Strategies of inanga (*Galaxias maculatus*) for surviving the environmental stressors of hypoxia and salinity change

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology

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ANOVA, analysis of variance

ASR, aquatic surface respiration

ATP, adenosine triphosphate

βNHE, beta sodium-hydrogen exchanger

BSA, bovine serum albumin

EST, expressed sequence tag

Hb, haemoglobin

Hct, haematocrit

ILCM, interlamellar cellular mass

LSD, least significant difference

MCHC, mean cellular haemoglobin content

MO_2, metabolic rate

MS222, 3-amino benzoic acid ethylester

NHE, Na^+/H^+ exchanger or antiporter

NKA, Na^+, K^+- ATPase

NKCC, $Na^+/K^+/Cl^-$ cotransporter

PCR, polymerase chain reaction

PO_2, partial pressure of oxygen

$PO_{2\ crit}$, critical O_2 tension of whole-animal O_2 consumption rate

$PO_{2\ out}$, external PO_2 that causes loss of equilibrium

RT-qPCR, reverse transcription real-time polymerase chain reaction

SD, standard deviation of the mean

SEM, standard error of the mean

VO_2, whole-animal oxygen consumption rate

‰, parts per thousand

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Abstract

Salinity and oxygen availability have long been recognised as important factors influencing animal physiology and therefore species distribution. The maintenance of appropriate cellular ion levels is critical for many essential physiological processes, but at the same time is energetically expensive. Since hypoxia is likely to impose aerobic limitations for ATP generation, the maintenance of salt and water homeostasis could be at risk during hypoxia. The amphidromous inanga (*Galaxias maculatus*) is well known for its salinity tolerance and its life cycle that involves several salinity-related migrations. During these migrations inanga also frequently encounters hypoxic waters, and therefore must maintain energy homeostasis when aerobic metabolism may be compromised. The present study has investigated behavioural, physiological, biochemical and molecular mechanisms by which inanga tolerate changes in salinity and hypoxia.

After 14 days of acclimation to salinities ranging from freshwater to 43‰, inanga showed physiological acclimation. This was evident by no changes in metabolic rates or energy expenditures through this salinity range. Energy balance seemed to be tightly and efficiently controlled by changes in the proportion of protein and lipids used as energy substrate. No mortalities and only minor changes in plasma osmolality also indicated salinity acclimation. The remarkable osmoregulatory capacity of inanga was also evidenced after a seawater challenge. The osmotic balance of inanga was only disrupted during the first 24 hours after the challenge, evidenced by an increase in plasma osmolality and plasma Na^+, and a decrease in muscle water content.

These physiological changes were correlated with changes at the molecular level. Different isoforms of the catalytic subunit of the Na^+,K^+-ATPase (NKA) were isolated, partially sequenced and identified in inanga. Phylogenetic analysis grouped inanga isoforms (α-1a, α-1b, α-1c) with their respective homologues from salmonids. Patterns of mRNA expression were also similar to salmonids, with α-1a being down-regulated and α-1b being up-regulated following seawater challenge. Previous to this study, NKA isoform switching was reported to occur only in salmonids and cichlids. The presence of NKA subunits that change with environmnetal salinity in inanga

indicates that this isoform switching phenomenon is much more widespread among teleost lineages than previously thought.

Aiming to elucidate the hypoxia tolerance of inanga, oxygen consumption rate as a function of decreasing external PO_2 was evaluated. At no point did inanga regulate oxygen consumption, suggesting that this species is an oxyconformer. This is the first robust demonstration of the existence of oxyconforming in fish. Evaluation of the scaling relationship between oxygen consumption and fish size in normoxia, showed that the exponent of this relationship fell within the range previously reported for fish. However, in hypoxic conditions the scaling relationship was less clear suggesting different size-related mechanisms for tolerating hypoxia. Analysis of the aerobic and anaerobic metabolism of small and large fish, showed that smaller inanga were able to sustain aerobic metabolism for longer than larger inanga, which instead relied on anaerobic metabolism for extending their survival. This knowledge is likely to be of value for the conservation of this iconic fish species, by incorporating these size-related differences in hypoxia tolerance in streams management.

In light of the unusual oxyconforming response of inanga, a study examining the behavioural responses of this species to declining dissolved oxygen was performed. Inanga did not display a behaviour that might reduce energy expenditure during oxygen limitation; instead swimming activity and speed were elevated relative to normoxia. As hypoxia deepened inanga leaped out of the water, emersing themselves on a floating platform. Once emersed, fish exhibited an enhanced oxygen consumption rate compared to fish that remained in hypoxic water. Although this emersion behaviour was hypothesised to be of physiological advantage, both aquatic hypoxia and emersion resulted in similar physiological and biochemical consequences in inanga. While in hypoxic water oxygen availability seemed to be the limiting factor, in air failure of the circulatory system was hypothesised to be the cause of a similar metabolic signature to that found in aquatic hypoxia.

Overall, inanga seemed to be not particularly well adapted to tolerate aquatic hypoxia. In light of the increasing likelihood of anthropogenic-induced hypoxia in inanga habitats, this is likely to have negative consequences for the future of inanga populations in the wild. Although this study provides the mechanisms behind the

exceptional salinity tolerance of inanga, its suceptibility to hypoxia is likely to impose further constraints for the osmoregulatory processes that guarantee inanga survival during life cycle migrations. The results of the present study are relevant for understanding and managing the fishery of this economically- and culturally-important fish species.

Chapter I

General introduction

1.1 Aquatic habitats

Life in water is not easy, even for a fish. Aquatic environments include ponds, lakes, rivers, streams, springs, estuaries, bays, the open sea, salt marshes and wetlands. These habitats have distinctly different bathymetric and geographic characteristics, which lead to vastly different physical characteristics of the water column (Cole, 1979). For example, parameters such as temperature, light, salinity and dissolved oxygen are highly variable in aquatic habitats (Kinne, 1971). Most aquatic life forms require specific optimal levels of these factors in order to ensure survival. As a consequence spatial and temporal variability in these parameters will challenge the survival of aquatic biota.

Aquatic habitats can also vary temporally. In summer, for example, high light levels lead to increases in the water temperature and stimulate photosynthesis. These two factors result in increased food availability and an increased metabolic rate, and can stimulate growth of aquatic organisms (increasing total biomass and thereby total oxygen consumption and organic wastes) (Cole, 1979). Conversely, the solubility of oxygen in water decreases with temperature, and oxygen consumption by the enlarged inhabitant population may deplete the amount of dissolved oxygen available. In winter, however, the opposite cascade occurs (Cole, 1979). Although water oxygen availability usually increases during winter, hypoxic conditions may develop when the freezing of lakes blocks oxygen diffusion from air to water. This, coupled with oxygen consumption of the indwelling fauna, can deplete the oxygen until hypoxic levels are reached, conditions that might persist for several months over winter (Nilsson and Renshaw, 2004). Oxygen levels in water further oscillate between day and night as a result of photosynthesis and respiration by aquatic biota. Salinity can also change temporally. For example the inflow of freshwater coming from rivers after heavy rains can significantly decrease the salinity in estuaries and coastal areas (Wolanski, 2007). Conversely, in summer, the evaporation of water can significantly increase the salinity of water bodies. At high latitudes, where the tides have higher amplitude, the salinity in the intertidal zone and in certain areas of estuaries can oscillate between nearly 100% freshwater and 100% seawater up to twice a day.

Living in a habitat such as water therefore offers a considerable challenge to aquatic organisms, and these animals must have developed adaptations in order to survive. Since maintaining homeostasis in a changing environment requires a considerable investment in energy and resources, even if survival is achieved it may incur at a cost. Consequently osmoregulation may also impact other vital functions such as escape from predators, food intake, reproduction, and growth (Petersen and Pihl, 1995; Boeuf and Payan, 2001; Urbina et al., 2010). This is particularly the case for fish that may migrate between waters that vary in their base physical properties.

1.2 Salinity

Salinity has long been recognised as an ecological master factor (Kinne, 1966), and is a major factor influencing the physiology of aquatic animals. These animals are bathed in a medium that may differ significantly in its osmotic concentration from the internal tissues. Unregulated movement of ions and water down concentration gradients across permeable body surfaces could therefore result in loss of salt and water balance. As the maintenance of appropriate cellular ion levels is critical for many essential physiological processes, such as cellular homeostasis (volume, energy levels, and acid-base balance; Sherwood et al., 2005), cardiovascular function (Olson and Hoagland, 2008), acid-base balance (Wright and Wood, 2012), nutrient availability (Frick and Wright, 2002) and metabolism (Bedford, 1981), ion imbalance could impair several vital functions, and may even result in death. Salinity can vary from less than 0.7‰ in freshwater rivers and lakes, to 33‰ in seawater (SW), or even higher in hypersaline waters from salt lakes, tidal pools or salt marshes (>50‰; Gonzalez, 2012). In estuaries salinity oscillates between nearly 100% FW during low tide to 100% SW in high tide as result of tidal cycles.

Depending on their salinity tolerance, teleost fish can be classified as stenohaline or euryhaline. Most fish are stenohaline (Nordlie, 2009), tolerating only small changes in environmental salinity and therefore inhabiting waters of a relatively constant salinity. Conversely, there are fewer euryhaline fish species, and they have the ability to tolerate and live in a wide range of external salinities. Euryhalinity is a remarkable

adaptation, and the maintenance of ionic homeostasis under changing salinities requires the proper functioning of several mechanisms including ion excretion/absorption, maintenance of water balance, and control over drinking and ingestion rates (Edwards and Marshall, 2013). Euryhalinity and the associated ability to acclimate to a wide range of external salinities is not only a complex phenomenon, but also has several associated costs. Most fish that perform salinity related migrations gain their osmoregulatory ability only at certain stages of their life cycle. For example, some fish species (e.g. some salmonids) migrate from the sea to freshwater streams to reproduce. After hatching juveniles migrate to the sea when they are physiologically ready to do so, a process called smoltification (Wedemeyer et al., 1980). This pattern of migration is called anadromy. Fish such as eels, display the opposite migratory pattern, termed catadromy (McDowall, 1997).

Both anadromy and catadromy are considered forms of diadromy (McDowall, 1997). Yet, there is another less common specialised form of diadromy, known as amphidromy. Amphidromous fish grow and reach maturity in freshwater and then migrate to estuaries to reproduce. The larvae then hatch in brackish water and subsequently migrate to the sea, where they spend several months feeding and growing. Juveniles then start their upstream migration back towards freshwater. Amphidromous species are not only exceptionally tolerant to changes in salinity, but they are likely to retain the ability to acclimate to a wide range of external salinities through their entire life (McDowall, 1997).

In freshwater, fish lose ions down a concentration gradient to their more dilute surroundings, while in ion-rich marine waters they are faced with the opposite problem- salt loading (Marshall and Grosell, 2005). Any osmotic impairment at an extracellular level would have an effect at intracellular level, if regulatory mechanisms were not in place. For example, if extracellular osmolality increased, cell volume would drop as result of passive water movement into the extracellular space (Sherwood et al., 2005). Conversely, if extracellular osmolality decreased, water would passively diffuse into the cell, increasing cell volume, and eventually causing cellular lysis (Sherwood et al., 2005). The challenge of osmoregulation is even greater in diadromous fish that experience salinity fluctuations due to migratory behaviours, and in euryhaline species that inhabit estuarine waters with salinities that may vary

considerably over the course of hours as result of the tidal cycle. Such osmoregulatory challenges would not be tolerated by stenohaline fish species. Euryhaline fish, however, manage the diametrically-opposed physiological challenges of ion loss and ion gain, surviving and acclimating to different external salinities.

Several mechanisms must be tightly regulated in order to successfully achieve ion homeostasis in different salinities. These include drinking and ingestion rates, and ion excretion and absorption (Wilson et al., 1996; Pyle et al., 2003; Gonzalez, 2012). In seawater, fish increase their drinking rates in order to replace the water lost by passive diffusion in this hyperosmotic medium (Wilson et al., 1996; Gonzalez, 2012). In seawater, this ingested water is of course of high osmolality, and therefore a mechanism to remove the salts in order to uptake the water must be enacted. Salts (primarily Ca^{2+} and Mg^{2+}) are precipitated in the gut by the secretion of HCO_3^- into the gut lumen. This raises the pH of the ingested fluid and most of the Ca^{2+} and Mg^{2+} ions precipitate, reducing the osmotic pressure (Wilson et al., 1996; 2002). Those precipitated salts are then eliminated in the faeces. Other ions such as Na^+, K^+ and Cl^- are excreted by the action of several transporters located mainly in the gills, driven by concentration gradients established by Na^+, K^+- ATPase (NKA) (see below). Once most of the salts have been removed from this ingested water, water will simply diffuse into fish cells driven by concentration gradients. Conversely in freshwater, fish drinking rates decrease and ions are retained aiming to compensate for the passive loss of ions and gain of water associated with this hypo-osmotic environment. Active and continuous ion uptake by several transporters in the gills also helps the fish to maintain homeostasis in freshwater. The kidney also plays a crucial role in osmoregulation, controlling the amount of salts and water excreted in the urine. In freshwater, for example, fish excrete large amounts of dilute urine, retaining ions. Conversely, in seawater, fish excrete small amounts of concentrated urine, retaining water (Masoni and Payan, 1974).

Osmotic and ionic balance is ultimately achieved by the action of a wide range of membrane transporters with properties that facilitate the selective movement of ions across the epithelia. The main site for ion regulation in fish is the gills, where the action of the ion transporters is greatly facilitated by the morphological characteristics of the gills. Gills, by virtue of a small diffusive distance, a counter-current blood-

water exchange system, and a large surface area, are the main osmoregulatory tissue in fish. Therefore gills are also the tissue where salinity-related changes are most evident (Evans et al., 2005). These transporters include ion-selective channels, exchangers and pumps, all of which work in concert to achieve ionic balance (Hirose et al., 2003). Of these the key transporter is NKA, which is responsible, either directly or indirectly, for regulating cellular water and ion balance. This basolateral transporter uses the cellular power of ATP to translocate three Na^+ from the cell to the extracellular space, in exchange for two K^+. The action of NKA is crucial for both ion excretion and uptake in teleost fish.

Seawater teleosts excrete the excess of Na^+ and Cl^- by specialised mitochondria-rich cells (MR cells) located in the gills, involving the linked transport of $Na^+/K^+/Cl^-$ via the so-called NKCC transporter and the action of the NKA. NKA generates a strong electrochemical gradient (i.e. low intracellular sodium and low intracellular positive charge) (Evans et al., 1999). This indirectly drives Cl^- uptake into the cell from the external medium by the action of NKCC. The accumulation of Cl^- in the intracellular space generates a gradient for the passage of Cl^- from the animal to the environment and Cl^- passes passively through the apical membrane via Cl^- channels (Cystic fibrosis transmembrane conductance regulator, CFTR) (Evans et al., 1999). The Na^+ excreted by NKA accumulates in the intercellular space. Here it builds up to levels whereby a gradient for the passage of the ion from the animal to the environment is created. Excretion is achieved when Na^+ moves paracellularly from the animal into the water across “leaky” tight junctions (Karnaky, 1986).

Conversely, freshwater teleosts need to absorb ions from the dilute medium. In the mitochondria-rich or pavement cells (depending on the fish species) located in the gills, protons are excreted by the V-H^+-ATPase, which drives the cellular uptake of Na^+ via sodium channels (ENaC) in the apical surface (Evans et al., 2005). Alternatively, another mechanism involving the action of an apical Na^+/H^+ exchanger (NHE) has been proposed (Wright and Wood, 1985). Extracellular Cl^- then enters the cell through a Cl^-/HCO^-_3 exchanger (Anion exchanger 1, AE1) maintaining a neutral electrochemical gradient. In the freshwater model, the net effect of these transporters is the uptake of NaCl.

1.3 Hypoxia

Oxygen availability is a key environmental factor. Oxygen is needed for ATP generation, and as such it sustains all physiological processes. However, dissolved oxygen can fluctuate in the natural environment. Hypoxia can be defined as when oxygen levels fall below 70% of saturation (~13.4 kPa) (Graham and Wegner, 2010). Different levels of hypoxia can also be identified. For example, a water PO_2 (partial oxygen pressure) between 70 and 40% (~7.7 kPa) of the saturation value is termed moderate hypoxia, between 40 and 23% (~4.4 kPa) is classified as severe hypoxia, and below 23% is extreme hypoxia. Anoxia refers to the absolute absence of oxygen (Graham and Wegner, 2010).

Several biological and physical factors can affect the availability of oxygen in water such as algal blooms, nutrient enrichment, temperature, and salinity. For example, during the day aquatic photosynthesis helps to maintain high levels of dissolved oxygen (normoxia > 15 kPa). Conversely, in small streams and pools during the night the oxidation of organic matter and biological respiration can quickly deplete the oxygen down to hypoxic levels (< 5 kPa) (Wilcock et al., 1998). Physical factors such as upwelling and thermal or haline stratification can also introduce and reinforce hypoxia in coastal, estuarine and freshwater ecosystems.

Human intervention has also contributed towards the depletion of oxygen content in natural waters. The release of water with high nutrient concentrations or high temperature may directly or indirectly result in hypoxic conditions in coastal and riverine waters (Diaz and Rosenberg, 1995). Nutrient enrichments produces organism proliferation increasing overall oxygen demand, but it also adds organic matter to water bodies, and the oxidation of this is itelf an oxygen consuming process (Cole, 1979). Furthermore, higher temperatures not only reduce the solubility of oxygen in water but also raise the oxygen demands of the indwelling organism by increasing their metabolism. Hypoxia has been extensively reported in marine, estuarine, and freshwater environments (Grantham et al., 2004; Almeida-Val et al., 2006; McNeil and Closs 2007; Neuenfeldt et al., 2009). In aquaculture, due to the high stocking

densities used, hypoxia has also been commonly reported (Boyd and Schmittou, 1999).

Hypoxia represents a significant challenge for fish, forcing them to develop adaptations and strategies to survive in oxygen-depleted waters. Teleost fish show a wide variety of responses to hypoxia at different levels. Behavioural responses are the first to be employed, and initially these can usually be grouped into one of two categories. Avoidance involves attempting to escape from hypoxic conditions, while quiescence aims to conserve energy by reducing non-essential movement (Diaz and Rosenberg, 1995). If these strategies are unsuccessful then further behavioural responses can be triggered, such as aquatic surface respiration (ASR). This involves the fish moving to the water surface where it aims to extract oxygen from the most oxygenated layer of water at the air-water interface (Graham 1990; Soares et al., 2006).

Physiological responses are also observed when fish encounter hypoxic waters. One of the first physiological mechanisms to be employed is an increase in the ventilatory frequency aiming to extract more oxygen from the medium (McNeil and Closs, 2007; Nilsson et al., 2007; Soares et al., 2006). Increases in blood haematocrit (Hct) and haemoglobin (Hb) concentration are also rapidly enacted in order to enhance the whole blood oxygen-carrying capacity (Mandic et al., 2009). Increases in the Hb-oxygen affinity may also enhance oxygen uptake after repeated or extended hypoxic episodes (Mandic et al., 2009). For species that may regularly encounter hypoxia, morphological adaptations that aid oxygen removal from the aquatic or aerial medium have also been observed. For example, modified swim bladders (*Arapaima gigas*, Brauner et al., 2004), enlargement of lower lips (*Colosomma macropomum*, Sundin et al., 2000), and increases in gill surface area (*Carassius carassius*, Sollid et al., 2003;2005; Nilsson, 2007; *Carassius auratus*, Sollid et al., 2005; *Gymnocypris przewalskii*, Matey et al., 2008) have been documented.

Metabolic and biochemical adjustments are also prevalent in fish exposed to hypoxic conditions. These primarily aimed to conserve and make efficient use of energy during hypoxia, optimising ATP utilisation and generation. Mechanisms employed by fish may include anaerobic ATP production that results in the production of ATP in

the absence of oxygen (Hochachka et al., 1973; Boutilier, 2001; Nilsson and Renshaw, 2004). Ethanol production and excretion can occur in some species, helping the fish avoid lactate poisoning (Nilsson and Renshaw, 2004). Some organisms may decrease the basal metabolic rate, an effect termed metabolic suppression, which considerably reduces energy expenditure (Hochachka, 1986; Lutz and Storey, 1997).

1.4 Emersion

If common responses of fish to hypoxia fail, more extreme measures may be initiated. Perhaps the ultimate step is for a fish to leave the water and rely exclusively on atmospheric oxygen for aerial respiration. A number of fish species have been shown to emerse, and survive long periods in the absence of water through aestivation or pseudo-aestivation. Aestivation is a state of dormancy or torpor, characterised by a lowered metabolic rate. Pseudo-aestivation, instead, is a state of inactivity or quiescence, but not accompanied by a significant decrease in metabolic rate. The most successful example of aestivating fish are lungfish (Smith, 1935). When their environment dries out they can aestivate for several days, or even months, in mud-lined cocoons. Gas exchange is maintained through diversion of the branchial circulation to the pulmonary gas exchange surfaces (Johansen et al., 1976; Abe and Steffensen, 1996). Mudfish (Eldon, 1979) and the mangrove rivulus (Taylor et al., 2007) also withstand emersion from days up to several weeks. Unlike lungfish, however, they do not truly aestivate. Instead these species remain quiescent in a basal metabolic state, where they are still capable of looking for better conditions or food (Eldon, 1979). Sculpins (Sloman et al., 2008) and mudskippers (Clayton, 1993) also have the ability to emerse for several hours or days, an activity mainly associated with tidal cycles. A common feature of all these fish is their habitation in environments that regularly experience hypoxia and dry out, either as part of the tidal cycle, or on a seasonal basis related to waterway desiccation.

Fish gills are exquisitely designed to extract oxygen from water. The small diffusive distance between the blood and external medium, a counter-current blood-water exchange system, and a large surface area are all characteristics that facilitate gas

exchange (Evans et al., 2005). Oxygen uptake, and transport from the gills to the tissues and organs, relies on several parameters. These include gill surface area, ventilation rate, perfusion, diffusion rate, and blood oxygen-affinity (Perry and McDonald, 1993). However, the effectiveness of the gill as a respiratory tissue in air may be limited. Without the support given by the aquatic medium, the gill filaments may collapse, reducing the surface area available for exchange (Graham, 1973). In some species this has led to the evolution of alternative mechanisms for aerial gas exchange. Among these is the modified swim bladder of *Arapaima gigas*, one the most aerially-dependent fish (Brauner et al., 2004). Air bubbles obtained by surfacing are stored in this modified gas bladder whereby oxygen is absorbed through a specialised lung-like tissue. Other body organs have also developed well-vascularised areas to extract oxygen from air. These include the gut of *Misgurnus anguillicaudatus* (McMahon and Burggren, 1987) and the buccal cavity of *Periophthalmodon schlosseri* (Aguilar et al., 2000). As in *A. gigas*, oxygen is absorbed in modified well-vascularised tissues (gut and mouth, respectively) from air bubbles obtained by surfacing. Some fish have primitive "lungs" facilitating aerial gas exchange (Graham, 1997).

Another potential route for aerial gas exchange in fish is the skin. Cutaneous oxygen uptake from water has been explored in several seawater (Nonnotte and Kirsch, 1978) and freshwater (Kirsch and Nonnotte, 1977; Nonnotte, 1981; Meredith et al., 1982) fish species. Nonnotte (1981) explored the net contribution of the skin to the total fish oxygen consumption (VO_2), concluding that only in the scaleless catfish (*Ictalurus melas*) could skin be considered a true respiratory organ. Interestingly, the highest cutaneous oxygen contribution to total uptake reported to date (43%) has been determined in a scaleless galaxiid fish, the Canterbury mudfish (Meredith et al., 1982).

Oxygen uptake is not the only problem for fish that emerse. Such species also have to deal with compromised water and ion balance, and nitrogen excretion. Metabolic end products such as ammonia are toxic (Randall and Wright, 1987), and if excretion is compromised, such as in the absence of available water for dilution, then it may accumulate to lethal levels (Frick and Wright, 2002). Even when water is present but in limited amounts, then ammonia excretion can be inhibited. Accumulation of

ammonia in the surrounding water eliminates the concentration gradient for diffusive ammonia loss, the major mechanism of ammonia excretion (Wright and Wood, 2009). Therefore, to avoid ammonia build-up, some fish can depress their rate of ammonia production (Ip et al., 2001, 2004a, b), actively transport ammonia against a concentration gradient (*P. schlosseri*, Randall et al., 1999; Ip et al., 2004b), volatilise ammonia (*Alticus kirki*, Rozemeijer and Plaut, 1993; *R. marmoratus,* Frick and Wright, 2002; and *M. anguillicaudatus*, Tsui et al., 2002), or alternatively excrete nitrogen in a less toxic form (urea, *Heteropneustes fossilis*; Srivastava and Ratha, 2010).

1.5 Osmorespiratory compromise

As previously discussed (section 1.4), gills are the main site for gas exchange in fish. However, some of the features of gills that enhance oxygen uptake can have the opposite effect on other gill functions, such as ion exchange. For instance, during hypoxia a high gill surface area maximises the oxygen uptake, but it also could promote passive water and ion exchange (Gonzalez and McDonald, 1992). Moreover, many of the processes involved ensuring adequate oxygen uptake under oxygen restriction, such as increased opercular frequency, can further affect ion and water exchanges by exacerbating passive diffusion (Mitrovic et al., 2009). This study has also shown that hypoxia modulates the abundance, distribution and size of MR cells, affecting Cl^- fluxes during and after exposure to hypoxia (Mitrovic et al., 2009). This suggests a further mechanism by which oxygen availability and salinity interact at the gills, and that their effects on fish physiology are linked.

Gill remodelling may also occur in order to facilitate changes in ion exchange requirements under changing salinities. At high salinities, for example, it has been proposed that some fish may increase gill surface area in order to expose more ion transporters to the external medium, and therefore maximise ion exchange (Gonzalez, 2012). The opposite occurs in freshwater, where the gill surface area is reduced in order to minimise passive ion loss (Turko et al., 2012; LeBlanc et al., 2010). While the consequences of gill remodelling for ion and water exchange at different salinities and under hypoxia are complex and still no so clear, the mechanisms used for

modifying gill surface area are known. One mechanism for controlling gill surface area is through the proliferation or reduction of the interlamellar cellular mass (ILCM). Increases in ILCM will cover a larger proportion of the lamellae, reducing its functional surface area and decreasing ion loss (LeBlanc et al., 2010; Turko et al., 2012). Conversely, decreases in ILCM will cover a smaller proportion of the lamellae, increasing surface area and facilitating ion exchange (LeBlanc et al., 2010; Turko et al., 2012).

Examining respiration as a function of environmental salinity is of particular importance since gas and ion exchanges are linked, and therefore changes that impact one of these exchanges may have consequences for the other. Fish have developed specific physiological systems that enable them to obtain oxygen from their environment (respiratory system), mechanisms allowing them to distribute oxygen to the tissues (circulatory system), and pathways that effectively maximise oxygen use (metabolism). Several of these processes can be affected or modified by external salinity. For example, salinity can affect the Hb-oxygen affinity (Jensen et al., 1993), impacting oxygen transport to the tissues. As oxygen is required for aerobic metabolism this could impose energetic restrictions on osmoregulation processes. This is particularly important given that the energetic cost of pumping ions by NKA is high, accounting for up to two thirds of ATP requirements during hypoxia (Buck and Hochachka, 1993).

1.6 The model species: inanga

Galaxioids comprise more than 50 fish species and are the most widespread and speciose of fish inhabiting the temperate waters of the Southern hemisphere. Galaxioids are closely-related to osmerids and salmonids of the Northern hemisphere, and inhabit similar ecological niches to these species (McDowall, 2002). The major family within the galaxioid grouping is Galaxiidae, to which inanga (*Galaxias maculatus*; Jenyns 1842) belongs. Inanga is a widespread scaleless fish species endemic to the Southern hemisphere. Its occurrence has been reported in New Zealand, Australia, South Africa, Argentina and Chile (McDowall and Eldon, 1980; Cussac et al., 2004). A coldwater fish, it is known by a variety of common names

including inanga (New Zealand), jollytail (Australia), puye (Chile), and puyen (Argentina). The juvenile stages of inanga and several other galaxiid species (known collectively as whitebait) are considered a luxury food, reaching prices between 28 and 83 $U.S. kg^{-1} (Dantagnan et al., 2002). In New Zealand the inanga also has cultural significance. It is an important traditional food source for the indigenous Maori population and, more recently, to New Zealanders in general. The great demand for this resource has increased the fishing pressure on inanga populations. Although New Zealand fosters ~20 galaxiid species in total, inanga is the main component of the whitebait fishery (McDowall, 1965; Rowe et al., 1992; Fulton, 2000).

Inanga are also threatened by other human actions. For example, deliberate introduction of salmonids for recreational purposes has led to the establishment of wild populations of salmon and trout (McDowall, 2003). Early life stages of these species cohabit with inanga, competing for food and space (Bonnet and McIntosh, 2004). Moreover, when salmon and trout reach sizes in excess of 150 mm, they become piscivorous preying directly on inanga (Mittelbach and Persson, 1998). Some recent studies have found that about 15% of the total stomach contents of salmon and trout could be native species, such as inanga (Arismendi et al., 2009). Other human activities such as agriculture, forestry and industry have modified the land and vegetation surrounding the rivers where inanga thrive, degrading the stream habitat. These disturbances change the light intensity, turbidity, nutrient flow, refuge availability and water variables such as pH and oxygen concentration. All these parameters may affect the dynamics of inanga populations (McDowall, 1984).

Under these joint scenarios of overfishing, competition and environmental degradation, inanga populations are under significant pressure. This is reflected in whitebait landings, which have been steadily in decline in both New Zealand (McDowall, 1984) and Chile (Campos, 1973). Although its commercial and cultural value has made inanga an attractive species for aquaculture, it is not currently commercially farmed. On an experimental scale, however, there have been some efforts in establishing inanga aquaculture in Chile (Dantagnan et al., 2002, Mardones et al., 2008; Encina-Montoya et al., 2011). To an extent the lack of success of this species in commercial farming is due to a lack of basic physiological knowledge.

The family Galaxiidae is considered a sister group of Salmonidae (McDowall, 2002) and representatives from both groups are renowned for their ionoregulatory capacity. Inanga is considered an amphidromous species, capable of living in a wide range of environmental salinities, with the ability to move freely between freshwater and seawater at several stages of its life cycle. Estuarine and landlocked populations have been also documented (Battini et al., 2000; Chapman et al., 2006; Barriga et al., 2002), suggesting a certain degree of physiological plasticity in regards to salinity tolerance. Although inanga are known for their exceptional ability to tolerate extreme changes in salinity (Chessman and Williams, 1975), even during the first hours of hatching (Mitchell, 1989), the mechanisms behind such ability have not been investigated to date.

Inanga possess a short but complex life cycle. Adult inanga inhabit freshwater where they feed, grow and reach sexual maturity. During autumn, sexually mature inanga migrate downstream to spawn in the upper tidal zone during the highest tide (McDowall, 1990; McDowall and Charteris, 2006). The fertilised eggs are deposited in the riparian vegetation, such that when the tide retreats the eggs remain emersed. Embryos usually develop exposed to air (emersed), however it has been shown that embryonic development can also be completed in water (Battini et al., 2000). This stage takes approximately one month, at which time the next high tide reimmerses the eggs, which then hatch, and the hatchlings migrate to the ocean. Here they reside for approximately five months. During spring juvenile inanga migrate from the sea to freshwater, where it takes a further six months for them to reach sexual maturity (McDowall and Eldon, 1980). It has been generally reported that inanga exhibit an annual life cycle, and thus die off after breeding. However, it has also been reported that a small percentage of the adults (<4%) remain in the stream and spawn a second year (McDowall, 1968), and an even smaller percentage may remain and spawn for a third year (Burnet, 1965). It is worth noting, however, that most inanga that are maintained in our aquaria without use live for up to three years, suggesting that more refined mechanisms of tracking individuals may lead to a revision of current thinking.

During its complex life cycle, inanga could be exposed to challenging environmental conditions. During their migrations (either as an adult or as a juvenile) mechanisms

for coping with different stressors, such as salinity and hypoxia, have to be enacted at the right time, or even in coordination, in order to achieve survival. For example, in estuaries and streams inhabited by inanga hypoxia may develop (Wilcock et al., 1998). Australian inanga populations have been discovered inhabiting waters with dissolved oxygen levels lower than 1 mg l^{-1} (~2.8 kPa; Chapman, 2003) and data obtained from New Zealand streams with resident inanga populations suggest dissolved oxygen may fall to levels as low as 3 kPa, and persist for up to 6 h (Chapter VII). In their upstream migration, juvenile inanga must somehow modify their ion excreting phenotype to an ion absorbing phenotype and therefore acclimate to this new freshwater existence. Conversely, adult inanga must modify their ion absorbing phenotype to an ion excreting phenotype and therefore acclimate to this new seawater/estuarine existence. Therefore, migratory inanga must not only cope with changing salinities, but must sometimes do so under sub-optimal oxygen conditions.

Possibly as vestigial adaptations to aerial embryonic development, inanga appear to have several features that would facilitate aerial oxygen uptake as adults. While gills perform the majority of oxygen uptake in most fish, in a few species skin may also play an important role in gas exchange. Galaxiids are one such grouping thought to be able to utilise the epidermis for oxygen uptake. In fact, all members of this family have characteristics advantageous for cutaneous oxygen uptake (Meredith et al., 1982; McDowall, 2001). However, to date, no studies have explored the cutaneous contribution to total oxygen uptake in inanga.

To be considered a model species, a fish must have several characteristics advantageous for its study. Inanga is easy to collect and maintain, quick to acclimate to the rearing and experimental conditions, and is adaptable to commercial food. Perhaps the most important characteristic of a model species is that it possesses biological characteristics that serve the question to be answered. August Krogh stated that “for such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied” (Krogh, 1929). This principle applies for inanga in the present study. As a consequence of its complex life cycle and the variety and extreme nature of the environments this species inhabits, inanga can be used as physiological model to explore how fish deal with stressors such as hypoxia, salinity and emersion. Both salinity variations and hypoxia are

commonly encountered in its habitat, sometimes even in combination. The small size and lack of scales both benefit cutaneous exchange, presenting a model to investigate the physiological role of fish skin in salinity acclimation and oxygen uptake under different environmentally-relevant scenarios. Furthermore, inanga emerse as a natural response to hypoxia, furthering the idea that these fish present a model to investigate the physiological role of skin in gas exchange.

1.7 Thesis scope and outline

In the present thesis inanga was used as a model species to understand the mechanisms by which fish are capable of responding to two of the most common environmental stressors they face in their natural habitat, salinity and hypoxia. Considering that animals can enact responses at behavioural, physiological, biochemical, metabolic and molecular levels, I chose to use an integrative approach which provides a more realistic approach from an ecophysiological perspective. For example, animal responses to a given stressor are triggered at different levels, and therefore evaluation at a single level of responses could offer no definitive conclusion as to the overall adaptive strategy of the animal. Physiologically, animal responses to a stressor will also depend on the timeframe and severity of the exposure. Therefore, inanga responses to salinity and hypoxia were evaluated after both short- and long-term exposures to the environmental stressors of interest.

Taking into account the environment where inanga lives, this thesis particularly focuses on salinity and hypoxia as main factors. However, it also briefly explores and discusses some of the potential interactions or synergistic effects of salinity and hypoxia on the physiology of inanga. Since inanga is a scaleless fish, and as a consequence of some of the natural behavioural responses of inanga during hypoxia exposure (whereby gills surface area was likely reduced), this thesis also explores the importance of skin for gas and ion exchange through several of the chapters. The effects of emersion on the physiology of inanga is also evaluated.

Considering the well known salinity tolerance of inanga (Chessman and Williams, 1975; Mitchell, 1989) and the salinity-related migrations present in inanga life cycle,

it was hypothesised that inanga possessed mechanisms for efficiently modifying its physiology at different salinities. Therefore, the first aim of this thesis was to *characterise the basic physiology of inanga across the environmental salinity gradient (freshwater to seawater) to which they are exposed in their natural habitat and determine the energetic costs associated with different salinities.* Chapter II evaluates the physiology and bioenergetics of inanga at different salinities after acclimation. Responses allowed evaluation of the physiological limits of inanga and also permitted identification of the mechanisms behind its exceptional salinity tolerance. The role and contribution of the skin in gas exchange and ammonia excretion was also assessed at different salinities.

Salmonids are also well known to successfully acclimate to different salinities during certain periods of their life cycle and this, in part, has been attributed to the presence of NKA isoform switching (Bystriansky et al., 2006). Based on the results of Chapter II, and in light of the fact that inanga is closely-related to salmonids and that both are euryhaline, it was hypothesised that inanga might also experience isoform switching and that this may partly explain its exceptional salinity tolerance. The next aim was, then, to *quantify the activity of NKA, and examine the potential existence and the mRNA expression of different isoforms of the NKA in inanga gills, gut, and skin across a salinity gradient, exploring its importance in maintaining osmotic balance.* Therefore Chapter III explores the physiological and molecular changes after a seawater challenge. Together, Chapter II and Chapter III provide the first study of the physiological mechanisms underlying the exceptional salinity tolerance of inanga.

Since hypoxia is also common in inanga habitats, it was hypothesised that inanga might have adaptations to overcome hypoxia. In order to explore such potential hypoxia adaptations in inanga, the first aim was *to determine the respiratory response of inanga to decreasing oxygen levels, explore the use of anaerobic metabolic pathways and investigate the role of the skin under oxygen restriction*. Chapter IV therefore examines the critical PO_2 ($PO_{2\ crit}$), which is the external PO_2 at which a fish can no longer regulate its oxygen consumption rate. However, since hypoxia tolerance has been reported to change with fish size, this possibility was explored in Chapter V which aimed to *evaluate how the aerobic metabolic rate scaled with fish size and explore if this relationship was affected by external* PO_2. If so, it was then

hypothesised that this relationship could be used a tool to elucidate differential size-related responses to hypoxia. Therefore, the second aim of Chapter V was to *determine if size-related differences evidenced in the scaling relationship could be correlated with actual indicators of hypoxia tolerance*. Chapter V, thus assessed the scaling of oxygen consumption in relation to fish size at different external PO_2 values, finding that the scaling relationship was affected by external PO_2. Since the scaling relationship suggested size-related differences in hypoxia tolerance, potential differences between small and large fish were further investigated in terms of aerobic and anaerobic metabolism, and hypoxia tolerance.

After evaluating the metabolic responses of inanga to hypoxia, the behavioural component of the hypoxia response was investigated in Chapter VI. The aim of this chapter was to *investigate any potential behavioural response to hypoxia that might be advantageous to survive aquatic hypoxia.* In these experiments the fish were provided with a floating platform as a route of escape. Surprisingly, inanga voluntarily leaped out of the water and emersed themselves in order to escape severe aquatic hypoxia. Chapter VI also describes some physiological aspects of this extreme emersion behaviour, such as opercular frequency and aerial oxygen consumption.

In light of this voluntary emersion behaviour it was, then, hypothesised that emersion might be advantageous for inanga relative to remaining exposed to aquatic hypoxia. Therefore Chapter VII aims to *evaluate the physiological and biochemical consequences of emersion and extended exposure to aquatic hypoxia.*

Finally, the main findings are discussed and summarised in Chapter VIII. Discussion is mainly focussed on salinity and hypoxia as natural stressors and how inanga physiologically cope with these factors. Implications for natural inanga populations, and whitebait fisheries and farming are also briefly discussed. This final chapter also highlights areas where more research is needed in order to widen our understanding of fish physiology. It also indicates specific bottlenecks that require further attention in order to deepen our knowledge in some specific mechanisms such as ion balance in hypoxia and the energy costs of isoform switching. This final chapter also provides an ecophysiologically relevant and integrated conclusion regarding the hypoxia and salinity tolerance of inanga, an iconic fish species. It is worth noting that since minor

but important variations exist among the methodologies used in each chapter, no specific methods chapter is included. Instead methods will be detailed within each chapter and consequently there may be some repetition in the 'Materials and Methods' sections through the thesis. All experiments and procedures described have been approved by the University of Canterbury Animal Ethics Committee.

Chapter II

Effect of environmental salinity on osmoregulation, metabolism and nitrogen excretion in inanga

2.1 Introduction

Environmental salinity directly affects the physiology of aquatic organisms and therefore is an ecological factor of considerable importance. If unregulated, changes in osmotic gradients between the environment and the animal can impair basic physiological processes and potentially cause death (Edwards and Marshall, 2013). The internal osmolality of teleost fish is maintained at a level approximately 30% that of seawater (Jobling, 1995; Nordlie, 2009). In freshwater, fish usually maintain plasma osmolality at ~281-310 mOsm kg^{-1} (Nordlie, 2009), significantly higher than that of their surroundings (<50 mOsm kg^{-1}). In seawater, plasma osmolalities of ~400 mOsm kg^{-1} are observed (Nordlie, 2009), significantly lower than that of the environment (~1050 mOsm kg^{-1}). Therefore life in most waters requires fish to maintain an osmotic balance, pumping ions against the concentration gradient, either into the body in freshwater, or out of the body in seawater (Marshall and Grosell, 2005).

The maintenance of this osmotic balance is, however, energetically expensive. For example, in muscle cells NKA pumping can account for up 80% of the resting cellular metabolic rate (Rolfe and Brand, 1996; Rolfe and Brown, 1997). In isolated tissues such as the rectal gland and gills, is has been quantified that the cost of NKA pumping represents about 60% and 25% of the total tissue VO_2, respectively (*Squalus acanthias*, Morgan et al., 1997). At a whole animal level the cost of NKA pumping is likely to be much lower than that in isolated muscle cells or tissues (< 1%; Morgan et al., 1997), but as there are many other transporters and processes involved in osmoregulation (e.g. protein synthesis; Pannevis and Houlihan, 1992), osmoregulation is still likely to be an expensive process. It has therefore been hypothesised that it would be energetically advantageous for a fish to remain in salinities close to its internal osmolality (i.e. near the isosmotic point) to minimise osmoregulatory costs (Zadunaisky, 1984; Boeuf and Payan, 2001). At salinities lower or higher than the isosmotic point the osmoregulatory costs are likely to increase, mainly owing to processes of ion pumping, gill remodelling and protein synthesis.

A metabolic rate reflects the sum of all the aerobic energy-consuming processes in an organism. Therefore it would be anticipated that costs associated with osmoregulation should be evident in measures of oxygen consumption. However, although most studies have reported increases in NKA activity as salinity increases (e.g. Kelly and Woo, 1999; Lin et al., 2004; Chang et al., 2007), the effects of salinity on metabolic rate are more variable. For example, metabolic rate has been reported to increase with salinity in the estuarine/marine *Leiostomus xanthurus* (Moser and Hettler, 1989), *Centropomus undecimalis* (Gracia-Lopes et al., 2006) and the freshwater/estuarine *Anabas testudineus* (Chang et al., 2007). However, metabolic rate has also been reported to decrease at salinities higher than 70‰ in the euryhaline *Aphanius dispar* (Plaut, 2000), higher than 40‰ in the euryhaline *Cyprinodon variegatus* (Nordlie et al., 1991) and higher than 55‰ in the marine *Chanos chanos* (Swanson, 1998). In salinities ranging from freshwater to seawater, metabolic rate has also been reported to remain unchanged in *Aphanius dispar*, irrespective of the salinity the fish was acclimated to (Plaut, 2000). Conversely, the metabolic rate of the estuarine *Micropogonias furnieri* has been reported to increase at both salinity extremes (low and high), with a minimum metabolic rate observed at an isosmotic salinity (Aristizabal-Abud, 1992). Consequently, no definitive conclusion regarding the effect of salinity on fish metabolic rate can be drawn.

