THE DEVELOPMENT OF ION REGULATION IN LARVAL RAINBOW TROUT, *ONCORHYNCHUS MYKISS*.

THE DEVELOPMENT OF ION REGULATION IN LARVAL RAINBOW TROUT, *Oncorhynchus mykiss.*

by

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ABSTRACT

The development of Na^+ and Ca^{++} transport, ammonia excretion, and respiration in larval rainbow trout were studied over the period of yolk sac absorption and shortly after the onset of exogenous feeding. The gills and the yolk sac epithelium of larvae were examined for any indication of the presence of mitochondria-rich cells and/or Na-K-ATPase activity.

Whole body Na^+ content of the larvae increased throughout yolk sac absorption and after the onset of exogenous feeding. During this period there was an increase in Na^+ influx, efflux, transporter capacity (J_{max}) and transporter affinity (K_m). J_{max} increased 4.6 times during yolk sac absorption, and increased another 2.8 times 248 degree·days after the onset of exogenous feeding. K_m decreased over the first 168 degree·days and stayed constant for the remainder of the experimental period. The gills were the primary site of Na^+ efflux. Na^+ accumulation from the water began the first day post-hatch.

Ca^{++} transport capacity increased throughout yolk sac absorption, but then decreased after the onset of exogenous feeding. $J_{max}^{Ca^{++}}$ exhibited a 15.5 fold increase over 257 degree·days, which then decreased to 30% of that value after exogenous feeding commenced. There was a 162 fold increase in Ca^{++} K_m over yolk sac absorption, which then decreased 15.6 times after the onset of exogenous feeding. Whole body Ca^{++} began to increase approximately 200 degree·days post-hatch and exhibited a large increase after the onset of exogenous feeding. It is possible that during yolk sac absorption the larvae does not begin to accumulate Ca^{++} from the external environment until its internal reserves are depleted, and with the onset of feeding the larvae then obtains its Ca^{++} from its diet.

Ammonia excretion in larval trout increased 3.6 times over yolk sac absorption, and increased another 2.8 times after exogenous feeding commenced. There was no evidence of a link between Na^+ uptake and NH_4^+ excretion in larval fish as NH_4^+ excretion was not stimulated in response to increasing external Na^+ concentrations. Measurements of the ammonium concentration at different surfaces of the larvae revealed a larger ammonium concentration next to the gills than next to the yolk sac and skin. This may indicate that the gills are the primary site for ammonia excretion in the larval fish.

Initially, branchial and cutaneous surfaces contributes to respiration, but as the gills grow and develop and the yolk sac decreases in size and the skin thickens, the contribution of the gills to this function increases.

The gills of first day hatchlings are comprised of gill arches and filaments. Lamellae do not begin to develop until a few days after hatching. The gill filaments contain mitochondria-rich cells and have Na-K-ATPase activity as determined through binding of the fluorescent dye anthroylouabain. The yolk sac epithelium contained cells with concentrated regions of mitochondria, but significant Na-K-ATPase activity was not detected when compared to branchial staining.

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CHAPTER 1: GENERAL INTRODUCTION

During yolk sac absorption, trout undergo rapid development: it is developing its skeleton, its gills are increasing in size and surface area, and it must switch from using endogenous food supplies to exogenous feeding. Several factors such as light intensity (Kwain, 1975), salinity (Blaxter, 1969), egg size (Murray and McPhail, 1988) and oxygen levels (Kalmer, 1992), affect the rate of development, but the most influential factor is temperature (Blaxter, 1969; Alderdice and Velsen, 1978; Crisp, 1981). It has been shown that incubation time to hatching and emergence (swim-up) is inversely related to temperature in chinook salmon (Alderdice and Velsen, 1978), pink and chum salmon (Murray and McPhail, 1988), and brown and rainbow trout (Stonecypher *et al.*, 1994).

Several mathematical models have been developed to determine which one most accurately represents this period of development in fish. The thermal sums equation developed by Wallich in 1901 states that for chinook salmon eggs the product of temperature (x) and incubation time (y) was a constant at 'normal temperatures',

$$x*y = k,$$

where 'normal temperature' is an assumption that development stops at the freezing point for freshwater (Alderdice and Velsen, 1978). A number of more recent studies (Alderdice and Velsen, 1978; Crisp, 1981) have compared Wallich's equation to more complex models, such as the log-inverse Bèlehrádek equation ($\ln P = \ln k + b \ln (x - c)$; where P is 100/y and y is time; k, b and c are constants; and x is temperature. Alderdice and Velsen (1978) concluded that at incubation temperatures near or above 4°C, either the unmodified thermal sums equation or the Bèlehrádek equation will provide

reasonable estimates of the length of development time. For simplicity sake, this study uses the thermal sum hypothesis to standardize the development of larval rainbow trout. In larval rainbow trout, the yolk sac is completely covered by the ectoderm in just under 252 degree·days post-hatch (36 days post-hatch at 7°C; 15 days post-hatch at 15°C). A degree·day is the thermal sums unit used to describe the age of the larval fish. It is the product of the average daily temperature and the age, in days, of the larval fish post-hatch. By using the thermal sums equation, it is possible to compare the development of larval rainbow trout reared at different temperatures.

At hatching, larval rainbow trout are usually transparent. Pigmentation increases with development. Opercula are thin and transparent and do not completely cover the gills. The branchial epithelium consists of gill arches and filaments (Holeton, 1971). The skeleton is cartilaginous and when skeletal ossification starts, it begins with the bones at the rostral region and progresses posteriorly (Heming, 1982). The yolk sac is large, in the chinook salmon making up approximately 59% of the total cutaneous area (Rombough and Moroz, 1990). As the yolk is metabolized the syncytium decreases in size, the ectoderm lays down scales and the skin pigmentation begins to migrate towards the ventral portions of the larva from the lateral line. There is still yolk remaining by the time the yolk sac is completely covered with the developing ectoderm.

Yolk content of eggs is inter- and intra-species dependent. Within a given species the yolk is affected by maternal age, weight and diet (Beacham and Murray, 1985). Based on dry weight, the yolk of rainbow trout eggs contains approximately 60% protein, 11% lipid, 0.6% carbohydrate and 4% inorganic material (Heming and Buddington, 1988). The yolk is surrounded by a specialized tissue called the yolk syncytium, which is responsible for yolk

absorption (Heming and Buddington, 1988). The syncytium is covered with a network of capillaries (the vitelline circulation), a mesoderm layer and an ectoderm layer of tissue. Absorption of the yolk is via phagocytosis by the syncytium, intrasyncytial digestion and the release of yolk metabolites to the vitelline circulation (Heming and Buddington, 1988; Kamler, 1992). After all of the yolk reserves are depleted, the yolk syncytium is reabsorbed (Heming and Buddington, 1988).

Growth is an increase in size resulting from the production of new tissues (Kamler, 1992). For endogenously feeding larvae, the yolk supplies the necessary materials for growth. However, the yolk also supplies the energy needed for metabolism, so growth is dependent on the conversion efficiency of yolk to tissue. The conversion efficiency is a measure of what proportion of the initial yolk reserves are converted to tissue, 100% efficiency is obtained when all of the yolk reserves are turned into tissue. Factors such as temperature, light conditions, water turnover rate and substrate contour all affect yolk conversion efficiency (Heming, 1982; Heming and Buddington, 1988; Kamler, 1992). The highest conversion efficiency is seen at lower temperatures (due to decreased metabolic costs), in dark lighting conditions with high water turnover and a substrate with a high surface area (Heming, 1982). Conversion efficiencies between 52% to 71% from fertilization to swim up have been reported for larval rainbow trout (Rombough, 1988a; Kamler, 1992).

The ion transport abilities of larval teleosts are not well documented. Ion kinetics, the location of ion activity, the cells responsible for ion movement and the mechanisms of ion movement are all part of ion transport. Some studies on larval fish have examined one or two aspects of ion transport, such as Na^+ uptake kinetics in Atlantic salmon (McWilliams

and Shephard, 1989), Ca^{++} balance in tilapia (Hwang *et al.*, 1994), and the development of yolk sac and branchial mitochondria rich cells in tilapia (Ayson *et al.*, 1994; Li *et al.*, 1995), but there has not been a study which has investigated a combination of these aspects. The purpose of this study was to create a comprehensive work on the development of the ion transport capabilities in larval rainbow trout, throughout yolk sac absorption and shortly after the onset of exogenous feeding.

Research Objectives

1. To determine the properties of the ion uptake mechanisms in larval rainbow trout and how they change over the period of yolk sac absorption. Kinetic analysis of Na^+ and Ca^{++} uptake over time were used to determine the transport capacity (J_{max}) and the affinity (K_m) of the ion transporters.
2. To establish the presence and functional state of chloride cells in the gill and yolk sac epithelium. Confocal microscopy and specific fluorescent dyes were used, in combination, to identity mitochondria-rich cells and Na-K-ATPase activity.
3. To elucidate the contribution of various epithelia to ion activity. Microelectrode and vibrating probe techniques were employed to follow changes in NH_4^+ and pH, Na^+ and Ca^{++} activity over time at the gills, the yolk sac and integument of the larval trout.

CHAPTER 2: ION TRANSPORT IN LARVAL RAINBOW TROUT

INTRODUCTION

A number of studies have suggested larval fish are capable of ion regulation (Shelbourne, 1957; Weisbart, 1968; Shen and Leatherland, 1978a; Steingraeber and Gingerich, 1991; Li *et al.*, 1995). Studying ion accumulation in larval brook trout exposed to acidic soft water, Steingraeber and Gingerich (1991) concluded that, at hatching, larvae had functioning compensatory mechanisms to reduce ion loss and establish ionic homeostasis which continued throughout the developmental period. They based this conclusion on their finding that, although larvae exposed to pH 6.5 had lower whole body ion concentrations than larvae exposed to pH 8.2, the net amount of ions accumulated by the fish in both groups were almost identical. Furthermore, Shen and Leatherland (1978a) found that larval rainbow trout maintained their osmotic and ionic internal environments independent of the external environment when exposed to water of varying degrees of salinity.

Whole body Na^+, Ca^{++}, K^+, Cl^-, Fe, and Sr increase in salmonid larvae throughout yolk sac absorption (Shen and Leatherland, 1978a; Shearer, 1984; Wood *et al.*, 1990; Steingraeber and Gingerich, 1991). In these studies, ion concentrations are expressed either per gram of wet weight or per gram of dry weight. However, throughout yolk sac absorption wet weight increases while dry weight decreases, which will, respectively, underestimate or overestimate the net accumulation of ions. Only Steingraeber and Gingerich (1991) reported the corresponding larval weights so the actual net ion accumulation could be calculated, and this calculation confirms an overall trend of net

accumulation of Na^+, Cl^-, Ca^{++}, K^+ and Mg^{++} in larval brook trout. In the present study ion concentrations have been expressed on both a per gram and a per fish basis.

Although not much is known of the actual ion uptake mechanisms in larval salmonids, McWilliams and Shephard (1989) demonstrated a carrier-mediated saturable transport system for Na^+ in Atlantic salmon embryos and larvae. Na^+ transport becomes more active through larval development with increases in J_{max}, while the affinity remains the same. The nature of the Na^+ uptake mechanism (i.e. Na^+/H^+ or Na^+/NH_4^+ exchange) has not been studied.

Skeletal ossification of the larvae begins shortly after hatching and continues throughout yolk sac absorption (Heming, 1982). Despite the importance of Ca^{++} for this process to occur, there is little known about the Ca^{++} uptake mechanism in salmonids during this period.

At hatching, larval rainbow trout excrete their nitrogenous waste as ammonia and urea (Wright *et al.*, 1995). Throughout yolk sac absorption, both ammonia and urea excretion increase. Although ammonia excretion increases throughout yolk sac absorption and after the onset of exogenous feeding, the relationship between ammonia excretion and ion regulation is not known.

Therefore, the objectives of this chapter are as follows: to study if, and the extent which, larval fish gain electrolytes from the environment, to study the development of Na^+ and Ca^{++} transport mechanisms, and to determine the relationship, if any, between Na^+ and NH_4^+ exchange.

MATERIALS AND METHODS

Experimental Animals

Eyed rainbow trout eggs were obtained from 4 hatcheries (Blue Springs Trout Hatchery, Durham, ON; Cedar Springs Trout Farm, Midland, ON; Rainbow Springs Trout Farm, Thamesford, ON; and Plymouth Rock Trout Farm, Plymouth, Massachusetts) (table 1). From the Ontario hatcheries, one egg lot was held at 10.00 ± 0.05 °C, a second was held at 8.0 ± 0.1 °C and the 10 remaining series were held at 7.00 ± 0.05 °C. All of the egg lots were held in subdued light in a 5-liter rectangular container supplied with a continuous flow of aerated, dechlorinated Hamilton tap water ($[Na^+]$: 0.6 mmol l^{-1}, $[Cl^-]$: 0.7 mmol l^{-1}, $[Ca^{++}]$: 1.0 mmol l^{-1}, $[HCO_3^-]$: 1.9 mmol l^{-1}, pH 7.9). Dead eggs were removed daily.

The egg lot from Plymouth Rock Trout Farm was held statically in a 40-liter aquarium filled with aerated, dechlorinated Woods Hole tap water ($[Na^+]$: 0.8 mmol l^{-1}, $[Ca^{++}]$: 0.8 mmol l^{-1}, pH 7.5). Water temperature was initially 7°C, but was gradually raised over seven days and held at 15°C. This temperature was chosen to increase the developmental rate of the larvae in order to examine the whole period of yolk sac absorption within a two week period. Dead eggs were removed daily.

Once hatching commenced the larvae were transferred to 0.5-liter cylindrical mesh-sided containers corresponding to the day they hatched. All of the containers were held in a wet table filled with aerated, dechlorinated tap water. Larvae were held in subdued light and the temperature was held constant throughout yolk sac absorption. Larvae were not fed during this period.

Table 2.1: A summary of the holding temperatures, initial weights and use of the egg lots obtained from various hatcheries. Initial wet weights are reported as means ± SE, (n). RS: Rainbow Springs Trout Farm; BS: Blue Springs Trout Farm; CS: Cedar Springs Trout Farm; PR: Plymouth Rock Trout Farm; WB: whole body.

Egg Lot	Date	Holding Temp (°C)	Initial Wet Weight (mg)	Source	Use
1	Oct. 1994	10	42 ± 2 (3)	RS	WB ions
2	Nov. 1994	8	95 ± 5 (5)	RS	pH & NH_4^+ measurements
3	Feb. 1995	7	73 ± 1 (10)	BS	not used, high mortality
4	Feb. 1995	7	82 ± 1 (40)	BS	not used
5	Mar. 1995	7	88 ± 4 (10)	BS	WB ions
6	Apr. 1995	7	82 ± 3 (10)	CS	WB ions, weights, NH_4^+ & pH measurements
7	May 1995	7		BS	confocal microscopy
8	Sept. 1995	15	59 ± 2 (12)	PR	WB ions, weights Na^+ & Ca^{++} measurements
9	Nov. 1995	7		BS	NH_4^+ & pH measurements
10	Jan. 1996	7	79 ± 2 (35)	BS	Na^+ kinetics, confocal microscopy
11	Apr. 1996	7	68 ± 2 (10)	BS	Ca^{++} kinetics, NH_4^+ & pH measurements
12	May 1996	7	78 ± 1 (56)	BS	Ca^{++} kinetics, confocal microscopy
13	June 1996	7	74 ± 3 (20)	BS	WB ions, weights

After yolk sac absorption was complete, larvae from the last 2 series were transferred to an 8-liter cylindrical opaque plastic container supplied with aerated dechlorinated tap water and feeding commenced. The fish were presented with approximately 10% of their body weight per day of starter feed (Zeilger). The ionic composition of the food was as follows: $[Na^+]$: 0.9 $mEq.g^{-1}$, $[Cl^-]$: 0.4 $mEq.g^{-1}$ and $[Ca^{++}]$: 0.1 $mEq.g^{-1}$. An automatic feeder was used to ensure the larvae were fed at 2 hour intervals.

Wet and Dry Weights

For measurements of wet and dry weights, larvae were sampled at weekly intervals throughout the period of yolk sac absorption. Fish were sacrificed by immersion in a lethal concentration of tricaine methanesulfonate (MS 222, 0.16 $g.l^{-1}$). To determine wet weight, individual larva were blotted dry and then weighed in a preweighed aluminum tray. To determine dry weight, the tray and the fish were covered with aluminum foil and placed in a 60°C oven for 48 hours, cooled in a dessicator, and weighed. Whole body water content was then calculated from the difference between the wet and dry weights.

Whole Body Ions

For measurements of whole body Na^+, Cl^- and Ca^+, larvae were sampled at 3-4 day intervals until yolk sac absorption was complete (approximately 274 degree·days post-hatch) and again at 424 degree·days post-hatch (150 degree·days after the onset of exogenous feeding). Six to ten fish from each sampling day were individually digested in 200 to 250 µl of 1.0 N H_2SO_4 at 60°C for 48 hours, with periodic mixing. The samples were vortexed, centrifuged and then stored for ion analysis.

The distribution of Na^+ and Cl^- between the yolk sac and remaining tissue of larval fish was determined in egg lot #6 (table 1). Seven to ten fish for both whole larvae and larvae without yolk sacs were sampled at 7, 56 and 156 degree·days post-hatch. Individual whole larvae were blotted dry, digested in 200 µl of 1.0 N H_2SO_4 and stored for later ion analysis. Yolk sacs were dissected from the larvae using a razor blade. Removal was quick to prevent any blood loss, and care was taken to remove the yolk sac without taking any additional body tissue. The yolk sac was discarded and the remaining tissue was digested and stored for later analysis of Na^+ and Cl^-. Yolk sacs could not be dissected from larvae older than 156 degree·days without removing excess fish tissue.

Na^+ Uptake Kinetics and Ammonia Excretion

Changes in the Na^+ transport mechanism with larval development were determined by the measurement of Na^+ uptake kinetics at periodic intervals. Larvae were sampled every 3-4 days throughout the yolk sac absorption period, and again at 249 degree·days after the onset of exogenous feeding. For each day sampled, a group of six larval fish were exposed to one of six different concentrations of $^{24}Na^+$ (ranging from 0.05 to 2.5 mmol l^{-1} NaCl in 1.0 mmol l^{-1} $CaCl_2$, pH was adjusted to 7.9 with KOH). $^{24}Na^+$ concentration in the radiolabelled media ranged from 0.037 kBq.ml^{-1} to 1.85 kBq.ml^{-1}. To prevent contamination of the radiolabelled media, larvae were rinsed briefly in deionized water to remove surface bound Na^+ before being placed in the media.

Water samples were taken at hourly intervals during the 3 hour exposure, starting at time 0. The water samples were used to determine the

specific activity of the radiolabelled media, the [Na^+] of the experimental solutions and the ammonia excretion rate over the 3 hour exposure period.

At the end of the exposure period, the larval fish were briefly rinsed in tap water to remove any surface $^{24}Na^+$, placed in individual scintillation vials with 20 ml of dechlorinated tap water, counted in a Minaxi auto gamma 5000 series gamma counter (Canberra-Packard Canada Ltd.) and weighed.

Effect of External [Na^+] on Ammonia Excretion

The above experiment showed that through yolk sac absorption there is an increase in ammonia excretion that parallels the increase in Na^+ uptake. Therefore, the purpose of this experiment was to determine if an acute i.e., 10 minute, increase in external [Na^+] would stimulate ammonia excretion.

Larval fish, sampled several times throughout yolk sac absorption, were exposed to a range of different Na^+ concentrations (from 0.05 to 2.5 mmol l^{-1} NaCl). Six fish were used for each concentration. The larvae were acclimated to the gently aerated bathing solution (1.0 mmol l^{-1} $CaCl_2$, pH adjusted to 7.9 with KOH, temperature held constant at 7°C) for 30 minutes. The solution was then spiked with the appropriate amount of 50 mmol l^{-1} NaCl to bring the bathing solution to the desired Na^+ concentration.

