THE RELATIVE TIMING OF SKIN-TO-GILL TRANSITIONS FOR SODIUM AND OXYGEN UPTAKE AND THE PLASTICITY OF GILL IONOREGULATORY DEVELOPMENT IN THE LARVAL RAINBOW TROUT, *Oncorhynchus mykiss*

by

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ABSTRACT

This study is the first to provide direct physiological evidence that larval fish gills play a primary role in ionoregulation considerably earlier than they do for gas exchange. Resting unidirectional sodium (Na^+) and oxygen (O_2) uptake across the skin and gills were measured simultaneously in rainbow trout *Oncorhynchus mykiss* between 0 to 18 days post-hatch (dph). The age at which the gills accounted for more than 50% of total uptake occurred at 15 and 16dph for Na^+ uptake while that for O_2 uptake occurred at 23 and 28 dph in soft and hard water, respectively. The finding that Na^+ uptake transitioned to the gills in about half the time required to do so for O_2 uptake suggests that the gills are required for ionoregulation prior to gas exchange in rainbow trout. The ratio of Na^+ to O_2 uptake rates (ion/gas ratio; IGR) increased with age indicating that the ionoregulatory components of the gills developed more rapidly than those for gas exchange.

The timing of transition for Na^+ uptake, gill Na^+, K^+-ATPase (NKA) protein concentration and gill NKA enzyme activity levels were not significantly different between larvae reared in soft and hard water. These results suggest that gill ionoregulatory development is non-plastic.

Approximately 90% (hard water) and 75% (soft water) of gill Na^+ uptake is estimated to have balanced diffusive Na^+ loss; the remaining was incorporated into the body. The apparent lesser Na^+ efflux in soft water suggests the presence of specific mechanisms that may reduce ion loss, such as tightening of paracellular junctions.

This investigation is the first to provide direct physiological evidence in support of the ionoregulatory hypothesis, challenging the long-held assumption that teleost gills develop initially for gas exchange. In rainbow trout, the gills clearly take on a primary role in

ionoregulation in advance of gas exchange, which suggests that ionoregulation may be the initial driving force for gill development in this species. Further investigation would be required to determine how wide spread this relationship may be in teleosts and more basal fishes.

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LIST OF ABBREVIATIONS

^{o}C	degrees Celsius
Ca^{2+}	calcium ion
$CaCO_3$	calcium carbonate
Cl^-	chloride ion
cpm	counts per minute
dpf	days post-fertilization
dph	days post-hatch
GABA	gamma-aminobutyric acid
HCO_3^-	bicarbonate ion
IGR	ion/gas ratio
K^+	potassium ion
Mg^{2+}	magnesium ion
MRCs	mitochondrial rich cells
MS-222	tricaine methane sulphonate
Na^+	sodium ion
$[Na^+]$	sodium ion concentration
^{22}Na	sodium-22
NaCl	sodium chloride
NKA	Na^+, K^+-ATPase
O_2	oxygen
ppm	parts per million
SEM	standard error of the mean

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Co-authorship Statement

Chapter Two of this thesis was co-authored. Research questions and experimental design were conducted by Clarice Hei Lok Fu under the co-supervision of Dr. Colin J. Brauner and Dr. Peter J. Rombough. The vast majority of research and data analysis was conducted by Clarice Hei Lok Fu. Dr. Jonathan Wilson conducted the study on NKA protein concentration. This thesis (with the exception of the methodology of the NKA protein concentration study) was written solely by Clarice Hei Lok Fu in consultation with Dr. Dr. Colin J. Brauner and Dr. Peter J. Rombough.

CHAPTER ONE: GENERAL INTRODUCTION

Adult teleost gills serve a number of functions including gas exchange, ionoregulation, osmoregulation, acid-base balance and nitrogenous waste excretion (reviewed in Evans, 2005). These functions have been well studied in adult gills, but relatively little is known about the functional ontogeny of larval gills. Existing literature is heavily biased toward early gill respiratory development; only recently has the potential role of larval gills in ionoregulation and acid-base balance been explored. This thesis investigates the relative role of the gills in gas exchange and ionoregulation during larval development to determine the initial function of the gills. I compared the timing at which sodium (Na^+) and oxygen (O_2) uptake transitioned from occurring predominantly ($\geq$50%) at the skin to predominantly ($\geq$50%) at the gills in the larval rainbow trout *Oncorhynchus mykiss*. The plasticity of ionoregulatory development was also evaluated by examining the effect of water hardness on the timing of the Na^+ transition and on Na^+, K^+-ATPase (NKA) protein concentration and activity. The remainder of this introduction summarizes what is currently known about gas exchange and ionoregulation in larval fish, with a focus on Na^+ and O_2 uptake in salmonids.

LARVAL DEVELOPMENT OF SALMONIDS

Salmonid eggs are deposited and fertilized in redds, in the gravel of freshwater streambeds (Kunz, 2004). Fertilized embryos are encased in an egg capsule which allows gases and ions to pass through, and protects the embryo from physical abrasion and pathogens (reviewed in Finn, 2007). Larvae hatch as alevins, with a large attached yolk sac that contains nutrients such as proteins, lipids and ions that support development until larvae are ready for exogenous feeding (Jobling, 1995; Kunz, 2004). Relative to other species, salmonids hatch at a

relatively advanced stage of development at which time the vascular system and fins are already clearly visible (Jobling, 1995). When yolk absorption is complete, larvae emerge from the gravel as free-swimming fry and begin to feed in the water column (Kunz, 2004).

WHY DEVELOP GILLS?

Since gills are immature at hatch, ionoregulation and gas exchange initially occur on the larval skin surface (Rombough, 1988). Rainbow trout, in particular, develop gill arches shortly after gastrulation (Morgan 1974), but the gills do not attain adult form until well after hatch (Shen and Leatherland, 1978). In the early stages of development, the exchange of gases and ions across the skin is sufficient due to a large surface area to tissue mass ratio. With growth, this ratio declines and the skin thickens, gradually limiting the efficacy of cutaneous exchange. At the same time, the gills increase in functional capacity and eventually displace the skin as the dominant site of these functions (Rombough, 1999).

Two main hypotheses prevail in the literature on the initial driving force for gill development: the widely accepted O_2 hypothesis and the recently proposed ionoregulatory hypothesis. The former maintains that the gills develop to supply the body's O_2 requirements (Krogh, 1941) and the latter suggests that the gills develop to satisfy ion demands (Li et al., 1995). Both hypotheses are discussed in light of developmental changes in the skin and gills, gas and ion demands of larvae, and existing morphological data, with particular reference to rainbow trout.

THE PHYSIOLOGY OF IONOREGULATION IN LARVAE

Larval fish, like adults, are hyperosmotic to freshwater, and thus have a tendency to gain water and lose ions through passive efflux across the skin and gills. Larvae must then counter water loading and ion loss by reducing epithelium permeability (Alderdice, 1988) and actively taking up ions (Kaneko et al., 2002). Ionoregulation in larval fish is not well-understood, but the physiology of ionoregulation is proposed to be similar to adults (reviewed in Rombough, 2004). The current model for Na^+ uptake in adult freshwater salmonids involves the serial placement of two ATPases and a Na^+ channel located primarily in mitochondrial rich cells (MRCs) of the gill epithelium. Apical H^+-ATPases drive Na^+ uptake into the cell via Na^+ channels by generating a favourable electrochemical gradient across the apical membrane (Wilson et al. 2000). The basolateral NKA then transports Na^+ from the MRC cytoplasm into the blood (Avella and Bornancin, 1989). MRCs have been detected on the gills, body and yolk sac epithelium of larvae (Kaneko et al., 2002). Their appearance on the body and yolk sac epithelium in advance of the gills has been reported in many species including rainbow trout (Shen and Leatherland, 1978; Rombough, 1999) ayu *Plecogossus altivelis* (Hwang, 1989), freshwater carp *Cyprinus carpio* (Hwang, 1989), chum salmon *Oncorhynchus keta* (Kaneko et al., 1995), and Mozambique tilapia *Oreochromis mossambicus* (Kaneko et al., 2002). Cutaneous MRCs are proposed to play a primary role in ionoregulation before the gills mature (Kaneko et al., 2002).

In rainbow trout, MRCs develop on the yolk sac epithelium as early as 18 days post fertilization, followed by appearance on the head and trunk 3-6 days before hatch (Rombough, 1999). The first gill MRCs were present only 3 days before hatch (Morgan, 1974; Rombough, 1999): first on the gill arches, and then the filaments (Gonzalez et al., 1996; Rombough, 1999). At hatch, MRC count on the skin is five-fold greater than the gills, but proliferation on the filaments is rapid so that by yolk resorption, 75% of all MRCs are located on the gills

(Rombough, 1999). By hatch, half of these gill MRCs appear fully differentiated (Morgan, 1974) and their similarity in morphology to mature adult MRCs may imply full functionality (Rojo and Gonzalez, 1999).

The ionoregulatory hypothesis proposes that ionoregulation is the initial driving force for gill development (Li et al., 1995). As larvae grow, ion uptake at the skin decreases in efficiency due to thickening of the epithelium and increasing distance of MRCs from blood vessels (Rombough, 1999). Gill development was proposed to compensate for this decrease in the skin's ionoregulatory capacity (Rombough, 1999). The pressure on the gills to ionoregulate is further intensified by increasing ion demands with growth. Firstly, uptake rate must increase with body size to supply ions to growing tissues. This is evident in the near doubling in whole body Na^+ content in rainbow trout during early development (Brauner and Wood, 2002a). Secondly, expansion of gill surface area exacerbates diffusive ion loss, which would require greater ion uptake to balance increased efflux (Evans et al., 1999). Due to the combined pressures of reduced skin exchange efficiency and increased ion demands, the gills are proposed to develop initially for ionoregulation (Li et al., 1995; Rombough, 2007).

THE PHYSIOLOGY OF GAS EXCHANGE IN LARVAE

As is the case with ionoregulation, respiratory gas exchange first occurs on the larval skin surface and gradually shifts to the gills (Rombough, 1988). The rate of diffusive gas exchange across these surfaces is described by the Fick equation:

$$M = K \cdot SA / \delta \cdot \Delta P,$$

where *M* is the rate of gas transfer, *K* is Krough's diffusion constant (similar between the skin and gills), *SA* is the surface area of the skin or gills, δ is the thickness of the skin or gills (i.e. the diffusion distance from the outside surface of the exchanger to the bloodstream), and Δ*P* is the

difference in partial pressure across the skin or gills (reviewed in Rombough, 2004). Thus, the skin and gills are good gas exchange surfaces as long as they are thin and have a large effective surface area (i.e. a large surface area to tissue mass ratio). The efficacy of gas exchange is also improved by increased perfusion of the exchange surface and adequate ventilation of water over the exchange surface (Rombough, 1988).

In embryos and early larval stages, the skin is a highly efficient gas exchange surface relative to adults. The skin is only one-cell thick in embryos (Kunz, 2004), and capillary beds develop close to the skin early in organogenesis (Rombough, 1988). The skin gradually thickens to 3 to 4 layers in larvae and grows more distant from blood vessels, so that the efficacy of gas exchange decreases (Kunz, 2004). In relatively sessile larvae such as rainbow trout, the boundary layer, or the layer of stagnant water adjacent to the skin surface, increases the diffusion distance significantly and is the major barrier to gas exchange (Rombough, 1992). Thickness of the boundary layer may be reduced by rhythmic movement of the pectoral fins, which ventilate water over the skin surface (Holeton, 1971).

In contrast with the skin, the gills decrease in thickness and increase in surface area, so that exchange efficiency increases with development. In rainbow trout, gill arches form shortly after gastrulation. With the proliferation of lamellae, the diffusion distance is halved from hatch to 90 days post-hatch (dph) as the epithelium transforms from being multilayered to only two cells in thickness (Morgan, 1974). The surface area of the gills increases approximately 16-fold from hatch to yolk resorption (Rombough, 1999). Furthermore, ventilatory capacity of the buccal and opercular pumps increases with development (Holeton, 1971). Overall, the gills' capacity for gas exchange increases significantly.

The oxygen hypothesis proposes that the gills develop to meet the O_2 demands of the growing body as the skin decreases in gas exchange efficiency (Krogh, 1941). Relative to body mass, larvae have a large surface area so that initially, exchange across the skin sufficiently

fulfills the body's metabolic demands (reviewed in Rombough, 2007). As larvae grow, surface area of the skin increases at a slower rate than body mass and metabolic rate (reviewed in Rombough, 2007) and diffusion distance increases as the skin thickens (Kunz, 2004). Overall, skin exchange efficiency is gradually reduced, and in compensation, the gills are proposed to develop as a specialized respiratory structure for gas exchange (Krogh, 1941).

THE IONOREGULATORY VS. THE O_2 HYPOTHESIS

Despite the lack of supporting evidence, the O_2 hypothesis has been widely accepted in the scientific community and has not been challenged since it was first proposed by Krogh (1941). A recent increase in understanding of the ontogeny of gill function has given rise to the ionoregulatory hypothesis as the more likely explanation for the driving force for initial gill development.

The decrease in surface area to tissue mass ratio and thickening of the skin with development has been argued to be limiting earlier for ionoregulation than for gas exchange (reviewed in Rombough, 2004). Gas exchange can occur across most of the skin surface while ion exchange can only occur where MRCs are immediately adjacent to blood vessels. In other words, a smaller fraction of the skin is available for ion than gas exchange at all developmental stages. Moreover, ionoregulation is more constrained by thickening of the skin; it becomes ineffective when MRCs are more than one cell away from the water and blood while gas diffusion can still occur at multiple cell thicknesses from the skin and blood, albeit at a lower diffusion rate (reviewed in Rombough, 2004).

The relative increase in demand for ions during development is greater than that for O_2. As ion stores in the yolk are depleted after hatch, there is an increasingly greater demand for ion uptake to compensate for diffusive ion efflux and to fulfill the ion demands of growing tissues.

For instance, whole body Na^+ content in rainbow trout doubled during the first 14 dph (Brauner and Wood, 2002a) and mass-specific Na^+ uptake rate increased over 3-fold from 2 to 9 dph (Brauner and Wood, 2002b). This suggests that ion flux rates increase significantly near hatching. By contrast, the relative increase in O_2 demand is relatively small and there is no sudden increase in routine metabolic rate during larval development (reviewed in Rombough, 2007). Thus, the relative increase in demand for ions appears to be more severe than for O_2 during early gill development (Rombough, 2007).

One of the major criticisms of the O_2 hypothesis is that the gills develop before they are required for gas exchange. Based on the relationship between skin surface area, O_2 uptake, and body mass, Rombough and Moroz (1997) calculated that O_2 uptake at the skin theoretically becomes limiting at about 100 mg of body mass. In all eight species considered, including rainbow trout, gills developed well before larvae reached 100 mg, i.e. before the gills are theoretically required to supplement skin exchange. This strongly suggests that the gills are performing other functions prior to gas exchange (Rombough and Moroz, 1997).

The most compelling evidence in support of the ionoregulatory hypothesis is that MRCs appear on the gills before lamellae. This has been observed in almost all species studied to-date including the brown trout *Salmo trutta* (Pisam et al., 2000), mummichog *Fundulus heteroclitus* (Katoh et al., 2000), Japanese flounder *Paralichthys olivaceus* (Hiroi et al., 1998), ayu *Plecoglossus altivelis* (Hwang, 1990), tilapia *Oreochromis mossambicus* (Li et al., 1995), sea bass *Dicentrarchus labrax* (Varsamos et al., 2002) and zebrafish *Danio rerio* (Rombough, 2002). In rainbow trout, MRCs appear on the gills as early as 15 days before the lamellae (Gonzalez et al., 1996). Moreover, the gills account for the majority of all MRCs earlier than for total respiratory surface area (Rombough, 1999). These findings currently provide the strongest evidence that the gills assume a primary role in ionoregulation before gas exchange. However, direct physiological measurements to support this are lacking.

MODEL SPECIES: THE RAINBOW TROUT *Oncorhynchus mykiss*

Rainbow trout, a member of the family Salmonidae, was selected as the model species to determine whether the ionoregulatory or O_2 hypotheses best describes gill development in larval fish. Rainbow trout larvae gill, skin and yolk sac morphology have been carefully quantified during development to address the O_2 and ionoregulatory hypotheses. While morphological data largely support the ionoregulatory hypothesis, no physiological data exist to quantify the relative role of the skin and gills in ion uptake. It is also unknown whether the trends in gill MRC and lamellar development that support the ionoregulatory hypothesis are indeed of physiological significance. Results on larvae can be compared to that of adults, for which ionoregulation and gas exchange has been studied extensively and is relatively well-understood. In addition, the respirometry technique employed has been proven successful on rainbow trout in a previous O_2 uptake partitioning study (Rombough, 1998). Finally, rainbow trout larvae are relatively easy to obtain and rear in the laboratory. Due to this combination of factors, rainbow trout was deemed the ideal candidate for this investigation.