The effects of salinity on other basic physiological measures, such as nitrogenous waste excretion, are even less clear in fish. No changes in the ammonia excretion rates at different salinities have been observed in the anadromous *Salmo trutta* (Dosdat et al., 1997), in the euryhaline *Allenbatrachus grunniens* (Walsh et al., 2004) and in the marine *Sphoeroides annulatus* (Perez-Robles et al., 2011). However, increases in the ammonia excretion rate at high salinity (35‰) have been reported in the euryhaline *Gambusia affinis*, and contrarily, decreases in the ammonia excretion rate at high salinity (25‰) have been reported in the freshwater *Danio rerio* (Uliano et al., 2010). To a certain extent these differences may be partially explained by differences in the nutritional status of the experimental animals, but in general responses seem to be species-specific. In other organisms, however, such as crabs the pattern seems to be clearer. Ammonia excretion rates generally increase at low salinities as a mechanism to regulate water and ion balance in a hypo-osmotic medium (Péqueux, 1995; Urbina et al., 2010).

As the osmoregulatory challenge increases there are several other transporters that increase their activity, a phenomenon that might help in generating an alternative explanation for the salinity-related pattern of nitrogenous waste excretion. One of the mechanisms involved in ammonia excretion in fish is through an indirect coupling between NH_4^+ efflux and Na^+ uptake (Wright and Wood, 2009). Therefore any increase in the NKA pump activity as a result of an osmoregulatory challenge may have an effect in ammonia excretion rates. NKA is not the only transporter that could co-transport NH_4^+. Na^+ uptake and NH_4^+ excretion are also linked via a complex metabolon that includes the Rh proteins (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger NHE-2 and/or NHE-3, Na^+ channel; Wright and Wood 2012). Therfore any change in environmental salinity that affects Na^+ fluxes will also affect the rates of NH_4^+ excretion.

Supporting this hypothesis, it has been shown that higher ammonia excretion rates occurred in freshwater for the estuarine *Centropomus undecimalis* (Gracia-Lopes et al., 2006), at 16‰ for the marine *Miichthys miiuy* (Zheng et al., 2008), and at 35‰ in the freshwater *Gambusia affinis* (Uliano et al., 2010). When ammonia excretion rate is determined along with oxygen consumption rate the O:N ratio can be calculated, a measure that can be used as an indicator of physiological stress (Widdows, 1978). The O:N index has been previously used as a stress index under varying saline conditions in fish (Zheng et al., 2008) and crabs (Urbina et al., 2010).

The euryhaline inanga is a scaleless fish species, known for its salinity tolerance (from near 0‰ to up to 48‰; Chessman and Williams, 1975), a tolerance even present during the first hours after hatching (Mitchell, 1989). However, to date, no study has attempted to elucidate the physiological mechanisms that allow inanga to inhabit different salinities. Furthermore, although inanga present some advantageous characteristics for cutaneous oxygen uptake, the potential role of cutaneous exchanges has not been explored in inanga. Cutaneous oxygen uptake in some freshwater air-breathing species has been reported to account for 10–24% of whole animal oxygen uptake (Graham, 1997). In another galaxiid, the Canterbury mudfish, cutaneous oxygen uptake has been reported to account for up to 43% of the whole animal oxygen uptake in water (Meredith et al., 1982). Fish skin has not only been shown to

have a role in gas exchange, it has also been reported to be actively involved in ion transport and ammonia excretion. Proliferation of MR cells has been reported in response to increasing salinity in the epidermis of the euryhaline *Aphanius dispar* (Chelouche et al., 1992) and in the opercular skin of the euryhaline *Rivulus marmoratus* (King et al., 1989). Furthermore, skin has also been reported to be involved in ammonia excretion (Morii et al., 1978). In fact, a recent study on goldfish (*Carassius auratus*) showed that when branchial surface area is compromised a larger proportion of the NH_4^+ is excreted cutaneously (Smith et al., 2012).

As discussed in Chapter I, many of the adaptations designed to achieve ion homeostasis impacts oxygen uptake, a trade-off termed osmorespiratory compromise (Gonzalez and McDonald, 1992). Given its exceptional osmoregulatory capacity and the possible importance of cutaneous gas exchange in inanga, studying the effects of salinity on the basic physiology of this fish could be insightful to elucidate the mechanisms of salinity adaptation. The potential use of skin for ion and gas exchange in inanga may be advantageous for salinity acclimation as functions could be distributed between gills and skin epithelia, thus minimising the osmorespiratory compromise. Specifically, the aims of this chapter were to 1) characterise the basic steady-state physiology of inanga after acclimation to different salinities, 2) evaluate the energetic costs of osmoregulation at different salinities, and 3) explore potential salinity-induced changes in the routes (branchial or cutaneous) used for oxygen uptake and ammonia excretion.

2.2 Materials and methods

2.2.1 Fish and rearing conditions

Juvenile inanga (*Galaxias maculatus*) were caught by seine nets in Canterbury streams, placed in plastic containers filled with water from the stream and then transported to the aquarium facility at the University of Canterbury, Christchurch, New Zealand. The fish were left to acclimate to captivity for a minimum of three weeks. During this period they were maintained in a 500 l aquarium (fish density ~2.4

kg/m^3), in flowing freshwater (17 mOsmol kg^{-1}, 315-330 µM Na^+, pH 6.8-7.2), at 14°C on a 12:12 day:night cycle. Fish were fed *ad libitum* on commercial flake food (Nutrafin®Max, Hagen). Food was withheld for two days before the start of the experiments.

2.2.2 Series I: Whole fish physiology and tissue sampling

2.2.2.1 Experimental design

After three weeks of acclimation, juvenile fish (n = 78; 0.35 ± 0.01 g, mean ± SD) were gradually acclimated from freshwater (17 mOsm kg^{-1}; ~0.6‰) up to a salinity of 43‰ (1230 mOsm kg^{-1}), at a rate no greater than 5‰ per day using a peristaltic pump (flow rate ~300 ml h^{-1}). The hypersaline treatment (43‰) was prepared by the addition of commercial marine salt (Red Sea salt®) to 100% seawater. This was performed in a static 70 l plastic tank, with air bubbling provided to assure normoxic conditions. Once each experimental salinity (0, 5, 10, 20, 33 and 43‰) was reached, a group of 13 fish was transferred to a separate 30 l plastic tank where the experimental salinity was held constant for a period of seven days. Partial water changes (50%) were performed daily with water at the appropriate salinity. Temperature, photoperiod and feeding regime were similar to that described in section 2.2.1. After seven days determination of oxygen consumption and ammonia excretion rates was performed, followed by blood and tissue sampling.

2.2.2.2 Oxygen consumption (MO_2)

Thirteen fish at each of the experimental salinities were individually placed in a glass respirometry chamber of 0.1 l volume and their oxygen consumption rates were determined by closed respirometry. Fish were left undisturbed to acclimate to the respiratory chambers for at least 5 h prior to the start of experiments. During this period chambers were supplied with a constant flow (1.2 l h^{-1}) of normoxic water at the corresponding salinity and 14°C. Chambers were submerged in a controlled-temperature bath at 14°C ± 0.1°C during both acclimation and incubation. After acclimation, the chambers were sealed and fish were left to deplete the oxygen inside

the respirometry chamber for about 40 min. During this period, the oxygen concentration did not decrease below 70% of the saturation (~110 mmHg) and the fish kept the water well mixed with gill ventilation and body movements. Oxygen concentration was quantified at the beginning and at the end of the incubation by sampling 1 ml of water in duplicate and determining oxygen content in an oxygen electrode placed in a water jacketed microcell, via a 781 oxygen meter (Strathkelvin), connected to a PowerLab/4SP unit for recording (ADI Instruments, Waverly, Australia). For each salinity treatment, three chambers lacking animals were used as controls to account for potential bacterial respiration. The oxygen electrode was calibrated daily with fully aerated water and a saturated sodium sulphite solution at the corresponding salinity equilibrated at 14°C. Atmospheric pressure and salinity were taken into account in each calibration.

2.2.2.3 Ammonia excretion

Ammonia excretion was measured in the same individual fish as that used for oxygen consumption. After finishing oxygen consumption determination, water was drained from the respirometry chamber and replaced by filtered water (0.22 µm syringe filter, Millipore; to remove any bacterial contamination), at the corresponding salinity. After rinsing (~30 s) water was drained again and replaced with 40 ml of clean, filtered water and fish were left undisturbed for a 1 h incubation period. Three containers without fish were used as controls and chambers were submerged in a controlled-temperature bath at 14 ± 0.1°C. At the beginning and at the end of the incubation 2 ml water samples in duplicate were withdrawn and the ammonia concentration was quantified spectrophotometrically at 635 nm by the indophenol method (Ivančić and Degobbis, 1984). Briefly, ammonia present in the water sample reacts with chlorine and phenolic compounds to form an indophenol dye, developing a blue coloured solution that can later be spectrophotometrically measured at 635 nm.

2.2.2.4 Blood and tissue sampling

After determination of oxygen consumption and ammonia excretion rates, fish were euthanised by an overdose of 3-aminobenzoic acid ethylester (MS222; 1 g l^{-1}). Fish

were then quickly rinsed in water at the appropriate salinity, water excess was gently removed by a tissue, and a blood sample was taken by caudal amputation. The blood was collected in a pre-cooled heparinised syringe (~16 units ml $blood^{-1}$; lithium salt, Sigma), transferred to a 100 µl tube and centrifuged at 12,000 *g* for 5 min at 4°C. Plasma osmolality was then measured in a vapour pressure osmometer (Wescor 5520; Wescor Inc., South Logan, UT, USA). This device works on the principle that the more solutes that are present in a sample the lower the vapour pressure, which is then measured through a thermocouple. While the blood sample was in the centrifuge, whole gill baskets were excised, placed in a 1.5 ml eppendorf tube and quickly frozen in liquid N_2 and stored at – 80°C for later NKA activity determination.

2.2.2.5 NKA activity

NKA activity was assessed using a modified microplate assay (McCormick, 1993). NKA activity was considered the ouabain-sensitive component of total gill ATPase activity. The absorbance due to the disappearance of reduced nicotinamide adenine dinucleotide (NADH) was measured every 20 s at 340 nm in a plate reader (Fluostar optima, BMG Labtech) during a 12 min period. An adenosine diphosphate (ADP) standard curve allowed the standardisation of the slope of the reaction by measuring the NADH formed from a known amount of ADP. The microplate and samples were kept on ice during addition of the reagents. NKA activity was then calculated by subtracting the slope of the sample with ouabain from the slope of the sample without ouabain Protein concentration was quantified in the remaining supernatant of each sample by the Bradford (1976) method and by constructing a calibration curve with bovine serum albumin (BSA). The final results were expressed as µmoles of ADP per mg $protein^{-1}$ h^{-1}. Due to reagent limitation, NKA activity was not assessed in samples obtained from fish in the 5‰ salinity group.

2.2.2.6 O:N ratio

The ratio between the oxygen consumed and the nitrogen excreted was calculated after converting the physiological rates to atomic equivalents following the procedure described by Widdows (1985). Briefly, mass-specific oxygen consumption and

ammonia excretion rates were converted to atomic equivalents by using the atomic weights (O_2 and NH_4^+), and the ratio was calculated per individual fish.

2.2.2.7 Energy cost at different salinities

The energy cost associated with different salinities was calculated using the data from *Series I*. Energy lost through oxygen consumption (R) and excretion (U) was calculated by converting the physiological rates to energy equivalents using the following conversion factors: 1 mg O_2 = 14.06 J (Gnaiger, 1983) and 1 mg NH_4–N = 24.87 J (Elliot and Davison, 1975).

2.2.3 Series II: Partitioned physiology, branchial and cutaneous contribution

2.2.3.1 Experimental design

Branchial and cutaneous oxygen uptake and ammonia excretion were investigated at three external salinities (0, 20 and 40‰) in a second group of fish (0.87 ± 0.06 g; mean ± SD; n = 42). Fish were randomly allocated to one of the experimental salinities (n=14 per treatment) and acclimated in the same way as previously described for *Series I*.

2.2.3.2 Oxygen consumption (MO_2)

A custom-built 90 ml transparent Perspex partitioned respirometry chamber was used to separate the head (forward of the pectoral girdle; containing gills) from the body (skin), with the two partitions separated by a sheet of dental dam through which the head of the fish was pushed. The dam was held stretched by the experimenter to ensure that once slotted in, and the dam was released, a tight seal was formed around the fish. The head compartment had a volume of 35 ml and the body 55 ml. Fish were placed in the unsealed respirometer and left to acclimate for at least 2 h while the chamber was immersed in a larger volume of aerated water at the corresponding salinity. Thereafter the compartments were filled with normoxic water and then sealed with rubber bungs (Fig. 2.1).

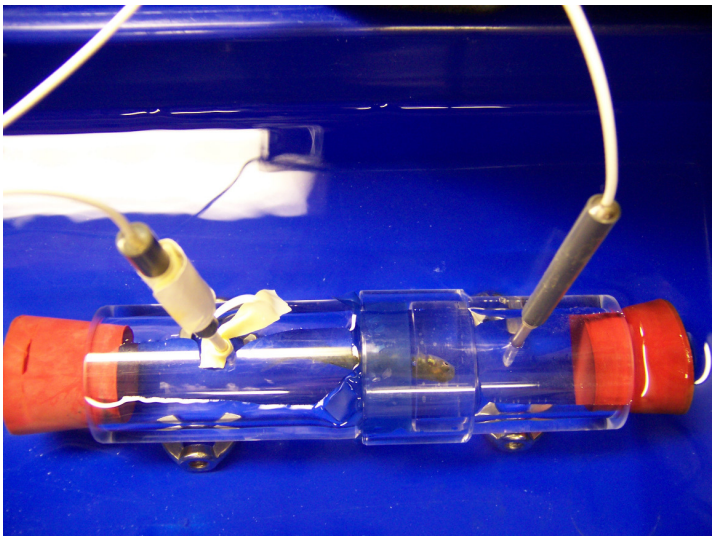

Figure 2.1: Custom-built 90 ml transparent Perspex partitioned respirometry chamber used to separate the head (forward of the pectoral girdle; containing gills) from the body (skin).

A small hole (3 mm) at the top of each compartment allowed the insertion of an oxygen microelectrode (MI-730, Microelectrodes Inc., USA), connected to a OM-4 oxygen meter (Microelectrodes Inc., USA). The oxygen electrodes were calibrated daily with fully aerated water and a saturated solution of sodium sulphite, at the corresponding salinity equilibrated at 14°C. Incubations lasted ~30 min and at least two consecutive measurements were conducted per fish. Since no statistical difference between the repeated trials was found, these were averaged to provide an oxygen consumption value for each fish (and compartment). Preliminary experiments showed that the system was hermetic from both the exterior of the chamber and between compartments. Three euthanised fish were used as controls, to account for potential oxygen diffusion around the sealing of the rubber dam and for passive diffusion through the fish.

2.2.3.3 Ammonia excretion

Once oxygen consumption measurements finished, oxygen electrodes were removed from both compartments and water was exchanged with fresh filtered oxygenated water at the corresponding salinity and temperature. Water samples (1 ml, in duplicate) were withdrawn at the beginning and at the end of the incubation with a 1

ml pipette from the hole at the top of each compartment. Fine tubing was inserted into this hole for gently bubbling air during the incubation (~1.5 h), to maintain chamber oxygenation. Ammonia was quantified as previously described for *Series I* (section 2.2.2.3).

2.2.4 Data treatment and statistical analysis

The effect of salinity on metabolic rate, ammonia excretion, O:N ratio, plasma osmolality, NKA activity and energy cost (R + U) (*Series I*) was evaluated by a one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) post-hoc assessment. The contribution of the gill and skin to total oxygen uptake and ammonia excretion (*Series II*) was evaluated by a two-way ANOVA (salinity and body compartment as factors), followed by a Tukey post-hoc test. All data were subjected to initial analysis of normality and homogeneity of variances by a Kolmogorov-Smirnov and Levene's median test respectively (Sokal and Rohlf, 1995). When parametric assumptions were violated, non-parametric tests were performed (NKA activity). Results were considered significant with a *p*-value lower than 0.05. All data are presented as mean ± standard error of the mean (SEM).

2.3 Results

2.3.1 Whole fish physiology

2.3.1.1 Oxygen consumption, excretion rates, and O:N ratio

No mortalities were recorded during either the acclimation or experimentation periods. Metabolic rate was not affected by external salinity (one-way ANOVA, p = 0.426; Fig. 2.2A). Fish acclimated to salinities ranging from freshwater to up to 43‰ had, on average, a metabolic rate of 11.1 ± 0.2 μmol O_2 g^{-1} h^{-1}. Ammonia excretion was, however, significantly affected by external salinity (one-way ANOVA, p = 0.007; Fig. 2.2B). In freshwater, fish had an excretion rate of 1.11 ± 0.08 μmol NH_4^+-N g^{-1} h^{-1}, a value that decreased as salinity increased up to 20‰, reaching a minimum

value of 0.65 ± 0.07 µmol NH_4^+-N g^{-1} h^{-1}, significantly lower than the freshwater value ($p < 0.001$). From this point, excretion rate increased again at the two highest salinities (20‰ *vs*. 33‰, $p < 0.001$; 20‰ *vs*. 43‰, $p = 0.004$). The average value of 1.01 ± 0.07 µmol NH_4^+-N g^{-1} h^{-1} at these two highest salinities was statistically indistinct from the freshwater value ($p = 0.731$, Fig. 2.2B).

Since metabolic rate remained constant through the experimental salinities and nitrogen excretion rate varied, the O:N ratio was significantly affected by salinity (one-way ANOVA, $p = 0.032$; Fig. 2.2C). In freshwater, an O:N ratio of 21.2 ± 1.7 was calculated. This value increased as salinity increased up to 20‰, reaching a maximum value of 34.2 ± 3.2, significantly higher than the freshwater value ($p = 0.002$). O:N ratio then decreased again at the two highest salinities presenting an average value of 23.9 ± 1.8, significantly lower than at 20‰ (20 *vs*. 33, $p = 0.009$; 20 *vs* 43‰, $p = 0.025$), and similar to the freshwater value ($p = 0.614$, Fig. 2.2C).

2.3.1.2 Energy losses

Metabolised energy (R) was the main component of energy loss, accounting for ~91 to 94% of the total energy lost across all experimental salinities, and was not affected by the acclimation salinity ($p = 0.386$, Fig. 2.3). Although excretion (U) represented only a small portion of the energy losses, it was significantly affected by salinity ($p = 0.007$, Fig. 2.3). The energy lost by nitrogenous waste excretion decreased as salinity increased reaching the lowest value at 20‰ ($p < 0.001$ and $p = 0.033$, compared to 0‰ and 5‰, respectively). At salinities higher than 20‰ energy lost by nitrogenous waste excretion then increased again being significantly higher at 33‰ ($p = 0.001$) and at 43‰ ($p = 0.004$), compared to values at 20‰. These values at both of the higher salinities were similar to the freshwater value ($p = 0.614$, Fig. 2.2C). Total energy losses (R+U) were not affected by salinity ($p = 0.190$), averaging 5.4 ± 0.1 J g $fish^{-1}$ h^{-1} across the entire salinity gradient (0‰ to 43‰, Fig. 2.3).

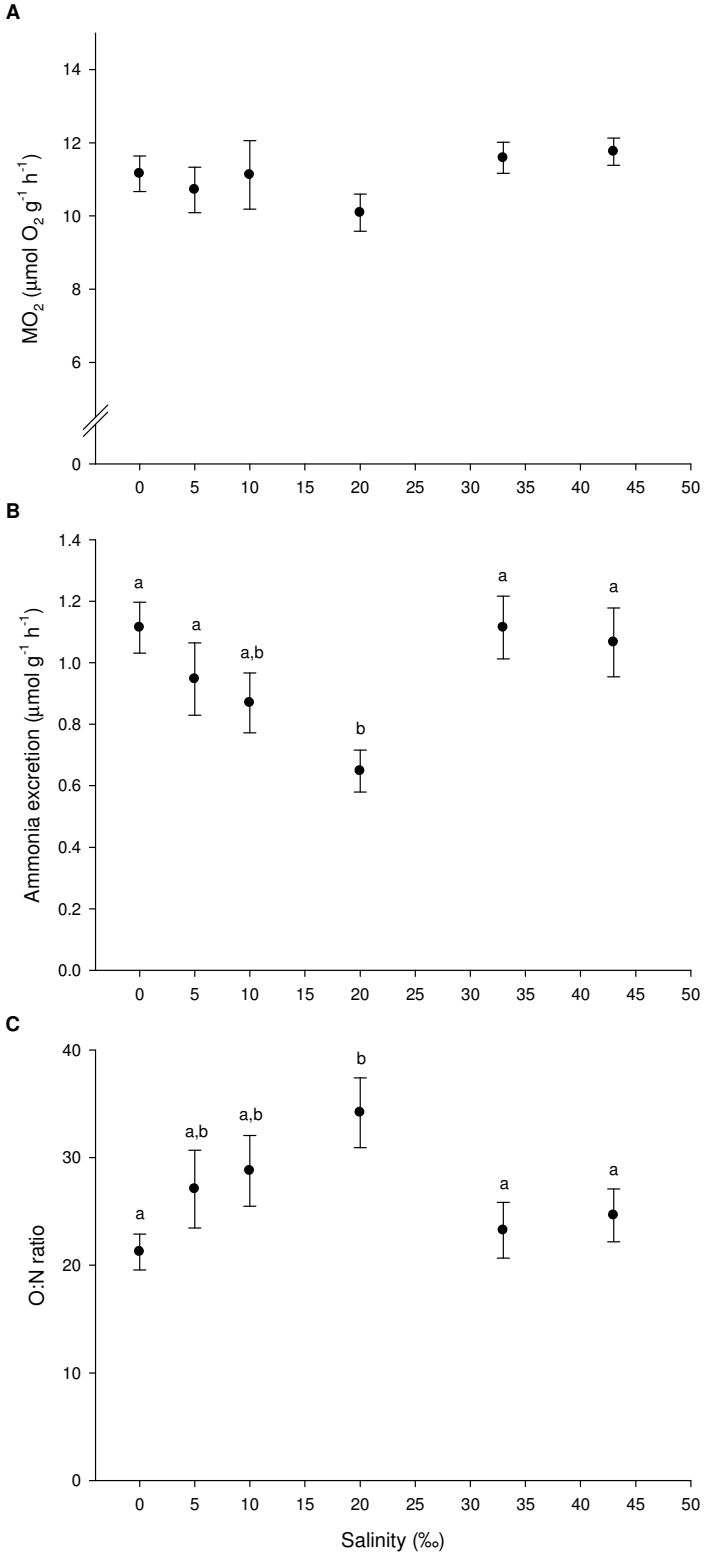

Figure 2.2: The effect of salinity acclimation on inanga A) metabolic rate (µmol O_2 g^{-1} h^{-1}), B) ammonia excretion (µmol NH_4^+-N g^{-1} h^{-1}), and C) O:N ratio. Plotted values represent mean ± SEM of 11-13 individuals. Values sharing letters are not significantly different as determined by one-way ANOVA, followed by LSD post-hoc assessment, tested at α = 0.05.

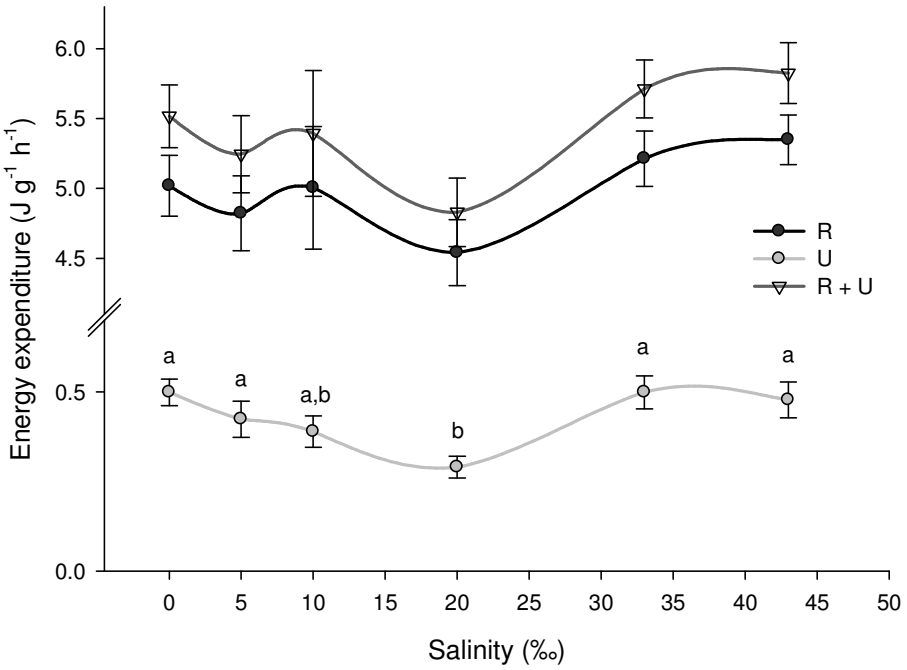

Figure 2.3: The effect of acclimation salinity on energy loss as nitrogenous waste (U, grey), metabolised energy (R, black) and total energy losses (R + U, white; all values J g fish^{-1} h^{-1}). Plotted values represent mean ± SEM of 11-13 individuals. Points sharing letters are not significantly different as determined by one-way ANOVA, followed by LSD post-hoc assessment, tested at α = 0.05.

2.3.1.3 Plasma osmolality

Plasma osmolality was successfully regulated by inanga across the entire salinity gradient studied. As a consequence plasma osmolality always differed from the external medium, except at 10‰ (*t*-test within each external salinity, plasma *vs.* water value). However, salinity still caused minor changes in plasma osmolality (one-way ANOVA, $p = 0.001$; Fig. 2.4). Freshwater fish showed a plasma osmolality of 301.3 ± 0.6 mOsm kg^{-1}, a value that decreased towards the isosmotic point. The lowest plasma osmolality (252.8 ± 5. mOsm kg^{-1}) was found at an external salinity of 10‰, and this value was significantly lower than plasma osmolalities found at 33‰ and 43‰ (both $p < 0.05$, Fig. 2.4). Plasma osmolality, then, increased with salinity. At 33‰, plasma osmolality had increased to a value similar to that in freshwater, 336.1 ± 5.1 mOsm kg^{-1} ($p > 0.05$). However, at the highest salinity (43‰) plasma osmolality reached

values statistically higher than the freshwater value (411.1 ± 14.2 mOsm kg^{-1}; $p < 0.05$, Fig. 2.4).

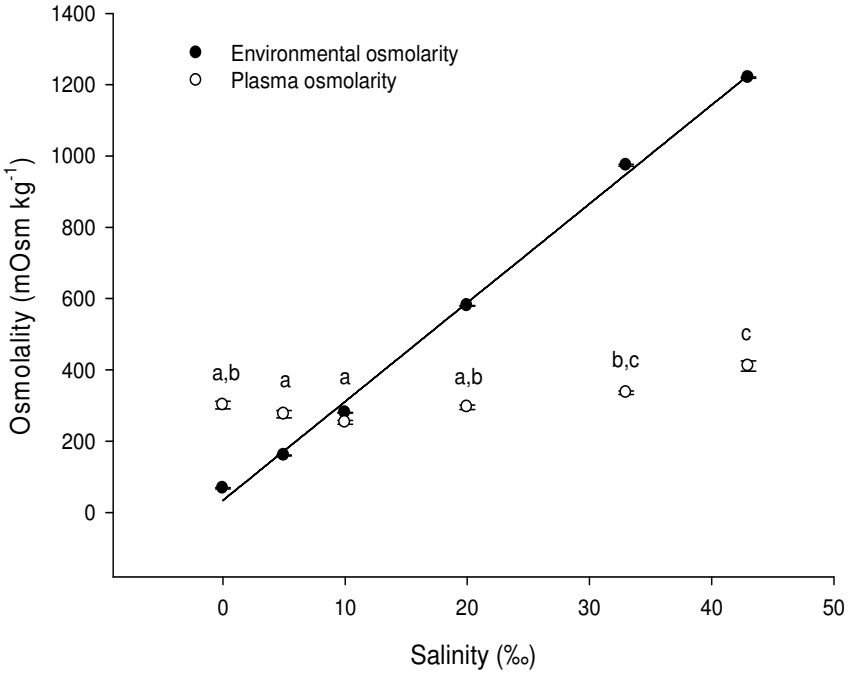

Figure 2.4: The effect of acclimation salinity on inanga plasma osmolality (mOsm kg^{-1}). Plotted values represent mean ± SEM of 11-13 individuals. Points sharing letters are not significantly different as determined by one-way ANOVA, followed by LSD post-hoc assessment, tested at α = 0.05. Filled circles and line represents the environmental osmolality.

2.3.1.4 NKA activity

The pattern of branchial NKA activity was similar to that of plasma osmolality, and activity was significantly affected by external salinity (one-way ANOVA, $p = 0.006$; Fig. 2.5). The lowest activity was measured at an intermediate salinity, 20‰ with a value of 1.2 ± 0.1 µmol ADP mg $protein^{-1}$ h^{-1}, although this value was not significantly different compared to the NKA activity in freshwater (1.9 ± 0.1 µmol ADP mg $protein^{-1}$ h^{-1}; $p > 0.05$, Fig. 2.5). However, at 43‰ NKA activity was significantly elevated (2.4 ± 0.3 µmol ADP mg $protein^{-1}$ h^{-1}, $p < 0.05$) relative to the value at 20‰, but was not significantly different from the freshwater value ($p > 0.05$; Fig. 2.5).

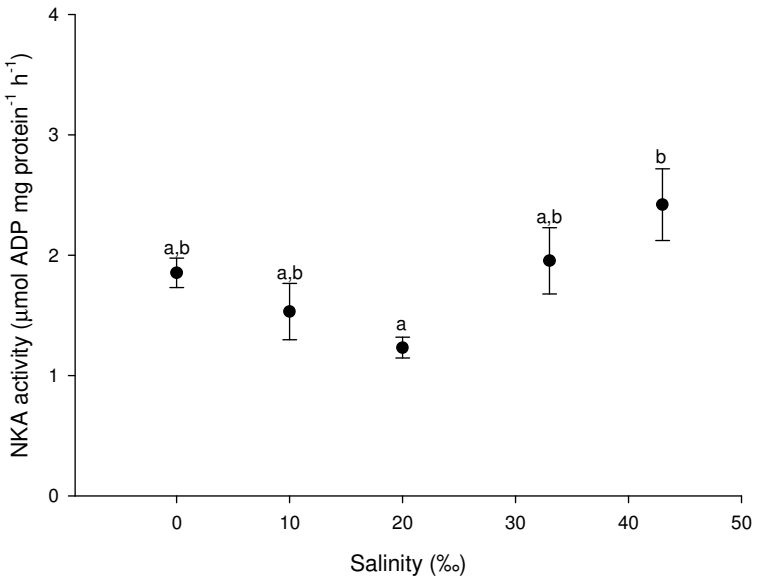

Figure 2.5: The effect of acclimation salinity on the branchial NKA activity of inanga (µmol ADP mg protein^{-1} h^{-1}). Plotted values represent mean ± SEM of 11-12 individuals. Points sharing letters are not significantly different as determined by Kruskal-Wallis ANOVA, followed by Dunn's post-hoc assessment, tested at α = 0.05.

2.3.2 Partitioned oxygen consumption and ammonia excretion rate

Total fish oxygen consumption was not significantly affected by external salinity (two-way ANOVA, p = 0.099, Fig. 2.6A). However, head and body compartments differed in their oxygen uptake (two-way ANOVA $p < 0.001$), and this was affected by environmental salinity (interaction term between salinity and compartment, two-way ANOVA; p = 0.025, Fig. 2.6A). The rates of oxygen uptake by the head compartment (gills) were higher than the oxygen uptake rates of the body compartment (skin) in all the experimental salinities (p = 0.011, $p < 0.001$, and $p <$ 0.001 for 0, 20 and 40‰, respectively). In the head compartment (gills) the rates of oxygen uptake were significantly higher at 20‰ and 40‰ salinities than in freshwater (p = 0.041, p = 0.004, respectively). In the body compartment, however, the oxygen uptake remained unchanged as external salinity increased (20‰, p = 0.997 and 40‰, p = 0.806, compared to the freshwater value).

Ammonia excretion was significantly affected by external salinity (two-way ANOVA, $p = 0.015$, Fig. 2.6B), and the head and body compartments differed in their excretion rates (two-way ANOVA, $p < 0.001$). Compartmental ammonia excretion, however, was not affected by environmental salinity (interaction term between salinity and compartment, two-way ANOVA; $p = 0.978$, Fig. 2.6B). Fish acclimated to 40‰ had a higher total ammonia excretion rate compared to fish acclimated to freshwater ($p = 0.01$), but similar to fish acclimated to 20‰ ($p = 0.23$). Gills (head compartment) were the main route for ammonia excretion in freshwater ($p= 0.028$) and in 40‰ ($p= 0.036$). There were no differences in the excretion rates between compartments at 20‰ ($p= 0.064$, Fig. 2.6B).

2.3.2.1 O:N ratio in the head and body compartments

Salinity alone did not significantly affected O:N ratio (two-way ANOVA, $p = 0.065$, Fig. 2.7). However, the head and body compartments differed in their O:N ratio (two-way ANOVA $p < 0.001$) and this was affected by environmental salinity (interaction term between salinity and compartment, two-way ANOVA; $p < 0.001$, Fig. 2.7). In freshwater, O:N ratio had a similar value in both compartments (~11; $p = 0.478$). However, as environmental salinity increased, O:N ratio differed between compartments, showing higher values in the head than in the body compartment at both 20 and 40‰ (both p-values < 0.001). Within the head compartment, O:N ratio was significantly higher at 20‰ than in freshwater ($p = 0.004$), but not significantly different from the value at 40‰ ($p = 0.107$). Within the body compartment, O:N ratio decreased as salinity increased reaching a significantly lower value at 40‰ than in freshwater ($p = 0.007$, Fig. 2.7).

2.4 Discussion

Exposure to a hypo- or hyperosmotic medium induces a concentration gradient between the environment and the animal. Unless efficient mechanisms of ion and water regulation are in place, then the exposure of fish to such conditions will be manifested by changes in plasma osmolality. For this reason, plasma osmolality is a

useful marker to determine the efficiency of osmotic regulation of a given fish species. Previous studies in an Australian population of inanga have shown that plasma osmolality is maintained constant at a value of ~267.9 mOsm kg^{-1} from freshwater to about 20‰ (Chessman and Williams, 1975). These authors classified

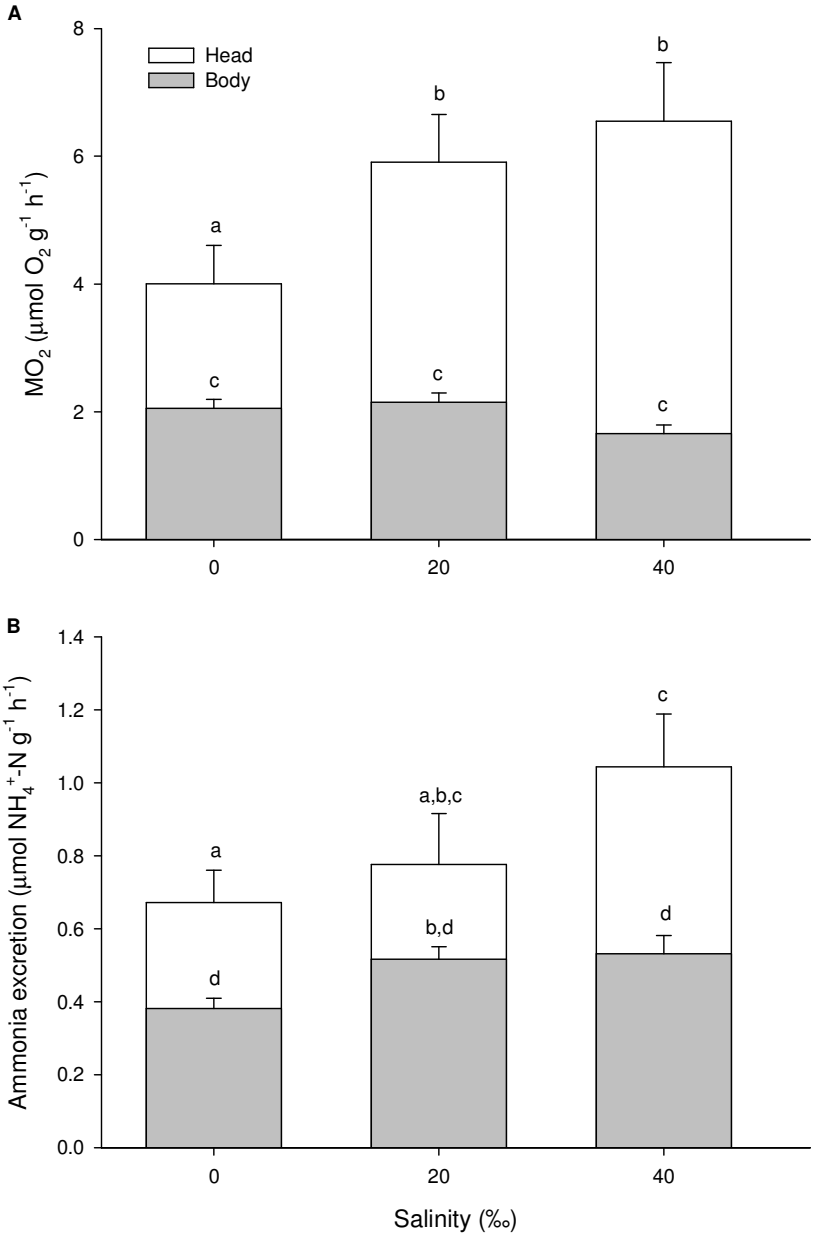

Figure 2.6: Branchial (head) and cutaneous (body) oxygen uptake (A), and ammonia excretion (B) rates, at three different acclimation salinities. Plotted values represent mean ± SEM of 14-15 individuals. Bars sharing letters are not significantly different as determined by two-way ANOVA, followed by a Tukey test, tested at α = 0.05.

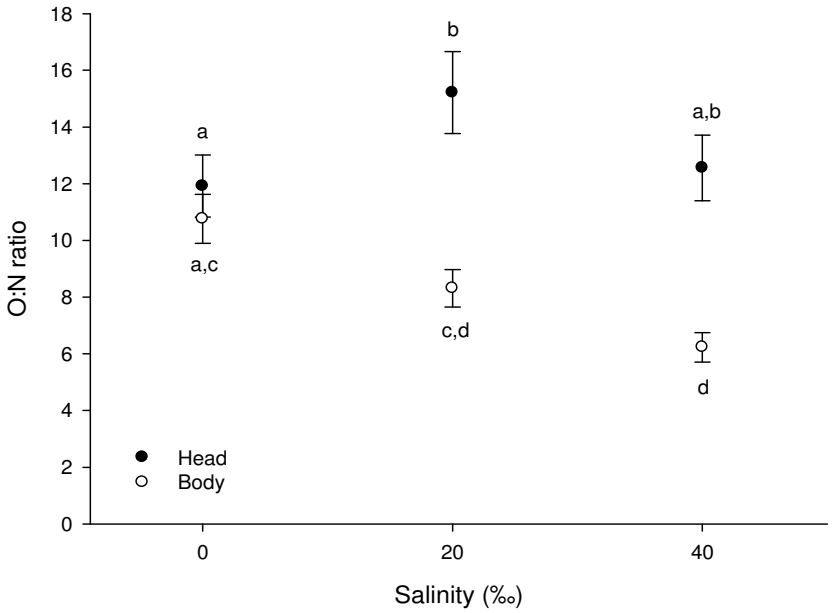

Figure 2.7: O:N ratio calculated for the head and body compartments at each of the experimental acclimation salinities (0, 20 and 40‰). Plotted values represent mean ± SEM of 14-15 individuals. Points sharing letters are not significantly different as determined by two-way ANOVA, followed by a Tukey test, tested at α = 0.05.

inanga as a "powerful osmoregulator". In the present study plasma osmolality was 301.3 ± 0.6 mOsm kg^{-1} in freshwater and 336.1 ± 5.1 mOsm kg^{-1} at 33‰, indicating good regulation over this range. However, in the present study at the highest salinity (43‰) plasma osmolality reached significantly higher values (411.1 ± 14.2 mOsm kg^{-1}). This does not necessarily mean that the fish was out of ion balance, but may simply signal a new equilibrium. An increase in plasma osmolality in fish acclimated to higher salinities is a common phenomenon (Nordlie, 2009), as it is thought to minimise the concentration gradient for ion influx. Consequently this elevated plasma osmolality value may simple represent the top end of the optimal range of plasma osmolalities in inanga.

Within the range of salinities tested, the lowest plasma osmolality was found at an external salinity of 10‰ (252.8± 5.2 mOsm kg^{-1}), close to the isosmotic point calculated from present data (9.1‰). The isosmotic point was calculated as the external salinity at which both curves, environmental and internal osmolality, intersect. This value is in agreement with previous reports (8.8‰; Chessman and

Williams, 1975). Therefore the isosmotic point in inanga, as in all teleost fish, is at osmolalities lower than that of seawater and higher than that of freshwater. The isosmotic point is very close to the average osmolality found in estuaries, which might be advantageous for a migratory fish such as inanga. The habitation of estuarine environments during their juvenile migration and then for reproduction when adults, might allow inanga to divert the energy saved from osmoregulation to growth or reproduction.

NKA plays a key role in regulating water and ionic balance. In the present series of experiments, NKA activity followed a similar pattern to that of plasma osmolality. NKA activity increased at both low and high salinities where the osmoregulatory challenges were greater, as a result of larger differences between internal and external osmolalities. Conversely, the lowest activity was found close the isosmotic point, at intermediate salinities where the osmoregulatory challenge was smaller. At the highest salinity (43‰) NKA activity was significantly higher than at 20‰. NKA activity increases have also been documented in the marine/estuarine *Sparus sarba* at 33‰ (Kelly and Woo, 1999), the freshwater/estuarine spotted green pufferfish at 35‰ (*Tetraodon nigroviridi*s; Lin et al., 2004) and in the freshwater/estuarine *Anabas testudineus* at 30‰ (Chang et al., 2007). These results suggest that NKA plays a crucial role in ion regulation not only in inanga, but in fish in general. Most previous studies have shown that NKA mainly increase at high salinities, yet the present results suggested that NKA increased at both salinity extremes in inanga (freshwater and seawater) which might partially contribute to the remarkable salinity tolerance of inanga.

