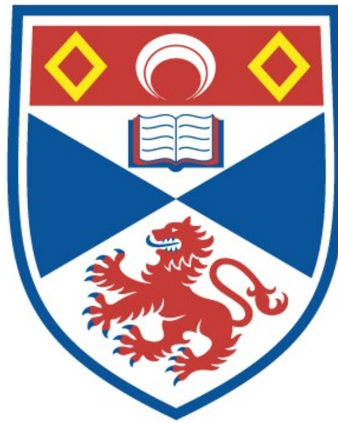
LIFE HISTORY STRATEGIES IN ANADROMOUS TROUT, 'SALMO TRUTTA L.' : WITH SPECIAL REFERENCE TO OSMOREGULATORY PHYSIOLOGY

Alan Melville Walker

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Life history strategies in anadromous trout, *Salmo trutta* L., with special reference to osmoregulatory physiology

A thesis submitted to the University of St Andrews for the degree of Doctor of Philosophy

by

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This thesis is dedicated to my family.

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Abstract

1. Juvenile trout, *Salmo trutta* L., from three parental groups - sympatric Sea trout and freshwater-Resident trout, and Isolated trout from above a waterfall impassable by upstream migrating anadromous trout - were reared under three ration regimes to manipulate growth rates. The development of seawater tolerance was studied by measuring drinking rates after periodic salinity challenges during the first two years of juvenile growth. No trout were observed to undergo the parr-smolt transformation in any of the parental form/ration combinations after two years in freshwater. However, a considerable proportion did mature during this time period. The proportion of maturing trout was directly related to ration level but was also influenced by parental form, with Isolated trout demonstrating a greater tendency to mature early. Seawater tolerance increased with age in all groups. However, mean drinking rates upon salinity challenge were generally lower, from Experiment 2 onwards, in Resident trout than in either of the other two groups.

2. Eight immature sea trout (finnock) were radiotracked in the River Eden, Fife, during September, October and November 1994. The individual finnock displayed considerable variation in patterns of movement; two remained in freshwater for at least 27 days whereas others moved downstream out of the river within days or even hours of release. In general, this highlighted the transient nature of the freshwater migrations of some finnock, indicating that they move in and out of rivers over brief periods of time and apparently do not necessarily remain in freshwater continuously throughout the winter.

3. The hypo-osmoregulatory ability of finnock during the winter was assessed in two experiments. The number of finnock was limited in Experiment 1. Therefore, this was designed as a preliminary assessment of the physiological response of finnock to acute freshwater-seawater transfer. Osmoregulatory abilities were assessed by measurement of drinking rates, plasma ion and plasma cortisol concentrations after acute freshwater-seawater challenge and compared with freshwater-adapted and seawater-adapted control groups. Finnock displayed physiological responses typical of euryhaline teleosts upon seawater challenge: a rapid increase in drinking rate, an increase in plasma ion concentrations (but only to levels similar to, or slightly greater than, those of seawater-adapted fish), and increased plasma cortisol concentrations. The second experiment, in which numbers of finnock were greater, made use of the same techniques to assess the longer term acclimation of finnock to both freshwater-seawater and seawater-

freshwater challenge, to establish whether finnock might suffer from a more subtle reduction in seawater-tolerance which would not have been necessarily apparent in the acute challenge of Experiment 1. Finnock did not appear to be physiologically compromised by seawater challenge during the winter months, and therefore, a breakdown in hypo-osmoregulatory abilities alone can not be considered a reason for finnock returning to estuaries and rivers during the winter.

4. The physiological effects of low to medium levels of infestation of the ectoparasitic copepod *Lepeophtheirus salmonis* (Krøyer) on wild sea trout post-smolts were assessed at intervals during the development of the parasite. A mean infestation level of 18 parasites caused significant disruption to the osmoregulatory ability of hosts, as demonstrated by significantly higher plasma osmolality and chloride ion concentrations when compared with naive post-smolts. In addition, since no skin lesions were apparent on the hosts, these physiological effects were considered to be the consequence of larval attachment to the gill filaments, thereby possibly puncturing the epithelia and also damaging vital branchial ion excretory cells.

5. The modern molecular genetic RAPD-PCR technique was used to screen DNA of *Lepeophtheirus salmonis* collected from wild and farmed salmonid hosts from around the Scottish coasts. This technique indicated markedly different patterns of genetic variation amongst *L. salmonis* of farmed and wild origin, and between different farms. A number of genetic markers were found to be exclusive to, or at considerably higher frequency amongst, sea lice collected from farmed salmonid hosts. This technique established the possibility of assigning provenance to *L. salmonis* collected from wild hosts.

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Chapter 1

General Introduction

1.1 Migratory strategies of Fishes

1.1.1 Diadromy

Historically, the most studied migrations of fishes are those which involve movement between the freshwater and seawater environments at different stages of the life cycle. These migrations usually result in a spatial and temporal bottleneck when the fish are most susceptible to predation/harvesting, and this is reflected in the bias towards commercially important and valuable species in our understanding of these migrations. The collective term for these directed migrations is diadromy, and is comprehensively reviewed by McDowall (1988). Diadromous fish are defined (Myers, 1949 cited in McDowall, 1988) as those which "normally, at regular and predictable phases in the life cycle, and for the majority of the population, migrate between marine and fresh waters". Note, however, that in many species commonly regarded as diadromous, there are populations, or even parts of populations, which deviate from this pattern: they fail to undergo migrations between freshwater and seawater, either because of physical barriers or differences in biological/environmental factors.

Present knowledge of the occurrence of diadromy within the fish taxa is limited, especially with regard to fish species from the tropics and the Southern Hemisphere. At present it is thought that at least 170 species display diadromy, ranging from lampreys to sticklebacks, but this is almost certainly an underestimate due to the paucity of knowledge regarding many species. However, those 170 species represent only 0.85% of the estimated 20,000 extant fish species described (McDowall, 1988).

Diadromy tends to occur primarily, and is strongly represented, in the more primitive and ancient fish groups such as the mordaciids (lampreys), acipenserids (sturgeons), anguillids (eels), the salmonids (salmon, trout and charrs) and clupeids (herrings). All are considered to be derived from ancient, basal stocks of fishes that date from the Mesozoic (180 mya) (Romer, 1966 cited by McDowall, 1988). Amongst the more recently diverged groups - paracanthopterygii, acanthopterygii - diadromy is a rare and highly intermittent phenomenon (McDowall, 1993). However, although all diadromous groups are 'ancient' or 'relic', the vast majority of these taxa are obligatory freshwater fishes (McDowall, 1988).

Within diadromy, migratory types are further split into three sub-categories; anadromy, catadromy and amphidromy. Although Meek (1916) probably was responsible for the wide use of the first two terms in the English literature, Myers (1949 cited in McDowall, 1988) is regarded as having established the current definitions of all three terms. Those definitions, as given by McDowall, are as follows, with diagrammatic representations of the migrations and examples given in Figure 1.1:

Anadromy - fishes which spend most of their lives in the marine environment and which migrate to freshwater to breed as fully grown, mature adults.

Catadromy - fishes which spend most of their lives in freshwater and which migrate to the sea as fully grown, mature adults for the purpose of breeding.

Amphidromy - fishes whose migration between freshwater and seawater, or vice versa, is not for the purpose of reproduction but occurs regularly at some other stage of the life cycle and is typified by a return migration of well-grown juveniles, which continue to feed and grow for months or even years prior to maturation and breeding. In amphidromous species occupation of fresh and marine waters varies widely.

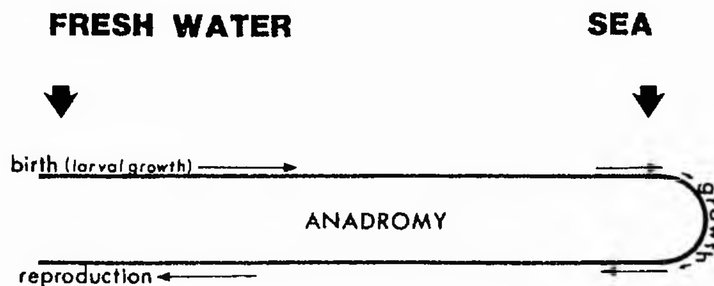
1.1.2 Strategic value of diadromy

Diadromy, like other migratory strategies, requires greater benefits than costs in order to persist in evolutionary terms. The potential advantages of migration include (1) increased food supply and growth opportunity, (2) avoidance of potentially harmful environmental conditions and/or a movement to more favourable conditions, (3) occupation of habitats which meet specific requirements, and (4) the availability of increased space. Costs incurred may include mortalities arising from (a) the migration itself, (b) physiological mechanisms required to adapt to changes in environmental conditions, e.g., osmoregulatory stress, (c) higher predation rates due to inexperience of a new habitat and/or predators, (d) new diseases, and (e) the possibility of becoming lost and unable to return to spawning grounds.

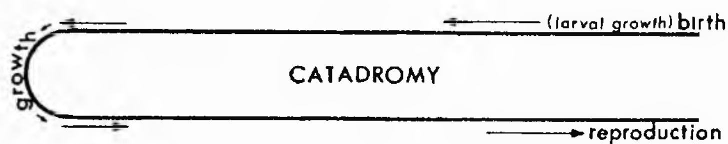
McDowall (1988) noted that all diadromous migrations involved the costs of osmoregulatory adaptation (see later), but that the advantages could be discussed under three headings: trophic, climatic and reproductive.

Figure 1.1

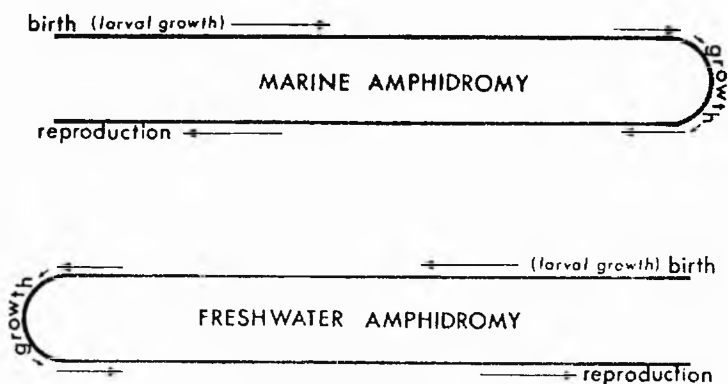
Figure 1.1 Outlines of life history patterns in anadromous, catadromous and amphidromous fishes, with examples of each.
After McDowall (1988).



e.g., some lampreys, sturgeons, salmonids, osmerids, salangids, retropinnids, some aplochitonids, clupeids, an engraulid, gasterosteids, a gadoid, some precichthyids, and some gobies



e.g., angullid eels, some clupeids, one each from the megalopids, centropomids, percichthyids, bovichthids, scorpaenids and galaxiids, and some of the cottids, mugilids, eletrids and pleuronectids



e.g., prototroctids, most diadromous galaxiids, one each of the plecoglossids and mugiloidids, and some eleotrids and gobiids

Trophic

Migration to a more abundant food supply confers the advantage of increased growth rates and thereby, increased fecundity (Bagenal, 1969) and increased breeding success through intrasexual dominance, allowing possession and defence of mates and territories (Gross, 1987). Such an advantage is apparent in the case of anadromy, involving movement from rather oligotrophic and unproductive freshwater habitats where food items tend to be relatively rare and large, to the more productive marine environment where food items are more abundant and smaller. The trophic benefits of catadromy are less clear for fish such as the European eel (*Anguilla anguilla*). Leptocephalus larvae migrate from the relatively nutrient-poor Sargasso Sea across the Atlantic Ocean to grow for several years in the oligotrophic freshwaters of western Europe, prior to returning to the Sargasso Sea to spawn.

A comparison of the relative productivity of marine and freshwater habitats at various latitudes with the distribution of diadromous species has suggested that anadromy occurs where food resources in the ocean exceed those of freshwater, and catadromy occurs where food resources are greater in freshwater than the surrounding seas (Gross, 1987), but this is somewhat of an oversimplification.

Climatic

It is often difficult to separate climatic from trophic migrations and probably most migrations involve a component of each. However, one example of the former is demonstrated by some fish (mostly salmonids) of the high Arctic which migrate into freshwater in winter to avoid low seawater temperatures. Although freshwater temperatures probably will probably be colder, some physiological mechanisms vital for seawater survival are ineffective at very low water temperatures (see Chapter 4). McDowall (1988) could find no reference to catadromous examples of climatic migrations.

Reproductive

Since all diadromous migrations involve return migrations, they can all be classified as involving a spawning component, whether it occurs immediately upon return or is delayed. The inherent advantage lies in congregating large numbers of large adult fish in areas which, while suitable for juvenile growth, would not support such numbers of large adults.

Section 1.2 Salmonidae

1.2.1 Origins of Salmonidae

Arguably the most widely known diadromous fishes, and certainly the most studied, are the anadromous salmonids. Salmonids are characteristic of cool and cold waters of all northern continents, occurring from the Arctic drainages of Europe, Asia and North America, as far south as the Mediterranean, northern Africa, some of the southern states of the U.S.A., and northern Mexico.

The family Salmonidae is comprised of three main genera; *Salmo* (2 species), *Oncorhynchus* (14 spp.), and *Salvelinus* (7 spp.) (Rounsefell, 1958; Rounsefell, 1962; Stearley and Smith, 1993). Previously, the Thymallidae (e.g., grayling, *Thymallus* spp.) and Coregonidae (e.g., Whitefish, *Coregonus* spp.) were included but currently are considered to be separate families (Maitland and Campbell, 1992).

The family Salmonidae is very ancient and evidence suggests divergence from other fish Families occurred around 100 million years ago (Holcik *et al.*, 1988). The genus *Oncorhynchus* may be more primitive than *Salmo*, based upon the five million year old fossil record of a giant salmonid, *Smilodonichthys,* with characteristics more akin to those of Oncorhynchids, especially the coho salmon (*Oncorhynchus kisutch*, Walbaum) (Cavendar and Miller, 1972).

Whether the salmonids have a freshwater or marine origin has been a matter of exhaustive, and inconclusive debate. Tchernavin (1939, cited by Hoar (1976)) concluded that salmonids were most likely of freshwater origin principally because while there are numerous wholly freshwater populations there no wholly marine species and all salmonid species spawn and incubate their eggs in freshwater. Further supporting evidence includes: their extremely broad distribution in freshwater; parallel anadromous and resident forms; a tendency to home to a natal freshwater system; and the presence of many features in the life of the freshwater forms which are present only during the juvenile stages of migrants.

In contrast, Thorpe (1982, 1984) suggested a marine origin based upon the following points. Some of the physiological and morphological changes associated with smoltification (see later) are observed simultaneously in resident parr, thereby

indicating endogenous control mechanisms independent of the smoltification process. Smoltification is the enhancement of these changes upon achievement of certain physiological thresholds and may render the fish unable to survive in freshwater, thus inducing migration to the sea. Accordingly, Thorpe (1982) suggested this process represented the re-emergence of ancestral marine adaptations which were suppressed during the evolution of a freshwater phase.

However, this view fails to address Tchernavin's two main lines of evidence (see above), and the consensus of current opinion is that salmonids are indeed of freshwater origin. Regardless of ancestral origin, however, the most important factor influencing the further evolution and speciation of salmonid fishes, within the last two million years, has been the geographic isolation and genetic divergence resulting from the multiple Pleistocene glaciations (Behnke, 1986; Ferguson, 1986; Hamilton *et al.*, 1989; Nyman, 1989).

1.2.2 Life Histories

Typically, salmonids bury their relatively large yolky eggs in a gravel stream bed during the late autumn and early winter months. Fully developed juveniles emerge from the gravel some weeks or months later, depending upon the local water temperature regime. As they grow, competition for space (territory) and food may result in juveniles migrating to larger, deeper areas of water. The epitome of this migration is a directed movement to the marine environment. Once at sea, anadromous salmonids spend a variable period of time growing quickly before returning to freshwater, commonly their birthplace, to mature and spawn. Pacific salmon (*Oncorhynchus* spp.) are semelparous in that they die after spawning. Most Atlantic salmon are semelparous also, but some individuals do survive to spawn more than once, whereas anadromous *Salmo trutta* are iteroparous in that they may survive to spawn in several seasons.

Within the salmonids, almost every possible variation in this general life history pattern is exhibited (Le Cren, 1985). The migratory behaviour of different salmonid species can be grouped into four classes in order of the decreasing strength of the freshwater base (adapted from Nordeng, 1989) (Table 1.1). To illustrate the flexibility of the salmonid life cycle, the life history of the trout, *Salmo trutta* L., will now be considered in detail.

Table 1.1

Table 1.1 Four classes of anadromous salmonid migratory behaviour, in order of decreasing strength of their freshwater base.
After Nordeng (1989).

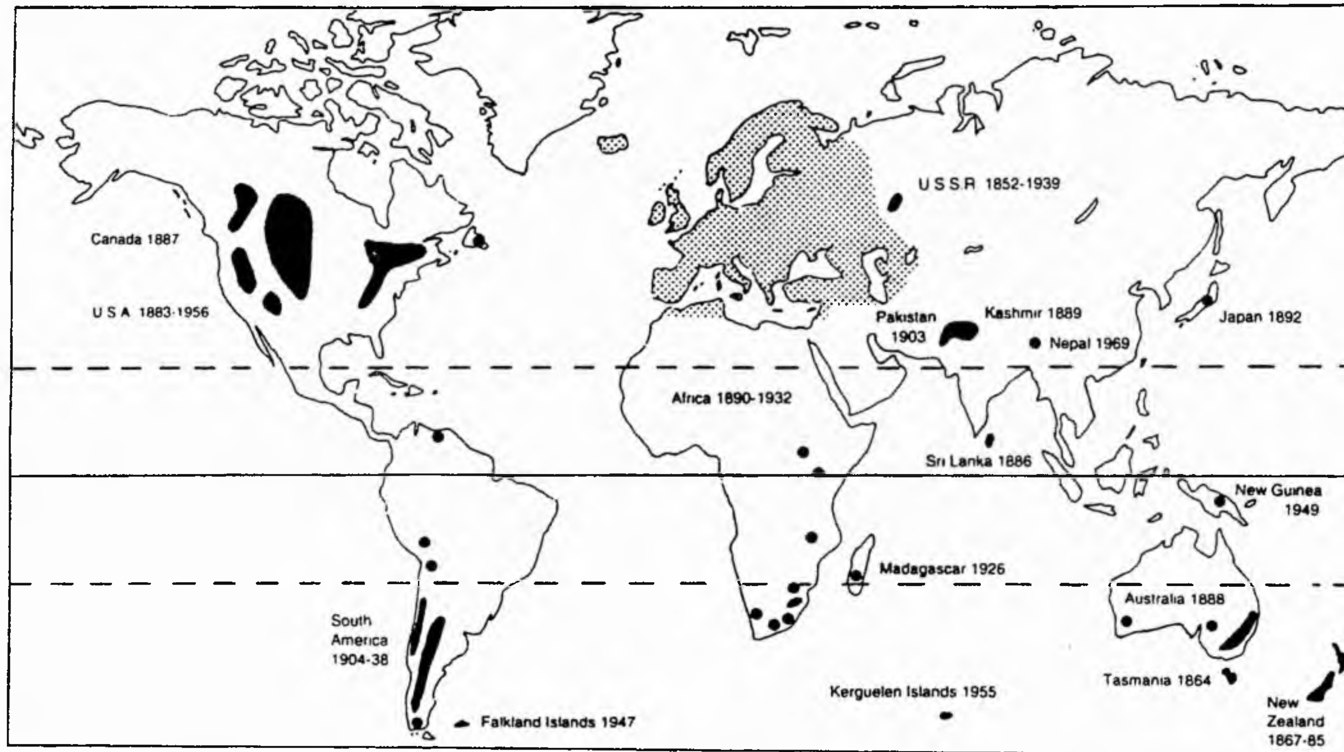
Class	Behaviour type	Smoltification ?	Examples
1	annual, coherent migration with permanent freshwater base consisting of individuals of all stages of development	Yes	Brown trout, Arctic charr (*Salvelinus alpinus*), Rainbow trout (*Oncorhynchus mykiss*)
2	annual migratory system with permanent freshwater base for juveniles and precocious parr of either both sexes,	Yes	Masu salmon (*Oncorhynchus masou*), Sockeye salmon (*Oncoryhnchus nerka*),
	or only males		Atlantic salmon, Coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*)
3	annual migratory system with weak freshwater base; all fry migrate to sea soon after hatching	No	Chum salmon (*Oncorhynchus keta*)
4	biennial migratory system with no permanent freshwater or marine base; all fry migrate to sea and return to spawn after one year	No	Pink salmon (*Oncorhynchus gorbuscha*)

2.3 *Salmo trutta*

The brown trout, or sea trout as the anadromous form is called, is indigenous to Europe, North Africa and North Western Asia. Furthermore, it has been successfully introduced to at least 24 other countries since the middle of the nineteenth century and is now found in all the continents except for Antarctica (see references in Elliott, 1989) (Figure 1.2). Thus, *Salmo trutta* is arguably one of the most successful salmonids as the extensive native and introduced ranges testify. Water temperature appears to be the primary limiting factor to its successful introduction. The thermal range for growth lies between 4°C and 19.5°C, with lower and upper survival limits of 0°C and 25°C – 30°C, respectively, depending upon acclimation temperature (Elliott, 1981; Elliott, 1982). Thermal limits for ova incubation are between 0°C and 15°C (Elliott, 1981) although mortality increases markedly above approximately 13°C (Frost and Brown, 1967).The general life history pattern of brown trout is represented by Figure 1.3, with various stages and morphs shown in Plates 1.1 to 1.9. The standard terminology of Allen and Ritter (1977) is used for the different life stages. Typically, adults lay eggs in the gravel beds of flowing streams and rivers in November and December (Plate 1.1). Some weeks or months later, the juveniles hatch as alevins with yolk sacs and remain in the gravel for several weeks, subsisting on their yolk reserves before emerging as fry (Plates 1.2 and 1.3). Mortality rates are high at this stage as the fry disperse, establish territories and learn to feed on drift particles. Soon they become parr, recognised by 8 - 10 dark stripes (parr marks) along each flank and a characteristic red or orange tip to the adipose fin (Plate 1.4). It is from this stage onwards that the marked plasticity of this species becomes apparent. Most parr spend the first year in the natal stream and, given sufficient space and food resources, some will remain and mature after 1 - 4 more years. However, where growth conditions are less suitable for larger juveniles, two migratory tactics are adopted; either parr migrate to larger streams, rivers or lakes to grow further and eventually mature (Plates 1.5 and 1.6), or juveniles, once they reach a sufficient size, migrate from freshwater to seawater. This latter migration usually is preceded by a coordinated series of external and internal changes which pre-adapt the juvenile to the marine environment. This pre-adaptation is called the parr-smolt transformation or smoltification (see below) (Plate 1.7). Further plasticity is displayed amongst those trout which migrate to sea. While some remain at sea for at least one winter before returning to freshwater to spawn, a variable proportion of juveniles return to estuaries and freshwater within a few weeks or months (Plate 1.8). These juveniles, with regional names such as finnock, school peal, whitling or herling, overwinter in

Figure 1.2

Figure 1.2 World distribution of brown trout. Stippled area represents the natural range while solid areas represent successful introductions. Dates refer to approximate period of introductions. After Elliott (1989).



Canada 1887
U S A 1883-1956
U S S R 1852-1939
Pakistan 1903
Kashmir 1889
Nepal 1969
Japan 1892
Africa 1890-1932
Sri Lanka 1886
New Guinea 1949
Madagascar 1926
Australia 1888
South America 1904-38
Tasmania 1864
Kerguelen Islands 1955
New Zealand 1867-85
Falkland Islands 1947

Figure 1.3

Figure 1.3 Schematic representation of the life cycle of the trout, *Salmo trutta.*

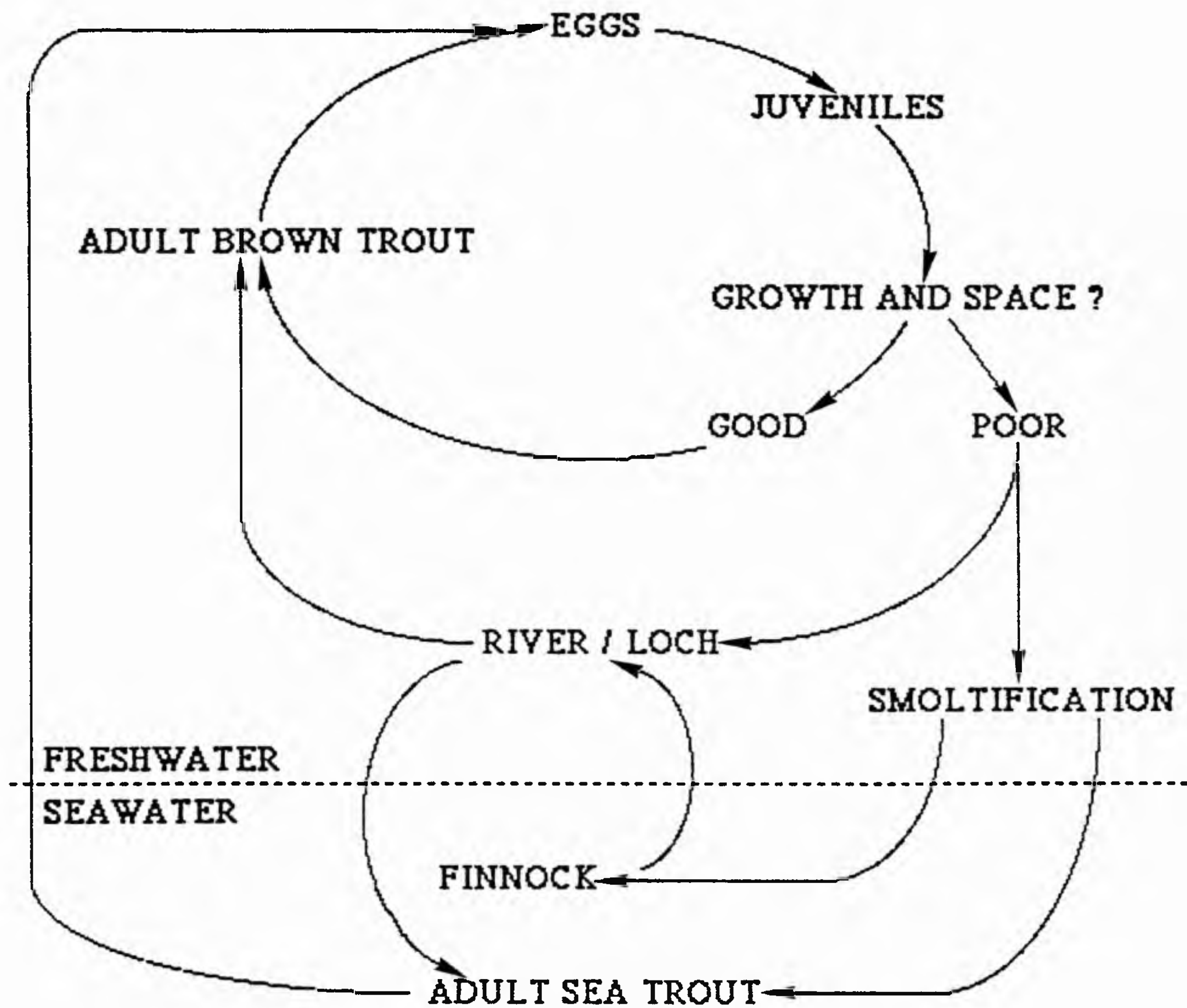

EGGS
JUVENILES
ADULT BROWN TROUT
GROWTH AND SPACE ?
GOOD
POOR
RIVER / LOCH
SMOLTIFICATION
FRESHWATER
SEAWATER
FINNOCK
ADULT SEA TROUT

Plate 1.1

Plate 1.2

Plate 1.1 Mature adult female trout being manually stripped of eggs.

Plate 1.2 Trout alevins emerging from gravel of spawning bed.

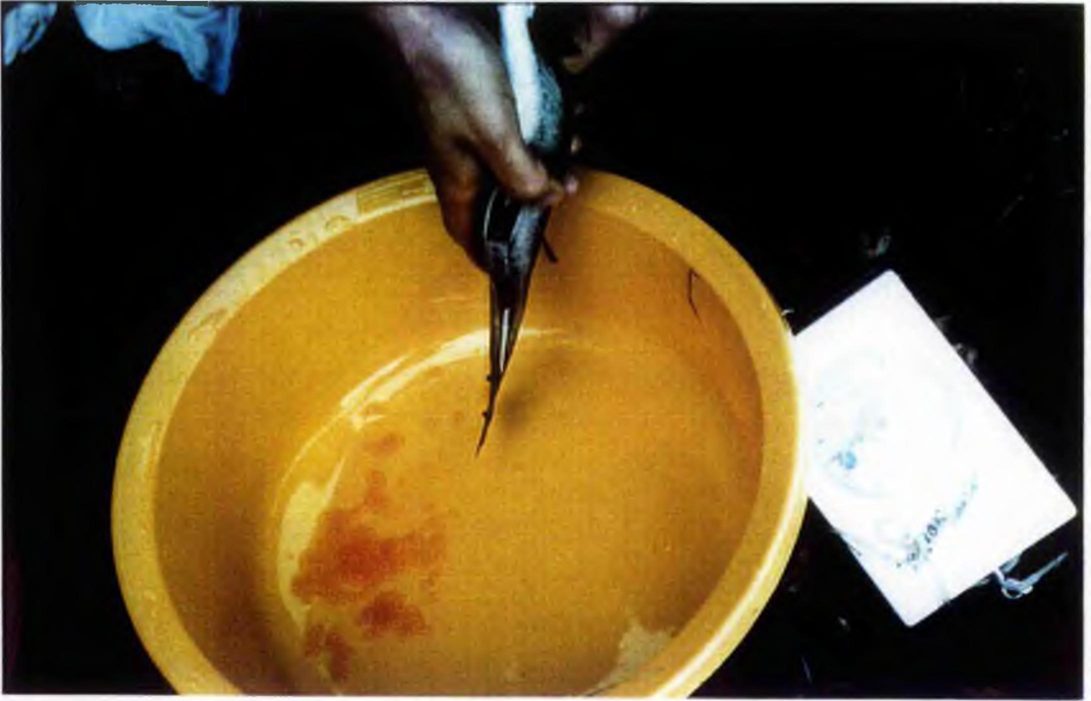


Plate 1.3

Plate 1.4

Plate 1.3 Trout fry.

Plate 1.4 Trout parr.

10

Plate 1.5

Plate 1.6

Plate 1.5 Adult freshwater-resident brown trout.

Plate 1.6 Adult freshwater-resident brown trout demonstrating the varied spot patterns and colouration.

Plate 1.7

Plate 1.8

Plate 1.7 Trout parr (upper two fish) and smolts (lower two fish).

Plate 1.8 Finnock caught in an estuary.

TROUT

freshwater or estuaries before returning to sea in the spring to continue growing and typically return to freshwater to spawn the following autumn (Plate 1.9).

Whether a trout adopts an anadromous strategy or matures in freshwater is determined by a combination of environmental and genetic factors that are discussed in Chapter 2. The plastic nature of the anadromous trout's life cycle, as typified by such variables as age at smolting, length of time spent at sea and multiple spawnings, results in spawning populations comprised of a heterogeneous mixture of age-classes. Furthermore, the inclusion of freshwater residents spreads the population distribution across a variety of environments. These variations from the general life history pattern may result in protection of the stock from local catastrophes, such as summer droughts, which might seriously deplete numbers of a species with a more rigid life history pattern, and allow trout to inhabit areas where other species could not. However, these are advantages conveyed upon the stock rather than necessarily selectively optimal for the individual.

1.2.4 The Parr-Smolt transformation

The parr-smolt transformation, or smoltification, is displayed in a variety of salmonid species prior to migration to the sea. The bulk of the scientific literature covering the parr-smolt transformation is based upon studies of salmon, particularly the Atlantic salmon and the summary below is necessarily drawn from such literature. That there are relatively few studies of this process in sea trout is perhaps indicative of an assumption of the similarity between the two species. However, studies of sea trout may be especially important given the broad differences in migratory behaviour, reproductive strategy and life history patterns between the two.

Smoltification is a gradual, progressive sequence of events and includes a combination of behavioural, morphological and physiological (cytological, biochemical and endocrinological) changes which culminate in active seaward migration. The parr-smolt transformation has been the subject of several review articles (Hoar, 1976; Folmar and Dickhoff, 1980; Wedemeyer *et al.*, 1980; Schreck, 1981; McCormick and Saunders; 1987).

Plate 1.9

Plate 1.9 Maturing sea trout caught soon after return to freshwater.

1.2.4.1 Behavioural changes

Salmonid parr living in streams and rivers are territorial and their spatial distribution is affected by dominance hierarchies based upon the ease of acquisition of food items and the availability of shelter from predators. In contrast, smolts display a diminishing territoriality and begin to form schools (Kalleberg, 1958). Rheotaxis, the stimulus to maintain station in a current, is characteristic of parr but is lost in developing smolts. They rise in the water column, possibly because of increased buoyancy due to internal changes (see below), and they appear also to lose the drive to swim at speeds sufficient to hold position in currents (McCleave and Stred, 1975; Thorpe and Morgan, 1978). They do not, however, become exhausted sooner than parr (Virtanen and Forsman, 1987).

1.2.4.2 Morphological changes

Parr are cryptic in coloration in order to hide amongst the stones and vegetation. The distinct silvering of smolts is due to the deposition of purines (guanine and hypoxanthine) in the dermis below the scales which hides the parr marks and spots. The fins also become darker. The degree of colour change varies between species. Trout smolts may still have faint parr marks and some spots, and dark yellow fins whereas salmon smolts are very silvery with black fins.

Rapid growth occurs during the transformation period but the increase in length is proportionally greater than weight gained and this results in a characteristic reduction in condition factor (K), an index of the length-weight relationship (see Chapter 2). Condition factor is also reduced as a result of increased use of metabolic reserves. Smolts display increased oxygen consumption (Baraduc and Fontaine, 1956, cited in Boeuf, 1993). A reduction in glycogen synthesis in the liver (Fontaine and Hatey, 1950, cited in Boeuf, 1993) and an increase in levels of glycogen phosphorylase (Sheridan *et al.*, 1985) leads to a decrease in lipid levels. There is also a change in the whole body lipid composition with an increase in the proportion of long chain polyunsaturated fatty acids (Sheridan *et al.*, 1985; Ogata and Murai, 1989). These changes in lipid composition were thought to increase the buoyancy of smolts but buoyancy also is elevated by increased volume of the swimbladder (Saunders, 1965; Pinder and Eales, 1969).

The main physiological changes of smoltification relate to the control of osmoregulation when the fish enters seawater. The ability to survive

osmoregulatory challenge is vital for smolts and is often used as a rudimentary test of the degree of transformation in reared smolts.

Section 1.3 Osmoregulation

As noted above, the major metabolic costs of all diadromous migrations are the necessary physiological adaptations demanded by crossing of the freshwater/seawater osmoregulatory barrier. In freshwater, all fish are hyperosmoregulators, i.e. their body fluids have a higher osmolality than the surrounding medium. The main consequences of this are (1) a constant influx of water to the body and (2) the simultaneous loss of salts (Figure 1.4). The primary source of these fluxes is the gills (branchial membranes), which have an extremely large surface area and high permeability (necessary to function efficiently as gas exchangers) and to a considerably lesser extent, the skin. In contrast, marine teleosts are hypo-osmotic to the surrounding medium, seawater having an osmotic concentration of approximately 1000 $mOsmol.kg^{-1}$ as compared to the 300 - 400 $mOsmol.kg^{-1}$ of marine teleost plasma. Consequently, teleosts lose water and gain salts, again primarily across the branchial epithelia (Eddy, 1982) (Figure 1.5). Water also is lost to a much lesser extent in a non-osmotic form via the renal pathway (Henderson and Brown, 1980; Rankin *et al.*, 1983). The successful transition from one environment to the other requires major changes in the mechanisms controlling a variety of osmoregulatory tissues.

1.3.1 Fluid Regulation

Freshwater teleosts compensate for passive fluid influx by producing large volumes of dilute, hypo-osmotic urine at an approximate rate of 0.15 - 0.4% body weight per hour (Eddy, 1982; Folmar and Dickhoff, 1981). This high urine flow rate is achieved by high glomerular filtration rate (GFR) (Evans, 1979). In contrast, marine teleosts must conserve water and, consequently, urine flow rate is minimal, reflecting reduced GFR (Rankin *et al.*, 1983) although urine concentration is maximally iso-osmotic with blood. However, reduced urine flow is itself insufficient to compensate for passive water loss and marine fish must drink the surrounding medium to replace lost fluids (Smith, 1930). Drinking does occur in freshwater teleosts but at a much reduced rate (Perrott *et al.*, 1992; Tierney *et al.*, 1995; Fuentes *et al.*, 1996) compared to that in marine teleosts (Table 1.2). The oesophagus, while highly impermeable to water and salts in freshwater fish, remains

Figure 1.4

Figure 1.4 Summary of ion exchange and osmoregulation in a freshwater teleost. From Jobling (1995).

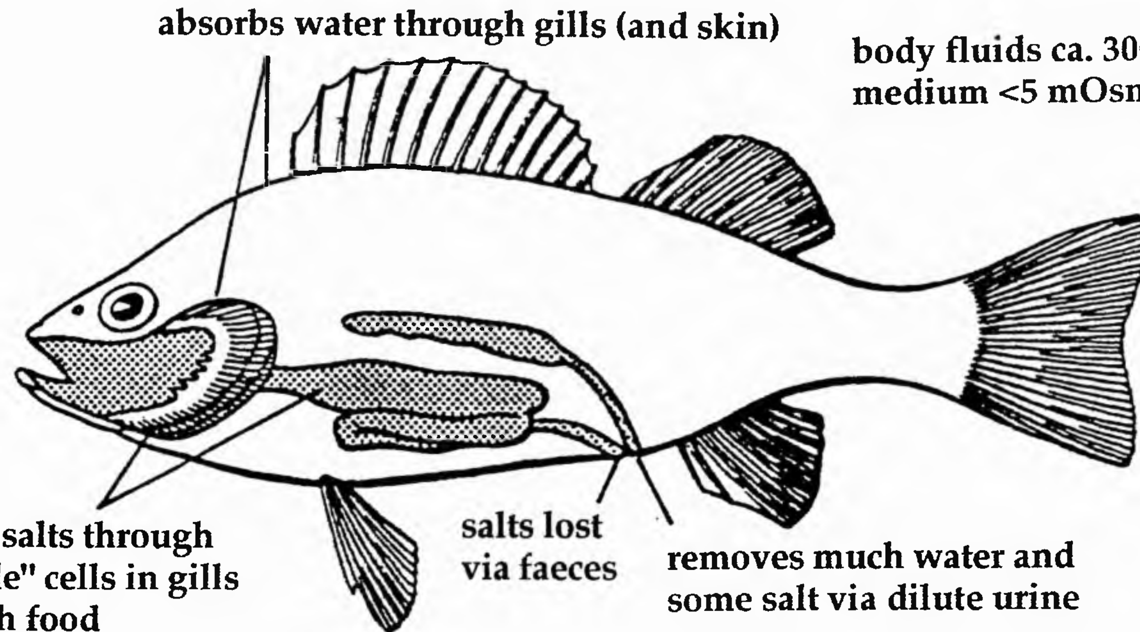

absorbs water through gills (and skin)
body fluids ca. 300 mOsm/kg,
medium <5 mOsm/kg
obtains salts through
"chloride" cells in gills
and with food
salts lost
via faeces
removes much water and
some salt via dilute urine

Figure 1.5

Figure 1.5 Summary of ion exchange and osmoregulation in a marine teleost. From Jobling (1995).

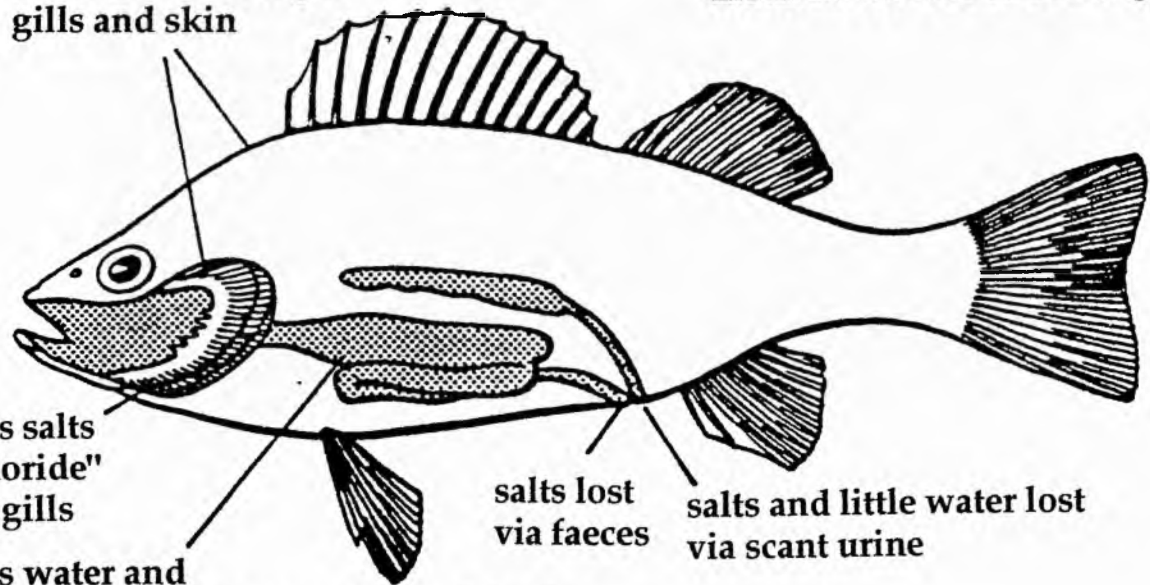

loses water through
gills and skin
body fluids ca. 350 mOsm/kg,
medium ca. 1000 mOsm/kg
removes salts
via "chloride"
cells in gills
salts lost
via faeces
salts and little water lost
via scant urine
gains water and
salts by swallowing seawater and food

Table 1.2

Table 1.2 Mean drinking rates in freshwater-adapted and seawater-adapted teleosts.
From Perrott *et al.* (1992).

Teleost species	Drinking rate (ml.kg $^{-1}$.hr $^{-1}$)
Freshwater	
Carp (*Cyprinus carpio*)	0.03 ± 0.01
Catfish (*Clarias gariepinus*)	0.21 ± 0.09
Eel (*Anguilla anguilla*)	0.09 ± 0.04
Flounder (*Platichthys flesus*)	0.04 ± 0.01
Rainbow trout (*Oncorhynchus mykiss*)	0.07 ± 0.02
Seawater	
Eel (*A. anguilla*)	1.00 ± 0.26
Flounder (*P. flesus*)	1.15 ± 0.15
Rainbow trout	1.42 ± 0.23
Plaice (*Pleuronectes platessa*)	2.52 ± 0.40
Dab (*Limanda limanda*)	3.60 ± 0.12
Whiting (*Merlangius merlangus*)	1.80 ± 0.24
Sea scorpion (*Myxocephalus scorpius*)	7.76 ± 0.75
Pogge (*Agonus cataphractus*)	2.21 ± 0.10
Wolf fish (*Anarhicas lupa*)	2.24 ± 0.23
Sand eel (*Ammodytes lanceolatus*)	2.96 ± 0.40

impermeable to water but is a major site for net salt uptake in fish in seawater and desalination of ingested seawater begins in this region (Hirano and Mayer-Gostan, 1976; Nagashima and Ando, 1994) (Figure 1.6). Intestinal fluid osmolarity is less than half that of sea water, but still hyperosmotic to plasma (Kirsch and Meister, 1982). Intestinal water uptake takes place as the passive consequence of active ion absorption against the osmotic gradient (Skadhauge, 1974; Hirano and Mayer-Gostan, 1976; Ando *et al.*, 1986) (see below).

However, this active ion absorption further compounds the ion loading of seawater fish resulting from passive influx across the branchial epithelia and skin. Complex iono-secretory mechanisms are present to compensate for this.

1.3.2 Ionoregulation

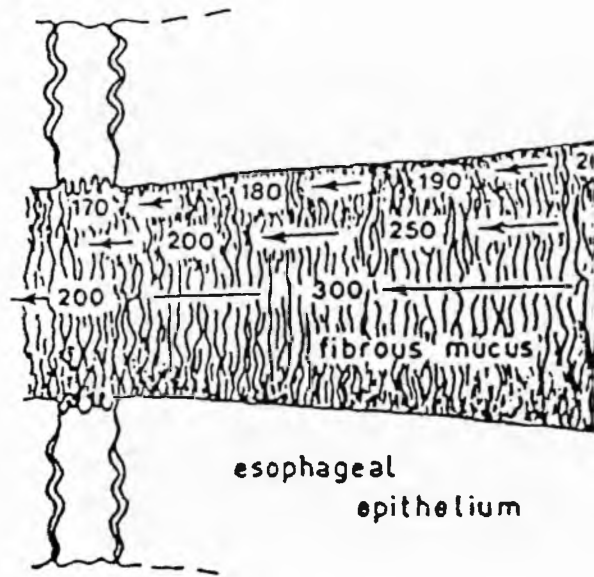
Sodium (Na^+) and chloride (Cl^-) are the two main extracellular ions (Table 1.3) and while divalent ions are excreted in the small amounts of urine, the major tissue for monovalent ion excretion in seawater fishes is the gill. Similarly, while dietary uptake compensates for the majority of ions lost in freshwater teleosts, the gill also is involved through active ion absorption from the surrounding media.

The teleost gill consists of four branchial arches extending from either side of the head and protected by the opercular plates. Perpendicular to each gill arch extend a double row of gill filaments, or primary lamellae, which, in turn, bear a row of secondary lamellae on either side. The gill is comprised of two types of epithelia: the primary epithelium covering the primary lamellae includes the interlamellar regions, and the secondary epithelium covers the free part of the respiratory lamellae (Laurent and Dunel, 1980). Blood supply to the gill involves two pathways, the arterio-arterial and the arterio-venous pathways supplying the secondary epithelium and primary lamellae, respectively (Laurent and Dunel, 1980).

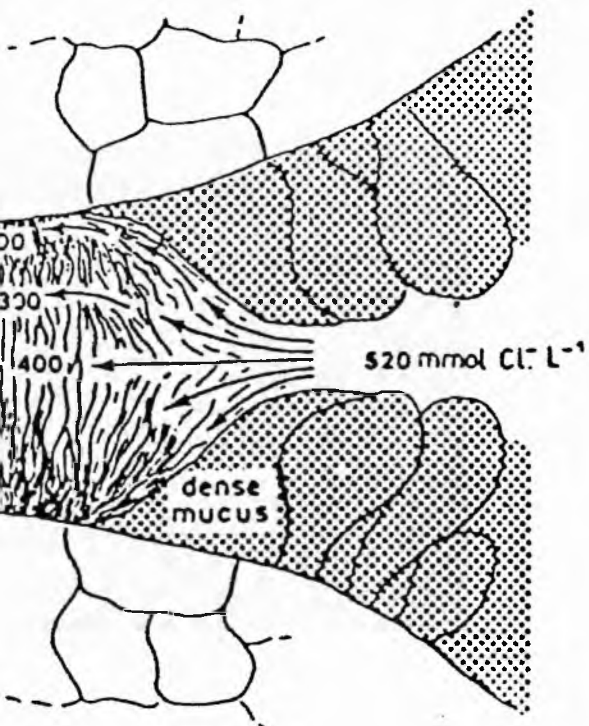
The branchial epithelium consists of at least four different cell types: pavement cells; chloride cells; mucus cells and neuroepithelial cells (Laurent, 1984). The site of ion transport across the gills, at least in seawater, generally is accepted to be the chloride cell (Motais and Garcia-Romeu, 1972; Zadunaisky, 1984), located predominantly in the primary lamellae but also in the secondary lamellae (Avella *et al.*, 1987). Chloride cells, first described by Keys and Willmer (1932), are characterized and recognised histochemically by the presence of numerous

Figure 1.6

Figure 1.6 Model of water and ion distribution in the oesophageal mucus. Numbers refer to local concentrations of Cl^- ions (mmol.l $^{-1}$): arrows have a length proportional to the local water flow.
From Kirsch (1978).



170
180
190
200
250
200
300
fibrous mucus
esophageal epithelium



300
400
520 mmol Cl^- L^{-1}
dense
mucus

Table 1.3

Table 1.3 Principle ionic components of seawater.

Ion	Concentration
Sodium (mM)	440
Potassium (mM)	10
Chloride (mM)	490
Calcium (mM)	10
Magnesium (mM)	51
Osmolality (mOsmol.kg $^{-1}$)	975-1000

mitochondria (hence also known as mitochondria-rich cells). Two sub-types of chloride cell, α and β, have been distinguished in the freshwater gill of the euryhaline guppy (*Lebistes reticularis*) and tilapia (*Oreochromis niloticus*) based on their location, shape and cytoplasmic staining, whereas only α cells are present in seawater-adapted fish gills, along with smaller accessory cells (Pisam *et al.*, 1987; 1990).

1.3.2.1 Freshwater ion exchange

Whereas some aspects of ion exchange in freshwater-adapted teleosts have been established, others still remain unclear. The active uptake of Na^+ and Cl^- occur as independent mechanisms (Krogh, 1939) and coupling between a Cl^- influx and HCO_3^- efflux was first indicated in goldfish (*Carassius auratus*) (Maetz and Garcia-Romeu, 1964) and subsequently confirmed (De Renzis, 1975). The mechanism of Na^+ uptake, however, is equivocal. A Na^+/NH_4^+ exchange mechanism was suggested (Maetz and Garia-Romeu, 1964) and supported by (i) the inhibition of Na^+ uptake by amiloride, an inhibitor of NH_4^+ efflux (Kirschner *et al.*, 1973) and (ii) a reduction in NH_4^+ efflux rates in Na^+ free media (Payan, 1978). However, amiloride also inhibits H^+ efflux (Kirschner *et al.*, 1973) while other studies found altering external Na^+ concentration affected H^+ but not NH_4^+ efflux and experiments with perfused head preparations suggested no coupling between Na^+ and NH_4^+ (Kersetter *et al.*, 1970; Avella and Bornancin, 1989). Thus, although Na^+/NH_4^+ exchange may be involved, a Na^+/H^+ exchange mechanism appears more important in Na^+ uptake.

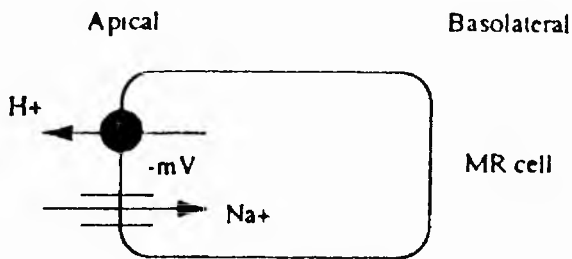
Avella and Bornancin (1989) suggested that Na^+ uptake occurred through Na^+ channels, driven by a H^+-ATPase (the proton pump), as occurs in the tight epithelium of the frog skin (Ehrenfeld *et al.*, 1985). This is supported by Sullivan *et al.* (1995) and Lin and Randall (1995). Three alternative models of Na^+ uptake through the Na^+/H^+, Na^+ channel mechanism, characterised by different cellular locations, are provided in Figure 1.7 (a-c). In model Ia, the transport mechanisms are located in the chloride cells (Avella and Bornancin, 1989) while in model Ib they are located in the pavement cells (Laurent *et al.*, 1994; Sullivan *et al.*, 1995). The third model (II) assumes that the Na^+ channel is located in the pavement cell but that the H^+ pump is located in adjoining chloride cells, as in the frog skin (Ehrenfeld *et al.*, 1985). However, none of these mechanisms are consistent with all the available experimental data (van der Heijden and Morgan, 1997).

Figure 1.7

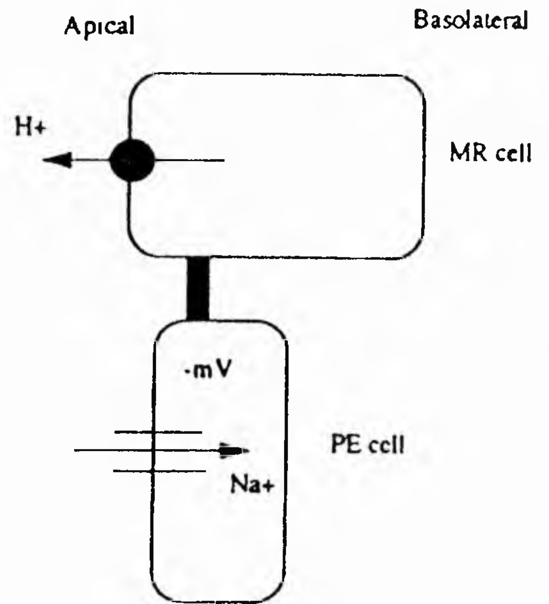
Figure 1.7 (a-c) Alternative models of Na^+ uptake across (1) the freshwater trout gill and (2) the freshwater frog skin, based on the H^+-pump/Na^+ channel mechanism.
From van der Heijden and Morgan (1997) and references therein.

I) Trout gills

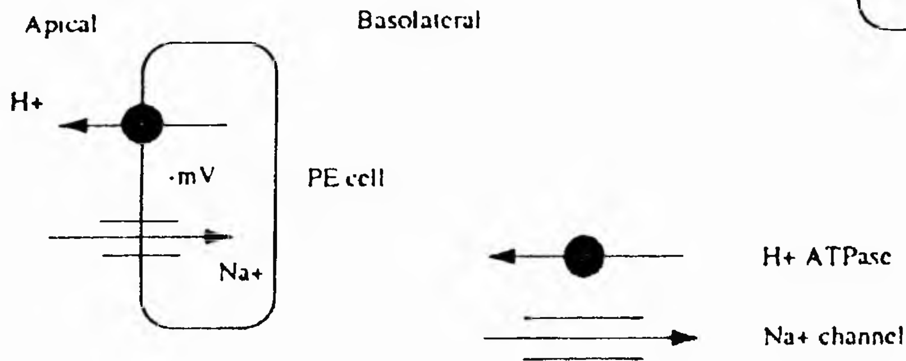
a) After Avella and Bornancin (1989)



II) Frog skin, after Harvey (1992)



b) After Sullivan et al. (1995)



As indicated by the possible three models of Na^+ uptake above, the site(s) of ionic absorption in the freshwater gill also remain to be established conclusively. Traditionally, chloride cells have been regarded as the site of ion absorption in the gills of freshwater teleosts, on the basis of the correlation between their distribution and gill ion uptake (e.g. Perry and Laurent, 1989), and chloride cell number and gill metabolic rate (Perry and Walsh, 1989). However, recent evidence suggests that Na^+ absorption may occur not in the chloride cell but in the pavement cell. Sodium influx was correlated with the development of pavement rather than chloride cells in brown bullhead (Goss *et al.*, 1992), proton pumps have been localized in the pavement cells (Laurent *et al.*, 1994; Sullivan *et al.*, 1995). Furthermore, given that cells involved in active transport of ions will display a brief perturbation in intracellular ion concentration before adaptive mechanisms compensate, Morgan *et al.* (1994) measured intracellular Na^+ and Cl^- concentrations in trout immediately after significant manipulations of apical concentrations. After high apical Na^+ concentration (1 mM), intracellular Na^+ concentration in pavement cells was double that of controls whereas chloride cells showed no change. This was assumed to indicate that pavement cells are the site of active Na^+ absorption. Alteration of external Cl^- concentration, using the same experimental design, resulted in significantly increased Cl^- concentrations in both pavement and chloride cells. However, external administration of thiocyanate (150 mM), a powerful inhibitor of Cl^- absorption (Kersetter and Kirschner, 1974; De Renzis, 1975), significantly reduced the intracellular Cl^- and Na^+ concentrations in the chloride cells but not the pavement cells (Morgan and Potts, 1995). This confirms that chloride cells are the site of active Cl^- uptake in the freshwater gill (van der Heijden and Morgan, 1997).

1.3.2.2 Seawater ion exchange

The site of ion exchange (secretion) in marine teleosts is universally agreed to be the chloride cell (Foskett *et al.*, 1983). When euryhaline teleosts migrate into seawater, chloride cells proliferate (Shirai and Utida, 1970; Utida *et al.*, 1971; Thomson and Sargent, 1977), although it has subsequently been shown that this proliferation involves only α-cells. B-cells degenerate and disappear over a few days (Pisam *et al.*, 1987). Morphological changes of the chloride cells also are associated with seawater adaptation. The tubular network and vesiculo-tubular system become more extensive, mitochondria numbers increase, tight junctions between adjacent chloride or accessory cells become shallower, and apical crypts deepen and narrow. The cytoplasmic processes of adjacent chloride and accessory cells become interdigitated especially in the region of the apical crypt (Karnaky, 1986; Pisam and

Rambourg, 1991). The function of accessory cells is presently unknown. Their appearance in seawater-adapted animals suggests a role in secretion of Na^+ and Cl^- (Cutler *et al.*, 1997) but they have been previously considered to be developing or degenerating chloride cells (Sardet *et al.*, 1979).

A model of ion excretion via chloride cells, modified from that first suggested by Silva *et al.* (1977), is presented in Figure 1.8. Chloride ions enter the cell from the serosa via furosemide-sensitive or bumetamide-sensitive $Na^+/K^+/2Cl^-$ co-transporters located in the basolateral membrane (Degnan *et al.*, 1977; Karnaky *et al.*, 1977; Zadunaisky, 1984; Davis and Shuttleworth; 1985; Karnaky, 1986; Zadunaisky *et al.*, 1995). An electrical gradient appropriate to the uptake and accumulation of intracellular Cl^- is maintained by active Na^+ exchange with K^+ from the basolateral tubular network via the ouabain-sensitive Na^+K^+-ATPase enzyme (Silva *et al.*, 1977) and K^+ recycling through barium sensitive K^+ channels (Degnan, 1985). Chloride ions subsequently move down an electrical gradient into the external milieu via chloride channels (Silva *et al.*, 1977). These channels may be the CFTR-like chloride channels but present evidence suggests these are baso-laterally, rather than apically, located (Riordan *et al.*, 1989; Anderson *et al.*, 1992). The exit of Cl^- produces a positive serosal gradient which is thought sufficient to allow passive movement of Na^+ paracellularly through 'leaky' intercellular junctions (Karnaky, 1986).

The Na^+K^+-ATPase enzyme is located in the gills (Epstein *et al.*, 1967; Kamiya and Utida, 1968) and it is the activity of this enzyme in cell suspensions which is the most commonly used measure of branchial iono-secretory ability in euryhaline teleosts. The enzyme consists of two subunits, α and β (Figure 1.9), both of which are necessary for function: expression of both subunits was demonstrated within three days of freshwater-seawater transfer of eels and parallel to a prolonged increase in enzyme activity (Luke *et al.*, 1994). The enzyme reaction leads to hydrolysis of ATP to produce ADP plus inorganic phosphate (Pi), with the translocation of three Na^+ into the extracellular compartment coupled to the transport of two K^+ to the intracellular compartment, per molecule ATP split (Schuurmans Slekhoven and Bonting, 1981) (Figure 1.9).

Figure 1.8

Figure 1.8 Schematic representation of the basic working model of ion transport in the secretory chloride cell of the branchial epithelium of marine teleosts first suggested by Silva *et al.* (1977).
From Cutler *et al.* (1997).

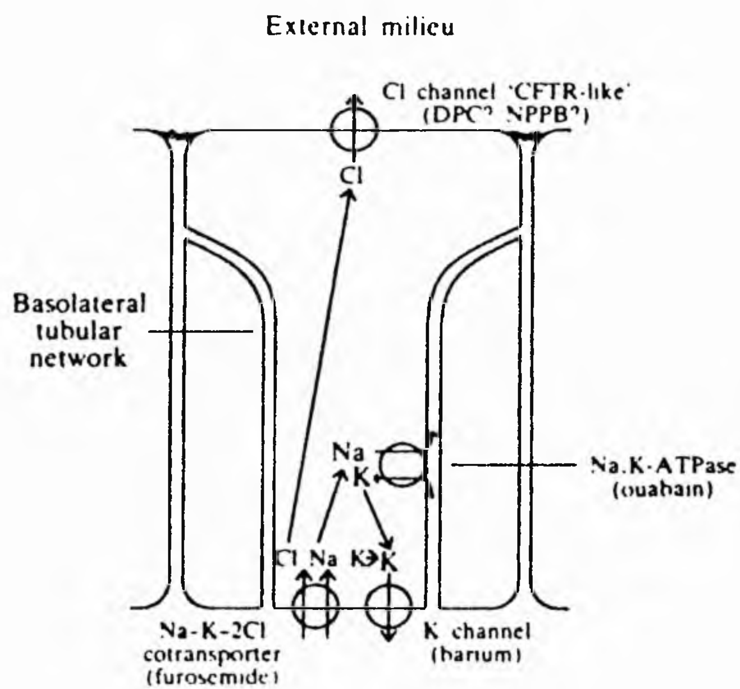
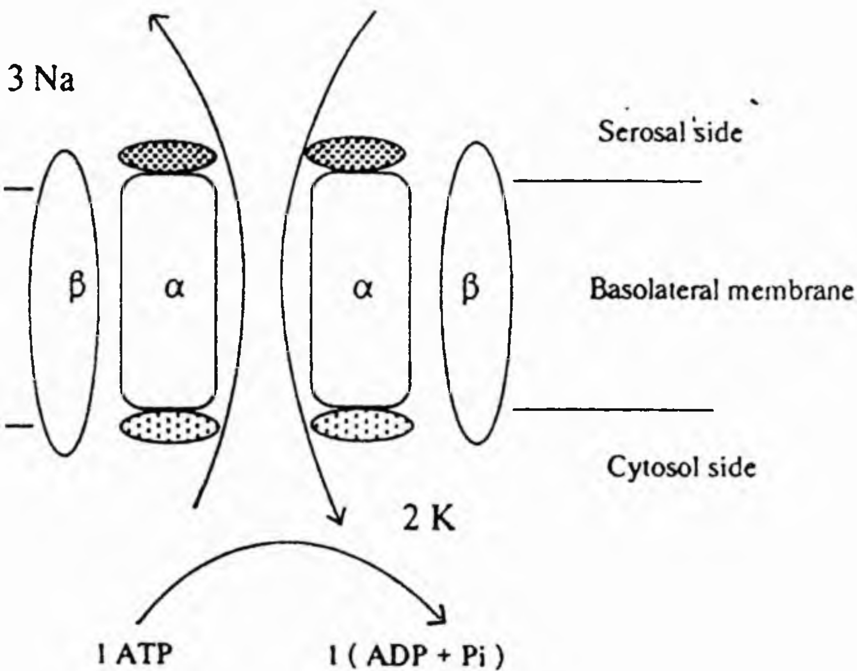

External milieu
Cl channel 'CFTR-like'
(DPC? NPPB?)
Cl
Basolateral
tubular
network
Na
K
Na.K-ATPase
(ouabain)
Cl Na K K
Na-K-2Cl
cotransporter
(furosemide)
K channel
(barium)

Figure 1.9

Figure 1.9 Schematic representation of the Na^+K^+-ATPase enzyme. From Tierney (1993).



Represents cardiac glycoside inhibitor sites.

Represents ATP binding sites.

1.3.3 Hormonal control of osmoregulation

The physiological processes involved in osmoregulation in freshwater and seawater, as outlined above, are controlled by the endocrine system. The teleost endocrine system is similar to that of tetrapods but with some important differences. Figure 1.10 presents a comparison of human and salmonid endocrine systems. Note the lack of parathyroids in teleosts and the presence of Stannius Corpuscles and the Caudal Neurosecretory System in teleosts.

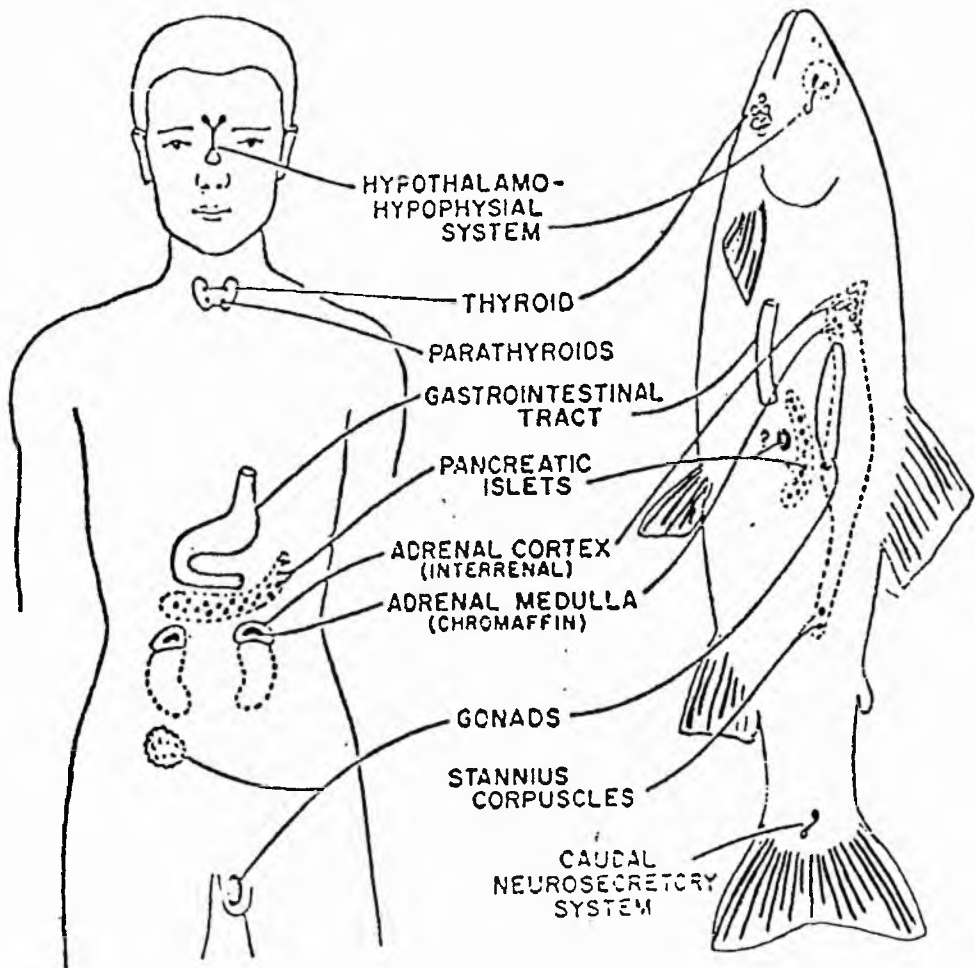
The pituitary organ or hypothalamo-hypophysial system accounts for a large proportion of hormones relevant to osmoregulation (Bern & Madsen, 1992) and consists of two principle tissue regions: the neurohypophysis and the adenohypophysis, the latter of which is further differentiated into the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI) (Figure 1.11).

1.3.3.1 Arginine Vasotocin

The neurohypophysis secretes arginine vasotocin (AVT). While its mammalian analogue, vasopressin, is an antidiuretic which acts on mammalian renal tubules, the identification of the function of AVT in teleost osmoregulation is equivocal. Manipulation, *in vivo*, of plasma AVT levels in freshwater-adapted euryhaline fish has profound effects on kidney function: large doses induce a diuresis due to the increase of vascular pressure (Babiker and Rankin, 1978) but lower, more physiologically appropriate, doses provoke an antidiuretic response (Henderson and Wales, 1974). A possible role for AVT in freshwater osmoregulation was indicated by greater plasma concentrations in freshwater-acclimated flounder and rainbow trout than in seawater-adapted fishes (Perrott *et al.*, 1991; Balment *et al.*, 1993), and a parallel relationship for levels of AVT mRNA in freshwater-adapted rainbow trout (Hyodo and Urano, 1991). However, the former may not be a seasonally consistent trend, at least in flounder. Warne and Balment (1995) demonstrated a two-fold increase in plasma AVT in seawater-adapted flounder between summer and winter whereas no significant difference was demonstrated between freshwater-adapted and seawater-adapted flounder pituitary AVT concentrations (Balment *et al.*, 1993).

Figure 1.10

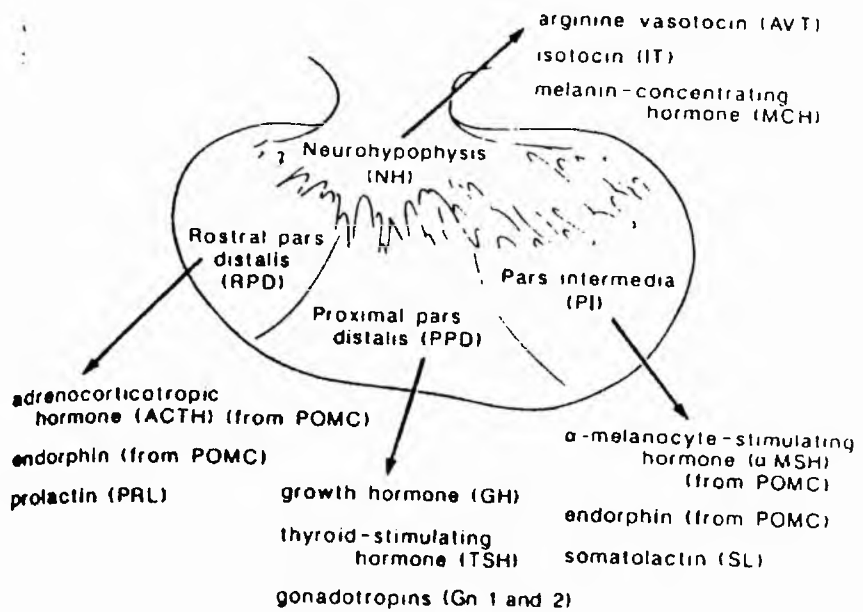
Figure 1.10 Comparative representation of the endocrine system of Man and a salmonid.



HYPOTHALAMO-
HYPOPHYSIAL
SYSTEM
THYROID
PARATHYROIDS
GASTROINTESTINAL
TRACT
PANCREATIC
ISLETS
ADRENAL CORTEX
(INTERRENAL)
ADRENAL MEDULLA
(CHROMAFFIN)
GONADS
STANNIUS
CORPUSCLES
CAUDAL
NEUROSECRETORY
SYSTEM

Figure 1.11

Figure 1.11 Schematic representation of the pituitary of trout showing sources of hormones from parts of the adenohypophysis (RPD, PPD, PI) and from neurohypophysis.
From Bern and Madsen (1992).


arginine vasotocin (AVT)
isotocin (IT)
melanin-concentrating hormone (MCH)
Neurohypophysis (NH)
Rostral pars distalis (RPD)
Pars intermedia (PI)
Proximal pars distalis (PPD)
adrenocorticotropic hormone (ACTH) (from POMC)
endorphin (from POMC)
prolactin (PRL)
growth hormone (GH)
thyroid-stimulating hormone (TSH)
gonadotropins (Gn 1 and 2)
α-melanocyte-stimulating hormone (α MSH) (from POMC)
endorphin (from POMC)
somatolactin (SL)

As such, a seawater-acclimating role for AVT was indicated by considerable elevation, or transient increase, of plasma levels in seawater-adapted eel (Henderson *et al.*, 1985; Balment *et al.*, 1993), a close correlation between plasma AVT levels and plasma osmolality and Na^+ concentration in seawater-adapted, but not freshwater-adapted flounder (Perrott *et al.*, 1991; Balment *et al.*, 1993), and diuresis in seawater-adapted eels after administering vasopressin, a competitive receptor antagonist of AVT (Babiker and Rankin, 1978).

In summary, whereas no consistent differences have been demonstrated in plasma AVT concentrations between long-term freshwater-adapted, and seawater-adapted euryhaline teleosts (Warne and Balment, 1995), the present consensus suggests AVT has a fluid conserving function because plasma AVT level is positively related to plasma osmolality, Na^+ and Cl^- concentrations, plasma AVT concentrations were significantly lower after volume expansion (Warne and Balment, 1995) and AVT stimulates receptors in the kidney (Harding and Balment, 1996). In addition, the gill vasculature is sensitive to AVT, which causes an increase in resistance to blood flow through the gill (Bennet and Rankin, 1986) although the physiological function, if any, of AVT at the gill still is not clear.

The two osmoregulatory hormones produced by the RPD are prolactin (PRL) and adrenocorticotrophic hormone (ACTH), converted from proopiomelanocorticotropin (POMC).

1.3.3.2 Prolactin

Two highly homologous forms of PRL have been found in salmon (Yasuda *et al.*, 1986), tilapia (Specker *et al.*, 1985) and eel (Suzuki *et al.*, 1991). PRL acts on a variety of osmoregulatory surfaces, including the gills, gut, kidney and urinary bladder (Hirano, 1986) and presently is regarded as the primary osmoregulatory hormone of most freshwater-adapted teleosts. Initial studies of PRL in fish were performed using ovine prolactin (oPRL) but more recent studies have demonstrated similar functional responses with teleost PRL (Kawauchi *et al.*, 1983; Prunet and Houdebine, 1984). In gills, PRL causes the reduction of epithelial permeability to water and ions by reducing the activity of the Na^+K^+-ATPase enzyme (Bern, 1975) and by initiating a de-differentiation of the chloride cell population (Foskett *et al.*, 1983). As outlined above, a characteristic feature of teleosts in freshwater is their copious production of urine. In the kidney, ovine PRL has been demonstrated to restore urine production after hypophysectomy (Stanley and Fleming, 1967) and to

reduce water reabsorption in the freshwater-adapted starry flounder, *Platichthys stellatus* (Foster, 1975). It has further been demonstrated to reduce bladder water permeability and increase Na^+ reabsorption from urine in seawater-adapted fish (Johnson *et al.*, 1972; Hirano *et al.*, 1973).

However, the role of PRL in hyperosmoregulation in salmonids is less clear. Plasma concentrations do increase after seawater-freshwater transfer (Prunet *et al.*, 1985), but pituitary prolactin secretion *in vitro* responds only to unphysiological reductions in medium osmolality (Gonnet *et al.*, 1988; Kelley *et al.*, 1990). Furthermore, hypophysectomised rainbow trout survive in freshwater (Donaldson and McBride, 1976), whereas other non-salmonid fish, e.g. killifish (*Fundulus heteroclitus*) die of osmotic imbalance (Pickford and Phillips, 1959).

Finally, PRL may interact with cortisol, an interrenal hormone important in hypo-osmoregulation (see later), but this relationship has yet to be defined. The presence of cortisol is required to demonstrate the effects of PRL (Clarke and Bern, 1980), *in vitro* studies have demonstrated that cortisol inhibits PRL release from the pituitary in a dose dependent manner (Borski *et al.*, 1991).

1.3.3.3 Growth Hormone

The PPD is responsible for the production of growth hormone (GH) and thyroid stimulating hormone (TSH) (see Section 1.3.3.6).

As the name suggests, GH is implicated in the promotion of growth in all classes of vertebrates. This effect is of particular osmoregulatory relevance to euryhaline teleosts migrating from freshwater to seawater because survival in seawater has been demonstrated to be positively size dependent (see Chapter 2). However, growth-independent effects on hypo-osmoregulation also have been demonstrated, particularly in salmonids. Transfer of salmonids to hypertonic media is associated with transient increases in plasma GH levels and in metabolic clearance rate (MCR), and administration of homologous GH to immature rainbow trout (*Oncorhynchus mykiss*) or chum salmon (*O. keta*) prevents an increase in plasma Na^+ concentration upon seawater transfer (Hasegawa *et al.*, 1987; Ogasawara *et al.*, 1989; Sakamoto *et al.*, 1990, 1991).

Various studies have demonstrated *in vivo* effects of GH including reduced plasma ion concentrations, increased gill $Na^{+}K^{+}$-ATPase activity, increased chloride cell size and density, and increased intestinal proline absorption (Table 1.4), thereby identifying the gill, gut and kidney as potential targets of GH osmoregulatory action (Sakamoto *et al.*, 1993).

1.3.3.4 Insulin-like Growth Factor 1

Recent evidence suggests that the effects of GH on seawater adaptation are mediated by insulin-like growth factor 1 (IGF-1) which has been demonstrated to improve ion regulation in rainbow trout after seawater transfer (McCormick *et al.*, 1991). Evidence suggests that GH may stimulate chloride cell differentiation through the local production of IGF-1, whereas systemic IGF-1 may act largely on undifferentiated cells (Sakamoto *et al.*, 1993). The mechanism of action of the GH-IGF-1 axis on the kidney and other osmoregulatory organs in fish is yet to be established.

GH also is associated with cortisol (see below). The two hormones act synergistically to further enhance hypo-osmoregulatory capabilities (Madsen, 1990) and GH has been demonstrated to enhance the response of coho salmon interrenal to ACTH, thereby increasing cortisol production (Young, 1988).

Amongst other hormones, the PI produces somatolactin, a peptide related to prolactin (Rand-Weaver *et al.*, 1992). However, an osmoregulatory role for this hormone is yet to be established.

The following section summarises the major osmoregulatory hormones of other endocrine tissues, and their functions.

1.3.3.5 Cortisol

Cortisol is the principal interrenal steroid (Sandor *et al.*, 1967) and has been demonstrated to stimulate ion pumps in the gill, intestine, kidney and bladder, as reflected by increased $Na^{+}K^{+}$-ATPase enzyme activity (Hirano and Utida, 1971; Mayer *et al.*, 1967; Pickford *et al.*, 1970). The increase in total branchial $Na^{+}K^{+}$-ATPase enzyme activity was found to be the result of chloride cell proliferation (McCormick and Bern, 1989), as observed in seawater adaptation (Sargent and Thompson, 1974). Cortisol treatment in freshwater-adapted rainbow trout

Table 1.4

Table 1.4 *In vivo* effects of growth hormone on salmonid osmoregulation.

[a] Effects could be consequences of growth-promoting actions of growth hormone.

[b] Effects were studied with homologous hormones. Hx, hypophysectomised.

[c] Hypophysectomised Coho salmon.

After Sakamoto *et al.* (1993).

Effects	Species	References
Decreased plasma osmolality and Na^+ and Cl^- concentrations	Sockeye salmon	Clarke et al. (1977)[a]
	Amago salmon	Miwa & Inui (1985)[a]
	Rainbow trout	Bolton et al. (1987)[b]; Collie et al. (1989); Madsen (1990)[a]; McCormick et al. (1991); Madsen & Bern (1992)
	Coho salmon[c]	Richman et al. (1987)
	Brown trout	Madsen (1990)[b]; Madsen & Bern (1992)
	Atlantic salmon	Boeuf et al. (1990)
Decreased plasma Ca^{2+} and Mg^{2+} concentrations	Rainbow trout	Bolton et al. (1987)[b]; Collie et al. (1987)
	Brown trout	Madsen & Bern (1992)
Increased branchial Na^+K^+-ATPase activity	Amago salmon	Miwa & Inui (1985)[a]
	Coho salmon	Richman & Zaugg (1987)[a]
	Coho salmon[c]	Björnsson et al. (1987)
	Rainbow trout	Madsen (1990)[b]; Madsen & Bern (1992)
	Atlantic salmon	
Increased chloride cell size and density	Rainbow trout	Madsen (1990)[a]
	Brown trout	Madsen (1990)[b]
Increased intestinal proline absorption	Coho salmon	Collie and Stevens (1985)[a]

significantly increased chloride cell number (Laurent and Perry, 1990) and stimulation of branchial Ca^{2+} uptake (Flik and Perry, 1989).

Increased renal Na^+ turn-over was demonstrated in seawater-adapted *Fundulus heteroclitus* treated with cortisol (Pickford *et al.*, 1970). However, although cortisol has also been demonstrated to increase urinary bladder water permeability and stimulate Na^+ and Cl^- reabsorption in the seawater-adapted goby (*Gillichthys mirabilis*) (Doneen, 1976), this was not the case in freshwater-adapted, or seawater-adapted starry flounder (Johnson *et al.*, 1972).

Although plasma cortisol levels have been demonstrated to rise upon seawater transfer, they are low or basal when measured in teleosts fully adapted to freshwater or seawater (Redding and Schreck, 1983; Redding *et al.*, 1984; Nichols and Weisbart, 1985; Franklin *et al.*, 1992). Transient peaks in plasma concentration have been demonstrated in response to acute stress such as handling (Pickering *et al.*, 1982; Waring *et al.*, 1992). It has been argued, therefore, that cortisol is not involved in continued adaptation to seawater but, rather, the transitory rise is indicative of general stress resultant from the transfer (Avella *et al.*, 1990). Cortisol has a variety of effects in fish. It is known to influence the intermediary metabolism of the fish (a glucocorticoid role) and it has also been shown to influence the maintenance of ionic and osmotic homeostasis (see above), thereby acting as a mineralocorticoid (Henderson and Kime, 1987). However, it has been demonstrated that, despite low plasma concentrations in long-term seawater-adapted fish, both MCR and blood production rate (BPR) were significantly elevated in the seawater-adapted compared to freshwater-adapted eel (Henderson *et al.*, 1974; Leloup-Hatey, 1974, Nichols, Weisbart and Quinn, 1985), thereby supporting the role of cortisol in hypo-osmoregulation.

Cortisol may also be involved in hyper-osmoregulation but evidence is inconclusive. Adrenalectomy of seawater-adapted eels caused a decrease in branchial Na^+ extrusion and a resultant increase in plasma Na^+ concentration, both of which were alleviated by cortisol injections. However, adrenalectomy in freshwater-adapted eels reduced urine flow rate and Na^+ reabsorption, both of which returned to resting levels after administration of cortisol (Mayer *et al.*, 1967).

1.3.3.6 Thyroid hormones

Two thyroid hormones, tri-iodothyronine (T3) and thyroxine (T4) have been studied with respect to osmoregulation. TSH stimulates the thyroid gland to produce and release T4 which is subsequently transformed into the active T3, a process which is mediated by GH (Nishioka *et al.*, 1982; McLatchy and Eales, 1990). Thyroid hormones are thought to play a role in metabolism. However, although many studies have demonstrated correlations between plasma levels of both hormones and various other hypo-osmoregulatory mechanisms, particularly during the parr-smolt transformation, there are few data unequivocally demonstrating an effect of thyroid hormones on osmoregulation (see Section 1.3.4)

1.3.3.7 Caudal secretory system

In teleost groups, neurosecretory cells in the caudal portion of the spinal cord project ventrally to a neurohaemal organ, the urophysis, from which at least two peptide factors are released. Urotensin I (U I) is a caudal neurosecretory hormone which is thought to be involved in seawater adaptation. Levels are elevated in the seawater-adapted flounder (Arnold-Reed *et al.*, 1991) and it acts both directly, on epithelial tissues to decrease water and Na^+ loss from the body (Loretz *et al.*, 1981), and indirectly, through the stimulation of cortisol release (Arnold-Reed and Balment, 1989). In contrast, urotensin II (U II) stimulates active Na^+ absorption through the urinary bladder and intestine, while inhibiting active Cl^- extrusion from secretory surfaces such as the branchial epithelia and skin. These processes imply that U II facilitates freshwater adaptation (Larson and Bern, 1987). However, this hormone also has been shown to inhibit the secretion of PRL (Loretz *et al.*, 1981), an important hormone in hyper-osmoregulation (see above).

1.3.3.8 Atrial Natriuretic Peptide

The presence of a peptide in mammalian atrial myocytes which caused diuresis, natriuresis and hypotension was first described by De Bold *et al.* (1981). It now is apparent that this principle extends throughout the vertebrates and encompasses a family of peptides. Thus, teleost natriuretic peptides have been isolated from atria, ventricles and brain in the Japanese eel and, while sharing a common structure, each is distinct (A, B and C types) (Takei *et al.*, 1989, 1990, 1991) (Figure 1.12). The first discovered and most studied natriuretic hormone is atrial natriuretic peptide (ANP) which acts on the mammalian vasculature, kidneys and adrenals to reduce

Figure 1.12

Figure 1.12 Amino acid sequence of some of the vertebrate natriuretic peptides characterized to data.
From Takei (1994).

Atrial (A-type) natriuretic peptide

eel	SK SS P CFG GKL DRIG SY SG L GC NS - R K
bullfrog	SS D CFG SRL DRIG AQ SG M GC -G - R RF
Rat	SLRR SS -- CFG GRM DRIG AQ SG L GC NSF R --Y
Man	SLRR SS -- CFG GRI DRIG AQ SG L GC NSF R --Y

Brain (B-type) natriuretic peptide

fowl	M MRD S G -- CFG RRI DRI GSL S GM GC NGS R KN
Dog	SPK - M MHK S G -- CFG RRL DRI GSL S GL GC NVL R KY
Rat	S - K - M AHS S S -- CFG QKI DRI GAV S RL GC DGL R LF
Man	SPK - M VQG S G -- CFG PRM DRI SSS S GL GC KVL R KH

C-type natriuretic peptide

dogfish	G P S R G -- C F G V K L D R I G A M S G L G C
eel	G W N R G -- C F G L K L D R I G S L S G L G C
killifish	G W N R G -- C F G L K L D R I G S M S G L G C
bullfrog	G Y S R G -- C F G V K L D R I G A F S G L G C
fowl	G L S R S -- C F G V K L D R I G S M S G L G C
man	G W N R G -- C F G L K L D R I G S M S G L G C

blood pressure and intravascular volume. It thereby is effective in removing excess water and Na^+ from the body. Injection of ^{125}I-labelled eel ANP into Japanese eel indicated that the major receptor bed was located in the gill, principally in the secondary lamellae, but receptors were also noted in the atrium, kidney, liver and bladder (Sakaguchi *et al.*, 1996). However, the osmoregulatory function of ANP in teleosts has yet to be established conclusively. A radio-immuno assay (RIA) using antiserum specifically raised against eel ANP indicated that levels are similar or lower in seawater-adapted than in freshwater-adapted eels (Takei and Balment, 1993), and Sakaguchi *et al.* (1996) noted a tendency for down regulation of ANP binding sites in seawater-adapted eel, especially in the gill. However, Kaiya and Takei (1996a) observed increased plasma ANP levels after seawater transfer of *A. japonica*, but not after seawater-freshwater transfer. Kaiya and Takei (1996b) also demonstrated elevated plasma ANP levels after increases in plasma osmolality, whether dependent or independent of NaCl concentration, cellular dehydration or increased blood volume. Thus, Kaiya and Takei (1996b) suggested the presence of two ANP receptor types: osmoreceptors that respond to cellular dehydration, and volume/stretch receptors which are activated by hypervolemia.