THESIS OBJECTIVES AND HYPOTHESES

The overall objective of this study was to seek physiological evidence in support of either the ionoregulatory or O_2 hypothesis to gain insight into the role of the gills during early development. There are two core objectives of this thesis:

Core objective #1: To determine whether ion or gas uptake transitions first from the skin to the gills in larval rainbow trout. To our knowledge, this is the first study to concurrently measure the partitioning of Na^+ and O_2 uptake between the gills and skin in larval fish. From this data, the time at which the Na^+ and O_2 uptake functions transition from the skin to the gills

was determined. It was hypothesized that Na^+ uptake would transition to the gills prior to O_2 uptake if the gills do indeed develop for ionoregulation prior to gas exchange.

Core objective #2: To assess the plasticity of gill ionoregulatory development.

Commonalities in MRC morphology suggest that the physiology of ionoregulation in larvae may be similar to that of adults (reviewed in Rombough, 2007). Previous studies have shown that MRCs proliferate on the gills of adult rainbow trout upon soft water transfer (Greco et al., 1996). Furthermore, branchial MRCs enlarge in the gills of adults acclimated to artificial freshwater with low sodium chloride (NaCl), presumably to aid in increasing NaCl uptake (Perry and Laurent, 1989). It was hypothesized that a similar plasticity may exist in larval gills so that ionoregulatory development can be accelerated if larvae are reared in softer water. This hypothesis was evaluated in two ways, including:

1) ***Assessment of the effect of water hardness on the timing of the Na^+ transition from the skin to the gills.*** This was determined by comparing the timing of Na^+ transition between larvae reared in soft and hard water. It was predicted that Na^+ uptake would shift to the gills sooner in soft water.

2) ***Assessment of the effect of water hardness on NKA protein concentration and activity***. It was predicted that NKA protein concentration and activity would be elevated in soft water larvae throughout development, reflecting accelerated gill ionoregulatory development.

This investigation is the first to reveal the relative role of the gills in ionoregulation and gas exchange in larval fish development. It is the first to compare the physiological timing of Na^+ and O_2 uptake transitions from the skin to the gills, which may provide insight into whether the initial function of fish gills is ionoregulation or gas exchange. This study is also one of the first to assess the plasticity of ionoregulatory gill development in larval fish in response to the water hardness of rearing environments. Overall, this thesis aims to gather solid physiological

evidence to differentiate between the ionoregulatory and O_2 hypotheses which will help us better understand functional development of the teleost gills.

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CHAPTER TWO: THE RELATIVE TIMING OF SKIN-TO-GILL TRANSITIONS FOR Na^+ AND O_2 UPTAKE AND THE PLASTICITY OF GILL IONOREGULATORY DEVELOPMENT IN LARVAL RAINBOW TROUT, *Oncorhynchus mykiss*

INTRODUCTION

The gills of adult fish serve a number of functions including gas exchange and ionoregulation (reviewed in Evans et al. 2005). However, developing fish initially have immature gills and these functions must occur elsewhere. The skin, with its large surface, appears to be the major site of these functions prior to the gills (Rombough, 1988). Specifically, gases diffuse across the skin from the water into the blood, while ions are primarily transported into the blood by MRCs located in the skin and yolk sac epithelium. Initially, gas and ion exchange at the skin surface is sufficient, due to the high surface area to tissue mass ratio of larvae. With development, the surface area to tissue mass ratio declines and the skin thickens so that the efficacy of skin ion and gas exchange is gradually reduced and eventually becomes limiting. At the same time that skin surface area is declining, the gills develop lamellae for gas exchange and MRCs for ionoregulation. Eventually, the gills displace the skin as the dominant site of these physiological functions (Rombough, 1999).

Since O_2 uptake is the most immediately critical function in adult fish, it has long been assumed to be the driving force for gill development during the embryonic and larval stages (Krogh, 1941). However, recent evaluations of the O_2 hypothesis, in combination with new

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evidence, suggest that ionoregulation may represent the initial driving force for gill development (Li et al., 1995; Rombough, 1999 and 2002).

One of the stronger arguments against the O_2 hypothesis is that the decrease in exchange capacity of the skin may be limiting for ionoregulation earlier than for gas exchange (reviewed in Rombough, 2004). Gas exchange can occur across most of the skin surface while ion exchange can only occur where MRCs are immediately adjacent to blood vessels. In other words, a smaller fraction of the skin is available for ion than gas exchange at all developmental stages. Furthermore, ionoregulation is more constrained by thickening of the skin; it becomes ineffective when MRCs are more than one cell away from the water and blood while gas diffusion can still occur at multiple cell thicknesses from the skin and blood (reviewed in Rombough, 2004).

The most compelling evidence in support of the ionoregulatory hypothesis is that the components for ionoregulation appear prior to those for gas exchange during early gill development. In all teleosts studied to-date including the brown trout, *Salmo trutta* (Pisam et al., 2000); mummichog, *Fundulus heteroclitus* (Katoh et al., 2000); Japanese flounder, *Paralichthys olivaceus* (Hiroi et al., 1998); ayu, *Plecoglossus altivelis* (Hwang, 1990); tilapia, *Oreochromis mossambicus* (Li et al., 1995); sea bass, *Dicentrarchus labrax* (Varsamos et al., 2002) and zebrafish, *Danio rerio* (Rombough, 2002), MRCs develop on the gills before lamellae. In the rainbow trout, *Oncorhynchus mykiss*, MRCs appeared on the gills as early as 15 days before hatch while lamellae only began to appear at hatch (Gonzalez et al., 1996). Furthermore, the gills account for the majority of total MRCs earlier than they account for the majority of total respiratory surface area (Rombough, 1999). Aside from these morphological findings, which may or may not reflect functionality, no direct physiological evidence exists to differentiate between the O_2 and ionoregulatory hypotheses.

The intention of this study was to seek physiological evidence in support of either the ionoregulatory or O_2 hypothesis and to gain insight into the relative role of the gills in these functions during early development. The rainbow trout was selected as the model species because larval gill morphology has already been characterized. This study had two objectives:

Objective 1: to determine whether ion or gas uptake transitions first from the skin to the gills in larval rainbow trout, which was calculated based on the relative contribution of the skin and gills to total uptake during development. To our knowledge, this is the first time ion and gas uptake partitioning between the skin and gills have been measured concurrently in larval fish. In light of the morphological findings that MRCs appear prior to lamellae in rainbow trout, we hypothesized that the Na^+ uptake transition to the gills would occur in advance of O_2 uptake.

Objective 2: to assess the plasticity of gill ionoregulatory development in larval rainbow trout. Studies on adult rainbow trout have shown that MRCs proliferate on the gills upon soft water transfer (Greco et al., 1996), and that adults acclimated to artificial freshwater with low sodium chloride (NaCl) have enlarged branchial MRCs, presumably to increase NaCl uptake (Perry and Laurent, 1989). It was hypothesized that a similar plasticity may exist in larval gills so that ionoregulatory development can be accelerated if larvae are reared in softer water. This hypothesis was evaluated in two ways, including:

1) ***Assessment of the effect of water hardness on the timing of the Na^+ transition from the skin to the gills.*** This was determined by comparing the timing of Na^+ transition between larvae reared in soft and hard water. It was predicted that Na^+ uptake would shift to the gills sooner in soft water.

2) ***Assessment of the effect of water hardness on NKA protein concentration and activity***. It was predicted that NKA protein concentration and activity would be elevated in soft water larvae throughout development, reflecting accelerated gill ionoregulatory development.

This study is the first to provide physiological evidence to aid the differentiation between the ionoregulatory and O_2 hypotheses by determining the order in which Na^+ and O_2 uptake shift to the gills. The contrasts between soft and hard water-reared larvae in the timing of Na^+ uptake transition, gill NKA protein concentration and NKA activity will shed light on the plasticity of gill ionoregulatory development in larval rainbow trout.

MATERIALS AND METHODS

Animals and Rearing

Rainbow trout, *Oncorhynchus mykiss,* were obtained as eyed embryos from Rainbow Springs Trout Hatchery in Thamesford, Ontario, Canada in March and April of 2008. Embryos were shipped by an air carrier and maintained between 3 to 8^oC for the duration of the 10 to12 hour transport. Upon arrival at the University of British Columbia, Vancouver, British Columbia, Canada, all embryos were disinfected by a 10-minute immersion in a 100 ppm free iodine solution (OVADINE, Syndel Laboratories Ltd.), and then acclimated to 10^oC. The soft water-acclimated group was reared in March 2008 in soft dechlorinated tap water from the City of Greater Vancouver (in mM: Na^+, 0.06; Cl^-, 0.05; Ca^{2+}, 0.03; Mg^{2+}, 0.007; K^+, 0.004; HCO_3^- unknown; alkalinity, 3.3 mg as $CaCO_3$/l; hardness 3.55 mg as $CaCO_3$/l; pH 6.2-6.7; Metro Vancouver, 2007), and the hard water-acclimated group was reared in April 2008 in artificial hard water made by adding salts to distilled deionised water (in mM: Na^+, 2.3; Cl^-, 0.1; Ca^{2+}, 0.9; Mg^{2+}, 1.0; K^+, 0.1; HCO_3^-, 2.3; alkalinity, 110-120 mg as $CaCO_3$/l; hardness 160-180 mg as $CaCO_3$/l; pH 7.6-8.0). Both groups were reared in the dark at a nominal temperature of 10^oC (actual temperature 9.5±0.1 ^oC) in an egg incubation tray supplied by a 40 L-recirculating system. A logger recorded the temperature of the set up every hour (iButton-TMEX, Dallas Semiconductor). Water changes were performed weekly and water quality was monitored

regularly to ensure ammonia, nitrate and nitrite levels were below 1.2 mg/l, 110 mg/l and 0.3 mg/l, respectively, as recommended for the maintenance of home aquaria (Hagen Liquid Division & H.A.R.S.). All experimental procedures involving animals adhered to UBC protocol A07-0080.

Respirometer design

Respirometers similar to those of Wells and Pinder (1996b) were designed to spatially divide the larvae into two compartments to enable *in vivo* measurement of O_2 and Na^+ uptake by the head and gills separately from the body and yolk sac. A respirometer consisted of two identical rectangular chambers made of acrylic plastic, each approximately 5 mL in volume. Each chamber was equipped with a micro stir bar, an air inlet and a O_2 probe/radioisotope injection port (Appendix 1).

Larvae positioning technique

Larvae were randomly selected for either oxygen (O_2) or sodium (Na^+) uptake measurements at 0, 6, 9, 12 and 18 dph. O_2 and Na^+ uptake measurements were performed on different individuals. Note that no uptake measurements were performed on 12 dph for soft water larvae due to technical difficulties.

Both types of measurements required positioning individual larva into a hole in a stretched rubber membrane of dental dam latex (SafeTouch 1255MG, Medicom, Quebec, Canada). Holes were burned in the rubber dam using a heating pen tool equipped with a tapered point (Creative HotMarks™, Walnut Hollow, Wisconson, USA). In order to minimize struggling and handling stress, larvae were first calmed with 100 ppm clove oil (Rougier Pharma, Quebec, Canada) for approximately 5 minutes to attain stage 5 anaesthesia (total loss of equilibrium and swimming motion with weak opercular motion and total loss of reactivity; Keene et al., 1998). Individual larvae were then drawn tail-first into the tip of a modified 1 mL

syringe, and then gently pushed head first through the hole in the dam, and positioned so that the dam was located immediately posterior to the pectoral fins. The two respirometer chambers were then closed around the dental dam containing the fish and secured by elastic bands. The respirometer was filled with water containing 10 ppm clove oil (prepared with soft or hard water, accordingly) to reduce struggling for the duration of the experiment. The respirometer was then aerated and submerged in a 10°C water bath (actual temperature 9.6±0.1^{o}C). Larvae were observed for a 1 h recovery period prior to the start of measurements. Larvae typically resumed normal ventilation and opercular movement within 30 minutes; those that did not recover within 45 minutes were not used for measurements and euthanized with 1000 ppm tricaine methane sulphonate (MS-222, Finquel, Argent Chemical Laboratories). After recovery, larvae were measured for either 1) Na^+ uptake rate in the anterior chamber, 2) Na^+ uptake rate posterior chamber, or 3) O_2 uptake in the anterior and posterior chambers simultaneously (see the following sections for both Na^+ and O_2 uptake measurement procedures).

This damming technique has been successfully employed in previous studies on rainbow trout (Rombough, 1998), Atlantic salmon (Wells and Pinder, 1996b) and chinook salmon (Rombough and Ure, 1991). These studies were used as references for anaesthesia, respirometer design, larvae positioning technique, respirometry technique, and recovery procedures.

Measurement of unidirectional Na^+ uptake rate

After the recovery period, either the anterior or posterior chamber (chosen randomly) was injected with 0.5 μCi of the radioisotope sodium-22 (^{22}Na; NEZ081, PerkinElmer Life And Analytical Sciences, Inc. Massachusetts, USA). Both chambers were mildly aerated and stirred for the duration of the 1.5 h flux period. Water samples of 25 μl were removed in duplicate from both chambers at 10 minutes post-injection and again at 1.5 h for measurement of water radioactivity and to ensure that there was no leakage of ^{22}Na across the dam. At the end of the

flux period, larvae were immediately removed from the dam and respirometer, rinsed three times with 5 mM NaCl to displace surface bound ^{22}Na, and then rinsed once with deionised water. Larvae were then recovered for 1 h in 3 ml of aerated, dechlorinated water in a small test tube. Water samples and the larva (with the associated test tube) were measured for radioactivity in counts per minute (cpm) using a gamma counter (Wallac 1470 Wizard, Perkin Elmer, Turku, Finland).

Na^+ uptake, expressed as μmol Na^+ g tissue weight $^{-1}$ hour^{-1}, was calculated as in Brauner and Wood (2002b):

Na^+ uptake = a / (SP · t · wt)

where *a* is the fish ^{22}Na activity in cpm, *t* is the total time of the flux period (1.5 h), *wt* refers to the wet tissue weight of the larva (g) and *SP* refers to the specific activity of the water calculated as:

$SP = [(cpm_i / [ion]_i) + (cpm_f / [ion]_f)] / 2$

where $_i$ refers to initial water samples taken 10 min after ^{22}Na injection and $_f$ refers to final water samples taken at 1.5 h. Water concentration of Na^+ was determined by flame atomic absorption spectrometry (Fast Sequential Atomic Absorption Spectrometer AA240FS, Varian Australia Pty Ltd., Victoria, Australia).

Measurement of O_2 uptake rate

Following the recovery period, residual air bubbles were removed and the chambers were topped up with fully aerated water containing 10 ppm clove oil. Needle-type housing fibre optic O_2 probes (Oxygen Microoptode, Loligo Systems ApS, Tjele, Denmark) were positioned above the ports of both chambers and connected to a data acquisition system (Microx TX3 and OXY-4 micro, Precision Sensing GMbH, Regensburt, Germany) to record the decline of O_2 in the water as the fish consumed O_2 . Sealing of the ports around the probes was not necessary due to

negligible O_2 exchange between the air and the water inside the respirometer. O_2 diffusion across the rubber dam separating the chambers was also negligible.

Anterior and posterior chamber O_2 consumption were recorded simultaneously as mg O_2 L^{-1} min^{-1} at 10^oC for 30 minutes. Water was continuously stirred in both chambers. After the completion of measurement, larvae were immediately removed from the dental dam and placed in aerated, dechlorinated water to recover for 1 h.

The rate of O_2 uptake was calculated from the slope at the longest linear portion of the recording and expressed as nmol O_2 g tissue wet weight $^{-1}$ $hour^{-1}$. The first 10 minutes of recordings were not used for calculations because the rate was not constant during this period. Tests without fish were conducted at the start and end of the day, and applied to fish data to correct for microbial O_2 consumption.

After the 1 h recovery period post-measurement, larvae were euthanized with 1000 ppm MS-222, and wet body weight (including yolk and without yolk) were determined to the nearest 0.1 mg. Data were discarded for individuals that failed to recover within 1 h or had visible physical damage. All chambers and dams were cleaned with detergent, disinfected with a 6% bleach solution and rinsed thoroughly prior to reuse to minimize radioactivity contamination and microbial accumulation.

Measurement of skin surface area

For each age group, mean percentages of total skin surface area in the anterior and posterior chambers were determined on preserved larvae. Animals were fixed in buffered paraformaldehyde solution (as described in Presnell and Schreibman, 1997) and phosphate buffered saline (pH 7.4, as described in Presnell and Schreibman, 1997) for 24 h at 4^oC, then stored in 70% EtOH at 4^oC until analysis. Because all samples were treated similarly and stored

for the same amount of time prior to analysis, shrinkage of the skin was assumed to be uniform across all samples and was not corrected for.

To measure total skin surface area, the body was divided into the head, pectoral fins, trunk and associated fins, tail and yolk sac regions. The head was first split along the medial line with a razor blade, the skin was removed of all attached tissue using fine-tipped tweezers, and both sides were gently pressed between a microscope slide and coverslip. Pectoral fins were mounted separately. The trunk (with associated fins) was halved, the skin removed and then mounted. The intact tail was flattened between two microscope slides. The yolk sac epithelium was dissected into 4-6 parts prior to mounting. All components were immersed briefly in Wright-Giemsa stain (SureStain™, Fisher Scientific, Pittsburgh, USA) and rinsed with deionised water prior to mounting to improve visibility for imaging. Slides were photographed with a Leica MZ16A dissecting microscope with a JVC KY-F75U digital camera using the software Auto-Montage Pro 5.01.0005 (Synoptics Ltd.). Surface areas were then measured using the Area Measurement function in trace mode using the software SigmaScan Pro Image Analysis 5.0.0 (SPSS).

