egg quality are fertilisation and hatching success (see reviews by Kjørsvik et al., 1990 and Brooks et al., 1997), then there needs to be a clear connection between abnormal cleavage

and hatching success in order to use the earlier indicator of cell cleavage as a reliable egg quality determinant. Manning and Crim (1998) suggested that abnormal cleavage can be used to estimate hatching success, but do not provide empirical evidence or a calculation to support their assertion. They did not record hatching success for their second year of study suggesting that they were relatively confident in the hatching rate and cell cleavage relationship. The assumption must be that no abnormal embryos survive to hatch, but that was not directly tested, and is inconsistent with the results of this study.

3.5. Summary

I conclude that larval condition (size) must be a major consideration in egg quality assessments as an egg that is fertilised and hatches may not necessarily produce a viable larva (Dushkina, 1975), and, conversely, abnormal embryonic development does not necessarily mean subsequent egg mortality or poor larval size. In addition, relationships between abnormal cell cleavage, over-ripening, bacterial contamination, and other factors must be taken into consideration as a correlation between abnormal cell cleavage. For example, over-ripening does not necessarily indicate that abnormal cell cleavage decreases egg quality. The current study shows that cell symmetry may be used as a reasonable and direct assessment of egg quality for yellowtail flounder.

3.6. References – Chapter 3

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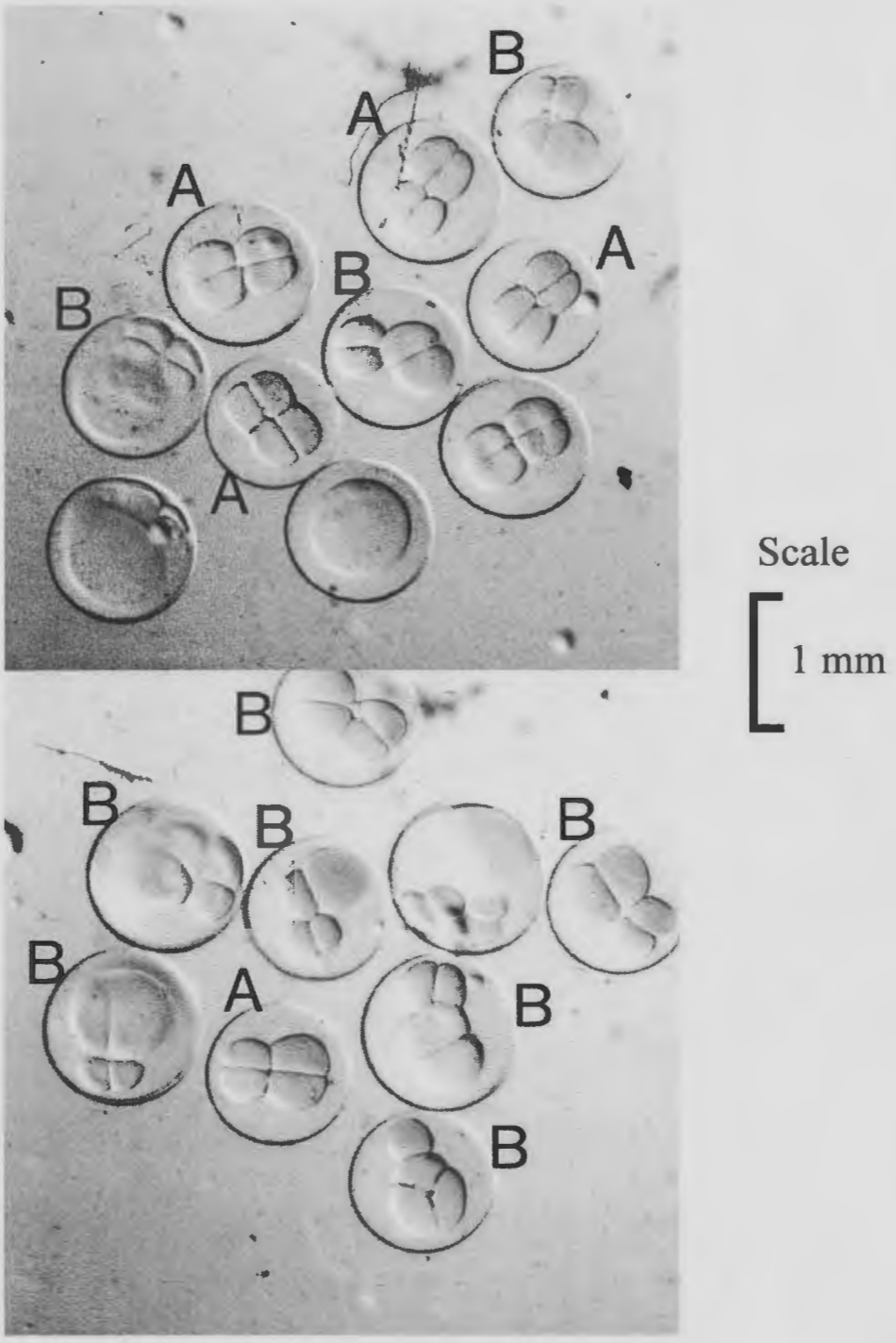

Scale

1 mm

Figure 3.1. Photograph of representative eggs of symmetrically cleaving (A) and asymmetrical cleaving (B) yellowtail flounder eggs. Notice the boxed formation of normally cleaving eggs at the 4 cell stage.

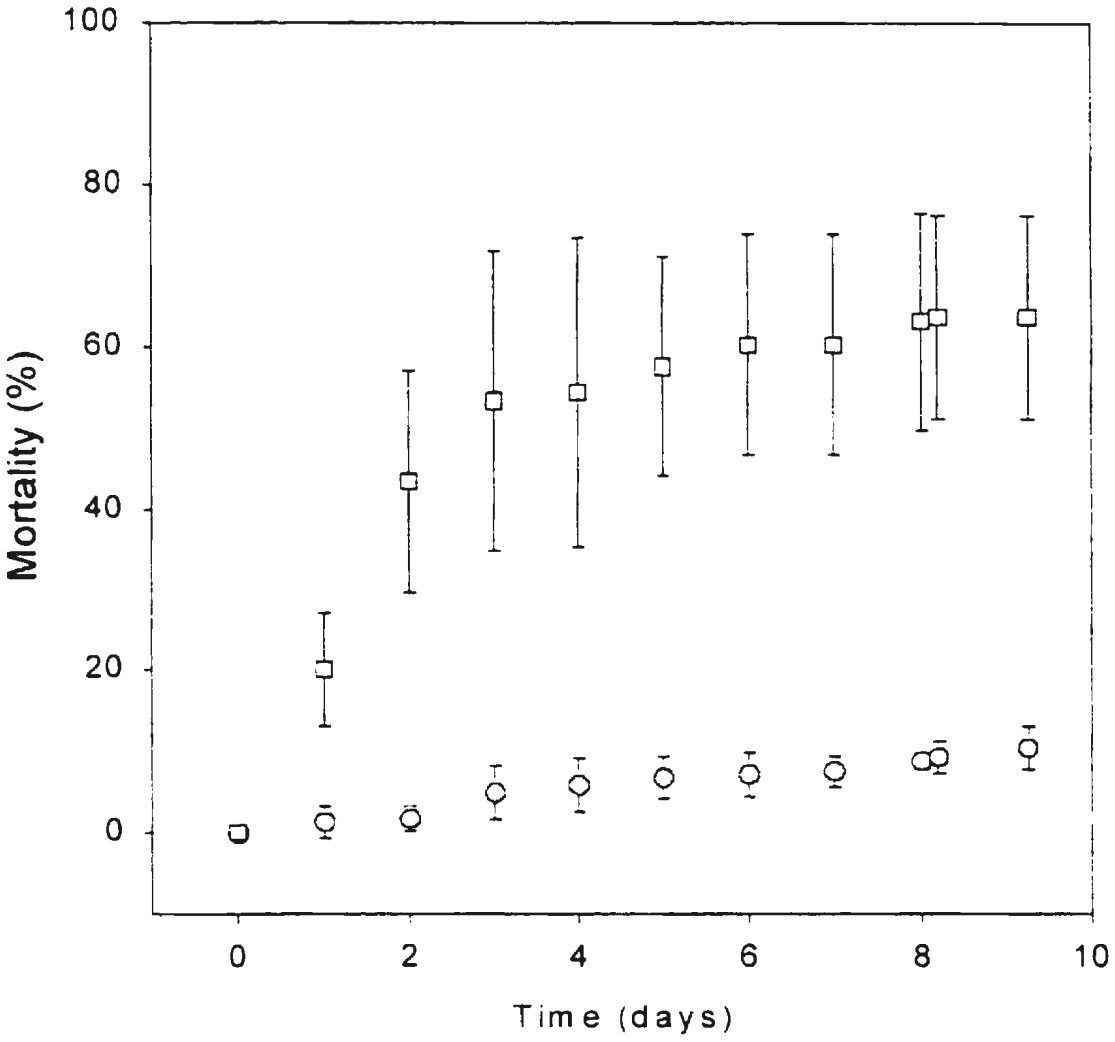

Figure 3.2. Cumulative mortality during embryogenesis of symmetrical cleaved (circles) and asymmetrically cleaved (squares) eggs at the 4-cell stage of cleavage. Data points represent the means (±SD) of 4 female yellowtail flounder.

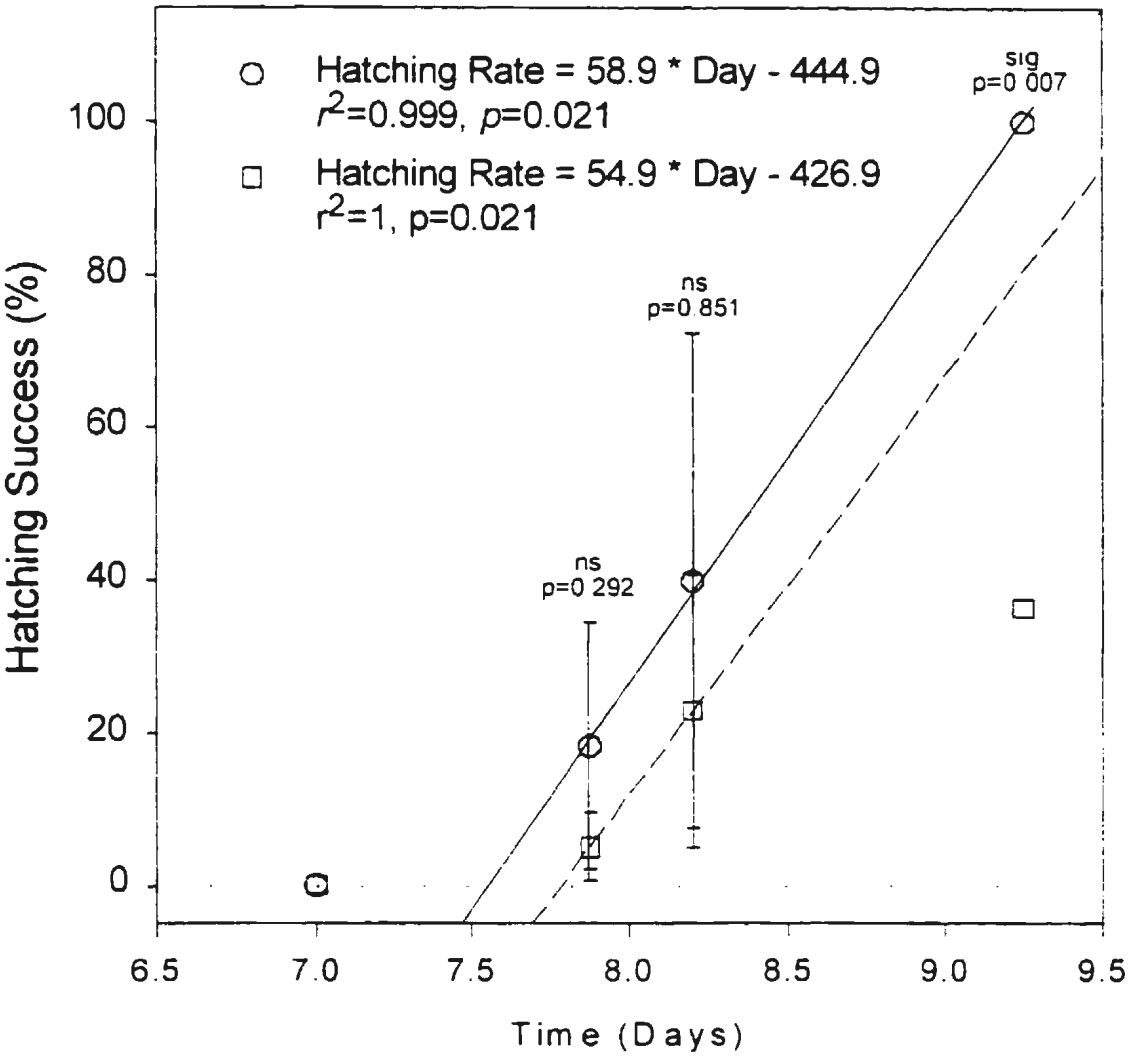

Figure 3.3. Hatching success of daily surviving yellowtail flounder larvae from symmetrically cleaved (circles) and asymmetrically cleaved (squares) eggs. Symmetry was determined at the 4-cell stage of development. Data points represent the mean (±SD) hatching success as a percentage of the number of eggs that survived to hatch. Significance values (p) indicate which pairs of values at each time are significantly different as determined through ANOVA. The regression through asymmetrically cleaved eggs is through the two mid-points only and extrapolated to illustrate the nearly equivalent slopes.