Water samples were taken before the fish were added to the bathing solution, after the 30 minute acclimation period and 10 minutes after the addition of NaCl. These samples were taken in order to determine ammonia excretion over the first 30 minutes and the change in ammonia excretion 10 minutes after the addition of NaCl. Group weights were taken after the Na^+ exposure and the fish were returned to their holding table.

Ca^{++} Uptake Kinetics

Ca^{++} influx throughout yolk sac absorption and shortly after the onset of exogenous feeding was followed by studying Ca^{++} uptake kinetics. Ca^{++} uptake kinetics were conducted using a method similar to the Na^{+} uptake kinetic experiments. Briefly, groups of 4 larval fish were exposed to 6 different concentrations of $^{45}Ca^{++}$, ranging from 0.005 to 1.25 mmol l^{-1} $CaCl_2$ in 0.65 mmol l^{-1} NaCl; pH was adjusted to 7.8 ± 0.1 using KOH. The radiolabelled media contained between 0.037 $kBq.ml^{-1}$ to 1.85 $kBq.ml^{-1}$ of $^{45}Ca^{++}$. Larvae were sampled weekly during the period of yolk sac absorption and again at 343 degree·days after the start of exogenous feeding.

At the end of the 3 hour exposure period, the fish were removed from the radiolabelled media and rinsed in 5 ml of 10 mmol l^{-1} ethyl-enediamine-tetraacetic acid (EDTA) with MS 222 (0.16 $g.l^{-1}$, pH adjusted to 7.4 with KOH), for 2 minutes to remove surface bound Ca^{++}. The EDTA solution was decanted from the larvae and placed in a scintillation vial to determine its $^{45}Ca^{++}$ activity. Whole fish were weighed, placed in individual scintillation vials with tissue solubilizer (Soluene-350, Packard-Canberra Canada Ltd.) and placed in a 60°C oven overnight to aid tissue digestion. $^{45}Ca^{++}$ activity was determined by adding a liquid scintillation fluor (Hionic-Fluor, Canberra-Packard, for the digested fish samples; BCS, Amersham Canada Ltd., for the water samples). The vials were shaken, wiped with acetone and placed in a beta counter (1217 RackBeta liquid scintillation counter, LKB Wallac, Fisher Scientific) for 30 minutes before counting was initiated.

Before the Ca^{++} uptake kinetics were performed, experiments to determine the amount of Ca^{++} bound to the surface of the larvae and the most effective agent for removing surface Ca^{++} were performed. Larval fish were exposed to $^{45}Ca^{++}$-labelled tap dechlorinated water for either 30 minutes,

1 hour or 2 hours. The larvae were rinsed for 2 minutes in one of the following solutions (in mmol l^{-1}): 1.0 EDTA, 10.0 EDTA, 0.1 $CaCl_2$, 1.0 $CaCl_2$ or 10.0 $CaCl_2$. The rinse solution was decanted into a scintillation vial. The larvae were weighed, placed in individual scintillation vials with tissue solubilizer and digested overnight. The $^{45}Ca^{++}$ activity of the rinse solution and the larvae was determine as described for the Ca^{++} uptake kinetics.

Data Analysis for Na^+ and Ca^{++} Kinetics

Na^+ and Ca^{++} influx rates ($J_{in}^{Na^+}$ and $J_{in}^{Ca^{++}}$) were determined based on the accumulation of radioactivity in the body of the whole larvae using the equation:

$$J_{in} = (CPF)/(SA * W * t)$$

where **CPF** was the number of counts per fish in cpm, **SA** was the specific activity of the water (cpm/nEq), **W** was the weight of the larval fish in grams, and t was the exposure time of the larval fish to the radioactive media in hours.

Kinetic parameters J_{max} and K_m were determined in SAS JMP using the following equation:

$$J_{in} = J_{max} * [X]_{ion} / (K_m + [ion])$$

where J_{in} was the Na^+ or Ca^{++} uptake rate and $[X]_{ion}$ was the external concentration of Na^+ or Ca^{++}.

Analytical Methods

For Na^+ analysis, 20 µl of the supernatant was diluted in 5 ml H_2O_{dd}; for Ca^{++} analysis, 10 µl of the supernatant was diluted in 10 ml of 0.2% $LaCl_3$; no dilutions were made for Cl^- analysis. Na^+ and Ca^{++} were analyzed using a

Varian AA-1275 Series atomic absorption spectrophotometer, and Cl^- was analyzed using a model CMT 10 chloride titrator.

For ammonia determinations, water samples were assayed using the micro-modification of the salicylate-hypochlorite assay for ammonia (Verdouw *et al.*, 1978).

RESULTS

Weights and Water Content

Initial weights of the larval fish lots ranged from 42 ± 2 mg to 95 ± 5 mg (table 2.1), with the majority of the larval weights falling between 60 and 85 mg. Initial water content determined in 3 of the egg lots varied between 37.8 ± 5.7 % to 62.8 ± 0.6 % wet weight. Even though there were significant differences in weights and water content amongst these groups they exhibited the same basic trends. Therefore, for simplicity's sake, one set of data (June 1996) has been chosen as representative.

Over the 250 degree·day period of yolk sac absorption wet weight increased 1.8 fold, while dry weight declined to half its initial value (figure 2.1A). Feeding commenced on 250 degree·days and, 172 degree·days later, larvae exhibited a further 2.2 fold increase in wet weight (figure 2.1A). The majority, if not all, of the weight gain over yolk sac absorption was due to a 2.2 fold increase in body water content as body water rose over yolk sac absorption from 37.8 ± 5.7 % to 83.8 ± 0.7 % of wet weight (figure 2.1B).

Whole Body Ions

Whole body Na^+, Cl^- and Ca^{++} content from 5 different egg lots were determined. There were significant differences in starting electrolyte content between egg lots. Initial Na^+ content of first day hatchlings ranged from 0.8 ± 0.0 $\mu Eq.fish^{-1}$ to 2.4 ± 2.2 $\mu Eq.fish^{-1}$, Cl^- content ranged from 2.1 ± 0.1 $\mu Eq.fish^{-1}$ to 4.6 ± 0.2 $\mu Eq.fish^{-1}$, and Ca^{++} content varied from 1.6 ± 0.0 $\mu Eq.fish^{-1}$ to 2.6 ± 0.2 $\mu Eq.fish^{-1}$. Comparisons of the electrolyte content with the corresponding wet weights of first day hatchlings revealed a significant linear relationship between body weight and content for the three ions studied (figure 2.2),

Figure 2.1: **A.** Representative relationship between wet and dry weights (mg) throughout yolk sac absorption and shortly after the onset of exogenous feeding. Wet weight: -□- ; Dry weight: --o--.

B. Relationship between water content (% wet weight) and time. Means ± SE, n= 10; *: exogenously feeding; Temperature: 7°C.

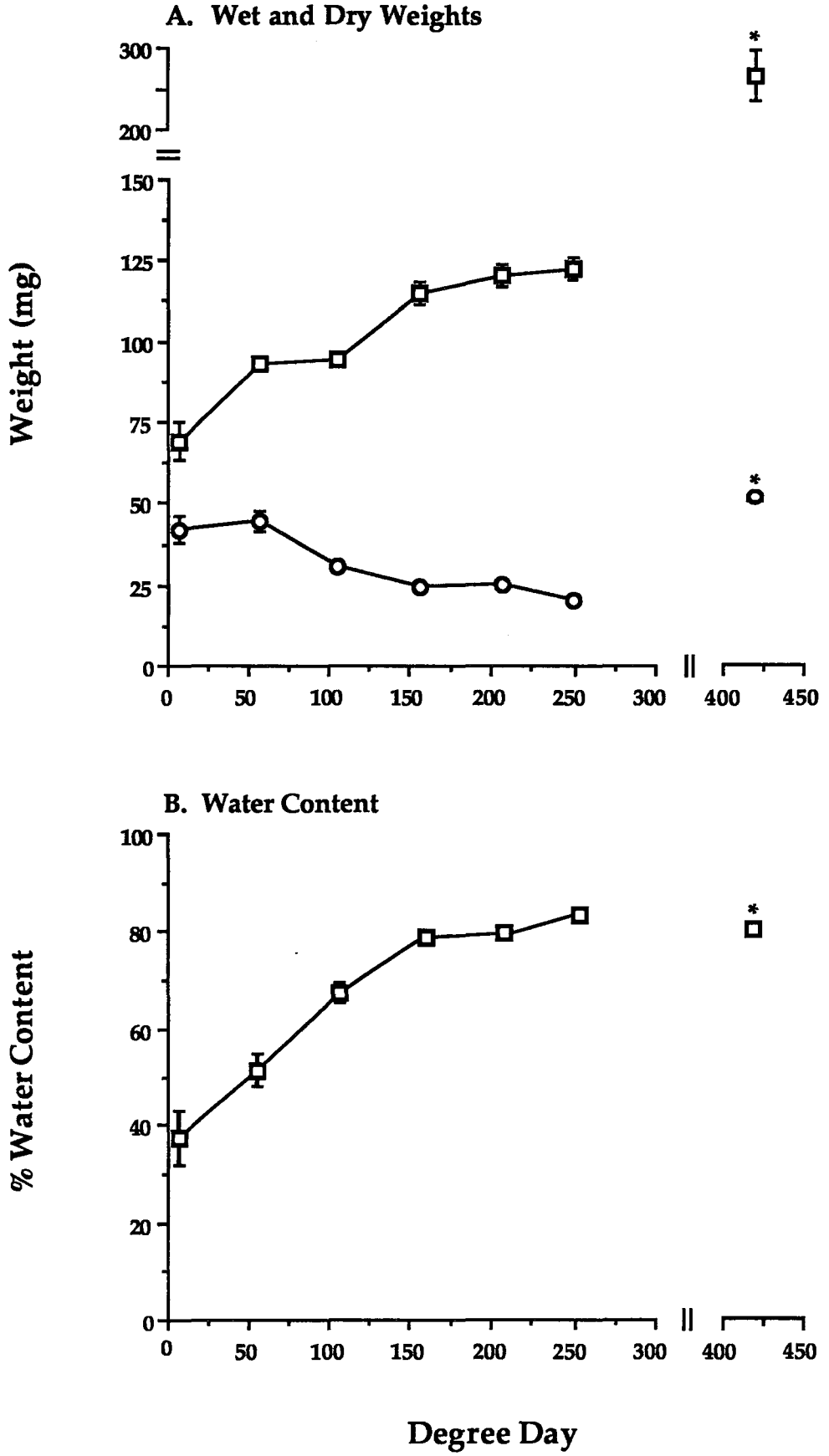
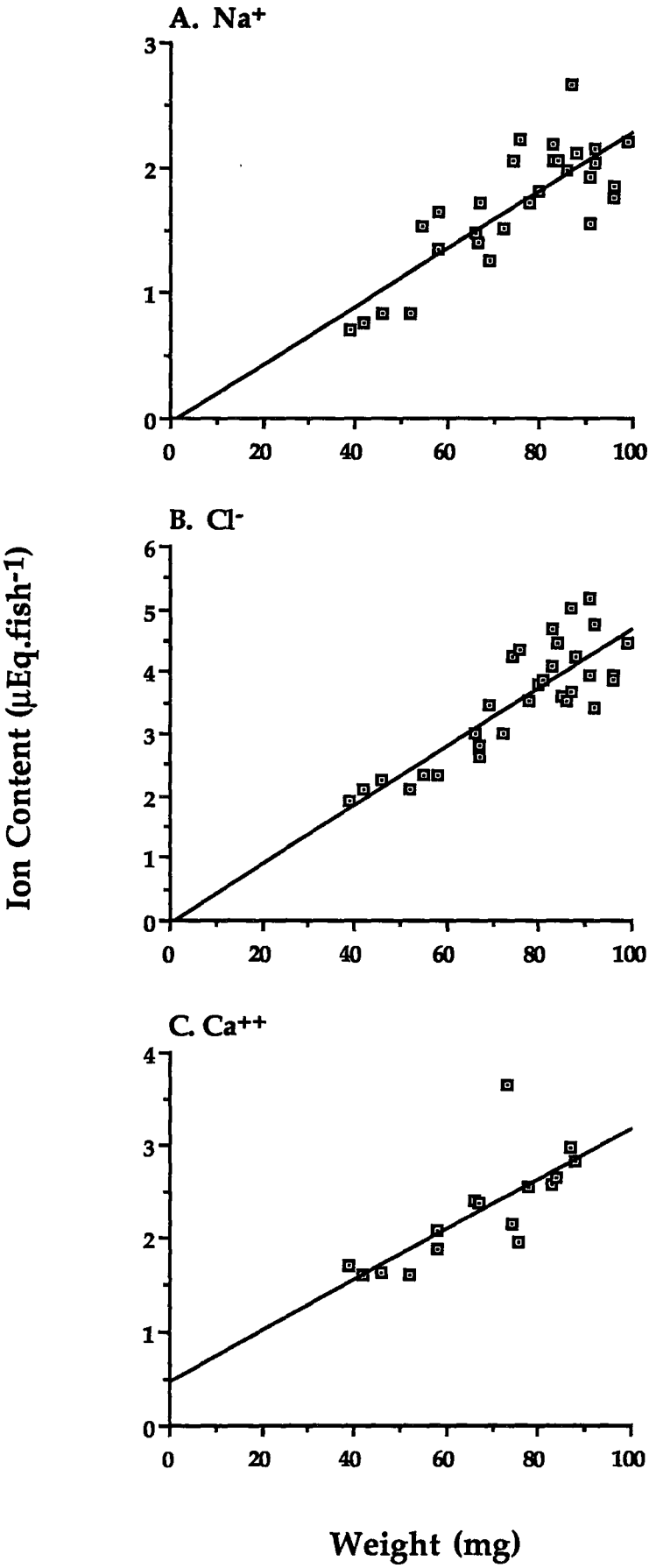

A. Wet and Dry Weights
Weight (mg)
300
200
150
125
100
75
50
25
0
0
50
100
150
200
250
300
400
450
*
*
B. Water Content
% Water Content
100
80
60
40
20
0
0
50
100
150
200
250
300
400
450
*
Degree Day

Figure 2.2: Relationship between whole body ion content ($\mu Eq.fish^{-1}$) of first day hatchlings and their initial wet weight (mg).

A. Na^+ relationship can be explained by the equation $y = 0.023x - 0.033$, $R^2 = 0.668$.

B. Cl^- relationship can be explained by the equation $y = 0.045x - 0.021$, $R^2 = 0.713$.

C. Ca^{++} relationship can be explained by the equation $y = 0.027x + 0.471$, $R^2 = 0.575$. Each point represents an individual fish.


A. Na+
B. Cl-
C. Ca++
Ion Content (µEq.fish-1)
Weight (mg)
0
1
2
3
4
5
6
0
20
40
60
80
100

with R^2 of 0.668, 0.713 and 0.575 for Na^+, Cl^- and Ca^{++}, respectively. In the three cases the intercept went through, or was very close to, zero. At hatching there was 23 nEq of Na^+, 47 nEq of Cl^- and 27 nEq of Ca^{++} per mg of wet fish tissue.

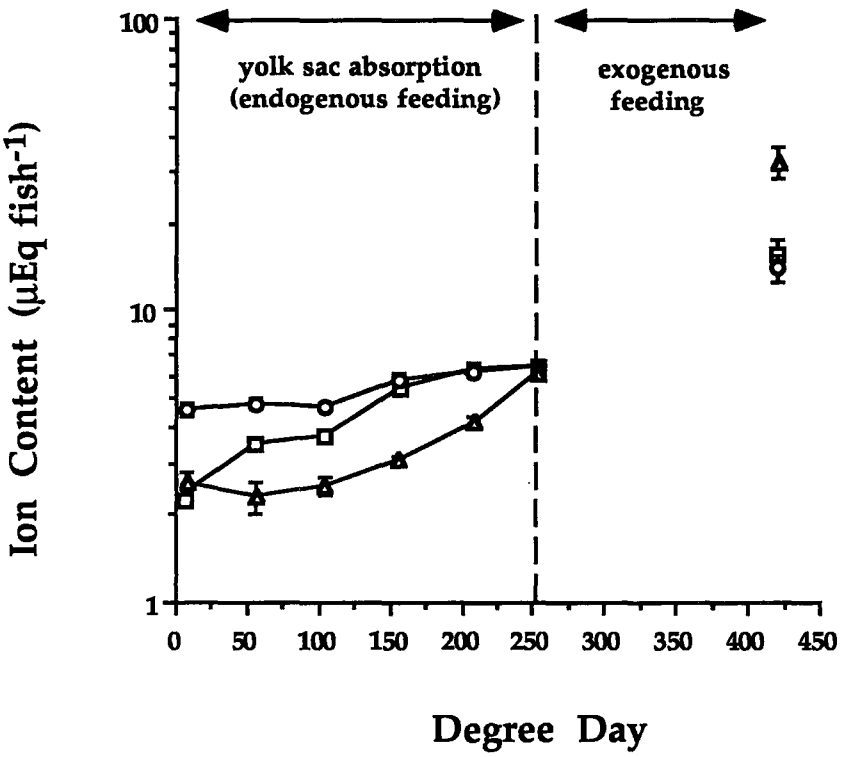
The rates of ion accumulation over the yolk sac absorption period were different for each lot, but followed the same basic trend. During the 250 degree·days of yolk sac absorption there were gradual linear increases in whole body Na^+ and Cl^-. The ion accumulation rates for Na^+ and Cl^- from the different lots are summarized in Table 2.2. Accumulation patterns for whole body Ca^{++} during yolk sac absorption were different from that of Na^+ and Cl^-. In the three lots examined (egg lots 1, 8 and 13), whole body Ca^{++} remained relatively constant for the first 200 degree·days before it started to significantly increase. Again, for the sake of simplicity, the June 1996 series has been chosen as representative in order to illustrate the ion accumulation trends throughout yolk sac absorption and after the onset of exogenous feeding (figure 2.3).

Larvae exhibited a 2.7 fold increase in Na^+ content from (2.4 ± 0.1 $\mu Eq.fish^{-1}$ to 6.5 ± 0.1 $\mu Eq.fish^{-1}$) throughout yolk sac absorption; Na^+ content increased another 2.4 fold by 172 degree·days after the onset of exogenous feeding (figure 2.3). Cl^- increased 1.4 fold throughout yolk sac absorption, with a further 2.2 increase after feeding began (figure 2.3). At hatching, Cl^- levels were consistently greater than Na^+ levels, however, by the end of yolk sac absorption the concentrations of the two ions were quite similar. Over the first 200 degree·days Ca^{++} increased 1.2 fold, however from 200 degree·days to the end of yolk sac absorption Ca^{++} increased an addition 2 fold. After exogenous feeding commenced, Ca^{++} increased another 5.2 fold to 32.4 ± 3.8 $\mu Eq.fish^{-1}$ (figure 2.3).

Table 2.2: Summary of Na^+ and Cl^- accumulation rates and their corresponding R^2 during yolk sac absorption from different egg lots. Initial wet weights are reported as means ± SE.

Group	Initial Wet Weight (mg)	Temp (°C)	Na^+		Cl^-	
			slope ($nEq.fish^{-1}.dd^{-1}$)	R^2	slope ($nEq.fish^{-1}.dd^{-1}$)	R^2
Oct. 1994	42 ± 2	10	10.9 ± 0.9	.706	6.7 ± 0.9	.468
Mar. 1995	88 ± 4	7	30.3 ± 1.8	.775	7.1 ± 1.0	.370
April 1995	82 ± 3	7	25.4 ± 1.4	.816	12.1 ± 0.7	.741
Sept. 1995	59 ± 2	15	9.5 ± 2.1	.428	7.0 ± 3.2	.152
June 1996	74 ± 3	7	17.8 ± 0.9	.866	8.7 ± 1.0	.595

Figure 2.3: Representative relationship between whole body ion content ($\mu Eq.fish^{-1}$) throughout yolk sac absorption and after the onset of exogenous feeding. Means ± SE, n = 10; Na^+: -□- ; Cl^-: --o-- ; Ca^{++}: --Δ--; Hatched line: start of exogenous feeding.


