The welfare and quality of farmed salmonids at harvest.

BYRNE, H.

2002

THE WELFARE AND QUALITY OF FARMED SALMONIDS AT HARVEST

THE
ROBERT GORDON
UNIVERSITY
ABERDEEN

HAZEL BYRNE

A thesis submitted in partial fulfilment of the requirements of
The Robert Gordon University for the degree of Doctor of Philosophy

OCTOBER 2002

ABSTRACT

Welfare and Quality of Farmed Salmonids at Harvest

Hazel Byrne, PhD thesis, 2002

Salmonid farming has become a firmly established industry within the UK. To maintain or expand current markets the industry needs to produce high quality fish by humane and environmentally sustainable methods. This study examined the effects of harvesting on the stress response and carcass quality of farmed salmonids with a view to recommending best practice.

Current salmonid harvesting techniques were determined *via* a questionnaire circulated to the industry. Commonly used killing/ stunning methods were tested for their effects on the welfare and quality of rainbow trout, (*Oncorhynchus mykiss,* Walbaum 1792), and Atlantic salmon, (*Salmo salar,* Linnaeus 1758). The effect of a 30-40 degree-day fast on rainbow trout quality was investigated.

A short-term fast was shown to increase fish freshness in rainbow trout. Killing/ stunning method caused a variable response in both salmonid species, which responded in a similar manner. There was less evidence of muscle activity in fish that were instantly stunned, by electricity (trout) or percussion (both species), compared with those that died naturally, in ice (trout) or air (both species), or had been anaesthetised by carbon dioxide (salmon). A control group of trout anaesthetised with benzocaine showed the *least* signs of muscle activity. Reduced muscle activity at harvest increased fish freshness.

The primary stress response data were more equivocal. Differences were not detected in plasma noradrenaline levels in either species. The death in ice slurry trout appeared to exhibit the lowest stress response with the least adrenaline and cortisol: it was hypothesised that reduced neural responses due to the low body temperature of these fish caused this effect. Adrenaline levels were high in electrically stunned trout, the carbon dioxide narcosis salmon, and the death in air fish (both species). Percussively stunned salmon had higher plasma cortisol levels than those in the fish left to die in the air.

When trout were anaesthetised before stunning/ killing compared with their non-anaesthetised counterparts they exhibited a decline in muscle activity (ice slurry fish) and plasma adrenaline levels (electrically stunned fish).

Recommendations to the industry were, in order to improve fish freshness, fish should be fasted before harvest and muscle activity reduced by stunning the fish quickly before killing.

ACKNOWLEDGEMENTS

Special thanks are due to Dr Neil Emmison who volunteered to be my Director of Studies: without whose chauffeuring, freeze clamping and fish phlebotomy skills this thesis would have been a lot shorter! Thank you for being both mentor and kindred spirit. I hope you enjoyed our Scottish 'jollies' as much as I did.

I also thank the other members of my supervisory team, Dr Paul Nesvadba and Professor Maureen Melvin, for giving me the opportunity to do this work, for their advice, and for spending their valuable time on essential, but essentially unexciting, administrative duties.

Thanks are also due to Alex Wilson for sharing his expertise on how to use the unfathomable to calculate the undeniable. Dr Anne Low for helpful suggestions concerning the catecholamine analysis and Dr Andrew Morrison for critical appraisal of this manuscript. The technical staff in School of Life sciences were, as ever, invaluable particularly: Peter Reid, who took the amazing 'tweezers' photograph; Allan MacPherson, who made the fiddly bits of equipment; and Iain Matthew, for sorting out the drips and pumps when I was in HPLC crisis.

Funding was provided by SHEFC Grant 80/1998. The EU project CT97-3127 gave unreserved access to much needed resources without which this work could not have been attempted. Additional funding came from Innes Walker Fish Harvester Ltd., and generous donations of salmon and staff time were given by Lighthouse of Scotland Ltd., and Scottish Sea farms Ltd.

Thanks are also due to my friends and colleagues for the buns and chocolate, not to mention the constructive discussions. Sam requires a special mention for her excellent cat sitting service and Chris for the loan of 'Old Smokey'.

Last, but not least, thanks are due to my parents for the 'Byrne Podiatric Multi Fish Gauge' and their continuing encouragement and support.

DECLARATION

I declare that the work submitted in this thesis is my own except where specifically acknowledged, and that neither the thesis nor the original work contained herein has been submitted to this or any other institution for an academic award.

Hazel Byrne

CONTENTS

LIST OF FIGURES

5 THE EFFECTS OF KILLING AND STUNNING TECHNIQUE ON THE *POST MORTEM* BIOCHEMISTRY OF ANAESETHETISED RAINBOW TROUT

6 THE EFFECTS OF FASTING ON FLESH AND PROCESS QUALITY OF FARMED RAINBOW TROUT

7 THE EFFECTS OF STUNNING AND KILLING TECHNIQUE ON THE WELFARE AND QUALITY OF FARMED ATLANTIC SALMON

8 COMPARISON OF SPECIES AND THE EFFECTS OF HARVESTING TECHNIQUE

APPENDIX 2

LIST OF TABLES

LIST OF ABBREVIATIONS

a*	colour measurement describing red - greenness
Ab	antibody
AC	alternating current
ACS	American Chemical Society
ACTH	adrenocorticotrophin hormone
ADP	adenosine diphosphate
AEC	adenylate energy charge
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
b*	colour measurement describing yellow - blueness
C*	chroma colour measurement indicating intensity/ opacity
CIE 1976	Commission Internationale d'Eclairage developed a system of measuring colour in 1976 using three co-ordinates L*a*b*
CK	creatine kinase
CP	creatine phosphate, phosphocreatine
Cr	creatine
DC	direct current
DEFRA	Department for the Environment and Rural Affairs, formerly MAFF
DFD	dark firm dry, meat condition caused by *ante-mortem* glycogen depletion
DHBA	3,4-dihydroxybenzylamine hydrobromide
DMA	dimethylamine
DPB	diphenylboric acid 2-aminoethyl ester
DSI	digestive somatic index
ECD	electrochemical detection
ECF	extra cellular fluid
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immunosorbant assay
FAWC	Farm Animal Welfare Council
FEAP	Federation of European Aquaculture Producers
FFA	free fatty acids
G6P-DH	glucose 6 phosphate dehydrogenase
HK	hexokinase
HPLC	high performance/ pressure liquid chromatography
HSI	hepatosomatic index
Hx	hypoxanthine
HxR	inosine
IMP	inosine monophosphate
K value	method of assessing freshness using metabolite degradation products
K' value	method of assessing freshness using metabolite degradation products
L*	lightness value, a colour measurement of how light or dark an object is
LDH	lactate dehydrogenase
MAFF	Ministry of Agriculture Fisheries and Food, now DEFRA
MS222	fish anaesthetic 2-methyl 4-sulpholnyl amino benzoate
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
PCA	perchloric acid
PEP	phosphoenolpyruvate
pH_{96}	pH 96 hours after death
pH_i	Initial pH, i.e. immediately after 'death'

P_i	inorganic phosphate ion
PK	pyruvate kinase
PSE	pale soft exudative, meat condition caused by rapid pH fall due to lactate production
RBC	red blood cell
RI	rigor index
RSPCA	Royal Society for the Prevention of Cruelty to Animals
SDA	specific dynamic action
SR	sarcoplasmic reticulum
SSGA	Scottish Salmon Growers Association
SSI	spleen somatic index
TBAB	tetrabutylammonium bromide
TEA	triethanolamine
TMA	trimethylamine
TMAO	trimethylamine oxide
TRIS	a buffer
TVB	total volatile bases
VER	visual evoked response
WBC/WHC	water binding or holding capacity

GLOSSARY OF FISH PROCESSING AND AQUACULTURE TERMS

all female	monosex population comprising only females
belly burn/burst	effect of gut enzymes on peritoneal wall *post mortem* causing discolouration or disintegration
bleed out	fish with severed gills left in water to bleed
bloodspotting	small areas of bleeding in the flesh
brining/dry salting	preliminary process in fish smoking/ curing
cold smoked side	large fish fillet that has been cured by smoking
condition factor	length weight relationship
crowded	increased stocking density in a tank/cage up to, or above the holding capacity of the water
degree-days	a combination of temperature and time
downgrading	classification of substandard fish
dress-out percentage	percentage carcass left after gutting
drip	water and soluble proteins lost from meat
exsanguination	bleeding
fork length	length from the nose to the fork in the tail
gaping	separation of muscle blocks in a fish fillet
grading	dividing a population of fish usually by size
gutted/eviscerated	a fish carcass with the peritoneal contents removed
iki-jime	killing method, inserting a spike into the brain
in the round	an intact fish carcass
kype	bony growth on the jaw of an adult male salmon, a secondary sexual characteristic
maturation or ripeness	season when adult fish contain ripening gametes
MT4	semi-automatic device for killing salmon by percussion
on-grown	feeding small juvenile fish so that they grow into a large adults
on ice	fish stored completely surrounded by ice
portion sized	a size of fish, one per meal/ plate
process quality	fish suitable for processing further than just gutting
process yield	amount of useable flesh taken from a carcass
ranching	culture system where juvenile hatchery fish are released into the environment to complete their growth and recaptured for harvest
S0+	smolts that can be transferred to sea water for ongrowing before they are one year old
slaughter	killing by bleeding
smolt	developmental stage of salmon where it converts from a freshwater to a sea water fish
stocking density	amount of fish per unit volume of water
taint	off taste in fish flesh caused by an exogenous factor i.e. muddy flavour in trout caused by algal biproducts
thaw rigor	stronger than usual *rigor mortis* contraction occurring after thawing fish frozen pre *rigor*
transfer	moving fish from one holding area to another by means of a net or pipe
transport	moving fish from one holding area to another by vehicle
trimming	cutting away unwanted tissue from fillets
triploid	animals whose cells contain three sets of chromosomes

1 INTRODUCTION

1.1 General introduction

One of the primary considerations in modern society concerning farming, is the protection from, and the elimination of pain in farm animals [62]. In 1979, the UK government established an independent advisory body, the Farm Animal Welfare Council (FAWC) to review farm animal welfare and advise on legislative change. It was not however, until 1992 that they were instructed to investigate the welfare of farmed fish [225]. The remit of the FAWC encompassed all aspects of aquaculture including fish harvesting procedures [225].

To maintain a competitive, sustainable salmonid farming industry in the UK, issues of welfare, quality and environmental impact need to be addressed. The present study investigated the welfare and quality of salmonids during harvesting.

1.2 Salmonid harvesting techniques

Harvest is a multistage process that converts fish living in the environment in which they were reared, into carcasses of marketable condition for human consumption. It consists of a selection of the following procedures: starving or fasting; crowding; transfer; transport; handling and restraint; and killing, or stunning and slaughter. The procedures used depend upon; the species, size, and quantity of fish to be killed; the rearing facilities and their location; and the intended product or customer. Fish welfare and product quality has the potential to be compromised during any of these procedures.

1.2.1 Starving or fasting

Harvest starts with a period of fasting or starvation, where food is withheld from the stock. The metabolic rate of an animal increases after feeding as it digests and stores the nutrients consumed. This rise in metabolic rate is known as specific dynamic action (SDA) [107]. Starvation occurs when the effects of SDA are no longer detectable, this can

be 2-3 days in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) [21]; up until that point, technically, the fish are fasted [220].

Starving/ fasting improves the keeping quality of fish. It is used for food hygiene reasons: minimising bacterial contamination and delaying microbial spoilage [97]. Autolytic degradation is also delayed due to the lower metabolic rate, and, in starved fish left 'in the round', belly burn is slower to develop as gut enzyme activity also declines [189].

The length of time food is withheld from the fish depends on the water temperature as well as the fish size and species. Water temperature is of particular importance as fish are ectotherms so the environmental temperature affects their metabolic rate. In the UK before harvest, farmed rainbow trout are commonly 'starved' for 30 degree-days e.g. three days at 10°C (spring) or two days at 15°C (summer), see Chapter 3. In Norway the recommended minimum period for fasting Atlantic salmon (*Salmo salar*, Linnaeus 1758) is seven days [34]. The FAWC suggested maximum fasting times of 72 and 48 hours for salmon and trout, respectively [225] they did not however relate these times to water temperature.

Maximum 'starvation' periods were recommended by the FAWC because fasting is considered to be a chronic stressor in animals [225]. However, the Atlantic salmon during spawning migration does not feed for several months [125] and so short-term 'starvation' may not stress these fish provided the rearing conditions are optimal [56]. However, concern is voiced, albeit by animal rights bodies, that, as farmed fish are usually fed to satiation, fasting fish before harvest is cruel [137].

The FAWC recommended that starving is permitted for gut evacuation but not for fat reduction [225]. Lipids are the most important energy source during long-term starvation [135]. Salmon are slow to release their muscle fat and so extended starvation periods are needed to reduce flesh lipid levels [214]. Prolonged fasting reduces fat levels in the carcass as a whole, and may therefore affect eating and process quality [135, 240, 272]. If rearing conditions are not optimal, aggressive behaviour in fish can increase during long term

starvation and/ or reduced feeding levels and the welfare of subordinate fish may be compromised [56].

Starved/ fasted fish are more easily handled as they produce less particulates, (vomit and faecal matter), and toxic excretory products, (ammonia), so respiratory stress and gill irritation in high-density situations is minimised. Starving reduces oxygen consumption, through the effects of SDA, as well as activity levels in fish, this facilitates crowding and transportation [263].

1.2.2 Crowding

Crowding is where; the density of fish in the cage/ net is increased to, or above, the carrying capacity of the water, and the density tolerance of the fish. It facilitates the movement of fish from the rearing area to the holding tank or slaughter facility. Rough crowding techniques can cause acute stress responses and physical damage to the stock [263].

1.2.3 Transport and transfer

If fish are to be killed at a place other than the rearing facility, they have to be moved, (transported), to the harvesting area. Well-boats or specially designed transport lorries are used to deliver them live to processing plants. Large numbers of fish are moved in small volumes of water, which can cause physical damage and acute stress [34, 263]. Transferring fish from the rearing facility to the transport vehicle or from the vehicle to a holding facility can also cause stress in fish [73].

1.2.4 Handling and restraint

The penultimate harvesting procedures are handling and restraint. Fish are removed from their holding facilities and either placed in the killing or stunning tank; or restrained manually to allow application of a stun. A handling technique known as live-chilling is employed by the salmon farming industry which lowers the pre-slaughter body temperature of fish to decrease muscle activity [217]. It is thought that this process delays *rigor mortis* and increases carcass quality [127, 217].

1.2.5 Stunning and killing

Stunning renders an animal unconscious so that it can be killed, while slaughter is a method of killing an animal using exsanguination [10]. The slaughter or killing of all UK farm animals is legislated by 'The Welfare of Animals (Slaughter or Killing) Regulations 1995' [10]. Fish however are not specifically mentioned in the Act, so although they are members of the Animal Kingdom it is uncertain whether they are covered by the legislation. The Act states that if animals are stunned, they should remain insensate until death occurs and that provided they are instantly killed or stunned, both manual and automated killing methods are permitted [10].

Trout in the UK are generally killed by anoxia either directly by removing them from the water, or indirectly following; a percussive stun, an electrical stun, a hypothermic shock (death in ice slurry) and more rarely carbon-dioxide narcosis (CO_2-narcosis). Salmon are most commonly slaughtered following CO_2-narcosis or a percussive stun, although iki-jime or exsanguination alone can be used, as detailed in Section 3.3.3.

1.2.5.1 Death in air

Death in air is the method commonly used for killing large numbers of portion sized rainbow trout [185]. Fish are netted from the holding facility into bins and left to die of anoxia [118]. Once removed from the water the gill filaments compact and gas exchange is prevented [184]. The oxygen demand of the tissue therefore dictates the time it takes for the fish to die, i.e. if they struggle, or if the environmental temperature is high, the metabolic rate is fast and the fish die quicker [119].

The advantages of the death in air method are that; it is inexpensive, it is not labour intensive, does not require a skilled workforce and also large quantities of fish can be despatched at one time. Physical damage can occur while the fish are struggling and if holding bins are over loaded, crush injuries are sustained. It is not easy to determine time of death or insensibility in fish killed by this method, especially in cold weather. If fish are eviscerated immediately after they have stopped struggling, the gutting process may be the

actual cause of death [184]. The FAWC state that this method is not acceptable for fish although it is yet to be covered by the legislation [225].

1.2.5.2 Death in ice slurry

A delay in the icing of fish reduces their freshness [51]. A killing method where live trout are added directly to ice, ice-water slurry or ice-saltwater slurry eliminates any such delay. If ice slurry is used, the fish are immersed for at least ten minutes and then the water drained (Steve Kestin, Bristol University, personal communication), and the fish die from anoxia while in the ice [118]. The low temperature reduces the metabolic rate of, and visible signs of struggling in, the fish but it extends the time it takes for them to die. Work by Kestin *et al.* [119] has shown that trout remain sensate whilst dying at these low temperatures. To reduce water uptake by the fish, salt is sometimes added to the ice slurry; this would lower the temperature further.

The FAWC were so concerned about the use of ice slurry as a killing method they recommended its prohibition [225]. Regardless of this concern however, the method is still widely used in the UK trout farming industry as the fish stop struggling quickly and so it appears humane. The method is not labour intensive nor does it require a skilled workforce and large numbers of fish can be despatched at once. It does however require copious supplies of ice on site.

1.2.5.3 Exsanguination

Exsanguination with or without a prior stun is a method used to slaughter salmon and large trout. One set of gill arches are severed either by pulling with the fingers, or cutting with a knife [9]. Once the gills have been cut, the fish are returned to the water to 'bleed out' to prevent blood clots occluding severed blood vessels. Bleeding is more efficient from cut than pulled gills, as stretch receptors in the arterial wall do not stimulate the muscle to contract and constrict the blood vessel. Research has shown cutting both sets of gills increases 'bleed out' in stunned fish [9]. Fish lose consciousness quicker the more vessels cut. However, in routine practice only between two and four gill arches are

severed [187]. Eventually death occurs from anoxia due to blood loss [187]. Activity hastens time of death by increasing the heart rate and depleting oxygen stores. During the dying process, which can take 5-10 minutes, vigorous muscle activity is often observed [187]. Gill cutting is thought to be stressful as fish shake their heads while bleeding, a possible sign of pain [187]. This method is labour intensive, it does not attempt to stun the fish, and therefore does not meet the legislative requirement for killing farm animals.

1.2.5.4 Iki-jime

Spiking or 'iki-jime', which means live killing in Japanese, is a harvesting technique that causes death by physically destroying the brain with a spike [103, 104]. This technique is only suitable for fish where the brain is large enough to locate quickly. The fish are restrained and the spike inserted into the brain through the dorsal surface of the skull. The spike is then moved around to ensure brain destruction. If performed correctly the animal stops struggling after insertion of the spike and death is instantaneous, it is possible however to miss the brain completely [8, 184]. Brain destruction or severance from the spinal column is thought to reduce muscle stimulation and so improve flesh quality [258]. This technique leaves a hole in the head of the fish and, as salmon are sold 'head on', there may be consumer resistance to fish killed by this method. An automated iki-jime machine for killing salmon was launched onto the market by Baader in 1998 [11] but currently is no longer for sale, (Tony Marsh, Fish Harvester Ltd., personal communication). Iki-jime is a labour intensive method and requires skilled labour and is not widely used in the UK.

