CIRCUMSTANCES OF ENERGETIC USE OF WHITE MUSCLE PROTEIN IN TWO FLATFISH SPECIES, HIPPOGLOSSOIDES PLATESSOIDES AND PLEURONECTES AMERICANUS: STARVATION, NATURAL VARIATION AND REPRODUCTIVE DEMANDS ON WHITE MUSCLE

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Circumstances of energetic use of white muscle protein in two flatfish species, *Hippoglossoides platessoides* and *Pleuronectes americanus*: starvation, natural variation and reproductive demands on white muscle.

by

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Abstract

White muscle moisture was examined in two species of flatfish indigenous to Newfoundland waters, the winter flounder (*Pleuronectes americanus*) and American plaice (*Hippoglossoides platessoides*), in relation to seasonal cycles and experimentally induced energetic stress. Natural elevation of white muscle moisture observed during the spawning season in *H. platessoides* reflects protein usage and is probably due, in part, to energetic demands imposed by batch spawning. The observation of developing yolky oocytes coinciding with evidence of recent spawning suggests the potential to increase fecundity during the spawning season by recruitment of immature oocytes for release in the current season. This might require further depletion of white muscle protein resulting in the significantly higher muscle hydrations observed at this time.

Experimental conditions which imposed energetic stress on *H. platessoides* as a result of high temperatures caused restricted gonad development if condition of the fish was less than 0.85 prior to setup and allowed maintenance of low white muscle moisture. Low condition fish which underwent reproductive development despite unfavourable conditions, experienced high muscle hydration and resorption of vitellogenic oocytes was evident upon termination. Those fish with high condition prior to setup developed their oocytes to a mature, vitellogenic state and maintained significantly lower muscle moisture than low condition fish which were in similar state of reproductive development at termination.

Depletion of white muscle protein is a reversible process. After only four

months of refeeding, a rebound in condition and lowered white muscle moisture was observed in winter flounder, *P. americanus*, in which low condition and high white muscle moisture had been induced by starvation. The ability to selectively use and rebuild white muscle suggests an energetic storage system which has been developed to deal with energetic demands the fish might face in the wild, including reproductive development and dealing with food shortages.

Glycerinated single fibre preparations were studied for contractile ability and sarcomere characteristics. Both *H. platessoides* and *P. americanus* have sarcomeres that are considerably shorter than those of mammalian tissue. Intrafibre sarcomere differences were also noted as sarcomeres near the insertion were shorter than those in the central fibre region. Comparisons between contractile ability of starved versus fed *P. americanus* were not quantitative since it was difficult to discern sarcomeres in fibres from starved fish. Contraction was observed in some fibres from starved flounder, probably the result of conservation of individual fibres or groups of fibres.

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Table of Contents **Page**

Abstract..... ii

Acknowledgements..... iv

List of Tables and Figures..... viii

General Introduction..... 1

Chapter 1- Seasonal variation in condition and gonadosomatic indices of American plaice, *Hippoglossoides platessoides*, with related changes in muscle moisture: implicating the use of white muscle as an energy store for maturing oocyte batches..... 5

1.1 Abstract..... 5

1.2 Introduction..... 7

1.3 Materials and Methods..... 12

1.4 Results..... 16

1.5 Discussion..... 22

Chapter 2- Starvation effect on white muscle moisture and gonad development in American plaice, *Hippoglossoides platessoides*..... 57

2.1 Abstract..... 57

2.2 Introduction..... 58

2.3 Materials and Methods..... 61

2.4 Results..... 64

2.5 Discussion..... 67

Chapter 3- Recovery of white muscle from a starvation induced high water state in winter flounder, *Pleuronectes americanus*, through refeeding..... 89

3.1 Abstract..... 89

3.2 Introduction..... 90

3.3 Materials and Methods..... 93

3.4 Results..... 96

3.5 Discussion..... 99

Chapter 4- Preliminary investigation of sarcomere characteristics in *Pleuronectes americanus* and *Hippoglossoides platessoides* using glycerinated single fibre preparations: sarcomere length and contractile ability..... 117

4.1 Abstract..... 117

4.2 Introduction..... 118

4.3 Materials and Methods..... 120

4.4 Results..... 122

4.5 Discussion..... 124

Conclusions..... 133

References..... 137

List of Tables and Figures **Page**

Figure 1.1: Map of sampling sites A through F as taken from American plaice, *Hippoglossoides platessoides*..... 33

Figure 1.2: Oocytes from *Hippoglossoides platessoides* in various developmental stages..... 35

Figure 1.3: Oocytes from American plaice, *Hippoglossoides platessoides*, showing advanced development..... 37

Figure 1.4: Photograph of a female American plaice, *Hippoglossoides platessoides*, killed in April..... 39

Figure 1.5: Prespawn and postspawn ovary characteristics of American plaice, *Hippoglossoides platessoides*..... 41

Figure 1.6: Ovary cross-section from an American plaice female killed in June with oocytes in various stages of development..... 43

Table 1.1: Means and ranges for mature female American plaice, *Hippoglossoides platessoides*..... 44

Table 1.2: Means and ranges for mature male American plaice, *Hippoglossoides platessoides*..... 44

Figure 1.7: Seasonal variation in gonadosomatic index (GSI) and percent white muscle moisture (%H_2O) in American plaice, *Hippoglossoides patessoides*, over time..... 46

Figure 1.8: Skipped spawning and adolescence in American plaice, *Hippoglossoides platessoides*..... 48

Figure 1.9: Seasonal variation in hepatosomatic index (HSI) and condition factor (CF) in American plaice, *Hippoglossoides platessoides*..... 50

Figure 1.10: White muscle from American plaice, *Hippoglossoides patessoides*, showing protein depletion in area of high water content..... 52

Figure 1.11: Indications of recent spawning accompanied by oocytes in various stages of development in *Hippoglossoides platessoides*..... 54

Figure 1.12: Length weight curve for American plaice, *Hippoglossoides platessoides*..... 56

Figure 2.1: Sampling sites from which muscle for histology and determination of moisture content were taken on American plaice, *Hippoglossoides platessoides*..... 72

Figure 2.2: Comparisons between fed and starved American plaice, *Hippoglossoides platessoides*..... 74

Figure 2.3: Gonadosomatic index (GSI) and Hepatosomatic Index (HSI) in fed versus starved American plaice, *Hippoglossoides platessoides*. 76

Table 2.1: Summary data for experimental American plaice, *Hippoglossoides platessoides*, ordered by increasing white muscle moisture..... 78

Figure 2.4: Water temperatures (°C) in experimental tanks over the course of experimental period..... 80

Figure 2.5: Ovaries from experimental American plaice which entered the experimental condition with different somatic condition..... 82

Figure 2.6: Ovary from American plaice, *Hippoglossoides platessoides*, that showed the highest muscle water content of 85.64%..... 84

Figure 2.7: Atretic breakdown of yolky oocytes in *Hippoglossoides platessoides*..... 86

Figure 2.8: Cross section of ovaries from experimental American plaice, *Hippoglossoides platessoides* which showed no evidence of atresia..... 88

Figure 3.1: Change in condition factor in winter flounder, *Pleuronectes americanus*, over the course of experimental starvation and refeeding..... 104

Figure 3.2: Moisture content of white muscle from winter flounder, *Pleuronectes americanus* determined at biopsy and termination..... 106

Figure 3.3: Relationship between white muscle water content and condition factor for experimentally starved and fed winter flounder, *Pleuronectes americanus*..... 108

Figure 3.4: Ultrastructural cross section of white muscle from experimentally fed (a) and starved (b) winter flounder, *Pleuronectes americanus*..... 110

Figure 3.5: Oocytes biopsied from a recrudescent winter flounder, *Pleuronectes americanus*, showing both previtellogenic (**pv**) and exogenously vitellogenic (**v**) oocytes..... 112

Figure 3.6: Gonads from flounder, *Pleuronectes americanus*, starved and subsequently refed..... 114

Figure 3.7: Atretic resorption of yolky oocytes in experimental *Pleuronectes americanus*..... 116

Figure 4.1: Sarcomeres from American plaice and rabbit psoas viewed under the light microscope..... 128

Figure 4.2: Graph showing change in sarcomere length following addition of ATP and metal salts solution to isolated, glycerinated muscle fibres from *Pleuronectes americanus*..... 130

Figure 4.3. Longitudinal section of white muscle from winter flounder, *Pleuronectes americanus*, viewed under the transmission electron microscope..... 132

General Introduction

It has been suggested that American plaice, *Hippoglossoides platessoides* and winter flounder, *Pleuronectes americanus*, have evolved different life-history strategies in order to deal with energetic constraints that may have to be dealt with in the natural environment. Plaice are reported to sacrifice somatic condition in case of energetic limitation, channelling energy into reproductive development for immediate reproductive gain (Roff 1982), while flounder are thought to suspend reproductive effort in order to preserve body condition thereby increasing the chance of survival until circumstances permit greater return on reproductive investment (Tyler and Dunn 1976). *H. platessoides* and *P. americanus* are flatfish species which inhabit the waters off Newfoundland. Plaice are generally found at greater depths offshore, on the Grand Banks, while winter flounder are an inshore, shallower water species (Scott and Scott 1988). Winter flounder undergo a natural period of starvation for approximately six months during the winter (Fletcher and King 1978, Kennedy and Steele 1971) and plaice are reported to reduce feeding in the winter compared to spring and summer consumption (Zamarro 1992a). Templeman and Andrews (1956) recorded large female American plaice with "jellied muscle" having water content as high as 96%. Pearcy (1961) stated that flesh of ripe adult winter flounder was "soft and watery" during the winter fast, and Mcleese and Moon (1989) suggest a cycle of muscle water content in Passomaquoddy Bay flounder. High white muscle water content is directly related to lower protein content (Love 1958).These species, then, may be exposed to situations in the natural environment which require the energetic use of white muscle protein.

The utilization of white muscle protein as a source of energy has been reported for several species of fish in times of starvation (Maddock and Burton 1994, Black and Love 1986, Kiran and Talesara 1985, Johnston 1981, Jobling 1980, Johnston and Goldspink 1973, Gas 1972, Greer-Walker 1971, Love 1958) and suggests the use of white muscle as a potential storage area drawn upon to deal with energetic demands over and above those met by traditional storage areas such as the liver. The depletion of protein from white muscle is likely detrimental to contractile function and Love (1980) was amazed at the ability of fish with extreme muscle water content to survive, much less function normally. The white muscle seems to be selectively targeted for protein usage (Maddock and Burton 1994, Kiran and Talesara 1985, Johnston 1981, Johnston and Goldspink 1973). As this muscle mass comprises the bulk of fish musculature, with red comprising a much smaller percentage of the total muscle mass, only 7% in *H. platessoides* (Greer-Walker and Pull 1974), white muscle is a much more considerable potential energy store. As well, red muscle is considered to be used primarily in the normal cruising activity, while white muscle is involved in burst activity needed for rapid tail flips used in predator evasion and prey capture. Although protein depletion likely compromises burst activity, it is the larger fish which were thought to reach worst states of muscle condition (Templeman and Andrews 1956) and these fish may be under less predation pressure than smaller fish might be. Another method of reducing the effects of protein removal from white muscle would involve conservation of areas of the musculature, at the level of muscle groups, motor units or of individual fibres,

allowing maintenance of some contractile function in the compromised state. The possibility that compromised fibres may retain contractile ability also remains to be seen.

The use of muscle protein for the purpose of gonad maturation is suggested for several species (Bradford 1993, Fisher *et al.* 1987, Dawson and Grimm 1980). American plaice are believed to sacrifice somatic condition in order to proceed with gonad development, instead of restricting energy input into gonad growth in the event of energetic crisis as undertaken in winter flounder, *P. americanus* (Burton and Idler 1987). The possibility of missed or skipped spawning in *H. platessoides* has been reported by Pitt (1966) and in the European subspecies *Hippoglossoides platessoides limandoides* by Bagenal (1957). Reproduction in local populations of *H. platessoides* has been characterized by Zamarro (1992b) as having group synchronous oocyte development with fecundity determined by May. A more complex pattern of oocyte growth and spawning strategy, including spawning of serial oocyte batches and the possibility that plaice can bring relatively undeveloped oocytes through vitellogenesis during the spawning season, as suggested in *Pleuronectes platessa* by Horwood (1990), might explain the need to draw more extensively on white muscle protein stores than in other species.

Rebuilding of white muscle protein following energetic consumption via reproductive or maintenance/survival pathways would be essential to maximize survival, growth and reproductive output. Recovery from a low protein state, indicated by increased white muscle moisture (Stirling 1976, Love 1958), is not

thought to occur in Dover sole, *Microstomas pacificus* (Fisher *et al.* 1987), and is a relatively slow process in Atlantic cod, *Gadus morhua* (Black and Love 1986).

The following investigations were undertaken in an attempt to better understand the energetic use of white muscle protein in *H. platessoides* and *P. americanus*; relationships between protein usage and reproductive development under natural and experimentally induced conditions; possibility of conservation of fibre integrity and function despite reduction in protein content; recovery of muscle from a high moisture state indicative of protein depletion; and effects of protein loss on fibre contractile ability.

Chapter 1

Seasonal variation in condition and gonadosomatic indices of American plaice, *Hippoglossoides platessoides*, with related changes in muscle moisture: implicating the use of white muscle as an energy store for maturing oocyte batches.

1.1 Abstract

Variation in condition factor (CF), gonadosomatic index (GSI), hepatosomatic index (HSI) and white muscle water content of American plaice, *Hippoglossoides platessoides*, was followed from December of 1993 until February of 1996. Spawning season was characterized by low GSI and higher white muscle moisture than at other times of the year. Female plaice displayed higher white muscle moisture than males during the spawning period. The largest increase in GSI occurred between January and February for females, and was maximum between January and April for males. Hepatosomatic index was lowest in the spawning season, suggesting cessation of feeding activity during spawning, but rose immediately in July and condition factor recovered from its low spawning level. Conservation of some white muscle fibres is suspected, but no regular pattern in area conserved was found.

Gonad development was advanced in December and by April some gonads were showing evidence of spawning. Ovaries from spawning females showed hydration patterns and histological detail consistent with batch or serial spawning strategy. Evidence of recent spawning activity, including the presence of post-ovulatory follicles, was found in ovaries that also contained oocytes that were

undergoing exogenous vitellogenesis. This may represent the ability of plaice to push oocytes through vitellogenesis from a previtellogenic condition during the spawning period. The increased white muscle moisture during June and July may reflect the use of muscle protein to bring oocytes through vitellogenesis for the current spawning season.

1.2 Introduction

Oocyte development and reproductive strategy have been described in many marine teleost species in an effort to understand the time course and energetic consequences of reproductive effort (Rickey 1995, N'Da and Déneil 1993, Htun-Han 1978, Foucher and Beamish 1977, Barr 1963). Oocyte growth from immature status to a mature yolky stage follows a general pattern. Oogonia, small cells (<4µm) identified by a large single nucleolus, grow into primary oocytes which are larger and have 1 to 5 central nucleoli (Barr 1963). Primary oocytes are diploid in nature, with a pause in meiosis for growth (Foucher and Beamish 1977) and are then distinguished by some authors as immature oocytes, which have multiple peripheral nucleoli (N'Da and Déneil 1993). This stage may be referred to as the "reserve fund" size (Foucher and Beamish 1977) and represents the store from which oocytes are matured. Immature ovaries are characterized by oocytes which have not progressed beyond the immature or perinucleolar stage (Rickey 1995). Primary vitellogenesis is defined by the appearance of a ring of vacuoles inside the cell membrane and appearance of the zona radiata (N'Da and Déneil 1993, Htun-Han 1978, Foucher and Beamish 1977). Also referred to as the cortical alveoli stage or endogenous yolk formation, the deposition of mucopolysaccharides is thought to occur in the cortical alveoli (Khoo 1979). True vitellogenesis, the deposition of vitellogenic yolk in the cytoplasm as yolk globules, occurs in secondary vitellogenesis or early true vitellogenesis, along with the appearance of the follicle layer (N'Da and Déneil 1993,

Htun-Han 1978). In late, or tertiary, vitellogenesis, the oocyte is packed with yolk and the zona radiata thickens (N'Da and Déneil 1993). The nucleus migrates to the animal pole prior to the breakdown of the nuclear membrane (Yamamoto 1956). Hydration precedes ovulation and the appearance of these hyaline oocytes is an indication of imminent spawning (West 1990). The follicle collapses after the oocyte has been released to form structures called post ovulatory follicles (POFs) which are indications of recent spawning and are not thought to persist for a long time. POFs in the red drum, *Sciaenops ocellatus* are thought to be less than 24 hours old, based on studies of captive fish (Wilson and Neiland 1994). Barr (1963) states that follicles are only seen as small bunches of thecal cells two months following spawning in the plaice, *Pleuronectes platessa*.

Spawning strategy may be classified based on the number of spawning episodes during the season, with some species spawning only once, or for a very short period during the spawning season as is seen in the winter flounder *Pleuronectes americanus* (Burton and Idler 1984). Other fish will spawn several times during the spawning season, releasing clutches or batches of eggs ovulated at discreet intervals. Atlantic cod, *Gadus morhua* (Kjesbu *et al.* 1991), Arrowtooth flounder, *Atheresthes stomias* (Rickey 1995), the European plaice, *Pleuronectes platessa* and *Solea solea*, the North Sea sole (Urban and Alheit 1988) are examples of serial, multiple or batch spawners. The advantages of batch spawning are thought to include increased fecundity by overcoming physical limitations of small body

cavities, spreading the risks from predators and impact on prey of the larvae, and increasing the chance that release of at least some of the batches will coincide with favourable conditions for hatching and survival of eggs and larvae (McEvoy and McEvoy 1992). Descriptions of the spawning period and fecundity (Zamarro 1992b, Pitt 1966, Bagenal 1957, Templeman and Andrews 1956) of *H. platessoides* do not include the spawning strategy undertaken in this species.

Batch spawners may be classified as having either indeterminate or determinate fecundity based on whether or not oocytes are brought through vitellogenesis during the spawning season. Fecundity is determinate if all of the oocytes to be released have reached a relatively advanced stage prior to spawning and no additional oocytes will undergo development once the spawning season commences. A gap in size distribution between immature and vitellogenic oocytes prior to spawning is indicative of determinate fecundity as seen in *Gadus morhua* (Kjesbu *et al.* 1991), *P. platessa* (Horwood 1990), and the red mullet, *Mullus surmuletus* (N'Da and Déneil 1993). Other species such as the red drum (Wilson and Neiland 1992), Atlantic mackerel, and Northern anchovy (Hunter and Leong 1981) are indeterminate spawners and do not show a gap in the size frequency of immature versus mature oocytes. These fish are capable of bringing oocytes from an immature condition through vitellogenesis during the spawning season.

Those fish that undergo gonad maturation during periods of lower food availability, such as *H. platessoides* and *P. platessa*, are thought to utilize somatic

energy reserves, particularly protein, for reproductive growth (Roff 1982, Dawson and Grimm 1980). The utilization of white muscle protein as a source of energy in times of starvation results in an increase in white muscle water content and breakdown of muscle integrity (Maddock and Burton 1994, Johnston 1981, Johnston and Goldspink 1973, and Love 1958) similar to the jellied muscle condition described by Templeman and Andrews (1956) in wild American plaice, *H. platessoides*. Red muscle, that used mainly for slow speed sustained swimming, is mostly conserved in starvation (Beardall and Johnston 1983, Johnston and Goldspink 1973). Conservation of discreet areas of fast muscle is also indicated in some species. White muscle degradation due to starvation does not appear to affect the central muscle mass (ie. that closest to the lateral line) of the sturgeon, *Acipenser transmontanus*, to the same degree as fibres further from the lateral line (Keissling *et al.* 1993). Fisher *et al.* (1987) also state that the muscle nearest the dorsal and anal fins in the Dover sole, *Microstomas pacificus*, is more susceptible to the jellied condition than the central muscle mass.

The spawning characteristics of American plaice, *Hippoglossoides platessoides*, from the Grand Banks of Newfoundland have been described by Zamarro (1992b) as having group synchronous oocyte development with fecundity for the following year determined by May. Spawning was recorded as occurring in the last part of March and April. Pitt (1966) studied *H. platessoides* from areas off the coast of Newfoundland and Grand Banks and records spawning temperatures

ranging from 1.1°C to 4.3°C, shows some females spawning in June and observes that some mature fish did not produce eggs every year. The aim of this study was to monitor the reproductive development of *H. platessoides* from Newfoundland waters and relate the white muscle moisture content and condition of the fish to the reproductive strategy.

1.3 Materials and Methods

Fish

American plaice were collected by the Department of Fisheries and Oceans (DFO) research vessels *Wilfred Templeman* and *Teleost* from NAFO areas 3NO between December 1993 and June 1995. One sample was collected from Trinity Bay, NAFO division 3L, in December of 1995 on board the DFO research vessel *Shamook*. Fish were obtained live and killed either with an excess of MS222 followed by transection of the spinal cord or by spinal transection alone. Some samples were obtained in frozen condition and were included in gonadosomatic index and length weight data but excluded from the white muscle water content study since freezing affected the moisture content of the muscle. Measurements taken included whole weight and fork length, used to determine condition factor, and gonad and liver weights, used to determine relative indices of these organs.

Samples of tissue, approximately $1cm^3$, were taken from six sites on the ocular and abocular surfaces of the plaice (Figure 1.1). In order to accommodate proportional differences in fish of varying size, sites were located using anatomical features which are proportional to fish size. Site a was defined as the midpoint between the lateral line and fin rays on the left side of the ocular surface at the level of the pectoral fin tip. Sites b, c, and d were sampled at a distance equal to the length of the pectoral fin away from the tip of the pectoral fin. Sites b and d were located midway between the vertebrae and fin rays on the left and right side of the

lateral line, respectively, while site c traversed the lateral line. The caudal sites were located 25 fin rays from the caudal peduncle and midway between the vertebrae and fin rays, site e on the right of the lateral line, and site f on the left. Muscle samples were taken to a depth of about 1cm or greater from both ocular and abocular surfaces, included the skin as a point of reference, and were preserved for histological examination. From each of the six sites on the ocular surface a piece of white muscle approximately $2mm^3$ was taken at a depth of 0.5cm or greater and was used to determine percent white muscle moisture ($\%H_2O$), by drying to constant weight at 60°C. On two occasions, six samples were taken from both ocular and abocular surfaces to compare muscle moistures.

Fish from some samples were measured to determine if the ocular muscle depth was similar to the abocular muscle depth. To accomplish this, the fish was sectioned completely at site C and the ocular and abocular muscle layer, from the lipid layer to the vertebral process, was measured to the nearest tenth of a centimetre. Comparisons were made using the Student's *t*-test with $\alpha=0.05$.

The posterior tip of the gonad were also preserved for histological examination.

Indices of Fish Condition

Condition factor was determined by the formula CF = 100 x whole weight (g) ÷ length $(cm)^3$. The state of gonad development was ascertained using the gonadosomatic index where GSI = 100 x gonad weight (g) ÷ whole weight (g). A

third measure of fish condition, the hepatosomatic index, was determined by HSI = 100 x liver weight (g) ÷ whole weight.

Histology

Samples for histological examination were fixed in Bouin's fixative (75:25:5 picric acid:strong formalin:acetic acid) for 48 hours then transferred to 70% ethanol. These samples were processed through an ethanol dehydration series (1 hour each in 90%, and 2 changes of 100%), cleared in xylene (1 hour) and embedded in Paraplast Plus® for sectioning. Cross-sections, approximately 7µm thick were cut on a rotary microtome. Sections were floated on water on slides smeared with Mayer's glycerine albumin for adhesion. They were dried at 37°C on a slide warmer before staining. The staining schedule involved removal of wax in xylene for 5 minutes, hydration through an ethanol series (5 minutes each in 100%, 90%, 70%, and 50%), 5 minutes in distilled water then staining in Ehrlich's haematoxylin and blueing with Scott's solution. After rinsing with distilled water, slides were partially dehydrated (5 minutes each in 50%, 70% and 90% ethanol) and counterstained with Eosin Y (30 seconds) before two changes of 100% ethanol (5 minutes each) completed dehydration. Slides were cleared in xylene and coverslips mounted with Histoclad®.

Slides were examined by light microscopy. Skin and muscle sections were examined to compare muscle and lipid status between sites on a fish and between fish to determine if sites are conserved in the case of high muscle moisture. Gonad sections were examined to determine the state of gonad development. In particular,

yolky oocytes indicated development was advanced while previtellogenic oocytes indicated an earlier state of development. Residual eggs in the lumen of the ovary and the presence of post-ovulatory follicles (POFs) were taken as evidence of recent spawning activity. Atretic oocytes were also noted.

Images

Images were attained from a Zeiss® microscope with Sony® videocamera attachment and Bravado® image capturing board. Measurements and calibrations were made with Mocha® video analysis software. Captured images were arranged and annotated in Coreldraw® and printed on a Lexmark Optra R® laserprinter at 1200dpi resolution.

1.4 Results

Maturation of the Ovary

Immature females are characterized by gonads with low GSI, previtellogenic oocytes, and very thin ovary wall (Figure 1.2a). Oocyte maturation proceeds through enlargement of previtellogenic oocytes to perinucleolar stage (Figure 1.2b), progresses to cortical alveoli stage (Figure 1.2c) and then begins exogenous yolk deposition. Yolk is deposited in yolk spheres and is visible histologically as eosinophilic material (Figure 1.3a). Migration of the nucleus from the central position occurs prior to its breakdown (Figure 1.3b), yolk granules fuse and hydration of the oocyte occurs before ovulation into the lumen from which the eggs are released in a spawning episode.

Gross observation of the gonad of spawning females, coupled with histological evidence, suggest that plaice do not spawn all of their oocytes in one episode, but instead release oocytes in distinct batches over a period of time. Figure 1.4 shows the ovary of a female caught in April. The gonad is large and well developed with ovulated hyaline oocytes grouped towards the vent, apparently ready to be released from the ovary. The interesting feature of this gonad, however, is the population of opaque, smaller, non-hyaline oocytes that comprise the remaining portion of the gonad. Histologically, evidence of batch spawning is seen in the presence of features associated with recent spawning concurrent with maturing, vitellogenic oocytes that are being prepared for release in the same

spawning season. A thick ovary wall results from shape changes that occur in the gonad after spawning, as prior to spawning the mature oocytes are large and stretch the ovary wall quite thin (Figure 1.5a) and post spawning shrinkage of the ovary results in a much thicker profile (Figure 1.5b). Post ovulatory follicles (POFs) are direct evidence of recent ovulation as the follicle which supported the developing oocyte is now broken down and resorbed (Figure 1.5c). Residual eggs in the lumen of the ovary are also an indication of a recent ovulation episode (Figure 1.5d). These post spawning features are not accompanied by the expected population of universally immature oocytes that one would expect in the post spawning period of a single episode spawner (non-batch spawner). These gonads contain oocytes that are in various stages of development, from immature, previtellogenic stage, through the cortical alveoli stage to vitellogenic oocytes that still have some vitellogenesis to conclude, but are sufficiently advanced that they could likely be released in this spawning season (Figure 1.6).