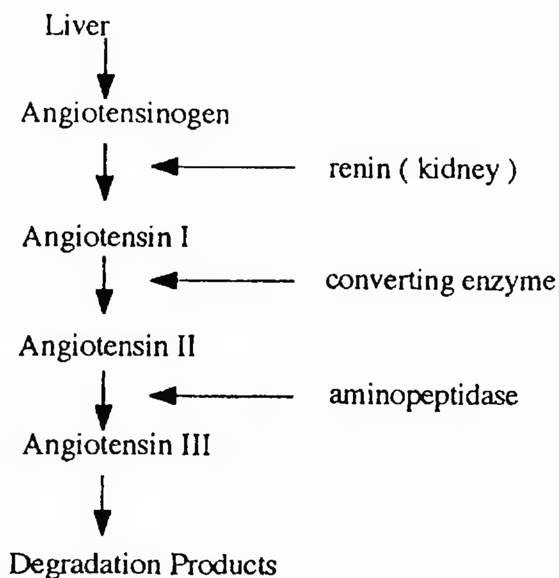
1.3.3.9 Angiotensin II

The renin-angiotensin system (RAS) is a major control system for salt and water balance in vertebrates. The principle biologically active component, angiotensin II (Ang II), is formed in the blood by the enzymatic actions of renin and converting enzyme (Figure 1.13). In mammals, Ang II induces water and Na^+ conservation by stimulating thirst and Na^+ appetite, increasing the intestinal absorption of water and Na^+ (Davies *et al.*, 1970; Nishimira, 1987), and decreasing GFR, while increasing tubular reabsorption of water and Na^+ (Nishimira, 1985).

In fishes, Ang II has been linked with increased drinking in all freshwater-adapted and seawater-adapted euryhaline species examined so far (Perrott *et al.*, 1992). The stimulatory effect of Ang II was abolished when Ang I to II conversion was inhibited by captopril, whereas drinking rate increased in seawater-adapted fish following stimulation of the endogenous RAS by the hypotensive agent papaverine (Perrott *et al.*, 1992). Angiotensin II acts also on the kidney to rapidly reduce GFR, urine flow and renal tubular transport, all of which result in the conservation of water and ions (Brown *et al.*, 1980).

Figure 1.13

Figure 1.13 The mammalian Renin-Angiotensin System.
From Balment and Henderson (1987).



Position of Peptide Cleavage

(Renin Substrate)

	1	2	3	4	5	6	7	8	9	10	11	12
Angiotensinogen	Asp-	Arg-	Val-	Tyr-	Ile-	His-	Pro-	Phe-	His-	Leu-	Leu-	Val

↑ renin (between Leu 10 and Leu 11)

Angiotensin I: Asp- Arg- Val- Tyr- Ile- His- Pro- Phe- His- Leu

↑ converting enzyme (between Phe and His)

Angiotensin II: Asp- Arg- Val- Tyr- Ile- His- Pro- Phe

↑ aminopeptidase (between Asp and Arg)

Angiotensin III: Arg- Val- Tyr- Ile- His- Pro- Phe

↑ (between Arg and Val)

Angiotensin IV: Val- Tyr- Ile- His- Pro- Phe

(3-8 AII)

In mammals, Ang II also stimulates the secretion of vasopressin and aldosterone, thereby indirectly promoting water and Na^+ conservation. Fishes, however, usually do not produce aldosterone, and cortisol serves as a mineralocorticoid as well as a glucocorticoid (see Section 1.3.3.5). In seawater fishes, Ang II stimulates the production of cortisol but, rather than being a Na^+ conserver, cortisol promotes ion extrusion (see Section 1.3.3.5).

1.3.3.10 Catecholamines

Catecholamines, such as epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine, are released into the circulation from the chromaffin cells in the wall of the posterior cardinal veins or adrenal medulla (Epple *et al.*, 1989) and are known to exert effects on branchial blood flow, on active ion transport mechanisms and on branchial permeability to water, ions and organic substances.

Evidence suggests that catecholamines may be involved in seawater adaptation: rainbow trout display reductions in GFR and urine volume upon seawater transfer which are partly blocked by the adrenergic blockers (catecholamine inhibitors) bretylium and phentolamine (Elger and Hentschel, 1983). The main branchial effects of catecholamines appear to be a greater blood flow through the lamellae (Steen and Kruysse, 1964; Richards and Fromm, 1969), the osmoregulatory significance of which is unclear, and an enlargement of the functional respiratory surface (Haywood *et al.*, 1977; Nilsson, 1984).

1.3.3.11 Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) exhibits a variety of biological functions in mammals, some of which are related to osmoregulation: inhibition of water and Na^+ absorption, along with stimulation of Cl^- secretion, of PRL release by the pituitary, of renin release by the juxtaglomerular cells, and of corticosteroid release from the adrenal (Rosselin *et al.*, 1982). In teleosts, whereas some of these processes would be involved in freshwater adaptation and some in seawater adaptation, the similarities between the effects of U I (see above) and VIP in fish suggest a role in seawater adaptation (Loretz *et al.*, 1981).

1.3.4 Hormonal control of smoltification

Smoltification is initiated by a combination of internal (energy store levels) and external (photoperiod and temperature) cues (Hoar, 1976, 1988) which act on the endogenous endocrine system to control the transformation. The plasma levels of many of the hormones discussed above have been demonstrated to vary during the smoltification process (Figure 1.14). Those which have received most attention and been implicated in the transformation are the thyroid hormones, growth hormone (and IGF-1) and cortisol.

Hoar (1939) first observed increased thyroid gland activity in smolting salmonids and many subsequent studies have demonstrated a surge in the plasma concentrations of both T4 and T3 (collectively TH) towards the culmination of the transformation. That such peaks in plasma TH concentration were of physiological significance, rather than being due to changes in plasma binding protein properties was confirmed by Boeuf *et al.* (1989). Thyroid hormones have been linked with a variety of smoltification processes (Table 1.5) including metabolic changes and growth, body silvering, and migratory behaviour where interestingly, peaks appear to coincide with lunar phases (Grau, 1981; Grau *et al.*, 1982; Boeuf and Prunet; 1985).

However, despite some reports of simultaneous peaks (Boeuf and Prunet, 1985; Virtanen and Soivio, 1985; Soivio *et al.*, 1989), no correlative link has been established between levels of TH and branchial Na^+K^+-ATPase activity (Saunders *et al.*, 1985; McCormick *et al.*, 1987; Richman III *et al.*, 1987) or between plasma TH levels and increased seawater tolerance (McCormick *et al.*, 1987; Boeuf *et al.*, 1989), and these changes appear to be independent in smoltification (Virtanen and Soivio, 1985).

Growth hormone and cortisol plasma concentrations both increase during transformation, to peak shortly before migration. Both hormones have been implicated in increased branchial Na^+K^+-ATPase activity (Sections 1.3.3.3., 1.3.3.5) and appear to play major roles in smolting and seawater adaptation (Boeuf *et al.*, 1989; Bisbal and Specker, 1991; Madsen and Korsgaard, 1991). As well as the direct effects described above (Section 1.3.3), GH may be involved also in control of the T4 - T3 transformation (Boeuf *et al.*, 1989). Administration of cortisol has been implicated in increased plasma T3 levels (Redding *et al.*, 1991) and therefore, the glucocorticoid role of cortisol (Section 1.3.5) implies that this hormone may be linked with TH in the metabolic changes described above (Section 1.2.4.2).

Figure 1.14

Figure 1.14 Schematic representation of hormone changes during smolting of salmonids.
From Boeuf (1993) and references therein.

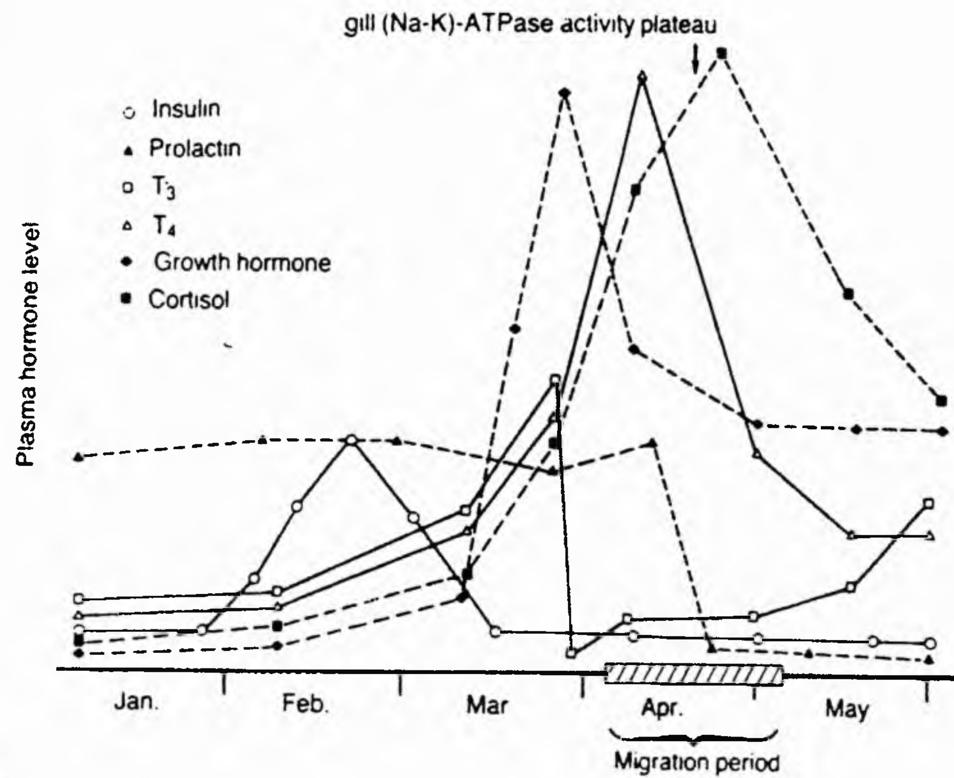

gill (Na-K)-ATPase activity plateau
Insulin
Prolactin
T3
T4
Growth hormone
Cortisol
Plasma hormone level
Jan.
Feb.
Mar
Apr.
May
Migration period

Table 1.5

Table 1.5 Established functions of elevated thyroid hormones during salmonid smoltification.
From Boeuf (1993).

Function	References
Control of growth	Fontaine (1975); Nishioka et al. (1982, 1985); Refstie (1982); McCormick and Naiman, (1984);Saunders et al. (1985); Boeuf and Gaignon (1989)
Stimulation of silvering	Fontaine (1975); Miwa and Inui (1983, 1985); Sullivan et al. (1987)
Stimulation of metabolism	Leloup et al. (1971); Narayansingh and Eales (1975); Bhattacharya et al. (1985); Madsen and Korsgaard (1989); Soengas et al. (1992)
Appearance of new haemoglobin forms	reviews: Sullivan et al. (1987); Hoar (1988)
Stimulation of migratory behaviour	Baggerman (1963); Godin et al. (1974); Fontaine (1975); Youngson et al. (1985); Iwata et al. (1989); Boeuf and Le Bail (1990)
Stimulation of imprinting	Leloup and Fontaine (1960); Fontaine (1975); Hoar (1976); Hasler et al. (1978); Scholz et al. (1985); Morin et al. (1989a, b)

Increased levels of plasma insulin probably reflect increased metabolic activity (Plisetskaya *et al.*, 1988) whereas the decrease in plasma PRL concentration may facilitate the increase in branchial $Na^{+}K^{+}$-ATPase activity because this hormone is considered to have an inhibitory effect on enzymatic activity rate (Section 1.3.3.2).

Section 1.4 Population changes in sea trout stocks

As described above, trout possess the necessary precise hormonal control mechanisms to control osmoregulatory adaptation which, in turn, allow successful migration between freshwater and seawater. Despite this inherent adaptability and flexibility, however, key indigenous populations on the Atlantic coasts of Scotland and Ireland have suffered a drastic decline in numbers in recent years. This species is of considerable ecological and commercial importance throughout the British Isles in both marine and freshwater habitats. The most recent survey in the U.K. revealed that sport angling in Scotland contributed £50 million to the economy (Anon., 1989), especially in remote rural communities where tourism and leisure are two of the most important sources of employment and angling contributes a considerable proportion of the local income.

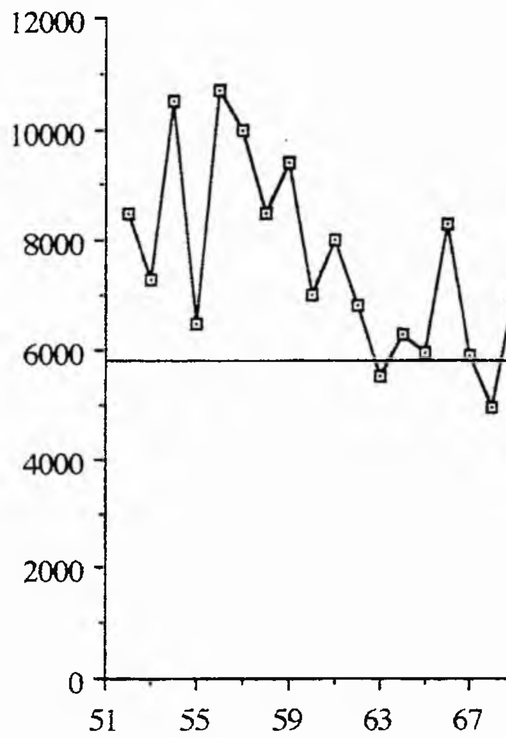
1.4.1 Scottish Trends

Catch returns for rod caught sea trout in the worst affected region of Scotland, the north-west, from 1955 demonstrate a long-term decline culminating in unprecedented lows in 1989 and 1990 (Figure 1.15). Catch figures for other Scottish areas show similar trends but with indications of a recovery from 1992 onwards. Note that catch statistics suffer from the error produced by fluctuations in fishing effort, compounded by the difficulty of accurately measuring fishing effort. Blank days are rarely recorded and there is no weighting for individual variation in success or for variations in fishing conditions. However, Scottish fisheries are statutorily required to report annual sea trout and salmon rod catches to the Scottish Office, Agriculture, Environment and Fisheries Department (S.O.A.E.F.D.), so these data ought to be suitable for examining general trends at least, providing the above sampling complications are borne in mind.

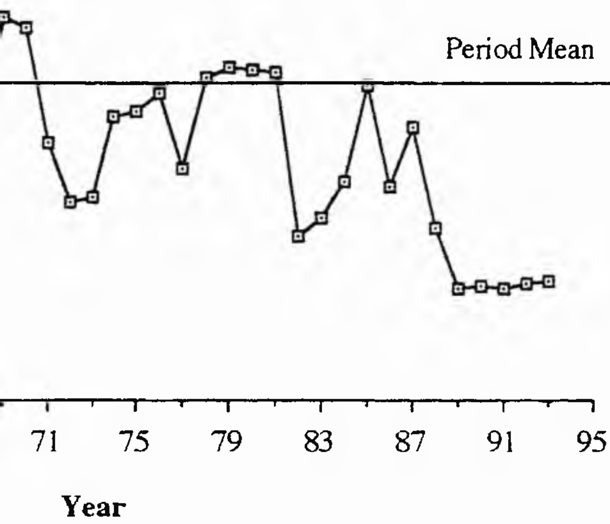
Summer droughts are not thought responsible for this regional crash in numbers because river flows were similar to the long-term average during 1989 to 1992. The

Figure 1.15

Figure 1.15 Annual rod and line sea trout catches for North-West Scotland between 1952 and 1993.
From Anon. (1994c).



No. Sea Trout Caught
12000
10000
8000
6000
4000
2000
0
51
55
59
63
67


Period Mean
71
75
79
83
87
91
95
Year

continuing low sea trout numbers of the north-west region imply that the general decline of the late 1980s was compounded by regional factors which have prevented the recovery apparent in other areas (Walker, 1994a).

An additional feature of the decline in north-west sea trout stocks is a reduction in the average size of mature adults (Figure 1.16), and a resultant drop in mean fecundity. Data from the few adults sampled suggest a marked reduction in the proportion of larger, older spawners and some evidence of reduced marine growth within year classes. Data from the River Ewe system (Wester Ross) suggest that the reduction in mean length of spawners would result in an approximate halving of the number of eggs available for deposition (Walker, 1994a). Thus, fewer returning adults are producing a relatively smaller number of eggs which further hampers any stock recovery, assuming the cause of the decline was removed.

1.4.2 Irish Trends

Sea trout catch statistics for the west and north-west of Ireland from 1985 to 1993 displayed a slow decrease to 1988 followed by a stock collapse in 1990 (Anon., 1993). During the same period, catch records from other areas of Ireland displayed considerable variation but not the dramatic collapse, and were considered 'satisfactory' in 1993.

Irish stock statistics benefit from the availability of a number of traps for upstream and downstream migrants on the Burrishoole System, Co. Mayo. This allows more accurate records of population numbers to be assessed without the error based on fishing effort. Figure 1.17 presents the approximate numbers of returning finnock and adult sea trout between 1971 and 1993 (Anon., 1993) and highlights the general decline followed by a population 'crash' with a failure to recover from 1989 onwards.

1.4.3 Possible causes of the declines

A variety of potential causes of these declines have been suggested and investigated both in Scotland and Ireland (Anon., 1994b; Whelan *et al.*, 1994). These causes can be divided into those centred around the freshwater or marine environments. Potential causes in freshwater include habitat destruction through afforestation and acid rain and a change in the proportion of juveniles which adapt to migrate to sea. The latter might be due to improved growth conditions brought about by increased

Figure 1.16

Figure 1.16 Growth at sea of sea trout from the River Ewe system, Wester Ross. From Walker (1994a).

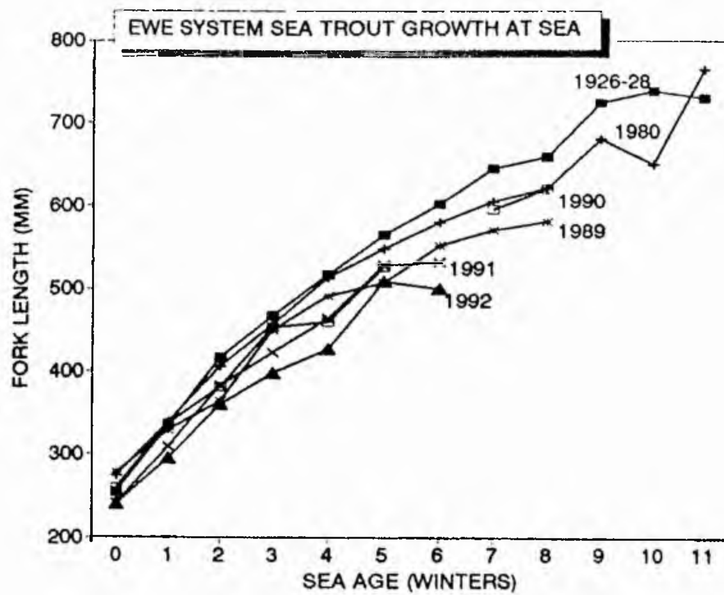
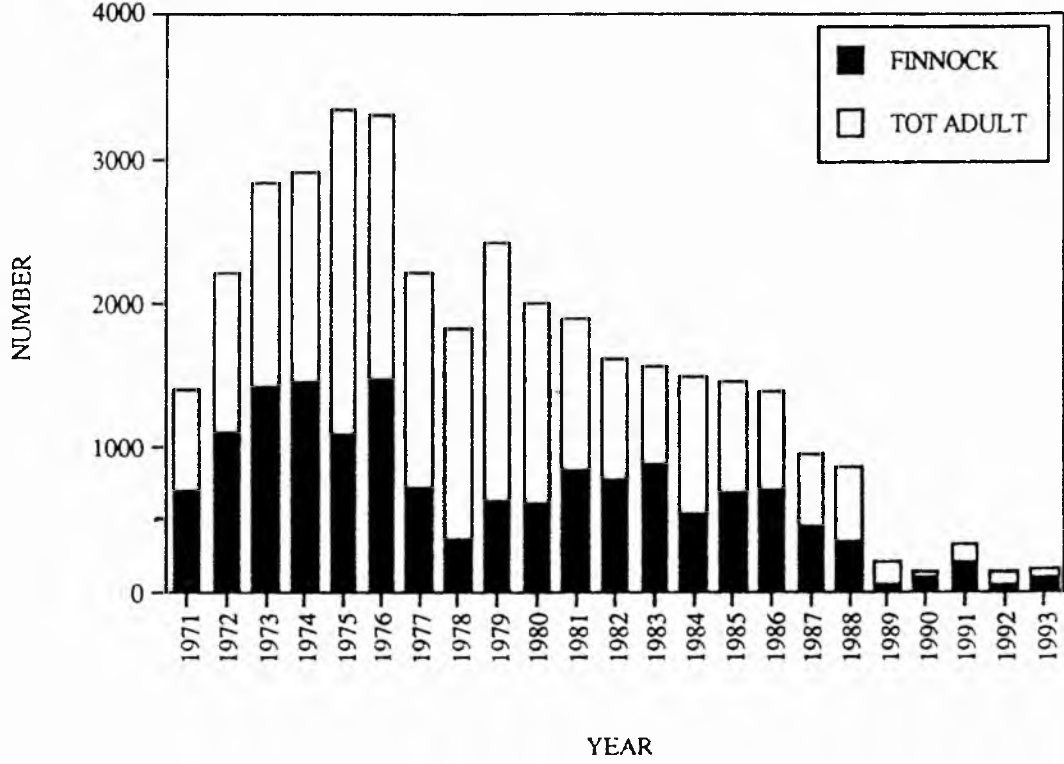

EWE SYSTEM SEA TROUT GROWTH AT SEA
FORK LENGTH (MM)
SEA AGE (WINTERS)
1926-28
1980
1990
1989
1991
1992
800
700
600
500
400
300
200
0
1
2
3
4
5
6
7
8
9
10
11

Figure 1.17

Figure 1.17 Number of upstream migrating finnock and adult sea trout counted through the traps at Burrishoole, Co. Mayo, between 1971 and 1993. From Anon. (1994c).


4000
3000
2000
1000
0
NUMBER
FINNOCK
TOT ADULT
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
YEAR

freshwater productivity as a consequence of the additional nutrients from agricultural run-off and sewage outflow and/or recent unusually mild winters. Such improved growth conditions might reduce the impetus of juvenile trout to migrate to sea (see Chapter 2). The major symptoms in the marine environment appear to be reduced growth, possibly due to overfishing of prey species, e.g. sand eels (*Ammodytes marinus*) and increased mortality. Factors resulting in increased mortality may include the poor hypo-osmoregulatory ability of smolts as a consequence of mild winters (see Chapter 2), increased poaching by gill net fisheries and/or the marked increase in the numbers of seals, both common (*Phoca vitulina*) and grey (*Halichoerus grypus*) in coastal waters (Hiby *et al.*, 1996).

The recent development of intensive salmonid aquaculture on the west coasts of Scotland and Ireland may also have contributed to increased mortality of wild salmonids. Salmon reared at high densities are particularly susceptible to stress and diseases (see McVicar *et al.*, 1994). Thus, cage systems could be a significant source of cross infection of disease between farmed and wild salmonids. In addition, the general immuno-deficient state of farmed salmon, combined with the use of chemotherapeutants to control disease, may result in new strains of diseases to which wild salmonids have poorer immunity. Just as fish farms can be the source of diseases, salmon in these cages also suffer from high infection intensities of ectoparasitic sea lice which release vast numbers of larvae into coastal waters where they probably infect wild salmonids.

Two species of ectoparasitic copepod sea lice are of particular importance in salmonid fish farming. *Caligus elongatus* (Nordmann) is a non-specific parasite and has been recorded infecting over 70 species of fish (Kabata, 1979) but has been observed in large numbers on juvenile sea trout (pers. obs.). In contrast, *Lepeophtheirus salmonis* (Krøyer) is specific to salmonid species. Sea lice feed on the mucus and dermal tissue of their hosts and congregations of *L. salmonis*, typically between the ventral fin and the tail or between the dorsal and adipose fins, can cause severe localised dermal erosion. The gills are the major external organ important in osmoregulation and so sea lice feeding in the areas noted above might not be considered to be of major physiological consequence. However, sea lice may indeed contribute to the osmoregulatory stress of the host as larvae have been found attached to the gills of salmonids on fish farms and in aquaria, and the natural infestations of adult sea lice can result in blood loss from localized areas. Whereas large adult salmonid hosts can probably cope with modest infestations of sea lice, the likelihood is that the smaller smolts which have recently migrated to sea and are

adapting to the marine environment, may be particularly susceptible to infection and significant physiological trauma.

1.5 Aims of this study

While the literature regarding *Salmo* biology is vast, most focuses on the Atlantic salmon to the extent that despite being very important commercially, large gaps remain regarding the basic physiology, behaviour and ecology of anadromous trout. There is, therefore, an urgent need to improve upon the current understanding of trout (particularly sea trout) biology, in order to help scientists and fishery managers protect and, hopefully, regenerate this economically and environmentally valuable resource. Therefore, the major objectives of this thesis were to investigate:

1. the influences of ration and parental strategy on life history choice in juvenile trout;
2. the behaviour of juvenile sea trout overwintering in freshwater;
3. the possible osmoregulatory stimulus for this overwintering behaviour;
4. the physiological consequences of *L. salmonis* infestation on post-smolt sea trout;
5. the potential link between sea-cage based salmonid aquaculture and *L. salmonis* on wild salmonids.

Chapter 2

Factors which induce trout to migrate to sea

2.1 Introduction

The factors that induce some juvenile trout to migrate to sea whereas others remain in freshwater are of great interest to fish biologists, fishery managers and those in aquaculture. In order to investigate these factors, it is first necessary to examine why some trout should migrate to sea at all.

2.1.1 Why should trout migrate to sea?

The general costs and benefits of anadromous migration have been outlined above (Section 1.1). The major benefit is improved growth through the increased availability of food resources. For example, the mean daily length increment of 60 sea trout post-smolts tagged and then recaptured in sampling of five Argyll sea lochs was calculated as 0.5 $mm.day^{-1}$ or a mean growth rate of 1% body $weight.day^{-1}$. This apparently represented a marked increase over the growth rates these fish had achieved during the previous two or three years in freshwater, even though the calculation may have been an underestimate due to the short period of time between captures (< 30 days) and the effects of tagging (Pemberton, 1976a).

For female trout, fecundity is directly related to body size (Walker, 1994a). Larger females produce greater numbers of eggs and the eggs are larger as well. More eggs increases the probability that some will survive to maturity and maintain the gene line. In addition, larger females result in bigger fry which can withstand starvation for longer and have a lower mortality rate than fry derived from smaller eggs (Bagenal, 1969; Elliott, 1988). Thus, producing larger eggs would appear to greatly increase the potential success of any spawning.

For male trout, where the investment in reproductive products is less than for the female, the advantages of being larger are less conclusive. Although larger males are able to defend spawning females from other males more effectively, small freshwater resident males, called precocious parr, have been observed attempting to contribute to the fertilisation of eggs. This tactic is not without risks, however, as considerable damage and fatalities are observed amongst precocious parr from attacks by larger adult males. In males there exists, therefore, a two-tier strategy: either migrate and grow larger, but with the resultant costs and risks of migration, or mature early at small size, to avoid the costs of migration but have a reduced chance of fertilising eggs.

The difference in the balance between costs and benefits of anadromy for the sexes is demonstrated by the fact that the sex ratio of sea trout populations commonly favours females (Le Cren, 1985). Examples include 2:1 females to males in trout caught in Argyll sea lochs (Pemberton, 1976a), 2.7:1 in smolts caught on the River North Esk, Angus (Pratten and Shearer, 1983a) and 1.58:1 for sea trout returning to the Findhu Glen Burn, Perthshire (Walker, 1990).

In addition, many trout populations exist where the majority of females migrate to sea while the male trout remain as freshwater residents. Campbell (1977) observed that the majority of maturing female trout ascending the Kirk Burn, a small tributary of the River Tweed, Borders, were sea trout whereas maturing males were predominantly river residents. Similarly, of 244 maturing brown trout captured in the Findhu Glen Burn, Perthshire prior to spawning time, only four were females (Walker, 1990). This difference between the migratory strategies of the sexes is of particular relevance in the declining sea trout populations of the west coast of Scotland where the vast majority of females are sea trout. In a sampling of several west Highland rivers and lochs, Walker (1994a) found a predominance of females amongst sea trout (321 males vs 413 females) and considerably greater numbers of maturing resident male versus female trout (381 males versus 15 females). Thus, the bulk of the trout eggs in these systems are produced by sea trout and a reduction in the number of returning sea trout females will have far greater impact on the subsequent juvenile population than in those populations where resident female brown trout can compensate for the reduced input of female sea trout.

2.1.2 What factors influence the choice to migrate to sea?

2.1.2.1 Freshwater growth

Since a considerable benefit of anadromy is to improve individual growth potential, it is not surprising that growth potential in freshwater should be a major factor in determining whether or not juvenile trout migrate to sea. Trout progeny of anadromous and resident parents have been shown to give rise to both sexes of sea trout and brown trout (Frost and Brown, 1967; Jonsson and Gravem, 1985; Walker, 1990), and several investigations have shown that the differentiation between resident and migrant behaviour depends on juvenile growth rate, or a physiological trait correlated with growth rate. In simplest terms, areas that provide good growth conditions ought to produce fewer sea trout than areas where growth is poor.

For example, sea trout juveniles transplanted into areas of stream with no indigenous trout, or parts of the River Tay system where sea trout were rare, possibly because of local chemical enrichment and periodic stock depletions due to pollution, grew faster and were more likely to remain as residents (Walker, 1990). Similarly, when eggs from these sea trout were transplanted to the area lacking indigenous sea trout, the vast majority of offspring became resident trout. Within aquaculture, growth rates may be increased so that the propensity to remain as freshwater-residents increases (Jonsson, 1989). Juvenile trout reared in an aquarium from sea trout parents produced a higher proportion of resident females than were found in the parent stream (Walker, 1990). Relative growth rates also influence the strategy of Arctic charr (*Salvelinus alpinus*). Parr of both sexes reared from anadromous, small or large freshwater-resident stocks manifested all three parental forms (Nordeng, 1983). Regardless of parental type (anadromous or resident), an increased amount of food during the parr stage increased the resident fraction in the population and reduced the fraction of smolts.

In contrast, systems with low productivity tend to produce more migrants. Zalewski *et al.* (1985 cited by Thorpe, 1990) found that the development rates of resident trout planted into a range of habitats in an upland stream in Poland were directly correlated with stream productivity but that in the least productive habitat, trout silvered and migrated rather than maturing.

As well as influencing the migratory strategy, growth rates also influence the proportion of precocious male parr. Precocious male trout were longer than immature parr in two streams in Sweden (Dellefors and Faremo, 1988). The incidence of parr males within streams was correlated to mean length and was higher in years of better growth. Similarly, in male salmon the frequency of precocious parr is related to growth rate with higher rates producing a greater proportion of precocious parr. The decision to mature or not in male Atlantic salmon parr is dependent on growth rates during a springtime 'window of opportunity' (Thorpe, 1986) as demonstrated by Adams and Thorpe (1989). Water temperature and photoperiod were manipulated to, respectively, increase growth rates and remove the 'window' in 0+ parr. Whereas 7.5% of males in the warmer, ambient photoperiod group matured as 1+, none of the males in the other groups matured.

2.1.2.2 Other factors

However, whether or not a trout migrates to sea is not only dependent on freshwater growth. Several other factors are involved, including the sex of the fish (discussed above), the stability of the freshwater environment and the genetic disposition towards migration or residency.

Trout in unstable freshwater environments are likely to migrate to sea whether freshwater growth rates are good or bad. For example, in the Black Bows Beck, a small Lake District stream, growth rates are good with juvenile trout growing at nearly maximal rates (Elliott, 1994). However, this stream is small and subject to sustained summer droughts which constrain the available habitat. As a consequence, nearly all the trout undergo smolting and migrate to sea after two years in freshwater.

The argument for a heritable component in the disposition towards anadromy amongst different populations of trout is based upon two lines of evidence, one of which is the presence of fast and slow growing sea trout smolts within a population. Not all sea trout smolts within a population that migrate in the same season are of the same age. A smolt run may include juveniles that have spent two, three, four or even more years in freshwater prior to migration. Typically, the younger smolts are smaller and migrate later in the season than do the older smolts (Pratten and Shearer, 1983a; Bohlin *et al.*, 1993; Bohlin *et al.*, 1996). However, to reach a size sufficient to smolt, these younger fish will have grown at a faster rate. Although older smolts are the largest, it would appear that the average growth rate of younger smolts is greater than that of older smolts (Pratten and Shearer, 1983a). Why then should fast growing parr undergo smolting earlier, if at all? Faster growing parr may become constrained by limiting factors such as available space or food. This would induce them to smolt at a younger age, not because of poor growth up to that point but because of the diminishing growth potential in freshwater as they become larger.

Furthermore, there are several studies that have demonstrated a different tendency to smolt or remain as residents amongst juveniles of anadromous or resident parentage. Alm (1959) demonstrated that juveniles reared from Swedish stream and lake trout under similar conditions maintained differences in age at maturity. Trout of both types matured earlier when grown faster but the relative difference in age at maturity remained.

Sea trout progeny tend to produce more sea trout, regardless of growth conditions. For example, Walker (1990) found that of the sea trout progeny stocked in a Scottish stream which normally produced few seaward migrants, 53.5% of the recaptures were sea trout. Jonsson *et al.* (1995) observed that, in general, trout of freshwater resident parentage displayed a higher degree of freshwater residency than anadromous stocks when released in the River Akerselva, Norway.

In charr, offspring of early maturing small resident charr produce more resident individuals and fewer smolts than do offspring of the later-maturing anadromous type (Nordeng, 1983). Offspring of anadromous parents transformed into smolts in higher proportions than did those of resident parents (31.7% vs 16.9% of recaptures), when transplanted into a freshwater system (Nordeng *et al.*, 1989).

Juveniles reared from trout caught above impassable falls display a reduced tendency to migrate (Jonsson, 1982, 1985; Svardstrom and Fagerstrom, 1982). By their nature, impassable falls act as migratory, and therefore genetic, barriers. Any fish that migrate downstream over the falls are unable to return to pass on their genes to the next generation. This would rapidly select against the tendency to migrate downstream within a population. Indeed, trout sampled from above impassable falls have been shown to possess a stronger rheotactic response and to resist greater downstream flow rates than trout sampled from below falls (Jonsson, 1982).

The studies cited above which demonstrated different tendencies to migrate compared trout from different freshwater systems or parts of freshwater systems. Genetic techniques have demonstrated a considerable degree of differentiation among trout populations of even neighbouring systems (see Ferguson, 1989; Stephen and McAndrew, 1990; Maitland and Campbell, 1992). However, little or no work has been made regarding the migratory tendency of juveniles reared from sympatric resident and anadromous trout. Most genetic studies to date have failed to show any genetic differentiation between sympatric resident and anadromous trout (see Hindar *et al.*, 1991; Cross *et al.*, 1992). This implies that there must be regular interbreeding between resident and migratory adults which is supported by evidence showing that in some systems producing both migratory and resident individuals, the vast majority of females are migratory while most of the males are resident (see above). Further support for the genetic results is provided by observations of both

sexes and both "types" mixing indiscriminately on the spawning grounds (Jonsson and Gravem, 1985).

However, a recent study of Scottish sea trout and river resident brown trout found significant differences between the sympatric resident and anadromous trout using mitochondrial DNA (mtDNA) analysis. Since mtDNA is inherited in a maternal fashion, this observation would suggest minimal crossing over of females between the two groups (Thompson, 1995). Therefore, it is of considerable interest to compare the migratory tendencies of such sympatric populations.

2.1.3 Relevance of such studies

The decline in sea trout numbers on the west coasts of Scotland and Ireland have been described already (Section 1.5). Providing the causes of marine mortality can be addressed, one favoured method of stock regeneration is to resupply rivers with hatchery-reared trout (Jonsson *et al.*, 1995). In order for such a regeneration programme to be cost-effective, it must maximise the proportion of sea trout produced from hatchery-reared juveniles. In addition, as it would be expedient to preserve local genetic variation amongst the sea trout populations, such regenerations should use locally derived broodstock. However, since in some rivers there are very few sea trout left (e.g. the Shieldaig system, Wester Ross (A.F. Walker, pers. comm.)), freshwater resident female trout may have to be used as broodstock.

On a wider scale, habitat enrichment and global warming have potential impacts on the numbers of sea trout migrating from river systems. The mean smolt age (MSA) of sea trout from the River North Esk, Angus declined between 1934 and 1979 (Pratten and Shearer, 1983a) and Fahy (1978) noted a similar trend in Irish sea trout smolts between 1930 - 1970. In addition, the peak number of smolts migrating downstream per day, generally became later in the season between 1970 and 1979 on the River North Esk (Pratten and Shearer, 1983a). A reduced MSA suggests that growth conditions are improving and juveniles are reaching a size suitable to smolt at an earlier age. A later peak in the annual smolt run might indicate that the younger smolts require a longer period of spring growth prior to smoltification (Pratten and Shearer, 1983a). Possible environmental changes causing an acceleration in the growth rates of parr include milder winters, and freshwater eutrophication through agriculture run-off resulting in improved freshwater productivity (Fahy, 1978).

Improved growth conditions in freshwater may reduce the number of sea trout by means of two mechanisms. As outlined above, improved growth conditions reduce the tendency of juvenile trout to migrate to sea and encourage more parr to remain in freshwater. Second, late migrating, younger sea trout smolts tend to be relatively small which may affect their subsequent marine mortality rate. Late migrating sea trout smolts contribute proportionally fewer returning adults (Le Cren, 1985). Body size is an important factor in the initial seawater tolerance of salmonids (see Discussion), and small smolts may be compromised physiologically. For example, late running sea trout smolts from the Afon Glaslyn (Wales) tended to spend the entire summer in the estuary rather than migrating out to sea (Brassington; in Le Cren, 1985). A similar situation is indicated by the presence of large numbers of small trout parr in the brackish waters at the head of the estuarine Kyle of Durness, Sutherland, during May to July (pers. obs.).

From the discussion above, it is clear that questions remain with regard to the interplay of environmental and genetic factors that influence the migratory strategy of juvenile trout and that further studies are required.

2.1.4 How to measure this choice to migrate to sea?

It is clear that both environmental and genetic factors have the potential to influence migratory strategy in trout. Rearing studies that vary the growth rates of trout of various genetic backgrounds comprise one strategy to investigate these factors. However, because most sea trout do not undergo the parr-smolt transformation until at least two years after they hatch, such an extended rearing period would be an expensive and slow way to provide data. The emphasis should, therefore, be on identifying parameters that indicate the individual migratory strategy at stages earlier than smoltification. Two parameters which provide prior indications of subsequent smoltification in some salmonid species are continued growth during the late autumn and winter months leading to a characteristic bimodal length frequency distribution, and seasonal peaks in branchial Na^+K^+-ATPase enzymatic activity.

2.1.4.1 Bimodal length frequency distributions

It is well established that stocks of Atlantic salmon parr reared under good growing conditions will segregate into two subpopulations towards the end of their first summer (Thorpe, 1977; Bailey *et al.*, 1980). A bimodal length frequency

distribution, apparent from late summer onwards (Metcalfe *et al.*, 1989) is the result of differential growth rates. During late summer/autumn, as water temperatures decline and photoperiod shortens, whereas the smaller parr (the lower modal group, LMG) exhibit reduced appetite (Metcalfe *et al.*, 1986) and depressed food intake (Higgins and Talbot, 1985), parr of the upper modal group (UMG) exhibit a brief increase in food consumption rate (Metcalfe *et al.*, 1989). This results in the bimodal distribution which is further differentiated by the virtual cessation of growth amongst the LMG during the winter whereas the UMG continue to grow at a reduced rate.

The UMG parr will undergo smoltification in the following spring while the parr of the LMG will be divided into those males which become precocious, and males and females which will grow in freshwater for another year before smolting (Skilbrei, 1991; Herbinger and Friars, 1992). As seawater tolerance increases with size (see below), it is thought that pre-smolts continue to feed and grow during the winter to maximise size whereas the LMG parr, which will have a further year's growth prior to smolting, enter pseudo-hibernation to preserve energy stocks and avoid predators.

However, such a bimodal distribution has not been reported in trout stocks (Tanguy *et al.*, 1993) and so may not be a suitable indicator of subsequent smolting in trout.

2.1.4.2 Seasonal peaks in branchial Na^+K^+-ATPase enzymatic activity

The first physiological signs that parr are preparing to smolt can also be seen in UMG salmon parr in the autumn/winter prior to smoltification. The gill Na^+K^+-ATPase enzyme is vital for ion extrusion in seawater. Enzymatic activity levels fluctuate seasonally while fish are still in freshwater (Lasserre *et al.*, 1978), apparently under the influence of an endogenous rhythm. Spring and autumn peaks in Na^+K^+-ATPase activity were noted in 0+ and 1+ coho salmon, thereby indicating changes in osmoregulatory physiology preparatory to seaward migration. However, it has been shown that UMG Atlantic salmon parr exhibit greater levels of enzyme activity in the autumn prior to smoltification. These larger parr also possess larger and more numerous chloride cells with higher levels of branchial succinic dehydrogenase (SDH) and Na^+K^+-ATPase activity in autumn prior to smoltification (Langdon and Thorpe, 1985).

However, such peaks in activity occur only in the autumn immediately prior to smoltification.

2.1.4.3 Seawater challenge

A third method by which to test the hypo-osmoregulatory abilities of developing juvenile trout is to challenge them with seawater (Hogstrand and Haux, 1985). Direct transfer from freshwater to seawater is accompanied by large physiological changes that must be accommodated successfully if fish are to survive and later adapt to the new medium. Acclimation to seawater can be separated into two distinct phases; an adjustive 'crisis' phase characterized by increased plasma osmotic concentration, and a regulative 'stabilization' phase during which control is established over the osmotic concentration (Jackson, 1981). The stabilization period begins after 24 - 48 hours and leads, over the next seven to ten days, to the complete adaptation of the fish. The salinity challenge focuses on the crisis phase, during which time fish must be able to survive long enough for adaptive mechanisms to begin re-establish physiological homeostasis.

2.1.4.4 Drinking rates

One of the most immediate measurable responses to seawater transfer is an increase in drinking rate (see General Introduction), as observed almost instantaneously in eels (Hirano, 1974), and within 4 - 6 hours in Atlantic salmon smolts (Usher *et al.*, 1988). The initiation of drinking (or increased rate of drinking) upon the seawater entry of eels has been variously attributed to increased tissue osmolality (Sharrat *et al.*, 1964), increased plasma chloride levels and decreased blood volume (Hirano, 1974). Stimulation of drinking in the flounder, *Platichthyus flesus*, appears to be due to dehydration and the consequent increased plasma osmotic and ionic concentrations (Carrick and Balment, 1983). The relatively slower response of salmon smolts was thought to indicate that increased plasma ion concentrations rather than the presence of an external ion were the triggering factor (Usher *et al.*, 1988).

Drinking in seawater-adapted eels was inhibited by infusion of large quantities of hypertonic saline or by distension of the stomach or intestine with isotonic mannitol solution (Hirano, 1974). After initiation, drinking rates are subsequently controlled by a negative feedback loop mechanism related to the degree of stomach distension (Evans, 1979).

2.1.4.4.1 Measurement of drinking rates

Measurement of drinking rate is very important in any study of osmoregulatory control. While it is relatively simple to measure the amount of water drunk by terrestrial vertebrates, it is much more difficult to measure that consumed by aquatic animals. There have been two types of method employed to measure drinking rates in fish. Direct measurement in eels has been attempted using an oesophageal cannula to collect all the imbibed fluid (Evans, 1968). However, this method has the distinct disadvantages of first, requiring a pre-experiment operation and second, preventing water entering the gut as normal. These factors result in artificially high drinking rates because blood loss during the operation will induce drinking to increase blood volume and none of the inhibitory mechanisms outlined above are stimulated. These disadvantages make this method suitable for measuring the delay between salinity transfer and onset of drinking but unsuitable for accurately measuring drinking rates. The method that gives a more accurate drinking rate involves a relatively straightforward dilution technique.

The fish is placed in a tank of water with a known concentration of dissolved marker substance. After a set period of time, chosen to ensure the marker has entered the gut but none has reached the anus, the fish is removed from the medium and killed. The gut is removed and the gut contents measured for the marker. This value is then divided by the concentration of marker in a known volume of medium, and the length of time the fish was exposed to the marker. For comparative purposes the drinking rate is also standardised to a fixed fish body mass, appropriate to the general size of the species investigated.

Initially, colorimetric measurement of phenol red was adopted (Smith, 1930; Motais and Maetz, 1965) but was difficult to use and was subsequently found to be less accurate than later methods. More accurate methods have resulted from the use of radioisotope markers. A variety of radioactive isotopes have been employed as markers but the ^{51}chromium-EDTA complex (Hazon *et al.*, 1989) has proven most appropriate and has several advantages over other radio-labels. ^{51}Chromium is a γ-emitter (Emax = 320 Kev; Half life = 27.7 days) and thus can be easily assayed, in contrast to ß-emitters such as ^{14}C-Inulin (Evans, 1967; Potts and Evans, 1967) and ^{35}Sulphate (Potts *et al.*, 1967). In addition, while the ^{125}I-labelled polyvinylpyrrolidine (PVP) complex (Evans, 1968; Balment and Carrick, 1985) also involves a large, inert molecule to prevent transport across epithelial linings, it is 'sticky' (N. Hazon, pers. comm.) that makes it difficult to decontaminate tanks and,

more importantly, makes it unreliable for determining drinking rates, particularly where rates are low, as in freshwater adapted fish. In all cases it is important that none of the marker is absorbed across the gut lining into the blood stream, i.e. that the marker compound is non-invasive. The EDTA bound to the ^{51}Cr ensures that the isotope remains in the gut contents (see Usher *et al.*, 1988). Absorption would have two deleterious consequences: it would lead to an underestimation of the drinking rate; and it would contaminate the blood stream and complicate any blood sampling and measurement of plasma ion concentrations.

2.1.5 Aim of this part of the study

The collapse of many of the sea trout populations on the west coasts of Scotland, Ireland and Norway has brought about the need for a much greater understanding of the developmental characteristics of juvenile trout. Provided that the issue of marine mortality can be addressed, restocking with hatchery reared trout, preferably from local stocks, is recognised as one of the more important potential solutions to the regeneration of failing stocks. However, even amongst progeny reared from sea trout parents, a significant proportion of the juveniles may not smolt but remain, instead, as resident brown trout.

Factors which alter growth potential in freshwater such as water temperature and food availability, the latter linked to some extent to the former, are the major environmental and biological contributors, respectively, to the "choice" of migratory strategy. At present it is unclear how potential changes in climatic conditions such as temperature and precipitation patterns brought about by global warming will affect the anadromous:resident ratio of trout populations. Recent surveys of juvenile trout populations from several systems on the west coast of Scotland have found little change in the expected ratio of female to male resident trout suggesting no significant change in the proportion of females migrating to sea (Walker, 1994b), and implying that the causes of the declines in stocks are more likely to be of marine origin. However, this does not mean that the continued environmental changes might not bring about such changes in the future.

The aim of this chapter, therefore, is to compare the development of hypo-osmoregulatory capabilities, measured by drinking rate after acute salinity challenge, between juvenile offspring of three parental groups (anadromous, river-resident and isolated trout) reared under three different feeding regimes.

2.2 Materials and Methods

2.2.1 Broodstock capture

Mature male and female trout (resident and anadromous) were captured by electrofishing from several sites in the River Eden and two larger tributary streams. A detailed description of the River Eden and its tributaries is provided in Chapter 3.

Electrofishing is probably the most time efficient technique for sampling a broad size range of live fish from burns or shallow rivers. Smooth DC current (c. 250 v) is supplied by a portable bank-based generator (500-1900 kv), plus transformer and control box, and applied to the water in short bursts via a handheld cathode on a pole (Moore, 1968). The circuit is completed by an anode, usually a wire mesh plate to increase surface area, placed in the water approximately 50 m away. The current stuns the fish and draws most of them towards the cathode where they can be netted and then kept in holding boxes or keepnets outwith the electric field. The technique requires a minimum of three staff. One person carrying the cathode and a landing net, one person carrying a bucket and a landing net and one person monitoring the generator for safety purposes (Plate 2.1). Electrofishing gear and trained staff were provided by the S.O.A.E.F.D. Freshwater Fisheries Laboratory, Pitlochry.

Twelve trips were made to the Eden system over a period of seven weeks from mid-October to early December, 1993. Seven mainstream, five tributary stream and two above-falls sites were fished, several on more than one occasion (Figure 2.1). A large number of maturing resident trout were caught, both below and above impassable falls (Plate 2.2), as well as 14 sea trout (12 females, mean fork lengths 441.6 mm ± 7.3 mm, and two males, 380 and 435 mm) caught below falls. All mature trout were transported live to the S.O.A.E.F.D. hatchery facility at Almondbank, Perthshire and held in freshwater until they were ready to spawn. Each trout was Pan-jet (Wright Dental Group, Dundee) tattooed with alcian blue dye at the base of one of the ventral fins according to site of capture to allow later identification. Fish were measured (mm) from the tip of the nose to the middle of the tail fork. Sea trout were distinguished from freshwater-resident brown trout by their morphology and by scale examination by F.F.L. staff.

Plate 2.1

Plate 2.2

Plate 2.1 Staff from the F.F.L. electrofishing for trout broodstock in the headwaters of the River Eden.

Plate 2.2 Dura Den Falls on the Ceres Burn - impassable to sea trout and brown trout from the River Eden.

Figure 2.1

Figure 2.1 Map of the River Eden with locations of electrofishing sites. After Anon. (1994d).

LOCATION MAP

FIRTH OF TAY
EDEN ESTUARY
GUARDBRIDGE
BAIRSIE BRIDGE
ST ANDREWS
CUPAR
KEMBACK
RIVER EDEN
AUCHTERMUCHTY
CERES
FALKLAND

SCALE
0 1 2 3 4 5
km

Approximately 10,000 eggs were stripped from females from each of the three parental groups, hereafter described as Sea trout, Resident trout and Isolated trout (sea trout, resident river trout and above-falls trout). Each batch of eggs was fertilised with milt from several males of the same trout type to maximise the genotypic diversity of the resultant offspring.

Stripped fish (kelts) were kept at the hatchery for several days to recover and then all resident trout were returned to their sites of capture while the sea trout kelts were transported live to the aquarium at the Gatty Marine Laboratory for a reconditioning trial.

2.2.2 Juvenile trout rearing conditions

The birthday of trout fry was taken to be 1 April and fish in any spring period thereafter were considered to be a complete number of years of age (Solomon, 1982). Fish in the summer, autumn and winter are referred to as X+ (e.g., 0+, 1+) according to their age at last birthday (Fahy, 1978). Eggs were initially placed in trays with flow-through water until the alevins had hatched and absorbed most of their yolk sacs. Once fry had begun to feed on commercial fry dust they were transferred to 1 m diameter circular tanks. Sea trout and Resident trout progeny were each split equally between six tanks. However, fewer Isolated trout were available and so these were split between four tanks. Three ration regimes (High, Medium and Low) were then set up and created by supplying food at the rate of approximately 5% body weight, 2.5% body weight and 1% body weight per day, respectively. Each ration regime was duplicated between two tanks (Figure 2.2). Fish were fed commercial trout pellets (EWOS) and the amount of food was controlled by a computer system linked to automatic feeders suspended above each tank. The food supply was reduced in the High and Medium ration groups during the winter as appetites declined with lower water temperature. Since there were only four tanks of Isolated trout, no Medium ration treatment was included for this group.

As the fish grew they were moved to larger tanks. However, batches of trout from each tank were returned to the river or burn of their origin after the September 1995 growth monitoring to prevent or reduce any density dependent growth inhibition. Care was taken to leave similar numbers of trout in all the tanks after this thinning procedure.

Figure 2.2

Figure 2.2 Schematic representation of the tank arrangement for the various parental group/ration treatments as detailed in the text.

Sea trout

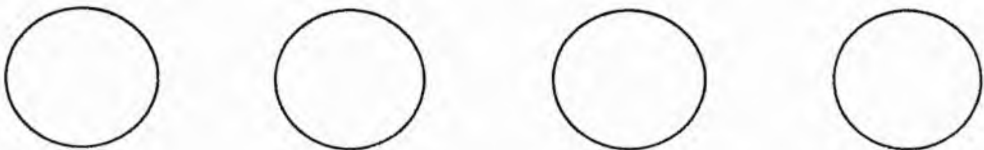
High ration | **Low Ration**

Resident trout

High ration | **Low ration**

Isolated trout

High ration | **Low Ration**



Medium ration

Sea trout | **Resident trout**

2.2.3 Growth monitoring

Growth was monitored by periodic sampling of all the fish in one tank from each of the parental group/ration treatments. Five samplings took place in August 1994, May 1995, September 1995, March 1996 and May 1996. These corresponded to approximate ages of 5, 14, 18, 24 and 26 months, respectively (Figure 2.3). A sample of 50 trout in Samplings 1 and 5, and 100 trout in Samplings 2 - 4 were first removed at random. After light anaesthesia in a dilute solution of benzocaine (25 - 30 ppm), fork length (mm) and wet weight (g) were measured. These trout were then allowed to recover in a spare tank. On the occasion of the first sampling, a further 250 trout from each tank were then measured for length alone. In subsequent samplings, all the trout remaining in each stock tank were measured for fork length. Condition factor (K) was then calculated with the following formula:

$$K = (\text{weight (g)} * 100) / \text{length (cm)}^3$$

2.2.4 Osmoregulatory physiology experiments

On four occasions throughout the experimental period (Figure 2.3), groups of trout from some or all of the parental form/ration treatments were transferred to the Gatty Marine Laboratory and challenged to a variety of salinities to investigate the effects of ration and parent migratory form on the development of hypo-osmoregulatory capabilities.

2.2.4.1 Experiment 1, November 1994 - 9 months old

The first set of salinity challenges was performed using the High ration trout of the three parental groups (Sea, Resident and Isolated trout). Thirty trout were collected at random from each of the two duplicate High diet tanks, giving a total of 60 trout per group, and transported to the Gatty Marine Laboratory aquarium in polythene bags filled with 20 l of water and inflated with an O_2 / CO_2-N_2 mix (95% / 5%). Each batch of fish was maintained in separate 400 l tanks of running freshwater for 21 days to recover from handling and transport. Fish were fed once daily with the same commercial fish food pellets as supplied by staff at the Almondbank Facility. The photoperiod was adjusted to simulate the natural photoperiod and the water temperature was ambient (7°C).

Figure 2.3

Figure 2.3 Calendar of the two years of juvenile trout growth with the months when growth monitoring samples were performed and when trout were collected for hypo-osmoregulatory challenges.

Year	Month	Age (months)	Growth Sample	Physiology Sample
1994	April	1		
	May	2		
	June	3		
	July	4		
	August	5	Sample 1	
	September	6		
	October	7		
	November	8		Experiment 1
	December	9		
1995	January	10		
	February	11		
	March	12		
	April	13		
	May	14	Sample 2	Experiment 2
	June	15		
	July	16		
	August	17		
	September	18	Sample 3	
	October	19		
	November	20		Experiment 3
	December	21		
1996	January	22		
	February	23		
	March	24	Sample 4	
	April	25		
	May	26	Sample 5	Experiment 5

Trout from each parental group were challenged by acute transfer to 20, 33, 50 and 75% seawater, and freshwater transfers were also performed to provide controls. Salinities greater than freshwater were produced by mixing freshwater and seawater in the appropriate proportions and confirmed with a salinity refractometer (Aquafauna Bio-Marine, Inc.).

Freshwater transfers of different parental groups were performed over two days but all subsequent transfers were carried out on the same day for each parent type group. In order to standardise the period of time in the labelled medium, transfers were staggered through the day to allow sufficient time to process a group before the next group was removed from the labelled medium. The first group was transferred at approximately 0900 hrs and the final group transferred at approximately 1300 hrs.

Drinking rate was measured by gut accumulation of the radioactive marker ^{51}Cr-EDTA. For each salinity transfer, groups of ten trout were placed in 5 l of continuously aerated water of the appropriate salinity with an approximate activity of 50 μCi.l $^{-1}$. After six hours in labelled medium, trout were removed individually and killed by an overdose of anaesthetic (2-phenoxyethanol, 2 ml.l $^{-1}$) and by severing of the spinal column. The gut was exposed, sutured at either end and removed to a pony vial. The activity of the gut contents, in counts per minute (c.p.m), was then measured on a gamma counter. Drinking rates were calculated by comparing this activity with the mean activity of two 1 ml aliquots of medium using the formula provided in Figure 2.4.

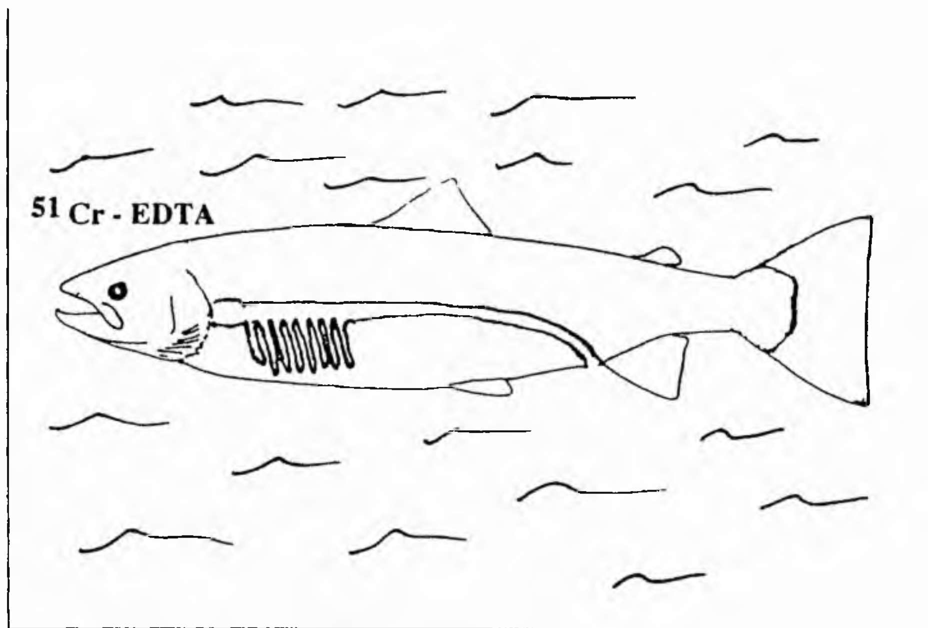
2.2.4.2 Experiment 2, May 1995 - 14 months old

The inclusion of Medium and Low ration groups resulted in eight separate groups of trout (three rations x three parental groups - Medium/Low). Due to the logistical complications of time (number of days and number of experiments performed each day) it was no longer practicable to perform the range of salinity transfers of Experiment 1 so transfers were limited to a freshwater control, brackish water (16 ppt) and seawater (32 ppt). Fish transport and acclimation procedures were similar to those outlined in Section 2.2.4.1. On this occasion only trout from High and Low diets were sampled.

Eighteen trout were netted at random from each of the duplicate tanks and combined to give a sample of 36 trout from each parental form/ration. Once at the Gatty Marine Laboratory, trout from each group were placed in six separate perforated

Figure 2.4

Figure 2.4 Schematic representation of the drinking rate measurement protocol.



$$\text{Drinking Rate} = \frac{C}{M * T * Wt} = \text{ml / kg / hr}$$

C = total gut count (cpm)

M = bathing medium isotope concentration (cpm / ml)

T = time in bathing medium (hours)

Wt = weight of fish (kg)

plastic holding boxes suspended in three 1 m diameter tanks. Each holding box was supplied with a separate water supply but the aeration was to the whole tank. Trout were fed commercial pellets (EWOS) twice daily and maintained at ambient water temperature (10°C) and photoperiod for three weeks prior to salinity transfers.

In this experiment, three circular 10 l tanks, each containing 5 l of either radiolabelled (75 $\mu Ci.l^{-1}$) freshwater, brackish water or seawater were used for the transfers. To avoid possible tank effects, each tank was further divided into three equal segments by a wooden frame and net mesh panels with the air supplied from the bottom of the central rod (Figure 2.5). Only two of the three tanks were used on any particular day to reduce the need to stagger transfer times across the whole day. All transfers began between 0900 hrs and 1000 hrs each day.

Each salinity was allocated a number range and a random number generator (RNG) was used to determine the two salinities that would be used on each day. Then each parental form/ration group was allocated a number range and the RNG used to determine the order in which the fish would be sampled. The experimental medium was replaced with clean water of the appropriate salinity after five experimental days.

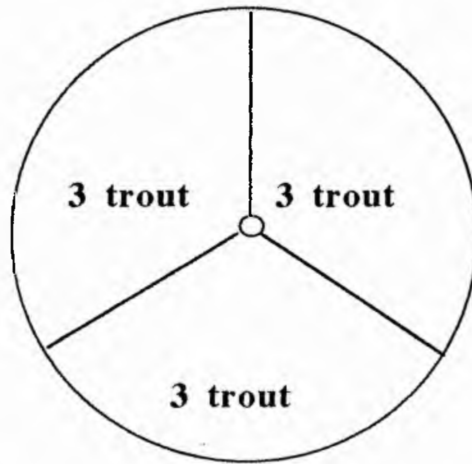
Drinking rates were measured in nine trout (3 groups of 3) from each group transferred to each salinity (0, 16 and 32 ppt). Drinking rate was measured as in Experiment 1.

2.2.4.3 Experiment 3, November 1995 - 20 months old

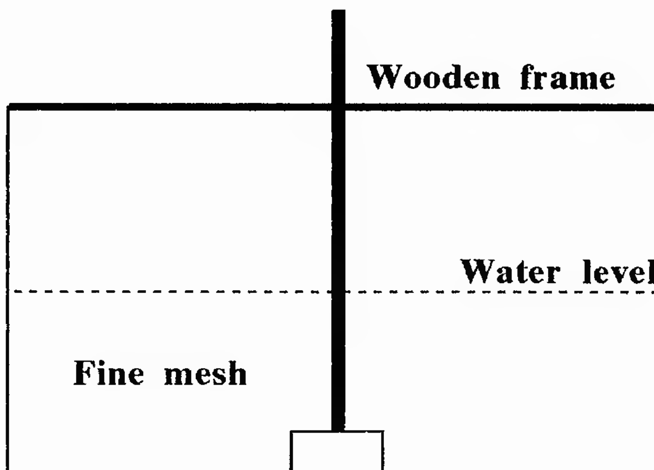
Groups of 12 trout from each of the parental form/ration groups were transported to the Gatty Marine Laboratory aquarium in a manner similar to that above. The trout were too large to be kept in separate holding boxes so each batch of fish was marked by a Pan-jet tattoo of alcian blue dye at the base of one of the pectoral fins. This was sufficient to identify groups of trout since they were split into four tanks; three tanks for the High and Low diets from each of the parental forms, and one tank for the two Medium diet groups (Figure 2.6). Trout were fed twice daily on commercial pellets and kept for four weeks prior to salinity transfers taking place. Water temperature was approximately ambient (7°C) and photoperiod adjusted to mimic natural conditions.

Figure 2.5

Figure 2.5 Sketch of the segmented experimental tank arrangement used in the Experiment 1 drinking rate measurements.



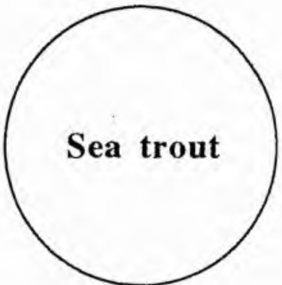
View from above

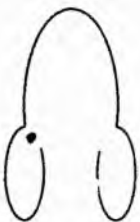


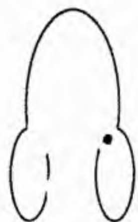
Side aspect

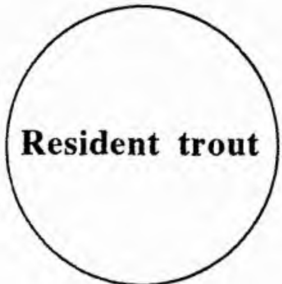
Figure 2.6

Figure 2.6 Schematic representation of the holding tank arrangement and panjet tattoo marks used for Experiments 3 and 4. Note that in Experiment 4, some trout also had the adipose fin removed as a distinguishing mark.

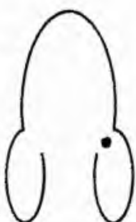

Sea trout

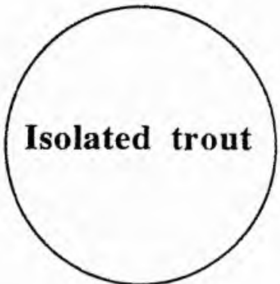

High
ration


Low
ration

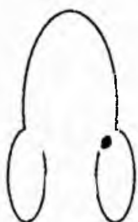

Resident trout

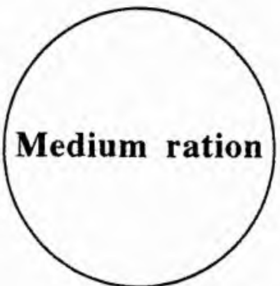
High
ration


Low
ration


Isolated trout

High
ration


Low
ration


Medium ration

Sea
trout

Resident
trout

Growth rates during the summer months of the second season were high and the fish were now too big to be held in the segmented experimental tanks in only 5 l of water. Three circular tanks (20 l capacity) containing 10 l radio-labelled water (40 $\mu Ci.l^{-1}$) were used, one for each salinity, and a maximum of five trout per tank. Therefore, only one group of fish could be transferred to each salinity on a particular day. Because a maximum of ten trout from each group were to be challenged to each salinity, two challenges of five trout were performed on separate days. The order of the salinity transfers was again randomised, as in Experiment 2 above, as much as possible. Experimental water was replaced after every second transfer.

Note that the radioactivity of labelled water was lower than that used in the Experiment 1. High radioactivity reduces the error inherent in measuring the minimal quantities of water drunk by small freshwater-adapted fish, but as the water volume is increased so the expense of maintaining such high activities becomes prohibitive. In larger trout that drink a quantitatively greater amount, an activity of 10 $\mu Ci.l^{-1}$ is adequate.

Measurement of drinking rates was performed in the same manner as described above. The sex and state of maturation of trout were noted where possible. Maturing males were defined as those with white, swollen testes rather than the empty translucent testes of immature males.

2.2.4.4 Experiment 4, May 1996 - 26 months old

It was expected that at this age some trout would be smolting in anticipation of migrating to sea. The plan for this last set of experiments was, therefore, to compare the hypo-osmoregulatory physiology of smolts versus resident parr from some or all of the groups. However, during the growth data samplings in March, and again in May, it was apparent that none of the trout from any of the parent forms or rations were becoming smolts. This was confirmed by staff of the F.F.L. Plates 2.3 and 2.4, which show wild sea trout smolts from the River North Esk (see Chapter 5) and trout reared for the present study, clearly demonstrate that the reared trout were not smolting. Therefore, the hypo-osmoregulatory capacities of groups of trout from each parental form/ration group were compared in a similar manner to the previous experiments.

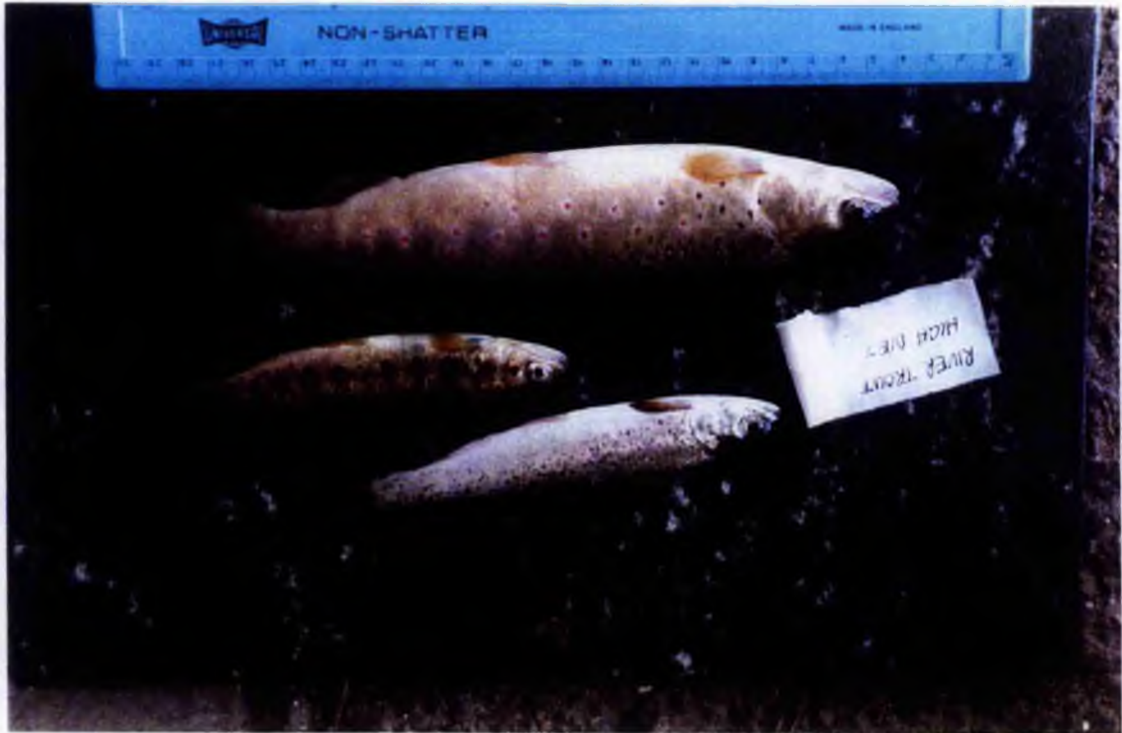
At the Almondbank hatchery, 20 trout were netted from each duplicate stock tank to give a total of 40 trout per group. Mature male trout, as indicated by their large size,

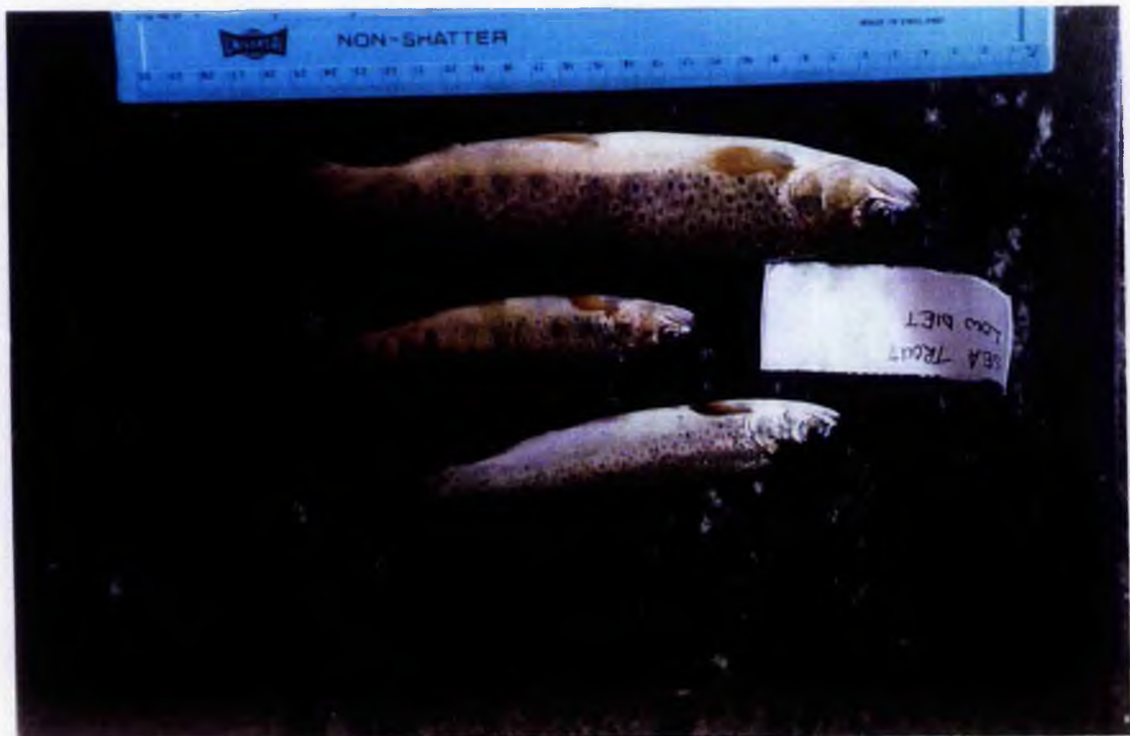
Plate 2.3

Plate 2.4

Plate 2.3 Two trout from the Low ration, Sea trout group and one wild sea trout smolt captured during migration from the River North Esk, Angus.

Plate 2.4 Two trout from the High ration, Resident trout group and one wild sea trout smolt captured during migration from the River North Esk, Angus.


NON-SHATTER
RIVER TROUT
HIGH DIET


NON-SHATTER
SEA TROUT
LOW DIET

sharp head shape, dark coloration and large adipose and ventral fins, were not included. Trout were transferred to the Gatty Marine Laboratory aquarium in the normal manner and then held in four 1 m circular tanks with flowing freshwater for seven days to recover from handling and transport. A combination of Pan-jet tattoos and adipose fin clips were used to distinguish between the different groups of fish within each stock tank. The groups of fish were separated in the same manner as Experiment 3. Freshwater-freshwater and freshwater-seawater challenges were performed in the same manner as above. Groups of eight trout were transferred to 75 l radio-labelled water (10 μCi.l $^{-1}$). Freshwater and seawater transfers were performed for each group on the same day. When all the groups had been challenged once, the labelled water was replaced and the process was repeated with further fish over the next 10 days to increase the sample sizes.

Drinking rates were measured in the same manner as before. In addition, blood was removed from the caudal vein by means of a heparinised needle and syringe, and stored in 1.5 ml Eppendorf tubes on ice until the end of the protocol. A small quantity of each blood sample was drawn into a heparinised capillary tube that was then stoppered at one end with putty. The tubes were centrifuged for three minutes at 13,000 rpm in a micro-haematocrit centrifuge (Hawksley, England), after which the haematocrit was measured as the volume of erythrocytes in proportion to the total blood volume. Whole blood was then centrifuged at 12,000 g for five minutes, after which plasma was transferred to another Eppendorf and stored at –20°C for later analysis. Plasma Cl^- concentration was measured in duplicate 20 μl samples by automatic titration (Chloride Analyzer 925, Corning).

After gut dissection for drinking rate measurement, the sex and state of maturity for each fish was recorded. Maturing females were defined as those with ovaries containing clearly differentiated eggs. Maturing males were defined as those with swollen, white testes as opposed to immature males in which the testes appeared empty and translucent for most of their length.

2.2.5 Statistical Analysis

2.2.5.1 Choice of statistical tests

The growth data in this study comprised eight groups (two forms x three rations, one form x two rations). The number of groups for which physiological data were obtained varied between experiments from 15 to 24. If the data for each variable

(length, weight, condition factor, drinking rate, plasma Cl^- concentration and haematocrit) were to be analysed by multiple between-means comparisons, this would involve 28 tests for each growth data set and between 105 and 276 separate tests for the physiology data, depending upon the experiment. Whereas this is feasible with computers, it is statistically incorrect. If a $p = 0.05$ (5%) level of significance is chosen for an analysis, statistical probability indicates that a Type 1 error will probably be incurred for one in every 20 tests performed. As the number of comparisons required for the analyses of the present study are all in excess of 20, some markedly so, the probability of mistakenly assigning significance to a test is unacceptably high. Analysis of variance (ANOVA) overcomes this difficulty by allowing comparisons to be made between any number of sample means, all in a single test. In addition, ANOVA can be used to simultaneously investigate the effect of more than one element (factor) on the data.

Before an ANOVA can be applied, the data must be assessed to ensure they conform to certain limitations. First, observations should be normally distributed around the appropriate mean and second, the variances of the samples should be similar. However, if these assumptions are not met, the data can be transformed to produce an approximate normal distribution and homoscedasticity.

2.2.5.2 Growth Data

Normal distribution of the datasets was investigated using the normality test feature of the StatView software package (version 4.5, Abacus Concepts Inc., Berkeley, CA, USA). This test creates an ideal normally distributed dataset with the same mean, count and standard deviation as the observed dataset. It then compares the frequency distributions of the observed and ideal datasets by the Kolmogorov-Smirnov X^2 test. A normally distributed observed dataset is implied by a p value significant at the 5% level ($p > 0.05$). All length, weight and condition factor datasets were found to be normally distributed (Table 2.6 a-e).

Several tests of heteroscedasticity (unequal variances) are available, e.g. F-max and Bartlett's. However, they are all adversely affected by non-normality, are low in power, or have other serious drawbacks (Zar, 1984). Because of the poor performance of these tests, and the robustness of ANOVA for multisample testing among means, Zar (1984) does not recommend that the former be performed as a test of the assumptions of the latter.

The means and variances of each sample were examined by eye, and heteroscedasticity was observed in several cases so the data were transformed to remove the dependence of the sample variances on the sample means. Data transformations were performed using StatView. Since the levels of the diet treatment had a proportional effect on the length and weight data, log transformation of these data makes the sample variances independent of the sample means and also makes the effects of the treatments additive. Log transformations are also suitable for data that are ratios and so condition factor (K) data was log transformed prior to data analysis.

Analyses of variance were performed using the SuperANOVA package (Abacus Concepts Inc., Berkeley, CA, USA). Length, weight and condition factor are all related to a greater or lesser extent and as such, could not be simultaneously applied to an ANOVA format. For this reason, each variable was tested separately against the two fixed factors (ration, parental group).

ANOVA requires a balanced experimental design, i.e. data are available for each combination of variable x factor. In the present study, however, the experimental design was unbalanced by the lack of Medium ration Isolated trout data for growth or physiological measurements. To balance the experimental design, ANOVAs were first performed with only the Low and High ration data. Medium ration data were then compared as a separate analysis.

2.2.5.3 Physiological Data

The sample sizes for physiological data were too small to allow confident assessment of the distribution around the mean but it was assumed that, given a sufficient sample size, the data would have been normally distributed. Data were transformed prior to analysis to remove the dependence of the variance and the mean. Drinking rate and plasma ion concentrations were log transformed while haematocrit proportion was arcsine transformed. Arcsine transformations are appropriate for distributions, such as proportions, where both tails are truncated because all values must lie on a scale with absolute limits of 0 and 1 (Fowler and Cohen, 1990). All transformations were performed with Statview software package (as above). All 2-way and 3-way ANOVAs and 2-factor ANCOVAs with log weight as a covariate were performed using the SuperANOVA software whereas 3-factor ANCOVAs with log weight as the covariate were performed using the SYSTAT (version 6.0 for WINDOWS) software PC package (SPSS Inc., Chicago, USA).

Data are presented in figures as the back-transformed sample means with upper and lower 95% confidence limits. Tables of the results of the statistical tests are presented at the end of the chapter.

2.3 Results

2.3.1 Growth

Trout grew throughout the experimental period (21 months) with increases in mean fork length and wet weight at each sampling point, regardless of parental form or ration level (length: Figure 2.7 a-c; weight: Figure 2.8 a-c). As expected, High ration trout were obviously longer and heavier than Low ration trout of comparable parental form at each sampling point from 12 months onwards but 2-way ANOVA indicated that a significant main effect of ration was present for log length and log weight at all sample times (Tables 2.7 to 2.16). The differences between High and Medium ration groups (Sea trout and Resident trout only) were considerably less and while log length appeared different at all sample points, log weight appeared similar from 18 months onwards. The Medium ration data were not included in the statistical analysis because of the unbalanced design of the full dataset (see above).

Visual examination of the slopes of lines between consecutive sampling points suggested that the maximum difference in log length and log weight between High and Low ration groups occurred during the second summer of growth (14-18 months). Note, however, that time periods between samplings were not equal and so the slopes of lines reflected an average increase over time and, as such, were not strictly comparable between different time intervals. A significant 2-way interaction between ration and parental form effects for log fork length was indicated by 2-way ANOVA of the 5 months sample data (Table 2.7) such that while High ration trout were longer than Low ration trout in all parent type groups, the difference between ration levels was least in Resident trout and greatest in Isolated trout. Such a significant interaction was neither reflected in the analysis of log weight data (Table 2.12), nor in any subsequent analyses of the log length or log weight data.

Significant main effects of parental form were indicated by 2-way ANOVA for log length in all subsequent sample times and for log weight between 24 months and 26 months. The pattern of parental form effects was consistent throughout, with Resident trout being larger than either Isolated trout or Sea trout groups. Isolated and Sea trout groups were similar in length and weight, except for the 18 months sample when Isolate trout were larger, bringing them closer to the Resident trout. This implied a comparatively higher growth rate in Isolate trout than other groups during the second summer of growth.

Figure 2.7 a

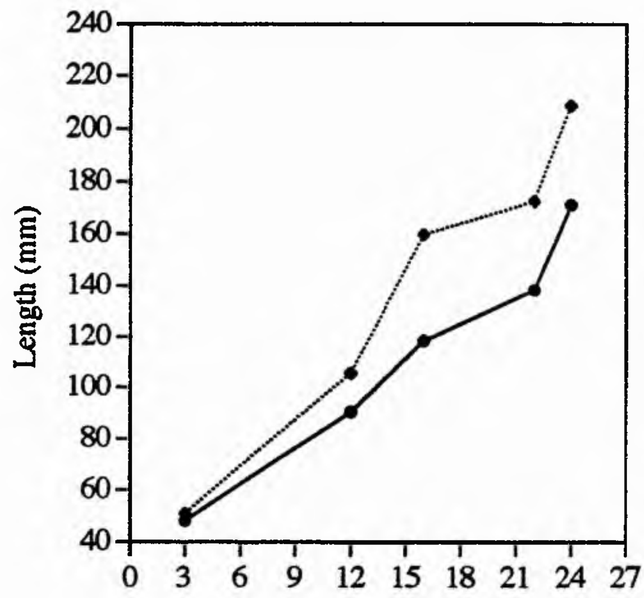
Figure 2.7 b

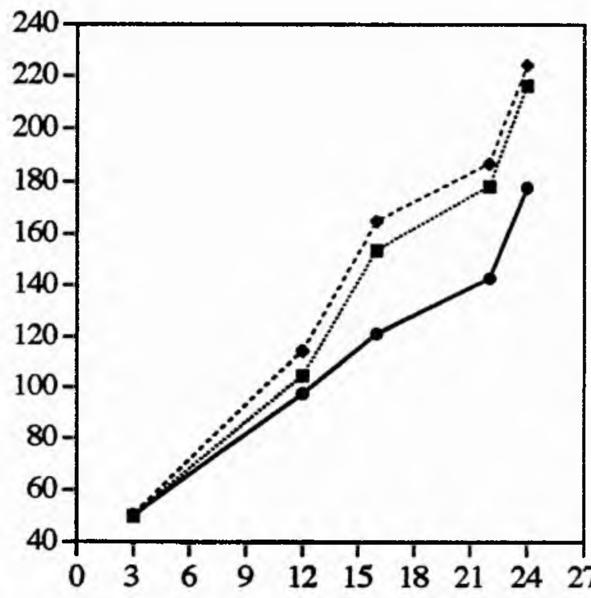
Figure 2.7 c

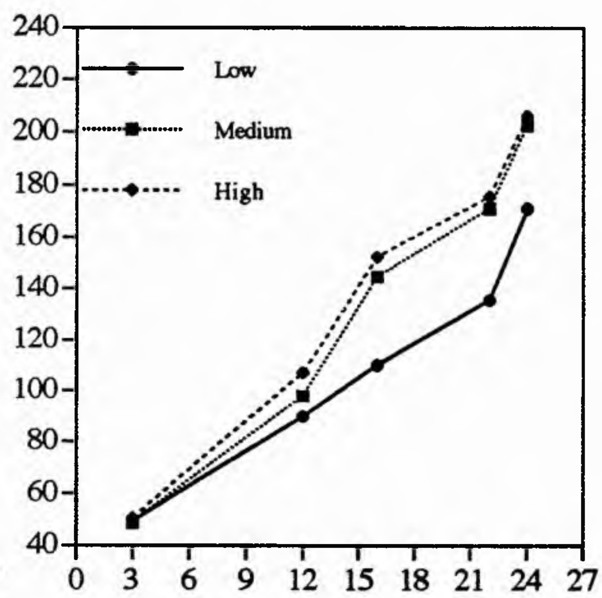
Figure 2.7a Back-transformed mean and 95% confidence limits of length (mm) for Sea trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.7 - 2.11 for sample sizes.

Figure 2.7 b Back-transformed mean and 95% confidence limits of length (mm) for Resident trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.7 - 2.11 for sample sizes.

Figure 2.7 c Back-transformed mean and 95% confidence limits of length (mm) for Isolated trout (Low and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.7 - 2.11 for sample sizes.







Age (Months)

Figure 2.8 a

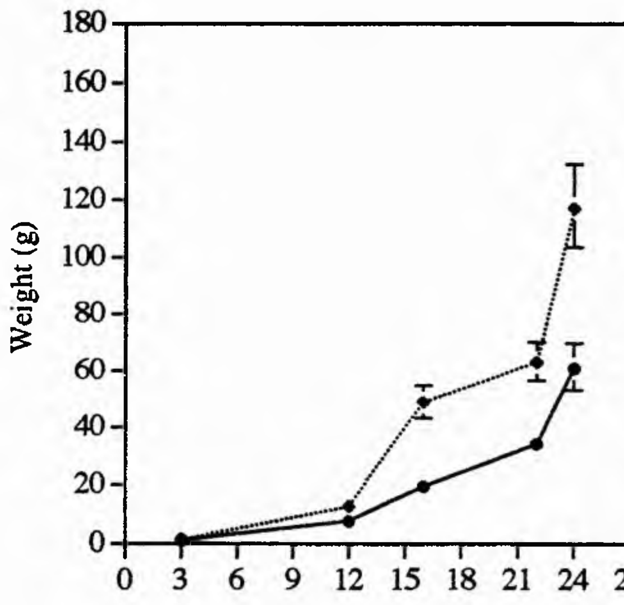
Figure 2.8 b

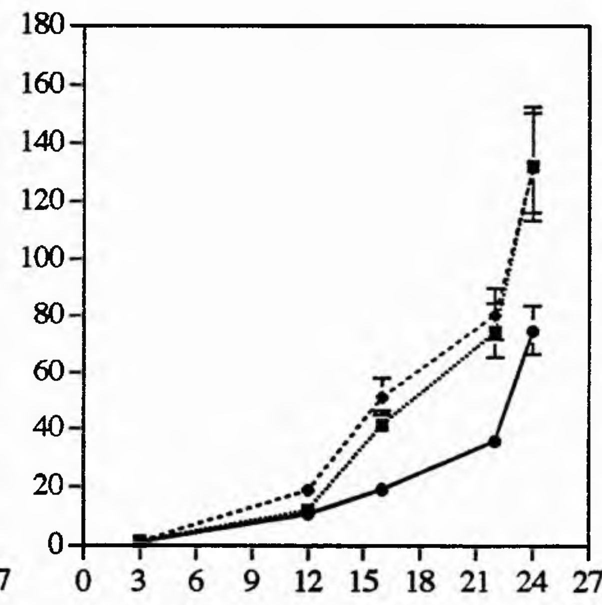
Figure 2.8 c

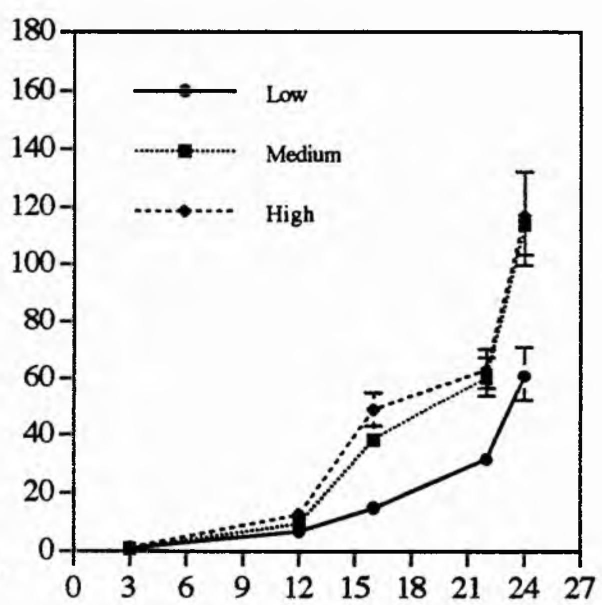
Figure 2.8 a Back-transformed mean and 95% confidence limits of weight (g) for Sea trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.12 – 2.16 for sample sizes.