An estimate of posterior skin surface area was obtained by summing the area of the trunk and associated fins, tail, and yolk sac. An estimate of total skin surface area was obtained by adding the remaining head and pectoral fin areas. The average percentages of total skin surface area in the anterior and posterior chambers were calculated for each age group (n=3 to 6 per group), with the exception of hard water larvae at 0 dph because they were too small to handle.

Calculation of gill and skin Na^+ and O_2 uptake rates

Rates of O_2 and Na^+ uptake in the anterior and posterior chambers were corrected to estimate uptake rates by the skin and gills using the following equations:

1. Gill uptake rate= $U_A - U_{SA}$,

2. Skin uptake rate = $U_P \cdot [A_A + A_P / A_P]$, where

U_A = total uptake rate in the anterior chamber,

U_{SA} = skin uptake rate in the anterior chamber, calculated as $U_P \cdot A_A / A_P$

U_P = total uptake rate (all of which is skin uptake) in the posterior chamber,

A_P = mean skin surface area in the posterior chamber, and

A_A = mean skin surface area in the anterior chamber.

Gill and skin absolute value uptake rates were also expressed as percentages of total uptake rates, where total O_2 uptake rate = anterior O_2 uptake rate + posterior O_2 uptake rate (obtained simultaneously on the same larvae), and total Na^+ uptake rate = mean anterior Na^+ uptake rate + mean posterior Na^+ uptake rate (measurements on different larvae). Gill and skin percentages of uptake were standardized to 100% if the sum of gill and skin uptake differed from 100%.

Measurement of NKA protein concentration

Five to seven larvae from each age group in soft and hard water were euthanized with 1000 ppm MS-222, immediately frozen intact in liquid nitrogen and stored at -80°C. Larvae were thawed on ice and the gills, yolk sac membrane and skin were dissected and refrozen separately in 100 ul SEI buffer (150 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 50 mM imidazole, pH 7.3). Tissues were homogenized by sonication on ice (40% intensity, two 2-second pulses; Sonics & Materials Ltd, Newtown, CT, U.S.A.), then centrifuged at 17 000 g at 4°C for 3 min. The supernatant was decanted and serially diluted tenfold in a four step series in 50 mM imidazole buffer (IB, pH 7.5) and dot blotted using a 96 well vacuum manifold (Convertible, Life Technology) onto PVDF membranes (Hybond P, GE HealthCare Inc, Carnaxide, Portugal). Wells were rinsed three times with IB and dried at 37°C for storage.

Membranes were rehydrated and blocked with 5% skimmed milk powder in TTBS (0.05% Tween 20, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and probed with a rabbit polyclonal anti-NKA α-subunit antibody (Wilson et al., 2007) diluted 1:10 000 in blocking buffer overnight at room temperature on an orbital shaker. Membranes were washed three times with TTBS and incubated with goat anti-rabbit horse radish peroxidase conjugated secondary antibody diluted 1: 100 000 in TTBS for 1 h at room temperature in an orbital shaker. Membranes were rinsed three more times with TTBS and incubated with ECL solution (Immobilon, Millipore) for 5 min. The signal was detected with a FujiFilm LAS 4000mini imager (FujiFilm, Porto). Membranes were stripped with 25 mM Glycine-HCl, 1% SDS, pH 2 for 30 min at room temperature and reprobed with a mouse monoclonal anti-actin antibody (clone AC-40, Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) diluted 1: 1000 in blocking buffer as described above with the exception of the use of a goat anti-mouse horse radish peroxidase conjugated secondary antibody diluted 1:50 000 in TTBS. Spot intensity from both NKA and actin dotblots were measured using Multi Gauge image analysis software (FujiFilm). Results are presented as a ratio of intensity of luminescence of NKA α-subunit to actin.

Measurement of NKA enzyme activity

Four to six larvae from each age group of the soft and hard water groups were euthanized with 1000 ppm MS-222, immediately frozen intact in liquid nitrogen, and stored at -80°C for analysis of NKA enzyme activity of the gills, body epithelium, and yolk sac epithelium. On the day of the assay, larvae were thawed individually, and samples of the gills (on larvae 6 dph and older), body epithelium, and yolk sac epithelium were homogenized by sonication (50% intensity, four 2-second pulses; Kontes Micro Ultrasonic Cell Disrupter, Mandel Scientific Co., Guelph, Canada) in 300 μl SEI buffer on ice. Samples were then centrifuged at 5000 g for 5 minutes. Activity was measured in the supernatant according to McCormick (1993) at 25°C

using a plate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, USA) and was expressed in μmol ADP mg $protein^{-1}$ h^{-1}. Protein content of the homogenate supernatant was measured using Bradford reagent (Sigma-Aldrich, USA) and bovine serum albumin as a standard and was expressed in mg.

Statistical analyses

O_2 and Na^+ uptake rates, NKA protein concentration and NKA enzyme activity were regressed against age. In general, the F test showed that linear regressions were a better fit to the data than second-order polynomial regressions (Prism 5.02, GraphPad Software, Inc, California, U.S.A). The F test was used to test for slopes significantly different from zero (SigmaPlot 10.0, Systat Software Inc., Germany). Significant differences between pairs of slopes were evaluated using the F test (Prism 5.02, GraphPad Software, Inc, California, U.S.A.).

Gill and skin O_2 and Na^+ uptake rates as percentages of total uptake rates were regressed against age. The age at which each pair of gill and skin lines intersected was determined. The mean and standard error for points of intersection were calculated by fitting data to the equation $Y = Ycross + (X – Xcross) \cdot slope$, where Xcross and Ycross were defined as shared parameters to find a best-fit value that applies to both the gill and skin lines. Pairs of crossing points were tested for significant differences using the Student's t-test (Prism 5.02, GraphPad Software, Inc, California, U.S.A.). Statistical significance was assumed at $P<0.05$. All values are reported as mean ± SEM.

RESULTS

Mean weight and skin surface area

Within the soft and hard water groups, larvae used for O_2 and Na^+ uptake measurements were similar in wet body weight both with and without yolk (Table 2.1). Soft water fish had statistically greater whole and tissue wet weights than their hard water age-matched counterparts ($P<0.001$, Table 2.1). Since mean percentages of skin surface area ($\%A_P$) in the posterior chamber was similar between soft and hard water groups, the $\%A_P$ of soft and hard water larvae were pooled. Mean $\%A_P$ declined with development from 86.1±1.7% at hatch to 72.6±1.6% by 18 dph (Table 2.1).

Na^+ uptake rate

Total Na^+ uptake rate in hard water was approximately double that of soft water at all ages. In general, both gill and skin Na^+ uptake rates were statistically greater in hard than in soft water ($P=<0.013$, Fig. 2.1 and Appendix 2). Total, gill, and skin Na^+ uptake rate increased with age in both soft and hard water (Fig. 2.1). The rate of increase in gill uptake was significantly greater than that of the skin ($P<0.02$), and was greater in hard than soft water ($P=0.005$; Fig. 2.1).

O_2 uptake rate

Gill O_2 uptake rates were not statistically different between soft and hard water at any age; skin O_2 uptake rates were statistically greater in hard water at 6 and 9 dph ($P=<0.045$, Fig. 2.2 and Appendix 2) In general, skin O_2 uptake rate was significantly greater than at the gills at all stages of development in both soft and hard water ($P=<0.001$, Fig. 2.2 and Appendix 2).

In both soft and hard water groups, total, gill, and skin O_2 uptake rates increased with age (Fig. 2.2). There were no significant differences between soft and hard water in the rate of

increase of total, gill, or skin O_2 uptake. The rates of increase in O_2 uptake between the gills and the skin were not significantly different (Fig. 2.2).

Relative contributions by the gills and skin to total Na^+ uptake rate

Water hardness had no significant impact on the contributions of the gills and the skin to total Na^+ uptake rates (Fig. 2.3). The skin's contribution was statistically greater than that of the gills in soft water at 0 and 6 dph ($P=<0.001$) and in hard water at 0, 6 and 9 dph ($P=<0.025$). By 18 dph, contribution by the gills in hard water was statistically greater than that of the skin ($P=0.023$, Fig. 2.3)

In both soft and hard water, the skin's contribution to total Na^+ uptake rate decreased linearly with age while the gills' contribution increased linearly (Fig. 2.3). At 0 dph, the gills contributed 9% to total Na^+ uptake rate in soft water and 14% in hard water; by 18 dph, the gills contribution had increased to 57% and 61%, respectively (Fig. 2.3). There were no significant differences between soft and hard water in the gills' rate of increase or the skin's rate of decrease in contribution to total Na^+ uptake rate (Fig. 2.3).

Relative contributions by the gills and skin to total O_2 uptake rate

Water hardness had no significant impact on the contributions of the gills and the skin to total O_2 uptake rates (Fig. 2.3). Gill and skin contributions to total O_2 uptake rates were not statistically different between hard and soft water groups at any age (Fig. 2.3). In both soft and hard water throughout development, the skin's contribution to total O_2 uptake rate was statistically greater than that of the gills (Fig. 2.3).

In soft and hard water, the skin's contribution to total O_2 uptake rate decreased linearly with age while the gills' contribution increased linearly (Fig. 2.3). At 0 dph, the gills accounted for 21% of total uptake rate in soft water and 25% in hard water; by 18 dph, its contribution had increased to 44% and 37%, respectively (Fig. 2.3). There were no significant differences

between soft and hard water in the gill's rate of increase or the skin's rate of decrease in contribution to total O_2 uptake rate (Fig. 2.3).

Intersection of gill and skin Na^+ and O_2 uptake regressions

The intersection between gill and skin Na^+ uptake rates (expressed as % of total uptake rates) occurred at 15±2 dph in soft water and at 16±2 dph in hard water (not statistically different; Fig. 2.3). Intersection points for O_2 uptake were extrapolated to occur at 25±2 dph in soft water and 28±3 dph in hard water (not statistically different; Fig. 2.3). The O_2 and Na^+ intersection points occurred significantly earlier for Na^+ than for O_2 in both soft and hard water ($P<0.003$; Fig. 2.3).

Gill ion/gas ratio

The gill ion/gas ratio (IGR) was calculated by dividing mean gill Na^+ uptake rate by mean gill O_2 uptake rate. IGR increased with development in both soft and hard water: in soft water, IGR increased from 5.9 to 87.4 pmol Na^+ nmol O_2^{-1}; in hard water, IGR increased from 60.8 to 184.3 pmol Na^+ nmol O_2^{-1} (Fig. 2.4). The rate of increase in IGR with age was not statistically different between soft and hard water groups; elevations were significantly different ($P=0.03$).

NKA protein concentration

Generally, NKA concentrations at each age were not statistically different between soft and hard water groups within tissue type (gills, body epithelium, and yolk sac epithelium). Gill NKA concentration ranged from 0.7±0.1 to 1.5±0.1 units (ratio of NKA α-subunit concentration to actin), body epithelium NKA concentration ranged from 0.5±0.0 to 1.2±0.3 units, and yolk sac epithelium NKA concentration ranged from 0.9±0.1 to 1.4±0.2 units (Fig. 2.5) during development.

Gill NKA concentration increased significantly with age in soft and hard water ($P=<0.003$) at similar rates (Fig. 2.5, Table 2.2). There was no correlation between body epithelium NKA concentration and age (Fig. 2.5). Yolk sac epithelium NKA concentration decreased with development (Fig. 2.5). There were no significant differences between soft and hard water in the rate of change of gill, body epithelium or yolk epithelium NKA concentration (Fig. 2.5). The rate of change in concentration was greater in the gills than the yolk sac epithelium in soft and hard water ($P=<0.001$) and greater in the gills than the body epithelium in hard water ($P=0.002$).

Gill Na^+ uptake rate was strongly positively correlated with gill NKA protein concentration (Fig. 2.6). The rate of increase in uptake rate with NKA protein concentration was not significantly different between soft and hard water groups (Fig. 2.6).

NKA enzyme activity

Generally, NKA activity at each age was not statistically different between soft and hard water groups at any age for any tissue type (Fig. 2.7). Gill NKA activity ranged from 1.43±0.14 to 2.13±1.15 µmol ADP mg $protein^{-1}$ h^{-1}, body epithelium activity ranged from 0.62±0.07 to 0.99±0.05 µmol ADP mg $protein^{-1}$ h^{-1}, and yolk sac epithelium activity ranged from 0.05±0.01 to 0.62±0.20 µmol ADP mg $protein^{-1}$ h^{-1}(Fig. 2.7) during development.

In both soft and hard water, gill NKA activity increased with age; body and yolk sac epithelium NKA activity remained constant with development (Fig. 2.7). There were no significant differences between soft and hard water in the rate of change of gill, body epithelium or yolk sac epithelium NKA activity (Fig. 2.7). In both soft and hard water, the rate of change in activity in the gills was significantly greater than at the body or yolk sac epithelium alone ($P<0.04$, Fig. 2.7). The rate of change in activity in the body and yolk were not significantly different (Fig. 2.7).

No significant relationships were detected between gill Na^+ uptake rate and gill NKA activity, or between gill NKA activity and gill NKA protein concentration (data not shown).

DISCUSSION

We found that the gills take on a primary role for Na^+ uptake in about half the time (15-16 dph) than the time required for O_2 uptake (23-28 dph) in rainbow trout. Furthermore, the timing of transition to the gills for Na^+ uptake, gill NKA protein concentration and gill NKA activity levels were similar between soft and hard water groups, suggesting that gill ionoregulatory development is unaffected by water hardness. These findings support the ionoregulatory hypothesis and have important implications for our current understanding of developmental physiology and the function of larval gills. In addition to gas exchange requirements, the need to ionoregulate also appears to strongly influence gill development in early life stages.

Measurement under calmed, resting conditions in normoxia

The measurement of Na^+ and O_2 uptake rates in resting, normoxic conditions likely underestimated uptake rates in the natural environment. Uptake rates were likely further depressed by the use of clove oil as a calming agent. These effects on measured uptake rates are first addressed.

Measurements of resting O_2 uptake rate in normoxia usually underestimate metabolic rate in the natural environment (Wieser, 1985), but this discrepancy is less of a concern in larval rainbow trout. Since rainbow trout larvae are relatively sedentary until yolk resorption, additional O_2 demand above resting levels is lower than larvae that are more active, such as cyprinids (Wieser and Forstner, 1986). Furthermore, the aerobic scope (the ratio of maximum to standard metabolic rate) in rainbow trout larvae is only 2.7, which is lower than that of fry (5.2;

Wieser, 1985) and adults (6 to 20; Shingles et al., 2001; Wilson et al., 1994). Thus, although all values reported here are for resting larval rainbow trout, maximal O_2 consumption rates would not be expected to be dramatically different from resting levels. Overall, resting measurements in larval rainbow trout may more closely represent levels in their natural habitats relative to juvenile and adult stages, as well as larvae of other species.

The use of clove oil as a calming agent is of concern because it can depress Na^+ and O_2 uptake rates. However, it was concluded from preliminary studies that a calming dose of clove oil must be administered during uptake measurements to prevent larvae from struggling and causing injury to themselves in the rubber dam. Previous O_2 uptake partitioning studies have used low doses of MS-222 (Rombough and Ure, 1991 and Rombough, 1998), but we found that even at a low level of 10 ppm, MS-222 suppressed Na^+ uptake by 49% while clove oil reduced Na^+ uptake by only 12% (statistically insignificant; Appendix 3). A previous study on rainbow trout larvae found that 50-75 ppm MS-222 had no significant effect of on O_2 uptake rate (Rombough, 1998), but clove oil reduced O_2 uptake rate by approximately 24% (Appendix 4). Since the determination of Na^+ uptake rate is of greater interest in this study due to its novelty, clove oil was deemed a more appropriate calming agent.

Since the effects of clove oil on O_2 uptake rate were greater than on Na^+ uptake rate, reported values for total O_2 uptake rate were likely a greater underestimation of values in uncalmed larvae than for total Na^+ uptake rate. We assumed that 10 ppm clove oil had the same effect on the gills and skin in O_2 or Na^+ uptake, and that these effects were consistent throughout development. Considering that clove oil is believed to be an agonist of gamma-aminobutyric acid (GABA), a neurotransmitter of the central nervous system (Aoshima and Hamamoto, 1999; Guénette et al., 2007), there is currently no basis to assume that 10 ppm clove oil will affect the relative contribution of the gills and skin to total uptake, and to affect them differently at

different stages of larval development. The use of clove oil may have affected the timing of the skin-to-gill transition for Na^+ and O_2 uptake, but likely only to a minor extent.