Seasonal Variation in Gonadosomatic Index

Tables 1.1 and 1.2 summarize mean values for samples taken over the course of this investigation.

Gonad development over the course of the year is reflected in the change in GSI (Figure 1.7). Maximum ovary size was observed in February of 1995, prior to the spawning season and decreased in March and April of that year, presumably as oocyte batches were shed. Lowest values were seen in June and July of 1994.

Testes are considerably smaller than the ovaries, comprising only 2% of the body weight at their largest, compared to the 17% of body weight that ovaries may attain in some females prior to spawning. Male plaice also show highest GSI in February, but maintain this level until April and by June GSI is at its lowest.

Possibility of Skipped Spawning

Histological observation of gonads in December suggest that both males and females may experience arrested maturation of reproductive products. One male showed testes that did not appear to be on track for the coming season and two females were quite undeveloped compared to the other females examined, showing the most advanced cells in the cortical alveoli stage and most oocytes immature (Figure 1.8).

Seasonal Variation in Condition

Condition factor and hepatosomatic index of mature female American plaice were also observed to vary seasonally (Figure 1.9), and may provide insight into the energy usage patterns of plaice over the year. Condition factor and hepatosomatic index were generally low at the onset of the spawning season, and increase post spawning. A coincident peak in condition and hepatosomatic index occurred in February of 1995 and corresponded to the maximum GSI attained during that season.

Male condition varies similarly, with HSI lowest in the spawning period, increasing rapidly after spawning to its maximum and having intermediate value

in December. Condition was lowest in June of 1995, recovered in July and peaked in December of 1993.

White Muscle Water Content

Muscle water content, measured at site c, in mature female American plaice was seen to increase from December through July (Figure 1.7) and reached its highest level during the spawning season when the gonadosomatic index reached its lowest value.

Male plaice also show an increase in white muscle water content during the spawning season, but levels were lower than those recorded for female fish.

Of the six ocular sites sampled for white muscle water content in each fish, there was a significant difference between sites on a given fish for one female from June and one July female. The largest fish taken in June of 1995 had a very large ovary, with a GSI of 37.10, a very good condition factor (CF=1.19) and a relatively low HSI. White muscle moisture ranged from 83-85% in four of the sites sampled. Site a showed a very high water content of 90% but site b was relatively conserved with moisture content of only 73%. Five of the six sites sampled on the July female had muscle hydrations of 85-86%. The aberrant site (b) had a muscle hydration of 92%. In cross-section, white muscle from this site showed fibres which appear severely compromised (Figure 1.10a). There were, however, fibres, immediately adjacent to these compromised fibres, which seemed to have been conserved (Figure 1.10b). This large female (57.5cm in length, 1937g) which showed the

highest muscle water content showed evidence of having spawned in the recent past, as did the June female. Post ovulatory follicles are evident (Figure 1.11a), as are vacant areas which may represent places that follicles have been completely resorbed, indicating a spawning event prior to that represented by the POFs. A thick ovarian wall also confirms previous spawning activity (Figure 1.11b). Also present in the July gonad are oocytes that are in various stages of development from previtellogenic, up to and including oocytes that contain exogenous yolk (Figures 1.11c). Given the low GSI of 2.99, it seems that these developing oocytes represent a final batch or two being prepared for release. The remainder of the fish showed no significant deviation from the mean water content between sites. As well, two fish, which were sampled on both the ocular and abocular surfaces to compare muscle moisture, showed no difference in water content on one surface or the other ($p>0.05$). It is interesting to note that plaice show a difference in muscle depth between ocular and abocular surfaces. The ocular muscle mass, measured in 24 fish from February and March, is significantly thicker ($p<0.0001$) than the abocular muscle mass with a mean difference of 0.34cm at both sites measured.

Length Weight Relationship

Figure 1.12 depicts the length weight relationship for American plaice described in this study and is typical of most growth curves. Three females in the March group appear to be heavier than expected for their lengths.

1.5 Discussion

Reproductive Development

Oocyte development in American plaice, *H. Platessoides*, follows the same basic progression as that described in other marine teleost fish (Rickey 1995, N'Da and Déneil 1993, Foucher and Beamish 1977, Htun-Han 1978, Barr 1963). Oogonia grow into immature, previtellogenic oocytes which characterize the immature ovary. The cortical alveoli stage follows, distinguished by the appearance of yolk vacuoles. Exogenous yolk deposition occurs in true vitellogenesis, peripheral migration and disappearance of the nuclear membrane precedes hydration and ovulation.

Evidence that American plaice are serial spawners, releasing discreet batches of eggs over an extended spawning season, include macroscopic and histological indications of recent spawning concurrent with mature vitellogenic oocytes. Gross observation of one gravid female killed in April (Figure 1.5) shows a pattern of oocyte hydration similar to that observed in the North Sea Sole, *Solea solea,* which is also a batch or serial spawner (Witthames and Greer Walker 1995). The anterior portion of the gonad in which the ovulated eggs wait to be spawned is called the hyaline window (Ibid.) while subsequent batches, still maturing in their follicles, remain opaque. For *Gadus morua,* Kjesbu (1989) suggests that hyaline oocytes, once ovulated, will remain in the ovary for a very short time prior to release. The next batch to mature will hydrate in as little as 35 hours and 10-15 batches will be spawned over a period of 35-70 days (Kjesbu 1989).

Histologically, gonads from June and July females show thick ovarian walls, compared to the thin wall seen in prespawn females. The shrinkage of the muscular wall following release of previous oocyte batches results in a thicker profile. Indications of recent spawning are the presence of ovulated eggs in the lumen of the ovary, the presence of post-ovulatory follicles (POFs), and the presence of atretic oocytes. In these ovaries which clearly have spawned in the recent past, there are also groups of exogenously vitellogenic oocytes which appear sufficiently advanced that they may be spawned during the same spawning season. American plaice, then, do not appear to release all oocytes in a brief breeding season as do the winter flounder, *Pleuronectes americanus* (Burton and Idler 1984), but spawn over a longer period, releasing a number of oocyte batches.

The first evidence of ripe ovaries with running eggs was seen in late April, corresponding to the spawning period for *H. platessoides* described by Zamarro (1992b) and Pitt (1966). There was evidence of spawning in June and July, probably the result of the ovulation of final batches since the GSI was relatively low during these times. Pitt (1966) records ripe females in June and partly spent females in July, supporting the idea of a protracted spawning period. The classification used by Pitt to macroscopically stage ovary development uses the presence and relative amount of hydrated oocytes to describe maturing gonads. All oocytes opaque (Mat A) was thought to precede the condition in which 1 to 50% of the oocytes were hydrated (Mat B) and a Mat C ovary with all hyaline oocytes was considered most

advanced. A gonad with no visible evidence of hydrated oocytes may, however, have already released one or two batches. A gonad with half of its oocytes hydrated may be preparing to release its first batch and so cannot be considered more advanced than the gonad with no evidence of hydration. Another complication of this method of staging involves fecundity estimation. The determination of fecundity by counting eggs from a Mat A gonad must be carefully monitored to ensure females which have already shed oocyte batches are not included in the analysis. Histological checking for evidence of recent spawning activity should be conducted to prevent an underestimation of fecundity.

The question of determinate versus indeterminate fecundity in American plaice was dealt with by Zamarro (1992b) who based a conclusion of determinate fecundity on the hiatus in size distribution of cortical alveoli and vitellogenic oocytes. Other authors distinguish between determinate and indeterminate spawners using a size difference between previtellogenic and maturing oocytes (N'Da and Déneil 1993, Urban and Alheit 1988). The larger size classes of truly vitellogenic oocytes compared to cells in the endogenous stage of development observed by Zamarro (1992b) may result from the maturation of the first batch or batches to be spawned.

In the present study, ovaries in the spawning season contained cells in many stages of development from immature through cortical alveoli stage to various stages of exogenous vitellogenesis including oocytes in advanced vitellogenesis. This is in contrast to December oocyte profiles which consisted largely of oocytes

in advanced tertiary vitellogenesis with few oocytes in the stages between immature and vitellogenic. Two explanations for the observed range of oocyte development observed in the spawning season are plausible based on the available information. The early stages of vitellogenesis observed during the spawning season may be the result of oocyte development for the following year. An alternative explanation is that batches spawned early in the season might go through a major phase of vitellogenesis several months earlier and later batches may be brought rapidly through vitellogenesis during the spawning season based on available energy reserves, including white muscle protein. If this is the case, differentiating between determinate and indeterminate oogenesis on the basis of the prespawning oocyte size distribution could be unsound and estimation of fecundity based on the vitellogenic oocyte population prior to spawning would result in inaccurate estimation of potential reproductive output, which might lead to an underestimation of larval mortality. Examination of post spawning ovaries, which were unavailable at the time of this investigation, to determine the nature of the oocyte population (ie. if all oocytes are immature and POFs not in evidence) would confirm fast tracking during the spawning season.

It is evident that oocyte growth from previtellogenic to true vitellogenesis occurs in a relatively short time and may be occurring immediately after spawning, if not during spawning. Zamarro (1992b) states that vitellogenesis begins in May and fecundity is determined at that time. If the vitellogenesis observed during May, June

and July is attributed to maturing batches to be released in the same year, the overlap in cortical alveoli stage and vitellogenic stage may actually support the ability of plaice to recruit oocytes into the vitellogenic stage. The size hiatus that develops between endogenous and exogenous oocytes in the post spawn period until April may be explained if the first batch (or first few batches) is matured initially and the recruitment of new batches in the spawning season is based on energy available to push oocytes through vitellogenesis at that time. Horwood (1990) observed, in some *P. platessa*, oocyte size frequency profiles that support a conclusion of indeterminate fecundity, while other fish showed the characteristic size frequency hiatus suggesting fecundity was determined prior to the spawning season. He describes the pushing of oocytes through vitellogenesis during the breeding season as a "mechanism for fine tuning of fecundity", with oocytes being matured as energetic reserves permit in order to maximize reproductive output.

Pitt (1966) stated that some American plaice females did not produce eggs every year. In this study, some females in the December sample were far behind the majority of females in ovarian development. Most females had oocytes in an advanced vitellogenic state. Two gonads, however, showed mainly previtellogenic cells with some oocytes in the cortical alveoli stage. Rickey (1995) states that oocytes of *Athoresthes stomias* with cortical alveoli were most frequently seen in spent or resting gonads and that resting females were seen throughout the year. A similar observation was made by Htun-Han (1978) in *Limanda limanda*. The poorly

developed December gonads in this study, may represent resting gonads that are not destined to spawn in the coming season, an indication that American plaice may forfeit reproduction in a given year. N'Da and Déneil (1993) found that the resting gonad of the red mullet, *Mulleus surmuletus*, contained only primary and immature oocytes. There is a lack of sufficient data to characterize the post spawned or resting ovary of the American plaice, but if similar to the red mullet, the less developed December females may be preparing to spawn for the first time and are actually juvenile or adolescent fish. If these females are indeed adolescent, they are insufficiently developed for the coming season so that ripening of the first reproductive products probably requires at least two years to develop from the immature condition.

Few male samples were attained over the course of this investigation, and due to this constraint, information on testis and sperm development is limited. Gonad maturation in male plaice does not result in the same degree of development of the gonad as that seen in females. The maximum GSI attained by any male was only 2.94, while one female produced gonads that, in June, attained 27.10% of her total body weight. The energetic cost of reproduction to the female is probably much greater than that imposed on the male. Some suggestion of reproductive retardation is seen in one large male killed in December with abnormal gonad characteristics. This male is not developed for the coming spawning season and may be non-reproductive.

Condition Cycles

Hepatosomatic Index (HSI) was highest in July of 1994 and maintained at an intermediate level in December of 1993. The minimum liver index was reached in April (females) and March (males) of 1994. Hepatosomatic Index is a good indicator of recent feeding activity (Tyler and Dunn 1976) and suggests that plaice may not feed as much during the spawning period. A minor peak in HSI was observed in female plaice in February- March of 1994 and may reflect the liver's involvement in growth of vitellogenic oocytes during this period.

Condition factor was lowest in June of 1994 for females and June of 1995 for males. *Mulleus surmuletus* also showed a minimum condition in the spawning season (N'Da and Déneil 1993). The same decline in condition is also recorded for the Long Rough Dab, the European subspecies *Hippoglossoides platessoides limandoides*, during the spawning period from March to mid-April (Bagenal 1957). The poorer condition is probably the result of mobilization of somatic energy reserves needed for reproductive development, and may be influenced by reduced feeding in the spawning period. Although Zamarro records maximum feeding activity of *H. platessoides* on the Grand Banks to occur in April, 40-45% of stomachs studied were empty in April and June and this value increased to 50% in July (Zamarro 1992a). A more useful indication of feeding activity would relate stomach content to reproductive status.

White Muscle Moisture and Histology

Increased white muscle moisture is an indication of protein depletion (Stirling 1976, Love 1958) and is one of the characteristics of starvation induced muscle deterioration observed in several species (Johnston 1981, Johnston and Goldspink 1973, Love 1958). White muscle water content in American plaice increased with reproductive development and was seen to increase to a greater degree in females. The mobilization of protein from white muscle is indicated during the spawning period and is probably the result of energetic demands that oocytes being matured would place on the female. For cod, larger females are more fecund (May 1967) and require more energy for reproductive development. This may explain the fact that in *H. platessoides*, the highest white muscle water contents achieved were recorded in very large females during the spawning period. Males also experience a cycle in white muscle moisture with peaks observed in July of 1994 and April of 1995. Levels do not reach the extreme seen in females, but it still indicates that males are capable of utilization of somatic protein as a source of energy.

Conservation of discreet areas of white muscle is not shown in this study, but the possibility that certain regions of musculature may be targeted more than others is inferred. In two females close to the end of spawning, extreme values of moisture (92% and 90%) were recorded in anterior peripheral samples while those taken closest to the lateral line and in posterior peripheral regions (sites E and F) were less hydrated, similar to results proposed by Keissling *et al.* (1993) for the sturgeon

and by Fisher *et al.* (1987) for Dover sole. Templeman and Andrews(1956) state that muscle closest to the dorsal and anal fins is most gelatinous. Conserving areas critical to maintaining some level of burst activity may be adaptive in lessening the impact of muscle protein loss on the ability of the fish to use the tail flip for food capture or predator evasion. Complicating the idea of conservation of the central muscle core was the occurrence of a less hydrated site (73% compared to the 83-84% found at the other 4 sites) at a peripheral location which indicates that maintenance of muscle integrity does not follow a simple pattern. Even in the regions of high hydration histological evidence of severe muscle breakdown occurs alongside fibres that appear normal. This suggests that a more distributive pattern of protein utilization may be at work, conserving some fibres in areas that are severely hydrated in order to preserve muscle tone in jellied areas, providing a muscular framework needed to keep somatic integrity from failing, and a basis on which recovering muscle may rebuild. Some level of contraction in the affected area might also be possible through the conservation of certain fibres in areas that are targeted for preferential protein depletion. No difference in the hydration of the ocular versus abocular muscle mass was noted. Templeman and Andrews (1956) describe jellied muscle in very large American plaice females in the wild. Few large females were attained during the course of this study and only a couple were killed during the spawning period. Jellied muscle occurred during the spawning period and

reached extreme levels at certain sites in the largest females. Further investigation of large females during the spawning and post spawning periods is warranted to determine the pattern of utilization and conservation of white muscle protein.

Figure 1.1: Map of sampling sites a through f as taken from American plaice, *Hippoglossoides platessoides*. Modification of original format from Scott and Scott (1988).

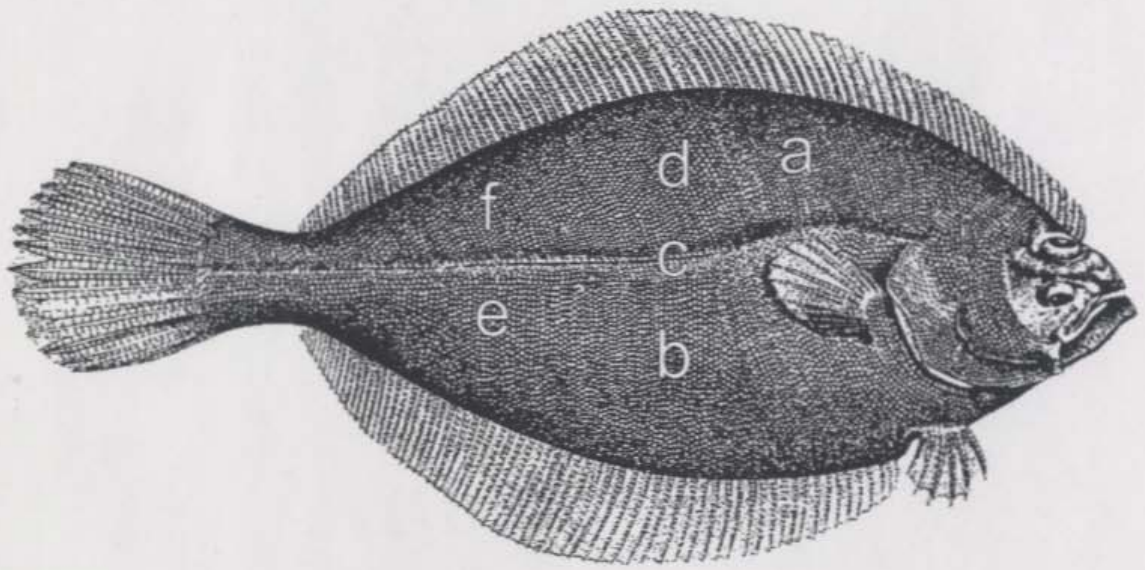

d
a
f
c
e
b

Figure 1.2: Oocytes from *Hippoglossoides platessoides* in various developmental stages. (a) Immature gonad with small, immature oocytes (**imm**) and thin ovarian wall (**w**). (b) Perinucleolar stage oocytes (**pn**) with nucleoli (**n**) arranged around the periphery of the nucleus (**nu**). Balbiani bodies (**b**) also visible. (c) Oocytes in endogenous yolk formation or cortical alveoli stage of development with yolk vacuoles or cortical alveoli (**ca**) around the cell periphery. All images at the same magnification. Scale bar = 100μm.

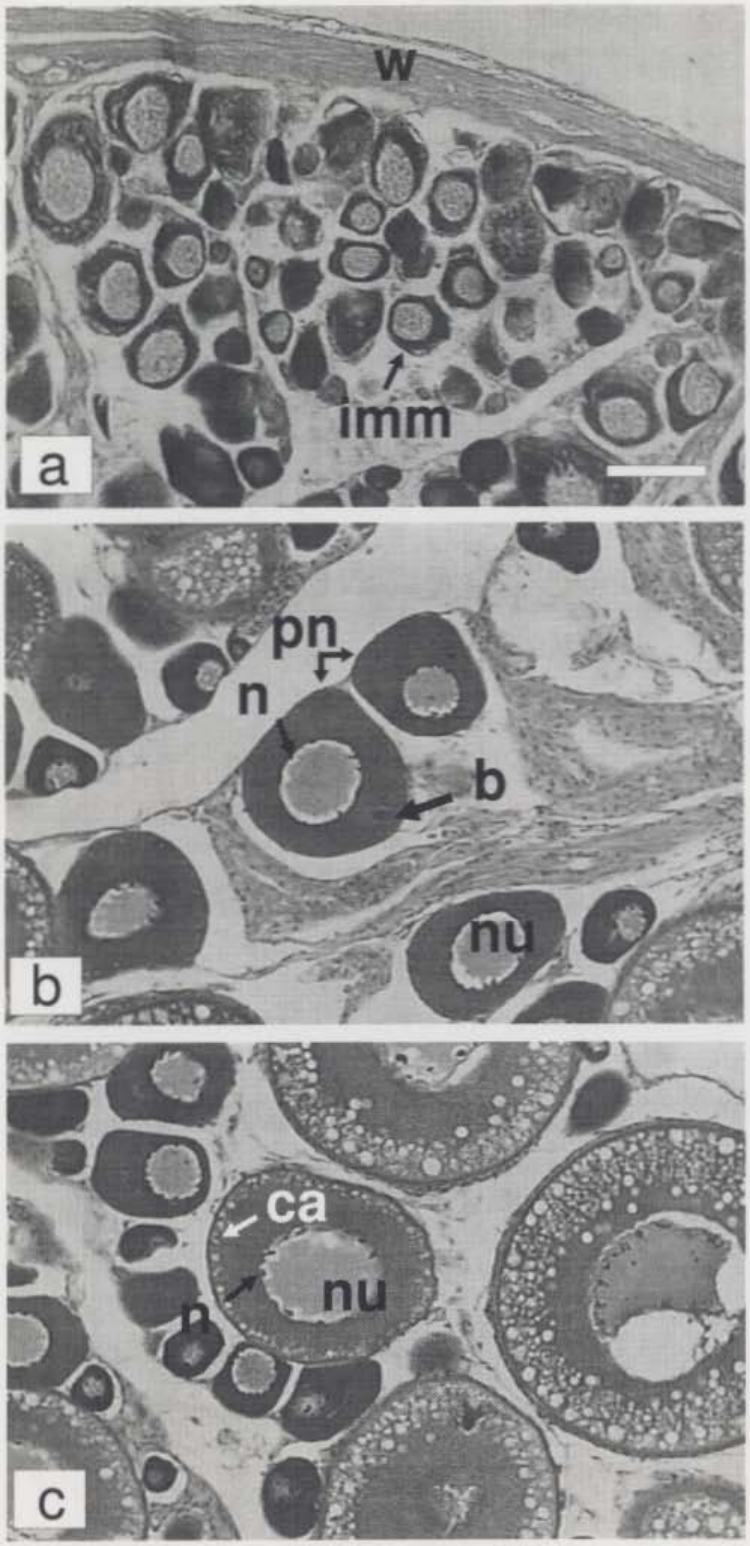
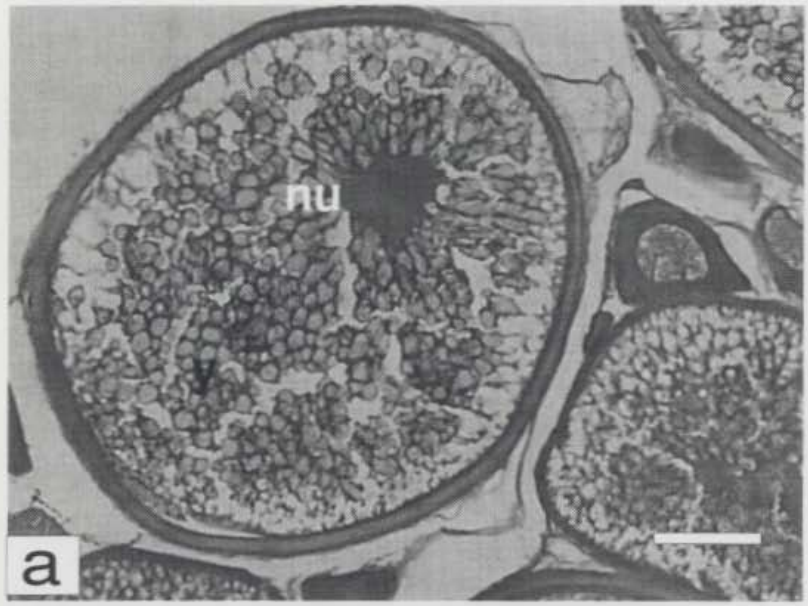

w
imm
a
pn
n
b
nu
b
ca
nu
n
c

Figure 1.3: Oocytes from American plaice, *Hippoglossoides platessoides*, showing advanced development. (a) Advanced oocytes in exogenous vitellogenesis show accumulation of yolk (**y**) and migration of the nucleus (**nu**) towards the animal pole. (b) Late vitellogenesis is characterized by cell enlargement, the coalescing of yolk and breakdown of the nuclear membrane. Both images were taken at the same magnification. Scale bar = 100µm.


nu
y
a

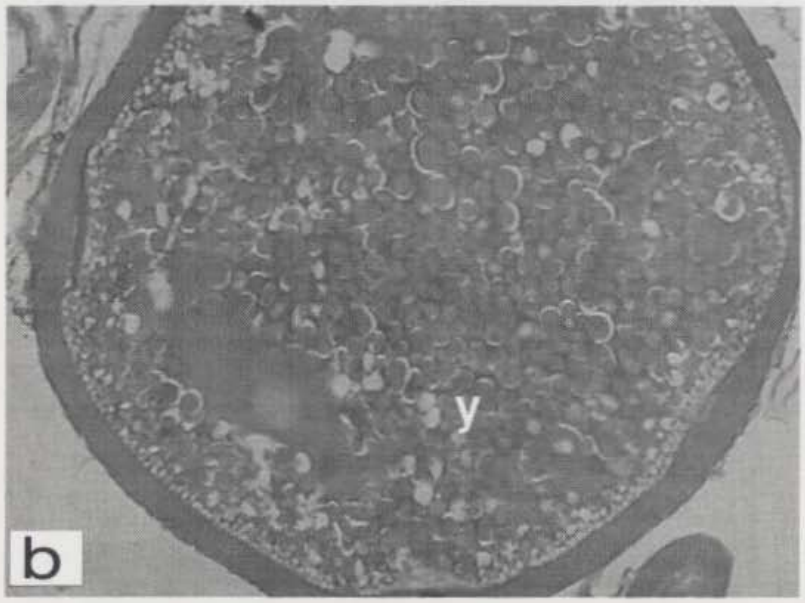

y
b

Figure 1.4: Photograph of a female American plaice, *Hippoglossoides platessoides*, killed in April. The ovary shows hydrated eggs which have been recently ovulated and were being held in the anterior lumen. The hydrated eggs represent a batch which was waiting to be shed and the opaque oocytes comprising the rest of the gonad were batches which would have been hydrated and shed during other spawning episodes during the spawning season.