Figure 2.8 b Back-transformed mean and 95% confidence limits of weight (g) for Resident trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.12 - 2.16 for sample sizes.

Figure 2.8 c Back-transformed mean and 95% confidence limits of weight (g) for Isolated trout (Low and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.12 - 2.16 for sample sizes.


Weight (g)
180
160
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27


180
160
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27
Age (Months)


Low
Medium
High
180
160
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27

In general, condition factor increased throughout the study from means of 1.00 - 1.08 at 5 months to 1.28 - 1.43 after 26 months (Figure 2.9 a-c). The exceptions were no change or a slight decrease observed in (1) Medium and Low ration Resident trout and (2) Medium and High ration Isolated trout over the first winter of growth (5 to 14 months). No reduction in mean condition factor was evident in any of the groups during 24 to 26 months when a reduction associated with smoltification might have been expected.

Statistical analysis of log condition factor data for Low and High ration groups at each sample point yielded significant 2-way interactions between ration and parental form at all but the first sampling (Tables 2.17 to 2.21). At 5 months old, whereas there was no significant main effect of ration, a significant parental form effect was indicated. Visual examination of the means when split by parental form alone, indicated that log condition factor for Resident trout and Isolated trout were similar while both were greater than that of Sea trout.

At 14 months, condition factor was greater in High than Low ration groups, greater in Resident trout than Sea trout or Isolated trout amongst the High ration groups but similar in all three Low ration groups. At 18 months, whereas the general ration effect remained, rapid increases in the High ration Sea trout and Isolated trout groups coincident with a relatively small increase in the Resident trout resulted in similar mean condition factor values for each. In the Low ration groups, however, mean condition factor increased considerably more in the Isolated trout group than the other two parental forms. A similar pattern to that of 14 months was observed at 24 months except that mean condition factor was greater in Low compared to High ration groups of each parental form. This was possibly indicative of the slow recovery in condition of precocious mature male trout that were present in Medium and High but not in Low ration groups. At 26 months, whereas no ration effects were apparent in Resident trout or Isolated trout groups, mean condition factor was once again greater in High than Low Sea trout groups.

2.3.2 Indicators of potential smolts

2.3.2.1 Bimodal length distribution

All length frequency distributions were found to be statistically normal in distribution. Therefore, no bimodal length frequency distributions were observed

Figure 2.9 a

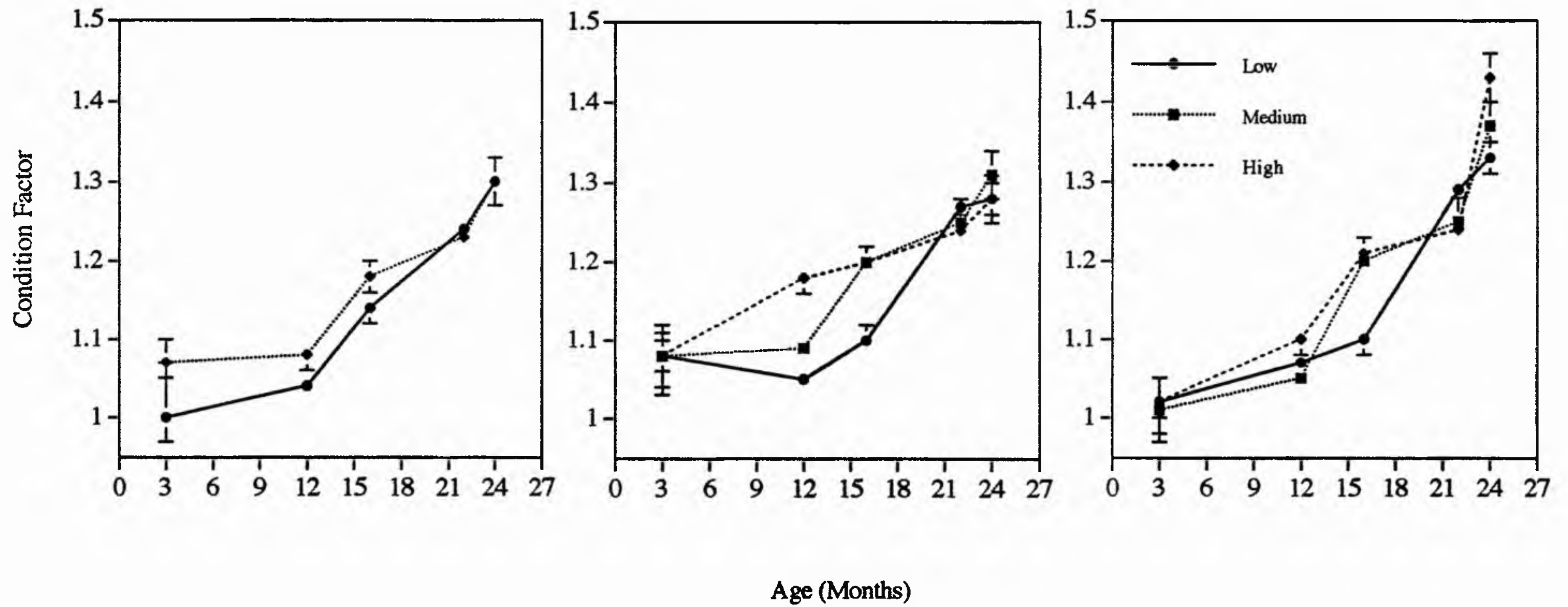
Figure 2.9 b

Figure 2.9 c

Figure 2.9 a Back-transformed mean and 95% confidence limits of condition factor (K) for Sea trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.17 - 2.21 for sample sizes.

Figure 2.9 b Back-transformed mean and 95% confidence limits of condition factor (K) for Resident trout (Low, Medium and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.17 - 2.21 for sample sizes.

Figure 2.9 c Back-transformed mean and 95% confidence limits of condition factor (K) for Isolated trout (Low and High rations) sampled on the five Growth Monitoring sample dates. See Tables 2.17 - 2.21 for sample sizes.



Condition Factor
1.5
1.4
1.3
1.2
1.1
1
0 3 6 9 12 15 18 21 24 27
Low
Medium
High
Age (Months)

amongst the various ration and parental form treatments throughout the study (Table 2.6).

2.3.2.2 Reduction in condition factor

As noted above, mean condition factor either remained at a similar level or increased in all groups between 24 and 26 months, the period when smoltification would have been expected to have become visually apparent (see Figures 2.9 a-c).

2.3.2.3 Silvering

Although some trout did appear to have an iridescent reflective quality during the March and May 1996 samplings, none were considered silver (see Plates 2.3 and 2.4).

2.3.3 Proportion maturing

Table 2.1 displays the proportion of trout sampled for drinking rates which were maturing during Experiments 3 (18 months). Since the data for Table 2.1 were obtained during the winter of 1995/1996 they indicated ration and parental form effects: fewer males matured in the Low ration groups compared to Medium and High rations, and fewer Sea trout matured when compared to Resident trout and Isolated trout groups.

As overtly maturing male trout were excluded from the experimental groups in Experiment 4, the proportion of mature fish amongst those sampled for drinking rates was biased. However, Table 2.2 gives the proportion of maturing females measured against the total number of females sampled and is, therefore, not biased by the exclusion of some males. The proportion of maturing females varied between 0.18 and 0.60 and, in Sea trout and Resident trout, was positively related to ration. Thus growth conditions, especially amongst the High and Medium ration groups but also to a lesser extent the Low ration groups, were sufficient to induce maturation in a proportion of both sexes of trout. This is of particular interest with respect to female maturation since females benefit more from the additional growth resultant from anadromous migrations and would, theoretically, require greater growth opportunity in freshwater to initiate maturation.

Table 2.1

Table 2.2

Table 2.1 Proportions of mature male trout amongst those sampled for Experiment 3 (20 months old). Note that whereas only maturing males were observed amongst these trout, the proportion is based on both sexes. Sex was recorded only for the maturing individuals so the proportion of maturing males versus immature males is not available. However, if one assumes that the sex ratio is 1:1 then the proportion given should be doubled to produce the approximate proportion of mature individuals amongst the males.

Table 2.2 Proportions of mature females amongst those sampled for Experiment 4 (26 months old).

	Sea trout		Resident trout		Isolated trout	
Ration	Proportion	n	Proportion	n	Proportion	n
High	0.12	26	0.33	33	0.34	32
Medium	0.00	29	0.31	26	na	na
Low	0.00	23	0.00	29	0.08	26

	Sea trout		Resident trout		Isolated trout	
Ration	Proportion	n	Proportion	n	Proportion	n
High	0.50	28	0.60	30	0.40	27
Medium	0.28	25	0.50	20	na	na
Low	0.18	17	0.40	15	0.52	21

2.3.4 Development of hypo-osmoregulatory physiology

2.3.4.1 Experiment 1, November 1994 - five months old

Figure 2.10 (a-c) displays the mean drinking rates from High ration Sea trout, Resident trout and Isolated trout, respectively after acute transfer to freshwater, 20%, 33%, 50% and 75% seawater. Both salinity and, to a much lesser extent, parental form had significant effects on log drinking rates but there was no significant interaction between these two effects (Table 2.22). When pooled with respect to parental form, mean drinking rate increased in proportion to salinity between freshwater and 50% seawater, but was lower in fish transferred to 75% seawater than in those transferred to 50% seawater.

The significant parental form effect was manifest by greater mean log drinking rate in Resident trout versus Sea trout and Isolated trout groups, the latter two being similar. On visual analysis of the mean drinking rate data split by salinity and parental form (Figure 2.10 (a-c)), it was apparent that this significant parental form effect was most probably due to a considerably greater drinking rate in Resident trout transferred to 50% seawater, and to a lesser extent, in those transferred to 33% and 20% seawater. A 2-way ANCOVA with log body weight as the covariate did not yield a significant log weight effect indicating that the parental form effect was not a consequence of differences in the influence of body size on drinking rates between trout types.

2.3.4.2 Experiment 2, April/May 1995 - 14 months old

Drinking rate was measured in response to several salinity challenges in May 1995 (Figure 2.11 (a-c)). In a 3-way ANOVA with salinity, ration and parental form as factors and the log drinking rate as the dependent value, there were significant 2-way interaction effects between parental form, ration and salinity (Table 2.23). Visual inspection of a plot of mean log drinking rates for parental form and ration treatments indicated that drinking rates for Low ration groups were similar while those for High ration groups were different, such that drinking rate was greatest in Sea trout, lowest in Isolated trout and intermediate in Resident trout. For the parental form versus salinity interaction, Resident trout and Sea trout drinking rates were similar in response to salinity so the significant interaction must have been due to the Isolated trout groups, in which there was a larger range between salinities. In

Figure 2.10 a

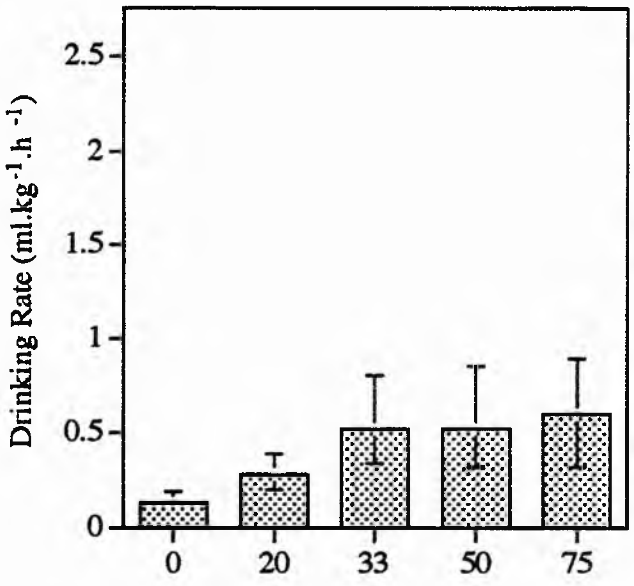
Figure 2.10 b

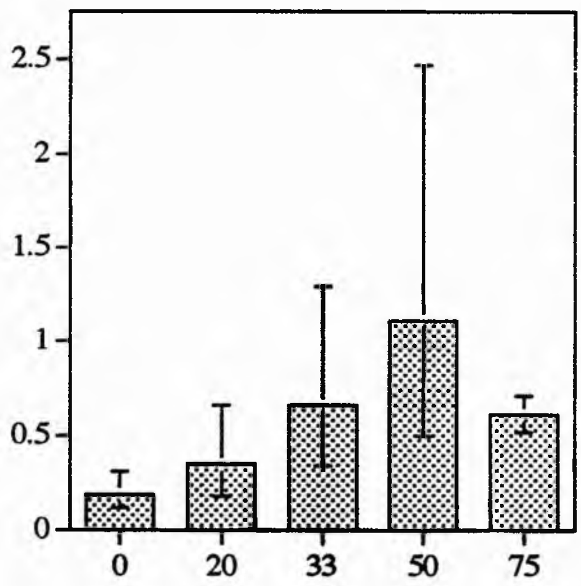
Figure 2.10 c

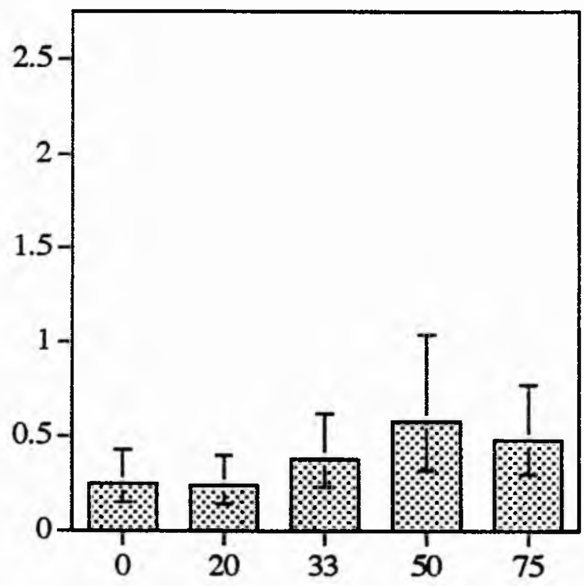
Figure 2.10 a Experiment 1. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of ten High ration, Sea trout parr after acute transfer to 0, 20, 33, 50 and 75% seawater.

Figure 2.10 b Experiment 1. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of ten High ration, Resident trout parr after acute transfer to 0, 20, 33, 50 and 75% seawater.

Figure 2.10 c Experiment 1. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of ten High ration, Isolated trout parr after acute transfer to 0, 20, 33, 50 and 75% seawater.







% Seawater

Figure 2.11 a

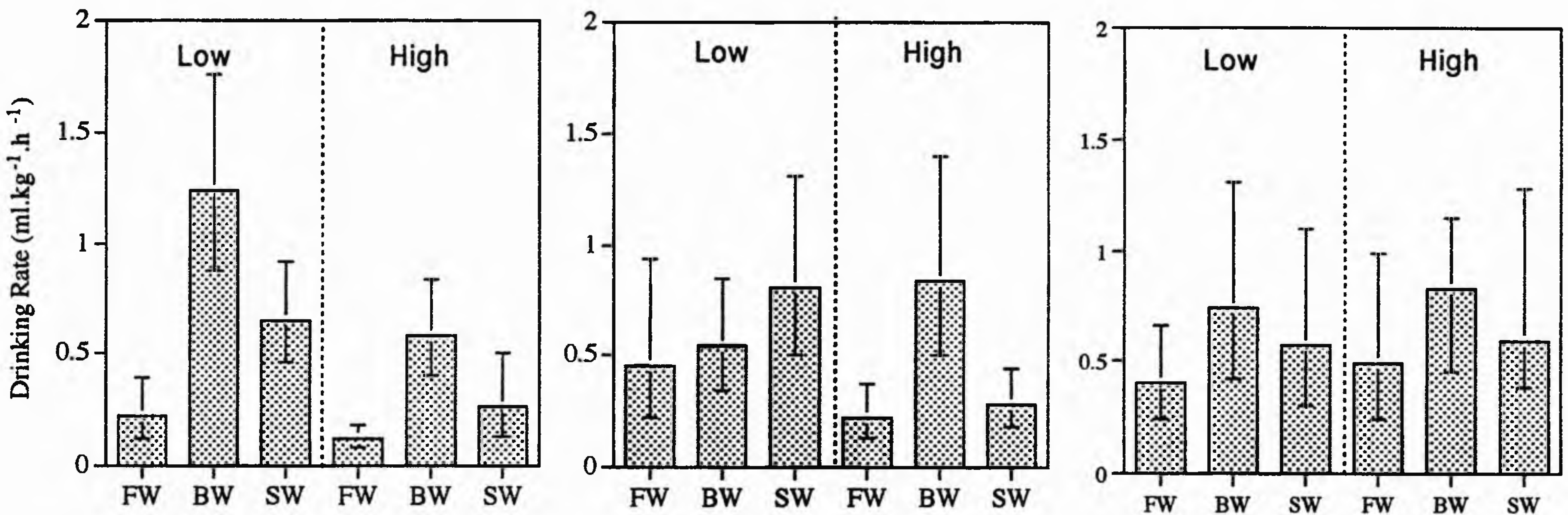
Figure 2.11 b

Figure 2.11 c

Figure 2.11 a Experiment 2. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of nine Sea trout parr (Low and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively).

Figure 2.11 b Experiment 2. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of nine Resident trout parr (Low and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively).

Figure 2.11 c Experiment 2. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of nine Isolated trout parr (Low and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively).


Drinking Rate (ml.kg-1.h-1)
2
1.5
1
0.5
0
Low
High
FW
BW
SW
FW
BW
SW
Salinity

addition, in overall terms, and in five of the six specific parental form/ration cases, the relative order of mean drinking rates was similar, such that drinking rate was greater in brackish water than seawater, whereas both were greater than freshwater. However, in the Low ration Resident trout group, drinking rate was similar, and certainly not greater, in brackish water than freshwater, whereas it was greatest in seawater. Whether this difference was real or an artefact was unclear but it was not due to the presence of statistical outliers (as determined by SYSTAT) nor to the presence of unusually high variability within these groups, since the 95% confidence limits were of similar magnitude to those of other data.

Given that there were significant interaction effects, main effects must be examined with caution but in general terms all three main effects were significant. The relationship of salinity effect, as described for the interactions above, was robust across ration and parental form. An examination of parental form effects indicated that mean log drinking rates were lowest in Isolated trout and highest in Sea trout groups. This could possibly have been due to effects of ration because a significant main ration effect was indicated. However, ration is confounded because body weight is a direct result of ration and hence, the two will co-vary. Therefore, a 3-way ANCOVA with the log body weight as the covariate was performed. The covariate term was highly significant and so body weight, *per se*, accounted for a large amount of the variation in drinking rate (Table 2.24). The main effect of ration was no longer significant, thereby indicating that ration covaried with body weight, as expected, and there was no body weight independent effect of ration. In addition, the 2-way interaction of salinity versus ration was now significant. Visual examination of the means indicated that this significant 2-way effect was primarily due to the response to the seawater challenge, where drinking rate was considerably higher in the Low than the High ration groups.

Some seawater challenged trout were moribund at the end of the experimental period. Moribund was defined as still alive but lying on one side rather than dorso-ventrally, as normal, and not reacting when netted from the bucket. This condition was noted in more High than Low Ration groups: 8/9 High, Isolated trout; 6/9 High, Resident trout; and 4/9 Low Sea trout. Perhaps this condition could have been a contributory factor in the low drinking rates of High ration trout.

This moribund condition was associated only with the second batch of seawater, but the nature of the experimental design (the groups of trout separate within buckets of water of various salinities) indicated that the repeated use of water (day sampled)

and something particular to the second bucket of seawater were probably not responsible for this effect. The former could be ruled out since moribund fish were noted on the first day when the second bucket of seawater was used and there was no effect of repeat use in groups of trout challenged with the first bucket of seawater. The lack of any moribund trout amongst the Low ration Resident trout or High ration Sea trout challenged to the same seawater as the Low ration Sea trout, of which 2/3 were recorded as moribund, did not support the suggestion that the moribund fish were due to a problem particular to the second bucket of seawater. Finally, the range of drinking rates amongst healthy and moribund trout were not considerably different (Table 2.3).

2.3.4.3 Experiment 3, November 1995 - 20 months old

Some of the male trout used for these drinking rate measurements were found to be mature when dissected (see Table 2.1). Maturation is reported to have deleterious effects on hypo-osmoregulation (see Discussion) and while the numbers of mature fish in this experiment were too small to allow appropriate comparisons with immature trout, the data from Experiment 4 indicated an effect of maturation on mean drinking rates. As a consequence, all mature individuals were removed from the following analyses.

Trout from Low, Medium and High ration regimes of Sea trout, Resident trout and Isolated trout parental form were challenged with brackish water and seawater and drinking rates compared with those of freshwater-adapted controls (Figure 2.12 a-c).

A 3-way ANOVA testing the effects of salinity and parent form on the log drinking rate (Table 2.25) indicated that there were no significant 3-way, or 2-way interactions but that there was a significant main effect of salinity, as would be expected. Visual examination of the log mean drinking rates when the non-significant factors were removed indicated that mean drinking rate was similar in brackish water and seawater but considerably lower in freshwater groups. Such a pattern was present in the original mean data although mean drinking rates appeared to be lower in seawater challenged trout than brackish water challenged trout in the Low ration Sea trout and both ration groups of Isolated trout (Figure 2.12 a-c).

A 2-way ANOVA comparing Medium ration log drinking rates with salinity and parental form (Sea trout and Resident Trout) as factors indicated a significant main

Table 2.3

Table 2.3 Range of drinking rates ($ml.kg^{-1}.h^{-1}$) in seawater-challenged trout which appeared healthy or moribund at the end of the six hour experimental period, Experiment 2 (14 months).

	max. drinking rate	min. drinking rate	n
Low, Sea trout			
Healthy	1.474	0.210	5
Moribund	1.295	0.150	4
High, Resident trout			
Healthy	0.346	0.146	3
Moribund	0.749	0.117	6
High, Isolated trout			
Healthy	0.840	na	1
Moribund	0.699	0.083	8

Figure 2.12 a

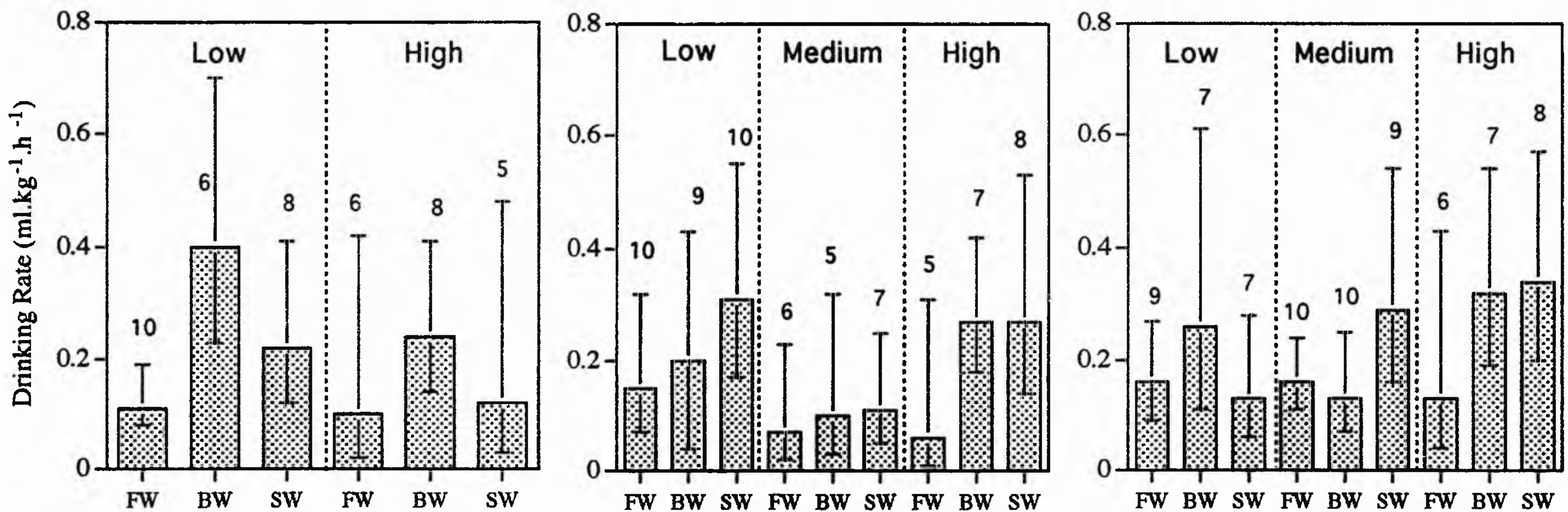
Figure 2.12 b

Figure 2.12 c

Figure 2.12 a Experiment 3. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature Sea trout parr (Low, Medium and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.12 b Experiment 3. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature Resident trout parr (Low, Medium and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.12 c Experiment 3. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature Isolated trout parr (Low and High ration groups) after acute transfer to freshwater, brackish water and seawater (FW, BW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.


Drinking Rate (ml.kg-1.h-1)
0.8
0.6
0.4
0.2
0
Low
High
Medium
FW
BW
SW
Salinity

effect of parental form but no significant interaction (Table 2.26). Visual examination of means based upon parental form indicated that drinking rate was greater in Sea trout than Resident trout.

A 2-way ANCOVA with log weight as the covariate indicated no significant covariance of body weight and drinking rate.

2.3.4.4 Experiment 4, May 1996 - 26 months old

2.3.4.4 Effects of maturation

Maturation is reported to have deleterious effects on hypo-osmoregulation as a consequence of increased production of sex steroids (see Discussion). This effect has been shown to exist in the summer following maturation the previous autumn (Lundqvist *et al.*, 1990) but the previous sexual status of trout examined in this study was unknown, except that obviously mature males were not sampled. Examination of the trout after dissection for drinking rate measurement indicated that between 78% and 94% of trout from the High and Medium ration groups were maturing, regardless of parent form, and most probably would have matured in the following autumn. Such high proportions of maturing fish resulted in physiological data being available for only a small number of immature fish within these ration levels and, as a consequence, no conclusions could be based upon such small datasets. In contrast, only between 47% and 66% of the Low ration trout were maturing: a meaningful analysis could, therefore, be based on a comparison between the Low ration mature and immature trout.

Mean drinking rates for Low ration immature and mature trout after transfer to freshwater and seawater are shown in Figure 2.13 (a-c). A 3-way ANOVA of log drinking rate data with salinity, state of maturity and parental form as factors indicated a significant salinity x state of maturity interaction effect but no significant 3-way or other 2-way interactions (Table 2.27). Visual examination of a plot of log mean drinking rates for the salinity x state of maturity interaction showed drinking rates were greater in seawater-challenged than freshwater-transferred groups, regardless of state of maturation and that there was little difference between freshwater-transferred groups but that drinking rate was lower in maturing than immature seawater-challenged trout. The presence of a significant 2-way interaction indicates that main effects should be treated with caution, as was demonstrated by the lack of a significant state of maturation main effect. However,

Figure 2.13 a

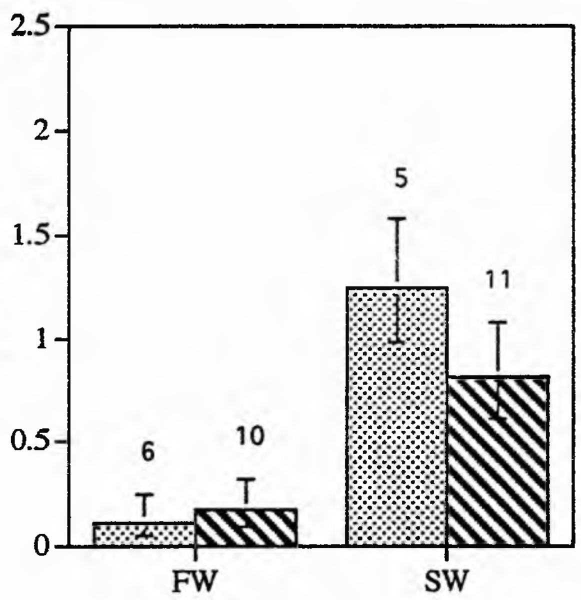
Figure 2.13 b

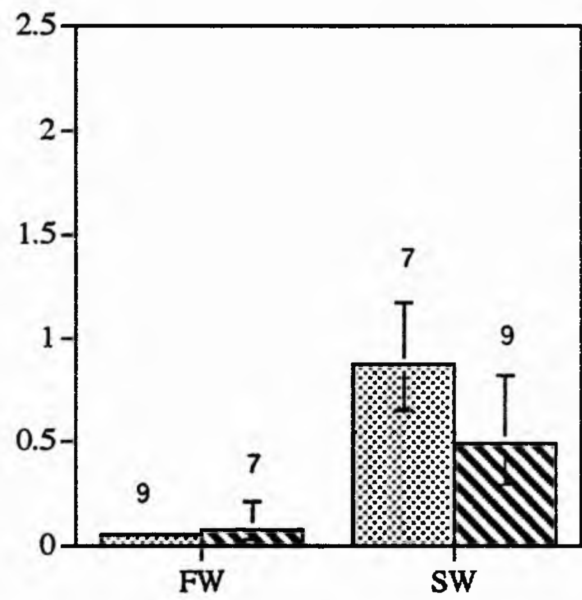
Figure 2.13 c

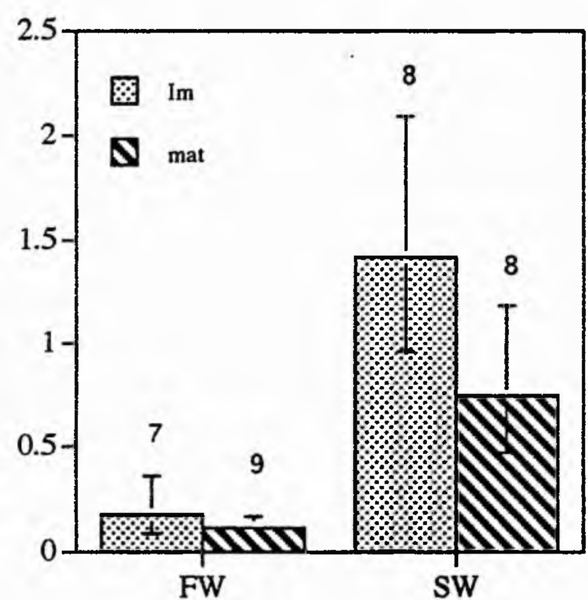
Figure 2.13 a Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature and mature Low ration, Sea trout parr after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.13 b Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature and mature Low ration, Resident trout parr after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.13 c Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of immature and mature Low ration, Isolated trout parr after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.







Salinity

a significant parental form main effect was noted and visual examination of the appropriate mean values indicated that drinking rates were similar for Isolated trout and Sea trout but lower for Resident trout.

There were considerable differences in mean fork length and mean weight, but not mean condition factor, between immature and mature trout of most parent form/ration groups; the exception was the freshwater-transferred Sea trout groups which were of similar mean length and weight (Tables 2.4 a-c). Note, however, that the differences in mean drinking rate were between the seawater-challenged groups so this similarity in size was not relevant to the model. A 3-way ANCOVA with log body weight as the covariate was applied to assess whether body weight could explain the differences in drinking rates between immature and mature trout. However, this indicated no significant log weight effect such that the parental type difference was not due to differences in body weight: the significant effects were not changed in the 3-way ANCOVA.

No clear differences were observed between immature and mature fish of any parent type in a visual examination of mean plasma Cl^- concentration or mean haematocrit between freshwater-transferred or seawater-challenged groups. However, since blood samples were not collected from the smallest fish because of practical difficulties, relatively few data are available from the Low ration groups. It would be inappropriate, therefore, to conclude that state of maturation had no effect on these parameters based on the available data.

Data for the immature Low ration trout of each parental form were used to compare parental form effects while the mature Low, Medium and High ration trout of each parental form were used to compare parental form and ration effects, under the assumption that maturation inhibited drinking rate in similar manner, regardless of parental form.

2.3.4.4.2 Parental form effects in the immature, Low ration trout

A 2-way ANOVA of log transformed drinking rate with salinity and parental form as factors indicated significant main effects but no significant interaction (Table 2.28). Subsequent visual examination of the interaction means indicated that drinking rate was greater in seawater-challenged than freshwater-transferred trout, and drinking rates were lowest in Resident trout and greatest in Sea trout. However, the parental

Table 2.4 a

Table 2.4 b

Table 2.4 c

Table 2.4 a Mean and standard errors for length, weight and condition factor (K) of immature and mature Low ration, Sea trout sampled in Experiment 4 (26 months old). See Figure 2.13 a for sample sizes.

Table 2. 4 b Mean and standard errors for length, weight and condition factor (K) of immature and mature Low ration, Resident trout sampled in Experiment 4 (26 months old). See Figure 2.13 b for sample sizes.

Table 2.4 c Mean and standard errors for length, weight and condition factor (K) of immature and mature Low ration, Isolated trout sampled in Experiment 4 (26 months old). See Figure 2.13 c for sample sizes.

	Freshwater		Seawater	
	Immature	Mature	Immature	Mature
Length (mm)	142.43 ±12.15	146.00 ±6.89	118.88 ±1.72	156.38 ±5.19
Weight (g)	46.86 ±9.75	46.56 ±6.40	26.38 ±1.22	57.00 ±4.98
K	1.51 ±0.05	1.42 ±0.04	1.57 ±0.06	1.50 ±0.03

	Freshwater		Seawater	
	Immature	Mature	Immature	Mature
Length (mm)	134.67 ±6.79	164.57 ±7.45	115.00 ±2.23	154.22 ±5.14
Weight (g)	38.00 ±4.63	69.00 ±8.40	24.29 ±1.74	56.56 ±4.20
K	1.51 ±0.04	1.50 ±0.05	1.58 ±0.05	1.53 ±0.06

	Freshwater		Seawater	
	Immature	Mature	Immature	Mature
Length (mm)	117.17 ±4.67	158.00 ±5.07	111.00 ±3.91	157.00 ±3.68
Weight (g)	24.50 ±2.17	60.60 ±6.05	20.60 ±2.14	57.73 ±4.19
K	1.51 ±0.06	1.47 ±0.02	1.49 ±0.02	1.46 ±0.03

form effect was not due to size effects as a 2-way ANCOVA with log body weight as the covariate gave no significant covariate effect.

2.3.4.4.3 Mature trout

As with the data from Experiment 3 above, the lack of a Medium ration Isolated trout group meant the experimental design was statistically unbalanced and could not be tested by ANOVA in its entirety. Therefore, in the first instance, the Medium ration data were removed and the reduced but balanced dataset analysed.

2.3.4.4.3.1 Drinking Rates

A 3-way ANOVA indicated no significant 3-way or 2-way interaction effects but significant salinity and parental form main effects (Table 2.29). Plots of mean values for the appropriate main effects yielded similar salinity and parental form patterns as those reported for immature versus mature trout above: drinking rates were greater in seawater-challenged than freshwater-transferred trout and whereas drinking rates were similar in Isolated trout and Sea trout, they were lower in Resident trout (Figures 2.14 a-c). A 3-way ANCOVA with log weight as the covariate indicated a significant body weight effect but the salinity and parental form main effects remained significant (Table 2.30). Thus, body weight had a significant effect on drinking rate but the salinity and parental form effects were still significant even when the effects of body weight had been removed from the analysis. Mean length, weight and condition factor for the various groups are provided in Table 2.5 (a-c).

2.3.4.4.3.2 Plasma Cl^- Concentration

Mean plasma Cl^- concentrations for the appropriate groups are presented in Figure 2.15 (a-c). While there were no significant 3-way or 2-way interactions, all the main effects were significant (3-way ANOVA) (Table 2.31). Visual examination of the interaction plots indicated that plasma Cl^- concentration was greater in seawater challenged trout than freshwater challenged trout (as expected), greater in High ration trout than Low ration trout, greatest in Resident trout and lowest in Isolated trout. Examination of the original data suggested the ration effect was due mostly to a relatively low mean concentration for seawater-challenged Low ration Resident trout.

Figure 2.14 a

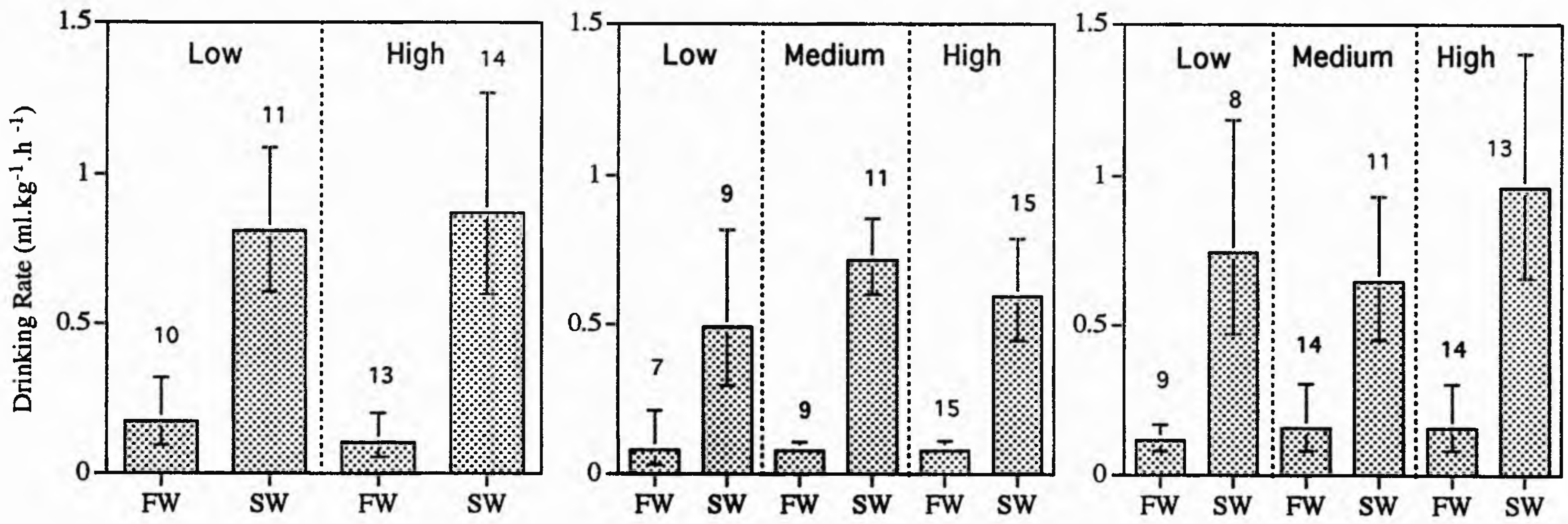
Figure 2.14 b

Figure 2.14 c

Figure 2.14 a Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of mature Sea trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.14 b Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of mature Resident trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.14 c Experiment 4. Back-transformed mean drinking rates ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, for groups of mature Isolated trout (Low and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.



Drinking Rate (ml.kg-1.h-1)
1.5
1
0.5
0
Low
High
Medium
FW
SW
10
11
13
14
7
9
9
11
15
15
9
8
14
11
14
13
Salinity

Table 2.5 a

Table 2.5 b

Table 2.5 c

Table 2.5 a Mean and standard errors for length, weight and condition factor (K) of mature Sea trout, reared under Low, Medium and High rations, sampled in Experiment 4 (26 months old). FW and SW correspond to the freshwater-transfer and seawater-challenge, respectively. See Figure 2.14 a for sample sizes.

Table 2.5 b Mean and standard errors for length, weight and condition factor (K) of mature Resident trout, reared under Low, Medium and High rations, sampled in Experiment 4 (26 months old). FW and SW correspond to the freshwater-transfer and seawater-challenge, respectively. See Figure 2.14 b for sample sizes.

Table 2.5 c Mean and standard errors for length, weight and condition factor (K) of mature Isolated trout, reared under Low and High rations, sampled in Experiment 4 (26 months old). FW and SW correspond to the freshwater-transfer and seawater-challenge, respectively. See Figure 2.14 c for sample sizes.

	Low		Medium		High	
	FW	SW	FW	SW	FW	SW
Length (mm)	146.00 ±6.89	156.38 ±5.19	170.21 ±7.39	168.09 ±6.80	176.14 ±6.48	177.77 ±7.79
Weight (g)	46.56 ±6.40	57.00 ±4.98	76.71 ±10.14	73.00 ±9.09	81.86 ±9.68	85.77 ±11.47
K	1.42 ±0.04	1.46 ±0.03	1.45 ±0.02	1.44 ±0.03	1.43 ±0.03	1.42 ±0.02

	Low		Medium		High	
	FW	SW	FW	SW	FW	SW
Length (mm)	164.57 ±7.45	154.22 ±5.14	180.11 ±10.59	172.18 ±7.44	182.33 ±6.56	176.00 ±7.76
Weight (g)	69.00 ±8.40	56.56 ±4.20	91.00 ±16.42	76.82 ±9.51	91.27 ±10.47	80.80 ±10.47
K	1.50 ±0.05	1.53 ±0.06	1.40 ±0.05	1.44 ±0.04	1.39 ±0.02	1.39 ±0.03

	Low		High	
	FW	SW	FW	SW
Length (mm)	158.80 ±5.08	157.00 ±3.68	181.46 ±6.65	170.57 ±6.63
Weight (g)	60.60 ±6.05	57.73 ±4.20	87.46 ±9.40	74.29 ±8.35
K	1.47 ±0.02	1.46 ±0.03	1.40 ±0.02	1.42 ±0.03

Figure 2.15 a

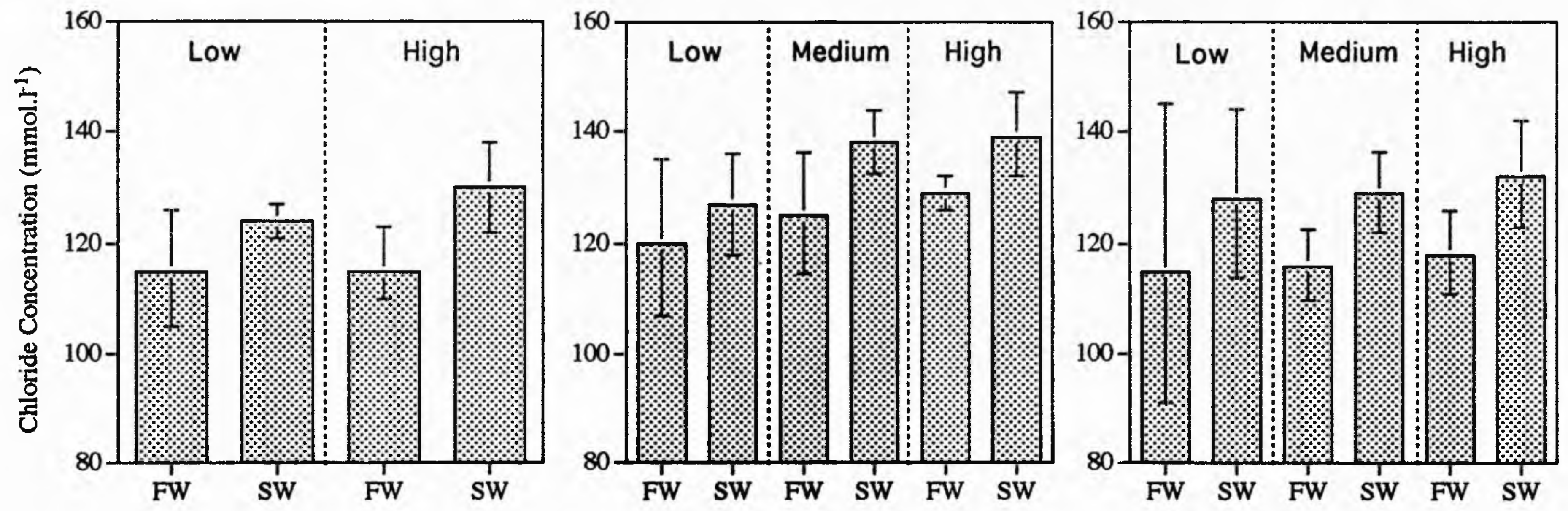
Figure 2.15 b

Figure 2.15 c

Figure 2.15 a Experiment 4. Back-transformed mean plasma Cl^- concentrations, with 95% confidence limits, for groups of mature Sea trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). See Figure 2.16 a for sample sizes.

Figure 2.15 b Experiment 4. Back-transformed mean plasma Cl^- concentrations, with 95% confidence limits, for groups of mature Resident trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). See Figure 2.16 b for sample sizes.

Figure 2.15 c Experiment 4. Back-transformed mean plasma Cl^- concentrations, with 95% confidence limits, for groups of mature Isolated trout (Low and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). See Figure 2.16 c for sample sizes.


Chloride Concentration (mmol.l-1)
160
140
120
100
80
Low
High
Medium
FW
SW
Salinity

However, when log weight was included in the model as a covariate (3-way ANCOVA), it was found to have a significant effect and the main ration effect was no longer significant, thereby suggesting that variation in body weight explained the majority of difference due to ration (Table 2.32).

2.3.4.4.3.3 Haematocrit

Mean haematocrit values are plotted in Figure 2.16 (a-c). A significant 3-way interaction and 2-way ration x parental form interaction were indicated by 3-way ANOVA of arcsine transformed haematocrit proportions (Table 2.33). Haematocrit means were generally higher in seawater-challenged trout than in the freshwater-adapted controls, except for the Low ration Resident trout where the order was reversed. This was most probably the cause of both the significant 3-way and 2-way interactions. The significant ration x parental form interaction suggested the possibility of a body size effect. A 3-way ANCOVA with log weight as the covariate confirmed the influence of body weight but the 3-way and 2-way ration x parent types were still significant (Table 2.34). In addition, the salinity main effect which was marginally significant in the initial ANOVA ($p = 0.051$) was significant in the ANCOVA.

2.3.4.4.3.4 Medium ration comparisons

As expected, salinity was shown to have a significant main effect on drinking rate but no significant effects were attributed to ration or the 2-way interaction (Table 2.35). Plasma Cl^- concentration was influenced by significant salinity and parental form effects (Table 2.36) and was greater in seawater-challenged trout and in Resident trout versus Sea trout but no significant interaction was indicated. No significant salinity or parental form main effects, nor any interaction were noted in mean haematocrit. No significant effect of log transformed body weight was indicated for any of the three dependent variables.

Figure 2.16 a

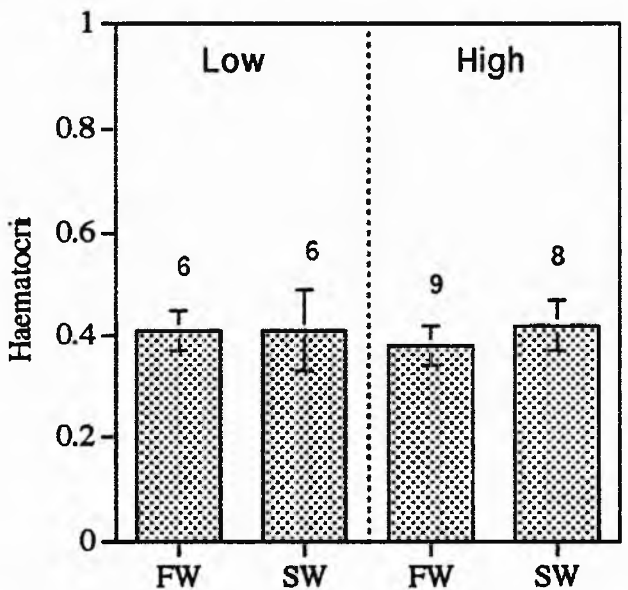
Figure 2.16 b

Figure 2.16 c

Figure 2.16 a Experiment 4. Back-transformed mean haematocrit, with 95% confidence limits, for groups of mature Sea trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.16 b Experiment 4. Back-transformed mean haematocrit, with 95% confidence limits, for groups of mature Resident trout (Low, Medium and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.

Figure 2.16 c Experiment 4. Back-transformed mean haematocrit, with 95% confidence limits, for groups of mature Isolated trout (Low and High ration) after acute transfer to freshwater and seawater (FW and SW, respectively). Sample sizes varied between groups and are indicated by numbers above the relevant bars.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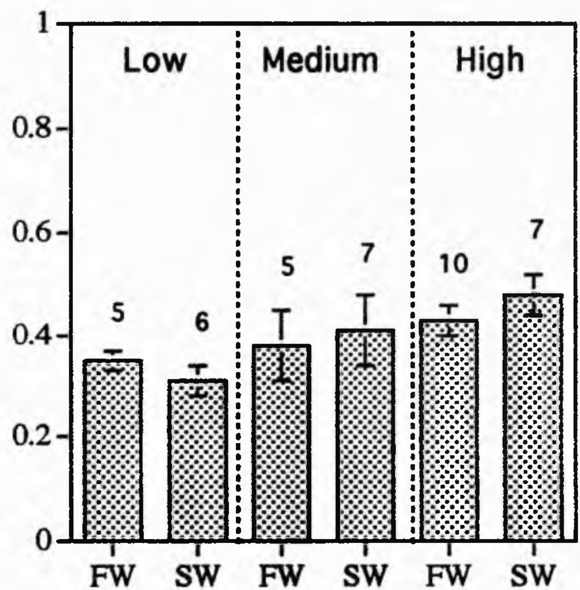
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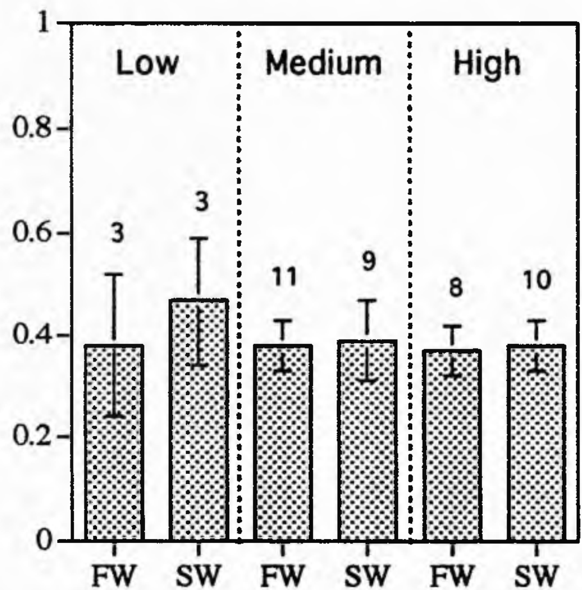
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2.4 Discussion

2.4.1 Summary of results

- Juvenile trout from sea trout and river trout were reared at three rations; juveniles from trout caught above impassable falls (Isolated) were reared at high and low rations
- Resident trout progeny grew larger than the other groups, within ration treatments
- After two years no trout had smolted
- Some males were mature at age 1+ whereas a proportion of both sexes were maturing at age 2. The proportion of maturing fish was related to ration and parent type
- Groups of trout were challenged to acute salinity increases at four times during the two years
- 9 months: drinking rates increased in trout transferred to 25%, 33% and 50% seawater, but declined again in sea trout and Isolate trout progeny transferred to 75% seawater
- 14 months: drinking rates were greatest in trout transferred to 50% seawater, ration effects were due to variations in body size
- 18 months: in immature trout, drinking rates were similar in trout transferred to 50% and 100% seawater. There were no ration or parent type effects
- 26 months: drinking rates increased upon seawater transfer but were lower in mature trout than immature trout. In general, drinking rates were lower in river trout than sea trout and Isolate trout, regardless of state of maturation. Plasma Cl^- concentrations displayed the opposite distribution.

As none of the trout in any of the parental form/ration treatments were undergoing the parr-smolt transformation at the completion of the experimental period, a discussion of the potential factors influencing the 'choice' to smolt cannot be made based upon the data of this study. However, juvenile trout of this age would be expected to adopt one of three strategies for the following year: smoltification; early (precocious) maturation; remain as immature freshwater-resident trout. Smoltification and early maturation are regarded as mutually exclusive processes (Thorpe and Morgan, 1980; Langdon and Thorpe, 1985) and so, while no comment can be made on the factors which induced trout to smoltify, the presence of maturing individuals allows a discussion of factors which influence this strategy.

Early sexual maturation will substantially affect the smolt production of any juvenile trout stock (Dellefors and Faremo, 1988).

2.4.2 Maturation Rate

Some of the male trout sampled in the 1995 - 96 winter and a larger proportion of both male and female trout in the spring of 1996 were found to be mature. The proportion of maturing trout amongst the sample populations appeared positively related to ration level but parental form was also a factor (more maturing in the Isolated trout group at lower rations). Early maturation rate in salmonids is dependent on growth. However, differences in the response of sea trout and salmon to good growth conditions probably reflect the different anadromous drive of these species. The growth rate of Atlantic salmon at sea is much greater than trout and thus, there are probably stronger selective pressures towards migration to the sea at the first available opportunity. In contrast, the facultative nature of anadromy amongst brown trout suggests less intense selection pressures (Okland *et al.*, 1993).

In trout, the fastest growing individuals will tend to mature early while the slower growing fish will either undergo smoltification or remain immature in freshwater until at least the following year. This is demonstrated by the observation that precocious parr were longer than immature parr of the same age (Dellefors and Faremo, 1988). In Norwegian trout, both within resident and anadromous stocks, more fish matured as 1+ in the fast-growing than in the more slow-growing groups, males matured earlier than females and the offspring of residents matured earlier than migrants, even though residents were more slow-growing than anadromous stocks (Jonsson, 1989). Thus, there appears to be a genetic difference in age at maturity between males and females, independent of their growth rates. This difference indicates that there is a higher selective premium on high growth rate for females than males.

Elliott (1988, 1989) found that the offspring of migrants were larger at all ages than those of residents in two small Lake District streams. This was due to differences in egg and fry sizes, rather than genotypic differences in growth rate. In contrast, however, the resident offspring in the present study were larger than migrant or isolated offspring at all times prior to the final sampling at age 2+.

2.4.2.1 When is the 'choice' made?

The effect of growth rate on decision to mature appears to vary depending upon season. Restricted ration regimes during spring reduced the proportion of mature male Atlantic salmon (Rowe and Thorpe, 1990; Berglund, 1991, 1995) but not if applied during the previous winter (Herbinger and Friars, 1992). If reduced spring growth were to inhibit maturation, increased spring growth might be expected to increase the proportion of maturing parr. A thinning operation to reduce fish density amongst the High and Medium ration groups during the second winter reduced the density of trout in these groups and may, therefore, have contributed further to the already good growth conditions which were inclining the trout towards early maturation rather than smoltification. A rapid increase in fork length and weight, as determined by the slope between mean March and May values, was observed in these groups during the final spring period in this study. Note, however, that this final sampling period was short and apparent growth rates during other, longer periods were likely to obscure greater temporal variation. However, Bohlin *et al.* (1994) observed that condition factor in autumn was greater in trout that were to subsequently mature than immature parr, indicating that the stimulus to mature began earlier, as in salmon. Indeed, the presence of mature male trout in the second winter sample indicates that growth conditions during the first and second summers were sufficient to drive some trout to maturation. Thus, the thinning procedure may have further augmented the already strong drive towards early maturation.

2.4.2.2 What factors influence the choice?

The effect of ration on maturation rate could be due to several factors, but three of the most likely are variation in growth rates, body size and nutritional state. The proportion of precociously maturing male parr has been positively related to growth rate in wild sea trout (Dellefors and Faremo, 1988), Atlantic salmon (Myers *et al.*, 1985) and in hatchery reared Atlantic salmon (Glebe and Saunders, 1986). Body size could be a factor as precocious male parr must be big enough to be sufficiently fecund but small enough to avoid attracting the attention of large, aggressive anadromous males (Berglund, 1995). The positive relationship with body size has been demonstrated in hatchery populations (see Bohlin *et al.*, 1994). However, body size alone cannot influence strategy in the wild as the proportion of sea trout parr maturing was not found to be associated with body length the previous autumn (Bohlin *et al.*, 1994). Fertility is affected by the amount of surplus energy stores so

levels above a threshold nutritional state could induce maturation. Sea trout that matured as parr had significantly higher condition factor than immature in the previous autumn (Bohlin *et al.*, 1994). Mean condition factor increased in all parental form/ration groups from the second summer period onwards to reach between approximately 1.29 and 1.42 by the end of the study. Such high condition factors would not be expected from wild fish and reflect the good growth conditions of the hatchery, thereby supporting the hypothesis that growth was sufficient to induce early maturation in favour of smoltification.

The proportion of maturing individuals also appears affected by the parental form, although this influence may be overshadowed by ration level since it was only clear in the Low ration groups where the proportion of maturing trout was greatest in Isolated trout. A genetic influence upon frequency of maturing male parr has been reported in hatchery reared Atlantic salmon (Glebe *et al.*, 1980; Thorpe and Morgan, 1980; Thorpe *et al.*, 1983; Glebe and Saunders, 1986). In sea trout, while there is a positive relationship between growth and the incidence of parr maturation within populations, the failure of this model to extend to between population comparisons indicates a genetic component to the choice (Dellefors and Faremo, 1988). The apparent increased propensity to mature at small size in Isolated trout is most probably the result of the lack of an anadromous tendency due to natural selection (impassable waterfall) and selection for maturation at smaller size because of the reduced space available in this stream when compared with the main river.

Of particular relevance to the current aims of this thesis, however, are the presence of maturing female trout. The majority of anadromous trout are females (see Introduction). Therefore, conditions that induce females to mature in freshwater and not migrate to sea will have a deleterious effect on the numbers of sea trout. Studies of the factors influencing early maturation have focused particularly on male salmon and female trout have received little attention. The presence of maturing females amongst the trout indicates that growth conditions in freshwater were favourable to freshwater-residence and that the advantages of anadromous migration might not have been sufficiently high to warrant smoltification.

2.4.3 Development of hypo-osmoregulatory capacity

2.4.3.1 General results

Throughout the experimental period, most groups of trout (with some unexplained exceptions) were observed to increase drinking rate in response to salinities up to 50% seawater. An increase in drinking rate is the typical response of a euryhaline teleost to hyperosmotic salinities but note, however, that drinking does occur in freshwater and in 20% seawater in Experiment 1. The physiological role of drinking in hypo-tonic conditions such as these remains unknown at present but the rate can be increased by activation of the RAS or by injection of Ang II (Perrott *et al.*, 1992). Drinking in alevins and larvae may be involved in calcium uptake or some aspect of feeding (Tytler *et al.*, 1990).

Increased drinking rates in salmonids transferred to hypertonic media are associated with dehydration (Evans, 1979) and elevated plasma ion concentrations (Usher *et al.*, 1988). Salmon fry (1 - 3 g) increased mean drinking rate from 0.6 $ml.kg^{-1}.h^{-1}$ in freshwater to about 0.9 $ml.kg^{-1}.h^{-1}$ when transferred to 50% seawater and also in response to RAS stimulation (1.4 $ml.kg^{-1}.h^{-1}$) via administration of vasodilatory sodium nitroprusside, a nitric oxide donor (Fuentes *et al.*, 1996). This indicates that the RAS system is functional in very young salmonids some considerable time before they would be expected to encounter hypertonic salinities. Note, however, that the administration of RAS inhibitors (ACE) did not reduce freshwater drinking rates in eel (Tierney *et al.*, 1995) or rainbow trout (Fuentes and Eddy, 1996) which suggests the possibility of an additional hormone system involved in drinking in freshwater (Fuentes and Eddy, 1997). In the current study, mean drinking rates of similar sized trout (*Salmo trutta*) ranged between 0.2 and 0.3 $ml.kg^{-1}.h^{-1}$ in freshwater to between 0.7 and 1.6 $ml.kg^{-1}.h^{-1}$ in 50% seawater. Freshwater-adapted rainbow trout drank at a similar rate but those adapted to 33% and 66% seawater for ten days drank at considerably higher rates (2.5 and 4.0 $ml.kg^{-1}.h^{-1}$, respectively) (Eddy and Bath, 1979) as measured by ^{125}I-PVP (Evans, 1968).

However, in the first two experiments (1 and 2, November 1994 and May 1995, respectively), the drinking response was either no greater than, or reduced in, those groups of trout transferred to salinities greater than 50% seawater. A drinking rate similar in trout transferred to full seawater or 50% seawater could indicate that transfer to the more dilute salinity stimulated the maximal drinking response and further salinity increases would have no accumulative effect. However, the apparent

reduction in drinking response suggests a deleterious effect. While such a reduced drinking response has not previously been reported in the literature, mean drinking rate for rainbow trout (5 - 20g) adapted to 50% seawater for ten days was greater (although apparently not statistically significant) than that of similar fish adapted to 75% seawater for a similar period (Eddy and Bath, 1979). In addition, salmon parr, with mean weight 22.6 g, displayed no statistically significant increase in mean drinking rate either two or 24 hours after seawater transfer (Smith *et al.*, 1991), although drinking rate was significantly greater than freshwater levels three days after seawater transfer. Smith *et al.* (1991) suggested this poor drinking response in parr was associated with relatively low and unchanged renin levels, possibly due to the inhibitory effects of high ANP levels. However, due to relatively large plasma volume requirements, the parr used to measure plasma renin and ANP levels were substantially bigger (365 g) than those in which drinking rates were measured and were post smolts which had been held in freshwater for two years after first smolting. In addition, a reduced endocrine response to seawater transfer would not explain the apparent 'normal' response to transfer to salinities up to 50% seawater.

Prior to smoltification, body size has been established as the major factor in the ability of salmonid juveniles to survive hyperosmotic transfer (e.g. Huntsman and Hoar, 1939; Parry, 1958; McCormick and Naiman, 1984; Salman and Eddy, 1990; Bjerknes *et al.*, 1992). A threshold size or length has been documented for survival after either (1) acute seawater transfer in, for example, Atlantic salmon parr (19g, 12 cm, Farmer *et al.*, 1978) and Arctic charr (15 cm, Delabbio *et al.*, 1988) or (2) even after gradual acclimation in Atlantic salmon parr (9.5 cm, Bjerknes *et al.*, 1992). This body size effect is probably due to the reduction in the gill surface area to body volume ratio as the fish increases in size, which results in less dehydration and branchial ion diffusion per unit weight in larger fish (Hughes, 1972, 1984).

The trout of Experiments 1 and 2 were of such size that they would not be expected to survive full seawater-challenge for an extended period of time. Rainbow trout (10.3 g) survival averaged five days in full seawater, even after stepwise salinity increase and acclimation for ten days (Eddy and Bath, 1979). Prior to death, these rainbow trout displayed large increases in plasma ion concentration and in whole body and muscle ion content. Similar large increases in plasma ion concentrations and marked tissue dehydration were observed in yearling brown trout (53 g) which subsequently suffered 50% mortalities within three days of transfer to 24.5 and 27.5 ppt brackish water (Madsen, 1990). Therefore, it is reasonable to assume that the trout in this study transferred to 75% and full seawater during Experiments 1-3 were

suffering at least the early stages of osmoregulatory breakdown and perhaps this might have affected the drinking response. An alternative to the low renin level hypothesis above, therefore, might be that this reduced drinking response reflected the inhibitory effect of excessively (dangerously) high plasma ion levels, although whether this inhibition could be dependent upon the rate of increase in ion concentration or a threshold level is unknown. Elevated plasma ion levels as a result of infusion of hypertonic saline solution inhibited the drinking response of the Japanese eel (*Anguilla japonica*) to seawater conditions (Hirano, 1974). Perhaps internal conditions became such that a more drastic mechanism, without the 'normal' increase in drinking rate, was adopted to enable short-term survival in hyperosmotic conditions. In the field, such a short-term mechanism might allow the fish sufficient time to return to less saline waters.

2.4.3.2 Parental form effects

Significant parental form effects on drinking rate after salinity challenge were observed throughout the study. However, whereas one might have expected an indication of higher hypo-osmoregulatory capacity amongst the trout of anadromous parentage (Sea trout), in most cases the mean drinking rate response to increased salinity was lowest in Resident trout and similar between Sea trout and Isolated trout. Resident trout were larger at all times after the first experimental sampling and since seawater tolerance is positively related to size, this might have explained the apparent differences. However, the significant parent type effect remained when body size was removed from the analysis by ANCOVA.

Several studies have compared the physiological capabilities of freshwater resident and anadromous salmonids. Gordon (1959) found no significant differences between the osmoregulatory capabilities of anadromous and resident adult brown trout transferred to seawater but the use of relatively large adult trout may have obscured any genotypic differences. Although resident brook charr did not show the characteristic silvering of migrating charr, there were no significant differences in plasma concentrations of T4, gill $Na^{+}K^{+}$-ATPase activity or haematocrit at time of migration in anadromous fish (McCormick *et al.*, 1985) and charr isolated in freshwater for at least 10,000 years (Hawswater, Lake District, U.K.) displayed a high degree of salinity tolerance characteristic of their anadromous ancestors (Roberts, 1971).

Other studies have demonstrated an apparent difference in hypo-osmoregulatory capability, specifically in activity of branchial Na^+K^+-ATPase activity. Soivio *et al.* (1989) measured levels of plasma hormones and gill Na^+K^+-ATPase activity in anadromous and lake-dwelling Finnish trout in freshwater and after seawater-transfer. Both stocks displayed spring peaks in plasma T4 and cortisol but only the anadromous stock showed a simultaneous rise in Na^+K^+-ATPase activity. However, both stocks tolerated seawater-transfer equally well between March and early June. A similar lack of seasonal peaks in Na^+K^+-ATPase activity was noted for non-migratory domestic brown trout and rainbow trout (Boeuf and Harache, 1982).

The offspring of resident and anadromous Arctic charr were reared under continuous light and salinity tolerance tested periodically by seawater transfer (Staurnes *et al.*, 1992). During the first summer, mortality rates after seawater-transfer were greater in resident offspring which also displayed higher plasma Na^+ levels and lower gill Na^+K^+-ATPase activity levels. In the second summer the anadromous offspring were able to adapt to seawater within ten days of transfer unlike the resident offspring. This adaptation of the former was accompanied by a two-fold increase in gill Na^+K^+-ATPase activity.

However, care must be taken to compare smolts with smolts and not smolts of anadromous trout with non-smolts of resident trout. For example, Tanguy *et al.* (1994) found that a decrease in plasma PRL during spring in anadromous non-migrant trout, but not in trout of resident parentage, was the only distinguishing feature between non-smolting anadromous and resident trout. No differences were observed in plasma ion profile after seawater transfer in either group, while other changes were related to the size of the fish rather than source: a decline in condition factor during spring and high survival after seawater-transfer in the larger trout was evident in both trout forms whereas variable survival after seawater-transfer in smaller trout of either form was related to body size rather than source. Finally, while the larger anadromous trout displayed an increase in gill Na^+K^+-ATPase activity levels, no increase was noted in smaller non-smolting anadromous or most resident trout, except for the small number of resident trout which did undergo smoltification, as indicated by silvering and gill Na^+K^+-ATPase activity levels. Thus, nearly all of the apparent differences between resident and anadromous trout could be explained by an incorrect comparison between smolts and non-smolts, i.e. differences in the proportion smolting. Such differences suggest a genetic component and this might have been especially so given the comparison of wild anadromous trout with domestic resident trout.

However, it must be noted at this point that the parental form alone does not fix the strategy of the offspring - the choice to migrate to sea or not is influenced both by genetic and environmental factors as demonstrated by the high proportion of maturing trout, of both sexes, in all three parent types reared at Medium or High ration levels.

2.4.3.3 Effects of hatchery rearing on hypo-osmoregulatory capacity

Hatchery rearing has been demonstrated to affect the timing and degree of smolting in some salmonids. Gill Na^+K^+-ATPase activity was lower in reared than wild smolting Atlantic salmon (Virtanen and Soivio, 1985). Peaks in Na^+K^+-ATPase and SDH activities of reared Atlantic salmon smolts lagged behind those of wild smolts by four to eight weeks (Langdon and Thorpe, 1985). Captive smolts may have lower thyroid activity than wild migrating smolts that may be due to steady flow conditions in tanks (Youngson and Simpson, 1984). Plasma levels of T3 and T4 decreased significantly in wild smolts after a week of captivity although gill Na^+K^+-ATPase did not change significantly (Virtanen and Soivio, 1985).

Other studies have shown that in wild smolts, physiological changes continue to develop during the downstream migration, and that the level of these changes depends on the distance travelled (related to the distance to be travelled rather than a measure of the distance covered). Thus, preventing the natural migration by rearing and transferring acutely between salinities may prevent the final optimisation of transformation. Bergstrom (1959) observed that Atlantic salmon smolts trapped during downstream migration had not completed the parr-smolt transformation. Of ten immediately transferred to seawater, two died within 18 hours and another three showed signs of great distress. The plasma Na^+ levels of survivors increased significantly but was restored within five days.

The rearing conditions at the Almondbank hatchery may have been too good to encourage the parr to undergo smoltification, either because the ration levels were too high or perhaps because the water temperature regime of this relatively low lying area enhanced growth. This highlights the need for further experiments to investigate the effects of varying water temperature regimes on smolting, both artificially in a hatchery and naturally by rearing the same stock at high and low altitudes.

Finally, the juveniles reared may not have been genotypically inclined to smolt in large numbers. The River Eden does support an appreciable sea trout stock and one batch of parr were the progeny of sea trout. However, this system is relatively nutrient rich due to the farm land through which it runs and the growth conditions for trout are good, as implied by the high condition factor of resident trout sampled.

2.4.3.4 Deleterious effects of maturation on hypo-osmoregulatory capacity

Mean drinking rates were significantly higher in immature trout, regardless of parent source, challenged with full seawater when compared with those of mature trout of both sexes in the final challenges (Experiment 4). Seawater-transferred immature trout were smaller than mature trout of each parent form. The difference in mean drinking rates might have been a size effect rather than due to state of maturation, such that smaller fish drink more in seawater. Drinking rates are expressed in weight specific terms in the current study but is this linear scaling sufficient to compare the drinking rates of fish which are twice as heavy as others? Gill surface area, of major importance with regard to whole body permeability and hence, osmoregulation and ionoregulation, scales to body weight by a factor of 0.67 rather than 1 (Hughes, 1966). The presence or absence of a size effect in weight specific drinking rates in salmonids has not been reported. However, when calculated on a weight-specific basis, no relationship was observed between body weight and drinking rate in dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) weighing between 1 and 150 g (Carroll *et al.*, 1995). Although the size differences are less, there does appear to be a ration effect amongst the length and weight data, but not condition factor, for mature trout in this study (see below). However, these size differences are not reflected in differences amongst drinking rates in trout transferred to seawater. This implies that the differences between mean drinking rates for immature and mature Low ration trout are not due to size effects.

Maturation has been demonstrated to have deleterious effects on seawater adaptation and to be counteractive to the parr-smolt transformation in several salmonid species. Significantly greater plasma Na^+ concentrations after seawater transfer were reported for mature versus immature sea trout despite the mature fish being larger (Dellefors and Faremo, 1988) and this also occurs in Atlantic salmon (Langdon and Thorpe, 1985; Lundqvist *et al.*, 1990; Berglund *et al.*, 1991). Mature male Atlantic salmon parr did not survive a 24 hour exposure to 40 ppt seawater, had low numbers of small chloride cells, low levels of branchial Na^+K^+-ATPase activity and the lowest whole-cell SDH activities of any fish recorded in spring

(Langdon and Thorpe, 1985). The mean seawater survival time was significantly lower in mature brook trout in comparison with immature males and females (McCormick and Naiman, 1984). The lack of any significant differences in mean plasma ion concentrations in precocious mature and immature chinook salmon (*O. tshawytscha*) in September (Bernier *et al.*, 1993) may have been due to the death of nearly 50% of the mature parr, both in freshwater and seawater, acting as a selection pressure for those with better hypo-osmoregulatory capacity, the 3 month period of seawater acclimation prior to sampling or a genus specific effect.

Such a reduced hypo-osmoregulatory response in maturing salmonids may be due to increased circulating levels of sex steroids. Oestradiol treated sea trout pre-smolts displayed poor ionoregulatory ability in freshwater, as indicated by lower plasma Na^+ concentration, and in seawater (28 ppt), as indicated by high mortality rates and high plasma ion concentrations (Na^+, Cl^- and Mg^{2+}) due, at least in part, to lower branchial Na^+K^+-ATPase activity (Madsen and Korsgaard, 1991). The fact that the deleterious effects were diminished within 14 days of treatment, probably as the dose decreased, and that simultaneous administration of GH and cortisol partly abolished these deleterious effects, indicates that oestradiol altered the general endocrine state of the trout and it was suggested that this might be through increased levels of PRL, a hormone which has been demonstrated to reduce branchial ionoregulatory mechanisms (see Section 1.3). However, circulating PRL levels were found to peak two months before peak levels of estrogens and androgens in maturing female Atlantic salmon (Andersen *et al.*, 1991).

While the deleterious effects of sex steroids are accepted, the timing of the inhibition is less well understood. Castration in March did not improve the subsequent hypo-osmoregulatory capacity of previously precocious mature male Atlantic salmon parr (Lundqvist *et al.*, 1990) and it was suggested that the deleterious effects were due to high levels of sex steroids in the previous winter. Berglund *et al.* (1991) suggest, however, that the poor seawater capacity of castrated parr might have been due to the continued presence of extragonadal sources of androgens, such as the head kidney.

The influence of sex steroids on drinking rate response to seawater transfer in sea trout has not previously been reported. Muscle water content was significantly lower in estradiol treated sea trout presmolts than control or GH/cortisol treated fish two days after seawater transfer, but had recovered to a level similar to control fish by day seven (Madsen and Korsgaard, 1991). This tissue dehydration was possibly

due to fluid transfer between tissue and serum to counteract the high plasma ion concentrations but may also be indicative of a reduced osmoregulatory capacity, i.e. reduced drinking rate response as observed in the present study. However, the reduced drinking rate response may have been a result of interaction between sex steroids and the RAS system that controls drinking rather than an inhibition by high plasma ion concentrations.

Mean plasma Cl^- concentrations in mature trout in the present study were 115 to 120 $mmol.l^{-1}$ in freshwater and 125 to 140 $mmol.l^{-1}$ in seawater-challenged trout. The freshwater values were similar to those of immature and mature freshwater adapted sea trout pre-smolts while the seawater concentrations were similar to those of immature pre-smolts two days after transfer to 28 ppt seawater (Madsen and Korsgaard, 1991). The lack of abnormal plasma Cl^- levels in mature trout in the present study may reflect plasma Cl^- concentrations not having peaked during the six hour salinity challenge performed. Plasma Cl^- concentration in Atlantic salmon parr, which reached a similar level after six hours, was still increasing two days after acute transfer (Stagg *et al.*, 1989). Hogstrand and Haux (1985) observed no differences in plasma ion concentrations between immature and male precocious mature sea trout parr after 24 hours salinity challenge but, since the salinity was only 20 ppt, this might not have been a sufficient physiological challenge to reveal differences in the response in relation to state of maturity.

Further study will be required to determine any differences in branchial Na^+K^+-ATPase activity and/or plasma concentrations of sex steroids, such as testosterone.

2.4.4 Concluding Summary

Increased ration resulted in faster growth in length and weight of trout of all three parental forms. Resident trout were, on average, larger than Isolated trout or Sea trout for most of the study. The proportion of maturing trout was positively related to ration but was also affected by parental form amongst the Low ration group in which Isolated trout were more prone to early maturation. Drinking rates increased with increasing salinity at all ontogenetic stages although they were greater in 50% seawater than 75% or full seawater during the first year, possibly due to the inhibitory effects of high plasma ion concentrations. Drinking rates were higher in immature than mature trout, regardless of parental form, on the final sampling and this was considered to indicate the general deleterious effects of sex steroids on hypo-osmoregulation. Drinking rate was not affected by ration type in the final

sampling but had been significantly affected when fish were smaller, even when size effects were taken into account. Parental form effects were observed at each sampling but were not consistent throughout the experimental period and, as a consequence, were difficult to explain. This suggests that early genotype effects were obscured when fish became large enough to withstand at least temporary acute seawater-transfer.

The presence of a large proportion of mature male and female trout in the final sampling, and the absence of any true smolts, indicates that conditions were too favourable for growth to induce smoltification. This may have been the result of both environmental and genetic influences.

2.5 Statistical Tables

Table 2.6 a Statistical results of Statview test of normality for the length, weight and condition factor (K) data for trout sampled in Sample 1.

Table 2.6 b Statistical results of Statview test of normality for the length, weight and condition factor (K) data for trout sampled in Sample 2.

Table 2.6 c Statistical results of Statview test of normality for the length, weight and condition factor (K) data for trout sampled in Sample 3.

Table 2.6 d Statistical results of Statview test of normality for the length, weight and condition factor (K) data for trout sampled in Sample 4.

	length		weight		K	
ration/parental form	X2	p	X2	p	X2	p
Sea trout, Low	3.527	0.343	1.000	>0.999	0.640	>0.999
Resident trout, Low	2.940	0.460	1.960	0.751	1.440	0.974
Isolate trout, Low	3.527	0.343	2.560	0.556	1.440	0.974
Sea trout, Medium	3.840	0.293	2.560	0.556	4.000	0.271
Resident trout, Medium	2.415	0.598	1.020	>0.999	2.000	0.736
Sea trout, High	2.167	0.677	3.306	0.383	0.653	>0.999
Resident trout, High	2.182	0.672	0.667	>0.999	1.042	>0.999
Isolate trout, High	3.538	0.341	1.020	>0.999	0.653	>0.999

	length		weight		K	
ration/parental form	X2	p	X2	p	X2	p
Sea trout, Low	3.204	0.403	2.000	0.739	0.320	>0.999
Resident trout, Low	1.117	>0.999	2.444	0.589	0.990	>0.999
Isolate trout, Low	2.765	0.502	2.880	0.474	0.500	>0.999
Sea trout, Medium	4.768	0.184	2.880	0.474	0.720	>0.999
Resident trout, Medium	1.653	0.875	1.636	0.883	0.727	>0.999
Sea trout, High	1.817	0.806	2.000	0.736	0.500	>0.999
Resident trout, High	3.682	0.317	2.444	0.589	0.505	>0.999
Isolate trout, High	4.436	0.218	4.545	0.206	0.990	>0.999

	length		weight		K	
ration/parental form	X2	p	X2	p	X2	p
Sea trout, Low	3.681	0.318	2.880	0.474	1.620	0.890
Resident trout, Low	2.706	0.517	2.880	0.474	0.500	>0.999
Isolate trout, Low	1.442	0.972	3.449	0.357	1.000	>0.999
Sea trout, Medium	2.113	0.695	1.280	>0.999	1.620	0.890
Resident trout, Medium	3.230	0.398	2.420	0.596	0.980	>0.999
Sea trout, High	4.088	0.259	1.280	>0.999	0.500	>0.999
Resident trout, High	1.948	0.755	5.172	0.151	1.293	>0.999
Isolate trout, High	2.909	0.467	1.280	>0.999	1.280	>0.999

	length		weight		K	
ration/parental form	X2	p	X2	p	X2	p
Sea trout, Low	3.063	0.432	3.881	0.287	0.970	>0.999
Resident trout, Low	0.678	>0.999	1.604	0.897	0.713	>0.999
Isolate trout, Low	2.649	0.532	1.604	0.897	0.713	>0.999
Sea trout, Medium	2.462	0.584	2.396	0.607	0.970	>0.999
Resident trout, Medium	2.930	0.462	0.990	>0.999	0.727	>0.999
Sea trout, High	4.325	0.230	1.267	>0.999	0.970	>0.999
Resident trout, High	1.744	0.836	1.636	0.883	0.727	>0.999
Isolate trout, High	2.645	0.533	2.420	0.597	0.500	>0.999

Table 2.6 e Statistical results of Statview test of normality for the length, weight and condition factor (K) data for trout sampled in Sample 5.

Table 2.7 Results of 2-way ANOVA of log length data for Sample 1 (5 months old). Data analyzed were for groups of 300 trout from two parental forms (Sea trout and Resident trout) reared under three ration regimes (High, Medium and Low) and one form (Isolated trout) reared under two ration regimes (High and Low).

Table 2.8 Results of 2-way ANOVA of log length data for Sample 2 (14 months old). See Table 2.7 for the various groups of trout and treatments. Sample sizes varied between 404 and 740.

Table 2.9 Results of 2-way ANOVA of log length data for Sample 3 (18 months old). See Table 2.7 for the various groups of trout and treatments. Sample sizes varied between 357 and 690.

Table 2.10 Results of 2-way ANOVA of log length data for Sample 4 (24 months old). See Table 2.7 for the various groups of trout and treatments. Sample sizes varied between 260 and 325.

Table 2.11 Results of 2-way ANOVA of log length data for Sample 5 (26 months old). See Table 2.7 for the various groups of trout and treatments. Sample sizes varied between 203 and 288.

	length		weight		K	
ration/parental form	X^2	p	X^2	p	X^2	p
Sea trout, Low	2.524	0.566	1.960	0.751	1.000	>0.999
Resident trout, Low	1.457	0.965	0.353	>0.999	0.980	>0.999
Isolate trout, Low	1.225	>0.999	1.042	>0.999	1.042	>0.999
Sea trout, Medium	3.712	0.313	1.440	0.974	0.640	>0.999
Resident trout, Medium	2.522	0.567	0.627	>0.999	0.627	>0.999
Sea trout, High	3.333	0.378	0.667	>0.999	1.042	>0.999
Resident trout, High	2.349	0.618	1.000	>0.999	3.240	0.399
Isolate trout, High	1.071	>0.999	1.000	>0.999	1.000	>0.999

Source	df	SS	MS	F	p
Ration	1	0.072	0.072	35.468	< 0.001
Parental form	2	0.012	0.006	3.054	0.048
Ration x parental form	2	0.028	0.014	6.944	0.001
Residual	1789	3.630	0.002		

Source	df	SS	MS	F	p
Ration	1	3.914	3.914	823.000	< 0.001
Parental form	2	0.714	0.357	75.090	< 0.001
Ration x parental form	2	0.008	0.004	0.892	0.410
Residual	3235	15.383	0.005		

Source	df	SS	MS	F	p
Ration	1	12.814	12.814	2123.00	< 0.001
Parental form	2	0.761	0.380	63.038	< 0.001
Ration x parental form	2	0.016	0.008	1.363	0.255
Residual	2900	17.501	0.006		

Source	df	SS	MS	F	p
Ration	1	5.047	5.047	7.91	< 0.001
Parental form	2	0.229	0.115	17.963	< 0.001
Ration x parental form	2	0.034	0.017	2.629	0.072
Residual	1722	11.024	0.006		

Source	df	SS	MS	F	p
Ration	1	2.980	2.980	598.000	< 0.001
Parental form	2	0.212	0.106	21.267	< 0.001
Ration x parental form	2	0.026	0.013	2.624	0.073
Residual	1509	2.524	0.005		

Table 2.12 Results of 2-way ANOVA of log weight data for Sample 1 (5 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 50 trout per group.

Table 2.13 Results of 2-way ANOVA of log weight data for Sample 2 (14 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group.

Table 2.14 Results of 2-way ANOVA of log weight data for Sample 3 (18 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group.

Table 2.15 Results of 2-way ANOVA of log weight data for Sample 4 (24 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group.

Table 2.16 Results of 2-way ANOVA of log weight data for Sample 5 (26 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 50 trout per group.

Table 2.17 Results of 2-way ANOVA of log condition factor data for Sample 1 (5 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 50 trout per group.

Table 2.18 Results of 2-way ANOVA of log condition factor data for Sample 2 (14 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group.

Source	df	SS	MS	F	p
Ration	1	0.071	0.071	2.892	0.090
Parental form	2	0.052	0.026	1.064	0.346
Ration x parental form	2	0.018	0.009	0.370	0.691
Residual	291	7.099	0.024		

Source	df	SS	MS	F	p
Ration	1	9.538	9.358	204.000	< 0.001
Parental form	2	3.521	1.760	37.687	< 0.001
Ration x parental form	2	0.186	0.093	1.986	0.1381
Residual	591	27.606	0.047		

Source	df	SS	MS	F	p
Ration	1	28.044	28.044	440.000	< 0.001
Parental form	2	0.921	0.460	7.222	< 0.001
Ration x parental form	2	0.138	0.069	1.083	0.339
Residual	592	37.742	0.084		

Source	df	SS	MS	F	p
Ration	1	14.362	14.362	249.000	< 0.001
Parental form	2	0.639	0.310	5.532	0.004
Ration x parental form	2	0.181	0.090	1.563	0.210
Residual	597	34.499	0.058		

Source	df	SS	MS	F	p
Ration	1	6.742	6.742	158.000	< 0.001
Parental form	2	0.248	0.124	2.907	0.0563
Ration x parental form	2	0.214	0.107	2.514	0.083
Residual	291	12.408	0.043		

Source	df	SS	MS	F	p
Ration	1	0.002	0.002	1.318	0.252
Parental form	2	0.033	0.016	10.667	< 0.001
Ration x parental form	2	0.001	0.001	0.326	0.722
Residual	291	0.449	0.002		

Source	df	SS	MS	F	p
Ration	1	0.094	0.094	136.000	< 0.001
Parental form	2	0.053	0.026	38.205	< 0.001
Ration x parental form	2	0.042	0.021	30.438	< 0.001
Residual	91	0.408	0.001		

Table 2.19 Results of 2-way ANOVA of log condition factor data for Sample 3 (18 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group

Table 2.20 Results of 2-way ANOVA of log condition factor data for Sample 4 (20 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 100 trout per group.

Table 2.21 Results of 2-way ANOVA of log condition factor data for Sample 5 (26 months old). See Table 2.7 for the various groups of trout and treatments. Sample size was 50 trout per group.