100
10
1
yolk sac absorption
(endogenous feeding)
exogenous
feeding
Ion Content (μEq fish-1)
0
50
100
150
200
250
300
350
400
450
Degree Day

There were differences in the Na^+ and Cl^- distribution patterns in the tissue of the developing larvae. From the first day of hatching the majority of whole body Na^+ was found in the larval fish tissue and not the yolk tissue (table 2.3). Na^+ content in the fish tissue increased throughout yolk sac absorption, while Na^+ in the yolk remained at negligible levels. Initially, whole body Cl^- was equally distributed between the fish tissue and the yolk tissue, but by 156 degree·days almost all of the Cl^- was found in the fish tissue (table 2.3).

Na^+ Uptake Kinetics

There was an increase in Na^+ influx throughout the period of yolk sac absorption, and an even greater increase after the fish had started feeding (figure 2.4). J_{max} increased approximately 4.6 fold throughout yolk sac absorption from 53.4 ± 16.2 $nEq.g^{-1}.h^{-1}$ the first day after hatching, to 248.4 ± 24.9 $nEq.g^{-1}.h^{-1}$ 258 degree·days after hatching. There was an additional 4 fold increase in J_{max} to 683.6 ± 70.2 $nEq.g^{-1}.h^{-1}$ after the fish had been fed for 249 degree·days (figure 2.5A).

Throughout yolk sac absorption, the transporter affinity increased as the K_m decreased from .668 ± .496 mEq l^{-1} to .155 ± .058 mEq l^{-1}. These values remained steady after the fish started feeding at .085 ± .038 mEq l^{-1} (figure 2.5B).

Ammonia Excretion

There was a 3.6 fold increase in ammonia excretion levels over the period of yolk sac absorption from 125.2 ± 23.2 $nmol.g^{-1}.h^{-1}$ at 7 degree·days to 453.2 ± 32.6 $nmol.g^{-1}.h^{-1}$ at 258 degree·days (figure 2.6). A further 3 fold increase in ammonia excretion was seen after the fish had started to exogenously feed (figure 2.6).

Table 2.3: The amount of Na^+ and Cl^- (μEq) found in larval fish tissue and yolk tissue at 7, 56, 156 and 225 degree·days post-hatch.

Degree Day	N	Weight of fish tissue without yolk (mg)	Amount of yolk removed (mg)	ΣNa^+ in remaining fish tissue (μEq)	Amount of Na^+ in yolk sac (μEq)	ΣCl^- in remaining fish tissue (μEq)	Amount of Cl^- in yolk sac (μEq)
7	7	35	46	2.0	0.3	1.9	1.6
56	10	61	32	3.0	0.0	2.2	2.1
156	10	108	11	5.5	0.1	4.4	0.6
225	10	126		7.6		6.1	

Figure 2.4: The relationship between the Na^+ uptake rate ($nEq.g^{-1}.h^{-1}$) and the external $[Na^+]$ ($\mu Eq\ l^{-1}$) in larval rainbow trout at different times throughout yolk sac absorption and after the onset of exogenous feeding. 7 degree days: -▯- , 124 degree days: -■-, 240 degree days: --o--, and 507 degree days: --●--. Means ± SE, n= 6; Temperature: 7°C; *: exogenously feeding.

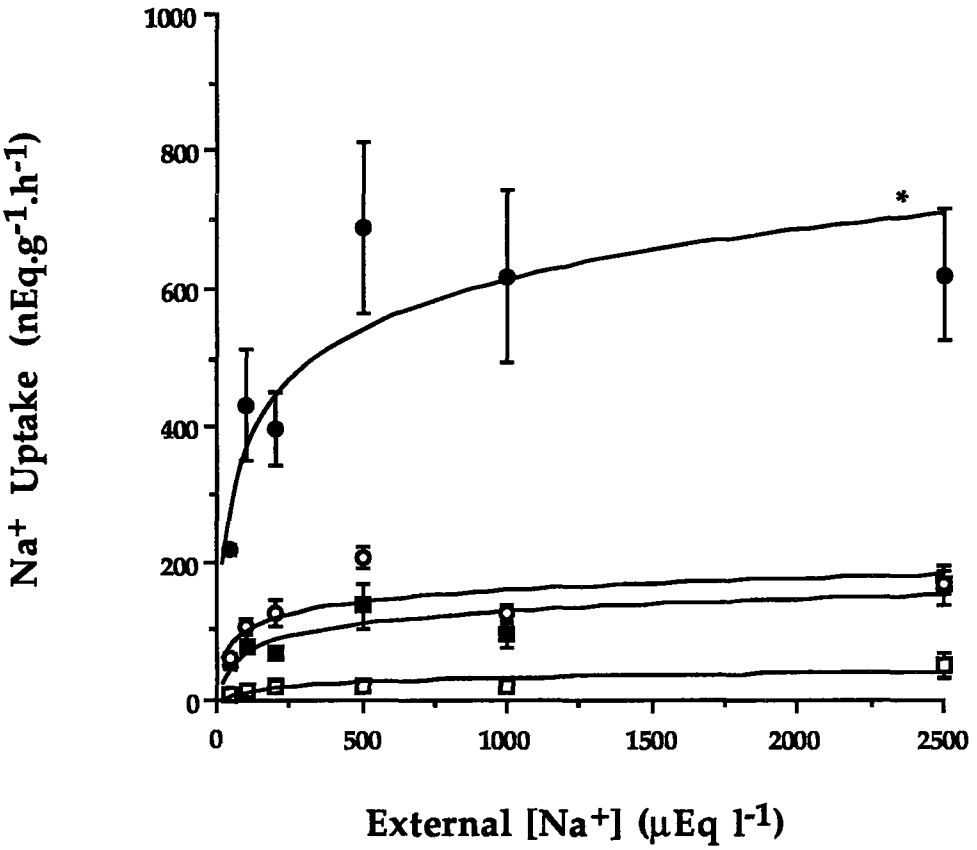

1000
800
600
400
200
0
0
500
1000
1500
2000
2500
*
Na+ Uptake (nEq.g-1.h-1)
External [Na+] (µEq l-1)

Figure 2.5: Na^+ uptake kinetics

A. The changes in maximum Na^+ transport capacity (J_{max}; $nEq.g^{-1}.h^{-1}$) in larval rainbow trout throughout yolk sac absorption and after the onset of exogenous feeding.

B. The changes in Na^+ transporter affinity (K_m; $mEq\ l^{-1}$) for the same time period. Means ± SE, n = 30; Temperature: 7°C; *: exogenously feeding.

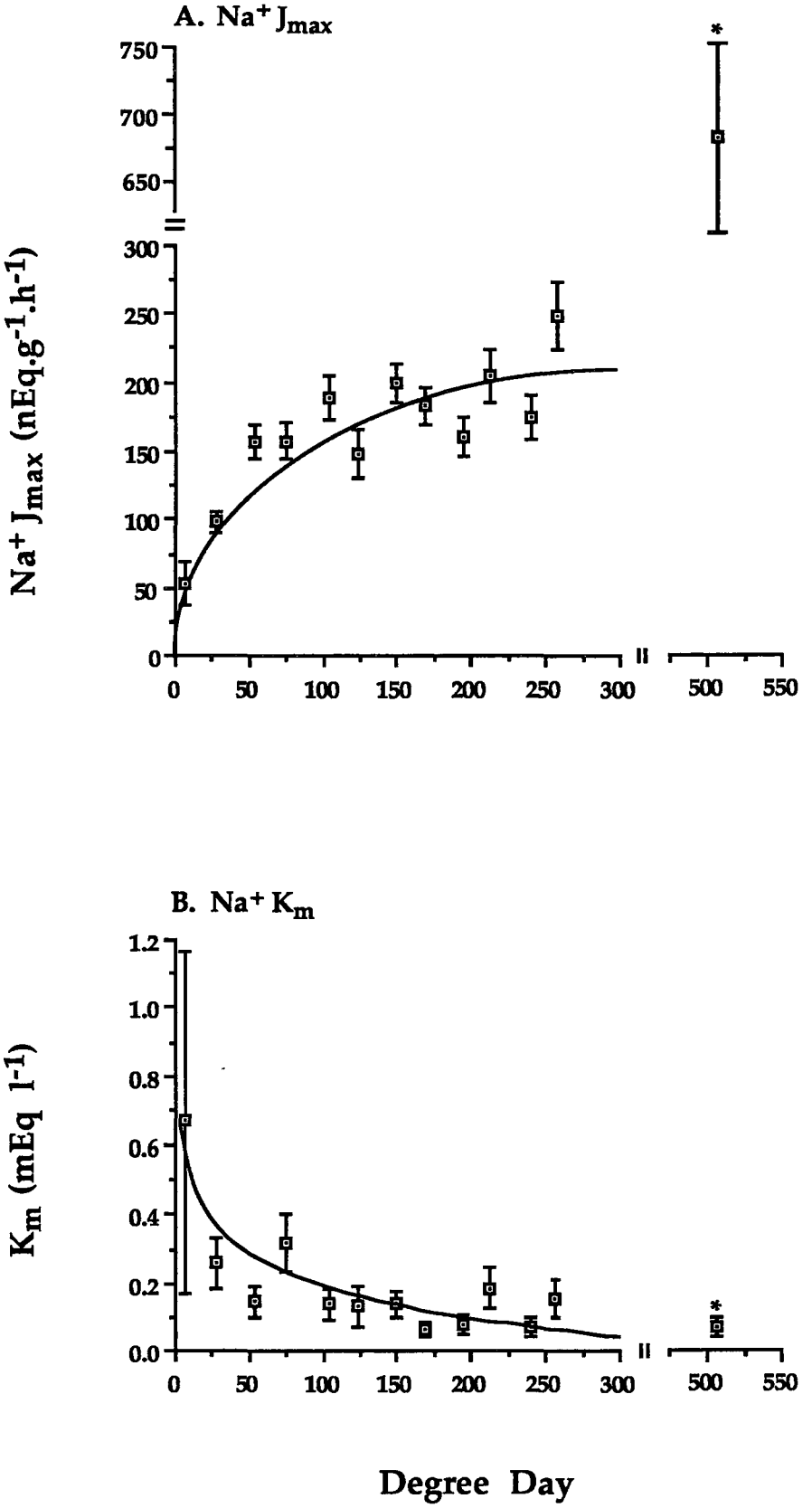
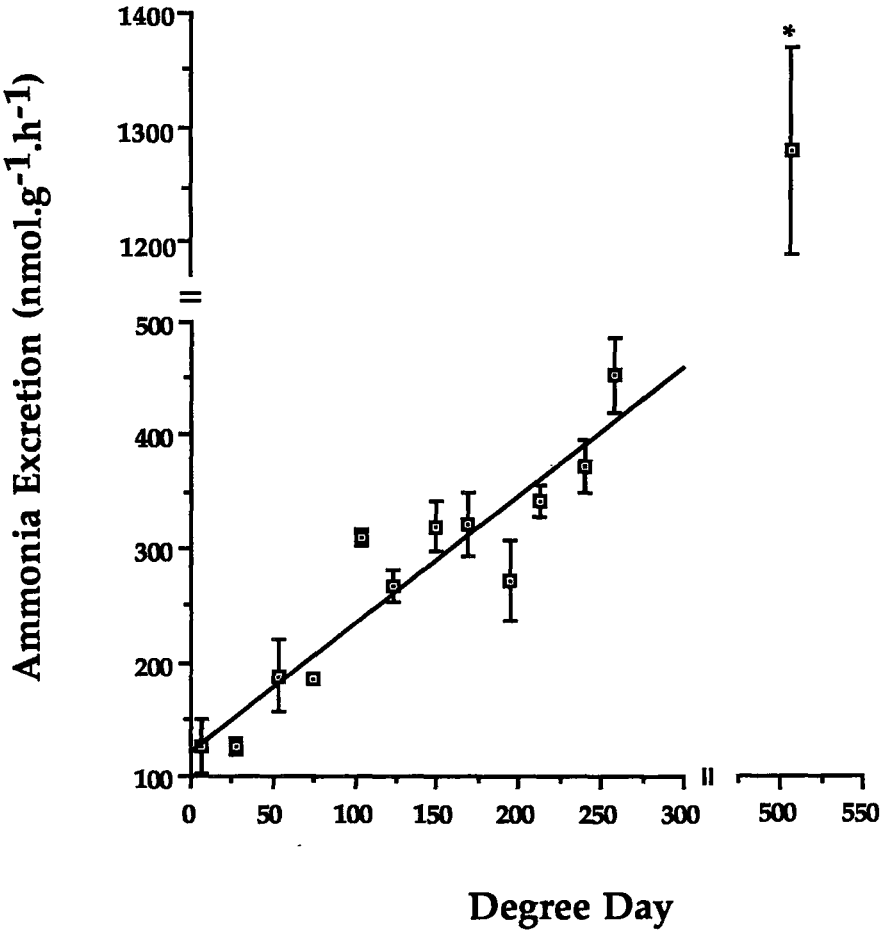

A. Na+ Jmax
Na+ Jmax (nEq.g-1.h-1)
750
700
650
300
250
200
150
100
50
0
0
50
100
150
200
250
300
500
550
*
B. Na+ Km
Km (mEq l-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
300
500
550
*
Degree Day

Figure 2.6: The changes in ammonia excretion ($nmol.g^{-1}.h^{-1}$) in larval rainbow trout throughout yolk sac absorption and after the onset of exogenous feeding. Means ± SE, n = 30; Temperature: 7°C; *: exogenously feeding.


Ammonia Excretion (nmol.g-1.h-1)
1400
1300
1200
500
400
300
200
100
0
50
100
150
200
250
300
500
550
*
Degree Day

Effect of External [Na^+] on Ammonia Excretion

Larval rainbow trout acutely exposed to 6 different concentrations of Na^+ showed no significant stimulation of ammonia excretion. On 7 and 208 degree·days there was no change in ammonia excretion in response to an acute increase in external [Na^+], there was a slight increase in ammonia excretion on 127 degree·days, and there was a slight decrease in ammonia excretion on 288 degree·days (figure 2.7). The R^2 for 7, 127, 208 and 288 degree·days were 0.001, 0.402, 0.000 and 0.875, respectively.

Ca^{++} Uptake Kinetics

Kinetics experiments reveal an increase in Ca^{++} influx throughout the 257 degree·days of yolk sac absorption. However, after feeding commenced (600 degree·days), the Ca^{++} influx decreased significantly (figure 2.8). These changes were seen as J_{max} increased from 15.7 ± 1.1 $nEq.g^{-1}.h^{-1}$ at 7 degree·days to 244.2 ± 53.5 $nEq.g^{-1}.h^{-1}$ at 257 degree·days, and then decreased to 73.6 ± 8.4 $nEq.g^{-1}.hr^{-1}$ after feeding (figure 2.9A).

The affinity of the Ca^{++} transporter decreased during the first 257 degree·days as the K_m increased from 9.2 ± 5.1 $\mu Eq\ l^{-1}$ to 1455.9 ± 688.0 $\mu Eq\ l^{-1}$, but then increased after the onset of exogenous feeding as the K_m decreased to 93.3 ± 48.8 $\mu Eq\ l^{-1}$ (figure 2.9B).

Surface bound Ca^{++} comprised 39% to 73% of the $^{45}Ca^{++}$ taken up by the larvae (table 2.4). The percentage of $^{45}Ca^{++}$ removed was inversely proportional to the exposure time. Since there was no significant difference in the amount of $^{45}Ca^{++}$ removed by the different rinse solutions, 10.0 mmol l^{-1} EDTA was used to remove surface bound Ca^{++} in the Ca^{++} uptake kinetics.

Figure 2.7: Relationship between ammonia excretion ($nmol.g^{-1}.h^{-1}$) in larval rainbow trout at several periods throughout yolk sac absorption in response to acute (10 minute) manipulation of the external [Na^+]. 7 degree days: -□- , 127 degree days: -■-, 208 degree days: --o--, and 288 degree days: --●--. Temperature: 7°C.

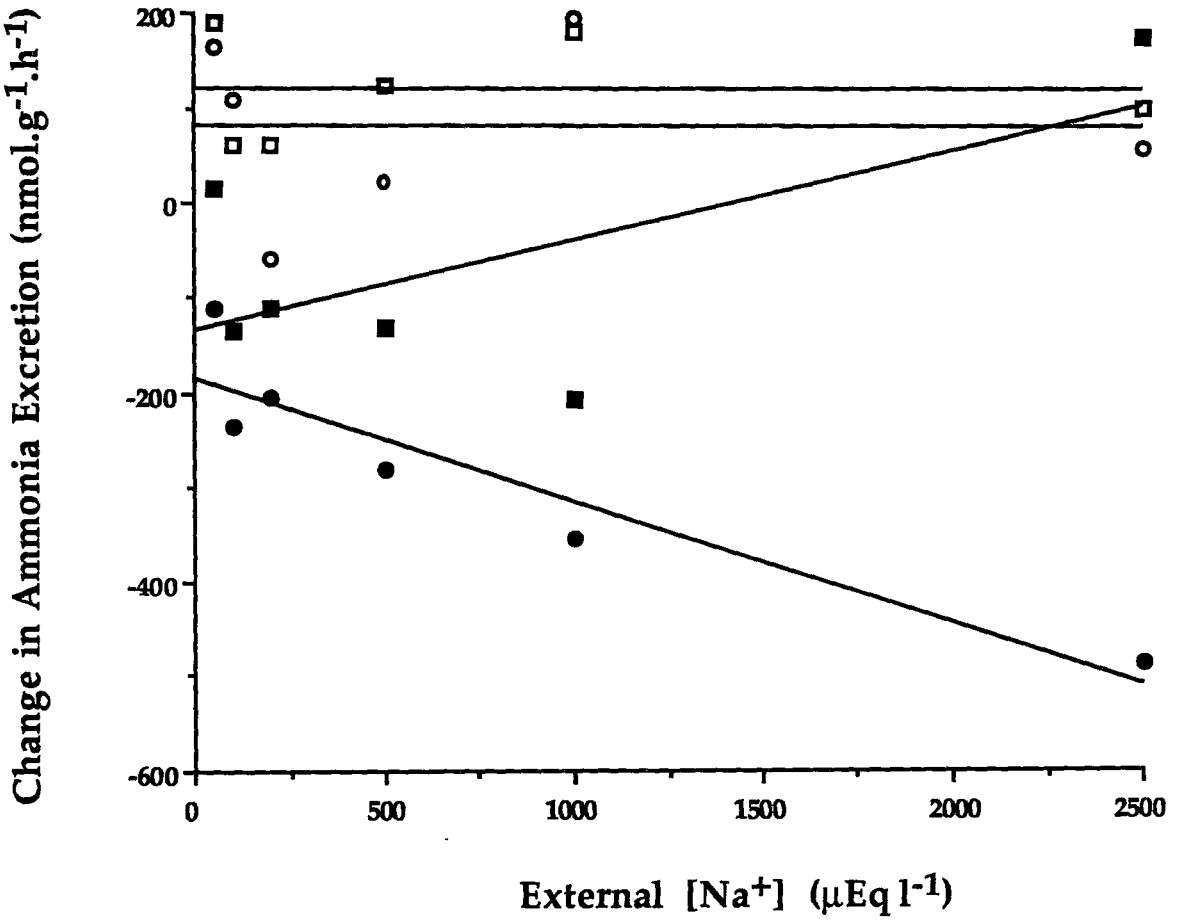

Change in Ammonia Excretion (nmol.g-1.h-1)
200
0
-200
-400
-600
0
500
1000
1500
2000
2500
External [Na+] (µEq l-1)

Figure 2.8: The relationship between the Ca^{++} uptake rate ($nEq.g^{-1}.h^{-1}$) and the external [Ca^{++}] in larval rainbow trout at different times throughout yolk sac absorption and after the onset of exogenous feeding. 7 degree days: –□–, 156 degree days: –▮–, 257 degree days: –o–, and 600 degree days: –●–. Means ± SE, n= 4; Temperature: 7°C; *: exogenously feeding.

