

THE ROLE OF SOCIAL RANK IN THE DEVELOPMENT, PHYSIOLOGY AND REPRODUCTIVE STRATEGIES IN SALMONIDS

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..... **JEFFERSON MURUA**

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ABSTRACT

Salmonids naturally organise into social hierarchies both in the wild and aquaculture. This thesis investigates how social rank influences the physiology and development of salmonids with different life strategies using Atlantic salmon (*Salmo salar*) as a model. In broad terms two types of studies were conducted. Firstly osmoregulatory traits of freshwater parr prior to smolting, maturing or remaining immature were investigated using Na⁺ gill uptake kinetics. Highly distinct patterns emerged, especially for Na⁺ uptake affinity, between future alternative phenotypes, which could potentially be used as an identification tool in otherwise visually identical fish. Examination of Na⁺ uptake kinetics from a social status perspective revealed that first and intermediate ranked fish, which received less aggression and had lower cortisol, were better prepared for sea water entry. In the second batch of studies brain serotonergic activity (5-HIAA/5-HT), a key regulator of agonistic behaviour in vertebrates, was examined in a range of social conditions. First, the stability of social ranks was tested by food manipulation. The most dominant fish were able to retain their high status even after being kept in nutrient poor conditions. High status was associated with a high standard metabolic rate (SMR) and low brain 5-HIAA/5-HT. Secondly, studies on hierarchies with marked bimodal size asymmetries showed that upper modal group fish (UMG) became dominant. Despite being subordinate lower modal group (LMG) individuals showed similar growth rates, serotonin turnover and cortisol to UMG fish, possibly due to high aggression and fin injury sustained by high rank fish fighting for dominance. Thirdly, the association between social dominance and developmental pathway was examined in size-matched groups of immature parr and precocious parr, with the latter obtaining higher social positions and showing higher aggression. Brain serotonin turnover revealed higher 5-HIAA/5-HT in immature parr, a phenotypic distinction that was also identified in immature salmonids in aquaculture. Plasma samples from alternative life histories (immature parr, precocious parr and smolts) were also used for a preliminary investigation of potential metabolite signatures utilising metabolomic techniques.

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ABBREVIATIONS

ACTH Adenocorticotrophic hormone
CNS Central nervous system
CRF Corticotrophin releasing factor
DA Dopamine
DOPAC 3,4-dihydroxyphenylacetic acid
E Epinephrine
5-HIAA 5-hydroxyindoleacetic acid
HPA Hypothalamus-pituitary-adrenal (axis)
HPI Hypothalamus-pituitary-interrenal (axis)
HR High responders
5-HT 5-hydroxytryptamine, serotonin
5-HTP 5-hydroxytryptophan
LMG Lower modal group
LR Low responders
L-DOPA 3,4-dihydroxyphenylalanin
MAO Monoamine oxidase
MRC Mitochondria rich cell
 Na^+, K^+ -ATPase Sodium, potassium ATPase
NE Norepinephrine
PNA Peanut agglutinin binding
SMR Standard metabolic rate
TRP Tryptophan
UMG Upper modal group

CHAPTER 1

GENERAL INTRODUCTION

1.1. The salmonid life cycle

Salmonids are amongst the best studied groups of fish worldwide due to their commercial importance and their highly contrasting life history characteristics including wide-ranging migrations and marked polymorphism (reviewed by Hutchings and Jones, 1998; Thorpe et al., 1998; Fleming and Reynolds, 2004). The life cycle of salmonids can be subdivided into several phases, as described by Jones (1959). In broad terms, three distinct phases may take place in a salmon's life: 1) an early juvenile period in fresh water, comprising the alevin to fry steps, thereafter reaching the parr phase after about a year. 2) While still at the immature parr stage at the length of c. 9-20 cm (Armstrong et al., 1997) some fish will either develop into a freshwater resident precocious mature parr or an immature smolt that will undergo an extensive oceanic migration. 3) Finally, anadromous adults will return with high fidelity to their natal river systems to reproduce during the breeding season, typically after 1 to 3 years at sea (Stabell, 1984).

It is in the Atlantic salmon, *Salmo salar*, where divergence in behavioural and phenotypic traits reaches its maximum expression with more than 20 combinations of life historical modes identified (reviewed by Hutchings and Jones, 1998; Marschall et al., 1998; Klements et al., 2003). Genetic and environmental factors have been investigated in the past to establish what causes these dramatic changes in life

histories of individuals originating from the same population (reviewed by Hutchings, 2002a). For example, environmental changes in biotic and physical factors within the river system can influence life history tactics, including displacement by flood or drought events, appearance of predators, shifts in feeding territories and opportunities, changes in appetite, alteration of metabolic rates, water temperature, photoperiod regime, and altitude (Metcalf et al., 1988; Sloman and Armstrong, 2002; Baum et al., 2004). Genetic elements also play a role as recently indicated by Piché et al. (2008) with work demonstrating the heritability of maturation thresholds. Therefore it is likely that sets of multiple variables influence the adoption of specific developmental pathways.

Early fish biologists such as Shaw (1840) recognised that maturing parr were present around female spawning grounds and that their milt could successfully fertilise female eggs. However, it was often assumed that due to their small size precocious parr were individuals of poorer quality (Day, 1885), unable to migrate to sea and return as large adult fish they had to adopt the alternative default strategy, in the case of salmon becoming a sneaker mature parr to ‘make the best of a bad situation’ (Maynard Smith, 1982) as they could not compete directly with stronger anadromous opponents. However, work by Rowe and Thorpe (1990a) found that in fact only individuals growing faster during the first year of life are able to mature as precocious parr, while individuals from the lower modal groups are forced to continue as immature parr for another season. What proportion of a population becomes precocious is likely to be influenced by both genetics and environment and is highly variable (e.g. 5% in Bagliniere and Maisse, 1985; 65% in Whalen and Parrish, 1999). The developmental conversion is conditional on exceeding a genetically predetermined energy or size threshold where the decision to adopt this route is

reached by mid-autumn, 12 months before spawning (Thorpe et al., 1998; Whalen and Parrish, 1999). The conversion can be halted if nutritional status is severely reduced shortly after this first switch point (Rowe and Thorpe, 1990b). The strategy of smolting is also common to salmonids that belong to the upper modal group (Metcalf et al., 1989), however the switch-point occurs by mid-summer, again about a year before migration. However, smolting is considered a negative decision by fish that previously failed to mature in that developmental cycle (Thorpe and Metcalfe, 1998).

Sneaker reproductive males, often referred to as opportunistic or parasitic (Taborsky 1994, 2001), were also thought of as inferior competitors relative to anadromous males during mating due to the large size difference between both phenotypes. However, novel genetic marker data has shown that in many cases early mature parr have a greater reproductive fitness in terms of genetic contribution to the population than anadromous fish (e.g. Atlantic salmon up to 89% - Moran et al., 1996; 86% - Martinez et al., 2000) and may be favoured by females as they are less aggressive (Watters, 2005). This parental contribution is especially important in rivers with declining populations because mature male parr can take over the reproductive role in the absence of significant numbers of returning adult anadromous males (García-Vazquez et al. 2001; Jones and Hutchings, 2001). Despite the importance of early maturity on ecological and economical aspects of population and aquaculture processes respectively, relatively little is known on how interactions between individuals with alternative life history strategies influence social hierarchies and physiological state.

1.2. Social hierarchies in salmonid fish

Linear dominance hierarchies form among salmonid fish due to differential competitive ability to acquire food and shelter (Jenkins, 1969; Bachman, 1984; Abbott and Dill, 1989; Metcalfe et al., 1989; Nakano, 1995a). Social dominance is not particular to salmonids, and has been described in a wide range of organisms (Huntingford and Turner, 1987). Salmon populations may be subdivided into groups within river sections, and within each portion several social hierarchies can be present (Watters, 2005). The size of social hierarchies ranges from simple dyadic dominant-subordinate forms demonstrated many times in the lab (Sloman and Armstrong, 2002), to intermediate-sized hierarchies (i.e. 5-20 individuals) probably more representative of wild populations (e.g. Nakano et al., 1995a,b; Sloman et al., 2008), and large sized groups such as those in aquaculture (e.g. Cubitt et al., 2008).

Salmon engage in social interactions shortly after hatching and naturally organise into social hierarchies both in the wild and in the lab (Bachman, 1984; Metcalfe et al., 1989; Aubin-Hort and Dodson, 2004). These hierarchies are formed as a result of sequential dyadic interactions between members of the group network (Chase et al., 2003). An animal is considered to hold dominant status over another when it performs agonistic behaviours (e.g. chasing, pushing, biting) and in return receives little or no aggressive reply, generally causing withdrawal of its opponent, thus demonstrating superior fighting ability or resource holding potential (Huntingford and Turner, 1987). Social interactions leading to hierarchy formation in juvenile groups of salmonids have been observed in the wild and replicated in the laboratory (reviewed by Sloman

<u>Behaviour</u>	<u>Description</u>
Hold position	Maintain position with minimal swimming movement. Energy saving behaviour.
Lateral display	Tensing body, elevating dorsal fin, and spread of other fins in response to approaching fish.
Chase	Aggressive pursuit of conspecific to attack rival.
Charge	Rapid approach towards another fish, usually the aggressor will ram the side of the opponent.
Bite	A fish will attack rival by using its mouth, sometimes inflicting considerable damage.

Table 1.1. Characteristic behaviours displayed by juvenile fresh water salmonids in social interactions. Listed in increasing order of severity of aggressive behaviour.

& Armstrong, 2002). A common method to assess social dominance in salmonids is to use some type of weighed behavioural scoring system (Metcalf et al., 1989; Johnsson et al., 1996; Sloman et al., 2001). This system assigns points to each fish according to position in the aquaria, food acquisition, and its social interactions, with active dominant behaviours scoring higher. Salmon display distinctive behaviours when interacting with conspecifics that may signal social status in a group (Table 1), and these activities can be used to determine the relative social rank of an individual within a group.

The costs of fighting are considered to be high, not only due to the direct energetic demand of the behaviour itself, but also associated expenditures such as increased risk of predation, lost time for other activities, reduced immune response, physical injuries, plus a range of physiological and endocrine disruptions (reviewed by Gilmour et al., 2005). Social hierarchies help buffer aggressive interactions among members of a group, ultimately promoting stability within populations and improving

food resource allocation (Gurney & Nisbet, 1979; Krebs & Davies, 1997). For instance, salmonids may signal social status through exogenous cues such as body colouration by darkening of their skin to indicate submission helping reduce aggression by the dominant individual (O'Connor et al., 1999; Höglund et al., 2000). During the formation of hierarchies by-standers monitor aggressive conflicts between other counterparts and assess the likelihood of winning a contest against the observed individuals (e.g. *Oncorhynchus mykiss*, Johnson & Akerman, 1998; *Betta splendens*, Oliveira et al., 1998). This assessment prevents energetically costly encounters to determine social rank. Subordination may result in a lower number of attacks directed towards an intruder and longer time between attacks (Lepage et al., 2005).

Salmonid hierarchies tend to be linear, meaning that if a first individual dominates another one, and this other one is dominant over a third counterpart, then number one is always dominant over the third fish too, and so on (Chase et al., 2002). In salmonids perfect linear hierarchies have been observed in groups with less than 10 members, while in larger groups there might be a greater chance that some social ranks may switch between individuals. This is because in larger group sizes, as in aquaculture conditions, hierarchies often are less rigid as it becomes increasingly difficult for a single fish to monopolise resources, and a group of several dominant fish might form (Alānarā and Brännäs, 1996). Nevertheless, overall, social hierarchies and dominant status are thought to be stable over time in the wild (e.g. Jenkins, 1969; Bachman, 1984) and in the lab (e.g. McCarthy, 2000; O'Connor et al., 2000). Territorial hierarchies may persist for over a year, as opposed to breeding hierarchies that usually last up to 3 weeks (Klemetsen et al., 2003; Watters, 2005). Dominant fish in simple experimental dyads also demonstrate high status in more complex social situations when transferred to high-density holding tanks (Metcalf et al., 1989,

1990). Similarly, competitively dominant individuals under moderate density conditions are also able to successfully hold resources in natural conditions where food is dispersed and unpredictable (Huntingford and García Leaniz, 1997).

Conspecifics in a group often compete for food and shelter and superior competitors gain preferential access to high quality territories which they tend to monopolise especially if resources are not spatially scattered (Metcalfé et al., 1989; Huntingford et al., 1990; Ryer and Olla, 1996; Reinhardt and Healey, 1997). Aggressiveness and ability to monopolise limited food resources are positively related (Adams et al., 1998). In addition, quality of the territory established progressively diminishes with lower social rank (Caron & Beaugrand, 1988). Therefore, growth rate of a fish can be strongly affected by its relative competitive ability (Huntingford & Turner, 1987). Dominants may not only exclude intruders from their territories, but may also actively invade territories defended by smaller fish (“partial territory” model; Greenberg, 1947). Size-related competitive exclusion from favourable food niches may lead to feeding suppression and growth depensation in subordinates (Jobling, 1985; Elliot, 1990; Metcalfé, 1991; Sandercock, 1991; Ryer & Olla, 1996). Fish in a cohort exhibiting poor growth may be more easily intimidated by other counterparts than larger fish (Metcalfé, 1991). Similarly, Huntingford et al. (1990) demonstrated that dominant juvenile parr made more feeding attempts and were more successful than subordinates, thus increasing the divergence in growth trajectories between high and low status fish. It has been suggested that larger size is a consequence, rather than a cause, of dominance (Huntingford et al., 1990; Metcalfé et al., 1992). Dominant fish despite having to sustain the costs of a higher metabolic rate, apportion a greater share of their overall resources to aggressive displays than feeding in comparison to submissive conspecifics, which helps to ensure the control

of the best feeding territories in the long-term (Cutts et al., 2002). Ultimately the relative social rank of fish may be the most important mediator in the timing and selection of life history strategies as it influences nutritional status, growth rates and welfare (Huntingford et al. 2006).

The formation of social hierarchies can be a stressful time for both the dominant and subordinate fish as reflected by stress indicators such as cortisol and serotonergic activity (Winberg and Nilsson, 1993). In most cases, at least under laboratory conditions, dominants regain basal levels of physiological parameters shortly after establishment of the hierarchy (Summers and Winberg, 2006). In contrast, subordinate individuals are only able to restore hormonal and neurotransmitter concentrations close to those prior to social subordination if they become isolated from dominants (Øverli et al., 1998). Thus in social hierarchies subordinates are subjected to chronic stress due to the constant threat of aggression and limited access to food resources caused by dominants (Abbott et al., 1985; Huntingford et al., 1990; Øverli et al., 1999a; Sloman and Armstrong, 2000).

1.3. Social rank, physiological correlates and reproductive strategies

The stress responses which help restore homeostatic equilibrium in the short-term may become maladaptive if activated for long-periods as is the case in subordinate fish under chronic stress (Wenderlaar Bonga, 1997). Over time consistent differences develop between dominant and subordinate fish, where the latter typically shows higher plasma glucocorticoid concentrations (Winberg and Lepage, 1998; Øverli et al., 1999b; Sloman et al., 2001a), higher serotonergic activity (Winberg et al., 1991; 1992a,b), lower body condition (Sloman et al., 2000a), lower growth rates (Li and

Brocksen, 1977; Pottinger and Pickering, 1992), lower hepatic energy reserves (Ejike and Schreck, 1980; Sloman et al., 2000a), higher gill sodium uptake (Sloman et al., 2007), and a reduced immune response (Peters et al., 1988) (Figure 1).

This section will concentrate on some of the effects of social rank on aspects of physiology including brain neurotransmitter activity, osmoregulation, endocrinology (in particular cortisol), and tertiary level growth rate responses.

1.3.1. Brain serotonergic activity in social fish

The brain serotonergic system interacts with neuroendocrine responses elicited during physiological or environmental challenges to maintain homeostasis (Winberg et al., 1997; Nelson and Chiavegatto, 2001). In many vertebrates, including fish, social subordination and other stressors have been reported to induce a general upregulation of brain neurotransmitter activity, perhaps serotonin being the most studied of all (Dunn, 1988; Dunn and Welch, 1991; Winberg et al., 1991, 1992a, 1993, 1996; Blanchard et al., 1993; Kaplan et al., 1994; Summers et al., 2005). Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter and neuromodulator synthesised from tryptophan (TRP) in a series of steps. TRP is first converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. Next 5-HTP is decarboxylated to 5-HT by aromatic L-amino acid decarboxylase (ADD) and free or released 5-HT is metabolised to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO). Dunn (1988) demonstrated that a transient reduction in stored 5-HT reflects a stress-induced increase in circulating serotonin which once released is readily metabolised. The ratio of metabolite (i.e. 5-HIAA) tissue concentration to that of the parent monoamine (i.e. 5-HT) is routinely used as an index of neural activity,

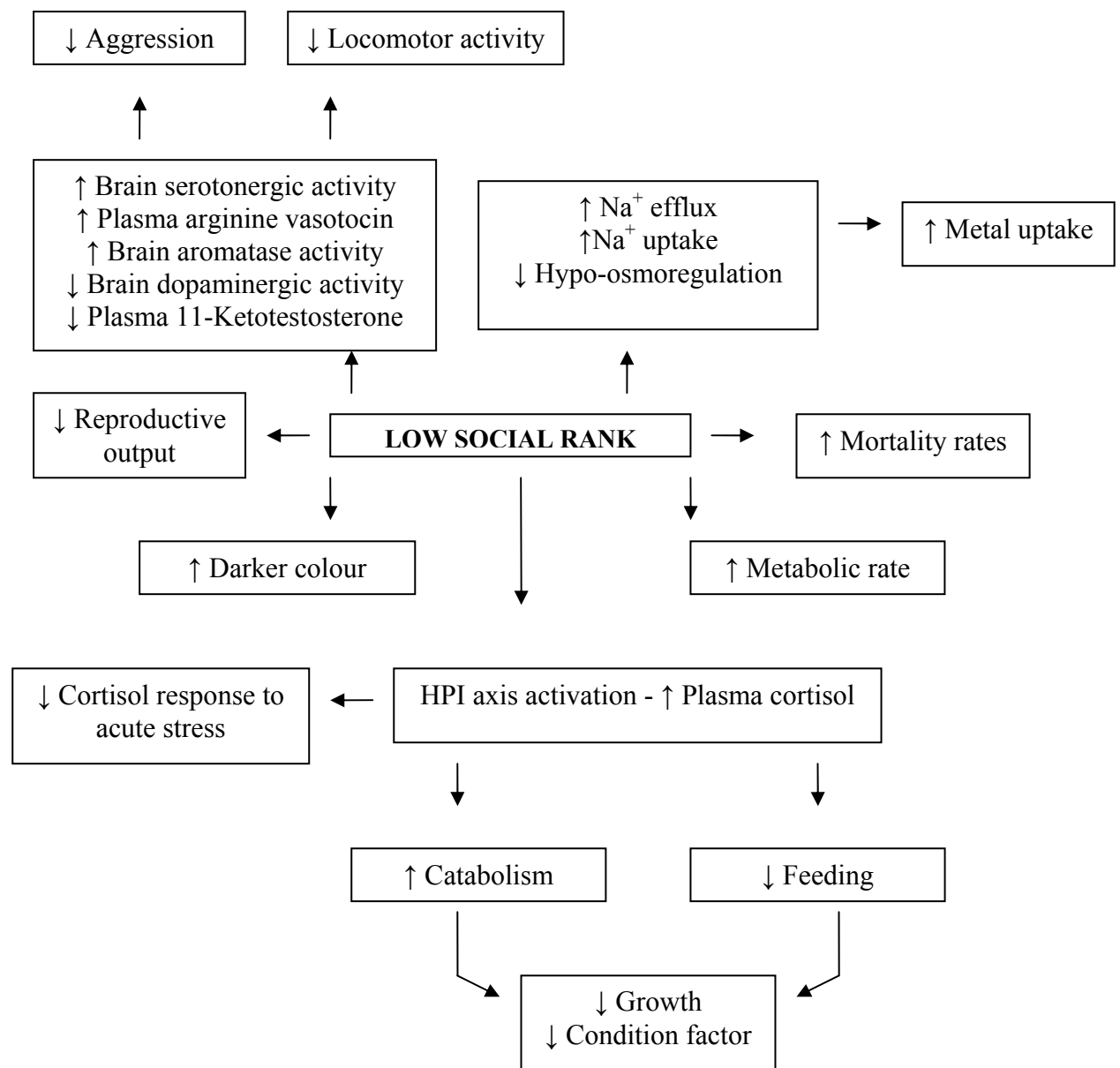


Figure 1.1. – Schematic diagram of physiological consequences of low social status in salmonids (adapted after Gilmour et al., 2005).

superior concentration of the metabolite being interpreted as an increase in release and turnover of the neurotransmitter (Shannon et al., 1986). This index has been shown to be remarkably sensitive to social stress and accurately reflect changes in social position within salmonid hierarchies (Winberg et al., 1991, 1992a).

In vertebrates the activation of the HPI axis by brain serotonergic activity appears to play a major role in the integration of behavioural, neuroendocrine and autonomic responses from the central nervous system (CNS) to external stimuli, in particular to stress (reviewed by Blanchard et al., 1993; Winberg and Nilsson, 1993). Social stress in animals appears to predictably produce the release of 5-HT in the major 5-HT innervated brain regions. The distribution pattern of the 5-HT cell bodies in the brain has been described as diffuse with axons projecting to a large number of regions in the CNS generally without a distinct contact on target dendrites (Törk, 1990, Jacobs and Azmitia, 1992). This configuration suggests that 5-HT might act as a neuromodulator, as well as a neurotransmitter, by diffusing to act on distant cellular targets (Fuxe and Agnati, 1991). Several high-affinity 5-HT receptor families have also been characterised in the brain of salmonids (Winberg and Nilsson, 1996). Winberg et al. (1997) found that administration of the specific 5-HT_{1A}-agonist 8-OH-DPAT resulted in a dose dependent elevation of plasma cortisol concentrations suggesting that the 5-HT system is part of the cortisol-induced regulatory pathway in the teleost HPI axis. Similarly, several authors have reported a highly significant correlation between brain 5-HIAA/5-HT ratios and cortisol and adrenocorticotrophic hormone (ACTH) plasma concentrations in subordinate salmonids (Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2000; DiBattista et al., 2005). However, the exact relationship between serotonin and cortisol appears to be complex where functions between neurotransmitter and hormone are related to a timeline

within the development of social hierarchies. During the initial more aggressive stages of a novel dyadic encounter (i.e. seconds to hours) both dominant and subordinate fish experience stress, inevitably resulting in 5-HIAA/5-HT and cortisol rising sharply but surprisingly with no apparent inhibition of aggression; whereas when social status is finally established and maintained (i.e. hours to days) only the subordinate individuals show chronically elevated 5-HT and glucocorticoids that promote aggression restraint (reviewed by Summers and Winberg, 2006). The rapid activation of norepinephrine (NE) and dopamine (DA) systems during initial social exchanges have been suggested as likely mechanisms that temporarily override the characteristic behavioural inhibition caused by 5-HT in the long term (Winberg and Nilsson, 1992; Höglund et al., 2001).

In social hierarchies low ranking individuals typically show signs of chronic stress with important implications for their physiological state (Gilmour et al., 2005). In this line of work stimulation of the 5-HT system in subordinate animals subjected to social stress is reported to shape behavioural motivation by eliciting the general inhibition of active responses such as feeding, aggression, reproduction or locomotion (Meyerson and Malmnäs, 1978; Olivier et al., 1989; Winberg et al., 1993b; De Pedro et al., 1998; Leibowitz and Alexander, 1998). For instance, elevation of telencephalic 5-HIAA/5-HT ratios in submissive fish has been repeatedly demonstrated in the telencephalon, a brain region primarily involved in the regulation and integration of aggressive behaviour in fish (Winberg et al., 1991; Winberg et al., 1992a).

One notable characteristic in the 5-HIAA/5-HT ratio of subordinate fish is that it reaches high levels quickly after exposure to a dominant fish, and the 5-HIAA/5-HT ratio will not decrease with time (e.g. social acclimation) until the presence of the dominant fish is removed (Winberg and Nilsson, 1993; Elofsson et al., 2000).

Winberg et al. (1993) reported that in adult male Arctic charr (*Salvelinus alpinus*) serotonergic activity levels were correlated with the number of aggressive acts conducted by dominants at the start of the social interactions, but not in later days; possibly indicating that a stable social hierarchy was established by overt aggressive behaviour by the dominant fish when social contact started and this structure remained thereafter without the need for further agonistic displays (Elofsson et al., 2000).

Stress incurred by subordinates can result from direct physical attacks by dominants, but may also derive indirectly such as through malnutrition caused by restrictions in access to food monopolised by dominant individuals (Jobling and Wandsvik, 1983; Abbot et al., 1985). Distinguishing which element or type of stress causes negative effects on the physiology of subordinates is difficult because stress induced changes, such as elevation in stress hormones (e.g. cortisol), occur usually as a general response (Øverli et al., 1998). Brain monoaminergic activity is a very useful physiological variable in this sense as stress caused by social environment and that originating from starvation can be distinguished. Although food deprivation has been reported to increase brain 5-HIAA levels in some mammals (Curzon *et al.* 1972; Kantak *etal.* 1978; Fuenmayor and Garcia, 1984), Winberg et al. (1992b) demonstrated that in salmonid fish stress induction by handling resulted in a consistent elevation of telencephalon and brain stem 5-HIAA concentration in fed and starved fish whereas starvation alone had no effect on 5-HIAA release.

Many studies have characterised brain monoaminergic patterns in relation to social rank within fish hierarchies (reviewed by Winberg et al., 2007). However, despite the strong association between social rank and developmental pathway adoption (Metcalf et al., 1998) and the fact that brain monoamines play a key role in

the establishment and maintenance of those social ranks little is known about the possible links between social status, serotonergic activity and phenotypic strategy in salmonid fish. Using genomics Aubin-Horth et al. (2005 a, b) showed that Atlantic salmon with alternative phenotypes (immature and mature parr) exhibit different gene brain profiles, where some were associated with behaviour. It would be interesting to establish if brain serotonergic activity follows a similar pattern in precocious and immature parr, as serotonin has also been shown to stimulate maturation in teleost fish (Khan and Thomas, 1992, 1993).

1.3.2. Fish hierarchies and cortisol

In teleost fish cortisol functions both as a glucocorticoid (i.e. metabolism) and mineralocorticoid (i.e. osmoregulation) hormone (Mommsen et al., 1999; Sloman et al., 2001a). Cortisol is regulated primarily through the action of corticotropin-releasing factor (CRF), a hypothalamic neuropeptide primarily expressed in the brain preoptic area. CRF stimulates the production of adrenocorticotrophic hormone (ACTH), and melanophore-stimulating hormone (α -MSH) from the anterior pituitary gland, which in turn leads to cortisol kidney synthesis by the interrenal cells (Wendelaar-Bonga et al., 1995). Cortisol receptors are mainly found in the gills, intestine, and liver, reflecting the important role of this hormone in hydromineral and metabolic regulation (Wendelaar-Bonga, 1997).

Cortisol has been extensively used as a physiological measure of welfare in salmonid fish as it is often correlated with social stress as consistent differences appear between fish of opposing social status (Øverli et al., 2002; Sloman et al., 2001b). Further, cortisol correlates with other physiological indicators associated with

dominance-subordinance relations such as brain monoamines (DiBattista et al., 2005; Øverli et al., 1999; Winberg and Lepage, 1998). In salmonids, significant cortisol elevation can occur even at very early stages of development. Barry et al. (1995) reported two week rainbow trout post-hatchlings to show a marked increase in plasma cortisol following acute stress. These responses may be different between ontogenic stages. For instance, Pottinger et al. (1995) described a stress-induced decrease in plasma cortisol in mature male rainbow trout compared to juvenile immature individuals and suggested that a lower threshold for the feedback mechanism between cortisol/ACTH in adult fish is responsible for this pattern. Shifts in stress sensitivity, measured as circulating plasma cortisol, can also be influenced by life history as Atlantic salmon are particularly sensitive to stress during the smoltification period (Carey & McCormick, 1997). Similarly, coho salmon subjected to handling stress exhibit a two-fold increase in plasma during the parr-smolt transformation compared to previous stages of their development (Barton et al., 1985).

Plasma cortisol is normally high following a short-lived distress, and its adaptive functional role has been related to energy reserve mobilization and increase of circulating plasma glucose (Gamperl et al., 1994). However when fish experience chronic social stress, as is the case of subordinates in strong social hierarchies, the adaptive function of cortisol may be lost and this raised state of physiological “alert” may result in negative physiological consequences (reviewed by Gilmour et al., 2005). Recent work by DiBattista et al. (2005) on rainbow trout described a reduction from 86 % to 40 % in the chances of becoming dominant in larger sized rainbow trout after administration of cortisol. In the same study, using size-matched trials, fish treated with cortisol were significantly more likely to become subordinate. Moreover, Sloman et al. (2001b) found that fish with higher plasma cortisol levels prior to

pairing in match-sized experiments subsequently became the subordinate individuals. Similar results have been confirmed by Dibattista et al. (2005). Continuous high levels of the corticosteroid within certain individuals of a group hierarchy can help perpetuate and maintain previously established social ranks. It is worth noting that dominant fish can also exhibit a partial elevation in plasma cortisol, suggesting a cost associated with the stress of holding a high social position (Øverli et al., 1999). The relationship between fish stress and cortisol can, in some instances, be complex and difficult to interpret. When McCormick et al. (1998) studied the effect of manual handling on the growth of Atlantic salmon (*Salmo salar*) parr, they found a higher growth rate in unstressed fish but no significant difference in cortisol levels between control and stressed groups. These authors suggest that desensitization or habituation to repeated stress could have led to lower cortisol production or an increase in the clearance rate of the hormone (Redding et al., 1984; Pottinger & Pickering, 1992). Nevertheless, the general consensus is that cortisol is a primary regulator of behaviour in fish, and high levels have been associated with various behaviours (i.e. feeding inhibition, lower movement activity) and physiology (poor growth rate, low condition factor and reduced food conversion efficiency) which appear with social subordination (Gilmour et al., 2005).

Cortisol also serves an important role in osmoregulation. It has been shown to elevate Na^+, K^+ -ATPase activity and cortisol treatment results in the partial restoration of the sodium pump activity at the gill in hypophysectomised coho salmon (Bjornsson et al., 1997). Administration of cortisol also results in the increase of surface area, number and activity of mitochondria-rich or MR (a.k.a. chloride) cells (Laurent & Perry, 1990; Perry et al., 1992). A rise in MR cell abundance can benefit anadromous fish by improving the capacity for ion transport across the gill and ultimately salt

secretion (Foskett & Scheffey, 1982; Bindon et al., 1994). Cortisol is responsible for the differentiation of the seawater form of the MR cell (McCormick, 1990), and enhances salinity tolerance levels in juvenile Atlantic salmon and brown trout (*Salmo trutta*) (Madsen, 1990; Bisbal & Specker, 1991). However, reports in the literature on osmoregulatory changes with plasma cortisol in teleosts show that it does not only mediate salt secretion. Lin and Randall (1993) suggested that cortisol is also responsible for the increase in activity of H⁺-ATPase in the gill, which is linked with sodium uptake in freshwater coho salmon. Cortisol has also been reported to decrease during acute sea water acclimation in brook trout (*Salvelinus fontinalis*) (Nichols et al., 1985). Similar findings have been reported in Mozambique tilapia (*Oreochromis mossambicus*) (Morgan et al., 1997). More recently, McCormick (2001) has proposed a dual function of cortisol in osmoregulation, whereby cortisol interacts with GH to enhance seawater acclimation and with prolactin during freshwater adaptation.

In summary, cortisol is a crucial hormone involved in a number of processes such as mediation of the social stress response and ionoregulatory balance both in fresh water and sea water, making it sometimes difficult to interpret the exact role it is playing. Nevertheless, high cortisol concentrations have been repeatedly found in fish with lower social ranks and provide a very useful tool to examine physiological status and welfare in teleosts.

1.3.3. Social ranks, developmental pathways and osmoregulation

Fish actively regulate their internal milieu in the face of changes in the external environment ionic concentration (reviewed by Evans et al., 1999, 2005; Marshall and Grosell, 2005). Teleosts living in fresh water hyperosmoregulate to overcome gain of

water across the gill and loss of ions due to diffusive forces. By having a substantial glomerular filtration and urine flow fish in dilute environments eliminate excess water. Loss of ions by diffusion through gill epithelia is offset by salt intake from food, tubular reabsorption of necessary ions and branchial active uptake mechanisms. In the gill Na^+ uptake is thought to be controlled by mechanisms involving apical membrane epithelial Na^+ channels electrogenically coupled to a proton pump (V-type H^+ -ATPase) in gill peanut lectin agglutinin negative (PNA⁻) α -mitochondria rich cells (MR cells), as opposed to lectin-binding (PNA⁺) β -MRCs less involved in fresh water Cl^- uptake (Reid et al., 2003). The proportion of the PNA subtype MR cells in the gill and the activity of ATPases within them also shift depending on the external ionic concentration of the environment (Hawkings et al., 2004). Basolateral Na^+, K^+ -ATPase (also known as the sodium pump) and V-type H^+ -ATPase extrude cations to generate an electrochemical gradient across the apical membrane that facilitate the intake of Na^+ from the fresh water in the environment (Katoh et al., 2003). Gill Na^+, K^+ -ATPase K_m values for Na^+ and K^+ are much lower (i.e. higher affinity) in freshwater acclimated rainbow trout compared to those of seawater-acclimated conspecifics, the lower intracellular Na^+ concentration inside further helping maintain a gradient for Na^+ uptake (Pagliarani et al., 1991). Apical $\text{Cl}^-/\text{HCO}_3^-$ exchange on the other hand appears to drive Cl^- uptake (Marshall, 2002). The current model for ionoregulation in the gill MR cells of sea water and fresh water fish is illustrated in Figure 1.2 and 1.3 (based on Marshall and Grosell, 2005). Still many elements in the mechanisms of sodium uptake that mediate acclimation to seawater are not fully understood.

In the past radio-labelled isotope ion transport experiments have provided a useful tool to characterise some of the gill osmoregulatory mechanisms (e.g. Maetz,

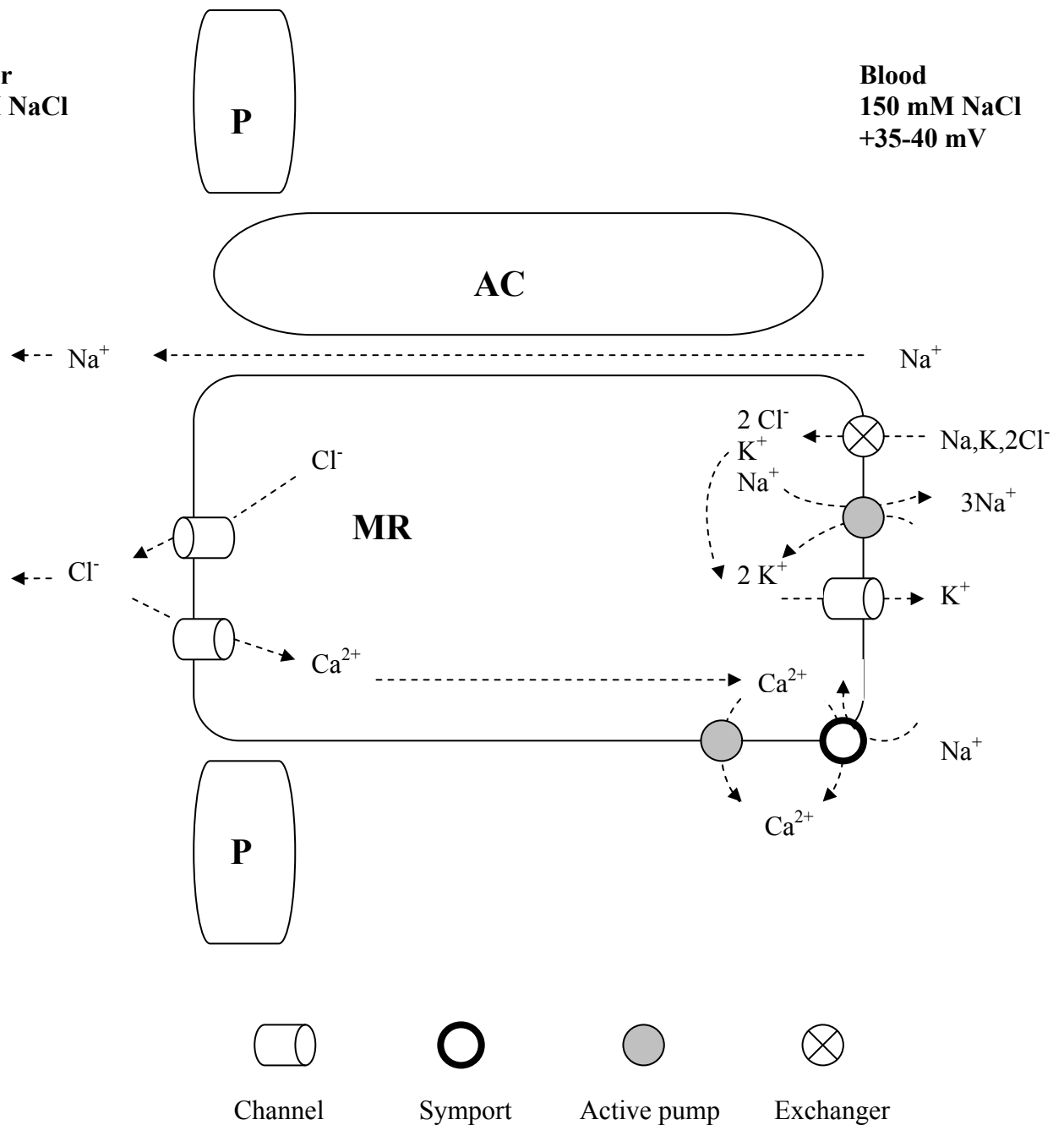


Figure 1.2. Model of salt-secreting cell characteristic of marine teleost gills with a mitochondria-rich (MR) cell and an adjacent accessory cell (AC) forming a cation selective (Na⁺) paracellular route, while pavement (P) cells create a diffusive barrier. NaCl is excreted actively through the Na⁺,K,2Cl⁻ cotransporter, while Na⁺,K⁺-ATPase actively recycles Na⁺ back to the plasma. This generates a transepithelial electrical potential that helps to move Na⁺ out via paracellular diffusion via the leaky tight junctions between MR and AC cells. After Marshal & Grosell (2005).