1.2.5.5 Percussion

A commonly used method of stunning fish is the percussive stun, which can be used in conjunction with exsanguination, or prior to evisceration. Fish are removed from the water, restrained, and a single or rapid multiple blows applied to the dorsal surface of the head with a club [9]. If the blows are applied correctly, the brain moves rapidly inside the skull creating shearing forces which, depending on the strength of the force applied, disrupt neural processes and renders the animal either dead or insensible [253]. If death is

not instantaneous, then later haemorrhaging into the brain causes death [253]. This method is labour intensive, can be hazardous to staff and has to be done correctly, which is not easy if the fish struggle and once the operatives are cold, wet, and tired. Quality problems can occur if blows are of excessive force (expulsion of eyes, and skull damage), or not correctly aimed (bruising), bruising may also occur from the restraint procedure [253].

Automated devices, which apply a mechanical stun to the head of fish are currently in use in the UK. One such device, the MT4 (Seafood Innovations Pty Ltd., Australia), relies on manual insertion of the fish into the machine, fully automated machines are being developed [93] but are not yet widely used in the UK, (Tony Marsh, Fish Harvester Ltd., personal communication).

1.2.5.6 Electrocution or electrical stunning

Electrocution or electrical stunning is a well-established harvesting method used by the trout farming industry. Batches of portion sized trout are electrocuted, (killed), or electrically stunned using specialised equipment which applies a current to the whole body. The fish are netted from the holding facility into a water filled chamber, and the current applied. The duration of the stun and the current applied varies with the equipment used. The fish lose consciousness due to disruption of neural processes in the brain, and possibly the blood supply to the brain due to arrhythmia [185]. At low currents, immobilisation of the fish may occur through exhaustion because of excessive muscle stimulation, and not from unconsciousness [184, 191].

Whole body electrical stunning of trout can cause carcass damage; reported injuries are fracture dislocation of vertebrae, haemorrhages and blood clots [185, 192] and are a result of strong uncoordinated muscular contractions caused by stimulation of the neuromuscular pathway or epileptic seizures [192]. Such quality defects can be avoided by passing the electricity directly across the head of the fish. However, head only stun requires the fish to be restrained and treated individually. The current required is higher than for whole body stun and so there are associated hazards for personnel, as a result this method is not

routinely used [118, 131, 132]. Whole body stun can be used to stun large quantities of fish at a time, is not labour intensive and does not require skilled workers, although specialist equipment and an on site electricity supply are required. A prototype, continuous flow, water bath, electrical stunning system has recently been developed which allows portion-sized trout to be harvested directly from a pond so negating pre-slaughter stresses. However, this machine is not yet in commercial production [131, 132].

The FAWC recommend that fish that have been stunned rather than electrocuted should be eviscerated before they recover consciousness and that the heart is removed [225]. Heart removal is stipulated because one of the clinical signs of recognition and confirmation of death is the permanent cessation of the heartbeat [50].

1.2.5.7 Carbon dioxide narcosis

Carbon-dioxide narcosis, while not generally used for killing rainbow trout, is currently one of the principal methods for stunning salmon in Scotland and Ireland [9, 81].

Carbon dioxide is a recognised method of killing non-aquatic animals and has been adapted for use with fish. The CO_2 is absorbed across the gill resulting in respiratory acidosis, which disrupts neural mechanisms that ultimately cause death [184, 270].

The technique involves dissolving CO_2 in the water of a holding tank, by diffusion through an air stone or 'leaky pipe'. The saturation point is reached when the pH is 4.5. A pH of approximately 5.0 gives a level of >250 torr which is sufficient to stun the fish in six minutes [9].

The use of CO_2 as an anaesthetic for the industrial slaughter of salmon is under some doubt [191], as extreme aversive behaviour has been observed during CO_2-narcosis in both Atlantic salmon [185], and rainbow trout, [118]. The FAWC state salmon can be exposed to CO_2 saturated seawater without an adverse response [225]. They add the proviso that if fish are stunned using this method that care should be taken to ensure that the fish have progressed from immobility to insensibility before exsanguination [225].

1.3 Welfare

1.3.1 Concerns for animal welfare

Media reporting of environmental and farm animal welfare issues has influenced consumer bodies and consumers in such a way that they are no longer only interested in the price and quality of food [53]. Bennett [25] elegantly demonstrated this in a study on the willingness of the public to pay a premium for free-range eggs. Gregory [84] proposed three main reasons for concern about animal welfare:

1. respect for animals and a sense of fair play
2. poor welfare leading to reduced product quality
3. loss of market share for products with a poor welfare image

Major retailers have long accepted that they have an ethical responsibility to provide safe, high quality, good value products [53]. More recently, they have responded to customer concerns for animal welfare. Retailers now supply organic meat and fish products and even apply the five freedoms paradigm to non-organic meat. The five freedoms, on which the principles of the FAWC are based, are:

1. freedom from thirst, hunger and malnutrition
2. freedom from discomfort
3. freedom from pain, injury and disease
4. freedom to express normal behaviour
5. freedom from fear and distress

Labelling schemes, initiated by independent bodies, provide consumers with information enabling them to chose meat reared to 'high' welfare standards. For example, the National Farmers Union's 'Farm Assured or red tractor' label and the RSPCA's 'Freedom Foods or blue label'. The Freedom Foods Label does not as yet, include farmed fish, but will once information on fish welfare becomes available (Martin Potter, RSPCA, personal communication).

1.3.2 Defining animal welfare

Concern for fish welfare has been less evident than for other farm animals, possibly because of their lower emotive potential. The delayed adoption of welfare issues to fish might also have been influenced by uncertainties about sentience and pain perception in fish [4].

It has been suggested that fish do not feel pain due to the primitive structure of their brains [190], but pain as defined in this instance was subjective, in that it had a cognitive element associated with it. This is not however the only definition. 'Objective pain' corresponds with activation of nociceptors by noxious stimuli [271]. Fish respond to, and exhibit avoidance behaviour to, pain stimuli [45]. They show learned behaviour to unavoidable stressors and modify subsequent responses [118, 164]. They therefore fulfil the criteria laid down by Zimmerman for defining pain in animals [273].

Whether fish have a sense of fear is another issue. Fear requires the ability to anticipate impending danger or pain and therefore requires cognition and the use of higher centres of the brain [42, 190]. If welfare is linked to the ability of fish to feel fear, as in the 'five freedoms', it has yet to be quantified.

Definitions of animal welfare generally fall into two schools of thought, feelings or function. The 'feelings group' define good welfare as when an animal experiences positive emotional states, (pleasure), and where negative emotional states, (suffering), are absent [61]. The biological function group however, define animal welfare as an animals 'state as regards its attempts to cope with its environment' [42]. The latter definition allows quantitation and separates welfare measurements from ethical questions as to whether the animal's state is acceptable. The acceptability of the animals condition can then be judged on objective evidence and societies moral values, so within this definition, an animal's welfare is poor when it is under stress. Stress is an environmental influence which over-taxes an animals regulatory systems and lessens its competence [42].

1.3.3 Biological indicators of stress in fish

Biological indicators of stress are divided into primary, secondary, tertiary and quaternary responses [263].

The endocrine system produces the primary response following perception of a stressful stimulus. The response consists of the acute (rapid) response, and the chronic response, which occurs in the medium to long term. The sympathetic nervous system stimulates release of stored catecholamines from chromaffin cells in the anterior kidney in the acute response [263], although release can be mediated through other mechanisms [115, 167, 168]. In the latter stages of acute stress, and in chronic stress, humoral stimulation of interrenal cells in the anterior kidney causes synthesis and subsequent release of cortisol. The hypothalamus initiates this response by releasing corticotrophin releasing factor, stimulating the anterior pituitary to produce adrenocorticotrophin hormone (ACTH), which stimulates the kidney to produce cortisol [263].

In general, hormones released in the primary response, act on tissues enabling the fish to deal with the stressor, this is the secondary stress response. The effects are; to increase heart rate, dilate blood vessels in gills and muscles and constrict vessels to 'non-essential' systems. Glucose production is increased and the ionic permeability of the gills may alter [37].

The tertiary stress response, is where the whole animal responds to the stressor. It can be a behavioural change such as an avoidance behaviour or disruption to a whole body system e.g. cessation of egg production [263].

Quaternary responses are those where entire populations or ecosystems respond e.g. year class recruitment failure and consequent population crash [169, 263].

1.3.4 Methods for measuring primary and secondary stress responses in fish

1.3.4.1 Haematological methods

1.3.4.1a Primary stress hormones

Quantitation of the primary stress response is the most valid indicator of physiological stress. The catecholamines responsible for the acute stress response, adrenaline and noradrenaline can be measured in blood plasma. Plasma cortisol levels indicate the chronic stress response, although some authors measure ACTH in preference to cortisol to determine the very early stages of the response [231, 262]. Catecholamines deteriorate easily in blood and so careful sample preparation is required. Antioxidants can be added to prevent catecholamine oxidation and the subsequent sample should be kept chilled, or if it is to be stored for any length of time, frozen to at least -80°C, to prevent deterioration [91]. The chemical and immunological techniques used for measuring these hormones are, however, expensive, time consuming and require specialist equipment; consequently, secondary stress response indicators are often used as an alternative [99, 100].

1.3.4.1b Secondary stress indicators in blood

The pH of blood can be used as a quick measure of metabolic and respiratory stress [172]. During severe stress, blood pH falls because of rising lactate levels, and H^+ extrusion from RBC's provided the fish are hypoxic [237]. Plasma glucose levels also rise due to the action of chronic stress hormones and so can be used as a measure of the secondary stress response [100]. However glucose is not as closely regulated in fish as in mammals [149] and a raised glucose level might therefore reflect nutritional status.

Haematological features such as haemoglobin content and haematocrit are also used to measure stress in fish [100]. They are inexpensive tests, which can indicate haemconcentration or haemdilution of the blood, which can suggest impairment of osmoregulation and RBC volume changes due to catecholamine stimulation. The effectiveness of osmoregulation can also be assessed by measuring the levels of sodium and chloride ions in the blood and plasma protein levels [100]. Plasma osmolarity is also used as

a stress indicator as it provides information on the efficacy of osmoregulation in combination with other factors such as raised glucose level [246].

1.3.4.2 Muscle energy status

As the primary stress response prepares skeletal muscle for activity, muscle energy-status can be used as a measure of activity/ stress. The amount of anaerobic glycolysis that has occurred in muscle can be estimated by measuring lactate levels and pH. The energy status of muscle can be quantified by measuring nucleotide levels. The adenylate energy charge (AEC), Equation 1.1, is an index of energy available for metabolism from the adenylate pool [180]. AEC varies from zero to one, larger values indicating higher energy status [180].

Equation 1.1

$$AEC=(0.5ADP+ATP)/(ATP+ADP+AMP)$$

Where ATP is adenosine triphosphate, ADP is adenosine diphosphate, and AMP adenosine monophosphate.

The percentage phosphorylation of the creatine pool, (sum of creatine phosphate (CP) and creatine (Cr)), and the CP: Cr ratio, are also used to indicate the remaining buffering capacity of creatine phosphate in the muscle. The ratio of creatine phosphate to ATP indicates how well an animal is coping with its conditions. Once the CP: ATP ratio falls below 1:1 the rate of decline of ATP in the cell is extremely rapid [259] and homeostatic mechanisms will soon fail. The ATP: IMP ratio is sometimes used to show energy status as IMP, (inosine monophosphate) is an early degradation product of ATP [71].

Changes in muscle metabolism can be followed *in vivo* using NMR techniques. This method gives an accurate picture of what is occurring in a live animal as the animal can be rested post handling and can therefore act as its own control. An NMR study using live common carp (*Cyprinus carpio*, Linnaeus 1758) has reported 80% phosphorylation of the creatine pool [251].

Invasive methods are less rigorous because of the techniques involved; handling and killing stress, the excision procedure, as well as sample preparation and measurement. In rainbow trout using enzymatic measurements of freeze clamped tissue, phosphorylation

levels around 39% or 45% are more common [58, 59, 63]. Although Wang *et al.* [256] stated that 80% phosphorylation is achievable with enzymatic techniques. However, the total creatine pool data exhibits in Wangs work differs from other studies, which might have artificially elevated the phosphorylation level. Invasive methods have the advantage in that they can be performed in the field making data collected more authentic. There are special difficulties associated with field sampling however due to the lack of controlled conditions and the need to store and transport samples before analysis.

To determine resting values, fish handling needs to be kept to a minimum, unconsciousness and death should be achieved quickly without undue struggling [91]. Use of anaesthetics can reduce muscle activity but induction of anaesthesia may evoke a stress response particularly if the animal becomes hypoxic [91].

When sampling tissue for nucleotide analysis it is vital to stop metabolic activity as soon as possible, and to prevent it occurring during subsequent storage, extraction and measurement. Metabolic processes continue *post mortem,* so dissection or biopsy must be completed as quickly as possible [91]. Time may limit the numbers of samples that can be taken, although Erikson *et al.* [72] found that in rested salmon the creatine phosphate levels were maintained for 5 h and actually increased after 2 h *post mortem.* This increase might however have been due to loss of muscle tone *post mortem* and so sampling over an extended period might increase the variability of the data.

Even when tissue is extracted immediately after excision, the metabolic reactions must be stopped during sampling, especially if the process being measured has a high reaction rate. Reactions can be halted within milliseconds by freeze clamping tissue. This involves cooling aluminium or copper tongs in liquid nitrogen to –196°C. The sample is clamped between the tongs, which flattens and freezes the tissue simultaneously. Snap freezing, placing the sample directly into liquid nitrogen, is less effective as the liquid nitrogen 'boils' when the sample is added and so the time taken to freeze the centre of the sample is prolonged [91]. The freezing rate is dependent on the distance to the core, the

temperature difference between the sample and the freezing media and the heat transfer coefficient of the tissue. The conductance of heat through water, (~80% tissue), is slow, so freezing rate is dependant on the sample size.

Sample excision needs to be fast and cause minimal damage, biopsy is faster than dissection but both methods cause tissue damage [91]. On cutting, damage will always occur at the cellular level, but more generalised damage also has detrimental effects. Severed nerves stimulate adjacent tissues *via* spinal reflexes and so the metabolic rate of the tissue can be increased [72].

Samples must remain frozen while being transported to the laboratory and during subsequent storage. Metabolites in frozen tissues are not stable at 'high' frozen temperatures e.g. –20°C. Enzymes can remain active at these temperatures so nucleotide and free fatty acid (FFA) levels can change [91]. To prevent sample deterioration the storage temperature should be as low as possible, at least -80°C preferably at liquid nitrogen temperatures [91].

Decomposition of nucleotides can occur during thawing and extraction [91], the frozen sample should therefore be pulverised with frozen extraction medium, the surface area of the pulverised sample should be maximal and it should be thoroughly mixed to minimise the time available for decomposition. The tissue needs to be pulverised into small and consistently sized particles to reduce diffusion barriers, break protein substrate bonds and to block enzyme activity. Even after extraction, metabolites may still be unstable so if the extract is not to be analysed immediately it should be frozen [91]. While the homogenate is thawing, glycolysis should be kept to a minimum. Glycolysis and hydrolysis of high energy bonds proceeds at higher rates between –0.8°C and –5°C than at room temperature [91]. This occurs because part of the tissue water is contained within ice crystals when frozen and so, as the tissue thaws, the enzymes, substrates, ions, and modulators are concentrated: consequently enzymatic reactions are enhanced. The ice crystals may also rupture membranes releasing Ca^{2+} ions into the cell from the sarcoplasmic

reticulum (SR) and Na^+ ions from the extracellular fluids. The tissue can be freeze-dried before extraction to prevent this enhanced activity, or EDTA and ethanol can be added to the frozen homogenate. Ethanol at 30% v/v melts the homogenate at –15°C, while the EDTA chelates divalent cations and so prevents many enzymatic activities [91].

Once the samples have been extracted, they should be kept chilled and measured as quickly as possible. Internal standards should be used where possible as samples contain unknown compounds, which may disturb the measurements [91]. Methods commonly used for determining metabolite levels in excised tissue are HPLC and enzymatic analysis with spectrophotometric detection.

1.3.4.3 Rigor mortis

Rigor mortis occurs when the muscle is depleted of ATP, see Section 1.4.1.1a. The time taken for the onset of *rigor mortis* to develop, the duration of *rigor mortis* and the strength of its contraction have been used as a measure of fish welfare at harvest. In fish that have been stressed, the onset of *rigor mortis* is earlier, the contractions are stronger, and the duration is shorter, than in rested fish [26, 157, 210]. The utilisation of ATP along the carcass is not necessarily uniform and so during the early stages of *rigor mortis* the stage of *rigor mortis* may differ with location [26]. This non-uniform ATP degradation may explain why the strength of *rigor mortis* contraction is higher in stressed fish. If all the cells enter *rigor mortis* simultaneously, then the contraction over the entire fish would be stronger than if individual cells went into *rigor mortis* at different times. The timing of r*igor mortis* development is not only dependent on endogenous factors such as degree of struggling before death, but also upon exogenous factors such as storage temperature.

The most common techniques of *rigor mortis* measurement for fish 'in the round' are 'rigor index' (RI) and 'degree of droop'. The original method for measuring *rigor mortis* was described by Cutting (1939) cited in Bito *et al.* [31]. The method was modified by Bito *et al.*, [31], and works on the principle of measuring the distance between a horizontal surface and the base of the tail of the fish laid on that surface, Figure 1.1. The anterior half of the fish

is placed on the surface and the posterior half allowed to sag over the edge. The RI is then calculated using Equation 1.2. Where L is the length measured from the horizontal surface to the base of the tail and L' is the length measured from the surface to the base of the tail at time zero.

Equation 1.2

$$RI = \frac{L - L'}{L} \times 100$$

The degree of droop measurement is similar to the RI method except it measures the angle the fish tail makes relative to the horizontal surface, Figure 1.1. Other modifications to the RI method have been used. Korhonen *et al.* clamped the fish vertically by the tail and measured the angle of sag [123], and Azam *et al.*, [16] used an image processing system to record the droop measurements.

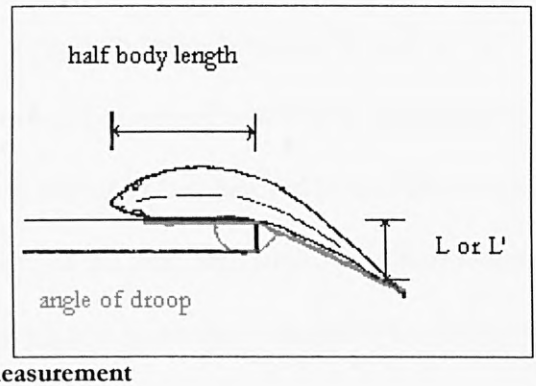


Figure 1.1 ***Rigor mortis*** **measurement**
Adapted from Bito *et al.* [31]

When measuring *rigor mortis* in freshly killed, rested fish, the initial measurement can be higher than the reading taken about an hour later. This might be due to the loss of underlying muscle tone present in live fish. After death, muscles are no longer stimulated by the sympathetic nervous system and so while ATP is being produced rapidly, and there is residual oxygen, they can fully relax, as detailed in Section 1.4.1.1a.

Handling fish can affect the duration and intensity of *rigor mortis* and therefore should be kept to a minimum [26]. To avoid this problem other methods have been developed to assess *rigor mortis* status; sensory panel [54, 55], texture measurements [224], or tension of excised muscle [103-105].

Measurements made on isolated muscle will be affected by the excision process, fibre location and orientation. There are two types of test: isometric and isotonic. Isometric tension tests measure the force required keep the muscle sample a constant length [103, 104, 123, 155, 156]. Isotonic tension tests measure the extensibility of the muscle by allowing it to shorten under a constant loading and unloading cycle. If the force applied is constant, the change in muscle length evaluates *rigor mortis* [204].

