Genetic Variation for Disease Resistance in Rainbow Trout
(Oncorhynchus mykiss)

By

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Dedicated to my parents, David and Linda Butterfield
Declaration

I declare that this thesis has been compiled by myself and is the result of my own investigations. It has not been submitted for any other degree, and all sources of information have been duly acknowledged.

Gareth Melgalvis Butterfield
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Abstract

Proliferative Kidney Disease (PKD) caused by the Malacosporean parasite *Tetracapsuloides bryosalmonae*, is presently the most economically damaging disease of British rainbow trout farming, costing the industry in excess of £2.5 million per annum in the UK alone. With no vaccine or prophylactic treatment available, and only management techniques currently adopted to minimise the stress and mortality associated with the disease, alternative approaches must now be considered. This document investigates if selective breeding for PKD resistance is possible by assessing the level of additive genetic variation, and calculating the subsequent estimates of heritability, for commercial strains of rainbow trout.

During a PKD outbreak on a commercial farm, 1500 communally reared juvenile rainbow trout from two strains (Houghton Spring and Isle of Man) were sampled on a single day, their body weight and fork length measured, and severity of kidney swelling scored according to the scale of Clifton-Hadley *et al.* (1987). Fish were assigned to individual families using microsatellite parentage assignment. Significant additive genetic variation was observed in the population, and families were ranked according to estimated breeding values. A combined estimate of heritability ($h^2 = 0.19 \pm 0.08$) for kidney score suggests the population will respond well to selective breeding for kidney score, which may be deemed a measure of resistance, whilst the favourable genetic correlations between kidney score and the production traits measured suggest simultaneous selection for kidney score and growth traits should also be effective. In order to support the findings of the initial research, controlled challenge experiments were conducted. Using the family EBV information on kidney score from the IoM strain (due to its certification as a disease-free site), four females, two with high and two with low response to PKD, were each crossed with a randomly selected neomale to produce twenty two families for PKD challenge experiments.

The PKD experimental challenges showed evidence of additive genetic variation to kidney score over an eleven week period, supporting initial findings. A low score was deemed as evidence of greater resistance to the parasite in this study. Although female EBV was taken into consideration in the statistical model, there was found to be no significant difference in resistance according to family. Immunohistochemistry stained kidney sections from each individual involved in the challenges proved kidney score correlated significantly to the number of parasites in the kidney, suggesting that the scale of Clifton-Hadley *et al.* (1987) is a sufficient and accurate basis on which to describe the severity of PKD, and infection level in rainbow trout.

Having discovered evidence that furunculosis, causative agent *Aeromonas salmonicida*, plays a major role in the mortality of fish suffering from PKD in the field, the bacterial disease was investigated to assess the resistance of the same families used in the PKD challenges. Twenty one of the families were used to discover that additive genetic variation for resistance to furunculosis is apparent when assessed as both a binary and longitudinal trait, suggesting significant genetic improvement can be made to increase resistance to furunculosis in the IoM stock. No significant correlation was observed between kidney score, EBV, and resistance to this bacterium, but there was a positive phenotypic correlation found between furunculosis resistance and size, suggesting simultaneous selection for performance and resistance is possible within this population.
Chapter 1: General Introduction
1.1 **Rainbow Trout**

1.1.1 **The family Salmonidae**

Rainbow trout are a member of the family Salmonidae, a small group of fishes containing species in several genera; *Oncorhynchus*, *Salmo*, *Salvelinus*, and *Thymallus*. Commonly known as salmon, trout, charr, and grayling, members of Salmonidae are naturally distributed throughout the northern hemisphere, being native to Europe, north west Africa, northern Asia, and North America (Muus and Dahlstrøm, 1971; Maitland, 1977, 2000), but widespread introductions - South America, India, Australia and New Zealand (Maitland, 1977, 2000) - now leaves Antarctica as the only continent not inhabited by salmonids (Brannon, 1991; Anon, 2006a).

Described by Watson (1993) as ‘primitive fish’ due to the fact they have changed little over a long period of evolutionary time, salmonids resemble their earliest known ancestors very closely. The evolutionary progress of salmonids is illustrated in Figure 1.1, below.

![Figure 1.1 - Ancestry tree of salmonids, illustrating evolutionary progress](image)

Source: Modified from Watson, 1993
Anatomically, their slender design compliments the predatory lifestyle; keen eyes, manoeuvrability, and sharp movement are skills of a true predator (Muus and Dahlstrøm, 1971), whilst internally the short digestive tract is typical of carnivorous fish species (Figure 1.2; Roberts and Shepherd, 1979).

Figure 1.2 - Internal and external anatomy of salmonid species  
Source: Modified from Shepherd and Bromage, 1988

1.1.2 Classification and distribution

As a member of Salmonidae, rainbow trout display all of the characteristics associated with the family’s taxonomy. However, classification to the genus and species level previously caused some confusion. Formerly recognised as *Salmo gairdneri* Richardson, 1836, rainbow trout were reclassified in 1989 as scientists concluded Pacific trout are more closely related to Pacific salmon than Atlantic salmon (see Smith and Stearley, 1989). The anadromous cousin of rainbow trout, the steelhead trout, was also reclassified following re-examination in 1992, and both forms are now recognised as *Oncorhynchus mykiss*, Walbaum, 1792.

Regardless of strain, rainbow trout are renowned for their ability to both grow and mature in a broad range of temperatures (Watson, 1993). This has led to a wide distribution from their indigenous areas of North America (Sedgwick, 1990; Brannon, 1991; Watson, 1993). Today, *O. mykiss* inhabit 39 of the 42 states in the United States of America alone (Watson, 1993). Out with the USA, they can be found as far north as Finland, throughout equatorial regions, and south, as far as
New Zealand (Brannon, 1991). It is believed some wild, self-sustaining populations have established (eg Shasta and Kamloops: Anon, 2006a) in such areas as Israel, (former) Yugoslavia, Madagascar, Sudan, Austria, Venezuela, Mexico, and Hawaii (Muus and Dahlstrøm, 1977; Sedgwick, 1990; Watson, 1993; Maitland, 2000). However, these populations are not a result of purposeful management practices, as attempts to establish free-living populations have generally been unsuccessful; it is expected that these populations derived from escapees of aquaculture (Muus and Dahlstrøm, 1977).

1.1.3  Rainbow trout in aquaculture

The farming of rainbow trout has a long history compared to other members of the Salmonidae family (Anon, 2006b). Following its initial introduction into foreign waters in 1874 (Anon, 2006a), the nineteenth century witnessed the first rainbow trout reared under artificial conditions (Roberts and Shepherd, 1979). As the longest serving member of the Salmonidae family in aquaculture, numerous authors now suggest rainbow trout are one of the few fish species that can be regarded as truly domesticated (Sedgwick, 1990; Anon, 2004). Consequently, the production cycle, industry, and market are well established for rainbow trout.

1.1.3.1  Production cycle

1.1.3.1.1  Broodstock and egg production

The commercial production of rainbow trout begins with the selection of suitable broodstock in a sex ratio of approximately one male to three females. During the spawning period daily checks for gravidity are undertaken. At maturation, females are removed from the holding unit, the flanks and vent area of the fish are dried to prevent water entering the collection bowl, and gentle pressure is applied along the sides of the fish causing eggs to flow freely from the urino-genital pore into a collection bowl. Recently, a more technologically advanced method has been applied on broodstock sites. Involving the use of a low pressure air compressor, a hypodermic needle is inserted about 10 millimetres into the female between the pelvic fins, and air pressure, at 2 pounds per square inch, is used to clear the body...
cavity of eggs. The method is reported to be less stressful to the fish (AquaNic, 2006), as it is faster and prevents excessive removal of the protective mucous and scales caused by continual massaging.

Once eggs are collected, milt is added to the bowl; extracted from males using the same manual stripping technique. Alternatively, where sex reversed females are used, milt extraction is lethal. Neomales are used to produce all-female (XX) offspring; beneficial as it prevents early maturation and associated aggression of males, which inevitably leads to disease. The functional males are produced by administering the hormone, 17-methyl testosterone, to females when first feeding begins. The females mature to grow large round testes but the vent to release milt is absent; hence fish are sacrificed in order to remove milt. From a marketing perspective the method is safe, as only parents are exposed to hormonal treatment. The offspring are completely marketable. The popularity of this method has grown so fast that Paaver et al. (2004) report all-female rainbow trout are now the main product of northern European fish farms.

To ensure good fertilisation, commercial sites use eggs from more than one female and milt from at least two males. Eggs and milt are mixed gently and water is added to activate the sperm. The eggs absorb water and become swollen and firm. This ‘water hardening’ takes approximately 20 minutes, during which time eggs increase in size by ~20%. At this stage, they are referred to as ‘green’ and can be moved for up to 48 hours. After 48 hours they should be left to incubate in complete darkness.

1.1.3.1.2 Egg incubation, hatching and first feeding

Three types of egg incubation system exist; hatching troughs, vertical flow incubators, and hatching jars (Anon, 2006a). The decision of which to choose is largely dependent on the availability of space, manpower, and water quality. In any system, eggs should be supplied with water circulation sufficient to provide enough oxygen and remove suspended particles that may lead to smothering, and subsequent death. Dead eggs quickly accumulate fungus, and egg picking is generally required. In extreme circumstances treatment may be necessary, eg 15
minute flushes of formalin (1:600 volume:volume, formalin:water) daily. When incubating eggs in jars, the flow should suspend and roll the eggs gently, whilst in troughs and trays, eggs should be stacked at no more than two layers deep.

Hatching of fertilised eggs is temperature dependent (~370 degree days). As alevins emerge (success rate: ~95%) empty shells should be removed from the holding unit to prevent an accumulation of waste products. At this time sac-fry should be kept at or below 10°C to prevent deformity and/or disease. Once the yolk-sac is almost fully used, fry swim to the surface. As 50% of the stock surface, first feeding begins. Feed is introduced on the surface 3 to 4 times daily until all fry are actively seeking food, at which point feeding rate should be increased to every 15 minutes where possible (but at least hourly). Weaning should be completed as soon as possible, as developing larvae will be susceptible to dust particles aggravating the gills from prolonged use of dust diets.

1.1.3.1.3 Ongrowing to market

Post-weaning fry can be classified as ongrowers. From this stage to market size, little changes in the way of handling and management practices. Sampling should occur weekly to allow estimations of food conversion ratios, production costs, uniformity, feed strategy and closeness to carrying capacity; essential considerations for good management practice (Anon, 2006a). Thinning of stocks may be required in some units to prevent overcrowding, but grading should be infrequent if the feeding strategy is managed correctly. Tank transfers may be necessary as the fish approach market size.

In the UK, market size (250 grams) is achievable in as little as 9 months (DPI, 2006), at which stage fish are harvested to supply one of two markets; table or restocking (Anon, 2006a; AquaNic, 2006). Fish intended for angling purposes are handled carefully, generally individually checked for fin quality, size, damage and external signs of disease. Table market fish are subject to less stringent examination due to the volume of individuals concerned. A small proportion of the stock may be kept as future broodstock. These fish generally exhibit characteristics that appeal to
the farmer, processor or consumer. The entire production cycle of rainbow trout can be summarised in a schematic diagram, as illustrated in Figure 1.3.

![Schematic diagram of the production cycle for rainbow trout](image)

**Figure 1.3 - Schematic diagram of the production cycle for rainbow trout**
Source: Modified from Anon, 2006a

1.1.3.2 **The industry and market**

The exponential growth of rainbow trout production (Figure 1.4) emphasises the popularity of the species. Inland culturing throughout Europe to supply domestic markets, and mariculture in cages throughout Norway and Chile for the export market, contribute to the vast increase in production witnessed in recent years (Anon, 2006a).
The second contributing factor is the species itself. Brannon (1991) describes rainbow trout as the most forgiving salmonid in terms of the abuse and displacement endured from management practices, with numerous advantages to account for the industrial growth; adaptability to culture environments, tolerance of immense amounts of environmental pressures, fast growth, ease of artificial spawning, short egg incubation phase, and ease of weaning (Brannon, 1991; Hardy, 2003; Anon, 2006a; Anon, 2006b). These factors represent a model species for aquaculture, and as a result rainbow trout became the most cultivated salmonid species in the world - a status maintained until 1994 (Hardy, 2003). By 2002, 64 countries reported the production of rainbow trout (Anon, 2006a), with primary culture based in Europe, North America, Chile, Japan, and Australia (Anon, 2006a). In 2006, over 53% of production could be attributed to Norway, Turkey, Italy, and Denmark (Table 1.1), with Britain contributing 17,600 tons to world production, ranking 8th in the league of European trout producing countries (CEFAS, 2008).

Table 1.1 - Rainbow trout production by major producing countries in 2006

<table>
<thead>
<tr>
<th>Country</th>
<th>Metric tons</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>48,750</td>
</tr>
<tr>
<td>Chile</td>
<td>42,656</td>
</tr>
<tr>
<td>Denmark</td>
<td>40,864</td>
</tr>
<tr>
<td>Italy</td>
<td>40,150</td>
</tr>
</tbody>
</table>

Source: Produced from CEFAS, 2008 data
In North America, Britain, Denmark, France, and Italy, production occurs mainly in freshwater, and although the number of fish produced in freshwater far exceeds that of saltwater production, due to the average weight of marine-produced individuals, overall marine production accounts for a large proportion of the industry. For example, in 2000, 150,000 metric tons of marine-reared rainbow trout were produced, accounting for approximately one third of global production.

1.1.3.3  **British trout farming**

1.1.3.3.1  **Scotland**

The production of rainbow trout in Scotland accounts for a large proportion of the UK market due to the advantage of ideal natural culture environments. In 2006, Scottish farms produced 7,492 tonnes of rainbow trout, 6,628 tonnes of which supplied the table market; the remaining 864 tonnes supplied the restocking market (CEFAS, 2008). From the previous year (2005), this was a production increase of over 7% (CEFAS, 2008), which is representative of the general and continuous overall increase in Scottish production since 1991 (Figure 1.5).

![Figure 1.5 - Annual Scottish production of rainbow trout from 1991 to 2006](image)

*Figure 1.5 - Annual Scottish production of rainbow trout from 1991 to 2006*

Source: Produced from FRS, 2002, 2005 and CEFAS, 2008 data

The production figures published by the FRS include fish produced in both freshwater and marine environments. Although the majority, if not all the rainbow trout produced in the UK is the non-anadromous strain, rearing still takes place in...
the marine environment. Saltwater production of rainbow trout in Scotland relies entirely on cage production. In freshwater production, 54% relied on cage farming in 2005, the balance are cultured on land-based farms (FRS, 2005).

1.1.3.3.2 England and Wales

Cage culture is extremely uncommon in England and Wales, and land-based systems dominate the production technique. Total production in England and Wales for 2006 exceeded 7000 tonnes, of which 67% was table market production. Restocking and ongrowing sectors of England and Wales appear significantly stronger than those of Scotland; 33% compared to <12% in 2006 (CEFAS, 2008). CEFAS (2008) report a continual decrease in rainbow trout production in England and Wales in recent years. Between 2005 and 2006, a significant drop of just under 16% was reported.

1.1.3.4 Current trends and the future for British trout farmers

Increased worldwide production of rainbow trout since the 1970’s has left the unit price comparatively low at the farm-gate. This has resulted in an increase in worldwide distribution of both processed and live products from countries with reduced labour costs. This is in addition to advancements in culture techniques, such as photoperiod manipulation, which allows hatchery strains to mature and spawn out of season, allowing a continuous and global supply of stock year-round (Anon, 2006a; AquaNic, 2006). Today, many British trout farms are forced to buy-in eggs to increase production, in an attempt to improve the economy of sales (AquaNic, 2006). In 2004, over 20 million eggs were imported into England and Wales (Figure 1.6; CEFAS, 2005).
To ensure their existence in the future, British trout farmers must consider alternative culturing techniques to improve their economic margins. Recent development in products and market expansion, as well as the continual demand for improved quality are just the beginning of a long and incessant trend. Rainbow trout farming has practically been forced to turn to genetic manipulation in order to meet current and predicted future demands for fish products. The opportunity of genetic improvement to improve such traits as growth rate, food conversion ratio, and disease resistance, will assist in bettering the species for aquaculture purposes. By producing fish that grow quicker, are better quality, and have a higher tolerance to commercially important diseases, more fish reach market size faster, in better condition, and at a higher quality. Biotechnological methods such as hybridisation, polyploidy, monosex populations and inbreeding are currently used as short-term investments in some aquaculture species to gain desired qualities, but only one technique offers long-term improvement for the rainbow trout industry; selective breeding for genetic improvement.
1.2 Genetic Improvement in Aquaculture

1.2.1 Selective breeding; the potential for genetic improvement in aquaculture

Friars (1998) believes aquaculture can be considered a component of agriculture and that the advances made in a long history of breeding technology in terrestrial animals and plants present a tremendous opportunity to the aquaculture sector. Progress in farmed stock through controlled mating and selection has accumulated on the strength of natural variation (Friars, 1998). Early estimates of genetic parameters and selection advances indicate that similar gains in aquaculture are possible. Additionally, there now exists the potential of merging new technologies with traditional breeding techniques, both at the molecular and cellular level, to assist in the selection of desirable traits in aquaculture stocks (see Gjedrem, 2005a).

The advances in genetic improvement of farm stock accelerated post World War II when knowledge and available technology improved; aquaculture was unable to benefit from such advances during this period, as the industry was in its infancy (Gjedrem, 1997). The significant increase in agricultural productivity over the last few decades is illustrated in Figure 1.7, below.

Figure 1.7 - Increased productivity in farm animals and finfishes following World War II
Source: Modified from Gjedrem, 1997
Although aquaculture appeared relatively far behind terrestrial species, Gjedrem (1997) suggested the steep incline in salmon production indicated existing potential for productivity to equal or even better the growth of that seen in terrestrial animals. By 2000, he supported the claim, explaining that the trend was changing as research found fish, carnivorous species especially, are two to three times more efficient than pigs and broilers in converting energy from food to edible protein for humans, suggesting aquaculture will become extremely competitive with terrestrial agriculture as the domestication of fish continues and efficiency improves (Gjedrem, 2000).

To date however, the majority of improvements seen in aquaculture has occurred through management; improved nutrition, health, welfare, and water quality. Important nonetheless, improving these factors is simply optimising the culture environment. Breeding strategies involve the animal and its genetics; any improvements made are biological. An optimum environment should be maintained, but selection for desirable traits, such as fast growth, low food conversion ratio, or disease resistance has the potential to culture fish more economically whilst building a cumulative effect on improved stocks (Tave, 1993).

1.2.2 Selective breeding in aquaculture; from past to present

Dunham et al. (2001) argue selective breeding in aquaculture began over 2000 years ago when Romans began breeding fish in ponds; without realising, selective breeding took place in the form of domestication. Domestication continues today in most, if not all aquaculture facilities. It is defined by Hale (1969) as the action where breeding, care, and the feeding of animals is ultimately controlled by man. It can be characterised by genetic changes in behaviour, morphology, or physiology, which occur throughout cultivation in artificial conditions (Gjedrem, 2005b). However, such changes went unnoticed or were not managed until the 1800’s, when conscious breeding strategies commenced in Japan as the culturing of patterned koi carp commenced. Then, in the twentieth century, commercial breeding programmes initiated as knowledge of breeding and inheritance improved (Dunham et al., 2001).
The application of genetic principles to aquatically reared species is therefore a relatively recent phenomenon with the majority of cultured aquatic organisms still extremely similar to their wild counterparts (Bartley, 2005). Today, a mere 1 to 2% of aquatically reared species are reported to be genetically improved (Gjedrem, 1997, 2000). Although genetic enhancement is a relatively new activity in aquatic species, the figure of 1 to 2% is still surprising, especially when considering the high reproductive capacity of aquatic species in comparison to farm animals, and the associated potential for selection this generates. Furthermore, the fecundity of fish makes it possible to produce progeny groups with many individuals in each group; an ideal scenario to research various traits in full- or half-sibling families (Refstie, 1990). These factors highlight the tremendous scope to increase productivity in aquatically reared species by applying genetic improvement techniques (Bartley, 2005).

1.2.3 Breeding goals

Selective breeding aims to produce animals that are more efficient in their use of available food, land, and water resources (Gjedrem, 2005b). Before applying selection to a breeding programme, it is important the goals of the scheme are recognised. Input from the industry, consumer, and processor is advised, and should be used to measure the value of selecting individual breeding goals. Even traits of extreme economic importance require careful consideration, as the difficulty and capital cost of selection may outweigh the benefits (Refstie, 1990). Important traits are generally those of economic value, and may include survival, food conversion efficiency, growth rate, meat quality, and disease resistance (Gjedrem, 1983, 1985; Refstie, 1990). However, some traits are immeasurable when the individual is alive. Traits requiring slaughter for assessment, such as market quality or disease resistance through challenges can only be assessed on family information (Friars, 1998). It is therefore imperative that all information and the desired outcome are discussed thoroughly before decisions are made in regard to the chosen traits and the optimum selection strategy to achieve the breeding goals (Refstie, 1990).
1.2.4 The basis of selection – variation

Gjedrem (2005b) defines animal breeding as a body of theory. In order to address the problem of finding individuals which produce the best offspring, generation after generation, knowing the variation among those individuals is essential. It is this variation or variance and its utilisation in breeding and selection strategies that are of the utmost importance for selective breeding. Selection is based on the desire to improve specific characteristics (traits), which can be categorised as qualitative or quantitative. Quantitative traits cannot be segregated into distinct categories; they have a continuous distribution where differences are a matter of degree rather than kind. They are assumed to be regulated by a large number of genes each with a small effect on the trait. Quantitative phenotypes are generally economically important for production, eg length and weight. Falconer (1981) partitions the phenotypic value into the influences of genotype and the environment, where genotype is the particular assemblage of genes possessed by the individual, and the environment is all non-genetic circumstances:

\[
\text{Phenotypic value (P) = Genotypic value (G) + Environmental deviation (E)}
\]

However, it is the variation associated with a particular trait that interests selective breeders, as the phenotypic variation \(V_P\) displayed is constructed of several components of variation (Tave, 1993):

\[
V_P = V_G + V_E + V_{G,E}
\]

where \(V_G\) is genotypic variance, \(V_E\) is environmental variance, and \(V_{G,E}\) is the interaction that exists between the genetic and environmental variance. Of primary interest to selective breeders is the genetic variance, as the object of any breeding programme is to alter the genetics of a population in order to improve its production. Genetic variation can be further divided into three components; additive, dominant, and epistatic variance:

\[
V_G = V_A + V_D + V_I
\]

The most important component of genotypic variation is additive variation \((V_A)\). Where, dominance variation \((V_D)\) results from the interaction between pairs of
alleles at single loci, and interaction variation \( (V_I; \text{epistasis}) \) results from the interaction of alleles between or within multiple loci, the additive effects of genes are inherited quantitatively from one generation to the next. The additive variance, which is also the variance of breeding values, is the chief cause of resemblance between relatives, and therefore the determinant of observational genetic properties of the population and of the response of the population to selection (Falconer, 1981; Gjedrem and Olesen, 2005). Consequently, the ratio of additive variation and phenotypic variation \( (V_A/V_P) \) gives an indication as to the extent at which phenotypes are determined by genes of the parents (heritability: \( h^2 \)), and the rate at which genetic progress is made (Falconer, 1981; Tave, 1993).

1.2.5 Heritability

Heritability is one of the most useful parameters in animal breeding. Defined by Gjedrem and Olesen (2005) as the proportion of the total phenotypic variation which is genetic in origin, it is the degree to which genetic variance influences the phenotype of a continuous trait. It is important to know the size of the heritability when planning a breeding experiment, as it can be used to predict the response to selection, or calculate the breeding value of individuals. Several techniques are used to estimate heritability, and it is important to know which technique is used in order to assess the calculated value. Generally, any technique used should produce equivalent results. For example, where differences occur in epistatic and dominance effects, with no non-additive genetic variation, these heritabilities should give equivalent results. However, it is important to consider that each technique can affect the accuracy of the estimate, with the increasing distance between relatives assessed for performance increasing the standard error associated with the calculated value. Falconer and Mackay (1996) provide the example of estimates based on half-sibling analysis, which can have a standard error of up to four times greater than that calculated from single parent regression, whilst Kinghorn (1983) describes the biases incorporated into estimations of full-sibling heritabilities as a result of dominance and environmental variation; family-related components of variation, eg maternal effects, can be especially detectable when estimations are based on dam variance as opposed to the sire component (Gjerde and Schaeffer,
Such maternal effects have been found to reduce with age in many fish species, which consequently increases the estimates of heritability (Kinghorn, 1983). Heritability can be defined and calculated in either the broad or the narrow sense. Where the proportion of genetic variation to phenotypic variation \( (V_G/V_P) \) is considered heritability in the broad sense, it often contains dominance and epistatic effects which do not contribute to selection. The utilitarian estimate of heritability is defined as the ratio of the additive genetic variance, \( V_A \), to the phenotypic variance, \( V_P \), and is therefore the proportion of the total variance that is due to the differences between the breeding values of individuals in the population, ie narrow sense heritability:

\[
h^2 = \frac{V_A}{V_P}
\]

The higher the heritability, the greater the genetic response that can be expected from selection. However, it is worth noting that the heritability is not a general and static characteristic of a breeding population. It is only relevant to the population from which measurements were taken (Gjedrem and Olesen, 2005). Heritabilities calculated out with the population may be inaccurate, as values can vary between individuals, strains, populations, and even location. Once calculated, the response to selection of that heritability can be predicted:

\[
R = Sh^2
\]

where \( R \) is response (gain or loss) in each generation, \( S \) is the selection differential (the superiority or inferiority of the broodstock over the population mean), and \( h^2 \) is the narrow sense heritability (Tave, 1993; Gjedrem and Thodesen, 2005). If the heritability is low, the selected trait will be slow to respond (Tave, 1993), and when high estimates of heritability are calculated, random drift, environmental trends, or inbreeding depression can be observed in the response to selection. It is therefore advised that at least two generations of trait information is collated before heritability conclusions are drawn (Kirpichnikov, 1981). A traditional method of monitoring the progress in any selective breeding programme was to establish a control population of fish, but Tave (1993) emphasises that both the selected and
control lines require identical conditions so that environmental fluctuations can be accounted for (Figure 1.8).

![Figure 1.8 - Example of control population used to calculate the genetic gain from selection](image)

Source: Modified from Tave, 1993

Today, good management techniques assist in monitoring the progress of selective breeding programmes; valid documentation of genetic progress is essential in larger breeding companies. Top producers often require evidence for marketing purposes to illustrate the success of the breeding programme implemented, especially in the main markets of the industry (Rye and Gjedrem, 2005). Unselected control lines are still used, but concerns relating to inbreeding and genotype by environment interaction has lead to alternative methods to assess genetic progress; repeated matings, average breeders, and genetic trend analysis can be used as alternative methods to monitor selective breeding progress (Rye and Gjedrem, 2005). Establishing procedures for monitoring and quantifying genetic changes in a breeding programme enables the progress to be recorded, but more importantly it
identifies if the realised gains do not meet the theoretical expectations. This allows adjustments to be made to the breeding strategy as necessary (Rye and Gjedrem, 2005).

The heritability therefore acts as an indication as to the potential success selection can accomplish in the population measured for that trait. As a rule of thumb, the larger the heritability the easier it is to change a population mean by selection. In aquaculture, heritabilities of \( \geq 0.25 \) have been effective for breeding programmes, whilst those of \( \leq 0.15 \) have proved difficult to change the population mean (Tave, 1993). However, various methods of selection are available in order to improve a population regardless of the heritability value. It is highly unlikely, and often only in extreme circumstances that genetic improvement could not be made through selective breeding.

1.2.6 Methods of selection

Tave (1993) defines selection as a breeding programme with the aim of choosing the most desirable individuals or families as broodstock in an effort to change the population mean in the next generation. By saving or culling individuals according to a predetermined cut-off value, it is predicted that offspring will display a mean and range similar to those of the chosen broodstock, instead of the original population. The first step is to measure, or record, the desired trait in the population, and then estimate the mean and standard deviation. Selection can then be conducted on superior individuals whose estimated breeding values can be calculated (Gjedrem and Thodesen, 2005). A prerequisite for running a sustainable selection programme is that a reasonable number of full- and half-sibling families are produced in a controlled and reliable manner. This allows estimations of genetic components to be calculated. It is therefore important that where the possibility of collecting eggs and milt separately is available, it should be utilised. In salmonids especially, the mating structure can be easily controlled due to the required human intervention. This means a large number of full- and half-sibling, maternal and/or paternal combinations are possible; mating designs are important as the genetic effects (ie (non-)additive genetic) can be calculated with unbiased and accurate parameter estimates, or predictions of breeding values (Gjerde, 2005). A mating
design commonly used in the selective breeding of rainbow trout is illustrated in Figure 1.9, below. This design is often utilised as both the maternal and paternal genetic components can be calculated having shared either the eggs of females or milt from (sexually reversed) males with more than one individual, producing various combinations of both full- and half-sibling families.

Figure 1.9 – Example of a common mating design used in selective breeding programmes for rainbow trout

Used as a tool to improve certain characteristics in every generation, selection represents a long-term genetic improvement strategy, described by Bartley (2005) as the best means to fully utilise the genetic resources of aquatic species. Historically, selection was based solely on phenotype, and although thought to represent superior genotypes, progress in genetics means genotypic data can now be incorporated into selection programmes, increasing the accuracy of selection. Using modern technology, genotypically superior individuals from a population can be selected to act as broodstock for subsequent generations; even where individuals are pooled, family information can be found through parental allocation, or in terminal studies the identification of relatives allows sibling selection, whilst the estimation of breeding values alone has improved the accuracy of which fish are selected. However, selection does not create new genes in a population, but rather it changes the gene frequencies. By increasing the frequency of alleles with a favourable effect on a specific trait and reducing alleles with unfavourable effects on that phenotype, it is expected to change the mean value of the population for the trait under selection (Gjedrem and Thodesen, 2005). It is therefore important to know the biological blueprint, heritability, recording methods, and nature of the selected phenotype, as well as the reproductive capacity of the species, as this makes it possible to determine which breeding programme to use in order to alter phenotypic, genotypic, and gene frequencies (Tave, 1993; Fjalestad, 2005b).
1.2.6.1 Individual (mass) selection

Individual, or mass, selection is based on individual performance or phenotype (Fjalestad, 2005b). Although classified together, mass selection is a term used when individuals are held *en masse* and superior individuals are selected from the population based only on their phenotype. Where individual selection experiments have taken place in fish a poor or even a negative response has been observed (e.g., Moav and Wohlfarth, 1976; Teichert-Coddington and Smitherman, 1988; Huang and Liao, 1990), but due to its simple design and the practicality of its use in commercial systems, it remains to be the most widely used method of selection for fish species (Tave, 1993; Fjalestad, 2005b). In successful selection experiments, the results have lacked either reliable control lines (Donaldson, 1968), or simply did not continue after the first generation of selection (Friars et al., 1990). The poor record of individual selection may be explained by the following areas:

- The low variability of the base populations; due to the high fecundity of most aquatic species, populations can be established with a limited number of breeders. This seems to be one of the main reasons for the failure of tilapia experiments (Teichert-Coddington and Smitherman, 1988; Huang and Liao, 1990) and of the carp Israeli experiment (Moav and Wohlfarth, 1974)

- Inbreeding may develop during selection experiments, and have an adverse effect on growth rate (-1.5% to -8% per 0.10 increase of the inbreeding coefficient, F; Chevassus 1989; Su *et al.*, 1996a; Pante *et al.*, 2001b). Again, the high selection intensities that are easy to apply in fish due to their fertility, leave them especially sensitive to inbreeding during selection

- Maternal effects can play a large role in the phenotypic variance between individuals. Differences in hatching time and/or egg size can have a dramatic effect on future performance (Chevassus 1976; Mor and Avtalion, 1988; Sin *et al.*, 1994; Vandeputte *et al.*, 2002)
However, efficient individual selection remains to be of interest to breeders since it is simple and cheap to establish in practical conditions (Chevassus et al., 2004). When the heritability is high for the trait of interest, individual selection is considered extremely effective, as the phenotypic values approximate the breeding values (Tave, 1993). This led to Falconer (1981) suggesting that its use should be adopted unless there are good reasons for preferring an alternative method. Additionally, individual selection can also be particularly useful in species that spawn communally. For example, Knibb et al. (1997, 1998) used mass selection in gilthead sea bream (Sparus aurata) due to the problems associated with group spawning, and achieved significant heritabilities for growth. However, in such species the method has been known to create disadvantages, as was found by Frank-Lawale (2005). When studying the mass spawning species, Hippoglossus hippoglossus, it was discovered that only half of the parents succeeded in contributing to the F₁ generation, resulting in the new population being comprised of a small number of large families. The skewed contribution of parents left the effective population size (Nₑ) at an unacceptable 8.11, where 100 is believed to be an optimum value (see Section 1.2.7), whilst the inbreeding coefficient, F, was calculated at 6.16% in the F₁ generation.

When spawning can be controlled in aquatic species, or when the heritability is low for the desired trait, or the trait can not be measured on live individuals (eg flesh colour or disease resistance), individual selection may not be the best method of selection, and family-based methods should be considered.

1.2.6.2 Family selection

Family selection differs from individual selection in that the decision to save or cull fish is made with reference to the family. The number of representatives per family contributes to the effectiveness of family selection; the greater the number, the closer the correlation between phenotypic mean and genotypic mean values (Falconer, 1981). Family selection is often preferred when the desired trait has a low heritability because most of the phenotypic variance observed among individuals is due to non-heritable sources of variance, ie an individual’s phenotypic value does not accurately represent its breeding value (Fjalestad, 2005b). It is
especially useful when the characteristics of selection cannot be measured on live fish, such as carcass traits or disease resistance (Refstie, 1990; Tave, 1993; Gjedrem, 2000). Family-based schemes are based on two components: between family selection that chose entire families on the basis of their means relative to the population mean, and within family selection that chose individuals based on their relative performance within families.

1.2.6.2.1 Between family selection

When numerous families are pooled into a single holding unit, successful selection relies on the environmental effect on all individuals cancelling each other out in the family mean, leaving the phenotypic mean as a good estimation of the genotypic mean. Then, the overall population mean is ignored, and selection is conducted in relation to family means alone. Families are ranked, those with the best means are saved, the remainder culled. This methodology requires a certain discipline; the temptation of retaining larger fish of families that require culling or disposing of smaller fish in selected families must be resisted. However, Tave (1993) points out that the entire family need not be saved; a random sample will suffice - but it must be random and in equal number per family to ensure no biases occur between the selected individuals.

1.2.6.2.2 Within family selection

Within family selection considers each family as a temporary sub-population with selection occurring simultaneously and independently within each sub-population. Top performing individuals from all families are saved based on their performance in comparison to the family mean. If families are held separately, the method is often used when environmental influences are uncontrollable, as deviations are felt at the family level instead of the individual level allowing unbiased selection overall (Tave, 1993). In species where sexual dimorphism occurs, care must be taken to avoid selecting an inappropriate sex ratio, and it is advised that selection is performed individually for each sex.
1.2.6.2.3 Comparing selection methods

When family means are compared much of the non-heritable variance, particularly environmentally-induced phenotypic variance, is reduced since deviations of individual values from family means negate each other. Thus, the family phenotypic means approximate their mean breeding values. This is particularly true if environmental variance has a greater effect on the individual level than on the family level. Within family selection is more efficient than individual or between family selection when major sources of non-heritable variance, particularly environmental variance, are common to all individuals of a family but are different among families, ie environmentally-induced variations at the family level. Examples include time of birth and age of female. When a large component of the phenotypic variance is due to environmental variance at the family level, within family selection will be more effective than between family selection, because the best families may be the best simply due to the environmental variance. In the case of individual selection, all fish selected are phenotypically the best, and where heritability is large, phenotypes approximate the breeding values. For example, if individual selection is based on weight and the desired value is 1 kilogram, all selected fish will be greater than 1 kilogram. When between family selection is used, the selected broodstock will consist of individuals with a broad range of phenotypic values, having been selected on the overall family means. When within family selection is used, the best fish in one family may be only half the weight of fish culled in other families.

The way to prevent such a scenario becoming a problem in breeding programmes is to combine selection methodologies. Tave (1993) gives the example of a two-staged selection programme where the first step is to use between family selection to select the best families, before using within family selection to utilise only the best fish of those families selected. However, Fjalestad (2005b) explains that combined selection is simply a combination of methods used together in a breeding plan to attain the desired result.
1.2.6.2.4 Combined selection

The decision of which methods to employ will rely largely on the outcome desired, the management of the farm, and the available information. By including all available sources of information, for example breeding values, phenotypic measurements, and the family structure (ie full- and half-siblings and contributing parents), an optimal way of improving desired traits can be established. Combined selection represents the general solution for obtaining the maximum rate of genetic gain; combined selection is therefore, in principle, always the best method of selective breeding for genetic improvement.

The simplest method generally adopted in aquaculture is an amalgamation of family and individual selection (Fjalestad, 2005b; Gjerde, 2005), where family deviations are taken into account in addition to the mean phenotypic value of individuals. Additionally, selection indices are used to improve the success of this method. Following the collection of information from the individual and its relatives, as well as from several commercially important traits, the information is combined into an index of merit, where traits are weighted according to their relative economic value (Fjalestad, 2005b). One of the most successful breeding schemes in the UK utilises combined selection in a similar manner. Using both within and between family selection and then selecting on individual performance, Landcatch pedigree breeding programme has had unprecedented success in salmonid aquaculture (Guy, 1998). By combining the two family methodologies, selection is based on between family selection first, and then within family selection; this ensures the most productive fish of the most productive families are selected as broodstock. The final broodstock are likely to be the top performers from those fish selected; individual selection (Tave, 1993).

A schematic diagram of the main areas involved in a selective breeding programme is illustrated in Figure 1.10. The nucleus of breeding programmes, as shown in the diagram, is generally based on commercial sites that have an interest in specific traits which improve overall production. Maturing fish are collected from production tanks and tagged so their identity is known. From these fish, breeding candidates are selected as breeders, in an appropriate sex ratio, having considered
the trait(s) of interest. A representative number of eggs from each of the families generated are retained separately in the breeding nucleus, whilst surplus eggs are either sold to fingerling producers (normally for a premium price due to the improved quality of the selectively bred fish), or to multipliers who assist in the programme by rearing the families and recording data on the stock, or who act as an intermediate by producing eggs and fry for sale to fingerling producers once maturity of the improved fish has been reached. Following the fingerling stage, adults are simply used as production fish for the table market, or are ongrown in the nucleus for subsequent selection generations. Finally, as a tributary to the information generated by the nucleus, challenge and field tests take place for the considered traits, allowing families/populations to be tested on their overall performance.

![Figure 1.10 - Schematic diagram demonstrating the main factors in a fish breeding programme](image)

Source: Modified from Gjerde, 2005
1.2.7 Risks in selection: inbreeding

Although individual selection is generally more effective than any family based method when heritabilities are large, there is no specific heritability value that identifies which selective breeding method will be more efficient (Tave, 1993), and alternative options may be sought, eg selection index or combined selection. Whichever methodology is chosen, it is imperative that controlled mating systems are conducted in order to prevent inbreeding (Friars, 1998). Inbreeding occurs when related individuals are bred together (Sonesson et al., 2005; Fjalestad, 2005a), which can result in inbreeding depression, where reduced heterozygosity and the loss of desirable alleles generally occurs, retarding or even negating the selection response (Knibb, 2000). Dunham et al. (2001) emphasise that it is as important to control inbreeding as it is to improve production through genetic gain. Traditionally in commercial systems, before the negative effects of inbreeding were fully understood, only a small number of parents were used to contribute to the next generation, creating a bottleneck effect and the loss of genetic variation (Mustafa, 1999; Fjalestad, 2005a; Gjedrem, 2005b). Today, the mating of related animals is used as a breeding tool in certain programmes, but poor management can result in potential hazards; ultimately a lack of overall fitness (Falconer, 1981). The detrimental effects of inbreeding are well documented in fish (Gjerde et al., 1983; Kincaid, 1983a; Refstie, 1990; Pante et al., 2001a).