Freshwater
1mM NaCl
0 mV

Blood
150 mM NaCl
+35-40 mV

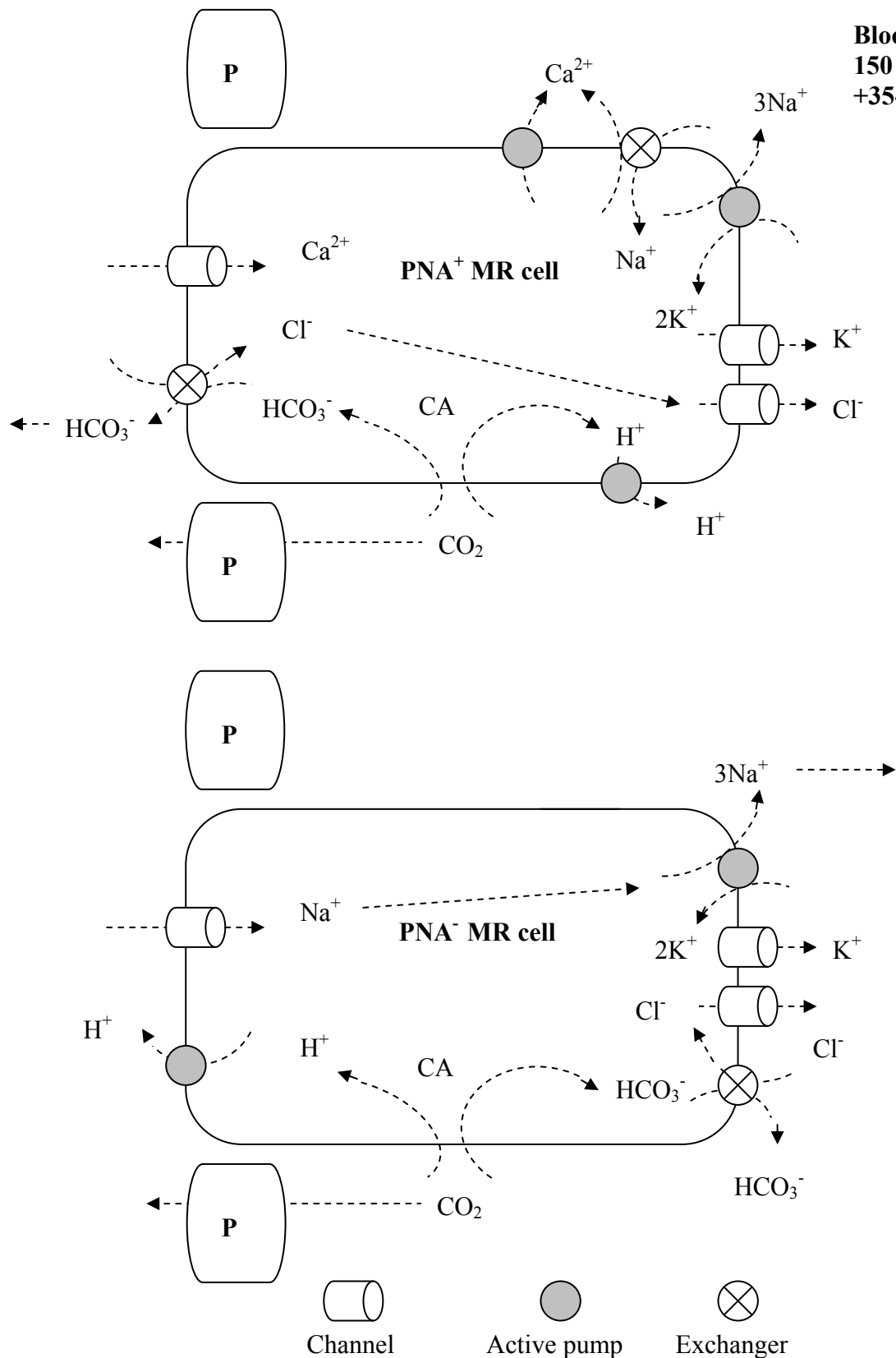


Figure 1.3. Two similar looking mitochondria-rich (MR) cell types of cells with distinct functions are present in the gills of freshwater teleost fish. The PNA⁺ (peanut agglutinin) cells secrete base apically and uptake Cl⁻, while PNA⁻ cells secrete acid and gain Na⁺. After Marshall & Grosell, 2005.

1971; Wood and Goss, 1990; Potts, 1994). This technique is particularly helpful as it enables non-invasive *in vivo* sampling at different stages of development. Further, ion fluxes offer a 10-400 fold greater sensitivity to detect variation in ionic transport compared to other methods using blood or tissue sampling (Wood, 1992). Despite these advantages, relatively few studies have examined the Na⁺ uptake kinetics of Atlantic salmon and those that have concentrated either on the very early fry (McWilliams & Shephard, 1989) or very late adult anadromous (Potts et al., 1985) stages of the life cycle. To our knowledge none have used isotopic techniques to investigate the potential differences in ion regulation between fish at the time of developmental conversion towards alternative life strategies.

Due to their anadromous nature most salmonids individuals undergo extensive oceanic migrations and later return to their natal river to spawn as fully grown adults (reviewed by Hinch et al., 2005). In certain cases such as in landlocked salmonid populations or river resident mature parr fish will only experience the freshwater environment (Jonsson, 1985; Verspoor and Cole, 1989). Salmon must reverse their osmoregulatory mechanisms when transferring between such distinct aquatic environments in order to survive (Shrimpton et al., 2005). Prior to seaward migration, while still in fresh water, a crucial developmental conversion sometimes referred to as the parr-smolt transformation takes place. Previously immature parr will start to smolt, a term used to describe a group of profound physiological changes, particularly observed in the remodelling of processes associated with ion turnover of branchial epithelial cells and changes in structure and activity of important ATPase proteins and other ion transporters to enable a successful entry into sea water (Hoar, 1988; Evans, 2002).

One important modification experienced in smolts is the gradual increase in Na^+,K^+ -ATPase (McCormick et al., 1985) a process that correlates positively with date of entry into sea (Nielsen et al., 2003, 2004). At this time mRNA expression of the predominant freshwater Na^+,K^+ -ATPase isoform (1 α a) switches to the seawater-responsive isoform (1 α b) to prevent large plasma osmolality shifts, hence promoting homeostasis during entry to sea water (Richards et al., 2003). A similar process occurs when returning to spawn during freshwater migration, also referred to as “reverse smoltification”, as adult fish show marked increases in mRNA expression of Na^+,K^+ -ATPase but this time of the 1 α a isoform (Bystriansky et al., 2007). The present study will concentrate on the physiological transformations taking place during sea water migration in juvenile salmonids.

The decision to smolt is taken approximately after mid summer in the year before the spring migration and is conditional to having a high growth trajectory that will surpass a certain threshold (Thorpe et al., 1980; Metcalfe et al., 1986, 1988, 1989). The actual process of smolting during which the osmoregulatory system is reorganised occurs in a much shorter period during spring time (McCormick et al., 1998). These complex endogenous developmental events are initiated and controlled by the endocrine system (reviewed by McCormick, 2001), with external environmental cues such as photoperiod and temperature affecting timing of smolting (Berglund et al., 1991; McCormick et al., 1995, 2000).

In salmonids movement from fresh water to seawater may represent a difficult time as this process is energetically demanding (Maxime et al., 1991; Tocher et al., 2000). Recently, Bystriansky et al. (2006) reported that Arctic charr transferred to sea water showed a rapid upregulation of muscle free amino acids and various associated enzymes to metabolise them, suggesting that increased energy production is required

for successful acclimation. In addition, during this period smolts appear to be particularly susceptible to stress and have higher cortisol plasma concentrations. In Atlantic salmon a 10-fold increase in plasma cortisol has been reported (Shrimpton & McCormick, 1998). Also upregulation of several hyperosmotic stress responsive genes take place during seawater transfer mediated by elevation in circulating cortisol (Mommsen et al., 1999). Reduced preparation for seawater transfer may lead to poor swimming performance (Brauner et al., 1992) and increased mortality (Saunders et al., 1985; Steffanson et al., 1993; Jonsson and Jonsson, 2009). Smolts experiencing poor nutritional status or with a low condition index may show higher serum osmolyte concentrations, decreased disease resistance and reduced survival after seawater entry (Berril, 2006; Rorvik et al., 2007). As preferential access to food, high growth rates and condition index are all positively correlated with dominance (Abbot and Dill, 1989; Metcalfe et al., 1989; Winberg et al., 1993) it could be hypothesised that smolts living in social hierarchies could show differential levels of preparation to sea water entry depending on their social position within the group. Many aspects of osmoregulatory traits resulting from socially-mediated mechanisms are still poorly understood.

1.3.4. Social position and standard metabolic rate

Basal metabolism is usually the largest component in the energetic budget of an animal. The standard metabolic rate refers to the minimal maintenance or resting metabolic rate of unfed animals below which physiological function is impaired (Brett and Groves, 1979; Priede, 1985). Metabolic rate may vary depending on factors such as stress, temperature, body size, feeding regime or activity levels (Jobling, 1981;

Clark and Johnston, 1999; Sloman et al., 2000b). The area between the upper or maximum metabolic rate (active metabolic rate, AMR) and lower limit (SMR) is known as the metabolic scope (Fry, 1947; Willmer et al., 2000). In theory a larger metabolic scope may mean that energetically expensive activities such as aggression can be conducted with fewer restrictions (Metcalf et al., 1995).

Recent studies on teleosts have established an association between having a high SMR and aggressive behaviour and dominance. For instance, fish with a higher SMR are more likely to be dominant compared to subordinates both in pairwise and larger group interactions (Metcalf et al., 1992, 1995; Yamamoto et al., 1998; Cutts et al., 1999a). Further, the probability of winning a fight and becoming dominant is directly correlated to the size of the asymmetry in relative SMR between opponents prior to social interaction (McCarthy, 2001). In this study the author reported that dominance was predicted by a higher SMR in the majority of dyadic interactions. Even in the few pairs having subordinates with the higher basal SMR, the larger the size difference between dominant (with the lower SMR) and the subordinate (with the higher SMR) the lower the competitive ability was in the latter.

In juvenile salmon dominance status is probably mediated through greater aggressiveness, an energetically costly behaviour enabled by a high SMR. Cutts et al. (2002a) showed that dominant fish when forming a new social hierarchy opted to fight at the cost of acquiring fewer food resources. This strategy paid in the long term as once social dominance was established these fish benefited from significantly higher growth rates.

Superior social rank associated with a high SMR has been demonstrated in a number of salmonid species including Atlantic salmon (Cutts et al., 1998, 1999a), Masu Salmon, *Oncorhynchus masou* (Yamamoto et al., 1998), rainbow trout,

Oncorhynchus mikiss (McCarthy, 2001), and Arctic char, *Salvelinus alpinus* (Cutts et al., 2001). Furthermore, the link between high SMR and aggressiveness may operate not only at the individual but also at the population level in salmonid fish (Lathi et al., 2002).

High SMR causes faster yolk absorption which leads to earlier first feeding, known to confer a competitive advantage in salmon in terms of the early establishment of feeding territories (Metcalf et al., 1995; Cutts et al., 1999a). This prior residence effect confers a greater chance of successfully defending a territory against novel individuals (Cutts et al., 1999b), thus having important consequences on future feeding opportunities. In juvenile salmon relative dominance during the first months after emergence are critical for subsequent adoption of strategies as rapid growth rates gained through initial high status are generally maintained or increased over time (Metcalf et al., 1989, 1990) and may enable the adoption of size threshold-dependent life histories such as early maturation and smolting (Metcalf et al., 1995; Metcalf, 1998). Upper modal group individuals typically show high SMR, high aggression levels, fast growth and earlier maturation and migration (Nicieza and Metcalf, 1999).

Having a high SMR has been generally viewed as a benefit rather than a cost. However, Cutts et al., (2002b) found that dominant fish with high relative SMR had a smaller metabolic scope than conspecifics with a low SMR. Therefore it may be detrimental to have a high SMR as it is associated with larger costs of maintenance, especially under poor or unpredictable feeding environments (Metcalf, 1986). It may be important to understand why salmonids show such high interspecific SMR variability, as depending on the microhabitat different SMR fitness costs will emerge (Metcalf, 2009).

Environmental factors such as starvation (O'Connor et al, 2000) or parasitic infection (Seppanen et al., 2008) can lower SMR whereas others such as stress may elevate metabolism (Sloman et al., 2000). Duration of the environmental factor may also be important, as for instance if the food shortage is temporally short high SMR dominant fish may be able to capitalise on their greater energy reserves accumulated during their higher growth in favourable feeding conditions to counteract the effect and hold their rank. Although animals may down regulate or up regulate their basal metabolic rate, they ultimately have little control over their SMR limits. To what extent an individual's SMR can vary is not totally understood. Several studies appear to indicate that relative metabolic rates within groups are maintained over long periods, whereby particular fish consistently have a higher SMR than others (McCarthy, 2000; Cutts et al., 2001). In addition, if food is restored after periods of starvation, the SMR of an individual returns to its previous relative position (O'Connor et al., 2000). Although the stability of SMR after periods of food shortage has been tested previously in salmonids, to our knowledge the link between feeding alterations and dominance stability using SMR has not been yet tested.

1.3.5. Specific growth rate and size asymmetries in salmonid fish

Size differences start at the egg stage, with egg size being positively correlated with size of fish at first feeding (Thorpe et al., 1984; Beacham & Murray, 1993; Fleming 1998). Fry originating from larger eggs, possibly mediated by maternal effects (Regnier et al., 2009), tend to be larger and commence first feeding earlier, with early and late feeders showing genetic differences (Pineda et al., 2003). Aubin-Horth & Dodson (2004) documented that size in Atlantic salmon 20 days after emergence from

the redd was greater in future mature male parr than in immature males, and this size advantage was still conserved after a year. It has been suggested that acquiring a good feeding territory before the rest of the fry emerge may confer a nutritional advantage, as prior residence favours dominance (Cutts et al., 1999). Although sometimes difficult to separate as a large size difference appears to confer a competitive advantage, fast growth rate and large size appear to be a consequence of dominance rather than its initial cause (Huntingford et al., 1990; Cutts et al., 2002a).

Under favourable feeding conditions, in laboratory and wild environments, growth during the first year of juvenile Atlantic salmon frequently shows a bimodal distribution with significant differences in size between upper modal group (UMG) and lower modal group (LMG) fish (Thorpe, 1977; Bailey et al., 1980; Metcalfe et al., 1988; Nicieza et al., 1991; Shrimpton *et al.*, 2000). Once established growth patterns appear to be conserved and few individuals appear to shift between modal groups (Saunders *et al.*, 1994), where the LMG formed primarily by fish with a lower competitive status (Metcalfe et al., 1989; Metcalfe and Thorpe, 1992). In salmonids many studies have demonstrated a positive relationship between size and high social status (Metcalfe et al. 1990; Thorpe et al. 1992; Holtby et al., 1993; Ryer & Olla, 1996; Huntingford et al. 1998). In addition, growth hormone levels have also been linked to dominant status (Johnsson and Björnsson, 1994; Johnsson et al., 1996). Fast growing has been associated with an active strategy, fighting and experiencing a more aggressive social environment compared to LMG subordinates adopting a passive feeding strategy (Nicieza and Metcalfe, 1999). In teleosts, as well as in mammals, two distinct stress coping strategies have been identified where dominant fish usually exhibit a proactive or low cortisol response (LR) whereas subordinate fish show a reactive or high cortisol response (HR) (Pottinger and Carrick, 2001). Under social

stress HR individuals show an increased 5-HT turnover (Øverli et al., 2001) and reduced appetite once isolated from the stressor (Øverli et al., 2002) relative to LR fish.

It is well established that a high social status and its associated rapid growth in early juvenile salmonids can lead to the adoption of the mature parr or the smolt strategy (Thorpe, 1977; Thorpe et al., 1982; Myers et al., 1986; Metcalfe et al., 1989; Okland et al., 1993; Bohlin et al., 1994, Hutchings and Jones, 1998). Both life histories are conditional upon reaching a certain genetically determined size or condition threshold well in advance of the developmental transformation into smolts (Elson, 1957; Kristinsson et al., 1985; Metcalfe et al., 1986, 1988) or mature parr (Rowe & Thorpe, 1990; Baum et al., 2004). Lipid reserves and growth rate have been proposed as early maturation predictors (Rowe et al., 1991; Simpson, 1992). Similarly, several authors have suggested that better growth opportunities directly raise the percentage of maturing parr in a population (Berglund, 1992; Letcher & Terrick, 1998). However, Aubin-Horth & Dodson (2004) reported that higher fork length at particular sites did not always translate into a higher proportion of mature parr. Meanwhile LMG fish typically will wait at least a further year in the river before migrating (Thorpe, 1977). Size differences between immature and precocious parr disappear after maturation when the latter invest energy into gonadal rather than somatic growth (Whalen and Parrish, 1999; Arndt, 2000). At the time of spawning precocious parr hierarchies will form close to breeding females and positive relation exists between mature parr size and reproductive output (Myers and Hutchings, 1987; Thomaz et al., 1997). Similarly, dominant anadromous males that successfully breed generally are larger than satellite males (Fleming et al., 1997, 1998).

Salmonid fish showing higher aggression levels often have greater growth rates (Fausch 1984; Huntingford et al. 1998; Nicieza & Metcalfe 1999; Lahti et al. 2001; but see Vollestad and Quinn, 2003). Aggressiveness and ability to monopolise resources are also positively related (Adams et al., 1998). Larger fish appear to be bolder and more willing to forage under risk of predation than smaller conspecifics (Johnsson 1993). This could be linked to the higher energetic demands incurred by larger fish associated with their higher SMR (Metcalfe et al., 1992, 1995). Territory size often increases with fish size in streams (Grant et al. 1989; Elliott 1990; Keeley and McPhail 1998; Keeley, 2001), possibly to provide sufficient food to maintain a high status. Mostly demonstrated in the lab using single point food sources (reviewed by Thorpe and Huntingford, 1992) but also in pools in the wild (e.g. Nakano et al., 1995a,b), dominant fish try to defend and monopolize food access through agonistic behaviour resulting in the highly biased distribution of food within the group that can result in high growth rates of dominants and reduced growth or even growth depensation in subordinates (Fausch 1984; Grant, 1990; Ryer and Olla, 1996). Additionally large fish may profit from the ability to consume a wider range of prey items (Bystrom & Andersson, 2005). In contrast, subordinate growth rates may be further decreased due to social stress experienced which may lead to characteristic physiological symptoms such as reduction in appetite and conversion efficiencies (Jobling and Wandsvik, 1983; Abbot and Dill, 1989). Fish growth is indeterminate and subordinates are able to deploy a growth-compensatory response after food deprived periods (e.g. Nicieza and Metcalfe, 1997), but there may be long-term costs associated with rapid catch-up growth (Metcalfe, 1998; Morgan and Metcalfe, 2001). Seasonal effects may also be important as small parr from the LMG that are not able to reach the size threshold to mature or smolt naturally reduce food intake during

winter (i.e. overwintering anorexia) regardless of external factors (Metcalf, 1986; Metcalfe and Thorpe, 1992; Simpson et al., 1996). On the other hand, larger fish that will mature or smolt remain relatively active in feeding during the winter (Metcalf et al., 1988; Morgan et al., 2000), maintaining higher lipid reserves and survival rates (Pickering and Pottinger, 1988; Meyer and Griffith, 1997). Therefore, apart from the important influence of social hierarchies, other environmental factors such as feeding history or developmental strategy adopted may need to be taken into consideration.

In dyads food monopolisation by the dominant is almost total, whereas as group size increases food distribution becomes more homogenous (Winberg et al., 1993; Jobling and Baardvik, 1994; McCarthy et al., 1999). In habitats with high spatio-temporal variability in resources or high risk of predation, both factors known to elevate the energetic costs of territory defence, the relationship between dominant status and growth rate may disappear (Grant & Kramer, 1992; Martin-Smith and Armstrong, 2002; Hardwood et al., 2003). In this sense it is important to take into account the social environment of the fish and the relative size and status differences, rather than the absolute values, because these asymmetries influence the outcome of social interactions (Abbott et al., 1985; Huntingford et al., 1990; Adams and Huntingford, 1996; Beaugrand et al., 1996; Cutts et al., 1999a; Johnsson et al., 1999). As described by Metcalfe (1990), absolute status refers to the inherent ability of an individual to dominate conspecifics in a large group, whereas relative status is representative of the ability to be dominant in a group, which is affected by member quality of certain traits (e.g. size, aggressiveness). For instance, in some cases being relatively large within a group, which has more large fish, can be a disadvantage as the most dominant fish may focus the majority of its aggression towards its closest rivals and can result in serious injuries in subdominants (McClean et al., 2001).

Similarly, Sloman et al. (2000a) reported that in groups of brown trout the second ranked fish, usually larger than most members, was also the recipient of aggression from the dominant fish and showed the highest stress levels as indicated by ionoregulatory disruption and reduction in condition. Other studies on fish provide further evidence that proactive subdominant fish may be worse off than subordinate fish (Winberg et al., 1991; Fernandes and Volpato, 1993). These findings may have been related to the active feeding strategy used by the subdominant yielding high energy returns with a high physiological cost, whereas most subordinates tend to adopt a low energy (i.e. remaining motionless and capturing drifting food close to them), low cost (i.e. low risk of injury) approach (Metcalf et al., 1986). Aggressive behaviour does not always equate to higher growth rates and condition, alternative feeding strategies lacking an agonistic approach may yield similar energetic returns depending on environmental conditions (Metcalf et al., 1986; Adams et al., 1998; Höjesjö et al. 2002). Perhaps the potential injury and energetic costs associated with being a proactive subdominant may be counterbalanced by the benefits of being next in line for dominance status. For instance, under environmental perturbations social hierarchies may be modified and it is the fish with the closest status to the dominant that more likely will replace it (Sneddon et al., 2005).

Social positions are usually decided rapidly when size asymmetries are large, often without physical contests as subordinate fish avoid fighting by escaping or hiding and signalling subordination by darkening of the skin (O'Connor et al., 1999, 2000). Work on rainbow trout by Abbott et al. (1985) showed that a size difference of over 5 % was sufficient to ensure that the larger fish became dominant. Game theory models hypothesise that aggression should be higher in groups formed by similar sized counterparts (Maynard Smith, 1982). This prediction seems to hold true in fish

hierarchies with small size differences (Sneddon et al., 2005). Accurately judging competitive ability of opponents becomes increasingly difficult and overt aggressive interactions may take place to establish ranks (Wong et al., 2008). In addition, social rank may not always be accurately judged by length or weight parameters as in a few studies size differences up to 20 % between members of a dyad were not sufficient for the larger fish to obtain dominance (Huntingford et al., 1990; Yamamoto et al., 1998). What makes some studies show strong size-mediated dominance-subordination status and others not is still not well understood. Previous growth trajectory, previous wins or losses in other fights, hormonal and neuroendocrine status or even life strategy adopted may influence the outcome of social bouts. A better understanding of what drives these size asymmetries in salmonids (i.e. bimodal size frequency) and how these affect social status and reproductive strategy would be desirable.

The aim of this thesis was to examine the impact social status has on the physiology, development and life strategies of salmonid fish to gain understanding on the mechanisms driving the behavioural ecology of these fish. In chapter 2 the stability of social rank in young Atlantic salmon was studied in response to manipulation of the feeding conditions. Both SMR and brain monoamines were examined as potential mechanisms involved in behavioural interactions. In chapter 3 the osmoregulatory mechanisms of juvenile parr about to smolt were investigated using a novel phenotypic trait, gill sodium uptake kinetics, to see if fish with different life histories show distinct signatures. In chapter 4 the same kinetic technique was used this time to establish if differences between fish with different ranks in a hierarchy show osmoregulatory similarities and how these might affect their entry to sea water. Chapter 5 looks at how fish with distinct bimodal sizes interact in social hierarchies and how their social rank might affect their growth rate and life history

selection. Brain serotonin turnover and cortisol measurements were used as physiological indicators to help characterise these social hierarchies. In Chapter 6 phenotypic differences in social status were studied between immature parr and precocious parr in complex hierarchies by measuring behavioural dominance and possible underpinning mechanisms, namely brain serotonin turnover. Finally, in Chapter 7 a preliminary attempt is made to characterise possible metabolic signatures associated with juvenile Atlantic salmon life strategies by using metabolomic techniques. Chapter 8 provides a general discussion of the experimental work in Chapters 2 to 7, in which I have attempted to focus on the wider implications of this work, and potential future directions, rather than reiterating the specific conclusions from each individual chapter.

Chapter 2

Social status stability and physiological correlates in young Atlantic salmon (*Salmo salar*) with alternative feeding environments.

ABSTRACT

The stability of social dominance hierarchies was studied in 0+ Atlantic salmon (*Salmo salar*) by manipulating an important environmental variable, feeding ration. Initial dominance ranks were established in a sequence of two dyadic combats, with winners of the first fight being matched against another winner, and the losers engaging in another dyadic combat. This resulted in four social ranks: a) most dominant (D+) winning both fights, b) subdominant (D-) winning the first but losing the second, c) subordinate (S+) losing its first combat but winning the second, and d) most subordinate (S-) losing in both interactions. Thereafter fish were kept together in mixed groups (with equal numbers of all 4 social ranks present). These mixed groups were fed either a high food ration or a low food ration. After 12 weeks fish from the most dominant rank (D+) and those of subdominant status (D-) from both equal and dissimilar nutritional conditions were paired and social status reassessed. Examinations of final body mass showed a clear effect of social status with mean body mass of D+ being consistently high, and also of feeding regime as social groups in the high ration were larger. Social status analysis showed that the majority of dominants, even those from the poorer feeding background, retained their superior social status against subdominants. Possible physiological mechanisms underlying the maintenance of social status were examined between dominant and subdominant fish. The highest ranked fish, D+, had a higher SMR than subordinates. However, low

ration D+ fish had a lower SMR than D+ high ration individuals, maybe indicating a limitation on the maintenance of a high SMR in a poor nutrient environment. Brain monoamine patterns showed that D+ fish had lower brain stem and telecephalon serotonergic activity, indicative of reduced social stress, relative to D- fish, although these differences were reduced when D- was kept in high feeding conditions.

Keywords: social dominance; standard metabolic rate; brain monoamines; feeding regime; Atlantic salmon.

INTRODUCTION

Environmental factors are known to influence social traits and ultimately developmental pathway in salmonid fish (Sloman and Armstrong, 2002; Sloman et al., 2001). However, to which degree social status may remain stable or change in response to environmental variables is not clearly understood. Perhaps one of the most important environmental factors in teleosts is food availability as it strongly influences growth, survival rate, life history adoption and reproductive success (Adams and Huntingford, 1997; Bohlin et al., 1994; Hutchings, 1993; Keeley and McPhail, 1998; Metcalfe et al., 1986).

Juvenile salmonids live in social groups and hierarchies develop as a result of agonistic interactions primarily to gain access to quality feeding territories (Keenleyside and Yamamoto, 1962). Dominant fish actively prevent access of lower ranked individuals to their feeding territories leading to poor growth (Fausch, 1984; Li and Brocksen, 1977) and possibly delay or even prevention of reaching certain life strategies (Nicieza and Metcalfe, 1999). In salmonids social differences in status develop at an early age. Alevins that consume their egg yolk faster and commence

first feeding at an earlier time usually attain dominant status over those that hatch later and are more likely to become smolts at a later stage (Metcalfé et al., 1992; Pineda et al., 2003). It is thought that characteristic physiological and behavioural traits such as fast growth, often as a result of higher aggressiveness, may be related to their high standard metabolic rate (SMR) (Cutts et al., 1998; McCarthy, 2001; Yamamoto et al., 1998). Under steady feeding conditions acquisition of food by these aggressive individuals leads to superior growth rates in dominants which is maintained and often reinforced as size asymmetries increase (Metcalfé, 1986; Øverli et al., 1998; Sloman et al., 2000). Fast growing juvenile salmon with a high SMR that reach a genetically determined size or condition switch-point are then able to adopt the precocious parr or the smolt life history (Metcalfé et al., 1995). Subordinate fish with a lower status on the contrary will have to defer their developmental conversion until the required condition threshold is met (Thorpe, 1977) and remain as immature parr for a further period (at least 1 year).

Despite the relative stability of SMR and social dominance (O'Connor et al., 2000; McCarthy, 2000), these traits are not completely fixed and may alter with shifts in environmental conditions. Under food fluctuations or restriction it is thought that dominant fish with a high SMR may be at a disadvantage over subordinates with a lower SMR as dominants will have higher energetic demands (Cutts et al., 2001). Fish living in high constant feeding regimes tend to form stable social networks where dominance correlates with higher growth (McCarthy et al., 1999). However, under variable food conditions, such as those encountered in natural streams, the relationship between dominance and growth may disappear (Smith et al., 2002; Harwood et al., 2003; Martin-Smith and Armstrong, 2002). On the other hand, recent physiological work suggest that dominance may be intrinsic to certain fish which

show suits of behavioural and physiological traits, often termed stress-coping styles, such as proactive behaviour, greater aggression, low-response to cortisol, lower brain serotonergic activity, or fast resumption of feeding (Øverli et al., 2007). How resilient these dominant-type individuals are to environmental variation such as temporal feeding shifts which may change their social status is still poorly understood.

In addition to SMR, brain monoamine activity has been used as a reliable indicator of dominance in teleost hierarchies (reviewed by Winberg and Nilsson, 1993) is a strong modulator of social behaviour via the hypothalamic-pituitary-interrenal axis (HPI) (Höglund et al., 2005; Winberg et al., 2007). In particular low brain serotonergic activity, measured as the ratio between 5-hydroxyindoleacetic acid (5-HIAA) to serotonin (HT), i.e. the ratio of released monoamine metabolite to parent monoamine, has been shown to positively correlate with modifications of social position in salmonids (Winberg et al., 1992a) (Øverli et al., 2001). Importantly brain serotonergic levels appear to be inversely correlated to social rank but independent of nutritional status unlike other physiological indicators of stress which show a more generalised response to stress (e.g. cortisol). For instance, in salmonid fish stress induction by handling results in a consistent elevation of telencephalon and brain stem 5-HIAA concentration in fed and starved fish whereas starvation alone has no effect on 5-HIAA release (Winberg et al., 1992b).

The aim of this study was to investigate the relationship between feeding environment and the stability of social status by testing the hypothesis that dominant juvenile Atlantic salmon are still able to hold a high social position after a period of food limitation against lower ranked fish kept in a benign feeding regime. Dominant and subordinate individuals were identified through dyadic interactions and matched again after being held in low and high food ration treatments to observe if social

position was altered. In addition growth rates, brain monoamines and SMR measurements were taken at the end of the experiment to investigate the physiological parameters associated with social rank in these fish.

METHOD

Fish husbandry

Over 200 fry of Atlantic salmon (*Salmo salar*) of the same size range fry (5-6 cm fork length) were obtained from an aquaculture facility in NE England (Yorkshire Salmon Ltd., Hebden) in June 2008 and reared in the aquarium system at Exeter University. Fish were kept in a stock tanks at constant temperature (11 ± 0.2 °C), and photoperiod (12L:12D) and were hand fed 2 % body mass day⁻¹ rations prior to experimental treatments.

Experimental protocol

To investigate the stability of dominance under variable feeding environments, first the social rank of Atlantic salmon juveniles was assessed. Fish were formed into a first set of pairs (n = 160 juveniles, to form 80 pairs), from which social status was unknown. This yielded a dominant and a subordinate individual from each pair (see behavioural observations section). Subsequently a second set of dyadic combats (n = 80 dominants, to form 40 pairs) was conducted by randomly pairing fish that had emerged as dominants in their initial fights, obtaining within each pair a winner and a loser. The same matching procedure was performed using subordinate fish that had lost their initial fight (n = 80 subordinates, to form 40 pairs). After this second dyadic encounter one of the subordinate fish would emerge as a winner. Thus after each fish had been involved in two dyadic encounters (first with a fish of unknown social status

and thereafter with a fish of matched rank) four social categories, with 40 fish in each, were established:

- 1) (D+): fish winning both fights.
- 2) (D-): subdominants which initially had won a fight but then lost to another dominant.
- 3) (S+): fish that lost their first fight, and thus categorised as subordinates, but won their second dyad against other subordinate individual.
- 4) (S-): most subordinate individuals which had lost both fights.

This protocol with two sequential dyadic combats was used because it provided a more robust chance of distinguishing between the more dominant (i.e. D+) and the most subordinate (i.e. S-) individuals (e.g. most proactive vs. reactive types *sensu* Pottinger and Carrick, 2001).

After the last set of dyadic interactions all fish were then transferred into one of four tanks (volume = 60 l) with 40 fish in each, which contained equal numbers of individuals ($n = 10$) for each social rank (D+, D-, S+ and S-) per tank. The high number of fish per tank was intended to reduce the chances of new strong social hierarchies developing in the stock tanks (Alänarä and Brännäs, 1996). Two of these tanks were fed low food ration (1 % body wt day⁻¹), and the other two tanks were fed a high food ration (3 % body wt day⁻¹) for a period of 12 weeks. Fish nutritional studies have previously used similar rations to categorise low and high food intake (e.g. Reinitz, 1983). Food was delivered in two daily rations that were hand-fed, rather than using an automatic feeder, to minimise food being predictable in space and time and therefore more easily monopolised by dominant individuals.

After this time fish were collected from each feeding treatment and standard metabolic rate (SMR) measured for fish belonging to the D+ and D- category (see

SMR section). Due to logistical time constraints only D+ and D- category fish were examined for SMR as it was anticipated that these groups would show the most interesting differences in metabolic rate. After metabolic measurement, fish dominance was tested by randomly pairing fish of the social ranks that had interacted in the last dyad examined (i.e. D+ with D-, and S+ with S-) prior to high and low food diet treatments. Behavioural observations and dominance was measured using the same protocol as in previous dyadic encounters.

Behavioural observations

Behavioural observations were conducted in a staggered manner, with 10 pairs each day introduced into separate tanks (60 x 15 x 20 cm) with an opaque divider in the middle and a small plastic shelter on each side and left to rest overnight. The next morning at 0900 h the divider was removed and fish behaviour for each dyad was recorded using digital video (Sony SE-90) for 15 min. Dominance usually was quickly resolved, with the subordinate fish receiving most agonistic acts and initially escaping from attacks and after a few minutes being found hiding under a shelter. Agonistic acts including biting, chasing, charging, lateral display and receiving aggression were scored with 4, 3, 2, 1 and 0 points respectively. Fish were left together for 4 hours and then food pellets were introduced (5 in total 1 at a time at 2 min intervals), fish scored 1 point for each pellet consumed. Competitive ability for food is a commonly used method to assess dominance (Metcalf, 1989; Metcalfe et al., 1990). In addition, fish colouration, usually being darker in subordinate fish; (Höglund et al., 2000; O'Connor et al., 1999), was annotated with dark fish scoring 0 points and pale fish 1 point. The average score for aggressive behaviour, the score for pellets obtained, and the score for skin colouration were added together for each fish

in the dyad to quantify dominance and the fish with the highest score was considered the winner. In most pairs there were very marked differences in behaviour between the winner and the loser.