Table 2.22 Results of 2-way ANOVA of log drinking rates for Experiment 1 (9 months old). Data analysed were drinking rates of groups of ten High ration trout of three parental forms (Sea trout, Resident trout and Isolated trout) after transfer to five salinities (0, 20%, 33%, 50% and 75% seawater).

Table 2.23 Results of 3-way ANOVA of log drinking rates for Experiment 2 (14 months old). Data analysed were from groups of nine trout of three parental forms (Sea trout, Resident trout and Isolated trout) reared under two ration regimes (High and Low) subsequently transferred to three salinity regimes (freshwater, brackish water and seawater).

Table 2.24 Results of 3-way ANCOVA of log drinking rates with log body weight as covariate for Experiment 2 (14 months old). See Table 2.23 for trout groups and sample sizes.

Source	df	SS	MS	F	p
Ration	1	0.156	0.156	186.000	< 0.001
Parental form	2	< 0.001	< 0.001	0.120	0.887
Ration x parental form	2	0.021	0.010	12.413	< 0.001
Residual	592	0.496	0.001		

Source	df	SS	MS	F	p
Ration	1	0.018	0.018	35.639	< 0.001
Parental form	2	0.012	0.006	11.876	< 0.001
Ration x parental form	2	0.006	0.003	6.008	0.003
Residual	597	0.300	0.001		

Source	df	SS	MS	F	p
Ration	1	0.008	0.008	7.628	0.006
Parental form	2	0.057	0.029	27.718	< 0.001
Ration x parental form	2	0.015	0.008	7.381	< 0.001
Residual	291	0.301	0.001		

Source	df	SS	MS	F	p
Ration	4	6.731	1.683	16.481	< 0.001
Parental form	2	0.628	0.314	3.141	0.046
Ration x parental form	8	0.889	0.111	1.112	0.359
Residual	134	13.390	0.100		

Source	df	SS	MS	F	p
Salinity	2	5.069	2.535	28.046	< 0.001
Ration	1	1.022	1.022	11.313	0.001
Parental from	2	0.919	0.460	5.086	0.007
Salinity x ration	2	0.426	0.213	2.356	0.098
Salinity x parental form	4	1.179	0.295	3.263	0.014
Ration x parental form	2	1.035	0.518	5.728	0.004
Salinity x ration x parental form	4	0.692	0.173	1.914	0.111
Residual	145	13.104	0.090		

Source	df	SS	MS	F	p
Salinity	2	5.210	2.605	32.115	< 0.001
Ration	1	0.186	0.186	2.291	0.132
Parental Form	2	1.483	0.741	9.140	< 0.001
Salinity x ration	2	0.563	0.282	3.473	0.034
Salinity x parental form	4	1.218	0.304	3.753	0.006
Ration x parental form	2	0.569	0.285	3.509	0.033
Salinity x ration x parental form	4	0.578	0.144	1.781	0.136
Covariate	1	1.424	1.424	17.561	< 0.001
Residual	144	11.680	0.081		

Table 2.25 Results of 3-way ANOVA of log drinking rates in Experiment 3 (20 months). Data analysed were from groups of immature trout of three parental forms (Sea trout, Resident trout and Isolated trout) reared under two ration regimes (Low and High) subsequently transferred to three salinity regimes (freshwater, brackish water and seawater). See Figure 2.12 a-c for sample sizes for each group.

Table 2.26 Results of 2-way ANOVA of log drinking rate data in Medium ration, immature trout for Experiment 3 (20 months). Data were analyzed from trout of two parental forms (Sea trout and Resident trout) transferred to freshwater, brackish water or seawater. See Figure 2.12 a-c for sample sizes.

Table 2.27 Results of 3-way ANOVA of log drinking rates for Low ration trout sampled in Experiment 4 (26 months old). Data analyzed were from groups of trout of three parental forms (Sea trout, Resident trout and Isolated trout), split by maturity (immature, mature), transferred to freshwater or seawater. See Figure 2.13 a-c for sample sizes.

Table 2.28 Results of 2-way ANOVA of log drinking rates for immature, Low ration trout sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout) transferred to freshwater or seawater. See Figure 2.13 a-c for sample sizes.

Table 2.29 Results of 3-way ANOVA of log drinking rates for mature trout sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High) transferred to freshwater or seawater. See Figure 2.14 a-c for sample sizes.

Source	df	SS	MS	F	p
Salinity	2	3.162	1.581	11.588	< 0.001
Ration	1	0.090	0.090	0.661	0.418
Parental Form	2	0.110	0.055	0.404	0.669
Salinity x ration	2	0.274	0.137	1.004	0.370
Salinity x parental form	4	0.785	0.196	1.437	0.226
Ration x parental form	2	0.575	0.288	2.108	0.126
Salinity x ration x parental form	4	0.733	0.183	1.344	0.258
Residual	117	15.965	0.136		

Source	df	SS	MS	F	p
Salinity	2	0.740	0.370	2.677	0.079
Parental form	1	1.078	1.078	7.803	0.007
Salinity x parental form	2	0.284	0.142	1.028	0.365
Residual	49	6.768	0.138		

Source	df	SS	MS	F	p
Salinity	1	18.821	18.821	250.008	< 0.001
Maturity	1	0.198	0.198	2.631	0.109
Parental Form	2	1.409	0.704	9.359	< 0.001
Salinity x maturity	1	0.484	0.484	6.436	0.013
Salinity x parental form	2	0.096	0.048	0.641	0.529
Maturity x parental form	2	0.243	0.122	1.616	0.205
Salinity x maturity x p. form	2	0.125	0.062	0.830	0.440
Residual	84	6.322	0.075		

Source	df	SS	MS	F	p
Salinity	1	11.192	11.192	198.000	< 0.001
Parental form	2	1.027	0.514	9.087	< 0.001
Salinity x parental form	2	0.173	0.086	1.529	0.231
Residual	36	2.035	0.057		

Source	df	SS	MS	F	p
Salinity	1	21.485	21.485	194.450	< 0.001
Ration	1	0.014	0.014	0.128	0.721
Parental Form	2	1.258	0.629	5.697	0.004
Salinity x ration	1	0.094	0.094	0.848	0.359
Salinity x parental form	2	0.008	0.004	0.038	0.962
Ration x parental form	2	0.269	0.135	1.220	0.299
Salinity x ration x p. form	2	0.109	0.054	0.491	0.613
Residual	126	13.916	0.110		

Table 2.30 Results of 3-way ANCOVA of log drinking rates for mature trout with log weight as the covariate, Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High) transferred to freshwater or seawater. See Figure 2.14 a-c for sample sizes.

Table 2.31 Results of 3-way ANOVA of log plasma Cl^- concentration for mature trout sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High), transferred to freshwater or seawater. See Figure 2.15 a-c for sample sizes.

Table 2.32 Results of 3-way ANCOVA of log plasma Cl^- concentration for mature trout with log weight as the covariate, sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High), transferred to freshwater or seawater. See Figure 2.15 a-c for sample sizes.

Table 2.33 Results of 3-way ANOVA of arcsine transformed haematocrit for mature trout sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High) transferred to freshwater or seawater. See Figure 2.16 a-c for sample sizes.

Source	df	SS	MS	F	p
Salinity	1	21.065	21.065	197.112	< 0.001
Ration	1	0.155	0.155	1.450	0.231
Parental Form	2	1.106	0.553	5.173	0.007
Salinity x ration	1	0.064	0.064	0.601	0.440
Salinity x parental form	2	0.011	0.005	0.050	0.951
Ration x parental form	2	0.340	0.170	1.591	0.208
Salinity x ration x p. form	2	0.119	0.060	0.557	0.574
Covariate	1	0.556	0.556	5.204	0.024
Residual	125	13.359	0.107		

Source	df	SS	MS	F	p
Salinity	1	0.029	0.029	30.466	< 0.001
Ration	1	0.006	0.006	6.524	0.013
Parental Form	2	0.010	0.005	5.106	0.009
Salinity x ration	1	0.001	0.001	0.566	0.455
Salinity x parental form	2	0.001	0.001	0.564	0.572
Ration x parental form	2	0.002	0.001	1.290	0.282
Salinity x ration x p. form	2	< 0.001	< 0.001	0.160	0.853
Residual	69	0.060	0.001		

Source	df	SS	MS	F	p
Salinity	1	0.030	0.030	34.238	< 0.001
Ration	1	0.002	0.002	1.716	0.195
Parental Form	2	0.008	0.004	4.761	0.012
Salinity x ration	1	< 0.001	< 0.001	0.420	0.519
Salinity x parental form	2	< 0.001	< 0.001	0.306	0.737
Ration x parental form	2	0.002	0.001	1.103	0.338
Salinity x ration x p. form	2	< 0.001	< 0.001	0.118	0.889
Covariate	1	0.005	0.005	5.675	0.020
Residual	68	0.060	0.001		

Source	df	SS	MS	F	p
Salinity	1	0.013	0.013	3.949	0.051
Ration	1	0.008	0.008	2.524	0.117
Parental Form	2	0.003	0.001	0.396	0.675
Salinity x ration	1	0.002	0.002	0.556	0.458
Salinity x parental form	2	0.006	0.003	0.997	0.381
Ration x parental form	2	0.122	0.061	18.377	< 0.001
Salinity x ration x p. form	2	0.027	0.013	4.029	0.022
Residual	71	0.235	0.003		

Table 2.34 Results of 3-way ANCOVA of arcsine transformed haematocrit for mature trout, with log weight as the covariate, sampled in Experiment 4 (26 months old). Data analyzed were from trout of three parental forms (Sea trout, Resident trout and Isolated trout), reared at two rations (Low and High) transferred to freshwater or seawater. See Figure 2.16 a-c for sample sizes.

Table 2.35 Results of 2-way ANOVA of log drinking rate data for Medium ration, mature trout sampled in Experiment 4 (26 months old). Data were analyzed from trout of two parental forms (Sea trout and Resident trout) transferred to freshwater or seawater. See Figure 2.14 a-c for sample sizes.

Table 2.36 Results of 2-way ANOVA of log plasma Cl^- concentrations for Medium ration, mature trout sampled in Experiment 4 (26 months old). Data were analyzed from trout of two parental forms (Sea trout and Resident trout) transferred to freshwater or seawater. See Figure 2.16 a-c for sample sizes.

Source	df	SS	MS	F	p
Salinity	1	0.017	0.017	5.937	0.017
Ration	1	< 0.001	< 0.001	< 0.001	0.991
Parental Form	2	0.004	0.002	0.780	0.462
Salinity x ration	1	0.002	0.002	0.524	0.472
Salinity x parental form	2	0.004	0.002	0.698	0.501
Ration x parental form	2	0.117	0.059	20.351	< 0.001
Salinity x ration x p. form	2	0.025	0.013	4.346	0.017
Covariate	1	0.034	0.017	11.666	0.001
Residual	70	0.201	0.003		

Source	df	SS	MS	F	p
Salinity	1	6.887	6.887	66.758	< 0.001
Parental form	1	0.179	0.179	1.733	0.195
Salinity x parental form	1	0.322	0.322	3.123	0.085
Residual	4	4.230	0.103		

Source	df	SS	MS	F	p
Salinity	1	0.014	0.014	14.562	< 0.001
Parental form	1	0.008	0.008	8.216	0.008
Salinity x parental form	1	< 0.001	< 0.001	0.106	0.747
Residual	28	0.026	0.001		

Chapter 3

Overwintering behaviour of juvenile sea trout

3.1 Introduction

3.1.1 Migratory strategies of sea trout

The benefits to the individual of any migration from one habitat to another must outweigh the costs if such a migration is to become selected for and a stable strategy. The advantages of the superior growth opportunities afforded anadromous salmonids have been described above (Section 2.1). When Atlantic salmon migrate to sea they remain there for at least one, if not several, winters before returning to freshwater to mature and spawn. In contrast, a variable proportion of many sea trout populations forego the growth opportunities of the marine environment by returning to estuaries and rivers within weeks or months of seaward migration and overwinter in fresh and/or brackish water prior to migrating seawards again in the following spring. These juvenile anadromous trout (Plate 3.1) are called finnock, herling, school peal or whitling, depending upon U.K. region, but will be described here as finnock.

Unlike Atlantic salmon, only a small but variable proportion of finnock mature during this first return to freshwater (see Table 3.1). There does appear to be a geographic effect on the proportion of finnock that mature. Fahy (1978) estimated the mean proportion of maturing finnock to be 6.15% and 8.76% in Scotland and Ireland, respectively, but 30.95% in Wales. In addition, 87% of the finnock sampled returning to the River Calonne, Normandy, 1990 were found to be maturing (Maisse *et al.*, 1991). This highlights the possible confusion over what should be regarded as a finnock. Pratten and Shearer (1983a) defined finnock as sea trout "smolts upon reaching the estuary.... until 31 March of the following year." I would argue that this definition should be tightened to include only those individuals that do not mature during this first return to freshwater. This definition will be used for the remainder of this thesis.

3.1.1.1 Present knowledge of finnock behaviour

Very few studies have reported details of finnock behaviour and what little is known is based upon either mark-recapture techniques (see below), periodic netting of estuaries and sea lochs or trap data. The following general descriptive pattern of finnock behaviour in systems on the east coast of Scotland is based upon studies of sea trout from the River Ythan, Aberdeenshire (Nall, 1927) and the River North Esk, Angus (Pratten and Shearer, 1983a, 1983b; Shearer, 1990).

Plate 3.1

Plate 3.1 Two finnock caught soon after entry into freshwater.

Table 3.1

Table 3.1 The proportion of finnock which were found to be maturing when sampled returning to various Scottish and Irish river systems.

River	Country	Year of study	% maturing	Reference
River Erriff	Ireland, west coast	1977	8 % females 13 % males	O'Farrell; in Le Cren (1985)
River Ythan	Scotland, east coast	1925-1926	approx. 10 %	Menzies and Nall (1932)
River North Esk	Scotland, east coast	1976-1980	0.20 % females 0.05 % males	Shearer and Pratten (1983a)
		1995	8 % females 0 % males	personal observations n = 96

Finnock begin to return to estuaries from as little as five to six weeks after migrating seawards as smolts. During the summer months they move in and out of estuaries along the coast, mixing with other stocks and some make brief migrations into freshwater. Sea trout smolts and finnock tagged in the River North Esk were subsequently recaptured in most rivers or estuaries between the Tweed and the Spey (Figure 3.1a and b). Most finnock are then thought to overwinter in estuaries and in the lower regions of rivers. During this time some continue to interchange with the sea and with other rivers while some make forays well upriver, joining the mature fish on the spawning redds. Note that nearly as many tagged North Esk sea trout were recaptured in the large estuary of the River South Esk, a few km further south. The North Esk has only a short, narrow estuary that is perhaps not suitable for overwintering.

Repeat sampling of five sea lochs in Argyll indicated that finnock return to sea lochs after a similar period at sea and congregate in the sea lochs through the summer and winter months (Pemberton, 1976a). Some finnock migrate into freshwater but trapping data would suggest these are only occasional, brief forays (Pemberton, 1976a). Finnock from the River Axe, Devonshire, also begin to return to the estuary within two months of migration however, according to trap data, most of these finnock do not return downstream to the estuary and the sea until the following spring (Potter; in Le Cren, 1985).

The general pattern for finnock in the U.K., therefore, is to spend the summer moving in and out of estuaries and to then overwinter in fresh or brackish water. The behaviour of finnock while in freshwater is even less well understood. Shoals of finnock appear in the River Tummel, 60 km from the estuary of the River Tay, Perthshire, during December and January but may be present for only a few days and do not appear every year (pers. obs.). More information regarding the behaviour of finnock overwintering in freshwater might provide greater insight into the reasons for this behaviour pattern.

3.1.1.2 Why are finnock important?

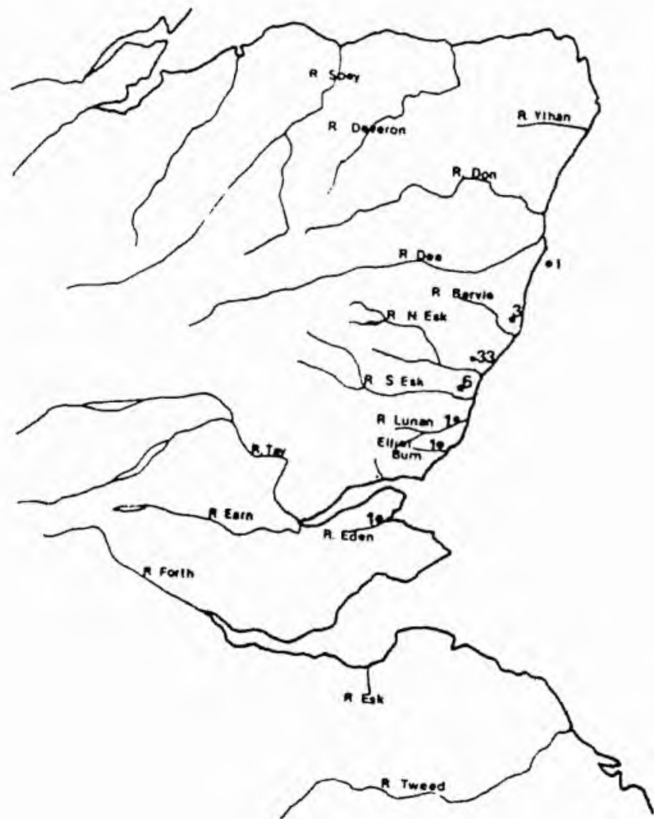
Traditionally, while finnock may have constituted a significant numerical proportion of sea trout caught on the west coast, larger, older sea trout produced most of the eggs and juveniles. In recent years, however, the mean size of mature sea trout in many west coast systems has decreased (Walker, 1994a).

Figure 3.1a

Figure 3.1b

Figure 3.1a Recapture sites of finnock tagged as smolts leaving the River North Esk during 1976 - 1980. The numbers beside river names refer to the number of recaptures. Total number smolts tagged = 12300. Note that other smolts were recaptured as adult sea trout in the years after tagging. From Pratten and Shearer (1983b).

Figure 3.1b Recapture sites of finnock tagged as finnock in the River North Esk during 1976 - 1980. The numbers beside river names refer to the number of recaptures. Total number finnock tagged = 2189. Note that other finnock were recaptured as adult sea trout in the years after tagging.
From Pratten and Shearer (1983b).



R Spey
R Deveron
R Ythan
R Don
R Dee
1
R Bervie
3
R N Esk
33
R S Esk
6
R Lunan
1
Elliot Burn
1
R Tay
R Earn
1
R Eden
R Forth
R Esk
R Tweed

1
R Spey
R Ugie
R Ythan
1
R Deveron
R Don
5
R Dee
R Cowie
1
1
R Bervie
20
R N Esk
77
R S Esk
61
R Lunan
8
Elliot Burn
2
1
R Tay
R Earn
R Eden
10
R Leven
1
R Forth
1
R Tweed

Smaller females produce fewer, smaller eggs so potential recruitment to the juvenile biomass is decreasing. Furthermore, smaller eggs result in smaller alevins with less yolk deposits which are, as a result, less well provisioned to survive periods of starvation (see Section 2.1). Thus, fewer eggs are being deposited and the resultant offspring may have a reduced survival rate. Finnock, and the survival of sea trout through this stage to subsequent maturity, are now far more important in terms of sea trout recruitment since there is no buffer of larger spawning adults to contribute to recruitment. It is imperative, therefore, that we increase our understanding of the causal factors, costs and benefits of this enigmatic behavioural strategy to assist fishery managers in attempting to regenerate depleted stocks. The present chapter will investigate the behaviour of finnock in freshwater in order that a better understanding of overwintering behaviour may highlight specific areas to be investigated with regard to the cause of this behaviour.

3.1.2 Methods of studying fish movements in the field

3.1.2.1 Mark-recapture techniques

Traditionally, studies of fish movements have employed mark-recapture techniques (Kipling and Le Cren, 1984). The fish are first captured, marked in some visible manner, released and then recaptured at a later date. This provides information regarding the distance travelled and the growth rate, provided there is sufficient time between capture and recapture, and that individual fish can be identified.

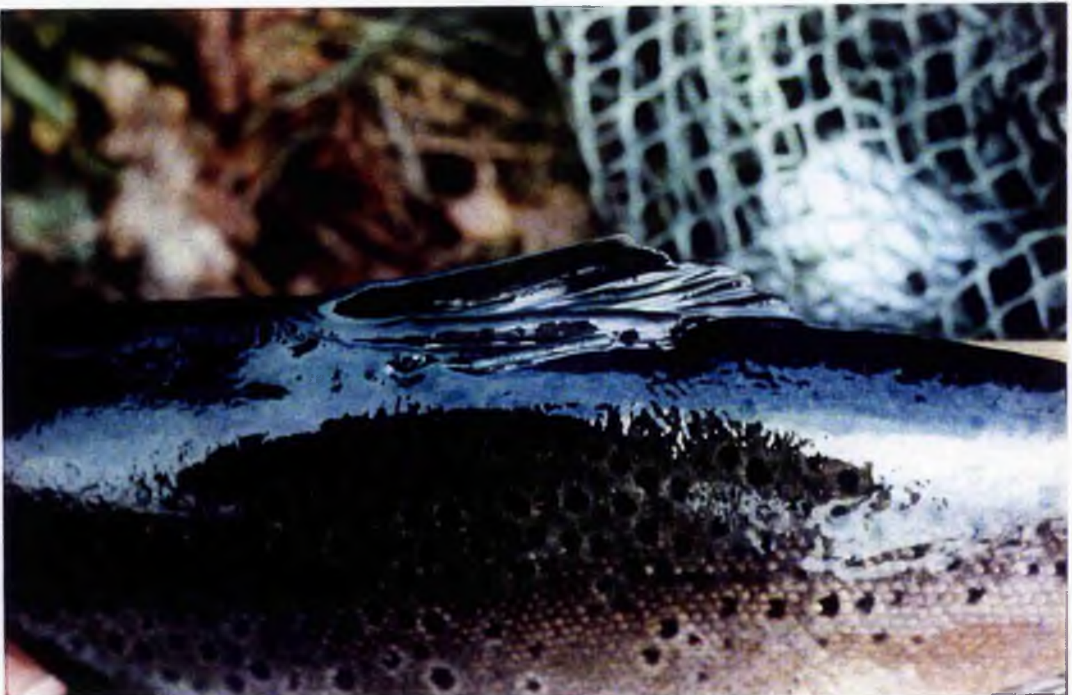
Methods of marking fish can be divided into two groups, depending upon whether the individual identity of the fish is a requirement of the study. Where such information is not necessary, the removal of a fin, typically the adipose fin, or the insertion of a visible dye at the base of one of the ventral fins is most often used to mark each fish. The removal of a fin such as the adipose is reported not to affect the subsequent growth or behaviour of the fish (Kipling and Le Cren, 1984), although the loss of the adipose for maturing male salmon, in which it increases in size and is a secondary sexual characteristic, may have some effect. If it is necessary to identify individual fish at a later date, each fish can be either externally tagged with a coded tag (Plates 3.2 and 3.3), internally tagged with a coded wire (Miles *et al.*, 1985) typically in the nose or visible implant tag in the transparent adipose eyelid tissue posterior to the eye (Haw *et al.*, 1990), or marked with a freeze-brand number tattoo applied in the same area. The externally mounted tags are printed with a unique

Plate 3.2

Plate 3.3

Plate 3.2 Sea trout smolt tagged with numbered Carlin-type tag attached through the dorsal fin rays.

Plate 3.3 Adult brown trout tagged with numbered Floy-type tag attached through the dorsal fin rays.



number to identify the fish and instructions for the return of the tag. The inserted tag or the freeze brand are very small and should not affect the behaviour of the fish but, conversely, this makes them difficult to spot by untrained fishermen who are often the major source of tag returns.

The advantages of such methods are that since each tag is relatively inexpensive, large numbers of fish can be marked with limited expenditure and the small size of modern tags and the use of implanted tags minimizes the possible tagging effects on the subsequent behaviour, growth and mortality of the fish.

However, there are some disadvantages. The major problem inherent in all mark-recapture methods is obtaining sufficient numbers of returned tags to allow conclusions to be made regarding the behaviour of the fish. A study of sea trout movements from the River North Esk, Angus between 1976 and 1980 found that only between 1.3% and 6.2% of tagged smolts were recovered using a variety of recapture methods, including commercial netting operations and rod and line (Pratten and Shearer, 1983b). Thus, very large numbers of fish must be marked initially. Pratten and Shearer tagged between 1089 and 6134 smolts annually. The proportion of recaptured fish is affected by the subsequent mortality of tagged fish and this mortality is related to the size of the fish when tagged. For example, the ranges of percent recaptured from fish tagged in the North Esk were 1.9 - 7.6, 5.5 - 10.2 and 15.8 - 21.6% of those tagged as smolts, finnock and adult sea trout. Another disadvantage is that, rather than reflecting the true distribution of tagged fish, the pattern of recaptures may reflect the distribution of fishing effort, e.g. the lack of sea trout recaptures in the open sea may well reflect the lack of fishing effort in this area rather than the lack of fish. Furthermore, recapture provides no information regarding the movements of the fish before recapture other than an estimate of the distance travelled over a known period of time.

3.1.2.2 Use of telemetry to study fish movements

The development of radio and acoustic tag technology has provided the opportunity to continuously track fish (Table 3.2) and a variety of other animals including crustacea, rodents, birds and mammals (see examples in Priede and Swift, 1992) thereby avoiding the problems noted above.

The basic principles of telemetric tracking remain similar to those of mark-recapture studies. The fish must still be captured, a tag attached and the fish released again.

Table 3.2

Table 3.2 Examples of studies of fish movements using radio and acoustic transmitters.

Species	Life history stage	Environment studied	Type of Tag	Objectives	References
Atlantic salmon (*Salmo salar*)	Adult	Coastal	Acoustic	Home river location	Hawkins *et al.* (1979)
		Estuarine / Freshwater	Radio/Acoustic (CART)	Upstream migration	Solomon & Potter (1988)
		Freshwater	Radio	Factors affecting migration	Webb (1989); Laughton (1991)
				Effects of catch and release policy	Walker & Walker (1992)
	Smolt	Estuarine	Acoustic	Seaward migratory patterns	Fried *et al.* (1978); Tytler *et al.* (1978)
Trout (*Salmo trutta*)	Sea trout	Freshwater	Radio	Upstream migration, spawning behaviour	Solomon (1982); Solomon & Storeton-West (1983); Sambrook (1990); Evans (1994)
	Smolt	Estuarine	Acoustic	Seaward migratory patterns	Moore & Potter (1994)
	Resident	Freshwater	Radio	Homing Patterns	Armstrong & Herbert (1997)

Continued over page

Table 3.2 continued

Species	Life history stage	Environment studied	Type of Tag	Objectives	References
Barbel (*Barbus barbus*)	Adult	Freshwater	Radio	Obstructions	Lucas & Frear (1997)
Coho salmon (*Oncorhynchus kisutch*)	Smolt	Estuarine	Radio & Acoustic	Seaward migratory patterns	Moser *et al.* (1991)
Bass (*Dicentrarchus labrax*)	Juvenile	Estuarine	Acoustic	Obstructions	Moore *et al.* (1994)

However, from then on the position of the tagged subject can be tracked continuously or periodically, either manually or automatically. The first tags to be used in the study of fish movements were designed to emit pulses of sound which were located by scientists using hydrophones (Mitson and Storeton-West, 1971). Acoustic signals are the only carrier able to travel any significant distance through saline waters but these acoustic tags have some limitations in their use. Acoustic signals do not travel well through air so tracking has to be done on water, requiring a large team of personnel and/or equipment and is, therefore, expensive in time and money. In addition, although acoustic tags are suitable for following fish in large bodies of open water and have been used for several studies of adult salmon (Stasko, 1975; Potter, 1988) and smolt migrations through estuaries (Fried *et al.*, 1978; Tytler *et al.*, 1978; Moser *et al.*, 1991; Moore and Potter, 1994). Acoustic signals can be severely attenuated by air bubbles and turbidity in streams so are not suitable for tracking fish in shallow freshwater systems.

An alternative to acoustic tags is the use of radio-transmitters, typically using VHF frequencies (wavelengths 1 m to 10 m) and unique pulse rates. Radiowaves from a submerged transmitter emanate from the surface of the water above the fish and can be received by a conventional aerial in air. Radio signals travel well through freshwater and air so tracking can be done from the riverbank or even from a plane, providing the signal is strong enough. Bearing is determined by listening for peaks or nulls in the signal level, depending upon the antenna design (Priede, 1992). The radio-tag identifies the individual by virtue of the transmitter frequency and the signal pulse rate. Radio transmitters use relatively little power so tags can be either large with a much longer battery life than accoustic tags (1 year), or very small to allow tracking of smaller species and of juvenile fish. However, radio signals are rapidly attenuated in saline waters so are not appropriate for tracking fish in brackish or marine environments.

A combined accoustic and radio transmitter, the CART tag, has been designed which offers the ability to track fish within the marine and estuarine environments for a short period of time and then continue to track the fish as they migrate into freshwater (Solomon and Potter, 1988). Clearly, this is of most use in the study of anadromous fish returning to spawn in freshwater. However, the large power requirements of such tags precludes their use in the study of smaller fish, at the present time.

3.1.2.2.1 Methods of tag attachment

Acoustic and radio tags can be attached to the fish in one of several ways. Tags can be attached to the external surface of the fish and are typically sutured in the area of the dorsal fin, an area where mark-recapture tags are also typically mounted (see Plate 3.2). This method has the advantage of allowing the use of an external antenna which greatly increases the detectable range in small tags. In addition, the use of dissolvable suture will allow the tag to fall off the fish after a predetermined amount of time. However, early developed externally mounted tags have been criticised for impeding movement, affecting growth rates, causing irritation and infection at the locus of attachment (Roberts *et al.*, 1973 a,b; McCleave and Stred, 1975; Greenstreet and Morgan, 1989) and increasing drag on the fish (Haynes, 1978).

A second method of attachment is to place the transmitter into the alimentary canal of the fish. This method, which is typically used for large salmonids, is not thought to adversely affect the behaviour of the fish (Evans, 1994). However, it is not recommended for fish that are expected to feed during the period of the study as this may result in regurgitation of the tag (Solomon and Storeton-West, 1983). In addition, the presence of a transmitter in the stomach of Atlantic salmon parr during the autumn severely reduces the food intake (Armstrong and Rawlings, 1993). This may be due to feeding inhibition through pressure sensors indicating the stomach is full (Toates, 1981). One point not previously considered is the possible inhibitory effects of stomach distension on drinking rate in tagged fish which return to seawater (Section 2.1.4.4).

The most recently developed method is to surgically place a small tag within the peritoneal cavity of the fish (Moore *et al.*, 1990). As with the intra-gastric method, this does not result in external protrusions but the size, and therefore detection range and lifespan, of the tag are limited. Furthermore, this method does not preclude host rejection of the tag, presumably through the body wall: the site of the expulsion is not always apparent (Moore *et al.*, 1990).

The use of radio/accoustic tags to study fish movements has several advantages over mark-recapture methods (Clarke and Gee, 1992). First, whereas fish tagged by any method are subject to the stress of capture and handling, radio-tagged fish do not subsequently have to enter a trap or pass over a weir to be recorded moving. Both of these obstructions may affect the behaviour of the fish. Second, information is not

limited to a single location (recapture site) in the river system. Third, direct assessments of stock management parameters, such as survival to spawn and maximal estimates of illegal catch, can be made independent of reporting of recaptures.

However, there are also some disadvantages to the use of radio or accoustic tags. Tags are expensive so most studies rely on a small number of tracks to provide qualitative rather than quantitative data. Second, whatever the method of study, it is not supposed to influence the behaviour of the fish which is being studied. There are inevitably questions as to the effects that tags have on the behaviour of the fish. However, a variety of studies using different fish species and different types of tags have observed tagged fish behaving similarly to untagged fish. Examples include: video footage of acoustic tagged saithe (*Pollachius virens*) shoaling normally amongst untagged fish around a small reef in Loch Ewe (Glass *et al.*, 1992); radio-tagged adult Atlantic salmon tracked to successful spawning in November after being tagged in July (Walker and Walker, 1992); no marked differences observed between the spawning behaviour of radio-tagged and untagged sea trout (Evans, 1994); radio-tagged finnock were not captured by a seal observed feeding within several metres of their location (the present study).

3.1.3 Aims of this part of the study

Given the advantages of radio and accoustic transmitters in the study of the behaviour of fish, they have become increasingly popular for studying a variety of fish behaviours (see examples in Priede and Swift, 1992). Despite the growing use of such transmitter technology in the study of fish behaviour, however, there have been relatively few studies of the movements of sea trout in freshwater (Solomon, 1982; Solomon and Storeton-West, 1983; Sambrook, 1990) and while some finnock have been tracked, no studies have focused directly on this part of the sea trout life cycle. Thus, whereas juvenile trout in freshwater and maturing adult sea trout returning to spawn have been extensively researched, the present study provides an opportunity to closely examine a less well understood, but no less important, aspect in the life cycle of sea trout. Therefore, the aims of this chapter were to use radio-tracking techniques to determine whether finnock migrations into freshwater were transitory or more committed and if long tracks could be obtained, to establish whether finnock behaved in a manner similar to upstream migrating maturing anadromous salmonids or freshwater-resident brown trout.

3.2 Materials and Methods

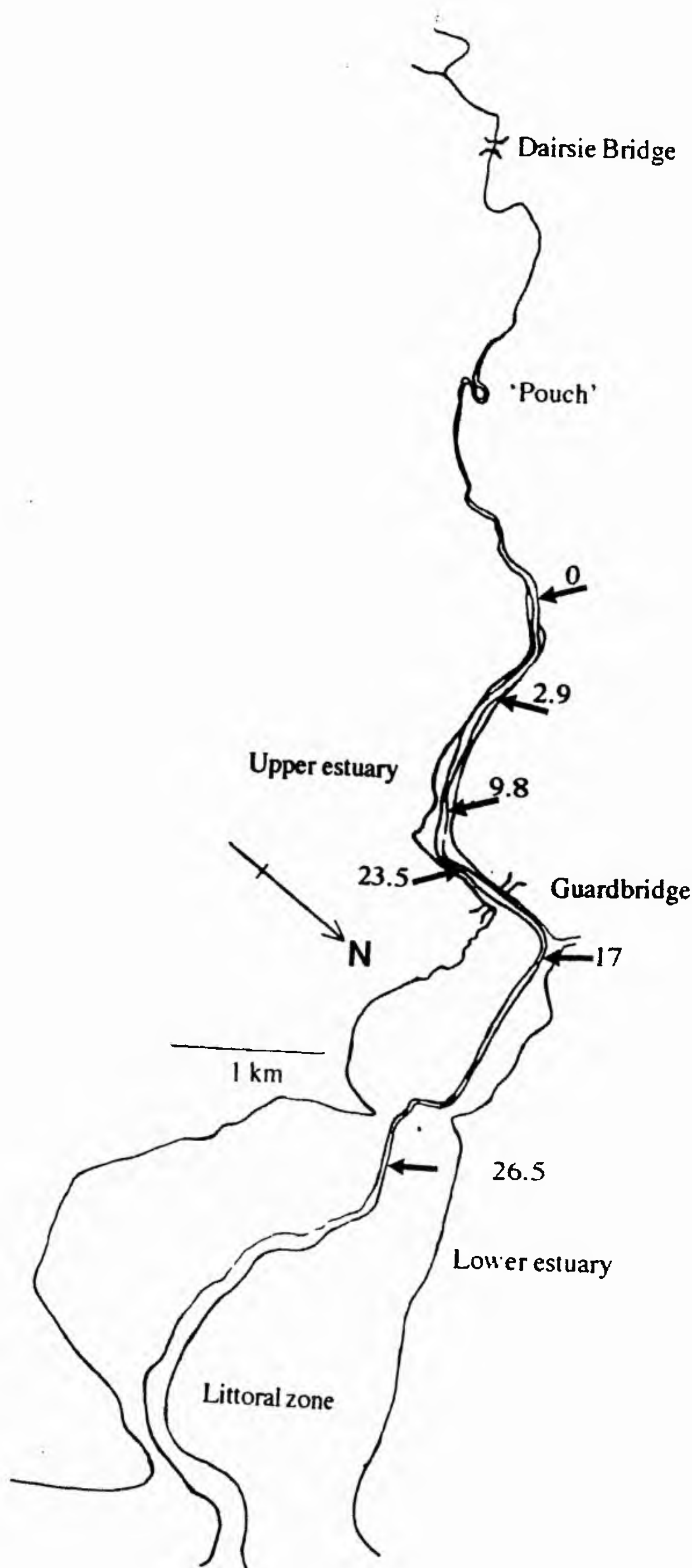
3.2.1 The study site

The following description is based upon information provided in Tytler *et al.* (1978) and Anon (1994c). The River Eden rises in the Lomond Hills at an altitude of 500m and flows eastward to enter the North Sea 4.5 km north of St Andrews and 8 km south of the mouth of the River Tay (Figure 2.1). The river and its tributaries drain a catchment area of some 320 sq.km and have a mean annual rainfall over an area of 307 km^2 of 790 mm (1967 - 1990). The major land use is arable farming, with 76% of the catchment providing prime agricultural land with fertile soils of freely or imperfectly drained brown forest and alluvial types. Water is abstracted from groundwater, the river and its tributaries in order to irrigate arable crops. A major feature of the water chemistry is the relatively high levels of nutrients consisting, in particular, of nitrates from agriculture and phosphates from sewage discharge. This results in extensive summer plant growth which often leads, in conjunction with areas of sluggish flow, to a large range in the levels of dissolved oxygen. An intensive study (1990) of the freshwater invertebrates as part of the National Survey of River Water Quality found invertebrate diversity and densities to be well below predicted levels, despite the results of chemical analyses of the water being regarded as satisfactory. Fish species found in the system are salmon, trout (resident and migratory), eel, minnow, stickleback, loach, brook and sea lamprey and flounder. Water quality does not appear to exclude salmonids from any part of the river although the Dura Den falls (Plate 2.2) on the Ceres Burn and the weir at Gateside on the main river both represent physical barriers for the upstream movements of salmon and migratory trout.

The highest point to which ordinary spring tides flow is 8.75 km inland from the mouth of the river and the estuary is split into two sections (Figure 3.2). The lower estuary (4.7 km) is characterized by broad and extensive mud flats where the salinity exceeds 25 ppt at high water. The upper estuary extends a further 3.2 km inland and has a narrow river channel (max. width 200 m) with a relatively small littoral zone composed of mud banks and flats. At low water the upper channel contains pure freshwater but at high water there is a salinity gradient of between 23.5 ppt at the lowest point (Guardbridge) to zero at a point 1.5 km further inland (Tytler *et al.*, 1978).

Figure 3.2

Figure 3.2 Map of the lower reaches of the River Eden, Fife. Salinities in the upper estuary were taken at High Water whereas those taken in the lower estuary were at 3 hours after High Water. The shaded areas represent the littoral zone of the estuary.
After Tytler *et al.* (1978).


Dairsie Bridge
'Pouch'
0
2.9
Upper estuary
9.8
23.5
Guardbridge
N
17
1 km
26.5
Lower estuary
Littoral zone
'St Andrews Bay'

3.2.2 Fish capture

Two batches (I and II) of finnock were tracked between September and November, 1994. Finnock for Exercise I were captured by rod and line using extremely light tackle (3 lb nylon, barbless No. 14 hooks) either by the author or members of the Stratheden Angling Club and the Eden Angling Association. Captured finnock were handled as little as possible and were held in a knotless mesh keepnet set in the river until tagging. All finnock were captured in one pool at the head of the tidal reach (water level is influenced by the tide but there is no saline influence).

As the angling season was closed at the time of Exercise II, finnock were captured by electrofishing using equipment described in Chapter 2. The river was electrofished from Dairsie Bridge (Figure 3.2) downstream over some 1400 m on 4 November. Electrofishing was carried out on foot with stop nets at the bottom of stretches to capture any fish which were deflected downstream ahead of the gear (although none were caught). Six finnock were collected, along with several resident brown trout. One finnock was subsequently killed as it had two dorsal puncture wounds, most probably stab wounds from a heron (*Ardea cinerea*). Six adult salmon (three pairs) were observed but not captured.

Finnock were held in a holding box submerged in the 'Pouch', a U-shaped pool at the head of the tidal influence (Figure 3.2) over the weekend and checked periodically. A further electrofishing survey took place on 7 November to attempt to increase the number of finnock. The remainder of the river down to the head of the 'Pouch' was fished but no finnock were caught. The fish were tagged in the late afternoon of 7 November but were held overnight and released the following morning (1130 hrs) as only a short period of daylight tracking would have been available the previous afternoon.

3.2.3 Radio-tagging technique

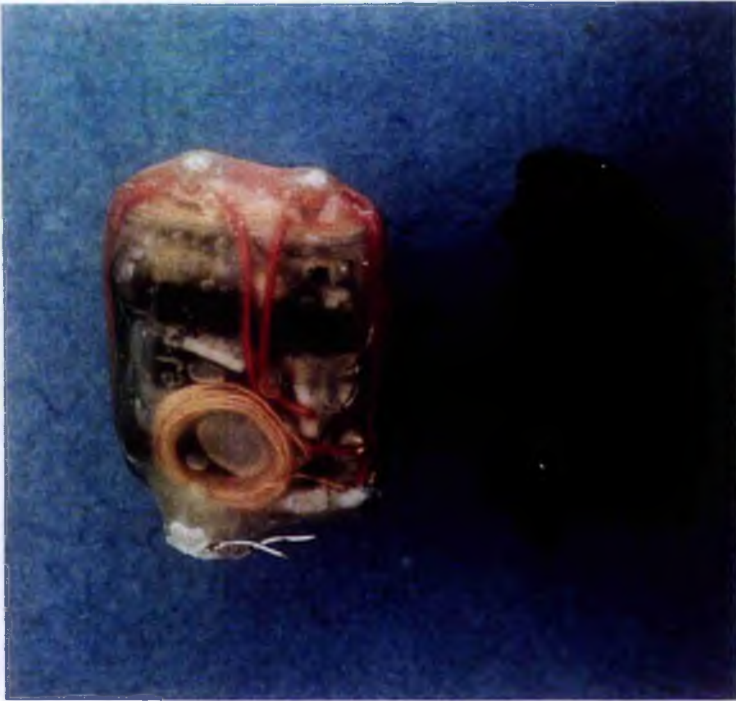
Finnock were tagged using miniature, externally mounted radio transmitters (Biotrak, Wareham, U.K.) (Plate 3.4). The transmitter circuitry and battery were sealed in black liquid plastic. Each tag transmitted a signal pulse on a slightly different radio frequency in the 173 MHz band. Tags had an effective life of up to 30 days and a maximum range of 200 m, depending on the bank-side terrain and the position of the tagged fish. All finnock were tagged under the provisions of the Home Office Personal Licence, No. 60/01186.

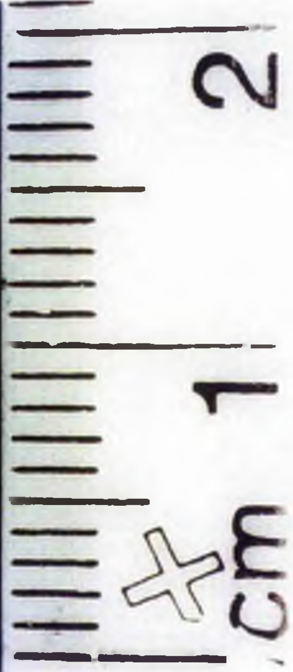
Plate 3.4

Plate 3.5

Plate 3.4 Biotrak radiotags similar to those used in the present study. The tag on the left is encased in clear plastic resin to show the internal circuitry whereas the tag on the right is encased in black resin, similar to that used in the present study.

Plate 3.5 Modified Yaesu receiver and folding antenna for locating radiotagged fish.





3.2.3.1 Tracking Exercise I

Three finnock were transferred to the licensed facilities of the Gatty Marine Laboratory for attachment of radio tags. Finnock were individually anaesthetized in a solution of 2-phenoxyethanol (0.5 ml.l $^{-1}$) until they lost the ability to maintain dorso-ventral position and opercular movements were slow and shallow (usually 2 - 3 min). Fork length and wet weight were noted and three scales were removed by fine nosed forceps from an area between the dorsal fin and the lateral line for ageing. Radio transmitters (1.4 g in air, 13 x 8 x 5 mm) were anchored by black braided surgical silk suture (EP 3 gauge, Davis and Geck, Hampshire, U.K.) ventrally to the dorsal fin, as with the carlin tag (see Plate 3.2), with a wire antenna extending towards the tail. The time to complete the tagging procedure was typically less than 60 s. Finnock were then allowed to recover from anaesthesia before transport back to the river.

Finnock were released from the point of capture in the manner suggested by Moore *et al.* (1990). Finnock were placed in a holding box (mesh cage) placed within the release pool in an area of slack water. After 60 minutes, the holding box was gently tipped onto its side and the lid removed. The finnock were then allowed to swim from the box of their own volition.

3.2.3.2 Tracking Exercise II

Five finnock were tagged (7 November) in a portable caravan sited within 100 m of the release point using the procedures described above, except that wet weight was not recorded. The release procedure was as before but the release point was the mid point of the 'Pouch'.

3.2.4 Tracking Methods.

The movements of tagged finnock during Tracking Exercise I were followed on foot using a modified Yaesu receiver (type FT 290R, pre-tuned to the tag frequencies) with a half wave length, folding aerial (Plate 3.5) (Solomon and Storeton-West, 1983). The position of each tagged finnock was recorded in relation to bankside features (pools, corners, trees, fences) and subsequently plotted on photocopy reprints of Ordnance Survey maps of the area (1914 Edition, 1/2500, sheet nos. VIII. 7, 10, 11, 13 and 14) to calculate distance travelled.

Movements of the finnock were tracked with high temporal frequency during the initial daylight periods after release (located every 5 - 15 min) and subsequently their position was recorded at least daily.

The movements of tagged finnock in Tracking Exercise II were also followed on foot but, in addition, an automatic listening station (ALSTN) was sited at the Pouch. This ALSTN was a prototype (Biotrak, Wareham, U.K.) on loan from Dr A.D.J. Johnstone of the Marine Laboratory, S.O.A.E.F.D., Aberdeen. The ALSTN consisted of a signal processor unit, a palmtop computer (Sharp PC3100), a disc drive and a receiver. This system is designed to continuously monitor up to 14 separate antennae and record the presence of several radio tags at each. At 10 min intervals the ALSTN searches through the pre-set radio frequencies at each antenna in turn and makes a record of any tag signal detected.

Thirteen antennae were located at approximately equi-distant intervals (20 - 30 m) around the Pouch (see Figure 3.12 below). The ALSTN antennae were shorter than the portable antenna used for manual tracking and, consequently, had a shorter detection range. This ensured that tagged fish would not be recorded on more than two antennae simultaneously. The purpose of this array was twofold. First, to detect any radio-tagged finnock which were leaving the river to move towards the estuary (note that this technology would not allow an assessment of whether any downstream migrating finnock remained in the estuary or migrated to sea) and, second, to monitor the movements of finnock within the Pouch with more temporal and distance detail including 24 hour monitoring (which was not possible on foot for reasons of safety).

The ALSTN was housed within a caravan situated close to the 'Pouch' and occupied during hours of darkness for reasons of security.

3.2.5 Environmental data

River flow was measured at the Tay River Purification Board (TRPB, now SEPA) monitoring station near Dairsie Bridge (OS grid reference NO 415158) and presented as daily mean flow rate in $m^3.s^{-1}$. Water temperature was automatically recorded every 15 min at the same location and provided by F.F.L., S.O.A.E.F.D., Pitlochry. Water temperature data are presented as mean daily temperature (°C).

3.3 Results

Eight finnock were radio-tagged and released into the River Eden during the period September to November 1994. Details of each fish are given in Table 3.3. For ease of presentation the fish will be separated into the two batches released on different dates. Finnock from Period I will be referred to as F1-F3 while those of Period II will be referred to as F4-F8.

3.3.1 Tracking Exercise I - 9 September onwards

3.3.1.1 Initial movements after release

Figure 3.3 depicts the release pool (Plate 3.6) and the Pouch above (Plates 3.7 and 3.8) with five areas (A - E) which were occupied by one or more finnock during the first daylight hours post-release. The movements of the finnock during this time will be described using these areas as reference points.

All three finnock (F1-F3) remained together initially after release at 1140 hrs. For the first 90 minutes after release, the finnock remained in the release pool, generally under cover of a large, overhanging tree but with one brief foray across the pool some 40 minutes after release, before returning to the cover. Ninety minutes after release, two finnock (F2 and F3) moved up over a shallow weir into the 'Pouch', prior to the rise in water from the incoming tide, while F1 stayed in the release pool and did not enter the 'Pouch' for another 120 minutes (210 minutes after release) just prior to the tide entering this pool. During this latter period F1 continued to periodically move around the release pool.

Although the finnock entered the 'Pouch' on two occasions separated by 120 minutes, their initial movements within this pool were similar. They moved quickly upstream to reach Areas D (F1 and F3) or E (F2) within five minutes but soon returned downstream to areas A and B where they remained for some time, except for occasional, brief forays to Areas C and D.

The tide entered the 'Pouch' some five hours after the finnock were released but this did not appear to influence the behaviour of the finnock. However, as the water level fell again some 50 minutes later, F1 and F3 both moved upstream to Area D. F1 remained for 60 minutes before returning to Area B while F3 returned to Area B within 10 minutes. F2 did not move at this time but did move up to Area E briefly

Table 3.3

Table 3.3 Details of radio-tagged finnock. Age: X.Y+ where X denotes the age at smolting and Y the post-smolt age. Thus, at least seven of the trout smolted after two years in freshwater, and all were finnock (.0+). R = replacement scales, the smolt age of this fish cannot be determined.

	Date released	Finnock code	Length (mm)	Weight (g)	Age
Tracking Exercise I	9 October	F1	291	216	2.0+
		F2	275	176	2.0+
		F3	280	186	2.0+
Tracking Exercise II	8 November	F4	309	na	R.0+
		F5	300	na	2.0+
		F6	296	na	2.0+
		F7	303	na	2.0+
		F8	313	na	2.0+

Figure 3.3

Figure 3.3 Diagram of the release pool and the 'Pouch' pool upstream. Areas A to E are referred to in the text as areas where radio-tracked finnock from Tracking Exercise I were located during the first day after release.

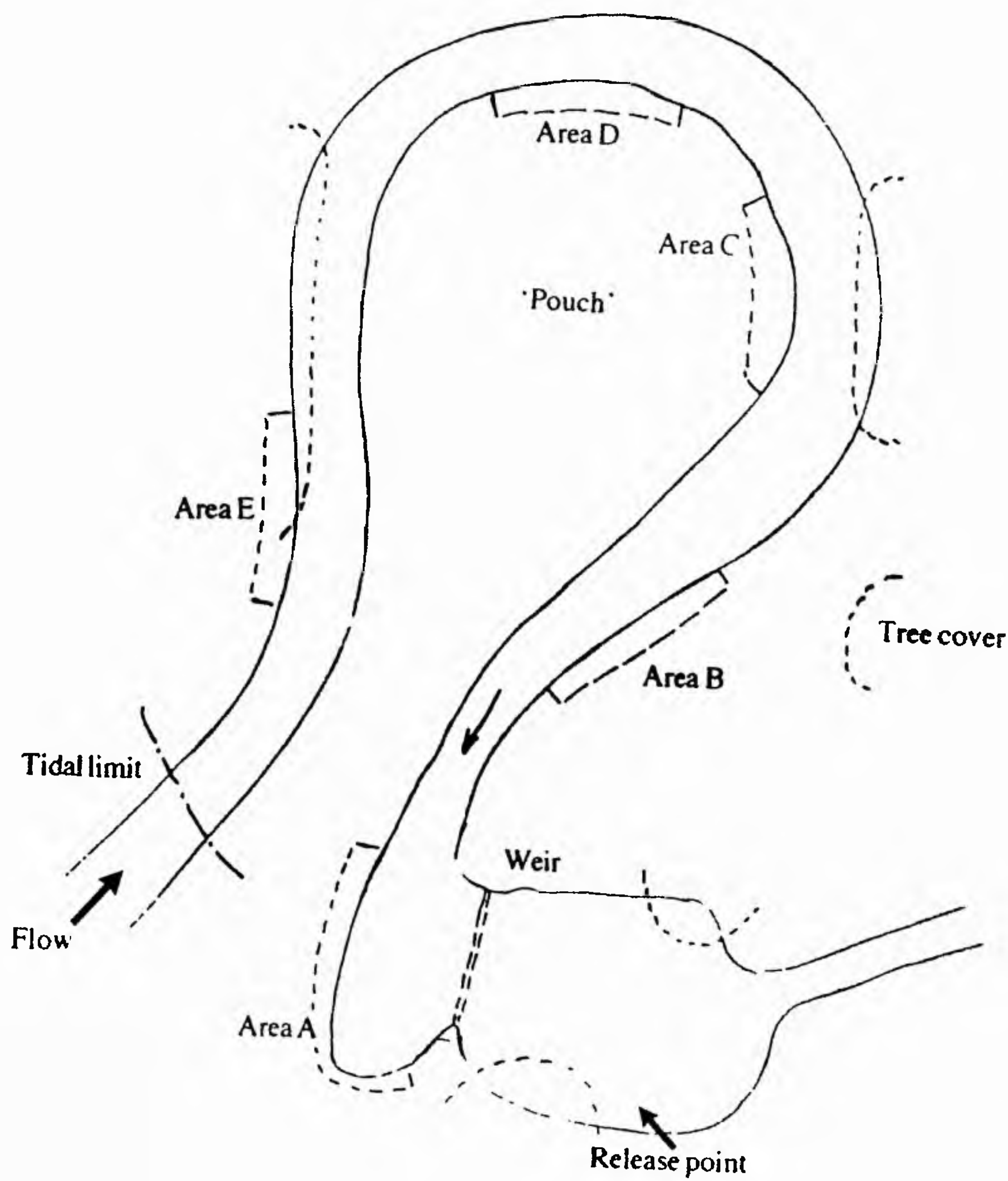

Area D
Area C
'Pouch'
Area E
Tree cover
Area B
Tidal limit
Weir
Flow
Area A
Release point
100 m

Plate 3.6

Plate 3.6 Pool where Finnock F1 - F3 were captured and subsequently released from in Exercise I. The weir and the tail of the 'Pouch' are located in the centre right of the frame.

Plate 3.7

Plate 3.7 Lower reaches of the 'Pouch' with Area A in the foreground. The weir to the release pool is out of frame to the centre right.

Plate 3.8

Plate 3.8 Areas D and E of the 'Pouch'. The release point for Tracking Exercise II is in the centre of the frame.

before dropping to Area D some 30 minutes after the tidal influence had diminished. F2 remained at Area D for a further hour before returning to Areas A and B.

3.3.1.2 Subsequent movements

While F2 and F3 were continually located in the Pouch (Areas A to C) until failing light prevented further tracking at 2135 hrs, F1 began to move upstream at 2010 hrs (8.5 hours after release) when it was located in Area C and could no longer be located in the Pouch by 2030 hrs. Subsequent locations of F1 are described with reference to Figures 3.4 and 3.5.

3.3.1.2.1 Finnock 1 (F1)

At 2056 hrs, F1 was relocated in the Top Copse area and had moved further upstream to the top of this area by 2104 hrs. This represented a distance of 480 m travelled in a maximum of 53 minutes indicating an average swimming speed of 0.5 body lengths per second (bl.s $^{-1}$). At 2109 hrs, F1 had dropped 20 m downstream to a deep pool where it remained until tracking ceased at 2130 hrs.

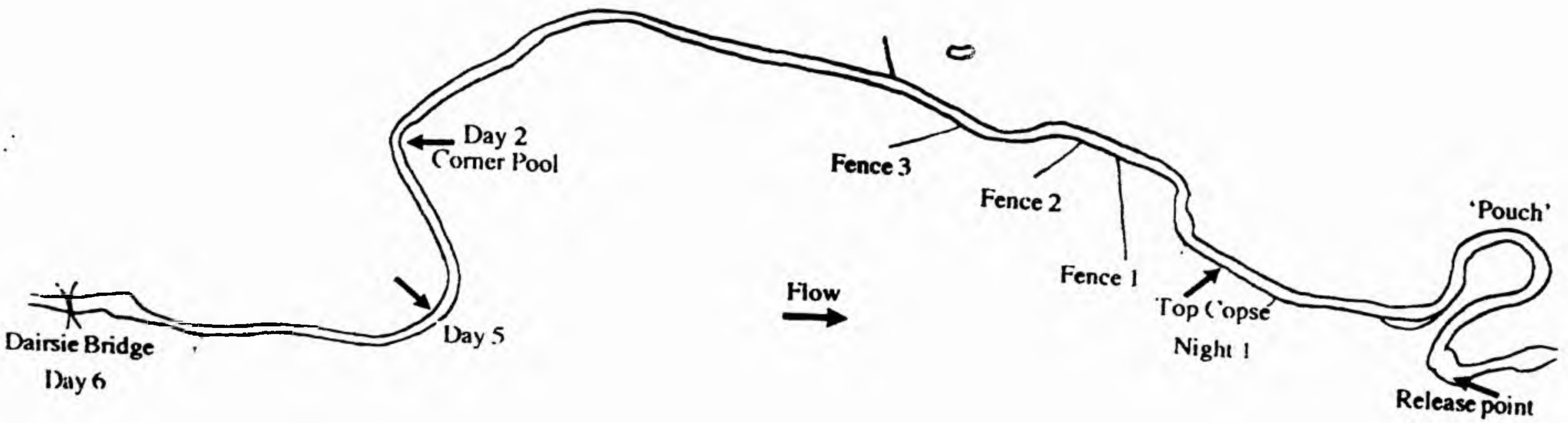
On Day 2, F1 was located at 0707 hrs under the overhanging bank of a corner pool some 1800 m from the release point (marked Day 2, Figure 3.5) (Plate 3.9). F1 remained in this location throughout the day. Its position was checked every five minutes for 100 minutes after first being located (0707 hrs - 0844 hrs), periodically throughout the day and again every five minutes from 2003 hrs to 2118 hrs. F1 remained in this pool for the next two days, typically in the same position, except for the morning of Day 3 when it briefly moved to another overhang some seven metres further up the pool.

On Day 5, F1 had moved a further 170 metres upstream to another overhang in the next corner pool where it was located at 1745 hrs (Figure 3.4, 3.5). However, it did not remain there long and was located in the pool below Dairsie Bridge (Plate 3.10) the following day at 1042 hrs. This represented a total upstream distance covered of 2490 metres in six days since release.

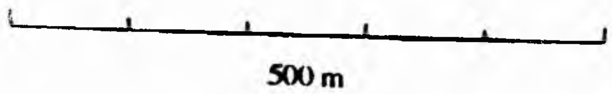
F1 was located in this pool every morning and evening for the next 20 days.

Figure 3.4

Figure 3.4 Map of the lower River Eden with areas where Finnock F1 was located during the tracking exercise.



Day 2
Corner Pool
Fence 3
Fence 2
Fence 1
'Pouch'
Top Copse
Night 1
Release point
Flow
Day 5
Dairsie Bridge
Day 6



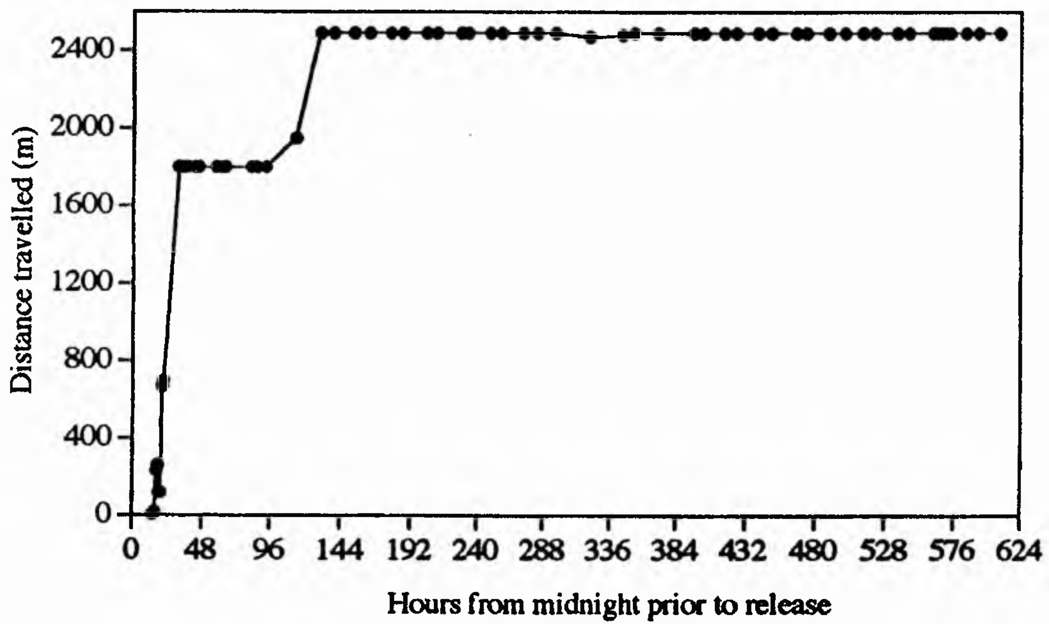
500 m

Figure 3.5

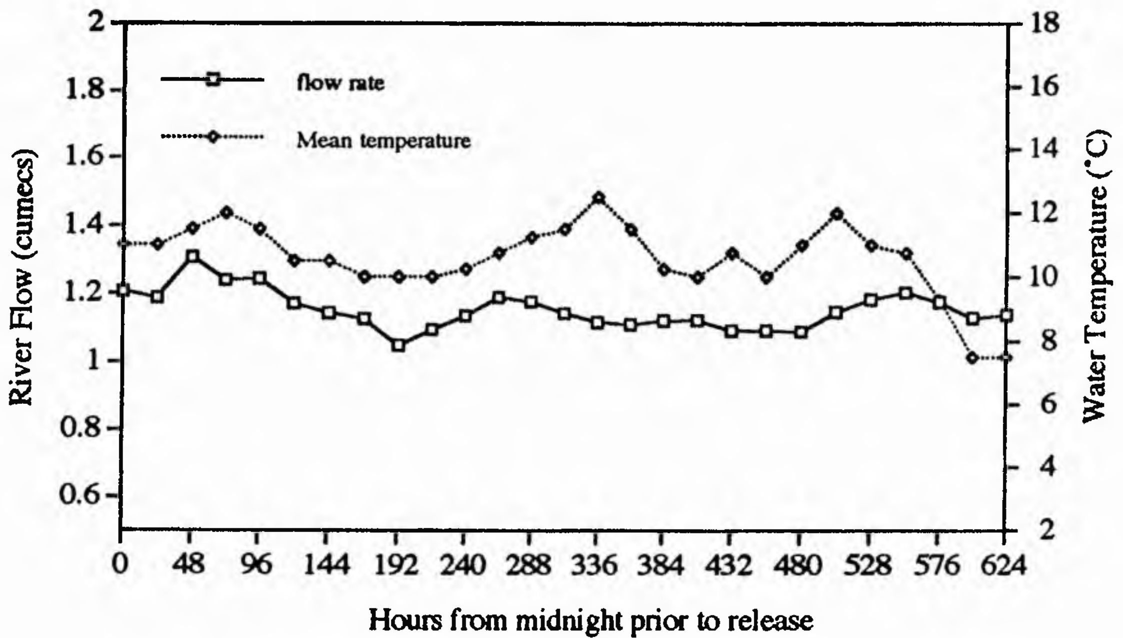
Figure 3.6

Figure 3.5 Movements of Finnock F1 expressed in metres travelled from release point.

Figure 3.6 Mean daily river flow (cumecs.s $^{-1}$) and mean daily water temperature (°C) during the period in which F1 was tracked.



Distance travelled (m)
2400
2000
1600
1200
800
400
0
0 48 96 144 192 240 288 336 384 432 480 528 576 624
Hours from midnight prior to release



flow rate
Mean temperature
River Flow (cumecs)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
Water Temperature (°C)
18
16
14
12
10
8
6
4
2
0 48 96 144 192 240 288 336 384 432 480 528 576 624
Hours from midnight prior to release

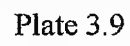


Plate 3.9

Plate 3.9 Corner pool 1800 metres upstream from the release point. F1 was located in this pool for four days.

Plate 3.10

Plate 3.11

Plate 3.10 Pool below Dairsie Bridge. F1 was located in this pool for 20 days, generally in the riffle area below the bridge.

Plate 3.11 Pool near Fence 1 and the Top Copse area in the centre right of the frame. F2 and F8 were both located briefly in this area during the tracking exercises.

While it was generally located in the tail of the riffle area below the bridge, some movement through the pool occurred on some days, e.g. it had dropped to the middle of the pool on Day 14 but had returned to the top of the pool by the morning of Day 15 and was mobile within the pool on the mornings of Days 19, 23 and 25. The tag was subsequently located in this pool for a further 30 days. However, the fish was not captured when this pool was electrofished on 4 November, nor did it move when electrofishing gear was passed very close to its supposed position. This indicated that the fish was no longer there and that the tag must have fallen off and become trapped amongst the rubble on the bottom of the pool. Therefore, the track of this fish was considered to end on the last day when it was recorded to have moved in an upstream direction (Day 25).

3.3.1.2.2 Finnock 2 (F2)

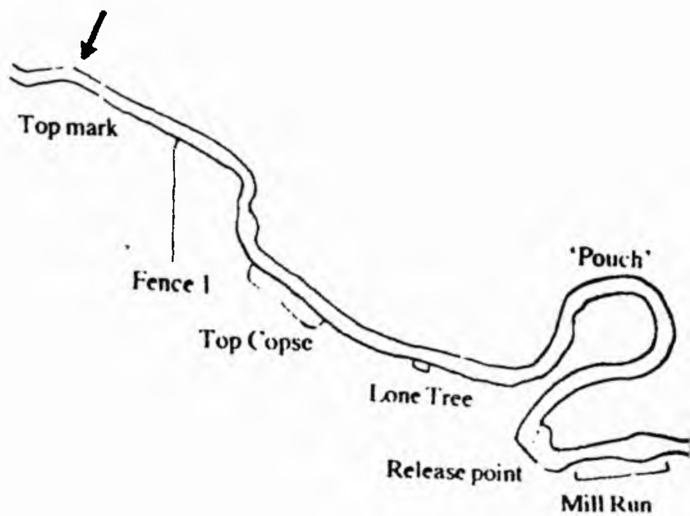
F2 was recorded in the 'Pouch' (Area B) at 2135 hrs on day 1. It moved upstream during the night and was located at the Top Copse, some 713 metres above the release point at 0703 hrs on Day 2 (Figure 3.7, Figure 3.8). It had moved a further 127 metres upstream by 0728 hrs when it was next located at the Top Mark. However, it soon turned back downstream and was subsequently located at Fence 1 (Plate 3.11) (1128 hrs), between the Top Copse and the Lone Tree (1206 hrs), and then at the top of the 'Pouch' (1317 hrs) where it remained until 2115 hrs when tracking ceased.

On Day 3, F2 was initially located in Area A of the Pouch at 0924 hrs but had moved upstream to Area B by 0932 hrs and was still there one hour later. However, this finnock had returned to Area E at the head of the Pouch by 1642 hrs and was still there at 1903 hrs when tracking ceased.

On Day 4 however, F2 was first located in the Mill Run, 120 metres below the release pool at 0932 hrs and was still there at 1001 hrs (the position was checked at 5 minute intervals). At 1516 hrs, F2 had dropped a further 140 metres downstream. At 1928 hrs it was located at the downstream end of Matt's Run, 390 metres from the release point, and was continually tracked as it moved downstream from this point. It reached the Slabs area at 1935 hrs and the Tatty Hole, 730 metres from the release point, at 1954 hrs. From 1928 hrs to this point, the high tide had been stemming the river flow such that there was very little flow in this region of the river. However, the tide turned at 1950 hrs and the downstream flow increased

Figure 3.7

Figure 3.7 Map of the lower River Eden with areas where Finnock F2 was located during the tracking exercise.


Top mark
Fence 1
Top Copse
Lone Tree
'Pouch'
Release point
Mill Run

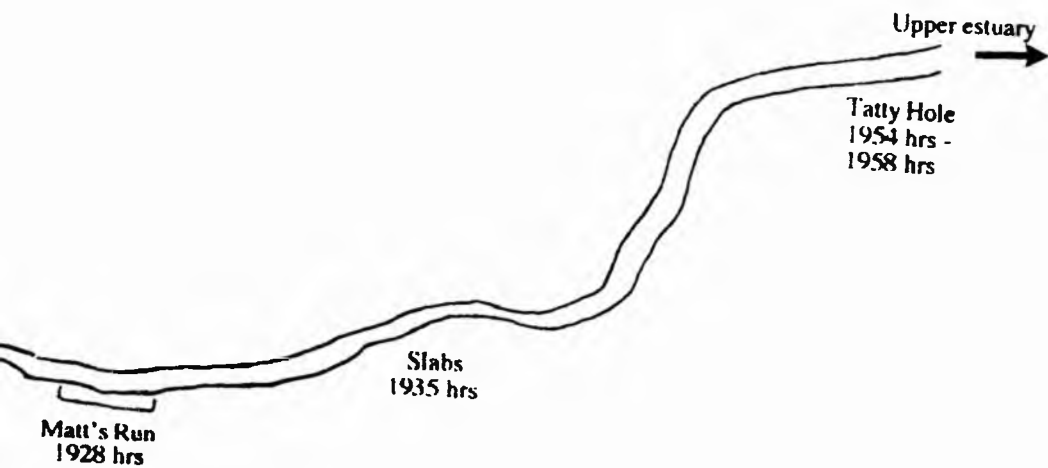
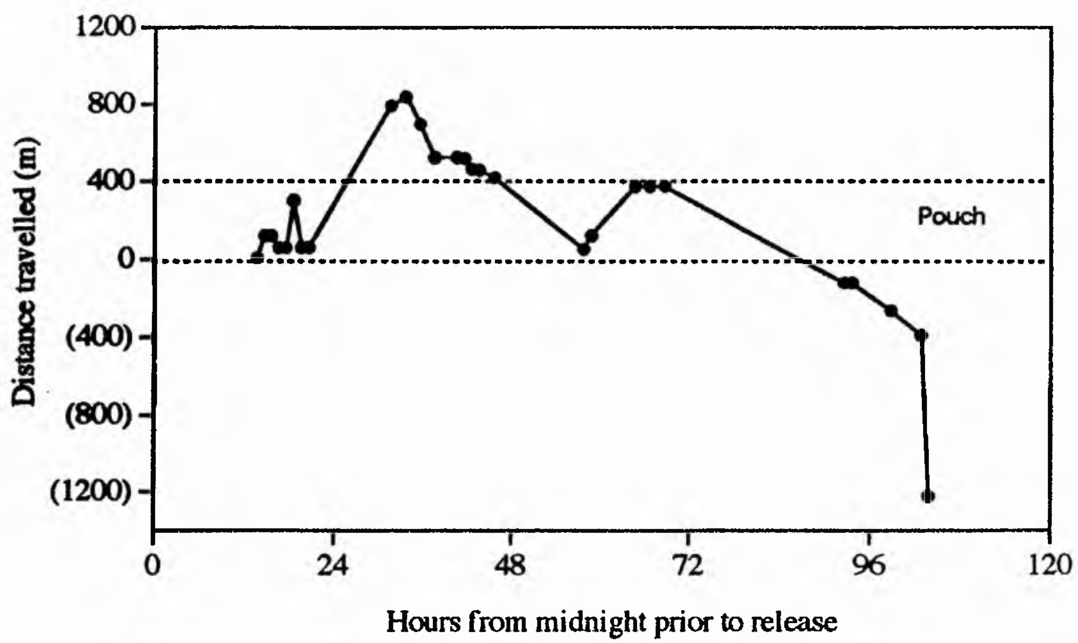

Upper estuary
Tatty Hole
1954 hrs -
1958 hrs
Slabs
1935 hrs
Matt's Run
1928 hrs
500 m

Figure 3.8

Figure 3.8 Movements of Finnock F2 expressed in metres travelled from release point.


1200
800
400
0
(400)
(800)
(1200)
Distance travelled (m)
Pouch
0
24
48
72
96
120
Hours from midnight prior to release

quickly. F2 continued to move downstream, leaving the Tatty Hole at 1958 hrs and was recorded a further 420 metres downstream only seven minutes later. Although the finnock was swimming with the flow of the river, this still represented an average speed of 3.33 $bl.s^{-1}$. F2 was not located again that evening, either downstream from the last location or upstream to the 'Pouch' and was considered to have left the river and entered the estuary (where the saline water would rapidly attenuate the radio signal). The following day, F2 was not located between Dairsie Bridge and Guardbridge, the start of the lower estuary. The 'Pouch' was checked for the presence of F2 daily for the following 21 days and the river was scanned on foot between Guardbridge and Cupar, 10 km from the estuary 10 days after F2 left the river but this tag was not subsequently located.

3.3.1.2.3 Finnock 3 (F3)

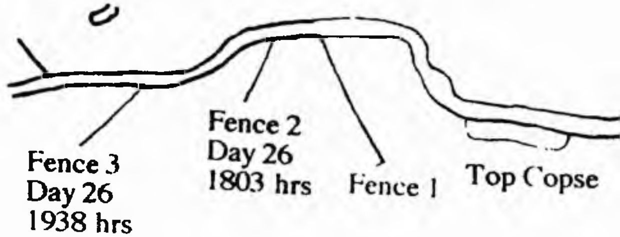
Unlike the other two finnock, F3 remained in the 'Pouch' for an extended period. Its movements therein will be described with reference to Figure 3.9 and distance travelled shown in Figure 3.10. At the end of Day 1, F3 was last located in Area C.

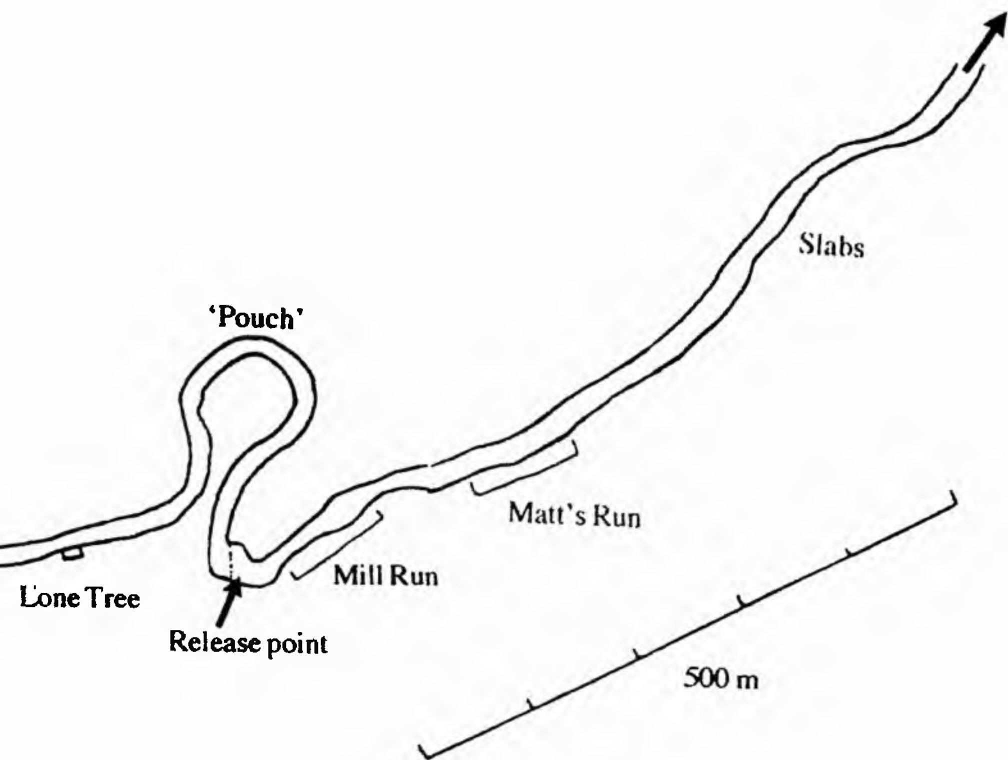
On Days 2 and 3, F3 was periodically located in Areas A to E, but with no apparent relationship to tidal influence or light levels. The position of F3 was then checked every morning and evening. From Day 5 to Day 25, F3 was always located in the lower section of the 'Pouch' but moved occasionally between Areas A and B, although not with any discernible pattern, such as morning and evening, or before and after tidal waters entered the pool. On Day 26, F3 was located in Area A in the morning (0836 hrs) but was not within the 'Pouch' at 1752 hrs. F3 was subsequently located at Fence 2 (Figure 3.9), 892 metres above the release point at 1803 hrs and at Fence 3, a further 150 metres upstream at 1938 hrs when tracking ceased due to failing light.

Overnight, F3 dropped downstream to between Fences 3 and 2 where it was located at 0900 hrs and again at 1734 hrs. However, F3 was not in this area on the morning of Day 28 and was not located between Dairsie bridge and 200 metres below the release point. Neither was it between the 'Pouch' and 4 km upstream during the afternoon. Over the next two days the main river was scanned between the 'Pouch' and 2 km upstream of Cupar but F3 was not located again.

Figure 3.9

Figure 3.9 Map of the lower River Eden with areas where Finnock F3 was located during the tracking exercise.


Flow
Eden Grove House
Fence 3
Day 26
1938 hrs
Fence 2
Day 26
1803 hrs
Fence 1
Top Copse



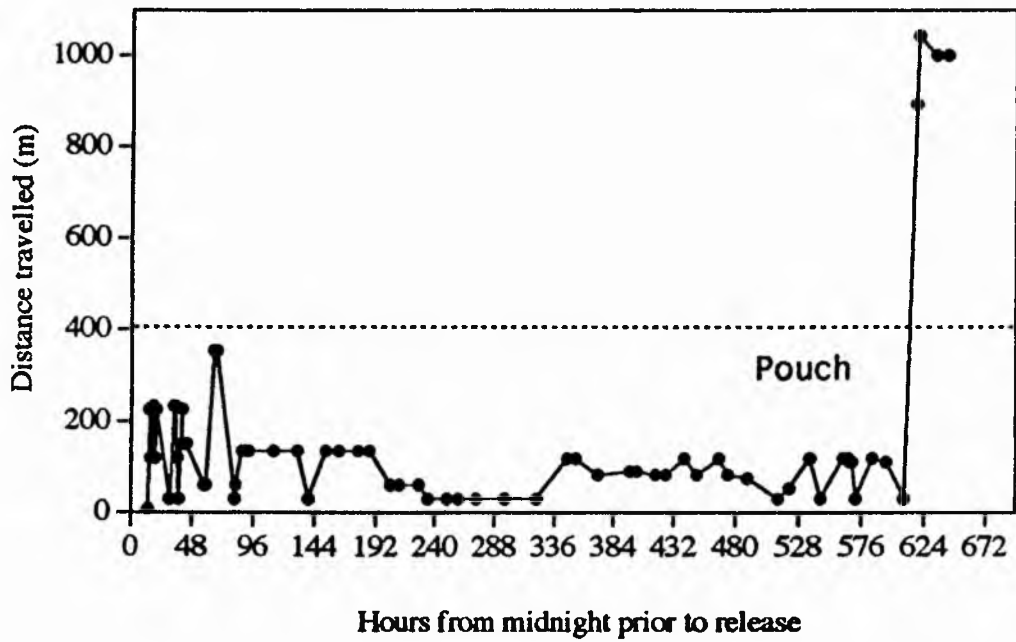
'Pouch'
Slabs
Matt's Run
Mill Run
Lone Tree
Release point
500 m

Figure 3.10

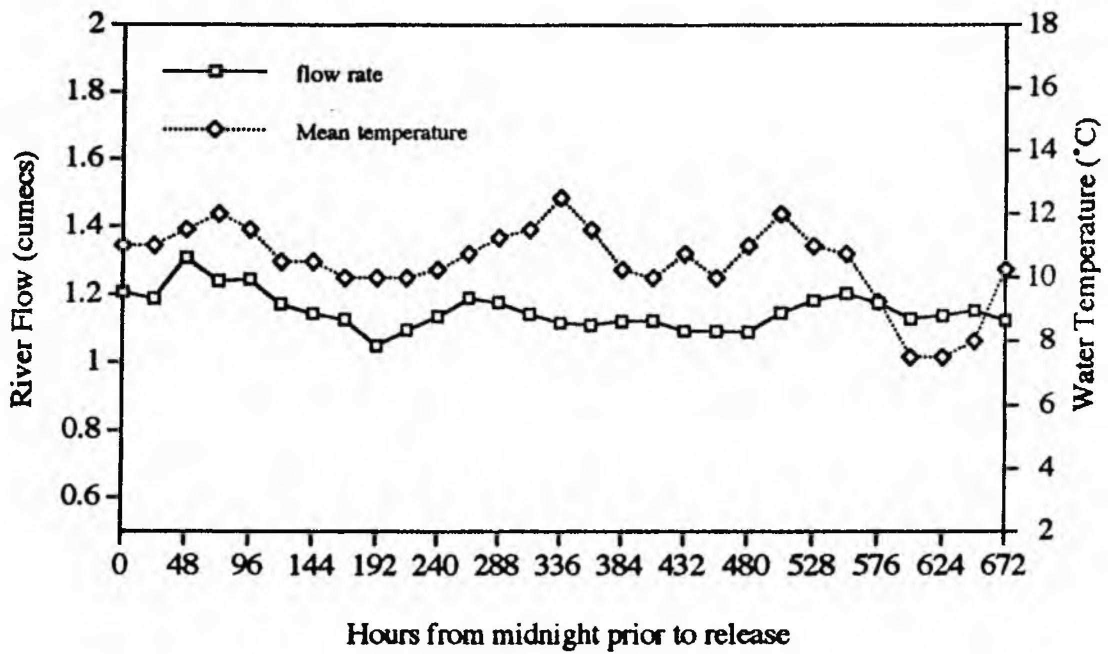
Figure 3.11

Figure 3.10 Movements of Finnock F3 expressed in metres travelled from release point.

Figure 3.11 Mean daily river flow (cumecs.s $^{-1}$) and mean daily water temperature (°C) during the period in which F3 was tracked.



Distance travelled (m)
1000
800
600
400
200
0
Pouch
0 48 96 144 192 240 288 336 384 432 480 528 576 624 672
Hours from midnight prior to release



flow rate
Mean temperature
River Flow (cumecs)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
Water Temperature (°C)
18
16
14
12
10
8
6
4
2
0 48 96 144 192 240 288 336 384 432 480 528 576 624 672
Hours from midnight prior to release

3.3.1.3 Effects of environmental factors

The appropriate mean river flow and water temperature data for F1 and F3 are presented in Figures 3.6 and 3.11, respectively. No environmental data are presented for F2 as the track was brief. Water temperature and river flow daily means did not vary a great deal throughout the study period: the range of mean flow rates was 1.091 - 1.308 $m^3.s^{-1}$ while the maximum for 1994 was 34.180 $m^3.s^{-1}$. Furthermore, data from only two finnock should not be used to infer a relationship between any environmental parameter such as water flow or temperature, and the movements of finnock in general. In the specific cases of F1 and F3 however, while both initiated upstream movements of several hundred metres coincident with a small drop in water temperature, these movements were not at the same time for each fish. No relationship between water flow and movements was apparent.

Similarly, there was no apparent consistent relationship between light levels and large scale movements. F1 and F2 both moved upstream and downstream during darkness or low light levels of dusk and dawn. However, F3 did not move from the Pouch when the other two finnock were moving and its upstream migration took place during the day, although it was lost during the following night.

3.3.2 Tracking Exercise II - 9 November onwards

Five finnock (F3 - F8) were released in the 'Pouch' at 1130 hrs on 9 November 1994. Tracking was by foot and also by ALSTN with a 13-antennae (A1 - A7, B2 - B7) array set-up around the 'Pouch' (Figure 3.12). The movements of each finnock will be described with reference to this Figure and two other figures showing some of the river both upstream and downstream from this pool.

For an initial 16 minute period after release, the five finnock all remained within 40 metres of the release point (between antennae B5 and B7) but did not shoal, either as a total group or as sub-groups. After this time the behaviour of each finnock was different and so will be described in turn.

3.3.2.1 Finnock 4 (F4)

This finnock remained within the 'Pouch' throughout Days 1 and 2 and was located in various areas during this period, generally between antennae B3 to B7 on Day 1 and between antennae A1 to A5 on Day 2. At 0907 hrs on Day 3, F4 was located in

Figure 3.12

Figure 3.12 Diagram of the 'Pouch' with the locations of the 13 ALSTN radio antennae used in Tracking Exercise II.

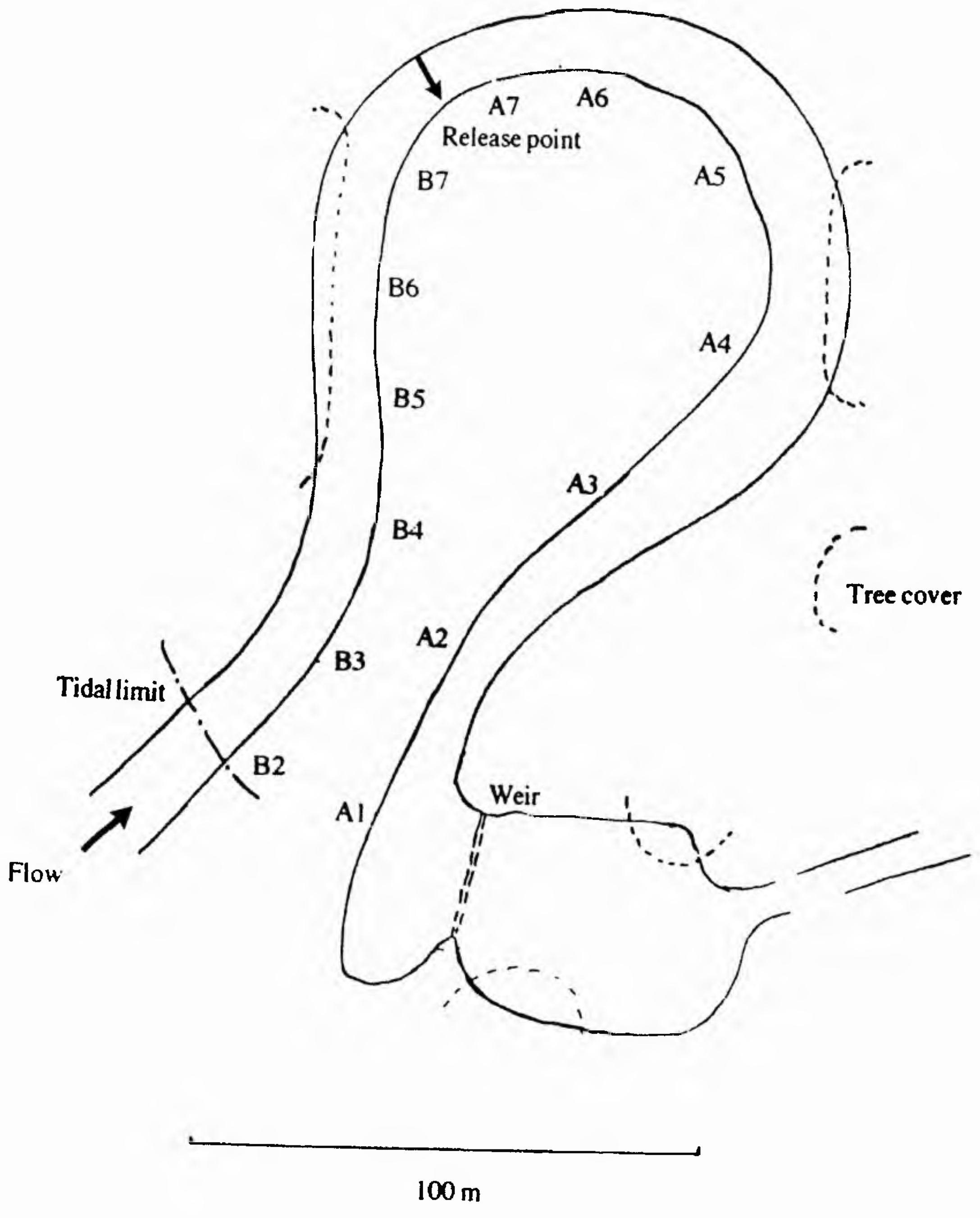

A7
A6
Release point
B7
A5
B6
A4
B5
A3
B4
Tree cover
A2
B3
Tidal limit
B2
Weir
A1
Flow
100 m

Figure 3.13

Figure 3.13 Map of the lower River Eden with areas where Finnock F4 to F8 were located during the tracking exercise.