1.3.5 Existing work on the welfare of farmed fish at harvest

1.3.5.1 Starving and exercise

The effects of 'starving' on fish welfare depends on factors such as fish size, species, environmental conditions, and the duration of the fast. Atlantic salmon naturally live without food while on the freshwater phase of their spawning migration [106]. They have adapted to endure long-term food shortages [52] and so fish reaching maturity may not be adversely affected by short periods of starvation. Starved fish may not however be able to respond to stressors as effectively as their fed counterparts as responding to a stressor involves a metabolic cost. In the wild, this would be disadvantageous. However, in farmed fish imminent for slaughter, a reduced stress response would have less significance. Whether a reduced response indicates a reduction in stress or an impairment of the response due to nutritional state is uncertain. Nakano and Tomlinson [154] hypothesised that the extent of post stress hyperglycaemia in rainbow trout was directly related to tissue glycogen levels. This was confirmed by Barton and Schreck [20] who demonstrated that the hyperglycaemic response of juvenile Chinook salmon (*Oncorhynchus tschawytscha*, Walbaum 1792) was lower in fish fasted for 20 days, compared with fed individuals. Work by Ruane *et al.* [194] demonstrated a reduced/ delayed cortisol response to confinement stress in common carp kept on a restricted ration. The restricted ration carp had lower cortisol levels after 30 min the significance of which was lost after one and three hours confinement. Plasma glucose and FFA levels were also lower, however the FFA levels in the starved fish were originally low [194, 195]. Conversely, Vijayan and Moon [250] found an

increased cortisol response to a three-minute handling stress in rainbow trout following 30 days starvation. The fed and starved groups had the same initial cortisol levels. It was suggested that the higher levels of cortisol were due to higher secretion rates in the starved fish, which increased sensitivity to the stressor. An alternative suggestion was the clearance of cortisol may have been reduced, but this was thought to be less likely.

Long-term starvation or reduced feeding to slow growth in cultured fish is however thought be a welfare issue in high-density situations [56]. Aggression, although normal fish behaviour, may become accentuated and severe fin nipping can result [56].

Exercise in fish can also initiate cortisol secretion. However, the level of the response depends on the degree of exercise and whether it is associated with other stressors [145, 238]. Work by Thomas *et al.* [238] in rainbow trout, found that stress and exercise elevated plasma cortisol compared with a control group, and, that the response was additive. The results were not however always reproducible, possibly due to a prior unnoticed stressor [238]. In Atlantic salmon stress and, stress with exercise, elicited a response while exercise alone did not [238].

Handling often increases muscle activity levels in fish. Work by Erikson *et al.* [71] compared fish killed immediately by blow to the head (unstressed), and fish which were chased round the tank and crowded before being killed (stressed), with anaesthetised fish. In the anaesthetised, unstressed and stressed groups, the muscle pH was 7.4, 7.2, and 6.8, the AEC was 0.90, 0.93, and 0.86, and the ATP: IMP ratio was 11.2:1, 15.5:1, 1.4:1, respectively. This suggests that exercised fish had a lower energy status and exhibited greater evidence of anaerobic glycolysis than rested fish.

1.3.5.2 Crowding

The effect of pre-slaughter crowding on Atlantic salmon was evaluated by Skjervold *et al.* [216]. After an 11-day fast, fish were kept for two hours at stocking densities of 50 or 300kg/m^3. Plasma cortisol, glucose and lactate levels were higher in the high-density group and there was an earlier onset and resolution of *rigor mortis* in these fish [216].

The advanced *rigor mortis* in the crowded group indicated that energy levels were lower in these fish, see Sections 1.3.4.3 and 1.4.1.1a.

Robb and Warriss [181] report that four hours crowding in a net caused a faster decline in muscle pH in Atlantic salmon compared with uncrowded fish. The faster decline in muscle pH indicated that there was more anaerobic activity occurring in the crowded fish and so their energy status would be correspondingly lower. Sigholt *et al.* [211] showed that even a short crowding stress of ten minutes was sufficient to affect Atlantic salmon before slaughter. Crowding lowered the pH as well as the energy status of the muscle. Crowding therefore appears to be a severe stressor to fish.

1.3.5.3 Transport, transfer, holding and restraint

Other harvesting procedures are also thought to contribute to pre-slaughter handling stress. For example, transportation was reported to evoke a stress response in fish [102].

Work by Flos *et al.* [80] investigated the effects of grading and transport in rainbow trout. The control fish had the lowest plasma cortisol levels one-hour post handling, fish that had been transported had the next lowest levels then grading, grading and transport combined produced the highest response. The difference in plasma cortisol between the groups had reduced three hours after handling and was back to normal after ten. The plasma glucose levels showed a similar pattern but did not distinguish between grading and transport and so cortisol was thought to be a more sensitive stress indicator than glucose. Iversen *et al.* [98] demonstrated a peak in plasma cortisol in Atlantic salmon smolts one hour after road transport and disturbances in osmoregulation in excess of 48 h. However, the work showed, that capture was a more severe stressor than transport *per se*.

Transport was also shown to have only a limited effect on stress in rainbow trout by Ostenfeld *et al.* [163]. Muscle pH increased, and lactate decreased in transported fish relative to the control, which indicates that the fish became calmer during transportation [163]. Work by Erikson *et al.* [73] also demonstrated that handling stresses can be minimal

when harvesting fish. In this study Atlantic salmon were transferred from a cage to a boat and transported to the processing plant for slaughter. The muscle pH of the fish was sampled at the cage (7.4), four hours after arrival at the plant (7.4) and after slaughter following CO_2-narcosis (7.0, significantly lower). This indicates that the fish were not greatly stressed by any of the handling procedures. The muscle energy status was also measured, the highest levels of creatine phosphate were found in the fish that were sampled four hours after transportation. The levels in these fish were significantly higher than those found at the cage edge, the lowest level of creatine phosphate was found in the fish after slaughter. The AEC was 0.91 at the cage, 0.94 after transport, and 0.91 after slaughter with no significant differences between the groups. This suggests that AEC is not as sensitive an indicator of energy status as creatine phosphate.

The literature suggests that the stress of capture might therefore have a greater effect on fish than transportation and transfer *per se,* and stress can be negated if fish are handled correctly.

An alternative method of producing rested fish for slaughter is to prevent capture stress. The anaesthetic Aqui-S™ has been shown to reduce pre-slaughter handling stresses in salmonids [181]. The fish become more handlable and exhibit less escape behaviour when anaesthetised. The anaesthetic delayed onset and reduced the contraction strength of *rigor mortis* in Atlantic salmon, indicating higher muscle energy status in the anaesthetised fish [181]. Fish exposed to Aqui-S™ had a higher initial pH than those subjected to crowding or CO_2-narcosis i.e. they showed less signs of anaerobic glycolysis. The muscle pH of fish killed by a percussive stun without exposure to Aqui-S™, however was even higher, suggesting that the anaesthetic *per se* influenced muscle activity [181]. Conversely, Davidson *et al.* [57] did not find Aqui-S™ alleviated a 30 minute crowding stress at a stocking density of 0.1kg/l. The stress response in this work may have been evoked by the rapid induction of anaesthesia, the fish partially lost equilibrium after only five minutes [57].

In Norway Atlantic salmon are chilled to approximately 1°C for an hour prior to stunning to improve product quality. *Rigor mortis* is delayed and weaker in fish that are live-chilled [217]. This is interesting, as the plasma cortisol levels in the live-chilled fish in the study detailed by Skjervold *et al.* [217] were higher than in fish that were not chilled. The reduced metabolic rate caused by the low temperature was therefore sufficient to mask the effects of the primary stress response. The stress response induced by the live-chilling was not as great as that induced by crowding. A combination of the two stresses produced the highest cortisol levels. The response was however, not completely additive [217].

1.3.5.4 Stunning, killing and slaughtering

1.3.5.4a Visually evoked responses

Determining whether a fish has lost consciousness or died is difficult. One method that has been developed to determine onset of brain death is electro-encephalography [119, 187]. This method evaluates the patency of visual pathways in the brain. Light perception is thought to be one of the last responses to cease functioning before brain failure. If the pathway fails, the animal is incapable of producing visual evoked responses (VER's) and is regarded as unconscious [187]. During deep anaesthesia, VER's are absent, and light anaesthesia produces a reduced response [119]. Work by Kestin *et al.* [119] investigated the VER's of rainbow trout left to die in air, in fish that had been acclimated to three water temperatures. The VER's were lost in 158±39s, 180±32s and 578±168s, at 20°C, 14°C and 2°C, respectively. Failure of VER's was earlier in fish that struggled violently. The time it took for all movements to stop in the fish were 676±98s, 1663±1022s and 11,858±4611s, at 20°C, 14°C and 2°C, respectively. Onset of brain failure was therefore faster at higher temperatures. This finding has implications for fish that are killed in ice, a procedure that appears to be humane because the fish cease to struggle quickly.

The work by Robb *et al.* [187] investigated the effectiveness of various slaughter methods on farmed Atlantic salmon using VER's. The stunning/ killing techniques

studied were exsanguination with or without prior stun by CO_2-narcosis, blow to the head, or iki-jime. Fish died immediately with the latter two methods provided they were performed correctly. Fish that were stunned with CO_2 lost their VER's after 300-554s while fish exsanguinated without a prior stun took 148-440s: aversive behaviour was observed during both these procedures. This work showed that of the methods tested, only blow to the head and iki-jime met welfare requirements [187].

1.3.5.4b *Rigor mortis*

Early onset of *rigor mortis* may indicate poor welfare in fish, as *rigor mortis* develops once energy supplies in the muscle are exhausted, and stress at harvest would increase energy utilisation. Azam *et al.* [15, 16] investigated the effects of slaughter on *rigor mortis* in rainbow trout. The stunning methods used were CO_2-narcosis, AC and DC electricity, percussive stun and ice slurry [15]. Onset of *rigor mortis* started first in the ice slurry fish and then in the fish stunned using CO_2-narcosis. The percussive stun group went into *rigor mortis* last and the electrically stunned fish fell between the two extremes. *Rigor mortis* resolved by 53-55 hours regardless of treatment, so the ice slurry fish remained in 'full' *rigor mortis* for the longest period [15]. The delay in *rigor mortis* with percussive stun was confirmed in a second study [16]. Here, resolution of 'full' *rigor mortis* in the percussively stunned fish was earlier and the fish came out of *rigor mortis* by 88-90 hours regardless of treatment [16]. It was concluded that stunning methods induced different degrees of stress which altered *rigor mortis* onset, but resolution was always the same [15, 16]. Other authors investigating the effects of stress on slaughter of Atlantic salmon have found earlier onset of *rigor mortis* in stressed fish, but where this occurred the duration of *rigor mortis* was shorter [181, 211]. The work by Robb and Warriss [181] found that in Atlantic salmon slaughtered following CO_2-narcosis *rigor mortis* was earlier and shorter compared with that of anaesthetised fish. This was also shown by Berg *et al.* [26] who reported that the maximal contraction strength was greater in stressed fish. The differences in duration of *rigor mortis* in Azam *et al.'s* work may not have been detectable for a variety of reasons. The sample

size was small 5-7 fish and the later sampling intervals were large. The differences might have been too small to detect if the fish had been stressed prior to sampling - a minimally stressed control group was not measured.

1.3.5.4c Primary stress response

The primary stress response has been used to assess the effectiveness of stunning technique/ anaesthesia and consequently the welfare of fish. Carbon-dioxide anaesthesia elevates plasma noradrenaline and adrenaline levels in rainbow trout [28], when used at levels lower than the lethal dose recommended by the SSGA [9]. A short AC electric shock also increases plasma cortisol and glucose in rainbow trout [19]. While DC continuous and pulsed shocks elevates cortisol in juvenile bull trout (*Salvelinus confluentus*, Suckley 1859) [18]. Analysis of stress related blood constituents in the work by Azam looking at killing/stunning technique [13], showed differences between rainbow trout killed by various methods and found stunning by blow to the head was the least stressful [13].

1.3.5.4d Muscle metabolites and pH

Post mortem muscle pH and metabolites indicate the degree of muscle activity that has occurred in fish: they have been used to assess welfare of fish at harvest. Proctor *et al.* [174] investigated the welfare of farmed Atlantic salmon by measuring muscle metabolites. The fish were killed by blow to the head either with or without a stun using electricity, CO_2, or the anaesthetic MS222 (2-methyl 4-sulpholnyl aminobenzoate), [174]. Differences were not detected between the anaesthetised and percussively stunned fish in levels of creatine phosphate, ATP or pH. The electrically stunned group had significantly lower levels of creatine phosphate and ATP relative to the percussive stun and anaesthetised fish but to a lesser extent than the CO_2-narcosis group. The muscle pH values were 7.1 for MS222, 7.08 percussive stun, 6.86 electrically stunned, 6.80 CO_2-narcosis [174]. Comparable results were obtained in a later study on wild Atlantic salmon where the fish were killed by the methods previously described with the omission of CO_2-narcosis [175]. The electrically stunned fish had significantly lower creatine phosphate and ATP levels than fish killed by

the other methods. The pH values were; 7.05 in percussively stunned fish, 6.88 in anaesthetised fish, and 6.61 in electrically stunned fish. This work demonstrates that *ante mortem* muscle activity can be affected by killing technique. The pH of unstressed salmon has been shown to be as high as 7.4 and it declines with stress [71, 73]. The anaesthetised fish in the work by Proctor *et al.* [174, 175] were therefore showing signs of stress but this did not over shadow the effects of killing method. The induction of anaesthesia was rapid and so might have triggered a stress response, and the electrocution method involved stunning the fish for 2-3min, which may have stimulated the muscle and thereby increased glycolytic activity.

Work by Berg *et al.* [26] on Atlantic salmon killed by blow to the head or by a commercial slaughter process which included CO_2-narcosis measured high-energy phosphates at one and three hours *post mortem*, respectively. A second measurement was taken when the fish were in *rigor mortis* at 19.5 and 3.5 hours in the percussion and CO_2 groups, respectively. In the percussion group the creatine phosphate levels were high pre *rigor mortis* and low at *rigor mortis*. The AEC fell from 0.88 to 0.64 and the ATP: IMP ratio fell from 2.7:1 to 0.5:1. In the CO_2-narcosis group, the creatine phosphate levels were always low, the AEC fell from 0.66 to 0.49 and the ATP: IMP ratio fell from 0.3:1 to 0.2:1. This indicated that CO_2-narcosis method induced more muscle activity than the percussive stun technique.

Exsanguination is a method used to slaughter salmon, Erikson *et al.* [72] investigated its effects on farmed Atlantic salmon. In the first of two experiments, the fish were either killed directly by blow to the head (unstressed) or chased for an hour before killing (stressed). These fish were compared with data from a second experiment where fish were exsanguinated and either stressed or left unstressed as previously described. An additional group of anaesthetised fish was provided as a control but not bled. The AEC of the unstressed fish (0.94) was similar to the anaesthetised fish (0.95) while the stressed fish value (0.88) was lower than both groups of bled fish (0.92 unstressed, 0.90 stressed). This

finding was mirrored in the CP: ATP ratio where anaesthetised (2.7:1) > unstressed (2.2:1) >>unstressed and stressed bled (0.4:1) >stressed fish (0.2:1). This indicates that pre-slaughter handling can mask the stress of exsanguination in fish [72].

The literature therefore shows that farmed fish are often stressed during harvest. Certain procedures, i.e. crowding and stunning with ice slurry or CO_2, can produce greater stress levels than other practices, i.e. transport and killing by blow to the head. It should therefore be possible to produce best practice guidelines for fish harvesting.

1.3.6 The relationship between welfare and quality

If stress is reduced in farm animals their welfare improves [42, 253]. Short-term stress, the fight-or-flight response, equips an animal for attack or escape by preparing its muscle for activity. This adaptation has the potential to influence meat quality, as muscle develops into meat *post mortem* [74], see Section 1.4.1.1a. Minimising capture and handling stresses immediately before death has the potential therefore to alter the characteristics of the meat product [211]. This may either decrease or enhance the final quality of the product, although Wall states 'good quality and good welfare go hand in hand' [253]. Meat quality improves in fish if *rigor mortis* onset is delayed and the carcass chilled as it develops [258].

1.4 Quality

Quality is not an absolute term. It has a multiplicity of meanings depending on who defines it [39]. It covers aspects as varied as ethics, safety, availability, process ability, as well as the universal interpretations of; freshness, nutritional and eating quality. The desirability of fish and its flesh characteristics are subjective and vary with the assessor. The significance of any particular attribute depends on the fate of the final product.

Fish producers define quality as attributes such as health, ripeness or 'maturation', external appearance, weight and length. They use colour, fat and texture to assess flesh quality, and in some instances, freedom from taint, taste, and succulence [213, 214, 243]. For fish processors the meaning of quality is much the same but also includes; freshness,

temperature on arrival at the plant, microbiological and processing properties [214]. Consumers are most influenced by appearance when deciding to purchase. Their decision is based on assumed freshness, fat content and colour [173], whilst on eating, quality is assessed by colour, flavour and texture [3, 171, 240].

1.4.1 Quality characteristics

The quality of fish flesh depends upon extrinsic and intrinsic influences on the fish before and after death. The producer can control some of the extrinsic factors influencing live fish, e.g. diet and feed ration, but not all e.g. weather and water conditions. Extrinsic elements have the ability to influence intrinsic factors such as muscle structure. In *post mortem* fish both intrinsic (e.g. autolysis) and extrinsic characteristics (e.g. storage conditions) have major effects on quality. As muscle turns into meat, the quality of the product improves as flavour and texture develops [74]. Then, as freshness is lost through biochemical and microbiological processes, quality declines, and with the onset of spoilage, off flavours develop [74].

Downgrading is the process whereby fish are rejected or classified as substandard or unsuitable for particular products. As much as ten percent of the annual volume through a processing plant can be affected [146]. The proportion of fish that are downgraded varies as the result of *peri mortem* handling procedures and season. In Atlantic salmon the principal causes of external downgrading are; ripening or 'maturation', misshapes, and lesions [146]. Internally, fish are downgraded because of; pale or soft flesh, gaping, bloodspotting, bruising, and as the result of wounds caused by primary processing, [146]. In rainbow trout, similar problems occur, fish are rejected as pale, out of specification size, misshapes and disease [116, 213, 214].

1.4.1.1 Rigor mortis, freshness and spoilage

The quality of fish flesh alters after death through autolytic and microbiological processes which occur independently and concurrently [51]. Initially, non-microbiological enzymatic changes predominate. These consist of degradation of high-energy phosphates

and their substrates. As meat ages and homeostasis mechanisms fail, bacteria or the enzymes they secrete are able to invade or diffuse into the flesh. Microbiological deterioration results and proteins and fats degrade leading to spoilage [51].

1.4.1.1a Muscle structure and conversion of muscle to meat

Muscle develops into meat *via* naturally occurring biochemical processes which develop as homeostasis fails in *post mortem* muscle [74]. Oxygen exhaustion is one of three processes that alters *post mortem* metabolism, the others being; depletion of substrate and enzyme inhibition [84].

Fish muscle is divided into 'W' shaped segments that run along the body [36], Figure 1.2. These segments or myotomes are separated by the perimycium or myocommata, which are attached to the vertebral column and to the connective tissue layer of the skin, they are used to transmit the skeletal muscle contractions [37].