It has been hypothesised that any increase in osmoregulatory costs associated with increased osmotic gradients should be reflected in metabolic rates of fish (Zadunaisky, 1984). Since one of the main cost of osmoregulation is ion pumping, it could be hypothesised that higher NKA activities should be reflected in measurements of metabolic rate. This has been shown in isolated tissues, such as in the rectal gland and gills of *Squalus acanthias*, where oxygen consumption was reduced after the inhibition of the NKA by addition of 0.5 mM ouabain (Morgan et al., 1997). However, to my knowledge, this correlation between oxygen consumption and branchial NKA activity has only been shown once at a whole organism level, in the

fish *A. testudineus* (Chang et al., 2007). In the case of inanga, the increases in the activity of the NKA at low and high salinities did not correlate with the metabolic rate of the fish. Instead, inanga maintained a near-constant metabolic rate, averaging 11.1 ± 0.2 μmol O_2 g^{-1} h^{-1} over the tested salinity range of freshwater to 43‰. This indicates that fish incurred no extra metabolic expenditures associated with either osmoregulation or salinity-induced stress, at least over the range of salinities used here. The metabolic rate reported here is also well in agreement with another report for inanga (~12.2 μmol O_2 g^{-1} h^{-1}, fish size = 1.2 g; Encina-Montoya et al., 2011).

Previous studies have attributed increases in the metabolic rate with an increased cost of osmoregulation at high salinities in the euryhaline *L. xanthurus* (Moser and Hettler, 1989) and the euryhaline *C. undecimalis* (Gracia-López et al., 2006). In agreement with these observations, minimum oxygen consumption rates have been reported close to the isosmotic point in the euryhaline *M. furnieri* (17 to 19‰; Aristizabal-Abud, 1992) and in the euryhaline *S. sarba* (15‰, Woo and Kelly, 1995). The following chapter (Chapter III) shows that significant changes at the physiological and at molecular level occur when inanga is quickly challenged with water at 28‰. This suggests that in the present study the exceptional osmoregulatory ability of inanga conferred complete acclimation over the course of the 14 day exposure. It is, however, possible that an initial spike in metabolic rate as the animal was adjusting to the new salinity may have been missed. However this would be difficult to measure as a rapid change in salinity is likely to cause an increase in activity as part of a stress response (Barton and Iwama, 1991; Uliano et al., 2010), which will then cause an increase in fish metabolic rate. Alternatively the lack of metabolic rate increase may suggest that inanga have generally low osmoregulatory costs (Morgan and Iwama, 1991). A further possibility is that inanga did incur a reduced metabolic cost at salinities where regulation, and thus costs of ion pumping, were minimised, but instead of reducing metabolic rate the "excess energy" was instead diverted to other processes such as growth and reproduction. Energy reallocation has been previously reported in fish in response to stress (Wendelaar-bonga, 1997). This latter hypothesis needs to be tested by a more complete and rigorous energy balance at different salinities.

In contrast to oxygen consumption, nitrogen excretion rate was influenced by acclimation salinity. It has been previously shown that at high salinities excretion rate decreased in the marine milkfish (*Chanos chanos*; Swanson, 1998), a response that has been attributed to a decreased amino acid catabolism (Swanson, 1998). However, in the mangrove rivulus (*Rivulus marmoratus*; Frick and Wright, 2002) ammonia excretion rate was reduced at 45‰, but was elevated at 30‰, compared with freshwater. Frick and Wright (2002), explained these differences by a complex response of changes in the membrane permeability to NH_4^+ and also by the use of amino acids as osmolytes. Results from other species have shown other patterns. For example, the common snook (*C. undecimalis*; Gracia-López et al., 2006) showed the highest excretion rates at 35‰ when feeding and in freshwater (0‰) during starvation. The change in the excretion pattern in the common snook with relation to external salinity and feeding might be reflecting a differential use of energy substrates as consequence of using energy reserves (starving) or an external energy substrate (feeding).

In the present study, the increase in excretion rate at both salinity extremes suggests an increase in amino acid catabolism, probably related to an interplay between energy available for osmoregulatory processes and somatic growth. The decrease in the nitrogen excretion rate at intermediate salinities, close to the isosmotic point, indicates less reliance on amino acid catabolism as the osmotic challenge decreased (Frick and Wright, 2002). This might signal that inanga switches the "excess energy" to anabolic processes such as growth and reproduction. Therefore instead of using amino acids for processes such as energy metabolism they are used for building tissue, and as such there is less production of nitrogen waste. Then the increases of the ammonia excretion rates at both low and high salinities, might indicate an increase in energy demands as proteins are catabolised for energy (Ballantyne, 2001). This hypothesis is also in agreement with the calculated O:N ratios (see below). Some crabs species are strong regulators when exposed to a large osmotic challenge, but when exposed to salinities closer to their isosmotic point they osmoconform, saving energy (i.e. *M. rosenbergii*; Castille and Lawrence, 1981; Freire et al., 2003). In the case of inanga, it seems that more than an energy saving strategy it is a matter of optimising energy substrate usage and diverting energy for fuelling other non osmoregulatory processes.

An alternative explanation for the observed increase in the ammonia excretion rates at both salinity extremes (freshwater and seawater), relates to the mechanism of ammonia excretion. It has long been suspected that ammonia excretion is coupled to the movement of other ions. For example, several studies have suggested a link between the transepithelial exchange of Na^+ and NH_4^+ (Maetz, 1973; Evans, 1980; Wright and Wood, 1985). These early findings have been subsequently accepted and included in the ion regulation/ammonia excretion model (Wright and Wood, 2009; 2012). Basically, Na^+ and NH_4^+ fluxes are linked by the action of several transporters such NKA and the more complex metabolon that includes the Rh proteins (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger NHE-2 and/or NHE-3, Na^+ channel) (Wright and Wood, 2009; 2012). Therefore any increase in the NKA pump as a result of an osmoregulatory challenge, or any change in environmental salinity that affects Na^+ fluxes will also affects the rates of NH_4^+ excretion.

The O:N ratio is thought to be indicative of the energy substrate being metabolised by an animal (Mayzaud and Conover, 1988). Values between 3 and 16 are suggestive of the use of protein as a substrate, while values between 50 and 60 are indicative of a mixed protein-lipid substrate. The O:N values calculated here for inanga ranged from ~20 (freshwater and seawater) to ~35 (20‰), suggesting that inanga mainly used a mix of protein and lipids as an energy substrate across the entire salinity range studied (freshwater to 43‰). This is in agreement with the values expected from the diet offered (45.0% protein and 13.2% fat; Nutrafin®Max, Hagen).

Changes in the energy substrate with environmental salinity have been previously reported. For example, at low salinities a preference for protein usage has been reported in *S. sarba* (Woo and Kelly, 1995) and in *C. undecimalis* (Gracia-López et al., 2006). Furthermore, Woo and Kelly (1995) also found a preference for carbohydrates and lipids at isosmotic salinities (15‰). The data presented here also support these findings. Although inanga always used a mix of proteins and lipids as energy substrate, a larger proportion of lipids (Mayzaud and Conover, 1988) was used at 20‰ (O:N ~35), and a larger proportion of proteins were used in fresh- and seawater (O:N ~20). Since fat catabolism liberates more energy than protein catabolism (9.5 kcal g^{-1} *vs* 4.5 kcal g^{-1}; Cook et al., 2000), bioenergetically it is more

efficient to maintain a higher proportion of lipids as an energy substrate. Interestingly, this seems to be the case for inanga (the present study) and for the crab *H. crenulatus* (O:N ~28, from fresh- to seawater; Urbina et al., 2010), both of which maintain a mix of proteins and lipids as the energy substrate and both successfully tolerate a wide range of salinities. In the few fish studies where O:N ratio has been calculated through a salinity gradient, protein has been found to be the main energy substrate used (the estuarine *Centropomus undecimalis,* Lopez-Gracia et al., 2006; and the seawater *Miichthys miiuy* Zheng et al., 2008), however, these species are less euryhaline than inanga. Potentially, the use of lipids as an energy substrate would be advantageous for fuelling osmoregulatory costs, and therefore leaving proteins for somatic growth.

The energy efficiency resulted from using a mix of proteins and lipids as fuel was also evident when the physiological rates (oxygen consumption and ammonia excretion) were converted to energy equivalents (Joules) and the energy cost at different salinities was calculated. Inanga maintained its expenditure constant with an average value of 5.4 ± 0.1 J g fish^{-1} h^{-1} recorded across the entire salinity gradient (0‰ to 43‰). As previously discussed, this might suggest that the costs of osmoregulation did not significantly increase at any particular salinity. Or, alternatively, it might indicate that inanga maintain its expenditures constant, but differentially allocate energy to fuel different processes when possible. Estimations of the cost of osmoregulation in seawater have been reported to range from 2.4% to 15% of the resting metabolic rate depending on the fish species (Potts et al., 1973; Kirschner, 1993; Swanson, 1998; Morgan and Iwama, 1999). Therefore, based on the fact that the energy expenditure remained near-constant across the entire salinity gradient studied, it could be hypothesised that the cost of osmoregulation in inanga is low, and therefore not evident in either the energy expenditures or in the metabolic rate. Further research is needed to test this hypothesis. It is worth noting that ingestion rate largely determines the final energy balance, at least in crabs (Urbina et al., 2010), and therefore measurement of feed intake as a function of environmental salinity may be important for future studies.

Partition experiments (*Series II*, section 2.2.3) showed a slightly different trend to that obtained in *Series I* (whole fish physiology, section 2.2.2) in response to increasing

salinity. In the partition experiments there was a small, but significant, increase in the oxygen consumption rate as acclimation salinity increased. Nitrogen excretion rate followed a similar pattern to that of oxygen consumption, increasing at higher salinities. These discrepancies with the results obtained in *Series I* (i.e. the lack of significant change in the oxygen consumption rate at any salinity, and a lowest nitrogen excretion rate at 20‰) are likely to be the result of an experimental artefact. The procedure of partitioning involves the placement of a dental rubber dam around the fish head, which could have caused dislodging of skin mucus, and therefore changes in skin permeability. Fish mucus forms an unstirred layer close to the fish surface which limits the diffusion of water, ammonia, ions and gases (Shephard, 1994). Therefore, if mucus was removed due to the partitioning procedure, this could explain changes in the ammonia and oxygen uptake rates compared with those obtained from whole fish exposures (*Series I*). If mucus was removed by the placement of the rubber dam, passive diffusion of salts would be exacerbated at high salinities. This would likely require an increase in ion pumping in order to maintain homeostasis, and this cost may have been evidenced in the elevated oxygen consumption rate. Ammonia excretion might have been facilitated by coupled exchanges as result of this net gain of salts in the absence of mucus. Is likely that mucus is particularly important in a scaleless fish such as inanga, that largely rely on mucus as a barrier.

During partitioning, fish were also restrained. This might have contributed to an increased stress level of the fish and therefore altered fish responses (Barton and Iwama, 1991). Metabolic rate rapidly increases after stress (Barton and Iwama, 1991), and this could have been exacerbated in some exposures by the synergistic effect of high salinity as a secondary stressor. Cortisol is a hormone known to be released under stress conditions (Barton and Iwama, 1991). Therefore, cortisol could have exacerbated an ion imbalance at high salinities, which might indirectly explain the observed changes in ammonia excretion, via an effect on coupled ion exchanges. Cortisol is also known to have an effect on ion regulation (Tipsmark at al., 2011), and therefore, its levels would be expected to be even higher levels at high salinities potentially exacerbating stress levels. Both an ion imbalance and stress are likely to result in an increased oxygen consumption rate as a compensatory mechanism aiming to restore homeostasis.

Oxygen consumption has been determined under partitioning conditions by using a rubber dental dam in another galaxiid fish, *Neochanna burrowsius* (Meredith et al., 1982), and also in inanga (Chapter IV). Results from those experiments have not differed from the oxygen consumption rates obtained with conventional respirometry, suggesting that the stress levels were not great enough to affect the measurements. Since partitioning was carried out following the same procedure in all experimental treatments (0, 20 and 40‰), the increased rates of ammonia excretion and oxygen uptake at high salinities are likely to be caused by salinity.

Although partition experiments showed a different pattern of oxygen consumption and ammonia excretion in response to increasing salinity compared to whole fish exposures, it was clear that the contribution of the gills as an exchange surface increased at high salinities. Although in the body compartment the oxygen consumption and nitrogen excretion rates remained unchanged in response to acclimation salinity (from freshwater to 40‰), in the head compartment both rates increased as salinity increased. Gills are the main site for ion exchange, but also function as the primary gas exchange surface in fish (Evans et al., 1999). Consequently there may be some trade-offs between gas and ion exchange, a phenomenon termed the osmorespiratory compromise (Gonzalez and McDonald, 1992). It has recently been proposed that one of the main mechanisms for fish to adapt to high salinities is by increasing the proliferation of MR cells, and the gills surface area, therefore enhancing ion-transporting capacity (Gonzalez, 2012). It seems that the present data support this hypothesis. Our data showed an increase in the oxygen consumption and ammonia excretion rates by the gills (head compartment) as salinity increased, a result that can be explained by the expected increase in transporters and in the gill surface area as hypothsised by Gonzalez (2012). In fact a recent immunofluorescence study has shown that in brackish water-acclimated *K. marmoratus* two important transporters involved in ammonia exchange (Rhcg1 and NHE3) are co-localised in MR cells of the gills (Cooper et al., submitted). Further exploration of gill morphology, effective surface area and MRC proliferation accompanied by determination of gas and ion exchanges, would be required to properly test this hypothesis.

The present chapter showed that inanga successfully acclimated to salinities ranging from freshwater to 43‰, proving that it is an excellent osmoregulator. At the physiological level minor changes in plasma osmolality, coupled with no significant changes in the metabolic rate, suggest that salinities within the range used here do not impose a significant stress on inanga. Successful homeostasis is likely to be obtained by rapid responses at the physiological level, accompanied by a bioenergetically efficient way to fuel salinity-related costs. Ammonia excretion is lowest at near isosmotic salinities. Together with the O:N data it suggests that there may be reduced costs at these intermediate salinities, but this excess energy is diverted to anabolic processes and accompanied by a protein sparing behaviour that reduces protein breakdown for energy.

This is the first time that the skin of inanga has been shown to take up oxygen and excrete ammonia. Athough the skin has significant importance for oxygen uptake and ammonia excretion, the main site for gas and ion exchange are the gills. This role is more important as environmental salinity increases.

Chapter III

Differential expression of Na^+, K^+-ATPase α-1 isoforms during seawater acclimation in the amphidromous inanga

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3.1 Introduction

The key transporter that drives ion exchange in animal cells is the sodium pump (NKA). Owing to the importance of the gill in ion regulation in fish, the NKA in this tissue is of particular significance for fish and their ability to deal with changes in environmental salinity. Situated on the basolateral membrane, NKA generates an electrochemical gradient that directly or indirectly drives all cellular ion exchange. In response to an increase in external salinity gill NKA activity generally increases (Johnston and Saunders, 1981; Madsen and Naamansen, 1989; Kelly and Woo, 1999; Lin et al., 2004; Chapter II). While quantitative changes in NKA are well recognised, it is only in recent years that it has been shown that NKA undergoes subtle qualitative changes in order to achieve ionic balance under different salinity conditions.

NKA is a multi-subunit enzyme. The α-subunit is considered to be the catalytic subunit as it contains the binding sites for ATP, Na^+, K^+ and ouabain, while the β-subunit is thought to be involved in facilitating placement of the α-subunit into the cell membrane (Blanco and Mercer, 1998). In recent years studies have identified the presence of multiple isoforms of NKA subunits in fish, with the catalytic α-subunit receiving the most attention (Cutler et al., 1995a,b; Guynn et al., 2002; Schonrock et al., 1991; Semple et al., 2002; Richards et al., 2003; Bystriansky et al., 2006; Tipsmark et al., 2011). It has been demonstrated that upon movement to waters of distinct salinities, changes in NKA α-1 subunit expression occur that result in an enhanced ionoregulatory capacity appropriate to the new environment. In several salmonid species it has been documented that the α-1a isoform is up-regulated in freshwater, suggesting a physiological role in ion uptake, while the α-1b isoform is up-regulated in seawater suggesting a role in ion excretion (*Oncorhynchus mykiss*, Richards et al., 2003; Bystriansky et al., 2006; *Salmo salar*; Mackie et al., 2005; Bystriansky et al., 2006; Madsen et al., 2009, and *Salvelinus alpinus*, Bystriansky et al., 2006). It has been shown that this salinity-dependent expression of NKA α-1 subunit isoforms is also present in the euryhaline cichlid Mozambique tilapia (*Oreochromis mossambicus*; Feng et al., 2002; Tipsmark et al., 2011) and in the cyprinid mummichog (*Fundulus heteroclitus*) a single isoform of the alpha subunit has also been shown to respond to changes in salinity (Scott et al., 2004) suggesting

that isoform switching is likely to have evolved several times in fish. However to date this phenomenon has not been characterised outside of the cichlids, cyprinids and the salmonids.

Although they belong to different orders, the family Galaxiidae is considered a sister group of Salmonidae (McDowall, 2002) and representatives from both groups are renowned for their ionoregulatory capacity. The amphidromous inanga (*Galaxias maculatus*), for example, undergo developmentally-related migratory transitions through waters of distinct salinities, and may also be subjected to diel fluctuations related to the tidal cycle (Chapter I). The physiological characteristics that permit amphidromy have been only rarely studied (c.f. McCormick et al., 2003). The ability of inanga to cope with salinity variations induced by distinct triggers, at different life stages, and over varying time scales suggests possession of an extraordinary ionoregulatory physiology, a hypothesis conformed by the results obtained in Chapter II of this thesis. These data not only suggest that NKA plays a crucial role during salinity acclimation in inanga, but also suggest that salinity acclimation is attained by an energetically-efficient mechanism.

By analogy with cichlids and salmonids, I hypothesise that the ability of inanga to cope with environmental salinity fluctuations, is facilitated by the differential expression of isoforms of the NKA α-1 subunit under different salinity conditions. The aims of the present chapter were to: 1) determine whether different isoforms of NKA exist in the inanga genome; 2) partially sequence NKA subunits and isoforms; 3) evaluate the expression of these isoforms after seawater challenge; and 4) evaluate how NKA activity, and internal ion and water balance, is modulated by salinity, and 5) to determine if changes in physiological indicators of salinity acclimation correlate with responses observed at the molecular level.

3.2 Materials and methods

3.2.1 Fish and rearing conditions

Juvenile inanga (*Galaxias maculatus*) (mean weight: 1.3 ± 0.8 g; n = 210) were collected, transported to aquarium facilities at the University of Canterbury, and maintained as previously described (Chapter II). Food was withheld for two days before the start of acclimation until after sampling on day 5 (120 h). Fish were fed again on days 5 (120 h) and 6 (144 h; see next section).

3.2.2 Whole animal exposures

3.2.2.1 Preliminary seawater exposure experiments

The existence of different isoforms of NKA in inanga was evaluated in preliminary experiments, where a rapid and a slow salinity challenge were conducted. In the rapid salinity challenge, groups of 6 fish were directly exposed to 10, 20 or 32.5‰ seawater, while a control group remained in freshwater. Three fish were sampled 24 and 72 h following transfer to each salinity. In the slow transfer acclimation, 12 fish were placed in a FW tank and 3 fish were immediately sampled. Thereafter, salinity was increased at a rate of 2.5‰ day^{-1} and groups of 3 fish were sampled when experimental salinities reached 10, 20 and 32.5‰ respectively. Sampling involved euthanasia of fish with an overdose of MS222 (1 g l^{-1}) and extraction of gill, gut, skin and kidney tissue, which were immediately frozen in liquid nitrogen, placed in RNAlater® (Sigma-Aldrich), and then stored at -20°C. These tissues were then shipped to the Zoology Department at the University of British Columbia (UBC), Vancouver, Canada, where RNA was isolated and cDNA synthesised (see Cloning and sequencing, below). This provided the data that were used for phylogenetic analysis and that described in the 'Expression of NKA α-1 isoforms in freshwater and seawater' section of the Results, section 3.3.1).

3.2.2.2 Seawater acclimation

Juvenile inanga (mean weight = 1.23 ± 0.51 g, n = 70) were challenged by abruptly changing the salinity from freshwater to 86% seawater (28‰). This was performed *in situ* by adjusting the freshwater and seawater flows into a tank previously receiving flow-through freshwater. The new experimental saline conditions were achieved in ~5

min. Groups of 8 fish were lethally sampled at time 0 (immediately prior to seawater challenge) and then at 8, 24, 48, 72, 120 and 240 h following salinity change. Whole gills, gut and skin tissue were quickly removed, frozen in liquid nitrogen and stored at -80°C. Gut samples, consisting of the stomach and anterior intestine, were quickly flushed with autoclaved water at the corresponding salinity to remove any potential food remnants. Skin samples were taken from the section of the body running from behind the pectoral fins to the caudal fin. These tissue samples were removed from -80°C and placed in RNAlater®, before being shipped to UBC, where they were used to generate the data for mRNA expression analysis of the different NKA isoforms before and after seawater challenge (see 'NKA α-1 isoform expression after seawater exposure' in Results, section 3.3.2).

A separate set of juvenile inanga (mean weight = 1.9 ± 1.1 g, n= 70) were treated in an identical manner to that described above, except blood, muscle and gill tissue were taken for analysis of physiological (plasma osmolality and Na^+, muscle water content) and biochemical (NKA activity) indicators of salinity acclimation status. Analysis of fish blood and muscle was conducted immediately. Gill samples were frozen in liquid nitrogen and stored at -80°C until NKA activity was assessed (<14 d after sampling; see 'NKA activity').

3.2.3 Analytical techniques

3.2.3.1 RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol® Reagent (Life Technologies), according to the manufacturer's recommendations. RNA quantity and purity was assessed spectrophotometrically by measuring the A260/A280 ratio on a Nanodrop Spectrophotometer (ND-1000; Nanodrop Technologies), and RNA quality was checked by electrophoresis on an agarose gel (1.2% (wt/vol) agarose-TAE (40 mM Tris-acetate, 2 mM EDTA)). First strand cDNA was synthesised from 1.5 µg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions.

3.2.3.2 Cloning and sequencing

Various isoforms of the α and β subunits of NKA were cloned and sequenced using degenerate primers designed from known sequences of fish NKA genes (see Table 3.1 and primer design section below). The resulting polymerase chain reaction (PCR) products were TA cloned into the vector pGEM T-easy (Promega, Fisher Scientific). Clones were isolated and sequenced using an automated DNA sequencer (3730S; Applied Biosystems). All products were sequenced in both directions from a minimum of 5 independent clones. The resulting sequences of the different subunits and isoforms of the NKA of *G. maculatus* were submitted to GenBank under the following accession numbers (Acc. No.): α-1a (JQ885968), α-1b (JQ885969), α-1c (JQ885967), α-2 (JQ885972), α-3a (JQ885970), α-3b (JQ885971) and β subunit (JQ885973).

3.2.3.3 Primer design

All reverse transcription real time polymerase chain reaction (RT-qPCR) primers were designed using Primer Express software (version 2.0.0, Applied Biosystems) and assessed for stability, primer-dimer formation and self binding using Genetool Lite (BioTools Inc.). RT-qPCR NKA isoform-specific primers were designed for the α-1 isoforms, using inanga α-1a, α-1b and α-1c sequences obtained for this purpose (see 'Preliminary seawater exposure experiments' above, section 2.2.2.3). Owing to the similarity of isoforms, areas where sequences differed were used for designing isoform-specific primers. Sequences were considered different where there were at least three base-pairs that were distinct. Such regions were usually at the end of the alignment. RT-qPCR primers for the reference gene were designed from the 18s rRNA sequence for inanga obtained from Genbank (Acc. No.: HQ615533). Melting curve analysis was performed following each reaction during heating. This procedure assesses the dissociation characteristics or melting temperature of the double stranded PCR products formed. A single peak is indicative of the formation of a single product, and therefore primer specificity. Furthermore, products from each primer pair were cloned and sequenced to confirm again the presence of only a single amplicon (10 independent clones were selected per primer pair). All RT-qPCR

primers are detailed in Table 3.2 and were purchased either from Integrated DNA Technologies (IDT) or Invitrogen.

Table 3.1: Conventional and degenerate PCR primers used for amplification of different isoforms of NKA. All base numbers based on *O. mykiss* α-1a, except * (based on *Anguilla anguilla* NKA β).

Isoforms	Sequence 5' - 3'	Region on mRNA (base no.'s).
α-1b, α-1c, α-3a and α-3b	F: 5'-CCC CHG AGT GGR TSA AGT TCT G -3'	301-1531
	R: 5'- GRG GAG GGT CAA TCA TGG ACA T-3'	
β	F: 5'-ACT ACA AGC CCA CAT ACC AGG AC-3'	312 -898*
	R: 5'-TGA TGG CCA CCA GAG GCT G-3'	
α-1a, α-1c	F: 5'- ACA AGA ACG TGA CKC CGG ASG AGT-3'	1222-2979
	R: 5'- GGC RAY RTC CAT YCC AGG RCA GTA-3'	
α-2	F: 5'-AAC CCC AGA GAT GCC AA-3'	1920-3060
	R: 5'-AAG GCA CAG AAC CAC CA-3'	
α-1a	F: 5'-RGC CGT CAT CTT CCT CAT-3'	701- 1820
	R: 5'- CGA GGC CGA CGA AGC AG -3'	
α-1b	F: 5'-CCC CHG AGT GGR TSA AGT TCT G -3'	301-1820
	R: 5'- CGA GGC CGA CGA AGC AG -3'	

Ambiguity codes: R, could be G or A; S, could be G or C; H, could be A, C or T; Y, could be T or C; and K, could be G or T.

3.2.3.4 Real-time quantitative PCR (RT-qPCR)

Gene expression was assessed using quantitative real-time PCR on an ABI Prism 7000 sequence analysis system (Applied Biosystems). Primers for all genes were designed using Primer Express software (version 2.0.0, Applied Biosystems; see Table 3.2). RT-qPCR reactions contained 1 µl of cDNA, 5 pmol of each primer, and 10 µl universal SYBR green master mix (Applied Biosystems) in a total volume of 21 µl. Cycling conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was performed following each reaction. Reactions with no cDNA template and with non-reverse-transcribed RNA were included in each plate, enabling determination of the level of background and genomic DNA contamination, respectively. Genomic contamination was always less than 1 in 10315 starting cDNA copies for all templates. A pooled

control, comprising a mixture of 16 gill samples, was used to construct a standard curve for each primer set, and was included in each plate. All results were expressed relative to these standard curves and normalised to the expression of 18s rRNA. This was shown to be a suitable housekeeping gene as its expression in gill, gut and skin did not change significantly following salinity acclimation (data not shown).

Table 3.2: RT-qPCR primers used to assess the expression of NKA isoforms α-1a, α-1b and α-1c, and the reference gene 18s ribosomal RNA in inanga.

Primer	Sequence
α-1a F	5' catcgtaacaggagtggaagagg 3'
α-1a R	5' aagggcgacatctctggaatc 3'
α-1b F	5' catgagagacaagtaccccaaagt 3'
α-1b R	5' actcctccggcgtcacg 3'
α-1c F	5' gacgacaactttgcctccatt 3'
α-1c R	5' tcgaagatcagacggccct 3'
18s F	5' cggtcggcggctaactt 3'
18s R	5' ttgctcaatctcgcgtgg 3'

3.2.3.5 Blood plasma osmolality and Na^+ concentration

Between 15 and 25 µl of blood was withdrawn with a pre-cooled heparinised syringe (~ 16 units ml $blood^{-1}$; lithium salt; Sigma) directly from the dorsal aorta in the caudal region and then kept on ice until centrifugation (~ 30 min). Blood plasma was separated by centrifugation at 13 000 *g* for 3 min at 4°C. A 2 µl plasma aliquot was diluted in 1400 µl of milli-Q water (>18 MΩ; Millipore Synergy® UV; Thermo Fisher Scientific), vortexed and then Na^+ concentration was assessed via flame photometry (Flame Photometer 410; Sherwood Instruments). Plasma osmolality was determined in 10 µl of the remaining sample in a vapour pressure osmometer (Wescor 5520; Wescor Inc.).

3.2.3.6 Muscle water content

Muscle samples from the dorsal region (~ 0.4 g) were excised, placed in pre-weighed pieces of aluminium foil and weighed. Samples were then dried for 48 h in a drying oven at 50°C, transferred to a desiccator for 5 min and subsequently reweighed. All samples were weighed to 0.0001 g precision using an analytical balance (UMX2;

Mettler-Toldeo). Muscle water content was calculated as the difference in weight between wet and dry samples, and expressed as a percentage of total muscle mass.

3.2.3.7 NKA activity

Gill NKA activity was assessed as previously described Chapter II. For ease and rapidity of sampling this assay was conducted on homogenised gill filaments. However, due to the small size of the fish a small proportion of gill arches (~30%) may have also been present. Protein concentration was also quantified as previously described Chapter II (section 2.2.2.5).

3.2.3.8 Phylogenetic analysis

Amino acid sequences were deduced from the nucleotide sequence of each isoform using Genetool Lite. Protein sequences or deduced amino acid sequences of other NKA α subunits were obtained from GenBank using the Entrez Genome Browser as follows: trout (*Oncorhynchus mykiss*) α1a (NM_001124461.1), trout α1b (NM_001124460.1), trout α1c (NM_001124459.1), trout α2 (AY319387.1), trout α3 (AY319388.1), stickleback (*Gasterosteus aculeatus*) α1a (ENSGACT00000018945), stickleback α1a-2 (ENSGACT00000018961), stickleback α2 (ENSGACT00000023416), stickleback α3-1 (ENSGACG00000009524), stickleback α3-2 (ENSGACG00000001959), *Fundulus heteroclitus* α1 (AY057072.1), *Fundulus heteroclitus* α2 (AY057073.1), *Oreochromis niloticus* α1-1 (XM_003446606.1), *Oreochromis niloticus* α1-2 (XM_003446604.1), *Oreochromis niloticus* α2 (XM_003447457.1), *Oreochromis mossambicus* α1(U82549.2) *Oreochromis mossambicus* α3 (AF109409.1), Human (*Homo sapiens*) α1 (X04297.1), Human α2 (BC052271.1), Human α3 (*NM_152296.4*), Norway rat (*Rattus norvegicus*) α2 (NM_012505.2), Norway rat α3 (NM_012506.1), Chicken (*Gallus gallus*) α1 (J03230.1), Chicken α2 (NM_205476.1), and Chicken α3 (NM_205475.1).

Phylogenetic trees were generated from translated protein sequences using MEGA 5.0 (Tamura et al., 2011). Neighbour joining (bottom up clustering), maximum parsimony (based on the assumption of the least evolutionary change possible to explain the phylogeny) and maximum likelihood (evaluates the most statistically probable

phylogenetic tree that explains the data) approaches all generated identical topologies. Bootstrap resampling (5000 replicates) was used to provide estimates of the reliability of the observed topologies. Note that the trees presented contain only the inanga α1 isoforms. Trees were constructed independently for the putative inanga α2 and α3 isoforms because the partial sequences of these isoforms did not overlap sufficiently with the sequences of the α 1 isoforms to allow a single tree to be constructed.

3.2.4 Data treatment and statistical analysis

The differences in isoform gene expression levels between tissues were analysed using a two-way ANOVA (using salinity and tissue as factors) followed by a Holm-Sidak post-hoc test. The mRNA expression of α-1a, α-1b and α-1c isoforms, blood plasma osmolality, plasma Na^+, muscle water content and NKA activity in the time course experiment after seawater transfer were analysed by a one-way ANOVA followed by either a Dunnett's post-hoc test (when comparing against the freshwater value), or by a Bonferroni t-test (for multiple comparisons; Sokal and Rohlf, 1995). When required, data were log-transformed to meet assumptions of normality and homogeneity of variances. All the statistical analyses were carried out using SigmaStat 3.5 software. Normal distribution and homogeneity of variances were tested with Kolmogorov–Smirnov and Levene's median test, respectively. Differences were considered significant with a p value less than 0.05. All data are presented as mean ± SEM.

3.3 Results

Several isoforms of the α and β subunits of the NKA were amplified, identified and partially sequenced from the inanga genome. Three α1 isoforms (a, b and c), which were isolated from gill, gut, kidney and skin were identified. The α-1a isoform was differentiated from the α-1c based on a specific amino acid substitution in the fifth transmembrane domain that has been previously documented by Jorgensen (2008) in *O. mykiss* and *S. salar*. The α-1c identified in inanga was so named as it showed highest similarity to the α-1c in salmonids. The α-1b isoform was identified by its

expression pattern after seawater challenge, a result that was further supported by the phylogenetic analysis. A single α2 subunit was found only in muscle tissue. Two isoforms of the α3 subunit were identified. An α-3a was isolated from gill, kidney and skin tissue, while an α-3b subunit was only found in kidney. Finally, a single β isoform was identified and isolated from gill, gut, kidney, and skin.

Figure 3.1 shows the phylogenetic relationships of the three α-1 subunits from inanga. The inanga α-1 isoforms form a well supported clade along with the α-1 isoforms from other fish species, birds, and mammals. Among fishes there are two major clades within the α-1 isoforms. One clade contains the isoforms named α-1a and α-1b for inanga and the α-1a and α-1b isoforms of rainbow trout, while the other clade contains the isoform named α-1c in inanga and the α-1c isoform of rainbow trout (Fig. 3.1). Similarly, both stickleback and Nile tilapia have an NKA isoform in each of these clades.

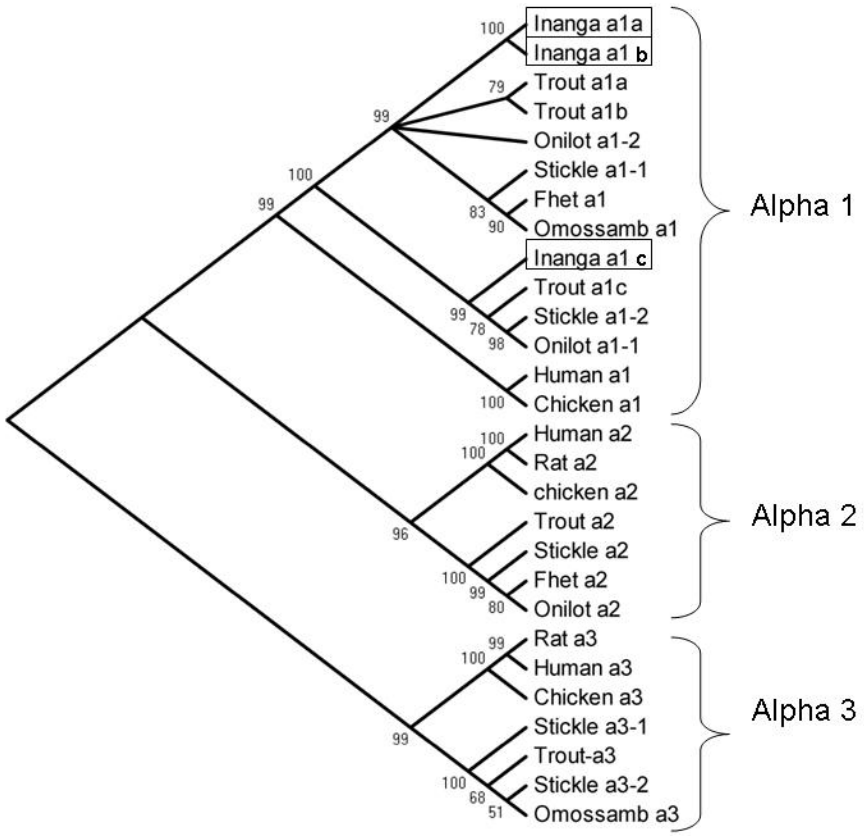

Figure 3.1: Phylogenetic relationships of the three α-1 subunits from inanga (*Galaxias maculatus*) with other fish, bird and mammal species.

3.3.1 Expression of NKA α-1 isoforms in freshwater and seawater

The expression of the α-1a isoform was higher in gills than in gut and skin in freshwater (Holm-Sidak, $p < 0.025$; $p < 0.05$, respectively). However, branchial α-1a isoform expression was significantly down-regulated after 10 d in seawater (Holm-Sidak, $p < 0.05$, Fig. 3.2A) and consequently differences in expression between tissues disappeared. The opposite pattern was observed for the expression of the α-1b subunit. No differences in expression among tissues were evident in freshwater, but after 10 d in seawater the branchial expression of the α-1b isoform was higher than that of gut and skin (Holm-Sidak, $p < 0.017$; $p < 0.025$, respectively, Fig. 3.2B). The α-1c isoform showed an expression pattern similar to that observed for the α-1a isoform, in that expression levels were significantly higher in the gill compared to the gut and skin in freshwater (Holm-Sidak, $p < 0.025$; $p < 0.05$, respectively). However, as a consequence of branchial down-regulation of expression after 10 d in seawater (Holm-Sidak, $p < 0.05$, Fig. 3.2C), these differences disappeared.

3.3.2 NKA α-1 isoform expression after seawater exposure

In agreement with the tissue-specific expression results, changes in mRNA expression of the NKA α-1a, α-1b and α-1c isoforms after seawater exposure were more dramatic in gills (Fig. 3.3) than in gut (Fig. 3.4) and skin (data not shown). Only minor changes in NKA isoform expression were detected in the gut (Fig. 3.4) and no significant changes in NKA isoform expression occurred in skin (data not shown). In gills, the mRNA expression of all α-1 isoforms was affected by challenging inanga with seawater exposure (ANOVA, $p < 0.001$ for α-1a, α-1b, and α-1c). The expression of the NKA α-1a isoform was progressively down-regulated after seawater exposure, an effect that reached a statistically-significant difference from the freshwater expression value after 72 h in seawater (Bonferroni *t*-test, $p < 0.05$; Fig. 3.3A). The expression of the NKA α-1b isoform showed the opposite pattern, exhibiting up-regulation 8 h following seawater transfer (Dunnett's, $p < 0.05$). The expression remained elevated up to 72 h (Dunnett's, $p < 0.05$; Fig. 3.3B) after exposure to seawater, and then decreased back to freshwater expression values. Similar to the α-1a, the α-1c isoform showed a down-regulation after seawater challenge, a value statistically significant

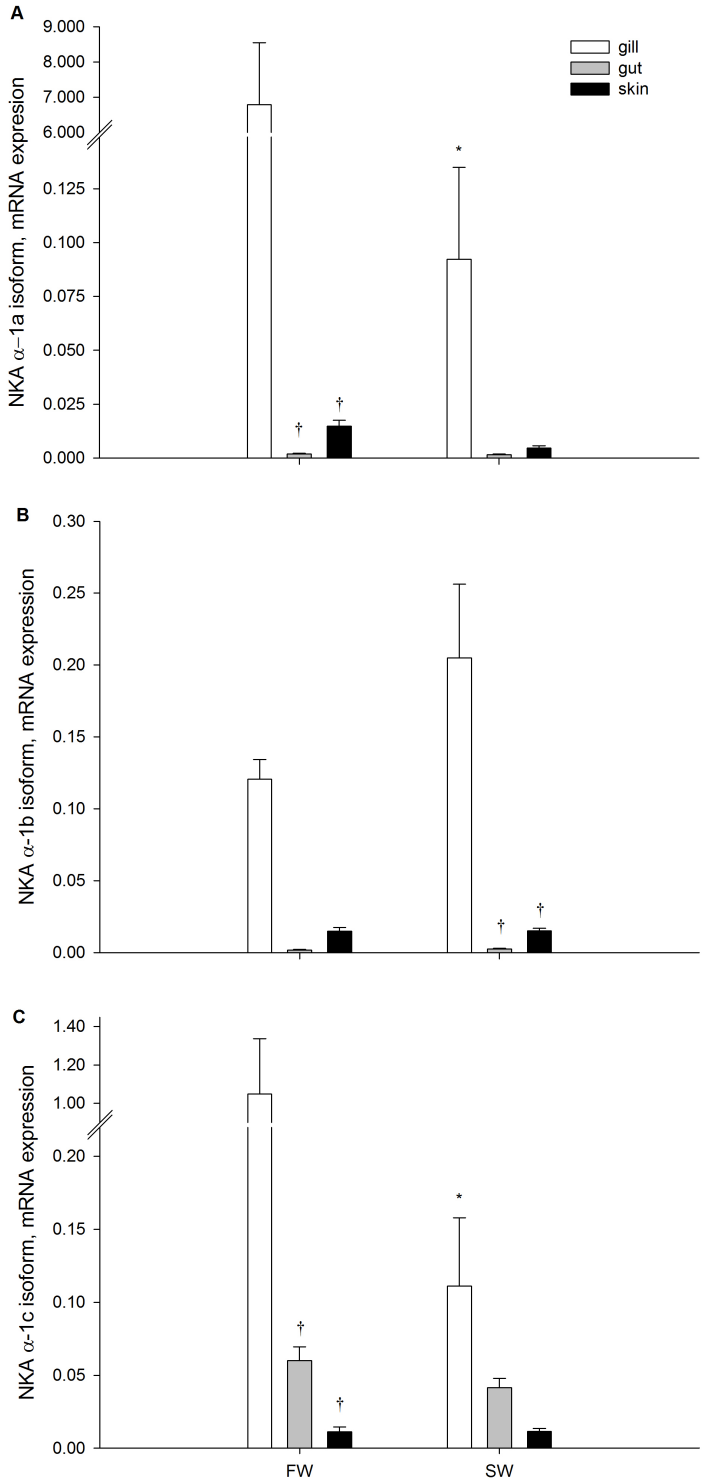

Figure 3.2: Tissue specific distribution of NKA α-1a (A), α-1b (B) and α-1c isoforms (C) in gill (white bars), gut (grey bars) and skin (black bars) in freshwater inanga (FW) and 10 days after transfer to seawater (SW). Plotted points represent absolute mRNA expression, mean ± SEM of 8 fish. Asterisks show statistical differences from the corresponding FW value and daggers show statistical differences from the gill expression value (within a salinity treatment), as determined by a two-way ANOVA, followed by a Holm-Sidak post-hoc assessment, tested at α = 0.05.