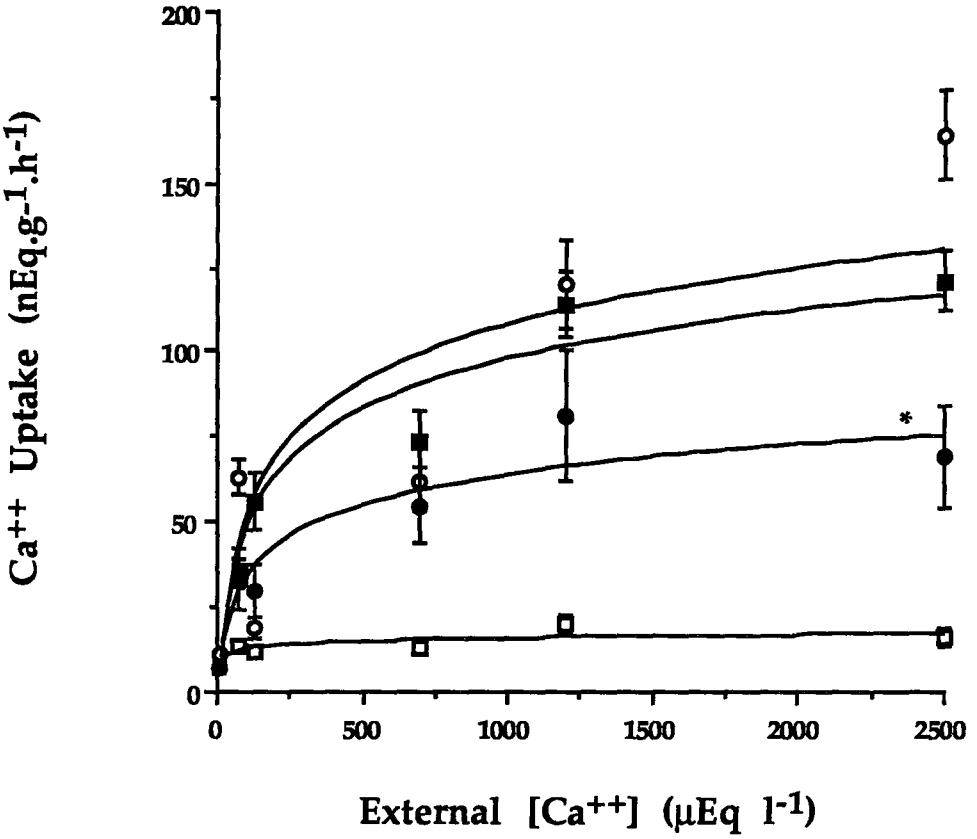

200
150
100
50
0
0
500
1000
1500
2000
2500
*
Ca++ Uptake (nEq.g-1.h-1)
External [Ca++] (µEq l-1)

Figure 2.9: Ca^{++} uptake kinetics

A. The changes in maximum Ca^{++} transport capacity (J_{max}; $nEq.g^{-1}.h^{-1}$) in larval rainbow trout throughout yolk sac absorption and after the onset of exogenous feeding.

B. The changes in Ca^{++} transporter affinity (K_m; $\mu Eq\ l^{-1}$) over the same time period. Means ± SE, n = 20; Temperature: 7°C; *: exogenously feeding.

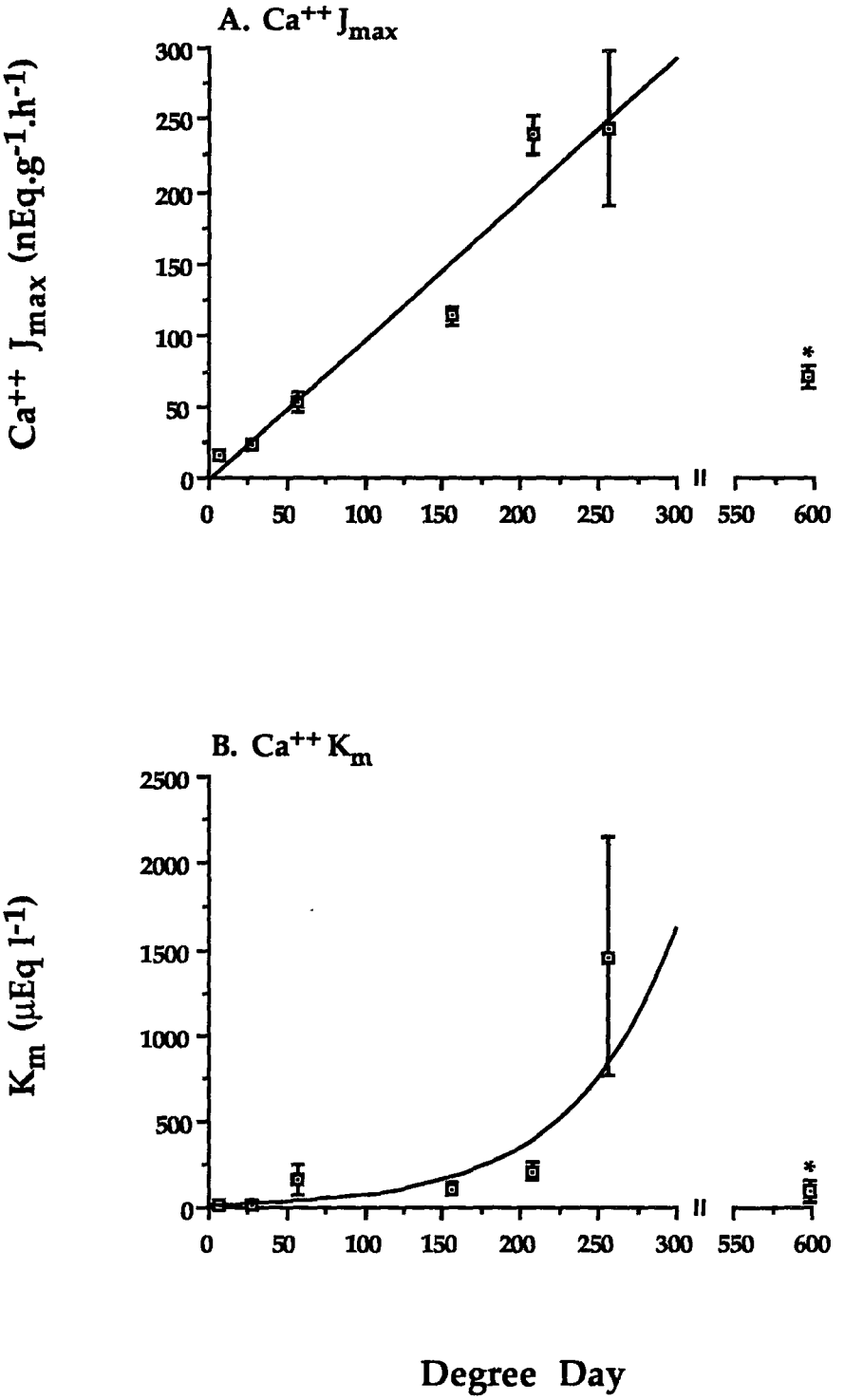

A. Ca++ Jmax
Ca++ Jmax (nEq.g-1.h-1)
300
250
200
150
100
50
0
0
50
100
150
200
250
300
550
600
*
B. Ca++ Km
Km (µEq l-1)
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
550
600
*
Degree Day

Table 2.4: A summary of the amount of $^{45}Ca^{++}$ bound to the surface of the larval rainbow trout removed by a 2 minute rinse in different concentrations of EDTA and $CaCl_2$ solutions.

Rinse Solution (mmol l^{-1})	Exposure Time (hours)	Larval Counts	Rinse Solution Counts	Total Counts	% Surface Bound
1.0 EDTA	.5	610	993	1603	62
	1	994	2690	3684	73
	2	1931	2033	3964	51
10.0 EDTA	.5	683	1055	1738	61
	1	805	1657	2462	67
	2	1847	1462	3309	44
0.1 $CaCl_2$	.5	756	998	1754	57
	1	1179	1984	3163	63
	2	2185	1406	3591	39
1.0 $CaCl_2$	.5	704	1347	2051	66
	1	1152	1487	2639	56
	2	2417	1695	4112	41
10.0 $CaCl_2$	.5	719	1848	2567	72
	1	1257	1108	2365	47
	2	1869	1855	3724	50

DISCUSSION

Weights, Water and Ion Content

There were variations in the initial wet weights amongst the different groups of larval trout. These differences can be attributed to differences in the maternal contribution to the egg and the conversion efficiency of yolk to tissue through embryonic development (Kamler, 1992). The mother's age and weight, in combination with her diet, affect the egg content and hence, weight of the egg (Beacham and Murray, 1985). Throughout yolk sac absorption the larvae exhibited an increase in wet weight and a decrease in dry weight as high density, low moisture yolk was converted to low density, high moisture tissue (Heming and Buddington, 1988).

Small eggs had lower electrolyte content than large eggs. Regression analysis revealed that initial Na^+, Cl^- and Ca^{++} content of first day hatchlings were proportional to the weight of the larvae (figure 2.2). These initial ion levels may be due to the maternal electrolyte contribution and to ion accumulation from the external environment before hatching. Studies on Atlantic salmon eggs noted their ability to accumulate Na^+ from the environment after the eyed stage (Rudy and Potts, 1969; McWilliams and Shephard, 1991). Rudy and Potts (1969) found that the eyed Atlantic salmon eggs accumulated Na^+ at a rate of 7.2% day^{-1}.

Despite the differences between large and small eggs, small larvae did not compensate their lower electrolyte content by increasing their ion accumulation rate during yolk sac absorption. The ion accumulation trends for each ion between egg lots were similar regardless of the initial weight and ion content.

During endogenous feeding, larvae have two ion reserves available to them: the yolk and the ambient water. From the first day post-hatch, and throughout yolk sac absorption, the larvae accumulated Na^+ and Cl^-, with further increases after the onset of exogenous feeding. The increase in whole body Na^+ and Cl^- throughout yolk sac absorption is similar to the increases seen in larval brook trout (Steingraeber and Gingerich, 1991). However, the Na^+ and Cl^- content in larval rainbow trout is greater than in larval brook trout, which could be a result of their smaller size. At hatching, the dry weight of larval rainbow trout is approximately 40 mg compared to 14 mg in larval brook trout (Steingraeber and Gingerich, 1991).

By comparing the Na^+ content in the fish tissue and Na^+ content in the yolk throughout yolk sac absorption, it appears that larval trout obtain 94% of their Na^+ from the external environment (table 2.3). Studies on endogenous feeding Atlantic salmon larvae determined that 65% to 70% of their Na^+ requirements were obtained from the ambient water (Rombough and Garside, 1984; Peterson and Martin-Robichaud, 1986). Cl^- content of larvae was initially equally distributed in the fish and yolk tissues, but increased in the fish tissue as it decreased in the yolk tissue (table 2.3). Throughout yolk sac absorption the increase in whole body Cl^- was greater than the amount of Cl^- available in the yolk tissue indicating that approximately 38% of whole body Cl^- was obtained from the environment.

The difference in the distribution patterns of Na^+ and Cl^- may be a function of their binding properties. Although Na^+ and Cl^- are both extracellular ions, Cl^- is an anion which binds with cations other than Na^+, such as Ca^{++}. Thus, the Cl^- in the yolk sac could be due to its binding with other cations present in the yolk and as these anions are metabolized or

transferred from the yolk to fish tissue, Cl^- is transferred to the fish tissue as well.

Whole body Ca^{++} began to increase significantly 200 degree·days post-hatch, with a further increase after exogenous feeding began. At hatching the skeleton of larval trout is cartilaginous with skeletal ossification beginning in the bones of the rostal region a few days later (Steingraeber and Gingerich, 1991). The small changes in whole body Ca^{++} over the first 200 degree·days indicate that the larval fish use their endogenous reserves to ossify their skeleton, switching to exogenous sources when further skeletal development is required. It has been suggested that the larval fish deplete their yolk sac Ca^{++} reserves before switching to the ambient water as a source (Nelson, 1982; Peterson and Martin-Robichaud, 1986). Peterson and Martin-Robichaud (1986) proposed that larvae do not take up Ca^{++} from the environment until a critical dry weight is reached which occurs when approximately 75% of the yolk reserves are depleted. Although a critical weight was not determined, this study confirms that larval rainbow trout do not take up Ca^{++} from the environment until the majority of its yolk is absorbed.

Na^+ Uptake Kinetics

First day hatchlings are capable of taking up Na^+ from the environment. Na^+ uptake in larval trout display saturable Michaelis-Menten kinetics (figure 2.4). That is, the rate of uptake is dependent on the external concentration up to a certain level (J_{max}) and is related to the affinity (K_m) of the transport carrier, where a decrease in K_m indicates an increase in the affinity (McWilliams and Shephard, 1989). This suggests that Na^+ transport in larval trout is carrier-mediated and/or involves a selective channel.

The J_{max} and K_m for Atlantic salmon non-feeding and feeding larvae were similar to the J_{max} and K_m values of the larval rainbow trout (table 2.5). The similarities in Na^+ transport properties suggest that larval salmonids develop these properties at the same rate. J_{max} in juvenile and adult rainbow trout were slightly lower than the newly feeding larvae, while the K_m of juvenile fish were similar to the larval fish (table 2.5). The similarities in J_{max} and K_m amongst the various life stages of rainbow trout suggest that at the end of yolk sac absorption the Na^+ uptake mechanism of larval fish has become fully developed.

J_{max} increased throughout yolk sac absorption and after the onset of exogenous feeding. Since J_{max} is proportional to the number of active transport sites (Goss *et al.*, 1992b), the increasing J_{max} for Na^+ transport indicates an increase in the number of transporters. The number of transporters will increase if there is an increase in the size of the transporting surface area, an increase in the number of Na^+ transporting cells, an increase in the number of Na^+ channels in the existing transporting cells, or any combination of the three.

Throughout larval development the branchial surface area increases significantly while the cutaneous surface area does not (McDonald and McMahon, 1977; Oikawa and Itazawa, 1985; Rombough and Moroz, 1990). Thus, the simple explanation for the increase in $J_{max}^{Na^+}$ is the increase in gill surface area. For example, the 4.6 fold increase in $J_{max}^{Na^+}$ over 258 degree·days in larval rainbow trout corresponds to the 5.3 fold increase in gill filament surface area of Arctic char during the same period (247 degree·days at 6.5°C). An increase in the area of the gill filaments and lamellae would result in an increase in the number of transporting cells. Both the pavement cell and the chloride cell in the branchial epithelium of adult teleosts have been

Table 2.5: A comparison of the Na^+ uptake properties, J_{max} ($\mu Eq.kg^{-1}.h^{-1}$) and K_m ($mEq\ l^{-1}$), at various life stages of rainbow trout and early life stages of Atlantic salmon. E: embryo; L: endogenously feeding larvae; F: exogenously feeding larvae; J: juvenile; A: adult.

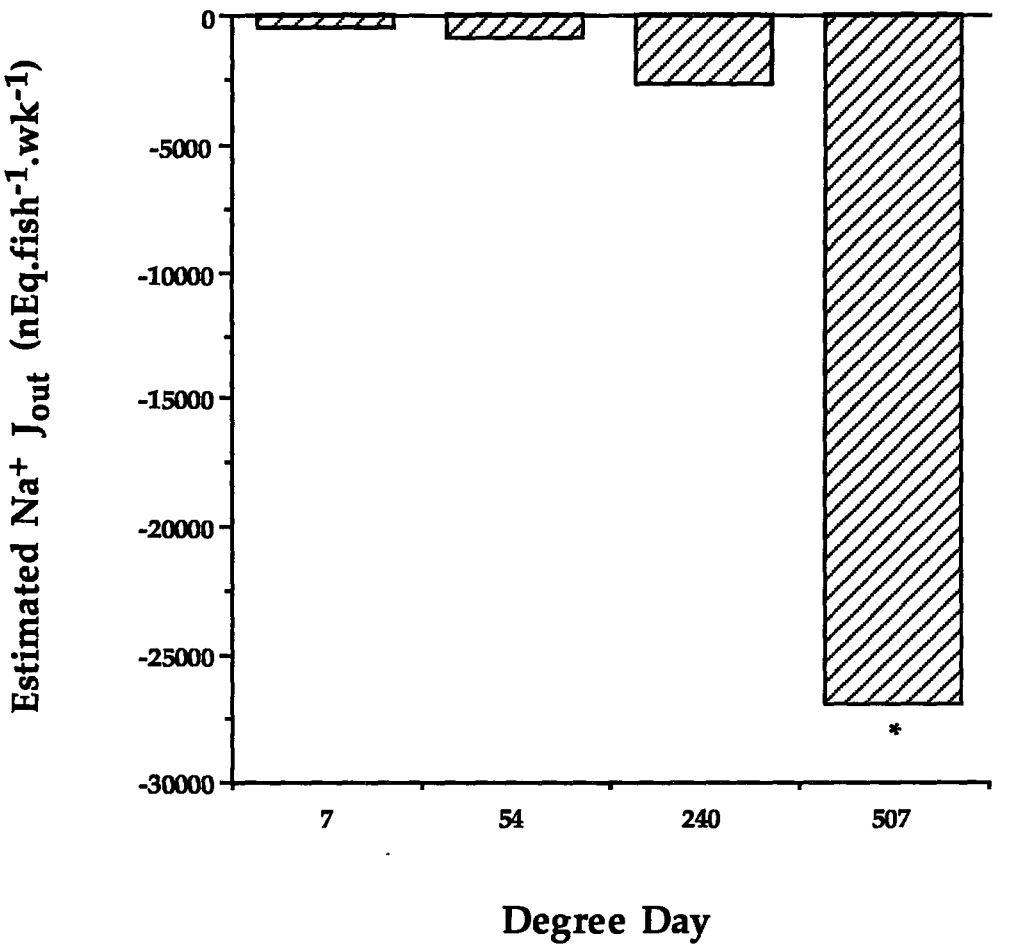
Species	Stage Tested	Temp (°C)	Weight (g)	J_{max} ($\mu Eq.kg^{-1}.h^{-1}$)	K_m ($mEq\ l^{-1}$)	Holding Condition ($mmol\ l^{-1}$)	Reference
Rainbow trout	L	7	.083 ± .001	187 ± 16	.14 ±.05	Na^+: .6	present study
	F	7	.261 ± .019	683 ± 70	.08 ± .03	Cl^-: .7	
						Ca^{++}: 1.0	
Atlantic salmon	E	1.8		130	.19	Na^+: .22	McWilliams & Shephard, 1989
	L	5.5		167	.20	Cl^-: .24	
	F	11.5		696	.17	Ca^{++}: .03	
Rainbow trout	J	15	11.7 ± 0.6	560 ± 55	.138 ± .056	Na^+: .60	Postlethwaite & McDonald 1995
						Cl^-: .80	
						Ca^{++}: 1.0	
Rainbow trout	A	11	150 - 400	~300		Na^+: .58	McDonald & Rogano, 1986
						Cl^-: .75	
						Ca^{++}: 1.1	

considered responsible for Na^+ uptake (Laurent and Dunel, 1980; Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989; Goss *et al.*, 1992a; Lin *et al.*, 1994). Pavement cells will increase in number as the gills develop and Shen and Leatherland (1978b) reported that the number of chloride cells on the branchial epithelium increases after hatching. The large increase in J_{max} after feeding suggests further development of the transporting mechanism even after yolk sac absorption. This increase in Na^+ uptake from the environment may be indicative of further gill growth and maturation, since any Na^+ requirements could have been met through the diet.