For identification purposes fish were lightly anaesthetised using a solution of tricaine methane sulphonate (MS222; 50 mg l⁻¹ buffered with NaHCO₃ and then vigorously aerated to restore normal pCO₂ levels) and unique colour combinations were applied to each individual using a biocompatible fluorescent elastomer (Visible Implant Elastomer, Northwest Marine Technology, Inc., Shaw Island, WA 98286). This marking method has been used previously to visually tag small sized fish and no significant effects have been reported on behaviour, growth or survival (Hoey and McCormick, 2006).

Standard metabolic rate measurement

Prior to behavioural observations metabolic rates were assessed by O₂ consumption measurements conducted by flow-through respirometry using a similar protocol to those detailed in other fish SMR experiments (e.g. Metcalfe et al., 1995, Sloman et al., 2000). Metabolic rates were measured from the reduction of O₂ concentration of fully aerated water flowing past a stationary fish (Steffensen, 1989). The system consisted of 20 cylindrical opaque Perspex chambers, 15 medium sized chambers (15 mm x 100 mm) and 5 larger ones (25 mm x 150 mm) to accommodate the full range of fish sizes, all maintained at a constant temperature (15 ± 0.1 °C). Due to the limited number of chambers SMR measurements were run over four consecutive days. Fish were placed in individual respirometer chambers fed with fully aerated water from a header tank, flow was adjusted to fish size to achieve approximately a 15 % reduction in oxygen content of the water running through the chamber. Flow rates through the

chambers were kept at a constant rate and measured gravimetrically (by weighing the amount of water flowing from the outflow of each chamber for 120 s). Measurements of blank water samples, with no fish in the tube, were conducted to test that bacterial respiration had no effect on reduction in oxygen concentration of the water. Fish were unfed for 24 h prior to measurements and kept in the tubes overnight to make sure that the fish had acclimatised to the new environment and a steady rate of O₂ consumption had been reached.

O₂ reduction in the chambers caused by fish respiration was measured by collecting with a hypodermic syringe a 5 ml water sample from the inflow and outflow, and injecting it into a thermostated cell (MC100; Strathkelvin Instruments Ltd., Glasgow, Scotland) containing the microcathode oxygen electrode (model 1302) which was connected to an O₂ meter (model 781). The reading from the oxygen meter for each sample was taken after 3 minutes, when the reading was fully stable. The electrode had previously been calibrated against both header tank air-saturated water and a zero oxygen solution of sodium sulphite in borax (0.01 mol sodium tetraborate l⁻¹).

The O₂ consumption measurement obtained is considered to be the standard metabolic rate i.e. the metabolism of an inactive fish that is not digesting food (Brett and Groves 1979). Three samples were collected per fish, allowing for 60 min between samples from the same fish. The average of these 3 measurements was used for analysis. If the variation between the 3 measurements was greater than 20 % a fourth measurement was conducted. All samples were collected between 0900 and 1300 to minimize effects of circadian rhythm on metabolic rate.

The O₂ consumption rates (*MO*₂) were calculated as:

$$MO_2 = (\Delta[O_2] \times V_w) / M$$

Where MO_2 is measured in $\mu\text{mol g}^{-1} \text{ h}^{-1}$, and change in O_2 concentration is measured in $\mu\text{mol l}^{-1}$, V_w is flow rate of water through the respirometer (l h^{-1}), and M is body mass (g).

The difference in O_2 concentration between the respirometer inflow and outflow was calculated as:

$$\Delta[O_2] = \Delta PO_2 \times \alpha_w O_2$$

Where ΔPO_2 is the difference in PO_2 (in mmHg) between the respirometer inflow and outflow and $\alpha_w O_2$ is the O_2 solubility in water at a given temperature and measured in $\mu\text{mol l}^{-1} \text{ mmHg}^{-1}$ (Boutilier et al., 1984).

Brain monoamine sampling

After 24 h of social interaction between fish in dyads, they were terminally anaesthetised (using buffered MS222, 200 mg l^{-1}), measured and weighed, and then rapidly decapitated and the brain dissected out (within 2 min) and separated into four parts: telencephalon (excluding the olfactory bulbs), hypothalamus (excluding the pituitary gland), optic tectum and brain stem (including the medulla and part of the spinal cord but excluding the cerebellum). Brain parts were immediately frozen in liquid nitrogen and stored at -80°C for later analysis of monoamines.

Brain monoamine analysis

Brain tissue was weighed and homogenized in 4 % (w/v) ice-cold perchloric acid containing 20 ng ml^{-1} of 3,4-dihydrobenzilamine (DHBA) as an internal standard, using a Potter-Elvehjem homogenizer. Samples were then centrifuged at $27,000 \text{ g}$ for 10 min at 4°C , and the supernatants used for analyses. Monoamine and monoamine

metabolites were determined using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Øverli et al. (1999a). Serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were examined. As a measure of serotonergic activity, the 5-HIAA/5HT ratio was calculated for each individual. The monoamines were quantified using standard solutions and corrected for recovery of the internal standard using HPLC software (CSW, Data Apex, the Czech Republic).

Statistical analysis

Data normality was examined using a Kolmogorov-Smirnov test for normality (Zar, 1996) and homogeneity of variance was evaluated using Levene's test (Schultz, 1983). Initial differences in body mass and fork length between social ranks at the start of the experiment was compared using a one-way ANOVA. Fish body mass and SMR data were examined using two-way ANOVA tests with status and food ration as factors, followed by *post hoc* tests (Tukey tests). Data for feeding regime with social status and also for brain serotonergic activity were examined using a nonparametric Wilcoxon signed-ranks test. Statistical analyses were conducted using SigmaStat (SigmaStat 3.0, PSS Inc., Chicago, IL, USA). Results are presented as means \pm standard error of the mean (SEM).

RESULTS

Social rank and size

There was no initial difference between social groups in body mass (one-way ANOVA; $F_{3,152} = 0.35$, $p = 0.789$) or in fork length (one-way ANOVA; $F_{3,152} = 0.432$,

$p = 0.73$). The two-way ANOVA used with body mass at the end of feeding regime experiment as the response variable showed both social status and food ration factors to be significant ($F_{3,152} = 50.87$, $p < 0.001$ and $F_{1,152} = 28.277$, $p < 0.001$ respectively), but not their interaction (social status x food ration; $F_{3,152} = 1.614$, $p = 0.189$) (Figure 2.1). *Post hoc* Tukey tests for mean body mass with social status showed that D+ individuals had a significantly higher body mass compared to D- (D+ v. D-, $p < 0.001$), S+ (D+ v. S+, $p < 0.001$), and S- had a lower body mass than the rest of the social groups (D+ v. S-, $p < 0.001$; D- v. S-, $p = 0.003$; S+ v. S-, $p = 0.002$). For feeding regime Tukey's tests revealed significant differences in mean body mass between fish kept on high and low rations ($p < 0.001$).

Feeding regime and social status

Analysis of social interactions between D+ and D- after the 12 week experimental period using Wilcoxon paired-tests showed that D+ individuals were more likely to win fights against D- fish in the examined feeding regime-social rank combinations (D+ high ration v. D- high ration; $Z = -2.236$, $n = 20$, $p = 0.048$; D+ low ration v. D- low ration, $Z = -3.13$, $n = 20$, $p = 0.004$; D+ high ration v. D- low ration, $Z = -2.683$, $n = 20$, $p = 0.017$), except in D+ low ration against D- high ration which showed non significance ($Z = -1.789$, $n = 20$, $p = 0.123$). Similarly, in the dyads between S+ and S- fish, again using Wilcoxon paired-tests, S+ won more fights in the examined feeding combinations over S- (S+ high ration v. S- high ration; $Z = -4.025$, $n = 20$, $p < 0.001$; S+ low ration v. S- low ration, $Z = -2.683$, $n = 20$, $p = 0.017$; S+ high ration v. S- low ration, $Z = -3.578$, $n = 20$, $p < 0.001$; S+ low ration v. S- high ration, $Z = -2.236$, $n = 20$, $p = 0.048$).

Standard metabolic rate

The relationship between juvenile salmon SMR (response variable) with social status (D+ and D- only) and feeding regime as factors was examined using a two-way ANOVA (social status, $F_{3,152} = 50.87$, $p < 0.001$; feeding regime, $F_{1,152} = 50.87$, $p < 0.001$; social status x feeding regime interaction, $F_{3,152} = 50.87$, $p = 0.002$) (Figure 2.2). Multiple pairwise *post hoc* comparisons using Tukey tests showed that within D+ status individuals showed significantly lower SMR when fed on lower diets (D+ high ration v. D+ low ration, $p < 0.001$), while SMR was similar in D- fish with either diet (D- high ration v. D- low ration, $p = 0.499$). Social status within feeding factor showed using Tukey tests that in the high ration regime D+ had a higher SMR than D- ($p < 0.001$), but this difference was insignificant when in the low ration ($p = 0.224$).

Brain serotonergic activity

Brain serotonin metabolism data from fish with a D+ or D- status was examined using Wilcoxon signed-ranks tests in each feeding treatment (Figure 2.3). Significant statistical differences emerged from D+ in low ration, showing lower 5HIAA/5-HT, compared to D- in low ration both in the brain stem ($Z = 2.395$, $n = 10$, $p = 0.014$) and the telencephalon ($Z = 2.09$, $n = 10$, $p = 0.037$). Similarly, comparison D+ high ration against D- low ration treatment was higher in the brain stem ($Z = 2.083$, $n = 10$, $p = 0.002$) and the telencephalon ($Z = 2.293$, $n = 10$, $p = 0.02$). However, where D- fish had been kept in high ration conditions serotonergic activity comparisons to D+ were found to be non-significant in the brain stem (D+ high ration v D- high ration, $Z = 0.357$, $n = 10$, $p = 0.77$; D+ low ration v D- high ration, $Z = 1.158$, $n = 10$, $p = 0.131$) and telencephalon (D+ high ration v D- high ration, $Z = 1.682$, $n = 10$, $p = 0.165$; D+ low ration v D- high ration, $Z = 0.051$, $n = 10$, $p = 0.989$).

DISCUSSION

This work highlights not only the importance of feeding conditions experienced by juvenile salmonids in nutrient rich or poor environments but also its interplay with social position (i.e. being dominant increases chances of obtaining a larger size compared to subordinates, even under low food rations) which is likely to be mediated by brain serotonergic activity and SMR.

Social dominance, feeding environment and growth patterns

Juvenile Atlantic salmon that showed the highest social status when all fish were reared in a uniform feeding environment, were also dominant after being kept in a poor feeding environment and interacting with fish with access to better feeding. This suggests that social dominance, at least under these temporary laboratory conditions, is stable over time even under adverse feeding situations. In the wild social hierarchies are thought to be stable overtime (Nakano, 1995), even when temporal and spatial food distribution is known to be variable in natural streams (Armstrong et al., 1999). These laboratory findings on dominance may be applicable to the stream environment as it has been shown that dominant salmonids in the laboratory also seem to perform well if moved to the wild (Höjesjö et al., 2002).

All fish were the same initial size at the start of the experiment but by the end of the 12 week feeding experiment, dominant individuals (D+) had obtained a higher body size advantage over more subordinate fish as indicated by the significant effect of social status. These results are in agreement with other studies considering large size a consequence rather than a cause of dominance (Huntingford et al., 1990; Yamamoto et al., 1998). Differences in body mass between individuals from the most

dominant category were significant in fish from the low and high ration treatments, growing faster in the latter. Despite this variation in growth within dominants in feeding treatments, D+ juveniles were consistently competitively superior to subdominant counterparts in high and low ration conditions. This pattern was also observed in the subordinates (S+) that had won their last fight. On the other hand the individuals from the S- rank, fish which had lost their social interactions at the start of the feeding period, SGR showed a lower growth relative to all other groups in both feeding conditions. Being enclosed for 24 hours in a confined space on a one to one basis with a fish that is aggressively dominant (which happened twice for S-) may have induced a stress-induced state of subordination leading to physiological traits such as reduced growth, high cortisol and elevation in serotonergic activity (Gilmour et al., 2005). Serotonin has been shown to reverse social status in dyads, as administration of the selective serotonin reuptake inhibitor sertraline leads to loss of dominance (Larson and Summers, 2001). Øverli et al. (1998) reported that subordinate Arctic char in dyads had higher serotonergic activity than their dominant counterpart and would cease feeding and reduce movement in the presence of the dominant fish. Even after the removal of the dominant fish subordinate individuals only resumed feeding very gradually and locomotory function remained low, showing that social defeat can have long lasting effects. Conversely, S+ fish winning its last social encounter may have had the opposite effect as they showed a final body mass similar to D- and higher than S- fish.

SMR and dominance relationships

The statistical analysis showed that both feeding regime and social status affected SMR and there was a strong interaction these factors. Individuals in D+ category

showed a significantly higher SMR compared to D- when under favourable feeding conditions but not when restricted. Mean while within social status comparisons indicated that the SMR was significantly lower in D+ low ration compared to D+ high ration individuals but SMR did not vary greatly between D- fish on either feeding treatment. In teleosts a high metabolism has often been associated with superior social rank (Røskoft *et al.*, 1986; Metcalfe *et al.*, 1992; Bryant & Newton, 1994; Cutts *et al.*, 1999). A high SMR can be a key motivator to increase dominant behaviours, such as active searching or aggressive displays, due to greater nutrient requirements to maintain basal functions (Cutts *et al.*, 1998). However, having a high metabolic rate is also costly and can become a burden if food is very scarce, thus it may pay D+ fish to maintain a lower SMR or they may not be able to physiologically maintain such an elevated SMR. Under suboptimal feeding conditions teleost fish may show partial reductions in their metabolic rates and these can vary highly between individuals (O'Connor *et al.*, 2000). The observed superior SMR and fast growth in dominant individuals held in favourable feeding tanks is typical of upper modal group juvenile salmonid fish acquiring a smolt or mature parr life history (Metcalfe *et al.*, 1995). Thus SMR appears to show some degree of flexibility in dominant fish in order to better acclimate to feeding regime experienced.

Brain serotonergic activity

Brain serotonergic metabolism showed that D+ fish had a lower 5-HIAA/5-HT ratio in the brain stem and telencephalon when compared to subdominant individuals. High serotonergic activity is a reliable indicator of social status in salmonid fish hierarchies (Höglund *et al.*, 2000; Winberg *et al.*, 1997), and is independent of starvation-induced stress (Winberg *et al.*, 1992b). There were no differences in either the serotonin levels

or serotonergic activity between the high and low ration individuals from each of the social ranks (which may support independence of feeding stress from social stress, although it is possible to argue that the serotonin reveals more about the last social outcome of a fight rather than if fish were fed a particular ration). Better feeding conditions in D- from the high ration treatment paired with D+ from the low ration did not result in lower serotonergic levels in these subdominants. The higher brain serotonergic activity in D- individuals may favour the persistence of submissive status as serotonin is known to inhibit key behaviours such as feeding, locomotion and aggression (Carpenter et al., 2009; Clotfelter et al., 2007; De Pedro et al., 1998; Øverli et al., 2002). Low final body mass showed by D- not only in the low ration conditions but also in the high ration may be regulated by high brain serotonergic levels initialised by losing fights in social encounters.

In summary, juvenile Atlantic salmon of higher social status were able to maintain their dominant rank despite prolonged (12 week) exposure to very different feeding conditions. The outcome of the last social encounter appeared to have a lasting effect during the length of the experiment. Dominant fish had faster growth rates compared to fish that lost their last social encounter. Physiological mechanisms underlying this social advantage could be through higher SMR and reduced ratios of serotonergic activity in the brain.

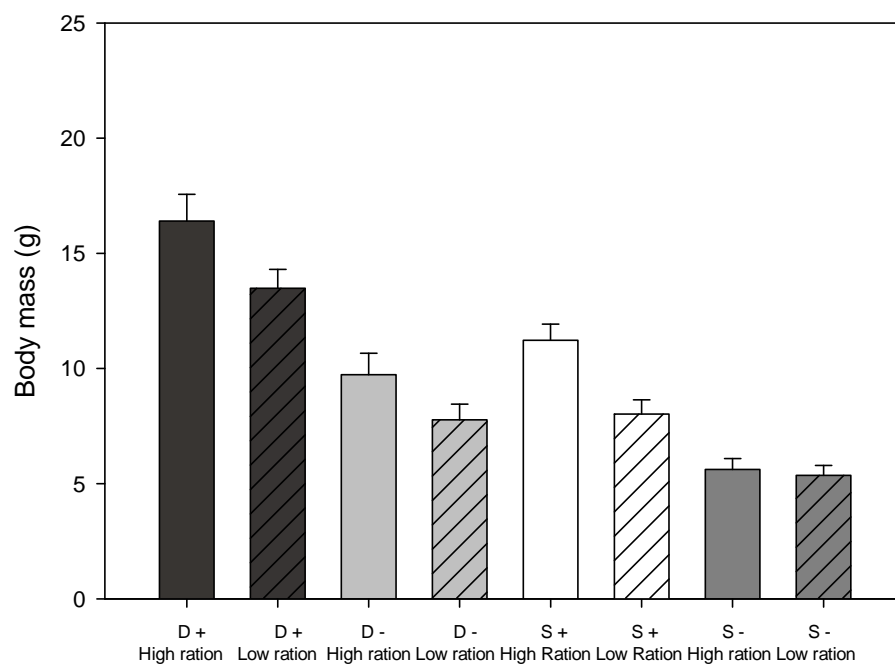


Figure 2.1. Final body mass (g) of juvenile Atlantic salmon with different social ranks (dominants (D+), subdominants (D-), subordinate (S+), and most subordinate (S-)) (n = 20 per group) held under a high food ration and a low food ration regime. Means \pm SEM.

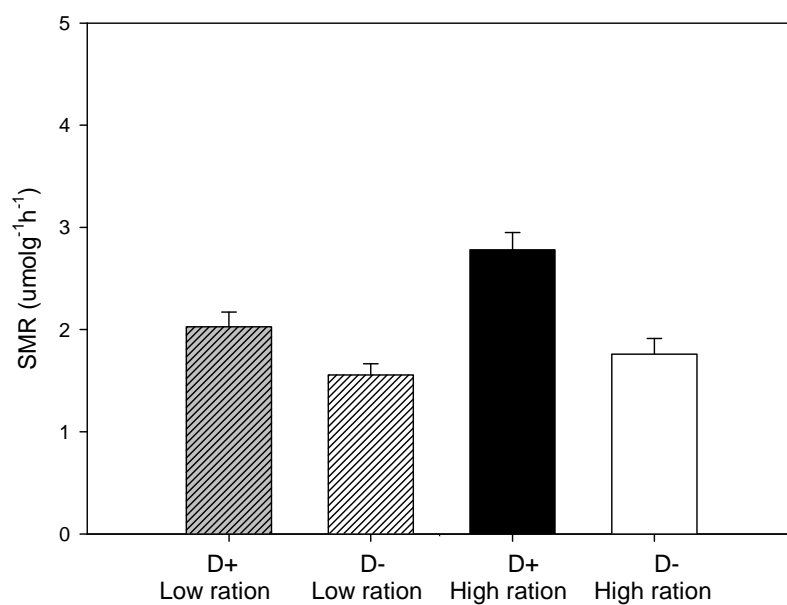


Figure 2.2. Specific metabolic rates (SMR) ($\mu\text{mol g}^{-1} \text{h}^{-1}$) of juvenile Atlantic salmon of dominant (D+) and subdominant (D-) status kept under a high food (HF) or a low food (LF) regime ($n = 20$ per group). D+ LF (▨), D+ HF (■), D- LF (▩), D- HF (□). Means \pm SEM.

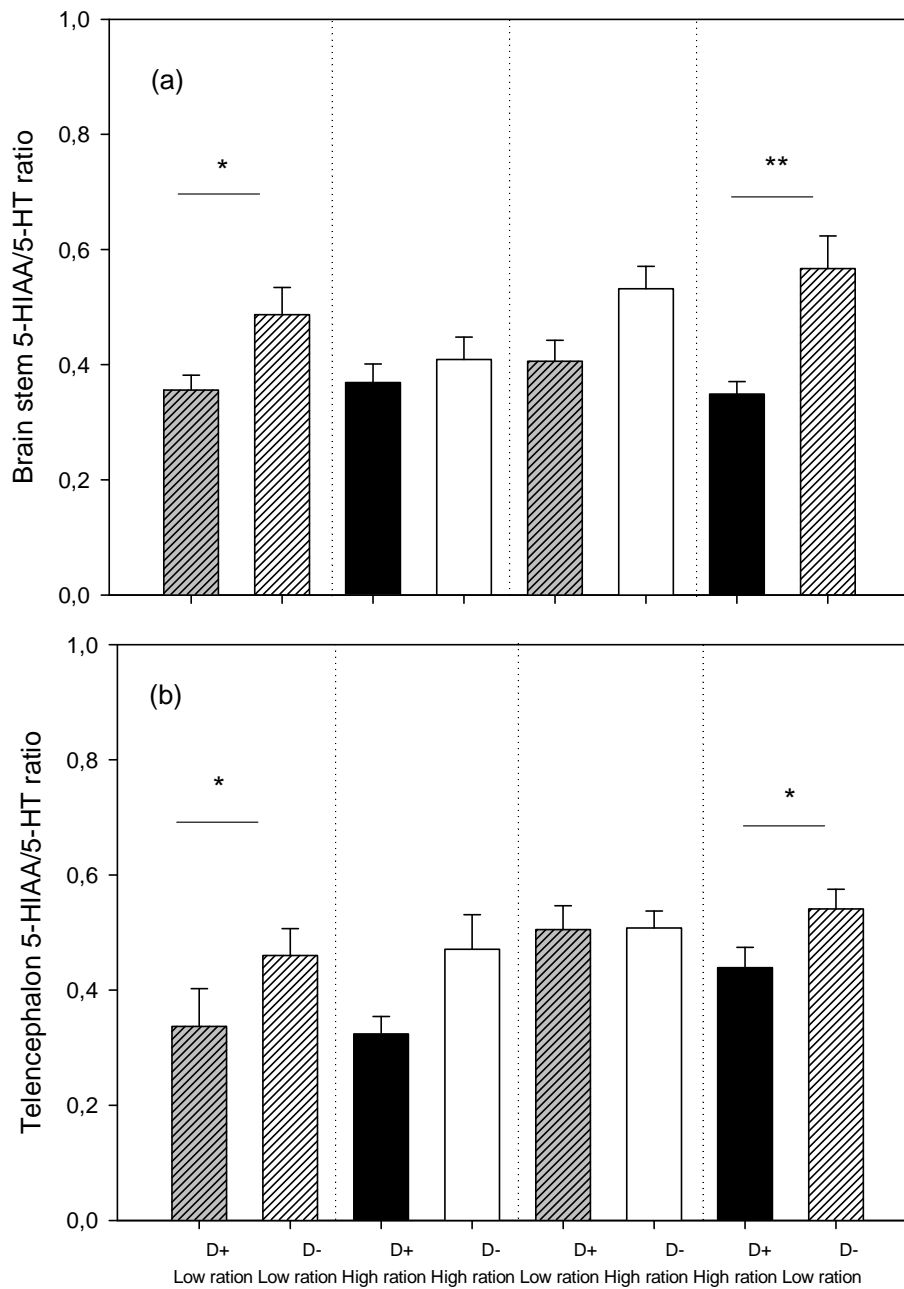


Figure 2.3. Brain monoamine 5-HIAA/5-HT ratios in (a) brain stem and (b) telencephalon from juvenile Atlantic salmon in dyadic social contests between previously dominant (D+) and subdominants (D-) fed on low food (LF) or high food (HF) rations (n = 20 per group). D+ LF (▨), D+ HF (■), D- LF (▧), D- HF (□). Means \pm SEM. Asterisks indicate a significant differences in 5-HIAA/5-HT ratios between groups (*p < 0.05, **p < 0.01).

Chapter 3

Differential sodium uptake kinetics in freshwater juvenile Atlantic salmon, *Salmo salar* L., with alternative life histories.

ABSTRACT

Juvenile Atlantic salmon (*Salmo salar*, L.) have three alternative life history strategies in fresh water; namely, freshwater precocious parr (also known as early-maturing parr), temporary freshwater residents (immature parr) and fish preparing for oceanic migration (smolts). I hypothesised that smolting would be associated with a loss of fresh water ionoregulatory characteristics (such as high affinity for gill Na^+ uptake), whereas parr (whether still immature or early maturing) would retain this ionoregulatory phenotype. In two experiments using Atlantic salmon juveniles from two different sources, we measured gill sodium uptake kinetics (affinity as K_m and maximum uptake capacity as J_{\max} , assessed from unidirectional ^{22}Na uptake rates) within fresh water in advance of the smolting process. In the first study three out of 18 fish sampled were precocious parr (no immature parr), the remainder being pre-smolts. In the second study, five fish were immature parr (none precocious) and the rest being pre-smolts. In the first study, pre-smolts had a two-fold lower J_{\max} ($291 \pm 45 \text{ nmol g}^{-1} \text{ h}^{-1}$) compared to precocious parr, and against the predicted trend, a lower K_m (higher affinity) for Na^+ uptake ($K_m = 118 \pm 69$ v. $439 \pm 127 \text{ } \mu\text{M}$, respectively). In study two, the immature parr exhibited the expected pattern of a higher affinity for Na^+ compared to pre-smolts ($K_m = 63 \pm 25$ v. $175 \pm 27 \text{ } \mu\text{M}$) but no difference in J_{\max} . There are therefore significant differences between the three freshwater phenotypes for physiological parameters relating to ion uptake kinetics. Despite these differences, all phenotypes appeared to be well suited for life in fresh water as reflected by their net Na^+ balance ($J_{\text{Na net}}$) and positive specific growth rates (SGR). Two months after

the Na⁺ uptake kinetic measurements, once salmon had clearly developed the exterior morphology of either a parr or smolt, a 24 h seawater challenge was conducted to examine the short-term hypo-osmoregulatory ability of each phenotype. The smolt phenotype consistently showed a better short-term acclimation to the seawater medium compared to both immature and early-mature parr, as indicated by i) lower plasma [Cl⁻] and less dehydration (percentage loss of body mass), and ii) higher total [CO₂] and presence of calcium carbonate precipitates within their intestinal fluid (associated with the marine osmoregulatory trait of drinking seawater). Ion uptake kinetics has the potential to be used as a non-lethal *in vivo* technique to predict future developmental phenotype in salmonids at a time at which they are still visually undistinguishable.

Keywords: Atlantic salmon; uptake kinetics; osmoregulation; life histories; smolt; early-maturing parr.

INTRODUCTION

Atlantic salmon (*Salmo salar*) are well-known for undertaking some of the most extensive migrations amongst aquatic species (McCormick et al., 1998). Initiating their journey from the natal river fresh water medium salmon must quickly acclimate to the high salt concentration of oceanic waters, whilst still maintaining an internal osmolality generally three times lower than that of sea water (Evans et al., 2005; Marshall & Grosell, 2005). An array of marine-adapting modifications occur during the parr-smolt transformation (smoltification) in salmonids, a process which involves profound modifications, including physiological (e.g. increase in hormones like

cortisol and changes in the activity and isoform expression of key gill enzymes such as Na^+/K^+ -ATPase), morphological (e.g. silvering of the skin, body lipid depletion) and behavioural (e.g. downstream migration) (McCormick et al., 1998). Smoltification generally occurs whilst still in the river in fish that previously were juvenile immature parr. The exact timing of these transformations is primarily concentrated during two or three months in the spring time (March-May) just before migration takes place (Whalen et al., 1999).

Not all individuals in a population will undergo smoltification which results in a considerable part of their lifecycle in the ocean. Many salmonid species have an alternative life history to this anadromous route, whereby a proportion of males will sexually mature early while still being parr (precocious) without migrating to sea, generally becoming permanent residents in their natal river (Orton et al., 1938). However, sometimes precocious male parr can smolt and migrate to sea in future seasons, but the fraction of fish that take this route is not clear and may vary among populations. Some authors have found a low incidence of mature parr that smolt in the next season (e.g. 7 %, Fleming, 1998), whereas others may regard this shift in life history as a more normal part of the mature parr's life cycle (e.g. Skilbrei, 1990). Typically during the pre-and early migration season three phenotypes are expected within an Atlantic salmon population: 1) smolting individuals metamorphosing to prepare for life at sea, 2) immature parr that will continue to live in fresh water until they reach a threshold condition to migrate in following seasons and 3) precocious or mature male parr many of which will permanently reside in the river.

One of the crucial physiological challenges that sea-running salmon must overcome during migration is to transform their fresh water Na^+ and Cl^- absorbing gill into a Na^+ and Cl^- excreting organ (Marshall, 2002). Na^+ and Na^+ are the most

abundant ions present in blood plasma and show the greatest flux rates across the gill epithelium (Wood et al., 1992). In freshwater osmotic balance is achieved through elevated glomerular filtration rates and high renal salt reabsorption that create a copious, dilute urine flow, coupled with active uptake of ions through the gill. In sea water this must change to a system based on ingestion of sea water and intestinal uptake of NaCl and water, together with NaCl extrusion through the gills and divalent ion excretion via the low urine volume excreted by the kidneys (Hoar, 1988; McCormick et al., 1989; Evans et al., 2005, Marshall & Grosell, 2005). The process of smoltification prepares freshwater fish for life in the marine environment, including reversing the ionoregulatory requirements of the gill (Lubin et al., 1989). Several mechanisms mediated by the endocrine system are thought to enable this transformation. For example, in the weeks prior to migration smolts show a dramatic rise in plasma cortisol concentration, which mediates the increase in gill Na^+, K^+ -ATPase activity, linked to the facilitation of salt extrusion when moving into the ocean (McCormick, 1994).

Perhaps the most widely used method to detect ionoregulatory changes to the gill during smolting has been the Na^+, K^+ -ATPase assay (McCormick, 1993), which has proven highly valuable in detecting differences between smolts and parr in terms of gill enzyme activity (e.g. Nielsen et al., 2003). However, this technique measures potential increases in capacity for salt transport through the gills via the Na^+, K^+ -ATPase enzyme, but can not reveal actual *in vivo* ion transfer rates nor even their direction. In addition, using changes in protein and mRNA expression, Richards et al. (2003) recently identified five Na^+, K^+ -ATPase α -subunits, which could be subdivided according to their freshwater and seawater response characteristics. For example, Shrimpton et al. (2005) have highlighted the existence of different isoforms of

Na⁺,K⁺-ATPase, one being up-regulated on entry to sea water (Na⁺,K⁺-ATPase - α 1b) and the other when in fresh water (Na⁺,K⁺-ATPase - α 1a). Similarly, Bystriansky et al. (2006) demonstrated in three species of salmonids, that the α 1a isoform decreased after entry in the sea, while α 1b increased sharply. These authors pointed out that most studies to date have not distinguished what proportion of each isoform makes up the total Na⁺,K⁺-ATPase activity measured in gill tissue. This is especially true in short-term experiments such as 24 h sea water challenges, where the α 1a isoform although declining is still present and α 1b is gradually rising. Furthermore, elevation in Na⁺,K⁺-ATPase activity can take several days until the gill epithelial cells have transformed and protein synthesis mechanisms are adjusted (D'Cotta et al., 2000), complicating the interpretation of gill ionoregulation patterns based on gill Na⁺,K⁺-ATPase assays in short sea water exposure experiments. As recent reviews on osmoregulatory aspects of the fish gill indicate (Evans et al., 2005; Evans, 2008; Hwang & Lee, 2007; Parks et al., 2008; Tresguerres et al., 2006), there is much debate over current models proposed to explain branchial ion uptake in freshwater teleosts and what is the precise role of Na⁺,K⁺-ATPase in basolateral Na⁺ transport. For example, although Na⁺,K⁺-ATPase pumps located on the basolateral membrane are crucial for gill transport system, there are other key trans-epithelial ion transport mechanisms including the apical proton pump (H⁺-ATPase), the Na⁺/H⁺, NH₄⁺-dependent and the Cl⁻/HCO₃⁻ dependent ATPases that may operate simultaneously (Goss et al., 1992; Evans et al., 1999; Clairborne, 2002; Tresguerres et al., 2006; Parks et al., 2008).

Measurement of branchial Na⁺ unidirectional transport rates using radio-labelled isotopes provides an effective technique to help characterise ion balance processes in fish because it can overcome some of the above mentioned problems. Using this

approach fish can be sampled *in vivo* non-invasively at any given time of their development. In addition, ion fluxes offer a 10-400 fold greater sensitivity to detect changes in ion transport functions compared to other conventional methods based on blood or tissue sampling (Wood, 1992). Furthermore, ion transport models based on the uptake of radioisotopic markers *in vivo* and *in vitro* have proven highly valuable in clarifying osmoregulatory mechanisms of teleosts in fresh water in particular (e.g. Maetz, 1971; Goss & Wood, 1990; Potts, 1994; Fenwick et al., 1999). Radio-labelled ion transport uptake kinetic techniques provide a valuable tool to better understand ion regulating systems and their mode of action under pre-selected environmental conditions. By measuring the disappearance of a radio-labelled ion (e.g. Na^+) from the water over a range of ambient concentrations, the transport kinetics can be estimated. When saturation kinetics are observed it is indicative of a carrier mediated active transport system in which the upper limit has been reached (Potts, 1994). Using the Michaelis-Menten equation, adapted from that commonly used for enzyme kinetic analysis, provides values for the kinetic variables K_m and J_{\max} . The K_m parameter is inversely proportional to the binding affinity of the active sites, including transport enzymes, carriers or channels, for the designated substrate. The J_{\max} value is calculated as the point of maximum uptake rate on the asymptotic curve described by ion substrate concentration (e.g. $[\text{Na}^+]$) and ion uptake (J_{in}) by the gills, and is indicative of the total number of operational transport sites (Perry & Wood, 1985).

Only a few studies have examined the Na^+ uptake kinetics of Atlantic salmon covering a specific stage of their life cycle. In particular, McWilliams & Shephard (1989) looked at Na^+ uptake during the earlier life stages of pre- and post-feeding Atlantic salmon fry, whereas Potts et al. (1985) investigated the later stages using adult anadromous salmon returning from sea water to a fresh water environment.

Furthermore, these two works did not set out to examine Na^+ uptake differences between phenotypes, but rather differential responses of one phenotype to different conditions in external variables (e.g. salinity). To our knowledge the present study is the first to investigate ion uptake kinetics in different phenotypes of the same species, and specifically with regard to juvenile salmonids during their critical differentiation into alternative freshwater and seawater forms.

In the present study we predicted that smolting salmon would start to lose their freshwater ionoregulatory characteristics (specifically manifested as a reduced affinity or higher K_m for Na^+) during this phase whilst still in fresh water, whereas precocious and immature parr that are not destined for seaward migration would not. To test this hypothesis the transport kinetics for gill Na^+ uptake were characterised in two separate experiments using juvenile salmon. By chance two of the three possible phenotypes of juvenile Atlantic salmon were found in the batches of fish used for each experiment. Consequently, in the first experiment pre-smolting salmon and male mature parr were examined, whilst in the second experiment pre-smolts and immature parr were studied. In both instances, short-term sea water adaptability of the same fish was examined later in the year, two months after Na^+ kinetics were characterised, when some fish were smolting and others were still at the parr stage.

METHODS

Fish keeping

Two separate experiments examining various aspects of osmoregulation in Atlantic salmon parr were conducted throughout the period of April to June 2001 and June to August 2006, respectively. The first experiment in 2001 was conducted on 18 parr,

this work was exclusively done by Dr. Rod Wilson. A similar protocol was followed by me in 2006 using 52 individuals as a follow -on experiment to gather more data. In these experiments the objective was to characterise the ion uptake kinetics of the three phenotypes present in freshwater juvenile Atlantic salmon before externally visible differentiation (i.e. smoltification) occurred and, 8 weeks later, to test ion regulating abilities of the three phenotypes in a 24 h sea water challenge. However concurrent investigation of the three phenotypes was not possible because in the first study only two of the phenotypes were found in the batch of salmon from Scotland, with no immature parr, whereas in the second study the batch of Devon salmon only contained the future smolt and immature parr phenotypes, and no precocious parr.