Measured by the inbreeding coefficient, F, inbreeding is the probability that two alleles at any locus are identical by decent, with values ranging from 0 to 1. The inbreeding coefficient expresses the amount of inbreeding that has accumulated from a specific point in the ancestry of a population. Estimates of the deleterious effects of inbreeding in aquaculture species generally involve salmonids, rainbow trout especially, due to their long history of culture (Toro and López-Fanjul, 1998; Lutz, 2002). For example, Gjerde et al. (1983) estimated reduced survival of up to 9% in rainbow trout fry with an inbreeding coefficient of 0.25. Over the three successive generations, this accumulated to a reduction of over 18%. Su et al. (1996a) found that a 10% increase in the inbreeding coefficient resulted in a delay in the spawning age of female rainbow trout by 0.53%, and a reduction in egg
number of 6.10%, whilst Pante et al. (2001a) studied the effect of inbreeding to find that an increase in the inbreeding coefficient of 10% resulted in a reduction of between 1% and 5% in the harvest weight of rainbow trout. Kincaid (1976ab, 1983a) also describes problems associated with inbreeding, where reductions in growth, survival, and reproduction of up to 30% have been observed in salmonid stocks.

When initiating a breeding programme, a broad genetic base is of the utmost importance. Synthetic populations have been manufactured in several selective breeding schemes in recent years to secure the genetic variability. This has resulted in research focusing on the maintenance of variation in subsequent generations, whilst considering the rate of inbreeding. In aquatic species it is important to have control over the rate of inbreeding, as opposed to level, due to the high fecundity, and possibility of high intensity selection. In aquaculture, research into constraining inbreeding is limited (Gjerde et al., 1996; Bentsen and Olesen, 2002), but numerous authors have concluded the optimum effective population size to be at least 100 (50 breeding pairs) to ensure the rate of inbreeding is maintained between 0.5% and ~1% per generation, which is thought to be an acceptable level (Gjerde et al., 1996; Bentsen and Olesen, 2002; Sonesson et al., 2005; Fjalestad, 2005a). In other research concentrating on the optimum number of parents and their contribution for maximising genetic gain, whilst constraining the inbreeding coefficient over a number of generations, numerous procedures have been documented (Wray and Goddard, 1994; Brisbane and Gibson, 1995). In 1998, an adaptation of the work by Meuwissen (1997) was completed by Grundy et al. (1998), who applied the theory of maximising genetic response whilst restricting the rate of inbreeding per generation to a predefined value, but refined the procedure so that the constraint was achieved in each generation of selection. Up until the year 2000 only Meuwissen and Sonesson (1998) had presented a method to maximise genetic progress whilst constraining the annual increase in inbreeding in the more complex situation with overlapping generations. However, having based their method on the control of the average increment in coancestry for discrete generations, the procedure is limited to annual constraints on the rate of inbreeding, where constraints per generation are considered to be more appropriate given the reduction of genetic variation and the
accumulation of mutations that occur per generation (Grundy et al., 2000). Grundy et al. (2000) present a procedure to optimise the genetic contributions of selection candidates for maximising the genetic progress, whilst the rate of inbreeding is constrained to a predefined value, all taking place in schemes with overlapping generations. The method is an extension of that described by Grundy et al. (1998) to overlapping generation structures, and allows for a restriction on the rate of inbreeding either per generation or per annum. The dynamic selection algorithm procedure gives the optimum number of individuals to be selected and the progeny they each produce, all of which results from the use of best linear unbiased prediction (BLUP) estimated breeding values, the augmented numerator relationship matrix, and lifetime breeding profiles. By considering all gene flow pathways, the optimisation procedure constrains the rate of inbreeding per generation to a predefined level across generations of selection, resulting in an improvement in the genetic response of up to 35% over standard truncation BLUP selection, at the same rate of inbreeding.

For most aquaculture traits, an inbreeding coefficient of 0.05 is deemed to be an acceptable rate, but this should be continually assessed throughout selection experiments. In selection programmes based on phenotype and without genotypic identification, high rates of inbreeding are inevitable with subsequent reproductive and production failures. This is exemplified in aquatic animals due to the reproductive capacity of most species (Davis and Hetzel, 2000). Through careful management and by maximising potential genetic gain using controlled management, the effects of inbreeding should be reduced. Modern DNA marker technologies are now available to calculate the heterozygosity of populations; used an indication as to the level of inbreeding. For example, Fishback et al. (1997) used microsatellite markers in order to monitor the level of inbreeding in a study on rainbow trout by assessing the number of alleles at specific loci in each generation. However, molecular techniques are not an outright solution to improving the performance of aquaculture stocks, and the best results are likely to come from a combination of marker technologies and selective breeding (Lutz, 2001).
Molecular markers in selective breeding

Molecular marker based knowledge emerged in the 1980’s, and since that time its use has increased dramatically (Dunham et al., 2001). Initial marker systems were based on protein polymorphisms and morphological characteristics (Davis and Hetzel, 2000). Traditional markers, such as isozymes (Liu et al., 1992), restriction fragment length polymorphism (RFLP; Miller and Tanksley, 1990), and mitochondrial DNA (mtDNA) analysis (Curtis et al., 1987), have now been replaced with several powerful new types of marker, including random amplified polymorphic DNA (RAPD); amplified fragment length polymorphisms (AFLP); simple sequence repeat (SSR), or, microsatellite markers; variable number tandem repeat (VNTR) markers; and single nucleotide polymorphism (SNP). Although each method has distinct properties and generates data for a variety of applications, the general principle of all these technologies is to detect variation at a single locus and nucleotide, or multiple loci in a single reaction (Davis and Hetzel, 2000). Today, DNA marker technologies enhance the way aquaculture genetics research is conducted (Liu and Cordes, 2004).

Genetic markers are necessary to locate genes on chromosomes, isolate genes, determine gene expression, study genomes, conduct gene-linkage mapping, and are essential for marker-assisted selection (Dunham, 2004). Today, the variety of biochemical and molecular markers available to study the genetics of aquatic species presents tremendous opportunities. For example, the construction of gene maps can now take a matter of months rather than years when traditional DNA technologies were used, such as RFLP (Liu and Cordes, 2004; Dunham, 2004). Further, the ability to monitor the DNA of an individual and evaluate its relationship to the population, such that family and pedigree can be established and included in the breeding strategy, is a great advantage in any programme aimed at genetic improvement. Using genetic markers for fingerprinting individual animals and parental assignment, or to determine the genetic diversity to assist in the selection of economically important traits provides great potential to selective breeding programmes and broodstock management (Lai, 2001). One particular molecular
tool has had unprecedented success in aquaculture due to it versatility; microsatellite markers.

1.2.8.1 **Microsatellite markers in selective breeding**

Microsatellite markers work by utilising the repetitive DNA which makes up 5% to 20% of the genome in some fish species (Elder and Turner, 1995; Twyman, 1998). Referred to as satellite DNA, regions of the genome that exhibit areas of tandemly repeated sequences are extremely polymorphic, and include regions of extremely short tandem repeats of 1 to 6 base pairs: microsatellites. Microsatellite markers are therefore manufactured as di-, tri-, or tetra-nucleotide repeats in order to flank the unique sequences and bind as primers to those sites at amplification when polymerase chain reaction is used (Dunham, 2004). Occurring frequently at $10^4$ to $10^5$ in fish (Park and Moran, 1995), microsatellites are an ideal molecular tool because they are highly polymorphic, codominantly inherited, extremely numerous, and evenly distributed throughout the genome (Dunham, 2004). The polymorphism and codominant inheritance allows precise genetic analyses and increased mapping accuracy, maximising the genetic information gathered and allowing lineages of individuals or families to be traced accurately (Waldbieser and Wolters, 1999). It is the number of alleles that broadly determine the degree of heterozygosity which means that markers are often selected for each application based on the number of alleles. Each allele can then be distinguished through differences in the size of polymerase chain reaction products generated. This versatility has lead to the successful application of microsatellite markers in such areas of aquaculture as relatedness determination (Herbinger et al., 1995; Norris et al., 2000), inbreeding (Su et al., 1996b; Pante et al., 2001a), and assessing diversity and variation in stocks (Bártfai et al., 2003; Overturf et al., 2003; Ward et al., 2003). However, they are not without their disadvantages, and Dunham (2004) warns that microsatellites require a great deal of time, effort, and expense in construction, screening, sequencing, and polymerase chain reaction primer analysis. They can also produce non-specific bands in the analysis of the polymerase chain reaction (Liu and Cordes, 2004).
1.2.9 Genetic improvement in aquaculture

Historically, the aquaculture industry has not adequately utilised breeding and selection knowledge to increase and improve aquaculture production, with many species continuing to rely on wild-caught broodstock or juveniles (Gjedrem, 2005b). Collaborations between research institutions and commercial partners have failed to exploit genetics as a tool for enhancing productivity and increasing competition in the industry (Knibb, 2000). However, recent years have seen an increase in the application of breeding technologies (Gjedrem, 1997; Gupta and Acosta, 2001; Bartley, 2005; Gjedrem, 2005b), and it is only recently that the economic importance of genetic improvement in aquaculture has been recognised; even slight improvements in productivity could result in millions of kilograms of additional production (Dunham and Smitherman, 1983), significantly improving the chance of meeting the future demands for fish produce (Gjedrem, 1997). Aquaculture genetics shows immense potential for improved production (Dunham et al., 2001).

With the ultimate goal of increased profitability, substantial opportunity exists, especially as progress made is cumulative and sustained (Davis and Hetzel, 2000; Powell, 2000). Today, aquaculture research institutes in most countries are researching selective breeding in order to improve and increase the efficiency and productivity of existing practices and, consequently, increase the rate of genetic improvement in aquaculture species (Gjedrem, 2005b).

1.2.9.1 Progress to date; salmonids

The fact that most fish reproduce externally allows human intervention to manipulate fertilisation and breeding strategies, including parental crossings and the segregation of stocks and/or families. Furthermore, the fecundity of fish, and large genetic variation often displayed allows increased selection pressure to be applied compared to most mammalian species (Gjedrem, 1997). Prior to 1970 genetic selection was seldom used in aquaculture. Today, a number of selective breeding programmes exist for aquatic species (Dunham et al., 2001).

The first selective breeding programme in fish species was established in 1971 by a Norwegian research institute for aquaculture. Although it took four years before
AKVAFORSK devised a breeding strategy, its success throughout the industry was renowned by 1985. By 1997 the programme involved 360 families of Atlantic salmon (*Salmo salar*) and rainbow trout (Gjedrem, 1997), increasing to 400 full-sibling families of Atlantic salmon and 120 families of rainbow trout by 2000 (Gjedrem, 2000). To date, the programme has achieved improvements in growth rate, sexual maturation, disease resistance, and flesh quality of greater than 20% per generation in some instances, highlighting the potential benefit of genetic improvement in the industry. In 2000 Gjedrem reported approximately 65% of Norwegian farmed salmon and trout to be genetically improved; a figure undoubtedly exceeded today. In Canada, a similar breeding programme to that of AKVAFORSK was established. Although not as large, the Canadian Atlantic Salmon Federation programme report significant gains in BKD resistance and growth since commencing in 1984 (O’Flynn *et al.*, 1999). In brown trout (*Salmo trutta*), gains in weight of 6.3% per generation have been attained through selection conducted by the PROSPER breeding programme (Vandeputte *et al.*, 2002), whilst in Coho salmon (*O. kisutch*) 60% improvements were made after only four generations of selection, following a selective breeding programme established by Washington University in 1977 (Hershberger *et al.*, 1990). With such positive results, it is obvious that the knowledge required for genetic improvement is already available in salmonid species (Gjedrem, 2000), however, the extent to which genetic improvement can be made, either in a single trait or over an index, is dependent on the genetic variation that exists for the trait or series of traits of interest (Davis and Hetzel, 2000).

In general, fish exhibit a greater genetic variation than mammals in most traits (Gjedrem and Olesen, 2005; Aquaflow, 2006). As the genetic variation allows the heritability to be calculated, this in turn gives the potential reach that can be obtained as the response to selection. For comparison of the potential gain between land and aquatic animals, or even across traits or species, a useful statistical parameter called, the coefficient of variation, can be used. The coefficient of variation defines how much reach is possible. A population with a large coefficient of variation can produce a large selection differential, even when heritabilities are low (Tave, 1993). When a trait is measured, the mean and standard deviation often
change in tandem, but the coefficient of variation is independent of the mean. In mammalian species, the coefficient of variation is generally 5% to 10%, whilst in fish a coefficient of variation of up to 80% has been observed in some traits (Gjedrem, 1998; Gjedrem and Olesen, 2005; Aquaflow, 2006). Numerous studies have shown positive results for selection in economically important traits in salmonid species (Gjerde and Schaffer, 1989; Hershberger et al., 1990; Gjerde et al., 1994; Martínez et al., 1999; Henryon et al., 2002; Quinton et al., 2002), with estimates of heritability typically high, ranging from 0.25 to 0.35 with coefficients of variation between 20% to 30%, implying that large responses to selection are possible (Gjedrem, 2000; Powell, 2000).

1.2.9.2 Potential for rainbow trout

A great deal of work has been completed on the potential for genetic improvement in rainbow trout. Promising results have been observed at both the scientific and commercial level. With increased understanding of genetics and methods of selection, aquaculturists are now more willing to cooperate with scientists in order to assess important traits within the commercial environment. For example, Fishback et al. (2002) studied a commercial rainbow trout aquaculture facility for three character sets; early progeny growth, later progeny growth at 8.5°C, and later progeny growth at 15°C. They found estimates of heritability ranging from 0.36 to 0.72 for the growth traits of total length, weight, and condition factor, as well as genetic correlations between the character sets of 0.86 ± 0.03, suggesting early progeny growth is a good predictor of later progeny growth. Traditionally, attention has been paid to the economically important characteristics of growth and reproduction; consequently, a great deal of work has been conducted in these areas. Perry et al. (2005) estimated the genetic (co)variance parameters of body weight, condition factor, and resistance to acute thermal shock, in a three generational rainbow trout pedigree. Estimates of heritability were calculated to be 0.46 ± 0.04, 0.52 ± 0.04, and 0.41 ± 0.07, respectively, suggesting substantial genetic potential exists for selection in the three traits. Similarly, Henryon et al. (2002) concluded selective breeding would be possible for the traits, body weight and body length, as
significant additive genetic variation was apparent, and subsequent estimates of heritability equalled 0.35 and 0.53, respectively.

However, not all traits or populations hold the capacity for improvement. At the University of California a selection experiment conducted by Su et al. (1996b) assessed body weight data from 1978 to 1986 to find heritabilities were low at 0.03 to 0.13. Although the estimates increased with age, they remained low; 0.05 at 168 days, and 0.10 at 364 days. Kause et al. (2002) calculated low estimates of heritability in body composition traits; percent abdominal fat, percent fillet protein, and ash and water, all producing estimates ranging from 0.02 to 0.06. Further, the genetic correlations between the body composition traits and body weight were negative ($r_A = -0.12$ to -0.36), suggesting the quality of fillets may be compromised through the selection for increased growth rate.

Although a number of studies have shown only low levels of additive genetic variation in some traits, to date, the most commercially important traits have generally shown outstanding potential for genetic gain through selective breeding, with moderate to high estimates of heritability frequently achieved. However, most research has focussed upon production traits in rainbow trout, with little attention paid to disease resistance. Recent years have seen an increase in the interest in selective breeding for disease resistance. At present only major companies have attempted selection for improved disease resistance in salmonids, but the results have been promising (ie AKVAFORSK, PROSPER, and Landcatch). In the United Kingdom, no scientifically based selection programs have been implemented for the genetic improvement of rainbow trout. As a large commercial sector, making a significant contribution to world rainbow trout production, it is important that research towards the improvement of rainbow trout stocks is implemented in the UK. In 2000, researchers at the Institute of Aquaculture, University of Stirling, collaborated with British trout farmers to establish what was believed to be the first selective breeding programme incorporating genetic information for rainbow trout in the UK. The project was initiated to assess levels of additive genetic variation in production traits, such as growth rate, yield, survival, and improved flesh colour; all of which produced significant heritabilities in the strains tested (Ureta-Scmidt, 2006).
unpublished work). However, in 2002 scientists revisited the farms involved to discuss the progress of the established LINK aquaculture project but were faced with concerns relating to major production losses encountered due to Proliferative Kidney Disease (PKD). With annual outbreaks of PKD resulting in up to 500 kilos of dead rainbow trout each day on a single site, and up to 1.2 million kilos across the English industry, the significance of the disease is obvious (Robinson, 2007 personal communication). Having discussed with the farms involved in the LINK project that avoidance and minimisation of losses were dominating husbandry practices, and without any viable vaccine or prophylactic treatment available at that time, it was clear that a programme to genetically improve the performance of rainbow trout to PKD was the only logical step to suppress its effects.

Coinciding with a natural outbreak of PKD occurring at the time, the opportunity was taken to sample 1500 rainbow trout from the two strains being tested for performance at the Test Valley Trout farm at Itchen Abbass. The trout were weighed, their length measured, and kidney assessed for inflammation caused by PKD, using the scale of Clifton-Hadley et al. (1987), whilst a fin clip was removed from each individual, which allowed parental assignment to one of the known families. For the farms already involved in the LINK aquaculture genetic project, an opportunity was presented to genetically improve their stocks for resistance to PKD, and from the data generated at the opportunistic sampling, the basis of this project was formed.
1.3 **Proliferative Kidney Disease**

1.3.1 **Affected species and geographical range**

Proliferative kidney disease, named by Roberts and Shepherd in 1974, has become highly documented in recent years. The first occurrence of the disease is unknown, but it is expected to pre-date the first reported identification in 1958 (Plehn, 1924; Schäperclaus, 1954; Besse, 1956; Hedrick *et al.*, 1984b). Today, PKD is recognised as one of the most serious and economically damaging diseases of salmonids. Principally affecting farmed, but also feral fish, the causative agent has been identified in rainbow and steelhead trout (D’Silva *et al.*, 1984; Feist *et al.*, 2001), Californian golden trout, *O. mykiss aquabonita* (Morris *et al.*, 2003b), brown trout (Peribáñez *et al.*, 1997), Atlantic salmon (Brown *et al.*, 1991), cutthroat trout, *O. clarki* (MacConnell and Peterson, 1992), sockeye salmon *O. nerka* (Higgins and Kent, 1998), Chinook salmon, *O. tshawytscha* (Kent *et al.*, 1995), Coho salmon (Hedrick *et al.*, 1984ab), grayling *Thymallus thymallus* (Bucke *et al.*, 1991), Arctic charr, *Salvelinus alpinus* (Brown *et al.*, 1991), and the non-salmonid, Northern pike, *Esox lucius* (Seagrave *et al.*, 1981). The natural distribution of these species has resulted in the reported incidence of PKD ranging throughout Europe, Canada and the USA (Bucke *et al.*, 1981; Seagrave *et al.*, 1981; Klontz and Chacko, 1983; Hedrick and Aronstien, 1987; Peribáñez *et al.*, 1997; AquaFlow, 2002; Henderson and Okamura, 2004) with some areas of Western Europe and North America now considered endemic (Hedrick *et al.*, 1993).

1.3.2 **PKD in the United Kingdom**

Within the United Kingdom, PKD has had a marked effect on the British trout industry. In recent years the level of incidence has escalated coinciding with increased production; in turn, the financial burden to aquaculture has grown (AquaFlow, 2002). In 1988 Alderman and Clifton-Hadley reported PKD to cost the British trout farming industry more than £1 million per year. Today, estimates fluctuate between £1.25 to 2.5 million per annum (AquaFlow, 2002; CEFAS, 2005; Hughes, 2005 personal communication). Such losses are accountable to the symptoms and associated mortality of the disease, where morbidity often reaches
100% (Sippel and Ferguson, 1983; Scott, 2002), and mortality can range from 20% to 100%, dependent on secondary infection (Ferguson and Needham, 1978; Hedrick et al., 1984a; Clifton-Hadley et al., 1986b). However, PKD is not thought to be an outright killer. The secondary infections that occur as the salmonid host is overcome by PKD are thought to be the true cause of death; an immunosupression associated with chronic PKD has resulted in numerous secondary pathogens being identified at disease outbreaks, including Infectious Pancreatic Necrosis (IPN) (Hoffman and Dangschat, 1981), Ichthyophthirius species, gill bacteria (Hedrick et al., 1985), Flexibacter columnaris (Hedrick et al., 1985; Foott and Hedrick, 1987), Saprolegnia, Costia (O’Hara, 1985) and furunculosis (see Section 1.4; Hoffman and Dangschat, 1981; Morris et al., 2003a).

As a seasonal summer disease in Britain, PKD generally affects freshwater fish in their first term (Ferguson and Needham, 1978; Foott and Hedrick, 1987; Alderman and Clifton-Hadley, 1988; Hoffman and El-Matbouli, 1994). In Northern Ireland and Scotland, the disease occurs from mid-July to early-September (Ferguson and Adair, 1977; Ellis et al., 1982). However, evidence of the disease has been recorded as early as May in the UK (Clifton-Hadley et al., 1986a). The outbreaks witnessed each summer coincide with increasing water temperature, initiating a severe development of the disease in affected fish (Ferguson and Needham, 1978; Foott and Hedrick, 1987). Although first year fingerlings (0+) on enzootic farms generally succumb to the disease due to a general susceptibility to opportunistic pathogens (Ferguson and Adair, 1977; Ferguson and Ball, 1979; Ellis et al., 1982), Hoffman and Dangschat (1981) describe the vulnerability of older fish (1+) that have had no previous exposure to the pathogen; age can not therefore be considered a protective measure in itself (Foott and Hedrick, 1987). Conversely, fish previously exposed to the disease tend to exhibit resistance to future infection (Ferguson and Ball, 1979).
1.3.3 Causative agent: discovering and classifying *Tetracapsuloides bryosalmonae*

Although identified for decades and characterised by Ferguson and Adair in 1977 the causative agent of PKD remained unclassified for many years due to the uncertainty of its taxonomic position (Hedrick *et al.*, 1984a, 1988b; Saulnier and de Kinkelin, 1996; McGurk *et al.*, 2003). Ascribed by Seagrave *et al.* (1980) as PK’X’, ‘proliferative kidney organism unknown’, numerous authors predicted its identity, ranging from an amoeba (Plehn, 1924; Ferguson and Needham, 1978) to a protozoan Haplosporea; PKX displays similarities to the oyster (*Ostrea edulis*) parasite, *Marteilia refringens* (Seagrave *et al.*, 1980). Kent and Hedrick (1985a) were the first to propose PKX to be a myxosporean, following studies that showed spore-like stages of the parasite which contained polar capsules with polar filaments; a characteristic of a myxozoan species (Kent and Hedrick, 1985ab, 1986). However, the authors could not categorise further as myxozoans are classified mainly on spore characteristics and only immature spore stages were found in PKD infected fish. In 1987 Feist and Bucke presented additional information on the structure of PKX, whilst Hedrick *et al.* (1988a) and Castagnaro *et al.* (1991) discovered the formation of secondary and tertiary cells, as well as the development of intratubular stages, all of which provided further evidence to support the myxozoan classification.

By 1999 Saulnier *et al.* proved the organism is a myxosporean. Its membership to the phylum Myxozoa was discovered through extracts of the parasite’s genomic DNA and small subunit (SSU) ribosomal DNA (rDNA) being amplified by polymerase chain reaction, cloned and sequenced. The evidence that PKX was a sister group of the phylum Myxozoa was provided following a tree reconstruction from PKX and 76 or 128 eukaryotic species post phylogenetical analysis on SSU rDNA. Kent *et al.* (1998) had already evaluated this theory but demonstrated, through 18S rDNA sequences, that the PKX organism collected from fish hosts is not closely related to any of the described myxozoans, all of which were classified in the orders Bivalvulida and Multivalvulida. Anderson *et al.* (1999a) analysed 18S rDNA and demonstrated a relationship of PKX with the previously described bryozoan parasite, *Tetracapsula bryozoides* (Canning *et al.*, 1999). Subsequently,
Anderson *et al.* (1999b) undertook a study of phylogenetic analysis using partial sequences of 18S rDNA sequences of myxozoan organisms from Bryozoa and salmonid fish, to find PKX and *Tetracapsula bryozoides*, although discrete species, formed a novel clade within Myxozoa; distinct from the existing orders Bivalvulida and Multivalvulida. As *Tetracapsula bryozoides* was originally classified in the class Myxosporea, order Multivalvulida (Canning *et al.*, 1999), the new class Malacosporea and order Malacovalvulida were established to accommodate the newly ascribed myxozoan within the family Saccosporidae and genus *Tetracapsula* (Canning *et al.*, 2000). Due to the morphological, behavioural, and molecular similarity between *Tetracapsula bryozoides* and PKX, PKX was positioned within the genus *Tetracapsula*, but as with *Tetracapsula bryozoides*, PKX was named according to the organism it parasitized: *Tetracapsula bryosalmonae* (Canning *et al.*, 1999). In 2000, a concurrent study suggested that the organism be referred to as *Tetracapsula renicola* to represent the target organ in affected fish, following an investigation of the disease in Arctic charr (Kent *et al.*, 2000). However, under the International code of Zoological Nomenclature, the first name ascribed has priority (Okamura *et al.*, 2001), and *Tetracapsula renicola* was made a junior synonym of *Tetracapsula bryosalmonae*.

In 2002 Monteiro *et al.* re-examined *Tetracapsula bryozoides* and made comparisons to the nematode-like parasite of freshwater bryozoa, *Buddenbrockia plumatellae*. They discovered that the rDNA sequences and morphological characteristics were almost identical between the organisms, leading Canning *et al.* (2002) to propose *B. plumatellae* and *Tetracapsula bryozoides* are in fact the same species of malacosporean, and that the name *B. plumatellae* should take historical preference. As such, *Tetracapsula bryozoides* was made a junior synonym of *B. plumatellae*, and the new genus *Tetracapsuloides* was established to accommodate *Tetracapsuloides bryosalmonae*, the causative agent of PKD.
1.3.4 Salmonids and PKD

Numerous authors have suggested salmonids are not the final host of *T. bryosalmonae* (Kent and Hedrick, 1986; Hoffman and El-Matbouli, 1994; Saulnier and de Kinkelin, 1996; Hedrick et al. 2004) due to the severe inflammation induced (Saulnier and de Kinkelin, 1996), and lack of transmission from fish to fish (Ferguson and Ball, 1979). Further, the occurrence of *T. bryosalmonae* in watercourses void of salmonids, suggests salmonids could be unnecessary hosts (Okamura et al., 2001), and may even display symptoms worse than the unknown definitive host (Hoffman and El-Matbouli, 1994). However, Morris and Adams (2006) proved salmonids to be an integral part of the parasite’s life cycle. By transmitting *T. bryosalmonae* from aquarium effluent containing infected brown trout to uninfected bryozoan, *Fredricella sultana*, and from there to rainbow trout via collected spores from the infected bryozoa, Morris and Adams (2006) successfully completed a full cycle of transmission, concluding that the parasite can cycle between these hosts indefinitely without requiring another organism to act as an additional host.

1.3.4.1 Response of salmonids to PKD

1.3.4.1.1 Internal symptoms

The early response of salmonids to PKD is believed to be cellular; proliferation of the haematopoietic cells in the interstitium of the kidneys (Castagnaro et al., 1991; MacConnell et al., 1989), followed by damage to vascular endothelium and the onset of lesions with severe diffuse granulomatous response occurring (MacConnell et al., 1989; Scott, 2002). Macrophages appear epithelial and gradually replace haematopoietic tissue resulting in a significant anaemia with haematocrits as low as 11%, where 40% would be considered normal (Ferguson and Needham, 1978). Increased iron deposits in the spleen (haemolysis) and hypoplasia of haematopoietic tissue are believed to contribute to the low haematocrit level (Ferguson and Needham, 1978). Uncontrolled proliferation of immune cells in various organs, particularly the kidney, results in the destruction of haemopoietic tissue, also contributing to the severe anaemia (Clifton-Hadley et al., 1985).
The main gross pathological symptom is excessive swelling of the kidney (Ferguson and Needham, 1978; Clifton-Hadley et al., 1983, 1984) often with greyish, bulbous ridges forming (Ferguson and Needham, 1978; Hedrick et al., 1984ab), but the reaction can extend along the full length of the kidney and into the anterior hematopoietic tissue (Ferguson and Needham, 1978; Hoffman and El-Matbouli, 1994). The chronic inflammation of the kidney characterising the disease (Kent and Hedrick, 1986; Hedrick et al., 1988b; Castagnaro et al., 1991) is caused by the presence of mononuclear cells and macrophages, often with partial fibrosis as the interstitial tissue converts to a granulomatous tissue (Hoffman and El-Matbouli, 1994). In severe cases the progressive renal swelling can lead to renal failure and displacement of the swimbladder (Alderman and Clifton-Hadley, 1988).

When the swimbladder is found to be laterally distorted, it creates longitudinal swelling, whilst abdominal swelling can be associated with peritoneal fluid; often blood stained (Ferguson and Needham, 1978; Sippel and Ferguson, 1983). Patches of greyish mottling, thought to be caused by granulomatous lesions are sometimes visible on the liver, and occasionally the spleen, beneath the capsule or throughout the stroma. The spleen can also be either reduced in size or massively enlarged (Ferguson and Needham, 1978; Sippel and Ferguson, 1983). The internal symptoms caused by PKD are illustrated in Figure 1.11, below.

Figure 1.11 - Internal symptoms of Proliferative Kidney Disease, including swollen kidney and spleen
Source: Photograph courtesy of C McGurk
1.3.4.1.2 External and behavioural symptoms

The inflammation of the kidney leaves heavily infected fish exhibiting distended abdomens with longitudinal swelling of the body wall, level with the lateral line (Ferguson and Needham, 1978). Darkened colouration (melanosisis) and exophthalmia can occur, either mono- or bilaterally (Ferguson and Needham, 1978; Hoffman and El-Matbouli, 1994; Scott, 2002). Feist et al. (2001) describe symptoms of frayed fins and haemorrhaging posterior to the adipose fin, with scale protrusion (O’Hara, 1985) and roughened skin; thought to be related to inflammatory nodules in the red muscle causing protuberances at the skin surface (Fernández-de-Luco et al., 1997). Behavioural changes include loss of appetite, introversion, apathy, and unresponsiveness (Hoffman and El-Matbouli, 1994). In the latter stages of disease, respiratory distress coincides with gill pallor due to pronounced anaemia (Klontz et al., 1986; O’Flynn and Mulcahy, 1995; Feist et al., 2001), whilst increased nervous agitation marks the inception of death (Ferguson and Needham, 1978; Sippel and Ferguson, 1983).

1.3.4.2 Biology of T. bryosalmonae within salmonids

Two developmental phases of the parasite have been described in infected fish;extrasporogenic and sporogenic stages (Morris et al., 1999). Extrasporogenic stages are found in the blood vessels and interstitium of the kidney at first (Foott and Hedrick, 1987; Fernández-de-Luco et al., 1997; Morris et al., 1999) before migrating to the lumen of the kidney tubules (Foott and Hedrick, 1987). Extrasporogenic stages are compiled of several secondary cells bound within a primary cell, whilst sporogenic stages are believed to be a result of the secondary cells being released from the primary cell following migration to the kidney tubule lumen (Kent and Hedrick, 1986). The sporogenic development without the presence of completely formed spores (Kent and Hedrick, 1986) and increasing number of parasites aggravate the kidney forming nodules (Fernández-de-Luco et al., 1997) which causes an intense inflammatory response (Foott and Hedrick, 1987; Kent and Hedrick, 1987; Fernández-de-Luco et al., 1997). Progression of the disease leads to parasites being transported around the body via the circulatory system to affect such tissues and organs as the spleen, liver, epithelium of the gills,
heart, brain, spinal canal, islets of Langerhans, striated muscle, intestinal submucosa and peritoneum (Fernández-de-Luco et al., 1997).

Clifton-Hadley et al. (1985) give a detailed account of the development of PKD, its symptoms and the histology of affected specimens over time. They describe how parasite appearance may take up to 8 weeks to occur, although the organism can be first detected in fish 3 to 4 weeks following exposure (MacConnell et al., 1989; Kent et al., 1995) or as little as 2 weeks in the kidney interstitium following exposure to enzootic waters (Kent and Hedrick, 1986; Clifton-Hadley et al., 1987). As little as 1 week later (week 9), clinical signs prevail lasting for 12 to 20 weeks (Clifton-Hadley et al., 1985) with weeks 6 to 20 thought to be most prominent (Kent et al., 1995). Parasites undergo degenerative changes 12 weeks after exposure up to week 20 when fish often make a complete recovery (Clifton-Hadley et al., 1985; Morris et al., 1999), and the infection in the kidney interstitium and associated lesions resolve (Kent et al., 1995). However, Clifton-Hadley et al. (1985) explain that the sequence of events may vary dependent on water temperature, disease prevalence, time of year, and location.

1.3.4.3 Variations in display

Normally associated with fish farms on soft, acidic waters (Ferguson and Adair, 1977; Ferguson and Needham, 1978), the prevalence and intensity of PKD has been reported to increase throughout summer (Fernández-de-Luco et al., 1997). Peribáñez et al. (1997) compared the renal prevalence and density of parasites in rainbow trout and brown trout held in the same environment. In rainbow trout renal prevalence and density of parasites peaked in July, but in brown trout renal prevalence and maximum density was reached in May and July, respectively. By January Fernández-de-Luco et al. (1997) observed no parasites in PKD recovering fish. Conversely, Foott and Hedrick (1987) observed later sporogonic stages of the parasite in rainbow trout for up to one year following initial infection.
1.3.4.4 Potential entrance sites and species-specific response

To date the method of entry into the host has remained in question (Okamura et al., 2001). Feist et al. (2001) discuss numerous sites for spore invasion. Although early invasive stages were repeatedly detected in the gills initially, the authors believe the most obvious entrance site would be via the skin. Ferguson and Needham (1978) describe *T. bryosalmonae* in the gills; throughout the capillaries and lamina propria of the second lamellae. Gills are an obvious entrance site for parasites, as they are extremely exposed organs and constructed of delicate tissue. Further, parasites could easily enter the bloodstream via the gills and use it as a transport system to other organs (Clifton-Hadley et al., 1985). While not detected in the epithelial cells of the gut mucosa, Ferguson and Needham (1978) observed parasites in the stroma of the liver, spleen, and kidney. Once the kidney has reached maximum capacity, the parasites have been known to invade the muscle (Fernández-de-Luco et al., 1997; Peribáñez et al., 1997). Peribáñez et al. (1997) and Fernández-de-Luco et al. (1997) first detected parasites in the muscle only when the kidney became saturated. Lesions, which principally affect kidneys, were found to occur in the striated muscle and macroscopical nodules in the red muscle in nearly 80% of surviving subyearling fish in the study by Fernández-de-Luco et al. (1997).

The presence of parasites in the muscle is thought to be exclusive to rainbow trout and that other species may have the potential to control the reproduction of *T. bryosalmonae* avoiding the subsequent spread to such areas (Peribáñez et al., 1997). However, MacConnell et al. (1989) observed an effective response to the parasite in rainbow trout and suggested that it interrupted the maturation of the organism, inhibiting the migration of *T. bryosalmonae* to the lumen of the kidney and the subsequent sporulation, when fish were experimentally induced with PKD. Although conflicting in terms of species ability to inhibit maturation, there appears to be a species-specific response to the disease, which is supported by the timing of *T. bryosalmonae*, its potency, and prevalence in different salmonids (Fernández-de-Luco et al., 1997; Peribáñez et al., 1997).
1.3.5 Detection of *Tetracapsuloides bryosalmonae*

As fish succumb to PKD the definitive cause of death is often difficult to distinguish due to the presence of secondary infection. In 1983, Clifton-Hadley *et al.* described how a presumptive diagnosis can be made simply by observation; symptoms, post mortem appearance, and referring to the history of the disease on site, whilst a positive diagnosis required histological examination, showing the characteristic proliferation of mononuclear cells in the renal interstitial tissue. However, the method was deemed costly in terms of consumables and time, and kidney impression smears were eventually introduced; found to be easier, quicker, and more efficient (Clifton-Hadley *et al.*, 1983). Similarly, squash preparations are a sufficient method to observe mononuclear lymphoid cells and macrophages surrounding primary cells (Clifton-Hadley *et al.*, 1983; Hoffman and El-Matbouli, 1994).

As technology advanced the methodology for diagnosis has modernised, and diagnosis by observation of *T. bryosalmonae* in kidney smears or histological sections is used as a basic tool only (le Gouvello *et al.*, 1999). More precise and sensitive methods have been designed; PCR (Saulnier and de Kinkelin, 1996; Kent *et al.*, 1998), monoclonal antibodies (Adams *et al.*, 1992; de Mateo *et al.* 1993; Saulnier and de Kinkelin, 1996), and lectins (Castagnaro *et al.*, 1991) allow early detection and response to PKD. The development of *T. bryosalmonae*-specific monoclonal antibodies (see Adams *et al.*, 1992) and the binding lectin, *Griffonia simplicifolia*, GS-1, allows staining of the extrasporogonic stages in tissue sections, through standard immunostaining techniques using peroxidase or fluorescein (Castagnaro *et al.*, 1991; Hedrick *et al.*, 1992).

The monoclonal antibodies developed against *T. bryosalmonae* allow a demonstration of the parasite’s antigenic characteristics which change throughout its development in fish (Adams *et al.*, 1992; Morris *et al.*, 1997; Saulnier and de Kinkelin, 1999). This change in antigenicity of the parasite implies monoclonal antibodies are limited for the study into the lifecycle, as well as the early detection of infections. However, a successful in-situ hybridisation DNA method that stains both developmental phases of the parasite has been designed, allowing the
identification of the genetic association between them. The method proved successful in preclinical and clinical infections in numerous organs, demonstrating the potential of the technique to identify non-clinical T. bryosalmonae in the salmonid host (Morris *et al.*, 1999).

1.3.6 **Freshwater Bryozoa and *Tetracapsuloides bryosalmonae***

Freshwater invertebrates, from the phylum Bryozoa (Class: Phylactolaemata), commonly known as ‘moss animals’ (McGurk *et al.*, 2003) are colonial, suspension-feeding invertebrates found in a wide variety of agnostic aquatic environments. They are generally overlooked due to their inconspicuousness, sessile nature, but can be found growing in subtle locations. Living on submerged branches, stones and macrophytes, as temperatures increase towards summer the organisms can be observed producing asexual propagules called statoblasts; tiny (<1 millimetre) seed-like structures of two chitinised valves enclosing dormant germinal tissues. These statoblasts are used to assess taxonomy as it is extremely difficult to differentiate between species due to phenotypic similarities of the colony form; taxonomy is based on morphological detail of the statoblasts (Okamura and Wood, 2002).

1.3.6.1 **Bryozoa; the primary host?**

Through DNA analysis, bryozoans were discovered to act as a host to T. *bryosalmonae* (Okamura *et al.*, 2001; Okamura and Wood, 2002). The prevalence of the parasite within numerous species of Bryozoa resulted in Canning *et al.* (2000) suggesting that bryozoans could be the primary host of *T. bryosalmonae*. From the numerous and poorly understood species of bryozoans, several are now considered to be natural hosts; phylactolaemate bryozoans ranging from primitive to derived genera, *Fredericella* species (*sultana* and *indica*), *Plumatella* species (*rugosa, magnifica, and emarginata*), and *Cristatella mucedo* are believed to be susceptible to *T. bryosalmonae*, with *F. sultana, F. indica*, and *P. emarginata* predominantly identified at disease outbreaks (Okamura *et al.*, 2001; Okamura and Wood, 2002).
Okamura and Wood (2002) briefly discuss bryozoans as the only host required in the lifecycle of *T. bryosalmonae*, whilst Okamura *et al.* (2001) considered infection may alternate between species of bryozoan depending on their seasonal availability, but this would not be obligatory as Okamura and Wood (2002) found only a single species of bryozoan at one site infected with *T. bryosalmonae*. Conversely, Tops *et al.* (2004) conducted experiments studying bryozoan to bryozoan transmission, reporting consistent failure, suggesting such transmission is precluded in malacosporean lifecycles. Morris and Adams (2006) successfully transmitted *T. bryosalmonae* from brown trout to bryozoans, and also from bryozoans to rainbow trout as found by Feist *et al.* (2001), but to date the transmission from rainbow trout to bryozoans remains inconclusive and requires further investigation (Tops *et al.*, 2004).

