Odorant receptor genes and their expression in migratory Atlantic saimon (*Salmo salar*, L.)

A thesis submitted to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy

by

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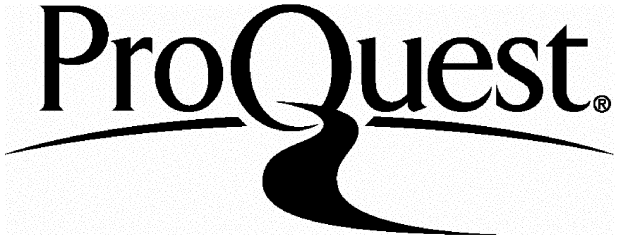

ABSTRACT

Being anadromous, the Atlantic salmon (*Salmo salar*, L.), spends part of its life cycle in fresh water and part of it at sea. They undertake return migrations of up to 4000km, negotiating complex oceanic environments, dendritic river systems and making numerous choices at river junctions to return to specific natal sites.

As the fish near coastal waters, olfaction has been shown to be pivotal in the selection of the appropriate estuary and identification of the natal stream.

Juvenile salmon are thought to imprint on biotic and/or abiotic environmental odours around the time of parr-smolt transformation (PST), and retain this information at least partly within the olfactory sensory neurons. These olfactory cues are then exploited with remarkable precision by adult migrants returning to the natal stream to spawn. Variation in olfactory receptors (OR) and pheromone receptors (or vomeronasal receptors: VNRs) expressed by these sensory neurons may therefore play a vital role in the maintenance of the structure of salmon populations, enabling numerous reproductively isolated communities to exist within one catchment area.

Here, the isolation and characterisation of both OR and VNR genes from *S.salar* has facilitated further elucidation of the olfactory changes associated with parr-smolt transformation. Both sets of primary receptors have representatives expressed in male germ cells as well as olfactory tissue. Real-time quantitative RT-PCR has revealed that a group of OR genes (*SORB*) is expressed at a higher level during the early stages of PST. One group of VNR genes (*SVRA*) however, shows a peak of expression later in PST. There were also expression differences observed between families of fish. Only one family showed a significant increase in expression of *SORB* and *SVRA*, the other family presumably using other receptor types not included in this study.

Molecular evidence therefore indicates that there is more than one incidence of specific-olfactory sensitivity involved in the smolting process. The stimulation of expression of two independent groups of chemosensory receptors indicates that both odours and semiochemicals play a role in olfactory imprinting. The odorant receptors involved in olfactory imprinting appear to vary between families of fish which suggests interfamilial differences in odour stimuli.

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1 HOMING IN SALMON - INTRODUCTION

1.1 GENERAL INTRODUCTION

The Atlantic salmon has always held a symbolic role in the culture of the peoples living around the North Atlantic coastline, representing the abundance of nature and the seasonality of life in both native American folklore and Celtic legend. The fish is depicted in French and Spanish cave art dating back approximately 25 millenia, and in Scots-Pictish stone carvings further north and many centuries later. The salmon is still revered to this day, but is now also considered an invaluable commercial commodity and the 'holy grail' of most anglers, and like many precious natural resources, has been cared-for, used, abused and mismanaged to varying degrees for centuries.

Much of the veneration this species is offered is due to its remarkable life history. Being anadromous, the salmon hatches in freshwater, later migrating down river systems to the sea for its major stage of growth and development before returning to its original river to spawn. The fact that the salmon's life passes through so many defined stages is highlighted by the variety of morphs that it acquires, earning each class a different name.

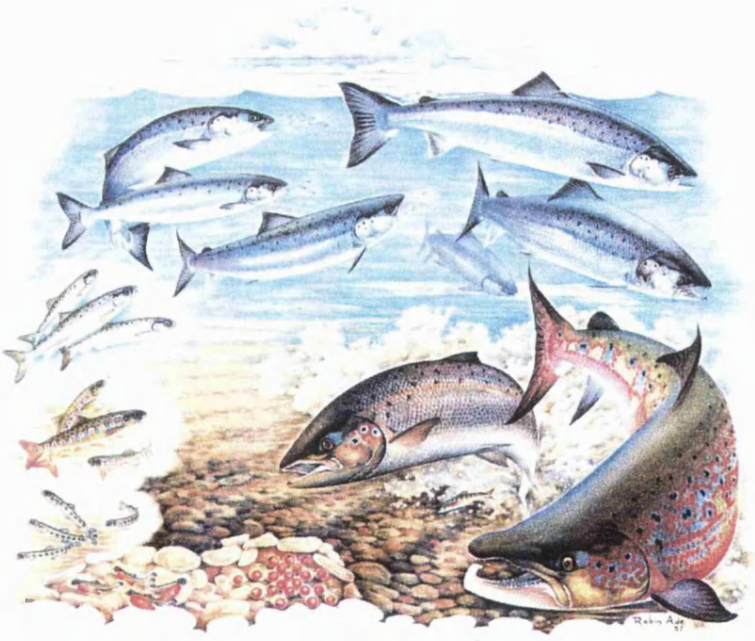

Figure 1.1 *Life Cycle of the Atlantic salmon (*Salmo salar, L.*).* Spawning adults lay eggs in a *redd*; the hatched *alevins* grow into *fry* and then develop into *parr*. After a period of growth, the parr migrate to sea as *smolts*. After up to five years feeding & developing at sea, adults return as either *grilse* or *salmon*. Any post-spawning adults may return to sea once more as *kelts*.

The adult spawners lay eggs within a gravel nest (*redd*), the hatched *alevins* grow into *fry* and then develop into *parr*, at which stage, a proportion of males may mature without going to sea at all. The fish may spend up to seven years in freshwater as parr before migrating to the sea as *smolt*. The marine development stage may last up five years (described as multi sea-winter fish, MSW), or the fish may return to its natal stream after a single sea-winter (SSW) commonly known as

grilse. If the salmon or grilse survives the rigours of spawning it may return to the sea as a *kelt* and spawn again in another year. Though only a few individuals survive to be kelts, most populations of Atlantic salmon contain some repeat spawners, unlike the Pacific species in which all adults invariably die after spawning (Barbour, 1992).

The cryptic coloration of the juvenile stages in freshwater provides remarkable camouflage, as does the spawning pigmentation of the adult after its return from the sea. During the feeding stage in the sea, however, the fish adopts a livery typical of many pelagic species, sporting an aquamarine back, silver flanks and white underside as the classic counter-shading.

Although most Atlantic salmon make the transition from freshwater to sea at some stage in their lives, there are exceptions to this rule. In some locations, none of the salmon population ever leave freshwater. These land-locked salmon are now rare in Europe, however, populations are still known to exist in Norway, Sweden and Russia, whereas many separate populations inhabit the lakes of eastern America and Newfoundland (Youngson & Hay, 1996). Land-locked salmon are not just restricted to habitats without access to the sea, but also occur in close proximity to sea-going populations, the two remaining discrete, reproductively isolated groups.

The hydrodynamic properties of the adult salmon's morphology affords remarkable feats of athleticism, particularly during the demanding homeward migration. For centuries it was thought that no fish could possibly leap so high without some supernatural assistance. It is this ability that earned the species its scientific name *Salmo salar*: salmon the leaper. The "scientists" of the day dispelled this belief in the salmon's paranormal abilities and explained the dynamics of the leap by stating that the salmon took its tail in its mouth and by rotating its body like a wheel was able to convolute over the most demanding waterfall (Dunfield, 1985 as cited by Shearer, 1992).

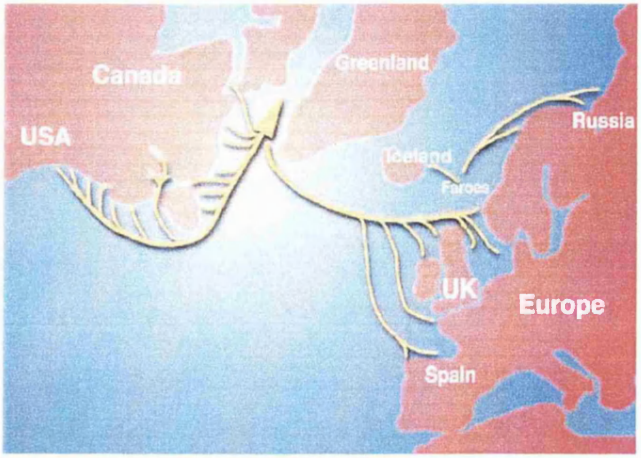

Figure 1.2 *Range map showing outward migration route of Atlantic salmon populations to the common feeding ground off the west coast of Greenland.* (Taken from Atlantic Salmon Federation website: http://www.asf.ca/)

All anadromous Atlantic salmon populations migrate to a common feeding ground, off the west coast of Greenland. Having mixed together during the marine

development stage, the populations then disperse and return to their natal river. These migrations of up to 4000km, involve negotiating open ocean, coastal waters, and dendritic river systems, and making numerous choices at river junctions to eventually return to the site of their emergence to spawn.

1.2 POPULATION GENETIC CONSEQUENCES OF HOMING

1.2.1 Differentiation

The philopatry that anadromous salmonids display has a pivotal role in the structuring of populations. As adult migrants on their spawning run return to their natal stream with such incredible precision, the convergence of sexually mature fish from the previous generation(s) will maintain genetically discrete populations within the same catchment area.

The rapid advancements in biochemical techniques over the past three decades has enabled the confirmation of population differentiation in salmonids. Before the advent of contemporary molecular techniques, electrophoresis of blood serum or tissue proteins predominated.

Nyman (1966), found significant differences in the serum proteins of *S. salar* from eastern Canada and Sweden, but was unable to detect any variation within each sample pool. Moller (1970), discovered three major patterns of transferrins, consisting of two molecular species, in plasma from hatchery-reared and wild Canadian Atlantic salmon. Using these three serotypes, similarities in gene frequencies were found in smolts and adults from the same locality. Statistical analysis revealed that significant differences existed between fish from various sample sites, lending support to the hypothesis of reproductive isolation maintaining inter-population variation. Within the Miramachi river, significantly more homozygotes than could be predicted by a Hardy-Weinberg distribution were found. Combined with other data acquired during the study, this finding strongly suggested the existence of several genetic populations within the one river.

A multiplicity of allozymes have also been used extensively as genetic markers to differentiate populations, including alcohol dehydrogenase (*Adh*), malic enzyme (*Me-1, 2, 3*), superoxide dismutase (*Sod*) (e.g. Ståhl, 1983); a number of loci suitable for electrophoretic analysis has previously been described for Atlantic salmon (Cross and Ward, 1980; Ståhl, 1981). An examination of 45 loci in naturally reproducing Atlantic salmon from northern Sweden revealed spatial genetic heterogeneity on a much finer geographical scale than was previously recognised (Ståhl, 1981).

Many of these allozyme studies were centred around the genetic variability of cultured salmon stocks and the impact of their (inadvertent or deliberate) release on the gene pool of naturally occurring wild populations. The first study of this nature concerning cultured salmon in Scotland was published in 1989 (Youngson *et al.*,). Twelve farmed strains of salmon were screened for six genetic protein loci commonly polymorphic in wild Scottish populations. It was found that strains of cultured fish differed genetically from each other, but this was to be expected given

the numerous wild sources from which the stocks were established. However, genetic differences were also found between four farmed strains independently founded from the adults of a single river.

Corroboration of these first indications of reproductive isolation, has since been achieved through contemporary genetic analytical techniques.

Microsatellites are hypervariable specific regions of DNA composed of a variable number of tandem repeats (VNTR), of two, three or four nucleotides, flanked by nonrepetive DNA (Nakamura *et al.,* 1987). The high level of allelic variation revealed at these loci make microsatellites potentially more useful markers to study population genetic structure than markers derived from allozymes, mitochondrial DNA (mtDNA) or neutral polymorphic protein loci.

Fontaine *et al.,* (1997) described an attempt to define the pattern of genetic exchange and isolation of Atlantic salmon populations of Quebec, Canada. Previous workers (e.g. Power, 1981) estimated that the salmon populations of this region had undergone 7000 to 13,000 years of selection for local conditions, resulting in three putative regional metapopulations between the 115 rivers. However, while the results were not consistent with Power's specific theory; the overall significant allelic heterogeneity among rivers led the authors to conclude that "...each population whether separated by thousands or tens of kilometres, should be considered and managed as a specific stock."

1.2.2 Local Adaptation

Reproductive isolation, combined with environmental control of phenotypic expression and variability among local environments may result in populations being distinguishable from one another in morphology, behaviour, physiology and life history (Taylor, 1991). If an attribute of a local population has a genetic basis, and it promotes the survival or reproductive success of individuals expressing the trait over those lacking it, then the trait is considered adaptive (Barker & Thomas, 1978). Local adaptation can be described as a process that increases the frequency of traits within a population that enhance the survival or reproductive success of those expressing such a trait. In order to study local adaptation it is therefore necessary to demonstrate that natural selection is responsible for the development of a trait within a population and to describe the nature of the genetic mechanism that has favoured it (Futuyma, 1986).

Investigations into the local adaptations of salmonid populations have concerned many aspects of fish biology. Morphometric differences among populations of Atlantic salmon and coho salmon (*Oncorhynhcus kisutch*) have been shown to be temporally stable and inherited; salmon residing in fast flowing or headwater streams are more streamlined and have longer paired fins than those

living in streams characterised by lower water velocities or being closer to the sea (Riddell *et al.,* 1981; Taylor and McPhail, 1985).

Behavioural-genetic studies by Raleigh (1971), Brannon (1972) and Kelso *et al.,* (1981) on sockeye salmon and rainbow trout have demonstrated differences in migratory behaviour among local populations that appear to be specialisations for the movement of fry from spawning and incubation sites to habitats suitable for feeding and development (usually lakes). Fry emerging in lake outlet streams typically migrate upstream, those from inlet streams move downstream, while multiple directional responses are evident in fry from more complex river systems (Brannon, 1972). Laboratory populations of chinook salmon (*Oncorhynchus tschawytscha*) show inherited differences in annual rheotactic rhythms that correlate well with differences in the timing of downstream smolt migration in nature (Taylor, 1990).

Studies of the genetic variation in Atlantic salmon populations from the British Isles have previously centred around the locus encoding malic enzyme 2 (MEP-2). The presence of this diallelic polymorphism has been reported throughout the species range (Cross & Healy, 1983; Ståhl, 1981; Verspoor, 1988; Verspoor and Jordan, 1989). The locus is expressed in the mitochondrial fraction of heart and skeletal muscle (Cross *et al.,* 1979) and the genetic basis of the variation has been determined through breeding studies (Johnson, 1984). The MEP-2 locus is amongst the few commonly polymorphic protein loci identified in Atlantic salmon, and as such, is expected to have a great influence on the levels of genetic differentiation among populations (Chakraborty & Leimar, 1987). Verspoor and Jordan (1989) reported that MEP-2 allele frequencies in salmon populations in North America and Europe show strong correlations with summer temperatures which suggests that this polymorphism may be maintained by selective pressures.

A subsequent study of the same locus (Jordan *et al.,* 1990) in sea run salmon of different age classes (both grilse and MSW fish), indicated that heterozygosity at MEP-2 may be associated with earlier maturation in adult salmon. The greater number of heterozygotes found amongst the grilse were not restricted to a single sample site, a single hatch-year class, or a single year of study, suggesting the action of some mechanism in the alteration of gene frequencies. The authors stated, however, that "..it can not be determined whether selection is acting directly on the MEP-2 locus or on some structurally linked or epistatic locus or loci." Previous findings (Skorkowski, 1988) that mitochondrial malic enzymes show higher levels in the activity in the muscle tissue of aquatic animals than terrestrial animals suggesting that these enzymes may have a greater role in the metabolism of aquatic animals. If this is true for *S. salar*, then it could be inferred that variations in the timing of maturity may result from kinetic differences between the MEP-2 allozymes. Conversely, this polymorphism may be effectively neutral and acts only as a marker

for a more extensive or chromosomal polymorphism (Jordan *et al.,* 1990).

Due to the obvious commercial importance to aquaculture practices, there have been numerous investigations of variability in disease resistance both between and within populations. For example, the work of Gjedrem and Aulstad (1974) suggests the importance of natural selection to differentiation among salmon populations in resistance to vibrio disease. Similarly, populations of steelhead trout, coho salmon and chinook salmon that have evolved sympatrically with the myxosporean parasite *Ceratomyxa shasta* in the Columbia river drainage are more resistant to infection than are coastal allopatric populations (Zinn *et al.,* 1977; Buchanan *et al.,* 1983; Hemmingsen *et al.,* 1986). A more recent study maintained *Gyrodactylus salaris* on isolated stocks from two Norwegian rivers, and a single Baltic population (Cable *et al.,* 2000). Age-specific mortality and fecundity of the parasite raised on the different stocks is consistent with exponential growth on the susceptible Norwegian stocks, whilst extinction of the parasite is likely on the resistant Baltic population. The close association between the presence of a pathogen within a watershed and the degree of resistance among populations for a range of species strongly implicates local adaptation as the major factor maintaining genetic variation in disease resistance among populations.

The coevolution of local adaptation with the precision of adult homing therefore perpetuates a system of population structuring within anadromous salmonid species.

1.3 MECHANISMS OF HOMING & ORIENTATION

Although relatively little is known about the salmon's adult marine life, the homing abilities of these fish have long been recognised. Tagging and marking studies were the original method of monitoring the return of salmon to their natal stream; these projects dating back to the mid 1600s (Shearer, 1992). Smolts are either tagged, or have their fins mutilated in an identifiable fashion as they leave freshwater, and are subsequently re-caught, when they return to the same place, usually some months later. However, much doubt surrounded the interpretations of these early studies, and it wasn't until the 1900s that conclusive evidence of the salmon's ability to return home became available, through tagging studies on the River Tay, Scotland (e.g. Calderwood, 1922). In the 20th Century, the precise return of the salmon to its original river was subjected to intense scientific observation. In the literature preceding the Second World War, the topic was discussed as the 'home-stream' or 'parent-stream' theory.

A broad discussion of the 'parent-stream' theory was initiated in the 1930's by Huntsman (1937, as cited by Stabell, 1984): "I have failed to find a single clear case of salmon returning to its natal river from distant places in the sea, that is, away from the neighbourhood of its river mouth". Following debate concerning the nature of his arguments, it was Huntsman himself who reported the evidence of the salmon's return from high seas, in 1942.

The phenomenon of homing in salmonids is one of the key factors in the maintenance of reproductively isolated populations, and thus the ecology and evolution of the species. Numerous hypotheses have been postulated through the years in attempts to explain the mechanism(s) governing anadromous salmonid navigation, creating a traditionally held division between navigation at sea (both open ocean and coastal waters) and navigation adjacent to or within the home river system.

During the oceanic phase of a salmon's life, many populations both converge and disband, meaning that individuals originating from the same natal river must plot different courses at critical points during their homeward journey. At some stage, the migration routes of these different populations and/or individuals must diverge; the most parsimonious explanation would suggest that salmon base their return course through the ocean on the memory of their outward journey. This seems unlikely as the fish would have to retrace the exact route, which could have taken up to four years. It is obvious that they must take advantage of certain environmental cues, however complex the signal processing and compensatory mechanisms may be.

1.3.1 Vision

Field studies conducted in 1958 (Hasler and Wisby) suggested that fish are able to use the sun as an aid for orientation. Subsequent laboratory studies confirmed that fish would maintain a consistent compass direction in sunlight regardless of the time of day and would behave similarly under artificial light, orienting in the 'artificial' direction, but were completely disorientated under an overcast sky.

Hasler *et al.,* (1958) concluded that both field and laboratory experiments suggested that the sun serves as a point of reference, and that the individual fish compensates for its movement using a 'biological chronometer'. The authors also proposed that orientation involving the use of a sun-compass could be involved in the homing behaviour of migrating fish. Two years later, Braemer suggested that a quantitative computation of the sun azimuth, affected by day length and the sun's altitude could be used in fish orientation. Hasler & Wisby (1958) were the first to indicate a flaw in their field experiments, though, suggesting that the differentiation between the two spawning areas was achieved by mechanisms other than celestial cues.

Neave (1964) highlighted one dramatic shortcoming in the use of celestial cues in salmonid orientation: a compass direction towards a small area of coastline may only be used for navigation if information on the position from which the course is taken is available. The 'chronometer' proposed by Hasler *et al.,* (1958) would have to be capable of determining not only the local time of day in open sea, but also comparing 'local' time with that of 'home' throughout the changing seasons. The use of such a complex bicoordinate system led the author to characterise the "chronometer" as purely speculative; Stabell (1984) added to the doubt surrounding Hasler's propositions by suggesting that a chronometer of this type would have to possess an accuracy far greater than any 'biological clock' as understood in contemporary scientific terms: "Even man had great problems determining longitude in seaward navigation before the invention of the first exact chronometer (Brown, 1956)." Further speculation also arises from the simple fact that salmon migrate at night as well as during the day, and through regions of total cloud cover (Royce *et al.,* 1968).

1.3.2 Magnetoreception

Iron has long been recognised as a common constituent of the body tissues of all vertebrates, and in some migrating species it is present in the form of the mineral magnetite, which when unrestrained, aligns with magnetic fields. In salmon, particles of magnetite have been identified in a number of tissues, particularly in

those along the length of the lateral line. It has therefore been proposed that these arrays form the basis of a sensory mechanism that permits magnetic orientation and compass navigation (Moore *et al.,* 1990). Youngson and Hay (1996) describe similar studies involving loggerhead turtles (*Caretta caretta*), a species which also overcomes similar navigational obstacles during its transatlantic migration.

Behavioural and electrophysiological responses to magnetic fields have been recorded in the rainbow trout (*Oncorhynchus mykiss*), and putative magnetoreceptor cells have been identified in the brain of this species (Walker *et al.,* 1997). Discrimination training was used to demonstrate robust behavioural responses by the trout to the presence or absence of an anomalous magnetic field. Single neurons within the superficial ophthalmic ramus (ros V) of the trigeminal nerve were identified as responding to changes in the magnitude, but not the direction of a magnetic field, and magnetite-based candidate magnetoreceptor cells were located using confocal laser scanning microscopy (CLSM). Within the same anatomical locality of these putative magnetoreceptors are also the lamina propria of the olfactory lamellae.

The proximity of the olfactory organs to the putative magnetoreceptive organs has implications for experiments on long-distance orientation in migratory vertebrate species (particularly salmonids), raising the possibility that olfactory impairment might also produce magnetic impairment, so disrupting long-distance orientation as well as coastal/freshwater orientation.

1.3.3 Rheotaxis

It was during an examination of the importance of olfaction in the homing of chinook salmon (*O. tshawytscha*) that it was revealed that in the absence of the homestream cue, (i.e. an upstream displacement from the home pond), the control fish would return downstream to the location at which the appropriate olfactory stimulation would elicit a positive rheotactic behaviour. In accordance with this, control fish released downstream of the home pond immediately displayed positive rheotaxis by making their way upstream (DeLacy *et al.,* 1969). Conversely, the olfactory-impaired fish used in these experiments were observed to continue downstream, certain individuals being caught in a salt water gill-net fishery several miles out to sea. Thus, it seemed that the tendency for downstream movement in the absence of olfactory cues was common to both the upstream displaced control fish and for those denied olfaction by the occlusion of the olfactory organs.

Within a river, various degrees of active swimming, aided by the directional movement of water will result in bringing negatively rheotactic fish downstream. In the ocean, a negative rheotaxis might contribute to a distribution of the fish within a

limited area (Stabell, 1984). It could therefore be inferred from the data of DeLacy *et al.,* (1969) as well as those from earlier studies (see section 1.3.4) that salmon employ an identical mechanism of orientation in the marine environment as in river systems, based on a positive rheotaxis to odorant cues.

1.3.4 Olfaction

DeLacy's study of 1969 is just one example of many experiments involving the elimination or debilitation of the olfactory sense in migratory salmonids, the seminal work being that of Craigie (1926). The olfactory nerves of 259 migrating sockeye salmon (*O. nerka*) were impaired, and the fish released along with 254 tagged controls in Deep Water Bay, Vancouver Island, Canada. Three of the control fish and 14 of the operated fish were recaptured in the same area. A further 62 controls and 28 operated fish were caught, of these, 59 controls and 23 treated individuals were taken in the Frasier River, from the estuary to the catchment area. These results led the author to conclude that the elimination of olfaction appears to affect the migration of the sockeye salmon.

Similar results were reported by Clemens *et al.,* (1936), but the authors gave credence to the fact that physiological disturbance may result from surgical intervention and concluded that the fish were able to navigate to the Frasier River without the olfactory sense.

More recently, Bertmar & Toft (1969) severed or cauterised the olfactory nerves of 191 Baltic *S. salar* grilse. One hundred of these impaired fish were released with 100 untreated controls 35 to 40 km north and south of the mouth of the home river. The traumatised fish were observed to orientate themselves towards the home river to the same extent of the controls, but most of the operated fish became disoriented and were recaught along the entire coastline of the study area. In total, only two neurotomised and six cauterised individuals homed. It was suggested that cauterisation was an inefficient method of disabling the olfactory organs, but the entire dataset indicated the importance of olfaction in coastal orientation and homing behaviour of Baltic salmon.

Toft continued the investigation of the role of olfaction and vision in spawning migrations, and confirmed the results of the previous study (1975). A total of 1276 *S. salar* grilse distributed among 5 treatment groups were used in displacement experiments in the Bothnian Gulf, 451 of which were recaptured. Tagged controls were observed to resume their spawning migration to the home stream whether released north or south of its estuary, or that of another river. Those treated by burning the dorsal side of the nasal rostrum behaved similarly. However, those fish with the olfactory sense eliminated homed at a significantly lower rate than the

controls, whether displaced within the estuary or 200km away. No significant deviations were observed in the homing behaviour of those grilse neurotomised on one side only, nor in the homing of the grilse with vision destroyed.

The olfactory-impairment of the subjects in all these experiments has been shown to reduce the homing accuracy of these salmon species. Unfortunately, only Toft (1975) controlled for visually aided navigation. In the review of Stasko (1971), the possibility that fish might be guided by other senses when olfaction is impaired, is highlighted (schooling is proposed as an alternative means of navigation for salmon released on a major migration route). Despite previous flawed experimental procedure, it can be inferred from these data that olfaction is mandatory for successful salmonid migration and homing. The necessity of olfaction and the non-requirement of vision for coastal navigation by salmonids has also been supported by studies on chum salmon (*O. nerka*) by Hiyama *et al.*, (1967, as cited by Stabell, 1984).

1.4 OLFACTORY IMPRINTING & PHEROMONE RECOGNITION

Although it now seems that several sensory mechanisms exploiting various environmental cues may be employed by salmonids during the oceanic stage of their life, olfaction was proposed as the predominant process through which the home river is identified.

Hasler and Wisby (1951) first demonstrated that bluntnose minnows (*Hyborhynchus notatus*) could be conditioned to respond to water from two Wisconsin creeks. The fish were observed to be unable to discriminate between the two water samples once the olfactory apparatus was destroyed, indicating an olfactory basis of discrimination. The fish were exposed to a series of distilled fractions of the creek water, resulting in a volatile organic substance being identified as the active odorant. This particular conditioning technique was employed in a later study on coho salmon fry (*O. kisutch*) by Wisby (1952, PhD thesis, as cited by Stabell, 1984), verifying olfactory discrimination in anadromous salmonids.

It was through this work, that the 'Imprinting Hypothesis' was modelled (Hasler & Wisby, 1951), proposing that natal river systems possess characteristic odours to which juvenile salmon become conditioned during development in fresh water. These 'imprinted' odours are then used as orientation cues when the fish return as adult migrants.

A further study by the same authors (1954) established that salmonids require the olfactory sense for navigation through their natal river system. Adult coho salmon were captured from both streams after a fork in Issaquah creek, Washington, during their upstream migration. Half of the fish in both groups had their olfactory pits occluded, and were released along with the untreated controls downstream of the junction in the creek. The olfactory-occluded fish were significantly less able to repeat their original choice at the stream junction than the untreated controls. Although this and many later studies (see Stabell, 1984) have confirmed the olfactory discrimination of stream water by fish, any connection with a previous imprinting event as proposed by the authors was purely speculative.

The association between cardiac response, thyroid activity and olfactory sensitivity during smoltification has now been established (Morin *et al.,* 1989a and 1989b; Morin and Døving, 1992). Fish are known to decelerate heart rate readily to novel chemical stimuli and this phenomenon has been used as an unconditioned response to assess olfactory memory (Hasler and Scholz, 1983). Salmonid juveniles injected with thyroid hormones become more sensitive to odours and have a greater learning ability (Gorbman, 1969; Scholz, 1980, both cited by Morin *et al.,* 1989b). Olfactory sensitivity peaks (low response threshold) at the beginning of smoltification and reaches a minimum (high response threshold) after smoltification (Morin and

Døving, 1992). Higher histological thyroid values were also detected concomitantly with this olfactory-sensitive learning period (Morin *et al.,* 1989b). Thyroid hormones are thought to facilitate olfactory learning during smoltification by increasing the excitability of the nervous system.

1.4.1 Olfactory memory involves peripheral mechanisms

Confirmation of olfactory imprinting came *via* the exposure of juvenile coho salmon to novel odorants (Nevitt *et al.,* 1992 and 1994). Evidence from these imprinting experiments suggested that learning in the olfactory system may be due to sensitisation of the peripheral sensory neurons as well as synaptic plasticity within the central nervous system. Dittman *et al.* (1997), imprinted juvenile coho salmon to the odorant β-phenyl ethyl alcohol (PEA) and examined the sensitivity of olfactory adenylyl and cytosolic guanylyl cyclases to PEA during development. The authors found that the stimulation of guanylyl cyclase activity by PEA was significantly greater in olfactory cilia isolated from PEA-imprinted individuals than that of PEA-naive fish only at the time of the homing migration, 2 years after PEA exposure. The imprinted fish were also shown to retain a memory specific to the imprinted odour: cyclic nucleotide activity was not significantly enhanced by exposure to the control odorant, L-serine.

This evidence suggested the existence of a peripheral mechanism of olfactory imprinting in salmon. Similar peripheral chemosensory imprinting mechanisms that have obvious adaptive advantages have been reported in other animals. The "Pregnancy block" in female mice induces termination upon exposure to the pheromones of an unfamiliar male and has been localised to the level of the olfactory bulb (Brennan *et al.,* 1990); newborn, weanling and adult rabbits that were exposed to juniper in the womb demonstrate an enhanced peripheral sensitivity for juniper odour (Hudson and Distel, 1998); and the moth, *Manduca sexta,* displays a peripheral mechanism for adapting the aversive behavioural response to harmless noxious compounds (Glendinng *et al.,* 2001).

1.4.2 Candidates for intrapopulation semiochemicals

The cellular mechanisms of olfactory imprinting may be becoming elucidated, but it is still not known on which kinds of odorants young salmon imprint. It has been proposed in the past that migrating salmon home using odorants emanating from their conspecifics (Døving *et al.,* 1973), a suggestion that became known as the 'Pheromone Hypothesis' (Stabell, 1984). The physiological experiments of Døving *et al.,* (1973) showed that fish produce substances that are potent stimuli to the olfactory organ and that there exists a neuronal basis for discrimination of different

strains of fishes (Døving *et al.,* 1974). The same author also described the olfactory sensitivity to bile acids in salmonids (1980). Bile acids have detergent properties and are used to aid lipid digestion in the vertebrate body, but some of them are readily degraded and would only have a short term effect in an aquatic environment. "..Others are more stable and are absorbed by organic matter and minerals in the water. This could give the fishes a possibility for recognizing their own territory." (Døving *et al.,* 1980).

Other biotic cues that have since been identified in eliciting electrophysiological responses from Atlantic salmonids include testosterone (Moore and Scott, 1991), 17α, 20β-dihydorxy-4-pregnen-3-one 20-sulphate (17, 20β-P-sulphate) the sulphated form of the teleost oocyte-maturation-inducing steroid (Moore and Scott, 1992) and urine (Moore *et al.,* 1994).

Testosterone was revealed to be a strong attractant to spermiating precocious salmon parr resulting in positive rheotactic and searching behaviour (Moore, unpublished observations), although the precocious parr appear to lose their olfactory sensitivity to the hormone some two weeks before spawning occurs in females from the same population. The hypothesis that the main role of testosterone, in female Atlantic salmon at least, may be as a pheromone, attracting the males to the females in the weeks leading up to spawning, was not substantiated by efforts to detect free testosterone in the urogenital fluids of ovulated female salmon. However, this may in turn suggest that either testosterone is released from the females by some route other than urine or ovarian fluid, or the females may not be the source of the testosterone at all. It is feasible that other male salmon may produce testosterone as a male:male pheromone to reduce milt production in conspecifics. It is also possible that the authors sampled the urines at an inappropriate time or that there is some alternative active substance in urine, which, although similar to testosterone, can not be detected by these experimental means (Moore and Scott, 1991).

Pre-exposure of precocious male parr to the urine of an ovulated female facilitates olfactory sensitivity to the steroid 17.20β-P-sulphate (Moore and Scott, 1992). In the same study, it was also found that ovulated female urine amplified the response of the olfactory epithelium to both testosterone and testosterone sulphate by a factor of two. The activating compound present in the urine of ovulated females does not appear to be present in that of either precocious male parr or immature females, indicating that it appears to be sex specific and related to sexual maturity in the female.

The ability of juvenile coho salmon to discriminate between the emanating odours of their own population and those of non-population members was demonstrated by Courtenay *et al.,* (1997). Not only did the salmon prefer water scented by the faeces of their own population over that of another, but they also

showed a preference for water conditioned by their own population over unscented water. This population-specific discrimination also appeared to be influenced by odour concentration, in so far as a higher concentration was preferred over the lower, at least within the range employed in the study. Common rearing during the embryonic, larval, and early free-swimming stages however, rendered families more attractive to each other than they were otherwise. This indicates that careful experimental planning is crucial to avoid bias owing to differences other than population-specific traits.

It was also demonstrated that Atlantic salmon parr have the olfactory ability to discriminate between the urine derived from sibling and non-sibling fish (Moore *et al.,* 1994). Parr were observed to move towards the source of sibling urine, but away from that of non-siblings and in response to both displayed behaviours typical of juvenile salmonids defending feeding territories. During the smolt stage (when Atlantic salmon are known to form shoals, before the outward migration), there was no such discrimination. Brown and Brown (1992, 1993) reported that Atlantic salmon parr prefer water conditioned by siblings over that conditioned by non-siblings and that both aggressive interactions and territory size were lower between sibling parr. The inference was that inclusive fitness benefits may be associated with territories near siblings against non-siblings. Moore *et al.* (1994) proposed that sibling discrimination in Atlantic salmon may be a special case of population recognition (Quinn and Hara, 1986), and that urine may therefore be the source of a specific odour or pheromone which the adults use during homing. "A continuous source of urine from salmon parr resident within a river would also provide an indication to homing adults of the suitability of the environment for growth and survival." (Moore *et al.,* 1994).

The ability of vertebrates to discriminate kin from non-kin has now been linked to the products of one of the largest gene superfamilies within the genome, the major histocompatibility complex (MHC) genes. The mammalian MHC is a set of linked genes spanning 2 megabase pairs, the products of which include those proteins involved in antigen presentation, primarily MHC class I and class II genes. It has been found that both mice and humans are able to distinguish between the odours of conspecifics with disparate MHCs (Yamazaki *et al.,* 1979; Wedekind *et al.,* 1995). There is also strong evidence that the active substance(s) involved in this discrimination is present in urine (e.g. Potts *et al.,* 1991). Since MHC genes have been found to fulfil a similar function in all vertebrates studied, including fish (Klein *et al.,* 1993), and given that there is genetic variation at the MHC, the rationale exists for the study of the influence of MHC specific odours on kin recognition in salmonids.

The seminal work was reported by Olsen *et al.,* (1998) in a study on Arctic charr (*Salvelinus alpinus*). Individual gentoypes of several fish were determined

using exon 2 of the MHC class II B gene, electrophoresed on a denaturing gradient gel (DGGE). Subsequently, it was observed during fluvarium tests, that when the fish had the choice between water scented by an MHC identical sibling and that scented by a sibling of different MHC genotype, they preferred that of the identical sibling. Likewise, water scented by an MHC different sibling was preferred to water from an MHC different non-sibling. Further still, no discrimination was observed when the test fish shared one allele with the non-sibling donor but no alleles with the sibling donor, indicating that MHC type has a significant influence on the odours used for kin recognition in juveniles of this salmonid species (Olsen *et al.,* 1998).

The Imprinting and Pheromone hypotheses should not be considered mutually exclusive as there is evidence for the "learning" of sibling odours in juvenile salmonids. The ability to recognise unfamiliar individuals as siblings or non-siblings could be explained by two mechanisms (Holmes and Sherman, 1983; Waldeman, 1988). "Phenotype matching" involves an individual recognising memorised phenotypic traits displayed by itself, siblings, or other close relatives with which it has been raised. These learned traits could subsequently be used as a template to which the traits of unfamiliar individuals can be compared, i.e. the imprinting of conspecific odours. Alternatively, the genetic mechanism (based on recognition alleles) as originally formulated by Hamilton (1964), does not involve learning.

A study involving fluvarium tests of Arctic charr both reared in isolation and together with siblings revealed that social experience sometime during the first 15 months is essential for the development of sibling preference in these salmonids (Winberg & Olsen, 1992). The fish reared in isolation were indifferent when given a choice between water scented by siblings and water scented by non-siblings, but preferred water scented by siblings to unscented tap water. When 15 months old, these same individuals were reared with siblings for 50-62 days, but were still indifferent when given a choice between water scented by siblings and water scented by non-siblings, indicating that sibling preference must develop sometime during the first 15 months of development.

1.4.3 The genetic component

There is well documented evidence from numerous transplantation studies that indicate the influence of genetics in the salmonids' ability to home. Marking of pre-smolt Atlantic salmon from Lundesokna hatchery, Norway, has been carried out since 1957 (Rosseland, 1965a). The eggs have been of different origins and the fish have been transplanted and released in a number of Norwegian rivers. Between the years of 1957-62, of 22675 fish marked and released, only 0.09% were recovered in

the stream of release. Similarly, of those 60795 fish marked and released during the period 1970-1976, only 0.16% returned. (Rosseland, 1978). These experiments show that a much lower percentage of transplanted fish are recovered in the stream of release compared to native, wild fish, indicating a reduced ability of correct 'homing' orientation in transplanted fish as compared to their wild, native counterparts. The fact that at least some transplanted fish return to the river of release has been used as an argument for imprinting, and against an influence of inheritance (e.g. Hasler *et al.,* 1978). Nordeng (1977), however, noted that return of fish transplanted into non-native rivers, might depend on association of the transplants with the migratory systems existing in the rivers of release, as salmonids migrate in schools.

