

ACID WATER TOLERANCE IN A NEW
ZEALAND NATIVE FRESHWATER FISH

by

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ABSTRACT

ACID WATER TOLERANCE IN A NEW
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Physiological experiments were conducted to measure the effects of low pH water upon Na^+ fluxes and swimming performance in east coast neutral water banded kokopu, and west coast banded kokopu living in naturally acid water. This allowed comparisons to be made regarding the relative acid tolerance between the two population stocks. A morphological study of the gills using scanning electron microscopy and transmission electron microscopy was carried out on a limited number of fish to compare gill structures of east coast acid and non acid exposed fish with west coast fish. East coast kokopu from neutral water showed a reduced sodium influx on the first day of exposure to pH4 water. This was accompanied by a reduction in passive loss or efflux. After 4 days at pH4 influx recovered allowing the fish to approach sodium balance. West coast fish showed no significant change in influx upon introduction to pH7 water and maintained influx values in pH4 water near those measured for control fish.

For east coast fish, swimming performance was significantly impaired on the 2nd swimming trial when pH was lowered from 7 to 4. On the third swim with the pH returned to 7, U_{crit} was similar to the initial value. West coast banded kokopu showed a significant increase in performance between trial one and three and were unaffected by pH7 water, matching performances measured in control fish.

Gills from east coast acid exposed fish resembled west coast fish in that the trailing edges of the lamellae were swollen due to the large numbers of chloride cells which

were present to a point approximately half way up the lamellar edge. Microridges were present on the apical surfaces of the chloride cells of east coast control fish which contrasted with the microvilli structures observed in west coast and acid exposed east coast fish. The microvilli structures present on the chloride cells of west coast and east coast acid exposed banded kokopu appeared to assist in the anchorage of mucus to the apical surface of the cell.

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Chapter 1

GENERAL INTRODUCTION

1. *Galaxias fasciatus* Gray, 1842

Thirteen species in two genera represent the Galaxiidae family of the order Salmoniformes in New Zealand (McDowall, 1970). This makes it the largest family of freshwater fish in New Zealand. Of these thirteen species five are diadramous, with the other eight species completing life cycles in freshwater (Main, 1988). The three kokopu species are included in the diadramous group. Banded kokopu (*Galaxias fasciatus* (Gray, 1842)), giant kokopu (*G. argenteus* (Gmelin, 1789)), and shortjawed kokopu (*G. postvectis* (Clarke, 1899)), are all 'large galaxiids'. They are the most deep bodied of the New Zealand Galaxidae and are present as juveniles in the annual New Zealand whitebait run. The other diadramous species are inanga (*G. maculatus* (Jenyns, 1842)), which makes up the largest proportion of the whitebait catch and koaro (*G. brevipinnis* Guenther, 1866), another of the 'large galaxiids'. This study focuses on the banded kokopu. This is the only kokopu species remaining in Canterbury, and fish collected from Banks Peninsula streams were used along with West Coast fish in a comparative study of environmental physiology.

The banded kokopu, *Galaxias fasciatus* (Gray), is endemic to New Zealand and has been markedly reduced in numbers this century (figure 1). Although unlike the giant and shortjawed kokopu, it is still found in Canterbury, its distribution is restricted to a few streams on Banks Peninsula. In Westland while relatively more abundant, populations have become disjunct and are associated with forested streams and naturally acidic water. Previous studies on distribution of the banded kokopu adult fish have shown an association with acidic water in Westland where brown water streams commonly measure pH 4 or lower but experiments carried out on kokopu whitebait (the

colonising stage) have indicated that juvenile banded kokopu (whitebait) enter low pH and neutral pH water equally often with no apparent preference (M. Main, 1988).

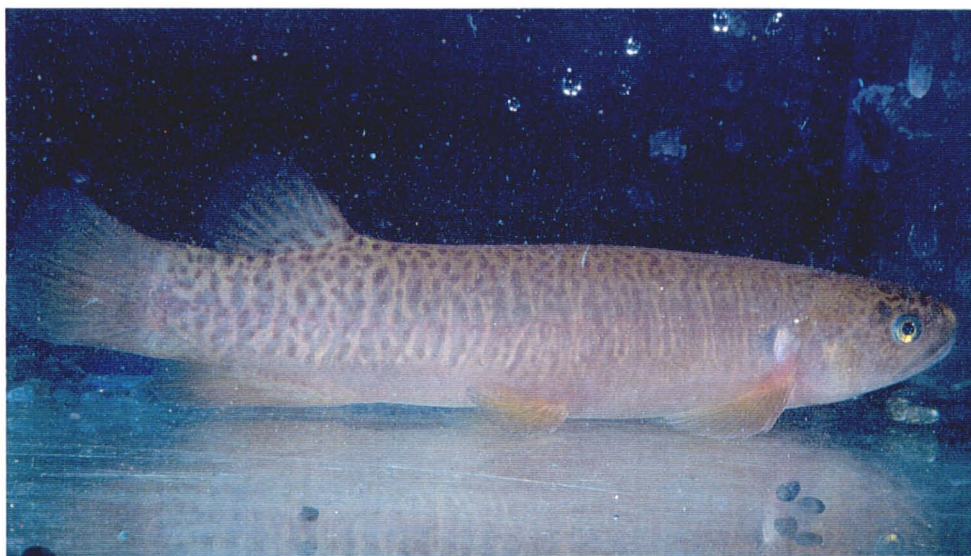


Figure 1-1: Lateral view of east coast banded kokopu (length = 30cm).

The banded kokopu is probably the best known species of *Galaxias* in New Zealand excluding the inanga. Like the giant kokopu it is sometimes known as Maori trout or native trout. Kopu is one of the Maori names used for the fish (Graham, 1956). The banded kokopu tends to inhabit small, stable streams with rocky-boulder beds and small cascades interspersed with small sandy pools (McDowall, 1970). Pools which offer cover from overhanging banks or logs are preferred as it is a very shy fish which takes cover quickly when disturbed. Although it is not uncommon to find several fish in one relatively small pool it is essentially a 'solitary' fish which does not school. The presence of forest cover is an important feature of the preferred habitat (Main, 1988), although populations may be found in stable streams that are deeply entrenched and heavily overgrown with stream-bank vegetation (McDowall, 1970). Although they appear to be more active at night, they are often visible during the day in dimly lit streams. Presence of forest cover may also provide food as they are mainly invertebrate predators and about 70% of invertebrates taken are terrestrial (Main, 1988).

Historically it appears that the banded kokopu and the giant kokopu have been confused in regards to taxonomy. Several scientific names have been applied including

reticulatus and *brocchus*, however the name *G. fasciatus* has been in use for over a hundred years. It would seem that this is the most appropriate as *fasciatus* means banded (Latin) (McDowall, 1970). The confusion between banded and giant kokopu is apparent when reading accounts written earlier this century. This extract from Graham (1956) probably includes giant, banded and possibly shortjawed kokopu judging from reported distribution and sizes.

“Reported that this freshwater fish seemed to be as abundant as ever. It is still found in all districts, in both high and low country. More specimens of this fish were sent to me and from a greater area than all other fish put together. Found from the mouths of rivers and creeks, to the very fountain-head, far above high waterfalls and away from the usual haunts of men. How these fish reach some of these localities is beyond my knowledge. In colour they considerably vary with the nature of the creek or lake, and are usually blackish or reddish brown with undulated more or less irregular light coloured cross-bars. My observations go to show that they vary with the nature of the habitat. Those found in dark, secluded creeks or ravines, where rotting logs are to be found, were fat and sluggish as compared with those living in stony and fast-running creeks with little or no vegetation overhanging the water. Kokopu, or Mountain trout grow to a length of 16 inches, and weigh up to 3 pound”
(Graham, 1956).

While the banded kokopu are still present in good numbers in many streams on the west coast it is obvious when reading further into Graham’s account that their distribution has have reduced in since that time. This is true to an even greater extent for the giant kokopu and probably also for the shortjawed kokopu although limited information is available for the latter. It may be that shortjawed kokopu have always been more rare than banded or giant kokopu.

“The fish were so abundant in those days it was no effort to catch them. Wherever there was one small water hole, especially a rocky one, the kokopu would be there in abundance”
(Graham, 1956).

The diet of the banded kokopu was also noted by Graham and its effectiveness in predated upon mosquito larvae was recognised as a possible controlling agent.

I repeatedly observed the absence of mosquito larvae from streams and lagoons inhabited by the kokopu. This was successfully tested in the laboratory and in field experiments. A well which contained 800 larvae per pint of surface water was almost completely cleared overnight upon addition of 8 kokopu. After 3 days they were completely absent while an adjacent well identical in all other respects continued with a flourishing population. Clearly, the kokopu could, with advantage, be transferred to mosquito-infested swamps, drains and streams" (Graham, 1956).

Kokopu were recognised by the Maori as an important food source. They were held as sacred fish and were caught on a hook and line as described in the following accounts.

The Maori were very fond of the kokopu, and if a fishing party caught the first kokopu it was seen as a good omen for the evening's work. If the fish escaped then a poor catch was foretold; indeed the party has been known to return home without further effort. The first fish caught was retained by the person who caught it and cast aside (not into the stream) as an offering to the god of fishing (Graham, 1956). A sacred method of catching kokopu was used by Maori tribes in the North-Auckland and Waiapu-Gisborne district. A short line of about one foot six inches long is dangled in the water from a long stick of six to eight feet long with a hauhan grub as bait. The following is recited to a gentle pulling motion: Tuna-tuna, parapara, kopukopu, kai mai ra ki taku matire; E mui a ana, e rongu ana; Tukia i to puku nu tukia, i to puku roa mau ka ahū mai ahū mai; Kia u. This formula is sacred to the catching of eels and kokopu, and is part of a larger karakia used formerly (W.J. Phillips, 1949).

The life history pattern of the banded kokopu is thought to be similar to that of the inanga (Main, 1988). In autumn adult inanga migrate to estuarine areas and spawn. This is stimulated by the high spring-tide. Egg development occurs in the air until

reimmersion on the next spring tide. Banded kokopu spawn on stream banks rather than estuaries and subsequent hatching is stimulated by flooding rather than spring tides (Mitchell and Penlington, 1982; Ots and Eldon, 1975). Upon hatching the fry swim to sea for a growing period of about six months (McDowall, 1968). In spring juveniles migrate into freshwater as whitebait.

Inanga are economically the most important species making up the greatest proportion of the whitebait catch in most areas. The importance of koaro and the kokopu species varied from nil in some areas, to 30% or more in some Westland rivers where they still made up a significant proportion of the catch (McDowall & Eldon, 1980).

A study on the distribution of kokopu and koaro was conducted in 1988 by Malcolm Main as part of a Masters of Science degree. He noted that distribution of what he termed the "large galaxiids" was disjunct. Deforestation had been suggested by McDowall (1980) and Main *et al.*, (1985) as a major determinant in population reduction in the case of the large galaxiids. Main (1988) considered that a number of limiting factors were associated with deforestation in affecting distribution. These factors included food, temperature, microhabitat and stream water pH. He also investigated possible competition with brown trout. Results of distribution studies showed that Westland kokopu species were usually found in waters of below neutral pH and often in very acidic water, as low as pH 4.2 (Main, 1988).

The inhabitation of acidic water does not appear to be attributed to 'choice' of low pH by kokopu whitebait. It is at the juvenile stage of the life cycle that streams and rivers are entered by the galaxiids hence whitebait were targeted for pH choice experiments. Main (1988) found kokopu whitebait to be unselective in channels ranging in pH values between 5.0 - 7.0. McDowall & Eldon (1980) found that kokopu whitebait entered both neutral and acidic water streams in South Westland in large numbers. Malcolm Main considered this, asking why adult kokopu were found in predominantly acidic waters on the west coast?

Banded kokopu inhabit neutral streams on Banks Peninsula and are relatively abundant in number in such streams. The lack of discrimination by kokopu whitebait suggests possible exclusion of adult kokopu from neutral water in Westland rather than any type of preference *per se* for acidic water. Factors excluding kokopu from neutral stream water on the west coast could be related to micro-habitat, or could result directly from competitive exclusion or predation from brown trout (*Salmo trutta*), a territorially aggressive fish with a low tolerance for acidic water.

The term 'acid water', is used in this thesis to describe water with an upper pH limit of 5.6, equivalent to the pH of pure water in equilibrium with atmospheric carbon dioxide (Turnpenny, 1989). In 1986 the UK Acid Waters Review Group defined three categories of water with respect to acidity.

- I. 'acid': pH occasionally < 5.6 , low alkalinity;
- II. 'never permanently acid': pH usually < 5.6 , alkalinity zero or close to zero;
- III. 'occasionally acid': pH never < 5.6 , well buffered.

The majority of research carried out on acid water and its effects on fish fauna has been conducted in the Northern hemisphere where acidification of waterways (streams, rivers and lakes) is predominantly due to acid deposition resulting from pollution. Acid rain, sleet and snow actually falling into waterways accounts for approximately one third of this deposition (Mason, 1989), with the majority being represented by dry state gases and small particles which reach waterways through a secondary means such as snow melt or water running off the catchment.

Brown water streams on the West Coast of the South Island, New Zealand, typically belong in the permanently acid (i) group by the above definitions. Recordings of stream water pH in some South Westland streams were as low as 3.8 (Main, 1988). My own observations from collection sites of banded kokopu indicated pH values as low as 4.01 in a Hokitika river tributary near Maitai. In the same stream following two days

of heavy rainfall, pH was found to have risen to pH 4.62, clearly remaining well within the permanently acid group. These recordings are in line with pH recordings for sampling areas by Main (1988). He found that kokopu species were predominantly associated with streams of pH < 5.0. These streams tend to be at low altitudes with associated forest cover. Forest cover may be required to maintain low stream water pH. Harriman & Morrison (1982), cited in Main (1988), described forested streams in Scotland which were found to be of lower pH than open streams with otherwise similar catchment attributes. The same authors discussed the process by which trees take up cations including calcium and magnesium. This removal of alkalinity contributes to stream water acidity. The process by which trees collect acid precipitation and subsequently release H⁺ ions into the water was also discussed, but is not relevant in New Zealand where acid precipitation is insufficient. Rainwater pH measures approximately 5.6 in New Zealand and sulphur deposition is about 40 times less than in the worst affected areas of Europe ((Holden & Clarkson, 1986) cited in Collier and Winterbourn, 1987). More importantly, the forest provides a means for which woody debris and other decomposing organic matter can contribute to low pH by being a source of carboxylic acids.

Fish species used in Northern Hemisphere studies have tended to be chosen due to their economic or recreational importance although other species have on occasion been used for comparison within a study. Many studies have focused around observed fish mortality in acid lakes and streams, usually with large numbers of fish killed over a short period of time. Fish deaths are usually attributed to the physical features of the waterway as close to the acid event as possible. This may include measurement of such parameters as pH, calcium and carbon dioxide concentration. Toxicity of aluminium is also related to pH and is moderated by the concentration of calcium present in solution (Brown and Sadler, 1989). The use of studies involving lethal exposure to acid water has been common in Northern Hemisphere fish where acid events frequently result in fish deaths. On the West Coast, it is assumed that exposure to low pH is long term (chronic) and sub-lethal for banded kokopu living in naturally acidic water. Potentially

lethal exposure below the natural pH tolerance for the fish would not occur in its natural environment. It was speculated that acid water which is within the natural pH range for west coast banded kokopu might have harmful effects on naïve (fish never exposed to acidic water) neutral water east coast fish which would normally not be exposed to low pH water. The possibility of genetic differences between east and west coast stocks was also considered due to the probability that during the juvenile (sea faring) phase of the life cycle it is unlikely that east and west coast stocks would mix, and the Southern Alps act as a natural barrier preventing inland mixing of adults. Therefore acid tolerant species may have become predominant on the west coast of the South Island as a result of natural selection which enabled banded kokopu to successfully exist in a habitat which was below the pH tolerance level of brown trout and other introduced freshwater species. This theory is considered with physiological data and in the absence of genetic studies remains only speculation. Reference to lethal studies in northern hemisphere fish will be used for comparative purposes as many of these studies were conducted with similar experimental protocol at comparable pH values.

Many lakes in Southern Norway and Sweden, parts of the U.K., Ontario and North East U.S.A., have reported a decline or loss of certain species of fish fauna since the 1930s. Long term accurate measurements of the magnitude of acidification for the same lakes over this period of time are not entirely reliable, however, it is apparent that there has been some decline in pH over this period and a correlation exists between low pH and "fishless" lakes. A sample of lakes in Southern Norway revealed that over half of those with a measured pH of < 5 were fishless compared with one in seven of those with a measured pH of > 5 (Mason 1989).

Freda and McDonald (1987) conducted studies in which three species of fish with varied tolerance to low pH were tested. Sensitivity to acidity differed between yellow perch; (*Perca flavescens*), (least sensitive), rainbow trout; (*Onchorynchus mykiss*), (intermediate),

and shiners; (*Notropis cornutus*), (least tolerant). Increased sensitivity was characterised by

- shorter survival times.
- greater loss of whole-body ions.
- more complete inhibition of Na^+ uptake.
- greater stimulation of Na^+ efflux.

Fish mortality resulting from acute acid exposure has been associated with a drop in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$. At a pH value of one or more below neutral the active uptake of Na^+ in rainbow trout is inhibited while passive loss is stimulated. The resulting effect is that net flux of both Na^+ and Cl^- becomes negative (Wood, 1989). Toxicity of acid water arises mainly from the disruption of ionic regulation at the gill which is automatically affected due to the epithelia facing and being in direct contact with the water (Heisler 1993). Previous studies on rainbow trout have indicated that influx is more sensitive to external acid than efflux, although a large initial efflux may cause most of the net loss (see chapter 3). The disruption of Na^+ influx is thought to be related to one or more of several possible mechanisms. Na^+ and H^+ may compete for transport sites and/or access channels to the carrier. An increased load on the pump or damage to the Na^+ ATPase at low pH are also possibilities (Wood, 1989).

The nature of the acid exposure, i.e. chronic and sublethal or acute may have some bearing on the mechanism by which the ionic balance of the fish is disturbed. Increased passive diffusion of ions to the medium is probably due to leaching of Ca^{2+} away from the paracellular channels in the branchial epithelium (McDonald *et al.*, 1989).

Simplified, this suggests that removal of Ca^{2+} under acidic conditions increases the 'leakiness' of these channels to Na^+ which is then lost passively. The mechanism by which net flux of Cl^- becomes negative has not been as well studied, therefore experiments measuring Na^+ fluxes were chosen to investigate acid tolerance in banded kokopu.

A drop in either plasma sodium or chloride ion concentrations of 30% or more results in death within a matter of hours. While a loss of this magnitude is not in itself enough to cause a fatal disruption in the physiology of nerve or muscle function, the associated haematological and fluid volume disturbances upset normal circulatory function (Wood, 1989). Death is thought to be closely associated with this ionic dilution.

Elevated heart rates and arterial blood pressure have been observed in trout surviving three day exposure to pH 4.3. Pharmacological evidence implicates catecholamine mobilisation or 'the stress response' as the cause and combined with changes to blood chemistry caused a doubling of blood viscosity (Wood, 1989). Cardiac workload increases dramatically in the face of increased peripheral resistance. This system would tend quickly toward collapse. Fish measured just prior to death had haematocrit levels above 70%, total viscosity had increased by three times, and blood volumes were less than half of those measured in control fish (Wood, 1989).

Chronic stress caused by sublethal acid exposure has been observed in acid events usually falling in the 'milder' pH range of 4.6-6.0. Studies of this type have grown in importance in the northern hemisphere where some water systems have been undergoing a more gradual pH drop over a number of years. Northern hemisphere trout collected from naturally acidified systems have shown a greater physiological resistance to acid exposure in the laboratory (McWilliams, 1980, 1982, 1983; Brown, 1981). Acid tolerance has been shown to be a heritable trait (Leivestad *et al.*, 1976), ((Swarts, Dunson & Wright, 1978) cited in Wood, 1989), and so tolerance to acidity could have been brought about by natural selection rather than acclimation.

In the brown water streams of Westland the acidity is natural and persistent natural selection would favour fish which were able to tolerate low pH. This could result in population stocks from these streams having a genetically derived acid tolerance. Conversely, acid tolerance may be an indication of acclimation in these populations

which would suggest that all banded kokopu were capable of such acclimation and the species as a whole were highly tolerant to acidity.

Studies attempting to produce an acclimation in brown trout, *Salmo trutta*, showed a recovery in plasma levels of Na^+ and Cl^- when fish were exposed to acidity for 42 days (McWilliams, 1980). This was due to Na^+ influx becoming more resistant to inhibition by acidity. The mechanisms by which the recovery occurs are thought to possibly be associated with a change in endocrine status and a recruitment/proliferation of chloride cells on the secondary lamellae (Wood, 1989). However, this ability to acclimate to acid water may be limited as studies were carried out in hard water which ameliorates the effects of acidity on ionic balance, and the acidic challenge was very mild (pH6) (McWilliams, 1980). Longer term studies (up to three months) were carried out on juvenile Atlantic salmon (*Salmo salar*) at pH levels of 4.5-5.2 in naturally soft water. Effects observed included “retarded growth and development, reduced branchial ATPase activities, impaired ionoregulation, and failure of smoltification”, (Saunders *et al.*, 1983; Wood, 1989). Although partial recovery was shown by surviving fish, $[\text{Na}^+]$ and $[\text{Cl}^-]$ in plasma were found to be depressed compared with control fish. This is consistent with studies conducted by Leivestad *et al.*, (1976) on brook trout, *Salvelinus fontinalis* which were exposed to pH 4.6 for a year and never fully recovered Na^+ and Cl^- to control values. Brook trout held at pH 5.2 (milder acid challenge) eventually show complete compensation and return to control levels of plasma ions.

This work aimed to monitor the effects of pH4 water on the Na^+ balance, swimming performance and gill morphology of banded kokopu from neutral water populations and populations from naturally acidic water. Acid exposed fish from Banks Peninsula were compared with a control group from the same stream which was not exposed to low pH. Banded kokopu from several west coast populations were collected from naturally acidic water and tested at their natural pH and also in neutral water in the laboratory for comparison with east coast fish.

Chapter 2

COLLECTION AND MAINTENANCE OF FISH

2. COLLECTION AREAS

EAST COAST

Banded kokopu were collected on Banks Peninsula from two adjacent streams. Wainui Valley stream in Akaroa harbour has a catchment of 10 km². Most of the catchment is forested although the lower reaches is pasture grazed by cattle, and willows are present. The stream flows for 4km and rises at 650m a.s.l (Malcolm Main, 1988).