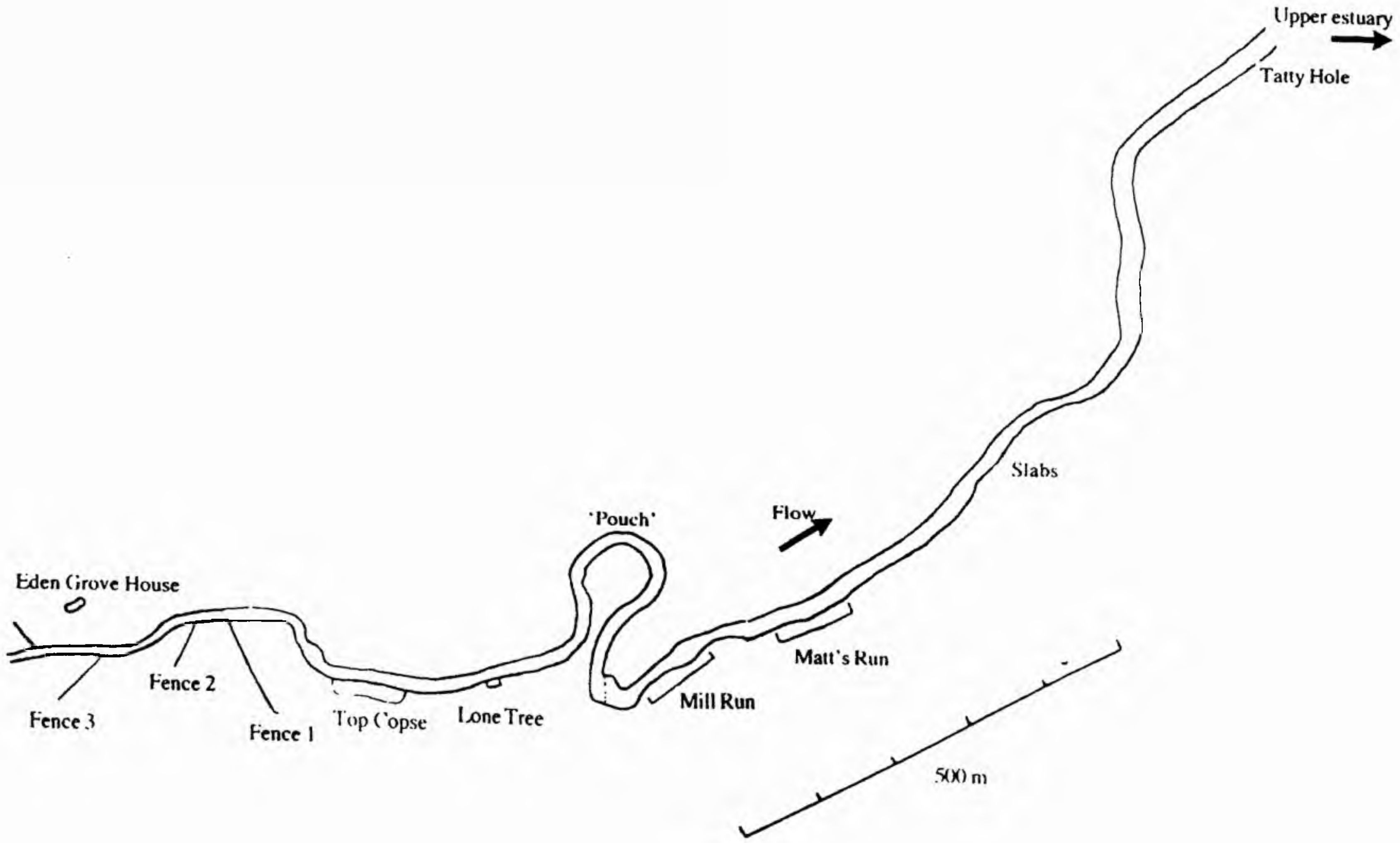
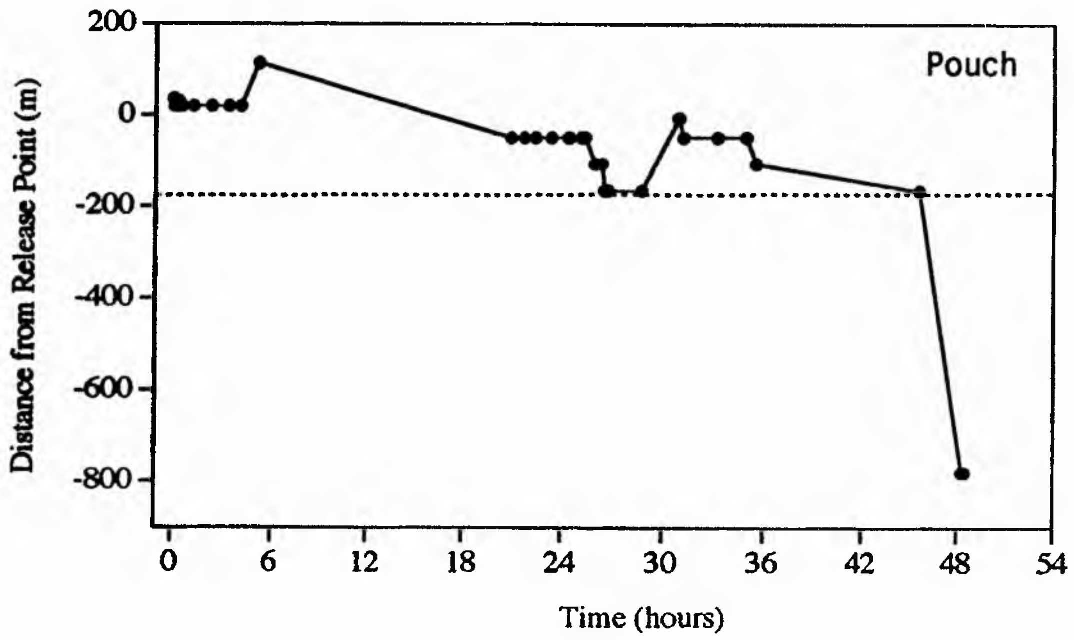

Upper estuary
Tatty Hole
Slabs
Flow
'Pouch'
Matt's Run
Mill Run
Eden Grove House
Fence 2
Fence 3
Fence 1
Top Copse
Lone Tree
500 m

Figure 3.14

Figure 3.14 Movements of Finnock F4 expressed in metres travelled from release point. The dotted line represents the weir at the tail of the 'Pouch'.


Pouch
Distance from Release Point (m)
200
0
-200
-400
-600
-800
0
6
12
18
24
30
36
42
48
54
Time (hours)

the area of A1 but moved downstream out of the pool and was next located at 1148 hrs in the Tatty Hole 780 m downstream from the release point (Figure 3.13, 3.14) where it remained until 1200 hrs when tracking ceased to locate the other fish. This finnock was not located in the river again, either manually or by the ALSTN. This is considered to indicate that this fish left the river to enter the estuary soon after it was last located.

3.3.2.2 Finnock 5 (F5)

F5 remained in the 'Pouch' for approximately 3.3 hours before dropping 280 metres downstream to the Mill Pool by 1525 hrs where it was located periodically over the next hour until manual tracking ceased for the day (Figures 3.13, 3.15). On Day 2, F5 was first located 50 metres further downstream (Matt's Run) at 0841 hrs and again at 0906 hrs and 1255 hrs on Day 2 and 1137 hrs on Day 3. On Day 4, however, F5 had moved upstream to the Mill Pool (1128 hrs). The final record for this finnock was 100 metres downstream from the Mill Pool at 0849 hrs on Day 5. After this, F5 was not located again, either manually or by the ALSTN. As with F4, this was considered to indicate that it had left the river for the estuary some time after the last record.

3.3.2.3 Finnock 6 (F6)

This finnock moved upstream from the 'Pouch' after 1206 hrs on the day of release and was next located 443 metres upstream of the release point at 1240 hrs (Figure 3.13, 3.16). This represents an average swimming speed of 0.67 bl.s $^{-1}$ over this distance. It was next located in this area at 1429 hrs but returned downstream to the head of the 'Pouch' by 1540 hrs, where it was recorded again at 1651 hrs and at 0822 hrs on Day 2. This was the last record of F6. As the ALSTN was functioning at this time, the loss of F6 may represent a tag failure.

3.3.2.4 Finnock 7 (F7)

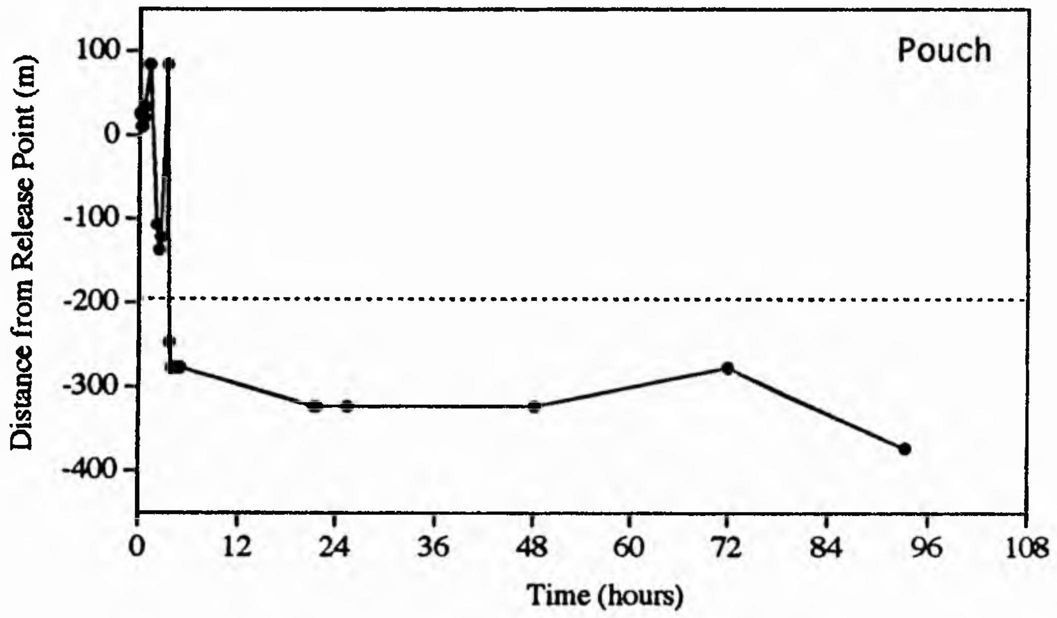
F7 remained within the 'Pouch' during Day 1 but became much more mobile towards the end of the afternoon, being located at A2 at 1452 hrs and then B3 at 1540 hrs and 1651 hrs (Figure 3.13, 3.17). This finnock was not located again after this time but since the ALSTN failed overnight, it is not clear whether it moved upstream or downstream. If F7 moved upstream then it must have travelled further

Figure 3.15

Figure 3.16

Figure 3.15 Movements of Finnock F5 expressed in metres travelled from release point. The dotted line represents the weir at the tail of the 'Pouch'.

Figure 3.16 Movements of Finnock F6 expressed in metres travelled from release point. The dotted line represents the weir at the tail of the 'Pouch'.


Pouch
Distance from Release Point (m)
100
0
-100
-200
-300
-400
0
12
24
36
48
60
72
84
96
108
Time (hours)

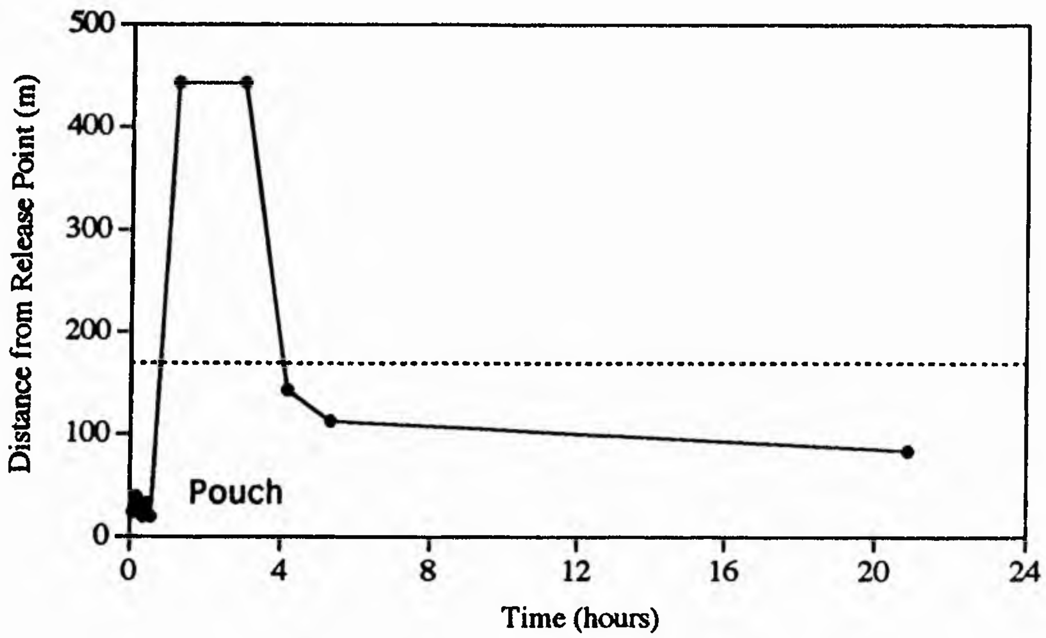
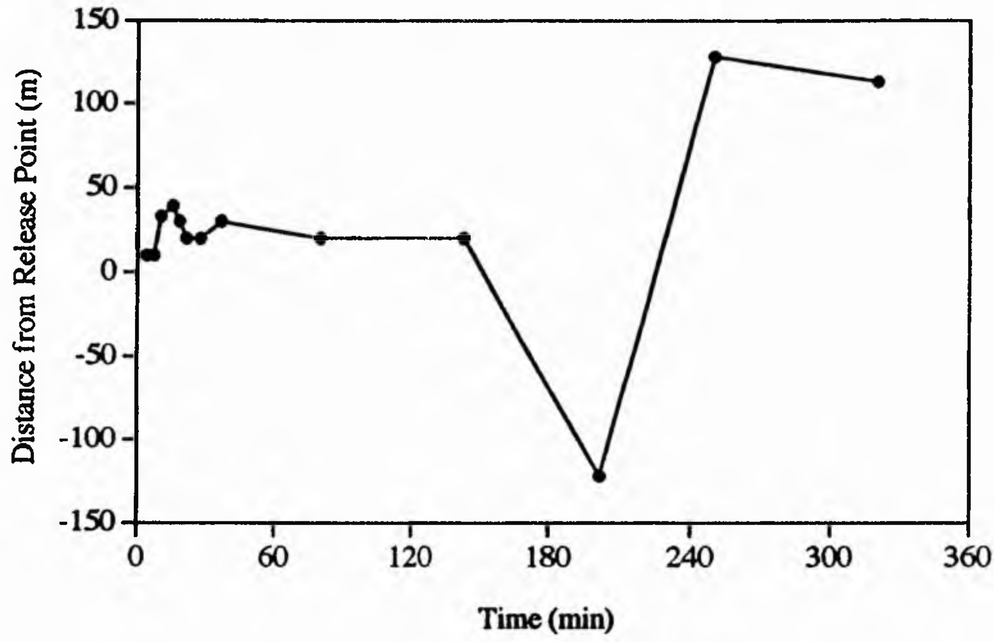

Distance from Release Point (m)
500
400
300
200
100
0
Pouch
0
4
8
12
16
20
24
Time (hours)

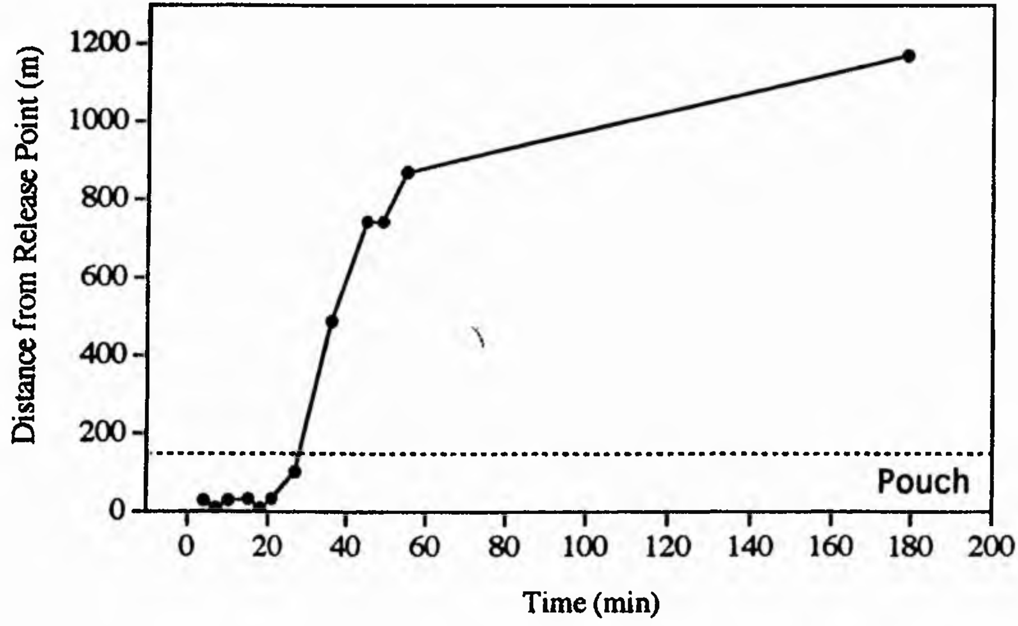
Figure 3.17

Figure 3.18

Figure 3.17 Movements of Finnock F7 expressed in metres travelled from release point.

Figure 3.18 Movements of Finnock F8 expressed in metres travelled from release point. The area below the dotted line represents the 'Pouch'.


Distance from Release Point (m)
150
100
50
0
-50
-100
-150
0
60
120
180
240
300
360
Time (min)


Distance from Release Point (m)
1200
1000
800
600
400
200
0
Pouch
0
20
40
60
80
100
120
140
160
180
200
Time (min)

than the 12 km of river scanned while if it moved downstream it must have left the river for the estuary.

3.3.2.5 Finnock 8 (F8)

F8 began to move upstream from the 'Pouch' within 20 minutes of release and had travelled from the area of B3 to the Top Copse, a distance of 385 metres, in 7 minutes (3 bl.s $^{-1}$) (Figure 3.13, 3.18). It was then located in the Top Pool between 1215 hrs and 1219 hrs (1 bl.s $^{-1}$), at Fence 1 at 1225 hrs (1 bl.s $^{-1}$) and 10 metres above Fence 3 at 1429 hrs, by which time the swimming speed had lessened (300 metres in 124 minutes). Tracking ceased at this time to locate the other finnock but F8 was not located again despite scanning approximately 12 km upstream from this point during the next three days. Whereas the fact that this finnock was last recorded moving upstream could indicate that it continued to do so and remained ahead of the trackers over the following three days, the failure of the ALSTN during the first night after the finnock were released means that F8 could have returned downstream and left the river without being tracked again. Other finnock had been shown to make brief upstream movements before returning downstream past the "Pouch' to leave the river.

3.3.2.6 Environmental data

The dissimilarity between movements of these latter five finnock and the brevity of all the tracks preclude any meaningful analysis of movements with respect to water temperature or flow rate.

3.3.3 Further attempts to track finnock

The lack of sufficient ALSTNs, coupled with the considerable distances over which finnock travelled in short periods of time, meant that very few finnock could be simultaneously tracked in the River Eden. Therefore, the Kenly Burn, 10 km south of St Andrews, was chosen as a second study site as finnock were reported to enter this stream in the summer and it was only 8 km long so if a tagged finnock was lost, the entire system could be checked in one day. In addition, there is no estuary because the stream runs straight into the sea, so the ALSTN could be sited a short distance upstream from the stream mouth with two antennae placed 100 m apart. Thus, any finnock last recorded travelling downstream past the ALSTN would have returned to the sea.

However, despite intensive electrofishing of the many larger pools in the lower 4 km of the stream over three days in December, 1995, no finnock were caught. A further intensive electrofishing of the River Eden between Dairsie Bridge and the Pouch in late February, 1996 did not locate any finnock. After this it was planned to electrofish further upstream but several heavy spates during the next month prevented electrofishing for six weeks, at which time other lab-based commitments prevented further tracking.

3.4 Discussion

3.4.1 Summary of results

- 3 finnock (F1 – F3) were released on 9 September
- F1 moved 2490 metres upstream over 5 days and then remained in one pool for the next 20 days
- F2 was tracked entering the estuary 4 days after release
- F3 remained near the release site for 26 days but was then lost
- patterns of movement were not related to changes in water temperature or river flow rate
- 5 finnock (F4 – F8) were released on 9 November
- F4 and F5 were lost downstream from the release site 3 and 5 days after release, respectively
- F6 moved 443 metres upstream but then returned to the release site and was not located after day 1.
- F7 was lost from the release site on day 1
- F8 moved 1200 metres upstream within 3 hours but was not located subsequently

3.4.2 Was the behaviour of finnock affected by the radio-tag?

An important proviso of any study of animal behaviour using conventional or telemetric tracking is that tagging does not adversely affect the behaviour of the animal in question. Radio-tracking fish with externally mounted tags such as those used in this study has the potential to affect fish behaviour, particularly swimming performance. However, the maximum recorded swimming speed of 3 $bl.s^{-1}$ against the current over a distance of 385 metres indicates that the attachment of tags did not seriously affect the potential swimming performance of finnock in this study. Radio-tagged resident Eden brown trout of similar size were recorded swimming at speeds up to 1.22 $bl.s^{-1}$ against the flow over distances of 675 - 1848 m (Armstrong and Herbert, 1997).

Another criticism of externally mounted tags is that they may increase the risk of predation, both through affecting the reaction behaviour (swimming ability) and by making the fish more obvious to predators. The former criticism is answered above but another observation made during this study indicates that the external tags did not significantly increase the visibility of the fish to predators.

Soon after the tide entered the 'Pouch', some five hours after the finnock of Exercise I were released, a seal was observed turning sharply underwater near the tail of the pool (Area B). This was considered to indicate that the seal was trying to catch fish. All three finnock (F1-F3) were within 4 metres of the seal when it was observed but since no tags left the pool when the seal did, and since each fish was subsequently tracked moving upstream, it is safe to assume that the seal did not catch any of the tagged finnock. Although anecdotal in basis, this evidence indicates that the tagging procedure had not severely affected the behaviour of the finnock or made them more susceptible to predation, and that they were sufficiently recovered from tagging and release to avoid such a predator.

Thus, the capture, handling and tagging procedures were not considered to have a lasting detrimental effect on the behaviour of the finnock in this study.

3.4.3 Did the finnock behave like mature migrating salmon and sea trout or resident immature brown trout?

The aims of this study were to discover how long finnock spent in the river before returning to the estuary and to ascertain whether finnock behaved in a manner similar to adult salmon and sea trout on spawning migrations, juvenile freshwater resident trout or in a manner different from either of these examples?

Radio-tagged finnock remained in the river for between a few hours and at least 27 days. Note, however, that no information was available regarding how long they had been in freshwater prior to capture. F1-F3 were caught in the tidal reaches earlier in the autumn while F4-F8 were caught further upriver later on. It is possible, therefore, that F1-F3 were caught early in their migrations into freshwater while the latter group had been in freshwater longer and were ready to return to the estuary. This might explain why more tagged finnock from batch II dropped out of the river soon after release.

3.4.3.1 Behaviour of adult salmon

The following general pattern of the upstream movements of adult salmon is based upon studies conducted on several Scottish rivers (Hawkins and Smith, 1986; Webb, 1989; Laughton, 1991; Walker and Walker, 1992). After freshwater entry there is an initial rapid upstream movement, the distance travelled varying with the time of

year, size of river system and flow conditions. Thereafter, most salmon enter a quiescent phase, occupying particular pools for long periods prior to spawning. Immediately before the spawning period, further upstream movement occurs, often associated with increased river flow, but once having reached the spawning grounds, considerable up and downstream movements can occur. Thus, maturing salmon move upstream through rivers in a step-wise rather than continuous manner (Hawkins and Smith, 1986).

3.4.3.2 Behaviour of adult sea trout

Maturing sea trout also migrate through rivers in a step-wise manner. Sea trout returning to spawn in the Afon Glaslyn (Wales) were observed (by acoustic tagging) to migrate upstream through the semi-tidal area without pausing and then hold up for long periods (3 - 79 days, mean 47 days) near the confluences of tributaries (Milner; in Le Cren, 1985). Similarly, radio-tagged adult sea trout in the River Fowey (Cornwall) completed most of their upstream migration within 2 - 3 weeks and then held up in areas for the remainder of the summer (Solomon and Sambrook; in Le Cren, 1985). Female sea trout radio-tagged a short time before spawning moved to holding pools after release and stayed there for 3 - 10 days before moving upstream to spawn and then dropping back down river (Evans, 1994).

3.4.3.3 Movements in relation to environmental factors

Radio-tagged adult salmon ascended the upper part of the River Tay estuary and the lower reaches of the river over a range of discharges (Webb, 1990). Most sea trout movements occur during the night but also during periods of high water or when the water is coloured (see Le Cren, 1985). Just prior to spawning, however, whereas female sea trout moved only under cover of darkness or coloured water, male sea trout were observed to move upstream from a holding pool in periods of non-elevated flow and regardless of photoperiod (Evans, 1994). Finnock in the present study moved in day and night in a period when the river flow rate was low.

3.4.3.4 Resident juvenile brown trout

Juvenile brown trout appear to possess home territories, displaying distinct homing behaviour when displaced from these areas. In a mark-recapture study of juvenile brown trout (85 - 292 mm) with four electrofishing surveys (7 - 49 day intervals) each year over two years, Harcup *et al.* (1984) found between 68% and 93% of

recaptures were within 15 m of previous capture site. In addition, 50% of trout displaced up to 75 m upstream or 111 m downstream returned to the site of capture within eight days.

Subsequently, a radio-tracking study of resident juvenile brown trout (206 - 288 mm) in the River Eden using the same radio-tracking technology as the present study, found that all six trout displaced downstream (1500 - 3400 m) and six of eight trout displaced upstream (500 m) homed to the areas where they were first captured (Figure 3.19) (Armstrong and Herbert, 1997). The median time to reach home range for trout displaced downstream was 3 days (range 0.5 - 14 days) and for those displaced upstream, 3.5 days (range 0.5 - 4 days).

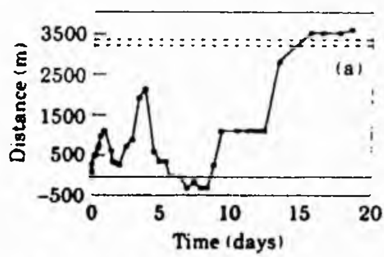
Maturing sea trout fitted with radio tags and displaced from spawning tributaries have also been observed to home to those areas again (Sambrook, 1990) but such homing has not been reported in immature finnock. Sambrook (1990) notes that migrating anadromous salmonids may migrate several km into freshwater before returning to sea and migrating further to the natal system. This behaviour is attributed to maturing fish seeking natal spawning grounds. Such a behaviour pattern in finnock might represent a search for a suitable river system in which to overwinter (Walker; in Le Cren, 1985). Several of the finnock in the present study did appear to make brief upstream migrations before returning downstream and possibly migrating out of the river.

3.4.4 What do the results indicate about finnock behaviour in freshwater?

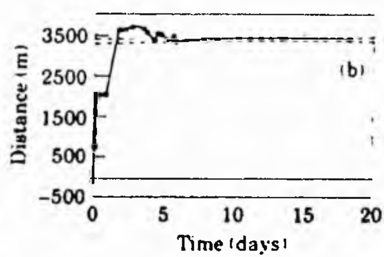
Finnock in this study displayed a variety of patterns of movement but, in general, were more akin to those of adult salmon and sea trout than juvenile brown trout, e.g., the step-wise upstream movements of F1 and the brief upstream movements followed by downstream movements out of the river displayed by several finnock. No obvious signs of homing behaviour were apparent in any of the finnock tracked. F1-3 were released in the same pool as capture and all moved out of this pool within 4 hours of capture. F4-8 were all displaced more than 2000 metres downstream from place of capture and although two finnock were tracked moving upstream, neither were ever located in the pools where they were first captured.

Figure 3.19

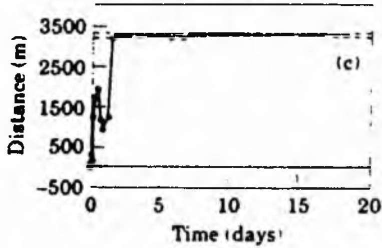
Figure 3.19 Movements of River Eden resident trout displaced downstream from site of capture. Each graph shows the movements of an individual fish. The stippled lines indicate the zone from which each fish was captured.
From Armstrong and Herbert (1997).



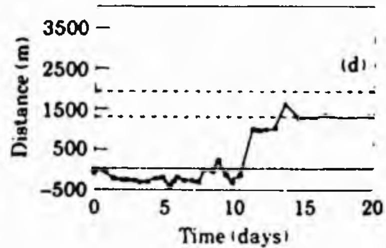
Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(a)



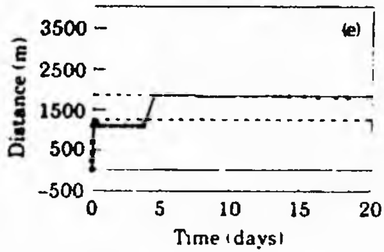
Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(b)



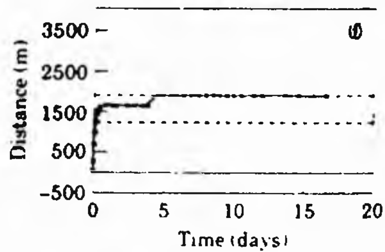
Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(c)



Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(d)



Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(e)



Distance (m)
3500
2500
1500
500
-500
0
5
10
15
20
Time (days)
(f)

The paucity of finnock caught in the lower region of the River Eden and their absence from the Kenly Burn during the winter indicate either that all the finnock were further upriver, perhaps on the spawning grounds or that finnock movements into freshwater are transitory in nature. If the finnock were not maturing then the only conceivable reasons for them to migrate up to the spawning grounds (which are often a considerable distance upstream in narrow, shallow sections, thereby further increasing predation risk), are either as a dummy spawning migration or to feed on loose fish eggs when adults are spawning. The latter is certainly plausible as eggs would provide a substantial source of nutrition during a period when food resources will be scarce. However, although Tracking Exercise II was performed in November just prior to the start of the spawning period of salmon (pers. obs.) when finnock might have been further upstream, the later electrofishing survey in February, long after spawning had ceased, did not capture any finnock either. There are quite a number of deep pools with extensive bankside cover in the region between Dairsie Bridge and the 'Pouch', the 'Pouch' itself being an excellent example, and tracking data have shown that finnock remain in some of these pools for several days at least. Why then should finnock migrate further upstream after the spawning season has ended?

Perhaps finnock migrations upriver are only transitory in nature and the fish soon return downstream to the estuary. One problem with radio-tracking is that whereas finnock can be recorded leaving the river to enter the estuary, no discrimination can be made between finnock which then remain in the estuary and those which continue out to sea.

The low river flows during the time when finnock were tracked were not considered normal for this time of year. One would normally expect at least one, if not several, large spates during the autumn. Since a considerable proportion of the river catchment is agricultural land, such spates tend to be very dirty and the river typically remains coloured for several days afterwards (C. D. Todd, pers. comm.). If there had been a spate then the behaviour of the tagged finnock might have been somewhat different and it is perhaps worth speculating as to what that behaviour might have included. Either the finnock would have remained within a localized area, as resident trout would be expected to do, the finnock might have moved upstream to the headwaters and spawning tributaries as adult spawning fish typically move up to the spawning areas during times of high water flow, or the finnock might have moved downstream and left the river for the estuary.

3.4.5 Final comments

Finnock appear to be highly mobile within the freshwater environment and certainly a proportion of them return to the estuary soon after tagging. However, as discussed earlier, this technology cannot determine whether the finnock continue this migration to the sea. The following chapter will discuss some of the potential reasons for this behaviour and investigate the osmoregulatory capabilities of finnock during the winter months.

Chapter 4

Why do finnock return to estuaries and overwinter in freshwater?

4.1 Introduction

As outlined earlier, the return of finnock to estuaries and rivers during the summer and their subsequent overwintering is somewhat enigmatic. The purpose of migrating to sea would appear to be to take advantage of superior growth conditions, as compared to freshwater, but does necessitate the physiological costs required of adaptation to different salinity environments. Obviously, these costs are multiplied by regular (or irregular) migrations from one environment to the other. Why, then, should finnock adopt this seemingly disadvantageous strategy? The immature status of the majority of finnock has already been discussed (Section 3.1). A number of other possible reasons for this migratory strategy are outlined below.

4.1.1 Predator avoidance

One probable cost of the migration between freshwater and seawater is an increased mortality due to novel, and increased numbers of, predators in the marine environment. Could returning to an estuary result in a reduced risk of predation? While concentrated within the confines of an estuary, especially at low tide, finnock must be at high risk of predation from seals, piscivorous birds, otters and mink, as well as susceptible to human angling activities. Predation will occur from seals and birds in the open seas, as well as from other cetaceans and larger fish species. However, the density of predators within the open seas will be much lower than within relatively enclosed estuaries. Thus, far from reducing predation risk, moving into an estuary during the summer months may increase the risk. However, returning to the relatively predator-free freshwater environment during the winter might be advantageous in avoiding more active homeothermic predators, such as seals, in the estuaries.

4.1.2 Feeding opportunities

Large estuaries are nursery areas for several other fish species as well as often having high densities of invertebrates. Personal observations of large numbers of shrimps, *Crangon crangon* L., and juvenile herring or sprats in seine net hauls in the River Eden estuary, together with observations of large numbers of mysids including the two major brackish water species, *Praunus flexuosus* (Müller) and *Neomysis integer* (Leach) (Isaac *et al.*, 1990) inhabiting the estuarine sediments, suggest that feeding opportunities for finnock in estuaries are excellent. Sea lochs are also considered important nursery areas for gadoids and clupeids (Gordon, unpubl. cited

by Pemberton, 1976a). Nall (1927) noted that many sea trout in the Ythan estuary moved in and out with the tides during the summer and grew rapidly on a mixed diet of sandeels, juvenile herring and sprats. Finnock from the Kyle of Durness and from the mouth of the River Laxford, Sutherland, were found to have large numbers of prey items including mysids, shrimps, small fish, crabs and terrestrial invertebrates (pers. obs.). The diet of sea trout is regarded as extremely varied; sea trout will consume anything that is available (Pemberton, 1976b). It is possible, therefore, that finnock inhabit estuaries during the summer months because of the high densities of available prey species.

However, not all finnock caught in estuaries or rivers during the summer are found to have been feeding. Of 46 finnock (150 - 310 mm fork length) examined for stomach contents from a larger sample caught in the upper estuary of the River North Esk, Angus in August 1995, 43 had empty stomachs and the remainder had only low numbers of food items each. The estuary of the River North Esk is short and narrow (see Pratten and Shearer, 1983b) and probably does not foster the high numbers and variety of prey species found in other, more expansive estuaries. However, less than 10 km to the south the estuary of the River South Esk provides the increased feeding opportunities of a large open estuary. Thus, good feeding may explain the presence of finnock in such large estuaries as the Eden, the South Esk or the Kyle of Durness but does not explain the presence of all finnock in brackish and freshwater environments.

During the winter, evidence suggests that although some finnock feed in freshwater (pers. obs.) this may be intermittent. Nall (1927) described finnock returning downstream from the Ythan river as "thin". Finnock present on the spawning beds may feed on loose eggs but spawning in salmonids only lasts a few weeks at most, so fish eggs cannot be considered a constant, reliable food source during the winter.

4.1.3 Increased seawater temperature

High summer seawater temperatures may cause physiological stress in salmonids and could, possibly, be implicated in the 'finnock' behaviour. Maisse *et al.* (1991), in a study of the sea trout of the River Calonne (Normandy), recorded peaks in numbers of upstream-migrating finnock during neap tides. They suggested that at such times the mixing of coastal waters with those from the cooler open sea was at a minimum, and the resultant rise in water temperature reduced dissolved O_2 levels while causing an increase in the average metabolic rate, and hence O_2 consumption

rate, of sea trout. Such conditions were thought to lead to discomfort for the sea trout in the estuary and induce them to return upstream to less saline and better oxygenated waters.

High mortality rates (60.7%, 80.4% and 64.8% between mid-June to mid-September, 1985 - 1987) in rainbow trout held in sea cages during the summer in Holland were suggested to be a consequence of the combination of increasing water temperature, particularly above 18°C, and high salinities (28 - 33 ppt) (Oorschot and Boon, 1993). The authors suggested that increased blood P_{CO_2}, as a result of temperature stress and the branchial Cl^-/HCO^{3-} ionoregulatory exchange mechanism (Section 1.2), favoured CO_2-haemoglobin binding which, in conjunction with the reduced concentration of dissolved O_2 in warmer water, resulted in mortalities.

However, in terms of finnock behaviour, neither of these studies explain why finnock should return to freshwater rather than moving further out into cooler, deeper seawater which would alleviate the temperature problem without further physiological stress from hyper-osmoregulation.

4.1.4 Parasite avoidance

When sea trout migrate to seawater they become susceptible to new parasites at a time when they are adapting to hyperosmoregulatory conditions and, therefore, in a potentially immuno-suppressed state. However, returning to freshwater could be a mechanism to reduce or remove any parasitic load acquired in the marine environment. The particular physiological consequences of ectoparasitic infection will be considered in Chapter 5.

4.1.5 Osmoregulatory physiology

Migration from freshwater to seawater requires a variety of physiological adaptations to enable finnock to not only survive but thrive in the marine environment. Many studies have demonstrated that sea trout and salmon smolts are well prepared for this migration by the process of smoltification, and that they rapidly adapt if transferred from freshwater to seawater. Furthermore, acoustic-tagging studies of smolts as they migrate through estuaries have found no evidence of any halt in migration upon increased salinity (Tytler *et al.*, 1978; Moore and Potter, 1994). However, if returning to freshwater some weeks or months later does not appear to have any distinct advantages, and indeed appears to confer distinct

disadvantages on the finnock, could this return be due to a deterioration in the hypo-osmoregulatory ability of finnock some time after their initial migration? If hypo-osmoregulation were compromised, the least energetically demanding environment in terms of osmoregulation would be the brackish areas of an estuary where the salinity of the water would be almost isotonic with the body fluids of the finnock.

It has been observed that not only trout and charr but even salmon return to freshwater during the winter months in the most northerly parts of Norway (Dahl and Sømme, 1944; Berg, 1964, both cited in Berg and Berg, 1989). Several studies have shown that salmonids have problems tolerating seawater conditions at very low water temperatures. Rainbow trout maintained in seawater over the winter near Murmansk, Russia were observed to have high plasma osmolarity, high intestinal osmolarity, decreased glycogen stores in the liver and a decreased osmotic resistance of erythrocytes (Belkovskiy *et al.*, 1991).

Such changes in osmoregulatory physiology consistent with a compromise of regulatory mechanisms are also observed when seawater-adapted salmonids are transferred to lower temperatures. Atlantic salmon post-smolts transferred from 5.6°C to 1.0°C displayed reduced drinking rates (0.58 to 0.24 ml.kg^{-1}.h^{-1}). Despite an apparent increase in the efficiency of intestinal water absorption (60 - 96%), the fish experienced a water deficiency at low temperature indicated by increases in plasma osmolarity (320 to 440 mOsmol.kg^{-1}) and tissue water concentration (75% - 69%) (Lega *et al.*, 1992).

Transfer of freshwater-adapted salmonids to seawater at low temperature also results in reduced or complete failure of the appropriate adaptive mechanisms. While the responses to seawater-challenge of Atlantic salmon pre-smolts were qualitatively similar at 10 and 1.5°C, the stabilization of plasma ions, muscle water content and blood mean cellular haemoglobin concentration occurred within two days at 10°C, but had yet to stabilize nine days post-transfer at 1.5°C (Virtanen and Oikari, 1984). Similarly, freshwater-adapted rainbow trout transferred to seawater at 1□C failed to stabilize plasma osmolality, exhibited marked tissue degradation and all died within seven days of transfer. By contrast rainbow trout transferred to seawater at 8°C exhibited the typical rise in plasma osmolality followed by rapid stabilization and no mortalities (Finstad *et al.*, 1988).

Temperature effects also are apparent when comparing teleosts adapted to less extreme temperatures. Flounder, *Platichthys flesus*, adapted to 6°C compared with

those adapted to 16°C, exhibited a disturbance of Na^+ balance indicated by an increased internal Na^+ space (Maetz and Evans, 1972). The increased Na^+ load was the result of impairment of the branchial Na^+-extrusion mechanism and was demonstrated by the reduction of the branchial Na^+/K^+ exchange activity to negligible levels while the passive Na^+ fluxes decreased by only a factor of two (Maetz and Evans, 1972). Similarly, dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) displayed significantly lower drinking rates and weight-specific Na^+ efflux rates when water temperature was decreased from 14 to 5°C, although the decrease was not significant when fish acclimated to 9°C were transferred to 5°C (Carroll *et al.*, 1995).

Low water temperatures also affect teleost ionoregulation in freshwater. Rainbow trout transferred to freshwater at 1°C exhibited a moderate reduction in plasma osmolality, Na^+ and Cl^- concentrations (Finstad *et al.*, 1988). However, the magnitude of the perturbations is much less than those exhibited by fish in seawater.

In summary, therefore, salmonids in more northerly clines may overwinter in freshwater or brackish water to avoid the combination of low temperatures and high salinities (Berg and Berg, 1989). However, such low seawater temperatures are rare in the coastal waters around Scotland. What other factor may cause changes in hypo-osmoregulatory capacity?

The size at which the sea trout smolt may be an important factor. Juvenile trout in the River Tweed (Borders), where finnock are rare, grow faster and are larger as smolts (R. Campbell, pers. comm.) than those of rivers further north along the east coast of Scotland, where finnock are commonplace. The migratory patterns of the sea trout from these two areas are quite different. As described in Chapter 3, finnock from the rivers of the north-east coast of Scotland spend the summer months migrating in and out of estuaries and the majority tagged as smolts are subsequently recaptured within the local area. In contrast, the sea trout smolts from the rivers of the north-east coast of England, from the River Tweed southwards, move south along the coast towards East Anglia before travelling out into the southern North Sea in an anti-clockwise rotation. This migration takes several months but upon reaching the English coast again the following spring, the sea trout migrate northwards until they locate their natal river (Le Cren, 1985; Potter, 1990).

Gudjónsson (1993) observed that the smaller sea trout, averaging 29.5 cm, strayed from the River Ulfarsa, Iceland to the neighbouring estuaries whereas the larger fish

migrated farther out to sea. In addition, the smallest anadromous Arctic charr migrating from the River Vardnes in Norway tended to spend the summer months in the brackish waters at the head of the estuary (Halvorsen *et al.*, 1993), as do the smaller sea trout smolts from the River Axe, Devonshire (Potter; in Le Cren, 1985). It is possible that the larger sea trout smolts must migrate further beyond the estuaries in order to find sufficient numbers of larger prey items to suit their demands as bigger fish. However, the important relationship between body size and the ability to survive salinity transfers, with smaller fish being less successful to salinity challenge than larger fish due to their greater surface area to volume ratio, has been discussed already (Section 2.4.2.1).

It is possible therefore, that finnock may possess a reduced ability to osmoregulate efficiently in seawater and that this induces them to return to brackish water and freshwater. If finnock do suffer from an inefficient hypo-osmoregulatory system, for whatever reason, then one would expect this to be most apparent during the winter months when water temperatures are low and finnock are least expected to be found in seawater. The following experiments were designed to investigate the physiological responses of finnock to acute and long-term salinity challenges during the winter. These experiments were carried out over two seasons. During the first season, only a small number of finnock were obtained so these were used in preliminary studies to establish experimental protocols and compare the hypo-physiological abilities of finnock with similarly sized freshwater-resident and hatchery-reared brown trout. Considerably larger numbers of finnock were available during the second season, allowing a larger investigation of the osmoregulatory adaptation of freshwater-adapted finnock transferred to seawater, and *vice versa*. Each Season will be considered separately.

4.2 Season One

4.2.1 Methods and Materials

4.2.1.1 Fish capture and maintenance

4.2.1.1.1 Estuarine finnock

Twenty-eight finnock were captured by beach seine netting in the Eden Estuary at or around low water on 25 August, 1993. These finnock were transported to the Gatty Marine Laboratory aquarium and held in a 2 m circular tank (1900 l) in running seawater. Staff from the F.F.L. provided the seine net, a boat and valuable technical assistance. An additional five finnock were captured in the same area on two further occasions, 21 October (4 fish) and 8 November (1 fish). These finnock were treated in an identical fashion.

Large numbers of juvenile herring or sprats were also caught on 21 October. These were collected and fed to the captive finnock. Finnock were also fed on shrimps (*Crangon crangon*) collected from the surf at low tide at the shore near the Marine Laboratory.

4.2.1.1.2 Hatchery-reared brown trout

Forty hatchery-reared brown trout of similar size to the finnock (mean length 250 mm) were acquired from the Howietoun Fishery, Stirling on 2 December, 1993 and transported to the aquarium where they were held in another 2 m diameter tank of running freshwater. These fish were fed commercial fish pellets (EWOS). Several suffered from fungal infections and were removed from the group. These fish were subsequently all found to be maturing males.

4.2.1.1.3 Freshwater-adapted finnock and wild brown trout

Eight finnock and eight similarly sized brown trout were collected from the lower reaches of the River Eden on 24 February, 1994. These fish were captured by electrofishing from a boat, again with equipment and assistance of staff from the F.F.L. Electrofishing was performed in a similar manner to that described in Section 2.2.1. These fish were transported to the Gatty Marine Laboratory aquarium and

acclimated to aquarium conditions in freshwater for two weeks prior to undergoing acute freshwater-seawater challenges (see below).

4.2.1.2 Salinity acclimations prior to experimental protocol

Figure 4.1 depicts the various salinity acclimations and experimental challenges. After two weeks in freshwater, 13 hatchery-reared trout were transferred to a smaller tank (400 l) and the salinity increased to seawater in a step-wise manner over a period of 48 hours (24 hrs at 11 ppt, 24 hrs at 22 ppt). Starting on the same day, 11 seawater-adapted finnock were acclimated to freshwater in a similar stepwise manner. While no finnock died post-transfer, four freshwater-adapted resident trout died; one each on 19, 21, 25 and 41 days after transfer to seawater.

The finnock transferred to freshwater and brown trout transferred to seawater were acclimated to new salinities for four weeks prior to commencing experimental salinity challenges (see below).

4.2.1.3 Experimental salinity challenges

Baseline osmoregulatory measurements of drinking rate, plasma ion concentrations and plasma cortisol concentration for long-term adapted trout were assessed in seawater-adapted, and freshwater-adapted 'estuary' finnock and hatchery-reared brown trout. Similar measurements were taken after acute freshwater-seawater transfer of hatchery-reared brown trout, wild brown trout and finnock (the latter two groups having been collected from the river). Ambient water temperature throughout the experimental period was 7°C.

4.2.1.4 Experimental procedure

On each sample day, three trout were collected at random from the appropriate group and transferred to an experimental tank of water of the appropriate salinity. The procedures for measuring drinking rates and collecting blood were as described for Experiment 4 in Chapter 2. After collection, whole blood was aliquotted into three 1.5 ml Eppendorf tubes (one containing 10 µl heparin (5000 units per ml) and two containing 50 µl protease inhibitors (0.05M 1,10-phenanthroline, 0.225M EDTA, 50 KIU aprotonin) and stored briefly on ice.

Figure 4.1

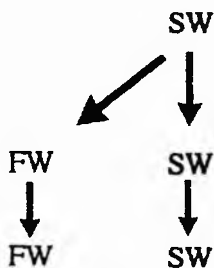
Figure 4.1 Flow diagram representing the various salinity acclimations and acute transfers performed in the Season One experiment protocols.

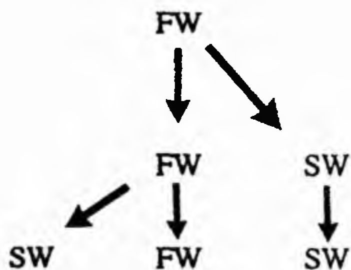
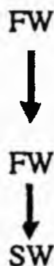
Finnock
(estuary)

Salinity in which the fish were obtained

Pre-experimental acclimation

Experimental transfer



Hatchery-reared brown trout	Wild brown trout and finnock caught in freshwater
	

At the end of the procedure for all three fish, the samples of whole blood were centrifuged at 13000 g for 4 minutes and plasma removed to fresh Eppendorfs and stored at -20°C for later analysis. Plasma osmolality was measured by freezing point depression (Roebling Automatic Osmometer, Camlab, Cambridge, UK), plasma Cl^- concentration was measured by end point titration (Chloride Analyser 925, Corning Ltd, Essex, UK) and plasma Na^+ and K^+ concentrations were measured by flame photometry (Eel Flame Photometer).

4.2.1.5 Measurement of plasma cortisol concentration

Cortisol was measured by RIA following the protocol of Waring *et al.* (1992). Cortisol standard was stored at 2mg.ml $^{-1}$ in 96% ethanol at -20°C. The ethanol was evaporated using a Genevac Centrifugal Evaporator and assay standards made up in RIA phosphosaline buffer containing disodium hydrogen phosphate (61.3 mM), sodium dihydrogen phosphate (39 mM), NaCl (154 mM) and Bovine Serum Albumin (BSA) (0.5%). ^{3}H-cortisol (Amersham International) with a specific activity of 92.7 Ci.ml $^{-1}$ was stored in 9:1 toluene/ethanol at -20°C. An aliquot of radioactive solution was removed, solvents evaporated off and resuspended in RIA buffer to give an activity of 20,000 decays per minute (d.p.m.) per 100 μl. Cortisol antiserum raised in sheep with a specificity of cortisol: 100%, corticosterone: 0.18%, cortisone: 0.07%, 11-deoxycortisol: 0.58%, 11-deoxycorticosterone: 0.03% (manufacturer's data), was obtained from the Scottish Antibody Production Unit (S.A.P.U., Law Hospital, Carluke, Lanarkshire, Scotland). Antibody was stored at -20°C in a 1:5 dilution.

4.2.1.5.1 Plasma extraction procedure

Six hundred μl 96% ethanol were added to 75 μl plasma in an Eppendorf tube. The contents were shaken in a vortex mixer for 60 seconds and then centrifuged at 13000 g for 3 minutes. The supernatant was then removed to a fresh tube and the pellet retained. The supernatant was diluted with 250 μl de-ionised water and 500 μl hexane was added prior to a further vortexing for 60 seconds. Fractions were allowed to separate over 15 minutes and then the upper hexane layer was aspirated off. A 700 μl aliquot of the solute was removed to a fresh tube, and the hexane procedure repeated on the pellet. Supernatant and pellet extractions were pooled and dried down in the Genevac and resuspended in an appropriate volume of RIA buffer.

Extraction efficiencies were calculated by the addition of ^{3}H-cortisol of known activity to plasma and following the extraction procedure as above. Extraction efficiency was 94 ± 1.5 % (n=6).

4.2.1.5.2 Standard assay procedure

Cortisol standards ranging from 5000 to 78 pg.ml $^{-1}$ in 250 µl were added to triplicate assay tubes, with duplicate 250 ng.ml $^{-1}$ samples to determine Non Specific Binding. Aliquots of 250 µl reconstituted extracted plasma samples were pipetted in duplicate into assay tubes. Five hundred µl of antibody at 1:200 dilution was added to each tube. Blank samples were also used to determine total counts added (950 µl assay buffer) and the zero point on the standard curve (450 µl assay buffer and 500 µl antibody). The tubes were briefly vortexed and allowed to stand at room temperature for 30 minutes after which 100 µl ^{3}H-cortisol were added and the contents vortexed briefly. The tubes were then incubated in a water bath at 37°C for 1 hour and then placed in a fridge at 4°C overnight.

Unbound cortisol was removed from the supernatant in the following manner. Two hundred µl dextran-coated charcoal (in RIA buffer) were added to all tubes, except the total counts tubes into which were added 200 µl assay buffer, and briefly vortexed before being placed on ice for 15 minutes. The tubes were then centrifuged at 2500 g for 15 minutes. Each supernatant was decanted into a separate pony vial and 4 ml scintillation fluid (Scintillator Plus Emulsifier, Packard) added. All the vials were shaken and the activity counted over five minutes in a liquid scintillation analyzer (Tri-Carb 2000, Packard). Results were calculated as percentage bound where

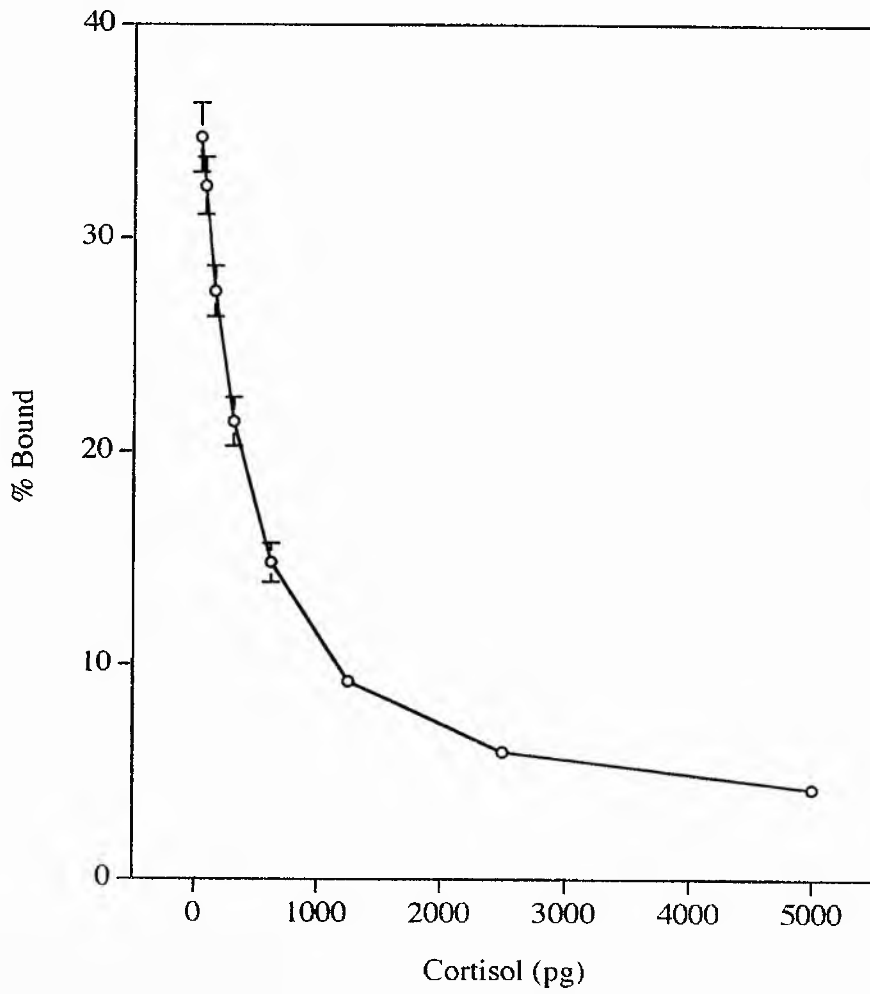
% Bound = (d.p.m. in sample tube / d.p.m. in total counts tube) x 100

A standard curve (see Figure 4.2) of percentage of ^{3}H-cortisol bound against cortisol concentration (pg.tube $^{-1}$) was plotted and sample cortisol concentrations determined from this.

Intra-assay variation [5.82% (n=12)] and inter-assay variation [16.22% (n=12)] were determined for repeat samples derived from a plasma pool.

Figure 4.2

Figure 4.2 Typical RIA standard curve for cortisol. Values, estimated from the percentage of total ^{3}H-cortisol bound to the antibody (ordinate) are means ± SEM expressed as pg.tube $^{-1}$ (abscissa). Number of determinations for each point is 8.


40
30
20
10
0
% Bound
0
1000
2000
3000
4000
5000
Cortisol (pg)

4.2.1.6 Statistical analysis

Statistical procedures were similar to those described in Section 2.2.5.3. The effects of salinity and trout form were assessed by 2-way ANOVA. The acute seawater-transfer data for the wild brown trout were not included in these analyses in order to maintain a balanced statistical design. Subsequently, a 1-way ANOVA was applied to test for trout form effects amongst the three acute seawater-transfer groups. Where 1-way ANOVA indicated significant differences, mean values were compared by Tukey-Compromise (TK-comp) post-hoc means comparison. This post-hoc comparison test was chosen as it is a compromise between the Student-Neuman-Keuls test (SNK) and the Tukey-Kramer test (T-K), both of which are recommended by Zar (1984).

The TK test determines the critical value that each means comparison must exceed in order to achieve significance. This critical value is a function of the total number of means involved in the collection of comparisons. The SNK test compares the two means with the greatest difference against the critical difference. If this comparison is significant then the two means with the second greatest difference are tested, and so on. This procedure is sometimes referred to as a step-wise or multilayered comparison procedure. While the TK tends to make Type I errors, the SNK tends to make Type II errors. The TK-comp test calculates the critical differences for a means comparison by both TK and SNK methods and then uses the average of these to assign a level of significance to the means comparison (User's Manual, Abacus Concepts, SuperANOVA).

The compromise is adopted rather than either of the two original tests as it allows for different sample sizes to be compared. The SNK version available with SuperANOVA requires equal sample sizes and the T-K test requires either equal samples sizes or sample sizes which are considerably different (sample sizes > 1:3). Data are presented in figures as back-transformed means and are displayed with the appropriate upper and lower 95% confidence limits.

All tables of statistical tests are given at the end of the chapter.

4.2.2 Results

4.2.2.1 Drinking rate

The mean drinking rates of acute seawater-challenged finnock and hatchery-reared brown trout were greater than those of the corresponding freshwater-adapted groups, but had not reached the mean rates of seawater-adapted groups during this six hour acute challenge (Figure 4.3 a,b). A 2-way ANOVA comparing log drinking rates of the finnock with those of the hatchery-reared brown trout indicated a significant main effect of salinity but not of trout form (Table 4.12). A 1-way ANOVA comparing log drinking rate data for the three acute seawater-challenged groups indicated no statistical differences.

4.2.2.2 Plasma ion concentrations

As with mean drinking rates, mean plasma osmolality increased between freshwater-adapted and acute seawater-challenged groups in both trout forms (Figure 4.4 a,b). However, mean plasma osmolalities in acute seawater-challenged groups were similar to or greater than those of seawater-adapted trout. A 2-way ANOVA indicated a significant interaction effect (Table 4.13) since the mean plasma osmolality in acute seawater-challenged hatchery-reared brown trout was greater than that of similarly treated finnock, but the order was reversed between the seawater-adapted groups. A significant main effect of salinity, but not trout form, was indicated but, given the presence of a significant interaction effect, main effects should be considered with caution. There were no significant differences between the mean plasma osmolality of the three acute seawater-challenged trout groups.

Because the major components of extracellular fluid composition are Na^+ and Cl^-, the mean plasma concentrations of these ions follow a similar pattern to those of plasma osmolality concentrations. Mean plasma Na^+ and Cl^- concentrations increased upon acute seawater-challenge in both trout forms to values which were similar to, or greater, than those of seawater-adapted groups (Figures 4.5 a,b and 4.6 a,b, respectively).

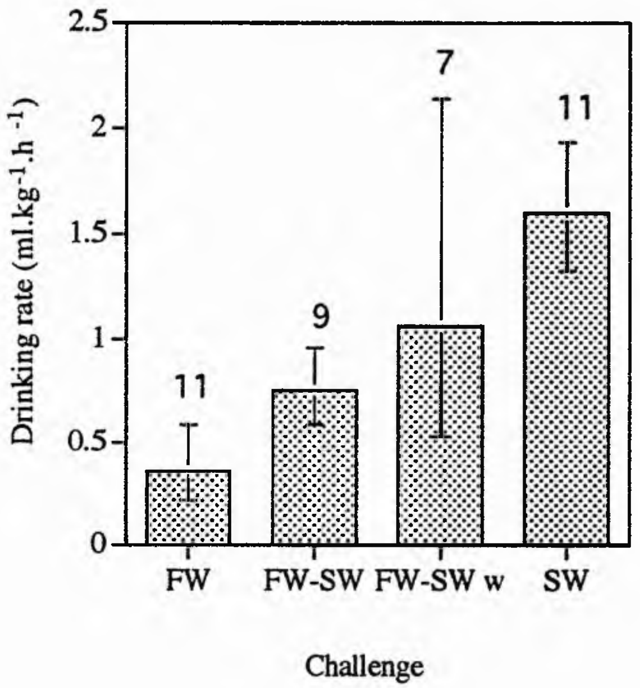
Two-way ANOVA of log Na^+ concentration indicated a significant interaction effect (Table 4.14). Visual examination of the means revealed a pattern closely similar to that of the mean plasma osmolality data. Significant main effects of salinity and

Figure 4.3a

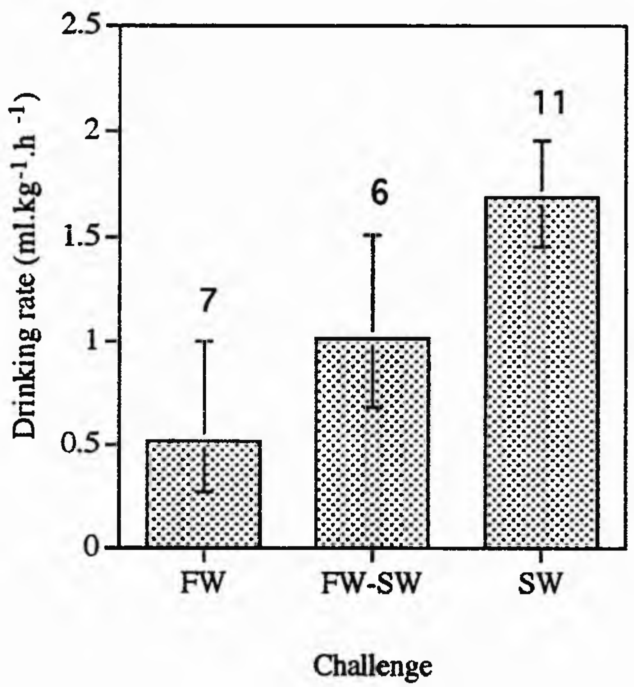
Figure 4.3b

Figure 4.3 a Back-transformed mean drinking rate ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) finnock. Sample sizes are indicated by the numbers above the appropriate bars.

Figure 4.3 b Back-transformed mean drinking rate ($ml.kg^{-1}.h^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) hatchery-reared brown trout, and acute seawater-challenged wild brown trout (FW-SWw). Sample sizes are indicated by the numbers above the appropriate bars.



Drinking rate (ml.kg-1.h -1)
2.5
2
1.5
1
0.5
0
11
9
7
11
FW
FW-SW
FW-SW w
SW
Challenge



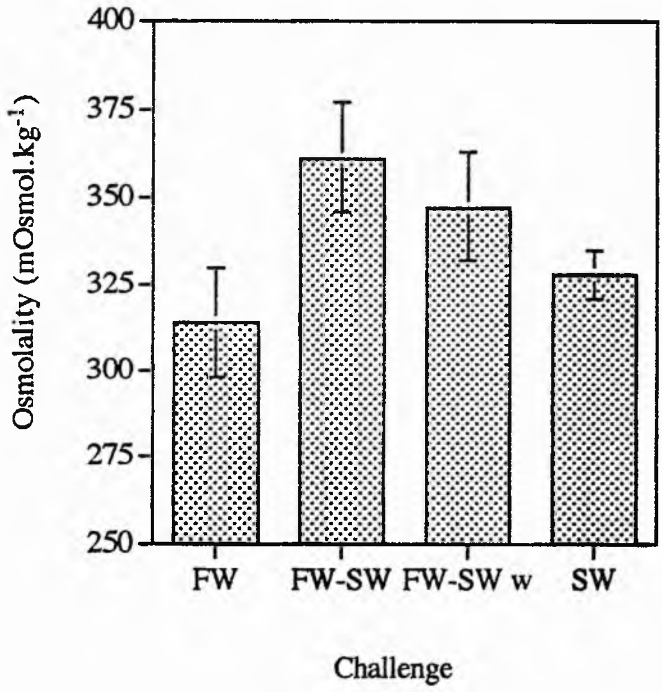
Drinking rate (ml.kg-1.h -1)
2.5
2
1.5
1
0.5
0
7
6
11
FW
FW-SW
SW
Challenge

Figure 4.4a

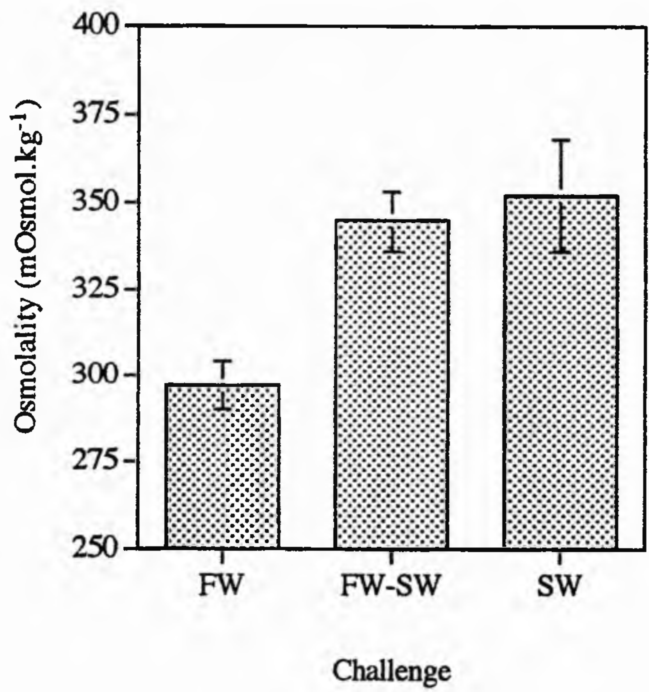
Figure 4.4b

Figure 4.4 a Back-transformed mean plasma osmolality (mOsmol.kg $^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) finnock. See Figure 4.3 a and 4.3 b for sample sizes.

Figure 4.4 b Back-transformed mean plasma osmolality (mOsmol.kg $^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) hatchery-reared brown trout, and acute seawater-challenged wild brown trout (FW-SWw). See Figure 4.3 a and 4.3 b for sample sizes.



Osmolality (mOsmol.kg-1)
400
375
350
325
300
275
250
FW
FW-SW
FW-SW w
SW
Challenge



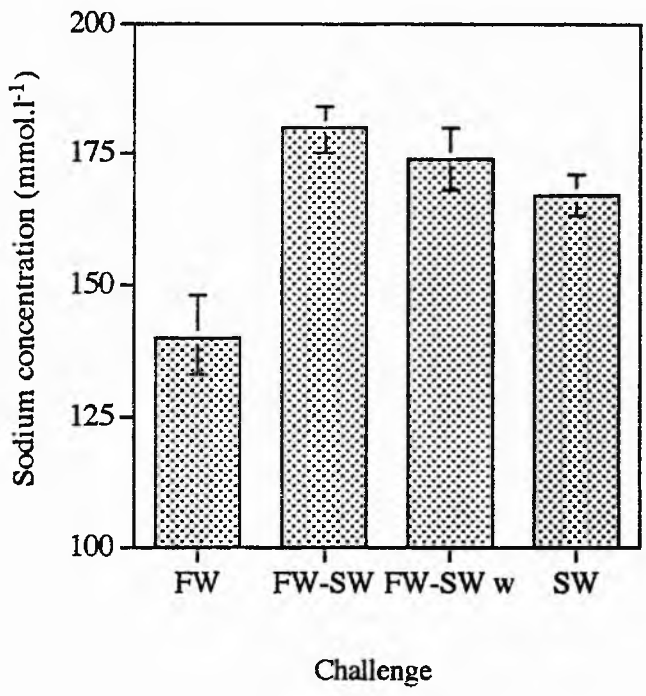
Osmolality (mOsmol.kg-1)
400
375
350
325
300
275
250
FW
FW-SW
SW
Challenge

Figure 4.5a

Figure 4.5b

Figure 4.5 a Back-transformed mean plasma Na^+ concentration ($mmol.l^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) finnock. See Figure 4.3 a and 4.3 b for sample sizes.

Figure 4.5 b Back-transformed mean plasma Na^+ concentration ($mmol.l^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) hatchery-reared brown trout, and acute seawater-challenged wild brown trout (FW-SWw). See Figure 4.3 a and 4.3 b for sample sizes.


Sodium concentration (mmol.l-1)
200
175
150
125
100
FW
FW-SW
FW-SW w
SW
Challenge

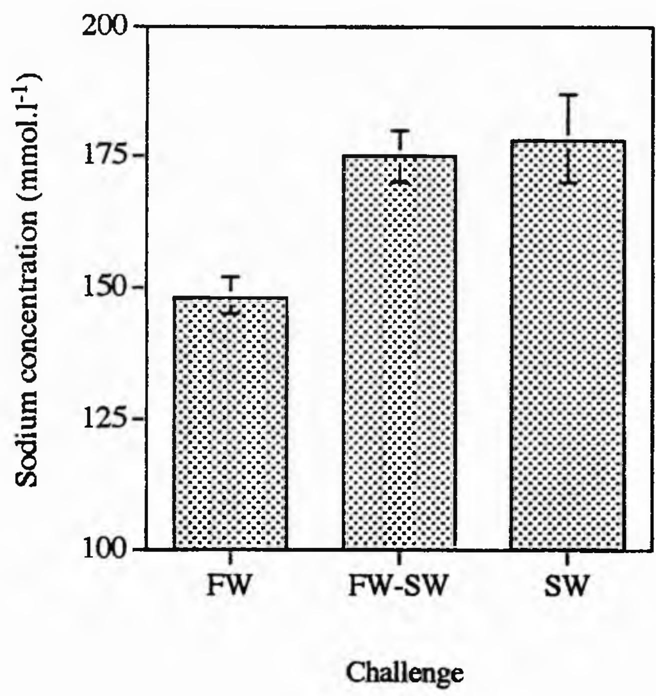
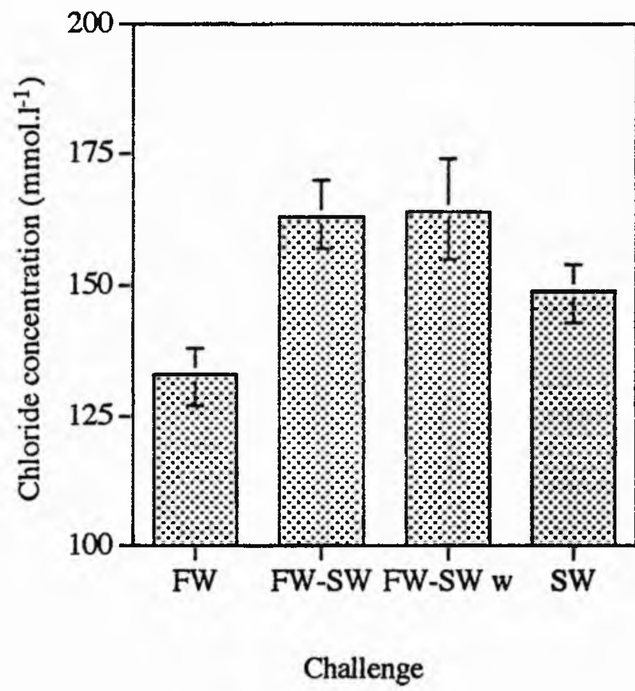

Sodium concentration (mmol.l-1)
200
175
150
125
100
FW
FW-SW
SW
Challenge

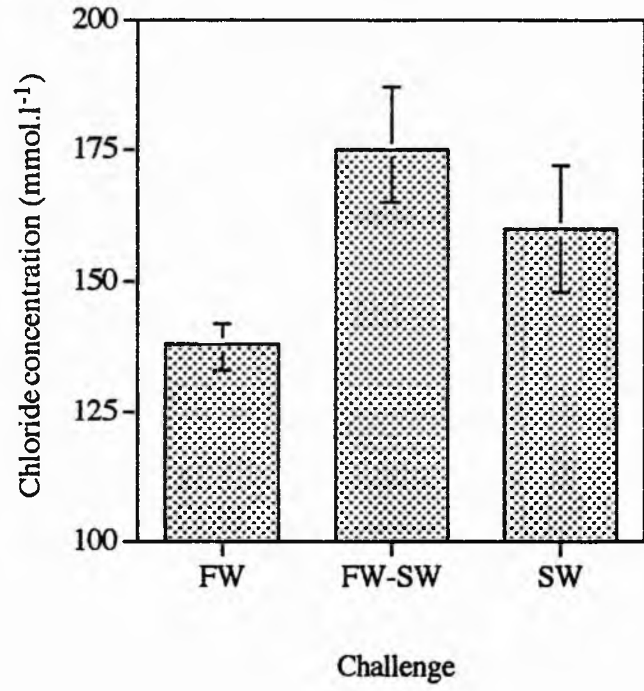
Figure 4.6a

Figure 4.6b

Figure 4.6 a Back-transformed mean plasma Cl^- concentration ($mmol.l^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) finnock. See Figure 4.3 a and 4.3 b for sample sizes.

Figure 4.6 b Back-transformed mean plasma Cl^- concentration ($mmol.l^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) hatchery-reared brown trout, and acute seawater-challenged wild brown trout (FW-SWw). See Figure 4.3 a and 4.3 b for sample sizes.


Chloride concentration (mmol.l-1)
200
175
150
125
100
FW
FW-SW
FW-SW w
SW
Challenge


Chloride concentration (mmol.l-1)
200
175
150
125
100
FW
FW-SW
SW
Challenge

trout form were indicated also and visual examination of the means pooled for each of the main effects in turn revealed that concentrations were similar in acute seawater-challenged and seawater-adapted groups, both of which were greater than the freshwater-adapted value, and greater in finnock than hatchery-reared trout. A 1-way ANOVA indicated no significant differences between the mean plasma Na^+ concentrations of the three acute seawater-challenged groups.

Two-way ANOVA of log plasma Cl^- concentration indicated significant main effects of salinity and trout form (Table 4.15). Visual examination of the means for salinity revealed that mean plasma Cl^- concentration was greatest in acute seawater-challenged trout and lowest in freshwater-adapted trout. Similar inspection revealed that mean plasma Cl^- concentrations were greater in finnock than hatchery-reared brown trout when pooled for trout form alone. There were no significant differences between the mean plasma Cl^- concentrations of the three acute seawater-challenged groups.

Mean plasma K^+ concentrations were similar in most groups and between salinities (Table 4.1). However, a 2-way ANOVA indicated a significant main effect of salinity (Table 4.16); visual examination of the means, pooled for this effect, revealed this to be due to a greater concentration in seawater-adapted trout than in freshwater-adapted or acute seawater-challenged trout, which were similar. Inspection of the mean data (Table 4.1) indicated that this effect was due mainly to a relatively high mean plasma K^+ concentration for the seawater-adapted hatchery-reared brown trout. There were no significant differences between the mean plasma K^+ concentrations of the three acute-seawater challenged groups.

4.2.2.3 Plasma cortisol concentration

Mean plasma cortisol concentrations were low in all groups except the acute seawater-challenged finnock and wild brown trout which were considerably greater than all other groups (Figure 4.7 a,b). Two-way ANOVA comparing finnock with hatchery-reared brown trout indicated a significant interaction effect (Table 4.17); inspection of the mean data (Figure 4.7 a,b) indicated this to be due to the reversal in order between comparisons of the freshwater-adapted and acute seawater-challenged means. A 1-way ANOVA comparing the three trout forms after acute seawater-challenge indicated also a significant trout effect (Table 4.18) and post-hoc comparisons confirmed that this was because the mean plasma cortisol concentration of the hatchery-reared brown trout was significantly lower than those

Table 4.1

Table 4.1 Back-transformed mean of plasma K^+ concentration, with 95% confidence limits (C.L.), for finnock, hatchery-reared brown trout and wild brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

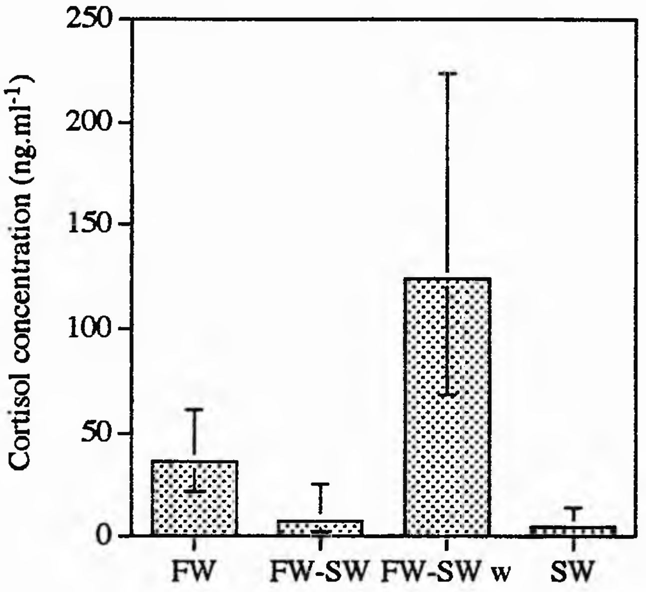
Salinity conditions	Trout form	mean	lower C.L.	upper C.L.
Freshwater-adapted	Finnock	2.2	-0.7	+0.9
	Hatchery-reared brown trout	2.6	-0.3	+0.4
Acute seawater-transfer	Finnock	2.4	-0.5	+0.7
	Hatchery-reared brown trout	2.5	-0.7	+1.1
	Wild brown trout	2.2	-0.8	+1.2
Seawater-adapted	Finnock	2.9	-0.4	+0.5
	Hatchery-reared brown trout	3.4	-0.4	+0.5

Figure 4.7a

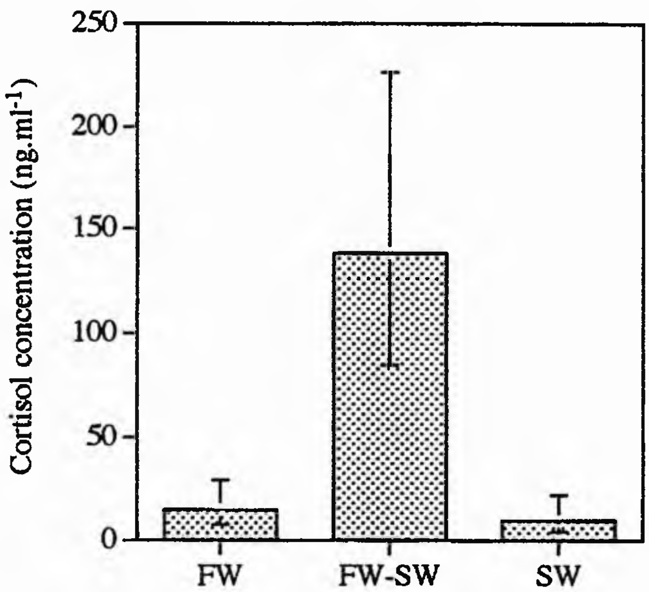
Figure 4.7b

Figure 4.7a Back-transformed mean plasma cortisol concentration ($ng.ml^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) finnock. See Figure 4.3 a and 4.3 b for sample sizes.

Figure 4.7b Back-transformed mean plasma cortisol concentration ($ng.ml^{-1}$), with 95% confidence limits, of freshwater-adapted (FW), acute seawater-challenged (FW-SW) and seawater-adapted (SW) hatchery-reared brown trout, and acute seawater-challenged wild brown trout (FW-SWw). See Figure 4.3 a and 4.3 b for sample sizes.



Cortisol concentration (ng.ml-1)
250
200
150
100
50
0
FW
FW-SW
FW-SW w
SW
Challenge



Cortisol concentration (ng.ml-1)
250
200
150
100
50
0
FW
FW-SW
SW
Challenge

of the finnock and wild brown trout, which were not themselves significantly different (Table 4.19).

4.2.3 Discussion of results

The aim of this study was to ascertain whether or not finnock display any physiological problems related to hypo-osmoregulation during the winter months; a period when they would be least expected to occur in the marine environment. The results of this preliminary study indicate that finnock display values for physiological parameters typical of seawater-adapted, euryhaline fish. Drinking rates were similar to those of seawater-adapted rainbow trout (Perrott *et al.*, 1992), Atlantic salmon parr (Smith *et al.*, 1991) and pre-smolts (Fuentes *et al.*, 1996). Usher *et al.* (1988) recorded mean drinking rates for seawater-adapted Atlantic salmon smolts three-fold greater (6.22 $ml.kg^{-1}.h^{-1}$) than other studies using the same radioisotopic technique. Similarly, plasma osmolality, Na^+, Cl^- and K^+ concentrations of seawater-adapted finnock were close to those reported for other euryhaline fish (see Table 4.2). Plasma cortisol concentrations were low in seawater-adapted finnock, as demonstrated in other studies (Avella *et al.*, 1990).

Furthermore, freshwater-adapted finnock transferred acutely to seawater displayed the predicted physiological responses to increased salinity. Drinking rates rose considerably within six hours of transfer. Note that freshwater-adapted finnock did drink a relatively small amount of medium. Early drinking rate studies suggested that freshwater-adapted fish did not drink (Smith, 1930; Shehadeh and Gordon, 1969) but later studies, using more accurate radiolabel methods have demonstrated that freshwater-adapted fish do drink, albeit at a relatively low rate (Perrott *et al.*, 1992, Tierney *et al.*, 1995; Fuentes *et al.*, 1996). The functional significance of drinking in freshwater is unclear at present. The drinking rates in freshwater-adapted finnock in the present study are somewhat higher than those of similarly acclimated rainbow trout (Perrott *et al.*, 1992). This may reflect a combination of temperature difference (approximately 5°C for the rainbow trout compared versus 7 - 9°C in the present study) and the use of ^{125}I-PVP by Perrott *et al.* (this marker does not distribute uniformly in water and, as a consequence, low drinking rates tend to be underestimated). A temperature effect on drinking rates has been reported previously. The drinking rates of dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) acclimated to 9°C during the winter were approximately half and two-thirds, respectively, those of summer fish acclimated to 14°C.

Table 4.2

Table 4.2 Typical plasma osmolality and ion concentrations for seawater-adapted euryhaline teleosts.

Species	Time in seawater	Mean plasma values			Reference
		Na^+ ($mmol.l^{-1}$)	Cl^- ($mmol.l^{-1}$)	osmolality ($mOsmol.kg^{-1}$)	
Atlantic salmon smolts	24 hours		154		Olsen *et al.* (1993)
Atlantic salmon juveniles	48 hours	170	155	360	Stagg *et al.* (1989)
Atlantic salmon smolts	48 hours		150	340	Soivio *et al.* (1989)
Atlantic salmon smolts	72 hours	190	185		Smith *et al.* (1991)
Atlantic salmon smolts	6 days			305	Prunet *et al.* (1989)
Sea trout parr	7 days	175	155		Madsen (1990)
Atlantic salmon smolts	10 days		135		Houston (1960)
Rainbow trout	12 days	150	148		Madsen and Naamansen (1989)
Sea trout pre-smolts	14 days	180	150		Madsen and Korsgaard (1991)
Rainbow trout	seawater-adapted			349	Perrott *et al.* (1992)

Eels increase drinking rate almost immediately after seawater-transfer, in response to increased Cl^- concentration in the medium (Hirano, 1974). Drinking rates in seawater-transferred Atlantic salmon smolts increase initially within 30 minutes, followed by a further increase from four hours onwards (Usher *et al.*, 1988) and are associated with dehydration (Evans, 1979) and elevated plasma ion concentrations (Usher *et al.*, 1988). These conditions stimulate the Renin-Angiotensin System (RAS) (Section 1.3) which, in turn, induces increased drinking, both in freshwater-adapted and seawater-adapted fish (Perrott *et al.*, 1992). Note, however, that in the present study, mean drinking rates after six hours in seawater had yet to reach those of fully seawater-adapted trout. Mean drinking rate peaked in Atlantic salmon smolts ten days after seawater-transfer (Usher *et al.*, 1988).

Plasma osmolality, Na^+ and Cl^- concentrations increased rapidly within six hours of seawater-transfer. This reflects both the passive influx of ions and osmotic loss of water across the integument and the increased body salt load due to ion uptake across the gut which facilitates fluid influx (see Section 1.2). Urine flow rate decreases to a very low level almost immediately after transfer (Eddy and Bath, unpubl. results cited in Bath and Eddy, 1979) so this will not affect plasma ion concentration. The observation in the present study that some plasma ion concentrations were greater than those of fully seawater-adapted groups indicated that branchial ion excretory mechanisms (Section 1.2) required longer than six hours to become fully functional after such an increase in external salinity.