Another salmonid species has also confounded the issue. Pink salmon (*O. gorbuscha*), migrate to sea immediately after hatching and return in the following year. This 2 year cycle means that spawning migration of separate populations occurs in even and odd years, and has therefore been used as an argument against 'pheromones' in salmonid migration, since rivers perpetuating only even or odd year stocks would contain no fry during migrant return. The significance of genetic influence on homing in pink salmon was demonstrated by Bams (1976). Comparison of return from the ocean between an introduced stock and a hybrid stock (a cross of females from the introduced stock with males of the local resident stock) revealed that large differences occurred between the genetic classes. Subsequent to passage through the inshore waters, the relative frequencies of numbers caught greatly favoured the hybrid fish. Some individuals from the introduced stock returned, but the hybrid stock with the addition of the local male genetic component showed better return to the natal stream. The influence of the native genes, was not, however, sufficient to achieve similar accuracy of homing as that of the native stock. The author concluded that genetic control of homing ability is clearly implicated in locating the home stream.

1.5 AIMS OF STUDY

The assumptions underpinning this studentship were based on those of both the *Imprinting* and *Pheromone* hypotheses:

Imprinting

(a) Juvenile salmonids undergo a process of olfactory imprinting during parr-smolt transformation (PST).
(b) The level of expression of olfactory receptors (ORs) varies throughout a salmon's life: olfactory imprinting at PST involves a change in the level of expression of ORs on sensory neurons within the olfactory epithelium.

Pheromones

(c) Each population or stock of Atlantic salmon produce their own bouquet of odours that enable kin recognition by members of that population.

At the start of this study, it was intended to isolate and characterise further olfactory receptor-like genes from Atlantic salmon and to verify the nature of the 5 families of genomic sequences identified in the pilot study (Deaville, 1998, unpublished). By elucidating the expression patterns of these candidate ORs, their possible function may be determined. OR sequences should only be expressed in olfactory sensory neurons, and potentially male germ cells. RNA was to be isolated from tissues obtained from Atlantic salmon and DNA synthesised from the transcribed genes in olfactory epithelium, testis and several other reference tissues. Expression of these particular ORs should then be revealed through screening with olfactory receptor-specific Polymerase Chain Reaction (PCR) and radiolabelled probe hybridisation.

Levels of OR gene expression relative to the housekeeping gene, β Actin, may then be quantitated from the olfactory tissues of individual salmon at varying stages of parr-smolt transformation. An increase in OR expression at PST would be consistent with the olfactory imprinting hypothesis.

Summary of Aims

1 Complete previously isolated OR gene fragments (Deaville, 1998, unpublished) and isolate further candidates.
2 Determine tissue-specific patterns of expression and infer putative gene function(s).
3 Establish a method of quantitating levels of gene expression.
4 Measure levels of gene expression in olfactory epithelium of fish before, during and after the smolting period.

2 CHARACTERISATION OF OLFACTORY RECEPTOR-LIKE GENOMIC SEQUENCES

2.1 INTRODUCTION - OLFACTORY RECEPTORS

Regardless of taxon or odour, all olfaction (or chemosensation) is mediated by the binding of the odorous ligand to its appropriate receptor protein. Preliminary indications that olfactory receptors are membrane proteins came from Getchell and Gesteland (1972) and Rhein and Cagan (1980). However, the seminal work that led directly to the characterisation of the receptors themselves was reported by Pace *et al.*, (1985): a cell-free preparation of rat olfactory cilia contained a highly active adenylyl cyclase with a very high sensitivity to odours, and this sensitivity depended on the activation of a G protein. This finding suggested that odours initially act through a G-protein-coupled receptor (GPCR), which activates the cyclic AMP (cAMP) second-messenger pathway.

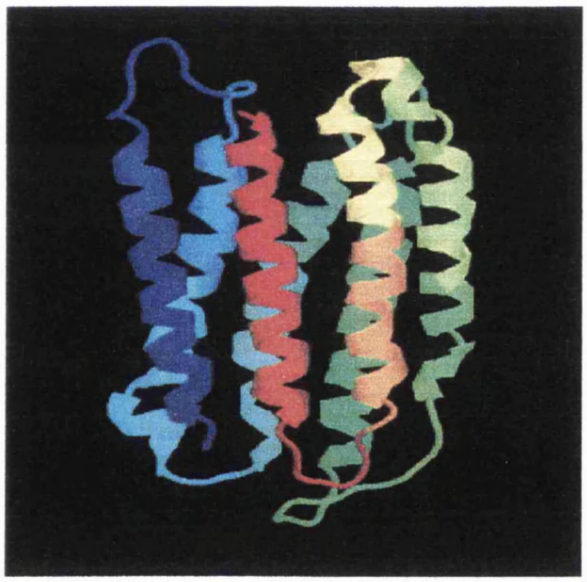

Figure 2.1.1 *Ribbon diagram of Secondary structure of a G-protein-coupled receptor.* The α-helical structure of the seven transmembrane domains, joined by alternating intra- and extracellular loops are illustrated.

Isolation and cloning of the components of the second-messenger pathway followed swiftly, based on the assumptions of Lancet (1986): (a) The vertebrate olfactory system can respond to a broad range of odour molecules; (b) individual OR expressing cells respond with varying degrees of excitation to a variety of odours; (c) there are no distinct categories of odorants; and (d) a minimum of 40 specific anosmias are known in humans, implying the existence of at least that many receptor proteins. Analogies were then drawn between these properties and those of the immune system, projecting three hypotheses: (a) ORs are likely to belong to a large family of receptor molecules, within the region of 100 to 1000 members; (b) a single

OR is likely to consist of a variable region involved in odour binding and a conserved region for second-messenger transduction; and (c) the receptor is likely to belong to the GPCR superfamily.

The subsequent isolation and cloning of OR genes based on these assumptions was achieved by Buck & Axel (1991), leading to the identification of a multigene family within the seven-transmembrane-domain GPCR superfamily. Screens of genomic libraries and Southern-based hybridisations of genomic DNA suggested that the gene family consists of at least 100 members, an estimate that has since been increased towards 1000 (Hildebrand & Shepherd, 1997). This OR gene family is distinguished from other members of the GPCR superfamily by the considerable residue diversity within transmembrane domains 3, 4 and particularly 5, thereby confirming the hypothesis that each receptor consists of a variable region able to interact with a variety of odorants.

The size of the mammalian OR gene family (the largest known in the genome) is accepted as strong evidence that these are odour receptors satisfying the criterion of diversity necessary to interact with an immense range of odorants. To date, OR genes gave been isolated from over thirty vertebrate species, and dendrograms have been constructed to represent inferred evolutionary relationships among them (Ben-Arie *et al.,* 1994; Firestein, 2001; Dukes *et al.,* 2002, in prep.). There is confusion, however, surrounding the nomenclature of these odorant receptors. The review of Bargmann (1997) sought to distinguish between the different classes of odour receptors. In mammals, the vomeronasal organ, which expresses a completely independent family of receptor proteins, (Dulac & Axel, 1995) is anatomically discrete from the olfactory apparatus, but no such physical distinction exists in fish. Vomeronasal sensory neurons are distinct from olfactory neurons in their morphology (vomeronasal neurons have microvilli, olfactory neurons have cilia) and in the signal transduction components they possess (Bargmann, 1997). Vomeronasal receptors (VNRs), respond to a different subset of odorous ligands including hormones, and appear to be linked with eliciting instinctive behaviours.

Guanylyl cyclase stimulation and increased levels of cGMP had previously been linked to odorant activation (Breer *et al.,* 1992, as cited by Dittman *et al.,* 1997), although the precise role of guanylyl cyclase in olfactory signal transduction remains largely elusive. Recent evidence suggests that particulate or membrane forms of guanylyl cylases act as primary receptors in olfaction (Fülle *et al.,* 1995; Yang *et al.,* 1996; Wedel and Garbers, 2001).

The size of the fish OR gene family is apparently small in comparison with that of mammals (perhaps 30-100, Ngai *et al.,* 1993b), suggesting that fish respond

to a limited range of odours, which has been supported by experimental evidence that the primary odours for these taxa are amino acids and bile salts (Caprio *et al.*, 1993).

Variation in the sizes of putative OR gene families provides a potential molecular basis, consistent with differences in sizes of OR expressing cell populations (Shepherd, 1992), for the difference between microsmatic and macrosmatic species (Hildebrand & Shepherd, 1997; Sharon *et al.*, 1999; Sullivan *et al.*, 2000).

It is due to the relative accessibility of the fish olfactory organs, the comparative simplicity of the fish OR gene family and the processing of olfactory information that has made fish (particularly salmonids) popular models for signal transduction and processing in chemoreception studies.

2.1.1 Receptor Expression & Regulation

Before the topographical distribution of olfactory receptors among olfactory sensory neurons was first elucidated, it was postulated that a combination of two cellular mechanisms might form the basis for odour discrimination. The initial step in olfactory discrimination requires the interaction of odorous ligands with specific receptors on olfactory sensory neurons. The first cellular mechanism suggested that selectivity might be achieved through each sensory neuron expressing only a limited suite of receptors, whilst the second relied on spatio-temporal patterns of action potentials being elicited from the olfactory epithelium to the olfactory bulb (Nef *et al.*, 1992).

Vassar *et al.* (1993) used *in situ* hybridisation to demonstrate that mammalian olfactory sensory neurons (OSNs) expressing distinct receptors are topologically separated into a small number of broad, yet discrete zones within the olfactory epithelium. Within each zone, however, olfactory neurons expressing a given receptor appear to be randomly distributed, rather than confined to a specific locality. The authors concluded that the complex mammalian olfactory system may therefore compartmentalise the epithelium into anatomically and functionally discrete units, so that each of these units will only express a subset of the entire receptor family.

Topographically distinct patterns of receptor expression were also observed by Ressler *et al.* (1993), in a similar study of mouse olfaction. The "expression zones" reported, exhibit bilateral symmetry in the two nasal cavities and are arranged along the dorso-ventral and media-lateral axes. The inferences from these observations are that within each zone, a neuron may select a gene for expression from a zonal gene set via a stochastic mechanism, and the pattern of expression produced may serve as an initial step in the organisation and coding of olfactory sensory information.

Understanding the role that receptor expression plays in the organisation and processing of olfactory information was further complicated by the effect of substitutions in coding regions of OR genes (Wang *et al.,* 1998). Deletions or nonsense mutations in the P2 OR gene in mice cause the axons of neurons expressing this gene to wander rather than target a specific glomerulus. The genes P2 and P3 share 75% amino acid sequence homology, however, when the coding region of P2 was replaced with that of P3, the axons of those neurons expressing this substitution projected to a glomerulus adjacent to the P3 wild type. In combination with the results from other mutation/substitution experiments, it was therefore proposed that although cells expressing a given receptor are randomly distributed throughout each zone, spatial order is restored further along the signalling pathway in the olfactory bulb where like axons converge on two positionally invariant glomeruli to create a topographic map encoding odour quality.

It is now accepted that each OSN express only one or two receptor types, *via* a complex mechanism of control exerted on the family of olfactory receptor genes. Only one of an allelic array encoding multiple receptor genes is active in any given OSN, and so expression derives exclusively from one allele (Chess, *et al.,* 1994; Breer and Strotmann, 1997). More recently in transgenic mice, the endogenous and transgenic OR genes have been differentially tagged, enabling the receptor type (transgenic or endogenous) expressed in each OR cell to be identified. It was found that the two genes are expressed in a mutually exclusive manner and that the two sets of OSNs expressing these labelled genes project their axons to separate glomeruli (Ishii *et al.,* 2001). This monoallelic expression however, is not a result of genomic imprinting (in which one allele - either paternal or maternal - is permanently silenced), but rather a form of allelic inactivation. Chess *et al.,* (1994) reported that five olfactory receptor genes from four independent loci are replicated asynchronously, and that the relative timing of replication is not allele specific. In some cells the paternal allele is replicated early and in other cells it is replicated late. This form of allelic inactivation bears the hallmarks of the regulation of immunoglobulin and T cell receptor genes.

Two hypotheses have been proposed concerning transcriptional control: a *cis*-regulatory element may activate a single gene from a linked array, or there may exist a single expression site, into which 'silent' genes may be introduced by a gene conversion event (Chess, *et al.,* 1994; Breer and Strotmann, 1997).

Conversely, in an attempt to address the question of whether an individual neuron expresses either one receptor (presumable broadly tuned to be activated by a range of odours), or a number of (finely tuned) receptors, cDNA was synthesised from a number of single rat olfactory neurons (Rawson *et al.,* 2000). From a total of five cells analysed, four were consistent with one OR cDNA per OSN, but the fifth

cell contained at least 3 OR sequences. Two of these sequences were confirmed by subsequent "rapid amplification of cDNA ends" (RACE) using degenerate OR primers. Double-label *in situ* hybridisation was also carried out, indicating that the sequences were coexpressed in a subset of 12 cells. These doubly-labelled cells were also located significantly nearer the apical surface of the olfactory epithelium (where the more mature cells reside), than those cells labelled with a single gene. This suggests that in at least a subset of OSNs, more than one receptor type may be transcribed, whether the message is then subsequently translated into a functional protein has yet to be determined. Support for the existence of more than one receptor per cell has been reported (Noé and Breer, 1998) also using single-cell molecular techniques. Cells responding to two different odour mixtures thought to activate different transduction mechanisms express elements of the two mechanisms, while cells activated by only one or the other odour mixture express elements of only one pathway.

It therefore appears that a subset of rat olfactory neurons are capable of expressing more than one receptor type and these receptors are activated by different ligands, adding a further dimension of odour-coding complexity operating at the level of the receptor cell.

2.1.2 Signal transduction

In vertebrates, there is now consensus that odorants activate olfactory sensory neurons (OSNs) in the main olfactory organ *via* G-protein-coupled receptors. An allosteric change in these receptors, and the associated G-protein then modulates the production of at least two intracellular secondary messengers, cyclic adenosine monophosphate (cAMP), and inositol 1,4,5-triphosphate ($InsP_3$). Different subsets of odorants have been demonstrated to elicit an increase of either cAMP or $InsP_3$ (Breer and Boekhoff, 1991; Ronneet *et al.,* 1993). In either case, the elevation of second messenger levels subsequently leads to changes in membrane conductances (reviewed in Schild and Restrepo, 1998; Dionne and Dubin, 1994; Ache and Zainazarov, 1995). Although these two secondary messengers represent individual transduction pathways, they do not work independently, but rather display a functional antagonism (Vogl *et al.,* 2000). A third candidate pathway involving cyclic guanosine monophosphate (cGMP) remains to be fully understood. Recent advances implicate the action of cGMP as a primary receptor as well as a secondary messenger (Fülle *et al.,* 1995; Yu *et al.,* 1997; Seimiya *et al.,* 1997; Takeda *et al.,* 1997; Suzuki *et al.,* 1999; Mantoku *et al.,*1999; Yamogami *et al.,* 1999; Lowe *et al.,* 1995).

2.1.2.1 cAMP pathway

The binding of an odorous ligand to its appropriate receptor, causes an allosteric change in the protein, "switching on" its associated G-protein (G_{olf}) by ejecting guanosine diphosphate (GDP) and replacing with guanosine triphosphate (GTP), thereby activating the protein. The G-protein in its active form can then diffuse away from the receptor and transfer the message to the next target downstream. In the case of cAMP mediated transduction, this is the membrane-bound enzyme, adenylyl cyclase. The G-protein is deactivated upon hydrolysis of its own bound GTP converting it back to GDP.

Cyclic AMP is a ubiquitous mediator of intracellular signalling, normal levels within a given cell being approximately 10^{-7} M, but intracellular concentration may be increased or decreased rapidly in response to extracellular signals. This rapid response requires that its rapid synthesis be buffered by its rapid degradation or removal, which occurs via one or more cyclic AMP phosphodiesterases to adenosine 5'-monophosphate (5'-AMP). In the cAMP-mediated transduction pathway in OSNs, cAMP is upregulated by stimulating the activity of adenylyl cyclase, increasing the synthesis of cAMP from adensoine triphosphate (ATP).

Heightened intracellular concentration of cAMP opens cAMP-gated ion channels (CNG), creating a influx of Na^{2+} that depolarises the membrane of the neuron. Depolarisation of the membrane, also opens voltage-gated Ca^{2+} channels, allowing the entry of Ca^{2+} into the cell. Ca^{2+} is another ubiquitous intracellular messenger, but it also interacts with the cAMP pathway at many levels. Ca^{2+} entering the cell through the CNG also activates a Ca^{2+} -dependent Cl^- channel in the olfactory cilia, which was originally described by Kleene (1993). This Cl^- current amplifies the primary odour induced cation current. It now appears that a substantial amount of the odour-induced current is due to this secondary Cl^- current (Ache and Zhainazarov, 1995, see Figure 2.1.2a.).

Rising $[Ca^{2+}]_i$ increases the activity of adenylyl cyclase but at the same time, decreases the sensitivity of the CNG channel to cAMP in a feedback loop. However, the inactivation and adaptation of the cAMP pathway are less well understood. The cascade can be downregulated by phosphorylation of the olfactory receptors by cAMP-dependent protein kinase-A (PKA - Boekhoff and Breer, 1992) and a G-protein coupled receptor kinase (β - adrenergic receptor kinase-2: βARK-2 - Dawson *et al.*, 1993; Boekhoff *et al.*, 1994). OSNs also express a Ca^{2+}/ calmodulin-activated phosphodiesterase (PDE - Borisy, *et al.*, 1992), which may play an additional role in signal termination.

2.1.2.2 $InsP_3$ pathway

The molecular components of the $InsP_3$ pathway are only just starting to be resolved. Munger *et al.* (2000), have provided molecular evidence for two components of the cascade from lobster olfactory neuron, a G protein α subunit of the G_q family and an $InsP_3$-gated channel or IP_3 receptor.

Again, intracellular Ca^{2+} is an integral part of the transduction cascade. A common mechanism for Ca^{2+} mobilisation appears to be the direct gating of a receptor ion channel (IP_3R) in the endoplasmic reticulum (ER) by $InsP_3$, enabling release of the ion from ER stores (Mikoshiba, 1997). $InsP_3$ and diacylglycerol (DAG) are produced by the hydrolysis of the membrane phospholipid PIP_2 (phosphoinositide 4,5-bisphosphate), by phospholipase C (PLC). The negative feedback mechanisms operating in OSNs have yet to be resolved, but as $InsP_3$ is found acting as a secondary messenger in many other cell-types, it is thought that it may be regulated in much the same way. Dephosphorylation of $InsP_3$ by specific phosphatases deactivates the signal, and Ca^{2+} that enters the cytosol is rapidly pumped out of the cell. However, not all of the $InsP_3$ is dephosphorylated; a certain amount is phosphorylated to 1,3,4,5- tetrakisphophate ($InsP_4$), which is thought to mediate more prolonged processes within the cell, or the immobilisation of Ca^{2+} in intracellular stores.

Taurocholic acid (TChA), a conjugated bile acid, has been shown to increase levels of PIP_2 degradation in extractions of Atlantic salmon olfactory epithelium (Lo *et al.,* 1994). Analysis of odorant amino acid binding indicated the possible interaction of TChA with a putative acidic amino acid receptor but no interactions of TChA with a putative neutral amino acid receptor. The authors concluded that olfactory discrimination between amino acids and bile acids in salmon occurs in part at the receptor level, while both classes of odour appear to share the same signal transduction mechanism: G-protein mediated activation of PLC.

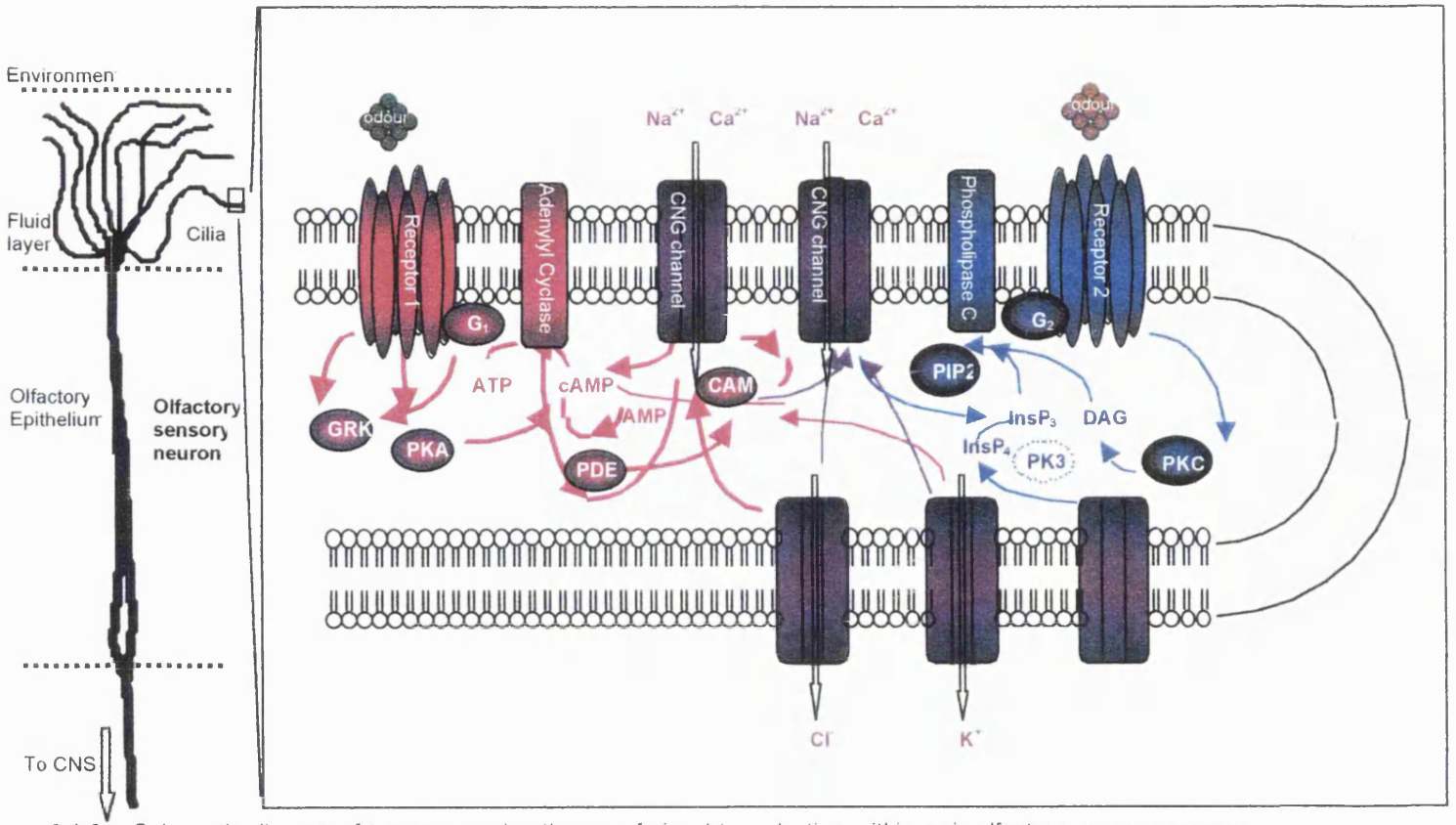

Figure 2.1.2a. *Schematic diagram of two proposed pathways of signal transduction within main olfactory sensory neurons.*
The cAMP (red) pathway is more clearly resolved: involving a receptor protein (R1), a GTP-binding protein (G1), an adenylate cyclase (AC) that produces cAMP, and a cation channel that is directly gated via cAMP (CNG). The alternative pathway (blue) involves a different receptor protein (R2), a different GTP-bing protein (G2), a phospholipase C that produces $InsP_3$ and diacylglycerol (DAG), and a cation channel that is directly gated by $InsP_3$. Each pathway can target more than one ion channel. Other ion channels implicated in olfactory transduction include a Cl^- selective channel, a K^+ selective channel, and a channel that is gated directly by InsP4, which is produced most directly from the action of protein 3-kinase (PK3) on $InsP_3$. Both pathways can also be modulated by several regulatory elements, including a phosphodiesterase (PDE), a G-protein coupled receptor kinase GPK, protein kinase A (PKA) and protein kinase C (PKC), and $Ca2^+$ / calmodulin (CAM). (Adapted from Ache and Zhainazarov, 1995).

2.1.2.3 cGMP pathway and the role of guanylyl cyclases

A third candidate, cGMP, has also been implicated as an odorant receptor. cGMP is produced by two different classes of enzymes (Garbers, 1992; Garbers *et al.,* 1994): soluble (or cytosolic) guanylyl cyclases (GCs) and particulate (or membrane) guanylyl cyclases. Particulate GCs contain a transmembrane domain and are activated by extracellular ligands (Garbers and Lowe, 1994).

Membrane GCs have now been isolated and cloned in several vertebrate and invertebrate taxa (Rat: Fülle *et al.,* 1995; *Caenorhabditis elegans*: Yu *et al.,* 1997; *Oryzias latipes*: Seimiya *et al.,* 1997; Takeda *et al.,* 1997; Suzuki *et al.,* Mantoku *et al.,* Yamogami *et al.,* 1999, Human: Lowe *et al.,* 1995). Sensory excitable tissues such as olfactory neurons and retina express specific isoforms of membrane GC. The first identified olfactory specific guanylyl cyclase (GC-D) was found to be restricted to a subpopulation of olfactory sensory neurons (Fülle *et al.,* 1995), but no ligand has yet been identified for the sensory-specific GCs.

Juilfs *et al.* (1997), discovered a subset of olfactory neurons in the nasal epithelium that express the cGMP-stimulated phosphodiesterase 2 (PDE2) and the olfactory-specific GC-D. The same neurons do not express the normally ubiquitous adenylyl cyclase III (AC III), PDE1C2 or PDE4A, additionally, the cells project to a distinct subset of glomeruli in the olfactory bulb. The authors proposed that these neurons utilise a signal transduction pathway that is different from that thought to be present in other MOE neurons and that cGMP may be an important second messenger in these neurons. Based on this, and additional neurochemical evidence, it is suggested that the function for this group of neurons may be in behavioural responses evoked by hormones or pheromones, rather than odours. Whether the cells possess additional odour-transducing machinery, such as an odorant receptor or a cyclic nucleotide gated channel, or whether GC-D functions as a pheromone receptor itself, has not yet been determined.

However in *C. elegans*, a GC (*gcy*-12) has been functionally expressed and shown to display guanylyl cylase activity (Yu *et al.,* 1997). Similarly, the authors fused GCY-X_1 to the extracellular and protein kinase region of GC-B, the mammalian receptor cyclase for C-type natriuretic peptide (CNP). Basal activity was recorded and the chimera was activated by CNP. The overall structural similarity to known mammalian receptors (each membrane form contains an apparent extracellular domain, a single membrane-spanning region and intracellular protein kinase-like and cyclase catalytic domains), the retention of conserved cysteine residues within the extracellular domain, the cellular localisation and the CNP-stimulated activity of these GCs led the authors to conclude that these membrane GCs function as

2.0 chemosensory/odorant receptors themselves. Proposed pathways are illustrated in Figure 2.1.2b.

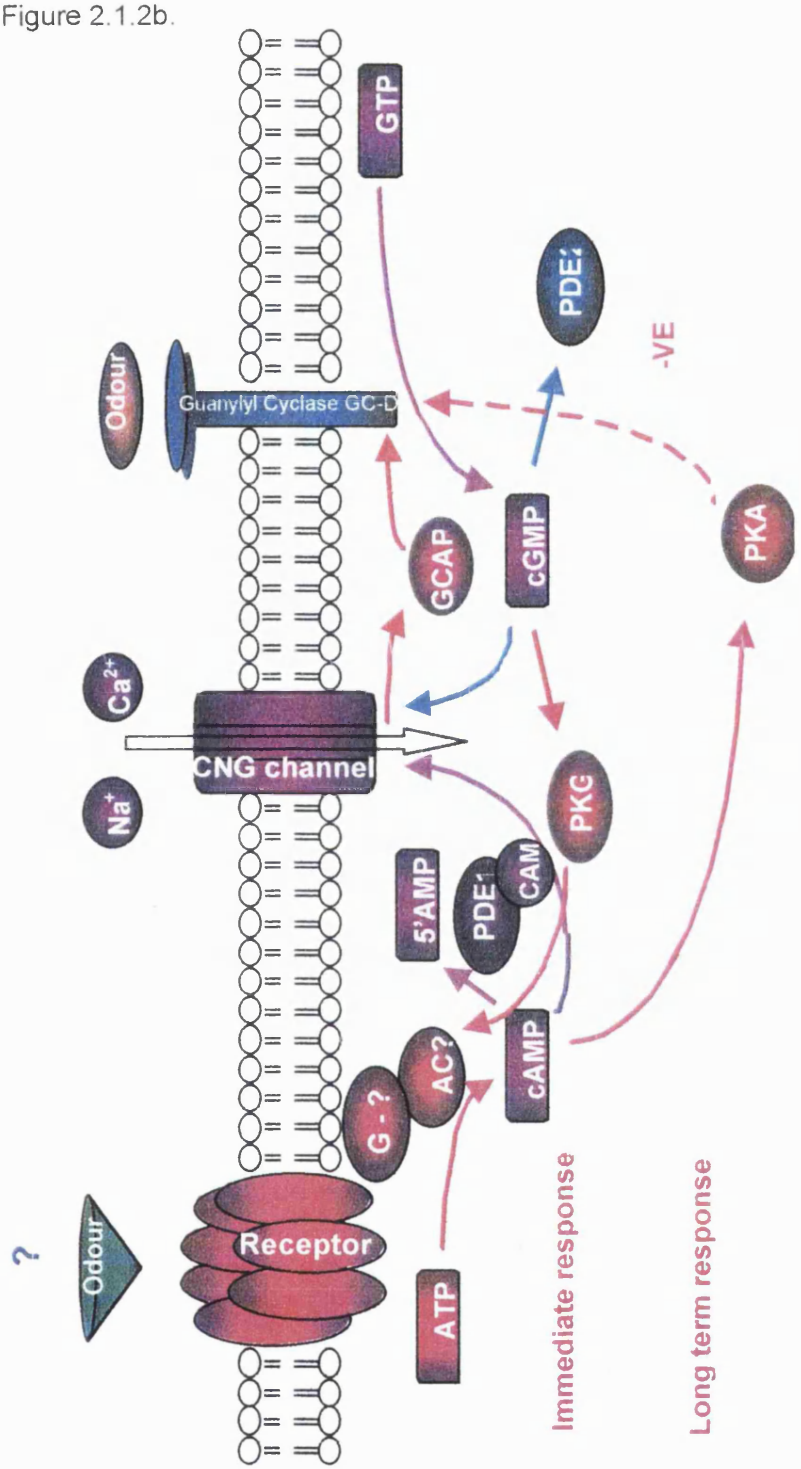

Figure 2.1.2b *A representation of two hypotheses of cGMP signalling in a subset of OSNs.*
(*Red*) An odorant receptor activates a cAMP signalling pathway, opening a CNG channel. Longer exposure to odorants stimulates membrane-bound GCs to produce cGMPvia GCAP and activate PKG, leading to a further increase in amount and duration of cAMP levels, which may activate PKA. As part of a negative feedback loop, PKA can inhibit ativation of GCand cAMP is degraded to 5'AMP by Calmodulin-dependent phosphodiesterase.
(*Blue*) Receptor guanylyl cyclase (GC-D) is activated by an odour to produce cGMP, which opens a cGMP-selective CNG channel, raising intracellular Ca^{2+}, cGMP may then be degraded by PDE2.
(After Yu *et al*, 1997; Moon *et al*, 1998; Meyer *et al*, 2000).

2.1.3 Patterns of Expression

Mammalian olfactory receptor genes have been reported in the past to be expressed not only in olfactory tissues, but also in male germ cells (Parmentier *et al.,* 1992). Northern blots of RNA extracted from dog testis were shown to contain transcripts of ORs belonging to a gene subfamily previously isolated from a human genomic DNA library and found to be expressed in the testis. Sequence comparison revealed that those clones isolated from the human library were the human counterpart of the putative rat receptors originally cloned by Buck & Axel (1991). The presence of the transcripts of these genes in both human and dog testis suggests that a common receptor gene family encodes ORs and sperm cell receptors that could be involved in chemotaxis during fertilisation.

Support for the role of ORs in sperm chemotaxis was generated through the immuno-blotting of transcripts derived from rat testis and spermatids using anti-odorant receptor antibodies (Walensky *et al.,* 1995). Immunohistochemistry identified two putative odorant receptors in elongating spermatids in the testis and in the midpiece of mature sperm. "The specific localization of odorant receptors to the respiratory center of mature sperm is consistent with a role for these proteins in transducing chemotactic signals." The mouse olfactory receptor gene *MOR23* is transcribed in both olfactory epithelium and testis. This gene has an intron within the 5'-untranslated region (UTR) upstream of the reading frame. In OSNs transcription of *MOR23* is initiated in a region upstream of this intron, whereas in testicular tissue, transcription starts within the intron (Asai *et al.,* 1996).

Sperm chemotaxis had been demonstrated in various invertebrate species (Garbers, 1989; Ward and Kopf, 1993, as cited by Vanderhaeghen *et al.,* 1997), before the authors attempted to estimate the diversity of OR genes expressed in the male germ cell line in a number of mammalian species. Sequence analysis of clones isolated from human, mouse, rat and dog testes revealed a striking diversity of gene transcripts, each displaying the characteristic sequence features of the OR gene family. This diversity suggests that these receptors recognise a multitude of ligands, but formal demonstration of the functional importance of these receptors is still required, as is the isolation of olfactory receptors from the male germ cells of non-mammalian vertebrates.

Transcription of a family of olfactory receptors has also been revealed in rat spleen (Blache *et al.,* 1998; Walensky *et al.,* 1998). The presence of a GPCR so closely related to previously isolated olfactory receptors in lymphomyeloid tissue, is thought to be due to the receptors playing a role in white blood cell maturation and/or migration. Sperm, olfactory cilia and immune system cells may therefore share common mechanisms for the detection of exogenous chemical signals.

2.1.4 Evolution of olfactory receptors

Analysing odorant receptor genes in both fish and mammals has revealed that receptors from both groups share some sequence motifs but show only moderate sequence conservation overall and are thought to form separate, non-overlapping receptor families. This distinction is also reflected in gene-family size and diversity. As many as 1000 different receptors exist in mammals (Buck & Axel, 1991), while the receptor repertoire in fish is thought to be reduced by a factor of ten (Ngai, *et al.,* 1993). Whether the structure and numerical differences between the two classes of olfactory receptor simply mirrors the phylogenetic distance between fish and mammals or is indicative of adaptive processes allowing fish to detect a limited array of water-soluble aquatic odours while their terrestrial counterparts exploit a more diverse spectrum of hydrophobic airborne odours is still unclear. However, evidence is mounting for the adaptive nature of olfaction in the different environments.

Freitag, *et al.* (1998), compared the olfactory receptor sequences isolated from aquatic and semiaquatic species representing different levels of vertebrate evolution: amphibia (*Rana esculenta* and *Xenopus laevis*), fish (*Carassius auratus* and *Latimeria chalumnae*) and the striped dolphin (*Stenella coeruleoalba*).

Fish, it seems, possess only Class I genes, whereas mammals are endowed selectively with Class II genes. Fish-like and mammalian-like receptors isolated from the amphibia were found to be expressed in two different compartments of the animals' nose, which are thought to be employed in the selective recognition of either water-soluble or air-borne odourants. Sequence analysis of amphibian ORs showed that both classes of receptors share a common secondary structure and share several highly-conserved amino acid residues, indicating that both classes originated from common ancestral genes. It is therefore conceivable that both classes may be specialised for recognising the distinct types of odorous ligands in the respective environment; class I for water-soluble, class II for airborne odourants.

It was also found that the coelacanth, *Latimeria chalumnae*, a species that has been described as one of the closest living relatives to the tetrapods (Betz, *et al.,* 1994), possesses both classes of receptor genes. However, in this "living fossil" most of the class II receptor genes represent non-functional pseudogenes, while no Class I pseudogene has yet been found. Hughes (1993) suggested that the presence of pseudogenes indicates the absence of selective pressure, therefore in *Latimeria,* class II receptors may not be of functional importance. Assuming that an ancient lobe-finned relative of the coelacanth was adapted to semi-aquatic life, this "loss of function" of class II genes may indicate a secondary adaptation to return to a totally aquatic lifestyle. Similarly, marine mammals have undergone a secondary

transition to a fully aquatic existence, having evolved from terrestrial ancestors (Carroll, 1988). *Stenella*, was found to completely lack Class I receptors, furthermore, the class II receptors this species does possess, exist exclusively as pseudogenes, suggesting that these receptors, again, lost their function during the adaptation to the aquatic environment.

The differentiation between "terrestrial" Class II and "aquatic" Class I receptors has however been recently besmirched by the unlikely discovery of abundant Class I ORs within the human genome (Glusman *et al.,* 2001). These receptors represent approximately 10% of the entire human OR count, are all confined to chromosome 11, and show a considerably lower pseudogene fraction than that observed for Class II (52% and 77%, respectively). Expression data currently exists for 6 Class I ORs, so it seems that they are under selective pressure to maintain functional motifs. Within the human genome, Class II families are all present in more than one chromosome each, and so the restriction of Class I receptors to chromosome 11 may indicate the regional control of expression of these genes. No such mechanism has been found for the Class II, and so this would represent a strong functional difference between the two classes.

In microsmatic terrestrial mammals (such as primates), a greater proportion of the OR repertoire is non-functional. Rouquier *et al.,* (2000), isolated and cloned OR sequences from a random sample of primate hominoids and prosimians and compared the percentage of pseudogenes between taxa with that of *Mus musculus*, a macrosmatic species. A dendogram of these sequences and those available in the literature from other species was constructed to determine any evolutionary relationships. The results suggested that from New World monkeys to hominoids, there is an increase in the percentage of OR pseudogenes, from ~0% to ~70% (Table 2.1.4).

Family/species	No. sequences	% ORF	% pseudogenes	Mean %
Hominoids				50
Human	99	30	70	
Chimpanzee	21	52	48	
Gorilla	18	50	50	
Orangutan	23	61	39	
Gibbon	22	59	41	
Old World monkeys				27
Macaque	20	65	35	
Baboon	21	81	19	
New World monkeys				2
Marmoset	19	100	0	
Squirrel monkey A	15	100	0	
Squirrel monkey B	15	93	7	
Prosimians				37
Lemur A	19	58	42	
Lemur B	16	69	31	
Rodents				0
Mouse	33	100	0	
Fish				0
Zebrafish	3	100	0	

Table 2.1.4 *Fraction of pseudogenes in the OR gene repertoire of primate species and mouse.* Taken from Rouquier *et al.* (2000).

Although hardly an exhaustive study (only 3 out of a possible 100 zebrafish genes were included), there is evidence of the existence a selective advantage for New World monkeys to retain a greater repertoire of ORs than their Old World counterparts. Similarly, an analysis of the OR cluster on human chromosome 17p13 and a selection of non-human primate orthologs (Sharon *et al.,* 1999) has indicated that a rapid decline (~10 million years ago, corresponding to the radiation of hominids) in the functional OR repertoire occured in mammals. The authors inferred that all OR genes within this cluster were fully functional before the divergence of orangutans from African apes. Furthermore, it appears that the pool of primate pseudogenes is still growing (Rouquier *et al.,* 1998).