Relative contributions of the gills to Na^+ and O_2 uptake rate

Studies on the function of larval gills have traditionally focussed on its role as a gas exchange organ; its importance in ionoregulation has been largely ignored. A comparison of the contribution of the gills to total Na^+ and O_2 uptake suggest that the gills develop more rapidly for ionoregulation than gas exchange. At hatch, the gills account for 21% (soft water) and 25% (hard water) of O_2 uptake rate (Fig. 2.3). These values are in agreement with published values of 27% in rainbow trout (Rombough, 1998), 26% in chinook salmon (Rombough and Ure, 1991) and 28% in Atlantic salmon (Wells and Pinder, 1996b). By contrast, the gills at hatch accounted for only 9% (soft water) and 14% (hard water) of Na^+ uptake rate (Fig. 2.3). However, the rate at which the gills increase in contribution to Na^+ uptake rate is significantly greater than that of O_2 uptake, so that the gills' relative contribution to Na^+ uptake rate exceeds that for O_2 uptake at 6 and 9 dph (Fig. 2.3). By 18 dph, the gills account for 57% (soft water) and 61% (hard water) of total Na^+ uptake rate but only accounts for 37% (hard water) to 43% (soft water) of total O_2 uptake rate (Fig. 2.3) This suggests that the gills increases its involvement in Na^+ uptake faster than for O_2 uptake rate.

Relative total surface area comprised of the gills has been suggested to underestimate respiratory capacity (Wells and Pinder, 1996b). Indeed, we found that the skin-to-gill transition for O_2 uptake occurred at 23 or 28 dph, yet the gills do not account for 50% of total respiratory surface area until about 35 dph (Rombough, 1999). At hatch, the gills accounted for 21% (soft water) and 25% (hard water) of total O_2 uptake rate (Fig. 2.3) yet only accounted for 7% of total respiratory surface area (Rombough, 1999). By 18 dph, the gills account for 37% (hard water) and 43% (soft water) of total O_2 uptake rate (Fig. 2.3) while constituting less than 30% of total

surface area (estimated from figure in Rombough, 1999). Similar contrasts have been reported for chinook salmon: by yolk resorption, the gills contribute 60% to total O_2 uptake (Rombough and Ure, 1991), yet account for only 28% of total respiratory surface area (Rombough and Moroz, 1990). This confirms suspicions that evaluation of the gill's contribution to total uptake based on relative surface area significantly underestimates physiological diffusion capacity (Rombough, 1999).

Similarly, MRC count appears to be a poor indicator of ionoregulatory capacity. At hatch, the gills contribute approximately 9-14% to total Na^+ uptake rate (Fig. 2.3), but account for 22% of all MRCs (Rombough, 1999). By 18 dph, the gills account for 57-61% of total Na^+ uptake rate (Fig. 2.3), but is the site of less than 50% of all MRCs (Rombough, 1999). It appears that evaluation of gill uptake capacity based on MRC count overestimates physiological contribution early in development, but underestimates it later on.

The discrepancies between morphological and physiological contribution of the gills to Na^+ and O_2 uptake may be due to factors affecting gas and ion uptake capacity other than relative surface area and MRC count, such as mean diffusion distance, degree of perfusion and efficiency of ventilation (Rombough and Moroz, 1990). With respect to the use of relative surface area to estimate respiratory capacity, total anatomical diffusion factor (ADF, the ratio of surface area to diffusion distance) has been proposed to be a better morphological indicator than surface area alone because it also takes into account diffusion distance (Wells and Pinder, 1996a).

Relative timing of skin-to-gill transitions for Na^+ and O_2 uptake

The timing at which Na^+ and O_2 uptake transition from the skin to the gills was defined as the age at which the gills account for 50% of total uptake rate. This was estimated as the intersection between the gills' and skin's contributions to total uptake rate (Fig. 2.3). As

predicted by the ionoregulatory hypothesis, the skin-to-gill transition occurred significantly earlier for Na^+ uptake than for O_2 uptake in both soft and hard water environments: the Na^+ transition occurred at approximately 15 dph in soft water and at 16 dph in hard water, while the O_2 transition occurred at 23 dph in soft water and at 28 dph in hard water (Fig. 2.3). Clearly, the gills are playing an important role not only in gas exchange, but also in ionoregulation during early development of rainbow trout. Similarly in zebrafish, *Danio rerio*, the gills were found to be essential for ionoregulation 7 days postfertilization (dpf) but were only essential for O_2 uptake by 14 dpf (Rombough, 2002). It appears that the gills play a major role in ionoregulation before doing so for gas exchange in both rainbow trout and zebrafish.

The occurrence of the transition for Na^+ uptake before O_2 uptake is consistent with existing morphological data in general terms. In a study on rainbow trout larvae, MRC count and surface area were selected as indicators of ionoregulatory and respiratory capacity, respectively. MRCs appeared on the gills as early as 15 days prior to the appearance of lamellae (Gonzalez et al., 1996). Furthermore, the gills accounted for 50% of all MRCs by 18 dph but accounted for 50% of total respiratory surface area only by 35 dph (Rombough, 1999). Upon comparison with our reported physiological Na^+ transition at 15-16 dph and O_2 transition at 23-28 dph, gill MRC count is a better predictor of the time of Na^+ uptake transition to the gills than gill surface area is to the time of O_2 uptake transition. As discussed previously, this is likely because surface area alone is a poor indicator of gas exchange efficiency.

Na^+ Uptake

Skin-to-gill transition

The skin-to-gill transition for Na^+ uptake occurred at 15-16 dph (Fig. 2.3), yet the gills account for 50% of all MRCs a little later, at 18 dph (Rombough, 1999). Since the timing of physiological transition is earlier than expected based on morphological data, ion uptake

capacity per MRC at the gills may be slightly greater than at the skin. Prior to this study, Li et al. (1995) found very high mass-specific gill NKA activity in larval tilapia, which led to the speculation that gill MRCs are more efficient than those of the skin (Rombough, 1999). Indeed, NKA activity, an indicator of Na^+ uptake efficiency, was greater at the gills than at the body or yolk sac epithelium throughout development (Fig. 2.7).

A gill functional ablation study on rainbow trout found that the skin alone is insufficient for ionoregulation by 15 dph, which coincides with our calculated Na^+ uptake shift to the gills at 15-16 dph (Rombough unpublished, reviewed in Rombough, 2007). This suggests that ionoregulation must transition to the gills by the time skin exchange alone becomes insufficient.

Contrary to our hypothesis, soft water did not accelerate the transition for Na^+ uptake. An early study on rainbow trout embryos and larvae reported that gill MRCs did not differ in number or appearance in larvae reared at different salinities (deionised water, 11 ppm and 13 ppm seawater; Shen and Leatherland, 1978). Coupled with our finding that NKA concentration and activity were generally not significantly different between soft and hard water groups (Fig. 2.5), water ion content appears to have no impact on the development of gill ionoregulatory development. Therefore, we propose that the timing of transition to the gills for Na^+ uptake is non-plastic.

Na^+ uptake rate partitioning between the gills and the skin

Total Na^+ uptake rate increased with development (Fig. 2.1). Total Na^+ uptake rate was lower overall and increased at a slower rate in soft water than in hard water, most likely because Na^+ uptake rate is dependent on water Na^+ content (Fig. 2.1 and Table 2.2; Kirschner, 1970). As was reported in Brauner and Wood (2002a), mass-specific total Na^+ uptake rate decreased with age. Mass-specific total Na^+ uptake rates at 18 dph (soft water, 392 nmol g^{-1} h^{-1} and hard water,

780 nmol g^{-1} h^{-1}) were comparable to that reported in Brauner and Wood at an equivalent developmental stage (350 nmol g^{-1} h^{-1}; 2002a).

Gill Na^+ uptake rate increased with age (Fig. 2.1). This is not surprising, since gill NKA concentration and activity increased (Fig. 2.5 and 2.7) and the number of MRCs per filament increased approximately 3-fold from 0 to 18 dph (Rombough, 1999). Na^+ uptake rate increased at a slower rate at the skin than the gills, suggesting that the ionoregulatory capacity of the gills increased more rapidly than the skin (Fig. 2.1). Ion uptake efficiency at the skin likely declines with age due to thickening of the skin, increasing distance of MRCs from blood vessels, and decreasing MRC density after hatch (Rombough, 1999 and 2004).

Demands for increased total Na^+ uptake rate with age

The observed increase in total Na^+ uptake rate with development in both soft and hard water (Fig. 2.1) may be attributed to two major demands for Na^+. Firstly, uptake rate must be elevated to supply ions to growing tissues. This is evident in the near doubling in whole body Na^+ content in rainbow trout during the first 14 dph (Brauner and Wood, 2002a). Secondly, expansion of respiratory surface area exacerbates diffusive ion loss at the lamellae, requiring greater ion uptake rates to balance increased efflux (Evans et al., 1999).

We estimated that the majority of Na^+ uptake compensates for diffusive Na^+ efflux. The proportion of Na^+ uptake incorporated into tissues was not measured in the current study, but was estimated from changes in whole body Na^+ content measured during development in larval rainbow trout obtained from the same hatchery, and reared under similar conditions (Brauner and Wood, 2002a) . Assuming that changes in whole body Na^+ were similar between larvae of the two studies, we estimated that approximately 10% (hard water) and 25% (soft water) of Na^+ uptake was incorporated into the body. The remaining 90% and 75% of Na^+ uptake, respectively, is presumed to have balanced diffusive Na^+ loss. These values are in

agreement with those calculated with Na^+ uptake rate obtained from Brauner and Wood (27% incorporated, 73% balancing efflux; 2002a). In all cases, most of the Na^+ uptake was used to compensate for ion losses while a smaller fraction of uptake supplied the ion demands of the growing body. Assuming that there were no differences in Na^+ incorporated into larvae reared in soft and hard water of the present study (which were not measured) Na^+ efflux must have been lower in soft water. The specific mechanisms that may reduce ion loss in a low ion content environment are not known, but may consist of tightening of paracellular junctions or reduction in gill perfusion. Note that the above values are only estimates, and were derived from data from another study. We recommend simultaneous measurement of Na^+ efflux and whole body Na^+ content (in addition to Na^+ uptake) in future studies to enable direct calculation of how Na^+ uptake is partitioned.

Given that the majority of Na^+ uptake appears to balance efflux, as described above, the relationship between Na^+ uptake rate and total gill surface area during development could prove interesting. Assuming that unidirectional Na^+ uptake is not environmentally limited (i.e. uptake rates are near V_{max}), and total gill surface area is an indication of potential Na^+ efflux, one might expect Na^+ uptake to scale to unity with total gill surface area. In adult fish gills, Na^+ uptake rate is maximal when environmental Na^+ is 3.0 mM due to saturation of NKA (Kirschner, 1973), which is fairly close to our hard water values of 2.3 mM (but far from our soft water values of 0.06 mM Na^+). In order for diffusive ion efflux to be proportional to functional respiratory gill surface area, gill perfusion is important (Perry and McDonald, 1993). In adult rainbow trout at rest, only about 60% of the gills are perfused (Booth, 1978); however, no data exist for larvae. In this study gill Na^+ uptake rate in larvae reared in hard water increased directly proportional to gill surface area (slope of 1.0), and thus uptake appears to be completely predicted by gill surface area which likely represents the potential for efflux (Fig. 2.8).

O_2 Uptake

Skin-to-gill transition

The age of O_2 uptake transition to the gills and body weight at the time of transition in rainbow trout larvae are compared to other species. Relative to other species, rainbow trout transitioned to the gills for O_2 uptake at an age closer to yolk resorption: rainbow trout transitioned at 80% (soft water) and 97% (hard water), Atlantic salmon at 33% (estimated from published figures; Wells and Pinder, 1996b), and chinook salmon at 50% to yolk resorption (Rombough and Ure, 1991). This may be because rainbow trout are smaller and utilize their yolk more rapidly than the other two species (Rombough, personal communication). Rainbow trout in soft and hard water were approximately 300 and 100 mg at transition, respectively, while transitions occurred at 120 mg in Atlantic salmon (interpolated from published figure; water hardness unknown; Wells and Pinder, 1996b) and at 281 mg in chinook salmon reared in hard water (Rombough and Ure, 1991). Given the differences in experimental parameters among these studies and the potential for errors, all transitions occur within the small weight range of 100 to 300 mg. The variation in weight at transition suggests that the shift of O_2 uptake to the gills is not strictly based upon limitations of surface area to tissue mass ratio as was previously proposed (Rombough and Moroz, 1990), but is likely driven by a combination of other factors that are species-specific, including the developmental stage of the gills at hatch and the rate of gill development and yolk resorption (Rombough, 2004).

O_2 uptake rate partitioning between the gills and skin

Total O_2 uptake rate increased with development at the same rate in soft and hard water (Fig. 2.2 and Appendix 2). Total O_2 uptake rates in weight-matched larvae (9 dph soft water, 340 nmol O_2 h^{-1} and 12 dph hard water, 566 nmol O_2 h^{-1}) were comparable to that reported by Rombough (~360 nmol O_2 h^{-1}; 1998). The trends of increasing total, gill and skin O_2 uptake rate

per larva with tissue mass were consistent with those reported for larval chinook and Atlantic salmon in earlier partitioning studies (data not shown; Rombough and Ure, 1991; Wells and Pinder, 1996b). From 0 to 18 dph, gill and skin O_2 uptake rates increased with development at similar rates (Fig. 2.2) yet mass-specific O_2 uptake rate at the gill remained constant while that of the skin decreased (data not shown). This is likely because gill surface area, hence gill uptake capacity, expanded at a faster rate than that of the skin relative to mass.

In adult fish, an increase in gill surface area is accompanied by a proportional increase in gas exchange capacity (Rombough and Moroz, 1990), but it is unknown whether this relationship exists in larvae. From 0 to 18 dph, gill surface area increased over 10-fold (Rombough, 1999) while gill O_2 uptake increased only 3 to 4-fold (Appendix 2). It appears that the proposed relationship between gill surface area and gas exchange capacity does not hold in larval stages, perhaps because skin exchange accounts for a significant proportion of total gas exchange. However, larvae likely do not utilize their full gas exchange capacity at rest in normoxia. If the demand for O_2 increases (e.g. during periods of increased activity), O_2 uptake at the gills can be increased by increasing perfusion as discussed by Perry and McDonald (1993). Hence, the proposed relationship between gill surface area and gas exchange capacity in larvae may not be clear based on measurements of O_2 uptake rate at rest in normoxia but may be apparent at maximum metabolic rates in more challenging environments (e.g. during exercise or hypoxia).

Gill ion/gas ratio

The gill ion/gas ratio (IGR) has been used to compare relative changes in Na^+ and O_2 fluxes in adult rainbow trout (Gonzalez and McDonald, 1992). Adult gill IGR during routine activity in hard water was intermediate to those found for soft and hard water larvae (Fig. 2.4; Gonzalez and McDonald, 1992). This is not surprising since Na^+ uptake is affected by external

Na^+ concentration (Kirschner et al., 1973), and that the water content of Na^+ in the study of Gonzalez and McDonald (0.6 mM; 1992) was in between that of our soft and hard water (0.06 and 2.3 mM, respectively).

The increase in IGR with development in both soft and hard water (i.e. Na^+ uptake rate increased at a greater rate than O_2 uptake; Fig. 2.4) further supports the proposition that ionoregulatory components at the gills develop more rapidly than those for gas exchange. Such components for ionoregulation may include NKA protein concentration and activity levels in the gills. The parallel increase in IGR between soft and hard water (Fig. 2.4) further implies that the development of ionoregulatory and gas exchange components are independent of water ion content, which further supports that gill development is non-plastic.

NKA protein concentration

The positive correlation between gill Na^+ uptake rate and gill NKA concentration suggests that the observed increase in gill Na^+ uptake rate is at least partially, if not all, attributed to increased NKA concentration (Fig. 2.6). Na^+ uptake rate was greater in hard water than soft water at any given NKA concentration level, most probably because NKA was nearly saturated in the former but not so in the latter (previously discussed). As a corollary, gill NKA concentration must be greater in soft water to achieve the same uptake rate as in hard water (Fig. 2.6).

Body and yolk sac epithelium NKA concentration declined or did not change with development (Fig. 2.5), yet skin Na^+ uptake rate increased (Fig. 2.1). Since body and yolk sac epithelium NKA activity did not change (Fig. 2.7), the increase in skin uptake rate may be due to other factors such as increased MRC count or density on the body and/or yolk sac epithelium. Indeed, MRC count and density from 0 to about 20 dph increased on the body epithelium, yet decreased on the yolk sac epithelium (Rombough, 1999). Thus, the increase in MRC count and

density on the body epithelium exceeded the decrease in the yolk sac epithelium and may be one of the reasons for the increase in overall skin Na^+ uptake rate.

Gill NKA enzyme activity

Larval gill NKA activity in rainbow trout reaches adult levels (approximately 2.0 μmol ADP mg $protein^{-1}$ h^{-1}; Richards et al., 2003) by 12 dph and exceeds it by 18 dph in both soft and hard water groups (Fig. 2.7). Similarly, gill NKA activity in tilapia, *O. mossambicus,* was reported to be greater in larvae at 10 dph than in adults (Li et al., 1995). Greater gill NKA activity in larvae on a mass-specific basis may be due to the fact that lamellae account for a smaller portion of total gill protein in early gills; as the gills mature, lamellar expansion contributes to total gill protein but not to ionoregulatory capacity. Thus, gill NKA activity is greater in larvae on a mass-specific basis.

Surprisingly, no correlations were found between gill NKA activity and gill Na^+ uptake rate, or between gill NKA activity and concentration. The lack of such relationships may be because *in-vitro* activity measured by the kinetic assay may not be an accurate reflection of activity *in-vivo*. Complicating developmental factors affecting NKA activity may have also obscured such relationships.