1.3.6.2 Environmental requirements and distribution

The environment in which infected bryozoan species have been found ranges from clear, cold streams to warm, eutrophic lakes, suggesting bryozoa, and therefore *T. bryosalmonae*, can inhabit a variety of environments indicating a wide geographical spread is possible (Okamura *et al.*, 2001). However, Okamura *et al.* (2001) found *T. bryosalmonae* to be inconsistent in distribution, both spatially and temporally. In the UK, *Plumatella* (probably *P. emarginata*) and *F. sultana* are the predominant species known to act as host to *T. bryosalmonae* (Longshaw *et al.*, 1999), whilst in many countries across Europe, Canada, and the USA records indicate the presence of *T. bryosalmonae* in various species of bryozoa (Tops and Okamura, 2003).

Although geographically dispersed Kent *et al.* (1998) discovered a maximum variation in sequences of only 0.8% among all *T. bryosalmonae* isolates across 764 base pairs, when specific primers were designed to amplify and sequence a portion of SSU rDNA extracted from kidney samples of infected salmonids in England, California, British Columbia, Washington, and Newfoundland. This strongly suggests *T. bryosalmonae* isolates from various regions across the world are closely related, or indeed, the same species. Henderson and Okamura (2004) discovered the greatest genetic diversity of *T. bryosalmonae* to be throughout North America. Colonies in southern Europe are thought to have established via distribution of this
North American clade (Henderson and Okamura, 2004). Although it remains unclear as to how colonies and the disease has spread, Henderson and Okamura (2004) believe the parasite was not distributed via fisheries, as colonisation significantly predated fisheries and aquaculture activity. Instead, it is suggested that waterfowl are the vectors of *T. bryosalmonae*, introducing the Malacosporean parasite from North America into European countries (Henderson and Okamura, 2004). However, new evidence describing the role of salmonids in the life cycle of *T. bryosalmonae* (Morris and Adams, 2006), suggests that salmonids should not be ruled out as a means of distributing the parasite.

Should alternative or obligate hosts become apparent, they will inevitably show a habitat requirement as extensive as the infective organism (Okamura and Wood, 2002). Potential final hosts discussed include, other fish; in particular, cyprinids (Kent and Hedrick, 1986; Hoffman and El-Matbouli, 1994; Okamura et al., 2001) and sticklebacks (*Gasterosteus aculeatus*) (Kent and Hedrick, 1986; Feist, 1988), invertebrates (Okamura et al., 2001), and numerous species of birds (Hoffman and El-Matbouli, 1994).
1.4 Furunculosis

1.4.1 Affected species and geographical range

Furunculosis received its name due to the characteristic boil-like lesions that resemble those observed in the human condition. As an aquatic disease, furunculosis has only been recognised for a little over 100 years, but in this time much emphasis has been placed on its control due to the detrimental effects witnessed in fresh and saltwater fishes (Munro and Hastings, 1993). Since its initial description in the late nineteenth century, the disease has been associated with major losses of both wild and cultured salmonid fish, which act as the predominant host (Emmerich and Weibel, 1894; Roberts and Shepherd, 1974; Munro and Hastings, 1993). Leitritz and Lewis (1976) describe the disease as a septicaemia; the causative agent is carried in the blood and collects in clumps in small blood vessels before rupturing and invading surrounding tissues. This produces lesions and sores which appear as swollen red spots beneath the skin, before destroying the surrounding tissue to enlarge as characteristic furuncles.

Although salmonids are the primary host of furunculosis, numerous other species are also known to be affected by the causative agent. Atypical strains of the bacteria have been linked with major diseases in marine and freshwater species; Atlantic cod (*Gadus morhua*), goldsinny wrasse (*Ctenolabrus rupestris*), carp (*Cyprinus carpio*), and goldfish (*Carassius auratus*) (see Munro and Hastings, 1993). However, in such diseases the symptoms and gross pathology generally differ from that observed in furunculosis of salmonids, for example in ulcerative disease of (non-)salmonid species, and erythrodematitis in carp (Munro and Hastings, 1993; Southgate, 1993).

The geographical spread of furunculosis mirrors the culture areas of salmonids, ranging across almost all continents. As the incidence of disease increases in wild and non-salmonid species, the distribution is now thought to be worldwide; endemic in Europe, North America, Japan, Korea, Australia, and South Africa (Holliman, 1993; Munro and Hastings, 1993). In Norway, the disease originated after several sporadic outbreaks in the Numedalslagen River between 1966 and 1977,
subsequently affecting fish farms by 1985; as the largest producer of salmonids in the world, furunculosis is recognised as one of Norway’s most economically damaging diseases (Nordmo, 1993). In the UK, Scottish salmon farms have also been hindered by furunculosis with many sites experiencing endemic outbreaks and losses of up to 20% of stock (Munro and Hastings, 1993; Wall, 1993).

1.4.2 Causative agent: discovering and classifying *Aeromonas salmonicida*

The first authentic report of the causative agent of furunculosis was given by Emmerich and Weibel in 1894 when it was discovered in a German trout hatchery. Although named bacillus of contagious trout disease (*Bacillus der Forellenseuche*) in Germany, it was referred to in English as *Bacillus salmonicida* until Griffin *et al.* (1953) suggested the taxonomy *Aeromonas* within the family Vibrionaceae. In 1963 Smith suggested a separate genus but this was disputed and the bacterium remained under the genus *Aeromonas* until classification was certified in 1978. By studying the homology between *A. salmonicida* and *A. hydrophila* McCarthy (1978) showed a 56% to 65% degree of binding, indicating a relationship at the generic level. Further homology studies between many isolates of *A. salmonicida* indicated three subspecies may be necessary (McCarthy, 1978; Belland and Trust, 1988). Based on epizootiological criteria, classification now includes; group one – *A. salmonicida*, subspecies *salmonicida* typically derived from salmonids; group two – *A. salmonicida*, subspecies *achromogenes* derived from salmonids as atypical strains, including the former *achromogenes* and *masoucida*; group three – *A. salmonicida*, subspecies *nova*, atypical strains associated with non-salmonid species (Belland and Trust, 1988).

1.4.3 Salmonids and furunculosis

Emerging as a septicaemia and often fatal, furunculosis can affect all species of salmonid at any stage of life (Munro and Hastings, 1993). Clinical manifestation varies from acute to chronic depending on the age and species of the fish concerned, as well as the environment (Southgate, 1993). Younger fish are more likely to suffer from the acute form of the disease when there may be few clinical signs other than behavioural changes, whilst more chronic cases display typical hemorrhagic
symptoms (Southgate, 1993). Rainbow trout are believed to be the least affected species, whilst Atlantic salmon and brown trout the most susceptible (Roberts and Shepherd, 1974; Leitritz and Lewis, 1976; Southgate, 1993). As an opportunistic pathogen, occurrences are generally stress-related and can be heavily influenced by the environment. Outbreaks of epizootic proportion have been known to occur at temperatures of 10°C to 15°C or more (Stevenson, 1987). The severity of the disease has led to morbidity of up to 100% with mortality exceeding 80%, especially in young fish (Stevenson, 1987). Such losses pose a real threat to the salmonid industry.

1.4.3.1  **Response of salmonids to furunculosis**

1.4.3.1.1  **Internal symptoms**

Depending on the stage of disease, the symptoms displayed may vary. Munro and Hastings (1993) explain that the time course of the disease is important as stocks may present a continuum of pathologies from acute to chronic. At the acute stage, internal inflammation of the intestine can be observed, as well as splenomegaly, liver pallor, soft kidneys, and petechial haemorrhaging in the pancreatic fat or musculature of the flanks (Holliman, 1993; Wall, 1993). Congestion of blood vessels in the body cavity is often observed, and the lining of the intestine may be inflamed with bloody discharge and mucous from the vent, especially after death. The spleen can be enlarged and cherry red, whilst the kidney is usually badly infected, often becoming liquefied. Death generally occurs before furuncules form, unlike that observed in the chronic form (Leitritz and Lewis, 1976).

In chronic cases the slow progression of infection results in a greater degree of bacterial localisation in visceral organs, commonly the kidney, spleen, blood vessel walls, and intestine, but also in the liver and gills (Munro and Hastings, 1993). Erythematous and petechial haemorrhaging may be apparent, but larger haemorrhaging occurs across the internal organs, especially the swimbladder, which appears swollen and cloudy. The kidney and liver turn greyish with necrotic areas, while blood vessels surrounding the lower intestine and pyloric caeca are inflamed. The peritoneum and pericardium often have bloody fluid accumulations and the
intestine is devoid of food and can have an exudate of blood, mucous, and cellular debris. The spleen is greyish to green in colour and the intestine may be filled with bloody food and faeces. Haematocrit measurements often show severely depressed red cell numbers, coinciding with gill and liver pallor, whilst furuncules grow on visceral organs, and the internal body wall (Stevenson, 1987; Munro and Hastings, 1993; Wall, 1993). Further development of furuncules within the skeletal muscle leads to the surrounding tissue being destroyed, eventually appearing as necrotic areas of muscle that burst through the exterior of the fish releasing a thick bloodstained material (Munro and Hastings, 1993; Wall, 1993).

1.4.3.1.2 External and behavioural symptoms

The display of furuncules occurs following a chronic outburst in older fish (Roberts and Shepherd, 1974). Chronic conditions present the majority of external and behavioural symptoms, as acute forms exhibit limited external symptoms (if any) prior to death; perhaps inappetence and melanosis. In addition to inappetance and melanosis, in chronic cases petechiation at the fin bases, lethargy, haemorrhaging, or anaemia of the gills can be apparent, before characteristic furuncules appear (Figure 1.12) (Leitritz and Lewis, 1976; Stevenson, 1987; Holliman, 1993; Munro and Hastings, 1993). Behaviourally, affected fish exhibit lethargy, aberrant swimming patterns, and a lack of shoaling (Wall, 1993).

Figure 1.12 - Atlantic salmon affected by Aeromonas salmonicida, showing classic 'furuncule'
Source: Modified from Southgate, 1993
1.4.4 Detection and treatment of *A. salmonicida*

Diagnosis of furunculosis is based on clinical signs, histopathology and the laboratory isolation of the bacteria from affected individuals. Post-mortem examination may show no gross pathological signs depending on the status of the disease (e.g. peracute). Similarly, histopathology differs with furunculosis development; in acute cases little more than limited necrosis of tissues is apparent, while sub-acute and chronic cases typically show the presence of accumulations of bacteria in many tissues with accompanying necrosis but little inflammatory response (Southgate, 1993). *Aeromonas salmonicida* has been identified as Gram-negative, facultative anaerobic rod with rounded ends, approximately 0.3 to 1.3 micron by 1.0 to 3.5 micron. Although members of the aeromonad family are generally motile, *A. salmonicida* is the only exception (Munro and Hastings, 1993; Southgate, 1993). Identification is possible through staining and observation of coccoid forms in tissue smears and culture plates. Plates containing suitable media require incubation at approximately 22°C for 24 to 48 hours, at which stage a brown diffusible pigment characteristic of *A. salmonicida* can be observed. Phenotypic identification is possible, but Munro and Hastings (1993) warn that colonies can vary dependent on the strain of *A. salmonicida*; the majority are extremely friable, with colonies capable of being pushed across the agar plate, whilst less common colonies are smooth looking and soft to touch. In broth cultures, the common strains autoagglutinate to produce a settlement at the base of tubes, whereas non-agglutinating smooth variants produce a uniform turbidity.

Historically, *A. salmonicida* would be isolated and its antibiotic sensitivity assessed in order to ascertain effective therapy (Southgate, 1993). In the 1950’s, the development of antibiotics showed promising results to control furunculosis, and by the 1970’s oxytetracycline, trimethoprim-sulphadiazine, oxolinic acid, and amoxicillin were regarded as effective treatments administered orally via feed. However, continued and prolonged use led to the pathogenic bacteria developing a resistance to the antibiotics. Initially, resistance was specific to one drug, so an alternative could be used, but eventually a general resistance occurred and by mid-1980 outbreaks of furunculosis increased dramatically. In time *A. salmonicida* has
evolved, leaving only amoxicillin as a curative. Today, British aquaculture continues to use amoxicillin, but even a resistance to this drug is occurring.

1.4.5 Vaccination

Attempts to produce an effective vaccine against furunculosis have been in progress since 1942. Essentially, vaccines are killed bacteria cultures that are tested for efficacy by immunising and challenging fish. Successful vaccines in other bacterial diseases of salmonids (e.g., Vibriosis and Enteric Red Mouth) were established relatively quickly compared to furunculosis. Due to limited knowledge of which antigen(s) are protective and in what quantity they are necessary, only modestly successful vaccines were developed for rainbow trout until recent years (Stevenson, 1987; Lutwyche et al., 1995; Siwicki et al., 2002). In the commercial production of Atlantic salmon, vaccines against furunculosis have been available for a number of years, but most have proved to be of limited use for rainbow trout. However, through improved knowledge and research into the disease, vaccines are becoming more and more effective. Today, numerous companies offer life long protection against furunculosis in rainbow trout, e.g., Pharmaq Vaccines, Norvatis Ltd, Schering-Plough Animal Health (Aquavetplan, 2001; Schering-Plough, 2007). Their role will inevitably play an important part in the future of preventing the disease (Southgate, 1993).

1.4.6 Control

Furunculosis is generally introduced to sites by the movement of infected fish on to a culture facility or by wild carriers shedding bacteria into the watercourse. Fish tissue, equipment, and escapees are also known to act as vectors (Southgate, 1993). However, even if present in the environment, it does not necessarily suggest that disease is inevitable. Furunculosis is highly dependent on the environment of the host, as stress is believed to be the primary factor resulting in outbreak. Poor environmental conditions, increased temperatures, trauma, and crowding resulting in physical damage, will increase the transmission and infection rate of the disease. Stressors therefore require careful consideration (Holliman, 1993; Munro and Hastings, 1993).
As the problems surrounding furunculosis continue, its impact on the industry is not to be underestimated; losses are increasing, multiple resistance to antibiotics is developing, and the distribution of farms is so widespread that no area is likely to be risk free. Although antibiotics can be administered with some degree of success, avoidance and vaccination are the preferred methods of control. With increased knowledge and interest in genetic techniques, selective breeding for resistant strains may well provide an alternative or additional opportunity to improve control measures, and help suppress the effects of the disease (Munro and Hastings, 1993).
1.5 Aims and objectives

The aim of this project was to test for evidence of additive genetic variation towards the commercially important disease Proliferative Kidney disease, via the scoring system of Clifton-Hadley *et al.* (1987), and to support any evidence of this variation by undertaking challenge tests with the causative agent, *Tetracapsuloides bryosalmonae*. The forthcoming chapters will consider:

- Testing for evidence of additive genetic variation towards kidney score, according to the scale of Clifton-Hadley *et al.* (1987), following a natural challenge in *T. bryosalmonae* enzootic water. Where evidence of variation exists, estimates of heritability will be obtained for the two rainbow trout strains involved. Additionally, the genetic correlations between the three traits measured (kidney score, fork length, and body weight) will be calculated.

- Experimentally inducing PKD in a certified disease-free strain of rainbow trout. Challenge tests on these distinct families is expected to provide information on the level of resistance to *T. bryosalmonae*, as all individuals involved will be subject to the Malacosporean parasite on a single day. The level of additive genetic variation for resistance to PKD will therefore be investigated, which will also act to support or dismiss the initial findings of the project. Additionally, the progression of PKD in individual families, and phenotypic trends between size and kidney score will be documented.
Chapter 2: General Materials and Methods
2.1 Molecular Biology; Genetics

2.1.1 DNA extraction

All DNA was extracted from tissue samples using one of two methods; phenol-chloroform or Chelex. Dependent on the tissue and storage time prior to extraction, samples were stored individually in 0.5 millilitre (ml) Eppendorf tubes (Thermofischer, UK) and either refrigerated immediately (frozen if necessary: kidney samples) or submerged in 95% ethanol (Fischer Scientific, UK) (fin clips) ready for extraction.

2.1.1.1 Phenol-chloroform method

The protocol used in the present project is specifically designed for the rapid extraction of salmonid DNA from fresh, frozen or ethanol preserved samples (Taggart et al., 1992). The expected quantity of DNA obtained using this method is dependent on the tissue used, eg liver, blood, adipose, muscle etc, and can vary significantly the amount of DNA obtained; ranging from 10 to 250 micrograms (µg) DNA. The size of tissue sample also affects DNA yield; standardisation was therefore important.

Dependent on the storage conditions of the tissue, samples were either thawed or were blotted free of ethanol and allowed to air dry before a biopsy punch (Kruuse, UK) of 3 millimetre (mm) diameter (Ø) was taken. Each sample was placed into an individually labelled autoclaved 0.5 ml Eppendorf tube containing 10 microlitre (µl) Proteinase K (ABgene, UK) at 20 milligram (mg)/µl and 340 µl of TEN buffer (0.2 molar (M) ethylenediaminetetra-acetic acid (EDTA) at pH 8.0 with 0.5% weight:volume (w:v) sodium dodecyl sulphate (SDS): Sigma, UK). The Proteinase K reduces proteins to their component amino acids, whilst SDS causes cells to rupture, initiating protein denaturation. The tubes were mixed briefly then placed in a rotating oven (Techne Hybridiser HB-1: Techne, UK) to incubate at 55°C for approximately 16 hours (hr). Following incubation 10 µl Rnase (Dnase free at 2 mg/µl: ABgene) was added to the solution and shaken vigorously. A further incubation period to digest and remove traces of RNA followed; 60 minutes (min) at
37°C. Phenol (Fischer Scientific) was added at 350 µl per tube to extract the denatured protein from the DNA solution. This was followed by vigorous shaking for 10 seconds (s) before gentle turning for 15 to 20 min. The process was mirrored with the addition of 350 µl of chloroform (Fischer Scientific) to each tube to absorb and eradicate traces of phenol, before the tubes were shaken for 10 s and turned gently for 15 to 20 min. New autoclaved Eppendorf tubes required labelling whilst the samples were centrifuged at 10,000 gravitational force (xG) for 5 min to create separation. Upon removal from the centrifuge a DNA-filled top aqueous layer was apparent, of which 300 µl was removed (using a wide bore pipette tip) and dispensed into the new tube. Nine hundred µl of 92% ethanol (volume:volume (v:v), distilled water) was added to the new tubes, which was then mixed vigorously to precipitate out the DNA pellet. A rest period of 2 to 3 min allowed the pellet to fall to the base of the tube, so that the 92% ethanol could be decanted, and replaced with 70% ethanol (v:v, distilled water). The tubes were then turned gently for at least 30 min, acting as a wash, before the ethanol was removed and the tube allowed to air dry for 5 to 10 min. The pellet was resuspended in TE buffer (10 mM electrophoresis purity reagent Tris (hydroxymethyl) – amino methane (Tris: Biorad, USA), 1 mM EDTA, pH 8.0) and left to dissolve for 24 to 48 hr. Aliquots of the stock solutions were transferred to 96-well plates (ABgene) and the concentration of extracted DNA determined using a 6405 UV/Vis spectrophotometer (Jenway, UK); additional TE was added where required to maintain a 100 µg/ml working concentration (protocol modified from Taggart et al., 1992). Both stock and working solutions were stored at -20°C.

Prior to using the working solution, the DNA was denatured by heating to 95°C for 15 min. Phenol-chloroform extracted DNA was preferred where high quality DNA was required and long periods of storage was necessary.

2.1.1.2 Chelex method

A 10% Chelex solution (1:10 w:v, (Chelex (Sigma) to TE buffer) at pH 8.0, plus 0.1% SDS) was warmed (approximately 60°C) and continuously spun using a Bibby HB502 centrifuge (Bibby, UK) to create an even distribution of beads to assist in their uptake. Using a wide bore pipette, 100 µl of solution was dispensed into the
number of required wells of a 96-well plate to which was added 3 µl of Proteinase K (20 mg/µl). As per the phenol chloroform method, tissue was removed from individually labelled Eppendorf tubes and blotted dry or allowed to thaw. Biopsies of the tissues were added to wells before the plate was sealed securely using adhesive PCR film (ABgene) to prevent evaporation at the incubation phase; 55°C for at least 3 hr in a T-gradient thermoblock (Biometra, Germany). At the end of incubation the plate was centrifuged at 258 xG for 1 min before being returned to the thermoblock for denaturation; 95°C for 15 min. A further spin of 258 xG for 1 min was performed. When the solution was not used immediately, samples were stored at -20°C, although attempts were made to use the DNA within two weeks of extraction.

2.1.2 Polymerase chain reactions (PCR) and primers

Three PCRs were used in this project, two of which utilised multiple loci due to the large amount of information required for successful parental allocation (Multiplexes 1 and 2). These PCRs required vast optimisation due to the intricate chemistry involved in the reaction. Where qualitative answers were required for the detection of *Tetracapsuloides bryosalmonae*, a single locus PCR was used.

2.1.2.1 Single locus PCR

2.1.2.1.1 Primer preparation

Designed by Kent *et al.* (1998) the primers, 5f (CCTATTCAATTGAGTAGGAGA) and 6r (GGACCTTACTCGTTTCCGACC), specific to the parasite *Tetracapsuloides bryosalmonae*, causative agent of proliferative kidney disease (PKD), were used in the ‘PKD-PCR’. The primers were dissolved in molecular biology grade (mbg) water (BDH Laboratory Supplies, UK) for 10 min at 55°C, resulting in a 100 micromolar (µM) concentration. Following a vortex, aliquots of 10 µl were stored at -20°C. When required, 2.5 µM working solutions (ws) were prepared by adding mbg water.
2.1.2.1.2 PKD-PCR

Alterations were made to the method of Kent et al. (1998) in the PCR preparation. The necessary amount of each solution per sample was pooled into a single 1.5 ml Eppendorf tube before being aliquoted into the required number of wells of a 96-well plate. Added into the tube were 8 µl mbg water, 1 µl of each primer, and 12.5 µl PCR master mix solution (2 x Reddymix, ABgene, UK; 1.25 units Thermoprime plus DNA polymerase, 75 mM Tris-HCL (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% Tween 20, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, precipitant and red dye for electrophoresis). The reaction volume totalled 25 µl, including 2.5 µl DNA template. Both positive and negative controls were included in each reaction (see Section 2.1.2.1.3).

The actual DNA amplification process followed that of Kent et al. (1998) where DNA denaturation lasted 3 min at 94°C, followed by 35 cycles of amplification; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, ending with a 5 min extension at 72°C, all of which took place in a T-gradient thermoblock. Samples were then mixed, pulse spun to condense the reaction mix in the bottom of the tube, and held on ice awaiting gel electrophoresis.

2.1.2.1.3 Gel electrophoresis

All PCR products were checked under ultra violet (UV) illumination (UVS white UV Syngene Ingenius transilluminator; Synoptics Ltd, UK) following electrophoresis on a 1.2% agarose gel containing Ethidium Bromide (EB). All agarose gels and their buffers were sodium boric acid (SBA) based, used at a 1x concentration. Although dependent on the required amount of buffer and size of gel, the majority were prepared by adding 80 ml of 25x SBA (1:100 w:v, 1 mol sodium hydroxide pearls (NaOH) with mbg water, pH to 8.5) to 1920 ml of mbg water making a 1x SBA solution. Two hundred millilitres of the solution was poured into a 500 ml flask and 2.4 grams (g) of electrophoresis grade agarose (Invitrogen, UK) added. This was mixed, heated in a microwave until boiling, then 4 µl EB (2 µl/100 ml) was added to the solution before being left to cool to handling temperature. Whilst cooling, a suitable gel caster (Biorad) was prepared and the
necessary combs positioned. Once cooled the solution was poured and allowed to set (approximately 30 min).

The gel was placed into a compatible electrophoresis machine (Biorad), and covered by the remaining 1800 ml of 1 x SBA solution. The wells were loaded with one of four solutions; 7 µl of negative PKD-PCR product, 7 µl of positive PKD-PCR product, 7 µl of PCR product requiring analysis, or 7 µl of DNA molecular weight marker (ØX 174 RF DNA Hae II, 11 bands, size 72 to 1353 base pairs (bp): ABgene). Due to the nature of SBA (see Brody and Kern, 2004), the gel could be used at 300 volts (v), 195 milliamps (mA) for 20 to 25 min allowing enough time for separation. Any positive bands were apparent at 435 bp; the size of which was determined from the nuclear weight marker.

2.1.2.2 Multiple locus PCR

2.1.2.2.1 Microsatellite loci

The microsatellite loci and subsequent multiplex PCRs in the present project were largely influenced by Fishback et al. (1999). In order to ascertain enough information to allow parental allocation, as many loci as possible were incorporated into one of two multiplex systems. A total of ten microsatellites were selected, seven comprising multiplex 1, and three in multiplex 2 (Table 2.1). Either the forward or reverse primer was labelled with one of three WellRED fluorescent dyes; dye 2PA (black), dye 3PA (green), or dye 4PA (blue) (Proligo primers and probes, USA).
2.1.2.2.2 Multiplex PCRs

The two multiplex systems were configured following a lengthy optimisation period. In the majority of instances PCR amplification took place in 0.2 ml 96-well plates, sealed with adhesive PCR film, using a total volume of 15 µl; 6 µl mbg water, 2.1 µl of fluorescently labelled primer and unlabelled primer (1:1, v:v), 280 µM of each dNTP (0.84 µl: ABgene), 2.0 µM MgCl₂ (1.2 µl: ABgene), 3 µl Buffer II (75 mM Tris-HCL; 20 mM (NH₄)₂SO₄, 0.01% (v:v) Tween® 20: ABgene), 3 Units Taq polymerase (ABgene), and 1.5 µl of genomic DNA, assumed to be ~100 µg.

As the loci used had various optimum annealing temperatures, it was necessary to use a T-gradient thermoblock touchdown PCR program, allowing simultaneous loci amplification and the suppression of any spurious artefact bands (Fishback et al., 1999). Multiplex 1 consisted of the following profile: 1 cycle of 95°C for 3 min, followed by 10 cycles of 95°C for 30 s, 65°C for 1 min (-0.5°C per cycle), and 72°C for 4 min, then 30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 4 min, ending in one cycle of 72°C for 45 min. Multiplex 2 followed the same sequence but used 12 cycles of 60°C for 1 min (-0.5°C per cycle) at the initial annealing stage.

### Table 2.1 - Primer and loci information for multiplex PCRs used in the project

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence</th>
<th>Dye</th>
<th>Range (bp)</th>
<th>No. Alleles</th>
<th>Multiplex</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmyFGT14TUF_R</td>
<td>5’ – AGAGGGTTACACATGCAACC – 3’</td>
<td>3PA</td>
<td>203 – 211</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>OmyFGT14TUF_F</td>
<td>5’ – TGAAGACTCAACAGTGACCGGC – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmyFGT10TUF_R</td>
<td>5’ – GCTCATGAACTGGGCTTCTC – 3’</td>
<td>3PA</td>
<td>148 – 178</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>OmyFGT10TUF_F</td>
<td>5’ – AAGCGAAAGTGTTAAGAAAGGC – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ssa20.19NUIG_R</td>
<td>5’ – CTAAGTTTCGAGGCAACGTC – 3’</td>
<td>4PA</td>
<td>66 – 92</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Ssa20.19NUIG_F</td>
<td>5’ – TCAACCTGTCTGCTTCGAC – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omy325UoG_R</td>
<td>5’ – CGGAGTCGCCATCTCCTCCC – 3’</td>
<td>2PA</td>
<td>104 – 150</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Omy325UoG_F</td>
<td>5’ – GAACCTTTGACTCCTATTGTGAG – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSOSL439_F</td>
<td>5’ – ATGTCAGGGGGGAGTGAAGGT – 3’</td>
<td>4PA</td>
<td>108 – 174</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>SSOSL439_R</td>
<td>5’ – TGCTGCTGGACATTAAGGGGAGAT – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One18ASC_R</td>
<td>5’ – AAACCCACACACACTGACCGCAA – 3’</td>
<td>2PA</td>
<td>166 – 186</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>One18ASC_F</td>
<td>5’ – ATGGGCTGCACTTAATGGAGAGTAA – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omy27DU_R</td>
<td>5’ – TTTATGGCTGGCAACTAATGT – 3’</td>
<td>3PA</td>
<td>99 – 131</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Omy27DU_F</td>
<td>5’ – TTTATGTCATGTCAAGCCAGTG – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmyFGT15TUF_R</td>
<td>5’ – GGTACACACAGCTTCATTGCA – 3’</td>
<td>2PA</td>
<td>145 – 169</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>OmyFGT15TUF_F</td>
<td>5’ – ATGTGGTACACTGGCAGATGC – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ots1BML_F</td>
<td>5’ – GGAAGAGCACAGTGTGTTGTT – 3’</td>
<td>4PA</td>
<td>162 – 272</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Ots1BML_R</td>
<td>5’ – TGAAGCAGACAGAAAGCA – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmyFGT23TUF_R</td>
<td>5’ – CTTATGGGGGTTGTGGTTTCCA – 3’</td>
<td>3PA</td>
<td>97 – 121</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>OmyFGT23TUF_F</td>
<td>5’ – ATTCCTGCGGTGTGTACGTG – 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and reduced from 30 cycles to 28 cycles at an annealing temperature of 54°C for 1 min.

Although the multiplex systems mimic closely the work of Fishback et al. (1999), significant modifications were made during optimisation. The predominant differences were the amount of, and way, primers were used. Primers were dissolved in biological grade TE to make a stock solution, before being pooled and diluted in mbg water to produce a working concentration of 10 µM. The following volumes (µl per sample) were added to each multiplex: multiplex 1 (7 loci), OmyFGT14TUF, 0.12; OmyFGT10TUF, 0.2; Ssa20.19NUIG, 0.12; Omy325UoG, 0.54; SSOSL439, 0.52; One18ASC, 0.40; Omy27DU, 0.38, and in multiplex 2 (3 loci), FGT15TUF, 0.30; Ots1BML, 0.11; OmyFGT23TUF, 0.80.

2.1.2.2.3 Microsatellite screening: genescanning

All microsatellite screening was undertaken on one of two Beckman Coulter CEQ 8800 machines (Beckman Coulter Inc, USA). By utilising a capillary electrophoresis-based machine possessing a plate changer, a high volume of samples could be analysed in a relatively short space of time; up to one hundred and ninety two samples in less than 24 hr. The chemistry required to load and run the samples was supplied by Beckman Coulter Inc, Fullerton, USA.

Slight adjustments were made to the fragment analysis method protocol supplied by Beckman Coulter to allow for variable dye concentrations present in the multiplex PCR products. Within a 96-well Beckman Coulter loading plate, 30 µl of sample loading solution (SLS) and 0.25 µl of size standard 400 (SS-400; 400 bp with WellRED dye D1) were added to each well already containing 0.9 µl of PCR product. The plate was then centrifuged at 258 xG for 1 min to mix, before each sample was covered with a drop of Sigma mineral oil to prevent evaporation. A separate Beckman Coulter 96-well buffer tray was filled with 125 µl CEQ separation buffer per well. Both sample and buffer plates were then loaded into a CEQ 8800 where onscreen instructions were followed. The option of running two plates simultaneously was selected, before a 20 ml separation gel cartridge (Genome LPA-I) was installed. Following setup, the CEQ 8800 was left to separate samples.
using the 33 centimetre (cm) 75 micron (µm) capillary array, whilst a wetting tray had been filled with mbg water to prevent the capillaries drying between analysis of rows.

The analysis parameters for microsatellite screening could be stored on the machine and analysis conducted whilst separation occurred, or data could be transferred to personal computers specifically maintained for Beckman Coulter data. One parameter requiring immediate setting was the analysis parameter Frag-3; used for fragment analysis of the size range 60 to 420 bp. All other parameters required manual optimisation from the default settings of the Beckman Coulter software.

2.1.2.2.4 Microsatellite screening analysis – genotyping

The parents of the offspring requiring analysis were used to set up all analysis parameters due to the fact any alleles present in the parental population could be present in the offspring. Therefore locus tags were compiled on the sire and dam information. Each tag required optimisation, but all followed the Beckman Coulter criteria of: search for stutter, a stutter detection window width of 1, detection of stutter longer and shorter than allele, spurious peak detection, as well as +A detection, including; apparent size includes +A, detection of +/-A, and use +A peak to call alleles. Optimised criteria included maximum relative stutter peak height (MRSPH), maximum height for spurious peaks (MHFSP) and the resultant allele confidence intervals (ACI) from the locus tags generated.

Further optimisation was required for fragment analysis. All data was subject to automatic analysis conditions of size standard 400, using a cubic model, dye mobility calibration phosphoramidite synthesis primers version one (PA ver 1), a slope threshold of 1, relative peak height threshold of 0%, the identification of short tandem repeat (STR) alleles, and tagging of STR loci; Omy27DU, Omy325UoG, One18ASC, OmyFGT14TUF, SSOSL439, OmyFGT10TUF, and Ssa20.19NUIG in multiplex 1; and OmyFGT23TUF, Ots1BML, and OmyFGT15TUF in multiplex 2. In every instance samples were manually checked in case of allele or loci miscalling. All analysis was completed using the CEQ 8800 version 7.0 software supported by associated training guides on fragment analysis, locus tag generation,
and allele list generation (Beckman Coulter, 2004ab). Resultant genotype lists were then compiled and exported to Microsoft Excel in preparation for parental assignment.

2.1.3 Parental assignment; software and analysis

The program used to determine parentage in the present project was family assignment program (FAP) version 3.1 (Taggart, 2000). Two other programs had been considered and tested (Cervus version 2.0: Marshall et al., 1998, and, Package for the analysis of parental allocation (PAPA): Duchesne, 2005) but due to the fact CERVUS assigns the two most likely parents by using one as a known parent, restricting the alleles available to match the second parent and PAPA assigns the most likely pair of parents regardless of sex, neither of these likelihood-based programs were used and FAP was selected as the most efficient for this project. Complementing the breeding design, FAP used exclusion principles in its predictive analysis, assuming a closed environment where individuals tested are the progeny of known parental combinations for which full genotypic data is available (Taggart, 2000).

All genotype data was configured to a six digit nuclear format for each locus (eg 122124). Where genotypic data could not be attained for a particular locus ‘000000’ was entered as a substitute. It was necessary to convert the Excel spreadsheets containing data into tab delimited text format to run FAP. Once data was entered correctly (for formatting see Taggart, 2000) the program used ten simple steps for analysis; enter parental genotypes, select locus tags to be considered, select not to include mt haplotypes in analyses, select ‘A’ for assignment analysis, enter progeny file name, name result file, select zero for allele size tolerance, and six for mismatch tolerance, before exiting to view the results file.
2.2 **Immunohistochemistry**

2.2.1 Supply, culture, and maintenance of monoclonal antibodies

Cryopreserved hybridoma cell lines, A8 (made in-house, Institute of Aquaculture, University of Stirling, and known to bind to secondary cells) and D41, known to produce effective anti-*T. bryosalmonae* monoclonal antibodies, were removed from liquid nitrogen and allowed to thaw (Morris *et al.*, 1997). Each vial was pipetted into a 40 ml flask (Nunc, Denmark) and had 10 ml of Dulbecco’s modified eagles medium (DMEM: Sigma-Aldrich, USA) added, plus additives (L-Glutamine (200µM), sodium pyruvate, penicillin/streptomycin; Sigma), and 10% foetal calf serum (Sigma). (For a detailed description of the processes leading up to the successful production of anti-*T. bryosalmonae* monoclonal antibodies, see Morris, 1996). Cells were checked every second day to ensure there was a plentiful supply of medium, no contamination had arisen, or a build up of acidic conditions occurred (identified by a colour change from red to yellow in the medium). For media requiring replacement or supplementation, DMEM with additives and 10% FCS was used.

To replenish the stocks of A8 and D41 used, aliquots of the cultured cell lines were cryopreserved. Cells were checked under a microscope to ensure a sufficient number were present. They were then harvested from flasks by pipetting media into 15 ml centrifuge tubes. These were pelleted by centrifuging at 10,000 xG for 7 min. As much media as possible was removed without disturbing the pellet before resuspending the cells in DMEM containing 20% dimethyl sulphoxide (DMSO; Sigma). The resuspended cells were aliquoted in labelled cryotubes (Nunc), and wrapped in insulation material to allow a slow freezing process; thought to prevent ice crystals forming in the cells, which would subsequently damage them. The vials were then stored at -70°C overnight before being transferred to liquid nitrogen.
2.2.2 Preparation of samples

Rainbow trout were over-anaesthetised in a 10% (w:v, ethanol) benzocaine solution (Sigma-Aldrich) before being euthanased by cutting the spinal cord. A ventral incision from jaw to anus was made and the posterior kidney and spleen were removed and placed in a vial of 10% neutral buffered formalin (NBF; sodium dihydrogen phosphate (monohydrate) 4 g/litre, Di-sodium hydrogen phosphate (anhydrous) 6.5 g/l, formaldehyde 40% 100 ml, mbg water 900 ml) for at least 24 hr to allow fixation to occur. Transects of the tissues were cut and placed within individually labelled cassettes (Surgipath Europe Ltd, UK) before being submerged in water. After a short period of soaking in water, samples were processed using a Shandon Citadel 2000 automatic tissue processor (Shandon, UK), and then embedded in paraffin wax (Tissue Tek Number 2, UK). Once set, blocks were trimmed and soaked in distilled water for 1 hr before sections of 5 µm were cut using a Reichert-Jung Biocut microtome. The sections were floated on to heated water (~45°C) and placed on poly-l-lysine coated slides (Surgipath, UK). The slides were labelled and placed in a drying oven at 60°C for at least 1 hr.

2.2.3 Immunohistochemistry methodology

Tissue sections were prepared following the protocol of Adams and Marin de Mateo (1994). In each assay kidney sections known to be positive and negative for *T. bryosalmonae* infection were included as controls. Slides were placed into slide racks (Surgipath) as sections required dewaxing via immersion in two washes of xylene for 5 min each. This was followed by rehydration in 100% ethanol for 5 min, 70% ethanol (v:v, distilled water) for 3 min, and distilled water for 5 min. Upon removal, sections were circled using a wax based liquid blocker (PAP pen, Daido Sangyo Co Ltd, Japan) to ensure localisation of reagents on the tissue sections during incubation periods. All incubation stages were conducted at room temperature (~22°C) in Hybaid omnislide slide racks within Hybaid omnislide wash sleeves (Hybaid Omnislide, UK) to prevent evaporation. The first incubation lasted 10 min using 10% (v:v) hydrogen peroxide in methanol (used to block endogenous peroxidise activity) before sections were rinsed and soaked for 3 min in Tris buffered saline (TBS; 20 mM tris, 0.5 M sodium chloride, pH 7.6; or phosphate buffer...
buffered saline, PBS; 20 mM phosphate, 0.15 M sodium chloride, pH 7.2). Non-specific binding was blocked when sections were incubated for 10 min with 10% (v:v, TBS) goat serum (Diagnostics Scotland, UK). The slides were tapped to remove the majority of goat serum, and a mixture (1:1) of the two anti-\textit{T. bryosalmonae} monoclonal antibodies, A8 and D41, were added to each section and left to incubate for 1 hr. Sections were then rinsed with TBS to remove the antibodies and a 1:50 solution (v:v, TBS) anti-mouse horse radish peroxidise (HRP: Sigma) was added to each section and allowed to incubate for 30 min. A further rinse in TBS was required before sections were covered with 3,3’-diaminobenzidine tetrahydrochloride (DAB; 20 µM DAB, 5 ml TBS, 100 µl of 1% hydrogen peroxide) for 15 min and left to incubate out with the wash sleeve.