2.1.5 Molecular Evolution

Important differences between the olfactory receptor protein family and the other seven transmembrane domain proteins, may be relevant to the function of these proteins in odour recognition. Structure-function experiments involving *in vitro* mutagenesis suggest that adrenergic ligands interact with their specific receptors by binding within the plane of the membrane (Strader, *et al.,* 1989). Unsurprisingly, small receptor families that bind the same class of ligands, such as the adrenergic receptor family, display maximum sequence conservation within the transmembrane domains. Conversely, ORs in the rat (Buck & Axel, 1991) show maximal diversity within the third, fourth and fifth transmembrane domains. This divergence in potential ligand-binding domains is consistent with the ability of this family of molecules to associate with a large number of odorants of diverse strucure.

A subfamily of ORs, displaying unique structural and expression characteristics (the genes contain an extended ECL3 and are expressed only on the apical turbinates), was subjected to secondary structure analysis (Kubick *et al.*, 1997). An insertion of six amino acids into the third extracellular loop, and the presence of common motifs conserved across all the members of this subfamily, result in ~50% of the amino acids in ECL3 being charged. Analysis showed that this domain adopts an α-helical structure of amphiphilic character. Several feasible functions were proposed for this region:

(a) A docking site for odorant-binding proteins (OBPs). OBPs are soluble proteins belonging to the lipocalin family which are associated with the transport of hydrophobic compounds in an aqueous environment and thus play a role in chemosensory perception (Pelosi *et al.*, 1994; Loebel *et al.*, 2000).

(b) A recognition site for the variable "address" of a ligand in order to achieve selectivity, or interaction with adhesion or chemotactic molecules during neurite outgrowth.

Whatever the function of this extended extracellular domain, the OR subfamily that possess this unique feature remain exclusive to mammals.

Ngai, *et al.*, (1993) also found a non-random distribution of amino acid differences among subfamily members of the aquatic-type class I receptors in catfish. Eighty percent of the amino acid changes occur within the transmembrane domains although these domains constitute less than 50% of the mass of the entire molecule. Again, this was suggested as evidence that each of the subfamily members may interact with different odorous ligands. The authors then subjected the most homologous genes within a subfamily to analysis for positive Darwinian selection. An estimation of the rates of nonsynonymous (replacement) substitutions and synonymous substitutions ("silent" mutations; d_S), provides an important means for understanding the mechanisms of molecular sequence evolution. If there is no postive or negative selection for amino acid sequence (neutral), then the number of nonsynonymous changes relative to the number of possible nonsynonymous changes (d_N) is expected to be approximately equal to the number of synonymous changes relative to the number of possible synonymous changes (d_S). Significant deviation from equilibrium between d_N and d_S reflect selection on the sequence. A value d_N greater than d_S indicates positive selection for a change in sequence, whereas a d_S value greater than d_N would reflect selection against a change (Tanaka and Nei, 1989). Ngai *et al.*, (1993) found that the d_N:d_S ratio in transmembrane domains three and four was 2, whereas the ratio in the remainder of the molecule was 0.3, a result consistent with a model in which transmembrane domains 3 and 4 have diverged to enable the recognition of several ligands with which this subfamily interacts. The other regions of the genes (including the normally

variable transmembrane domain 5) have been conserved, which, the authors inferred, agree with the suggestion that this receptor gene subfamily recognises structurally related odourous ligands.

The signature of positive selection has also been detected in a 450-Kb cluster of OR genes within the human genome (Gilad *et al.,* 2000). Comparisons were made of both the ratio of polymorphism to divergence and the d_N:d_S ratio between functional genes, pseudogenes and introns. Nucleotide diversity was higher for the pseudogenes and the introns than for the intact OR genes. A lower rate of polymorphism to divergence was found in genes compared to pseudogenes or introns, and high non-synonymous substitution rates were found in functional genes. The authors claimed that OR genes (probably constituting the largest multigene family in vertebrates), experience a unique class of evolutionary constraints and pressures, and that individual OR genes may seldom be essential for survival, but the general expansion and diversification of the olfactory repertoire may confer a selective advantage.

2.1.6 Evolution of the olfactory repertoire

Following the completion of the sequencing of the *Caenorhabditis elegans* genome, evolutionary analysis of all the nematode's chemosensory seven-transmembrane domain receptors (*str*) has been possible, revealing frequent gene movements both within and between chromosomes, in some cases resulting in the expansion of new gene lineages on other chromosomes (Robertson, 2001). The *str* family of *C. elegans* consists of 189 genes and 74 pseudogenes, the related *srj* family consists of 39 genes and 18 pseudogenes, residing on 6 chromosomes, although the majority are confined to chromsome V. Assuming that the large chromosome V was the ancestral residency for chemosensory receptor genes, 14 movements to chromsome IV, six to X, three to II, two to III and one to I were inferred from the construction of a neighbour-joining tree. One thousand replicates were used to bootstrap the phylogeny and all gene subfamilies were supported at >70%. Of the newly recognised D(SP) subfamily, 20 members are located on chromosome IV, and all appear to be the result of the duplication of an ancestral gene that moved from chromsome V. Four members of this subfamily are now located on chromosome V, but each involved a separate movement and are spread throughout chromosome V (Robertson, 2001). Similarly, movement of genes within chromosome V appears to have been frequent, presumably resulting from the same mechanism as movement between the chromosomes. These movements and duplications, resulting in the formation of (usually) new pseudogenes, seem to be kept in check by large deletions (determined from comparison with a sister species, *C.briggsae*), which appears to

maintain the small size of the nematode genome.

Similarly, in the mouse genome, OR genes have been found to be widely spread: 11 distinct regions have been identified on 7 different chromosomes (Sullivan *et al.,* 1996). Multiple genes were found to map to 8 of the regions, indicating that the majority contain clusters of OR genes. Each OR cluster seems to be related to others by linkage to members of one or more gene families. Instead of individual genes being tandemly repeated, it appears that large chromosomal regions have been duplicated, creating novel OR clusters.

Both Sullivan *et al.,* (1996) and Glusman *et al.,* (2000, 2001) have shown that in mammals (mouse and human, respectively) highly similar OR genes can be interspersed with genes from different OR gene subfamilies. Within the human genome, the largest cluster of OR genes (on chromosome 11) comprises both Class I and II. The reverse seems to be the case in teleost genomes - so far only closely related OR genes are found in tightly linked groups in the zebrafish (Dugas and Ngai, 2001). In mammals, closely related OR genes can be found on more than one chromosome (Sullivan *et al.,* 1996). In primates, extensive regions of DNA containing multiple OR-like sequences have been duplicated onto several chromosomes (Rouquier *et al.,* 1998), but in zebrafish, a single cluster appears to contain all the members of two OR subfamilies (Dugas and Ngai, 2001). This difference in chromosomal distribution of closely related OR genes between zebrafish and mammals probably reflects a divergence in the mechanisms used to amplify the OR gene repertoire in vertebrates. It is possible that the large scale duplication of chromosomal regions originated to enable the rapid expansion of the OR gene family in terrestrial vertebrates.

2.2 AIMS

The object of this study was to isolate OR-like sequences from the Atlantic salmon (*Salmo salar*) and to establish patterns of their expression in a range of tissues as a means of elucidating their possible function. It was also intended to classify salmon OR-like sequences, based upon their amino acid sequence in the context of OR genes isolated from other taxa, with particular reference to teleost species. Five families of olfactory receptor-like sequences were identified in the pilot study (Deaville, 1998, unpublished), all other work reported here was carried out by myself as part of the studentship. A polymerase chain reaction (PCR) approach was employed, using PCR primers homologous to conserved regions of OR loci to isolate OR-like sequences. As alignments of *OR* gene sequences have revealed few conserved areas for primer design (Mombaerts, 1999), published primer sequences which had proved successful in previous amplifications of OR genes in fish and other species were used. OR sequences were amplified under low-stringency PCR conditions from total genomic DNA from a relatively small number of individuals sampled from different populations in Scotland, and a large number of independent clones (20-50) from each individual was examined. This approach was adopted since individual fish are estimated to possess as many as 50-100 OR loci in their genome (Ngai *et al.,* 1993a; Barth *et al.,* 1996; Korsching *et al.,* 1997).

2.2.1 MATERIALS & METHODS

Genomic DNA extracted from ten Atlantic salmon samples from each of three sites in Scotland was used as template in a PCR based protocol. The samples used were representative of the range of this species in Scotland: from the southwest (River Nith), through the northeast (River Oykel), to the southeast (River Tweed). All thirty samples were used in initial optimisations of amplification from various primer pairs.

PCR primers used previously to isolate *OR* sequences from zebrafish, *Danio rerio* (Byrd *et al.,* 1996), *Xenopus laevis* (primers X2.4 and OR7.1; Freitag *et al.,* 1995) and catfish, *Ictalurus punctatus* (Ngai *et al.,* 1993a) were used in various combinations in an attempt to amplify as diverse a selection of *OR* sequences as possible from Atlantic salmon genomic DNA. All primers were degenerate (i.e. containing inosine or ambiguous base positions), and corresponded to amino acid motifs conserved across a wide range of *OR* molecules (Mombaerts, 1999). (see Fig. 2.2).

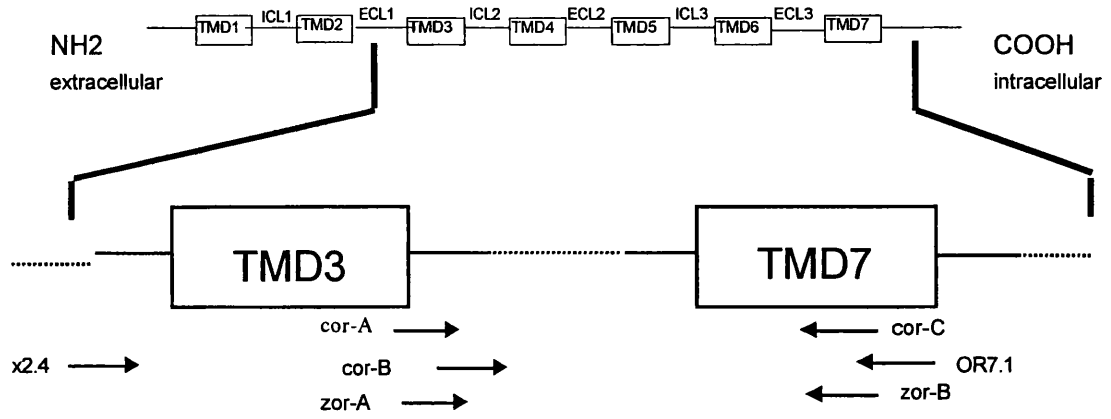

Figure 2.2. *Position of PCR primers (relative to protein structure) for amplification of OR genes from Atlantic salmon. 3' primers:* cor-A, cor-B and zor-A were based on an amino acid motif spanning the junction of TMD3 and ICL2 which is conserved in OR molecules from a variety of species. The x2.4 primer was based on an amino acid motif (IAKYWF) which is highly conserved in ECL1 of "fish-like" OR genes in *Xenopus laevis* (Freitag, *et al.,* 1995), and which is similar to a motif [I(A/S)RY(W/L)F] present in catfish and zebrafish sequences (Ngai, *et al.,* 1993; Barth, *et al.,* 1997). *5' primers:* All 5' primers (cor-C, zor-B, and OR7.1) correspond to different areas of a semi-conserved amino acid motif in TMD7.

Following optimisation, one individual sample per site, i.e. that showing the highest specific yield of the expected size product, was used for further cloning and sequence analysis. Subsequently a single adult salmon caught in Scottish coastal waters was used for isolation of *OR* sequences using the catfish degenerate primers. PCR products were ligated into an appropriate vector, and the inserts sequenced using dideoxynucleotide chain-termination methods, with radio-nucleotide labelling and ABI PRISM®, Big Dye™ Terminator Cycle Sequencing.

2.2.2 PCR amplification, cloning and sequencing

Amplifications were optimised in 25 μl reaction volumes containing 2.5-25 ng genomic DNA in standard buffer conditions [1.2 μM each primer, 0.1 mM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Nonidet P40, 10 mM Tris-HCl (pH8.8) and 0.5 units *Taq* polymerase]. Buffer conditions (particularly $MgCl_2$ concentration), duration of temperature steps, annealing temperatures, numbers of cycles, and ramp rates between steps were varied during PCR optimisation. PCR products were excised from agarose gel and purified using the GeneClean III kit (Amersham), before ligation into the pCR2.1 vector (Original TA cloning kit®, Invitrogen). Partial sequences from one strand were used as the basis for identification of each insert (through BLAST alignments with Genbank sequences; Altschul *et al.,* 1997), but complete sequences reported here resulted from sequencing of both strands.

The sequences obtained were grouped into three classes according to results of BLAST searches: (a) sequences showing high levels of homology to

putative OR genes, (b) a single highly-represented sequence which had a high level of homology to a number of putative G-protein coupled receptor (GPCR) genes, and (c) sequences with no strong homology to any sequences in GenBank. Those sequences belonging to groups (b) and (c) were omitted from further analyses.

Specific primers for the OR-like sequence families *SORA*, *SORB* and *SORD*, and *SORF* were designed and used in PCR amplifications of 1st strand DNA synthesised from a range of tissues taken from the single Scottish salmon (see above), to determine the expression pattern.

2.2.3 Examination of tissue expression patterns

Although partial OR-like sequences were isolated during the pilot study of this project (Deaville, unpublished), primers designed to amplify these sequences in genomic DNA had not yet been successful in detecting the expression of all *SOR* families in olfactory tissue. In order to determine which genes are expressed in the olfactory epithelium (OE), cDNA was synthesisied from the RNA isolated from the olfactory tissue of an Atlantic salmon.

RNA Isolation

Using samples previously collected from a single male grilse, total RNA was extracted from 4 reference tissues: testes (T), brain (Br), liver (Li), anterior kidney (AK), as well as olfactory epithelium (OE) using TRIZOL™ reagent (Life Technologies, Gibco BRL). Genomic DNA was also subsequently purified from the by-product of RNA isolation, following the manufacturer's instructions.

Synthesis of 1st Strand cDNA

First strand cDNA was generated from the mRNA present in these isolates using RT-PCR (First Strand cDNA Synthesis Kit, Pharmacia Biotech). The mRNA:cDNA heteroduplex produced was subsequently used as a template for PCR.

PCR Screening of 1st Strand cDNA

β Actin is consitutively expressed in animal cells as it is involved in the transport of intracellular components and cell division. Unlike OR genes, β actin genes contain introns, areas which will be amplified from a genomic template, giving a significantly larger PCR product, than from a cDNA template. Using β actin as a control not only verifies the success of the PCR reaction itself, but also indicates any contamination of cDNA isolates by genomic DNA. Mouse β actin primers (degenerate) were obtained, the forward primer complementary to a portion of sequence on exon 2 and the reverse complementary to a portion on exon 4; giving an expected product of approximately 550bp from cDNA.

Despite intensive optimisation (including varying annealing temperature, ramp rate, template concentration etc.) it was only possible to obtain the appropriately sized PCR product from the anterior kidney, liver and testis cDNAs, and so a more specific primer set was obtained.

Salmon specific β actin primer sequences were acquired (S.A.M. Martin, pers. comm.) giving an expected size product of approximately 240bp from cDNA. A low yield (as visualised on an ethidium bromide stained agarose gel) was obtained in the first reaction, but using a 1:5 dilution of template, robust products were amplified from each cDNA. Standard buffer conditions [12.5pM each primer, 1.8mM each dNTP, 1.5mM $MgCl_2$, 50mM KCl, 20mM Tris-HCl (pH 8.4) and 0.5 units *Taq* polymerase] were used with a thermocycling profile of 30 cycles of 95°C for 1 minute, 57°C for 30 seconds and 72°C for 1 min.

Target gene	Primer set (5' – 3')	Amplicon length
β Actin	Forward: ATGGAAGATGAAATCGCCGC Reverse: TGCCAGATCTTCTCCATGTCG	~ 240 bp (cDNA) ~ 560 bp (gDNA)
SORA	Forward: GAGCTTTCCTAATACCTTTGGAC Reverse: GACCTCTGCATTATCAA ACTGTC	468 bp
SORB	Forward: TGCCACCTTCCTGTAGTCTG Reverse: CCCCTGCGATATTCCATC	470 bp
SORD	Forward: AGTGAAACATAATGGACGCC Reverse: CAAAATGCTGGTGAAGCTG	470 bp
SORF	Forward: GGAAGGGGCTGCTTGCTCGA Reverse: CTGTCTACTGAGGTATAACT	484 bp

Table 2.2.3 *Primer sequences used to determine tissue specific expression of olfactory receptor families*

Once the control gene had been established an attempt was made to amplify the OR-like sequences using primers specific to each gene family (Table 2.2.3) Standard buffer conditions were used with the following thermocycling profile: 2 cycles of 95°C for 30 secs, 60°C for 45 secs and 72°C for 45 secs, followed by 30 cycles of 95°C for 30 secs, 59°C for 45 secs and 72°C for 1 min.

An agarose gel of PCR products was Southern blotted using a standard protocol (Sambrook *et al.*, 1989) onto positively charged nylon membrane (Hybond™ N+, Amersham), and probed using random-primed radiolabelled (Boehringer Mannheim / Roche) OR inserts excised from the plasmid vector.

2.2.4 Phylogenetic analysis

Sequences of OR genes from teleosts were obtained from GenBank. Alignments of deduced amino acid sequences were made using Clustal X (Thompson *et al.*, 1997) prior to phylogenetic analysis using PAUP* (Swofford, 1998). The OR

sequence phylogeny was reconstructed by neighbour-joining (Saitou and Nei, 1987) using mean character difference as a genetic distance measure. Estimates of the robustness of the nodes of the tree were obtained by bootstrapping (1000 replicates) (Felsenstein, 1985).

2.3 RESULTS

2.3.1 PCR amplification, cloning and sequencing

Between 20-59 clones per individual (total 150 clones) were partially sequenced, and based on the results of BLAST searches, 38 of these clones (8-11 per individual) were completely sequenced on both strands. The OR-like sequences obtained were classified into 6 families on the basis of nucleotide sequence, with between 1-4 members identified within each family (Figure 2.3.4). A difference of at least two base substitutions from any other sequence was used as an arbitrary criterion for the identification of a novel member of a family. These sequence families have been designated salmon olfactory receptor (*SOR)A-F*, and the sequences have been deposited in GenBank (Accession Numbers AY005449-AY005460, AY005824-AY005826).

SORB, *SORF, SORD2* and *SORD3* sequences have uninterrupted open reading frames. However, within each *SORA* sequence there are four stop codons, and one stop codon within *SORD1*. At least for *SORA* sequences, these stop codons do not appear to result from PCR or sequencing artefacts since the same mutations were observed in all clones and from all individuals sampled. The 2 bp difference in length of *SORA* compared to *SORB/D* sequences also suggests a frameshift deletion in *SORA* sequences, but it is not possible to identify the position of the indel from current data. Further work is required to confirm that the stop mutation in *SORD1* is real and not a PCR or sequencing artefact as at present it is known from a single clone only. Similarly, both the truncated sequence of *SORC1*, and the sequence of SORF are also known from only one clone apiece. However, the truncated *SORE* sequences (obtained independently from two individuals) were truncated at the same point in all clones and displayed relatively high levels of polymophism, suggesting that they represent pseudogene sequences.

The OR-like sequences were classified into 5 families on the basis of nucleotide sequence: salmon olfactory receptor (*SOR)A - F. SORA* and *SORB/D* sequences were approximately of the expected length, 468bp and 470bp long respectively. *SORC* and *SORE* sequences were much smaller in length and appear to represent truncated sequences homologous to the 5' end of the *SORA*, *B*, and *D* products. Little polymorphism exists in each family, with the exception of the *SORE* family.

On examination of deduced amino acid sequences, it was found that each *SORA* sequence contains four stop codons, indicating that this family may represent pseudogenes. Using deduced amino acid sequences facilitates the determination of relationships among protein-coding sequences as the position of the twenty possible amino acids gives more information than the nucleotide level. It was found that *SORB*

and *SORD* sequences are very similar along their entire length, with the truncated *SORC* sequence also clearly related at the 3' end. *SORA* sequences form a distinct group, as do *SORE* sequences, but a number of amino acid residues are conserved across the families (Figure 2.3.4b).

2.3.2 Patterns of expression

Robust PCR products of approximately 240bp and 560 bp were generated from first strand cDNA and genomic DNA respectively, using salmon-specific β actin primers. A difference in size of 300 bp is clearly resolved on an ethidium bromide-stained agarose gel (see Fig. 2.3.2), and so a successful reaction control and contamination control was established.

The pseudogene status of the *SORA* sequences was verified by the subsequent failure to amplify any sequence using cDNA as template; amplification was successful using genomic DNA controls extracted from the same individual.

SORB primed amplification gave a strong product from testes-derived cDNA of approximately 470bp, the same as from genomic controls, as expected for intronless genes, but a low yield product from OE cDNA (see Fig 2.3.2).

The *SORD* primer pair gave a low yield product from testes cDNA only (buffer conditions and thermocycling as *SORB*).

Both *SORB* and *SORD* primers also produced a low yield product from the amplification of anterior kidney cDNA.

The *SORF* primers amplified a product from olfactory epithelium only.

An agarose gel of these PCR products (see Fig. 2.3.2) was Southern blotted using a standard protocol (Sambrook *et al.,* 1989) onto positively charged nylon membrane (Hybond™ N+, Amersham), and probed using random-primed radiolabelled (Boehringer Mannheim / Roche) OR inserts excised from the plasmid vector. The resulting autoradiograms confirmed the initial RT-PCR results (data not shown).

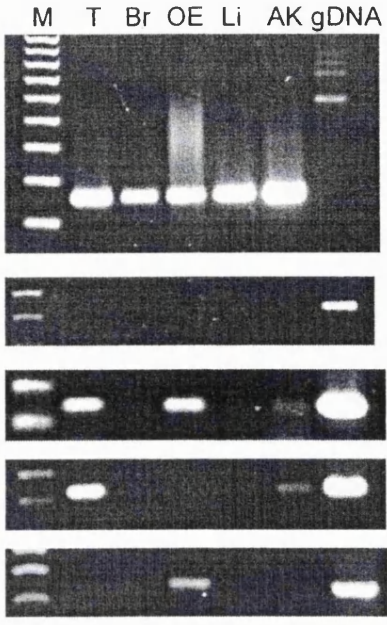

Figure 2.3.2 *Gel photographs depicting bands amplified using gene-specific RT-PCR from 5 different tissues from a single individual.* T = testis, Br = brain, OE = olfactory epithelium, Li = Liver, AK = anterior kidney, gDNA = genomic DNA from the same individual, M = 100 bp marker.

2.3.3 Phylogenetic analysis

When the "phylogeny" of teleost *OR*-like sequences was reconstructed, a "star" topology was obtained (i.e. internal branch lengths were generally short in comparison to external branches) (Figure 2.3.3). However, whilst most of the lineages were strongly supported by bootstrap analysis, the star topology gave little evidence for distinct lineages. The lack of distinct species specific lineages makes identification of paralogous loci difficult, whilst the patchy interspersion of species with others renders the identification of orthologues problematic.

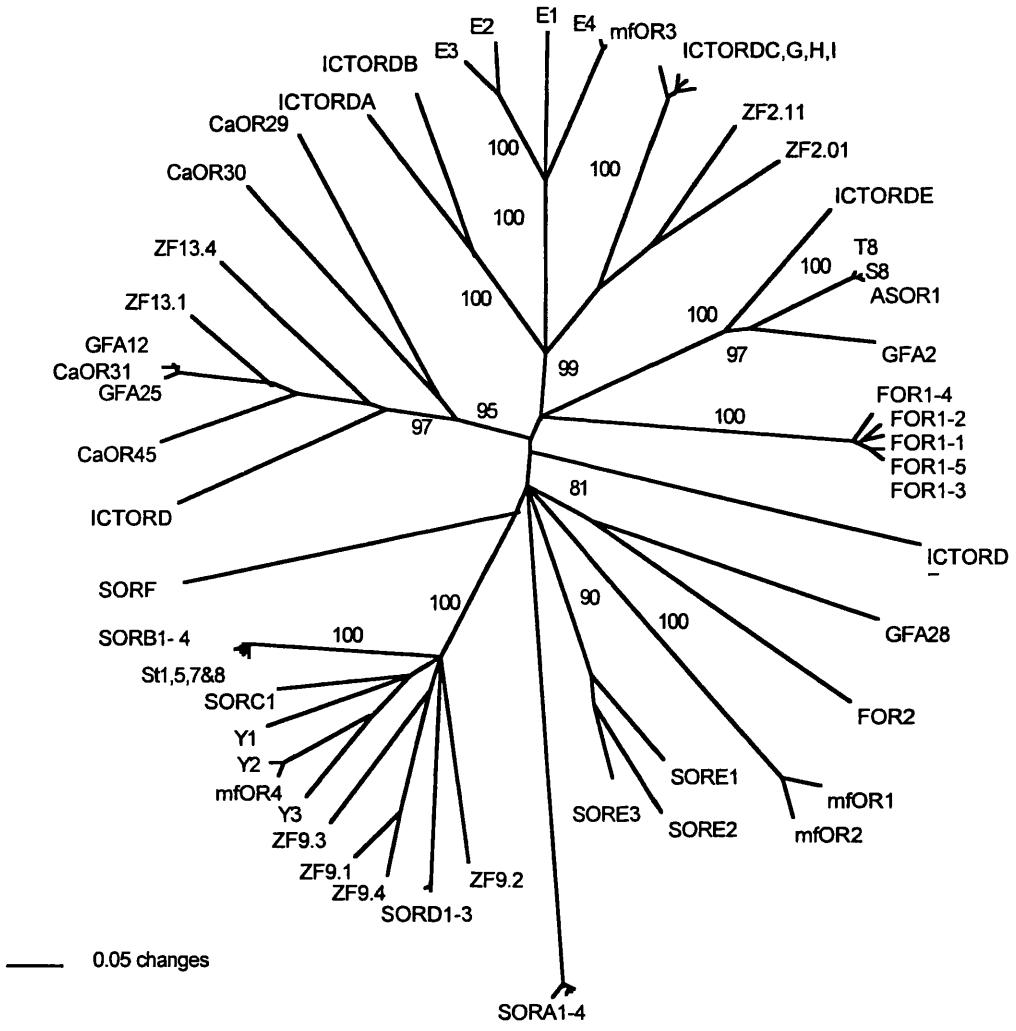

Figure 2.3.3. *Bootstrapped Neighbour-Joining tree of all putatative olfactory receptor sequences from Teleost fish.*
All teleost putative OR sequences available in GenBank were used to construct this neighbour-joining tree. 1000 replicates were used and examples of bootstrap values obtained are indicated.
ICTORDA-I: L09217-L09225 Ngai *et al.*, 1993 - Channel catfish (*Ictalarus punctatus*); *ZR-10, 9, 6, 2, 13, 1*: U42392, U42394-U42398, Barth *et al.*, 1996; *ORZF1A, 1L, 39* = U44439-U44441, Byrd *et al.*, 1996; *ZOR1-10* = U72683-U72692, Weth *et al.*, 1996 - Zebrafish (*Danio rerio*); *GFA2, 12, 25, 28* = AF083076-AF083079, Cao *et al.*, 1998; CaOR29-31, 45 = AJ233785-AJ233788 Freitag *et al.*, 1998 Goldfish (*Carassius auratus*); *mfOR1*-mfOR4 = AB029474-AB029480, Sun *et al.*, 1999; *mfORE1-3, Y1*-3 = AB029474-AB029480, Yasuoka *et al.*, 1999 - Medaka fish (*Oryzias latipes*); *FOR1.1-1.5, 2* = AB031380-AB031385, Asano-Miyoshi *et al.*, 2000 - Pufferfish (*Fugu rubripes*). *ASOR1* = AY007188, Wickens *et al.*, 2001.

2.4 DISCUSSION

In the analysis of *SOR* expression patterns, only *SORA, SORB, SORD* and *SORF* were examined, as unlike *SORC* and *SORE*, these were not extensively truncated in the clones derived from genomic DNA. The interruption of *SORA* putative coding regions by multiple stop codons suggested that they would not be expressed at the protein level, although transcription of the sequences was possible (i.e. "processed pseudogenes"). However, there was no evidence of *SORA* transcription in any of the tissues screened. The presence of these pseudogenes, and two other putative pseudogenes (*SORC* and *SORE*) in a sample of six OR-like families represents a potentially high incidence of *OR* pseudogenes in the Atlantic salmon genome. While pseudogenes are common in primates and marine mammals (Freitag *et al.,* 1998; Mombaerts, 199b; Sharon *et al.,* 1999; Rouquier *et al.,* 2000, Glusman *et al.,* 2001), they have not yet been shown to be common in the genomes of other teleosts.

Transcription of *SORB* and *SORD* in testis was not unexpected given than transcription of *OR*-like sequences has been found in the male germ cells of a range of mammals (Vanderhaeghen *et al.,* 1997) in the channel catfish (Ngai, *et al.,* 1993a), and now in humans (Goto *et al.,* 2001). However the presence of *SORB* and *SORD* transcripts in the anterior kidney was not initially anticipated. This is an intriguing result in the light of recent evidence for OR-like sequence transcription in the periarterial lymphatic sheath of rat spleen and the speculation that OR molecules might function as chemoreceptors on cells of the immune system (Blache *et al.,* 1998; Walensky *et al.,* 1998). The anterior kidney in teleost fish is a lymphomyeloid tissue, and therefore rich in lymphocytes. Whether these transcripts represent members of an alternative *SORD*-like testis-specific and kidney-specific OR families, or whether they are homologous to those transcripts in olfactory epithelium has yet to be determined (expression in testicular or olfactory tissue may be dependent on differential start sites or 5' splicing, Walensky *et al.,* 1998). Results from other studies suggest that pseudogenes and sequences expressed in tissues other than olfactory epithelium are often closely related to sequences expressed on olfactory neurons. The same species of OR may also be expressed in a number of tissues (Parmentier *et al.,* 1992; Barth *et al.,* 1997, Vanderhaeghen, *et al.,* 1997).

Of those isolated in this study, *SORB* and *SORF* sequences appear to be the best candidates for genes coding for olfactory receptor proteins. Efforts were therefore concentrated on these sequences to determine gene regulation during the olfactory-sensitive period of parr-smolt transformation.

OR-like sequences were previously thought to fall into two phylogenetically distinct groups: terrestrial and aquatic (Freitag *et al.,* 1995, 1998; Zhou *et al.,* 1997;

Sun *et al.,* 1999). However, Class I receptors (aquatic) have now been shown to be functional in both rat (Raming *et al.,* 1998) and humans (Reingold *et al.,* 1999; Glusman *et al.,* 2001). Functional work on *Xenopus* (Mezler *et al.,* 2001) has shown that Class I receptors are activated by water-soluble odorants, whereas Class II receptors are activated by volatile compounds, which confounds the issue further. In order to simplify the evolutionary analysis the position of *SOR* sequences within a phylogeny of only teleost OR-like sequences was resolved. Short internal branches within the phylogeny suggest rapid radiation of the *OR* genes early in the vertebrate lineage (Zhou et al., 1997), which makes identification of orthologous and paralogous *OR* sequences from the phylogeny difficult (Fig 2.3.3). There was no indication of species-specific lineages nor of lineages related to specific aquatic environment (sequences from the only truely marine fish in the analysis, *Fugu rubripes* were found throughout the phylogeny). Olfactory receptors are now known to reside in either multigene family clusters or remain as discrete, solitary genes, and when only a limited number sequences is available from different species the identification of orthologous loci is dubious.

SOR sequences were also not confined to one "clade" within the teleost *OR* phylogeny; sequences from other species were dispersed throughout. Like the sequences from *Fugu*, salmon isolates probably represent only a fraction of the *OR* diversity found in teleosts, as they are not as dispersed as those from catfish, medaka and zebrafish. It may be that the strategy for isolation used here identified only a fraction of the *OR* diversity present in Atlantic salmon, despite adopting the same approach that has previously produced a larger repertoire of genes from other fish species. OR-like sequences were only successfully amplified using two pairs of degenerate primers based on sequences from zebrafish and channel catfish; it could be argued that designing more primers to conserved motifs among sequences isolated from alternative teleosts may yield a greater number (and more divergent) *SOR*s. Indeed, the publication of a novel salmon olfactory receptor sequence, *ASOR1,* (Wickens *et al.*, 2001) illustrates this limitation. *ASOR1* groups with sequences from goldfish and catfish rather than *SOR* sequences, but was also amplified using degenerate primers based on the catfish sequences of Ngai *et al.* (1993a).

OR genes reside in clusters in vertebrate genomes (Barth, *et al.,* 1997; Ben-Arie *et al.,* 1994; Rouquier *et al.,* 1998, 2000). Novel OR gene subfamilies have recently been sequenced in zebrafish, (Dugas and Ngai, 2001), and a comparison of these sequences to the primers used by the authors in previous screens revealed several mismatches. The fact that the novel subfamilies were not previously amplified by degenerate primers illustrates that OR genes omitted by homology-based approaches may be identified by sequencing the genomic regions adjacent to known

OR genes. The limited diversity observed among the *SOR* may simply be a result of similar mismatches; further families/subfamilies of *SOR* may reside in genomic sequences proximal to those already identified.

However, the independent cloning of closely related sequences from the same species by different research groups (e.g. ZR-13/ZOR8, ZR-2/ORZF39, mfOR4/mfORY2 and mfOR3/mfORE4; Figure 2.3.3) suggests that current strategies for isolating *OR* sequences are limited in their ability to isolate a wide range of sequence types. Fish have been estimated to possess in the region of 100 OR genes (Ngai *et al.*, 1993b), and upon the completion of the zebrafish genome project, there may be a sudden increase in the number and diversity of teleost OR sequences identified.