CONCLUSION AND FUTURE DIRECTIONS

Until the ionoregulatory hypothesis was proposed (Li et al., 1995), it was assumed that the gills develop for the purpose of gas exchange, and that the function of ionoregulation was acquired secondarily. Our findings on rainbow trout provide convincing physiological evidence in support of the ionoregulatory hypothesis. Indeed, the gills assume a primary role in Na^+ uptake in half the time required to do so for O_2 uptake. In environments that differ markedly in ion content (hard vs. soft water), there were no differences in the timing of skin-to-gill transition

for Na^+ uptake during development in rainbow trout. Furthermore, no differences in gill NKA protein and activity during development were observed between soft and hard water rearing environments indicating that the ontogeny of ionoregulation at the gills may be non-plastic.

Further investigation is required for a more complete evaluation of the relative importance of ionoregulation and gas exchange in early gill development of the rainbow trout. Uptake partitioning studies on other important ions such as Cl^- and Ca^{2+} and studies in different conditions such as hypoxia, exercise, and at varied temperatures would reveal whether ionoregulation consistently shifts to the gills in advance of gas exchange and would also shed light on the plasticity in the timing of these transitions. The role of larval gills in acid-base regulation should also be assessed, considering that acid-base regulation is a form of ionoregulation and that the two functions are intimately linked (McDonald et al., 1991).

Thus far, research on larval gill function has been heavily biased toward its role in gas exchange. Studies on its involvement in ionoregulation by contrast, are severely lacking. Our novel findings in rainbow trout emphasize the need to consider the different functions of larval gills in other species (e.g. larval species of different size, larvae in marine habitats) before we can make generalizations on functional development of the gills.

The findings of our study may have important implications on our current understanding of gill evolution. It is commonly assumed that gills evolved from the branchial baskets of protochordates to complement O_2 uptake across the skin as O_2 demands increased due to the evolution of more active, predatory lifestyles and increased body size (reviewed in Coolidge et al., 2007). Such pressures are similar to those encountered by larvae during gill development (Rombough, 2004). It has been suggested that exchange at the skin became limiting for ionoregulation before gas exchange during the course of gill evolution, as is believed to be the case for the gills during development (Rombough, 2004). This hypothesis is supported by studies on the gill function of primitive fishes. Studies on gill function in marine hagfishes,

extant members of the earliest fish lineage, suggest that the first function of the vertebrate gills may be acid-base regulation (Evans, 1984; Mallat et al., 1987; reviewed in Evans et al., 2005). It has been proposed that the mechanism of acid-base relevant NaCl exchange at the gills were an exaptation for ionoregulatory functions that became important when fish invaded freshwater habitats (Evans, 1984; reviewed in Wright, 2007). In subsequently evolving species including lampreys (reviewed in Wright, 2007), elasmobranchs (reviewed in Evans et al., 2005) and basal teleosts such as sturgeons (reviewed in Wright, 2007), the gills are also believed to play an important, if not dominant role in acid-base regulation and/or ionoregulation. Unfortunately, the relative involvement of the gills in ionoregulation and gas exchange in primitive fishes and basal teleosts is unclear. The evolutionary progression of branchial ionoregulation in particular awaits further investigation.

In summary, we emphasize the need to investigate the functions of both larval and adult gills in phylogenetically varied species. The role of the gills in ionoregulation is a good starting point for future research. We can then make a well-informed hypothesis on the relative strength of the forces that drive ontogenic gill development, and on a larger scale, the selective pressures that may have influenced the evolution of the vertebrate gill.

CHAPTER SUMMARY

1. Regarding the relative timing of skin-to-gill transitions for Na^+ and O_2 uptake in rainbow trout larvae:

Na^+ uptake transitioned from the skin to the gills in half the time required to do so for O_2 uptake in rainbow trout larvae. Transitions occurred at 15-16 dph and 23-28 dph, respectively.

Our findings support the ionoregulatory hypothesis.

2. Regarding the plasticity of gill ionoregulatory development in rainbow trout larvae:

i) Water hardness had no effect on the timing of the Na^+ uptake transition from the skin to the gills.

ii) Water hardness had no effect on gill NKA protein concentration and NKA activity.

It appears that gill ionoregulatory development is unaffected by water hardness, suggesting the ontogeny of gill ionoregulatory function is non-plastic.

3. Regarding the partitioning of Na^+ uptake between ion demands in rainbow trout larvae:

The majority (75-90%) of Na^+ uptake balanced diffusive losses; only a small portion (10-25%) of Na^+ uptake was incorporated into the body.

This suggests the presence of mechanisms that reduce diffusive ion loss in softer water.

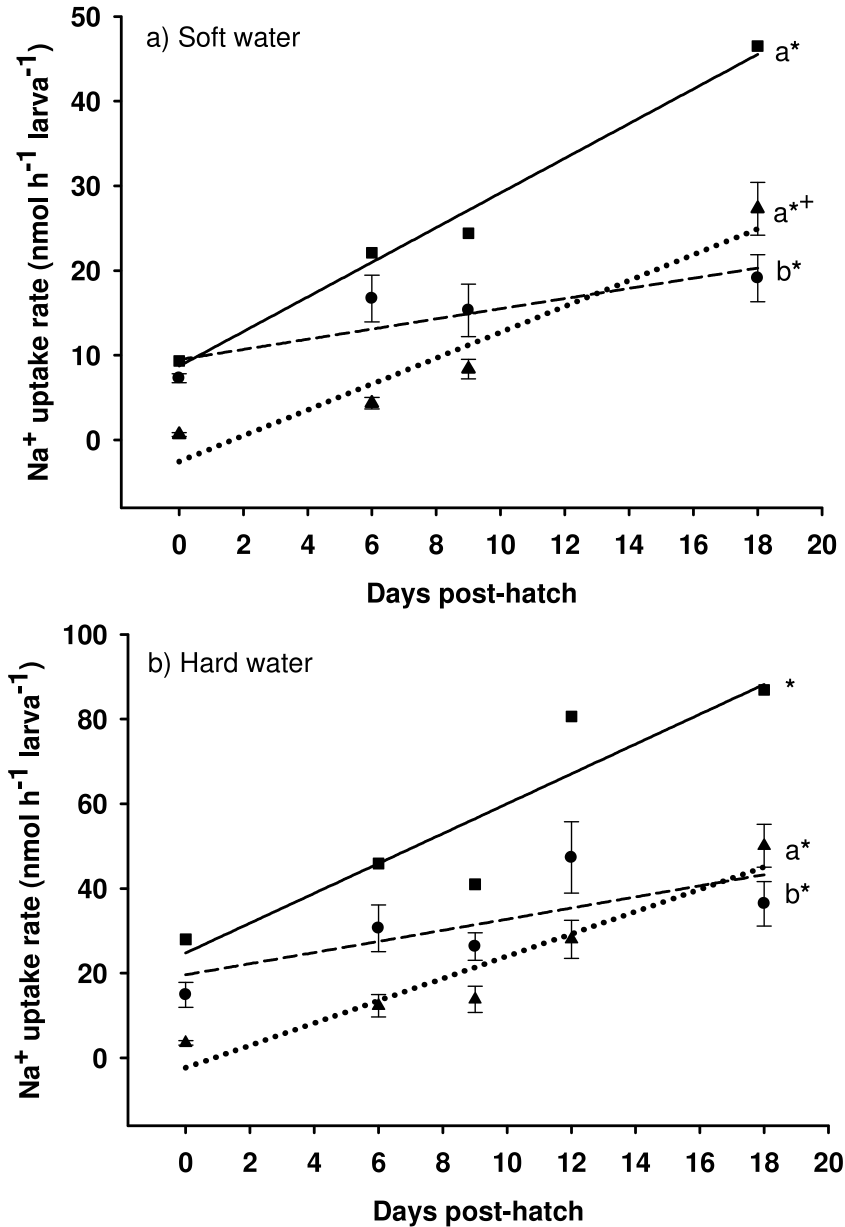

Figure 2.1. Total (■, solid lines), gill (▲, dotted lines), and skin (●, dashed lines) Na^+ uptake rates in rainbow trout reared in a) soft water and b) hard water from 0 to 18 days post-hatch. Linear regressions are based on raw data. Regression statistics are presented in Table 2.2. Asterisks (*) denote slopes significantly different from zero. Letters denote significant differences in slopes within soft and hard water groups. Plus (+) denote significant differences in slope between soft and hard water gill or skin regressions ($P<0.05$). Note vertical scale in hard water is two-fold of soft water. Data shown are mean ± SEM.

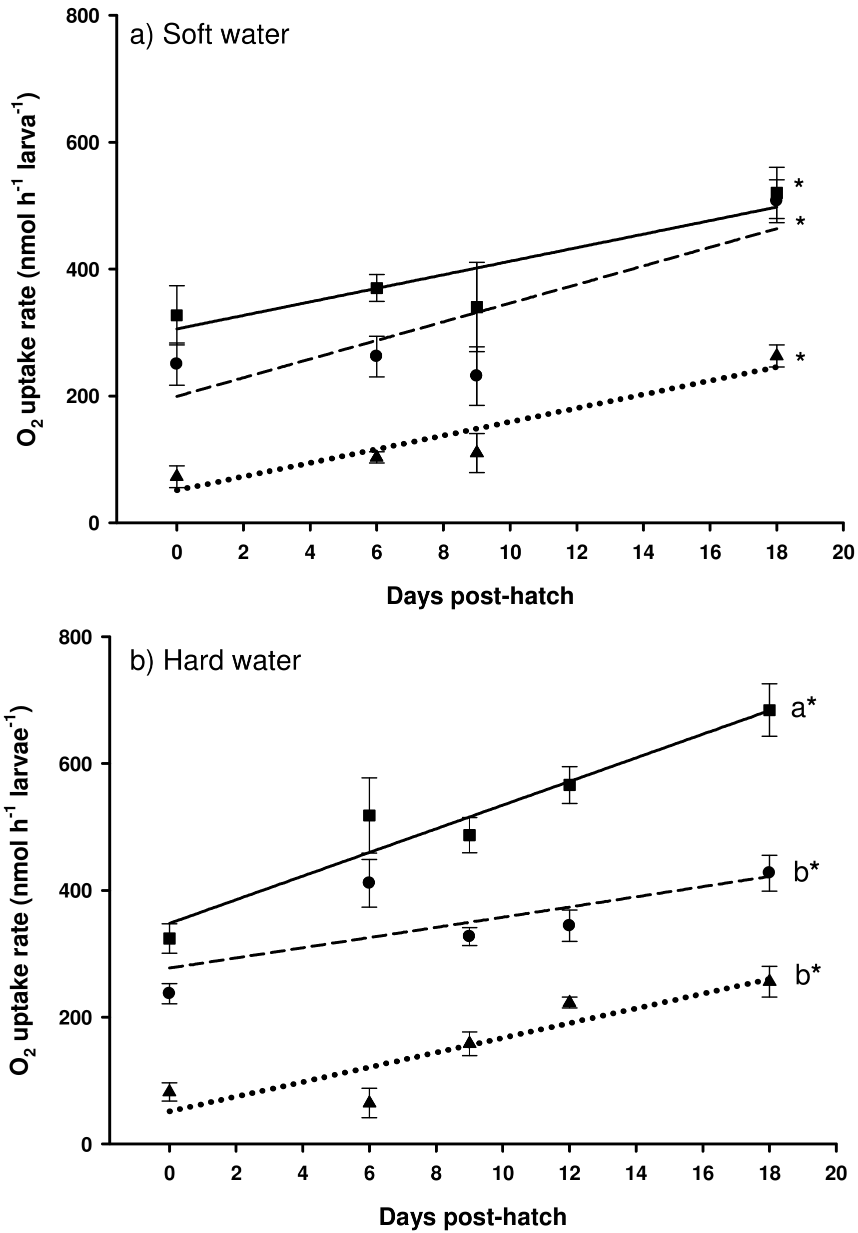

Figure 2.2. Total (■, solid lines), gill (▲, dotted lines), and skin (●, dashed lines) O_2 uptake rates in rainbow trout reared in a) soft water and b) hard water from 0 to 18 days post-hatch. Refer to the caption of Fig. 2.1 for all other details.

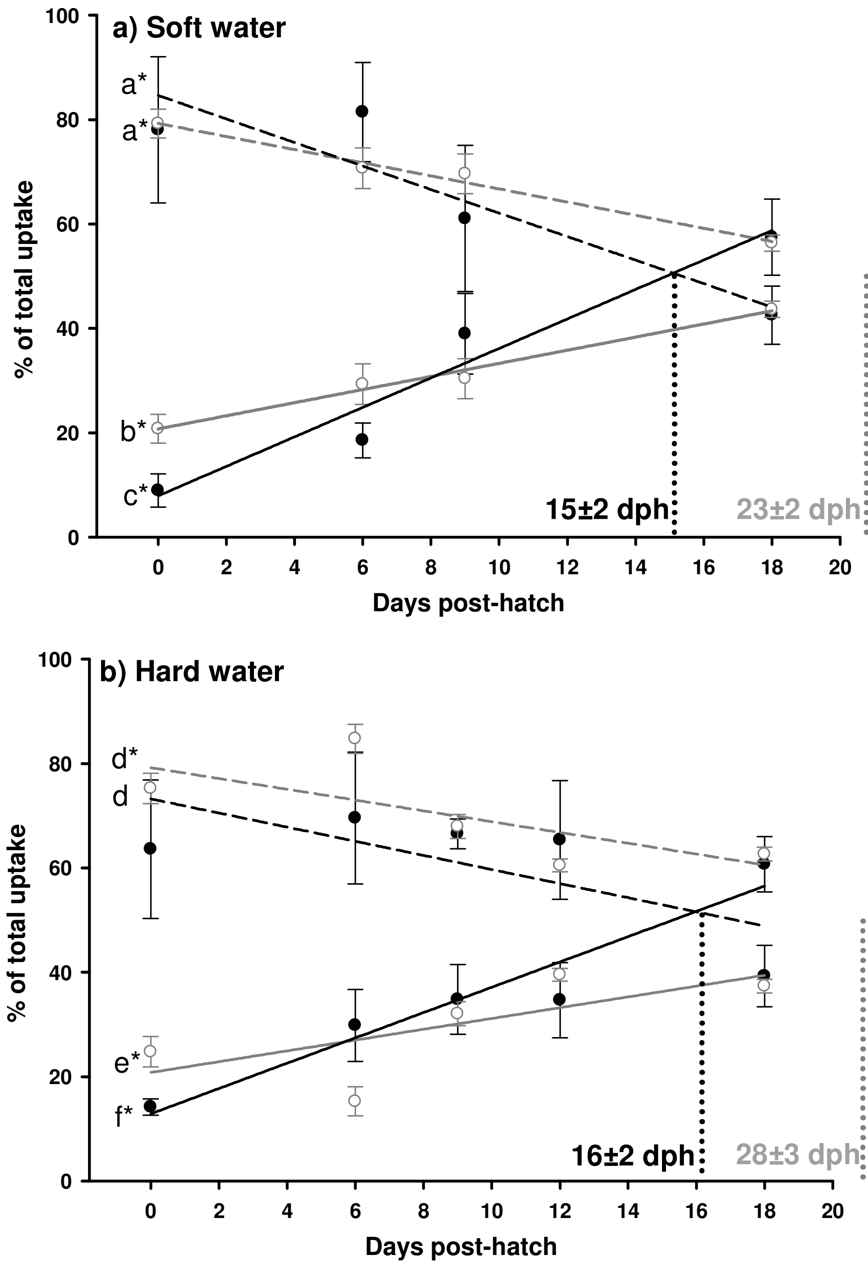

Figure 2.3. Percent contributions of the gill (solid lines) and skin (dashed lines) to total Na^+ (●) and O_2 (○) uptake rates in rainbow trout reared in a) soft water and b) hard water from 0 to 18 days post-hatch (dph). Calculated skin-to-gill transitions are indicated by vertical dotted lines. Times of Na^+ or O_2 transition are not significantly different between soft and hard water; times of Na^+ and O_2 transition are significantly different within soft and hard water (P=0.003). Refer to the caption of Fig. 2.1 for all other details.