Ohinepaka Bay stream is adjacent to and just south of Wainui Valley stream. It has a catchment of about 3km² and is dominated by broadleaf forest consisting of mainly kowhai and pigeonwood. It runs for 3.5km and rises to 700m a.s.l (Malcolm Main, 1988).

Both of these clearwater streams have a circum-neutral pH.

WEST COAST

West coast fish were collected from various brown water streams near the Hokitika River and the Lake Kainere area. All streams were naturally acidic with a pH range of 4.01 to 4.80. West coast streams used in collection tended to be low lying and slow flowing (except after rainfall). Collection sites were all forested with dominant species including kowhai (*Sophora microphylla*), broadleaf (*Griselinia littoralis*), and rimu. A large amount of woody debris was typically present in the streams as a result of falling branches in rainwater runoff washing the dead wood into the waterway. Other streams

were surveyed for available species such as Kaituna stream. This was wider and swifter flowing than the streams mentioned above and was found to contain giant kokopu rather than banded kokopu as well as introduced trout.

2.1 COLLECTION METHODS

Banded kokopu were almost always collected at night. This was due to their apparent preference for nocturnal activity. It was possible to see fish on some occasions during the day but this would tend to be in patches of stream shaded by forest cover or containing large amounts of woody debris. Fish would be spotted with the aid of a handheld torch and caught with a small handheld net (basket approximately 50cm wide). A powerful halogen spotlight was initially used but this was later traded for a smaller 'maglite'. The more powerful light while providing better illumination would often startle all fish in a pool causing them to hurriedly take cover before they could be netted. This type of disturbance would often muddy the pool eliminating any chance of further fishing there or directly downstream until the water cleared again. As most fishing was done with only myself in the water a smaller torch allowed greater freedom with my hands and was held together with a second small net which would block the front escape route for the fish while the other net was brought up behind the fish.

Usually the fish would be 'stalked' individually by approaching it from behind with slow deliberate movements. Any quick movements would also cause the fish to take cover in the stream banks or underneath large immovable boulders. When the fish was within netting distance the first net would be slowly placed behind and slightly above the fish would usually be resting on its fins in a relatively shallow area of the pool. Once the net was positioned the second net would be introduced slowly into the water forward of and slightly above the fish. Once both nets were in position the net at the anterior would be brought down quickly toward the head while the other net would be lowered. Most often the fish would about turn and 'burst' swim into the back net or otherwise accelerate forward into the net placed over the head. Several variations of this technique were used depending on the size and depth of the pool of collection and

the proximity of the stream bank to the fish. It was almost always successful although it required a large time investment in stalking the fish.

Particularly in the east coast streams, certain fish were found to occupy the same pools in the same streams often being in exactly the same position in the pool as a previous visit. This would enable a second attempt after a failed capture and also allowed return of the fish to their original pool of capture. The larger kokopu tended to occupy the most favourable territory of a particular pool and would usually be found in shallow water where flow was at its slowest velocity. They would typically be found in a resting position probably indicative of a 'sit and wait' predatory style. Smaller kokopu tended to be more active, usually maintaining station in the more central parts of a pool which probably indicated that they were lacking territory. This theory is supported by my own observations on several occasions of smaller banded kokopu being chased in a burst type swimming action by larger individuals after venturing to the edge of the pools. After chasing off smaller fish, the dominant adults would usually return to their original position after several revolutions at cruising speed around the pool, provided no further disturbances prompted them to remain swimming or take cover.

Once caught, fish would be stored in large plastic containers filled with water from the collection site. Buckets would be covered with a lid which would be weighted down with a heavy object to prevent escape as banded kokopu proved themselves capable of dislodging lids by jumping out of the water and hitting it with their snout. Banded kokopu were also capable of very coordinated movement on land in a sinusoidal motion and could easily move back to the water after escaping from a bucket. Care was taken to keep water in the buckets fresh while further collection continued and further changes of water were carried out during the journey back to the laboratory with water brought from the site. Before and during the journey back to the laboratory the water was gassed with a battery powered air pump which maintained high oxygen conditions. If possible, fish were returned to the laboratory immediately after collection finished as this was likely to lessen stress on the animals and so reduce mortality.

2.2 MAINTENANCE OF FISH

Fish were held in the wet laboratory and in the temperature controlled aquarium room. Water pH always corresponded to the approximate pH of the collection site from which they were captured, i.e. pH4 for west coast fish, pH7 for east coast fish. All fish were maintained at their 'home' pH until and during the first stage of every experiment to allow initial measurements of physiological parameters to be as near as possible to basal or normal measurements. For west coast acid water fish, manual addition of hydrochloric acid into the aquaria was usually required daily as pH tended to drift slightly upward (see materials and methods section for more information on this).

West coast fish were held in 60 litre static water aquaria which each had biological filtration and aeration systems operating. East coast neutral water fish were usually held in the flow through tanks with water flowing at a rate of 1-2 litres per minute. All aquaria were supplied with shingle, rocks or plastic pipes which were used as shelter by the banded kokopu. Large fish were housed alone for territorial reasons and when held together in an aquarium would tend to occupy diagonally opposite areas of the tank and often different heights in the vertical water column with the larger fish usually resting on the bottom. Smaller fish were usually housed 2-3 to a tank and no territorial behaviour was observed as often smaller fish were found lying close together in the tank.

Fish were typically fed after one day of recovery following capture except for the exercise groups which were not fed in favour of moving more quickly into experiments resulting in a shorter duration in the laboratory and a quicker return to the stream. Food which was not consumed within 24 hours was cleaned out of the tank to prevent decay. Food included trout pellets, tubifex worms, earthworms, cockroaches and locusts. Larger fish would only take live food and tended to feed off the surface of the water as observed by Malcolm Main (1988).

2.3 TREATMENT OF DISEASE

Banded kokopu were very difficult fish to keep in the laboratory, an observation which has been supported by Malcolm Main and NIWA staff (personal consultations). Fish tended to develop a fungal infection which was likely to be *Saprolegnia sp.* if held in the laboratory for longer than two weeks. Although infection was probably normally due to skin abrasions through handling or interactions with other fish, often the fungus would develop overnight in fish which appeared very healthy the previous day. Several fish from east coast populations were observed to carry this infection in the wild possibly as a result of high rainfall increasing water current and causing abrasive damage against rocks and other debris in the stream. These fish were not brought back to the laboratory. In general east coast fish were more susceptible to infection than west coast fish although this could be purely related to the larger average size of east coast fish as larger fish were more susceptible than smaller ones.

Infected fish were isolated and treated with malachite green bathing solution for several hours in a static water tank. The recommended concentration used by Adrian Meredith (1985) during his work on Canterbury Mudfish *Neochanna burrowsius* (Phillips) was 0.05 mg/l for three days. Due to the rapid onset of infection and the speed at which the banded kokopu succumbed to the fungus a more highly concentrated dose for a shorter duration was adapted with better results. This concentration was not measured as such, but was monitored by experience with the colour of the water. Fish would often recover from such infections although the skin was sometimes scarred and/or raw in the affected areas and secondary infections often occurred unless the area healed rapidly. Fish with such infections were not used in experiments.

Overall the incidence of disease and general mortality was largely reduced by ensuring that time in captivity was kept to a minimum. For this reason long recovery times after capture were concluded to be impractical due to the adverse effects on the fish outweighing the possible benefits in regard to experimental procedure.

Chapter 3

SODIUM BALANCE

3. INTRODUCTION

Freshwater fish are hyperosmotic regulators. They are required to actively take up ions from a dilute medium to maintain body tissue and fluid ions at much higher concentrations than are present in the surrounding water. Active uptake of ions is required to counter the passive loss across ion permeable membranes or through ion leaky junctions. Brown trout exposed to pH4 lose the ability to regulate ionic levels of body tissues and body fluids (Leivestad *et al.*, 1976). Severe ionic imbalance affects fundamental physiological processes including, but not limited to, nervous conduction and enzymatic reactions.

“Most measurements of the effects of acid toxicity on aquatic animals concentrate upon changes in body fluid pH and the flux of ions between water and blood”, (Thomas, 1989). External acidity which resulted in a lower blood pH could potentially cause acidification of tissues and a deviation from the optimum pH of intracellular enzymes (Thomas, 1989). Heisler (1989) also states that deviations from certain set-points may result in reduced metabolic performance, due to the enzyme activity of metabolic energy-producing processes having pronounced pH optima. Heisler concluded that there were three general mechanisms available for fish acid-base regulation; buffering, adjustment of P_{CO_2} by changes in ventilation, and branchial ion transfer processes. The limitations of branchial ion transfer mechanisms (in regard to acid-base regulation) mainly reside in the availability of external counter ions, which may be low in naturally soft, low pH water.

3.1 LOW pH AND ACID-BASE BALANCE

McDonald (1983) studied the responses of rainbow trout to low pH at different levels of water hardness [Ca^+]. At a mean pH of 4.3 the physiological disturbances in the trout were dependent on the level of calcium in the environment, and not the levels of other ions. High [Ca^+] was associated with a relatively small plasma ionic disturbance; however a marked blood acidosis was measured. The reverse was observed at low [Ca^+]. This is consistent with results collected by Wood (1989) who stated "while acidosis can confidently be dismissed as a cause of death during pure acid stress in natural soft water, this may not be entirely true in hard water".

The mechanisms by which a differential acidosis may occur between hard and soft water is related to the acid-base theory that solutions separated by membranes (e.g. blood and external water) are only able to interact in acid-base terms "by processes which alter the values of their independent variables, which are the difference between strong cation and anion concentration (SID), the PCO_2 , and the total weak acid present (mainly protein in blood)" (Stewart, 1978). Wood writes that the latter two are minor in their involvement in a situation of acid challenge in fish (1989). Relative movements of strong cations and anions are the important factors due to the automatic positive or negative net H^+ flux they create regardless of how the movements occur. This need not involve the actual movement of H^+ across the gills due to water being a source of acidic equivalents. The acid gradient between the blood and the environment is immaterial to this flux (Wood, 1989).

McDonald noted that disturbances to acid-base regulation and to oxygen delivery only occur when calcium levels are elevated (hard water) and combined with low pH. This suggests that although branchial acid-base regulation mechanisms may be limited by non availability of counter ions in soft water, it is unlikely that acid-base status would be challenged in soft water in a resting fish although plasma ionic levels may show greater disturbance. When [Ca^{2+}] levels are elevated (hard water) and pH is low the leakage pathways for ions are cation selective which constrains net H^+ uptake. In soft

water under low pH conditions, the overall permeability is increased in relation to diffusive loss but is anion selective. This results in a negligible H^+ uptake or even excretion of H^+ . The mechanisms by which $[Ca^{2+}]$ might modulate relative anion to cation losses in acid water remain unknown (Wood, 1989). Internal acidosis in resting fish resulting from low pH high $[Ca^{2+}]$ water would be unlikely to occur in the wild as calcium serves to buffer acidity and generally prevents low pH values from being achieved.

Wood (1989) attempted to correct the misconception that external acidity must cause internal acidosis in fish stating that more studies need to be conducted in soft water. Mortality in acid sensitive fish usually ensues as a result of ionic imbalance and the associated osmotic problems which follow. In physiological studies of rainbow trout at low pH in soft water Wood found no internal acidosis was brought about through internal acid-base disturbance. No significant change in pH_i in any measured compartment was measured in soft water while in hard water the brain, liver, white muscle, and red muscle were all well regulated in the face of extracellular acidosis. There were small but significant changes observed in the heart ventricle myocytes and the red blood cells. Cameron (1989) wrote that acid-base regulation was only indirectly related to low external pH. Cameron states that a change in the acid-base status may result from two possible mechanisms: the permeabilities of various other strong ions (Na^+ , Cl^- , etc.) are changed leading to imbalances of these ions which will often result in changes in the acid-base status; and many of the transport proteins in external membranes have hydrophilic tails whose configuration and affinity are strongly affected by pH ((Philpott, 1989) cited in Cameron, 1989). He writes that it is "somewhat surprising that the effects of external pH are no larger than they are". Many workers now regard the acid-base disturbance as secondary in importance to ion and water imbalances (Wood, 1989). Wood states that while acid-base balance may be upset to some degree in very hard water, the extent of the resulting acidosis is likely to contribute only as a secondary factor to mortality. Rarely has Wood seen a hard water acid death which did not have an accompanying 30% electrolyte loss (1989).

3.2 LOW pH AND IONOREGULATION

The principle effect on fish of chronically toxic acid exposure is an ionoregulatory disturbance. Understanding how acid environments interfere with branchial ionoregulatory mechanisms is of central importance to understanding the effects of low pH.

The effect of low pH on branchial Na^+ flux of banded kokopu from neutral and acid water was chosen as a physiological parameter for study. The maintenance of Na^+ balance is essential to optimal physiological function and it has been shown to be sensitive to pH change and therefore a good indicator of relative acid tolerance in fish. Measurement of Na^+ flux allows non invasive techniques to be used eliminating the need for potentially harmful blood sampling and/or catheterisation. This enabled fish to be returned to the wild following experimentation in my study which was consistent with the conservation based aims of the project. Inhibition of Na^+ influx is instantaneous and irrespective of external Na^+ (Maetz, 1973). As noted above it is more highly disturbed in soft, poorly buffered waters and under these conditions also gives good indication of Cl^- balance due to the close stoichiometry between these two ions in soft water, while being less complicated to measure than Cl^- . Losses in soft water of Na^+ and Cl^- are nearly equimolar (McDonald, 1983).

3.3 MATERIALS AND METHODS

ANIMALS

Fish ranging in weight from 18.0g to 183.1g ($n=20$) were collected from Banks Peninsula as described in the collection methods chapter. West coast fish ranged in weight from 12.2g to 47.7g ($n=12$). Sex was not distinguished in this experiment and was therefore assumed to be random.

EQUIPMENT

For sodium flux experiments fish were contained in 'flux boxes'. The boxes contained a known water volume which was determined by weighing the boxes and subtracting the dry weight of the box, lid, screws etc, and the wet weight of the fish. Between experiments water flow was maintained by means of a recirculated flow system. The system contained a header tank to generate a water pressure gradient through the flux boxes and a bottom tank which received water from the boxes and held a submerged pump which worked continuously to return water to the header tank. In addition both the header and the bottom tanks contained two biological filters to control ammonia and other nitrogenous waste. An overflow pipe from the header tank to the bottom tank ensured that no water was lost from the system when the pumped water volume exceeded that flowing through the flux boxes. Aeration of water occurred in each of the flux boxes and in the four biological filters in the holding tanks. Containment of fish in the flux boxes for the duration of the experimental period lessened the amount of stress to the fish through excess handling and activity in the laboratory. Experiments were carried out in soft water, ie with a hardness of less than 0.5 mM CaCO₃ (described as the general boundary delimiting 'hard' and 'soft' water by (Marier *et al.*, 1979) cited in McDonald *et al.*, 1989).

The concentration of sodium ions in the water contained in the flux boxes ranged from 0.38 mmol.l⁻¹ to 0.84 mmol.l⁻¹. To monitor influx of Na⁺ the radioactive isotope ²²Na was used. During the three hour experimental period the water flow was stopped leaving approximately 1500mls inside the boxes. Bungs were inserted into the inflow and outflow of each box. This effectively sealed the boxes with the only exception being an air hole on the lid to prevent build up of air pressure from the constant aeration. The air hole was contained in a bung in the lid, which was removed at time of sampling to allow entry of a 5ml Gilson pipette. A 5ml sample was removed from each of the four boxes prior to addition of the radioisotope. This provided a radioactive 'blank' (ie background radiation count) for each of the four boxes. Following addition of the radioisotope a five minute 'mixing' period was allowed to ensure homogenous

distribution of the ^{22}Na . A sample ($t=0$) was then taken from the medium surrounding the fish and was followed by further sampling every 30 minutes until the experiment was completed ($t=180$). The samples were stored in a rack and labelled according to the time at which they were sampled for each fish.

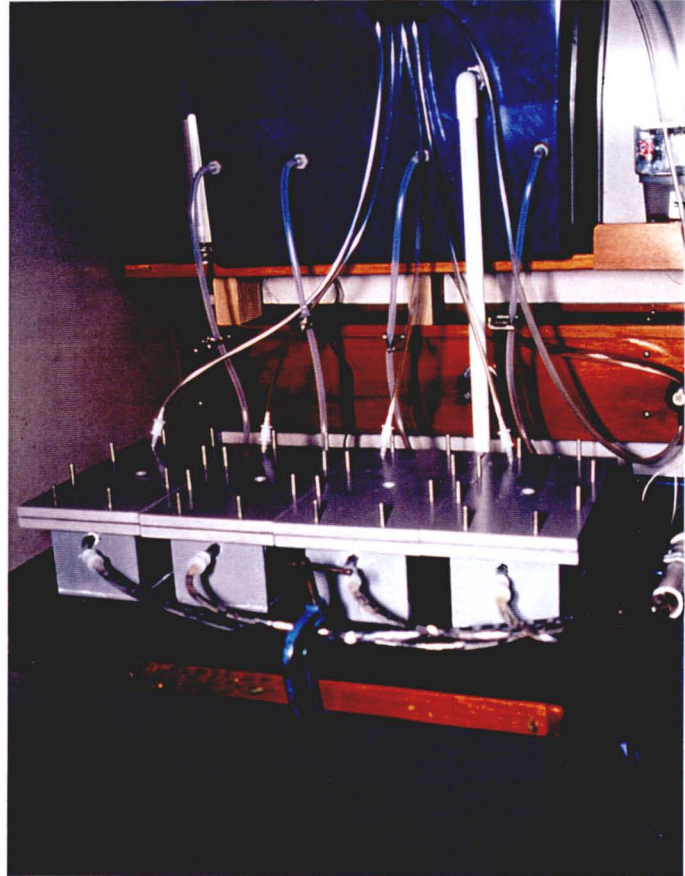


Figure 3-1: Flux boxes in which fish were enclosed for the duration of the experiment. Water flow pressure was generated by the header tank. Water flow would be stopped during the experimental trials to allow Na^+ fluxes to be measured over a three hour period.

Addition of the isotope in to the medium provided a means of tracking the influx of sodium ions through its disappearance into the fish. Points representing the disappearance of radioactivity over time were plotted on a graph and a regression line was drawn according to the best r^2 value. The regression output at a known external Na^+ concentration allowed the influx for each fish to be calculated in $\text{mmol}/100\text{g}/\text{hour}$. Due to the external concentration of sodium ions being insignificantly small in comparison to the total amount of sodium in the fish, it was assumed that during the three hour sampling period no significant backflux of radioactive sodium would occur.

Because of the small quantity of isotope taken up and the 'noise' when measuring radioactivity, no correction was made to allow for the removal of samples. Net flux was determined by flame photometry and atomic emission spectrophotometry which allowed the change in Na^+ concentration of the water over time to be measured. The efflux was then calculated as the difference between net flux and influx.

Previous studies by Wood (1989) on rainbow trout used a similar approach with the addition of a bladder catheter to measure relative contributions to ionic regulation of the gill and kidney. Urine was collected and analysed separately allowing the assumption that all environmental ionic exchanges in the medium were a result of branchial process only. Catheterisation was not considered in this experiment due to banded kokopu being much smaller than rainbow trout and likely to suffer effects detrimental to the health of the fish and therefore the results of the experiment. It is important to note that in Woods' results, he concluded that in soft water the kidney's contribution to net flux is negligible (Wood, 1989). We did not expect physiological disturbances in naïve (never exposed to acid) banded kokopu to be as severe as those measured in rainbow trout. The rainbow trout is recognised as a very acid sensitive fish while west coast banded kokopu have been observed living in naturally acidic water with a pH value similar to that which was often fatal in rainbow trout. Wood also states that under any environmental conditions, the gill accounts for 80% of the major electrolyte loss and all of the net acidic uptake. Therefore it is a reasonable assumption to expect that measured environmental changes in Na^+ concentration were due primarily to branchial exchanges and the need for invasive catheterisation techniques would not have served to improve the methods enough to justify the potentially harmful effects on the limited fish supply. From Curtis and Wood's (1992) experiments on rainbow trout we can calculate that a similar rate of urinary Na^+ loss in banded kokopu would raise external Na^+ concentration by c. 1% over the three hour experimental period. This is at the limits of detection in our system. Urinary loss was not considered problematic to results with Na^+ influx not being affected due to influx

measurements being dependant on the disappearance of the radioisotope, and ion loss through urine being an event which would be measured if contributions to the total loss were significant. There was no effort made to isolate the relative contribution of the gills and kidney in ion loss.

Water pH was controlled within a pre-determined range by manual addition/dilution of acid. HCl was used due to its strong acidic properties and due to the low toxicity of the Cl⁻ anion. The water flow through the flux boxes was halted at the time of addition of acid to header and bottom tanks when changing pH from 7 to 4. This was to prevent toxic shock which could result from inadequate mixing of concentrated HCl added to the bottom tank. The pump served to mix the acid thoroughly between the bottom and header tank and the water flow was opened to the flux boxes again when the desired pH was achieved in both the header and the bottom tank. Due to a slight tendency for pH to drift over time due to alkanisation, addition of acid to low pH water was typically required once daily to maintain pH within 0.2 of a pH unit which was considered acceptable to the experimental protocol and was within the control range used for similar studies. Experiments usually took place during the mid-morning - early afternoon period although on a few occasions they were started earlier to accommodate swimming trials scheduled for the same day which took place during late January 1996 through to the end of March 1996. Na⁺ flux measurements were carried out over two periods being May 1995 to September 1995 and November 1995 to early March 1996. Water temperature in the system was maintained between 14.9°C and 15.4°C for the winter period and between 16.9°C and 17.4°C for the summer period.

STATISTICAL TREATMENT

Data are reported as means \pm 1 SEM (n) within each experimental treatment for all groups of fish. The paired Students t-test was used to compare means of the 1st set of data with the 2nd, 3rd, and 4th flux experiment for each experimental group. All significant differences were at the 99% (marked on graph with **) or 95% confidence level (marked on graph with *).

3.4 RESULTS

All flux results are stated in units equivalent to mmol/100grams/hour.

WAINUI TEST GROUP

Figure 4.2 shows mean sodium ion flux results for the Wainui test group. Upon introduction to pH4 water on day four, mean influx was reduced sharply from 0.062 to 0.024. This reduction in mean influx is significant at the 99% confidence level. Mean efflux is also reduced from 0.137 to 0.065 which is significant at the 99% confidence level. The overall effect of the reduction in mean efflux is to lessen the net sodium ion loss to the medium which is occurring due to reduced sodium ion mean influx. The fish appeared to remain in negative net sodium balance however despite this reduced efflux. After four days in pH4 water mean influx was increased slightly while mean efflux remained at a similar rate. This further reduced the net sodium loss. After being returned to pH7 for four days, influx had been further increased from trial three, achieving a value not significantly different from that measured in trial one. Mean efflux remained similar for trials two, three, and four, and remained significantly lower than measured for trial one.