The limited diversity of *SOR* genes identified in this study nevertheless provides a platform for further investigations into olfaction in salmonids. Atlantic salmon are known to imprint on odours of the homestream during parr-smolt transformation (PST) which they then use as cues during the spawning migration some years later. This olfactory memory is apparent within the peripheral sensory neurons (Nevitt *et al.*, 1994), but the mechanisms underlying the formation of this long-term memory are still unclear. The putative olfactory receptors *SORB*, *SORF*, and *SORD* are all candidates for the study of the molecular and cellular basis of olfactory imprinting in salmon, a process that in turn may aid elucidation of memory formation.

```
SORD1   ---------- ---------- ---------- ---------- CAACCCTCTG CGATACGCCA
SORD2   .......... .......... .......... .......... .......... ..........
SORD3   .......... .......... .......... .......... ......C... ..........
SORC    .......... .......... .......... .......... ......C... ..........
SORB4   .......... .......... .......... .......... ......C... .....TT...
SORB1   .......... .......... .......... .......... ......C... .....TT...
SORB2   .......... .......... .......... .......... ......C... .....TT...
SORB3   .......... .......... .......... .......... ......C... .....TT...
SORE2   .......... .......... .......... .......... TT.T.....A .AG..TAA..
SORE3   .......... .......... .......... .......... TT.T.....A .....TAAT.
SORE1   .......... .......... .......... .......... TT.T...... T.G..TAA..
SORA2   .......... .......... .......... .......... T.GA...... .AT..TCAA.
SORA4   .......... .......... .......... .......... T.GA...... .AT..TCAA.
SORA3   .......... .......... .......... .......... T.GA...... .AT..TCAA.
SORA1   .......... .......... .......... .......... T.GA...... .AT..TCAA.
SORF    CGGAATTCGT GATGGCGTAT GATCGGTATA TCGCAATCTG .TGT.TA... A.G..TAA.T

SORD1   CCATCATGAA CAACAAAATG CTGGTGAAGC TGTGTGTGTC TGCCTGGTGA GTGTCGTTTG
SORD2   .......... .......... .......... .......... .......G.. ..........
SORD3   .......... .......... .......... ........C. .......G.. ..........
SORC    .........C .....GG... A.C.CA..A. ..AC...T.T .......G.G TCTG.C...C
SORB4   T........C .......... A.TA.C.G.T ...CG..... .......G.G ...G.CA.A.
SORB1   T........C .......... A.TA.C.G.T ...CG..... .......G.G ...G.CA.A.
SORB2   T........C .......... A.TA.C.G.T ...CG..... .......G.G ...G.CA.A.
SORB3   T...A....C .......... A.TA.C.G.T ...CG..... .......G.G ...G.CA.A.
SORE2   AT..T....C ACCT....CC A.T-..TG.. .TAA.TCTA. ....CCAA.T ..A.TC...T
SORE3   AT..T....C ACCT.....A G.T-..TG.. .TAA.TCTA. ....CT.G.T ..A.TC...T
SORE1   GT..T....C ACCT.....C A.T-..TG.. .TAA.TCTG. ..T.AT.GTT ..A.TC...T
SORA2   .TG......C A.GAGT.GCA A.CTGC...T ..CTCA.T.T CATT...CTG T..C.A.G..
SORA4   .TG......C A.GAGT.GCA A.CTGC...T ..CTCA.C.T CATT...CTG T.TC.C.G..
SORA3   .TG......C A.GAGT.GCA A.CTGC...T ..CTCA.T.T CATT...CTG T..C.A.G..
SORA1   .TG......C A.GAGT.GCA A.CTGC...T ..CTCA.T.T CATT...CTG T.TC.C.G..
SORF    .........G TTC..GT.AC T..A....AA .CATCA.T.T CATG...CTT T..GTCA..A

SORD1   TCTTTGTGGG GGTCCTTATA AGCCTCTCGA TCCGCTTATC ACGCTGCAGG TCACAAGTCA
SORD2   .......... .......... .......... .......... .......... ..........
SORD3   .......... .......... .......... .......... .......... ..........
SORC    .GC....... ......CC.G G.....A.AC ..A..C.G.. ...A.....A ..TG..A..T
SORB4   ....A..... .A....GC.G G.....A.C. .....C.G.. .......... ..GACCA.AT
SORB1   ....A..... .A....GC.G G.....A.C. .....C.G.. .......... ..GACCA.AT
SORB2   ....A..... .A....GC.G G.....A.C. .....C.G.. .......... ..GACCA.AT
SORB3   ....A..... .A....GC.G G.....A.C. .....C.G.. .......... ..GACCA.AT
SORE2   .TCA------ ---------- ---------- ---------- ---------- ----------
SORE3   AACA------ ---------- ---------- ---------- ---------- ----------
SORE1   .TCA------ ---------- ---------- ---------- ---------- ----------
SORA2   ATACAACA.. A..G.CC..T TCAA.AAT.C ..A.G.ATC. T.TT..TG.A .....CA.AG
SORA4   AAACAACA.. A..G.CC..T TCAA.AAT.C ..A.G.ATC. T.TT..TG.A .....CA.AG
SORA3   ATACAACA.. A..G.CC..T TCAA.AAT.C ..A.G.ATC. T.TT..TG.A .....CA.AG
SORA1   ATACAACA.. A..G.CC..T TCAA.AAT.C ..A.G.ATC. T.TT..TG.A .....CA.AG
SORF    C.C.GA.T.T ...T..GC.G GCT..GGTC. CT.....CAA GAT......A A..AC.A.AG

SORD1   TCAACCCTTT TTGCGACAAC CCATCATTAT TCAAACTTTC CTGTGACAAC TTGTTCATCA
SORD2   .......... .......... .......... .......... .......... ..........
SORD3   .......... .......... .......... .......... .......... ..........
SORC    ---------- ---------- ---------- ---------- ---------- ----------
SORB4   .A.......C C.....T... G.C..G.... ....G..C.. ...C..G.G. G.........
SORB1   .A........ C.....T... G.C..G.... ....G..C.. ...C..G.G. G.........
SORB2   .A........ C.....T... G.C..G.... ....G..C.. ...C..G.G. G.........
SORB3   .A........ C.....T... G.C..G.... ....G..C.. ...C..G.G. G.........
SORE2   ---------- ---------- ---------- ---------- ---------- ----------
SORE3   ---------- ---------- ---------- ---------- ---------- ----------
SORE1   ---------- ---------- ---------- ---------- ---------- ----------
SORA2   A.TGA.TC.. ...TC.A... TGGG....GA .-.G....G. T..CC.AG.G GACA.TG.T.
SORA4   A.TGA.TC.. .C.TC.A... TGGG....GA .-.G....G. T..CC.AG.G GACA.TG.T.
SORA3   A.TGA.TC.. ...TC.A... TGGG....GA .-.G....G. T..CC.AG.G GACA.TG.T.
SORA1   A.TGA.TC.. ...TC.A... TGGG....GA .-.G....G. T..CC.AG.G GACA.TG.T.
SORF    .GG.TATA.A ...TA....T .........G .G.GG..CAT T.....TG.. ACACGT..A.

SORD1   ACCAAATCTA TGGTCTCTTT TTCACCGCTG TATTCTTTAT TGCCTCCATG GGGAGTGTAG
SORD2   .......... .......... .......... .......... .......... ..........
SORD3   .......... .......... .......... .......... .......... ..........
SORC    ---------- ---------- ---------- ---------- ---------- ----------
SORB4   ..A.C..G.. ...C...ACC ......AT.. .CC....C.. CT....A..T ..C..CA.C.
SORB1   ..A.C..G.. ...C...ACC ......AT.. .CC....C.. CT....A..T ..C..CA.C.
SORB2   ..A.CG.G.. ...C...ACC ......AT.. .CC....C.. CT....A..T ..C..CA.C.
SORB3   ..A.C..G.. ...C...ACC ......AT.. .CC....C.. CT....A..T ..C..CA.T.
SORE2   ---------- ---------- ---------- ---------- ---------- ----------
SORE3   ---------- ---------- ---------- ---------- ---------- ----------
SORE1   ---------- ---------- ---------- ---------- ---------- ----------
SORA2   .A..T..TGT CAA.GGG.C. ...T---T.. .G.GG....A .TTACTTG.A .CATT.C.TA
SORA4   .A..T..TGT CAA.GGG.C. ...T---T.. .G.GG....A .TTACTTG.A .CATT.C.TA
SORA3   .A..T..TGT CAA.GGG.C. ...T---T.. .G.GG....A .TTACTTG.A .CATT.C.TA
SORA1   .A..TG.TGT CAA.GGG.C. ...T---T.. .G.GG....A .TTACTTG.A .CATT.C.TA
SORF    ..A.CTA... ...GT.G..G A.A..A.... .C..TCAGGG ..TA..GT.. ATAGTG..TA
```

```
SORD1          CTCTGACATA CATTAGCATC GCCATAGTGT GTGTGAGAAG TAAGAGCAAG TCGCTAAACA
SORD2          .......... .......... .......... .......... .......... ..........
SORD3          .......... .......... .......... .......... .......... ..........
SORC           ---------- ---------- ---------- ---------- ---------- ----------
SORB4          .A.....C.. ...G.AG... ..AGCC.A.. .CT.T.TC.. .....A.... G.TA.G....
SORB1          .A.....C.. ...G.AG... ..AGCC.A.. .CT.T.TC.. .....A.... G.TA.G....
SORB2          .A.....C.. ...G.AG... ..AGCC.A.. .CT.T.TC.. .....A.... G.TG.G....
SORB3          .A.....C.. ...G.AG... ..AGCC.A.. .CT.T.TC.. .....A.... G.TA.G....
SORE2          ---------- ---------- ---------- ---------- ---------- ----------
SORE3          ---------- ---------- ---------- ---------- ---------- ----------
SORE1          ---------- ---------- ---------- ---------- ---------- ----------
SORA2          T.A.CT.... TG.C.AA..G ATTG.T.CT. .--.TG.C.T .CCC.AA..A ..AAC.C.A.
SORA4          T.A.CT.... TG.C.AA..G ATTG.T.CT. .--.TG.C.T .CCC.AA..A ..AAC.C.A.
SORA3          T.A.CT.... TG.C.AA..G ATTG.T.CT. .--CTG.C.T .CCC.AA..A ..AAC.C.A.
SORA1          T.A.CT.... TG.C.AA..G ATTG.T.CT. .--.TG.C.T .CCC.AA..A ..AAC.C.A.
SORF           TAT.T..C.. T..CCAG... CTGC.CACC. ....C.TG.A ....TCGTCT GATGCCCGG.

SORD1          GCAAAGCCCT GCAGACCTGC TCTACACACC TGGC--TGTA TACTTCATTA TGCTGATGAC
SORD2          ....G..... .......... .......... .......... .......... ..........
SORD3          ....G..... .......... .......... .......... .......... ..........
SORC           ---------- ---------- ---------- ----.----- ---------- ----------
SORB4          .T..G..... .A........ AGC.....TT ..A......G .....G..C. .......C.G
SORB1          .T..G..... .A........ AGC.....TT ..A......G .....G..C. .......C.G
SORB2          .T..G..... .A........ AGC.....TT ..A......G .....G..C. .......C.G
SORB3          .T..G..... .A........ AGC.....TT ..A....... .....G..C. .......C.G
SORE2          ---------- ---------- ---------- ----.----- ---------- ----------
SORE3          ---------- ---------- ---------- ----.----- ---------- ----------
SORE1          ---------- ---------- ---------- ----.----- ---------- ----------
SORA2          TTT.T.T.-. A.TTGTT... CTC.TTT.AT A.C.CT...C A....T.... .TT.A.CA.T
SORA4          TTT.T.T.-. A.TTGTT... CTC.TTT.AT A.C.CT...C A....T.... .TT.A.CA.T
SORA3          TTT.T.T.-. A.TTGTT... CTC.TTT.AT A.C.CT...C A....T.... .TT.A.CA.T
SORA1          TTT.T.T.-. A.TTGTT... CTC.TTT.AT A.C.CT...C A....T.... .TT.A.CA.T
SORF           ....G...A. T.....A..T GG......T. .T.T..A..G AT.A.AT.CT .AGA.T.C.A

SORD1          AGGTTTCGTC ATTGTCTTTC TTCATCGGTA CCCACAGTGG TCAAACCAC- --AGGACAG-
SORD2          .......... .......... .......... .......... .......... ..........
SORD3          .......... .......... .......... .......... .......... ..........
SORC           ---------- ---------- ---------- ---------- ---------. ..--------.
SORB4          T....C.A.. ..CA..A.C. .G..C..T.T T..T..T.AC ...G..T... ......AG..
SORB1          T....C.A.. ..CA..A.C. .G..C..T.T T..T..T.AC ...G..T... ......AG..
SORB2          T....C.A.. ..CA..A.C. .G..C..T.T T..T..T.AC ...G..T... ......AG..
SORB3          T....C.A.. ..CA..A.C. .G..C..T.T T..T..T.AC ...G..T... ......AG..
SORE2          ---------- ---------- ---------- ---------- ---------. ..--------.
SORE3          ---------- ---------- ---------- ---------- ---------. ..--------.
SORE1          ---------- ---------- ---------- ---------- ---------. ..--------.
SORA2          .TT.GAT.GT ..G.A..C.. .GTT.G.A.C .AT.G.TG.T C....GGTAT T.....A..C
SORA4          .TT.GAT.GT ..G.A..C.. .GTT.G.A.C .AT.G.TG.A C....GGTAT T.....A..C
SORA3          .TT.GAT.GT ..G.A..C.. .GTT.G.A.C .AT.G.TG.T C....GGTAT T.....A..C
SORA1          .TT.GAT.GT ..G.A..C.. .GTT.G.A.C .AT.G.TG.T C....GGTAT T.....A..C
SORF           C.CC.G...T .GCC.GA.AT C......A.T .GAG..AGCA G.CCCTTC.T TG....GG.C

SORD1          ---TGGCGTC CATTATGTTT CACTTGGTTC CACCTTGCC- ---------- ---------
SORD2          .......... .......... .......... .......... .......... .........
SORD3          .......... .......... .......... .......... .......... .........
SORC           ...------- ---------- ---------- ---------. .......... .........
SORB4          .......AG. .C.GC.C..C ...A.CA.C. .CAGGA.... .......... .........
SORB1          .......AG. .C.GC.C..C ...A.CA.C. .CGGGA.... .......... .........
SORB2          .......AG. .C.GC.C..C ...A.CA.C. .CGGGA.... .......... .........
SORB3          .......AG. .C.GC.C..C ...A.CA.C. .CGGGA.... .......... .........
SORE2          ...------- ---------- ---------- ---------. .......... .........
SORE3          ...------- ---------- ---------- ---------. .......... .........
SORE1          ...------- ---------- ---------- ---------. .......... .........
SORA2          TCA..T.AGT TGC...TA.. GT.A.CCC.. ..TT.A---. .......... .........
SORA4          TCA..T.AGT TGC...TA.. GT.A.CCC.. ..TT.A---. .......... .........
SORA3          TCA..T.AG. TGC...TA.. GT.A.CCC.. ..TT.A---. .......... .........
SORA1          TCA..T.AGT TGC...TA.. GT.A.CCC.. ..TT.A---. .......... .........
SORF           TTT...A..A TCAGT.A.GG TGT.CCC.C. ..TG..CAAC CCCTTCATCT ATCTAGAGC
```

Figure 2.3.4a *Alignment of nucleotide sequences of OR-like sequences isolated from* S. salar.
Alignment created using Clustal X (Thompson *et al.,* 1997) and PHYLIP 3.57c (Felsentsein, 1995). The symbol "–" indicates a gap, while "." represents identical residues.

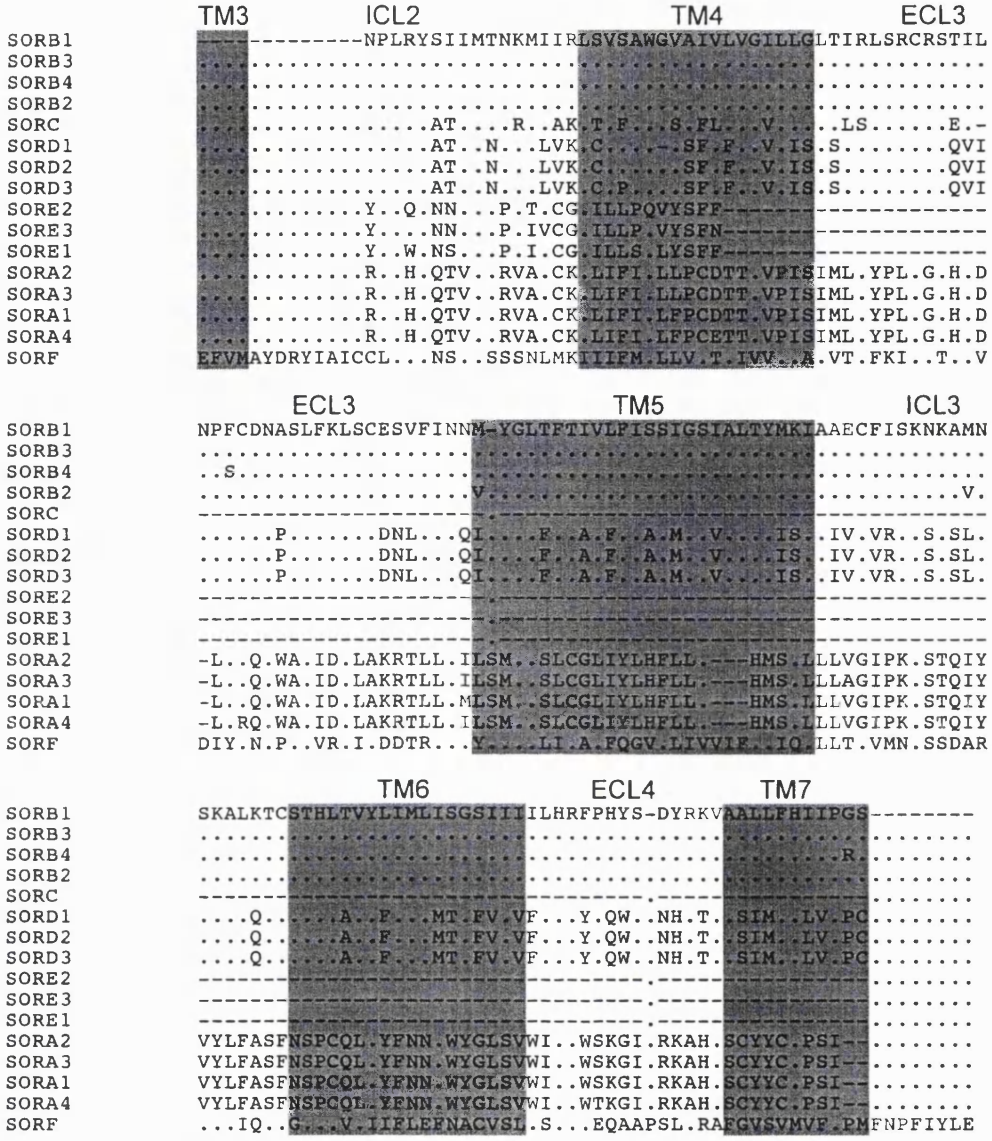

Figure 2.3.4b *Alignment of amino acid sequences of OR-like sequences isolated from* S. salar.
Alignment created using Clustal X (Thompson *et al.*, 1997) and PHYLIP 3.57c (Felsentsein, 1995). Possible transmembrane domains are highlighted in grey. The symbol "–" indicates a gap, while "." represents identical residues.

3 CHARACTERISATION OF PHEROMONE RECEPTOR-LIKE SEQUENCES

3.1 INTRODUCTION

The mammalian olfactory system comprises two distinct organs: the main olfactory epithelium (MOE) and the vomeronasal or Jacobson's organ (VNO), the principle function of the VNO being pheromone reception. While olfactory sensory neurons (OSNs) are ciliated in structure, vomeronasal sensory neurons (VSNs) are microvillar.

In mammals, the VNO comprises a tubular structure in the nasal cavity, enclosed within bilateral bony capsules of the ventral septum (Jacobson, 1811). Axonal projections from the VNO conjoin to form the vomeronasal nerve and terminate at target cells within the accessory olfactory bulb. Halpern's report (1978) of neuronal tracing experiments revealed that the VNO is exclusively connected to specialised centres of the limbic system, including specific nuclei of the ventromedial hypothalamus involved in reproduction and aggression. In this way, pheromone perception, unlike the main olfactory system, elicits stereotyped responses that do not involve higher cognitive centres of the brain. Such stereotyped behaviour has called into question whether higher primates, including humans, may possess a functional VNO, or whether pheromonal communication is limited to "lower" vertebrates. The presence/absence of the human VNO has been the subject of intense discussion in recent years (see the reviews by Keverne, 1999; Meredith, 2001).

Chemical cues broadly defined as pheromones are present in urine, sweat and other bodily secretions and are involved in chemical communication among animals (reviewed by Halpern, 1987; Wysocki, 1989). Pheromones convey information concerning the species, gender, reproductive state and identity of the animal and trigger behavioural and endocrine responses often sustaining reproduction and hierarchical order in an animal group. These responses are primarily mediated by the VNO, with a contribution from the main olfactory system.

Fish, crocodiles and chameleons only possess a single olfactory organ. In fish, two types of sensory cell generally exist, ciliated and microvillar, implying that the single organ is used for both pheromone and olfactory reception.

The only vertebrate in which production, release, sensory detection and biological function of a pheromone has been thoroughly characterised is the goldfish, *Carassius auratus.* Similarly, there are several insects (*Manduca sexta, Heliothis spp.*) from which the chemical composition of the pheromonal blends, the ethology of both intra- and interspecific chemical communication, and the neural circuits that

enable the reception, preliminary processing and discrimination of the chemical cue is known. Indeed, many parallels between the discrimination of pheromonal cues in fish and insects are now being drawn (see review by Sorensen *et al.,* 1998).

Candidates for both sex and migratory pheromones are now being isolated in fish (Moore and Scott, 1992; Li and Sorensen, 1997; Sorensen and Goetz, 1993; Shoji *et al.,* 2000). The former appear to derive from hormonal products, the latter from bile acids, at least in lamprey. In salmonids, it seems, amino acids are now the more likely candidates as home-stream odorants (Shoji *et al.,* 2000). However, amino acids have also been identified in eliciting feeding behaviour in juvenile salmonids (Valentincic *et al.,* 1999). Whether amino acids are detected by ciliated and microvillous chemosensory neurons in salmonids has yet to be established.

3.1.1 Pheromone Receptors

Differential expression studies using cDNA libraries generated from single VNO neurons enabled the isolation of a new receptor gene family (VNR or V1R; Dulac and Axel, 1995). Subsequent sequence analysis suggested that they are likely to adopt a structure similar to that of the seven transmembrane receptors, but VNO receptors do not share any of the conserved sequence motifs exhibited by members of the previously identified superfamily. Two years later, a second family of receptors (V2R) was identified (Herrada and Dulac, 1997; Ryba and Tirindelli, 1997; Matsunami and Buck, 1997) and are structurally related to the calcium sensing receptor (CaSR; Hebert and Brown, 1995) and metabotropic glutamate receptor (mGluR; Tanabet *et al.*, 1992) families. A functional calcium sensing receptor has since been characterised in *Fugu*, (Naito *et al.,* 1998). Six classes of *Fugu* VNR were also identified (I –VI) and it was demonstrated that the gene structure of the type II *Fugu* VNRs is identical to that of the Ca^{2+}-sensing receptor. Type III and IV have an extra intron in the extracellular domain; V and VI also have extra intons in the TM domain as well as other differences in the extracellular domain. The common intron in the TM domain of types V and VI are also found in two *Fugu* mGluR genes. The authors also found that these VNRs, like the ORs in other genomes, are clustered in tandem arrays and that some of these linked genes show high amino acid similarities suggesting a recent duplication within the genome. Not only do V2R genes contain introns (unlike V1Rs and ORs) but they also have an extremely long N-terminal extracellular domain. In V2Rs, this extended domain may interact with the ligand, whereas in ORs and V1Rs, ligand binding may occur in a pocket formed by the transmembrane domains (Matsunami and Buck, 1997). Strong evidence therefore exists for the independent evolution of two extremely divergent chemosensory systems.

Matsunami and Buck (1997) observed that about two-thirds of the V2R cDNAs only encode partial proteins, with missing sequences or stop codons that disrupt their coding regions. Of the 100 or so V2R genes detected by hybridisation, perhaps only 35 are functional, the rest being processed pseudogenes. However, the authors could not exclude the possibility that some variant V2Rs are functional; they proposed that some truncated V2Rs that lack transmembrane domains could conceivably be secreted pheromone-binding proteins.

The complexity of the pheromone detection system was elevated further by the discovery of a third group of VNRs. The mouse genome is thought to encode over 100 V3Rs, a group of genes that do not share the amino acid signatures of the V1Rs, nor the extended N-terminal extracellular loop of the V2Rs. They are thought to be distant relatives of the V1Rs and the gustatory (taste) receptors, the T2Rs

(Pantages and Dulac, 2000). Again, this large group of closely related genes are expressed by a distinct subset of vomeronasal neurons, and combined with the multiple signature sequences exclusive to the V3Rs, and the disparate comparisons with other putative pheromone receptors, a sharp contrast between the intricacy of the pheromone detection system and the relative simplicity of the main olfactory system can be seen.

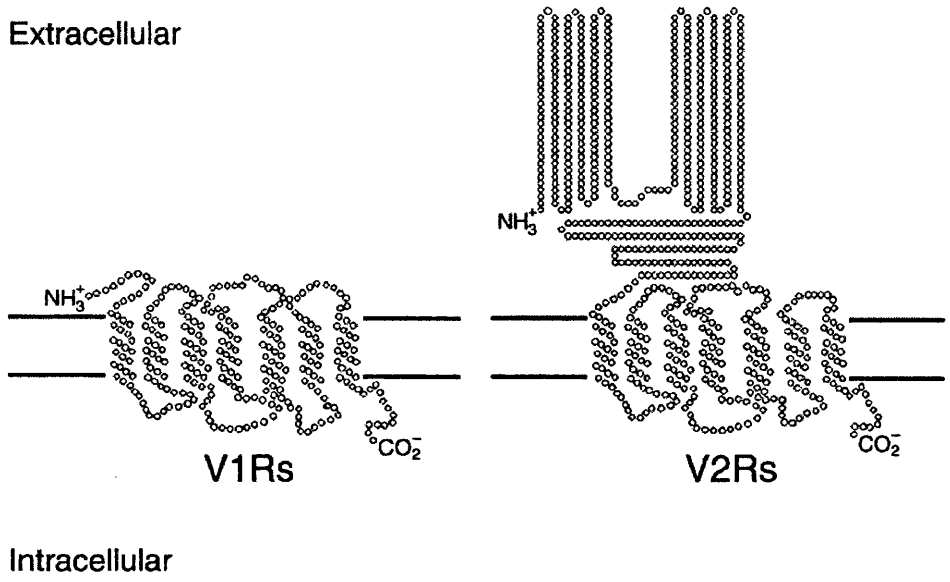

Figure 3.1.1 *Proposed topology of vomeronasal organ receptors.* (Taken from Tirindelli, *et al*, 1998)
V1Rs and V2Rs share the same putative seven transmembrane structure of other G-protein coupled receptors, however, V1Rs share no other structural feature related to other transmembrane receptors. V2Rs possess a large extracellular N-terminal domain similar to those of Ca^{2+} sensing receptors and metabotropic glutamate receptors, which is thought to be associated with ligand binding

3.1.2 Sexual dimorphism

In addition to the molecular isolation of a novel family of putative pheromone receptors, Herrada and Dulac (1997) also showed a sexually dimorphic pattern of expression of the gene G_o-*VN2*, using *in-situ* hybridisation. In female rats, G_o-*VN2* appeared to be expressed in a large and centrally located region comprising about one third of the neuroepithelium on the VNO, whereas in males, expression of the same gene was confined to a small population of cells in the most apical side of the neuroepithelium. The emergence of receptor expression was also followed throughout postnatal development, preliminary data indicating that sexual dimorphic expression is undetectable at 6 weeks after birth in the rat and emerges only in late postnatal development, reaching its definitive pattern at sexual maturity. Such dimorphism has yet to be reported in fish.

3.1.3 Signal transduction

Dulac and Axel (1995) first elucidated the divergent nature of the vomeronsal and main olfactory transduction pathways. Assuming that the MOE and VNO shared a common evolutionary origin, the authors discovered that low stringency hybridisations of MOE receptor probes to rat VNO cDNA libraries, PCR using conserved motifs from both the OR gene family and the superfamily of known seven transmembrane domain receptors were consistently unsuccessful. The components of the main olfactory signal transduction cascade: olfactory-specific G protein, G_{olf}, (Jones and Reed, 1989); the olfactory-specific adenylate cyclase (Bakalyar and Reed, 1990); and one subunit of the cyclic nucleotide-responsive ion channel, (Dhallan *et al.*, 1990) were also not detectable in VNO neurons by *in situ* hybridisation. Liman and Corey (1996), also showed that VNO neurons show no response to cyclic nucleotides.

Two G-protein α subunits, $G_{\alpha o}$ and $G_{\alpha i2}$, cosegregate with the V1R and V2R receptor families in nonoverlapping subpopulations of VNO neurons and have been suggested to mediate pheromone receptor transduction (Halpern *et al* 1995; Berghard and Buck, 1996). Since phototransduction in *Drosophila spp.* and chemosensation in *C. elegans* involve members of a transient receptor potential (TRP) family of ion channels in a cyclic-nucleotide-independent G-protein mediated pathway, Liman *et al.* (1999), reasoned that a TRP homologue might be involved in vertebrate pheromone transduction. Indeed, a specific TRP ion channel, rTRP2, was identified and found to be exclusively expressed in VNO neurons and specifically localised to the VNO sensory microvilli - the proposed site of pheromone transduction. In *Drosophila* phototransduction, a G-alpha protein of the G_q class is activated, triggering a phosphotidyl inositol ($InsP_3$) cascade that eventually leads to the opening of the cation-selective channels dTRP and dTRPL (Scott and Zuker, 1998).

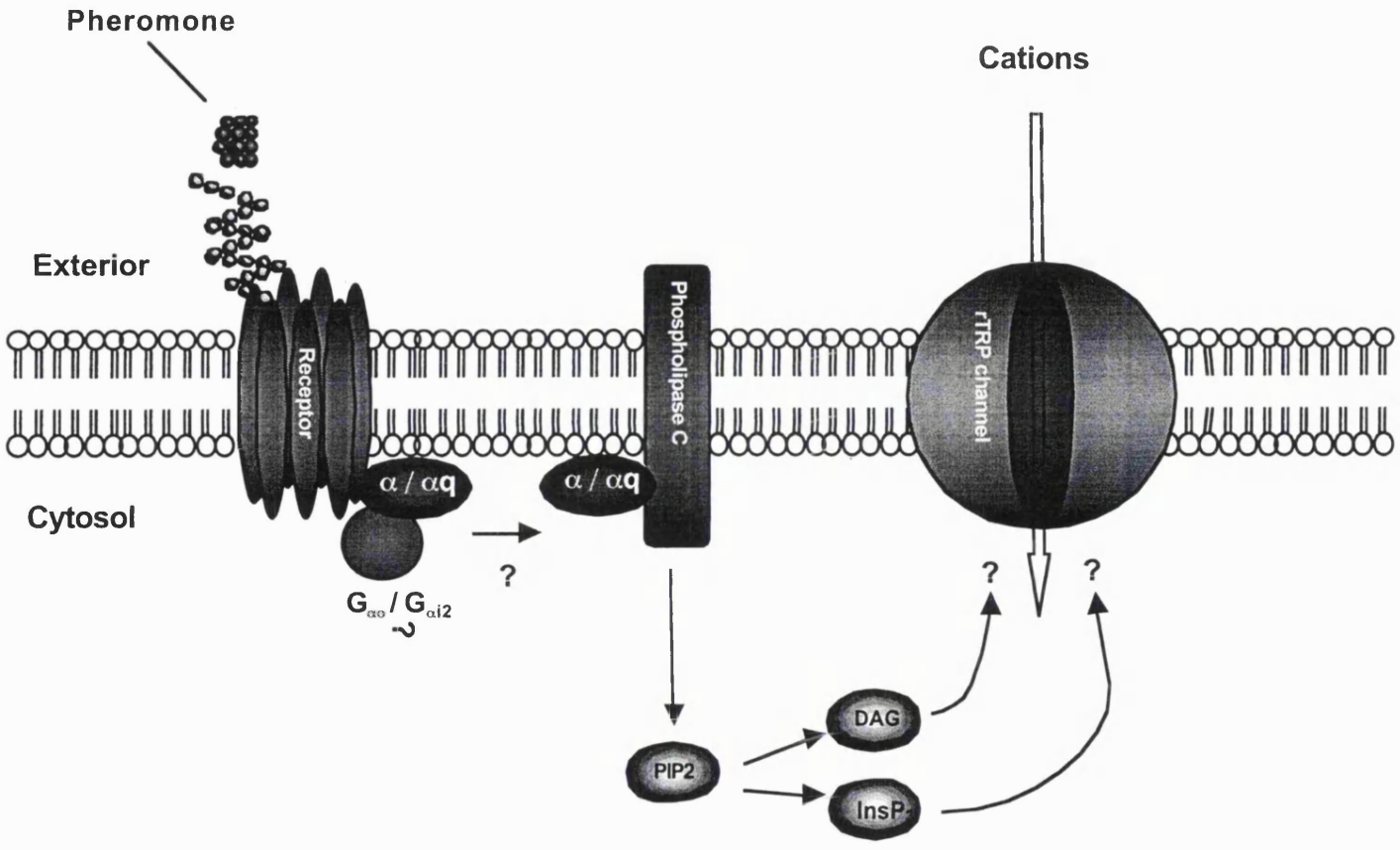

Figure 3.1.3 *Proposed Schematic model of Pheromone-induced signalling in the microvilli of VNO sensory neurons.*
The activation of Gao/Gai2by pheromone receptors and the subsequent release of bg or the activation of G would stimulate a phospholipase C, leading to the hydrolysis of PIP2 to InsP3 and DAG, which in turn, through an as yet unclear mechanism, would lead to the opening of the TRP2 channel and an alteration in membrane potential.
After Liman *et al* (1999).

3.2 AIMS

The *Imprinting Hypothesis* (Hasler and Wisby, 1951), proposes that natal river systems possess characteristic odours to which juvenile salmon become conditioned during development in fresh water. These 'imprinted' odours are then used as orientation cues when the fish return as adult migrants.

However, it has also been proposed that migrating salmon home using odorants emanating from their conspecifics (Døving *et al.*, 1973), a suggestion that became known as the *Pheromone Hypothesis* (Stabell, 1984). The physiological experiments of Døving *et al.* (1973) showed that fish produce substances that are potent stimuli to the olfactory organ and that there exists a neuronal basis for discrimination of different strains of fishes (Døving *et al.*, 1974). The same authors also described the olfactory sensitivity to bile acids in salmonids (1980). The detergent properties of bile acids are used to aid lipid digestion, but some of them are readily degraded and would only have a short term effect in an aquatic environment. "..Others are more stable and are absorbed by organic matter and minerals in the water. This could give the fishes a possibility for recognizing their own territory." (Døving *et al.*, 1980).

Olfaction has been shown to be a vital mechanism for the navigation of river systems during the spawning run in adult salmon, yet confusion remains concerning the nature of the odorants involved. As previously described (Chapter 1), there is strong evidence for a period of olfactory imprinting during parr-smolt transformation (PST), whether or not this process involves abiotic and/or biotic factors, remains to be seen. A rise in the level of OR gene expression at this sensitive stage would seem consistent with the imprinting of either odour-type, (environmental odours or excreted odours, such as bile acids - see Chapter 1). Alternatively, a change in the level of VNR gene expression would be consistent with the hypothesis that the young salmon are imprinting on pheromones produced by their cohorts/conspecifics, i.e. population-specific odours.

By isolating and sequencing pheromone receptor genes using the same approach as described for the OR genes (Chapter 2), the potential exists for the simultaneous quantitation of VNR and OR expression during smolting

The object of this part of the study was to isolate VNR-like sequences from the Atlantic salmon (*Salmo salar*) and to establish patterns of their expression throughout a range of tissues in order to infer their possible function. It was also intended to classify salmon VNR-like sequences, with respect to the three classes of VNR genes isolated from mammals and the sequences available from teleost species.

3.3 MATERIALS and METHODS

3.3.1 PCR amplification, cloning and sequencing

Published degenerate primers (F1, F2, R1 and R2) designed from an alignment of human (X81086), rat (U20289), and bovine (S67307) Ca^{2+} sensing receptors (Naito, *et al.*, 1998) were used to amplify PCR fragments from both genomic DNA and cDNA isolated from the olfactory epithelium of a single adult salmon caught off the coast of Scotland.

PCR was optimised in 25μl reaction volumes containing 0.2-0.4μg DNA in standard buffer conditions [12.5pM each primer, 1.8mM each dNTP, 1.5mM $MgCl_2$, 50mM KCl, 20mM Tris-HCl (pH 8.4) and 0.5 units *Taq* polymerase]. Forty cycles of 95°C for 30 seconds, 57°C for 55 seconds and 72°C for 45 seconds was successful in generating sufficient product (as visualised on an Ethidium Bromide-stained agarose gel) to use as template for a second round of PCR. Products obtained from both genomic DNA and 1st strand DNA synthesised from testes, brain, olfactory epithelium, liver and anterior kidney (primer pair F1 and R1) and from olfactory epithelium (primer pair F2 and R2), underwent a second round of PCR.

Second round PCR products were excised from the agarose gel (Fig 4.3a), purified using QIAquick Gel Extraction Kit (QIAGEN), then ligated, transformed and cloned using the Original TA cloning kit® (Invitrogen®). All sequencing was performed using ABI PRISM®, Big Dye™ Terminator Cycle Sequencing.

3.3.2 Examination of tissue expression patterns

RNA Isolation and cDNA Synthesis

A range of tissues (olfactory epithelium, brain, anterior kidney, liver, and skeletal muscle) were obtained from an adult Atlantic salmon caught off the Scottish coast, and total RNA extracted from approximately 100 mg of each tissue using TRIzol™ reagent (GibcoBRL). Residual DNA was removed by treatment with DNase I (Pharmacia Biotech), and cDNA produced using an oligo dT primer (1st Strand cDNA Synthesis Kit; Pharmacia Biotech).

PCR screening of 1st Strand cDNA

Primers for amplification of Atlantic salmon β-actin sequences were used to check for presence of cDNA and absence of genomic DNA contamination. The primers were designed to span the first intron of the β-actin locus; amplification products from cDNA and genomic DNA are therefore easily distinguishable on the basis of size. Tissue expression patterns of *SVR* sequences were then established

using PCR primers specific to individual families of salmon vomeronasal receptor (*SVR)* sequence (see Table 3.2.2)

Target gene	Primer set (5'→3')	Amplicon length
β Actin	Forward: ATGGAAGATGAAATCGCCGC Reverse: TGCCAGATCTTCTCCATGTCG	~ 240 bp (cDNA) ~ 560 bp (gDNA)
SVRA	Forward: ATGGCCTTCAGGGCTACGCT Reverse: AGGCAGCTTCCGAGCCAGAA	285 bp
SVRB	Forward: ATAGCTTTCCAGGCCACAAT Reverse: AGGCAGCTTCCGAGCCAGAA	282 bp
SVRC	Forward: GCTGTGTTTAGGACCTCTAA Reverse: TGGAAGATTCCTGGCCAGGA	288 bp
SVRD	Forward: CTTCTAGTGTTCGAAGCCAA Reverse: TGGCAGTTTTCGTGATTTAA	285 bp

Table 3.2.2 *Primer sequences used to determine tissue specific expression of vomeronasal receptor families*

Once the β Actin control had been established, the VNR-like sequences were PCR amplified under standard buffer conditions using a thermocycling profile of 10 cycles of 94°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by 25 cycles of 94°C for 20 seconds, 59°C for 30 seconds and 72°C for 30 seconds.

To confirm the identity of the PCR products, they were Southern blotted from agarose gels onto nylon membrane, and probed by hybridisation to cloned representatives of each of the family of sequences using standard techniques (Sambrook *et al.*, 1989).

3.3.3 Phylogenetic Analysis

Sequences of VNR, Ca^{2+}-sensing-like and putative pheromone receptor genes from both teleosts and mammals were obtained from GenBank and Swiss-Prot. Alignments of deduced amino acid sequences were made using Clustal X (Thompson *et al.*, 1997) prior to phylogenetic analysis using PAUP* (Swofford, 1998) and PHYLIPv3.57c (Felsenstein, 1995). A relationship between these *VNR*-like sequences and main olfactory receptor genes was estimated by neighbour-joining (Saitou and Nei, 1987) using mean character difference as a genetic distance measure. Estimates of the robustness of the nodes of the tree were obtained by bootstrapping (1000 replicates) (Felsenstein, 1985).

3.4 RESULTS

3.4.1 Amplification, cloning and sequencing

PCR products of between 300 bp and 400 bp were obtained from the cDNA of all tissues for the first primer pair (*naito1*), but amplification products of *naito2* primers were only obtained from olfactory epithelium cDNA and genomic DNA (~ 400 bp each).

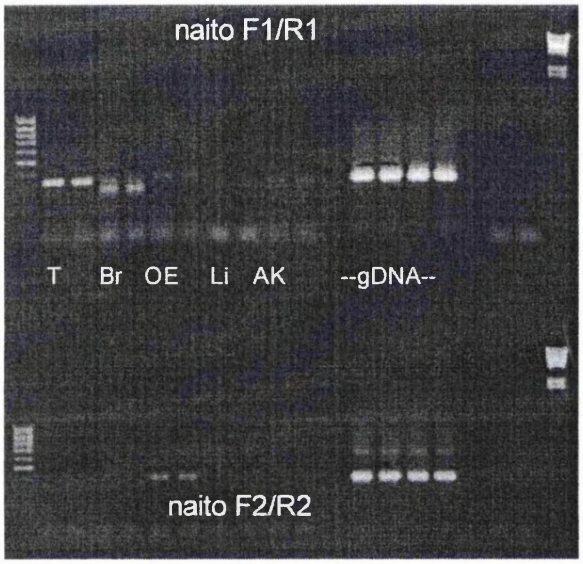

Fig 3.4.1 *Agarose gel photograph depicting PCR products used for ligation & cloning*

T = Testis
Br = Brain
OE = Olfactory Epithelium
Li = Liver
AK = Anterior Kidney
gDNA = Genomic DNA

After plasmid cloning of the PCR products, restriction analysis (EcoR 1) showed that of the 80 colonies picked, 50 contained inserts. Sequencing and a subsequent BLAST search of these ~371bp and 400bp inserts revealed that 16 clones shared amino acid motifs with either putative pheromone receptors or extracellular calcium ion-sensing receptors isolated from *Carassius auratus* and *Fugu rubripes* (goldfish and puffer fish). These 16 clones were derived from PCR products generated from the cDNA of olfactory epithelium and from genomic DNA.

The sixteen clones were translated and aligned with other amino acid sequences identified as showing the greatest similarity through BLAST. The sequences included in the alignment consist of Ca^{2+}R-like receptors and putative pheromone receptors from *C. auratus* and *F. rubripes*, *Rattus sp.*, and *Mus sp.*

These 16 putative VNR sequences can be seen as belonging to 4 distinct groups (denoted *SVRA-D*), with between one and six sequences per group (see nucleotide alignment, Figure 3.4b), *SVRA* sequences are either 353 or 354 bp long (except *SVRA6*, which is truncated at the 5' end, giving 126 bp), *SVRB* was 353 bp long, SVRC are 357 bp long and *SVRD* are 321 bp long. All 16 sequences represent a portion of the open reading frame encoding from the third transmembrane (TM3)

domain to the sixth transmembrane domain (TM6, see Figure 3.4a). *SVRA*-C seem to show more homology with pheromone receptor-like sequences in GenBank, while SVRD seems more similar to extracellular calcium receptors

Within families A and D, certain members have obvious mutations, sometimes resulting in frameshifts, which may indicate the presence of pseudogenes

SVRA1 has a deletion at base 180, and *SVRA4* has a deletion at base 10, both resulting in nonsense mutations. Both have therefore been omitted from further analyses of translated sequences.

SVRD3 and *4* both contain in-frame stop codons at the same position in the sequence: at nucleotides 59-61 - part of the region that would encode the second intracellular loop, and have therefore also been omitted from subsequent analysis of translations.

Both the *SVRA* and the *SVRD* family are however, also represented by members with intact reading frames. *SVRB* was identified from a single sequence from a single clone, and bears more similarity to *SVRA* than the other families identified.