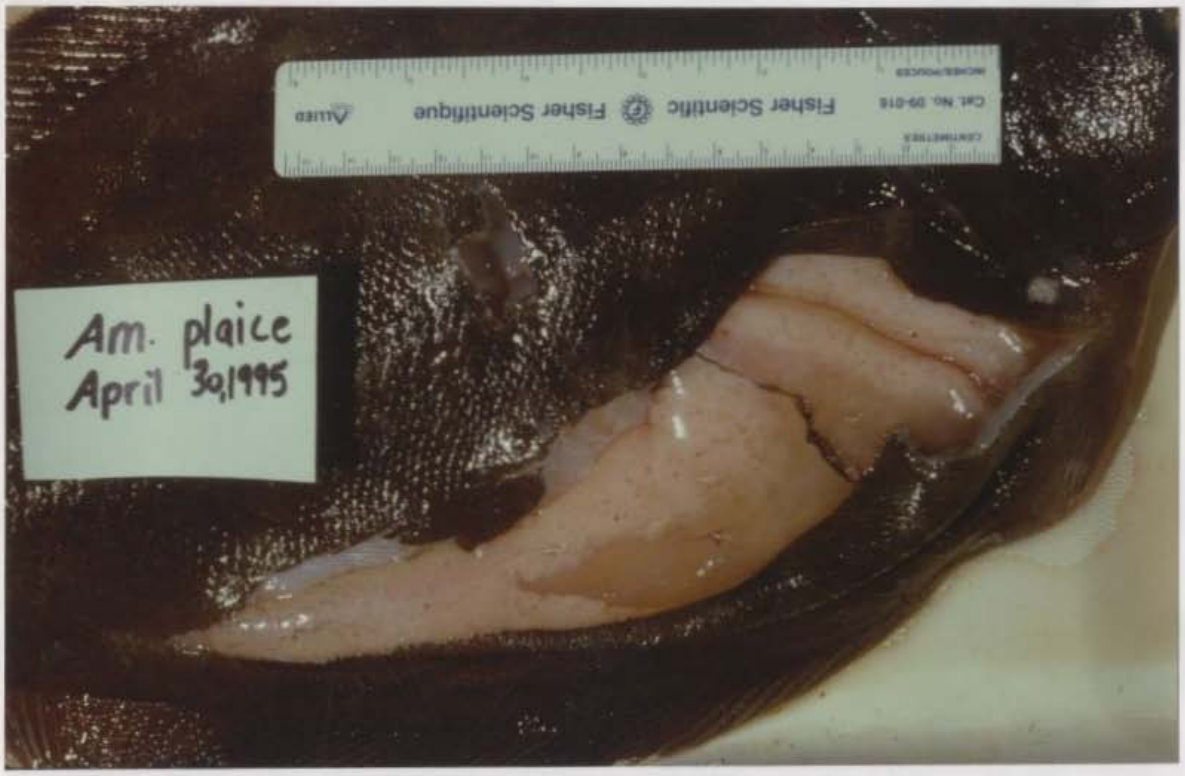

Am. plaice
April 30,1995
Fisher Scientific
Fisher Scientific

Figure 1.5: Prespawn and postspawn ovary characteristics of American plaice, *Hippoglossoides platessoides*. (a) Prespawning ovary from *Hippoglossoides platessoides* showing advanced vitellogenic oocytes and thin ovarian wall (**w**). (b) Postspawned ovary of *H. platessoides* showing a much thicker wall. (c) Post-ovulatory follicle (**POF**) from a female plaice killed in June. (d) Residual egg (**re**) in the lumen of an ovary taken in June. Both (c) and (d) are indications of a recent spawning episode. All images were taken at the same magnification. Scale bar = 100µm .

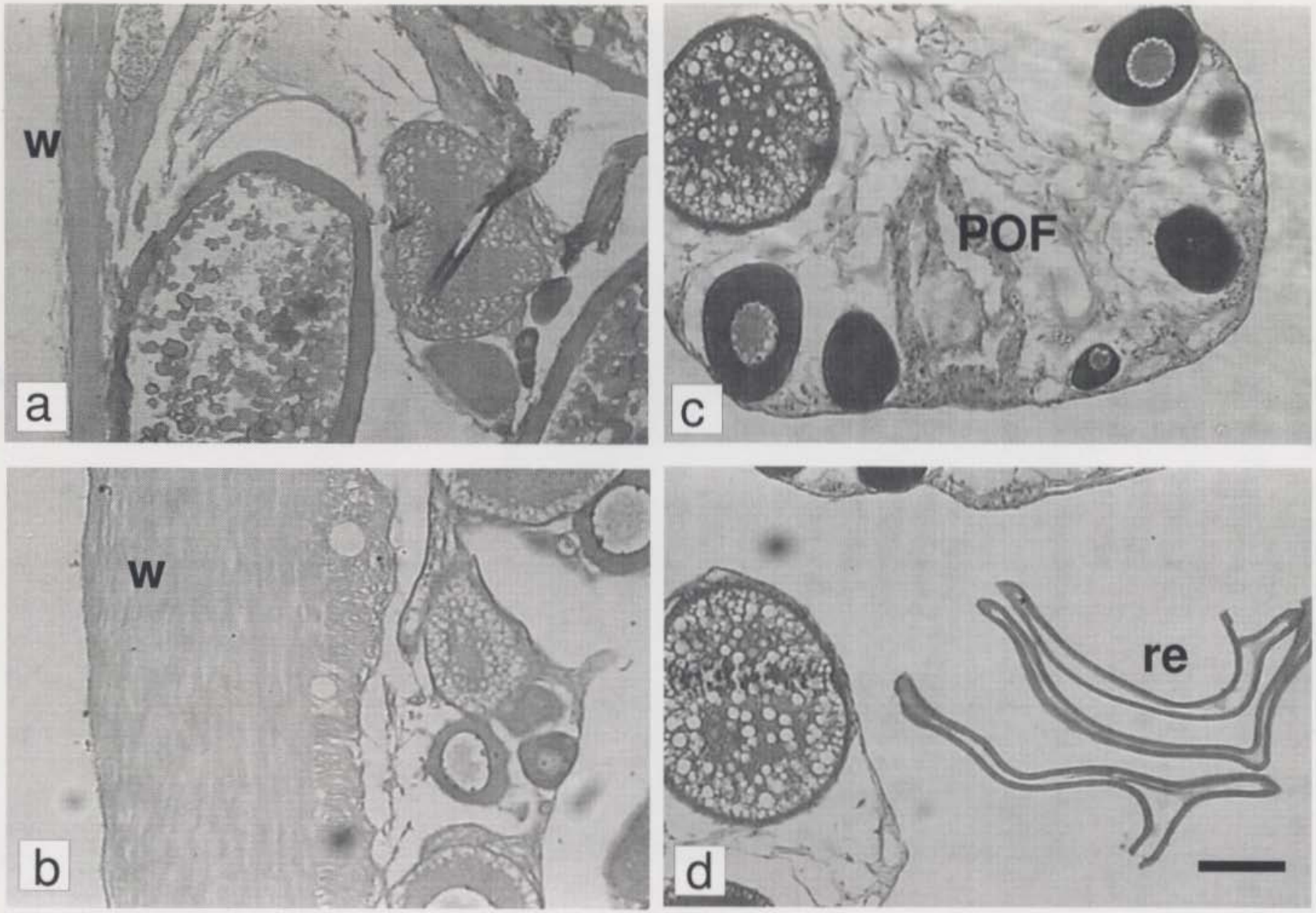

w
a
POF
c
w
b
re
d

Figure 1.6: Ovary cross-section from an American plaice female killed in June with oocytes in various stages of development. Immature (**imm**), perinucleolar (**pn**) and cortical alveoli (**ca**) stages and oocytes in exogenous vitellogenesis (**vit**). Scale bar = 100µm.

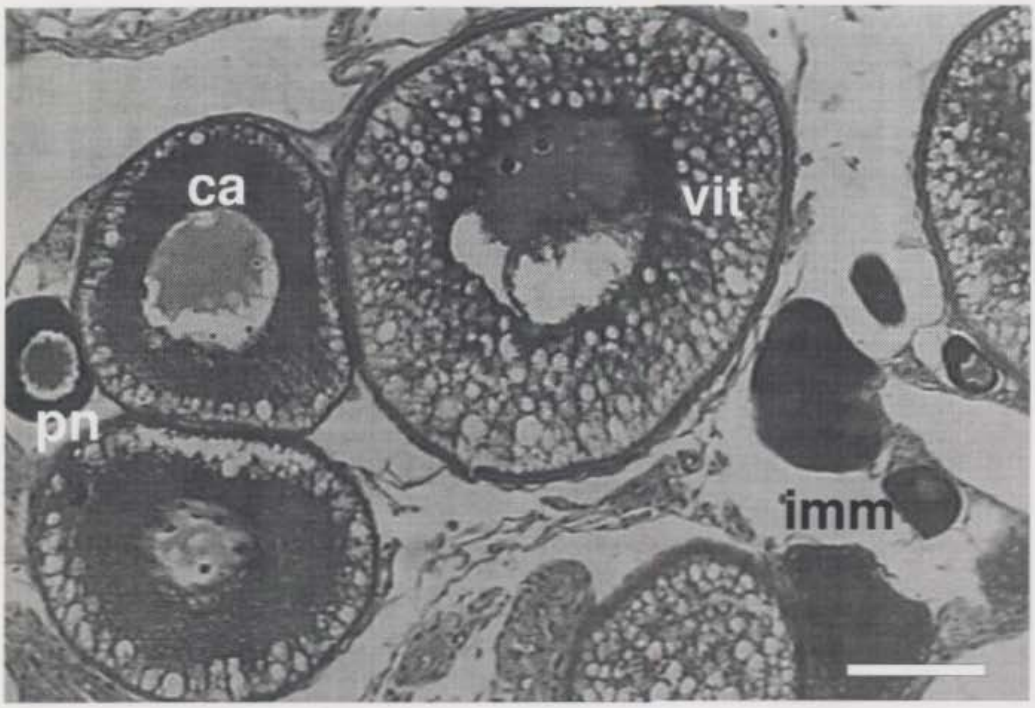

ca
vit
pn
imm

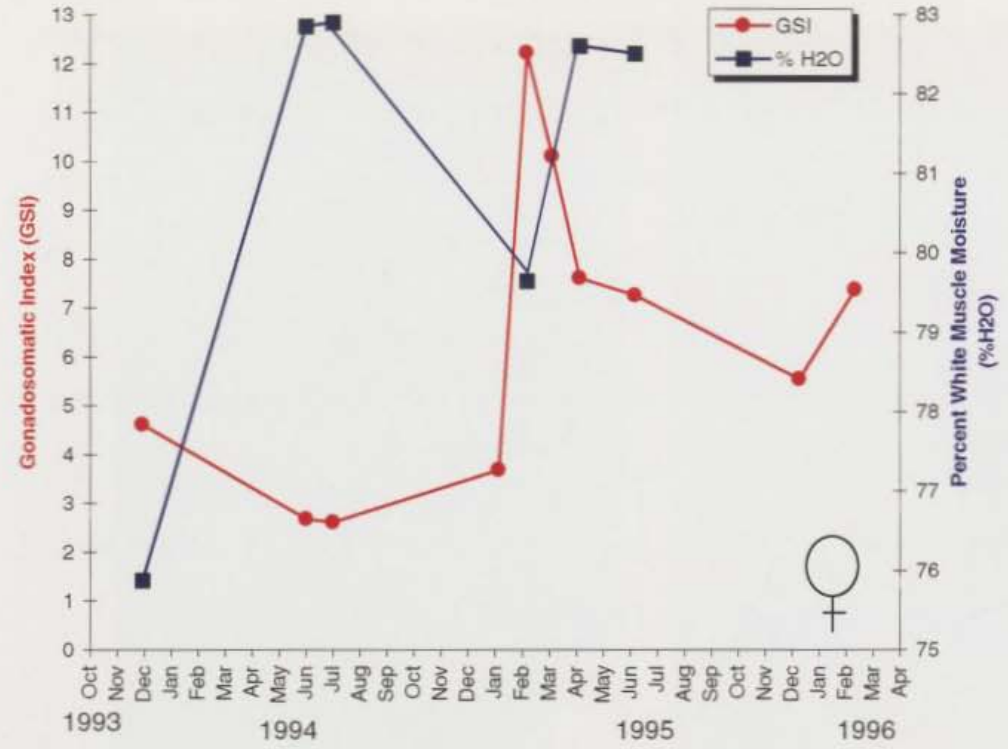
Table 1.1. Means and ranges for mature female American plaice, *Hippoglossoides platessoides*. Where frozen samples were taken (F), measurement of white muscle moisture was excluded due to changes in moisture content caused by freezing. *Denotes information not obtained from the sample. N= sample size, CF= Condition Factor, GSI= Gonadosomatic Index HSI= Hepatosomatic Index, and $\%H_2O$= percent white muscle water content.

Month	N	Length	Weight	CF	GSI	HSI	$\%H_2O$
Dec-93	5	35.0 30.0-40.2	396.8 210.0-619.0	0.88 .78-.95	4.61 3.83-5.35	2.98 1.99-3.96	75.88 73.52-77.26
Jun-94	5	45.02 39.8-51.6	767.2 462.0-1235.0	0.80 .71-.90	2.68 1.86-4.21	1.28 1.0-1.52	82.86 80.63-85.33
Jul-94	5	53.3 44.7-57.7	1532.8 852.0-1937.0	0.98 .94-1.05	2.61 1.76-3.15	5.83 4.23-8.05	82.90 80.71-86.65
Jan-95	1(18F)	35.6 26.7-49.8	443.0 128.0-1287.0	0.88 .67-1.07	3.69 .74-9.63	0.98 .43-2.25	NA
Feb-95	2	45.0 38.5-51.4	1006.0 540.0-1472.0	1.02 .95-1.08	12.21 8.52-15.9	1.99 1.77-2.22	79.65 77.12-82.18
Mar-95	8F	46.46 41.5-54.9	1059.6 603.0-1718.0	1.03 .84-1.31	10.10 7.35-16.82	1.45 .33-2.04	NA
Apr-95	4	41.3 36.6-48.4	658.0 372.0-1209.0	0.87 .75-1.07	7.60 .81-16.38	0.58 .40-.73	82.61 77.76-83.74
Jun-95	16	39.17 29.9-49.6	562.4 210.0-1450.0	0.87 .76-1.19	7.25 .66-37.1	1.22 .88-1.76	82.51 80.46-85.33
Dec-95	12	34.8 25.6-44.0	417.0 146.0-828.0	0.91 .8-1.0	5.54 1.6-9.2	2.63 2.0-3.6	*
Feb-96	19	47.0 32.0-61.0	1095.0 255.0-2520.0	0.92 .78-1.21	7.37 1.11-16.14	*	*

Table 1.2. Means and ranges for mature male American plaice, *Hippoglossoides platessoides*. Where frozen samples were taken (F), measurement of white muscle moisture was excluded due to changes in moisture content caused by freezing. N= sample size, CF= Condition Factor, GSI= Gonadosomatic Index, HSI= Hepatosomatic Index, and %H2O= percent white muscle water content.

Month	N	Length	Weight	CF	GSI	HSI	$\%H_2O$
Dec-93	8	26.2 20.6-31.0	168.0 63.0-321.0	0.89 .69-1.42	1.77 1.18-2.8	2.63 1.69-4.67	77.52 73.12-79.6
May-94	4	33.5 31.1-36.4	311.3 243.0-377.0	0.83 .78-.90	0.73 .33-1.23	1.06 .53-1.54	80.33 79.74-80.81
Jul-94	5	44.3 41.6-50.0	737.0 852.0-1937.0	0.84 .79-.92	0.98 .59-1.63	4.01 3.46-4.86	81.21 80.5-82.39
Jan-95	1(8F)	29.1 24.8-43.1	234.0 133.0-689.0	0.84 .77-.91	2.07 .74-2.94	0.83 .36-1.62	76.37
Feb-95	2	32.5 31.5-38.9	363.0 273.0-453.0	0.82 .77-.87	1.83 1.1-2.56	0.70 .66-.73	79.52 78.02-81.01
Mar-95	2F	39.8 39.4-40.1	509.0 466.0-551.0	0.81 .72-.91	2.05 .64-3.45	0.41 .18-.64	NA
Apr-95	3	42.6 42.0-43.5	640.0 531.0-704.0	0.83 .72-.90	2.07 1.7-2.45	0.77 .56-1.17	81.57 80.27-83.93
Jun-95	3	34.4 32.9-37.1	305.0 249.0-416.0	0.73 .69-.81	0.43 .40-.48	0.99 .88-1.2	79.96 77.94-81.33

Figure 1.7: Seasonal variation in gonadosomatic index (GSI) and percent white muscle moisture (%H_2O) in American plaice, *Hippoglossoides platessoides*, over time.


GSI
% H2O
Gonadosomatic Index (GSI)
Percent White Muscle Moisture (%H2O)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
75 76 77 78 79 80 81 82 83
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
1993
1994
1995
1996

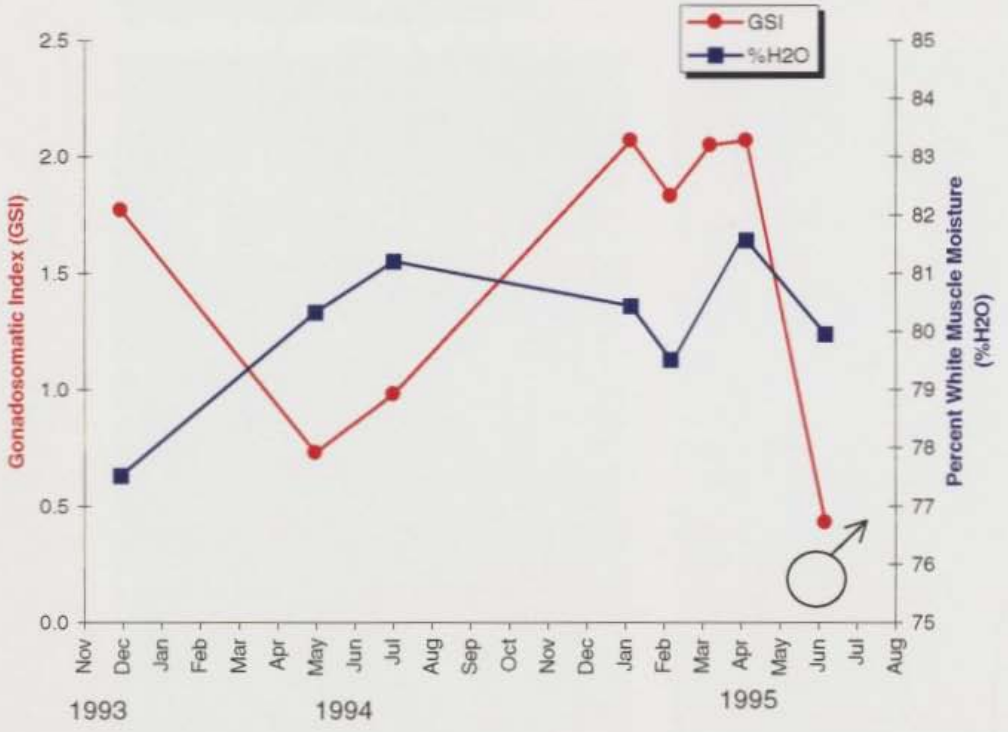

GSI
%H2O
Gonadosomatic Index (GSI)
Percent White Muscle Moisture (%H2O)
0.0 0.5 1.0 1.5 2.0 2.5
75 76 77 78 79 80 81 82 83 84 85
Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug
1993
1994
1995

Figure 1.8: Skipped spawning and adolescence in American plaice, *Hippoglossoides platessoides*. Testes from a plaice killed in December (b) showed spermatagonia (**sg**) but did not show evidence of sperm development (**s**) and lobule breakdown which would be expected in a fish developing to spawn in the coming season as seen in (a). This female (c), killed in December, was not as advanced as other females killed at the same time (d), but did have some oocytes in the cortical alveoli stage (**ca**) and the ovarian wall was quite thin. Images (a) and (b) were taken at the same magnification, scale bar = 50µm. Images (c) and (d) were taken at the same magnification, scale bar = 100µm. (**ev**) indicates exogenous vitellogenesis.

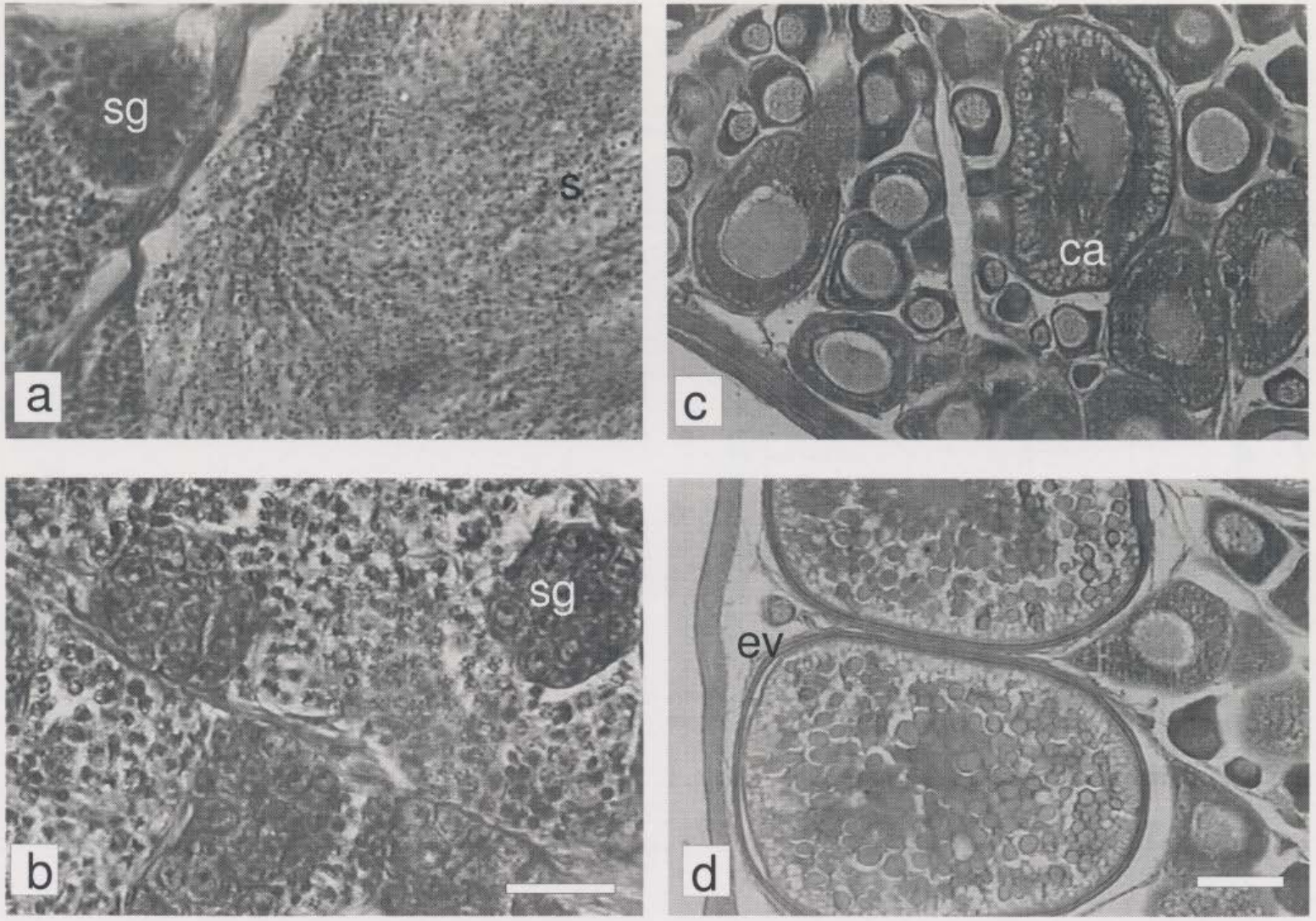
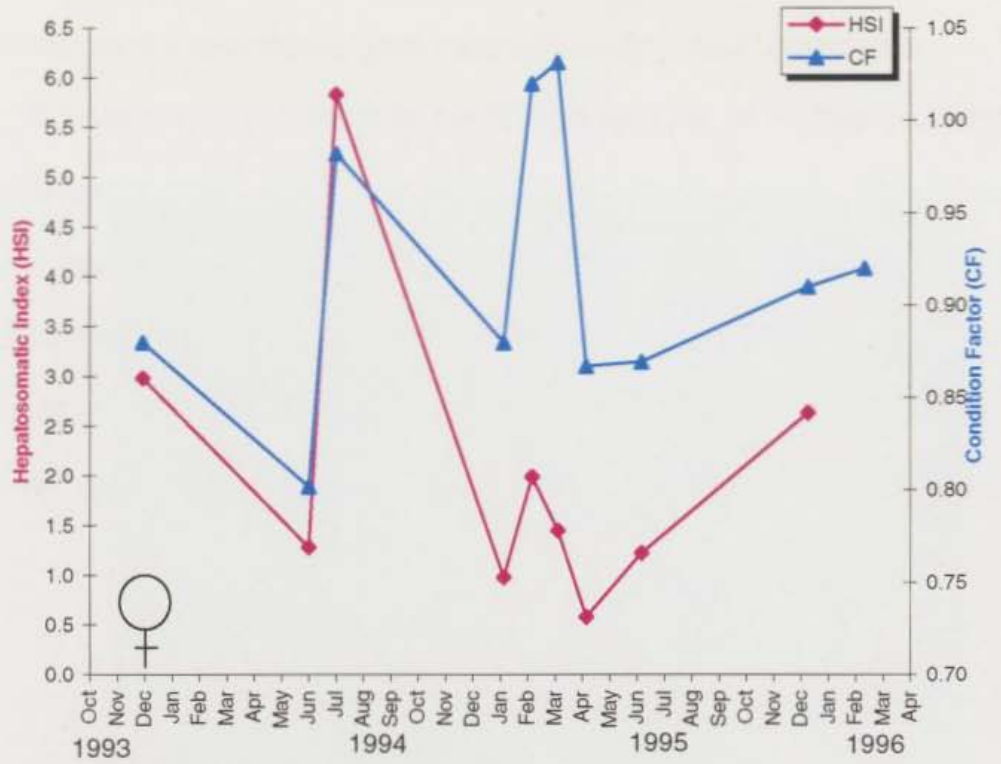

sg
s
a
c
ca
b
sg
ev
d

Figure 1.9: Seasonal variation in hepatosomatic index (HSI) and condition factor (CF) in American plaice, *Hippoglossoides platessoides*.


HSI
CF
Hepatosomatic Index (HSI)
Condition Factor (CF)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
1993
1994
1995
1996

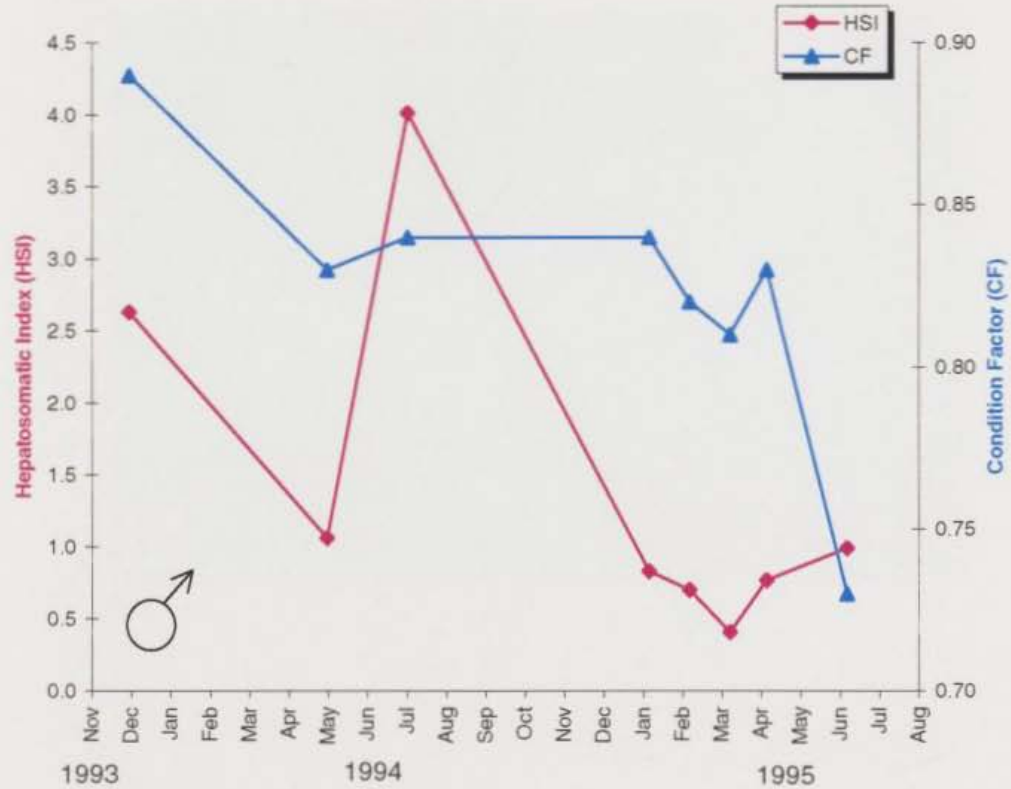

HSI
CF
Hepatosomatic Index (HSI)
Condition Factor (CF)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.90
0.85
0.80
0.75
0.70
Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug
1993
1994
1995

Figure 1.10: White muscle from American plaice, *Hippoglossoides platessoides*, showing protein depletion in area of high water content. (a) Cross-section of compromised white muscle fibres from a female plaice, *Hippoglossoides platessoides*, killed in June. Muscle moisture immediately adjacent to this site was measured to be 92%. Conservation of white muscle fibres is suggested by the occurence of fibres which do not appear to be compromised (b) right next to fibres which are in poor shape. Staining is with haematoxylin and eosin. Scale bar = 100µm.