In study one juvenile Atlantic salmon were grown from eggs obtained from a farmed origin (Landcatch, Salmon Farm, Argyll, Scotland). Fish (body mass = 47.6 ± 3.3 g) were divided equally in groups of 5 in 60 l stock tanks (data for two fish were not included in the final analysis as Na^+ uptake kinetics showed extremely high J_{max} and K_m values). Tanks were supplied with running dechlorinated Exeter tap fresh water at ambient water temperature (range 12 – 16 °C) and light/dark regime was continuously adjusted to latitude 52N conditions. Exeter water composition is described in detail in Scott & Wilson (2007) (average composition \pm s.e. : $[\text{Na}^+] = 412 \pm 15$, $[\text{K}^+] = 55 \pm 3$, $[\text{Ca}^{2+}] = 624 \pm 18$, $[\text{Mg}^{2+}] = 139 \pm 7$, titratable alkalinity (TAlk) = $969 \pm 42 \mu\text{mol l}^{-1}$, pH = 7.46 ± 0.04). Fish were allowed to settle for several weeks before PIT tagging for identification purposes prior to experimental procedures. In study two juvenile Atlantic salmon were reared in a semi-natural pond in Dartmoor National Park (Devon, UK) and collected at the parr stage (body mass = 40.4 ± 1.8 g) in March 2006. The fish were transported to the Exeter aquarium and held in similar conditions to study one. A week later after arrival, all fish were PIT tagged, divided

into 8 groups of 7 individuals and moved into similar stock tanks to the previously described ones. All experiments were conducted in accordance a UK Home Office Project Licence under the Animals (Scientific Procedures) Act 1986, following permission from the University of Exeter Ethical Review Group.

Na⁺ uptake kinetics

In April 2001 and June 2006 unidirectional Na⁺ uptake kinetics were conducted on all fish from the two studies described above. The system used for conducting the Na⁺ flux experiments is similar to that described in more detail in Scott & Wilson (2007). Flux chambers were used to measure Na⁺ uptake in individual freshwater salmon. The chambers were opaque to minimize stress and chambers holding various volumes were used (between 600 ml and 1000 ml) to provide a small ratio of fish mass (g) to water volume (ml) to maximise flux measurement sensitivity (e.g. 1:10 to 1:30). Prior to the experiment fish were left without food for 48 h to stabilise ammonia excretion rates (Brett & Zala, 1975) and fish were moved from the stock tank to the chambers 24 h hours before the fluxes began in order to reduce stress during the experiment.

A temperature controlled (Grant RC 1400G chiller) 150 l volume re-circulating system was used to feed the flux chambers with fresh water. The reservoir water was adjusted to pH 7.5 by addition of 0.1 mol l⁻¹ KOH using a pH controller (Hanna HI 8710E). At the start of each experiment, each flux chamber was flushed thoroughly at $\approx 400 \text{ ml min}^{-1}$ for 5 minutes with Na⁺-free water. This was made up similar to the method described by Goss & Wood (1995) by adding adequate amounts of CaCO₃ and (MgCO₃)₄.Mg(OH)₂.5H₂O salts to deionised water to closely match the Ca²⁺, Mg²⁺ and titratable alkalinity (TAlk) composition to that of Exeter tap water. Excess water exited each chamber via an overflow hole, thus enabling water

replacement. A water sample was obtained from a representative number of chambers and Na^+ content immediately analysed using a flame photometer (Corning 410). Once the Na^+ concentration fell below 20 μM then Na^+ -free water flow to the chambers was stopped and the overflow hole was sealed with a bung. This method minimised fish disturbance.

To measure unidirectional Na^+ flux rates at different ambient ion concentrations, a stock solution of NaCl was added at 7 time intervals to obtain Na^+ concentrations that nominally doubled at each increment (i.e. 20, 40, 80, 160, 320, 640, and 1280 μM). Radio-labelled ^{22}Na was simultaneously added to chambers so that activity also increased with ambient $[\text{Na}^+]$. These solutions were pipetted through a small hole in the chamber lid to minimise disturbance of the fish. At each time interval the same procedure was repeated as follows. After waiting 10 minutes for the added Na^+ to completely mix with the flux chamber water, an initial 30 ml water sample was taken, followed by a second (final) 30 ml water sample after 0.5 h. This short time period was chosen to minimise backflux of the ^{22}Na isotope from the fish into the water (Maetz, 1956).

At the end of the experiment fork length (cm) and body mass (g) of all fish was recorded and then fish were returned to their original stock tanks. The initial and final activities (counts per minute; cpm) of ^{22}Na in each flux chamber at all time intervals were estimated from triplicate 2 ml water samples using a gamma counter (Packard Cobra B5002). Additional sample was used for flame photometry (Corning 410) to establish total $[\text{Na}^+]$ in the water.

Unidirectional Na⁺ flux parameter estimation

Net Na⁺ flux ($J_{\text{Na net}}$) was calculated from the change in Na⁺ concentration of the water in each chamber over 0.5 h using the following equation from Preest et al. (2005):

$$J_{\text{Na net}} = V \times ([\text{Na}^+]_0 - [\text{Na}^+]_1) / (M \times T)$$

where V is the water volume in the chamber (l), $[\text{Na}^+]_0$ and $[\text{Na}^+]_1$ represent Na⁺ concentrations ($\mu\text{mol l}^{-1}$) in the bath at the beginning and end of the flux period, respectively, M is the mass of the fish (g), and T is the duration of the flux period (h). The Na⁺ influx was calculated from the disappearance of ²²Na isotope from the water and the average Na⁺ concentrations of the water during the flux period using the following equation from Gonzalez and Dunson (1987):

$$J_{\text{Na in}} = (\ln Q_{\text{out}0} - \ln Q_{\text{out}1}) \times Q_{\text{out}} / (M \times T)$$

where $Q_{\text{out}0}$ and $Q_{\text{out}1}$ are the total counts per minute in the flux chambers at the beginning and end of the flux period, respectively, Q_{out} is the average amount of Na⁺ in the flux bath during the flux period, M is the mass of the fish (g), and T is the duration of the flux period (h). The resulting $J_{\text{Na in}}$ values are given in $\text{nmol g}^{-1} \text{h}^{-1}$

In addition because the substrate (Na⁺) is added sequentially at increasing concentrations, the efflux rate ($J_{\text{Na out}}$) can be calculated at each experimental period (Wood, 1992) as the difference between influx ($J_{\text{Na in}}$) and net flux ($J_{\text{Na net}}$). The plot of the relationship between $J_{\text{Na in}}$ and the external concentration ($[\text{Na}]_{\text{ext}}$) generally results in a hyperbolic shape, which can be examined using a modified form of the Michaelis-Menten equation for substrate reactions:

$$J_{\text{Na in}} = (J_{\text{max}} \times [\text{Na}]_{\text{ext}}) / (K_m + [\text{Na}]_{\text{ext}})$$

J_{max} yields the maximum influx rate and tends to be interpreted as the number of operational sites available (i.e. the number of functional transporters in the gill), while

K_m is calculated as the value of $[\text{Na}]_{\text{ext}}$ at $\frac{1}{2} J_{\text{max}}$, and represents the index of the binding affinity of the operational site of transport. These values help characterise the type of transport system operating within an individual fish. Values of J_{max} and K_m were obtained from plots of sodium influx *versus* sodium concentration (illustrated in Figure 3.1) using Sigmaplot (Systat Software Inc), where each curve represents the sodium influx of an individual at each of 7 sodium concentrations.

Sea water challenge

A 24 h seawater challenge was conducted 8 weeks after each of the Na^+ uptake kinetic experiments to analyse the osmoregulatory response of salmon on transfer to a hyper-osmotic environment (33 and 21 salinity, respectively) once their developmental pathway was decided (e.g. smolts showed an obvious silvery exterior). The experiment was carried out in the summer months (June and August, respectively), when Atlantic salmon adopting the migratory phenotype should be well prepared for life at sea. The fish were terminated humanely using MS222 anaesthetic dissolved in buffered sea water (200 mg l^{-1}). Blood was taken by caudal puncture using heparinised syringes and centrifuged at $3000 \times g$ for 2 minutes; plasma was then removed and kept on ice for ion analysis on the same day. Plasma osmolality was measured using a vapour pressure osmometer (Wescor 5520), and Cl^- using an automatic chloride titrator (Corning 925). Intestinal contents were examined for precipitates of calcium carbonate (CaCO_3) and remnant fluid (both indicative of seawater acclimation). Total CO_2 (TCO_2) in intestinal fluid was measured when sufficient volume was retrieved, as elevated values are indicative of seawater acclimation (Wilson et al., 1996, 2002, 2009), using a total carbon dioxide analyser

(Mettler Toledo 965D). Gonad maturation stage and gender were examined visually. Percentage body mass loss, an indicator of dehydration in fish, was calculated as:

$$((M_i - M_f) / M_i) \times 100$$

where M_i is initial body mass (g) one week prior to the 24 h seawater challenge and M_f represents body mass (g) after the seawater test.

In addition, specific growth rate (SGR, % body mass per day) of each phenotype was calculated between the time of Na^+ uptake kinetics trials and the seawater transfer (approximately 2 months). Individual specific growth rates (SGR) were calculated as follows:

$$\text{SGR} = 100 (\ln M_2 - \ln M_1) / t,$$

where M_2 and M_1 are the body mass (g) at the end and start of the experiment respectively and t is the number of days between measurements. Data normality was examined using a Kolmogorov-Smirnov test for normality (Zar, 1996) and homogeneity of variance was evaluated using Levene's test (Schultz, 1983). A non-parametric one sample t-test or a non-parametric equivalent was used to examine if sodium fluxes were in balance (i.e. different from zero), while t-tests for parametric and Mann-Whitney U-test for non-parametric data were utilised to examine uptake kinetic measurements (K_m and J_{\max}), SGR (on arcsine square root transformed data), Cl^- and Na^+ concentrations, plasma osmolality, and weight loss after sea water entry. Statistical analyses were conducted using SigmaStat (SigmaStat 3.0, PSS Inc., Chicago, IL, USA). Results are presented as means \pm standard error of the mean (SEM).

RESULTS

Experiment 1 - Smolts v. Precocious Parr

In April 2001 18 fish were examined, 15 being pre-smolts (body mass = 52.1 ± 3.3 g), and three precocious parr (body mass = 29.2 ± 3.6 g). Significant differences in K_m (Mann-Whitney U-test, $Z = 48$, $n = 18$, $p = 0.024$) and J_{\max} (t-test, $t_{16} = -4.452$, $p < 0.001$) were detected between fish with alternative life histories (Figure 3.2.a). Fish that later became smolts had a K_m for Na^+ uptake ($K_m = 118 \pm 69$ μM) almost four-fold lower than precocious parr ($K_m = 439 \pm 127$ μM). The J_{\max} of future smolts ($J_{\max} = 291 \pm 45$ $\text{nmol g}^{-1}\text{h}^{-1}$) was less than half that of freshwater resident precocious parr ($J_{\max} = 787 \pm 110$ $\text{nmol g}^{-1}\text{h}^{-1}$).

Examination of fluxes measured at the same $[\text{Na}^+]$ as their prior freshwater holding conditions (Figure 3.3.a) showed that net Na^+ fluxes that were essentially in balance (i.e. not significantly different from zero) for both smolts ($J_{\text{Na}\text{net}} = -9 \pm 43$; one sample t-test, $t_{14} = 0.209$, $p = 0.837$) and precocious parr ($J_{\text{Na}\text{net}} = -35 \pm 196$ $\text{nmol g}^{-1}\text{h}^{-1}$; one sample t-test, $t_2 = 0.183$, $p = 0.872$).

During the experimental period smolts showed a higher body mass SGR compared to precocious parr (0.82 ± 0.3 versus 0.47 ± 0.2 % body mass day^{-1} ; t-test, $t_{17} = -3.143$, $p = 0.006$). Following the 24 h seawater challenge test, precocious parr had much higher plasma ions than smolts ($[\text{Cl}^-] = 179 \pm 7$ versus 156 ± 3 mM, respectively; t-test, $t_{16} = 4.652$, $p < 0.001$) and greater loss of body mass due to dehydration (body mass change = -13.4 ± 0.9 and -9.2 ± 0.5 %, respectively; t-test, $t_{16} = 3.016$, $p = 0.008$) as expected (see Table 3.1). There was also no detectable fluid in the intestine of precocious parr, whereas 13 out of 15 smolts had visible gut fluid and

in 11 of these there was enough sample volume to measure total CO₂ which was highly elevated (TCO₂ = 80 ± 4 mM).

Experiment 2 - Smolts v. Immature Parr

During the second experiment a high number of juvenile Atlantic salmon parr (n = 52) were used to investigate Na⁺ influx across the gills. All parr examined (n = 5) were sexually immature (body mass = 18.4 ± 1.0 g), none were precocious parr, with the remainder (n = 47) being future smolts (body mass = 44.6 ± 2.5 g). Immature parr had significantly lower K_m values ($K_m = 63 \pm 25 \mu\text{M}$) than smolts ($K_m = 174 \pm 27 \mu\text{M}$) in this experiment (Mann-Whitney U-test, $Z = 69$, $n = 52$, $p = 0.048$). The J_{\max} values for immature parr ($564 \pm 96 \text{ nmol g}^{-1} \text{ h}^{-1}$) and future smolts ($954 \pm 73 \text{ nmol g}^{-1} \text{ h}^{-1}$) were also found to be significantly different (Mann-Whitney U-test, $Z = 65$, $n = 52$, $p = 0.038$). Examination of fluxes measured at the same [Na⁺] as their prior freshwater holding conditions showed higher influx than efflux, resulting in a positive mean value for the non-parametric net Na⁺ flux data in the presmolts (Wilcoxon signed-rank, $Z = 806$, $n = 45$, $p < 0.001$), while in the immature parr there was a similar trend but it was non-significant (Wilcoxon signed-ranks test, $Z = 15$, $n = 5$, $p = 0.059$) (Figure 3.3.b).

Statistical analysis of SGR (on arcsin transformed data) revealed a significantly higher growth in smolts (SGR smolts = 0.85 ± 0.1 ; immature parr = 0.71 ± 0.1 % body mass day⁻¹; t-test, $t_{44} = 2.258$, $p = 0.029$). After the 24 h seawater challenge test analysis of examined plasma ions in immature parr (Cl⁻ = $148 \pm 3 \text{ mM}$; Na⁺ = $159 \pm 4 \text{ mM}$) was higher for both Cl⁻ (Mann-Whitney U-test, $Z = 223$, $n = 52$, $p = 0.003$) and Na⁺ (t-test, $t_{47} = 2.518$, $p = 0.015$) than smolts (Cl⁻ = $138 \pm 1 \text{ mM}$; Na⁺ = $145 \pm 5 \text{ mM}$). Consequently total plasma osmolality was found to be significantly

higher in immature salmon ($351 \pm 11 \text{ mOsm kg}^{-1}$) than in smolts ($320 \pm 2 \text{ mOsm kg}^{-1}$) (Mann-Whitney U-test, $Z = 211$, $n = 47$, $p = 0.007$).

Analysis of percentage body mass change following the seawater challenge showed a significant and almost two-fold greater loss in immature parr ($-6.3 \pm 1.4 \%$) compared to smolts ($-3.7 \pm 1.6 \%$) (t-test, $t_{48} = -3.498$, $p < 0.001$). When intestinal fluid was extracted it was either present in very low amounts or absent in many fish, and thus difficult to obtain a representative sample. However, it is worth pointing out that either intestinal fluid and/or calcium precipitates were present several immature parr ($n = 3$) and smolts ($n = 25$).

DISCUSSION

This work shows for the first time that salmon with alternative life history phenotypes appear to possess different Na^+ uptake kinetics in their freshwater phase before exterior morphological differences develop, and may reflect distinct ion transport mechanisms. The experiments examined an unusually high number of fish ($n = 70$) compared to previous salmonid studies using this particular technique (e.g. Potts et al., 1985; McWilliams and Shepard, 1989). The differences in Na^+ uptake for K_m and J_{max} were marked and observed across both experiments holding different phenotypes, although the expected ionoregulatory trend between precocious parr and future smolts was somewhat unexpected.

The present study hypothesized that individuals preparing for life at sea will present different ionoregulatory characteristics to those fish that are destined to remain in fresh water for at least another year. In the past, several physiological differences between salmonids with alternative phenotypes have been reported. In

particular, endocrine dissimilarities have received most attention, with plasma concentrations of hormones such as 11-ketotestosterone, testosterone, oestrogens, cortisol, gonadotropin-releasing hormone and growth hormone varying according to phenotype (Heath et al., 1997; Bjornsson, 1997; Antonopoulou et al., 1999; Shrimpton & McCormick, 2002). Other biological indicators associated with life histories include body mass and somatic lipid levels (Thorpe et al., 1998; Jonsson & Jonsson, 2005), gill cell membrane lipid content (Bystriansky and Ballantyne, 2007), energetic investment to gonads (Gage et al., 1995), and skin colouration (Duston, 1995). Thus, the existence of differential ion uptake kinetics in Atlantic salmon with alternative life histories may be yet another example of physiological disparity. Several studies have reported total branchial Na^+/K^+ -ATPase activities (Nielsen et al., 2003) that reveal unique ionoregulatory capacities that distinguish pre-migratory smolts from parr (but not between freshwater immature and precocious parr). The present data on Na^+ uptake kinetics add a new dimension to these findings, providing an additional physiological distinction between fish which are about to adopt alternative strategies before distinctive external characters emerge, and could potentially be used in conjunction with other predictive parameters (e.g. hormone levels or gill enzymes) to distinguish between phenotypes that are otherwise visually identical. Although the analysis of Na^+ uptake kinetics is not as straightforward to perform and has obvious safety implications (use of radioisotopes) that would probably limit its use to research laboratories, importantly this technique appears to distinguish between salmon with alternative reproductive tactics at the whole organism level and without the need for invasive sampling. Furthermore, the distinctive Na^+ uptake kinetics may reflect differential mechanisms of transport (or individual components of the transport process), rather than simply overall transport

capacity. Distinct ion uptake kinetics may potentially also be found in other salmonids that also have a precocious parr and an anadromous stage in their life cycle (e.g. *S. trutta*, Titus and Mosegaard, 1992; *Oncorhynchus masou*, Koseki & Maekawa, 2000; *O. kisutch*, Spidle et al., 1998). Indeed, this may even be applicable to non-salmonid fishes showing alternative strategies associated with a movement between distinct water masses (e.g. yellow and silver eels; Fontaine et al., 1995; McCleave & Arnold, 1999).

Both experiments in the present study followed a very similar experimental protocol. However, the variability introduced by differences in the origin of fish (e.g. genetic makeup, environmental rearing conditions) and the timing of experiments (e.g. April and June) could have affected the Na^+ uptake kinetics in salmon (Potts et al., 1985; McWilliams and Shepard, 1989). As expected J_{max} which represents the number of operational sites for Na^+ uptake, was higher in precocious parr destined to stay in fresh water than in fish developing into smolts. Surprisingly, precocious parr showed lower affinity (higher K_m) for Na^+ uptake in fresh water than the pre-migratory smolts. This contrasted with our initial expectation that pre-migratory smolts would start to lose the high affinity ion uptake systems as part of their preparation for leaving freshwater. Despite the large number of fish examined for Na^+ uptake kinetics ($n = 70$ across both experiments) we found a low incidence of precocious parr, which can be common in the wild (e.g. Bagliniere and Maisse, 1985). It is worth noting that the relatively high variation in measured parameters within this phenotype is partly due to there actually being 4 precocious parr found in Study 1, but only 3 of these have been included in the K_m and J_{max} data shown. The Na^+ uptake kinetics in this additional fish followed the same trend as the other precocious parr (high K_m and J_{max}), but the Michaelis-Menten curve was virtually a straight line

within the $[\text{Na}^+]$ range we used. This resulted in extremely high values ($K_m = 8269 \mu\text{M}$ and $J_{\max} = 11230 \text{ nmol g}^{-1} \text{ h}^{-1}$) being estimated by the Sigmastat software which are inappropriate to include because the uptake rate had not yet reached an asymptote on the Michaelis-Menten curve. Nevertheless, it is worth reiterating that all four of these precocious parr displayed a similar pattern and certainly distinct from the smolts.

It is also important to observe that the uptake kinetics were always run while fish were still being maintained in fresh water, thus pre-smolt parr would still require an active uptake of Na^+ and Cl^- through the gills to compensate for any diffusive ion losses. Freshwater fish must actively take up Na^+ to overcome the >100-fold difference between their plasma levels and that of their hypo-osmotic environment. When smolting juvenile salmon are prevented from migrating into the ocean they will continue to survive in fresh water, unlike maturing adult salmon which cannot remain in sea water indefinitely and must return into fresh water (Shrimpton et al., 2000). It is possible that our sampling was conducted just before the narrow time-window during which parr-smolt transformation occurs (4-8 weeks) and adaptive changes to a marine environment had not been fully initiated at the time of the Na^+ fluxes in all fish.

Pre-smolts in the second study had a higher K_m (lower affinity) for Na^+ than immature parr. Fish in sea water will only require sodium uptake for acid-base regulatory purposes (e.g. proton excretion via Na^+/H^+ exchange) and the strong inward Na^+ concentration gradient in the marine environment would clearly not necessitate a high affinity Na^+ uptake transport system. It was therefore expected that individuals preparing for oceanic migration would show a tendency for reduced Na^+ uptake affinity. Analysis of maximum carrying capacity transport (J_{\max}) showed that values were different in pre-smolts and immature parr. In these two phenotypes J_{\max} is

first reached at an ambient sodium concentration ($\sim 700 \mu\text{M}$), which is higher than that of the fresh water in which they lived ($412 \pm 15 \mu\text{M}$). Thus, both phenotypes would not be operating near their maximum Na^+ uptake capacity (J_{max}) under these conditions.

When looking across experiments a pattern emerged in terms of sodium affinity, with K_m being lowest in immature parr, with smolts in both experiments showing K_m values approximately double that, and finally precocious parr showing an 8-fold higher K_m (i.e. even lower affinity). Comparison between immature parr and precocious parr in J_{max} was also different, thus it appears that changes may be happening both in number of operational sites for Na^+ as well as in affinity for this ion. Certainly within salmonids the upregulation of Na^+ intake rates can be modified quite rapidly (within 24 hours; Postlethwaite & McDonald, 1995). This is the case in fish subjected to social stress, with subordinates experiencing a higher loss of ions due to leakage through the gills leading to a higher affinity or intake rate just to keep Na^+ balance (see review by Sloman, 2007). As in salmonids individuals with different developmental pathways are associated with a particular social status (i.e. immature parr tend to be subordinate, Metcalfe et al., 1989), it is possible that in social hierarchies stress undergone through social interactions could strongly influence ionoregulatory traits such as ion affinity.

Despite the differences in Na^+ uptake kinetics in fresh water, during both experiments all phenotypes appeared to be in Na^+ balance with net flux either being non-significantly different from zero or slightly positive, and all fish had positive specific growth rates. This suggests that the various ion transport systems found worked sufficiently well in fresh water, including in the pre-smolts that were clearly preparing for migration to a seawater environment. Atlantic salmon are recognised as

one of the most competent and flexible osmoregulators amongst salmonids, and it could be that they are capable of a faster or greater up- or down-regulation of ion transport mechanisms depending on their environmental requirements than other species. For example, Bystriansky et al. (2006) showed that compared to rainbow trout (*O. mykiss*) and Arctic char (*Salvelinus alpinus*), Atlantic salmon regained normal plasma osmolality faster after seawater transfer, possibly aided by a three-fold higher $\alpha 1b$ isoform of gill Na^+, K^+ -ATPase activity and protein content than the other species studied. Also, immature parr and precocious parr, which in the wild would not leave their freshwater environment, if subjected to a seawater challenge will show high survival and growth rates after an initial adaptation period (Skilbrei, 1990), thus emphasizing the ion balance plasticity of juvenile Atlantic salmon.

Two months after the Na^+ uptake kinetics analysis, smolts acclimated better to the seawater transfer than did either the precocious parr (experiment 1) or immature parr (experiment 2), as indicated by their lower plasma ions and % loss of body mass. The higher total CO_2 concentration and presence of calcium carbonate precipitates in the intestinal fluid is also a good indicator of better seawater acclimation following the initiation of drinking (Grosell, 2006; Wilson et al., 1996; 2002; Wilson, 1999). Unfortunately, different salinities were used for the seawater challenges in Experiments 1 and 2. This prevents any direct comparisons of the degree of seawater acclimation between fish in these two parts of the study. Nevertheless no mortality was present in any of the phenotypes and precocious parr are generally capable of full adjustment to marine water if given enough time (Skilbrei, 1990). In the study of Bystriansky et al. (2006) it took 10 days for seawater-transferred Atlantic salmon to appropriately regulate their internal osmotic concentration to previous basal levels in fresh water and the α -1b isoform of gill Na^+, K^+ -ATPase was still increasing after 30

days (total duration of their sampling). Thus, internal osmotic adjustments caused by movement from fresh water to sea water appear to be gradual and may extend over weeks.

These results add an interesting detail to our understanding of the physiological processes expressed within the different salmonid life history phenotypes on top of those already known. This work identifies the existence of differences in ion uptake dynamics within juvenile salmon in fresh water with diverging strategies, but the precise mechanisms (i.e. low affinity vs. high affinity systems) that underpin these differences are difficult to characterise, and beyond the scope of the current study. It is known that freshwater teleosts take up Na^+ from their surrounding environment against strong concentration gradients (Perry, 1997) but the mechanisms driving this physiological observation are not fully understood. Sodium uptake is currently explained by mechanisms involving apical membrane epithelial Na^+ channels electrogenically coupled to a proton pump (V-type H^+ -ATPase) in α – mitochondrial rich cells (MRCs) of the gill (Reid et al., 2003). Cation transporting pumps, such as the V-type H^+ -ATPase on the apical gill membrane and Na^+, K^+ -ATPase located basolaterally can generate an electrochemical gradient across the apical membrane driving Na^+ inside from the surrounding external fresh water. Using approaches similar to those that have helped for example to advance our understanding of metal toxicants on fish gill ion transport processes (e.g. Bury and Wood, 1999; Grosell and Wood, 2002), combining ion uptake kinetics with the use of selective ion transport inhibitors (e.g. ouabain, amiloride, EIPA). This could advance our mechanistic knowledge and may help characterize and integrate the physiological responses of fish to Na^+ uptake disrupting events such as social stress (Sloman et al., 2004) or metal toxicity (Grosell et al., 2002) at the phenotypic level.

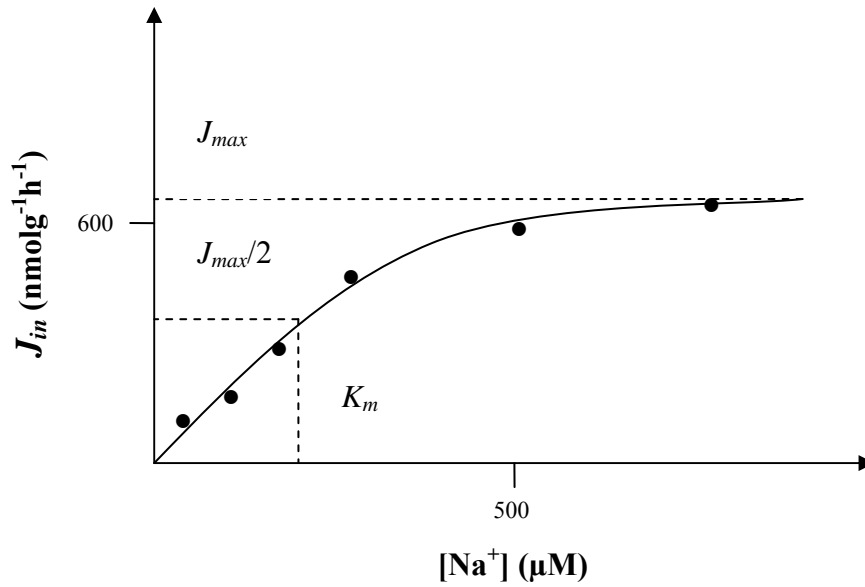


Figure 3.1. Diagram representing an adaptation of the Michaelis-Menten curve to estimate Na^+ uptake kinetics by plotting substrate concentration, in this case external $[\text{Na}^+]$, against a measure of reaction velocity such as Na^+ influx (J_{in}). The value for J_{max} represents the point of maximum influx and K_m , a measure of substrate affinity, is reached at $J_{max}/2$.

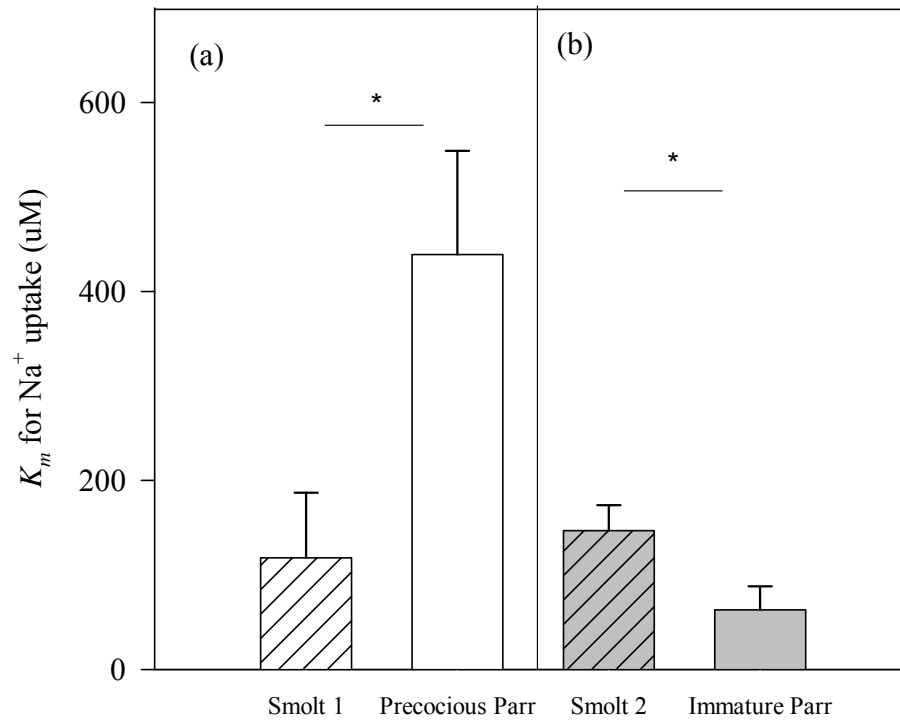


Figure 3.2. Na^+ unidirectional influx values for K_m (μM) in juvenile Atlantic salmon (a) pre-smolts ($n = 15$) and precocious parr ($n = 3$) in study one and (b) pre-smolts and ($n = 47$) and immature parr ($n = 5$) parr in study two. Values are means \pm SEM. Asterisk indicates significant differences in K_m between phenotypes (* $p < 0.05$).

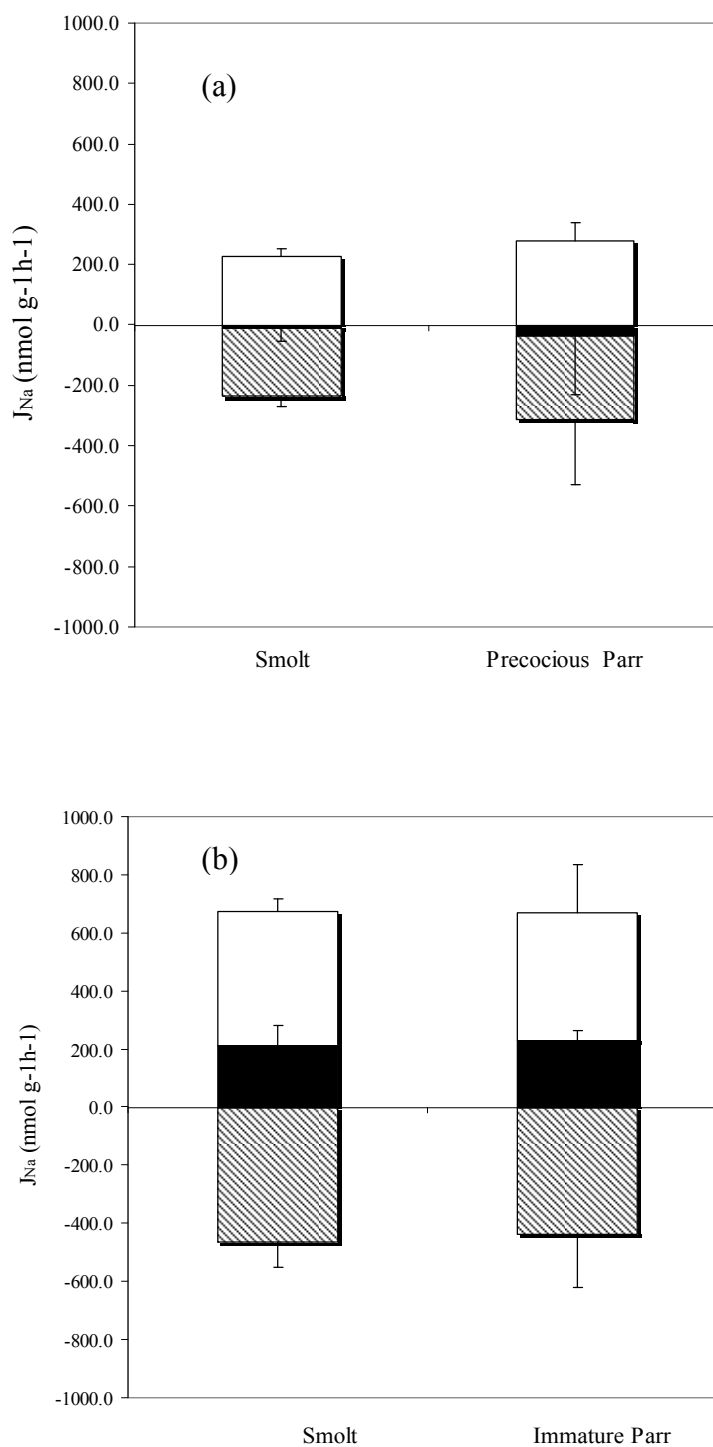


Figure 3.3. Na^+ fluxes [$J_{\text{Na},\text{in}}$ (\square), $J_{\text{Na},\text{net}}$ (\blacksquare), $J_{\text{Na},\text{out}}$ (\boxtimes)] measured at the same $[\text{Na}^+]$ as their prior fresh water holding conditions in juvenile Atlantic salmon with different life history phenotypes in (a) study one and (b) study 2. Mean \pm SEM values represented with positive and negative signs indicating a gain or loss to the fish, respectively.

	Study 1		Study 2	
	Smolts	Precocious parr	Smolts	Immature parr
<u>Freshwater Growth Period</u>				
SGR (% body mass day⁻¹)	0.82±0.04	0.47±0.07**	0.85±0.02	0.71±0.08
<u>24 h post-SW transfer</u>				
Plasma Cl⁻ (mM)	156±3	179±7***	138±1	148±3**
% Body Mass Loss	9.2±0.5	13.4±0.9**	3.7±1.6	6.3±1.4***
Gut Fluid TCO₂ (mM)	80±4	None present	+	+
% Fish with gut CaCO₃ precipitate	33	0	36.2	20

Table 3.1. Atlantic salmon specific growth rates (SGR) during the 2-month holding period in fresh water (FW), and osmoregulatory parameters measured following a 24 h seawater (SW) transfer. The latter includes plasma Cl⁻ (mM), % body mass loss, gut fluid TCO₂ (mM) and % fish with gut calcium carbonate precipitates (+ = gut fluid present but insufficient volume for TCO₂ analysis in experiment 2). In study 2, plasma osmolality (mM) and Na⁺ (mM) were also measured in smolts and immature parr, see text for details. Asterisk indicates a significant difference between osmoregulation variables measured (** p < 0.01, ***p < 0.001).

Chapter 4

Sodium uptake kinetics in smolt and immature parr Atlantic salmon (*Salmo salar*) living in social hierarchies.