The slides were washed with tap water in the wash sleeve and left to soak for 3 min. Once removed and transferred from the Hybaid slide racks back to Surgipath slide racks, counterstaining was performed using Mayer’s haemotoxylin (3 mM haemotoxylin, 1 mM sodium iodate, 0.1 M aluminium potassium sulphate dodecahydrate, 5 mM citric acid, 30 mM trichloroacetalddehyde hydrate) for 3 min, followed by a 10 min flush in running tap water. The sections were then subject to alcohol dehydration of a minimum 3 min in 70% ethanol and then 100% ethanol. Finally, sections were rinsed in two separate submersions of xylene for at least 5 min, before being coverslipped using Pertex (Sigma) and left to dry overnight. The following day sections were viewed using light microscopy (Olympus CH, x40 objective: Olympus optical Co, UK), and if present, \textit{T. bryosalmonae} appeared brown in colour.

### 2.2.4 Counting parasites

In order to assess the parasite load of infected kidneys, and subsequent correlations between parasite number and kidney score (according to Clifton-Hadley \textit{et al.}, 1987), individuals used in the PKD challenges (Chapter 4) of this project had the number of parasites within the kidney tissue section counted. Following the methodology described by Higgins and Kent (1998) and Morris \textit{et al.} (2003a), sporogonic and extrasporogonic stages of \textit{T. bryosalmonae} within the kidney sections were counted under 11 random fields of view at x400 magnification (0.45
mm diameter) under light microscopy. The total parasite count was converted to number per mm$^2$, which was then plotted against the individuals kidney score following transformation of the dataset.
2.3 **Bacteriology**

2.3.1 **Source and quantification of *Aeromonas salmonicida***

The strain of *A. salmonicida* used throughout the study was isolated from a natural outbreak of furunculosis at a Marine Harvest site in 2005 (*A. salmonicida* MH; Crumlish, 2006 personal communication). This was obtained from the Bacteriology department at the Institute of Aquaculture, University of Stirling. Being an extremely virulent strain, it was decided that passaging the bacteria in fish was not required prior to challenge experiments. Instead a direct culture from a slope was inoculated on to horse blood agar and incubated at 22°C for 48 hr in preparation of a standard curve (Appendix 1). Horse blood agar plates were prepared and sealed using Nescofilm (Alfresa Pharma corporation, Japan) in anticipation of the challenges. The standard curve (concentration versus optical density) produced an $R^2$ value of 0.931.

2.3.2 **Challenge preparation and culture**

A subculture was taken from the culture grown on the horse blood agar and grown on tryptone soya agar (TSA). This was incubated at 22°C for 96 hr before several colonies were removed with a loop and placed in tryptone soya broth (TSB; 50 ml) for 24 hr. After 24 hr, the bacteria were harvested by centrifuging the TSB culture at 3000 xG for 10 min to produce a pellet. The supernatant was removed and replaced with 5 ml of sterile 0.85% (w:v, distilled water) saline solution. At this point, the purity of the bacterial suspension was checked by inoculation on to a TSA plate to ensure *A. salmonicida* was the only bacteria present. Using the equation obtained from the standard curve, $y = 4.0061x$, an optical density (O.D) of 0.25 was calculated to achieve a $1 \times 10^8$ solution. From here serial dilutions in 0.85% saline allowed the desired concentration to be made for injection into test fish. Drop counts were made on TSA plates at $10^4$, $10^5$, and $10^6$ to quantify the actual dosage injected. Although a 24 hr incubation period was required before drop counts could be examined and actual concentrations calculated, it was assumed that an injection of 0.1 ml of a particular solution produced the desired (although approximate) dose rate; ie 0.1 ml injection of $1 \times 10^4$ produces a dosage of $1 \times 10^3$ per fish.
2.3.3 Injection and bacteria recovery

Every fish used in Chapter 5 was injected intraperitoneally (ip) with either 0.1 ml of 0.85% saline (control fish) or 0.1 ml of the \(A. \text{salmonicida}\) suspension (test fish) at the desired concentration. Following preparation, solutions were immediately stored on ice to equalise methodology, but more importantly to ensure shifts in bacteria morphology and/or number were minimised. The solution containing bacteria was therefore used within 1 hr following its preparation. Prior to injection fish were anaesthetised using 10% benzocaine at 4 mg/l, and post injection were recovered in highly aerated water.

As mortality or morbidity occurred, fish were removed from tanks (euthanased if necessary) and sampled for the presence of \(A. \text{salmonicida}\). Using sterile equipment, an incision along the ventral surface from jaw to anus was made. The internal organs were removed and a second sterile incision allowed access to the head kidney. Using a sterile plastic loop (TSC technical service consultants, UK) the sample was collected, inoculated on to a TSA plate, and incubated at 22°C for 48 hr in preparation of analysis. \(Aeromonas \text{salmonicida}\) was identified, if present, using pigmentation analysis, morphological identification, Gram’s staining, and agglutination tests.

2.3.4 Pigmentation analysis and morphological examination

As \(A. \text{salmonicida}\) produces a brown diffusible pigment and due to the fact fish used in challenges were certified disease free by a qualified veterinarian prior to the study, it was highly unlikely that any brown pigment found diffusing on TSA plates would be caused by anything other than \(A. \text{salmonicida}\). However, due to limited experience with bacteria, \(A. \text{hydrophila}\), also known to produce a brown diffusible pigment, and potentially the only bacteria that could be confused with \(A. \text{salmonicida}\), was grown to allow comparisons to be made. Marked differences could be observed in the growth rate and morphology of the two species. With increased confidence in the ability to identify \(A. \text{salmonicida}\), all samples collected were visually checked using pigmentation analysis and morphological examination. Additionally, Gram’s staining and agglutination tests were conducted. Due to the
limited requirement for the two methodologies (ie the disease-free certification of the fish involved and the fact \textit{A. salmonicida} had been injected into test fish), as well as time constraints, and the expense of consumables only 10\% to 20\% of all samples taken were analysed using Gram’s staining and agglutination testing.

2.3.5 Gram staining and agglutination testing

The following Gram’s staining method was used throughout the project: first a loop of sterile 0.85\% saline was aseptically placed on to a clean microscope slide. Then, one or two single colonies of \textit{A. salmonicida} were removed from the agar plate using a sterile loop. The bacteria were then emulsified evenly into the saline and across the slide, before being left to air dry for a few minutes. The slide was passed through a Bunsen burner to heat fix, and left to cool in preparation of the staining protocol; immerse in crystal violet solution for 1 min, wash with tap water, immerse in iodine for 1 min, decolourise with acetone solution for 2 to 3 s, rinse under tap water, immerse in Safranin solution for 2 min, wash with tap water, remove excess moisture, and allow to air dry. Once dry, the slides were microscopically examined under x40 or x100 objective, using magnification oil. All observations were recorded, eg red/pink stained coccibaccili.

During pre-challenge experiments limited success was achieved in agglutination tests with the rabbit polyclonal anti-\textit{A. salmonicida} antisera developed at the Institute of Aquaculture, University of Stirling. To ensure a distinction could be made between positive and negative results, a rapid agglutination test kit for \textit{A. salmonicida} was used (BioNor AQUA Mono-As, Norway). The kit was supplied with testing cards which made recognition of positive and negative samples simple. To use, a drop of the test reagent was positioned on each test area before a colony of bacteria was aseptically removed from the culture plate and mixed into the solution. After a few moments of tilting the test card, agglutination was obvious. For each group of tests performed, negative controls were included, using the same methodology but replacing the test reagent with the control reagent provided.
Chapter 3: Proliferative Kidney Disease Cross Section
3.1 Introduction

The importance of Proliferative Kidney Disease (PKD) as an economically damaging disease has been recognised for many years. In 2002 British trout farmers gathered to discuss the significance and severity of the losses associated with the parasitic infection. Ranging from 25% to 100% mortality and up to 100% morbidity in most circumstances, recent years have seen the most severe outbreaks of PKD and as a result, the greatest production losses, costing the industry up to £1.8 million in Britain alone; a figure which continues to grow. As British rainbow trout production increases and climatic change continues to elevate summer water temperatures (known to increase the development of the causative agent, *T. bryosalmonae*; Ferguson, 1981; Clifton-Hadley *et al.*, 1986a; Gay *et al.*, 2001), the situation is unlikely to improve.

At present, no functional and viable chemical, available vaccine, or prophylactic treatment is available, leaving avoidance and minimisation of losses dominating current husbandry and management practices on farms. Although husbandry, maintenance of equipment, optimising environmental parameters, and minimising handling and husbandry stressors can greatly reduce associated losses (Seagrave *et al.*, 1981; Le Gouvello *et al.*, 1999), management practices alone can not be relied upon for long term control. To date, the only method of control is to naturally immunise fish by delaying their exposure to clinically infected waters until late summer, as water temperature and PKD development decline. Fish exposed at this time, effectively build a resistance in the following year (Ferguson, 1981; Ellis *et al.*, 1982; Hedrick *et al.*, 1985; Foott and Hedrick, 1987; Morris *et al.*, 2003a). This natural approach will inevitably be preferred over chemical treatment or immunisation in an ever-increasing environmentally-conscious society, but it dramatically reduces the productivity on affected farms, increasing the time it takes to produce market-sized fish. Further, the long-term efficacy and required management are far from ideal. As advances are made in the understanding of the complicated lifecycle of *T. bryosalmonae*, the likelihood of an effective vaccine grows closer, but until such a time, alternative methods must be sought.
The development of rainbow trout strains less susceptible to PKD is currently the only logical step to suppress the disease in the British rainbow trout industry. To date, the level of tolerance in cultured populations has yet to be assessed. Natural selection through the mating of survivors has not been attempted; segregation within the industry means broodstock are kept on different sites to production fish, and are therefore rarely exposed to the parasite, as a result no information of tolerance is available at the individual, family, or even strain level. Immunity may be apparent in strains with limited exposure to the disease. However, and conversely, farmed strains may have developed a genetic resistance due to continual exposure, as discussed by Brown et al. (1991) in feral landlocked salmon. Both suggestions imply selective breeding may be a future possibility.

For this reason, the objective of the current study was to detect the level of additive genetic variation displayed in two commercial populations of rainbow trout. Following exposure to a natural challenge, the information gathered was used to calculate estimates of heritability for preliminary use in the development of a selective breeding programme against PKD.
3.2 Materials and Methods

3.2.1 Broodstock and mating design

In the winter of 2002 broodstock at the two farms involved in the LINK project (Houghton Spring Hatchery, Hampshire (HS) and Glenwyllin fish farm, Isle of Man (IoM)) were crossed to generate families for a large scale performance trial under commercial conditions. The relationship among broodstock was unknown. Consequently, they were assumed to be unrelated. Sires and dams were mated using a partial factorial design. Each sire was mated with four to eight dams, and each dam to four sires, resulting in 540 full- and half-sibling families.

IoM utilised two lines, A and B, to generate 160 families, using 20 females (FI) and 20 neomales (NI) per line, whilst HS produced 380 families, generated by crossing 95 females (FH) with 50 neomales (NH). IoM broodstock were crossed to achieve four strains; females were mated with neomales of the same strain (ie AFI x ANI or BFI x BNI) as well as crossed between strains (ie AFI x BNI and BFI x ANI), whilst HS families were produced from a single strain (ie FH x NH; Figure 3.1). The mating design of IoM is illustrated in Figure 3.2.

At stripping, female broodstock were subject to adipose fin clipping to allow DNA extraction (phenol-chloroform method), genotyping, and subsequent parental assignment of their offspring, as described in the General Materials and Methods (Chapter 2, Section 2.1). Females had passive integrated transponder (PIT) tags injected into the body cavity for individual identification purposes, so they could be identified for future selective breeding. Due to the lethal methodology used in extracting milt from neomales only fin clips were required for genotypic identification.
Figure 3.1 - Specific crosses of Houghton Spring broodstock used in the PKD Cross Section study
Figure 3.2 - Specific crosses within and between strains of the IoM broodstock used in the PKD Cross Section study
3.2.2 Offspring: incubation to growout

Fertilisation, shocking, and hatching differed between the farms by less than one month. In IoM families, fertilisation took place on 22/11/2002. The eggs were incubated as separate families following IoM commercial practices. Water temperature was elevated in order to increase egg development, with shocking 11 days post fertilisation once the eyed stage had been reached. Houghton Spring also used heated water. Following fertilisation on 17/12/2002 HS families were maintained separately according to HS commercial conditions. Eggs were shocked at the eyed stage (31/12/2002).

In the IoM, after shocking, individual family sizes were assessed using an automatic egg counter. Each family was split into three equal batches to form three communal populations. Once eyed, one of the egg batches was transported from IoM to Iwerne Spring, a fingerling rearing site, where complete hatch occurred by 17/12/2002 - 25 day incubation period. Once hatched, the families were transferred to growout tanks and reared to a normal fingerling size, before being transported to Test Valley Trout farm, Itchen Abbas (TVT), where they were held in a single tank. Fifteen hundred of these fish were PIT tagged (~5 to 10 g), the balance were used in commercial production on this farm. The HS families, which hatched after a 30 day incubation period, on the 16/01/2003, were ongrown to fingerling size using standard commercial practice, before being transported to the TVT site at ~2 to 5 g. Fifteen hundred from this population were held in a separate tank until PIT tagged, the remainder were mixed with the IoM fish and ongrown under normal commercial conditions.

3.2.3 PKD data collection

During the summer of 2003 (July to September), all fish at the TVT site were naturally challenged with PKD via exposure to enzootic river water. Fish were assumed to be infected due to the history of the disease on site, behavioural and external symptoms, as well as the onset of mortality in some stocks on site, where dissected fish displayed internal symptoms of PKD. On a single day in September 2003, 1500 fish were collected from the mixed untagged population, having been
randomly selected from three tanks in proportionate numbers to the stocking regime; Tank 1 HS stock only, 250 individuals; Tank 2 HS stock only, 250 individuals; Tank 3 HS and IoM mixed (1:4 ratio), 1000 individuals. The fish were euthanased, and one person was allocated to assign fork length, body weight, and kidney score to each individual. Fin clips were taken and stored in individually marked, ethanol filled Eppendorf tubes for DNA extraction (Chelex method), and subsequent genotyping, and parental assignment (as described in Chapter 2, Section 2.1).

PKD scoring was based on the scale of Clifton-Hadley et al. (1987); Grade 0, kidney with no apparent lesions; Grade 1, kidney with slight enlargement, especially at the posterior end, but maintaining a dark red colouring as for Grade 0 kidneys; Grade 2, kidney obviously enlarged along its length, the capsule corrugated over renal tissue, which has a mottled red and grey appearance; Grade 3, kidney approximately six times its normal volume, with marked corrugation of the kidney surface and the capsule having a blue sheen; Grade 4, kidney mottled pink and grey, further swollen due to oedema, with clear fluid running from cut surfaces and gelatinous fluid adhering to the underside of the kidney capsule.

3.2.4 Statistical analysis

3.2.4.1 Genstat

Genetic parameters were estimated using linear mixed models performed in Genstat Release Version 9.1 (VSN International, UK). Restricted maximum likelihood (REML) analysis was used to provide genetic variance estimates and subsequent heritabilities using a linear mixed model:

\[ y_{ijklmq} = \mu_i + tank_{ij} + hs_{ik} + b_1.i \cdot dam_{ijklmq} + b_2.i \cdot pa_{ijklmq} + b_3.i \cdot dab_{ijklmq} + sire_{iko} + dam_{ikp} + e_{ijklmq} \]

where \( y_{ijklmq} \) is the observational phenotypic value for trait \( i \) (ie kidney score, fork length, or body weight) of individual \( q \) kept in tank \( j \), and with sire \( o \) and dam \( p \) from origin \( k \). \( \mu_i \) is the population mean for trait \( i \), \( tank_{ij} \) is the fixed effect of tank \( j \)
for trait $i$, $h_{sk}$ is a fixed effect with $k$ representing fish strain (ie origin; HS or IoM) for trait $i$; sire$_{iko}$ is the random effect of sire $o$ from origin $k$; and dam$_{kp}$ is the random effect of dam $p$ from origin $k$, whilst $e_{ijklmnq}$ represents the residual error connected to trait $i$. To model the lines within the IoM strain three further terms were fitted as regressions with coefficients $b_{1i}$, $b_{2i}$, and $b_{3i}$ for trait $i$: on the maternal line origin (dam$A_{ijklmnq} = 1$ if the dam was from line A, or 0 otherwise); on the additive effect of line A (pa$A_{ijklmnq} = 0$, 0.5, or 1 if the parental contribution was from neither, one, or both parent(s) of line A); and on the dominance deviation (dab$A_{ijklmnq} = 1$ if AxB or BxA, or 0 otherwise). Values of 0 were given to the regression variables for HS fish.

The random effects of sire$_{io}$, dam$_{ip}$, and residual error ($e_{ijklmnq}$) were considered to be independent random normal variables with mean zero, and variances denoted $\sigma_s^2$, $\sigma_d^2$, and $\sigma_e^2$, respectively. Total phenotypic variance was denoted $\sigma^2$, and was estimated as $\sigma^2 = \sigma_s^2 + \sigma_d^2 + \sigma_e^2$. Genstat estimates of heritability are expressed as narrow sense heritabilities, ie the ratio of the additive genetic variance to the total phenotypic variance, which was calculated using the VFunction procedure in Genstat, and following formulae from Falconer (1981):

$$\frac{4(\sigma_s^2)}{\sigma^2}, \text{ from the sire variance component } h_s^2$$

$$\frac{4(\sigma_d^2)}{\sigma^2}, \text{ from the dam variance component } h_d^2$$

Combined estimates, relying on information from both parents, is more precise and therefore favoured over single parent estimates. However, combined estimates assume that the dam component contains no other sources of variance other than additive genetic (as is assumed to be the case for the sire). To test this, a likelihood ratio test was conducted to look for evidence that the dam component was larger than the sire variance. This is most easily carried out by defining sire and dam components as a ratio with the residual error variance, $\gamma_s = \sigma_s^2/\sigma_e^2$ and $\gamma_d = \sigma_d^2/\sigma_e^2$ and testing a null hypothesis $H_0$: $\gamma_s = \gamma_d$ against an alternative hypothesis, $H_1$: $\gamma_s \neq \gamma_d$. This was completed within Genstat for each trait. The $\gamma$ values for sire and dam were constrained to be equal within Genstat, before manually entering appropriate...
values to 5 decimal places to minimise the deviance value. Once the value that
minimised the deviance was attained, the value of the deviance obtained from the
constrained model was compared to the deviance of the original model where \( \gamma_s \) and
\( \gamma_d \) were not constrained to be equal. The difference in the deviance was the
likelihood ratio test statistic for testing \( H_1 \) against \( H_0 \) and was compared to \( \chi^2 \) at 1
degree of freedom, using 95% significance level (3.84). Ninety-five percent
confidence intervals (95% CI) were defined by the minimum deviance values
required to reject the null hypothesis. For all variates the null hypothesis was
accepted, and the equation, \( h_{cf}^2 = \frac{4\gamma}{2\gamma+1} \), was used to estimate combined
heritability.

3.2.4.2 ASReml

Data was also processed using ASReml version 1.1 software (VSN International,
UK) to identify genetic (co)variation, and subsequent correlations. ASReml takes
into account all relationships between the analysed individuals as well as the crosses
between the sires and dams. It therefore provides a more direct estimate of
heritability without the need for the manual iteration. With more complex pedigree
structures it is also capable of producing a more accurate estimate of heritability.
Although results from Genstat are displayed within the results section, the final
statistics are derived from ASReml. In particular ASReml provides a means of
estimating genetic correlations (\( r_A \)) between the traits. These differ from phenotypic
correlations as they are estimates of correlations between breeding values. This
requires multivariate analyses, which can be handled by ASReml. The analysis
fitted the same fixed effects as the univariate model, but the random effects of sire
and dam were replaced by an individual term \( u_{ijklmnoq} \), plus an additional term for the
dams. This term was considered random with a (co)variance matrix given by \( \sigma_A^2 \).
Estimates of heritability were then estimated by \( h^2 = \frac{V_A}{V_P} \).

To calculate \( r_A \), the multitrait analyses fitted a (co)variance matrix for the additive
genetic variance \( \Sigma_A \) across traits, and a matrix for the residual effects \( \Sigma_E \). For two
traits, eg y and z, \( r_A \) was estimated by \( \sigma_{A,y,z} / (\sigma_{A,y} \sigma_{A,z}) \), where \( \sigma_{A,y}^2 \) and \( \sigma_{A,z}^2 \) are the
additive genetic variances for y and z, and \( \sigma_{A,y,z} \) is their additive genetic covariance,
all obtained from \( \Sigma_A \).
3.3 Results

3.3.1 Molecular biology

Having successfully optimised the two multiplex PCRs it was noted that locus OmyFGT10TUF was uninformative. Its removal from the multiplex was tested with negative results. In order to prevent a delay in data collection, its use was continued in the system, although its information was not. This lowered the total loci used to nine. The optimised genotyping criteria for each locus are displayed below in Table 3.1.

Table 3.1 - Analysis information produced by Beckman Coulter software for locus tags used in the multiplex systems

<table>
<thead>
<tr>
<th>Locus</th>
<th>MRSPH</th>
<th>MHFSP</th>
<th>ACI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmyFGT14TUF</td>
<td>30%</td>
<td>30%</td>
<td>0.60</td>
</tr>
<tr>
<td>OmyFGT10TUF†</td>
<td>75%</td>
<td>75%</td>
<td>0.72</td>
</tr>
<tr>
<td>Ssa20.19NUIG</td>
<td>80%</td>
<td>80%</td>
<td>0.74</td>
</tr>
<tr>
<td>Omy325UoG</td>
<td>85%</td>
<td>85%</td>
<td>0.70</td>
</tr>
<tr>
<td>SSOSL439</td>
<td>75%</td>
<td>75%</td>
<td>0.83</td>
</tr>
<tr>
<td>One18ASC</td>
<td>75%</td>
<td>75%</td>
<td>0.43</td>
</tr>
<tr>
<td>Omy27DU</td>
<td>60%</td>
<td>60%</td>
<td>0.83</td>
</tr>
<tr>
<td>OmyFGT15TUF</td>
<td>60%</td>
<td>60%</td>
<td>0.50</td>
</tr>
<tr>
<td>Ots1BML</td>
<td>65%</td>
<td>65%</td>
<td>0.60</td>
</tr>
<tr>
<td>OmyFGT23TUF</td>
<td>65%</td>
<td>65%</td>
<td>0.41</td>
</tr>
</tbody>
</table>

†Locus proved to be uninformative, and its use in analysis was removed; MRSPH – Maximum relative stutter peak height; MHFSP – Maximum height for spurious peaks; ACI – Allele confidence interval

The FAP program used for parental allocation successfully allocated 86.14% of the 1500 offspring genotyped for this study. The confidence in the allocation is high due to previous testing conducted. Although six mismatches was selected for analysis, results data relied only on zero or one mismatch information. To ensure the reliability of this data, the zero and one mismatch individuals were tested; specific alleles were changed to the most common allele of a particular locus. In the 100 zero mismatch individuals tested, the majority changed to one mismatch as expected or did not change at all. In a minority of cases, the results showed one mismatch with multiple families, or multiple families with no mismatches. In the 100 one mismatch individuals, 13 were found to be incorrectly called at a single locus. Therefore, 25 individuals were subject to PCR using only their weakest locus (Omy325UoG, SSOSL439, or Ots1BML). At locus Omy325UoG, 29% were
miscalled, at locus SSOSL439, 25% were miscalled, and none were miscalled at locus OtI1BML. Overall 67% of the one mismatch individuals subject to single locus PCR were miscalled. When the most common alleles at specific loci were replaced, the result remained the same (one mismatch; but not necessarily at the same locus), changed to two mismatches or to no mismatches, all of which kept the same single family; in only 2% of the hundred tested, the family changed. This implied that both zero and one mismatch individuals were reliable, providing only single family matches were used.

3.3.2 PKD resistance

Data and/or pedigree information was available from 1501 individuals, including 1287 offspring and 214 parents; 89 sires and 125 dams. Of the 1287 offspring, 1029 were allocated to a parental crossing with zero mismatches, whilst 258 were allocated with a single mismatch. Representatives of HS comprise 60.36% of the data, the remainder coming from IoM. The proportion of fish sampled taken from each tank equated to 214 from T1 (HS stock only), 214 from T2 (HS stock only), and 859 from T3 (HS and IoM mixed, 1:4 ratio); 343 HS, and 516 IoM. The data overall, and divided into distinct categories, are summarised in Table 3.2, below.

<table>
<thead>
<tr>
<th>Table 3.2 - Structure of the PKD Cross Section data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of individuals with pedigree and/or data</td>
</tr>
<tr>
<td>Total number of offspring with pedigree and data (Overall; HS, IoM)</td>
</tr>
<tr>
<td>Total number of parents with pedigree</td>
</tr>
<tr>
<td>- of which were dams (Overall; HS, IoM)</td>
</tr>
<tr>
<td>- of which were sires (Overall; HS, IoM)</td>
</tr>
<tr>
<td>Number of full-sib families represented (Overall; HS, IoM)</td>
</tr>
<tr>
<td>Number of dams per sire (Mean; range)</td>
</tr>
<tr>
<td>Number of sires per dam (Mean; range)</td>
</tr>
<tr>
<td>Progeny per full-sib family (Overall mean; range)</td>
</tr>
<tr>
<td>Progeny per HS full-sib family (Mean; range)</td>
</tr>
<tr>
<td>Progeny per IoM full-sib family (Mean; range)</td>
</tr>
</tbody>
</table>

Offspring displayed the full range of kidney scores; 0 to 4 inclusive. Mean values between HS and IoM differed significantly (P<0.001) with HS having a mean kidney score of 0.46 lower than IoM. IoM fish were both longer and heavier, statistically, than those of HS, with differences in mean values between the stocks of
10.92 mm and 13.91 g, respectively. No difference was found between the A- and B-only lines of the IoM strain or the diallele crosses. Overall mean values - where data was calculated from all 1287 offspring - were 2.19 (kidney score), 150.95 mm (fork length), and 62.29 g (body weight). Means ± their standard errors (SE), as well as the range for each trait are illustrated below in Table 3.3.

Table 3.3 - Mean kidney score, fork length (mm), and body weight (g) overall, and per strain ± SE for PKD Cross Section data

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean ± SE</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall kidney score</td>
<td>2.19 ± 0.03</td>
<td>0.00 – 4.00</td>
<td>1287</td>
</tr>
<tr>
<td>Overall fork length</td>
<td>150.95 ± 0.51</td>
<td>40.00 – 204.00</td>
<td>1287</td>
</tr>
<tr>
<td>Overall body weight</td>
<td>62.29 ± 0.59</td>
<td>11.10 – 189.40</td>
<td>1287</td>
</tr>
<tr>
<td>HS kidney score</td>
<td>2.01 ± 0.04</td>
<td>0.00 – 4.00</td>
<td>771</td>
</tr>
<tr>
<td>HS fork length</td>
<td>146.57 ± 0.60</td>
<td>40.00 – 195.00</td>
<td>771</td>
</tr>
<tr>
<td>HS body weight</td>
<td>56.71 ± 0.63</td>
<td>11.10 – 121.50</td>
<td>771</td>
</tr>
<tr>
<td>IoM kidney score</td>
<td>2.47 ± 0.05</td>
<td>0.00 – 4.00</td>
<td>516</td>
</tr>
<tr>
<td>IoM fork length</td>
<td>157.49 ± 0.82</td>
<td>100.00 – 204.00</td>
<td>516</td>
</tr>
<tr>
<td>IoM body weight</td>
<td>70.62 ± 1.02</td>
<td>18.30 – 189.40</td>
<td>516</td>
</tr>
</tbody>
</table>

Following the identification of additive genetic variation, estimates of heritability were calculated, and found to be moderate to high in all cases (Table 3.4). When calculated for each strain, estimates differed by ≥ 0.10 in all traits; higher in the IoM stock. The estimates of heritability for HS were 0.25, 0.38, and 0.33, for kidney score, fork length, and body weight, respectively, whilst IoM displayed estimates of 0.35, 0.53, and 0.50 for the three traits. These estimates were against the overall estimates of 0.31, 0.44, and 0.41, respectively (Table 3.4, below). (All estimate of heritability to this point calculated using Genstat).

Table 3.4 – Overall estimates of heritability ± 95% Confidence Interval (CI) for kidney score, fork length (mm), and body weight (g), calculated using Genstat for PKD Cross Section data

<table>
<thead>
<tr>
<th>Trait</th>
<th>Lower 95% CI</th>
<th>( \hat{h}^2 )</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney score</td>
<td>0.20</td>
<td>0.31</td>
<td>0.41</td>
</tr>
<tr>
<td>Length</td>
<td>0.31</td>
<td>0.44</td>
<td>0.58</td>
</tr>
<tr>
<td>Weight</td>
<td>0.29</td>
<td>0.41</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Genetic correlation between fork length and body weight was high and positive; 0.98. The calculated genetic correlation between size and kidney score was found to be moderate and negative; against body weight, -0.34 ± 0.16, and between fork length and kidney score, -0.36 ± 0.15. The estimates of heritability calculated using
ASReml ± SE, and correlations between the traits (genetic and environmental) are highlighted in Table 3.5, below.

**Table 3.5 – Overall estimates of heritability ± SE, genotypic, and environmental correlations calculated using ASReml for PKD Cross Section data**

<table>
<thead>
<tr>
<th></th>
<th>Fork length</th>
<th>Body weight</th>
<th>Kidney score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fork length</td>
<td>0.43 ± 0.08</td>
<td>0.89</td>
<td>-0.41</td>
</tr>
<tr>
<td>Body weight</td>
<td>0.98</td>
<td>0.42 ± 0.08</td>
<td>-0.34</td>
</tr>
<tr>
<td>Kidney score</td>
<td>-0.36 ± 0.15</td>
<td>-0.34 ± 0.16</td>
<td>0.19 ± 0.08</td>
</tr>
</tbody>
</table>

Genetic correlations below, and environmental correlations above the diagonal values of heritability (calculated using ASReml)

The additive genetic covariance was high for length and weight, and moderate for kidney score. The genetic and environmental covariances used to calculate the phenotypic correlations of fork length and body weight, 0.93; fork length and kidney score, -0.38; and body weight and kidney score, -0.33 are illustrated in Table 3.6, whilst the phenotypic trend of larger size equalling lower kidney score is shown in Table 3.7. Both the average fork length and average body weight of rainbow trout measured display the trend, with minimal kidney swelling (kidney score: 0) displayed in the larger fish of average fork length, 182.50 mm, and average body weight, 103.80 g, whilst the most severely affected fish (kidney score: 4) are distinctly smaller at an average fork length of 143.45 mm, and average body weight of 55.94 g.

**Table 3.6 - Genetic and environmental covariation for the traits, fork length (mm), body weight (g), and kidney score from individuals in the PKD Cross Section data**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Fork length</th>
<th>Body weight</th>
<th>Kidney score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fork length</td>
<td>---</td>
<td>175.80</td>
<td>-5.04</td>
</tr>
<tr>
<td>Body weight</td>
<td>145.0</td>
<td>---</td>
<td>-4.78</td>
</tr>
<tr>
<td>Kidney score</td>
<td>-1.90</td>
<td>-1.97</td>
<td>---</td>
</tr>
</tbody>
</table>

Genetic covariation below, and environmental covariation above diagonal.

**Table 3.7 - Average fork length (mm) and body weight (g) of fish assessed in the PKD Cross Section using the scale of Clifton-Hadley et al. (1987)**

<table>
<thead>
<tr>
<th>Kidney Score</th>
<th>Fork length (mm)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>182.50 ± 4.30</td>
<td>103.80 ± 6.41</td>
</tr>
<tr>
<td>1</td>
<td>156.29 ± 0.79</td>
<td>67.37 ± 1.03</td>
</tr>
<tr>
<td>2</td>
<td>151.88 ± 0.88</td>
<td>62.30 ± 1.00</td>
</tr>
<tr>
<td>3</td>
<td>146.08 ± 1.19</td>
<td>57.90 ± 1.24</td>
</tr>
<tr>
<td>4</td>
<td>143.45 ± 1.26</td>
<td>55.94 ± 1.38</td>
</tr>
</tbody>
</table>
3.4 Discussion

This study was possible due to known pedigree structures on the farms involved and the careful management used to maintain family integrity, as well as the genetic technology available to distinguish families using microsatellite markers. The objective to test for the existence and level of additive genetic variation for PKD score within the farmed populations of rainbow trout involved in the project proved to be successful. The two populations display a moderate level of additive genetic variation for PKD score, as well as high levels of additive genetic variation for body weight and fork length. When genetic correlations were produced, an expected high and positive relationship between the two production traits was found, whilst in the case of either production trait and PKD score, a moderate and negative genetic correlation was observed. Estimates of heritability were calculated for all traits, and appear to be paralleled by other salmonid populations (Kinghorn, 1983; Gjedrem, 1983, 1992, 2000; Gjerde, 1986; Gjedrem et al., 1991; Henryon et al., 2002; Pante et al., 2002; Perry et al., 2004). The additive genetic variation detected in the present study indicates selective breeding for PKD score can be successful within the HS and IoM strains.

Challenges under commercial conditions may be viewed as a more realistic synopsis than experimental testing, as fish are exposed to a vast range of additional pathogens in the culture environment, and in unknown quantities. However, it could be argued that the results of the present study are masked due to effects of the culture environment, secondary pathogens, or holding unit; despite the inclusion of model terms to account for such sources of variance where possible (ie tank). Kolstad et al. (2005) noted that infection in the wild is highly variable in time and magnitude when conducting a field experiment to improve the resistance of Atlantic salmon to the sea louse, *Lepeophtheirus salmonis*, recommending that challenge experiments should be used where selective breeding is to be implemented. In order to support the preliminary findings of this study, it is necessary to reproduce the results under controlled experimental conditions. Additionally, the validity of the scoring system may be open to question. Although one person conducted all measurements for kidney score, and the scoring system of Cliftion-Hadley et al.
(1987) is very comprehensive and can be followed easily, it must be stressed that observational characters are extremely subjective, and can vary from person to person, or even over time. It is therefore imperative to consider observational results, such as those in the present study, specific to the population tested (Kolstad, 2005). Comparisons are possible, as differences should be a matter of degree rather than kind, but in each instance the measurement technique and population from which results came should be described.

The phenol-chloroform DNA extraction method used in the present study has had limited success with some species (ie perch *(Perca fluviatilis)*, roach *(Rutilus rutilus)*, and stickleback), but the technique has proved successful with most salmonids. Phenol-chloroform extraction was used for the parents in the present study, due to the high quality of DNA produced and low risk of degradation in storage; an advantage when archives are required or when genotyping errors occur, as was often the case here. Chelex extracted DNA does not share the advantages of phenol-chloroform extracted samples, as the DNA is of poorer quality and storage is limited to only a few months even when at -20°C. However its use is compensated by the simple, fast, and safe extraction protocol involved. All the reagents used in the Chelex extraction process pose no immediate danger to the user, it is simpler as the substances are added to a single Eppendorf tube or 96-well plate well before adding the tissue, and it is faster due to the fact a thermocycler can be used for incubation and denaturing stages. Consequently, Chelex was ideal for the offspring analysis, where a high throughput of samples was required in a short period of time. Further, Chelex is advantageous for microsatellite analysis. Requiring DNA of relatively low molecular weight (<3 kilobase pairs), because sequences of interest are short (<500 bp), the shorter fragments produced from Chelex extraction improves the binding of primers with the target sequence, excluding the purification process needed in many other protocols, saving time.

The microsatellite analysis used in the study was selected based on the high level of inter- and intra-specific polymorphism displayed in the markers, as well as the advantageous Mendelian codominant inheritance displayed for microsatellites. The number of markers available within the genome makes them an ideal tool for
determining parentage (Wikipedia, 2007). In rainbow trout, the methodology has already been applied successfully in aquaculture projects (Herbinger et al., 1995; Fishback et al., 2002; McDonald et al., 2004), with up to eight primers combined in a single multiplex for salmonid analysis (Fishback et al., 1999). The increased and improved development of fluorescently labelled microsatellite markers and automated sequencing machines seen in recent times, means loci with overlapping product sizes can now be differentiated at the analysis stage. Although this is time consuming in preparation, due to the fact primers are generally designed in isolation and annealing temperatures vary between loci considerably, the development of successful multiplexes used in this study saved both time and resources over the course of research. By pooling primers together in one solution, it reduced the number of PCR reactions required to achieve the same result. The uninformative locus left the number of loci suboptimum for the project, but those used were sufficient to provide enough information to allow parentage assignment.

Parental assignment was previously based on exclusionary power using a small number of loci, but improved statistical analysis using computer based likelihood methods has resulted in the ability to increase the number of loci used, improving the confidence level of the parental match. The success of assignment in this study is similar to the levels of allocation in other projects involving aquatic species (Norris et al., 2000; Rodzen et al., 2004; Sekino et al., 2005), including rainbow trout (Herbinger et al., 1995; Fishback et al., 2002). Vandeputte et al. (2006) discuss how assignment by exclusion, using multiple and polymorphic loci, can create genotyping errors in parentage assignment due to mismatching, whilst Jones and Arden (2003) describe how exclusionary assignment can be extremely fragile as a single mismatch between parent and offspring is enough to exclude one of the parents. In the present study the FAP program used for parentage assignment ensured that both parents were taken into consideration as the mating design, and parental combinations could be entered into the program prior to analysis.

Additionally, problems associated with null alleles occurred in the analysis which required attention. Null alleles can be caused by poor primer annealing due to nucleotide sequence divergence in one or both flanking primers, differential
amplification of size variant alleles (Wattier et al., 1998), or because of the competitive nature of PCR, where alleles of short length often amplify more efficiently than larger ones, such that only the smaller of the two alleles might be detected from a heterozygous individual (Dakin and Avise, 2004). Null alleles can often be eradicated by increasing the sample load, adjusting the contrast of the analysis screen, or by improving the template quality (Dakin and Avise, 2004); 'partial null' alleles were often present in the genotyping data here, but these were easily eradicated by the suggestions made by Dakin and Avise (2004). Finally, the addition of a nucleotide, principally adenosine, caused by Taq DNA polymerase catalysing non-templated DNA (Brownstein et al., 1996), in PCR reactions was prevented using an extension time in both multiplexes, whilst the testing and use of only zero and one mismatch individuals in the study maintained confidence in the parental allocations.

As one of, if not the first study reporting on the potential of PKD resistance in any species of fish, it is impossible to compare the results here with other disease resistance studies involving T. bryosalmonae. Further, due to vast contrasts in the aetiologies of fish diseases, to make comparisons with the results of studies involving other fish pathogens would be impractical. Given previous reports of genetic resistance against fish pathogens (Gjedrem et al., 1991; Hard et al., 1997; Gjedrem, 2000), it was plausible to assume that additive genetic variation would exist for T. bryosalmonae within the populations tested. In other salmonid studies additive genetic variation has been found for resistance to specific, and even multiple pathogens (Gjedrem, 2000; Henryon et al., 2005). It is therefore worth noting that the results of this study, in terms of the presence of additive genetic variation and subsequent moderate estimate of heritability, are in agreement with other studies involving various diseases of salmonids (Henryon et al., 2002, 2005; Perry et al., 2004).