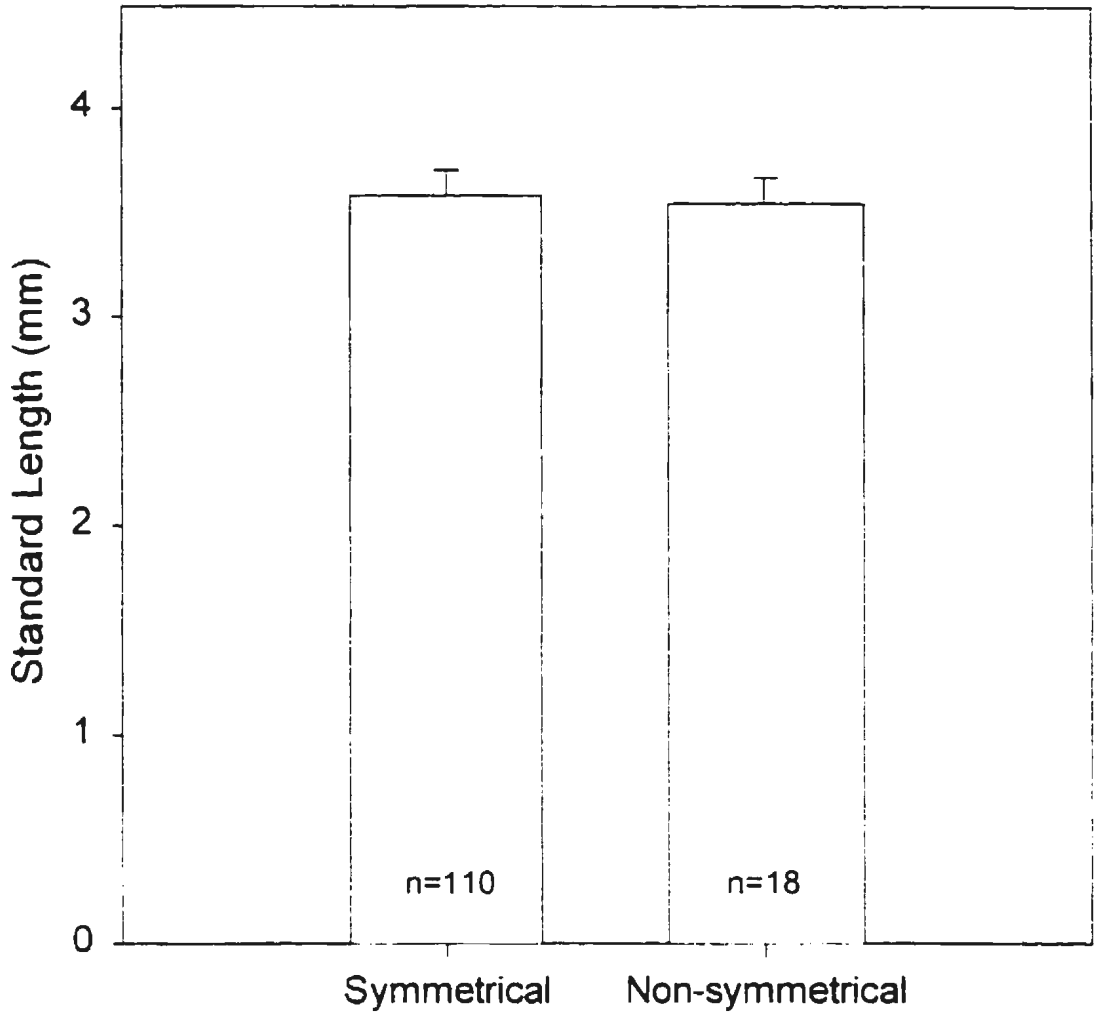

Figure 3.4. Mean standard lengths (±SD) of yellowtail flounder larvae from symmetrically and asymmetrically cleaved eggs. Symmetry was determined at the 4-cell stage of development. Means are not significantly different after accounting for female and date of sampling in a nested ANOVA (p = 0.286).

CHAPTER 4

MATERNAL AND PATERNAL EFFECTS ON EARLY LIFE-HISTORY TRAITS OF YELLOWTAIL FLOUNDER, *PLEURONECTES FERRUGINEUS* (STORER)

4.1. INTRODUCTION

Variation in early life-history (ELH) traits described for one species cannot be unconditionally applied among populations of the same species or to other species. Aggregating data from individual to population levels obscures the phenotypic variability among individuals upon which natural selection acts (Chambers, 1993). Phenotypic expression is ultimately observed at the level of the individual and it is at this level that factors contributing to ELH traits and their variation must be studied. Few studies have quantified variation at the individual level even though variation in larval growth rates is widely thought to be a central feature of recruitment (year-class formation) (Leggett and Deblois, 1994) and recruitment variability has been shown to be linked to characteristics of early life-history stages (Pepin and Myers, 1991). The importance of investigating variation was summarised by Houde (1987) who stated that small variations in mortality rates, growth rates, or stage duration in the early life history of fish could cause ten fold or greater fluctuations in fish recruitment.

As the global supply of fish stocks declines and the world population rises, the need for cultured fish becomes more important. Currently about 27% of

global fish consumption comes from aquaculture and it is predicted that aquaculture will have to supply the global shortfall in seafood over the next 10 years (Masood, 1997). Moreover, marine teleost species account for only about 3–4% of total fish culture (Planas and Cunha, 1999) and selecting broodstock for aquaculture so that maximum viability can be achieved will undoubtedly become more important as the percentage of cultured fish rises. Maximum viability is generally assessed through egg and larval quality where egg quality is generally considered to be determined by female condition (e.g., Chambers and Leggett, 1996; Kjesbu et al., 1996) and larval quality is primarily considered to be under environmental control and more dependent on larval genotype especially later in ontogeny (see Chambers, 1993; Howell et al., 1998). Moreover, empirical studies at the individual level (e.g., Miller et al., 1995; Chambers and Waiwood, 1996; Bertram et al., 1997; Benoît and Pepin, 1999) and reviews that recognise and properly assess aggregate levels of past data (e.g., Chambers, 1993; Pepin and Miller, 1993; Heath and Gallego, 1997) have shown that year to year variation in recruitment is linked to ELH variability. Understanding the factors that contribute to variability in ELH traits and the covariance of these traits is important to predict larval recruitment in nature and larval quality in aquaculture.

Although fertilisation success or 'the egg's potential to produce viable fry' has recently been regarded as the definition of egg quality (Kjørsvik et al., 1990), that definition is much narrower than historical definitions (see Zuromska and Markowska, 1984). A broader approach to egg and larval quality is resurfacing as more genetic and environmental information is compiled. Egg size, over-

ripening, larval size, larval growth and development time have recently received more attention (e.g., Brooks et al., 1997; Manning and Crim, 1998; Benoît and Pepin, 1999) with the majority of studies focussing on egg and larval size (both length and mass) as key indicators of quality. However, interactions among ELH traits affected by multiple agents are rarely considered (Kolok, 1999) although variability in many ELH traits is probably the result of numerous genetic and environmental factors contributing in concert to the phenotype (Chambers, 1993).

One of the main reasons for revisiting egg and larval quality is the realisation that aggregate levels of data (mean values scaled upward from individual data) do not usually represent individual-level relationships (Chambers, 1993). For example, relationships between female size and egg and larval traits at the female level, and between egg size and larval traits at the individual larvae level are not directly transferable between levels or to population levels (Chambers, 1993). Studies utilising distribution, variance component, and coefficient of variation analyses have been useful in determining what factors are significant at the individual level and for comparisons among higher level aggregates of data (e.g., Benoît and Pepin, 1999). Unfortunately, some researchers still discard "outlier" groups that may reveal the most about reproduction and optimal egg theory as they relate to evolution (Bernardo, 1996). In addition, maternal effects have continued to receive the majority of attention as variability is considered at different levels (e.g., Pepin and Miller, 1993; Chambers and Waiwood, 1996). What are often not considered are genetic

effects (Bernardo, 1996) despite suggestions that genetics may stabilise environmental (temperature) effects more than previously thought and that egg size differences over a season may be controlled by innate genetic rhythms under hormonal control (see Kjesbu et al., 1996).

For example, although many relationships that have reached "paradigm" status, such as the "bigger is better" hypothesis (see Litvak and Leggett, 1992), are generally repeatable, one must look at the underlying assumptions and conditions (Bernardo, 1996). Inter-specific relationships should not be extrapolated to intra-specific or individual levels (Chambers, 1993; Pepin and Miller, 1993) without first evaluating the surrounding factors such as environment, species behaviour and genetics (such as mating pairs) (Bernardo, 1996). When predation effects and limits on food availability are removed, and food quality (and possibly size) is constant such as in aquaculture situations, the three main arguments for the bigger is better hypothesis become irrelevant. Specifically, larger larvae swim faster and (by doing so) avoid predators more easily, larger larvae can capture more and larger prey, and larger larvae can survive food shortages longer (Blaxter and Hempel, 1963; Hunter, 1981; Knutsen and Tilseth, 1985). In this case, the definition of what constitutes a viable larva becomes a function of a controlled environment and the degree of influence of that environment on larval traits can depend on the underlying genotype (e.g., Iwamoto et al., 1986). In a similar manner, localised natural environments may also be quite different and may affect genetic expression, and thus larval quality, and ultimately recruitment. For example, Gisbert et al. (2000)

found that under favourable conditions egg size of Siberian sturgeon (*Acipenser baeri*) did not provide any survival advantage to young fish, and that smaller larvae were able to grow at the same relative growth rates as larger larvae. Furthermore, the bigger is better hypothesis is based largely on aggregate data (e.g., Petersen and Wroblewski 1984) and may not accurately reflect processes and relationships at the individual level (Chambers, 1993). So why does genotype not take a more central role in egg and larval quality studies in fishes?

Genetic contributions to variability in ELH traits is often confounded by fertilising different females or batches of eggs with different males (e.g., Norberg et al., 1991; Chambers and Waiwood, 1996; Kjesbu et al., 1996), or by combining sperm from more than one male (e.g., Larsson et al., 1997; Manning and Crim, 1998). However, sperm quality and competition is important and may be related to male condition (Trippel and Neilson, 1992; Hutchings et al., 1999; Rakitin et al., 1999). The obvious conclusion to be drawn from behaviour such as mate choice and reproductive phenomena such as sperm competition is that the underlying genotype of an individual is evolutionarily important. Genetic influence, particularly through male contributions, may be greater than previously thought and empirical studies focussing on the variance and covariance of ELH traits in regard to males could aid in understanding fish population ecology (Chambers, 1993). Moreover, the use of aggregate data and the lack of covariance analyses of ELH traits have left gaps in the current knowledge base.

Fish culture and predictive recruitment models would be better served if a firm grasp of male contributions to variability in ELH traits were known. Maternal

effect studies dominate the literature (e.g., Buckley et al., 1991a; Chambers and Leggett, 1996; Kjesbu et al., 1996; Manning and Crim, 1998; Benoît and Pepin, 1999), but it has been shown that males may contribute substantially to larval traits later in larval development and that these traits are heritable (Gjerde, 1989; Gjerde and Schaeffer, 1989). Furthermore, few paternal studies have been completed on marine teleost species at the order of the individual and even fewer on multiple batch-spawning species such as the flounders. For example, Chambers and Waiwood (1996) did not have any batch-spawning species with which to compare their Atlantic cod (*Gadus morhua*) variance component data.

This study will primarily concentrate on the contribution of males to the phenotype of larvae. Specifically, I will investigate the relative contribution of females, males and female · male interactions on early life-history traits of eggs and larvae. Close attention will be given to the level at which these data are aggregated (larval, female, family, and population) using a comparative statistical analyses approach. To this end, egg and larval quality and all relationships among groups are determined through univariate and multivariate analyses of ELH traits and their affecting factors. Where possible, all data are recorded at the individual level and compared to aggregate levels of data from the same samples. This study will use yellowtail flounder because it is an important commercial groundfish species and have recently been investigated as a potential aquaculture species.