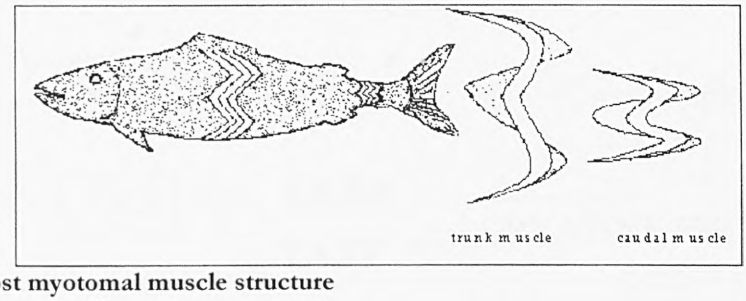


Figure 1.2 Teleost myotomal muscle structure
Adapted from Bone *et al.* [37].

The fine structure of skeletal muscle in fish is similar to that of mammals although the myosin head structure may differ slightly [66]. Each muscle cell comprises many myofibrils which contain the contractile proteins actin (thin) and myosin (thick) and the regulatory proteins troponin and tropomyosin [209].

In salmonids the muscle predominately consumed as meat is the white (glycolytic) muscle [111]. The oxygen supply of white muscle is quickly consumed *post mortem* as the tissue is poorly vascularised because, in life, it is used for burst activity. In life, muscle contraction is powered by free energy released from the conversion of ATP to adenosine diphosphate (ADP) with the consequent release of inorganic phosphate (P_i) [209, 230]. In the presence of oxygen, ATP is produced by the mitochondrial respiratory chain through

oxidative phosphorylation, Figure 1.3. Oxygen depletion occurs rapidly *post mortem,* particularly if the animal has been exsanguinated. Consequently, these aerobic processes are no longer involved in the restoration of ATP levels. ATP is replenished from creatine phosphate stored in the muscle, which donate a phosphate group to ADP, resulting in the production of ATP and creatine while using a proton, Figure 1.3. Once ATP can no longer be produced in sufficient quantities because oxygen and creatine phosphate supplies are exhausted, it is made by anaerobic glycolysis, the end-product of which is lactate, Figure 1.3. The build up of lactate reduces the *post mortem* muscle pH to such an extent that glycolytic enzymes resynthesising ATP can no longer work fast enough to maintain ATP levels needed. ATP is required for both the formation and the release of linkages or cross-bridges between the actin and myosin filaments and so once ATP is no longer available *rigor mortis* develops.

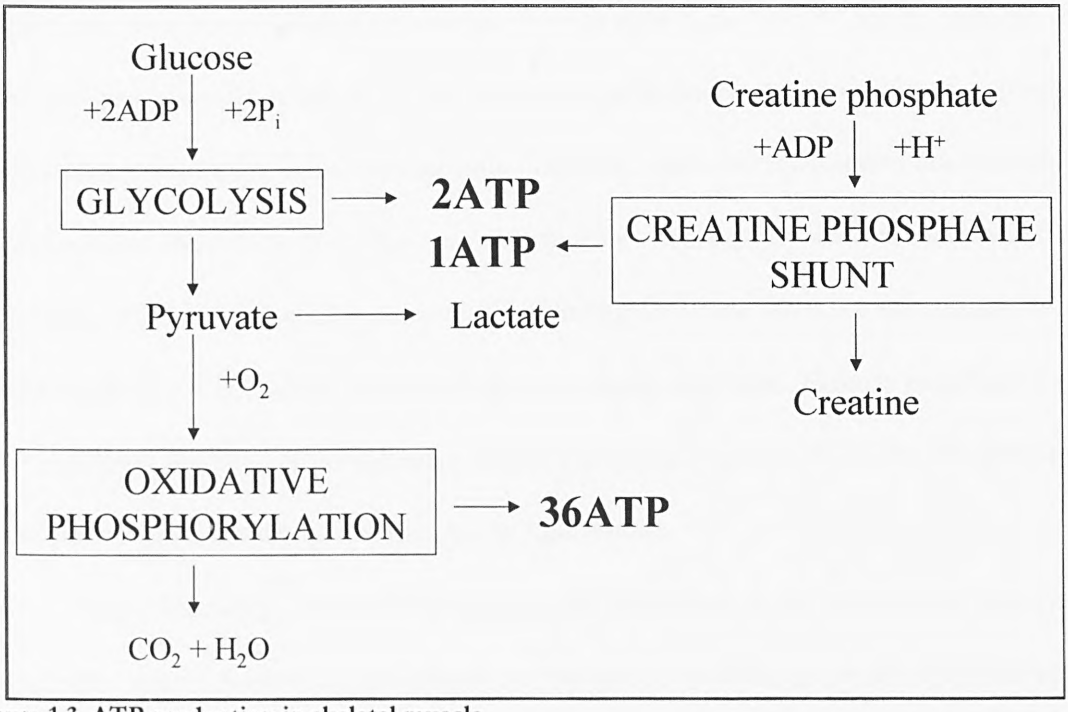


Figure 1.3 ATP production in skeletal muscle

Muscle contraction is achieved by the actin and myosin filaments sliding over each other by cross-bridge cycling. In cross-bridge cycling, one of the myosin heads is energised by myosin-ATPase, which hydrolyses the ATP bound to the ATP binding site, into ADP and P_i. The myosin undergoes a conformational change into a high-energy state and is

capable of binding to actin forming a cross-bridge between the thick and thin filaments. In the absence of Ca^{2+} ions, the myosin remains energised and does not bind to actin because tropomyosin blocks access to the binding site of actin. When Ca^{2+} ions are released from the sarcoplasmic reticulum (SR) in response to membrane depolarisation, they bind to troponin. This causes tropomyosin to translocate and uncover the binding sites of actin. The energised myosin-binding site then attaches to the exposed actin-binding site and actomyosin is formed. The energy stored by the bound myosin head is released, and ADP and P_i are released. The myosin molecule relaxes, the head swivels causing the thick and thin filaments to slide across one another and the sarcomere shortens. The second myosin head binds to actin while the first myosin head binds ATP, which causes the actomyosin to unbind from the first head. The second myosin head releases its ADP and P_i, rotates and the fibre shortens again. The second myosin head binds to ATP, the actin and myosin unbind, the head is re-energised and is ready for the next cycle [209, 230]. In live cells the cross-bridge cycle stops when Ca^{2+} ions are no longer bound to troponin as tropomyosin translocates to cover the actin-binding sites. The Ca^{2+} ions are sequestrated into the SR by ion pumps and the muscle returns to the resting state. During *rigor mortis* the levels of Ca^{2+} ions in the cell are high because ion pumps removing Ca^{2+} ions from the sarcoplasm are no longer fuelled by ATP, so the actin-binding sites remain exposed. There is insufficient ATP to release the myosin cross-bridge and so it remains bound to the actin filament and the muscle is permanently contracted, i.e. in *rigor mortis*.

Resolution of *rigor mortis* is not true muscle relaxation, as the actomyosin filaments do not slide apart. A variety of processes are thought to resolve *rigor mortis*; proteases split the fibres by solublising the linkages between sarcomeres, the actomyosin junction weakens, and connective tissue between the myofibres degrade [234]. These mechanisms are thought to be initiated by the increase of Ca^{2+} ions in the cell.

1.4.1.1b Rigor mortis and quality

Rigor mortis does not develop evenly throughout the carcass. In salmonids it is generally accepted that it starts behind the head and progresses to the rest of the body [70, 140]. The onset and duration of *rigor mortis* varies as it is dependent on the potential of each muscle cell to produce ATP as well as its rate of depletion. High levels of muscle activity prior to death reduce oxygen levels and therefore ATP production potential through mitochondrial oxidative phosphorylation, creatine phosphate stores will also be depleted. In animals that have been active or stressed during harvesting therefore, the level of ATP falls rapidly and *rigor mortis* sets in early [84]. High *post mortem* storage temperature also advances *rigor mortis,* as enzymatic activity is enhanced and consequently the rate of ATP depletion is faster [104]. The strength of the *rigor mortis* contraction is maximal when ATP depletion is rapid. In this situation, most of the cells are depleted of ATP at the same time. This precludes relaxation of the filaments, and as the majority of the cells are contracting at once, more muscle shortening occurs. Severe muscle shortening during *rigor mortis* can damage the cell myocommata junctions and cause gaping, see Section 1.4.1.5, [240]. For this reason handling, packing, processing or freezing should be avoided while fish are in *rigor mortis* [26].

Gutting and packing before onset of *rigor mortis* reduces gaping and fish reach the retailer in a better condition [6]. Filleting of pre-*rigor mortis* fish can however cause quality defects. Special handling techniques are required for pre *rigor mortis* fillets as *rigor mortis* develops after filleting. Myofibrillar contraction when not resisted by the skeleton causes severe shrinkage and subsequent toughening of the flesh [223]. If pre *rigor mortis* fillets are frozen before onset of *rigor mortis*, *rigor mortis* develops on thawing. This process, known as 'thaw rigor', can cause stronger than normal contractions. During freezing ice crystals remove water from the tissue and so effectively concentrate enzymes and substrates, additionally the SR membrane ruptures releasing Ca^{2+} ions into the cell. Thaw rigor can be prevented by storage at 'high' frozen temperature e.g. >-5°C or thawing at -2-0°C [70]. This

allows the muscle to pass through *rigor mortis* while still frozen [143]. Salting or brining is an important process in the production of smoked fish. Salt uptake in pre *rigor mortis* muscle is low compared with that of post *rigor mortis* muscle [254]. Therefore using pre *rigor mortis* flesh for producing smoked fish will affect the taste and microbiological safety of the end-product. It is vital therefore for fish processors to know whether fish they receive have passed through *rigor mortis*.

1.4.1.2 Drip losses and shrinkage

The water binding capacity (WBC) or water holding capacity (WHC) of meat is its ability to retain water. Fish with a higher WBC are classified as more juicy and tasty [97]. Water is bound electrostatically to the surface of myofibrillar proteins and is loosely associated with them in pockets [161]. In living tissue, the volume of extracellular fluid (ECF) is low, as 85% of water is located inside the cells. Changes in the pH and ionic strength of a cell *post mortem* alter the electrical charge of its proteins. Proteins near their isoelectric point lose their charge and their ability to bind water, which is released into the sarcoplasm. The osmotic potential of the cell is then reduced and so water is lost from the cell. The isoelectric point of fish myosin, the major protein constituent of muscle, is pH 5.4 [5]. As lactate levels increase in *post mortem* muscle the pH falls, as detailed in Section 1.4.1.1a, the WBC decreases and water may be lost as 'drip'. As the tissue loses water, the myofibrils shrink. This shrinkage is thought to occur laterally, not along the length of the sarcomere as cross bridge formation prevents the contractile proteins from sliding across each other. Shrinkage is thought to be caused by the fall in pH, attachment of cross bridges between actin and myosin and the denaturation of myosin (decreased solubility). Shrinkage is restrained by the cells osmotic potential, the cell membrane, intracellular structures and the collagen that links cells together and to skeletal attachments [161, 162]. Drip contains soluble proteins and so the nutritional quality as well as the yield of the meat decreases with increasing drip.

1.4.1.3 Colour

Colour perception relates to how incident light reflects from the surface of meat as well as the pigments it contains [133]. The flesh of fresh fish is translucent while that of stale fish is opaque [51].

When the WBC of muscle is low and the myofibrillar proteins release their associated water, the cut surface of the meat becomes less uniform. The scatter of light from its surface increases, and subtle colour changes result in the flesh appearing paler Figure 1.4A, [258].

The translucency of the flesh also alters subtly with time in that it becomes increasingly opaque as the sarcoplasmic proteins precipitate due to denaturation. These insoluble proteins cause incident light to be reflected out of the tissue, so less light is transmitted through the flesh, and it appears more opaque, Figure 1.4B. Incident light is transmitted diffusely in translucent objects, Figure 1.8C, whereas light is reflected diffusely in opaque ones, Figure 1.4B, [95].

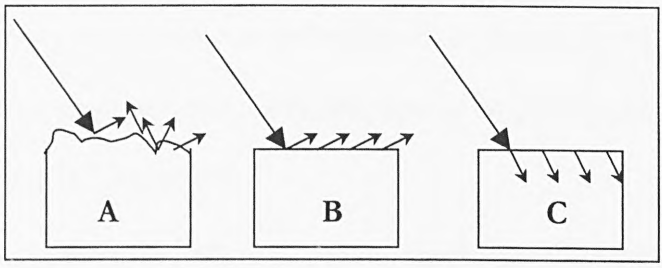


Figure 1.4A-C Effect of transmission of light on colour perception
A: Increased scatter from an uneven surface causing a paler colour. B: Uniform reflection from an even surface influencing opacity. C: Uniform refraction from an even surface influencing translucency

The colour of salmonid flesh is however primarily effected by the pigments it contains, the intensity of the colour depending on the pigment levels. The diet of farmed salmonids contains the carotenoids astaxanthin and canthaxanthin [215]. Astaxanthin produces a red colour while canthaxanthin's colour is more orange [218]. These pigments are important both in the marketing of salmonid flesh and in fish health as the fish are unable to synthesise the pigments and require them as antioxidants [159]. The pigments bind to hydrophobic sites on actomyosin and produce the red, orange and yellow colours

of salmonid flesh [228]. Flesh pigment levels are primarily determined by diet although genetic make up, muscle growth rates, cellularity, and maturation also play a role [112]. Astaxanthin is incorporated into fish food in higher proportions than canthaxanthin, as in rainbow trout it binds more strongly to muscle proteins and produces a stronger red colour [159, 203]. However, recent work suggests that canthaxanthin may pigment Atlantic salmon more successfully than rainbow trout [17]. The aim of pigmentation is to produce a strong, uniform, red colour which is what consumers prefer [112].

1.4.1.4 Texture

The texture of raw fish is influenced by both collagen and muscle fibres, however, after cooking, collagen no longer plays a role as it is has been denatured [86]. Fish texture is an important processing as well as eating characteristic. A common criticism of farmed fish is that it is soft [229]. The causes of softness are multifactoral and not clearly understood.

Softness may be misinterpreted as excess fat [213]. Muscle fat is inversely proportional to moisture, so fish with low fat levels can be spongy due to high water content [214]. It has been suggested that the distribution of fat plays an important role in texture as well as the actual fat levels [76].

Husbandry procedures can influence texture, small textural changes following short-term starvation are thought to be caused by the reduction of sarcoplasmic proteins. The proteolytic enzymes in the cell hydrolyse the structural proteins that contribute to flesh texture in fish. Fed fish have greater amounts of sarcoplasmic proteins than starved fish as sarcoplasmic proteins and glycogen are initially used as fuel in preference to the myofibrillar proteins [22]. This reduces the quantity of proteases available for the degradation of the structural components which causes softening of the flesh. A pre-harvest fast therefore is thought to firm fish flesh. Cross-link formation in collagen also plays a role in the texture of fish. It can occur while fish are being fasted or growing slowly, so low growth rates can be associated with firmer texture [68]. Firm texture is also

associated with high fibre density [96]. This is seen in the firmer flesh of the caudal region where cells are smaller and in higher densities [112].

1.4.1.5 Gaping

Gaping is where the muscle blocks (myotomes) comprising a fillet, separate leaving gaps in the tissue, Figure 1.5AB. Severe collagen breakdown can cause the myotomes to disassociate from the myocommata (connective tissue) [247]. It is uncertain if this disassociation occurs from weakening of sarcomere attachment to the sarcolemma, weakening of linkages between basal lamina and sarcolemma membrane, or disintegration of the extracellular matrix structure [29]. Gaping is a serious problem in the salmon industry as fillets that gape are unattractive to the consumer, difficult to process and unsuitable for producing smoked sides [6].

The causes of gaping are multifactoral and interrelated: the strength and duration of *rigor mortis*, muscle pH, muscle cellularity and distribution of fat are all thought to contribute to gaping [113, 240]. Fish muscle is more susceptible to gaping than red meat as it contains less collagen, which has fewer cross-links [111]. The collagen of fast growing and young fish has fewer cross-links than that of slow growing adults, and so has a greater tendency to gape [213]. Lactate accumulation due to anaerobic activity in the muscle reduces muscle pH which can weaken the collagen cross-links and induce protease leakage from cell lysosomes [265]. The proteases then denature the weakened collagen.

To reduce gaping it is recommended to minimise handling at harvest [211], use 'rested' fish [105], and to transport fish rapidly to market [6].

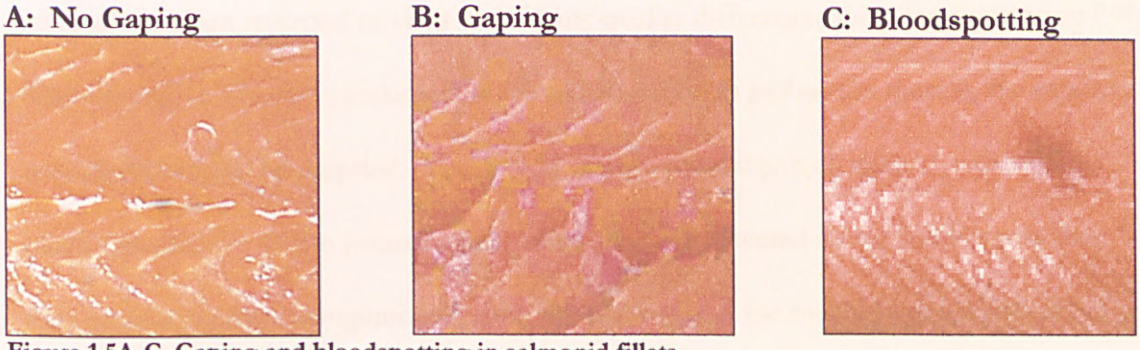


Figure 1.5A-C Gaping and bloodspotting in salmonid fillets
Figures A and B, in-house pictures, Peter Reid. Figure C, Courtesy of EU project CT97-3127.

1.4.1.6 Haemorrhagic lesions

Quality defects caused by haemorrhagic lesions include bloodspots and bruises.

Bruises occur where physical injury has caused diffuse bleeding into an area without breaking the skin. If the injury were recent, i.e. received during harvesting, then blood would still be present in the tissue. In rainbow trout, older injuries, where the blood is no longer present, are often still visible [126]. Bloodspotting, see Figure 1.5C, is thought to occur from catecholamine induced rise in systemic blood pressure leading to rupture of blood vessel [192, 193].

Blood spots and bruises downgrade fish destined for production of fillets and 'cold smoked sides'. To produce merchandise of acceptable appearance they need to be 'trimmed' out of fillets, consequently yields are reduced and labour costs are increased. Exsanguination of fish at harvest is thought to reduce the incidence of fillet bloodspotting and help to maintain quality during long-term frozen storage by reducing lipid oxidation potential [126, 170, 227, 241]. Product specifications may include exsanguination, and in some countries e.g. Norway, it is a legal requirement [72].

Exsanguination of fish without prior stunning is thought to be beneficial for end-product quality by maximising 'bleed out' as the heart continues to beat. However, in red meat species it has been shown that the heart does not need to pump for efficient 'bleed out'. Correct cutting of arteries is the most important factor [257, 258]. The majority of the blood in salmonids is located in the viscera and so gutting immediately on death would remove the most of the blood, and therefore gill cutting may not be necessary [193]. Farmed rainbow trout are reported to show little if any quality differences with exsanguination [126]. White muscle is poorly vascularised, so to reduce blood in *post mortem* muscle, the capillary bed would need to be emptied. This would require contraction of skeletal rather than cardiac muscle [97]. If the primary stress response were elevated in the fish because they were sensate during exsanguination, then blood vessels in the muscle would dilate in preparation for fight-or-flight. Therefore, exsanguination without prior stun, if it enhances

the primary stress response, might reduce rather than enhance the efficiency of 'bleed out' in fish.