To date, the work conducted on parasite resistance in fish, although limited, has shown promising results (Gleeson et al., 2000; Hedrick et al., 2001; Karvonen et al., 2005; Kolstad et al., 2005; Gilbey et al., 2006). The majority of parasitic research in fish has focused on acquired resistance following initial exposure. Many
studies have found an improved tolerance after the first encounter with the causative organism (Gleeson et al., 2000; Karvonen et al., 2003, 2005; Gilbey et al., 2006; Cable and Van Oosterhout, 2007), as is displayed with *T. bryosalmonae* (Foott and Hedrick, 1987; Morris et al., 2003a). For example, Karvonen et al. (2005) found an acquired resistance to the trematode parasite, *Diplostomum spathaceum*, improved infection rate at the second exposure by up to 89.1% in rainbow trout, but the presence of innate resistance to the same pathogen was minimal, potentially zero. Numerous parasites have been used to test potential innate immunity in fish species, including *Gyrodactylus* species, *L. salmonis*, *Cryptobia* species, *Trypanosoma* species, *Ceratomyxa shasta*, *Myxobolus cerebralis*, and *Kudoa thyrsites*. However, such studies generally fail to clarify and/or differentiate the relative roles and degree of innate and acquired immunity against parasitic infection in fish (Jones, 2001).

In studies testing for innate resistance, Kolstad et al. (2005) concluded the potential for improving resistance to *L. salmonis* in Atlantic salmon through selective breeding has potential following an experiment of 350 full-sibling families. Estimates of heritability ranged from 0.02 ± 0.02 to 0.14 ± 0.02 in the field (dependent on the methodology of parasite counting), with a higher estimate of 0.26 ± 0.07 following experimental challenge. The authors also detected a favourable, moderate genetic correlation (0.32 to 0.37) between body weight and the number of lice; a correlation of similar magnitude (but of a negative sign) has been found in the present study – both suggest it is possible to improve body weight and resistance to the parasites, *L. salmonis* or *T. bryosalmonae* simultaneously through selection, in the respective populations. In another salmonid study, Gilbey et al. (2006) found resistance to *G. salaris* was heritable, and hypothesised a polygenic mechanism of control. By identifying ten regions associated with heterogeneity in both innate and acquired resistance, they were able to explain 27.3% of the total variation in parasite loads, with both the innate and acquired parasite resistance deemed to be under polygenic control in Atlantic salmon. They concluded that Atlantic salmon would be highly suited to a selection programme to improve resistance to *G. salaris* in either wild or farmed populations. Glover et al. (2005) studied the susceptibility of Atlantic salmon to the sea lice, *L. salmonis* and *Caligus elongatus*, to find additive genetic variation was apparent for both species - with a significant difference in *L.*
salmonis resistance between families - indicating selective breeding for improved resistance would be possible in both organisms. Mustafa and MacKinnon (1999) studied 73 full-sibling families to attain an estimate of heritability of 0.22 for the susceptibility of Atlantic salmon to C. elongatus, whilst in myxozoan parasite research several studies have identified the potential for genetic selection towards M. cerebralis and C. shasta resistance. In 2001 Bartholomew et al. experimented with the susceptibility of F1 progeny crosses of rainbow trout parents resistant and susceptible to C. shasta. It was found that groups with at least one parent of the resistant strain resulted in less than 5% mortality, compared to up to 98% mortality from a susceptible parent cross, leading the authors to conclude that resistance to C. shasta is a dominant trait, which would respond to selective breeding. In M. cerebralis research numerous authors have experimented with susceptible and non-susceptible strains of rainbow trout (Hedrick et al., 2003; Severin and El-Matbouli, 2007). With the strain-specific susceptibility for this infection well established, attention has turned towards the underlying genetic basis for the variation expressed within (non-)susceptible strains (Severin and El-Matbouli, 2007).

A broad genetic basis has been suggested as a key element for parasite resistance, with heterozygous individuals assumed to detect and present a wider range of pathogen-derived antigens due to a larger number of different Major Histocompatibility Complex (MHC) molecules (Langefors et al., 2001). In a study by Hedrick et al. (2001) it was discovered that populations from different sources showed variable, but not statistically different responses to an exotic fluke from guppies (Poecilia reticulate) on the endangered Gila topminnow (Poeciliopsis occidentalis). They found the most homozygous population to carry the greater infection and mortality, with homozygotes for a MHC gene displaying lower (although not statistically different) survival compared to heterozygotes. Further, an inbred line from one of the populations showed lower survival and higher infection compared to an outbred control, leaving the authors to conclude that low genetic variation in general, or for the important MHC genes, and populations with a history of inbreeding are more likely to suffer detrimental effects from this parasitic infection. Similarly, Gleeson et al. (2000) discovered distinct populations of rainbowfish (Melanotaenia species) differed significantly in their susceptibility to
Ichthyophthirius multifiliis in a controlled environment. In the same experiment they produced intraspecific hybrids which demonstrated increased tolerance, leading to the same hypothesis; there may be a link between heterozygosity of populations and their innate ability to resist parasitic infection. Cable and Van Oosterhout (2007) studied the tolerance of guppy populations from different areas of the Aripo River in Trinidad to G. turnbulli to find a superior resistance both initially and across subsequent challenges, suggesting the immunocompetence for this organism in the Aripo River guppy population has a heritable genetic basis.

With a multitude of parasite species in the aquatic environment, it is perhaps unsurprising that the impact on the host results in different physiological effects. The immune reaction depends on both the parasite and fish species involved, as well as the immunogenetic background of the host. Represented by MHC alleles, the specific and adaptive immune system in fish can defend against parasitic invasion in a number of ways. Genes of the MHC represent the most polymorphic genes in the vertebrate genome (Marsh et al., 2000; Marieb, 2004). The MHC genes encode cell surface glycoproteins responsible for the presentation of self and foreign peptides to T lymphocytes (T cells). Generally, foreign peptides produced by the degradation of intracellular pathogens are bound by MHC class I molecules and are presented to cytotoxic T cells, whilst foreign peptides derived from extracellular pathogens are bound by MHC class II molecules, which are presented to helper T cells (Rammensee, 1995; Marieb, 2004). In the majority of circumstances, presentation and recognition of foreign peptides produces a humoral or cell mediated immune response, where the highest level of polymorphism observed in the MHC genes is concentrated within the peptide-binding regions (PBR). Polymorphism within the PBR enables different allelic variants to bind and present unique sets of antigenic peptides. However, some pathogens may escape recognition by certain MHC molecules as their peptides are not presentable by the MHC, leading to variation in susceptibility to certain pathogens. Alternatively resistance may be derived through a high affinity binding of certain peptides by specific MHC alleles (Marieb, 2004).

To date, the specific immune response during PKD is unknown, and requires further investigation. However, it has been identified that the MHC genes in teleost species
are considered to be primitive compared to humans, having the unusual feature of non-linkage between the classical class I and class II genes (Sato et al., 2000; Shum et al., 2002). This led to the genes being renamed MH genes in Atlantic salmon (Stet et al., 2002). However, the class II alpha and beta loci (DAA and DAB) have remained linked in salmonids, with evidence of a single dominantly expressed locus of both the classical class I and class II genes in Atlantic salmon and rainbow trout (Shum et al., 2002; Stet et al., 2002). The polymorphic nature of the MHC has made it possible to associate certain alleles or genotypes to increased tolerance to infectious pathogens in numerous animals. For example, in Soay sheep, MHC polymorphism has been significantly associated with juvenile survival and nematode parasitism (Paterson et al., 1998), whilst in chickens, one MHC haplotype is known to be significantly associated with resistance to Marek’s disease (Bacon, 1987). In salmonid research, Grimholt et al. (2003) have shown a highly significant association between MH polymorphism and resistance to furunculosis and Infectious Salmon Anaemia (ISA) in Atlantic salmon, whilst Langefors et al. (2001) concluded fish from high and low resistance families of Atlantic salmon displayed significant differences in MHC class II beta alleles, which helped to produce a large variation in resistance to furunculosis infection. Wynne et al. (2007) found a significant association between specific MHC alleles and the susceptibility to Amoebic Gill Disease (AGD) in Atlantic salmon, while Miller et al. (2004) describe an increased resistance to Infectious Haematopoietic Necrosis (IHN) in Atlantic salmon associated with certain MHC alleles. In the viral disease, ISA, Kjøglum et al. (2006) identified specific MHC alleles that influence resistance. Although genotyping was conducted in the present experiment, its use was solely for the purpose of parental allocation. Future research into PKD resistance would benefit from the identification of specific loci that provide information on the immune reaction of the host to *T. bryosalmonae*, which identifies the alleles that provide beneficial results for selection.

Having discovered additive genetic variation exists for PKD score within the two farmed stocks, estimates of heritability for each strain were calculated, and found to be moderately high in both populations. However, age differences and discrepancies in early rearing left comparisons between the two strains confounded;
as a result the overall estimate is thought to be more reliable. The overall heritability calculated for kidney score from combined components was medium, suggesting significant additive genetic variation is present in both populations, which would respond to selective breeding and result in improved PKD score, and potentially resistance. Improving resistance to PKD has numerous benefits for the British rainbow trout industry. By selectively breeding for PKD resistance, a cumulative improvement can be observed in each generation of selection. The advantages of such improvements are obvious; less fish will be lost due to the direct mortality associated with the disease, less feed will be wasted in fish that die, there will be a reduced requirement for management, more fish will make it to harvest, and the general welfare on the farm will be improved. However, it must be considered that the causative agent is likely to evolve to survive in the host over selection generations. Although evidence is available to support this claim in higher vertebrates (Nicholas, 1987), to date, no information is available on the evolution of fish pathogens. However, any increase in resistance of the pathogen will inevitably offset at least some of the progress made for PKD resistance within the fish. Additionally, the current limited knowledge of interactions between rainbow trout as a host and the causative agent, means any evolutionary progress of T. bryosalmonae will most likely go unnoticed for the first few generations of selection; such considerations are undoubtedly challenges facing selection for PKD resistance.

The heritability estimate for body weight is in agreement with other studies involving sub-yearling salmonid species (Henryon et al., 2002; Perry et al., 2004, 2005). Estimates of heritability for fork length are limited for sub-yearling salmonids in the literature, but the heritability calculated here is similar to that described by Henryon et al. (2002), and is expectedly high. Similarly, the genetic correlation between body weight and fork length is expectedly high and positive (Gunnes and Gjedrem, 1978). Between either performance trait and kidney score, the genetic correlation is moderate and negative. The negative correlation between size and kidney score has two implications. It could be suggested that more resistant fish are less stressed and so continue to feed throughout the epidemic gaining weight and being larger at the time of sampling, or, having been exposed to
the parasite for some two to three months, certain individuals may have been more affected at different times; those affected earlier recovering earlier and subsequently feeding, being larger at the time sampling took place. Alternatively, larger fish may simply be less susceptible to the disease. The fact that larger fish have a greater kidney size may affect the rate of development of PKD. Where smaller fish (ie smaller kidneys) require fewer parasites to cause the profuse swelling seen throughout the progression of PKD, larger fish are affected only when the same parasite to kidney ratio is reached. However, this does not necessarily mean that these fish are less susceptible overall as the affect of the disease may simply be delayed. In the present study, data was collected as a cross section over a single day, ie a snapshot in time of the disease status. If disease progression could be followed in larger and smaller fish, it may simply show that larger fish are affected later rather than less. This in itself has implications; if larger fish take longer to become affected, size alone could be incorporated into current management strategies. Where natural vaccination (Ferguson, 1981; Foott and Hedrick, 1987; Morris et al., 2003a) is utilised, managing fish size according to water temperature may provide a reduced impact by stocking more resistant, larger fish earlier in the summer when PKD is more prolific, and smaller fish later in the summer as temperature (and PKD development) decline. With the average body weight at the lowest kidney score almost twice that at the highest kidney score (103.80 grams compared to 55.94 grams), it suggests that this may be a feasible practice even at commercial levels. At the intermediate grades (1, 2, and 3), although the trend continues, the degree of difference is reduced. Between grades 1 and 4, the difference is a mere 11.43 grams, but between grades 0 and 1, the difference is 36.43 grams, which would be sufficient to categorise fish when grading on farms. By using a cut off average weight of approximately 70 grams (for the HS and IoM populations), and stocking fish above this threshold earlier, and smaller fish later, a reduction in the effects of PKD may be seen. However, this could only be viable on commercial farms where fish could be held in a PKD-free environment until reaching the threshold size. Further, it is worth noting that, although greater size is shown to display more resistance in correlation terms, it is not represented in the mean size traits and kidney score data of each farm; IoM representatives have the larger average kidney score, yet are both longer and heavier than HS
representatives. As preliminary data in terms of PKD resistance and size in this population, no stringent conclusions can be drawn here, and further work is required.

Considering the estimate of heritability calculated for kidney score, it is predicted that significant gains can be made towards genetic improvement for PKD score, whilst the favourable correlation between body weight or fork length and kidney score suggests it is possible to improve the two production traits and resistance to *T. bryosalmonae* simultaneously through selection. Perry *et al.* (2005) suggest pedigreed selection may provide specific sires and dams with advantageous genotypic combinations (see Kause *et al.*, 2003); a hypothesis tested in Chapter 4. Nevertheless, the additive genetic (co)variation detected here highlights the potential to successfully implement a breeding programme for rainbow trout in the UK industry. The estimates provide an indication of the magnitude of additive genetic variation associated with each trait, as well as the genetic correlations between them. As a result, these preliminary findings form a basis on which to develop a suitable breeding programme for PKD resistance in the UK trout industry, beginning with HS and IoM strains.
Chapter 4: Proliferative Kidney Disease Challenges
4.1 Introduction

The relatively high proportion of existing genetic variation in metric characters has resulted in numerous studies concentrating on the genetic control of production traits (Bolivar and Newkirk, 2002; Goyard et al., 2002; Pante et al., 2002). The level of progress made in improving commercially important characteristics is dependent on the amount of genetic variation displayed in the trait (Marsden et al., 1996). Until recently, improvements in characteristics of economic value in fish species had focused on growth and performance traits, but emphasis is now moving towards selective breeding for disease resistance (Argue et al., 2002; Fishback et al., 2002; Henryon et al., 2005). Mortalities from specific pathogens in aquaculture have indicated considerable genetic variation exists between fish at the inter- and intra-specific level; as such several reviews of selective breeding for disease resistance are now available (Kinghorn, 1983; Chevassus and Dorson, 1990). By incorporating disease resistance into selection indices, improved economic returns are inevitable, through minimising losses and reducing disease incidence (see Henryon et al. 2002).

In areas endemic to Proliferative Kidney Disease (PKD), sites would benefit from genetically improved stocks. Now considered the most costly disease to the British rainbow trout farming industry, its incorporation into selective breeding programmes will be welcomed. The progress made in disease resistance is dependent on the level of genetic variation exhibited in relation to the causative pathogen within the population tested; this being most beneficial when variation is large (Marsden et al., 1996). Chapter 3 discussed the existing variation in Houghton Spring Hatchery (HS) and Isle of Man (IoM) populations, suggesting significant potential is available to produce strains of rainbow trout with lower PKD scores. However, to ensure the genetic variation is real, it is necessary to reproduce the results under experimental conditions, alleviating any environmental or management practices that may have created biases under commercial conditions (Kolstad et al., 2005).
The present chapter focuses on challenge experiments used to test if genetic differences are apparent in the resistance to PKD between different commercial families produced from the disease-free site, IoM. From the information gathered in Chapter 3, a challenge was established to assess known high and low responding families in an attempt to support the evidence described previously that additive genetic variation exists to PKD score. Timeframes for each family to assess the development of PKD will be incorporated into the study. These could potentially be used as a useful tool when integrated into current management practices, ie artificial vaccination. Finally, parasite counts from immunohistochemistry-stained kidney sections may provide evidence of a significant and positive correlation between kidney score and parasite load that will justify the continued use of the Clifton-Hadley et al. (1987) scale used to categorise the severity of PKD.
4.2 Materials and Methods

4.2.1 Broodstock and mating design

From the information gathered in Chapter 3, estimated breeding values (EBVs) were calculated for each family. Based on the EBV, female siblings of the naturally challenged fish were then used as broodstock in the present study. Equal numbers of high and low responding females were selected from a total of 500 mature females, previously identified and ranked according to EBV position; see ‘Female ID’ and ‘EBV Position (1-500)’ of Table 4.1, which illustrates all information relating to the generation of the experimental families. The 28 selected females were then crossed with 7 randomly selected neomales; two high and two low responding females were crossed with each neomale so that sire effect could be calculated. However, it is important to note that, as the broodstock matured at different times (see ‘Fertilisation date’ in Table 4.1), selection intensity for actual high and low ranking females was hindered; those selected are therefore considered to be nominally high and low responding females. Although ‘Response (EBV)’ is documented as high and low in Table 4.1, the EBV value is also included as the analysis of data uses regressions on EBV of the dams rather than the nominal line, as this considers the variation as deviance from the regression line.

Only IoM families were used in the experiment due to their disease-free certification. Families were transported to the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling in two batches at the eyed egg stage. Spawning, fertilisation, and incubation until this time were conducted according to IoM commercial techniques. Sufficient numbers of eggs were sent to allow for mortality during the culture period.
Table 4.1 - Family generation and pedigrees of fish used in the PKD Challenge trials

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<th>Female ID</th>
<th>EBV Position (1-500)</th>
<th>Response (EBV)</th>
<th>Neomale</th>
<th>Family</th>
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<td>307</td>
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<td>High (2.87)</td>
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<td>ARF 3</td>
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‘Family’ is the experimental name given prior to challenge (see below for further detail), and ‘n’ refers to the number of eggs sent per family.

4.2.2 Batch 1 - ARF

Eyed eggs representing 16 families arrived at the Institute of Aquaculture, University of Stirling on the 25/01/2006, at an age of 253 degree days (dd). Upon arrival, the distinct families remained separated, and were stocked into egg trays suspended inside 10 l holding units. Flow rates and aeration were adjusted to supply enough oxygen and clear debris, whilst the light regime was 12 h light:12 h dark. Over a 6 day period water temperature was increased from ambient (~6°C) to a maintained 10°C (± 2°C). Water filtration was minimal (charcoal filter only) due to the use of mains tap water. Egg incubation was normal in terms of development and mortality, but due to a system failure tanks were subject to critical water temperatures resulting in a complete loss of ARF 11 and severe reduction of ARF 4.
Following 100% hatch and first feeding to a size suitable for handling, all groups were transferred to larger (100 l) circular tanks (18/03/2006). Water temperature remained at 10°C (± 2°C). As ambient temperatures increased towards summer, >10°C, water supply was switched. Inflows were adjusted to a minimum of 0.5 litres per min (l/min) and airstones provided additional oxygen and water circulation. The light regime remained at 12h light:12h dark. On the 07/04/2006, fish number was reduced to 300 per family, with the exception of family 4, where only 65 fish remained. Average weights ranged from 0.574 g to 0.920 g. On the 26/04/2006 symptoms of Costia occurred. All 15 families were subject to a 1 h bath of formalin at 200 parts per million (ppm). No future symptoms were observed. Average weights were calculated at two intervals prior to challenge; 15/05/2006, overall average weight 3.06 g; and, 02/06/2006, overall average weight 5.31 g. Test fish were required to exceed 5 g to ensure the immune system was developed. Feeding to 5 g was *ad libitum* before reduced to maintenance ration until the trial began.

4.2.2.1 Tagging

Tagging was completed over a two day period (28/06/2006 and 29/06/2006). One hundred and ten fish per family (excluding family 4) were randomly selected and subject to tagging using Visible Implant Elastomere (VIE, Northwest marine technology (NMT), USA) of two colours, pink and green. Injection took place in one of 10 locations; head, belly, left or right; eye, dorsal, flank, or jaw (Figure 4.1); these combinations allowed identification to the family level (Table 4.2).
Fish were removed from the holding unit, anesthetised in 10% benzocaine (0.4 ml/l), injected with VIE, and placed into an aerated recovery bin. Tagged fish were transferred to duplicate 100 l circular holding units, and labelled as experimental fish. Mortality following tagging was negligible.

<table>
<thead>
<tr>
<th>Family Number</th>
<th>Tagging Location and Colour</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF 1</td>
<td>Right flank – pink</td>
<td>RF – P</td>
</tr>
<tr>
<td>ARF 2</td>
<td>Left flank – pink</td>
<td>LF – P</td>
</tr>
<tr>
<td>ARF 3</td>
<td>Right jaw – pink</td>
<td>RJ – P</td>
</tr>
<tr>
<td>ARF 4</td>
<td>Adipose fin clipped</td>
<td>Adipose</td>
</tr>
<tr>
<td>ARF 5</td>
<td>Left jaw – pink</td>
<td>LJ – P</td>
</tr>
<tr>
<td>ARF 6</td>
<td>Left jaw – green</td>
<td>LJ – G</td>
</tr>
<tr>
<td>ARF 7</td>
<td>Belly – green</td>
<td>B – G</td>
</tr>
<tr>
<td>ARF 8</td>
<td>Right flank – green</td>
<td>RF – G</td>
</tr>
<tr>
<td>ARF 9</td>
<td>Left flank – green</td>
<td>LF – G</td>
</tr>
<tr>
<td>ARF 10</td>
<td>Left dorsal – green</td>
<td>LD – G</td>
</tr>
<tr>
<td>ARF 12</td>
<td>Left eye – green</td>
<td>LE – G</td>
</tr>
<tr>
<td>ARF 13</td>
<td>Head – green</td>
<td>H – G</td>
</tr>
<tr>
<td>ARF 14</td>
<td>Left eye – pink</td>
<td>LE – P</td>
</tr>
<tr>
<td>ARF 15</td>
<td>Head – pink</td>
<td>H – P</td>
</tr>
<tr>
<td>ARF 16</td>
<td>Right eye – green</td>
<td>RE – G</td>
</tr>
</tbody>
</table>
4.2.3 Batch 2 - BRF

Twelve distinct families of eyed eggs arrived at the Institute of Aquaculture, University of Stirling on the 02/02/2006 at an age of 252 dd. The families were transported to Buckieburn research facility (BRF), where they were disinfected using buffadine and laid down in egg troughs within 1.5 m circular tanks. Flow rates, sourced from Buckieburn Dam, were set to 2 l/min to supply oxygen and clear suspended solids. Ambient light and water temperature regimes were followed. At times of poor water quality, eggs were checked and flushed as necessary to prevent smothering.

After hatching, problems occurred in almost all holding units resulting in mass mortality in the majority of groups. Coinciding with the presence of *Costia* and/or *Trichodina*, formalin treatments ensued; flushes on the 6th, 10th, 13th and 18th April, and baths on the 21st, and 24th April. However, mortality persisted, resulting in fish veterinarian Richard Collins (Institute of Aquaculture, University of Stirling) observing and sampling stocks: “A definitive cause of the problem among fry remains uncertain. The possibility of lipid-related nutritional deficiency among the ova, only manifesting significantly at lower incubation temperature, is not to be excluded”. Mortalities due to the ‘condition’ resulted in the complete loss of families BRF 1, BRF 7, BRF 11, and BRF 12.

On the 13/07/2006 families were reduced to 300 fish per holding unit, duplicated in case of further losses. They were maintained at the BRF until sufficient tank space was available for transfer to the ARF. Feeding was maintained *ad libitum* until transfer. On the 31/08/2006, families were moved to the ARF. The groups of 300 were transported in well-oxygenated plastic bags. However, families BRF 2 and BRF 3 died in transit, possibly due to a lack of oxygen and/or acute stress; they were replaced with their duplicate groups. Due to higher than expected average weights, families were divided into two 100 l holding units upon arrival. Feeding was reduced to maintenance ration in anticipation of challenge.
4.2.3.1 Tagging

Tagging took place over a single day (18/10/2006). At least 60 representatives were selected at random and subject to family marking using VIE. The family markings are illustrated below in Table 4.3.

**Table 4.3 - Tagging location and code for experimental BRF families used in challenges**

<table>
<thead>
<tr>
<th>Family Number</th>
<th>Tagging Location and Colour</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRF 2</td>
<td>Left jaw – pink</td>
<td>LJ – P</td>
</tr>
<tr>
<td>BRF 3</td>
<td>Head – pink</td>
<td>H – P</td>
</tr>
<tr>
<td>BRF 4</td>
<td>Right jaw – pink</td>
<td>RJ – P</td>
</tr>
<tr>
<td>BRF 5</td>
<td>Left flank – pink</td>
<td>LF – P</td>
</tr>
<tr>
<td>BRF 6</td>
<td>Right jaw – green</td>
<td>RJ – G</td>
</tr>
<tr>
<td>BRF 8</td>
<td>Left flank – green</td>
<td>LF – G</td>
</tr>
<tr>
<td>BRF 9</td>
<td>Left jaw – green</td>
<td>LJ – G</td>
</tr>
<tr>
<td>BRF 10</td>
<td>Head – green</td>
<td>H – G</td>
</tr>
</tbody>
</table>

4.2.4 Challenges

4.2.4.1 Screening

Prior to the challenges taking place, representatives from each family were screened for the presence of *Tetracapsuloides bryosalmonae* using the same techniques described for the actual challenges (see Section 4.2.4.4). Using the equation of Otte (2007):

\[ n = z^2 \times p(1-p) / a^2 \]

where \( n \) is the required sample size, \( z \) is the appropriate value from the normal distribution for the desired confidence (95%), \( p \) is the anticipated prevalence of disease (0.01), and \( a \) is the desired precision (0.05). The resultant sample size was divided by the number of families; 15 ARF families, and 8 BRF families. The low prevalence, \( p \), was used due to both batches being certified disease-free and no history of PKD at the two holding sites. All fish tested negative for PKD.
4.2.4.2 Design

Both PKD challenges were conducted at the ARF; a home office licensed premises to perform experimental tests. The consecutive challenges were completed so comparisons could be made. Both used three 100 l holding units, with flows of ≥2 l/min, plus additional aeration. Light regime remained at 12 h light:12 h dark, whilst feed was given at 2% body weight per day. Water temperature was adjusted, ie heated when necessary, to maintain temperatures above 12.5°C (± 2°C). After tagging, test fish were allowed at least 21 days recovery time to ensure the VIE had solidified. Movement to the test tanks took place at the time of injection (21/07/2006; day 0) for PKD challenge 1 (ARF fish), and 3 days prior to injection (06/11/2006) for PKD challenge 2 (BRF fish, 9/11/2006; day 0). Test fish in PKD challenge 2 were given time to acclimatise and recover from tank transfer due to experiences of a general susceptibility to stress, possibly related to the earlier condition. In PKD challenge 1, each replicate consisted of 14 fish per family, 210 fish per tank (total: 630), whilst in PKD challenge 2, 20 fish per family were used, 160 fish per tank (total: 480). Representatives were weighed into test tanks to ensure no biases in replicate weight.

4.2.4.3 Induced infection

Intraperitoneal injection followed the protocol of McGurk (2005); for each replicate, six heavily PKD infected Artic charr were euthanased and kidney removed aseptically. Following impression smears and Rapi-diff staining (Appendix 2) to ensure infection was sufficient, five of the six infected kidneys were added to 40 ml of sterile PBS and homogenised; repeated for each replicate and kept on ice to prolong use. Fish were anaesthetized (4 mg/l 10% benzocaine) before being ip injected with 0.1 ml homogenate, and released or returned to test tanks.

4.2.4.4 Sampling

The sampling regimes for the two challenges were identical, but sampling numbers differed. At 3 weeks post injection (pi), sampling took place to ensure *T. bryosalmonae* had transmitted successfully. Using a VIE light (NMT, USA) to
identify individuals, 2 (PKD challenge 1) or 3 (PKD challenge 2) representatives per family per replicate were selected, euthanased, fork length and body weight taken, and kidney score assigned, before the spleen and a section of posterior kidney were removed and placed in 10% neutral buffered formalin for immunohistochemistry, as described in Chapter 2, Section 2.2. A small section of kidney was stored on ice for PKD-PCR (Chapter 2, Section 2.1) at 3 weeks pi only.

The next sampling occurred at 6 weeks pi following the same protocol but without PCR analysis. This continued weekly until 11 weeks pi, taking 96 fish per week in PKD challenge 1 (6 fish per family), and 72 fish each week in PKD challenge 2 (9 fish per family) where possible, until no fish remained in test tanks.

4.2.4.5 Kidney scoring as a measure of resistance

Using the entire scale of Clifton-Hadley et al. (1987), each sacrificed fish was allocated a score from 0 to 4 inclusive, in addition to healing (grade H) where appropriate; kidney with advanced signs of healing, often containing either discrete cream-coloured patches or spherical nodules up to 10 mm in diameter surrounded by dark red renal tissue. Measuring the severity of inflammation was believed to provide a measure of the degree of resistance, ie the smaller the swelling, the greater the resistance. Further, the analysis of representatives from each family every week allowed a timeframe of PKD progress within each family.

4.2.4.6 Parasite counting

Following immunohistochemistry, the parasite load of sampled kidneys was recorded. The methodology used is described by Higgins and Kent (1998) and Morris et al. (2003a), where sporogonic and extrasporogonic stages of *T. bryosalmonae* within the kidney sections were counted under 11 random fields of view at x400 magnification (0.45 mm diameter) under light microscopy. The total parasite count was then converted to number per mm$^2$ for each fish and plotted against the individual’s kidney score in order to establish if any relationship was apparent.
4.2.5 Statistical analysis

4.2.5.1 Summary Statistics

Data from the challenges were entered into Microsoft Excel in preparation of analysis. Where summary statistics were desired, formulas in Excel were used to calculate the basics; mean, minimum, maximum, count etc. The majority of graphs presented were generated using Microsoft Excel. All other analysis was conducted using Genstat Release Version 9.1.

4.2.5.2 PKD resistance, survival, and estimates of heritability

Genetic parameters were estimated using a single linear mixed model in Genstat Release Version 9.1. All traits were analysed using the REML model:

\[ y_{ip} = \mu_i + b_i \cdot \text{EBV}_{ip} + \text{sire}_{ik} + \text{dam}_{il} + \text{tank}_{im} + e_{ip} \]

where \( y_i \) is the vector for trait \( i \), when the variable \( y_i \) is the observed value of fish \( p \), with random effects of sire \( k \), dam \( l \), and tank \( m \) for trait \( i \). The residual error connected to trait \( i \) is represented as \( e_{ip} \). \( \mu_i \) is the population mean for trait \( i \), and \( b_i \cdot \text{EBV}_{ip} \) is the regression on the estimated breeding value of dam \( l \), treated as a fixed term.

Survival was assessed using this model as a binary trait (ie survived/died), where \( y_i = 0 \) was allocated if death occurred and \( y_i = 1 \) if the individual survived. Other traits included kidney score, fork length, body weight, and parasite/mm\(^2\). Further, evidence of genetic variation, effect of EBV, and estimates of heritability were calculated on a week by week basis for each challenge using this model, by restricting the data appropriately.

The random effects of sire\(_{ik}\), dam\(_{il}\), and residual error \( (e_{ip}) \) were assumed to be independent random normal variables with mean zero, and variances denoted \( \sigma_s^2 \), \( \sigma_d^2 \), and \( \sigma_e^2 \). Total phenotypic variance was denoted \( \sigma_t^2 \), and was estimated as \( \sigma_t^2 = \sigma_s^2 + \sigma_d^2 + \sigma_e^2 \). The genetic information therefore comes from three sources: (i) the regression on the EBV of the dam; (ii) the dam variance \( (\sigma_d^2) \), which represents the
variance of deviations from the regression on EBV; and, (iii) the sire variance ($\sigma_s^2$). Estimates of heritability were based on the narrow sense heritability, and could be obtained from the sire variance using the formula:

$$\frac{4(\sigma_s^2)}{\sigma_t^2}$$

from the sire variance component $h_s^2$

The variance between dams, in principle, contains other non-genetic maternal effects, but in this study, given its small size, was interpreted as being wholly genetic, with the estimates of heritability obtained from the dam variance using the formula:

$$\frac{4(\sigma_d^2)}{\sigma_t^2}$$

from the dam variance component $h_d^2$

However, it is important to note that part of the genetic variance attributable to dams was removed by the regression on EBV.

In every instance the significance of the random variables were tested using a likelihood ratio test. Using the above REML model, the term of interest (sire, dam, or tank) was dropped and the deviance compared to the original deviance. If the difference was greater than $\chi^2$ at 1 degree of freedom (likelihood ratio test statistic at 95% CI: 3.84), the variable was considered to be significant.

Additional analysis was conducted on survival. A Kaplan-Meier estimate of the survivor function, including graph, was completed based on the mortality occurring due to anything other than sampling. Using trial (factor), tank (factor), female (factor), EBV (variate), sire (variate), and survival (variate), the data was input into the estimate with actual time points considered (measured as days post injection to death) and survival censored for sampling weeks.
Prior to the challenges, families were weighed into test tanks. In order to ensure no differences were apparent between families, a similar REML model was used, but modified to include the term *trial*:

\[ y_{ihq} = \mu_i + b_i \cdot EBV_{ihq} + \text{trial}_{ih} + sire_k + dam_l + \text{tank}_{m} + e_{ihq} \]

where \( y_i \) is the bulk weight of family \( q \) involved in the challenge, and \( \text{trial}_{ih} \) is included as a fixed effect to assess differences between PKD Challenge 1 and PKD Challenge 2 fish stocks.
4.3 Results

From the 1109 individuals involved in the challenges, information was available on 1106. However, only 1032 were used for the analysis of PKD resistance as many individuals were unidentifiable to the family level, victims of mortality between sampling weeks, or simply lost from the study; most likely due to cannibalism. Overall mean values for kidney score ranged from 0.64 ± 0.09 to 2.46 ± 0.19 within full-sibling groups, against an overall mean of 1.48 ± 0.03. The information gathered per family, per challenge, and overall is summarised in Table 4.4.

At 3 weeks pi, 85.19% of individuals tested using the PKD-PCR demonstrated successful transmission of T. bryosalmonae. In PKD Challenge 1, 85 of the 90 fish showed successful transmission at week 3 (94.44%), whilst PKD Challenge 2 demonstrated a lower transmission of 73.61%. An example of the 435 bp positive bands obtained in the PKD-PCR is illustrated below in Figure 4.2.

![Ethidium Bromide stained agarose gel image](image.png)

**Figure 4.2 - Example of Ethidium Bromide stained, 1.2% agarose gel image. Chelex extracted DNA (source: kidney) from (non-)infected rainbow trout. Lanes 1 to 4 negative for PKD (no bands), lane 5 ØX 174 RF DNA Hae II ladder, lanes 6 to 9 positive for PKD. Bands apparent at 435 bp**
<table>
<thead>
<tr>
<th>Family</th>
<th>Kidney score ± SE</th>
<th>Length (cm) ± SE</th>
<th>Weight (g) ± SE</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF 1*</td>
<td>2.05 ± 0.19</td>
<td>13.21 ± 0.42</td>
<td>28.13 ± 1.78</td>
<td>37</td>
</tr>
<tr>
<td>ARF 2*</td>
<td>1.94 ± 0.25</td>
<td>12.67 ± 0.24</td>
<td>27.08 ± 1.58</td>
<td>33</td>
</tr>
<tr>
<td>ARF 3*</td>
<td>2.46 ± 0.19</td>
<td>13.35 ± 0.31</td>
<td>33.08 ± 2.16</td>
<td>37</td>
</tr>
<tr>
<td>ARF 4*</td>
<td>1.68 ± 0.20</td>
<td>13.10 ± 0.21</td>
<td>28.43 ± 1.43</td>
<td>37</td>
</tr>
<tr>
<td>ARF 5*</td>
<td>1.51 ± 0.14</td>
<td>13.15 ± 0.28</td>
<td>30.99 ± 1.88</td>
<td>41</td>
</tr>
<tr>
<td>ARF 6*</td>
<td>2.21 ± 0.22</td>
<td>12.19 ± 0.30</td>
<td>23.83 ± 1.65</td>
<td>29</td>
</tr>
<tr>
<td>ARF 7*</td>
<td>2.28 ± 0.23</td>
<td>12.02 ± 0.27</td>
<td>22.94 ± 1.56</td>
<td>36</td>
</tr>
<tr>
<td>ARF 8*</td>
<td>1.84 ± 0.22</td>
<td>12.26 ± 0.26</td>
<td>23.20 ± 1.39</td>
<td>37</td>
</tr>
<tr>
<td>ARF 9</td>
<td>1.29 ± 0.14</td>
<td>12.90 ± 0.24</td>
<td>28.81 ± 1.48</td>
<td>41</td>
</tr>
<tr>
<td>ARF 10*</td>
<td>1.13 ± 0.14</td>
<td>12.59 ± 0.20</td>
<td>26.59 ± 1.25</td>
<td>39</td>
</tr>
<tr>
<td>ARF 12*</td>
<td>1.35 ± 0.17</td>
<td>12.13 ± 0.25</td>
<td>23.43 ± 1.33</td>
<td>40</td>
</tr>
<tr>
<td>ARF 13*</td>
<td>1.47 ± 0.17</td>
<td>12.34 ± 0.22</td>
<td>24.81 ± 1.21</td>
<td>36</td>
</tr>
<tr>
<td>ARF 14*</td>
<td>1.67 ± 0.17</td>
<td>11.75 ± 0.24</td>
<td>22.42 ± 1.41</td>
<td>39</td>
</tr>
<tr>
<td>ARF 15*</td>
<td>1.38 ± 0.16</td>
<td>12.01 ± 0.26</td>
<td>23.11 ± 1.63</td>
<td>34</td>
</tr>
<tr>
<td>ARF 16</td>
<td>1.13 ± 0.12</td>
<td>12.77 ± 0.27</td>
<td>27.96 ± 1.90</td>
<td>40</td>
</tr>
<tr>
<td>BRF 2</td>
<td>1.35 ± 0.11</td>
<td>13.00 ± 0.14</td>
<td>27.21 ± 0.93</td>
<td>60</td>
</tr>
<tr>
<td>BRF 3</td>
<td>1.67 ± 0.10</td>
<td>13.21 ± 0.14</td>
<td>27.23 ± 0.97</td>
<td>60</td>
</tr>
<tr>
<td>BRF 4</td>
<td>1.45 ± 0.09</td>
<td>13.12 ± 0.15</td>
<td>28.06 ± 0.93</td>
<td>60</td>
</tr>
<tr>
<td>BRF 5</td>
<td>1.13 ± 0.10</td>
<td>13.59 ± 0.17</td>
<td>30.34 ± 1.15</td>
<td>60</td>
</tr>
<tr>
<td>BRF 6</td>
<td>1.68 ± 0.16</td>
<td>12.68 ± 0.22</td>
<td>25.86 ± 1.36</td>
<td>60</td>
</tr>
<tr>
<td>BRF 8*</td>
<td>1.33 ± 0.10</td>
<td>12.49 ± 0.26</td>
<td>24.34 ± 1.54</td>
<td>58</td>
</tr>
<tr>
<td>BRF 9*</td>
<td>0.64 ± 0.09</td>
<td>14.37 ± 0.17</td>
<td>36.18 ± 1.34</td>
<td>59</td>
</tr>
<tr>
<td>BRF 10*</td>
<td>0.76 ± 0.09</td>
<td>13.26 ± 0.17</td>
<td>29.69 ± 1.16</td>
<td>59</td>
</tr>
<tr>
<td>PKD Challenge 1 Overall</td>
<td>1.67 ± 0.05</td>
<td>12.78 ± 0.21</td>
<td>26.41 ± 0.43</td>
<td>556</td>
</tr>
<tr>
<td>PKD Challenge 2 Overall</td>
<td>1.25 ± 0.04</td>
<td>13.21 ± 0.07</td>
<td>28.62 ± 0.46</td>
<td>476</td>
</tr>
<tr>
<td>Combined Overall</td>
<td>1.48 ± 0.03</td>
<td>12.98 ± 0.12</td>
<td>27.43 ± 0.31</td>
<td>1032</td>
</tr>
<tr>
<td>Unidentified</td>
<td>1.78 ± 0.21</td>
<td>13.41 ± 0.43</td>
<td>33.08 ± 2.89</td>
<td>18</td>
</tr>
</tbody>
</table>

*Total mortality PKD Challenge 1 53
*Total mortality PKD Challenge 2 3
The timecourse of PKD development throughout the challenge periods was calculated for every family. The progression of the disease for a number of families is shown in Figure 4.3, which illustrates not only the variation in peaks and troughs between the mean PKD score of families recorded at each sampling week (eg ARF 4 and BRF 2), but also the overall difference between families, for example ARF 7 and BRF 9 (which is also representative of the overall mean scores).