4.2. Materials and Methods

Yellowtail flounder were collected by SCUBA divers or by trawling from offshore fishing vessels and were held at the Ocean Sciences Centre for at least 2 years prior to the study. Females were held in a common, 1 m square, 400-L tank supplied with degassed, filtered seawater with additional aeration. A natural photoperiod was maintained. Temperature was regulated once ambient temperature reached 10°C by the addition of cooled seawater. The seawater temperature was ~7–8°C during egg collection. This temperature range is in agreement with the minimum temperature observed for the onset of spawning (about 6°C) and the maximum temperature (about 12°C) for reliable egg collection in captive yellowtail flounder (Larsson et al., 1997; Manning and Crim, 1998). All fish received the same commercial moist feed in the period leading up to the commencement of the study. Fish were not fed during manual stripping to prevent faecal contamination of the eggs.

From a pool of five females and nine males (Tables 4.1 and 4.2), four females and four males were selected at random every 2–3 days. Females were stripped when ripe (about every second day) whether they were used or not to minimise the chances of overripe eggs (Manning and Crim, 1998). If a female was not ripe or a male would not produce sperm of >50% motility (see Nagler et al., 2000) on any given day, another was randomly selected. Ninety-six families (mating pairs) were initiated from 9 July to 22 July, 1998. All females were stripped on more than one day, therefore, date of stripping and egg batch were

essentially equivalent and confounding factors for those instances and were considered one factor in the statistical analyses. Batch numbers were not necessarily sequential because of the random selection of the females (Table 4.1). All batches were assessed for initial egg viability (VIA) using commonly cited methods [spherical, clear, floating and with no perivitelline space before fertilisation (McEvoy, 1984; Kjørsvik et al., 1990)]. Those batches that did not exceed minimum (50%) viability measures were not used (Table 4.1). Egg diameters (ED) were measured for each batch using an Olympus SRZ microscope at 40x magnification.

Individual egg batches were split into four 5-mL (yielding about 10,000 eggs) aliquots and each aliquot was fertilised with 30 µL of milt from one of the four males according to the methods of Larsson et al. (1998). Aliquots were then randomly placed in incubation vessels suspended in a flow-through water bath maintained at 6.1 ± 1.1°C. The incubation vessels were 500 mL Nalgene beakers with Nitex screen (mesh size: 0.7 mm) bottoms to allow air and seawater exchange from the water bath. All vessels were cleaned twice daily by siphoning dead eggs from the bottom of each vessel and from the bottom of the water bath.

The flow-through system consisted of filtering ambient seawater through three filters (minimum pore size: 1 µm), then delivering the filtered seawater through titanium tubes suspended in the water bath that acted as a pre-cooler, then through a Neslab temperature control unit to a degassing system and finally filling a header tank. Nissling et al. (1998) showed that antibiotics and aeration in

small container incubation of Atlantic halibut (*Hippoglossus hippoglossus*) eggs was not necessarily important, but as a precautionary measure, and because of past incubation successes of yellowtail flounder with added antibiotics (Manning and Crim, 1998), 100 mg streptomycin L^{-1} and 60 mg penicillin L^{-1} were added daily to the header tank daily to reduce bacterial contamination. The header tank delivered water through six tubes with randomly placed outflows that were moved to different locations in the water bath three times a day. The entire incubator system was contained in a temperature-controlled room maintained at 4.5°C.

After fertilisation, the contents of each Nalgene vessel were stirred and three 10-mL aliquots were removed using a Stempel pipette. These sub-samples were incubated separately in petri dishes at 6°C in a Hotpack incubator (Hotpack Corp. model # 352602) for determination of fertilisation success (FER) and the proportion of FER that had non-symmetrical cleavage patterns (abnormally cleaving) (FERNS). Both FER and FERNS were determined from the mean of three aliquots and these mean values were used in the analyses. Rapid egg development from the 4–32 cell stage prevented counting dead eggs on the bottom of the petri dish for all fertilisation success counts, therefore, some counts were based on the number of viable eggs rather than the total number of eggs. Fertilisation success (number of eggs fertilised / total number of eggs) and viability fertilisation success (FERVIA, number of fertilised eggs / total number of viable eggs) were not combined for any analysis.

Five larval traits were measured; four quantitative traits; standard (notochord) length (SL), total body depth at insertion of anus (TBD), body depth from top of notochord to insertion of anus (NBD) and yolk volume (YV), and one qualitative trait (Q). All measurements were done on an Olympus SRZ microscope at 40x magnification. Yolk volume was determined by measuring the longest (a) and shortest (b) axis of the yolk sac and applying the formula for a oblate spheroid (volume = $4/3 \cdot \pi \cdot a^2 \cdot b$). The quality or categorical trait consisted of a general assessment of each larva scored via a dummy variable from 5 to 1 based upon criteria that were commonly employed during past rearing trials of yellowtail flounder at the Ocean Sciences Centre (Table 4.3). Pigmentation was included as one part of the quality criteria because it was suggested by Yevseyenko and Nevinskiy (1982) as a possible larval quality indicator for yellowtail flounder and because of other possible links to developmental-dependent traits (Seikai et al., 1987) and normal function of the brain and retina of fishes (Pagliarini et al., 1986; Mourente et al., 1991; Sargent et al., 1993). Although pigmentation may only affect larvae later in ontogeny (Rainuzzo et al., 1994; Reitan et al., 1994), malpigmented flatfish have shown physiological alterations particularly pertaining to vision (Heap and Thorpe, 1987). Later in ontogeny, pigmentation seems to affect larvae disproportionately more (Howell et al., 1998), so it was given less weight.

Initially about 25 larvae from each family were assessed on either 2, 3 or 4 days post hatch (DPH) as preliminary studies showed that 2 DPH would be a sufficient time to allow the head to separate from the yolk sac and the body to

straighten. However, this was not the case for all larva and measuring at 4 DPH predominated as the study proceeded. Four DPH was in agreement with Yevseyenko and Nevinskiy (1982) for yellowtail flounder for a similar SL. Because too few larvae on 3 DPH (n = 63) were measured to allow for reasonable statistical inference, only 2 (n = 455) and 4 DPH (n = 990) data were used. Both individual-based and mean-based analyses of both groups were completed and compared.

All factors were considered random effects in the statistical analysis thereby providing a quality assessment that is population (female and male contributions) and temporally (date of spawning or batch effects) applicable rather than applicable only to differences between specific individuals or dates used in this study (Sokal and Rohlf, 1995). This random effects analysis should not be confused with species, population, and individual level aggregates of data. Females and males were selected to be of similar size in both the female and male pools (Tables 4.1 and 4.2). Univariate (ANOVA) and multivariate (MANOVA) analysis of variance were compared and used to determine the relative maternal and paternal contributions and their interactions using a suite of five traits in an unbalanced design with unequal replication. Partial correlation coefficients from MANOVA's were compared between 2 and 4 DPH larvae to provide an insight into early growth. Variance components (VC) of all random factors were determined with the VARCOMP procedure of SAS/STAT (Version 6.12) to determine the relative contributions of factors. Interaction plots were constructed to illustrate the complexity of interactions between females, males

and the date of mating (or batch effect). Both the likelihood ratio test (Wilks' Lambda) and the Bartlett-Nanda-Pillai Trace test (Pillai's Trace) were used as critical test statistics for the MANOVA's. Wilks' Lambda has more power than Pillai's Trace, nonetheless, Pillai's Trace was included as an alternative since the derivation of the two critical test statistics is fundamentally different (Anderson, 1984). Nested ANOVA was used to determine initial batch characteristic differences among females (Table 4.1).

Tank effects were tested on a number of occasions through comparisons of additional batch splitting (5 or 6 equal splits rather than 4) fertilised with the same male and compared for mean larval size differences. It was determined through the test samples that there were no tank effects as found previously using the same equipment in the same facility. This undoubtedly results from the randomisation of family placement in tanks, the uniform light, temperature and water movement around tanks, and the measurement of larvae during endogenous feeding only (tank effects have been noted in exogenous feeding growth experiments; e.g., Herbinger et al., 1999). Data transformations were not required as the assumptions of both the univariate and multivariate were met as judged by residual analyses. As a further, more comprehensive comparison, all univariate analyses were completed on log transformed and on ranked (non-parametric) data to satisfy proponents of these methodologies (Appendix 2). The results showed no appreciable differences among the three statistical methods in magnitude of *p*-values. These results confirm that transformation of the data was not necessary.

Linear and multiple linear regressions were performed to determine the relationship between mean SL (MSL), mean VIA (MVIA), mean FER (MFER), mean FERVIA (MFERVIA), mean FERNS (MFERNS), mean Q (MQ), and mean ED (MED). Data (2 and 4 DPH) were combined in cases where the variables of interest were all measured prior to hatching. A forward stepwise procedure (REG procedure in SAS/STAT version 6.12) with α = 0.15 was employed for multiple linear regressions. A significance value of 0.15 was selected in order to provide the model that provides the best prediction since all variables have previously been show to have some predictive capability.

4.3. Results

Both males and females were listed in order of use (Tables 4.1 and 4.2). All larvae died in some families, therefore, fewer than four families could be used in larval analyses for some split batches (Table 4.1). Consequently, of the original 96 mating pairs, only 58 families remained for larval analyses. ED, VIA and fertilisation measurement data for families that did not have surviving larvae or that had too few larvae for meaningful larval analyses, were included in all other analyses (up to 88 families). The inconsistent number of male crosses for individual males was primarily due to inconsistent milt production (Table 4.2).

With the exception of MED, individual batch characteristics were not different from the female batch means as judged by the respective standard deviations (italicised numbers; Table 4.1). However, there were considerable differences in individual (used) batch viabilities among females. ED's were

significantly different among females (uppercase characters; F = 232.97, p < 0.001, n = 1347), among batches within females (F = 13.21, p < 0.001), and between used and mean batches within females (lowercase characters; α = 0.05) (Table 4.1). Females accounted for 35% of the variation in egg diameter and batches within females accounted for a further 10%. In particular, female FA had significantly larger and FC significantly smaller eggs, respectively, although FC was the second largest female in both length and mass. Furthermore, no significant relationship was found between female length and MED (F = 1.40, r^2 = 0.091, p = 0.256, n = 16), or female mass and MED (F = 3.55, r^2 = 0.202, p = 0.081, n = 16) at α = 0.05. There was also no significant relationship between MED and MVIA when all batches from all females, including those not used in the larval analyses, were considered together (Table 4.4).

Two and 4 DPH were considered separately because of the fundamental differences between MFER and MFERVIA, and because a preliminary ANOVA showed that DPH (growth) significantly affects SL (p < 0.05). MSL at 4 DPH and a larger sample size was significantly related to MQ, MFERVIA, MVIA and MED using multiple regression analysis, but these four variables combined only explained about 50% of the variation (Table 4.4). Partial correlation coefficients for MVIA and MED were low and suggest that the test criteria of α = 0.15 was too robust. Overall, MQ accounted for the majority of the relationship for both 2 and 4 DPH. MVIA and fertilisation success also showed significant relationships to MSL, but not consistent across development time.

Univariate (ANOVA) and multivariate (MANOVA) analysis of variance were completed on five (T5) and three (T3) larval traits at the level of the individual. Fewer larvae from T5 were measured compared to T3 because fewer TBD and NBD measurements were made than SL, Q, and YV (Tables 4.5 and 4.6). Within T5 only four differences (17%) existed between 2 and 4 DPH; two (SL at Day and TBD at Male) changed from significant to non-significant, and two (SL at Female · Male and TBD at Female · Male) became significant (Table 4.5). A similar overall pattern was shown when the sample size was increased by dropping TBD and NBD; three deviations at 2 DPH (SL at Male, Q at Female, and YV at Female) and one at 4 DPH (Q at Day) (Table 4.5). Within T3 six differences (25%) existed between 2 and 4 DPH; two were the same changes as in T5 (SL at Day and SL at Female · Male), and the other four occurred most probably because of increased sample size (Table 4.5). However, most of these differences should not be considered at face value because of the existence of significant interaction terms in many of the ANOVA's. They are shown only to illustrate statistical differences that occur when sample sizes are increased and during ontogeny, and for comparison to previous studies.