1.4.1.7 Lesions caused by mechanical damage

Lesions caused by mechanical damage such as abrasions, scale loss, skin loss, bruising and wounds reduce the commercial value of fish [146]. Damage can occur during handling if fish are roughly treated, and/ or they are over-crowded during the harvesting process. Primary processing may cause further damage e.g. flesh wounds caused by gutting machines [146]. Quality schemes, such as the Shetland Seafood Quality Control Company's scheme, grade fish before sale [8]. Damaged carcasses, such as those with more than ten-percent scale loss, are downgraded and not sold for a premium [8]. Careful handling at all stages of harvesting and processing can reduce levels of downgrading.

1.4.1.8 Ripening or 'maturation'

The appearance and composition of a fish alters as it prepares for reproduction. In females, proteins, lipids and pigments are sequestrated for egg production, at the expense of flesh quality. In males, spawning colours are adopted by the relocation of pigments from the flesh to the skin, and additional bony growth such as kype formation may occur [207].

The trout farming industry has overcome this problem by selling the majority of their production as 'portion sized' i.e. one fish per person per meal. Modern fish diets produce portion trout within one year [213]: farmed female trout generally mature in their second year while a proportion of males mature in their first [207]. The use of all female stock therefore eliminates maturation in portion sized trout production. To avoid maturation in larger trout (two and three year olds) triploid stocks are used [215]. Triploid trout contain one paternal and two maternal sets of chromosomes and the females do not mature [129, 207].

The approach of the salmon farming industry to maturation has been different due problems of deformities in triploid Atlantic salmon [199, 252]. Salmon are often harvested

after two sea winters and so the potential for maturation is present, the use of late maturing strains to reduce this problem is common [124].

1.4.2 Methods used to measure carcass quality characteristics

Carcass quality can be assessed subjectively by sensory analysis or objectively using a variety of instruments. Although subjective measurements may not be as reliable as instrumental methods, as the ultimate destination of fish is the plate, sensory measurements may have a greater validity.

1.4.2.1 Rigor mortis

Fish that are in *rigor mortis* are perceived as fresh by the consumer and so *rigor mortis* can be used as a quality indicator for freshness. Methods for assessing *rigor mortis* in fish were described in Section 1.3.4.3.

1.4.2.2 Drip loss and water binding capacity

The conformational state of muscle protein, which affects *rigor mortis*, also influences the muscles ability to bind water, and therefore potential drip loss. Methods for measuring this expressible water have been reviewed in the literature [242]. Techniques measure water loss from different fluid compartments and so may not be directly comparable [161, 162]. The water found between the fibre bundles and the perimycium is lost first as it has a shorter distance to travel than the water from between the fibres and the endomycium. The amount of water present in either of these two compartments will be affected by the binding ability of the proteins inside the cell.

The most commonly used techniques for measuring water-binding capacity are the press and centrifugal methods. The press method expresses water by pressing the sample between two filter papers at a constant force for a known time. The quantity of drip released is dependent on the force applied and is measured either by, the increase in weight of the filter papers, or by measuring the wet area relative to the sample size. This method is quick but can only be used with small homogeneous samples, evaporative losses from small samples can cause great variability in the data [242].

Centrifugal methods for determining expressible water use either high, (5000 - 40000*g*) or low (200-800*g*) speeds. Water released is measured directly by weight or weight loss of the sample. Low speeds cause less microstructure damage to the sample, and filters can be used to separate the sample from released water so preventing re-uptake [161, 162].

1.4.2.3 Flesh colour

Visual colour is also influenced by the conformational state of the muscle proteins. Colour is one of the major quality attributes that influence consumer selection of fish products for purchase [215]. It can be measured visually or using various instrumental techniques.

1.4.2.3a Visual assessments

The salmonid farming industry score colour using the Roche Colour Card™ or more recently the Roche *Salmo*Fan™, Figure 1.6A-B. These systems help to standardise measurements and ease communication with customers. However, colour is not uniform along a fillet and alters with time, so sampling sites and timings must be noted when describing colour. Visual assessments of colour should always be undertaken under standard lighting conditions as lighting affects colour perception [95]. Observers should be trained and tested for colour blindness and acuity of colour vision [183].

A: Roche Colour card™ **B: Roche *Salmo*Fan™**

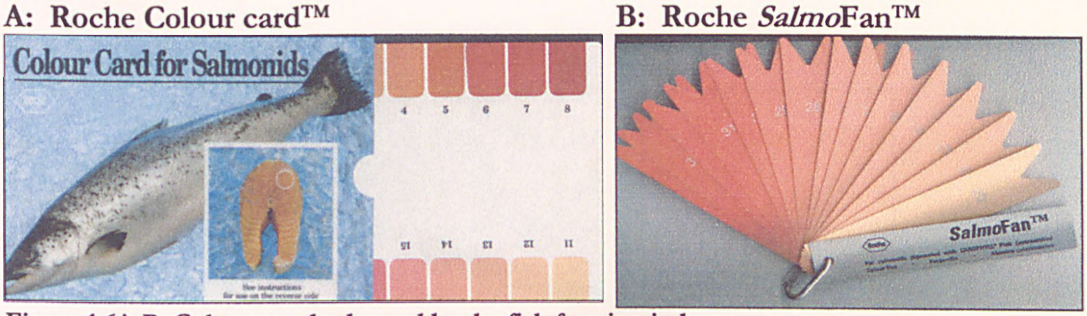


Figure 1.6A-B Colour standards used by the fish farming industry

1.4.2.3b Instrumental measurements

Objective colour measurements are made with a variety of instruments, which use many systems to describe colour. The CIE L*a*b* 1976 colour system is the one that most closely relates to human perception [183]. It is measured using a tristimulus colorimeter

which gives L*a*b* readings.

- *Lightness*

The L* co-ordinate, or lightness value, describes how light or dark an object is, it goes from zero (dark) to 100 (light). When the a* and b* values are low then a L* value of zero is black and 100 is white, Figure 1.7, [39].

- *Angle of Hue*

The 'colour' of the object e.g. red or blue is known as hue, and is described by the a* and b* readings. The a* value measures red-greenness, negative values correspond to green and positive to red. The b* value relates to yellow-blueness, the positive numbers indicate yellow and the negative blue, Figure 1.7, [39]. These readings are combined into one calculated number known as the angle of hue, Equation 1.3.

Equation 1.3: Angle of Hue in degrees

$$(\tan^{-1}\frac{b^*}{a^*}) * 57.295$$

- *Chroma*

The intensity of the colour of an object, its brightness to greyness, is known as chroma (C*) [95], and is calculated from the a* and b* scores, Equation 1.4. The larger the C* the more intense the colour.

Equation 1.4: Chroma

$$\sqrt{a^{*2} + b^{*2}}$$

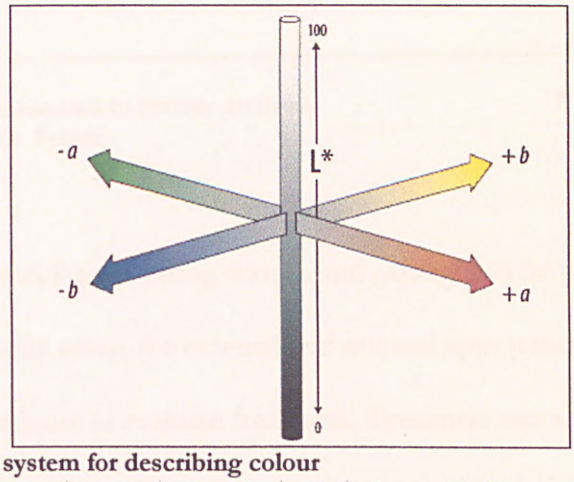


Figure 1.7 CIE 1976 L*a*b* system for describing colour
Taken from http://www.adobe.com/support/techguides/color/colormodels/images/CIELAB.gif

1.4.2.4 Texture and gaping

Sensory panels are used to assess texture using both the finger press method and mouth feel scores [239]. Gaping in fish fillets can also be assessed by sensory methods that visually score the degree of damage in the tissue [6].

Instrumental methods for evaluating texture measure a combination of shear, compressive and tensile forces, Figure 1.8. The main tests are puncture, shear force, compression and tensile strength [60]. The principles are the same for all tests, probes are attached to a force measuring device and pressed into the sample. The texture of fish is difficult to interpret and results are often conflicting because the intrinsic qualities of a fish are highly variable and extrinsic factors associated with measurements effect texture. For example: storage time and temperature; sample location, geometry and preparation method [212, 240, 247]. Texture profile analysis is an instrumental test that seeks to relate force measurements with sensory attributes [41]. It relates the areas under force time plots from a double compression test to sensory attributes such as resilience (elasticity), cohesiveness (strength retained after first deformation) and hardness on first bite.

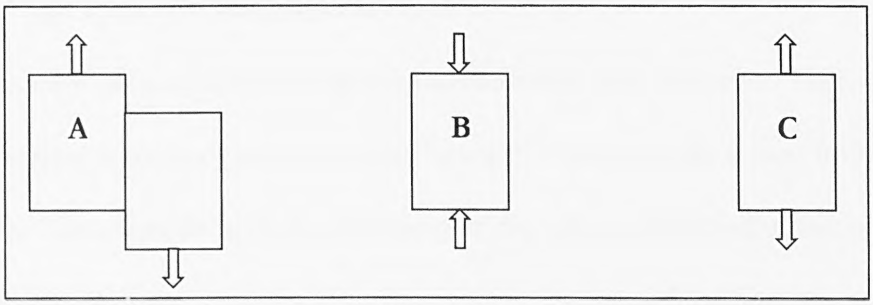


Figure 1.8 Types of stress measured in texture analysis
A: Shear, B: Compressive, C: Tensile

1.4.2.5 Freshness

Sensory panels as well as assessing texture and gaping, can be used to appraise fish freshness. They can visually assess the external and internal appearance of fish and taste panels use flavours and odours to evaluate freshness. Freshness can also be measured by electronic meters or chemical tests [51].

Electronic meters, e.g. the Torrymeter, are non-destructive and measure the electrical properties of fish skin and muscle [101]. These properties alter as fish undergo *post mortem* spoilage [39]. They can be correlated with autolysis and or bacterial spoilage although not caused directly by it [138]. The resistance and capacitance of the tissue are measured and converted to a numerical 'freshness reading' [46]. The Torrymeter gives readings between 0-16 with lower values corresponding to fewer days shelf life. Consecutive Torrymeter readings in pre *rigor mortis* tissue rise, but once *rigor mortis* starts to resolve the values decline and can be related to loss of freshness. Readings can however vary with species, fat content and handling. The readings relate to the quantity of free ions in the various fluid compartments. When there is insufficient ATP to power the ion pumps, the ionic concentrations of the intercellular fluid and ECF alter. The ion concentrations influence the electrical impulse that the Torrymeter passes through the fish and the returning signal sensed. The Torrymeter does not work on fish that have been frozen or brined [46, 101, 138]. The readings on frozen fish read at or near to zero because ice crystals formed during freezing rupture the cell membranes. Salted or brined fish do not produce readings because of high ion concentrations in the ECF's.

Chemical tests are traditionally used to determine fish freshness. They are however destructive, time consuming and laboratory based. To interpret the results from these methods the characteristic patterns of metabolite degradation have to be known for the fish being tested. This can vary with species, season, location of catching, stage of spoilage, type of processing and storage as well as method of analysis [138].

The major chemical used in freshness testing is total volatile basic nitrogen, (TVB). TVB is a general term that includes TMA (trimethylamine), DMA (dimethylamine) and ammonia (NH_3) [39]. TMA is produced by spoilage bacteria during ice storage, NH_3 is produced by the deamination of amino acids by bacteria or by autolysis of AMP during ice storage, while DMA is produced during frozen storage by autolytic enzymes. TVB measures the late stages of spoilage and so is used to condemn samples. It does not

indicate the mode of spoilage and results are very dependent on the method of analysis [2, 39]. TMA is associated with the fishy smell of spoiling fish, it is produced from TMAO by specific spoilage bacteria. TMA levels do not relate directly to total numbers of bacteria on the flesh. However total viable counts that count the numbers of all viable bacteria on the flesh and do not differentiate fish spoilage bacteria are sometimes used to evaluate fish spoilage. DMA is produced in fish that contain the enzyme TMAO-dimethylase, which converts TMAO to DMA and formaldehyde during frozen storage. Formaldehyde causes toughening of muscle proteins and its presence can be used to deduce temperature fluctuations during frozen storage [2, 39, 51].

Rancid and off-flavours may also develop during long-term frozen storage due to lipid oxidation. Methods used to detect lipid oxidation in fish are TBA (thiobarbituric acid) and PV (peroxide value) [39, 51].

Biogenic amines have also used to determine the freshness of fish. They are formed from the decarboxylation of amino acids by bacterial enzymes under acidic conditions. Histamine, putrecine and cadaverine are examples of the amines produced but their absence from fish does not ensure the product is not spoilt [188]. They are however heat stable and so are useful in determining how fresh fish were before canning [2, 39].

In the early stages of deterioration nucleotide degradation products can be used to determine freshness [39]. In many species, the sequence of degradation is as follows: - ATP→ADP→AMP→IMP→Inosine (HxR)→Hypoxanthine (Hx) [117]. Levels of each compound rise and fall in the tissue as freshness declines, so analysis of the complete nucleotide profile is necessary to assess freshness. Concentrations of these compounds also vary with location in the fish so consistency of sampling sites is vital if these methods are to be used to indicate fish freshness [44]. The K value or ratio of the sum of the breakdown products of ATP was first used by Saito *et al.* [201] to estimate the freshness of fish [198, 245], Equation 1.5. The K value later became modified to the K' value due to the rapid degradation of ATP, ADP and AMP [114], Equation 1.6. For species where the K

value reaches 100% after only a few days and where they are still suitable for consumption Luong *et al.* [136] proposed the use of the H value, Equation 1.7.

Equation 1.5

$$K = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx}$$

Equation 1.6

$$K' = \frac{HxR + Hx}{IMP + HxR + Hx}$$

Equation 1.7

$$H = \frac{Hx}{IMP + HxR + Hx}$$

1.4.2.6 Muscle pH

Post mortem muscle pH initially relates primarily to the amount of lactic acid produced by anaerobic glycolysis, see Section 1.4.1.1a. The lactic acid formed dissociates into lactate and a proton and so the pH of the tissue falls. If measured immediately after death, pH can be used as a measure of *ante-mortem* anaerobic muscle activity. When measured during long-term ice storage a rise in pH indicates onset of spoilage due to TVB production [39].

Methods for measuring meat pH either measure directly using spear tipped electrodes or indirectly in a homogenate. Care must be taken when interpreting the data as a spear electrode can give higher readings compared with a homogenate [211]. However, homogenisation methods can produce reliable measurements. Solomon [221] compared three methods homogenising the muscle in iodoacetate, homogenising the muscle in de-ionised water and measuring the pH within 3-5 seconds with a spear electrode. Comparable results were obtained with all three methods. Method selection for measuring pH will therefore depend on the time available and type of study being performed.

1.4.3 Existing work on the influence of harvesting technique on fish quality

1.4.3.1 Starving and exercise

The effect of starving/ fasting on fish flesh quality varies with species and duration of the starvation period. Some authors report measurable differences in carcass quality [68],

while others do not [12].

Higher enzyme activities in the muscle of fed fish, due to the effects of SDA, would enhance autolysis and thereby influence freshness. The K value would be expected to develop faster, in fed fish and so they would be classified as less fresh than fasted fish of the same *post mortem* age. Higher K values were found in fed salmon by Einen and Thomassen, and Einen *et al.* [68, 69], while Blokhus [34], presents evidence of a faster decline in freshness in fed compared with starved salmon using the Torrymeter.

Changes in the rate of ATP degradation between fed and starved fish would lead to different concentrations of important flavour compounds in the flesh at any particular point in time. IMP, an early degradation product of ATP, is a known flavour enhancer which sweetens flesh, whereas hypoxanthine a later degradation product, has a bitter flavour [130]. The fat content of fish also affects its taste, as fat acts as a carrier for flavour molecules and plays a role in 'mouth feel' [23]. Under commercial conditions i.e. <18 days 'starving', the fat content of rainbow trout fillets does not decline [75]. Therefore, the short-term fasting of farmed fish as practised by the fish farming industry may not influence eating quality. In rainbow trout starved for two months however, a reduction in taste, odour and juiciness has been reported [108]. Deterioration in eating quality of rainbow trout with starvation has also been reported by Warriss and Robb [258] but they do not state for how long food was withheld. They cite the work of Wiseman (1993) who investigated the effects of starving and exercise on eating quality in rainbow trout. Flavour was influenced mainly by starving and texture by exercise. Both factors adversely affected eating quality and were additive [258].

The aquaculture industry has long observed muscle softening in inadequately fasted fish. Work by Regost *et al.* [179] report firmer flesh in brown trout (*Salmo trutta*, Linnaeus 1758) after two months starvation. Other studies, using Atlantic salmon, show increased firmness with starvation in triploid fish starved for as little as seven days [83], and in diploid fish starved for 58 days [67, 68]. The work by Faergemand *et al.* [75] however did not detect

textural differences after six or 18 days starvation in rainbow trout.

Long-term starvation and exhaustive exercise deplete muscle glycogen stores. Muscle glycogen was depleted on the day of slaughter in Atlantic salmon starved for five days at 17°C [217]. Muscle with a low glycogen content is incapable of synthesising large amounts of lactate *post mortem* and so the pH does not decline as far or as fast as tissue with a high glycogen content. Drip losses are therefore minimised so the tissue remains firmer, this is seen by the meat industry in DFD meat [84]. This effect was demonstrated in Atlantic salmon by Einen and Thomassen [68] in fish starved for 86 days at 4°C.

Another mechanism that can increase firmness in the flesh of starved fish is collagen cross-linking. Work by Gómez-Guillén *et al.* [83], showed decreased collagen solubility (increased linkage) in fish starved for 30 days with a concurrent increase in muscle firmness. The effects of a 14-day fast on the texture of Atlantic salmon were investigated over 24 days by Veland and Torrissen [247]. The initial measurements showed the muscle of the starved fish was softer. However, the muscle of fed fish lost its strength faster, so the differences were not detectable after the first two days *post mortem*. The initial difference was explained by the lower energy status of the starved group completing the early *post mortem* changes earlier. The faster softening of the fed group occurred due to the effects of SDA, see Section 1.2.1. The work of Einen and Thomassen [68] found prolonged starving caused the texture of raw Atlantic salmon fillets to become firmer while cooked fillets became softer. This can be explained by the loss of the collagen influence on texture with cooking. Texture can also be related to body size and the prolonged fish lost weight and so were smaller. The fed cooked fillet was firmer than the starved one probably due to increased numbers of muscle cells or an increased myofibrillar component of the cells. Although both sarcoplasmic and myfibrillar proteins are utilised during long term starvation in fish, the sarcoplasmic proteins are taken in preference [22]. In short to medium term starvation, therefore, texture differences due to changes in the myofibres may not be expected.

The texture of fish can also be altered by forced swimming, as shown by the work of Tachibana *et al.* [233] in cultured red sea-bream (*Pagrus major,* Temminck and Schlegel, 1843). Johnston and Moon cited in [111] found increased hyperplasia in saithe (*Pollachius virens* Linnaeus 1758) exercised at two body lengths a second for three weeks. Hyperplasia would increase the firmness of the tissue through increased numbers of small diameter fibres [96] and the additional contractile element of the new fibres. Work by Sigurgisladottir *et al.* [212] showed that rearing practices have the capacity to alter texture: ranched salmon had a firmer texture than sea cage or tank reared fish which would have had less opportunity for exercise.

