THE DEVELOPMENT OF EMBRYOS AND LARVAE OF THE ATLANTIC COD, Gadus morhua, WITH PARTICULAR EMPHASIS ON THE ONTOGENY OF CHLORIDE CELLS

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THE DEVELOPMENT OF EMBRYOS AND LARVAE OF THE ATLANTIC COD, *Gadus morhua*, WITH PARTICULAR EMPHASIS ON THE ONTOGENY OF CHLORIDE CELLS.

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ABSTRACT

Chloride cells are mitochondria-rich ion-transporting cells (ionocytes) that secrete chloride from epithelial surfaces. The origin and differentiation of presumptive chloride-secreting cells in Atlantic cod (Gadus morhua) were studied by a combination of light and electron microscopy.

By examination of available literature data, in conjunction with data collected from the present study, estimates of the effect of temperature on hatching time in the Atlantic cod were made. Subsequently, methods for the localization of chloride cells were developed, and compared with classical methods of staining ionocytes. Ionocytes were stained using conventional mitochondrial dyes, potential-sensitive fluorescent mitochondrial dyes (including styryl and rhodamine dyes, and a novel aldehyde-fixable rhodamine derivative), a fluorescent probe for the Na^+, K^+-ATPase (the ouabain derivative, anthroyl-ouabain), a cardiolipin-specific probe (nonyl-acridine orange), tetracycline, a lipophilic membrane probe ($DiOC_6(3)$), and conventional precipitation methods for ion localization.

Staining methods were used to examine the gradual differentiation of ionocytes from mitochondria-rich precursor cells. This information was applied to the analysis of chloride cell differentiation from the embryonic stages through to maturity.

The development of Atlantic cod embryos and larvae was then observed under various salinity and temperature conditions, and in the presence of hormone or vitamin-supplemented media, to determine the potential role of exogenous factors on the timing of chloride cell differentiation.

A variety of secretory cells, including sacciform, goblet and ionocytes, developed in the epidermis of cod embryos and larvae. In the embryo, ionocytes first appeared on the yolk sac and buccal/gill (oropharyngeal) cavity epithelium. Such cells were lenticular, and often had a large multilobed nucleus. Some appeared to be binucleate. Beyond yolk sac absorption, ionocytes appeared on the gill filament epithelium, and were retained on the oropharyngeal epithelia, but not the abdominal region. In postmetamorphic stages, oropharyngeal ionocytes become elongate and traversed epithelia that were several cells thick.

Although some experimental treatments delayed or accelerated rates of embryo development, yolk sac chloride cell differentiation appeared to occur after epiboly completion in all cases. The pattern of distribution of chloride cells in the later embryonic and larval stages appeared to be a result of migration of undifferentiated

cells over the surface of the yolk sac and into the pharyngeal cavity. This migration probably occurs through pharyngeal 'pores', the embryonic precursors of the openings to the adult gill chambers, and is ultimately responsible for the recruitment of epithelial cells (including chloride cells) by the developing gill filaments.

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Seasonal seawater temperature data were provided by Ross Wilson, and salinity standards were supplied by Jack Foley.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CMTR-H_2	Chloromethylated tetramethylrosamine (reduced form)
CMXR	Chloromethylated X-rosamine (oxidized form)
CMXR-H_2	Chloromethylated X-rosamine (reduced form)
DASPEI	Dimethylaminostyrylethylpyridiniumiodine
DASPMI	Dimethylaminostyrylmethylpyridiniumiodine
$DiOC_6(3)$	3, 3'-dihexyloxacarbocyanine iodide
DMSO	Dimethylsulfoxide
NAO	Nonyl acridine orange
NPP	Nitrophenylphosphate
Rh-6G	Rhodamine 6G
Rh-123	Rhodamine-123
SW	Seawater
Tris	Tris[hydroxymethyl]aminomethane = 2-amino-2-(hydroxymethyl)-1,3-propanediol

CHAPTER 1. GENERAL INTRODUCTION AND HISTORICAL OVERVIEW

General Aspects of Ion Transport and Osmoregulation

In most animals, intracellular fluids differ substantially in electrolyte composition from extracellular fluids. These ionic gradients are maintained by a variety of passive and active transport mechanisms (Stein, 1990).

The maintenance of ionic gradients is of critical importance to the survival of animal cells and tissues. High concentrations of intracellular K+ and low intracellular Na^+ and Ca^+ must be maintained for cells to function. Although the Na^+, K^+-ATPase pump has evolved as the basic mechanism of active ionic regulation between the intracellular and extracellular compartments (Macknight et al., 1994), the same enzyme system is also used to move ions and other solutes between major body compartments and, in aquatic animals, the aqueous habitat. This is achieved by certain types of compartment barriers, commonly referred to as *transporting epithelia*. In such epithelia, two features are typically observed: relatively 'tight' cell junctions to reduce passive solute and water movements over most of the epithelial surface, and the presence of specialized cells containing high concentrations of Na^+, K^+-ATPase, usually associated with infoldings of the basolateral cell membranes. In some situations, other permeases may complement or replace Na^+, K^+-ATPase (Berridge and Oschman, 1972; Stein, 1990).

The evolutionary complexity of ion-regulation involves historical transitions between oceans and freshwater, and is further compounded by the diversity of habitats occupied by aquatic animals. These range from almost pure water to saturated brines (Potts and Parry, 1963), and include semi-terrestrial habitats occupied by adult amphibia and intertidal animals (Rankin and Davenport, 1981). In addition, some animals have secondarily invaded aqueous habitats. In fact, many examples of elaborate salt glands, which contain ionocytes, are found among the marine reptiles and birds (Schlatter and Greger, 1988).

The considerable expenditure of metabolic energy observed in biological systems undergoing active transport of ions is a clear indication of the adaptive value of ionic regulation. It may be argued that such processes must be of value to animals if so much of their energy budget is dedicated to ionic regulation. It has been estimated that during resting metabolism, a human being uses 50 % of its energy reserves to maintain ion gradients across cell membranes (Keynes, 1975). It can be seen that an aquatic animal living in a hypotonic or hypertonic medium faces the additional energetic burden of regulating ion movement between extracellular fluids and the aqueous medium. Among aquatic animals, it is estimated that hyposmotic regulators use up to 15% of their standard metabolism for maintaining ion homeostasis, although this may

be a conservative estimate (Kirschner, 1993). In such animals transporting epithelia constitute at least a part of the body covering and, in many cases, the gill epithelium and gut lining (Berridge and Oschman, 1972).

Ion Transport by Ionocytes in Teleost Fishes

The hyperosmotic environment of marine teleosts requires that they drink seawater to compensate for water loss, the latter occurring primarily through the permeable gill surface. Passive salt leakage over the gill epithelium, as well as salts ingested while drinking, necessitate the active removal of sodium and chloride extrarenally, through specialized chloride cells in the gill (Hoar, 1983; Potts and Hedges, 1991). Similar mitochondria-rich (MR) cells are found in a wide variety of transporting epithelia in terrestrial and aquatic animals, including freshwater (Laurent and Perry, 1990) and marine (Woodhead and Woodhead, 1959) teleosts, where they are sometimes referred to as 'ionocytes' (Conte, 1980).

Mitochondria-rich ion-transporting cells have been described from a variety of epithelial tissues in fishes, amphibians and other vertebrate animals, and are variously referred to as flask cells, beaker cells or clear cells (amphibian skin), chloride cells or Keys-Willmer cells (fish gill or buccal epithelium), mitochondria-rich cells (various epithelia), intercalated cells (vertebrate renal tubules), or ionocytes (various tissues). Such cells may be found in the

amphibian urinary bladder (DiBona et al., 1969; Choi, 1963), skin (Brown et al., 1981; Ilic and Brown, 1980; Larsen, 1988), and oropharyngeal epithelium (Clemen and Opolka, 1992); in teleost buccal, skin, and gill epithelia (Nonnotte et al., 1979; Hootman et al., 1987; Marshall, 1988), and even in the tubules of the mammalian kidney (Larsen, 1991).

While cellular transport is a commonplace event, and high levels of active ionic transport occur in many types of specialized cells, a distinguishing property of the ionocyte is readily recognized in transporting epithelia. In contrast with other cells, such as neurons, which engage in active ion transport to fulfil their own ionic requirements, ionocytes are involved in active transport of ions and other solutes across epithelial barriers separating physiological compartments. Frequently, as in many osmoregulating aquatic animals, ionocyte transport is required for bulk transport of ions between the animal and its environment. In at least the majority of cases, it is likely that the electrochemical gradients created during ion-pumping by membrane ATPases is coupled to other forms of ionic and non-ionic transport, and is ultimately responsible for providing most of the energy required for moving solutes and water across epithelia.

The absence of morphologically and functionally distinct ionocytes from certain types of transporting epithelia, however, is a clear indication that other strategies are

involved in epithelial transport. One possible explanation for this discrepancy is that a larger proportion of the epithelial cell population is involved in transport, obviating the need for more intense metabolic activity by a subpopulation of specialized cell types.

While many cells, such as neurons or muscle fibres, are specialized for activities (eg. in association with generation of the action potential, or in contractile responses) that require the transport of particular ions, these cells generally do so to fulfil their own ionic requirements. Cells also transport ions to regulate their volume and pH. In contrast, specialized epithelial ion-transporting cells actively move ions between major body compartments, and sometimes to and from the external body surface. This movement requires the expenditure of metabolic energy, which is furnished through the hydrolysis of mitochondrial ATP by membrane-bound ATPases. Ionocytes, therefore, exhibit a close coupling between cellular metabolism and electrolyte transport. While other epithelial cells often participate in transport, these processes usually appear to be energized by the activity of ionocytes (Harvey, 1992).

For these reasons, all cells sharing in common the ultrastructural chararacteristics of mitochondria-rich cells, and presumed to be actively engaged in the transport of ions will be referred to as ionocytes in the present review. This

practice represents a slight departure from the convention followed in most other reviews of ion-transporting cells which traditionally have dealt with specialized types of ionocytes, such as 'chloride' (Degnan et al., 1977) or 'mitochondria-rich' (Brown et al., 1981) cells. Later chapters in the present work will focus on a specialized type of ionocyte, the teleost chloride cell.

Ionocytes, therefore, may be regarded as cells that have taken chemiosmotic coupling (*sensu* Mitchell; reviewed by Tyler, 1992) to an extreme, effectively linking mitochondrial respiration and ATP synthesis with bulk transport of solutes across epithelial barriers.

Ionocytes have a common morphology, usually being somewhat columnar, and are often attached basally to a basement membrane, with an apical pit facing a lumen or the external medium. The cells are generally larger than the other 'pavement' cells of the epithelium (up to about 20 μm), and contain high densities of mitochondria. A system of membranous tubules (the so-called vesiculotubular system) connects the basolateral cell membrane to the cytoplasm. This membrane system usually contains permeases, such as Na^+, K^+-ATPase (Firth, 1983) and H^+-ATPase (Larsen, 1991), and ion currents are generally detectable at the surface of the apical pit (Larsen, 1991). MR (mitochondria-rich) cells also generally contain high concentrations of carbonic anhydrase (Scott et

al., 1974), and have been implicated in the transport of ions, such as Na^+, Cl^-, H^+, and Ca^{2+} (Flik et al., 1993; Larsen, 1991).

It had long been suspected that chloride secretion in the gills of marine fishes occurred through a large, mitochondria-rich, acidophilic cell, originally described by Keys and Willmer (1932), and named the Keys-Willmer cell. Although this cell is now generally referred to as the 'chloride' cell, its recent implication in the transport of other ions has led to the use of the name, 'ionocyte', particularly for such cells occurring in freshwater fishes, which absorb ions from the external medium. This may require a revision of the terminology (Marshall et al., 1992; Flik et al., 1993). Definitive identification of this cell type as the active transporter of chloride ion was only made in recent years using microprobe recordings of individual chloride cells (Foskett and Scheffey, 1981). Although teleost chloride cells were originally described from seawater-adapted eel gills by Keys and Willmer (1932), they have since been observed in the gill, buccal and skin epithelia of adult seawater-adapted, freshwater and marine teleosts (Nonnotte et al., 1979; Marshall and Nishioka, 1980), and on the skin and yolk sac of embryonic and larval teleosts (Hwang and Hirano, 1985). The structure and function of the teleost chloride cell has been reviewed by Pisam and Rambourg (1991).

The role of the chloride cell has been clarified in both hyperosmotic and hyposmotic regulation by teleost fishes. In the former case, freshwater teleosts must maintain hyperosmotic tissue fluids in an ion-poor medium by active uptake of ions, while marine teleosts are faced with the problem of actively removing ions and avoiding water loss by osmosis (Hoar, 1983). In these two different environments, it has been concluded that the chloride cell is active in chloride ion transport. Evidence for this hypothesis comes from several sources.

In both teleost fishes and anuran amphibia, chloride currents have been localized to the apical surface of MR cells using microprobe techniques (Foskett and Scheffey, 1981; Katz and Scheffey, 1986). In addition, salinity transfer often results in changes in chloride cell densities and apical surface areas, and in Na^+, K^+-ATPase levels (Ilic and Brown, 1980; McCormick, 1990a; Laurent and Perry, 1991; Yoshikawa et al., 1993).

In marine teleosts, chloride transport by chloride cells has been indicated by several procedures, including localization of ouabain-inhibitable Na^+, K^+-ATPase, and a correlation between chloride cell densities and *in vitro* short-circuit currents (Degnan et al., 1977; Karnaky et al., 1984; Marshall and Nishioka, 1980). Similarly, chloride uptake by anuran MR cells has been shown using microprobe techniques

to measure local ion currents (Katz and Larsen, 1984; Katz and Scheffey, 1986; Harvey and Larsen, 1993).

Recent models of chloride transport in 'tight' epithelia have been reviewed by Kristensen and Üssing (1985), and by Wright (1991). In these epithelia, ions are actively transported through the apical surface of the so-called chloride cells against electrochemical gradients using energy derived from ATP. While freshwater and marine fishes transport ions in opposite directions, in accordance with their requirements for homeostasis, the basic mechanism in both groups of teleosts appears to be similar.

The effects of ambient conditions on the morphology and function of teleost gill chloride cells has recently been summarized by Laurent and Perry (1991).

In adult fishes, ionocytes have been reported to occur in the epithelium of the inner operculum, and in the skin and gills.

Changes in ionocyte density, distribution and ultrastructure have also been described as a result of temperature (Woodhead and Woodhead, 1959), salinity or hormone treatment (Sakamoto et al., 1993). For example, in the rainbow trout, Oncorhynchus mykiss, cortisol injections stimulate gill chloride cell proliferation and increases in the apical cell surface area (Laurent and Perry, 1990). Similarly, when many euryhaline fishes are transferred to seawater, cell

densities, apical surface areas and Na^+, K^+-ATPase activity are seen to increase (Laurent and Perry, 1991). Comparable responses to low pH levels, ion-poor water and even 'xenobiotics', have also been described (Laurent and Dunel, 1980; Leino and McCormick, 1984; Leino al., 1987; Hwang and Hirano, 1985; Perry and Laurent, 1989; Laurent and Perry, 1991).

It had long been suspected that chloride secretion in the gills of marine fishes occurred through a large, mitochondria-rich, acidophilic cell, originally described by Keys and Willmer (1932). Although this cell is now generally referred to as the 'chloride' cell, its recent implication in the transport of other ions, particularly in fishes other than marine teleosts, has led to considerable confusion in the literature. This situation is partly responsible for the revised terminology used in the present paper, although the term 'chloride cell' will be used to refer to this cell type in marine and brackish-water teleosts. Definitive identification of the chloride cell as the active transporter of chloride ion in marine fishes was only made in recent years using microprobe recordings of individual chloride cells (Foskett and Scheffey, 1981). Although the teleost chloride cell was originally described from seawater-adapted eel gills by Keys and Willmer (1932), it has since been observed in the gill, buccal and skin epithelia of adult seawater-adapted,

freshwater and marine teleosts (Foskett, 1987), and on the skin and yolk sac of embryonic and larval teleosts (Hwang and Hirano, 1985). The structure and function of the teleost chloride cell has recently been reviewed by Pisam and Rambourg (1991).

Among the teleosts, ionocytes have been described from a variety of tissues, including gills, kidney tubules, and buccal cavity (oro-pharyngeal) epithelium of adult fishes (Foskett, 1987), and larval and embryonic yolk sac and skin (Hwang, 1989).

Although few studies of the effects of physical factors other than salinity on ionocytes have been performed on other vertebrates, Laurent and Perry (1991) have shown that a wide variety of factors influence ionocyte densities in the gills of freshwater fishes, and suggest that these cells are involved not only in calcium and sodium chloride uptake, but also participate in acid-base balance.

Similar responses to changing salinity have been noted in the adult African clawed toad, Xenopus laevis (Ilic and Brown, 1980). Under conditions in which animals would be expected to require increased ion uptake rates, namely in ion-deficient water, ionocytes increase in density. At higher salinities, ionocytes appear to undergo morphological changes associated with a loss of transport properties (Ilic and Brown, 1980). It can be concluded that the early and adult stages of fishes and

amphibians respond to salinity changes by changing both the numbers and activities of ionocytes in their epithelia.

Fewer studies (eg. Hwang and Hirano, 1985) have examined the differentiation and function of chloride cells in the embryos and larvae of fishes.

Development of Ionocyte Transport Systems and Differentiation of Ionocytes

The development of the larval Artemia salt gland (Holliday et al., 1990), and of salt-secreting structures in aquatic insect larvae, such as mosquito, mayfly and caddis larvae (Komnick et al., 1972; Komnick and Stockem, 1973; Wichard and Komnick, 1973; Komnick, 1977) are well-known, although differentiation of these systems has not been examined in detail.

Among the vertebrates, little more progress has been made toward understanding mechanisms of ionocyte differentiation. However, a number of approaches that may yield further information in the future include studies of wound repair in fish epithelia (Bereiter-Hahn, 1986; Iger and Abraham, 1990), adaptive responses to altered salinity, pH and temperature, reconstruction of dissociated epithelia (Naito and Ishikawa, 1980), organ culture methods and endocrine treatment of cell and tissue cultures (McCormick, 1990b), studies of normal ionocyte differentiation in epithelial renewal (Conte, 1980) or in molting amphibians (Masoni and Garcia-Romeu, 1979), and

study of the development of amphibian tadpoles (Cox, 1979), fish embryos and larvae (Hwang and Hirano, 1985).

Hwang and Hirano (1985) have shown that seawater adaptation in the early stages of fishes may be related to the formation of specialized intercellular junctions. These authors have also noted the presence of a small form of chloride cell which occurs in close proximity to the more typical chloride cell. This so-called 'accessory cell' may represent an intermediate stage of cell differentiation, where epithelial cells are being recruited to form chloride cells under increasing osmotic stress. However, there is, as yet, no convincing demonstration of this function, aside from the common appearance of accessory cells in the gills of seawater-adapted euryhaline fishes (Hootman and Philpott, 1980; Hwang and Hirano, 1985). Accessory cells often exhibit interdigitations with neighbouring chloride cells, and have more electron-dense cytoplasm, giving them a darker appearance (Hwang and Hirano, 1985). While there has been some speculation that chloride cell differentiation is stimulated by hormones, such as cortisol and prolactin (Hwang and Hirano, 1985), recent studies have confirmed that cortisol has a direct effect on differentiation of gill chloride cells (McCormick, 1990a).

Subject and Scope of the Present Study

The present study was undertaken to investigate the

timing of chloride cell differentiation in Atlantic cod, Gadus morhua, relative to the main developmental events involving adaptations for osmoregulation.

By examination of available literature data, in conjunction with data collected from the present study, estimates of the effect of temperature on hatching time in the Atlantic cod were made (chapter 2).

Subsequently, methods for the localization of chloride cells were developed, and compared with classical methods of staining ionocytes (chapter 3). The developmental state of ionocytes was determined using fluorescent probes for the Na^+, K^+-ATPase, as well as potential-sensitive mitochondrial dyes and conventional precipitation methods for ion localization. This information was applied to the analysis of chloride cell differentiation from the embryonic stages through to maturity (chapter 4).

The development of Atlantic cod embryos and larvae was also observed under various salinity and temperature conditions, and in the presence of hormone or vitamin-supplemented media, to determine the potential role of exogenous factors on the timing of chloride cell differentiation (chapter 5).

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CHAPTER 2. THE RELATION BETWEEN HATCHING TIME AND TEMPERATURE FOR THE ATLANTIC COD, Gadus morhua

ABSTRACT

Hatching (= 'incubation') times for embryos of the Atlantic cod, Gadus morhua, were determined at several incubation temperatures, and were compared with data assembled from a survey of the literature from 1880 to the present (1995).

Models were fitted to data for hatching times at 'constant' incubation temperatures. Development time for cod embryos from separate genetic stocks, incubated at a variety of salinities, showed a similar relationship to incubation temperatures. Data were consequently pooled to derive a general, predictive model relating hatching time to incubation temperature.

The model giving the best fit (smallest sum of squares, least heteroscedasticity) was the relation :

$$Ti = 8.0791 + 30.8927 \cdot e^{(-0.2574 \cdot Te)},$$

where, Ti = incubation time, in days, from fertilization to hatching, and, Te = incubation temperature, in °C.

No evidence was found to indicate that the embryos of Atlantic cod from different populations hatch at different times when incubated at the same temperatures. Therefore, although variable rates of hatching and survival sometimes occur at extreme temperatures, the time to hatching for Atlantic cod can be predicted if the eggs are incubated at a known constant temperature.

INTRODUCTION

The relation of incubation time to temperature is of particular interest to workers studying the early development of fishes, especially for the prediction of hatching time in laboratory studies or hatcheries (Crisp, 1981; 1988). By manipulating incubation temperature for poikilotherm embryos, hatching time can be controlled and estimated from the established relationship between temperature and incubation time (Deeming and Ferguson, 1991; Stonecypher et al., 1994).

In addition, recent studies on the physiology of development at near-zero temperatures indicate a potential application of thermal physiology to short-term gamete preservation (Cloud et al., 1988), to the study of the ecological physiology of marine teleosts at high latitudes (Valerio et al., 1992), and in the analysis of the effects of thermal pollution (Jungwirth and Winkler, 1984).

A variety of theoretical models have been used to describe development rate in fishes as a function of incubation temperature. Although early studies used linear models to describe the relationship between incubation time and temperature, this approach is now generally believed to be inappropriate for species that tolerate a wide range of incubation temperatures (Jungwirth and Winkler, 1984). In a few cases involving temperature-sensitive tropical species or temperate species developing within a narrow range of

temperatures (Crisp, 1988), the linear approximation for incubation time (Ti) at a constant temperature (Te) may be encountered (where K is a constant),

$$Ti = K \cdot Te \qquad 1.$$

Early studies of development in fishes (eg. see Johansen and Krogh, 1914) employed the thermal unit ('Tagesgrade' or 'day-degree') of Wallich (1901), Reibisch (1902) and Apstein (1909), which in turn had originated among botanists studying plant development (Johansen and Krogh, 1914). This approach, which incorporates the 'thermal sums' concept, treats the product of time and temperature as a constant whose units are 'day-degrees'. This yields the formula for a simple 2-parameter hyperbolic function :

$$Ti \cdot Te = K \qquad 2.$$

One unfortunate consequence of this approach is that the Celsius scale yields a "biological zero" for development time at 0°C. In fact, since division by zero is undefined, it is not possible to deal with development rates at zero degrees. However, even the use of the Kelvin scale does not circumvent the problem of describing development, since thermal units are not constant over a wide range of temperatures (Johansen and Krogh, 1914; Barlow, 1961). Similarly, calculated values for Van't Hoff's Q_{10} (eg. see Bělehrádek, 1930) are not constant over wide ranges of temperature, but decrease as temperature increases (Johansen and Krogh, 1914).

Moreover, the assumption of a cessation of development at sub-zero temperatures (Blaxter, 1956) is now known to be invalid (Blaxter, 1992). Since Dannevig's (1895) studies of development in Atlantic cod embryos at subzero temperatures, it has been assumed by many workers that development can proceed at temperatures above the freezing point of the 'water' medium. Blaxter (1969) has criticized the application of several models on the grounds that the mathematically defined biological zero may be below the freezing point of tissue fluids. In reality, normal embryonic development may proceed in some species well below the freezing point of the tissue fluids, since some embryos can apparently supercool, even in the presence of external ice crystals (Williams, 1975; Valerio et al., 1992). Therefore, the assumption of a non-zero temperature threshold, as proposed by Reibisch (1902), at least partly solves the problem of describing development rates. It may be assumed that embryonic development can proceed as long as cellular organization (eg. microtubule stability) and function are unimpaired.

The inadequacy of the 'thermal sums' model for describing the relationship between hatching time and temperature in fishes is most apparent for species exhibiting normal development over a wide range of temperature. For example, although mortalities may be somewhat higher at temperature extremes, successful hatching has been reported for the eggs

of winter flounder, Pleuronectes americanus, incubated in the laboratory at -1.8 °C (Williams, 1975) to about 14 °C (Williams, 1975; Rogers, 1976). The eggs of the Atlantic cod, Gadus morhua, may also develop normally at temperatures from -1.5 °C (Makhotin et al., 1986) to 14°C (Thompson and Riley, 1981).

In a modification to the thermal sums equation (Alderdice and Velsen, 1978), a new term, **c**, may be introduced, corresponding to the lower temperature threshold for development,

$$Ti = K/(Te - c) \qquad 3.$$

This equation may be rewritten in the form proposed by Bělehrádek (Alderdice and Velsen, 1978), and is sometimes referred to as the Bělehrádek equation (Alderdice and Velsen, 1978), or as a power law with a temperature correction term, **c** (Jungwirth and Winkler, 1984),

$$Ti = K/(Te - c)^b \qquad 4.$$

It may be appreciated that equation **4** is equivalent to a power law (Humpesch, 1985) when c = 0,

$$Ti = K/(Te)^b \qquad 5.$$

The power law (**5**), hyperbola (**2**) and Bělehrádek's equation (**4**) may be generalized to the following equation (Humpesch, 1985):

$$Ti = K(Te - c)^{-b} \qquad 6.$$

If b=1, and c is the 'biological zero' or threshold

temperature for development, then the 2-parameter hyperbolic function (equation 2) is obtained, and the 'thermal sums' principle applies. When the temperature correction, c, is zero, then equation 6 reduces to the 2-parameter power law described by equation 5. Algebraic rearrangement of equation 6, of course, yields equation 4.

These classical approaches are still occasionally used, particularly where narrow temperature ranges justify a linear approximation or the use of constant thermal units (see reviews by Alderdice and Velsen, 1978; Blaxter, 1969; Hempel, 1979).

However, many recent studies have discarded the classical theoretical models and have adopted an empirical approach to model-fitting. Among these models are the polynomial functions (Alderdice and Forrester, 1971; Crisp, 1981),

$$Ti = a + b_1 \cdot Te + b_2 \cdot Te^2 + \ldots + b^n \cdot Te^n \qquad 7.$$

exponential functions (Pepin, 1991),

$$Ti = a + b \cdot e^{(c \cdot Te)} \qquad 8.$$

and logistic functions (Alderdice and Velsen, 1978),

$$Ti^{-1} = a/(1 + e^{(b+c \cdot Te)}) \qquad 9.$$

In several studies, these empirical models have been fitted after logarithmic transformation (Pauly and Pullin, 1988) or algebraic manipulation (Alderdice and Velsen, 1978). For example, the inverse logistic curve,

$$Ti = (1 + e^{(b+c \cdot Te)})/a \qquad 10.$$

has been used to model hatching time in salmonid fishes by Alderdice and Velsen (1978). This model, however, is equivalent to an exponential model, and can be obtained by algebraic rearrangement of equation 8.

The application of polynomial, exponential and power functions to models of hatching time have been reviewed by Humpesch (1985) and Page and Frank (1989).

Egg incubation temperature may influence a variety of physiological and structural characters, in addition to incubation time. At higher latitudes, where ocean temperatures are lower, the eggs of marine teleost fishes have a larger size, take longer to develop to the hatching stage, and produce adults with higher numbers of meristic characters, such as vertebrae. In some species, intra-specific variation in egg size is related to the time of spawning (Barlow, 1961; Blaxter, 1969; 1988), and could potentially influence incubation time if more yolk is available to the developing embryo.

Variables such as seawater salinity (Forrester and Alderdice, 1966; Holliday, 1969; Laurence and Rogers, 1976), illumination (MacCrimmon and Kwain, 1969; Solberg and Tilseth, 1984), oxygen tension (Garside, 1966; Hempel, 1979; Latham and Just, 1989), and egg size (Pepin, 1991) may have a minor influence on the rate of embryonic development in fishes, although the extent of this effect has often been the subject

of debate amongst fish embryologists (eg. Humpesch, 1985). More significant effects on development rate may be observed in the presence of toxins (Braaten et al., 1971), although both premature hatching and delayed hatching have been observed in experiments with fish eggs exposed to toxins (Kühnhold, 1974).

Despite the variety of factors known to influence incubation time in fishes, temperature appears to play a more significant role in determining the time to hatch than any other single factor (Blaxter, 1988).

The Atlantic cod, Gadus morhua, is found in the North Atlantic ocean and adjacent waters from Hudson Strait and West Greenland in the west to Iceland and Spitzbergen in the east, and as far south as North Carolina in the west and the Bay of Biscay in the east (Leim and Scott, 1966). Spawning appears to occur throughout most of its range (Schmidt, 1909; Colton et al., 1979).

Since cod eggs are sometimes released in relatively deep water and are buoyant, they are probably exposed to a wide range of temperatures during their vertical ascent. In addition, eggs are exposed to a variable thermal regime as a result of both horizontal displacement by water currents, and to seasonal variation of ocean temperatures. Laboratory studies of development in the Atlantic cod are commonly performed, and recent experiments with large-scale cultivation

of eggs and larvae have proved successful (Kvenseth and Oiestad, 1984).

Since geographically separate, and genetically distinct, populations of cod are exposed to different ocean climates, and may show variations in thermal responses, the present study was undertaken to summarize the results of previously published reports of development in relation to temperature, with particular emphasis on controlled laboratory studies.

Few workers have attempted to fit models to experimental data for Atlantic cod embryonic development times at different temperatures. Laevastu and Hayes (1981) fitted a nonlinear model to the data of Earll (1880) and Dannevig (1895), while scatterplots and data from several other early studies have been presented by Johansen and Krogh (1914), Russell (1976), Hardy (1978), Ellertsen et al. (1986), and Page and Frank (1989).

A comparison of hatching times at 'constant' incubation temperatures should reveal whether different populations of cod respond in a compensatory fashion to temperature. Alternatively, a general model of incubation time might be applicable to all populations of Atlantic cod, and may allow a reasonable estimate of hatching time from spawning dates and ocean temperatures. Although ambient temperatures during the spawning season are frequently quite variable, such information could still be helpful in comparing times of

hatching in wild cod populations.

MATERIAL AND METHODS

Maintenance of Adult Cod and Collection of Eggs

Adult cod used for spawning stock (>40 cm) were obtained during a Department of Fisheries and Oceans cruise by the *Chinook* in Trinity Bay, Newfoundland during June, 1993. Fish were maintained in aquaria until egg collection during the next spawning season (from March to July, 1994).

Embryo Incubation Conditions

All eggs were collected within 24 hours of spawning, and were incubated under ambient conditions of salinity, temperature and photoperiod in rectangular plastic tubs according to procedures described elsewhere (Valerio et al., 1992; see also chapters 3, 4 and 5).

During the incubation period for all the egg batches, temperature varied from 0.3°C (March) to 9.0°C (July), while salinity varied from 32.15 ppt (March) to 32.80 ppt (July). For individual batches, temperature changes were smaller.

Additional batches of embryos were incubated in 1-liter flasks at a constant temperature (± 0.5°C) in a cold room or refrigerator in seawater containing penicillin (100 U/ml) and streptomycin sulfate (0.1 mg/ml). At these concentrations, antibiotics do not influence hatching time (chapter 5). Salinities and temperatures were measured as described previously (chapter 5).

Source of Data

Experimental data from the present study were pooled with data assembled from a survey of the literature from the 1880s to the present (1995). Data for cod stocks from as many regions as possible were used, including studies from the European and North American literature. Wherever possible, the original publications were examined (table 2.1). A total of 46 studies, representing 176 paired time/temperature data points, were included in the analysis.

Types of data

Data was compiled from tabulated values of incubation time and temperature, or values were estimated from published graphs, and Fahrenheit measurements converted to degrees Celsius (table 2.1).

Several workers have observed a prolonged period of hatching for the eggs of the cod (over a period of several days), even if eggs are obtained from a single spawning from one parent, and are incubated at a constant temperature. The time from the beginning to the end of the protracted hatching period for a single batch of eggs can be as long as several days (Laurence and Rogers, 1976). Consequently, a more appropriate measure of hatching time may be the time required for 50% of the eggs to hatch at a constant temperature (Crisp, 1988; Humpesch, 1985).

Optimally, therefore, data is for 50% (median or 'peak')

hatching time at a constant (small standard error of the mean) temperature.

Data taken from studies involving treatment of embryos with toxins were only used if available for control batches subjected to 'normal' conditions.

Designation of Cod Stocks

The classification system used to designate the source of cod used in laboratory or field studies was adapted from the ICES Study Group on Cod Stock Fluctuations (ICES, 1990), and reflects an emphasis on regional variation in hydrological conditions, particularly seasonal means of water temperature. To some extent, this system also identifies genetically distinct populations:

1 = Arcto-Norwegian Cod: Bergen, north to the Arctic Ocean.

2 = White Sea Cod: Russia (subsp. G. morhua marisalbi).

3 = North Sea Cod: Including the Irish Sea, western Scotland and southern Norway (the Skagerrak region).

4 = Northern Cod: Labrador to Nova Scotia (present study).

5 = Southern Canada/ USA Shelf Cod: New Brunswick, Maine, Massachusetts, etc ...

6 = Icelandic / West Greenland Cod.

7 = Baltic Sea Cod.

Model Fitting

For some cod stocks (eg. Icelandic / West Greenland Cod), only a limited range of temperature data was available. Models

were fitted to pooled data for all stocks.

Models were fitted using linear least squares (linear model only), or Gauss-Newton iteration (all other models) to estimate model parameters using the SAS 'NLIN' nonlinear model-fitting procedure (SAS, 1988). Models were compared by examining residual distributions, confidence limits for parameter estimates, and residual sums of squares.

The following models were fitted to the data : the linear approximation (equation 1, above), the modified thermal sums equation (equation 3), the generalized Bělehrádek equation (equation 6), a second-order polynomial function (equation 7), and the exponential equation (equation 8).

RESULTS

Models were fitted to data pooled from all cod stocks (table 2.2). The exponential model is plotted in figure 2.1. Although incubation times for the eggs of fishes have been shown to depend on variables other than temperature, the fitted curves all exhibit a close fit for the simpler models, with only temperature as an independent variable. That is, the relationship between incubation time and temperature appears to vary little between genetically distinct stocks, and between studies done at different salinities, or with eggs of different size.

Although insufficient information is available from most studies to perform a statistical test of differences for all

relevant parameters, most of the data points deviate very little from the fitted curve.

In comparing the residual sums of squares for the various models (table 2.2), the exponential function yielded the closest fit to the data.

DISCUSSION

Determination of Empirical Models

Wherever possible, a description of data acquisition methods were considered. However, the wide range of publication dates and variety of methodology used in all of the studies may influence the quality of data in a manner that is not amenable to statistical treatment. In particular, instruments capable of maintaining a constant temperature have evolved substantially over the past hundred years, and therefore some consideration must be given to the magnitude of the variance when using mean temperatures.

Fitted models should also be interpreted judiciously for the following reasons :

1. The condition and survival ability of hatched larvae may depend on the egg incubation temperature.
2. There are relatively few data available for extreme temperatures, since most workers have been interested in optimizing incubation conditions. In addition, at extreme temperatures, there may be selection for particular rates of development in surviving embryos.

3. Some of the data represent less than ideal measurements of time and temperature - eg. the midpoint of a range of varying temperature, or a range of hatching times, particularly for earlier studies using less effective methods of controlling incubation temperatures, or for data collected from studies of development under ambient conditions.
4. Some of the data are approximate estimates of incubation time and temperature from field observations of cod eggs.
5. In many cases, additional variables have not been controlled, including salinity, light intensity, oxygen and photoperiod.

While the 'pooling' of data for various fish stocks does have its drawbacks in formulating a statistical model, this approach has proved useful in previous studies of development rate in fishes (Crisp, 1981), and presents at the least, a starting point for more detailed investigation of each separate stock.

In comparing models fitted in the present study (table 2.3) with those of Laevastu and Hayes (1981; based on the data of Earll [1880], and of Dannevig [1895]), parameters for the fitted exponential model are seen to differ little.

Temperature fluctuations during the embryonic period probably contribute the largest source of variation to measured hatching times. However, in some studies, temperature

varies in an approximately linear fashion during the incubation period, and therefore the midpoint of the temperature range is approximated as the 'average' incubation temperature. This assumption, while useful, may contribute an additional source of error to the analyses.

For Newfoundland 'northern' cod embryos incubated at ambient temperatures early in the season, mean incubation temperatures were calculated from a range of temperatures. The temperature rose from about 0.8 °C to 3.8°C during the period of incubation. These data points (figure 2.1) appeared to show a faster development than for cod embryos from other populations incubated at similar, but perhaps more constant temperatures (figure 2.1).

The omission of data for other factors that influence development rate in fishes may be justified on the grounds that such information is rarely available. In addition, most other environmental conditions have a relatively minor effect on development rate compared to temperature, and reported effects appear to be somewhat inconsistent.