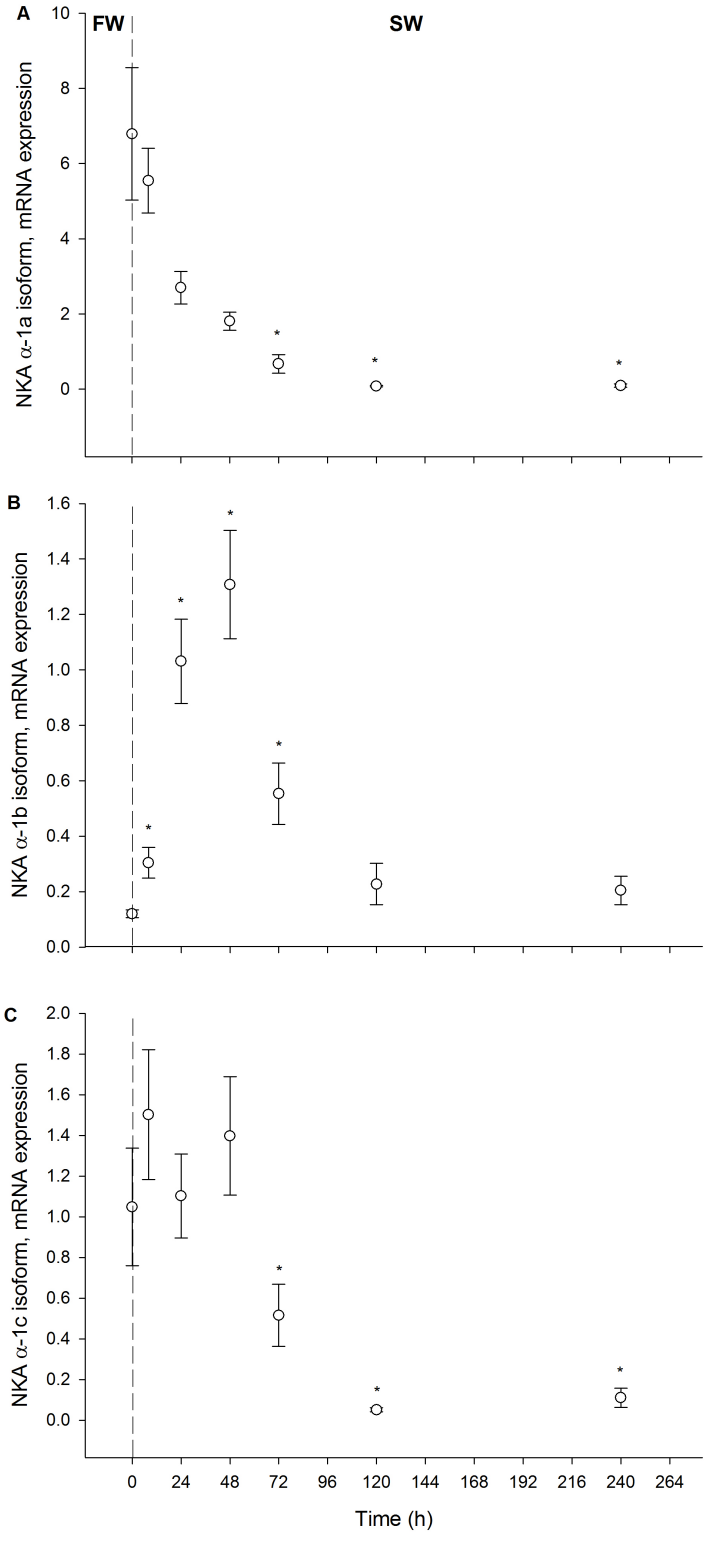

Figure 3.3: Effect of transfer from freshwater (FW) to seawater (SW) on inanga gill mRNA expression of NKA α-1a (A), α-1b (B) and α-1c isoforms (C). Plotted points represent absolute mRNA expression, mean ± SEM of 8 fish. Asterisks show statistical differences from the FW value, as determined by a one-way ANOVA followed by a Bonferroni *t*-test post-hoc assessment, tested at α = 0.05.

from the freshwater value after 72 h in seawater (Bonferroni *t*-test, $p < 0.05$; Fig. 3.3C).

The expression of the α-1a isoform in the gut of inanga exposed to seawater followed a similar trend to that noted for gill (downregulation), although this effect was not statistically significant (ANOVA, $p = 0.272$). NKA α-1b and α-1c isoforms were significantly affected by seawater exposure (ANOVA, $p < 0.01$; $p < 0.001$, respectively. Fig. 3.4), and displayed an expression pattern similar to that exhibited for these isoforms in the gill, α-1b was upregulated and α-1c was downregulated. Although a significant overall effect of seawater exposure on the α-1b isoform expression in gut was detected via ANOVA, the effect could not be attributed to a specific time-point by post-hoc analysis. A similar finding was noted in the expression of the α-1b isoform in skin (data not shown). The NKA α-1c isoform was upregulated after 8 h (Bonferroni *t*-test, $p = 0.03$; Fig. 3.4C), but expression decreased thereafter back to levels observed in freshwater.

3.3.4 Physiological correlates of ion and water balance after seawater exposure

Seawater exposure caused a relatively rapid change in plasma osmolality (ANOVA, $p = 0.016$), plasma Na^+ concentration (ANOVA, $p = 0.003$) and muscle water content (ANOVA, $p < 0.001$). Blood plasma osmolality in freshwater was 274.3 ± 6.4 mOsm kg^{-1}, and by 8 h after seawater exposure this value had increased to 356.8 ± 24.5 mOsm kg^{-1} (Dunnett's, $p < 0.05$), and remained elevated up to 24 h following seawater exposure (Dunnett's, $p < 0.05$; Fig. 3.5A). From 48 h onwards, plasma osmolality decreased to a "seawater acclimated" value of 312.0 ± 0.8 mOsm kg^{-1}, a value that was not statistically different from that in freshwater.

Plasma Na^+ concentration also increased after seawater exposure. In freshwater, plasma Na^+ concentration was 130.3 ± 5.6 mmol l^{-1}, a value that progressively increased to reach 157.6 ± 7.9 mmol l^{-1} after 24 h in seawater (Dunnett's, $p < 0.05$; Fig. 3.5B). After this time point, plasma Na^+ concentration decreased to reach values similar to those in freshwater, and was maintained at this level (129.4 ± 0.2 mmol l^{-1}) for the duration of the seawater exposure.

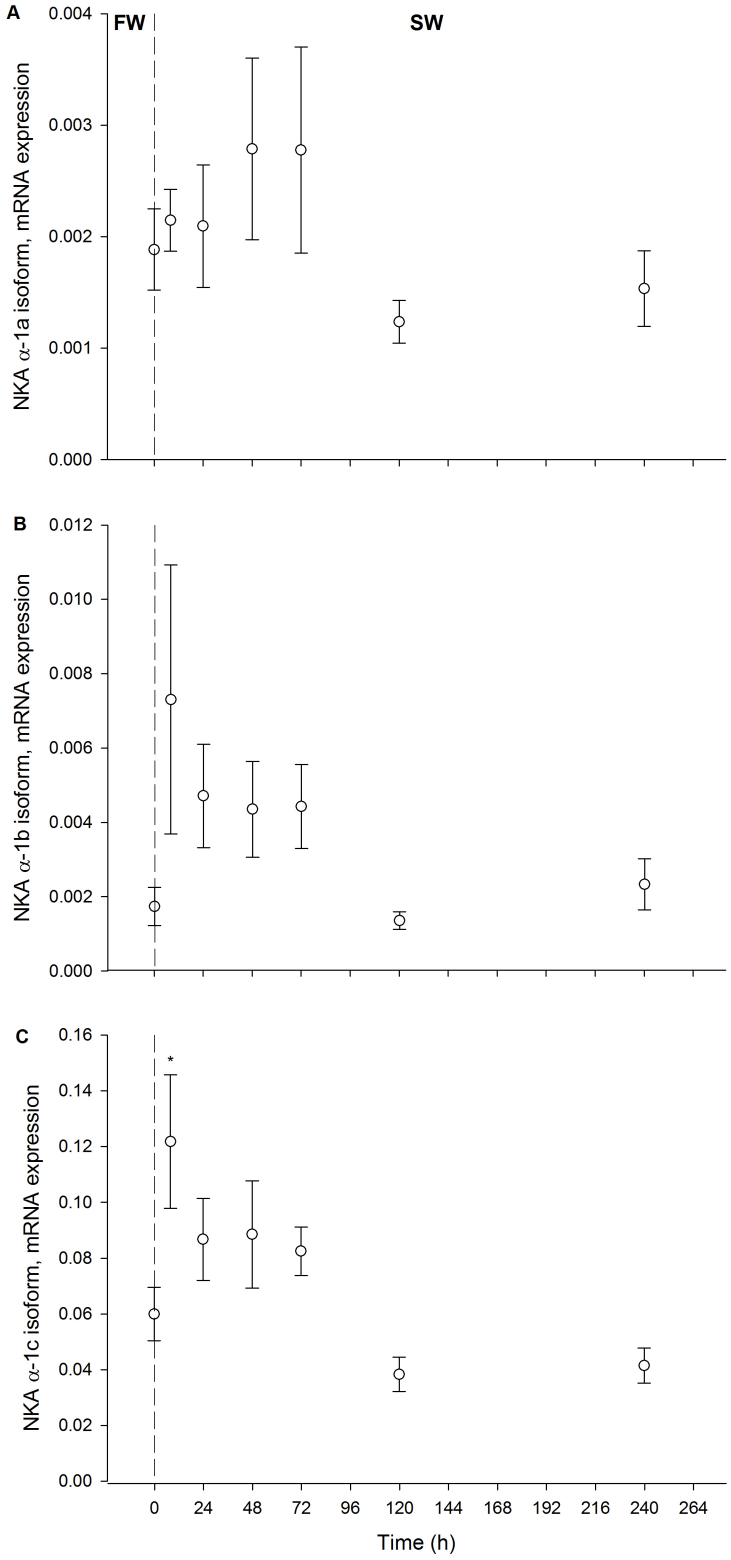

Figure 3.4: Effect of transfer from freshwater (FW) to seawater (SW) on inanga gut mRNA expression of NKA α-1a (A), α-1b (B) and α-1c isoforms (C). Plotted points represent absolute mRNA expression, mean ± SEM of 8 fish. Asterisks show statistical differences from the FW value, as determined by a one-way ANOVA followed by a Bonferroni t-test post-hoc assessment, tested at α = 0.05.

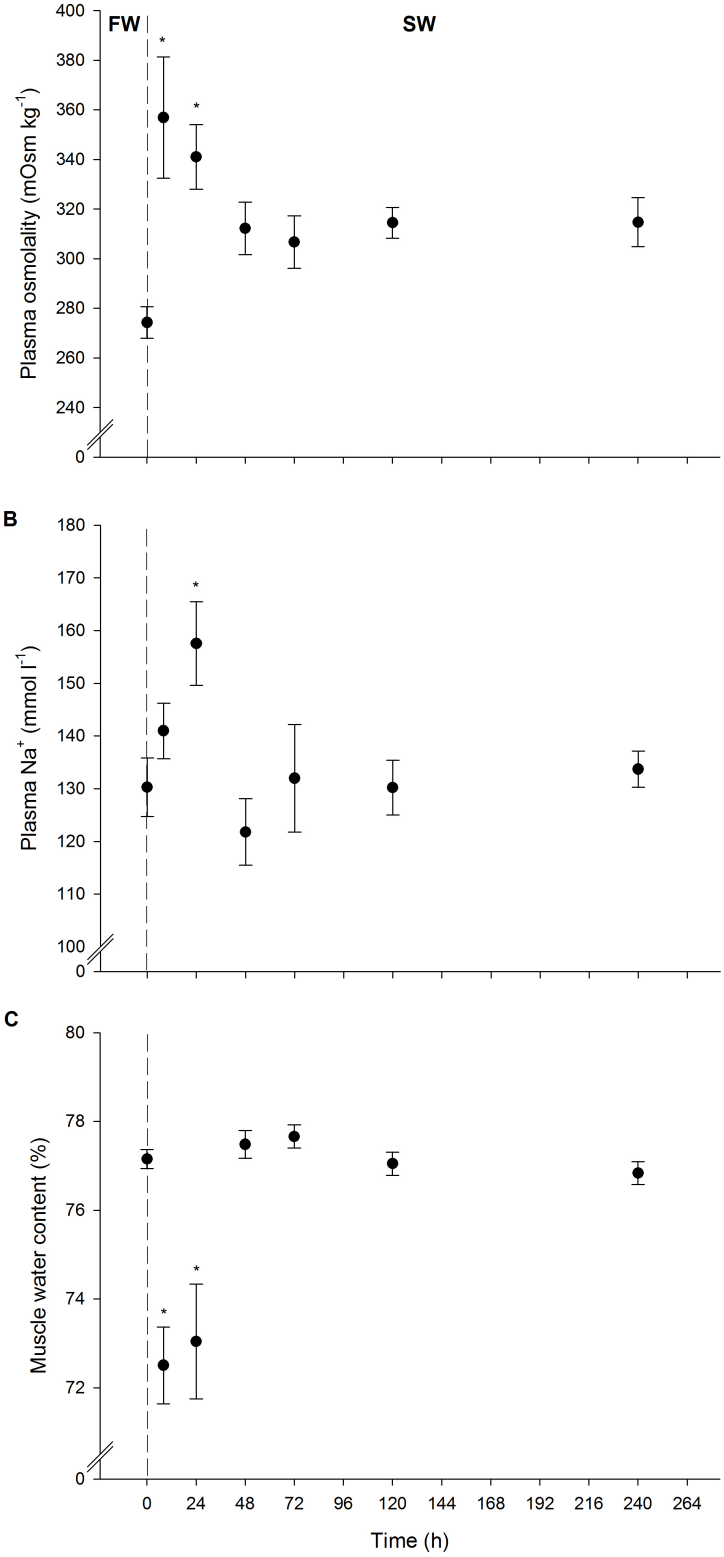

Figure 3.5: Plasma osmolality (A), plasma sodium (B), and muscle water content (C) before (FW) and after direct exposure of inanga to seawater (SW). Plotted points represent, mean ± SEM of 8 fish. Asterisks show statistical differences relative to the FW value, as determined by a one-way ANOVA followed by a Dunnett's post-hoc assessment, tested at α = 0.05.

Muscle water content in inanga after seawater exposure showed the opposite pattern to that exhibited by plasma osmolality and plasma Na^+. In freshwater the water content of inanga muscle was 77.2 ± 0.2%, which decreased significantly to 72.8 ± 0.2 % within 8 h of seawater exposure (Dunnett's, $p < 0.05$), remaining at this level for 24 h (Dunnett's, $p < 0.05$, Fig. 3.5C). Thereafter, the water content of the muscle increased to 77.2 ± 0.02 %, almost identical to its freshwater value.

3.3.5 NKA activity after seawater exposure

NKA activity of the gill was significantly affected by seawater exposure (ANOVA, $p < 0.001$). In freshwater gills the NKA activity was 2.5 ± 0.4 µmol ADP mg protein^{-1} h^{-1}, which rapidly decreased over the first 72 h following seawater exposure, to an average activity of 1.3 ± 0.03 µmol ADP mg protein^{-1} h^{-1}. This decrease was not statistically different from the freshwater value (Bonferroni *t*-test, $p = 0.25$, Fig. 3.6). After 120 h NKA activity increased, reaching a similar activity to the pre-seawater transfer value, then at 240 h following seawater exposure NKA activity had increased to 3.29 ± 0.34 µmol ADP mg protein^{-1} h^{-1}. This value was significantly higher than that observed between 8 and 72 h post seawater exposure (Bonferroni *t*-test, $p < 0.05$, Fig. 3.6), but not statistically different from the freshwater value.

3.4 Discussion

3.4.1 NKA isoforms in inanga

Several NKA subunits and isoforms were expressed in inanga, of which the α-1a, α-1b and α-1c isoforms were differentially-expressed depending on external salinity. Several NKA isoforms have been previously identified in salmonids (Richards et al., 2003; Bystriansky et al., 2006), European eel (*Anguilla anguilla*; Cutler et al., 1995a), notothenioids (*Trematomus bernacchii*; Guynn et al., 2002), mummichog (*Fundulus heteroclitus*; Semple et al., 2002), zebrafish (*Danio rerio*; Rajarao et al., 2001), and cichlids (*Oreochromis mossambicus*; Hwang et al., 1998; Tipsmark et al., 2011). In

the present work, three isoforms of the α-1 subunit were identified, designated as α-1a, α-1b and α-1c, which correspond to the α-1a, α-1b and α-1c in salmonids. A single α-2 subunit with homology to the α-2 in salmonids, and two isoforms of the α-3 subunit, designated as α-3a, α-3b, that share a high level of conservation to the α-3 in salmonids (Richards et al., 2003), were also identified.

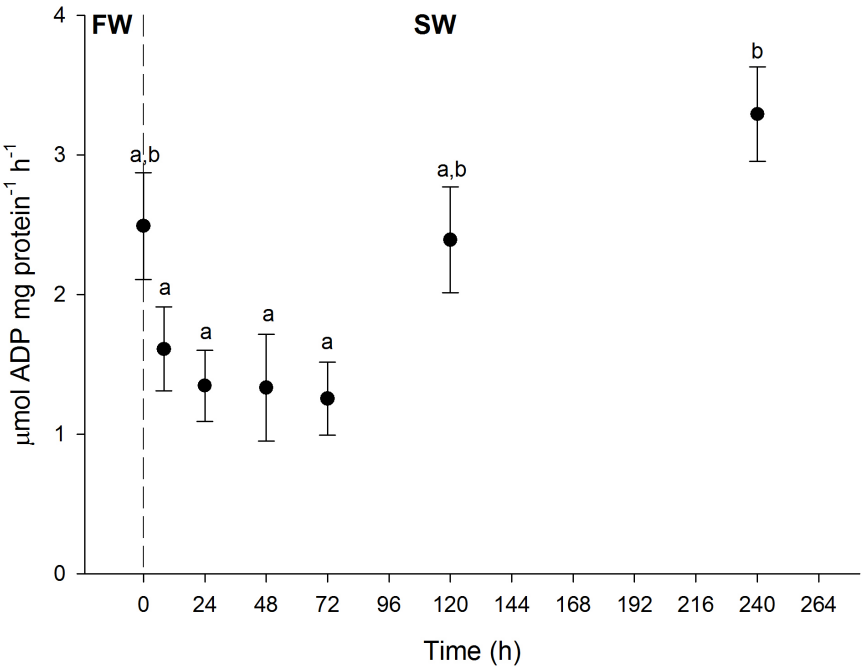

Figure 3.6: Branchial NKA activity before freshwater (FW) and after direct exposure of inanga to seawater (SW). Plotted points represent, mean ± SEM of 8 fish. Values with different letters are significantly different ($p < 0.05$), as determined by a one-way ANOVA followed by a Bonferroni *t*-test post-hoc assessment.

Examining the position of the identified inanga isoforms within the phylogeny, the α-1a and α-1b are members of a large, well-supported clade within the α1 isoforms of fishes. This clade includes the α-1a and α-1b isoforms of rainbow trout and other salmonids that have been shown to undergo the phenomenon of "isoform switching" in response to changes in external salinity. This clade also includes two isoforms from tilapia (here labelled *O. niloticus* α1-2 and *O. mossambicus* α1). Although the "isoform switching" ability of these specific isoforms is unknown, Tipsmark et al. (2011) used two short expressed sequence tag (EST) fragments (GR645170.1 and

GR644771), which were highly similar to the complete *O. niloticus* α1-2 and the *O. mossambicus* α1 sequences, to demonstrate that these isoforms undergo switching in response to salinity change in Mozambique tilapia. Note that these short EST sequences were not included in the phylogenetic analyses because of limited overlap between these sequences and the partial sequences for inanga. However, taken together, these data strongly suggest that the isoforms responsible for the previously observed isoform switching in cichlids and salmonids are located within this one clade. It is important to note, though, that these isoforms do not group together within the clade, suggesting that the phenomenon of isoform switching may have evolved independently in these groups. Alternatively, the lack of grouping could be the result of sufficient reversion and back mutation such that the evolutionary pattern of relationships is no longer detectable in these sequences. Interestingly, in inanga we also observed changes in the expression of the α-1c isoform in response to salinity change. This isoform is not salinity-responsive in trout, where salinity acclimation is only obtained by switching between the expresion of the α-1a and α-1b isoforms. In inanga, the inclusion of the NKA α-1c in the isoform switching phenomenon supports the hypothesis of multiple independent evolutionary origins of isoform switching among fishes, where different fish groups might have developed different variants of the NKA isoform switching phenomenon to reach salinity acclimation.

3.4.2 Molecular responses of NKA isoforms to seawater exposure

Studies in several salmonid species such as *O. mykiss* (Richards et al., 2003; Bystriansky et al., 2006), *S. salar* (Mackie et al., 2005; Bystriansky et al., 2006; Madsen et al., 2009; McCormick et al., 2009), *S. alpinus* (Bystriansky et al., 2006), in the euryhaline cyprinid *F. heteroclitus* (Scott et al., 2004) and in the euryhaline cichlid *O. mossambicus* (Tipsmark et al., 2011) have reported changes in the expression level of NKA isoforms following salinity transfer. It has been shown that after seawater transfer the freshwater isoform α-1a is down-regulated, while the seawater isoform α-1b is up-regulated, suggesting that each isoform has a particular physiological role in maintaining ion homeostasis in these different salinity environments. In the present study, the mRNA expression of all the α-1 isoforms identified in inanga was modulated by seawater transfer, but in differing directions.

This strongly suggests a physiological role of these isoforms in the exceptional salinity acclimation of inanga, supporting the findings of Chapter II.

In agreement with previous studies, the α-1a isoform in inanga was rapidly down-regulated after seawater transfer, suggesting that this isoform plays a specific role in the ion-absorbing phenotype present in freshwater. Also in agreement with previous studies, the α-1b isoform identified in inanga was rapidly up-regulated after seawater transfer. It is anticipated that this isoform may play an important role in ion homeostasis in high salinity. Bystriansky et al. (2006), reported an increase in the mRNA expression of the α-1b NKA isoform after 1, 2 and 4 d of seawater exposure for Arctic char, rainbow trout, and Atlantic salmon respectively. These were the first sampling points for each of these species, so it remains possible that NKA α-1b isoform was up-regulated more rapidly after seawater transfer than these numbers suggest. In the present study, increases in the mRNA expression of the α-1b isoform were evident as soon as 8 h following seawater transfer. This might be indicative of the exceptional osmoregulatory ability of inanga (Chessman and Williams, 1975) and reflect its amphidromous lifestyle.

The inanga NKA α-1c exhibited molecular properties similar to those previously described for the seawater isoform (α-1b) in other fish species (Jorgensen, 2008). However, expression of α-1c was not up-regulated after seawater transfer. Instead the inanga α-1c isoform showed a similar trend to that of the α-1a isoform, and was down-regulated in seawater. Although the expression of the α-1c isoform is rarely evaluated, a previous study reported no changes in its expression level in *O. mykiss* after seawater transfer (Richards et al., 2003). This is therefore the first study reporting salinity-induced changes in the expression of NKA α-1c and suggests a physiological role for this isoform in inanga acclimated to freshwater. The presence of an α-1c isoform in inanga, and its modulation by salinity, might be related to the exceptional salinity tolerance and efficient osmoregulation found in Chapter II. Further research should be conducted in order to elucidate the physiological role of the α-1c in salinity acclimation. Exploring the presence and salinity modulation of homologous α-1c isoforms in other euryhaline species might contribute to our understanding of the exceptional salinity tolerance of euryhaline fish.

Tissue-specific distribution was only measured in three key tissues hypothesised to have a role in osmoregulation (gill, gut and skin), and only for the NKA isoforms proven to have a physiological role in salinity acclimation. Preliminary studies used to survey expression profiles in response to salinity changes highlighted specific patterns of isoform expression. The α-1 isoforms were isolated from gill, gut, kidney and skin tissues, while the α-2 subunit was found only in muscle tissue. This suggests that α-2 functions mainly in a non-osmoregulatory role, which is consistent with the findings of previous studies in rats (Mobasheri et al., 2000) and salmonid fish (Richards et al., 2003). One of the α3 isoforms (α-3a) was found in gill, kidney and skin, while the α-3b subunit was only found in kidney.

Although ion and water balance in fish is achieved by the action of several osmoregulatory tissues, gills are recognised as the osmoregulatory tissue of greatest importance (Evans et al., 2005). Consequently the gill has been the focus of most studies examining the role of NKA isoforms in salinity acclimation. Preliminary data showed a relatively high NKA activity in inanga skin and gut, and therefore we hypothesised that these tissues may also play an important role in the physiological adjustments that occur during exposure to seawater. Analysis of the expression of α-1a, α-1b and α-1c isoforms in inanga gill, gut and skin showed that although each isoform was present in these tissues, all were expressed more highly in the gills, reinforcing the importance of the gill as an osmoregulatory tissue.

3.4.3 Physiological correlates of seawater acclimation

From a survey of plasma osmolalities from ~20 diadromous teleost species, Nordlie (2009) calculated an average plasma osmolality of 311 mOsm kg^{-1} and a mean plasma/serum Na^+ value of 150.0 ± 3.9 mmol l^{-1} in freshwater and an average plasma osmolality of 403 mOsm kg^{-1} and a mean plasma/serum Na^+ value of 173.8 ± 3.8 mM in seawater (Nordlie, 2009). The present data show that inanga has slightly lower than average levels of plasma osmolytes and Na^+ in both freshwater (274.3 ± 6.4 mOsm kg^{-1}, 130.3 ± 5.6 mmol l^{-1}) and seawater (10 days; 312.0 ± 0.8 mOsm kg^{-1}; 133.7 ± 3 mmol l^{-1}). Similar plasma osmolality values were found in Chapter II and also have been reported in an Australian inanga population after a slow seawater acclimation

(Chessman and Williams, 1975). Plasma osmolality and Na^+ did reach higher levels in the present study (348.9 ± 3.1 mOsm kg^{-1}; 157.6 ± 7.9 mM), but only during the "osmotic stress stage", within the first 24 h following seawater exposure. Results from Chapter II showed slightly higher increases in plasma osmolality (411.1 ± 14.2 mOsm kg^{-1}), but after 14 days at a much higher salinity (43‰). Bystriansky et al. (2006) showed that plasma osmolality rose from ~ 315 mOsm kg^{-1} in freshwater to a value of ~415 mOsm kg^{-1} (a ~27% increase) between 2 and 4 days following seawater transfer in Arctic char and rainbow trout. Conversely, these authors reported only a 4% increase in plasma osmolality in *Salmo salar*, a species considered to be a good osmoregulator. It has been also shown that plasma Na^+ levels increase and remain high for 15 days following transfer to 80% SW in *O. mykiss* (Richards et al., 2003). Bystriansky et al. (2006) also found that Na^+ plasma levels remained high after seawater transfer for 30 d in Arctic char, 10 days in *O. mykiss* and up to 4 days in *S. salar*. Overall, inanga displayed relatively small increases in plasma osmolality and Na^+ levels after seawater exposure, and these changes recovered rapidly (< 48 h). As such inanga can be classified as good osmoregulators.

Despite this good osmoegulatory capacity, the present results clearly showed that osmotic homeostasis in inanga was impaired during the first 24 h of seawater exposure. During this "osmotic stress period", the lack of the necessary "ion excreting" molecular architecture (appropriate NKA isoform and accessory transporters) likely impeded the capacity of the fish to balance salt and water exchange. This resulted in passive ion gain and water loss, leading to an increased plasma osmolality and Na^+ concentration. This osmotic stress period, however, is likely to be less dramatic in the natural environment of inanga. In estuaries, for example, salinity might change from nearly 100% freshwater (~7‰) to nearly 100% seawater (~28‰) over a period of six hours (tidal cycle), while in our experiment salinity was quickly changed within 5 minutes. Furthermore, it has been reported in Australia that juvenile inanga slowly migrate from the sea to the estuaries, taking ~5 days, and then taking more than 30 days to complete their upstream migration (Jung et al., 2009). This slow migration is likely to contribute to the progressive acclimation of juvenile inanga for the expected salinity challenges and therefore minimising, or may be even avoiding, this "24h osmotic stress period". However, although no

mortalities occurred during or after this osmotic stress period, this osmotic stress might have other sub-lethal consequences, such as relocation of energy from somatic growth to osmoregulation. A large proportion of the osmoregulation is achieved by active pumping and therefore incurring in energy expenditures, would affect the energy budget available for growth. Interestingly, it have also been reported low growth rates during the first 21 days of migration (Jung et al., 2009), which is likely to be associated to the increased cost of osmoregulation.

In agreement with the increases in inanga plasma osmolality and Na^+ concentration, muscle water content decreased from 77.2 ± 0.2% in freshwater to 72.8 ± 0.2% during the same osmotic stress period (up to 24 h following seawater transfer). This decrease in muscle water content was probably the result of passive water diffusion to the extracellular milieu, caused by increased plasma osmolytes. In sturgeon (*Acipenser naccarii*) a freshwater muscle water content of ~76.5% reduced to ~73.5% in seawater (Martinez-Albares et al., 2002), with the sheepshead minnow (*Cyprinodon variegatus*) displaying a similar change (from 74.5% in freshwater to 72.0% in seawater; Nordlie et al. 1995). Similar changes have also been documented in the marine silver sea bream (*Sparus sarba*), from 77.1 ± 0.3% in freshwater to 75.8 ± 0.5% in seawater (Kelly and Woo, 1999). The magnitude of these changes is similar to that observed for inanga in the present study, and is consistent with a short-term perturbation in osmoregulatory capacity. In inanga this disturbance is then corrected as the molecular machinery switches to adjust to the new salinity environment, as demonstrated by the return in seawater muscle tissue water content back to the pre-transfer (freshwater) value.

3.4.4 mRNA turnover and NKA activity

During the first 72 h period of rapid adjustment in isoform gene expression following seawater exposure there was a trend of decreasing NKA activity. This corresponded with the down-regulation in gene expression of the α-1a and α-1c isoforms and the up-regulation of α-1b isoform expression. Previous research has shown differences in the biochemical activity of NKA in freshwater- and seawater-acclimated fish. Lower K_m values for K^+ and Na^+ were reported in freshwater-acclimated fish compared to

those acclimated to seawater (Pagliarani et al., 1991). Jorgensen (2008) rationalised this change in terms of a switch in NKA isoforms, in particular the result of a lysine substitution in the transmembrane region 5 (TM5) of the α-subunit. This amino acid substitution is believed to alter ion binding sites for Na^+ and K^+ (Jorgensen, 2008) and changes at this locus would therefore support the biochemical differences found by Pagliarani and co-workers (1991). This also implies that the phenomenon of differential isoform expression observed in the present study is likely to confer changes in NKA activity. The trend of a decrease in NKA activity observed during the first 72 h in seawater may therefore represent the isoform switching period (deactivation, degradation and ubiquination of the existing NKA transporters) to an isoform with ion binding characteristics unsuitable for the new environmental salinity, and that the recovery of activity represents the fully fuctioning of an isoform with kinetic properties more favourable for the seawater setting.

All changes at the gene expression level were completed within 120 h following seawater exposure. At this time-point NKA activity had returned to values similar to those exhibited in gills from freshwater inanga. Although a new molecular equilibrium appeared to have been established 5 d following transfer to seawater, it was only after 10 d in seawater that an increase in NKA activity was observed in the present study. An increase in the NKA activity at 43‰ compared to that at 20‰ was also found in Chapter II, after 14 days of acclimation. This could indicate the time required for *de novo* synthesis of the salinity-specific NKA isoform. A similar time lag between mRNA expression level and increases in NKA activity has been previously reported in several salmonid species (Madsen et al., 1995; D'Cotta et al., 2000; Bystriansky et al., 2006). I propose, therefore, that seawater acclimation is first mediated by a rapid initial deactivation, degradation and ubiquination of the existing NKA transporters. Thereafter *de novo* synthesis of the salinity-specific NKA isoforms occurs, taking up to 10 days to re-establish levels of transporters optimal for the new environmental salinity. The mechanism by which plasma Na^+ concentrations returned to freshwater values after 48 h of seawater exposure without changes in NKA requires further research.

Data from present chapter confirms the findings from Chaper II, supporting the concept that inanga are excellent osmoregulators. This ability is the result of rapid

activation of physiological and molecular responses to salinity change. Different isoforms of the α-1 subunit are expressed by inanga and the exact expression profiles of each of these isoforms in the gill (and to a lesser extent also in the gut) are dependent on the environmental salinity. The expression of α-1a, α-1c in freshwater, and of α-1b in seawater is likely to facilitate switching of the osmoregulatory phenotype from ion absorbing in freshwater, to ion excreting in seawater. This is confirmed by patterns of gene expression for these isoforms that match indicators of physiological tolerance to salinity change. These results indicate that differential expression of NKA isoforms in fish is much more widespread than previously recognised, and that this ability is likely to have occurred multiple independent times during the evolution of teleosts. The occurrence of NKA isoform switching in multiple extant teleost fish lineages implies that this phenomenon is of significant importance in facilitating acclimation to changes in environmental salinity.

Chapter IV

A novel oxyconforming response in the freshwater fish *Galaxias maculatus*

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4.1 Introduction

Inanga breed in near-coastal environments and, as adults, inhabit lowland streams. These are both habitats that are threatened by anthropogenic eutrophication and, consequently, hypoxia (McDowell and Wilcock, 2008). Hypoxia represents a significant challenge to organisms, such as fish, that inhabit these waters. Oxygen is the fuel that drives aerobic metabolism, and therefore when oxygen is limited strategies must be developed that allow fish to maintain a balance between ATP production and ATP use. Since aerobic ATP production is much more efficient than anaerobic metabolism, most fish attempt to maintain a constant oxygen consumption rate, both during normoxia, and when exposed to decreasing dissolved oxygen levels. This response is termed oxyregulation (Prosser, 1973). However, at some point the fish will not be able to maintain oxygen consumption, and this measure will therefore decrease linearly as oxygen tension (PO_2) falls, a response termed oxyconformation (Prosser, 1973). The environmental PO_2 below which the fish can not maintain a constant oxygen consumption is known as the critical PO_2 ($PO_{2\ crit}$) (Pörtner and Grieshaber, 1993) and is indicative of hypoxia tolerance. The lower the $PO_{2\ crit}$, the more hypoxia-tolerant a species is considered. For oxyregulators, the dependence of oxygen consumption rate (MO_2) on external PO_2 below $PO_{2\ crit}$ signals the onset of anaerobic metabolism (Pörtner and Grieshaber, 1993). In animals that oxyconform the diminishing MO_2 with declining environmental PO_2 suggests that anaerobic metabolism may be used to meet energy requirements, or that these species exhibit metabolic suppression whereby certain functions are turned down, or even switched off as environmental PO_2 declines (Hochachka, 1986; Lutz and Storey, 1997).

In Chapter II the oxygen consumption of inanga was examined as function of environmental salinity, showing that salinity had no statistically-significant effect on inanga oxygen consumption. The examination of fish oxygen consumption as a function of other environmental stressors, such as low PO_2, is also important for understanding the capacity of fish to respond to environmental changes. The vast majority of literature shows that teleost fish are oxyregulators (Ultsch et al., 1981; Perry et al., 2009). There have been a few reports of fish that exhibit both oxyregulating and oxyconforming patterns, with the strategy adopted depending upon

factors such as water flow (Hall, 1929; Hughes and Umezawa, 1968; Piiper et al., 1970; Steffensen et al., 1982; McKenzie et al., 2007). Historical reports of oxyconforming in fish (e.g. Hall, 1929; Marvin and Heath, 1968; Piiper et al., 1970) have been questioned based on experimental design and/or methodological limitations (Prosser, 1973; Ultsch et al., 1978). To my knowledge there is no unequivocal study illustrating obligate oxyconforming in a teleost fish.

In the present chapter, a series of experiments were conducted in order to determine the $PO_{2\ crit}$ of inanga. Using a variety of approaches to overcome methodological artifacts associated with determining oxygen consumption under laboratory conditions, it was determined that inanga are oxyconformers, and that there is no evidence that these species oxyregulate.

4.2 Materials and methods

4.2.1 Fish and rearing conditions

Juvenile and adult inanga were caught, transported to the aquarium facility in the School of Biological Sciences at the University of Canterbury and maintained as described in Chapter II (section 2.2.1). No mortalities were recorded during the acclimation period. Food was withheld two days before the start of experimentation.

4.2.2 Experimental design

In all the respirometry experiments described below, control chambers (i.e. lacking fish), flow rate and normoxic conditions during acclimation, and temperature were identical to those described in Chapter II (section 2.2.2.2). However, the acclimation period used here was longer and individual fish were left undisturbed to acclimate to the respiratory chambers for at least 12 h prior to the start of experiments. Respirometers, electrodes, and calibration were identical to those described in Chapter II (section 2.2.2.2).

4.2.2.1 Series 1: Oxygen consumption determination by closed respirometry

Following the methodology described in Sloman et al. (2006), oxygen consumption of inanga was determined in 67 fish (0.1-4.5 g). Individual fish were placed in respirometry chambers of 0.1, 0.3 or 0.5 l volume, depending on fish size, and chambers were submerged in a controlled-temperature bath at 14°C ± 0.1°C. Gill ventilation and body movements kept the water well mixed. After acclimation, the chamber was sealed and fish were left to deplete the oxygen concentrations inside the respirometry chamber. Respirometry was stopped when fish exhibited signs of distress, a process that took between 4 and 10 h (~4.5 kPa). Oxygen concentration was monitored throughout the experiment by sampling 0.7 ml of water at 30 min intervals and determining oxygen content via a 781 oxygen meter (Strathkelvin®, Glasgow, Scotland). The number of data points obtained per individual fish varied from 9 to 20.

4.2.2.2 Series 2: Oxygen consumption determination by semi-closed respirometry

Owing to data suggesting that different respirometry techniques can induce different oxygen consumption responses (Burggren and Randall, 1978), a second experiment was conducted. Oxygen consumption by semi-closed respirometry was evaluated in a distinct set of seven fish (mean weight 1.7 ± 0.5 g). Fish were individually placed in a glass respirometry chamber with a volume of 135 ml, and oxygen consumption was continuously recorded by placing a Strathkelvin oxygen electrode inside the chamber. As for Series 1 (section 4.2.2.1), temperature was maintained at 14°C by placing the chamber in a controlled-temperature water bath. Recordings were made over 15 min intervals, at dissolved oxygen levels of 18.3, 14.5, 10.3, 8.0, 4.7, 3.7 and 3.0 kPa. The chamber was connected to a reservoir of water such that following oxygen consumption measurement at one dissolved oxygen level, the chamber could be slowly flushed (8 min for complete exchange) with water that had been pre-equilibrated at the next experimental oxygen level. After a 2 min equilibration, the chamber was sealed and oxygen consumption was determined at the new PO_2. Stopping or starting the water flow through the respirometry chamber had no evident effect on fish behaviour. Oxygen consumption was measured starting at the highest

dissolved oxygen level (representing normoxic water; 18.3 kPa) and proceeded in sequence through to the lowest, hypoxic, level (3.0 kPa). At the conclusion of this series, a second determination of oxygen consumption at normoxia was carried out. As the fish oxygen consumption rate did not differ between the two normoxic treatments (repeated measures ANOVA, $p > 0.1$), these values were averaged.

4.2.2.3 Series 3: Oxygen diffusion across the head and body compartments in hypoxia

A partitioned respirometry chamber was used to separate the head (forward of the pectoral girdle; containing gills) and body (skin) of inanga in order to determine the oxygen consumption of each compartment (Chapter II, section 2.2.3.2). The total volume of the chamber was 90 ml, with the head compartment having a volume of 35 ml and the body 55 ml. A total of seven fish were subjected to partitioned respirometry (mean weight 2.5 ± 0.5 g). Oxygen tensions were manipulated such that the head of the fish was exposed to normoxic water (~19.5 kPa), while the body portion was exposed to hypoxic water (~2.6 kPa). The water dissolved oxygen levels were then switched such that the head was hypoxic and the body was normoxic. This was performed in order to evaluate the hypothesis that under aquatic hypoxia the fish may be losing oxygen via diffusion by either the skin or the across the gills.

Fish were gently placed in the rubber dental dam, and then in the partition chamber as decribed in Chapter II. After placement fish were left in the unsealed respirometer and left to acclimate for at least 2 h while the chamber was immersed in a larger volume of aerated freshwater. Thereafter the compartments were filled with water at a PO_2 of either 19.5 kPa (normoxia) or 2.6 kPa (hypoxia), and then sealed. A small hole (3 mm) at the top of each compartment allowed the insertion of an oxygen microelectrode as described in Chapter II (section 2.2.3.2). Preliminary experiments showed that routine metabolic rates were established within one hour of handling, and that the system was hermetic from both the exterior of the chamber and between compartments. Three euthanised fish were used as controls.

4.2.3 $PO_{2\ crit}$

For this study, $PO_{2\ crit}$ was defined as the lowest PO_2 that a fish can maintain its MO_2, which is the point where the fish ceases to oxyregulate. Since complications for determining $PO_{2\ crit}$ are well known (see discussion of this chapter) and different data analyses could lead to different results, two distinct approaches for calculating $PO_{2\ crit}$ were used for *Series 1*. All individual data points for fish MO_2, expressed as µmol O_2 g^{-1} h^{-1}, were plotted against the specific PO_2 at which they were obtained. Data were then also collated, averaged and plotted against 12 different PO_2 values, spaced evenly every 1.6 kPa. For the data from *Series 2* fish, MO_2 values, expressed as µmol O_2 g^{-1} h^{-1}, were averaged at each of the experimental PO_2 values. In an attempt to calculate $PO_{2\ crit}$ both the pooled fish averages and the values for each individual fish were used. These analyses yielded very similar results in that neither permitted the calculation of $PO_{2\ crit}$.

4.2.4 Data treatment and statistical analysis

Individual raw data values were subjected to incremental regression analyses (SigmaPlot ver. 11.0; Systat Software), in order to determine the best fit for the data. This analysis evaluated each polynomial order equation starting at zero and then increasing to the third order. This permitted a mathematical assessment of whether the data best fitted a single linear relationship (i.e. the fish were oxyconforming), or whether a $PO_{2\ crit}$ value could be determined as the intersection point of two distinct linear relationships (one at hypoxic oxygen concentrations, the other at normoxic; i.e. oxyregulation). The best fit was chosen on the basis of the *p*-value of incremental analysis. All data subjected to this analysis were initially tested for normality and homogeneity of variances by a Kolmogorov-Smirnov and Levene's median test respectively. Fish MO_2 values from *Series 1* (collated) and from *Series 2* were evaluated by Friedman repeated measures ANOVA, using PO_2 as a factor. MO_2 /oxygen diffusion rates of the head and body portions at normoxia and hypoxia (*Series 3*) were evaluated by a Friedman two-way repeated measures ANOVA. In both cases normal distribution and homogeneity of variances were tested by a Kolmogorov-Smirnov and Levene's median test, respectively. Differences among treatments were tested by a Holm-Sidak post-hoc test (Sokal and Rohlf, 1995).

Results were considered significant with a *p*-value lower than 0.05. All data are presented as means ± standad deviation of the mean (SD).

4. 3 Results

4.3.1 Patterns of oxygen consumption with decreasing ambient oxygen

Although fish moved intermittently within the respirometry chambers used for the determination of MO_2, they were apparently unstressed during the experiments (*Series 1* and *2*), and only exhibited evident signs of distress at the lowest PO_2's tested. Furthermore, no changes in activity were noted during the experiment. In *Series 1* (closed respirometry) the metabolic rate of inanga decreased linearly with decreasing external PO_2. This trend was the same irrespective of whether individual data points or pooled averages were examined (Fig. 4.1).