These results show that the effect of pH4 water was a significant reduction in mean influx which had recovered to some extent after four days exposure. Mean efflux was also reduced in pH4 water which probably helped offset the reduction in mean influx and reduced the net loss of sodium ions to the water. Mean influx was fully recovered to a statistically similar value as measured for trial one upon return to pH7 water with mean efflux levels significantly lower than trial one. This meant the fish were on average closest to sodium balance during trial four in pH7 water.

WAINUI CONTROL GROUP

Figure 4.3 shows mean sodium ion flux rates for the Wainui control group for four trials at pH7. Wainui control fish showed a much reduced mean efflux rate (0.075) compared to the test group on the first trial (day four in pH7 water). Mean influx was

measured at 0.040 during trial one. For the second trial (after 4 days in pH7 water), there had been no significant change to efflux or influx. On the proceeding third trial, there was no significant change in mean influx values although mean efflux was slightly reduced to 0.039. This was followed during trial four by a significant increase in mean influx and efflux to 0.079 and 0.125 respectively.

The control group of fish showed that the absence of acid challenge resulted in no depression of mean influx values over the whole experimental period. Fish remained slightly in a negative sodium ion balance except for trial three where mean influx and efflux values were not significantly different. The large mean influx value measured for the fourth trial is almost solely attributable to one fish which had an influx measurement approximately ten times the average influx values for the previous trials. The same individual fish was also largely responsible for the accompanying large mean efflux values although one other fish had its largest measured efflux in the fourth trial. This experiment supports the view that reduction in mean influx values does not simply result from containment in the flux boxes and supports the proposal that acid challenge caused the reduction in the east coast test group.

WEST COAST GROUP

Figure 4.4 shows sodium ion flux rates for the west coast group. Influx did not change significantly during any of the four experimental trials. Mean efflux fluctuated more showing a significant increase in trial two in pH7 water. Efflux was reduced when returned to pH4 water on trial four to a measurement not significantly different to that measured at pH4 in trial one. Overall the only significant change in any measured mean flux value was that measured for mean efflux in trial two (pH7) which showed an increase compared to trial one which was significant at the 95% confidence level. Fish were in a slightly negative sodium balance for the entire experimental period as was observed for other groups. Net flux values were on average less negative than for the east coast test group.

This experiment suggests that mean influx values of west coast banded kokopu are not affected by change in water pH under the experimental conditions. Overall the average values for influx and efflux were of a similar magnitude when expressed as flux/100grams/hour despite the average size difference between the east and west coast fish.

AVERAGE SODIUM ION INFLUX AND EFFLUX FOR EAST COAST TEST GROUP FOR FOUR TRIALS
AT pH7, pH4, pH4, and pH7

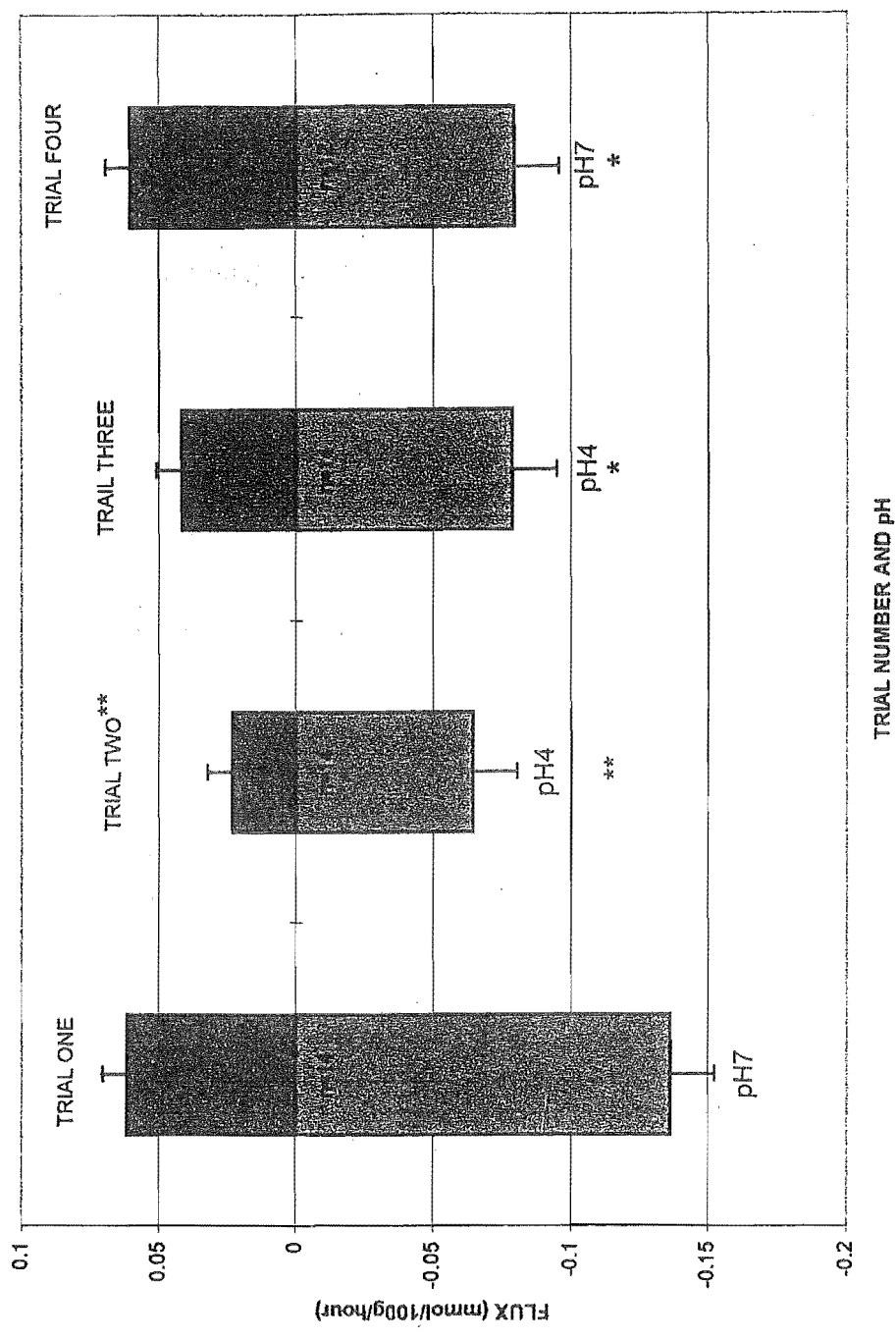


Figure 3-2: East coast test group showed a significant reduction in average Na⁺ ion influx and efflux in east coast fish upon exposure to pH4 water in trial two compared to trial one. (** = significant at the 99% confidence level). After four days in pH4 influx recovered to near initial values. Efflux remained significantly lower than trial one (* = significant at the 95% confidence level). Na⁺ balance was nearly achieved after four days in pH7 water (trial 4). n = 14 for all trials. Trial one = 4 days in pH7 water; Trial two = 1 day in pH4 water; Trial three = four days in acid water; Trial 4 = four days in neutral water. All trials were concurrent.

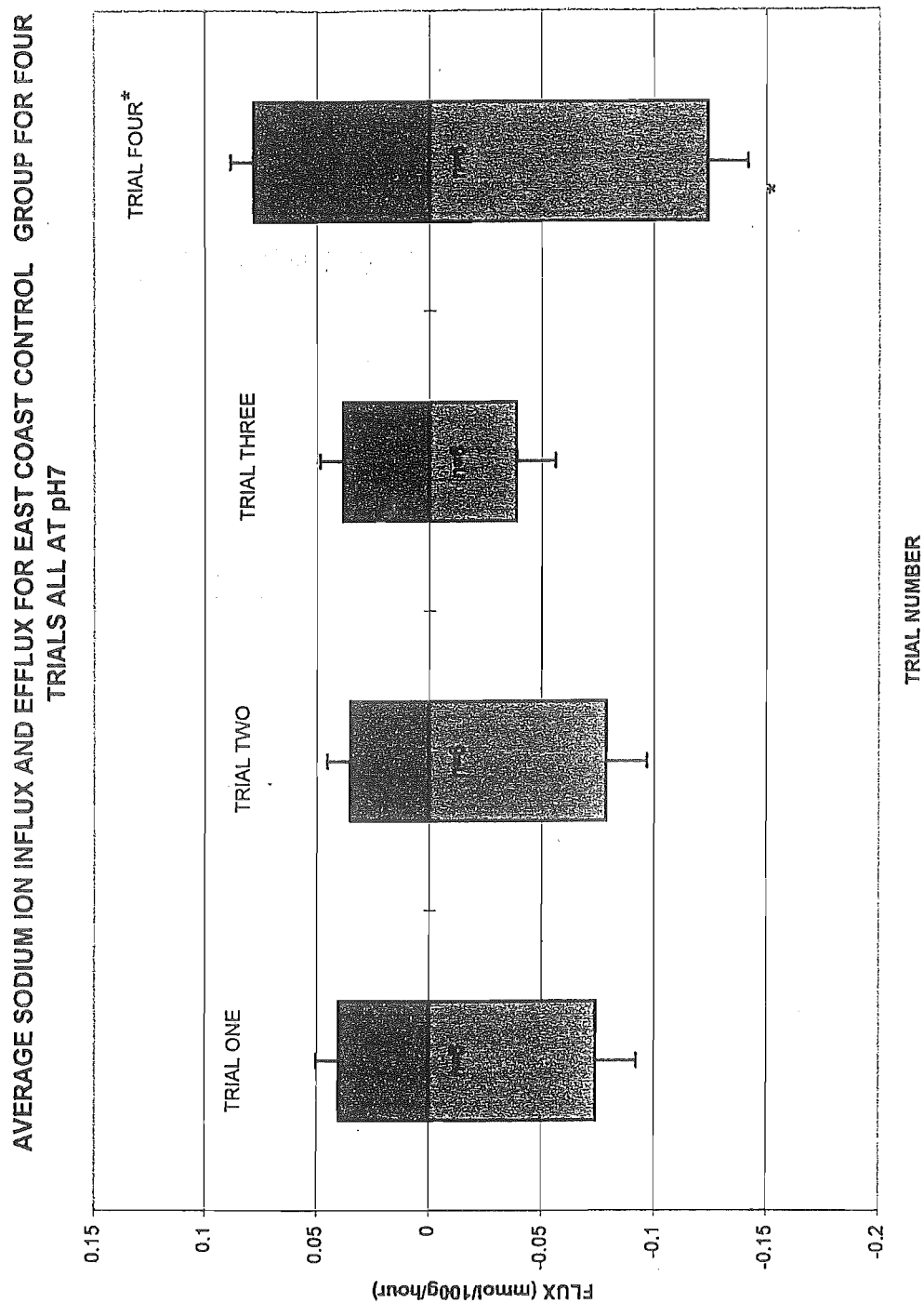


Figure 3-3: East coast control fish showed no reduction in average Na^+ influx over the four trials in pH7 water. Trial four showed a significant increase in influx and efflux (* = significant at the 95% confidence level). The control group showed that the reduction in average sodium influx and efflux demonstrated by the test group in pH4 water was not due to being contained in the flux boxes. $n = 6$ for all trials. All trials were at pH7 on the same days as the east coast test group.

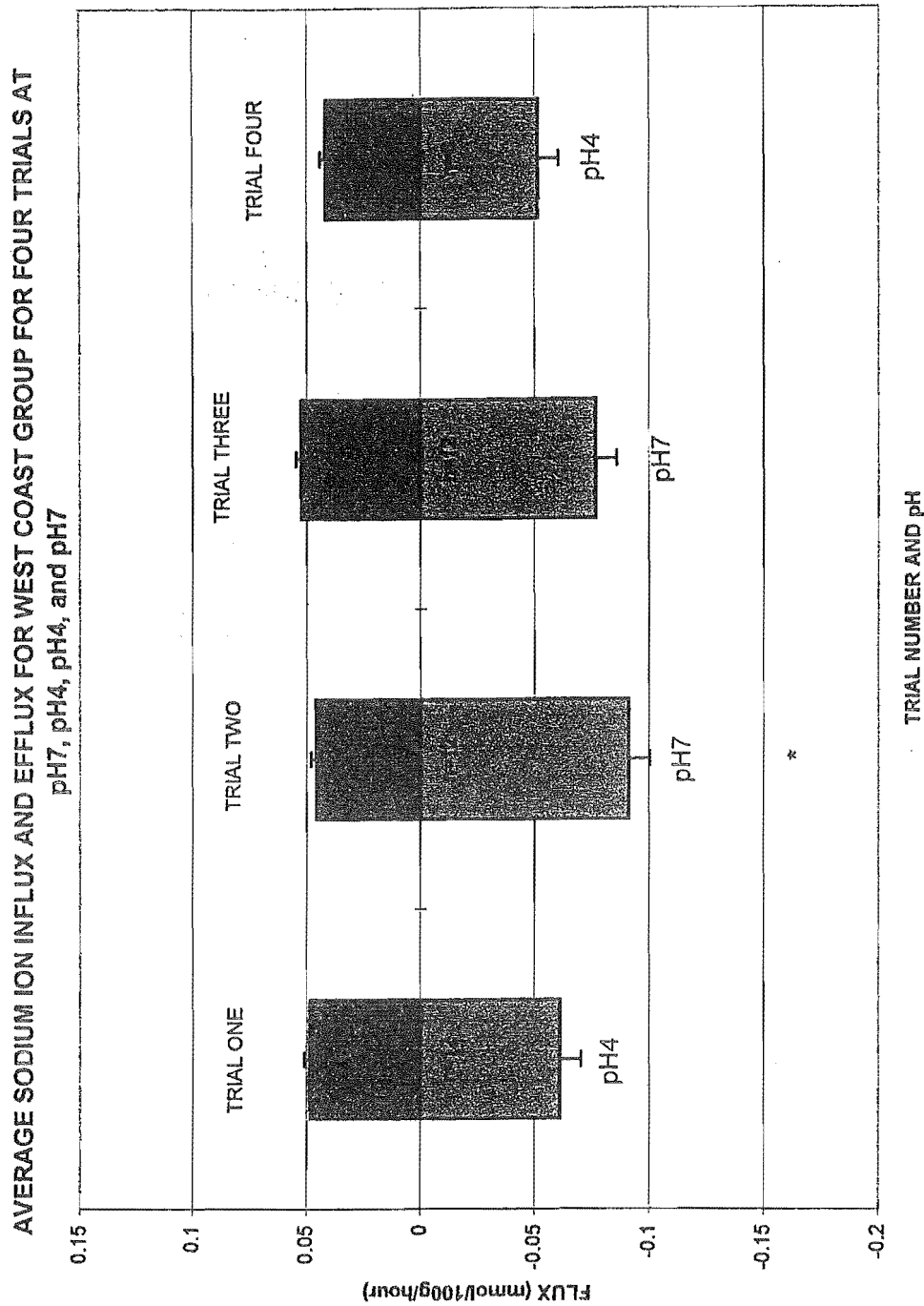


Figure 3-4: West coast banded kokopu showed no significant changes in average Na^+ influx over the four trials. Efflux increased significantly on trial two at pH7 (* = significant at the 95% confidence level). No other changes in efflux were significantly different from trial one. $n = 12$ for trials one, two and three. $n = 8$ for trial four (group of four fish developed fungus and were removed from trial four). Trial one = 4 days in pH4; Trial two = 1 day in pH7; Trial three = 4 days in pH7; Trial four = 4 days in pH4.

3.5 DISCUSSION

The west coast banded kokopu showed a relative insensitivity to pH change during this experiment. There was no significant change in influx between any of the trials at pH4 or pH7 and only a minor fluctuation in efflux which increased slightly for the pH7 trials. The increase in efflux for the pH7 trials was consistent with the Wainui test group which showed a reduced efflux at pH4. West coast banded kokopu would be expected to encounter little difficulty with the acidic challenge they were exposed to in this experiment as it was within the pH range of their natural habitat from which they were collected. It was also anticipated that west coast banded kokopu living in low pH streams would frequently encounter higher pH values due to the consistent rainfall associated with the west coast. In the northern hemisphere acid precipitation serves to add to the acidity of a waterway and lower the pH. Acid precipitation is not a significant feature in New Zealand and therefore rainfall on the west coast of the South Island is likely to be closer to neutral and have the effect of diluting the natural acidity of west coast brown water streams and swamps.

It was predicted that a fish which was tolerant to life at low pH would not suffer any detrimental effects in neutral water. The possibility was considered that acid tolerant (west coast) fish might improve average performance as a result of enhanced sodium ion uptake capabilities and reduced efflux due to lower gill permeability. However this was not observed in this study as neutral water trials showed no significant change in sodium ion influx.

The Wainui test group exposed to pH4 water showed depressed Na^+ influx rates and an overall negative net Na^+ flux on the second trial (first day pH4 water). This 'acute' effect has been demonstrated in many studies on freshwater teleosts and along with the loss of body electrolytes is thought to be one of the primary physiological responses to acid challenge (Wood, 1989; Muniz and Leivestad, 1980; McDonald and Wood, 1981; McDonald, 1983). These authors have reached a consensus that death of fish exposed to acid water is caused by a chain of events, beginning with the loss of sodium ions, and

leading to osmoregulatory and cardiovascular failure. It is apparent that neutral water banded kokopu show some degree of tolerance to acid challenge. Reduction of influx upon acid exposure was not accompanied by an increased efflux which would have been expected for an intolerant species with no pre-exposure to low pH water, as shown by Wood (1989) with rainbow trout. A decrease in efflux was shown on the first day in pH4 water. This contrasted with results from rainbow trout exposed to similar pH values in other experiments (Freda and McDonald, 1987; Wood, 1989) which showed a large increase in measured Na^+ efflux upon acid exposure.

The need to limit water volume to ensure small fluxes of Na^+ could be measured could potentially have caused much stress on fish being confined to such a small space for the duration of the experimental period. It is possible that this stress would have been greater for larger fish but due to limited numbers available, preferential sampling for a particular size was not viable. Stress effects were difficult to account for and due to having (relatively) low sample sizes, individual variation may have had a significant effect on the groups overall average.

The purpose of the non acid exposed group in these experiments was to determine whether being confined in the flux boxes for an extended time and having water samples taken under 3 hour stopped flow conditions for 4 procedures, had any effect on measured sodium fluxes. The results from this group suggest that there was little change in sodium fluxes over time in the absence of a pH4, acidic challenge and changes in the east coast test group were due to pH4 exposure.

East coast neutral water banded kokopu showed a greater tolerance to acid challenge when compared to similarly naïve rainbow trout exposed to pH4 water. Data from Freda and McDonald (1987) is shown in table 4.1 to compare the acute exposure to pH4 water in common shiners, *Notropis cornutus*; rainbow trout, *Oncorhynchus mykiss*; yellow perch, *Perca flavescens*; and naïve east coast banded kokopu. The data from Freda and McDonald has been adapted from the original units ($\mu\text{Eq Kg}^{-1} \text{h}^{-1}$) to mmol

100grams⁻¹ h⁻¹ to allow direct comparison with the results of this study. Results have been taken from a graph with no table and so the accuracy is limited by the interpolation.

TABLE 3.1

GRAPH OF SODIUM ION INFLUX AND EFFLUX FOR FOUR FISH
EXPOSED TO ACUTE pH4 CHALLENGE

FISH SPECIES	pH	INFLUX (mmol.100g ⁻¹ .h ⁻¹)	EFFLUX (mmol.100g ⁻¹ .h ⁻¹)
SHINER	4	0	0.60000
RAINBOW TROUT	4	0	0.2800
YELLOW PERCH	4	0.0120	0.10000
E.COAST BANDED KOKOPU	4	0.024	0.065

Banded kokopu are shown from this comparison to have a greater ability to take up Na⁺ ions and control Na⁺ efflux under acid challenge than rainbow trout, shiner and yellow perch. Bearing in mind that the probable major disturbance in the physiology of acid exposed fish in soft water was the mechanisms of ion regulation, it is logical to expect that mechanisms allowing tolerance of acid water would be related to reducing or negating this disturbance.

Freda and McDonald (1987) concluded that control of efflux of sodium ions during acid challenge was the most important factor in determining survival. Naïve east coast banded kokopu demonstrated a significantly *reduced* sodium ion efflux when exposed to pH4 water compared with initial measurements in pH7 water, and no mortality was observed in this group which could be directly associated with pH4 exposure.

Rainbow trout are shown to have zero influx upon exposure to pH4. Wood (1989) also showed that sodium ion influx in rainbow trout was virtually obliterated during pH4 exposure and together with greatly stimulated efflux of sodium ions acted within

minutes to cause a net loss of ions to the water. McDonald (1983) described the rapidity of ion loss, rather than the absolute amount of loss as being the key factor to mortality. Therefore slowing down the net ion loss by reducing the efflux and maintaining some degree of influx is advantageous and probably the difference between fish mortality or survival at least for short term exposure. This conclusion is supported by Freda and McDonald (1987) who stated that the differences in tolerance between species is related to their rate of ion loss. Furthermore they state that “variation among species in acid tolerance is not so much related to the *basic mechanisms* of H^+ toxicity, but at the *threshold* at which large ion losses occur” (my emphasis).

Fish exposed to acid water are thought to go through a ‘shock’ phase during which influx of ions is nearly completely inhibited and large increases in ion efflux result in a rapid net ionic loss to the environment. The following recovery phase seen in surviving animals involves a partial to complete recovery of efflux and a recovery of influx which is slower and less complete. Efflux reduction is thought to reflect a reduced branchial permeability to ions during the recovery phase.

Acid tolerant species (or less tolerant species undergoing a ‘mild’ acid challenge), would be expected to survive the initial shock phase and enter the recovery phase. As survival rates become more prolonged persistent inhibition of influx becomes more important as efflux recovers. If influx remains depressed due to pH inhibition, the net loss of ions will be likely to continue. This situation would probably lead to a more ‘chronic’ physiological disturbance such as a failure of sodium dependant enzymatic and nervous processes as plasma ionic levels drop below minimum thresholds (Leivestad, 1976).

Naïve banded kokopu give no indication that a ‘shock’ phase was entered into at all. Trial two measured sodium ion influx and efflux over the *first hours* of exposure to pH4 water and no stimulated efflux was observed. Inhibition of influx was measured during the first three hours of exposure but at no time was it completely blocked (as in shiners or rainbow trout), or even reduced to the level measured for the more resistant yellow

perch (Freda and McDonald, 1987). After four days of pH4 exposure sodium ion influx was increased to a value slightly less than, but not significantly different from trial one in pH7 water, while efflux of the ion remained reduced.