A dependence of incubation time on salinity has not been noted by all workers. Although Laurence and Rogers (1976) showed an effect of salinity on both hatching length and incubation time in the Atlantic cod, and von Westernhagen (1970) reported retardation of development in the Baltic Sea cod at low salinities, other workers have not confirmed these

results. For example, Pickova and Larsson (1992) reported that salinity variation between 20 and 30 ppt did not influence hatching in the Atlantic cod stocks from the Baltic Sea. In some fishes, hatching time may be delayed by both high and low salinity extremes (Battle, 1930). Some workers have argued that the slight, apparent effect of salinity on hatching time is merely an artefact due to protracted hatching (longer duration of hatching) of weakened larvae (Alderdice, and Velsen, 1978). Under extreme environmental conditions, selective mortalities may also influence the mean incubation time for surviving embryos.

The effect of light intensity and photoperiod on egg incubation time is probably slight. Various effects have been reported, from none at all (Iglesias et al., 1991) to slightly accelerated development at very high light intensities (Kwain, 1975).

Reduced oxygen levels have been shown to influence hatching time both through general effects on delaying development, and by direct triggering of hatching in advanced stages of development (Hempel, 1979; Latham and Just, 1989). However, these responses are usually only seen at abnormally low oxygen tensions, and hypoxia appears to have a much more profound effect on embryonic survival than on development rate (Ohldag et al., 1991).

In addition, egg densities in culture vessels may

indirectly influence incubation time by altering the physico-chemical environment, particularly in static, or closed systems that lack flowing water. The accumulation of waste products from embryos, and of metabolites from microbial growth, as well as oxygen depletion and changes in pH may all influence incubation time, particularly through direct effects of pH on the activity of fish chorionase, or hatching enzyme (Yamagami, 1988). Nevertheless, temperature appears to be the dominant factor determining development rate in poikilotherms.

Since time to hatching depends upon the time required for the embryo to develop fully-differentiated hatching glands, this event ultimately determines the embryonic incubation time. Under certain conditions, active hatching glands may appear prematurely, and larvae hatch before embryogenesis is complete. Alternatively, hatching gland cells may not be sufficiently active in temperature-accelerated embryos to allow hatching (chapter 5). This phenomenon may partly explain the low survival rates sometimes reported for extreme incubation temperatures.

Temperature and Early Larval Survival

Although development to hatching may be observed over a wide temperature range, the size and percentage of hatching larvae may vary for different incubation temperatures. In fact, many authors have reported limited success in hatching the eggs of the cod at extreme temperatures, while others have

shown normal development at very high or very low temperatures. These disparities may be explained in part by genetic variation between cod populations studied by different workers.

Although marine teleost embryos are probably protected from freezing by an ice impermeable chorion (Valerio et al., 1992), the studies reviewed in the present literature survey suggest a lower lethal temperature of -1.5 to 0 °C for the cod embryo, since mortalities are often high at these temperatures and little data is available for hatching times (figure 2.1). In some cases, mortality may occur as a result of hatching failure due to abnormal development of hatching glands (Yamagami, 1988).

Clearly, some care must be taken in interpreting the significance of developmental rates at extreme temperatures in the absence of data on survival. Low hatching rates, or low percentages of 'healthy' larvae may indicate where the limit of thermal adaptation has been exceeded, and physiologically determined incubation times may be altered by selection for fast or slow-growing embryos at temperature extremes.

Future studies should ascertain the thermal limits for successful embryonic development in the Atlantic cod, particularly for populations spawning at high latitudes where water temperatures may sometimes remain below zero for much of the incubation period. Further, mechanisms allowing normal

development at extreme temperatures should be further investigated.

Development Rate of Eggs in The Natural Habitat of the Cod

At higher latitudes, the Atlantic cod tends to spawn later and the eggs take longer to develop at the lower water temperatures (Schmidt, 1909; Colton et al., 1979).

However, regardless of water temperature at the spawning site, cod eggs are usually subjected to a range of temperatures during their incubation period. These variations in temperature result from the displacement of eggs during their vertical ascent in the water column and from horizontal transport by water currents, but are complicated by the gradual increase in water temperatures during the spring.

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TABLE 2.1. DATA USED FOR MODEL FITTING

** See methods section (above) for stock classification codes.
*** Estimated from graphed data.
F Field data for temperature on the spawning grounds.

Temp. (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
0.00	41.00				
1.00	34.00				
2.00	28.00				
3.00	24.00				
4.00	20.00	--	3	--	Apstein, 1909
5.00	18.00				
6.00	16.00				
7.00	15.00				
8.00	14.00				
9.00	12.00				
12.00	8.50				
10.00	9.00				
8.00	11.50	--	5	32.31	Bonnet, 1939.
6.00	17.20				
5.50	17.00	--	1	--	Braaten et al., 1971.
-0.56	50.00				
0.00	40.00				
1.11	31.00				
2.20	25.00	--	?	--	Brice, 1887 (cited by Altman and Dittmer, 1966).
4.40	17.00				
6.10	14.00				
8.30	10.00				

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
3.30	21.50	--	?	--	Brice, 1898.
6.10	14.50	--	?	--	Brice, 1898.
7.00	13.00	--	5	--	Buckley, 1981.
7.50	12.00	--	3	--	Cunningham, 1885.
3.50	22.50	--	?	--	Dannevig, 1910.
-1.00	42.00				
3.00	23.00				
4.00	20.50				
5.00	17.50				
6.00	15.50	1.39	3	--	Dannevig, 1895.
8.00	12.75				
10.00	10.50				
12.00	9.67				
14.00	8.50				
5.00	20.00	--	1	34.00	Davenport et al., 1981.
-0.56	50.00				
0.56	34.00				
1.11	31.00				
2.22	24.00	--	5	--	Earll, 1880.
3.33	20.00				
5.00	16.00				
7.22	13.00				

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
7.50	12.00				
3.00	25.00	1.42	3	--	Ellertsen et al., 1981.
5.10	19.00				
7.20	9.75	1.41	6	--	Fridgeirsson, 1978.
5.00	16.00	--	1	--	Fyhn et al., 1987.
-0.60	50.00				
0.00	40.00				
3.30	21.00				
3.90	19.00				
4.40	17.00	--	?	--	Howell, 1921.
5.60	15.00				
6.10	14.00				
7.20	12.00				
7.80	11.00				
12.00	7.00				
10.00	9.00				
8.00	10.00	--	1	35.25	Iversen and Danielssen, 1984 (***).
6.00	14.00				
5.00	17.00	--	1	--	Kjorsvik, 1986.
5.00	19.00	1.14	1	34.00	Kjorsvik and Lonning, 1983.

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
5.00	18.00			34.00	
5.00	18.00	1.40	1	41.00	Kjorsvik et al., 1984.
5.00	18.00			48.00	
5.10	17.00	1.28	5	34.50	Knutsen and Tilseth, 1985 (***).
0.00	43.00				
0.10	43.00				
0.40	40.00				
1.30	35.00				
1.50	28.00				
5.10	20.00	--	2	--	Kuftina and Novikov, 1986.
5.40	18.00				
5.70	17.00				
6.00	16.00				
6.10	16.00				

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
2.00	24.00	--	5	26.00	
2.00	22.00	--	5	28.00	
2.00	21.00	--	5	30.00	
2.00	22.00	--	5	32.00	
2.00	21.00	--	5	34.00	
2.00	24.00	--	5	36.00	
4.00	18.00	--	5	26.00	
4.00	18.00	--	5	28.00	
4.00	17.00	--	5	30.00	
4.00	13.00	--	5	32.00	
4.00	13.00	--	5	34.00	
4.00	13.00	--	5	36.00	
6.00	12.00	--	5	26.00	
6.00	12.00	--	5	28.00	
6.00	12.00	--	5	30.00	
6.00	12.00	--	5	32.00	
6.00	12.00	--	5	34.00	
6.00	12.00	--	5	36.00	
8.00	10.00	--	5	26.00	Laurence and Rogers,1976
8.00	10.00	--	5	28.00	
8.00	11.00	--	5	30.00	
8.00	10.00	--	5	32.00	
8.00	11.00	--	5	34.00	
8.00	10.00	--	5	36.00	
10.00	9.00	--	5	26.00	
10.00	9.00	--	5	28.00	
10.00	9.00	--	5	30.00	
10.00	9.00	--	5	32.00	
10.00	9.00	--	5	34.00	
10.00	9.00	--	5	36.00	

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
12.00	8.00	--	5	26.00	
12.00	8.00	--	5	28.00	
12.00	8.00	--	5	30.00	Laurence and Rogers,1976
12.00	9.00	--	5	32.00	(continued)
12.00	9.00	--	5	34.00	
12.00	8.00	--	5	36.00	
5.00	18.00	--	1	--	Lonning et al., 1988.
-1.50	47.50	--	2	29.00	Makhotin et al.,
4.00	13.00	--	2	29.00	1984. (F)
-1.50	47.50	--	2	30.00	Makhotin et al.,
8.00	13.00	--	2	30.00	1986.
5.00	16.00	--	1	34.00	Mangor-Jensen and Fyhn, 1987.
5.50	12.00	--	3	--	Meek, 1924.
5.00	17.00	--	7	20.70	Ohldag et al., 1991.
5.00	14.00	--	7	13.00	Ohldag et al., 1991.
5.00	16.00	1.50	3	--	Oppenheimer, 1955.
11.00	9.75	--	7	--	Pickova and Larsson, 1992.
6.00	14.00				
8.00	12.00	--	3	--	Radtke, 1989.
10.00	10.00				

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
4.00	19.00	--	5	21.50	Radtke and Waiwood, 1980.
-0.60	50.00	--	?	--	Reibisch, 1902.
5.00	16.00				
5.00	22.00	--	?	--	Rognerud, 1889.
7.50	16.50				
3.33	20.00	--	5	--	Ryder, 1884.
8.05	11.50	--	5	--	Ryder, 1886.
5.00	18.00	--	3	34.00	Serigstad, 1987a.
5.00	17.00	--	3	34.00	Serigstad, 1987b.
5.00	18.00	--	1	--	Solberg and Tilseth 1984.
5.00	18.00	--	1	33.00	Stene and Lonning, 1984.
5.50	16.00	--	3	33.00	Swedmark and Granmo, 1981.

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
1.70	26.99				
3.35	22.50				
4.65	19.50				
6.53	16.50				
7.93	14.50	--	3	--	Thompson and Riley, 1981
8.84	12.60				
10.03	11.74				
11.59	9.50				
2.0	33.00	--	2	--	Timeyko, 1986 (***).
9.7	15.00				
9.5	14.00				
10.6	9.00				
9.9	12.00				
7.8	12.00				
10.1	13.00				
1.5	18.00				
1.5	22.00				
6.4	15.00				
6.4	16.00				
5.0	17.00	--	4	33.15 to 32.80	Present study.
3.8	19.00				
10.2	10.00				
7.0	13.00				
7.0	13.00				
7.0	15.00				
9.7	14.00				

TABLE 2.1. DATA USED FOR MODEL FITTING (CONTINUED)

Temp (°C)	Time (d)	Diam. (mm)	**Stock	Salinity (ppt)	Source
9.0	14.00				
7.0	15.00	--	4	33.15 to 32.80	Present study.
6.4	16.00				
6.0	17.00				
8.30	10.00	--	3	--	Woodworth et al.,1946.
6.75	20.00	1.41	3	32.50	Yin and Blaxter, 1986.

TABLE 2.2. MODELS FITTED TO COD INCUBATION DATA

(Ti = time, in days, from fertilization to hatch; Te = temperature, in °C)
(Ti_5 = predicted incubation time, in days, at 5°C)

SS = sum of squares: (terminology used by SAS computer program)

A = model
B = error
1 = regression
2 = residual

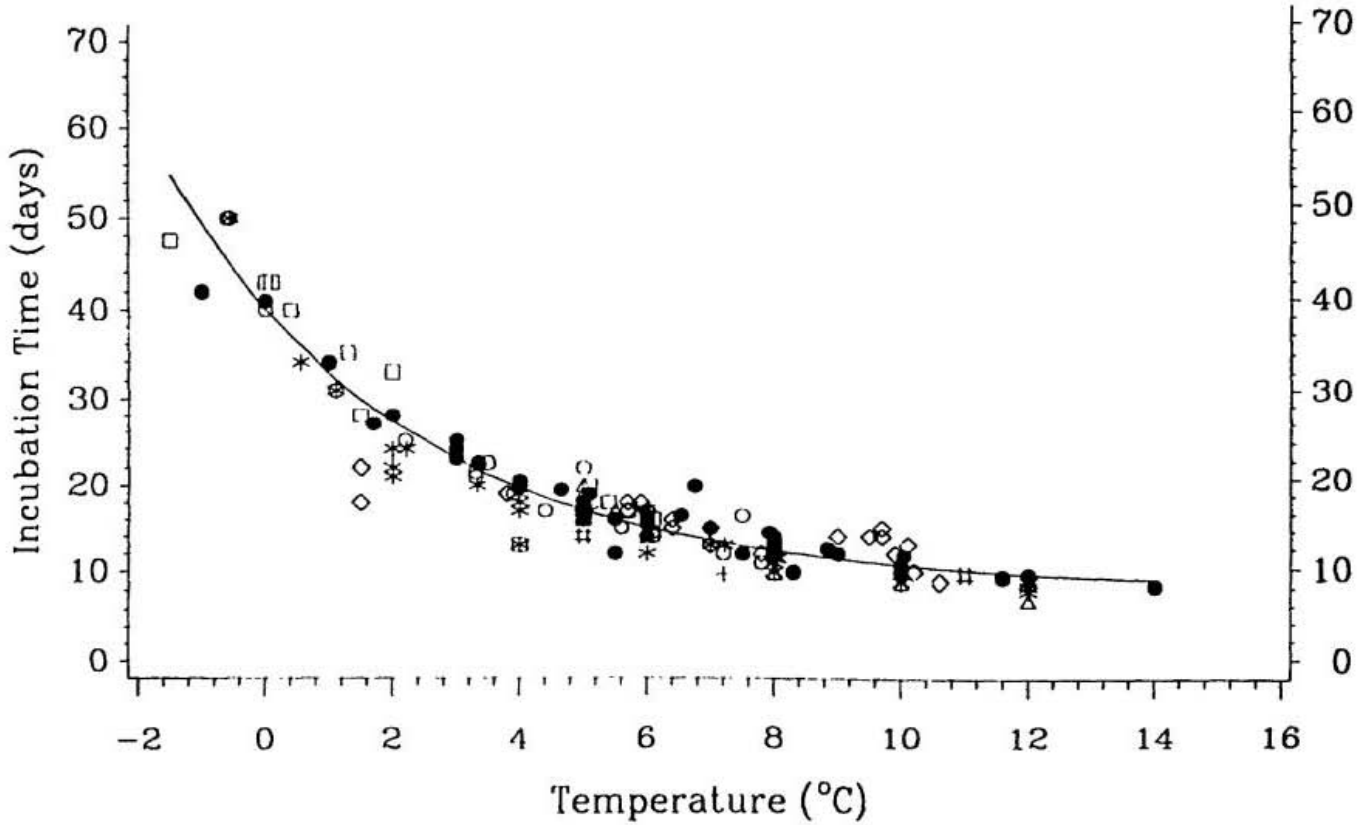
Equation	Model	SS	Ti_5
$Ti = a + b \cdot Te$	Linear	A. 11370.49	19.90
$Ti = 32.0863 - 2.4374 \cdot Te$		B. 4265.10	
$Ti = a \cdot (Te - c)^{-1}$	Modified thermal sums	1. 71013.75	17.02
$Ti = 157.4375 \cdot (Te + 4.2496)^{-1}$		2. 1536.09	
$Ti = a \cdot (Te - c)^{-b}$	Bělehrádek equation	1. 71188.68	17.03
$Ti = 1553.67 \cdot (Te + 8.38)^{1.74}$		2. 1361.16	
$Ti = a + b_1 \cdot Te + b_2 \cdot Te^2$	Polynomial	1. 70869.99	17.02
$Ti = 38.374 - 5.6697 \cdot Te + 0.2799 \cdot Te^2$		2. 1679.85	
$Ti = a + b \cdot e^{(c \cdot Te)}$	Exponential	1. 71241.78	16.61
$Ti = 8.0791 + 30.8927 \cdot e^{(-0.2574 \cdot Te)}$		2. 1308.06	

TABLE 2.3. COMPARISON OF EXPONENTIAL MODELS FOR ATLANTIC COD INCUBATION TIME.

MODEL	SOURCE
$Ti = 7 + 30.3 \cdot e^{[-0.215(Te)]}$	Laevastu and Hayes, 1981. (Based on data of Earll, 1880, and of Dannevig, 1895)
$Ti = 8.0791 + 30.893 \cdot e^{-0.2574(Te)}$	Present study, all data.

Figure 2.1.Incubation Time (days) for Atlantic Cod Embryos Reared at Various Temperatures (°C)

Fitted line : Time = 8.079 + 30.892 exp(−0.257(Temp)); number of observations=176.



CHAPTER 3. MICROSCOPIC LOCALIZATION OF CHLORIDE CELLS IN FISH EPITHELIA

ABSTRACT

Ionocytes functioning in chloride secretion, 'chloride cells', exist within the epithelial tissues of marine teleost fishes. Such cells are morphologically and metabolically specialized for ion secretion, and exhibit typical staining properties.

The present study was undertaken to compare methods of visualizing chloride cells in fish epithelia for light microscopy. In particular, newer methods of localizing mitochondrial components and ion-transporting enzymes were compared with classical techniques for staining mitochondria-rich cells. Experiments were conducted with isolated cells and intact epithelia from embryonic, larval and adult marine fishes.

In addition to the use of standard potential-sensitive stains, aldehyde-fixable chloromethyl derivatives of X-rosamine (CMXR) and tetramethylrosamine (CMTMR) were tested as stains for mitochondria-rich ion-transporting cells ('ionocytes') in whole fish embryos and larvae, and in cell suspensions isolated from adult fish epithelia.

It was shown that several techniques (eg. DASPEI) used to visualize chloride cells underestimated cell numbers - for example, due to selective staining of cells with higher than average mitochondrial potentials. Other methods yielded inflated estimates of chloride cell numbers due to staining of other epithelial cell types. Overstaining was particularly noticable with fixable chloromethyl derivatives, although these dyes may be of value where samples are only available seasonally and the preservation of staining patterns in live tissues must be achieved by chemical fixation.

INTRODUCTION

The differential staining of epithelial cells that are morphologically and metabolically specialized for ion secretion presents some interesting challenges to the microscopist (Firth, 1983). Ionocytes in fish buccal, pharyngeal (including gill), and skin epithelia are believed to be involved in the secretion or absorption of various salt ions, including chloride (Keys and Willmer, 1932). Traditionally, these cells are referred to as 'chloride' cells in marine teleost fishes, where secretory chloride currents have been localized to cell apical surfaces (Foskett and Scheffey, 1981). Similar cells in freshwater fishes are believed to be ionocytes concerned with salt uptake from the medium (Marshall et al., 1992), and may resemble the salt transporting cells of amphibian epidermis (Harvey, 1992).

Such cells characteristically possess an elaborate cytoplasmic membrane system (Berridge and Oschman, 1972) enriched with transporting enzymes (McCormick, 1990a). Their high levels of metabolic activity are often indicated by the presence of numerous mitochondria whose membrane potentials are elevated (Bereiter-Hahn, 1976; Chen, 1989). In addition, ion currents at cell apical surfaces may be correlated with transport activity (Foskett and Scheffey, 1981; Katz and Scheffey, 1986).

Consequently, microscopists have endeavoured to identify

such cells in transporting epithelia using a variety of techniques, including enzyme histochemistry, staining with potential-sensitive dyes and ion precipitation methods, and using electrophysiological recording (Firth, 1983).

Many of the cytological techniques used to identify ionocytes are concerned with mitochondrial localization. The differential staining of mitochondria has its origins in the late nineteenth and early twentieth centuries with the work of such pioneers as Altmann, Benda, Bensley, Kull, Regaud and others (Gabe, 1976; Tyler, 1992) using acid fuchsin in combination with various counterstains. Michaelis's introduction of Janus green B, a potential-sensitive vital dye, allowed the localization of mitochondria in living cells for the first time (Tyler, 1992). Other so-called redox probes, such as the tetrazolium dyes were introduced for the histochemical study of mitochondrial oxidoreductase activity in cells (Mellors, 1959). A number of additional, related methods for mitochondrial enzyme localization have since been introduced (Karnaky et al., 1984; Loveland et al., 1992).

While such classical methods are still frequently used, more recent studies have utilised antibodies to mitochondrial components (Hevner and Wong-Riley, 1993), potential-sensitive fluorescent probes (Irion et al., 1993) and DNA probes for staining mitochondrial nucleoids (Kuroiwa and Kuroiwa, 1992). Although numerous methods for ultrastructural localization of

mitochondria by electron microscopy are also available, it is still frequently advantageous to stain mitochondria-rich (MR) cells for light microscopy, especially where patterns of MR cell distribution must be correlated with gross tissue structure, or where living cells must be examined.

Potential-sensitive dyes are generally lipophilic, cationic dyes that accumulate within mitochondria in approximate proportion to the potential difference across the inner mitochondrial membrane (Chen, 1989). This potential, in turn, is a consequence of the high levels of aerobic respiration in certain cells. For ionocytes, these elevated potentials may be due to the high rates of ATP synthesis required to sustain intense transport activity. In support of this hypothesis, such cells also contain high concentrations of transporting enzymes, the ATPases (Firth, 1983).

Potential-sensitive probes include such compounds as the fluorescent styryl dyes, DASPMI and DASPEI (Bereiter-Hahn, 1976; Karnaky et al., 1984), and various rhodamine derivatives (Johnson et al., 1980; Zhang et al., 1990), among others.

While most potential-sensitive probes are removed from tissues during fixation and processing for microscopy, recent work has led to the synthesis of potential-sensitive dyes that can be permanently retained in cells following chemical fixation (the chloromethylated rosamine derivatives, termed MitoTracker™ dyes, from Molecular Probes, Inc., Oregon) or

photo-induced cross-linking (Hahn et al., 1993).

The membranous internal structure of the chloride cell also lends itself to staining by such techniques as Karnovsky's ferrocyanide-reduced osmium method (Pisam and Rambourg, 1991) or the Champy-Maillet stain (McCormick, 1990b).

The present study was undertaken to compare methods for visualizing fish ionocytes by light microscopy, using a variety of classical staining methods and novel fluorescent probes.

MATERIALS AND METHODS

Collection and Maintenance of Fish

Embryonic, larval and adult Atlantic cod, Gadus morhua, were maintained as described in a previous study (Valerio et al., 1992). Adult cunner, Tautogolabrus adspersus and winter flounder, Pleuronectes americanus, were maintained in flowing seawater aquaria under ambient temperature and photoperiod (Valerio et al., 1990). Freshwater fishes, including goldfish, Carassius auratus, and Arctic char, Salvelinus alpinus were obtained from commercial suppliers and maintained in freshwater under ambient photoperiod at 20°C (goldfish) or 12 °C (char).

Dissection of Epithelia and Preparation of Cell Suspensions

Chloride cell preparations were examined from embryonic, larval and adult stages of fishes using several types of

preparations:

a) Intact epithelia (all species).

b) Intact gill filaments (all species).

c) Dispersed epithelial cells (cod and flounder).

d) Whole embryos and larvae (cod only).

Samples of intact epithelia were obtained by peeling off the outer epithelial layer, then mincing the tissue in saline or Karnovsky's fixative with a razor blade. These samples were used to observe the *in situ* orientation of chloride cells.

For staining of live cells, the various epithelia were removed with fine forceps and transferred to an isotonic saline without further dissection. All procedures were performed at 2-4°C. A buffered saline formula was used for all marine fish tissues. The saline composition was based on reported analyses of Atlantic cod plasma (Fletcher, 1978; Fletcher et al., 1982). Although the composition of cod plasma varies seasonally and between populations of fish (Fletcher et al., 1982), the basic formula appeared to be adequate for the short-term maintenance of tissues, embryos and larvae of the various marine species.

A liter of the saline (formula I) contained 8.77 g NaCl (150 mM), 2.94 g $NaHCO_3$ (35 mM), 0.60 g NaH_2PO_4 (5 mM), 0.37 g KCl (5 mM), 0.33 g $CaCl_2$ (3 mM), 0.12 g $MgSO_4$ (1 mM) and 0.70 g glucose (70 mg% or 4 mM). The pH was adjusted to 7.8 with 1 M NaOH. The final osmolality was 349 mOsm. This saline was

diluted to approximately 300 mOsm with distilled water for use with the tissues of freshwater (goldfish and char) species (formula II: 60 ml formula I diluted to 70 ml with distilled water).

A small piece of epithelium (< 1 cm^2) was transferred to the stain solution on ice, and after rinsing in saline, the tissue was mounted on a slide with a coverslip. Gill filament fragments were similarly trimmed, stained, washed and mounted.

Gill chloride cells were obtained by dissecting whole filaments into saline, or by dispersing cells with brief EDTA saline treatment, followed by enrichment of chloride cells on discontinuous (Hootman and Philpott, 1978) or continuous (Sargent et al., 1975) Ficoll density gradients. Although density gradient methods did not yield highly purified cell fractions, increased numbers (estimated as 10 to 20% by haemocytometer counts) of chloride cells were obtained for staining procedures.

Fish were killed by spinal section, and the gills were perfused with 10 to 20 ml isotonic Mg^{++} - free and Ca^{++} - free saline containing 2 mM EDTA. One liter of this EDTA saline (formula III: formula I with magnesium and calcium salts replaced with NaCl, and 2 mM EDTA added) contained 9.09 g NaCl (156 mM), 2.94 g $NaHCO_3$ (35 mM), 0.60 g NaH_2PO_4 (5 mM), 0.37 g KCl (5 mM), and 0.70 g glucose (70 mg% or 4 mM), 0.186 g EDTA (2 mM). The unadjusted pH was 7.2.

Perfusion was accomplished by injecting fish through the *conus arteriosus* using a 21 gauge needle. The gill arches were then removed with a scalpel, rinsed in saline and incubated with slow agitation in 50 ml EDTA saline for 2 hours on ice.

A scalpel was used to scrape cellular material from the gill epithelium after EDTA incubation. Cells were resuspended in 20 ml EDTA saline, by gently pipetting with a pasteur pipette. The tube of resuspended cells were then shaken gently for an additional 10 minutes to dissociate cell aggregates before filtering through 150 μm nylon mesh netting. The netting was then washed with a further 10 ml of chilled EDTA saline and the collected fraction was spun at 3,000 RPM for 5 min. The resulting pellet was resuspended in 30 ml saline, and counted in a haemocytometer to determine the required dilution for cell isolation experiments using Ficoll (Sigma) gradients (see below).

Similar treatment was given to epithelia from the mouth roof, inside operculum, and cleithral region (the area posterior to the gill opening, overlying the cleithral bone). However, only a few chloride cells were removed intact from these epithelia in salines lacking calcium and magnesium. This suggested a need for a more effective treatment than that used for gill cell isolation. For these epithelia, the dissected tissue was incubated in 10 ml of 2 mM EDTA saline containing an additional 0.25% (2.5 mg/ml, Sigma cell culture tested,

1300 BAEE units per mg) porcine pancreatic trypsin (Waymouth, 1982). Epithelial samples were floated (dermal side down) in petri dishes containing this formulation (formula IV: formula III saline with 0.25% trypsin added) for 2 hours with gentle agitation on a shaker bath. Cells obtained from such epithelia were resuspended in EDTA saline (formula III) containing 2.5 mg/ml chicken egg white trypsin inhibitor (Sigma, cell culture tested), then were respun and resuspended in saline for vital staining, or in Karnovsky's fixative for fixation. After 2 hrs, fixed cells were again pelleted and resuspended in cacodylate buffer for storage at 4°C.

Intact epithelia, dispersed cells, and whole embryos and larvae were embedded in Unicryl (British Biocell International, Cardiff), a hydrophobic acrylic resin (Scala et al., 1992), or Spurr's epoxy resin (Spurr, 1969). Acrylic samples were polymerized with UV light using a 365 nm Spectroline® model X-15A UV lamp (Spectronics Corp., Westbury, New York) at 4 °C, and sectioned at 1-2 μm with a Sorval MT-1 ultra-microtome using a glass knife.

General Aspects of Staining

Intact tissues and sectioned material were stained and rinsed by immersion in staining solutions or saline at 0-2°C (live material) or 20-24°C (fixed material), while cell suspensions were treated by centrifuging the cells through the staining medium.

Unstained control samples of tissues in isotonic saline were always examined using phase contrast microscopy or autofluorescence for comparison with stained samples.

Cell nuclei were counterstained with either DAPI or ethidium bromide (Sigma Chemical Co.), for dead cells, or Hoechst 33258 (Sigma Chemical Co.) or dihydroethidium (Molecular Probes, Inc.), for living cells. Working concentrations of nuclear stains were: 5 μM (DAPI), 1 μM (Hoechst 33258=bisbenzimide), 10 μM (dihydroethidium), or 10 μM (ethidium bromide).

While it is often difficult to avoid subjective criteria in cytological studies, an attempt was made to perform a qualitative comparison of staining methods based upon the consistency of staining patterns, and the specificity of stains for chloride cells. The chloride cell type was defined as a cell having a granular cytoplasm, containing large numbers of mitochondria, and having an exposed apical surface. Such cells may be elongate in thick epithelia or flattened in thin yolk sac epithelia (chapter 4).

Staining of Plastic Sections

Spurr resin sections were stained with 1% alkaline toluidine blue (Millonig, 1960), methylene blue/azure II (Humphrey and Pittman, 1974) or basic fuchsin (Huber et al., 1968). Acrylic sections were stained with Harris' haematoxylin (Bell et al., 1986). Samples fixed in Regaud's fixative or

treated by postchromatization (Gabe, 1976) after fixation with Karnovsky's fixative were stained with acid fuchsin according to a modification of Altmann's procedure (Gabe, 1976).

Specimens fixed according to the Champy-Maillet method (McCormick, 1990b) were lightly counterstained with toluidine blue (Millonig, 1980) after sectioning.

Various Potential-Sensitive Mitochondrial Dyes

Potential-sensitive probes were generally applied by immersion of dissected epithelia (adult or juvenile cod) or intact embryos and larvae in isotonic saline solutions containing the dyes. For embryos, the chorion and yolk sac were punctured with a glass needle to allow stain penetration into the perivitelline fluid and tissue fluids. Specimens were placed into a plastic tube with a 0.20 mm nylon mesh base, and the tube was lowered into the stain solution in a beaker on ice for 5 to 20 minutes before rinsing in stain-free saline.

Various fluorescent dyes, including 2-(4-dimethylamino-styryl)-1-ethylpyridinium iodide (DASPEI), MitoTracker™ dyes (CMTR-H_2, CMXR-H_2, and CMXR), rhodamine-123 (Rh-123), and rhodamine-6G (Rh-6G) were applied to living tissues to target high mitochondrial potentials in chloride cells (Bereiter-Hahn, 1976; Karnaky et al., 1984; Chen, 1989).

In some cases, tissues stained with DASPEI were subsequently washed and restained with Rh-123 to differentiate low and high potential mitochondria in cells.

Fluorescent Membrane Probes

Additional membrane probes, including 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and nonyl acridine orange (NAO) were used to stain mitochondrial membrane components (Terasaki et al., 1984; Petit et al., 1992).

$DiOC_6(3)$ has been shown to act as a potential-sensitive dye at low concentrations, but may bind to other membrane components, including the endoplasmic reticulum at higher concentrations (Koning et al., 1993). Its utility as a mitochondrial dye may depend on both staining mechanisms in some cases. Nonyl acridine orange (NAO) is a phospholipid probe with a high affinity for cardiolipin, a phospholipid found in the inner mitochondrial membrane (Petit et al., 1992). This probe was used to stain mitochondria in both living and aldehyde-fixed tissues.

Silver Staining of Intact Epithelia

A modification of the silver staining technique of Holliday and coworkers (Holliday et al., 1990) was used to localize chloride ion. Precipitated salts were identified by energy dispersive x-ray microanalysis (eg. see Philpott, 1965; Gupta and Hall, 1979). A Tracor-Northern TN5500 system and Hitachi 570 scanning electron microscope were used to simultaneously view salt deposits by backscatter electron imaging, and to identify element peaks in spectra by energy dispersive x-ray microanalysis.

Anthroylouabain Localization of Na^+,K^+-ATPase

A fluorescent derivative of the glycoside ouabain (g -strophanthin), anthroylouabain, was used to localize Na^+,K^+-ATPase in the tubular membrane system of chloride cells from cod epithelia according to the methods of McCormick (1990a). Since ouabain- binding of the phosphorylated enzyme is inhibited by potassium (Yoda and Yoda, 1988), a low-potassium saline was used (McCormick, 1990a).

One liter of this saline (formula V) contained 8.77 g NaCl (150 mM), 2.94 g $NaHCO_3$ (35 mM), 0.60 g NaH_2PO_4 (5 mM), 0.015 g KCl (0.2 mM), 0.33 g $CaCl_2$ (3 mM), 0.12 g $MgSO_4$ (1 mM) and 0.70 g glucose (70 mg% or 4 mM). The pH was adjusted to 7.8 with 1 M NaOH. The final osmolality of the saline was 332 mOsm.

Tissues were dissected from fish and placed on ice in isotonic saline (formula I) in petri dishes until ready for staining (usually within 15 to 30 minutes). Fragments of opercular membrane or gill filaments were incubated in a buffered saline solution (formula V) containing 2 μM anthroylouabain at 4 °C for 30 min. The staining solution was prepared from a stock solution containing 2 mM anthroylouabain in ethanol.

The tissues were then rinsed twice in cold saline, and examined in a Zeiss Axiovert 35 microscope modified for epifluorescence with a 50 W HBO illuminator, a 365 nm

excitation filter, a 395 nm chromatic beam splitter, and a 420 nm longwave-pass filter (McCormick, 1990a; 1990b).

Aldehyde-Fixable Potential-Sensitive Dyes

A drawback to the use of potential-sensitive fluorescent mitochondrial probes, as noted by Adoff (1986) and others, is the tendency of these dyes to be washed out of the tissues during processing for microscopy of whole or sectioned material. This problem has now been overcome with the availability of aldehyde-fixable derivatives of rhodamine dyes. The MitoTracker™ dyes are a class of novel aldehyde-fixable potential-sensitive mitochondrial probes (Molecular Probes, Inc., Eugene, OR.). They were used to follow the appearance of chloride cells in fixed embryos with fluorescence microscopy. While ordinary potential-sensitive probes are washed out of tissues during processing, the MitoTracker™ dyes can be fixed in place by aldehyde treatment. All of the MitoTracker™ dyes (50 μg) were dissolved in 50 μl DMSO (dimethylsulfoxide) and divided up into 5 X 10 μl aliquots before storing at -20 °C. When thawed for use, each of these aliquots were diluted to a total of 0.1 μM with saline and stored at 4 °C. Embryos and larvae were stained by immersion in 0.1 μM CMXR for 15 min, then were washed in saline, then fixed in 10% formalin in buffered, isotonic saline at 4°C.

The new aldehyde-fixable MitoTracker™ dyes were compared

with other potential-sensitive dyes. The limited permeability of the reduced forms of these dyes, chloromethylated X-rosamine (CMXR-H_2) and chloromethylated tetramethylrosamine (CMTR-H_2) prohibited their use as mitochondrial stains in the present study. However, the oxidized form of chloromethylated X-rosamine (CMXR) yielded an acceptable staining reaction. This dye also emits in the red range, contrasting well with green-yellow aldehyde-induced autofluorescence when using longpass barrier filters or allowing the use of a narrow-band barrier filter beyond the range of tissue autofluorescence.

All of the MitoTracker™ dyes were dissolved at 2.0 mM in DMSO (dimethylsulfoxide) and were divided up into aliquots for storage at -20 °C. When thawed for use, each of these aliquots were diluted to a total of 50 ml (0.2 μg/ml) with saline and stored at 4 °C.

Stock and staining solutions of the dyes were prepared as outlined in table 3.1, and tissues were stained by immersion for the specified times (table 3.1) at 4°C. Washing was performed by incubation in saline solution (formula I, above) for 15 min at 4 °C. To achieve good staining results with the MitoTracker™ dyes, it was necessary to destain embryos for 2-3 hours in a saline wash before fixing them. For cell suspensions a shorter wash time is possible.

Photomicrography

Embryos and larvae stained with fluorescent dyes were

viewed and photographed with a Zeiss Axiovert 35 inverted microscope modified for epifluorescence with a 50W HBO illuminator. Specimens were viewed with either a 450-490 nm band-pass excitation filter, a 510 nm chromatic beam splitter, and a 515-565 nm bandpass filter, or a 365 nm excitation filter, 395 nm chromatic beam splitter, and a 420 nm longpass filter (table 3.1) Photographs were then taken using a Contax 167 MT camera with 400 ISO color Kodak film.

Additional photographs of stained embryos and larvae were obtained with an Olympus BHS microscope equipped with an Olympus PM-10ADS photomicrographic System and BHS-RFC reflected light fluorescence attachment.

Specimens stained with non-fluorescent methods were viewed and photographed with a Nikon N2000 camera attached to a Nikon Diaphot-TMD inverted microscope fitted with phase contrast optics.

Scanning and Transmission Electron Microscopy

Most specimens of epithelia, embryos and larvae were fixed as intact samples or dispersed cells, as described above. However, some were fractured for scanning electron microscopy (SEM) according to the method of Humphreys and coworkers (Humphreys et al., 1976). Other epithelial samples were enzymatically dissociated in a Ca^{++}, Mg^{++}-free saline (formula III) containing 2 mM EDTA and 0.25% (2.5 mg/ml) porcine pancreatic trypsin (Waymouth, 1982) at 4°C for 30 min prior to fixation to expose internal structure (formula IV).