3.4.2 Patterns of Expression

Robust PCR products of approximately 240 bp and 560 bp were amplified from first strand cDNA (derived from 5 different tissues) and genomic DNA respectively, using salmon-specific β actin primers. A difference in size of ~300 bp is easily resolved on an agarose gel, and as the primers span an intron, any contamination of the cDNA with genomic DNA would have been detected.

Once this control was established the SVR-specific primers were used in PCR amplifications using first strand cDNA as template.

SVRA primers gave a strong product from olfactory epithelium cDNA and the genomic control, as did the *SVRB* and *SVRC* primers. Primers specific to the *SVRD* family, however, also amplified a robust product from testis cDNA, as well as olfactory epithelium and genomic DNA.

In order to ensure that these RT-PCR bands were products of the appropriate gene family, and not simply spurious amplifications of non-specific sequences, the bands were southern blotted onto nylon membrane and hybridised with appropriate probes (Fig. 3.3.2). Southern hybridisation confirmed the initial RT-PCR results, that *SVR* products were amplified from olfactory epithelium cDNA, and genomic controls by all *SVRA*, *B*, and *D* primers, and that the *SVRD* primer pair also generated a product from testis cDNA.

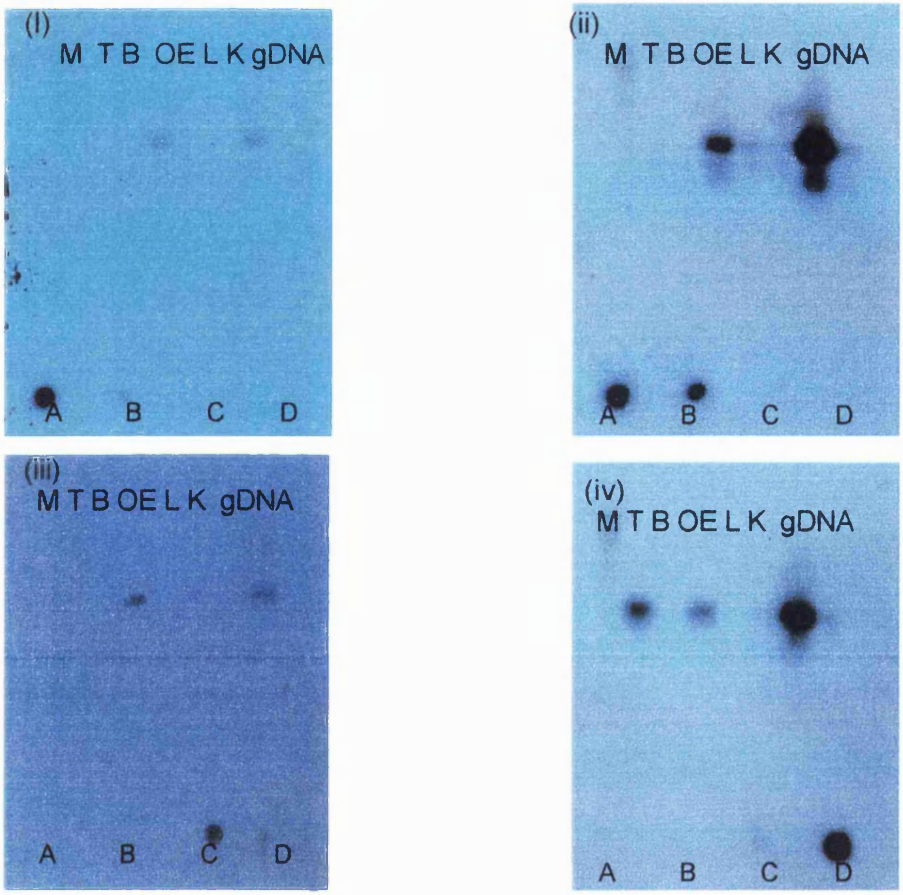

Figure 3.3.2 *Southern Blots of RT-PCR products amplified from 5 different tissues, then hybridised with SVR probes.*
(i) = *SVRA*, (ii) = *SVRB*, (iii) = *SVRC*, (iv) = *SVRD*
M = Molecular weight marker, *T* = testis, *B* = brain, *OE* = olfactory epithelium, *L* = liver, *K* = anterior kidney, *gDNA* = genomic DNA from same individual.
A, B, C and *D* = dot blotted unlabelled SVRA-D clones used as positive controls.

3.4.3 Phylogenetic Analysis

As previously mentioned (section 3.4.1), those sequences containing nonsense mutations and stop codons have not been used in the analysis of protein sequences.

Translated protein sequences of all potentially functional *SVR*s were included in the construction of a Neighbour Joining tree along with a subset of putative pheromone receptors representing all three groups (V1R, V2R and V3R) isolated from both mammals and teleosts. (Cao *et al.*, 1998; Naito *et al.*, 1998; Speca *et al*, 1999; Herrada and Dulac, 1997; Dulac and Axel, 1995; Pantages and Dulac, 2000 - see Fig 3.3.3b). The three groups of VNRs have separated in the phylogeny to form distinct clusters, with all fish vomeronasal receptors grouping with Type II.

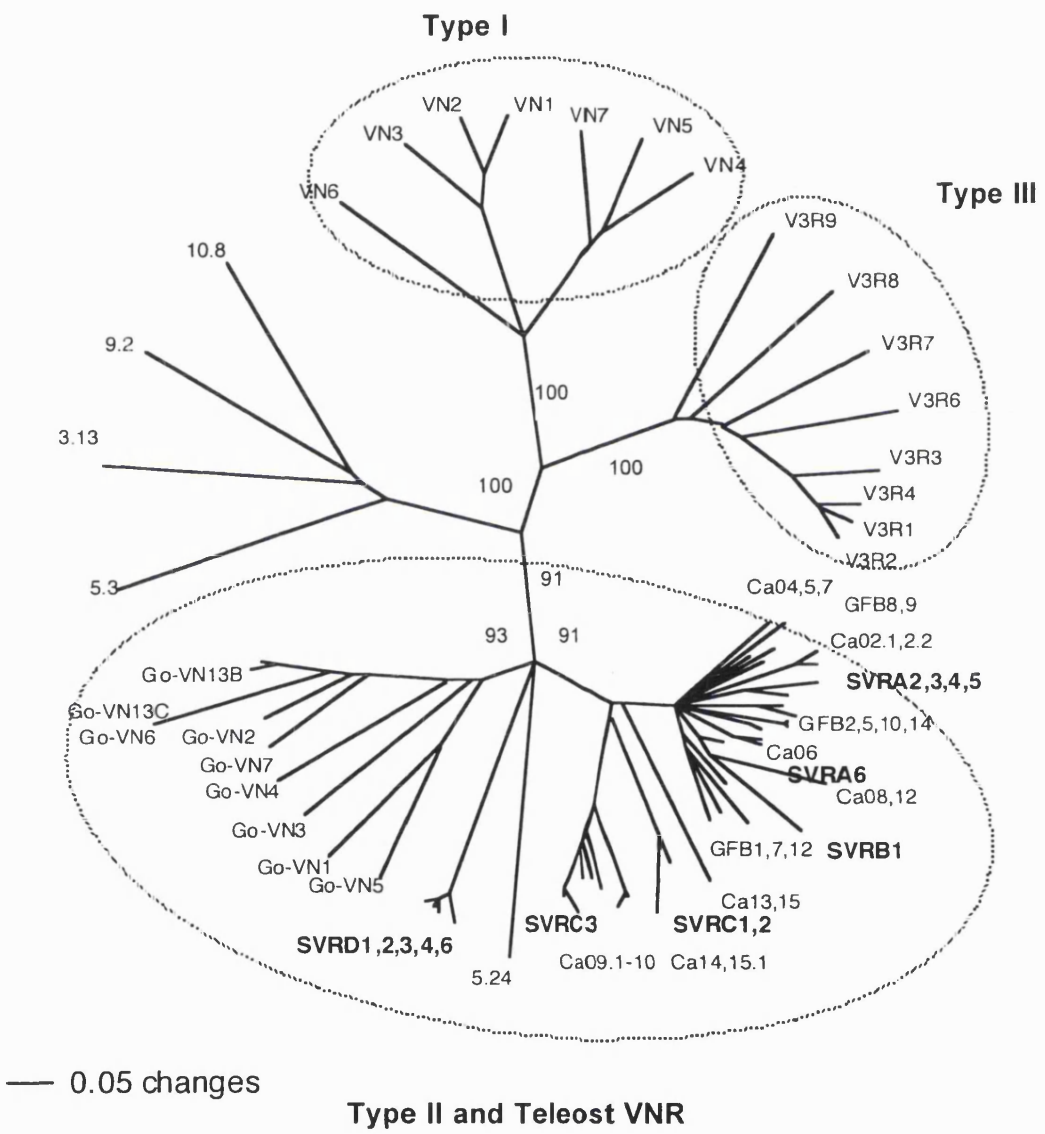

Figure 3.4.3a *Neighbour-Joining tree of protein sequences of the three groups of functional VNR receptor genes (Bootstrapped 1000 replicates)*
SVR = salmon sequences; *GFB* = Goldfish, (Cao *et al.*, 1998); *Ca* = Medaka fish (Naito *et al.*, 1998); *3.13-10.9* = Goldfish (Speca *et al*, 1999), *Go-* = Type II Rat (Herrada and Dulac, 1997); VN = Type I, Rat (Dulac and Axel, 1995), *V3R* = mouse (Pantages and Dulac, 2000)

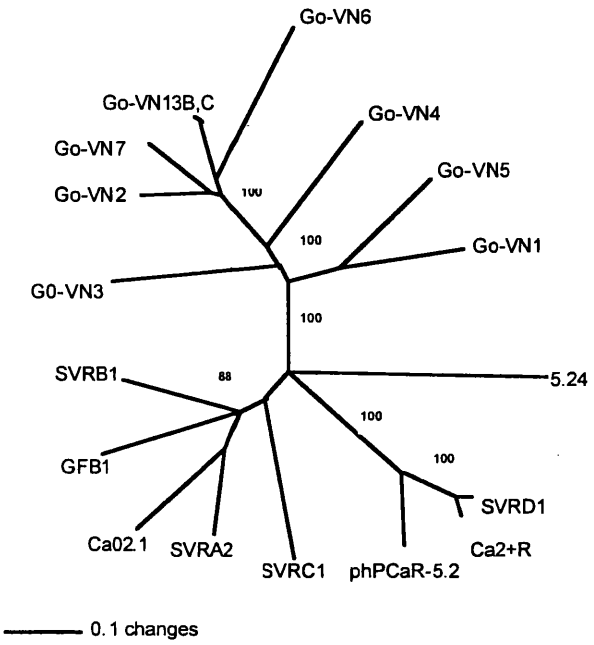

Figure 3.4.3b *Unrooted Neighbour-Joining tree of representative teleost VNR and type II terrestrial VNR amino acid sequences (Boostrapped x1000).* *SVR* = salmon sequences; *GFB* = Goldfish, (Cao *et al*, 1998); *Ca* = Medaka fish (Naito *et al*, 1998); *3.13-10.9* = Goldfish (Speca *et al*, 1999), *Go-* = Type II Rat (Herrada & Dulac, 1997); *pHPCaR-5.2* = human calcium receptor (Garrett *et al*, 1995)

3.5 DISCUSSION

Sixteen different putative pheromone receptor sequences have been isolated from the genomic and olfactory cDNA from a single Atlantic salmon. These sequences have been classified into four families (designated SVRA to D), represented by between one and six sequences. Two of these familes (*SVRA* and *D*) contain potential pseudogenes, indicated by the presence of nonsense mutations and stop codons within the reading frames.

SVRA6, a sequence amplified from olfactory epithelium cDNA, is truncated, resembling the 3' end of other members of the A family, however, it only differs by a single nucleotide creating a single amino acid change. As this was represented by two clones this nucleotide change is not likely to result from a PCR or sequencing artefact. It is possible, however, that the truncation of this PCR product is an artefact of reverse transcription or due to degradation of mRNA prior to reverse transcription.

RT-PCR and subsequent hybridisation have shown that members of *SVRA*, *B*, *C* and *D* families are expressed in olfactory epithelium, but members of *SVRD* are also expressed in testis. The V1R type pheromone receptors have recently been found to be expressed in the developing germ cells in mouse testis (Tatsura *et al.*, 2001). Testicular pheromone receptors (TVRs) were localised in subsets of the

seminifierous tubules, suggesting that they are being expressed by selective subsets of spermatids. In addition, the authors found that each sperm expresses multiple pheromone receptors. It is suggested that they, like testicular olfactory receptors, may occupy a role in the migration and/or maturation of sperm. These salmon sequences however, seem to belong to the type II VNRs (Figure 3.3.3a) and are also closely related to the goldfish receptor 5.24 (Speca *et al.,* 1999). This may be the first example of type II pheromone receptors and/or teleost pheromone receptors being expressed in male germ cells. From the phylogenetic analysis (Figs 3.3.3a and b), it can be seen that *SVRD* sequences fall out with CaSRs isolated from both *Fugu* and human. Calcium-sensing receptors are involved in regulating systemic Ca^{2+} and as such are expressed by calcium-regulating homeostatic organs such as kidney. Non-homeostatic tissues that require changes in local Ca^{2+} in order to modulate cell function, such as neuronal tissues, are also expected to express CaSRs (Herbert and Brown, 1995), but *SVRD* expression was not found in either brain or anterior kidney in Atlantic salmon. This family of genes shows an amino acid similarity of >86% with the CaSR isolated from *Fugu*, a sequence that shows greatest expression in the brain and kidney in *Fugu* (Naito *et al.,* 1998). These data indicate a connection between the evolutionary pathways of VNRs, and both systemic and neuronal CaSRs, but the nature of this relationship is yet to be resolved.

Among the main olfactory receptors (ORs), there is a ubiquitous amino acid motif Acidic-Arginine-Aromatic which is located a few amino acids downstream of TM3, and is thought to be necessary for G-protein coupling. This feature does not appear to be shared by the CaSR-like VNRs (see Fig 3.4a and the alignment of Speca *et al.,* 1999), which may be a reflection of the differences in signal transduction mechanisms.

The three types of terrestrial VNRs have clustered neatly into 3 strongly supported clades (bootstrap values >90) within the "phylogeny"; with all teleost sequences falling out with the Type II clade (see Fig. 3.3.3a). Within this VNR II clade, the sequences belonging to fish form a distinct lineage from those of Type II rat genes (Herrada and Dulac, 1997), there is no interspersion of mouse or rat genes with teleost sequences, which may reflect the difference between pheromonal ligands in aquatic and terrestrial environments. It is emerging that pheromonal detection and perception in fish are very different from that of terrestrial vertebrates and actually show more similarity to arthropods (Sorensen *et al.,* 1998). Fish have no discrete vomeronasal organ – vomeronasal neurons are localised near the apical surface of the olfactory epithelium and teleost VNRs show greater sequence similarity to CaSRs than V2Rs.

The isolation of 16 independent sequences from the cDNA and genomic DNA of a single salmon is an indication of the potential abundance and diversity of vomeronasal receptors in this species. The complex life history of the Atlantic salmon hinges upon the fish's ability to recognise odours of the natal stream and to perceive pheromones that trigger certain behavioural and physiological changes prior to spawning (Stabell, 1984). Whether the period of olfactory imprinting during parr-smolt transformation (Nevitt *et al.*, 1994) involves the formation of memory to population-specific odours rather than that of the stream itself, and whether these odours may constitute pheromones, is the subject of broad discussion (Hasler and Wisby, 1951; Døving *et al.*, 1974; 1980; Moore and Scott, 1991; Moore *et al.*, 1994; Stabell, 1984).

The identification of four families of functional putative pheromone receptors in Atlantic salmon, provides a molecular basis for the continued investigation of olfactory imprinting during PST, and potentially for the basis of kin-recognition in this species.

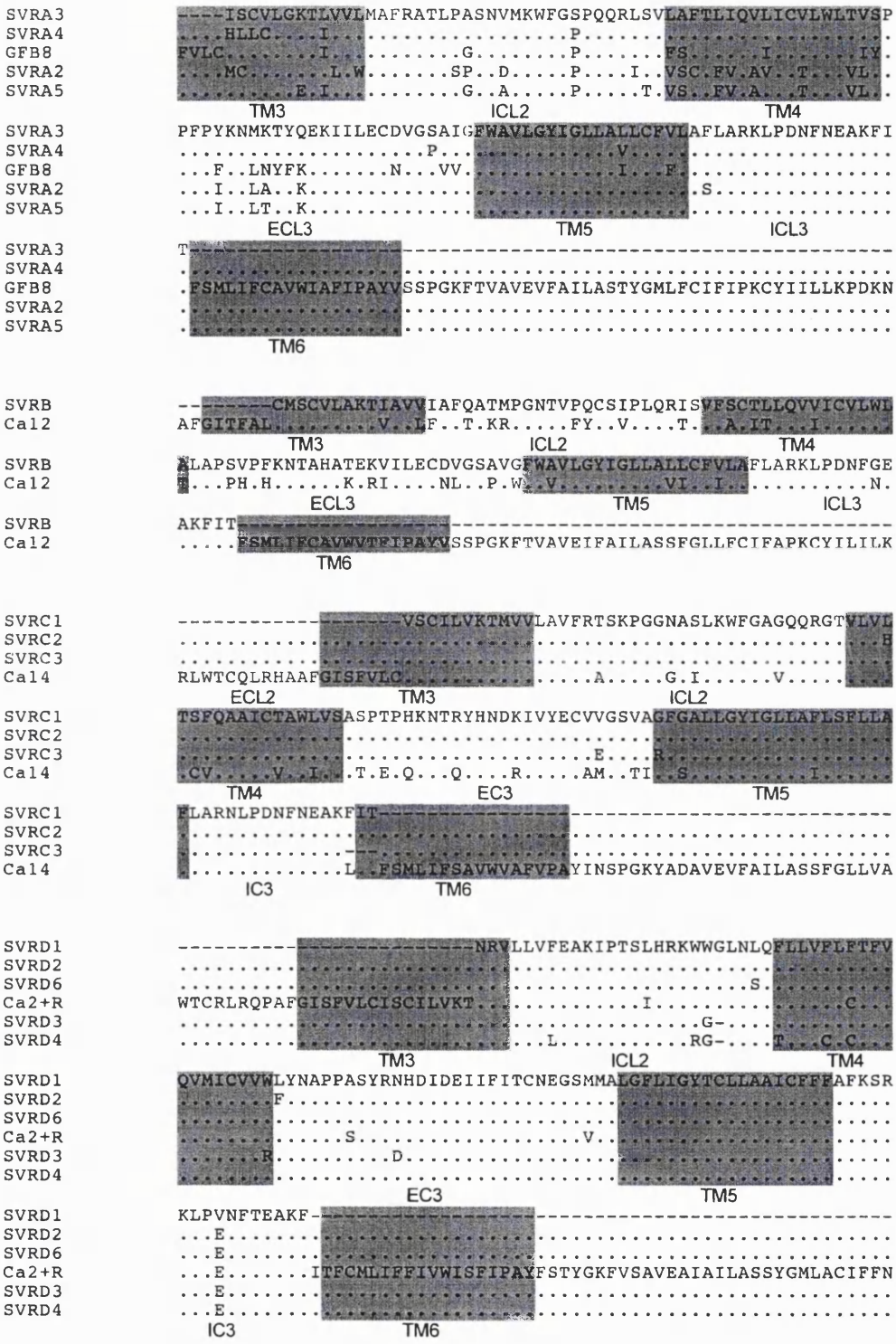

Figure 3.4a *Alignment of amino acid sequences with that showing the greatest similarity by BLAST search*
GFB8 = Putative goldfish VNR : AF083081 (Cao *et al.*, 1998); Ca12 and Ca14 = Putative *Fugu* VNR: AB008860, AB009043; Ca2+R = Putative calcium-sensing receptor from *Fugu* (Naito *et al.*, 1998). Areas highlighted in grey represent possible transmembrane domains. The symbol "–" indicates a gap, while "." represents identical residues.

```
SVRA1   --CATCCCTT GTGTTCTGGG GAAAACAATA GAGGAGTTGA TGGCCTTCAG GGCTACACTT
SVRA5   ......T... .......... .G........ .T..T...A. .......... ..........
SVRA2   .....GTG.. .......... .......T.. TT..T..G.. .......... ......G...
SVRA3   ......T... .......... .......T.. .T..T..... .......... ......G...
SVRA4   ......T... ....-..... A......... .T..T..... ....A..... ......G...
SVRC1   ..TG..T.C. ..A.A....T ...G.....G .T..T.C..G CT.TG..T.. .A.CT.TAAG
SVRC3   ..TG..T.C. ..A.A....T ...G.....G .T..T.C..G CT.TG..T.. .A.CT.TAAG
SVRC2   ..TG..T.C. ..A.A....T ...G.....G .T..T.C..G CT.TG..T.. .A.CT.TAAG
SVRA6   ..-------- ---------- ---------- ---------- ---------- ----------
SVRB    TGT..GT.C. ....C....C ...G..C... .C..T.G... .A..T...CA ...C...A.G
SVRD3   ..-------- ---------- ------T.AC CGA.TAC.TC .A.TG...GA A..C.AGA.C
SVRD4   ..-------- ---------- ------T.AC CGA.TTC.TC .A.TGC..GA A..C.AGA.C
SVRD1   ..-------- ---------- ------T.AC CGA.TAC.TC .A.TG...GA A..C.AGA.C
SVRD2   ..-------- ---------- ------T.AC CGA.TAC.TC .A.TG...GA A..C.AGA.C
SVRD6   ..-------- ---------- ------T.AC CGA.TAC.TT .A.TG...GA A..C.AGA.C
SVRD5   ..-------- ---------- ---------- ---------- ---------- ----------

SVRA1   CCAGGCAGTA ATGCCA---T GAAATGGTTT GGGCTTCCAC AGCAGAGATT GACTGTAGTG
SVRA5   .......... .......... .......... ..T.C..... .......... A.........
SVRA2   T..CC..... ...AT..... .......... ..T.C..... ........A. C.G.......
SVRA3   ....C..... ...T...... .......... ..TTC..... ........C. C.G...TT..
SVRA4   ....C..... ...T...... .......... ..T.C..... ........C. C.G...TT..
SVRC1   ..C..GG.C. ......GCC. ...G...... ..TGC.GGT. ........GG A..A..CC..
SVRC3   ..C..GG.C. ......GCC. ...G...... ..TGC.GGT. ........GG A..A..CC..
SVRC2   ..C..GG.C. ......GCC. ...G...... ..TGC.GGG. ........GG A..A..CC..
SVRA6   ---------- ------...- ---------- ---------- ---------- ----------
SVRB    .......A.. CA.TTC...C CC.G..C.C. ATT.C.---. TC......A. C.G...GT.C
SVRD3   ..CAC....C TCCATC...G T..G....GA .....AAACT T....TTCC. .TTA..GT.C
SVRD4   ..CAC....C TCCATC...G T..GA...GA .....AAACT T.....CCC. .TTA..GTGC
SVRD1   ..CAC....C TCCATC...G T..G....GG .....AAACT T....TTCC. .TTA..GT.C
SVRD2   ..CAC....C TCCATC...G T..G....GG .....AAACT T....TTCC. .TTA..GT.C
SVRD6   ..CAC....C TCCATC...G T..G....GG .....AAACT C....TTCC. .TTA..GT.C
SVRD5   ---------- ------...- ---------- ---------- --...TTCC. .TTA..GT.C

SVRA1   TTCTTCACGT TTGTCCAGGC TTTGATATGC ACTCTGTGGT TGGTCCTGTC CCCTCCCTTC
SVRA5   .C........ .......... .......... .......... .......... ..........
SVRA2   .C..G..... ....A..... GG........ .......... .......... ..........
SVRA3   GCT.....TC .CA.A....T CC....C..T GT...T.... .AACAG.C.. G.....T...
SVRA4   GCT.....TC .CA.A....T CC....C..T GT...T.... .AACAG.C.. G.....T...
SVRC1   G.TC....C. CAT....A.. .GCT..C..T ..AGCC...C ....TTCAG. .T....AACT
SVRC3   G.TC....C. CAT....... .GCT..C... ..AGCC...C ....TTCAG. .T....AACT
SVRC2   G.TCA...C. CAT....... .GCT..C... ..AGCC...C ....TTCAG. .T....AACT
SVRA6   ---------- ---------- ---------- ---------- ---------- ----------
SVRB    AG..GT..CC .CC.A....T GG.T.....T GT...C.... .A.CT..CG. ....T.AG.T
SVRD3   C.G.....A. ....G..A.T GA.......T GTGG.CC..C .TTA.AATG. T.....GGCG
SVRD4   C.G.G...A. ....G..A.T GA.......T GTGG.C...C .TTA.AATG. T.....GGCG
SVRD1   C.G.....A. ....G..A.T GA.......T GTGG.C...C .TTA.AATG. T.....GGCG
SVRD2   C.G.....A. ....G..A.T GA.......T GTGG.C.... .TTA.AATG. T.....GGCG
SVRD6   C.G.....A. ....G..A.T GA.......T GTGG.C...C .TTA.AATG. T.....GGCG
SVRD5   C.G.....A. ....G..A.T GA.......T GTGG.C...C .TTA.AATG. T.....GGCG

SVRA1   CCCAT-AAAA ACCTCACTAC CTACAAGGAA AAGATCATTC TTGAGTGTGA TGTGGGTTCA
SVRA5   .....T.... .......... .......... .......... .......... ..........
SVRA2   .....T.... .....G.... .......... .......... .A........ ..........
SVRA3   ...TAC..G. ..A.G.AA.. ...TC..... .....A.... .A........ ..........
SVRA4   ...TAC..G. ..A.G.AA.. ...TC..... .....A.... .A........ .......C..
SVRC1   ..ACAC.... ..ACGCGCTA .C.T..T..T .....TG.AT A........T G.....G..G
SVRC3   ..ACAC.... ..ACGCGCTA .C.T..T..T .....TG.AT A........T G.A...G..G
SVRC2   ..ACAC.... ..ACGCGCTA .C.T..T..T .....TG.AT A........T G.....G..G
SVRA6   -----.---- ---------- ---------- ---------- ---------- ----------
SVRB    ..AT.C.... ..ACAG.CCA TGC..CT... ...G...... .A........ ..........
SVRD3   AG.TAC.GGG ..---CA.GA .ATTG.C..G .TA..TT.CA ..ACA..CA. ..A...C..T
SVRD4   AG.TAC.GG. ..---CA.GA .ATTG.T..G .TA..TT.CA ..ACA..CA. ..A...C..T
SVRD1   AG.TAC.GG. ..---CA.GA .ATTG.T..G .TA..TT.CA ..ACA..CA. ..A...C..T
SVRD2   AG.TAC.GG. ..---CA.GA .ATTG.T..G .TA..TT.CA ..ACA..CA. ..A...C..T
SVRD6   AG.TAC.GG. ..---CA.GA .ATTG.T..G .TA..TT.CA ..ACA..CA. ..A...C..T
SVRD5   AG.TAC.GG. ..---CA.GA .ATTG.T..G .TA..TT.CA ..ACA..CA. ..A...C..T

SVRA1   GCTATTGGTT TCTGGGCTGT GTTGAGCTAT ATTGGTCTCC TGGCTCTCTT GTGCTTTGTG
SVRA5   ........C. .......... ....G..... .....A.... .......... ..........
SVRA2   .......... .......... ....G..... ..A..A.... .......... ..........
SVRA3   .......... .......... ....G..... ..A..A.... .......... ..........
SVRA4   .......... .......... ....G.G... ..A..A.... .....G.... ..........
SVRC1   .TAGCA..G. ..G.A..GT. ...AG....C ..C..C..T. ....GT..C. TA....CC.C
SVRC3   .TAGCAC.G. ..G.A..GT. ...AG....C ..C..C..T. ....GT..C. TA....CC.C
SVRC2   .TAGCA..G. ..G.A..GT. ...AG....C ..C..C..T. ....GT..C. TA....CC.C
SVRA6   ---------- --..T..... ....G..... ..A..A.... ..T....... ..........
SVRB    ...G...... .......... ....G.G... ..A..A.... .......... ..........
SVRD3   ATG..G.CGC .TG.CTTCC. AA.TG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
SVRD4   ATG..G.CGC .TG.CTTCC. AA.TG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
SVRD1   ATG..G.CGC .TG.CTTCC. AA.TG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
SVRD2   ATG..G.CGC .TG.CTTCC. AA.TG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
SVRD6   ATG..G.CGC .TG.CTTCC. AA.CG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
SVRD5   ATG..G.CGC .TG.CTTCC. AA.TG.G..C .CAT.C..G. ....AGC.A. A.....CT.C
```

```
SVRA1          CTGGCTTTTC TGGCTCGGAA GCTGCCTGAT AACTTCAATG AGGCCAAATT CATCACCTT-
SVRA5          .......... .......A.. .......... .......... .......... ..........
SVRA2          .......C.. .......A.. .......... .......... .......... ..........
SVRA3          .......... .......... .......... .......... .......... ..........
SVRA4          .......... .......... .......... .......... ....A..... ..........
SVRC1          T.......C. ....CA.... T..T..A..C .........C. .......G.. ..........
SVRC3          T.......C. ....CA.... T..T..A..C .........C. .......G.. ----------.
SVRC2          T.......C. ....CA.... T..T..A..C .........C. .......... ..........
SVRA6          .......... .......... .......... .......... .......... .........C
SVRB           .......... .......A.. .......... ......GG.. .......... ..........
SVRD3          T.T..A...A AAT.A..A.. A.....A..G .....T.C.. ....T..G.. ...-------.
SVRD4          T.T..A...A AAT.A..A.. A.....A..G .....T.C.. ....T..G.. ...-------.
SVRD1          T.T..A...A AAT.A..A.. A.....A.TG .....T.C.. ....T..G.. ...-------.
SVRD2          T.T..A...A AAT.A..A.. A.....A..G .....T.C.. ....T..G.. ...-------.
SVRD6          T.T..A...A AAT.A..A.. A.....A..G .....T.C.. ....T..G.. ...-------.
SVRD5          T.T..A...A AAT.A..A.. A.....A..G .....TCC.. ....T..G.. ...-------.

SVRA1          ---------- --------
SVRA5          .......... ........
SVRA2          .......... ........
SVRA3          .......... ........
SVRA4          .......... ........
SVRC1          .......... ........
SVRC3          .......... ........
SVRC2          .......... ........
SVRA6          AGCATGCTCA TATTCTTA
SVRB           .......... ........
SVRD3          .......... ........
SVRD4          .......... ........
SVRD1          .......... ........
SVRD2          .......... ........
SVRD6          .......... ........
SVRD5          .......... ........
```

Figure 3.4b *Alignment of all VNR-like nucleotide sequences isolated from* S.salar. Alignment created using Clustal X (Thompson *et al.*, 1997) and PHYLIP 3.57c (Felsentsein, 1995). The symbol "–" indicates a gap, while "." represents identical residues.

4 ODORANT RECEPTOR GENE EXPRESSION DURING PARR-SMOLT-TRANSFORMATION

4.1 INTRODUCTION

Around the time of parr-smolt transformation (PST), salmonids undergo a period of increased olfactory sensitivity associated with surges in thyroid activity (Morin *et al.*, 1989a and b) which is thought to constitute an imprinting episode on odours characteristic of their natal site. This memory is thought to be retained at least in part, within the peripheral nervous system (Nevitt *et al.*, 1994). The isolated cilia of olfactory sensory neurons (OSNs) from juvenile coho salmon that had been imprinted on the odorant β-phenyl ethyl alcohol (PEA) exhibited significantly enhanced stimulation of olfactory adenylyl and guanylyl cyclase upon exposure to the imprinted odorant over non-imprinted control fish. This heightened olfactory sensitivity was also inferred to be specific to the imprinted odour; cyclic nucleotide activity in response to exposure to L-serine (an odorous amino acid) was not significantly increased in PEA-imprinted fish (Nevitt *et al.,* 1994). Similarly, a twofold increase in guanylyl cyclase activity was observed in PEA-imprinted mature coho salmon prior to spawning (Dittman *et al.*, 1997). The fish were exposed to PEA during PST, and olfactory cilia were isolated throughout the development of the fish until the spawning season 17 months later, but it was only in this brief window of development that such an enhancement in activity was displayed.

Recently, further studies of peripheral olfactory memory in other species have been reported including the adaptation of aversive behavioural responses in the moth *Manduca sexta*, to harmless noxious compounds (Glendinning, *et al.*, 2001; see also Yee and Wysocki, 2001; Hudson and Distel; 1998 and the review of olfactory learning by Hudson, 1999). The "pregnancy block" observed in mice involves the formation of a long-term olfactory memory of the mate's pheromones within the recently mated female that stimulates termination upon exposure to the scent of an unfamiliar male. Pharmacological and lesion studies indicate that this memory is formed at the level of synaptic connections of mitral cells within the vomeronasal olfactory bulb (Brennan *et al.*, 1990).

Two mechanisms of odour-induced sensitivity within the primary levels of

olfactory processing in salmon were proposed by Nevitt *et al.,* (1994):

(i) Binding of specific ligands to receptors in the presence of the appropriate hormone(s) (thyroxine, T4 being the primary candidate) may precipitate an increased functional expression of those receptor proteins.

(ii) Alternatively, hormones may promote neurogenesis of generic olfactory receptor neurons and only those most active (responsible for detecting homestream odours) would survive to find synaptic targets within the olfactory bulb, resulting in a clonal expansion of only a subset of neurons.

Main olfactory receptors (ORs) and vomeronasal receptors (VNRs) will be collectively referred to as odorant receptors throughout the report of this study.

The latter hypothesis was maintained as being the most likely mechanism of imprinting during PST, with the olfactory memory being maintained silently until maturation of the fish upon which there may be a hormone-induced up-regulation of specific olfactory receptors linked to guanylyl cyclase (Dittman *et al.*, 1997).

The roles of the thyroid hormones (3,5,3'triiodo-L-thyronine [T3] and thyroxine [T4]) in development, homeostasis, cellular proliferation and differentiation have been broadly investigated. The hormones are known to promote neurogenesis and cyto-architectural changes in peripheral olfactory systems of other vertebrates while hypothyroidism depresses turnover of olfactory receptor neurons (Paternostro & Meisami, 1996a,b). Thyroid hormones modulate functional expression of a multiplicity of genes throughout the nervous system, including membrane receptors (see Thompson and Potter, 2000 for a review), and the mechanisms of transcriptional control are now reasonably well understood (see Wu and Koenig, 2000).

The results of patch clamp studies on coho salmon (*O. kisutch*), show consistent differences in outward current conductances in OSNs isolated pre- and post-smolt, which may be a consequence of differences in receptor cell populations (Nevitt and Moody, 1992).

Unpublished work (cited by Nevitt *et al.*, 1994), has suggested a four-fold increase in OSN cell number during smolting and Bertmar (1973) reported twice as many primary lamellae in smolts than in parr. It is now known that ORs are only functionally expressed in postmitotic olfactory neurons (Fan and Ngai, 2001): it seems that the descendants from individual olfactory neuron stem cells are not committed or specified to express a particular odorant receptor gene. Olfactory receptor expression is governed by temporally regulated cues during development (Barth *et al.*, 1996) and

genes from a single cluster may be activated at different times (Barth *et al.*, 1997). Furthermore, a receptor phenotype is selected regardless of whether the olfactory bulb is present (Fan and Ngai, 2001), although it is still not clear what role the glomeruli play in determining OR-type: OSNs expressing the same OR converge upon spatially conserved glomeruli (Ressler *et al.*; Vassar *et al.*, 1994). This evidence provides for a model whereby new postmitotic OSNs generated during PST in salmon may select a receptor type based upon an activity-dependent mechanism. An alternative model may be derived from that of Nevitt *et al.,* (1994): the thyroxine surges observed during PST may promote an activity-dependent increase in olfactory neurogenesis, so that only neurons that project to the most active glomeruli proliferate. Whatever the mechanism, single OSNs show a change in outward current during PST (Nevitt and Moody 1992), and an increased responsiveness to imprinted odours over non-imprinted odours (Nevitt *et al.*, 1994).

To investigate whether this olfactory-sensitive period of parr-smolt-transformation in Atlantic salmon could involve a change in odorant receptor expression, relative quantitative RT-PCR was performed on RNA isolated from the olfactory epithelium of fish before, during and after PST. Two full-sibling families of juvenile Atlantic salmon were hatchery reared throughout the period of smolting (Spring in Scotland, April to June) and were sampled before, during and after this period of time. Gene-specific primers for main olfactory receptors (ORs) and vomeronasal receptors (VNRs) were used to PCR amplify transcripts, and relative quantitation was achieved by concurrent amplification of the housekeeping-gene, β actin.

4.2 MATERIALS & METHODS

4.2.1 Study Design

Two full-sibling families of Atlantic salmon were reared at Almondbank Hatchery (Perthshire, Scotland). All tanks were supplied directly with homestream water, and the fish were offspring of wild broodstock.

Sampling of the olfactory epithelia began in January (before PST); 20 individuals were taken from each family. During smolting, another 20 individuals were sampled at four week intervals (April, May and June). Finally, 20 fish that had completed PST were sampled in August. At each sampling episode, the lengths of a representative sample of each family (100 individuals) were also taken.

Cultured salmon are known to display a bimodal distribution in body size during this stage of the life cycle (Thorpe, *et al.*, 1982; see Fig. 4.2.1). Larger individuals are more likely to undergo smolting. However, in culture, once the larger, more aggressive individuals are removed from the population, the previously smaller, subordinate individuals may then exploit the opportunity to occupy a more dominant role and may themselves enter PST (Metcalfe, *et al.*, 1990). Once dominant individuals are removed from a population, it is impossible to predict the physiological destiny of those remaining. It was therefore ensured that all samples were taken from fish belonging to the upper mode of body length and in order to compare the expression data to a "null" state, values obtained from the pre-smolt samples (collected in January) and those obtained from post-smolts (August) served as an effective control. All expression values obtained from subsequent sampling episodes were calculated relative to those of January.

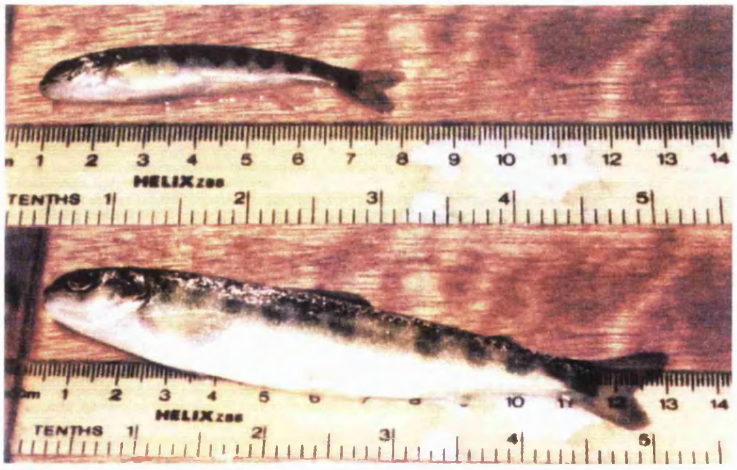

Figure 4.2.1
Photograph to show the gross difference between members of both modes within a single family
Note the silvering flanks of the larger individual

4.2.2 Sample collection

Twenty individuals from each family were isolated, then killed, measured (length: nose to fork of tail) and the required tissues removed and immediately snap-frozen on dry ice. From the first six individuals of each family, testis/ovary, brain, liver, muscle and anterior kidney were taken as reference tissues and/or for tissue banking. Muscle and olfactory epithelium only were taken from the rest of the fish. Only individuals from the larger mode of each family were sampled (see Fig 4.2.2).