Comparisons of efflux values measured in east coast test and control groups for trial one in pH7 water show some disagreement, being approximately twice as large on average for the test group (figs 3.1-3.2). There is no clear reason why this is so as fish were of similar size, were from identical collection sites and were subjected to identical trials in pH7 water for this trial. Although this could potentially place doubt upon the real magnitude of the observed efflux reduction when the test group were exposed to pH4 water in trial two, the argument that efflux is controlled at low pH is still substantiated as it was approximately equal to the 'normal' influx value for the ion measured in trial one in neutral water.

Gills are the primary target for H^+ ions. Leakiness of the gills to ionic loss is largely a function of the physical dimensions of the gills; the diffusion distance, the thickness of the outer mucus layer and the intrinsic properties of tight junctions which seal adjacent cells (McDonald, 1983; Freda and McDonald, 1987). The concentration of calcium in the external medium (water hardness) has been shown to modulate the gill permeability and resulting ionic loss in less tolerant species, but had no effect upon yellow perch which were the least sensitive fish species tested by Freda and McDonald (1987). Our studies were conducted in soft water of low $[Ca^{2+}]$ (see *materials and methods*, this section), and so the protective effect of Ca^{2+} upon the gills would be lessened if banded kokopu were affected by water hardness. Results from these experiments appear to be in line with conclusions made by Freda and McDonald (1987) for yellow perch, and suggest that low $[Ca^{2+}]$ had little effect on acid toxicity in banded kokopu.

Although comparative studies in soft and hard water would be needed to conclude this without doubt, the superior acid tolerance measured in banded kokopu as compared to yellow perch coupled with no mortality and recovery from ionic disturbances support

this argument. This conclusion only extends to ionic disturbances in *resting fish* within the *measured pH range*, and over the *four days* of exposure. The conclusion drawn by McDonald and Freda (1987) that the mechanism of toxicity is the same for tolerant species at a lower pH threshold suggests that at lower pH ($\text{pH} < 4$), the protective role of Ca^{2+} should not be discounted. Furthermore $[\text{Ca}^{2+}]$ may significantly effect acid tolerance in exercising fish (discussed in chapter 4), and protect gills from morphological damage due to prolonged low pH exposure (discussed in chapter 5). It should be noted however, that naturally acidic brown water streams of the west coast are low in $[\text{Ca}^{2+}]$ (Collier and Winterbourn, 1987), and acid exposure is chronic. Therefore it is unlikely, at least for west coast banded kokopu, that $[\text{Ca}^{2+}]$ plays any protective role at all in their natural environment. In the absence of $[\text{Ca}^{2+}]$ playing a role in decreasing permeability to ionic loss, other mechanisms such as increased mucus production and/or morphological changes in the gill structure should be considered (discussed in chapter 5).

In conclusion, the experimental results indicate that ion fluxes in west coast banded kokopu are not significantly altered by interchanging pH between 7 and 4. Naïve neutral water east coast banded kokopu show an initial reduction in influx, but demonstrate control of efflux and recovery of influx over four days of pH4 exposure. Measurements of ionic fluxes in naïve east coast acid exposed banded kokopu indicated that they are more tolerant to acid challenge than three species of fish tested by Freda and McDonald (1987). A control group of east coast fish were subjected to duplicated trials all at pH7 and indicated that the reduction in average sodium ion influx was due to the pH4 exposure and not prolonged enclosure in the flux boxes. Mechanisms of acid tolerance in these fish are likely to involve a decreased gill permeability to ionic loss which is probably independent of external $[\text{Ca}^{2+}]$.

Conclusions in this chapter are restricted to the severity and duration of pH challenge used in the experimental procedure, disturbances to ionic regulation, and are relevant only to resting fish. The effect of low pH upon exercising fish and other possible disturbances are considered in the next chapter.

*Chapter 4***EXERCISE****4. INTRODUCTION**

Swimming performance is critical to fish survival. Once a fish has survived beyond its larval stage the most important biological factor determining its survival is usually predator avoidance ((Pitcher & Hart, 1982), cited in Reidy *et al.*, 1994). This may have been less important to the adult banded kokopu during pre-European history before the introduction of trout. Since the introduction of freshwater fish species such as brown trout it is possible that the banded kokopu has come under increased competition or predation pressure. This was recognised by writers as early as Phillips (1949) who wrote "The introduced trout is its worst enemy and like many of our native fishes it is at its best in a stream where trout do not live". It is advantageous to perform maximally with regard to swimming to ensure successful competition for prey and escape from predation. Inhabitation of low pH water could potentially have deleterious effects on the physiological processes essential for maintaining exercise at optimal levels.

In the previous chapter, ionic disturbances in response to low pH in *resting* fish were measured. Results suggested that disturbances were not lethal in naïve fish and had corrected to a large extent after four days exposure at pH4. Many of the disturbances in the physiology of resting fish exposed to low pH water were similar to responses seen after strenuous exercise. Disturbances in plasma electrolyte concentration (soft water), blood acidosis (hard water), and haemoconcentration were described in resting fish in low pH conditions (Wood, 1989), and similar responses have also been described in fish following exhaustive exercise (Graham *et al.*, 1982). It is likely that the two conditions together would have an additive effect resulting in compounded disturbances to the

physiology of fish. Exercise has been shown to exert a significant effect on acid toxicity in rainbow trout under both hard water and soft water conditions with the exercise groups surviving for significantly less time between pH 3.4 and 4.2 (Graham and Wood, 1981).

At low swimming speeds the fish primarily exercises aerobically, however anaerobic processes are recruited as the speed increases (Reidy *et al.*, 1994). In this study U_{crit} was used as the protocol to measure swimming performance. This is the critical swimming speed protocol which has become the performance test of choice for many physiologists due to the large amount of work which has been directed toward calibrating the U_{crit} procedure (Reidy *et al.*, 1994). In the absence of simultaneous measurements of oxygen consumption the U_{crit} method does not allow assessment of relative importance of aerobic and anaerobic components in isolation during the test period. However from other studies and the vigorous swimming observed close to U_{crit} it is almost certain that anaerobic metabolism was employed at high swimming speeds.

As described in the previous chapter, the mechanisms of acid toxicity are complex and exercise potentially further complicates the processes which are already modulated by other environmental variables such as water hardness. Exhaustive exercise is in itself a potentially lethal event. Experiments in which rainbow trout were swum to exhaustion resulted in 40% post exercise mortality (Wood, *et al.*, 1983). Fish which died were found to have a significant post-exercise intracellular acidosis compared to fish that survived. The acidosis was related solely to an accumulation of lactic acid which evidently was too great for the fish to unload to the external medium causing plasma pH to remain low.

This acidosis effect is thought to be potentiated in acid water due to the higher external $[H^+]$, which makes unloading of H^+ ions to the water more difficult. The transient storage of surplus H^+ ions in the ambient water has been demonstrated in many species following exercise (Heisler, 1993). As lactic acid is metabolised, surplus H^+ ions are

transferred back into the animal. This will usually prevent intracellular acidosis and lowering of blood pH under normal (neutral) environmental pH conditions.

In acid water the transfer of H^+ to the external water is compromised and in turn may affect the duration of exercise and/or the recovery of the fish following exercise.

Lowering of blood pH causes a Root effect which lowers the O_2 carrying capacity of the blood. Other predicted mechanisms of post-exercise lethality in fish exposed to low pH water have included interference with O_2 uptake (branchial mucus production and/or damage to gill epithelial surface) and elevated ionoregulatory costs (Graham and Wood, 1981). Ionoregulatory disturbances, while controlled and recoverable in resting fish, may not be recoverable in exercising fish due to the higher cost of maintenance.

A comparison of performance in acid and neutral water was made between banded kokopu from Banks Peninsula neutral water streams and west coast acid water. As fish of a similar size were not available from both coasts, standardisation of the U_{crit} procedure was of primary importance.

4.1 MATERIALS AND METHODS

ANIMAL MAINTENANCE AND EQUIPMENT

Adult banded kokopu were collected from January to March by the methods described in chapter 1. As soon as possible after capture, fish were transferred to holding aquaria in the laboratory. This meant returning in the early hours of the morning from Banks Peninsula and the west coast following sampling which resulted in a much reduced period of stress from transportation. Water from the stream of collection was used to transport fish and transport containers were aerated with battery powered air pumps. Fish were selected from those caught according to body length to provide the greatest possible variation for the experimental group with the rest being immediately returned to the field. Fish were not fed before or during the experimental procedure. They were maintained in holding tanks in the aquarium at a pH value corresponding to the stream

water of the collection site. Due to the tendency for experimental fish to succumb to fungal attacks during captivity, the acclimation period prior to the first experimental swim was minimal at two days. For the same reason the recovery time between each experimental trial was set at 48 hours to enable completion of three trials for each fish within six days. This procedure resulted in the fish being in captivity for little over a week and ensured low mortality rates and a high rate of successful returns to the field.

To measure swimming performance, fish were tested in a Blazka-type respirometer. The diameter of the swim tube measured 19cm with a length of 67cm. It contained a water reservoir of approximately 90 litres. It was constructed from clear perspex which allowed observation of the fish while exercising. The tube contained a perspex impeller at one end, drawing water through the tube from the reservoir. Water velocity was controllable by a variable resistor which controlled the amount of current supplied to the motor and therefore the water current velocity.

A light was positioned at the downstream end while the upstream end of the tube was darkened by a black cloth. This served to dissuade fish from resting against the back grid of the tunnel. Banded kokopu are light sensitive and swam to avoid illumination. Most often several flashes of a powerful halogen spotlight would keep the fish swimming in the desired area of the swim tunnel.

Water temperature was maintained at 14.0 - 14.2 degrees Celsius. Initial water temperature was controlled with the addition of melted ice prior to introduction of the fish and during the experiment as required. The water was continuously aerated with an air line connected to an air stone which remained in the water outside of the swimming tunnel. Water flow in the chamber was not turbulent and water velocity (cm.s^{-1}) versus propeller revolutions (hertz), gave a linear relationship when plotted as a calibration line.

MEASUREMENT OF CRITICAL SWIMMING SPEEDS (U_{crit})

Prior to an exercise trial, a fish was weighed and its body length measured. It was then transferred to the swim tunnel from the holding aquarium. Entry to the swim tunnel was through a 30cm length of plastic tubing which allowed the fish to slide through the entrance at the top of the tunnel. The fish typically settled quickly on the bottom of the swim tunnel at the darkened end. A period of one hour was allowed before the exercise trial began. To initiate an experiment water speed was set at an initial velocity of 1 body length. s^{-1} which was increased every 15 minutes by 0.5 body lengths. s^{-1} until the fish was exhausted. Time of exhaustion was defined as the time when the fish did not respond to repeated stimulation from the spotlight and continued to rest on the back of the grid or was unable to swim away from the back of the chamber. At this point the water flow was slowed to the initial velocity of 1 body length. s^{-1} and the timing clock was stopped. When timing stopped the fraction of the 15 minute increment that the fish had achieved was multiplied by the swimming velocity in lengths. sec^{-1} and this value was added to the previously completed increments to give U_{crit} .

TABLE 4.1

EXPERIMENTAL pH CONDITIONS FOR ALL GROUPS OF FISH

GROUP	pH SWIM 1	pH SWIM 2	pH SWIM 3
WEST COAST	4	7	4
EAST COAST (TEST)	7	4	7
EAST COAST (CNTR)	7	7	7

Swim one represents exercise in *natural pH conditions* for all fish. Swim two was conducted after two days in pH7 water for west coast fish and two days in pH4 for the east coast test group. Swim three was conducted after two further days with pH values being returned to the natural pH for west and east coast test groups. The east coast control group was subjected to three trials at pH7 with the same recovery periods as the test groups.

The U_{crit} measured for the first swim was plotted against fish length. This was done for every individual fish from all three groups as all fish were swimming in their natural pH for swim one. U_{crit} measurements (body length. sec^{-1}) were also converted into total bodylengths swum to give a clearer indication of the greater distance weighting of the higher U_{crit} values (ie 15 minutes at 2.5 body lengths. sec^{-1} equals a much greater distance than 15 minutes at 2.0 body lengths. sec^{-1}).

The calculation of total distance swum in bodylengths was performed using the following equation:

$$(U_1 \times T_1) + (U_2 \times T_2) \dots \text{etc}$$

$$U_1 = 1.0 \text{ (bodylength} \cdot \text{sec}^{-1}\text{)}$$

$$U_2 = 1.5 \text{ (bodylength} \cdot \text{sec}^{-1}\text{)}$$

$T_{1,2}$, = time of increment (900 seconds) other than at U_{crit} when the clock was stopped and T was measured as a proportion of a full increment.

STATISTICAL TREATMENTS

Data are reported as means \pm 1 SEM (n) within each experimental treatment for all groups of fish. Repeated measures analysis of variance (Instat 2) was performed on the data, with a post-hoc Tukey Kramer Multiple Comparisons Test to compare performances of the first trial with the second and third trials. Significant differences were at the 99% (indicated by **) or the 95% (indicated by *) confidence level.

4.2 RESULTS

WEST COAST FISH

Nine fish ranging in length from 10.0cm to 15.5cm (average length 13.6cm), were subjected to three trials as described above. West coast fish swam an average distance of

9322 body lengths in trial one at pH4. This increased to 9833 body lengths in trial two at pH7 and further increased in trial three at pH4 to 10590 body lengths (figure 4.1). The increase in performance between trial one and three was significant at the 99% confidence level.

EAST COAST ACID EXPOSED FISH

Eight fish ranging in length from 20.0cm to 26.5cm (average length 21.7cm), were subjected to three trials as described above. East coast fish swam an average distance of 6583 body lengths in trial one at pH7. This significantly decreased to 4429 body lengths in trial two at pH4. In trial three at pH7 fish swam an average of 6075 body lengths which was not significantly different from the trial one value (figure 4.2). The decrease in performance for swim two was significant at the 95% confidence level. Performances for swims one and three were not significantly different.

EAST COAST NON ACID EXPOSED FISH

Seven fish ranging in length from 20.0cm to 24.0cm (average length 20.4cm) were subjected to three trials as described above. East coast control fish swam an average distance of 5475 body lengths in trial one. This increased to 6722 body lengths in trial two at and increased further in trial three to 8015 body lengths (figure 4.3). The increase in performance between trial one and three was significant at the 99% confidence level.

U_{crit} VERSUS LENGTH

A plot of U_{crit} versus length shows that smaller fish achieved higher U_{crit} values when critical swimming speed was measured in bodylengths sec^{-1} (figure 4.4). Regression output: $y = -0.1147x + 5.3884$, $R^2 = -0.6481$

BODYLENGTHS SWUM VERSUS pH IN WEST COAST FISH FOR THREE SWIMMING TRIALS AT pH4, pH7, and pH4

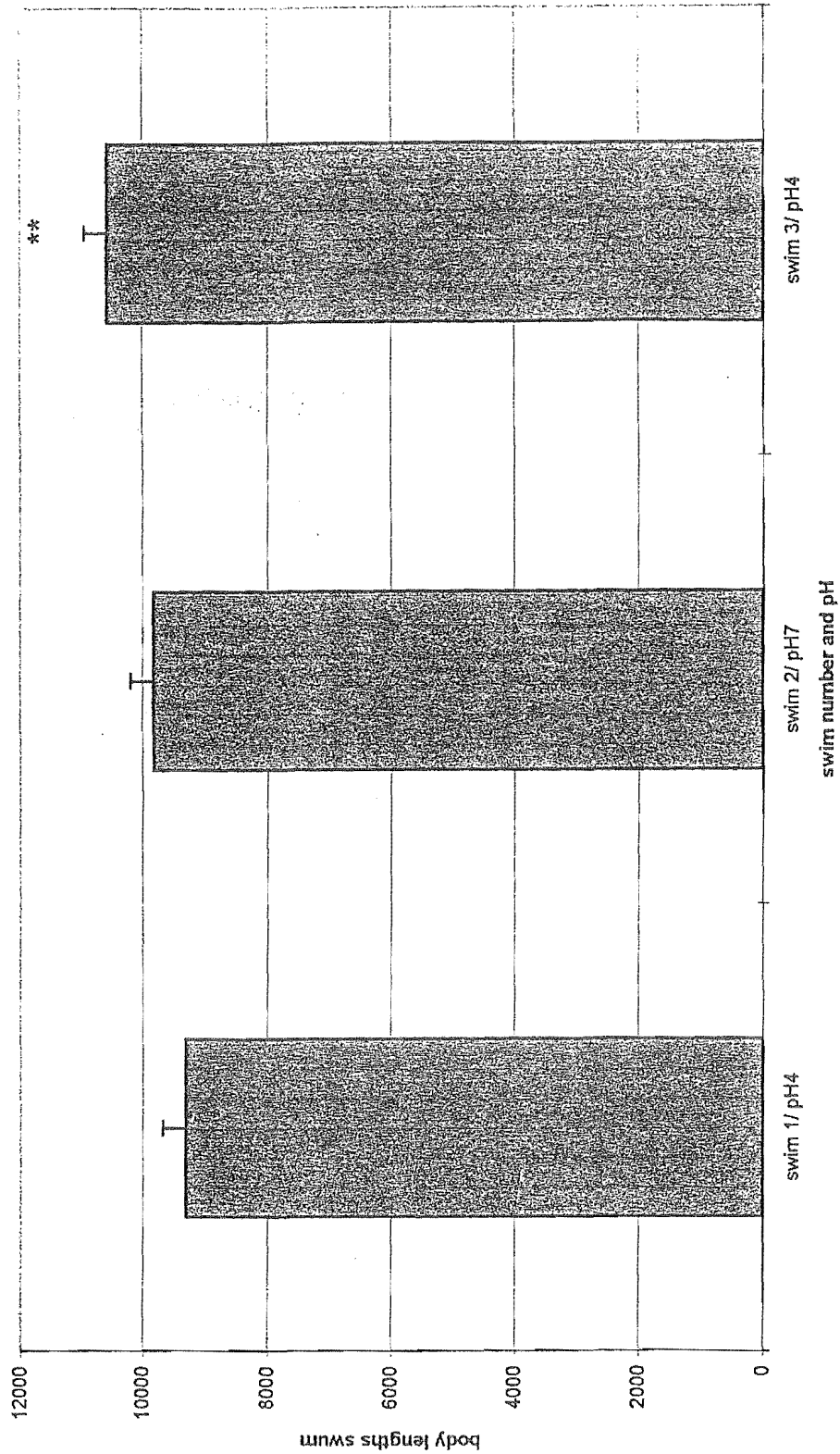


Figure 4-1: West coast fish showed a significant increase (** = significant at the 99% confidence level) in performance between swim one and swim three. $n = 9$ for all trials. Trial one = 2 days at pH4; Trial two = 2 days at pH7; Trial three = 2 days at pH4. All trials were sequential.

BODYLENGTHS SWUM VERSUS pH FOR EAST COAST ACID EXPOSED FISH FOR THREE SWIMMING TRIALS AT pH7, pH4, and pH7

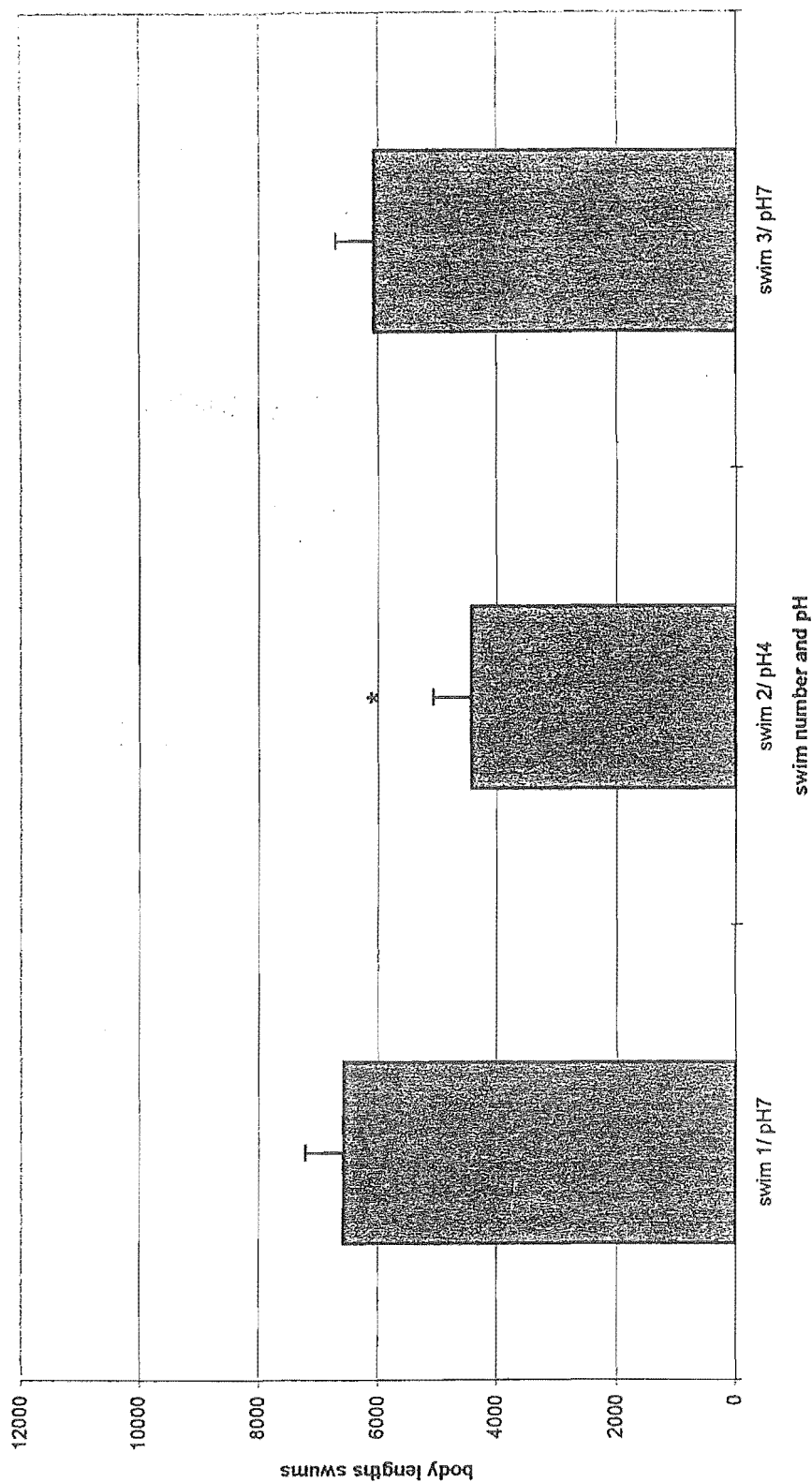


Figure 4-2: East coast acid exposed fish showed a significant reduction (* = significant at the 95% confidence level) in swimming performance for trial two in pH4 water. $n = 8$ for all trials. Trial one = 2 days in pH7; Trial two = 2 days in pH4; Trial three = 2 days in pH7.