K_m is a measure of the transporters affinity for the ion, it is the concentration at which 50% of the transporters are saturated. Any changes in K_m are usually related to changes in the active site of the enzyme (Goss *et al.*, 1992b). During yolk sac absorption the K_m decreased revealing a heightened affinity of the Na^+ transporter. The affinity reached its greatest level approximately 168 degree·days post-hatch and did not change significantly for the remainder of the yolk sac absorption period, or with the onset of exogenous feeding.

Comparison of whole body Na^+ content vs. $J_{max}^{Na^+}$ reveals a discrepancy between the amount accumulated and the rate of uptake; based on $J_{max}^{Na^+}$, the amount of whole body Na^+ accumulated by the larvae should be greater. This indicates that as $J_{in}^{Na^+}$ increased, there was a simultaneous increase in $J_{out}^{Na^+}$. An estimate of $J_{out}^{Na^+}$ using the known J_{max} and the actual Na^+ content of the larval fish shows this to be case (figure 2.10). This large increase in $J_{in}^{Na^+}$ and corresponding increase in $J_{out}^{Na^+}$ may indicate that the purpose of the Na^+ transporter during this developmental period is not only for Na^+ accumulation. The rapid development of the Na^+ transporter indicates activity in another function, such as whole body acid-

Figure 2.10: Changes in Na^+ efflux in larval rainbow trout throughout yolk sac absorption and after the onset of exogenous feeding estimated from whole body Na^+ content and $J_{max}^{Na^+}$. *: exogenously feeding.


Estimated Na^+ J_{out} ($nEq.fish^{-1}.wk^{-1}$)
0
-5000
-10000
-15000
-20000
-25000
-30000
*
7
54
240
507
Degree Day

base balance. The high uptake rates would allow the larval fish to quickly correct an internal acidosis by Na^+/H^+ exchange, while the high efflux rate would allow the fish to lose the added Na^+ load.

Ammonia Excretion

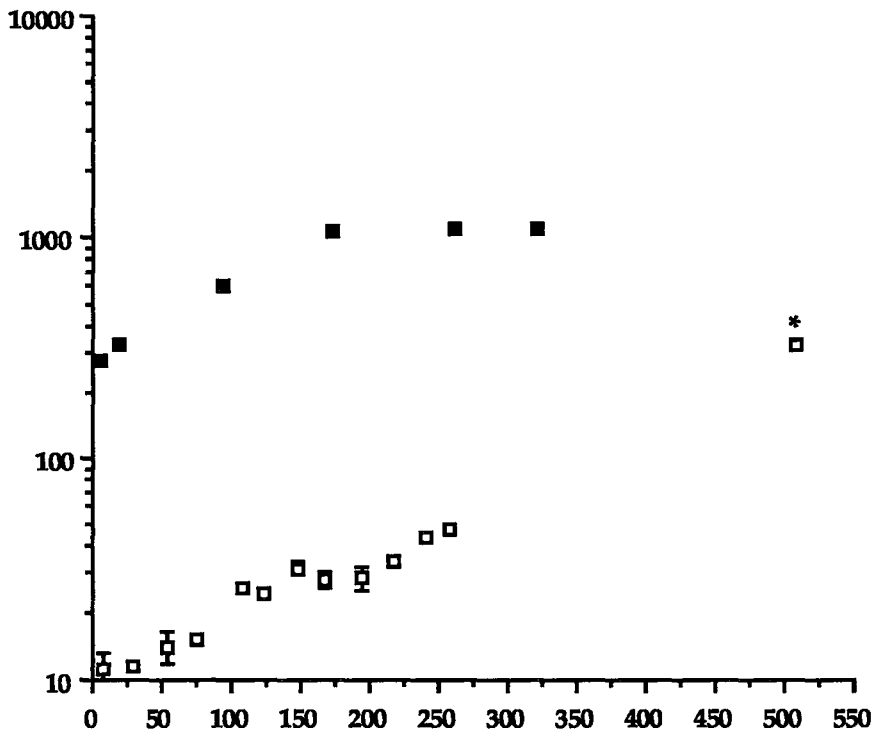
Ammonia excretion increases linearly over yolk sac absorption with a significant increase after the onset of the exogenous feeding. As the larva metabolizes the protein supply in its yolk sac, it produces nitrogenous waste. Due to its toxicity, the nitrogenous waste must be excreted; either as ammonia or urea.

The increase in ammonia excretion could be due to an increase in metabolism, or an increase in ammonia permeability or both. Throughout yolk sac absorption the increase in ammonia excretion follows the increase in oxygen consumption (figure 2.11, Rombough, 1988a). On the other hand, Wright *et al.* (1995) found whole body ammonia levels at hatching of larval rainbow trout to be quite high, approximately 3.0 mmol l^{-1} compared to whole body levels of approximately 0.5 - 0.7 mmol l^{-1} in older fish (Randall and Wright, 1987), and remained elevated for approximately 10 days. Therefore, initial ammonia excretion is limited by the relative impermeability of branchial and cutaneous surfaces to ammonia.

Effect of External [Na^+] on Ammonia Excretion

There have been a number of mechanisms proposed for Na^+ transport across the gills. The three mechanisms proposed for Na^+ uptake at the gills are: (i) via a Na^+/H^+ exchange, (ii) via a Na^+ channel coupled with an H^+ pump powered by H^+-ATPase and (iii) via a Na^+/NH_4^+ exchange (Kerstetter *et al.*, 1970; McDonald and Prior, 1988; McDonald *et al.*, 1989; Goss *et al.*, 1992b).

Figure 2.11: Comparison between ammonia excretion (nmol.fish^{-1}.h^{-1}; 7°C) of larval rainbow trout and oxygen consumption (nmol O_2.fish^{-1}.h^{-1}; 6°C) of larval steelhead trout (from Rombough, 1988a) throughout the period of yolk sac absorption. Ammonia excretion: -□- ; Oxygen consumption: -■- ; *: exogenously feeding.



Degree Day

Recently, H^+-ATPase has been localized on the apical membranes of both chloride cells and pavement cells in the gill epithelia of rainbow trout (Lin *et al.*, 1994). A recent study (Wilson *et al., 1994*) has discounted the presence of a Na^+/NH_4^+ exchange, however this conclusion was made after a long term exposure (i.e. greater than 3 hours) to various external $[Na^+]$. Since ammonia excretion occurs by a variety of routes, a change in ammonia excretion due to external $[Na^+]$ manipulations should only be detectable after a short term exposure. The short exposure will capture any changes in ammonia excretion before a steady state is re-established using another route. In rainbow trout and brook trout, McDonald *et al.* (1989) found that acutely varying, i.e. less than 30 minutes, the external $[Na^+]$ produced a 1:1 relationship between Na^+ uptake and ammonia excretion. These results strongly support a Na^+/NH_4^+ exchange in these fish. If a Na^+/NH_4^+ exchange exists in larval rainbow trout, the same trends in Na^+ uptake and ammonia excretion should be evident in response to acute manipulations of the external $[Na^+]$. However, the lack of stimulation of ammonia excretion in response to an acute exposure to different $[Na^+]$, over all the days examined, suggests that there is no evidence of a Na^+/NH_4^+ exchange in larval rainbow trout.

Ca^{++} Uptake Kinetics

Ca^{++} uptake kinetics in larval rainbow trout displayed Michaelis-Menten characteristics (figure 2.8). $J_{max}^{Ca^{++}}$ increased over the period of yolk sac absorption, but decreased with the onset of exogenous feeding. Akin to the explanation for the increase in $J_{max}^{Na^+}$, this increase may be the result of an increase in the gill surface area, an increase in the number of transporting cells, an increase in the number of Ca^{++} transporters per cell, or any combination of the reasons mentioned.

Ca^{++} uptake in the adult teleost occurs at the gills and the intestine, with little contribution from extrabranchial tissue (Marshall *et al.*, 1992; Flik *et al.*, 1986; Flik and Verbost, 1993; Perry *et al.*, 1992; Flik *et al.*, 1996). Branchial Ca^{++} transport occurs through the chloride cell (Payan *et al.*, 1981; Perry and Wood, 1985; Perry *et al.*, 1992) via diffusion down its electrochemical gradient. Ca^{++} ions are then pumped out of the cell across the basolateral membrane by high affinity Ca^{++}-ATPase and/or Na^{+}/Ca^{++} exchange (Flik *et al.*, 1985; Flik and Verbost, 1993; Verbost *et al.*, 1994). Intestinal Ca^{++} uptake occurs through enterocytes via a Na^{+}/Ca^{++} exchanger located on the basolateral surface (Flik *et al.*, 1990; Flik and Verbost, 1993; Schoenmakers *et al.*, 1993).

The large increase in whole body Ca^{++} after the onset of exogenous feeding, despite the decrease in $J_{max}^{Ca^{++}}$, may indicate that the larval fish is able to meet its Ca^{++} demand through its diet. Dietary acquisition of Ca^{++} would explain the decrease in K_m after the onset of exogenous feeding. K_m is related to the enzymatic activity of the transporter (Goss *et al.*, 1992b). If Ca^{++} requirement were met through the diet, the down-regulation of the enzyme would be advantageous from an energetic point of view.

Estimates of $J_{out}^{Ca^{++}}$ over yolk sac absorption using the $J_{max}^{Ca^{++}}$ and Ca^{++} content reveal an increasing Ca^{++} efflux (figure 2.12), which is then abolished after the onset of exogenous feeding. This implies that in order for the larval fish to accumulate the amount of Ca^{++} that it did during exogenous feeding, the Ca^{++} must have come from a source other than the ambient water. Ca^{++} levels in non-feeding larval fish past 250 degree·days show a slight increase, but not in proportion to the increase seen in feeding fish (figure 2.13). Ichii and Mugiya (1983) found that goldfish exposed to a calcium deficient diet for 56 days had similar whole body Ca^{++} levels to the control

Figure 2.12: Changes in Ca^{++} efflux in larval rainbow trout throughout yolk sac absorption and after the onset of exogenous feeding estimated from whole body Ca^{++} content and $J_{max}^{Ca^{++}}$. *: exogenously feeding.

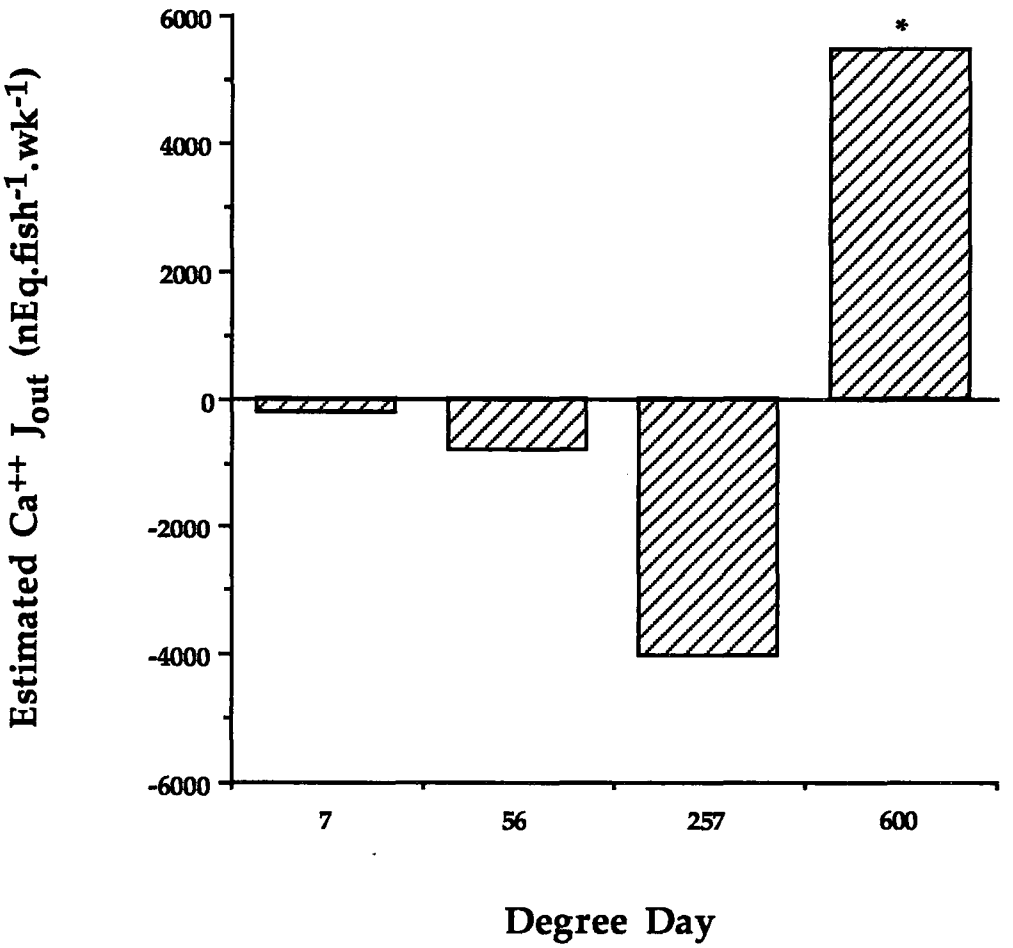
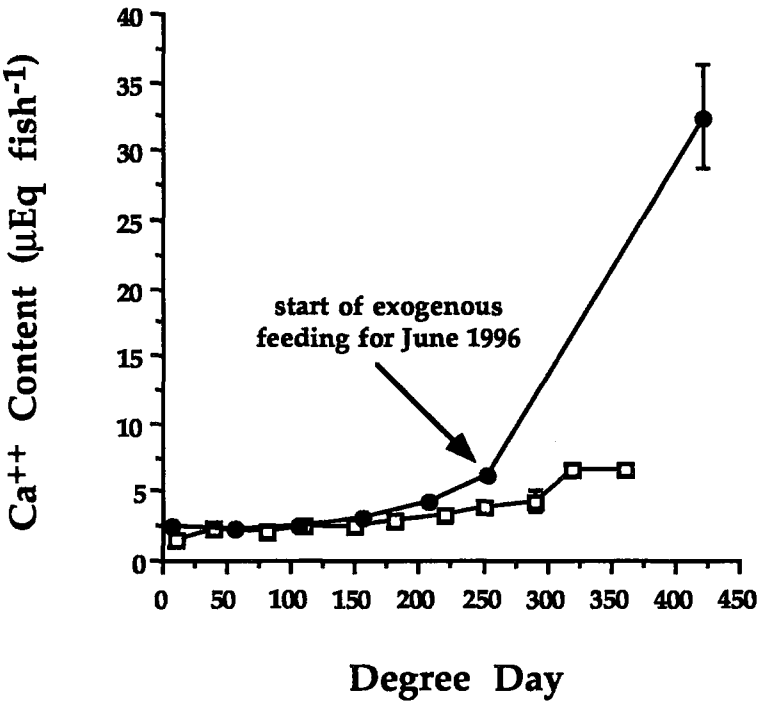

Estimated Ca++ Jout (nEq.fish-1.wk-1)
6000
4000
2000
0
-2000
-4000
-6000
*
7
56
257
600
Degree Day

Figure 2.13: Differences in whole body Ca^{++} content ($\mu Eq.fish^{-1}$) in fed and unfed fish. October 1994: -□-, June 1996: -●-. Means ± SE, n = 6 for October 1994 and n = 10 for June 1996; *: exogenously feeding.


Ca++ Content (µEq fish-1)
40
35
30
25
20
15
10
5
0
start of exogenous
feeding for June 1996
0
50
100
150
200
250
300
350
400
450
Degree Day

fish, but the $^{45}Ca^{++}$ activity of the calcium deficient fish was greater than the activity in the control fish. This suggests that the goldfish compensated for the low dietary calcium by increasing Ca^{++} uptake from the external environment. It would be interesting to see what would happen to $J_{max}^{Ca^{++}}$ for newly exogenous feeding rainbow trout on a calcium deficient diet. If Ca^{++} uptake was stimulated in the fish exposed to the low calcium diet it would indicate that the larval fish are able to maintain Ca^{++} homeostasis by changing the method of ion acquisition, suggesting that these larval fish have the ability to ionoregulate.

Conclusions

- Whole body Na^+ and Cl^- increased linearly throughout yolk sac absorption. Whole body Ca^{++} began to increase after 200 degree·days.
- Na^+ and Cl^- are not equally distributed in the larval fish. At hatching the fish tissue contains almost all of the Na^+ in the larvae, while Cl^- is evenly distributed between the fish and yolk tissue.
- First day hatchlings take up Na^+ and Ca^{++} from the environment. Na^+ and Ca^{++} J_{max} increase throughout yolk sac absorption. After exogenous feeding commenced, $J_{max}^{Na^+}$ increased while $J_{max}^{Ca^{++}}$ decreased. These differences may indicate that Na^+ requirements are met from the external environment, while the Ca^{++} requirements are met through the diet.
- The increase in ammonia excretion throughout yolk sac absorption is due to an increase in routine oxygen consumption and/or an increase in ammonia permeability.
- There is no evidence of a Na^+/NH_4^+ exchange in larval rainbow trout as determined by acute manipulations of the external $[Na^+]$.

CHAPTER 3: THE LOCALIZATION OF ION ACTIVITY IN LARVAL RAINBOW TROUT

INTRODUCTION

In Chapter 2, it was shown that significant changes in whole body ion content, in the transporting properties and in ammonia excretion of larval rainbow trout occurred throughout yolk sac absorption. During this period whole body Na^+, Cl^- and Ca^{++} increased, the maximum transport rate (J_{max}) for Na^+ and Ca^{++} increased, and ammonia excretion increased. These findings led to the questions concerning the sites of these processes in larval trout and whether these sites change over time. Because of the small size of the larvae, traditional methods to determine the location of ion activity (i.e., Perry and Wood, 1985) could not be utilized. Instead, this study used confocal microscopy and electrophysiology (using static and vibrating ion-selective microelectrodes), to answer this question. Confocal microscopy of fluorescent stained specimens was used to determine the presence of Na-K-ATPase activity in the gills and yolk sac at hatching and throughout yolk sac absorption. Ion selective microelectrodes were employed to determine the activities of NH_4^+, pH, Na^+ and Ca^{++} at the gills, the yolk sac and the skin throughout yolk sac absorption.

Respiration and Ammonia Excretion in Larval Fish

Gills of adult fish are responsible for gas exchange, acid-base balance, nitrogenous waste excretion and ion regulation (Laurent 1984; Kültz and Jürss, 1993). However, it is believed that the gills of larval trout are not

capable of performing these functions until a few weeks after hatching (Holeton, 1971; Shen and Leatherland, 1978b; Guggino, 1980).

At hatching, the branchial epithelium of larval rainbow trout consists of gill arches and filaments (Holeton, 1971; Morgan, 1974b; Shen and Leatherland, 1978b). The time of lamellar development was variable between the three studies with lamellae being noticed before hatching and a few days after hatching (Morgan, 1974a; Shen and Leatherland, 1978b). Morgan's morphological study (1974a) on the development of secondary lamellae concluded that, at hatching, the gills of larval rainbow trout possess most of the features of the adult gill and the trout hatches with a fully functional respiratory system. However, Holeton's study (1971) of the respiratory and circulatory systems of larval rainbow trout found that the gill filaments of the first day hatchlings were small, the buccal and opercular pumps were not fully developed, and the yolk sac was large and well vascularized. These observations led him to conclude that the yolk sac was the principal site of gas exchange just before and shortly after hatching. Studies on the large surface to volume ratio of larvae have resulted in the belief that cutaneous respiration through the body (including the yolk sac) and fins play an important role during the early stages of larval life (Oikawa and Itazawa, 1985; Rombough, 1988a and b; Rombough and Moroz, 1990; Wieser, 1995) and that this importance decreases within the first few weeks of hatching as the gills and respiratory pumps rapidly grow and develop (Holeton, 1971; Rombough and Moroz, 1990).