ABSTRACT

Salmonid fish form linear social hierarchies with dominance and subordinate status influencing key physiological traits and ultimately life history selection. In the present study we examined the behaviour of undifferentiated juvenile Atlantic salmon (*Salmo salar*) parr during the smolting stage until fish became either smolts or remained as immature parr. The relationship between social status, growth rates, gill sodium uptake kinetics and plasma cortisol was investigated in social hierarchies and several patterns emerged. Firstly, the most dominant fish (rank 1) exhibited a higher specific growth rate (SGR) and lower affinity for gill sodium uptake (higher K_m), both recognised as advantageous for life at sea. The most subordinate fish (rank 5) showed poor growth rates and higher affinity for sodium, which may be socially mediated. Plasma cortisol, often used as an indicator of stress, was lower in the most dominant fish (rank 1) and intermediate status individuals (ranks 3-4) in the groups compared to subdominant fish (rank 2) and most subordinates (ranks 5-7). Subdominant fish also showed higher sodium uptake affinity which could have resulted from social stress.

Keywords: Sodium uptake; osmoregulation; social hierarchies; subordination; behaviour; salmon.

INTRODUCTION

Social status strongly influences welfare and life history pathway in fish that form hierarchies, where the physiology of dominants and subordinates can be extremely different (Metcalf, 1998; Gilmour et al., 2005). One critical physiological variable that is modified in relation to social position is the ability to regulate internal ionic concentrations in the face of life in a hypo-osmotic freshwater environment. The gills are the main organ for compensatory ion uptake in freshwater fish (Evans et al., 2005) and Sloman et al. (2002a) demonstrated that in paired trials of rainbow trout (*Oncorhynchus mykiss*) subordinates had higher branchial sodium uptake rates, possibly as an effect of stress. This is important as lower ranked salmonids appear to be more susceptible to toxicity from copper and silver because these metals largely enter the animal via some of the same branchial transporters as sodium (reviewed by Sloman, 2007). However, in complex salmonid hierarchies, subdominants (i.e. second ranked fish in a hierarchy) can become the primary recipients of aggression from dominants, and may also experience social stress like that of lower ranked subordinate fish, displaying similar physiological responses (Sloman et al., 2000a, 2008).

Many aspects of socially mediated alterations in osmoregulatory function remain poorly understood; especially the effect of social rank on the ion regulation of fish living in hierarchies formed by individuals with alternative life strategies. Within a cohort of juvenile Atlantic salmon (*Salmo salar*) parr distinct phenotypes can be found in freshwater streams prior to seaward migration, namely immature parr, precocious parr and smolts (Fleming, 1996). They must all compete for shelter and food usually within a linear hierarchical structure (Jenkins, 1969; Bachman, 1984).

The adoption of each of these life histories is thought to be strongly controlled from an early stage of their development by the relative social position within their social groups, which determines growth patterns (Metcalf et al., 1988, 1989). In this way fish that are socially dominant and grow fast surpassing a genetically determined size threshold before critical time periods (usually during their first summer and autumn at age 0+) become smolts or precocious parr respectively, whereas subordinate immature parr in the lower modal group will remain in the river at least until the following season (Thorpe et al., 1998).

Given the high plasticity in salmonid life histories the interactions between behaviour, body size, phenotype and ion regulation can be complex (Klementsen et al., 2003). For example the life history strategy for immature parr that will be freshwater residents for at least another year, could increase their potential for exposure to toxicants in a river environment, as compared to smolts that migrate to sea (Hutchings, 2002). On the other hand, during smolting salmonids are known to be especially sensitive to stress while they undertake critical osmoregulatory modifications (such as increased gill Na^+, K^+ -ATPase, mitochondria-rich cells, etc.) which enables their future acclimation to seawater (Carey and McCormick, 1998). Thus socially derived ionoregulatory disruptions at this stage may have a greater impact on their welfare. Sodium and chloride are the most important ions involved in gill osmoregulatory processes in teleosts, and gill uptake kinetics using radioisotopes has proven a useful technique in the characterisation of *in vivo* osmoregulatory mechanisms (e.g. McWilliams and Shephard, 1985; Potts et al., 1985; McWilliams, 1993) and socially mediated ionoregulatory disruptions (e.g. Sloman et al., 2002, 2003, 2004) in salmonid fish.

I hypothesised that dominant individuals would belong to the smolt phenotype and that social subordination would result in lower growth rates and poorer osmoregulatory ability. In the present study we therefore examined the social behaviour and sodium uptake kinetics of juvenile Atlantic salmon parr living in groups during the smolting stage. Individuals were kept in the same social groups until life strategy, in this case immature parr or smolt (no precocious parr occurred), was clearly established. The relationships between social rank, sodium ionoregulation, plasma cortisol, and growth relative to phenotype were investigated.

METHODS

Fish keeping

Juvenile Atlantic salmon, *Salmo salar*, were reared in a semi-natural pond in Dartmoor National Park (Devon, UK) and collected at the parr stage (body mass = 40.4 ± 1.8 g; mean \pm s.e.m) in March 2006. The fish were transported to the University of Exeter aquarium and held in two 60-l cylindrical, bottom-draining stock tanks supplied with Exeter dechlorinated tap water (flow rate 6 l min^{-1}) at ambient water temperature (range $12 - 16$ °C) and light/dark regime was continuously adjusted to latitude 52N conditions. The average Exeter water composition is described in detail in Scott & Wilson (2007) with the typical $[\text{Na}^+]$, the ion of interest, being $412 \pm 15 \text{ } \mu\text{mol l}^{-1}$. Fish were hand fed daily a 2 % ration (food mass/wet body mass) of commercial salmon pellets (Skretting Ltd., UK). In May 2006 fish were anaesthetized in a solution of tricaine methane sulphonate (MS222, 100 mg l^{-1} , buffered with NaHCO_3 followed by vigorous aeration to restore normal CO_2 levels) and PIT tagged, body mass (M) and fork length (FL) recorded and fish divided into 8

groups of 7 individuals and moved into stock tanks similar to those previously described. Recording of fish behaviour was not commenced until 2 weeks later when fish had fully settled and social hierarchies were well established (see behavioural observations section for details). At the start of the experiment phenotypic identity of fish was unknown as no parr had yet started showing external signs of smolting. All experiments were conducted in accordance with a UK Home Office Project Licence under the Animals (Scientific Procedures) Act 1986, and with permission from the University of Exeter Ethical Review Group.

Na⁺ uptake kinetics

In June 2006 unidirectional Na⁺ uptake kinetic analysis was conducted on each individual fish before any externally visible differentiation (i.e. smoltification) had occurred. A total of 52 individuals were examined for the kinetic work. The system used for conducting the Na⁺ flux experiments is similar to that described in more detail in Scott & Wilson (2007). Flux chambers were opaque to minimize disturbance and chambers holding various volumes were used (600 to 1000 ml) to provide an appropriate ratio of fish mass (g) to water volume (ml) to maximise analytical sensitivity (e.g. 1:10 to 1:30). Prior to the experiment fish were left without food for two days to stabilise ammonia excretion rates (Brett & Zala, 1975) and avoid faecal contamination of flux chambers and fish were transferred from the stock tank to the individual chambers 24 h hours before the fluxes began to allow fluxes to stabilise.

A temperature controlled (Grant RC 1400G chiller) 150 l volume re-circulating system was used to feed the flux chambers with fresh water. The pH of the reservoir water was maintained at pH 7.5 by addition of 0.1 mol l⁻¹ KOH using a pH controller (Hanna HI 8710E). At the start of each experiment, each flux chamber was

flushed thoroughly at $\sim 400 \text{ ml min}^{-1}$ for 5 minutes with Na^+ -free water. This medium was prepared using appropriate amounts of CaCO_3 and $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ salts added to deionized water to closely match the Ca^{2+} , Mg^{2+} and titratable alkalinity (TAlk) composition of Exeter tap water (Goss and Woods, 1990; Scott and Wilson, 2007). During the flushing period, excess water in each chamber exited via an overflow hole in a side wall, and flushing continued until Na^+ concentrations in the chambers fell below $20 \mu\text{M}$. Water flow was then stopped and the overflow hole was sealed with a bung.

To measure unidirectional Na^+ flux rates at different ambient ion concentrations, a stock solution of NaCl was added at 7 time intervals (A-G) to obtain Na^+ concentrations that nominally doubled at each increment (range = 10 to $2361 \mu\text{M}$). Water samples (20 ml) were collected at each Na^+ concentration increment from the flux chambers to verify that concentrations were within the desired range. The sodium radioisotope, ^{22}Na , was simultaneously added to chambers so that activity also increased with ambient $[\text{Na}^+]$. These solutions were pipetted through a small hole in the chamber lid to minimise disturbance of the fish. At each time interval the same procedure was repeated as follows. After waiting 10 minutes for the added Na^+ to completely mix with the chamber water, an initial 30 ml water sample was extracted using a syringe with a short (20 cm) piece of silicone tubing attached to the tip. After 0.5 h a final 30 ml water sample was collected using the same technique. This short time period was chosen to minimise backflux of the ^{22}Na isotope from the fish into the water (Maetz, 1956). Due to the large number of individuals, sodium kinetic sampling was divided into 4 trials conducted over a short time period of 10 days between the first and the last trial of this batch of fish.

At the end of the experiment fork length (cm) and body mass (g) of all fish was recorded and then returned to their original stock tanks. The initial and final activities (counts per minute; cpm) of ^{22}Na in each flux chamber at all time intervals were estimated from triplicate 2 ml water samples using a gamma counter (Packard Cobra B5002). Additional sample was used for flame photometry (Corning 410) to establish total $[\text{Na}^+]$ in the water.

Unidirectional Na^+ flux parameter estimation

Net Na^+ flux ($J_{\text{Na}}^{\text{net}}$) was calculated from the change in Na^+ concentration of the water in each chamber over 0.5 h using the following equation from Preest et al. (2005):

$$J_{\text{Na}}^{\text{net}} = V \times ([\text{Na}^+]_0 - [\text{Na}^+]_1) / (M \times T)$$

where V is the water volume in the chamber (l), $([\text{Na}^+]_0$ and $[\text{Na}^+]_1$ represent Na^+ concentrations ($\mu\text{mol l}^{-1}$) in the bath at the beginning and end of the flux period, respectively, M is the body mass of the fish (g), and T is the time of the flux period (h). The Na^+ influx was calculated from the disappearance of ^{22}Na isotope from the water and the average Na^+ concentrations of the water during the flux period using the following equation from Gonzalez and Dunson (1987):

$$J_{\text{Na}}^{\text{in}} = (\ln Q_{\text{out}0} - \ln Q_{\text{out}1}) \times Q_{\text{out}} / (M \times T)$$

where $Q_{\text{out}0}$ and $Q_{\text{out}1}$ are the total counts per minute in the flux chambers at the beginning and end of the flux period, respectively, Q_{out} is the average amount of Na^+ in the flux bath during the flux period, M is the mass of the fish (g), and T is the duration of the flux period (h). The resulting $J_{\text{Na}}^{\text{in}}$ values are given in $\text{nmol g}^{-1} \text{h}^{-1}$

In addition Na^+ efflux ($J_{\text{Na}}^{\text{out}}$) values were calculated as the difference between influx ($J_{\text{Na}}^{\text{in}}$) and net flux ($J_{\text{Na}}^{\text{net}}$). The plot of the relationship between $J_{\text{Na}}^{\text{in}}$ and the concentration outside the fish ($[\text{Na}]_{\text{ext}}$) generally results in a hyperbolic shape, which

can be examined using a modified form of the Michaelis-Menten equation for substrate reactions:

$$J_{\text{Na}}^{\text{in}} = (J_{\text{max}} \times [\text{Na}]_{\text{ext}}) / (K_{\text{m}} + [\text{Na}]_{\text{ext}})$$

where J_{max} yields the maximum influx rate and tends to be interpreted as the number of operational sites available (i.e. the number of functional transporters in the gill), while K_{m} calculated as the value of $[\text{Na}]_{\text{ext}}$ at $\frac{1}{2} J_{\text{max}}$, represents the index of the binding affinity of the operational site of transport. These values help characterise the type of transport system operating within an individual fish. Values of J_{max} and K_{m} were obtained from plots of sodium influx *versus* sodium concentration using Sigmaplot (Systat Software Inc), where each curve represents the sodium influx of an individual fish at each of 7 sodium concentrations.

Behavioural observations

Behavioural observations were made during 4 days prior and 4 days after each sodium uptake kinetic sampling, three times daily (08:00-08:20, 12:00-12:20, 17:00-17:20). These times were chosen to obtain a representative sample of salmon behaviour throughout the day as salmonid studies indicate that some fish, depending on their life strategy, may show distinct behavioural patterns during the diurnal cycle (Metcalf et al., 1998b). Fish behaviour was recorded using two static video cameras (Sony KIR-040) mounted on a frame approximately 80 cm above the tanks (each camera could record 4 tanks at a time). The footage was recorded on a digital video-recorder; the system was remotely operated to avoid any disturbance. Aggressive acts were counted with higher scoring behaviours being those that potentially can inflict a more severe injury to the recipient, with biting, chasing, charging, lateral display and receiving or avoiding aggression scores being 4, 3, 2, 1 and 0 points respectively (Gallardo &

Neira, 2005). At the end of the experiment behavioural scores were added for each fish, the most dominant being the individual with the highest score. Due to the difficulty in identifying the most subordinate individuals of each group, which exhibited very similar low levels of activity, data from fish ranked 5, 6 and 7 were pooled together, a practice used in multimember fish group behavioural studies (e.g. Sloman et al. 2008).

SGR measurement

Individual specific growth rates (SGR) were calculated as follows: $SGR = 100 (\ln M_2 - \ln M_1)/t$, where M_2 and M_1 are the body mass (g) at the end and start of the experiment respectively and t is the number of days between measurements.

Cortisol analysis

In August 2006 fish were terminally sampled after 24 h by anaesthetizing them in a solution of tricaine methane sulphonate (MS222, 250 mg l⁻¹). Blood was collected from the caudal vasculature with a heparinised needle and syringe, centrifuged at 13,000 g for 4 minutes and plasma aliquots were frozen in liquid nitrogen and stored at -80° C.

Plasma cortisol concentrations were measured with a commercial ELISA (DGR Diagnostics, Marburg, Germany) which has been used in other fish studies previously (e.g. Sloman et al., 2008). Cortisol concentrations were determined from plasma samples run in parallel to the standard curve and recovery of cortisol plasma from the spiked samples was $92 \pm 6 \%$.

Statistical analysis

Data used for parametric analysis was examined using a Kolmogorov-Smirnov tests for normality (Zar, 1996), and homogeneity of variance was evaluated using Levene's test (Schultz, 1983). One-way repeated measures (RM) ANOVA or a non-parametric equivalent (Friedman repeated measures test) were used for examination of social ranks differences in body size, cortisol and sodium uptake data with rank as the repeated measure within each tank, followed by SNK *post hoc* comparisons. Statistical tests were conducted using Sigmastat (SigmaStat 3.0, SPSS Inc., Chicago, IL, USA). Results are presented as means \pm standard error of the mean (SEM).

RESULTS

Initial size, social rank and SGR

Statistical analysis using a Friedman nonparametric repeated measures test indicated that fish of high social rank had a higher body mass at the start of the experiment ($\chi^2 = 23.572$, d.f. = 4, $n = 52$, $p < 0.001$) (Figure 4.1). Fish of different social ranks also showed significantly different growth rates (Friedman nonparametric repeated measures test, $\chi^2 = 12.3$, d.f. = 4, $n = 52$, $p = 0.01$), with the most dominant individual having a higher SGR than the rest of the individuals (Figure 4.2).

Sodium uptake kinetics and plasma ions

Comparison of the K_m across social ranks using a one-way RM ANOVA showed significant differences ($F_{4,28} = 12.061$, $p < 0.001$) where the most dominant fish within groups showed a significantly higher mean K_m as indicated by *post hoc* examination compared to the lower ranked individuals except for fish ranked 3 (SNK, $p =$

0.252) (Figure 4.3.). When J_{\max} or the maximum rate of sodium uptake was compared against social position no significant differences were detected between individuals in the tank groups (one-way RM ANOVA, $F_{4,28} = 0.323$, $p = 0.811$).

Plasma cortisol and social rank

Cortisol concentrations in fish of different social ranks (1-4) revealed significantly lower cortisol compared to fish from the most subordinate rank (one-way RM ANOVA, $F_{4,28} = 24.516$, $p < 0.001$) (Figure 4.4). *Post hoc* SNK showed that rank 1 salmon had a lower cortisol in the plasma than rank 2 (rank1 v. rank2, $p = 0.035$) and rank 5 (rank1 v. rank 5, $p < 0.001$). Similarly rank 3 fish had a lower mean cortisol than rank 2 (rank2 v. rank 3, $p = 0.035$) and rank 5 (rank3 v. rank5, $p < 0.001$).

DISCUSSION

The present experiment reports the occurrence of differences in osmoregulatory patterns, namely Na^+ uptake kinetics, and plasma cortisol which are related to social status. Importantly, these physiological traits did not follow a simple linear relationship with fish rank within the multi-member hierarchy. This is different from typical dyad studies where dominant and subordinate fish show clearly contrasting physiological responses (Gilmour et al., 2005). The results here suggest that being most dominant (rank 1), under the tested laboratory tank conditions, can be beneficial in terms of growth, osmoregulation and cortisol levels. However, being rank 2 fish may not follow this trend and may exhibit physiological characteristics often seen in more subordinate fish, whereas intermediate ranked fish (i.e. rank 3) may avoid these costs.

Dominance and uptake kinetics

The sodium uptake kinetics of the most dominant individual in the group and rank 3 fish showed a significantly higher K_m , or lower affinity for Na^+ , relative to subordinates which could be indicative of an appropriate preparation for future migration to seawater. In salmonids reorganisations of gill ionoregulatory processes appear weeks in advance of migration (Nielsen et al., 2004), whereby pre-smolts switch from the Na^+/K^+ -ATPase isoform- $\alpha 1a$ (freshwater responsive form) to the $\alpha 1b$ (sea water responsive form) whilst still in freshwater (Richards et al., 2003). Gill Na^+/K^+ -ATPase (or sodium pump) K_m values for Na^+ are lower in fresh water-acclimated than in sea water-acclimated rainbow trout (Pagliarani et al., 1991). Because fish are hypo-osmotic to sea water it would be advantageous to maintain a low sodium intake to prevent high plasma osmolality, through reduced uptake affinity and/or higher extrusion rates of salts. Even in the absence of smoltification social subordination leads to a significant increase in sodium uptake rates in salmonids secondary to the stress-induced changes in branchial permeability and increased sodium loss (Sloman et al., 2004). The higher affinity for sodium observed in subdominant (rank 2) and most subordinate (rank 5) animals may be related to the fact that subordinates tend to require a higher sodium influx to counteract the higher sodium efflux caused by social stress (Sloman et al., 2002; 2003). Plasma cortisol, which is one of the most used indicators of stress, was higher in fish ranked 2 and rank 5 fish and may be implicated in social ionoregulatory disruptions. Subordinate fish both in dyads and larger groups tend to show higher plasma cortisol (Ejike and Schreck, 1980; Pottinger and Pickering, 1992). Osmoregulatory challenges are controlled by numerous hormones (e.g. prolactin, growth hormone, vasotocin, insuline-like growth factor) and their interactions can be complex (Mancera and

McCormick, 2007). For instance, there is also evidence for the reverse role of cortisol in the maintenance of transport proteins involved in ion uptake (McCormick, 2001) and seasonal elevation in the key sea water acclimating proteins such as Na^+, K^+ -ATPase often occurs prior to any measurable increase in cortisol. Thus the influence of stress-induced cortisol elevation on ion regulation in subordinate fish warrants careful investigation.

Examination of J_{max} or maximum capacity for sodium uptake was not different between social ranks, suggesting that social position did not modify (or if it did it was equally across ranks) the number of operational sites for sodium uptake. Thus if elevation in metabolic rate due to subordination (Sloman et al., 2000) and its associated increase in ventilation rate (Millidine et al., 2008) result in a greater loss of branchial Na^+ through enhanced paracellular leakage in low ranked fish this process may be counteracted by increasing affinity for this ion (i.e. step up of active proteins for uptake, rather than the number of cells or operational sites (i.e. MRC proliferation). Other factors such as diet may also influence ion uptake requirements, as lower food intake by subordinates should reduce dietary intake of sodium leading to a higher gill uptake to balance this deficiency (Sloman and Wilson, 2006).

Physiological costs of subdominance

The second ranked or subdominant individual showed higher sodium uptake affinity compared to most dominant and intermediate ranked fish which could be mediated by stress induction as plasma cortisol showed significant differences between the dominant and subdominant individuals, but not fish ranked 3 and 4. Recent work by Sneddon et al. (2006) has shown that subdominant (i.e. rank 2) three-spined sticklebacks (*Gasterosteus aculeatus*) were the receivers of most aggression from

dominants. In salmonid social hierarchies fish with high status often aim agonistic actions towards direct contenders that may pose a threat to their rank (Nicieza and Metcalfe, 1997; McClean et al., 2001). For instance, work by Sloman et al. (2000) using rainbow trout living in groups showed that the 2nd ranked fish adopted a more active feeding approach than lower ranked fish resulting in more attacks from the dominant. As a result the subdominant fish was the worse off rank in physiological terms, measured as decreases in condition factor and the need for high sodium uptake rates. Other workers have also reported negative physiological effects resulting from subdominant status in fish hierarchies (Winberg et al., 1991; Fernandes and Volpato, 1993) including in the wild (Sloman et al., 2008). Previous work in rainbow trout has shown that individuals in the middle of the hierarchy exhibit the lowest interrenal activity, an indicator of stress (Noakes & Leatherland, 1977), and the highest levels of immunocompetence (Thompson, 1993), possibly reflecting the benefits of having a relative high rank but without the high costs associated with directly contending for dominance status.

Surprisingly despite the high levels of aggression received, 2nd ranked fish did not appear to adopt a more passive approach which could have helped reduce injury chances and energy expenditure. In this study the high cost-high energy strategy was profitable in that the subdominant fish was able to maintain its size and show a positive SGR. This differed from other studies where subdominant fish showed a low growth rate or condition (Sloman et al., 2000; Sneddon et al., 2006). This discrepancy perhaps could be explained by differences in experimental methodology. In our study food was distributed by hand in round shaped tanks, whereas an automatic feeder located at the upstream section of each tanks was used in the work by Sloman et al. (2000). Food scattering makes food monopolisation more difficult compared to point

source feeding methods that tend to increase the nutritional intake and size divergence between the dominant and the rest of the group (Ryer and Olla, 1996). Second, fish from the same cohort were chosen randomly but not size-matched, meaning that differences in size already existed between individuals. Initial size was significantly correlated with dominance status in the groups in our experiment, size being a strong determinant of high social rank in numerous salmonid fish studies (e.g. Noakes, 1980; Abbot et al., 1985; McCarthy *et al.*, 1992; Adams et al., 1998, 2000). This may have conferred the subdominant individual an advantage to gain preferential access to food over other fish except the dominant. Size-selective mortality processes have been suggested in smolts (Mathews and Ishida, 1989; Henderson and Cass, 1991). Similarly, Holtby et al. (1990) reported that large size conferred a significant advantage in years of poor marine conditions. These benefits may be enough to offset the increased chance of injury (usually non-lethal) from dominants and poorer sodium regulation at sea water entry observed in confined social groups. Furthermore, under environmental disturbances that are common in the wild (i.e. flooding, drought) fish hierarchies may reorganise (Sloman et al., 2001; Sneddon et al., 2006), and subdominant fish have the highest chances of obtaining dominant status when this social position is altered. Dominance assessment methods such as serial removal appear to prove this point as once the most dominant fish is removed, the previously second most dominant fish often acquires the vacant highest social position (e.g. Metcalfe et al., 1989; Huntingford and García Leaniz, 1997). Thus, it may pay to remain in contention for the dominant status despite negative physiological trade-offs, such as ionoregulatory alterations, associated with this position.

Subordination and physiological correlates

In this subordinate fish had initially a lower size than any other of the higher ranked fish (rank1-4) and final SGR. Smaller smolts often are more susceptible to predation (Reitan et al. 1987; Kennedy and Greer 1988; Feltham 1990), disease (Cusack 1986; Jansen and Bakke 1993*a*, 1993*b*), and ionoregulatory fluctuations at sea water entry (Bjerknes et al. 1992; Thorpe and Metcalfe 1998; Berril et al., 2006) than larger counterparts. Furthermore, even after prolonged periods at sea size differences between large and small smolts are maintained (Nicieza and Braña 1993), large smolts reach maturity in a faster time and show higher survival (Lundqvist et al. 1994; Mangel, 1996; Friendland et al., 1992, 1997). In addition this study showed an unfavourable K_m for seawater preparation, which has never been reported before, and high cortisol concentrations which is in agreement with previous work showing negative effects of subordination on the physiology of salmonid fish (Sloman and Armstrong, 2002). A growing body of evidence points towards an important role of social rank mediating physiological differences in dominant and subordinate fish including ionoregulatory status (reviewed by Gilmour et al., 2005; Johnsson et al., 2006), although many aspects of the mechanisms driving this variation in osmoregulation remain poorly understood. In this study we present evidence supporting differences in ionoregulatory abilities in complex social hierarchies of juvenile Atlantic salmon and the cost incurred by subdominants and highly subordinate members in terms of higher Na^+ uptake affinity which may be a response to increased Na^+ loss by gill paracellular leakage in stressed fish and may reduce ionoregulatory acclimation during entry to sea and present cortisol data which shows a similar pattern in relation to social status.

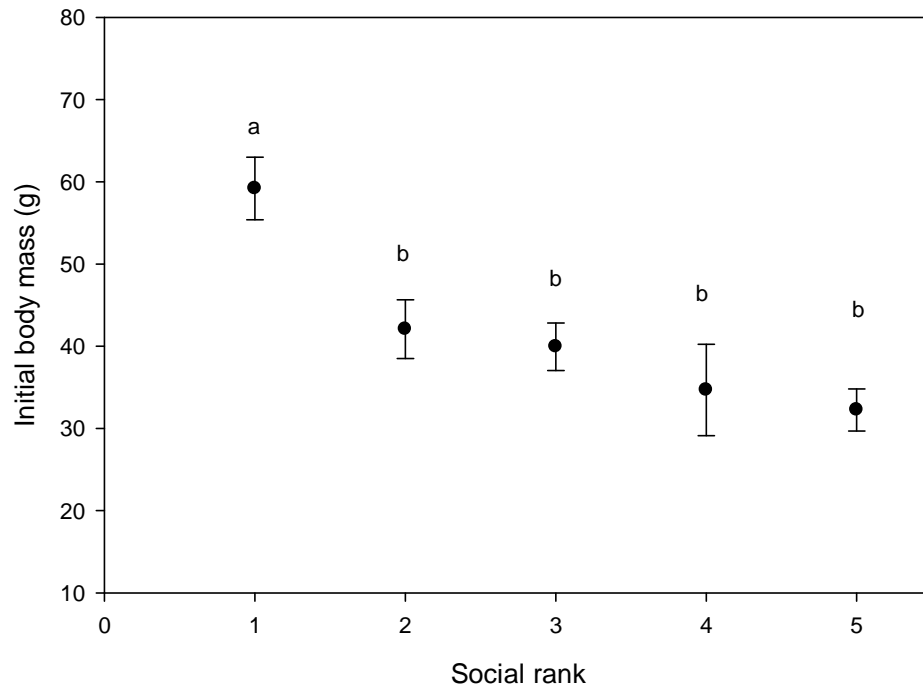


Figure 4.1. Relationship between social rank and initial body mass (g) ($n = 8$ individuals per rank) of juvenile Atlantic salmon. Statistical differences are indicated by letters ($p < 0.001$), ranks sharing the same letter are not significantly different.

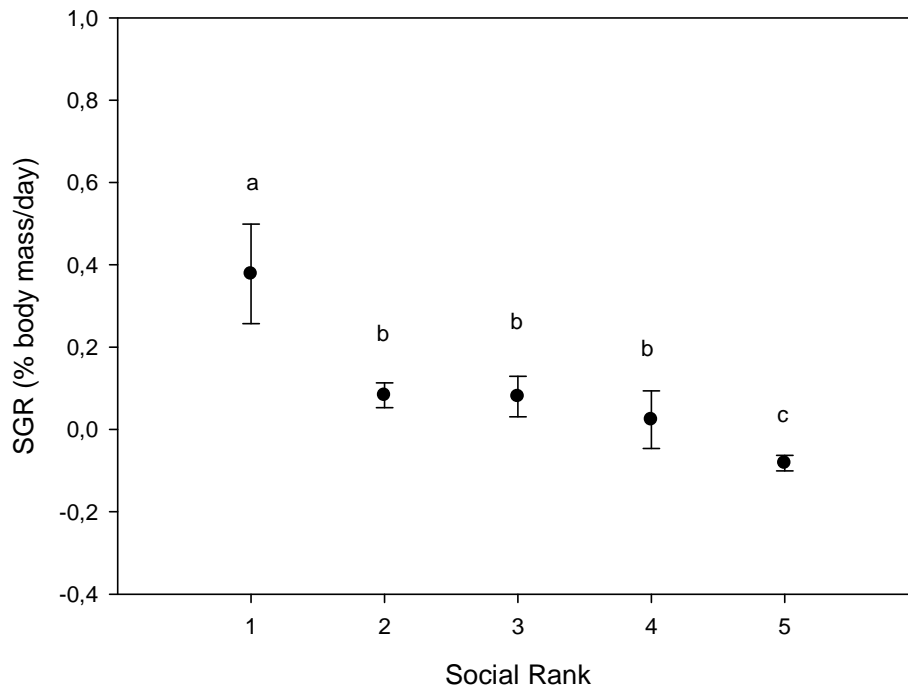


Figure 4.2. Specific growth rate (% body mass day⁻¹) with social rank (n = 8 per rank) of juvenile Atlantic salmon. Data shown are means (\pm SEM). Statistical differences ($p < 0.01$) are indicated by letters, ranks sharing the same letter are not significantly different from one another.

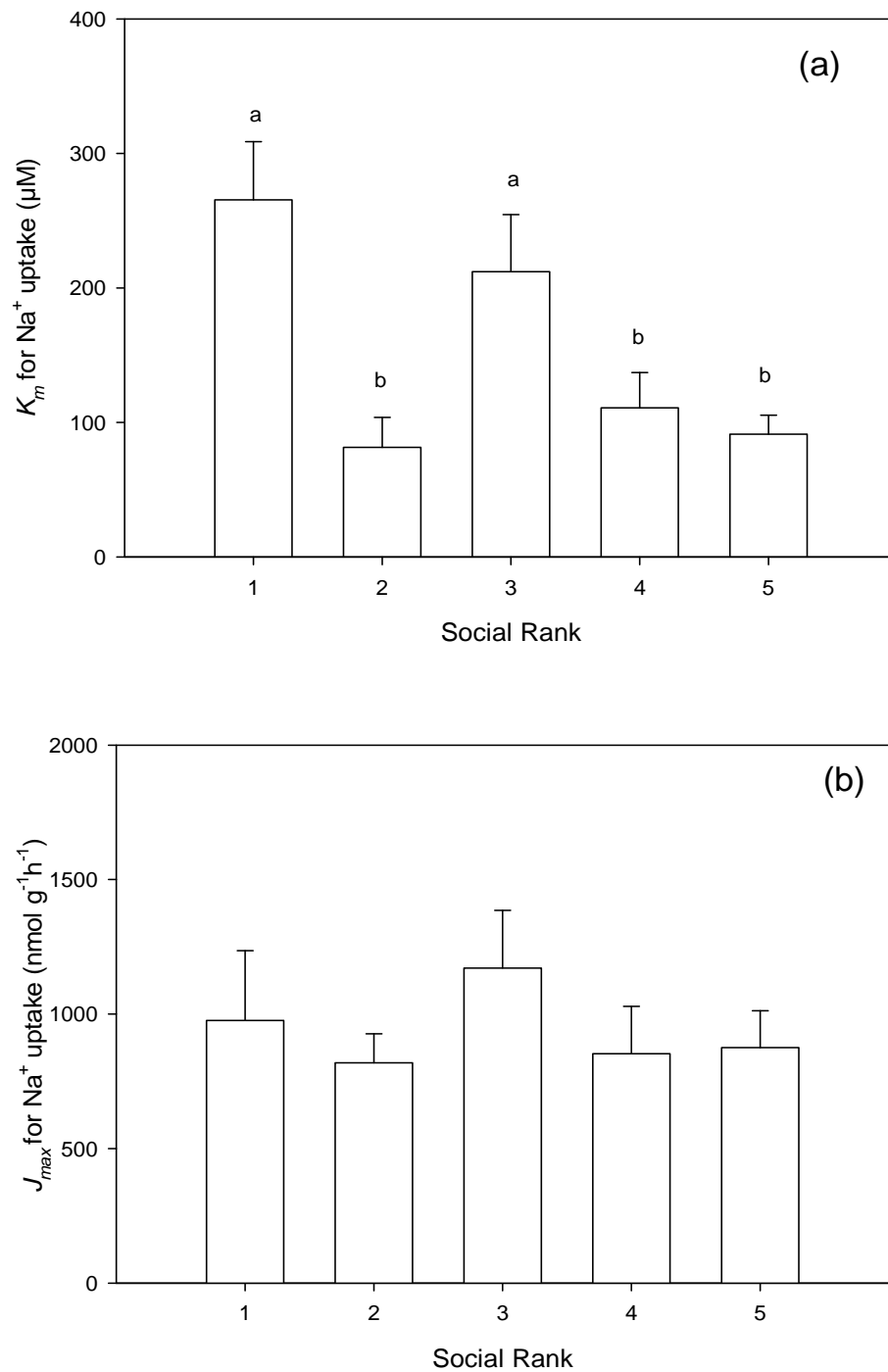


Figure 4.3. Na⁺ unidirectional uptake kinetics for (a) K_m (μM) and (b) J_{max} (nmol g⁻¹h⁻¹) from Atlantic salmon with different social ranks (n = 8 fish per rank). Data shown are means (±SEM). NS differences in J_{max} .

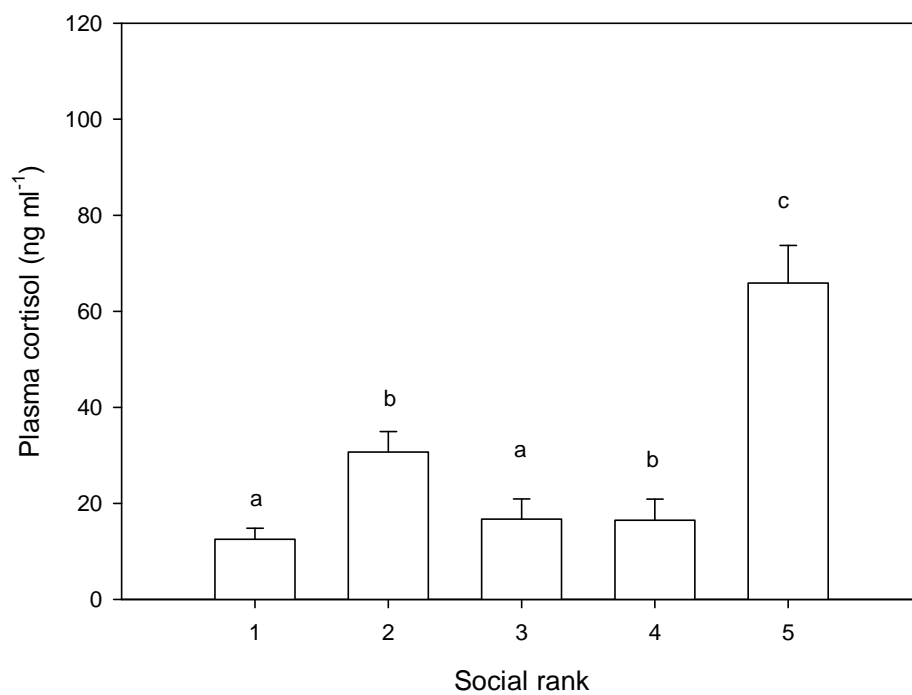


Figure 4.4. - Relationship between social rank and plasma cortisol concentration (ng ml⁻¹) in juvenile Atlantic salmon (n = 8 fish per rank). Data shown are means (\pm SEM). Statistical differences ($p < 0.001$) are indicated by letters, tanks sharing the same letter are not significantly different from one another.