**BODYLENGTHS SWUM FOR EAST COAST CONTROL GROUP FOR THREE SWIMMING TRIALS ALL
AT pH7**

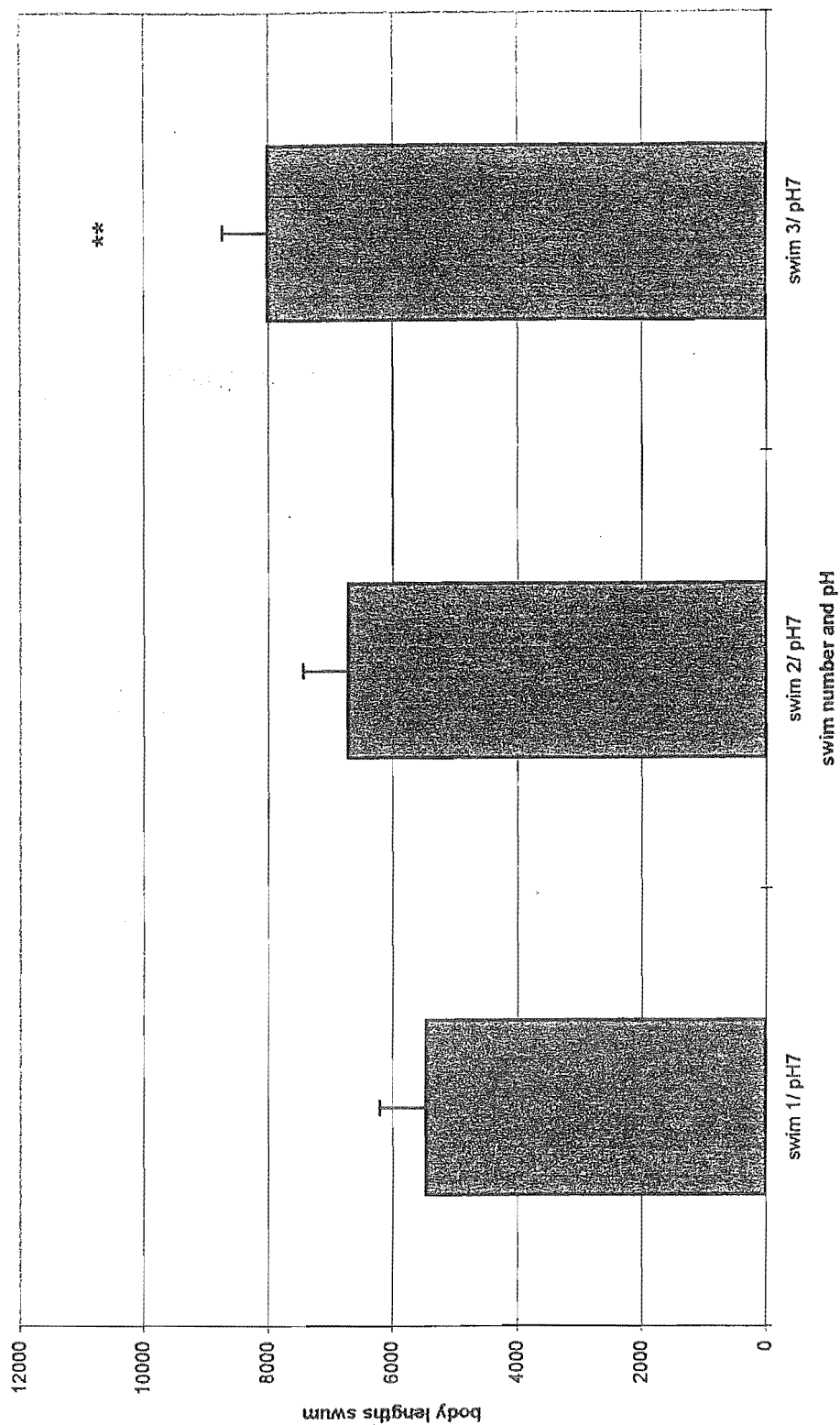


Figure 4-3: East coast non acid exposed fish showed a significant increase (** = significant at the 99% confidence level) in performance between swim one and swim three. n=7 for all trials. pH = 7 for all trials.

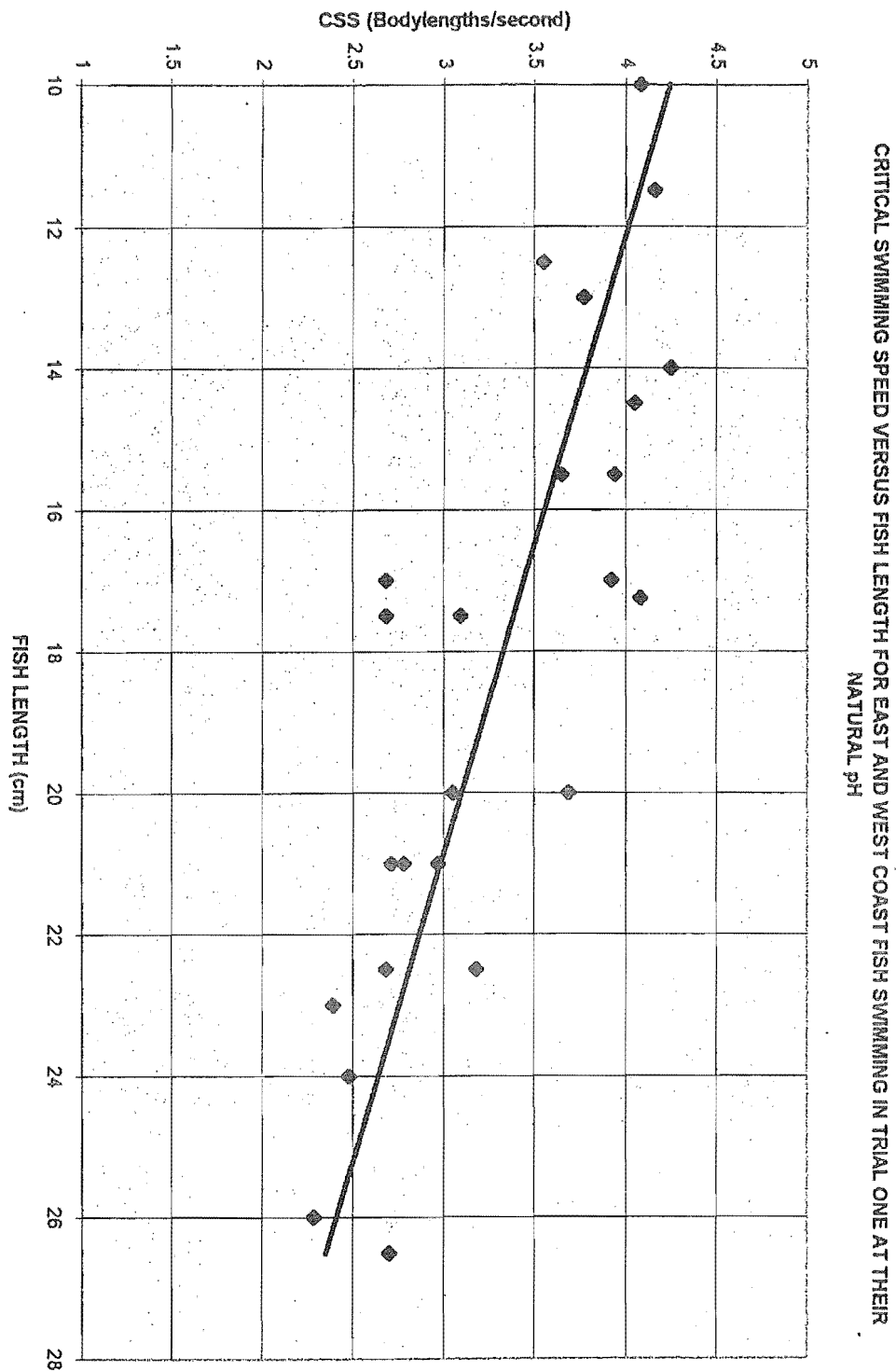


Figure 4-4: Critical swimming speed (bodylengths/second) versus fish length (cm). An inverse relationship is shown between critical swimming speed and body length of fish. $n=24$.

4.3 DISCUSSION

The ability to exercise optimally is essential to a variety of normal behaviours in fish. Feeding, predator avoidance, spawning, migration etc, all require a high level of scope for activity and are important activities in assuring the successful survival capability of a wild population. The method used in this study was similar to that used by Graham and Wood in 1981. The U_{crit} test in the swim tunnel allowed standardisation of experimental procedure, and as close as possible to a uniform treatment for all fish. This allowed a comparative study to be made between the different treatments and quantification of the effect of pH change on critical swimming speed. The U_{crit} test, however, may not give a real indication of the effect of low water pH on exercise in the natural habitat of the fish. For the greater part of the test the fish is assumed to be performing aerobically with an anaerobic component becoming more significant at higher swimming speeds (Reidy *et al.*, 1994). Once migration up the stream is completed and banded kokopu reside in a pool their behaviour is largely territorial (my observation).

The method of prey capture in adult fish mainly resembles a sit and wait type of predator in small, shallow backwaters of a pool where water current is absent or low. Banded kokopu will usually not maintain water station but rather rest on the stream bed upon their pectoral fins. When prey is detected they will sprint from this position often taking the prey from the top of the water column with a head snapping motion before returning to their initial position or taking cover to devour it. If disturbed by sudden movement they will sprint for cover under rocks, stream debris or holes in the bank. Very little time is spent cruising around pools, which are often very small considering the size of the fish occupying it. Rather, most activity of larger adult banded kokopu is confined to 'burst type' swimming either for catching prey or avoiding predators.

Smaller banded kokopu (5 - 8cm) are more active, constantly involved in cruise type swimming and maintaining station in the water column (my observations). This may be related to favourable territory within the pools being secured by the older dominant fish.

The implication here is that although low pH may affect exercise in a U_{crit} type test with a large aerobic component, this effect may be negligible upon anaerobic burst type exercise which appears to be predominant in adult banded kokopu in the wild if the nature of the disturbance is related to impaired oxygen delivery. Anaerobic exercise performance is largely independent of oxygen delivery (Wood *et al.*, 1977).

No measurements of O_2 uptake or any other physiological processes were taken in this experiment. This was due to the problems associated with the high sensitivity of respirometer readings required to measure potentially small differences in the concentration of partial pressure of oxygen in a lone fish swimming trial and the large water volume of the respirometer. A smaller respirometer would have been more suitable for this type of measurement but would have had the disadvantage of potentially compromising the swimming ability of the fish due to size constraints. Measurements of other physiological parameters would have required invasive techniques which, as described in the previous chapter, were not used due to their potentially harmful nature.

In using U_{crit} as the sole measure of swimming performance, conclusions drawn which involve physiological parameters not measured are only speculative. There are, however, many studies conducted using a similar protocol from which comparisons and suggestions can be made. This is especially true when combined with results from other chapters in this thesis in which the effects of acid exposure upon Na^+ balance and gill morphology have been measured. Any disturbances which were measured in these studies would be likely to be also represented and perhaps exacerbated in fish subjected to an equivalent pH challenge while forced to undergo exhaustive exercise.

Comparing the swimming ability of banded kokopu from east and west coast populations has given some insight as to the ability of each population to perform in their natural pH and in the other population's pH. Although the tests were conducted under laboratory conditions, the implications are important when considering the relative tolerance between the different populations to pH challenge and how exercise is affected. If west coast banded kokopu live in sub-optimal conditions in the wild populations due to competitive pressure from brown trout, it might be expected that when forced to exercise at their natural low pH (swim one), performance would be lower than that achieved for the east coast fish swimming at their natural neutral pH (swim one). This would have indicated that inhabiting low pH water may result in chronic sub-lethal acid stress which while being tolerated, was having a deleterious effect upon exercise. This was not the result measured in the U_{crit} test which indicated that west coast fish in low pH water performed at least as well as east coast fish in neutral water for the first swim and did not show an improvement in performance for trial two in pH7 water above that which was expected from the training effect. In this respect west coast fish have been shown to be as well adapted to their naturally acidic waters as east coast fish are to neutral waters.

A reduced performance was measured for east coast fish maintained in acid water for two days. As this result was in the opposite direction to the east coast control group which increased performance during the second trial this result is attributable solely to the lowered water pH which was the only experimental condition to be altered between the two groups. The mechanism by which the acidity caused a reduction in performance cannot be established from this experiment alone as no parameters other than critical swimming speed were measured. We can however, discuss possible mechanisms using information from the previous and following chapters. Other studies that have concentrated on salmonid fish also give useful information as to the mechanisms of acid toxicity on exercising fish.

Graham and Wood (1981) measured a reduction in the critical swimming speed (CSS) in response to acid exposure in fingerling rainbow trout. CSS was found to vary in an approximately linear fashion with pH, declining by about 4% per 0.1 pH unit. The reduction in CSS was thought to be an indicator of *sub-lethal acid stress*. This is also likely to be true for the east coast acid exposed banded kokopu in light of the indication that naive banded kokopu appear to be more acid tolerant than rainbow trout when comparing sodium balance results in response to lowered pH. The drop in swimming performance in the banded kokopu was markedly less than that measured for rainbow trout over a larger pH drop which adds weight to the argument that banded kokopu are more acid tolerant than rainbow trout under soft water laboratory conditions. Graham and Wood (1981) hypothesised that an interference with O₂ uptake caused by acid stress would be a likely cause for a reduction in exercise performance and a dependence of CSS on environmental pH may well occur. Interference with O₂ uptake in rainbow trout exposed to acid water may be a result of morphological changes at the gills, e.g., sloughing of gill epithelial tissue and increased branchial mucus production clogging gills. O₂ delivery to body tissues may be compromised by a Root effect reducing the capacity of the blood to carry O₂.

They conclude that acid probably kills fish by more than one mechanism. O₂ uptake disturbances were thought to be the major factors limiting performance of rainbow trout at very low pH (3.0-3.2) with less severe pH values resulting in a more gradual disturbance in ionic regulation and acid-base balance. The complexity of the mechanism is appreciated when considering that ionic regulation is the major disturbance in soft water, but is much less pronounced in hard water where acid-base balance is more likely to be disturbed. Furthermore these mechanisms are all probably dependent upon the O₂ demand of the fish (Graham and Wood, 1981).

When attempting to assess the relative importance of these mechanisms and how they may apply to banded kokopu it is necessary to acknowledge that the actual higher acid tolerance of the banded kokopu compared to the rainbow trout requires that the pH

values associated with lethal and sub-lethal acidity be redefined. From these results it is unlikely that west coast banded kokopu are affected by any of the above disturbances in low pH water over and above what might be expected during a normal exercise trial (compare results of west coast fish and east coast control fish). The mechanism by which low pH water caused a reduced performance in east coast acid-exposed fish may not involve all of the processes documented for rainbow trout.

A recovery on swim trial three to a level which was not significantly different from trial one suggests that the disturbances which resulted from exercising in acidic water in trial two were remedied in part, if not fully during the 48 hour recovery period in neutral water which proceeded. This suggests that significant damage to gill structure did not occur as repair would probably take longer than the 48 hour recovery period, and this would probably be indicated by a prevailing lower swimming performance in swim trial three (however, see chapter 5). The most likely disturbance of low pH water in combination with exercise in naïve banded kokopu would be elevated ionoregulatory costs and a Root effect due to inability to dump H^+ ions to the environment which lowers blood pH and in turn lowers the O_2 carrying capacity of the blood. There was no mortality in naïve fish following this experiment which is further indication of their acid tolerance.

Rainbow trout exposed to pH 4.0 in soft water experience physiological disturbances which are likely to represent a severe and probably fatal acid challenge (Wood, 1989). Na^+ balance results from the previous chapter indicate that disturbances are probably less severe even for naïve neutral water banded kokopu and it is likely that the physiological effect of low pH upon exercise could be less severe than those experienced by rainbow trout under similar conditions.

Wood (1989) stated that exposure to low pH (4.3) naturally soft water did not result in a significant internal metabolic acidosis in rainbow trout. The major physiological events to occur during acid challenge were ionoregulatory disturbances. Other

disturbances were suggested to be secondary effects resulting from the removal of strong ions such as Na^+ and Cl^- . He measured approximately equal branchial losses of Na^+ and Cl^- and only a small H^+ uptake in soft water. In hard water there was a large net H^+ uptake at the gills and an excess Na^+ loss over Cl^- from the plasma. The major conclusion drawn in relation to blood metabolic acidosis was that it was greatest at the highest water $[\text{Ca}^{2+}]$ and there was *no blood acidosis at all* (original emphasis) after three days exposure to $\text{pH} = 4.3$ in $0.2 \text{ mmol L}^{-1} [\text{Na}^+]$ used as the boundary between soft and hard water in his and this study (Wood, 1989). The important point here is that these measurements are relevant for resting fish and not necessarily for fish which are forced to undergo exhaustive exercise, where lactic acid is generated in muscle cells and enters the plasma.

A fish exercising at a normal environmental pH level may be able to limit the extent to which the pH of its blood drops by shuttling H^+ ions to the environment across the gills (Cameron, 1989; Heisler, 1993). This may not be possible when surrounded by an acidic medium and gill exchanges of H^+ have been altered. Furthermore, ion and proton exchange processes are by definition sensitive to the availability of an appropriate counter ion in the water. The lack of a counter ion for H^+ , ie (Na^+), particularly in the face of increased external $[\text{H}^+]$, may limit the transfer of ions relevant to acid-base regulation. In the northern hemisphere this is particularly relevant in soft waters where ion levels are low and acid toxicity is most often encountered (Heisler, 1989).

The higher U_{crit} values measured for smaller fish accounts for the overall higher U_{crit} values measured for the west coast group as they were much smaller fish on average (see tables). The increase in performance measured in west coast banded kokopu over the three trials is a training response which is also shown in east coast control fish. Although fish are regarded to be much less plastic than mammals in response to increased performance through training (Davison, 1989), a similar response has been measured in other teleost fish (McLeod, 1967). A time dependent increase in U_{crit} has

been measured in many studies with the increase in performance being linked to a lower O₂ consumption rate at a given swimming speed (Woodward and Smith, 1985). It was concluded that the increase in performance was probably due to an increase in the actual efficiency of O₂ consumption and a reduction in the levels of stress hormones which would adversely affect performance (Woodward and Smith, 1985).

The pH change to neutral water appeared to have no adverse or heightening effect on the swimming performance of the west coast fish. This could be expected when considering the natural environment of the west coast banded kokopu. A stream which had a pH in the permanently acid range (see general introduction) could be expected to become less acidic during the plentiful periods of rain experienced on the west coast. Rain water has a pH of approximately 5.6 in New Zealand and a large fall would raise the pH of a stream, potentially reaching a similar pH value as the rain. Although this was not observed during my own measurements it must be pointed out that rainfall on the west coast will often endure for many more days than the periods I took measurements over.

The effect of this would be that west coast acid adapted fish must be regularly exposed to waters of higher pH. East coast fish however, would have been unlikely to encounter low pH water in their natural environment due to the lack of naturally acid streams on the peninsula and the absence of acid pollution.

Lakes in Scandanavia and Ontario have recently become more acidic (pH < 4.8) and it is likely that the exercise capabilities of fish surviving in these lakes have been reduced (Graham and Wood, 1981). However, not all studies have come to this conclusion. Graham and Wood in a later study (1982) found that acute exposure to acid conditions in hard water did not exacerbate the physiological disturbances associated with exercise in rainbow trout. They related this to results from McDonald *et al.*, (1980) which concluded that the physiological effects of acid toxicity develop slowly in hard water. The exercise protocol used in their later study (1982) differed from that used in

measuring the critical swimming speed (1981), in that chase protocols were employed to achieve burst performance levels and to swim the fish to exhaustion. Therefore it is possible that post-exercise physiological disturbances were not increased in acidic conditions simply because less swimming was done by the fish at low pH to achieve exhaustion. This was recognised by the authors who concluded that there was no guarantee that all fish received the same treatment and suggested that a swim tunnel capable of achieving burst performance levels would ensure a uniform work load on all fish.

In summary these data draw attention to the detrimental effect of acidic stress upon naive exercising fish. It also emphasises the problems encountered with interpreting data when using experimental methods which attempt to lessen stress upon the animal. Acid toxicity has been shown in many studies to have a complex mechanism and the likely mechanisms of action have been discussed here. Only by measuring gas exchange and ion and proton fluxes in exercising fish can these mechanisms be further characterised and conclusions drawn as to the nature and extent of the disturbances caused by low pH upon the physiology of the banded kokopu.

If methods of keeping banded kokopu in the laboratory bring greater success in future longer studies which look at parameters other than critical swimming speed would be possible, over longer periods than were allowed in this work. If a larger, more viable population was available, 'riskier' studies with more invasive techniques could be warranted and studies could look at comparing lethal limits with other salmonids in which this has been done (Daye and Garside, 1975). This study has been useful however in showing that differences between the exercise performance under acid stress do exist and further investigation is warranted. The study also demonstrates that the west coast fish are able to perform as well as east coast fish in exercise tests, despite living in low pH water.

*Chapter 5***GILL MORPHOLOGY****5. INTRODUCTION**

Physiological mechanisms enable fish to survive in freshwater systems which may undergo large fluctuations not only in pH, but also in temperature, oxygen, carbon dioxide, and dissolved ion concentrations (Perry & Laurent, 1993). The gill being located between the external and internal environments acts as an interface at which gas-transfer, acid-base balance and ionic regulation occur. It is anticipated that being the primary organ involved in the above processes and having direct contact with the external and internal environment, any morphological adjustments in response to environmental change would be shown to some degree in the gill. McDonald (1982) wrote that "the gills are the primary target of elevated hydrogen ion activity in the environment. Thus, many of the effects of low pH upon freshwater fish can be more fully understood by appreciating the nature of the interference of H^+ with normal gill functions".

Generally morphological adjustments in response to environmental stimuli are considered to be adaptive if they enable a fish to survive an environmental challenge. However some morphological changes may actually serve to impair function in the gill. This is related to its multi-functional nature where amelioration of a particular physiological function may serve to compromise another (Perry & Laurent, 1993). The large diffusive area of the gills while serving to enhance O_2 uptake from the water also provides a larger site for the diffusive loss of ions.