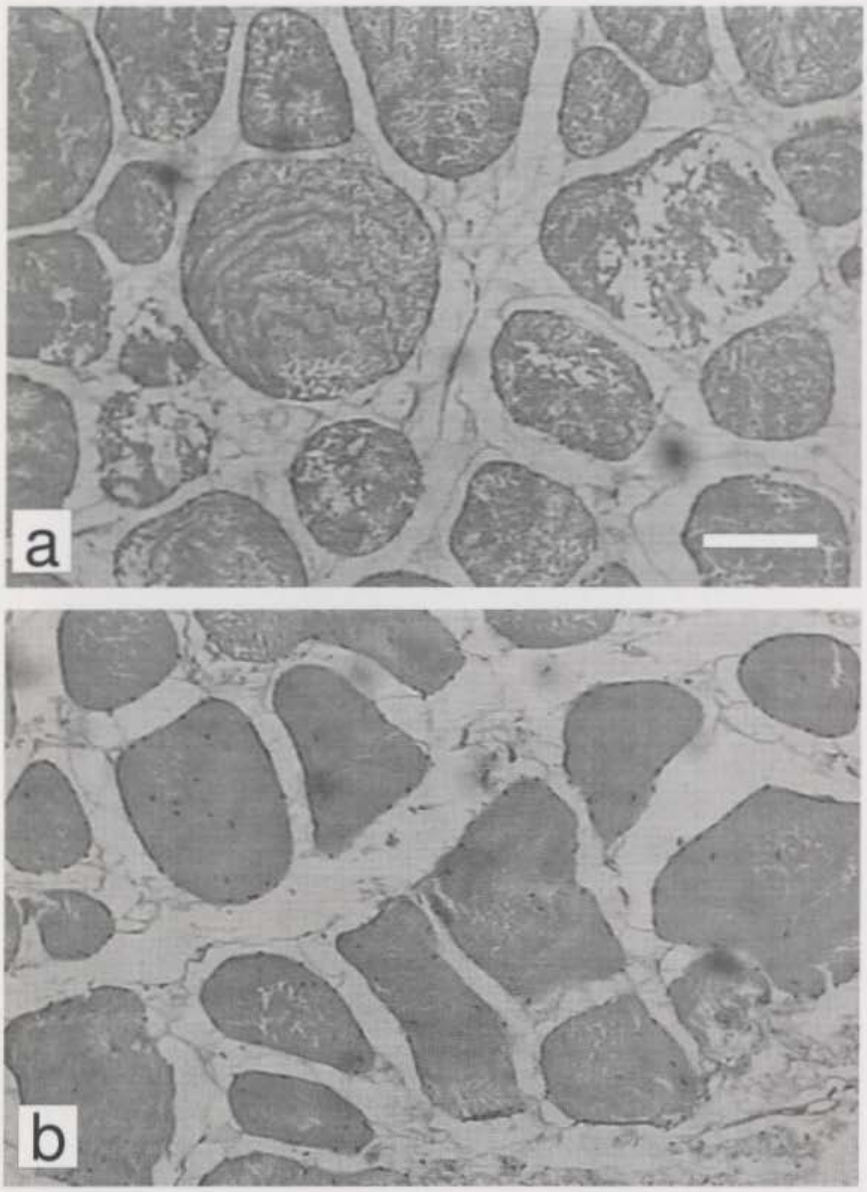

a
b

Figure 1.11: Indications of recent spawning accompanied by oocytes in various stages of development in *Hippoglossoides platessoides*. Post ovulatory follicles (**POFs**) (a), evidence of recent spawning, and a thick ovarian wall (**w**) (b) indicate previous spawning. Present in the same ovary are oocytes in various stages of development from previtellogenic (**pv**) to exogenous yolk deposition (**ev**). All images were taken at the same magnification. Scale bar = 100µm.

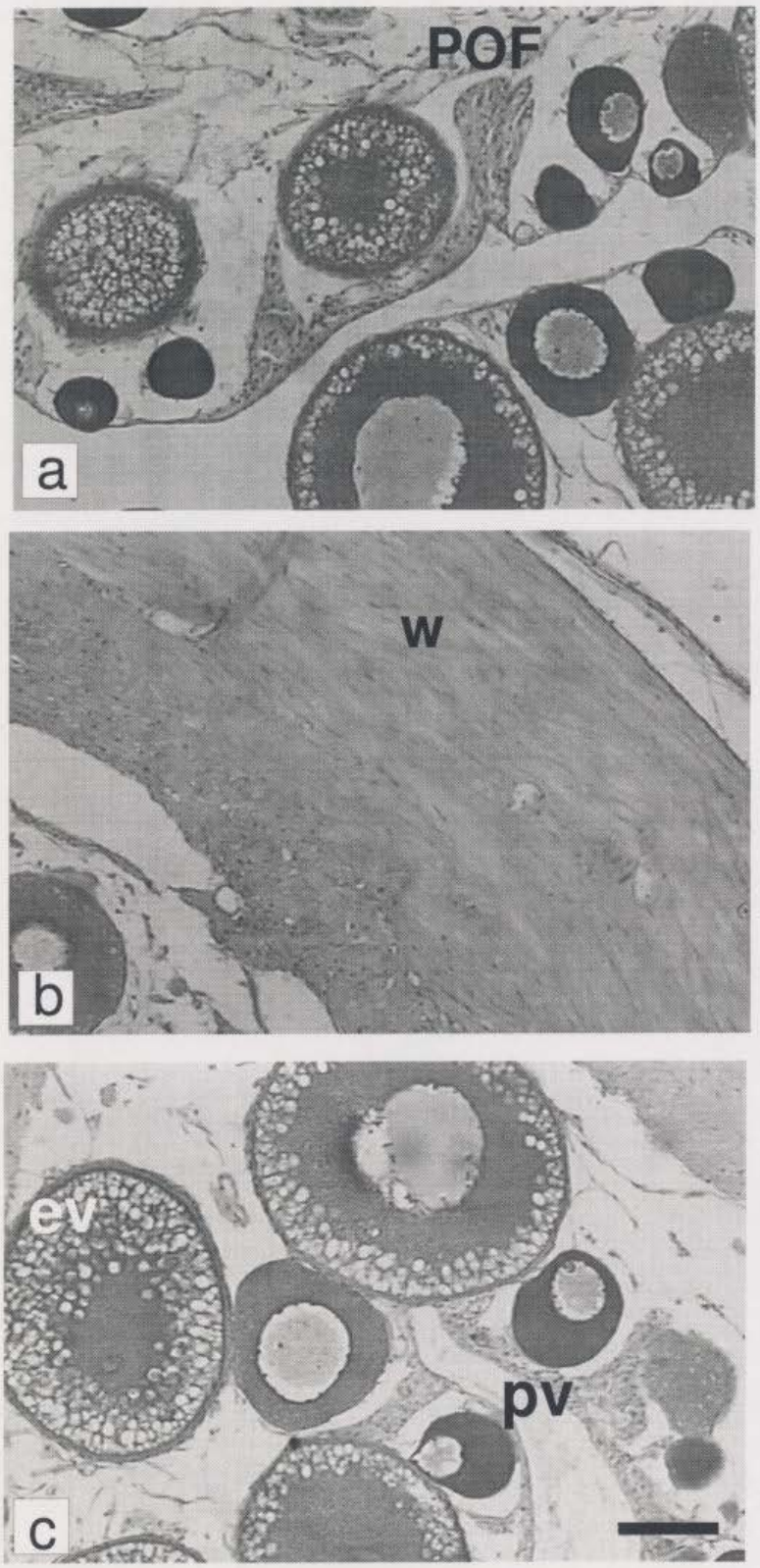
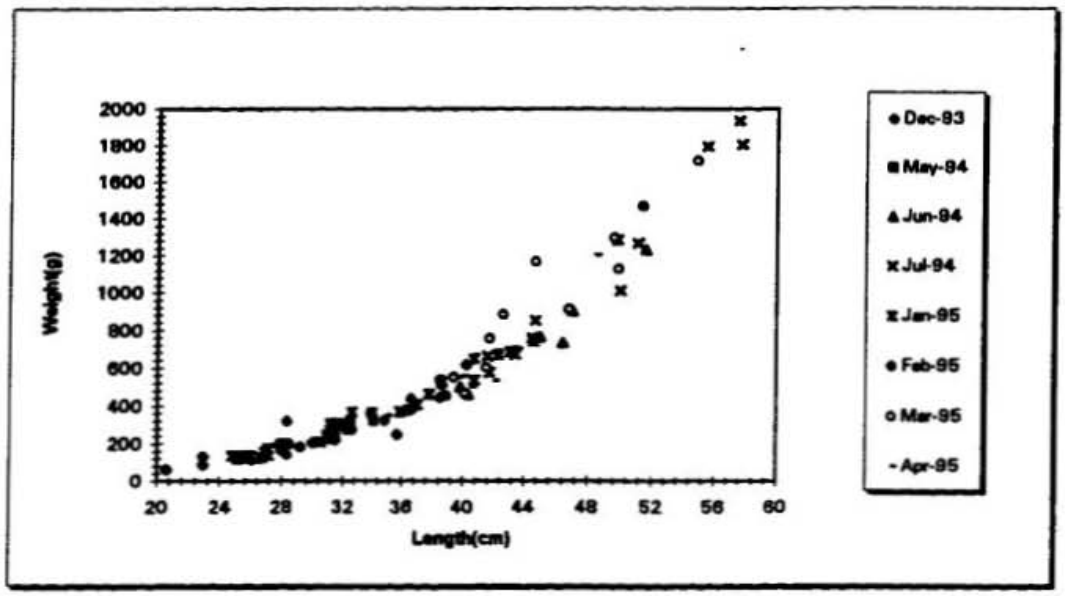
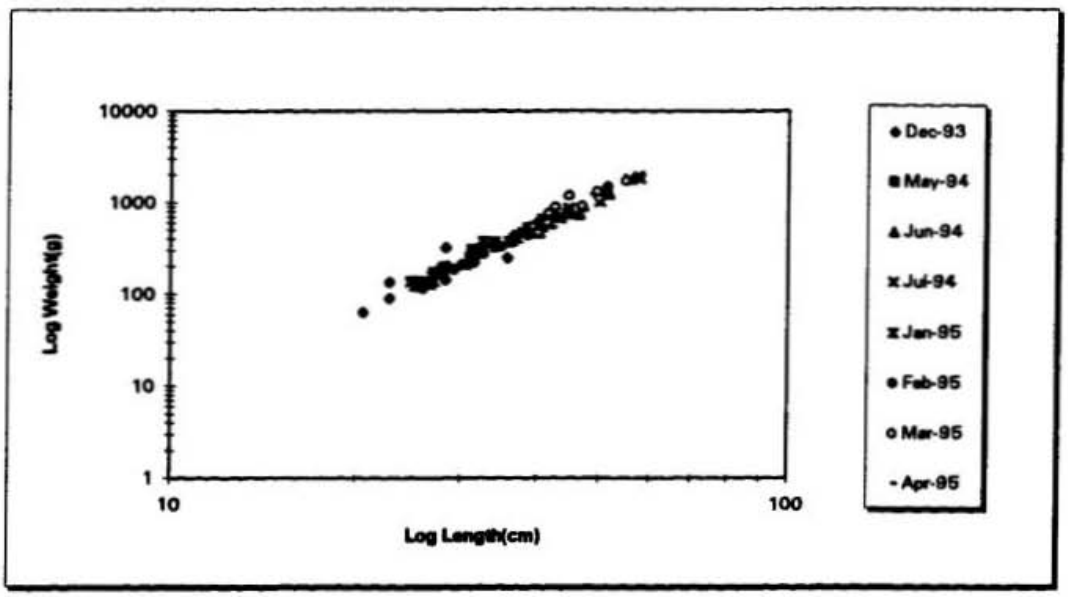

POF
a
w
b
ev
pv
c

Figure 1.12: Length weight curve for American plaice, *Hippoglossoides platessoides*.


Weight(g)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
20
24
28
32
36
40
44
48
52
56
60
Length(cm)
Dec-93
May-94
Jun-94
Jul-94
Jan-95
Feb-95
Mar-95
Apr-95


Log Weight(g)
10000
1000
100
10
1
10
100
Log Length(cm)
Dec-93
May-94
Jun-94
Jul-94
Jan-95
Feb-95
Mar-95
Apr-95

Chapter 2

Starvation Effect on White Muscle Moisture and Gonad Development in American plaice, *Hippoglossoides platessoides.*

2.1 Abstract

The intent of this study was to determine the effect of starvation on the condition (condition factor, hepatosomatic index and percent white muscle moisture) and reproductive development of American plaice, *Hippoglossoides platessoides*. The experimental design attempted to maintain water temperatures at natural levels by using chilled water, but due to limitations of the water supply system, water temperatures reached a maximum of 16°C. The fact that plaice can survive at these extreme temperatures was a surprising finding. The expected differences in white muscle moisture and condition at termination between starved and fed fish did not occur, although a significant difference in hepatosomatic index was noted.

High temperatures may be responsible for reproductive problems observed in fish from both starved and fed groups. Increased metabolic activity may have increased energetic demands requiring atretic recovery of reproductive products. There are indications that plaice can omit spawning in the face of energetic crisis, as some females showed gonads that were not on track for the next spawning season and were resorbing previtellogenic oocytes. Interestingly, even though they entered the experiment with low condition, these fish were able to maintain a significantly lower white muscle moisture than those fish with similarly low initial condition that had developed their gonads to a more advanced yolky state.

2.2 Introduction

The effects of starvation on muscle condition have been documented for several fish species, including winter flounder, *Pleuronectes americanus* (Maddock and Burton 1994), European plaice, *Pleuronectes platessa* (Johnston 1981, Johnston and Goldspink 1973), and Atlantic cod, *Gadus morhua* (Black and Love 1986, Love 1958). The utilization of white muscle protein in the starved state as a source of energy results in high white muscle water content, decreased protein content, decreased cross-sectional fibre area, and increased myofibrillar spacing. Similar states of muscle degradation are found in natural populations. Templeman and Andrews (1956) describe large female American plaice, *Hippoglossoides platessoides*, with muscle moisture reaching 96%, and the previous chapter describes natural cycles of muscle moisture in this species throughout the season. The natural occurrence of high water content muscle in plaice suggests the use of white muscle as a source of energy as a result of energetic demands faced in the wild. Maintenance requirements and energy needed for development of reproductive products are the major sources of energetic demand.

Some fish species have the ability to cease gonad development in the face of energetic crisis in order to preserve body condition. In particular, winter flounder have been shown not to proceed with production of gametes if starved during a critical period (Burton and Idler 1987). European plaice, *Pleuronectes platessa*, maintained on low rations, were seen to neglect reproductive advancement in 12 of

28 fish compared to a well fed group in which suspended reproduction was not evident (Horwood *et al.* 1989). American plaice, however, were thought to undertake a different reproductive strategy. Roff (1982) suggests that plaice sacrifice somatic condition in order to proceed with gonad development. The fact that plaice are batch spawners and may be capable of using white muscle for energy to push final oocyte batches through vitellogenesis (see previous chapter), may better explain the poor condition observed in some spawning females. Observation of adult female plaice that did not produce eggs every year by Pitt (1966), indicates that plaice probably do have the ability to assess energetic reserves and withhold gonad production if conditions are unfavourable. Bagenal (1957) also speculated that *H. platessoides* might not produce eggs every year. Records of such skipped spawning may be scanty due to the difficulties in distinguishing gonads that are not developing from those that have already spawned and are in a resting state prior to beginning maturation for the next spawning episode.

The effects of temperature on gonad products must also be considered as the temperature range to which the experimental plaice were exposed was considerably above that at which plaice are thought to dwell and breed. Pitt (1966) gives spawning temperatures for several areas around Newfoundland that plaice were captured, with a range of -1 to 3.5°C. American plaice on the Scotian Shelf, however, experience temperatures in the range of 0-13°C (Scott 1982). Morgan (1993) describes temperature preference experiments in which starved plaice

actively sought out lower temperatures than those fish that were well fed thereby lowering metabolic activity and conserving energy. Rapid increases in temperature during the spawning season are believed to interrupt or delay spawning in the English sole, *Parophrys vetulus* (Kruse and Tyler 1983). Conversely, mass resorption of oocytes through atretic processes in Greenland halibut, *Reinhardtius hippoglossiodes*, was thought to be caused by a decrease in temperature (Federov 1971).

To test the idea that plaice would continue to develop their gonads at the expense of protein reserves in the white muscle, this experiment investigates the reproductive response of American plaice to imposed starvation in relation to condition and muscle moisture. Analysis of muscle and gonad condition following experimental termination takes unusually high temperature into account.

2.3 Materials and Methods

Fish

American plaice were obtained from the Department of Fisheries and Oceans (DFO), Northwest Atlantic Fisheries Centre. Fish were weighed and their lengths measured to obtain initial condition factor before being tagged and separated into two groups. One group was fed to satiation twice weekly on chopped male capelin beginning in July of 1994, the other group was not fed at all for the duration of the experiment. Fish were maintained in tanks of aerated, filtered, circulating seawater and kept at natural photoperiod at the Ocean Sciences Centre (OSC), Logy Bay. Temperature varied with ambient surface temperatures. The experiment was terminated in September of 1994 at which time all plaice were killed with an excess of MS-222 followed by transection of the spinal cord.

Measurements taken at termination include whole weight and fork length, liver weight and gonad weight. Samples, consisting of skin, lipid layer and muscle, were taken from the six sites depicted in Figure 2.1 and preserved for histological examination. From each site on the ocular surface, a small piece of white muscle, taken near the vertebrae to ensure exclusion of red muscle, was dried to constant weight at 60°C to determine percent white muscle moisture (%H_2O).

The posterior tip of the gonad was also preserved for histological examination.

Indices of Fish Condition

Indices of fish condition were determined by the following:

Condition Factor (CF) = 100 x whole weight (g) ÷ length $(cm)^3$

Gonadosomatic Index (GSI) = 100 x gonad weight (g) ÷ whole weight (g)

Hepatosomatic Index (HSI) = 100 x liver weight(g) ÷ whole weight (g)

Comparisons were made using the Student's *t*-test with level of significance taken to be α=0.05.

Histology

Samples for histological examination were fixed in Bouin's fixative (75:25:5 picric acid: strong formalin: acetic acid) for 48 hours then transferred to 70% ethanol. These samples were processed through an ethanol dehydration series (1 hour each in 90%, and 2 changes of 100%), cleared in xylene (1 hour), infiltrated with and embedded in Paraplast Plus® for sectioning. Sections, approximately 7µm thick, were cut on a rotary microtome, floated on water on slides smeared with Mayer's glycerine albumin for adhesion, and dried at 37°C on a slide warmer before staining. The staining schedule involved removal of wax in xylene for 5 minutes, hydration through an ethanol series (5 minutes each in 100%, 90%, 70%, and 50%), 5 minutes in distilled water then staining in Ehrlich's Haematoxylin and blueing with Scott's solution. After rinsing with distilled water, slides were partially dehydrated (5 minutes each in 50%, 70% and 90% ethanols) and counterstained with Eosin Y (30 seconds) before two changes of 100% ethanol (5 minutes each) completed

dehydration. Slides were cleared in xylene and coverslips mounted with Histoclad®.

Muscle was examined for indications of white muscle breakdown and gonad sections were examined for reproductive status. Reproductive development for the next spawning season was indicated by the presence of vitellogenic oocytes and the absence of such signified failed reproduction.

2.4 Results

Effect of Treatment

The fed females had white muscle moisture content that ranged from 78.99% to 83.84%, not significantly different from the starved group with range 81.01% to 85.64% (Figure 2.2a). Initial and final condition factors were also consistent between treatments (Figures 2.2b and 2.2c) as was gonadosomatic index (Figure 2.3a). A significant difference ($p=0.0034$) was noted, however, in the hepatosomatic index at termination (Figure 2.3b). Table 2.1 depicts experimental data ordered by increasing muscle water content.

Temperature

Temperatures were quite high over the course of the experiment, registering around 11°C at setup and gradually increasing to a maximum of 15.7°C mid August (Figure 2.4).

Ovary histology and changes in somatic condition

If the fish are grouped regardless of feeding regimen taking both condition and state of ovary development at termination into account, some interesting trends are noted. Fish which enter the experiment in good condition (initial condition factor > 0.85, fish a, 1 and 4) or greatly increased condition over the course of the experiment (fish b) (Figure 2.2b,c), developed their oocytes to a mature yolky stage (Figure 2.5a) and maintained a relatively low white muscle moisture content ranging from 78.99% to 82.66%. Of those individuals that began the experiment with a

relatively low condition (< 0.85), two outcomes were observed with respect to gonad development and white muscle moisture. One group of low condition fish (N=3, fish 5, d and e) maintained a muscle water content similar to the high condition plaice ranging from 81.01% to 81.13% (p=0.58). These females had gonads that were at an early stage of development with all oocytes in a previtellogenic perinucleolar stage (Figure 2.5b). Other low condition females (N=2, fish 2 and c) showed significantly higher white muscle moisture (83.81% to 84.06%) than the high condition fish (p=0.03) and the low condition, pre-vitellogenic group (p=0.03). The gonads from these fish showed evidence of advanced oocyte development through the presence of yolky oocytes, residual eggs, or atresia of vitellogenic oocytes.

The female that showed the highest degree of muscle protein breakdown (reference 3), with a water content of 85.64%, showed evidence of recent spawning with residual oocytes in the lumen of the ovary, thickened ovary wall and very little in the way of gonad tissue remaining in the ovary (Figure 2.6).

Atresia

Most experimental plaice show evidence of various degrees of reproductive atresia. The lowest degree of atresia is seen in the female with highest final condition and lowest water content of all the experimental plaice (fish b). High condition, low muscle water content fish are undergoing resorption of their mature yolky oocytes and atresia can be followed through its early stage of thickening of the zona pellucida (Figure 2.7a) through to the convolution and yolky breakdown seen

in the advanced stage of atresia observed in some females (Figure 2.7b). Pre-vitellogenic oocytes of the low condition fish which apparently did not proceed with gonad development are also susceptible to atretic resorption (Figure 2.7c).

Two females showed no evidence of atresia in sections examined. One of these fish had a gonad with most advanced oocytes in the cortical alveoli stage and thin ovarian wall (Figure 2.8a). The other female had large vitellogenic oocytes and no evidence of atretic resorption at the time of termination (Figure 2.8b).

2.5 Discussion

The expected differences in white muscle water content and condition factor between starved and fed plaice did not result from the imposed starvation. Instead, both groups had statistically similar condition factors and muscle moistures as those observed in wild populations during the spawning season (see previous chapter). Spawning females have significantly higher muscle moisture during the spawning season, reaching 83% (compared to prespawning December levels of 76%) corresponding to energetic depletion of protein from white muscle. High experimental temperatures, reaching ~16°C, certainly created additional energetic stress to which both groups had to adapt. The effects of feeding may have been negated by the increased metabolic demand that digestion and assimilation at such extreme temperatures would impose (Jobling and Spencer Davies 1980). The high muscle moistures in both groups are likely the result of increased metabolic activity demanding the use of muscle protein to meet energetic demands. The dual role of white muscle protein, that as a reproductive fuel as well as an energy source in times of nutritional deprivation, is an interesting capability and may result from two separate schemes of cues and mobilization pathways. The only benefit of feeding observed was the significantly greater hepatosomatic index in the fed plaice ($p<.01$, Figure 2.3b). This may reflect the post-prandial activity of the liver, but the corresponding increase in condition that would indicate nutritional benefit of feeding is not demonstrated.

Considering the lack of beneficial effects of feeding on condition and muscle moisture, the observed reproductive states of the experimental fish are treated as the result of a nutritionally deprived state, compounded by temperatures bounding on lethal. The condition of the fish upon entering the experimental setup may be decisive in whether the fish proceeds with oocyte development with minimum muscle protein depletion. The fish which enter the unfavourable situation with condition factor above 0.85 are able to develop their oocytes to an advanced vitellogenic state while those that are in worse condition (<0.85) are susceptible to higher white muscle moisture if oocytes are developed to such a mature state.

There is also the indication that plaice can decide to repress gonad development if condition is poor when adverse conditions are encountered, as one group of initially low condition plaice was characterized by undeveloped oocytes (Figure 2.7). The removal of the energetic demand placed on a fish by reproductive development allows the sparing of white muscle protein which may be needed to meet maintenance requirements should conditions worsen. The observed response to starvation of low condition plaice is quite similar to the effects observed in European plaice, *P. platessa*, maintained on low rations (Horwood *et al.* 1989). A portion of *P. platessa* in the low ration group did go on to develop oocytes for the coming season, but were less fecund with a lower GSI and smaller oocytes than those fish maintained on higher rations. Information on initial condition is not clear in this paper, but it may be that those fish that did continue with gonad development

were in better initial condition than those that ceased reproductive development. The ability of *H. platessoides* to assess energetic stores and suspend gonad growth based on poor condition is indicated, confirming suggestions that plaice may skip spawning seasons due to naturally imposed energetic crises (Pitt 1966, Bagenal 1957).

Significant levels of atresia were observed in many of the experimental plaice. Atresia in *P. platessa* is considered to occur at the relatively low rate of 2.7% despite nutritional deprivation (Horwood *et al.* 1989). Assuming atresia is also naturally uncommon in *H. platessoides*, which is suggested by observation of oocyte development in wild populations (see previous chapter), the degree of atretic resorption seen in the experimentally maintained plaice (Figure 2.7) is likely due to the extreme temperatures to which the fish were exposed. Atresia in the low condition plaice that suspended oocyte development is evidenced by the breakdown of previtellogenic oocytes in the perinucleolar stage. This suggests that energy reserves in the body were at such low levels that resorption of such early oocytes was necessary for maintenance requirements. Atretic oocytes in developing gonads were observed in various stages of resorption. In exogenously vitellogenic oocytes, thickening of the zona pellucida appears to be an initial characteristic, followed by convolution of the chorion and breakdown of the yolky material. Witthames and Greer Walker (1995) describe similar stages of atretic resorption in *Solea solea*. The high temperatures may have forced the experimental plaice into an energetic

situation in which any means of saving or recovering energy would have to be undertaken, including resorption of any reproductive products.