Chapter 5

Serotonergic activity in juvenile Atlantic salmon (*Salmo salar*) social hierarchies with alternative life histories and size asymmetries

ABSTRACT

The effects of size asymmetries and social status on the physiology of juvenile salmon (*Salmo salar*) were studied in social hierarchies in stream tanks during 5 months at a time of developmental conversion. Tanks held size-mixed groups of 6 fish, 3 belonging to the upper modal group (UMG) and the other 3 to the lower modal group (LMG) of the cohort. Behaviour was analysed and related to physiological variables including growth patterns, brain serotonergic activity, plasma cortisol and fin damage. During the experiment initially undifferentiated Atlantic salmon developed one of the three freshwater life-history strategies: immature parr, precocious parr or smolts. Individuals that started with a larger size (UMG) held higher social ranks in the hierarchies, while fish from the LMG occupied the lower subordinate positions. Trajectories of the specific growth rate (SGR) between UMG and LMG were similar, with no increase in size difference (i.e. growth depensation) between large and small fish over time. Fish in the UMG developed primarily into smolts (70%), followed by precocious parr (20%) and immature parr (10%), whereas LMG individuals remained as immature parr (60%) or became precocious parr (40%). Brain serotonergic activity, measured as the ratio between the metabolite 5-hydroxyindoleacetic acid (5-HIAA) and its precursor 5-hydroxytryptamine (5-HT, serotonin), and typically higher in

subordinate individuals, was measured in the brain stem, telencephalon, optic tectum and hypothalamus. Only the most dominant (i.e. rank 1) and most subordinate (i.e. rank 6) fish in the hierarchy exhibited significantly different brain stem 5-HIAA/5-HT ratios. No significant differences were detected between 5-HT, 5-HIAA and 5-HIAA/5-HT in fish grouped as UMG and LMG. Plasma cortisol concentrations were also similar between modal groups. Proportion of overt attacks received and fin damage, usually indicators of received aggressive behaviour, were prevalent among the UMG individuals. Fish with high fin erosion had higher plasma cortisol than injury-free fish. Competition through aggression for dominance status and food monopolisation may escalate among high ranked members leading to reduced physiological benefits such as higher physical injury and serotonergic profiles similar to those of lower ranked conspecifics. As social groups become more complex, (or perhaps under certain social structures such as strong size bimodality) the rewards of being dominant may not be as marked as in dyadic encounters whilst subordinates may experience social stress to a lesser degree.

Keywords: social hierarchies; social behaviour; growth rate; brain neurotransmitters; Atlantic salmon

INTRODUCTION

Many teleosts organise into social groups where relative differences in body size and social status between members shape competitive ability and life history selection (Humphries, 1999; Buston, 2003). Understanding how social interactions influence

growth dynamics over time is a complex subject in fish as they exhibit indeterminate growth that can lead to periods of rapid compensatory growth if limiting environmental conditions cease (Nicieza and Metcalfe, 1997; Metcalfe and Monaghan, 2001). Among teleosts the Salmonidae family is one of the most extensively studied in terms of social behaviour and what effect this has on growth and adoption of reproductive strategies (reviewed by Hutchings and Jones, 1998; Metcalfe, 1998).

Salmon organise into linear social hierarchies in the wild and the lab (Jenkins, 1969; Bachman *et al.*, 1984; Metcalfe *et al.*, 1988; Winberg *et al.*, 1991). These are established through the outcome of successive dyadic interactions (Chase *et al.*, 2003) and stable dominance hierarchies are thought to benefit all individuals in the group by reducing aggressive encounters between members (Krebs and Davies, 1997). Salmonid fish with preferential access to best feeding or mating grounds are typically regarded as dominant (Metcalfe *et al.*, 1989; McCarthy *et al.*, 1992; Winberg *et al.*, 1993) and social dominance is often associated with larger relative size (Kallenberg, 1958; Noakes, 1980; Metcalfe *et al.*, 1995). However, the exact nature of the relationship between dominance and size is not clearly understood, as greater size may be a consequence of status, rather than its cause (Huntingford *et al.*, 1990; Adams and Huntingford, 1996; Cutts *et al.*, 2001). Knowledge of the relationship between size, behaviour and phenotypic development is important as juvenile salmon growth in cohorts under benign feeding conditions typically show a bimodal size distribution with an upper modal (UMG) and a lower modal (LMG) group (Thorpe, 1977; Bailey *et al.*, 1980; Bagliniere and Maisse 1985) and developmental pathways adopted (i.e. immature parr, precocious parr and smolt) appear to depend upon

reaching a particular size or condition threshold (Kristinsson et al., 1985; Metcalfe et al., 1989; Rowe and Thorpe, 1990a).

Most current knowledge on salmonid social behaviour derives from laboratory based studies using size matched designs (reviewed by Sloman and Armstrong, 2002); however the nature of the social structure in groups with dissimilar size composition may be quite different. For example, the incidence of overt acts of aggression in teleosts is reduced when size differences are large (Symons, 1968; Wankowski & Thorpe, 1979), as subordinates reduce food intake and compete less in the presence of larger dominant fish (Abbot et al., 1985; Sneddon et al., 2006). In addition, growth depensation may result when the initial size difference between the dominant and the subordinate fish increases over time due to chronic social stress in low rank fish as a result of aggression and limited access to resources (Jobling, 1985). Studies using fish groups with size inequalities have primarily focused on growth patterns (Jobling and Wandsvik, 1983; Jobling and Reinsnes, 1986; Wallace and Kolbeinshavn, 1988). Contrasting results have emerged from some of these studies, some showing size disparity to favour overall growth among the group members (e.g. Seppa et al., 1999; Adams et al., 2000; Lahti and Lower, 2000) while others have not (e.g. Jobling and Reinsnes, 1987; Baardvik and Jobling, 1990).

Fewer studies have focused on neuroendocrine physiological mechanisms mediating social environment in fish groups with important size asymmetries. Brain serotonergic turnover is an important physiological parameter, correlated with plasma cortisol levels, which has been associated with social dominance in salmonids (Winberg and Nilsson, 1993; Winberg and Lepage, 1998; Øverli et al., 1999). Brain neurotransmitters such as serotonin are thought to play a key role in the integration of the stress response in teleosts via the hypothalamus-pituitary-interrenal (HPI) axis

(reviewed by Winberg et al., 1997; Sørensen et al., 2007), and have been linked to appetite and body weight regulation (Winberg et al., 1992; Alänarä et al., 1998; Winberg et al., 2001), as well as inhibition of aggressive behaviour (Summers and Winberg, 2006). Chronically subordinate fish tend to show higher brain serotonergic activity compared to dominant fish (Winberg et al., 1991; Winberg and Lepage, 1998; Höglund et al., 2000; Øverli et al., 2004). Similarly, elevated plasma cortisol concentrations are characteristic of fish subjected to social stress (Sloman and Armstrong, 2002). In addition, external indicators of welfare and social rank, such as fin damage can provide valuable information on agonistic behaviour within fish groups (Moutou *et al.*, 1998; Latremouille, 2003; Hoyle et al., 2007; Ellis, 2008).

In the present study we examined the relationship between size asymmetries, behaviour and life history selection in groups of juvenile Atlantic salmon, particularly focusing on its relationship to brain neurotransmitters and plasma cortisol. Based on typical dyadic social-subordinate results, we hypothesised that fish with a high relative size in the group (i.e UMG individuals) should be socially dominant over LMG fish, leading to greater specific growth rates and lower brain serotonergic activity compared to smaller individuals. In addition UMG fish should predominantly adopt the smolt or precocious parr life strategies which require a minimum size threshold and is characteristic of faster growing fish.

METHOD

Fish husbandry

Atlantic salmon originating from a small brood stock of wild salmon from the river Delphi (Ireland) and hatched in February 2007 at Sparsholt College facilities (UK) were reared in the lab at Exeter University (UK) from March 2007 onwards shortly after first feeding. Approximately 200 alevins were held in a stock tank (volume 20 l) continuously supplied with aerated dechlorinated Exeter tap water at ambient temperature (14 ± 1.6 °C). Tanks contained gravel for shelter and low light was provided by covering the tank with a semi-opaque net. Fish were hand fed 5 times a day on commercial fry food (Skretting Ltd., UK). In June 2007 fry were moved into larger holding tanks (volume 60 l) and kept under a 12h:12h L:D photoperiod. Fish were hand fed twice a day (2% body wt day⁻¹). During the month prior to the experiment the feeding method was changed to using automatic fish feeders (Fish Mate F14) delivering the same quantity of dry pellets as before at 4 h intervals to adjust fish to this method of feeding.

Experimental protocol

Two weeks prior to the experiment 90 fish were anaesthetized in a solution of tricaine methane sulphonate (MS222; 100 mg l⁻¹, buffered with NaHCO₃ and vigorously aerated to restore normal CO₂ levels), PIT tagged for identification, and fork length (L_F) and body mass (M) recorded ($L_F = 11.6 \pm 0.3$ cm; $M = 20.1 \pm 0.4$ g; mean \pm s.e.m). Size frequency distribution of L_F was plotted, which yielded a distinct bimodal distribution with fish falling into an UMG and a LMG. Fish that were not clearly in

either the large or the small size categories were not selected for experiments. Three fish from each size group were randomly allocated to one of 10 replicate stream tank raceways (flow rate = 6 l min⁻¹; dimensions = 150 x 58 x 30 cm), providing in terms of relative size a combination of three large and three small juvenile parr (n = 6 fish per tank), all still with an undifferentiated external appearance (i.e. no smolting had begun yet). A week before the start of the experiment individuals were anaesthetised (as described above) and visually marked using a small grey sequin sutured to the skin. Preliminary experiments had shown that sequin tagged fish recovered quickly from marking and the tag had no measurable effect on fish social behaviour or growth. Similar methods using small tags attached with a suture thread have been successfully used in other juvenile salmon studies (e.g. Metcalfe et al., 1989). Sequins were used as tags because they provided a clear and large enough visual marker (sequin radius = 2 mm) for fish identification as opposed to other marking methods such as visual implant elastomers which would have required large amounts of dye to be injected subcutaneously for effective marking.

These social networks with size asymmetries were reared whilst behavioural interactions were recorded for a period of more than 5 months (3rd May-28th October 2008). Unidirectional water current was maintained in each raceway using water pumps (Eheim 1250) fitted with submerged custom-built spray bars immediately upstream of a grid at the end of each raceway. At the top of each raceway a fish feeder was placed which delivered food 4 times a day (6 am, 12 am, 6 pm, 12 pm) amounting to 2 % wt day⁻¹. The food would drop at the top of the raceway and drift towards the back end following the current direction. Fish occupying the front section of the tank had preferential access to food pellets.

Fish behaviour was recorded using four static video cameras (Sony KIR-040) placed approximately 140 cm above the tanks, which were linked to a digital video-recorder; the system was remotely operated to avoid any disturbance. Aggressive acts were counted during two daily observations at 08:00 and 12:00 each lasting 10 minutes. These observations were recorded on two different days of the week, giving a total of 68 observations per raceway. The following agonistic acts were recorded, with higher scoring behaviours being those that potentially can inflict a more severe injury to the recipient, with biting, chasing, charging, lateral display and receiving or avoiding aggression scoring 4,3,2,1 and 0 points respectively (Gallardo & Neira, 2005). In addition the closeness of each individual in relation to the defendable fixed food source, another indicator of status, was recorded at 0 min, 5 min and 10 min of each observation with fish holding position at the front third (closest to feeder) of the stream tank scoring 3 points, middle area 2 points and back third (further away from food) 1 point. Social rank was calculated by averaging the scores of these behaviours for each fish. Dorsal fin damage was also examined at the end of the experimental period and recorded following the protocol of Hoyle et al. (2007), which divides fin condition into 5 categories, representing progressively increasing degrees of fin erosion (i.e. category 1 no damage to category 5 very severe fin damage). Number of bite events, the most overt aggressive action between fish and potential cause of fin damage, performed and received by both UMG and LMG fish were counted to investigate behavioural patterns of agonistic behaviour in modal groups.

Blood and brain monoamine sampling

At the end of the experimental period fish from each stream tank were netted simultaneously and terminated with an overdose of buffered anaesthetic (MS222, 250

mg l⁻¹), measured and weighed. Blood samples were drawn from the caudal vasculature into heparinised needles and syringes, and placed on ice. Blood samples were quickly taken and plasma was separated by centrifugation and stored at -80 °C for later analysis of hormone concentrations. The fish were decapitated and the brain rapidly dissected (within 2 min) and separated into four parts: telencephalon (excluding the olfactory bulbs), hypothalamus (excluding the pituitary gland), optic tectum and brain stem. Brain parts were immediately frozen in liquid nitrogen and stored at -80° C for later analysis of monoamines.

Brain monoamine analysis

Brain tissue was weighed and homogenized in 4 % (w/v) ice-cold perchloric acid containing 20 ng ml⁻¹ of 3,4-dihydrobenzilamine (DHBA) as an internal standard, using a Potter-Elvehjem homogenizer. Samples were then centrifuged at 27,000 g for 10 min at 4 °C, and the supernatants used for analyses. Monoamine and monoamine metabolites were determined using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Øverli et al. (1999). Serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were examined. As a measure of serotonergic activity, the 5-HIAA/5HT ratio was calculated for each individual. The monoamines were quantified using standard solutions and corrected for recovery of the internal standard using HPLC software (CSW, Data Apex, the Czech Republic).

Cortisol analysis

Plasma cortisol concentrations were measured with a commercial ELISA (DGR Diagnostics, Marburg, Germany) as used previously in fish studies (e.g. Sloman et al.,

2008). Cortisol concentrations were determined from plasma samples run in parallel to the standard curve and recovery of cortisol plasma from spiked samples was $91 \pm 3\%$.

Specific growth rate and condition index

Individual specific growth rates (SGR) were calculated as follows:

$$\text{SGR} = 100 (\ln M_2 - \ln M_1)/t,$$

where M_2 and M_1 are the body mass (g) at the end and start of the experiment respectively and t is the number of days between measurements. Somatic measurements were taken 4 times in total (at the start on 3rd May, and thereafter on 10th July, 2nd September, and 28th October 2008).

Condition index (K) was calculated as:

$$K = 100M_2L_F^{-3}$$

where M_2 is final body mass (g) and L_F is fork length (cm).

Statistical analysis

Data normality was examined using a Kolmogorov-Smirnov test for normality (Zar, 1996), and data transformed if appropriate. Homogeneity of variance was evaluated using Levene's test (Schultz, 1983). Repeated measures ANOVA or non-parametric equivalent (Friedman test) were used for social rank comparisons including brain monoamine activity (followed by SNK *post hoc* tests) and cortisol data with rank as the repeated measure within each tank. Ranked data including fin erosion and aggressive behaviour on pooled UMG and LMG fish were compared using a Mann-Whitney U-test. Statistical tests were conducted using Sigmastat (SigmaStat 3.0, SPSS Inc., Chicago, IL, USA). Whenever possible the data was transformed to

achieve a normal distribution. Results are presented as means \pm standard error of the mean (SEM).

RESULTS

Social status, SGR and life history

Fish initially belonging to the UMG obtained superior social ranks (i.e. ranks 1-3) compared to fish from the LMG in the stream tank groups (Mann-Whitney U-test, $Z = 426$, $n = 56$, $p < 0.001$). The UMG maintained their size gap relative to LMG fish and still held a significantly larger fork length by the end of the experiment (t-test, $t_{54} = 9.82$, $p < 0.001$). Only 5% of LMG individuals showed a marked change in relative size and ended up as one of the 3 largest fish of their tank. Despite this no signs of growth depensation (i.e. size differences increasing over time between dominants and subordinates) were detected as UMG and LMG fish showed very similar final SGR both in body mass (Mann-Whitney U-test, $Z = 725.5$, $n = 56$, $p = 0.238$) and fork length (Mann-Whitney U-test, $Z = 750$, $n = 56$, $p = 0.436$). At the start of the experiment shortly after the first month in July 2008 UMG fish were showing a significantly higher body mass SGR (Mann-Whitney U-test, $Z = 1188$, $n = 60$, $p < 0.001$) but after this period SGR differences were not present in the following two sampling times in September (t-test, $t_{58} = -1.307$, $p = 0.196$) and October 2008 (Mann-Whitney U-test, $Z = 863$, $n = 56$, $p = 0.291$) (Figure 5.2.). Precocious parr from the UMG had a higher K than smolts (Mann-Whitney U-test, $Z = 111.5$, $n = 26$, $p = 0.036$), whereas LMG precocious parr showed a K similar to the immature parr in the same size group (Mann-Whitney U-test, $Z = 172.5$, $n = 28$, $p = 0.556$). By the end of the experiment fish phenotypic outcome in the UMG was made up by the smolt

strategy (n = 19), followed by the precocious parr (n = 7) and immature parr (n = 2), while the LMG class consisted of immature parr (n = 17) and also small precocious parr (n = 11).

Brain serotonergic activity and plasma cortisol

Comparison of brain stem neurotransmitter activity, measured as 5-HIAA/5-HT, in fish of different social rank revealed a significant difference (One-way ANOVA repeated measures test $F_{5,53} = 2.668$, $p = 0.036$), with post-hoc analysis showing that a statistical difference only existed between rank 1 and rank 6 fish (SNK, $p = 0.015$). Data for 5-HIAA/5-HT activity with social rank showed no significant differences in telencephalon (Friedman nonparametric repeated measures test; $\chi^2 = 8.175$, d.f. = 2, n = 54, $p = 0.147$), optic tectum (One-way ANOVA repeated measures test $F_{5,53} = 1.586$, $p = 0.186$), and hypothalamus (Friedman nonparametric repeated measures test; $\chi^2 = 3.349$, d.f. = 2, n = 54, $p = 0.646$). Brain serotonergic activity in juvenile salmonids of different modal groups was examined by comparing 5-HT, 5-HIAA and 5-HIAA/5HT between combined data within the UMG and LMG fish, again showing no differences in any of the brain parts (Table 5.1.). Analysis of data using social status revealed no differences in circulating cortisol concentration between ranks (Friedman nonparametric repeated measures test; $\chi^2 = 5.414$, d.f. = 2, n = 56, $p = 0.367$). Plasma cortisol for pooled data on concentrations of fish belonging to the UMG compared to the LMG were similar (Mann-Whitney U-test, $Z = 856$, n = 56, $p = 0.346$). Comparison of plasma cortisol between UMG with high (score > 2) and UMG with low fin damage (score ≤ 2) revealed that fish with higher fin erosion had higher cortisol levels (Mann-Whitney U-test, $Z = 216$, n = 28, $p < 0.001$).

Fin damage

Incidence of serious fin erosion, commonly used a sign of social aggression, was significantly higher among fish from the UMG in the social hierarchies (Mann-Whitney U-test, $Z = 934$, $n = 56$, $p = 0.006$). Behavioural observations confirmed this pattern whereby comparison of overall proportion of severe attacks (e.g. behavioural score > 2) performed (Mann-Whitney U-test, $Z = 155$, $n = 20$, $p < 0.001$) and received (Mann-Whitney U-test, $Z = 154$, $n = 20$, $p < 0.001$) in each of the stream tanks was highly polarized towards the UMG (Fig 5.4.b). Severity of fin injury within the UMG was significantly higher in the fish in tanks (pooled data) where at least one precocious parr was present compared to those without them (Mann-Whitney U-test, $Z = 250$, $n = 28$, $p = 0.02$). Comparison of agonistic behaviour showed a non-significant tendency towards a higher mean score for aggression acts in the stream tanks holding at least one UMG precocious parr (Mann-Whitney U-test, $Z = 5069$, $n = 136$, $p = 0.072$).

DISCUSSION

Size asymmetries and life strategies

Juvenile Atlantic salmon initially belonging to the UMG held their size status throughout the duration of the experiment. Maintaining their size difference relative to LMG fish could have been mediated through the higher social positions being held in the stream tanks by the UMG individuals. Our findings are in agreement with other studies showing that large size disparity can strongly influence the outcome of dominance status, typically through superior fighting and resource monopolisation ability (Noakes, 1980; Abbott et al., 1985; Winberg et al., 1991; Johnsson and

Bjornsson, 1994; Adams and Huntingford, 1998; Beaugrand et al., 1996; Cutts et al., 1999; Johnsson et al., 1999). UMG fish were able to hold the best feeding positions upstream closer to the food source in the stream tank. Similarly, in the wild dominants are able to hold areas near favourable feeding grounds resulting in preferential access to food (Nakano, 1995).

By the end of the study larger socially dominant juvenile salmon had mostly developed into smolts or precocious parr, with a minority of UMG fish remaining as immature parr. Choice of life history strategy in Atlantic salmon is importantly influenced by relative social status (Metcalf et al., 1990; Thorpe et al., 1992), where dominant ranks with preferential access to food grow faster and are able to reach the threshold sizes required to trigger smolting or maturation (reviewed by Metcalfe, 1998). It is likely that life history strategy outcome was determined prior to the start of our experiment as key condition-dependent checkpoints that enable adoption of certain developmental strategies occur at an early stage. Specifically in Atlantic salmon smolting and parr maturation are triggered 10 to 12 months prior to the developmental conversion (Thorpe, 1977; Metcalfe et al., 1986; Thorpe, 1994). Thus, possibly environmental conditions experienced during the experiment did not determine the outcome of the observed phenotypes. This was not the aim of our study, but rather understanding how hierarchies influence physiological variables across social groups with two clearly differentiated modal groups. Surprisingly many precocious parr were found among the LMG fish despite the fact that high growth rates during the first year of life increase chances of early maturation (Myers et al., 1986; Rowe and Thorpe., 1990b). Perhaps these precocious parr had already reached the necessary size threshold at the start of the experiment, which is usually done by their first summer (Metcalf et al., 1998), and thereafter the other fast growing fish

would have surpassed them once mature parr started to divert most energy towards gonadal rather than somatic development (Simpson, 1992). Despite the fact that most precocious parr were found in the LMG, in at least half the tanks one precocious parr was present in the UMG. Individuals having the precocious parr strategy can show strong dissimilarities both in size and competitive ability, with dominant precocious parr usually being larger in size and consistently obtaining a closer position to reproductive females to sneak during fertilization events (Thomaz et al., 1997; Hutchings and Jones, 1998), in a similar way to what dominant anadromous males do with respect to satellite anadromous counterparts. It may be that large more dominant precocious parr are predisposed to be bolder, since in the wild they must not only assert their social position within the mature parr hierarchy but also show a proactive behaviour when sneaking for female fertilizations in the presence of much larger aggressive anadromous fish, which can lead to severe injury or even death (Hutchings and Myers, 1987). Future work testing this hypothesis examining its physiological basis (i.e. brain monoamines) may help understand better how precocious parr and anadromous salmon networks function in the wild.

Specific growth rate patterns

The size status between the UMG and LMG fish in stream tank groups appeared to be fairly conserved, with very little interchange between members of each modal group (i.e. very few LMG fish ended up in the top 3 sized fish of their tank). In line with this Bjorklund et al. (2003) found that variation in growth trajectories was minimal in Arctic charr monitored throughout a 2.5 year period, showing that fish that were initially small remained in the low size ranks till the end of the study, and large fish did likewise. On the other hand no signs of growth depensation (*sensu* Jobling, 1985),

where small size differences become larger over time, were detected between modal groups in the long term. The use of a monopolised point-source food supply that is easily defensible by dominants in theory could have helped increase the disparity in food acquisition between UMG and LMG fish (McCarthy et al., 1992; Ryer & Olla, 1996). The pattern of SGR between modal groups was not uniform in time. In the first weeks of the experiment UMG salmon increased their size advantage over LMG fish. This is in agreement with many studies showing a substantial difference in growth rates between dominants and subordinates (Huntingford and Turner, 1987), probably caused by a more pronounced inhibition in feeding by subordinates occurring during the more stressful initial stages of the hierarchy formation. A slow down of body mass growth in UMG compared to LMG fish thereafter could have been due to developmental conversion of juvenile salmon phenotypes at this time as fish going through smolting tend to lose condition and become more streamlined (Stefansson et al., 1991), while precocious parr divert gained energy towards gonadal rather than somatic growth (Simpson et al., 1996).

Long term studies have reported growth trajectory similarities between large and small fish in spite of the considerable initial size differences (i.e. lines of growth regression against time showing lines with similar slope despite differences in their elevation or intercept) (Seppa et al., 1999; Bjorklund et al., 2003). This work highlights the importance of the time scale in behavioural/physiological research as many studies operate in a short term scale (i.e. hours to weeks), whereas in the longer term physiological patterns may be very different (i.e. the social order may remain the same but the benefits of high status may decline). Recent metabolomic work examining long-term stress responses in juvenile Atlantic salmon supports this hypothesis by demonstrating that over the first two weeks of the study concentrations

of stress-related metabolites increased significantly in stressed fish compared to controls, but at three weeks and thereafter these differences declined substantially (Karakach et al., 2009).

In general terms the smaller fish in the tanks adopted a less aggressive feeding method than large fish by waiting for food moving downstream instead of actively competing for pellets near to the automatic feeder. This strategy is effective when food availability is not in short supply as it affords a low risk of injury while still achieving positive growth (Kadri et al., 1996; Alanärä et al., 1998). On the other hand UMG fish gained food by actively competing for the best feeding position, as indicated by their closeness to the feeding area and higher aggression towards each other. This energetically expensive strategy is characteristic of dominant fish in salmonid linear hierarchies (Adams et al., 1998; Nicieza and Metcalfe, 1999). Increased aggression and motivation to compete in dominant fish is thought to be mediated by higher maintenance costs associated a higher standard metabolic rate (SMR) (Cutts et al., 1998; Millidine et al., 2009). Smaller individuals would have been able to grow using fewer food resources as metabolic demands are scaled down with size, and benefit from lower energy expenditure in costly behaviours such as fighting (Persson, 1985; Bystrom et al., 2004). Meanwhile, fish incurring fin injuries during active feeding may incur higher social stress as indicated by plasma cortisol levels.

Brain monoamines and plasma cortisol in complex social hierarchies

Differences in the serotonergic activity in brain stem were detected between fish with the highest and the lowest social rank. The most subordinate (i.e. rank 6) individual in the group showed increased activation of the serotonergic system, measured as the

ratio between the metabolite 5-HIAA and the parent neurotransmitter 5-HT. Differences in brain serotonergic function in fish, as well as other vertebrates, have been linked to social status and an elevation in 5-HIAA/5-HT ratios in subordinate individuals promotes feeding and locomotory inhibition (Winberg et al., 1991, 1992; Øverli et al., 1998; Höglund et al., 2001). There were no significant differences in 5-HIAA/5-HT metabolism in the rest of the members of the hierarchy in any of the four brain parts examined. As the serotonergic system is highly sensitive to stress, including social stress (Prunet et al., 2008), the uniformity observed among most ranks, except for the first and last fish in the hierarchy, may indicate similar levels of stress experienced, although its cause is probably different (it could be argued that it also could be caused by differential affinity/number of 5-HT receptors however I could find no evidence for this in the literature).

Contrary to our expectation that UMG fish would have significantly higher plasma cortisol and lower serotonergic ratios compared to LMG, given that these larger fish were consistently holding the higher social ranks in the hierarchies (i.e. rank 1-3), no differences in neither 5-HT, 5-HIAA, nor 5-HIAA/5-HT were observed. Alanärä et al. (1998) reported that in groups of 8 Arctic charr 5-HIAA/5-HT did not differ between most dominant and intermediate rank fish, but were significantly higher in subordinate ranks relative to dominant ones. One possibility for the lack of contrast in brain serotonergic turnover and plasma cortisol between UMG and LMG fish in this experiment could be related to the fact fish sizes were clearly bimodal (i.e. not showing a bell shaped normal distribution), whereas in Alanärä's et al. (1998) study fish showed a wider spread in the range of sizes. In this study most of the aggressive acts observed occurred within rather than between modal groups, highlighting the importance of giving consideration to the size composition on social

interactions in hierarchies. In teleosts large relative size differences helps contests to be resolved quickly, very often with no need for aggression (Sneddon et al., 2006; Höjesjö et al., 2007). Smaller subordinate fish received low levels of aggression from dominants; instead the most competitive contests occurred between high ranked individuals from the UMG fighting for the best feeding positions in the tank. Having half the members of the hierarchy with a similar relative large size, which is positively correlated to competitive ability, may have triggered the strong agonistic levels observed in the UMG as aggressive behaviour is both positively correlated with large relative size (Noakes, 1980; Adams et al. 2000) and size similarity between contestants (Brännäs et al., 2002; Sneddon et al., 2006).

It is likely that the nature of the social stress experienced by dominants (e.g. aggressive fights for resources) and subordinates (e.g. social intimidation, food restriction) could be different. Nevertheless, in our experiment the end result at the circulating plasma cortisol and brain 5-HIAA/5-HT level between UMG and LMG fish appeared to be similar. Key physiological mediators of the stress response including cortisol and brain serotonergic activity show a general response to stressful events regardless of their origin (i.e. fighting, handling, predation) (reviewed by Winberg et al., 1997). In dyadic trials during the initial stages of the establishment of dominant-subordinate status (i.e. minutes to hours) both combatants will show elevation in serotonergic activity, however brain 5-HIAA/5-HT rapidly decreases in the winner whereas in the loser this stress-related index becomes chronically high, as well as other physiological stress responses such as high cortisol, low growth rates or ionoregulatory alterations (Gilmour et al., 2005). However, in multimember salmonid hierarchies in the wild these physiological consequences of social dominance or subordination observed in dyads in the laboratory become less clear and keeping

control over high quality feeding territories against other high status competitors may be energetically costly for dominants (Martin-Smith and Armstrong, 2002; Sloman et al., 2008). Acknowledging the limitations of our setup which lacked some of the elements found in the wild (e.g. shelter, predation), the use of more complex social hierarchies (e.g. with a group size in the range of social hierarchies in nature and important size differences between members) held in wild stream-mimicking settings (e.g. water flow in circulatory stream tanks) may lead to closer findings to those reported in wild populations. For instance, Sloman et al. (2008) also found no marked physiological correlation of dominance with serotonergic activity in several social hierarchy groups (size 8-10 fish) of wild brown trout living in the wild. Given more time and resources a design controlling for the exact proportion of precocious versus immature parr (e.g. groups of 6 fish with 3 individuals of each type), perhaps by selecting fish at the prime time of gonadal development to ensure enough precocious parr with running milt are collected for experimental replication. Also, ideally if the experiments were conducted in sections of wild streams where fish may have a wider range of behavioural choices (e.g. escape far enough from dominants), this could help avoid some of the criticisms associated with behavioural laboratory experiments (e.g. increase of the physiological differences between dominant and subordinate fish).

Fin erosion, size and life strategy

Fin erosion was minimal in the smaller fish within the stream tanks. While many studies have reported greater aggression and fin damage towards most subordinate individuals (e.g. Abbott and Dill, 1989; Noble et al., 2007) other workers have found dominant individuals to be more aggressive towards fish that pose a direct challenge to their status (i.e. subdominants). Overt agonistic behaviours have often been

associated with dominant status in salmonid fish (Nakano and Furukatawanaka, 1994; Adams et al., 1998; Brännäs, 2009). In this study overt attacks (i.e. biting) were more frequently observed in the UMG fish, which generally held high social positions within the stream tank. Similarly, work by Nicieza and Metcalfe (1999) showed greater levels of aggression in the UMG than in the LMG in juvenile Atlantic salmon, with attacks by larger individuals being highly skewed towards fish in their same upper size range. MacLean et al. (2000) reported that the largest salmon in the tank were up to six times more likely to present fin damage than smaller ones suggesting it was due to the aggressive competition taking place between dominant fish compared to the passive low-risk strategy adopted by smaller subordinates. The authors in that study demonstrated that the effect was related to the size range of the fish in the tank rather than absolute size per se (MacClean et al., 2000). This effect has also been reported in salmonids held under commercial production densities where individuals with the higher food consumption, usually larger fish, are the recipients of more attacks and fin erosion (Adams et al., 1998; Moutou et al., 1998). Thus our findings concur with the theory that fish with a close size range competing for higher social positions are more aggressive towards each other and likely to incur in fin damage. Cortisol analysis showed that UMG with strong fin erosion had higher plasma concentrations of the hormone than non-injured UMG fish and even the LMG individuals.

In addition a higher level of fin damage was observed in groups holding at least one precocious parr in the large size range, (but had no relation to the number of mature parr from the LMG, which showed submissive behaviour). It is known that during spawning precocious parr arrange themselves in hierarchies and body size appears to importantly determine which ones will have the closest position and access

to mates (Myers and Hutchings, 1987; Jones and Hutchings, 2001). Similarly, Thomaz et al. (1997) reported a significant positive correlation between parr size and reproductive success. Presumably these larger individuals gain dominance by winning social fights, in a similar way to that by dominant anadromous salmon against other satellite males. Unlike the smaller subordinate precocious parr in the experiment, those found in the UMG were highly aggressive. Hormonal changes, particularly during the breeding season, can have a strong influence on aggressiveness in teleosts (Briffa and Sneddon, 20007). At the time of final sampling precocious parr had large testes occupying most of their internal cavity and filled with freely running milt (pers. obs), possibly indicating the ripe condition of gametes. Compared to other life strategies maturing parr have higher levels of 11-ketotestosterone and testosterone when gonads are ripe (Mayer et al., 1990; Shrimpton and McCormick, 2002), both these hormones closely associated with modulation of aggressive behaviour and dominance in teleosts (Oliveira et al., 2002). In brown trout precocious parr have a bolder behaviour and are more likely to subject themselves to higher risk of predation when food is limited (Dannewitz & Peterssen, 2001). Thus it may be that large precocious parr could be more likely to engage in agonistic interactions if the relative size difference between dominant type opponents is moderate. There was a tendency, almost significant, for UMG precocious parr to be more aggressive than smolts which could help explain the greater fin damage recorded in tanks with these two phenotypes. Phenotypic differences in levels of fin erosion have been reported elsewhere between groups of precocious parr and immature parr, being significantly greater in the later strategy (Mork et al., 1989). During smolting circulating thyroxine levels increases have been linked with a reduction in aggressive behaviour in this strategy (Hutchinson and Iwata, 1998), but other studies have reported strong

aggressiveness between Atlantic salmon of this strategy prior, during and after the smolting conversion (e.g. MacLean et al., 2000; Noble et al., 2007, 2008). In the stream tanks with smolt only UMG fish occurrence of fin damage and combative behaviour was also present, thus supporting the existence of smolt agonistic encounters (perhaps migratory non-aggressive behaviour is more probable in the wild and during a limited time during downstream migration).

In summary, juvenile Atlantic salmon initially in the UMG became socially dominant over those in the LMG. Most large fish adopted either the smolt or the precocious parr life strategy. Despite an initial higher SGR in UMG fish overall size differences between both modal groups were maintained but not increased over the 5 month experiment. Brain serotonergic activity differences were only detected between both extremes of the social range, most dominant and most subordinate fish. No significant differences in brain neurotransmitter activity or cortisol concentrations were detected between the UMG and LMG fish, possibly reflecting reduced benefits in large fish due to higher aggression and fin injury. Phenotypic composition of the social hierarchy may also influence to some extent the nature of social encounters and aggression levels. However, further studies investigating the effects social hierarchies have on individuals in groups of higher structural complexity (i.e. where dominants are challenged by opponents and some low ranked individuals experience being dominant over other more subordinate fish) are necessary if we are to characterise physiological and ecological responses that more accurately reflect conditions in the wild.

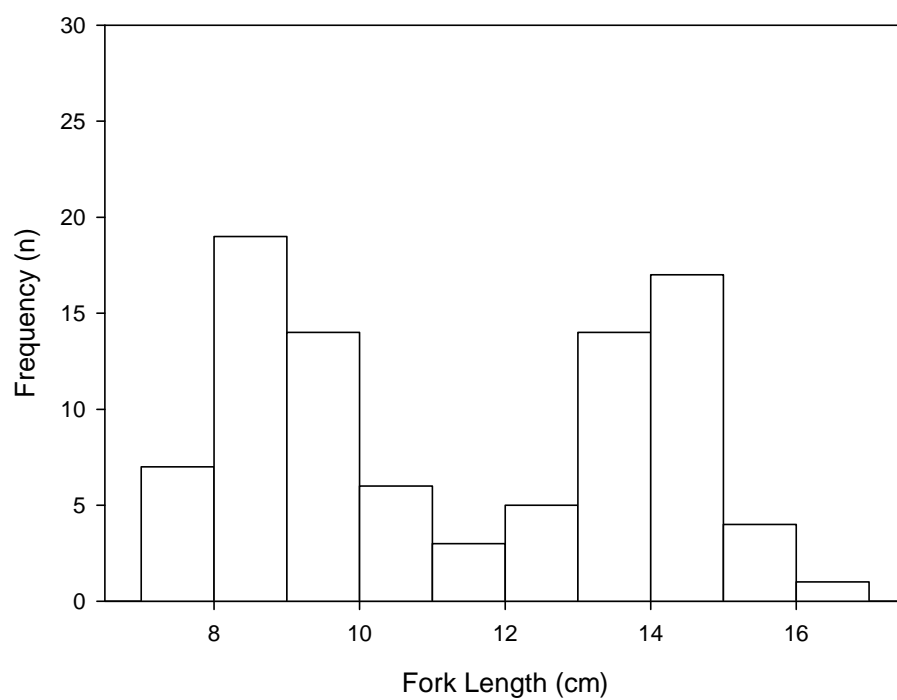


Figure 5.1. Bimodal length-frequency distribution of the stock of juvenile Atlantic salmon ($n = 90$) from which UMG ($n = 30$) and LMG ($n=30$) individuals were collected in May 2008.