1.4.3.2 Crowding, transport and live-chilling

The effect of pre slaughter handling stress on flesh quality has been found to influence product quality.

The effects of capture stress on Atlantic salmon has been shown to increase K value, i.e. decrease freshness, over a 14-day period [71]. The effect was however only statistically significant two days *post mortem.* The effects of confinement stress (ten minute crowding before stunning) on quality was investigated by Sigholt *et al.* [211]. This short-term pre-slaughter handling stress produced an earlier onset of *rigor mortis* and muscle softening in the fish but no difference was detected in K value. Crowding was also found to advance *rigor mortis* in Atlantic salmon in work by Skjervold *et al.* [216]. However the crowding stress, which lasted for two hours, increased flesh firmness and was almost statistically significant on day five *post mortem.* Skjervold *et al.* [216] related this toughening to DFD type meat caused by the long-term stress, while the short-term stress in Sigholt *et al.* [211] produced PSE type meat. In PSE meat the pH declines rapidly after death and reaches a very low value, due possibly to abnormally fast Ca^{2+} leakage from the SR. This decreases the WBC of the proteins, so drip loss increases and the flesh appears paler because of increased light scatter from its surface; see Sections 1.4.1.2 and 1.4.1.3.

Limiting muscle activity during harvest therefore preserves the energy status of the muscle and can alter the eating characteristics of fish. The use of food grade anaesthetics to reduce crowding and netting stresses has lead to improved carcass quality in rainbow trout compared with that of fish electrostimulated after death [186]. In the electrostimulated group, onset of *rigor mortis* was earlier, the flesh was paler, less red and more opaque. The Roche *Salmo*Fan™ score also indicated inferior colour in the electrostimulated fish, which were also more susceptible to gaping. Live-chilling of Atlantic salmon prior to slaughter has also been shown to affect product quality. It delayed resolution of *rigor mortis*, increased flesh firmness and the incidence of gaping five days *post mortem* [217].

However not all authors have detected differences in *post mortem* quality due to stress during harvest. Bonnet *et al.* [38] found colour, WBC, and texture seven days *post mortem* not strongly related to fasting or adrenaline induced experimental stress in brown trout.

1.4.3.3 Restraint, killing, stunning and slaughter

Slaughter is where an animal is killed by bleeding, and is used to improve the quality of meat [84]. The evidence for the effectiveness of exsanguination on improving fish quality however is more equivocal. Work by Tretsven and Patten [241] showed exsanguination significantly improved the appearance, and reduced rancidity in rainbow trout fillets after eight months frozen storage. A delay of 20minutes between death and arterial severance however reduced the efficiency of 'bleed out'. This finding was in direct contrast to work on sockeye salmon (*Oncorhynchus nerka*, Walbaum 1792) by Porter *et al.* [170], where exsanguination did not effect lipid oxidation and hydrolysis during one years frozen storage. Work by Roth *et al.* [193] reports that bleeding of salmonids is unnecessary for chilled products provided the fish are gutted immediately after death. However if the fish are handled roughly during *rigor mortis* when not exsanguinated then quality defects occur [193].

The influence of stunning method, in combination with exsanguination, on rainbow trout quality was investigated during 15 days chilled storage by Azam *et al.* [14]. The fish were stunned using electricity, CO_2, or by a blow to the head. Initially, the percussively stunned fish had a higher muscle pH, caused by lower levels of lactic acid, this suggested that they were the least metabolically active. Spoilage, as evaluated by bacterial numbers on the flesh, increased with time, but the small differences seen between stunning methods were discounted as being within errors inherent in the plating technique [14]. The flesh of all the fish softened with time, but textural differences were not detected between treatments in either raw or cooked tissue. The conclusions made from the work were that biochemical, textural and microbiological parameters were not influenced by stunning method [13, 14]. Conversely, textural differences were found between killing methods in the work by Gómez-Guillén *et al.* [83] where muscle softening occurred in stressed (exsanguinated without stunning) compared with 'unstressed' (CO_2 'anaesthetised') Atlantic salmon.

The quality attribute freshness, can be influenced by muscle activity during harvest. An increase in freshness was observed in rainbow trout that died 'tranquilly', (percussive stun) compared with fish that struggled during asphyxiation [268].

Electrical killing / stunning methods have also been reported to influence quality. Cary (1995) cited in Warris and Robb [258] looked at whole body electrical stunning in rainbow trout and reported an increased incidence of haemorrhagic lesions compared with fish that died following a percussive stun. Quality defects have been reported in electrically stunned fish by other authors. Marx *et al.* [141] found electrically stunned trout were least acceptable in a sensory assessment of cooked meat compared with fish killed by CO_2-narcosis and blow to the head [141]. Blood spots were seen along the vertebral column in 6.8%, 2.9%, and 1.4%, of the fish killed following an electrical stun, percussive stun and CO_2-narcosis, respectively. 'Colour' was poor in 32.8%, 20.8% and 15.9% of the fish killed following an electrical stun, percussive stun and CO_2-narcosis, respectively. Tests on raw

fish quality indicated that death by blow to the head was the most acceptable method for all species investigated (trout, carp, and eel (*Anguilla anguilla* Linnaeus, 1758)), [141]. However with trout, CO_2-narcosis was, and with eel, electrical stun was, as good as blow to the head. However the pH data for all three species showed that CO_2-narcosis caused a significantly greater fall in pH at death, and percussion showed the smallest decline [141]. Water holding capacity at death was lowest in the CO_2 group of all species [141]. In common carp and eel CO_2-narcosis advanced *rigor mortis*, percussive stun delayed *rigor mortis* in all species [141].

The effect of killing/ stunning technique on flesh quality of channel catfish (*Ictalurus punctatus* Rafinisque 1818) has also been investigated [35]. The killing/ stunning methods examined were; exsanguination, DC and AC electrical stun, CO_2-narcosis and packing in ice. No statistically significant differences were detected between the groups in the qualities evaluated; process ability and yield, colour, firmness and sensory attributes. However, there was subjective evidence that the exsanguination-only fish bled more efficiently and were the easiest to skin. The CO_2-narcosis group were the next easiest to skin and had a good appearance as they had the least haemorrhages. While muscle contractions occurred in the electrically stunned fish and they did not bleed well, the AC shock method produced the worst appearance and flavour was rated lowest in the DC stunned fish. The fish that died in ice also bled poorly and were the hardest to skin. These results, although showing some similarities with other fish species suggest that quality attributes may be species dependent.

Methods used to stun and kill farmed salmonids may in some instances, have profound effects on quality. Harvesting procedures might therefore be able to be tailored to make the best of the inherent quality of farmed fish.

1.5 Future of the UK salmonid farming

When MAFF/ DEFRA reviewed the UK trout farming industry in 1999 four constraints to growth were identified; structural, input, production and markets [148]. Structural constraints included the EU and UK legislative framework, which encompassed welfare, water abstraction and discharge. Input constraints were supply and quality of; water, stock, and feed stuffs. Production constraints, included disease and its treatment, stock enhancement, diversification and welfare [148]. Finally, markets constraints, covered competition from overseas and other farmed fish species, and public concern over environmental impact and animal welfare [148]. Fish welfare was therefore identified as an important constraint on the industry, and it must therefore be taken into consideration for a diverse, competitive, sustainable salmonid farming industry to be maintained within the UK.

Supply of trout has remained static over the last ten years and salmon production increased by only a small tonnage [77]. The ready availability of farmed fish has resulted in falling prices [77], and loss of luxury image [49]. The quality of farmed fish must improve to regain and enhance its market position. The market is no longer supply driven and producers and processors must alter current practices to fit into the market place ensuring continuity of supply and consistent quality [139]. Quality assurance and branding schemes may reassure public concerns over food safety and quality. Farms converting to organic status may alleviate public concerns about animal welfare, sustainability and environmental impact [139].

1.6 Aims

EU and UK legislation require farm animals to be killed or slaughtered without being subjected to undue stress, although not specifically referred to in the legislation, there is increasing awareness that this should include farmed fish [225]. Objective measurement of the effects of stunning and killing methods on the welfare of captive fish are required

before guidelines for fish slaughter and killing can be formulated. Links between the acute primary stress response in farmed fish during harvest, endogenous fuel utilisation and carcass quality need to be established. The aim of the present study is to investigate the effects of fasting, and stunning/ killing methods on the welfare and carcass quality of farmed salmonids.

2 GENERAL METHODS

2.1 Experimental animals and facilities

2.1.1 Ethical issues

Home Office approval was sought prior to performing the field studies as they involved killing animals by methods other than those listed under Schedule 1 to the 'Animals (Scientific Procedures) Act 1986'. The Home Office waived the requirement for licences, provided the work was only performed in the field, on farms that were not sites designated for animal experimentation, see Appendix 1. The sites used for the present study complied with these recommendations.

2.1.2 Animals

The rainbow trout used were reared at the Mill of Elrick Fish Farm, Auchnagatt, Aberdeenshire, UK. The fish were hand fed and reared in freshwater tanks and ponds. The Atlantic salmon were on-grown post smoltification in seawater cages by Lighthouse of Scotland Ltd., Argyll, UK, (pilot study) and Scottish Sea Farms Ltd. (commercial harvest).

2.2 Materials

Enzymes, coenzymes, nucleotides and substrates were purchased from Roche Diagnostics (Lewes, East Sussex, UK). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd., (Walkerburn, Scotland, UK). The water used was ultra pure Milli-Q 18.2 M Ω (Millipore UK Ltd., Watford, Hertfordshire, UK). Other chemicals were ACS reagent grade obtained from Sigma –Aldrich Co. Ltd., (Poole, Dorset, UK).

2.3 Biochemical analysis

The most valid estimation of welfare determinable in these studies was the primary stress response as discussed in Section 1.3.4.1a. The primary stress hormones adrenaline,

noradrenaline and cortisol were selected for quantitation, and this required blood sampling. Secondary stress responses were also evaluated in blood and in muscle. The muscle response was measured as it links welfare to meat quality. A freeze clamping technique was chosen for collection of muscle samples, as it was the most appropriate method and could be adapted for use in the field.

2.3.1 Sample collection

2.3.1.1 Blood

Samples were collected within 2 minutes of 'death' to determine the early stages of the stress response. Blood was extracted from the caudal vein using a 10ml syringe with a 21 gauge needle either 38mm or 50mm long, for trout and salmon, respectively. The needle was inserted at an angle of about 45° through the ventral surface of the caudal-peduncle, posterior to the ventral fin, Figure 2.1 and Appendix 2, Figure 9. The vein runs inferior to the spinal column and was located by detecting the spine with the needle and withdrawing slightly. The samples were immediately dispensed into 0.5ml tubes (for haematocrit) or 2 ml tubes (for plasma) containing 0.75mg or 1.5 mg of the anticoagulant EDTA dissolved in 10µl or 20µl of water, respectively. The tubes were mixed by gentle inversion, stored on ice and transported to the laboratory.

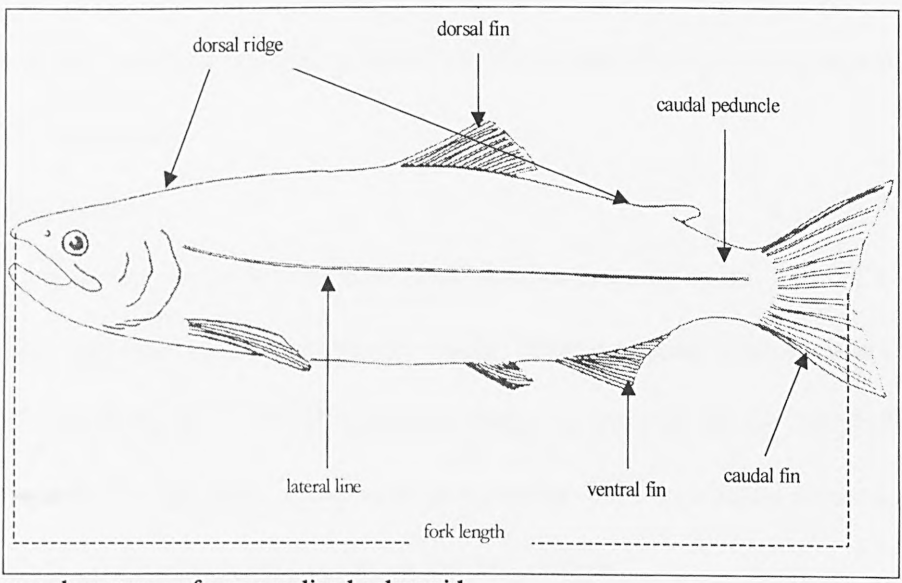


Figure 2.1 External anatomy of a generalised salmonid

2.3.1.2 Muscle

2.3.1.2a Pre rigor mortis

To minimise degradation of unstable metabolites, muscle samples were taken within 2.5 minutes of 'death' from separate fish to those used for blood samples. A deep incision was made through the dorsal ridge from the neck to the caudal-peduncle, Figure 2.1. The epiaxial, (above the lateral line), white muscle exposed was excised from the region inferior to the dorsal fin or immediately adjacent to it. An 8mm-diameter biopsy punch (Stiefel Laboratories Ltd., High Wycombe, Bucks., UK) was used to cut the samples which were immediately freeze clamped.

The freeze clamps consisted of two cylindrical aluminium blocks (38mm diameter by 28mm depth) secured to 'fisher pattern' stainless steel tongs 46cm long (Merck Ltd., Poole, Dorset, UK). They were pre-cooled in liquid nitrogen, (-196°C), and the sample placed between the blocks and simultaneously compressed and frozen. The samples were immersed in liquid nitrogen until wrapped in pre-labelled aluminium foil. They were transported to the laboratory under vapour phase liquid nitrogen, (-150°C), in 'dry shippers' (Biotrek 3, Statebourne Cryogenics Ltd., Washington, Tyne and Wear, UK). The freeze clamping procedure is shown in Appendix 2, Figures 1-8. The samples were stored under vapour phase liquid nitrogen for a maximum of fourteen days prior to preparation and analysis for nucleotides.

2.3.1.2b Post rigor mortis

Fish were stored 'on ice' on stainless steel benches in a chill room, 2.0±0.4°C (mean±sd), until they had passed through *rigor mortis*. Muscle samples were excised as described above; omitting the freeze-clamping procedure as the high-energy metabolites would have degraded by this time. Samples were stored at -80°C in labelled aluminium foil for a maximum of 16 months prior to preparation and analysis.

2.3.2 Sample preparation

2.3.2.1 Blood

2.3.2.1a Haematocrit

For the trout studies, (Chapters 4 and 5), within nine hours of blood sampling, duplicate haematocrit tubes were filled by capillary action from a chilled EDTA blood sample. They were centrifuged at 9400*g* for 2min at ambient temperature in a Centra 4-X centrifuge (International Equipment Company, Dunstable, Bedfordshire, UK). The packed cell volumes were measured using a haematocrit reader and results expressed as a % of total volume. Plasma was prepared from the remaining blood samples.

2.3.2.1b Plasma preparation

Biochemical analysis was performed on plasma to prevent interference from blood cells. Within nine hours of collection, the chilled trout blood samples were centrifuged in the cold room at 14000*g* at 2°C for 60s (model A14, Jouan Ltd., Ilkeston, Derbyshire, UK). The resultant plasma was aspirated and transferred to clean eppendorf tubes and stored at -80°C. The chilled salmon blood samples were centrifuged at ambient temperature. In the pilot study they were centrifuged within three hours of collection and frozen at -20°C for 24 hours before storage at -80°C. In the salmon commercial harvesting study, the samples were centrifuged within one hour of collection and then transported to the laboratory on ice, 9h, before storage at -80°C.

2.3.2.1c Plasma deproteinisation

Catecholamines, glucose and lactate were measured in deproteinised plasma. Samples were brought to melting point and 125µl of ice cold 0.6M perchloric acid (PCA) was added to a 250µl aliquot of plasma. The resultant mixture was vortexed, then neutralised with 25µl of ice cold 2M $NaCO_3$ and left on ice for 30min allow precipitate formation. They were then centrifuged at 14000*g* at 4°C for 10min (model PK 121R, ALC®, Winchester, Virginia, USA). The supernatant was analysed for catecholamines immediately, whilst the remaining sample was frozen at -30°C for less than four weeks, for

subsequent analysis of lactate and glucose.

2.3.2.2 Muscle

2.3.2.2a Dry weight determination

Muscle metabolites were expressed as μmol/g dry wt, as the water content of fish is variable. Muscle portions of approximately 0.2 g were excised from the caudal-peduncle region between 4 and 7 days *post mortem*, depending on the experiment, (analytical balance, model AC 100, Mettler Toledo Ltd., Leicester, UK). Water content measurements were made in duplicate by drying samples to constant weight (48h) in a freeze dryer at -52°C, -1 bar (Edwards Modulyo Freeze dryer, Edwards High Vacuum International, Crawley, West Sussex, UK).

2.3.2.2b Pre rigor mortis muscle extraction

The extraction was performed in the chill room, (2°C±0.4) to minimise sample deterioration. The acid homogenisation was performed under liquid nitrogen in a freezer mill (model 6750, Glen Creston Ltd., Stanmore, Middlesex, UK) so that enzymatic activity on thawing would be negligible. Approximately 0.6 g of frozen muscle tissue was pulverised with six times its weight of frozen 0.6M PCA, in the freezer mill. The grinding vials had 4ml polycarbonate centre cylinders with stainless steel end-plugs and impactor, (model 6751, Glen Creston Ltd., Stanmore, Middlesex, UK). The freezer mill cycle was ten impacts per second with 0.5min pre and intermediate cool and three, 2min grindings. The resultant homogenate was rapidly brought to melting point in a heating block set at 50°C (model QBT2, Grant Instruments, Cambridge, Cambridgeshire, UK). The thawed homogenate was immediately decanted into two, 2ml tubes and centrifuged at 14000*g* at 2°C for 5min. The supernatant was decanted in two pre-chilled, 1.5ml eppendorf tubes and neutralised with ice cold 3M potassium hydroxide (KOH), and left for 30min for precipitation of potassium perchlorate. The effectiveness of neutralisation was monitored with narrow range, pH 6-8, universal indicator paper (Whatman International Ltd., Maidstone, Kent, UK). The neutralised extract was centrifuged at 14000*g*, at 2°C for 5min

and the supernatant transferred to clean eppendorf tubes and stored on ice until analysed. Within 6h of preparation the samples were analysed in order for creatine phosphate, creatine, creatinine and ATP. An aliquot of the neutralised sample was stored at -80°C for subsequent determination of lactate.

2.3.2.2c Post rigor mortis muscle extraction

Muscle portions of approximately 0.2 g were excised from the caudal-peduncle region between 4 and 7 days after 'death' and stored at -80°C for later determination of K value and/ or lactate. The extracts for these analyses were prepared from approximately 0.6 g of frozen muscle, which was homogenised with five times its weight of ice cold 0.6M PCA as described by Ryder [198]. The sample was kept on ice while being homogenised to reduce sample degradation. Homogenisation was achieved using three, 6s bursts with an Ultra Turrax® homogeniser set at 24000 rpm (model T25 basic, dispersing tool S25N-8G, IKA Works, GmbH, Staufen, Germany). The homogenate was centrifuged at 14000*g* at 4°C for 10min. The supernatant was neutralised with ice cold 1M KOH and left for 30min on ice. It was then centrifuged at 14000*g* at 4°C for 5min stored on ice and analysed within 3h for the K value components. An aliquot of the neutralised sample was stored at –80°C for subsequent analysis of lactate.