There were only three interaction terms that were not significant in both T5 and T3, none of which were in the 4 DPH (higher n) group(s) (Table 4.5). Because of the interaction terms, only the day factor can be assessed directly. However, that factor was primarily included as a blocking factor and will receive little discussion. Both the female and male effects must be assessed via simple effects diagrams or by other multiple comparison methods. Since I was primarily

interested in whether a male and (or) a female · male interaction existed, rather than which female, male or female · male combination produce better larvae, multiple comparison tests were not done. Instead, graphically, it was apparent that interactions were occurring between females and males (Figures 4.1 and 4.2). For example, the SL of females FC and FE on day 2 reversed their relative position when fertilised by male MD (Fig. 4.1). However, the relative positions of YV remained the same (Fig. 4.2). Similar individual level patterns can be seen for other mate pairs on various spawning days. The combined day data suggest that males MC and MG produce the most variable SLs, but that trend is mirrored in YV for MC only.

In applying a univariate analysis to the data, there was no accounting for the covariance of the traits. A MANOVA considers the covariance between variables and should provide a more powerful test of overall larval quality. Multivariate tests at the individual level of both T5 and T3 showed that all variables were significant for both 2 and 4 DPH (Table 4.6) using both Wilks' Lambda and Pillai's Trace. Most notably, the significant interactions between females and males were confirmed, and the significance in the day factor was more pronounced.

The matrices of partial correlation coefficients (PCC's) confirm that covariance between traits existed and, in many cases (70%), were significant (Table 4.7)00. No differences in significance were present between corresponding T5 and T3 PCC's, and most corresponding values were relatively equal (Table 4.7). Significant partial correlation between standard length and

quality (Q) measures confirm the MQ and MSL multiple regression relationship. The high correlation between TBD and NBD was expected, confirming that the MANOVA calculations are producing reasonable results.

In comparison to the individual level analyses, univariate and multivariate mean values for 8 traits (T8) were calculated by day, female and male and analysed in the same manner as individual level T5 and T3 groups. Too few samples were available for a meaningful test of MED, MVIA and MFERVIA for 2 DPH so they were dropped from the analyses. Male, and female · male interactions were left in the MED and MVIA ANOVA's to keep the analyses consistent and as a check of statistical effectiveness although it was apparent that fertilising males cannot affect egg diameter or viability. The highly non-significant male and female · male interactions for both MED and MVIA confirm this truth (Table 4.8).

Nearly half (43%) of *p*-values changed significance in comparing the individual level univariate analyses of T5 to the corresponding mean level factors, but only 21% changed when comparing T3 to T8 (Table 4.8). Most notably, nearly all of the interaction terms were non-significant when using mean values. This result is fundamentally different from the results of the individual level analyses. These differences were reflected in the multivariate analyses where non-significant day, male and interaction terms were apparent using Pillai's Trace as a test criteria (Table 4.6). However, the more powerful Wilks' Lambda confirmed the individual level results of T5 and T3 groups at all levels except the mean level female · male interaction.

Fifty percent of the PCC's changed significance at 2 DPH when comparing individual level to mean level; all becoming non-significant at the mean level. Principally, the changes in the SL to MSL coefficients all became non-significant when using means. In comparison, only 30% of the PCC's had changed significance from individual to mean level at 4 DPH with no changes within the SL/MSL coefficients (Tables 4.7 and 4.9). Furthermore, 60% of the significant PCC's were higher in magnitude at the mean level at 4 DPH. The change from a positive to a negative correlation in the case of SL–YV to MSL–MYV is of particular interest as it is a plausible correlation in either case. Again, the consistency of the MTBD–MNBD PCC confirmed that the calculations were reasonable.

Additional PCC's generated by MED, MVIA and MFERVIA were included at the mean level at 4 DPH only. Mean egg diameter was not correlated with any variable, and MVIA was correlated only with MYV. In contrast, MFERVIA was significantly correlated with all variables except MED (Table 4.9). This result confirms the multiple regression analyses. The univariate results showed that for MED and MVIA, females were significant factors while for MFERVIA females and day were both significant.

The interpretation of univariate and multivariate analyses is complicated by the variance associated with each factor. A factor may well be statistically significant, but if it does not account for a reasonable amount of the variance, in comparison to the other factors for a given trait, then it may well not be biologically significant. Variance components are an applicable tool to show the

variance contributed by each factor. The VC's were in general agreement with the ANOVA and MANOVA. A low VC corresponded to non-significance and a high VC corresponded to significance within each factor at both 2 and 4 DPH (compare Table 4.5 to Table 4.7). The only deviation from the general trend was in the day factor for TBD, NBD and YV and in the day factor for TBD at 4 DPH where a significant univariate value corresponded to a very low VC. This result suggests that statistical significance does not necessarily mean biological significance.

The VC's of mean values were considerably different than at the individual level (Table 4.10). Of all female · male interactions, 70% had 0% of the VC compared to as high as 23% at the individual level (Table 4.7 and 4.10). As well, 90% of the female VC's increased in magnitude at the mean level. There was a general increase in error variance from 2 to 4 DPH in both tables. However, where there was a decrease in error variance (MTBD and MYV) the other VC's increased proportionally. The opposite trend was shown when comparing individual level components to mean level components where 80% of the error VC's decreased. For the most part, the relative VC's within a factor remained constant between individual and mean level analyses.

The additional VC's from MVIA, MED, MFER, MFERVIA and MFERNS generally showed higher day factor components compared to the other mean variables (Table 4.10). As well, the univariate significance check generated by leaving the male and interaction terms in the ANOVA for MED and MVIA were confirmed by the zero or relatively near zero VC's for both male and female ·

male interactions (Table 4.10). In relation, the high day and female factors confirm that there is a significant day and/or female effect for variables that should not be influenced by the male.

4.4. Discussion

Hempel and Blaxter (1967) and Hislop (1988) found that egg mass was significantly related to female size in Atlantic herring (*Clupea harengus harengus*) and haddock (*Melanogrammus aeglefinus*), respectively, but only when younger, smaller fish were included in the analyses. Similarly, Kjesbu et al. (1996) found smaller eggs in 2-year old, first-time spawning captive Atlantic cod. Bernardo (1996) suggests that there may be morphological constraints [e.g., diameter of the oviduct, (Congdon and Gibbons, 1987)] in younger organisms that prevent eggs from attaining larger sizes. This relationship relates to optimal egg size theory; a topic that is outside the scope of this study, but with which the majority of pelagic spawning marine fish seem to comply (Wootton, 1994). Although egg diameter was found to be significantly different among females and batches within females using analysis of variance, no significant regression relationships were found between either female length or mass and egg diameter. Benoît and Pepin (1999) also found significant significant maternal effects on egg diameter (82.5% for females), but they only had one batch per female.

All females in the current study had spawned at least once previously and were 50–100% larger than 2–3 year old stock that were being raised in captivity

at the time of the study (pers. obs.), therefore, it is unlikely that I had very young fish in the sample. Bengston et al. (1987) found no relationship between female size, egg diameter and larval length in the Atlantic silverside (*Menidia menidia*) and Hinckley (1990) similarly found no female size to egg diameter relationship in walleye pollock (*Theragra chalcogramma*), but Marteinsdottir and Steinarsson (1998) found that female size, condition and age of Icelandic Atlantic cod (*Gadus morhua*) were significantly correlated to egg and larval size. Although . Benoît and Pepin (1999) found that females contributed significantly to the variation in egg diameter, they did not attempt to determine the relationship further such as the regression analyses performed herein. This finding suggests that maternal influences on early life history may be species specific. Contradictory results among and within species (see Hinckley, 1990) suggest that the relationship between female size and egg size is obscured by effects beyond maternal control or that biochemical condition may be more important than size *per se* (see Chapter 2). Generally, there is only a weak relationship between female size and egg size in pelagic spawning marine teleosts within a given species (Buckley et al., 1991b; Pepin et al., 1997) and comparisons among marine species and other taxa such as Salmonidae (Bromage et al., 1990) are probably irrelevant (Hislop, 1984; Miller, 1984; Parker and Begon, 1986; Duarte and Alcaraz, 1989). The lack of a wide range of female sizes and, thus, possibly ages may account for the lack of a regression relationship of female length or mass to MED (reviewed in West, 1990). However, this result was ideal for the current study as a female to MED relationship would have

confounded the results for other factors such as male effects that were of primary interest.

MSL was significantly related to MED, but only within a multiple regression analysis with a weak partial correlation coefficient. A relationship, although sometimes weak, has also been shown in other marine teleosts such as Atlantic cod (e.g., Solemdal, 1970; Knutsen and Tilseth, 1985; Pepin et al., 1997), walleye pollock (Hinckley, 1990), and herring (Blaxter and Hempel, 1963). In comparison, no correlation has been shown in carp (*Cyprinus carpio*) (Zonova, 1973), channel catfish (*Ictalurus punctatus*) (Reagan and Conley, 1977) and Midas cichlids (*Cichlasoma citrinellum*) (Lagomarsino et al., 1988) although all of the latter teleosts are freshwater species. Furthermore, Marteinsdottir and Able (1992) found that larger eggs produced larger larvae, but their study compared two sub-species and four populations.

Population and species level comparisons of egg size and larval size is more common than individual level comparisons (e.g., Bengston et al., 1987; Duarte and Alcaraz, 1989), yet until recently, the extrapolation of inter-population and inter-specific relationships to intra-specific, intra-population and individual based studies was common (see comments by Chambers, 1993; Pepin and Miller, 1993; Wootton, 1994; Chambers and Waiwood, 1996; Bertram et al., 1997; Heath and Gallego, 1997). Unfortunately, I was unable to compare individual egg sizes with corresponding larvae, but the PCC's from the multivariate analyses on mean values were calculated taking all factors into account and provide a good indication that SL and ED are not related. However,

even if relationships are found at levels higher than the individual, they may have little or no significance for assessing larval quality.

Heath and Gallego (1997) state that the mean properties of a population observed over time cannot be used to infer average changes in individuals. Regardless, my main interest revolved around determining whether males contributed to variability within and among the families. I acknowledge the difficulties in assessing quality criteria at the individual level, but, if only for comparisons to other studies, have extrapolated the individual based analyses in some instances to mean level aggregates At this population[1] level, the inference associated with the analysis revolves around the plasticity of traits in multiple environments rather than the variation in and among traits (Chambers, 1993). In summary, the resolution of the current study may not be sufficient to determine egg size to larval size relationships particularly if female effects contribute substantially to egg size (e.g. Kjesbu, 1989; Kjesbu et al., 1996; . Benoît and Pepin, 1999) and, in turn, egg size is related to larval size (e.g. Chambers et al. 1989; Miller et al., 1995).

On an individual basis, Chambers et al. (1989) showed a significant correlation between length at hatching and initial yolk volume (similar to egg diameter) in the capelin (*Mallotus villosus*) although the correlation coefficient was only 0.31. Miller et al. (1995) showed that a correlation coefficient generally under 0.5 was not statistically significant when determining individual egg size and larval size relationships. Of 12 separate samples in their study, 50%

[1] Population, in this context, follows the definition of Chambers (1993): specifically, any grouping below the species level and above that of the individual

produced a correlation that was not significant, however, this result was primarily due to low sample sizes. In general, Miller et al. (1995) showed that a correlation did exist. However, these relationships are not as axiomatic as historically thought (see Bernardo, 1996; Chambers and Waiwood, 1996), probably because the most compelling evidence comes from population and species level means (Duarte and Alcaraz, 1989; Pepin, 1991) or from studies that incorporate salmonids.

A further example of a mean change was that of the partial correlation between standard length and yolk volume. The correlation was significant at both the individual and mean levels at 4 DPH, but the relationship went from a positive (individual level) to a negative (mean level) correlation. Both results are reasonable. At the individual level, larger larvae could have larger yolk sacs because they developed from larger eggs, if that relationship is true (e.g. Blaxter and Hempel, 1963 and various reviews). In contrast, a longer development time or higher metabolic costs, on average, could produce larvae of a similar size, but with smaller yolk sacs. This is supported to some degree by the rapid development during the early life of pelagic larvae, and more so by the fact that larvae consume yolk reserves during embryogenesis (a term that generally signifies within egg development, but that more correctly signifies the period of endogenous feeding; Balon, 1984). Embryonic metabolism is genetically influenced, therefore, it is not unreasonable that larvae from different families will have different metabolic rates and thus, will utilise yolk reserves more (or less) efficiently resulting in more (or less) yolk reserves available for growth (see

Bengston et al., 1987; Chambers et al., 1989). Furthermore, the time of larval measurement was not completed at a saltatory ontogenetic event, but within an ontogenetic period, namely endogenous feeding, that is affected by many epigenetic and environmental stimuli (Balon, 1984; Chambers, 1993).