Tissues were transferred directly to Karnovsky's fixative at 4°C (Karnovsky, 1965). In some cases, the primary fixation step took several months, as specimens were stored in the fixative at 4°C. Tissues were then washed in cacodylate buffer (Glauert, 1975), and postfixed in potassium-ferrocyanide-reduced osmium tetroxide (Karnovsky, 1971) at 4°C over a 2 hour period. Prior to dehydration through an ethanol series, the samples were rinsed in distilled water. For scanning electron microscopy (SEM), the tissues were then critical-point dried with liquid CO_2 in a Polaron E 3000 critical point drying apparatus. Dried specimens were attached to aluminum stubs with silver paint, then were gold-coated with an Edwards S150A Sputter Coater.

Specimens were examined with a Hitachi S570 scanning electron microscope operated at an accelerating voltage of 20

kV. Stereopairs were taken with an 8 degree tilt angle difference.

Transmission electron microscopy (TEM) was performed on samples embedded in either Spurr's low-viscosity resin (Spurr, 1969), or in Unicryl (British Biocell International, Cardiff), a hydrophilic acrylic resin (Scala et al., 1992). Sectioning was performed on a Sorval MT-1 ultra-microtome using glass knives. Semithin sections (1 μm) were examined after staining with toluidine blue (Millonig, 1980), or a modification of Altmann's method for mitochondria (Gabé, 1976). Ultrathin (< 1μm) acrylic sections were mounted on butvar-coated grids and were viewed with a Zeiss EM 9A transmission electron microscope at 60 kV.

RESULTS

Although similar results were obtained for all species examined (see Methods section), results are presented only for cells and tissues from Atlantic cod, cunner, and winter flounder.

Comparison of Classical Histological Methods and TEM

Examination of alternate semithin (light microscopy) and ultrathin sections (TEM) of gill tissue stained by Altmann's technique and Karnovsky's ferrocyanide-reduced osmium method revealed that the light microscopical methods stained only a few of the mitochondria-rich cells identifiable as chloride

cells by TEM.

In contrast, the method of Champy-Maillet, which is specific for phospholipids, and therefore can be used to identify cells with an extensive membrane system (McCormick, 1990b), was found to give an unacceptably high background staining for cell types other than presumptive chloride cells. For example, in gill epithelia, many pavement epithelial cells with relatively small numbers of mitochondria stained similarly to chloride cells.

Scanning Electron Microscopy (SEM) versus Silver Staining

Surface views of intact epithelia often revealed the presence of numerous secretory pits corresponding to the apical surfaces of goblet (mucus), sacciform or chloride cells (figure 3.1, a and b; compare with TEM, figure 3.1c).

Silver-stained epithelia also exhibited localization of reaction product to secretory cell pits (figure 3.2), as well as the intercellular space. The precipitate was identified as AgCl by energy dispersive X-ray microanalysis.

Although neither method was capable of resolving secretory cell types, examination of silver-stained epithelia by phase contrast microscopy revealed that some of the stained cell apices belonged to mitochondria-rich cells.

Potential-Sensitive Mitochondrial Dyes

Buccal and pharyngeal epithelia from cod, winter flounder, cunner, char and goldfish all had columnar MR cells

that stained positively with potential-sensitive mitochondrial stains, including Janus green B, rhodamine-123, rhodamine-6G, DASPEI (figure 3.3 and 3.4), and the MitoTracker™ dyes (figure 3.5 and 3.6).

With one exception, DASPEI, these dyes all produced considerable levels of 'background' staining. That is, epithelial cells other than MR cells exhibited positive staining to some extent. On the contrary, DASPEI-stained chloride cells appeared to represent only a fraction of the total chloride cell population of the tissues. This effect was most pronounced in thin, yolk sac epithelia, since all MR cells are readily observed by autofluorescence of mitochondrial pyridine nucleotides in UV light, which can be used to verify cell identity (figure 3.7).

A related phenomenon was observed in epithelia stained sequentially with DASPEI, then rhodamine-123. Two populations of chloride cells were seen : a weakly-staining group that stained green with the rhodamine dye, and a strongly DASPEI-positive group that stained yellow with DASPEI, but did not appear to stain with the subsequent application of the rhodamine dye. This response may be due to an intrinsic difference in membrane potential between cells, or may be due to morbidity of cells heating on the microscope stage. This phenomenon was consistently observed with DASPEI staining, while other potential-sensitive dyes tended to overestimate

the number of chloride cells, rather than staining a sub-population of high potential cells.

Although most stains, including rhodamine dyes and membrane probes passed through the chorion of the cod embryo relatively easily, DASPEI staining was only possible with dechorionated or chorion-punctured embryos (figure 3.8). Tests of chorion permeability to various fluorescent and non-fluorescent stains indicate that chorion permeability may be a function of both molecular size and charge (Valerio, unpublished data).

Aldehyde-Fixable Potential-Sensitive Dyes

The reduced forms of the chloromethyl derivatives of X-rosamine (CMXR-H_2) and tetramethylrosamine (CMTMR-H_2) did not appear to penetrate cell membranes readily. However, the oxidized form of X-rosamine, CMXR, produced a more acceptable staining reaction (figure 3.5 and 3.6).

Fixation of stained epithelia did result in retention of stain, although a decrease in stain intensity within chloride cells resulted in reduced contrast (figure 3.9 and 3.10).

Fluorescent Membrane Probes

Additional experiments with other fluorescent probes, including tetracycline (figure 3.11) and the membrane probes NAO (figure 3.12) and $DiOC_6(3)$ (figure 3.13), showed some staining specificity for chloride cells, particular in yolk sac epithelia, but with high background compared to DASPEI and

even the rhodamine dyes.

Anthroylouabain Localization of Na^+,K^+-ATPase

Anthroylouabain staining showed that Na^+, K^+-ATPase concentrations appeared to be higher in mitochondria-rich cells. This is consistent with their identification as chloride-secreting cells. Similar results were obtained using other mitochondrial stains, including rhodamine-123 (figure 3.14 and 3.15) or rhodamine-6G in conjunction with anthroylouabain.

DISCUSSION

Epithelial Surface Pores

All of the methods for localizing the apical surface of chloride cells appeared to be incapable of distinguishing the several types of secretory cells seen in transporting epithelia. While some attempt can be made to distinguish between goblet (mucus) cells and chloride cells, the problem may be confounded further in certain epithelia that are rich in other secretory cells, including the sacciform cell or various related cell types (Adoff, 1986; Morrison, 1993). It is apparent that the presence of surface pores is in itself not a sufficient condition for the identification of chloride cells.

A number of the staining methods purported to yield specific localization of ion-transporting cells may be, in fact, based upon the reaction or behaviour of stains at sites

of increased permeability in otherwise 'tight' epithelia. Thus, since chloride ion tends to leak passively out of an epithelium through surface pores at the apical surfaces of secretory cells, staining reactions might misleadingly indicate these regions as sites of active ion transport (see below). This may be particularly true for cases where such secretory cells are attached to adjacent cells by 'leaky' junctions.

Thus, while the staining of secretory cells by silver precipitation of chloride ions was consistent with the apparent success of the microanalytical method as reported by earlier workers, the interpretation of such staining patterns is probably in error. For example, while Shelbourne (1957) hypothesized that reticulate epithelial staining patterns in marine flatfish larvae indicated secretory activity, a more plausible explanation for this effect is that extracellular chloride ion reacts with silver ion to form a precipitate which delineates pavement epithelial cells. It is unlikely that the silver method can resolve the 'background' chloride in intercellular fluids, and in fluids adherent to sacciform pits, from chloride originating from active secretion. However, local concentrations of chloride ion can apparently be detected by such methods in some freshwater animals and terrestrial plants (Läuchli, 1975; Van Steveninck and Van Steveninck, 1978; Gupta and Hall, 1979), perhaps due to their

having a less complex epithelial cell population.

Variable Tissue and Cell Permeability to Stains

A number of factors confound identification of chloride cells in epithelia when potential-sensitive dyes are used. In the early embryonic stages of cod, mitochondrial potentials are low and such stains are ineffective. In the later stages of marine fishes, there may be considerable variation in staining intensity between individual chloride cells, perhaps reflecting different levels of cellular differentiation or metabolic activity.

While further work is necessary to explore the mechanisms of stain permeation of tissues and cells, the present study indicates that permeability varies between tissues and between different stages of development. Gill epithelia were particularly permeable to most stains, while at the other end of the spectrum, embryonic and larval skin were relatively impermeable to stain solutions.

Where restricted junctional permeability was circumvented by perforating epithelia before incubation, stain permeation appeared to be somewhat enhanced. This strategy is probably the simplest solution, when it is not practical to disperse cells by mechanical or enzymatic methods.

An additional artefact frequently occurs where cells must be destained by rinsing them in stain-free saline. For intact tissues or even large cells, stain diffusion may be

slower than for other, isolated cells, and false positives may occur. This effect may have contributed to part of the stain retention by isolated gill chloride cells. This phenomenon is likely to be of more importance in determining the success of staining methods for relatively thick epithelia, such as intact buccal or pharyngeal epithelial preparations.

The gradual acquisition of staining properties by chloride cells during development is probably an indication of their differentiation at the molecular level. The absence of mitochondrial fluorescence in early blastula cells treated with styryl dyes indicates that the organelles are not sufficiently differentiated at this point to engage in the high levels of ATP synthesis required for transport activity beyond that required for normal metabolism. Consequently they have low membrane potentials. This phenomenon may also explain why early stages rely upon passive barriers to ion influx, while elevated levels of transport activity develop later on.

As epiboly nears completion, rates of ATP synthesis rise, and proton gradients become sufficiently steep to allow staining by fluorescent potential-sensitive probes. The differentiation of the chloride cell involves not only mitochondrial development, but also the elaboration of an extensive vesiculo-tubular membrane system, and the synthesis of unusually high levels of ATPase and ATP. Presumably, chloride cells also manufacture hormone receptors for

modulation of their activity by endocrine factors (McCormick, 1990b).

Criteria For Stain Selection

Where chloride cell shape, size and orientation are distinctive enough to allow recognition without a highly specific form of staining, the type of stain is obviously not as critical. However, in many instances, cell shape and size may be obscured, as in relatively thick, intact epithelia. Where accurate quantification of chloride cell densities is required, enumeration of dispersed cells may be the only recourse.

More uniform results may be obtainable with cell suspensions, since dye uptake is not limited to the apical cell surface. However, the absence of other cell types and autofluorescent connective tissues may also simplify matters.

Utility of Fixable CMXR Dyes

The restricted seasonal availability of many types of biological samples makes their storage and preservation essential for some studies. The use of fixable mitochondrial dyes would appear to offer a solution for these situations.

The results of our preliminary tests with the MitoTracker™ dyes indicate that they may have some utility in preserving staining patterns for certain epithelia, particularly for dispersed cells, or permeable epithelia, such as gill.

However, CMXR staining of intact epithelia, larvae or embryos may be limited by the permeability of epithelia to the dye, and by the slow rate of diffusion of stain and fixative through the tissues. As may be expected, even where mitochondria were well-stained by the dye, it was necessary to fix the dye in place before cells died, potentials were lost, and stain began to diffuse from the mitochondria. This effect was readily observed with any potential-sensitive dye, where dying cells heated with a microscope lamp or exposed to fixatives rapidly lost their membrane potentials, and mitochondrial dye diffused into the cytoplasm.

In the case of the Mitotracker™ dyes, this effect was particularly pronounced for thick pieces of tissue. If cell suspensions or thin (<3-4 cells thick) epithelia were stained with CMXR, reasonable staining differentiation of mitochondria-rich cells was obtained. However, thicker samples were slower to pick up fixatives, and consequently, the dye tended to leak out of dying cells, staining the entire tissue sample uniformly. For the tissues of cold-water teleosts, temperature sensitivity is likely more of a problem than for tissues from warm-blooded animals. A more rapid fixation may sometimes be obtained by scraping off epithelial cells, or by perforating epithelia to allow basal entry of the dyes.

Other Fluorescent Probes

An alternative stain for mitochondrial membranes used in

the present study was nonyl-acridine orange (NAO), a fluorescent probe that targets cardiolipin, a phospholipid found in the inner mitochondrial membrane. NAO fluorescence has been used as a quantitative index of mitochondrial mass, since its fluorescence does not depend upon membrane potential (Petit et al., 1992). Some caution should probably be exercised in its application to the study of embryonic cells, however, since it is not yet clear whether cardiolipin synthetic activity also varies during embryonic development and cell differentiation. In the present study, NAO staining was diffuse, as the dye apparently leaked out of mitochondria into the cytoplasm.

The staining of yolk sac chloride cells with tetracycline has not been described before. The mechanism of tetracycline staining in the present study is unclear, although tetracycline has been used to label calcified tissues in vertebrates (Culling, 1974), and to stain cells in tumours (Vassar et al., 1960).

CONCLUSIONS

Variations in staining reactions with the various techniques described in the present study indicate the need for some caution in identifying or enumerating mitochondria-rich cells. Where possible, it is probably advisable to apply several techniques to confirm cell identity.

The fixable mitochondrial dyes appear to be a useful

addition to the methodology for studying ionocytes, particularly where tissue samples may be scarce or limited by seasonal availability.

Generally, ionocytes in fish epithelia can be recognized by a number of criteria:

1) Elongate (bucco-pharyngeal) or lenticular (yolk sac) cell shape.
2) Intrinsic NADH/NADPH fluorescence in UV light.
3) Similar staining pattern to gill filament controls.
4) Absence of similar cells in control epithelia (eg. from cornea, fin, etc ...).
5) Positive staining reaction with anthroylouabain (for Na^+, K^+-ATPase).

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TABLE 3.1. DYE CONCENTRATIONS AND STAINING TIMES

Stain	Stock solution (mM)	Working solution (μM)	Staining time (min)
Anthroylouabain	2.0	10.0	30
CMTR-H_2	2.0	0.1	15-30
CMXR	2.0	0.1	15-30
CMXR-H_2	2.0	0.1	15-30
DASPEI	80.0	10.0	30
$DiOC_6(3)$	1.0	10.0	15
Janus green B	1.0	10.0	20
NAO	1.0	0.1	10
Rhodamine-123	0.1	10.0	30
Rhodamine-6G	2.0	10.0	30

FIGURE 3.1a. SECRETORY PORES IN THE ADULT COD OPERCULAR EPITHELIUM

Adult cod opercular epithelium; thin arrow = occluded pore; thick arrow = open pore; note prominent microridges on pavement epithelial cell [*] apical surfaces.

Hitachi S570 SEM; magnification: 2,000X.

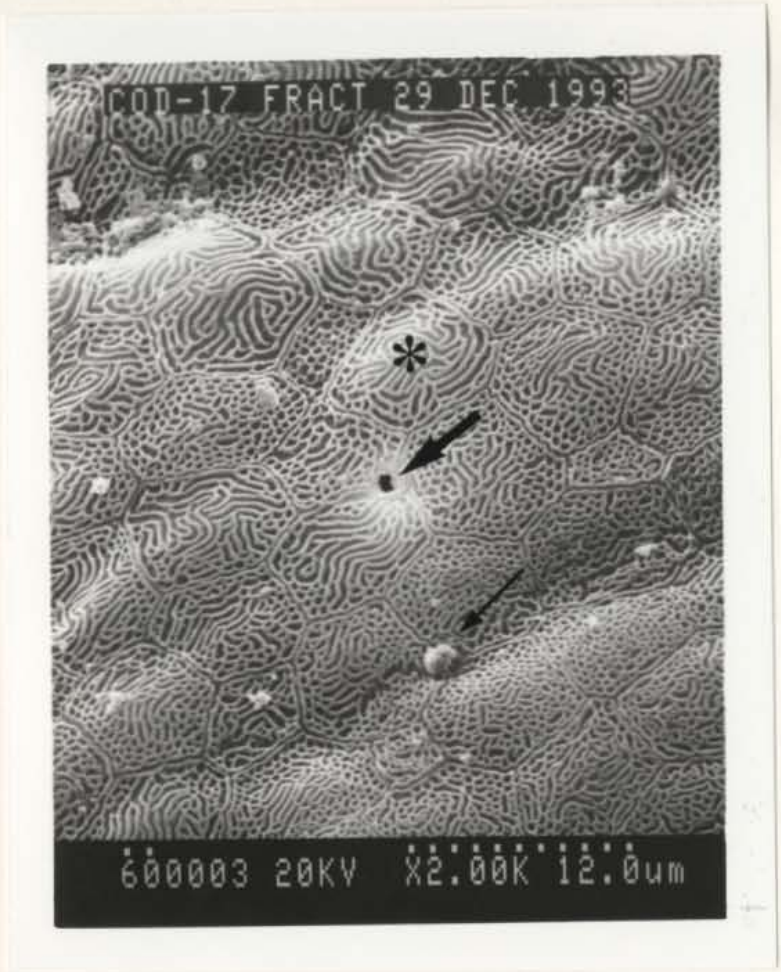


FIGURE 3.1b. SECRETORY PORES IN THE LARVAL COD YOLK SAC

Cod larva at hatch; rectangular area on yolk sac below is further magnified by 10X in the top half of the figure; arrow indicates position of a pore.

Hitachi S570 SEM; magnification: below, 70X; above, 700X.

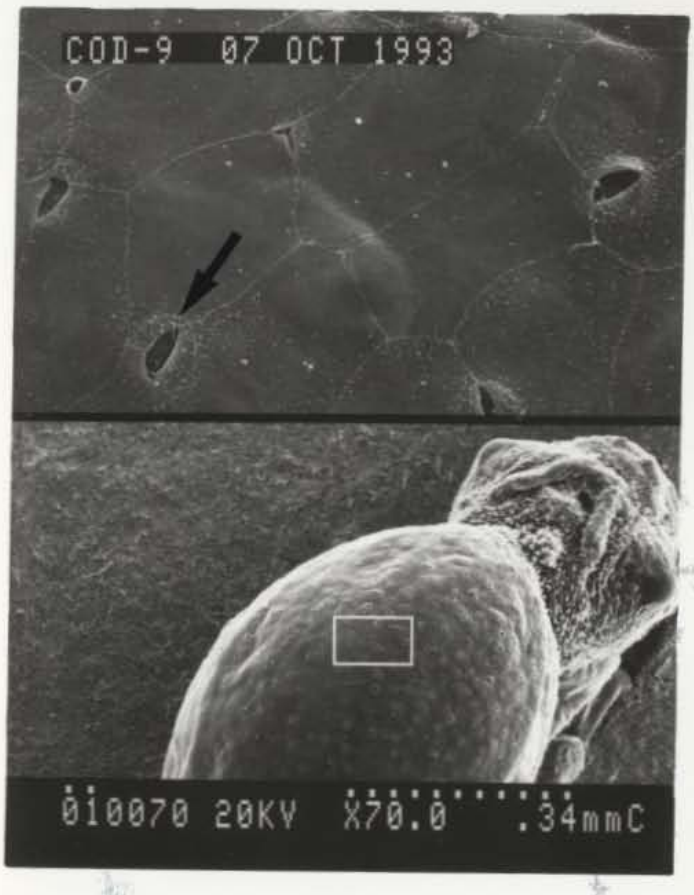


FIGURE 3.1c. EPITHELIAL SECRETORY CELLS FROM ATLANTIC COD VIEWED BY TEM

(arrows indicate apical side of cell)

Transmission electron microscopy (TEM) was performed on samples embedded in Spurr's low-viscosity resin (Spurr, 1969). Sectioned (1 μm) with a Sorval MT-1 ultra-microtome using a glass knife. Zeiss EM 9A transmission electron microscope at 60 kV.

3.1c.1. Goblet Cell.

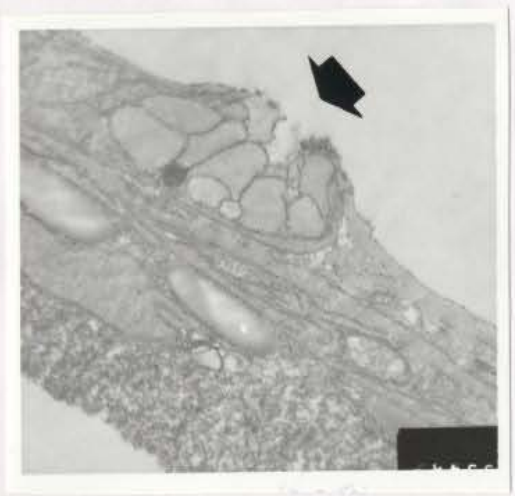
3.1c.2. Sacciform Cell.

3.1c.3. Chloride Cell

FIGURE 3.1c. EPITHELIAL SECRETORY CELLS FROM ATLANTIC COD VIEWED BY TEM

(arrows indicate apical side of cell)

Transmission electron microscopy (TEM) was performed on samples embedded in Spurr's low-viscosity resin (Spurr, 1969). Sectioned (1 μm) with a Sorval MT-1 ultra-microtome using a glass knife. Zeiss EM 9A transmission electron microscope at 60 kV.



3.1c.1. Goblet Cell.

3.1c.2. Sacciform Cell.

3.1c.3. Chloride Cell

FIGURE 3.2. ATLANTIC COD SILVER-STAINED YOLK SAC EPITHELIUM

Cod larva at hatch; silver stain; arrow indicates apical pit of a secretory cell stained dark brown by precipitated silver salt; the asterisk (*) shows the position of one of the pavement epithelial cells that comprise the superficial epithelial surface.

Nikon Diaphot-TMD inverted microscope; magnification: 400X.

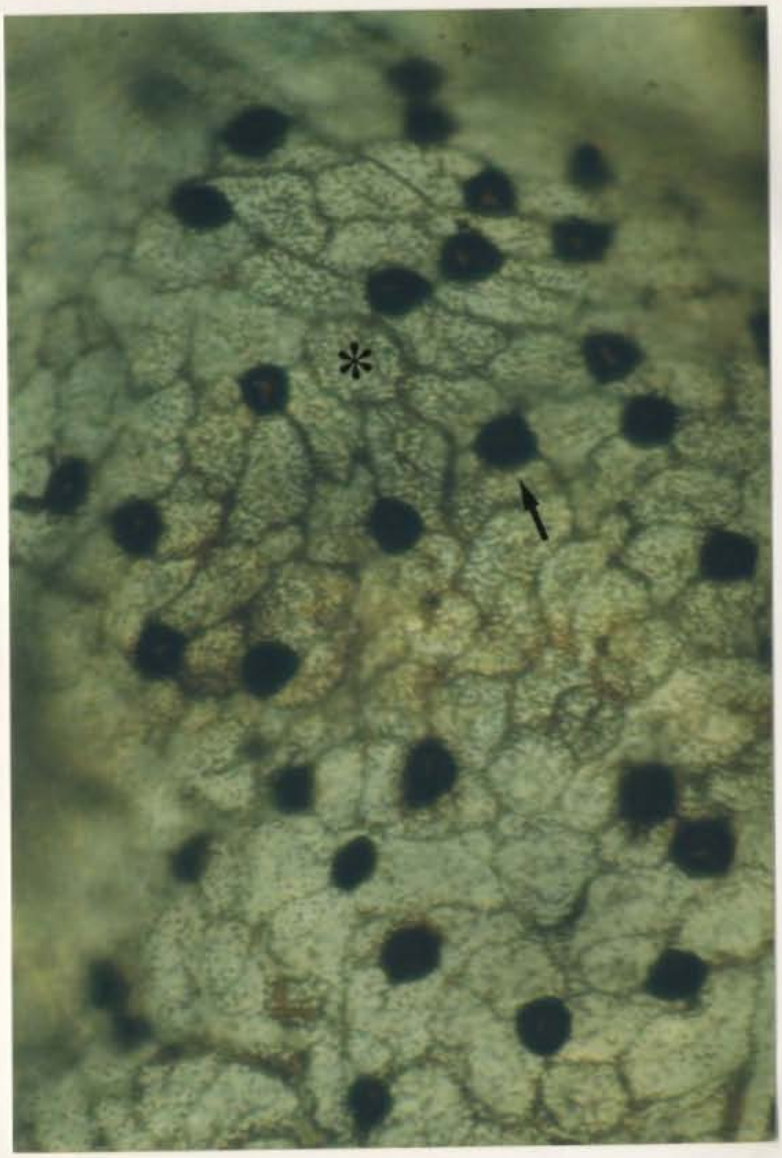


FIGURE 3.3. WINTER FLOUNDER OPERCULAR EPITHELIAL CHLORIDE CELLS

The winter flounder opercular epithelium contains DASPEI-positive (yellow cell indicated by thin arrow) chloride cells, while the underlying dermis has large, dark melanophores (thick arrow); note that, depending on their orientation, some of the chloride cells can be seen to be elongate and club-shaped; most cells, however, are viewed along their long axis, and appear circular or oval in cross-section.

Zeiss Axiovert 35 microscope; magnification: 200X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

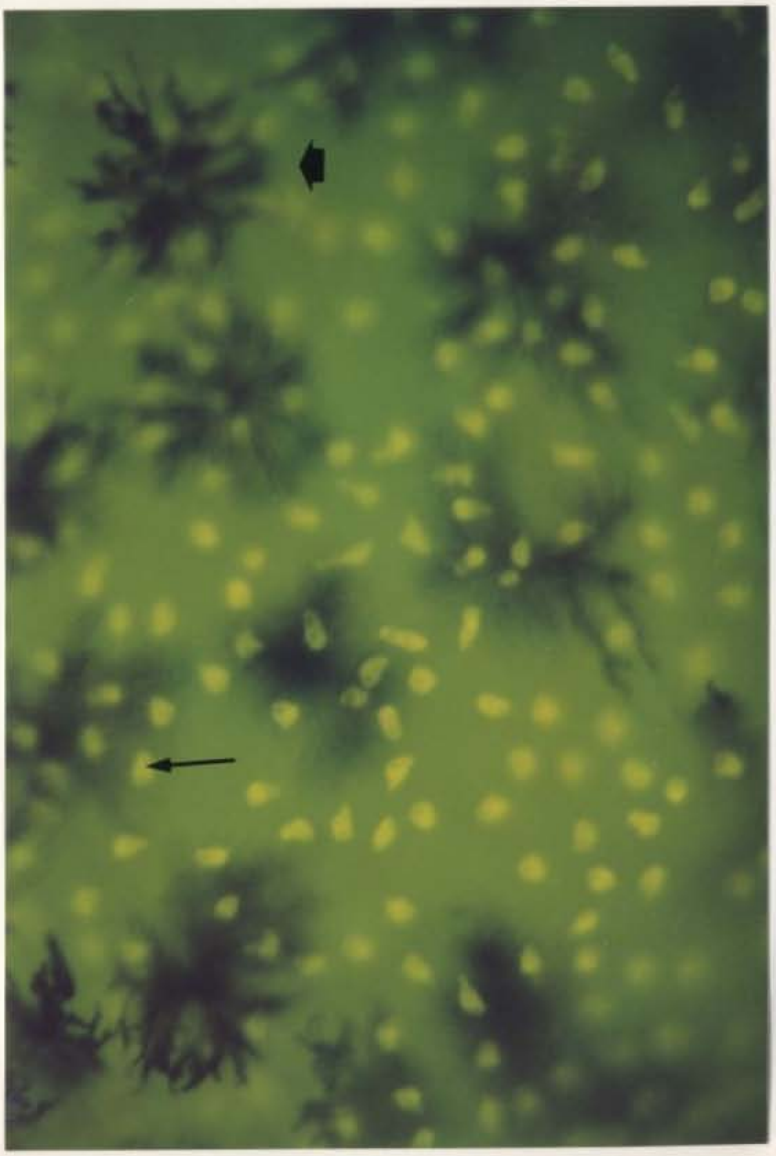


FIGURE 3.4. CUNNER OPERCULAR EPITHELIAL CHLORIDE CELLS

DASPEI-positive chloride cells in cunner opercular epithelium; note low densities of fluorescent chloride cells (thin arrows), but high concentrations of dark goblet cells (thick arrows); goblet cells contain mucin granules, do not stain with DASPEI, and appear as dark shadows outlined by dye in the interstitial fluids (thick arrows).

Zeiss Axiovert 35 microscope; magnification: 200X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

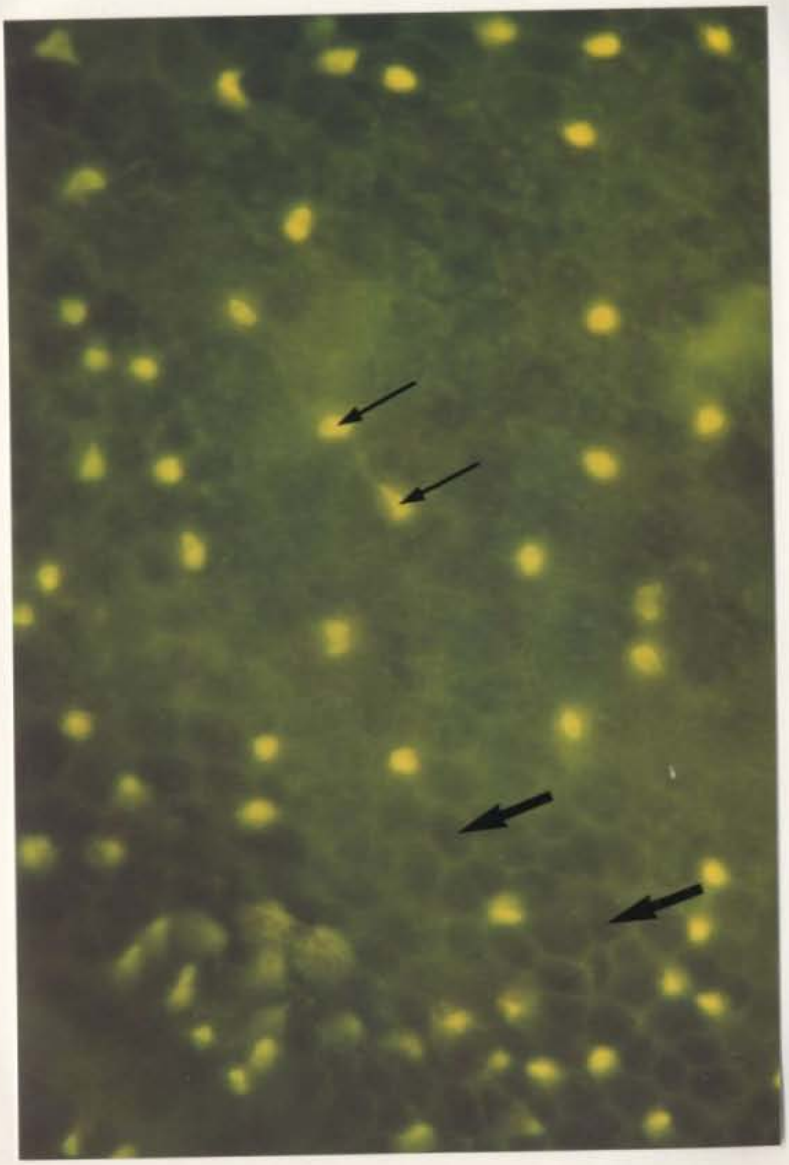


FIGURE 3.5. COD EMBRYO SHOWING GENERAL POSITION OF YOLK SAC

Cod embryo (18 d, 6°C) removed from chorion; yolk sac (Y) has collapsed; although viewed without fluorescence, the barrier filter admits only red transmitted light and thus the specimen appears to be red in color; the following figure (figure 3.6) shows the same specimen viewed with fluorescence to reveal the position of specifically labelled yolk sac chloride cells.

Olympus BHS microscope; magnification: 200X.

Green fluorescence filter set: 20BP545 excitation filter, DM570 dichroic mirror, and 20R610-W22 barrier filter.

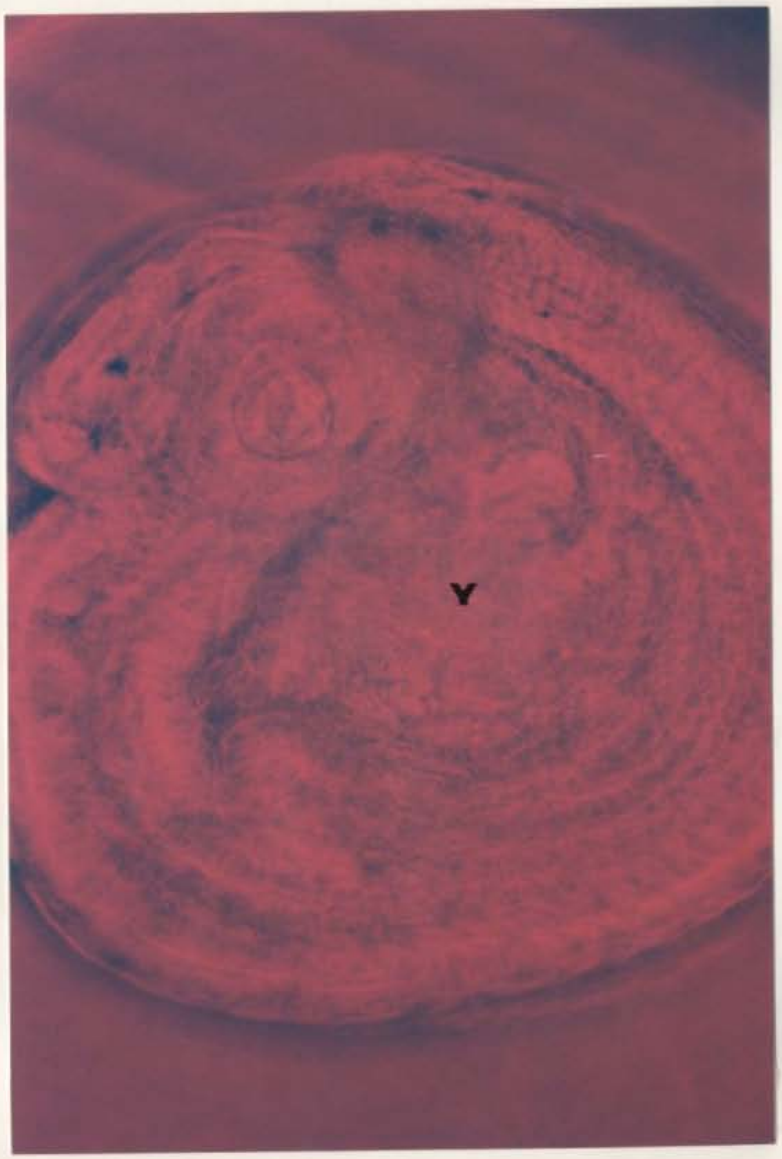


FIGURE 3.6. COD EMBRYO SHOWING CMXR STAINING OF YOLK SAC CHLORIDE CELLS

Cod embryo (18 d, 6°C) removed from chorion; yolk sac (Y) has collapsed; arrow points to a chloride cell labelled with the fixable mitochondrial dye, CMXR; this is the same specimen as used for figure 3.5;

Olympus BHS microscope; magnification: 200X.

Green fluorescence filter set: 20BP545 excitation filter, DM570 dichroic mirror, and 20R610-W22 barrier filter.

FIGURE 3.7. CHLORIDE CELL MITOCHONDRIAL AUTOFLUORESCENCE

Cod larva at hatch showing autofluorescent yolk sac (Y) chloride cells (arrow); note the individual mitochondria within the chloride cell are clearly resolved; the bright blue autofluorescence in the lower left corner (G) is due to the gut of the larva.

Olympus BHS microscope; magnification: 400X.

UV fluorescence filter set: 20UG1 excitation filter, DM400 dichroic mirror, and 20L435-W22 barrier filter.

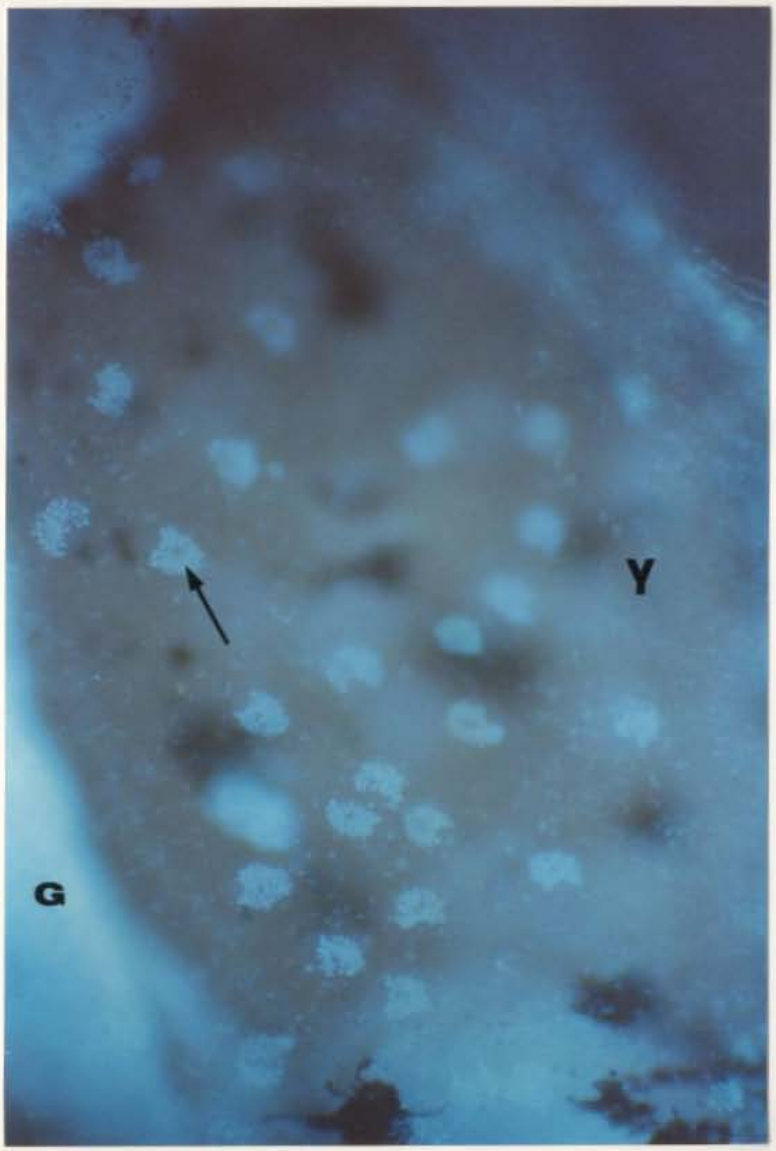


FIGURE 3.8. COD EMBRYO SHOWING DASPEI STAINING OF YOLK SAC CHLORIDE CELLS

DASPEI-positive chloride cells on the yolk sac (Y) of the cod embryo (16d, 2°C); the embryo has been removed from the chorion for the purpose of staining the chloride cells (arrow).