Ammonia excretion in larval fish increases during yolk sac absorption, but for the first 10 days post-hatch, ammonia tissue levels remain elevated (Wright *et al.*, 1995). The gills are the primary site of ammonia excretion in adult teleosts, but whether this is the same for larval fish is not known.

Ion Regulation in Larval Fish

Pavement cells and chloride cells have been postulated as the possible sites for ion regulation in adult gills (Goss *et al.*, 1992a; Goss *et al.*, 1994). Pavement cells are the most abundant; making up approximately 90% of the total number of cells in the branchial epithelium.

Although less abundant (less than 10%) the chloride cell has all the characteristics of a transporting epithelium: it has a large quantity of mitochondria (and is often referred to as the mitochondria rich cell), an extensive tubular network and an apical crypt with microvilli extending into the external media (Karnaky, 1986; Battram *et al.*, 1989; Hwang, 1989; King and Hossler, 1991; Pärt *et al.*, 1993). Chloride cells in adult freshwater teleosts are found as unicellular complexes located on the lamellar and filament epithelial surfaces, concentrating on the trailing edges of the filament epithelia (Perry *et al.*, 1992).

In early life stages, chloride-type cells have been found in the branchial epithelium prior to hatching (Morgan, 1974a; Shen and Leatherland, 1978b). Although no quantitative result was given, Morgan (1974a) stated that larvae possess a greater number of chloride cells than adults. However, Shen and Leatherland concluded that there did not appear to be enough chloride-type cells in the gills to play a significant role in ion regulation. Chloride cells have been found on the skin and yolk sac of several larval teleosts such as the plaice (*P. platessa*), the puffer fish (*F. niphobles*), the killifish (*F. heteroclitus*), the Atlantic herring (*S. caerules*), the tilapia (*O. mossambicus*) and the rainbow trout (*O. mykiss*) (Shelbourne, 1957; Shen and Leatherland, 1978b; Hwang and Hirano, 1985; Somasundaram, 1985; Ayson *et al.*, 1994). It has been suggested that the yolk sac is a possible site of ion regulation in the

larval fish (Shelbourne, 1957; Weisbart, 1968; Guggino, 1980; Ayson *et al.*, 1994), but there is no clear evidence to support this.

The methods used in this study were confocal microscopy and electrophysiology. Confocal microscopy was used in order to elucidate the functional state of the gills and yolk sac epithelium, and electrophysiology was used to determine the areas of ion activity throughout yolk sac absorption.

Fluorescent Dyes and Confocal Microscopy

Over the past few years the use of fluorescent dyes in identifying cells and cellular processes has become increasingly popular. A number of specific dyes have been created to visualize the locations of organelles within a cell. Dimethyl-amino-styrylmethyl-pyridinium-iodine (DASPMI) is a vital stain which accumulates specifically in mitochondria (Bereiter-Hahn, 1976). Rhodamine 123 (R123, [methyl-o-6-amin-3'-imino-3H-xanthen-9-yl benzoate monohydrochloride]) is a positively charged, non-toxic dye which is also sequestered in actively respiring mitochondria (Shinomiya *et al.*, 1992). The positive charges of both DASPMI and R123 are attracted to the negative potential inside the mitochondria (Shinomiya *et al.*, 1992). Anthroylouabain (AO) is a fluorescent derivative of ouabain, made by a reaction between anthracene and ouabain (Fortes, 1977). AO binds to the active, or phosphorylated, form of Na-K-ATPase inhibiting its activity (Moczydlowski and Fortes, 1980; Yoda and Yoda, 1982). After exposure to the dye, fluorescence is achieved by exciting the fluorophore with the appropriate wavelength of light which can be done using a confocal microscope or a conventional light microscope fitted with epi-fluorescence. Confocal microscopy is able to reduce the out-of-focus blur that is inherent in

conventional microscopes permitting high resolution through optical sectioning (Shotton and White, 1989; Shotton, 1989).

Ion Selective Electrodes

Ion selective electrodes can be used as a non-invasive, extracellular method for measuring specific ion movements, in and out of a biological specimen, in an aqueous environment (Smith, 1994). They contain a liquid ion sensor (the ionophore), the electrolyte filling solution and a $AgCl_3$ coated silver wire. Acting like a semi-permeable membrane, the ionophore contains a neutral carrier that shuttles the ion from the extracellular medium to the electrolyte filling solution. Voltage differences are a result of differences in the ion distribution across the ionophore (Vaughan-Jones and Aickin, 1988). These voltage changes can then be converted into an ion concentration. The principle behind the static and vibrating ion selective microelectrodes used in this study is the same except that the vibrating electrode has a greater resolution than the static electrode due to noise reduction and signal averaging.

The ion concentration is not the total concentration because electrodes do not measure the total concentration of the ion, rather they measure the activity of the ion. The ion activity is the effective concentration of the ion that is available to participate in chemical reactions (Vaughan-Jones and Aickin, 1988). In this study, the ion concentration of NH_4^+, pH, Na^+ and Ca^{++} next to the surface of the larvae at different locations were measured.

MATERIALS AND METHODS

Experimental Animals

Eyed eggs for confocal microscopy and NH_4^+ and pH measurements were obtained from Blue Springs Trout Farm (Durham, ON) (see Table 2.1) and held in subdued light in a covered 5-liter container supplied continuously with aerated, dechlorinated Hamilton tap water ($[Na^+]$: 0.6 mmol l^{-1}, $[Cl^-]$: 0.7 mmol l^{-1}, $[Ca^{++}]$: 1.0 mmol l^{-1}, $[HCO_3^-]$: 1.9 mmol l^{-1}, pH 7.9), temperature was held constant at 7°C. Dead eggs were removed daily.

Eyed eggs for the Na^+ and Ca^{++} measurements were obtained from the Plymouth Rock Trout Hatchery (Plymouth, Mass.) and held in a 40-liter static aquarium with aerated, dechlorinated Woods Hole tap water ($[Na^+]$: 0.8 mmol l^{-1}, $[Ca^{++}]$: 0.8 mmol l^{-1}, pH: 7.5). Temperature was increase from 7°C to 15°C over the course of a week and held constant at 15°C for the remainder of the experimental period. This temperature was chosen in order to increase larval development so that the whole period of yolk sac absorption could be examined within a two week period. Dead eggs were removed daily.

Once hatching commenced, the larvae were transferred to 0.5-liter, mesh sided containers held in subdued light in a water table filled with the same water that was supplied to the eggs. Larvae were not fed during the yolk sac absorption period.

Experimental Series

1. Confocal Microscopy of the Gills and Yolk Sac of Larval Trout

i. *in vivo* Exposure

Larvae were sampled at weekly intervals after hatching and immersed in rhodamine 123 (R123, [methyl - o - (6 - amino - 3 '- imino - 3H - xanthen - 9

- yl benzoate monohydrochloride]) solution (2 $\mu g.ml^{-1}$ of holding water) for 7 hours. After the exposure period, the larvae were sacrificed with an overdose of MS 222 (0.16 $g.l^{-1}$) and lightly fixed in 2.0% glutaraldehyde in phosphate buffered saline (PBS, pH 7.8) for 30 minutes, under low light and on ice. The samples were then rinsed three times over 20 minutes in PBS. The gills and yolk sac epithelium were dissected from the specimens. A shallow well with a diameter of 10 mm was made on a microscope slide with nailpolish. The dissected tissues were placed in the well and embedded using concentrated gelatin. A coverslip was placed on top of the tissue and secured with nailpolish.

The slides were kept in the dark at 4°C until examined (the next day) at the University of Western Ontario, London, ON. Slides were examined using a confocal laser scanning microscope equipped with an argon-ion laser and mounted on a Nikon Diaphot microscope using BioRad MRC-600 FITC filter set.

ii. *in vitro* **Exposure**

Larvae from 7, 28, 60 and 105 degree·days post-hatch were sacrificed with an overdose of MS 222 (0.16 $g.l^{-1}$). The gills and yolk sac epithelium were dissected and rinsed in a phosphate buffered saline (PBS, pH 7.8), for 5 minutes. The dissected tissues were exposed to either R123 (5$\mu g.ml^{-1}$ in PBS), or anthroylouabain (AO; 2 $\mu mol.l^{-1}$ or 10 $\mu mol.l^{-1}$ in PBS) for 30 minutes, after which the tissues were removed and rinsed in PBS for 30 minutes. The tissues were lightly fixed for 30 minutes using 2.0% glutaraldehyde in PBS and were rinsed (3 times over 20 minutes) in PBS. The tissues were mounted and examined using the same methods as described in the *in vivo* exposure.

ll. NH_4^+ and pH Measurements

NH_4^+ and pH at the surface of the larvae were measured simultaneously using a double-barrel microelectrode. Measurements were made every 3 - 4 days from 7 to 291 degree·days post-hatch. Larvae were lightly anaesthetized with MS 222 (0.06 $g.l^{-1}$) in 0.5 mM $CaCl_2$, 0.5 mM Na_2CO_3, ammonia-free water; pH was adjusted to 7.9 with HCl. Due to the high levels of ammonia occurring in bath water NH_4^+ measurements and standards were made in ammonia free water in order to increase electrode resolution and detect NH_4^+ levels in the $\mu mol.l^{-1}$ range. Ammonia free water for the larval measurements was prepared by passing $H2O_d$ through Dowex-50 ion exchange resin (Sigma) to remove the ammonia.

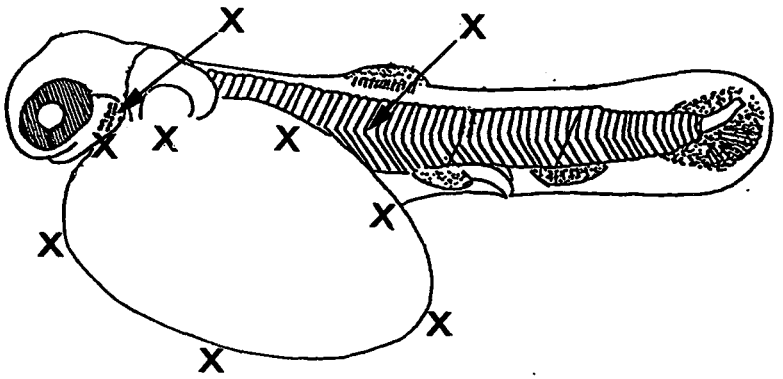
The larvae were placed on their right side in a small petri dish lid and held in position with small dissecting pins embedded in a layer of parafilm. This prevented the larvae from moving out of position during the experiment. NH_4^+ and pH measurements were made at the operculum, the gills, several places on the yolk sac and the skin (mid lateral line) (figure 3.1A). For each measurement, the electrode was placed 10 - 20 µm away from the surface of the larvae. After each measurement a bulk water measurement was taken > 1 cm away from the specimen. Electrode placement was achieved by visualizing the electrode through a 40X dissecting microscope and manually moving the electrode with micromanipulators. The electrode was positioned as close as possible to the specimen without touching it. The reference electrode remained > 1 cm away from the larvae. Each experiment took 20 - 30 minutes to complete, after which the larvae were returned to their holding containers to recover from the anaesthetic.

Figure 3.1: Locations of ion measurements

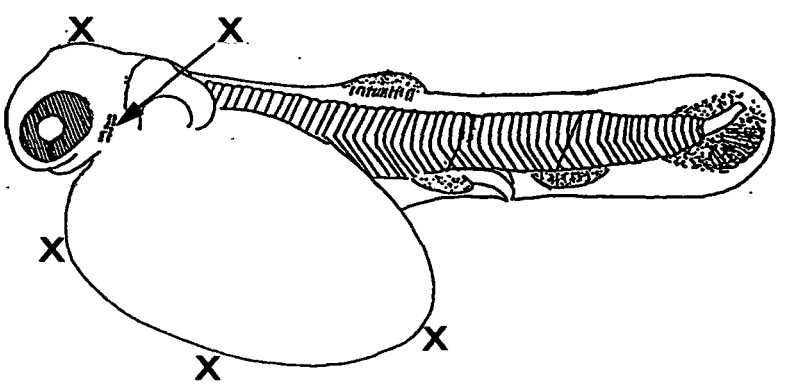
A. Areas of NH_4^+ and pH measured on the larval rainbow trout using the static microelectrode.

B. Areas of Na^+ and Ca^{++} measured on the larval rainbow trout using the vibrating microelectrode.

A.



B.



III. Na^+ and Ca^{++} Measurements

Na^+ and Ca^{++} at the surface of the larvae were made on separate occasions using a single-barrel microelectrode. Larvae from 15, 60, 120, 165 and 225 degree·days post-hatch were lightly anaesthetized with MS 222 (0.06 $g.l^{-1}$), pH adjusted to 7.8 with KOH in the regular holding water. They were positioned and secured in a petri dish lid in the same manner as described above. Before ion measurements were conducted, the opercular membrane from the left side of the fish was removed in order to allow access to the gills with the vibrating probe. Measurements from 3 different locations on the yolk sac (anterior dorsal, middle dorsal and posterior dorsal), the gills and from the skin (the top of the head) were recorded (figure 3.1B). These areas were chosen because they allowed the probe to be aligned parallel with the area of interest in order for the probe to vibrate perpendicular to its long axis (figure 3x). During the experiment the electrodes were placed 10 - 20 μm away from the tissue surface and were vibrated an excursion of 90 μm away from this position. The larvae and the electrode were visualized using a 40X dissecting microscope. Electrode placement was achieved via computer control of the micromanipulators. The reference electrode was > 1 cm away from the specimen at all times. It took approximately 20 minutes to complete the measurements on one fish.

Manufacture of Electrodes

I. Static Microelectrodes

Electrodes were made from pulled acid-washed double-barrel glass capillary tubes (AM Systems, Inc.). The electrodes were silanized to help stabilize the hydrophobic ionophore cocktail in its tip i.e., preventing the experimental water from entering the tip of the electrode and displacing the

ionophore. Electrodes were placed in a covered petri dish on a heating plate and exposed to dimethylchlorosilane vapor for 5 minutes. The petri dish lid was removed for 5 minutes to allow the vapors to dissipate and the process was repeated. After the second exposure the electrodes were left, uncovered, on the heating plate for an hour to dry and then stored in a desiccator.

For ammonium electrodes, the NH_4^+ ionophore (7.5% nonactin, 2.5% sodium tetraphenylborate and 89% nitrophenyloctoether) was introduced to the tip of the barrel and the remainder of the barrel was back-filled (filled from the blunt end) with 0.5 mM NH_4Cl. The electrode was calibrated to 3 different concentrations of NH_4Cl (1.0 μM, 10 μM and 100 μM).

For pH measurements, the pH ionophore based on the neutral carrier tridodecylamine (H^+ Ionophore Cocktail B, 60962 Fluka) was introduced to the tip of the other barrel and the rest of the barrel was filled with a pH cocktail containing 100 mM NaCl and 100 mM sodium citrate, pH 6.0. The electrode was calibrated to two different pH solutions, with a pH difference of 1 unit.

Each barrel of the double-barrel electrode was connected to a preamplifier using a $AgCl_3$ coated silver wire to make the electrical connection. The reference electrode was made from filamented single barrel, 1 mm glass capillary tubes (World Precision Instruments Inc.) and filled with 3 M KCl.

II. The Vibrating Microelectrode

Electrodes were made using an electrode puller (Model BB-CH Mecanex) from 1.5 mm diameter borosilicate glass capillary tubes to produce a tip approximately 2 to 3 mm long, with a tip diameter between 2 to 4 μm. The electrodes were silanized by drying them at 180°C for an hour and exposing

them for 15 minutes to N,N-dimethyltrimethylsilylamine vapor (41726, Fluka Chemical, Ronkonkoma, New York). After 15 minutes, the remaining silane vapor was allowed to escape and the electrodes were covered and dried for at least one hour. Electrodes were stored over dessicant in airtight containers.

For Na^+ measurements, the electrodes were back-filled using 100 mM NaCl and then front-filled with the Na^+ ionophore (Sodium Ionophore II-Cocktail A, 71178, Fluka Chemical). The electrodes were calibrated using 3 different concentrations of solutions, differing by 10 fold i.e., 0.1 mM, 1.0 mM and 10.0 mM NaCl.

For Ca^{++}, the electrode was back-filled using 100 mM $CaCl_2$ and then front-filled with the ionophore (Calcium Ionophore I- Cocktail A, 21048, Fluka Chemical). The electrodes were calibrated using 3 different concentrations of solutions, differing by 10 fold i.e., 0.1 mM , 1.0 mM and 10.0 mM $CaCl_2$.

The Na^+ and Ca^{++} electrodes were mounted to a preamplifier and the electrical connection was made with a $AgCl_3$ coated silver wire . Reference electrodes were made from L-shaped borosilicate glass capillary tubes filled with 3 M KCl in 1% agar.

Data Acquisition

I. The Static Electrode

The electrode and the $AgCl_3$ coated silver wire were connected to a pH/ion amplifier (model 2000, A-M Systems). The signal was fed through an analog to digital board (TL1 DMA Interface, Axon Instruments) and then displayed and recorded on a PC-based data acquisition system (AXOTAPE, Axon Instruments).

ll. The Vibrating Electrode

The ion selective electrode and $AgCl_3$ coated silver wire were mounted on an orthogonal array of stepper motors and connected to a preamplifier. In order to minimize electrical and mechanical interference the electrode and the preamplifier were vibrated together. Between the preamplifier and the computer is another amplifier, with a gain of 1000, and a low pass filter, which removed signals higher than 30 Hz. The resulting signal was fed through an analog to digital board (DT 2800 series; Data Translations) to the computer where the software (DVIS; National Probe Facility) sampled, compared and displayed the voltage changes.

Data Analysis

l. NH_4^+

The ammonium concentration next to the surface of the larvae ($[NH_4^+]$ in μM) was calculated using the equation:

$$[NH_4^+] = [NH_4^+]_{ref} * 10\text{^}(\Delta mV/ S)$$

where $[NH_4^+]_{ref}$ was the ammonium concentration from a reference NH_4^+ reading (μM), ΔmV was the difference between the reference NH_4^+ reading and the unknown NH_4^+ reading (mV), and S was the slope from the calibration curve corresponding to the mV value of the unknown NH_4^+ reading ($mV.\mu M^{-1}$).

ll. pH

The pH at the surface next to the larvae (pH) was determined by using the equation:

$$pH = pH_{ref} - (\Delta mV/ S)$$

where pH_{ref} was the pH from a reference reading, ΔmV was the difference between the reference pH reading and the unknown pH reading (mV), and S was the slope from the calibration curve corresponding to the mV value of the unknown (mV/pH unit).

III. Na^+ and Ca^{++} Concentrations Next to the Surface

The ion selective electrode measured voltage changes in the ion of interest. This voltage change was converted into an ion concentration using the equation:

$$C_{ion} = [(2.3 * \Delta V * C_B) / S] * E$$

where C_{ion} was the concentration of the ion ($\mu mol.cm^{-3}$), ΔV was the measured voltage difference (mV), C_B was the background ion concentration ($\mu mol.cm^{-3}$), S was the slope obtained from a 3 point calibration of static measurements of 3 different concentrations, differing by a decade change (mV), and E was the efficiency of the electrode (Smith *et al.*, 1994). Electrode efficiency is determined by measuring the ion efflux from a source of a known concentration at a specific frequency and comparing the measured value with the actual value. For Ca^{++} measurements the efficiency of the ionophore was determined to be 50% at the frequency of vibration used, while the efficiency of the Na^+ electrode was unknown and was assumed to be 100%.