Two females showed no evidence of recovery of reproductive products. One of these was probably an adolescent female, perhaps in a suspended state of development. The other female, with advanced oocytes, which showed no atresia on slides examined, might have started vitellogenesis earlier than most of the other experimental fish. Allowed to continue, this female might have showed similar resorption of vitellogenic oocytes.

Faced with energetic shortages, the plaice probably go through a series of decision making steps. The first solution would be to move to an area of higher food supply. The next step might be to move to an area of lower temperature, maybe deeper water, as a means of lowering metabolic demand on stressed energy stores. If this is not an option, or if demands persist, the choice to avail of protein from white muscle might be initiated. Once some critical level of protein depletion is reached, suspension of reproductive growth would decrease energetic requirements further. If reproductive development had already begun before conditions were critical, development would probably complete, as is seen in *P. platessa* in low ration feeding experiments (Horwood *et al.* 1989). In these cases, energetic recovery of gametes through atresia would be an option for survival requirements.

Figure 2.1: Sampling sites from which muscle for histology and determination of moisture content were taken on American plaice, *Hippoglossoides platessoides*. Modified from Scott and Scott (1988).

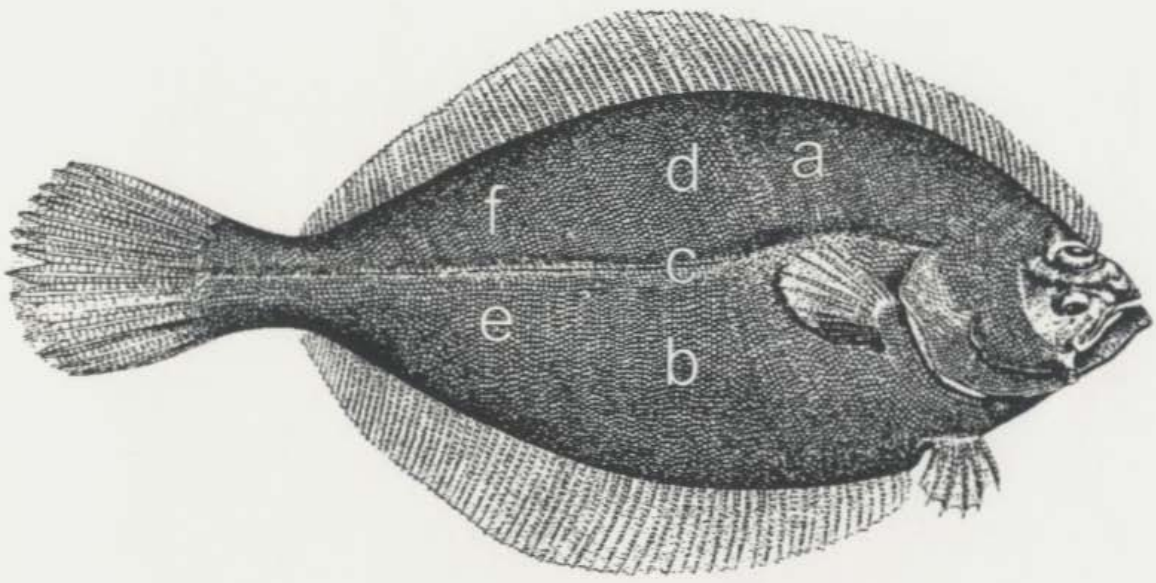

d
a
f
c
e
b

Figure 2.2: Comparisons between fed and starved American plaice, *Hippoglossoides platessoides*. (a) White muscle moisture content, (b) initial condition factor and (c) final condition factor. Letters and numbers refer to individual fish and are consistent between plots.

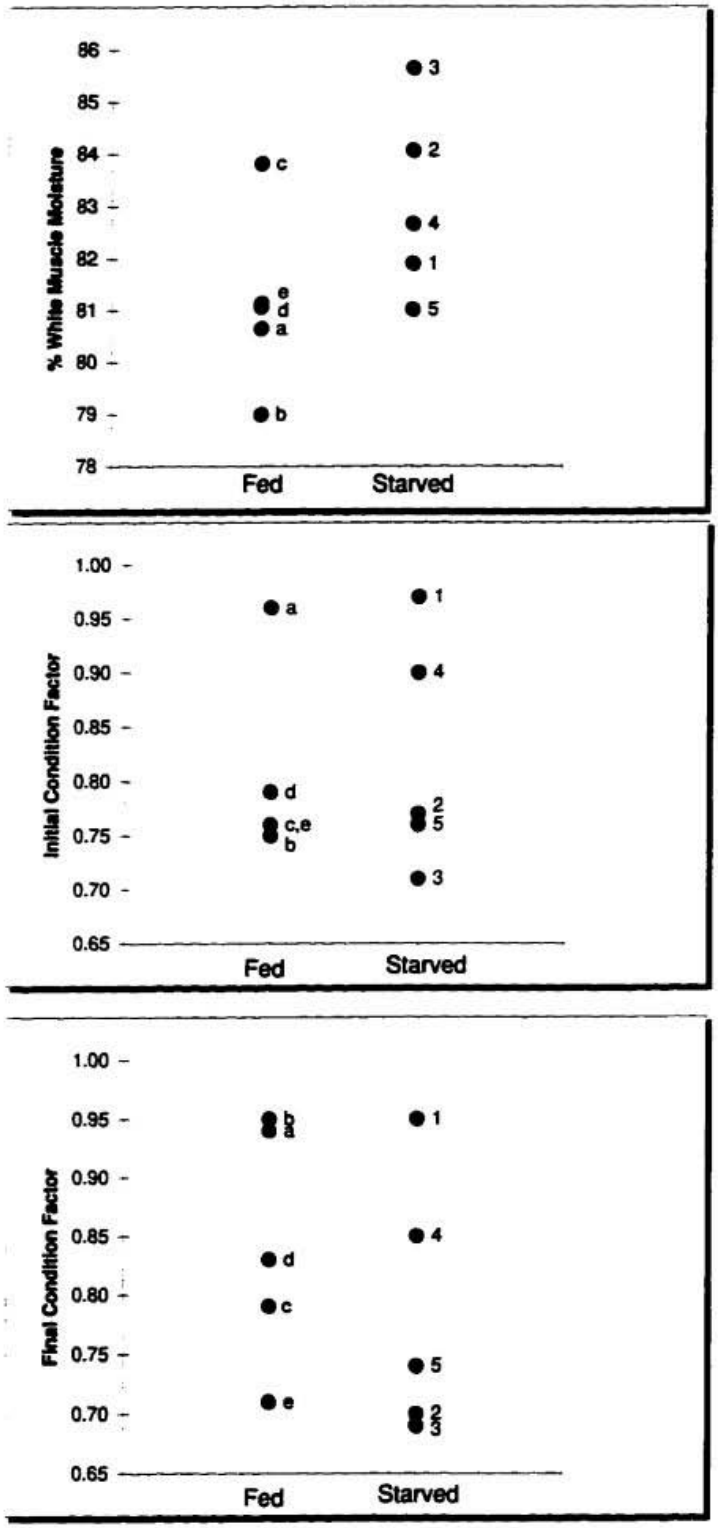

% White Muscle Moisture
86
85
84
83
82
81
80
79
78
c
e
d
a
b
3
2
4
1
5
Fed
Starved
Initial Condition Factor
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
a
d
c,e
b
1
4
2
5
3
Fed
Starved
Final Condition Factor
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
b
a
d
c
e
1
4
5
2
3
Fed
Starved

Figure 2.3: Gonadosomatic index (GSI) and Hepatosomatic Index (HSI) in fed versus starved American plaice, *Hippoglossoides platessoides*. HSI differs between treatments, significant at $\alpha = 0.05$ using Student's *t*-test. Letters and numbers refer to individual fish and are consistent with Figure 2.2.


Gonadosomatic index
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
c
a
d
b
e
2
1
4
5
3
Fed
Starve


Hepatosomatic index
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
c
a
e
d
3
5
1
4
2
Fed
Starved

Table 2.1: Summary data for experimental American plaice, *Hippoglossoides platessoides*, ordered by increasing white muscle moisture. Reference column refers to labels for individual fish used in Figures 2.2 and 2.3. Letters denote fed fish and numbers mark starved plaice.

Tag	Reference	Initial CF	Final CF	GSI	HSI	Mean %H2O
[illegible]	b	0.75	0.95	1.08	2.38	78.99
[illegible]	a	0.96	0.94	2.65	2.24	80.64
P686	5	0.76	0.74	1.87	1.12	81.01
[illegible]	d	0.79	0.83	1.30	1.30	81.06
[illegible]	e	0.76	0.71	0.91	2.13	81.13
O801	1	0.97	0.95	3.56	0.99	81.90
O870	4	0.90	0.85	3.51	0.91	82.66
[illegible]	c	0.76	0.79	4.18	2.30	83.81
P693	2	0.77	0.70	7.88	0.72	84.06
P692	3	0.71	0.69	1.44	1.15	85.64

	Fed Plaice
	Starved Plaice

Note: Final CF has been modified if length at termination was shorter than that at setup by using setup length in the calculation of final condition.

Figure 2.4: **Water temperatures (°C) in experimental tanks over the course of experimental period.**

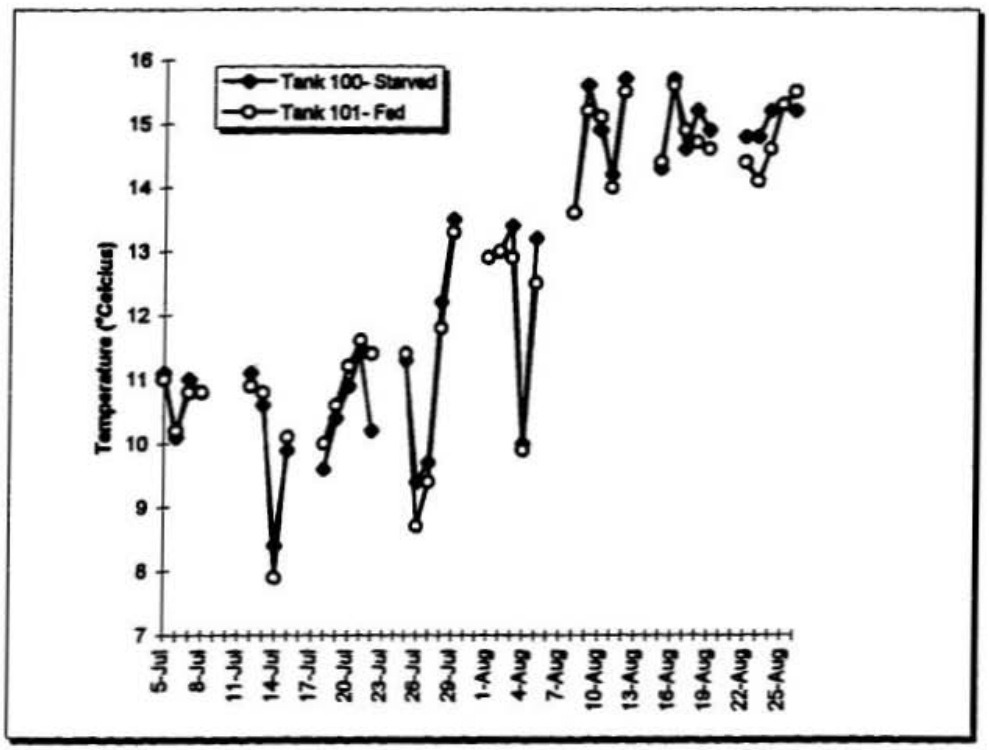

Tank 100- Starved
Tank 101- Fed
Temperature (°Celcius)
16
15
14
13
12
11
10
9
8
7
5-Jul
8-Jul
11-Jul
14-Jul
17-Jul
20-Jul
23-Jul
26-Jul
29-Jul
1-Aug
4-Aug
7-Aug
10-Aug
13-Aug
16-Aug
19-Aug
22-Aug
25-Aug

Figure2.5: **Ovaries from experimental American plaice which entered the experimental condition with different somatic condition. (a) Cross section of ovary from an American plaice that entered the experimental conditions with high condition. Vitellogenic oocytes (v) were on track for the upcoming spawning season. (b) Cross section of ovary from a plaice that entered experimental conditions with a low condition and did not proceed with gonad development. All oocytes were previtellogenic (pv), one showing a Balbiani body (b). w = ovarian wall. Both images captured at the same magnification, scale bar = 100µm.**

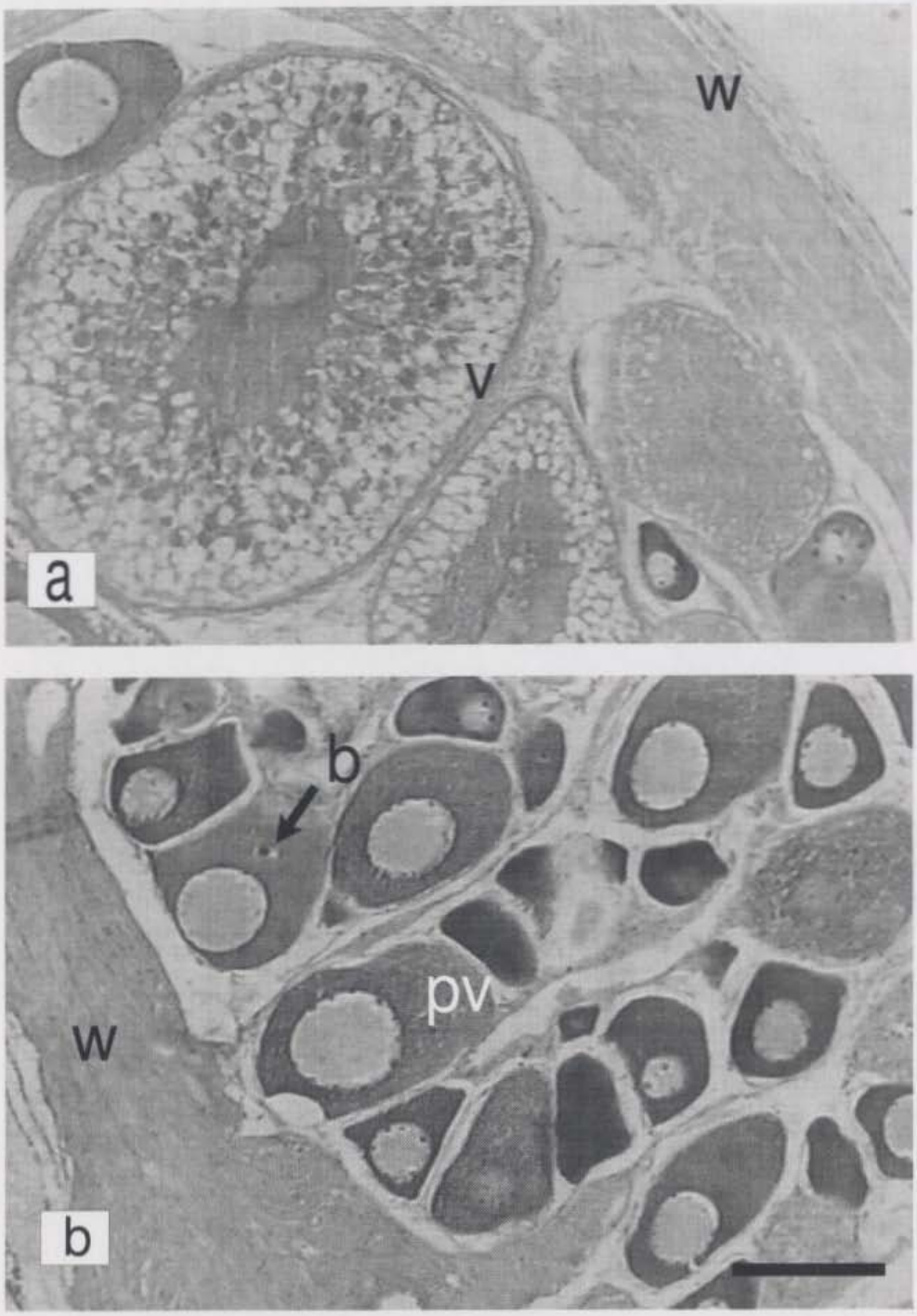

w
v
a
b
pv
w
b

Figure 2.6: Ovary from American plaice, *Hippoglossoides platessoides*, that showed the highest muscle water content of 85.64%. Evidence of prior spawning activity includes (a) a thickened ovarian wall (**w**), with only immature oocytes remaining, and (b) residual eggs (**re**) in the lumen of the ovary. Both images were taken at the same magnification, scale bar = 100µm.

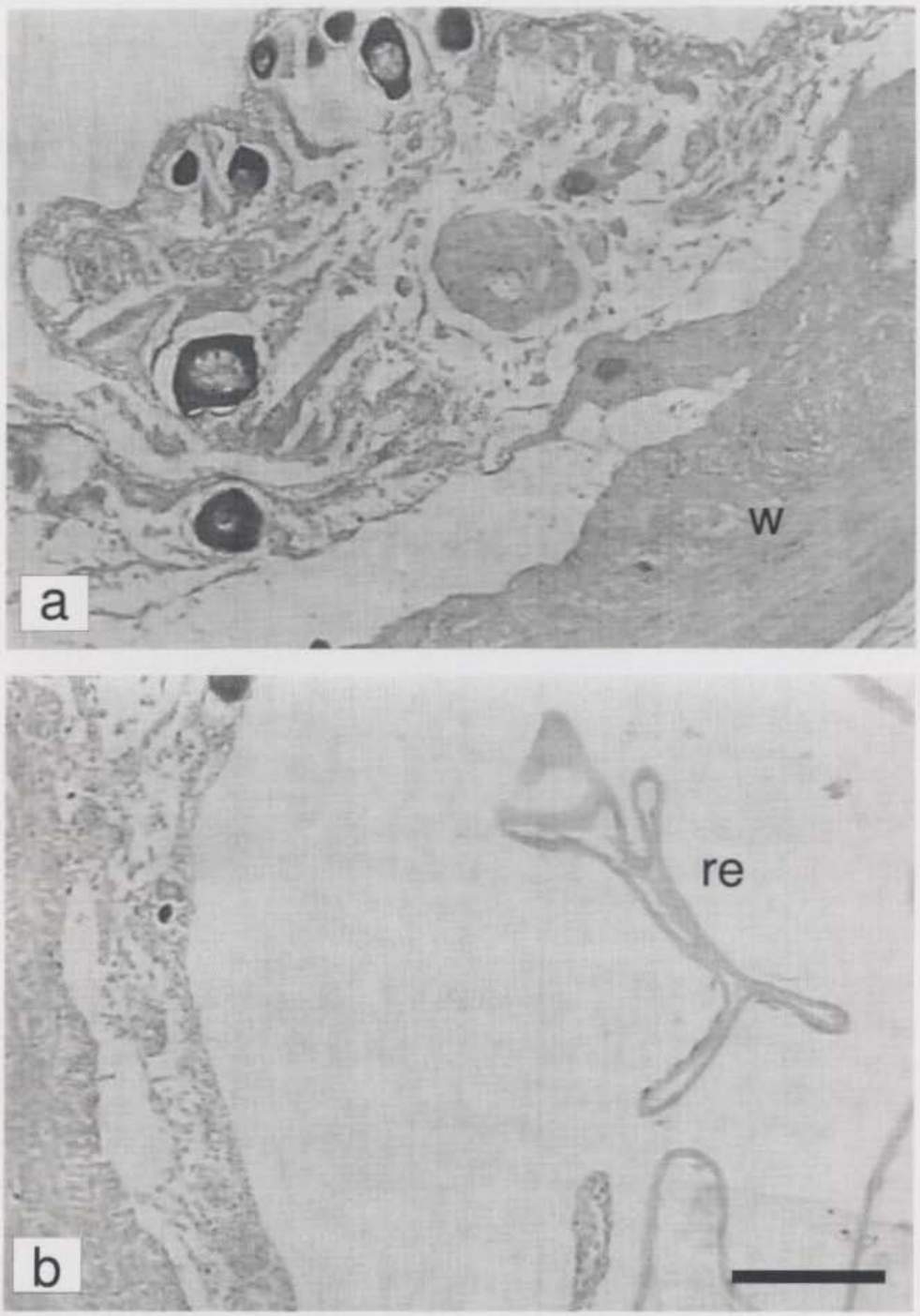

w
a
re
b

Figure 2.7: Atretic breakdown of yolky oocytes in *Hippoglossoides platessoides* shows (a) initial thickening of the zona pellucida (**zp**) and degradation of yolk (**y**). (b) Late atresia is characterized by a convoluted appearance of the zona pellucida (**zp**) and most of the yolk has been resorbed. (c) In plaice that did not proceed with gonad development, atretic recovery of previtellogenic oocytes results in an atretic body (**A**) that is quite different from normal perinucleolar oocytes (**pn**). **nu** = nucleus, **n** = nucleolus. Images (a) and (b) captured at the same magnification, scale bar = 100µm. Image (c) is at higher magnification, scale bar = 50µm.

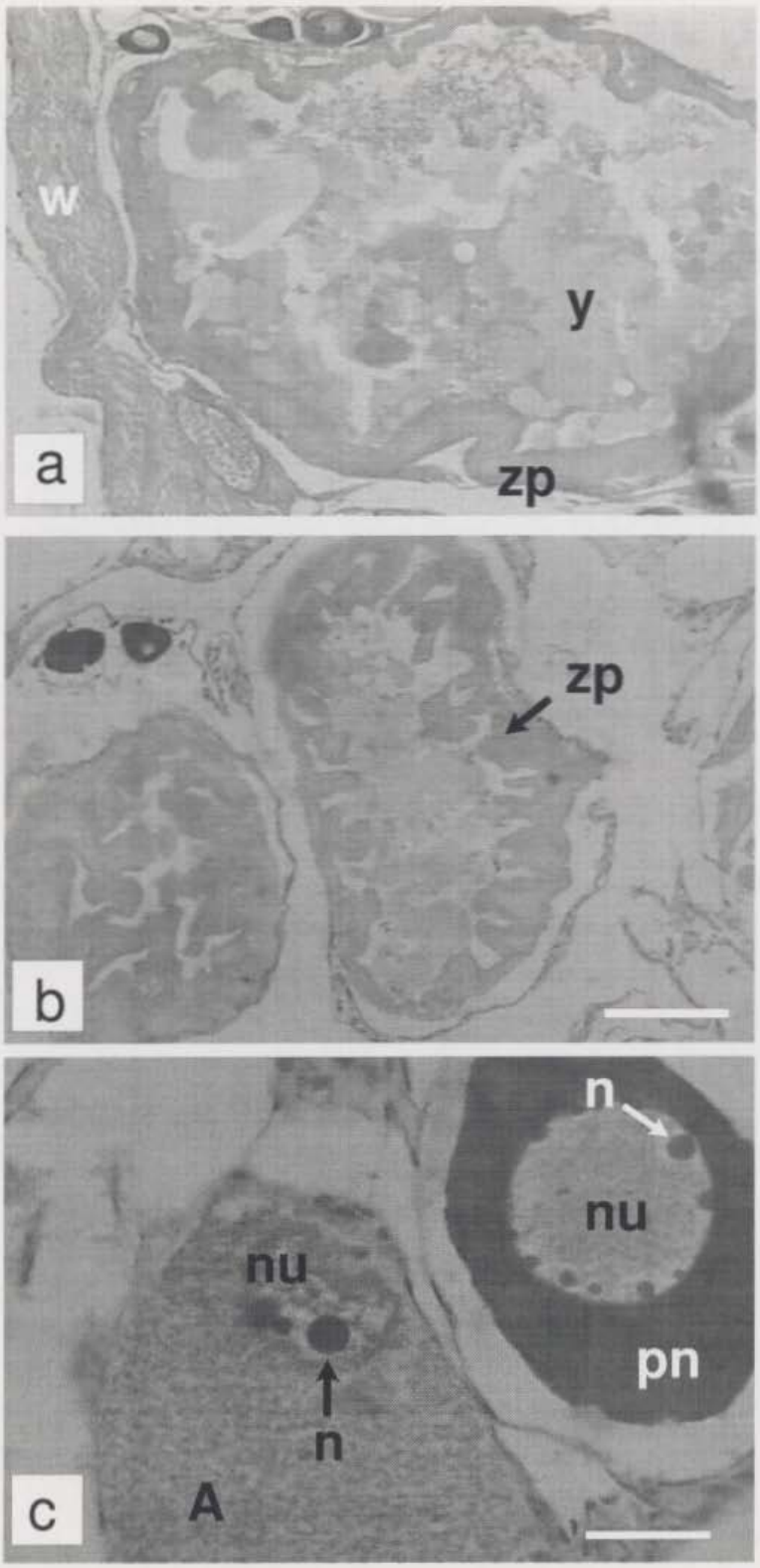
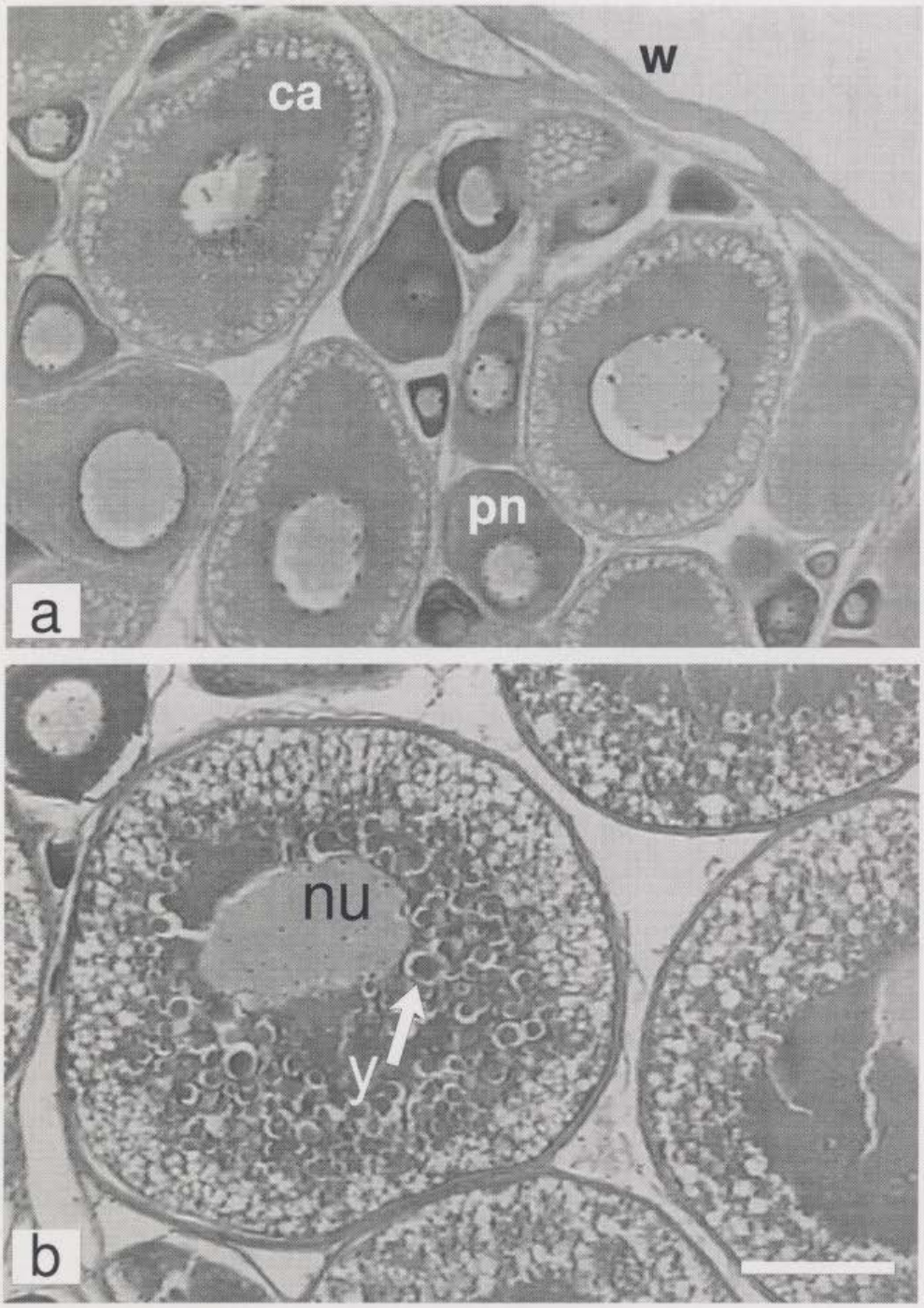

w
y
a
zp
zp
b
n
nu
nu
pn
n
A
c

Figure 2.8: Cross section of ovaries from experimental American plaice, *Hippoglossoides platessoides* which showed no evidence of atresia. (a) Ovary from a plaice which entered experimental setup with good condition and maintained the lowest muscle water content shows most oocytes in the cortical alveoli stage (**ca**) and others in immature and perinucleolar (**pn**) stages. The ovarian wall (**w**) is relatively thin and coupled with the relatively undeveloped state of the oocytes, suggests development for the first time. (b) In comparison, these oocytes are much more developed with yolk (**y**) deposition advanced. Atresia was not noted in these two gonads. Both images were captured under the same magnification, scale bar = 100µm.


w
ca
pn
a
nu
y
b

Chapter 3

Recovery of white muscle from a starvation induced high water state in winter flounder, *Pleuronectes americanus*, through refeeding.