Emphasis should be placed on both the multivariate analyses and variance components over univariate tests since univariate analyses do not account for correlation among traits (Steel and Torrie, 1980; Anderson, 1984; Sokal and Rohlf, 1995). In all analyses, multivariate statistics generally showed a female · male interaction on overall larval quality. A result that is not apparent with univariate analyses. These results are reflected in most cases at the mean level, but is less important when assessing larval quality as it pertains to recruitment (e.g., Heath and Gallego, 1997). Regardless of which level is considered, male effects are statistically apparent. The proportion of the variance associated with paternal effects in relation to other VC's should be more indicative of the male contribution to overall variability.

Individual level VC's generally confirm the multivariate results, but not for all cases. More residual (error) variance was present at 4-DPH and probably reflects rapid and variable larval growth, some of which was probably due to varying genotypes. At 2-DPH female effects dominated, but after only 2 days, variance components were much more diverse and distributed. The egg size variance component (2-DPH 78%, 4-DPH 83%) was comparable to that for capelin (71%; Chambers et al., 1989), but higher than that for winter flounder (46%; Chambers and Leggett, 1996) and Atlantic cod (35%; Chambers and

Waiwood, 1996). However, Chambers and Waiwood (1996) showed a combined variance component for egg size and batches of 61%, which is comparable considering I did not attempt to determine batch differences.

Females seem to be most important overall accounting for up to 70% of the variance early in ontogeny and consistently registering high VC's throughout individual and mean level analyses. The current female VC for SL of 29% (2 DPH) and 20% (4-DPH) is in close agreement with Benoît and Pepin (1999) of 22% for the same species. Moreover, Benoît and Pepin (1999) showed a residual VC of about 65% that is comparable to the range of residual VC's here (43–88%) at the individual level. Males seem to be particularly important in NBD and female · male interactions suggesting that males contribute significantly to larval development.

Changes in VC's over time (2–4 DPH) and from individuals to means emphasise the rapid changes occurring during early development. The low VC's of males is an indication of a lack of a male effect. Metabolic effects attributed to genotype may also be influencing larval YV and this may explain why MYV, MSL and MED show an inconsistent relationship. Further evidence to suggest that genotype may be more influential than previously thought is demonstrated in studies of compensatory growth by larvae where it seems that phenotype is not thoroughly expressed or does not dominate until later in development.

Individual level VC results are not carried to the mean level. Strong proportions (female at SL/MSL, female and female · male at YV/MYV, and NDB/MNDB) are relatively consistent at 4-DPH, but most other male and female

· male contributions are not present at the mean level. Furthermore, MVIA, MED and fertilisation measures all seem to be predominately under female control. The vast majority of fish studies support this result, but not directly as few studies have taken males into consideration when determining female differences thereby effectively confounding female and male effects. Of particular importance, larval length and egg traits (size and viability) have relatively low VC's at the temporal level (day) suggesting that time of spawning and (or) batch (because of confounding) does not greatly affect these traits. This result is also seen in the relatively stable egg diameters among batches within females. Conversely, fertilisation, and in particular asymmetrical fertilised eggs, was highly affected by day of spawning. Predominant maternal effects may mask environmental effects that are responsible for differences in time of spawning. Finally, traits that are thought to be tightly integrated with maternal effects (MED, MVIA) showed no male or female · male interaction variance components.

Although temperature was regulated, closely monitored and the mean temperature almost identical among trials, there were short periods that incubation temperatures would be relatively higher or lower than the mean temperatures. Changes in temperature may have significant effects on rates of development (Pepin et al., 1997) or on timing of ontogenetic events (Chambers and Leggett, 1987; Fuiman et al., 1998). At temperatures near the extremes for a given species, metabolic processes may be more important (Bengston et al., 1987; see also Pepin et al., 1997) and smaller larvae would result because of disproportionate use of resources for respiration rather than growth.

Balon (1984) provides a theoretical framework of saltatory ontogeny that encompasses similar ideas, albeit in more detail than the simplistic example above. He proposes two developmental models. "(1) does development proceed via a continuous accumulation of inconspicuous small changes, or (2) is it a sequence of rapid changes in form and function, separated by prolonged accumulation and canalisation of complex structures, developing at different rates, that render the next rapid change possible?". If small changes in temperature are considered in the first instance, then short temperature increases would be offset by short temperature decreases, on average, and all larvae would be subjected to the same rate of development, on average. In the second instance, temperature changes at specific "accumulation and canalisation" events could possibly "render the next rapid change" faster in some larvae than others.

Arguments have been put forth that multivariate and distribution analyses are superior to univariate analyses and from a holistic egg and larval quality perspective this may be true. However, univariate analyses are also statistically valid especially when effects at specific levels of data aggregation, within particular groups, or between similar studies (comparisons of only particular traits) are of interest. Therefore, an account of the univariate analyses is useful and, in agreement with the multivariate analyses, show considerable evidence that female · male interactions did exist at least at the larger sample size, and hence more powerful, individual level analyses. For example, only YV had a

significant female · male interaction when mean values were used and this was only for the 4 DPH group.

A high number of significance value changes (*p*-values changing from significant to non-significant) were recorded between individual and mean levels. The lower sample sizes, and therefore less powerful tests, probably accounted for some effect in the 2-DPH group, but a reasonable number of samples were used at 4-DPH (n = 40) and it was the 4-DPH group that showed the most differences when compared to the individual level analyses. This suggests that using means may mask relationships that really exist most probably because mean values do not take into consideration the distributions of variables (e.g., Chambers, 1993; Benoît and Pepin, 1999). For example, Rice et al. (1993) showed that in predation models dispersion and distribution of sizes and ages during saltatory ontogeny might be of equal or greater importance in growth rate and survival. Caution must also be used when comparing between aggregations since the results at different aggregations correspond to fundamentally different questions (Chambers, 1993; Pepin and Miller, 1993).

Day of spawning does not seem to affect egg diameter or viability. This result was consistent with recent work on yellowtail flounder (Chapter 2). In contrast, Atlantic cod (*Gadus morhua*) show decreasing MED's over the spawning season (e.g., Kjesbu et al., 1996; Chambers and Waiwood, 1996), although the relationship was not as well defined. Only 50% of females in Chambers and Waiwood (1996) had decreasing MED's most likely because the size range of individuals used was narrow. Because of the confounding of day

and batch within this study, it is impossible to determine what particular component caused the significant result.

Visual inspection (Q) should not be discounted as a useful larval quality criterion. Because it is a reliable indicator of the combined effect of the other four variables, then it would be the most practical trait to use in assessing larval quality either for a quick assessment in the field or for aquaculture. An analogue to Q was used by Buckley et al. (1991a) where egg dry mass was found to be more highly correlated than any single class of biomolecule. On the other hand, measuring Q is subjective at best and an objective metric would be more applicable for aquaculture (Fuiman et al., 1998). In this context, SL may be the best overall metric to use for larval quality for yellowtail flounder as it is relatively easy to measure, objective, tangible, and it was significantly correlated to most other variables and had the second lowest CV (4.1 ± 1.1%). Fuiman et al. (1998) concluded that a length based metric was best for Atlantic menhaden (*Brevoortia tyrannus*) and red drum (*Sciaenops ocellatus*) for intra-specific comparisons because it was objective and showed the lowest intrinsic variability. A view that is supported by the close relationship between body size and physiological rates (Calder, 1984).

4.5. Summary

The considerable variation in ELH traits and the correlation among traits in yellowtail flounder larvae show that one single trait might not adequately reflect larval quality and that a suite of traits may be necessary to determine larval

quality. However, if a single criterion had to be chosen, one based on a linear metric such as standard length may be the most useful. Males seem to have some influence on larval quality most likely through genetic linked contributions, but the majority of variability seems to be associated with females and the date of spawning. However, the link between females and maternal effects on egg size, viability and fertilisation were not overly apparent and further studies using a wider range of female sizes is warranted. Changes in significance in correlation coefficients, variance components and ANOVA results were probably due to rapid development (growth) effects between 2 and 4 DPH, an effect shown in the early larval stages of many marine fish (e.g. Nissling et al., 1998; Hinckley, 1990). It is hypothesised that these rapid changes are due, in part, to genetic contributions from the male and that the intrinsic physiological effects, such as changes in metabolism and respiration, may strongly affect egg and larval development especially if yellowtail flounder are sensitive to environmental changes.

Comparisons of individual and mean level data confirm the findings of Chambers (1993), Pepin and Miller (1993) and others in that conclusions drawn at one aggregate level cannot generally be applied to other levels. Mean values effectively eliminate the variance upon which natural selection acts and tend to obscure or smooth relationships that may show the most about why larvae succeed to the next life-history stage. It is the elucidation of causes of variability that needs to be brought into focus in order to develop accurate recruitment models or to select the best broodstock for aquaculture.

4.6. References – Chapter 4

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Table 4.1. Female and egg batch characteristics.

	Female	Mass (g)	Total Length (cm)	Max Body Depth (cm)	Batch #	Batch Volume (mL)	Viability (%)	Egg Diameter (mean ± SD)		Number of Families
	FA	414	36	15	3	30	66	0 956 ± 0 011	ba	3
					4	35	78	0 951 ± 0 031	b	4
A					6	25	87	0 973 ± 0.019	ca	3
					8	30	56	0 972 ± 0 017	ca	4
					10	*28 6 ± 18 9*	*63 ± 24*	*0 966 ± 0 041*	a	
	FB	242	30 5	13 5	4	42	51	0 929 ± 0 023	b	4
B					6	53	50	0 922 ± 0 013	ba	3
					10	*33 2 ± 15 9*	*64 ± 14*	*0 922 ± 0 022*	a	
	FC	337	34	14	3	53	54	0 911 ± 0 028	b	4
					6	73	84	0 911 ± 0.016	b	4
C					7	57	79	0 905 ± 0 022	b	4
					8	45	53	0 922 ± 0 018	a	4
					9	*41 3 ± 21 9*	*67 ± 14*	*0 930 ± 0 043*	a	
	FD	249	29	13 5	8	44	94	0 968 ± 0 011	c	3
					10	25	92	0 927 ± 0 019	b	4
D					11	35	79	0 923 ± 0 015	b	4
					12	*33 3 ± 20 0*	*68 ± 15*	*0 939 ± 0 035*	a	
	FE	267	31	14	6	45	93	0 922 ± 0 013	b	2
					7	42	91	0 930 ± 0 015	a	4
D					9	28	97	0 937 ± 0 018	a	4
					10	*32 8 ± 10 1*	*74 ± 25*	*0 936 ± 0 035*	a	

Notes: Number of families represents the number of split batches that were fertilised by a different male for a given egg batch. Italicised numbers are the total number of batches, and overall mean (±SD) for batch volumes, viability and egg diameters for all batches of eggs from each female. Uppercase, bold letters of the same character between female and mass values did not have significantly different overall MED's among females. Lowercase letters of the same character adjacent to egg diameters did not have significantly different MED compared to MED *within* females.

Table 4.2. Male characteristics for crossing study.

Male	Mass (g)	Total Length (cm)	Max. Body Depth (cm)	Number of Families
MA	209	29	12	8
MB	n/a	n/a	n/a	4
MC	235	30	12.5	13
MD	226	29	12	5
ME	309	32	13	3
MF	407	34	14.5	9
MG	265	29	12.5	8
MH	313	30	13.5	5
MI	389	33	14.5	3

Table 4.3. Qualitative larval rating; 5 being the best and 1 the worst.

Rating	Definition
5	Straight, symmetrical, no abnormalities in morphology or pigmentation.
4	Slight lateral body curvature (lordosis), or minor abnormalities in morphology or pigmentation.
3	Any combination of lordosis, slight tail or fin abnormalities, or abnormal pigmentation.
2	Combinations of curved body, moderate tail, fin or gastrointestinal tract (yolk sac, stomach and anal region) abnormalities, and/or pigment abnormalities.
1	Major deformities in morphology.

Table 4.4. (a) Linear regression of mean viability (MVIA) regressed against mean fertilisation (MFERS, MFERNS, MFERVIA) and mean egg diameter (MED). (b) Significant variables of a forward stepwise multiple regression of mean standard length (MSL) regressed against mean quality (MQ), mean fertilisation, mean viability and mean egg diameter.