GILL STRUCTURE, CELL TYPES AND THEIR FUNCTIONS

A detailed description of gill morphology and ultrastructure of teleost gills is given in Laurent (1989). The side of the throat is divided into gill arches by gill slits. Filaments project from the arches toward the opercula and secondary lamellae are found projecting from the filaments. The trailing edge of the lamellae is the last site of exchange as water leaves the gill and moves into the opercular cavity. The secondary lamellae serve to increase the functional surface area of the gill. There are three predominant cell types on the freshwater teleost gill epithelium. They are the pavement cell, the chloride cell, and the mucus cell.

The filament epithelium typically is multi-layered while the lamellar epithelium is normally composed of a double layer of cells separated by extracellular spaces. Inside the pavement cell mitochondria are sparse in distribution which contrasts with the abundance of rough endoplasmic reticulum, numerous vesicles and well developed Golgi apparatus. The absence of mitochondria suggests that pavement cells are not involved in the active transport of ions. Occluding tight junctions join pavement cells to each other or adjacent chloride cells. Pavement cells generally cover about 90% of the filament and lamellar epithelium (Perry & Laurent, 1993).

The majority of work on fish gill cell types has been carried out on chloride cells. The chloride cell has been suggested to be the site of Na^+ and Cl^- in freshwater fish from the morphological observations that the characteristics of chloride cell populations change when transferred between different ionic environments. Freshwater fish have been shown to have fewer chloride cells than seawater adapted fish, however the size of each cell is decreased in seawater resulting in no real significant difference in the total combined cell apical surface area (Laurent and Hebibi, 1989). Chloride cells usually comprise less than 10% of the total gill surface area (Perry and Laurent, 1993). These cells are characterised by abundant mitochondria, an extensive tubular network within the cell due to an amplification of the basolateral membrane, and a well developed vesicular system in the apical regions of the cell.

Mucus cells are found on both the lamellar and filamental epithelial surfaces. Mucus is a polyanionic glycoprotein and suggested functions have included defence against pathogens and a role in osmotic and ionic regulation (Handy, 1989).

The gills serve a variety of functions in fish. A large, permeable surface area is required to enable their first and foremost function, respiratory gas exchange. As noted earlier, this area also provides a site for the diffusional loss of ions in freshwater, the principal components of these losses being Na^+ and Cl^- .

It was therefore anticipated that a study on the gill morphology of east coast neutral water, non acid exposed control fish, and acid exposed fish may provide some insight as to the nature of: 1) The processes employed to compensate to reduce the loss of ions to the medium in acid exposed fish or, 2) Possible damage caused by acid exposure. The gills of west coast fish from naturally acidic water were also examined as a further comparison in light of the possible pre-adaptive mechanisms which could be present in these fish to cope with low pH water. Morphological differences, if present between east and west coast banded kokopu, could reflect a genetic difference between east and west coast populations which may have arisen as a result of natural selection.

The aim of this chapter was to undergo some preliminary investigations of the gill morphology of east coast acid exposed kokopu and non acid exposed kokopu, and make comparisons with gills from west coast kokopu. Due to the lethal nature of this study only limited numbers of fish were used with two fish from each of the three groups used for S.E.M. and two more used for T.E.M. Therefore it must be emphasised that this was not a quantitative gill study but rather an attempt to observe whether the gill morphology reflected or was complementary to any of the changes measured in the physiological studies.

5.1 MATERIALS AND METHODS

Several fish were killed from each group by immersion in benzocaine (conc. 200mg.l⁻¹). Gill arches were removed immediately using scissors and placed in normal fish Ringer. For S.E.M., the arches were gently cleaned with a soft paint brush to remove any congealed blood and debris. The arches were placed in the 1^o fixative: 2.5% glutaraldehyde, buffered in 0.1M sodium cacodylate, (pH=7.3) for 3 days at 4°C. After washing in buffer solution to remove excess glutaraldehyde, gills were post-fixed in 2% buffered osmium tetroxide overnight at 4°C. The specimens were then dehydrated in a graded alcohol series: 30%, 50%, 70%, 80%, 90%, 95%, 100% ethanol, for a minimum of 2 hours in each solution. After a fresh change in 100% ethanol overnight, the gills were passed through an amyl-acetate (AA) series, diluted with 100% ethanol: 25%AA, 50%AA, 75%AA, 100%AA. All steps were for a minimum of 2 hours at room temperature. The samples were then placed into fresh 100% AA overnight prior to critical point drying, using CO₂ as the transitional fluid. Dried specimens were mounted on aluminium stubs with conductive carbon paint, either as whole arches or sections of hemibranches containing 3-4 filaments, oriented for different views. The stubs with gills were then sputter coated with 60nm of gold and examined in a LEICA S440 S.E.M. at accelerating voltages of 12-20kv. Images were photographed using Ilford Pan F (50ASA) film.

Gill arches for T.E.M. examination were dissected from each other and only the second and third arches were processed further. These arches were cut and pieces from the mid-section only were used, each possessing 2-4 filaments. They were placed in 2.5% glutaraldehyde buffered as for S.E.M. schedule, for 4 hours at 4°C. Three washes of buffer over a 30 minute period followed, then samples were post-fixed in 2% buffered osmium tetroxide for 2 hours at 4°C. After a brief wash in buffer solution, the gills were dehydrated in a graded ethanol series, 15-20 minutes only in each solution. They were further dehydrated in absolute acetone in preparation for embedding in Spurr's epoxy resin. The impregnation schedule involved three steps: (resin:acetone) 50%:50%, 70%:30% (for 2-3 hours), 90%:10% (overnight). The next day, after a final period in

pure resin for 1 hour, the gills were carefully placed in fresh 100% resin in plastic moulds and cured in an oven at 65°C for 18 hours.

Semi-thin and ultrathin sections were cut using an LKB 8800 ultramicrotome. For correct orientation and consistency, the same relative area of the gill was sectioned for each of the fish groups: mid region of the filament and at 45° to its long axis. The thicker sections were 2µm and were stained with 1% toluidine blue (in borax). Gold and silver sections (70-90nm) were cut and collected on 200 mesh copper grids. These were stained with 5% uranyl acetate (in distilled water) for 30 minutes, followed by lead citrate for 15 minutes. They were observed in a JEOL 1200-EX TEM at an accelerating voltage of 80kV.

5.2 RESULTS

WEST COAST FISH

S.E.M. micrographs revealed a proliferation of chloride cells on the trailing edge of the gill filament and lamellae compared to the leading edge (figure 5.1, 5.2). Epithelial chloride and surrounding pavement cells appeared swollen on the trailing edge which was possibly due to activity of chloride cells beneath the epithelium. Chloride cells on the filament were concentrated near or at the base of the lamellae. Those on the trailing edge of the lamellae were present within a region approximately half way between the base of the lamella and the point most distal from the filament (figure 5.3). The surface of the chloride cells was markedly different from the surface of the surrounding pavement cells with microvilli being tightly arranged on the apical surface of the chloride cells (figure 5.3). The dense arrangement of microvilli appeared to aid anchorage of mucus to the chloride cells. Chloride cells were absent from the epithelial surface of the leading edge of the filament and lamellae (figure 5.4). Mucus was not

apparent on any of the gill epithelial surface with the exception of where it was anchored to chloride cells. The mucus did not provide a blanket covering of the chloride cell, but rather individual droplets were anchored to the tightly compacted microvilli (figure 5.5). Overall, gill structure was intact and appeared normal with no visible damage to the gill epithelium. Hypertrophy of mucus cells was not observed and there was no indication that fusion of lamellae and other corrosional damage had occurred.

T.E.M. micrographs showed the apical membrane of chloride cells to be neither sunken nor raised compared to surrounding pavement cells. Microvilli were prevalent and protruded above the profile of the microridges on surrounding pavement cells (figure 5.6).

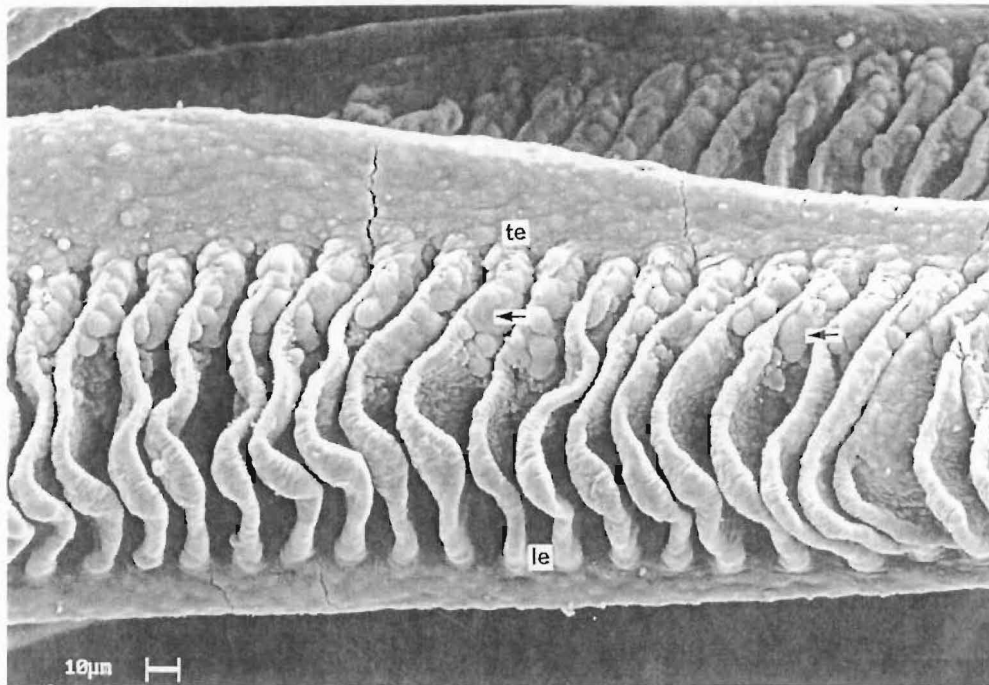


Figure 5-1: West coast banded kokopu. S.E.M. micrograph of the mid-region of a filament showing the swollen tissue (arrows) of the trailing edge (te) of the lamellae compared to the leading edge (le). The swelling is probably due to proliferation of chloride cells.

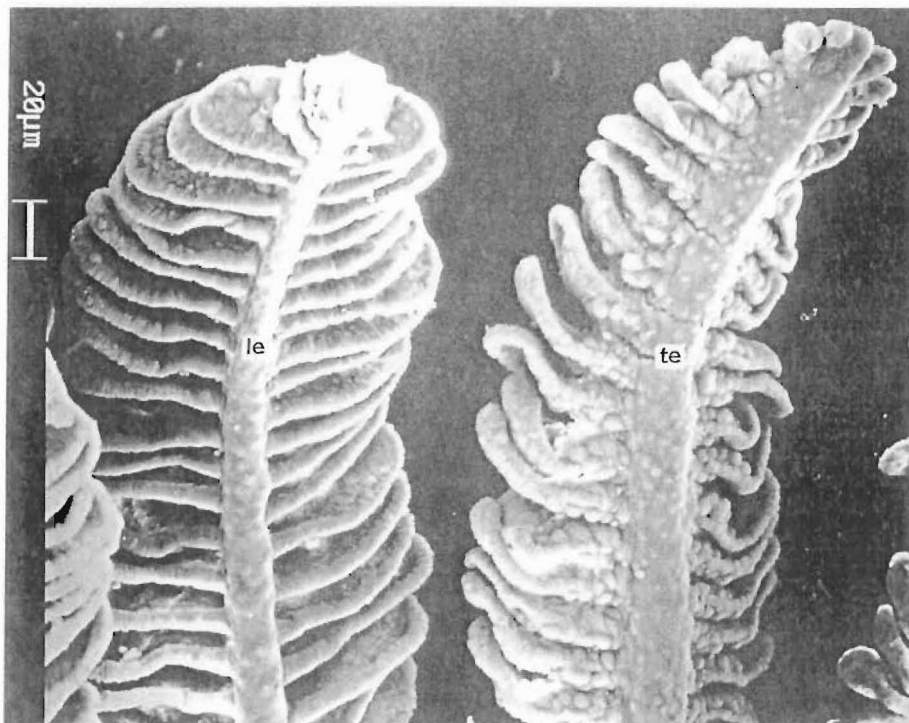


Figure 5-2: West coast banded kokopu. S.E.M. micrograph of two hemibranchs clearly showing the difference in tissue swelling on the leading (le) and trailing (te) edges of the filaments.

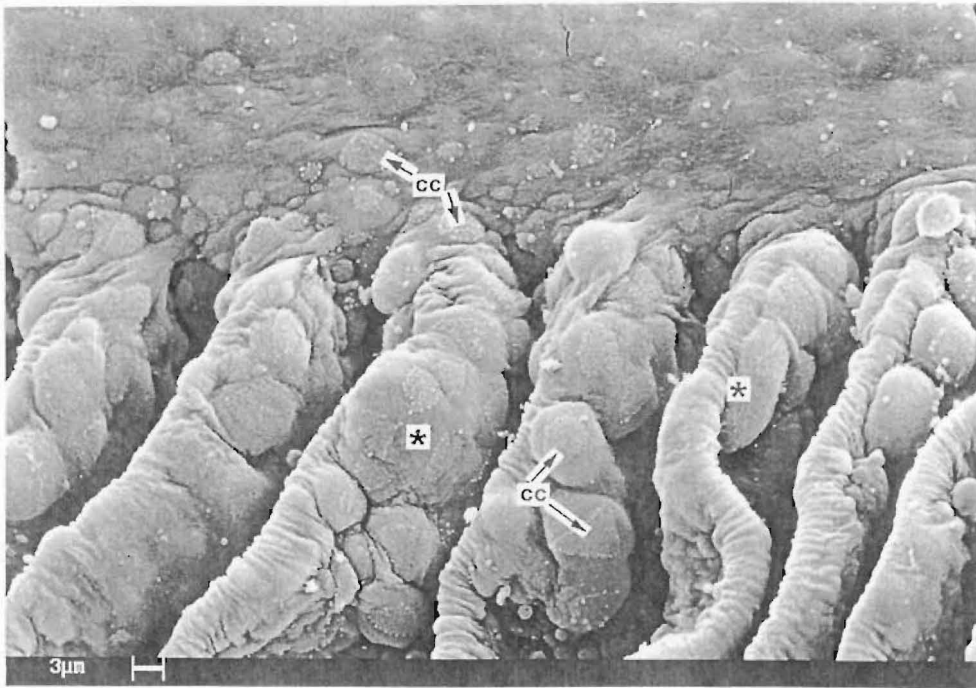


Figure 5-3: West coast banded kokopu. S.E.M. micrograph of an area of the trailing edge of the filament observed in Figure 5-1, but more highly magnified. The chloride cells (cc) are clearly visible, as is the swollen nature of the lamellar tissue (*).



Figure 5-4: West coast banded kokopu. S.E.M. micrograph showing the leading edge of the filament, as seen in Figure 5-1, but at a higher power. The leading edge shows a comparative absence of chloride cells; the microridges of the pavement cells (pc) being most apparent.

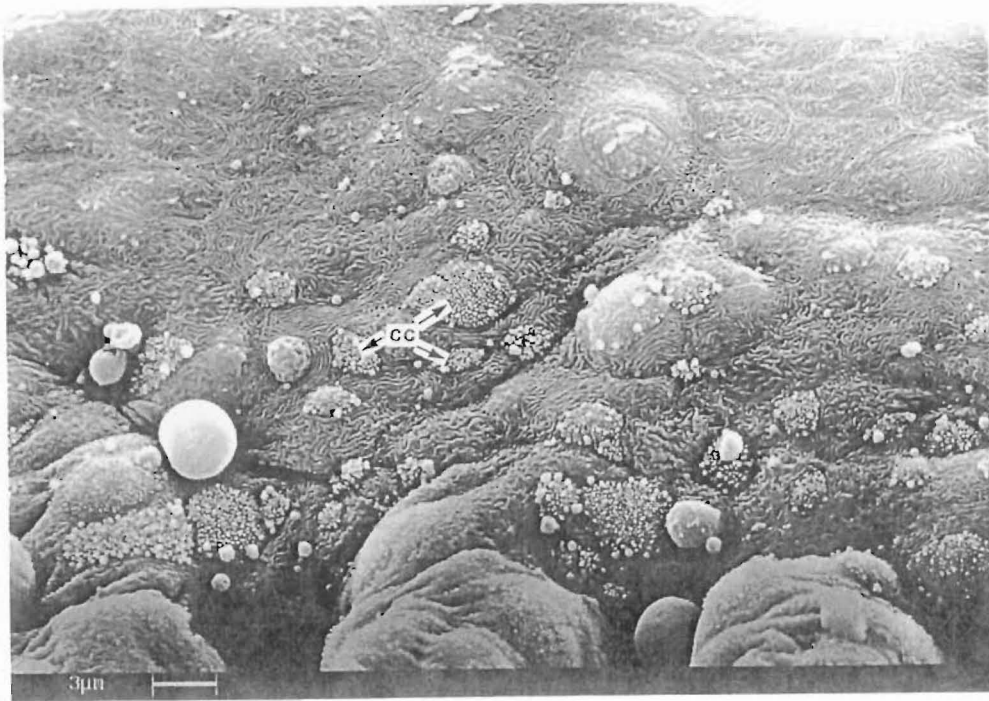


Figure 5-5: West coast banded kokopu. S.E.M. micrograph of the epithelial surface of the trailing edge of the filament near the base of the lamellae. Chloride cells (cc) are prolific and their microvilli are covered with numerous droplets of mucus (mc).

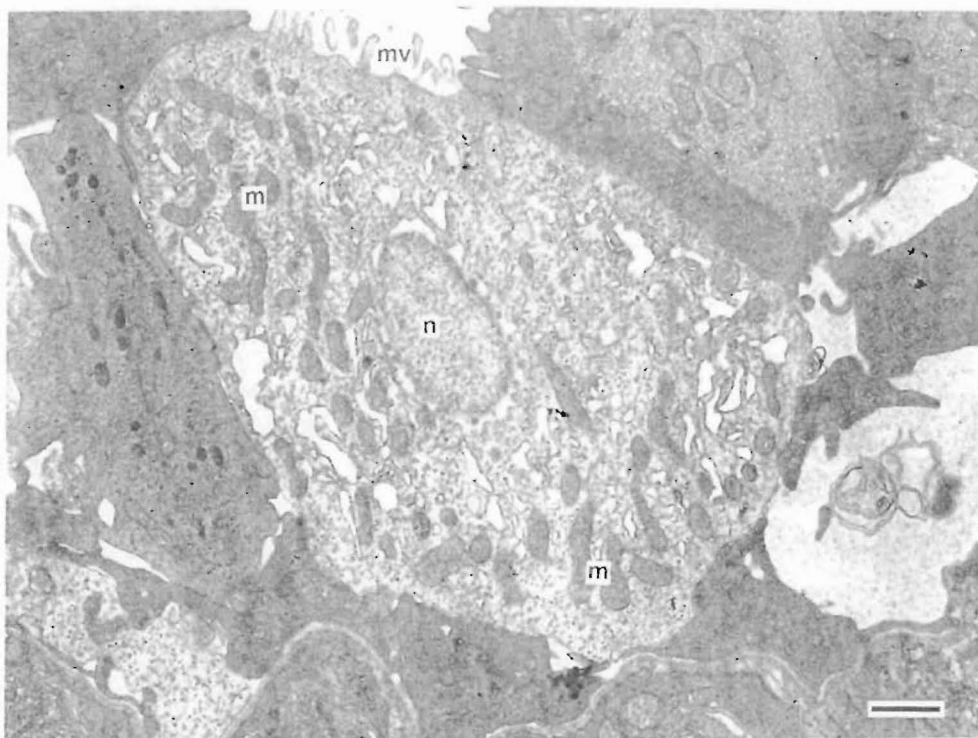


Figure 5-6: West coast banded kokopu. T.E.M. micrograph of a chloride cell at the surface of the filament. Mitochondria (m) are abundant amongst the smooth endoplasmic reticulum (ser) of the cytoplasm. Microvilli (mv) at the apical surface of the cell are apparent. n = nucleus. Scale bar = 1µm.

EAST COAST NON ACID EXPOSED FISH

S.E.M. pictures revealed chloride cells on the trailing edge of the lamellae (figure 5.7). The swelling in this area appeared to be less marked than that observed on the trailing edge of the gills for the west coast fish. Overall the thickness of the leading and trailing edges of the lamellae was more homogeneous than observed in the west coast fish. The chloride cells were markedly different in appearance from those observed on the west coast fish, being more raised on the surface (figure 5.8). Upon closer observation of individual chloride cells the bulbous appearance was obvious (figure 5.9).

Also visible were differences in the pattern of microridges on the apical surface of the chloride cells. These strongly contrasted with the microvilli structures observed on the apical surface of the chloride cells of the west coast fish. Overall the microridges were less densely arranged than those observed on the west coast chloride cells. Furthermore they appeared to be more sparse than those on the adjacent pavement cells. Little mucus was observable anchored to the surface of the chloride cells, their structure being less favourable for the anchorage of mucus. Chloride cells were present on the trailing edge of the lamellae only, up to a point approximately halfway to the distal tip from the filament.

T.E.M. examination of non acid exposed east coast fish showed chloride cells on the filament and lamellae. The microvilli of the apical region of the chloride cell of figure 5.10 was raised consistent with observations under S.E.M. However the other east coast non acid fish did not show this (figure 5.11).

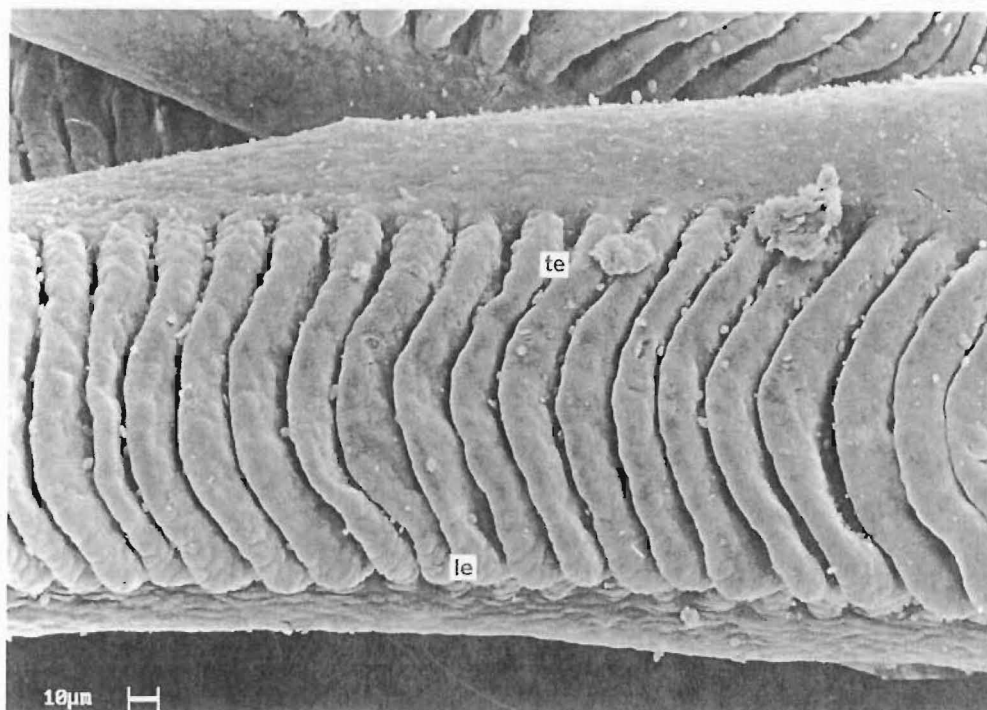


Figure 5-7: East coast non acid exposed banded kokopu. S.E.M. micrograph of a filament (mid-region) showing leading edge (le) and trailing edge (te) of the lamellae. The leading and trailing edges are homogeneous in thickness.