2.3.3 Sample analysis

2.3.3.1 Endpoint enzymatic assays

Endpoint enzymatic analysis was selected for quantitation of metabolites as it allowed contemporaneous measurement of the unstable muscle metabolites. Creatine phosphate, creatine, creatinine, ATP, lactate and glucose were determined using enzymatic analysis. Methods were adapted from Bergmeyer [27] for use in a microtitre plate reader. Plates were incubated at room temperature, standards and reagent blanks were used on all plates. Triplicate wells were used per reading and duplicate readings were made per sample and sample blank. The pipetting procedure was, unless otherwise stated; sample or standard, main reagent, and then the reaction starting enzyme, all solutions were kept on

ice. Plates were read on a microtitre plate reader (model MRXII, Dynex Technologies, Middlesex, UK) using the appropriate wavelength filter. The software used was Revelation version 4.02, (Dynex Technologies, Middlesex, UK). Standards were prepared in aqueous solution over the ranges shown in Figure 2.2. The fitted regression lines had R^2 values between 0.97 and 1.00.

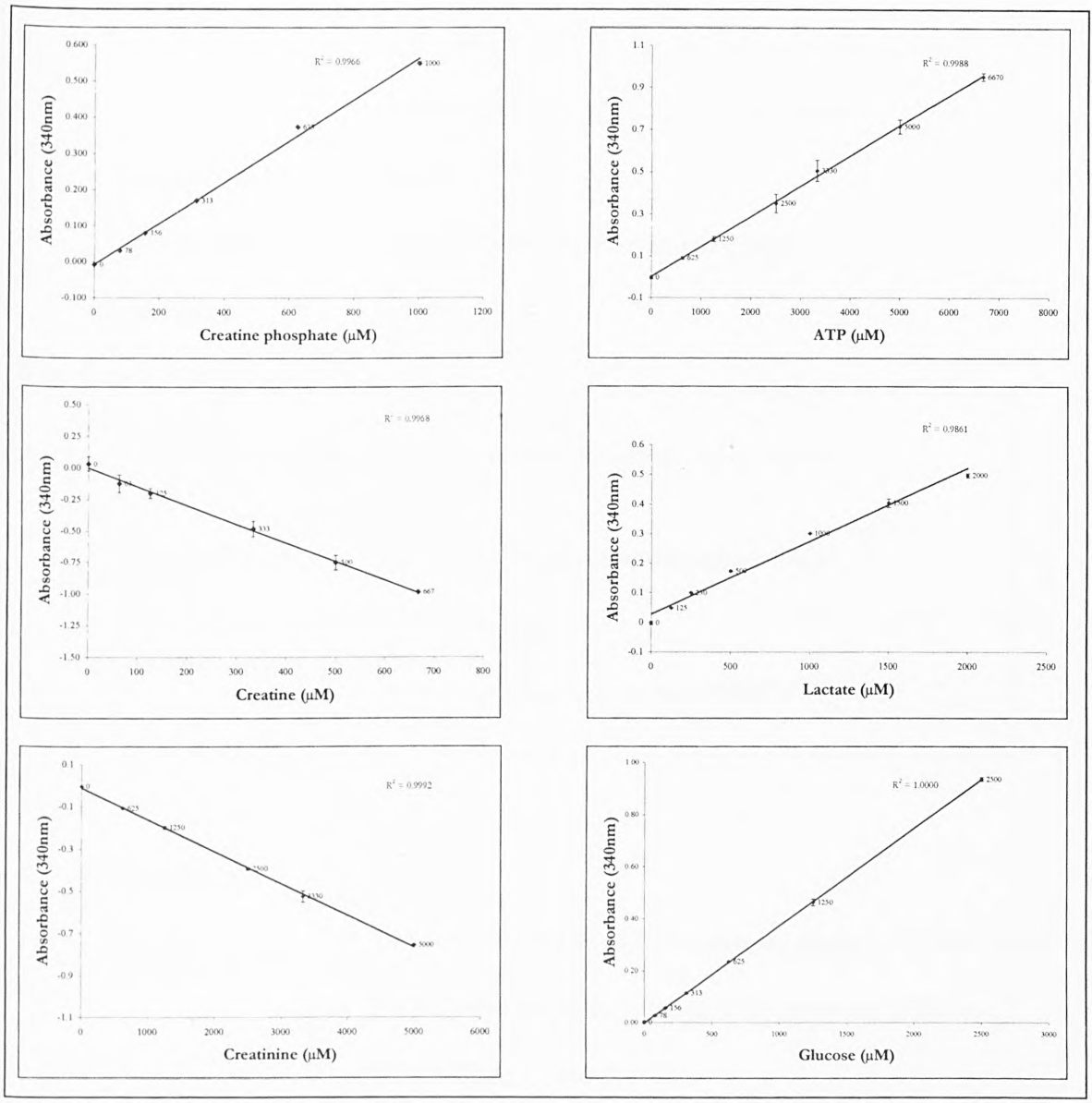
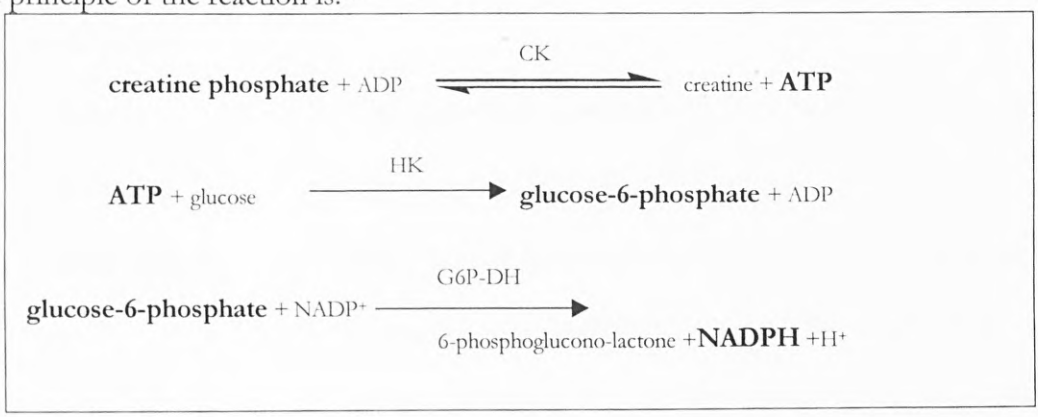


Figure 2.2 Endpoint enzymatic assays standard curves
Standards were prepared in aqueous solution and measured in triplicate on each microtitre plate.

2.3.3.1a Creatine phosphate

Sample:	40µl, neutralised perchlorate extract, diluted 1:6 with water
Standard:	40µl, range 0-1000µM
Main reagent:	185µl (38.5mM triethanolamine (TEA) pH 7.5, 0.24mM nicotinamide-adenine dinucleotide phosphate (NADP), 9.6mM $MgCl_2$, 48mM glucose, 0.2mM adenosine-diphosphate (ADP), 1U/ml Glucose-6-phosphate dehydrogenase (G6P-DH), 1U/ml Hexokinase (HK))
Pre incubation:	15min
Start reagent:	25µl (29U/ml Creatine kinase (CK))
Incubation:	60min

The principle of the reaction is: -



2.3.3.1b Creatine

Sample:	200µl neutralised perchlorate extract diluted 1:14 with water pH adjusted to between 8 and 9 by dropwise addition of 0.21M TEA, 1.16M K_2CO_3
Standard:	200µl, range 0-667µM
Main reagent:	30µl (5mM phosphoenolpyruvate (PEP), 200mM $MgCl_2$, 125mM glycine, 3.3mM reduced nicotinamide-adenine dinucleotide (NADH), 8.3mM adenosine-5-triphosphate

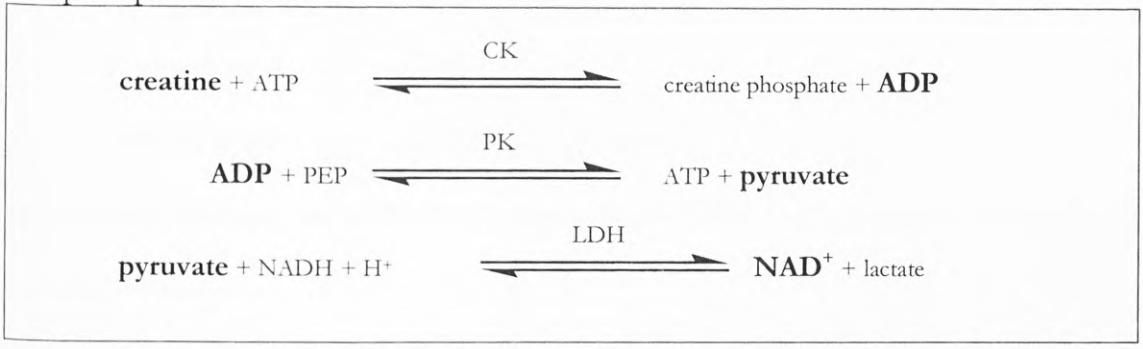
(ATP), 12U/ml lactate dehydrogenase (LDH), 4U/ml pyruvate kinase (PK))

Pre incubation: 20min

Start reagent: 20µl (25U/ml CK)

Incubation: 60min

The principle of the reaction is: -



2.3.3.1c Creatinine

Sample: 10µl neutralised perchlorate extract diluted 1:2 with water.

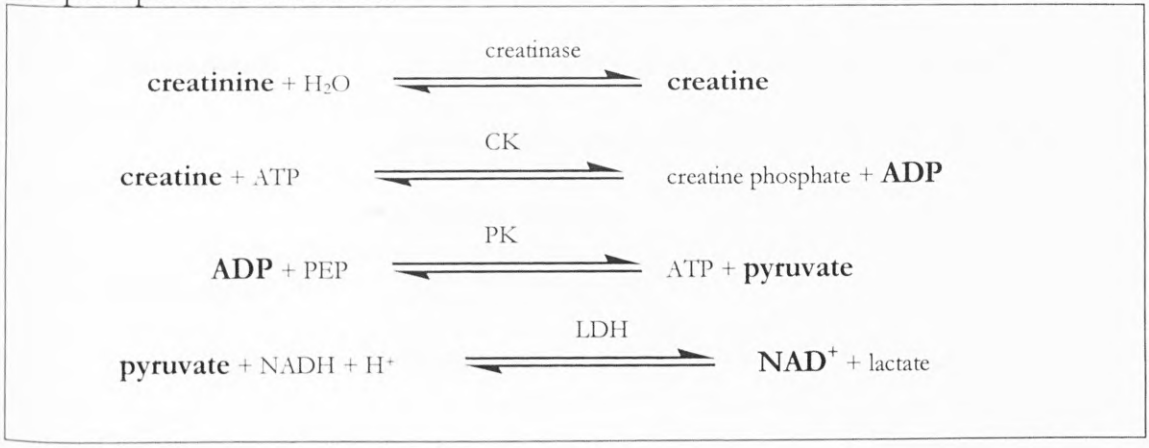
Standard: 10µl, range 0-5000µM

Main reagent: 200µl (96mM glycine pH 8, 72mM $KHPO_4$, 2.2mM $MgCl_2$, 0.25% (v/v) TritonX100, 1.44mM ATP, 48mM PEP, 0.23mM NADH, 9U/ml CK, 4U/ml PK, 9U/ml LDH)

Start reagent: 40µl (5U/ml creatinase)

Incubation: 60 min

The principle of the reaction is: -



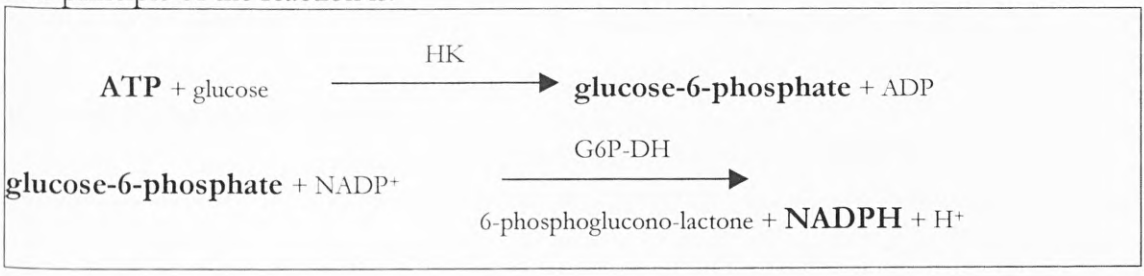
2.3.3.1d ATP

Sample:	10µl, neutralised perchlorate extract, undiluted
Standard:	10µl, range 0-6670µM
Main reagent:	185µl (44mM TEA pH 7.5, 0.39mM NADP, 7.8mM $MgCl_2$)
Pre incubation:	5min
Priming reagent 1:	10µl (1U/ml G6P-DH)
Pre incubation:	10min
Priming reagent 2:	25µl (500mM glucose)

Immediately following the addition of priming reagent 2 the start reagent was added to start the incubation

Start reagent:	20µl (2U/ml HK)
Incubation:	25min

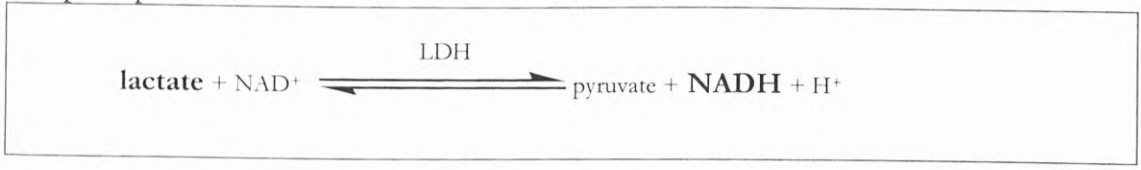
The principle of the reaction is: -



2.3.3.1e Lactate

Sample:	25µl neutralised perchlorate extract diluted with water 1:6 (muscle), 1:8 (plasma)
Standard:	25µl, range 0-2000µM
Main reagent:	75µl (71mM TRIS pH 9.3, 177mM NaCl, 3.1mM nicotinamide-adenine dinucleotide (NAD^+), 1% (v/v) hydrazine hydrate)
Start reagent:	150µl (1U/ml LDH)
Incubation:	60min

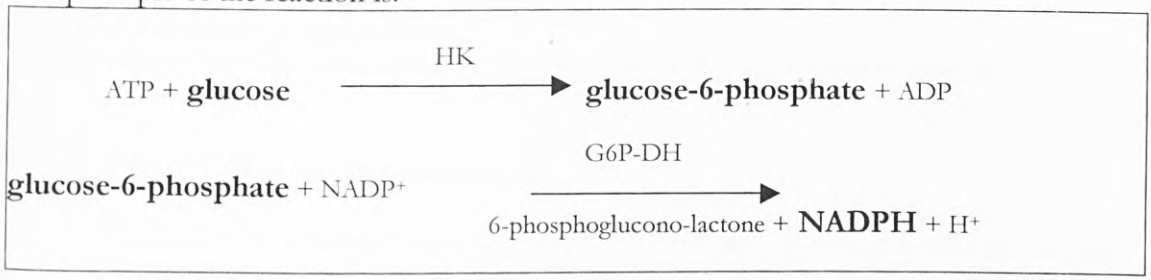
The principle of the reaction is: -



2.3.3.1f Glucose

Sample:	25µl neutralised deproteinised sample diluted 1:4 with water
Standard:	25µl, range 0-2500µM
Main reagent:	200µl (300mM TEA, 4mM $MgSO_4$, 1mM ATP, 900µM NADP, 1U/ml G6P-DH)
Pre incubation:	5min
Start reagent:	25µl (2U/ml HK)
Incubation:	30min

The principle of the reaction is: -



2.3.3.2 Protein Assay

Plasma protein was determined using a Protein Assay Kit (no P5656, Sigma Diagnostics, Poole, Dorset, UK). It is based on the Folin-Lowry principle where, under alkaline conditions, copper ions in the reagent bind to nitrogen in the protein. The protein copper complex reduces the reagent, the resulting blue colour, is proportional to the amount of protein present in the sample [85]. The kit was adapted for use on the microtitre plate reader. Reagents were made up as directed, standards were prepared in aqueous solution over the range shown in Figure 2.3, and run on each plate. To 100µl of diluted plasma sample or standard, 100µl of 'Lowry reagent' was added. The reagents were mixed vigorously on the microtitre plate reader for 60s and then during the 20min incubation on a

rocking platform at 30rpm, (model STR9, Stuart Scientific, Redhill, Surrey). After incubation, 50µl of Folin and Ciocalteu's phenol reagent was added. Following another vigorous shake, the plate was incubated for a further 30min on the rocking platform, and then read on the microtitre plate reader at 560nm. Plasma samples were diluted 1:250 with water, and had been through three freeze thaw cycles prior to analysis.

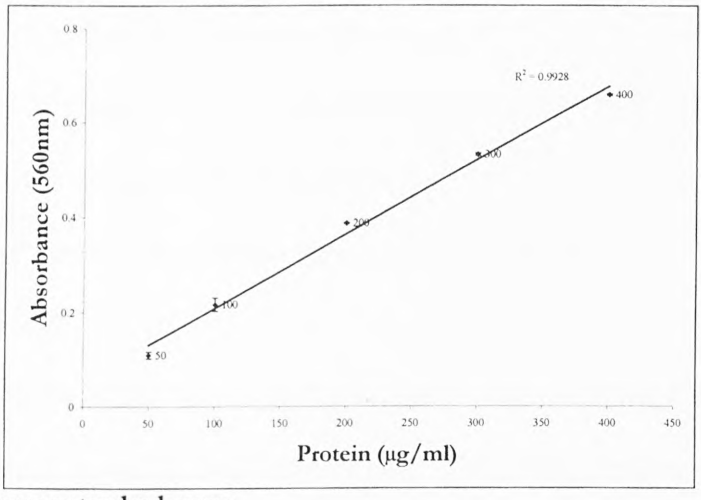


Figure 2.3 Protein assay standard curve
Standards were prepared in aqueous solution and measured in triplicate on each microtitre plate.

2.3.3.3 Enzyme Linked Immunosorbant Assays

ELISA's were selected for the quantitation of the primary stress hormones on the grounds of sensitivity, safety, speed and convenience. ELISA kits were brought to room temperature before use.

2.3.3.3a Cortisol

Plasma samples that had been through two freeze thaw cycles were analysed for cortisol using a DRG Cortisol ELISA kit (IDS Ltd. Boldon, Tyne and Wear, UK). The kit worked on the principle of direct competition, 200µl of horseradish-peroxidase labelled cortisol (HRP-cortisol) and 20µl of plasma sample competed for a fixed limited number of antibody (Ab) sites on microtitre plate wells, incubation time 60min. Following the competitive immunoreaction, any unbound HRP-cortisol was removed by washing four times with the buffer provided. Care was taken to remove all the residual fluids at this stage. The bound HRP-cortisol was then converted to a blue compound by addition of

100µl of a chromogen solution which contained 3,3'-5,5' Tetramethylbenzidene and hydrogen peroxide. This enzymatic reaction was stopped after 15min with 100µl of 0.5M sulphuric acid, the test solution turned yellow. The absorbance was measured at λ450nm, reference filter 650nm, and was inversely related to the concentration of cortisol in the sample. Unknowns were calculated from a standard curve constructed by plotting the absorbance of the standards expressed as a percentage of the zero standard, against the log of their concentrations, Figure 2.6. The plates were incubated at room temperature, hand washed, mixed on the rocking platform at 30rpm and read on the microtitre plate reader. The lowest level of cortisol detectable using the kit was 2.5ng/ml.