Figure 4.2 uses the same data presented in Figure 4.1 but follows the relationship between PO_2 and MO_2 for individual fish across the range of PO_2's tested. This figure includes data for all fish used, which varied in mass from 0.1 to 4.5 g, and shows that there was no evidence of a scaling effect in the relationship between MO_2 and external PO_2. The routine metabolic rate of inanga measured by closed respirometry did not differ from the metabolic rate obtained by semi-closed respirometry at any PO_2 level (all p values > 0.064, Fig. 4.3). In both closed and semi-closed respirometry, the routine metabolic rate of inanga decreased as external PO_2 was reduced (both $p < 0.001$, Fig. 4.3).

Inanga mass-specific MO_2 decreased to 83.5% of the normoxic value at 14.6 kPa ($p < 0.05$) in closed respirometry, and in semi-closed respirometry mass-specific MO_2 decreased to 79.0% of the normoxic value at 14.5 kPa ($p < 0.05$, Fig. 4.3). Thereafter, fish MO_2 continued to decrease as PO_2 was reduced, and at 3.7 kPa MO_2 had decreased to 33.9% of the normoxic value ($p < 0.05$) in closed respirometry. A similar

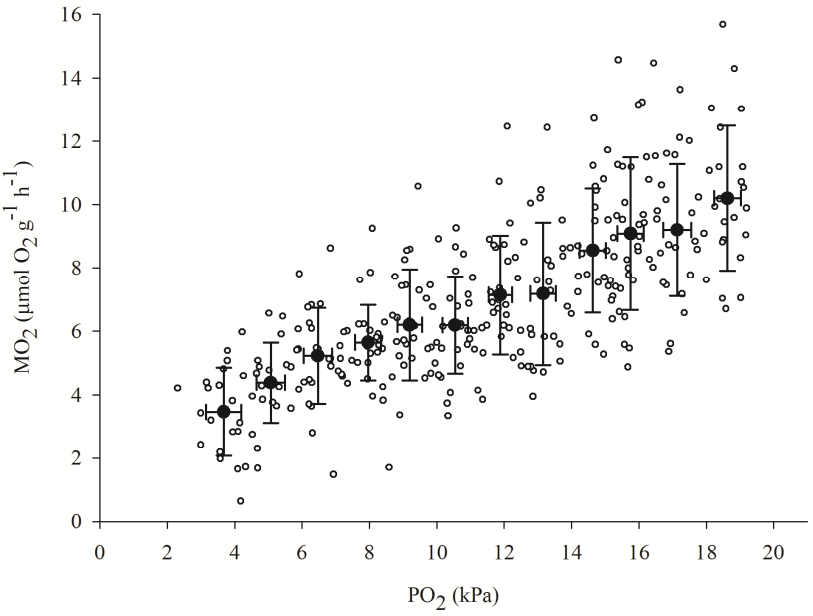

Figure 4.1: Routine MO_2 of inanga as a function of decreasing PO_2 determined via closed respirometry, graphed as all individual measurements. Individual data points are represented by unfilled points, with mean data (± SD) represented by filled points. The graphed data represents measurements made from 67 fish.

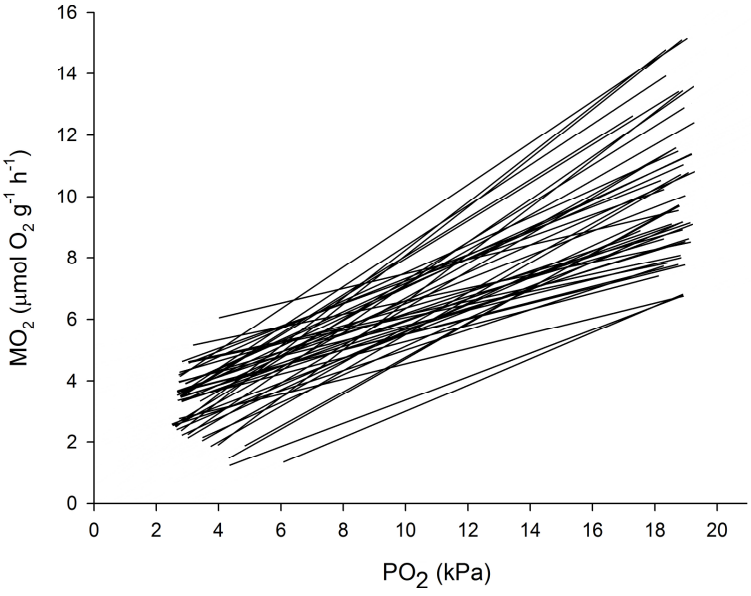

Figure 4.2: Routine MO_2 of inanga as a function of decreasing PO_2 determined via closed respirometry, best-fit linear regression line for individual fish. The graphed data represent measurements made from 67 fish.

value was attained in semi-closed respirometry at 3.0 kPa, where the fish reduced its MO_2 to 33.3% of the normoxic value ($p < 0.05$, Fig. 4.3).

The best-fit curve to describe the relationship between MO_2 and external PO_2 was a straight line for both individual ($r^2 = 0.52$) and pooled values ($r^2 = 0.98$) from *Series 1* (both $p < 0.001$, Fig. 4.1). Furthermore, there was no statistical support for justifying two lines in the plot (i.e. no break-point where a regulation pattern was superseded by a conforming pattern), using all data points (incremental analysis, $p = 0.457$) or using average values (incremental analysis, $p = 0.447$). A similar result was obtained using semi-closed respirometry. There was no statistical basis for defining two curves in the plot (incremental analysis, $p = 0.071$), and again the best-fit curve when regressing the data was a straight line ($p < 0.001$, $r^2 = 0.77$, Fig. 4.3).

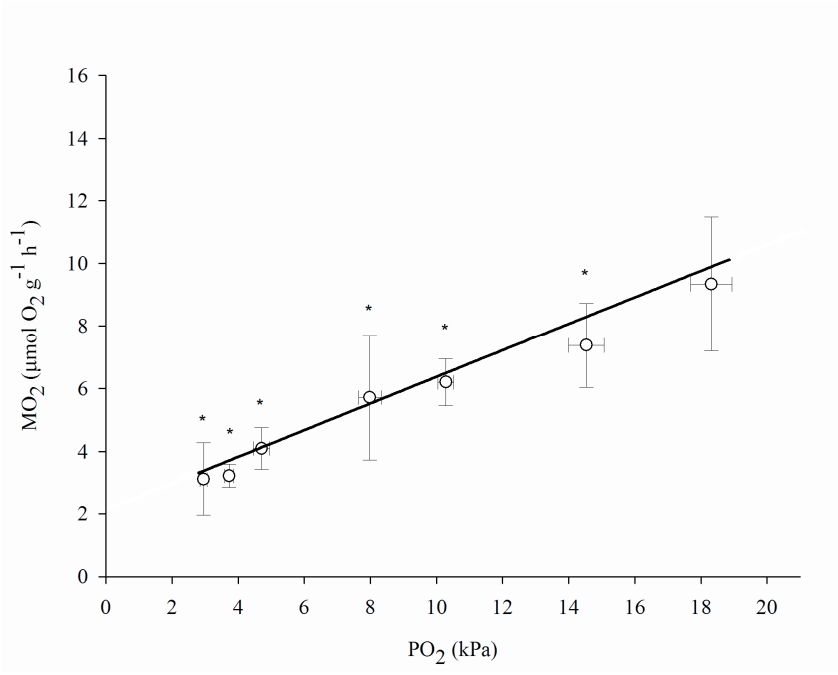

Figure 4.3: Routine MO_2 of *G. maculatus* as a function of decreasing PO_2. Data from closed (best fit regression line, data from Fig. 4.1) and semi-closed respirometry (empty circles). Data from semi-closed respirometry presented as means ± SD, and asterisks indicate significant differences from MO_2 at normoxia.

4.3.2 Diffusion of oxygen across the skin and gills in hypoxia

Oxygen uptake in inanga is achieved by a combination of both gills and skin (Chapter II). When both sections of the fish were independently exposed to normoxic water (~19.5 kPa), rates of 6.3 ± 1.1 and 2.9 ± 0.6 μmol O_2 g^{-1} h^{-1} were attained for the head and the body, respectively (Fig. 4.4). Almost identical values were attained for concurrent exposure of both compartments to high dissolved oxygen (data not shown). This indicates that 69% of total oxygen uptake occured via the head (gills) and 31% by the body (skin) when the fish was in normoxia. Conversely, when sections were exposed to hypoxia (~ 2.6 kPa) MO_2 was significantly reduced relative to the same compartment in normoxia (Holm-Sidak post-hoc, $p < 0.05$, Fig. 4.4), consistent with the observations made in Figures 4.1 and 4.2. There were no significant differences in oxygen consumption between the two compartments under these hypoxic exposure conditions. When either the head or the body was exposed to normoxia, and the other compartment was hypoxic, there was no net loss of oxygen from either the head or body to the ambient water across the hypoxic compartment (Fig. 4.4). In the controls without fish, there was no exchange of oxygen between the two compartments or between the respirometer and the environment.

4.4 Discussion

In this chapter data showed that inanga were not able to maintain their normoxic oxygen consumption rate when external PO_2 was decreased, but instead oxygen consumption rate was decreased as external PO_2 declined. This dependence of the oxygen consumption rate upon external PO_2 classifies inanga as an oxyconformer.

4.4.1 Metabolic rate and external PO_2

The routine metabolic rate of inanga at normoxia was consistent across the different experimental methods used (closed respirometry, 10.2 ± 2.3 μmol O_2 g^{-1} h^{-1}; semi closed respirometry, 9.3 ± 2.1 μmol O_2 g^{-1} h^{-1}; and partitioned head + body portions, 9.2 ± 1.3 μmol O_2 g^{-1} h^{-1}). These values are also in agreement with results from Chapter II (11.07 ± 0.24 μmol O_2 g^{-1} h^{-1}).

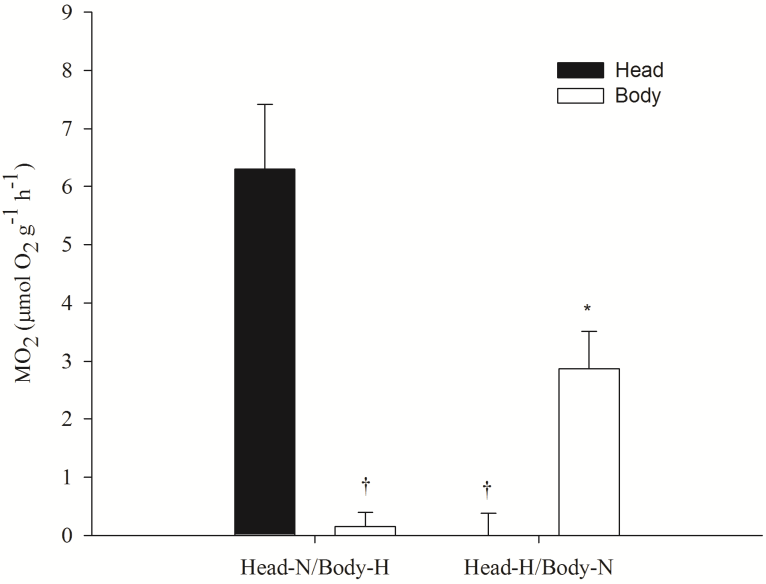

Figure 4.4: Partitioned oxygen consumption of inanga. The head was immersed in normoxic water (~19.5 kPa) while the body was immersed in hypoxic water (~2.6 kPa) (Head-N/Body-H), and then the body was immersed in normoxic water while the head was immersed in hypoxic water (Head-H/Body-N). Data are presented as mean ± SD. Asterisks indicate statistical differences ($p < 0.05$) between body portions at a given PO_2 (either normoxia or hypoxia), and daggers indicate statistical differences between PO_2 treatments within compartments (either head or body).

When exposed to decreasing external PO_2 the majority of vertebrates oxyregulate, maintaining MO_2 constant until a critical PO_2 is reached. At this point oxygen consumption rates decline. Co-ordinated functioning of respiratory and circulatory systems mediate O_2 and CO_2 exchanges in fish and ensure that oxygen uptake and tissue delivery are maintained (Prosser, 1973; Burggren and Randall, 1978). There are, however, reports of fish that do not maintain a constant MO_2, and whose metabolic rates instead scale with environmental oxygen levels (i.e. oxyconforming; see discussion in *Methodological approach* below). However, the results of the current study indicate that inanga do not regulate MO_2, but instead have metabolic rates that conform to the oxygen content of the waters in which they reside. The mechanisms that facilitate this metabolic control and the implications for fish behaviour deserve further investigation.

4.4.2 Methodological approach

Previous reports of oxyconforming in fish have been questioned owing to methodological limitations. For example, Burggren and Randall (1978) reported oxyconforming in the sturgeon *Acipenser naccarii* when the fish were assessed under conditions where movement was restricted. However, recently McKenzie et al. (2007) examined MO_2 in sturgeon that were gently exercised and showed that these fish oxyregulated, and they were able to derive a $PO_{2\ crit}$ of 4.46 kPa. Although the sturgeon is not a ram ventilator, it is nevertheless hypothesised that swimming might make a significant contribution to oxygen uptake in this species (McKenzie et al., 2007). The importance of ram ventilation on oxygen uptake in inanga has not been evaluated, but it is unlikely to have a significant effect on the response to declining environmental oxygen. In fact, adult inanga generally inhabit still or gently flowing waters (Jowett, 2002). Furthermore, the respirometry chambers used for *Series 1* and 2 were approximately 60 times the volume of the fish, and allowed fish to move during the experiments. This would have afforded them the capacity to regulate by increasing flow across the gills, if this was a necessary component of their response to declining PO_2. However no evidence of increased activity was found until the lowest PO_2 levels were reached.

In the present study two distinct methodologies for examining MO_2 as a function of environmental oxygen content were used. Both closed and semi-closed respirometry provided evidence for oxyconforming in inanga. A potential limitation of closed respirometry is the accumulation of CO_2 as a waste product of metabolism. In many vertebrates CO_2 has a well-described role in controlling the respiration reflex, and increases in CO_2 levels may lead to an increase in MO_2 (Pörtner et al., 1998). In fish, however, the influence of CO_2 on MO_2 appears to vary on a species by species basis (for reviews see: Gilmour, 2001; Ishimatsu et al., 2008). The fact that both closed (where CO_2 build-up could have occurred) and semi-closed respirometry gave identical results suggests that alterations in dissolved CO_2 levels are unlikely to have influenced the observations made.

4.4.3 Why do inanga oxyconform?

Gills perform the majority of oxygen uptake in most fish. Respiratory control is mainly achieved by regulation of water flow over the gill epithelium via increasing opercular frequency and/or ventilatory volume. In a few species skin also plays an important role in gas exchange (Kirsch and Nonnotte, 1977; Meredith et al., 1982; Graham, 1997; Maxime et al., 2000). Inanga appear to rely significantly on the skin as a respiratory tissue, with approximately 31% of oxygen uptake in normoxia occurring across the cutaneous surface (Chapter II). This uptake would be mainly mediated by the diffusion gradients between the external and internal media.

It is hypothesised that the importance of cutaneous uptake might have a role in the unique oxyconforming pattern observed in inanga. The skin surface of galaxiids is scaleless (Dean and Richardson, 1999), an adaptation that clearly facilitates gas exchange. Though many fish take up oxygen through the skin (Kirsch and Nonnotte, 1977; Graham, 1997), Nonnotte (1981) showed that of six freshwater teleosts, only one, the scaleless catfish *Ictaluras melas*, showed a small, net transcutaneous flux. In the other species the metabolism of the cutaneous tissues themselves consumed any oxygen crossing the skin. The small size and elongate form of galaxiids provides a much higher surface area to volume ratio than any of the species measured by Nonnotte. In the Canterbury mudfish, closely related to inanga, cutaneous oxygen uptake is proportionately one of the highest of any aquatic vertebrate (Meredith et al., 1982; Feder and Burggren, 1985).

It is not known if oxygen uptake via the skin is regulated in inanga as it is in Anura (Burggren, 1988), or in the tail of other fish species (Cooper et al., 2012). If peripheral blood perfusion can be altered to maximise cutaneous exchange, such a mechanism is unlikely to be comparable in magnitude to the control over uptake exerted by alterations in gill perfusion and irrigation. It could therefore be hypothesised that a fish that depends significantly on the skin for oxygen uptake would have less control over oxygen uptake and therefore less capacity to oxyregulate. Supporting this hypothesis is research examining the response of the polychaete *Nereis pelagica* to changing environmental oxygen levels (Tschishka et al., 2000). In this animal the reliance on the body surfaces for oxygen uptake was concluded to be the major driver

for the observed oxyconforming strategy. Further studies are underway on other galaxiid fish to determine if they also oxyconform (R. White, pers. comm.).

Another possible explanation for the oxyconforming strategy observed in inanga could be that the relatively high permeability of their skin to oxygen resulted in an equilibration between the oxygen content of their body and that of the environment. This was tested in *Series 3*, where the head (containing the gills as a gas exchange surface), and the body (skin as a gas exchange surface) were isolated. The exposure of the head to normoxia did not result in an increase in ambient oxygen content in the hypoxic body compartment, and the same was true when the oxygen profiles were swapped so that the body was normoxic and the head hypoxic. This suggests that diffusive loss of oxygen is unlikely to explain oxyconformation in inanga.

The skin of inanga appears to play an important role in oxygen uptake (Chaper II and Fig. 4.4). This capacity for cutaneous gas exchange may explain the observation of oxyconforming in this fish. Inefficient oxygen removal by the gills, and a greatly reduced skin oxygen uptake, both caused by a reduction in the diffusion gradient, could be reasons why this fish is unable to regulate oxygen consumption as ambient PO_2 decreases.

Chapter V

Relationship between fish size and metabolic rate in the oxyconforming inanga, *Galaxias maculatus*, reveals size-dependent strategies to withstand hypoxia

5.1 Introduction

The metabolic rate of an animal is the summation of multiple physiological processes, and can be influenced by numerous intrinsic and extrinsic factors. Among intrinsic factors body mass is known to be important. The effect of body mass on metabolic rate, and its implications for bioenergetics from the individual level through to the population scale are well recognised (Nagy, 2005; Bangert et al., 2008). For example, quantification of metabolic rate allows precise predictions of the maximum stocking densities, water flow, and feeding requirements in aquaculture (Sims, 1996; Encina-Montoya et al., 2011).

The scaling of resting metabolic rate (VO_2) with body mass (W) can be described by an allometric equation, $VO_2 = a\ W^{\ b}$, where *b* represents the scaling exponent and *a* is the intercept. For several decades the numerical value of this scaling exponent has been subject to debate. Values of 0.67 (based on the surface area to volume relationship; White and Seymour, 2003) and 0.75 (based on transport through a branching network; West et al., 1997) are the most common theoretically-supported values for the scaling exponent, although recent studies suggest that the value is species-specific and oscillates between 0.5 and 1 (Bokma, 2004; Glazier, 2005). In general, the measured relationship between body mass and metabolic rate in teleost fish ranges between 0.79 and 0.88 (Winberg, 1960; Brett and Groves, 1979; Goolish, 1995; Peters, 1983; Clarke and Johnston, 1999; White et al., 2006).

Body size is not the only factor that affects metabolic rate, as extrinsic factors such as environmental temperature and oxygen availability can also exert an effect (Lardies et al., 2004; Everett and Crawford, 2010; White et al., 2012). Oxygen availability can be a particularly important factor shaping metabolic rate in aquatic animals given the variability of oxygen in water bodies, and the potential for this to directly impact MO_2. Consequently for animals such as fish, metabolic rate may be dependent on oxygen uptake strategies, and how effectively these deal with environmental oxygen fluctuations. Furthermore, oxygen uptake strategies may differ with fish size (Sloman et al., 2006). Metabolic rates in fish may therefore represent the interplay between

intrinsic factors such as size, and extrinsic factors such as environmental oxygen (Verberk and Bilton, 2011; Verberk et al., 2011).

The interaction between size and hypoxia tolerance in fish has been previously investigated (for a review see Nilsson and Ostlund-Nilsson, 2008). Evidence to date generally suggests that bigger fish are better equipped than smaller fish to tolerate periods of suboptimal oxygen conditions. This is attributed to larger stores of glycogen to fuel anaerobic metabolism and greater reservoirs for accumulation of toxic anaerobic end products (Almeida-Val et al., 2000; Sloman et al., 2006; Nilsson and Ostlund-Nilsson, 2008; Everett and Crawford, 2010). Arguing against this pattern, however, is evidence of higher hypoxia-related mortality with increasing fish size (sharpsnout seabream, *Diplodus puntazzo*; Cerezo and Garcia, 2004) and inhibition of ingestion rate at higher dissolved oxygen levels in larger fish compared to smaller conspecifics (Nile tilapia, *Oreochromis niloticus*; Tran-Duy et al., 2011). Behaviourally, it also has been shown that larger fish avoid hypoxic waters before small fish (Burleson et al., 2001; Robb and Abrahams, 2003; Sloman et al., 2006). Consequently there remains controversy regarding the influence of body size on tolerance and responses to hypoxia in fish.

Inanga inhabit waters that are increasingly prone to eutrophication and, consequently, hypoxia (McDowell and Wilcock, 2008). Habitation of such waters occurs at different lifestages, and thus, body sizes. Furthermore, they are unusual in that they oxyconform, failing to maintain a constant oxygen consumption rate as environmental oxygen levels decline (Chapter IV). Oxygen consumption in inanga is therefore modulated by external PO_2 and is not subject to overt physiological regulation. As a consequence inanga provide a model system for investigating the effect of fish size on hypoxia tolerance by analysing how metabolic rate scales with mass at different PO_2's, eliminating the complication associated with physiological maintenance of metabolic rate. Furthermore, given the cultural and socioeconomic importance of this fish species (McDowall, 1990), an understanding of the tolerance of different size classes to hypoxia may contribute information critical to conservation efforts.

The present chapter aimed to explore the relationship between body mass and oxygen consumption in an oxyconforming fish, and to determine how this relationship

changes with environmental PO_2. These data were then analysed to determine whether metabolic rate at normoxia and body size were possible predictors of hypoxia tolerance in inanga. Anaerobic metabolism was also evaluated in two inanga size classes to elucidate whether tolerance of hypoxia was related to measures of anaerobiosis.

5.2 Materials and methods

5.2.1 Fish and rearing conditions

Inanga were caught as previously described (Chapter II, section 2.2.1), from streams where dissolved oxygen ranged from 0.5 to 9.6 mg O_2 l^{-1} during a typical summer day. Fish were transported to the aquarium facility in the School of Biological Sciences at the University of Canterbury and maintained as described in Chapter II. Feeding was stopped two days prior to the commencement of experiments.

5.2.2 Respirometry

As described in Chapter IV, inanga were individually placed in glass respirometry chambers of 0.1, 0.3 or 0.5 l volume depending on fish size. Temperature, length of the acclimation period, flow rate, normoxic freshwater characteristics, control chambers, equipment, calibration and sampling were identical to those described for Chapter IV (Section 4.2.2.1). Owing to the matching of volume chamber to fish size, experiments were conducted over similar exposure times for all fish, ~7-9 h. In Chapter IV (Fig. 4.3) it was shown that under these conditions respiration in inanga was not affected by the accumulation of metabolites such as CO_2.

5.2.3 Effect of body mass on metabolic rate

Metabolic rate as a function of body size in normoxia (dissolved oxygen = 17.9 kPa) was evaluated in 78 fish ranging in mass from 0.137 to 11.275 g wet weight. A total

of 67 of these fish (ranging in mass from 0.137 to 4.530 g wet weight) were then used to examine the effect of body size on metabolic rate in declining environmental oxygen concentrations (17.9, 16.6, 13.9, 11.3, 9.9, 6.7, and 3.9 kPa). This size range represents the natural range of fish that inhabit Canterbury waters (personal observation). As an indicator of hypoxia tolerance, the PO_2 at which the fish showed the first signs of distress was also examined ($PO_{2\ out}$; Nilsson and Ostlund-Nilsson, 2004). In the present study, distress was defined as the loss of equilibrium. Once $PO_{2\ out}$ was reached, fish were removed, weighed (wet weight to 0.001 g precision) and returned to the aquarium system. To evaluate size-related responses, seven small (0.5 ± 0.1 g) and seven large (3.0 ± 0.2 g) fish were selected from this data set and oxygen consumption rates were averaged for a size-class comparison.

5.2.4 Effect of body mass on anaerobic metabolism

Lactate accumulation in fish muscle at $PO_{2\ out}$ was quantified in a different set of two fish size-classes, small (0.63 ± 0.06 g, n=8) and large (6.60 ± 0.85 g, n=7). Fish were individually placed in respirometry chambers and $PO_{2\ out}$ was determined under an identical protocol to that described above (see *Respirometry*, section 5.2.2). These data were also included in the analysis of $PO_{2\ out}$ and fish size. Once $PO_{2\ out}$ was reached, the water PO_2 was recorded, fish were killed by cephalic blow and weighed before being snap-frozen in liquid nitrogen. Samples were subsequently stored at -80°C until analysis (less than 6 d). Muscle lactate and glucose were then quantified. Briefly, a muscle sample from the medial dorsal area (~130 mg) was weighed and homogenised on ice in 300 µl of ice-cold 1 M perchloric acid for 30 s, using a Polytron tissue homogeniser at the highest speed. A 50 µl sample of the homogenate was neutralised with ice-cold 2 M KOH, centrifuged at 12,000 *g* for 5 min at 4°C, and lactate was quantified in the supernatant using an enzymatic kit (K-LATE, Megazyme, Ireland), following the manufacturers instructions. The basis of this assay is that lactate is first oxidised to pyruvate and nicotinamide adenine dinucleotide by lactate dehydrogenase and then pyruvate is further converted to D-alanine and 2-oxoglutarate by the action of D-glutamate pyruvate transaminase. The total amount of NADH released in those reactions is proportional to the initial amount of lactate in the sample. Estimations of whole fish lactate were carried out by simply scaling the

lactate concentration per gram of fish muscle, to 2/3 of the total fish weight (the proportion of fish mass accounted for by muscle; Randall, 2012). Glucose was quantified from the same supernatant as that used for lactate assessment, via a glucose kit (Gluco-quant, Roche Diagnostics GmbH, Germany) following manufacturer instructions. The principle of this assay is that hexokinase catalyses the conversion of glucose to glucose-6-phosphate, which is then oxidised by glucose-6-phosphate dehydrogenase. The total amount of NADPH formed is directly proportional to the initial glucose concentration in the sample.

5.2.5 Calculation of the relationship between mass and oxygen consumption

All oxygen consumption data, expressed as μmol O_2 h^{-1}, were regressed against fish wet weight (kg) in order to examine the relationship between these two variables. Data were examined in the raw form, and also following log transformation. This analysis was performed at a number of dissolved oxygen levels (17.9, 16.6, 13.9, 11.3, 9.9, 6.7, and 3.9 kPa). Log-transformed data at each oxygen level were subjected to a regression analysis to extract the scaling parameters and construct the following equation (Prosser, 1973):

$$VO_2 = a\ W^{\ b}$$

where VO_2 is total oxygen consumed per unit of time, W is the fish body wet weight, *b* is the slope of the regression (log-log) and represents the scaling exponent, and *a* is the intercept of the regression (Table 5.1).

Individual $PO_{2\ out}$ values (kPa) recorded during the experiments conducted under declining dissolved oxygen levels were also regressed against fish weight and fish standard MO_2 in normoxia to elucidate relationships between $PO_{2\ out}$, fish size, and MO_2.

5.3.6 Data treatment and statistical analysis

All individual VO_2 data were regressed against fish weight and the scaling relationships were calculated by a least-squares regression method. Differences between the scaling slope obtained at normoxia and the theoretical values of 0.66 and 0.75 were assessed using a slope test function (R Development Core Team, 2010), which allow to modify the null hypothesis from being equal to 0 to being equal to the theoretical slope against testing is desired. The effect of body mass on metabolic rate at different PO_2 values was determined using a Generalised Linear Mixed Model (GLMM) followed by a Tukey post-hoc test. All individual data for normoxic MO_2 and fish weight were regressed against $PO_{2\,out}$. Regression analyses were performed in SigmaPlot (ver. 11.0; Systat Software), after testing for normal distribution and homogeneity of variances by a Kolmogorov-Smirnov and Levene's median test, respectively (Sokal and Rohlf, 1995). Differences in the VO_2 at each of the external PO_2 levels analysed were tested by an ANOVA after assessment of normality and equal variance. Muscle lactate and glucose between small and large fish were tested by a *t*-test after assessment of normality and equal variance. Since estimations of total fish lactate did not meet normality assumptions, a Mann-Whitney rank sum test was performed. All data are presented as means ± SEM, and were considered significant with a *p*-value less than 0.05.

Table 5.1: Scaling exponent, intercept, fish weight range, n values and values generated from the best fit regressions between log VO_2 and log fish wet weight at different oxygen levels.

PO_2 (kPa)	*n* *	Weight range (g)	Scaling exponent (*b*)	Intercept (*a*)	*p*	r^2
17.9	78	0.137 – 11.27	0.82	0.93	< 0.0001	0.929
16.6	42	0.137 - 4.52	1.00	0.96	< 0.0001	0.964
13.9	37	0.137 - 4.52	1.02	0.88	< 0.0001	0.936
11.3	36	0.137 - 4.52	1.05	0.80	< 0.0001	0.924
9.9	36	0.137 - 4.52	1.04	0.76	< 0.0001	0.919
6.6	27	0.230 - 4.52	0.92	0.66	< 0.001	0.901
3.9	14	0.321 - 4.52	0.70	0.49	0.0018	0.571

* As $PO_{2\,out}$ differed for each fish, n values decreased as the experiment progressed.

5.3 Results

No mortalities were recorded during the acclimation period or during the experiments. During respirometry fish were mostly quiescent, interspersed with occasional bouts of gentle swimming. The exception was immediately prior to $PO_{2\ out}$, when fish exhibited short periods of burst swimming followed by loss of equilibrium.

5.3.1 Relationship between oxygen consumption and body mass at normoxia

In normoxic conditions oxygen consumption increased with body weight in inanga, such that larger fish showed higher oxygen consumption rates than smaller fish (Fig. 5.1A). The same trend was evident, but clearer, when the data were log-transformed (Fig. 5.1B). The relationship between oxygen consumption and body weight was described by the following power equation: $VO_2 = 0.9318\ W^{0.8214}$, derived from the log-transformed data ($p < 0.0001$, $r^2 = 0.93$, Fig. 5.1B). The scaling exponent (0.82) obtained for inanga was significantly higher than both of the proposed theoretical values, 0.66 ($p < 0.001$) and 0.75 ($p = 0.008$). Using this scaling relationship it was calculated that a fish of 0.1 g wet weight would have a metabolic rate of 12.8 µmol O_2 g^{-1} h^{-1}, while a 10 g fish (100 times larger) would have a metabolic rate of 5.8 µmol O_2 g^{-1} h^{-1} (slightly less than half that of a 0.1 g fish).

5.4.2 Relationship between oxygen consumption and body mass with declining PO_2

Inanga oxygen consumption was reduced as external PO_2 decreased, a pattern that held for all fish sizes (Fig. 5.2A). Although the scaling of oxygen consumption with fish weight was observed at all tested PO_2 levels (all p-values < 0.0018, and all $r^2 >$ 0.57, Fig. 5.2B, Table 5.1), the scaling relationship was significantly affected by external oxygen tension (GLMM, $p < 0.001$). The scaling exponent (b) was 0.83 in normoxia, which then increased to ~1 and was maintained at this value until environmental PO_2 reached 9.9 kPa. Below this point, the scaling exponent decreased to 0.92 at 6.7 kPa and subsequently to 0.70 at 3.9 kPa (significantly lower than at all other PO_2's; all p values < 0.0038).

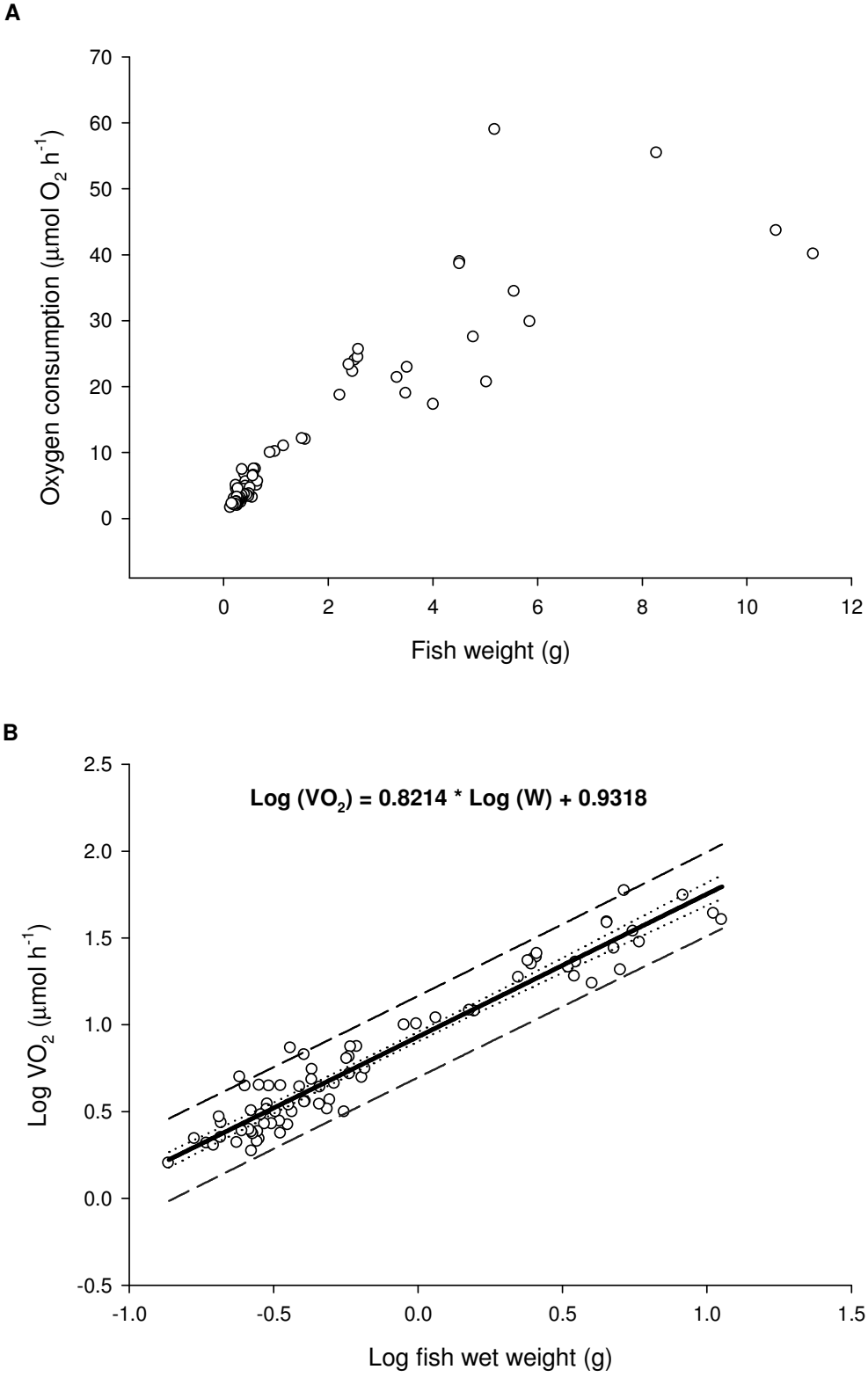

Figure 5.1: Oxygen consumption in inanga (n = 78) ranging from 0.137 to 11.275 g wet weight under normoxic conditions (PO_2 = 17.9 kPa) using raw (A) and log-transformed (B) where the plotted line represents the best fit regression with 95% confidence interval) data.

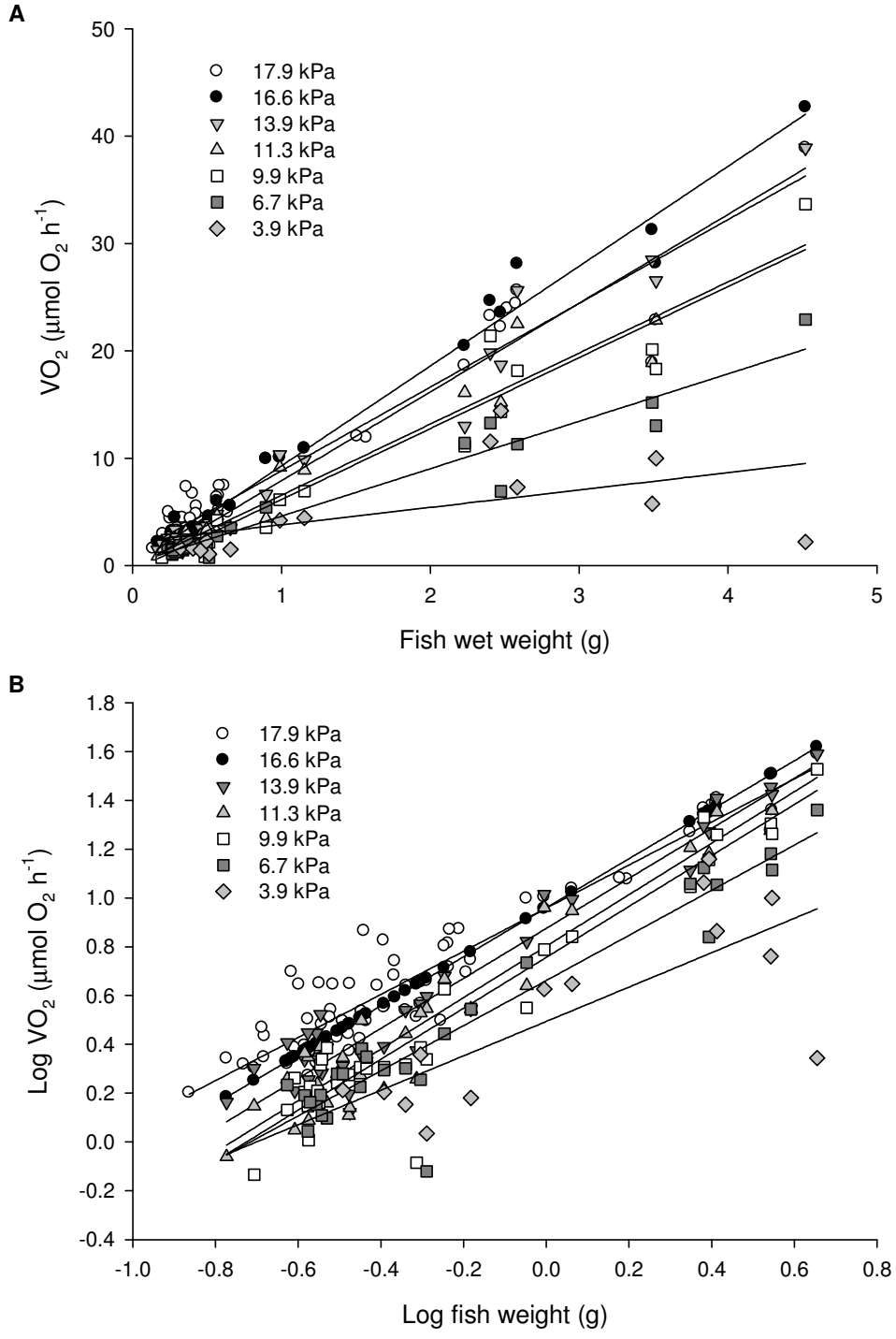

Figure 5.2: Effect of water PO_2 on oxygen consumption in inanga as a function of weight, plotted using raw (A) or log-transformed (B) data. Plotted lines represent best fit regressions for each tested oxygen tension (n = 67-78). Exponents, intercepts and regression values are shown in Table 5.1.

5.4.3 Effect of normoxic metabolic rate and body size on $PO_{2\ out}$

No significant association was found between the normoxic metabolic rate of the fish and their corresponding $PO_{2\ out}$ (p = 0.136; Fig. 5.3A). However, $PO_{2\ out}$ showed an effect of fish size (p = 0.006; Fig. 5.3B), larger fish having a lower $PO_{2\ out}$ than smaller fish.

5.4.4 Oxygen consumption in different fish size classes

As environmental PO_2 decreased an oxyconforming pattern (linear decrease in oxygen consumption with declining environmental PO_2) was evident in both small (~0.5 g) and large fish (~3 g) (Fig. 5.4). At all tested PO_2 values the VO_2 of the larger inanga was significantly higher than that of the smaller fish.

5.4.5 Measures of metabolic status as a function of fish size

Muscle lactate accumulation at $PO_{2\ out}$ was significantly dependent on fish size (Fig. 5.5A). Small fish accumulated 7.9 ± 0.8 μmol of lactate g muscle^{-1} at $PO_{2\ out}$, while larger fish accumulated 12.5 ± 1.1 μmol of lactate g muscle^{-1}. Thus larger fish accumulated about 58% more lactate per gram of muscle than small fish (p = 0.005). Extrapolating the muscle lactate concentration to a total fish lactate content showed that large fish can store about 16.6 times more lactate than small fish (Mann-Whitney, $p < 0.001$, Fig. 5.5B). Muscle glucose levels, however, were similar in small and large fish (t-test, p = 0.783, Fig. 5.5C).

5.4 Discussion

During respirometry inanga exhibited occasional gentle swimming movements and for this reason the measurements of oxygen consumption could best be considered representative of standard metabolic rate. Therefore, unless otherwise stated, literature

values for standard metabolic rate were used for comparison to those obtained in the present study.