Another 80 fish from each family were measured to give an indication of the length frequency distribution of the families. (Following the third sampling episode, families were divided into the two modes and reared in separate tanks following standard aquaculture practice).

4.2.3 RNA extraction and analysis

RNA was extracted from each pair of olfactory rosettes using 250µl TRIZOL™ reagent (Life Technologies, Gibco BRL), the total RNA resuspended in 12µl RNase-free water, 2µl of which were run on an RNA check gel. After DNAse treatment, 2µl total RNA was used in a 10µl reaction to synthesise first strand cDNA (TaqMan® Gold RT-PCR kit, PE Biosystems).

Both a qualitative and quantitative control for gene expression had already been established (see Chapters 1 and 2). Salmon-specific primers were used to amplify regions of the ß actin gene from all RNA samples taken and used as a reference for relative quantitation of expression of target genes.

4.2.4 Candidate genes

In an attempt to isolate additional candidate genes a cDNA library was constructed from the olfactory epithelia of five adult salmon (see Appendix). Although three different approaches to screening the library were adopted (a digoxigenin-based hybridisation, a radio-labelled hybridisation technique and a PCR-based screening protocol), all clones sequenced were false positives.

It was also intended to isolate the membrane form of guanylyl cyclase from Atlantic salmon olfactory epithelium. All amplifications using degenerate primers based on alignments of rat (GC-D: Fülle *et al.*, 1995), and medaka fish, *Oryzias latipes* (Seimiya *et al.*, 1997; Takeda *et al.*, 1997; Mantoku *et al.*, 1999; Suzuki *et al.*, 1999;

Yamagami *et al.*, 1999) sequences were unsuccessful. Therefore only main olfactory receptors and putative vomeronasal receptors characterised in Chapters 2 and 3 were included in this study.

SORB is a family of olfactory receptor (OR) genes that have been shown to be expressed in olfactory epithelium as well as testis (see Chapter 2), and was therefore chosen as the first main OR candidate for screening in this study. *SVRA* is a family of putative pheromone receptors (VNR) identified previously as being transcribed in olfactory epithelium, and was therefore used as the first candidiate for VNR screening. Fish are thought to possess approximately 100 main OR genes (Ngai, *et al.*, 1993b), and 30-40 VNR genes (Naito *et al.*, 1998), and so it was necessary to quantitate more than one candidate gene for each receptor type. In order to prevent bias towards what maybe only a fraction of the true diversity of salmon OR genes, it was decided to use a recently published Atlantic salmon OR gene, *ASOR1* (AY007188; Wickens *et al.*, 2000). The second VNR to be screened, was *SVRC*, a family of salmon VNRs that share approximately 44% sequence similarity to *SVRA* at the amino acid level (see Chapter 3).

4.2.5 Real-time RT-PCR detection systems

In recent years, a quick and accurate technique for measuring PCR-product accumulation during the exponential phase of the reaction has been successfully exploited (Overbergh *et al.*, 1999). Several Real-time quantitative PCR instruments now exist (e.g. ABI prism 7700®/7900® sequence detection systems: TaqMan®; Perkin Elmer/Applied Biosystems and the Light-Cycler, Roche).

All systems operate along similar basic principles (see Fig. 4.2.5). A non-extendible fluorogenic probe is used to monitor PCR product formation continuously during the amplification reaction. This probe is an oligonucleotide, labelled with both a reporter and a quencher dye, specific to a sequence downstream from the forward primer.

The fluorescent emission of the reporter dye (e.g. FAM, 6-carboxyfluorescein), is absorbed by the quencher (e.g. TAMRA, 6-carboxytetramethylrhodamine) whilst the probe remains intact. The probe and primers bind to the denatured template in the standard way during the annealing phase of PCR amplification. During extension, however, the hybridised probe is hydrolysed by the 5' nuclease activity of Taq polymerase, dissociating the reporter and the quencher. Separation of the two molecules produces an increase in the fluorescent emission of the reporter, which is quantitative for the initial amount of template (Overbergh *et al.*, 1999).

Real-time measurement of the fluorescent spectra of all 96 wells (SDS 7700®) or 384 wells (SDS 7900®) of the thermocycler is possible using the ABI instrumentation. Amplification plots are then constructed from the fluorescent emission data. Data collected can be visualised on a linear plot of ΔR_n (normalised fluorescence values) vs. cycle number; all emission until 2 cycles before amplification begins are considered baseline. Threshold cycle (C_T) values are calculated by determining the point at which the amplification reaction is within its exponential phase (emission is approximately ten times that of the baseline). The software calculates C_T values for all samples, which may be plotted onto a standard curve with known dilution (for relative quantitation) or known concentration (for absolute quantitation) to determine the original amount of template in an unknown sample. Alternatively, once a standard curve has been calculated for a primer/probe set, C_T values may be subjected to mathematical modelling (see section 4.2.7).

Although this method combines PCR amplification, product detection and template measurement in one step, the different phases of the process must first be

optimised.

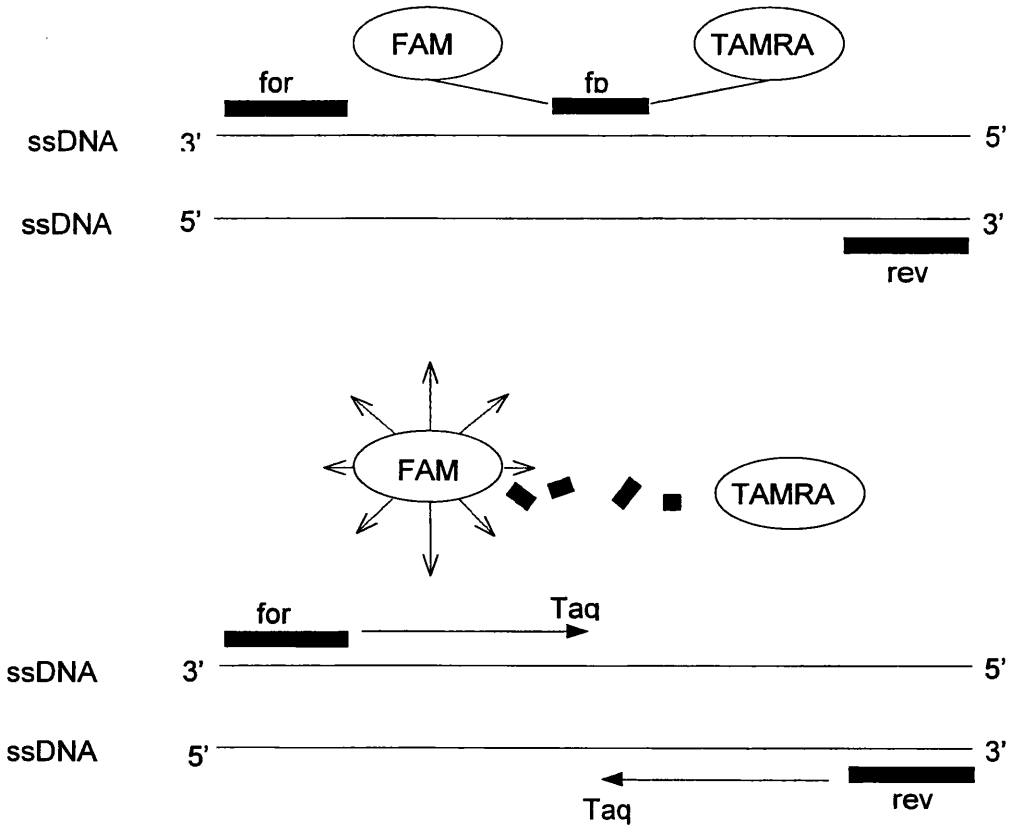

Figure 4.2.5. *Schematic representation of the* TaqMan *principle* (after Overbergh, *et al.*, 1999) During PCR annealing, primers and fluorogenic probe anneal to the denatured template. As long as the probe is intact the quencher dye (TAMRA) conjugated at the 3' end, absorbs the fluorescence of the reporter dye (FAM) at the 5'. During extension, the probe is cleaved by the 5' nuclease activity of Taq polymerase, dissociating the reporter and quencher molecules, producing an increase in fluorescence of the reporter signal. *for = forward primer, rev = reverse primer, fp = fluorogenic probe.*

4.2.6 Relative quantitative assay design and analysis

In order to design primers and probes for both the endogenous reference gene, and the target gene(s), the manufacturers of the 7900® SDS recommend that the Primer Express® software be used.

There are definitive guidelines to primer/probe design for quantitative assays (Table 4.2.6a), which must be adhered to in order to maximise amplification efficiency and subsequent statistical power of the data.

TaqMan® Probe Guidelines	**Sequence Detection Primer Guidelines**
Select the probe first & design the primers as close as possible to the probe without overlapping it (amplicons of 50-150 bp are strongly recommended)	
Keep the G/C content in the 20-80% range.	
Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided	
When using Primer Express® software, the Tm should be 68-70°C	When using Primer Express® software, the Tm should be 58-60°C
No G on the 5' end	The five nucleotides at the 3' end should have no more than two G and/or C bases
Select the strand that gives the probe more C than G bases	

Table 4.2.6a *Guidelines used to design primers and probes for quantitative RT-PCR.* Taken from "Sequence Detection Systems Quantitative Assay Design and Optimisation" PE Biosystems.

Primers are in large molar excess during the exponential phase of amplification and by independently varying the initial concentrations of both forward and reverse primers, their effective T_ms can be adjusted by as much as +/- 2°C. Any improvement in annealing efficiency can compensate for both non-specific primer-binding and inaccuracies in Tm estimation by the Primer Express® software. Primers were optimised in 20μl reactions, using 1μl of cDNA pooled from all individuals from family A (diluted 1:20 as template) and using the matrix of concentrations given in Table 4.2.5b.

Reverse Primer (nM)	**Forward Primer (nM)**		
	50 *(-2°C)*	300 *(0°C)*	900 *(+2°C)*
50 *(-2°C)*	50/50	300/50	900/50
300 *(0°C)*	50/300	300/300	900/300
900 *(+2°C)*	50/900	300/900	900/900

Table 4.2.6b *Primer concentrations used in optimisation*

The optimal combination of concentrations is that which produces the lowest C_T and the highest ΔR_n i.e. the most efficient amplification.

The optimisation of probe concentration is only necessary for reducing operational costs. Initial concentrations of probe is varied between 50nM and 250nM, to determine at which point above 50nM the C_T remains unchanged, allowing a lower concentration to be used. However, when intending to detect low copy numbers, it is necessary to avoid probe limiting concentrations and maximise ΔR_n, and so a 250nM initial concentration was employed in this study.

Target Gene	Primer Set (5' → 3')	Probe (5' → 3')
β Actin	For: CCAGATCATGTTTGAGACCTTCAA Rev: CCGGAGTCCATGACGATACC	CCTGTACGCCTCTGGCCGTACCA
SORB	For: CTCACCTTCACCATTGTCCTCTT Rev: AGCACTCGGCTGCGATCT	CAATTGGCAGCATCGCACTGACCTA
SVRA	For: TGGGCTTCCACAGCAGAGAT Rev: GGGACAGGACCAACCACAGA	TCACGTTTGTCCAGGCTTTGATATGCA
ASOR1	For: TGGCCTCTAGCCTGCCTTAC Rev: AGCTGTTGCTAGTTTCCTGTTGAA	TGCTGTGAGCACGGCCCTGTATACA
SVRC	For: CAGGGTTCGGAGCGTTGT Rev: TCGTTGAAGTTGTCTGGAAGATTC	CGGCCTTCTGGCGTTCCTTAGCTT

Table 4.2.6c *Taqman® Primer and Probe sequences.*
All OR/VNR primers and probes specific to more than one member of the gene family. All probes labelled with FAM and TAMRA.

Table 4.2.6c gives the primer and probe sequences used in all amplifications. All primers and probes designed may amplify more than one member of a gene family. All amplifications used 1μl of cDNA (diluted 1 in 10), in a 10μl reaction and were run in triplicate. The buffer conditions were as standard for the TaqMan® Gold RT-PCR kit, and the thermocycling profile was the default in the SDS 2.0 software (50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). For each primer and probe set, two negative controls were also amplified in triplicate. The first consisted of non-reverse transcribed total RNA that had been treated with DNase I (as a control for contamination by genomic DNA) and the second consisted of no template, to control for any contamination of the reagents.

4.2.7 Data Analysis

Once the primers and probe were optimised, a ten-fold dilution series of the template was amplified under optimal conditions for the endogenous reference gene, (β Actin) and all target sequences. By plotting the normalised C_T value given for each

dilution against its logarithmic value (1:10 dilution = 0.1, 1:100 dilution = 0.001 etc) a standard curve is constructed. The slope of the curve and the R^2 value is indicative of the efficiency of amplification for each primer/probe set. Subtracting the slope of the reference gene from that of the targets gives a threshold by which the analytical method is determined. If the differential is <0.1, then the amplification efficiencies are sufficiently similar to use the $\Delta\Delta C_t$ calculation method (see Equation 1). A differential of >0.1 indicates that the amplification efficiencies are too disparate to use $\Delta\Delta C_t$, and a standard curve must be constructed for each amplification when running the assay. Equation 1 illustrates the $\Delta\Delta C_t$ calculation method as recommended by the instruments' manufacturers.

ΔΔCt Method

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

Where

$\Delta C_t = C_t$ [target] $-C_t$ [ref]

$\Delta\Delta C_t = \Delta C_t$ [target] - ΔC_t [calibrator]

Since amplification efficiencies were all equal (data not shown), the $\Delta\Delta C_t$ calculation method was employed. In order to test whether the mean ΔC_t values obtained from each sampling episode were statistically equal, a simple single-factor analysis of variance (ANOVA) was performed. As analysis of heterogeneity showed unequal variance, *post hoc* analysis was carried out used Dunnet's test (SPSS software).

All graphical data reported are the results of the $\Delta\Delta C_t$ model, i.e., normalised to the values of January. All significance levels displayed result from the statistical analysis of non-normalised data.

4.3 RESULTS

4.3.1 Body size frequency distribution

All fish from which olfactory epithelium was taken belonged to the upper mode of body length, and were therefore considered likely to complete PST (see fig. 4.2.2). A Kolmogorov-Smirnov Z Test (two sample) showed that the bimodal distributions differed between the families (data given in Appendix). The distribution of body size in family A (Fig 4.2.2 A) was heavily skewed towards the upper mode, while the distribution shown by family B (Fig 4.2.2 B) seemed more uniform, with an approximately equal number of individuals belonging to each mode.

4.3.1 Gene Expression

From figures 4.3.1-4.3.4 and tables (Appendix II), it can be seen that for *SORB*, Family A showed a massive increase in relative expression ($p<0.021$), during April. Family B showed no such increase in relative expression, instead showing a decrease in relative expression during May (non-significant), which then recovered to presmolt values by June.

Relative expression of *SVRA* genes surged in Family A during June ($p<0.027$) then returned to presmolt levels in August. No significant change was observed in Family B.

In Family A, *SVRC* showed a non-significant increase in relative expression during June. Again, no change in expression was observed in family B.

During the optimisation stages of *ASOR1* primers and probe, no repeatable amplification was obtained. Optimisation stages were repeated using fresh reagents and resuspended primers and probe, but product was only obtained after cycle 38, 39 and 40 in a small number of reactions. It was decided that this was insufficient for analysis and so further screening for this gene was abandoned.

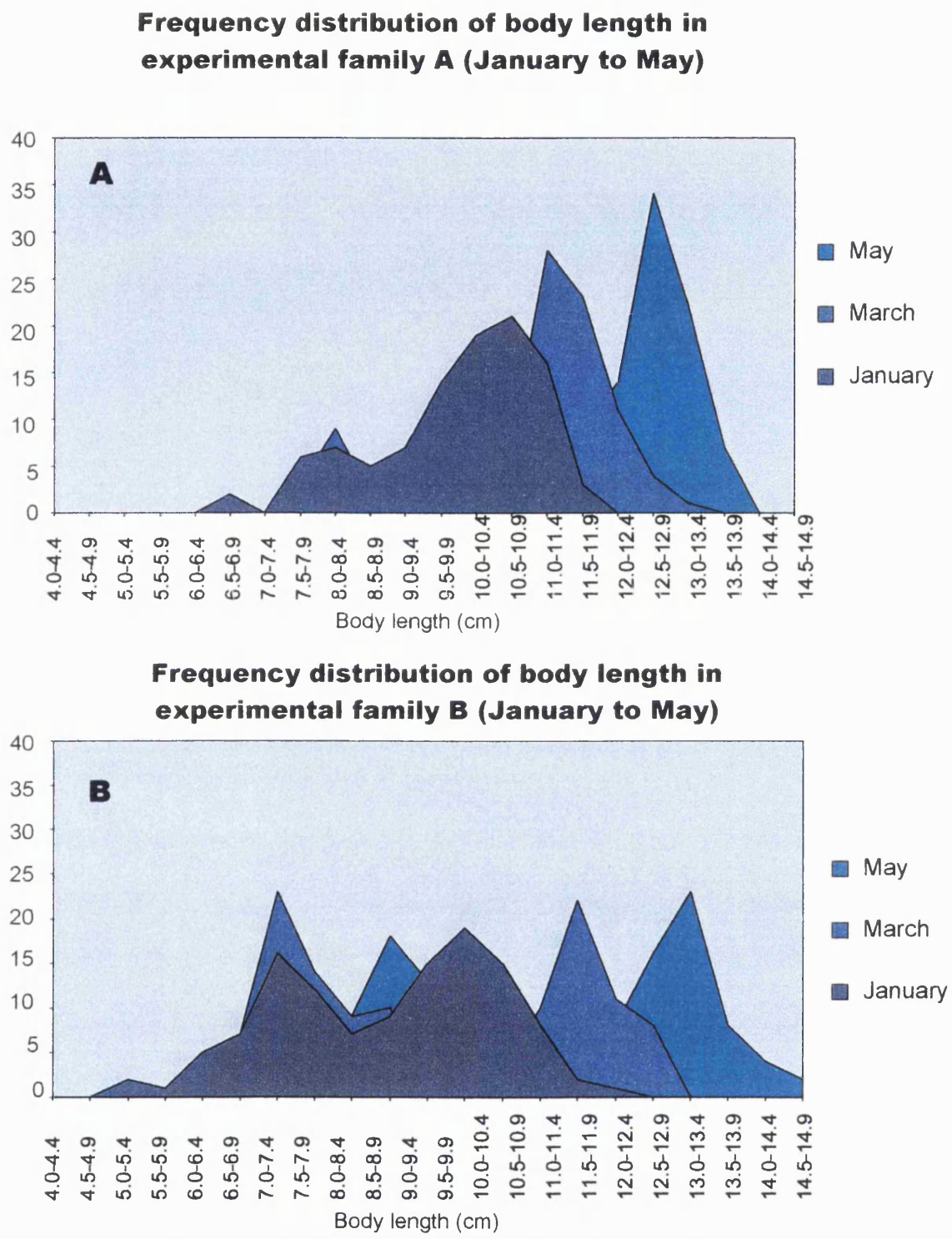

Figure 4.2.2 *Frequency distributions to show body length in both families of experimental fishfor the months January, April and May.*
A: Frequency distribution of body length in family A, **B**: Frequency distribution of body length in family B.

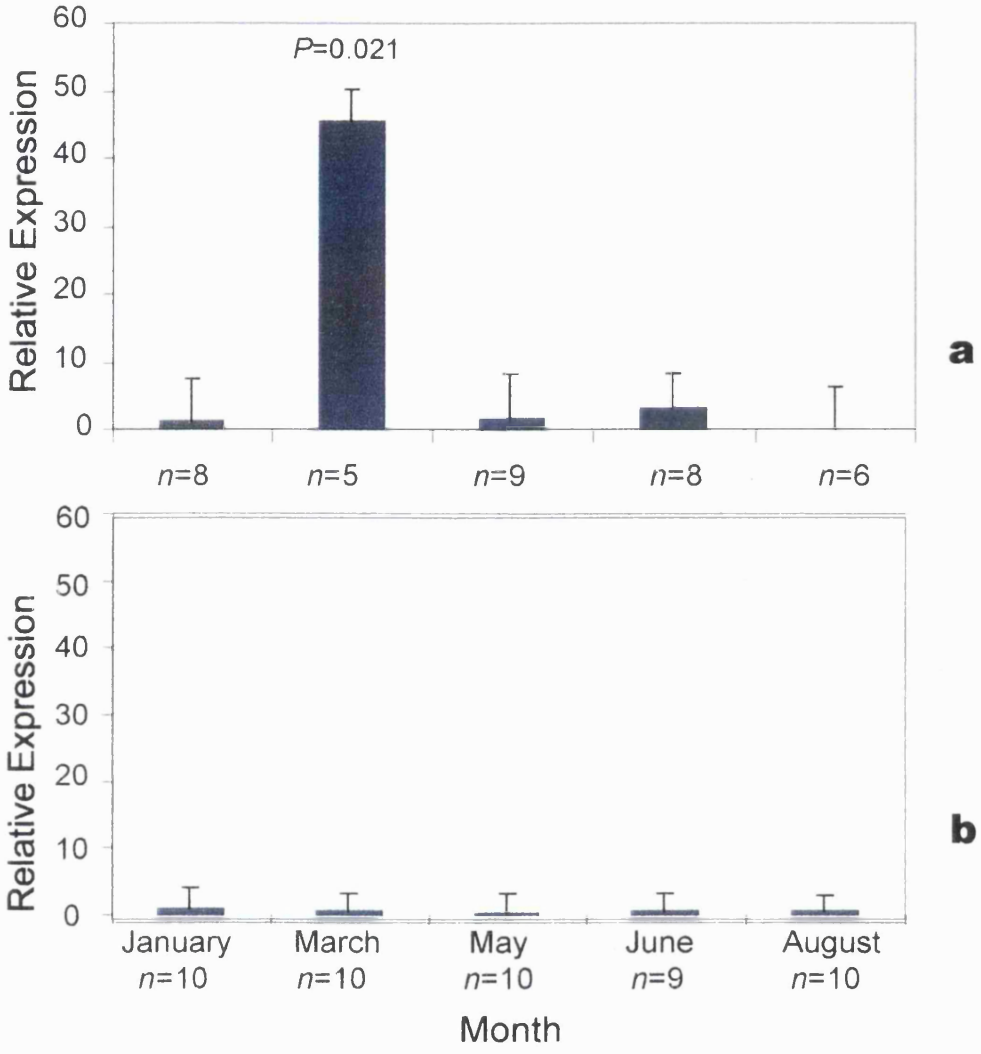

Figure 4.3.1a and b Relative SORB expression during Parr-Smolt Transformation in Atlantic salmon.

a, expression by Family A, **b,** *SORB* expression by Family B. All expression values are normalised to that of January using the ΔΔCt method (Applied Biosystems). P value indicates relative expression levels that are significantly different from presmolt (January) or postsmolt (August) values, as determined by Dunnet's test. (Error bars given by SD = $\sqrt{(sd(actin)^2+sd(target)^2)}$.

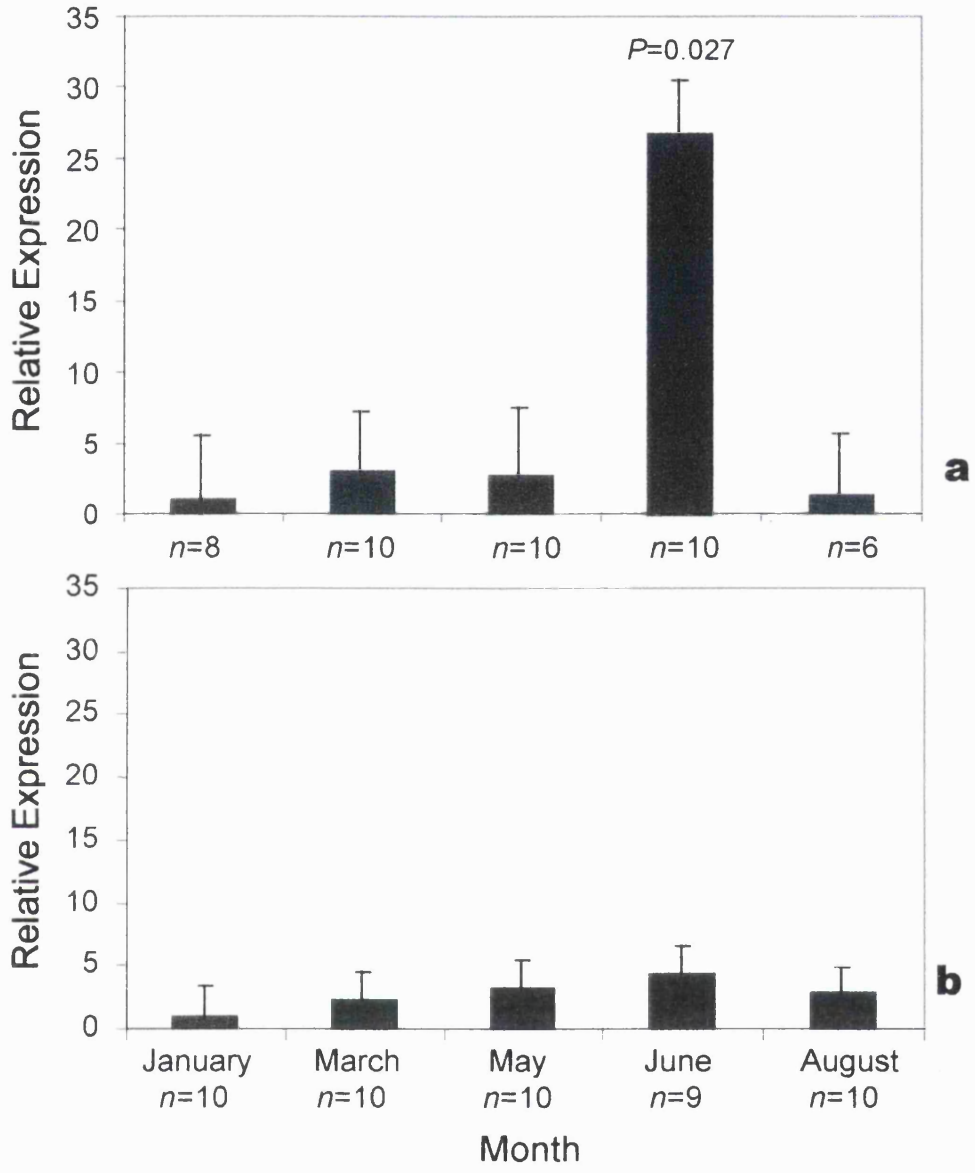

Figure 4.3.2a and b Relative SVRA expression during Parr-Smolt Transformation in Atlantic salmon.

a, expression by Family A, **b,** expression by Family B. All expression values are normalised to that of January using the ΔΔCt method (Applied Biosystems). P value indicates relative expression levels that are significantly different from presmolt (January) or postsmolt (August) values, as determined by Dunnet's test. (Error bars given by SD = $\sqrt{(sd(actin)^2+sd(target)^2)}$.

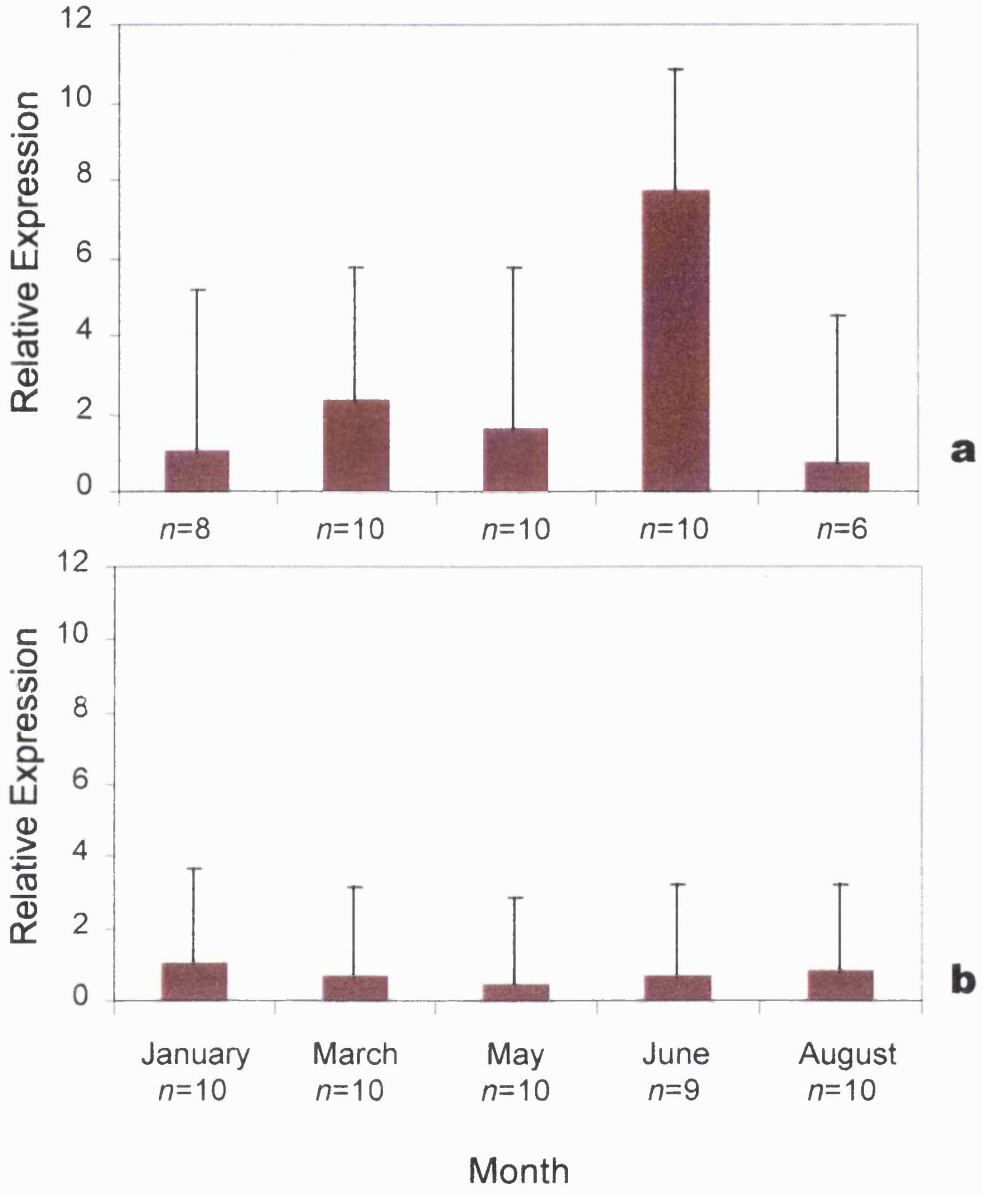

Figure 4.3.3a and b Relative SVRC expression during Parr-Smolt Transformation in Atlantic salmon.

a, expression by Family A, **b,** expression by Family B. All expression values are normalised to that of January using the ΔΔCt method (Applied Biosystems). P value indicates relative expression levels that are significantly different from presmolt (January) or postsmolt (August) values, as determined by Dunnet's test. (Error bars given by SD = $\sqrt{(sd(actin)^2+sd(target)^2)}$.

4.4 DISCUSSION

4.4.1 PST involves a change in odorant receptor expression

This is the first report of which I am aware, of a change in odorant receptor expression associated with a period of olfactory learning, and the interfamilial differences in profiles of expression are further evidence of odour-specific imprinting and / or genetic differences in the smolting process. These findings are also, apparently, the first example of a change in primary receptor expression associated with a period of sensory learning of any form. Whether primary receptor expression is likely to change during episodes of learning associated with the other senses is clearly debatable.

When the data is compared with the presmolt values of January, family A showed a fifty-fold increase in relative expression of main olfactory receptors (*SORB*) during April. The same family of fish also demonstrated a change in expression of two groups of vomeronasal receptors (*SVRA* and *SVRC*, although non-significant), in June, at which time relative expression increased by thirty-fold and seven-fold, respectively. The second family of fish did not show the same patterns of expression of any odorant receptor. The months of April and June are both important with respect to olfactory activity in Family A. These findings are consistent with a sensitive period of olfactory imprinting (SPOI) during parr-smolt transformation as identified by Morin *et al.* (1989a, b).

It could therefore be inferred that *SORB* and *SVRA* (and potentially *SVRC*) encode receptors that also play an important role in olfactory imprinting within family A. The lack of demonstrable change in the expression of these genes in family B is an indicator that they are not involved in the imprinting process within this particular family. Smolting salmon enter a window of time within which they must learn odours specific to their population and/or natal stream in order to use them later as orientation cues during the spawning migration. The variation in expression profiles between the two fish families may be due to the response to interfamilial differences in odour stimuli.

4.4.2 The meaning of relative expression data

Bertmar (1973) reported twice as many primary lamellae in smolts than in parr and this increase in surface area of epithelium was thought to be related to an increase in number of OSNs during PST. Unpublished anatomical studies cited by Nevitt *et al.* (1994) have also suggested a quadrupling in olfactory receptor cell number during smolting. If OSN numbers are increasing during PST, then actin expression must increase accordingly. Therefore we expect to see a decrease in relative expression of target genes, if the number of cells expressing the target remains constant (scenario *c* Fig. 4.4a). An active down regulation (a reduction in transcription) would not be definitively recorded by RT-PCR method using actin as endogenous reference (scenario *d*, Fig. 4.4a), unless the gene ceases to be transcribed at all. Scenarios *c* and *d* can not be distinguished by this method. However, an active up regulation would be detected, as the ratio of target to actin increases (scenario *b*, Fig. 4.4a). An active up regulation may also be seen by the relative expression remaining constant: actin is constantly increasing and a target that maintains the same ratio with actin will either indicate that receptors per cell are increasing in a small subset of neurons, or that the numbers of target-specific neurons are increasing (scenario *a*, Fig. 4.4a). Relative expression data unfortunately gives no indication of whether this increase in expression is due to increased transcription per OSN, or clonal expansion of specific neuronal subsets.

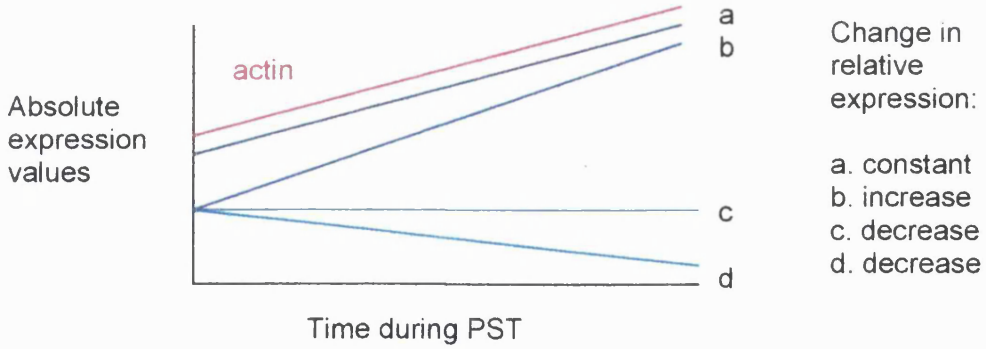

Figure 4.4a *Model to show four possible scenarios of gene expression and their effect on relative expression data.*
a. target gene increases parallel with actin = increase in number of receptors per cell OR increase in receptor-specific cell number.
b. target increases relative to actin = as in *a.*
c. target expression remains at constant level, but target: actin ratio decreases = no active downregulation
d. target actively downregulated, and target:actin ratio rapidly decreases.

All primer and probes sets were retrospectively found to align with more than one member of a gene family. Other members of the *SORB*, *SVRA* and *SVRC* created mismatches with the primers and/or probes. It may therefore be the case that more than one locus is amplified in each reaction. If more than one locus per gene family is expressed, this may result in an artificially high increase in expression. Similarly, if one member of a gene family ceases to be transcribed, while related loci continue at the same level, a decrease in relative expression may be seen. Comparing the levels of change in expression (e.g. seven-fold versus fifty-fold) would therefore be imprecise: those fish showing a seven-fold increase may only be expressing one locus, whereas those showing a fifty-fold increase may be expressing multiple loci. For this reason, only a qualitative change in expression will be discussed.

Despite the lack of change in expression observed in Family the substantial increase in expression displayed by family A during April is evidence that this group of genes has an important role to play during imprinting within these fish.

4.4.3 Mechanisms of odorant receptor regulation during PST

The question of whether the observed changes are due to clonal expansion of specific OSNs or the upregulation of receptors per cell may only be resolved by more anatomically-based techniques, such as *in situ* hybridisation. Previous studies implicate thyroid hormones in the stimulation of cytoarchitectural changes within peripheral neuronal tissue and the regulation of transcription of a vast array of genes (reviews by Wu and Koenig, 2000; Thompson and Potter, 2000). Smolting salmon are known to experience elevated T4 levels in complex patterns that may be partly seasonal or developmental but also include transient responses to environmental cues also associated with migratory activity. These transient responses have been demonstrated to result from increases in stream flow-rate (Youngson, 1984; Youngson *et al.*, 1986), the progress of the lunar cycle (Grau *et al.*, 1981) and are associated with periods of olfactory sensitivity (Morin *et al.*, 1989b).

PST and the associated development of migratory behaviours involve many complex phenomena stimulated by a multiplicity of exogenous and endogenous factors (McCormick *et al.*, 2000) and so the associated period of olfactory imprinting is also likely to be an intricate process. Some of these changes are clearly very transient, involving brief alterations in gene expression, which may bring about long term effects.

Dissecting such an intricate system inevitably attracts problems associated with oversimplification, and so any experiments intended to shed further light on the interplay of environmental cues, endocrinology, gene expression and olfactory imprinting must be carefully designed.

4.4.4 Different families of fish show different expression profiles

The use of two full-sibling families in this experiment was to allow experimental replication. In an attempt to ensure genetic homogeneity within families and environmental homogeneity between them, both families were the offspring of wild-caught broodstock from the river Almond, both were raised in tanks of the same size in the same hatchery under the same conditions (maintained in river water, same light/dark cycle), which meant that they may have undergone PST with some degree of synchronicity.

The bimodal distribution of body size was observed in both families (see 4.2.2) as were the anatomical indicators of PST (silvering of the flanks etc.). The families nonetheless, showed a disparity in the expression of specific odorant receptors. Only family A shows a significant increase in expression in any of the genes amplified. This does not, however, show that Family B was not undergoing a period of olfactory imprinting. Family B may simply not require an increase in *SORB, SVRA* or *SVRC* expression during PST, but instead uses alternative receptor-types that are specific to odours produced by family B cohorts. Alternatively, it may be that the PST of family A and B are not precisely synchronised, so by adopting an experimental approach that involves sampling at monthly intervals, the corresponding peaks of *SORB, SVRA* and *SVRC* expression by family B were not observed. Genetic influence on the intensity and timing of smolting and seaward migration has now been confirmed (Nielsen *et al.*, 2001), so it is unsurprising that interfamilial differences were observed in this study.