No significant differences were observed between finnock and wild brown trout in any of the parameters measured after acute seawater-transfer. Gordon (1959) also found no significant differences in hypo-osmoregulatory abilities between adult sea trout and brown trout. This indicates that the physiological responses necessary for preliminary adaptation to hyperosmotic conditions are present in both freshwater-resident and anadromous trout of this size, and further emphasizes the plasticity of the life history strategy of this species (see Chapter 2).

However, some physiological differences were found between wild and hatchery-reared trout. The only statistically significant difference upon acute seawater-transfer was in mean plasma cortisol concentration. The basal concentrations in freshwater-adapted and seawater-adapted trout indicate that the experimental procedure did not unduly stress the fish. A rapid increase was observed in cortisol concentration of finnock and wild brown trout upon acute seawater-challenge and has previously been reported in a variety of fish species including Atlantic salmon

(Nichols and Weisbart, 1985), sockeye salmon (Franklin *et al.*, 1992), and eel (Ball *et al.*, 1971). However, no such increase was observed in the hatchery-reared trout in the present study.

Cortisol is recognised as a hormone important in seawater acclimation, particularly through its facilitation of ionoregulation (see Section 1.3.3.5). The lack of an increase in plasma cortisol concentration in seawater-transferred flounder (Warne, 1994) was thought to be the consequence of artificially high concentrations in freshwater-adapted flounder rather than the lack of a response to seawater-challenge. Plasma cortisol concentrations remain elevated in salmonids which fail to adapt to hyperosmoregulatory conditions (Avella *et al.*, 1990; Franklin *et al.*, 1992). The lack of an increase in acute seawater-challenged hatchery-reared trout, albeit within six hours of transfer, is therefore intriguing. No statistically significant differences in drinking rate or plasma ion concentrations were observed between acute seawater-challenged reared and wild trout, although the mean drinking rate was lower and mean plasma osmolality higher in the former group. Whereas the role of cortisol in controlling drinking is unclear, administration of cortisol to freshwater-adapted Atlantic salmon pre-smolts resulted in higher drinking rates than sham operated fish after seawater-challenge (Fuentes *et al.*, 1996b).

Whereas no conclusions can be based upon an analysis of plasma hormone levels without a simultaneous assessment of metabolic clearance rate, if the results of the present study indicate a genuine inability of these hatchery-reared trout to increase plasma cortisol concentration in response to increased salinity, it is conceivable that this condition could be related to the artificial rearing of these fish. While cortisol is recognised as an important hormone in seawater acclimation, it is also regarded as a good indicator of general stress. However, sustained high levels of plasma cortisol are known to induce an immuno-suppressed state and increased mortalities due to disease (Pickering and Pottinger, 1985, 1989). Fish reared in hatcheries are subject to repeated stresses such as confinement and disease. Thus, hatchery conditions, over generations, may select for those fish that display reduced increases in plasma cortisol, i.e. the interrenal gland is less sensitive to all factors that stimulate cortisol release. A heritable component of the stress response has been tentatively established (Fevolden and Røed, 1993) such that plasma cortisol increases following confinement stress were lower in rainbow trout selected from parents which also displayed lower cortisol peaks after similar stress. However, although lower, the cortisol peaks were still significantly greater than basal levels. Thus, the concept that a possible reduced interrenal sensitivity, selected for by the artificial

environment, may result in trout that do not increase plasma cortisol levels in response to increased salinities, remains to be established.

To conclude, these results indicate that finnock are able to maintain 'normal' physiological levels of osmoregulatory parameters if held in full seawater during the winter and that they display some of the expected rapid physiological responses to acute seawater-challenge. However, while this may suggest that finnock do not return to estuarine and freshwater environments in winter because of a physiological problem in seawater, the effects of any such physiological compromise might be too subtle to be revealed by a brief six hour challenge. Less dramatic effects might be more apparent in a study of the long-term adaptation of finnock to seawater. The following experiment was designed to investigate this long-term adaptation of finnock to seawater and freshwater during the winter.

4.3 Season Two

4.3.1 Materials and Methods

4.3.1.1 Fish capture and maintenance

Ninety-three finnock were caught by seine net in a lower pool of the River North Esk, Angus on 2 August, 1995, with help of fishermen from the commercial netting company Joseph Johnson's, Montrose, and staff from the F.F.L. These finnock were transported live to the Gatty Marine Laboratory, St Andrews.

Upon arrival, all finnock were lightly anaesthetised (2-phenoxyethanol, 0.5 $ml.l^{-1}$) to facilitate handling. Measurements of wet weight (g) and fork length (mm) were taken, a small sample of scales was removed and ectoparasitic sea lice, *Lepeophtheirus salmonis*, were counted and removed. Finnock were then transferred to a large circular tank (Tank A) containing 1900 l of flowing water maintained at approximately 16 ppt by supplying freshwater and seawater at equal flow rates. Salinity was monitored daily with a salinity refractometer which was zeroed with distilled water prior to sampling.

A circular flow was established by means of a mains-driven submersible pump (PJP 3001, Project, Italy). The outflow from this pump was directed into a vertical pipe (2.5 cm diameter) which was closed at the top but had eight holes (5 mm) drilled at regular intervals along its length. The pipe was long enough to extend above the surface of the water and the top three holes directed water jets across the surface. The pump and drilled outflow pipe created the circular flow in the tank, supplemented the dissolved oxygen levels created by two compressor-fed airstones and provided some extra cover for the finnock.

An additional 95 finnock were collected from the same pool on the River North Esk on 9 August, 1995 and transported to the Gatty Marine Laboratory aquarium where they were placed in a second large circular tank (Tank B). These finnock were Pan-jet tattooed at the base of the left pectoral fin with alcian blue dye to distinguish them from those of Tank A when finnock were combined for later experiments.

Finnock were fed daily on a ration of diced sand eel and squid (Ammodytes Company Ltd, Cornwall). Seawater temperature was ambient but photoperiod was

controlled by a time switch and altered periodically to maintain a close simulation of natural photoperiod.

Soon after capture, finnock began to die at a rate of one or two per day. Most showed no obvious symptoms but one did have a large ulcer, 1 cm in diameter, on its flank. A small number of recently dead finnock were transported on ice to the Fish Cultivation Unit (Fish Diseases Section) at the S.O.A.F.E.D. Marine Laboratory, Aberdeen where tissue and blood samples were taken for analysis. Results indicated that the finnock had furunculosis, an infection of the blood caused by the bacterium *Aeromonas salmonicida*, and staff advised an appropriate antibiotic treatment. All remaining finnock were lightly anaesthetised and given a weight-dependent dose of amoxycillin trihydrate (trade name: Duphamox LA) (Solvay Duphar Veterinary, Southampton, U.K.). Although 12 trout died within 24 hours of treatment, no further mortalities were recorded thereafter, indicating that the initial mortalities were due to the stress of the procedure and that the dose of antibiotic was sufficient to combat the bacterial infection.

4.3.1.2 Salinity manipulations

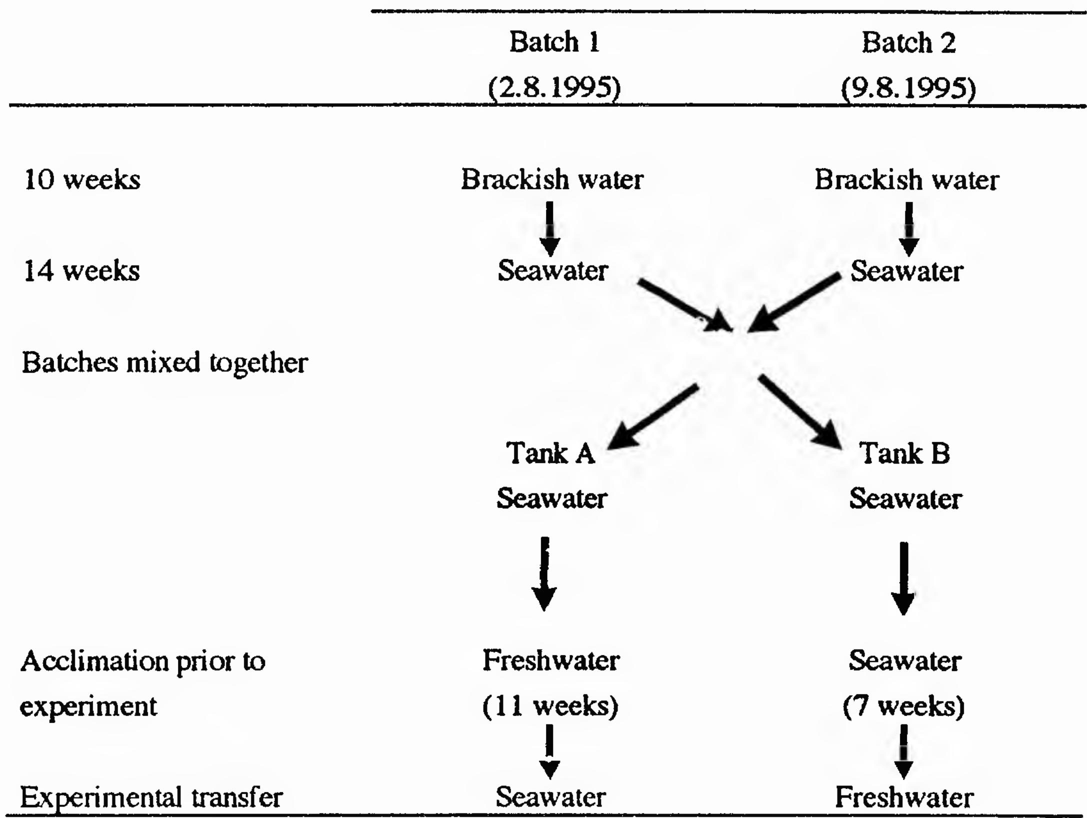
Figure 4.8 summarises the combination of the two groups of finnock between the two tanks and the various subsequent salinity manipulations which will be described below.

After ten weeks the freshwater flow to each tank was halted and salinities increased to full seawater (34 ppt) over four hours. Fish were then maintained at this salinity for a further 14 weeks to ensure all had a suitably long period for physiological adaptation to a similar salinity.

After 14 weeks in seawater the finnock from tanks A and B were lightly anaesthetised and fork length and wet weight measured. The Panjet marks of the Tank B finnock were checked and reinforced if necessary. Finnock from both batches were then divided equally between the two tanks. Five days later (26 January) the salinity of Tank A was gradually reduced to 0 ppt in a stepwise manner over 21 days by increasing the freshwater inflow to the tank. Again, salinity and temperature were monitored daily. Prior to each salinity change, Tank A was drained to 25% volume (475 l) to accelerate the reduction in salinity and facilitate the easy capture of finnock for blood sampling (see below). Tank B was drained to

Figure 4.8

Figure 4.8 Flow diagram depicting the various tank transfers, salinity acclimations and challenges of the Season Two experiment.



Batch 1
(2.8.1995)
Batch 2
(9.8.1995)
10 weeks
Brackish water
Brackish water
14 weeks
Seawater
Seawater
Batches mixed together
Tank A
Seawater
Tank B
Seawater
Acclimation prior to experiment
Freshwater
(11 weeks)
Seawater
(7 weeks)
Experimental transfer
Seawater
Freshwater

a similar depth and some finnock removed and replaced several times with a small net to simulate the experimental procedures of Tank A.

4.3.1.2.1 Measurement of freshwater-adaptation

During the salinity transfer procedure in Tank A, blood samples were taken from six randomly sampled finnock from each of the two batches to investigate whether there were any differences in acclimation. Fish were lightly anaesthetised (as above) to facilitate handling and 600 μl of whole blood removed from the caudal sinus using a fresh heparinised syringe and needle for each finnock. Whole blood was transferred to a heparinised Eppendorf tube and centrifuged at 13000 g (Microcentrifuge, MSE) for four minutes. Plasma was removed to a fresh tube and stored at -20°C. Each sampled finnock was given another dye mark at the base of the right pectoral fin to indicate that it had been sampled. Plasma osmolality and Cl^- concentrations were measured as in Section 4.2.1.4.

4.3.1.3 Acute salinity challenge procedure

4.3.1.3.1 Transfer protocol

Acute salinity transfers began on 11 March, 1996. The protocol for the acute seawater-freshwater transfer of finnock in Tank B is described. Eight finnock were netted at random and transferred to a 1 m diameter tank of running seawater (400 l) to provide a seawater-adapted control group. Note that while the volume of this control tank was smaller than that of the stock tank, the biomass in each tank was similar. A further eight finnock were transferred immediately to radiolabelled freshwater for measurement of drinking rate, etc. (see below). The remaining trout were transferred acutely to freshwater within Tank B. The tank volume was drained to 25%, the seawater turned off and freshwater turned on. The tank drain remained open to maximise the turnover of water. Salinity was measured at five minute intervals for the first hour and then at greater intervals (10, 15 and 30 minutes) as the transfer progressed. Salinity was reduced from 34 ppt to 17 ppt within 45 minutes, to 8 ppt within 63 minutes and to 0 ppt within 220 minutes. Further groups of eight finnock were then sampled randomly at 24, 48, 72 hours and 6 days after seawater-freshwater transfer. The mean freshwater temperature over this period was 5.9°C (range 5.1 - 6.8°C).

Eight finnock died 72 hours after seawater-freshwater transfer, two more the following day and of those remaining after the 144 hour sampling, all died nine days after they were first transferred to freshwater. This prevented the final sampling planned for ten days after freshwater-transfer. The cause of these mortalities was thought to be unusually high levels of dissolved chlorine in the freshwater supply. This incident will be discussed further in the Discussion below.

Although none of the freshwater-adapted finnock (Tank A) died during the period noted above, 22 died within the following six days, presumably also from the chlorine poisoning. As a consequence, there were fewer freshwater-adapted finnock available for the seawater challenge than had been planned. Therefore, groups of seven finnock were sampled at 6, 24 and 72 hours, 6 and 10 days after freshwater-seawater transfer. Acute freshwater-seawater transfer was performed in a similar manner to that described above, and began on 8 April. The mean seawater temperature over this period was 8°C (7.1 - 9°C).

4.3.1.3.2 Physiological measurements

The analytical procedures for drinking rates, plasma ion and cortisol concentrations were conducted in the same manner as Section 4.2.1.4, with the additional measurement of wet weight and the following analyses.

4.3.1.3.3 Additional analysis to preliminary experiment (Season One)

4.3.1.3.3.1 Haematocrit

Haematocrit was measured in the same manner as described in Section 2.2.4.4.

4.3.1.3.3.2 Na^+K^+-ATPase enzyme activity assay procedure

After gut dissection, gill arches were dissected out, placed in labelled pony vials, snap frozen in liquid nitrogen and then stored at -80°C for later analysis of Na^+K^+-ATPase activity.

4.3.1.3.3.2.1 Tissue preparation

Gill filaments were removed from the gill arches with a clean scalpel blade and then homogenised by polytron (Kinematica, Kriens-Luzern, Switzerland) for one minute

in homogenisation buffer containing 50 mM Hepes, 1 mM EDTA (disodium salt), 1mM phenylmethylsulphonyl flouride (PMSF), initially dissolved in 96% ethanol to a concentration of 333 mM (18 mg in 3 ml ethanol), and at a pH of 7.4. The homogenate was then aliquotted into 1 ml volumes and stored at -70°C.

4.3.1.3.3.2.2 Standard Assay Procedure

All steps were carried out on ice unless otherwise stated. Homogenate aliquots were defrosted and 10 μl of 10% deoxycholic acid (sodium salt) added. Homogenates were vortexed briefly and filtered through four layers of sterile absorbent gauze BP (The Boots Company) before being diluted with de-ionised water to produce a protein concentration of approximately 0.5 $mg.ml^{-1}$. Three hundred μl aliquots of diluted homogenate were incubated at room temperature (19 - 22°C) for one hour in buffer containing final concentrations of 120 mM NaCl, 8 mM MgCl, 30 mM histidine, 20 mM KCl and 3 mM ATP. Three sets of four incubations were carried out for each sample: set 1, "time zero", also included 500 μl ice-cold 10% trichloroacetic acid (TCA) to calculate the background hydrolysis of ATP; set 2, "plus ouabain", had 50 μl of 20 mM ouabain added to inhibit ouabain-sensitive Na^+K^+-ATPase enzyme activity; and set 3, "minus ouabain", had 50 μl de-ionised water added. At the end of the incubation period the reaction was stopped by the addition of 500 μl ice cold 10% TCA. Tubes were vortexed briefly, transferred to a refrigerator at 4°C and, after at least 15 minutes, centrifuged at 2400 g for five minutes at a temperature of 2°C (Mistral 3000, MSE).

After centrifugation, 200 μl of supernatant were removed to a fresh tube to which were added 750 μl de-ionised water, 200 μl ammonium molybdate and 50 μl stannous chloride (1 M solution in 12 N HCl, further diluted to 20 mM in de-ionised water) to create the colour reaction. Each reaction mixture was transferred to a 0.5 ml plastic semi-cuvette and the light absorbance of the contents measured at 650 nm on a UV-visible spectrophotometer (UV-1601, Shimadzu) after 15 to 30 minutes. The absorbance reading was compared against those of a standard curve based upon a range of inorganic phosphate (Pi) standards ranging from 1 mM to 10 μM, produced by diluting a 10 mM stock solution of monopotassium phosphate in 5% TCA.

4.3.1.3.3.2.3 Measurement of protein concentration

Fifty µl of diluted homogenate was pipetted into a clean tube and 1 ml Bradford's Reagent added [Coomassie Brilliant Blue G, (1.2 mM), (initially dissolved in 96% ethanol at a concentration of 24 mM) and 8.5% (w/v) orthophosphoric acid, filtered through Whatman No. 1 filter paper]. The mixture was transferred to a 0.5 ml plastic semi-cuvette and the light absorbance measured at 595 nm after approximately 30 minutes. The absorbance reading was compared against those of a range of protein standards ranging from 2 mg.ml $^{-1}$ to 90 µg.ml $^{-1}$ [BSA dissolved in de-ionised water].

4.3.1.3.3.2.4 Calculation of ouabain-sensitive Na^+K^+-ATPase activity

The amount of phosphate released from each individual assay sample was determined by direct comparison with the standard curve. The amount of phosphate released by samples incubated in the presence of ouabain were subtracted from those incubated without ouabain to determine the ouabain-sensitive component of the phosphate released, i.e. the Na^+K^+-ATPase-dependent ATP hydrolysis. Division of the ouabain-sensitive phosphate production rate in nmoles Pi.ml $^{-1}$.hour $^{-1}$ by the amount of protein present in the assay reaction (protein concentration in mg.ml $^{-1}$ x 0.06) produced the final value of ouabain-sensitive phosphate released in µmoles Pi.mg protein $^{-1}$.hour $^{-1}$.

4.3.1.4 Statistical analysis

Data transformations and statistical analyses were performed in similar manner to those described in Section 2.2.5.3. The Student's t-test was used to compare finnock length, weight and condition factor before and after mixing of the two batches, and plasma osmolality and Cl^- concentration of finnock sampled a each salinity during the step-wise seawater-freshwater acclimation. Plasma osmolality and Cl^- concentrations for finnock sampled at each salinity were compared by 1-way ANOVA, followed by the Tukey Compromise post-hoc means comparison. Data are presented in figures and tables as back-transformed means with the appropriate upper and lower 95% confidence limits (C.L.). Tables of the results of statistical tests are presented at the end of the chapter.

4.3.2 Results

4.3.2.1 Analysis of similarity in size between the two batches of finnock

Mean and 95% C.L. values for fork length, wet weight and condition factor for the two batches of finnock (Tanks A and B) before, and after, they were mixed and evenly divided between the two tanks are presented in Tables 4.3 and 4.4. The finnock in each tank were not significantly different in size, either before, or after, the mixing took place.

4.3.2.2 Stepwise seawater-freshwater adaptation of finnock in Tank A (21 days)

No significant differences were found between the mean plasma osmolality of the two original finnock batch sub-samples during stepwise salinity transfer from seawater to freshwater. Similarly, no significant differences were found between the plasma Cl^- concentrations of the two batches in seawater, 75% seawater or 50% seawater. The mean plasma Cl^- concentration for the Batch I finnock sampled at 25% seawater was significantly higher than that of the Batch II group (Table 4.20). No biological reason for this statistical difference could be established. Therefore, both sets of data were pooled for further analysis (Table 4.5). As expected, mean plasma osmolality and Cl^- concentration began to decrease at 50% seawater and were significantly lower than seawater values in finnock adapted to 25% and freshwater (osmolality: Tables 4.21 and 4.22; Cl^-: Tables 4.23 and 4.24).

4.3.2.3 Acute salinity transfers

4.3.2.3.1 Growth data

The back-transformed mean and 95% C.L. values for fork length, wet weight and condition factor data for freshwater-adapted and seawater-adapted finnock, measured when finnock were sampled for drinking rates, are presented in Table 4.6. Both groups of finnock increased in mean length and weight during the seven (Tank B) to eleven (Tank A) week period between salinity acclimation and acute salinity transfer (see Table 4.4). Whereas the mean length and weight for the two tanks of finnock were similar, the mean condition factor of the freshwater-adapted group was lower than that of the seawater-adapted group, and had decreased in the eleven weeks since

Table 4.3

Table 4.4

Table 4.3 Back-transformed mean and 95% confidence limits (C.L.) of length (mm), weight (g) and condition factor (K) for two tanks of finnock (A and B) prior to mixing.

Table 4.4 Back-transformed mean and 95% confidence limits (C.L.) of length (mm), weight (g) and condition factor (K) for two tanks of finnock (A and B) after mixing.

	Tank A (n = 92)			Tank B (n = 71)		
	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
Length (mm)	264	-8	+8	265	-12	+13
Weight (g)	191	-18	+19	196	-27	+31
K	1.04	-0.02	+0.02	1.05	-0.02	+0.02

	Tank A (n = 82)			Tank B (n = 81)		
	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
Length (mm)	262	-11	+10	267	-9	+10
Weight (g)	186	-22	+25	200	-21	+24
K	1.04	-0.02	+0.02	1.05	-0.02	+0.02

Table 4.5

Table 4.5 Back-transformed means with 95% confidence limits (C.L.) for plasma osmolality (mOsmol.kg $^{-1}$) and Cl^- concentration (mmol.l $^{-1}$) for pooled batches of finnock sampled after acclimation to different salinities during the stepwise seawater-freshwater acclimation.

% seawater	Number of days	osmolality ($mOsmol.kg^{-1}$)			chloride ($mmol.l^{-1}$)		
		mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
100	87	333	-9	+8	145	-8	+7
75	4	336	-8	+7	145	-5	+6
50	8	326	-6	+6	145	-5	+4
25	6	319	-6	+6	137	-5	+6
0	3	316	-6	+8	129	-3	+4

Table 4.6

Table 4.6 Back-transformed mean and 95% confidence limits (C.L.) of length (mm), weight (g) and condition factor (K) for two tanks of finnock (A and B) when sampled for drinking rate measurement.

	Tank A (n = 42)			Tank B (n = 47)		
	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
Length (mm)	294	-12	+11	288	-9	+10
Weight (g)	230	-28	+30	241	-27	+29
K	0.91	-0.03	+0.03	1.01	-0.04	+0.04

these finnock were transferred to freshwater. This reduction in mean condition factor may have been partly due to a qualitative reduction in the amount of food consumed, but the physiological trauma and recovery associated with the high chlorine incident may have contributed also to this result.

4.3.2.3.2 Physiological data

4.3.2.3.2.1 Freshwater to seawater transfer (Tank A)

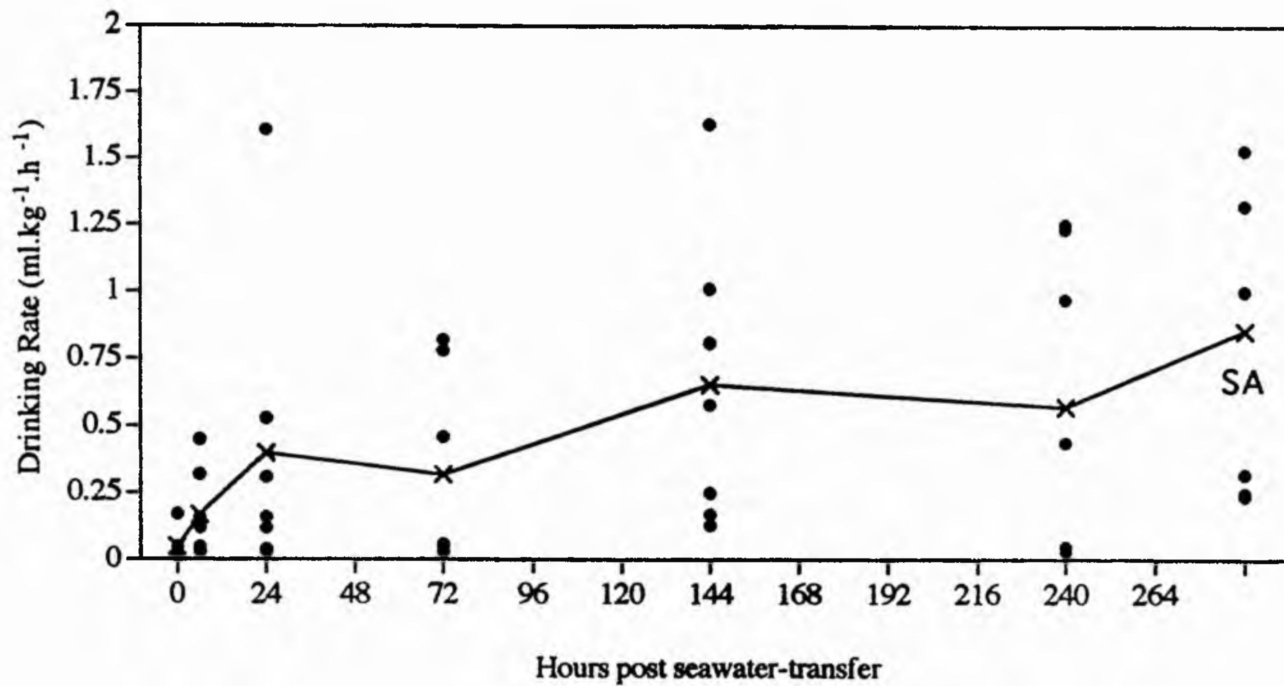
Figure 4.9 presents summary data for drinking rates for groups of seven freshwater-adapted finnock at time 0 and 6, 24, 72, 144 and 240 hours after acute challenge to seawater, with data from the seawater-adapted group for comparison. Large variations were observed between the individual drinking rates of finnock within separate time samples, thus, for example, it would appear that even 240 hours post-transfer, some finnock were drinking at very low levels. This variation may have been due burst drinking amongst these finnock rather than the finnock not drinking in seawater (see Discussion). Statistical analysis of these drinking rate data was therefore complicated by this variation. It is impossible to separate the maximal and minimal drinking rates from intermediaries and as such, statistical comparisons between time points would be ambiguous. However, for illustrative purposes, a scatter plot of the drinking rates at each time sample, with the mean drinking rates superimposed is presented in Figure 4.9. It is difficult to accurately describe the changes in drinking rate after seawater-transfer because this variation obscured any reference points on which to base comparisons. However, the mean values will be considered as a qualitative indicator. Mean drinking rates increased within six hours of seawater-challenge but appeared to require in excess of 72 hours to reach a level similar to fully seawater-adapted finnock.

Mean plasma osmolality increased significantly within six hours of transfer from freshwater to seawater and remained significantly greater than the freshwater-adapted mean at all subsequent time points (Figure 4.10, Tables 4.25 and 4.26). Mean plasma osmolality peaked at 72 hours after transfer, at which time it was significantly greater than the six hour mean, and then declined once more, although no further significant differences were indicated (Table 4.26).

As expected, mean plasma Cl^- concentration displayed a pattern similar to that of mean plasma osmolality (Figure 4.11). Mean Cl^- concentration increased

Figure 4.9

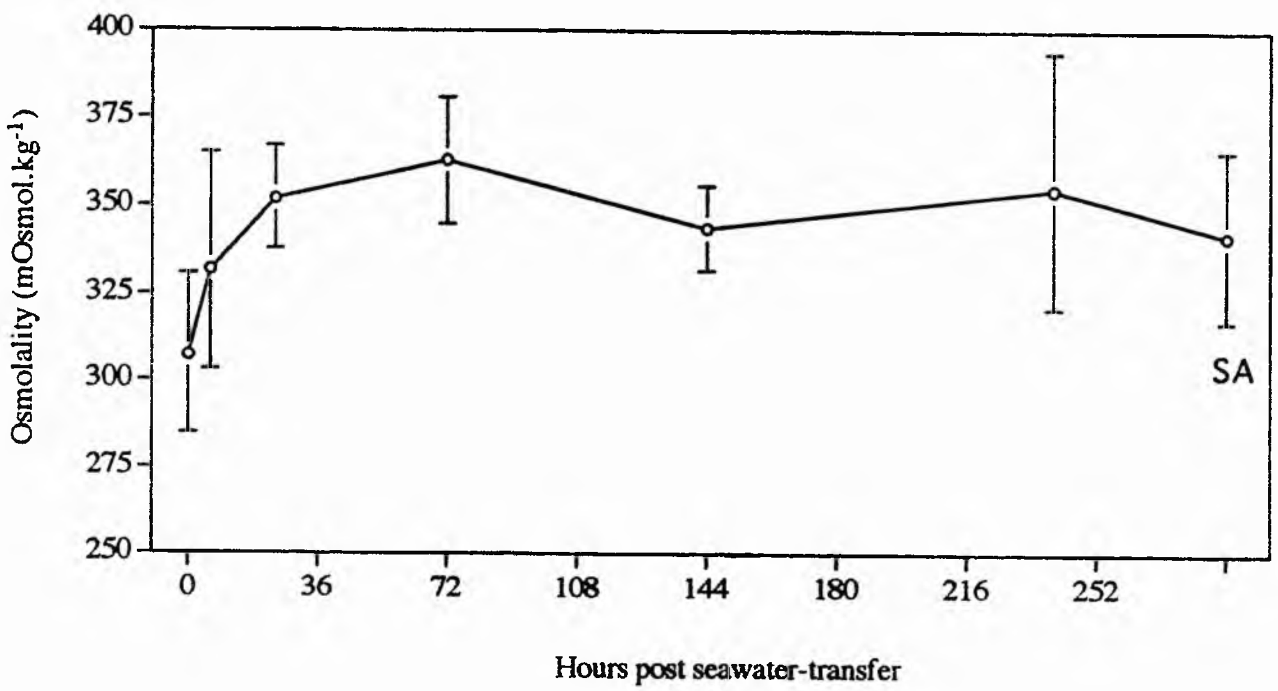
Figure 4.9 Change in mean drinking rate (ml.kg $^{-1}$.h $^{-1}$) of groups of seven freshwater-adapted finnock measured prior to, and at 6, 24, 72, 144 and 240 hours after, acute seawater-transfer. The final group are seawater-acclimated finnock (SA). The mean value are represented by the 'x', and the scatter of solid circles above and below each 'x' represent the drinking rates of individual finnock at that time point.



Drinking Rate (ml.kg-1.h-1)
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0
24
48
72
96
120
144
168
192
216
240
264
SA
Hours post seawater-transfer

Figure 4.10

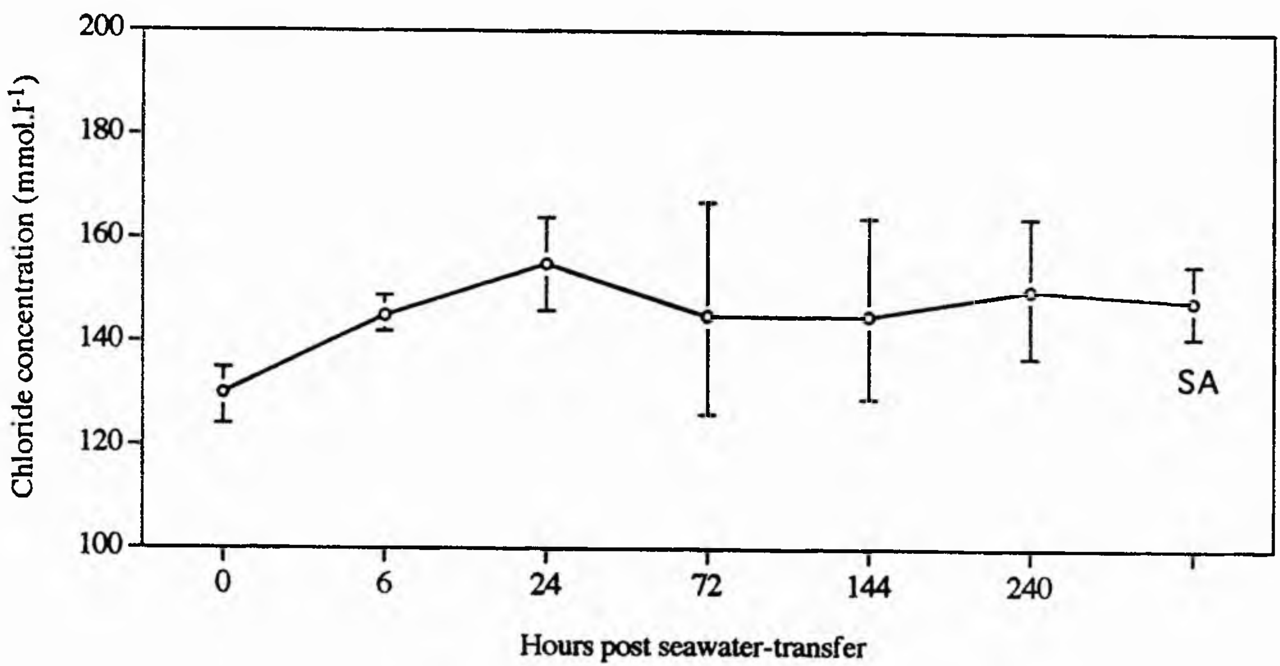
Figure 4.10 Change in mean plasma osmolality (mOsmol.kg $^{-1}$) of groups of seven freshwater-adapted finnock measured prior to, and at 6, 24, 72, 144 and 240 hours after, acute seawater-transfer. The final group are seawater-acclimated finnock (SA).



Osmolality (mOsmol.kg-1)
400
375
350
325
300
275
250
0
36
72
108
144
180
216
252
Hours post seawater-transfer
SA

Figure 4.11

Figure 4.11 Change in mean plasma Cl^- concentration ($mmol.l^{-1}$) of groups of seven freshwater-adapted finnock measured prior to, and 6, 24, 72, 144 and 240 hours after, acute seawater-transfer. The final group are seawater-acclimated finnock (SA).


Chloride concentration (mmol.l-1)
200
180
160
140
120
100
0
6
24
72
144
240
SA
Hours post seawater-transfer

significantly above that of the freshwater-adapted groups within six hours post-transfer and was significantly greater than the freshwater-adapted mean at all subsequent time points (Table 4.27 and 4.28). The mean value peaked at 24 hours post-transfer before stabilizing at a lower value from 72 hours onwards, although there were no statistically significant differences between means at any time points after seawater-transfer.

Mean branchial $Na^{+}K^{+}$-ATPase enzymatic activity demonstrated a slight increase above that of the freshwater-adapted value at 144 hours post-transfer (Table 4.7) but was not significantly greater than the freshwater-adapted control value until 240 hours post-transfer (Tables 4.29 and 4.30). No relationship was visually apparent between branchial $Na^{+}K^{+}$-ATPase activity and any of the other physiological parameters measured in freshwater-adapted finnock acutely transferred to seawater.

Mean haematocrit values did not vary significantly different throughout the experiment (Table 4.8).

Plasma osmolality and Cl^{-} concentration and haematocrit for those finnock which displayed uncharacteristically low drinking rates in seawater were similar to those of finnock which had higher drinking rates.

Mean plasma cortisol concentration peaked 72 hours after seawater-transfer before declining by 144 hours post-transfer (Table 4.9). The mean concentration of seawater-adapted finnock was considerably greater than those sampled at any other time points. However, individual variation within groups was considerable and, as a consequence, 1-way ANOVA did not indicate any significant differences.

4.3.2.3.2.2 Seawater to freshwater challenge (Tank B)

Mean drinking rate fell significantly within six hours post-transfer to a low level, not significantly different from the freshwater-adapted group, for the remainder of the experimental period (Figure 4.12, Tables 4.31, 4.32).

Mean plasma osmolality decreased within six hours of transfer (Figure 4.13) and had continued to decrease slightly by 24 and 48 hours post-transfer to a mean concentration similar to that of the freshwater-adapted group, although none of these means were significantly different from that of the seawater-adapted group.

Table 4.7

Table 4.7 Back-transformed mean and 95% confidence limits (C.L.) of branchial Na^+K^+-ATPase enzymatic activity in groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after transfer to seawater, along with those from freshwater-adapted and seawater-adapted finnock. Units of enzymatic activity are μmoles Pi.mg protein $^{-1}$.h $^{-1}$.

Time after transfer (hours)	mean	lower C.L.	upper C.L.
Freshwater-adapted	1.016	-0.518	+1.064
6	0.979	-0.528	+1.144
24	1.626	-0.813	+1.617
72	1.208	-0.603	+1.202
144	1.972	-0.905	+1.676
240	2.831	-1.572	+3.522
Seawater-adapted	4.046	-2.743	+8.845

Table 4.8

Table 4.9

Table 4.8 Back-transformed mean and 95% confidence limits (C.L.) of haematocrit values in groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after freshwater-seawater transfer, along with those from freshwater-adapted and seawater-adapted finnock. Haematocrit is expressed as a proportion.

Table 4.9 Back-transformed mean and 95% confidence limits (C.L.) of plasma cortisol concentration ($ng.ml^{-1}$) in groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after freshwater-seawater transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Time after transfer (hours)	mean	lower C.L.	upper C.L.
Freshwater-adapted	0.32	-0.04	+0.04
6	0.34	-0.04	+0.06
24	0.30	-0.04	+0.03
72	0.35	-0.05	+0.05
144	0.29	-0.05	+0.05
240	0.29	-0.07	+0.07
Seawater-adapted	0.33	-0.03	+0.03

Time after transfer (hours)	mean	lower C.L.	upper C.L.
Freshwater-adapted	32	-22	+74
6	45	-20	+38
24	41	-14	+22
72	73	-24	+32
144	45	-24	+39
240	37	-21	+49
Seawater-adapted	97	-41	+72

Figure 4.12

Figure 4.12 Change in mean drinking rate ($ml.kg^{-1}.h^{-1}$) of groups of eight seawater-adapted finnock measured prior to, and at 6, 24, 48, 72 and 144 hours after, acute freshwater-transfer. The final group are freshwater-acclimated finnock (FA).

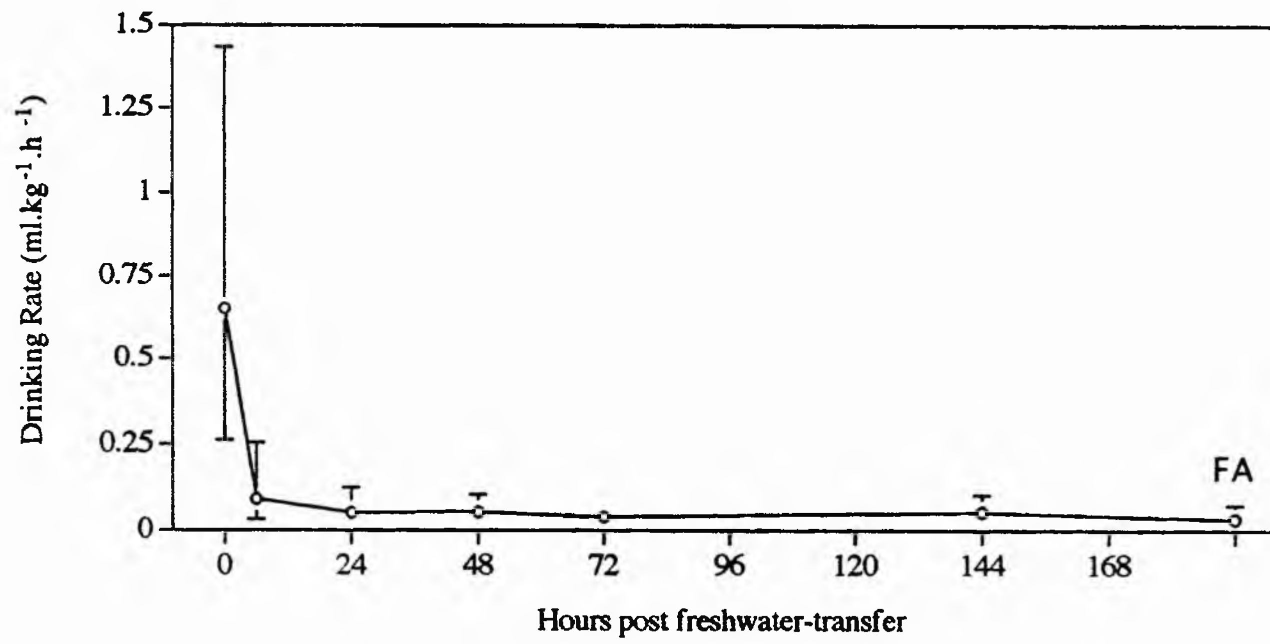
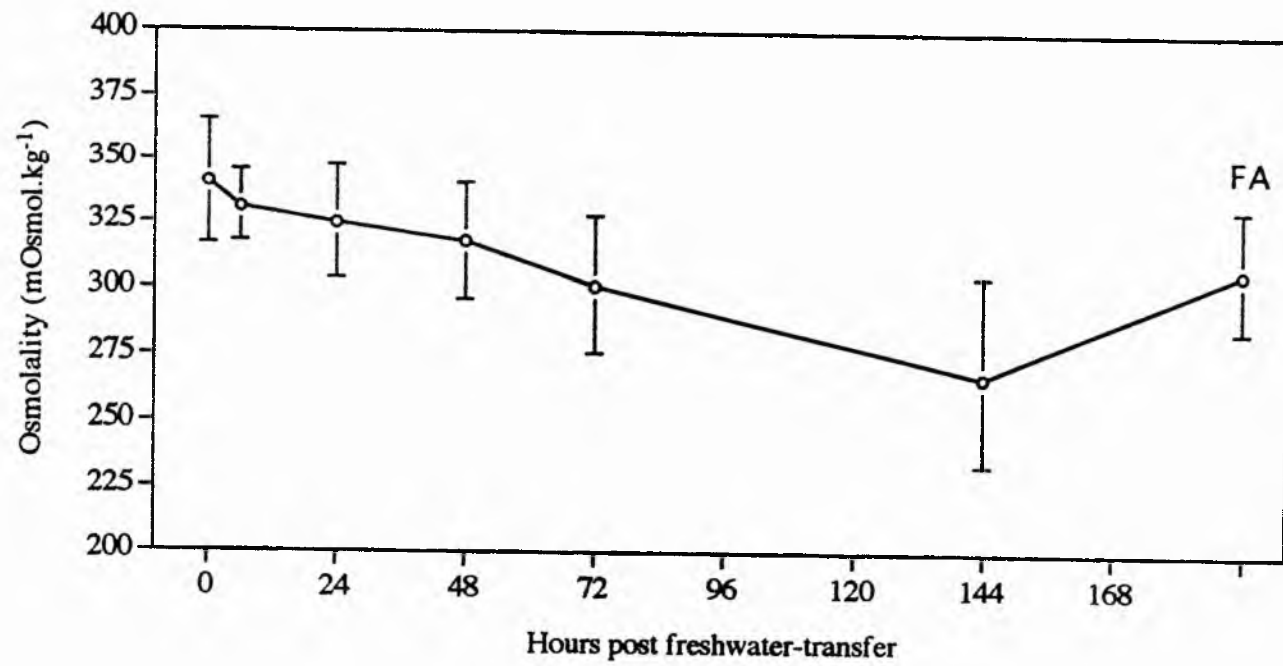

1.5
1.25
1
0.75
0.5
0.25
0
Drinking Rate (ml.kg-1.h-1)
FA
0
24
48
72
96
120
144
168
Hours post freshwater-transfer

Figure 4.13

Figure 4.13 Change in mean plasma osmolality (mOsmol.kg $^{-1}$) of groups of eight seawater-adapted finnock measured prior to, and at 6, 24, 48, 72 and 144 hours after, acute freshwater-transfer. The final group are freshwater-acclimated finnock (FA).


400
375
350
325
300
275
250
225
200
Osmolality (mOsmol.kg-1)
0
24
48
72
96
120
144
168
Hours post freshwater-transfer
FA

However, 1-way ANOVA did indicate a significant difference between means when comparing the complete dataset (Table 4.33): mean osmolality concentration continued to decline at 72 hours post-transfer and displayed a further marked decrease at 144 hours to reach a level significantly lower than all other time points including the freshwater-adapted group (Table 4.34). As noted above, all the remaining acute seawater-freshwater transferred finnock died prior to the planned 10 days sample. The uncharacteristically low mean osmolality at 144 hours post-transfer presumably indicated the initial signs of severe, and ultimately fatal, physiological trauma.

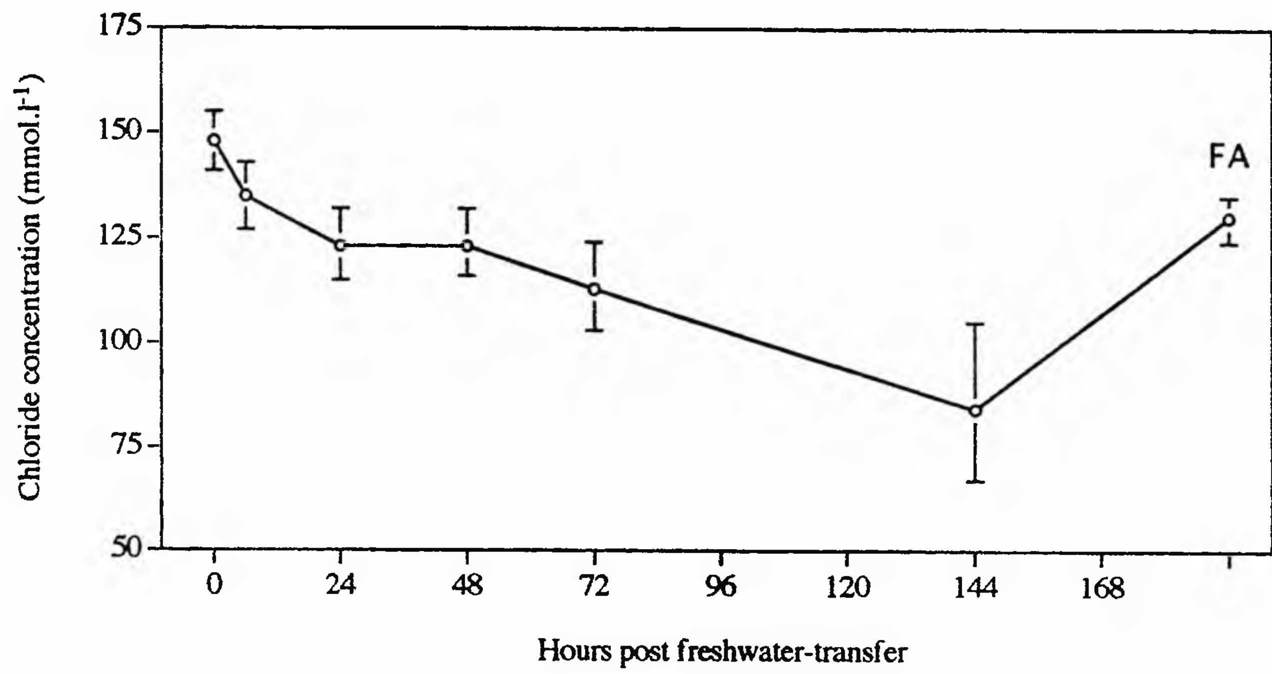
Mean plasma Cl^- concentration displayed a similar pattern to that of mean plasma osmolality described above (Figure 4.14). Mean plasma Cl^- concentration fell within six hours of transfer and reached a level significantly lower than the seawater-adapted group (Table 4.35, Table 4.36), and not significantly different from that of the freshwater-adapted group, within 24 hours of freshwater-transfer. Mean concentration appeared similar between 24 and 48 hours post-transfer but began to decline once more between 48 and 72 hours post-transfer to reach a minimum level at 144 hours post-transfer, at which time the mean concentration was significantly lower than at all other sample points (Table 4.36).

Mean branchial Na^+K^+-ATPase enzymatic activity decreased initially at six hours after freshwater-transfer but had returned to a level similar to that of the seawater-adapted group within a further 18 hours (Table 4.10). The mean level of activity had declined once more by 48 hours post-transfer to a level qualitatively similar to that of the freshwater-adapted group. However, 1-way ANOVA did not indicate any significant differences amongst the means during the experimental period. This was probably due to the considerable variation in activity levels within sample groups.

Mean haematocrit values displayed significant differences during the experimental period (Table 4.37). The mean value increased above that of the seawater-adapted group during the initial 24 hours post-transfer (Figure 4.15), the difference being significant at 24 hours (Table 4.38). The mean value then declined once more by 48 hours post-transfer to return to a level not significantly different from that of either the seawater-adapted or freshwater-adapted groups. However, the mean value increased again at 72 hours post-transfer and reached a level significantly greater than the freshwater-adapted group at 144 hours post transfer.

Figure 4.14

Figure 4.14 Change in mean plasma Cl^- concentration ($mmol.l^{-1}$) of groups of eight seawater-adapted finnock measured prior to, and at 6, 24, 48, 72 and 144 hours after, acute freshwater-transfer. The final group are freshwater-acclimated finnock (FA).



175
150
125
100
75
50
Chloride concentration (mmol.l-1)
FA
0
24
48
72
96
120
144
168
Hours post freshwater-transfer

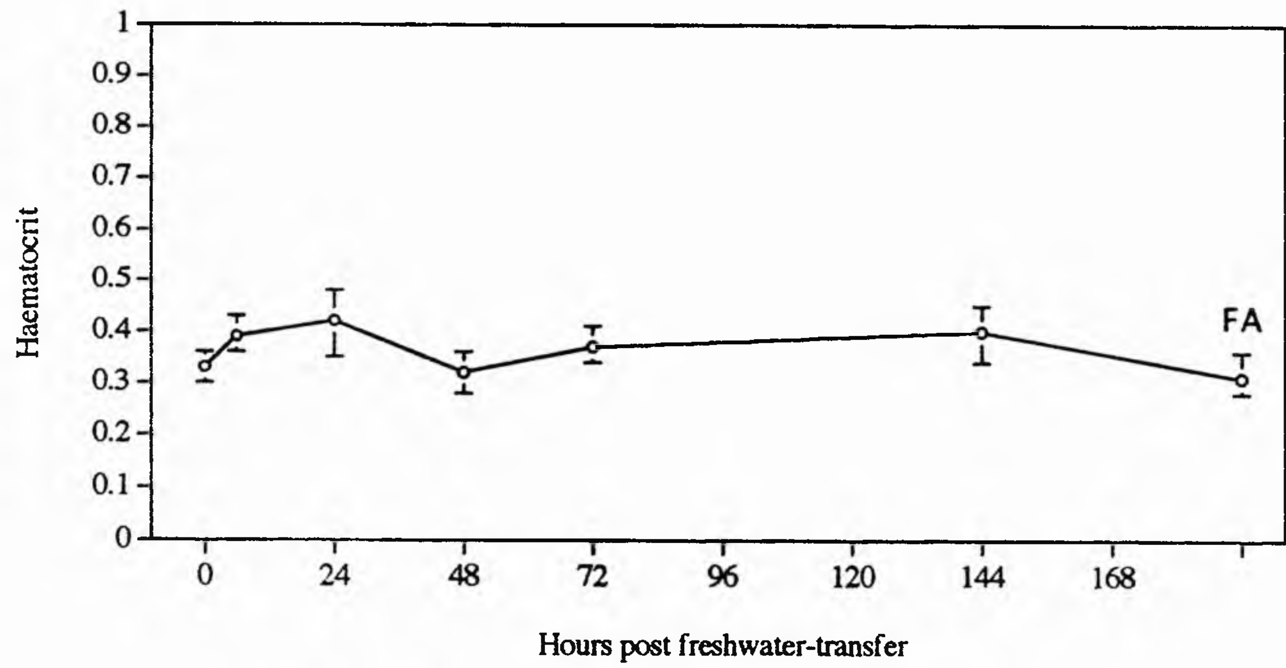
Table 4.10

Table 4.10 Back-transformed mean and 95% confidence limits (C.L.) of branchial $Na^{+}K^{+}$-ATPase enzymatic activity in groups of eight finnock sampled at 6, 24, 48 and 72 hours after seawater-freshwater transfer. Units of enzymatic activity are μmoles Pi.mg protein $^{-1}$.h $^{-1}$

Time after transfer (hours)	mean	lower C.L.	upper C.L.
Seawater-adapted	4.05	-2.75	+8.48
6	2.33	-1.67	+5.91
24	3.95	-3.29	+19.60
48	1.57	-1.02	+2.90
72	1.62	-1.46	+15.32
Freshwater-adapted	1.02	-0.52	+1.06

Figure 4.15

Figure 4.15 Change in mean haematocrit of groups of eight seawater-adapted finnock measured prior to, and at 6, 24, 48, 72 and 144 hours after, acute freshwater-transfer. The final group are freshwater-acclimated finnock (FA). Haematocrit is expressed as a proportion.


Haematocrit
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
FA
0
24
48
72
96
120
144
168
Hours post freshwater-transfer

Mean plasma cortisol concentration decreased during the first 48 hours post-transfer (Table 4.11) but rose again to a considerably higher level by 144 hours post-transfer. One-way ANOVA indicated a significant difference, at least between the highest and lowest means, e.g. the 48 hour and 144 hour means (Table 4.39). However, post-hoc means comparisons did not support this analysis, presumably because of the greater statistical power of the ANOVA compared to post-hoc means comparisons, and the considerable variation in cortisol concentration within sample groups.

Table 4.11

Table 4.11 Back-transformed mean and 95% confidence limits (C.L.) of plasma cortisol concentration ($ng.ml^{-1}$) in groups of eight finnock sampled at 6, 24, 48, 72 and 144 hours after seawater-freshwater transfer.

Time after transfer (hours)	mean	lower C.L.	upper C.L.
Freshwater-adapted	95	-52	+117
6	80	-21	+28
24	36	-22	+59
72	24	-11	+18
144	39	-24	+61
240	104	-67	+189
Seawater-adapted	32	-22	+74

4.3.3 Discussion

4.3.3.1 Summary of results

- Freshwater-adapted finnock, hatchery-reared resident trout and wild resident trout were transferred to seawater for 6 hours
  - All three groups displayed osmoregulatoy changes typical of euryhaline teleosts
  - Increases in drinking rates, plasma osmolality, concentrations of plasma Na^+ and Cl^-. Plasma cortisol concentration increased in finnock and wild resident trout but not in hatchery-reared resident trout
- Freshwater-adapted finnock were transferred to seawater and sampled periodically over 10 days
  - Drinking rates were unusually variable but there was a trend to increase within 6 hours and to have stabilised at a higher rate after 72 hours. Plasma osmolality and concentrations of Cl^- and cortisol had stabilized at new levels within 72 hours of transfer whereas branchial Na^+K^+-ATPase activity did not increase significantly until day 10.
- Seawater-adapted finnock were transferred to freshwater and sampled periodically over the next 6 days
  - Drinking rates decreased to basal within 6 hours whereas plasma osmolality and concentrations of Cl^- and cortisol fell after transfer to stabilize within 48 hours. There was no significant change in branchial Na^+K^+-ATPase activity.
  - Finnock transferred to freshwater suffered suspected chlorine poisoning 48 hours after transfer which was manifested by further decreases in plasma osmolality and Cl^- concentration, an increase in plasma cortisol concentration and the death of all remaining finnock between days 6 and 9.

4.3.3.2 Acclimation to seawater

The general aim of this study was to ascertain whether finnock demonstrated any physiological problems when challenged to seawater during the winter, in order to confirm or refute the possibility that the freshwater overwintering behaviour of finnock was due to hypo-osmoregulatory problems. The results of Section 4.2 indicated that finnock could survive in seawater during the winter months and displayed typical drinking rates, plasma ion levels and plasma cortisol concentrations when acclimated to seawater and after acute freshwater-seawater

transfer. The aim of this second experiment was to investigate further the seawater acclimation of finnock over a longer period during the winter to ascertain whether more subtle physiological problems would become apparent.

Acclimation of euryhaline fish to seawater can be separated into two distinct phases. An adjustive 'crisis' phase characterized by increased plasma osmotic concentration as fish gain ions and lose fluid to the surrounding environment (Houston, 1960), followed by a regulative 'stabilization' phase during which control is established over the osmotic and ionic concentration (Jackson, 1981). This latter phase begins usually some 24 - 48 hours post-transfer and leads, over the next seven to ten days, to the successful adaptation of the fish to the new environment. The data presented in this chapter indicate that finnock, in general, demonstrate such typical euryhaline responses to changes in environmental salinity during the winter months when most inhabit the freshwater or estuarine environments and few have been observed in the sea.

Marine teleosts must drink seawater to replace fluids lost by osmosis while excreting ions to compensate for the influx of ions. In the present study, upon freshwater-seawater transfer, large variations in drinking rates were observed between individual finnock within groups sampled at each time point: some finnock drank at the expected rate of approximately 1 $ml.kg^{-1}.hr^{-1}$ whereas others had drunk considerably less over the six hour experimental period. Such variation was also apparent in the seawater-adapted finnock, although not to such an extreme.

Plasma osmolalities, Cl^- concentrations and haematocrit values for those finnock with low drinking rates were within the ranges of those drinking at the expected rates. In addition, no mortalities occurred during the experimental period. These observations indicate that the finnock with low drinking rates were apparently not suffering any extra physiological stress, but were 'burst' drinking. Rather than drinking continuously, these finnock appear to drink relatively large quantities of medium over short periods of time, after which further drinking is inhibited, most probably by the presence of stretch receptors in the intestine, as demonstrated in the Japanese eel (Hirano, 1974), and does not commence again until much of the ingested water has been absorbed into the body. As such, if this period between bursts of drinking is greater than the experimental period (six hours) then some finnock might appear to drink very little during this time. Other finnock, which have started, or ceased, drinking during the experimental period would display intermediate drinking rates. This is proposed as an explanation for the apparent low

drinking rates of some finnock transferred from freshwater to seawater. The variation could have been reduced experimentally by increasing the time period adopted for the drinking rate measurement.

The presence of such large variation complicates any statistical analysis and even any accurate description of the changes in drinking rate with time after transfer. If the data were split into those finnock which were drinking at expected rates and those which were drinking considerably less then the latter could be excluded from the analysis. However, the presence of drinking rate data between the two extremes and the inability to discriminate between maximal and intermediate values precludes this selection of the data. As a consequence, the data were presented as mean values superimposed upon a scatter plot and the mean data will be discussed, with the understanding that the mean values are not as representative as one would normally expect.

Mean drinking rates increased rapidly over the first 24 hours post-transfer and, as implied by Season One data, continued to increase until 144 hours post-transfer. Atlantic salmon smolts transferred from freshwater to seawater required a similar period of time to equilibrate drinking rate at a new, higher level (Usher *et al.*, 1988).

In the present study, a slight decline in mean drinking rate at 72 hours post-transfer was followed by a further increase at 144 hours post-transfer. A similar brief decline in drinking rates was observed in Atlantic salmon smolts after 48 hours in seawater (Usher *et al.*, 1988). Hirano (1974) demonstrated the brief inhibition of drinking in seawater-adapted eel after the increase of extracellular ion concentrations by single injection of hypertonic NaCl. Mean plasma Cl^- concentration in the present study increased rapidly after transfer to peak at 24 hours post-transfer. This implies that the rapid increase in drinking rates upon seawater-transfer would quickly be inhibited, although not completely, by the similar rapid increase in plasma ion levels. Drinking rates then increased again between 72 and 144 hours post-transfer, coincident with lower plasma ion concentrations.

The mean drinking rates recorded in this study are not strictly comparable with those of other studies because of the presence of drinking rates approaching zero. However, the pattern of increase is similar to that of Atlantic salmon transferred to seawater (Usher *et al.*, 1988; Smith *et al.*, 1991) and the maximal drinking rates in the current study are similar to the mean values for seawater-adapted rainbow trout in Perrott *et al.* (1992).

As indicated above, plasma osmolality and Cl^- concentrations increased rapidly after seawater-transfer to peak at 24 hours before declining to stabilize at lower concentrations by 72 hours post-transfer, although still significantly greater than freshwater-adapted finnock. This pattern of recovery is similar to that reported for other salmonids (Hogstrand and Haux, 1985; Hughes *et al.*, 1986; Usher *et al.*, 1992; Franklin *et al.*, 1992; Halvorsen *et al.*, 1993).

Increasing plasma ion concentrations are the result of the passive influx of ions from the hypertonic media and the active ion absorption across the gut to facilitate the absorption of water (see Section 4.2.3). In seawater-adapted fish, excess monovalent ions are excreted via branchial processes while divalent ions are excreted via the renal pathway (see Section 1.2). High levels of branchial Na^+K^+-ATPase activity have been associated with seawater-adaptation in a variety of teleosts (Sargent *et al.*, 1975; Langdon and Thorpe, 1984). However, levels were not observed to increase significantly for several days after seawater-transfer in sockeye salmon (Franklin *et al.*, 1992), or Atlantic salmon smolts (Langdon and Thorpe, 1984). The reduction in drinking rates indicated above will reduce the body salt load to some extent but other temporary compensatory mechanisms must be involved prior to the established increase in branchial effluxes.

The permeability of the gill membrane is reduced by pH changes at the membrane surface, and the interaction of Ca^{2+} with the superficial membrane sites associated with monovalent ion transport (see Folmar and Dickhoff, 1980). A further temporary compensatory mechanism which has been reported to reduce the rate and magnitude of plasma ion increases upon seawater-transfer is a phase shift of ions to the musculature, which act as a buffer system until more efficient and appropriate ionoregulatory mechanisms can be initiated.

More recently, a rapid increase in Na^+K^+-ATPase activity has been demonstrated in the eel within six hours of seawater-transfer (Luke *et al.*, 1994). The three-fold increase in enzymatic activity above that of freshwater-adapted eel then declined over the following 18 hours. As there was no increase in mRNA expression at this time, the mechanism of upregulation was considered to be either the recruitment of pre-formed sequestered units or direct activation by phosphorylation/ dephosphorylation. However, a third possible mechanism is implied by the presence of Ang II receptors in the gill of rainbow trout (Cobb and Brown, 1992) and in the eel (Tierney, 1993; Marsigliante *et al.*, 1997). Using immunocytochemical methods,

Marsigliante *et al.* (1997) demonstrated that these receptors were present at three-fold higher concentration in the chloride cells of seawater-adapted versus freshwater-adapted eels. In addition, perfusion of seawater-adapted gills for 30 minutes with 0.1, 10 or 100 nM Ang II resulted in a dose dependent increase in $Na^{+}K^{+}$-ATPase activity in freshwater gills whereas a similar perfusion protocol in seawater gill produced a biphasic response in which enzymatic activity was significantly increased.

The subsequent increase in $Na^{+}K^{+}$-ATPase activity some 4 - 12 days after seawater-transfer is considered to be due to increase in enzyme units, in chloride cell number and in chloride cell hypertrophy (Folmar and Dickhoff, 1981; Langdon and Thorpe, 1984; Madsen and Naamansen, 1989; Franklin *et al.*, 1992). Clearly, such effects require marked changes or modifications of the gill epithelium at the cellular level. Changes in the structure and relative distribution of different chloride cell subtypes take place but these changes take longer than the 48 - 96 hours required for euryhaline teleosts to reach ionic and osmotic homeostasis. *De novo* synthesis of gill epithelial proteins requires four to six days in coho salmon (Conte and Lin, 1967). Chloride cell diameter was unaffected by 24 hour seawater-transfer in Atlantic salmon smolts but had increased to the size observed in long-term seawater-adapted smolts by day 14 (Langdon and Thorpe, 1984). The percentage yield of chloride cells was unchanged at day 14 but had nearly doubled by day 28 (Langdon and Thorpe, 1984). It is clear, therefore, that the chloride cells are not the only site of significant ion extrusion in the teleost gill and other epithelial cell types such as pavement cells are currently under investigation.

Mean haematocrit values displayed small increases at 6 and 72 hours post transfer but, in general, displayed little variation throughout the experimental period. Haematocrit has been reported to increase (Franklin *et al.*, 1992) or decrease (Bath and Eddy, 1979) after seawater transfer. Exercise to fatigue results in an increase in haematocrit in freshwater-adapted coho parr but a decrease in seawater-adapted parr (Brauner *et al.*, 1992). An increase in haematocrit can constitute a stress response in fish but since an increase in haematocrit can occur due to such a variety of circumstances, e.g. a decrease in plasma volume (associated with dehydration), by recruitment of erythrocytes from the spleen, and/or by the swelling of erythrocytes (Nikinimaa, 1986; Wells and Weber, 1990), it would be improper to base conclusions about osmoregulation on variation in haematocrit alone.

The control of these adaptive and regulatory mechanisms is due to the function of a variety of hormones. Section 1.3 summarizes the role and effects of various hormones implicated in seawater-adaptation. One hormone of particular importance in seawater-adaptation is cortisol, which has, for example, been implicated in chloride cell proliferation and hypertrophy in freshwater-adapted rainbow trout (Laurent and Perry, 1990). Levels of plasma cortisol increase after acute freshwater-seawater transfer, as observed in Section 4.2.2, but are typically low in freshwater-adapted, and seawater-adapted fish. In the present study, however, mean plasma cortisol concentrations in seawater-adapted and freshwater-adapted groups were considerably higher than those described in Section 4.2.2.

The basal levels of plasma cortisol concentrations in the freshwater-adapted and seawater-adapted finnock in Season One indicate that the experimental protocol should not have resulted in undue stress which would have explained the high plasma cortisol concentrations.

The finnock to be sampled after control seawater-seawater transfer were moved to a smaller tank (400 l) at the beginning of the seawater-freshwater transfer protocol. This might have resulted in a brief period of confinement stress. However, the control seawater-seawater experimental transfer using these finnock took place 30 days after they were transferred to the smaller tank. Such a period of time would be considered sufficient to allow the finnock to acclimate to the new tank conditions and, therefore, confinement within the smaller tank was unlikely to have contributed to the high plasma cortisol concentrations. In addition, the control freshwater-freshwater transfer group was sampled from the freshwater-adapted stock group (Tank B) before the salinity was increased to seawater in this tank. These finnock also, which were not transferred to a smaller tank, demonstrated unexpectedly high plasma cortisol concentrations.

The freshwater-freshwater finnock and those transferred from freshwater to seawater in the present study were the survivors from the previous chlorine contamination of the freshwater supply. Perhaps, therefore, the unexpectedly high levels of circulating cortisol could have indicated physiological mechanisms attempting to repair the damage caused by the chlorine, especially to the gills. However, this is speculative and would not explain the high plasma cortisol concentrations observed in some of the seawater-adapted group, which were not exposed to the chlorine in the freshwater supply. Therefore, no obvious reason(s)

are apparent at this time for the unexpectedly high mean plasma cortisol concentrations.

However, despite these high levels of circulating cortisol, the data do qualitatively indicate a rise in plasma concentration up to 72 hours post-transfer followed by a further decline, as would be expected in the typical euryhaline response to freshwater-seawater transfer.

4.3.3.3 Acclimation to freshwater

4.3.3.3.1 Stepwise acclimation

Finnock transferred in step-wise manner from seawater to freshwater demonstrated only small changes in plasma osmolality and Cl^- concentrations between different salinities. This indicated, as expected, that finnock were capable of responding physiologically to small incremental changes in salinity.

4.3.3.3.2 Acute transfer

As previously described in Section 1.2, the body fluids of freshwater-adapted teleosts are more concentrated than the surrounding medium and as a consequence, gain water and lose ions passively. The major osmoregulatory mechanisms involved in freshwater are the copious production of dilute urine and the active uptake of ions from the diet and the environment through branchial mechanisms. Thus, finnock that are transferred from seawater to freshwater must reverse hypo-osmoregulatory mechanisms in order to adapt to the new hypotonic conditions.

In the present study, mean drinking rates declined rapidly to reach basal levels within 24 hours of transfer. Once again, the data demonstrated that freshwater-adapted finnock do drink but that the rate is significantly lower than that of seawater-adapted finnock. Drinking rates in salmonids after seawater-freshwater transfer have not previously been reported in the literature. However, the rapid reduction in drinking rate of finnock to basal level similar to that of freshwater-adapted finnock indicates an efficient adaptive, inhibitory mechanism. Drinking rates in freshwater were inhibited during the initial (10 - 15 minute) hypertensive effect of bolus injection of Ang II to eels (Hirano and Hasegawa, 1984) and trout (Fuentes and Eddy, unpubl. cited in Fuentes and Eddy, 1997) but were stimulated by the subsequent hypotensive effect of Ang II in both studies. Drinking rates were

inhibited in eel transferred from seawater to freshwater, but only if the eel had been in seawater for less than 24 hours (Hirano, 1974). Drinking rates in seawater-adapted eels have been inhibited by increase in extracellular fluid concentration and stomach and intestinal distension (Hirano, 1974). However, neither condition would be expected after seawater-freshwater transfer.

Fluid regulation after seawater-freshwater transfer requires not only decreased drinking rates but also increased urine flow rate to excrete the excess water. A similar time scale of adaptation to that of drinking rates has been demonstrated for urine flow rate. Talbot *et al.* (1989) found that urine flow rates remained low for at least 25 hours after adult Atlantic salmon were transferred to freshwater, but increased considerably after 60 hours and had reached a typical freshwater-adapted rate after eight days.

However, such an increase in urine flow rate must be coupled with decreased urine ion concentrations in order to conserve ions in response to the passive losses to the environment across the integument. In the same study, Talbot *et al.* (1989) demonstrated that urine Cl^- and Mg^{2+} concentrations fell rapidly within 60 hours post-transfer (Cl^-: 139 to 13 mmol.l $^{-1}$, Mg^{2+}: 123 to 5 mmol.l $^{-1}$) and that Mg^{2+} levels had fallen further (0.5 mmol.l $^{-1}$) after long-term adaptation. The decrease in urine Na^+ concentration was slower, taking eight days to decline from 31 to 26 mmol.l $^{-1}$, but had declined further to 12 mmol.l $^{-1}$ by three months post-transfer. The slow renal response, as indicated by the lag to reduce urine Na^+ concentrations may be advantageous to species which move between salinities on a regular or frequent basis, given the hormonal and morphological changes, and the consequent energetic costs, of full renal adaptation to freshwater. Thus, temporary changes in salinity could be tolerated by other processes without a committed change in renal function (Talbot *et al.*, 1989). Alternatively, this may simply reflect the importance of the gills and/or gut rather than the kidney in maintaining plasma Na^+ levels.

In the present study, mean plasma osmolality and Cl^- concentration declined rapidly within six hours post-transfer before stabilizing at lower levels, similar to those of fully freshwater-adapted finnock, between 24 and 48 hours post-transfer. Such an initial decrease in plasma ion concentrations is due to the reversal of the osmotic gradients between the fish and the environment resulting in an increase in body fluid volume and the passive loss of ions across the integument.

In the present study, although the variation within sample groups was high, mean branchial Na^+K^+-ATPase enzymatic activity appeared to have qualitatively declined to a level lower than that of the seawater-adapted group by 72 hours post-transfer. In immature Atlantic salmon post-smolts, reversal of net whole Na^+ loss, gain of body water and decrease in plasma and muscle ions occurred after three days exposure to freshwater (Talbot and Potts, 1989). These immature salmon were of similar size (100-150g) to the finnock used in the present study but would not naturally have been expected to encounter freshwater until several months, if not one year, later. The pattern of response in adult Atlantic salmon was similar except that branchial electrolyte uptake was immediate whereas salmon post-smolts (Talbot and Potts, 1989) and small rainbow trout (Battram and Eddy, 1990) took several days to reach the same level. This suggests that the electrolyte pump is stimulated prior to freshwater entry in adults but not in post-smolts. However, salmon post-smolts do show a small increase in Na^+ uptake, possibly due to size/volume effects, almost immediately after freshwater transfer (Talbot and Potts, 1989).

Plasma prolactin levels are low in seawater-adapted finnock and do not return to freshwater-adapted levels immediately so it is unlikely that this hormone, which is considered the major freshwater hormone (Avella *et al.*, 1990), is involved in any change in ion transport while still in seawater (Potts *et al.*, 1989).

Although no mortalities occurred during the experimental protocol, eight finnock in the stock tank died 72 hours after seawater-freshwater transfer, two at 96 hours and all the finnock remaining in this tank died nine days post freshwater-transfer. Whereas none of the long-term freshwater-adapted finnock had died prior to this, 22 died over the following six days.

Mean plasma osmolality and Cl^- concentration declined again 48 hours after seawater-freshwater transfer to reach unexpectedly low means of 267 $mOsmol.kg^{-1}$ and 84 $mmol.l^{-1}$, respectively, at 144 hours. Such a further decline was not observed in either immature or adult Atlantic salmon (Potts *et al.*, 1989; Talbot and Potts, 1989; Battram and Eddy, 1990). The mortalities in the stock tank and the second decline in plasma osmolality and Cl^- concentrations were coincident with an increase in the dissolved chlorine level in the freshwater supply. The Water Board recorded the dissolved chlorine level as 0.08 ppm, more than twice the normal range (0.02 to 0.06 ppm). Some of the dead finnock had pale patches along the dorsal surface which might indicate chemical burns, although this is speculative.

Decreases in plasma ion concentrations have been observed in freshwater-adapted finnock following exposure to toxicants such as heavy metals, pesticides and chlorinated hydrocarbons (Jobling, 1995). An increase in the permeability of gill lamellae seems to be the major contributory factor and would lead to an increased efflux of ions and an enhancement of the osmotic uptake of water. An increase in water influx would, in turn, lead to increased urine production and thereby further exacerbate the loss of ions. In addition, the inhibition of active ion uptake by the cells in the gill epithelium that accompanies exposure to a range of toxicants contributes further to the negative ion balance.

Mean haematocrit rose initially after freshwater transfer but had returned to a level similar to that of fully adapted finnock within 48 hours. However, the mean value increased once more from 72 hours post-transfer onwards. Mean plasma cortisol concentration also declined during the first 48 hours post-transfer, although the variation within sample groups was considerable, as in the freshwater-seawater transferred groups. However, mean plasma cortisol concentration displayed a considerable increase once more by 144 hours post-transfer. Both plasma cortisol concentration and haematocrit have been suggested as indicators of stress in fish and such increases were most probably due to the high levels of chlorine in the freshwater supply. However, cortisol is also regarded as having a role in the control of branchial ionoregulatory mechanisms and therefore, the increased plasma cortisol concentrations may have indicated a physiological response to perturbations in ionoregulatory control, as indicated by the uncharacteristically low plasma osmolality and Cl^- concentrations.

The considerable variation in plasma cortisol concentration between finnock in each of the sample groups might have been due, in some part, to the individual variation in response to the chlorine pollution. The fact that mortalities amongst the freshwater-challenged and freshwater-adapted groups each occurred over a period of six days indicates the considerable variation in ability to withstand the pollution event.

The acute seawater-freshwater challenged finnock most probably succumbed to the pollution earlier than the freshwater-adapted finnock because the former were suffering from the combined physiological stresses of hyperosmoregulatory adaptation and the chemical pollution.

4.3.4 Conclusions

To summarise, wild finnock display typical euryhaline responses to acute and long-term seawater-challenges during the winter, although the variation in response between individuals is large. The response to freshwater-challenge was typical with rapid stabilization of plasma ion concentrations, prior to an elevated dissolved chlorine incident. Thus, a problem with hypo-osmoregulation would not appear to be a causal factor in the return of most finnock to estuarine and freshwater conditions. However, the finnock used in these experiments were cleaned of external parasites soon after capture. Recent evidence implicates ectoparasitic sea lice species in the premature return of post-smolts to freshwater, by way of osmoregulatory failure (see Chapter 5). The following chapter will investigate the physiological effects of sea lice infestations on wild post-smolt sea trout.

4.4 Statistical Tables

Table 4.12 Results of 2-way ANOVA comparing log drinking rates for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.13 Results of 2-way ANOVA comparing log plasma osmolality for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.14 Results of 2-way ANOVA comparing log plasma Na^+ concentrations for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.15 Results of 2-way ANOVA comparing log plasma Cl^- concentrations for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.16 Results of 2-way ANOVA comparing log plasma K^+ concentrations for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.17 Results of 2-way ANOVA comparing log plasma cortisol concentrations for finnock and hatchery-reared brown trout in freshwater, seawater and after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Table 4.18 Results of 1-way ANOVA comparing log plasma cortisol concentrations for finnock, hatchery-reared brown trout and wild brown trout after acute freshwater-seawater transfer. See Figure 4.3 a and 4.3 b for sample sizes.

Source	df	SS	MS	F	p
Salinity	2	3.103	1.551	35.077	< 0.001
Trout form	1	0.144	0.144	3.247	0.078
Salinity x trout form	2	0.050	0.025	0.561	0.5742
Residual	49	2.170	0.044		

Source	df	SS	MS	F	p
Salinity	2	0.036	0.018	29.771	<0.001
Trout form	1	< 0.001	< 0.001	0.474	0.494
Salinity x Trout form	2	0.009	0.004	7.181	0.002
Residual	49	0.029	0.001		

Source	df	SS	MS	F	p
Salinity	2	0.079	0.039	71.636	< 0.001
Trout form	1	0.002	0.002	4.382	0.042
Salinity x Trout form	2	0.004	0.002	3.728	0.031
Residual	49	0.027	0.001		

Source	df	SS	MS	F	p
Salinity	2	0.078	0.039	40.973	< 0.001
Trout form	1	0.009	0.009	9.263	0.004
Salinity x Trout form	2	0.001	< 0.001	0.364	0.697
Residual	49	0.047	0.001		

Source	df	SS	MS	F	p
Salinity	2	0.171	0.086	4.357	0.018
Trout form	1	0.035	0.035	1.799	0.186
Salinity x Trout form	2	0.010	0.005	0.259	0.773
Residual	49	0.963	0.020		

Source	df	SS	MS	F	p
Salinity	2	4.774	2.387	8.105	< 0.001
Trout form	1	2.163	2.163	7.345	0.009
Salinity x Trout form	2	5.629	2.814	9.555	< 0.001
Residual	49	14.432	0.295		

Source	df	SS	MS	F	p
Trout form	2	2.490	1.245	10.435	<0.001
Residual	24	8.614	0.359		

Table 4.19 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma cortisol concentrations for finnock, hatchery-reared brown trout and wild brown trout after acute freshwater-seawater transfer (Table 4.18).

Table 4.20 Significant t-test statistic for the comparison of mean plasma Cl^- concentrations for groups of six finnock from Batches I and II acclimated to 25% seawater.

Table 4.21 Results of 1-way ANOVA comparing log plasma osmolality for groups of 12 finnock acclimated to seawater, 75% seawater, 50% seawater, 25% seawater and freshwater. See Table 4.5 for number of days at each salinity.

Table 4.22 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma osmolality for groups of finnock acclimated to various salinities (Table 4.21).

Table 4.23 Results of 1-way ANOVA comparing log plasma Cl^- concentration for groups of 12 finnock acclimated to seawater, 75% seawater, 50% seawater, 25% seawater and freshwater. See Table 4.5 for number of days at each salinity.

Table 4.24 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma Cl^- concentration for groups of finnock acclimated to various salinities (Table 4.23).

Table 4.25 Results of 1-way ANOVA comparing log plasma osmolality for freshwater-seawater transferred finnock. Data analyzed are from groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after transfer to seawater, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.26 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma osmolality for freshwater-seawater transferred finnock (Table 4.25).

Means comparison		Diff.	Crit. Diff.
Hatchery-reared brown trout vs.	Finnock	1.228	0.722
	Wild brown trout	1.276	0.793

Mean Diff.	df	t	p
0.033	10	2.544	0.029

Source	df	SS	MS	F	p
Salinity	4	0.006	0.001	6.521	< 0.001
Residual	55	0.012	< 0.001		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	Seawater-adapted	0.021	0.017
	75% seawater	0.025	0.017
25% seawater	Seawater-adapted	0.018	0.016
	75% seawater	0.022	0.017

Source	df	SS	MS	F	p
Salinity	4	0.023	0.006	8.766	< 0.001
Residual	55	0.036	0.001		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	50% seawater	0.048	0.028
	Seawater-adapted	0.050	0.029
	75% seawater	0.050	0.030

Source	df	SS	MS	F	p
Time in seawater	6	0.023	0.004	4.919	0.011
Residual	33	0.025	0.001		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	24 hours	0.059	0.051
	240 hours	0.063	0.052
	72 hours	0.072	0.053

Table 4.27 Results of 1-way ANOVA comparing log plasma Cl^- concentrations for freshwater-seawater transferred finnock. Data analyzed are from groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after transfer to seawater, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.28 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma Cl^- concentrations for freshwater-seawater transferred finnock (Table 4.27).

Table 4.29 Results of 1-way ANOVA comparing log branchial Na^+K^+-ATPase activity for freshwater-seawater transferred finnock. Data analyzed are from groups of seven finnock sampled at 6, 24, 72, 144 and 240 hours after transfer to seawater, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.30 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log branchial Na^+K^+-ATPase activity for freshwater-seawater transferred finnock (Table 4.29).

Table 4.31 Results of 1-way ANOVA comparing log drinking rates for seawater-freshwater transferred finnock. Data analyzed are from groups of eight finnock sampled at 6, 24, 72 and 144 hours after transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.32 Results of significant post-hoc means comparisons subsequent to significant 1-way ANOVA comparing log drinking rates for seawater-freshwater challenged finnock (Table 4.31).

Table 4.33 Results of 1-way ANOVA comparing log plasma osmolality for seawater-freshwater transferred finnock. Data analyzed are from groups of eight finnock sampled at 6, 24, 72 and 144 hours after transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Source	df	SS	MS	F	p
Time in seawater	6	0.024	0.004	2.533	0.035
Residual	42	0.067	0.002		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	24 hours	0.077	0.066

Source	df	SS	MS	F	p
Time in seawater	6	1.850	0.308	2.961	0.020
Residual	33	3.436	0.104		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	Seawater-adapted	0.616	0.608
6 hours	Seawater-adapted	0.599	0.597

Source	df	SS	MS	F	p
Time in freshwater	6	8.185	1.364	8.676	< 0.001
Residual	46	7.233	0.157		

Means comparison		Diff.	Crit. Diff.
Seawater-adapted	Freshwater-adapted	1.292	0.630
	72 hours	1.215	0.619
	24 hours	1.103	0.606
	144 hours	1.091	0.588
	48 hours	1.081	0.563
	6 hours	0.853	0.521

Source	df	SS	MS	F	p
Time in freshwater	6	8.185	1.364	8.676	< 0.001
Residual	46	7.233	0.157		

Table 4.34 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma osmolality for seawater-freshwater transferred finnock (Table 4.33).

Table 4.35 Results of 1-way ANOVA comparing log plasma Cl^- concentrations for seawater-freshwater transferred finnock. Data analyzed are from groups of eight finnock sampled at six, 24, 72 and 144 hours after transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.36 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing log plasma Cl^- concentrations for seawater-freshwater transferred finnock (Table 4.35).

Table 4.37 Results of 1-way ANOVA comparing arcsine transformed haematocrit for seawater-freshwater transferred finnock. Data analyzed are from groups of eight finnock sampled at six, 24, 72 and 144 hours after transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Table 4.38 Results of significant post-hoc comparisons subsequent to significant 1-way ANOVA comparing arcsine haematocrit proportions for seawater-freshwater transferred finnock (Table 4.37).

Table 4.39 Results of 1-way ANOVA comparing log plasma cortisol concentrations for seawater-freshwater transferred finnock. Data analyzed are from groups of eight finnock sampled at 6, 24, 48, 72 and 144 hours after transfer, along with those from freshwater-adapted and seawater-adapted finnock.

Means comparison		Diff.	Crit. Diff.
144 hours	72 hours	0.051	0.047
	Freshwater-adapted	0.060	0.051
	48 hours	0.75	0.053
	24 hours	0.85	0.055
	6 hours	0.093	0.056
	Seawater-adapted	0.105	0.057

Source	df	SS	MS	F	p
Time in freshwater	6	8.185	1.364	8.676	< 0.001
Residual	46	7.233	0.157		

Means comparison		Diff.	Crit. Diff.
144 hours	72 hours	0.128	0.064
	48 hours	0.163	0.069
	24 hours	0.166	0.072
	Freshwater-adapted	0.188	0.074
	6 hours	0.205	0.076
	Seawater-adapted	0.244	0.077
72 hours	6 hours	0.077	0.077
	Seawater-adapted	0.116	0.076
48 hours	Seawater-adapted	0.081	0.074
24 hours	Seawater-adapted	0.078	0.072

Source	df	SS	MS	F	p
Time in freshwater	6	285.814	47.636	4.880	< 0.001
Residual	45	439.221	9.760		

Means comparison		Diff.	Crit. Diff.
Freshwater-adapted	6 hours	4.844	4.827
	144 hours	4.972	4.929
	24 hours	6.148	5.020
48 hours	24 hours	5.803	4.929
Seawater-adapted	24 hours	5.257	4.822

Source	df	SS	MS	F	p
Time in freshwater	6	2.691	0.449	2.887	0.019
Residual	42	6.526	0.155		

Chapter 5

Effects of *Lepeophtheirus salmonis* infestation on the hypo-osmoregulation of wild sea trout post-smolts

5.1 Introduction

The recent drastic declines in sea trout populations in many river systems of the western regions of Scotland and Ireland have been outlined in Section 1.4. Several possible causes have been investigated but evidence suggests an increased marine mortality during the post-smolt phase of the life cycle is a major contributing factor (Anon, 1993; Anon, 1994d; Anon, 1995; Whelan, 1993; Walker, 1994a). The two most plausible reasons for an increased mortality rate would seem to be an apparent increase in the numbers of sea lice, *Lepeophtheirus salmonis* and *Caligus elongatus*, particularly the former, infesting wild sea trout (Tully and Whelan, 1993; Tully *et al.*, 1993; Walker, 1994b; McVicar *et al.*, 1993, 1994), and recent unusually mild winters, which may have physiologically or behaviourally affected the downstream migrating smolts (Bohlin *et al.*, 1993; Whelan, 1993) (see Chapter 2). The current controversy that exists regarding the possible contributory role of sea cage based fish farming in the apparent increase in sea lice numbers will be discussed in Chapter 6.