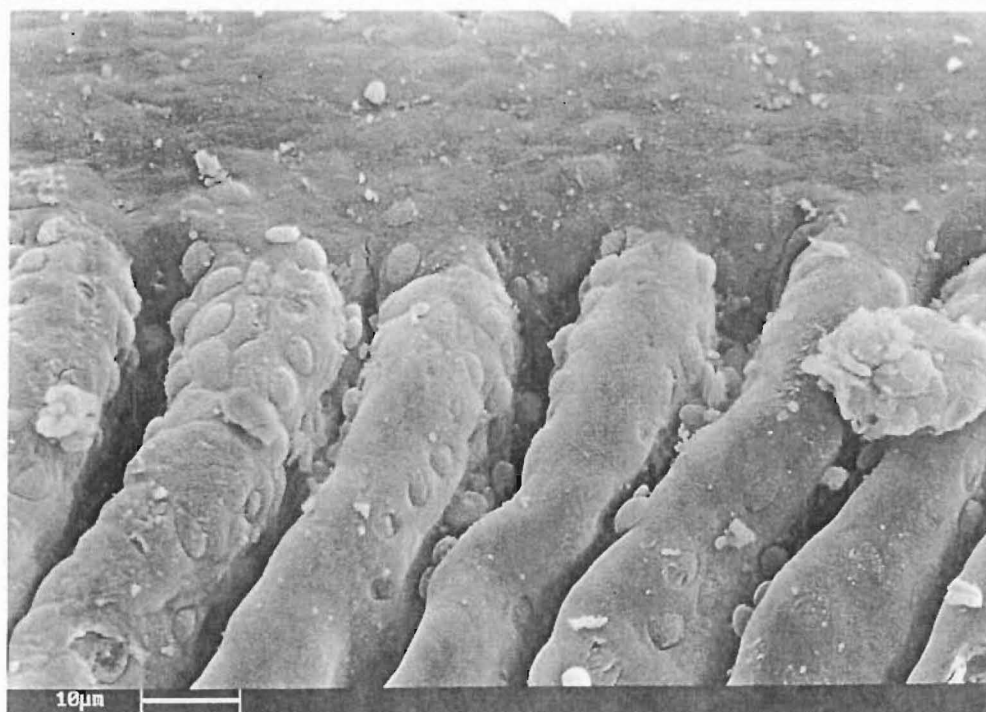


Figure 5-8: East coast non acid exposed banded kokopu. S.E.M. micrograph of the trailing edge of the gill. There is less swelling present on the trailing edge compared to west coast fish.

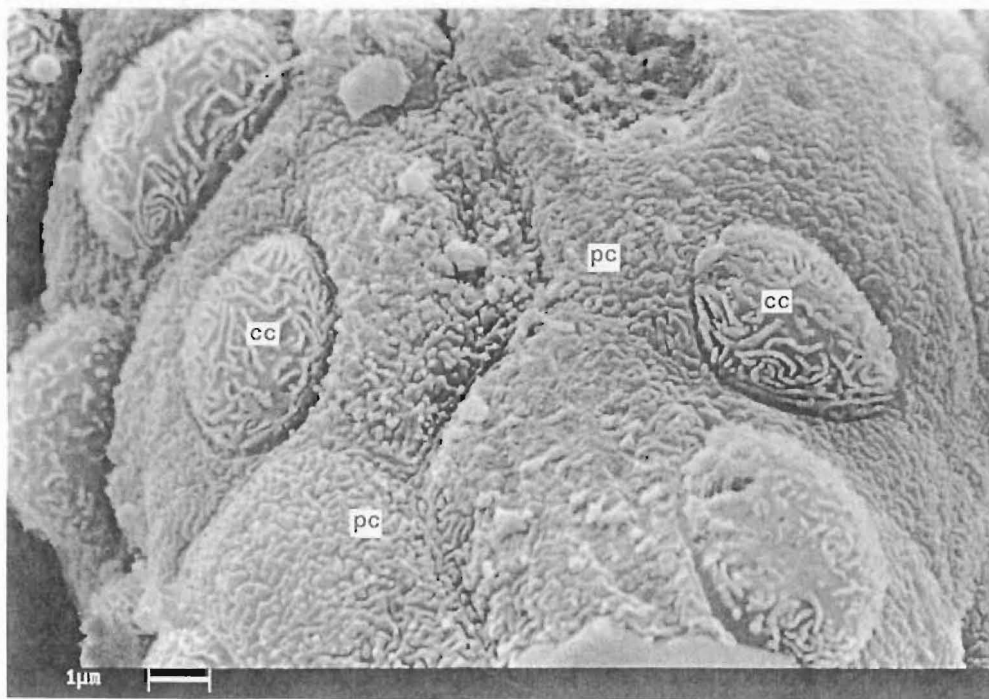


Figure 5-9: East coast non acid exposed banded kokopu. S.E.M. micrograph showing a higher power view of Figure 5-8. The chloride cells (cc) are markedly different in appearance from those of west coast fish. The apical surface is slightly raised and has a microridge structure more similar to the surrounding pavement cells (pc). These do not appear to assist in the anchorage of mucus.

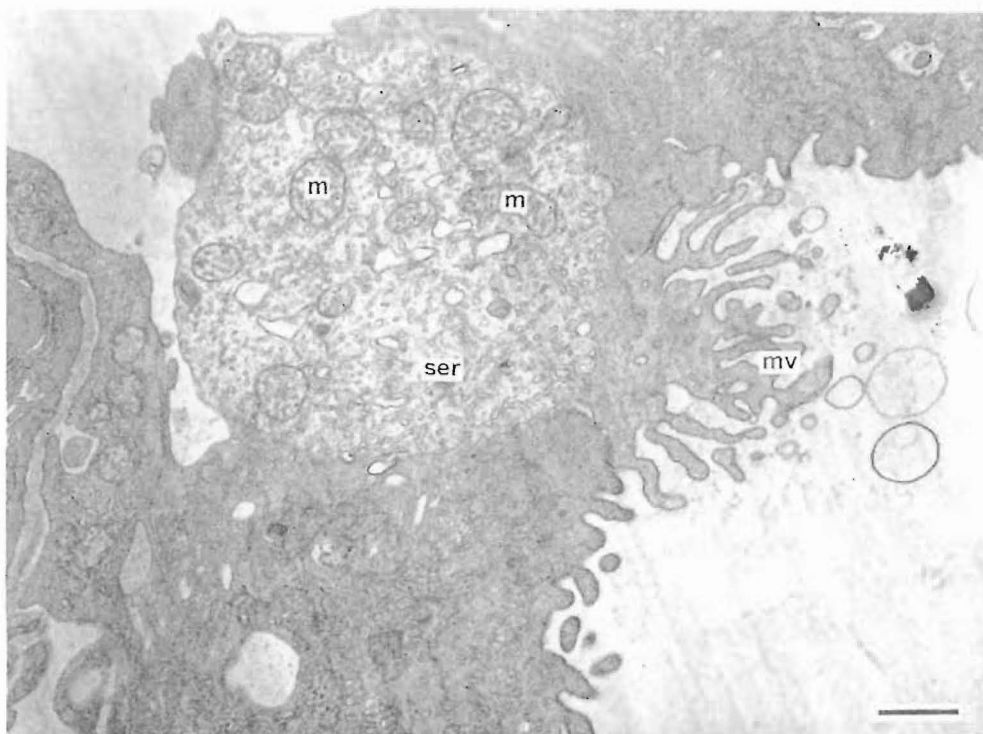


Figure 5-10: East coast non acid exposed banded kokopu. T.E.M. micrograph of a chloride cell of a lamella. The apical surface of the chloride cell is raised in this fish. mv=microvilli; m=mitochondrion; ser=smooth endoplasmic reticulum. Scale bar = 1 μm.

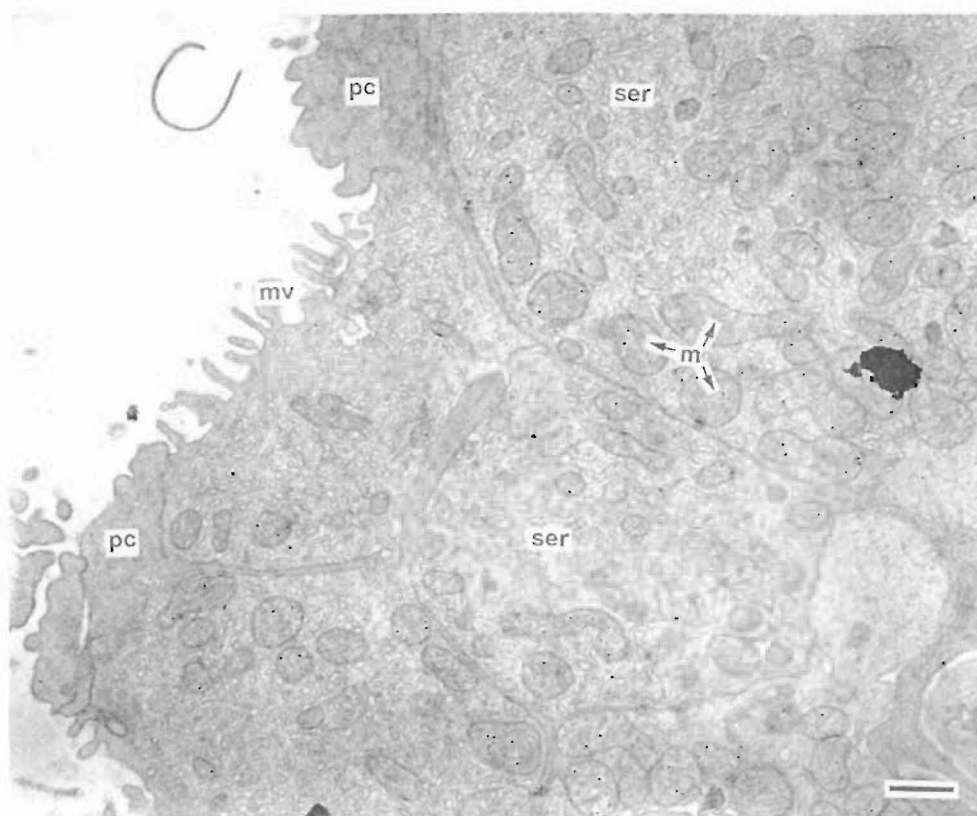


Figure 5-11: East coast non acid exposed banded kokopu. T.E.M. micrograph of several chloride cells of a filament. The apical surface is not raised in this fish but the microvilli (mv) are elongated. Mitochondria (m) and cisternae of smooth endoplasmic reticulum (ser) are abundant. pc = pavement cell. Scale bar = $1\mu\text{m}$.

EAST COAST ACID EXPOSED FISH

S.E.M. micrographs showed swelling on the trailing edge of the gill lamellae possibly due to the presence of chloride cells (figure 5.12). Chloride cells on the gill filament had an abundance of microvilli on the epithelial surface which were tightly compacted to the microridges of the surrounding pavement cells (figure 5.13). Mucus particles appeared to be anchored to the microvilli as in west coast fish. Mucus was droplet like in appearance rather than a smooth uniform coating possibly reflecting some precipitation due to low pH. Overall the chloride cells and the S.E.M. micrographs in general appear to most closely resemble the west coast fish.

T.E.M. micrographs supported the observations made under the S.E.M. that the apical surface of the chloride cell was neither sunken nor raised and the microvilli were noticeably elongated and more densely arranged compared to surrounding pavement

cells (figure 5.15). Chloride cells were observed to be densely packed with mitochondria (figure 5.14). Mucus cells were often located in close proximity to the chloride cells, as can be seen in figure 5.14. The microvilli structures were more pronounced than the microridges on pavement cells rising above the profile of the cell surface supporting observations made from the S.E.M. micorgraphs.

The gills of one acid exposed east coast fish can be observed in figure 5.16. Gill deformations were present in this fish including fusion of lamellae which resulted in 'clubbed' gills. This swelling and fusion originated at the tip of the filament distal from the gill arch and incorporated approximately quarter of the filament length in the most extreme observable case. This probably represents a loss of lamellar surface area due to the loss of lamellae as they are incorporated into the swollen region (figure 5.17).

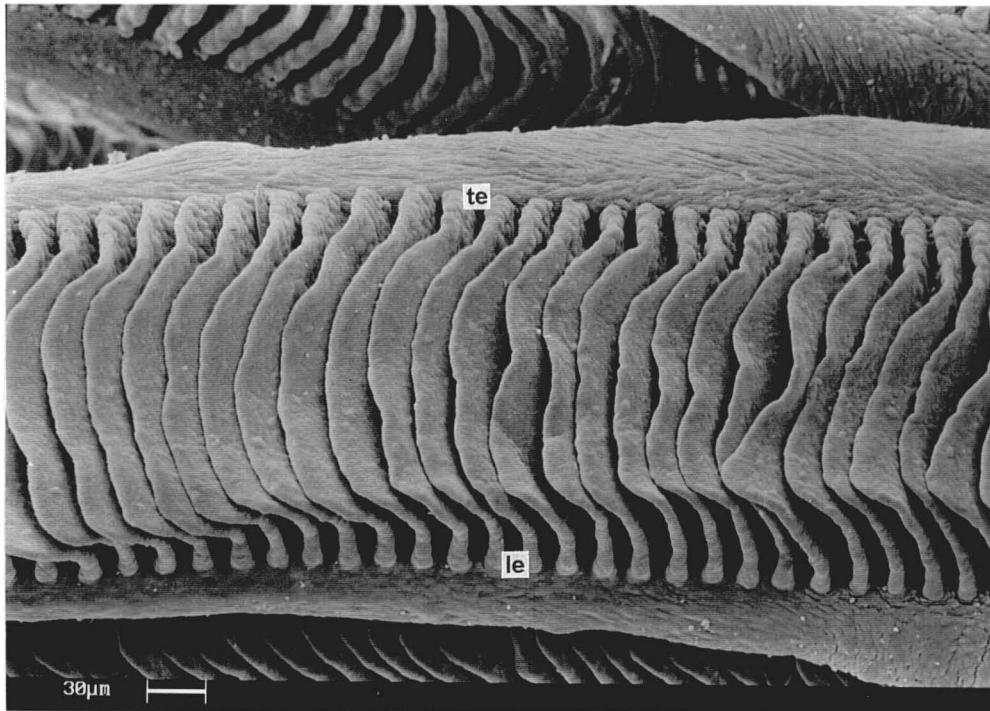


Figure 5-12: East coast acid exposed banded kokopu. SEM micrograph of the mid-region of a filament together with lamellae. The trailing edge (te) is more swollen than the leading edge (le) due to the presence of chloride cells. This micrograph resembles the west coast fish, but the swelling is less extreme.



Figure 5-13: East coast acid exposed banded kokopu. Higher power view of the filament surface on the trailing edge. Chloride cells (cc) have microvilli on their apical surfaces which are tightly compacted and resemble those of the west coast fish. Mucus is anchored to the microvilli. pc = pavement cell.

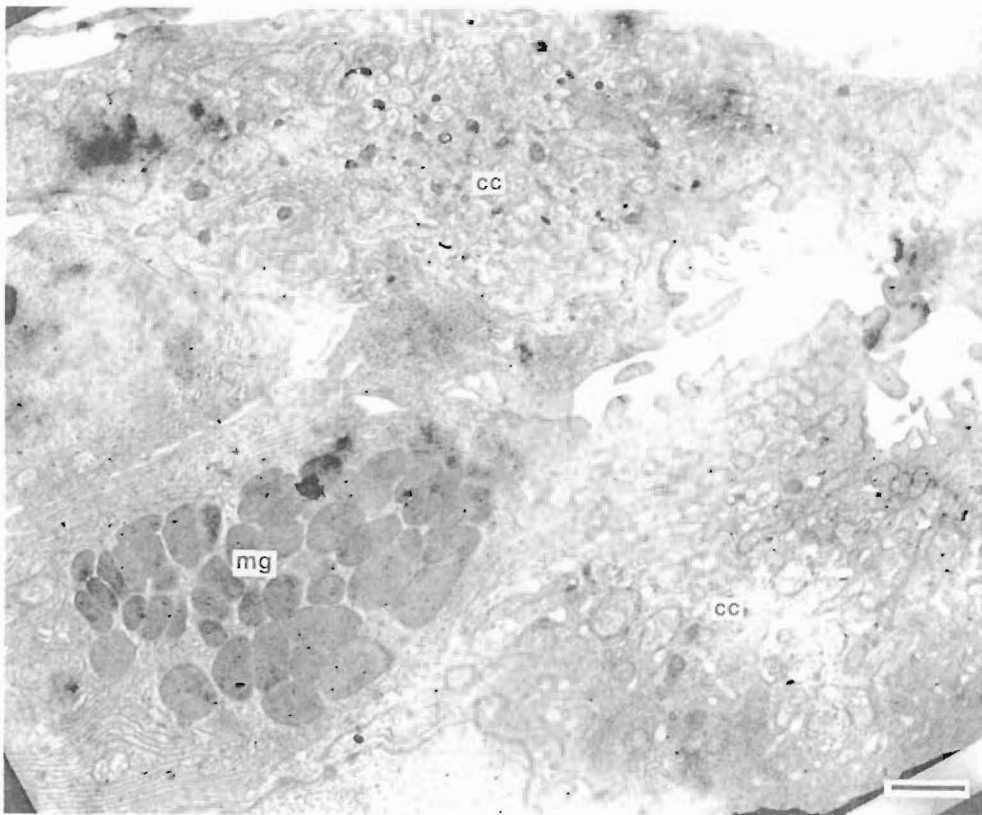


Figure 5-14: East coast acid exposed banded kokopu. T.E.M. micrograph showing a mucus gland (mg) flanked by two chloride cells (cc) on the trailing edge of the filament near the base of a lamella. Scale bar = $1\mu\text{m}$.

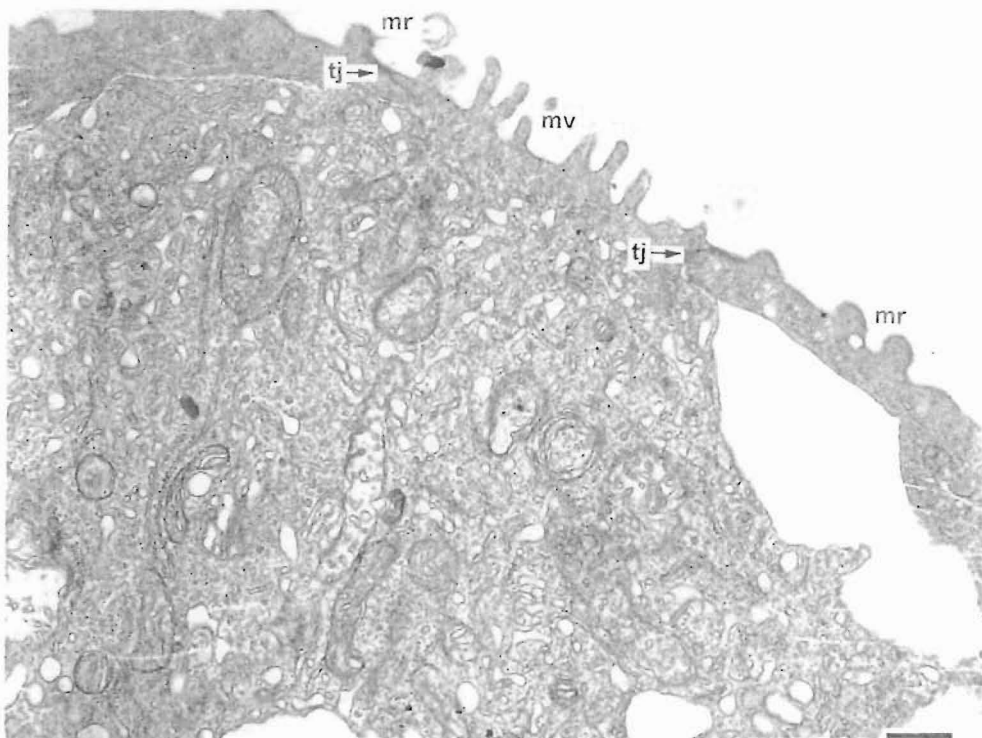


Figure 5-15: East coast acid exposed banded kokopu. T.E.M. micrograph of a chloride cell of a lamella. The microvilli (mv) extend above the profile of the surrounding pavement cells. Note the wider, flattened form of the microridges (mr) of the pavement cells. Tight junctions (tj) between chloride and pavement cells are indicated. Scale bar = 500nm .