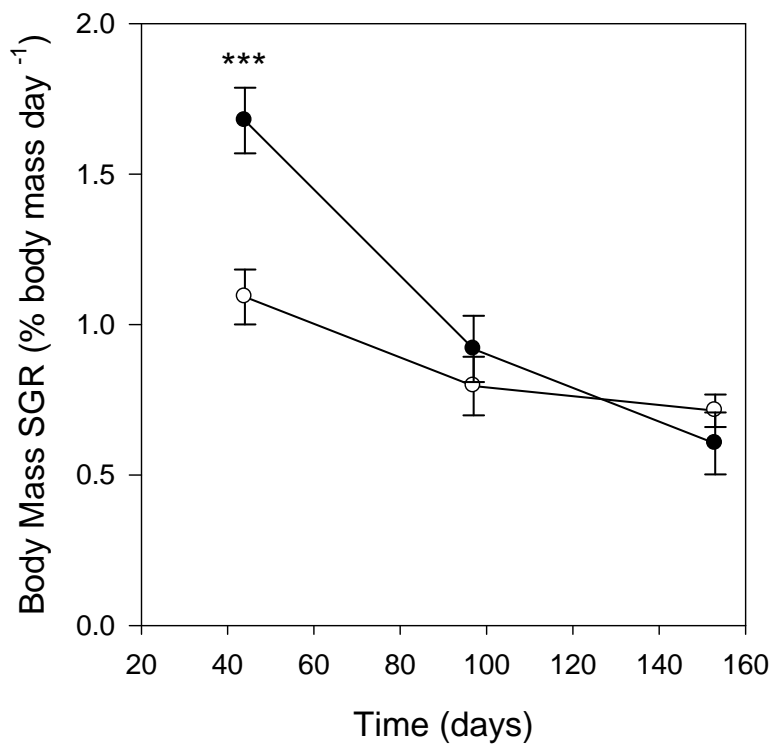


Figure 5.2. Body mass SGR (% body mass day⁻¹) of upper modal group (UMG) (closed circles) (n = 28) and lower modal group (LMG) (open circles) (n = 28) juvenile Atlantic salmon. Data shown are means (\pm SEM). Asterisks denote statistical differences in SGR (***p < 0.001).

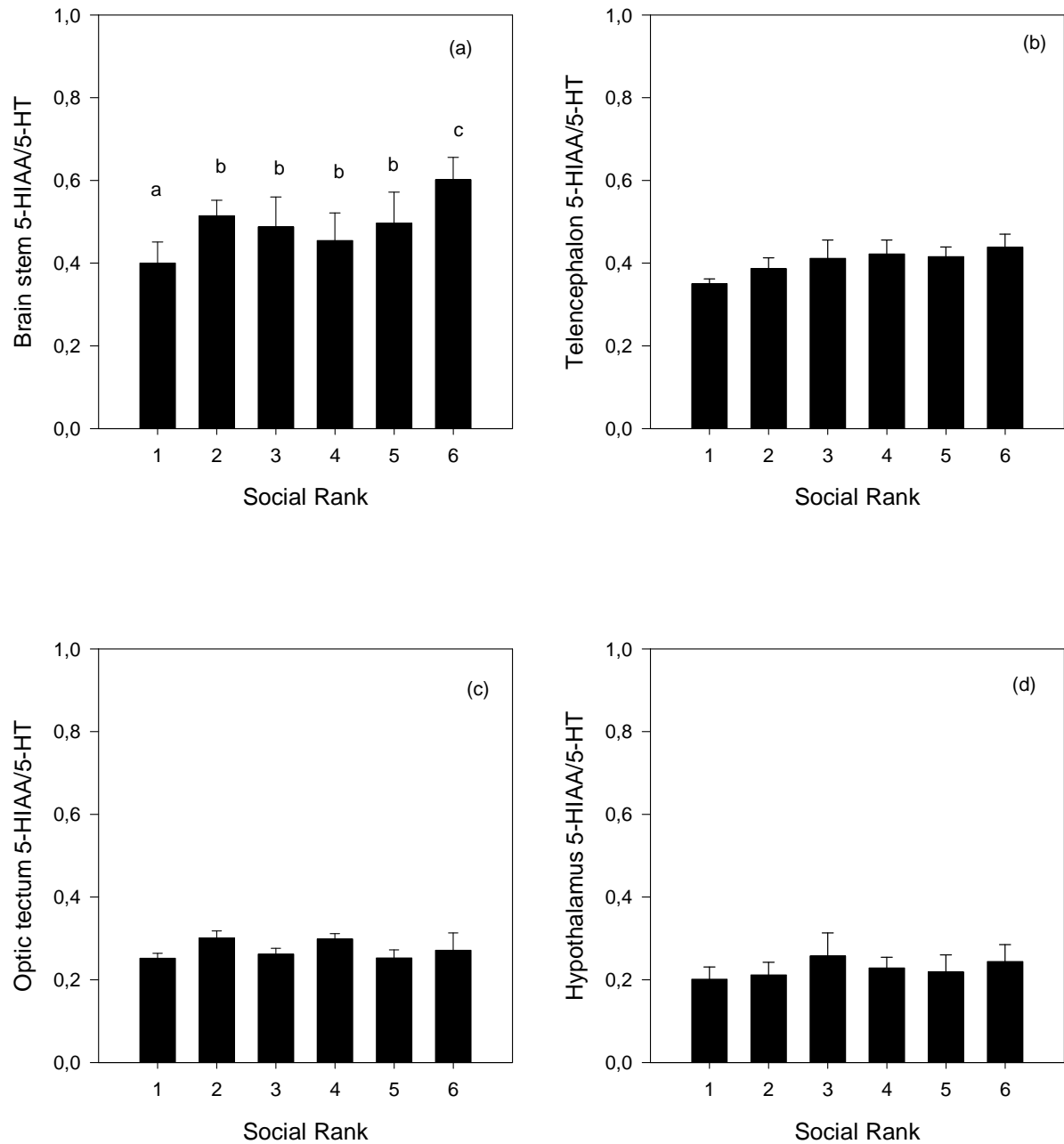


Figure 5.3. Metabolite/monoamine ratio (5-HIAA/5-HT) in the (a) brainstem, (b) telencephalon, (c) optic tectum, and (d) hypothalamus of Atlantic salmon with different social ranks (n = 10 per rank). Means \pm SEM. Different letters denote statistical differences between social ranks ($p < 0.05$), absence of letters indicates NS.

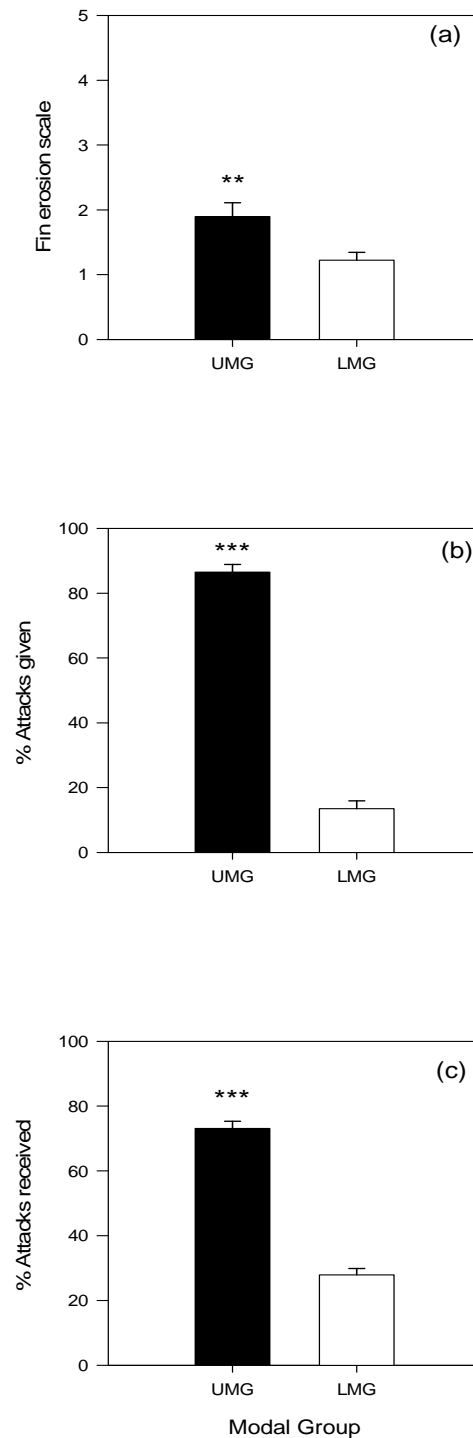


Figure 5.4. Comparison of (a) fin erosion scores for upper modal group (UMG, closed bars) ($n=28$) and lower modal group (LMG, open bars) ($n=28$) (b) percentage of attacks given and (c) received by UMG and LMG juvenile Atlantic salmon. Data shown are means (\pm SEM). Asterisks indicate a statistical difference between groups (** $p < 0.01$, *** $p < 0.001$).

Brain region	UMG	LMG
Brain stem		
5-HT	267.23 ± 15.21	298.46 ± 29.18
5-HIAA	127.25 ± 9.04	147.0 ± 20.00
5-HIAA/5-HT	0.49 ± 0.03	0.52 ± 0.06
Telencephalon		
5-HT	482.17 ± 34.10	521.8 ± 37.76
5-HIAA	179.81 ± 12.75	220.58 ± 17.25
5-HIAA/5-HT	0.38 ± 0.02	0.40 ± 0.02
Optic tectum		
5-HT	163.52 ± 8.83	172.91 ± 6.81
5-HIAA	44.09 ± 2.50	46.30 ± 2.63
5-HIAA/5-HT	0.27 ± 0.01	0.27 ± 0.01
Hypothalamus		
5-HT	835.74 ± 46.49	905.12 ± 73.71
5-HIAA	164.63 ± 7.97	186.6 ± 24.7
5-HIAA/5-HT	0.22 ± 0.12	0.23 ± 0.02

Table 5.1. Tissue concentrations of monoamine and metabolite, 5-HT and 5-HIAA (ng g^{-1}), and the corresponding metabolite/monoamine ratio (5-HIAA/5-HT) in brain stem, telencephalon, optic tectum and hypothalamus of UMG and LMG Atlantic salmon ($n = 56$). Values are means \pm SEM. All comparisons were NS at the $p < 0.05$ level.

Chapter 6

Social rank and brain serotonergic activity in immature and precocious Atlantic salmon (*Salmo salar*) parr

ABSTRACT

The relationship between social rank, brain monoamine neurotransmitters and phenotype in juvenile Atlantic salmon (*Salmo salar*) was examined in immature and precocious parr. Groups of 6 fish of mixed phenotypes or immature parr only were held in stream tanks for 3 weeks and their behaviour examined. Dominants within mixed phenotype groups were consistently found to be precocious, occupying the best feeding areas and showing the most aggressive behaviour compared to immature parr. Brain monoamine activity measured as the ratio of 5-hydroxyindoleacetic acid (5-HIAA) to serotonin (5-HT) correlated with social rank and was lower, typical of dominant individuals, in the brain stem and telencephalon of precocious parr than immature parr. Despite their dominant status, specific growth rate was inferior in precocious parr, presumably as a result of energy allocation to gonadal development. In groups with only immature parr, high ranked fish also showed a lower serotonergic activity, but a different pattern of behaviour and growth emerged with less intense aggression and higher growth rates. In addition, a subsample of precocious and immature parr reared in aquaculture rearing conditions was examined for serotonin. The 5-HIAA: 5-HT ratios were again lower in the telencephalon of precocious fish. This work highlights the importance of understanding the nature of social interactions

and its neuro-physiological effects on alternative phenotypes, precocious and immature parr, which commonly compete for resources in the wild and aquaculture.

Keywords: social behaviour; precocious; immature parr; phenotype; brain monoamines; serotonin

INTRODUCTION

In teleosts with alternative life strategies, differences in behavioural, neuroendocrine and physiological traits between phenotypes can be remarkable (Oliveira, 2006). Understanding these phenotypic effects on fish populations is becoming a key objective in conservation ecology (Watters et al., 2003; Young et al., 2006). This is especially important in salmonids as they exhibit a great variety of life histories (Klements et al. 2003 for review) and wild populations are threatened by overfishing and introduction of farmed fish (Flemming et al., 1994; Parrish et al., 1998; McGinnity et al., 2003).

Despite the fact that many aspects of the alternative life histories of salmonids have been well researched (Flemming, 1996; Metcalfe, 1997; Hutchings et al., 1998 for reviews), surprisingly few studies have addressed specifically the social interactions between the two phenotypes that most commonly coexist both in fish farms and as residents in the river competing for resources all year-round, the precocious (or early-mature parr) and immature parr. Perhaps the shortage of *a priori* behavioural experiments using pre-selected groups of immature and mature parr is due to the difficulty in differentiating these types externally as precocious parr lack secondary sexual characters. The few studies that have specifically examined

behavioural patterns using mature and immature parr salmonids have found differences between them. Dannewitz and Petersson (2001) reported that during spring precocious brown trout (*Salmo trutta*) would return to a patch where it had been presented with a simulated predator sooner than immature individuals. The authors argue that this “boldness” of mature parr resulted in superior growth rates and condition factor. Yambe et al (2005) reported that immature parr of masu salmon (*Oncorhynchus masou*) actively avoided entry into a Y-maze channel conditioned with mature male urine compared to the control. In addition, precocious parr show higher growth rates up to maturation (Thorpe et al., 1983; Rowe and Thorpe, 1990) and lower rates of fin damage when kept with immature parr (Mork et al., 1989). Even though circumstantial evidence points towards a higher social rank of precocious parr compared to immature parr, direct support for this theory and its physiological basis is lacking.

Social status plays a very important role in the physiology and well being of fish, as dominant individuals tend to have access to the best food resources, higher growth rates, aggression and locomotor activity, improved immune function, lower plasma cortisol, stress and fin damage (Gilmour et al., 2005 for review). Brain neurotransmitters have proven a useful tool in trying to understand the effects social context has on fish welfare as they serve a crucial role in mediating behavioural and physiological stress responses through activation of the hypothalamic-pituitary-interrenal (HPI) axis with a subsequent elevation of plasma cortisol (Winberg et al., 1997; Winberg and Lepage, 1998; Øverli et al., 1999a, 1999b). In vertebrates serotonin (5-hydroxytryptamine, 5-HT) appears to integrate behavioural and physiological stress responses like those caused by social agonistic interactions (McIntyre et al., 1979; Winberg and Nilsson, 1993), primarily through an inhibitory

role on aggression (Huntingford and Turner, 1987). Turnover of 5-HT measured as the ratio between brain 5-HT and its principle catabolite, 5-HIAA, is a sensitive and reliable indicator of social status as 5-HIAA release is elicited during stressful situations and socially subordinate fish consistently show a pattern of high 5-HIAA/5-HT ratio relative to dominants (Winberg et al. 1992, 1993). Using Arctic char (*Salvelinus alpinus*) Winberg et al. (1991) demonstrated that brain serotonergic activity was socially induced as brain 5-HIAA/5-HT ratios were related to the last social position experienced by an individual, regardless of a dominant or subordinate status in prior hierarchies. Despite the body of literature on brain monoamines and social behaviour in vertebrates, and salmonids in particular (reviewed by Winberg and Nilsson, 1993; Summers and Winberg, 2006), the influence of phenotypic identity of juvenile salmon on social status and how it relates to brain neurotransmitter profiles is currently unknown. As serotonergic activity is increasingly being used as an indicator of animal welfare (Øverli et al., 2007), understanding social interactions between salmonid phenotypes from a brain monoaminergic perspective may help improve management practices both in aquaculture and conservation.

In the present study the hypothesis that precocious and immature parr Atlantic salmon show different social traits and that these reflect on aspects of their physiology and neuroendocrinology was tested by keeping size-matched mixed groups of both phenotypes in stream tanks and monitoring their behaviour, growth and brain serotonergic activity. We also examine if a link between phenotype and brain monoamines holds in fish living in larger groups under fish farming conditions.

METHOD

Fish husbandry

Juvenile 1+ Atlantic salmon originating from a small brood stock of wild salmon from the river Delphi (Ireland) and subsequently reared under fish farm conditions (Hampshire, UK) were transported to the laboratory in July 2007 and kept indoors in holding tanks (stocking biomass 10.4 g l^{-1}) continuously supplied with aerated dechlorinated Exeter tap chilled water at $11 \pm 1 \text{ }^{\circ}\text{C}$ and under a 12h:12h L:D photoperiod for six months before running the experiment that compared mature and immature parr. A proportion of the immature parr used in the second trial were collected from the same cohort in a second trip in September 2007 and kept under the same conditions in the lab. Throughout this period fish were hand-fed twice daily a total of 2 % body mass per day with a commercial salmon pellets (Skretting Ltd., UK), except for the last 3 weeks prior to the experiment when food was delivered using fish feeders (Fish Mate F14) at 4 h intervals to acclimatise fish to this method of feeding.

Experimental protocol

Two weeks prior to the experiments fish were anaesthetized in a solution of tricaine methane sulphonate or MS222 (100 mg l^{-1} , buffered with NaHCO_3 followed by vigorous aeration to restore normal CO_2 levels), PIT tagged for identification, and fork length (L_F) and body mass (M) recorded ($L_F = 16.1 \pm 0.1 \text{ cm}$; $M = 55.1 \pm 0.6 \text{ g}$; mean \pm SEM). A small sub-sample was stripped manually to identify precocious salmon but none showed signs of running milt. As there is no simple visual way of telling precocious parr apart from immature parr we used condition index (K) as a

possible indicator of maturity as several reports suggest that in salmonids early maturing parr have a superior K due to their higher lipid content (Bohlin et al., 1994; Shearer and Swanson, 2000). Three individuals from the lower and other three from the higher half of the K distribution of the originally stocked fish were transferred to individual stream tanks ($n = 6$ fish per tank) hoping to obtain a good mixture of precocious and immature parr. Note that after concluding the experiment and examining fish phenotype many immature parr were found in initial upper K modal group, indicating that condition was not a reliable indicator of parr maturity. Prior to the start of the experiment individuals were visually tagged after anaesthetising with buffered MS222 (100 mg l^{-1}) using a small grey sequin tied with a surgical knot. Preliminary experiments had shown that sequin tagged fish recovered almost immediately from marking and the tag had no effect on fish social behaviour or growth. Similar methods using small tags attached with a suture thread have been successfully used in other juvenile salmon studies (e.g. Metcalfe et al., 1989).

Fish in each tank were size-matched (i.e. within 5 % L_F of each other). In total 12 experimental stream tank raceways (flow rate = 6 l min^{-1} ; dimensions = $150 \times 58 \times 30 \text{ cm}$) were used per trial ($n = 72$ fish per trial). Unidirectional water current was maintained in each raceway using water pumps (Eheim 1250) fitted with submerged custom-built spray bars immediately upstream of a grid at the end of each raceway. At the top of each raceway a fish feeder was placed which delivered food 4 times a day (6am, 12 am, 6 pm, 12 pm) amounting to 2 \% wt day^{-1} . The food would drop at the top of the raceway and drift towards the back end following the current direction. Fish occupying the front section of the tank had preferential access to food pellets.

Fish behaviour was recorded using four static video cameras (Sony KIR-040) positioned approximately 140 cm above the tanks and linked to a digital video-

recorder; the system was remotely operated to avoid any disturbance. Aggressive acts were counted during two daily observations at 08:00 and 12:00 each lasting 10 min. Each experimental trial lasted 21 days giving a total of 42 observations per tank. The following agonistic acts were recorded, with higher scoring behaviours being those that potentially can inflict a more severe injury to the recipient, with biting, chasing, charging, lateral display and receiving or avoiding aggression scoring 4,3,2,1 and 0 points respectively (Gallardo & Neira, 2005). In addition the closeness of each individual in relation to a static food source, another indicator of status, was recorded at 0 min, 5 min and 10 min of each behavioural observation and determined by the fish being either in the front third (closest to feeder), middle or back third (further away) of tank. Fish with the highest behavioural scores were considered dominants.

Due to the limited number of available stream tanks the experiment was divided into two trials which were run consecutively following the same protocol. In the first trial there was a good mixture of precocious ($n = 41$) and immature parr ($n = 28$) and three fish died, while in the second trial most individuals were immature parr ($n = 70$) and only 2 fish were precocious parr.

Brain sampling

At the end of the experiment fish in individual groups were netted simultaneously and lethally anesthetized with MS222 (200 mg l⁻¹). Final L_F and M were recorded. The brains were quickly removed (within 3 min) and divided into telencephalon, hypothalamus, and brain stem. The tissues were frozen in liquid nitrogen and stored at -80°C until brain monoamine analysis.

Hatchery fish sampling

Juvenile Atlantic salmon age 1+ kept under commercial rearing conditions in a hatchery (Basingstoke, UK) were sampled for brain monoamine analysis. Fish of precocious and immature parr phenotype ($n = 30$ of each) and similar size (precocious parr $L_F = 16.6 \pm 1.2$ cm; $M = 39.1 \pm 2.0$ g; immature parr $L_F = 15.9 \pm 0.7$ cm; $M = 38.3 \pm 2.2$ g; mean \pm SEM) were netted from 3 tanks (volume = 3.43 m^3 ; flow rate = 100 l min^{-1} ; stock density = 25 kg m^{-3} ; total fish number per tank = 4000-5000). Fish were killed using a lethal dose of buffered MS222 (200 mg l^{-1}) and brain removed immediately, frozen in liquid nitrogen and kept in a freezer at -80°C until further analysis.

Brain monoamine analysis

Brain tissue was weighed and homogenized in 4 % (w/v) ice-cold perchloric acid containing 20 ng ml^{-1} of 3,4-dihydrobenzilamine (DHBA) as an internal standard, using a Potter-Elvehjem homogenizer. Samples were then centrifuged at $27,000 \text{ g}$ for 10 min at 4°C , and the supernatants used for analyses. Monoamine and monoamine metabolites were determined using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Øverli et al. (1999a). Serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were examined. As a measure of serotonergic activity, the 5-HIAA/5HT ratio was calculated for each individual. The monoamines were quantified using standard solutions and corrected for recovery of the internal standard using HPLC software (CSW, Data Apex, the Czech Republic).

Physiological measurements

Individual specific growth rates (SGR) were calculated as follows:

$$\text{SGR} = 100 (\ln M_2 - \ln M_1)/t,$$

where M_2 and M_1 are the body mass (g) at the end and start of the experiment respectively and t is the number of days between measurements.

Condition index (K) was calculated as:

$$K = 100M_2L_F^{-3}$$

where M_2 is final body mass (g) and L_F is fork length (cm), and gonadosomatic index as:

$$I_G = 100 M_G M_2^{-1}$$

where M_G is gonad mass (g).

Statistical analysis

Data normality was examined using a Kolmogorov-Smirnov test for normality (Zar, 1996) and homogeneity of variance was evaluated using Levene's test (Schultz, 1983). Due to the low incidence of precocious parr in trial two (2 out of 72 fish) their data was removed from analysis in order to better assess physiological trends in immature parr. Repeated measures ANOVA or non-parametric equivalent (Friedman test) were used for social rank comparisons between brain monoamine activity (followed by SNK *post hoc* tests), with rank as the repeated measure within each tank. Behavioural scores, and somatic indexes were compared using Mann-Whitney U-tests. Differences between brain monoamines in pooled data for phenotypes were tested using t-tests or a non parametric equivalent. Significance was set at $p < 0.05$. Statistical analyses were conducted using SigmaStat (SigmaStat 3.0, PSS Inc.,

Chicago, IL, USA). Results are presented as means \pm standard error of the mean (SEM).

RESULTS

Dominance hierarchies

Linear social hierarchies rapidly developed and were maintained throughout the experiment. In trial 1 with mixed groups of both phenotypes, rank 1 and 2 fish were primarily precocious, taking the most dominant status (rank 1) in 10 out of 12 tanks. Comparison of social ranks obtained by precocious and immature parr were significantly different (Mann-Whitney U-test; $Z = 1341.15$, $n = 69$, $p = 0.016$). Precocious parr in trial 1 showed higher mean behavioural scores per observation (i.e. more intense aggression) than immature parr (Mann-Whitney U-test; $Z = 2381$, $n = 84$, $p < 0.001$).

SGR, condition index and gonadosomatic index

Combined data for precocious parr in trial 1 showed a lower body mass SGR than immature parr after 21 days (t-test; $t_{67} =$, $p < 0.001$) (Figure 6.1.a). Despite lower body mass growth rates, precocious parr showed a higher K (Mann-Whitney U-test; $Z = 810$, $n = 69$, $p = 0.009$) (Figure 6.1.b), and had significantly greater I_G than immature parr (Mann-Whitney U-test; $Z = 417$, $n = 69$, $p < 0.001$) (Figure 6.1.c).

Brain monoamines and social rank

Repeated measures ANOVA showed a significant effect of social rank in 5-HIAA/5-HT in the brain stem ($F_{5,52} = 9.055$, $p < 0.001$) and telencephalon ($F_{5,52} = 4.602$, $p <$

0.001) of trial 1, but not in the hypothalamus ($F_{5,52} = 0.564$, $p = 0.727$) . *Post hoc* SNK comparisons between social ranks revealed that the differences were between the most dominant and subordinate ranks 5 and 6 individuals (Figure 6.2). In trial 2 brain stem showed significant differences between social ranks for serotonin ratios (one-way RM ANOVA; $F_{5,53} = 2.518$, $p = 0.041$) but not telencephalon (Friedman nonparametric repeated measures test; $\chi^2 = 10.429$, d.f. = 5, $n = 70$, $p = 0.064$) and hypothalamus (one-way RM ANOVA; $F_{5,53} = 1.095$, $p = 0.374$).

Brain monoamines and phenotype

Comparison of brain monoamines between fish with alternative phenotypes in trial 1 showed a significantly higher 5-HIAA/5-HT activity in the immature parr's brain stem (t-test; $t_{65} = -3.626$, $p < 0.001$) and telencephalon (t-test; $t_{65} = -3.73$, $p < 0.001$), but not in the hypothalamus (t-test; $t_{65} = -0.234$, $p = 0.816$) (Figure 6.3).

Brain monoamines in aquaculture fish

Precocious and immature parr sampled for brain tissue analysis in high density commercial conditions had a similar body mass (t-test; $t_{58} = 0.304$, $p = 0.762$) and fork length (t-test; $t_{58} = 0.19$, $p = 0.85$). The two phenotypic groups sampled living in aquaculture facilities showed a brain monoamine/metabolite telencephalon pattern similar to those of fish kept in the laboratory stream tanks, with immature parr having again a higher 5-HIAA/5-HT ratio compared to precocious parr (t-test; $t_{58} = -2.462$, $p = 0.017$) (Figure 6.4.). The I_G in precocious parr was significantly higher than in immature parr (Mann-Whitney U-test; $Z = 11.0$, $p < 0.001$) (Figure 6.1.c).

DISCUSSION

This study shows for the first time divergent behavioural and brain monoaminergic patterns in salmonids with alternative life tactics. Higher aggression and lower serotonergic activity were observed in precocious compared to immature parr in size-matched mixed groups, both at the laboratory and at the finfish production level. Salmonid phenotypic identity may be important when considering conservation or aquaculture management decisions.

Dominance hierarchies

In the present study mature parr and immature parr readily organised into dominance hierarchies based upon the outcomes of agonistic interactions. During the experiment precocious parr in general were better competitors ranking higher than immature parr. This would be in agreement with Jarvi and Pettersen (1991) who found that sexually-mature parr were superior competitors and were able to defend better food patches than immature parr. In early life stages salmon with better growth rates and belonging to the upper modal groups, a characteristic of more dominant fish, tend to select the precocious parr or smolt strategy rather than remain as immature parr (Thorpe et al., 1983; Metcalfe et al., 1989). We found that the higher social status in precocious parr was achieved through agonistic behaviour of greater intensity towards subordinate fish, using more active aggressions (i.e. bites and chases) rather than warning behaviours (i.e. lateral displays) which were observed in the groups consisting of only immature parr. Mork et al. (1989) reported a high incidence of dorsal fin damage in hatcheries holding mixed groups of precocious and immature parr, and discovered that immature parr had a higher incidence of dorsal fin damage, which is commonly

used as an index of received aggression and lower social rank. High levels of intraspecific aggressive behaviour has deleterious effects on finfish welfare and production as subordinate fish suffer higher injury levels, decreased appetite and reduced immunity to disease (Davies and Olla, 1987; Gilmour et al., 2005).

Specific growth rate

In salmonids higher aggression and social rank are usually associated with superior growth rate (Huntingford et al., 1998; Nicieza & Metcalfe, 1999; Lahti and Lower., 2000). Despite a higher average social rank of precocious parr in the phenotype mixed groups, based on proximity to feeders and agonistic interactions, this did not translate into a superior growth rate. In fact, immature parr showed higher body mass growth, although the SGR was low compared to that of other studies (e.g. Metcalfe and Thorpe, 1992; Bacon et al., 2005). One possibility is that at this stage of their development precocious parr were diverting most energy towards gonadal growth. In Atlantic salmon mature parr will exhibit fast somatic growth from the hatchling to the parr stage (Thorpe et al., 1983; Aubin-Horth & Dodson, 2004) but once they reach a size threshold which depends upon genetic and environmental factors, the greater proportion of their energy is devoted towards gonadal development (Koseki & Maekawa, 2002). Although generation of gonadal mass is energetically costly to produce, most mature parr we examined had a very high GSI. During the later stages of gonadal maturation precocious parr may enter a period of slow growth relative to immature parr (Berglund, 1992; Tveiten et al., 1995; Arndt, 2000). In addition, Tucker & Rasmussen (1998) demonstrated that precocious salmon parr needed a food intake of 1.5 times higher than non-mature fish for a relatively smaller increase in weight, confirming that maturation is a costly strategy. In the second trial, where

virtually all fish were immature parr, a significant positive trend emerged between social rank and body mass SGR, with dominant fish achieving higher growth. Thus, it appears that if growth rate is being used to infer group or population dynamics in fish that have alternative life histories (e.g. river or aquaculture surveys on fish size distribution or growth) results should be interpreted with caution if phenotypic identity is not taken into account.

Brain monoamines and social rank

Brain ratios of 5-HIAA /5-HT showed neuroendocrine differences between fish of high and low social rank within both trials of Atlantic salmon parr. In dyadic dominance-subordinance experiments contrasting behavioural and physiological responses can occur, while the use of larger social groups (e.g. 5-15 fish) appears to produce a milder and wider range of responses (reviewed by Sloman and Armstrong, 2002). We found that in both trials significant differences in 5-HIAA/5-HT ratios only occurred between most dominant ranks (1-2) and most subordinate ones (5-6), with middle ranking fish attaining access to relatively good feeding areas while still avoiding high levels of aggression. Similarly, Alanärä et al. (1998) using comparable sized groups to ours reported three social categories of Arctic char with one or two aggressive dominant fish, a small group of one to three intermediate ranked fish and the rest of individuals falling into the subordinate category. They also found that significant brain serotonergic differences appeared between char from the most dominant and most subordinate categories, while the intermediate category fell somewhere in between.

Brain monoamines and phenotype

Significant differences in brain serotonergic activity were detected between the two juvenile salmon phenotypes. Pooled data for immature parr showed a higher mean 5-HIAA/5-HT ratio in brain stem and telencephalon, characteristic of social subordination, or other stressors (Winberg et al., 1992). Indeed, immature parr ranked lower than precocious parr, making the greater proportion of the lower ranks in the mixed group trials. Other studies have not found behavioural differences between precocious and immature parr (e.g. Johnsson et al., 2001; Martin-Smith and Armstrong, 2004). The reason for this divergence is not well understood, but perhaps could be related to the timing of peak circulating androgen levels associated with male parr sexual maturation. Several studies show that a peak in plasma testosterone (T) takes place a short time before spermiation, while 11-keto testosterone (11-KT) peaks during spermiation (Scott et al., 1980; Kime & Manning, 1982) with both these androgens correlating with dominance in salmonids (Cardwell et al., 1996; Elofsson et al., 2000). The time while androgen concentration in precocious parr is high relative to immature parr is limited. Shrimpton and McCormick (2002) found that in Atlantic salmon high androgenic activity occurred during approximately two months in late autumn, thereafter sharply decreasing to levels comparable to immature parr. In their study the authors reported that sperm stripping was only possible during the peak in androgens, but not before or after this short period (Shrimpton and McCormick, 2002). In our study although we did not measure androgen levels precocious parr appeared to be in prime reproductive state judging from their very high I_G and easy milt release.

It would be of interest to test if early-maturing and immature parr belong to the stress low-responders (LR) and high-responders (HR) respectively, as described

by Pottinger and Carrick (1999; 2001) which in rainbow trout, *Oncorhynchus mykiss*, is also associated with changes in the function of brain monoaminergic systems (Øverli et al., 2001). Experiments describing higher “boldness” and aggression by precocious parr (e.g. Jarvi and Pettersen, 1991; Dannewitz and Petersson 2001) and the fact that fish later adopting the precocious parr phenotype achieve elevated growth rates and condition factor from an early stage in life (Bohlin et al., 1994; Shearer and Swanson, 2000; Aubin-Horth and Dodson, 2003) may support this notion. On the contrary, immature parr tend to belong to the lower modal group, subordinate positions or both (Metcalf et al., 1989) and are usually less bold or proactive. For example Metcalfe (1989) reported that the presence of a simulated competitor resulted in an 18-fold reduction in feeding efficiency in immature parr but had no effect in fast-growing bolder counterparts.

Brain monoamines in aquaculture salmonids

A trend in brain serotonergic metabolism similar to that of laboratory held fish was found in the sub-sampled population of mature and immature parr living under commercial rearing conditions. A lower ratio of 5-HIAA/5-HT was detected in the telencephalon of precocious parr as in our stream tank fish. Incidentally sampled precocious parr were again readily spermiating when stripped. Although social linear hierarchies tend to weaken with high rearing densities such as those seen in fish farms (Brown et al., 1992) agonistic interactions and social dominant-subordinate positions still exist. Recently, Cubitt et al. (2008) have demonstrated that in Atlantic salmon grown under high-density commercial rearing conditions brain tissue 5-HIAA /5-HT analysis is sensitive enough to detect dominant and subordinate relationships and serotonin profiles are started to being used as a tool to improve animal welfare (Øverli

et al., 2007). If the higher brain serotonergic turnover detected in immature parr in this experiment is a reflection of chronic stress caused by social subordination as it happens in smaller groups (Winberg et al. 1991, 1992; Øverli et al. 1998), then the deleterious impact of precocious parr on aquaculture industry could be two-fold as not only would they (a) stop growing at a small size while still consuming resources but also (b) their level of aggression and dominance may have a negative impact on production and welfare of the subordinate immature parr. In addition, given the higher incidence of precocious maturation in fish farms relative to wild streams a skewed introduction of early mature parr either through restocking programs or escapes may change the phenotypic make up of the native populations with important ecological consequences (e.g. Garant et al., 2003).

In summary, precocious parr and immature parr held in groups in the laboratory diverged in social rank and we report for the first time brain serotonergic differences between salmonids with alternative life strategies. Precocious parr showed higher intensity of agonistic encounters and lower 5-HIAA/5-HT ratios, characteristic of dominance. On the other hand in the tanks containing only immature parr aggressiveness was lower and, although physiological differences still existed between most dominant and subordinate members, a lower overall serotonin turnover was observed compared to groups containing precocious parr as well. In high-density aquaculture rearing conditions, despite the less strict social linear hierarchies, Atlantic salmon brain 5-HIAA/5-HT ratios were still dissimilar and followed the same trend, with precocious parr showing lower brain serotonergic activity characteristic of socially dominant and less stressed individuals. Thus, it may be important to take into account phenotypic identify of salmonids when planning river restocking programs or managing aquaculture enterprises to maximise fish welfare.