Variable	n	Parameter Estimate	r^2	F	p
(a) *Mean viability (MVIA)*					
Intercept		4.69E+01 ± 4.25E+00			
MFERS	30	8.11E-01 ± 1.60E-01	0.479	25.8	<0.001*
Intercept		3.90E+01 ± 5.83E+00			
MFERVIA	30	6.11E-01 ± 9.84E-02	0.355	38.5	<0.001*
Intercept		6.98E+01 ± 3.46E+00			
MFERNS	30	-5.27E-01 ± 3.90E-01	0.061	1.83	0.187
Intercept		-6.85E+00 ± 1.45E+02			
MED	30	8.44E+01 ± 1.55E+02	0.010	0.294	0.592
(b) *Mean standard length (MSL)*					
2 DPH (with MQ, MFER, MFERNS, MVIA and MED)					
Intercept		2.61E+00 ± 2.99E-01			
MQ		1.40E-01 ± 6.71E-02	0.301 (partial)	6.90	0.018*
MFER		6.96E-03 ± 3.96E-03	0.120 (partial)	3.10	0.099**
Total	18		0.421		
4 DPH (with MQ, MFER, MFERNS, MVIA and MED)					
Intercept		1.88E+00 ± 5.81E-01			
MVIA		2.67E-01 ± 1.16E-01	0.369 (partial)	4.09	0.083**
MQ		4.30E-03 ± 1.44E-03	0.296 (partial)	5.30	0.061**
Total	9		0.665		
2 DPH (with MQ, MFERVIA, MVIA and MED)					
Intercept		2.60E+00 ± 3.44E-01			
MQ		1.69E-01 ± 7.43E-02	0.271 (partial)	5.20	0.039*
Total	16		0.271		
4 DPH (with MQ, MFERVIA, MVIA and MED)					
Intercept		1.29E-01 ± 6.06E-01			
MQ		2.68E-01 ± 6.71E-02	0.180 (partial)	8.35	0.006*
MFERVIA		-2.56E-03 ± 9.63E-04	0.138 (partial)	7.48	0.009*
MVIA		3.20E-03 ± 9.13E-04	0.078 (partial)	4.63	0.038*
MED		2.14E+00 ± 4.90E-01	0.101 (partial)	7.04	0.012*
Total	40		0.497		

Notes: * significant at α=0.05; ** significant at α=0.1
All days post hatch values were combined for MVIA regressions as viability, fertilisation success and egg diameters are measured before eggs hatch.

Table 4.5. Univariate analysis of variance on 2 and 4 days post hatch (DPH) for 5 and yellowtail flounder larval traits at the individual level.

Univariate									
Trait	Factor	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*
					Low Sample Number (5 traits)				
			2 DPH (n=254)				4 DPH (n=544)		
Standard Length									
	Day	2	238	14.4	<0.001*	3	515	0.370	0.775
	Female	3	7.98	23.6	<0.001*	4	22.7	22.7	<0.001*
	Male	5	6.21	2.07	0.198	6	18.8	1.87	0.139
Female	male	5	238	1.48	0.196	15	515	1.56	0.081**
Quality									
	Day	2	238	0.560	0.572	3	515	0.748	0.524
	Female	3	5.60	3.20	0.111	4	21.7	1.67	0.189
	Male	5	5.25	1.09	0.460	6	18.4	0.222	0.965
Female	male	5	238	6.78	<0.001*	15	515	1.77	0.036*
Yolk Volume									
	Day	2	238	14.1	<0.001*	3	515	25.2	<0.001*
	Female	3	5.70	3.84	0.080**	4	16.0	3.51	0.031*
	Male	5	5.30	1.56	0.312	6	15.5	1.34	0.297
Female	male	5	238	5.80	<0.001*	15	515	10.6	<0.001*
Total Body Depth at Anus									
	Day	2	238	7.94	<0.001*	3	515	6.30	<0.001*
	Female	3	7.39	17.5	0.001*	4	18.3	8.28	<0.001*
	Male	5	5.98	3.36	0.087**	6	16.7	1.47	0.249
Female	male	5	238	1.82	0.110	15	515	3.40	<0.001*
Notochord Body Depth at Anus									
	Day	2	238	4.37	0.014*	3	515	12.3	<0.001*
	Female	3	7.22	11.8	0.004*	4	18.2	5.22	0.006*
	Male	5	5.92	3.23	0.094**	6	16.6	3.12	0.031*
Female	male	5	238	1.94	0.088**	15	515	3.52	<0.001*

Table 4.5 Cont.

Univariate Trait	Factor	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*
					High Sample Number (3 traits)				
			2 DPH (n=455)				4 DPH (n=988)		
Standard Length									
	Day	2	439	21 0	<0 001*	3	959	0.118	0 950
	Female	3	9.02	47 3	<0 001*	4	18.3	15.6	<0 001*
	Male	5	6.5	6.23	0.019*	6	16.7	1 40	0.270
Female	male	5	439	1 16	0 327	15	959	3 23	<0 001*
Quality									
	Day	2	439	1 39	0.251	3	959	12.85	<0.001*
	Female	3	5 45	5.35	0 045*	4	18 5	1 42	0.267
	Male	5	5 18	1 96	0 234	6	16 9	0.574	0.746
Female	male	5	439	9 05	<0.001*	15	959	3.02	<0.001*
Yolk Volume									
	Day	2	439	14 3	<0 001*	3	959	42 3	<0.001*
	Female	3	5.32	2.89	0 136	4	15 6	3 62	0 028*
	Male	5	5.13	1 22	0 413	6	15 3	1 18	0 365
Female	male	5	439	12.8	<0 001*	15	959	16 3	<0 001*

Notes: * significant at α=0.05; ** significant at α=0.1
Increased sample numbers were realised by elimination of total and notochord body depth measurements (see text for details). (Ndf=numerator degrees of freedom; Ddf=denominator degrees of freedom; F=F statistic; *p*=*p*-value)

Table 4.6. Multivariate statistics at 2 and 4 days post hatch (DPH) for 5 (low sample number) and 3 (high sample number) yellowtail flounder larval traits at the individual and mean level of aggregation.

Multivariate		Individual Level								Mean Level			
		Low Sample Number (5 traits)				High Sample Number (3 traits)							
Factor	Statistic	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*
		2 DPH (n=116)				2 DPH (n=217 5)				4 DPH (n=1 5)			
		MSL, MQ, MYV, MTBD, MNBD				MSL, MQ, MYV				MSL, MQ, MYV, MTBD, MNBD			
Day	Wilks' Lambda	10	468	8 07	<0 001*	6	874	13 5	<0 001*	15	19 7	2 95	0 013*
	Pillai's Trace	10	470	8 03	<0 001*	6	876	13 3	<0 001*	15	27 0	1 92	0 068**
Female	Wilks' Lambda	15	646	14 2	<0 001*	9	1064	28 4	<0 001*	20	24 2	8 19	<0 001*
	Pillai's Trace	15	708	13 3	<0 001*	9	1317	25 9	<0 001*	20	40 0	3 38	<0 001*
Male	Wilks' Lambda	25	870	4 84	<0 001*	15	1207	11 9	<0 001*	30	30 0	2 49	0 007*
	Pillai's Trace	25	1190	4 69	<0 001*	15	1317	11 7	<0 001*	30	55 0	1 54	0 083**
Female male	Wilks' Lambda	25	870	3 66	<0 001*	15	1207	8 43	<0 001*	75	37 7	1 74	0 031*
	Pillai's Trace	25	1190	3 50	<0 001*	15	1317	8 23	<0 001*	75	55 0	1 32	0 141
		4 DPH (n=254 5)				4 DPH (n=477 5)				4 DPH (n=1 5)			
		MSL, MQ, MYV, MTBD, MNBD				MSL, MQ, MYV				MSL, MQ, MYV, MTBD, MNBD, MED, MVIA			
Day	Wilks' Lambda	15	1411	8 14	<0 001*	9	2329	16 7	<0 001*	24	12 2	2 18	0 079**
	Pillai's Trace	15	1539	7 71	<0 001*	9	2877	15 8	<0 001*	24	18 0	1 51	0 185
Female	Wilks' Lambda	20	1696	17 3	<0 001*	12	2532	30 2	<0 001*	32	16 3	14 9	<0 001*
	Pillai's Trace	20	2056	16 5	<0 001*	12	2877	29 2	<0 001*	32	28 0	7 56	<0 001*
Male	Wilks' Lambda	30	2046	5 88	<0 001*	18	2707	7 11	<0 001*	48	23 7	1 89	0 049*
	Pillai's Trace	30	2575	5 58	<0 001*	18	2877	6 96	<0 001*	48	54 0	0 962	0 552
Female male	Wilks' Lambda	75	2452	3 79	<0 001*	45	2844	7 14	<0 001*	120	40 7	1 34	0 141
	Pillai's Trace	75	2575	3 61	<0 001*	45	2877	6 83	<0 001*	120	88 0	0 982	0 541

Notes: * significant at α=0.05, ** significant at α=0 1

Trait acronyms follow that of the text. Both Wilks' Lambda and Pillai's Trace statistics are provided. (Ndf=numerator degrees of freedom; Ddf=denominator degrees of freedom; F=Fstatistic; *p*=*p*-value)

Table 4.7. Partial correlation coefficient matrices and *p*-values (*p*) for multivariate analysis of variance on 2 and 4 days post hatch (DPH) for 5 and 3 yellowtail flounder larval traits at the individual level.

Multivariate	2 DPH				4 DPH			
	Q	TBD	NBD	YV	Q	TBD	NBD	YV
	Low Sample Number (5 Traits)							
	(df=238)				(df=515)			
SL	0.442	0.180	0.111	-0.050	0.262	0.393	0.298	0.149
p	<0.001*	0.005*	0.087**	0.440	<0.001*	<0.001*	<0.001*	<0.001*
Q		0.154	0.069	0.138		0.183	0.207	0.157
p		0.018*	0.283	0.037*		<0.001*	0.283	<0.001*
TBD			0.747	0.038			0.760	-0.006
p			<0.001*	0.563			<0.001*	0.901
NBD				0.080				0.064
p				0.216				0.150
	High Sample Number (3 Traits)							
	(df=439)				(df=515)			
SL	0.418	-	-	0.060	0.351	-	-	0.145
p	<0.001*	-	-	0.207	<0.001*	-	-	<0.001*
Q		-	-	0.170		-	-	0.214
p		-	-	<0.001*		-	-	<0.001*

Notes: * significant at α=0.05, ** significant at α=0.1
(SL=standard length (mm); Quality=qualitative criteria; TBD=total body depth at anus (mm); NBD=body depth from top of notochord to anus (mm); YV=yolk volume (μL)).

Table 4.8. Univariate analysis of variance on 2 and 4 days post hatch (DPH) for 5 (2 DPH) and 8 (4 DPH) yellowtail flounder larval traits at the mean level.

Univariate		2 DPH (n=19)				4 DPH (n=40)			
Variable	Factor	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*
Mean Standard Length									
	Day	2	3.00	7.84	0.064**	3	11.0	0.019	0.996
	Female	3	7.56	40.5	<0.001*	4	26.0	10.1	<0.001*
	Male	5	7.92	7.18	0.008*	6	24.1	0.997	0.450
Female	male	5	3.00	0.392	0.831	15	11.0	0.466	0.915
Mean Quality									
	Day	2	3.00	7.69	0.066**	3	11.0	4.36	0.030*
	Female	3	5.06	5.91	0.042*	4	23.6	1.04	0.409
	Male	5	5.03	1.91	0.247	6	20.5	0.453	0.834
Female	male	5	3.00	58.4	0.003*	15	11.0	0.937	0.557
Mean Total Body Depth at Anus									
	Day	2	3.00	0.464	0.667	3	11.0	1.30	0.322
	Female	3	6.19	6.51	0.024*	4	26.0	5.31	0.003*
	Male	5	7.72	1.32	0.348	6	24.0	1.27	0.309
Female	male	5	3.00	0.216	0.934	15	11.0	0.481	0.907
Mean Notochord Body Depth at Anus									
	Day	2	3.00	0.217	0.816	3	11.0	2.80	0.090**
	Female	3	6.74	4.32	0.053**	4	25.2	3.48	0.022*
	Male	5	7.95	1.20	0.391	6	22.1	2.56	0.049*
Female	male	5	3.00	0.270	0.904	15	11.0	0.674	0.765
Mean Yolk Volume									
	Day	2	3.00	2.14	0.265	3	11.0	13.5	<0.001*
	Female	3	6.68	2.30	0.168	4	16.9	3.38	0.033*
	Male	5	5.97	1.00	0.488	6	16.0	1.15	0.382
Female	male	5	3.00	1.99	0.303	15	11.0	5.18	0.045*
Mean Egg Diameter									
	Day	-	-	-	-	3	11.0	0.502	0.689
	Female	-	-	-	-	4	24.9	52.7	<0.001*
	Male	-	-	-	-	6	25.8	0.073	0.998
Female	male	-	-	-	-	15	11.0	0.302	0.983
Mean Viability (MVIA)									
	Day	-	-	-	-	3	11.0	4.23	0.032
	Female	-	-	-	-	4	25.8	18.5	<0.001*
	Male	-	-	-	-	6	23.1	0.188	0.977
Female	male	-	-	-	-	15	11.0	0.561	0.852

Table 4.8 Cont.