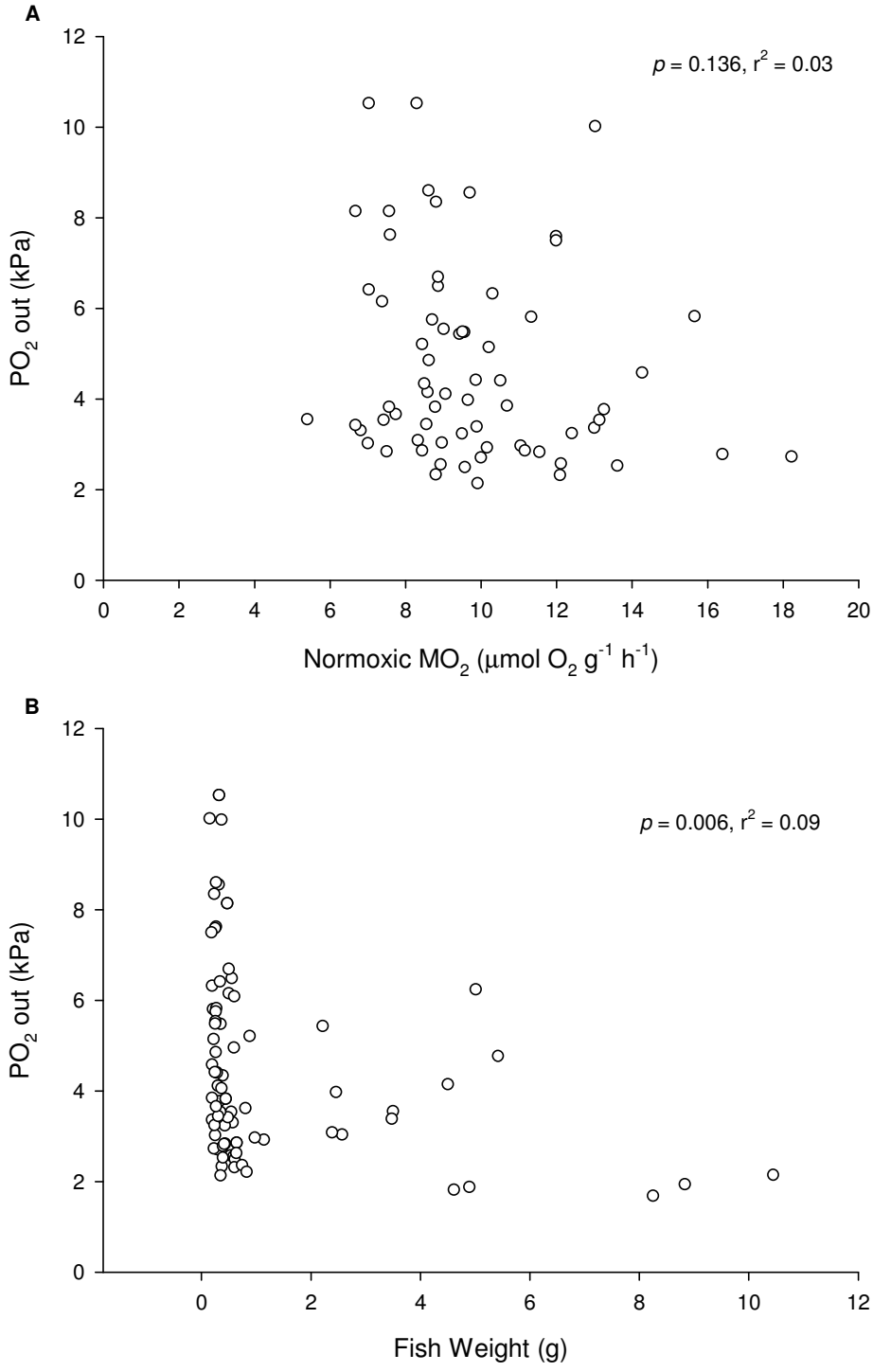

Figure 5.3: Relationship between normoxic mass-specific metabolic rate and $PO_{2\ out}$ (A; n = 67), and fish body mass and $PO_{2\ out}$ (B; n = 82).

5.4.1 Relationship between body mass and oxygen consumption

The present data set, using the complete natural mass range of inanga, is the first to comprehensively examine the relationship between fish size and metabolic rate for this, or any galaxiid, species. The Galaxiidae are an important and widespread group in the Southern hemisphere, and display a number of unusual biological characteristics. These include oxyconforming in response to a declining environmental PO_2 (Chapter IV), and a scaleless integument (Dean and Richardson, 1999). Under normoxic conditions the relationship between oxygen consumption and fish mass was described by a power function ($VO_2 = 0.9318\ W^{0.8214}$), and can be classified as a Type I scaling function, whereby the metabolic rate scales linearly with body mass, but at a value less than isometry (Glazier, 2005). Using this scaling relationship it could be calculated that a fish of 0.1 g wet weight (e.g. a juvenile fish) would have a metabolic rate of 12.8 µmol O_2 g^{-1} h^{-1}, while a 10 g fish (100 times bigger) would have a standard metabolic rate of 5.8 µmol O_2 g^{-1} h^{-1}, less than a half of that of a 0.1 g fish.

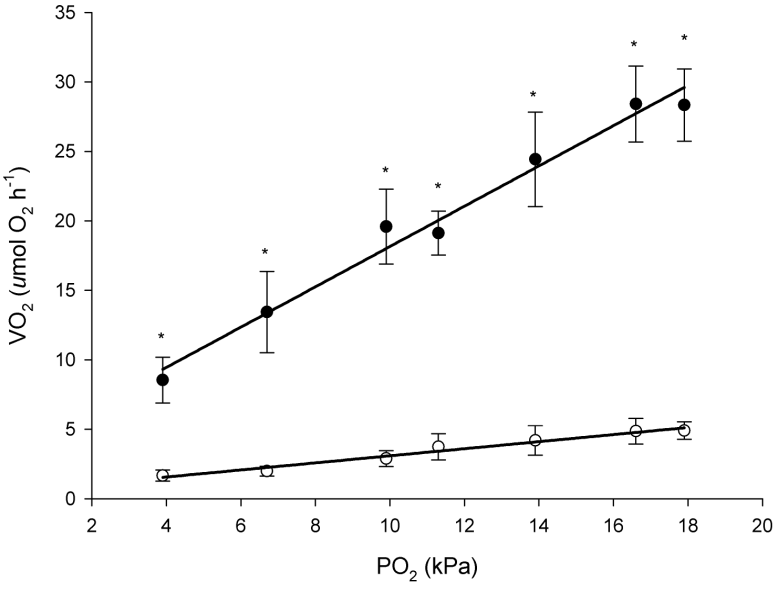

Figure 5.4: Effect of varying PO_2 on oxygen consumption rates in small (0.54 ± 0.10 g, empty circles, n= 7) and large (3.03 ± 0.21 g, black filled circles, n= 7) inanga. Data are presented as means ± SEM. Asterisks represent statistical differences between VO_2 values from larger fish and those of smaller fish at each PO_2 value, as determined by a *t*-test (all *p*-values < 0.016).

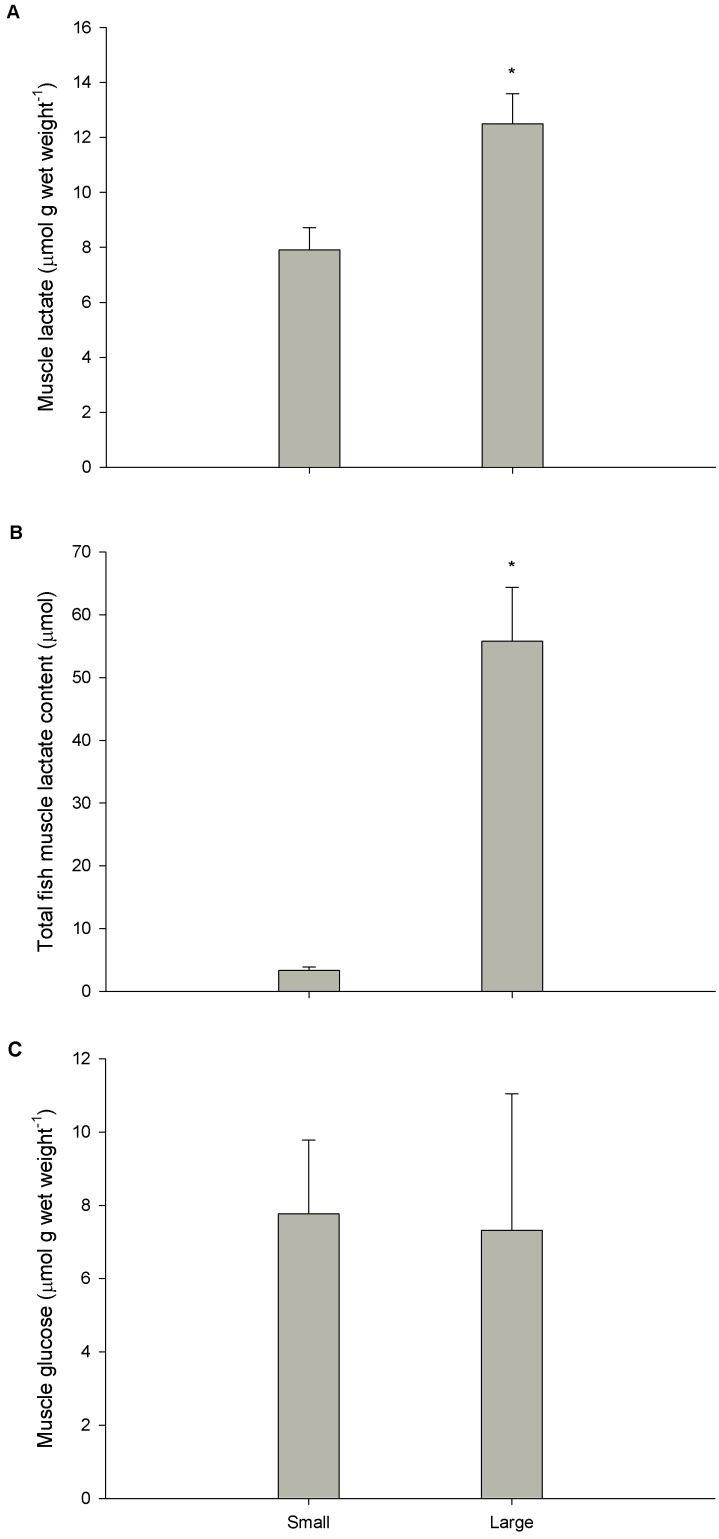

Figure 5.5: Muscle lactate concentration (A), total muscle lactate accumulation (B) and muscle glucose (C) at $PO_{2\ out}$ in small (0.6 ± 0.1 g, n=8) and large (6.6 ± 0.9 g, n=7) inanga. Data are presented as means ± SEM. Asterisks indicate significant differences between size classes as determined by a *t*-test at an α level of 0.05.

These calculated values are well in agreement with the metabolic rate of inanga obtained in previous chapters (Chapter II, 11.07 ± 0.24 μmol O_2 g^{-1} h^{-1}, fish size = 0.35 ± 0.01 g; Chapter IV, 10.2 ± 0.3 μmol O_2 g^{-1} h^{-1}, fish size = 0.78 ± 0.1 g). Results are also in agrrement with other studies (~ 12.2 μmol O_2 g^{-1} h^{-1}; fish size = 1.2 g; Encina-Montoya et al., 2011).

Scaling exponents reported in fish have historically been documented to fall between 0.79 and 0.88 (Brett and Groves, 1979; Goolish, 1995; Peters, 1983; Clarke and Johnston, 1999; White et al., 2006). Recently, Killen et al. (2010) used data from 89 teleost species and reported a similar range of exponent values, extending from ~0.70 for pelagic species to ~0.86 for bathyal fish. The normoxic scaling exponent calculated here for inanga (0.82), is in the range reported for fish, and together these data fit with the observation that scaling slope is higher in ectotherms than in endotherms (Glazier, 2005; White et al., 2006). This is likely to be a consequence of the extra costs associated with thermoregulation.

5.4.2 Effect of reduced PO_2

Although the importance of the relationship between metabolic rate and body size is well recognised, the existence of a universal scaling exponent is controversial (Hochachka et al., 2003; Suarez and Darveau, 2005; Glazier, 2005; Agutter and Tuszynski, 2010). It is, however, increasingly accepted that there is unlikely to be a scaling exponent that fits all data, probably due to intrinsic (i.e. physiological) and extrinsic (i.e. environmental) constraints (Hochachka et al., 2003; Suarez and Darveau, 2005; Glazier, 2005). It is been shown that several extrinsic factors affect the relationship between metabolic rate and body size. Oxygen tension is one such factor previously shown to have an impact (Atkinson, 1973; Everett and Crawford, 2010). The results recorded here, across a range of PO_2 values, showed variation in scaling exponents between 0.70 to 1.05, supporting the hypothesis that factors such as environmental oxygen levels can influence the relationship between body size and metabolic rate.

In the current study PO_2 was modulated in order to determine its impact on the relationship between fish mass and oxygen consumption. Given that inanga lacks overt physiological control of oxygen consumption rates (i.e. they oxyconform), a constant scaling exponent with declining PO_2 may have been anticipated. The present data set showed instead that the relationship between body mass and oxygen consumption varied with altered oxygen tension. A previous study has shown a similar phenomenon in the killifish, *Fundulus grandis* (Everett and Crawford, 2010). In this study the authors noted a loss in scaling (b = 0.37) with body size at low PO_2 (1.8 kPa), and hypothesised that this was due to either a reduction in overall metabolism or an enhanced reliance on anaerobic metabolism. This latter explanation holds for *G. maculatus* in the present study (see below).

To confirm that the scaling component changes with PO_2, VO_2 data was evaluated separately in two size classes of inanga. The data obtained showed that although both size classes responded in the same general manner to decreasing PO_2, the reductions in VO_2 were larger in large fish. As a consequence differences between the absolute VO_2 values of small and large fish became smaller as external PO_2 decreased, modifying the scaling exponent. This suggests that larger fish failed to meet aerobic metabolic demands during hypoxia as readily as smaller fish. Given this finding, it may be expected that larger fish would begin to show signs of distress at higher PO_2 levels than smaller fish. However, the opposite was found. Data for $PO_{2\ out}$ was not related to metabolic rate of the fish at normoxia, but was inversely related to fish size. Larger fish presented lower $PO_{2\ out}$ values than smaller fish.

These data indicate that inanga of different mass have distinct strategies for dealing with hypoxia. It has been shown that the enzymatic capacity for anaerobic metabolism scales with fish size in *Astronotus ocellatus* (Almeida-Val et al., 2000), and that $PO_{2\ crit}$ decreases with fish size in both *A. ocellatus* (Sloman et al., 2006) and *F. grandis* (Everett and Crawford, 2010). An increased capacity for anaerobic metabolism and an ability to maintain aerobic metabolism for longer in light of falling environmental oxygen levels are both advantageous for hypoxia tolerance. Furthermore, larger fish have larger stores of glycogen/glucose to fuel anaerobic metabolism, and also larger reservoirs to buffer toxic anaerobic end products such as lactate and H^+ (Nilsson and Ostlund-Nilsson, 2008). This would also increase their tolerance to hypoxia.

Later in this thesis (Chapter VII) it was observed that adult inanga can depress their aerobic metabolism by 49% under hypoxia (3 kPa), and that 33% of the resulting metabolic rate was satisfied by anaerobic metabolism. It may therefore be hypothesised that as environmental oxygen tensions decline, oxygen consumption is depressed, and a switch to anaerobic metabolism occurs at a higher PO_2 value in larger fish than in smaller fish (as evidenced by the changing scaling components and Fig. 5.3). This ability to use anaerobic metabolism was also reflected in the point where fish become distressed ($PO_{2\ out}$), with larger fish having lower $PO_{2\ out}$. Indeed these data showed that larger fish produced more lactate than small fish (12.50 ± 1.10 and 7.92 ± 0.80 μmol of lactate g muscle^{-1}, respectively), suggesting that larger inanga are capable of extending their survival in hypoxia by an increased capacity for anaerobic metabolism. Smaller inanga appear to primarily rely on fuelling their metabolism via the aerobic pathway at reduced PO_2's. Muscle lactate concentration does not, however, provide a full picture of anaerobic capacity. Based on an assumption that muscle accounts for two thirds of the body weight (Randall, 2012), the total amount of lactate accumulated in the fish was estimated. Owing to their size and higher lactate production rates, larger inanga can accumulate up to 16.6 times more lactate than small fish. Therefore, these data suggest that the capacity for anaerobic metabolism is size-dependent, a finding that has been previously reported in other fish species (Almeida-Val et al., 2000; Nilsson and Ostlund-Nilsson, 2008).

Several other factors could also set the limit for hypoxia tolerance in fish. These include H^+ accumulation, ATP synthesis, energy substrate availability, and ion homeostasis (Hochachka, 1986; Boutilier, 2001; Nilsson and Ostlund-Nilsson, 2008). Of these factors, data are only available regarding the energy substrate, glucose. These data showed no differences between muscle glucose in small and large fish sampled at $PO_{2\ out}$. Results also showed that at $PO_{2\ out}$ there was still glucose available for fuelling anaerobic metabolism. Therefore, at least in inanga, depletion of the energy substrate during hypoxia is not likely to be responsible for setting the limits of hypoxia tolerance.

The present study supports the view of a species-specific scaling relationship (Darveau et al., 2002; Hochachka et al., 2003; Bokma, 2004), and provides strong evidence for the importance of extrinsic factors in its regulation. Specifically, the relationship between metabolic rate and body mass was depressed at low PO_2 values, suggesting that either inanga of different sizes used different physiological strategies during hypoxia, and/or that sensitivities to low PO_2's were different between small and large fish. This knowledge is likely to be of great value in protecting this iconic and widely distributed fish species (McDowall, 2000), particularly given its known sensitivity to hypoxia (Chapter IV). Evaluating changes in the scaling relationship seems to be an accurate tool for exploring differential size-related responses to environmental factors. The present study showed that larger fish were more tolerant to low oxygen tensions than small fish, and demonstrated that the capacity for anaerobic metabolism scaled with fish size. The capacity for anaerobic metabolism and/or the ability to tolerate high levels of metabolic end products appear to be the physiological tools used by inanga to extend its survival during hypoxia. While the mechanistic basis underlying the cause of death of inanga in hypoxia remains unknown, the results of the present study indicate that this is not due to the depletion of the energy substrate glucose.

Chapter VI

Leap of faith: Voluntary emersion behaviour and physiological adaptations to aerial exposure in a non-aestivating freshwater fish in response to aquatic hypoxia

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6.1 Introduction

Fish are mobile animals that may exhibit behavioural responses to avoid hypoxic events (Diaz and Rosenberg, 1995). The behavioural strategy adopted by a fish depends on several variables. These will include habitat characteristics, the nature of the hypoxic event (i.e. size, severity, and duration of the hypoxic water mass) (Schurmann and Steffensen, 1994; Chabot and Claireaux, 2008), and the presence and success of physiological and biochemical adaptations for hypoxia tolerance (Schurmann and Steffensen, 1994; Chabot et al., 1999; Chabot and Claireaux, 2008). Of all possible behavioural approaches for dealing with hypoxia, perhaps the most extreme is to emerse and exploit the oxygen rich air. A number of fish species have been shown to emerse, or survive for significant periods in the absence of water through aestivation. These include lungfish (Smith, 1935), sculpins (Sloman et al., 2008), mudskippers (Clayton, 1993), mudfish (Eldon, 1979), and mangrove rivulus (Taylor et al., 2007). A common feature of all these fish is their habitation in environments that regularly experience hypoxia, either as part of the tidal cycle or on a seasonal basis related to waterway desiccation.

As stated in previous chapters, inanga can regularly encounter low levels of dissolved oxygen (Chapter I, IV and V; Chapman, 2003). Furthermore, given the life-history characteristics of emersion, coupled with the lack of metabolic regulation as PO_2 decreases (Chapter IV, Fig. 4.1), I hypothesised that adult inanga may have latent adaptations for dealing with more recent anthropogenically-induced hypoxia. Characterising behaviour in hypoxia could be an insightful approach, not only for exploration of hypoxia tolerance, oxygen sensing, and respiratory and metabolic adaptations in fish, but also for informing the potential ecological impacts of hypoxic episodes.

In this chapter adult inanga were presented with an "escape route" into the air as the dissolved oxygen of the water in which they were held was reduced. A variety of behaviours that might be considered adaptive under hypoxia exposure (Soares et al., 2006), including changes in activity and ventilation, use of aquatic surface respiration

and air gulping (Mandic et al., 2009; Dean and Richardson, 1999; Gee and Gee, 1991), were explored.

6.2 Materials and methods

6.2.1 Fish and rearing conditions

Adult inanga were caught, transported to the aquarium facility in the School of Biological Sciences, and maintained as described in Chapter II. Two days before the experiments, individual weights and lengths of fish were recorded (means 1.19 ± 0.64 g, 5.82 ± 0.58 cm, n=70). They were then returned to the acclimation tank and fasted until the experiments.

6.2.2 Experimental design

One set of fish (n=34) was used for the behaviour experiments and a second set of fish (n = 36) was used for the physiology experiments. No differences were found in the weights or total lengths of the fish groups assigned to each experiment and treatment (ANOVA, $p > 0.05$). A 20 l glass aquarium (30 x 21 x 32 cm: length x width x height) was placed partially submerged in a temperature-controlled bath at 14°C. A fine bubble air stone was fixed at the bottom and central part of the aquarium. At one side of the aquarium, a small piece (10 x 21 x 1 cm) of foam mat was placed on the water surface, acting as a platform for emersion. Three sides of the aquarium were covered with black plastic sheeting to minimise disturbance to the fish. The fourth, unobstructed, side was demarcated with a 2 cm x 2 cm grid pattern and a digital video camera (PAL MV 400i, Canon) was positioned to record fish behaviour. A switch, joined by tubing, allowed interchange between air and nitrogen bubbling without perturbing the fish. Preliminary experiments without fish were conducted to determine the time and gas flows required to develop a slow-onset hypoxia within the aquarium set-up. Water oxygen tension ranged from ~20 kPa (normoxia) to ~3 kPa (hypoxia). An oxygen microelectrode (connected to a meter and

subsequently to a PowerLab/4SP unit, Chapter II, section 2.2.3.2) was placed in the upper part of the water column inside the aquarium, allowing recording of the oxygen tension throughout the experiment. Preliminary experiments showed that the combination of gas bubbling and fish movement resulted in a homogeneous PO_2 throughout the aquarium. Before each trial the oxygen electrode was cross-calibrated with an oxygen electrode (IL 1302) connected to a 781 oxygen meter (Strathkelvin®), with with fully aerated water and a saturated sodium sulphite solution (Chapter II).

6.2.3 Behavioural monitoring

In natural environments inanga are found in shoals (Mitchell, 1989), and consequently experiments were conducted in groups (17 fish) to reduce stress and avoid unnatural responses. In preliminary experiments fish behaviour was analysed at 0, 5, 11 and 12 h following transfer to the experimental set-up. These investigations showed that natural behaviour was established within 5 h following transfer to the experimental aquarium. In the experiments presented here fish were transferred and left for 12 h to acclimate. During acclimation the water was continuously exchanged with fresh water at an approximate exchange rate of 100% per h, a procedure that did not create any appreciable currents. Following acclimation, fish behaviour was recorded for 45 min under normoxic conditions, in order to establish a baseline. Subsequently, air bubbling was switched to N_2 bubbling, and the PO_2 decreased in an exponential fashion over a 90 min period until water PO_2 reached ~3 kPa. The PO_2 was maintained at this level of hypoxia for 30 min. Any fish exhibiting loss of equilibrium was removed from the aquarium and transferred to a well-oxygenated tank. Thereafter, the N_2 bubbling was switched to air bubbling and the oxygen concentration was increased until normoxia was restored (~40 min). Individual fish behaviour and observations of the opercular frequency were recorded throughout the experiment. Following exposure fish were returned to acclimation tanks where feed was restored and survival evaluated during a 7 d post-hypoxia period. This procedure was replicated twice, with different fish in each experiment.

6.2.4 Digital video analysis

All the video records were transferred to a computer, and matched against the continuous record of oxygen content allowing a correlation between fish behaviour and water PO_2. Fish behaviour was analysed during a 5 min period initiated at the following water oxygen levels: 19.9, 14.5, 10.3, 6.2, 4.3 and 3.1 kPa (hereafter rounded to 20, 15, 10, 6, 4 and 3 kPa). Individual fish were identified at the beginning of the observation period, and then followed through the experiment. Fish that were unable to be tracked for the extent of the observation period were excluded from the analysis, therefore n values ranged from 21 to 27. Horizontal and vertical swimming speeds were determined following the procedure described by Soares et al. (2006), and expressed as body lengths per min (BL min^{-1}). Briefly, vertical and horizontal displacements were divided by the time that the movement lasted. Total swimming speed was calculated trigonometrically. Fish movements with vectors in the X and Y axes were used to calculate swimming speeds, acknowledging that these calculations could slightly underestimate the real swimming speed of the fish (because of the omission of the fish displacement in the third dimension, Z plane). Therefore, the term apparent swimming speed will be used. The opercular frequency was quantified over a minimum of three min for each fish during hypoxia induction, emersion and during recovery (up until PO_2 of 14 kPa, due to recording limitations), and was expressed as beats per min (beats min^{-1}). The water column was divided in three equal sections (each representing a 10 cm vertical distance): upper, middle and lower and the time that each fish spent in those areas was recorded, and expressed as a proportion of the observation time (5 min). Likewise, the time that individual fish were active (when the fish were swimming), and performing ASR (when the fish made contact with the upper 3 millimetres of water (Kramer and McClure, 1982), were recorded and expressed as proportion of the observation time. Avoidance behaviour (defined as attempted emersion), successful emersion (fish that voluntarily remained out of the water on the floating platform) and loss of equilibrium were also recorded at each of the monitored oxygen levels and expressed as absolute frequencies (number of fish). There was a distinct difference between avoidance and emersion behaviour, aside from the successful emergence from the water. Avoidance involved a rapid vertical exit from the water, whereas emersion was preceded by contact with the platform and involved a horizontal 'flop' out of the water. Opercular frequency was the only variable quantified in emersed fish. Once emersed inanga remain relatively subdued, and there was no attempt made to quantify activity patterns in these fish.

6.2.5 Oxygen consumption experiment

Inanga were randomly assigned to one of three experimental treatments: normoxic water (20 kPa; n=12), hypoxic water (3 kPa; n=12) and air (emersion) (n=12). In all the treatments the fish were individually placed in respirometry chambers (~0.3 l). Acclimation period lasted at least 12 h (Chapter IV, section 4.2.2). Water characteristics, controls, chamber temperatures, and flow rate were identical to those previously described (Chapter II, section 2.2.1). For aquatic exposures the incubation period was 40 min. One ml water samples were withdrawn at the beginning and at the end of the incubation period for assessment of oxygen consumption (see below). Following incubation, water flow was re-established for 15 min to avoid potential accumulation of CO_2 or any metabolic products, and the respirometry chambers were closed for a second quantification of the oxygen consumption rate as described above. The consumption rate of the two trials was always similar and they were averaged to provide an oxygen consumption value for each fish. In the hypoxic treatment the same procedure was used, but prior to the respirometry chambers being closed, the water was exchanged for hypoxic water previously bubbled with nitrogen (and left to equilibrate for 15 min). In the emersion treatment, chambers were filled with air following acclimation, and fish were incubated for 40 min as described above. In these experiments, 8 ml of air was sampled at the start and the end of the incubation period in order to obtain a stable reading. All samples were withdrawn using glass gas-tight syringes (Trade Van Mark), and in water care was taken of not including bubbles in the samples. The air volume removed was replaced with water to maintain pressure in the respirometer. In normoxic and hypoxic aquatic conditions, oxygen content in the water samples was assessed as previously described (Chapter II, section 2.2.2.2). For aerial respirometry, samples were read in a ML 205 Gas analyser (ADI Instruments, Austalia), connected to a PowerLab/4SP unit (ADI Instruments, Waverly, Australia). The gas analyser was calibrated with air, nitrogen, a gas mixture of 5% of CO_2 in air, and a gas mixture of 5% of CO_2 in O_2.

6.2.6 Data treatment and statistical analysis

The responses of individual fish in independent behaviour trials were compared using ANOVA within each oxygen level. As no differences were found between the trials (all p values > 0.2), data from replicate experiments were pooled. Swimming speeds and opercular frequency are reported as absolute values. Swimming activity, ASR and water column distribution are reported as the proportion of time that an individual fish was engaged in the behaviour or found in the tank position. Avoidance behaviour, loss of equilibrium and emersion behaviour are reported as absolute frequencies. All the statistical analyses were carried out using SigmaStat 3.5 software, in either absolute values or absolute frequencies. Normal distribution and homogeneity of variances were tested with Kolmogorov-Smirnov and Levene's median test, respectively. A repeated measures ANOVA (RM ANOVA) was used to evaluate the impact of dissolved oxygen on the following behavioural parameters: apparent swimming speed, opercular frequency, swimming activity, ASR and water column distribution. Analyses were performed on animals during onset of hypoxia, and within the 30 minute period where the water was held at a constant hypoxic level (3 kPa). When the data did not meet the criteria of normal distribution or homogeneity of variances (ASR, water column distribution, vertical and horizontal swimming speed), a Friedman repeated measures ANOVA on ranks (RM ANOVA on ranks) was performed (only fish that were identifiable in all the treatments were used, n=21). For both parametric and non-parametric RM ANOVAs, a Tukey post-hoc test was used to examine specific pairwise comparisons. A quadratic non-linear regression was used to describe the relationship between opercular frequency and oxygen concentration. Regression analyses were performed using SigmaPlot (ver. 11.0; Systat Software), using all the individual raw data and the best fit was chosen on the basis of the r^2 value after an incremental analysis. Avoidance behaviour, loss of equilibrium and emersion at the different oxygen levels were evaluated by applying a Pearson X^2 analysis (Sokal and Rohlf, 1995), which tested the observed frequency of each behaviour relative to its expected frequency. A Kruskal-Wallis one way ANOVA followed by a Tukey post-hoc test was used to evaluate the oxygen consumption of fish in normoxic water, hypoxic water and air (emersed). Differences and relationships were considered significant with a p value lower than 0.05. All data, unless otherwise stated, are expressed as mean ± SEM.

6.3 Results

No mortalities were recorded during the experiments, recovery, or after the 7 d post-experiment period. During the trials no inanga lost equilibrium at oxygen tensions higher than 6 kPa, though, on average, 2 fish lost equilibrium at each of the lowest oxygen tensions (4 and 3 kPa). Although in nature inanga live in shoals (Mitchell, 1989), no schooling behaviour was observed in the present study, likely owing to the lack of appreciable current in the experimental system.

6.3.1 Apparent swimming speed

Inanga increased their apparent swimming speed as PO_2 decreased. This increase was consistent across both dimensions, horizontal swimming speed ($p = 0.013$, Fig. 6.1) and vertical swimming speed ($p < 0.001$, Fig. 6.1). When exposed to the lowest PO_2 (3 kPa) fish moved horizontally 2.7-fold faster than when at 10 kPa ($p< 0.01$, Fig. 6.1). Vertical swimming speed showed a similar pattern, increasing as oxygen concentration decreased. Thus, 2.4- ($p < 0.05$, Fig. 6.1) and 2.7- ($p < 0.01$, Fig. 6.1) fold increases were found when the fish reached 4 and 3 kPa, respectively, compared to PO_2's in excess of 10 kPa. Consequently, total apparent swimming speed was also significantly impacted by water PO_2 ($p < 0.001$, Fig. 6.1). Inanga increased their total apparent swimming speed 2.5-fold at 3 kPa, compared to when they were at 10 kPa ($p < 0.001$, Fig. 6.1). An increase in the total apparent swimming speed was also observed between low PO_2s, with fish swimming 1.4 and 1.3 times faster at 3 kPa than at 6 and 4 kPa, respectively ($p < 0.05$, Fig. 6.1).

6.3.2 Swimming activity

Swimming activity of inanga increased when the PO_2 dropped to 15 kPa ($p < 0.001$, Fig. 6.2), but then remained constant until the end of the experiment. At 20 kPa individual fish spent, on average, a third of their time swimming (33.8 ± 3.3%), while below PO_2 levels of 15 kPa, the fish spent about 53% of their time swimming, remaining at this level independent of the water oxygen content ($p > 0.05$, Fig. 6.2).

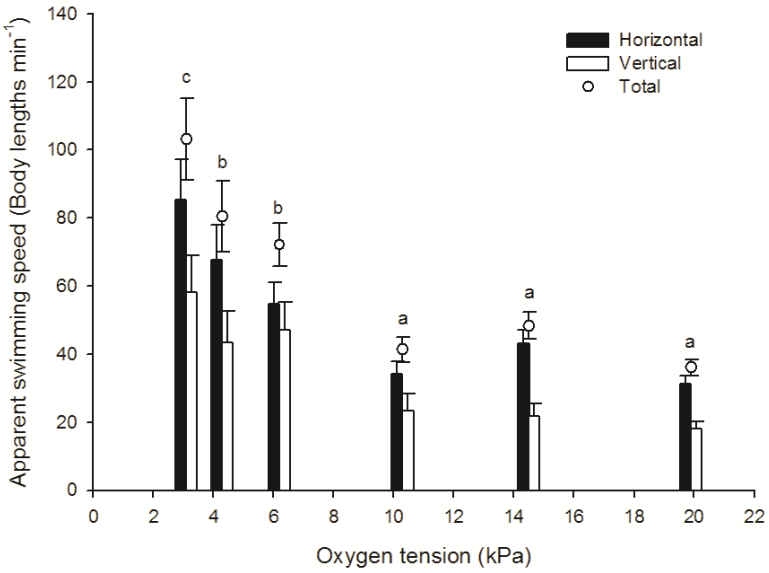

Figure 6.1: Horizontal, vertical, and total apparent swimming speed expressed as body lengths per minute, in inanga at different dissolved oxygen concentrations. All data are presented as mean ± SEM. Plotted points sharing letters are not significantly different (α = 0.05) as determined by repeated measures ANOVA followed by post-hoc Tukey analysis. Statistical indicators are only shown for total apparent swimming speed.

6.3.3 Water column distribution

Inanga distribution in the water column (upper and lower) was significantly affected by the water PO_2 (upper: $p < 0.001$; lower: $p < 0.001$). In normoxia (20 kPa), individual fish spent, on average, 66.5 ± 4.5% of the time in the lower part of the water column, and only spent 3.1 ± 1.0% of the time in the upper part of the water column (Fig. 6.3).

Then, as oxygen concentration started to decrease (15 kPa), a subsequent net movement of fish occurred from the lower to the upper part of the water column (Fig. 6.3) (20 kPa vs 15 kPa, $p < 0.05$). After this initial response to reducing oxygen, a slow net migration of fish from the upper to the lower part of the water column was recorded, as the PO_2 decreased to 3 kPa (15 vs 3 kPa, upper: $p < 0.05$; lower $p < 0.05$). Therefore, as with the situation in normoxia, during severe hypoxia the fish spent most of the time in the lower part of the water column (Fig. 6.3). In some fish

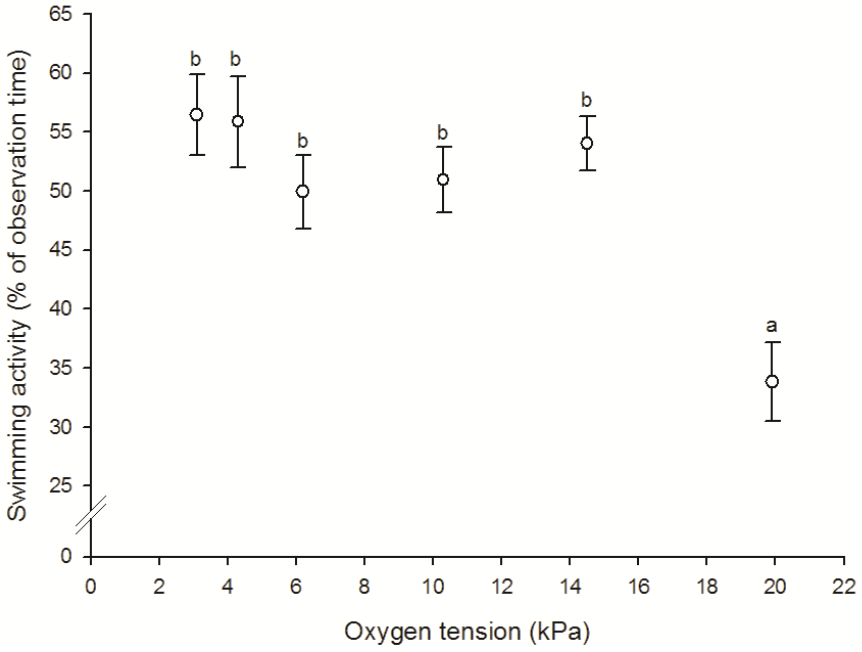

Figure 6.2: Swimming activity (percentage of time individual fish spent active) in inanga at different dissolved oxygen concentrations. Data are presented as mean ± SEM. Plotted points sharing letters are not significantly different (α = 0.05) as determined by repeated measures ANOVA followed by post-hoc Tukey analysis.

the occupation of the lower water column was followed by avoidance behaviour (see below). The proportion of time that fish were located in the middle part of the water column remained unchanged ($p = 0.058$) throughout the course of the experiment.

6.3.4 Aquatic surface respiration (ASR)

The time that inanga spent performing ASR increased at low PO_2 ($p < 0.001$, Fig. 6.4). At 20 kPa, very few fish performed ASR, and no significant change was observed until a water PO_2 level of 4 kPa was reached. At this level the fish spent an average 16.4 ± 1.0% of the time performing ASR ($p < 0.05$, Fig. 6.4), and at 3 kPa this value increased to 29.0 ± 2.6%, significant compared to the ASR recorded in normoxia ($p < 0.05$, Fig. 6.4). Although at a PO_2 of 3 kPa inanga spent most of the time in the lower part of the water column, they made frequent brief visits to the surface to perform ASR.

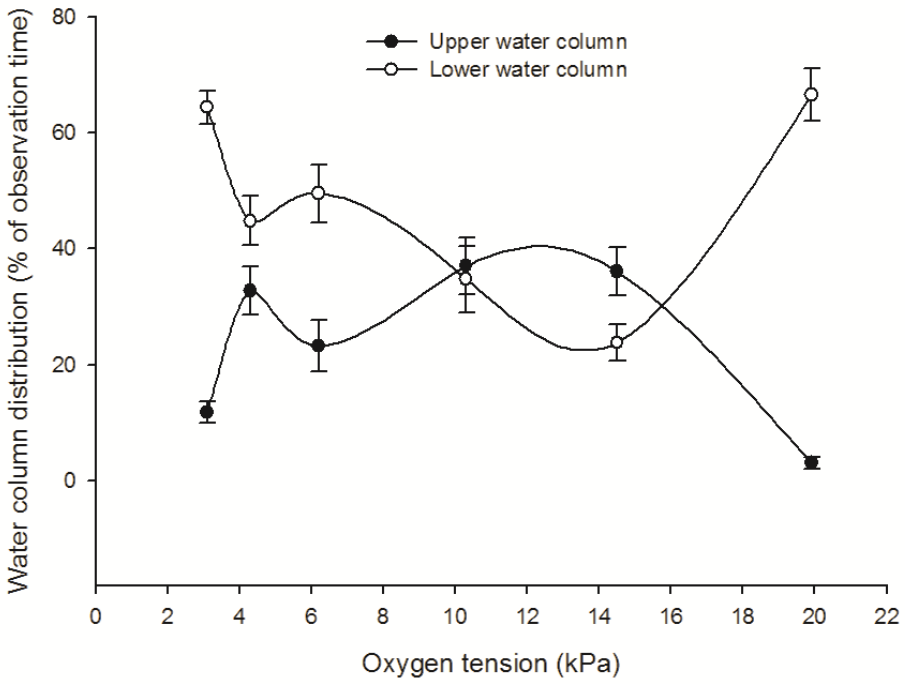

Figure 6.3: Distribution of inanga (as percentage of time an individual spent at each section) between the upper and lower portions of the water column at different dissolved oxygen levels. Data are presented as mean ± SEM.

6.3.5 Avoidance and emersion behaviour

Avoidance behaviour, defined as when inanga tried to jump out of the aquarium, was only observed at the lowest oxygen concentrations (< 6 kPa). This behaviour was characterised by the fish swimming to the lower reaches of the aquarium, before shooting up at speed to exit the water. An average of 12 and 16 fish (~70 and ~94%) exhibited avoidance behaviour at 4 and 3 kPa respectively ($p < 0.001$). On the other hand, emersion behaviour, defined as inanga succeeding in jumping onto the floating platform, was only exhibited at 3 kPa, where on average 4 fish (24%) voluntarily left the water ($p < 0.05$). As the fish emersed, they flopped a couple of times with the upper body to ensure complete emersion from the water. After that, they remained subdued during emersion, except from opercular movement and an occasional shifting of position. Fish were emersed for a maximum of 60 min (entire hypoxic period and the duration of the normoxic restoration period).

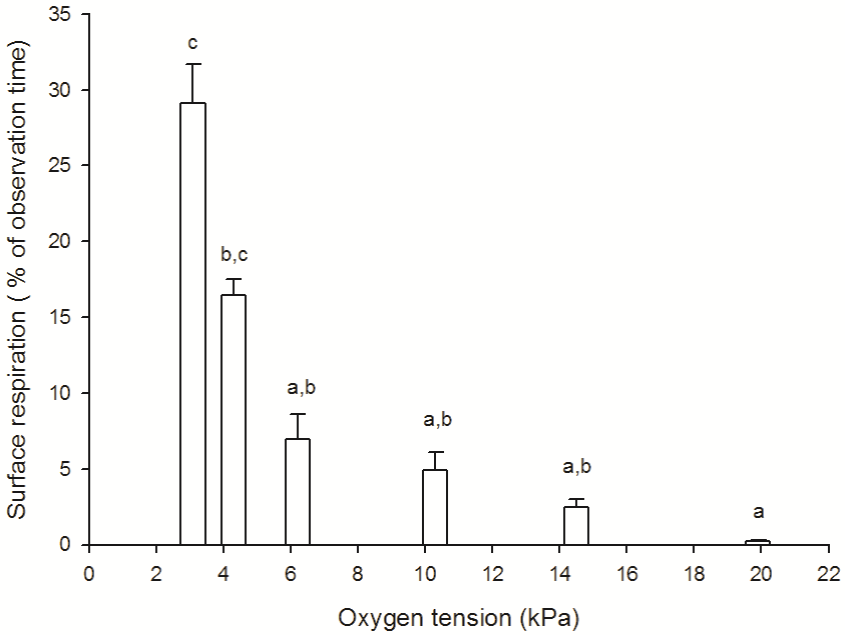

Figure 6.4: Aquatic surface respiration (ASR; as percentage of time spent performing ASR) in inanga at different dissolved oxygen concentrations. Data are presented as mean ± SEM. Plotted points sharing letters are not significantly different (α = 0.05) as determined by repeated measures ANOVA followed by post-hoc Tukey analysis.