3.1 Abstract

Winter flounder, *Pleuronectes americanus*, starved over the normal summer feeding period, showed higher white muscle moisture and poorer condition factor than fed fish. Ultrastructurally, white muscle from starved fish showed less glycogen and fewer oleosomes than white muscle from fed fish. Flounder with condition factor and muscle moisture comparable to the starved group were fed during the following feeding season and were observed to recover their condition and muscle moisture to the same level as those of the fed group within five months. Refeeding also restored reproductive development, and although not as advanced as the fed group, starved fish had begun exogenous vitellogenesis by September. Mass atresia of yolky oocytes was recorded in most fed flounder and was likely temperature related.

3.2 Introduction

Changes in white muscle due to starvation have been described in several species (Beardall and Johnston 1983, Johnston 1981, Johnston and Goldspink 1973, Gas 1972, and Love 1958) including the winter flounder, *Pleuronectes americanus*, (Maddock and Burton 1994). Changes in muscle structure described by these authors include decreased cross sectional area of white muscle fibres, increased intercellular spacing, and decreased myofibrillar content. Love (1958), working on cod, *Gadus morhua*, and Stirling (1976) working on *Dicentrarchus labrax*, related increased moisture content of the white muscle with decreased protein allowing inferences on protein depletion based on a relatively simple measure of water content of the muscle. Gas (1972) describes ultrastructural detail of starved *Cyprinus carpio* showing depletion of myofilaments and disorganization of the sarcomere. The preferential utilization of white muscle and conservation of red muscle in starvation is documented for *Pleuronectes platessa* (Johnston and Goldspink 1973) and suggested in winter flounder through maintenance of cross sectional area of red muscle following starvation (Maddock and Burton 1994).

Winter flounder are believed to sacrifice reproductive development in the face of nutritional deprivation in order to preserve somatic condition (Tyler and Dunn 1976). Experiments undertaken to investigate starvation effects on reproductive development of *P. americanus* have shown that starvation during a critical period will effectively turn off the production of gametes for the subsequent breeding

season (Burton 1994). Description of non-reproductive winter flounder from the wild (Burton and Idler 1987, Ibid. 1984) suggest that conditions of nutritional shortage in the natural population require conservation of energy via shutdown of reproductive growth.

Alpha atresia or breakdown of maturing oocytes prior to ovulation has been described in *Pleuronectes platessa* by Witthames and Greer Walker (1995). Initial breakdown of yolky oocytes is identified by a thickening of the zona pellucida which also takes on a wavy appearance. Breaks in the zona radiata externa allow yolk vesicle discharge toward the follicle, the zona pellucida collapses toward the center of the cell and yolk is eventually resorbed completely before the follicle hypertrophies. Mass atresia of reproductive products in the Greenland halibut, *Reinhardtius hippoglossoides*, was thought to be induced by hydrological conditions of the spawning grounds (Federov 1971).

Pearcy (1961) described *P. americanus* from winter samples to have flesh which was "soft and watery" in comparison to fish collected in the summer, suggesting that the use of protein from muscle is necessary to survive the winter fast. Starvation of this species has resulted in very high levels of white muscle moisture, with levels reaching 95% (Maddock and Burton 1994). Love (1958) questioned the ability of fish which have undergone protein depletion to such extremes to survive at all, and Fisher *et al.* (1987) suggested that the jellied state, at least in *Microstomas pacificus*, did not improve once imposed. Refeeding of

Atlantic cod from a starvation induced high white muscle water state resulted in decreased water content, but levels remained higher than control fed fish even after 200 days of refeeding (Black and Love 1986). Reversible utilization of white muscle protein in order to endure episodes of starvation in the wild is a useful survival technique. This study attempts to monitor white muscle recovery from a starvation induced high moisture state in winter flounder, *P. americanus*, through refeeding. A biopsy technique allows tracking of muscle condition in individual fish over a period of starvation and subsequent refeeding.

3.3 Materials and Methods

Fish

Winter flounder, *Pleuronectes americanus*, from Witless Bay and Harbour Main were collected by divers from the Ocean Sciences Centre. The fish were divided equally into two groups, each of which was maintained in tanks of filtered, circulated, aerated seawater and held at ambient temperature and seasonal photoperiod at the OSC., Logy Bay. One group, beginning in June of 1994, was fed twice weekly to satiation on chopped capelin. The other group was not fed at all over the normal summer feeding season in 1994 (June through December). Feeding in the control group was suspended from December 1994 until April of 1995 corresponding to the natural starvation period. Feeding was recommenced in the fed group in April of 1995. Due to high mortality of fish in the starved group, low condition winter flounder from another experimentally starved group were introduced into the setup in May 1995 for the purpose of refeeding. The experiment was terminated in September 1995 at which time all fish were killed with an excess of MS222 followed by transection of the spinal cord. Measurements taken included whole weight (g), total length (cm), liver weight (g) and gonad weight (g). The posterior tip of one gonad was fixed for histological observation and a muscle sample was dried to constant mass at 60°C to ascertain moisture content (%H_2O). Gonadosomatic Index was calculated using the formula GSI = 100 gonad weight (g) ÷ total weight (g).

Biopsy Technique

In December of 1994 muscle samples were obtained from the experimental flounder using a biopsy technique (Mair 1989). Fish were anesthetized using MS222 prior to biopsy. Incision was made at midbody dorsal to the lateral line, consistent with the site c sampled in Chapter 2 to allow for comparison. Muscle samples approximately $1mm^2$ were taken as deep as possible to ensure that white muscle was obtained. One sample was dried to constant weight at 60°C, one was preserved for histology and another was preserved for transmission electron microscopy. Total length and weight were taken at this time to determine condition factor using the formula condition factor (CF)= whole weight(g) ÷ total length$(cm)^3$. Statistical comparisons were made using the Student's *t*-test with a significance level of 0.05.

In fish that showed overt sign of ovary development an attempt was made to obtain a sample of ovarian tissue by gently inserting a thin plastic catheter into the vent and threading it up the oviduct into the right ovary. A syringe was then used to draw tissue into the tubing and once removed, the tissue was fixed and processed for histological analysis.

Histology

Muscle and gonad samples were fixed in Bouin's solution (75:25:5 picric acid:strong formalin:acetic acid) for 24-48 hours and then transferred to 70% ethanol. Samples were processed through an ethanol dehydration series (1 hour each in 90% and 2 changes of 100%; before being cleared for 1 hour in xylene and

embedded in Paraplast Plus® for sectioning. Sections were cut at approximately 7µm thick on a serial microtome and mounted on slides smeared with Mayer's glycerine albumin for adhesion.

Staining was with Ehrlich's haematoxylin and Eosin Y following wax removal in xylene and hydration through an ethanol series (5 minutes in each of 100%, 90%, 70%, and 50% and 5 minutes in distilled water). Blueing was with Scott's solution, dehydration through the reverse ethanol series noted above was interrupted prior to the absolute ethanol for counterstaining in Eosin Y for 30 seconds, and two changes of 100% ethanol preceded xylene clearing prior to mounting coverslips with Histoclad®.

Ultrastructure

White muscle, minced to approximately 1 mm^3, was fixed in Karnovsky's primary fixative, rinsed three times in cocadylate buffer and post-fixed in 1% osmium tetroxide in cocadylate buffer before dehydration through an ethanol dehydration series (25%, 30%, 50%, 70%, 80%, 95% and 3x100%). Tissue was then infiltrated with Spurr's resin, placed in moulds and polymerized at 70°C. Thin sections were stained with uranyl acetate followed by lead citrate and viewed with a transmission electron microscope. The relative presence of glycogen granules was used to infer glycogen depletion and fat depletion was indicated by a decrease in oleosomes.

3.4 Results

Figure 3.1 depicts the change in condition of starved, low condition and fed winter flounder over a fifteen month period. Starvation over the normal summer feeding season, from June through December 1994, resulted in a decrease in condition of starved flounder to levels significantly lower than the condition factors of fish from the fed group ($p=0.001$). At the end of the starvation period (December 1994), white muscle moisture of starved fish was higher ($p=0.009$) than that of the fed group (Figure 3.2). High white muscle moisture and low condition characterized starved flounder while fed fish were in better condition and had lower white muscle water content (Figure 3.3) Biopsy samples of white muscle from starved fish, processed for viewing by transmission electron microscopy, showed less glycogen and fewer oleosomes than in white muscle from fed flounder (Figure 3.4).

Reproductive development of starved flounder appeared restricted since the ovarian development observed in fed females as swelling of the ovarian region was not evident in the starved group. An attempt to ascertain the reproductive status of each fish following the starvation period, using a catheter to remove ovarian tissue, was successful only in two of the developing females. Insertion of the tubing was not effective in fish that were not developing and attempts were abandoned for fear of further stress to the starved fish. Developing oocytes from fed females were

extracted and sectioned, confirming reproductive development for the 1995 spawning season, and show immature and vitellogenic oocytes in the extracted tissue (Figure 3.5).

Substitution of low condition factor fish from another experimental setup became necessary in May of 1995 due to high mortality in the starved group over the winter fasting period. The low condition fish were not significantly different from the starved fish with respect to post-starvation condition ($p=0.064$) and white muscle moisture ($p=0.51$) upon their introduction to the experimental setup (Figure 3.3). Refeeding of the experimentally induced low condition fish occurred during the 1995 summer feeding season, from May through September at which time the experiment was terminated. Recovery of white muscle from its high water state was indicated and by September, the refed flounder had white muscle moistures that were not significantly different from the fed group ($p=0.078$, Figure 3.2) and condition, although still lower than the fed group, was not statistically different ($p=0.16$, Figure 3.1).

Gonad development at the time of termination showed low condition fish with ovaries that contained primarily previtellogenic and cortical alveoli stage oocytes with some evidence of yolk deposition (Figure 3.6). The mean GSI of the low condition females was 3.59 ± 1.41 while the mean GSI for the fed group females was much higher at 19.01 ± 9.57. Fed flounder showed oocytes that were much more advanced than low condition fish. Their gonads contained large, advanced,

vitellogenic oocytes, some of which showed nuclear migration. Although oocyte development for the next season had advanced to the late vitellogenic stage, the fed flounder appeared to be undergoing atresia of vitellogenic oocytes (Figure 3.7). The one male flounder included in this study as a member of the starved group, showed testes that were probably on track for the coming season and appeared to have secondary spermatogonia.

The muscle biopsy technique was useful in tracking the recovery of muscle condition from the starvation induced high moisture condition in individual fish following refeeding.

3.5 Discussion

Starvation of *P. americanus* over the normal summer feeding season resulted in lower condition and higher white muscle moisture compared to a control group of fed fish. These results are similar to those obtained in previous experiments using this species (Maddock and Burton 1994) and in Atlantic cod, *Gadus morhua* (Black and Love 1986). The depletion of protein from white muscle of Atlantic cod, *Gadus morhua*, is accompanied by an increase in water (Love 1958) and suggests the utilization of somatic protein as a source of energy needed for maintenance during periods of nutritional deprivation. The ability to monitor changes in muscle moisture in an individual fish is a useful means of tracking protein depletion and recovery over the course of experimental manipulation of nutrition.

Gas (1972) indicates that myosin is preferentially depleted in starvation of *Cyprinus carpio.* Depletion of muscle glycogen and lipid reserves during starvation in flounder is indicated by ultrastructural examination, but it is unclear which protein fraction, myosin or actin is targeted, or whether there is a general depletion of several types of proteins.

Using the protein from white muscle as a source of energy can only benefit if this process is reversible, allowing rebuilding of fibre integrity in order to preserve contractile ability once conditions support allocation of energy into somatic regrowth. Contrary to the idea that jellied flatfish do not recover their muscle condition (Fisher *et al.* 1987), refeeding of starved fish over a period of four months resulted in a

rebound in condition and a lowering of white muscle water content to values statistically similar to control fish indicating that protein depletion is reversible when food is available. Recovery of white muscle in *G. morhua* following starvation was indicated by a decrease in white muscle moisture, but even after 200 days of refeeding, moisture levels were still significantly higher than the control group of fed fish (Black and Love 1986). The fact that winter flounder seem to be able to recover more rapidly from protein depletion than cod may be related to life strategy differences between these two species. Winter flounder are known to undergo a period of fast in the wild for up to six months of the year (Kennedy and Steele 1971) while cod may experience periods of low food availability, but are not thought to cease feeding completely (Burton *et al. in press*). Mcleese and Moon (1989) report an increase in muscle moisture in *P. americanus* corresponding to the fasting season, and Pearcy (1961) describes winter flounder taken during the fasting season as having soft, watery flesh. Poor condition upon entering fasting conditions may require use of white muscle protein for energy and the development of a quick recovery system would allow flounder to maximize the benefit of the short feeding season during which time somatic recovery or growth and reproductive effort must be addressed before the onset of the next fast. It is interesting that muscle protein is the priority in recovery of cod from starvation, in that liver lipids did not start recovery until white muscle moisture had dropped below 82% (Black and Love 1986). This strategy allows muscle, needed for burst activity important in prey

capture, to rebuild first before lipid storage is initiated.

Reproductive development is not as advanced in the refed flounder when compared to the control group. Although development has proceeded to the stage of exogenous yolk deposition, oocytes are not as advanced as those of the fed group (compare Figures 3.6 and 3.7a). Oocyte maturation might have been delayed in the starved fish, perhaps due to allocation of energy reserves first into recovery of white muscle and somatic condition before reproductive development could begin. Roughly one month's advantage in feeding was given to the control group, which may explain the variation.

Oocytes from most females of the fed group had reached an advanced stage by September 1995. Large yolky cells with nucleus already peripheral indicate that fed fish were more advanced than the starved females. One female, however, showed oocytes that were similar in developmental stage to the starved fish. Exogenous yolk deposition had begun, but oocytes were not as mature as in the other fed flounder. This particular female showed no sign of gonad development at the time of initial setup (in fact, she was actually mistaken for a male based on visual assessment of the gonad). The lack of gonad development at that time of year might reflect an early spawner or an adolescent fish. It may simply reflect variation in timing of reproductive development, which would also explain the less developed females in the refed group, and suggests that reproductive effort may have been restored in the refed group as the result of excellent nutritional status in the current

feeding season. The substantial increase in condition over the course of refeeding confirms the benefit of refeeding (Figure 3.1).

Atresia of advanced oocytes was noted in the fed flounder at termination. The initial stage of thickening of the zona pellucida precedes yolk vesicle breakdown and collapse of chorion results in an infolded structure similar to the breakdown of yolky oocytes described by Witthames and Greer Walker (1995) in *Solea solea*. The breaks in the zona radiata externa and extrusion of yolk vesicles described by these authors is not observed in atretic oocytes examined here in *P. americanus*. The breakdown of mature reproductive products may be the result of unusually high temperatures, reaching 16°C at one point in the experiment. Chapter 1 describes similar resorption of yolky oocytes in experimental American plaice, *Hippoglossoides platessoides*, which were maintained on the same water source at that time. Federov (1971) implicates complex hydrographic conditions of the breeding area with mass atresia and failure to spawn in *Reinhardtius hippoglossoides*, perhaps temperature changes could be involved in atretic resorption of advanced oocytes.

Recovery of white muscle, through refeeding, from a starvation induced state of high water content is indicated in *Pleuronectes americanus*, contradicting the idea that this jellied state of muscle is maintained once imposed (Fisher *et al.* 1987). The delay in reproductive development of starved fish following refeeding may result from energetic allocation into somatic recovery before gonad development is initiated, or may simply reflect variation in individual timing of gonad growth.

Figure 3.1: Change in condition factor in winter flounder, *Pleuronectes americanus*, over the course of experimental starvation and refeeding. The yellow bar on the x-axis indicates the period of experimental feeding/starvation, pink bar indicates period of natural winter fast, during which no fish were fed, and green bar indicates period of refeeding of both experimental groups. Data are mean ± 1 SD. N starved= 4, N low CF= 5, N Fed= 5.

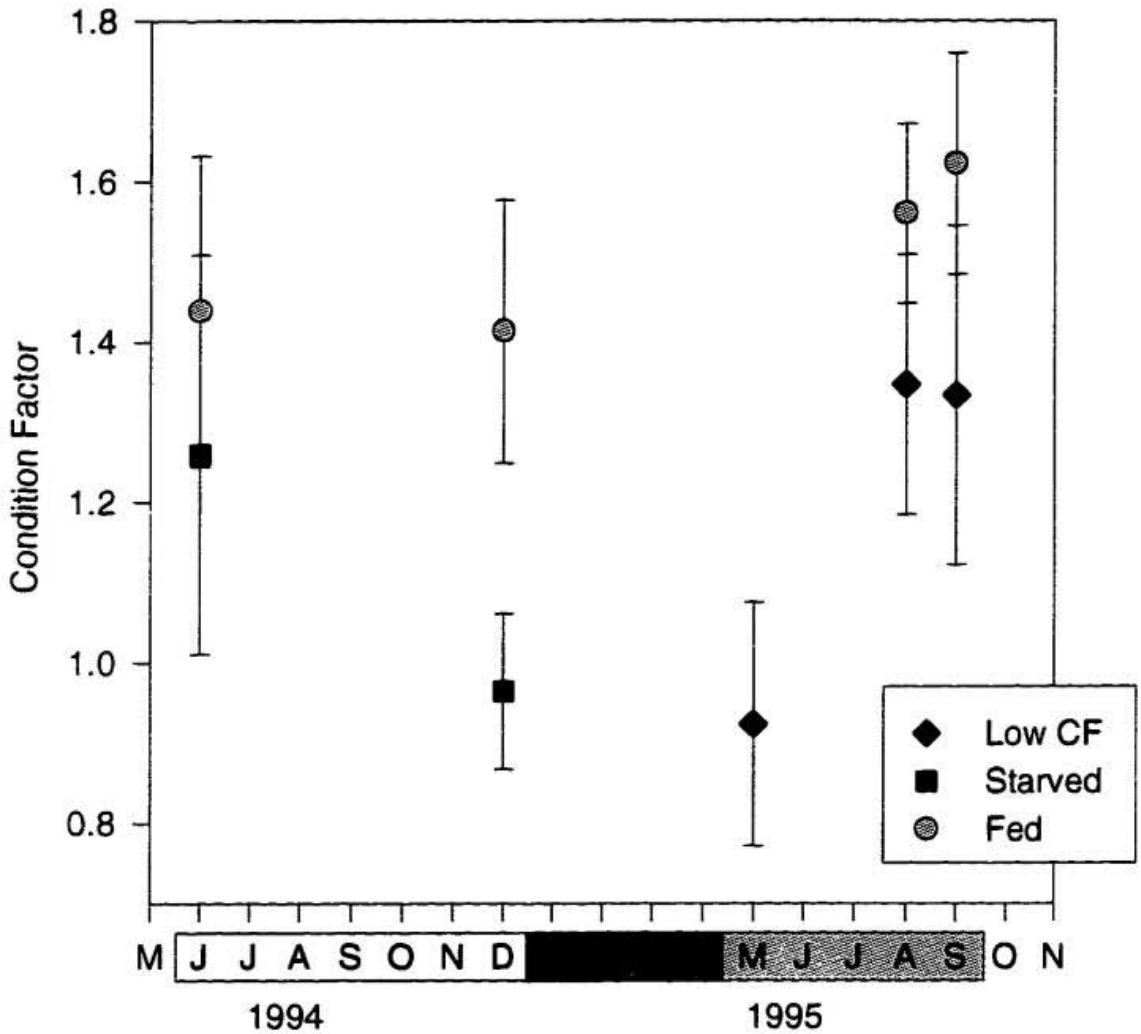

1.8
1.6
1.4
1.2
1.0
0.8
Condition Factor
Low CF
Starved
Fed
M J J A S O N D M J J A S O N
1994
1995

Figure 3.2: Moisture content of white muscle from winter flounder, *Pleuronectes americanus*, determined by biopsy and at termination. Biopsy followed experimental starvation or feeding and experimentally starved flounder were refed prior to termination. N starved= 4, N low CF= 5, N Fed= 5.

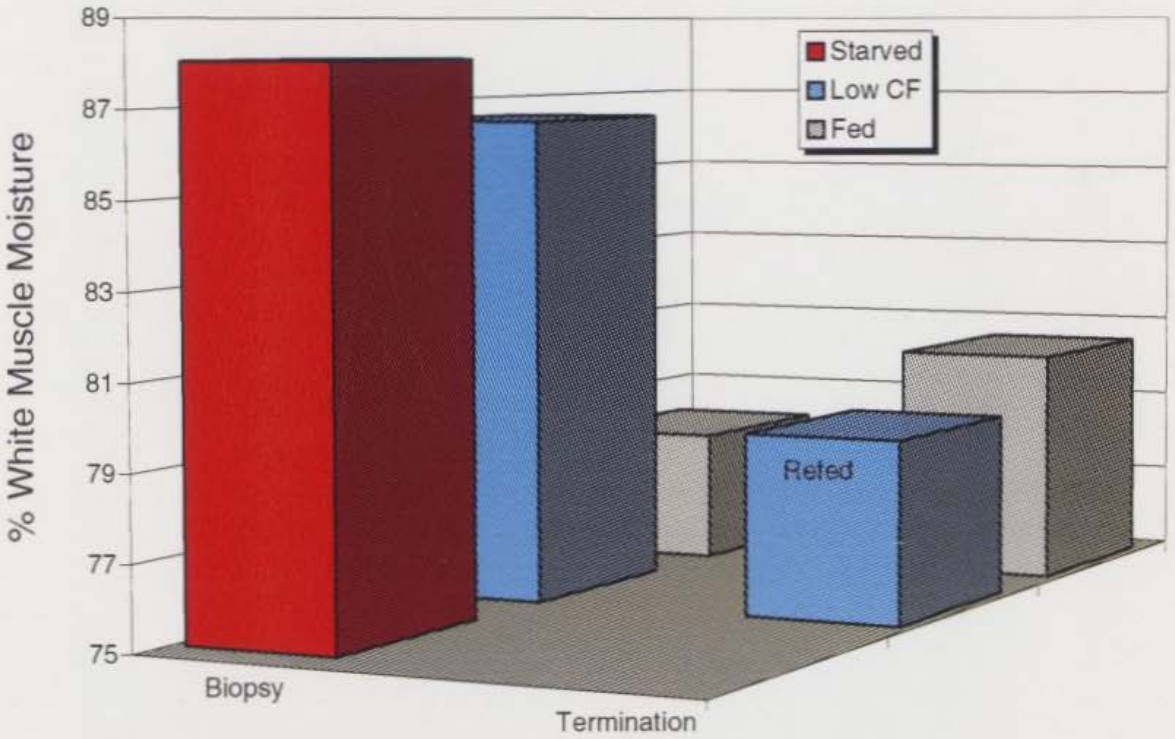

% White Muscle Moisture
89
87
85
83
81
79
77
75
Starved
Low CF
Fed
Refed
Biopsy
Termination

Figure 3.3: Relationship between white muscle water content and condition factor for experimentally starved and fed winter flounder, *Pleuronectes americanus*. Symbols represent values for one fish.