Univariate		2 DPH (n=19)				4 DPH (n=40)			
Variable	Factor	Ndf	Ddf	F	*p*	Ndf	Ddf	F	*p*
Mean Fertilisation of Viable Eggs									
	Day	-	-	-	-	3	11 0	6.37	0.009*
	Female	-	-	-	-	4	25 7	58.6	<0 001*
	Male	-	-	-	-	6	25.1	0.468	0 826
Female	male	-	-	-	-	15	11 0	0.375	0.960

Notes: * significant at α=0.05, ** significant at α=0.1
Day, female and male factors were all considered random effects.
(Ndf=numerator degrees of freedom; Ddf=denominator degrees of freedom; F=F statistic; *p*=*p*-value)

Table 4.9. Partial correlation coefficient matrices and p-values (p) for multivariate analysis of variance on 2 and 4 days post hatch (DPH) for 5 (2 DPH) and 7 (4 DPH) yellowtail flounder larval traits at the mean level.

Multivariate	2 DPH (df=3)				4 DPH (df=11)						
	MQ	MTBD	MNBD	MYV	MQ	MTBD	MNBD	MYV	MED	MVIA	MFERVIA
MSL	0 016	0 484	0 477	-0 650	0 837	0 900	0 874	-0 522	0 086	0 493	0 774
p	0 984	0 517	0 523	0 350	<0 001*	<0 001*	<0 001*	0 082**	0 791	0 103	0 003*
MQ		0 559	0 491	0 392		0 745	0 641	-0 243	0 063	0 285	0 705
p		0 441	0 509	0 609		0 005*	0 025*	0 447	0 846	0 370	0 010*
MTBD			0 996	-0 531			0 932	-0 534	0 161	0 476	0 633
p			0 004*	0 469			<0 001*	0 074**	0 617	0 118	0 027*
MNBD				-0 589				-0 416	0 258	0 382	0 562
p				0 411				0 178	0 418	0 221	0 058**
MYV									-0 088	-0 921	-0 588
p									0 786	<0 001*	0 044*
MED										0 063	-0 235
p										0 844	0 462
MVIA											0 586
p											0 045*

Notes: * significant at α=0.05; ** significant at α=0.1
(MSL=mean standard length, MQ=mean qualitative criteria; MTBD=mean total body depth at anus; MNBD=mean body depth from top of notochord to anus; MYV=mean yolk volume; MED=mean egg diameter; MVIA=mean viability).

Table 4.10. Variance components of contributing factors for 5 yellowtail flounder larval traits at the individual and mean level of aggregation.

		Individual Level						Mean Level					
		2 DPH			4 DPH			2 DPH			4 DPH		
Variable	Factor	n	Var Comp	%	n	Var Comp	%	n	Var Comp	%	n	Var Comp	%
Standard Length		455			990			19			40		
	Day		1 21E-02	22 7		0 00E+00	0 0		9 55E-03	29 9		0 00E+00	0 0
	Female		1 53E-02	28 7		5 72E-03	20 4		1 63E-02	51 1		5 53E-03	48 6
	Male		2 81E-03	5 3		1 26E-04	0 5		3 33E-03	10 4		1 53E-04	1 3
	Female male		1 27E-04	0 2		1 33E-03	4 8		0 00E+00	0 0		0 00E+00	0 0
	Error		2 29E-02	43 0		2 08E-02	74 3		2 73E-03	8 6		5 69E-03	50 0
Quality		455			990			19			40		
	Day		3 28E-03	0 7		9 21E-03	5 5		0 00E+00	0 0		1 00E-02	30 1
	Female		6 94E-02	14 3		1 74E-03	1 0		1 01E-01	41 6		2 04E-03	6 1
	Male		2 39E-02	4 9		0 00E+00	0 0		3 67E-02	15 1		0 00E+00	0 0
	Female male		8 39E-02	17 2		9 46E-03	5 7		1 03E-01	42 4		0 00E+00	0 0
	Error		3 06E-01	62 9		1 47E-01	87 8		2 32E-03	1 0		2 13E-02	63 8
Total Body Depth at Anus		455			545			19			40		
	Day		7 98E-06	0 1		0 00E+00	0 0		0 00E+00	0 0		0 00E+00	0 0
	Female		2 35E-03	33 0		8 24E-04	15 6		2 52E 03	37 5		9 12E-04	30 2
	Male		4 39E-04	6 2		1 05E-04	2 0		3 04E-04	4 5		1 46E-04	4 8
	Female male		2 12E-04	3 0		4 86E-04	9 2		0 00E+00	0 0		0 00E+00	0 0
	Error		4 11E-03	57 7		3 87E-03	73 2		3 89E-03	57 9		1 96E-03	64 9
Notochord Body Depth at Anus		254			545			19			40		
	Day		0 00E+00	0 0		6 21E-05	5 9		0 00E+00	0 0		7 74E-05	13 9
	Female		7 47E-04	34 6		8 70E-05	8 3		7 19E-04	38 6		1 05E-04	18 8
	Male		1 40E-04	6 5		7 66E-05	7 3		5 49E-05	2 9		8 01E-05	14 4
	Female male		7 14E-05	3 3		9 88E-05	9 4		0 00E+00	0 0		0 00E+00	0 0
	Error		1 20E-03	55 6		7 25E-04	69 1		1 09E-03	58 4		2 95E-04	53 0

Yolk Volume		254		988		19		40		
	Day	0 00E+00	0 0	5 45E-03	7 3	0 00E+00	0 0	5 10E-03	14 0	
	Female	4 53E-02	27 7	1 16E-02	15 5	4 22E-02	48 1	1 13E-02	30 9	
	Male	0 00E+00	0 0	0 00E+00	0 0	0 00E+00	0 0	0 00E+00	0 0	
	Female male	3 38E-02	20 7	1 76E-02	23 5	1 98E-02	22 6	1 51E-02	41 2	
	Error	8 43E-02	51 6	4 01E-02	53 6	2 57E-02	29 3	5 08E-03	13 9	
Egg Diameter						19		40		
	Day	.	.	.	.	6 35E-05	10 4	0 00E+00	0 0	
	Female	.	.	.	.	4 79E-04	78 1	5 42E-04	82 5	
	Male	.	.	.	.	2 60E-05	4 2	0 00E+00	0 0	
	Female male	.	.		.	0 00E+00	0 0	0 00E+00	0 0	
	Error	.	.	.	.	4 46E-05	7 2	1 15E-04	17 5	
Mean Viability						19		40		
	Day	.	.	.	.	4 19E+01	13 2	5 22E+01	14 5	
	Female	.	.	.	.	2 23E+02	70 3	2 06E+02	57 3	
	Male	.	.	.	.	1 39E+01	4 4	0 00E+00	0 0	
	Female male	.	.	.	.	0 00E+00	0 0	0 00E+00	0 0	
	Error	.	.	.	.	3 82E+01	12 1	1 01E+02	28 2	
Mean Fertilised of Viable Eggs						16		40		
	Day	.	.	.	.	8 64E+00	3 8	1 11E+02	34 5	
	Female	.	.	.	.	1 30E+02	57 0	1 78E+02	55 4	
	Male	.	.	.	.	0 00E+00	0 0	0 00E+00	0 0	
	Female male	.	.		.	0 00E+00	0 0	0 00E+00	0 0	
	Error	.	.	.	.	8 94E+01	39 2	3 26E+01	10 1	

Mean Fertilised of all Eggs						18				
	Day						7 36E+00	8 7		
	Female						5 86E+01	69 6		
	Male						1 82E+01	21 7		
	Female male						0 00E+00	0 0		
	Error						0 00E+00	0 0		
Mean Non-symmetrical Fertilised						18				
	Day						9 94E+00	92 5		
	Female						6 38E-01	5 9		
	Male						1 71E-01	1 6		
	Female male						0 00E+00	0 0		
	Error						0 00E+00	0 0		

Notes: Absolute and percentage of total values are provided for 2 and 4 days post hatch larvae.

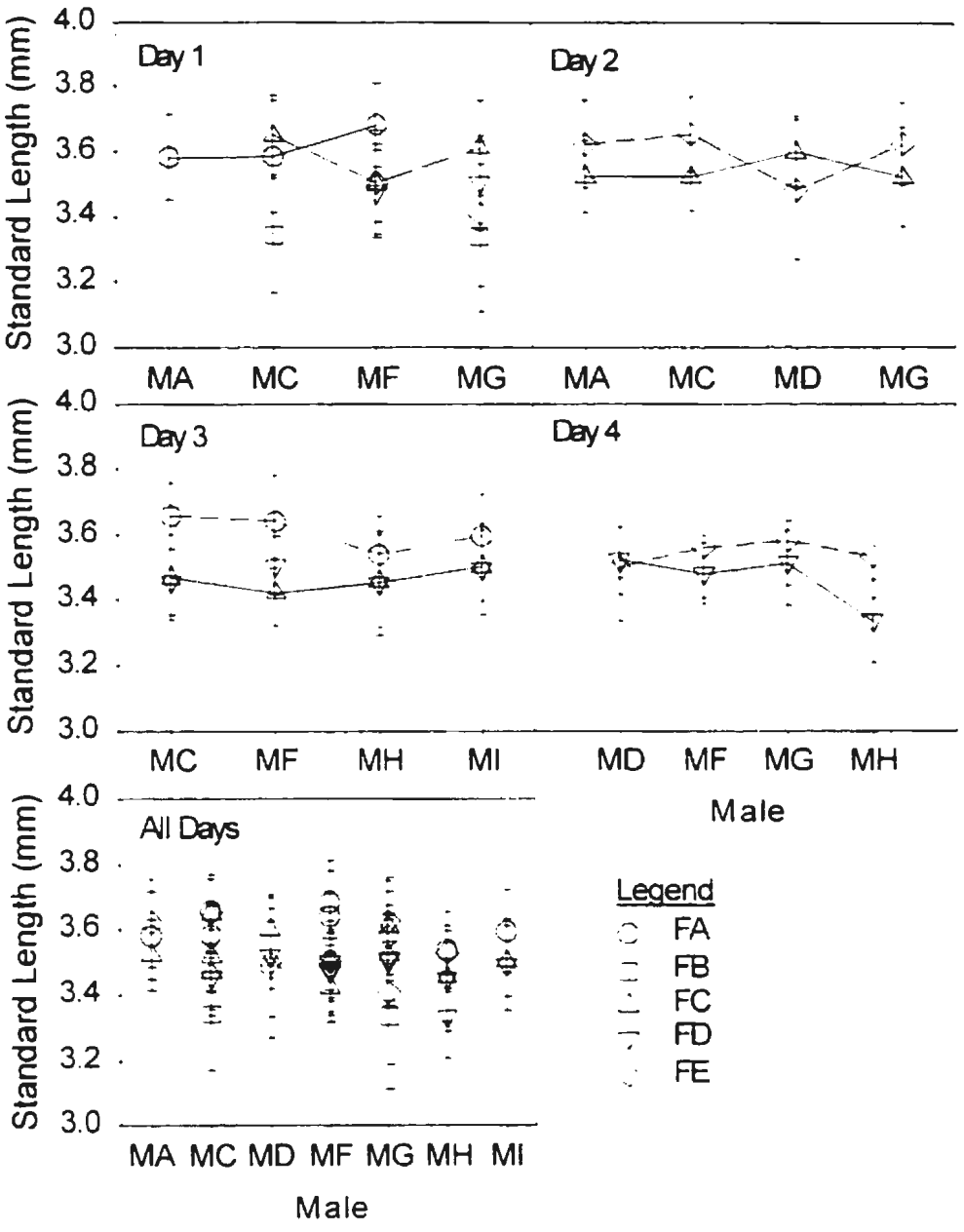

Figure 4.1. Larval standard length interaction diagrams (simple plots) for each mating day for the 4 DPH group only. Data points represent means (±SD) for each family of yellowtail flounder. Symbols represent different females (FA – FE) and letter-codes different males (MA – MI). Solid and dashed lines are meant only to enhance display and correspond to larval yolk volume lines of Figure 4.2.