![Figure 4.3](image)

**Figure 4.3 - Timecourse of kidney scores for rainbow trout over sampling weeks in the PKD Challenges; demonstrated using only six families (three ARF and three BRF) to highlight variation**

The mean fork length and body weight of fish involved in the challenges demonstrated a general overall increase throughout the trial period, as can be seen in Figures 4.4 and 4.5.

![Figure 4.4](image)

**Figure 4.4 - Mean body weight (g) for rainbow trout used in PKD Challenge 1 and PKD Challenge 2**
As illustrated in Table 4.4, it can be seen from Figures 4.4 and 4.5 that the fish involved in PKD Challenge 2 are both longer and heavier than those involved in PKD Challenge 1, which is constant throughout the challenges (with the exception of a small crossover in Week 11 for weight, and Weeks 7 and 11 for length; none of which are significant – data not shown).

![Figure 4.5 - Mean fork length (cm) for rainbow trout used in PKD Challenge 1 and PKD Challenge 2](image)

**Figure 4.5 - Mean fork length (cm) for rainbow trout used in PKD Challenge 1 and PKD Challenge 2**

Phenotypic correlations between the three traits were calculated, with an expected high and positive trend between fork length and body weight ($r=0.962$, $P<0.001$), and low and negative trends between either production trait and kidney score. Correlation between fork length and kidney score was significant; $r=-0.217$, $P=0.039$. The phenotypic trend of larger size and lower kidney score is shown in Table 4.5. Both the average fork length and average body weight of rainbow trout measured display the trend, with the most severe kidney swelling (kidney score: 4) displayed in fish at an average length of 11.95 cm and average weight of 23.67 g, whilst the least severely affected fish (kidney score: 0) are larger at an average fork length of 13.38 cm, and average body weight of 30.14 g.

<table>
<thead>
<tr>
<th>Kidney score</th>
<th>Fork length</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.38 ± 0.12</td>
<td>30.14 ± 0.85</td>
</tr>
<tr>
<td>1</td>
<td>12.89 ± 0.09</td>
<td>27.29 ± 0.53</td>
</tr>
<tr>
<td>2</td>
<td>12.91 ± 0.09</td>
<td>27.78 ± 0.55</td>
</tr>
<tr>
<td>3</td>
<td>12.30 ± 0.13</td>
<td>24.48 ± 0.77</td>
</tr>
<tr>
<td>4</td>
<td>11.95 ± 0.18</td>
<td>23.67 ± 1.10</td>
</tr>
</tbody>
</table>

*Table 4.5 - Mean fork length (cm) and body weight (g) of fish assessed in the PKD Challenges using the scale of Clifton-Hadley et al. (1987)*
Throughout the challenge period, the overall mean measurements of kidney score (n=1050; 1032 of known pedigree, 18 unknown) were recorded for each challenge by week; illustrated in Figure 4.6, below. The kidney scores of fish sampled in PKD Challenge 1 are consistently greater than those of PKD Challenge 2 throughout the entire study period. The two datasets follow a similar trend with a sharp increase between weeks 3 and 6 (the greatest duration between sampling weeks), before peaking at week 7 in PKD Challenge 1, and over a three week period in PKD Challenge 2 (weeks 7, 8 and 9), before a reducing trend occurs in the last weeks of the disease. The kidney scores of mortalities from PKD Challenge 1 were recorded where possible, and having been segregated to fit within sampling periods, are also illustrated in Figure 4.6. The mean kidney scores of mortalities at each sampling period are constantly greater than those of fish sampled, but the low number of observations leaves the statistical significance questionable.

Figure 4.6 - Mean kidney score for rainbow trout at each sampling week ± SE for PKD Challenge 1 (n=556), PKD Challenge 2 (n=476), and mortalities of PKD Challenge 1 (n=53; NB Week 10 mortality is a single observation; standard error could not be calculated)

Overall, mortality during the challenges was relatively minimal at 5.30%. PKD Challenge 1 displayed the greater mortality, 53 fish from 627 died (8.45%); of those possible to examine, 64.15% displayed signs of PKD, whilst in PKD Challenge 2, only 3 fish died over the course of the trial (0.61%), none of which exhibited signs
of PKD. The number of deaths that occurred varied between 11 and 22 in the three replicates of Challenge 1, with a minimum dpi to death of 9 and maximum of 69. In PKD Challenge 2, the 3 mortalities died at 17, 19, and 20 dpi, one from each replicate. The number and time at which death occurred for each challenge, and by replicate, are shown in Table 4.6. The number of mortalities within each family selected for the study is illustrated in Figure 4.7. Colour-coordinated according to high and low EBV values, the greatest mortality in any family with the female parent having a low EBV was 11 (ARF 6), whilst from a female parent having a high EBV was 6 (ARF 7). Conversely, the lowest mortality of 1 fish was shared among 7 families, 4 having a female parent of low EBV, the remainder high.

### Table 4.6 - Summary of mortality, in days post injection to death, in PKD Challenges

<table>
<thead>
<tr>
<th></th>
<th>Tank 1</th>
<th>Tank 2</th>
<th>Tank 3</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKD 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>10</td>
<td>28</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Maximum</td>
<td>69</td>
<td>55</td>
<td>55</td>
<td>69</td>
</tr>
<tr>
<td>Mean</td>
<td>38.36</td>
<td>39.55</td>
<td>34.45</td>
<td>37.45</td>
</tr>
<tr>
<td>Count</td>
<td>11</td>
<td>22</td>
<td>20</td>
<td>53</td>
</tr>
<tr>
<td>PKD 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPI to death</td>
<td>17</td>
<td>19</td>
<td>20</td>
<td>mean: 18.67</td>
</tr>
</tbody>
</table>

**Figure 4.7 - The number of mortalities per family for both PKD Challenge 1 and 2.** The colour blue indicates families with parental female of low EBV to PKD, whilst those coloured in pink represent families with parental female of high EBV.
A Kaplan-Meier estimate of the survival function was conducted for the challenges overall; Figure 4.8, below. Although the mortality experienced was low overall, the greatest loss was observed between days 20 and 41, with the rate levelling until the trial terminated at 77 dpi, when the overall survival equalled 94.95%.

![Figure 4.8 - Kaplan-Meier estimate of the survival function (Overall) for the duration of the two challenges (77 days) of rainbow trout with *Tetracapsuloides bryosalmonae*; − Survivor function, □ Censored observations](image)

Family weights between the two groups (ARF and BRF) at the beginning of the trial and water temperatures between the two challenges differed significantly (P<0.001). The summary of the water temperatures (including means ± their SE) for the challenges is displayed in Table 4.7, whilst the temperature profiles, showing a constant lower temperature in PKD Challenge 2, as well as the time at which mortality occurred in the challenges is shown in Figure 4.9.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>PKD Challenge 1</th>
<th>PKD Challenge 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>15.00</td>
<td>12.50</td>
</tr>
<tr>
<td>Maximum</td>
<td>19.00</td>
<td>16.00</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>16.59 ± 0.10</td>
<td>14.21 ± 0.08</td>
</tr>
</tbody>
</table>
As a binary trait, there was no significant difference in the survival between the challenges, and the EBV showed no significant effect. However, a likelihood ratio test demonstrated a significant dam effect when analysis was conducted overall. Analysis of PKD Challenge 1 alone, followed the same trend, but PKD Challenge 2 showed no parental effect following the likelihood ratio test.

The genetic components were extremely variable between the two challenges, but also between each sampling week. From sires, the genetic component for PKD score varied from a bound estimate to 0.359 over the two challenges, whilst the greatest component from females came from PKD Challenge 1 in week 7 (0.390), the lowest being bound. Only six of the parental components were deemed to be significant following likelihood ratio tests, whilst the regression of kidney score on EBV was never found to be significant, although the majority did display a positive sign. All the genetic components related to the random terms, sire, dam, and tank effect, as well as all residual variance, whether EBV was significant, and the regression on EBV are illustrated in Table 4.8, below.
Table 4.8 - Summary of genetic components for PKD score from the PKD Challenges, by week

<table>
<thead>
<tr>
<th>Trial/Week</th>
<th>( \sigma^2 ) sire</th>
<th>( \sigma^2 ) dam</th>
<th>( \sigma^2 ) replicate</th>
<th>( \sigma^2 ) residual</th>
<th>Significant EBV</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>0 bound</td>
<td>0.049 ± 0.026</td>
<td>0.001 ± 0.005</td>
<td>0.106 ± 0.018</td>
<td>X</td>
<td>0.013 ± 0.187</td>
</tr>
<tr>
<td>1/6</td>
<td>0.032 ± 0.060</td>
<td>0.121 ± 0.031</td>
<td>0.014 ± 0.047</td>
<td>1.000 ± 0.166</td>
<td>X</td>
<td>-0.317 ± 0.325</td>
</tr>
<tr>
<td>1/7</td>
<td>0.061 ± 0.126</td>
<td>0.390 ± 0.229</td>
<td>2.056 ± 0.319</td>
<td>X</td>
<td>0.008 ± 0.545</td>
<td></td>
</tr>
<tr>
<td>1/8</td>
<td>0.027 ± 0.063</td>
<td>0 bound</td>
<td>0 bound</td>
<td>0.605 ± 0.430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/9</td>
<td>0.164 ± 0.260</td>
<td>0.088 ± 0.135</td>
<td>1.342 ± 0.222</td>
<td>X</td>
<td>0.345 ± 0.412</td>
<td></td>
</tr>
<tr>
<td>1/10</td>
<td>0.042 ± 0.090</td>
<td>0.166 ± 0.170</td>
<td>1.083 ± 0.200</td>
<td>X</td>
<td>0.501 ± 0.455</td>
<td></td>
</tr>
<tr>
<td>1/11</td>
<td>0.071 ± 0.133</td>
<td>0 bound</td>
<td>0.971 ± 0.232</td>
<td>X</td>
<td>0.719 ± 0.442</td>
<td></td>
</tr>
<tr>
<td>2/3</td>
<td>0 bound</td>
<td>0.016 ± 0.024</td>
<td>0.233 ± 0.041</td>
<td>X</td>
<td>0.300 ± 0.211</td>
<td></td>
</tr>
<tr>
<td>2/6</td>
<td>0.021 ± 0.045</td>
<td>0.012 ± 0.030</td>
<td>0.008 ± 0.021</td>
<td>X</td>
<td>0.194 ± 0.230</td>
<td></td>
</tr>
<tr>
<td>2/7</td>
<td>0.087 ± 0.136</td>
<td>0.009 ± 0.026</td>
<td>0.046 ± 0.058</td>
<td>X</td>
<td>-0.091 ± 0.218</td>
<td></td>
</tr>
<tr>
<td>2/8</td>
<td>0.109 ± 0.173</td>
<td>0 bound</td>
<td>0.104 ± 0.126</td>
<td>X</td>
<td>-0.139 ± 0.264</td>
<td></td>
</tr>
<tr>
<td>2/9</td>
<td>0.131 ± 0.263</td>
<td>0.165 ± 0.147</td>
<td>0.524 ± 0.091</td>
<td>X</td>
<td>0.141 ± 0.522</td>
<td></td>
</tr>
<tr>
<td>2/10</td>
<td>0.359 ± 0.647</td>
<td>0.348 ± 0.264</td>
<td>0.557 ± 0.433</td>
<td>X</td>
<td>0.482 ± 0.704</td>
<td></td>
</tr>
<tr>
<td>2/11</td>
<td>0.312 ± 0.536</td>
<td>0.125 ± 0.184</td>
<td>0.874 ± 0.211</td>
<td>X</td>
<td>0.182 ± 0.577</td>
<td></td>
</tr>
</tbody>
</table>

† denotes significant effect when tested using a likelihood ratio test; X denotes a negative result for significance of EBV.

The estimates of heritability for PKD score display a vast range, 0.05 to 1.25 from either parent. Due to limited variance in some instances, estimates were not possible, and due to the inequality of sire and dam components, combined estimates are not appropriate. From sires, the estimates range from 0.05 to 1.05, and from dams 0.09 to 1.25 for kidney score, whilst for the size traits, estimates ranged from 0.004 to 0.72 from the sire component, and 0.05 to 1.14 from the dam component. Where estimates obtained are greater than 1.00, the value has been reduced to the maximum theoretical value achievable. Estimates of heritability, from both the sire and dam component, for all three traits measured are displayed in Table 4.8, below.

Table 4.9 – Estimates of heritability ± SE (from both the sire and dam components) for each trait measured in the PKD Challenges - kidney score; KS, fork length (mm), and body weight (g)

<table>
<thead>
<tr>
<th>Trial/Week</th>
<th>( h^2 ), KS</th>
<th>( h^2 ), KS</th>
<th>( h^2 ), Length</th>
<th>( h^2 ), Length</th>
<th>( h^2 ), Weight</th>
<th>( h^2 ), Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>---</td>
<td>1.00</td>
<td>0.06 ± 0.14</td>
<td>0.05 ± 0.30</td>
<td>0.004 ± 0.05</td>
<td>---</td>
</tr>
<tr>
<td>1/6</td>
<td>0.21 ± 0.37</td>
<td>0.77 ± 0.46</td>
<td>0.16 ± 0.30</td>
<td>0.59 ± 0.43</td>
<td>0.13 ± 0.27</td>
<td>0.73 ± 0.46</td>
</tr>
<tr>
<td>1/7</td>
<td>0.17 ± 0.34</td>
<td>1.00</td>
<td>0.07 ± 0.14</td>
<td>---</td>
<td>0.27 ± 0.42</td>
<td>0.23 ± 0.34</td>
</tr>
<tr>
<td>1/8</td>
<td>0.05 ± 0.12</td>
<td>---</td>
<td>0.18 ± 0.29</td>
<td>---</td>
<td>0.16 ± 0.27</td>
<td>0.12 ± 0.33</td>
</tr>
<tr>
<td>1/9</td>
<td>0.40 ± 0.57</td>
<td>0.22 ± 0.33</td>
<td>0.11 ± 0.20</td>
<td>---</td>
<td>0.08 ± 0.18</td>
<td>0.31 ± 0.37</td>
</tr>
<tr>
<td>1/10</td>
<td>0.13 ± 0.27</td>
<td>0.51 ± 0.49</td>
<td>0.05 ± 0.18</td>
<td>---</td>
<td>---</td>
<td>0.24 ± 0.42</td>
</tr>
<tr>
<td>1/11</td>
<td>0.27 ± 0.48</td>
<td>---</td>
<td>0.004 ± 0.25</td>
<td>1.00</td>
<td>---</td>
<td>1.00</td>
</tr>
<tr>
<td>2/3</td>
<td>---</td>
<td>0.25 ± 0.38</td>
<td>0.56 ± 0.83</td>
<td>0.14 ± 0.32</td>
<td>0.72 ± 0.94</td>
<td>---</td>
</tr>
<tr>
<td>2/6</td>
<td>0.25 ± 0.51</td>
<td>0.14 ± 0.35</td>
<td>1.00</td>
<td>0.14 ± 0.68</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2/7</td>
<td>0.93 ± 1.13</td>
<td>0.09 ± 0.27</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2/8</td>
<td>0.72 ± 0.91</td>
<td>---</td>
<td>0.74 ± 0.54</td>
<td>---</td>
<td>0.48 ± 0.46</td>
<td>---</td>
</tr>
<tr>
<td>2/9</td>
<td>0.59 ± 1.03</td>
<td>0.74 ± 0.62</td>
<td>0.14 ± 0.77</td>
<td>1.00</td>
<td>0.19 ± 0.78</td>
<td>1.00</td>
</tr>
<tr>
<td>2/10</td>
<td>1.00</td>
<td>1.00</td>
<td>0.19 ± 0.48</td>
<td>0.30 ± 0.43</td>
<td>0.51 ± 0.78</td>
<td>0.10 ± 0.31</td>
</tr>
<tr>
<td>2/11</td>
<td>1.00</td>
<td>1.00</td>
<td>0.24 ± 0.53</td>
<td>---</td>
<td>0.24 ± 0.53</td>
<td>---</td>
</tr>
</tbody>
</table>

--- denotes a 'bound' estimate due to limited or no variation displayed in the character by one of the parents; 1.00 denotes an estimate attained above a value of 1 - value reduced to maximum theoretical value achievable; \( s \) and \( d \) are the estimates calculated from the sire and dam component, respectively.
Following observation of the kidney sections taken, 755 of the 1032 (73.16%) fish displayed *T. bryosalmonae*; stained sporogonic and extrasporogonic stages of *T. bryosalmonae* following immunohistochemistry of a kidney section are shown in Figure 4.10, below.

![Figure 4.10 - Sporogonic and extrasporogonic stages of *Tetracapsuloides bryosalmonae* within a kidney section (stained brown in colour) following immunohistochemistry using the anti-*T. bryosalmonae* monoclonal antibodies A8 and D41](image)

Overall, the mean number of parasites per mm$^2$ observed in immunohistochemistry stained kidney sections was 23.45, with PKD Challenge 1 fish displaying a mean of 26.66, and PKD Challenge 2 fish, 17.83 overall. The maximum number of parasites for any individual was 319.61 per mm$^2$ from an individual in PKD Challenge 2 (Week 8). Both the lowest and highest percentage of individuals with parasites observed was in PKD Challenge 1, with 92.22% of the fish displaying *T. bryosalmonae* in Week 6, and only 55.68% of fish displaying parasites in Week 11. Overall, parasites were present in 71.22% of the fish in PKD Challenge 1, and in 75.42% of the fish in PKD Challenge 2. In relation to kidney score, there was a significant and strong positive correlation between the two terms, $r=0.607$, $P<0.001$. 
The trend of increasing parasites/mm$^2$ in relation to kidney score is shown in Figure 4.11. At each increasing kidney score the number of parasites/mm$^2$ increases also. Although a linear regression model should not be fitted to ordered categorical data, for the purpose of illustration, the $R^2$ value here gives an indication as to the correlation calculated as statistically significant.

![Figure 4.11 – Parasites/mm$^2$ (*Tetracapsuloides bryosalmonae*) observed at each assigned kidney score; numbers displayed relate to the mean parasite/mm$^2$ (pink line) and number of observations (n) at each kidney score](image)

The genetic components in relation to parasites per mm$^2$ were variable both within and between the two challenges. In relation to the sire and dam, components range from bound estimates to 748 in PKD Challenge 1, and from bound to 441 in PKD Challenge 2. The significance of the regression of parasites per mm$^2$ on EBV is also illustrated, with four of the fourteen analyses proving to be significant; the majority of the signs being negative. The genetic components related to the random terms, sire, dam, and tank effect, as well as the associated residual variance are presented in Table 4.10, below. The significance of EBV as well as the regression of parasite count per mm$^2$ on EBV are also illustrated.
Table 4.10 – Summary of genetic components relating to parasite number per mm² from the PKD Challenges, by week

<table>
<thead>
<tr>
<th>Trial/Week</th>
<th>σ² sire</th>
<th>σ² dam</th>
<th>σ² replicate</th>
<th>σ² residual</th>
<th>Significant EBV</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>1.69 ± 3.01</td>
<td>0 bound</td>
<td>2.97 ± 4.67</td>
<td>49.04 ± 7.70</td>
<td>✓</td>
<td>-6.928 ± 2.157</td>
</tr>
<tr>
<td>1/6</td>
<td>84.0 ± 148</td>
<td>179 ± 166</td>
<td>48.0 ± 92.0</td>
<td>1294 ± 216</td>
<td>X</td>
<td>13.360 ± 14.557</td>
</tr>
<tr>
<td>1/7</td>
<td>138 ± 241</td>
<td>375 ± 261†</td>
<td>136 ± 187</td>
<td>1533 ± 254</td>
<td>X</td>
<td>17.420 ± 18.347</td>
</tr>
<tr>
<td>1/8</td>
<td>10.0 ± 52.0</td>
<td>0 bound</td>
<td>0 bound</td>
<td>3127 ± 485</td>
<td>✓</td>
<td>36.790 ± 16.767</td>
</tr>
<tr>
<td>1/9</td>
<td>63.0 ± 122</td>
<td>75.0 ± 190</td>
<td>188 ± 263</td>
<td>2079 ± 356</td>
<td>X</td>
<td>21.390 ± 15.342</td>
</tr>
<tr>
<td>1/10</td>
<td>59.0 ± 165</td>
<td>748 ± 447†</td>
<td>52.0 ± 116</td>
<td>1503 ± 281</td>
<td>X</td>
<td>27.870 ± 23.875</td>
</tr>
<tr>
<td>1/11</td>
<td>0 bound</td>
<td>0 bound</td>
<td>0 bound</td>
<td>628.9 ± 153</td>
<td>X</td>
<td>13.070 ± 11.133</td>
</tr>
<tr>
<td>2/3</td>
<td>3.36 ± 3.52</td>
<td>5.16 ± 6.53†</td>
<td>32.66 ± 5.87</td>
<td>X</td>
<td>1.393 ± 2.543</td>
<td></td>
</tr>
<tr>
<td>2/6</td>
<td>3.50 ± 32.2</td>
<td>39.4 ± 48.5</td>
<td>33.8 ± 47.7</td>
<td>323.8 ± 59.1</td>
<td>X</td>
<td>0.039 ± 9.203</td>
</tr>
<tr>
<td>2/7</td>
<td>34.7 ± 56.9</td>
<td>0.40 ± 15.4</td>
<td>42.2 ± 50.9†</td>
<td>208.5 ± 37.5</td>
<td>X</td>
<td>-3.877 ± 5.311</td>
</tr>
<tr>
<td>2/8</td>
<td>281 ± 490</td>
<td>0 bound</td>
<td>67.0 ± 173</td>
<td>2498 ± 435</td>
<td>✓</td>
<td>-37.18 ± 18.194</td>
</tr>
<tr>
<td>2/9</td>
<td>441 ± 649†</td>
<td>0 bound</td>
<td>49.9 ± 79.5</td>
<td>708.0 ± 122.3</td>
<td>✓</td>
<td>-23.23 ± 9.765</td>
</tr>
<tr>
<td>2/10</td>
<td>348 ± 566</td>
<td>126 ± 141</td>
<td>0 bound</td>
<td>857.7 ± 151.6</td>
<td>X</td>
<td>-4.592 ± 16.304</td>
</tr>
<tr>
<td>2/11</td>
<td>84.6 ± 176</td>
<td>0 bound</td>
<td>0 bound</td>
<td>948.0 ± 212.0</td>
<td>X</td>
<td>-6.749 ± 13.978</td>
</tr>
</tbody>
</table>

† denotes significant effect when tested using a likelihood ratio test; X denotes a negative result for significance of EBV; ✓ denotes a positive result for significance of EBV
Discussion

Striving to test if significant differences were apparent between families for resistance to PKD, evidence of additive genetic variation, whether the regression of kidney score on EBV of selected females was significant, and calculating timeframes of families (that may assist in natural vaccination procedures), only additive genetic variation was detected and timecourses calculated. No family demonstrated a significantly higher tolerability to PKD regardless of the high or low EBV rankings, and any regressions calculated were not significant. Additive genetic variation was detected every week from at least one parent relating to PKD score, and in the majority of weeks for fork length and body weight. Estimates of heritability were calculated from both the sire and dam component, and show a very diverse range; the majority suggesting that moderate to strong genetic improvement to PKD resistance is possible within the families selected for this study. Due to biases caused from maternal effects, the sire component (and therefore its estimate of heritability) is thought to be more reliable (Falconer and Mackay, 1996; Heath et al., 1999; Kolstad, 2005). For this reason, all estimates of heritability discussed from this point onwards will be referring to those calculated from the sire component, unless otherwise stated. Finally, the timecourse of disease progression was calculated for every family involved in the challenges; a vast difference between family disease progression was observed. Such timeframes could potentially assist farmers in stocking regimes to minimise the effects of PKD, ie when and which families to stock in the production cycle.

Due to limitations of experimental facilities in the ARF at the Institute of Aquaculture, University of Stirling, the families sent from the IoM were reared at different sites; 15 ARF families, 8 BRF families. Although the fertilisation date differed between these two groups by nine days, approximately 90 dd, this could have been overlooked if comparisons were required. However, Dunham (2004) explains that differences in spawning time and ultimately age of experimental fish can result in such environmental deviations that the genetic effects within the population can be masked. Although considered, the age difference was believed to be of no great concern but exposure to very different environments and rearing
techniques, left numerous differences between the ARF and BRF families; combining their data would leave the results confounded. The greatest difference at the time of challenge was the statistically significant size variation between the two groups. Further, BRF fish encountered numerous pathogens at the commercial site prior to challenging, two of which (*Costia* and *Trichodina*) required treatment on multiple occasions. ARF fish were also affected and treated for *Costia*, but only on one occasion. Also, the BRF families suffered mortality, chronic in some instances, due to a ‘condition’ which left some families so depleted they were unable to be used in the study. As a result, the two groups were analysed separately. Another area of concern was tagging. The inexperience of using visual implant elasomere left many fish in PKD Challenge 1 unidentifiable to the family level. The groups most affected included ARF 2, ARF 6, and ARF 15; losses of up to 31% of the family information were experienced. Also, limitations of experimental tanks and fish number hindered the challenge design. The design used was deemed to be the most efficient way of gathering the data required to assess for PKD resistance and associated additive genetic variation. Finally, although kidney score was used as a measure of resistance here, other parameters have been identified as good indicators (assessed as correlated marker traits) of resistance to other diseases in fish species (Refstie, 1982; Røed *et al.*, 1990, 1992, 1993; Salte *et al.*, 1993; Fevolden *et al.*, 1994; Strømsheim *et al.*, 1994ab; Lund *et al.*, 1995). In PKD research it is understood that there is a good humoral antibody response to *T. bryosalmonae* by rainbow trout at approximately seven weeks post infection, which would suggest that serology could be a useful tool to help with measuring and understanding resistance levels of rainbow trout to PKD. Although the collection of serum was discussed in the present study, time limitations on sampling days meant serum was never collected. Future studies would benefit from serum sampling as well as Enzyme-Linked ImmunoSorbent Assays (ELISAs) being conducted in family research towards PKD resistance.

Having assessed the regression of kidney score on EBV of females selected for this study, none were found to be statistically significant. However, the majority of weeks display a positive sign for the regression of kidney score on EBV, supporting the hypothesis that PKD score is positively related to the EBV of the selected
females demonstrating high and/or low response to PKD. Further, as each sampling was considered a subsample of the population, and therefore independent each week (ie fish were slaughtered to assess kidney swelling and could therefore not be used as repeated measurements), there was justification for using a one-sided test to assess the significance of each regression. The one-sided test reduces the critical level that requires to be exceeded for the regression to be considered significantly different from zero; however, even after this analysis, none were found to be statistically significant, assessed either individually or when pooled (data/results not presented).

As the majority of weeks display a positive sign for regression of kidney score on EBV, the philosophy that EBV plays a role on kidney score cannot be dismissed, but, due to the limited power of this experiment, it is difficult to draw stringent conclusions. Further, the distance between the individuals assigned an EBV for this study, and the actual broodstock used to produce this population may have weakened any regressions; EBVs were calculated from the offspring of Chapter 3 and assigned to the respective parents, yet it was the siblings of the offspring involved in Chapter 3 that were used as broodstock in the present study. Future research in this area would benefit the British trout farming industry, as well as improve knowledge towards PKD. Further studies involving the regression of kidney score on EBV should use a greater number of individuals per family, a larger number of families, and also families that exhibit greater extremities in the high and low lines of susceptibility to PKD; these factors could potentially support and strengthen the stated hypothesis and positive relationship identified here.

The estimates of heritability calculated for resistance to PKD are so diverse that comparison to other disease resistance studies would be ineffective. Ranging from 0.05 to 1.25, the estimates cover almost all other estimates of disease resistance cited in the literature (Gjøen et al., 1997; Perry et al., 2004; Kolstad et al., 2005; Henryon et al., 2005). With no previous work conducted on the genetic component of PKD resistance (with the exception of Chapter 3 in the present project), no literature is available for comparison. However, the evidence that additive genetic variation is available at any stage during the timecourse of the disease and from either parent suggests that selection, and therefore genetic improvement is possible, regardless of the time selection takes place, disease progress, or parental component
No trends are apparent in relation to the estimates of heritability over the timecourse suggesting there is not an optimum week in which selection would yield a better genetic response from either group involved. Although some weeks demonstrate a genetic component above what could realistically be referred to as an estimate of heritability (ie 1.25), the realism is that additive genetic variation is present and selection for improved PKD resistance is possible, supporting the findings of Chapter 3. From the sire component, although a low estimate is apparent; 0.05 (PKD Challenge 1, week 8), all remaining estimates are moderate to strong implying selection for disease resistance measured by kidney score will provide positive results from the families involved here.

The fact that no differences were observed between high and low responding females, or between families, according to the term EBV in the statistical model, suggests that selection based on EBV will provide no additional benefit towards PKD resistance over other selection methods within the population tested here. However, the families displayed a range of average kidney scores from 0.64 to 2.46. Should at least some of the additive genetic variation be held within those families with lower average kidney scores, a greater improvement in PKD resistance than that estimated may be observed over selection generations. Variation was also expressed between the families for the timecourse relating to the development of PKD. Figure 4.3 illustrates the PKD score timecourse of a number of families used in the experiment; including all families on one graph would be somewhat confusing, hence a subsample of representatives have been selected to demonstrate the differences between groups. As previously mentioned, timecourses could give farmers the opportunity to stock particular families at different times in the production cycle to naturally minimise the effects of PKD, but the practicalities of implementing such a stocking regime on commercial farms must be addressed. Further, the significant negative correlation between fork length and kidney score again suggests that fish size plays a role in PKD resistance. In Chapter 3, a genetic correlation between size and kidney score was detected and discussed. It was suggested that individuals may have been affected at different times, with those affected earlier surpassing the epidemic, recovering and beginning to feed to
become larger. As all fish in the present study were infected at the same time, this could not be the case here, although size variation within or between families may still play a role. Secondly it was suggested that more resistant fish are less stressed and so continue to feed gaining weight throughout the disease, and being larger at the time of sampling. Although fed on maintenance ration throughout the challenges, this theory cannot be dismissed either, as hierarchical differences within the tank may have occurred so that larger fish dominate at feeding times increasing their size. Finally, it was suggested that larger fish may simply be less susceptible or take longer to reach the epidemic stage, possibly due to a parasite to kidney ratio. Unfortunately, these claims cannot be dismissed either, and further work towards fish size in relation to PKD resistance may benefit the industry, where sizes are practical enough to be useful.

The correlation between fork length and kidney score, although statistically significant, is weak and below what could be considered practical to use in commercial production. The difference between the extreme grades of kidney score and fork length is only 1.43 cm, which is not enough to utilise in commercial management practices. However, having shown on two separate occasions within this project that fish size is correlated to kidney score, should correlations be strong between production and physiological traits, by combining the family information (ie mean kidney score and timecourse) as well as fish size (either as fork length or body weight), additional benefits may be presented to the farmer; ie by stocking larger, and therefore more resistant fish and late ‘peakers’ early in the summer when PKD is most prolific, and smaller, less resistant fish and early ‘peakers’ later in the summer as water temperatures and PKD development decline - the benefit to the farmer is two-fold. However, in practice, commercial farms may not benefit from segregating families and/or fish of differing sizes due to the time and management required in doing so. The significant (albeit low) correlation between fork length and kidney score indicated here is based on phenotypic data. However, it is likely that the genetic correlation would mirror the phenotypic correlation fairly closely, as demonstrated in Chapter 3. To date, numerous authors have suggested that phenotypic correlations are generally indicative of genetic correlations, but only for pairs of production traits (Mousseau and Roff, 1987; Roff and Mousseau, 1987;
Little is known of the predictive value of phenotypic correlations in relation to genetic correlations for production and physiological traits (see Roff, 1997; Perry et al. 2004).

Where fork length and body weight demonstrated a continual and rising trend over time (Figures 4.4 and 4.5), PKD score illustrated a trend that peaked and troughed according to disease progression in each challenge. As expected, at 3 weeks post injection kidney scores were at their lowest as the parasite began to infiltrate the kidney (D’Silva et al., 1984; MacConnell et al., 1989). Over time, development increased. In PKD Challenge 1 kidney scores peaked at 7 weeks post injection (which appears not to be statistically different from weeks 6 and 8, according to the standard error), whilst in PKD Challenge 2 the highest overall kidney score was at week 9 (which appears not to be statistically different from weeks 7 and 8, according to the standard error). In PKD Challenge 1, following week 7, a general and overall decrease in the development of the disease occurred, demonstrated by the lowering average kidney scores, until the trial was terminated at week 11. In PKD Challenge 2, weeks 7, 8, and 9 combined are the peak in disease progress, before a reduction in kidney score is seen at week 10. The observed trends in both challenges are similar to those observed in other studies (D’Silva et al., 1984; Clifton-Hadley et al., 1985, 1986; MacConnell et al., 1989). Although a slight increase in kidney score was observed in week 11 over week 10 in both challenges, due to fewer individuals involved at week 11, increasing the standard error associated with the sampling, the two weeks are thought not to be statistically different. Overall, the two challenges follow a similar trend in relation to kidney score over time with PKD Challenge 2 consistently lower than PKD Challenge 1 at every time point. This is believed to be related (ultimately) to water temperature. Similarly, the reduced transmission in PKD Challenge 2, 73.61% compared to 94.44% in PKD Challenge 1, is believed to be a result of lower water temperatures. With the development of PKD known to be temperature related (Ferguson, 1981; Clifton-Hadley et al., 1986), the lower and significant difference in water temperature between challenge 1 and 2 inevitably created differences between the two trials. Although temperature alone could be the cause of the differences seen week on week, and in transmission, the possibly of husbandry stressors in earlier
life must not be dismissed. Varsamos et al. (2006) found temperature variation and husbandry-associated stress in early life rearing later reduced the tolerance of juvenile sea bass (*Dicentrarchus labrax*) to disease, whilst Saeij et al. (2003) demonstrated that handling stress in the early life stages led to much more susceptible carp later in life when infected with *Trypanoplasma borreli*. Such incidences suggest a negative impact. Potentially, a reverse effect may have occurred in the present study. Due to more severe stress in earlier life, BRF fish may have accustomed a greater tolerance to stress, leaving them more able to withstand PKD, but this is unlikely as a greater general susceptibility to stress was noted in the stock (see Section 4.2.4.2), supporting the findings of Varsamos et al. (2006) and Saeij et al. (2003). However, having been exposed to various parasitic infections on multiple occasions, a general immune response against *T. bryosalmonae* may have occurred. This has certainly been the case for re-exposure to the same parasitic infection (Gleeson et al., 2000; Karvonen et al., 2003, 2005; Cable and Van Oosterhout, 2007). It cannot be ruled out that temperature, husbandry, and previous exposure to disease had an effect on both the progress of PKD, and also the mortality experienced in the two challenges.

In PKD Challenge 2 mortality was negligible, whilst in PKD Challenge 1 mortality was much higher than anticipated. Numerous publications describe mortalities associated with PKD in salmonids (Ferguson and Needham, 1978; Ferguson, 1981; Ellis et al., 1982), but the actual cause of death is often confounded due to the presence of other pathogens (Ellis et al., 1982; Foott and Hedrick, 1987), previous exposure to the disease (Foott and Hedrick, 1987; Hedrick and Aronstien, 1987), management techniques (Ellis et al., 1982), or environmental stressors (Brown et al., 1991). To compare the mortality of the present study to those from field experiments is impracticable, and in the laboratory situation where fish are induced with PKD, having had no previous exposure to the disease, few records exist that report associated mortality. D’Silva et al. (1984) report mortality having conducted an 8 week study using naïve rainbow trout exposed to *T. bryosalmonae* using the same inducing method as used in the present study (ip injection with homogenates of PKD-infected kidney), and where temperatures were similar to those of the present study (18°C ± 2°C); the mortality experienced was much greater than that
found in PKD Challenge 1, 35.48% compared to 8.45%. Conversely, in an experiment by Morris et al. (2005), naïve rainbow trout induced via ip injection of PKD-infected kidney homogenate, and also held at 18°C ± 2°C, experienced no mortality over a 40 week period. Both experiments used fish of comparable age (0+) and size to those of the present study. Undoubtedly, the disease itself contributed to death, as the average kidney scores of mortalities are consistently higher than the average kidney scores of sampled fish (Figure 4.6), but PKD alone is thought not to be the sole reason for death. To compare mortality in different salmonid species is not practical, as the effect of PKD is species-dependent (Ferguson and Needham, 1978; Ellis et al., 1982; Hedrick and Aronstien, 1987; Brown et al., 1991). However, temperature has been discussed as a contributing factor where mortality has been associated with experimental T. bryosalmonae-related mortality (Ferguson, 1981; Clifton-Hadley et al., 1986a).

Since the two challenges were conducted in an identical manner in terms of holding unit and the sampling procedure, it is assumed that the ARF group were either more susceptible to stress, or the temperature profile in PKD Challenge 1 bordered, if not exceeded the upper critical level for this population of fish. As water temperature rises, the metabolic rate of fish increases (Fry, 1971). To accommodate the increase in metabolism, fish increase their oxygen consumption. However, with rising water temperature comes a depression in the solubility of oxygen in water, presenting an unfavourable and degenerative cycle to fish living within such conditions. During brief periods of high metabolic demand, the fish may be able to compensate by resorting to anaerobic metabolism, however Ojolick et al. (1995) warn that this may not be possible during chronic conditions of high metabolic demand, for example when disease is combined with high water temperatures, as was the case in PKD Challenge 1. Temperatures of 21°C ± 1°C have been described as chronically high for rainbow trout following a study of diploid individuals at 21°C for 3 weeks: 39% mortality was experienced (Ojolick et al., 1995). In the same study, it was noted that a marked increase in mortality occurred when water temperatures exceeded 18°C. This is in agreement with Myers and Hershberger (1991) who discuss mortality in rainbow trout at an averaged water temperature of 18.4°C. Mortalities have also been recorded in rainbow trout associated with a constant water
temperature as low as 17°C (Blanc et al., 1992). This suggests that there is no single optimum or critical temperature range for all rainbow trout, and that the tolerance of any population measured could differ due to water chemistry (e.g., oxygen or ammonia), previous trauma (e.g., disease, stress, or suboptimal conditions), or the genetics of the stock. With water temperature known to have a significant effect not only on production traits, but also on disease resistance (Dunham, 2004), several studies have concentrated on the potential of selectively breeding for increased temperature tolerance in rainbow trout (Ihssen, 1986; Linton et al., 1998; Molony et al., 2004), with Perry et al. (2005) discussing genetic differences for upper thermal tolerance between different populations within a single experiment. Molony et al. (2004) compared domesticated and naturalised lines of rainbow trout to find that domestication through passive selection left the stock more tolerant to water temperatures in excess of 25°C. In the present study, the IoM population have had no (known) previous selection pressure in relation to temperature tolerance. However, from the range experienced it is unlikely that temperature alone was the cause of mortality in the PKD challenges, and elevated water temperature, handling stress, overcrowding, and hierarchical dominance, or a combination thereof, may have contributed to the mortality witnessed.