Zeiss Axiovert 35 microscope; magnification: 100X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

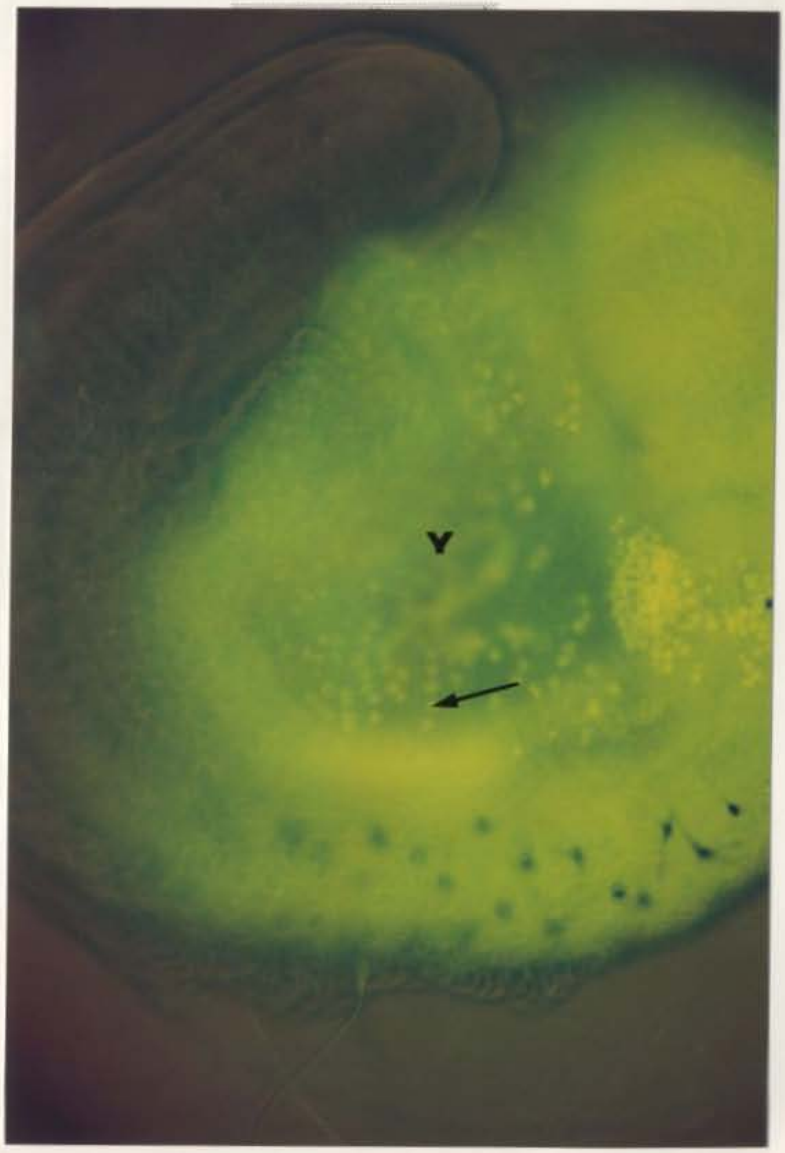


FIGURE 3.9. ADULT COD GILL FILAMENT LAMELLAE STAINED WITH CMXR AND FIXED IN KARNOVSKY'S FIXATIVE

Adult cod gill lamellae (L) with chloride cells stained by CMXR (arrow); this is the same specimen as used for figure 3.10;

Olympus BHS microscope; magnification: 100X.

Green fluorescence filter set: 20BP545 excitation filter, DM570 dichroic mirror, and 20R610-W22 barrier filter.

FIGURE 3.10. ADULT COD GILL FILAMENT LAMELLAE STAINED WITH CMXR AND FIXED IN KARNOVSKY'S FIXATIVE

Adult cod gill lamellae (L) with chloride cells stained by CMXR (arrow); this is the same specimen as used for figure 3.9, but at a higher magnification.

Olympus BHS microscope; magnification: 200X.

Green fluorescence filter set: 20BP545 excitation filter, DM570 dichroic mirror, and 20R610-W22 barrier filter.

FIGURE 3.11. COD EMBRYO YOLK SAC CHLORIDE CELLS STAINED WITH IN TETRACYCLINE

Tetracycline-positive chloride cells (arrow) on the yolk sac (Y) of a newly-hatched cod larva.

Zeiss Axiovert 35 microscope; magnification: 200X.

'UV' fluorescence filter set: 365 nm excitation filter, 395 nm chromatic beam splitter, and 420 nm barrier filter.

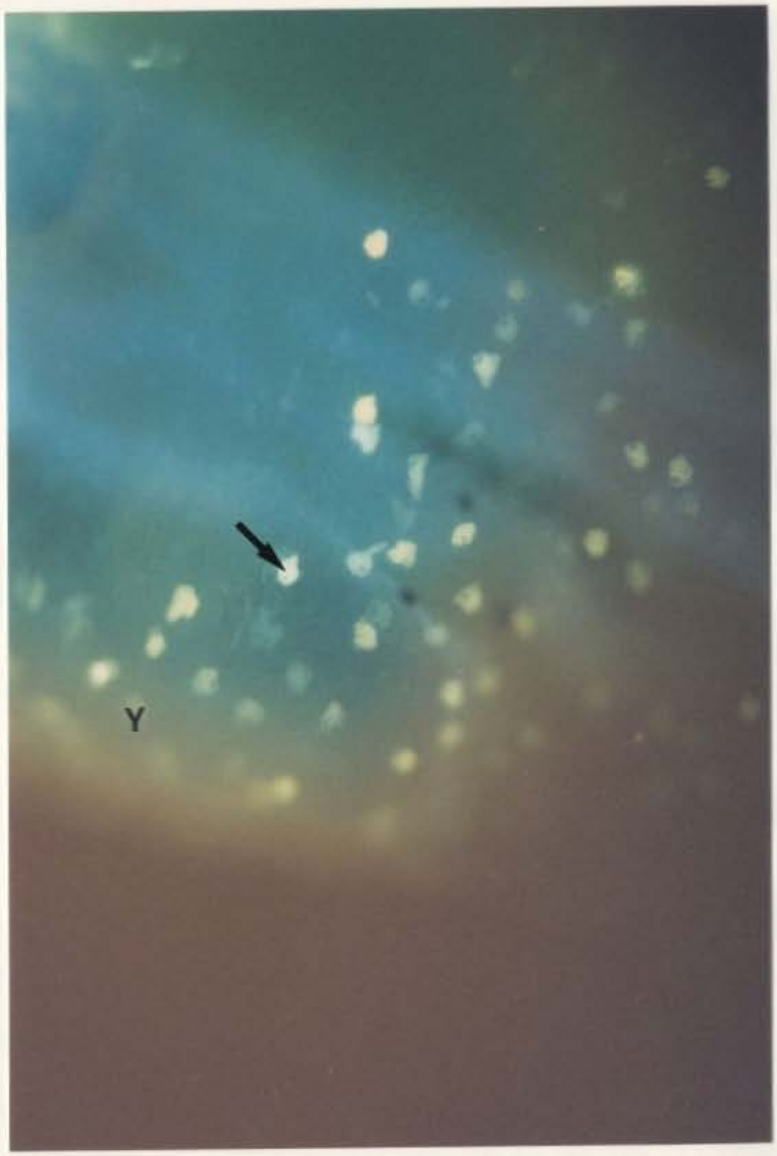


FIGURE 3.12. COD EMBRYO YOLK SAC CHLORIDE CELLS STAINED WITH NAO

Chloride cells (arrow) on the yolk sac of a cod larva at hatching; stained with NAO.

Zeiss Axiovert 35 microscope; magnification: 400X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

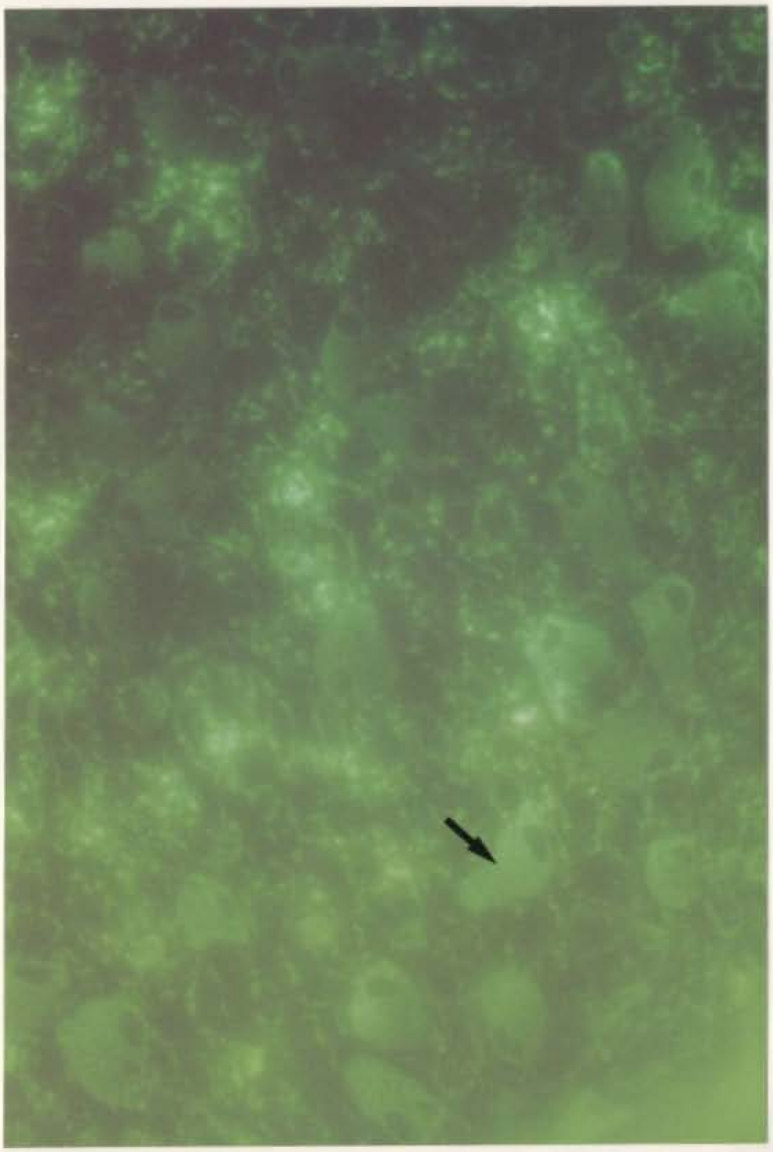


FIGURE 3.13. COD EMBRYO YOLK SAC CHLORIDE CELLS STAINED WITH $DIOC_6(3)$

Chloride cells (arrow) on the yolk sac of a cod larva at hatching; stained with $DIOC_6(3)$.

Zeiss Axiovert 35 microscope; magnification: 200X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

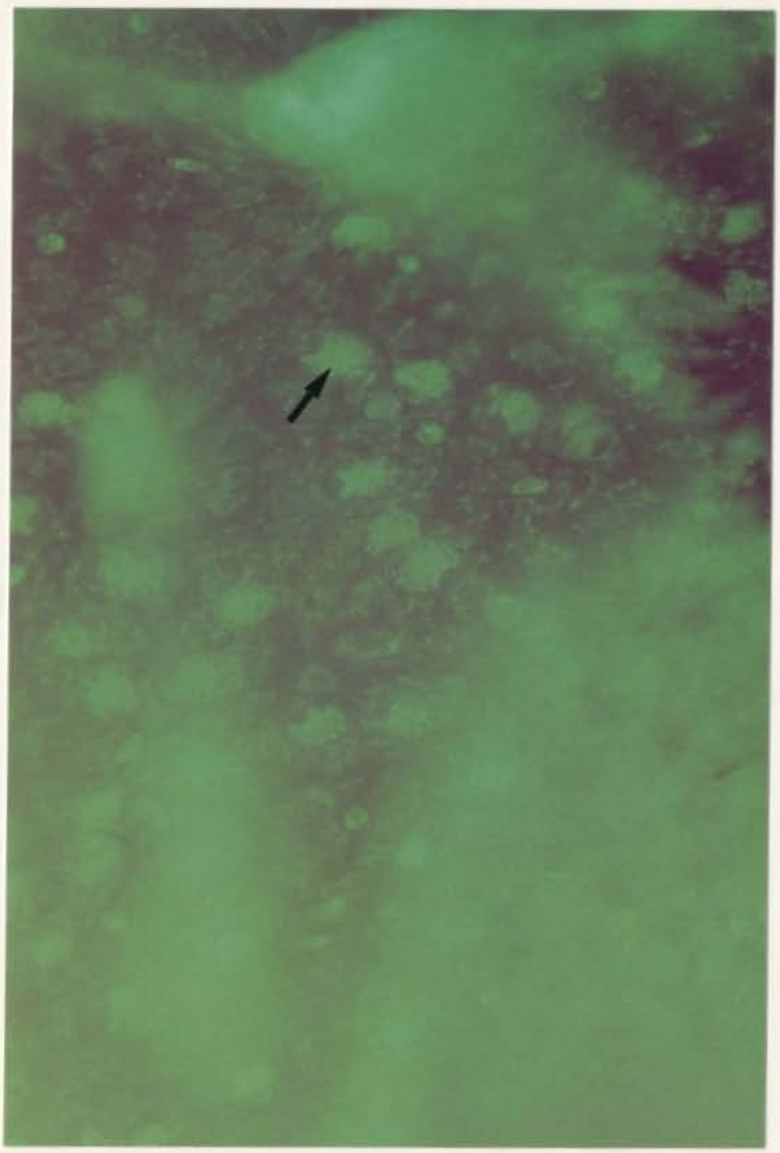


FIGURE 3.14. COD EMBRYO PHARYNGEAL CHLORIDE CELLS STAINED WITH RHODAMINE-123

Chloride cells (arrow) from the pharynx of a cod larva at hatching; this cell mass has been extruded from the pharynx by applying pressure to the cover slip over the head region of a larva on a microscope slide; the cells have been triple-stained with a mitochondrial dye, rhodamine-123 (green-yellow), a nuclear counterstain, ethidium bromide (orange), and a stain for Na^+, K^+-ATPase, anthroylouabain (blue, see next figure, 3.15); the arrow points to the orange nucleus of a chloride cell; this same cell is labelled in figure 3.15.

Zeiss Axiovert 35 microscope; magnification: 400X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

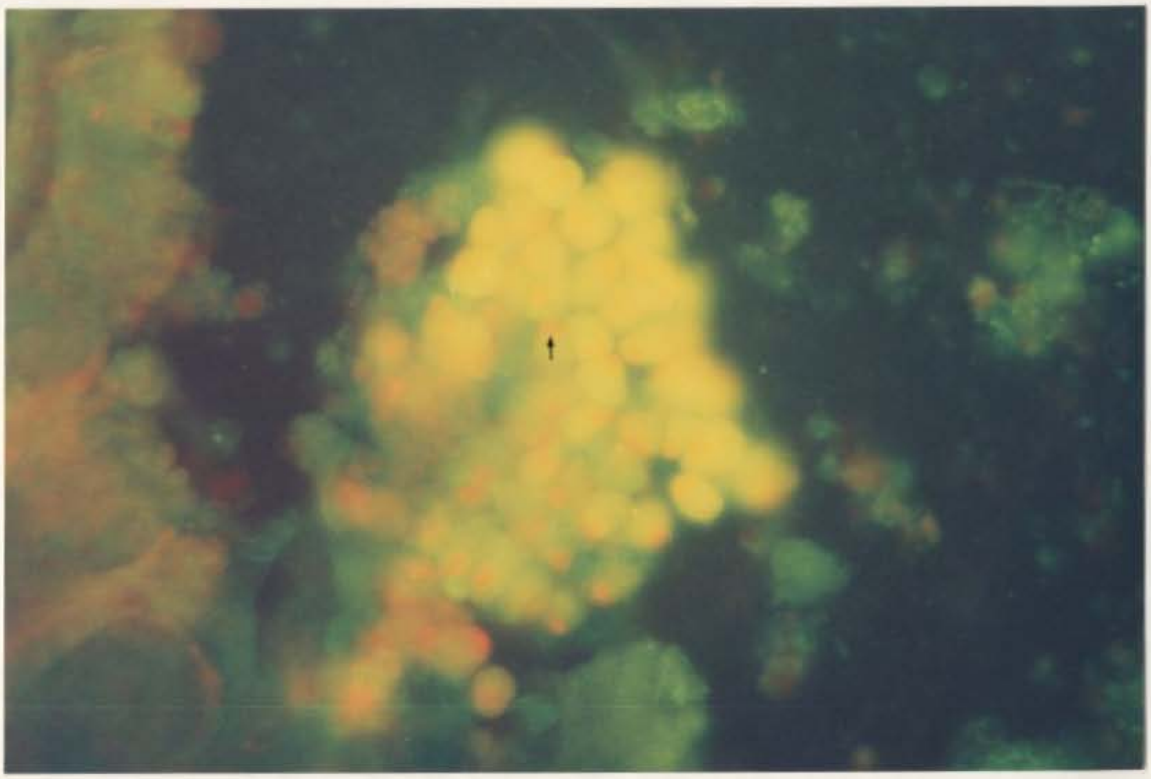
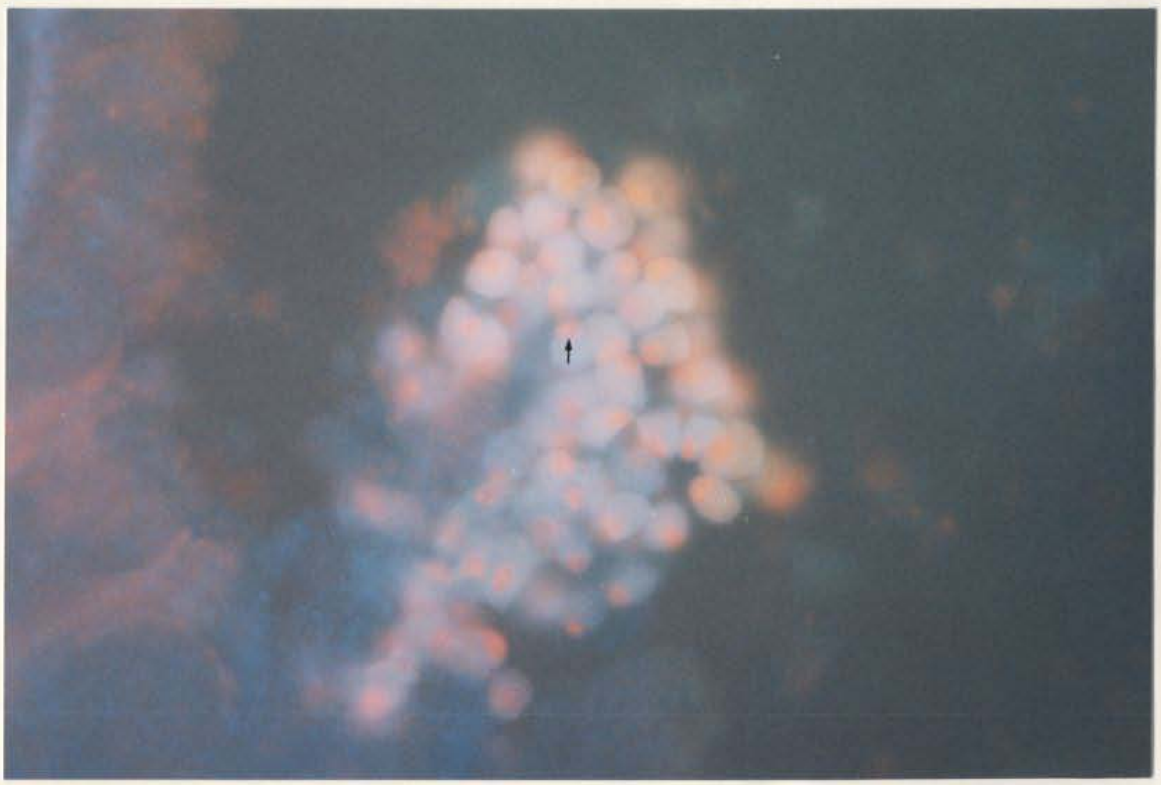


FIGURE 3.15. COD EMBRYO PHARYNGEAL CHLORIDE CELLS STAINED WITH ANTHROYLOUABAIN

Chloride cells (arrow) from the pharynx of a cod larva at hatching; this cell mass has been extruded from the pharynx by applying pressure to the cover slip over the head region of a larva on a microscope slide; the cells have been triple-stained with a mitochondrial dye, rhodamine-123 (green-yellow, see previous figure, 3.14), a nuclear counterstain, ethidium bromide (orange), and a stain for Na^+, K^+-ATPase, anthroylouabain (blue); the arrow points to the orange nucleus of a chloride cell; this same cell is labelled in figure 3.14; note that figures 3.14 and 3.15 indicate that chloride cells, which accumulate mitochondrial dyes, are also rich in Na^+, K^+-ATPase.

Zeiss Axiovert 35 microscope; magnification: 400X.

'UV' fluorescence filter set: 365 nm excitation filter, 395 nm chromatic beam splitter, and 420 nm barrier filter.



CHAPTER 4. THE ONTOGENY OF CHLORIDE CELLS IN THE ATLANTIC COD, Gadus morhua

ABSTRACT

Chloride cells are mitochondria-rich ion-transporting cells (ionocytes) that secrete chloride from epithelial surfaces. The origin and differentiation of presumptive chloride-secreting cells in Atlantic cod, Gadus morhua, were studied by a combination of light and electron microscopy. Ionocytes were stained using conventional mitochondrial dyes, potential-sensitive fluorescent mitochondrial dyes (including styryl and rhodamine dyes), a fluorescent ouabain derivative (anthroylouabain), a cardiolipin-specific probe (nonyl-acridine orange), tetracycline, and a lipophilic membrane probe ($DiOC_6(3)$).

A variety of secretory cells, including sacciform cells, goblet cells and ionocytes developed in the epidermis of cod embryos and larvae. In the embryo, ionocytes initially appeared on the yolk sac epithelium at the completion of epiboly. Following epidermal migration into the mouth and pharynx, chloride cells later appeared on the buccal/gill (oropharyngeal) cavity epithelium. Such cells were lenticular, and often had a large multilobed nucleus. Some appeared to be binucleate. Beyond yolk sac absorption, ionocytes appeared on the gill filament epithelium, and were retained on the oropharyngeal epithelia, but not the abdominal region. In postmetamorphic stages, oropharyngeal ionocytes become elongate and traversed epithelia that were several cells thick.

The gradual appearance of chloride cells in the yolk sac, pharyngeal, gill filament and buccal epithelia suggests that the chloride cells are ultimately derived from ectodermal tissue that invades the oropharyngeal cavity through a pair of small pores. These pharyngeal pores appear to be the precursors of the opercular openings that later lead to the gill cavities in the adult fish.

Active drinking of seawater by cod larvae did not appear to occur until after hatching, when the buccal and pharyngeal cavities become confluent, although the oesophagus and pharynx developed at a much earlier stage.

The appearance of chloride cells in the embryo at the end of epiboly when epithelia become leakier suggests that active salt secretion is required at an early stage in development.

INTRODUCTION

Ionocytes are cells that are metabolically and morphologically specialized for active secretion of salt ions against an electrochemical gradient using metabolic fuel. Such cells are mitochondria-rich, and have a system of cytoplasmic membranes with high levels of transporting enzymes, such as Na^+, K^+-ATPase. This cell type has been reported from a wide variety of secretory epithelia from invertebrate and vertebrate animals (Berridge and Oschman, 1972), and can be involved in salt transport *per se*, or in the production of electrochemical gradients for coupled transport processes (Stein, 1990). Their high mitochondrial membrane potentials are probably a consequence of the elevated respiration rates required to fuel ATP synthesis for active ion transport. Such potentials also form the basis for a number of staining reactions involving the intramitochondrial accumulation of lipophilic, fluorescent, cationic dyes (Chen, 1989; Schneider et al., 1994).

In adult marine teleost fishes, ionocytes occur in the renal tubules, in the gill tissues, gut epithelia, and oropharyngeal epithelium (Berridge and Oschman, 1972; Marshall, 1988). Ionocytes associated with the buccal cavity and pharynx, including the gill and opercular epithelium are recognized as mediators of sodium and chloride transport (Conte, 1980; Marshall, 1988). These so-called 'chloride

cells' appear to be required for the maintenance of osmotic balance in the marine environment (Komnick, 1986). While similar cells in freshwater fishes, amphibia, and non-teleost marine fishes may be concerned with salt ion uptake (Flik et al., 1993), or even proton secretion (Mallatt et al., 1987; Harvey, 1992), there is considerable evidence to support the chloride-secreting and absorbing functions of the marine teleost chloride cell and of certain mitochondria-rich cells from amphibians. For example, chloride currents can be localized to the apical surfaces of such cells in both amphibia (Katz and Scheffey, 1986) and fishes (Foskett and Scheffey, 1981).

The differentiation of ionocytes has previously been examined in several amphibian and teleost fish species. In anuran and caudate amphibian tadpoles, ionocytes initially differentiate in the gill epithelium (Dietz and Alvarado, 1974; Cox, 1979). As the gills are gradually resorbed during metamorphosis, ionocytes then appear on the skin, and in the urinary bladder (DiBona et al., 1969). Adult amphibia, like other vertebrates, also possess mitochondria-rich kidney tubule cells that transport ions (Berridge and Oschman, 1972). More recent studies also indicate the transient occurrence of ionocytes in the pancreatic ducts of amphibian tadpoles (De Zarate et al., 1993).

In teleost embryos and larvae, ionocytes have generally been referred to as 'chloride cells', even in the absence of direct physiological measurements of ion secretion. Circumstantial evidence, however, indicates that mitochondria-rich epithelial cells in marine teleost embryos and larvae are responsible for chloride ion secretion (Shirai and Utida, 1970; Hwang, 1989; 1990; Hwang and Hirano, 1985). Physiological studies have shown that the early stages of marine fishes, like the adults, have tissue fluids that are hyposmotic to the seawater medium (Mangor-Jensen and Adoff, 1988; Brown and Tytler, 1993). The marine teleost, therefore, must actively excrete salts and retain and/or acquire water to maintain an osmotic balance (Guggino, 1980; Brown and Tytler, 1993).

Both the timing of appearance of chloride cells, and their tissue distribution during the early development of fishes are unclear. Chloride cells reportedly first appear on the yolk sac and gill cavity epithelia (Adoff, 1986; Hwang, 1989), and later in the gill filament epithelium (Morrison, 1993). However, they have also been described from the skin of marine fish larvae at metamorphosis (Pfeiler and Lindley, 1989), or from the pericardium (Dépêche, 1973; O'Connell, 1981). Some studies have reported their appearance only after hatching (Lasker and Threadgold, 1968), while others claim that they differentiate in the embryo at the completion of, or

during, epiboly (Alderdice, 1988). Adoff (1986) claimed that mitochondria-rich cells visible in the blastula stage of the Atlantic cod prior to epiboly were chloride cell precursors.

The present study was undertaken to investigate the origin and proliferation of chloride cells in the Atlantic cod, Gadus morhua, using ultrastructural methods and specific probes for light microscopic localization of mitochondrial components and ion-transporting enzymes. The distribution and morphology of chloride cells in juvenile and adult cod were also examined for comparison with those of the early stages. Cod embryos and larvae are known to possess specialized, mitochondria-rich cells similar to those implicated in salt excretion from the gills of adult marine fish (Morrison, 1993). However, little is known about the origin and tissue distribution of such cells in the early stages of development (Adoff, 1986; Morrison, 1993).

METHODS AND MATERIALS

Maintenance of Adult Cod and Collection of Eggs

Adult cod used for spawning stock (>40 cm) were obtained during a Department of Fisheries and Oceans cruise by the *Chinook* in Trinity Bay, Newfoundland during June, 1993. Some additional fish were obtained from the 'Sea Forest Plantations' aquaculture facility at Bay Bulls, Newfoundland in 1992. Fish were transported to the marine laboratory at Logy Bay, Newfoundland.

Bay Bulls fish spawned from mid-April of 1993 to late June, 1993. Trinity Bay fish spawned in the same year, from the time of collection, in late June, 1993 into the fall of 1993. However, Trinity Bay fish were maintained in aquaria until egg collection during the next spawning season (from March to July, 1994).

Embryo Incubation Conditions

All eggs were collected within 24 hours of spawning, and were incubated under ambient conditions of salinity, temperature and photoperiod in rectangular plastic tubs according to procedures described elsewhere (Valerio et al., 1992).

During the incubation period for all the egg batches, temperature varied from 0.3°C (March) to 9.0°C (July), while salinity varied from 32.15 ppt (March) to 32.80 ppt (July). For individual batches, however, temperature changes were smaller.

Additional batches of embryos were incubated in 1-liter flasks at a constant temperature (5 to 6°C) in a cold room in seawater containing penicillin (100 U/ml) and streptomycin sulfate (0.1 mg/ml).

Processing of Epithelia for Staining

Intact embryos were viewed in a small chamber created by applying a ring of silicone vacuum grease (Dow Corning) to a microscope slide. The 'well' was filled with stain-free

isotonic saline, and the embryo was transferred to it before applying a cover-slip. Additional specimens were viewed on a plain slide after dissecting the yolk sac free from embryos using fine insect pins (Fine Science Tools, Inc., Vancouver).

In some cases, chloride cells were isolated from adult cod gill filaments (Hootman and Philpott, 1978; Perry and Walsh, 1989; Pärt et al., 1993), then were stained while suspended in saline solutions containing dyes.

Staining Protocols for Light Microscopy

Chloride cells were stained using protocols described in detail in the previous chapter (chapter 3). Staining methods included Altmann's stain (Gabé, 1976), the Champy-Maillet method (McCormick, 1990a), silver-precipitation methods (Philpott, 1965; Van Steveninck and Van Steveninck, 1978; Holliday et al., 1990), fluorescent membrane probes: nonyl acridine orange and $DiOC_6(3)$ (Terasaki et al., 1984; Petit et al., 1992), potential-sensitive mitochondrial dyes: DASPEI; rhodamine-6G and rhodamine-123; MitoTracker™ dyes (Chen, 1989), and others, including anthroylouabain (McCormick, 1990b) and tetracycline.

Reduced intramitochondrial pyridine nucleotides fluoresce when they absorb UV light at about 365 nm (Hackenbrock, 1966), so that some fluorescence could be observed without fluorescent stains. For example, the NADH emission maximum is

about 450 nm (Tokuka et al., 1992). These fluorescence signals were also used to verify the location of MR cells.

Photomicrography

Embryos and larvae stained with fluorescent dyes were viewed and photographed with a Zeiss Axiovert 35 inverted microscope modified for epifluorescence with a 50W HBO illuminator. Specimens were viewed with either a 450-490 nm band-pass excitation filter, a 510 nm chromatic beam splitter, and a 515-565 nm bandpass filter, or a 365 nm excitation filter, 395 nm chromatic beam splitter, and a 420 nm longpass filter. Photographs were then taken using a Contax 167 MT camera with 400 ISO color Kodak film.

Additional photographs of stained embryos and larvae were obtained with an Olympus BHS microscope equipped with an Olympus PM-10ADS photomicrographic System and BHS-RFC reflected light fluorescence attachment.

Specimens stained with non-fluorescent methods were viewed and photographed with a Nikon N2000 camera attached to a Nikon Diaphot-TMD inverted microscope fitted with phase contrast optics.

Scanning and Transmission Electron Microscopy

Specimens viewed by electron microscopy were collected and processed as described in chapter 3.

Use of Extracellular Markers to Trace Body Cavities

Since the development of an active ion-regulating mechanism might be expected to coincide with increased exposure to the hypertonic seawater medium (Riis-Vestergaard, 1984), additional experiments were performed to examine the development of drinking behaviour in the cod, and to assess the stage of development of the mouth, gills and pharynx before and after hatching.

Fluorescently-labelled extracellular markers, including tetramethylrhodamine-labelled dextran (MW = 70,000; Molecular Probes, Inc.), FITC-labelled gamma globulin (Sigma Chemical Co.), and 1 μm fluorescently-labelled latex particles (Molecular Probes, Inc.) were used to trace the opening of the stomodeum, pharynx and oesophagus in the cod embryo and larva. Dechorionated embryos and hatched larvae were incubated for times ranging from 5 minutes to 24 hours in saline containing dextran (0.25mg/ml), gamma-globulin (1:100 [V/V] dilution), or latex particles (at approx. 0.002% [W/V] solids).

Enumeration of Chloride Cells.

Profile counts of chloride cells were performed using fresh embryos and larvae. Chloride cells were identified under UV light by mitochondrial autofluorescence, and both pharyngeal and yolk sac chloride cell counts were determined by counting the number of chloride cells on one side of each individual. Therefore, pharyngeal and yolk sac chloride cell

counts represent one half of the chloride cells observed in the pharynx and on the yolk sac. Mean pharyngeal and yolk sac counts were determined from 12 individuals sampled periodically from batches of embryos and larvae cultured at 6 °C. For individuals beyond yolk sac absorption, 'yolk sac' cell counts actually refer to chloride cells persisting over the abdominal region (from the posterior limit of the pericardial cavity, back to the anus, and dorsally to the upper limit of the gut).

Although such counts are estimates of cell numbers on curved surfaces, by adjusting the microscope focus it was possible to obtain consistent (low standard error of the mean) counts of cells. Since mean counts did not differ statistically between left and right sides, mean counts were based upon pooled data for left and right sides of embryos and larvae.

RESULTS

Detailed comparisons of chloride cell staining methodology have been described in the previous chapter (chapter 3). The results presented here will focus primarily on the general trend in chloride cell distribution during the development of the cod, and the relation of these events to the development of various related osmoregulatory adaptations, including the development of the mouth, pharynx, gut and kidney.

General Aspects of Staining Reactions

Generally, potential-sensitive dyes gave positive results with several types of mitochondria-rich cells, including neuromast cells, hepatocytes, renal epithelial cells, and chloride cells. However, chloride cells were readily identifiable as large, mitochondria-rich cells with high-potential mitochondria and an apical secretory surface exposed to the surrounding medium. The identity of the cells was confirmed by the presence of numerous cytoplasmic tubules in specimens prepared for TEM, and by additional staining reactions for light microscopy.

The most useful methods for the analysis of chloride cell distribution included the use of potential-sensitive stains, phase contrast microscopy, mitochondrial autofluorescence, anthroylouabain staining and the membrane probes, $DiOC_6(3)$ and nonyl acridine orange (NAO).

Numbers of MR cells visible with classical staining methods, such as Altmann's method, were fewer than that for sections from the same specimen viewed by TEM. The specificity of many staining methods, including the Champy-Maillet method, Altmann's technique, and techniques of silver-staining were deemed to be insufficient for routine work with chloride cells in cod tissues.

Potential-sensitive Mitochondrial Probes

Initial tests with the reduced forms of the chloromethylated derivatives of X-rosamine (CMXR-H_2) and tetramethylrosamine (CMTMR-H_2) revealed limited penetration through the cell membrane. However, the oxidized form of chloromethylated X-rosamine (CMXR) gave more promising results. CMXR staining was, however, quite pronounced for the mitochondria in several other types of secretory cells, including chloride cells, particularly at high concentrations or before washing in dye-free saline. It was believed that this resulted from a more rapid penetration of such cells through the apical secretory surface, as pavement epithelial cells were not markedly stained at the same dye concentration. When combined with phase contrast microscopy, CMXR staining was a useful method for identifying chloride cells in fixed epithelia.

Epithelia from embryonic, larval, juvenile and adult cod contained chloride cells that stained positively with conventional potential-sensitive mitochondrial stains, including rhodamine-6G, rhodamine-123, and DASPEI (figure 4.1).

Na^+, K^+-ATPase Staining

Simultaneous treatment of isolated cells or intact tissues with potential-sensitive probes and anthroylouabain

appeared to show that Na^+, K^+-ATPase levels are higher in chloride cells than in other epithelial cells.

Fluorescent Membrane Probes and Mitochondrial Autofluorescence

Yolk sac chloride cells were also readily stained by membrane probes, including $DiOC_6(3)$ and NAO. Tetracycline also produced a staining reaction in chloride cells (see figures 3.11, 3.12 and 3.13 in chapter 3).

Autofluorescence in UV light, due to NADH and other pyridine nucleotides, was also useful for examining chloride cell distribution in live embryos, although background tissue autofluorescence sometimes obscured this effect.

Scanning Electron Microscopy (SEM)

The adult cod gill and oropharyngeal epithelium exhibited surface pores that could be identified as the apical surfaces of sacciform, chloride and goblet cells in sectioned (TEM), fractured (SEM) or enzymatically dissociated (SEM) material (figures 4.2 and 4.3). However, some of these secretory pores were also associated with goblet and sacciform cells (Morrison, 1988).

Transmission Electron Microscopy (TEM; see chpt. 3, fig. 3.1c)

Numbers of MR cells visible with classical staining methods, such as Altmann's method, were fewer than for sections from the same specimen viewed by TEM. In addition, Altmann's method yielded a high 'background' staining reaction.