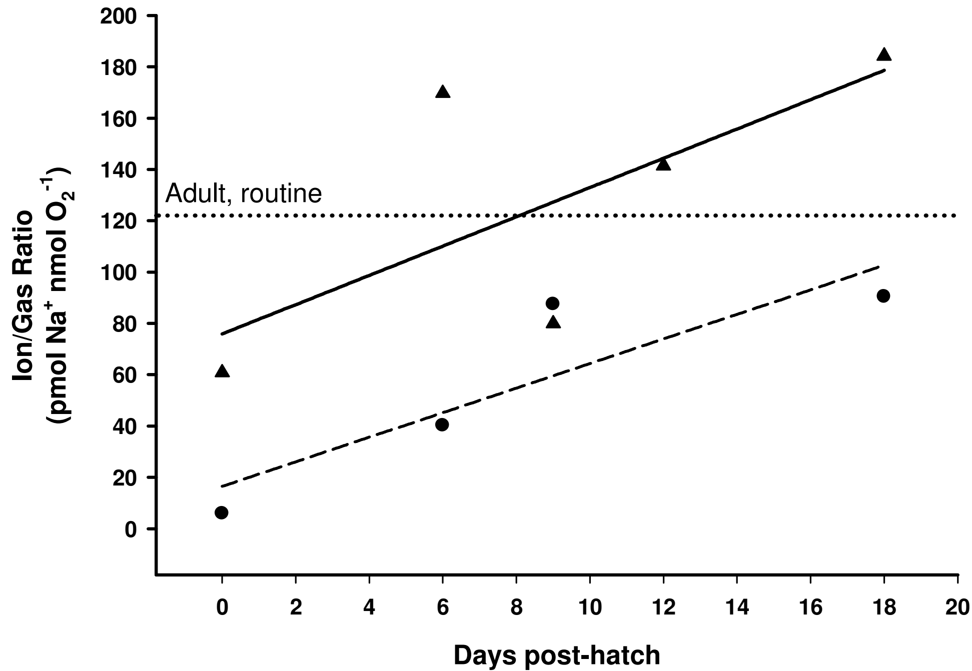

Figure 2.4. Changes in mean gill ion/gas ratio (IGR) in rainbow trout reared in soft water (●, dashed line) and hard water (▲, solid line) from 0 to 18 days post-hatch. The equation for hard water is y= 5.7x + 75.9 (r^2=0.32); the equation for soft water is y= 4.8x + 16.5 (r^2=0.68). Slopes are not statistically different from zero. Slopes are not statistically different from each other, but intercepts are significantly different (P=0.03). Routine IGR of adult rainbow trout reared in hard water is shown as a dotted line (from Gonzalez and McDonald, 1992).

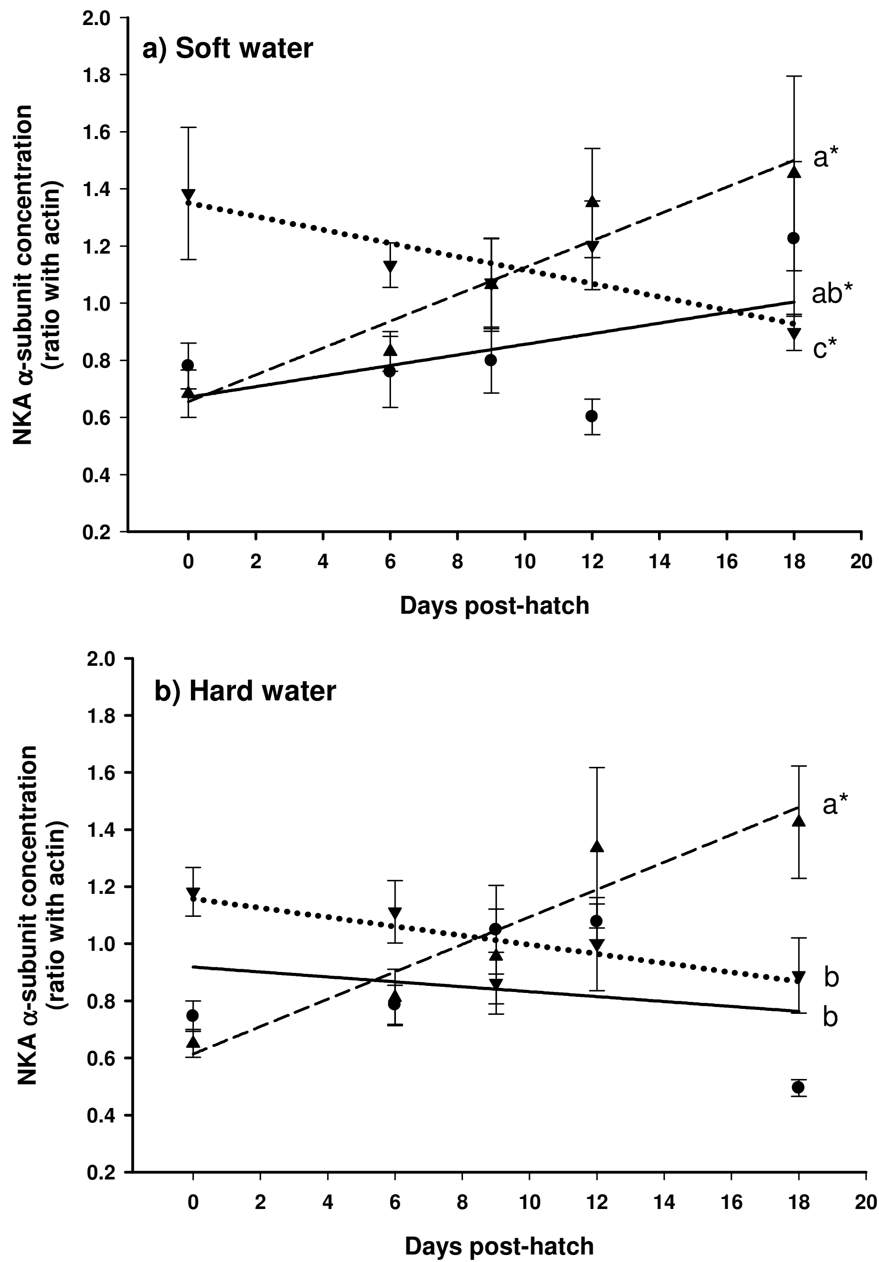

Figure 2.5. NKA α-subunit concentration at the gills (▲, dashed lines), body epithelium (●, solid lines), and yolk sac epithelium (▼, dotted lines) in rainbow trout reared in a) soft water and b) hard water from 0 to 18 dph. Refer to the caption of Fig. 2.1 for all other details.

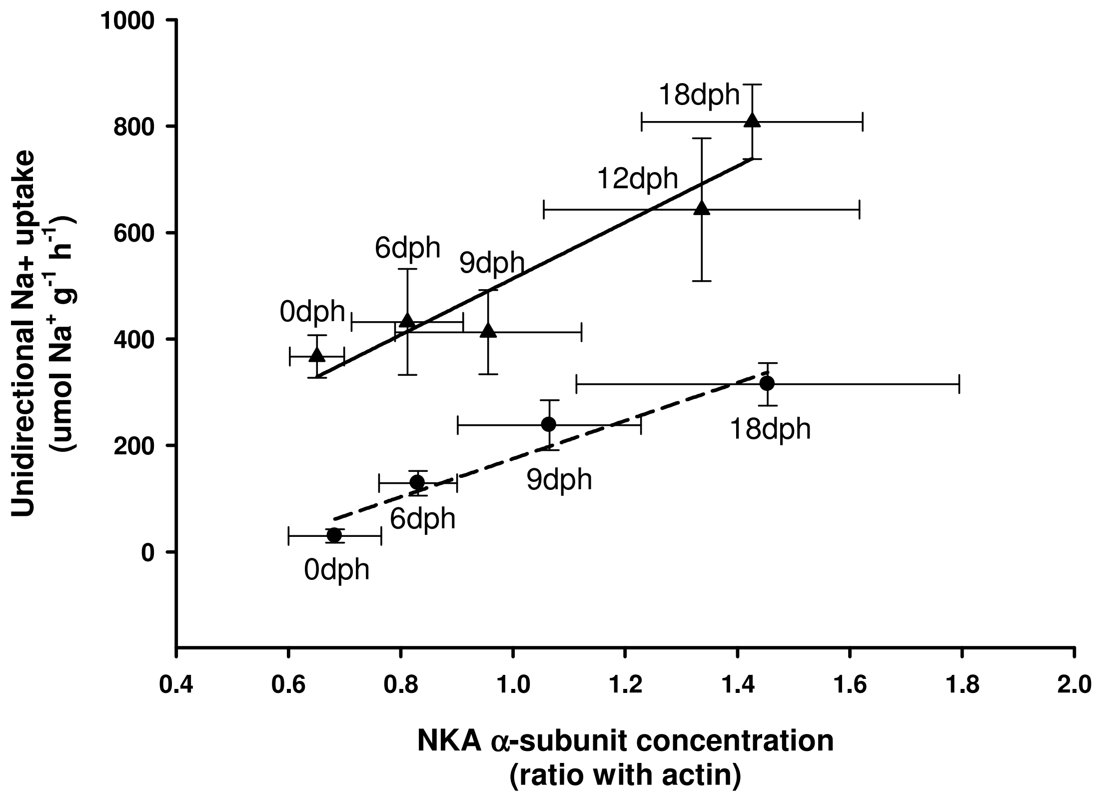

Figure 2.6. Relationship between gill Na^+ uptake and NKA concentration in rainbow trout reared in soft water (●, dashed line) and hard water (▲, solid line) from 0 to 18 dph. Linear regressions are based on means. The equation for hard water is y= 529x -15 (r^2=0.86); the equation for soft water is y= 358x + 183 (r^2=0.89). Slopes of both regressions are significantly different from zero, but are not significantly different from each other (P>0.05). Data points are mean ± SEM.

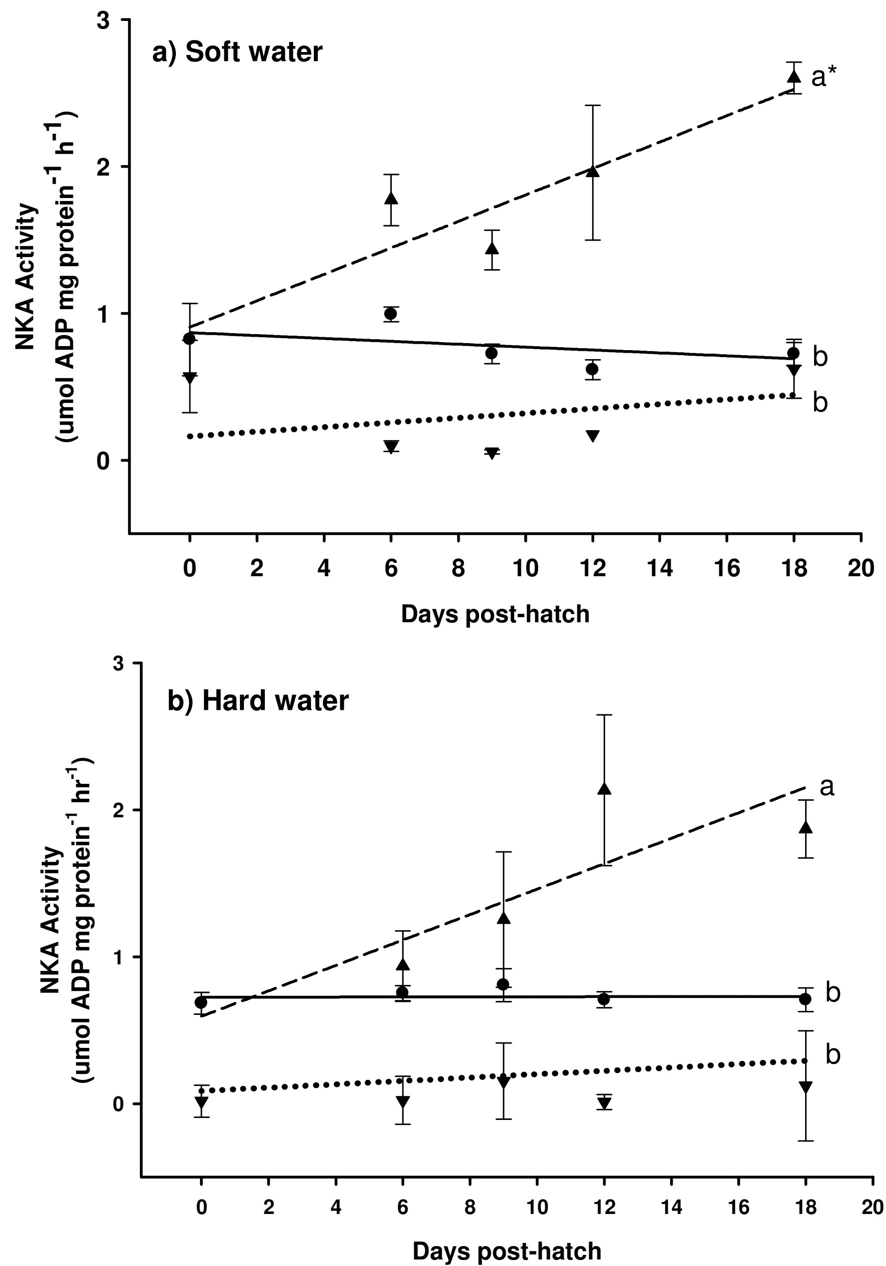

Figure 2.7. NKA activity of the gills (▲, dashed lines), body epithelium (●, solid lines), and yolk sac epithelium (▼, dotted lines) in rainbow trout reared in a) soft water and b) hard water from 0 to 18 days post-hatch. No data is available for the gills at 0 days post-hatch. Refer to the caption of Fig. 2.1 for all other details.

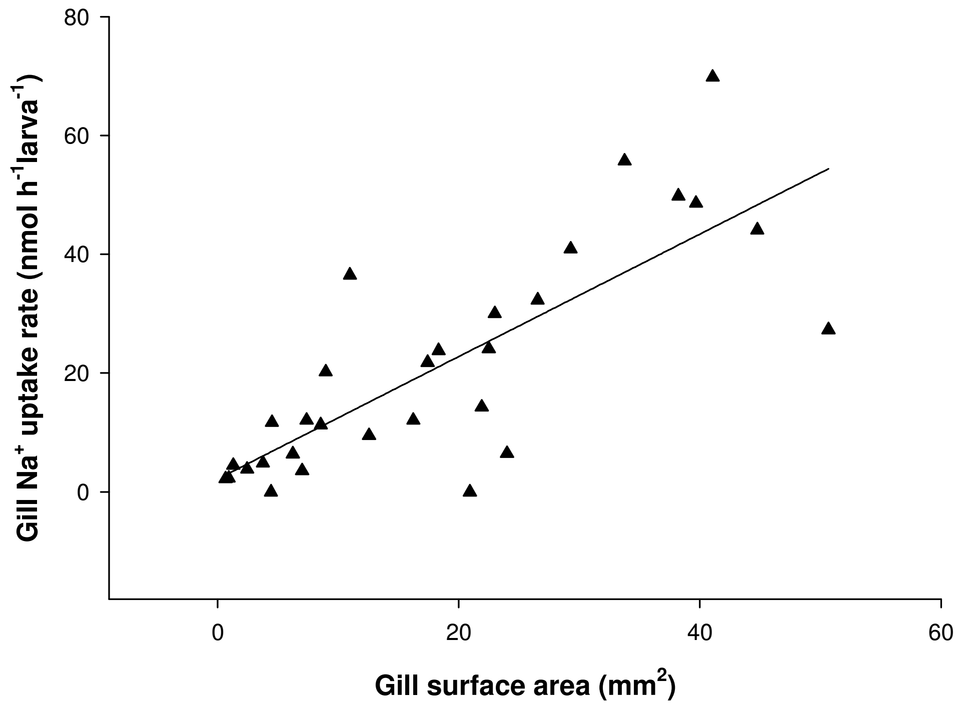

Figure 2.8. Relationship between gill Na^+ uptake rate and gill surface area in rainbow trout reared in hard water from 0 to 18 dph. The linear regression is y=1.03x + 2.18 (r^2=0.61). Gill surface area was obtained from the allometric relationship with wet body weight (without yolk) reported in Rombough, 1999.

Table 2.1. Wet body weights with and without yolk, and the percent of cutaneous surface area in the posterior chamber in rainbow trout larvae reared in soft and hard water from 0 to 18 days post-hatch. Asterisks (*) denote statistical differences between soft and hard water wet body weight with or without yolk (P<0.05). Data are mean ± SEM.

	SOFT WATER			HARD WATER			Cutaneous Surface Area	
Age (dph)	**N**	**Wet weight with yolk (g)**	**Wet weight without yolk (g)**	**N**	**Wet weight with yolk (g)**	**Wet weight without yolk (g)**	**N**	**% in posterior chamber**
0	19	0.081±0.001*	0.020±0.001*	17	0.062±0.001	0.012±0.002	3	86.1±1.7
6	19	0.089±0.002*	0.031±0.002	18	0.076±0.001	0.031±0.002	6	80.5±0.8
9	18	0.096±0.002*	0.040±0.002*	18	0.077±0.001	0.034±0.002	6	78.6±0.6
12	n/a	n/a	n/a	18	0.085±0.002	0.045±0.003	5	76.5±0.6
18	18	0.128±0.002*	0.081±0.003*	18	0.098±0.001	0.064±0.002	6	72.6±1.6

TABLE 2.2. Linear regression statistics of gill, skin and total Na^+ and O_2 uptake rates (as absolute values and as percent of total uptake), NKA concentration and activity as functions of age in rainbow trout larvae reared in soft and hard water from 0 to 18 days post-hatch. Refer to Appendix 2 for calculated mean total, gill and skin Na^+ and O_2 uptake rates at each age. Data are mean ± SEM.