RESULTS

Confocal Microscopy of the Gills and Yolk Sac

There were mitochondria-rich cells in the gill epithelium of first day larvae as determined by R123 fluorescence. The gills were comprised of gill arches and filaments and general fluorescence was seen throughout the tissue. There were no lamellae on the gill filaments at hatching. Since all cells contain some mitochondria, all of the branchial epithelium exhibited some fluorescence. However, along the edge of the filaments were isolated patches of intense fluorescence, indicating a large concentration of mitochondria at that location (figure 3.2A). By 105 degree·days post-hatch lamellae were beginning to develop on the filaments. Fluorescence of the tissue resembled first day gills with patches of intense fluorescence occurring on either side of the filament and at the base of the lamellae (figure 3.2B).

The yolk sac epithelium stained with R123 had regions of light fluorescence and areas of no fluorescence. The light fluorescence was the result of cellular mitochondria and the area of no fluorescence could be areas of cells, such as the nucleus, which do not take up any of the stain. When focusing through the yolk sac epithelium, packets of intense fluorescence appeared in some of the non-fluoresced areas (figure 3.2C). These packets could be concentrated areas of mitochondria within the cells that are located deeper within the cell and not at the surface. This image was typical of R123 stained yolk sac epithelium over all of the days examined (figure 3.2C).

Anthroylouabain (AO) fluorescence in the gills of first day hatchlings indicate the presence of Na-K-ATPase activity by the isolated regions of intense fluorescence appeared along the edge the gill filament (figure 3.3A). When stained with AO, the yolk sac epithelium exhibited a general

Figure 3.2: Gills and Yolk Sac Epithelium Stained with R123

A. Confocal laser scanning micrograph of the gill filament of a first day rainbow trout after *in vivo* labeling with R123. Notice the isolated patches of intense fluorescence along the edge of the filament. F: gill filament; Arrow head: mitochondria-rich cell; Scale bar = 25 μm.

B. Confocal laser scanning micrograph of the gill filament and developing lamellae of a larval rainbow trout 105 degree·days post-hatch after *in vivo* labeling with R123. Notice the regions of intense fluorescence on either side of the filament and at the base of the lamellae. F: gill filament; L: lamellae; Arrow head: mitochondria-rich cell; Scale bar = 25 μm.

C. Confocal scanning micrograph of the yolk sac epithelium of a rainbow trout 105 degree·days post-hatch after *in vivo* labeling with R123. Notice the regions of intense fluorescence in the middle of non-fluoresced areas. This was a representative micrograph of the yolk sac epithelium throughout yolk sac absorption. YSE: yolk sac epithelium; N: non-fluoresced cell; Arrow: region of concentrated mitochondria; Scale bar = 25 μm.

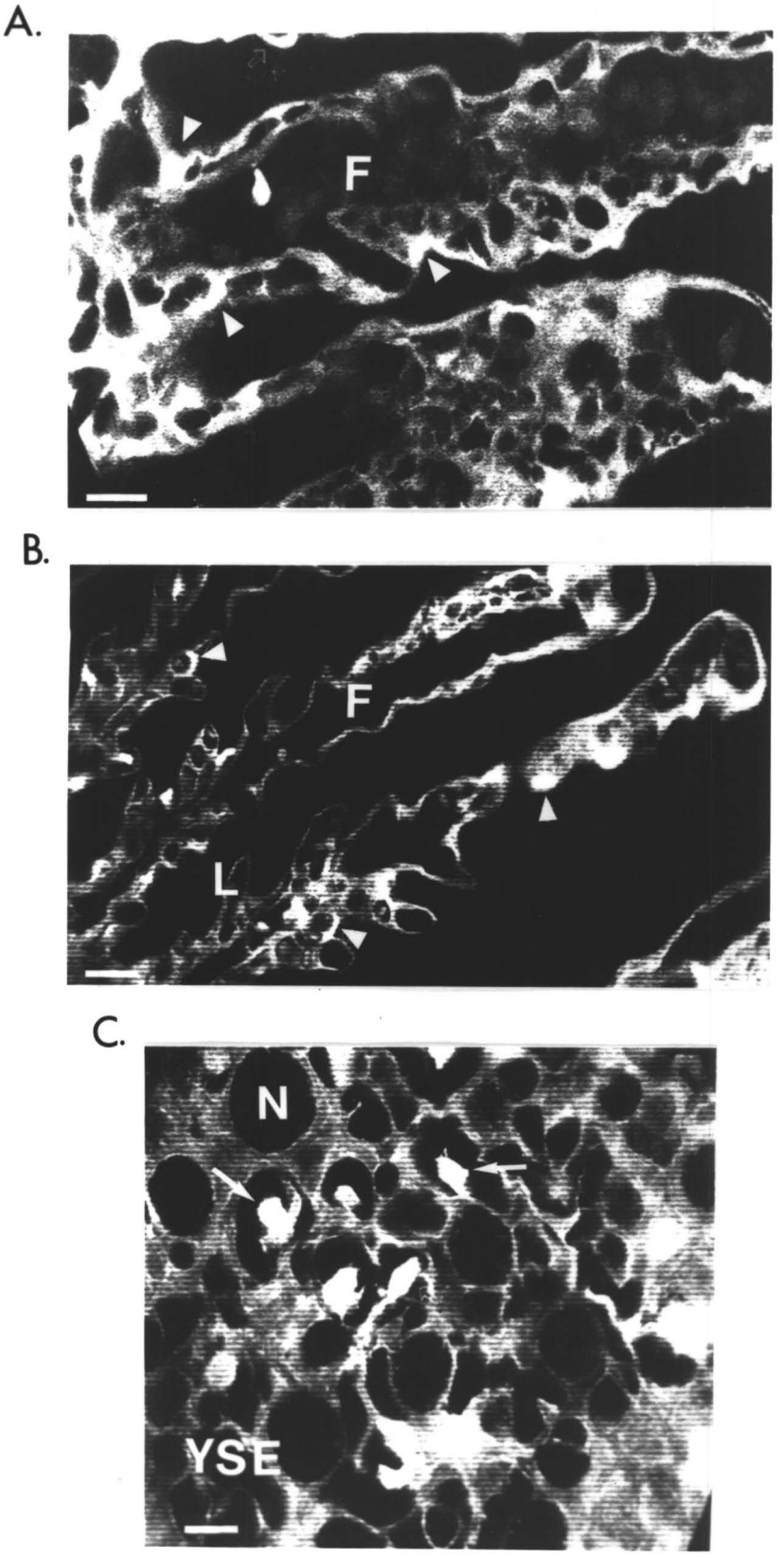
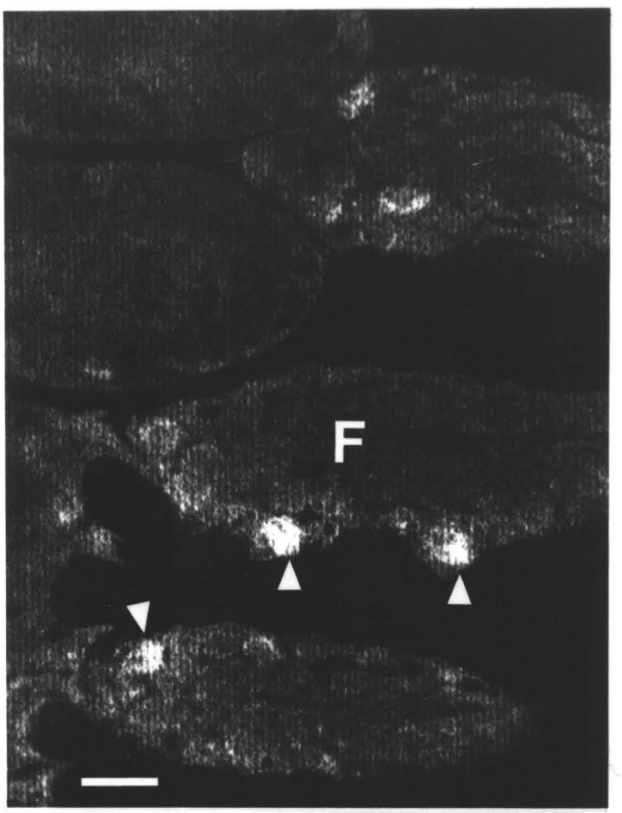

A.
F
B.
F
L
C.
N
YSE

Figure 3.3: Gills and Yolk Sac Epithelium Stained with AO

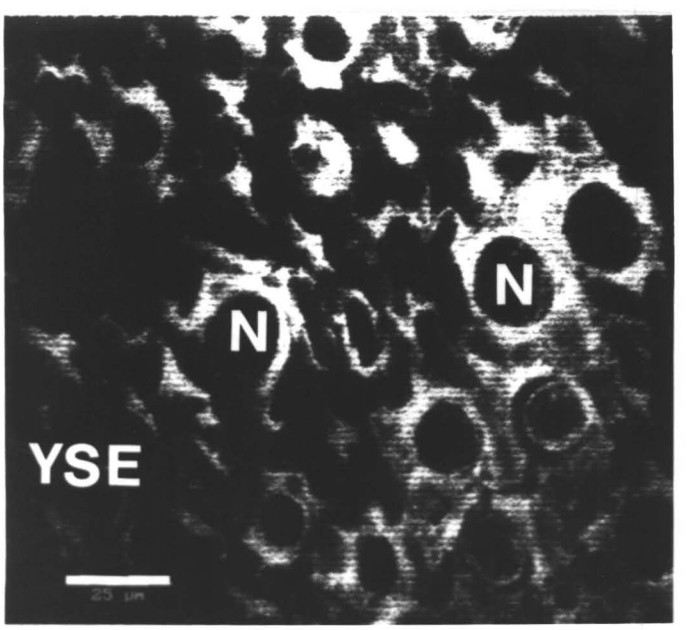
A. Confocal laser scanning micrograph of the gill filament of a first day rainbow trout after *in vitro* labeling with AO. Notice the regions of intense fluorescence along the edge of the gill filament. F: gill filament; Arrow head: region of Na-K-ATPase activity; Scale bar = 25 μm.

B. Confocal scanning micrograph of the yolk sac epithelium of a first day rainbow trout after in vitro labeling with AO. Notice the absence of intense fluorescent within the non-fluoresced areas. YSE: yolk sac epithelium; N: non-fluoresced cell; Scale bar = 25 μm.

A.



B.



fluorescence with areas of no fluorescence in a manner similar to the R123 stained tissue (figure 3.3B). However, focusing through the tissue did not produce any regions of intense fluorescence on the yolk sac of newly hatched larvae.

$[NH_4^+]$ Next to the Surface

Throughout larval development the $[NH_4^+]$ next to the surface of the gills was consistently higher than the concentration next to the surface of the other areas studied. The $[NH_4^+]$ next to the surface of the gills (in excess of the bath $[NH_4^+]$) remained relatively constant during the first 125 degree·days, but then increased 2-3 fold over the remainder of the sampling period (figure 3.4). The $[NH_4^+]$ next to the surface of the operculum, the yolk sac epithelium and the skin remained constant throughout yolk sac absorption. There was a greater $[NH_4^+]$ next to the surface of the skin than the surface of the yolk sac epithelium and the surface next to the operculum (figure 3.4).

pH Next to the Surface

Throughout yolk sac absorption the water next to the surface of the larvae was more acidic than the bath water as determined by microelectrode analysis (figure 3.5). At 8 degree·days there was a large difference in pH which then decreased by degree·day 28. The acidity then gradually increased to peak levels at 125 degree·days where the pH levels stayed relatively constant for the remainder of the yolk sac absorption period. The increase in acidity was consistently greater at the surfaces next to the gills and the operculum than at the surfaces next to the yolk sac and the skin throughout yolk sac absorption, but the differences were not as great as for those seen with the NH_4^+ electrode.

Figure 3.4: Differences in the Δ $[NH_4^+]$ (difference between the surface $[NH_4^+]$ and the bath $[NH_4^+]$; μmol l^{-1}) next to the surface of various locations of the larval rainbow trout throughout yolk sac absorption. **A.** Gills, **B.** Operculum, **C.** Yolk Sac and **D.** Skin. Bath $[NH_4^+]$ was < 10 μmol l^{-1}. Means ± SE.

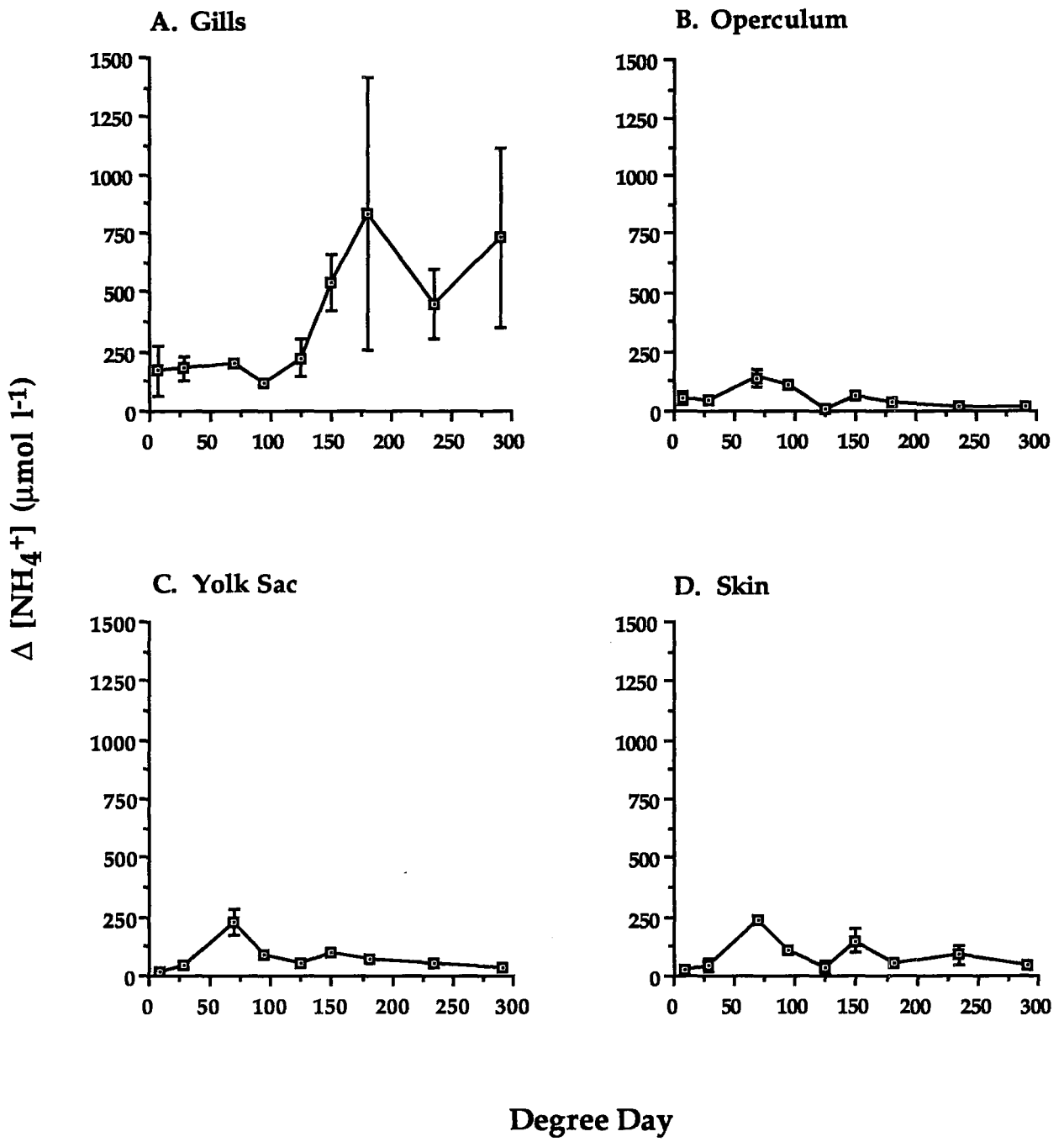
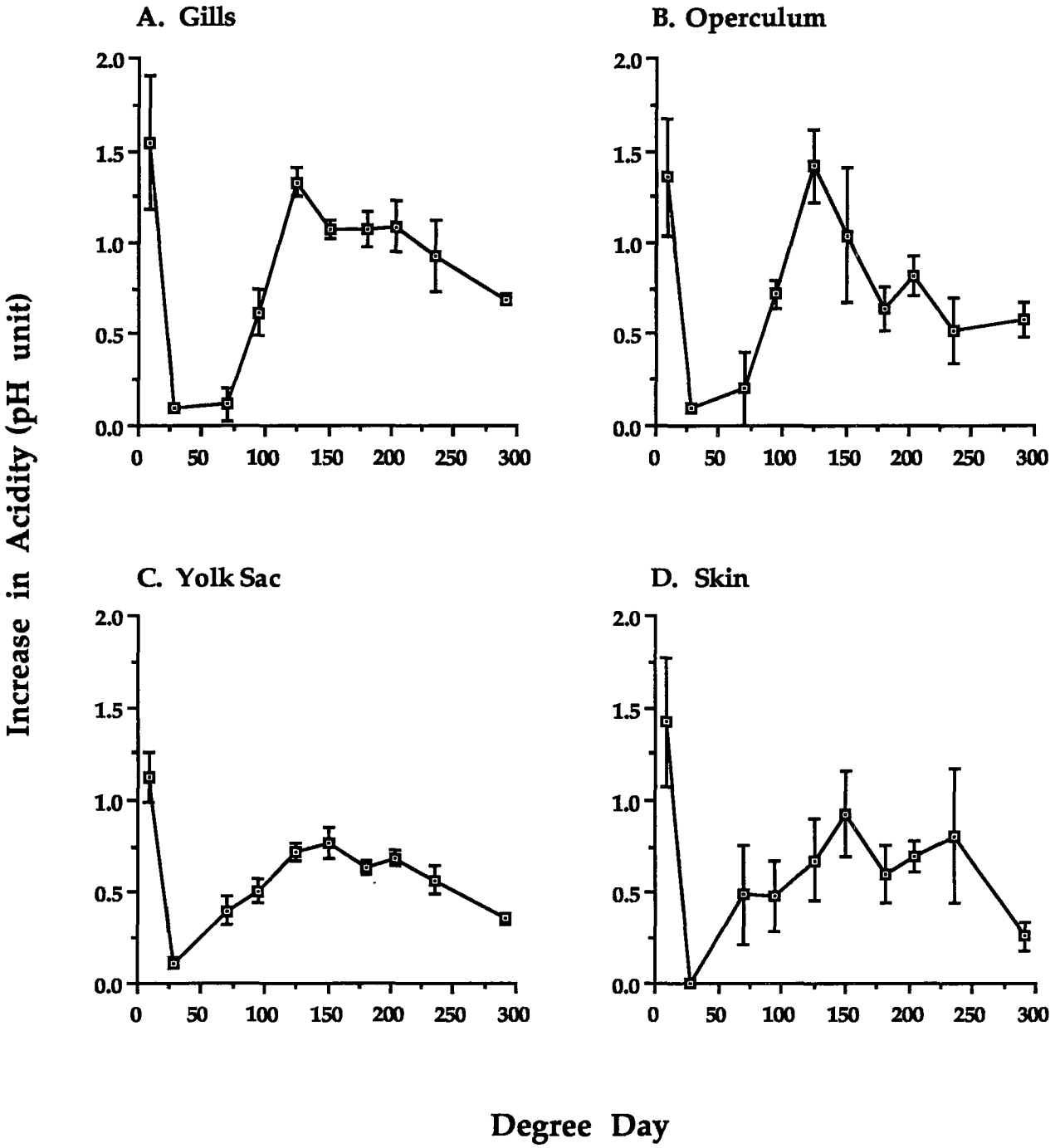

A. Gills
B. Operculum
C. Yolk Sac
D. Skin
Δ [NH4+] (µmol l-1)
Degree Day
0
250
500
750
1000
1250
1500
0
50
100
150
200
250
300

Figure 3.5: Changes in acidity with respect to the bath water measured at various location of the larval rainbow trout throughout yolk sac absorption. **A.** Gills, **B.** Operculum, **C.** Yolk Sac and **D.** Skin. Bath pH was 7.9. Means ± SE.