The first indication that sea lice might be the cause of increased mortality of sea trout came from Ireland where adult and, in particular, post-smolt sea trout were observed to return prematurely to the estuarine areas of a number of mid-west fisheries from 1989 onwards. Post-smolts were generally in poor physical condition and a large percentage of adults and post-smolts carried heavy recent infestations of largely juvenile sea lice (Anon, 1991; Tully *et al.*, 1993 a,b; Whelan, 1993). In Scotland, the heaviest infestations have been recorded from the west and northwest, those areas which have suffered the most drastic declines in sea trout numbers (Sharp *et al.*, 1994; Walker, 1994b). In Norway, wild sea trout post-smolts, as well as salmon and sea-going charr (*Salvelinus alpinus*), have been captured with heavy infestations of sea lice (Finstad *et al.*, 1994): post-smolts ascending the Lonningdalselven River, western Norway, displayed a median intensity of 206 *Lepeophtheirus salmonis*, most of which were chalimus and juvenile stages (Birkeland, 1996).

Sea lice belong to the copepod sub-order Siphonostromatoidea, family Caligidae (Kabata, 1979). All caligids are parasitic, having a flattened body and prehensile appendages that allow them to cling to the body surface of the host. *Lepeophtheirus salmonis* has a circumpolar distribution in the northern hemisphere, and is a host specific parasite, occurring on most species of the genera *Salmo*, *Oncorhynchus* and *Salvelinus* during the marine phase of their life cycles (Kabata, 1979). Small numbers (0 - 3) of mobile stages have been observed on saithe, *Pollachius virens*, in

the vicinity of a salmon farm (Bruno and Stone, 1990). However, this is thought to reflect artificial conditions as in an experiment where adult and pre-adult stages (total = 37 lice) were placed on 42 saithe in a tank with 42 naive Atlantic salmon, only eight sea lice (five adults and three pre-adults) remained on the saithe after four days, and no *L. salmonis* were recorded on saithe caught from open waters.

The life cycle of *Lepeophtheirus salmonis* has recently been described (Johnson and Albright, 1991a; Schram, 1993) and like other caligids, comprises five phases and ten stages, each separated by a moult (Figure 5.1, Plate 5.1). Two free-swimming nauplius stages and one free-swimming infective copepodid stage precede host encounter and are followed by four attached chalimus stages, two unattached pre-adult stages and the vagile reproductive adult (Kabata, 1972). While the precise details of host location and larval attachment are unclear, copepodids have been demonstrated to respond with swimming activity to light, pressure waves, chemicals and water currents (Bron *et al.*, 1993).

The copepod sea lice *Lepeophtheirus salmonis* and *Caligus elongatus*, are the primary ectoparasites of farmed and wild salmonids. They feed on the mucus, skin, and blood of host salmonids (Kabata, 1974; Brandall *et al.*, 1976), and may seriously debilitate or kill farmed (Wootten *et al.*, 1982; Pike, 1989) and wild salmon (White, 1940; Johnson in Tully and Whelan, 1993) particularly when freshwater migration is delayed by low river flow and returning adults are forced to congregate in inlets: sockeye salmon in such circumstances developed severe lesions and suffered high mortalities, both considered a result of *L. salmonis* infestations (Johnson *et al.*, 1996). In addition, whereas a large proportion of those adult sockeye salmon entering freshwater had lesions, very few lesions were recorded amongst fish on the spawning grounds, suggesting additional pre-spawning mortalities. Excessive grazing of mucus and dermal tissue causes haemorrhages and sores, and may erode the skin and expose the underlying tissue, in severe cases even revealing the bones in the skull roof (Berland, 1993). Breaches in the epidermis may lead to secondary infections (Wootten *et al.*, 1982) and the parasite itself may be a vector for other pathogens (Nylund *et al.*, 1993).

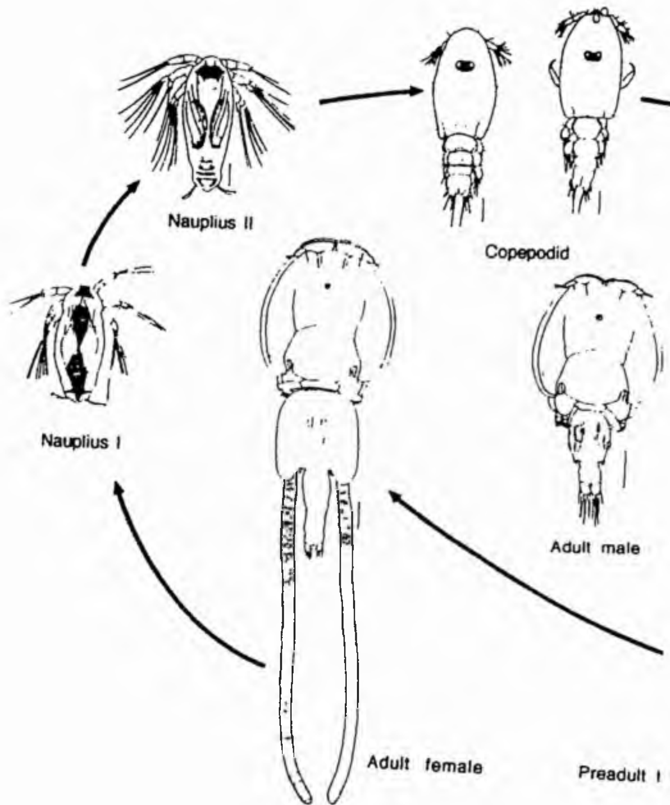
It has been postulated that skin damage, even from attached chalimus stages of sea lice, may compromise the osmoregulatory ability of the host (Wooten *et al.*, 1982; Anon., 1992; Tully *et al.*, 1993 a,b), and that this may be a factor causing the premature return of post-smolt sea trout to estuarine and freshwater conditions

Figure 5.1

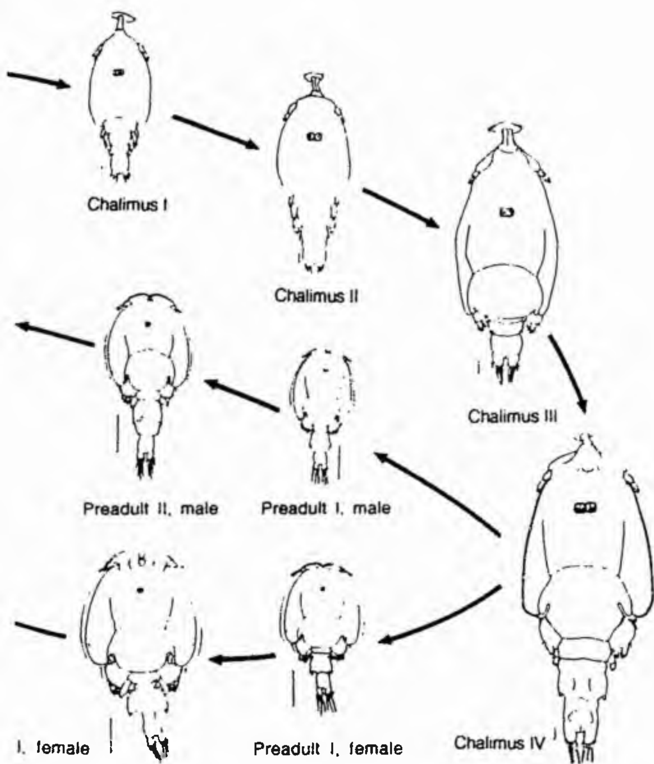
Figure 5.1 Life cycle of *Lepeophtheirus salmonis*. Scale bars: nauplius to chalimus = 0.1 mm, pre-adult to adult = 1 mm.
From Schram (1993).

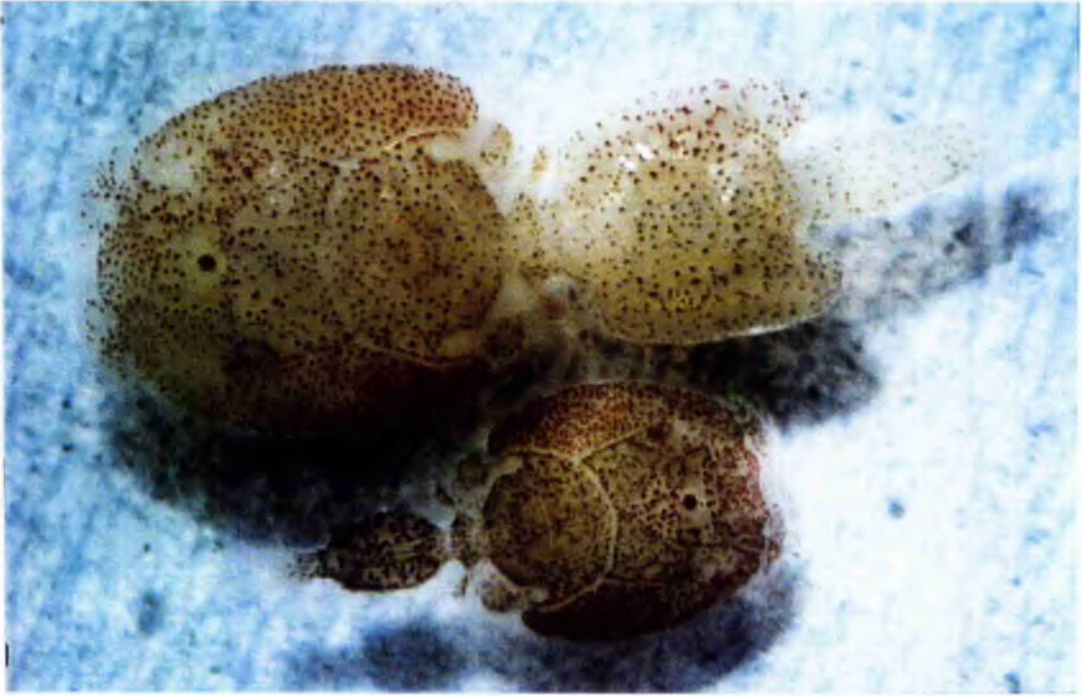
Plate 5.1

Plate 5.1 Pair of mature adult *Lepeophtheirus salmonis*. The female is the larger individual.



Nauplius II
Copepodid
Nauplius I
Adult male
Adult female
Preadult I


Chalimus I
Chalimus II
Chalimus III
Preadult II, male
Preadult I, male
Chalimus IV
I, female
Preadult I, female



5 mm

(Anon., 1992; Tully *et al.*, 1993 a,b). Norwegian sea trout smolts artificially infested with *Lepeophtheirus salmonis* suffered from osmoregulatory failure in seawater and returned to freshwater within two days of release from sea cages, at which time the median intensity of infestation was 62.5 sea lice, most of which were chalimi and late juveniles (Birkeland and Jacobsen, 1997). Previously naive control smolts also began returning to the estuary within four days of release and displayed a median intensity of 150 copepodids, indicating rapid and heavy infestations. Data suggest that adult *L. salmonis* cannot survive in low salinity waters and drop off returning salmonids within a few days of the hosts entering freshwater (McLean *et al.*, 1990). Returning to freshwater could, therefore, be advantageous to the host in the consequent shedding of any sea lice infestation, albeit at a probable cost of reduced growth and reproductive output in the short-term (Tully *et al.*, 1993a; Birkeland, 1996).

However, a recent investigation of the physiological effects of sea lice on hatchery-reared Atlantic salmon smolts observed no significant physiological effects of high infestations (30 - 250 lice) of chalimi (Grimnes and Jacobsen, 1996). Significant physiological trauma and mortalities were observed, however, when sea lice reached mobile pre-adult stages. This would suggest that high levels of infestation of chalimi, as recorded on prematurely returning post-smolts, would not be the root cause of this aberrant behaviour. However, that study was performed using hatchery-reared Atlantic salmon smolts whose responses to infestation may be different from wild individuals for a variety of reasons (see Discussion). It is important, therefore, to investigate the physiological effects of sea lice infestation on wild sea trout smolts to gain further insight into the possible links between sea lice infestation and the current drastic decline of sea trout in western regions of Scotland and Ireland.

In the following investigation, wild sea trout smolts were adapted to seawater and subsequently infested with *Lepeophtheirus salmonis* larvae from wild female parasites collected from wild salmon and sea trout. Groups of fish were then sampled periodically, infestation parameters were recorded and physiological parameters were compared with uninfested controls.

5.2 Materials and Methods

5.2.1 Fish capture and maintenance

Two hundred wild sea trout smolts were trapped during their downstream migration to the sea from the River North Esk, Angus on 6 June and 8 June 1996, at the peak of the smolt run. A sketch diagram of the S.O.A.E.F.D. trap at Kinnaber is given in Figure 5.2. Fish are diverted from the main river into a lade and then into a large concrete holding tank. The trap is checked every morning and afternoon during the smolt season (late March through June) and collected smolts are counted and released again every morning. Smolts were transported back to the Gatty Marine Laboratory in a specially designed transporter tank with O_2 supplied continuously from an externally mounted pressurised cylinder.

After transfer to the Gatty Marine Laboratory, the smolts were maintained initially in brackish water, 16 ppt, to acclimate them to aquarium conditions. Tank conditions were similar to those for the finnock experiments described previously (see Chapter 4). After two weeks, to ensure the smolts had recovered from the stress of capture and transport, the salinity was increased to full strength seawater. Smolts were initially fed a mixed diet of chopped squid (Ammodytes Company Ltd) and chopped whitebait (Kerachers Fish Merchants, St Andrews), both of which were consumed voraciously. Commercial fish food pellets (EWOS) were offered by hand but initially not eaten. However, after several weeks of persistent offering of pellets as well as the squid and whitebait, some of the fish, now classed as post-smolts began to take pellets. The proportion of post-smolts feeding on the pellets increased rapidly as did the amount of pellet food eaten. From two grading exercises, however, it was clear that a small proportion of the post-smolts did not eat pellets and so the mixed diet was continued throughout the experimental period.

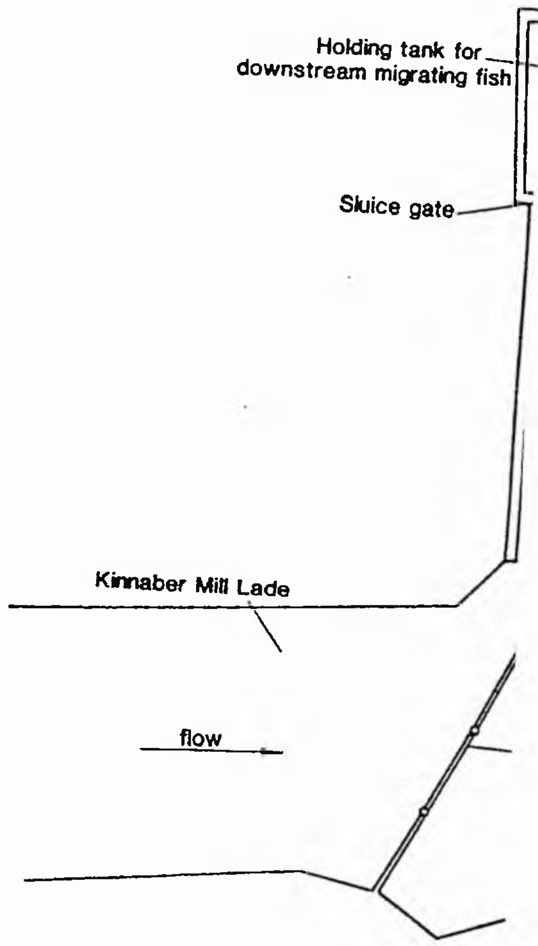
Water temperature was ambient and photoperiod was adjusted to mimic the natural L:D cycle throughout the experiment.

5.2.2 Sea lice capture and maintenance

Ovigerous female sea lice were collected from recently netted salmon and sea trout from the mouth of the River Tweed, Borders. Sea lice were removed from fish with fine nosed forceps and placed in empty film cassettes along with a small amount of

Figure 5.2

Figure 5.2 Diagram of Kinnaber Mill fish trap, River North Esk, Angus. From Shearer (1990).



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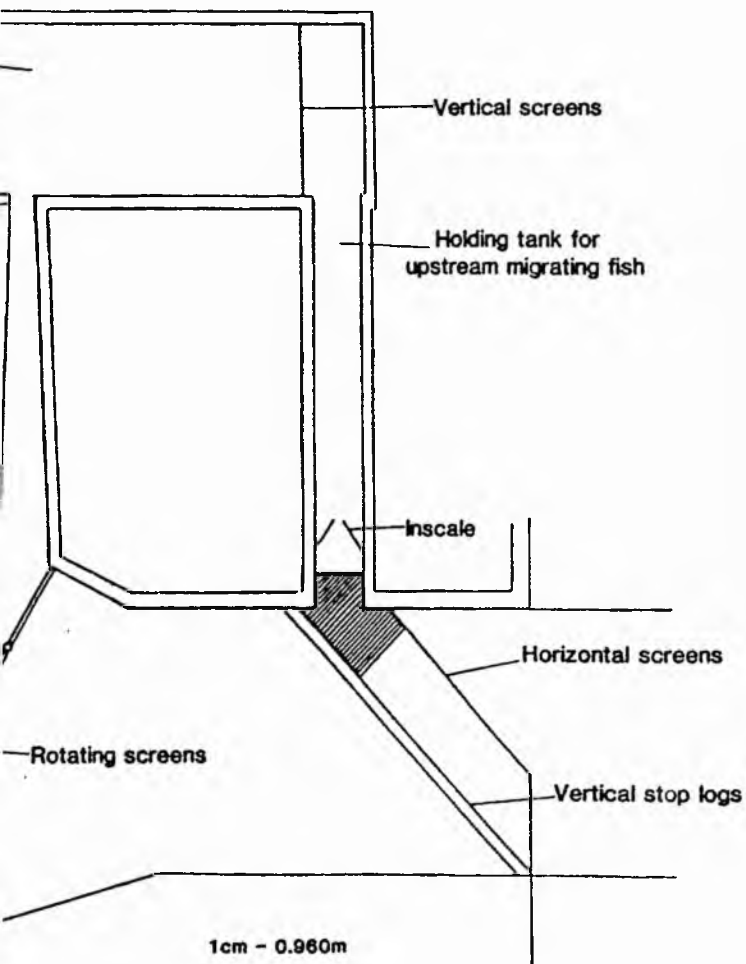

Vertical screens
Holding tank for upstream migrating fish
Inscale
Horizontal screens
Rotating screens
Vertical stop logs
1cm - 0.960m

seawater to keep them damp. The cassettes were sealed and placed on ice prior to transport back to St Andrews. All sea lice survived the handling and transporting procedures.

Egg strings were removed from female sea lice and placed in beakers of filtered seawater which were aerated continuously and kept at a constant 10°C. Nauplii began hatching from some of the egg strings within 24 - 48 hours. Once all the mature egg strings (as shown by pigmented eggs) had ruptured, the water was passed through a 40 μm filter, the nauplii collected and removed to 1 l of clean, aerated seawater. The seawater was replaced every 48 hours. Small numbers of larvae were sampled daily and examined under a microscope to monitor the rate of larval development. Approximately 80% of the nauplii had moulted to copepodids within seven days of hatching at 10°C.

5.2.3 Experimental design

After the grading (see above) the post-smolts were split between four 1 m diameter tanks with flowing seawater (see Figure 5.3).

After a further two weeks, the post-smolts in the four experimental tanks were examined again. Fish were lightly anaesthetised with 2-phenoxyethanol (0.5 $ml.l^{-1}$) to ease handling and then weight and fork length measured. Any post-smolts that appeared especially thin or in poor condition were removed from the experiment and returned to the stock tank. Finally, the number of fish per tank was adjusted to ensure, as near as possible, an equal number in each of the four tanks. This resulted in two tanks of 36, and two tanks of 37 post-smolts. Fish resumed feeding on chopped squid within 30 minutes of recovery from the anaesthesia, indicating that they were not unduly stressed by the anaesthetic and sampling procedures.

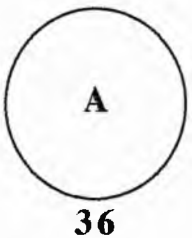
5.2.4 Infestation protocol

Infective copepodid larvae were concentrated in approximately 100 ml seawater by filtering the larger volume through a 40 μm mesh. The total number of larvae was estimated by extrapolating from the number of larvae in four 1 ml samples of water drawn from the beaker after the water had been mixed to distribute the copepodids evenly throughout the water column. The total number of copepodids was 3736 ± 212 (S.E.). After this estimate of number of copepodids, the water was mixed again

Figure 5.3

Figure 5.3 Schematic representation of the tank arrangement and experimental design.

Infested



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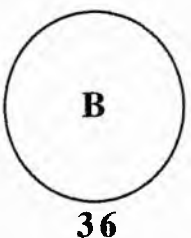

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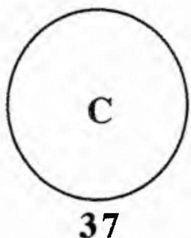
Uninfested



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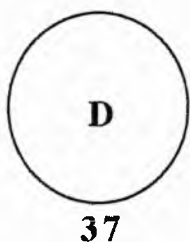

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Infestation parameters

5 post-smolts sampled 3, 7, 13 and 18 days p.i.

Physiological parameters

10, 7, 7 post-smolts sampled 5, 13 and 17 days p.i.

NB. 8 post-smolts sampled from Tank A on Day 20

Control groups

10, 7 and 7 post-smolts sampled on days 5, 13 and 17 p.i., 3/8 post-smolts sampled on Day 20

and then split between two beakers, each containing approximately 1868 copepodids. The beakers were then placed in water from the experimental tanks for 30 minutes to allow the copepodids to acclimate to the higher aquarium water temperature (15°C) prior to the infestation.

Infestation of the post-smolts was performed in a manner similar to that of other studies (Johnson, 1993). The volume of water in tanks A and B was reduced to 93 litres and the water supply turned off. The water was continuously aerated and the air stones were fixed to the central drains so that rising bubbles would create a circulating current in the water column to facilitate mixing of the copepodids throughout the tank. The copepodids were then introduced and after five hours the unattached copepodids were flushed from the tanks by increasing the volume to 200 l and then draining to 93 l. This procedure was repeated and then the tanks were filled up to normal volume (400 l) overnight. The following morning, tanks A and B were again drained to 93 l to further reduce the number of unattached copepodids. This ensured that all sea lice subsequently observed on smolts had attached to their hosts within a 12 hour period and negated the complications involved in analysing the developmental rates of sea lice which attached on different days.

Tanks C and D, the control "uninfected" tanks, were treated in the same manner as above, both during the infestation period and at all times afterwards. For example, if fish were removed from an infested tank for sampling, a similar number of net dips would be applied to each of the control tanks, and the other infested tank.

5.2.5 Sampling protocol

The infested post-smolts from Tank A were sampled in groups of five at 3, 7, 13 and 18 days post infestation (p.i.) to record the developmental rate and infestation success of the sea lice. For the osmoregulatory study, ten infested post-smolts from Tank B were sampled on Day 5 p.i., along with ten post-smolts from each of the control Tanks (C and D). Because of mortalities in some of the tanks (see below), the sample sizes were reduced to seven for the next two samplings at 13 and 17 days p.i. At the final sampling, on Day 20 p.i., no infested post-smolts from Tank B remained, although there were 12 infested post-smolts in Tank A. In addition, whereas one control tank contained eight post-smolts, the other contained only three. Therefore, all the control post-smolts were sampled, along with eight infested post-smolts from Tank A.

5.2.6 Plasma analysis protocol

Blood sampling was carried out in a manner similar to that described previously in Section 2.2. Post-smolts were netted at random from a tank. Each fish was individually killed by overdose of anaesthetic followed by two sharp blows to the back of the head and then placed individually in a labelled bag. In the case of infested post-smolts the dip net and the bucket of anaesthetic were checked for any loose sea lice. Where sea lice could be matched to individual post-smolts they were returned to the plastic bag. Any sea lice that could not be matched to a particular post-smolt were noted for subsequent analysis.

Plasma osmolality, Cl^- concentration and haematocrit were measured using the techniques described in Chapter 2.

5.2.7 Infestation analysis

All post-smolts, whether infested or controls, were measured for fork length and wet weight and a sample of scales removed from the flank between the posterior of the dorsal fin and the lateral line. Post-smolts infested with sea lice were examined under a binocular microscope and the developmental stage, position (fins, skin or gills) and sex of attached sea lice were recorded. Developmental stage and sex were determined according to the descriptions of Johnson and Albright (1991a). Chalimi were separated into stages I/II and III/IV, whereas pre-adult stages I and II of both sexes were pooled. Some post-smolts were examined soon after death but most were frozen in their bags and examined at a later date. The prevalence of infestation (proportion of fish which were infested), infestation intensity (number of sea lice per fish) and infestation density (number of sea lice x fish length $^{-3}$) were calculated according to Margolis *et al.* (1982).

5.2.8 Data analysis

Statistical tests were carried out using the STATVIEW and SuperANOVA software packages (Abacus Concepts, Inc., Berkeley, CA). The infestation intensity of *Lepeophtheirus salmonis* on cultured salmon is reported to demonstrate an approximately normal distribution, or have slight skew (Tully, 1992), although the present sample sizes (5 - 12) were too small to confirm this. As such, infestation intensity and infestation density data were log transformed to correct for skew and remove the positive relationship between the variance and the mean. Fork length,

wet weight, condition factor and physiological parameters were transformed as described in Chapters 2 and 4, prior to ANOVA. A significant result was considered to be indicated by $p < 0.05$. The results of significant statistical tests are presented as tables at the end of the chapter.

Neither infestation intensity nor density differed significantly between the two groups of infested fish when compared at sample dates 3 - 7, 13 and 17 - 20 days p.i. Sample dates were grouped since fish were sampled from the two tanks on different days post-infestation. The lack of significant differences between tanks was used to justify the pooling of infestation parameters results from both Tanks A and B.

No significant differences were observed in mean length, weight or condition factor between the two infested groups at any of the three sample groupings outlined above. This similarity of various measures and infestation parameters between the two infested groups justifies, on a preliminary basis, the inclusion of the final group of infested fish from Tank A in the physiological study.

5.3 Results

5.3.1 Observations of host responses to sea lice infestation

An epidermal reaction was observed on some post-smolts (five to six per tank) within 24 hours of infestation. Small black spots appeared on the dorsal surface of the fish, commonly between the dorsal and adipose fins and around the head. No black spots occurred on the control fish throughout the experimental period.

At one hour post-infestation the behaviour of the infested and control fish was similar; most fish were holding position in the water flow around the central drain. At two hours post-infestation, infested post-smolts appeared generally more active and took longer to settle after being disturbed by the presence of the observer. Some post-smolts were observed to have resumed feeding the morning following infestation and all post-smolts were feeding as normal by Day 3 p.i.

5.3.2 Rate of sea lice development

Sea lice stage data from both infested tanks were pooled for presentational purposes. Only chalimus stages were observed from Day 3 to Day 10 post-infestation. Stages I and II were observed exclusively during Days 3 to 5 while stages III and IV were apparent from Day 7 onwards. A combination of late chalimi and pre-adults of both sexes were recorded on Day 10. By Day 13, chalimi were no longer present, pre-adult males and females predominated, although some adult males were recorded. A combination of pre-adult females and adults of both sexes were recorded on Days 17 and 18 but adults predominated. Finally, all sea lice recorded on Day 20 post-infestation were adult males and females.

5.3.3 Areas of infestation

The distribution of sea lice found on different host body areas varied with time and/or stage of development. Table 5.1 presents the mean values of total lice infestation found on the body, fins and gills at sample times throughout the experimental period. The majority of chalimi were found on the fins, with lesser numbers on the body and attached to the primary filaments of the gills (Days 3 to 10). Mobile stages were only recorded from the body surface (Days 10 to 20). A similar distribution is presented for fish examined soon after death (Table 5.2).

Table 5.1

Table 5.2

Table 5.1 Distribution (% of total recorded) of *Lepeophtheirus salmonis* on the body surfaces of sea trout post-smolts sampled during the experimental period.

Table 5.2 Distribution (% of total recorded) of *Lepeophtheirus salmonis* on the body surfaces of sea trout post-smolts removed as mortalities during the experimental period.

Day p.i.	Body	Fins	Gills	No. sea lice	No. hosts
3	5	86	9	57	5
5	13	66	21	184	10
7	27	68	5	119	5
13-20	100	0	0	490	31

Day p.i.	Body	Fins	Gills	No. sea lice	No. hosts
4-7	16	75	9	133	7
10	60	14	26	35	2
14-20	100	0	0	54	4

5.3.4 Fish growth and mortality

Measurements of length, weight and condition factor for fish from Tank A were included in this analysis. Parameters were pooled for the duplicate groups for analysis and presentation. Tables 5.3 and 5.4 display back-transformed mean and 95% confidence limits for the control and infested groups for each sample day (note that for the infested group, samples from Days 3, 5 and 7 are pooled and described as Day 5 while samples from Days 17 and 18 are pooled and described as Day 17). Whereas the mean wet weights of the control group displayed no significant variation throughout the experiment, mean fork length of the Day 20 sample was significantly greater than that of the Day 13 sample (Tables 5.10, 5.11). Condition factor differed significantly amongst the control group also (Table 5.12); the mean condition factor of the Day 13 sample was greater than that of the Day Five sample (Table 5.13). Because there were no trends in these significant differences, they were considered to reflect small sample sizes rather than be of biological significance. Amongst the infested group, fork length, wet weight and condition factor did not vary significantly throughout the experimental period.

Despite these significant differences amongst control groups, no significant differences were observed between the fork lengths, wet weights or condition factors of the control and infected groups on any sample date.

Several fish died in all four tanks before, during and after the experimental period. Cumulative mortalities for each tank during the experimental period are presented in Figure 5.4. Mortality rates amongst the infested groups showed no consistent relationship with sea lice development. A small number of dead post-smolts were examined for disease infection at the S.O.A.E.F.D. Marine Laboratory, Aberdeen, in a similar manner to that described in Chapter 4. However, no significant disease agent was identified.

5.3.5 Infestation rates

Prevalence was 100% throughout the experiment. Mean infestation intensity and infestation densities for each sample day are presented in Table 5.5. Neither parameter varied significantly over the experimental period, nor were there significant differences for these parameters between live fish and recovered mortalities when compared over Days 1 to 7.

Table 5.3

Table 5.3 Growth data for uninfested sea trout post-smolts during the experimental period.

	Fork length (mm)			Wet weight (g)			Condition Factor			Sample size
Days P.I.		95% C.L.			95% C.L.			95% C.L.		
	mean	lower	upper	mean	lower	upper	mean	lower	upper	
5	174.58	-6.31	+6.97	49.20	-6.64	+7.55	0.93	-0.05	+0.05	20
13	169.96	-14.00	+6.64	47.21	-8.04	+11.00	1.04	-0.02	+0.06	14
17	171.79	-12.20	+12.71	50.58	-9.46	+11.65	1.01	-0.05	+0.05	14
20	187.50	-10.90	+12.06	63.83	-12.07	+15.06	0.97	-0.07	+0.08	11

Table 5.4

Table 5.4 Growth data for *Lepeophtheirus salmonis* infested sea trout post-smolts during the experimental period. Note that sample data (1) for Days 3, 5 and 7 have been pooled and are presented as Day 5 and (2) for Days 17 and 18 have been pooled and presented as Day 17.

	Fork length (mm)			Wet weight (g)			Condition Factor			Sample size
Days P.I.		95% C.L.			95% C.L.			95% C.L.		
	mean	lower	upper	mean	lower	upper	mean	lower	upper	
5	168.66	-9.07	+9.58	50.47	-8.49	+10.20	1.05	-0.08	+0.08	20
13	162.93	-8.40	+8.86	46.24	-6.70	+7.71	1.07	-0.06	+0.06	12
17	164.82	-9.22	+9.76	44.77	-6.93	+8.20	1.01	-0.06	+0.05	12
20	176.60	-18.47	+20.64	50.82	-13.75	+18.85	0.92	-0.18	+0.15	7

Figure 5.4

Figure 5.4 Cumulative record of dead infested (Tanks A and B) and uninfested (Tanks C and D) sea trout post-smolts.

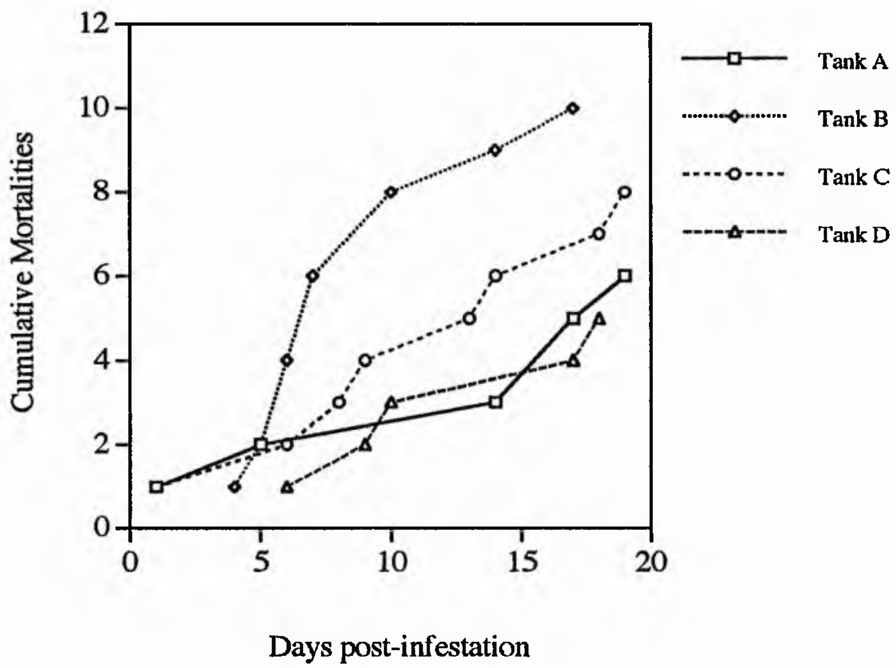

Cumulative Mortalities
Days post-infestation
Tank A
Tank B
Tank C
Tank D
0
2
4
6
8
10
12
0
5
10
15
20

Table 5.5

Table 5.5 Back-transformed mean and 95% confidence limits (C.L.) for *Lepeophtheirus salmonis* Infestation Intensity and Density on sea trout post-smolts (see Table 5.4 for details of which samples were pooled and sample sizes). Infestation intensity = number of sea lice per host. Infestation Density = Infection Intensity x host length^{-3}.

		Infestation Intensity			Infestation Density	
Day	mean	lower (C.L.)	upper (C.L.)	mean	lower (C.L.)	upper (C.L.)
5	16.14	-3.20	+4.00	16.26	-3.20	+4.02
13	14.52	-4.00	+5.53	14.62	-4.03	+5.52
17	14.42	-3.20	+4.12	14.52	-3.22	+3.14
20	14.83	-4.74	+6.90	14.93	-4.77	+6.95

5.3.6 Blood parameters

Back-transformed mean and 95% confidence limits for plasma osmolality and Cl^- concentration for post-smolts from the uninfected groups sampled during the experiment are given in Tables 5.6 and 5.7, respectively. Significant differences in mean plasma osmolality between the two groups were indicated by t-tests on Days 13, 17 and 20 (Table 5.14). Significant differences in mean plasma Cl^- concentration between the two groups were also indicated for Days 5, 13, 17 and 20 (Table 5.15). However, no group displayed consistently higher ion concentrations throughout the experimental period. The possible influence of an unidentified disease agent on the variation observed in plasma parameters amongst the control groups is addressed in the Discussion. Therefore, plasma osmolality and Cl^- concentration data from the two control groups were pooled and compared with those of the infested group on the appropriate sample day.

Mean plasma osmolality of sea lice infested post-smolts was significantly greater than that of the uninfested control group on all sample days (Figure 5.5, Table 5.16). Mean plasma osmolality did not vary significantly in either group throughout the experimental period. Mean plasma Cl^- concentration was significantly greater in infested groups than control groups from Day 13 onwards (Figure 5.6, Table 5.17). Mean plasma Cl^- concentration in the control group did not vary significantly throughout. One-way ANOVA did indicate a significant difference between at least the largest and smallest mean plasma Cl^- concentration in the infested group during the experimental period (i.e. between Days 5 and 20 (Table 5.18)) but this difference was not supported by a post-hoc means comparison. Significant differences between the mean haematocrit of the infested and control groups were indicated for Days 13 and 17 (Tables 5.8 and 5.19). However, there was no consistent trend over time. Whereas mean haematocrit did not vary significantly in the control group during the experimental period, a significant difference amongst the mean haematocrit of the infested group was indicated (Table 5.20). The post-hoc comparison revealed mean haematocrit on Day 13 to be significantly lower than on Days 17 and 20 (Table 5.21).

No correlation was found between infestation density and any of the blood parameters throughout the study. Neither was any significant correlation found between infestation density or intensity and fish length when data from all sample days were combined.

Table 5.6

Table 5.7

Table 5.6 Back-transformed mean and 95% confidence limits (C.L.) for plasma osmolality (mOsmol.kg $^{-1}$) of uninfested sea trout post-smolts sampled from the two control tanks during the experimental period. Sample sizes were 10, 7, 7 and 3 for Tank C and 10, 7, 7 and 8 for Tank D for samples collected 5, 13, 17 and 20 days p.i.

Table 5.7 Back-transformed mean and 95% confidence limits (C.L.) for plasma Cl^- concentration (mmol.l $^{-1}$) of uninfested sea trout post-smolts sampled from the two control tanks during the experimental period. See Table 5.6 for sample sizes.

	Tank C			Tank D		
Days p.i.	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
5	367	-15	+16	370	-17	+18
13	382	-29	+31	352	-8	+10
17	375	-11	+12	356	-15	+16
20	352	-8	+13	366	-14	+14

	Tank C			Tank D		
Days p.i.	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
5	144	-7	+7	148	-9	+10
13	160	-11	+13	143	-7	+7
17	155	-8	+8	153	-7	+8
20	141	-15	+16	151	-11	+12

Figure 5.5

Figure 5.5 Back-transformed mean plasma osmolality ($mOsmol.kg^{-1}$), with 95% confidence limits, for sea trout post-smolts infested with *Lepeophtheirus salmonis* compared with uninfested control groups. Sample sizes were 10, 7, 7 and 8 infested post-smolts and 20, 14, 14 and 11 uninfested post-smolts for days 5, 13, 17 and 20, respectively.

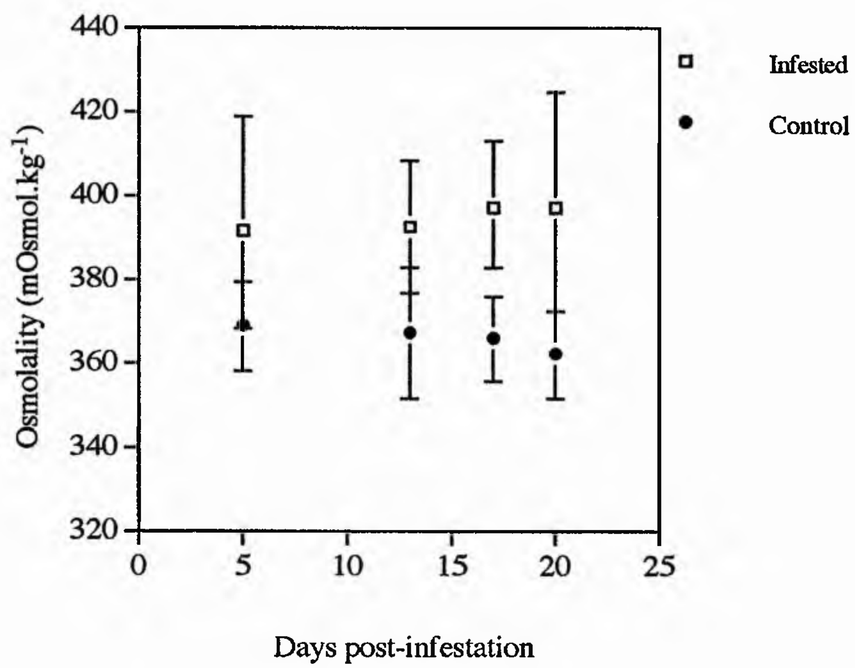

440
420
400
380
360
340
320
Osmolality (mOsmol.kg-1)
0
5
10
15
20
25
Days post-infestation
Infested
Control

Figure 5.6

Figure 5.6 Back-transformed mean plasma Cl^- concentration ($mmol.l^{-1}$), with 95% confidence limits, for sea trout post-smolts infested with *Lepeophtheirus salmonis* compared with uninfested control groups. Sample sizes were 10, 7, 7 and 8 infested post-smolts and 20, 14, 14 and 11 uninfested post-smolts for days 5, 13, 17 and 20, respectively.

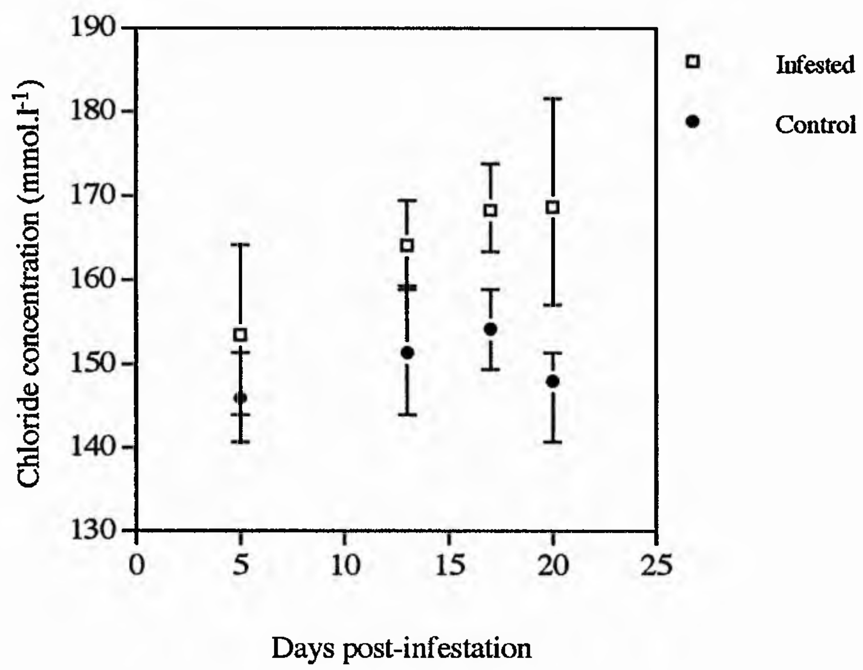

190
180
170
160
150
140
130
Chloride concentration (mmol.l-1)
0
5
10
15
20
25
Days post-infestation
Infested
Control

Table 5.8

Table 5.8 Back-transformed mean and 95% confidence limits (C.L.) of haematocrit values for sea trout post-smolts infested with *Lepeophtheirus salmonis* compared with uninfested controls. See Figure 5.5. for sample sizes. Note that no haematocrit samples were measured on Day 5.

Days P.I.	Infested			Control		
	mean	lower C.L.	upper C.L.	mean	lower C.L.	upper C.L.
13	32.56	-2.63	+2.69	37.19	-2.27	+2.29
17	40.87	-3.75	+3.79	37.14	-1.64	+1.66
20	35.59	-2.99	+3.04	34.33	-2.03	+2.05

5.4 Discussion

5.4.1 Summary of results

- Wild sea trout post-smolts were infested with *Lepeophtheirus salmonis* copepodids (mean number per fish = 14.5) and the osmoregulatory physiology compared with uninfested post-smolts during the life cycle of the parasite
- No effects of infestation were apparent in changes in fish length, weight or condition factor
- Mean plasma osmolality and Cl^- concentration were greater in infested versus uninfested post-smolts throughout the experimental period

5.4.2 Plasma parameters

It is possible that the unidentified disease infecting some or all of the post-smolts, was responsible, to some extent, for the variation recorded amongst the plasma ion data in the control groups. If this were the case then the sea lice infested post-smolts ought to have been equally affected. That mean plasma osmolality and Cl^- concentrations were elevated in infested groups throughout the study indicates that sea lice infestation caused an osmoregulatory problem for the fish which was apparent even above the possible background effects of the unidentified disease agent. Plasma osmolality and Cl^- concentrations for seawater-adapted post-smolt sea trout are rare in the literature but some data are available for other salmonids (see Table 4.10); control values are similar to those of other salmonids. Sea lice graze on the epidermal tissues of the host and studies have shown that even the early attached chalimus stages cause epidermal erosion (Jones *et al.*, 1990; Jónsdóttir *et al.*, 1992). This erosion would mechanically disrupt the epidermis, thereby altering the permeability and facilitating increased fluid loss and ion gain from the surrounding seawater, both of which would raise plasma ion and osmolality concentrations.

Although mean haematocrit values did differ significantly between infested and control groups during the study period, no consistent trend was apparent. Grimnes and Jacobsen (1996) attributed a significant decrease in mean haematocrit in infected groups to increased plasma ion concentrations causing shrinkage of erythrocytes and, possibly, reduced erythrocyte counts due to leakage from lesions. No lesions were observed on infected post-smolts in the present study and while significant increases in plasma ion concentrations were observed, these increases

were smaller in magnitude than those in the Norwegian study. It may be that the experimental conditions were not sufficiently severe to cause significant differences in haematocrit. However, haematocrit levels can fluctuate in response to a wide variety of physiological factors (erythrocyte number and volume, changes in erythrocyte production from the spleen, as well as changes in extracellular fluid concentration) and, as such, it is extremely difficult to attribute levels to a particular causal factor (see Chapter 4).

Plasma osmolality in infested groups displayed no significant variation over the experimental period. Mean plasma Cl^- concentration increased significantly during the experimental period. Grimnes and Jacobsen (1996) reported a similar increase in mean plasma Cl^- concentration with time post-infestation and considered this to reflect the progressive increase in dermal erosion as the sea lice developed, partly due to their increasing size but also as a result of the lice becoming mobile and grazing over a larger surface area. However, such a result might be due to the gradual breakdown in the osmoregulatory mechanisms of the infested host due to the damage caused by the initial infestation of copepodids.

Whereas mean plasma osmolality values in this study were significantly greater at Day 5 in infested fish, when compared to control groups, Grimnes and Jacobsen (1996) observed no physiological response of Atlantic salmon post-smolts prior to sea lice developing to mobile stages. In the present study, the majority of chalimi were found on the fins but in excess of 25% were found attached to the gill filaments. Grimnes and Jacobsen (1996) did not report the areas from which sea lice stages were recovered. The majority of attached sea lice stages are normally found on the fins of farmed salmon (Wooten *et al.*, 1982; Jaworski and Holm, 1992; Jackson and Minchin, 1993), wild salmon (Finstad *et al.*, 1994) and sea trout (pers. obs.). However, Johnson and Albright (1991b) reported that 53% of recorded copepodid/chalimus stages were attached to the gills in a laboratory study of *Lepeophtheirus salmonis* infestation of Atlantic salmon smolts. Previously, the damage due to attachment of chalimii has not been considered sufficient to cause significant physiological stress since the area of damage is small (Jones *et al.*, 1990). However, osmotic stress could be increased considerably in those fish where copepodids and chalimi attach to the gills, quite probably puncturing the filaments, allowing leakage of blood products and damaging vital ion transport cells.

Settlement on the gills appears to be a feature of aquarium and cage conditions because chalimi are rarely found on the gills of salmonids sampled in the wild. This

difference in settlement pattern may reflect reduced water flow over the gills in tanks or attractive currents derived from respiratory movements (Bron *et al.*, 1991) or could be an artefact of the greater numbers of copepodids passing over the gills in artificial conditions due to the high densities of copepodids within confined volumes of water. Little is known regarding how a copepodid finds a host and even less regarding choice of attachment site. Cues such as light, chemical, pressure and water flow/vibration have been implicated in determining the directed movement and host-seeking behaviour of copepodid larvae (see Bron *et al.*, 1993). Homing to water currents may explain the preference of *Lepeophtheirus salmonis* for the fins; alternatively, the fins might provide shelter from water currents and allow the larvae to attach (Bron *et al.*, 1991).

Johnson (1993) reported all *Lepeophtheirus salmonis* stages between chalimus and pre-adult males and females on the gills of Atlantic and chinook salmon. However, in general, those larvae which attach to the gills are thought to move out onto the skin as they develop. The results of the present study support this as intensity of infestation did not decline significantly between chalimi and mobile stages (note that the variation in intensity may have occluded this). The continued elevation of plasma ions in infected smolts after all chalimi had developed and moved from the gills suggested that, if the attachment to the gills did induce the physiological differences, either the damage to the gills was not repaired during the experimental period or the mobile stages continued to disturb the fish through grazing on other parts of the body surface. The lack of lesions on the skin of infested post-smolts in the present study would tend to support the former hypothesis.

Plasma cortisol concentration was not measured in the present study. This hormone has been implicated both in hypo-osmoregulation and the general stress response of fish (see Section 1.4). In addition elevated plasma cortisol levels have been implicated in immuno-suppression (Ellis, 1981) which may make the host more susceptible to secondary disease infections (Wooten *et al.*, 1982). Clearly, further studies ought to measure the plasma concentration of this hormone in infested fish over time.

5.4.3 Fish growth and mortality

Mortalities were neither restricted to, nor greater in number, in the infected groups and occurred throughout the experimental period. This is in contrast to the study of Grimnes and Jacobsen (1996) in which mortalities only occurred in the infected

groups and only after *Lepeophtheirus salmonis* had reached mobile stages. Mean intensity of infestation in the Norwegian study was 42 pre-adults as opposed to 18 in the present study, thereby suggesting that infestation level in the present study was at a sub-lethal level. The post-smolt sea trout were transferred to full strength seawater several weeks prior to the beginning of the experimental period and plasma osmolality and Cl^- ion concentrations did not vary significantly in the control groups throughout the experimental period. This suggests that an inability to acclimate to seawater was not a factor in the mortalities. Some dead post-smolts displayed haemorrhaging at the base of paired fins, an indication of blood bacterial infection. A small number of mortalities were examined at the Fish Cultivation Unit, S.O.A.E.F.D., Aberdeen but no evidence of a significant pathogen was found. However, salmon and sea trout from the river from which the smolts were obtained, the River North Esk, Angus, are known to suffer heavily from furunculosis (R. Shelton, pers. comm.). As described in Chapter 4, finnock collected from this river suffered mortalities when brought to the aquarium but mortalities ceased completely within two days of prescribed treatment with the antibiotic amoxycillin trihydrate (duphamox LA) (Solvay Duphar Veterinary).

The effects of disease agents on plasma parameters would have been equally distributed amongst the infected and control groups and would not, therefore, account for the differences observed between these groups. It is possible, however, that the background levels of disease may have made the post-smolts particularly susceptible to further physiological stress resultant from parasitic infestation. Thus, the use of wild sea trout smolts with natural levels of this disease may well provide a more accurate depiction of the natural system where several stressors may combine to severely compromise smolts at a particularly susceptible stage in the life cycle.

5.4.4 Rate of sea lice development

Pre-adults of both sexes and adult males were present by 13 days post-infestation on sea trout post-smolts in this study and adult females were present from 18 days post-infestation. Development rate is known to be strongly influenced by water temperature in copepods such as *Caligus elongatus* (Pike *et al.*, 1993) and in *Lepeophtheirus salmonis*. Rate of egg production, rate of planktonic stage development (Johnson and Albright, 1991b) and generation time are negatively correlated with water temperature (Tully, 1989, 1992). The development rate of sea lice in the present study was faster than those of *L. salmonis* infesting other salmonids but most other investigations have studied sea lice development to adult

stages at 10°C as opposed to the 15°C of the present study. For comparative purposes therefore, the sea lice development time in the present study and others are presented as degree days in Table 5.9.

Even when adopting degree days, however, the development rate of the *Lepeophtheirus salmonis* in the present study was somewhat faster than those of other studies. This may reflect the non-linear nature of growth rate at different temperatures and imply that the use of degree days is insufficient to properly compare development rates at different temperatures. However, in a simultaneous study of the growth rates of *L. salmonis* on Atlantic salmon and chinook salmon smolts, Johnson (1993) attributed the greater development time of *L. salmonis* on chinook salmon to non-specific host defence mechanisms which interfere with the parasite's feeding activities, hence slowing growth. Parasite fecundity also indicated host species specific differences. Copepods collected from Atlantic salmon produced, on average, twice as many eggs as those collected from chinook salmon. Similar differences may exist between Atlantic salmon and sea trout that could account for the accelerated development rate of *L. salmonis* on sea trout. From unpublished data it is clear that adult *L. salmonis* are smaller on post-smolt sea trout and finnock than on adult salmon. Thus, these wild sea trout post-smolts could have a poorer defence response towards sea lice infestation than Atlantic salmon and, if so, this could account for the lack of significant parasite mortalities and for the increased parasite development rate in the present study.

Infection intensities in this study did not vary significantly throughout the experimental period. Previous studies have reported declines in infestation intensity with time post-infestation and attributed these to parasite mortality (Johnson, 1993; Grimnes and Jacobsen, 1996) and/or loss through mobile stages moving off hosts and being unable to find a new host prior to flushing from the tank (Jaworski and Holm, 1992). Mean intensity values were lower in the present study compared to those of Grimnes and Jacobsen (1996) (median infestation intensity of pre-adults was 42 lice compared with 18 in the present study). Little is known of the interactions amongst sea lice on individual hosts or of the density dependent and independent factors involved in determining loadings. The intensities of the present study might have been too low to induce density-dependent effects.

Table 5.9

Table 5.9 Comparison of *Lepeophtheirus salmonis* development rate on different salmonid hosts, recorded in degree days.

Host species	Water temperature (°C)	
		Copepodid-pre.adults
S. trutta	15	195
S. salar	10	250
S. salar	10	187
O. tshawytscha	10	187
S. salar	10	200

Degree days		Reference
Copepodid-adult males	Copepodid- adult females	
195	270	this study
310	340	Grimnes & Jacobsen (1996)
234	330	Johnson (1993)
379	444	Johnson (1993)
280	400	Johnson & Albright (1991b)

5.4.5 Relevance to the natural situation

High numbers (>30) of the mobile stages of *Lepeophtheirus salmonis* have been demonstrated to be fatal to post-smolt farmed Atlantic salmon due to physiological trauma (Grimnes and Jacobsen, 1996) and high numbers of chalimi are thought to induce post-smolt sea trout to return to freshwater prematurely, possibly due to hypo-osmoregulatory failure. The results of this study indicate that even relatively low infestation levels have a significant effect on wild sea trout post-smolt physiology and support the hypothesis that hypo-osmoregulatory failure probably occurs in heavily infested post-smolt sea trout as well as salmon. Although little information is available regarding the behaviour of salmonids in the sea, salmon smolts are thought to rapidly depart from coastal waters whereas sea trout tend to remain closer to shore (Pemberton, 1976a; Jonsson *et al.*, 1994; Johnstone *et al.*, 1995). The densities of sea lice in different regions of coastal waters are even less well understood but it is thought that infective larvae numbers are 'concentrated' in inshore areas, particularly in semi-enclosed bodies of water such as estuaries and sea lochs. This would make sea trout post-smolts particularly susceptible to repeated infestation by sea lice.

5.4.6 Marine mortalities

While no mortalities in this study could be conclusively attributed to sea lice infestation, it is important to note that significant numbers of sea trout smolts died during the acclimation and experimental periods. This is most certainly not a common occurrence in this laboratory and, as such, is considered to reflect the natural situation of these wild smolts. Smolts were acclimated to brackish water for two weeks prior to transfer to seawater. This implies that hypo-osmoregulatory failures were not the cause of the mortality in control fish. However, it is not possible to discount this completely since plasma parameters were not measured for mortalities and the survivors would naturally be those which could osmoregulate efficiently. If these mortality rates did reflect the natural situation, and were caused by disease rather than physiology, then a significant proportion of the annual sea trout smolt migration from the River North Esk might die at sea from disease infections. This mortality rate could be increased by sea lice infestations but, due to the lack of knowledge regarding the additive or synergistic effects of disease factors and sea lice infestations on wild sea trout smolts, this mortality rate cannot be presently assessed. This fact makes it imperative that further studies using wild hosts are performed in the near future.

Finally, a note about experimental design. This experiment, and others cited, challenged post-smolts to *Lepeophtheirus salmonis* infestation some weeks after seawater transfer at a time when the hosts would have been fully acclimated to seawater. In the natural situation, by contrast, smolts suffer from the stress of salinity challenge and sea lice infestation simultaneously. Smolts have been recorded with median infestation intensities of 150 chalimi within four days of seawater entry (Birkeland and Jacobsen, 1997). Thus, the results of these physiological tests must be viewed as a 'best case scenario' of the physiological effects of infestations. The present study has tried to mimic natural conditions more than previous studies by using wild sea trout smolts rather than hatchery-reared smolts. However, this must be advanced further by challenging wild migrating smolts to *L. salmonis* infestation and increased salinities simultaneously. Only then can we begin to fully understand the potentially significant effects such parasitic infestations can have on post-migratory mortalities.

5.4.7 Conclusions

1. This study has demonstrated that intensities of sea lice infestation, which could be considered within the natural range, do have a physiological effect on sea trout post-smolts, although this level of infestation did not appear to be lethal in the short-term. Further studies are required to investigate the long term effects of such infestation levels on such parameters as growth rates. However, it is clear that even at sublethal loadings, *Lepeophtheirus salmonis* have a significant effect on post-smolt sea trout and therefore any increase in infestation levels will have a significant impact on survival and growth of sea trout post-smolts.

2. The physiological state of the host and the development rate of the parasite both indicate significant differences in the host-parasite relationship between salmon and sea trout. As such, while studies using salmon and hatchery-reared post-smolts provide valuable insights into the physiological effects of *Lepeophtheirus salmonis* infestations, only studies using wild sea trout post-smolts can begin to accurately assess the impact of infestation with regard to the natural situation and the decline of sea trout in various countries.

3. Finally, although the physiological data from this and other studies implicate sea lice as a possible factor in the decline of sea trout, another important question,

particularly in light of the apparent increase in sea lice numbers, is the source or sources of these sea lice. Sea-cage based salmonid aquaculture has been heavily criticised as a major contributor of sea lice larvae to coastal waters. The following chapter describes the development of a tool to identify the source(s) of sea lice on wild sea trout.

5.5 Statistical Tables

Table 5.10 Results of 1-way ANOVA comparing log length of control post-smolt sea trout sampled on Days 5, 13, 17 and 20 from the beginning of the experimental period. Sample sizes were 20, 14, 14 and 11, respectively.

Table 5.11 Results of significant post-hoc means comparison subsequent to significant 1-way ANOVA comparing log length data for control groups of sea trout post-smolts (Table 5.10).

Table 5.12 Results of 1-way ANOVA comparing log condition factor of control post-smolt sea trout sampled on Days 5, 13, 17 and 20 from the beginning of the experimental period. Sample sizes were 20, 14, 14 and 11, respectively.

Table 5.13 Results of significant post-hoc means comparison subsequent to significant 1-way ANOVA comparing log condition factor data for control groups of sea trout post-smolts (Table 5.12).

Table 5.14 Results of significant t-tests comparing plasma osmolality values between the two control groups of uninfested sea trout post-smolts during the experimental period. See Table 5.6 for sample sizes.

Table 5.15 Results of significant t-tests comparing plasma Cl^- concentrations between the two control groups of uninfested sea trout post-smolts during the experimental period. See Table 5.6 for sample sizes.

Table 5.16 Results of significant t-tests comparing plasma osmolalities between *Lepeophtheirus salmonis* infested sea trout post-smolts and uninfested control groups. See Figure 5.5 for sample sizes.

Source	df	SS	MS	F	p
Days	3	0.018	0.006	3.122	0.033
Residual	55	0.106	0.002		

Means comparison	Diff.	Crit. Diff.
Day 13 to Day 20	0.053	0.044

Source	df	SS	MS	F	p
Days	3	0.024	0.008	3.805	0.015
Residual	55	0.115	0.002		

Means comparison	Diff.	Crit. Diff.
Day 5 to Day 13	0.052	0.046

Days p.i.	Mean Diff.	df	t	p
13	0.034	12	2.330	0.038
17	0.023	12	2.447	0.031

Days p.i.	Mean Diff.	df	t	p
13	0.051	12	3.282	0.007

Days P.I.	Mean diff.	df	t	p
5	-0.027	28	-2.258	0.032
13	-0.029	19	-2.226	0.038
17	-0.036	19	-3.971	< 0.001
20	-0.041	16	-3.488	0.003

Table 5.17 Results of significant t-tests comparing plasma Cl^- concentrations between *Lepeophtheirus salmonis* infested sea trout post-smolts and uninfested controls. See Figure 5.5 for sample sizes.

Table 5.18 Results of 1-way ANOVA comparing the log plasma Cl^- concentrations for *Lepeophtheirus salmonis* infested sea trout post-smolts during the experimental period of 20 days post-infection. Sample sizes were 10, 7, 7 and 8, respectively.

Table 5.19 Results of significant t-tests comparing haematocrits between *Lepeophtheirus salmonis* infested sea trout post-smolts and uninfested controls. See Figure 5.5 for sample sizes.

Table 5.20 Results of 1-way ANOVA comparing the arcsine transformed haematocrit values of *Lepeophtheirus salmonis* infested sea trout post-smolts during the experimental period of 20 days post-infestation. Sample sizes were 10, 7, 7 and 8, respectively.

Table 5.21 Results of significant post-hoc means comparisons subsequent to significant 1-way ANOVA comparing arcsine transformed haematocrit data of *Lepeophtheirus salmonis* infested sea trout post-smolts during the experimental period of 20 days post-infestation (Table 5.20). Sample sizes were 10, 7, 7 and 8, respectively.

Days P.I.	Mean diff.	df	t	p
5				ns
13	-0.035	19	-2.303	0.033
17	-0.039	19	-3.973	< 0.001
20	-0.057	16	-3.349	0.004

Source	df	SS	MS	F	p
Days P.I.	3	0.010	0.003	3.596	0.030
Residual	27	0.024	0.001		

Days P.I.	Mean diff.	df	t	p
5				na
13	2.785	19	2.768	0.012
17	-2.185	19	-2.438	0.025
20				ns

Means comparison	Diff.	Crit. Diff.
Day 13 to Day 20	1.832	2.543
Day 13 to Day 17	4.944	2.790

Source	df	SS	MS	F	p
Days P.I.	2	87.479	43.740	10.459	0.001
Residual	18	75.274	4.182		

Chapter 6

Patterns of genetic variation of sea lice, *Lepeophtheirus salmonis*, on wild and farmed salmonids

6.1 Introduction

Parasitic sea lice (*Lepeophtheirus salmonis*) infestations cause serious, and potentially lethal, physiological trauma to post-smolt salmon and sea trout in the laboratory (Grimnes and Jacobsen, 1997; this thesis, Chapter 5) and may be expected to have a similar impact in the wild. Although much of the evidence is anecdotal, numbers of sea lice infesting sea trout on the west coasts of Scotland and Ireland appear to have increased in the last 10 years (Sharp *et al.*, 1994; Tully and Whelan, 1993; Tully *et al.*, 1993 a,b), coincident with the dramatic decline in sea trout populations in these areas. Those Scottish and Irish areas which have seen the greatest declines in wild sea trout coincide geographically and temporally with the development of sea cage based salmon aquaculture and in those areas where wild fish are most heavily infested, salmonids cultured in sea cages also suffer from sea lice infestation (Costello, 1993). This has led to the suggestion that fish farms may be the source of this increase in sea lice numbers on wild salmonids and possibly a cause, whether directly or indirectly, of the decline in sea trout stocks. An ICES workshop was convened in Edinburgh in late 1996 to discuss "Interactions between salmon lice and salmonids" (Anon, 1997).

Salmon farming commenced in Scotland in 1971 and by 1976 the need to control infestations of sea lice by neurotoxic organophosphates such as dichlorvos (now 'Nuvan') became evident (Roth *et al.*, 1993; Sharp *et al.*, 1994). However, sea lice quickly developed some resistance to organophosphates (Jones *et al.*, 1992; Bron *et al.*, 1993; Roth *et al.*, 1993). More recently applied chemotherapeutants include hydrogen peroxide (Johnson *et al.*, 1993a; Thomassen, 1993) and orally administered ivermectin (Johnson & Margolis, 1993; Johnson *et al.*, 1993b; Smith *et al.*, 1993). A variety of other, non-chemical control methods including the use of cleaner wrasse (various species), vaccines and lice pathogens have been tested (see Costello, 1993). Despite chemical control methods, however, it has become apparent that salmon farms can be self-reinfesting with sea lice (Bron *et al.*, 1993; Costello, 1993) and a combined chemotherapeutic/good husbandry practice approach is now recognised as the most effective method of control. Good husbandry practices centre around fallowing and rotation of marine culture sites and restrictions on mixing of age classes of cultured salmon within the confines of given sea lochs (the "all in - all out" principle) (Grant & Treasurer, 1993).

No study has yet shown conclusively whether or not the recently reported increase in sea lice numbers present on wild salmonids is due to sea lice larvae emanating from

fish farm cages. Studies primarily in Ireland have attempted to draw correlations between the presence of fish farms in an area and increased numbers of sea lice infesting wild sea trout (Anon., 1993; Tully & Whelan, 1993; Tully *et al.*, 1993a,b). However, recent independent reviews (commissioned by the Irish Salmon Growers Association) of some of the Irish analyses suggested failings both in data collection (Cowx, 1997) and statistical analyses contained in the original reports (Cowx, 1994; Murphy, 1994). A subsequent independent review of this independent review supported most of the criticisms therein, but did find some failings in the review analysis as well (Rothschild, 1997). It is perhaps noteworthy that this second independent review did not examine the original reports or data.

Another approach has been to attempt to map the dispersal of sea lice larvae from fish farm cages but, again, there are technical problems. Costelloe *et al.* (1996) showed rapid dilution of sea lice larvae with increased distance from farm cages but the analysis was flawed by the removal of the larval concentration data for the sample point closest to the cage. Furthermore, while the data indicated some retention of larvae within a salmon farm cage, although they distinguished the post-hatching nauplius larval stages from the infective copepodid stage in their open water samples, no such distinction was made for larvae captured within the cage. Given the almost intractable difficulties of directly tracking marine invertebrate larvae in the field (Levin, 1986), perhaps one of the more fruitful indirect approaches to addressing the question of provenance of sea lice on wild salmonids is to examine the genetic structure of *Lepeophtheirus salmonis* populations over a geographic range to ascertain the degree of population differentiation. No evidence of differentiation would indicate a single panmictic population over that range and that the sea lice on wild and farmed salmonids must be considered genetically homogeneous. However, if significant differentiation were to be observed, that would point to some degree of genetic isolation and perhaps even disjunct populations of *L. salmonis* around the coastline and/or between sea lice found on farmed and wild salmonids.

For marine invertebrates the deduction commonly made is that the inclusion of a planktonic larva in the life cycle confers dispersal potential, extensive gene flow between populations and therefore genetic homogeneity (e.g. Johnson & Black, 1982; Hunt & Ayre, 1989; Liu *et al.*, 1991; Hunt, 1993; Williams & Benzie 1993; Benzie, 1994; Borsa *et al.*, 1994; Silberman *et al.*, 1994; Ayre, 1995). The life cycle of *Lepeophtheirus salmonis* was described in Chapter 5 (Figure 5.1) but of particular relevance to the patterns of population variation are the three planktonic larval stages (nauplii I and II and the infective copepodid). The egg-to-egg generation time

is related to seawater temperature, and can be as short as three weeks but possibly as long as 17 weeks (Tully, 1989, 1992; Johnson & Albright, 1991b). The likelihood is, therefore, that sea lice larvae are planktonic for at least four days (nauplius) and perhaps as long as 14 days (nauplius + copepodid) before infecting a host (Johnson & Albright, 1991b).

Sea trout tend to remain in coastal waters (see Section 3.1) and therefore, are not as wide-ranging in the marine environment as Atlantic salmon: multi-sea winter salmon may migrate across the full extent of the North Atlantic (Turrell and Shelton, 1993). In addition, except at spawning time, sea trout do not necessarily show such a high fidelity to their natal river. For example, tagged Scottish sea trout from the River North Esk (Angus) have been found temporarily to ascend the Rivers Tweed (Borders), some 100 km to the south and Spey (Grampian) 210 km to the north (Pratten & Shearer, 1983b). Given the planktonic period of *Lepeophtheirus salmonis* and the high mobility of migrating host fish, the likelihood is that there ought to be considerable genetic exchange amongst sea lice populations on wild salmonids around the Scottish coasts. Adult sea lice have been observed in large aquarium tanks to switch sea trout hosts by detaching, swimming and reattaching (pers. obs.) and Bruno & Stone (1990) observed transfers between Atlantic salmon post-smolts within aquarium tanks. Although no data are available regarding drift distances for the planktonic *L. salmonis* larval stages, the foregoing suggests that it is likely that there ought to be considerable gene flow and thus little (if any) genetic differentiation of *L. salmonis* around the coasts of Scotland (see also Todd *et al.*, 1994). However, the fish farm environment cannot be considered to be natural. The high densities of possibly immuno-deficient hosts may lead to rapid cycling between parasite generations and the frequent use of chemotherapeutants to control these parasites may engender selection pressures not found amongst the sea lice of wild salmonids. If fish farms are truly self-reinfesting then such conditions might conceivably lead to genetic differentiation between sea lice found on wild and farmed salmonids.

6.1.2 Genetic techniques

6.1.2.1 Allozyme electrophoresis

There are two types of modern population genetic analysis: allozyme electrophoresis and DNA electrophoresis. Isozymes are functionally similar but separable forms of enzymes (proteins), encoded by one or more loci (Markert and Moller, 1959). Isozymes that are products of different alleles at the same locus are termed

allozymes. Allozyme electrophoresis has been the mainstay of quantitative population genetics over the past 20 years and can provide powerful data on genetic variation because it allows indirect quantification of allele frequencies amongst populations. The technique is relatively inexpensive, requires little specialized equipment, is a relatively rapid procedure to perform on a large scale and allows screening of a large number of unlinked loci dispersed throughout the genome (Pasdar *et al.*, 1984). However, resolution is not always adequate for detecting differences between populations and individuals (see Park and Moran, 1995). Due to the redundancy in the DNA code that dictates protein sequences, all changes in a gene may not result in a change of the protein expressed. Thus many genetic variants are not detected by protein electrophoresis. Furthermore, protein electrophoresis is limited to detecting genetic changes that affect genes that actively express proteins detectable with a histochemical stain. These genes constitute only a small proportion of the genome.

Thus, in a survey of *Lepeophtheirus salmonis* from wild and farmed salmonids around the coasts of Scotland, Todd *et al.* (1997), found only two polymorphic loci for 20 loci screened whereas at least four are considered prudent for statistical analysis of population differentiation. A similar study of *L. salmonis* infesting six Norwegian salmon farms analysed four polymorphic loci (of 16 screened) but only one displayed marked differences in allele frequencies (Isdal *et al.*, 1997). Marked differences in allele frequencies have been observed between *L. salmonis* collected in Norway when compared with some collected in Japan (Åre Nylund, pers. comm. 1997) but this is hardly surprising given the huge geographic separation (Atlantic vs Pacific hosts).

Furthermore, allozyme electrophoresis poses problems for sample collection and storage. Some proteins are only available from internal organs (e.g. diaphorase (Dia-1) and lactate dehydrogenase (Ldh-5) which are extracted from trout eyes (Stephen and McAndrew, 1990)), and therefore, require that the subject is killed. In addition, allozyme electrophoresis requires active proteins so samples must be frozen quickly and stored at or below temperatures of –70°C.

The potential amount of genetic variation detectable by modern DNA methods vastly exceeds that detectable by protein electrophoresis because DNA sequences are assayed directly. Perhaps, therefore, a modern technique which examines variation at the DNA level would provide sufficient detail for the purpose of this study.

6.1.2.2 DNA techniques

6.1.2.2.1 Principles common to most studies of DNA variation

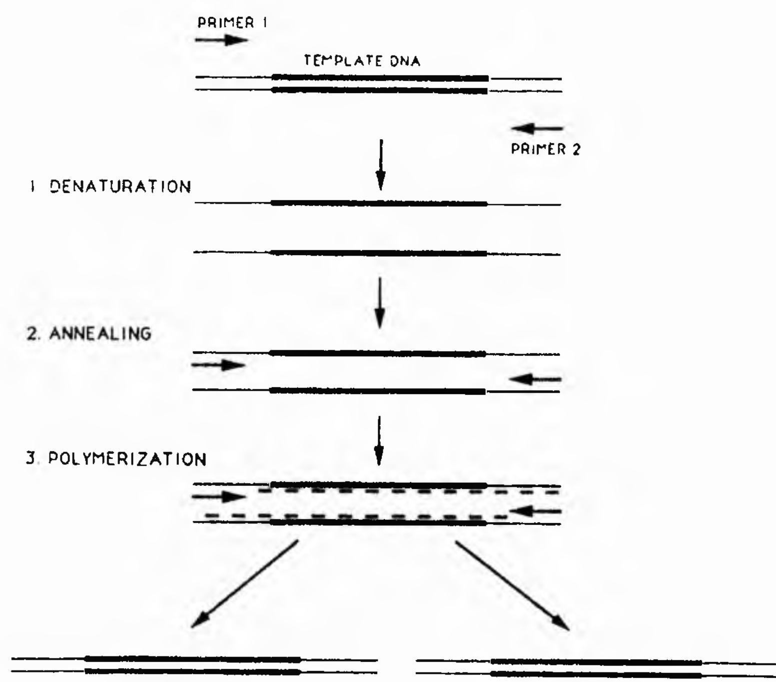
Normally, DNA exists as a double strand of nucleotides (G, C, A, T) (bases) in a helical matrix (Watson and Crick, 1953). However, the hydrogen bonds which maintain this helix can be broken (denatured) by heating and since no chemical changes take place, the complementary strands will join (anneal) together again as the DNA is cooled (Doty *et al.*, 1960). This principle is fundamental to modern molecular genetic analysis of DNA variation, particularly in DNA sequence amplification using polymerase enzymes.

Polymerase enzymes catalyse the synthesis of new strands of DNA using the denatured (single) strand DNA as a template but can only do this in the presence of a 'primer' - a short DNA sequence bound to a target area of the template DNA - and a supply of individual nucleotides (bases). The first polymerase enzymes used were also destroyed by the high temperatures required to denature the target DNA and initial amplification techniques required manual addition of fresh polymerase enzyme after each denaturation step. Thermally stable polymerase enzymes (originally isolated from geothermal vent organisms), which can withstand the temperatures required to denature the DNA helix, are now used to facilitate automatic sequence multiplication using the polymerase chain reaction (PCR) (Mullis *et al.*, 1986; Saiki *et al.*, 1988).

In PCR, a very small amount of template DNA can be used to amplify specific or anonymous nucleotide sequences. The template DNA is combined with two primer sequences and the four nucleotide subunits in the presence of a thermostable polymerase (Figure 6.1). The target DNA is denatured by heating and as it is cooled again, primers anneal to complementary sequences on the template. The polymerase then adds nucleotides to the primer in the correct code, as determined by the template, and the DNA is 'zipped up' again into two double helices. Most primers are designed so that they are complementary to sequences that flank the region to be amplified. One primer is made complementary to one strand, while the second primer is complementary to the opposite strand. Since amplification can only occur in the 5' to 3' direction, it occurs across the region of interest and towards the other primer, forming two new double stranded DNAs from the original template. The entire process is repeated some 20 - 40 times with the newly synthesized strands becoming templates for subsequent cycles. Because the number of target sequence regions is doubled with each cycle, the amplification proceeds

Figure 6.1

Figure 6.1 Diagrammatic representation of the polymerase chain reaction (PCR). The denaturation, annealing and polymerization steps are repeated 20 - 40 times, thus producing over a millionfold amplification of the original target sequence.
From Hoelzel and Green (1992).


PRIMER 1
TEMPLATE DNA
PRIMER 2
1 DENATURATION
2. ANNEALING
3. POLYMERIZATION

exponentially and 30 cycles results in more than a million copies of the target sequence. This technique allows researchers to study DNA sequences when only a small amount of DNA is available such as in valuable archive material and also, unlike allozyme electrophoresis, does not necessarily require the sacrifice of the specimen of interest. For example, DNA can be extracted from the adipose fin removed from a trout or salmon and the fish released afterwards (Taggart *et al.*, 1992).

A variety of modern molecular techniques are available to study genetic variation at the DNA level and all have distinct advantages and disadvantages, depending on time available, number of samples, expertise and equipment required and financial restrictions (Tables 6.1 and 6.2). A detailed description of the various techniques can be found in Hoelzel (1992).

6.1.2.2.2 RAPD technology

The recently developed randomly amplified polymorphic DNA (RAPD) technique (Welsh & McClelland, 1990; Williams *et al.*, 1990) was adopted in the present study because it is conveniently adapted to new species both in terms of training and procedures, it samples a large proportion of the genome at random and it is relatively inexpensive amongst DNA techniques (Hadrys *et al.*, 1992).

While most PCR techniques use specific primer sequences which anneal to pre-identified target regions of the genome, the RAPD method utilises short (10 - 12 bases long) primers to amplify random segments of DNA, hence the quick development time without the initial sequencing of target regions and construction of primers. A 10-mer will anneal to a random sequence approximately every million base pairs (Hoelzel and Green, 1992) and a small number of fragments (usually 5 - 30) will be amplified when the oligo anneals on each strand over a length range that can readily be amplified by PCR (usually less than 3 - 4 kb). Fragments are then size separated by agarose gel electrophoresis and visualized, typically by UV fluorescence of ethidium bromide bound to the double helix, as a series of bands. Individuals are scored for the presence and absence of these bands. Changes in the nucleotide sequence, either through base deletion, addition or substitution may remove existing primer sites, produce new ones, or increase or decrease the size of the amplified fragments, thereby accounting for the absence of bands or the occurrence of new bands.

Table 6.1

Table 6.1 Qualitative comparisons of several common molecular methodologies used to examine several different classes of DNA. Evaluations of practical issues are based upon general cases and do not apply to all specific cases. Various approaches were also evaluated for their utility in addressing more specific applications. Evaluations of mitochondrial DNA (mtDNA), single-copy nuclear DNA (scnDNA), and interspersed repeats are given for three different techniques. 'Tissue requirements' refers to constraints placed upon tissue collection for use with a given method: stringent (S) reflects a number of constraints (large amounts of tissue, multiple tissue types, large molecular weight DNA, etc.), relaxed (R) indicates relatively few constraints. NS indicates that a technique is generally not suitable for a given application.

1 = RFLP analysis; 2 = PCR/RFLP; 3 = PCR/sequencing.

VNTR = variable number tandem repeats. M = moderate. O = often; Y = yes; N = no.

From Park and Moran (1995).

	Protein methods	mtDNA[*] 1 2 3	scnDNA[*] 1 2 3	Interspersed repeats 1 2 3	VNTRs Single	VNTRs Multi	Microsatellites	RAPDs
Practical issue								
Tissue requirements[†]	S	M R R	M R R	M R R	S	S	R	R
Sacrifice of specimens[‡]	O	O N N	Y/N N N	Y/N N N	Y/N	N	N	N
Percent of genome	< 5	< 10^{-4}	40–70	~20	5–20	5–20	5–20	Unknown
Number of loci	10^2–10^3	1	> 10^6	10^5–10^6	10^4	10^4	10^4–10^5	Unknown
Relative rate of evolution among categories	Moderate	Rapid	Variable	Moderate/rapid	Moderate/rapid	Moderate/rapid	Rapid	Rapid
Type of study								
Pedigree analysis	Fair	NS	Good	NS	Excellent		Fair	Good
Population genetics	Excellent	Good	Good	NS	Good		Excellent	Fair
Hybrid zones	Good	Good/excellent	Fair	Good	NS		NS	NS
Phylogenetics	Fair	Excellent	Good	Excellent	NS		NS	NS

Table 6.2

Table 6.2 Salient features of some molecular genetic tools used in population analysis. Nature of the information considers whether the data can provide direct tests on genotype distributions (Hardy-Weinberg paradigm), the ability to provide measures of gene flow, and the probability of variants being under selective constraints. Comparisons of screening costs are based on costs per individual. VNTR = variable number tandem repeats. SLP = single-locus probes.
From Carvalho and Hauser (1995).

Molecular tools	Nature of information				Relative cost	
	Degree of polymorphism	Hardy–Weinberg distributions	Gene flow detected	Coding/non-coding (probability of selective constraints)	Start-up	Sample screening
Allozymes	Low–moderate	Yes	Yes	Coding (some loci, high)	Low	Low–moderate
Mitochondrial DNA						
Probing	Low–moderate	No	Female only	Some coding (low–moderate)	High	Moderate–high
PCR-based	Low–moderate	No	Female only	Some coding (low–moderate)	Moderate	Moderate
Nuclear DNA (VNTR)*						
Probing	High	Yes (SLPs)†	Yes	Non-coding (low)	High	Moderate–high
PCR-based	High	Yes (SLPs)	Yes	Non-coding (low)	Moderate	Moderate

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However, while the RAPD technique is advantageous for the present study for the reasons above and has been used to study genetic variation in, amongst others, invertebrates (Okamura *et al.*, 1993), birds (Haig *et al.*, 1994) and plants (Huff and Peakall, 1993; Yu and Pauls, 1993; Vicario *et al.*, 1994), there are also some constraints (reviewed by Hadrys *et al.*, 1992). Bands are dominant markers and preclude the discrimination of heterozygotes and homozygotes, thereby negating the use of some traditional population genetic statistical analyses. The banding patterns are sensitive to changes in amplification conditions, such as the concentration of template DNA or MgCl, which may give rise to potential problems of repeatability. In addition, since the sequence of bands is unknown, the possibility of unrecognised DNA contamination is greater than other techniques. However, Grosberg *et al.* (1996), in a comprehensive and objective review of the technique and its applications, addressed these disadvantages and concluded that, "while RAPDs are unsuited to the characterization of breeding systems, the calculation of population genetic parameters such as F-statistics, or the inference of phylogenetic relationships above a species level, providing care is taken in both the generation and interpretation of markers, RAPDs hold as yet unmatched promise for characterizing hierarchical genetic structure and close genetic relationships in large samples".

6.1.3 Aims of this part of the study

This study reports on RAPD analyses of sea lice sampled from the east, north and west coasts of Scotland. The samples included sea lice from wild salmon and sea trout and sea-farmed salmon and rainbow trout. As such, the aims of this study were as follows:

(1) to establish whether or not sea lice found on wild and farmed salmonids around the coasts of Scotland are genetically panmictic;

(2) if not, whether any genetic differences can be used to indicate farm or wild provenance of parasites.

6.2 Materials and Methods

6.2.1 Sources of sea lice sample material

Sea lice were collected from both wild (four river catchments) and farmed (four locations) salmon, and wild sea trout (four river catchments; one embayment) from the east, north and west coasts of Scotland (see Tables 6.3 to 6.5; Figure 6.2). For convenience, fish hosts are identified with particular river systems, but with two exceptions (wild salmon from the Rivers Dionard and North Esk) these fish were actually taken from the river estuaries. Other than the "Argyll" sea trout which were captured in July 1994, all sample material of adult and pre-adult sea lice was obtained between July and November 1995.

6.2.2 RAPD analysis

An initial screening of 10 individuals (5 males and 5 females) from each of eight populations (Table 6.6) with 10 RAPD primers (Operon, California, USA) (Experiment 1) gave results at variance with previous allozyme data from this laboratory (Todd *et al.*, 1997) and called for a more detailed analysis with sea lice samples from more sources, particularly from west coast wild salmonids. Of necessity, therefore, a further series of polymerizations (Experiment 2) for eight additional samples of sea lice was undertaken (Table 6.7), but including only six primers selected from the seven primers that revealed polymorphisms in Experiment 1. The seventh polymorphic primer was not included as it was not recognised as polymorphic during preliminary visual examination of Experiment 1 gels. In view of the small sample sizes, primers were considered to reveal genetic polymorphism if the frequency of particular bands was zero in one or more populations and at least 0.40 in another. Inspection of the data suggested this was a conservative but sensible subjective criterion.

6.2.2.1 DNA extraction and PCR conditions

Total DNA was extracted using a standard phenol-chloroform procedure, as described by Taggart *et al.* (1992) but modified to suit the sample tissues (Figure 6.3), and DNA concentrations were determined by gel electrophoresis. Preliminary experiments showed that the template DNA concentration used for PCR could be varied over a 100-fold range and still produce consistent banding patterns. For routine PCR, approximately 6 ng of template DNA was used and optimal resolution

Table 6.3

Table 6.4

Table 6.3 Sources of farmed salmonids (Atlantic salmon unless otherwise stated) from which sea lice were collected during 1995.

Table 6.4 Sources of wild salmon from which sea lice were collected during 1995.

Farm Location	Date Collected	Host weight range	No. sea lice collected
Farm 1 (Sutherland)	Sample B (28 Aug.)	2.5 - 3.5 kg	120 - 200
	Sample C (6 Nov.)	2.5 - 3.5 kg	120 - 200
Farm 2 (South Uist)	30 October	2.5 - 3.5 kg	40
Farm 3 (Wester Ross)	6 November	0.3 kg	38
Farm 4 (Argyll)	Sample X (3 July)	mean 3.5 kg	98
	Sample Y (25 Oct.) Rainbow trout	mean 0.7 kg	40
	Sample Z (3 Nov.) FO - cages.	no data	72

Location	Date Collected	Host weight range	No. sea lice collected
River Tay (Perthshire)	6 July	50 Grilse and 16 Salmon	513
River South Esk (Angus)	12 July	Salmon	51
	20 July	Grilse	55
River North Esk (Angus)	7 November	Salmon (6) (4 - 8 kg)	18
River Dionard (Sutherland)	30 August	2.5 kg (1 fish)	6

Table 6.5

Table 6.5 Sources of wild sea trout from which sea lice were collected (1995 unless otherwise stated).

Location	Date Collected	Host weight range (kg)	No. fish	No. sea lice collected
River South Esk (Angus)	12 July	1 - 3	no data	27
	20 July	1 - 3	no data	37
	10 August	1.05 - 1.40	6	11
River North Esk (Angus)	2 August	0.062 - 0.202	132	22
River Dionard (Sutherland)	11 - 14 June	0.120 - 0.474	7	12
	22, 27 July	0.150 - 0.180	3	15
	28 - 30 August	0.050 - 0.343	11	32
River Gruinard (Wester Ross)	7 July	0.062 - 0.170	4	13
Dunstaffnage Bay (Argyll)	25 July 1994	0.096 - 0.222	3	10

Figure 6.2

Figure 6.2 Outline map of Scotland, showing the river systems and districts referred to in the text.