		SOFT WATER				HARD WATER			
		N	**y-intercept**	**slope**	**adjusted r^2**	**N**	**y-intercept**	**slope**	**adjusted r^2**
Absolute uptake rate (umol h^{-1})	***Na^+***								
	Gill	25	-2.5±1.6	1.5±0.2	0.82	28	-2.3±3.5	2.6±0.3	0.72
	Skin	25	9.5±2.0	0.6±0.2	0.31	28	19.6±5.2	1.3±0.5	0.20
	Total	4	8.7±1.8	2.0±0.2	0.98	5	24.8±9.7	3.5±0.9	0.78
	O_2								
	Gill	25	51±16	11±1	0.68	29	51±19	12±2	0.60
	Skin	25	199±33	15±3	0.48	29	277±26	8±2	0.26
	Total	25	306±36	11±3	0.28	29	348±33	19±3	0.56
% of total uptake rate	***Na^+***								
	Gill	19	7.91±5.43	2.82±0.51	0.61	28	12.89±5.01	2.42±0.45	0.51
	Skin	19	84.62±8.97	-2.25±0.80	0.27	28	73.22±8.11	-1.35±0.73	0.08
	O_2								
	Gill	25	20.74±2.25	1.26±0.21	0.59	28	20.81±2.81	1.03±0.25	0.36
	Skin	25	79.26±2.25	-1.26±0.21	0.59	28	79.19±2.81	-1.03±0.25	0.36
NKA concen-tration (ratio with actin)	Gill	26	0.66±0.14	0.05±0.01	0.32	28	0.61±0.16	0.05±0.01	0.26
	Body epithelium	26	0.67±0.12	0.02±0.01	0.06	28	0.92±0.10	-0.01±0.01	0.00
	Yolk sac epithelium	26	1.35±0.12	-0.02±0.1	0.12	28	1.16±0.11	-0.02±0.01	0.06
NKA activity (umol ADP mg $protein^{-1}$)	Gill	18	0.91±0.25	0.09±0.2	0.53	18	0.60±0.54	0.09±0.1	0.13
	Body epithelium	23	0.87±0.10	-0.01±0.01	0.01	25	0.72±0.06	0.0±0.1	0.00
	Yolk sac epithelium	20	0.16±0.17	0.02±0.01	0.01	24	0.09±0.07	0.01±0.1	0.09

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Chapter Three: General Discussion

Results of this investigation are the first strong physiological evidence in support of the ionoregulatory hypothesis. The finding that Na^+ uptake transitioned to the gills in half the time that it took for O_2 uptake suggests that the gills are required for ionoregulation prior to gas exchange in rainbow trout. Furthermore, the finding that water hardness had no effect on the timing of the Na^+ transition to the gills, gill NKA protein concentration and gill NKA activity suggests that gill ionoregulatory development is non-plastic.

This chapter presents additional considerations regarding our findings on Na^+ uptake rate including evaluation of factors necessitating increased Na^+ uptake rate with age and the effect of water ion concentration on Na^+ fluxes. Several issues addressed in the previous chapter warrant a more in-depth discussion and will also be presented here, including the use of clove oil, the NKA activity assay, future studies, and the potential evolutionary significance of the ionoregulatory hypothesis.

Factors affecting Na^+ flux rates

Since gill NKA protein concentration and activity at each age and their respective rates of increase did not differ between soft and hard water groups (Fig. 2.5 and 2.7), I suspect that the greatly reduced Na^+ uptake rate in soft water (Fig. 2.1) was primarily due to low water Na^+ concentration, and to a lesser extent, differences in the development of ionoregulatory components at the gills, e.g. MRC proliferation and density. The effect of water Na^+ and calcium concentration ($[Na^+]$ and $[Ca^{2+}]$) on Na^+ fluxes in the gills of adult rainbow trout have been well-documented; their potential impacts on Na^+ flux in larval gills must also be evaluated. Note that most cited literature in the following discussion are of studies on adult fish. Due to

limited equivalent literature on larvae, I have assumed that the kinetics of Na^+ flux are similar in larvae. Future studies are recommended to determine the effect of [Na^+] and [Ca^{2+}] on gill Na^+ uptake and efflux rates in rainbow trout larvae.

Effect of [Na^+] on gill Na^+ uptake and efflux rate

In adult rainbow trout, gill Na^+ uptake rate increases curvilinearly in Michaelis-Menten fashion between 0 and 3 mM Na^+, and reaches a maximum rate (V_{max}) at 3 mM Na^+, presumably due to saturation of the Na^+ carrier (Kerstetter et al., 1970). Thus, gill Na^+ uptake rate was likely near maximum in hard water (2.3 mM Na^+), but was significantly lower in soft water (0.06 mM Na^+). The curvilinear relationship predicts a 3-fold difference in uptake rates, yet we observed only a 2-fold difference between soft and hard water groups. A combination of factors may contribute to this discrepancy, including 1) greater gill MRC proliferation in soft water, and 2) different concentration effects of Ca^{2+} on Na^+ uptake or efflux.

MRC proliferation in soft water

Based on studies in adult rainbow trout, Laurent (1985) proposed that soft water with less than 0.1 mM Na^+ may trigger gill MRC proliferation. Other studies have reported that 0.017 mM Na^+ (Perry and Laurent, 1989) and 0.055 mM Na^+ (Greco et al., 1996) elicited gill MRC proliferation. I thus speculate, albeit cautiously, that [Na^+] of 0.06 mM in our soft water may have been "low" enough to elevate gill MRC proliferation above levels in hard water. Samples are currently being analyzed to determine whether this is the case.

In a study contrasting the effects of [Na^+] on Na^+ uptake in deionised (0 mM Na^+) and freshwater (0.2 mM Na^+), Avella et al. (1987) reported no difference in K_m and an elevated V_{max} in deionised water. According to Michaelis-Menten enzyme kinetics, this represents no change in carrier efficiency, but an increase in the total number of Na^+ transporters, respectively. Indeed, I observed no difference between soft and hard water in gill NKA activity (a reflection

of capacity; Fig. 2.7). If total gill NKA content was indeed elevated in soft water (not measured), it may have been achieved by greater MRC proliferation at the gills. Immunohistochemistry studies will follow to verify differences in gill MRC proliferation (e.g. total count, morphology and density) between soft and hard water-reared larvae.

Effect of [Ca^{2+}] on gill Na^+ uptake and efflux rate

The rate of Na^+ efflux may have differed between soft and hard water due to differences in [Ca^{2+}] ([Ca^{2+}] = 0.03 and 0.9 mM, respectively). Ca^{2+} in water binds to membrane-bound junctional proteins, and is required for control of diffusive ion efflux by maintaining low ion permeability at paracellular tight junctions (Hunn, 1985). If ambient Ca^{2+} is low, Ca^{2+} is leached from tight junctions, increasing ion permeability at the gill epithelium, which in turn increases passive ion loss (Gonzalez et al., 1998). According to a study on adult brown trout, *Salmo trutta*, gill Na^+ efflux decreases as [Ca^{2+}] increases from 0 to 1.0 mM (McWilliams, 1982). The [Ca^{2+}] of soft water and hard water used in this study occur at the low and high ends of this range. Thus, it is likely that lower [Ca^{2+}] in soft water led to greater Na^+ efflux at the gills.

The concentration of Ca^{2+} in the water may also affect gill Na^+ uptake, but its effects are more equivocal. A study on goldfish, *Carassius auratus*, reported that removal of Ca^{2+} from the water with chelating agents was correlated with increased gill Na^+ uptake (Cuthbert and Maetz, 1972). This increase may have been accomplished by elevated gill MRC density to compensate for greater diffusive ion loss at leaky junctions (Çalta, 2000). Indeed, a study on brown trout larvae noted increased gill MRC density in water with 0.01 mM Ca^{2+} compared to 0.9 mM Ca^{2+} (identical [Na^+]; McWilliams, 1982). However, Na^+ uptake decreased slightly (though not statistically significant) with decreased [Ca^{2+}] (McWilliams, 1982). Due to differences between these published studies, we will not speculate on the effect of [Ca^{2+}] on Na^+ uptake in rainbow trout larvae. A future study is recommended to determine whether a relationship exists.

The timing of the skin-to-gill transitions for Na^+ uptake in larval rainbow trout was almost identical in soft and hard water (Fig. 2.3) despite the potential impact of [Na^+] and [Ca^{2+}] on Na^+ uptake and efflux, and despite the fact that the soft and hard water groups were reared and experimented one month apart. The concurrent transition due to coincidence is unlikely. The finding that ion environment had little impact on the ionoregulatory transition to the gills strongly suggests that the timing of ionoregulatory transition is non-plastic.

THE USE OF CLOVE OIL

Prior to data collection, it was first necessary to establish whether anaesthesia was required during the damming process and during uptake measurements. Previous studies involving the damming method on rainbow trout (Rombough, 1998) and chinook salmon larvae (Rombough and Ure, 1991) employed MS-222, but a study on Atlantic salmon did not require anaesthetics (Wells and Pinder, 1996). In my preliminary studies, rainbow trout larvae exhibited visible signs of stress (i.e. struggling) during damming and while positioned in the rubber dam in the absence of anaesthetic. Larvae were also less active than normal after a 1 h recovery period and none survived beyond 24 h. Therefore, it was imperative to employ an anaesthetic or calming agent to prevent self-injury from struggling in the dam. The two candidates for calming agents were MS-222 and clove oil. Distilled from the clove tree *Eugenia aromatic*, clove oil has recently gained popularity as a fish anaesthetic (Guénette et al., 2007). It has been recommended for use on adult rainbow trout as an alternative to MS-222 because it can induce anaesthesia more rapidly at lower concentrations and is more effective at reducing short-term handling stress (Keene et al., 1998). Juvenile and adult rainbow trout exposed to acute doses of 40-120mg/l clove oil did not differ from controls in subsequent swimming performance, suggesting that minimal physiological disturbance is caused by this concentration range (Anderson et al., 1997).

Furthermore, it has been shown that doses as low as 30mg/l effectively induces and maintains deep anaesthesia in adult rainbow trout for invasive surgical procedures (Prince and Powell, 2000). The mechanism of its neuroprotective effect is still under investigation, but the anaesthetic properties of eugenol, the active ingredient in clove oil, may be attributed to its agonistic effect on GABA, the main inhibitory neurotransmitter of the central nervous system (Aoshima and Hamamoto, 1999; Guénette et al., 2007).

Studies on MS-222 and clove oil were first conducted to determine minimum doses needed to 1) immobilize larvae within 5 minutes of exposure, required for positioning larvae into the rubber dam (stage 5 anaesthesia, total loss of equilibrium and swimming motion with weak opercular motion and total loss of reactivity; Keene et al., 1998), and 2) to achieve a calming effect for the duration that larvae are in the rubber dam. A larva was considered 'calmed' if it did not struggle in the dam and maintained regular opercular movement during the measurement period. Doses of 100 ppm MS-222 and 100 ppm clove oil immobilized larvae, while 10 ppm MS-222 and 10 ppm clove oil achieved the same desired calming effect.

The more suitable calming agent was then selected based on how significantly Na^+ and O_2 uptake rates were affected. Na^+ uptake rate was reduced by 49% in 10 ppm MS-222 and by 12% in 10 ppm clove oil (Appendix 3). Previous studies on chinook salmon and rainbow trout larvae report that MS-222 had no significant effect on O_2 uptake rate (Rombough and Ure, 1991; Rombough, 1998), but 10 ppm clove oil reduced O_2 uptake rate by approximately 24% (Appendix 4). Since the determination of Na^+ uptake rate was of greater interest due to its novelty, clove oil was deemed a more appropriate calming agent. Note that the 100 ppm immobilizing dose of clove oil had a negligible effect on Na^+ uptake rate following a 1 h recovery period (Appendix 3).

Prior to conducting similar uptake partitioning studies on rainbow trout or other larvae, we recommend a more thorough investigation of the effects of clove oil to validate the

assumption that the gills and skin are affected to the same degree in Na^+ or O_2 uptake rate throughout development. In addition, it was assumed that the impact of the selected dosages on Na^+ and O_2 uptake rate does not change with development; the dosages were thus kept constant for all age groups. However, I recommend that dose-response relationships be established for all larval stages of interest so that dosages can be adjusted if necessary to achieve a similar depth of calming at different ages.

INTERPRETATION OF NKA ACTIVITY DATA

Surprisingly, no correlations were found between gill NKA activity and gill Na^+ uptake rate as would be expected. Furthermore, no relationship between gill NKA activity and gill NKA protein concentration was detected. This was also the case in zebrafish, in which gill NKA activity increased despite no change in protein levels in the first 3 days of soft water exposure (Craig et al., 2007). The lack of clear relationships between Na^+ uptake or NKA protein levels and NKA activity may be due to complicating developmental factors or limitations of the techniques employed.

The potential relationships between NKA activity and expression or Na^+ uptake may have been obscured by limitations of the NKA enzyme activity assay. The method employed by McCormick (1993) is widely used to assess maximal NKA activity under saturating conditions *in-vitro*. Since conditions *in-vivo* are likely less than optimal, actual NKA activity is probably not at maximum. Two conditions of the assay clearly differ from those *in-vivo*. First, the temperature of the assay was set at 25°C to optimize the activities of all reagents, which was significantly higher than the rearing and experimental temperature of 10°C. Second, in the tissue homogenizing process of the assay, NKA was presumably removed from its native environment, potentially changing the dynamics of its functioning. These assay conditions likely led to

overestimation of true NKA activity. However, assuming that increased temperature and displacement of the enzyme from its native membrane had the same effect on NKA activity for all homogenates regardless of age and tissue type, the reported trends in NKA activity and comparisons of trends between tissue types should still be reflective of relationships *in-vivo*.

Several factors have a less predictable effect on measured NKA activity including the activity of intracellular stores of NKA and normalization of activity to total protein content. The tissue homogenization process may have exposed NKA located in intracellular stores that were not involved in ionoregulation *in-vivo* but may have contributed to measured activity. Estimations based on immunofluorescent localization in cultured chick skeletal muscle cells suggest that intracellular stores of NKA may account for 60% of total NKA of the cell (Wolitzky and Fambrough, 1986). Unfortunately, it is unknown how much intracellularly stored NKA contributes to total NKA activity measured in larval fish, and whether the quantity of these stores differs among tissues and changes with larval development. This problem may be compounded by the possibility that intracellular and membrane-bound NKA differ in kinetic properties (Omatsu-Kanbe and Kitasato, 1990). Normalizing activity to protein content may also be problematic, considering that protein content of the gills and the skin likely increases with development, and does so at different rates. Determination of whole gill and skin NKA activities may have been more informative, but was not feasible due to the difficulty in removing complete gills and skin in younger larvae. The effects on NKA activity associated in intracellular NKA stores and protein content are difficult to estimate, but may have obscured potential relationships between NKA activity and protein concentration or Na^+ uptake rate.

FUTURE DIRECTIONS

Further investigation on rainbow trout is required to verify that ionoregulation precedes gas exchange in shifting to the gills in other conditions. A study of particular interest is to determine the effect of hypoxia on the timing of O_2 and Na^+ transition to the gills. Studies on the crucian carp *C. carassius* revealed coincident lamellar proliferation and intralamellar cell mass retraction in adult gills as a strategy to increase O_2 uptake in hypoxic conditions (Sollid et al., 2003). Interestingly, exposure of larval Arctic char *S. alpinus* to hypoxia retarded lamellar growth initially (McDonald and McMahon, 1977). It has been proposed that in larvae, gill ventilation is energetically costly due to the high viscosity of water relative to body size. In normoxia, larvae may favour exchange across the skin due to lower costs, but in hypoxia may elevate gill involvement in gas exchange by increasing the force and regularity of gill ventilation (Rombough, 1988). Thus, in hypoxia, the O_2 transition to the gills may be accelerated solely due to elevated ventilation. O_2 and Na^+ uptake partitioning studies on larval rainbow trout reared in hypoxia can reveal 1) the effects of varied gill ventilation rate on the gill's contribution to total O_2 uptake, 2) whether limited O_2 supply will retard or promote lamellar development, and 3) whether the timing of the O_2 and Na^+ transitions will be affected.

The effects of temperature on the involvement of the gills in gas exchange and ionoregulation are also of great interest because the relative impacts are more difficult to predict. Gill O_2 uptake may differ with temperature due to impacts on metabolic rate and water gas solubility (reviewed in Finn, 2007). Gill Na^+ uptake may also change since salt solubility, thus water ion concentration, is dependent on temperature (reviewed in Finn, 2007). In the adult crucian carp *C. carassius* and goldfish *C. auratus*, metabolic rate decreased and lamellae became embedded in intralamellar cell masses as temperature decreased. The presumed subsequent reductions in O_2 uptake capacity and diffusive ion loss (Sollid et al., 2005) may affect the

relative involvement of the gills in ionoregulation and gas exchange. Since larvae are generally more stenothermic than juveniles and adults (reviewed in Finn, 2007), changes in temperature may have more pronounced effects on the gills' involvement in ion and gas uptake and the time at which these functions transition to the gills. Should the age of transition for Na^+ uptake occur at 15-16 dph once again, the proposition that the development of gill ionoregulatory components is non-plastic would be further supported. Unfortunately, the rate of larval development depends on temperature and is not directly proportional to accumulated thermal units (temperature x days) as was once believed (Jobling, 1995). Hence, a system of expressing equivalent developmental age must first be established to enable comparisons between larvae reared at different temperatures.

The relative timing of skin-to-gill transitions may also be related to variations in larval sizes of different species. Skin gas and ion uptake in smaller larvae such as zebrafish would presumably suffice until relatively later in development, based on the reasoning that their surface area to tissue mass ratio becomes limiting at a later stage. By contrast, the surface area to tissue mass ratio in larger larvae such as chinook salmon would become limiting earlier in development. These contrasts may be manifested as advanced or delayed gas and ion uptake skin-to-gill transitions and will reveal the effect of body size on gill functional development.