The changes observed in odorant receptor expression are clearly transient, and only further work involving a much more frequent and intensive sampling regime will determine their onset and longevity. A study of this nature may also reveal the turnover of OSNs during this olfactory sensory period, which in turn may relate to the longevity of these episodes of elevated expression.

A high level of variation was observed in both experimental families (as indicated by the error bars in figs. 4.3.1B-4.3.3B). This means that the expression levels differed greatly between the fish within each cohort: certain fish may be showing an

increase in expression while others do not. This may be an effect of sampling, or may be inherent biological variation. Inter-individual variation in hippocampal mRNA expression has been shown to be as much as twenty-fold in inbred rats (Alfonso *et al.*, 2002). The authors also showed that this inter-individual variation could not simply be due to sampling and handling variability.

As outlined in section 4.2.1, the act of removing individual fish from a population may result in altering the physiological outcome of those individuals remaining. The "destiny" of the fish involved in this experiment can not be predicted, and it may be the case that not all of the fish sampled would enter the period of olfactory imprinting or complete PST. It can also be noted that although these fish were derived from wild broodstock, they have by no means experienced natural conditions during rearing. Deformed or diseased individuals that would probably not survive under natural conditions can persist in artificial rearing environments. Fish that could never smolt and migrate to sea that survive artificial rearing conditions would have the effect of increasing intrafamilial variation.

Similarly, these fish have not experienced changes in flow-rate, water quality (including salinity) and many other environmental factors that would otherwise be the norm in the wild.

4.4.5 Temporal variation in odorant receptor expression

The work of Morin *et al.* (1989a and b), showed two peaks of olfactory sensitivity associated with two peaks of plasma thyroxine (T4). The first peak was described as being the SPOI (sensitive period for olfactory imprinting) and the second as a period for olfactory learning, with a "..high capacity for odor learning but low capacity for odor memory". These two periods of olfactory sensitivity were recorded in fish of age groups 3 and 6 (612-619 days and 642-649 days), and so there existed an interval of merely three weeks between the two episodes. In this study, an increase in main olfactory receptor expression (*SORB*) is observed in April, while the increase for pheromone receptor expression (*SVRA*) is only observed in June, which constitutes an interval of nine weeks. Nevertheless, it may be argued that this late peak in VNR expression reflects this second period of olfactory sensitivity. Certainly, in Scottish conditions, the changes in behaviour and physiology expressed as active migration among wild salmon smolts are evident over a similarly extended period (Youngson, pers. comm.). Moreover, the fish in Morin *et al.*'s studies, were artificially induced to enter PST by

raising the water temperature from 6.4°C to 12.6°C, and by increasing the photoperiod. The fish sampled in this present study were kept at the temperature of the river water, as for wild fish, throughout the experimental period. Whether the artificial induction of smoltification *via* raising temperature accelerated the smolting process and therefore reduced the interval between the two olfactory-sensitive period can only be resolved by further experimentation. By inducing PST through raising temperature and/or increasing photoperiod and recording the resultant expression of ORs/VNRs with concomitant titres of plasma thyroxine, the interplay of thyroid activity, olfactory sensitivity and receptor expression might be elucidated.

Regardless of whether the early expression of OR(s) and the late expression of VNRs reflect the two periods of activity recorded in previous studies, it still remains unclear why this temporal variation may exist between these two types of chemoreceptor. During April, Almondbank fish are well into the smolting period, and by June should be entering the end of the freshwater phase, at which point the fish revert from territorial to shoaling behaviour (Hoar, 1988). The samples from June may therefore be representative of the end of the freshwater stage. If the fish spend at least a year growing and developing in the vicinity of their redds, it is logical to conclude that they have a sufficient period of time to learn the relevant odours of the natal site. A study involving fluvarium tests of Arctic charr both reared in isolation and together with siblings revealed that social experience sometime during the first 15 months is essential for the development of sibling preference in these salmonids (Winberg & Olsen, 1992). This presmolt odour learning could easily be extrapolated to include odours of the natal stream. It would therefore be only when seaward migration commences that the smolts encounter novel odours as they make their way down through the river system and into the estuary. During the journey, novel odours whether biotic or abiotic, will become more numerous and the fish will only have a brief period of time to lay down olfactory information *en route*. The period of olfactory imprinting may therfore conceivably begin at the start of smolting and end when the fish enter the sea. The entire process would thus have to be extremely plastic in order to be effective in such a constantly changing environment. Increased water temperatures (Solomon, 1978), discharge (Hansen and Jonsson, 1985) and turbidity (Greenstreet, 1992) are all known to have a rapid stimulatory effect on the initiation of downstream migration. Variation in imprinting between populations that spawn at different altitudes would therefore be probable.

Empirical support for the 'Sequential imprinting hypothesis' is still required. In a

study aimed at investigating the homing behaviour of farmed 'escapees', Hansen *et al.* (1987) also claimed their results supported the sequential imprinting theory of Harden Jones (1968). The greater homing accuracy displayed by a group of smolts (raised throughout their lives in homestream water) over that shown by a group of fish that had been transferred to sea pens, allowed to sexually mature and then released elsewhere was given as evidence for the requirement of a smolting migration. A later study showed that grilse home to the river from which they emigrated as 2-year-old smolts rather than that in which they were hatchery-reared. Additionally, translocated post-spawners (kelts) home to the river from which they emigrated as smolts, rather than the river from which they emigrated as kelts, implying that the PST-associated imprinting process maybe fixed, and not plastic.

Clearly, the seaward migration plays an important role, however, careful experimental planning is required to demonstrate whether or not smolting fish depend on a sequence of imprinting episodes in order to establish sufficient olfactory memory for accurate homing.

Depending on the timing of seaward migration within the families involved in this study, the increase in OR expression during April could potentially represent the olfactory imprinting episode proper, when the smolting fish learn the odours of their natal river system. The later expression of VNRs in June may alternatively represent the transition between territoriality and schooling behaviour (Hoar, 1988), although it is not yet known whether the shoals of smolts are population-specific, or mixed. It would be necessary to collect expression data of additional chemoreceptors from other families of smolts coupled with behavioural observations in order to verify whether this duality reported by Morin *et al.*, (1989a and b) is manifest in wild fish or is merely the observation of only two of several periods of heightened olfactory sensitivity.

Temporal changes in olfactory receptors have previously been reported in mouse (Nef *et al.*, 1992) zebrafish (Barth *et al.*, 1996), chicken (Nef and Nef, 1997) and *Xenopus* (Mezler *et al.*, 1999). Transient expression was observed in the developing olfactory system of all four organisms and the ontogenic role of olfactory receptors was discussed. Expression of olfactory receptors in immature neurons has since been associated with axonal guidance (Wang *et al.*, 1998). The transient rise in expression of *SORB*, *SVRA* and *SVRC* in juveniles in this study may be an example of odorant receptors occupying a similar primary role in the formation of odour memory.

4.4.5 Further work

Many questions have been raised by this study, and in order to address these, much experimental work is required.

In order to resolve whether the increases in odorant receptor expression that have been observed in this study result from increased neurogenesis of *SORB / SVRA*-specific OSNs and/or increased transciption and presentation of receptors by the cells themselves requires a neuranatomical approach. *In situ* hybridisation may allow the number of receptor-specific OSNs within the olfactory epithelium to be recorded from fish at different stages of smolting. Alternatively, new techniques such as single-cell RT-PCR can enable the quantity of receptors transcribed within an individual cell to determined.

To establish general patterns of change in olfactory sensitivity during PST, a greater number of fish families from different populations need to be be screened for a greater number of genes. By increasing sample sizes in this way it may be possible to show that there are rigid differences in both the temporal expression of odorant receptors and the profiles of expression displayed by different populations during PST.

In concert with the quantitation of plasma levels of thyroxine from the sampled fish, observations in cellular and molecular changes would allow the role of this hormone in regulating neurogenesis and/or receptor expression to be revealed. By repeating these experiments using PST-induced fish, it may be determined whether these two peaks of olfactory sensitivity are genuine and associated with the two types of chemoreceptor.

Temporal variations in odorant receptor expression may also be due to the fish undergoing a sequence of imprinting episodes during the seaward migration. Depriving groups of fish homestream odours at different stages of smolting (transferring them to dechlorinated tap water for instance), and then releasing them in the estuary may reveal a lengthy process of imprinting. A relationship between longevity of experience of homestream odours during PST and subsequent homing accuracy may appear.

A further complication to the regulation of receptor expression during PST may involve the odours themselves. Does exposure to specific odorants directly affect expression of their associated receptors? Recent work with *C.elegans* (Peckol *et al.*, 2001) has shown that the exposure to dauer pheromone has a direct effect on the transcription of certain chemoreceptors, suppressing expression of one receptor gene, and inducing transcription of another, which has dramatic effects on the animals'

subsequent behaviour and development. Whether chemosensory experience can directly affect the expression of odorant receptors in vertebrates has yet to be defined in this way. Functional expression studies that identify the ligands to specific *SORs* or *SVRs* are the first step in determining the role of odour exposure on receptor regulation.

Regulation of odorant receptor expression may also be pivotal at further stages in the life cycle of salmon. As previously mentioned, the increase in VNR expression in June could (speculatively) be associated with less aggressive, more social behaviour in post smolt salmon. Salmon migrate to sea in shoals, a behaviour that is also observed at the marine feeding grounds where many populations converge, and the possibility exists that odorant receptor expression may play a role in maintaining shoals.

More obviously, during the spawning migration, olfaction is known to be vital in selecting the correct branches of complex river systems. The role of odorant receptors during this remarkable stage of the life cycle must therefore be of utmost importance, and may involve the regulation of those receptors that were transcribed at the highest levels during PST. Investigating chemoreceptor expression patterns in migrating adults and comparing them with the patterns shown by their siblings during PST (which may have occurred 4 or 5 years previously), may show similarities to the regulation of genes involved in the immune response to previously encountered antigens. It may prove to be the case that as migrating adults encounter previously imprinted odour(s), the neural activity elicited by specific ligands may encourage the maturation and/or growth of neurons expressing the appropriate receptors. Equally, ligand-specific neural stimulation may evoke an upregulation of the associated receptors by the primary neurons themselves. An experiment on this scale however, would be lengthy and logistically complex.

4.4.6 Summary

Molecular evidence indicates that there are changes in odorant receptor expression coincident with parr-smolt transformation. Smolting juveniles are known to undergo a period of olfactory imprinting in order to learn the odours associated with the homestream and the seaward journey. There is more than one transient increase in odorant receptor expression during the smolting period, which may represent a variability in function involving discrete intervals of olfactory sensitivity. Differences in expression profiles between families of fish suggests interfamiilal differences in odour stimuli.

The stimuli that elicit such increases in transcription, and the mechanisms through which the receptor population expands are still unknown. Whether smolting salmon consistently undergo brief periods of odour-learning until they reach the sea also remains to be determined. This is the first report of a change in odorant receptor expression associated with a period of olfactory imprinting, and the interfamilial differences in profiles of expression are further evidence of odour-specific imprinting and / or genetic differences in the smolting process. To my knowledge, this represents the first example of a change in primary receptor expression associated with a period of sensory learning of any form.

5 DISCUSSION

5.1 FULFILMENT OF AIMS

The original aim of this studentship was to investigate the role of olfactory receptor (OR) genes in the period of olfactory imprinting in the migratory Atlantic salmon (*Salmo salar*). The first step was therefore to isolate potential olfactory receptor-like sequences of genomic DNA. During the pilot study, five families of partial OR-like genes were identified (*SORA-E*; Deaville, unpublished), and it was on this preliminary data that the studentship was based. The following sections are aimed at drawing together the observations made throughout the project.

5.1.1 Main Olfactory Receptor Genes

A further OR gene family (*SORF*) was added to this dataset, and patterns of expression of selected OR-like sequences throughout tissue types were examined.

The presence of four stop codons with *SORA* sequences and the lack of amplification from the cDNA of any tissue indicated that this family of sequences constitutes pseudogenes. *SORA* could therefore not be used in further investigations. Likewise *SORC* and *SORE* were excluded from any more detailed study as they were both represented by truncated clones. *SORB* and *SORF* however, were found to be transcribed in olfactory tissue and therefore were identified as potential candidates for analysis of expression. Unlike *SORB*, the transcription of *SORF* was confined to olfactory epithelium.

Transcription within tesiticular tissue was observed in the analysis of *SORB* and *SORD*, the latter family of genes being expressed exclusively in male germ tissue. Expression of ORs in testis was not entirely unexpected as olfactory receptor-like sequences are now believed to occupy a role in sperm chemotaxis in several taxa (Vanderhaeghen *et al.,* 1997; Ngai, *et al.,* 1993a; Goto *et al.,* 2001).

The amplification of *SORB* and *SORD* from cDNA synthesized from anterior kidney was not intially expected. Recent evidence for OR-like sequence transcription in the periarterial lymphatic sheath of rat spleen suggests that OR molecules might function as chemoreceptors on cells of the immune system (Blache *et al.,* 1998; Walensky *et al.,* 1998). The anterior kidney in teleost fish is a lymphomyeloid tissue, and therefore rich in lymphocytes.

Each of these three families of genes (*SORB*, *SORD* and *SORF*) consisted of between one and four family members. The genomes of teleosts are believed to contain between 30 and 100 different loci (Ngai *et al.*, 1993b) and so the approach adopted in this part of the study had succeeded in identfying only a proportion of the possible OR "subgenome" in Atlantic salmon, as illustrated by the recent publication of the *ASOR1* sequence (Wickens *et al.*, 2001).

Phylogenetic analysis confirmed the limited diversity of *S.salar* ORs. Salmon sequences were not confined to one "clade", but they were not as dispersed throughout the phylogeny as those isolated from other teleosts.

Although these sequences were isolated as potential candidates with which to search for molecular evidence of olfactory imprinting, the potential size of the Atlantic salmon OR repertoire meant that it was necessary to attempt to identify genes that may be involved in homestream recognition.

5.1.2 Vomeronasal Receptor Genes

Sequences encoding putative pheromone receptors, or vomeronasal receptors (VNR) were increasingly being reported in the literature (from 1997 onwards) including those isolated from teleosts. It was therefore decided that to maximise the possibilty of identifying receptors specific to population, or homestream odours, pheromone receptors from Atlantic salmon should also be included in the study.

Four families of pheromone receptor genes (*SVRA-D*) were isolated from the genomic DNA and olfactory epithelial cDNA of an Atlantic salmon, using a similar approach to that of the ORs. All four families were found to be expressed in olfactory epithelium. Transcription of *SVRD* was also observed in testis. This is the first report of the expression of vomeronasal receptor-like sequences in the male germ tissue of a teleost.

Evolutionary analysis of *SVR* sequences with the three types of VNR isolated from mammals and those available from teleosts, revealed that fish VNRs group with type two VNRs. Type two mammalian VNRs share sequence similarity to metabotropic glutamate receptors (mGluRs) and calcium ion sensing receptors (CaSRs) (Tanabe *et al.*, 1992; Herbert and Brown, 1995). It was shown that *SVRD* genes show greater similarity to the more ubiquitous CaSRs than the other *SVR*s. Whether or not this family of genes represents receptors involved in genuine pheromone recognition or the binding of ligands more associated with calcium homeostasis can only be determined

by functional analysis. For this reason, this family of genes was excluded from expression studies.

5.1.3 Odorant receptor gene expression during PST

The isolation and characterisation of both groups of chemosensory receptors provided a battery of candidates with which to screen olfactory tissues obtained from fish at different stages of parr-smolt transformation (PST). The publication of a further putative OR from Atlantic salmon (Wickens *et al.*, 2001) added to this array.

Two families of full-sibling salmon (denoted family A and B) were hatchery-reared in separate tanks fed by homestream water. Olfactory tissue was taken from parr (presmolt) and then sampling during PST was performed monthly, with a final episode post-smolt.

Real-time quantitative RT-PCR revealed transient increases in odorant receptor expression (relative to β actin) during the smolting period, compared with presmolt values. This is the first demonstration of a change in odorant receptor expression associated with a period of olfactory imprinting. To my knowledge, this constitutes the only example of a rise in primary receptor expression associated with any form of sensory learning.

A rise in *SORB* expression was observed during April in family A, but a similar rise was not displayed by family B.

An increase in pheromone receptor expression occurred two months later. Family A displayed a significant increase in *SVRA* expression, but the expression of *SVRC* did not change significantly in either experimental group.

These results are consistent with a sensitive period of olfactory imprinting (SPOI) during smolting (Morin *et al.*, 1989a and b). The two variables observed in expression profiles (family differences and temporal differences) demand explanation.

Interfamilial differences in odorant receptor expression are likely to be due to the genetic differentiation of the families. A genetic basis for the timing and intensity of smolting between populations of *S.salar* has recently been identified (Nielsen *et al.*, 2001), and so if the two families of fish were smolting asynchronously, any equivalent rise in *SORB* or *SVRC* expression by family B may have been omitted by sampling at monthly intervals. However, the variation in expression profiles between family A and B may be due to the response to differences in odour stimuli produced by the two families.

The temporal differences in the expression of the two types of odorant receptor may parallel transient variation that characterises other aspects of the PST. More specifically, these transient changes in odorant receptor expression may be a reflection of the observations made by Morin *et al.* (1989a and b). Two peaks of olfactory sensitivity associated with similar surges in plasma thyroxine were reported by these authors. The rise in OR expression seen in April and the subsequent rise in VNR expression in June may constitute corroboration of these earlier findings.

However, the process of PST is a highly convoluted metamorphosis involving a range of environmental cues (photoperiod, temperature, discharge, odours etc.), producing transient endocrine responses that mediate the physiological response (McCormick *et al.*, 2000). Olfactory learning associated with kin-recognition has also been shown to occur in the first 15 months in another salmonid (Arctic charr, Winberg and Olsen, 1992), and so it is not unreasonable to expect the olfactory imprinting process to occur over an extended period during which the fish encounter novel odours. The downstream migration of smolts will result in the fish experiencing a constantly changing environment as they progress through the river system towards the sea. The fish will encounter many novel odours but will only have a limited period of time in which to lay down a long-term memory, and so it would be unsurprising to discover that several intervals of olfactory imprinting occur *en route*.

A sequential imprinting hypothesis was first proposed by Harden Jones (1968), as an extension of Hasler and Wisby's imprinting hypothesis (1951). Indirect experimental corroboration of sequential imprinting was reported by Hansen *et al.* (1987). The authors' claim that the greater homing accuracy observed in a group of smolts (maintained in homestream water throughout their lives) over that of fish transferred to sea pens, allowed to sexually mature, and released elsewhere is evidence for the requirement of a smolting migration. Each group of fish was reared to smolting at the hatchery, and then transferred to the different treatments for a further year of growth. This means that each group had experienced homestream odours throughout the first year. Those fish transferred to seapens effectively experienced two treatments: seawater and sexual maturity, rather than being deprived of homestream odours. A later study (Hansen and Jonsson, 1994) showed that grilse home to the river from which they emigrated as 2-year-old smolts rather than that in which they were hatchery-reared. Additionally, translocated post-spawners (kelts) home to the river from which they emigrated as smolts, rather than the river from which they emigrated as

kelts, implying a certain "fixed" nature of the PST-associated imprinting process.

The seaward migration therefore plays an important role, however, substantial empirical support is still required to demonstrate whether or not smolting fish depend on a sequence of imprinting episodes in order to establish sufficient olfactory memory for accurate homing.

5.2 FUTURE WORK

The results of this studentship raise many questions and provide a substantial platform for future work. The following sections outline several hypotheses born from this work and potential approaches that could be used to test them.

5.2.1 Imprinting involves the clonal expansion of specific OSNs

In order to determine the mechanistic process(es) involved in the increase in odorant receptor expression a neuroanatomical approach needs to be adopted. *In situ* hybridisation may allow the number of receptor-specific OSNs within the olfactory epithelium to be recorded from fish at different stages of smolting.

Either whole-mount immunocytochemistry using an antibody raised to olfactory receptors and/or vomeronasal receptors, or *in situ* hybridisation of olfactory epithelium from fish at different stages of smolting using labelled RNA probes may be possible. The number of cells within the olfactory rosettes expressing these receptors at successive episodes during smolting may then be compared.

This approach may also provide an index of the turnover of olfactory neurons during smolting. Previous observations have indicated a massive increase in neurogenesis during smolting, but the longevity of OSNs at this time is not known. The approach may also give an insight into the appropriate sampling period (e.g. monthly or weekly) for olfactory imprinting studies.

5.2.2 Imprinting involves increased transcription of receptors per OSN.

Alternatively, new techniques such as single-cell RT-PCR can enable the quantity of receptors transcribed within an individual cell to determined. Real-time relative quantitative PCR may be performed using primers designed to conserved regions of ORs and VNRs. The use of intercolating fluorescent dyes such as SYBR Green allow the detection of all PCR products, and mean that locus-specific primers and probes need not be designed. The OR/VNR specific to the cell in question would therefore not need to be identified prior to the study.

Regardless of the method, an approach needs to be adopted to distinguish between the clonal expansion of a subset of cells and the number of receptors expressed per cell.

5.2.3 T4 increases receptor expression in presence of odours during imprinting.

Does T4 directly induce the increase in expression of odorant receptors? Whether or not the celluar mechanism has been identified (section 5.2.1 and 5.2.2), it may be possible to determine if the exogenous application of T4 results in an increase in receptor expression.

By quantifying receptor expression following the application of exogenous thyroid hormones to parr, the direct action of T4 on the regulation of these genes may be determined. Fish maintained in homestream water and treated with T4 may be compared with treated fish maintained in dechlorinated tap water (and untreated controls). This will show whether or not the presence of odours is required for any T4-induced olfactory sensitivity.

Exposure of *C.elegans* (Peckol *et al.*, 2001) to dauer pheromone has a direct effect on the transcription of certain chemoreceptors, suppressing expression of one receptor gene, and inducing transcription of another, which has dramatic effects on the animals' subsequent behaviour and development. Whether exposure to odorants can directly affect the expression of odorant receptors in vertebrates has yet to be defined in this way. Functional expression studies using complete *SOR* and *SVR* sequences to determine these receptors' ligands may not be necessary if a method can be established that can detect changes in expression of any form of chemoreceptor.

5.2.4 T4 increases receptor expression in presence of odours during adult homing.

Regulation of odorant receptor expression may also be pivotal at further stages in the life cycle of salmon. During the spawning migration, olfaction is known to be vital in selecting the correct branches of complex river systems. The role of odorant receptors during this remarkable stage of the life cycle must therefore be of utmost importance, and may involve the regulation of those receptors that were transcribed at the highest levels during PST.

It may prove to be the case that the elevated levels of T4 experienced by adult migrants (Youngson and Webb, 1993) may be sufficient to upregulate transcription of the appropriate receptors before the fish encounter the imprinted odours. This would

allow fish entering coastal and/or estuarine waters to be primed for odour recognition. Alternatively, a combination of raised T4 levels and the presence of imprinted odours may be required for any increase in transcription to take place. This would mean that the fish would have to re-encounter an odour before any increase in the expression of the appropriate receptor(s) could take place. This latter scenario would show more parallels with immunological memory. A feedback mechanism may operate whereby an olfactarily "primed" adult encounters an imprinted odour, and therefore orientates towards it. This leads to the fish encountering the odour at greater concentration which stimulates increased expression of its appropriate receptor(s), thereby increasing the olfactory sensitivity of the fish further.

In order to test this, relative expression data would have to be obtained from adult fish at different stages during homing. Do fish in coastal waters show the same expression profiles as fish in increasingly higher reaches of the river system?

5.2.5 Sequential imprinting is necessary for accurate homing.

In an effort to address the requirement of several episodes of olfactory imprinting during the seaward migration of smolts, experiments must be carefully designed.

The artificial rearing of smolts in homestream water until they reach the age of 'natural' migration may constitute the equivalent experience to that of a natural smolting migration. However, the treatment of experimental groups 'deprived' of a seaward migration are of utmost importance if comparisons are to be made. If fish raised throughout their lives in homestream water and then released into the estuary are considered the 'wild' model, experimental treatments should include the removal of the homestream element at sequential times during the smolting process. It could be possible to transfer experimental groups of fish to dechlorinated tap water (at the same temperature, light:dark cycle etc.) at different stages during smolting, and then release all experimental groups at the same place in the estuary.

Recapture rates could then be correlated with the amount of homestream experience each group of fish underwent during smolting.

5.2.6 Receptor expression profiles differ between populations.

Interfamilial variation in gene expression was observed during this study. It was

proposed that differences in expression of odorant receptors may be due to differences in interfamilial odour production. It may prove to be the case that certain receptor combinations are expressed by specific populations, or that populations maintain specificity of odour detection in an alternative manner. Population differentiation through odours may prove to be quantitative rather than qualitative. Each population may possess the same alleles, but express them in a specific manner, alternatively different populations may show different alleles.

The coding of olfactory information seems to be organised on a combinatorial scale: one OR recognises multiple odorants and one odorant is recognised by multiple ORs (Malnic *et al.*, 1999). Interestingly, the production of odours also seems to be due to combinative assortment. Yamazaki, *et al.* (1999), gave the basis of a comprehensive view of odortypes. Polymorphic genes, including major histocompatibility complex, are seen as agents of normal variation, which bestow quantitative variation in the production of odorants. So a slight change in odour between one population and another, may require a different combination of receptors to detect.

If the differences between populations are therefore based upon patterns of odorant receptor expression rather than qualitative differences in the receptors themselves, a study of population genetic consequences of olfactory imprinting and homing would be complex.

In order to establish fine-scale differences between populations, rather than genotyping each member of a sample of a population (as is the norm for population genetic studies) the pattern of OR expression for each individual would have to be determined. Such recent techniques as microarrays or suppressive subtractive hybridisation (SSH) may prove suitable. Differential expression studies of this type, however, would be lethal to the individuals involved.

It may be possible to detect olfactory differences in a non-lethal manner between populations on a larger scale. Discrete populations from different catchments may display qualitative differences in the OR genes themselves. This should be the pilot study for any population genetic studies based upon these genes. If qualitative differences (polymorphisms) are not observed between divergent populations, then they are not likely to be found on a finer scale.

In conclusion, there may be two population genetic consequences of olfactory imprinting and subsequent homing. Polymorphic OR loci may potentially be discovered by orthodox genotyping, but if population-odour specificity is detected by assortative

combinatorial expression of the same suite of receptors, then a *de novo* method of screening populations must be established.

5.2.7 Proposed model of olfactory imprinting and homing

Smolting is mediated endogenously by complex endocrine mechanisms. Although PST may occur *via* endogenous mechansims, it is stimulated or precipitated in parr by environmental cues (water temperature, photoperiod, lunar phase etc.). One of the prime candidates for the induction of physiological change are the thyroid hormones (particularly T4). Plasma T4 increases in response to increased discharge (Youngson *et al.*, 1986) and is thought to stimulate downstream migration (Youngson and Simpson, 1894). Thyroid hormones are also responsible for the stimulation of cytoarchitectural changes within peripheral neuronal tissue and the regulation of transcription of a vast array of genes (reviews by Wu and Koenig, 2000; Thompson and Potter, 2000). Odorant receptor expression has now been seen to increase transiently during smolting.

I therefore present a model of olfactory imprinting and subsequent homing. The endogenously or environmentally-triggered induction of PST results in alteration of endocrine status, including raised T4 levels in parr. This hormone, and others stimulate the anatomical, physiological and behavioural changes associated with smolting, including an increase in odorant receptor expression. When the smolting fish experience a locally-relevant trigger which may reportedly be a transient increase in stream flow-rate, raised stream temperature, or the progression of the lunar cycle, the disposition to downstream migration is triggered. The associated transient changes in T4 during the periods of increased migratory activity, particularly in conditions of raised discharge, facilitate the memory formation of homestream and novel odours, *via* an activity-dependent mechanism of cytoarchitectural change, involving an increase in primary receptor expression.

After the smolting process, receptor expression returns to presmolt levels, but differences in cellular architecture within the peripheral and/or central nervous sytem maintains the olfactory memory until maturity.

The rise in T4 levels experienced by migrating adults stimulates the expression of odorant receptors specific to previously imprinted odours. These olfactory-primed adults then begin to re-encounter these odours, and orientate towards them. This results in an activity-dependent feedback mechanism increasing odour sensitivity.

Increased sensitivity and positive rheotaxis combine to increase the chances that the fish will be able to follow the odour plume back to the natal site.

It is still inappropriate to speculate at this stage what nature of cytological changes result in memory formation. Future work may show that an increase in receptor expression is mediated by the clonal expansion of neurons and / or a change in the transcription of receptors per cell. This change in expression may also be seen to represent only the first stage in odour memory formation. By increasing the olfactory system's sensitivity to odours, the ability to form memory may be enhanced. The maintenance of odour-memory throughout the rest of the fishes' life is likely to occur at higher levels within the nervous system, but upon the return migration trigger the periphery of the system to become sensitised to the same odours once again.

5.3 CONCLUSIONS

The isolation and characterisation of both OR and VNR genes from *S.salar* has facilitated further elucidation of the olfactory changes associated with parr-smolt transformation. Both sets of primary receptors have representatives that are expressed in male germ cells as well as olfactory tissue.

Molecular evidence indicates that there is more than one incidence of specific-olfactory sensitivity involved in the smolting process. The olfactory imprinting process associated with PST concerns odours detected by both main olfactory receptors and vomeronasal receptors. The stimulation of expression of two independent groups of chemosensory receptors indicates that both odours and semiochemicals play a role in the imprinting process. The odorant receptors involved in olfactory imprinting vary between families of fish which suggests interfamilial differences in odour stimuli.

Further experimentation is required to determine the stimuli responsible for these changes in gene expression, the mechanism through which they arise, and the occurrence of olfactory changes at subsequent changes in this remarkable life cycle.

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APPENDIX I

CONSTRUCTION OF cDNA LIBRARY

Collection of Tissue Samples

The olfactory organs ("rosettes") were dissected from the olfactory pits of six adult Atlantic salmon, aged approximately 18 months, supplied by Hydroseafoods fish farm, near Connel, (Argyll, Scotland). The tissues were immediately stored on dry ice, then subsequently at -80°C before further processing.

Extraction of Total RNA

Total RNA was extracted from the olfactory epithelium of 5 of the farmed fish using TRIZOL™ reagent (Life Technologies, Gibco, BRL) according to the manufacturer's instructions. The resulting RNA suspensions were then pooled and used to create a cDNA library representative of a 'mixed population'.

Isolation of mRNA

Oligo dT cellulose (0.3g) was suspended in sterile Column Loading Buffer (CLB; 20mM Tris-HCl pH 7.6, 0.5M NaCl, 1mM EDTA, 0.1% SDS) and a 2.5ml chromatography column prepared by plugging a sterile syringe with DEPC-treated glass wool. The column was washed with 3 volumes 0.1M NaOH/5mM EDTA in DEPC H_2O, followed by 5 volumes of sterile CLB and the resulting effluent checked to ensure a pH of less than 8.

The pooled total RNA (15μl of which was retained as a control) was heated to 65°C for 10 minutes, an equal volume of 2X sterile CLB added, and the solution allowed to cool to room temperature before being applied to the column. The effluent from the column was collected, reheated to 65°C for 2 minutes and reapplied to the column. This stage was repeated so that the RNA passed through the column a total of four times. The effluent was retained and stored at -70°C for future use as a control, and the column washed with 10 volumes of sterile CLB, collecting the effluent as 10 separate fractions. These fractions were stored at -70°C for later UV spectrophotometric quantitation, in order to verify the complete elimination of $polyA^-$ RNA from the column.

The $polyA^+$ RNA was then eluted from the column in 3ml DEPC-treated H_2O and 6 X 500μl fractions collected. In order to precipitate the mRNA, 50μl 3M NaAc (pH 5.2) and 1ml cold ethanol was added to each fraction and incubated overnight at -20°C.The precipitates were then centrifuged at 14,000 rpm for 15 minutes, the supernatant

carefully discarded, the pellets washed in 500µl cold 70% ethanol and then resuspended in a total of 50µl DEPC-treated H_2O.

Synthesis, preparation and packaging of cDNA

A ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit was obtained from Stratagene. The manufacturer's protocol was strictly followed: first and second strand cDNA synthesis, blunting the cDNA termini, ligation and phosphorylation of *Eco*R1 adapters, *Xho*1 digestion, size fractionation and packaging.

The λ phage provided in the ZAP-cDNA® Gigapack® III Gold Cloning Kit, also contains pBluescript SK (-) plasmid DNA enabling the in vivo excision and recircularisation of the cDNA via the interaction of a variety of proteins, including f1 bacteriophage-derived proteins. The f1 phage proteins recocgnise a motif normally serving as the f1 bacteriophage origin of replication, containing both the sites of iniation and termination of DNA synthesis. This region of the target phage is made accessible to the f1 proteins through simultaneous infection of *E. coli* with both the lambda vector and the f1 bacteriophage.

Once the single stranded synthesis product is formed, it is recircularised by the gene II product from the f1 phage. The circular DNA resulting contains all DNA between the iniator and terminator, including the pBluescript phagemid and any insert.
During circularisation, a functional f1 origin is recreated producing a "subcloned" functional plasmid which can then be sequenced using the tradtional method.

SCREENING OF cDNA LIBRARY

In order to screen the library for OR genes, a protocol was designed, based upon that of Israel (1994).

The library is amplified in an array of 8x8 wells using a 96 well microtitre plate, at a titre of 1000pfu per well. The columns and rows are pooled, diluted 1:1 with sterile distilled water and used as a template for PCR with primers specific for the sequence in question. The agarose gel is then blotted and hybridised using probes directed at an internal portion of the sequence.

Any positive well can be identified using the row and column pools as coordinates. The positive well is then amplified at a lower titre (e.g. 30pfu per well),

pooled, and a second round of PCR and hybridisation performed in the same manner. Positive wells are then amplified at a lower titre still (e.g. 2 pfu per well), pooled, and a third round of PCR and probing carried out. The positive clones are then grown as plaques and can be individually analysed by PCR in order to verify their identity before the phagemid is excised, recircularised as a plasmid and then sequenced.

RESULTS & DISCUSSION

A total of 1,270,000 plaque forming units were produced by two packaging reactions. The resulting library, once the packaging reactions were combined and amplified (titre of 2.06 x 10^{11} pfu/ml), was stored at 4°C, with 500µl aliquots in DMSO maintained at -70°C.

Preliminary Screening using Actin Primers.

An overnight culture of host cells was infected and the library amplified at an initial titre of 1000pfu per well (x 64 wells). The rows and columns of wells were pooled and diluted 1:1, and 2µl removed from each of the 16 pools to be used as PCR template. The reaction conditions for amplification followed those optimised previous previously.

Four of the pooled templates gave a robust product of approximately 240bp, indicating that between two and four of the initially amplified wells putatively hold clones containing β actin cDNA.

Screening with SOR B primers.

Preliminary amplifications and rounds of PCR screening using SOR B specific primers (as described above) began unsuccessfully. However, the use of a primer specific to an internal portion at the 3' end of SOR B, and the primer, T3, specific to the 3' flanking region of the cloning site in the phage, a robust product of approximately 450bp was amplified. Since cDNAs are directionally cloned using ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit (Stratagene), it was thought that this 450bp product was from the 3' end of the gene, upstream of the SOR B sequence. However, upon aligning the primers and sequences (using SEQUENCHER™), it was realised that the pair of primers being used for PCR screening were actually complementary to the same template strand and therefore any amplified product was likely to be either single stranded and/or randomly amplified

Screening with SOR D primers

As screening efforts using the SOR B primers proved unsuccessful, efforst were directed towards the SOR D family. Evidence from the tissue expression pattern study suggested that SOR D sequences were confined to testicular tissue, at least within the experimental individual. However the possibility exists that SOR D-like sequences could be expressed in the olfactory epithelium of fish from another population. To this end, attempts were made at isolating OR genes using SOR D primers.

Wells containing positive clones were found throughout the PCR screening; verification through hybridisation was thought to be expensive in terms of labour and consumables and was therefore deemed unnecessary for this protocol. Positive clones were plated out as individual plaques after the tertiary screen and 20 plaques were separately screened. All showed bands of the appropriate size. The 20 plaques were cored in order to be "rescued" as a phagemid using *in vivo* single-clone excision, as supplied in the ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit (Statagene).

The underlying principles of phagemid excision are based upon the interaction of helper phage derived proteins on the lambda phage during the simultaneous infection of a host cell. (see *Synthesis, preparation and packaging of cDNA*).

Single plaques giving the appropriate sized products from SORD-primed PCR were excised in the manner outlined in the appendix and sequenced using ABI PRISM®, Big Dye™ Terminator Cycle Sequencing. The results of a BLAST search of GenBank indicated that these clones were false positives, implying that the screening protocol was inadequate.

Alternative cDNA library screening protocols were also attempted, based on hybridisation with Digoxigenin-labelled probes and isotopically labelled probes.

Finally, in an attempt to determine whether or not any of the candidate genes could be amplified from the library, T3 and T7 primers (priming sites flank the cloning region of the vector) were used in a PCR containing serial dilutions of the cDNA library as template. The PCR products generated were then southern blotted and hybridised with appropriate probes (*SORB*, *SORD*, β Actin and labelled cDNA library itself). Figure 5.3.1 shows that the signal of hybridisation was detected from only the positive controls.