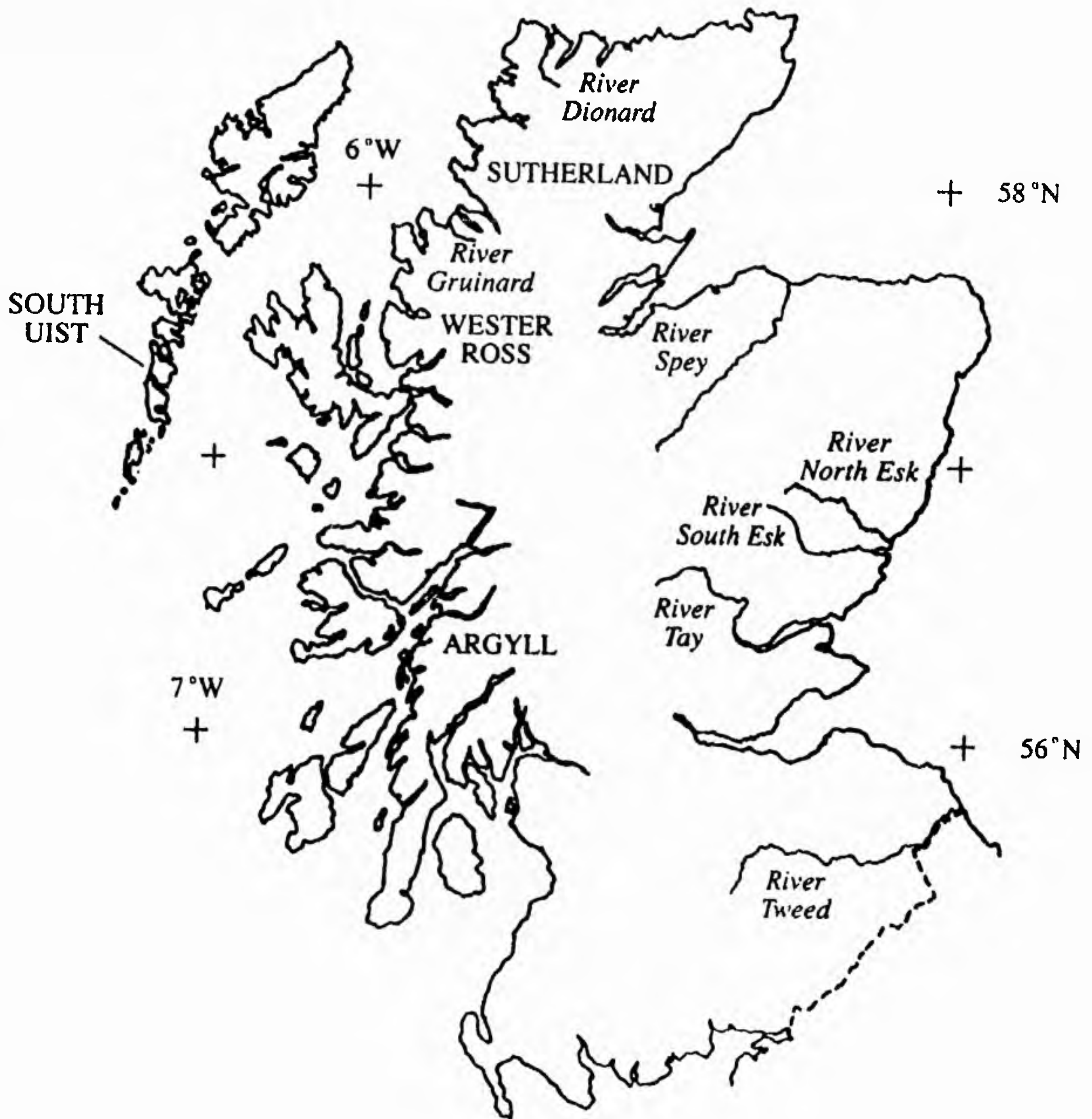

River
Dionard
6°W
SUTHERLAND
58°N
River
Gruinard
SOUTH
UIST
WESTER
ROSS
River
Spey
River
North Esk
River
South Esk
River
Tay
ARGYLL
7°W
56°N
River
Tweed

Table 6.6

Table 6.7

Table 6.6 Details of number of host fish and number of sea lice screened in Experiment 1 from various wild and farmed salmonid sources.

Table 6.7 Details of number of host fish and number of sea lice screened in Experiment 2 from various wild and farmed salmonid sources.

Host species	River/Farm	No. fish sampled	No. sea lice screened
Wild Atlantic Salmon	R. Tay (Perthshire)	5	10
	R. South Esk (Angus)	8	10
	R. Dionard (Sutherland)	1	6
Wild Sea trout	R. South Esk (Angus)	5	6
	R. North Esk (Angus)	6	9
	R. Dionard (Sutherland)	7	10
Farmed Salmon	Farm 1 (Sutherland)	na	10
	Farm 4 (Argyll)	na	10

Host species	River/Farm	No. fish sampled	No. sea lice screened
Wild Atlantic Salmon	R. North Esk (Angus)	6	10
Wild Sea trout	R. Griunard (Wester Ross)	3	12
	Dunstaffnage Bay (Argyll)	3	10
Farmed Salmon	Farm 1 (Sutherland)	na	10
	Farm 2 (Western Isles)	na	10
	Farm 3 (Wester Ross)	na	10
	Farm 4 - FO cages (Argyll)	na	10
Farmed Rainbow trout	Farm 4 (Argyll)	na	8

Figure 6.3

Figure 6.3 Sea lice DNA extraction protocol modified from that described in Taggart *et al*. (1992).

Sea lice DNA extraction protocol (based upon Taggart *et al*., 1992)

DAY 1

1. Remove genital segments from adult louse with scalpel blade and place carapace in a 1.5 ml Eppendorf tube.
2. Add 375 μl CTAB buffer and 6 μl DNAase free RNAase (10 mg/ml, Boehringer).
3. Grind with small grinder - 10 strokes.
4. Vortex briefly and incubate for 1 hour at 37 °C.

DAY 2

5. Add 400 μl phenol to each tube.
6. Vortex for 10 s then rest for 10 s, repeat 4 times.
7. Add 400 μl chloroform/isoamyl alcohol (24:1) and repeat step 6.
8. Centrifuge at 13000 RPM for 5 minutes.
9. Using a wide bore pipette (P1000) carefully remove top aqueous layer to a new tube.
10. Repeat steps 5 - 9.
11. Add 800 μl ice cold 96% ethanol to the aqueous layer and vortex briefly followed by storage at -20 °C for 15 minutes to allow DNA to precipitate.
12. Centrifuge for 10 minutes.
13. Carefully decant off ethanol and add 1 ml ice cold 70% ethanol to the pellet.
14. Vortex briefly and store at -20 °C for 30 minutes.
15. Decant off ethanol and remove last drops from inverted tube with glass pipette.
16. Allow DNA partially dry at room temperature 5 - 10 minutes.
17. Dissolve DNA in 50 μl TE buffer solution (at least overnight).

Buffer Recipes.

CTAB (2X):	0.1M Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% CTAB, 1% PVP + distilled H2O
TE Buffer:	10 mM Tris, 1 mM EDTA; pH 8.0
Dynazyme optimised:	10 mM Tris-HCl (pH 9.0 at 20 °C), 50 mM KCl, 2 - 5 mM MgCl, buffer (Flowgen) 0.1% Triton X100

of the PCR products was achieved with a final MgCl concentration of 1.5 mM. Negative PCR controls were produced by replacing template DNA with distilled water: although fragments typically were resolved for some of the negative controls, on no occasion did they conform to bands in the experimental samples. Further positive controls in Experiment 2 were included by running repeat PCR of eight individuals from three of the populations from Experiment 1.

Each PCR reaction included template DNA, free nucleotides, distilled water, a single 10-mer primer, Dynazyme optimised buffer (Flowgen) and Dynazyme-DNA polymerase (Flowgen). The PCR programme, using a PTC 100 thermal cycler (MJ Research, Inc.), was as follows: one denaturation at 94°C for 3 min; 45 cycles of (i) 30 s at 94°C, (ii) 45 s at 40°C, (iii) an increase to 72°C at the rate of $0.4°C.s^{-1}$, and (iv) 90 s at 72°C; a final 4 min at 72°C. PCR products were resolved on 1.4% agarose gels containing ethidium bromide and were photographed under UV light. A molecular weight ladder was run alongside each gel lane to allow the weight of bands to be estimated, where appropriate.

All RAPD photographs were scored by two observers independently and the data remained uncoded until scoring was complete. Only those bands that were consistent across both gels within an experiment were scored and only those bands which could be resolved over both experiments (see below) were analyzed. On some occasions, bands would appear faint in some gels but bright in others. The presence of faint bands often meant that scorers could not be confident of recording a presence or absence for some samples. In these circumstances, the band was not included. This did result in some apparently informative bands necessarily being dropped from the analysis.

6.2.3 Statistical methods

RAPD band variation (band presence/absence) for individuals within and between the populations screened was analysed by AMOVA, using the program WINAMOVA (Excoffier *et al.*, 1992; Huff *et al.*, 1993), which allows partitioning of the within-population and between-population variance. The latter component, termed phi_{ST}, is analogous to Wright's F_{st} (1969) in that it provides a measure of the proportion of the overall variation attributable to population differentiation, albeit based not on allele frequencies but on phenotypic band sharing. Since WINAMOVA (Excoffier, 1993) would not accommodate missing values (which arose from occasionally failed PCR for various individuals and primers), the analytical data set was reduced to include only those individuals for which all primers were successful.

Band-sharing between all individuals was calculated using the Euclidean distance as advised by Excoffier *et al.* (1992). With this distance matrix WINAMOVA performs an analysis of variance and the computed variance components provide an overall estimate of phi_{ST} (the variance between populations) in addition to pairwise phi_{ST} values between the various populations (Excoffier *et al.*, 1992; see also Yeh *et al.*, 1995). Although based on phenotypes, phi_{ST} here is broadly analogous to Weir & Cockerham's θ (1984) which commonly is directly estimated from allelic allozyme data. The significance of the pairwise phi_{ST} values for RAPDs was assessed at the conservative level of $p < 0.01$ in comparing the probability that a random distance is greater than the observed distance.

As a final illustrative procedure, the final 120-individual RAPD data set (six primers; 41 RAPD bands) was subjected to bootstrapping (Felsenstein, 1985) using the SEQBOOT routine of PHYLIP (Felsenstein, 1993). The latter resamples the original traits (bands) across all individuals with replacement, until 41 resampled traits are assembled. Consistency of the patterns found in these data sets indicates the robustness of the information within the original data matrix. Pairwise distance matrices were constructed for each resultant data set using a similarity index calculated as the number of matches (shared presence or absence of bands) divided by the total number of bands scored. This was used in preference to the more conventional Nei & Li band sharing index (Nei & Li, 1985) because, at the low genetic distances studied here, the extra information gained is likely to outweigh errors introduced by non-homology of shared band absences. Distances were calculated as 1-similarity using RAPDPLOT (Black, 1995). Twenty such bootstrapped matrices were used for neighbour-joining tree construction (input order randomized), which is more robust than UPGMA to assumptions concerning the rate of band replacement, and these trees were subject to consensus analysis (Felsenstein, 1993).

6.3 Results

6.3.1 Experiment 1

These data encompass a minimum geographic range (headland to headland) of approximately 890 km between the Tay estuary and Argyll (Figure 6.2). The ten primers used in Experiment 1, and the number of bands scored (in parentheses), were OPA2 (13), OPA3 (15), OPH1 (7), OPH2 (4), OPH11 (13), OPH14 (5), OPH15 (12), OPH17 (2), OPH18 (12) and OPH19 (5). Seventeen of the 88 RAPD bands scored were monomorphic throughout the eight populations (Plate 6.1). One primer revealed an apparently sex-specific marker pair but was not considered polymorphic on the criteria given above: OPH11 showed two large fragments (Table 6.8: OPH11.a, OPH11.b; 2.4 and 1.8 kb respectively) which were lacking in all 31 of the males scored, but were both present in 37 of 38 females scored (Plate 6.2). The one apparent misclassification was not for the presence of unexpected bands in a male, but for a female in which the bands were apparently absent.

Table 6.8 summarizes the frequencies of polymorphic RAPD bands for sea lice from salmon, sea trout or rainbow trout from each location. The overall pattern of RAPD polymorphism for these eight populations is very clear, despite the small sample sizes and sporadic incidence of missing values for individual sea lice. For Experiment 1 there were 710 possible phenotypes (71 individuals x 10 primers). The 27 missing values reduced these to 683 individual results, amongst which there were 94 instances of primers revealing polymorphism. Seventy of the 94 polymorphisms were recorded amongst the 20 sea lice from the two farmed salmon populations. Nine of the 15 polymorphic bands recorded for Experiment 1 were exclusive to these farmed populations (e.g., Plate 6.3) while four (OPA2.4, OPA2.9; OPH1.6, OPH1.7) were predominantly associated with farms: these bands are hereafter described as 'putative farm marker' bands. Only two bands (OPH11.4; OPH14.a) were exclusive to sea lice on wild fish. Putative 'farm marker' bands were recorded for four individual sea lice from wild hosts (two from River Tay salmon and one each from River North Esk and River South Esk sea trout). Of the two markers absent from the salmon farms, OPH14.a might be an east/north coast marker, having been recorded for sea lice from both wild salmon and sea trout.

Plate 6.1

Plate 6.2

Plate 6.3

Plate 6.1 RAPD fingerprint gel (OPH 18) demonstrating the consistency of banding patterns amongst individuals and the presence of monomorphic bands. Lanes are numbered from left to right. Two populations of sea lice were screened: Population 1, lanes 1-10; Population 2, lanes 11-20. Lane 22 is a 1 kb molecular ladder (lane 21 is empty).

Plate 6.2 RAPD fingerprint gel (OPH 11) demonstrating the sex-specific banding patterns. Lanes are numbered from left to right. Individuals in lanes 5 - 8, 10, 12 - 14 and 20 are females, as indicated by the bright band second from the top of lane, as well as several other fainter bands.

Plate 6.3 RAPD fingerprint gel (OPH 15) for 18 individual *Lepeophtheirus salmonis* sampled from two salmon farms. Two RAPD bands are diagnostic for Farm 1 (lanes 2 - 9) and one band is diagnostic for Farm 2 (lanes 10 - 19). None of these bands were recorded from parasites on wild salmonids. Lanes 20 and 21 are control negative and positive polymerisations, respectively.

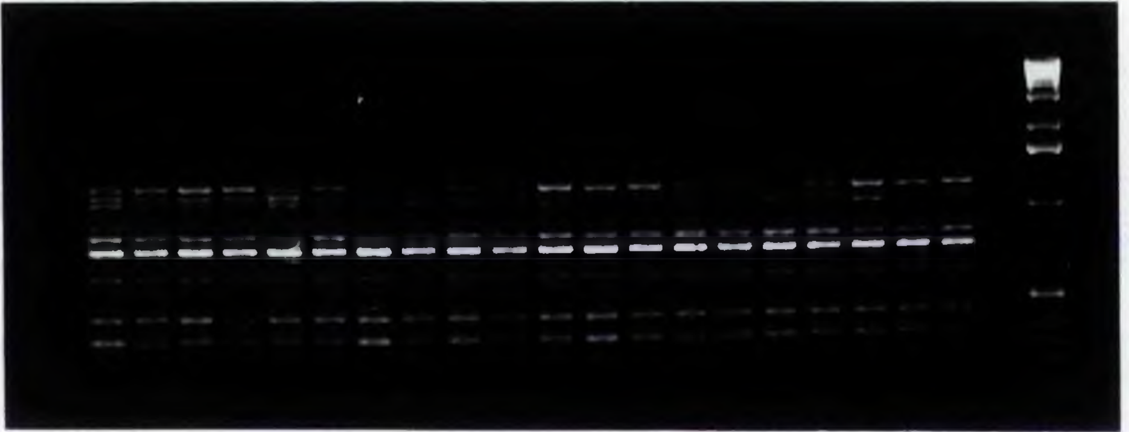


Table 6.8

Table 6.8 Frequencies of RAPD phenotypes for the polymorphic bands scored across Experiments 1 and 2. RAPD bands are numbered in decreasing order of fragment size. Lettered (un-numbered) bands denote RAPD markers that had been scored in Experiment 1, but which necessitated exclusion from the overall AMOVA analysis because they could not be unequivocally scored in Experiment 2. The tabulated data include observations for all individuals in each population and take account of missing values for given primers. The data for each population included in the AMOVA analysis therefore differ slightly from these tabulated frequencies for certain populations (e.g. River Gruinard sea trout, for which only three individuals comprised complete data sets for all primers). (FO) = Farm Ocean cages; S, ST and RT = salmon, sea trout and rainbow trout, respectively; * = one missing value; ** = two missing values; † = five missing values; †† = six missing values;
nd = no data, primer not run.

River/Farm	Tay (A)	South Esk	South Esk	North Esk	North Esk	Dion-ard
Region	East	East	East	East	East	North
Species (S, ST, RT)	S	S	ST	S	ST	S
Wild (W)/ Farmed (F)	W	W	W	W	W	W
Experiment No.	1	1	1	2	1	1
PRIMER. band number/letter						
OPA2.e	0	0	0	nd	0	0*
OPA2.1	0	0	0	0	0	0*
OPA2.2	0	0	0	0	0	0
OPA2.4	0.10	0	0	0	0	0
OPA2.9	0	0	0.17	0	0	0
OPA2.10	0	0	0	0	0	0
OPH1.6	0.10	0	0	0*	0.11	0*
OPH1.7	0.10	0	0	0	0.11	0
OPH11.a	0.40	0.50	0.67	nd	0.56	0.80*
OPH11.b	0.40	0.50	0.67	nd	0.56	0.80*
OPH11.1	0	0	0	0.10	0	0*
OPH11.4	0	0	0.50	0	0.11	0.20
OPH14.a	0.70	0.20	0.67	nd	0	0.25**

Dion-ard	Farm 1 (B)	Farm 1 (C)	Farm 2	Farm 3	Gruin-ard	Farm 4	Farm 4 (FO)	Farm 4	Argyll
North	West	West	West	West	West	West	West	West	West
ST	S	S	S	S	ST	S	S	RT	ST
W	F	F	F	F	W	F	F	F	W
1	1	2	2	2	2	1	2	2	2
0*	0.40	nd	nd	nd	nd	0.10	nd	nd	nd
0*	0.90	0.11*	0.80	0	0††	0	0.11	0	0.10
0	0.90	0	0.80	0	0	0	0.11	0	0
0	0	0	0	0	0	0.40	0	0.13	0
0	0.50	0.22	0	0	0	0.50	0.33	0	0.10
0	0	0	0.60	0	0	0	0	0	0
0*	0.22*	0.11*	0	0.40	0.33**	0.30	0.50*	0.63	0*
0	0.22	0.11	0	0.40	0.33	0.40	0.50	0.75	0.11
0.56*	0.50	nd	nd	nd	nd	0.50	nd	nd	nd
0.56*	0.50	nd	nd	nd	nd	0.50	nd	nd	nd
0*	0.30	0.80	0.10	0*	0.11**	0.90	0.25*	0	0*
0.11	0	0	0	0	0.78	0	0	0	0.56
0*	0	nd	nd	nd	nd	0	nd	nd	nd

Table 6.8 continued

River/Farm	Tay (A)	South Esk	South Esk	North Esk	North Esk	Dion-ard
Region	East	East	East	East	East	North
Species (S, ST, RT)	S	S	ST	S	ST	S
Wild (W)/ Farmed (F)	W	W	W	W	W	W
Experiment No.	1	1	1	2	1	1
PRIMER band number/letter						
OPH15.2	0	0	0	0	0	0
OPH15.3	0	0	0	0	0	0
OPH15.4	0	0	0	0	0	0
OPH15.5	0	0	0	0.10	0	0
OPH18.a	0	0	0	nd	0	0
OPH2.3	0*	0*	0	0	0	0

Dion-ard	Farm 1 (B)	Farm 1 (C)	Farm 2	Farm 3	Gruin-ard	Farm 4	Farm 4 (FO)	Farm 4	Argyll
North	West	West	West	West	West	West	West	West	West
ST	S	S	S	S	ST	S	S	RT	ST
W	F	F	F	F	W	F	F	F	W
1	1	2	2	2	2	1	2	2	2
0*	0.90	0*	0.90	0*	0†	0	0.25*	0	0
0	0.90	0	0.80	0	0	0	0.25	0	0
0	0	0	0	0	0	0.90	0.25	0	0
0	0	0.11	0	0	0	0.10	0.50	0	0
0	1.00	nd	nd	nd	nd	0	nd	nd	nd
0**	0.80	0	0	0*	0**	0	0*	0	0

Overall, there was an apparent east/north coast vs west coast differentiation of sea lice populations but the pattern of polymorphism may not be simply geographic because of the admixture of farmed and wild populations included. Nearly all Scottish mainland salmon farms are confined to the west coast and the only two west coast samples in Experiment 1 were restricted to the two farms initially sampled. No data were therefore available for west coast populations of sea lice from wild hosts. RAPD Experiment 2 partially addressed this specific issue in order to provide a more representative data set for more detailed analysis.

6.3.2 Experiment 2

All banding patterns of the repeated 'control' individuals in Experiment 2 complied with those recorded for those same individuals in Experiment 1, but not all bands which had been reliably scored in Experiment 1 could be scored for Experiment 2. Accordingly, the RAPD data statistical analyses for all 16 populations sampled across the six chosen primers were confined to those bands which were reliably resolved in both experiments. Among the bands excluded from the overall analysis was one apparent 'farm marker' band from OPH18. The numbers of bands scored for the respective primers in Experiment 2 were 11 (OPA2), 8 (OPH1), 5 (OPH2), 6 (OPH11), 7 (OPH15) and 4 (OPH18). In common with 18 other bands over these six primers, the two sex-specific OPH11 bands from Experiment 1 could not be unequivocally resolved for all individuals in Experiment 2 and consequently were excluded from the final data set. In total, 29 individuals across all six primers were deleted from the analyzed data set, resulting in information extending to 120 individuals (16 populations) and 41 RAPD bands. The overall phi_{ST} value for the 120-individual data set was 0.362, indicating that 36% of the overall variation was due to between-population differences. Sea lice samples from all the farmed salmon/rainbow trout populations were significantly different from the wild populations (Table 6.9), but there was no marked geographic or species specific differentiation between sea lice populations from wild hosts.

Figure 6.4 shows the neighbour-joining RAPD phenogram for all 120 individuals. One individual was arbitrarily chosen as the outgroup. Since this individual is fixed for all the bootstrapped replicates it spuriously appears well supported (see Felsenstein, 1993). Because this is a consensus tree of the bootstrapped data, it is not possible to place a precise scale of genetic distance on the diagram. The genetic distance between the most differentiated pair of individuals (23rd and 24th from the top of the figure) and the basal cluster of five nodes (excluding the outgroup node) was, however, approximately 0.18, indicating an overall genetic similarity of the

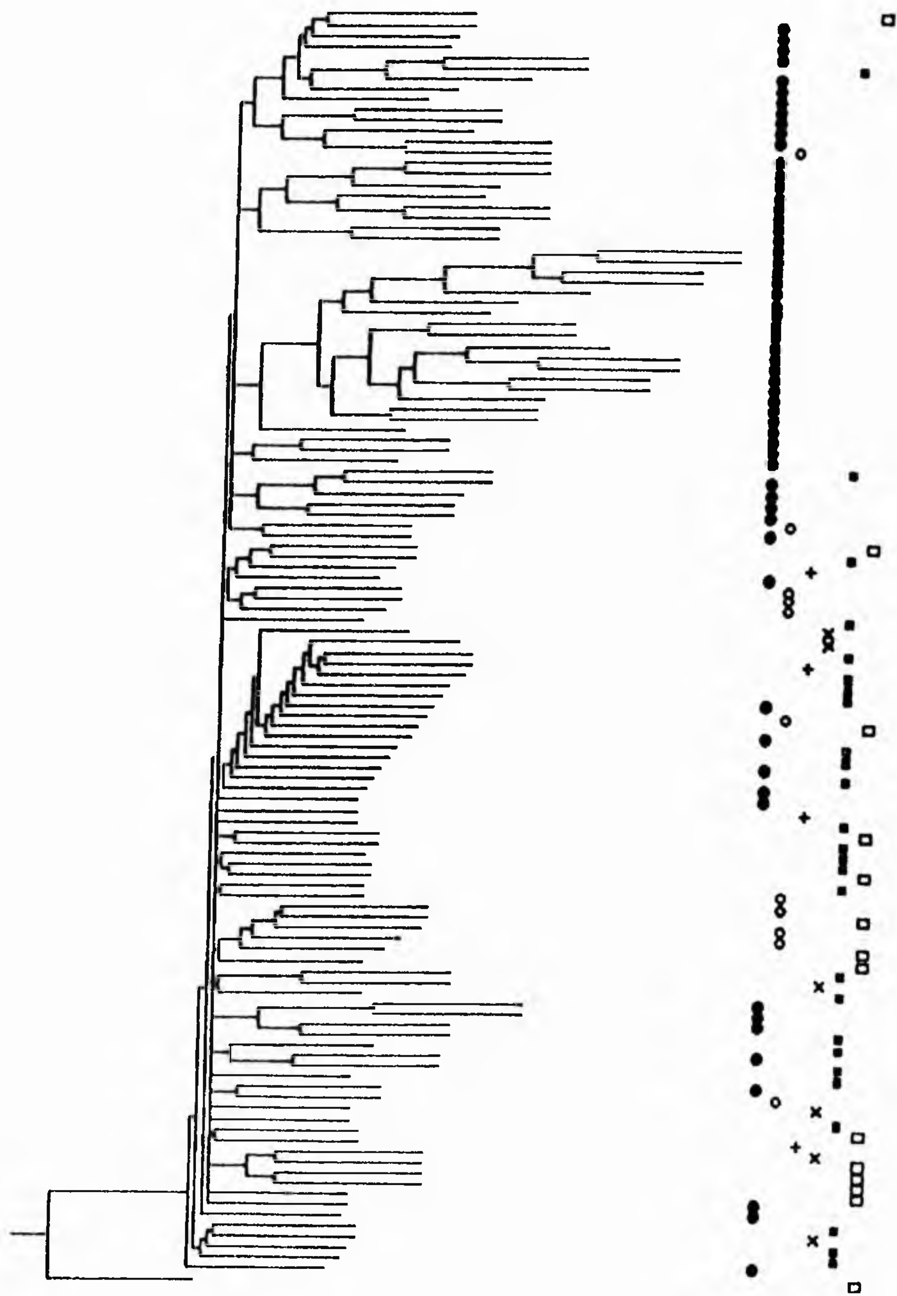
Table 6.9

Table 6.9 Matrix of pairwise phi_{ST} from AMOVA (below diagonal) of RAPD phenotypes and the probability that the observed distance is different from a random distance (above diagonal). Significant differences (S) are indicated at the $P < 0.01$ level. It is important to note, in relating these phi_{ST} values to the tabulated band frequencies in Table 6.8, for example, that all individuals for which there were one or more missing values were excluded from the AMOVA analysis.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Tay salmon		.21	.07	.59	.84	.83	.23	S	S	S	.16	.10	S	S	.03	S
2. S. Esk salmon	0.01		.06	.38	.14	.49	.23	S	S	S	.10	.06	S	S	S	S
3. S. Esk sea trout	0.08	0.11		.09	.51	.93	.25	S	S	S	.73	.03	S	S	S	.34
4. N. Esk salmon	-0.03	-0.01	0.09		.85	.77	.12	S	.04	S	.11	.01	S	S	S	.02
5. N. Esk sea trout	-0.07	0.04	-0.01	-0.06		.90	.14	S	.03	S	.33	.12	S	.02	.02	.05
6. Dionard salmon	-0.08	-0.05	-0.13	-0.09	-0.14		.37	S	.09	S	.65	.17	.08	.08	.02	.43
7. Dionard sea trout	0.03	0.03	0.03	0.07	0.06	0.00		S	.02	S	.22	.18	S	.02	S	.10
8. Farm 1A salmon	0.61	0.67	0.57	0.64	0.60	0.57	0.60		S	S	S	S	S	S	S	S
9. Farm 1B salmon	0.15	0.27	0.21	0.13	0.13	0.12	0.23	0.57		S	.02	S	S	.02	S	S
10. Farm 2 salmon	0.48	0.55	0.47	0.52	0.48	0.44	0.47	0.27	0.48		S	S	S	S	S	S
11. Griunard sea trout	0.10	0.22	-0.13	0.17	0.05	-0.09	0.09	0.58	0.24	0.45		.07	S	.10	.02	.81
12. Farm 3 salmon	0.08	0.16	0.17	0.18	0.09	0.11	0.08	0.61	0.25	0.51	0.18		S	.17	.23	.02
13. Farm 4 salmon	0.36	0.45	0.37	0.40	0.36	0.34	0.38	0.58	0.23	0.55	0.37	0.35		S	S	S
14. Farm 4 (FO) salmon	0.15	0.23	0.18	0.18	0.13	0.12	0.16	0.45	0.16	0.36	0.14	0.07	0.18		.02	S
15. Farm 4 rainbow trout	0.16	0.34	0.27	0.29	0.18	0.21	0.25	0.59	0.28	0.52	0.28	0.04	0.33	0.12		S
16. Argyll sea trout	0.12	0.16	0.01	0.16	0.12	0.00	0.09	0.58	0.25	0.47	-0.08	0.15	0.39	0.22	0.22	

Figure 6.4

Figure 6.4 Neighbour-joining phenogram of the RAPD phenotypes for the 120 individual data set (all missing values excluded) from Experiments 1 and 2. For clarity, individual parasites have been coded into six categories only, according to the provenance of the host fish: solid circle = west coast, farmed salmon; open circle = west coast, wild sea trout; + = north coast, wild salmon; x = north coast, wild sea trout; solid box = east coast, wild salmon; open box = east coast, wild sea trout.



120 individuals of >0.82. None of the nodes were consistently supported among the bootstrapped data set, reflecting the overall high genetic similarity.

For illustrative clarity the individuals for the 16 populations included in Figure 6.4 were combined into six categories only (farmed salmon/rainbow trout, wild sea trout (east, north and west coasts)) and wild salmon (east and north coasts) and are coded accordingly. The tree shows that the pattern of differentiation is one of a generally clear distinction between sea lice originating from farmed salmon and those from wild salmon and sea trout. There was, however, a small proportion of sea lice sampled from farms which were phenotypically indistinguishable from those sampled from wild fish. The patterns of polymorphism were complex and, for the one farm with repeat samples separated by 3 months (Farm 1; samples B and C), temporally and/or spatially variable to a considerable extent (Table 6.8).

Band OPH11.4 was recorded for 18 individual *Lepeophtheirus salmonis*, of which only one was parasitic on wild salmon (River Dionard), with the remainder on wild sea trout from both east and west coasts. Notwithstanding the absence of west coast wild salmon samples, this may indicate some genetic differentiation between sea lice on wild sea trout and farmed salmon, and possibly between wild sea trout and wild salmon.

It is necessary also to emphasize the effects of deletion of individuals from the AMOVA analysis due to missing values for some polymerizations. Table 6.8 included all results (149 individuals), whereas the final data set for AMOVA (Table 6.9) and individual band-sharing analysis (Figure 6.4) had been reduced to the 120 individuals for which the 6-primer data set was complete. The wild sea trout sample of 11 sea lice from the River Gruinard produced only three individuals with a complete set of results for all six primers; sea lice collected from River Gruinard sea trout showed 47/66 missing values whereas the ten sea lice from "Argyll" sea trout showed only 2/60. Of the ten sea lice screened from "Argyll" sea trout, one carried OPA2.1 and OPA2.9 and another carried OPH1.7. Similarly, for the River Gruinard sea trout, two sea lice carried OPH1.6 and OPH1.7, one OPH1.6 and OPH11.1 and another OPH1.7. These OPA markers were otherwise found exclusively on sea lice collected from farmed salmonids and the OPH markers were predominantly recorded from sea lice collected from farmed salmonids (see Table 6.8). Thus, at least six of these 21 *Lepeophtheirus salmonis* sampled from west coast wild sea trout carried markers otherwise predominantly or exclusively associated with farms. This might, therefore, indicate a farm provenance for some of the sea lice found on west coast wild sea trout.

6.3.3 Analysis of possible host DNA contamination

PCR techniques amplify small amounts of target DNA to produce in excess of a million copies of the target region. However, this process is susceptible to contamination by foreign DNA which might also be amplified. Since RAPD bands are anonymous, such a contamination event might result in foreign bands which are indistinguishable from source bands. The possibility of contamination can be guarded against by following good lab practices and using sterile instruments and chemicals for PCR. In addition, the source of bands can be ascertained by subsequently sequencing the band in question and using this information to create a band specific primer to screen further samples. However, adopting this approach from the beginning negates most of the advantages of using RAPDs. In this study, a separate analysis of the possibility of salmonid DNA contamination was performed.

Blood products are often visible in the gut of adult female sea lice, most noticeably in the mid-gut and hind-gut regions. From visual inspection the (much smaller) adult males seldom showed evidence of blood in the gut. In the study described above, the genital segments from all female sea lice were removed and only the carapace used for PCR - this was done to avoid possible problems with stored spermatozoa DNA and also to reduce the amount of possible host blood in the louse material. However, the possibility remained that the presence of host blood in the anterior louse gut might pose a DNA contamination risk.

The following is a summary of several tests carried out to investigate the possibility of salmonid DNA contamination in the sea lice PCR reactions. Female sea lice were used because they were observed to contain visible quantities of host blood and therefore more likely to be susceptible to host contamination products. Throughout the tests, where available, the egg strings of sea lice were screened together with the respective female's carapace. Since egg strings are not directly connected to the gut system they act as a contamination-free control but the RAPD phenotypes will be complex due to the possibility of multiple paternity.

6.3.3.1 Comparison of banding patterns between known host and parasite

This is perhaps the most direct test because the potential contaminant host RAPD profiles are known. Sea trout post-smolts from the River North Esk were infected with sea lice larvae reared from ovigerous females collected from wild River Tweed salmon (see Chapter 5). Five adult female sea lice, three with egg strings, were

collected from one trout and screened for the seven polymorphic primers (see above) along with blood, muscle, skin scrapings and mucus swipes collected from the host after it had been killed in the manner described in Chapter 2. All sea lice were observed to have been feeding on the blood of the host.

None of seven polymorphic primers produced shared bands between the sea lice and the host trout.

Five sea lice were removed from an anaesthetised sea trout post-smolt and placed on an anaesthetised naive post-smolt salmon. After 18 days four sea lice were collected from the salmon and screened against blood sampled from the host. All sea lice were observed to have been feeding on host blood despite only apparently superficial skin damage. Blood samples were also taken from four other salmon and three sea trout to compare within-species polymorphisms. DNA extractions were screened using the seven polymorphic primers.

Only OPH 14 produced an apparently shared band between sea lice and sea trout but this band was subsequently observed in RAPD products from DNA extracted from egg strings, indicating that the bands were of similar size but did not indicate host contamination.

Host skin scrapes and mucus swipes failed to produce detectable quantities of DNA or any PCR products. Thus they are very unlikely to be a source of contamination.

6.3.3.2 Artificial host DNA contamination

A range of dilutions of salmon and sea trout DNA were added to sea lice DNA prior to PCR-RAPD screening to investigate the proportion of contaminant DNA necessary to produce foreign bands. Note these were artificial combinations of pristine DNA extracted from individual fish and combined with individual sea louse extractions. Contaminant DNA proportions of 25%, 14%, 9%, 4%, 2% and 1% were assessed. DNA extracts from sea lice collected from wild sea trout, wild salmon and farmed salmon were contaminated with DNA from either a wild sea trout or a wild salmon and all screened using primer OPH 15. This primer was chosen as it had previously (1) produced bright, consistent bands for sea lice and salmonids and, (2) revealed easily recognised sea lice polymorphisms.

Salmonid DNA produced poor RAPD results in all three cases but the conservative result was that a minimum of 9% contaminant DNA was required before even faint

contaminant bands began to appear and that a minimum of 14% contaminant DNA was necessary in order to affect the brightness of the sea lice bands and to produce contaminant bands clear enough to score. Since the hind gut and genital segments, which contain the majority of blood products, are not included for extraction, it is unlikely that this amount of host DNA would be available during the PCR reaction. Furthermore, contamination was performed with pristine DNA - even slight enzymatic degradation of the fish blood DNA in the louse gut would markedly reduce the PCR results.

6.3.3.3 Further comparison of banding patterns from salmonids and sea lice

Sea lice collected from wild and farmed salmon were screened against blood and muscle extractions from wild sea trout and salmon using the seven primers listed above. Where available, egg strings were also extracted and screened.

The OPH 14 band which was apparently shared between lice and salmonids was also produced in the egg strings of some sea lice. Since the egg string extractions are blood contaminant free this indicates that this band in sea lice is not a contamination product.

6.3.3.4 Procedural steps to reduce the possibility of contamination

The genital segments of female sea lice were routinely removed from the carapace prior to DNA extraction. This not only removes the majority of blood products but also opens the foregut allowing other products to leak out. The following tests were performed to investigate whether the blood products in the genital segments would result in contaminant bands and whether clearing of the foregut contents prior to extraction would reduce the number of bands.

Sea lice were placed individually on paper towel and the egg strings removed. The sea louse was then held at the mouth of an Eppendorf and the genital segments cut from the carapace with a scalpel blade such that leaking gut contents were collected in the Eppendorf with the genital segments. Then the carapace was either placed in another Eppendorf and extracted as normal or placed ventrally on a piece of absorbent tissue, the foregut contents squeezed out by the flat of a scalpel blade and then washed in CTAB buffer several times before DNA extraction.

Six female sea lice with three sets of egg strings (from the South Uist farm), six males from the same farm, five sea lice with one set of egg strings (from the

Sutherland farm) and six sea lice with three sets of egg strings collected from River Tweed salmon were screened against one salmon and one trout blood sample for all seven polymorphic primers. Again, apart from the "shared" band of OPH14, no other co-occurring bands were observed in sea lice (carapace or genital segments) and fish. This implies that contamination from the blood products in the genital segments is extremely unlikely, probably because host DNA has been degraded by digestive enzymes, and that squeezing out of the foregut contents did not result in fewer bands from the carapace extractions.

6.4 Discussion

6.4.1 Summary of results

- sea lice collected from wild and farmed salmonids were screened with between 6 and 10 RAPD primers
- 8 RAPD bands were exclusive to parasites collected from farmed salmonids
- 7 RAPD bands were found at much higher frequencies in parasites collected from farmed versus wild salmonids
- 2 RAPD bands were exclusive to parasites collected from wild salmonids
- there was no evidence of host blood contamination of the PCR reactions
- at the sample level, parasites from farmed salmonids were different from those collected from wild salmonids and were different between fish farm sites

6.4.2 Discussion of results

The use of primers to amplify anonymous DNA sequences in RAPD-PCR does allow the potential for unrecognised foreign DNA contamination to be scored and included in subsequent analyses. However, the results of the comprehensive series of tests of host DNA contamination indicate that such an event was extremely unlikely to occur given the protocols and stringent lab practices adopted in the present study. Therefore, one can conclude that the patterns of genetic variation apparent amongst sea lice samples collected from farmed and wild salmonids are genuine.

The RAPD data (Tables 6.8 and 6.9; Figure 6.4) showed clearly that most of the sea lice from the four salmon farms sampled were genetically distinct from those collected from wild salmonids. In addition, there were considerable distinctions between the farms: in Experiment 1, for example, six of the nine putative 'farm marker' bands from the six primers ultimately analysed were private to Farm 1, one private to Farm 4 and two shared. Two other marker bands were absent in sea lice collected from salmonids sampled on the west coast. For Experiments 1 and 2 combined there were 402 (67 individuals x 6 primers) possible RAPD phenotypes for sea lice from farmed salmon and rainbow trout. For eight of these individuals there were missing values, but 56 of the 67 sea lice from the four farms each carried at least one, and up to nine, putative 'farm marker' bands while 48 carried two or more marker bands. Of the 11 individuals which showed no 'farm marker' bands, six were from Farm 3.

The major feature in these RAPD data to be explained is the genetic differentiation of the farm populations over perhaps only 120-150 generations since salmon farming began in Scotland. Two possible explanations are founder effect and selection or, more likely, a combination thereof. Founder effects could arise from small numbers of initial colonist larvae, perhaps of rare RAPD phenotypes, increasing to epizootic levels on fish as a result of some degree of localized reinfestation of farm cages. Founder effect may, therefore, explain the occurrence of a small number of bands at high frequency in farm populations (and low frequency in the wild), but this alone probably would not explain the observed high number of separate 'farm marker' bands and their typically high frequencies within farms. There would have to be additional and persistently high levels of immigration of extremely rare RAPD phenotypes into the sea lice populations of fish farms for founder effect alone to produce the patterns observed in the present study. Given the extreme levels of differentiation of sea lice from Farms 1, 2 and 4, and the high number and frequency of putative 'farm marker' bands recorded, it is likely that the combined effects of founder events and selection would be commensurate with the genetic patterns observed amongst the sea lice collected from the various farm samples included here. An obvious potential selection agent in the farm environment might be chemotherapeutant treatments applied to control parasite numbers. The development of resistance to such chemicals amongst sea lice populations (e.g. Costello, 1993) may indicate an intense selection effect is possible.

Farms 1-3 had adopted differing chemotherapeutic husbandry strategies and the patterns of variation amongst the *Lepeophtheirus salmonis* on all four farms sampled were complex. Farms 1 and 2 showed extreme levels of differentiation but there also were high levels of spatial/temporal variation amongst the samples from Farm 1, with bands OPA2.2, OPH15.2 and OPH15.3 declining from frequencies of 0.90 (August) to zero (November). The consistent banding of the repeated 'control' individuals in both experiments indicated that these results were not a reflection of unreliable PCR. These samples were obtained from different cages held within the same raft and, therefore, the data cannot be used to distinguish between small-scale spatial, or temporal, heterogeneity as possible explanations. Farm 3, by contrast, showed RAPD phenotypes closely similar to wild-type sea lice, with 'farm marker' bands present only in 4 of the 10 individuals. This may be attributable to those samples having been collected from only juvenile salmon (mean weight 300 g). Sea lice collected from Farm 4 salmon and rainbow trout, which had received no direct chemotherapeutic treatment, not only showed high frequencies of putative 'farm

marker' bands, but, like Farm 1, also displayed high levels of spatial/temporal heterogeneity. Irrespective of chemotherapy, therefore, it may be that the very nature and uniformity of the farm environment itself also provides some selection pressures. The confinement of hosts at high density may select for those sea lice that are most compatible with farmed salmon and that are able to settle out early in their developmental life history. It has, for example, been proposed that farm cage conditions enhance the passive uptake of planktonic stages of sea lice via the gill filaments and that these lice then migrate to other areas of the fish prior to attaining the first (attached) chalimus stage (Anon., 1994b). None the less, the incidence of 'farm marker' bands (especially for OPH1 and OPH15) at Farm 4 might be at least partially explained by the fish being infested by larvae emanating from adjacent farms (at least 8 km distant) at which chemotherapeutants had been routinely applied. If infestation of farmed fish is indeed essentially attributable to copepodids deriving from farmed rather than wild stocks, interaction between founder events and further selection may well lead to the patterns of differentiation implied by the present data.

The *Lepeophtheirus salmonis* sampled from "Argyll" wild sea trout were netted only 1 km from the Farm 4 raft cages and showed RAPD phenotypes closely similar to sea lice from east coast wild hosts: only one out of those ten sea lice carried two 'farm marker' bands. The "Argyll" sea trout were captured in the previous summer to the Farm 4 salmon and rainbow trout samples, and yet the farm has been continuously active for many years. Those sea trout should have been vulnerable to copepodids deriving from Farm 4 or indeed other adjacent farms. Of the 21 *L. salmonis* typed from the two west coast wild sea trout populations (10 "Argyll"; 11 River Gruinard), six individuals carried putative 'farm markers'. Neither of those two "Argyll" sea lice carried the putative wild sea trout marker (OPH11.4), whereas all four from River Gruinard did so. On the assumption that the aforementioned RAPD markers are indicative of a farmed origin, this result might suggest that OPH11.4 is not a true wild sea trout louse population marker, or that these particular *L. salmonis* could have derived from a fertilization between parents from wild and farmed fish. Irrespective of this complication, and given the assumption that these markers are inherited as Mendelian factors, the important deduction can be drawn that some degree of gene flow between *L. salmonis* populations on salmon farms and wild sea trout hosts appears likely.

6.4.2 Further work

Clearly, much more extensive and comprehensive samples and controlled breeding data on the heritability of these putative farm and wild salmonid marker bands are required to confirm the general patterns of genetic differentiation of *Lepeophtheirus salmonis*. Furthermore, it will be essential to confirm the homology of the 'farm marker' bands and those occurring amongst sea lice on wild hosts. Detailed time-series samples from separate cages within farms starting at the naive post-smolt stage of the host life cycle rather than sampling fish generally only at harvest will be required as a first step towards identifying the cause of the spatial/temporal heterogeneity noted above. In addition, regular sampling before and after chemotherapeutic treatment, and after treatment of different types, will be required to establish whether chemotherapeutants are possible selective agents.

Whatever the cause(s) of the observed patterns on farms, a major objective must be to explain the incidence of putative 'farm markers' amongst sea lice on wild hosts. Whereas some of the RAPD marker bands appear from the present samples to be exclusive to sea lice collected from the farmed salmonids (i.e. OPA2.2, OPA2.10, OPH15.2-4, OPH2.3), others (e.g. OPA2.4, OPH15.5) also showed low frequencies in those sampled from wild host populations, distant from the west coast farms (e.g. Rivers Tay and North Esk). For these particular sea lice, assuming that the patterns shown here primarily result from founder events and selection in the farm environment, the difficulty remains in discriminating these as being representative of low background frequencies of alleles in the wild population, as opposed to being indicators of infestation of wild fish by sea lice emanating from the farms. The recording of single marker bands will, in itself, be insufficient to attribute a farm origin for those particular lice. However, on the basis of much larger samples, comparisons of multi-locus genotype frequencies of the various marker bands in farmed and wild populations of *Lepeophtheirus salmonis* will allow probabilistic statements about the likelihood of their emanating from farms to be made with some confidence.

6.5 Concluding remarks

RAPD analysis revealed 14 putative 'farm marker' bands for *Lepeophtheirus salmonis*. Two additional RAPD markers apparently were absent from sea lice parasitizing farmed salmon. Although the AMOVA showed that populations of *L. salmonis* on wild salmon and sea trout are not significantly different from one another overall, there are indications from one primer of subtle levels of

differentiation between them. The overall data indicates that a proportion of the individual parasites sampled from west coast sea trout probably are of farmed origin, but more comprehensive data are required before it will be possible to ascertain with confidence the levels of gene flow and any possible implications in local sea trout stock collapses.

Chapter 7

General Discussion

Trout, *Salmo trutta*, are a highly adaptable species of salmonid with the ability to exploit a variety of environmental conditions, as is demonstrated by the large number of their successful introductions worldwide over the past 150 years. This adaptability is due, to a large extent, to the considerable flexibility or plasticity exhibited within the trout life cycle. Within the freshwater environment, for example, such plasticity is reflected by the considerable size range attainable by adult trout. Campbell (1971) reported a two year old, sexually mature female brown trout measuring only 10.7 cm in length captured together with two mature males measuring only 7.9 cm and 11.2 cm respectively. In contrast, piscivorous 'ferox' trout can grow to in excess of 10 kg (Walker, 1994). In addition, of course, trout are not necessarily restricted to the freshwater habitat. As is the case for most salmonid species, some trout adopt an anadromous life history strategy. These 'sea trout' migrate from freshwater to spend a variable period of time in the marine environment, presumably benefiting from the superior growth opportunities therein. In addition, plasticity is also apparent amongst sea trout themselves: whereas some will remain at sea for at least one year before returning to freshwater to mature and spawn, others - described as finnock in the present thesis - return to estuaries and freshwater within only weeks or a few months of migration to sea.

However, despite the apparent adaptability of this species, and the considerable flexibility in life history pattern demonstrated by individuals, some ancestral stocks of sea trout have suffered severe declines in population numbers over approximately the last decade. Investigations regarding the cause(s) of these population crashes have been hampered by the fact that, traditionally, most scientific research on *Salmo* species has focused on the Atlantic salmon rather than the trout. This is certainly due in considerable part to the greater commercial value ascribed to the Atlantic salmon. This fish has traditionally been regarded by anglers as one of, if not the, prize fish to catch because of its potential size - the present British record is 64 lbs or 29.03 kg. Atlantic salmon is also regarded as a valuable food item with products such as smoked salmon attracting luxury item status. As a consequence, Atlantic salmon have also been the focus of considerable and extensive development in aquaculture in the last 26 years, both in freshwater and, in particular, the marine environment. This commercial development has led to considerable research investigating various aspects of the salmon life cycle in order to benefit the fish farming industry. One avenue of research which has obvious commercial implications and which has received particular attention, is the ability of salmon to survive transfer between freshwater and seawater at particular stage in the life cycle. Of interest here is the commercial expedient of reducing the culture period in

freshwater so as to maximize farm output in the marine environment. Successful manipulation of smoltification in Atlantic salmon now produces some juveniles which can be placed at sea at least six months prior to the earliest smoltification that might be encountered in the natural environment (see below).

Fish inhabiting the freshwater environment are hyper-osmoregulators, i.e. their body fluids are more concentrated than the surrounding medium, and as a consequence, these fish are constantly gaining water and losing ions. Physiological mechanisms are in place to maintain homeostasis in such circumstances. In contrast, the body fluids of fish in the marine environment are more dilute than the surrounding medium, and as a consequence, they constantly losing water and gaining ions. Salmonids and other diadromous fishes, must reverse these physiological mechanisms if they are to survive and adapt after migration to seawater, or from seawater to freshwater.

In most anadromous salmonids, migration between freshwater and seawater is preceded by a series of coordinated physiological, morphological and behavioural changes which pre-adapt the juvenile to survival in the marine environment. This series of changes is called the parr-smolt transformation, or smoltification. Smoltification in salmon has been one of the major avenues of research encouraged by the development of aquaculture described above. Two aspects of smoltification have received particular emphasis. One aspect is the identification of those factors which induce salmon to undergo this transformation, in order to produce fish which smolt at as early an age as possible. Salmon grow much faster in seawater than in freshwater. The typical length of a two year old salmon smolt is between 12 and 15 cm whereas salmon returning after one year in the sea can measure between 50 and 70 cm in length. Thus, it is commercially advantageous to produce smolts as young as possible. Presently, reared juvenile salmon can be induced to undergo smoltification as soon as six months after hatching. The other main avenue of research regarding smoltification has been to identify the optimal time at which to transfer juvenile salmon from freshwater to seawater. Smoltification in wild salmonids is a continuous series of developmental processes that occur over a period of several months and culminate in migration to the marine environment. In aquaculture, where smolts are artificially transferred from freshwater to seawater, it is imperative to establish that the smoltification process is complete prior to this transfer. Transfer either before the transformation is complete, or after physiological changes have begun to regress, often results in poor growth and stunting, if not death. Therefore, because it is essential for fish farmers to be able to

identify when the transformation is complete, a considerable amount of research has focused on characterizing the physiological processes involved in smoltification and the various internal and external factors by which it is coordinated.

A further aspect of the salmon life cycle which is of particular importance in aquaculture is the control of, or more specifically, the delay of maturation. Maturation alters the flesh quality and considerably reduces the market value of the product. Early maturing salmon, which in wild stocks, return to freshwater after only one year at sea, are unwanted in aquaculture for two main reasons. They may represent a reduced return on the investment since they are relatively small when harvested. Furthermore, they constrain the supply to the market as they must be sold prior to maturation, regardless of the market demand (and price) at the time.

As well as the considerable commercial impetus to focus scientific research on Atlantic salmon rather than trout, the gulf of knowledge between the two species has probably been widened by the apparent misconception that the results of research on Atlantic salmon could be directly extrapolated to include trout as well. However, the considerable differences in the life history patterns, reproductive strategies and migratory patterns between the two species suggest that this is certainly not necessarily a valid assumption. Various differences between the two species will be highlighted in the appropriate sections of the following discussion.

It is apparent, therefore, that research into both anadromous and freshwater-resident trout has been limited to the extent that large gaps exist in our understanding of the basic ecology, population dynamics, physiology and behaviour of this species. The general aim of this thesis, therefore, was to investigate some of aspects of sea trout biology and, in particular, focus attention on those factors relevant to the drastic decline of some wild sea trout populations.

One facet of sea trout biology which is certainly not fully understood is the combination of and interplay between, factors that induce some juvenile trout to adopt the anadromous life history pattern whilst others remain in freshwater for their entire lives. For example, anadromous and resident trout have been observed spawning together (Johnson and Gravem, 1985), both forms have been produced from offspring of either form (Frost and Brown, 1967; Johnson and Gravem, 1985; Walker, 1990) and with one recent exception (Thomson, 1995), no genetic distinction has been established between sympatric anadromous and resident stocks (see Ferguson, 1989). Growth in freshwater is a factor in the 'choice' of whether or

not to migrate to sea because several studies have demonstrated that fewer migrants are produced under conditions of good growth (Jonsson, 1989; Walker, 1990). However, a genetic component to the tendency to become anadromous is also indicated (Alm, 1959; Walker, 1990).

The present study manipulated growth rates of juvenile trout by means of three ration levels. In addition, this ration treatment was duplicated in juvenile trout of three parental groups - sympatric Sea trout and freshwater-Resident trout, and Isolated trout from above a waterfall impassable by upstream migrating anadromous trout. The development of seawater tolerance was studied by measuring drinking rates after periodic salinity challenges during the first two years of juvenile growth.

No smolts were observed in any of the parental form/ration combinations after two years in freshwater. Sea trout smolts from the east coast of Scotland are typically two or three years old at the time of their first migration (Pratten and Shearer, 1983a). However, a considerable proportion of the experimental trout did mature during this time period. Reproduction is considered to take priority over growth in salmonids (Thorpe, 1989, 1990). In the present study, only mature male trout were evident in the second autumn of growth (age 1+) whereas both males and females were maturing when sampled towards the end of the following spring. This reflects the difference in reproductive strategy between the sexes where the advantages of growth before maturation are considerably greater for females than males, and hence, males tend to mature at an earlier age and smaller body size. The proportion of maturing trout was, as expected, directly related to ration levels but was also influenced by parental form, with Isolated trout demonstrating a greater tendency to mature early. This was considered to reflect genetic difference in reproductive strategy selected for by life in a small stream.

Seawater tolerance increased with age in all groups. This was not unexpected because seawater tolerance is related to body size in small salmonids (McCormick and Naiman, 1984). However, mean drinking rates upon salinity challenge were generally greater in Resident trout than in either of the other two groups. The Resident trout were typically larger than the others throughout the study and this body size difference might have been a factor in the drinking rate data. However, analysis of the data with the influence of body weight removed still supported this parental group effect. The reasons for this difference remain unclear at the present time but warrant further study to establish whether such differences would persist over a longer period of salinity challenge and acclimation, and if so what effects this

would have on the subsequent survival and growth rates of these trout in the marine environment?

With the current severe decline in numbers of sea trout returning to many river systems on the west coasts of Scotland and Ireland, one possible remedial mechanism to restore natural populations is to supplement juvenile recruitment with hatchery-reared sea trout smolts. However, it is apparent from the results of the present study and other investigations (Jonsson, 1989; Walker, 1990) that the inherent plasticity in the life cycle of trout may preclude the production of smolts in many areas because of the difficulty in artificially creating the conditions necessary to induce juvenile trout to adopt an anadromous strategy. Two years of rearing to produce no smolts would be a costly and wasteful investment of resources.

Perhaps, therefore, juvenile trout should be planted into streams at a much earlier age, possibly as early as the fry stage, with the intention that the natural conditions will induce a greater proportion to smolt than would do so under hatchery conditions. However, this option has several problems associated with it especially given that the decline of wild sea trout populations appears related to mortality problems at sea, rather than production of juveniles in the freshwater environment (Whelan, 1993; Walker, 1994b). Stocking fry into areas of stream which already contain high densities of juveniles would, most probably, result in considerable mortality of the hatchery-reared fry due to competition for resources. Therefore, if this approach were to be adopted, fry should be planted into areas of stream which presently have unnaturally low densities of juveniles. However, previous studies have demonstrated that the stocking of trout into such areas tends to result in a high proportion of freshwater-resident fish rather than anadromous migrants (Walker, 1990; Jonsson *et al.*, 1994), presumably due to the good growth conditions resulting from low competition for resources. Thus, stocking of fry might be most fruitful in areas where trout densities were lower than that which the resources could support. In such circumstances, stocking fry might not only increasing the number of potential migrants, but increase also the competition for resources, thereby perhaps stimulating a greater proportion of native juveniles to migrate to sea. Recent studies of juvenile trout densities in various Scottish west coast river systems have, however, indicated that although densities are lower than the national average. This is not surprising given low productivity of the freshwater habitat in these areas. The indications are, however, that stocking fry in many areas would not be a cost-effective measure to increase numbers of returning sea trout (Walker, 1994a). Thus,

one must return to the concept of stocking systems with hatchery-reared smolts and investigate further mechanisms to maximise the proportion of smolts produced.

It has been established that the major physiological decisions of smolting and sexual maturation in Atlantic salmon occur during brief periods of development, typically during spring and early summer, and depend on the metabolic performance of the fish at that time (Thorpe, 1986, 1989; Adams and Thorpe, 1989). If similar 'windows of opportunity' extend to trout then further research should focus on the effects of ration, and other environmental factors, on smoltification at these times.

A great deal of research has focused on the effects of altering photoperiod and water temperature on the growth (Thorpe *et al.*, 1989), development and subsequent seawater tolerance of Atlantic salmon smolts. Some changes characteristic of the parr-smolt transformation are influenced by these factors while others appear to be independent of environmental influence or manipulation. Constant photoperiod and water temperature throughout juvenile life does not affect cyclical changes in juvenile morphology such as silvering, darkening of the fins and the reduction in condition factor (Johnston and Eales, 1970; Eriksson and Lundqvist, 1982), but does result in non-existent or reduced peaks in branchial Na^+K^+-ATPase activity (McCormick *et al.*, 1989; Olsen *et al.*, 1993), and poor seawater survival (McCormick *et al.*, 1987). It is apparent that changes in photoperiod act as cues to entrain the endogenous rhythms which underlie smoltification. Juvenile Atlantic salmon subjected to decreasing daylength in late winter and spring demonstrated high condition factor in freshwater at the time of expected smolting and stunting after transfer to seawater (Saunders and Henderson, 1970). However, juvenile Atlantic salmon reared under constant photoperiod and then returned to simulated natural photoperiod (SNP) in the autumn demonstrated similar seawater tolerance the following spring to smolts reared under SNP, whereas those returned to SNP in December demonstrated delayed (and possibly diminished) seawater tolerance (McCormick *et al.*, 1987). In the context of the present study, however, the ability to accelerate or delay the onset of smoltification is not as important as inducing juvenile trout to adopt the choice of smolting in the first place. The rearing of juvenile Atlantic salmon at elevated water temperatures has been shown to increase growth and the proportion of juveniles undergoing smoltification (Johnston and Saunders, 1981). However, as discussed above, such good growth conditions have the opposite effect of inducing trout to mature rather than become smolts.

An additional example of the paucity of information regarding the sea trout is our poor understanding of all aspects of the biology of finnock - those sea trout which return to estuaries and/or freshwater within a few weeks or months of migrating to sea. In a similar manner to the greater attractiveness of the salmon when compared to the sea trout, finnock have been the most neglected stage in the sea trout life cycle, probably because they have always been a part of many sea trout populations but were considered of little more than nuisance value by anglers. As a consequence, very little is known regarding the behaviour of finnock or the causes behind this enigmatic life history and migratory strategy.

Confusion still remains as to the definition of a finnock. Elliott (1994), in his review of *Salmo trutta* ecology, implied that since finnock were predominantly males, their early return to freshwater ensured that spawners were from at least two successive year-classes, in a similar manner to that of coho salmon. Coho salmon males which return to spawn after six months at sea are called 'jacks' whereas the remainder of males do not return to spawn until they have been at sea for at least 18 months and are called 'hooknoses' (Gross, 1985). One major difference between coho salmon and sea trout, however, is that whereas coho salmon are semelparous (spawn once and then die), sea trout are iteroparous in that they may return to spawn on several occasions. For example, a sea trout from a spawning tributary of Loch Maree, Wester Ross, was estimated to be 19 years of age and to have spawned 11 times (McLaren, 1989).

Elliott (1994) also noted that only a small proportion of finnock actually mature during their first return to freshwater (see Fahy, 1978; Pratten and Shearer, 1983a; Le Cren, 1985). Thus, the finnock tactic cannot be directly classed as a reproductive strategy. Elliott's illustrative study of the trout of Black Bows Beck, in which the majority of finnock mature, is atypical of finnock from most other British sea trout populations. A relatively high proportion of finnock ascending the River Orne, Normandy are maturing (Maisse *et al.*, 1991) but this obscures the distinction between a finnock and a sea trout. Accordingly, I have suggested that the definition of Pratten and Shearer (1983a) be modified to include only those trout which do not mature during this migration.

The status of the finnock component of sea trout populations is of particular relevance to the dramatic decline of sea trout stocks on the west coasts of Scotland, Ireland and Norway. In Scotland, at least, the evidence points not only to increased marine mortality, but also reduced growth at sea and the virtual disappearance of the

larger, older sea trout (Walker, 1994a). This makes finnock of great importance to stock recovery as the present spawning stocks are based more on those that survive the finnock stage from year to year. A considerable section of this thesis was dedicated towards investigating the behaviour of and possible causal factors involved in this stage of the sea trout life cycle.

Previous reports of finnock movements are rare and those available focus on movements in estuaries and between rivers rather than within freshwater (Pemberton, 1976a; Pratten and Shearer, 1983b; Le Cren, 1985). In this present study, the movements of eight radio-tagged finnock in the River Eden, Fife, were recorded during September, October and November 1994. The individual finnock displayed considerable variations in patterns of movement. Two remained in freshwater for at least 27 days whereas others moved downstream out of the river within days or even hours of release. In general though, this study highlighted the transient nature of the freshwater migrations of some finnock, indicating that they move in and out of rivers over brief periods of time and apparently do not remain in freshwater continuously throughout the winter. Once again, this clearly demonstrates the flexibility of the trout life cycle.

However, several technical problems were encountered in this study and, as such, the main benefit of the present study was as a preliminary assessment of the practicality of using such methods to study finnock behaviour. If such an investigation were to be repeated, the present study has highlighted several necessary improvements. The most obvious requirement of further studies would be a greater number of individual finnock to track. A small sample size is probably one of the most recurrent criticisms of any radio-tracking study. Tagging sufficient numbers of finnock relies upon catching them first. Anglers using very light tackle would be a suitable source of undamaged and minimally stressed finnock during the summer and autumn months. An advantage of using rod-caught finnock would be that they could be released soon after capture, without the possibly extended delay resulting from trapping fish. However, another aim of further tracking studies would be to compare the movements of finnock during the summer and winter months to establish whether finnock found in freshwater during the late winter remained in freshwater for longer periods than those caught in the summer. The requirement to capture finnock outwith the angling season would necessitate the use of either electrofishing or a trap. The former was not very successful in the present study. However, the latter would require regular attention and monitoring, both to process captured finnock and to remove debris. In addition, a trap containing

finnock would attract the unwanted attentions of predators (seals, otters and mink) and also poachers.

Providing sufficient finnock can be caught and radio-tagged, the next requirement, which is directly related to tracking large numbers of animals simultaneously, would be the efficient deployment of several ALSTNs. In the present study, finnock were considerably more mobile than freshwater-resident brown trout radio-tracked in the same river (Armstrong and Herbert, 1997). This made it difficult to track several fish on foot simultaneously. Furthermore, the inability to continue tracking during the hours of darkness reduced the amount of data gathered and increased the likelihood of finnock moving during the night and not being subsequently relocated. The use of a series of ALSTNs located at regular intervals throughout the river system, and a short distance upstream from the confluence of each major tributary, would facilitate the location of missing tagged fish and reduce the amount of time spent routinely scanning the river.

Financial considerations would ultimately restrict the number of ALSTNs available but of additional importance would be the necessary requirement of a secure site for each unit. However, the number of ALSTNs should be determined by the study objective. First, if the objective is simply to establish how long finnock remain in freshwater then one, or preferably two, ALSTNs situated towards the mouth of the river below the site of release should be sufficient. Second, if the objective is to expand the study to investigate where the finnock actually are in freshwater, specifically whether they move to the spawning grounds or remain in the main stem of the river, then sufficient ALSTNs ought to be available to site one at the mouth of each spawning tributary. Tagged finnock which are recorded by these ALSTNs can then be located on foot if finer resolution of data is required.

As discussed in Chapter 3, however, the detail which a radio-tracking study cannot provide is whether finnock which move downstream out of the river but remain in the estuary or continue onwards and move out to sea again. A further tracking study of finnock in a smaller stream, the Kenly Burn, south of the main study site was attempted. This stream flows directly into the sea without an estuary. Therefore, any finnock tracked leaving the stream would necessarily be returning to full seawater. However, only two finnock were caught in this stream and neither were tagged due to technical difficulties. Thus, whereas the absence of an estuary might have resulted in more conclusive results regarding their behaviour, the scarcity of

finnock, possibly due in some part to the absence of an estuary suitable for overwintering, precludes the suitability of such a system for present purposes.

Studies of the behaviour of finnock in estuaries during the winter months would seem an appropriate extension of radio-tracking studies in freshwater. If finnock do remain in estuaries during the winter months, an important aspect of their behaviour might be whether or not they seek areas of water of a particular salinity or temperature range. A study which combined the use of conventional acoustic tags to follow the movements of finnock within an estuary, with the use of acoustic tags which were able to measure salinity and relay this information to a series of detectors moored throughout the estuary, would seem to be the most effective approach to ascertaining the estuarine behaviour of finnock. Tags which measure environmental salinity and then telemeter this information to a bankside recorder have been used for adult salmon (Priede, 1982) but the availability of such tags of a suitably small size for finnock is not known at present.

One of the possible reasons for finnock returning to estuaries and freshwater during the winter is, perhaps, that their hypo-osmoregulatory abilities are compromised in some manner and that returning to lower salinity conditions alleviates this physiological problem. The radio-tracking study described above was designed, in particular, to establish whether finnock remained in freshwater during the winter months. As explained, however, finnock certainly appear to be able to move between freshwater and brackish water, although whether they then moved to areas of full seawater could not be established.

To further investigate the hypothesis of a seasonal compromise in the hypo-osmoregulatory abilities of finnock two sets of experiments (Chapter 4) were performed to establish whether finnock displayed any reduced hypo-osmoregulatory capacity during the winter. The first experiment, in which the number of finnock was limited, was designed as a preliminary assessment of the physiological response of finnock to acute freshwater-seawater transfer. Osmoregulatory abilities were assessed by measuring drinking rates, plasma ion concentrations and plasma cortisol concentrations in acute freshwater-seawater challenged finnock, and by comparing them with freshwater-adapted and seawater-adapted control groups. Cortisol is known to facilitate seawater adaptation by stimulation of branchial ion excretory mechanisms (Richman and Zaugg, 1987; Madsen, 1990).

Finnock displayed physiological responses typical of euryhaline teleosts upon seawater challenge: a rapid increase in drinking rate; an increase in plasma ion concentrations (but only to levels similar or slightly greater than those of seawater-adapted fish); increased plasma cortisol concentrations.

The second experiment, in which numbers of finnock were greater, was designed to assess the longer term acclimation of finnock to both freshwater-seawater and seawater-freshwater challenge during the winter months, to establish whether finnock might suffer from a more subtle reduction in seawater-tolerance which would not have been necessarily apparent in the acute challenge of Experiment 1. Groups of finnock were acclimated to freshwater and seawater for several weeks and then osmoregulatory abilities were measured at various time periods after acute seawater and freshwater challenge, respectively. In general, finnock displayed typical euryhaline acclimation to both salinity challenges, with drinking rates and plasma ion concentrations reaching levels similar to those of fully adapted fish within 2 - 4 days (Usher *et al.*, 1988). However, an unexpected result was the presence of a subsample of seawater-challenged finnock which apparently were only drinking at a very low rate, even several days after transfer. The normal plasma ion concentrations of these finnock was considered as evidence that the seemingly low drinking rates were a consequence of these finnock imbibing the medium in short bursts with an interval between bursts greater than the length of time fish were kept in the experimental medium. Such a drinking rate response has not previously been reported in any study of drinking in fish and, as such, warrants further attention both in terms of the mechanics and the control of this drinking response.

The conclusion was that finnock did not appear to be physiologically compromised by seawater challenge during the winter months, and therefore, a breakdown in hypo-osmoregulatory abilities alone can not be considered a reason for finnock returning to estuaries and rivers during the winter.

A greater understanding of the movements of sea trout post-smolts and finnock in estuaries, in particular whether they make directed movements to remain within bodies of water of a certain range of salinities, would be invaluable in assessing their susceptibility to repeated infestation by the ectoparasitic sea lice *Lepeophtheirus salmonis* and *Caligus elongatus*. Sea lice graze on the mucus, skin and blood of hosts (Kabata, 1974; Brandall *et al.*, 1976), and high infestations are known to debilitate or even kill both wild and farmed salmon (Wooten *et al.*, 1982; Pike, 1989; Whelan, 1993). Heavy infestations of sea lice, in particular *L. salmonis*,

have been implicated in the premature return to freshwater of post-smolt sea trout (Tully *et al.*, 1993a,b) which is thought to be symptomatic of increased mortality of post-smolt sea trout within the marine environment.

It has been demonstrated that infestation levels as low as 30 *Lepeophtheirus salmonis* can be fatal to hatchery-reared Atlantic salmon (Grimnes and Jacobsen, 1996). However, since the problems of sea lice infestation and stock declines in general appear to be far greater in sea trout than salmon, it was deemed important to establish the physiological effects of *L. salmonis* infestation on wild sea trout smolts. Infestation of wild sea trout post-smolts with *L. salmonis* in the present study indicated that, although not fatal, a mean infestation level of 18 parasites caused significant disruption of the osmoregulatory ability of sea trout smolts, as demonstrated by significantly higher plasma osmolality and chloride ion concentrations when compared with naive post-smolts. In addition, since no lesions were apparent on these infested post-smolts, these physiological effects were considered to be the consequence of larval attachment to the gill filaments, thereby puncturing the epithelia and possibly also damaging vital branchial ion excretory cells. However, whereas developmental stages of *L. salmonis* up to and including pre-adults have been observed on the gill filaments of hosts in laboratory-based studies (Johnson and Albright, 1991b; Johnson, 1993; this thesis), no such pattern of distribution has been reported on wild smolts. At present, it is unclear whether or not this result is an artefact of the laboratory-based studies. Clearly, further comparative investigations of larval attachment are required to ensure that the physiological data is relevant to the wild, as well as the farmed, environment.

The susceptibility of smolts to infestation by sea lice highlights other considerable differences between the behaviour of Atlantic salmon and sea trout smolts. Atlantic salmon smolts tend to migrate to sea several weeks earlier than sea trout smolts (Pratten and Shearer, 1983a). By migrating later, when the water temperature may be somewhat warmer, sea trout smolts may encounter more sea lice larvae since the parasite generation time is negatively correlated with water temperature (Tully, 1989, 1990). Furthermore, whereas sea trout smolts remain in inshore waters, often very close to shore, salmon smolts leave the sea lochs and estuaries rapidly (Pemberton, 1976a; Johnstone *et al.*, 1997) and move out into deeper waters on their migration to the feeding grounds in the North Atlantic. Thus, sea trout smolts may be more susceptible to repeat infestation by sea lice because they remain in inshore areas where the density of larvae is thought to be greater.

It should be noted, however, that although because of their migratory behaviour, sea trout post-smolts would appear more susceptible to ectoparasitic infestation, the flexibility demonstrated in the migratory behaviour of this species allows the opportunity to return to freshwater where sea lice die within a few days (McLean *et al.*, 1990). It is possible, therefore, that returning to freshwater to shed sea lice might be one of the reasons for this behaviour in finnock. Just as Atlantic salmon smolts which migrate quickly out to sea are less susceptible to infestation than sea trout smolts which remain in inshore waters, those sea trout smolts which migrate farther out to open waters, i.e. those from the rivers of the east coast of England where finnock are rare, might also be less susceptible to infestation and therefore, not induced to return to freshwater. However, this is speculative and it is also very difficult to differentiate between the cause and effect. Finnock may remain in inshore waters and move in and out of freshwater because they are infested with sea lice, or because finnock are particularly susceptible to sea lice infestation they remain more in inshore waters.

In contrast, salmon smolts, which are not inherently driven to return to freshwater, might also be dying at sea from sea lice infestations. Post-smolt Atlantic salmon captured in the waters over the continental shelf within weeks of migration to sea were found to be carrying *Lepeophtheirus salmonis* (A.F. Walker, pers. comm.). They are most probably infested during the short period of time that they migrate through inshore waters. Whereas this briefer period of susceptibility might have historically limited the level of parasitic infestation to low levels, the recent apparent increase in the numbers of infective larvae in the environment, as implied by the apparent increase in the numbers of sea lice infesting sea trout, may mean that salmon smolts are becoming infested with considerably greater numbers of larvae as they migrate. Grimnes and Jacobsen (1997) observed that as few as 30 pre-adult *L. salmonis* could be fatal for salmon post-smolts. Therefore, perhaps salmon smolts are becoming infested with potentially fatal numbers of sea lice but because they are unable to return to freshwater, they are dying in open waters where no-one can see it.

This phenomenon of 'missing fish' also applies to trout and is a general problem for any study of the distribution of a lethal agent, such as a disease or parasitic infestation, amongst a wild population of fish where the survey can usually only sample those fish which survive long enough to return to an area where they can be sampled. In the case of the sea trout, post-smolts with the heaviest infestations of sea lice might be dying before they can return to freshwater or become available for

sampling. This is an important consideration for the effects of other diseases as well. A considerable number of the sea trout smolts collected from the River North Esk for the study of the physiological effects of sea lice infestation died either before or during the experimental period from an unidentified disease agent. This indicates that a considerable proportion of the sea trout smolts migrating from this river might be dying at sea where their deaths will be unnoticed. Thus, future studies must focus on solving this problem of the missing fish.

One study which attempted to assess this hidden mortality kept wild sea trout post-smolts in sea cages in three sea lochs on the west coast of Scotland (McVicar *et al.*, 1994). This study was aimed particularly at the effects of ectoparasitic infestation and the possible poor osmoregulatory ability of sea trout smolts, but did also examine samples of post-smolts for signs of significant disease. No significant diseases were found. However, such an experimental design might be appropriate for the sea trout smolts from the River North Esk in order to establish what proportion of the sea trout smolts, and of the salmon smolts, might be dying soon after migration.

To summarize, therefore, sea lice infestations have a significant physiological effect on sea trout smolts and post-smolts and these may be one of the causes of the apparent increase in marine mortality symptomatic of the decline of sea trout from the west coasts of Scotland, Ireland and Norway. If the apparent increase in numbers of sea lice infesting sea trout were simply a natural occurrence resultant from, perhaps, a gradual increase in marine water temperatures in these areas accelerating parasite generation times, then scientists and fishery managers would be powerless to prevent this. However, evidence suggests that sea cage-based salmonid aquaculture may be a considerable extra source of sea lice. Sea lice are the major ectoparasite in salmonid marine aquaculture, where high densities of hosts held in confinement provide excellent conditions for rapid cycling between parasite generations and conceivably contribute vast numbers of sea lice larvae to the inshore environment. In addition, it cannot be ignored that those regions in which sea trout numbers have suffered drastic declines have also been the areas of major aquacultural development at the same time.

If the fish farms could be identified as a major contributory source of the increased numbers of sea lice infesting wild salmonids then this could be used not to close fish farm sites, since fish farming is a very important part of the economy in rural areas where there are relatively few other employment opportunities, but to encourage fish

farms to take all steps possible to reduce the numbers of sea lice infesting farmed salmon. Such steps might include increasing the development of novel sea lice control methods. In addition, if the farm origin of sea lice could be established then this might be of benefit to fish farms as well in identifying sources of infestation of smolts soon after they are transferred from freshwater to sea cages. There have been several cases in recent years of considerable numbers of smolts being killed by sea lice infestation within only a few weeks of their transfer to sea. If it were established that such infestations were from other fish farms then the distance between farms could be used to establish how large zones of cooperation would need to be.

In contrast, if it were to be established that fish farms were not a considerable source of sea lice found on wild salmonids then scientists and fishery managers could focus their attention on other possible factors in the decline of sea trout populations. Whereas fish farms are likely to be the source of considerable numbers of sea lice larvae, perhaps these larvae could be selected for infestation of hosts within the fish farm environment where hosts are confined, relatively slow swimming and possibly in an immuno-suppressed state, all of which might facilitate host location and attachment. However, larvae of fish farm origin might be unable to successfully locate and attach to wild hosts.

Despite considerable anecdotal evidence suggesting the significant contribution of fish farms to the increase in numbers of sea lice infesting wild sea trout, however, no conclusive proof has been supplied. The use of genetic techniques to screen for markers particular to sea lice of either farmed or wild origin would appear to be one of the more fruitful approaches to establishing what proportion of sea lice on wild hosts might originate from fish farms.

The modern molecular genetic RAPD-PCR technique was used to screen sea lice collected from wild and farmed salmonid hosts from around the Scottish coasts. This technique indicated markedly different patterns of genetic variation amongst sea lice of farmed and wild origin, and between different farms. A number of genetic markers were found to be exclusive to, or at considerably higher frequency amongst, sea lice collected from farmed salmonid hosts. Although only 21 sea lice collected from a small number of west coast sea trout were screened, approximately 28% of these sea lice were found to display more than one of these genetic markers which were predominantly found otherwise on lice of farmed origin. This would suggest the possibility that 28% of the sea lice on wild sea trout in the two areas

where these fish were sampled, were of fish farm origin. However, this proportion should be regarded with caution, given the small sample sizes.

The use of RAPD technology has allowed a quick and relatively inexpensive preliminary assessment of the patterns of genetic variation amongst populations of *Lepeophtheirus salmonis* infesting wild and farmed salmonids around the coasts of Scotland. Other DNA techniques would have required a considerably greater investment of time and finance and, as a consequence, a study adopting other DNA techniques might not have been considered financially viable. However, RAPDs have some limitations (see Chapter 6), not least of which are the presence of some bands which may be amplified strongly in one PCR reactions but weakly in others and, as a consequence, should not be included in subsequent analyses and the inability to distinguish heterozygous individuals from homozygous individuals, therefore precluding the enumeration of allele frequencies or an evaluation of whether the data fit the Hardy-Weinberg Equilibrium Principle. The lack of allelic data precludes the application of many conventional analytical protocols (e.g. Wright's F-statistic) in assessing population differentiation.

Since differences in patterns of genetic variation between *Lepeophtheirus salmonis* populations infesting wild and farmed salmonid hosts have been indicated, it would now be prudent to expand the assessment using additional DNA techniques which resolve some or all of the limitations of RAPDs. Two techniques in particular show promise for further examining patterns of variation: sequence characterized amplified regions (SCAR) and microsatellites.

SCARs are a further development of RAPD technology (Paran and Michelmore, 1993; Weising *et al.*, 1995). RAPD fragments which are suitable for distinguishing between populations of interest, e.g. putative farm or wild markers, are cloned and sequenced and then 24-mer primers are designed to complement these bands. Samples can then be screened specifically for these bands. The main advantages of this technique are that it uses bands which have already been demonstrated to differentiate between populations of interest, thereby avoiding an extended period of time researching new techniques to find those suitable for the study in question, that amplifying only one band in the PCR reaction reduces the probability of competitive amplification producing bands of variable brightness between gels, and that screening for the presence of only one band considerably simplifies the scoring. However, SCARs either retain the dominant segregation behaviour of the original

RAPD fragment or are converted into a co-dominant markers, the former of which would prevent assessment of allele frequencies, as in the RAPDs.

Microsatellites are short length Variable Number Tandem Repeats (VNTRs), typically of di, tri or tertranucleotide repeats arranged in tandem or a combination of repeats (Tautz, 1989). Each locus is flanked by a unique sequence and primers designed to be complementary to these 'flanking regions' can be used to amplify the tandem array using PCR. The advantages of microsatellites, as summarized by Wright and Bentzen (1994) include the following:

1. They are highly abundant with a typical frequency of between 10^3 and 10^5 copies in the eukaryote genome (Wright, 1993).

2. Allele variants are evident as different sizes of PCR product and they are co-dominant markers inherited in Mendelian fashion which allows the reliable calculation of population genetic estimates and parameters such as F_{ST}.

3. Some microsatellites have extremely high levels of variation. Variation is based upon their high susceptibility to length mutations, considered to be the result of slip-strand mispairing or slippage during DNA replication (Wright, 1993, 1994). Mutation rates are high at an estimated rate between 10^{-3} and 10^{-4} (Huang *et al.*, 1992; Kwiatkowski *et al.*, 1992), much greater than those of allozymes (10^{-6}, Nei, 1987). Variation at a microsatellite loci is typically considerably greater than that for allozymes, e.g. microsatellite probes revealed between 5 and 20 alleles per locus in Atlantic salmon (Taggart and Ferguson, 1990; Prodöhl *et al.*, 1994).

Such high levels of variation should allow discrimination between recently derived or geographically proximate populations when genetic distances are limited. This is of particular relevance to the present study of sea lice populations on wild and farmed hosts since sea cage-based aquaculture only began in Scotland in 1971 (Sharp *et al.*, 1994), and the populations are unlikely to be isolated physically.

However, because not all microsatellites have such high levels of allelic variation, they offer the ability to choose those which provide a level of variation appropriate to the study in question, e.g. high levels of variation where discrimination between individuals is required and lower levels to discriminate between populations.

4. Microsatellites are appropriate for PCR amplification so only minute amounts of DNA are required. The use of PCR amplification is of particular relevance to the present study as the sea lice which are of most interest are those which infest the prematurely returning post-smolt sea trout. These sea lice are predominantly the small chalimus stages.

5. Finally, although the initial development of microsatellites is relatively expensive in terms of time and finance when compared to RAPDs, e.g. a typical estimate of the period of time required to develop microsatellites for any study is 4 - 6 months (Wright and Bentzen, 1995) whereas an entire RAPD study may be completed within this time, the microsatellite technology provides subsequent gains in efficiency because large numbers of samples can be screened. Their highly variable allelic nature means that more information is provided per unit assay than from most other marker systems. They show also greater reliability with which they can be scored across gels and changes in laboratory procedures.

However, without further studies of the physiological effects of sea lice on hosts, information regarding the proportion of sea lice infesting a sea trout which are of farmed origin is of limited value other than to incriminate the fish farms in the decline of sea trout on a general basis. For example, if 25% of the sea lice on a wild host are of farmed origin, what does this actually mean for the host? Does this represent an increased intensity of infestation above the 'natural' level? At present, no information is available regarding the relationships between individual parasites on a host, other than the observation that adult female sea lice and adult males are commonly located on separate areas of large adult salmon - females tend to be located between the dorsal and adipose fins, and between the ventral fin and the tail whereas males tend to be located around the head. It is conceivable that space will be a factor limiting the number of adult sea lice which can effectively infest a host. However, such limitations may not be relevant in the numbers of juvenile stages of sea lice.

Furthermore, if these lice do represent an increase above the natural intensity of infestation, what additional effects does this have on the host - is the increase potentially lethal? Whereas the present study has established that infestation of even relatively low numbers of sea lice on post-smolt sea trout have a physiological effect, further study is required to establish the longer term consequences of such non-lethal levels of infestation.

Concluding remarks

Within the past decade, sea trout populations in some ancestral regions have suffered severe declines in population numbers. Sea trout, the anadromous form of the freshwater-resident brown trout, are of considerable commercial and economic value in terms of tourism and leisure industries, and especially so in those rural areas where sea trout numbers have crashed. The decline has highlighted the general lack of scientific knowledge regarding even the fundamental elements of sea trout ecology, behaviour and physiology. These areas must be addressed if the causes of these declines are to be understood and steps taken to reverse the declines.

Of considerable relevance to the ecology of sea trout is an understanding of the interplay of factors, both environmental and genetic, which induce some juvenile trout to undergo smoltification and migrate to sea whilst others remain in freshwater. The high proportion of maturing trout produced from those reared under three ration regimes in the present study emphasizes the difficulties which exist in attempting to supplement dwindling sea trout stocks with hatchery-reared smolts. In addition, even if mechanisms could be established with which to maximize smolt production, evidence suggests that a major symptom of the decline in sea trout stocks has been reduced growth and increased mortality in the marine phase of the life cycle. If this is indeed the case, therefore, restocking with smolts may be a rather futile remedial measure. The migratory behaviour, and its induction in post-smolts and finnock, are particularly poorly understood. Radio-tracking finnock in freshwater indicated that excursions into freshwater are typically brief when compared to those of maturing adult salmonids. Furthermore, assessment of the osmoregulatory capabilities of finnock during the winter months indicated that they do possess the necessary physiological mechanisms to adapt rapidly to a range of environmental salinities. Therefore, the patterns of movements and physiological abilities of finnock both suggest that overwintering in freshwater is not because of a seasonal breakdown in their ability to survive in hypertonic conditions and that other potential factors must now be investigated.

Increased numbers of sea lice infestations have been implicated in the apparently unusually high rates of marine mortality but, to date, studies have focused on the physiological impact of sea lice on reared Atlantic salmon smolts, whereas the problem of high infestation intensities is most apparent in wild sea trout. The present study has established that even comparatively low numbers of sea lice have a significant physiological impact on wild sea trout post-smolts, even after they have

been allowed sufficient time to become fully acclimated to seawater. The source(s) of this increase in parasite numbers provides a provocative and controversial debate. Considerable attention has been focused on fish farms because salmonids in sea cages suffer from high numbers of sea lice and the areas where declines in wild sea trout populations have been most severe are also areas where there has been considerable development of aquaculture. A modern molecular genetic technique - RAPD-PCR - indicated that sea lice from farmed salmonids could be distinguished from those infesting wild salmonids by the presence of several DNA 'markers' which were exclusive to, or found at much greater frequencies amongst, sea lice from fish farms. The RAPD technique has provided encouraging indicative information that the provenance of sea lice can be identified, although other techniques, such as microsatellites, would be more appropriate for further, more comprehensive studies and would allow the provenance of parasites infesting wild salmonids to be determined with greater confidence. However, even when such techniques are established, it is almost inevitable that the results will indicate that sea lice on wild sea trout are derived from both wild and farmed sources. This will, therefore, require that a value judgement be made regarding whether or not the input of fish farms has a significant effect on the hosts. Bearing in mind that anadromous salmonids and sea lice have probably co-existed since the end of the last ice age, it is likely that the hosts are able to cope with the physiological effects of low to mid-level parasitic loadings. The input from the fish farms might, however, increase parasitic loadings above a presently undetermined physiological threshold, thereby contributing significantly to the apparent reduced growth rates and increased mortality rates of sea trout in the marine environment.

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