A. Gills
B. Operculum
C. Yolk Sac
D. Skin
Increase in Acidity (pH unit)
Degree Day
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300

[Na^+] Next to the Surface

The [Na^+] at the surface next to the gills (in excess of the bath water) increased 24 fold over yolk sac absorption while the concentration in the surface next to the yolk sac epithelium and the skin decreased (figure 3.6). Initially, [Na^+] was slightly greater at the skin than at the yolk sac epithelium and gills. This changed at 60 degree·days when [Na^+] was greater at the surface next to the gills than at the surfaces next to the yolk sac epithelium and the skin, and remained this way for the remainder of the yolk sac absorption period (figure 3.6). The [Na^+] at the surface of the gills also increased with time. The [Na^+] at the surface next to the yolk sac and the skin were not significantly different from each other and gradually decreased throughout yolk sac absorption.

[Ca^+] Next to the Surface

The [Ca^{++}] next to the surface of the larvae (in excess of the bath water) stayed fairly constant throughout yolk sac absorption. Except for the measurements conducted at 15 degree·days where the [Ca^{++}] was greatest at the surface of the gills, the [Ca^{++}] next to the surface of the yolk sac was slightly larger than the [Ca^{++}] next to the surfaces of the gills and the skin over yolk sac absorption (figure 3.7).

Figure 3.6: Changes in Δ [Na^+] (difference between the surface [Na^+] and the bath [Na^+]; μEq l^{-1}) measured at various locations of the larval rainbow trout throughout yolk sac absorption. **A.** Gills, **B.** Yolk Sac and **C.** Skin. Bath [Na^+]: 0.8 mmol l^{-1}. Means ± SE.

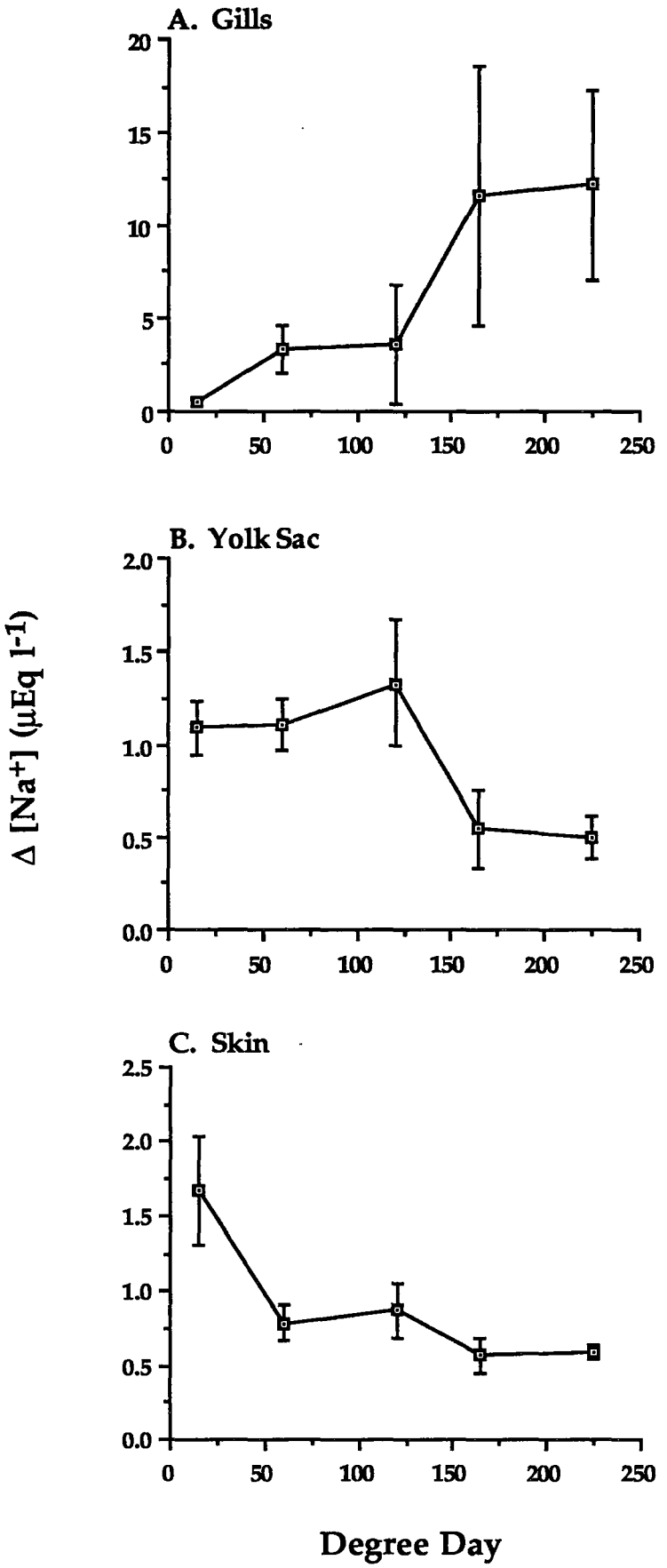
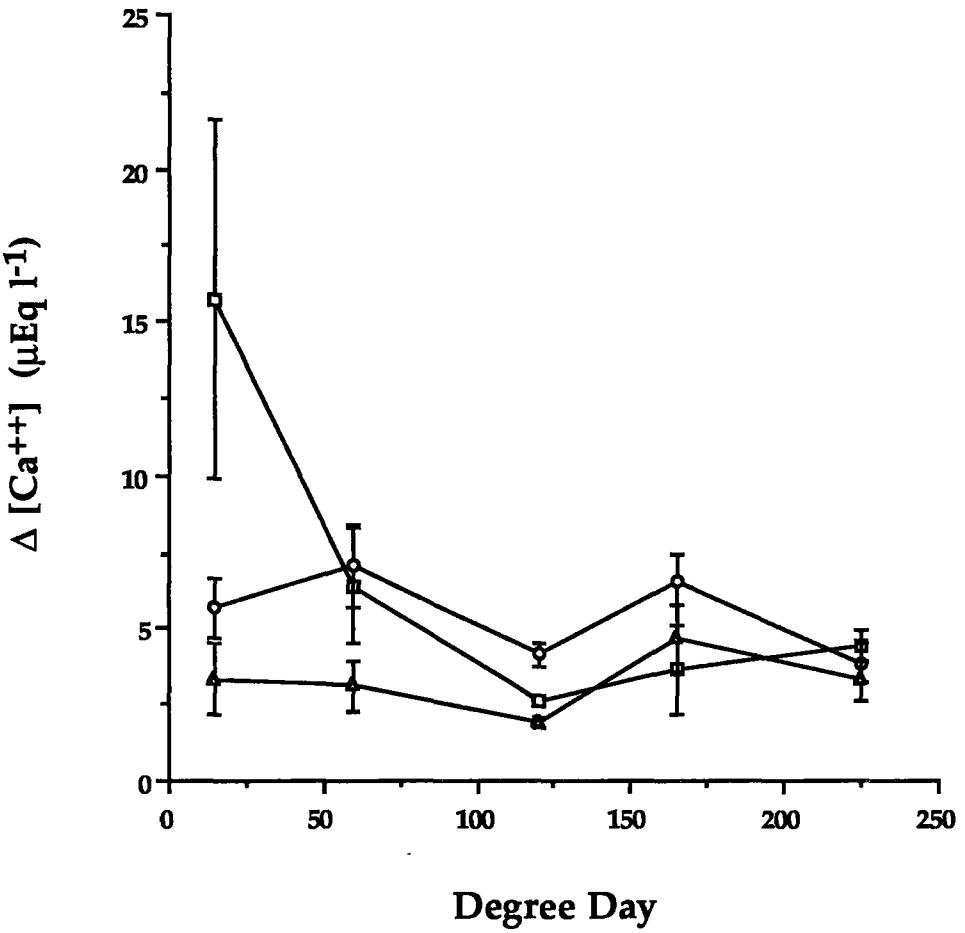

A. Gills
B. Yolk Sac
C. Skin
Δ [Na+] (μEq l-1)
Degree Day
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
200
250

Figure 3.7: Changes in Δ [Ca^{++}] (difference between the surface [Ca^{++}] and the bath [Ca^{++}]; $\mu Eq\ l^{-1}$) measured at various locations of the larval rainbow trout throughout yolk sac absorption. Gills: --□-- ; Yolk Sac: --o-- ; Skin: --Δ--. Bath [Ca^{++}]: 0.8 mmol l^{-1}. Means ± SE.


25
20
15
10
5
0
0
50
100
150
200
250
Δ [Ca++] (μEq l-1)
Degree Day

DISCUSSION

The Gills and Yolk Sac Epithelium of Larval Rainbow Trout

The development of rainbow trout branchial epithelia through embryonic and initial larval development has been documented (Morgan, 1974a; Morgan, 1974b), but their functions in gas exchange, acid-base balance, ammonia excretion and ion regulation are still in debate (Holeton, 1971; Morgan, 1974b; Wright *et al.*, 1995). In this study, the gills of first day hatchlings consisted of gill arches and filaments; lamellae began to develop a few days later (figure 3.2B). These results are consistent with those of Shen and Leatherland (1978b) who noted lamellae development 5 days after hatching, but not Morgan (1974a) who stated that developed lamellae were present at hatching. It is not easy to determine why these differences exist since the rearing temperatures in both Morgan's and Shen and Leatherland's study were the same. In an extensive morphological study McDonald and McMahon (1977) found lamellar development in larval Arctic char began 8 days after hatching. By two weeks post-hatch, the lamellar surface area comprises 10% the total gill surface area which increases to 23% of the total surface area by 47 days post-hatch. Lamellae number and surface area increased as the gill filament length increased (McDonald and McMahon, 1977).

A number of studies have showed that AO does stain mitochondria-rich cells by colocalizing DASPMI and AO fluorescence in the chloride cell (McCormick, 1990; Li *et al.*, 1995). In this study, confocal microscopy work on the gills using the fluorescent dyes R123 and AO, specific for mitochondria and phosphorylated Na-K-ATPase respectively, suggest that at hatching there are mitochondria-rich cells on the gill filaments, and that gill Na-K-ATPase

activity is also present. These results suggests that the gills of larvae trout are capable of Na^+ transport at the gills as soon as they hatch. Using DASPMI and AO, Li *et al.* (1995), identified chloride cells with Na-K-ATPase activity in the branchial epithelium of 3 day old tilapia. Based on the absence of lamellae until the 3rd day, they concluded that the gills of larval tilapia are responsible for ion regulation before they are responsible for gas exchange (Li *et al.*, 1995).

Confocal microscopy study using R123 of the yolk sac epithelium indicated regions of vast numbers of mitochondria (figure 3.2C). However, there was no indication of the presence of Na-K-ATPase as staining with AO failed to produce any intense fluorescence as seen in the gills (figure 3.3A and B). Mitochondria-rich cells have been identified on the yolk sac epithelium of larval tilapia (Ayson *et al.*, 1994), however, the functional state of these cells are unknown.

[NH_4^+] Next to the Surface

Total ammonia excretion is the amount of ammonia (NH_3) and ammonium (NH_4^+) excreted (Randall and Wright, 1989). In freshwater adult teleost the gills are the major site of ammonia excretion (Randall and Wright, 1987; Avella and Bornancin, 1989), with >90% of the ammonia excreted originating from the blood (Cameron and Heisler, 1983). The remainder of is comprised of ammonia synthesis in the gills (Cameron and Heisler, 1983). There have been a number of ammonia excretion mechanisms proposed: (i) NH_3 diffusion down a partial pressure gradient (Randall and Wright, 1987; Wilson *et al.*, 1994), (ii) H^+/NH_4^+ or Na^+/NH_4^+ electroneutral exchange (Kerstetter *et al.*, 1970; Cameron and Heisler, 1983; Randall and Wright, 1987), and (iii) NH_4^+ diffusion down an electrochemical gradient (Cameron and Heisler, 1983; Randall and Wright, 1987). Since NH_4^+ diffusion to the water is

considered to be negligible under normal conditions (McDonald and Prior, 1988) and there is no evidence of a Na^+/NH_4^+ exchange in the larval fish, the NH_4^+ detected by the microelectrode is a result of an H^+/NH_4^+ exchange and/or the protonation of excreted NH_3. An acidic layer next to the surface will donate a H^+ and trap NH_3 as NH_4^+, facilitating NH_3 excretion by maintaining a gradient (Randall and Wright, 1987, 1989).

NH_4^+ measured at the surface next to the fish indicate that ammonia excretion occurs at all of the locations measured on the larvae, albeit at varying degrees of intensity. The initial low concentrations at the locations may be due to low tissue catabolism rates. As the yolk sac is metabolized its metabolites are synthesized into tissue instead of being used for energy. NH_4^+ levels at the operculum, yolk sac and skin remain fairly constant over yolk sac absorption, while the concentration at the gills begins to increase after 125 degree·days. This increase suggests an increase in larval metabolism (table 2.5) and/or an increase in gill permeability to ammonia. By the end of the yolk sac absorption period it appears that the gills play a more important role in ammonia excretion than the cutaneous locations examined.

pH Next to the Surface

Fish generally excrete 10 times more CO_2 than ammonia (Randall and Wright, 1989). When metabolic CO_2 is excreted it is hydrated to form bicarbonate (HCO_3^-) and hydrogen (H^+) ions, the latter which acidifies the surrounding water (Randall and Wright, 1989). The hydration of CO_2 to HCO_3^- and H^+ is catalyzed by carbonic anhydrase found in the mucus (Shephard, 1994). The pH at the surface next to the gills, the yolk sac and the skin is more acidic than the bath water which indicates CO_2 excretion is of similar intensity at all 3 locations. The large pH difference on the first day of

hatching may be the result of a large transient increase in metabolic activity due to the exertion needed for the larvae to free themselves from their chorion and/or due to the activity associated with repayment of an oxygen debt. Oxygen consumption has been shown to increase at hatching, along with a decrease in respiratory quotient and lactic acid content. These are all indicative of oxygen debt repayment (Kamler, 1992). Oxygen consumption over yolk sac absorption in unfed larvae follows a parabolic model, initially increasing, then decreasing as endogenous food supplies become limiting (Rombough, 1988b). The activity of the larvae decreased significantly after hatching as they laid motionless on the bottom of their holding containers metabolizing their yolk sac and synthesizing new tissue. This could explain the decrease in the pH difference over the next 86 degree·days. Routine activity increases with increasing body weight (Wieser, 1985) and routine metabolic rates for larvae reared at 6°C and 9°C increase 4.1 and 3.4 fold, respectively, over yolk sac absorption (Rombough, 1988a). The larvae began to spontaneously swim while still absorbing its yolk sac, resulting in an increase in its metabolic demands, this is reflected in the increase in pH difference for the remainder of yolk sac absorption.

Although pH differences with respect to the bath water were found at the branchial and the cutaneous locations, the gill surface was consistently more acidified than the cutaneous surface. The difference in acidity between the gills and the cutaneous surfaces increased throughout yolk sac absorption. This could be indicative of the relative contributions of the branchial and cutaneous locations to gas exchange. Initially both the branchial and the cutaneous locations are important for gas exchange, but as development progresses the gills become more important in this function. This could be the result of the increase in the available gill surface area (McDonald and

McMahon, 1977; Oikawa and Itazawa, 1985), the increase in the development of the respiratory pumps (i.e. the buccal and opercular pumps) and the increase in the diffusion distance between the skin and water as the skin thickens and scales develop (Holeton, 1971). These results support other studies which have been done on the large surface to volume ratio of larvae resulting in the belief that cutaneous respiration through the body (including the yolk sac) and fins play an important role during the early stages of larval life (Oikawa and Itazawa, 1985; Rombough, 1988a; Rombough and Moroz, 1990; Wieser, 1995).

[Na^+] Next to the Surface

[Na^+] next to the gills increased throughout yolk sac absorption, but decreased next to the yolk sac epithelium and skin. Since the [Na^+] measured is the concentration above the [Na^+] in the bulk water, the larger concentration next to the surface of the larvae is the result of a Na^+ efflux from the fish and/or due to Na^+ accumulation in the mucus from the bulk water. Bulk water [Na^+] remained constant throughout the experimental period so if the microelectrode was measuring Na^+ attracted to the mucus from the bulk water no difference in the [Na^+] at different locations, or at the same location over time should be measured. Thus, changes in [Na^+] at the branchial and cutaneous surfaces of the larvae suggest changes in Na^+ efflux over yolk sac absorption. As determined in Chapter 2, Na^+ efflux does increase over yolk sac absorption following the increase in Na^+ uptake (figure 2.10). From this study it appears that Na^+ efflux from the gills increased throughout yolk sac absorption, while the Na^+ efflux from the yolk sac and the skin decreased. Therefore, in larval fish, the gills appear to be the primary site of Na^+ efflux.

[Ca^{++}] Next to the Surface

The [Ca^{++}] at the surface of various locations on the larvae were similar to each other and remained constant over yolk sac absorption. Again, the measured [Ca^{++}] was not the absolute concentration because the ion selective microelectrode measured only ionic, or free, Ca^{++}. The measured [Ca^{++}] could be the result of Ca^{++} efflux from the fish and/or any Ca^{++} attracted to the mucus from the bulk water. Chapter 2 revealed an increase in larval Ca^{++} efflux throughout yolk sac absorption (figure 2.12) and a large component of surface bound Ca^{++} (table 2.4) due to Ca^{++} unique binding properties with mucus.

The mucus coat is secreted by mucous cell found in the gills and the skin (Fletcher *et al.*, 1976; Lumsden *et al.*, 1994). Although it's exact function is unknown, it is believed to form a protective barrier from parasites, fungi and bacteria, a lubricating layer to decrease friction with water, and an ion attractant to contribute to ion regulation (Eddy and Fraser, 1982; Lumsden *et al.*, 1994; Shephard, 1994). Mucus is comprised of proteins, carbohydrates and glycoaminoglycans, the latter having large ion and water binding capacities (Van de Winkle *et al.*, 1986). Calmodulin, a Ca^{++} binding protein, is also present in mucus (Shephard, 1994). Despite the large binding capacity of mucus, it has a relatively low binding affinity as it does not slow down the diffusion rate for Ca^{++} (Pärt and Lock, 1983).

The pattern of [Ca^{++}] at each location and over time suggests that the mucus binding properties do not change at different areas of the fish. The ability of mucus to attract and loosely bind Ca^{++} may facilitate Ca^{++} uptake at the surface because of the persistent availability of the ion. The similar [Ca^{++}] at the gills, yolk sac and skin may indicate that all locations are involved in

Ca^{++} uptake. Perry and Wood (1985) found that Ca^{++} uptake at the gills and skin in rainbow trout occurred at approximately equal rates *in vivo*.

Conclusions

- The gills of first day hatchlings consist of gill arches and filaments. They contain mitochondria rich cells and have Na-K-ATPase activity. Lamellae are not present until 105 degree·days post-hatch.
- The yolk sac epithelium of first day hatchlings contains cells with regions of concentrated mitochondria, but no Na-K-ATPase activity was detected.
- The gills are the primary site of ammonia excretion throughout yolk sac absorption as determined by NH_4^+ measurements at the gills, the operculum, the yolk sac and the skin.
- Initially, the entire fish contributes to respiration, but as the gills grow and develop and the yolk sac decreases in size and the skin thickens, the contribution of the gills to this function increases.
- The gills are the primary site of Na^+ efflux which increases throughout yolk sac absorption.
- The site of Ca^{++} efflux was not determined due to binding of Ca^{++} with fish mucus.

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