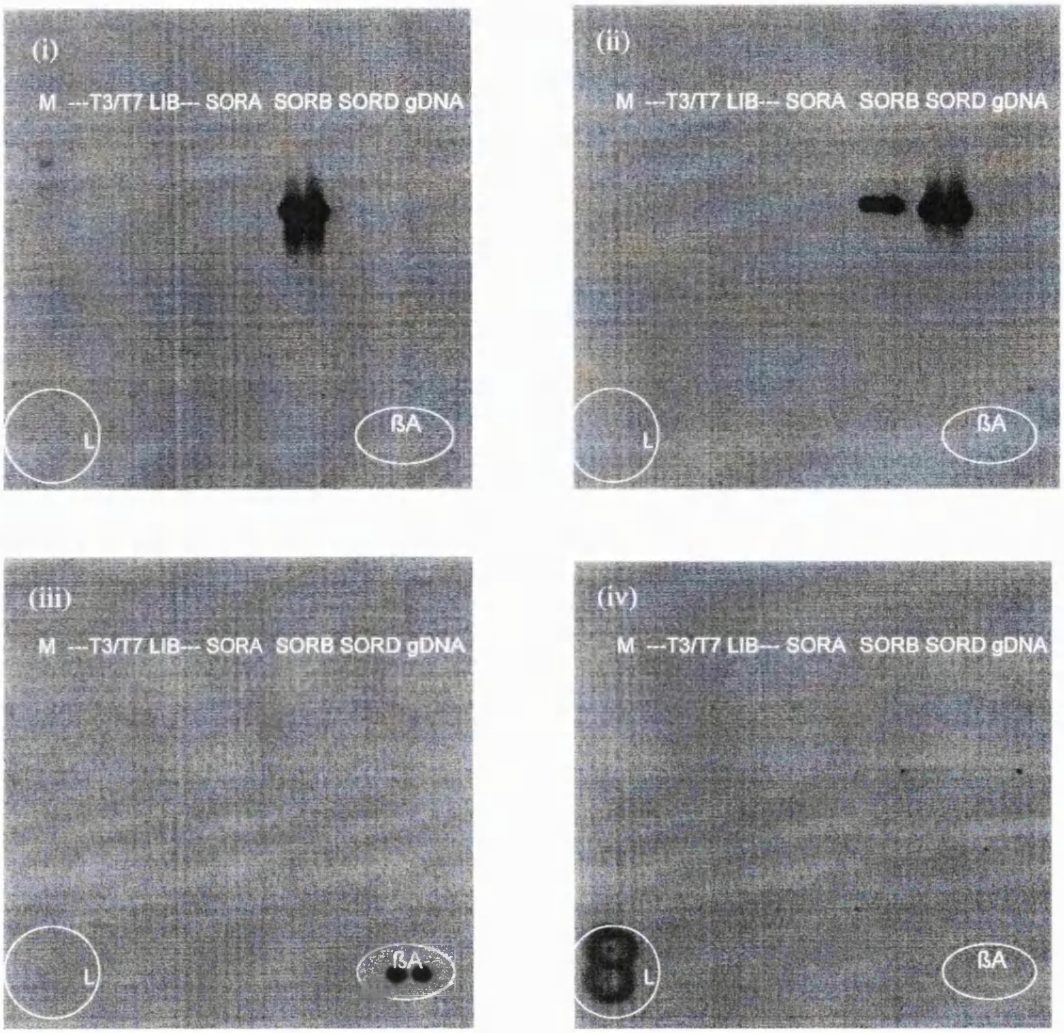


Figure 5.3.1 ***Hybridisation of T3/T7 amplified cDNA library with α-P^{32} labelled probes***
(I) = SORB probe hybridies only to southern-blotted SORB PCR products
(ii) = SORD probe hybridises with southern-blotted SORD PCR products, but also cross-hybridises with SORB PCR products
(iii) = β Actin probe hybridises only with dot-blotted β Actin PCR products
(iv) = Labelled library hybridises only with dot-blotted library
M = Molecular weight marker, βA = dot-blotted ß Actin PCR product, L = dot-blotted cDNA library

The "housekeeping" gene, β Actin was used as an absolute control to determine the presence of any transcripts within the cDNA library. β Actin PCR products were dot-blotted as postive controls, and used to generate a probe. Again, a strong positive signal was seen from the postive controls, but not from the amplified library, suggesting that the library did not actually contain any gene transcripts.

The phenol/chloroform extract of the library was also dot-blotted onto the same membrane and then subsequently labelled and used in hybridisation. The labelled library bound with the dot-blotted control, but not with the amplified library, the SOR products, nor the ß Actin PCR products.

The lack of hybridisation seen from the T3/T7 amplified library may simply be due to the inadequate transfer of DNA onto the membrane. However, when the library was used as a probe itself, no signal was seen from the ß Actin or SOR fragments dotted onto the membrane, indicating that these transcripts may not be present.

Verification of the quality of the cDNA library has to date, not been possible. Screening of false positives has resulted in the sequencing of random *S.salar* genes, as identified by BLAST searches (e.g. Transposases), individual plaques have been PCR amplified using T3/T7 primers showing inserts of >1Kb, so transcripts of the appropriate size are present. Whether the difficulty in isolating genes of interest lies in the inadequacy of the screening protocol or the inherent nature of the lamda phage library itself is yet to be resolved.

APPENDIX II

Kolmogorov-Smirnov Z test output of body length data

Testing for differences in the frequency distributions of body lengths between the experimental families.

January
Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Length	218	9.2399	1.4706	5	11.9
Family	218	1.5413	0.4994	1	2

January
Frequencies

	Family	N
Length	A	100
	B	118
	Total	218

January
Test Statistics

		Length
Most Extreme Differences	Absolute	0.376
	Positive	0.376
	Negative	0.000
Kolmogorov-Smirnov Z		2.765
Asymp. Sig. (2-tailed)		0.000

March
Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Length	236	9.8924	1.8601	5.8	13.0
Family	236	1.5508	0.4985	1.0	2.0

March
Frequencies

	Family	N
Length	A	106
	B	130
	Total	236

March
Test Statistics

		Length
Most Extreme Differences	Absolute	0.372
	Positive	0.372
	Negative	0.000
Kolmogorov-Smirnov Z		2.844
Asymp. Sig. (2-tailed)		0.000

May
Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Length	231	11.3766	1.8969	1.9	14.00
Family	231	1.5281	0.5003	1	2.00

May
Frequencies

	Family	N
Length	A	109
	B	122
	Total	231

May
Test Statistics

		Length
Most Extreme Differences	Absolute	0.327
	Positive	0.327
	Negative	-0.025
Kolmogorov-Smirnov Z		2.484
Asymp. Sig. (2-tailed)		0.000

APPENDIX III

ANOVA

Testing for a difference between the ΔCP values for each month

SORB Family A

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	195.749	4	48.937	3.204	0.025
Within Groups	488.767	32	15.274		
Total	684.516	36			

Reject Null Hypothesis.

SVRA Family A

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	126.959	4	31.740	3.779	0.011
Within Groups	327.528	39	8.398		
Total	454.487	43			

Reject Null Hypothesis.

SVRC Family A

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	58.91	4	14.727	2.909	0.034
Within Groups	197.416	39	5.062		
Total	256.326	43			

Reject Null Hypothesis.

ANOVA

Testing for a difference between the ΔCP values for each month

SORB Family B

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41.456	4	10.364	3.766	0.010
Within Groups	121.076	44	2.752		
Total	162.532	48			

Reject Null Hypothesis.

SVRA Family B

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.145	4	6.036	2.725	0.041
Within Groups	97.459	44	2.215		
Total	121.604	48			

Reject Null Hypothesis.

SVRC Family B

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.926	4	1.732	0.643	0.635
Within Groups	118.56	44	2.695		
Total	125.486	48			

Accept Null Hypothesis.

Post Hoc Tests

Pairwise comparison of mean ΔCt for each month to determine which months' values are significantly different.

Dunnet's T3

SORB Family A

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	5.5068	2.228	0.085	-.6160	11.6296
	May	0.6262	1.854	1.00	-6.4210	7.6735
	June	1.6133	1.954	0.987	-4.7141	7.9406
	Aug	-2.7186	2.111	0.909	-10.4296	4.9924
Mar	Jan	-5.5068	2.228	0.085	-11.6296	.6160
	May	-4.8806	2.141	0.094	-10.3538	.5927
	June	-3.8935	2.228	0.060	-7.9164	.1293
	Aug	-8.2254(*)	2.367	0.021	-15.0915	-1.3593
May	Jan	-0.6262	1.854	1.0	-7.6735	6.421
	Mar	4.8806	2.141	0.094	-.5927	10.3538
	June	0.9870	1.854	1.0	-4.8087	6.7828
	Aug	-3.3448	2.018	0.746	-10.7512	4.0616
June	Jan	-1.6133	1.954	0.987	-7.9406	4.7141
	Mar	3.8935	2.228	0.06	-.1293	7.9164
	May	-0.9870	1.854	1.0	-6.7828	4.8087
	Aug	-4.3318	2.111	0.328	-11.2209	2.5572
Aug	Jan	2.7186	2.111	0.909	-4.9924	10.4296
	Mar	8.2254(*)	1.367	0.021	1.3593	15.0915
	May	3.3448	2.018	0.746	-4.0616	10.7512
	June	4.3318	2.111	0.328	-2.5572	11.2209

* The mean difference is significant at the 0.05 level.

Dunnett T3

SVRA Family A

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	1.5345	1.375	.953	-3.1477	6.2168
	May	1.4398	1.375	.981	-3.5845	6.4642
	June	4.7473(*)	1.375	.027	.4817	9.0129
	Aug	0.3756	1.565	1.0	-5.2706	6.0218
Mar	Jan	-1.5345	1.375	.953	-6.2168	3.1477
	May	-.09474	1.296	1.0	-4.6426	4.4532
	June	3.2127	1.296	.078	-.2496	6.6750
	Aug	-1.159	1.496	.994	-6.4767	4.1587
May	Jan	-1.4398	1.375	.981	-6.4642	3.5845
	Mar	0.094739	1.296	1.0	-4.4532	4.6426
	June	3.3075	1.296	.147	-.7413	7.3562
	Aug	-1.0642	1.496	.998	-6.6198	4.4913
June	Jan	-4.7473(*)	1.375	.027	-9.0129	-.4817
	Mar	-3.2127	1.296	.078	-6.6750	.2496
	May	-3.3075	1.296	.147	-7.3562	.7416
	Aug	-4.3717	1.496	.103	-9.5256	.7821
Aug	Jan	-0.3756	1.565	1.0	-6.0218	5.2706
	Mar	1.159	1.496	.994	-4.1587	6.4767
	May	1.0642	1.496	.998	-4.4913	6.6198
	June	4.3717	1.496	.103	-.7821	9.5256

* The mean difference is significant at the 0.05 level.

Dunnett's T3

SVRC Family A

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	1.2286	1.067	.954	-2.8160	5.2732
	May	0.6920	1.067	1.0	-3.8141	5.1981
	June	2.9464	1.067	.185	-1.0291	6.9218
	Aug	-0.395	1.215	1.0	-5.0758	4.2858
Mar	Jan	-1.2286	1.067	.954	-5.2732	2.8160
	May	-0.5366	1.006	1.0	-4.0044	2.9312
	June	1.7178	1.006	0.119	-.2919	3.7275
	Aug	-1.6236	1.162	0.741	-5.4904	2.2433
May	Jan	-0.6920	1.067	1.0	-5.1981	3.8141
	Mar	0.5366	1.006	1.0	-2.9312	4.0044
	June	2.2543	1.006	.277	-1.0398	5.5485
	Aug	-1.087	1.162	.990	-5.4034	3.2295
June	Jan	-2.9464	1.067	.185	-6.9218	1.0291
	Mar	-1.7178	1.006	.119	-3.7275	.2919
	May	-2.253	1.006	.277	-5.5485	1.0398
	Aug	-3.3413	1.162	.093	-7.2319	.5492
Aug	Jan	0.395	1.215	1.0	-4.2858	5.0758
	Mar	1.6236	1.162	.741	-2.2433	5.4904
	May	1.087	1.162	.990	-3.2295	5.4034
	June	3.3413	1.162	.093	-.5492	7.2319

* The mean difference is significant at the 0.05 level.

Dunnett's T3

SORB Family B

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	-.6356	0.742	.994	-3.227	1.9558
	May	-2.72	0.742	.079	-5.6427	.2028
	June	-.855	0.762	.959	-3.4744	1.7645
	Aug	-.8107	0.742	.925	-3.1913	1.5699
Mar	Jan	0.6356	0.742	.994	-1.9558	3.227
	May	-2.0844	0.742	.162	-4.6575	.4887
	June	-.2194	0.762	1.0	-2.3824	1.9435
	Aug	-.1751	0.742	1.0	-1.9455	1.5953
May	Jan	2.72(*)	0.742	.079	-.2028	5.6427
	Mar	2.0844	0.742	.162	-.4887	4.6575
	June	1.8650	0.762	.271	-.7367	4.4666
	Aug	1.9093	0.742	.149	-.4495	4.268
June	Jan	0.855	0.762	.959	-1.7645	3.4744
	Mar	0.2194	0.762	1.0	-1.9435	2.3824
	May	-1.865	0.762	.271	-4.4666	.7367
	Aug	0.04429	0.762	1.0	-1.7974	1.8859
Aug	Jan	0.8107	0.742	.925	-1.5699	3.1913
	Mar	0.1751	0.742	1.0	-1.5953	1.9455
	May	-1.9093	0.742	.149	-4.286	.4495
	June	-0.04429	0.762	1.0	-1.8859	1.7974

* The mean difference is significant at the 0.05 level.

Dunnett's T3

SVRA Family B

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	1.1976	0.666	.644	-1.0739	3.4691
	May	1.6403	0.666	.282	-.6550	3.9356
	June	2.0964	0.684	.087	-.1967	4.3895
	Aug	1.4815	0.666	.251	-.5511	3.5141
Mar	Jan	-1.1976	0.666	.644	-3.4691	1.0739
	May	0.4427	0.666	.999	-1.7603	2.6458
	June	0.8988	0.684	.868	-1.3026	3.1002
	Aug	0.2839	0.666	1.0	-1.6291	2.1970
May	Jan	-1.6403	0.666	.282	-3.9356	.6550
	Mar	-.4427	0.666	.999	-2.6458	1.7603
	June	0.4561	0.684	.999	-1.7704	2.6825
	Aug	-.1588	0.666	1	-2.1050	1.7874
June	Jan	-2.0964	0.684	.087	-4.3895	.1967
	Mar	-.8988	0.684	.868	-3.1002	1.3026
	May	-.4561	0.684	.999	-2.6825	1.7704
	Aug	-.6149	0.684	.963	-2.5635	1.3337
Aug	Jan	-1.4815	0.666	.251	-3.5141	.5511
	Mar	-.2839	0.666	1.0	-2.1970	1.6291
	May	.1588	0.666	1.0	-1.7874	2.1050
	June	.6149	0.684	.963	-1.3337	2.5635

* The mean difference is significant at the 0.05 level.

Dunnett's T3

SVRC Family B

Month (1)	Month (2)	Mean Difference (1-2)	SE	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Jan	Mar	0.63	0.734	.993	-3.115	1.8549
	May	1.1437	0.734	.752	-3.56	1.2727
	June	0.5398	0.754	.998	-3.1329	2.0533
	Aug	0.371	0.734	1.0	-2.8038	2.0619
Mar	Jan	-0.63	0.734	.993	-1.8549	3.115
	May	0.5136	0.734	.996	-2.7003	1.673
	June	0.090211	0.754	1.0	-2.3085	2.489
	Aug	-0.259	0.734	1.0	-1.9477	2.4657
May	Jan	-1.1437	0.734	.752	-1.2727	3.56
	Mar	-0.5136	0.734	.996	-1.6730	2.7003
	June	-0.6038	0.754	.991	-1.7221	2.9298
	Aug	-0.7727	0.734	.930	-1.3464	2.8917
June	Jan	-0.5398	0.754	.998	-2.0533	3.1329
	Mar	-0.09021	0.754	1.0	-2.489	2.3085
	May	0.6038	0.754	.991	-2.9298	1.7221
	Aug	-0.1688	0.754	1.0	-2.1747	2.5123
Aug	Jan	-0.3710	0.734	1.0	-2.0619	2.8038
	Mar	0.2590	0.734	1.0	-2.4657	1.9477
	May	0.7727	0.734	.930	-2.8917	1.3464
	June	0.1688	0.735	1.0	-2.5123	2.1747

* The mean difference is significant at the 0.05 level.

APPENDIX IV
PROTOCOLS

TRIZOL Reagent: *Total RNA Isolation Reagent*

1. Homogenisation

Tissues.

Macerate tissue in liquid N_2 in RNAse-free pestle and mortar. Transfer macerated tissue to a fresh eppendorf and homogenise tissue samples in 1 ml TRIZOL reagent per 50-100 mg of tissue.

2. Phase Separation

Incubate the homogenised samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml chloroform per 1 ml TRIZOL reagent. Cap sample tubes securely. Shake tubes vigourously by hand for 15 secs and incubate them at 15 to 30°C for 2 or 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 mins at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourles upper aqueous phase. RNA remains exclusively in this aqueous phase - the volume being about 60% of the TRIZOL used for homogenisation.

3. RNA Precipitation

Transfer aqueous phase to a fresh eppendorf and retain organic phase for isolation of DNA and/or protein. Precipitate RNA from the aqueous phase by mixing with Isopropanol. Use 0.5 ml isopropanol per 1 ml of TRIZOL reagent used for original homogenisation. Incubate samples at 15 to 30°C for 10 mins and centrifuge at no more than12,000g for 10 mins at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA Wash

Remove the supernatant. Was the RNA pellet once with 75% ethanol, adding at least 1 ml 75% EtOH per 1 ml TRIZOL originally used. Mix the sample by vortexing and centrifuge at no more than 7,500g for 5 mins at 2 to 8°C.

5. Resuspending RNA

Remove supernatant carefully. Briefly dry RNA pellet (air-dry, or vacuum-dry for 5-10 mins). Do not dr the RNA by centrifugation under vacuum. It is important not to let the pellet dry completely as this will greatly reduce the solubility of the RNA. Partially dissolved RNA samples have a $A_{260/280}$ ratio of < 1.6. Dissolve RNA in Rnase-free water 0r 0.5% SDS solutuion by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.

6. Running an RNA check gel

50 ml, 1.5% GEL:		20 µl SAMPLE:	
DEPC-Treated H_2O	36.5 ml	RNA	4 µl
10 X MOPS	5.0 ml	Deion. Formamide	10 µl
Agarose	0.75 g	EtBr (500 ng/µl)	1 µl
		Loading Buffer	2 µl
		DEPC-Treated H_2O	3 µl

Add MOPS, water and agarose to a conical flask, boil the mixture, then cool to hand-warm, then in a fume hood, add 8.5 ml Formaldehyde. Swirl to mix, then pour into gel tray and add comb

Heat samples to 65°C for 10 mins, then snap chill on ice before loading.

REAGENTS:

10 X MOPS:
400mM Morpholinopropanesulphonic acid pH7.0
100mM Sodium Acetate
10mM EDTA pH 7.0 (make up using DEPC-treated water)

Formamide Deionize by passing through mixed bed resin until pH is neutral.

LOADING BUFFER (100 ml):

50 ml Glycerol
1mM EDTA
o.4 % Bromophenol Blue
Make up to 100 ml with DEPC-treated water

DEPC-TREATED WATER

Add 100 µl of diethylpyrocarbonate (DEPC) to 1 l MilliQ double-distilled H_2O. Let stand over-night and autoclave.

FIRST STRAND cDNA SYNTHESIS KIT: *Amersham Pharmacia Biotech*

1. Place a Dnase-treated RNA sample in an eppendorf and add Rnase-free water, if necessary to bring the RNA to the appropriate volume (8 µl or 20 µl, see below).
2. Heat the RNA solution to 65 °C for 10 minutes, then chill on ice.
3. Gently pipette the **bulk first-strand cDNA reaction mix** to obtain a uniform suspension. Add the appropriate volume of mix to a sterile eppendorf. To this tube add 1µl of **DTT solution**, 1µl of the chosen primer at the appropriate concentration (see below) and the heat-denatured RNA. Pipette up and down several times to mix.
4. Incubate at 37 °C for 1 hour.
5. The completed first-strand cDNA reaction product is now ready for immediate second-strand cDNA synthesis or PCR amplification.

Table 1. Volumes of components in first-strand reaction

Bulk first-strand reaction mix	Primer[a]	DTT solution	RNA	Final volume first-strand reaction
11µl	1µl	1µl	20µl	33µl
5µl	1µl	1µl	8µl	15µl

Table 2. Recommended quantities of primer for first-strand synthesis (in 1µl)

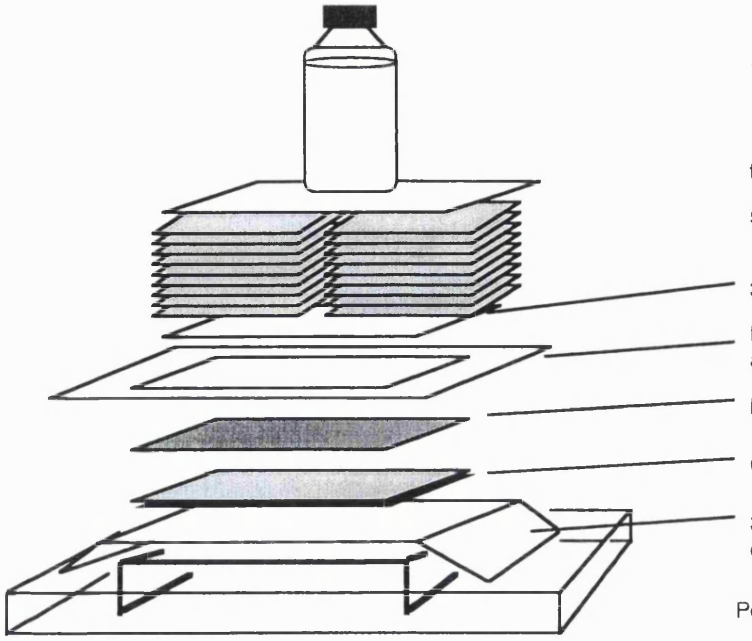
	Intended application	
First-strand cDNA primer	Second-strand synthesis	PCR-amplified synthesis
pd $(N)_6$	0.2-0.02 µg[b]	0.2 µg
Not I-d$(T)_{18}$	5 µg	0.2 µg[c]
Specific primer	40-400 pmol	20-40 pmol

[a] The primer must be added to the reaction in a volume of 1 µl; in some cases, dilution may be required (see above). If dilution is required, perform the dilution using **Rnase-free water**, and use 1 µl of the diluted primer for first-strand cDNA synthesis.
[b] undiluted → 1:10 dilution. When using random primers to prime first-strand synthesis prior to Gubler-Hoffman second-strand synthesis, the size of the cDNAs obtained will depend on the amount of pd $(N)_6$ primer added. In general, the more pd$(N)_6$ added, the shorter the cDNAs produced.
[c] 1:25 dilution - required if 1st-strand cDNA is used as template for PCR.

DNA TRANSFER FROM AGAROSE GELS

Ammonium Acetate Transfer Method

1. Set up the box, bridge and first 3 layers of filter paper as shown in the figure. Wet the filter paper with 1 M ammonium acetate/0.04 M NaOH. remove bubbles from the underside of the paper with a glass rod. Leave sevel mm of liquid on top of the paper.
2. After electrphoresis, cut through the centre of the wells with a scalpel blade and discard the gel behind the lanes.
3. Soak the gel in a tray containing 1.5 M NaCL, 0.5 M NaOH twice for 15 minutes each.
4. Soak gel in 1 M ammonium acetate/0.04 M NaOH twice for 15 mins each.
5. Carefully flip gel over so the smooth bottom of the gel is facing up and slide it onto the filter paper wicks on the transfer bridge.
6. Gently squeeze out bubbles from beneath gel with glass rod.
7. Have a nylon membrane cut to the appropriate size, labelled at the bottom in pencil, with a nick cut in the top left corner and dampened in dH_2O.
8. Place the membrane on the gel with the label side downwards and the nick at the well end. Be sure to put it on as straight as possible as moving the membrane around on the gel may create shadow bands.
9. Repeat step 6.
10. Place a plastic film over the membrane with a window cut out to the size of the gel.
11. Add 3 layers of 3MM paper wetted with 1 M ammonium acetate/0.04 M NaOH, squeeze out air bubbles, and carefully pile on stacks of paper towels. Weigh down towels with a litre bottle of water on a tray, to ensure good contact between the gel, the membrane and the wicking paper.
12. Leave overnight for the transfer to proceed and top up transfer solution as required.
13. Remove wet towels and 3MM paper and discard.
14. Lift off nylon membrane and neutralise in ~200ml 2 X SSC for several minutes.
15. Blot excess moisture from membrane and leave to dry.
16. Cross-link DNA by baking membrane at 80°C for 2 hours (protected by envelope of 3MM paper) or in UV cross-linker for 30 seconds.



Random Primed DNA Labelling Kit (Roche, Boehringer Mannheim)

1. Denature the DNA by heating for 10 mins at 100°C and subsequent snap-chilling on ice.
2. Add the following to a screw-cap eppendorf on ice and make up to a final volume of 20 µl:
   25 ng denatured DNA
   1 µl dATP (solution 2)
   1 µl dGTP (solution 4)
   1 µl dTTT (solution 5)
   2 µl Reaction Mixture (solution 6)
   5 µl 50 µCi [α^{32}P] dCTP, 3000 Ci/mmol, aqueous solution
   1 µl Klenow enzyme (solution 7)
3. Incubate for 1 hour at 37°C.
4. Stop the reaction by adding 2 µl 0.2 M EDTA, (pH 8.0), and/or by heating to 65°C for 10 min.

Hybridisation

Reagents required:
20% SDS
10% BSA
0.5 M Na-phosphate buffer (0.5 M Na_2HPO_4 + 4ml 85% H_3PO_4 / litre)
0.5 M EDTA (pH 8.0)

Prehybridisation

1. Make up 2 x 20 ml of Church Prehyb/Hyb buffer in 50 ml Falcon tubes:

For 20 ml:	
7 ml	20% SDS
10 ml	0.5M Na-phosphate buffer
2 ml	10% BSA
1 ml	H20
40 µl	0.5M EDTA (pH 8.0)

   Use one lot for prehybridisation and the second for hybridisation.
2. Place southern blot membrane on top of mesh and dampen with ~5ml prehyb. buffer.
3. Roll mesh & blot tightly into tube shape and place in hybridisation cylinder - allow outside edge of mesh to adhere to glass cylinder and slowly turn cylinder so that mesh unrolls against the inside surface.
4. Add remainder of prehybridisation buffer and place tube in rack in hybridisation oven, with balance tube directly opposite.

5. Set temp to 65°C and turn drive on. Prehyb. for 2 hours - overnight.

Hybridisation

1. Place hybridisation buffer in oven for 30 mins to bring it up to temperature.
2. 2 Heat labelled probe at 95 - 100 °C for 10 mins to denature. Place on ice immediately.
3. Quickly spin probe down to bottom of tube & replace on ice.
4. Pour of prehybridisation buffer from cylinder, add fresh hybridisation buffer, and then add labelled probe.
5. Hybridise at 65°C for 12 - 48 hours depending on target DNA type (PCR products = shorter time, single copy sequences - longer)

Washing

1. Make up Church washing Buffer:
   Per litre:
   40 ml 0.5 M Na-phosphate buffer
   5 ml 20 % SDS
2. Place 2 x 20ml washing buffer in 50 ml Falcon tubes at 65°C for 30 mins to bring it to temperature.
3. Pour off hybridisation buffer, and add 20 ml washing buffer. Incubate in hybridisation oven at 65°C for 30 mins.
4. Pour off first wash and repeat stage 4.
5. Remove mesh and blot from cylinder into plastic box, wrap in Saran wrap, and tape into position on old piece of X-ray film in cassette
6. Expose to fresh X-ray film for 2 hours - 1 week depending on strength of signal.
7. Develop film.

U.S. Dept Commerce/NOAA/NMFS/NWFSC/Molecular Biology Protocols

Ribonuclease Protection Assay

contributed by James McCaughern-Carucci, Yale University

Most RNase Protection protocols require an overnight hyb with numerous subsequent clean-up steps. This method requires a maximum hyb of four hours, and the clean-up steps are the barest minimum, yet still produce nice images. It is strongly recommended to titer RNase concentrations with probes prior to running experiments...some require more RNase than others.

Part I: In Vitro Transcription

In a sterile 1.5ml microfuge tube, combine the following:
(all reagents obtained from Promega)
4 µl 5x Transcription Buffer
2 µl 0.1M DTT
4 µl 2.5mM NTP's (A, C, G)
0.8 µl RNasin RNase Inhibitor (25U/ul) (Promega)
2.4 µl of 100uM cold UTP (Note: Use 1mM UTP for loading control probes e.g. B-actin)
1 µl of 1 ug/ul linearized DNA template
5 µl of 10 uCi/ul P32 UTP (800Ci/mmol) (Dupont NEN - #NEG507X), or 1 µl for loading control probes
1µl RNA polymerase SP6,T7 or T3 (concentration varies by vendor)
Total Volume ~20µl.

Incubate 1 hour @ 37C.
Add 2µl of DNase I (Promega) to each transcription, incubate 20 minutes @ 37C.

Part II: Probe Purification

Purify probes using QIAGEN QIAquick Nucleotide Removal Kit or Boehringer Spin Columns (G50 Sephadex).
Check 1µl in scintillation counter, P32 channel. A good probe will be ~ $5x10^5$ to $1x10^6$ cpm.

Part III: Hybridization

Turn heatblock on to 95C.
For samples in water or ethanol, dry down appropriate amount of RNA, and include a tube with 1µl of tRNA or Glycogen (Sigma), this is the negative control.
Each sample should have the following:
24µl Formamide
2µl 0.6M PIPES
2.4µl 5M NaCl
0.3µl 0.1M EDTA
2 $x10^5$ cpm main probe
5 $x10^4$ cpm loading control probe
DEPC water

Total Volume 30µl

Mix samples well. Heat @ 95C for 10 minutes. Incubate 4 hours @ 55C.

Part IV: RNase Digestion

RNase Digestion Buffer:
300mM NaAc
10 mM TRIS
5 mM EDTA
To each sample add 350µl Digestion buffer.
Add 1µl of 4mg/ml RNase A and 0.4µl of 10u/µl RNase T1.
Incubate @ 30C for 45 minutes to 1 hour.

Part V: Proteinase K

To each sample add 10µl of 20% SDS and 2.5µl of 10mg/ml Proteinase K. Incubate @37C for 15-20 minutes.

Part VI: Clean-Up

Extract once with 400µl of Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
Transfer the supernatant to a new tube. Add 1000µl of 100% ETOH and 1µl of 10mg/ml of Glycogen. Mix well.
Incubate samples at -70C for 30 minutes or in a dry ice/ETOH bath for 10 minutes.
Spin in microfuge for 15 minutes. Aspirate ETOH. Allow pellets to air dry.
Resuspend in 8ul of Formamide based loading dye. Allow to sit at RT for 5-10 minutes, with frequent mixing.

Part VII: Polyacrylamide Gel Analysis

Heat samples for 5 minutes @ 100C. Load onto a 5% polyacrylamide/7M Urea denaturing gel with 2000cpm of a molecular weight marker (dCTP labelled pBR 322 MspI digest works nicely).
Run gel @ 38-42 mA. Dry gel, expose to film O/N at -70C with an intensifying screen.

Troubleshooting and Notes

1. No Discrete Bands, Only Smears :
RNA degraded, or the the RNase Digestion was too harsh.
Try reducing the concentration of RNase A to 1 mg/ml or digest for a shorter period of time. Check your RNA for condition.
2. Bands Too Large, High Molecular Weight Artifacts:
The RNase Digestion was inefficient and unable to effectively trim down the RNA:RNA duplexes.
Try RNasing longer or increasing the RNase concentration.
3. Incompletely linearized template DNA. Bands in the Negative Control Lane:
Inefficent RNase Digestion (see above).
4. Sense template contaminating riboprobe:
Insufficent DNase digestion of riboprobe.
5. Many Lower Molecular Weight Bands Under the Main Band:
There can either be premature stop sites in the probe leading to smaller probe sizes, therefore smaller products. This can also stem from overdigestion by RNase A which will break-up the duplexes if the concentration or digestion time is too long.
6. No Signal At All:
You did generate an antisense probe right?

DNA ISOLATION FROM ORGANIC PHASE OF TRIZOL RNA ISOLATION

1. DNA Precipitation with Ethanol

Add 0.3 ml of 100% ethanol (EtOH) per 1 ml TRIZOL used, and mix by inversion. Incubate samples at 15 to 30°C for 2 to 3 mins and then centrifuge at no more than 2,000 x g for 4 mins at 4°C.

2. DNA Wash

Remove Phenol-Ethanol supernatant carefully and retain for possible protein isolation. Wash the pellet in 0.1 M Sodium Citrate / 10% EtOH, using 1 ml solution per 1 ml TRIZOL used in original tissue homogenisation. Shake tube periodically whilst incubating at 15 - 30°C for 30 mins, then centrifuge at 2,000 x g for 5 mins.
Repeat this process 3 times for tissue-derived samples.
Wash the pellet in 1.5 ml 75% EtOH, incubate at 15 - 30°C for 10 to 20 mins, then centrifuge at 2,000 x g for 5 mins at 4 °C.

3. Resuspending DNA

Carefully remove the EtOH supernatant, then air-dry or vacuum-dry for 5 to 10 mins.
Redissolve the pellet in 500 µl 8mM NaOH, to a give a [DNA] ~ 0.2 - 0.3 µg / µl.

QIAquick Gel Extraction Kit Protocol

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimise the size of the gel slice by removing excess agarose.
2. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Incubate at 50°C for 10 minutes, or until the gel slice has completely dissolved. To help dissolution, vortex the tube every 2 to 3 mins during the incubation.
4. After the gel slice has completely dissolved, check that the colour of the mixture is yellow. If the colour is violet, add 10 µl of 3M sodium acetate, pH 5.0. The colour should return to yellow.
5. Add 1 gel volume of isopropanol to the sample and mix. Do not centrifuge the sample at this stage.
6. Place a QIAquick spin column in a 2ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min. The max. volume of the reservoir is 800 µl. For samples in excess of 800 µl, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. This step removes all traces of agarose and is only required for direct sequencing, *in vitro* transcription or microinjection.
10. To wash, add 0.75 ml of PE buffer to QIAquick column and centrifuge for 1 min. *N.B.* If the DNA is to be used for salt-sentive applications, such as blunt-ended ligation and direct sequencing, let the column stand for 2-5 mins after addition of PE before centrifuging.
11. Discard the flow-through and centrifuge the QIAquick column for an additional minute at 10,000 x g (~13,000 rpm). Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Place the QIAquick column into a fresh 1.5 ml eppendorf.
13. To elute DNA, add 50 µl buffer EB (10mM Tris-Cl, pH 8.5) or H2O to the centre of the QIAquick column and centrifuge for 1 minute at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the centre of the QIAquick column, let stand for 1 minute and then centrifuge at maximum speed.

Original TA Cloning® Kit (pCR®II, pCR2.1) INVITROGEN®

Production of PCR products

1. Amplify PCR products using your own protocol.
2. Ananlyse PCR sample by gel electrophoresis.
3. Extract PCR product from gel & quantify DNA.

Cloning into pCR® vector

1. Ligation Reaction

Sterile Water		5 µl
10X Ligation Buffer	1 µl	
pCR® vector (25 ng/µl)		2 µl
Fresh PCR product (~10 ng)	1 µl	
T4 DNA ligase		1 µl

2. Incubate ligation reactions at 14°C for at least 4 hours (preferably overnight).
3. Centrifuge the ligation reactions briefly and place on ice.

Transformation

1. Thaw appropriate number of vials of One Shot™ cells on ice.
2. Pipette 2 µl of 0.5 M β-ME into each vial of One Shot™ cells and mix by stirring gently with a pipette tip.
3. Pipette 1-2 µl of each ligation reaction into cells and stir gently with a pipette to mix.
4. Incubate the vials on ice for 30 minutes.
5. Heat shock for exactly 30 seconds in a 42°C water bath. Do not mix.
6. Place the vials on ice for 2 minutes.
7. Add 250 µl of SOC medium to each vial.
8. Shake the vials at 37°C in a shaker at 225 rpm for exactly 1 hour. Place the vials with the transformed cells on ice.

Analysis

1. Plate 50 µl and 150 µl from each transformation on an LB plate containing 50 µg/ml ampicillin and X-Gal. Add IPTG if using TOP10F' cells.
2. Incubate at 37°C for at least 18 hours. Shift plates to 4°C for 2 to 3 hours for colour development.
3. Analyse 10 white transformants for the presence and orientation of insert by restriction mapping or sequencing.

Setting up Mini-Prep cultures

LB per litre:
10 g

1. Add 5ml LBAmp (100 μg / ml) to sterile culture vessel (15 ml Falcon tube).
2. Using aseptic technique, inoculate each 5 ml culture with a SINGLE white transformant colony, and flame neck and top of Falcon before replacing top.
3. Incubate overnight at 37 °C, shaking at 225 rpm.

Purification of Plasmid DNA (*ABI Alkaline Lysis*)

Reagents:

GTE buffer (50 mM Glucose, 25 mM Tris pH8.0, 10mM EDTA pH8.0)
0.2 N NaOH / 1% SDS (freshly made)
3.0 M Potassium Acetate, pH 4.8
Rnase A (Dnase-free, 10 mg / ml)
Chloroform
Isopropanol
4.0 M NaCl
13% PEG_{8000} (autoclaved)

1. Pellet 1-5 ml aliquots of overnight culture for 1 min in a microcentrifuge.
2. Remove supernatant completely and resuspend bacterial pellet in 200 μl of GTE buffer by pipetting up and down.
3. Add 300 μl freshly prepared 0.2 N NaOH / 1% SDS, mix by inversion and incubate on ice for 3 mins.
4. Neutralise by adding 300μl of 3.0 M Potassium Acetate, pH 4.8, mix by inversion and again incubate on ice for 3 mins.
5. Remove cellular debris by centrifuging for 10 min at room temp, and then transfer supernatant to a clean tube.
6. Add 2 μl Rnase A (10 mg / ml) and incubate at 37°C for 20 mins.
7. Extract supernatant twice with 400 μl chloroform. Mix the phases by hand for 30 secs after each extraction. centrifuge the tube for 1 min to separate the phases and remove upper aqueous phase to a fresh tube.
8. Precipitate the total DNA by adding an equal volume of 100% isopropanol and immediately centrifuging the tube for 10 mins at room temp.
9. Wash the pellet with 500 μl EtOH and then dry under vacuum for 5 min.
10. Dissolve the pellet in 32 μl dH_2O and precipitate the plasmid DNA by first adding 8μl of 4 M NaCl and then adding 40 μl of autoclaved PEG_{8000}
11. After thorough mixing, incubate the sample on ice for 20 min and then pellet the plasmid DNA by centrifugation for 15 min at 4°C.
12. Carefully remove the supernatant and rinse the pellet with 300μl of 70% EtOH. Dry the pellet under vacuum for 5 minutes and then resuspend in 20 μl dH_2O - store at -20°C.

Restriction Analysis of Transformants

1. For each clone add the following:

To a total volume of 20 μl:	
10 X Eco *R*1 buffer	2 μl
Eco *R*1 enzyme	1 μl
Plasmid DNA	2 μl
dH_2O	15 μl

2. Incubate reactions at 37 °C for 1 hour.
3. Add 2 μl loading buffer, then load onto 2% agarose gel.

SEQUENCING PROTOCOL

Following identification of positive transformants via restriction analysis:

1. From ABI prism kit (with which you will get a protocol), add per sample:

BIG DYE Terminator reaction mix 4ul
Internal sequencing/PCR primer (3.2pmol/ul) 1ul
Plasmid template (check first on agarose gel - need approx 250ng per rxn.) usually between 1 μl and 2.5μl
Make up to 10 μl l with MQ H2O

2. Amplify using thermocycling profile as specified in kit:
25 cycles of:
Rapid thermal ramp to 96°C
96°C for 10 sec.
Rapid thermal ramp to 50°C
50°C for 5 sec.
Rapid thermal ramp to 60°C
60°C for 4 min.
Rapid thermal ramp to 4°C and hold until ready to purify.

Purification of Extension Products

1. Transfer entire vol. of amplification prduct from above to eppendorf and add to each sample:

3M NaOAc	1 µl
Etoh	25 µl

2. Vortex and place on ice for 10 mins, then spin 13000rpm for 20mins.
3. Carefully remove all supernatant and rinse pellet with 125µl 70% Etoh
4. repeat spin from 4
5. dry pellets in speedvac
6. retain dry pellet for loading onto gel.