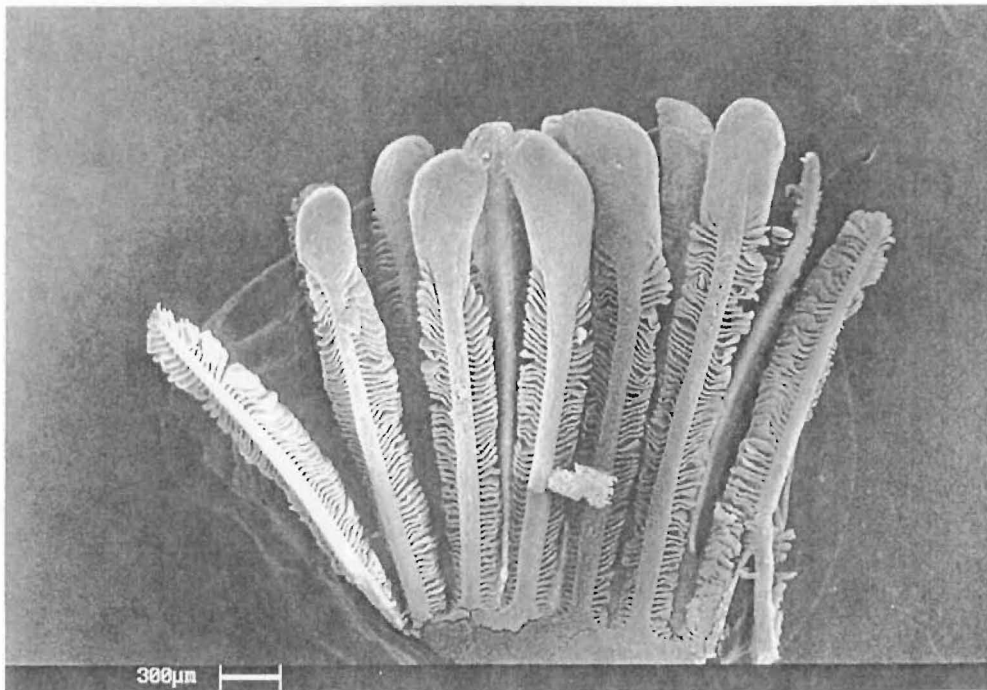


Figure 5-16: East coast acid exposed banded kokopu. S.E.M. micrograph of 'clubbed' deformation of the distal tips of the filaments of two hemibranchs.

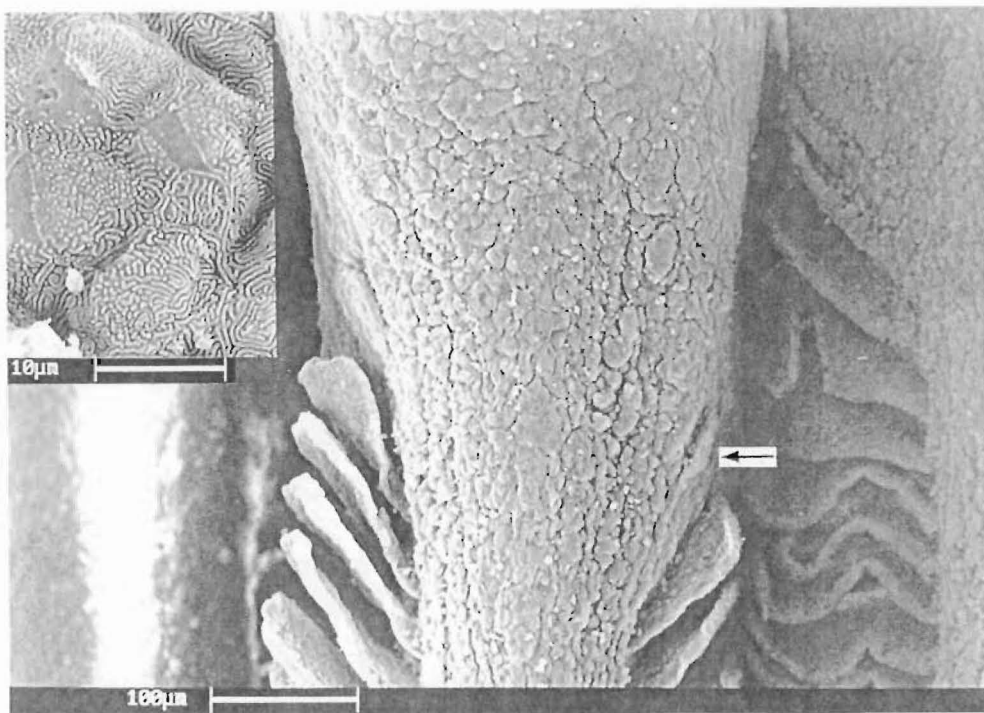


Figure 5-17: East coast acid exposed banded kokopu. S.E.M. micrograph showing Figure 5-16 at magnification. Lamellae are swollen and fused together at the distal tip of the filament. Several lamellae in this micrograph are becoming incorporated in the swollen region (arrow). Inset: Higher magnification of the epithelial surface.

5.3 DISCUSSION

Chloride cells were found to be present on the trailing edge only of the gill filament and lamellae in all fish. West coast and east coast banded kokopu showed a proliferation of chloride cells on the trailing edge which was not matched to the same degree in east coast non acid exposed fish. The appearance of chloride cells on the trailing edge of the filament is consistent with other reports on the gills of freshwater fish (Perry and Laurent, 1993), but these authors noted that chloride cells were only normally present on the base of lamellae. Perry and Laurent also noted that chloride cells were only normally present on the base of lamellae, usually being limited to the filament and interlamellar regions. Fish in this study all showed the additional presence of chloride cells along the trailing edge of the lamellae to a point at least half way to the distal tip. There are a number of reports that indicate an increase in lamellar chloride cells as a response to ion-poor water (Perry and Wood, 1985; McDonald *et al.*, 1991; Perry and Laurent, 1993). Acidified water may also represent an ion poor medium. This is due to the increased concentration of H^+ ions which directly compete for Na^+ transport sites on the gill epithelium (Wood, 1993). The morphological findings in banded kokopu contrasts with studies on rainbow trout exposed to pH4. Rainbow trout showed a 35% decrease in the number of chloride cells in pH4 water (Audet and Wood, 1993). Rainbow trout as discussed earlier, are far less tolerant of acid water than banded kokopu and would not necessarily show the same changes in gill morphology as pH4 would present a serious and probably fatal acid challenge. Rainbow trout did show increased mucus upon the gill epithelium in acid exposed fish and this was consistent with observations in this investigation.

Hypertrophy of mucous cells and increased mucus production has been observed in fish exposed to acidic water (McDonald, 1983). In this investigation it is not clear whether the increased mucus anchored to the surface of chloride cells in west coast and east coast acid exposed fish is a function of increased mucus production, or solely attributable to the higher binding capacity for mucus by the microvilli structures on these cells

compared with the microridges present on adjacent pavement cells. Microridges increase the surface area of the pavement cells which is beneficial to O_2 uptake. Pavement cells have been suggested to have a major role in the diffusive uptake of O_2 (Perry and Laurent, 1993), and their predominance on the gill epithelial surface indicates that they probably are involved with the primary function of the gill (gas exchange). Mucus probably has a detrimental effect on oxygen uptake and it would not be desirable to have mucus accumulation on the gas transfer interface. The chloride cells on the east coast non acid fish gills were more like microridges than microvilli. As suggested above these may be less inclined to anchor mucus than microridges due to their less raised profile and decreased density compared to microridges present on the chloride cells of the other groups. The structures on the surface of the chloride cells in the east coast non acid exposed fish were less compacted than the microridges on the surrounding pavement cells.

Anchoring of mucus to epithelial chloride cells has been suggested to have a restraining effect on the diffusional losses of Na^+ and Cl^- (Hughes, 1980). Some other workers do not support this argument and Marshall (1978) demonstrated that mucus had very little ability to impede ionic diffusion, having permeation characteristics which were not significantly different from water. However these studies were *in vitro* and other workers have shown that the mucus layer of the gill epithelium accumulated Na^+ and Cl^- (Bierther, 1970) which suggested some role in lessening the net loss of sodium and chloride ions. Perry and Laurent (1993) wrote that mucus may be important in establishing a microenvironment next to the gill epithelium which is abundant in the enzyme carbonic anhydrase. This enzyme is suggested to have a beneficial effect maintaining carbon dioxide excretion in acid water. Accumulation of Na^+ and Cl^- ions in a stable environment next to the gill epithelial ion uptake mechanisms may increase their uptake by favourably increasing their relative concentrations with respect to competing H^+ ions. These suggestions could possibly explain why mucus was preferentially anchored to chloride cells in west coast and east coast acid exposed banded kokopu. Unfortunately no conclusions can be drawn from the small sample

size in this investigation. Further studies could potentially support this argument. The possibility must also be addressed that mucus may solely be an undesirable product of a stress response which impedes normal gill functions such as oxygen uptake and serves no adaptive purpose.

Structural damage to the gills has been documented in many species. McDonald and Wood (1992) proposed that for acclimation to a particular toxicant to occur, there would first have to be significant structural damage to the gill epithelium. This is related to their hypothesis which states that branchial mechanisms of acclimation are a function of damage repair. Results from Na⁺ flux tests and exercise trials in pH4 water for naïve east coast banded kokopu suggest that physiological disturbances were sub-lethal, and possibly correctable at least for Na⁺ fluxes. The physiological results do not support the suggestion that the gill deformations observed in an acid-exposed fish were a result of pH4 exposure. While in appearance it is very similar to the examples of gill hypertrophy and fusion of lamellae reported in acid exposed brown trout by Peuranen *et al* (1994) and in other studies [McDonald, 1982; Daye and Garside, 1976; Perry and Laurent, 1993, it cannot be concluded in this thesis to be a direct result of acid exposure due to the relatively small numbers of fish used, the lack of similar observations in the other acid exposed fish and the lack of supporting evidence from the physiological data. It is worthwhile to note however, that the observed effect is very similar to that which is described in other studies (McDonald, 1982; Daye and Garside, 1976; Perry and Laurent, 1993). Fusion of lamellae and hypertrophy of the epithelial cells has been shown to significantly increase the diffusion distance for efflux of ions across the gills. This could have the net effect of lowering the diffusional loss of ions to the medium which could be seen as adaptive when the ability to balance ion loss is disturbed in low pH. However this would also have a negative effect on oxygen uptake due to the increase in distance for oxygen diffusion and would not necessarily be adaptive. While gill 'clubbing' has been demonstrated to occur in acid sensitive rainbow trout at low pH, it was observed in control fish which were not exposed to acid water with the same frequency (Audet and Wood, 1993). Deformations of the gill could be a result of a an

infection, and due to its delicate structure and direct association with the external medium, the gill is vulnerable to all water borne pathogens. Further investigation of this gill damage would be desirable especially noting that studies of this type on tilapia (*Oreochromis mossambicus*), which is known to be more acid tolerant than rainbow trout, showed substantial initial gill damage followed by pronounced repair and a prominent proliferation of chloride cells during pH4.5 exposure (Audet and Wood, 1993). This supports the argument that the reduction in chloride cells exhibited by rainbow trout when exposed to low pH water is not necessarily typical for more acid tolerant species.

These results do not suggest mechanisms that might reduce the Na^+ efflux for banded kokopu transferred to pH4 water. However, this reduction was apparently instantaneous, and morphological damage to the gill would not be expected to be observed immediately after pH4 exposure. Future studies could look to the tight junctions for a possible mechanism of reducing efflux as it is through these paracellular pathways that ions seem to escape. In summary this preliminary investigation suggests that further exploration into the possible differentiation of gill structure in acid exposed and non acid exposed rainbow trout is warranted and desirable if the mechanisms which aid acid tolerance are to be characterised. Observations from this small group suggest that chloride cell proliferation, the role of mucus, and the apparently assisted anchorage of mucus preferentially to chloride cells and the implications for ion balance and oxygen uptake of this binding are possible avenues of future study.

Chapter 6

GENERAL DISCUSSION

The main intention of this study was to make predictions based on physiological experiments in the laboratory as to whether populations of the New Zealand banded kokopu from the east coast would be able to survive in low pH water and populations from the west coast in neutral water. Different tolerances of fish from the two collection areas would raise the questions of a possible genetic difference in the population stocks, which may have been genetically separated due to the Southern Alps acting as a physical barrier between east and west coast populations. It is not known if fry from one coast can be carried to the opposite coast of the South Island, but that seems unlikely. If the populations have different physiological abilities then, for example, attempts to restock east coast rivers with west coast stock would have less chance of being successful. Malcolm Main (1988) performed pH choice experiments upon banded kokopu whitebait in which the juvenile fish were presented with channels of different pH and their choice was recorded. Juvenile banded kokopu were found to be unselective in their choice between neutral and acid water channels, entering both in similar numbers. Another galaxiid species, koaro, was found to enter neutral water channels more often than low pH channel and was therefore concluded to be selective in the pH of the water it colonised. This observation for koaro was consistent with distribution of adults which were usually found in water close to neutral pH. Adult banded kokopu from the west coast however were found to be associated with low pH water. This result suggests that the association of west coast banded kokopu with low pH water is a secondary effect of exclusion from neutral water.

The questions asked at the beginning of this study were concerned mainly with the inhabitation of acid water by west coast banded kokopu and its effects on optimal

survival. Therefore it is desirable to reflect on the studies detailed within this thesis and to provide answers to these questions. The physiological studies conducted were indicative of the quality of life for the fish. While they were not exclusive indicators, nor absolute in their indication as to the success of these fish in acid water, they were selected due to their importance at a basic physiological level and relevance to survival in their micro-habitat. Na^+ flux experiments demonstrated the effects of low pH water upon balance of that ion. Maintenance of high ionic levels in their body compartments compared to the dilute medium is important for all freshwater fish and has been demonstrated to be compromised in acid sensitive species. Ionic imbalance has an effect on enzymatic and nervous processes which like all physiological systems have optimal conditions or 'set points' at which their function will be maximised. West coast banded kokopu performed equally well at pH4 as at pH7 in this experiment. Similarly, exercise experiments showed west coast banded kokopu in pH4 were able to swim at least as well as east coast banded kokopu in pH7 and increased performance over the three swimming trials with pH fluctuating from pH4 to pH7 to pH4 in each trial respectively. Exercise performance is critical to food capture and evasion of predators as well as migration upstream during the colonising stages of the life cycle. Gill morphology observations, while limited, indicated no gill abnormalities or damage due to acid exposure in the lab or field. The overall picture constructed of the physiology of the west coast banded kokopu is one of extreme tolerance to low pH conditions, with physiological parameters considered in this thesis being maintained at least as well as east coast banded kokopu in neutral water.

McDonald (1982) ranked seven major effects of low pH on the gills in order of their importance with declining pH: inhibition of Na^+ and Cl^- uptake mechanisms, increased ion permeability and diffusional ion efflux, increased hydrogen ion permeation of the gills, enhanced mucus production and release, mucus coagulation and precipitation, inhibition of gas transfer across the gills, and damage to and separation of the gill epithelial layers. This list was compiled largely on the basis of observations on trout which are related to galaxiids. H^+ ions may have similar effects on resistant and

intolerant species. Resistant species however may require a more severe exposure to produce the same effects.

The seemingly lower tolerance to low pH water of the neutral water east coast requires some care in interpretation. Experiments were all of short duration due to the problems encountered when trying to hold fish in captivity for extended periods of time. Therefore it is difficult to conclude the amount of stress caused by captivity and its effect on the experimental results. It is possible that 'basal' physiological levels were not achieved in all or any captive fish. An improvement over time may have been possible in east coast fish had experimental procedure allowed for longer periods at low pH. Due to the standardised procedures used between east and west coast populations however, it is acceptable to make direct comparisons between the groups and it is apparent that west coast fish were more successful at low pH in the experiments used in this study. East coast fish however were able to maintain average Na^+ influx at pH4 at a level which was above that measured by Freda and McDonald (1987) for shiner (least acid tolerant), rainbow trout (intermediate), and yellow perch (most acid tolerant). Furthermore the average Na^+ efflux measurements for east coast banded kokopu at pH4, while being higher than west coast banded kokopu were less than that measured for shiner, rainbow trout, and yellow perch. Bearing in mind that the experiments conducted by Freda and McDonald (1987) were very similar in procedure to the experiments in this thesis (with the possible inclusion that banded kokopu were under greater stress due to shorter time given to recover from captivity), it is apparent that east coast banded kokopu showed greater acid tolerance than all species with the exception of the west coast banded kokopu. A similar conclusion can be made when comparing the reduction in critical swimming speed in east coast banded kokopu in pH4 water to the reduced critical swimming speed of rainbow trout in low pH water. Below pH 4.4-4.6, critical swimming speed in the rainbow trout was found to decline linearly by 4% per 0.1 pH unit (Graham and Wood, 1980). East coast banded kokopu showed reduced critical swimming speed between pH 7.0 and 4.0 of approximately 30% in total which possibly suggests that as in Na^+ balance, naïve banded kokopu are less

affected by low pH than rainbow trout. Overall the results suggest it is likely that east coast banded kokopu do have an inherent tolerance to low pH water which is not due to prior exposure. This tolerance indicated in their physiology may be a genetic artefact from pre-European history which would have seen streams on Banks Peninsula as having more complete forest cover and perhaps lower pH due to the greater forest presence.

Why would west coast banded kokopu be excluded from neutral water which is suitable and indeed preferred by another galaxiid species koaro, as well as other freshwater species of fish such as trout? As observations of populations on Banks Peninsula and physiological tests in the laboratory show, adult banded kokopu are capable of living successfully in neutral water, and are unlikely to be limited in any physiological parameters in neutral water. This is not a startling observation as neutral water is not expected to upset the physiology of a freshwater fish, but it is an important observation as it suggests that some other excluding factor must be present in neutral water streams which are not occupied by banded kokopu. I suggest that the above is at least part of the answer. Introduced brown trout and koaro preferentially occupy neutral water. Being far stronger swimmers with a critical swimming speed of almost 8 bl s^{-1} (Moffat and Davison, 1986), and due to brown trout being much larger and more territorially aggressive than banded kokopu it is probable that they are able to outcompete the banded kokopu for food and territory, and it is possible that brown trout presents some predation pressure. Therefore banded kokopu are most likely to survive into adulthood in a microhabitat which is not preferred by other fish, particularly brown trout. This contests the theory that west coast banded kokopu prefer acid water *per se* and as suggested by Main (1988), population of low pH water may be a direct result of competitive exclusion from neutral water.

It is worth noting that during my own observations of banded kokopu in the wild, there were no sightings of brown trout or any other kokopu species in the same area. The same was not true of some giant kokopu which were observed in higher pH water

(5.8) in a stream near Kaituna. In the same stream I observed several brown trout in the immediate vicinity and this sighting was confirmed by locals who lived nearby. It is likely that these species co-exist due to the ability of the giant kokopu to repel threats from brown trout (Main, 1988) and probably compete more successfully.

When extrapolating any results from laboratory study to wild populations, care must be taken so as not to make conclusions on wild populations when laboratory studies may be under a set of conditions not indicative of the natural environment of the fish. This is true of the experiments detailed within this thesis where it was only possible to carry out the prescribed physiological tests in the laboratory. Due to the nature of the studies performed (Na^+ balance physiology, exercise physiology, and gill morphology) and the environmental factor influencing these physiological and morphological parameters (pH) it is possible to isolate the areas in which problems are likely to arise from differences in laboratory conditions compared to the natural environment and account for these to some extent. The major problems in the laboratory for these studies which could introduce inconsistencies when comparing to the natural environment are related to greater fish stress in the laboratory resulting from HCl used for acidification rather than organic acids, lack of feeding during experimental periods, lower water hardness in the laboratory than some streams overstating the toxic effects of acid water and containment in aquaria. All of these factors are likely to have a similar effect on the results of laboratory work, probably overstating the effect of low pH water on the fish. In reality banded kokopu may have a greater tolerance for acid water than the laboratory tests demonstrated.

The laboratory had the advantage however of allowing standardisation of procedure which not only made comparison between east and west coast populations possible, but also allowed work on other fish species to be used for comparative purposes by adopting a similar experimental procedure. The actual physiological mechanisms of acid tolerance in banded kokopu was not investigated in any real depth in this thesis and so is open for further investigation. Mechanisms of acid toxicity/tolerance may

differ to some extent or even completely to those discovered in salmonids which are not as tolerant to low pH. What has been shown as acclimation in some studies may be present in banded kokopu as adaptation at a genetic rather than physiological level. This is another possible avenue for investigation in the future. When approaching a study such as this it is important especially when comparing data to other studies to continually recognise that the environmental acidity discussed in this thesis is natural and not a result of pollution. Examples of low pH polluted streams are also present on the west coast of the South Island. These streams which have been subjected to runoff from mining and/or deforestation tend to have clear water and are usually fishless (Mike Winterbourn, personal communication) so there is no implication that the New Zealand banded kokopu could survive in the low pH polluted waters of the northern hemisphere. The conclusion that under laboratory conditions, banded kokopu show greater acid tolerance for the parameters measured in this thesis than other salmonids tested in the northern hemisphere is reasonable however, and has conservation and environmental implications for management of the species within New Zealand. Many large areas of the South Island are no longer populated with the large galaxiid species and while populations are still present in high numbers in some areas, they tend to be disjunct with an absence from many waterways. Translocation of fish from plentiful populations to struggling or absent areas could be a future option. For this to be successful prior indication of pH tolerance/preference is beneficial when waterways in the South Island differ so greatly in pH. I have attempted to build on the work by Malcolm Main in 1988 by isolating an environmental factor associated with the distribution of the banded kokopu and testing its effect on physiological parameters essential for optimal survival. There are still many questions unanswered. Why are west coast acid water banded kokopu significantly smaller than those collected from neutral water on Banks Peninsula? I made many attempts to sample for large specimens on the west coast but the largest fish collected was 17cm long. When I sampled selectively for larger fish during exercise experiments the average size of the fish caught which were the largest I could gather was 13.6cm compared to 21cm for east coast fish. It is likely that this is indicative of the larger populations of the west coast presenting an

abundance of younger fish in the areas sampled but it is interesting that Malcolm Main did not recall collecting large banded kokopu from acid water streams (personal communication) when presumably during his study on distribution of the large galaxiids he would have travelled more extensively up waterways and observed many more populations than I was able to. Reduced growth rates have been reported as one indicator of sub-lethal chronic stress in fish (Wood, 1989) but this would require a large and complex investigation. Giant kokopu which also inhabit low pH water of comparable acidity to banded kokopu have been observed to grow to very large sizes which negates a reduced growth rate theory for that species.

Finally, further study is desirable to raise the public concern and awareness in regard to our native fish species. Preservation of New Zealand's waterways for fish while becoming increasingly widespread is often for the benefit of sport and recreational fishing of introduced trout species. This may be to the detriment of some native species which may be required to seek a habitat not inhabited by a flourishing and successful trout population which are larger, faster and more aggressive. There is no doubt considerable knowledge to be gained from locals in places such as the west coast as to the whereabouts and characteristics of local populations of kokopu. To some locals however, 'whitebait are whitebait'. While visiting the house of a resident of Kaituna I asked permission to collect on his property and was given directions as to where to find the 'small black trout' and the 'large trout'. The man had been resident on his farm for 65 years and had whitebaited and angled for most of that time. He had considerable knowledge of the local whitebait and fish and found the idea of the 'small black trout' being adult whitebait to be very funny. "I think you've got your ideas wrong there my boy" were his words and I argued no further as it was his stream after all! The small black trout turned out to be giant kokopu and some of the largest I had seen (approximately 40cm) and were the adult fish of at least one of the whitebait species he caught every season.

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