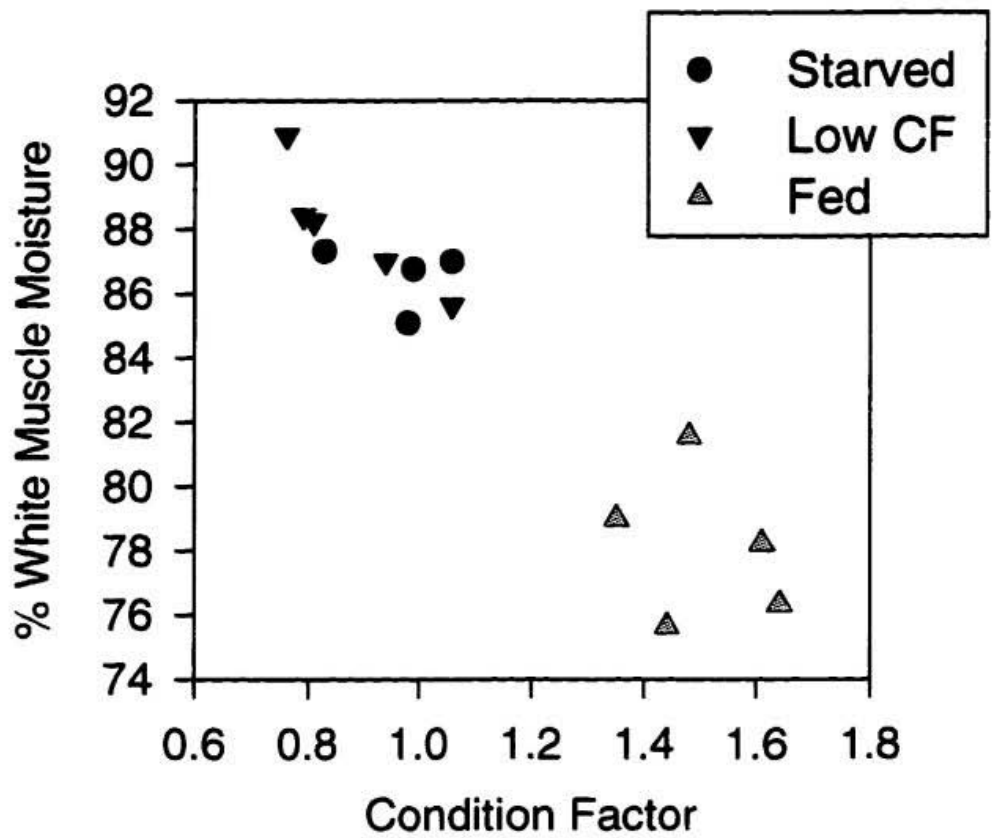
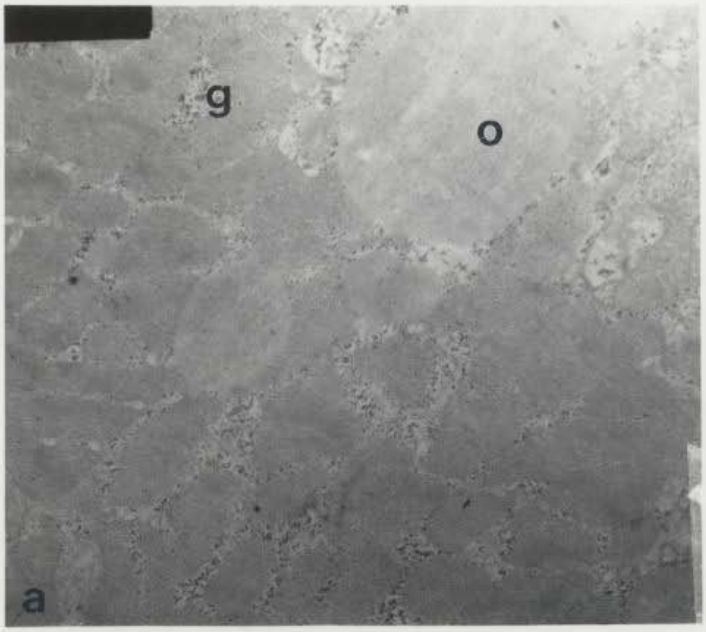

Starved
Low CF
Fed
% White Muscle Moisture
92
90
88
86
84
82
80
78
76
74
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Condition Factor

Figure 3.4: Ultrastructural cross section of white muscle from experimentally fed (a) and starved (b) winter flounder, *Pleuronectes americanus*. Glycogen (**g**) and oleosomes (**o**) are reduced in the starved state. Magnification at 10 575X.


g
o
a

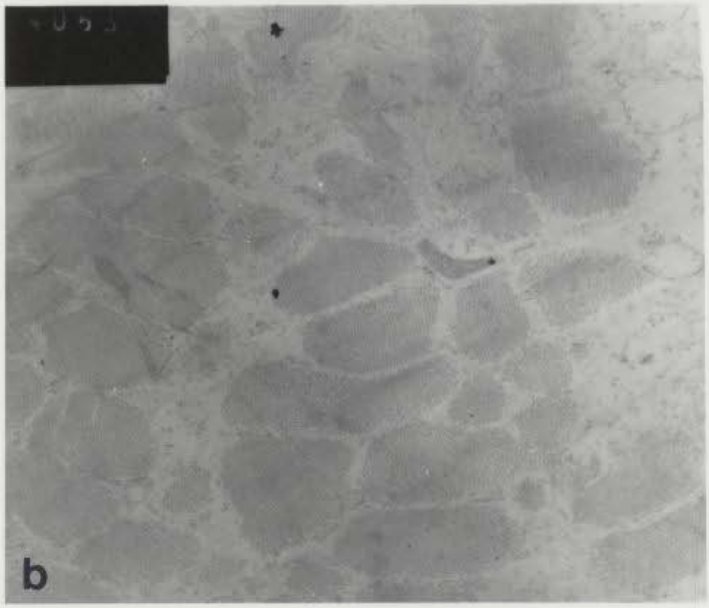

b

Figure 3.5: Oocytes biopsied from a recrudescent winter flounder, *Pleuronectes americanus*, showing both previtellogenic (**pv**) and exogenously vitellogenic (**v**) oocytes.

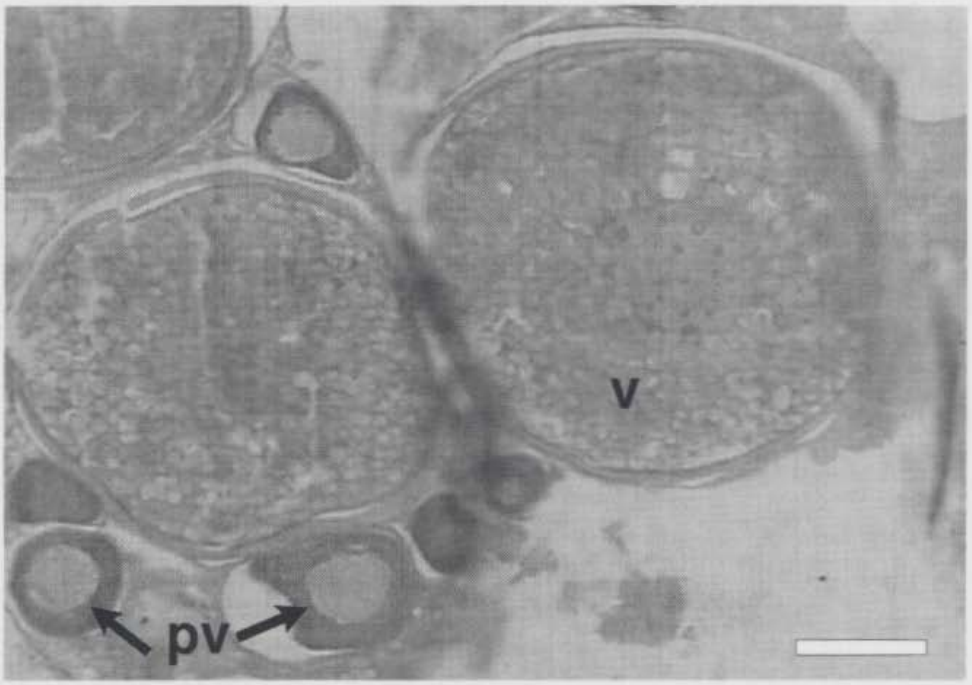

v
pv

Figure 3.6: Gonads from flounder, *Pleuronectes americanus*, starved and subsequently refed, show oocytes that appear to be developing for the coming spawning season. **pv** = previtellogenic oocytes, **ca** = cortical alveoli stage oocytes, **ev** = cells in exogenous vitellogenesis. (a) and (b) are ovaries from two starved flounder in slightly different stages of development.

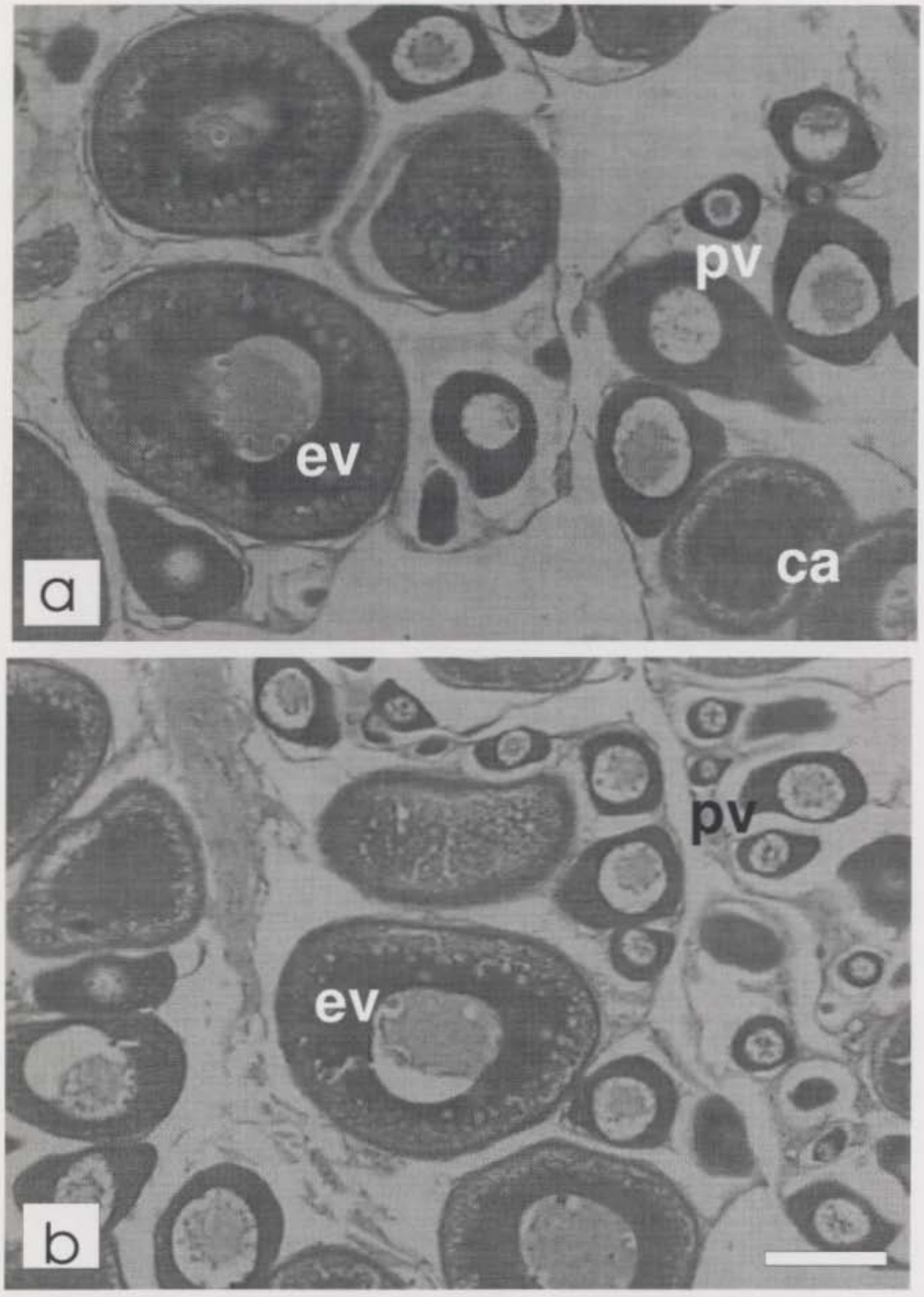
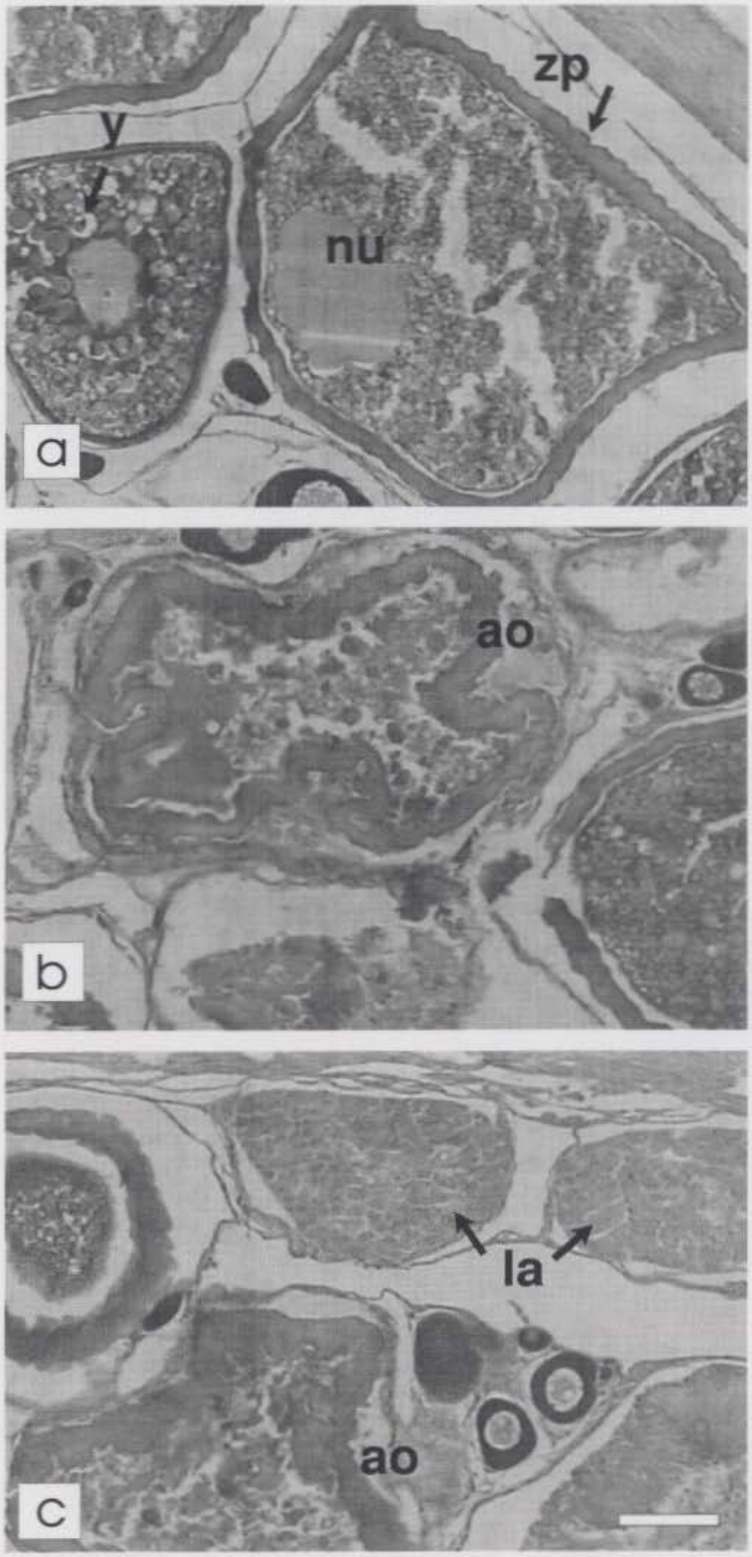

pv
ev
ca
a
pv
ev
b

Figure 3.7: Atretic resorption of yolky oocytes in experimental *Pleuronectes americanus*. (a) Gonad from a fed winter flounder showing advanced oocyte development with yolk deposition (**y**) and nuclear migration (**nu**). The beginning of atretic breakdown of the larger cell is indicated by the thickening of the zona pellucida. (b) An atretic oocyte (**ao**) in a more advanced state of degeneration with a convoluted chorion and yolk breakdown evident. (c) Late atresia (**la**) is characterized by complete yolk resorption.


zp
y
nu
a
ao
b
la
ao
c

Chapter 4

Preliminary investigation of sarcomere characteristics in *Pleuronectes americanus* and *Hippoglossoides platessoides* using glycerinated single fibre preparations: sarcomere length and contractile ability.

4.1 Abstract

Preliminary experiments indicate that contractile ability may be studied in glycerinated single fibres using light microscopy and video analysis to measure change in sarcomere length following addition of ATP and metal salts solution to induce contraction. In the fish species studied, sarcomeres are shorter than the 2.0-2.5μm reported for mammals. Muscle from American plaice, *H. platessoides*, had sarcomeres that measured 1.7μm maximum and, upon addition of ATP and metal salts solution, contracted to a minimum of 1.0μm. Fibres from winter flounder, *P. americanus*, measured approximately 1.4μm, confirmed with ultrastructural detail of the flounder sarcomere. Regional difference in sarcomere length is suggested, with sarcomeres at the ends of the fibres shorter than those in the middle. The ability of compromised fibres, from starved fish with high water content, to contract is still uncertain. Some fibres from starved fish showed contractile ability similar to fibres from fed fish. It was not possible to measure other starved fibres due to the lack of sarcomere resolution under the light microscope, presumably due to degradation of muscle protein in the starved state.

4.2 Introduction

Muscle is composed of bundles of fibres which, in turn, are made up of myofibrils. Each myofibril consists of protein filaments (actin, myosin and associated proteins) arranged to form repeating units called sarcomeres. It is the sarcomere that is the functional unit of contraction. The banding pattern observed in muscle fibres under the light microscope are the result of differences in refractive properties of different areas, or bands, of the sarcomere. The A- band is a dark band representing the overlap of actin and myosin filaments. The lighter areas that separate adjacent A- bands are known as I- bands. In the center of the I- band is the Z- line, a partition that defines the sarcomeres boundary.

Much of the base work for muscle structure and function was carried out using mammalian skeletal muscle. The frog sartorius and rabbit psoas were highly exploited for study since they were easily attained. The length of the sarcomere in resting muscle, likely referring to mammalian tissue, is given as approximately 2.5μm in Wilkie (1970) and for frog striated muscle in the resting state, the sarcomere length was reported to be between 2.0 and 2.25μm (Gordon *et al.* 1966). Experiments on fish suggest sarcomere lengths somewhat shorter than these values, with a maximum length of 1.82μm in white muscle from the coalfish, *Gadus virens* (Patterson and Goldspink 1972) and ultrastructural detail of *Cyprinus carpio* (Gas 1972) suggests sarcomere length considerably shorter than 2.5μm. Johnston and his co-workers, however, set the sarcomere length to 2.3μm for glycerinated

preparations used in contraction experiments on several species (Johnston and Altringham 1987, Johnston and Wokoma 1986, Johnston and Harrison 1985, Johnston and Salamonski 1984).

There is also an idea that sarcomere lengths are not consistent within a single fibre. Keynes and Aidley (1991) state that sarcomeres at the end of a fibre may "take up lengths different from those in the middle". The implication that terminal sarcomeres are different in length might influence sarcomere measurement and measurements, therefore, should keep as much as possible to consistent locations for comparison purposes.

The use of single fibres for contraction experiments is a useful tool for studying the possibility of conservation of contractile ability in cases of high white muscle moisture, when protein has been removed from the fibre. Glycerination removes the fibre membrane and allows the contractile mechanism to be engaged simply by the addition of adenosine triphosphate (ATP). The extracted fibres behave similar to live muscle with respect to contractile force, maximal shortening upon contraction, and other contractile properties (Rüegg 1971).

4.3 Materials and Methods

In January, one male American plaice (247g, 31.0cm) with a muscle hydration of 81.77%, collected as outlined in Chapter 1, was additionally sampled for single fibre experiments. Winter flounder muscle was biopsied from fish experimentally starved and fed as described in Chapter 3. White muscle was taken from site c (see Chapter 1) and placed in 50% glycerol which was kept in the freezer. A sample of white muscle from *Pleuronectes platessa* was also examined for comparison. Ultrastructure of starved and fed winter flounder muscle was examined following methods outlined in Chapter 2.

Single fibres were gently teased apart under a dissecting scope with dissecting needles and placed in 50% glycerol on a microscope slide onto which a coverslip was placed. The fibre was located with low power on the light microscope and magnification was increased to 1000x so that sarcomeres were distinguishable. For contraction studies, 20 to 25 sarcomeres in the center of the fibre were measured. The microscope was linked through a video camera to an image capturing board in a computer. The image could be viewed live on the monitor and captured or grabbed for measurement using Mocha video analysis®.

To study the ability of fibres to contract, ATP and metal salts solution (0.25% ATP + 0.05M KCl + 0.01M $MgCl_2$) was dropped onto the slide next to the coverslip while the fibre was still in view on the monitor, and a small square of paper towel was placed on the opposite edge of the coverslip to draw the liquid across the slide

and into contact with the fibre. Computerized images of the sarcomeres were captured using a Bravado® image capturing board before addition of ATP and metal salts and at intervals following the treatment.

To determine if sarcomere length was consistent over the length of the fibre, sarcomeres were measured in the middle and towards the insertion in 20 fibres from winter flounder, *P. americanus*.

Measurements of sarcomere length were made using Mocha® video analysis software through Sony® video link to the microscope. The sarcomere length was defined as the distance between neighboring light bands as viewed under high power (1000x) of a Zeiss light microscope. Comparisons were made using the Student's *t*-test with a level of significance of 0.05. Images were captured and printed as described in Chapter 1. Sarcomere length was also calculated from ultrastructural detail of white muscle in longitudinal section. The length of the myosin was used to calculate resting sarcomere length based on proportions given in Gordon *et al.* (1966).

4.4 Results

Figure 4.1a depicts the sarcomere from a white muscle fibre of an American plaice as seen under high power (1000x), before and after contraction. The dark band is thought to represent the myosin/actin overlap and the sarcomere length is measured from the centre of one light band to the centre of the neighbouring light band. In comparison, a fibre from rabbit psoas muscle (Figure 4.1b) shows sarcomeres that are larger, with mean of 2.47μm, and the banding patterns are more distinct. Fibres from *P. platessa* were also measured and showed sarcomeres that were somewhat larger than American plaice or winter flounder, with an mean uncontracted length of 1.97μm and a mean contracted length of 1.57μm.

Figure 4.2 shows the change over time of sarcomere length following addition of ATP and metal salts solution to fibres from American plaice. Fibres were observed to contract from 1.7μm and 1.6μm to 1.35μm and 1.2μm respectively while a third trial showed initial sarcomere length consistent with contracted measurements. In one trial sarcomere length appeared to increase again at 12 minutes following initial stimulation. A second treatment of ATP and metal salts at 16 minutes in two trials did not appear to affect sarcomere length further and at 24 minutes in the third trial was also ineffective.

Ultrastructural detail (Figure 4.3) of winter flounder white muscle shows sarcomere length to be approximately 1.4μm.

Sarcomeres at the fibre insertion were found to be shorter ($p=0.03$) in winter

flounder, *P. americanus*, than those in the middle of the fibre, based on measurement of 15 to 20 sarcomeres at each location from 20 fibres.

Fibres from starved and fed winter flounder were examined for contractile ability. One flounder with a white muscle moisture content of 83.57% showed significant contraction ($p=0.025$) following addition of ATP and metal salts, from a mean resting sarcomere length of 1.43μm to a mean contracted length of 1.18μm ($n=10$). A starved flounder, with a white muscle water content of 88.94%, did show contractile ability of the sarcomere, but the initial mean resting sarcomere length measured 1.32μm and contraction resulted in a shortening to a mean length of 1.21μm. Other fibres from starved flounder could not be measured since the sarcomeres were not distinguishable.

4.5 Discussion

These preliminary experiments on sarcomere characteristics and contractile ability of single fibres from *H. platessoides* and *P. americanus* show that it is possible to induce contraction in isolated glycerinated preparations and observe individual sarcomeres before and after contraction. It was found that sarcomeres are shorter in *H. platessoides* and *P. americanus* than in mammalian muscle, measuring 1.4 to 1.7μm in the isolated preparations. This agrees with work on the coalfish, *Gadus virens* (Patterson and Goldspink 1972) and the carp, *Cyprinus carpo* (Gas 1972) which also showed sarcomeres shorter than in mammalian muscle. Using the length of the myosin filament, measured in ultrastructural preparation, it is possible to calculate the resting length of the sarcomere of the winter flounder based on the proportions given in Gordon *et al.* (1966). This calculation takes the possibility of contraction prior to preparation into account, but assumes that similar proportions exist between mammalian sarcomeres and those of the species under study. A calculated length of 1.4μm agrees strongly with measurements taken from the glycerinated preparations. Contraction trials on a fibre from *P. platessa* showed sarcomere length to be somewhat larger than winter flounder or American plaice, with a resting length of 1.97μm and contracted length of 1.57μm.

It must be noted that fibres were assumed to be relaxed but it is possible that mechanical stimulation of the fibres during separation from surrounding tissue caused some degree of contraction. To try to minimize the effect of this, fibres with

maximum sarcomere length were chosen for contraction trials. This may introduce a bias into an investigation, but it allows the investigator to observe the ability of apparently relaxed fibres to contract upon addition of stimulant.

The difference in sarcomere length between central and terminal muscle fibre regions should also be kept in mind when measuring contraction. As suggested by Keynes and Aidley (1991), the sarcomeres at the end of fibres from winter flounder, *P. americanus*, were shorter than those in the middle. In this study, sarcomeres were observed in the center of the fibre to reduce the effect of this additional variable.

Some fibres from starved winter flounder, with high water content, were observed to contract, but not to the same degree as fibres from fed fish, which were considerably less hydrated. Percent contraction of fibres from low water content fish was calculated to be 17.5% while a fibre with higher moisture experienced only 8.3% contraction. The low number of fibres measured was due to the scarcity of fibres in suitable condition for measurement, and perhaps the measurable fibres were partially contracted prior to addition of the contractile stimulant. Other fibres from starved flounder could not be measured since the sarcomeres could not be distinguished under the light microscope. This is probably the result of protein depletion from the fibres due to energetic demands imposed by starvation. The inability to observe cellular detail may be the direct result of protein loss, or it may reflect the more fragile condition of the fibre as the result of myofibrillar degradation.

The process of separating single fibres may have caused the damage observed, but it is still evidence of the breakdown of muscle integrity, since it is apparent that fibres from fish with lower water content do not suffer the same damage. The fact that some fibres did contract and had maintained integrity is consistent with the idea that some areas of white muscle may be conserved in high water states.

These observations are preliminary in answering questions concerning the contractile properties of fibres which may be compromised due to energetic removal of protein for reproductive development or survival. It is apparent, however, that experiments using glycerinated single fibres, which may be biopsied from fish under long term study, may provide insight into the use of white muscle protein as an energy source. Although measurement of sarcomere contraction does not seem feasible in the case of compromised tissue, perhaps an approach that measured contractile force would shed light on the ability of compromised muscle to function, even in a reduced capacity, to minimize the detrimental effects of protein depletion from white muscle.

Figure 4.1: Sarcomeres from American plaice (a) and rabbit psoas (b) viewed under the light microscope. Inset in (a) was captured following addition of ATP and metal salts solution which caused contraction. Arrows denote sarcomere length.