MR cells , identified as chloride cells, were visible on gill and oropharyngeal epithelia. The ultrastructure of MR cells differed somewhat, even within the same section of tissue. In some cases, large cells with numerous lightly-stained mitochondria were visible in the same section as smaller cells that had more darkly-stained mitochondria. In all cases, such cells had numerous cytoplasmic tubules, readily identified as part of the tubulovesicular membrane system (Pisam and Rambourg, 1991).

Tissue Distribution of Chloride Cells

A variety of secretory cells, including sacciform, goblet and chloride cells, developed on the epidermis of cod embryos and larvae. The absence of high mitochondrial potentials in some (MR) mitochondria-rich cells complicated their identification as chloride cells or their precursors. However, when MR cells were found at tissue sites that later harboured chloride cells, it was concluded that these MR cells were likely candidates for chloride cell precursors.

Chloride cells first appeared on the yolk sac, and in the epithelial lining of the embryonic pharynx (figure 4.4). The progressive changes in chloride cell distribution during development of the Atlantic cod (at 6 °C) are outlined as follows:

a. Fertilization to Blastula Formation (0-2 days)

As the blastula formed by division of the first blastomeres, a surface layer of tightly-joined cells, [1]referred to here as the enveloping layer, developed. This layer covered the deeper layer of blastomeres. The enveloping layer of the early cod blastula consisted of largish, bulging cells with numerous small vacuoles and mitochondria in the cytoplasm. Mitotic figures were frequently evident in semi-thin sections, particularly in the deep blastomere layer (not shown in figures). These early MR cells had very little autofluorescence (due to pyridine nucleotides, such as NADH), and did not stain with potential-sensitive dyes. In fact, most staining reactions were absent until near the completion of epiboly (see below), when the MR cells in the yolk sac and pharyngeal cavities began to exhibit high levels of metabolic activity. These changes were reflected in cell autofluorescence and staining by various fluorescent probes.

In the early blastula, the periblast was readily discerned in the transmission electron microscope as an

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[1]Other authors have been inconsistent in their use of terminology for this layer. For example, Bouvet (1976) referred to the earliest superficial layer as the peripheral layer, the term 'enveloping layer' being reserved for a later stage, when the outer cells are more tightly apposed, and they no longer contribute to the formation of the deeper cell layers by mitosis and cell migration.

electron-dense region at the yolk surface. Scattered nuclei and secretory vesicles were seen throughout this yolk layer.

b. Onset of Blastoderm Migration (epiboly) (2-3 days)

During epiboly, some of the MR cells in the second cell layer became flattened, although did not yet contact the perivitelline fluid at an exposed apical surface. Surface views of the embryo at this stage (figure 4.5), as well as sectioned material do not reveal the presence of any secretory pits connecting the subsurface cell layer with the embryonic surface.

As the blastoderm migrates over the yolk sac, the outermost cells, 'the enveloping layer', develop prominent surface ridges, or microplicae. At this time, the enveloping layer forms a typical pavement cell epithelium, with tight junctions between the flattened pavement cells. Desmosomes and hemidesmosomes (see Kelly, 1966) were abundant in the outermost cell layer.

Prior to the completion of epiboly, mitochondria-rich cells appeared over the blastoderm and in the future gill cavities. At this stage the cells do not exhibit high mitochondrial potentials, as indicated by the absence of positive staining by mitochondrial probes such as DASPEI or the rhodamine derivatives, Rh-123 and Rh-6G.

c. Completion of Epiboly (4-5 days)

As the time of blastopore closure approaches, a pair of pharyngeal 'pores' (Guggino, 1980), precursors of the gill cavity openings, appeared posterior to the embryonic head (figure 4.6). These pores were round, and lacked associated opercular flaps, since the embryonic mouth (stomodeum) was closed, gills were absent and the embryo was incapable of ventilating. At this stage, the pharyngeal and buccal cavities are not yet confluent, but the pharynx is lined by chloride cells (figure 4.7). Although the mouth was closed until just before or just after hatching (depending on the temperature), the open pores probably allowed the fluid of the pharyngeal cavity to become isotonic with the perivitelline fluid that surrounded the embryo. As the embryo developed, the pores advanced anteriorly to their final position in the adult fish. At this stage, the anterior part of the cavity, the stomodeum or primordial buccal cavity, is separated from the pharynx by a septum, the oropharyngeal membrane (Morrison, 1993). MR cells appeared to be confined to the posterior pharynx.

By the time the blastoderm had almost covered the entire yolk surface, the embryo was already well-developed. It was at this stage that the mitochondria in the chloride cells began to fluoresce brightly when the embryo was stained with potential-sensitive styryl dyes, such as DASPEI (figure 4.8). At this time, mitochondrial autofluorescence also increases.

The outer epithelium, referred to as the periderm from now until hatching, lies over 2 to 3 deeper cell layers. The chloride cells normally occur in the second cell layer, just below the periderm. Their basal surface only rarely reached the basement membrane of the embryonic skin, while the apical surface was exposed to seawater through a perforation in the periderm. This situation is similar to that for the early stages of the Pacific sardine, Sardinops caerulea, as described by Lasker and Threadgold (1968), although it differs markedly from that described by Roberts and coworkers (Roberts et al., 1973) for larvae of the plaice, Pleuronectes platessa. In the plaice larva, chloride cells have been reported to rarely make contact with the body surface, and have small, round nuclei (Roberts et al., 1973). This observation, however, may be based on small numbers of samples, since most TEM sections through chloride cells do not go through the small, apical secretory surface.

Chloride cells on the yolk sac and skin surface were lenticular, and often had a large multilobed nucleus (figure 4.9). Chloride cells in the pharyngeal cavity were more irregularly-shaped. In all regions, a few cells appeared to be binucleate (figure 4.10). Mitochondria were numerous and the apical surface was exposed to the external medium through a gap in the periderm (figure 3.1c, chapter 3).

However, such 'pits' were not always the secretory surface of chloride cells, since sacciform and goblet cells were also exposed to the external medium (= perivitelline fluid). These observations were consistent with the findings of Adoff (1986) and Morrison (1993). Surface views of the periderm revealed several types of pores, corresponding to the apical surfaces of goblet (mucous), sacciform and chloride cells (figure 4.11). Initially, goblet cells were found infrequently, but later predominated as the epidermal secretory cell of the adult fish. In the early stages, the dominant secretory cell was the sacciform cell.

During the remainder of development little change in the distribution of chloride cells was observed in the embryo, although cell numbers tended to increase toward the time of hatching.

d. Peak (50%) Hatching (16 days)

At hatching, the yolk sac chloride cells fluoresced intensely when stained with DASPEI, particularly if the yolk sac was punctured to allow basal entry of the dye into the cells (see Methods section). At hatching, the gill cartilage was formed, but filaments were absent.

Chloride cell distribution at hatching was essentially unchanged from that after the completion of epiboly, with cell being found in the pharyngeal cavities, and on the yolk sac surface (figure 4.12).

At hatching, the shallow stomodeum, formed by invagination of ectodermal epithelium (Manner, 1975), did not contain chloride cells. At this stage, the pharynx is still separated from the stomodeum by the oropharyngeal membrane (figure 4.12). Experiments with fluorescent dyes revealed that the membrane persisted for one day after hatching, but that after one day at 6°C, the gut was filled with dye during drinking activity.

e. Yolk Sac Absorption (23-25 days)

Although chloride and sacciform cells continue to predominate as the secretory cell types of the larval epidermis, occasional goblet (mucus-secreting) cells are observed. Abdominal chloride cells persist beyond yolk sac absorption, although their numbers diminish as the abdominal surface area increases. At the same time, orophayngeal chloride cell numbers are increasing (figure 4.13).

f. Formation of Gill Filament Epithelium (20-50 days)

Although occasional chloride cells occur on the gill arch from about 20 days (at 6°C), at this stage the gill filaments and their lamellae are absent. The formation of an epithelial covering of pavement cells on the gill filaments occurs relatively late in the cod, at about 40 to 50 days. Chloride cells tend to occur on the filament near the base of the lamellae, as in the adult cod. At the same time, abdominal chloride cells gradually disappear.

These events more or less parallel the process of metamorphosis, during which the larval cod acquires morphological and behavioural characteristics of the juvenile stage. As the cod larva metamorphoses, the gill and buccal cavities become distinct, the gill opening becomes elongate, and the gill cavity is covered with an opercular flap. Each of these epithelial surfaces is lined with chloride cells, as are the gill epithelia.

g. Juvenile (> 40 days) and Adult Cod (> 3 years)

At the juvenile stage, chloride cells are found primarily in the oropharyngeal cavity, including the gill filament epithelium. A few cells are occasionally seen on the gill lamellae, and even the arches. Some of the chloride cells lining the oropharyngeal cavity, or their progeny, persist into the adult stage as chloride cells that line the mouth and gill cavities, the inside of the opercular flap, and the scaleless region of skin just posterior to the gill cavity (Marshall et al., 1992), referred to here as the 'cleithral' epithelium, since it covers the region where the cleithral bone is found. It may be noted, however, that there is no distinct association between chloride cells and the cleithral region early in development, although this is one of the first bones to appear in the larval cod.

Examination of abdominal skin from juvenile and adult cod did not reveal the presence of MR cells or chloride cells. The

patterns of chloride cell distribution in the Atlantic cod are summarized in figure 4.14.

DISCUSSION

Are Chloride Cells Polyploid or Binucleate?

The apparent existence of binucleate chloride cells in the early stages of the cod suggests the possibility of a transitional tetraploid state for this cell type. As with chloride cells on the yolk sac of cod larvae, chloride cells from adult cod epithelia are sometimes binucleate, perhaps indicating active nuclear division (endomitosis) in the fully differentiated state. This, however, does not resolve the question of whether chloride cells are recruited from other cells in the epithelial cell population, or arise from fully differentiated chloride cells.

Although binucleate and mononucleate tetraploid cells have been occasionally described in the somatic tissues of developing vertebrates, their function is not well understood (Brodsky and Uryvaeva, 1977; Davies et al., 1993; Meyer and Nagl, 1993). For example, in the liver of weaning rats, tetraploid binucleate cell frequencies peak at about 50% of the hepatocyte population, thereafter declining (Davies et al., 1993). However, most chloride cells (> 90%) in cod embryos and larvae appeared to possess a single, multilobed nucleus. These 'polymorph-like' cells may in fact be polyploid, although this question may not be resolved without

the use of flow cytometric methods (Davies et al., 1993). Similar 'binucleate' cells resembling chloride cells have been observed by TEM in the pseudobranch of Atlantic cod (Morrison, 1988).

An additional possibility is that the multilobed nucleus represents a stage in the gradual fragmentation of the nucleus during apoptotic cell death. Apoptotic nuclear fragmentation in cells has been widely reported under both pathological conditions and during normal development (Pesce and De Felici, 1994; Re et al., 1994). Similar degenerative changes have also been described from the gill chloride cells of euryhaline fishes when they are transferred to seawater (Pisam et al., 1987; Pisam and Rambourg, 1991).

Chloride Cell Shape Reflects Epithelial Thickness

Chloride cells in the 'skin' of embryonic and larval stages of the cod are lenticular ('flattened ellipsoids', in the terminology of Threadgold and Lasker, 1967; and Lasker and Threadgold, 1968) when they occur in the intermediate cell layer of the embryonic skin epithelium, but somewhat more irregularly-shaped when found in the lining of the primordial gill cavity. It may be noted, however, that columnar-shaped skin chloride cells have been described in larval teleosts that have a relatively thin epidermis (Hwang, 1990).

Variation in chloride cell shape was explained by Hwang (1989) as a consequence of the thickness of the epithelium in

which the cells reside. This hypothesis is probably correct, since thicker epithelia, such as that of the adult cod inner operculum contain long, columnar chloride cells with their long axis oriented perpendicularly to the plane of the epithelium. When sectioned through their long axis, most opercular chloride cells appeared to extend throughout the epithelium, so that the basal surface rested on the epithelial basement membrane, and the apical surface was exposed to the seawater. Since chloride cells in the later stages of teleosts tend be more columnar, it is possible that their shape is changeable. Isolated columnar chloride cells from the buccal and pharyngeal epithelia of the Atlantic cod tend to round up and become spherical within a few hours in saline media (chapter 3).

Tissue Distribution of Chloride Cells

Chloride cells in the gill, buccal, cleithral and opercular epithelia are probably descendants of the pharyngeal chloride cell population in the embryonic cod.

In some teleost fishes, including the Atlantic cod, functional gills are not present in the earliest developmental stages, and the differentiation of gill chloride cells does not occur until some time after hatching (Hwang, 1990; Valerio et al., 1992; Morrison, 1993). This pattern seems to be typical of other teleosts, such as the ayu, Plecoglossus altivelis, flounder Kareius bicoloratus and carp, Cyprinus

carpio (Hwang, 1989). However, a more advanced state of differentiation has been described for the gill epithelium in freshwater-adapted trout, Salmo gairdneri (Morgan, 1974a; 1974b), which contains discernible chloride cells at hatching (31 days postfertilization at 10°C), and in higher numbers than found in the adult gill. These differences may be due to the higher incubation temperatures used for trout eggs, although chloride cells do not appear in the gills of the goby, Chaenogobius urotaenia, until about two weeks after hatching (10 to 20 °C rearing temperature), according to Hamada (1968). They are also absent from newly-hatched ayu reared at 17 to 20°C (Hwang, 1990).

Contrary to Dépêche's (1973) claim that teleost embryos have chloride cells in the pericardium, no chloride cells were ever observed in the serous internal membrane or external covering of the pericardial sac of adult, larval or embryonic cod. Numerical estimates of chloride cell densities given by Dépêche (1973) appear to be based upon counts of secretory surface pits viewed by SEM, a technique that was found to be inappropriate for localizing chloride cells in the present study, due to the presence of numerous surface pits of sacciform and mucus cells in epithelia.

Similarly, O'Connell's (1981) description of chloride cells in the 'pericardio-coelomic cavity' of the northern anchovy, Engraulis mordax, appear to be a misidentification of

the pharyngeal chloride cells (figure 1A in O'Connell, 1981). In fact, counts of chloride cells in the lateral abdominal epidermis (overlying the pericardial cavity) of cod embryos and larvae were noted, and found to average close to zero during the entire period of development. This would appear to exclude the heart and its associated tissues as sites of chloride cell activity. Various, reported claims of an association between vitelline blood vessels and chloride cells (Alderdice, 1988) were not supported by the present study. It may be noted, however, that the embryonic and larval stages of the cod lack an extensive vitelline circulation, as found in some other teleosts (Morrison, 1993), perhaps accounting for the discrepancy.

There is some evidence that chloride cells found in the skin during early development (Lasker and Threadgold, 1968; Leonard and Summers, 1976; Hwang and Hirano, 1985; Pfeiler and Lindley, 1989; Hwang, 1990) may persist over the general body surface in adult fishes, perhaps in an inactive form as precursor cells whose differentiation is activated under certain conditions (Henrikson and Matoltsy, 1968; Merrilees, 1974; Schwerdtfeger and Bereiter-Hahn, 1978; Korte, 1979; Nonnotte et al., 1979; Philpott, 1980). While abdominal chloride cell densities decreased during development as a result of both increased skin surface area and apparent loss of chloride cells, it was not clear whether some of these

cells were retained in the adult stage. Since examination of abdominal skin from adult cod did not reveal the presence of MR cells or chloride cells, it is assumed that they are either extensively 'diluted' by pavement cell proliferation, or are lost in the adult cod by epithelial sloughing, or 'de-differentiation' (Baron, 1993) to a more generalized epithelial cell type. The loss of the differentiated condition also appears to be a typical response of isolated chloride cells to culture conditions (W.S. Marshall, personal communication), and might indicate the involvement of some endocrine factor in the maintenance of the differentiated state.

These observations may be contrasted with the chloride cells found in some intertidal teleosts. Here, apparently functional chloride cells are found in the integument, perhaps as an adaptation for life in hypersaline environments (Nonnotte et al., 1982; Yoshikawa et al., 1993).

Do Chloride Cells Have a Common, Ectodermal Origin?

The origin and migration of chloride cells appeared to be strongly influenced by the mode of formation of the various epithelia covering the yolk sac and lining the buccal and pharyngeal cavities (figure 4.12).

In the vertebrates, the pharyngeal gill slits originate by the dual action of an outward movement of internal pharyngeal endoderm, the gill pouch, combined with an

invagination of external ectodermal tissue, the gill cleft (Morgan, 1974a; 1974b). Simultaneously, an external pore, the precursor of the opercular opening, is formed by ectodermal invagination. While these events are occurring, the buccal cavity is formed by anterior ingrowth of ectoderm to form the stomodeum (figure 4.12). Eventually, the septum separating the buccal and pharyngeal cavities (the buccopharyngeal or oropharyngeal membrane) ruptures, and the oropharyngeal cavity is formed (figure 4.12). In fishes, this membrane ruptures relatively late in development. In the present study, SEM studies revealed that the membrane is intact at hatching, and remains so for about one day at 6°C. Thus, the gill bars, and the lining of the oropharyngeal cavity are probably comprised of cells from both ectodermal and endodermal sources, while the gill lamellae are later derived from the ectodermal tissues (Goodrich, 1958; Romer, 1970; Morgan, 1974a; 1974b).

The absence of chloride cells from the anterior head region during initial formation of the mouth strongly argues against chloride cell recruitment into the anterior pharynx from the stomodeal ectoderm. Rather, it is likely that yolk sac chloride cells, or their precursors, invade the posterior pharynx via the pharyngeal pores at the time of the formation of the pores, and that these cells act as a source for the later colonization of the gill filaments, mouth roof epithelium and inner opercular epithelium. As the yolk sac is

absorbed, abdominal chloride cells gradually disappear during the larval stage, and examination of abdominal skin from juvenile and adult cod did not reveal the presence of MR cells or chloride cells.

An additional patch of chloride cells persists in the scaleless region of skin which connects the last, partially fused, gill arch to the skin overlying the cleithral bone (Marshall et al., 1992). This region, referred to here as the 'cleithral' epithelium, appears to harbour ionocytes in a number of teleost species (Marshall et al., 1992; chapter 3).

These observations tend to suggest that chloride cells in teleosts have an ectodermal origin. The appearance of the chloride cells follows the general movement of ectodermal tissues from the yolk sac, as described above. In addition, this hypothesis supports the notion of a single source of stem cells that are recruited to form chloride cells (Conte, 1980).

According to this point of view (figure 4.12), mitochondria-rich (MR) cells in the blastoderm first migrate over the yolk surface during epiboly. As epiboly nears completion, the blastoderm (of ectodermal origin), invaginates anterolaterally on the embryo at the positions of the gill slits to form the external oropharyngeal pores, and, in combination with pharyngeal endoderm, the internal gill slits. The MR cells, or their progenitors, migrate inward to form part of the lining of the pharyngeal cavity. At this stage,

the mouth (stomodeum, lined by ectoderm) is separate from the pharynx, and the primordial gill arches are little more than strands of cartilage cells. At the completion of epiboly, mitcchondrial membrane potentials become elevated, and the chloride cells become fully functional as mediators of chloride secretion in the pharynx and on the yolk sac. It is presumed that the later appearance of chloride cells in the buccal cavity (mouth roof), gill filament and cleithral epithelia is a consequence of cell migration from the pharynx.

Further work is required to confirm this hypothesis. In particular, new methods of cell labelling may allow tagging the blastomeres with microinjected fluorescent tracers to observe the course of chloride cell migration and differentiation. The potential-sensitive dyes are of limited use for this purpose. While the rhodamine dyes tend to be toxic, long-term experiments with alternative dyes like DASPEI are not readily performed without dechorionating embryos. Preliminary experiments, however, (Valerio, unpublished data) show that adult cod chloride cells in opercular epithelia labelled with DASPEI may be stored for several days using 'organ culture' techniques (McCormick, 1990b).

Gradual Development of Chloride Cell Activity, and of Related Osmoregulatory Structures

Since DASPEI fluorescence is not observed in mitochondria of cells in embryos undergoing epiboly, it appears that the

mitochondria are involved in only low levels of ATP synthesis, coupled to rather weak respiratory proton gradients. This is consistent with the observation (Riis-Vestergaard, 1984) that early embryonic stages of marine fishes maintain osmotic balance mainly as a result of the tightness of the epithelial layer, rather than by active salt secretion. This conclusion is further supported by the early appearance of tight junctions, and the absence of the numerous epidermal secretory pores observed later in development.

The outermost layer of squamous epithelial cells in the cod embryo, the periderm, is typical of the epithelial coverings found in fishes. In contrast to the periderm (epitrichium) of terrestrial vertebrates, which is morphologically (Hoyes, 1968; O'Rahilly and Müller, 1992) and functionally (Wickett et al., 1993) distinct from other squamous epithelia, the teleost periderm appears to function in a manner similar to the tight epithelial barriers on the body surface of the teleost larva and adult animal. Unlike the periderm of other vertebrates (Sengel, 1976), and contrary to Bouvet's (1976) observations of fish development, the periderm of the cod embryo does not appear to be shed at a specific stage of development. It is more likely that cells are gradually exfoliated as the superficial layers are replenished by migrating cells from the deeper regions of the skin, including stem cells which recruit the sacciform, goblet,

chloride and pavement epithelial cell populations. Microridges (microplicae) are prominent on the surfaces of the 'pavement' epithelial cells, and raised areas occur over the cell junctions. This appearance, and the absence of any obvious specialized ion-secreting cells, confirms that osmoregulation in the early teleost embryo is achieved primarily by the presence of effective passive barriers to ion influx, as shown by Riis-Westergard's (1984) study of water transport in the Atlantic cod embryo.

The absence of any extensive change in the secretory cell population of the epidermis at hatching is consistent with the similar composition of the seawater medium and perivitelline fluid, (Alderdice, 1988).

It has been suggested that teleost fishes possess an embryonic cavity whose fluid contents are maintained isotonic with the blood plasma (Guggino, 1980). In embryos of the euryhaline teleosts, Fundulus heteroclitus, and Fundulus bermudae (Guggino, 1980), this cavity apparently connects with the external medium via a pair of openings that connect the chamber posteriorly with the perivitelline fluid, while the anterior portion of the chamber is confluent with the pharynx via the gill slits. This embryonic cavity is said to disappear shortly after hatching (Guggino, 1980). In Fundulus embryos the oropharyngeal membrane blocks intake of water through the mouth from the perivitelline fluid. Since water apparently

enters through the lateral openings of the embryonic cavity, it is unclear how the composition of this fluid is regulated, as claimed by Guggino (1980). A more likely explanation is that the fluid within the cavity (interpreted in the present study as being the pharyngeal cavity) is continuous with, and isosmotic to, the perivitelline fluid. Therefore, for teleost embryos developing in seawater, only fluid within closed, *internal* body cavities is likely to be hyposmotic to the perivitelline fluid. The latter would include fluid from the pericardial cavity and from the subdermal space or sinus (Armstrong and Child, 1965; Morrison, 1993). In this interpretation, it is assumed that the small amounts of water intake observed during the embryonic stage (Guggino, 1980) are due to leakage through the oesophagus from fluid in the pharynx, while true drinking behaviour is only initiated later in hatched larvae, when the mouth is open and the swallowing reflex has developed. This is consistent with both published quantitative estimates of water flux in developing embryos and larvae (Guggino, 1980; Brown and Tytler, 1993; Tytler and Ireland, 1994), and the observed behaviour of fluorescent markers in the present study. This osmoregulatory strategy may be prevalent in the embryonic stage of other teleosts, although few species appear to have been investigated (Alderdice, 1988).

Guggino's (1980) estimate for water intake through the pharyngeal pore/oesophageal route for embryonic Fundulus, is of the order of 600 pL/mg/h for 7-8 day embryos at 25 °C. Later larval stages of marine teleosts (Brown and Tytler, 1993; Tytler and Ireland, 1994) have considerably higher drinking rates (0.20 μL/mg/h for 255 degree-day turbot larvae). These results suggest that, although there are no morphological barriers to the drinking of perivitelline fluid by embryos once the oesophagus is confluent with the pharynx, behavioural factors probably play a significant role. The swallowing reflex, required for bulk seawater ingestion, does not appear to be elicited until after hatching when the oropharyngeal membrane ruptures.

The fact that gut intake of seawater is delayed, while the embryonic skin and pharynx contact a hypersaline solution (the perivitelline fluid) throughout development, may indicate that the gut mucosa is relatively permeable to salts and water, and that seawater ingestion would be a liability to the embryo before the kidneys are adequately developed. Similarly, elaboration of an extensive gill epithelium might incur unacceptable water losses and salt influx if it occurred before the gills were equipped with chloride cells for active salt secretion.

Comparison of Amphibian and Fish Ionocyte Differentiation Patterns

It has been observed that during amphibian metamorphosis, osmoregulatory ion transport by gill ionocytes (de Zarate et al., 1993) is gradually replaced by transport due to cutaneous mitochondria-rich cells as the gills of the larva are resorbed during metamorphosis (Dietz and Alvarado, 1974; Cox, 1979; Gabbay et al., 1992).

However, amphibia retain cutaneous chloride cells as adults, while fishes generally lose the cutaneous chloride cells, or they become sparsely distributed on the skin.

CONCLUSIONS

The pattern of chloride cell development observed in the Atlantic cod correlates well with its life history. Early stages of the cod do not appear to rely extensively upon active secretion for osmoregulation, but develop this strategy gradually as osmotic barriers become less effective, and water intake becomes necessary. Thus, the differentiation of chloride cells is tied closely to the development of the mouth, pharynx and gills.

It is concluded that, while kidney and gut epithelia may play a role in larval osmoregulation in cod, the primary osmoregulatory structures in embryos and early larvae are the chloride cells of the yolk sac and pharyngeal epithelium. Prior to feeding, the larvae ingest seawater, which probably

results in salt influx and water efflux through the gut epithelium. Re-absorption of water and secretion of salt through the kidney may also begin to occur at this time.

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FIGURE 4.1. CHLORIDE CELLS IN ADULT COD GILL FILAMENT

Chloride cells (arrow) in adult cod gill filament; stained with DASPEI.

Zeiss Axiovert 35 microscope; magnification: 100X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

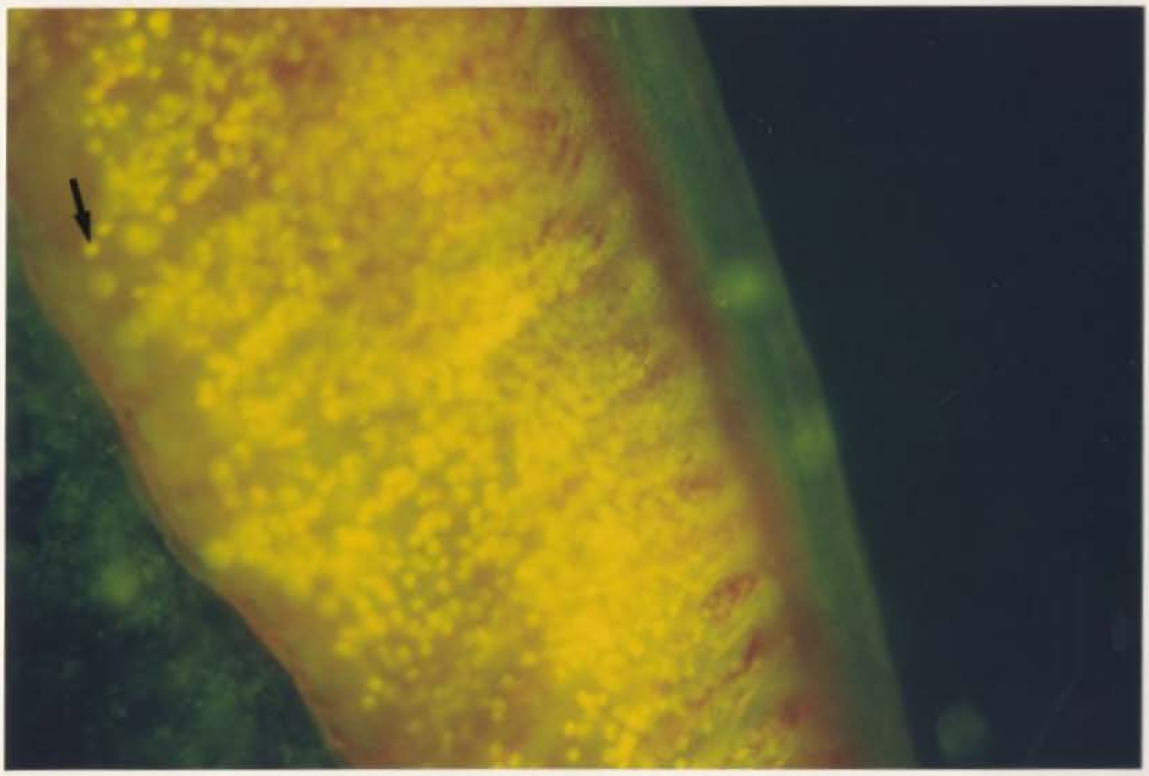


FIGURE 4.2. CHLORIDE CELLS IN ADULT COD OPERCULAR EPITHELIUM

Adult cod opercular epithelium treated with proteases to dissociate cells; rectangular area below is further magnified by 5X in the top half of the figure; arrow indicates position of a chloride cell; note the elongate, club-shape of the cell.

Hitachi S570 SEM; magnification: below, 400X; above, 2000X.

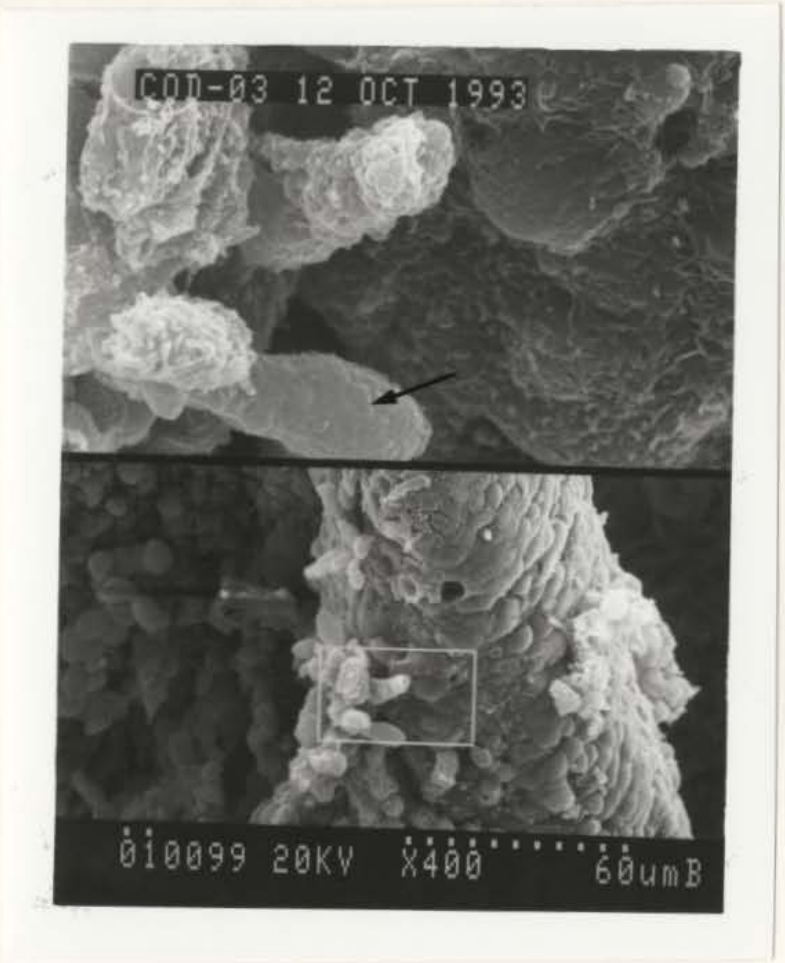


FIGURE 4.3. CHLORIDE CELLS IN ADULT COD OPERCULAR EPITHELIUM

Adult cod opercular epithelium treated with proteases, as in figure 4.2, to dissociate cells; rectangular area below is further magnified by 5X in the top half of the figure; arrow indicates position of a chloride cell; note elongate shape of chloride cells compared with other spherical epithelial cells; the latter are pavement epithelial cells that have rounded up.

Hitachi S570 SEM; magnification: below, 400X; above, 2000X.

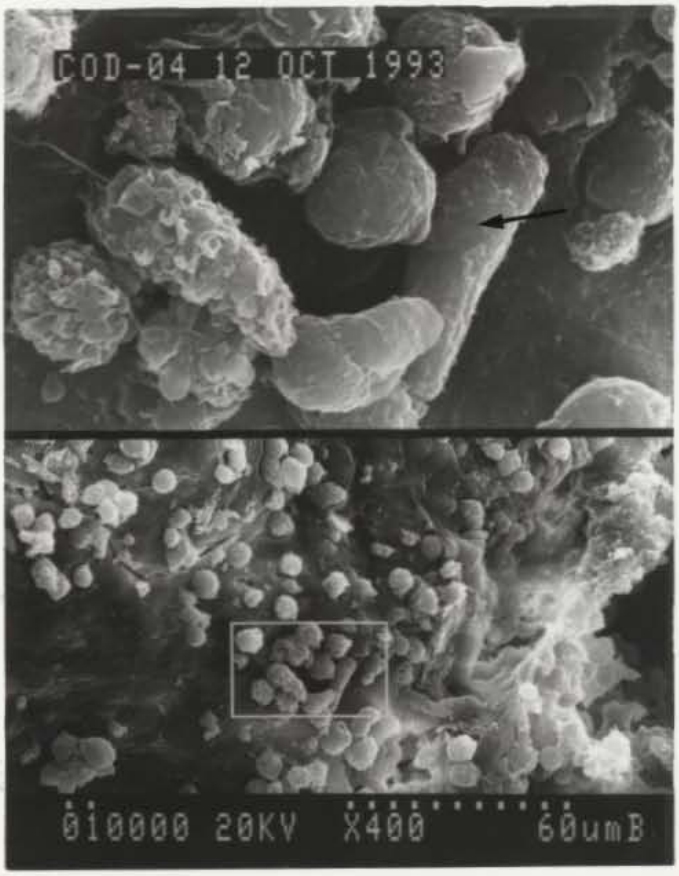


FIGURE 4.4. DASPEI-STAINED CHLORIDE CELLS IN COD YOLK-SAC LARVA

Chloride cells in cod larva at hatching; chloride cells occur mainly over the yolk sac (Y), and in the pharynx (P); note also the intense autofluorescence from bile pigments in the gut (G) and gall bladder (B); DASPEI stain.

Zeiss Axiovert 35 microscope; magnification: 100X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

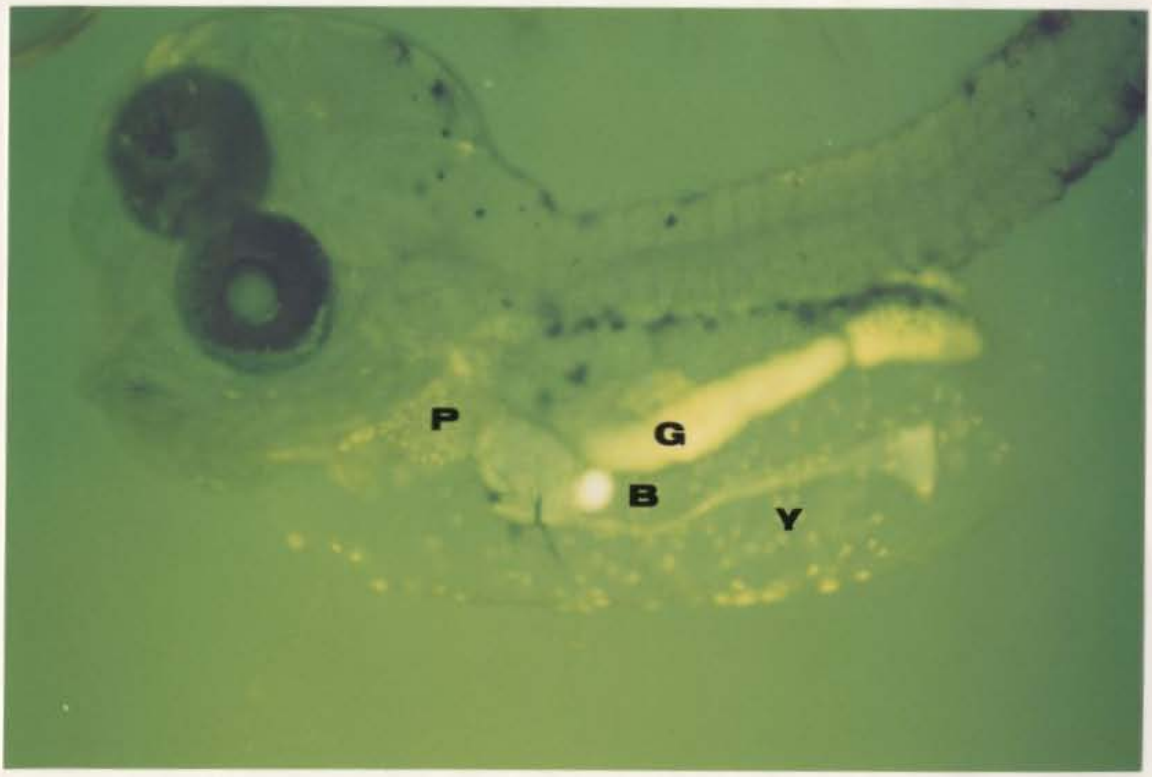


FIGURE 4.5. BEGINNING OF EPIBOLY

Dechorionated embryo; rectangular area over the cellular region of the blastodisc (B) indicates the actively migrating cells of the early embryo; the noncellular edge (E) of the embryo is the marginal periblast, which also moves over the yolk (Y) surface; the yolk sac surface is wrinkled due to fixation-associated shrinkage of the yolk mass.