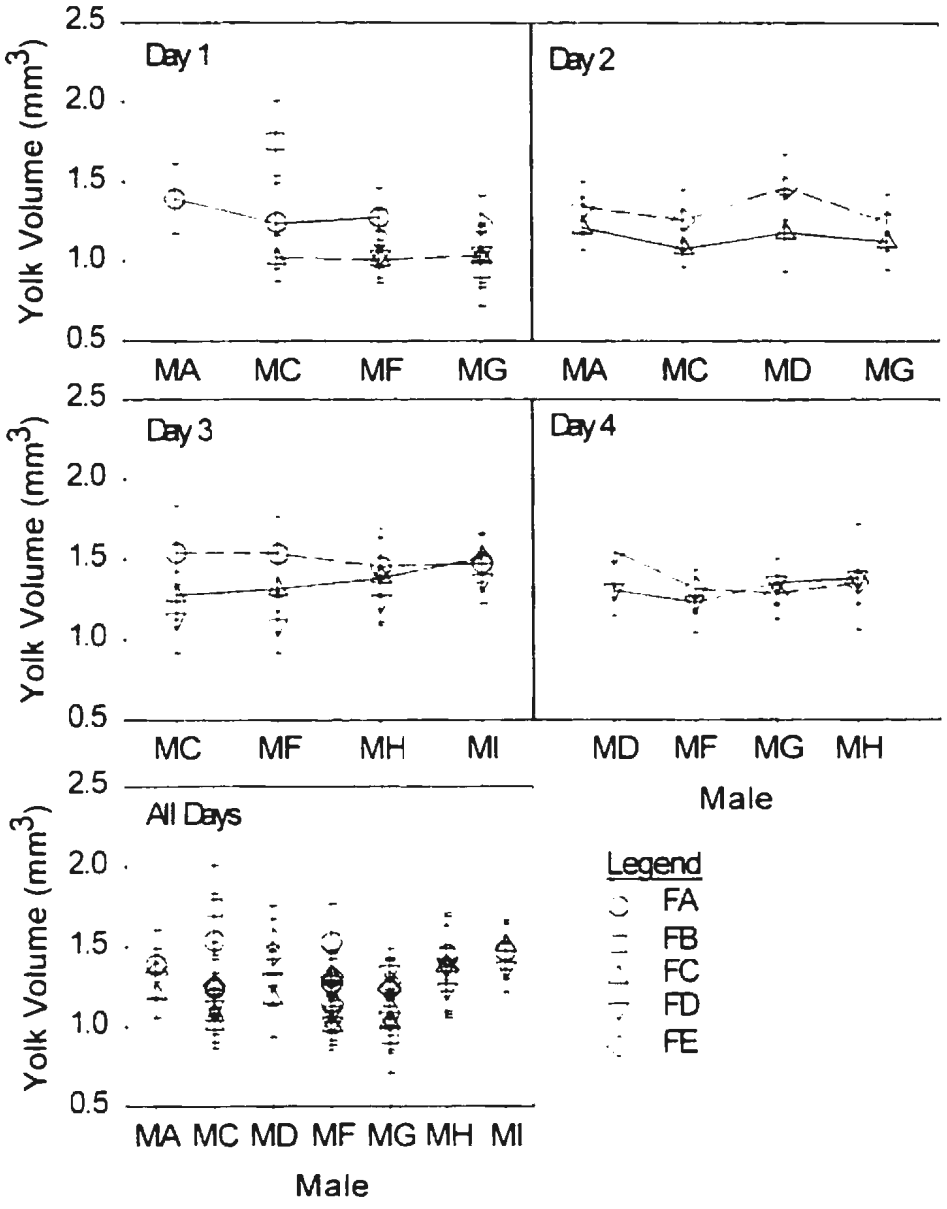

Figure 4.2. Larval yolk volume interaction diagrams (simple plots) for each mating day for the 4 DPH group only. Data points represent means (±SD) for each family of yellowtail flounder. Symbols represent different females (FA – FE) and letter-codes different males (MA – MI). Solid and dashed lines are meant only to enhance display and correspond to larval standard length lines of Figure 4.1.

Summary

It was found that yellowtail flounder produce highly variable egg batches that seem to be influenced more by females than males and that significant interactions between females and males predominated when larvae were analysed at the individual level. Females accounted for the majority of variability in larval assessment based on coefficients of variation followed by female · male interactions. Males contributed less; generally less than 10% of the total, but sometimes as high as 30% of female contributions such as in the quantitative quality measure (Q). This trend in coefficients of variation is repeated when larval data are scaled to mean levels. However, mean-level aggregate data analysed univariately showed significant changes in all female · male interactions. In almost all cases, those that were significant became not significant. This result shows conclusively that problems associated with scale do exist and that population biologists must extrapolate small-scale studies to wild populations cautiously. In that respect, these results strengthen the views of Chambers (1993), Pepin and Miller (1993), and Heath and Gallego (1997).

Multivariate analyses were found to be significantly more effective in determining overall larval quality and relationships between ELH traits than either univariate analyses or multiple regression analyses. Univariate analyses were consistently variable within and among factors both temporally and under changes in sample size or changes in the number of ELH traits that were included in the analyses. Multiple regressions were marginally more effective

except that they were unable to handle qualitative data. It is recommended that multivariate approaches to data analyses be utilised whenever possible as they are more effective in assessing overall egg and larval quality and because they can encompass many traits.

This research has shown that univariate data analyses are much more sensitive to changes in sample size, larval growth, and whether data are analysed at different scales. The biological significance of using multivariate analyses is obvious. An individual is composed of many traits that are affected by environment, physiology, and genetics that cannot generally be summed up in one or two traits (see review by Bernardo, 1996). Therefore, the use of egg diameters and larval standard lengths as the two premier quality criteria are suspect because they may not adequately reflect the influence of many factors acting in concert.

Effective and proper use of statistical tools are crucial for biologists in order to understand biological systems. Recruitment variability may be the single most important issue surrounding groundfish fisheries in Atlantic Canada. Population biologists must refrain from using loose interpretations of data, summary statements, and extrapolated data in critical recruitment calculations. The interpretation and summary of scientific thought, generally administered through scientific reviews of a subject, must not lose the qualifications under which the studies were performed. The resulting generalities are often of little use to the application of the science.

Curiously, the axiomatic view that females are the most important factor in egg and larval quality is not beyond speculation for yellowtail flounder. Female traits such as size and mass did not correlate well with early egg quality indicators such as viability and egg size in Chapter 2 or 4. Furthermore, viability was not strongly related to egg size. Egg size appears to be a speculative indicator of egg quality within yellowtail flounder and possibly other multiple batch spawning fish such as Atlantic cod and halibut. This view is supported by inconsistencies and general lack of knowledge with egg hydration processes in multiple batch spawning flatfish, and the lack of evidence to support that egg size (diameter or volume) is closely related to actual egg content. However, strong evidence was found that viability, fertilisation success, and larval standard length are highly correlated and follow the general insights provided in reviews by Kjørsvik et al. (1990) and Brooks et al. (1997).

Overall, the data and results of the four studies are in agreement with other work on yellowtail flounder (Larsson et al., 1997; Manning and Crim, 1998; Benoît and Pepin, 1999). Egg batches are highly variable within and among females, and female effects comprise the majority of the variation. What is not known is how the variability is manifested or controlled. I have presented some results that suggest genetics may play a larger role than previously thought in larval quality through male contributions. These contributions may not be immediately apparent, but the early ontogeny results are encouraging.

Genetics may also play a larger role in female contributions although the early indication is that females are more plastic to environmental influences. In

particular, the processes surrounding vitellogenesis and hydration are not completely understood. Induced ovulation seems to affect mortality during embryogenesis suggesting that some important egg constituents are laid down in the brief period between ovulations. Larger eggs may not necessarily contain more nutrients or produce larger larvae, but may be produced in response to environmental cues such as water temperature and salinity. These environmental cues may influence hydration in order to maintain a neutral buoyancy. Egg size may also be affected by the timing of spawning through photoperiod, temperature or physiological cues such as decreases in vitellogenin.

The research herein is applicable to large-scale aquaculture. Mortality during egg and larval rearing is severe and is the biggest hurdle to overcome for large-scale aquaculture operations to become successful. Broodstock selection has received little attention, but has been shown herein to be an area that requires further research. Maternal effects appear to dominate egg and larval quality, however, maternal effects are still present.

References – Overview, Summary & Appendices

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Appendix 1.

A MORTALITY ESTIMATION TECHNIQUE BASED ON DRY MASS OF FILTERED DEAD EGGS IN INCUBATION SYSTEMS

Chapter 1 was primarily undertaken to elucidate some of the differences between mortality and maternal effects of SO and IO eggs of yellowtail flounder. In order to accomplish this task, a methodology had to be devised to accurately assess mortality.

The number of eggs remaining in an incubator is often overestimated because of the small size of the eggs compared to the large surface area of the incubator. Determining the number of eggs remaining in an incubator by visual inspection is crude at best and highly inconsistent if more than one person is performing the task. Using a dry mass technique (DMT), as outlined in Chapter 1, eliminates these two sources of variability from mortality estimations. Briefly, the DMT is performed as follows: 1) the known number of eggs in a given volume is generally taken from unfertilised eggs by pipetting a volume of eggs and counting them, 2) Standard regression analysis is then done using various pipetted volumes of eggs, and 3) The number of eggs in the filtrate is then estimated with the standard regression relationship. The drawback of the DMT is that the technique requires prior knowledge of the number of eggs in a given volume since the dry mass calculations are based on actual egg counts. Given that, the required standard linear regressions and corresponding egg counts only have to be done periodically to confirm the standard relationships. There may

also be problems associated with the logistics of providing a calculated or scaled-up estimation.

The main reason for employing a DMT stems from the typical practise of estimating dead eggs by directly equating filtrate volume to known numbers of eggs in a volume of ovarian fluid when the matrix of particles in the siphoned filtrate and eggs in ovarian fluid are substantially different. The siphoned material can be thought of as a suspension of particles with a high and variable water content whereas the unfertilised eggs pack together tightly forming a more cohesive mass that is held together by ovarian fluid rather than suspended in it. Thus, the calculation of egg number in the filtrate would be overestimated based on the volume counts within ovarian fluid if the counts were done directly. A water content factor could be used to reduce the overestimation, but this would have to be used on a per-siphoned sample basis since the water content would vary. This process would be time consuming and may not be cost effective.

The earlier that high quality egg batches (those that have reduced mortalities) can be determined, the more beneficial it is to the fish culturist. The DMT seems to give a good indication of egg quality at around day two to day three of development. Using this technique for fish culture, a mortality level can be established based on previous egg batches and modified as new batches are reared. Any batch that exceeds the maximum mortality limit in a given amount of time (e.g., three or four days) would be discarded and a new batch of eggs started thus providing quick turnover for valuable incubator space, and ensuring the potential for high quality larvae.

In comparison to the DMT, more indirect mortality estimation methods involve relative visual mortality estimates, where mortality is tallied as low, medium, or high (Wanzenbök and Wanzenbök, 1993), or visual estimates of the percent surface coverage of floating eggs where dead eggs sink. Both indirect methods lack accuracy and precision for large scale incubation processes and are subjective relying, to some extent, on the experience of the observer. The most accurate method of determining egg mortality involves subtracting the number of dead eggs or the number of hatched larvae from an initial, known number of eggs (e.g., Laurence and Howell, 1981; Marteinsdottir and Able, 1992). However, hatching success can not be determined until the eggs hatch.

Two assumptions arise with the DMT. 1) The total biomass of material siphoned from the bottom of the incubators was from dead eggs only. This assumption is reasonable since the incubating system was static, the incubator water was filtered through a 1 μm glass wound filter and under ultraviolet light, and antibiotics were added, and 2) Egg quality based on mortality is related to larval survival which needs to be assessed in further detail for yellowtail flounder.

Appendix 2.

COMPARISON OF PARAMETRIC, NON-PARAMETRIC, AND LOG TRANSFORMED DATA ANALYSES AS PERFORMED BY GENERAL LINEAR MODEL (GLM) PROCEDURES. DATA ARE TAKEN FROM TABLE 4.5.

	2 DPH			4 DPH		
	Parametric	Non-Parametric	Log Transform	Parametric	Non-Parametric	Log Transform
Standard Length						
Day	<0.001	<0.001	<0.001	0.775	0.703	0.798
Female	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Male	0.198	0.284	0.204	0.139	0.107	0.142
Female Male	0.196	0.140	0.176	0.081	0.070	0.088
Quality						
Day	0.572	0.572	0.572	0.524	0.524	0.524
Female	0.111	0.111	0.111	0.189	0.189	0.189
Male	0.460	0.460	0.460	0.965	0.965	0.965
Female Male	<0.001	<0.001	<0.001	0.036	0.036	0.036
Yolk Volume						
Day	<0.001	0.066	0.001	<0.001	<0.001	<0.001
Female	0.080	0.122	0.117	0.031	0.026	0.034
Male	0.312	0.421	0.439	0.297	0.213	0.282
Female Male	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Notochord Body depth at Anus						
Day	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Female	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Male	0.087	0.058	0.079	0.249	0.301	0.265
Female Male	0.110	0.155	0.110	<0.001	<0.001	<0.001
Total Body depth at Anus						
Day	0.014	0.034	0.016	<0.001	<0.001	<0.001
Female	0.004	0.002	0.003	0.006	0.004	0.007
Male	0.094	0.082	0.088	0.031	0.014	0.039
Female Male	0.088	0.081	0.104	<0.001	<0.001	<0.001