6.3.6 Opercular frequency

Opercular beat frequency showed an increase as PO_2 declined ($p < 0.001$, Fig. 6.5). At 20 kPa opercular frequency was 53.9 ± 5.4 beats min^{-1}, while at 3 kPa it had increased to 144.3 ± 2.1 beats min^{-1} (2.7-fold increase, $p < 0.001$, Fig. 6.5). Conversely, during recovery, as the PO_2 increased, opercular frequency decreased ($p < 0.001$, Fig. 6.5). Emersed fish showed an opercular frequency slightly higher than fish in normoxic water (73.1 ± 4.7 beats min^{-1}; $p = 0.04$, Fig. 6.5). During recovery from hypoxia, opercular frequency trended similarly to opercular frequency measured during hypoxia induction, albeit slightly higher. The opercular frequency during recovery at 8 kPa was 150.9 ± 4.8 beats min^{-1}, 14.6% higher than at 8 kPa during hypoxia induction (value calculated from the regression equation: opercular frequency = $-0.2684*PO_2^2 + 1.289*PO_2 + 137.98$, Fig. 5; $p = 0.015$). Opercular frequency was also

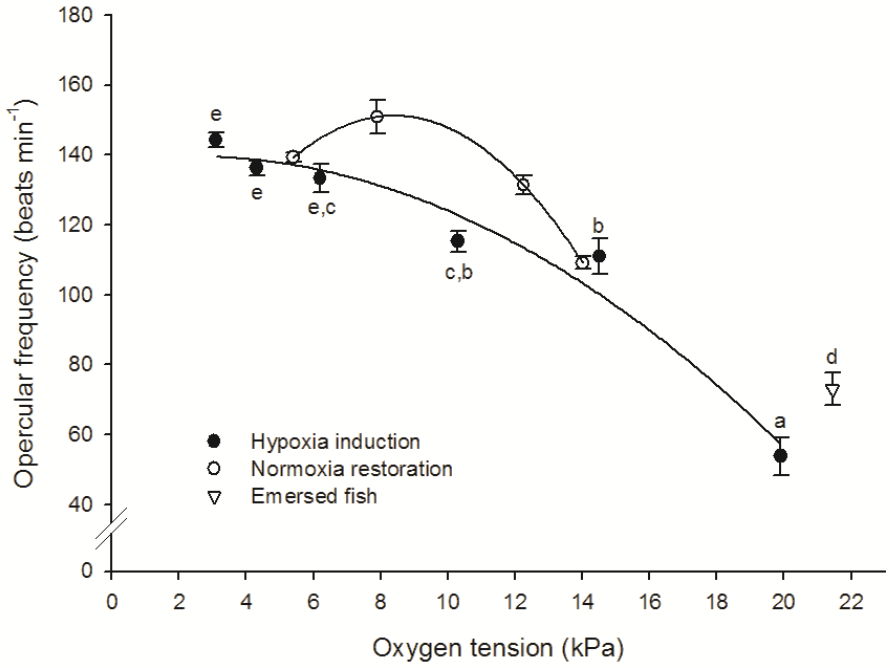

Figure 6.5: Opercular frequency in inanga (beats per minute) at different dissolved oxygen levels during hypoxia induction, restoration of normoxia and in emersed fish. Data are presented as mean ± SEM. Plotted points sharing letters are not significantly different (α = 0.05) as determined by repeated measures ANOVA followed by post-hoc Tukey analysis.

examined in fish performing ASR *versus* those that were not performing ASR at 3 kPa. There was no difference in gill ventilation rates between these two groups (p = 0.709).

6.3.7 Changes within the hypoxic period

Changes in behaviours during the hypoxic period, and the statistical tests used to assess these behaviours, are detailed in Table 6.1. Although the apparent swimming speed of inanga remained unchanged as hypoxia progressed, a marked reduction in the time that the fish spent active was recorded. Therefore, after 30 min of hypoxia exposure, the time that inanga spent active had reduced by about 50% relative to the start of the 3 kPa period. This was accompanied by a decrease in the number of fish trying to escape the water, which dropped to 4 compared to the 16 at the initiation of the 3 kPa period (Table 6.1). Although not significant, over the 30 min of hypoxia

Table 6.1: Apparent swimming speed, swimming activity, water column distribution, ASR, opercular frequency, avoidance behaviour, loss of equilibrium and emersion behaviour in inanga after a progressive decrease in PO_2 until 3 kPa (initiation of hypoxic period), and then after 30 min of hypoxia (3kPa).

Parameter	At initiation of hypoxia (3 kPa)	After 30 min hypoxia (3 kPa)	Statistical test	*p* value
Apparent swimming speed	103.2 ± 12.1	88.9 ± 10.8	RM ANOVA	0.328
Swimming activity	56.5 ± 3.4	29.1 ± 3.2	RM ANOVA	<0.002
Upper water column distribution	11.8 ± 1.9	36.3 ± 8.6	RM ANOVA	0.057
Middle water column distribution	23.7 ± 1.9	28.1 ± 3.5	RM ANOVA	0.241
Lower water column distribution	64.4 ± 2.9	35.5 ± 7.1	RM ANOVA	0.011
ASR	29.1 ± 2.6	29.5 ± 6.5	RM ANOVA	0.967
Opercular frequency	144.3 ± 2.1	144.7 ± 2.3	RM ANOVA	0.863
Avoidance behaviour	16 ± 1	4 ± 1	Pearson X^2	< 0.001
Emersion	4 ± 0.5	8 ± 0.5	Pearson X^2	> 0.05
Loss of equilibrium	1.6 ± 0.4	3.5 ± 0.5	Pearson X^2	> 0.05

Data are presented as mean ± SEM. Apparent swimming speed is expressed in body lengths min^{-1}; swimming activity, water column distribution, and ASR are expressed as the proportion of time that individual fish was engaged in the behaviour or found in the tank position; opercular frequency is expressed in beats min^{-1}; avoidance behaviour, loss of equilibrium and emersion behaviour are reported as absolute frequencies.

exposure the number of fish that emersed doubled, and fish that were already emersed did not return to the water over this period. Fish spent about 45% less time in the lower part of the water column compared to the start of the 3 kPa period (Table 6.1). No other measured behaviours changed significantly over the thirty minute period of hypoxia evaluated (Table 6.1).

6.3.8 Oxygen consumption

The oxygen consumption rate (VO_2) of inanga in normoxic water (20 kPa), hypoxic water (3 kPa) and air, was significantly different ($p < 0.001$, Fig. 6.6). The oxygen consumption of the normoxic fish averaged 9.5 ± 0.4 μmol O_2 g^{-1} h^{-1}, while in hypoxia it was significantly lower, with a mean value of 3.9 ± 0.2 μmol O_2 g^{-1} h^{-1} ($p < 0.05$). The oxygen consumption of the emersed fish was 6.3 ± 0.5 μmol O_2 g^{-1} h^{-1}, lower than in normoxic water ($p < 0.05$), but higher than in hypoxia ($p < 0.05$, Fig. 6.6).

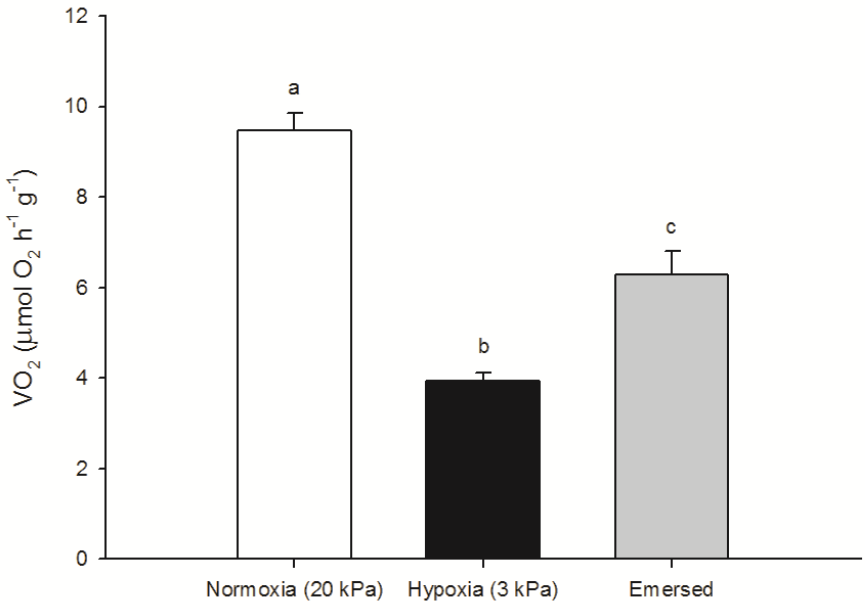

Figure 6.6: Oxygen consumption rate in inanga (μmol O_2 g^{-1} h^{-1}) while in normoxic water (20 kPa), hypoxic water (3 kPa) and emersed. Data are presented as mean ± SEM. Plotted points sharing letters are not significantly different (α = 0.05) as determined by a one way ANOVA followed by post-hoc Tukey analysis.

6.4 Discussion

Inanga did not exhibit behaviours consistent with an energy-saving strategy in response to acute aquatic hypoxia, but instead showed a complex and graded behavioural escape repertoire that culminated in emersion. This emersion resulted in

an enhanced capacity to acquire oxygen relative to remaining in hypoxic water. Despite the severity of the hypoxic exposure used in the present work, no mortalities were recorded either during the experiments or after a 7 day recovery period, suggesting that behaviours observed were largely in response to physiological drivers, caused by hypoxia.

6.4.1 Inanga do not display depressed activity-related behaviour in response to acute hypoxia

An increase in apparent swimming speed as the oxygen concentration decreases has been documented in several small- to medium-sized tropical fish (e.g. Soares et al., 2006; Soares and Junk, 2000), and recently in migratory weakfish (Brady et al., 2009). In particular, an increase in the horizontal swimming speed has been proposed to be related to the onset of ASR as this enhances intake of oxygenated surface water (Soares et al., 2006). The present results showed a similar increase in horizontal swimming speed with decreasing PO_2, thus supporting the idea that this facilitates ASR once the fish is at the water surface. At the lowest oxygen tensions inanga were observed to swim towards the bottom of the tank, before returning rapidly towards the surface in an apparent attempt to leave the water. Surprisingly the average apparent swimming speed recorded at 3 kPa PO_2 was ~0.15 m s^{-1}, which is lower than the sustained swimming speed reported for inanga (0.19 to 0.36 m s^{-1}, Mitchell, 1989; Boubee et al., 1999), and much lower than the reported maximal swimming speeds (0.47 to 1.3 m s^{-1}, Mitchell, 1989; Boubee et al., 1999; Nikora et al., 2003). The present findings suggest that even though an increase in the swimming speed was evident, the fish were not exceeding their routine swimming speed. The advantage of this is that an increase in speed within the routine range would not add greatly to oxygen consumption. This is a consequence of the relatively lower slope of the relationship between routine swimming speed and oxygen consumption (compared to slow and fast extremes) that has been reported in other fish species (Farrell et al., 2003). Diversion of oxygen supply from the gut, for example, might be sufficient to fuel locomotor activity without altering overall oxygen consumption (Thorarensen, 1993). Therefore, if the observed increase in swimming speed within the routine range occurs without an increase in oxygen consumption, this would be an effective strategy

to increase survival time. It is, however, possible that the restricted size of the tank (4 to 6 body lengths) limited swimming speeds, although clearly this did not compromise the capacity to emerse.

A decrease in swimming speed during hypoxia has been observed in dogfish (*Scyliorhinus canicula*; Metcalfe and Butler, 1984), eelpout (*Zoarces viviparous*; Fischer et al., 1992) and Atlantic cod (*Gadus morhua*; Chabot and Dutil, 1999; Herbert and Steffensen, 2005). The contrast with inanga could indicate different physiological adaptations or energetic strategies to tolerate hypoxia. It might also reflect the duration and specific nature of the hypoxia exposure (Chabot and Dutil, 1999). Differences in behavioural approaches to hypoxia may also reflect differences in hypoxic habitats (Schurmann and Steffensen, 1994; Chabot and Claireaux, 2008). As an example, for *G. morhua*, a marine fish living at depth, horizontal migration is a more suitable option than moving to the surface to avoid hypoxia. Furthermore, the spatial spread of hypoxia distributed by marine currents (Chabot and Claireaux, 2008), may favour the adoption of an energy-saving strategy over an avoidance response. In the case of some tropical fish species (Soares et al., 2006) and inanga, which inhabit shallow waters, active avoidance of hypoxia, reflected by the increase in swimming speed, might be worthwhile. It is worth noting that inanga did not increase their apparent swimming speed within the period of constant hypoxia, in direct contrast to the increase in this parameter during progressive hypoxia.

The water column distribution of inanga showed a complex behavioural response to hypoxia, but one that was consistent with the results for swimming speed. Initially, possible escape behaviour at 15 kPa (movement towards the surface) might be indicative of avoidance of potential physiological stress. Subsequently, as this did not relieve the hypoxia, inanga slowly returned to their initial water column distribution as PO_2 decreased. Below an oxygen tension of 15 kPa the proportion of time the fish spent swimming did not change, and remained constant down to the lowest PO_2, whereupon active fish showed an increase in apparent swimming speed and ASR. Below a PO_2 of 4 kPa fish spent most of the time in the lower part of the water column, but made frequent short visits to the water surface to perform ASR or to engage in avoidance behaviour. This suggests that inanga may delay the amount of time spent in the upper water column until such point that hypoxic stress overcomes

the risk of predation associated with a higher position in the water column. It is important to note that the nature of these experiments cannot eliminate the possibility that chronic exposure to hypoxic waters would eventually promote a physiological energy-conservation response, should avoidance or emersion be unsuccessful. However, despite the use of a hypoxia level that induced equilibrium loss in inanga, there is no evidence from the present study that inanga employ such a strategy.

6.5.2 Responses to diminishing dissolved oxygen

As the proximity of atmospheric oxygen results in a higher PO_2 in the water surface layers, ASR has evolved in different fish families as a mechanism to improve oxygen uptake under hypoxic conditions, and has been extensively documented in a variety of aquatic habitats and latitudes (e.g. Gee and Gee, 1991; Soares et al., 2006; Mandic et al., 2009). Furthermore, ASR has been previously documented in inanga (Dean and Richardson, 1999) and in another galaxiid fish, *Galaxias rostratus* (McNeil and Closs, 2007). The present study confirms that inanga use ASR as a strategy when faced with diminishing PO_2 levels. Under the present experimental conditions, there was insufficient evidence to conclude whether ASR brings a physiological relief to inanga, although it is noted that at 3kPa gill ventilation rates (opercular frequencies) were equivalent between fish performing ASR and those that were not. Furthermore, as PO_2 levels progressed the appearance of avoidance and emersion behaviours suggests if enhanced oxygen uptake did occur as a result of ASR it was insufficient to completely relieve hypoxic stress.

The onset of ASR was recorded at 4 kPa, in exact agreement with the value reported for the closely-related *G. rostratus* (McNeil and Closs, 2007). Dean and Richardson (1999), using similar methodology to that employed in teh current study reported that adult inanga moved to the surface after 12 h at 3 mg $O_2\,l^{-1}$ (~6 kPa), contrasting with the almost immediate response recorded here. One possible explanation for this difference could be the observer position. In the current study the video camera was placed at one side of the aquarium, while in the study of Dean and Richardson (1999) the observer was above the aquarium, and thus the fish could have delayed ASR due to the perceived risk of predation. Aerial predation on galaxiid fish is known to occur

from examination of stomach contents from coastal bird species (Falla and Stokell, 1945). Predation risk has been shown to inhibit surface use (Sloman et al., 2008), and to cause reductions in the surfacing rate (Herbert and Wells, 2001) and onset of surfacing (Shingles et al., 2005).

Under oxygen limitation increases in opercular frequency help the fish to remove more oxygen from the water by increasing water flow across the gills (Nilsson and Renshaw, 2004; Hughes, 1973, Maxime et al., 2000). The specific increase in opercular frequency during oxygen depletion and the fall in this measure during oxygen repletion is indicative of a specific response of this parameter to PO_2. The early and marked increase of the opercular frequency at 15 kPa, accompanied by an increase in the fish activity and surface migration, is suggestive of an ability of inanga to detect minor changes in PO_2 levels and respond to modest oxygen depletions. On the other hand, it has been proposed that an elevated opercular frequency can indicate physiological stress (Kramer, 1987; Petrosky and Magnuson, 1973). However, detection of diminishing PO_2 values has been reported in other fish species at levels significantly above the physiological stress thresholds (Brady et al., 2009; Herbert and Steffensen, 2005) or known to cause impairment to parameters such as growth (Chabot and Dutil, 1999). Further data are required to determine whether the gill ventilation responses of inanga to a slight desaturation of PO_2 (from 20 to 15 kPa) represents the onset of physiological stress or whether this is a behavioural response triggered to facilitate hypoxia avoidance that safeguards against future physiological stress.

The maximum opercular frequency has previously been found to occur when, or soon after, the fish start using ASR (McNeil and Closs, 2007; Gee and Gee, 1991). Similarly, in the present study the maximum opercular frequencies were recorded at the lowest oxygen tensions (4 and 3 kPa), coincident with the onset of ASR. McNeil and Closs (2007) reported that *G. rostratus* was unable to maintain ASR during severe hypoxia and anoxia, and ASR was accompanied by a sustained high opercular frequency and followed by loss of equilibrium (McNeil and Closs, 2007). In the present experiment, once the fish emersed, the opercular frequency decreased and remained constant at a rate similar to that shown between 20 and 15 kPa oxygen tensions. This finding could indicate physiological relief and suggests that aerial

respiration was capable of better meeting the fish oxygen requirements than remaining in hypoxic water. This is also supported by the oxygen consumption data (Fig. 6.6).

Quantification of the oxygen consumption rates in inanga under normoxic conditions (9.5 ± 0.4 μmol O_2 g^{-1} h^{-1}) were well in agreement with previous measurements (11.1 ± 0.2 μmol O_2 g^{-1} h^{-1}, Chapter II; 10.2 ± 0.3 μmol O_2 g^{-1} h^{-1}, Chapter IV; ~ 12.2 μmol O_2 g^{-1} h^{-1}, Encina-Montoya et al., 2011). Oxygen consumption rates were also similar to closely-related species, i.e. *Lepidogalaxias salamandroides* (~13 μmol O_2 g^{-1} h^{-1}) and *Galaxiella nigrostrata* (~21 μmol O_2 g^{-1} h^{-1}) (Thompson and Withers, 1999). Under hypoxia, oxygen consumption decreased to about 40 % of the normoxic value, attaining a value similar to that recorded for *L. salamandroides* at a similar level of hypoxia (~4.5 μmol O_2 g^{-1} h^{-1}, Thompson and Withers, 1999; *versus* 3.9 ± 0.2 μmol O_2 g^{-1} h^{-1}, present study). Values under hypoxia were also well in agreement with those obtained in Chapter IV. This observed decrease could be indicative of metabolic depression or may represent an inability of the fish to extract sufficient oxygen from the water. Arguing against metabolic depression is the fact that in hypoxia, inanga maintained activity at levels similar to, or even higher than, those in normoxia. Furthermore, results from Chapter V showed that inanga lost their equilibrium at an external PO_2 that was always above 2 kPa. This suggests that the inability of inanga to extract oxygen from hypoxic water was the cause of reduced oxygen consumption. In emersed fish, the elevated oxygen consumption rate relative to hypoxic fish represents a physiological advantage, and explains the likely benefit of performing such an extreme behaviour at greatly reduced water oxygen levels. The oxygen consumption values reported in emersed inanga (6.3 ± 0.5 μmol O_2 g^{-1} h^{-1}, at 14°C), in the present study, are well in agreement with values previously reported following emersion in a related species (*G. nigrostrata* ~6.7 μmol O_2 g^{-1} h^{-1}, at 20°C, Thompson and Withers, 1999). This therefore suggests that inanga are better able to extract oxygen from air than from oxygen-limited aquatic environments.

As discussed in Chapter IV (section 4.4.3), inanga present several characteristics likely to be advantageous for cutaneous gas exchange. Cutaneous oxygen uptake is likely to be of greater importance during aerial ventilation, as gill lamellae in all but a

few aerially-respiring fish, are adapted for aquatic ventilation and tend to collapse upon exposure to the less dense medium of air (Graham, 1973). In the present study, although inanga decreased their ventilatory frequency while emersed, they were able to increase oxygen uptake, suggesting that cutaneous oxygen uptake may play an important role in the emersion strategy of inanga exposed to hypoxic waters.

When restoring the oxygen conditions (i.e. bringing PO_2 from hypoxia back to normoxia), inanga opercular frequency decreased as the oxygen concentration increased, highlighting the ability of inanga to recover from hypoxic episodes. However, relative to hypoxia induction, opercular frequencies were significantly higher during this recovery, suggesting that during hypoxia part of the energy requirements were satisfied using anaerobic metabolism, and consequently an oxygen debt was accumulated. This likely required relatively higher levels of oxygen uptake during recovery to service this debt. Higher opercular frequencies during post-hypoxia recovery have been previously associated with reoxidation of anaerobic metabolic end products in other fish species (e.g. *Scophthalmus maximus*; Maxime et al., 2000).

Based on the results of the present study, it its proposed that inanga has the ability to respond to small degrees of water oxygen desaturation (from 20 to 15 kPa). Once a decrease in PO_2 is detected, physiological responses such as increased ventilation rate and behavioural responses such as increased activity and swimming speed are induced, followed by ASR, avoidance and emersion. Oxygen concentrations below 6 kPa, in which the fish showed a high opercular frequency and swimming speed, will demand a great physiological effort to maintain homeostasis, therefore potentially impairing other fundamental functions such as feeding, reproduction and predator avoidance. Furthermore, it is proposed that waters with oxygen tensions below 4 kPa impose metabolic restrictions in inanga, which maintain high levels of activity in light of reduced oxygen consumption rates. However, inanga seem to be physiologically adapted, at least in the short term, to emerse from hypoxic water and exploit the air as a source of oxygen to better meet its energy demands. The potential physiological advantages of emersion, compared to remaining in aquatic hypoxia, are further explored in the next chapter.

Chapter VII

Should I stay or should I go?: Physiological, metabolic and biochemical consequences of voluntary emersion upon aquatic hypoxia in the scaleless fish *Galaxias maculatus*

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7.1 Introduction

Data from Chapter IV showed that the scaleless inanga displayed an unusual pattern of oxygen consumption as environmental oxygen declined. Instead of oxyregulating as the majority of fish do (Ultsch et al., 1978; Ultsch et al., 1981; McKenzie et al., 2007), inanga oxyconform, such that there is a linear relationship between oxygen consumption and environmental oxygen content (Chapter IV). Furthermore, the previous chapter (Chapter VI) showed that an extreme behavioural mechanism may also play a role in the response of this species to declining environmental oxygen. Upon exposure to severe hypoxia (~3 kPa) in a laboratory setting, inanga voluntarily leapt from the water, emersed themselves on floating platforms, and respired in air (Chapter VI). This suggests that the oxyconforming strategy of inanga hinders their ability to deal with extreme hypoxia, and instead this fish can more easily meet its metabolic requirements by exploiting the oxygen-rich air. The aim of the present study was to evaluate the physiological, metabolic and biochemical responses of inanga when exposed to aquatic hypoxia or when emersed. It is hypothesised that emersion is energetically and biochemically favourable, relative to remaining exposed to aquatic hypoxia.

7.2 Materials and methods

7.2.1 Fish and rearing conditions

Adult inanga were caught, transported to the aquarium facility in the School of Biological Sciences at the University of Canterbury and maintained as described in Chapter II. No mortalities were recorded during the acclimation period. Feeding was stopped two days before the experiments started.

7.2.2 Experimental design

A total of 48 fish (mean weight ± SD, 6.3 ± 2.2 g) were randomly allocated to one of 6 experimental treatments (each n = 8): normoxia (control; PO_2 ~19 kPa), progressive aquatic hypoxia (slow decrease in PO_2 from 19 kPa to 3 kPa over ~45 min), aquatic hypoxia (PO_2 ~3.1 kPa) for 1 h following progressive hypoxia, aquatic hypoxia (PO_2 ~3.1 kPa) for 6 h following progressive hypoxia, emersion for 1 h following progressive hypoxia, and emersion for 6 h following progressive hypoxia. There were no differences in the weights or total lengths of the fish groups assigned to any of the experimental treatments (ANOVA, $p > 0.05$).

The experimental chamber consisted of a 5 l plastic aquarium (18 x 18 x 20 cm) partially submerged in a temperature-controlled bath at 14°C. The tank had a continuous recirculating flow (50 l h^{-1}) fed from a 10 l glass reservoir tank equipped with a fine bubble air stone fixed at the bottom. No floating platforms or structures that allowed emersion were provided. Continuous monitoring of oxygen in the experimental tank, oxygen meters, calibrations, acclimation, flow rates, water characteristics and switching between air and N_2 was conducted as described in the previous chapter (Chapter VI). This closed system underwent a 50% water change twice daily. In natural environments inanga are a social species (Mitchell, 1989), and therefore acclimation was conducted in groups of four fish in order to reduce stress and any subsequent non treatment-related metabolic alterations. To avoid pseudoreplication, each of the four fish within a given trial was allocated to a distinct treatment. As there were six experimental groups and only four fish per trial, not all experimental treatments were represented in each trial. Overall there were twelve trials run (four fish per trial to give the total n value of 48; n = 8 for each of the six experimental treatment).

The following text describes an example experiment detailing the manipulation of fish assigned to the normoxic control, progressive hypoxia, aquatic hypoxia and emersion treatments. One of the four acclimated fish (hereafter known as the normoxic control) was removed and lethally sampled using an overdose of anaesthetic (MS222, 1 mg l^{-1}) and spinal cord transection. A blood sample (~25 µl) was withdrawn from the caudal aorta using a pre-cooled heparinised syringe (~16 units ml $blood^{-1}$; lithium salt, Sigma) and used for the analyses described below. Tissue samples were then obtained from muscle, gill and liver, quickly frozen in liquid nitrogen, and transferred to a -

80°C freezer until metabolite analysis (section 7.2.5). Following sampling, air bubbling was switched to nitrogen and the oxygen concentration in the tank holding the three remaining fish was decreased from normoxic to hypoxic levels. Hypoxia induction took ~45 min, and when hypoxia was reached (3.1 kPa) one further fish (progressive hypoxia-exposed) was gently removed and sampled as previously described for the normoxic control. Concurrently, a third fish was removed and emersed in a 250 ml Schott glass bottle, containing moist tissue that covered one third of the inside of the bottle to keep the fish and air humid (mirroring natural river bank conditions; Hickford and Schiel, 2011). This emersion chamber was partially submerged in the same temperature-controlled bath used for the other treatments (14°C). The remaining fish was maintained in aquatic hypoxic conditions (3.1 kPa). After one hour of both emersion (fish 3) and aquatic hypoxia (fish 4), fish were euthanised and blood and tissue were sampled as previously described. For fish that were either emersed or exposed to aquatic hypoxia for 6 h, the same exposure and sampling procedure was followed, except with the extended period of exposure. To exclude the possibility that any changes observed were related to time spent in the exposure apparatus, rather than treatment, an additional experiment was conducted under identical conditions, involving two sets of control (i.e. normoxic) fish, one sampled after the 12 h acclimation period (n = 4) and the other sampled after an additional 6 h (n = 4). No differences in any of the variables measured (Hct, Hb, MCHC, blood pH, plasma sodium concentration) were found between these two groups (all p-values ≥ 0.2; data not shown).

7.2.3 Blood parameters

For each fish, ~25 µl of blood was available for analysis. Immediately after blood was withdrawn, a subsample of 2 µl was diluted in 500 µl of Drabkin's Reagent (Sigma), vortexed and stored in the dark (24 h at 4°C) for total Hb quantification. Briefly, the method consist in rapidly converting total hemoglobin in cyanoderivative at alkaline pH, which then present a peak in absorbance at 540 nm. Samples were read in duplicate via a microplate reader (FLUOstar OPTIMA, BMG Labtech). In parallel, pH was measured in a second blood subsample of 19 µl by using a pH microelectrode

(MI 414, Microelectrodes Inc., USA), connected to a PowerLab/4SP unit (ADI Instruments, Waverly, Australia). This electrode was calibrated daily using three pH standards at 14°C and a reference pH solution (pH 7.442; Radiometer, Copenhagen) was read between samples.

After pH determination, the pH subsample was pooled with the remaining blood (~5 µl to give ~24 µl) and Hct was determined following centrifugation of capillary tubes at 10,000 *g* for 5 min in a haemofuge (Heraeus Sepatatech, GmbH, Germany). MCHC was calculated as the ratio of Hb to Hct. Subsequently, blood plasma was removed from the capillary tube, transferred to a 100 µl centrifuge tube and was again centrifuged (500 x g, at 4°C for 5 min). The plasma Na^+ concentration was then assessed via flame photometry (Sherwood Instruments, Flame Photometer 410) after diluting and vortexing 2 µl plasma in 1400 µl of milli-Q water (18 MΩ; Millipore Synergy® UV).

7.2.4 Branchial Na^+, K^+-ATPase (NKA) activity

Analysis of NKA activity was performed using the modified microplate assay of McCormick (1993). Protein was determined via the Bradford (1976) assay. Details of these analyses are provided in Chapter II (section 2.2.2.5).

7.2.5 Muscle and liver metabolites

Muscle from the medial dorsal area (~100 mg) and approximately half of the liver (~60 mg) were weighed and homogenised as described in Chapter V (section 5.2.4). Glucose and glycogen analyses were also determined as described in Chapter V (section 5.2.4).

7.2.6 Data treatment and statistical analysis

Normal distribution and homogeneity of variances were tested with Kolmogorov–Smirnov and Levene's median test, respectively. All data passed these assessments and were therefore analysed by a one-way ANOVA followed by a LSD post-hoc test when differences occurred (Sokal and Rohlf, 1995). All the statistical analyses were carried out using SigmaStat 3.5 software. Differences were considered significant with a p value lower than 0.05. All data are presented as mean ± SEM.

7.3 Results

No mortalities were recorded in any of the experimental treatments. During progressive hypoxia fish increased their activity, moved to the surface of the tank, actively performed ASR and occasionally tried to jump out of the experimental tank. In the aquatic hypoxia treatments (1 and 6 h) fish continued performing ASR. Conversely, emersed fish were largely quiescent, with fish only occasionally adjusting position by whole body 'flopping' movements. Although opercular frequency was not quantified, observations suggested that this measure was lower in emersed fish than in fish that remained exposed to aquatic hypoxia.

7.3.1 Blood parameters

All blood parameters measured in inanga were altered during either progressive hypoxia or during hypoxia/emersion exposures (ANOVA, Hct, $p < 0.001$; Hb, $p = 0.022$; MCHC, $p = 0.003$; pH, $p = 0.004$; and plasma Na^+, $p = 0.031$, Fig. 7.1, Fig. 7.2 and Fig. 7.3). In the normoxic treatment inanga showed a Hct of 24.9 ± 1.6%, a value that increased to 34.3 ± 1.8% after progressive hypoxia. Hct remained elevated during emersion periods of both 1 h ($p < 0.001$) and 6 h ($p = 0.001$, Fig. 7.1A). Hct also remained high during the first hour of aquatic hypoxia exposure (37.2 ± 1.4%, $p < 0.001$, Fig. 7.1A), but after 6 h of hypoxia this value had decreased to 28.0 ± 2.0%, similar to the normoxic value ($p = 0.212$) and significantly lower than the value after a similar time of emersion ($p < 0.030$). Hb concentration showed a similar pattern to Hct (Fig. 7.1B). In normoxia Hb concentration was 49.0 ± 4.7 mg ml^{-1} which

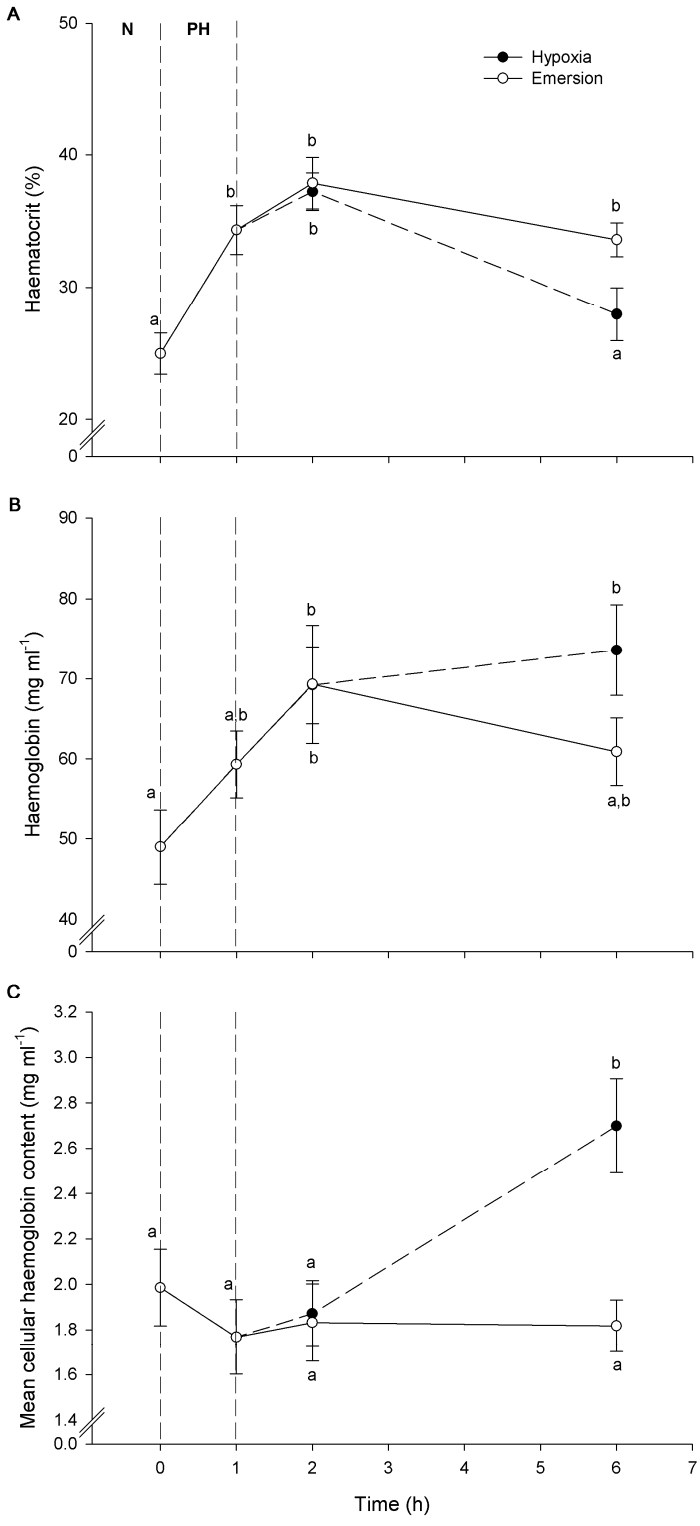

Figure 7.1: Blood Hct (A), Hb (B) and MCHC (C) in inanga in normoxia (N; 0 h), after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a LSD post-hoc test.

increased throughout progressive hypoxia before reaching a significantly higher value after 1 h of aquatic hypoxia (69.2± 4.8 mg ml^{-1}; $p = 0.009$) or 1 h of emersion (69.3 ± 7.4 mg ml^{-1}; $p = 0.009$, Fig. 7.1B). Hb concentration after 6 h of aquatic hypoxia (73.6 ± 5.7 mg ml^{-1}) was statistically unchanged from the 1 h hypoxia level. Although fish exposed to 6 h of emersion trended towards a decrease in Hb concentration (60.9 ± 4.2 mg ml^{-1}) this value was not significantly different from the value attained after 6 h of hypoxia exposure ($p = 0.114$, Fig. 7.1B) nor from the normoxic value ($p = 0.129$).

MCHC is a measure of the amount of Hb per red blood cell. This parameter remained unchanged among all treatments with the exception of the 6 h aquatic hypoxia group, where the decrease in Hct resulted in an elevated mean cellular Hb content of 2.7 ± 0.2 mg ml^{-1}, a value significantly higher than that calculated for all other treatments (all p values < 0.004, Fig. 7.1C).

Progressive hypoxia induced a fall in blood pH to a value of 7.16 ± 0.09 from the normoxic level of 7.49 ± 0.08 ($p < 0.001$, Fig. 7.2). Blood pH did not differ between hypoxia exposure and emersion for the two exposure periods examined (1 h, $p = 0.605$; 6 h, $p = 0.503$) and, similar to progressive hypoxia, both treatments maintained a significantly lowered pH value relative to normoxia (all p values < 0.012, Fig. 7.2).

Plasma Na^+ concentration in normoxia was 120.8 ± 7.3 mmol l^{-1} a level that remained unchanged after progressive hypoxia and after 1 h of hypoxia exposure/emersion (all p values > 0.703, Fig. 7.3). After 6 h both hypoxia exposure and emersion resulted in an increase in plasma Na^+ concentration from the normoxic value to 143.2 ± 4.1 mmol l^{-1} ($p = 0.012$) and 138.8 ± 2.2 mmol l^{-1} ($p = 0.046$), respectively (Fig. 7.3).

7.3.2 Branchial NKA activity

There was an apparent trend whereby NKA activity appeared to decrease during progressive hypoxia and during hypoxia exposure/emersion. However, this effect was not significant (ANOVA, $p = 0.356$, Fig. 7.4).

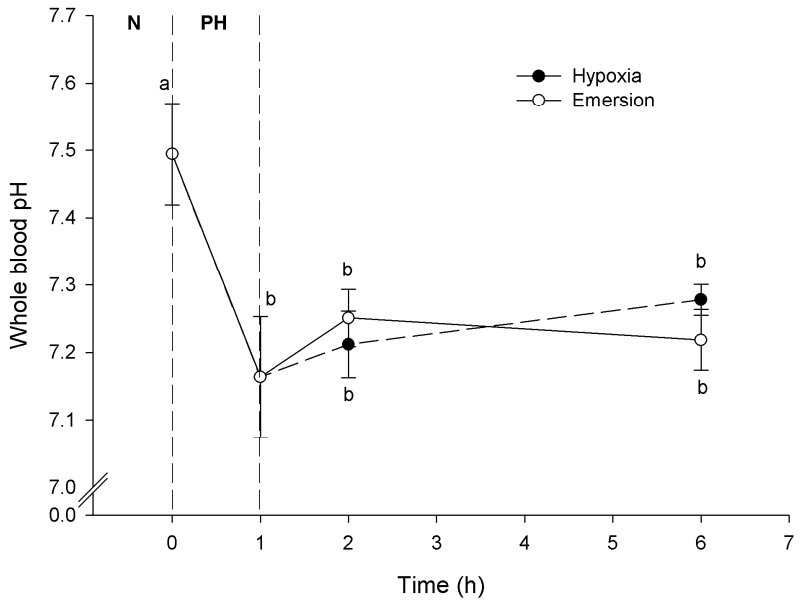

Figure 7.2: Whole blood pH in inanga in normoxia (N; 0 h), after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a LSD post-hoc test.

7.3.3 White muscle metabolites

Muscle lactate, glucose and glycogen levels in normoxia were 4.5 ± 0.7, 1.9 ± 0.4, and 6.8 ± 0.8 μmol g^{-1}, respectively (Fig. 7.5). Muscle metabolite levels remained unchanged during progressive hypoxia (lactate, $p = 0.339$; glucose $p = 0.650$; and glycogen, $p = 0.964$, Fig. 7.5), after 1 h of hypoxia exposure (lactate, $p = 0.646$; glucose $p = 0.398$; and glycogen, $p = 0.132$), and after 1 h of emersion (lactate, $p = 0.720$; glucose $p = 0.455$; and glycogen, $p = 0.213$, Fig. 7.5). However, after 6 h of aquatic hypoxia exposure lactate and glucose levels rose to 6.3 ± 0.5 ($p = 0.04$, Fig. 7.5A) and 4.0 ± 0.8 μmol g^{-1} ($p = 0.004$, Fig. 7.5B), respectively. A similar response was caused by 6 h of emersion, where lactate and glucose levels increased to 7.3 ± 0.2 ($p = 0.002$, Fig. 7.5A) and 5.1 ± 0.5 μmol g^{-1} ($p < 0.001$, Fig. 7.5B), respectively. As would be expected, glycogen levels decreased after 6 h of hypoxia exposure/emersion reaching values of 2.8 ± 0.9 ($p = 0.001$) and 1.8 ± 0.6 μmol g^{-1} ($p < 0.001$), in aquatic hypoxia and emersion treatments, respectively (Fig. 7.5C).

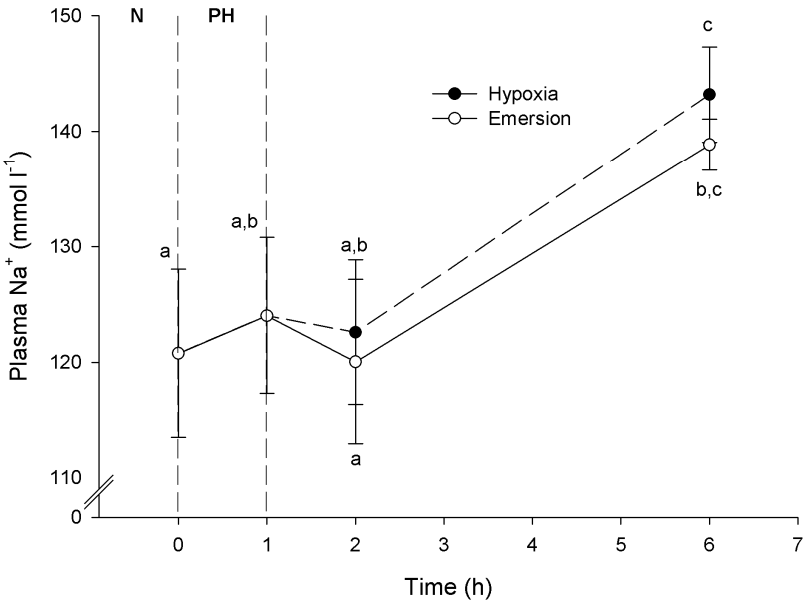

Figure 7.3: Plasma Na^+ concentration in inanga in normoxia (N; 0h), after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a LSD post-hoc test.

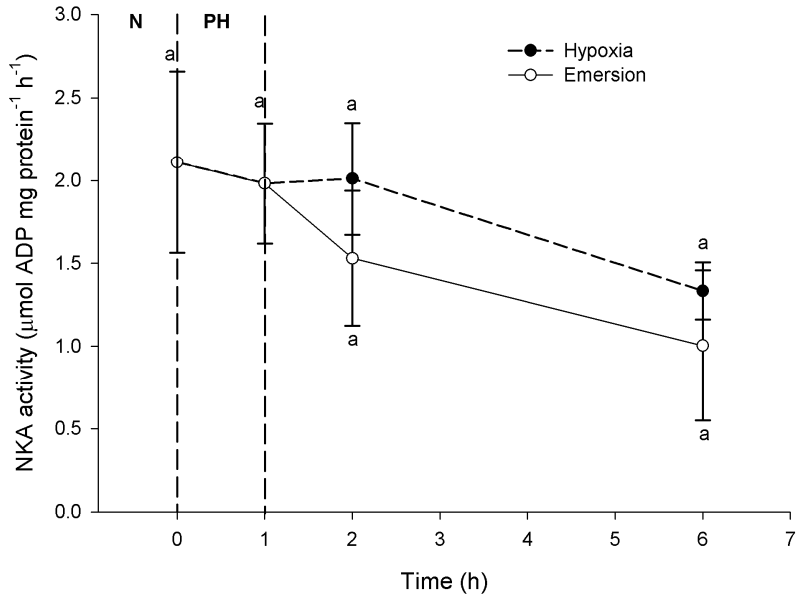

Figure 7.4: Branchial NKA activity in inanga in normoxia (N; 0 h), after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a LSD post-hoc test.