Hitachi S570 SEM; magnification: 110X.

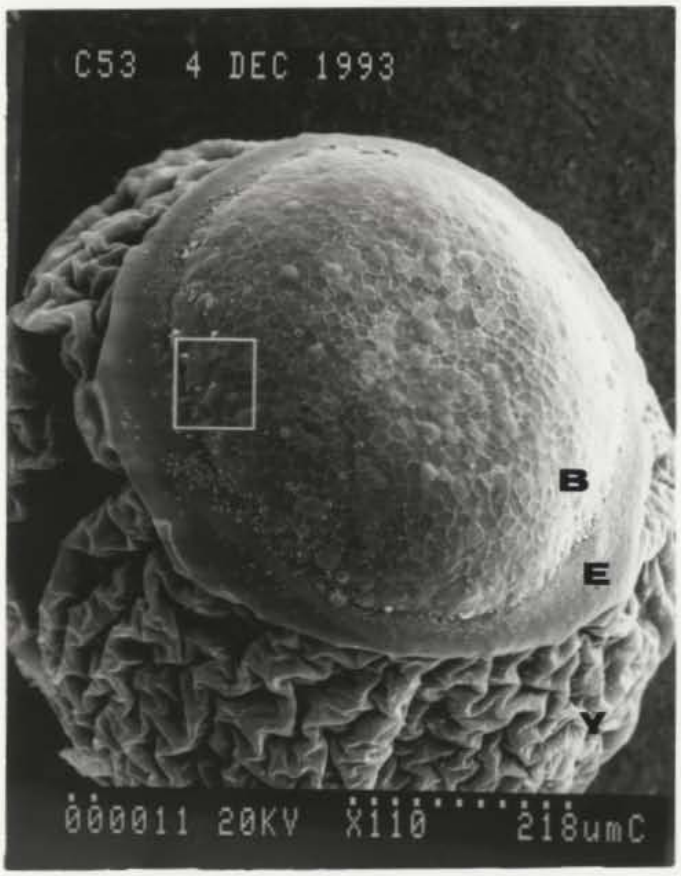


FIGURE 4.6. PHARYNGEAL PORES

Cod embryonic pharyngeal pores as they appear at the completion of epiboly; rectangular area below, on the right side of the embryo (E), is further magnified by 10X in the top half of the figure; note how far back from the head (H) region the openings are, and their location between the embryo (E) and yolk (Y) sac.

Hitachi S570 SEM; magnification: below, 50X; above, 500X.

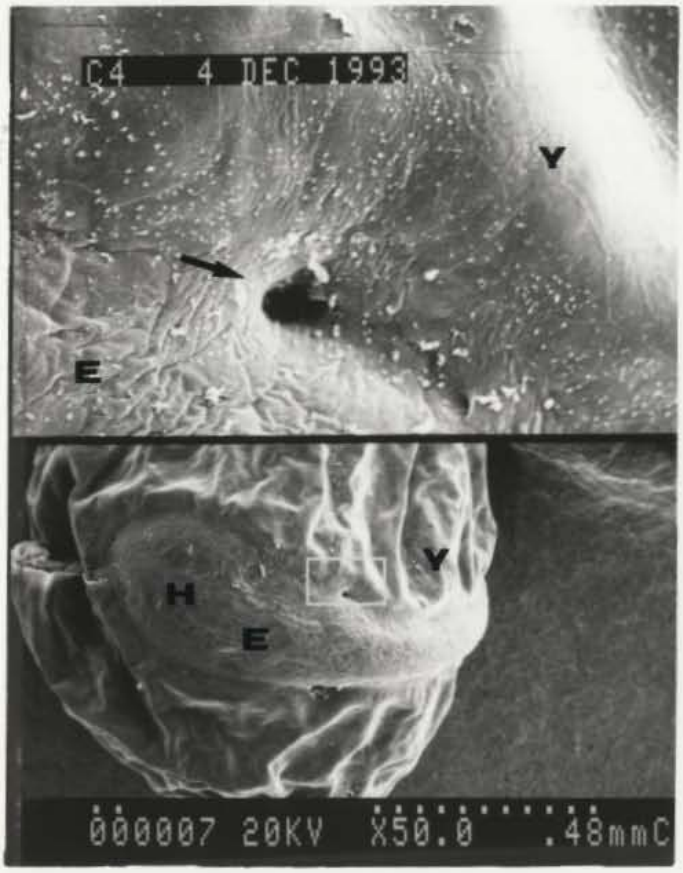


FIGURE 4.7. PHARYNGEAL CHLORIDE CELLS

Cod larva (22 d, 7 °C) stained with anthroylouabain to show a sheet of chloride cells lining one of the pharyngeal pores; the opening of the pore is indicated by the thick arrow (right), while the cup-shaped sheet of chloride cells is indicated by the left (thin) arrow.

Olympus BHS microscope; magnification: 400X.

UV fluorescence filter set: 20UG1 excitation filter, DM400 dichroic mirror, and 20L435-W22 barrier filter.

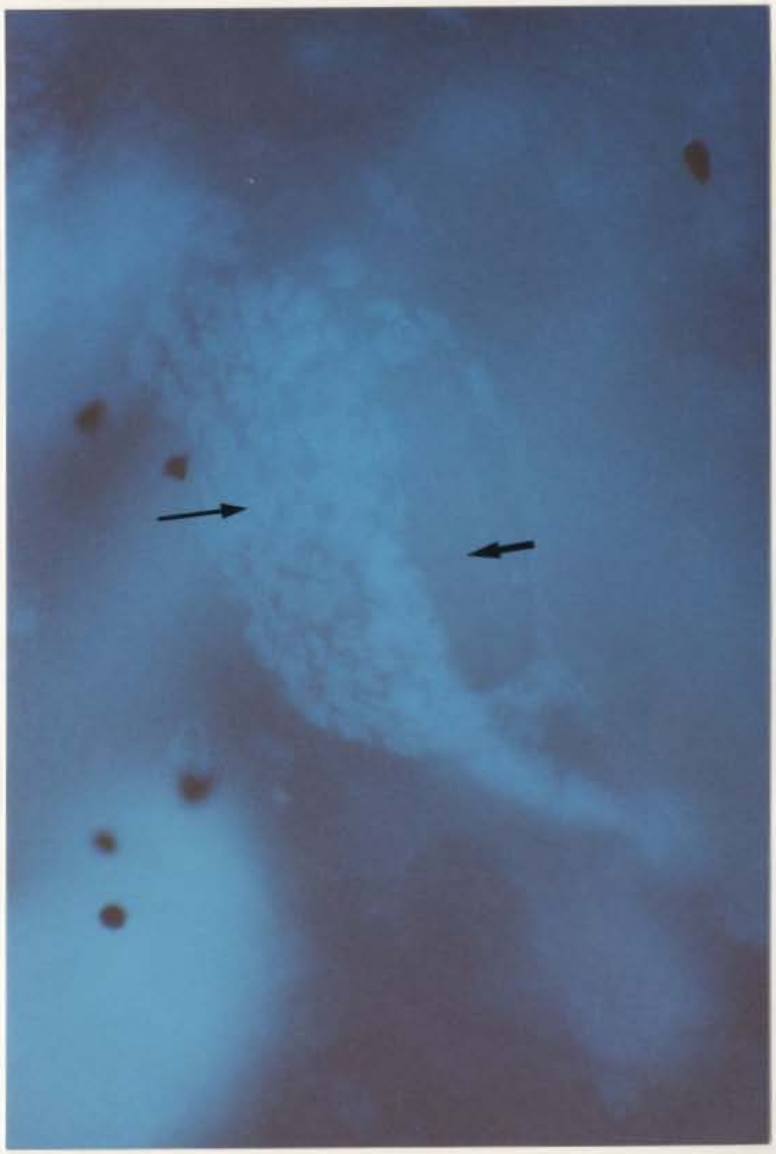


FIGURE 4.8. PHARYNGEAL AND YOLK SAC CHLORIDE CELLS

The same general pattern of chloride cell distribution is evident in the cod embryo during the entire period of time from shortly after the completion of epiboly to hatching; chloride cells occur mainly over the yolk sac (Y, collapsed at centre; thick arrow points to chloride cells), and in the pharynx (thin arrow); DASPEI stain; 16 d at 0°C.

Zeiss Axiovert 35 microscope; magnification: 100X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

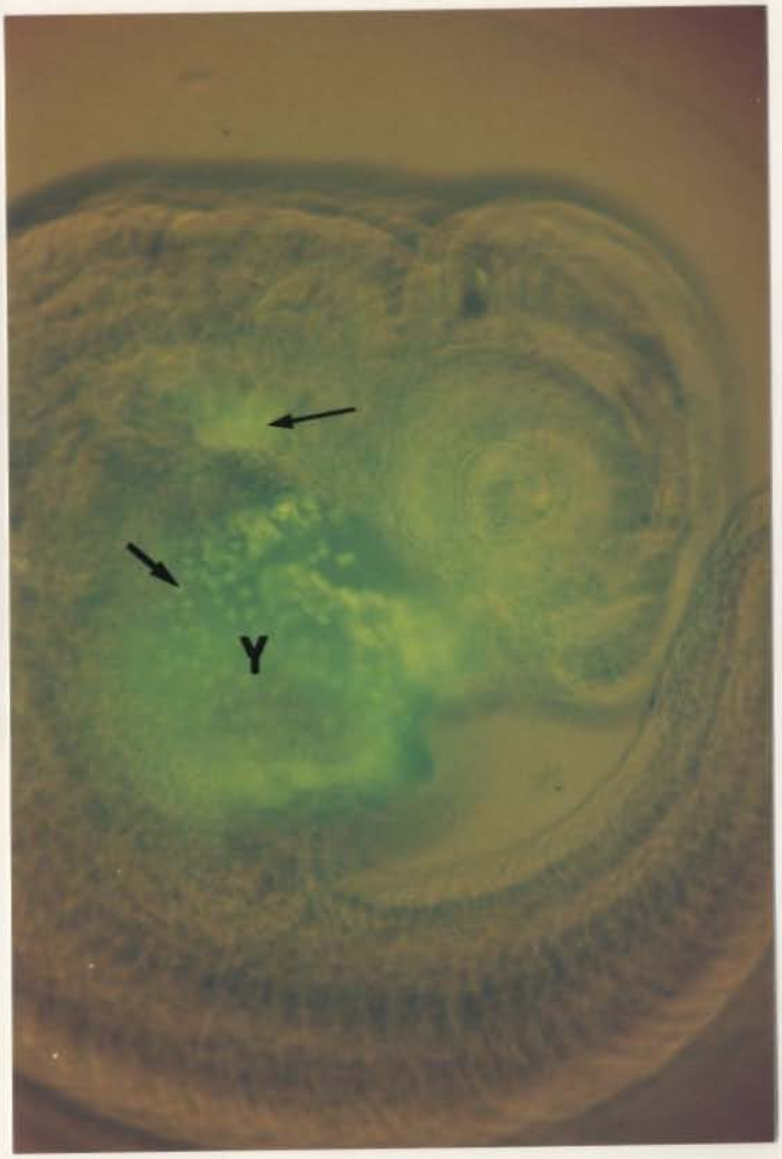


FIGURE 4.9. CHLORIDE CELLS WITH LARGE, LOBED NUCLEI

Large, lobed nuclei are evident in these isolated pharyngeal chloride cells removed from a larva at hatching; the cytoplasm (C) of the cells has been stained with the Na^+, K^+-ATPase probe, anthroylouabain, while the nuclei (orange, indicated by small arrows) have been counterstained with dihydroethidium.

Zeiss Axiovert 35 microscope; magnification: 100X.

'UV' fluorescence filter set: 365 nm excitation filter, 395 nm chromatic beam splitter, and 420 nm barrier filter.

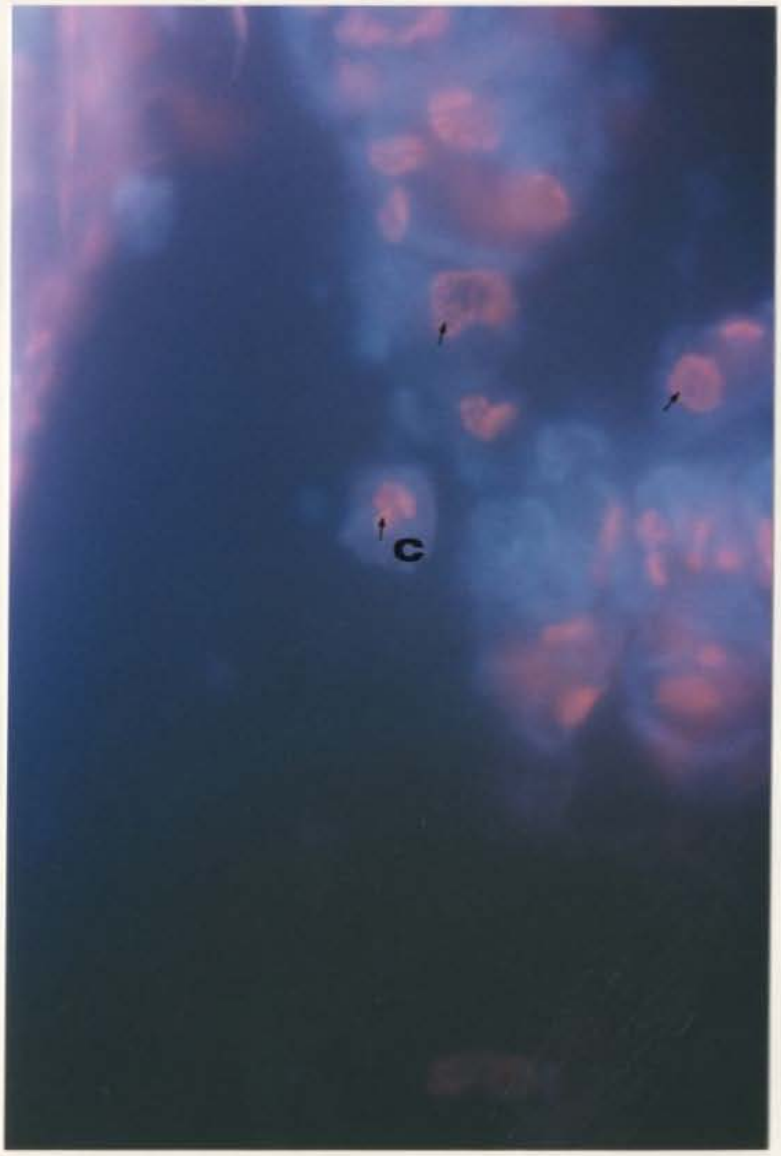


FIGURE 4.10. RHODAMINE-123 STAINED YOLK SAC CHLORIDE CELLS

Dispersed yolk sac chloride cells that have been removed from a cod larva at hatching; note the apparent binucleate (arrows) condition; it is unclear whether this condition is a consequence of apoptotic nuclear fragmentation during naturally-occurring or dye-induced cell death, or whether it represents a natural multinucleate cell state; rhodamine-123.

Zeiss Axiovert 35 microscope; magnification: 400X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

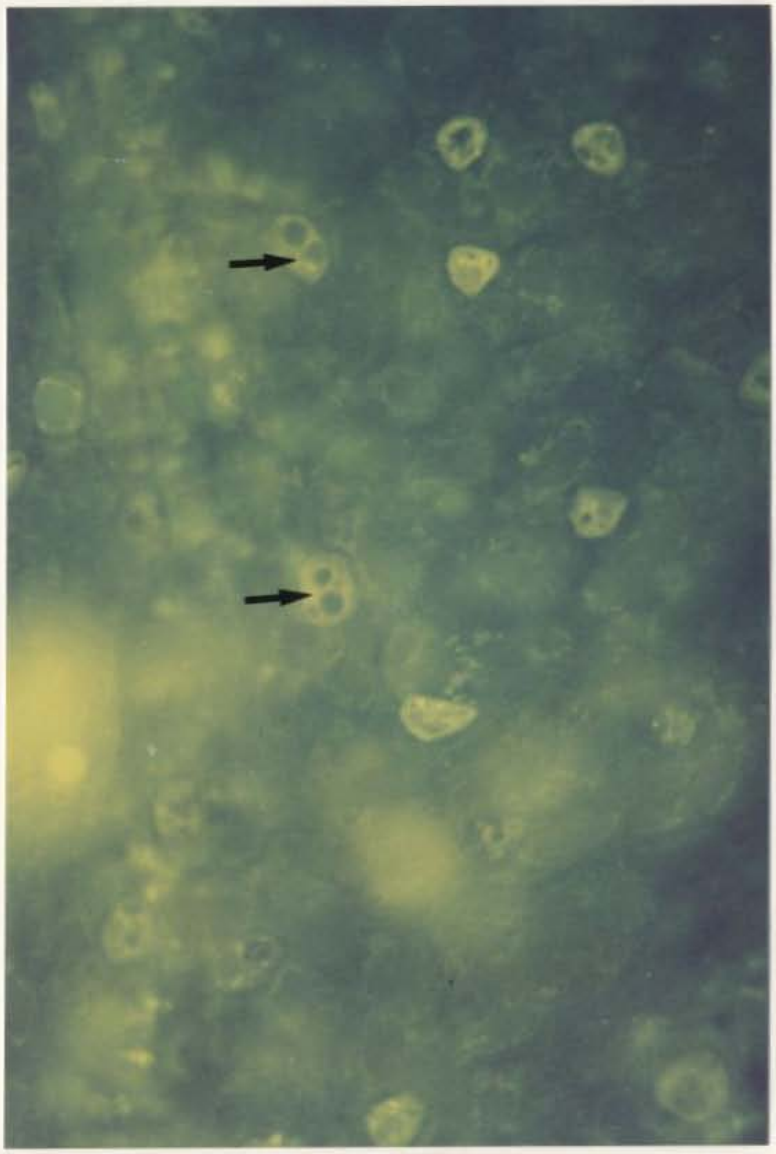


FIGURE 4.11. SECRETORY PORES IN THE PERIDERM OF THE COD EMBRYO.

Epithelial pores (arrows) occurring over the yolk sac of a cod larva at hatching; although these pores are invariably associated with underlying secretory cells, it is impossible to reliably determine the identity of the secretory cell without using additional methods (eg. stains specific for chloride cells); in the absence of further information, it can be assumed that the pores are the apical secretory surfaces of goblet (mucus-secreting), sacciform or chloride cells.

Hitachi S570 SEM; magnification: 700X.

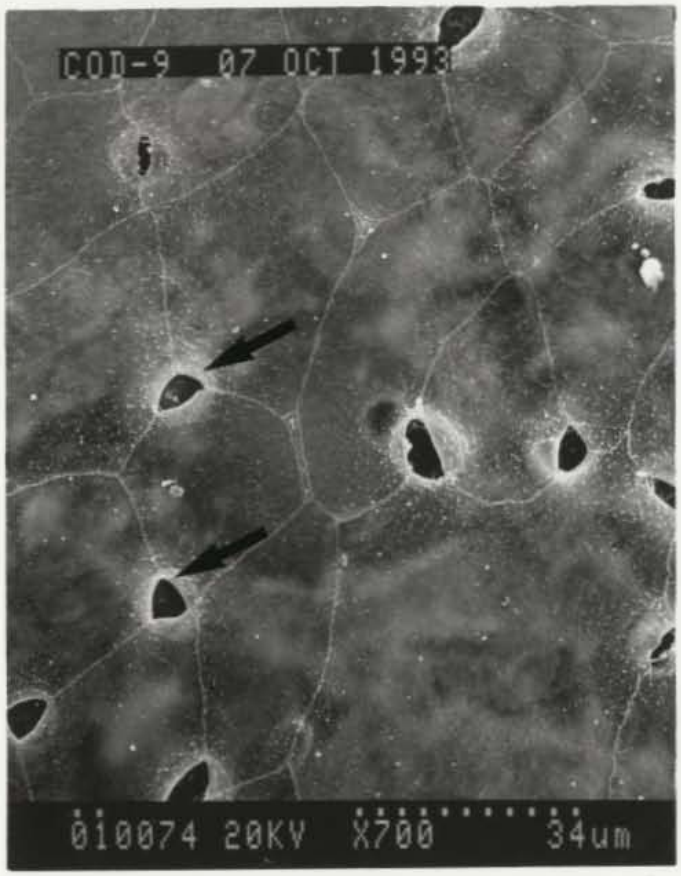
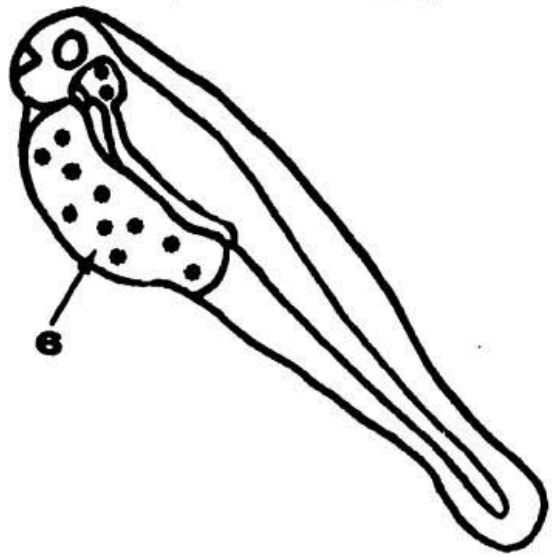


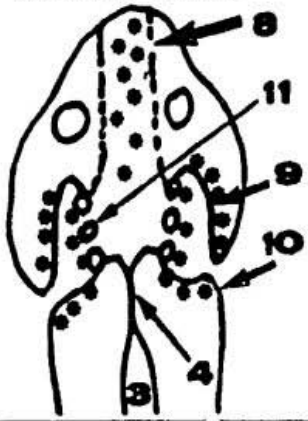
FIGURE 4.12. CHANGES IN CHLORIDE CELL DISTRIBUTION DURING COD DEVELOPMENT (* = chloride cell)

4.12.a.1. Embryo Near Hatch
(dorsal view)

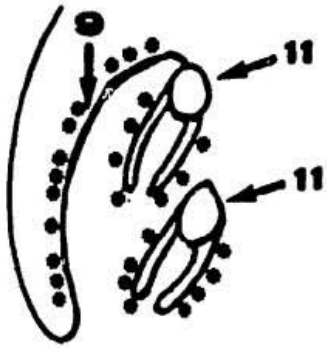
4.12.a.2 Hatched Larva
(left side, lateral view)



4.12.b.1. Adult Cod Chloride Cells
(dorsal view)

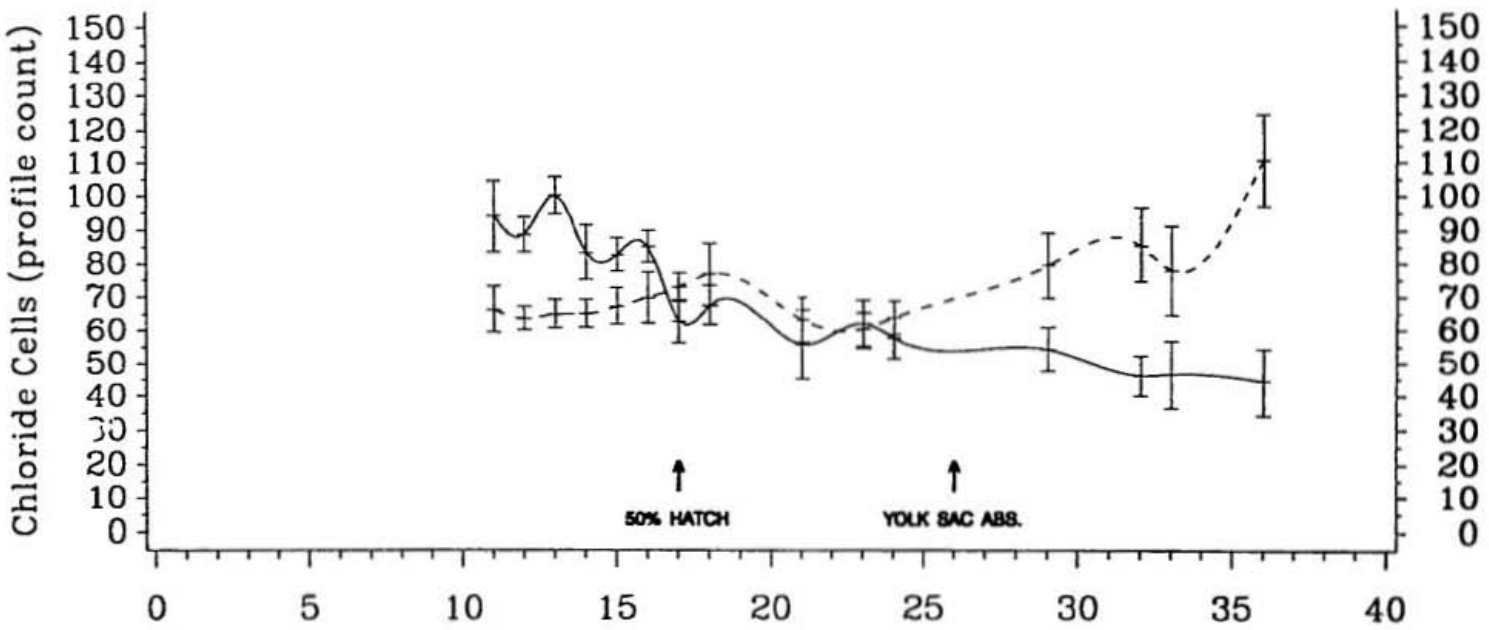


4.12.b.2 Adult Cod Gill Arch and Filaments
(left side, dorsal view)



1. Stomodeum
2. Pharynx
3. Gut
4. Oesophagus
5. Pharyngeal pores
6. Yolk sac
7. Oropharyngeal membrane
8. Mouth roof
9. Inner opercular epithelium
10. 'Cleithral' epithelium
11. Gill arch and filaments

Figure 4.13. Changes in Yolk Sac and Oropharyngeal Chloride Cell Numbers.
(arrows indicate hatch and yolk sac absorption times at 6 degrees Celsius)
After yolk sac absorption, 'yolk sac' cell numbers refer to
chloride cells that persist in abdominal region – see 'Methods'.



(Yolk sac cell numbers decrease with age: solid line)
(Gill cavity cell numbers increase with age: dashed line)

FIGURE 4.14. SUMMARY OF CHLORIDE CELL DIFFERENTIATION IN ATLANTIC COD (at 5-6°C).

(MR cells = mitochondria-rich cells; in this case, the term is used to denote MR cells with 'low-potential' mitochondria, viewed here as likely precursors of the fully differentiated and functional chloride cell that exhibits high-potential mitochondria)

Days	STAGE	Appearance of MR Cells and Chloride Cells
0	OVUM	**Maternally inherited mitochondria.**
0	ZYGOTE	**Maternally inherited mitochondria.**
1-2	BLASTULA	**MR cells in dividing blastomeres pre-epiboly).**
2-3	EPIBOLY	**New MR cells appear in migrating yolk sac epithelium; mitochondrial potentials increase.**
4-5	COMPLETION OF EPIBOLY	**Chloride cells in yolk sac epithelium and in common gill/buccal (= 'oropharyngeal') cavity.**
7	BEGINNING OF HEARTBEAT	**Chloride cell numbers increase.**
16	HATCHING	**Yolk sac chloride cells at maximum numbers.**
24	YOLK SAC ABSORPTION	**Yolk sac chloride cell numbers decrease; abdominal chloride cell numbers decrease.**
20-50	GILL ARCH (20-30 d) AND FILAMENT (40-50 d) 'EPITHELIALIZATION' (Metamorphosis)	**Chloride cells on gill filaments.**
> 40	JUVENILES	**Abdominal chloride cells disappear.**
> 3 Yr.	ADULT COD	**Chloride cell pattern of juvenile stage retained (oropharyngeal, 'cleithral', gill, opercular).**

CHAPTER 5. THE EFFECT OF INCUBATION CONDITIONS ON THE PHYSIOLOGY AND DEVELOPMENT OF ATLANTIC COD, Gadus morhua, EMBRYOS AND LARVAE

ABSTRACT

The effects of incubation conditions on the development of gross morphological characters in the embryos and early larvae of the Atlantic cod, Gadus morhua, were investigated in the laboratory. Temperature, salinity and drug effects on development rate, survival and cardiac function were examined in a static culture system, and under ambient conditions in flowing seawater.

Embryos were transferred to incubation media containing variable concentrations of salts, cortisol, retinol or antibiotics, and changes in development rate, heart rate, somite number, morphology, and chloride cell differentiation were recorded.

It was found that development rate was changed by some, but not all treatments. The embryonic heart began to beat weakly at an earlier stage than reported for Norwegian populations of the Atlantic cod cultured at similar salinity and temperature.

The anterior part of the coelomic cavity was noted to expand under conditions that would lead to dehydration of the tissues, namely in seawater containing high salt concentrations or ethanol, the carrier used for drug treatments.

Although the timing of embryonic events was altered by some incubation conditions, yolk sac chloride cell development was always found to occur shortly after the completion of epiboly, even in the presence of drugs (cortisol and retinol) that are known to influence epithelial cell differentiation. The timing of chloride cell differentiation, therefore, appears to be relatively inflexible.

INTRODUCTION

The laboratory culture of marine fishes has long been of interest to fisheries biologists and students of comparative anatomy (Earll, 1880; Ryder, 1884; Meek, 1924). Several studies have focussed on the physiological significance of variation in morphological characters during development, particularly under varied conditions of salinity and temperature (Dannevig, 1895; Battle, 1930; Barlow, 1961; Laurence and Rogers, 1976; Davenport et al., 1981; Alderdice, 1988; Blaxter, 1992). However, further work is required to establish the adaptive significance of such responses. In addition, a better understanding of physiological ontogeny may lead to improvements in methods of applied aquaculture and in the bioassay of toxins and drugs (Thomas, 1975; Stene and Lonning, 1984a; Solberg and Tilseth, 1987; von Westernhagen, 1988).

Among the changes in physiological and morphological characters occurring in altered incubation conditions, those associated with osmoregulatory functions (Davenport et al., 1981; Alderdice, 1988) are of critical importance to the survival and development of marine fishes. This conclusion is based on the diversity of responses to salinity change in developing marine teleost fishes. Salinity is known to influence both survival (Holliday and Blaxter, 1960) and hatching time (Forrester and Alderdice, 1966; Laurence and

Rogers, 1976). Effects on meristic characters have also been reported by some workers (Garside, 1966; Lindsey, 1988).

It is known that active chloride secretion develops gradually in marine fish embryos. Although chloride cells appear in the yolk sac of the early embryo, differentiation of the gut and kidney only occur in the hatched larval stage, and gill chloride cells do not appear until well after yolk sac absorption. It is likely that salt and water intake are balanced eventually by salt secretion from renal and oropharyngeal epithelia.

This suggests that earlier stages might differ from the more advanced larval stage in their ability and/or strategy for tolerating extreme salinity changes. For example, post-epiboly embryos may be more dependant on osmoregulation, since they cannot rely as much on integrity of the epithelial barrier (Riis-Vestergaard, 1984; 1987) for their survival. A study of ouabain-tolerance in metamorphosing marine teleost larvae supports the hypothesis that active salt excretion becomes more important at later stages of development, since the development of active transport as the predominant osmoregulatory strategy is accompanied by an increase in ouabain-inhibitable Na^+, K^+-ATPase activity measured from embryo extracts (Conte et al., 1991). Some workers have also reported ultrastructural changes in the chloride cells of euryhaline fishes when transferred to high salinities, such as

changes in cell junctions and the cytoplasmic tubular system (Hwang and Hirano, 1985; Hwang, 1990). Chloride cell densities in adult fish gill epithelia have also been shown to respond to salinity treatment (Laurent and Perry, 1991).

Although the eggs of marine fishes are frequently cultivated in flowing seawater, even for toxicity testing (Solberg and Tilseth, 1987; Solberg et al., 1987), it is usually necessary to culture embryos or larvae in closed systems for antibiotic, drug or pollutant treatments (Abedi and McKinley, 1967). In these situations it is simpler and more economical to explore the effect of changing environmental conditions on the developing embryo, particularly where the effects of dissolved substances are being investigated, and solute concentrations must be maintained at a constant value. In addition to the practical application of such studies, the small size and restricted mobility of the teleost embryo and early yolk sac larva makes these stages particularly suitable for testing the effects of ambient conditions on developmental parameters.

In a previous chapter (chapter 3) the embryonic origin and development of chloride cells were described in the Atlantic cod. The present chapter describes the effects of culture conditions on the relative timing of various morphological changes in the cod embryo and larva, with particular emphasis on features of significance for

osmoregulation and transport. The effects of varying culture conditions on the development of transport-related phenomena in Atlantic cod embryos and early larvae were determined by incubating cod embryos and larvae in solutions of known composition, and in ambient seawater. The short-term effects of temperature, salinity and antibiotics were tested by examining survival and hatch rates, cardiac development and function, and gross morphological changes, such as the development of the mouth and coelomic cavity, and the differentiation of the yolk sac chloride cells.

In addition, the reported roles of cortisol (McCormick, 1990) and retinol (Hardy, 1992) in vertebrate epithelial differentiation and transport were investigated by culturing embryos in media supplemented with these compounds. Treated embryos were then examined to determine the effects of treatment on the time of differentiation of yolk sac chloride cells, and on other developmental events associated with osmoregulation.

METHODS AND MATERIALS

Collection and Maintenance of Adult Fish

Adult cod used for spawning stock (>40 cm) were obtained during a Department of Fisheries and Oceans cruise by the *Chinook* in Trinity Bay, Newfoundland during June, 1993. Fish were transported to the marine laboratory at Logy Bay, Newfoundland.

Trinity Bay fish were maintained in aquaria until egg collection during the next spawning season, from March to July, 1994 (see methods, chapter 3). For most of the experiments, eggs taken from the collector were probably spawned from more than one pair of fish, and all were collected within 24 hrs of spawning. The six female cod in the spawning tank produced a total of about 60 egg batches over the spawning season (from April to June, 1994).

Embryo Incubation and Larval Rearing

Several culture methods were used for embryo and yolk sac larvae, including static seawater media at constant salinity and temperature, and flowing seawater aquaria under natural photoperiod, temperature, and salinity.

Eggs exposed to ambient conditions of salinity, temperature and photoperiod were incubated in rectangular plastic tubs as described elsewhere (Valerio et al., 1992). Additional batches of eggs were incubated at various 'constant' (± 0.5°C) temperatures in large (0.4 to 2 liter) beakers with static seawater in a temperature-controlled room. Although ambient temperatures and salinities were not constant during the term of egg incubation and larval rearing, comparisons were made between groups of individuals at different mean temperatures. Over the course of the spawning season, the range of ambient salinity did not exceed 1 ppt in the embryo incubation tanks.

General Aspects of Short-term Incubation Experiments

Embryos were drained of seawater on a nylon mesh screen (250 μm), then were quickly rinsed and transferred by plastic pipette to incubation media without removing the chorion. Initially, incubation experiments were conducted with 400.0 ml plastic (polypropylene) beakers. However, later experiments were scaled down to use 30.0 ml plastic (polystyrene) petri dishes. A wide range of stocking densities (1.2 to 39.8 individuals per ml) was used for short-term incubation experiments in artificial seawater or supplemented seawater. Although the duration of incubation was brief, stocking density was also included in the data analysis to determine its effects, if any. In long-term experiments where larvae were reared to yolk sac absorption in natural seawater (in static or flowing systems), densities were lower (2-3 individuals per ml).

The effect of density, salinity, and temperature, drug type and concentration, and initial stage of development on survival, hatching rate, heart rate, somite number were tested statistically, using SAS statistical software (SAS, 1988).

Salinity Experiments

Salinity experiments were performed using commercially prepared sea salt (Forty Fathoms® Bio-Crystals Marinemix, Baltimore, Maryland) dissolved in aerated distilled water (Bidwell and Spotte, 1985). For comparison, filtered (0.45 μm

millepore filter) seawater was also used as an incubation medium for some of the embryos. All solutions contained antibiotics at a standard (1X) dose, as described below ('**Antibiotic Treatments**').

Embryos were transferred at various densities (see results), at 1, 4 and 5 days after fertilization. Control transfers were performed with ambient seawater and 32 ppt artificial seawater, while experimental groups were incubated in 0, 4, 8, 16, 40, 48, 56, 64, and 72 ppt artificial seawater.

Salinities were determined using an osmometer (Fiske 'One-ten' freezing point osmometer) to measure osmolalities. A standard seawater sample was initially used to calibrate a Guildline 'Autosal' model 8400 salinometer, which yielded a salinity value for seawater samples taken from aquaria. A dilution series of these seawater samples was then prepared to derive a relationship between salinity and osmolality. Subsequent samples of seawater for embryo incubation were used for osmolality determinations, and for chloride titrations with a CMT-10 chloride titrator. Seasonal changes in ambient seawater were determined from samples stored in 20 ml glass vials at room temperature, while seawater temperatures were recorded periodically during the experiments using a Cole-Parmer (Chicago, Ill.) Quick® digital thermometer.

The pH of incubation media was checked at the incubation temperature before and after adding supplementary compounds, then again after the experiment was conducted. All incubation experiments were of short duration (2-10 days; see below).

At the completion of the experiments, embryo hatching rate, stocking density, survival and heart rates were determined, and larvae were preserved in 10% buffered formalin for later microscopic examination. Subsequent somite counts and descriptive data were then collected for preserved specimens.