A kidney score was allocated to every fish sampled for PKD resistance, according to the scale of Clifton-Hadley et al. (1987). Over the eleven week period, each fish had a section of kidney removed which was used for immunohistochemistry. In order to numerate the infection level, the extrasporogonic and sporogonic stages of *T. bryosalmonae* were stained using anti-*T. bryosalmonae* antibodies, A8 and D41. A significant positive correlation between kidney score and parasite load was found, suggesting that it is feasible to assume that the scale of Clifton-Hadley et al. (1987) is representative of the infiltration of parasites to the kidney, which may also be defined as a method of calculating resistance. Although high (*r*=0.607), the correlation is not as strong as expected. This could be due to a number of factors. Firstly, the kidney score assigned is extremely subjective. Although the grading system of Clifton-Hadley et al. (1987) is detailed and comprehensive, there are only five possible categories in which to position a swollen kidney. In the case of parasite load, the numbers could show an extensive range; in this study alone counts
of one, up to five hundred and fifty nine were observed over the eleven fields of view. The correlation could be stronger if more categories were added to the Clifton-Hadley et al. (1987) scoring system, but as a long standing and simple approach, this is unlikely to change. Other areas that could affect the correlation include the methodology in preparing sections, ranging from the quality of section cut to the successful staining of parasites, as well as the choice of antibodies and their efficacy (which could also vary dependent on culture and storage conditions). It was noted in the present study that the majority of procedures (including cutting and staining) improved over time as competence increased; that is not to say earlier samples are less reliable, simply harder to observe. The storage and culture of the cells producing the antibodies should be conducted so that variation is minimised. In the present study, cultures were prepared in bulk before being aliquoted into manageable volumes, and storage was equal both within and between the antibody-producing hybridoma cell lines.

Although every attempt was made to ensure that a similar transect of kidney was used for immunohistochemistry from each fish, it was inevitable that some variation would occur. It was noticed when counting parasite numbers that sections with a greater density of kidney tubules had lower parasite numbers; the majority of sections densely packed with tubules produced zero to less than ten parasites over the eleven areas studied (personal observation). Clifton-Hadley et al. (1987) reported a similar observation following a histological study of 0+ rainbow trout on a PKD infected farm. The fact that *T. bryosalmonae* destroy kidney tubules, suggests that disease progression to a chronic state will inevitably increase the number of parasites, and therefore the rate at which tubules are destroyed, explaining the number of parasites to tubule ratio in both this and the study of Clifton-Hadley et al. (1987). However, as kidney tubules condense towards the posterior of the kidney, the variation seen in the present study may be due to the area of kidney taken for immunohistochemistry. However, the theory that fish possessing a greater density of tubules within the kidney may be more tolerant to the infiltration of *T. bryosalmonae* must not be overlooked. The implications of such a correlation could provide numerous advantages to the industry and research. Resistance could be selected based on tubule number rather than kidney score or
parasite count, reducing the need for artificial challenging or collating data from natural outbreaks. The techniques required to count tubules are more affordable and simpler than for immunohistochemistry, especially as the requirement for antibodies would be removed. Further, farmers could use production fish at harvest to gather the required information, saving both time and money on trials and/or sacrificing healthy or young fish. Further research to support or dismiss these claims would benefit the industry.

In order to fully understand if kidney score is a good indicator of parasite load, and in turn resistance, further research is required in this area. The present study has provided a firm basis on which to develop future work, suggesting that kidney score is a good measure of parasite infestation, and in turn a representative measure of resistance to PKD. However, with areas requiring further attention in parasite counting, strict guidelines need to be developed, standardised, and adopted in relation to the section of kidney taken for immunohistochemistry, the protocols used in the cutting and staining procedure, as well as the number of tubules within the kidney section taken into account.

Finally, when parasites/mm$^2$ was assessed for the regression on female EBV, the result was extremely variable; this is further identified by the graphical display of parasites at each kidney score (Figure 4.11). Most notably, the sign for regressions in the majority of weeks throughout PKD Challenge 2 is negative. As kidney score was hypothesised to relate significantly to the number of parasites in the immunohistochemistry stained kidney sections taken for each individual (which was proved with some degree of success in this study), it is perhaps surprising that a similar result to kidney score regression on EBV was not found in PKD Challenge 2, ie the majority of regressions being positive. Further, of all the regressions of parasite/mm$^2$ on EBV calculated, unlike kidney score, four were deemed to be statistically significant; three of which demonstrated a negative value. However, the regressions in PKD Challenge 1 are in agreement with the philosophy that parasites/mm$^2$ has a positive relationship with the EBV of females, and in turn, the relationship between kidney score and parasites/mm$^2$ is strengthened. Although the regression in week 3 in PKD Challenge 1 is negatively significant, it is important to
note that the other significant regression is positive in week 8. In PKD Challenge 2, the significant regressions are in weeks 8 and 9, and although negative in PKD Challenge 2, this timing is believed to be an important stage in PKD progression, and the aetiology of this disease during this time should be focussed upon in future research. With the mean kidney scores in both trials peaking at or around weeks 7 to 9, and the timecourse of individual families showing vast variation seven weeks post infection, as well as the humoral antibody response to *T. bryosalmonae* at approximately seven weeks post infection already identified, it is obvious that this stage of infection may hold the key to understanding the resistance of rainbow trout to PKD.

Having identified additive genetic variation for PKD score exists within the IoM population (Chapter 3), the present chapter was established and proved to support these findings. Although families of high and low tolerance to the disease were selected for the study, no significant difference occurred between the families, or between the groups held at different locations; ARF and BRF. However, additive genetic variation leading to significant estimates of heritability was calculated from both the sire and dam components in the study, suggesting selection for improved resistance to PKD is possible within the families used here. Additionally, the variation in average PKD scores of each family indicates that selection may be improved above that predicted by selecting families with lower scores, but only if the additive genetic variation calculated is held within those families. The numerous positive regressions of kidney score on EBV, although not significant, indicate that future research may support the theory that broodstock of high and low response to PKD relates significantly to kidney score. The timeframes calculated for each family illustrates the variation of the effect of PKD for each group. The demonstration of peaks and troughs of PKD score at different times may provide a useful tool for farmers seeking to minimise the effects of PKD through management practices, ie stocking regimes assisting with natural vaccination. Finally, the significant and positive correlation found between PKD score and parasite number within kidney sections suggests that scoring according to the scale of Clifton-Hadley *et al.* (1987) is representative of the infiltration of parasites to the kidney throughout the course of the disease, reassuring that kidney score is a good measure
of PKD resistance. However, with many areas questionable as to the reliability of the counting, no strict conclusions can be drawn here, and it is suggested that further research is conducted. Overall, this study has supported the claims that additive genetic variation exists within the IoM population that would respond well to selection for improvement to PKD resistance. This strengthens the initial findings, and provides further evidence that a selective breeding programme for PKD resistance in the British rainbow trout industry is not only possible, but also feasible.
Chapter 5: Furunculosis Challenges
5.1 Introduction

Scientists at the Institute of Aquaculture, University of Stirling, consistently report the presence of *Aeromonas salmonicida* in fish affected by *Tetracapsuloides bryosalmonae* in the field situation or in naïve fish experimentally induced from field affected fish. Although no literature is available in support of this claim, acting as an opportunistic pathogen following the infection of *T. bryosalmonae*, it is perhaps unsurprising that *A. salmonicida* takes advantage of immunocompromised species, suggesting Proliferative Kidney Disease (PKD) acts as a precursor for the bacterial disease. In PKD challenges where naïve fish have been injected with the kidney homogenate of fish infected with *T. bryosalmonae* from a commercial site, evidence of furunculosis is often recorded in the experimental fish. However, the symptoms of the bacterial disease are rarely witnessed in the fish used to transmit PKD, suggesting that infection of PKD often leads to a carrier state of *A. salmonicida* in rainbow trout (Morris *et al.*, 2003a and subsequent unpublished laboratory work). To date, numerous pathogens have been reported at field sites affected by PKD (Hoffman and Dangschat, 1981; Hedrick *et al.*, 1985; O’Hara, 1985), but the presence of *A. salmonicida* as a secondary pathogen has been described at only one (Hoffman and Dangschat, 1981). In the majority of circumstances rainbow trout are considered the salmonid least affected by furunculosis, but under the extreme pressures of PKD, especially in the culture environment, an opportunity is presented to overcome the host.

Controlling furunculosis has historically been problematic. Although some evidence of treatment through the use of therapeutic antimicrobials has been possible, the evolution of drug resistant strains has and continues to hinder the salmonid industry (Munro and Hasting, 1993). Advances in fish vaccine design have been made in recent years (Munn, 1994; Lutwyche *et al.*, 1995; Siwicki *et al.*, 2002) and oil-adjuvated vaccines against furunculosis have produced encouraging results in the field (Press and Lillehaug, 1995; Midtyling *et al.*, 1996). Although numerous companies offer vaccines to control the disease (Aquavetplan, 2001; Schering-Plough, 2007), there continues to be increased interest in selective breeding to produce fish stocks tolerant to furunculosis; as a result substantial work
has been conducted in this area (Marsden et al., 1996; Gjøen et al., 1997; Nordmo et al., 1998; Perry et al., 2004). Selection for furunculosis tolerant strains on farms endemic to PKD will help to alleviate at least one of the associated stressors contributing to mortality. Where genetic correlations between PKD and furunculosis resistance are found to be favourable, a suitable selection index can be designed to utilise families with the greatest resistance to both diseases. Conversely, the discovery of a negative relationship, most likely due to differing aetiologies of the diseases, as found by Henryon et al. (2005) when studying the bacterial diseases Enteric Red Mouth (ERM) and Rainbow Trout Fry Syndrome (RTFS), and Viral Haemorrhagic Septicaemia (VHS), means alternative approaches may be necessary, eg sequential selection.

The object of the present study is to test the tolerance of the Isle of Man (IoM) commercial families to furunculosis under experimental conditions. By estimating the degree of genetic variation that exists in these commercial families following exposure to the causative agent, the subsequent data will indicate as to whether genetic improvement to furunculosis resistance is possible. The estimates of heritability calculated can then be incorporated into the previous results attained for PKD resistance. With information gathered for both diseases, data will be available in anticipation of a forthcoming breeding programme for the farms involved in this project.
5.2 Materials and Methods

5.2.1 Broodstock and mating design

The same families of fish used in the PKD challenges were used for the furunculosis challenges in order to assess and compare the resistance between the two commercially important diseases. The broodstock, mating design, rearing, and tagging are therefore identical to those seen in Chapter 4, Section 4.2. The only exception is the absence of ARF 4 in the furunculosis challenge due to a limited number of representatives available.

5.2.2 Challenges

5.2.2.1 Screening

Prior to any challenge taking place, representatives from each family were screened for the presence of *A. salmonicida* using the same equation described in Chapter 4, Section 4.2.4.1. However, sample size required was divided by 14 ARF families, not 15 as described in Chapter 4. Again, the low prevalence (0.01) was used due to both batches being certified disease-free and no history of furunculosis at the two holding sites. All fish tested negative for furunculosis. The techniques used to screen fish mirrored those of the actual challenges described below.

5.2.2.2 Design

Both furunculosis challenges were conducted at the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling due to its Home Office licensed status. The challenges were completed consecutively and design remained the same so comparisons could be made. The challenge involved four test tanks and four control tanks, each assigned at random. Each tank was 100 l in volume, with flows of ≥2 l/min plus aeration. Light regime was standard to the ARF; 12 h light:12 h dark. Feed was not given. Water temperature remained at or above 15°C (± 2°C) in both challenges. Movement to the test tanks took place 5 days prior to injection (17/08/2006) for Furunculosis Challenge 1, ARF families (22/08/2006; day 0), and 1 day prior to injection (19/09/2006) for Furunculosis Challenge 2, BRF...
families (20/09/2006; day 0). In Furunculosis Challenge 1, each replicate consisted of 10 fish per family, equating to 140 fish per tank (total: 1120). In Furunculosis Challenge 2, 20 fish per family were used, 160 fish per tank (total: 1280). Representatives were weighed into tanks to ensure no biases in replicate weight for each challenge.

5.2.2.3 **Bacterial preparation**

The Marine Harvest strain of *Aeromonas salmonicida* used for challenging fish was prepared following the Institute of Aquaculture, University of Stirling standard curve protocol (Appendix 1). The virility of the bacterial strain and unknown response of the population to be tested meant pre-challenge experiments were conducted to calculate the required dose to achieve 50% mortality. The 14 ARF families were used in pre-challenge experiments.

5.2.2.4 **Pre-challenges**

Performed in three replicates, 3 fish per family were ip injected with $1.15 \times 10^3$, $10^4$, and $10^5$ colony forming units (CFU), equating to 42 fish per replicate (a total of 126 fish). This was the most efficient way to calculate the desired dose rate without excessive use of fish, whilst maintaining a representative number per family. Mortality in each replicate exceeded the desired 50% mark with $1.15 \times 10^3$, $10^4$, and $10^5$ CFU producing 62%, 83%, and 90%, respectively, over a 9 day trial period. A further pre-challenge using two replicates was performed to assess dose rates of $5 \times 10^2$ and $1 \times 10^3$ CFU. Over the second nine day challenge period, mortalities of 45% and 55% were obtained, respectively, and $1 \times 10^3$ CFU was selected as the challenge dose.

5.2.2.5 **Actual Challenges**

The infection method used throughout challenges was ip injection. Test fish were subject to a 0.1 ml injection of $1 \times 10^4$ CFU of *A. salmonicida*-MH to achieve a dose rate of $1 \times 10^3$ CFU per fish, whilst control fish were treated identically but injected with 0.1 ml of 0.85% saline solution only. The day of injection was defined as day 0, and both trials lasted 21 days.
5.2.2.6 Observation and examination

Tanks were observed twice daily until the inception of death, when observation was increased to four times daily, in accordance with Home Office regulations. Dead and moribund fish were removed each day. Fish were identified to the family level, had their fork length recorded, and were examined bacteriologically for the presence of *A. salmonicida*. Anterior kidney smears were plated on TSA, and incubated for 48 hr prior to examination. After 48 hr, isolates of *A. salmonicida* were identified as typical colonies (morphological examination) producing brown diffusing pigment (pigmentation analysis). Each day, a random sample of at least 10% of the isolates were examined by Gram’s staining and agglutination testing; positive *A. salmonicida* showed Gram-negative coccibaccili bacteria, and reacted positively by agglutination test with the test serum BioNor AQUA Mono As (BioNor, Norway); for protocols see Chapter 2, Section 2.3.

5.2.3 Statistical analysis

5.2.3.1 Summary statistics

All data relating to the challenges were entered into Microsoft Excel in preparation of analysis. Where basic statistics (mean, count, standard error etc) were required Microsoft Excel was used. Some graphs are also products of Microsoft Excel. All other analysis was conducted using Genstat Release Version 9.1. Mortality of the fish following challenge is presented as the number of mortalities, number surviving, cumulative mortality within each full-sibling group, as well as per trial, and overall. The average length at time of death, and average number of days to death is also presented.

5.2.3.2 Furunculosis resistance, survival, and estimates of heritability

Resistance was assessed as both a binary trait (ie survived/died) and longitudinal trait (ie time until death following challenge). The statistical model used is described below. As a binary trait, resistance was assessed as a binary variable, Yi, where Yi is the observed value of fish *p* (yi*)*, allocated the value yi = 0 if death occurred during the 21 days following injection and yi = 1 if it survived. Fish that
survived were assumed to be more resistant than those that died. As a longitudinal trait, resistance of fish \( p \) was assessed as the time (days post injection; dpi) to death. Having aimed for 50% mortality rate, it was assumed that all fish surviving to day 21 would recover from \( A. \) salmonicida infection; these fish were assigned as censored observations. Measuring resistance as the time until death provides a measure of the degree of resistance; the longer it takes for a fish to die, the greater the resistance. The study also looked at the proportion of each family that died over the 21 day challenge period; this was thought to give a further indication as to the level of resistance within each family. Fish size was measured, but due to problems associated with measuring body weight in challenge trials where no feed is administered (ie cannibalism), fork length was taken as a more accurate measure.

The REML model allowed individuals or entire families to be assessed on their challenge performance. Further, estimates of heritability could be calculated from the resultant genetic components:

\[
y_{ihp} = \mu_i + b_i\text{EBV}_{ihp} + \text{trial}_{ih} + \text{sire}_{ik} + \text{dam}_{il} + \text{tank}_{im} + e_{ihp}
\]

where \( y_{ih} \) is the vector for the trait of interest \( (i) \) of fish \( p \). \( \mu_i \) is the population mean for trait \( i \), \( b_i\text{EBV}_{ihp} \) is the regression on the estimated breeding value of dam \( l \), treated as a fixed term, \( \text{trial}_{ih} \) is the fixed effect to assess any differences between ARF and BRF fish (ultimately Furunculosis Challenge 1 and 2), and \( \text{sire} k, \text{dam} l, \) and \( \text{tank} m \) are random effects for trait \( i \), whilst \( e_{ihp} \) represents the residual error associated with trait \( i \).

As per Chapter 4, the random effects of \( \text{sire}_{ik}, \text{dam}_{il}, \) and residual error \( (e_{ihp}) \) were considered to be independent random normal variables with mean zero, and variances denoted \( \sigma_s^2, \sigma_d^2, \) and \( \sigma_e^2 \). Total phenotypic variance was denoted \( \sigma_t^2 \), and was estimated as \( \sigma_t^2 = \sigma_s^2 + \sigma_d^2 + \sigma_e^2 \). The genetic information for this study comes from: (i) the dam variance \( (\sigma_d^2) \), which represents the variance of deviations from the regression on EBV; and, (ii) the sire variance \( (\sigma_s^2) \).
Estimates of heritability were based on the narrow sense heritability, and could be obtained from the sire variance using the formula:

\[ 4(\sigma_s^2) / \sigma_t^2, \text{ from the sire variance component } h_s^2 \]

Whilst the dam variance, in principle, contains other non-genetic maternal effects, in this study, given its small size, it was interpreted as being wholly genetic. The estimates of heritability were obtained from the dam variance using the formula:

\[ 4(\sigma_d^2) / \sigma_t^2, \text{ from the dam variance component } h_d^2 \]

However, it is important to note that part of the genetic variance attributable to dams was removed by the regression on EBV.

In order to determine if random variables were significant, they were assessed using a likelihood ratio test. Following the REML model, the term of interest (sire, dam, or tank) was removed and the resultant deviance compared to the original. The term was deemed significant if the difference was greater than \( \chi^2 \) at 1 degree of freedom (likelihood ratio test statistic at 95% CI: 3.84).

The rate at which fish died is also demonstrated using Kaplan-Meier estimates of the survival function for the 21 day challenges (Kaplan and Meier, 1958), where factors included trial, tank, and female, whilst variates included dpi, EBV, and survival.
5.2.3.3 Bulk weights

As in Chapter 4, the bulk weights of families weighed into test tanks were assessed for any significant differences. The REML model used to assess the bulk weight of families was:

\[ y_{ihq} = \mu_i + b_i \cdot \text{EBV}_{ihq} + \text{trial}_{ih} + \text{sire}_{ik} + \text{dam}_i + \text{tank}_{im} + e_{ihq} \]

where \( y_{ih} \) is the bulk weight of family \( q \) involved in the challenge, and \( \text{trial}_{ih} \) was included as a fixed effect to assess differences between ARF and BRF fish (i.e. Furunculusis Challenge 1 and 2).
5.3 Results

5.3.1 Furunculosis resistance

Overall the challenges produced a mortality rate close to the anticipated (50%), 52.04%; Furunculosis Challenge 1, 45.74%, and Furunculosis Challenge 2, 57.37%.

Of the 1204 test individuals, 1201 were available for testing, but only 1178 could be identified to the family level. For these individuals, mean dpi to death was 4.57 ± 0.06 overall, with a range of 3.61 ± 0.10 (BRF 10) to 5.60 ± 0.31 (ARF 3) within full-sibling groups. Cumulative mortality expressed a range from 22.50% (ARF 5 and ARF 6) to 96.25% (BRF 10) within groups; mean 48.86%. Fish unidentifiable to the family level were included in analyses where possible, ie Kaplan-Meier estimates overall and by challenge. The data collated in the challenges is summarised according to family and overall in Table 5.1, below.

Table 5.1 - Summary of furunculosis challenge data collected over a 21 day period for rainbow trout of different families

<table>
<thead>
<tr>
<th>Family</th>
<th>Mortalities</th>
<th>Survivors</th>
<th>Cum. Mort %</th>
<th>Morts %</th>
<th>Mean dpi ± SE</th>
<th>Length ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF 1</td>
<td>15</td>
<td>26</td>
<td>37.5</td>
<td>36.59</td>
<td>4.60 ± 0.32</td>
<td>11.91 ± 0.22</td>
</tr>
<tr>
<td>ARF 2</td>
<td>21</td>
<td>17</td>
<td>52.5</td>
<td>55.26</td>
<td>5.00 ± 0.34</td>
<td>11.69 ± 0.19</td>
</tr>
<tr>
<td>ARF 3</td>
<td>10</td>
<td>30</td>
<td>25</td>
<td>25.00</td>
<td>5.60 ± 0.31</td>
<td>12.64 ± 0.63</td>
</tr>
<tr>
<td>ARF 5</td>
<td>9</td>
<td>31</td>
<td>22.5</td>
<td>22.50</td>
<td>5.11 ± 0.20</td>
<td>11.96 ± 0.23</td>
</tr>
<tr>
<td>ARF 6</td>
<td>9</td>
<td>32</td>
<td>22.5</td>
<td>21.95</td>
<td>5.00 ± 0.33</td>
<td>11.64 ± 0.48</td>
</tr>
<tr>
<td>ARF 7</td>
<td>14</td>
<td>25</td>
<td>35</td>
<td>35.90</td>
<td>5.50 ± 0.90</td>
<td>11.45 ± 0.23</td>
</tr>
<tr>
<td>ARF 8</td>
<td>22</td>
<td>18</td>
<td>55</td>
<td>55.00</td>
<td>4.32 ± 0.23</td>
<td>11.75 ± 0.48</td>
</tr>
<tr>
<td>ARF 9</td>
<td>16</td>
<td>24</td>
<td>40</td>
<td>40.00</td>
<td>4.69 ± 0.30</td>
<td>10.92 ± 0.23</td>
</tr>
<tr>
<td>ARF 10</td>
<td>24</td>
<td>16</td>
<td>60</td>
<td>60.00</td>
<td>4.71 ± 0.39</td>
<td>11.66 ± 0.29</td>
</tr>
<tr>
<td>ARF 12</td>
<td>19</td>
<td>17</td>
<td>47.5</td>
<td>52.78</td>
<td>5.47 ± 0.65</td>
<td>11.25 ± 0.27</td>
</tr>
<tr>
<td>ARF 13</td>
<td>29</td>
<td>10</td>
<td>72.5</td>
<td>74.36</td>
<td>4.66 ± 0.31</td>
<td>11.51 ± 0.18</td>
</tr>
<tr>
<td>ARF 14</td>
<td>21</td>
<td>18</td>
<td>52.5</td>
<td>53.85</td>
<td>4.71 ± 0.23</td>
<td>11.44 ± 0.30</td>
</tr>
<tr>
<td>ARF 15</td>
<td>14</td>
<td>18</td>
<td>35</td>
<td>43.75</td>
<td>4.79 ± 0.52</td>
<td>11.42 ± 0.14</td>
</tr>
<tr>
<td>ARF 16</td>
<td>24</td>
<td>11</td>
<td>60</td>
<td>68.57</td>
<td>4.67 ± 0.38</td>
<td>11.05 ± 0.17</td>
</tr>
<tr>
<td>BRF 2</td>
<td>44</td>
<td>36</td>
<td>55</td>
<td>55.00</td>
<td>4.70 ± 0.23</td>
<td>10.75 ± 0.16</td>
</tr>
<tr>
<td>BRF 3</td>
<td>41</td>
<td>39</td>
<td>51.25</td>
<td>51.25</td>
<td>4.88 ± 0.23</td>
<td>10.52 ± 0.21</td>
</tr>
<tr>
<td>BRF 4</td>
<td>45</td>
<td>35</td>
<td>56.25</td>
<td>56.25</td>
<td>4.33 ± 0.16</td>
<td>10.13 ± 0.13</td>
</tr>
<tr>
<td>BRF 5</td>
<td>32</td>
<td>48</td>
<td>40</td>
<td>40.00</td>
<td>4.50 ± 0.30</td>
<td>10.16 ± 0.16</td>
</tr>
<tr>
<td>BRF 6</td>
<td>31</td>
<td>49</td>
<td>38.75</td>
<td>38.75</td>
<td>4.61 ± 0.19</td>
<td>9.65 ± 0.21</td>
</tr>
<tr>
<td>BRF 8*</td>
<td>33</td>
<td>47</td>
<td>41.25</td>
<td>41.25</td>
<td>4.82 ± 0.40</td>
<td>9.75 ± 0.37</td>
</tr>
<tr>
<td>BRF 9</td>
<td>63</td>
<td>17</td>
<td>78.75</td>
<td>78.75</td>
<td>4.43 ± 0.13</td>
<td>10.73 ± 0.10</td>
</tr>
<tr>
<td>BRF 10*</td>
<td>77</td>
<td>1</td>
<td>96.25</td>
<td>98.72</td>
<td>3.61 ± 0.10</td>
<td>10.39 ± 0.11</td>
</tr>
</tbody>
</table>

Challenge 1 families 247 293 45.74 4.85 ± 0.11 11.54 ± 0.07
Challenge 2 families 366 272 57.37 4.39 ± 0.07 10.33 ± 0.06
ALL families 613 565 52.04 4.57 ± 0.06 10.82 ± 0.05
Unknown ARF 8 13
Unknown BRF 0 0

* Non-furunculosis deaths 2
The rate at which fish died was highest from days 3 to 5 over both challenges following injection with *A. salmonicida*. The Kaplan-Meier estimate of the survival function indicates that over 10% of fish challenged with *A. salmonicida* died on these days when data was combined, with the greatest mortality on day 4: 20.57%. Very few fish died after day 7. The rate at which fish died over the challenge period is illustrated in Figure 5.1.

![Figure 5.1 - Kaplan-Meier estimate of the survival function (Overall) for 21 days following challenge of rainbow trout with *Aeromonas salmonicida*; − Survivor function, □ Censored observations](image)

The Kaplan-Meier account of mortality over the 21 day period (illustrated in Table 5.2, below) details the time and number of deaths of fish involved in the study. It illustrates the initial number of fish as ‘No. at risk’, which reduces accordingly as fish succumb to the injected *A. salmonicida*. The Kaplan-Meier estimates, as well as the respective upper and lower estimates, are given to three decimal places on each day that mortality occurred during the challenge period.
Table 5.2 - Kaplan-Meier output for the two furunculosis challenges combined

<table>
<thead>
<tr>
<th>Time of Death</th>
<th>No. of deaths</th>
<th>No. at risk</th>
<th>Lower estimate Kaplan-Meier</th>
<th>Upper estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>1201</td>
<td>0.993</td>
<td>0.998</td>
</tr>
<tr>
<td>3</td>
<td>123</td>
<td>1199</td>
<td>0.877</td>
<td>0.896</td>
</tr>
<tr>
<td>4</td>
<td>247</td>
<td>1076</td>
<td>0.663</td>
<td>0.690</td>
</tr>
<tr>
<td>5</td>
<td>137</td>
<td>829</td>
<td>0.548</td>
<td>0.576</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>692</td>
<td>0.496</td>
<td>0.525</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
<td>630</td>
<td>0.473</td>
<td>0.502</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>603</td>
<td>0.468</td>
<td>0.497</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>597</td>
<td>0.462</td>
<td>0.490</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>589</td>
<td>0.459</td>
<td>0.487</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>585</td>
<td>0.458</td>
<td>0.486</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>584</td>
<td>0.456</td>
<td>0.485</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>582</td>
<td>0.455</td>
<td>0.484</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>581</td>
<td>0.454</td>
<td>0.483</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>580</td>
<td>0.454</td>
<td>0.482</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>579</td>
<td>0.453</td>
<td>0.481</td>
</tr>
<tr>
<td>Total</td>
<td>Failed</td>
<td>Censored</td>
<td>% Censored</td>
<td></td>
</tr>
<tr>
<td>1201</td>
<td>623</td>
<td>578</td>
<td>48.13</td>
<td></td>
</tr>
</tbody>
</table>

The time (dpi) and number (cumulative mortality) of mortalities within each family following exposure to A. salmonicida are shown in Figure 5.2, below, which highlights the variation in susceptibility to A. salmonicida. It can be seen from Figure 5.2 that certain families succumb to the pathogen quickly resulting in a larger cumulative mortality (eg BRF 9 and BRF 10, which may be perceived as resistance assessed as a binary trait), whilst others show a greater resistance initially but mortalities persist over a longer period of time (eg ARF 7 and BRF 8, which may suggest a greater resistance assessed as a longitudinal trait).

The two trials are segregated in Figure 5.3 to provide comparable Kaplan-Meier estimates of the survival function for both Furunculosis Challenge 1 and Furunculosis Challenge 2. Displaying similar trends in mortality over the callenge periods, including the final level of mortality, the subtle difference between the two challenges lies with the initial death rate, being larger and in a greater number in Furunculosis Challenge 2.
Figure 5.2 – Cumulative mortality of all families involved in the Furunculosis Challenges up to 18 days post injection with *Aeromonas salmonicida*
Figure 5.3 - Kaplan-Meier estimate of the survival functions (by challenge) for 21 days following challenge of rainbow trout with *Aeromonas salmonicida*; – Survivor function, □ Censored observations

The Kaplan-Meier estimate of the survival function shown as Figure 5.4 illustrates the survival divided into individual groups, according to female, highlighting the variation between families. This vast variation demonstrates survival according to female ranging from less than 5% to over 75%. Most females demonstrate a similar trend in relation to cumulative mortality over this time period, but in certain families over 70% of the family is lost by 6 dpi.

A Kaplan-Meier estimate of the survival function is also presented to illustrate the variation between neomales (Figure 5.5). Again, a similar pattern has occurred, where not only a large difference in overall survival can be observed, for example between neomale 2 (over 60% survival) and neomale 7 (less than 15% survival), but also the rate at which fish die according to neomale.
Figure 5.4 - Kaplan-Meier estimate of the survival functions (by Female) for 21 days following challenge of rainbow trout with *Aeromonas salmonicana*; – Survivor functions, □ Censored observations

Figure 5.5 - Kaplan-Meier estimate of the survival functions (by Neomale) for 21 days following challenge of rainbow trout with *Aeromonas salmonicana*; – Survivor functions, □ Censored observations
Although completed at different times the temperature profiles between the two challenges did not differ significantly, but the bulk weights of families between the groups (ARF and BRF) did (P<0.001).

5.3.2 Genetics

Overall, neither the binary nor the longitudinal traits uncovered significant differences between the two challenges, or between the females selected for their known high and low response to PKD (model term: EBV). However, in both binary and longitudinal analyses, a significant genetic effect was present for sire and dam. Analysed separately, the two challenges displayed no significant effect for EBV, but a significant dam effect was evident for both binary and longitudinal traits in both challenges, following the likelihood ratio test. A weak but significant correlation between individual fish size, measured as fork length, and timing of mortality (dpi to death) was observed in the stock (r=0.09, P<0.05). The phenotypic trend of increasing fork length equating to greater resistance can be seen as dpi to death and mean fork length in Table 5.3, where smaller fish, eg 10.64 cm and 10.56 cm die due to furunculosis at 3 and 4 dpi, respectively, whilst larger fish, eg 11.57 cm and 11.51 cm succumb to the disease after a longer period of time, 8 and 9 dpi, respectively.

Table 5.3 - Average fork length of fish that died on specific days following injection with *Aeromonas salmonicida* in the Furunculosis Challenges

<table>
<thead>
<tr>
<th>Days post injection to death</th>
<th>Fork length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10.64 ± 0.11</td>
</tr>
<tr>
<td>4</td>
<td>10.56 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>11.19 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>11.19 ± 0.16</td>
</tr>
<tr>
<td>7</td>
<td>11.24 ± 0.26</td>
</tr>
<tr>
<td>8</td>
<td>11.57 ± 0.48</td>
</tr>
<tr>
<td>9</td>
<td>11.51 ± 0.25</td>
</tr>
</tbody>
</table>

NB Only dpi with 4 or more observations used

Estimates of heritability for fork length were calculated to be 0.09 ± 0.15 from the sire component, and 0.23 ± 0.10 from the dam component. There was a significant difference (P=0.005) in the fork length of fish between the two challenges, as well as a significant dam effect for fork length when tested using a likelihood ratio test.
The proportion of fish that died in each family was calculated and compared to the EBV of females selected for the study (Figure 5.6). From the EBVs, ranging from 1.63 up to 2.83, the corresponding value of the proportion of the family that died was plotted. Although a negative trend is apparent, as can be seen from the low EBV values corresponding to the greater proportion dead percentage, it is not significant \((r=-0.139, p=0.196)\). However, a significant sire and dam effect was found when analysed overall using the likelihood ratio test, but no effect from the fixed model term, EBV, occurred. When analysed by challenge, a significant dam effect was found in both Furunculosis Challenge 1 and Furunculosis Challenge 2.

![Figure 5.6 - 2D scatter graph displaying the proportion of each family that died due to infection with *Aeromonas salmonicida* over a 21 day period, against the Estimated Breeding Value of the females selected for the study.](image)

The Kaplan-Meier estimate of the survival function below (Figure 5.7) illustrates survival according to females selected as high and low responders to PKD, ie with high and low EBV values, respectively. As described for Figure 5.6, the females categorised as Low responders (ie with low EBVs) demonstrated a greater number of mortalities throughout the challenge period (represented as a lower survival in Figure 5.7), whilst females that were selected as high responders to PKD show a greater survival after 21 days.
Figure 5.7 - Kaplan-Meier estimate of the survival functions (by High and Low responders to PKD selected for the study) for 21 days following challenge of rainbow trout with *Aeromonas salmonicida*; – Survivor functions, □ Censored observations

The estimates of heritability could be calculated from both the sire and dam components from the information attained. The overall estimates of heritability were similar when calculated as binary and longitudinal traits, but were considerably higher when calculated from the proportion of fish that died. All of the estimates displayed in Table 5.4, below, showed a significant genetic effect when tested by the likelihood ratio test.

**Table 5.4 - Estimates of heritability ± SE calculated from Furunculosis Challenges**

<table>
<thead>
<tr>
<th>Estimate</th>
<th>$h_s^2$</th>
<th>$h_d^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary trait</td>
<td>0.14 ± 0.21</td>
<td>0.33 ± 0.13</td>
</tr>
<tr>
<td>Longitudinal trait</td>
<td>0.15 ± 0.23</td>
<td>0.37 ± 0.14</td>
</tr>
<tr>
<td>Proportion dead</td>
<td>0.72 ± 0.96</td>
<td>1.00</td>
</tr>
<tr>
<td>Fork length</td>
<td>0.09 ± 0.15</td>
<td>0.23 ± 0.10</td>
</tr>
</tbody>
</table>

1.00 denotes an estimate attained above a value of 1 – value reduced to maximum theoretical value achievable; $s$ and $d$ are the estimates calculated from the sire and dam component, respectively.
5.4 Discussion

This experiment was designed to investigate whether significant differences and additive genetic variation exists in susceptibility to *Aeromonas salmonicida* between families specifically selected for high and low tolerance to PKD. The EBVs of females selected to produce the families used in the experiment demonstrated no difference in resistance to furunculosis. However, the additive genetic variation and subsequent estimates of heritability indicate selective breeding for furunculosis resistance is possible within these specially selected lines. Although calculated from both the sire and dam component, due to maternal effects that can create biases in disease resistance studies in fish species (Mor and Avtalion, 1988; Sin et al., 1994), all estimates of heritability discussed will be based on the sire component only, as a more reliable estimate (Kolstad, 2005). An estimate of heritability for fork length was also obtained. The precedence of measuring fork length was to assess the effect of size on furunculosis resistance; as a result, a positive and significant phenotypic correlation was found between the length of fish and days to death following challenge with *A. salmonicida*.

As previously discussed in Chapter 4 (Section 4.2.7), there was limited availability of experimental facilities to hold all families at one site and challenge simultaneously. This led to marked differences between the two stocks. The 22 families were therefore held as two groups; ARF stock, consisting of 14 families, and BRF stock, consisting of 8 families. Although separation of the two challenges was accounted for in the statistical model (term: trial), certain areas should not be overlooked; age difference, the exposure to and incidence of multiple diseases in the BRF families prior to challenge (as well as the single incidence of disease in ARF fish), the ‘condition’ suffered by BRF families (which resulted in 100% mortality in some families), and the size difference between the groups. Many, if not all of these areas have been shown to have an effect on disease resistance in fish. Numerous authors have reported an increase in resistance to reinfection following re-exposure to the same pathogen (Ferguson, 1981; Ellis *et al.*, 1982; Gleeson *et al.*, 2000; Karvonen *et al.*, 2003, 2005; Gilbey *et al.*, 2006) but there is also evidence of immunodepression following parasitic infection, which can increase the
susceptibility of fish to other pathogens (see Woo, 1992). The stocks used in the present trial were exposed to and affected by *Costia* (ARF and BRF) or *Trichodina* (BRF). To stipulate whether or not the previous exposure affected the overall result is impossible to conclude; to assess such effects would require research in itself. However, with the two groups experiencing very different levels of parasitic infection (both intensity and number), it is suggested that different responses may be apparent. Ideally, any experimental testing should use naïve fish that have been exposed to the same environment. When experimental facilities are lacking, where possible, terms should be incorporated into the statistical model to account for differences. Further, the ‘condition’ experienced by BRF fish, thought to be related to the environment in which they were kept, described as a “lipid-related nutritional deficiency among the ova, only manifesting significantly at lower incubation temperature”, may also have had adverse effects on the susceptibility of the BRF trout (see Saeij *et al.*, 2003; Varsamos *et al.* 2006). Finally, age and size difference has also been noted to affect the results of challenge experiments (Glover *et al.*, 2004). However, due to the nature of the disease, the design of the experiment, and the way data was compiled, it was decided to combine the results from both groups to attain an overall perspective of furunculosis resistance in the IoM fish.

An additional problem encountered throughout Furunculosis Challenge 1 was the loss of tags. Initially it was expected that fish from the family ‘Belly – green’ (ARF 7) would be a problematic group as many of the recovered mortalities had their underside cannibalised due to starvation throughout the challenge. However, it was found that ARF 7 had only one record missing from its data. The most problematic tags were ‘Left eye – green’, ‘Head – pink’, and ‘Right eye – green’, belonging to families ARF 12, ARF 15, and ARF 16, each losing 10%, 20%, and 12.5% (respectively) of their data due to missing tags. The main reason for the loss of tags was inexperience using elastomere tagging, which is supported by the number of unknown individuals in Furunculosis Challenge 2; zero. Finally, the challenge design could have been improved. Although the four test tanks provided sufficient information to assess any differences between families as well as calculate additive genetic variation, the results will have undoubtedly strengthened had the number of control tanks been reduced from four to two, increasing the test tank number from
four to six. However, the challenge design was standardised across the two experiments.