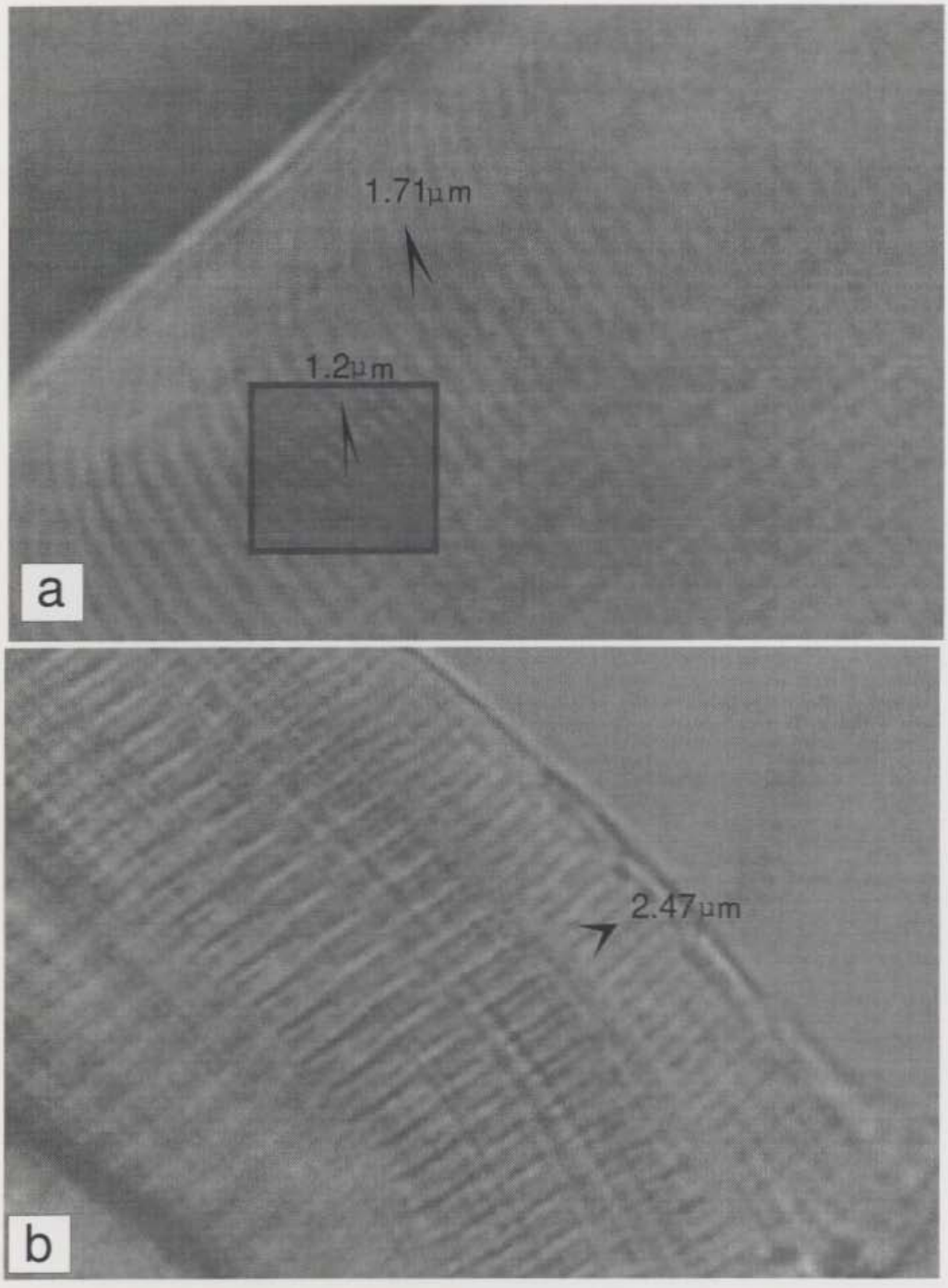

1.71μm
1.2μm
a
2.47μm
b

Figure 4.2: Graph showing change in sarcomere length following addition of ATP and Metal Salts solution to isolated, glycerinated muscle fibres from *Pleuronectes americanus*. ATP and metal salts solution was added at time 0 and images were grabbed (captured) every 2 or 4 minutes thereafter for measurement purposes. Each plot denotes a separate experimental trial conducted on a single fibre.

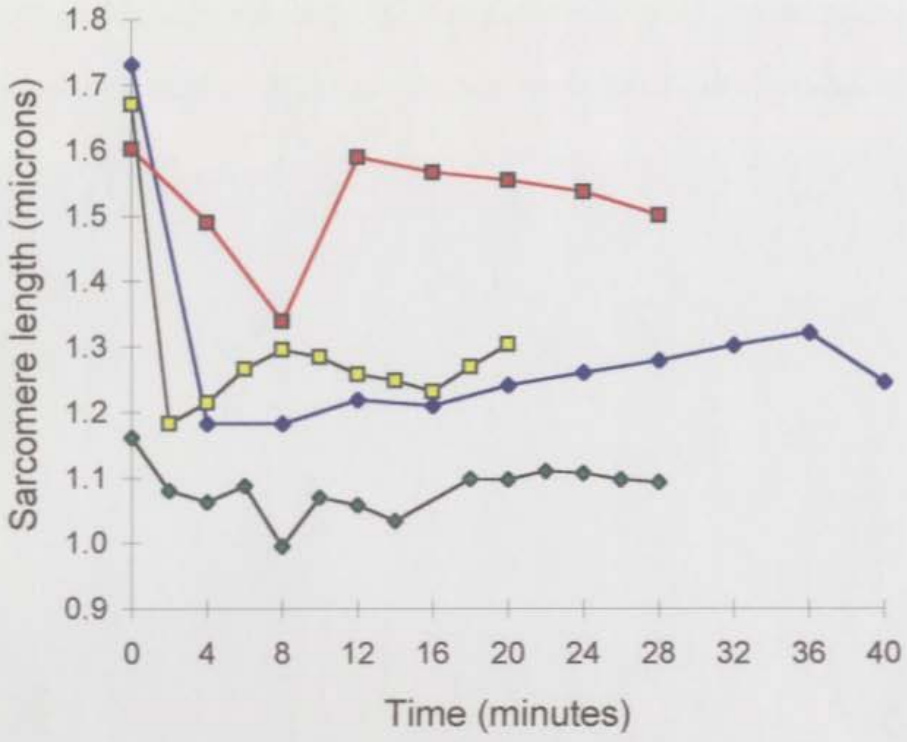
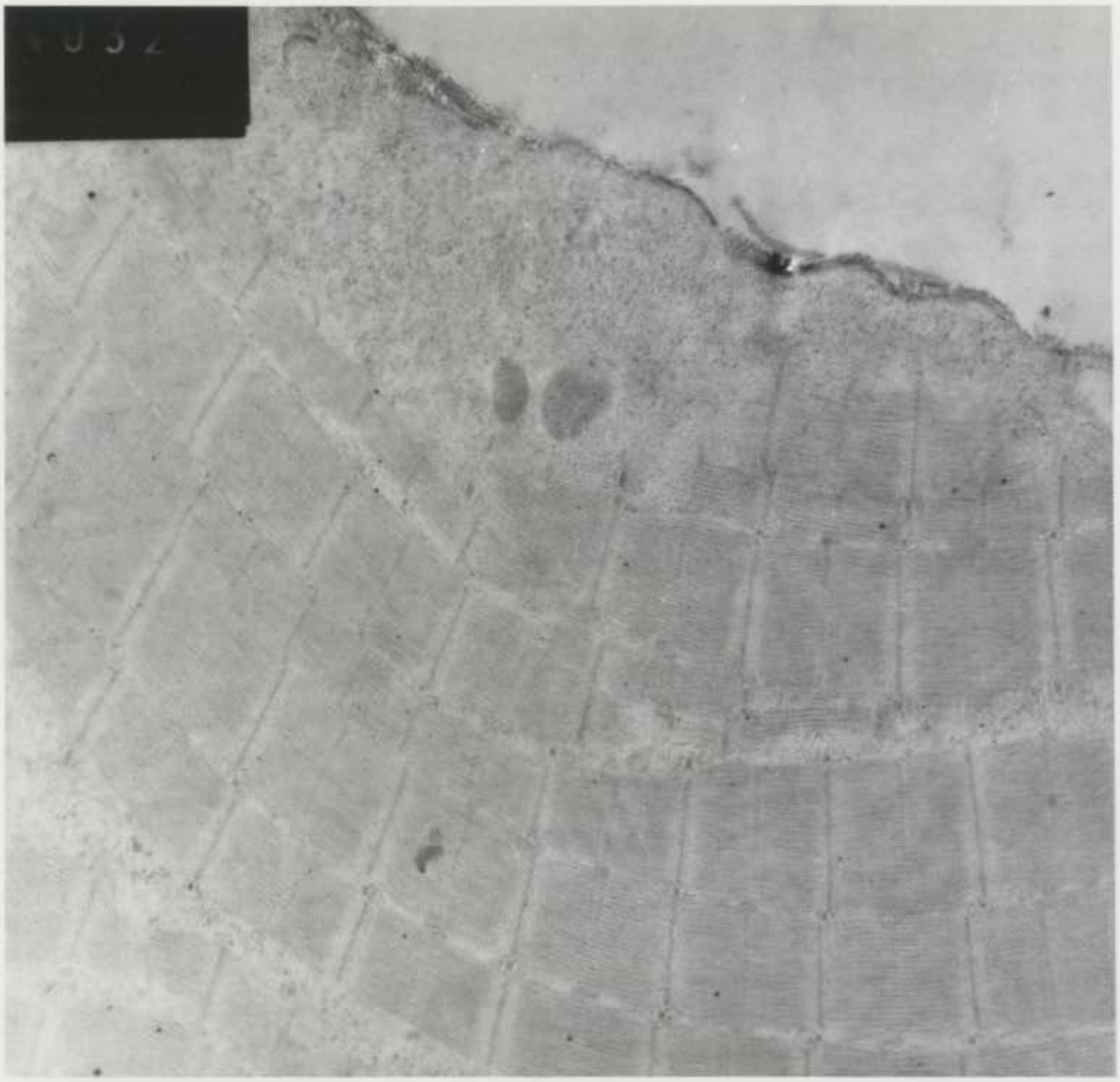

Sarcomere length (microns)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0
4
8
12
16
20
24
28
32
36
40
Time (minutes)

Figure 4.3. Longitudinal section of white muscle from winter flounder, *Pleuronectes americanus*, viewed under the transmission electron microscope. Sample was taken from the central region of the fibre. Magnification = 17003X.



Conclusions

The seasonal variation observed in white muscle water content in American plaice, *Hippoglossoides platessoides*, is likely due to the spawning strategy undertaken in this species. In contrast to winter flounder, *Pleuronectes americanus*, which spawn all oocytes in a very short period, the American plaice experience a protracted spawning season, releasing batches of eggs at intervals over a period of several weeks. It may be the energy required to complete batch development and hydration which demands the use of white muscle protein as an energy source, or it may be due to the growth of oocytes from a previtellogenic state to exogenous vitellogenesis during, or shortly after, the spawning season.

The question of the fate of these vitellogenic oocytes present in the gonad during spawning season has not been fully resolved. They may be the result of development for next year's spawning, or they may represent late batches being rapidly pushed through vitellogenesis for release during the current season. This development is an energetically demanding task and is probably the main source of protein depletion from white muscle during the summer months. The characterization of the postspawned gonad would shed some light on this issue. If the completely spawned female shows a gonad with only immature, previtellogenic oocytes, and there is no evidence of recent spawning, then it may be concluded that fast tracking of oocytes is possible. If this is the case, it would have serious implications in the estimation of fecundity and the ability to classify fish as

determinate or indeterminate spawners. Using the method of oocyte size distribution to determine spawning strategy might overlook the possibility that fish may be capable of increasing their reproductive output during the spawning strategy based on somatic condition and energy reserves, including white muscle protein. Based on oocyte size classes, Zamarro (1992b) classified American plaice as having determinate fecundity. If they, instead, increase their reproductive yield during the spawning season, then their fecundity may better be described as quasi-determinate (semi-determinate?) with a minimum fecundity determined prior to the spawning season and late oocyte batches developed as somatic energy reserves permit.

Experimental starvation of American plaice did not produce differences in white muscle moisture and condition which might be expected if the starved group was not able to meet somatic or reproductive demands for energy. The inability to maintain experimental water temperatures at ambient bottom temperatures may account for the unexpected but interesting findings outlined in Chapter 2. Condition of the fish upon entering compromising situations is important in the individual's ability to maintain muscle protein stores. American plaice seem to deal with the energetic pressure imposed by unusually high temperatures by shutting down reproductive development if condition is poor, thereby reducing energy requirements. This strategy allows conservation of white muscle protein which may be better allocated into surviving the period of stress. It appears that a critical period is involved in the ability of plaice to restrict oocyte maturation. If gonad development

has already been initiated prior to the critical period then maturation will continue and white muscle protein will be utilized. Energy can be recovered, however, in these cases, through resorption of vitellogenic oocytes. Atresia is also noted in previtellogenic oocytes and represents an effort to recover as much energy as possible to deal with energetic constraints. The ability of some plaice to forego reproductive development has implications for natural populations and should be considered when assessing the potential fecundity of a population.

Starvation induced high white muscle water content is a recoverable situation in winter flounder, *Pleuronectes americanus*, through refeeding. The ability of flounder to improve condition, replenish protein and continue with reproductive development despite poor condition and reproductive failure the previous season, suggests that the utilization of muscle protein as an energy store is a normal and reversible process. Natural use of white muscle protein as an energy source might result from a poor feeding season preceding the natural winter fast experienced in this species.

Preliminary experiments on contractile ability of glycerinated single fibres from *H. platessoides* and *P. americanus* were encouraging. Contraction of single fibres can be observed, and change in sarcomere length measured, following addition of a contractile stimulant such as ATP and metal salts. Sarcomeres in plaice and flounder were found to be shorter than those of mammalian striated muscle, and terminal sarcomeres were shorter than their central counterparts. The

question of the ability of compromised fibres to contract could not be answered directly, but observations suggest that sarcomere structure is disrupted or weakened, possibly leading to damage upon dissection. It was found that some fibres from high moisture muscle could contract, adding strength to the idea that conservation of individual fibres or motor units might lessen the detrimental effects of protein depletion and provide the framework on which recovering muscle would be rebuilt once energy surplus was experienced. The next step might be to measure contractile strength of individual or groups of fibres to determine if high moisture muscle retains any contractile ability, even if a particular fibre has experienced protein removal.

White muscle seems to be an energy store which may be tapped in times of energetic demand. The demand may result from reproductive need to push oocytes through vitellogenesis in a relatively short period, or it may be due to nutritional deprivation and ensuing survival requirements for energy. The ability to shut down reproductive development is reported for winter flounder (Burton and Idler 1987) and is suggested here, as well, for American plaice, *H. platessoides*, as a means of conserving energy. If energetic needs are not met by this strategy, somatic reserves of protein are available to sustain the animal until conditions improve. Conservation of discreet areas of muscle is possible, as is conservation of fibres in a more distributive pattern to maintain some degree of muscle tension and provide a framework against which recovering muscle may build.

References

Bagenal, T.B. 1957. The breeding and fecundity of the long rough dab *Hippoglossoides platessoides* (Fabr.) and the associated cycle in condition. J. mar. biol. Ass. U.K. 36:339-375.

Barr, W.A. 1963. The endocrine control of sexual cycle in the plaice, *Pleuronectes platessa* L. I.Cyclical changes in the normal ovary. Gen. Comp. Endocrinol. 3:197-204.

Beardall, C.H. and Johnston, I.A. 1983. Muscle atrophy during starvation in a marine teleost. Eur. J. Cell Biol. 29:209-217.

Black, D. and Love, M. 1986. The sequential mobilisation of energy reserves in tissues of the Atlantic cod during starvation and refeeding. J. Comp. Physiol. B 156:469-479.

Bradford, R.G. 1993. Differential utilization of storage lipids and storage proteins by Northwest Atlantic herring *(Clupea harengus harengus*). J. Fish Biol. 43:811-824.

Burton, M.P.M. 1994. A critical period for nutritional control of early gametogenesis in female winter flounder, *Pleuronectes americanus*. J. Zool. Lond. 233:405-415.

Burton, M.P., and Idler, D.R. 1984. The reproductive cycle in winter flounder, *Pseudopleuronectes americanus* (Walbaum). Can. J. Zool. 62:2563-2567.

Burton, M.P., and Idler, D.R. 1987. Variability of reproductive status in a population of *Pseudopleuronectes americanus*. Proc. V Congr. europ. Ichthyol., Stolkholm 1985, pp. 207-212.

Burton, M.P.M., Penney, R.M. and Biddiscombe, S. *in press*. Time course of gametogenesis in Northwest Atlantic cod (*Gadus morhua*). Can. J. Fish. Aquat. Sci. 54.

Dawson, A.S., and Grimm, A.S. 1980. Quantitative seasonal changes in the protein, lipid and energy content of the carcass, ovaries and liver of adult female plaice, *Pleuronectes platessa* L.. J. Fish Biol. 16:493-504.

Federov, K.Y. 1971. The state of the gonads of the Barents Sea Greenland halibut [*Reinhardtius hippoglossoides* (Walb.)] in connection with failure to spawn. J. Ichthyol. 11:673-682.

Fisher, R.A., Fritzche, R.A., and Hendrickson, G.L. 1987. Histology and ultrastructure of the 'jellied' condition in Dover sole, *Microstomus pacificus*. Proc. V Congr. europ. Ichthyol., Stolkholm 1985, pp. 345-350.

Fletcher, G.L., and King, M.J. 1978. Seasonal dynamics of Cu^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} in gonads of winter flounder (*Pleuronectes americanus*): evidence for summer storage of Zn^{2+} for winter gonad development in females. Can. J. Zool. 56:284-290.

Foucher, R.P. and Beamish, R.J. 1977. A review of oocyte development in fishes with special reference to Pacific Hake (*Merluccius productus*). Fish. Mar. Serv. Tech. Rep. 755:16p.

Gas, N. 1972. Altérations structurales du muscle blanc de Carpe (*Cyprinus carpio* L.) au cours du jeûne prolongé. C.R. Acad. Sc. Paris 275:1404-1406.

Gordon, A.M., Huxley, A.F. and Julian, F.J. 1966. The variation in isomeric tension with sarcomere length in vertebrate muscle fibres. J. Physiol., London 184:170-192.

Greer Walker, M. 1971. Effect of starvation and excercise on the skeletal muscle fibres of the cod (*Gadus morhua* L.) and the coalfish (*Gadus virens* L.) respectively. J. Cons. int. Explor. Mer. 33:421-426.

Greer-Walker, M. and Pull, G.A. 1975. A survey of red and white muscle in marine fish. J. Fish Biol. 7:295-300.

Horwood, J.W. 1990. Fecundity and maturity of plaice (*Pleuronectes platessa*) from Cardigan Bay. J. Mar. biol. Ass. U.K. 70:515-529.

Horwood, J.W.,Greer-Walker M., and Witthames, P. 1989. The effect of feeding levels on the fecundity of plaice (*Pleuronectes platessoides*). J. Mar. biol. Ass. U.K. 69:81-92.

Htun-Han, M. 1978. The reproductive biology of the dab *Limanda limanda* (L.) in the North Sea: seasonal changes in the ovary. J. Fish Biol. 13:351-359.

Hunter, J.R. and Leong, R. 1981. The spawning energetics of female northern anchovy, *Engraulis mordax*. Fish. Bull. (U.S.) 79:215-230.Patterson, S. and Goldspink, G. 1972. The fine structure of red and white myotomal muscle fibres of the coalfish (*Gadus virens*). Z. Zellforsch. 133:463-474.

Jobling, M. 1980. Effects of starvation on proximate chemical composition and energy utilization of plaice, *Pleuronectes platessa* L. J. Fish Biol. 17:325-334.

Jobling, M. and Spencer Davies, P. 1980. Effects of feeding on metabolic rate, and the Specific Dynamic Action in plaice, *Pleuronectes platessa*. J. Fish Biol. 16:629-638.

Johnston, I.A. 1981. Quantitative analysis of muscle breakdown during starvation in the marine flatfish *Pleuronectes platessa*. Cell Tissue Res. 214:369-386.

Johnston, I.A. and Altringham, J.D. 1988. Muscle contraction in polar fishes: experiments with demembranated muscle fibres. Comp. Biochem. Physiol. 90B(3):547-555.

Johnston, I.A., and Goldspink, G. 1973. Some effects of prolonged starvation on the metabolism of the red and white myotomal muscles of the plaice *Pleuronectes platessa*. Mar. Biol. 19:348-353.

Johnston, I.A. and Harrison, P. 1985. Contractile and metabolic characteristics of muscle fibres from Antarctic fish. J. exp. Biol. 116:223-236.

Johnston, I.A. and Salomonski, J. 1984. Power output and force-velocity relationship of red and white muscle from the Pacific blue marlin (*Makaira nigricans*). J. exp. Biol. 111:171-177.

Johnston, I.A. and Wokoma, A. 1986. Effects of temperature and thermal acclimation on contractile properties and metabolism of skeletal muscle in the flounder (*Platichthys flesus* L.). J. exp. Biol. 120:119-130.

Keissling, A., Hung, S.S.O., and Storebakken, T. 1993. Differences in protein mobilization between ventral and dorsal parts of white epaxial muscle from fed, fasted and refed white sturgeon (*Acipenser transmontanus*). J. Fish Biol. 43:401-408.

Kennedy, V.S., and Steele, D.H. 1971. The winter flounder (*Pseudopleuronectes americanus*) in Long Pond, Conception Bay, Newfoundland. J. Fish. Res. Board Can. 28:1153-1165.

Keynes, R.D. and Aidley, D.J. 1991. Nerve and Muscle second edition. Cambridge University Press, Cambridge.

Khoo, K.H. 1979. The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. Can. J. Zool. 57:617-626.

Kiran, S. and Talesara, C.L. 1985. Prolonged starvation effects on myotomal muscles of two freshwater teleost fishes- Murrel (*Channa punctata*) and Catfish (*Clarias batrachus*). Ind. J. Exp. Biol. 23:17-22.

Kjesbu, O.S. 1989. The spawning activity of cod, *Gadus morhua* L.. J. Fish Biol. 34:195-206.

Kjesbu, O.S., Klungsøyr, J., Kryvi, H., Witthames, P. and Greer Walker, M. 1991. Fecundity, atresia, and egg size of captive Atlantic cod (*Gadus morha*) in relation to proximate body composition. Can. J. Fish. Aquat. Sci. 48:2333-2343.

Kruse, G.H. and Tyler, A.V. 1983. Simulation of temperature and upwelling effects on the English sole (*Parophrys vetulus*) spawning season. Can. J. Fish. Aquat. Sci. 40:230-237.

Love, R.M. 1958. Studies on the North Sea cod. III. Effects of starvation. J. Sci. Fd. Agric. 9:617-620.

Love, R.M. 1980. The Chemical Biology of Fishes, Vol. 2: Advances 1968-1977. Academic Press, Toronto.

Maddock, D.M. amd Burton, M.P.M. 1994. Some effects of starvation on the lipid and muscle layers of the winter flounder, *Pleuronectes americanus*. Can. J. Zool. 72:1672-1679.

Mair, G.C. 1989. A technique for sampling muscle from live fish. J. Fish Biol. 35:159-160.

May, A.W. 1967. Fecundity of Atlantic cod. J. Fish. Res. Bd Canada 24(7).

McEvoy, L.A. and McEvoy, J. 1992. Multiple spawning in several commercial fish species and its consequences for fisheries management, cultivation, and experimentation. J. Fish Biol. 41(B):125-136.

McLeese, J.M., and Moon, T.W. 1989. Seasonal changes in the intestinal mucosa of winter flounder, *Pseudopleuronectes americanus* (Walbaum), from Passamaquoddy Bay, New Brunswick. J. Fish Biol. 35:381-393.

Morgan, M.J. 1993. Ration level and temperature preference of American plaice. Mar. Behav. Physiol. 24:117-122.

N'Da, K. and Déneil, C. 1993. Sexual cycle and seasonal changes in the ovary of the red mullet, *Mullus surmuletus*, from the southern coast of Brittany. J. Fish Biol. 43:229-244.

Patterson, S. and Golkdspink, G. 1972. The fine structure of red and white myotomal muscle fibres of the coalfish (*Gadus virens*). Z. Zellforsch. 133:463-474.

Pearcy, W.G. 1961. Seasonal changes in osmotic pressure of flounder sera. Science 134:193-194

Pitt, T.K. 1966. Sexual maturity and spawning of the American plaice, *Hippoglossoides platessoides* (Fabricius), from Newfoundland and Grand Bank areas. J. Fish. Res. Bd Can. 23(5):651-672.

Rickey, M.H. 1995. Maturity, spawning and seasonal movement of arrowtooth flounder, *Atheresthes stomias*, off Washington. Fish. Bull. 93:127-138.

Roff, D.A. 1982. Reproductive strategies in flatfish: a first synthesis. Can. J. Fish. Aquat. Sci. 39:1686-1698.

Rüegg, J.C. 1971. Experiments on glycerinated muscle fibres. Exp. in Physiol. and Biochem. 4:203-217.

Scott, J.S. 1982. Depth, temperature and salinity preferences of common fishes of the Scotian Shelf. J. Northw. Atl. Fish. Sci. 3:29-39.

Scott, W.B. and Scott, M.G. 1988. Atlantic Fishes of Canada. Can. Bull. Fish. Aquat. Sci. 219:731p.

Stirling, H.P. 1976. Effects of experimental feeding and starvation on the proximate composition of the European bass *Dicentrarchus labrax*. Mar. Biol. 34:85-91.

Templeman, W., and Andrews, G.L. 1956. Jellied condition in the American plaice *Hippoglossoides platessoides* (Fabricius). J. Fish. Res. Board Can. 13:147-182.

Tyler, A.V., and Dunn, R.S. 1976. Ration, growth, and measures of somatic and organ condition in relation to meal frequenc in winter flounder, *Pseudopleuronectes americanus*, with hypotheses regarding population homeostasis. J. Fish. Res. Board Can. 33:63-75.

Urban, J. and Alheit, J. 1988. Oocyte development and cycle of plaice, *Pleuronectes platessa*, and North Sea sole, *Solea solea*. ICES C.M. 1988/G:52 Demersal Fish Committee.

West, G. 1990. Methods of assessing ovarian development in fishes: a review. Aust. J. Mar. Freshwater Res. 41:199-222.

Wilkie, D.R. 1970. The Institute of Biology's Studies in Biology no. 11: Muscle. William Clowes and Sons Ltd., London.

Wilson, C.A. and Neiland, D.L. 1994. Reproductive biology of the red drum, *Sciaenops ocellatus*, from the neritic waters of the northern Gulf of Mexico. Fish. Bull. 92:841-850.

Witthames, P.R. and Greer Walker, M. 1995. Determinancy of fecundity and atresia in sole (*Solea solea*) from the Channel, the North Sea and the Irish Sea. Aquat. Living Resour. 8:91-109.

Yamamoto, K. 1956. Studies on the formation of fish eggs. I. Annual cycle in the development of ovarian eggs in the flounder, *Liopsetta obscura*. J. Fac. Sci. Hokkaido Univ. Ser. VI. 12:362-374.

Zamarro, J. 1992a. Feeding behavior of the American plaice (*Hippoglossoides platessoides*) on the southern Grand Banks of Newfoundland. Netherlands Journal of Sea Research 29(1-3):229-238.

Zamarro, J. 1992b. Determination of fecundity in American plaice (*Hippoglossoides platessoides*) and its variation from 1987 to 1989 on the tail of the Grand Banks. Netherlands Journal of Sea Research 29:205-209.