Antibiotic Treatments

The effects of antibiotics on embryo development were tested by transferring embryos at the blastula stage (2 days, at 6 °C), or at the beginning of the heartbeat (7 days, at 6 °C), to petri dishes containing 30.0 ml of filtered (0.45 μm millepore) ambient seawater (32-33 ppt) containing 0X, 1X, 2X, 4X, 8X or 16X strength antibiotic formula. The basic (1X) antibiotic formula, which was also used for other embryo incubation experiments, was 100 mg streptomycin sulphate and 60 mg penicillin G (1650 Units/mg) in 1.0 liter of filtered (0.45 μm) seawater.

At the completion of the incubation period, embryo hatching rate, survival and heart rates were determined, and larvae were preserved in 10% buffered formalin for later examination.

Hormone and Drug Treatments

The pH of hormone or vitamin-supplemented media did not change by more than 1 %, even at the highest concentration tested, 1:100 dilution of the stock cortisol or retinol.

A stock cortisol (Sigma Chemicals) solution was prepared by dissolving cortisol at 1.0% (W/V) in absolute ethanol. All incubation media were prepared fresh from stock solutions (stored at -20 °C) by diluting with filtered seawater. A dilution series of the stock was prepared as for antibiotics (see above). Dilutions tested were: 0 (control = filtered seawater alone), 1:100, 1:1,000, 1:10,000, and 1:100,000 of the stock cortisol solution.

A similar series of dilutions were prepared from a stock solution of 1% (W/V) retinol (Sigma Chemicals) in absolute ethanol. Again, stock dilutions tested were: 0 (control = filtered seawater alone), 1:100, 1:1,000, 1:10,000, and 1:100,000.

For both cortisol and retinol incubations, parallel control incubations were performed with ethanol alone in seawater at the same concentrations used for experimental incubations (ie. at 1:100, 1:1,000, 1:10,000, and 1:100,000). Embryos were transferred to incubation media as described above for salt and antibiotic solutions.

Heart Rate Measurements

Embryos or larvae were gently pipetted from the rearing containers to a petri dish containing ambient seawater. The dish was placed on ice in a larger petri dish, then left for 20 minutes. After this time the temperature of the seawater in the petri dish had stabilized at 3-4 °C, at which temperature all heart rates were determined. Heart rates were counted by viewing on a Nikon N2000 camera attached to a Nikon Diaphot-TMD inverted microscope fitted with phase contrast optics.

Initial tests were performed to determine whether oxygen was a factor limiting cardiac function in the embryonic or larval cod under the incubation conditions used in the present study. It was found that when embryos or larvae were transferred to a beaker of seawater oxygenated with 95% O_2, no change in the mean heart rate was observed, compared with either pretreatment samples, or non-treated controls. It was concluded that heart rates determined from embryos and larvae were typical of healthy specimens. However, it was necessary to allow the animals to remain on ice for the full 20 minutes to allow them to recover from the stress of transfer to the microscope stage, at which time heart rates stabilized. With these precautions, it was possible to compare heart rates between different treatment groups. Over the time required to make counts of heart rates in each sample (approximately 30 min), it was determined that heart rates did not exhibit an

acclimatory response. This is consistent with the observation that longer periods of time (several days) are required to elicit acclimatory changes in the adult teleost heart (Bailey and Driedzic, 1990).

A comparison was made between embryos and larvae cultured at different temperatures or in different types of media (see above, '**Salinity Experiments**', '**Antibiotic Treatments**', '**Hormone and Drug Treatments**').

Light Microscopy, Somite Counts, and Heart Rates

Photographs were taken with a Zeiss Axiovert inverted compound microscope fitted with an ocular micrometer, Contax 167 MT camera, mercury lamp, and phase contrast/fluorescence optics. Additional photographs of non-fluorescent material were taken with a Nikon N2000 camera attached to a Nikon Diaphot-TMD inverted microscope, or an Olympus BHS microscope equipped with an Olympus PM-10ADS photomicrographic System.

Somite counts were performed with intact embryos or larvae, or, in the case of advanced embryos, with fixed, dechorionated specimens.

Staining of embryos and larvae was performed as described previously (chapters 3 and 4). Data were analyzed using SAS statistical software (SAS, 1988).

Chorion Permeability Experiments

In addition to cortisol, retinol, ethanol and seawater salts, a number of other additives were used to examine the permeability of the chorion to solutes.

Permeability tests were performed by incubating embryos in seawater and saline solutions containing various dyes. Solutes were dissolved at various concentrations (10 μM to 0.1 M; see table 5.1).

Permeation was determined by examining embryos with transillumination to observe staining of the perivitelline fluid, or by viewing with fluorescence microscopy. In cases where the chorion itself was stained, the embryos were dechorionated to reveal whether stain had penetrated the perivitelline fluid.

RESULTS

Significant variations between egg batches were observed in many experiments, and suggest a major parental component to drug and salinity effects. For this reason, comparisons between treatments performed on aliquots of a single batch of eggs are more appropriate than comparisons between batches. For short-term incubation experiments, densities did not appear to influence any of the developmental parameters, and so analyses were performed on pooled data.

The terminology used to describe developmental stages is based partly on the works of Apstein (1909), Pelluet (1944), and Hisaoka and Battle (1958).

Some of the main developmental events, and their time of appearance are shown in figure 5.1.

Effects of Temperature on Development Rate

The most pronounced effect of elevated temperature on development was to accelerate most, but not all, developmental events relative to the time of hatching. Hatching times, and the time of appearance of most characters, were delayed by lower temperatures.

When somite numbers were examined at age, the first appearance of the somites was seen to occur at a later age for the lower incubation temperatures (figure 5.2). Although the slower development of cod embryos at low temperatures appeared to result in higher numbers of somites when compared with high-temperature embryos at the same chronological stage of development, this was only the case at the early developmental stages. Ultimately, the final somite number did not differ between high and low temperature groups. The final, or maximum, somite numbers attained by cod embryos were generally reached by the time of hatching, regardless of development rate (see chapter 2). Changes in somite numbers at age for all temperatures and batches pooled together, and for a single

batch cultured at two different temperatures, are shown in figure 5.2.

The initial heartbeat was very weak (ie. the amplitude of contraction was small), and consequently heart muscle contractions were only visible at 200X to 400X magnification. At 6 °C rearing temperature, the heartbeat began at about 8 or 9 days after fertilization, and increased steadily toward hatching, at 17 days (figure 5.3a). A comparison of the scatterplots for embryo heart rates (figure 5.3a) revealed that embryos raised at higher temperatures had a faster heart rate, even when compared at the same measurement temperature (see 'Methods' section, above).

Some evidence is available to suggest that elevation of the heart rate was due mainly to the accelerating effect of temperature on developmental rate, rather than by a direct action of temperature on the heart. While embryos raised at elevated temperatures were indeed more advanced when compared at the same chronological age, heart rates at the same developmental stage were similar, particularly for later stages of development (figure 5.3b). Similar stages, relative to hatching, were calculated as a percent of incubation time (figure 5.3b), based on estimates of time to hatch (chapter 2).

Effect of Salinity on Development

In some experiments, high mortalities were observed in both control (ambient seawater or 32 ppt artificial seawater) and experimental groups. Data for these experiments were not included in the analysis, since stress-related mortalities or mortalities due to poor egg quality might bias results. However, most of the transfer experiments showed a consistent pattern of mortality (figure 5.4). Survival was relatively high throughout the 24 ppt to 64 ppt salinity range, but dropped off at both higher and lower salinities. No survivors were ever seen after 2 or more days incubation in distilled water, regardless of stocking densities, while embryos at 4-16 ppt or 56-64 ppt salinity frequently exhibited a general tissue opacity, presumably as a consequence of tissue necrosis.

Both unhatched embryos and hatched larvae exposed to high salt concentrations (> 56 ppt) exhibited a distended anterior portion of the coelomic cavity at the anterior end of the yolk sac, apparently filled with tissue fluid, but separate from the pericardial cavity. This effect was noted from the end of epiboly to the end of the yolk sac stage in hatched larvae. At low salt concentrations (4 and 8 ppt), the cavity appeared to be almost nonexistent (figures 5.5a, 5.5b and 5.5c).

Effect of Antibiotics on Development

At moderately high (4X) antibiotic concentrations, survival was slightly elevated, relative to low concentrations. At concentrations above 4X, survival rates declined somewhat, perhaps as a result of antibiotic toxicity.

For the various strengths of antibiotic-supplemented seawater media (in parentheses), survival rates (mean percent survival for 3 replicates ± standard errors) were as follows: 35.36 ± 4.97 (0X), 42.34 ± 3.65 (1X), 46.10 ± 4.88 (2X), 65.93 ± 3.62 (4X), 63.90 ± 2.69 (8X), and 50.89 ± 2.95 (16X).

Antibiotics did not significantly alter the pH or osmolality of the incubation medium.

Effect of Retinol and Cortisol on Development

A comparison of embryo survival rates at various retinol and cortisol concentrations, revealed that survival rates were generally high for short-term exposure to most concentrations of the drugs (data not shown).

High concentrations of retinol (0.1 mg/ml), however, were absolutely lethal (100% mortality rate) to cod embryos transferred to incubation media at the stage (7 days at 5 °C) where the heartbeat begins. However, all other concentrations of either retinol or cortisol did not appear to affect the survival rates when compared with control groups treated with ethanol alone.

Interestingly, the ethanol vehicle, used for both retinol and cortisol stock solutions, and for control groups, did appear to have a number of toxic effects when the different doses of ethanol were compared. In particular, high concentrations of ethanol (1% [V/V] in seawater) were associated with major changes in gross morphology, regardless of whether the alcohol was used as a drug vehicle or not.

The coelomic distension noted (see above) in embryos and larvae cultured at high salt concentrations was also seen in embryos and larvae exposed to high ethanol concentrations (figure 5.6). Simultaneously, high ethanol concentrations appeared to delay the normal elongation and differentiation of the lower jaw tissues for larvae transferred to ethanol solutions after hatching (21 days at 5°C). In untreated larvae, Meckel's cartilage elongated to form the typical undershot lower jaw (Ellertsen et al., 1980).

A comparison of somite numbers and heart rates between the different ethanol doses did not reveal a general growth-retarding effect of the alcohol. This suggests that the mechanism of retardation of jaw development is attributable to a more direct action of ethanol. However, survival rates were significantly lower at high retinol or ethanol concentrations.

Heart rate, percent hatch and somite number showed no systematic variation with salt, drug or antibiotic concentration.

Effects of Culture Conditions on Chloride Cell Development

Chloride cells appeared to be fully differentiated on the yolk sac at the completion of epiboly (figure 5.7), regardless of culture conditions. However, the time at which epiboly was completed (at blastopore closure) was delayed under conditions that resulted in a general slowing down of developmental events, namely at low temperatures.

Chorion Permeability to Various Solutes

The results of permeability tests from the present study, together with data from the literature, are summarized in table 5.1 and figure 5.8. Generally, solute permeation of the teleost chorion appeared to be a function of molecular size, with the cutoff being found around 320 or more molecular weight (figure 5.8).

DISCUSSION

General Aspects of Culture Technique

The use of a fairly wide range of stocking densities for some of the short-term incubation experiments in the present study did not appear to cause significant differences in development rate, survival, or other developmental parameters. This may not be true for cultures maintained at higher (> 7°C) temperatures, or where antibiotics are not used. The presence of low concentrations (1X formula, see Methods) of antibiotics in culture media probably did not affect embryonic development

adversely, since embryos did not exhibit a dose-dependant response to antibiotics.

Although embryos and larvae were enumerated as 'live' or 'dead', many live specimens may not have been equally healthy. Fish embryos and larvae cultured under ambient conditions tend to be quite transparent, and have a typical posture, heart rate and mobility. Some specimens cultured under extreme conditions of temperature or salinity, however, had necrotic tissues that appeared opaque with transillumination, or whitish in reflected light. These animals occasionally exhibited an abnormal negative buoyancy at high (> 32 ppt) salinity, indicating osmoregulatory problems. However, most appeared to be otherwise normal. The opacity of necrotic tissues in morbid fish embryos has been observed by several workers (Battle, 1930; Oppenheimer, 1955; Skidmore, 1966), and is probably due to the precipitation of soluble proteins in tissue fluids.

The use of a simple, dichotomous classification (dead vs. live) system may obscure some effects of incubation conditions, particularly long-term effects, where larvae with substantial tissue necrosis may not survive. However, in the absence of a reliable method for quantifying degrees of tissue damage, it is probably still worthwhile to consider the acute effects of incubation conditions on survival.

Effect of Incubation Conditions on the Heart Rate

Although heart rate in adult teleost fishes is not closely correlated with metabolic rate variation between species (Priede and Tytler, 1977), embryonic and larval heart rates in any vertebrate species gradually increase during development, apparently in response to increasing oxygen demands (Burggren and Pinder, 1991).

The heartbeat of the Atlantic cod embryo was observed to begin earlier, and with a lower frequency, than reported for similar temperatures in other studies. For example Serigstad (1986), who found no effect of crude oil extracts on the heart rate of Norwegian cod, reported an initial rate of 30 BPM, starting on day 12 at 5 °C. At 25 days, this had increased to 60 BPM. Solberg and coworkers (1982) found that the heart began to beat about one week before hatching (17d-7d = 10d after fertilization, at 5°C), increasing to a stable rate of 55 BPM after hatching. Although these differences might be attributable to the higher magnification (200X-400X) and use of a compound, rather than dissecting microscope in the present study, the higher initial and final rates found in the present study might suggest population differences between Norwegian and North American populations. However, the early heartbeat has a very low amplitude, and its presence would be almost impossible to detect with the low magnifying capability of the dissecting microscope.

The more erratic values for larval heart rates observed in the present study were probably due to the greater sensitivity of later stages for environmental disturbances. However, their rates were also faster than either embryonic heart rates of the present study, or larval rates reported for Norwegian cod larvae at the same stage and temperature.

It is necessary to consider the procedure used to compare embryonic characters at the same developmental stage. Some workers have attempted tc do so by comparing characters at the same chronological age relative to hatching, or at the percentage of time to hatch (eg. Brooks and Johnston, 1994). While this approach appears to solve the problem of comparing embryos that develop at different rates, it may be noted that the differential response of embryonic characters to temperature complicates matters somewhat. That is, some environmental variables, such as temperature are found to selectively alter the rate of development of particular organ systems (Johnston, 1993).

For example, at high temperatures cod embryos can prematurely develop jaw cartilage and musculature, and are capable of drinking seawater before hatching. In the absence of specific information on the mechanism involved, it could be argued that differentiation or activity of the hatching enzyme cells (Yamagami, 1988) are not stimulated as much as the development of other tissues. Therefore, for some characters,

percentage of development time is not a sufficient criterion for comparing embryos.

Variation in the Somite Number

Although embryonic cells may become committed early to their future fate as somite constituents (Kimmel et al., 1988), the time of appearance of distinct somite furrows generally occurs after gastrulation has occurred. As in other teleost fishes, such as the zebrafish, Brachydanio rerio, somites in the Atlantic cod were observed to form shortly after epiboly completion, with the trunk somites (trunk myotomal precursors) appearing first. These are gradually followed by a rostrocaudal (head to tail) progression of somite differentiation (Kimmel et al., 1988). Somite formation in the Atlantic cod was observed to begin at the end of epiboly in the present study, regardless of culture conditions. Relative to epiboly completion, somite formation occurred at approximately the same time as the appearance of melanophores on the body, and of chloride cells on the yolk sac.

It has long been speculated that the fundamental mechanisms governing somitogenesis in fish embryos are ultimately responsible for the number of discrete skeletal elements in the adult fish (Lindsey, 1988; Brooks and Johnston, 1994). Thus, when high numbers of somites form in the developing fish embryo, the adult fish may subsequently

exhibit high numbers of fin rays, vertebrae, and other repeated skeletal elements (ie. meristic characters). Aside from the important implications for species and stock recognition, the effects of environmental and genetic factors on somitogenesis are often relevant under culture conditions, where temperature and dissolved solutes could influence meristics (Laale, 1982).

Many workers have reported a decrease in meristic counts when embryos are reared at high temperatures. Although most studies have reported counts of skeletal elements, such as vertebrae, some workers have observed a similar result with embryonic somite counts (eg. Brooks and Johnston, 1994). Although variable results have been obtained by varying salinity (Lindsey, 1988), some workers have suggested that any condition, including extreme salinities, that causes slower developmental rates will increase meristic counts (Garside, 1966). However, this generalization has been largely abandoned due to the large number of exceptions to the rule (Lindsey, 1988).

The present results for temperature treatments were not consistent with the principle of increased meristic counts as a result of development at low temperature. However, this finding does not preclude the possibility that later developmental stages might exhibit a correlation between vertebral or fin ray counts and rearing temperature.

Effects of Cortisol and Retinol on Embryonic Development

Vitamin A, or retinol, is a lipid-soluble vitamin found in high quantities in avian egg yolk, and in lesser concentrations in vertebrate plasma. It is believed to function in the maintenance of epithelial tissues, among other things (Hardy, 1992). Its presence in the plasma of adult bony fishes is well-documented (Ganguly, 1989; Berni et al., 1992). Similarly, the steroid hormone cortisol, is believed to function in the regulation of epithelial salt transport in adult fishes (McCormick, 1990).

In the present study, neither cortisol nor retinol treatment of embryos was found to affect the timing of chloride cell development. Rates of development of gross morphological features also appeared to be unaffected.

Other studies have examined the effects of retinoids on the development of the zebrafish, Brachydanio rerio. While deformations of the eye have been noted after retinoic acid treatment, retinal and retinol do not seem to exert a similar effect (Hyatt et al., 1992).

A number of technical problems remain to be resolved in these types of experiments, however. There is practically no information available at present to allow an estimate of extracellular fluid volume in embryonic and larval fishes. Therefore, drug doses in incubation media are based on known plasma concentrations of hormones and vitamins for

(unstressed) adult fishes (eg. Avella et al., 1990), and are, at best, only approximate.

In addition, for most drugs of biological interest little is known about chorion penetration, transport through epithelial cell membranes and intercellular junctions, and therefore routes of administration, as well as drug stability in incubation media need to be more carefully examined. It is likely that the chorion is impermeable to molecules as large as cortisol (MW, 362.5), or that diffusion rates are extremely slow. This might account for the lack of toxicity of high cortisol (0.01 % W/V) concentrations to unhatched embryos, while similar concentrations (0.01 % W/V) of retinol were lethal to all embryos. Hydrocarbon toxicity levels (Falk-Petersen and Kjorsvik, 1987) and solvent permeation rates (Harvey and Ashwood-Smith, 1982) have previously been correlated with molecular size and chorion porosity.

Although the role of endocrine factors in embryonic and larval teleost physiology has been little studied, recent work has shown that the embryonic stage of some teleosts, such as the Japanese flounder, Paralichthys olivaceus contain cortisol and thyroid hormones (de Jesus et al., 1991). However, the role of these compounds in the early development of fishes remains a largely unexplored area of research.

The rudimentary state of differentiation of the teleost embryo's pituitary gland (Blaxter, 1969; Leatherland and Lin,

1975), as well as the apparent lack of a stimulating effect on chloride cell differentiation by cortisol, might suggest that the embryonic stage may regulate chloride cell differentiation and function differently from the adult fish (McCormick et al., 1991). This conclusion appears to be supported by the absence of effects of other factors on the relative timing of chloride cell development.

Effects of Ethanol and Salts on Body Cavities

The 'normal' cod embryo, cultured in 32-33 ppt seawater, or in similar concentrations of artificial seawater, exhibited a shallow, elongate coelomic cavity. In the embryo at epiboly completion, the cavity extended posteriorly from just in front of the head to just behind the gut. However, at high ethanol or salt concentrations, the cavity was distended, particularly in front of the head.

This observation is of particular interest, since a similar cavity has been reported from Fundulus heteroclitus by Armstrong and Child (1965), who assumed the 'vesicle' represented a precursor of the pericardial sac. Similarly, Battle (1930) reported an enlargement of the pericardial cavity for embryos of the marine teleost, Enchelyopus cimbrius. This was clearly not the case in the present study, since the pericardial sac of the cod embryo could be seen anterodorsal to the enlarged coelomic cavity. Other workers have also briefly described this cavity in normal fish embryos

(Guggino, 1980). Fluid accumulation and enlargement of the yolk sac of thermally shocked Atlantic salmon alevins has also been reported (Peterson et al., 1977), as has a similar edematous condition in yolk sac larvae of the Atlantic halibut, Hippoglossoides hippoglossoides, exposed to low oxygen pressures (Jelmert and Naas, 1990).

Effects of Ethanol and Temperature on Jaw Development and Oropharyngeal Membrane Rupture

When cod larvae at the early yolk sac stage (21 days at 5 °C) were transferred to seawater containing high ethanol concentrations (1 % V/V), jaw development appeared to be inhibited or slowed down. In the delayed larvae, the lower jaw cartilage was rounded and did not extend beyond the snout, a characteristic that is typical of earlier developmental stages. Control larvae, however, had the typical 'undershot' jaw of the normal cod larva preparatory to exogenous feeding. These larvae were not followed beyond the yolk sac stage, and so the ultimate effect on jaw function and feeding is unknown.

Interestingly, although other workers have reported ethanol toxicity to fish embryos, concentrations of 1% or less have not generally been considered to exert a toxic effect (Abedi and McKinley, 1967; Laale, 1971; Stene and Lonning, 1984a).

Incubation of embryos, from fertilization onwards, at high temperatures (> 9°C) had a different effect on jaw

development, as well as on the development of other organs, namely an acceleration of development rate for the lower jaw. High-temperature embryos had large, gaping mouths, and an undershot lower jaw, indicating that not only had the oropharyngeal membrane ruptured prematurely, but elongation of the lower jaw cartilage had progressed even before hatching. Experiments with fluorescent dyes have shown that such larvae are capable of drinking seawater at hatching, in contrast to larvae hatched at lower temperatures (eg. at 5 °C), which undergo oropharyngeal membrane rupture within about 24 hours of hatching, and have much slower jaw development (chapter 4). The drinking behaviour of newly hatched cod larvae, therefore, probably depends on the temperature at which they are hatched, as well as the time elapsed since hatching (Mangor-Jensen and Adoff, 1987). Since temperature affects the rate at which chloride cells appear on the yolk sac, as well as the rate of mouth and gut development, and since chloride cells appear before active drinking occurs, the hatched larva is always prepared to actively excrete salt ions acquired from ingested seawater. The absence of any significant alteration in the relative chronological sequence of events in development is likely of adaptive value to fishes. Perturbations that affect development rate do not alter the preparedeness of the embryo as it enters each successive phase of development. In contrast to true toxicological reactions, in which specific organ

systems are targeted for damage, rate modifications may be survived by the embryo.

Permeability of the Chorion to Solutes

The permeability of embryonic membranes is frequently an issue in studies of embryonic anatomy, physiology and metabolism (Limbourg and Zalokar, 1973; Harvey et al., 1983; Valerio et al., 1992; Strecker et al., 1994). The teleost chorion is known to be permeable to a variety of small molecules and ions, such as water (Gray, 1932; Potts and Eddy, 1973), salt ions (Eddy et al, 1990) and respiratory gases (Rombough, 1988; Latham and Just, 1989). However, limits to the passage of larger molecules have not been extensively investigated.

Previous work on the effects of antibiotics on marine fish embryos (Oppenheimer, 1955) has shown that penicillin-streptomycin combinations tend to be non-toxic, while other antibiotic treatments involving chloromycetin (= chloramphenicol) and terramycin (= oxytetracycline) are quite lethal. Similarly, chloramphenicol has been shown to exert a toxic effect on the early development of the zebrafish, Brachdanio rerio (Anderson and Battle, 1967).

At least part of the reason for such differences in toxicity may be due to differences in chorion permeability. It is known that the marine teleost chorion generally has a low porosity and that this may result from the 'hardening' process

that occurs after cortical granule exocytosis (Donovan and Hart, 1986). Thus, occlusion of the pores of the zona radiata, and closure of the micropyle both contribute to a marked decrease in chorion porosity after activation of the egg and fertilization. Experiments with radiolabelled cryoprotectant agents (Harvey and Ashwood-Smith, 1982), and with dyes (Lonning and Davenport, 1980; present study) have revealed that the teleost chorion can obstruct the passage of quite small molecules. This finding has important consequences for the study of solute effects on embryo incubation.

The review of chorion permeability, together with data from the present study, suggest that, although the chorion appears to have a molecular weight cutoff around 320 or more (figure 5.8), compounds with a molecular weight in the range of 300 to 400 might vary considerably in their rate of transport into the perivitelline fluid and embryonic epithelia as a result of variation in other parameters, including molecular charge and hydrophobicity.

Fish eggs are not usually invaded by microbial cells through the chorion, unless the chorion, or perhaps the micropyle, is damaged by prior enzymatic digestion (Subasinghe and Sommerville, 1988). Consequently, improvement of fish embryo survival by antibiotics probably occurs by reducing the effect of microbial metabolism on the pH and oxygen content of the incubation medium, and by preventing them from digesting

the proteinaceous chorion (Subasinghe and Sommerville, 1988). Generally, therefore, large molecular weight antibiotics do not contact embryonic epithelia, and thus present no hazard to the developing fish.

An exception to this principle may be found in terramycin (oxytetracycline), whose molecular weight (table 5.1) should preclude entry into the teleost embryo from the incubation medium (Oppenheimer, 1955). In such cases, break-down products with smaller molecular weights may form during prolonged incubation and contribute to mortalities. Similarly, although penicillin (MW, 356.4) and DDT (MW. 354.5) have almost identical molecular weights, the toxicity of DDT to fish embryos (Dethlefsen, 1975) may be due to the entry of smaller molecular weight DDT metabolites, rather than to the direct action of DDT entering the embryonic capsule. Further work with radiolabelled drugs is required in this area to clarify routes of drug metabolism in fish embryos.

According to the results of most toxicity studies with fish embryos (figure 5.8) it can be surmised that streptomycin entry is unlikely, due to its high molecular weight. However, penicillin transport may occur at low rates, and is perhaps metabolized rapidly enough to prevent it from being toxic to embryos.

Although it has been assumed that treatment conditions have not influenced chorion porosity in any toxicology

studies, it should be noted that chorion porosity may increase at low pH, perhaps by vacuolation of the normally occluded pores of the zona radiata (Kügel et al., 1990). None of the incubation media in the present study were found to drop below pH 7.0, however.

CONCLUSIONS

The short-term culture of marine fish eggs in closed systems appears to provide a simple method for assaying the effects of ambient conditions, including exposure to biologically active or xenobiotic compounds. However, some precautions are necessary when interpreting the results of such studies. Many effects arise by acceleration of development rate, rather than by specific action on developmental processes.

Chorion permeability and stability of administered substances should be further investigated to determine whether target tissues are indeed exposed to effective concentrations of drugs.

Although other studies have not reported teratological effects from treatments with ethanol at concentrations as low as 1%, the present study shows that such concentrations cause at least a slowing down of jaw development. This effect may be of interest to workers using ethanol as a vehicle for alcohol-soluble drugs and vitamins in culture experiments.

The rudimentary state of differentiation of most endocrine organs in embryonic teleost fishes, and the apparent inflexibility of the relative timing of chloride cell differentiation, suggests that the regulation of the developing fish osmoregulatory system may be achieved by an alternative mechanism to that found in the adult fish.

Often, embryological and larval structures are treated by the researcher as either of vestigial (past) or rudimentary (future) significance, without regard to possible present function. It is, however, likely that the embryonic body cavities in marine fishes are directly involved in the maintenance of electrolyte homeostasis. The possible morphological and functional links between the coelomic space, yolk sac compartment, pericardial cavity and subdermal space, and their role in osmoregulation and transport should be further investigated, since little work on their fluid volume or possible role in transport has been done.

The small size of embryonic marine fishes, their seasonal availability and the lack of basic knowledge regarding physiological parameters, such as extracellular fluid space or blood volume, makes these animals a challenging subject for study by the experimental physiologist.

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TABLE 5.1. COMPARISON OF CHORION PERMEABILITIES IN VARIOUS FISH SPECIES

Permeability [Perm.]: '+' = permeable; '-' = impermeable; '?' = unknown; Formula weights are shown in parentheses in the third column.

Species	Compound	Perm.	Assay	Source
Brachydanio rerio, various salmonids.	Dimethyl-sulfoxide (DMSO)	+ (78.1)	Cryoprotection, isotopic label.	Harvey and Ashwood-Smith, 1982; Harvey et al., 1983.
"	Methanol	+ (32.0)	"	"
"	Glycerol	+ (92.1)	"	"
Gadus morhua	Water	+ (18.0)	Isotopes	Riis-Westergaard, 1984.
Brachydanio rerio	Water	+ (18.0)	Isotopes	Harvey and Chamberlain, 1982.
"	Ethanol	+ (46.1)	Toxicity	Laale, 1971.
"	Chloramphenicol (chloromycetin)	+ (323.1)	Toxicity	Anderson and Battle, 1967.
"	Retinol	? (286.5)	None	Hyatt et al., 1992.
"	Retinoic acid	+ (300.4)	Toxicity	"
"	Retinal	? (284.4)	None	"
"	Retinoic acid	+ (300.4)	Toxicity	Holder, and Hill, 1991.
"	Ethyl carbamate (urethan)	+ (89.1)	Toxicity	Battle and Hisaoka, 1952.
Various species	Various metal salt ions	+ (23.0-65.4)	Toxicity	Stockard, 1907; Swedmark and Granmo, 1981.

TABLE 5.1. COMPARISON OF CHORION PERMEABILITIES (Cont.)

Permeability [Perm.]: '+' = permeable; '-' = impermeable; '?' = unknown; Formula weights are shown in parentheses in the third column.

Species	Compound	Perm.	Assay	Source
Gadus morhua	Retinol (all-trans)	+ (286.5)	Toxicity	Present study.
"	Cortisol	? (362.5)	None	"
"	DASPEI	- (380.3)	Fluorescence	"
"	CMXR	- (531.5)	Fluorescence	"
"	Rhodamine-123	- (380.8)	Fluorescence	"
"	Rhodamine-6G	- (479.0)	Fluorescence	"
"	Nonyl-acridine orange	- (472.5)	Fluorescence	"
"	Ethidium bromide	- (394.)	Fluorescence	"
"	Dihydro-ethidium	+ (315.4)	Fluorescence	"

TABLE 5.1. COMPARISON OF CHORION PERMEABILITIES (Cont.)

Permeability [Perm.]: '+' = permeable; '-' = impermeable; '?' = unknown; Formula weights are shown in parentheses in the third column.

Species	Compound	Perm.	Assay	Source
Gadus morhua	Janus Green B	- (511.1)	Color	Present Study.
"	Neutral red	+ (288.8)	Color	"
"	$DiOC_6(3)$	- (572.5)	Fluorescence	"
"	Glutaraldehyde	+ (100.1)	Toxicity	"
"	Formaldehyde	+ (30.0)	Toxicity	"
"	Streptomycin	- (1361.0)	None	"
"	Penicillin	? (356.4)	None	"
Gadus morhua	Chloromycetin (chloramphenicol)	+ (323.1)	Toxicity	Oppenheimer, 1955.
"	Terramycin (Oxytetracycline)	+ (498.5)	Toxicity	"
"	Xylene	+ (106.2)	Toxicity	Kjorsvik, 1986.
Gadus morhua and Hippoglossoides platessoides limandoides	Janus Green B	- (511.1)	Color	Lonning and Davenport, 1980; Davenport et al., 1981.
	Toluidine blue	+ (305.8)	Color	
	Acridine orange	- (301.8)	Color	
	Dinitrophenol	+ (184.1)	Color	

TABLE 5.1. COMPARISON OF CHORION PERMEABILITIES (Cont.)

Permeability [Perm.]: '+' = permeable; '-' = impermeable; '?' = unknown; Formula weights are shown in parentheses in the third column.

Species	Compound	Perm.	Assay	Source
Gadus morhua	2-methyl-naphthalene	+ (142.2)	Toxicity	Stene and Lonning, 1984a.
"	naphthalene	+ (128.2)	Toxicity	Stene and Lonning, 1984b.
"	2-methyl-naphthalene	+ (142.2)	Toxicity	"
"	1, 3-dimethyl-naphthalene	+ (156.2)	Toxicity	"
"	Hydroxy-naphthalenes	+ (144.2)	Toxicity	"
"	Dimethyl-phenols	+ (122.2)	Toxicity	Falk-Peterson and Kjorsvik, 1987.
"	DDT	+ (354.5)	Toxicity	Dethlefsen, 1975.
"	Benzo(a)pyrene	+ (252.3)	Toxicity	Solbaaken et al., 1984.
"	Phenanthrene	+ (178.2)	Toxicity	"
Clupea harengus pallasi	Benzene	+ (78.1)	Toxicity	Struhsaker et al., 1974.

Figure 5.1. Timing of Developmental Stages at 5 to 6 °C.