The estimate of heritability calculated from binary data is somewhat lower than other estimates for disease resistance in salmonids (Gjøen et al., 1997; Henryon et al., 2005), while assessed as a longitudinal trait is comparable (Henryon et al., 2002, 2005) or lower (Perry et al., 2004). Such research indicates that selection for disease resistance is possible within salmonid populations, but it is impracticable to compare the results of the present study with work conducted on other pathogens. The research that has been compiled on A. salmonicida in salmonids has shown promising results (Marsden et al., 1996; Gjøen et al., 1997; Nordmo et al., 1998; Perry et al., 2004). As early as 1925 selection experiments proved how successful genetic improvement towards furunculosis resistance could be as Embody and Hyford (1925) reported an increased survival rate from 2% to 69% after three generations of selection when studying brook trout. Additionally, Ehlinger (1977) successfully reduced mortality rates in both brown trout and brook trout following selection for furunculosis resistance. Since that time, a significant genetic component has been documented for resistance to furunculosis in many salmonid species. Gjedrem et al. (1991) estimated heritability at 0.40 in Atlantic salmon, whilst Gjøen et al. (1997) calculated heritabilities of 0.53 and 0.38 for Atlantic salmon in challenge and field experiments, respectively. Ødegård et al. (2007) calculated a heritability estimate of 0.43 ± 0.02 for furunculosis resistance in Atlantic salmon following challenge tests, whilst Perry et al. (2004) report an estimate of heritability of 0.51 in an experimental challenge using Arctic charr. Glover et al. (2004) found no significant difference between stocks of Atlantic salmon from farmed, hybrid, and wild parentage; any differences observed were deemed to be due to size of the fish in each group. There has been limited research conducted on the difference in susceptibility to furunculosis at the family level, however Kjøglum et al. (2005) identified differences in susceptibility (prevalence of death) to furunculosis between three families of Atlantic salmon studied, whilst Dahle et al. (1996) observed family but not population differences to furunculosis susceptibility in the same species. Although the estimates of heritability calculated here are moderate, and somewhat lower than those reported elsewhere, there
remains to be potential to improve the selected lines for furunculosis resistance. Further, the extreme variation in response to the disease illustrated between the families (Figure 5.4 and Table 5.1) suggests that some families could be disregarded from selection. By selecting families that demonstrate a stronger resistance to *A. salmonicida*, the response to selection may be improved above that estimated from the heritability, providing the additive genetic variation detected lies within those families. Although it is often difficult to select neomales according to family information due to the commercial management strategies used on farms, the graph illustrating the Kaplan-Meier estimate of survival by neomale highlights the variation that can occur between randomly selected fish. By improving the management of neomale broodstock and selecting those with a greater resistance to the disease of interest based on family information, the resultant offspring should display a greater resistance overall. Between the PKD high and low resistant lines, there is very little variation (Figure 5.7). This supports the notion that the females selected for this project should be considered nominally high and low responding females as discussed in Chapter 4. Finally, the estimate of heritability calculated from the proportion of each family that died, although promising, due to the limited number of families involved it is somewhat unreliable, as indicated by the large standard error associated. Additionally, no other estimates of heritability for disease resistance in salmonids have been calculated this way, leaving comparisons to other work unavailable.

It did not seem to matter whether resistance was assessed as a binary or longitudinal trait, as indicated by the estimates of heritability. This is favourable for trout farmers, as it implies that the way resistance is assessed is not crucial to the overall result attained. The trout were moderately challenged in this study, with mortality at ~50% in each challenge. Most of the trout died within 8 days of challenging, presumably due to the high concentration of the causative agent administered. Challenge by intraperitoneal injection allows the bacterium to bypass initial lines of innate immune defence. However, from a breeding perspective, challenging the trout in this way should have provided a suitable means to assess resistance. Firstly, intraperitoneal injection meant a standardised amount of the bacterium was given to all challenged fish. Secondly, it allowed the additive genetic variation for resistance
to be detected; although it is likely other forms of challenge would allow the genetic variation to be detected. Many studies have shown that detection of additive genetic variation can be successful at a range of mortality rates and challenge methods for disease resistance. For example, Gjedrem et al. (1991) challenged Atlantic salmon with *A. salmonicida* via cohabitation, resulting in mortality of 68%, and heritabilities of 0.32 to 0.48. Henryon et al. (2002) immersed rainbow trout in the causative agent of VHS, resulting in almost 85% mortality and a heritability estimate of 0.13, whilst in 2005 Henryon et al. challenged rainbow trout with three commercially important diseases, induced via intraperitoneal injection (ERM and RTFS) or immersion (VHS), and resulting in 86% to 90% mortality with successful detection of additive genetic variation, and subsequent heritabilities ranging from 0.07 to 0.57.

Although standardised, the amount of causative agent injected into the fish requires consideration. The fact that fish in the present study expressed a range of sizes, especially between the two groups (ARF and BRF, significant difference), suggests individuals were exposed to different amounts of bacterium in relation to size. This is potentially the reason why a significant correlation between fork length and days to death occurred, with size found to have a bearing on resistance of the trout to furunculosis; the trend suggesting that smaller fish are more susceptible to *A. salmonicida*. Glover et al. (2006) found the reverse to be true as a significant, albeit weak, negative correlation was detected between length and time until death when Atlantic salmon were challenged with *A. salmonicida*; however, when fish of less than 12 cm were removed from the data, correlations were no longer significant. In the present study, it is assumed that the (weak) significant positive correlation is due to the ratio of fish size to the amount of bacterium; smaller fish displaying a faster response to *A. salmonicida*. Using young/small fish in challenge experiments is beneficial for a number of reasons. More representatives per holding unit can be used, less feed and management is required prior to challenge, and small scale experimental facilities can provide results as strong as large commercial operations utilising natural outbreaks. Having used smaller and relatively young rainbow trout in the present study, enough data was collated to produce estimates of heritability for furunculosis resistance. Using young fish in challenge experiments may provide
an insight into the resistance of adult rainbow trout. The early selection for disease resistance would be a useful tool in breeding strategies. However, it must be ensured that there is sufficient genetic correlation between the early and late trait measurements so that the indication given in early life is representative as the fish age. To date, this has been the case for some production traits in salmonid species (Fishback et al., 2002; Su et al., 2002), but such correlations are yet to be measured for disease resistance (Perry et al., 2004), and further work is required.

In addition to quantitative genetic experiments calculating significant heritability (as demonstrated here), there has been research conducted that demonstrates a link between allelic variation at the Major Histocompatibility Complex (MHC) class I and II loci and susceptibility to furunculosis in studies involving salmonids (Langefors et al., 2001; Lohm et al., 2002; Grimholt et al., 2003). Langefors et al. (2001) tested the importance of genetic variation in the MHC class II beta in Atlantic salmon for survival following challenge with A. salmonicida. Fish from high and low resistance families were screened for their MHC class II beta genotypes to find certain alleles were more prevalent relating to high or low resistance; the study was the first of its kind to find a correlation between MHC class II allelic variation and resistance to a bacterial pathogen in a lower vertebrate. Later, Lohm et al. (2002) strengthened the claim of disease resistance and an association with the MHC when a strong survival advantage was detected for individuals carrying high-resistance MHC alleles against A. salmonicida; however, following exposure of numerous families, there was no difference between the groups displaying varying levels of hetero- and homozygosity. Additionally, Grimholt et al. (2003) demonstrated the association between MHC class I and class II alpha and resistance against A. salmonicida in Atlantic salmon. Having discovered a significant genetic component exists for susceptibility to furunculosis (Marsden et al., 1996; Gjedrem, 2000), and that Langefors et al. (2001) detected significant differences between the distributions of MHC class II beta alleles between families with high and low resistance to infection with A. salmonicida, it may have been expected to find differences between the families studied here. However, in agreement with the work of Lohm et al. (2002) the families involved in the present study displayed no difference in susceptibility to furunculosis relating to
the EBV. This is perhaps unsurprising considering the families are of a similar origin, and have had comparable histories of exposure to the bacteria, ie none. However, it does appear that families with low EBVs are more affected by *A. salmonicida* than those of high EBV, but this effect is not statistically significant.

As the first study to test for additive genetic variance in relation to furunculosis resistance in families specifically selected for high and low PKD tolerance, an overall positive result was observed. Although no difference was indicated in relation to the female EBV, the additive genetic variation detected suggests significant gains in resistance to *A. salmonicida* can be made in the families used in this study. It also suggests that other families from the IoM population may hold the potential for improvement to furunculosis resistance, as female EBV played only a minor role, if any. Having calculated estimates of heritability of a similar magnitude from both binary and longitudinal traits, it appears the methodology used to calculate resistance shows no bias; an advantage to the farmer. Finally, the families were assessed only against furunculosis in the present study. Having already been assessed for PKD resistance (Chapters 3 and 4), and the data here suggesting a negative effect between the two diseases based on EBV, where a low EBV suggests a low resistance to furunculosis and high resistance to PKD, ie potentially a negative correlation (although not statistically significant), the data can be collated and used to provide information to the farms involved. This will assist in establishing a suitable selective breeding programme incorporating the two commercially important diseases.
Chapter 6: General Discussion
Initially, the sole aim of this project was to examine and identify if additive genetic variation exists within farmed populations of rainbow trout to the commercially important disease, Proliferative Kidney Disease (PKD); causative agent *Tetracapsuloides bryosalmonae*. However, as research continued into the disease, it was noted that the bacterial pathogen, *Aeromonas salmonicida*; the causative agent of furunculosis, may play an important role in the mortality experienced at farm outbreaks. Under the belief that PKD alone is not enough to cause mortality, it has been assumed that the effects of *T. bryosalmonae* in the latter stages of PKD (Holland *et al.*, 2003) combined with additional infection from other rainbow trout disease(s) compromise the immune system of fish resulting in mortality (Hoffman and El-Matbouli, 1991, 1994; Foott and Hedrick, 1990). With only two publications highlighting *A. salmonicida* at PKD outbreaks (Hoffman and Dangschat, 1981; Morris *et al.*, 2003a) one of which is deemed to be related to the drug used rather than PKD (Morris *et al.*, 2003a), this extended investigation into the relationship between PKD and furunculosis is based on the observations of scientists at the Institute of Aquaculture, University of Stirling, who continually report the presence of *A. salmonicida* at PKD epidemics. This evidence is based on observations from the field, via commercial practices or field trials, or following the injection of kidney homogenate from PKD infected fish from the field into naïve experimental trout, often resulting in outbreaks of furunculosis in the injected fish, suggesting a carrier state of *A. salmonicida* in the transmission individuals. In only one instance has PKD been reported to improve the immune response to another pathogen (Foott and Hedrick, 1990); the general consensus, supported by numerous authors reporting the presence of secondary infection where mortality occurs (Hoffman and Dangschat, 1981; Hedrick *et al.*, 1985; O’Hara, 1985), is that opportunistic pathogens are the critical factor resulting in death where PKD is apparent, hence the importance of considering secondary pathogens of commercial importance, such as *A. salmonicida*.

To test for a genetic component to PKD resistance, initial research involved a natural challenge test, where commercial populations of 0+ rainbow trout were exposed to the causative agent in the field situation. The 1500 juveniles sacrificed on a single day were assigned kidney scores, from 0 to 4 inclusive, according to the scale of Clifton-Hadley *et al.* (1987). From the data generated, it was discovered
that significant genetic (co)variation exists within the farmed populations to PKD score, and therefore potentially resistance, where the theory that the lower the kidney score the higher the resistance is considered, additive genetic variation for this trait could prove beneficial to the industry for selective breeding purposes. A combined estimate of heritability for the two farms involved, Houghton Spring Hatchery (HS) and Isle of Man (IoM), was moderately high, suggesting that significant gains can be made towards the genetic improvement of PKD. Further, the favourable genetic correlation between kidney score and fish size (fork length or body weight), as well as the additive genetic variation detected for the two production traits indicates that improvement to all three characters can be conducted simultaneously through selection. Management techniques and genetic technology allowed the individuals to be assigned to specific families, as pedigree structures had been previously generated for a LINK project working on the genetic improvement of production traits in the populations. This resulted in families being ranked (1 to 500) based on their estimated breeding values (EBVs are based on the same principal of kidney score); those with low values were used in production, whilst representatives of both high and low resistance families were retained so that challenging under experimental conditions could take place in order to support or dismiss the findings of the initial research. The opportunistic challenge therefore presented the first insight into the potential of selectively breeding for PKD resistance in the British trout farming industry.

The PKD experimental challenge was established using known high and low responding females to PKD, calculated from the initial study. Four females (two high, and two low) were crossed with a single neomale so that the sire component could be calculated. However, it was deemed that the high and low responding females were only nominally high; hence the regression of kidney score on female EBV was incorporate into the data. The study resulted with the evidence of additive genetic variation to PKD score being supported, and having based the kidney score in the experimental challenges on the afore mentioned theory, that a lower score is deemed to be higher resistance, it suggests that the additive genetic variation is represented as PKD resistance also. Although results were confounded due to differences in families held at different locations, additive genetic variation for PKD resistance was found to exist at each sampling week over an 11 week period. The
additive genetic variation and subsequent estimates of heritability demonstrate the potential of selectively breeding for PKD resistance within the IoM families used. It also suggests that a genetic component may well be present in other production fish at the IoM site. The regression of kidney score on EBV generally demonstrated a positive sign, suggesting that kidney score and EBV do hold a relationship, but the statistical insignificance at each sampling week (and overall) means no conclusions can be drawn here; further work is required to support the hypothesis. The EBV was also included as a model term to assess family performance, but no family demonstrated a significantly better resistance to PKD. The usefulness of incorporating EBV into a selection programme for PKD resistance in this population is therefore questionable; any programme developed should be based on the fact that additive genetic variation exists within the population that would respond favourably to selection. Further, by recording family performance in relation to the desired trait, numerous options exist as to what selection methodology could be employed. The timeframes produced for each family may prove useful to farmers to assist in stocking regimes so that the effects of PKD can be minimised, ie natural vaccination. Where this could be employed practically, and the segregation of families could be maintained, families which PKD score peak later could be stocked earlier in the summer when PKD is most prolific, whilst those which peak earlier could be stocked later in the year as water temperature and PKD development decline. Further, by incorporating the timeframe data with the negative correlation between fork length and kidney score (where size variation is sufficient to be practical, as seen in Chapter 3), it may be possible to stock larger fish earlier in summer, and smaller fish later to help suppress the effects of PKD. Finally, PKD score was found to relate significantly to the number of parasites in the kidney suggesting that the scale of Clifton-Hadley et al. (1987) is indeed sufficient and accurate to describe the degree of infection in rainbow trout. However, certain areas in relation to T. bryosalmonae counting require attention.

The same families, with the exception of one, were used in the furunculosis challenges. The experimental design differed significantly to that of the PKD challenge, but the design was sufficient to detect additive genetic variation for resistance to furunculosis. Assessed as both a binary and longitudinal trait, estimates of heritability calculated for survival were moderate suggesting significant
genetic improvement can be made within these families for resistance to furunculosis. No families illustrated a higher tolerance to the disease according to the EBV but as additive genetic variation was detected it suggests the IoM production stock may also hold potential resistance to the disease. Similarly to the previous two studies, a favourable correlation was found between size and resistance, suggesting these traits can be used in selection simultaneously. However, having analysed the same families for the two separate commercial diseases, it was discovered that the EBV of females showed an opposing result to each disease. Females with low EBVs, calculated from initial data suggests that a higher degree of resistance to PKD should be demonstrated, and that higher EBVs would produce a more severe PKD reaction (although this was not statistically significant in the PKD challenges). When EBV was plotted against days to death or high and low responding lines were assessed using a Kaplan-Meier estimate following challenge with *A. salmonicida*, although not statistically significant, females of lower EBV were found to respond quicker to the bacteria, indicating a lower resistance to furunculosis, and overall, a potentially negative correlation between the two diseases.

To date, the results of other studies have noted various responses to research between two diseases in salmonids. There is evidence of both favourable (Ødegård *et al.*, 2007) and unfavourable (Gjøen *et al.*, 1997) genetic correlations between viral and bacterial diseases, a favourable response between two bacterial diseases (Gjøen *et al.*, 1997), and inconclusive evidence in a rainbow trout population studied by Henryon *et al.* (2005), who discovered only a weak genetic correlation between two bacterial diseases, Enteric Red Mouth (ERM) and Rainbow Trout Fry Syndrome (RTFS), and the viral disease, Viral Haemorrhagic Septicaemia (VHS); it was hypothesised that a positive and strong genetic correlation between ERM and RTFS, and a unfavourable correlation between the two bacterial diseases and VHS would be apparent. In terms of production traits and disease resistance both favourable (Standal and Gjerde, 1987; Gjedrem *et al.*, 1991; Nilsson, 1992; as well as Chapter 3, and phenotypically in Chapter 4) and unfavourable (Henryon *et al.*, 2002) genetic correlations have been identified. Ødegård *et al.* (2007) are critical of some of the work conducted on genetic correlations in disease resistance, suggesting that the work of Gjøen *et al.* (1997) does not indicate a truly antagonistic genetic
relationship, stating the low negative correlation calculated may have been the result of sampling error. The same suggestion is made by Ødegård et al. (2007) toward the work of Henryon et al. (2005), who are self-critical of their result and suggest that the weak-to-moderately strong genetic correlation may be due to uncertainty of the estimates attained. They (Henryon et al., 2005) continue by stressing how it is important that reliable estimates of genetic correlations are required through assessing large numbers of families for resistance. Although the correlation is not statistically significant in the present study, there remains to be an indication that the two diseases conflict. Having only a limited number of families to assess, as well as a limited number of observations within those families, the error associated with the result here is enough to mask any real genetic relationship that may be occurring. Based on the discussions of previous authors, it is therefore important that further research is conducted towards the relationship between PKD and furunculosis, or any other important commercial disease that may be contributing to mortality when PKD occurs.

As far as is known this is the first study to take into account a bacterial and parasitic disease within the same population of rainbow trout, and although tentative, it is also the first to report any form of correlation between resistance to parasitic and bacterial diseases. Due to the different aetiologies of aquatic diseases, it is expected that certain pathogens will conflict when genetic studies take place. The mechanisms involved in fighting pathogens will undoubtedly play a considerable role in any correlations detected. This very assumption led Henryon et al. (2005) to explain how the different pathogens of ERM, RTFS, and VHS are believed to have invoked different immune responses from the trout, and that the response mechanisms were under independent genetic control. In the present study, the ideal situation would have been a significant and positive correlation between the two diseases, which would benefit the industry so that selection could be conducted simultaneously resulting in a faster genetic improvement to both PKD and furunculosis. The result here implies that progress can be made towards resistance in both diseases simultaneously or over time without a severe negative impact occurring in resistance to the opposing disease, suggesting that sequential selection or a selection index could be used in a breeding programme. Sequential selection allows the genetic improvement towards resistance of one disease first before the
second is added to the selection programme, whilst a selection index uses families based on a moderate response to both diseases, and all traits are taken into account, given an appropriate weight according to the heritability and phenotypic and/or genetic correlation between them (Gjedrem and Thodesen, 2005).

Due to the statistical insignificance of the trend between the two diseases shown here, it is suggested that the effect of selecting for one disease will not have a detrimental effect on the other. However, as *A. salmonicida* is considered a secondary pathogen in this situation as it emerges only as water temperatures increase towards summer inflicting additional stress on the trout, and because the host is immunocompromised due to the effects of PKD, it would be more beneficial to employ sequential selection to the population used in the present study.

Sequential selection requires careful management, and it is important to record the progress of PKD resistance as discussed in the General Introduction (Chapter 1, Section 1.2.5). By selecting for high tolerance to PKD first, an improvement in the effects of the disease may result in less secondary infections affecting the host, especially where such secondary infection(s) can be vaccinated against, as is the case for furunculosis, ERM, and Vibriosis in rainbow trout. This approach could be incorporated into future research towards PKD resistance, as diseases that already have well-developed vaccines require less attention in regards to selective breeding towards their resistance. That is providing PKD does not have an adverse effect on the effectiveness of the vaccine administered, especially in the case of furunculosis as British farms are presently not allowed to booster; this area will undoubtedly require consideration in future research. Where PKD resistance is improved through selection, the effect of *A. salmonicida* (and other pathogens acting as secondary infections) should be reduced, but it is important to re-estimate genetic parameters periodically in a selection programme to account for any changes in the genetic parameters initially measured; this is especially important when simultaneous selection is being applied (Gjedrem and Thodesen, 2005). Following selection for PKD resistance, additional research on furunculosis resistance is advised prior to its inclusion in the breeding programme. This does not mean the research conducted here has been uninformative. The furunculosis data attained has provided evidence of additive genetic variation in the population, an indication as to the potential resistance of families, as well as identifying a potential relationship
between PKD and furunculosis resistance. Further, the information gathered can be retained as reference material so that the techniques and challenge design used can be mirrored in the future, allowing comparisons to be made.

The favourable genetic and phenotypic correlations calculated between disease resistance, either in PKD or furunculosis, and production traits, as fork length or body weight, in the present project suggests it is feasible that selection for resistance will not create an unfavourable response in the production traits. Although the correlations were not of a great magnitude, it would be beneficial if selection for a production characteristic improved disease resistance in the population. Falconer and Mackay (1996) state that indirect selection has great potential providing there is sufficient genetic correlation between the two characters, and that the production trait holds a substantially higher heritability than the disease resistance trait. As disease resistance traits are often expensive or difficult to measure, Fjalestad et al. (1993) had considered indirect selection to improve the efficiency of selection for disease resistance. In order to reduce costs it was suggested that correlated or marker traits could be used to measure disease resistance, such as lysozymes (Røed et al., 1992, 1993; Lund et al., 1995), immunoglobulin levels (Strømsheim et al., 1994a; Lund et al., 1995), plasma α2-antiplasmin activity (Salte et al., 1993), antibody titres (Lund et al., 1995; Strømsheim et al., 1994b), haemolytic activity (Røed et al., 1990, 1992, 1993), and cortisol levels (Refstie, 1982; Fevolden et al., 1994); all of which have shown genetic variation and a genetic correlation to survival. However none of the genetic correlations exceeded ±0.37, suggesting the correlated response to improve disease resistance would not be particularly large (Gjedrem and Thodesen, 2005). However, if an estimate of heritability is found to be particularly high in a production or correlated or marker trait, because the measurements can be taken from live individuals (ie the breeding candidates, and/or their full- or half-siblings), it would present an opportunity to use individual selection in disease resistance studies, or other studies where the methods for measuring the trait are critical (Fjalestad, 2005b). Further, it would increase the opportunity to improve (and combine) selection methods, and therefore the response to selection.
To understand fully the interaction between PKD and furunculosis, a challenge using both infectious agents at the same time should be conducted. This had been suggested in the present project, but due to time limitations, it did not take place. The very different effects seen in the two challenges could be masked or heightened by challenging with two diseases simultaneously; potentially leading to very different results. Challenging with both infectious agents may provide a more representative situation of what occurs in the field situation where fish are first affected by PKD then furunculosis. Future research should consider such a challenge.

Also, a ‘natural’ PKD challenge was conducted in this project on the BRF stock. The challenge involved administering *T. bryosalmonae* via a peristaltic pump to naïve rainbow trout to assess the transmission and effect of PKD using this method. The *T. bryosalmonae* were sourced from *T. bryosalmonae*-infected bryozoa cultured at the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling. As the bryozoa released spores into the surrounding water of their holding unit, the infected water was pumped into tanks holding rainbow trout. This method was believed to mimic more closely what occurs in the farm situation. However, due to an extremely low transmission rate, the number of infected fish was negligible and the data was not used. There was however, evidence of PKD, apparent as swollen kidneys, and the presence of *T. bryosalmonae*, determined by PKD-PCR of kidney samples. Although the direction of transmission from infected bryozoa to rainbow trout has been completed previously (Feist *et al.*, 2001; McGurk *et al.*, 2006; Morris and Adams, 2006), this method of transmission has rarely been used. The method of using a peristaltic pump from *T. bryosalmonae*-infected water is believed to have been the downfall of the challenge. The length of tubing from the peristaltic pump to some tanks is believed to have had an effect on the success of transmission. With no other reports publicising these materials and methods, it was difficult to predict such an outcome. Since the failure of the natural challenge, the problem has been reported in another study (Morris and Adams, 2007). A number of factors are considered as causing the failure in transmission, including; parasites dying due to a lack of oxygen or increased temperature because of prolonged periods within the tubing; agglutination to the tubing, so that the organism never reached the outlet to infect the host; a filtering effect caused by the
build up of waste materials and algae within the tube, or due to the type of outlet tap connected to the tubing; or, areas of concentration where parasites have congested causing a filtering effect or blockage within the tube. Having observed a similar problem when attempting to transmit *T. bryosalmonae* to rainbow trout, D. Morris (Institute of Aquaculture, University of Stirling) simply shortened the length of tubing used and was able to successfully transmit the parasite using a peristaltic pump and the same tubing, which evidently resulted in chronic PKD (Morris, 2007 personal observation).

Considering its effect on the industry and the fact it has been evident for over 90 years (Plehn, 1924; Schäperclaus, 1954; Besse, 1956; Hedrick *et al.*, 1984b), it is perhaps surprising that there are no feasible, legal, or environmentally-friendly methods for the control of PKD to date. It is also surprising that this is the first study to consider the genetic component related to PKD resistance, considering previous work conducted on the genetics of disease resistance in rainbow trout (Gjøen *et al.*, 1997; Henryon *et al.*, 2002, 2005; Ødegård *et al.*, 2007). Historically, work on PKD has focussed on chemical and environmental methods of control only. For example, the use of Cyzine (Klontz, 1984), salination (O’Hara, 1985), malachite green (Clifton-Hadley and Alderman, 1987), fumagillin (Hedrick *et al.*, 1988b; Wishkovsky *et al.*, 1990; le Gouvello *et al.*, 1999), and its analogue, TNP-470 (Higgins and Kent, 1998; Morris *et al.*, 2003a) have all been discussed with some degree of success. However, the disadvantages associated with each chemical far outweigh the potential benefit to repress PKD; most are associated with health-related issues, either to the fish or to the consumer. In the case of salination, its use demonstrated great potential until the disease was observed in anadromous species and the presence of *T. bryosalmonae* in saltwater was confirmed (Kent *et al.*, 1995). As a natural approach, aeration is used to increase the level of oxygen in the water, helping to minimise stress during endemic outbreaks, but temperature continues to be the only environmental factor that can minimise the effects of PKD directly. Known to be imperative to the development of PKD (Ferguson, 1981), many authors suggest the disease is most prolific at 15°C or higher (Ferguson, 1981; Hedrick *et al.*, 1984b; Clifton-Hadley and Alderman, 1987, 1988), with increased severity and development between 12°C and 20°C (Brown *et al.*, 1991). However, Gay *et al.* (2001) proved water temperatures as low as 8°C can be sufficient to
induce *T. bryosalmonae* infestation. A reduction in water temperature will undoubtedly slow the development and at least some of the effects of PKD, but the majority of production sites would find controlling water temperature impossible. Additionally, as poikilotherms, a severe reduction in growth would be observed in fish with reduced water temperature, resulting in major production losses throughout summer months when PKD is most prolific. Presently, the only feasible way to utilise water temperature is to delay the exposure of fingerling fish to infected water with temperatures in excess of 15°C for prolonged periods; greater than 2 months. This effectively vaccinates the fish, as a mild dose of PKD is contracted allowing an immune response to occur the following season (Ferguson, 1981; Ellis *et al*., 1982; Hedrick *et al*., 1985; Foott and Hedrick, 1987; Morris *et al*., 2003a). Foott and Hedrick (1987) speculate that resistance following exposure may be more prominent when clinical disease is experienced at 15°C or higher, but Morris *et al*. (2003a) warn that timing must be vigilant for the technique, and that the method is not suitable for year-round production fish. With the information gathered in the present project in terms of the timecourse of disease progression in families, and also size in relation to the effects of PKD (as discussed in the relevant chapters), additional techniques may now be available to the farmer to coincide with using natural vaccination.

Out with chemical and environmental factors to control PKD, genetics and general farm husbandry have been considered. Brown *et al*. (1991) found that feral landlocked salmon may have produced a genetic resistance to the disease due to continual exposure in the wild, indicating the potential to selectively breed for resistance to *T. bryosalmonae*, and having supported this theory through the identification of additive genetic variation to PKD resistance in the present project, the benefits to the British trout farming industry are obvious. However, genetic control should not be a substitute for good husbandry practice, as Le Gouvello *et al*. (1999) observed increased mortality due to poor husbandry and the condition of equipment at PKD epidemics. By minimising handling and husbandry stressors, as well as optimising environmental parameters, the mortality witnessed at PKD epidemics should be considerably reduced (Seagrave *et al*., 1981).
Additional genetic opportunities exist for research into both PKD and furunculosis resistance in rainbow trout via the Major Histocompatibility Complex (MHC) and Quantitative Trait Loci (QTL). As the understanding behind the genetics of resistance to disease in fish improves, numerous studies are beginning to concentrate on such areas, especially in salmonids. Because the physiological and/or biochemical mechanisms responsible for disease resistance can have a strong genetic basis and show significant heritabilities (Kaastrup et al., 1991; Yamamoto et al., 1991), the ability to associate major genes with these traits would permit marker-assisted genetic selection for increased disease resistance in cultured fish (Lande and Thomson, 1990; Kono et al., 2000; Quillet et al., 2001). In Atlantic salmon, numerous projects have studied specific alleles and genotypes relating to the effects of infectious agents on the MHC (Langefors et al., 2001; Lohm et al., 2002; Kjøglum et al., 2006; Wynne et al., 2007). For example, Langefors et al. (2000) studied 120 full-sibling families for resistance to *A. salmonicida*. Having identified high- and low-resistance families, their MHC class II beta genotypes were screened to find a significant association between specific alleles and furunculosis resistance, whilst Kjøglum et al. (2006) used 1966 fish from 7 families to assess the relationship between the MHC and the viral disease, Infectious Salmon Anaemia (ISA). It was found that specific alleles of MHC class I demonstrated a significant resistance to the disease, while other alleles of MHC class I and alleles of class II alpha showed significantly more susceptibility to the disease. It was concluded that specific genotypes in relation to MHC class I and class II alpha alleles can influence the effects of ISA in Atlantic salmon. In rainbow trout, Palti et al. (1999) conducted a study to assess candidate DNA markers associated with Infectious Haematopoietic Necrosis (IHN) resistance in backcrosses of rainbow and cutthroat trout. From the 33 restriction fragment length polymorphism markers detected between mortalities and survivors, 17 were tested to find a significantly higher frequency of markers among fish more susceptible to IHN. Although not directly related to the MHC, the rainbow trout study further identifies the potential for genetic information to be used in selective breeding programmes for disease resistance. Similar studies have been conducted on the marine species, *Dicentrarchus labrax* (Gornati et al., 2004, 2005). As research continues, there will undoubtedly come a time when information is available on the MHC of rainbow trout in relation to disease resistance.
detected MHC genes will serve as good starting point to search for related QTL (Dunham, 2004).

An extension of the characterisation of resistance to Infectious Pancreatic Necrosis (IPN) by Okamoto et al. (1987, 1993) was used by Ozaki et al. (2001) to report the first mapping of a QTL associated with disease resistance in rainbow trout. Using microsatellite markers, two chromosomal regions associated with IPN virus resistance/susceptibility were identified, suggesting that marker-assisted selection can be used to improve the resistance of rainbow trout to the IPN virus. In another study, Moen et al. (2004) reported a QTL with significant effects on resistance to ISA in Atlantic salmon. Such studies demonstrate the suitability of models for the genetic characterisation of complex traits like disease resistance. The development of genomic tools for rainbow trout research has progressed rapidly in recent years, with genetic linkage maps already available (Young et al., 1998; Sakamoto et al., 2000; Nichols et al., 2003). Over 250 polymorphic microsatellite markers have already been developed for rainbow trout, and the addition of those designed for Atlantic salmon that work for rainbow trout, leaves the total in excess of over 500, providing substantial opportunity to research. As the number of libraries concentrating on the rainbow trout genome continues to rise, the high-density genetic map and completed sequencing of the genome is expected in coming years, which will be extremely useful resources in rainbow trout breeding programmes. To date, most effort has been directed towards mapping QTL using markers from the genetic linkage maps. By improving high density maps, required for fine mapping QTL with economic importance, such as growth rate, feed conversion, and disease resistance, the application of marker-assisted selection and gene introgression into rainbow trout breeding programmes is undoubtedly imminent. However, Hayes and Andersen (2005) warn that it is important to understand the magnitude of a proposed experiment to detect QTL. Although QTL experiments have been successful in some livestock species, the QTL often displays only comparatively small effects even for those QTL displaying the larger effects for the trait (see Carlborg et al., 2003). This suggests powerful mapping will be required. For QTL detection, the reproductive capacity and existing lines of the species is important. For example, Ozaki et al. (2001) used backcross and F₂ populations in fish from the intercrossing of different lines to map a QTL relating to disease.
resistance; such populations are powerful resources for QTL mapping (Hayes and Andersen, 2005). The recent and future developments in molecular genetics present the possibility of using genotype selection for performance traits (Gjerde, 2005). The tools and information surrounding QTL and the MHC system in rainbow trout will undoubtedly be of immense benefit to the British trout farming industry. The optimum use of this information would require current breeding programmes to be modified in design (Gjerde, 2005), but where the opportunity of marker-assisted selection exists, especially as the rate of genetic gain is expected to be improved by 10% to 50% in comparison to traditional animal breeding programmes (Kincaid, 1983b; Weller, 1994; Ferguson and Danzmann, 1999; Danzmann et al., 1999; Davis and Hetzel, 2000), the integration of molecular technology should be considered, and further investigation is advised.

The research conducted here is only part of a large sector that requires attention relating to PKD in the British trout farming industry. Although furunculosis has been repeatedly witnessed at outbreaks, other pathogens, including IPN virus (Hoffman and Dangschat, 1981), *Ichthyophthirius, Flexibacter columnaris*, gill bacteria (Hedrick et al., (1985), *Saprolegnia*, and *Costia* (O’Hara, 1985) have all been identified at PKD epidemics. Undoubtedly, farmers would like resistance to all pathogens (ie a general resistance), and given that additive genetic variation has been demonstrated for the two diseases considered in this project, it is conceivable that resistance to other pathogens is possible; this has been the case for pathogens investigated to date (see reviews by Kinghorn, 1983; Gjedrem, 1992, 1997, 1998, 2000; Gjerde, 1986; Chevassus and Dorson, 1990; Fjaæstad et al., 1993). Henryon et al. (2002) believe additive genetic variation to disease resistance is likely to exist within most, if not all, salmonid populations due to the successful history in its detection. Where additive genetic variation exists, focus must be towards the most economically damaging diseases encountered in commercial production. For example, in this project PKD was discussed and recognised as the greatest limiting factor to the production of rainbow trout in the Hampshire farms involved, whilst the evidence of furunculosis consistently present at sites suffering from PKD meant the two diseases selected here were deemed to be the most problematic. With this in mind, populations should be tested for the presence of additive genetic variation to the most prominent disease, and where genetic variation exists, families should
be selected so that production generates fish with a higher, and general, tolerance towards the most damaging diseases.

Further, it would be beneficial if earlier disease resistance was indicative of later disease resistance as a trait. Future research in this area relating to the two diseases covered here would also benefit the industry. Another potential advantage would be indirect selection for disease resistance via production traits. Having provided evidence of correlations between disease resistance and fish size in the current project, indirect selection for disease resistance via size could eliminate the difficulty and expense of disease testing when such traits have a high and favourable genetic correlation (see Falconer and Mackay, 1996), and also where the economic risk of disease outbreak exceeds the cost of progeny testing (Perry et al., 2004), which is generally the case on sites suffering from PKD. For the populations used in this project, specific management guidelines should be developed and a breeding plan implemented where the most efficient course of action is taken to alleviate the effects of PKD, and subsidiary effects of furunculosis. Numerous selective breeding programmes have already shown the potential for the genetic improvement to commercially important diseases (Hershberger et al., 1990; Gjedrem, 1997, 2000; O’Flynn et al., 1999; Dunham et al., 2001; Vandeputte et al., 2002). By considering the data presented in this thesis and discussing the results with the farms involved, the British Trout Association, scientists at the Institute of Aquaculture, and ongrowers interested in PKD and furunculosis resistant stock, there is every opportunity to initiate a selective breeding programme to improve the resistance to these two commercially important diseases within the British rainbow trout farming industry.
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Appendix 1

Institute of Aquaculture Standard Curve Procedure

Materials and Equipment
TSA Agar Plates
0.85% sterile saline (w/v)
Bacterial isolate to be tested (sample)
Bacterial loop
Bunsen
Sterile pipettes
Sterile pipette tips
Discard jar
Sterile plastic cuvettes
Sterile plastic Pasteur pipettes
Marker pen
Spectrophotometer (wavelength 520nm)
Centrifuge (bench top)
Centrifuge tubes (sterile)

Procedure
Preparation of bacteria
1. Prepare a bacterial broth suspension at log-phase growth (24-48 hours for most aquatic bacterial pathogens) in centrifuge tubes.
2. Centrifuge the culture at 3,500 rpm (maximum) for 15 min at 4°C.
3. Switch on the spectrophotometer and follow instructions (green sheet on wall).
4. Once the centrifugation is finished, remove the supernatant carefully and resuspend the pellet using 5 ml of sterile physiological saline or sterile PBS.
5. It may be necessary to repeat step 2 if you are using the bacterial suspension for fish challenge work.
6. Place 1-2mls of sterile saline or PBS in a sterile cuvette and use this as the blank. Set the wavelength (610 nm for most aquatic bacteria and 520nm for the cytophaga-like organisms).
7. Remove 1-2mls of the resuspended bacterial suspension (from step 4) and place this into another sterile cuvette. Place this into the spectrophotometer and record the optical density (OD).
Performing drop counts

1. Dilute the bacterial suspension until you have an OD = 1.00. Remove 0.5mls of this and place it into the 1st dilution. Put the tip into the discard jar and then invert the 1st dilution and remove 0.5mls and place this into the 2nd dilution and remove the tip. Continue this until you have completed 1st to 6th dilution (Table 1).

Table A1 - Dilutions

<table>
<thead>
<tr>
<th>1st Dilution</th>
<th>2nd Dilution</th>
<th>3rd Dilution</th>
<th>4th Dilution</th>
<th>5th Dilution</th>
<th>6th Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>$10^{-2}$</td>
<td>$10^{-3}$</td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
<td>$10^{-6}$</td>
</tr>
</tbody>
</table>

2. Mark 3 agar plates into 6 sections and label them OD = 1, $10^{-4}$, $10^{-5}$, $10^{-6}$.
3. Remove 20ul of the 4th dilution and place this onto each of the six segments. Remove the tip and do the same for the 5th and 6th dilutions. Keep these agar plates flat and then seal them and incubate them at the correction temperature. This is called drop counts.
4. Dilute the bacterial suspension at OD = 1 to OD = 0.9 and repeat steps 7-11, but this time label the 3 agar plates at OD = 0.9 $10^{-4}$, $10^{-5}$, $10^{-6}$.
5. Continue doing this until you have the OD values and have performed the drop counts for OD 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1.
6. Incubate all of the drop count plates overnight, maximum for 2 days and then remove then and count the colonies.
7. Normally only count the 5th dilution with confidence. Count the number of colonies in each of the 6 segments. Then find the average number of colonies and multiply this by 50 and by the dilution factor. This will give you the total number of viable colonies at that OD value.

Example

Calculation: average of 20 cfu X 50 = 1000 X dilution factor ($10^{-5}$) = 100000000 cfu at OD = 1

This means that there were $1 \times 10^8$ cfu per ml at the OD = 1
<table>
<thead>
<tr>
<th>OD wanted</th>
<th>OD actual</th>
<th>Dilution factor</th>
<th>cfu per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.100</td>
<td>$10^{-5}$</td>
<td>$1 \times 10^5$ cfu</td>
</tr>
<tr>
<td>0.9</td>
<td>0.903</td>
<td>$10^{-5}$</td>
<td>$1 \times 10^7$ cfu</td>
</tr>
</tbody>
</table>

8. The standard curve is produced by plotting the actual OD values against the number of cfu per ml using an Excel spread sheet.

9. Right click on the line and click “add trendline”.

10. Then click on options and tick the 3 lower boxes. This will give you the $R^2$ value of the line and display the line and equation on the graph. The closer the $R^2$ value is to 1 the better the standard curve.

11. Use the equation to calculate the number of colonies required or the OD value required to obtain a certain number of colonies.

12. Record all results in lab book.
Appendix 2

- Euthanase fish and remove section of posterior kidney
- Using forceps repeatedly ‘dab’ the cross-section on to a glass slide until excess blood no longer remains
- Allow to air dry
- Dip slide five times in fixative solution 1 (1 dip per second)
- Dip slide five times in red staining solution; solution 2 (1 dip per second)
- Dip slide five times in blue staining solution; solution 3 (1 dip per second)
- Rinse in buffered water (pH 7.0)
- Blot dry, and place in Xylene
- Mount
- Observe under microscope