7.3.4 Liver metabolites

Hepatic lactate levels were 9.0 ± 2.1 μmol g^{-1} in normoxia and remained unchanged following progressive hypoxia (9.2 ± 2.1 μmol g^{-1}, p = 0.908) and after 1 h of emersion (11.5 ± 1.1 μmol g^{-1}, p = 0.275, Fig. 7.6A). Conversely, after 1 h of aquatic hypoxia lactate levels increased to 15.2 ± 2.4 μmol g^{-1} (p = 0.01, Fig. 7.6A). Then, after 6 h of aquatic hypoxia exposure or emersion lactate levels dropped to a very similar value in both treatments (~5.3 μmol g^{-1}, p = 0.916), a value not significantly different from that in normoxia (hypoxia, p = 0.099; emersion, p = 0.136) but significantly lower than the value after 1 h of hypoxia exposure and emersion (both p < 0.001, Fig. 7.6A).

Hepatic glucose levels were 16.7 ± 2.9 μmol g^{-1} in normoxia and remained unchanged during progressive hypoxia (17.0 ± 2.1 μmol g^{-1}, p = 0.927, Fig. 7.6B). Although glucose levels trended downwards after 1 h of hypoxia exposure (14.5 ± 1.9 μmol g^{-1}) and 1 h of emersion (13.3 ± 2.5 μmol g^{-1}), values were not statistically different from the preceding treatments (hypoxia, p = 0.455; emersion, p = 0.244, Fig. 7.6B). However, after 6 h of aquatic hypoxia exposure and 6 h of emersion glucose levels had dropped significantly to 8.0 ± 0.3 (p = 0.005) and 9.7 ± 0.8 μmol g^{-1} (p = 0.026), respectively (Fig. 7.6B). Hepatic glycogen levels remained unchanged, with an average value of 82.1 ± 4.6 μmol g^{-1} recorded throughout the experiment (Fig. 7.6C).

7.4 Discussion

In response to aquatic hypoxia, several physiological and biochemical changes were enacted by inanga. Surprisingly, emersion, a behaviour that inanga perform in response to aquatic hypoxia (Chapter VI), evoked similar physiological and biochemical responses to fish that remained in aquatic hypoxia. The only significant differences between emersion and aquatic hypoxia after 6 h were a reduced MCHC and an elevated Hb in the emersed fish. No fish showed any sign of terminal distress

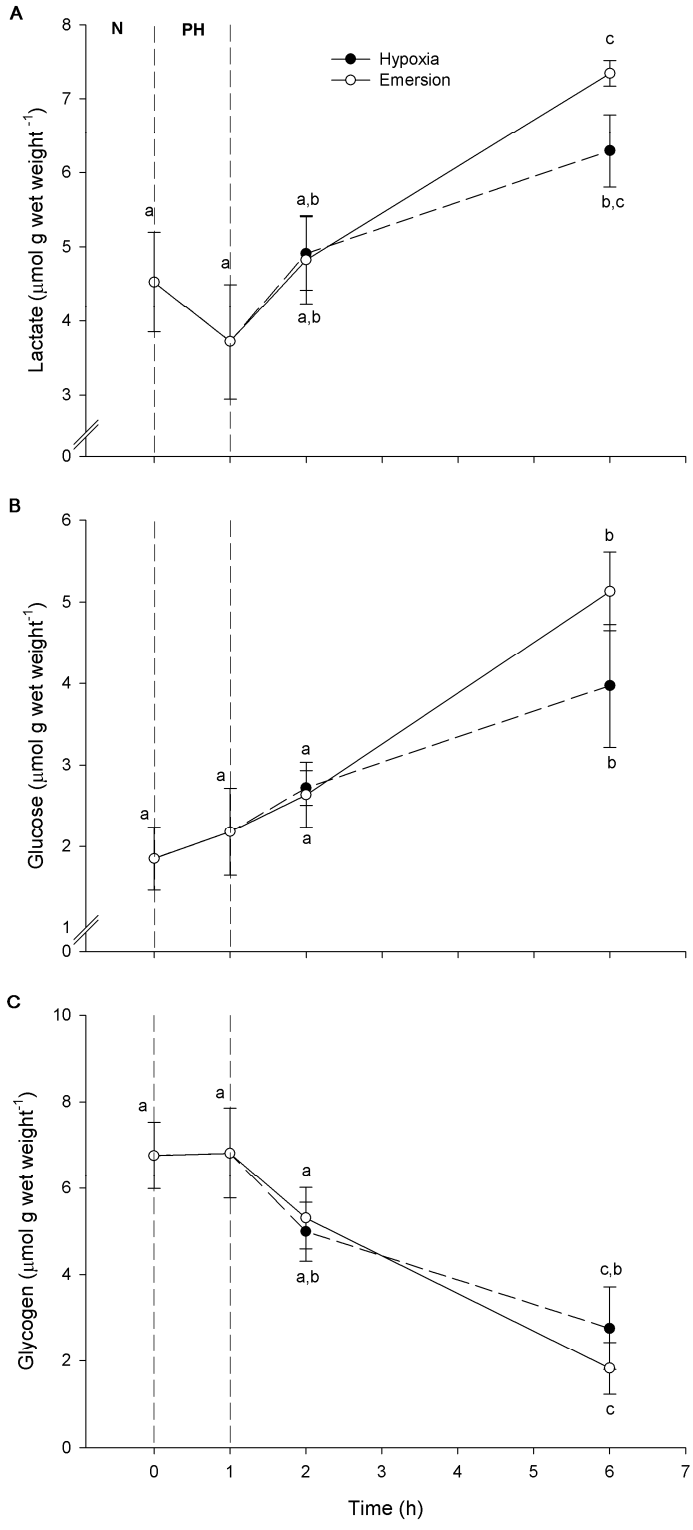

Figure 7.5: Muscle lactate (A), glucose (B) and glycogen (C) in inanga in normoxia (N; 0 h), after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a Fisher LSD post-hoc test.

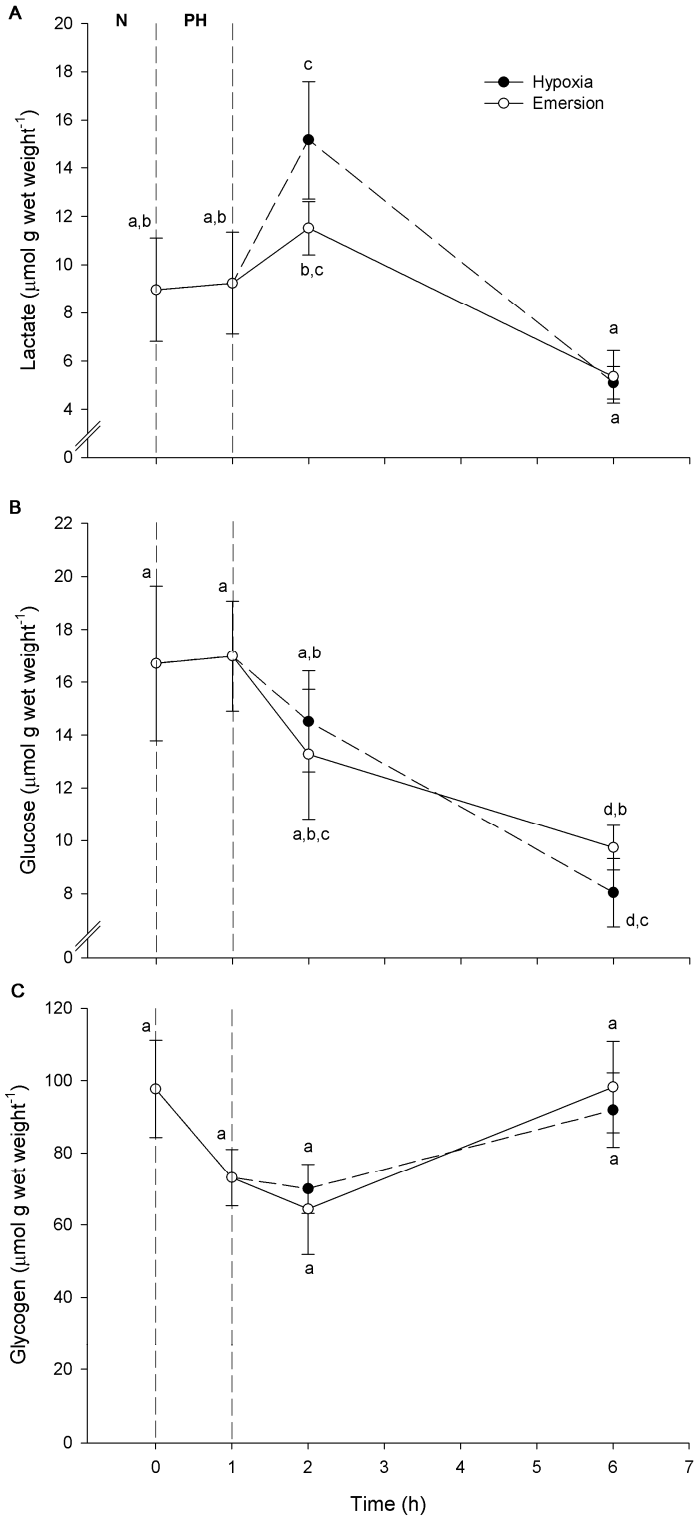

Figure 7.6: Liver lactate (A), glucose (B) and glycogen (C) in inanga in normoxia (N; 0 h) after progressive hypoxia (PH; 1 h), and after 1 h or 6 h of either aquatic hypoxia (black filled circles, dashed line) or emersion (empty circles, continuous line). Values are mean ± SEM; n= 7-8. Plotted points that do not share letters are significantly different ($p < 0.05$) as determined by a one-way ANOVA followed by a LSD post-hoc test.

and no mortalities were recorded during the experiment suggesting that inanga were able to endure at least 6 h of either aquatic hypoxia or emersion.

7.4.1 Blood responses to aquatic hypoxia/emersion

In response to the onset of hypoxia, fish display a number of immediate behavioural and physiological changes which maintain O_2 uptake and delivery to the tissues. Of the physiological responses, increases in ventilatory frequency, altered blood flow, elevation in Hct, and increased Hb content of blood (Randall and Perry, 1992) are first to occur. In Chapter VI increases in ventilatory rate within minutes of hypoxia induction were documented, while the present results showed significant increases in Hct after progressive hypoxia and increased Hb after 1 h of aquatic hypoxia/emersion. This is indicative of a rapid response of this species to increase the oxygen-carrying capacity of blood. This rapid increase in Hct is likely the result of either spleen contraction mediated by catecholamine release (Randall and Perry, 1992) or the result of a reduction in plasma volume due to water movement to surrounding tissues (Jensen et al., 1993). The data showing an increase in plasma Na^+ seems to support the latter explanation, although this effect was not present until 6 h of aquatic hypoxia/emersion, indicating splenic release of red blood cells was the most likely mechanism for Hct increase.

The responses of inanga to hypoxia and emersion seemed strikingly similar after 1 h of exposure. However, after 6 h Hct levels remained elevated in emersed fish, but decreased in fish exposed to aquatic hypoxia. In concert with the decrease in Hct, fish exposed to aquatic hypoxia for 6 h sustained Hb levels that were elevated following progressive hypoxia, resulting in an increased MCHC. The fall in Hct after 6 h suggests that an elevated Hct might have been an issue for fish that remained immersed. An increase in Hct is usually associated with a rise in blood viscosity, and hence an increase in the work that must be done by the heart to perfuse the tissues (Wells and Baldwin, 1990). At some point the additional metabolic cost of elevated Hct will outweigh the benefits of higher blood oxygen-carrying capacity. The carrying-capacity of the blood is ultimately environmentally-determined and consequently the “metabolic tipping point” will occur more readily in a fish that is

immersed in an oxygen-poor environment, than one which is in an oxygen-rich environment such as air. The pattern observed in the current study (maintained high Hct in emersed inanga after 6 h, compared to a Hct reduction in inanga exposed to aquatic hypoxia), supports this idea. The ability of an emersed fish to maintain an elevated Hct could therefore be the physiological trigger that drives the behavioural response of emersion in response to aquatic hypoxia.

The present study showed a significant decrease in blood pH over the 1 h of progressive hypoxia. This increase in blood proton level was maintained throughout the experiment, irrespective of treatment. Acidosis is a commonly reported phenomenon following hypoxia exposure in fish (e.g. Holeton and Randall, 1967). Although plasma lactate was not measured in the present study, it is likely that the acidosis observed was metabolic in origin, confirmed by the accumulation of lactate in muscle that appeared after 6 h.

Most fish haemoglobins display a marked Root effect (Berenbrink et al., 2005). This is characterised by a decrease in pH that results in a decrease in the Hb-oxygen binding capacity and facilitates the release of oxygen to the tissues, where pH is generally lower owing to metabolic acid production (Wells, 2009). The consequence of this effect is to reduce the blood oxygen-loading capacity and therefore oxygen uptake. The presence of a Root effect in inanga has not been specifically tested, but it could explain the observation of inanga as oxyconformers (Chapter IV). If acidification of the blood occurs linearly with declining oxygen, then this would result in a decline in oxygen uptake and may result in an oxyconforming pattern of oxygen utilisation.

There are two important caveats to this hypothesis. The first is that inanga red blood cells would need to lack the adrenergically-regulated sodium-hydrogen exchanger (βNHE). This transporter modulates intracellular pH and therefore would maintain oxygen-binding properties at the gill as oxygen declines. The βNHE is present in most teleost fish, including Salmoniformes and Esociformes (Berenbrink et al., 2005), which are considered to be closely related to galaxiid fish such as inanga (Li et al., 2010). The hypothesis of a Root effect contributing towards oxyconforming would therefore require the specific loss of the βNHE from the inanga lineage. The βNHE is

thought to have been secondarily lost at least five independent times during teleost fish evolution (Rummer et al., 2010), so such a loss is not without precedence. The presence of the red blood cell βNHE in inanga requires further investigation.

It is important to note that most fish exhibit a Root effect at pH values significantly lower than those which could be anticipated during hypoxia (Regan and Brauner, 2010). Thus the second caveat regarding the hypothesis of a hypoxia-induced acidosis leading to oxyconformation in inanga, is the presence of an unusual Hb with a Root effect onset at a plasma pH value close to that found in normoxia. Oxyconforming is a very rare characteristic among fish. It has, however, been previously noted in two species of sturgeon (Crocker and Cech, 1997; McKenzie et al., 2007). White sturgeon display a higher Root effect onset pH (~6.7) relative to other primitive fish (e.g. ~5.8-6.3 for gar and paddlefish; Regan and Brauner, 2010). However this onset pH is still significantly lower than the blood pH predicted for an acidosis in sturgeon, based on other studies of acidotic fish. It therefore remains to be determined whether a high Root effect onset pH might be related to oxyconforming in fish. Consistent with this concept, however, Wells (2009) suggested that fish that exhibit marked metabolic acidosis would not successfully tolerate hypoxic environments unless they used air as a source of O_2. This premise also fits well with the emersion response of inanga to declining environmental oxygen levels (Chapter VI).

Plasma ion balance was impaired by hypoxia exposure and emersion after 6 h, with an increase in plasma Na^+ concentration observed. This is similar to the finding of Richards et al. (2007), in the hypoxia-adapted cichlid *Astronotus ocellatus* exposed to severe hypoxia (0.37 mg O_2 l^{-1}) for 20 h. One explanation for this effect is that an increase in anaerobic metabolites in tissues and interstitial spaces, raised the osmolalities of these compartments, and caused osmotic movement of water from the plasma into the active tissues. Although this would consequently increase plasma Na^+, as observed in the present study, it would also be expected that Hct would increase. However, no elevation in Hct was recorded as aquatic hypoxia progressed (Fig. 7.1A). Changes in plasma Na^+ and pH are often mediated by the actions of the erythrocytic βNHE, which moves sodium into the red blood cell in exchange for protons (Nikinmaa et al., 1990). The actions of the βNHE would be expected to both lower plasma Na^+ and increase Hct (Nikinmaa et al., 1990), inconsistent with the

observations here. The mechanism for the change in plasma Na^+ therefore remains undetermined.

7.4.2 Tissue responses to hypoxia/emersion

The present results indicate that the metabolic demands of inanga white muscle are at least partially satisfied by the use of anaerobic metabolism during aquatic hypoxia and emersion. As hypoxia and emersion progressed, there was a decrease in white muscle glycogen and a resulting increase in glucose. This was related to an increase in muscle lactate concentration, which is consistent with the expected increased anaerobic metabolic activity in inanga white muscle (Hochachka et al., 1973).

Relative to muscle, the liver is of less importance in terms of a potential fuel depot for anaerobic metabolism. Although the liver comprises < 3% of fish mass, it can, however, still play a critical role in anaerobic metabolism as a store of glycogen (White et al., 1964). Hepatic lactate concentration increased only during the first hour in fish exposed to aquatic hypoxia but did not change in emersed fish. This reinforces the concept that emersion provides access to a richer oxygen source, and which therefore delays the onset of anaerobic metabolism, relative to fish remaining in aquatic hypoxia.

While the pattern of muscle metabolites illustrates an active tissue, the pattern in the liver is suggestive of a relatively inactive tissue. Hepatic lactate was not elevated and glycogen levels were maintained at normoxic levels. Unlike the muscle, where glucose levels increased with emersion/aquatic hypoxia, hepatic glucose levels declined. Together these patterns are suggestive of an inhibition of metabolic work by the liver under oxygen limitation. This finding is in agreement with the depression in liver metabolic activity that has been documented in *Cyprinus carpio* (Van Raaij et al., 1994) and *Solea solea* (Dalla Via et al., 1994) under hypoxic conditions.

7.4.3 Anaerobic contribution to metabolism

There are two main strategies for surviving oxygen limitation. Animals may decrease energy requirements and/or they may utilise pathways that do not require oxygen for ATP generation (Hochachka et al., 1973; Boutilier, 2001). Success in hypoxic waters will depend on the capacity of a fish to perform these processes.

Muscle in fish accounts for nearly two-thirds of the body weight (Randall, 2012) and white muscle typically comprises up to 90% of the myotomal muscle mass (Ellerby, 2011). Therefore lactate accumulation in white muscle could be used as a simple proxy of anaerobiosis. Muscle lactate data from normoxia and hypoxia could be used to assess the ability of fish to perform anaerobic metabolism and to depress their metabolism. The VO_2's of inanga are 9.33 ± 2.14 and 3.11± 1.16 μmol O_2 g^{-1} h^{-1} in normoxia and hypoxia (3 kPa), respectively (Chapter IV). At this same hypoxic exposure level lactate accumulation in white muscle was ~0.5 μmol g^{-1} h^{-1} (Fig. 7.5A). This accumulation level can be converted to O_2 equivalents by assuming that 3 μmol of O_2 would be required to alternatively catabolise pyruvate via the citric acid cycle (Finn et al., 1995). Consequently, it can be calculated that inanga depress their metabolism to 49% of the normoxic value under hypoxia (from 9.33 ± 2.14 to 4.61 μmol O_2 g^{-1} h^{-1} when the costs of aerobic conversion of pyruvate are added to the measured hypoxic value of oxygen consumption) and 33% of this is satisfied by anaerobic metabolism. As a comparison, at a higher environmental temperature (28.3 °C) and more severe hypoxia (~1.3 kPa) the extremely hypoxia-tolerant Amazonian cichlid *Astronotus ocellatus,* decreased its metabolism in hypoxia to ~74% of that in normoxia, and ~72% of this was satisfied by anaerobic metabolism (based on data from Sloman et al., 2006; Richards et al., 2007). In another study, Richards et al. (2008), exposed the hypoxia-tolerant mummichog, *Fundulus heteroclitus*, to hypoxia at 20°C, measuring MO_2 and white muscle lactate build up in normoxia and hypoxia (~1.3 kPa). Based on these data hypoxic *F. heteroclitus* displayed a small change in metabolic rate (~95% of normoxic value), but exhibited a large anaerobic contribution (87%) to maintain its energy demands.

The data above suggest that *A. ocellatus* uses a mix of metabolic depression and anaerobic metabolism to satisfy energetic demands under hypoxia. Conversely, *F. heteroclitus* maintains a 'normoxic metabolism' relying almost exclusively on

anaerobic metabolism. These two strategies differ, but both represent a mechanism for withstanding hypoxia. Inanga exhibit decreased oxygen demand with hypoxia and also switch to anaerobiosis. In this regard the response is similar to *A. ocellatus*. However the magnitude of the anaerobic contribution to the total metabolism is far less than that demonstrated by the hypoxia-tolerant species, and underlies the fact that inanga are not especially well-adapted for survival in hypoxic waters.

The present chapter has shown that inanga were able to withstand up to 6 h of aquatic hypoxia and/or emersion by a series of physiological and biochemical responses. At the start of the hypoxic/emersion period inanga rapidly enhanced blood oxygen-carrying capacity. Then after 6 h, inanga displayed increased anaerobic metabolism, coinciding with previous data showing a decrease in oxygen metabolism in hypoxia (Chapter IV and VI). Only a minor proportion of energetic demand (33%) was satisfied by anaerobic metabolism during hypoxia/emersion, which was mainly fuelled by glycogenolysis/glycolysis. Considering the rapid depletion of muscle glycogen stores (Fig. 7.5C), emersion behaviour (Chapter VI), and oxyconforming (Chapter IV), it is proposed that inanga are not well-adapted to survive prolonged periods of hypoxia.

Chapter VIII

Perspectives and Conclusions

8.1 General summary

This thesis examined molecular, biochemical, physiological and behavioural strategies and adaptations that inanga use for tolerating two environmental stressors encountered in its natural habitats; salinity fluctuations and hypoxia. Salinity and oxygen availability have long been recognised as important factors shaping animal physiology (Kinne, 1971). While osmoregulation is crucial to maintain ion and water homeostasis in an environment that varies in salinity, oxygen availability is crucial for maintaining ATP generation and therefore energy homeostasis. Since osmoregulation is an energy consuming process, it could be at risk during hypoxia. Inanga is well recognised for its salinity tolerance, but since hypoxia has also been recently documented in its habitat, inanga present a good model to understand how fish adapt to two different but inter-related stressors.

The salinity tolerance of inanga (Chessman and Williams, 1975; Mitchell, 1989) was explored in Chapters II and III. Results supported the concept that inanga is an exceptionally good osmoregulator, an ability that is the result of rapid enactment of both physiological and molecular responses, accompanied by a bioenergetically efficient mechanism of fuelling salinity-related costs in order to maintain homeostasis. Inanga successfully acclimated to salinities ranging from freshwater to 43‰ (Chapter II). At the physiological level, minor changes in plasma osmolality, ammonia excretion rate and energy substrate usage, and no changes in the metabolic rate and energy expenditures, suggested that salinities ranging from freshwater to 43‰ did not impose a significant stress on inanga (Chapter II). Inanga seemed to rely on a mix of proteins and lipids as an energy substrate, to fuel osmoregulation without detrimental energetic consequences. It is proposed that when osmoregulatory costs decrease, such as in salinities close to the isosmotic point, this “excess energy” is diverted to anabolic processes such as growth and reproduction. An alternative hypothesis suggesting that osmoregulatory costs are low in inanga, based on the existence of an energetically-inexpensive mechanism of maintaining ion and water homeostasis is further discussed in '*Future perspectives*' (section 8.4). Chapter II is also the first study to evaluate the importance of cutaneous gas exchange and

nitrogenous waste excretion in inanga. These findings showed that skin was a major route for oxygen uptake and for nitrogenous waste excretion.

Salinity-induced changes at the molecular level were explored in Chapter III. NKA isoform switching has been previously reported to occur in several salmonid species (*Oncorhynchus mykiss*, *Salmo salar* and *Salvelinus alpinus*, Bystriansky et al., 2006), but has only rarely been explored outside of this grouping. Different isoforms of the NKA α-1 subunit were shown to be expressed by inanga and the exact expression profiles of each of these isoforms in the gill (and to a lesser extent also in the gut) were dependent on the environmental salinity (Chapter III). The expression of α-1a, α-1c in freshwater and of α-1b in seawater are likely to facilitate switching of the osmoregulatory phenotype from ion-absorbing in freshwater, to ion-excreting in seawater. This was further confirmed by the match between the patterns of gene expression of these isoforms and the indicators of physiological acclimation after a salinity challenge (Chapter III). The α-1c isoform has been rarely evaluated in the past, and the data presented in Chapter III were the first to show salinity-induced changes in the expression of this isoform, suggesting a novel physiological role for NKA α-1c in euryhaline fish acclimated to freshwater. This work expands the presence of the isoform switching phenomenon into a new grouping, indicating that it may be more widespread than previously thought. It further supports a physiological role for this phenomenon in salinity acclimation.

Adaptations or responses to hypoxia were examined in Chapters IV-VII. The ability to sustain metabolic rate as external PO_2 decreases is considered adaptative for hypoxia tolerance. Therefore, the lower the $PO_{2\ crit}$, the better adapted to hypoxia a species is considered (Ultsch et al., 1981; Pörtner and Grieshaber, 1993). Chapter IV aimed to determine the $PO_{2\ crit}$ of inanga, yet no signs of oxyregulation were found. This species instead oxyconformed, suggesting it might be poorly adapted to tolerate aquatic hypoxia. Oxyconforming is a very rare response in fish, and although previously suggested, this is the first robust report of it existence. The role of cutaneous gas exchange in the oxyconforming response of inanga was also investigated. Cutaneous oxygen uptake is mainly diffusion-mediated and thus was shown to be largely dependent on the environmental oxygen concentration. As cutaneous oxygen uptake plays such an important role in inanga (see Chapter II) these

data suggested a greatly reduced skin oxygen uptake may explain why inanga was unable to regulate oxygen consumption as ambient PO_2 decreased (see *Future perspectives*, section 8.4).

Based on the oxyconforming response found in Chapter IV, and therefore a lack of respiratory regulation as PO_2 decreased, I rationalised that inanga would be a good model to explore the effects of hypoxia on the relationship between metabolic rate and body mass (i.e. scaling). It was hypothesised that size-related differences in hypoxia tolerance and/or in the mechanism of dealing with hypoxia might exist. Results from Chapter V not only supported the view of a species-specific scaling relationship (Darveau et al., 2002; Hochachka et al., 2003; Bokma, 2004), but also proved that this relationship was altered during hypoxia. This suggests that either inanga of different sizes used different physiological strategies during hypoxia, and/or that sensitivities to low PO_2's were different between small and large fish. This is the first time that the scaling relationship has been used to elucidate intraspecific differences in hypoxia tolerance and then shown to be correlated with anaerobic capacity (Chapter V). It was further found that larger fish were more tolerant to hypoxia than small fish, a result explained by a greater size-specific capacity for anaerobic metabolism.

Based on the oxyconforming response of inanga (Chapter IV) and its unclear advantage during aquatic hypoxia, potential behavioural responses to hypoxia were explored in Chapter VI. Inanga presented a complex repertoire of behaviours as hypoxia progressed, enacting a graded series of responses such as increased ventilation rate, enhanced swimming activity and swimming speed. These were followed by ASR, avoidance and emersion. No signs of an energy-conserving strategy were evident. Instead this response suggested that inanga strongly rely on behaviour to escape hypoxia. This extreme emersion behaviour upon aquatic hypoxia was associated with a decreased opercular frequency and an enhanced oxygen uptake compared to fish that remained exposed to aquatic hypoxia (Chapter VI). This response supported the results obtained in Chapter IV, and further suggested that inanga is poorly adapted to withstand aquatic hypoxia. This emersion response is likely to be advantageous for inanga, allowing it to escape or mitigate hypoxia, at least over short time-frames. Although emersed inanga can successfully exploit

oxygen-rich air via the skin (Chapter VI), maintaining cutaneous permeability may also facilitate evaporative water loss, which is potentially dangerous (Sayer and Davenport, 1991). Therefore, although beneficial, the emersion response of inanga may only be a short-term response to escape aquatic hypoxia.

The use of skin as a fully competent gas exchange organ is likely to be widespread among the Galaxiidae family. The importance of skin for aquatic oxygen uptake and ammonia excretion in inanga was shown in Chapter II, and results from Chapter VI suggested that its role might be even more important during emersion. As previously discussed (section 1.6), characteristics advantageous for cutaneous oxygen uptake are shared by all galaxiids. Furthermore, several mudfish species within the family Galaxiidae are known to pseudo-aestivate during prolonged droughts (Eldon, 1979). Since most fish gills usually collapse out of water (Graham, 1973), those galaxiids that withstand long periods of emersion are likely to rely on the skin for most gas exchange (O_2 and CO_2). Further exploration of cutaneous exchanges within members of the family Galaxiidae is likely to reveal further insights in the role of skin not only during emersion, but also as a fully competent organ for gas exchange.

The hypothesised physiological advantage of emersion in inanga was examined in Chapter VII. Surprisingly it was found that aquatic hypoxia and emersion engendered similar effects in inanga at a physiological and biochemical level. However, Chapter VII also proved that inanga were able to survive up to 6 h of emersion/hypoxia without mortality. A series of rapid changes enhancing whole blood oxygen-carrying capacity were triggered. These included increases in Hb and Hct. Thereafter energy requirements were met in part by anaerobic metabolism, evidenced by muscle lactate accumulation and a drop in blood pH. Decreases in glycogen and increases in glucose suggested that anaerobic metabolism was fuelled by glycogenolysis/glycolysis. Some evidence also suggested that an energy-saving strategy was enacted, including a hypothesised reduction in oxygen consumption by organs such as the liver.

Based on the oxyconforming response (Chapter IV), emersion behaviour (Chapter VI), rapid depletion of glycogen stores (Chapter VII), and a small storage capacity of the liver (~2% of the body weight, Boy et al., 2009), long-term survival of inanga based on anaerobiosis is unlikely to be successful. As such it can be proposed that

inanga is poorly adapted to withstand long-term aquatic hypoxia. Furthermore the data presented in this thesis suggest that low oxygen concentrations are likely to impose challenges on ion and water homeostasis.

8.2 Environmental implications

Owing to a number of factors, including habitat modification and the introduction of salmonids, inanga populations appear to be threatened. In order to sustain this important resource, an understanding of how inanga deal with environmental stressors is critical. This thesis looked at two stressors that are thought to be important in inanga habitats. Hypoxia is likely to increase in streams inhabited by inanga due to eutrophication caused by nutrient enrichment sourced from inputs such as intensive dairy farming (Jenkins, 2010; Morgan et al., 2002). With modification of wetlands, changes in the saltwater wedge may occur, pushing saline waters further upstream into adult waters that are usually unimpacted by salinity (Callander et al., 2011). Consequently, both salinity and hypoxia are environmentally relevant factors. The data contained in this thesis suggest that inanga are unlikely to be threatened by salinity changes, but are potentially at risk from hypoxia.

The salinity tolerance of inanga not only suggests a capacity to deal with changes in the salinity profiles of water, but also suggests a possible mechanism of avoiding competition and predation. As mentioned in Chapter I, the introduction of salmonids into New Zealand for recreational purposes has now lead to the establishment of wild populations. When small, salmon compete with inanga for food and habitat (Bonnet and McIntosh, 2004), and then when adults, salmonids prey directly on inanga (Mittelbach and Persson, 1998; Arismendi et al., 2009). Although most salmonids are also euryhaline species, they can only tolerate changes in salinity at certain points of their life cycle (Wedemeyer et al., 1980; Seidelin et al., 2000). Results from the present study suggest that inanga would be able to acclimate to different salinities at any time during its life cycle. Thus by moving between streams and estuaries inanga populations could avoid predation by salmonids, but also to have access to new food sources. It has previously been hypothesised that a high tolerance to low pH waters

has aided galaxiid fish in avoiding salmonids in acid-rich streams of New Zealand's West Coast (Olsson et al., 2006).

The salinity tolerance of inanga and the existence of a marine larval stage imply an enhanced ability to colonise new environmnets. In fact this has been proposed as one of the main factors for the biogegraphical spread of galaxiids across the Southern hemisphere (McDowall, 1988; 1990). Given the competition with salmonids and anthropogenic habitat degradation (McDowall, 1984), this ability to disperse and avoid polluted and salmon-populated streams is likely to be advantageous.

Streams and estuaries in the West Coast of New Zealand are habitats of major importance for inanga populations, but they are also at risk from metal pollution (Greig et al., 2010; Hogsden and Harding, 2012). One of the main mechanisms of metal toxicity in fish is by impairing ion transport at the gill (Wood, 2012). For example metals such as copper and silver inhibit sodium uptake at the gill and therefore prevent fish from maintaining osmotic homeostasis (Grosell et al., 2002). A recent study has demonstrated that inanga in freshwater have a high affinity of ion transport and high sodium turnover rates (Glover et al., 2011), both of which would potentially mean that any inhibition in ion exchange will have a significant impact on the ability of inanga to withstand metal pollutants. The salinity tolerance of inanga might, therefore, be advantageous for avoiding exposure to such polluted conditions by migrating upstream or downstream to estuaries in seeking less polluted conditions and/or waters of higher salinity. In fact data from Chapter II suggest that metals would have a less deleterious effect in estuaries, at salinities close to the isosmotic point, where inanga do not need to rely as significantly on osmoregulation. Furthermore, since metals are less toxic in saline conditions, owing to enhanced competition between the higher ion content and the metals for binding to the sites of toxic effect, moving to more saline waters could also potentially decrease the risk posed by metals (Wood, 2012).

The exceptional salinity tolerance of inanga is due partly to rapid changes at the molecular level (Chapter III). These allow inanga to express and translate a protein that biochemically favours either ion uptake or ion excretion depending on the external medium (Jorgensen, 2008). Pagliarani and co-workers (1991) previously

showed differences in the biochemical activity of NKA in freshwater- and seawater-acclimated fish. Lower K_m values for K^+ and Na^+ were reported in freshwater-acclimated fish compared to those acclimated to seawater (Pagliarani et al., 1991). This not only supports the view that isoform switching is biochemically beneficial for optimising ion exchange, but also suggests that different biochemical properties of the NKA are likely to confer different sensitivities to metal contaminants. I hypothesise, therefore, that fish acclimated to freshwater will differ in their metal tolerance compared to fish acclimated to seawater. To date, the problem of metals contaminants at different salinities has only been considered from the water chemistry perspective, by considering metal availability (USEPA, 2003; Bianchini et al., 2004), yet differences in animal physiology are rarely acknowledged (Lee et al., 2010). Further research is needed to understand ion exchange in fish, and also to establish environmentally-relevant limits for pollutants, which might well differ between fresh- and seawater, from both a water chemistry and an ionoregulatory physiology perspective.

While inanga appear highly tolerant to salinity change, the data presented here suggest that they are poorly adapted to withstand aquatic hypoxia. In the Canterbury region, farming and dairying activities have proliferated in recent years (increasing 3.6-fold since 1995; Jenkins, 2010). These activities not only reduce oxygen availability in the streams by the input of organic matter and fertilisation, but also by decreasing the water levels due to irrigation purposes (Morgan et al., 2002). These streams are inhabited by inanga, consequently placing these populations at risk. As shown in Chapter V the risk is greatest for small inanga, such as those that arrive in these streams following their migration from the ocean. Furthermore, farming and dairying activities usually involve changes in, or removal of, riparian vegetation. Since one of the few hypoxia adaptations of inanga is to emerse, a lack of riparian vegetation is likely to make inanga more vulnerable to predation and desiccation (Chapter VI). Therefore, under such conditions, emersion might not longer be useful for escaping aquatic hypoxia. Based on the results of Chapter V and VI, particular care should be taken to ensure proper environmental management to maintain riparian vegetation and to minimise nutrient inputs into streams, and therefore minime the risks of hypoxia to inanga. Overall, the present findings indicate that increasing

degradation of near-coastal waters may greatly impact the long-term sustainability of this economically-important and culturally-iconic fish species.

8.3 Methodological considerations

In Chapter II all variables, including MO_2, were measured after acclimation had been achieved (14 days). One aim of this study was to examine whether there were different energy costs associated with the habitation of different salinities. However, by measuring MO_2 only after acclimation was achieved the costs associated with acclimation were not determined. Based on the results from Chapter III, which showed molecular changes at the mRNA level as rapidly as 8 h following seawater challenge, a different pattern of MO_2 might be anticipated if measurements had been performed during salinity acclimation. In fact, an increase in fish metabolic rate would be expected to be observed, until acclimation was completed. This increase would likely to attributed to increased stress levels (Barton and Iwama, 1991), ion pumping (Kelly and Woo, 1999; Morgan et al., 1997), protein synthesis associated with *de novo* synthesis of new transporters (Frick and Wright, 2002), and degradation and ubiquination of the existing transporters inappropriate for the new environment (Richards et al., 2003; Bystriansky et al., 2006).

A second example of the importance of sample timing is with respect to the changes at mRNA level found in Chapter III. In this chapter, the mRNA expression of the NKA α-1b isoform was upregulated 8 h after seawater challenge (Chapter III, Fig. 3.3). However, the rapidity of these changes in inanga were hard to compare with the existing literature, since sampling has usually been conducted after 24 h (Richards et al., 2003; Bystriansky et al., 2006). On a related note, it is also worth noting that changes at the mRNA level, such as the ones reported in Chapter III of this thesis, are not always related to changes at the protein level (Scott et al., 2004). Validating the changes observed at the mRNA level by measuring actual protein levels (Western inmunoblotting) would be an important next step (Scott et al., 2004; Bystriansky et al., 2006). Furthermore, future development of isoform specific antibodies would

greatly contribute in elucidating the physiological role of each isoform in salinity acclimation.

In the present thesis several chapters explored the effect of hypoxia on the physiology of inanga. It is worth noting that since the experimental set-ups sometimes differed, the mechanism of hypoxia induction also differed. For example in Chapter IV (*Series I*, section 4.2.2.1) and in Chapter V hypoxia was induced by the allowing the fish to deplete the oxygen levels in the repirometer. This method for hypoxia induction was different to that used in other experiments (Chapter IV, section 4.2.2.2.; Chapter VI, section 6.2.5) where N_2 bubbling was the mechanism for lowering PO_2. However, despite the different methods for inducing hypoxia same respiratory response was found (Fig. 4.3; Fig. 6.6). The potential problem of inducing aquatic hypoxia by having the fish deplete oxygen in the chamber is the accumulation of metabolic end products, such as CO_2. Although it has been shown that CO_2 controls the respiration reflex in vertebrates in order to maintain blood CO_2 levels within certain limits (Pörtner et al., 1998), in fish the effect of CO_2 on MO_2 appears to be species-specific (Gilmour, 2001; Ishimatsu et al., 2008). Therefore, this suggests that CO_2 levels did not have a significant effect on MO_2 in inanga, at least under the experimental conditions used in the studies reported here.

8.4 Future perspectives

A key finding of the thesis was the capacity of NKA, the main facilitator of ion transport processes, to undergo isoform switching. This phenomenon might be an energetically-efficient way of maintaining ion and water homeostasis. The expression of a protein isoform that biochemically favours either ion uptake or ion excretion depending on the external medium (Jorgensen, 2008) may save ATP relative to other strategies such as increasing the amount of a less-efficient isoform. This hypothesis would not only be consistent with the previous observations of no change in the metabolic rates of some euryhaline fish species (Morgan and Iwama, 1991; Haney and Nordlie, 1997), but would be also be in agreement with unchanged calculated energy costs at salinities ranging from freshwater to 43‰ seawater for inanga

(Chapter II). Although the exploration of osmoregulatory costs in fish has received some attention, it would greatly benefit from further exploration in different fish models. For example, a comparative study evaluating the osmoregulatory costs among fish that exhibit only NKA α1-a, and α1-b isoform switching, those that exhibit NKA α1-a, α1-b and α1-c isoform switching, and those that do not exhibit NKA isoform switching might be of value. There are, of course, several technical and biological issues that make this comparison complex such as difficulties to perform real time physiological measurements (MO_2 and nitrogen excretion), validity of performing an energy balance in such short periods of time, and unnatural fish responses associated with salinity challenges and handling stress. Despite these difficulties, further research in this direction is likely to contribute to our understanding of salinity acclimation in fish and the ecophysiological relevance of different salinity acclimation strategies.

Another aspect that would benefit from future research is a wider exploration of the oxyconforming response in fish. Respiratory control is mainly achieved by the regulation of water flow over the gill epithelium via increasing opercular frequency and/or ventilatory volume (Burggren and Randall, 1978; Burggren, 1988). Therefore, the presence of advanced respiratory and circulatory systems in fish makes it difficult to rationalise the phenomenon of oxyconforming. In fact, the vast majority of literature shows that teleost fish are oxyregulators (Ultsch et al., 1981; Perry et al., 2009). In some studies oxyconforming has been described, but appears to depend mainly on the presence of certain prevailing factors such as water flow (Hall, 1929; Hughes and Umezawa, 1968; Piiper et al., 1970; Steffensen et al., 1982; McKenzie et al., 2007). Historical reports of oxyconforming in fish (e.g. Hall, 1929; Marvin and Heath, 1968; Piiper et al., 1970) have been questioned based on experimental design and/or methodological limitations (Prosser, 1973; Ultsch et al., 1978) such as measurements only in a limited range of normoxic conditions or during asphyxia. To my knowledge the data presented in Chapter IV is the first unequivocal study illustrating oxyconforming in a teleost fish. Most of previous studies reporting oxyregulation have been conducted in relatively large, scaled fish. These are both characteristics that do not facilitate cutaneous oxygen uptake. In light of cutaneous oxygen uptake in the small, scaleless inanga, it is possible that further studies of similar fish may elucidate further examples of oxyconforming. Many galaxiid fish, for example, present similar characteristics to inanga and are likely candidates for further

exploration of oxyconforming. In the Northern hemisphere there also are several small fish species that inhabit environments similar to inanga. Some of these species are similar to inanga in that they emerse in response to aquatic hypoxia or other detrimental aquatic conditions (e.g. *Kryptolebias marmoratus*; Taylor et al., 2008). Since these species, to a certain extent, rely on cutaneous oxygen uptake (Cooper et al., 2012), they might well present an oxyconforming response as aquatic PO_2 decreases.

In the partitioning experiments (Fig. 2.6), it was shown that skin played an important role in gas exchange and ammonia excretion in inanga (Chapter II). Preliminary experiments found high NKA activity in skin tissue, however, no major changes occurred at a molecular level in response to changes in salinity (Chapter III). This suggests that NKA in the skin was probably associated with its basal physiological role in cellular homeostasis, but not directly involved in salinity-related ion regulation. The results showed, however, that the gills were adaptable in terms of their molecular response to changing salinity, confirming that the gill plays the major role in ion regulation in inanga. The contribution of the skin to ion regulation requires further attention to determine whether changes in NKA observed are relevant to salinity acclimation. It was nevertheless clear from this thesis that the skin performs an important role in gas exchange and nitrogenous waste excretion. In general, however, the plasticity of the skin in response to different environmental conditions seemed to be limited, and gills appeared to be more functional and adaptable to meet fish requirements (Chapter II, Fig. 2.6). During emersion, this might well change. Further exploration of the role of fish skin under different environmental conditions might contribute to our general understanding of the role of different epithelia in maintaining homeostasis.

IX

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