(Ranges are from beginning to end of each stage, ionocytes appear at 100% epiboly completion)

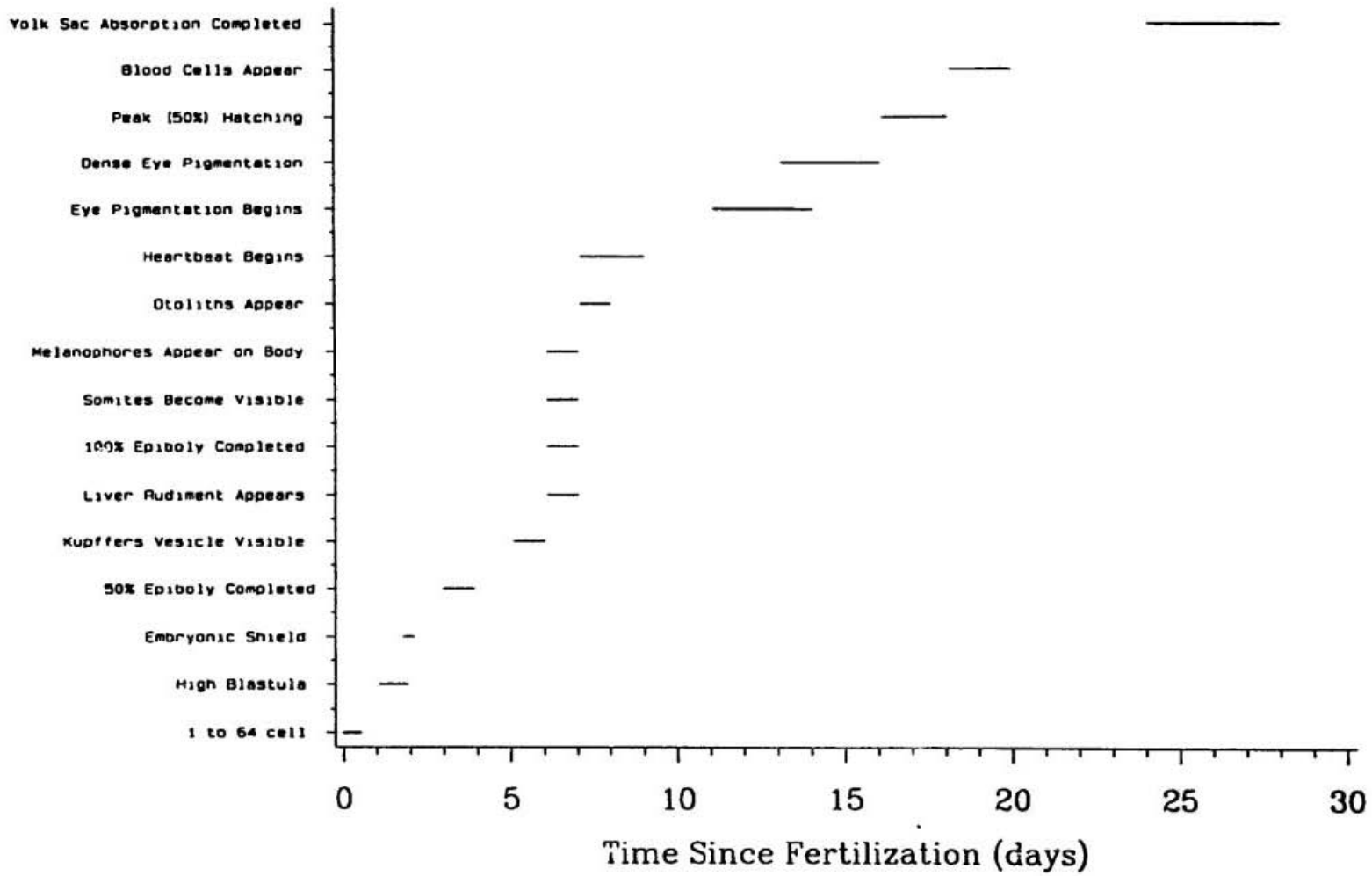


Figure 5.2. Somite Number As a Function of Age (chronological age)

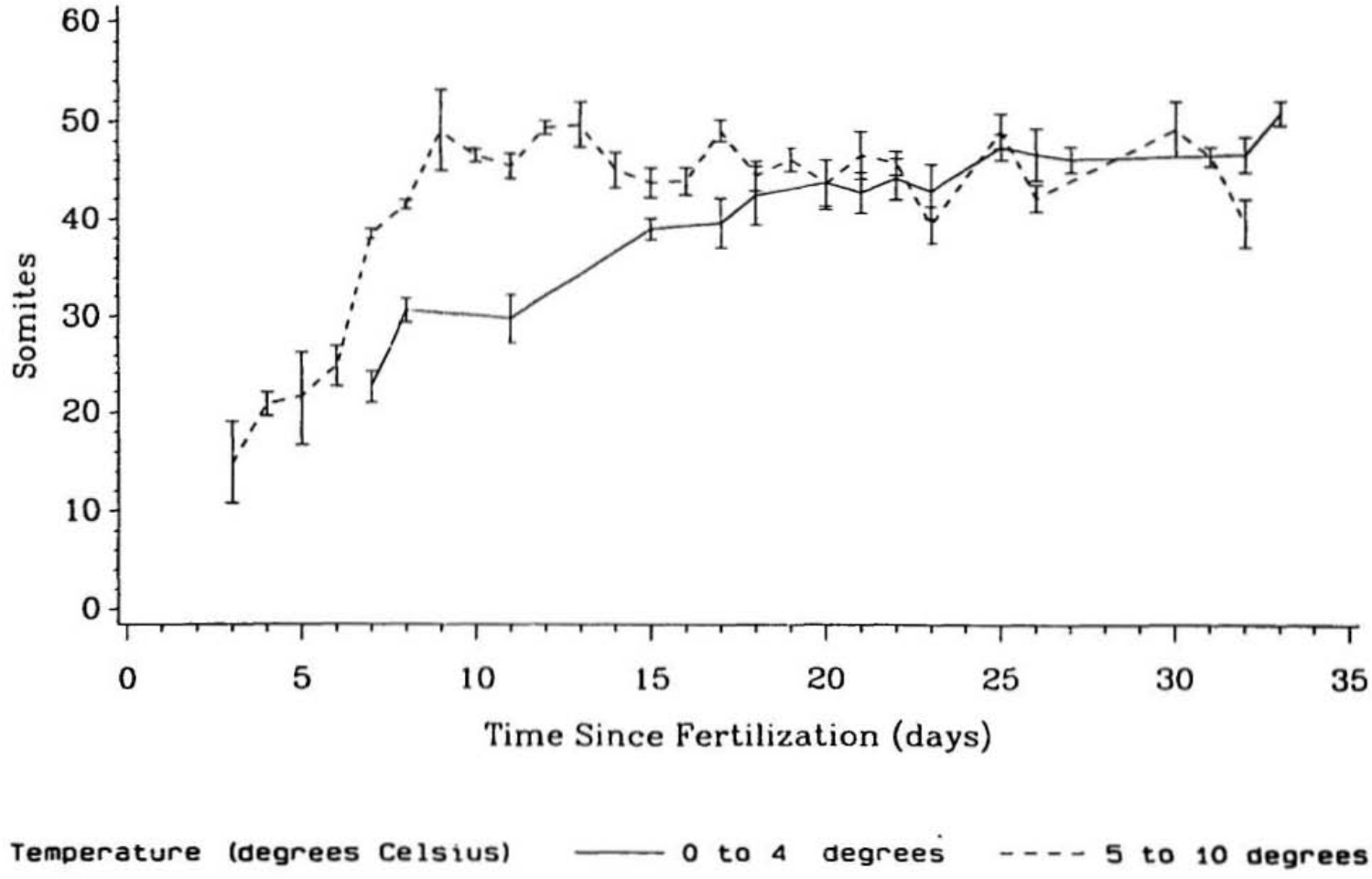


Figure 5.3a. Embryonic Heart Rates (chronological ages)

(plotted lines are for three different incubation temperatures)

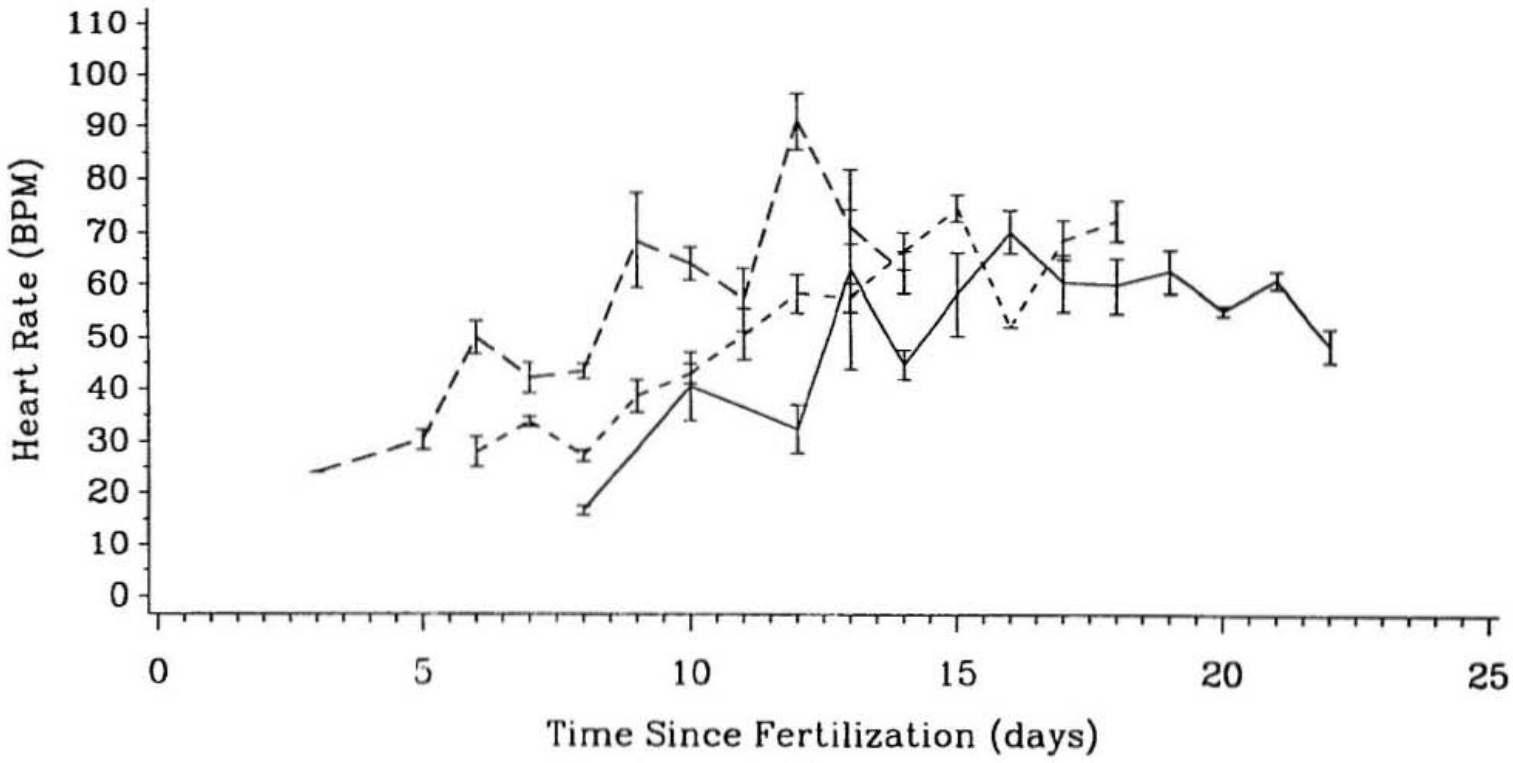
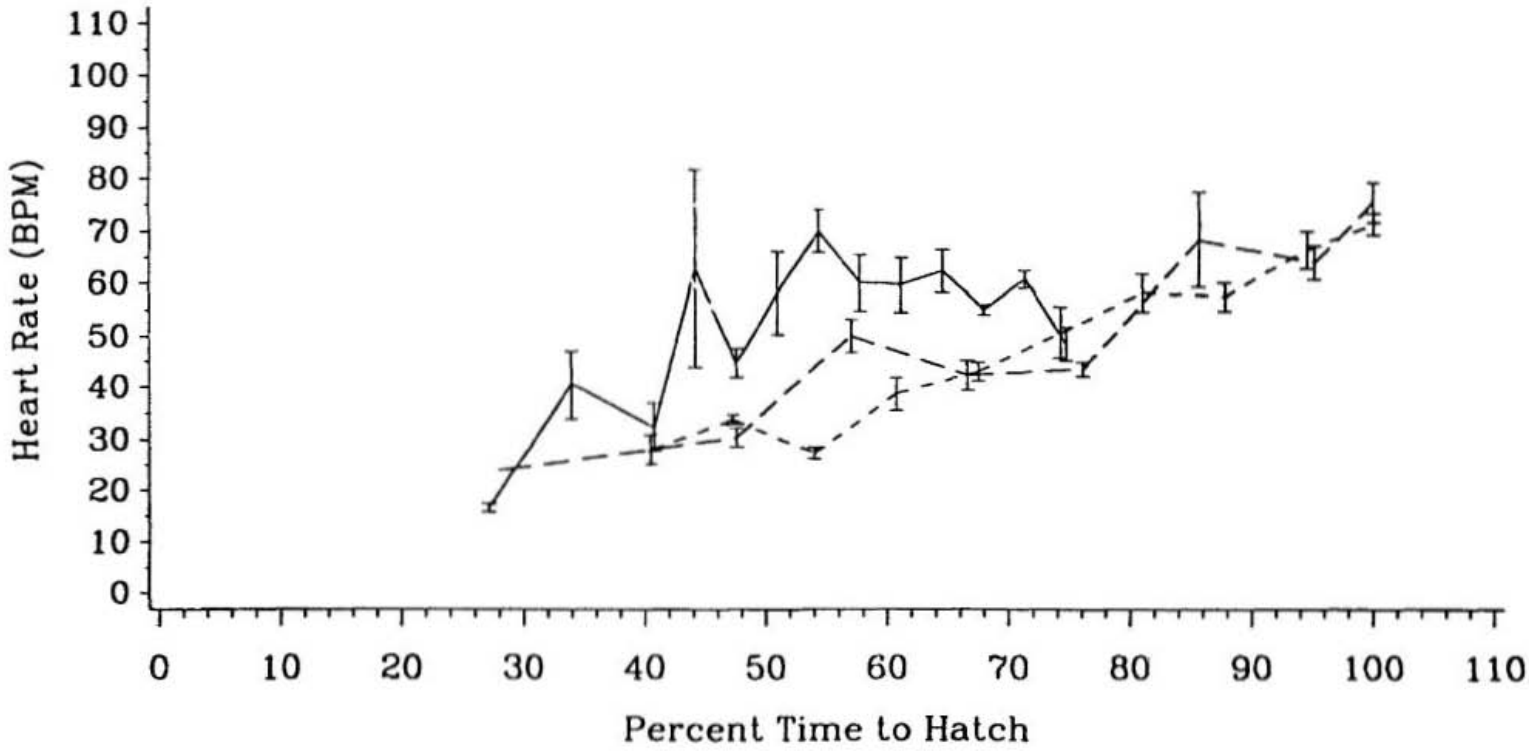


Figure 5.3b. Embryonic Heart Rates (relative ages)

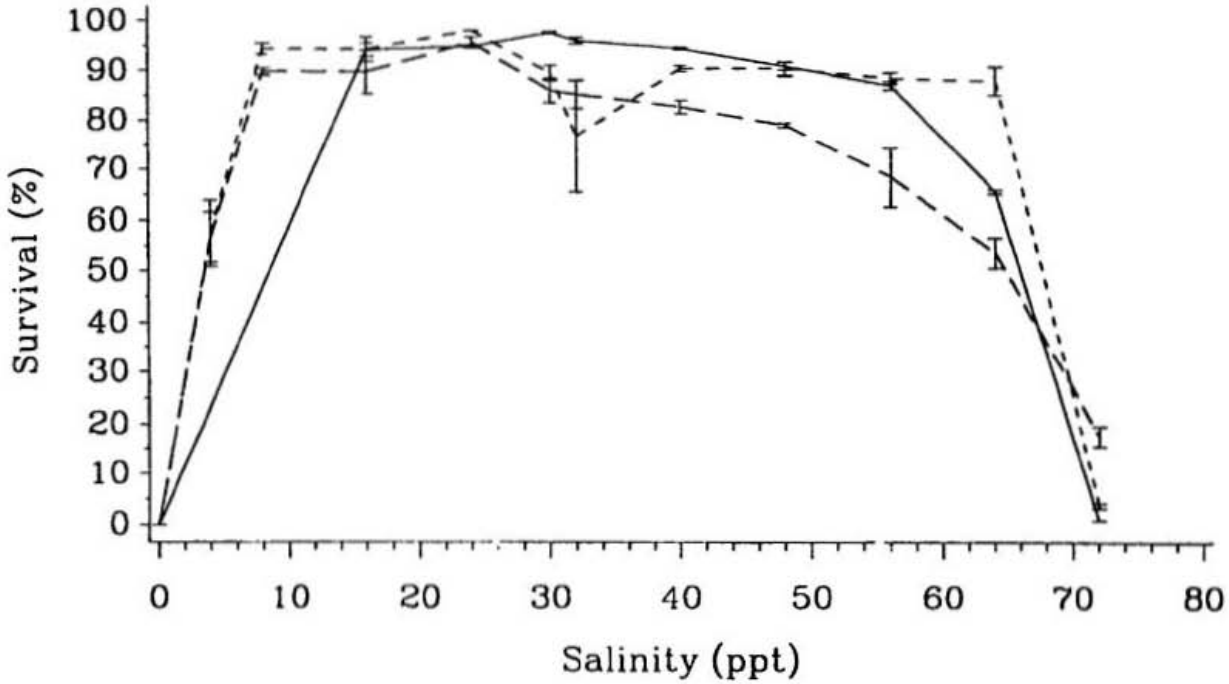
(plotted lines are for three different incubation temperatures)



Temp. (degrees Celsius) : ——— 0 to 5.5 - - - - 5.5 to 7.5 — — — 7.5 to 12

Figure 5.4. Cod Embryo Survival at Various Salinities

(Embryos were transferred from 32 ppt seawater to the test salinity at 1, 4 or 5 days after fertilization)
(Survivors were counted after 3 days incubation; ie. at 4, 7 or 8 days after fertilization)



Initial Transfer Stage ——— 1 day - - - - 4 day — — — 5 day

FIGURE 5.5a. NORMAL (SEAWATER-INCUBATED) COD LARVA

Cod larva at hatch; phase contrast; note the pitted surface of the yolk sac, which is a consequence of the presence of numerous secretory cells, including sacciform, goblet and chloride cells; the three arrows indicate the long, narrow coelomic space between the gut (G) and yolk sac (Y);

Nikon Diaphot-TMD inverted microscope; magnification: 100X.

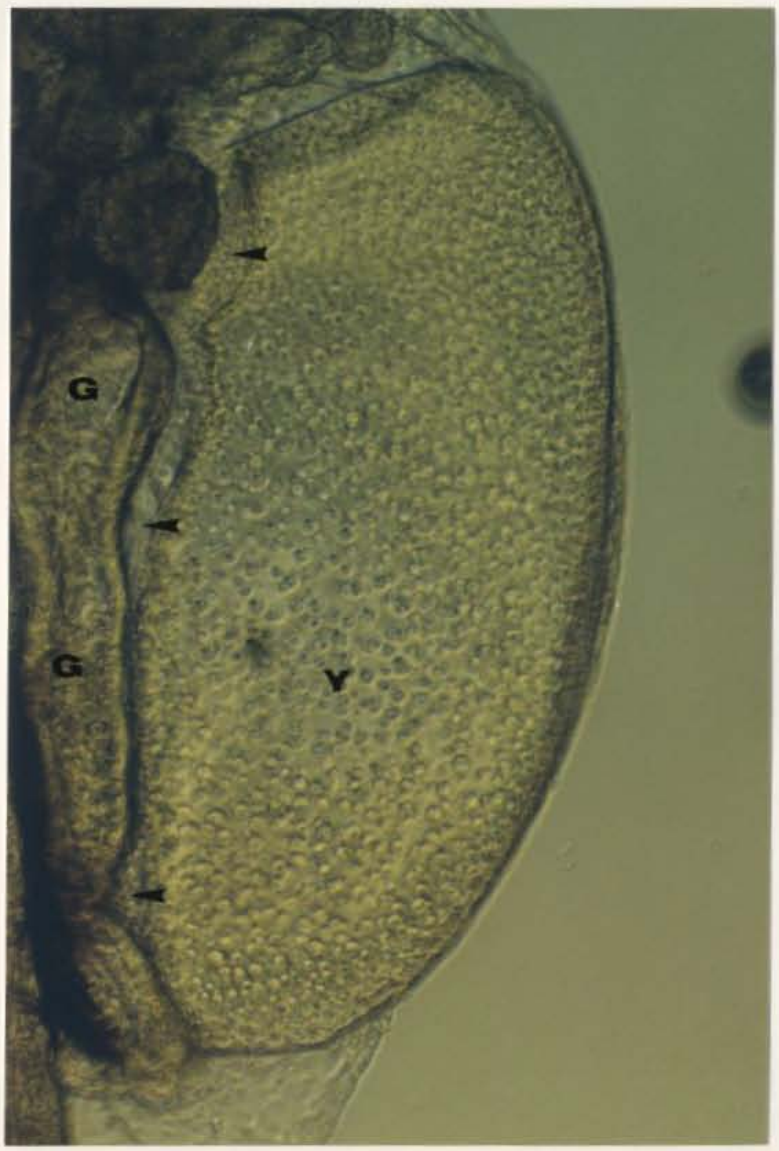


FIGURE 5.5b. COD LARVA CULTURED IN 8 PPT SEAWATER

Cod embryo observed at 8 days after fertilization. Embryo was cultured at 32 ppt salinity and 7°C for 5 days, then transferred to 8 ppt salinity and 7°C from 5 days to 8 days after fertilization. Unstained with chorion intact; showing embryo (E) closely apposed (arrow) to the yolk sac (Y).

Olympus BHS microscope; magnification: 100X.

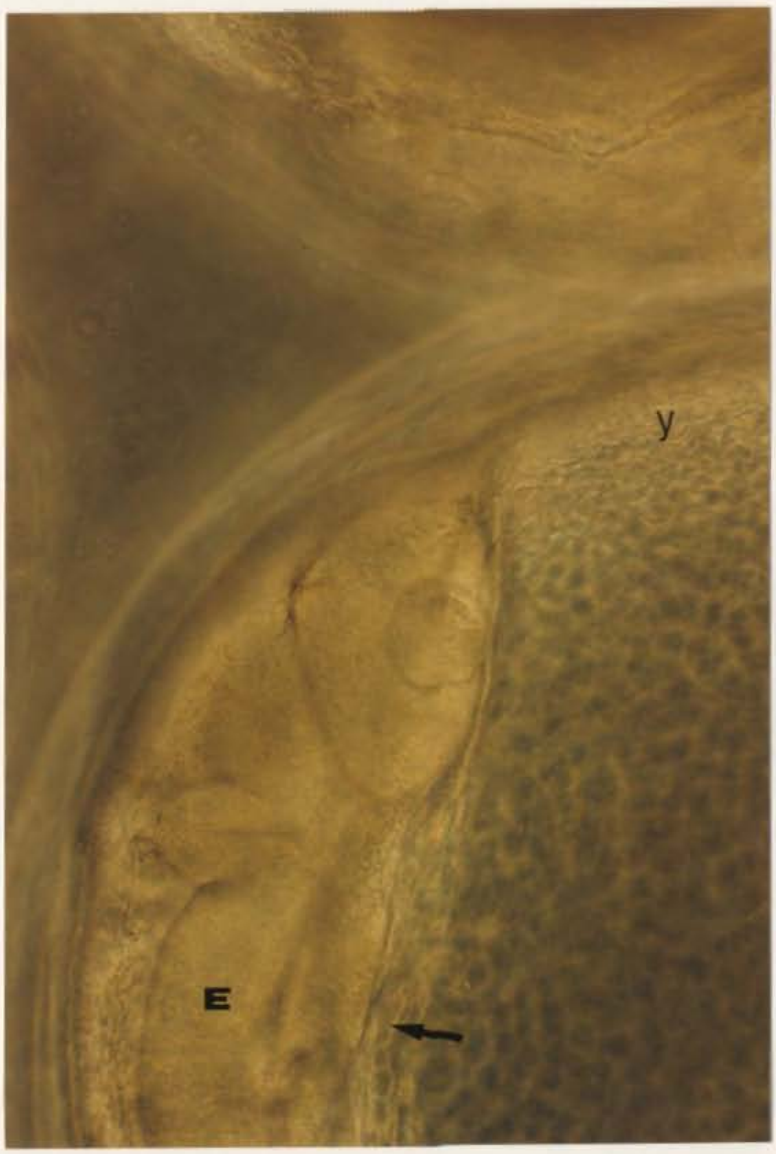


FIGURE 5.5c. COD LARVA CULTURED IN 72 PPT SEAWATER

Cod embryo observed at 8 days after fertilization. Embryo was cultured at 32 ppt salinity and 7°C for 5 days, then transferred to 72 ppt salinity and 7°C from 5 days to 8 days after fertilization. Embryo (E) is separated from the yolk sac (Y) anteriorly by a large cavity (arrow).

Olympus BHS microscope; magnification: 100X.

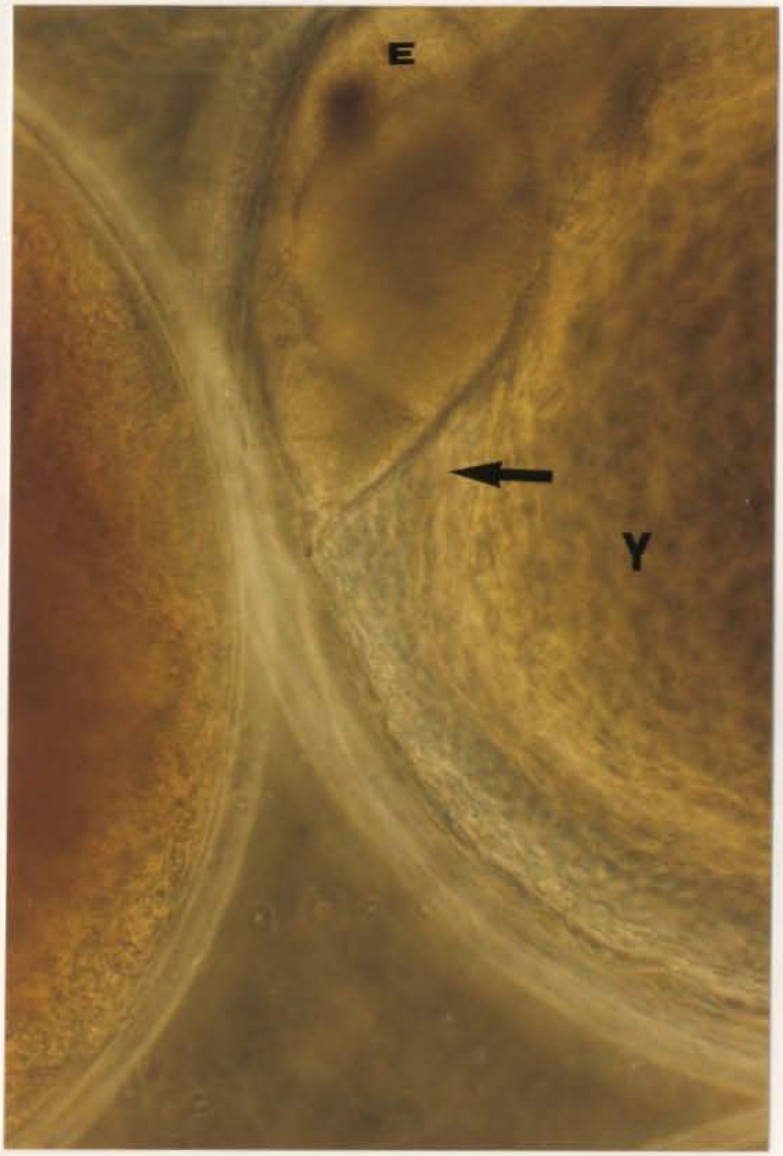


FIGURE 5.6. COD LARVA CULTURED IN 1% (V/V) ETHANOL

Cod embryo observed at 13 days after fertilization. Embryo was cultured at 32 ppt salinity and 7°C for 8 days, then transferred to 1% ethanol in 32 ppt salinity and 5°C from 8 days to 13 days after fertilization, unstained with chorion intact; showing a large anterior cavity (C) between yolk (Y) and pericardial cavity (P).

Olympus BHS microscope; magnification: 100X.

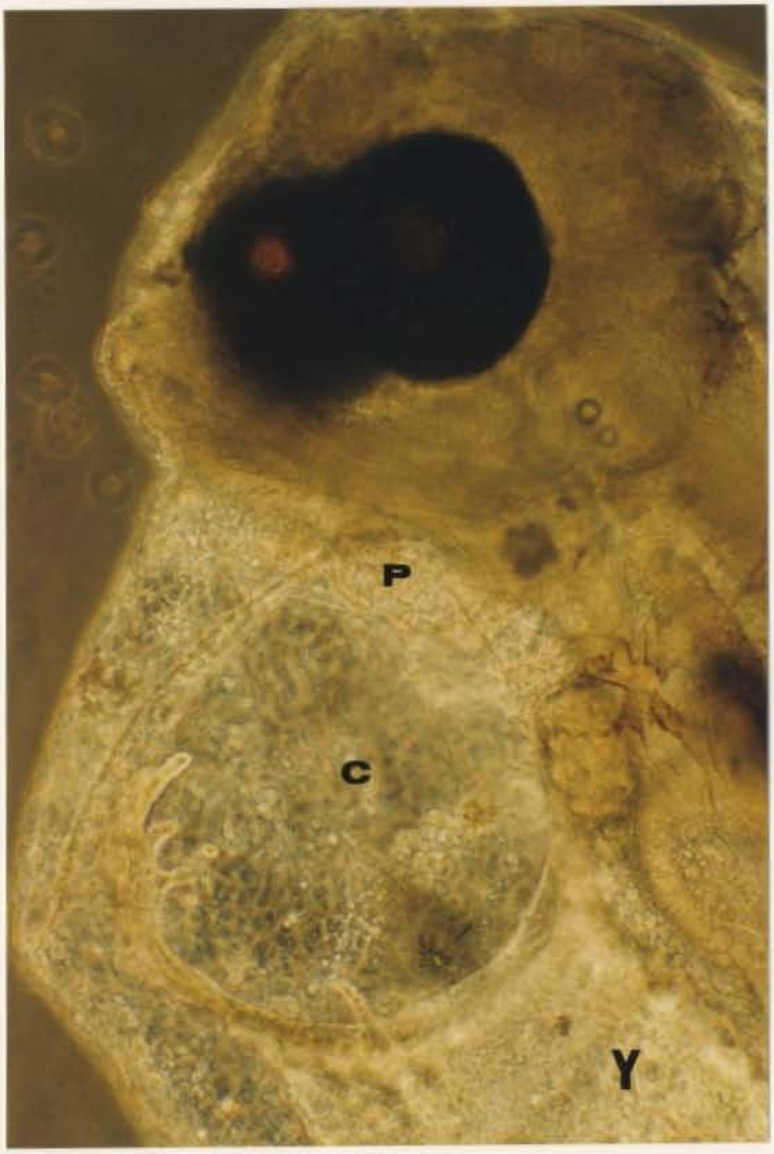


FIGURE 5.7. COD LARVAL YOLK SAC CHLORIDE CELLS

The occurrence of yolk sac (Y) chloride cells (thin arrow) and pharyngeal chloride cells (thick arrow) persists from shortly after the completion of epiboly until hatching; DASPEI stain; 16 d at 0°C.

Zeiss Axiovert 35 microscope; magnification: 100X.

'Blue' fluorescence filter set: 450-490 nm band-pass excitation filter, 510 nm chromatic beam splitter, and 515-565 nm bandpass filter.

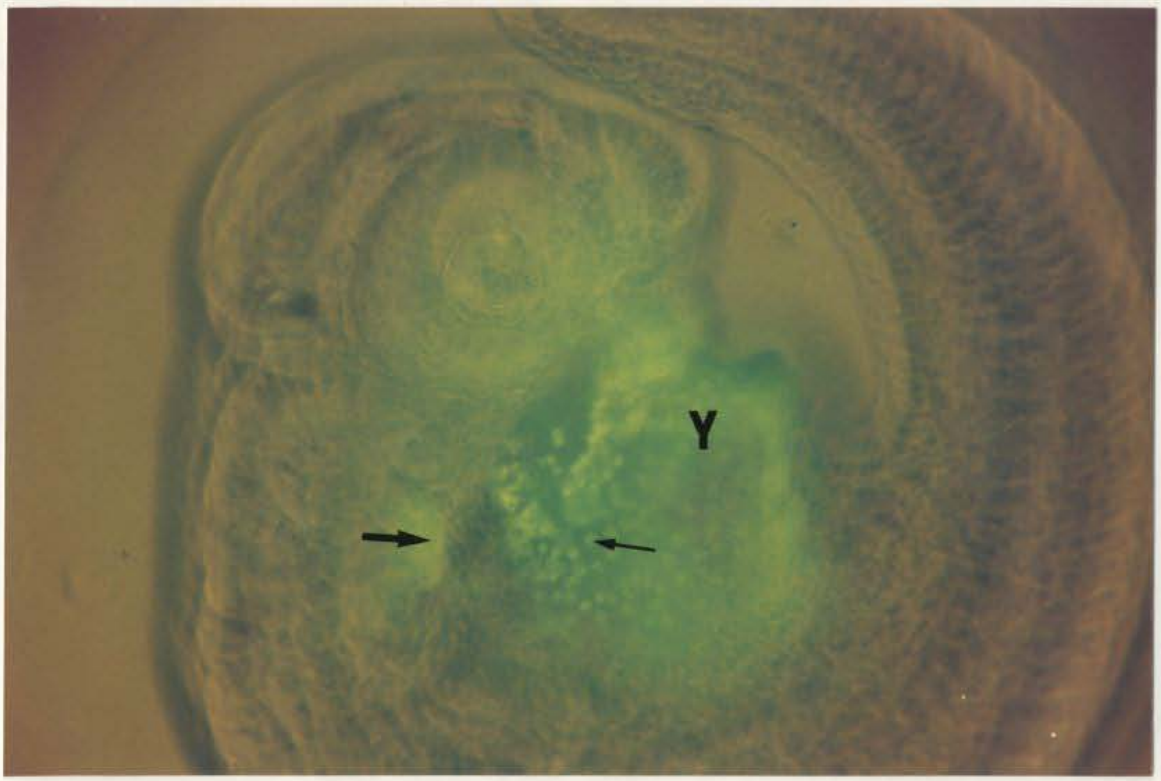
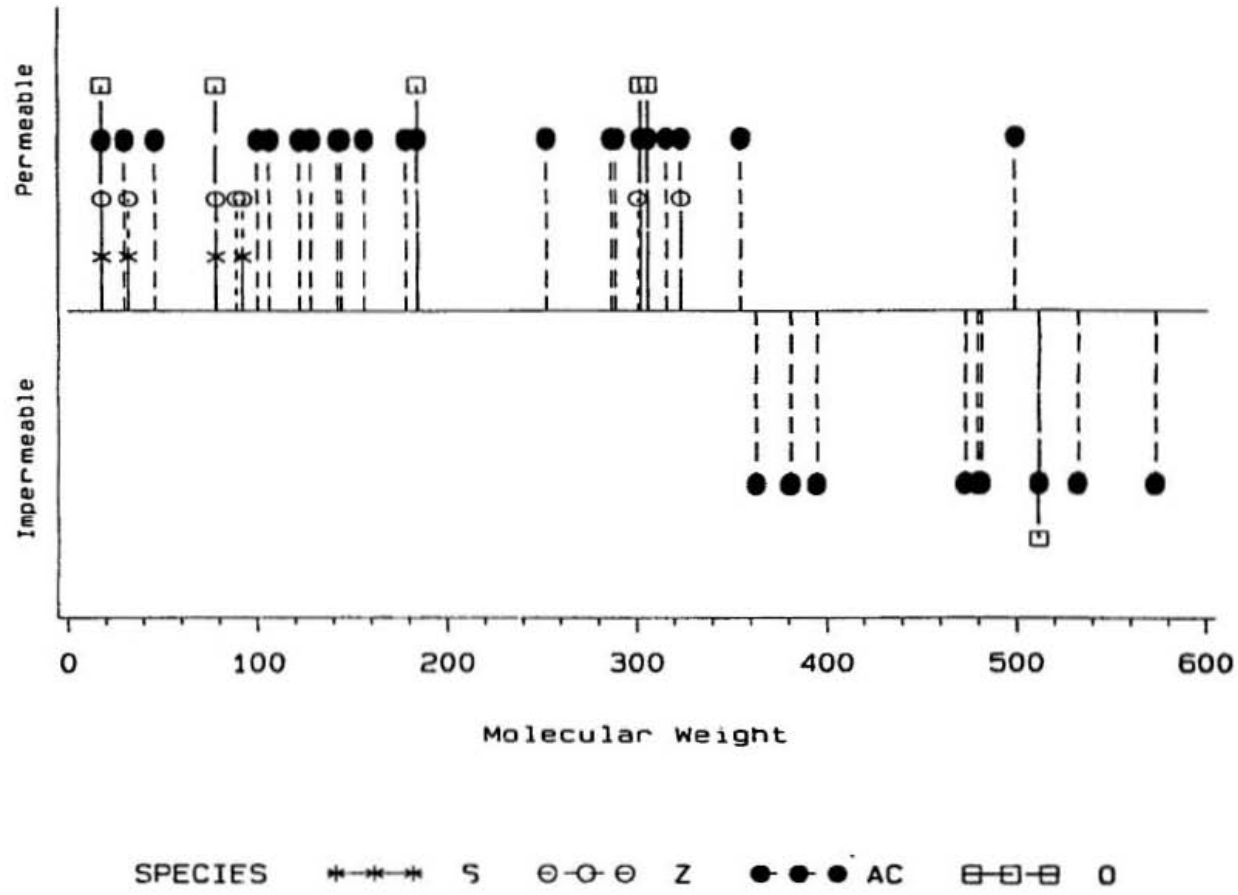


Figure 5.8. Permeability of the Fish Chorion as a Function of Molecular Weight.

(Note: height of vertical lines does not indicate relative permeability)
(Legend: S = Salmonids; Z = zebrafish; AC = Atlantic Cod; O = other)



CHAPTER 6. GENERAL CONCLUSIONS

General Pattern of Ionocyte Differentiation in Atlantic Cod

It has been noted (chapter 4) that chloride cell differentiation in the Atlantic cod coincides with the completion of epiboly, when the pharynx opens to the perivitelline fluid, and epithelia may become somewhat more leaky (Riis-Vestergaard, 1984). The presence of apparently functioning chloride-secreting cells prior to hatching indicates that active osmoregulation is a characteristic of the developing cod embryo.

Although Adoff (1986) suggested that mitochondria-rich cells in the early blastula stage were perhaps precursors of the chloride cells, the present study does not support this contention. The absence of high-potential mitochondria and intense mitochondrial autofluorescence due to pyridine nucleotides is indicative of low levels of metabolic activity in early blastomeres. The absence of mitochondrial fluorescence in early blastula cells treated with styryl dyes indicates that the organelles are not sufficiently differentiated at this point to engage in high levels of ATP synthesis, and consequently have low membrane potentials. This is consistent with the view that vertebrate mitochondrial DNA contains only information for the synthesis of some of the proteins of the electron transport chain (Poulton, 1992; Tyler, 1992). Until the transport chain machinery has been

completely assembled, respiratory activity of the mitochondria may be at the minimum required for cell maintenance. This phenomenon may also explain why early stages rely upon passive barriers to ion influx, and active transport processes develop later.

Effects of Environmental Conditions on Chloride Cell Differentiation

Many marine teleost larvae are known to survive wide fluctuations in salinity (Holliday and Blaxter, 1960; Jones et al., 1966). This adaptation is probably due to their frequent occurrence in the upper layers of inshore waters and estuaries where salinities can be extremely variable. Since the outermost layer of the planktonic egg stage in teleosts is noncellular and allows passive movements of salt and water, the perivitelline fluid which contacts the outer surface of the embryo must exert an osmotic stress on the embryo, in proportion to the salinity level of the seawater medium. This effect has been observed in numerous physiological studies of larval teleost osmoregulation (Alderdice, 1988).

However, contradictory results have been obtained as regards morphological evidence of salinity adaptation. While Jones and coworkers (Jones et al., 1966) reported no changes in the integument of larval herring, Clupea harengus, at different rearing salinities, chloride cells were not examined. In other studies of chloride cell morphology in

relation to environmental salinity, noticeable changes in the chloride cell population have been recorded (Hwang, 1990; Hwang and Hirano, 1985). As embryos or larvae are exposed to higher salinities, chloride cell numbers could, in theory, increase, or ultrastructural changes in the cytoplasm of chloride cells might be observed. According to Hwang (1989), the skin epithelia of marine teleost larvae has a more extensive junctional structure than that of their freshwater counterparts.

Since the chloride cell population turns over like other epithelial cell populations, it appears that this cell type is derived from some other epithelial component, and gradually degenerates, before sloughing and replacement. Further work is required to determine the mechanisms of cell differentiation, and to clarify the factors regulating this process.

One major technical problem with these types of studies is the difficulty in quantifying cytological changes. Previous attempts (eg. Dépêche, 1973) to express chloride cell densities in terms of the number of secretory pores in the gill epithelia must be carefully interpreted. In the Atlantic cod embryo, larva, and adult, a variety of secretory cells exist, including sacciform, goblet and chloride cells, confounding any attempt to count cells based upon superficial appearances.

Similarly, quantitative analyses of enzyme activity in the early stages of fishes have seldom been done (Conte, 1980), probably due to the low levels of enzymes present, and the requirement for extracting relatively large numbers of individuals at the same developmental stage.

The absence of an effect on the relative timing of chloride cell differentiation by changing environmental conditions has interesting implications for embryonic osmoregulation. Embryonic stages of the Atlantic cod appear to be capable of active salt and water regulation over a wide range of salinities and temperatures. Although development rate can be markedly altered by temperature, most events, including the differentiation of chloride cells, maintain their relative position in the sequence of developmental stages. Therefore, whether larvae hatch at an advanced stage of development or not, pharyngeal chloride cells differentiate before the mouth has opened, and before active drinking by larvae. The timing of chloride cell differentiation appears to be tightly regulated by events associated with the migration of ectodermal tissues over the yolk sac, rather than by environmental factors.

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