It would also be interesting to determine whether the rate of gill functional development and the timing of transitions differ between freshwater and marine species, considering how they differ in ionoregulatory strategies. In the limited morphological data that is available on marine larvae, MRCs also appear earlier than lamellae (sea bass, *Dicentrarchus labrax;* Varsamos et al., 2002). If morphology reflects physiological function in marine larvae as was the case in rainbow trout, it is possible that ionoregulation also transitions to the gills prior to gas exchange in marine species.

Since ionoregulation and acid-base regulation are tightly linked in adult fish gills, the involvement of larval gills in acid-base regulation should also be explored. Studies have shown that the two functions are linked by Na^+/H^+ and Cl^-/HCO_3^- exchangers at the gills, so that changes in the activities of these exchangers affects both ion uptake and acid-base excretion in freshwater fish (McDonald et al., 1991). As is the case for gas exchange and ionoregulation, pH regulation of body fluids is believed to occur across the skin during early gill development (Lin et al., 2006). To our knowledge, the relative roles of the skin and gills in acid-base regulation and the shift of the function to the gills have not been studied.

EVOLUTIONARY SIGNIFICANCE

I close this discussion with a consideration of the potential implication of our findings on current hypotheses regarding vertebrate gill evolution. It is generally assumed that vertebrate gills evolved to facilitate O_2 uptake (Gans, 1989). The modern teleost gills are believed to be homologous to the branchial basket of filter-feeding protochordates (reviewed by Coolidge et al., 2007). Protochordates led a sessile lifestyle and relied on suspension feeding facilitated by branchial baskets; cutaneous respiration alone was enough to meet metabolic demands (reviewed by Coolidge et al., 2007). With the evolution of jaws and increased feeding efficiency, the branchial basket was freed from its function in feeding, and organisms evolved more active lifestyles (Mallat, 1996). The new predatory lifestyles required more efficient O_2 uptake that exceeded the capacity of cutaneous exchange. This is commonly believed to be the selective pressure driving evolution from the branchial basket to modern gills (reviewed by Coolidge et al., 2007).

It has been suggested that the forces that drove gill evolution may be comparable to those experienced during ontogenic development, based on the commonality that during both

evolution and ontogeny, organisms become larger and more active and thus exchange at the skin alone is increasingly insufficient (Rombough, 2004). Thus it is possible that ionoregulation may be a greater driving force than gas exchange not only during early gill development (as our data on rainbow trout suggests), but also in the course of gill evolution (Rombough 2004). What little is known about the gill's involvement in ionoregulation and gas exchange in extant forms of ancestral vertebrates appears to be consistent with this hypothesis, assuming that physiological characteristics of these gill functions were conserved in evolution.

The protovertebrate amphioxus, believed to be a vertebrate ancestor, possess primitive gill bars primarily used for filter feeding. Its gills are believed to play a small role in gas exchange due to their low diffusing capacity (Schmitz et al., 2000). The investigators of this study further argued that these gills were likely not a major respiratory organ. The degree to which the amphioxus gill bars are involved in ionoregulation is unknown, but such a role is likely limited since the circulatory system of these animals appears to be poorly designed for ion and gas transport (Schmitz et al., 2000). Gills at this stage of evolution would have still served a feeding function and would likely not have developed mechanisms for gas exchange and ionoregulation.

The gill functions in primitive fish and basal teleosts are poorly understood yet this knowledge is critical to understanding the evolution of gill ionoregulatory and gas exchange mechanism. What is known is largely speculative. Hagfish, considered the most primitive of vertebrates, are considered osmoconformers, yet some studies have detected ionic gradients between their body fluids and their marine environment, suggesting some degree of ionoregulation (reviewed in Evans, 1993). The ions, Na^+ and Cl^- in particular, seem to be higher and lower in concentration than that of seawater, respectively, and these ions are proposed to be exchanged primarily at the gills (reviewed in Evans, 1993). Indeed, MRCs have been found in hagfish gills, and the abundance of NKA, Na^+/H^+ and Cl^-/HCO_3^- exchangers in branchial MRCs

has been associated with acid-base regulation (Evans, 1984; McDonald et al., 1991). These ion exchangers are proposed to be an exaptation of ionoregulatory strategies that would later become selectively advantageous as fish invaded freshwater (Evans, 1984). Since the burrowing and feeding habits of hagfish likely implicate an anoxic lifestyle, the role of hagfish gills in acid-base regulation (and associated ionoregulatory activities) may be crucial to survival (Evans, 1993). Indeed, recent studies indicate that hagfish have an unparalleled ability for pH regulation of the blood during exposure to hypercarbia (Baker and Brauner, unpublished). By contrast, hagfish gills were estimated to account for less than 20% of total O_2 uptake (Steffensen et al., 1984). If extant hagfish gills retained features of its proposed common ancestor with teleosts, these findings suggest that gills first evolved for acid-base/ionoregulation.

The gills of other primitive fishes such as lampreys and basal teleosts such as sturgeon are also proposed to be the dominant site of ionoregulation (reviewed in Wright, 2007). Unfortunately, the relative involvement of the gills in acid-base/ionoregulation and gas exchange is not well-studied and awaits future investigation.

Inadequate understanding of the phylogenetic ancestry of teleosts and the evolution of gill functions currently limits our evaluation of the hypotheses on why gills evolved. Future investigations should be aimed at determining the functions of the gills in primitive fish species and more basal teleosts. Nonetheless, the hypothesis that O_2 uptake was the driving force for gill evolution now seems much too narrow given the multifunctional role of the gills (Evans et al., 2005). Consideration of other potential driving forces such as ionoregulation and acid-base balance would add great value to ideas of the evolution of the modern teleost gills.

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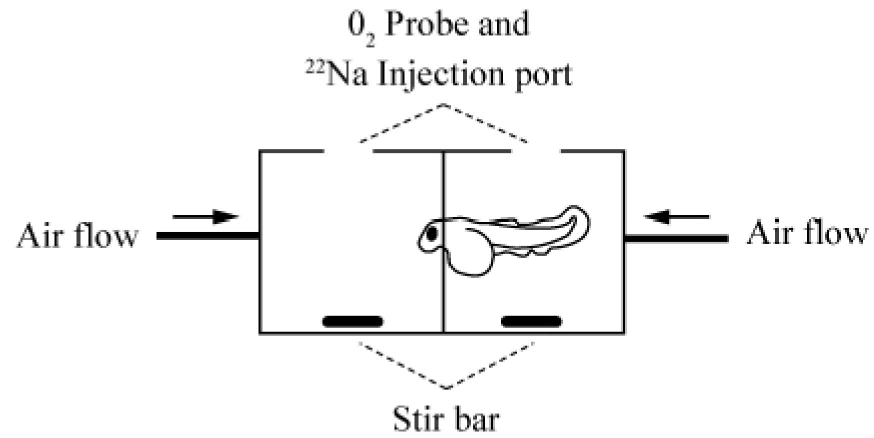
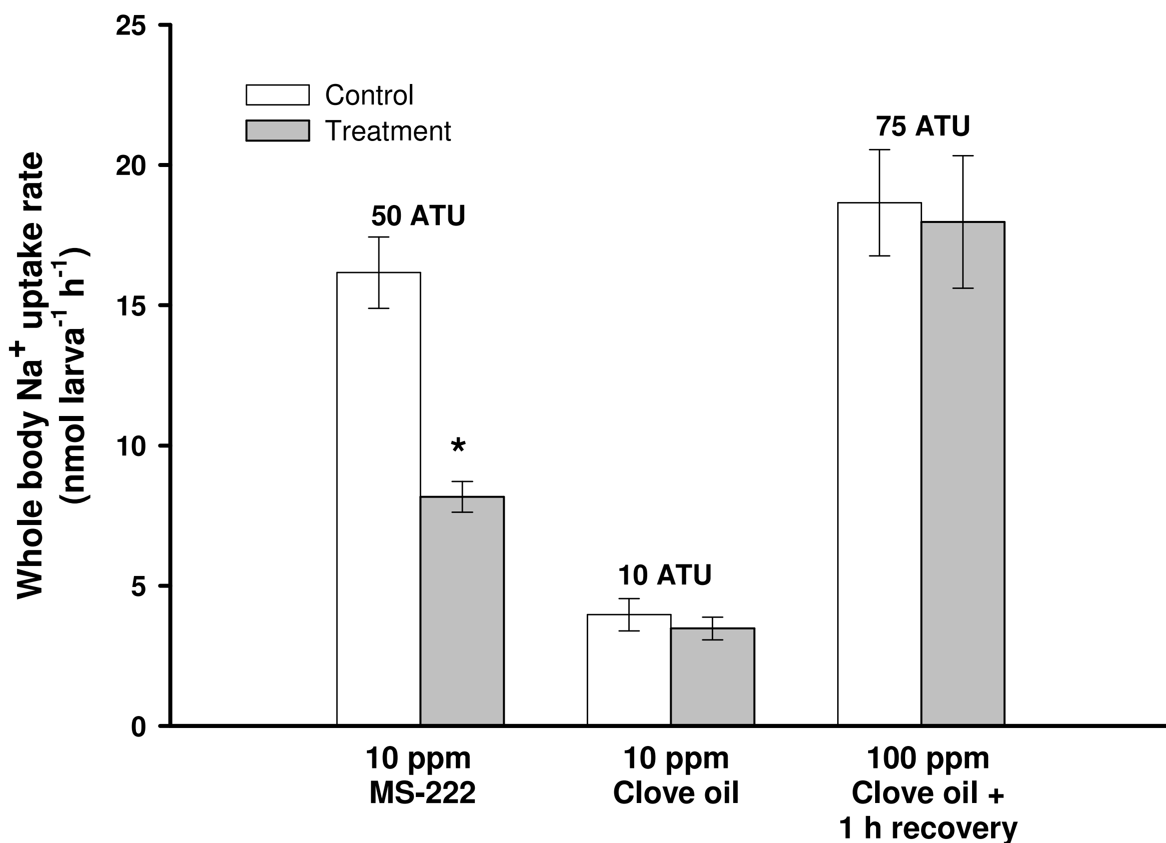
APPENDIX 1. Respirometer design**.** Na^+ and O_2 uptake were partitioned by a rubber dental dam between the anterior chamber (containing the head and gills) and posterior chamber (containing the rest of the body). Fish were positioned so that the rubber dam was immediately posterior to the pectoral fins. The total inner volume of the respirometer was 10 ml.

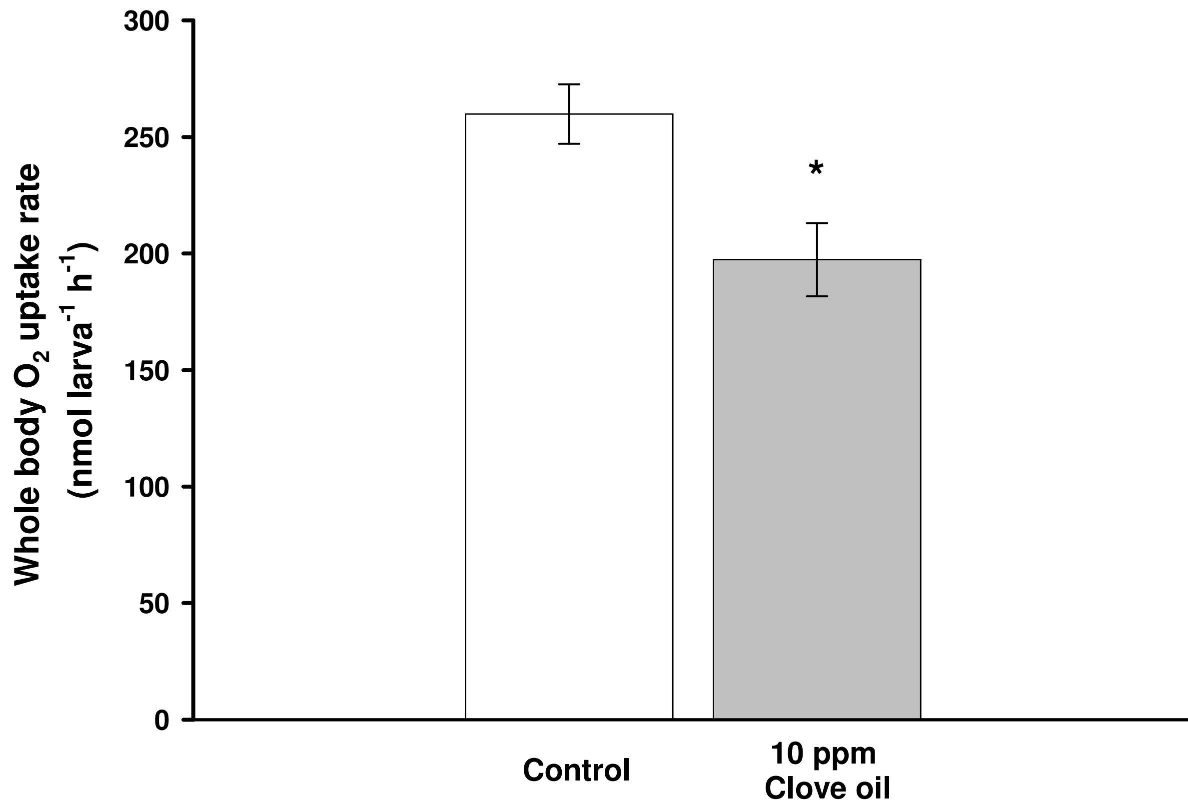


APPENDIX 2. Measured anterior and posterior chamber, calculated total, gill and skin Na^+ and O_2 uptake rates (nmol h^{-1}). Plus (+) denote significant differences between soft and hard water uptake rates within age and tissue type. Asterisks (*) denote significant differences between gill and skin uptake rates at that age within soft or hard water groups. N are indicated in brackets. Data are mean ± SEM.

		Age (dph)	Measured Anterior Chamber Uptake	Measured Posterior Chamber Uptake	Calculated Total Uptake	Calculated Gill Uptake	Calculated Skin Uptake
SOFT WATER	Na^+	0	1.9±0.2 (5)	6.3±0.5 (6)	9.3	0.6±0.2 (5)+*	7.3±0.5 (6)
		6	7.1±1.3 (5)	13.5±2.2 (5)	22.1	4.4±0.7 (5)+*	16.7±2.8 (5)
		9	11.4±1.3 (6)	12.7±1.8 (5)	24.4	8.4±1.2 (6)	15.3±3.1 (5)+
		18	33.0±3.2 (5)	13.9±2.0 (6)	46.5	27.3±3.1 (5)+	19.1±2.8 (6)+
	O_2	0	112±19 (7)	215±29 (7)	327±47 (7)	72.5±17 (7)*	250±34 (7)
		6	159±12 (6)	211±26 (6)	370±21 (6)+	103±9(6)*	262±32 (6)+
		9	158±36 (5)	182±36 (5)	340±70 (5)	110±31 (5)	231±46 (5)+
		18	277±20 (7)	243±25 (7)	520±40 (7)+	263±18 (7)*	507±34 (7)
HARD WATER	Na^+	0	3.9±0.6 (5)	12.6±2.1 (5)	28	3.5±0.5 (5)*	14.9±3.0 (5)
		6	17.1±2.6 (5)	24.7±4.4 (6)	45.9	12.3±2.7 (5)*	30.6±5.5 (6)
		9	19.6±3.5 (6)	20.7±2.6 (5)	41	13.8±3.1 (6)*	26.3±3.3 (5)
		12	43.4±5.4 (6)	36.2±6.4 (6)	80.6	28.0±4.5(6)	47.3±8.4 (6)
		18	58.9±5.3 (6)	26.5±3.8 (6)	86.9	50.1±5.1 (6)	36.4±5.3 (6)
	O_2	0	119±12 (5)	204±14 (5)	324±23 (5)	82.1±14.4 (5)*	237±16 (5)
		6	187±33 (5)	331±30 (5)	518±59 (5)	64.5±23.3 (5)*	411±37 (5)
		9	230±20 (7)	257±11 (7)	487±28 (7)	158±19 (7)*	327±14 (7)
		12	303±12 (6)	263±19 (6)	566±29 (6)	223±9 (6)*	344±25 (6)
		18	375±22 (6)	310±21 (6)	684±41 (6)	256±24 (6)*	427±28 (6)



APPENDIX 3. The effect of 10 ppm MS-222 and 10 ppm clove oil on whole body Na^+ uptake rate. The effect of an immobilizing dose of 100 ppm clove oil on whole body Na^+ uptake rate after a 1h recovery is also shown. N= 6 to 7 for all groups. Numbers above bars indicate age of larvae in accumulated thermal units post-hatch (ATU = mean daily temperature x days). Asterisks (*) denote a significant difference between control and treatment groups (Student's t-test, $P<0.05$). Bars show mean ± SEM.



APPENDIX 4. The effect of 10 ppm clove oil on whole body O_2 uptake rate. N= 7 (control group) and 8 (clove oil treatment group). Larvae were 50 ATU post-hatch (accumulated thermal units = mean daily temperature x days). The asterisk (*) denotes a significant difference between the control and clove oil treatment groups (Student's t-test, $P < 0.05$). Bars show mean ± SEM.