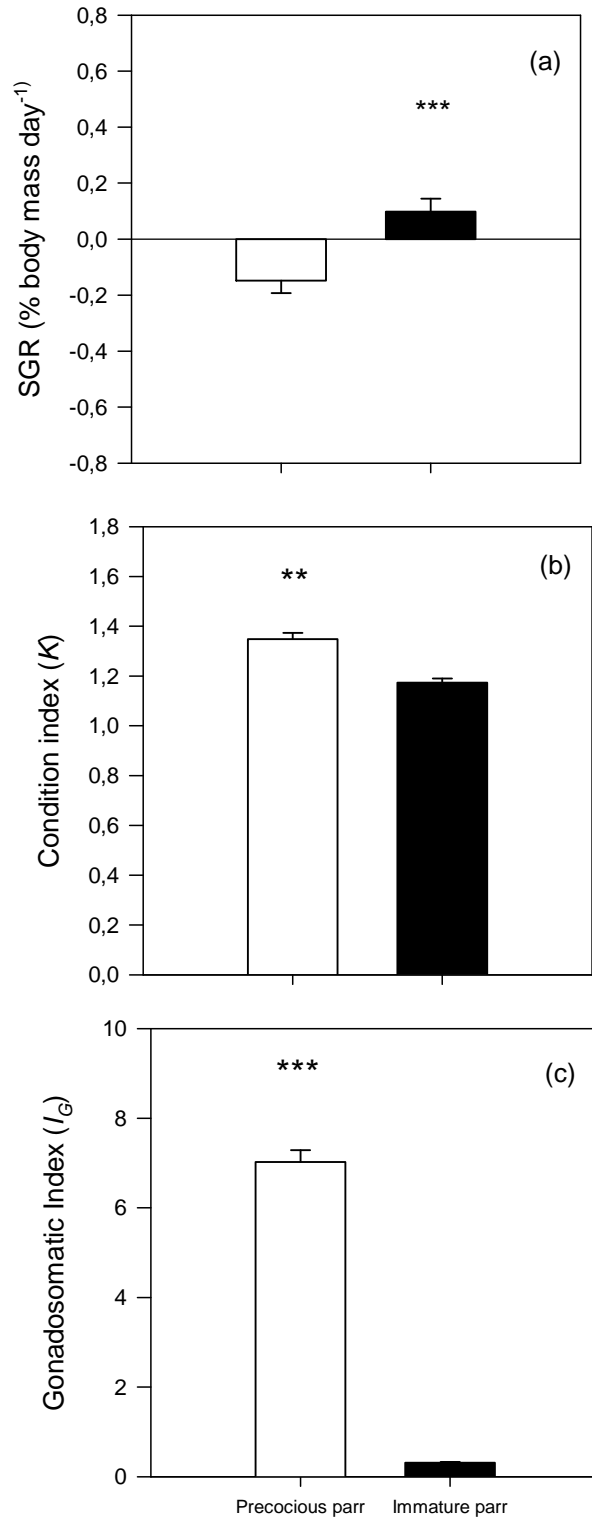


Figure 6.1. Somatic variables of (a) body mass specific growth rate, (b) condition index (K), and (c) gonadosomatic index (I_G) of trial 1 precocious (open bar) ($n = 41$) and immature parr (closed bar) ($n = 28$) phenotypes. Data shown are means (\pm SEM). Asterisk indicate significant differences between phenotypes (** $p < 0.01$, *** $p < 0.001$).

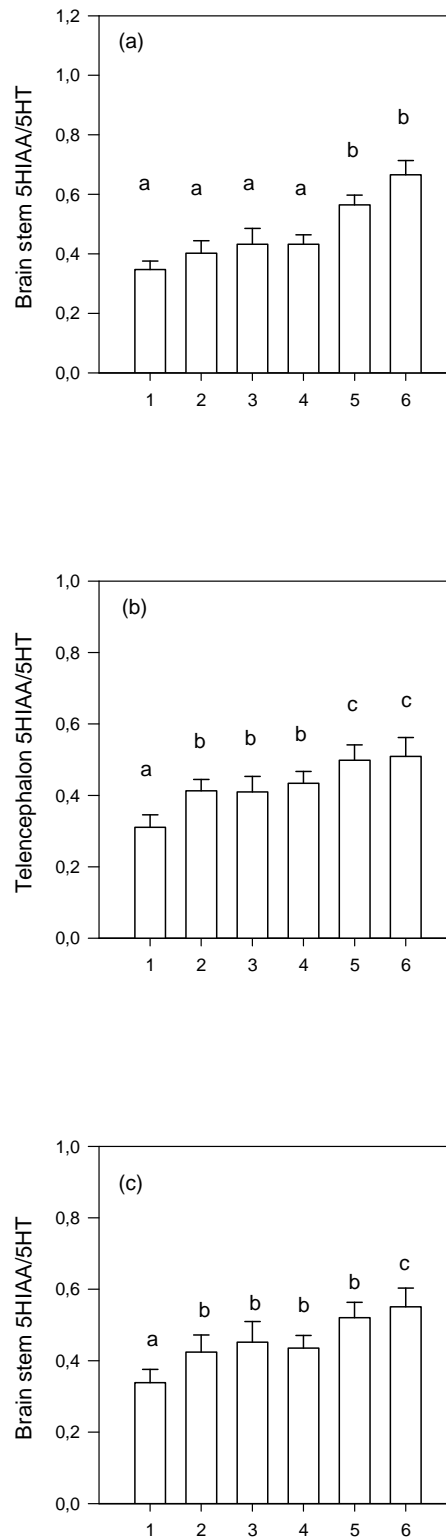


Figure 6.2. Brain 5-HIAA/5HT ratios in (a) brain stem and (b) telencephalon from trial 1 ($n = 69$) and (c) brain stem from trial 2 ($n = 70$) of Atlantic salmon with different social ranks ($n = 12$ per rank). Data shown are means \pm SEM. Different letters denote statistical differences between social ranks, see text for details.

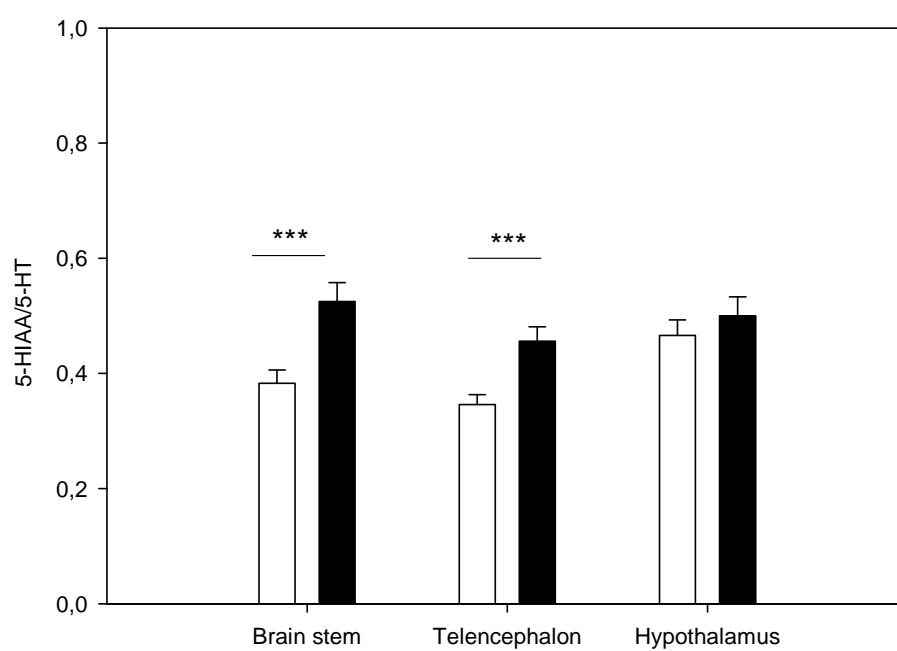


Figure 6.3. Brain neurotransmitter 5-HIAA/5-HT ratio in brain stem, telencephalon and hypothalamus of precocious parr (open bars) (n = 41) and immature parr (closed bars) (n = 28) in Trial 1. Data shown are means \pm SEM. Asterisk indicates a significant difference between phenotypes (***) ($p < 0.001$).

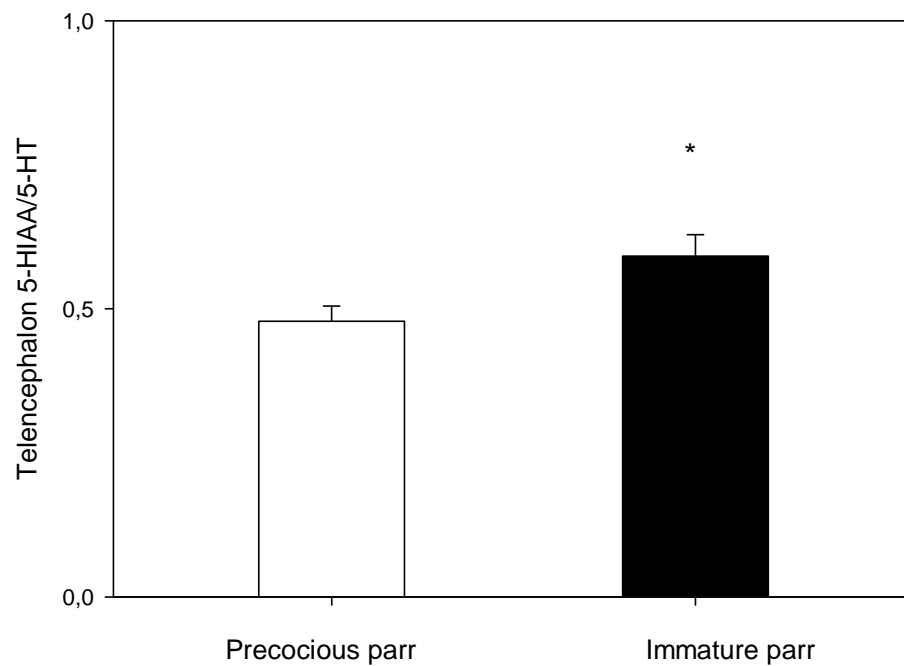


Figure 6.4. Telencephalon brain monoamine metabolite/monoamine ratios of 5-HIAA/5-HT for precocious parr (open bars) ($n = 30$) and immature parr (closed bars) ($n = 30$) from commercial reared conditions. Data shown are means \pm SEM. Asterisk indicates significant difference between phenotypes ($*p < 0.05$).

Chapter 7

A preliminary study into the metabolomic signatures of juvenile Atlantic salmon (*Salmo salar*) with alternative life histories

ABSTRACT

Freshwater Atlantic salmon (*Salmo salar*) with alternative life histories present many differences in neuroendocrine, osmoregulatory and genetic traits. In this study we utilised a metabolomic approach to try to establish if juvenile fish with an immature parr, precocious parr or smolt life history selection presented distinct metabolome signatures. Despite a trend towards separation between groups with alternative strategies these differences were not significant. We discuss possible reasons for this finding and future improvements.

Keywords: metabolomics; Atlantic salmon; life history;

INTRODUCTION

Atlantic salmon populations in their juvenile fresh water phase can be categorised into one of three distinct alternative life histories: immature parr, precocious parr or smolts (Fleming, 1996). Individuals from these diverging strategies can vary markedly in their reproductive, osmoregulatory and behavioural traits (Armstrong et al., 2001; McCormick et al., 2007; Metcalfe, 1998). Numerous studies have demonstrated

dissimilarities in key ionoregulatory enzymes like Na^+, K^+ -ATPase (Ewing et al., 2001; Nielsen et al., 2006; Olsen et al., 1993) and hormonal plasma concentrations such as cortisol, testosterone (T) or 11-ketotestosterone (11-KT) (reviewed by Oliveira, 2006) in fish with alternative strategies. It is important to acknowledge that many of these disparities in physiology vary seasonally and their time scale may be relatively short (e.g. weeks). For example significant elevation in the peaks of T and 11-KT in precocious parr relative to immature parr are found only during the spawning season, being similar among all fish the rest of the year (Mayer et al., 1990).

As current research is aiming towards a more integrative line to underpin complex biological processes novel techniques such as genomics and proteomics provide a holistic view of the interacting processes at the whole organism level (Perry and Burggren, 2007; St-Cyr and Aubin-Horth, 2009; Sweetlove and Fernie, 2005). Recent genomic studies have proved successful in differentiating genetic signatures associated with alternative salmon phenotypes. Juvenile Atlantic salmon show up to a 15 % difference in brain genes expressed between developmental strategies, some of these genes are involved in processes such as growth, reproduction and behaviour (Aubin-Horth et al., 2005a). Further, genomic studies have helped distinguish between genetic and environmental influences on the developmental strategy of salmonid fish (Aubin-Horth et al., 2005b).

Metabolomics, one of the most recent “x-omics”, is the study of metabolite profiles from biological samples including tissue and biofluids (Viant, 2007). There are important advantages to this approach as it shows the actual complete snapshot of the cell by-products (or metabolome) including processes such as cell signalling and energy transfer (Schmidt, 2004). It can also be linked with results of other techniques

such as genomics, transcriptomics or proteomics as it shows the final result of the gene expression, then converted into proteins and enzymes which eventually will be broken down in the cell into an end product or metabolite. If salmonids with different life histories vary in the genes they express as demonstrated by genomics, it could be interesting to find if this also reflects in the metabolites produced by these fish. The purpose of the present study was to investigate if Atlantic salmon with alternative strategies showed consistent metabolic signatures that separated them apart, and if so to identify the metabolites involved to better understand the underlying molecular mechanisms at the cell level involved in these developmental transformations.

METHOD

Experimental fish

Juvenile 1+ Atlantic salmon reared in a river re-stocking facility in Hampshire (UK) were used for plasma collection in July 2007. Samples were obtained onsite by netting individuals of similar size which were immediately anaesthetised with a lethal dose of buffered MS222 (250 mg l⁻¹). Blood samples were drawn from the caudal vessels into heparinised syringes, and placed on ice. Blood samples were quickly taken and plasma was separated by centrifugation and kept at -80 °C for later metabolomic analysis. Body mass (27.98 ± 3.2 ; mean \pm SEM) and fork length (13.41 ± 0.52 ; mean \pm SEM) were also annotated. Two smolts from the same cohort were sampled at a later date (December 2007) to complete the data set. In total 30 male individuals, 10 from each phenotype (immature parr, precocious parr and smolt) were examined.

Metabolomic techniques

Samples were sent to Birmingham University where metabolomic and data analyses were performed exclusively by Dr. Mark Viant and Dr. Adam Hynes using previously described techniques (Viant et al., 2003). The following text represents a verbatim description of the techniques followed in this paper: one dimensional (1D) ^1H nuclear magnetic resonance (NMR) spectra of Atlantic salmon plasma samples were measured at 500.11MHz using an Avance DRX- 500 spectrometer (Bruker, Fremont, CA). Acquisition parameters consisted of a 9-s (60°) pulse, 7-kHz spectral width, 2.5-s relaxation delay, 200 transients collected into 32 k data points at 295 K, requiring a 13-min total acquisition time. The residual water resonance was presaturated during the relaxation period. All 1D data sets were zero-filled to 64,000 points, exponential line-broadenings of 0.5 Hz were applied before Fourier transformation, phase and baseline corrected, and then calibrated (TMSP peak at 0.0 ppm) using XWINNMR software (Version 3.1; Bruker). Selected peaks were assigned by comparison to tabulated chemical shifts (Fan, 1996) and confirmed by 2D NMR. Each spectrum was then segmented into 1960 chemical shift bins between 0.2 and 10.0 ppm, corresponding to bin widths of 0.005 ppm (2.5 Hz), using custom written *ProMetab* software (Viant, 2003) inMATLAB (The MathWorks, Natick, MA). The area within each spectral bin was integrated to yield a 1×1960 vector containing intensity-based descriptors of the original spectrum. Bins representing the residual water peak (from 4.70 to 5.10 ppm) and acetate (from 1.90 to 1.95 ppm) were removed, the latter due to extreme variability in the peak intensity. Next, four groups of bins from 3.75 to 3.80, 7.02 to 7.15, 7.85 to 8.03, and 8.06 to 8.23 ppm were each compressed into a single bin (this captures peaks that shifted due to slight variations of the sample pH into a

single bin). The total spectral area of the remaining 1767 bins was normalized to unity to facilitate comparison between the spectra.

Unsupervised PCA of the pre-processed NMR data was performed using PLS Toolbox (Version 2.1; Eigenvector Research, Manson, WA) within MATLAB. PCA calculates new variables (the PCs) that are linear combinations of the original intensitybased descriptors (chemical shift bins), such that the first PC captures the most variance between the spectra. PCA serves to reduce the dimensionality of the data and summarize the differences between multiple NMR spectra. After PCA, the sample labels were added to the PCA scores plot and the coordinates of these points along the PC1 and PC2 axes were analyzed using one-way ANOVAs followed by Tukey–Kramer post hoc tests using Number Cruncher Statistical System (2001 Edition; NCSS Statistical Software, Kaysville, UT). PCA loadings plots were then used to interpret which peaks in the NMR spectra changed intensity as a result of developmental strategy. A false discovery rate (FDR) test was used to adjust p-values resulting from multiple tests. Putative metabolite ID were obtained by trying to connect a chemical formula that fits the m/z peak identified and then searching on the KEGG (Kyoto Encyclopaedia of Genes and Genomes) online database for possible fitting metabolites.

RESULTS

Comparison of all strategies using PCA analysis showed some overlap between groups. There was a non significant trend in mature parr to have higher PC2 scores compared to smolt individuals (Figure 7.1.). A highly significant trend along the PC6 axis was found in the immature parr versus smolt plot suggesting metabolic

differences. The PC6 component accounted for a small proportion of the overall metabolite variability (7.2 %). A small number of metabolites ($n = 4$) present in immature parr and smolt samples showed a two-fold to four-fold increase in the later. However, after ANOVA p-value correction using false discovery rate applied for multiple sets, no significant differences in p-values were found between metabolites belonging to these strategies. Some of the putative metabolites identified in the Atlantic salmon metabolome are presented in Table 7.1. However, most KEGG searches performed to identify those metabolites with the greatest contribution towards variation between smolts and immature parr proved unsuccessful.

DISCUSSION

This has been the first attempt to investigate disparity in metabolite signatures between teleosts (or vertebrates) with alternative life histories. To date published metabolomic studies are scarce compared to genomic and proteomic work. The majority of metabolomic work on vertebrates has focused on environmental research (i.e. effects of toxicants) and disease monitoring (i.e. characterisation of disease by-products) (Stentiford et al., 2006; Tiziani et al., 2009; Viant et al., 2006; Williams et al., 2009). The reason for this technique being more amicable to these kinds of tests is that knowledge on by-products of the toxic substance or previously mapped metabolic pathways in the disease provide identifiable target metabolites to look for. The comparison of the metabolome of fish with alternative life histories on the other hand was a much more open approach. In this sense this trial was a preliminary exploration to find if any obvious disparities between life histories were reflected in the metabolite signature given the distinct hormonal and ion regulatory changes

undergone through developmental conversion in each phenotype (McCormick, 2001; Stefansson et al., 1991; Young et al., 1995).

Despite a tendency towards separation between the mature parr and smolt group and a significant difference in PC6 between immature parr and smolts, there was a great amount of overlap and within group variation. Smolts had a slightly higher upregulation of some metabolites implicated in growth processes (e.g. L-Glutamine) compared to immature parr. However, (and perhaps surprisingly), no significant differences were obtained from the metabolite comparisons after p-values were adjusted. Perhaps a higher number of replicates per phenotype used ($n = 10$) could have helped reduce variability and obtain more robust results. In addition, a number of samples presented haemolysis signs and might have influenced quality of the readings. Readings from biofluid metabolites tend to be more variable and less stable compared to tissue samples which provide an alternative option (Lindon et al., 2000).

It was also presumed, as fish experienced the same environment, that metabolomic discrepancies or similarities should be attributed to life strategy alone. However, other factors known to affect metabolism such as social status or disease (Metcalf et al., 1995; Seppanen et al., 2008), something we had no information on for these particular samples, could have influenced the metabolic profile of these fish. Also variation within groups may mask some of the metabolomic differences between phenotypes as metabolic rates, peaks in endocrine parameters or expression of ionoregulatory molecules can range dramatically both in the timing and scale within fish of the same life history. It may be that clear metabolite differences are not present at certain times of the year, when most individuals operate in a similar manner. In this sense it is important to understand the biology of the fish and when

key metabolic pathways are likely to be upregulated or downregulated such as during the parr-smolt transformation window and seawater entry for smolts or spawning season for mature parr.

It cannot be ruled out either that at this point in time the techniques used are not advanced enough to detect distinct metabolomes between life strategies. Despite advantages in using NMR techniques such as its reproducibility and being able to analyse samples directly, it has an important drawback which is its low sensitivity and it is estimated that only about 10 % of the total amount of metabolites are picked up (Lin et al., 2006). In this line, metabolites resulting from lipid-based steroids, such as those critically implicated in early parr maturation and parr-smolt transformation (i.e. T, 11-KT, oestrogen, cortisol) are not detectable with NMR. Metabolomics is still a very recent area of research (started in the early 1990's) and most likely advances in equipment and techniques should overcome some of these shortfalls in the future. An integrative approach using genomics, transcriptomics and metabolomics (i.e. gene expression leading to protein formation and its breakdown into by-products at the end of the pathway) at different periods of the developmental conversion would help provide a more detailed account of the processes that modulate it and physiological dissimilarities resulting from this transformation. In summary, this work investigated possible differences in the metabolome of juvenile Atlantic salmon with alternative life histories and showed a non-significant trend towards a separation between phenotypes, especially the smolt versus immature parr groups. Future attempts may benefit from using a more selective approach, zooming in on possible target metabolites, a larger sample size to reduce variation and knowledge of the previous social environment encountered by the fish.

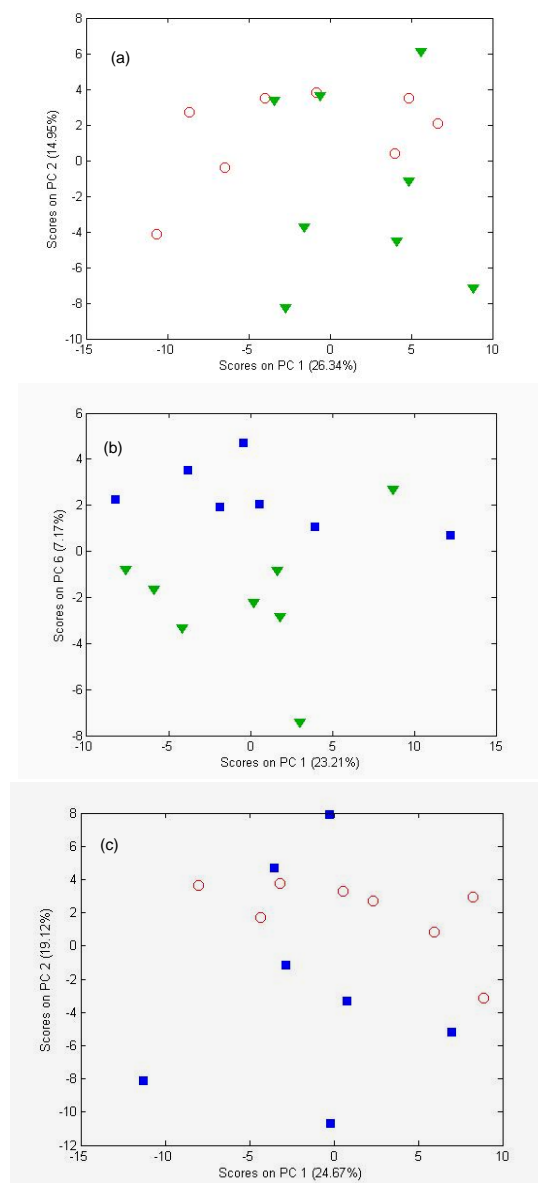


Figure 7.1. Principal Component Analysis (PCA) scores plot from analysis of the NMR metabolomic fingerprints of plasma from juvenile Atlantic salmon with alternative life histories. (a) immature parr (▼) versus precocious parr (○), (b) immature parr versus smolts (■), (c) smolts versus precocious parr.

Putative metabolite	Non-adjusted p-value	Fold change smolt/immature parr	Fold change smolt/mature parr
D-Ornithine hydrochloride	0.0001	1.66	1.31
L-Glutamine	0.004	1.57	1.22
3-(4-Hydroxyphenyl)lactate	0.001	1.73	1.72
2-Methylcitrate	0.01	1.04	0.60

Table 7.1. Putative metabolites (KEGG database) identified as contributors to Principal Component Analysis (PCA) separation between juvenile Atlantic salmon with alternative life strategies.

Chapter 8

General discussion

This thesis set out to test if social status played a role in the physiology, development and reproductive tactics in salmonid fish. This point has been supported by the experimental findings presented throughout this work showing evidence of socially-mediated alterations to the osmoregulatory, hormonal and brain serotonergic systems in Atlantic salmon living in groups. For example, chapter 3 showed for the first time distinct *in vivo* ion uptake kinetics, indicative of differential ion mechanisms, in juvenile Atlantic with alternative life strategies by examining Na⁺ uptake kinetics with radioactively marked isotopes in an unusually high number of fish for this kind of study. This technique has the potential to be used as a tool for both differentiation between parr destined for different life histories before this is externally identifiable for instance when subsampling salmon parr to know how many fish will become smolts or mature parr for river restocking purposes, and also can help underpin the complex reorganisation of ionoregulatory processes prior to smoltification. Chapter 4 followed these findings a step further and found that ion uptake kinetics not only are influenced by phenotypic development but are also affected by social position. Again this is the first time that the physiological variable of Na⁺ uptake kinetics has been shown to vary with social rank in Atlantic salmon. Importantly it showed a non-linear fashion between social rank and Na⁺ uptake kinetics, as the ion uptake kinetics of second ranked fish was more similar to that of the most subordinate individuals in the hierarchies rather than the dominant fish. This ionoregulatory similarity between second ranked and most subordinate fish was also observed in plasma cortisol,

commonly used as an index of stress in salmonids. Chapter 2, 5 and 6 used different scenarios to investigate the role of social rank during the development of Atlantic salmon in fresh water and related these findings to brain monoaminergic activity patterns. The brain serotonergic system is a key regulator of aggressive and inhibitory social behaviour in teleost fish and other vertebrates (Winberg et al., 1997). In chapter 2, most dominant individuals showed high growth even when kept in low feeding conditions, and this may have been mediated by their higher SMR, which was subject to feeding regime, and lower serotonergic activity (this is the first time these two important physiological variables have been examined in conjunction in teleosts). Similarly, although brain serotonergic activity has been investigated in numerous fish studies in relation to social context, here I report for the first time brain monoamine patterns in fish hierarchies with structured size asymmetries (chapter 5). This is important if we are to understand how salmonid social networks operate both in the wild and aquaculture given the pronounced size bimodality showed in natural streams and in captivity. Multi-member complex social networks may result in more physiological similarities (at least under laboratory conditions) between dominants and subordinates than dyadic interaction studies would suggest given that fish in pairs tend to show opposite extremes in physiology (e.g. plasma cortisol, serotonin, SGR). In addition, in chapter 6 this thesis shows for the first time distinct brain monoamine activity in size-matched animals in relation to alternative life strategies, with precocious parr showing lower brain 5-HIAA/5-HT ratios, higher social ranks and aggressive behaviour compared to immature parr in the laboratory. Similar brain serotonin patterns were observed in high density aquaculture conditions. This finding highlights the importance of acknowledging phenotypic identity of individuals in restocking programs (e.g. what are the proportions of fish of each type introduced and

how this might affect their growth and survival chances?) for successful reintroduction to the wild.

This thesis has tried to contribute, if only in a modest way, to better understand how social status interplays with some components of the physiology and development of salmonids, using the Atlantic salmon as a model organism. The studies have focused on providing an integrative approach trying to connect the dots between social behaviour and diverse physiological elements including hormones, brain monoamines, ion uptake or metabolism. In addition the experiments have tried to incorporate relatively complex social environments (i.e. above dyads of dominant-subordinate set ups) to try to better mimic natural hierarchies and the degree of physiological alteration imposed by compound social interactions. For example, in chapters 3-6 similar group sizes (6 fish) were used, which is in the range of some of the observed salmonid groups in the wild (e.g. 6-10 fish in Sloman et al., 2008). Even in chapter 2 where dyads were used, sequential fights were used to add a degree of social complexity yielding four social categories.

A general theme identified during most of the chapters was that larger fish (at least under the laboratory conditions described) appear to attain the highest ranks in the groups. This was particularly clear in groups which started out the experiment with an initially large size advantage, like in chapter 4 and chapter 5. However, the benefits of being a high ranked fish did not yield equally in all experiments. For example there seems to be some contradictions between the higher SGR in large versus small fish in chapters 2 and 4, while this is not observed in chapter 5. It may be that apparently similar social networks (i.e. groups of 6 juvenile salmon) may vary considerably in their behaviour and physiology according to relative size differences within these groups. This is what chapter 5 suggests as strong size asymmetries may

polarize aggressive behaviours between high ranked fish of similar size. It is not size *per se* which determines behavioural outcomes but rather the relative size between individuals in the group.

Because some variables changed between experiments (i.e. phenotypic composition of the group, origin of the fish, time of the year, and duration of the trials) it is difficult to establish direct comparisons between some of the chapters. A more constant set of laboratory conditions (i.e. equal tank design) or experimental layout (i.e. always the same age or size of fish) between experiments may have eliminated some of that experimental “noise”. As always, in hindsight many improvements could have been made to the design and execution of some of these experiments. However, one must be realistic about the restriction on budgets, aquaria space, obtaining large number of fish from one source or time issues. This last point is especially important when trying to conduct experiments on the life histories of animals, like salmon, that take months to years before reaching the desired short lived window of developmental pathway (i.e. smoltification during 4-8 weeks in any given year) that is intended to study. In addition controlling the proportion of fish that will reach certain life history is at present impossible. For example despite using over 50 fish for the second experiment in the Na^+ uptake kinetics none of them turned into precocious parr. Perhaps in the future advancement in genetic techniques or others (i.e. proteomics, metabolomics) may make this task easier.

Some of the findings using multiple member groups may apply to the fish farming industry, as observed in the similar brain serotonergic patterns observed in the immature and precocious parr in the lab and the aquaculture facilities. Focus has been on the freshwater phase because as well as logistic experimental advantages (i.e. handling smaller fish and smaller holder facilities requirements), this is also the time

when social behaviour and their consequences start to develop, sometimes leading to dramatic physiological metamorphosis in these fish.

Integrative approaches are desirable as behaviour is known to affect many aspects of physiology ranging from primary to tertiary responses. Although many studies on salmonid social behaviour have a history of working on some of the more common physiological correlates of social status such as cortisol, no doubt further physiological parameters will emerge in the future to create a map of many reliable social indicators. For example, hormones affecting feeding such as ghrelin or neuromodulators such as neuropeptide Y and their relation to behaviour might prove productive (Eva et al., 2006; Abizaid, 2009). Identification of novel differentially expressed biological compounds may originate from studies applying overall views on physiological responses such as the “omic” techniques (i.e. genomics, proteomics, metabolomics). In this respect the few studies such as those of Aubin-Horth et al. (2005), Sneddon et al. (2005) or St-Cyr and Aubin-Horth (2009) linking genomic signatures to behaviours and life histories in salmonids to uncover their mechanistic basis are highly informative. Zooming in on the genetic components that have been identified as differentially expressed between fish with alternative life strategies in these studies would be the next step. Blocking or stimulating some of the genes/hormones/neuropeptides thought to be involved with life history adoption may prove useful.

Advancements on the characterisation of ionoregulatory mechanisms in teleosts can contribute to explain how fish prepare for movement between different water masses. Differential ionoregulatory profiles as those found in this study can help predict the future phenotypic outcome of fish destined for alternative histories. Despite being somewhat expensive and time consuming sodium uptake kinetics may

currently be the only non-invasive method for making such a prediction. This could perhaps be used at least as a research tool, and if cheaper and/or safer alternative methods (e.g. stable instead of radioactive isotopes) could be developed, then perhaps it could even be used for example in restocking programs using a subsample of fish to estimate the proportion of fish from each phenotype that will be introduced into the river. In addition, following the observation of life history associated ion uptake patterns this kinetic technique would be useful to test if individuals of a certain strategy cope better or worse with osmoregulatory challenges (e.g. acidification, contamination events). Sodium kinetic analysis also showed that social hierarchies influenced ion regulation and these differences may be important if subjected to disrupting events such as trace metal contamination as demonstrated in other studies (reviewed by Sloman et al., 2007). Future work should aim at identifying if it is the social position *per se* that most influences the osmoregulatory patterns (i.e. being most dominant) or it is the amount of stress experienced the key determinant. For example would top rank fish in highly stressed groups (e.g. subjected to predation or handling) suffer a higher ionoregulatory disruption than lower ranked fish in a less stressful environment? These results would suggest so given the ionoregulatory similarities found between highly subordinate fish and subdominant individuals receiving most aggression from dominants and experiencing high stress levels as indicated by cortisol concentrations. However, the differential affinity of ion transporters in different ranked fishes will also need to be assessed for their relative affinities for non-target ions, especially toxic metals.

Given that juvenile salmonids that will reach early precociously maturation or smolting often show a high social rank in early life that is usually maintained throughout development, as we found in our UMG fish, it would be pertinent to ask if

these fish have a certain “personality” (*sensu* Sneddon 2003), bold or shy fish, or cortisol high-responsive (HR) or low-responsive (LR) traits as suggested by the studies of Pottinger and co-workers (Øverli et al., 2001; Pottinger and Carrick, 2001; Schjolden et al., 2005) that favour specific life historical outcomes (i.e. are smolts HR and immature parr LR?). The differences in brain monoamines between Atlantic salmon and associated physiological responses in our studies, such as the stability of dominant status against changing feeding conditions in chapter 2, would favour this hypothesis.

The finding of brain monoamine profiles differences in Atlantic salmon with alternative reproductive strategies, namely immature parr and precocious parr, may also apply to other salmonids species with alternative life histories. Further, in fish social systems with alternative life histories brain monoamine studies may help understand the behavioural ecology of these systems (e.g. parasitic versus bourgeois, non-territorial versus territorial). Teleosts alone show the widest range of reproductive modes of any vertebrates (Thresher, 1984), and mechanisms of brain monoamine activity perhaps may differ between fish with cooperative or aggressive courtship reproduction. Given the highly conserved structure of the brain serotonergic system in vertebrates and the existence of socially-induced reproductive strategies in many animals (e.g. birds of paradise, naked mole rats) it might be also appropriate to investigate brain monoamines mechanisms that may apply across taxa.

Currently one of the biggest challenges faced by fish biologists working on the behaviour and physiology of social species is to bridge the gap in the findings between laboratory and field based experiments. Conducting laboratory studies is the first stepping stone to try to start understanding how social dominance relationships work in salmonid fish as it enables manipulation and control of certain variables (i.e.

predation, feeding conditions, size structure, number of fish in a hierarchy, etc). On the downside it may introduce artefacts into the interactions, such as magnification of the difference in physiological stress responses found in dyadic studies, less natural feeding conditions or limited options to escape a contestant (see review by Sloman and Armstrong, 2002). Behavioural studies of fish in their wild streams are notoriously difficult however, and advances in tracking and recording equipment may prove important in the future for successful field studies. Meanwhile, trying to add layers of complexity in terms of number of members in a hierarchy, provision of cover to escape attacks, predation situations, and other elements which may help collect more “real life” data to understand the behavioural ecology and how physiological control mechanisms such as brain monoaminergic and neuroendocrine systems operate in the wild must be the way forward.

From a practical standpoint understanding more about the behavioural patterns in salmonid phenotypes may help reduce deleterious physical effects such as fin injuries or reduced growth rates in aquaculture (e.g. separating aggressive precocious parr from other phenotypes). In addition, given the increasing practice of salmonid fish river restocking our results point towards a more careful consideration to the proportions of fish from each phenotype introduced into the wild as it can have deep ecological implications. For instance, introducing a large number of male precocious parr into a river system not only will mean that most fish will not migrate that year and potentially fewer migratory anadromous females than expected will be obtained; but also, these precocious parr in high condition will put a strain on river resources (e.g. good feeding territories) which they may gain through greater aggression and dominance status from native immature parr, therefore reducing the growth chances and jeopardising the future developmental conversion of the latter.

To conclude, this thesis has tried to contribute to the current understanding of the connections between salmonid social status in complex hierarchies and their physiological consequences. Novel findings reported include the strikingly distinct Na^+ uptake kinetics of salmon parr prior to developmental differentiation, the importance of accounting for fish composition in hierarchies (i.e. size asymmetries) which can lead to dominants and subordinates showing similar stress physiology, and the differential patterns of brain serotonergic activity associated with a particular life history in salmonid fish.

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