2.3.3.3b Catecholamines

IBL Cat Combi ELISA kits (IDS Ltd. Boldon, Tyne and Wear, UK) were assessed for measuring plasma adrenaline and noradrenaline. The catecholamines were extracted from the plasma by an immune reaction before quantitation. As the extraction procedure was not expected to be 100% effective, the standards and controls were also processed. The catecholamines bind to Ab adsorbed to the wells of a macrotitre plate. The wells were then washed four times and the bound catecholamines acylated and then released to form the clean sample. Adrenaline and noradrenaline levels were then determined by two separate 'antibody sandwich' ELISA's. The wells of microtitre strips come pre-coated with an Ab directed towards an epitope of either adrenaline or noradrenaline. An aliquot of the cleaned sample was incubated in the well with an enzyme conjugated second Ab, which binds to a different region of the catecholamine molecule (E-Ab). After incubation any unbound E-Ab was washed off. The amount of bound E-Ab is proportional to the concentration of the hormone in the sample. For detection, an amplification and visualisation reagent was added. The resulting colour intensity is proportional to the hormone concentration in the sample, and was measured at λ490nm with reference filter λ650nm. Standards were used to construct a calibration curve against which the unknowns

were determined. The lowest detectable level of the kit is 10 and 20pg/ml for adrenaline and noradrenaline respectively.

The ELISA kit used for catecholamine analysis proved unreliable. Sample and standard replicates were not reproducible; the standard curves produced are shown in Figure 2.4. An alternative technique was therefore sought for catecholamine analysis.

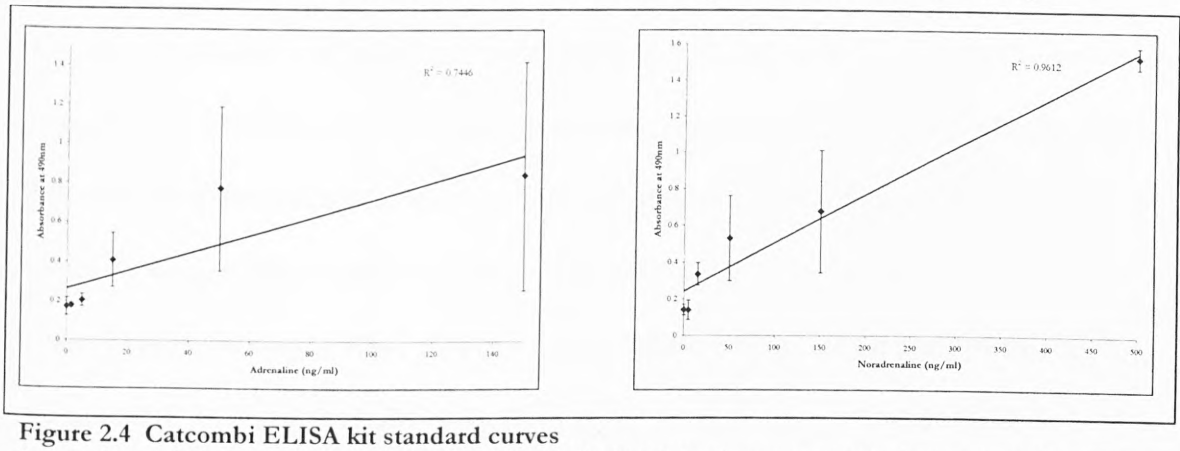


Figure 2.4 Catcombi ELISA kit standard curves
Results from triplicate measurements in the sandwich ELISA following extraction by immunoreaction. This method was abandoned, as it was not reliable.

2.3.3.4 High performance liquid chromatography

2.3.3.4a Catecholamines -Electrochemical detection

HPLC with electrochemical detection (ECD) is a sensitive, well-documented method for the determination of catecholamines. It was assessed for the quantitation of plasma adrenaline and noradrenaline levels following the method proposed by Raggi *et al.* [177] and adapted for amperometric detection.

- Apparatus and chromatographic conditions

The HPLC apparatus used was; a Spectra series P200 pump, and a Spectra Physics SP8750 organiser (Thermo Separation Products Ltd., Hemel Hempstead, Hertfordshire, UK). The ECD detector was a Model 400 (EG&G Princeton Applied Research, Princeton New Jersey, USA). The electrodes were; working electrode, 3mm single glassy carbon, and reference electrode, silver/silver chloride. The working electrode potential against the reference electrode was 710mV. Other detector conditions were; mode DC, current range 20nA/V, and time constant 5.0s. Chromatographic separation was achieved at ambient temperature using a reverse phase Waters Symmetry C8 5µm column 4.6x150mm with

guard column (Waters, Elstree, Hertsford, UK). The column and working electrode were equilibrated for five hours before use to obtain a background current of <2nA. The peak area was calculated by a Chromajet integrator (Thermo Separation Products Ltd., Hemel Hempstead, Hertfordshire, UK).

The mobile phase was prepared fresh daily and comprised of a 25:975 mixture of methanol and solution 1. Solution 1 contained: 10.5g/l citric acid, 20mg/l EDTA and 20mg/l 1-octanesulfonic acid sodium salt; and was made to pH 2.9 with 1M NaOH. The flow rate through the column was 1ml/min, samples were injected onto the column manually using a 100µl Hamilton syringe *via* a 100µl loop. Solution 1 was degassed and filtered under vacuum to 20µm through a nitro-cellulose filter. The mobile phase was sparged with helium during elution to prevent dissolution of gases. Standards and samples were filtered to 45µm through cellulose acetate syringe filters and stored on ice until injected onto the column, run time was approximately 10min. Standard curves were created from single samples, range 4-500ng/ml.

- Extraction procedure

The method of Raggi *et al.* [177] recommended extraction of the catecholamines from the plasma prior to quantitation to remove interfering compounds and increase the sensitivity of the technique.

Catecholamines were extracted using Oasis™ HLB (Hydrophilic -Lipophilic Balance) extraction cartridges, 30mg, 1ml (Waters Elstree, Hertsford, UK) as suggested by Raggi *et al.* [177].

In order to extract the catecholamines they have first to be complexed with diphenylboric acid 2-aminoethyl ester (DPB) as otherwise they are not retained by the cartridge. This can be achieved at room temperature under alkaline conditions. After loading and washing the cartridge, the catecholamines can be eluted by changing the pH to acidic conditions. This releases the catecholamines from the diphenyl boric acid catecholamine complex to form the cleaned sample.

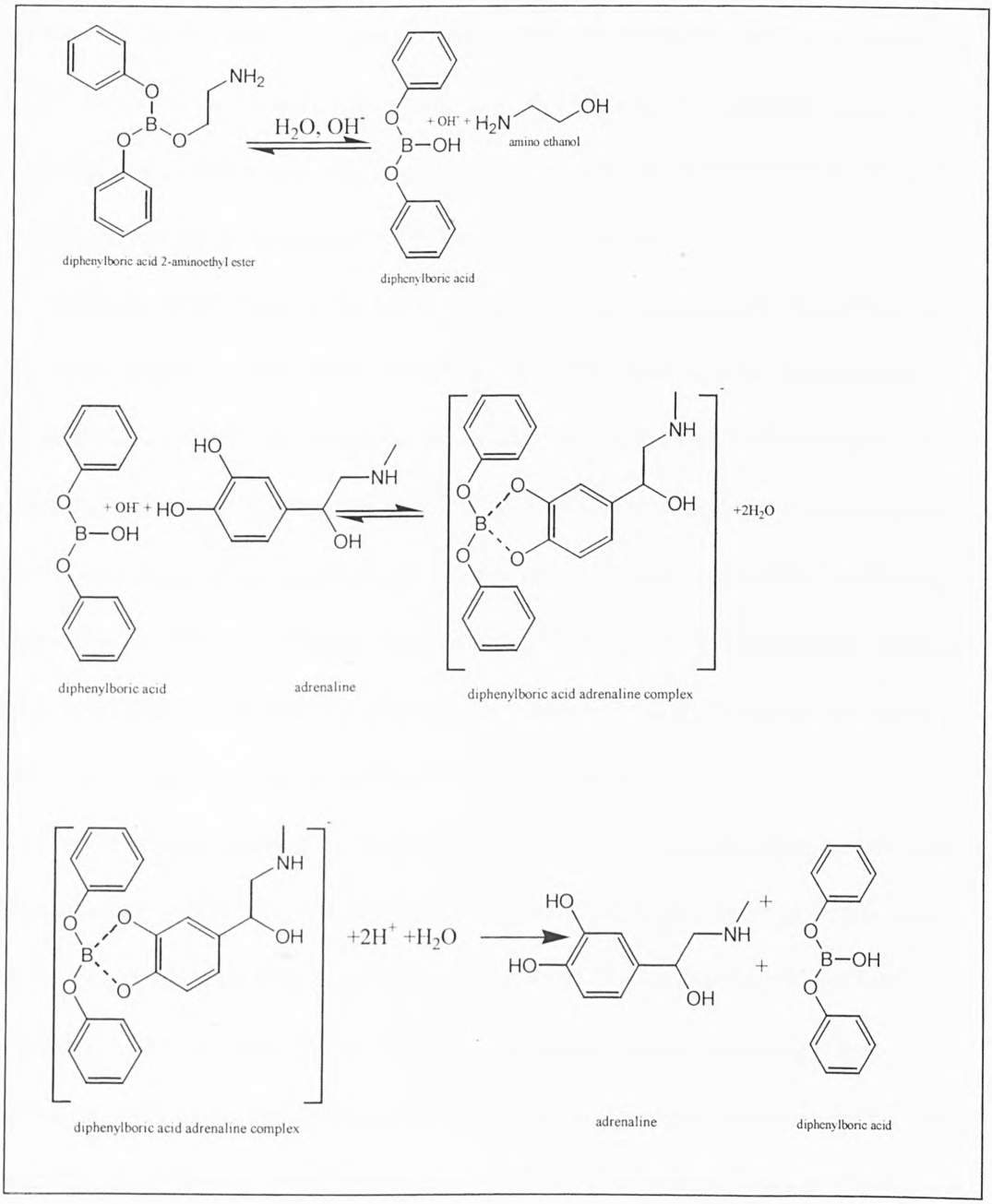
The reagents required for the extraction procedure were:

Buffer 1: made to pH 8.5 with 30% ammonia. DPB 2g/l, tetrabutylammonium bromide (TBAB) 2g/l, EDTA 5g/l, and NH_4Cl 107g/l.

Buffer 2: a 1:1 dilution of methanol and the following solution made to pH 8.5 with 30% ammonia. TBAB 4g/l, EDTA 0.5g/l, and NH_4Cl 10.7g/l.

The combined standards stock solution contained; adrenaline 1mg/ml, noradrenaline 1mg/ml, sodium metabisulphite 1mg/ml, NaCl 8mg/ml, and 37% HCl 5µl/ml.

The principle of the extraction process is: -



A flow rate of approximately 0.5ml/min through the cartridges was achieved by using a vacuum manifold (model IST Vacmaster, International Sorbent Technology Ltd., Ystrad Mynach, Hengoed, Mid Glamorgan, Wales, UK) attached to a water vacuum system. The cartridges were conditioned by passing through two column volumes of methanol, water, and buffer 1, in that order. They were not allowed to dry out during conditioning. Thawed plasma (250µl) was then mixed with 750µl of buffer 1 and DHBA as an internal standard. The resulting mixture was left for 10min for the catecholamine-diphenylborate complex to form. The prepared sample was then loaded onto the previously conditioned cartridge, which had not been allowed to dry out. Once loaded, the cartridge was washed with two column volumes of buffer 1 then buffer 2, and thoroughly dried. The catecholamines were then eluted from the cartridge by lowering the pH to 2.9 with 500µl of the mobile phase, which was passed through the column in three aliquots. Analytes were kept on ice and analysed within 2h of extraction.

A plasma sample from a fish left to struggle for five minutes and then killed by blow to the head gave no detectable adrenaline, 18ng/ml noradrenaline and 39ng/ml DHBA (130%) recovery. The extraction procedure was clearly not 100% effective. To determine the percentage recovery of noradrenaline and adrenaline from the solid phase extraction procedure, (SPE) standards were extracted. This was achieved by loading the cartridges with a solution containing 750µl of buffer 1, 125µl of artificial plasma, 125µl of standards and DHBA. The artificial plasma was comprised of; KCl 0.2g/l, NaCl 8g/l, KH_2PO_4 0.2g/l, Na_2PO_4 1.15g/l, and bovine albumin 40g/l.

The percentage recovery from the SPE procedure was tested at 50ng/ml adrenaline and noradrenaline with a 10ng/ml DHBA internal standard spike. The recoveries from five extractions performed simultaneously were 52.4±11.1% adrenaline, 40.5±17.8% noradrenaline, and 3.7±1.3% DHBA. The SPE procedure as well as having a low percentage recovery of catecholamines, broadened the peaks of the chromatograms. The percentage recovery from the SPE procedure varied with the amount loaded: 1250ng/ml

gave, 73.5% adrenaline, 60% noradrenaline; 625ng/ml gave, 74.8% adrenaline, 61.1% noradrenaline; while 250ng/ml gave, 58.6% adrenaline, 55.0% noradrenaline. No improvement was achieved by using Strata X 33u 30mg/ml cartridges (Phenomenex, Macclesfield, Cheshire, UK). The extraction procedure was therefore deemed unreliable.

To test the necessity of extracting the catecholamines before analysis, the plasma was deproteinised by adjusting the to pH 2.9 (the same pH as the mobile phase) with 0.6M PCA. It was then centrifuged at 14500*g* at 4°C for 10min and the supernatant injected onto the HPLC column. The catecholamines eluted in the 'solvent peak' and the sensitivity of the electrode declined to approximately 20% of its original value after only two injections.

The HPLC with ECD method for catecholamine analysis was therefore abandoned.

2.3.3.4b Catecholamines -Fluorometric detection

A method for the fluorometric detection of catecholamines separated by HPLC was then investigated. Mitsui *et al.* [147] tested various plasma clean-up techniques prior to using this technique for the detection of catecholamines. Although the work recommended using SPE because the sample could be concentrated and the limits of detection improved, a method without extraction was described and was chosen for this study due to problems previously encountered with SPE.

- Apparatus and chromatographic conditions

The HPLC apparatus was as described in Section 2.3.3.4a with the following alterations. The detector was a Linear™ fluorometric detector, (model Fluor LC304, Linear Instruments, Reno, Nevada, USA). It had a 2x4mm flow-cell with 3µl illuminated volume. Chromatographic separation was achieved at ambient temperature on a reversed phase C18 µBondapak™ stainless steel column 125Å, 10µm, 3.9x300mm, with sentry guard column (Waters, Elstree, Hertsford, UK). The mobile phase at pH 7.0, comprised acetonitrile: 50mM Tris-HCl in a ratio of 450:560, filtered and degassed as described in Section 2.3.3.4a. The flow rate through the column was 1ml/min and samples were

injected manually *via* a 100µl loop. The detector conditions: were λ350nm excitation λ480nm emission, PMT voltage 600, lamp flash rate 100Hz, filter rise time 10, output offset 1%, chromatogram range 0.1 and 0.02. The run time was 30min. Signals were detected using chart recorders (model SE 120, Asea Brown Boveri, Zurich, Switzerland and model Graphic 1002, Lloyd Instruments Ltd., Fareham, Hampshire, UK). Peaks were integrated manually using Equation 2.1.

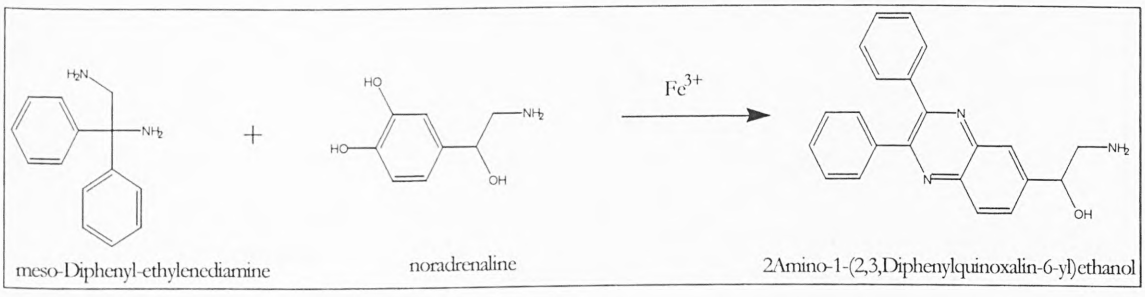
Equation 2.1

peak area = 1.06 × peak height × peak width @ 0.5peak height

- Derivatisation procedure

The standards and samples were converted to their corresponding fluorescent compounds with meso-diphenylethylenediamine (DPE). The reaction occurs under mild conditions, pH 6-7, 0-50°C, in the presence of potassium ferricyanide and can be accelerated with acetonitrile [147].

The derivatisation reaction between DPE and noradrenaline was as follows.



meso-Diphenyl-ethylenediamine noradrenaline 2Amino-1-(2,3,Diphenylquinoxalin-6-yl)ethanol

Samples were deproteinised as reported in Section 2.3.2.1c. The neutralised, deproteinised plasma was then derivatised with DPE. To 150µl of the sample, 10µl of 20mM potassium ferricyanide was added, and 6µl of the internal standard isoproterenol (IP) at 1000ng/ml. Acetonitrile, 150µl, was then added and finally 50µl 100mM DPE which had been dissolved in 0.1M HCl. The resultant mixture was vortexed and incubated in the dark for 40min at 37°C in a fan assisted incubator (size 2, Sanyo-Gallenkamp, Loughborough, UK). After incubation, derivatised samples were centrifuged at 14500*g* at 4°C for 10min to remove precipitated material. They were stored on ice until manually injected on to the HPLC column with a 100µl Hamilton syringe within 3h of preparation.

Typical standard curves and a chromatogram showing separation of the catecholamines are presented in Figures 2.5 and 2.6.

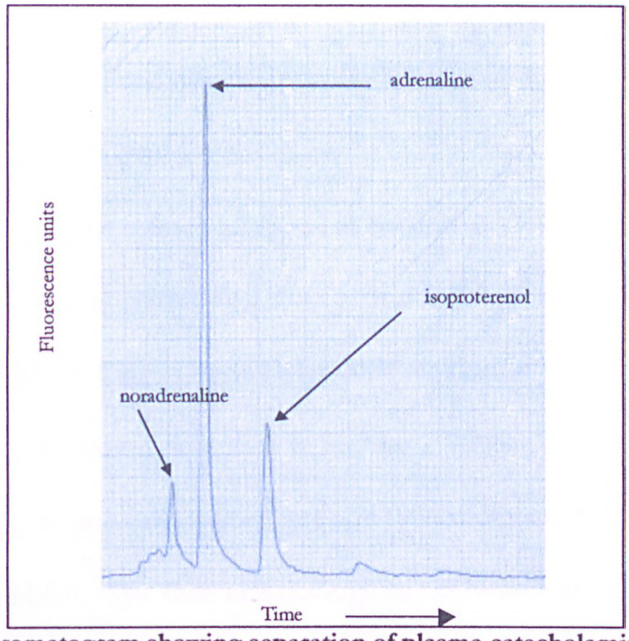


Figure 2.5 Typical chromatogram showing separation of plasma catecholamines
Separation of noradrenaline and adrenaline in deproteinised fish plasma following derivatisation with DPE.

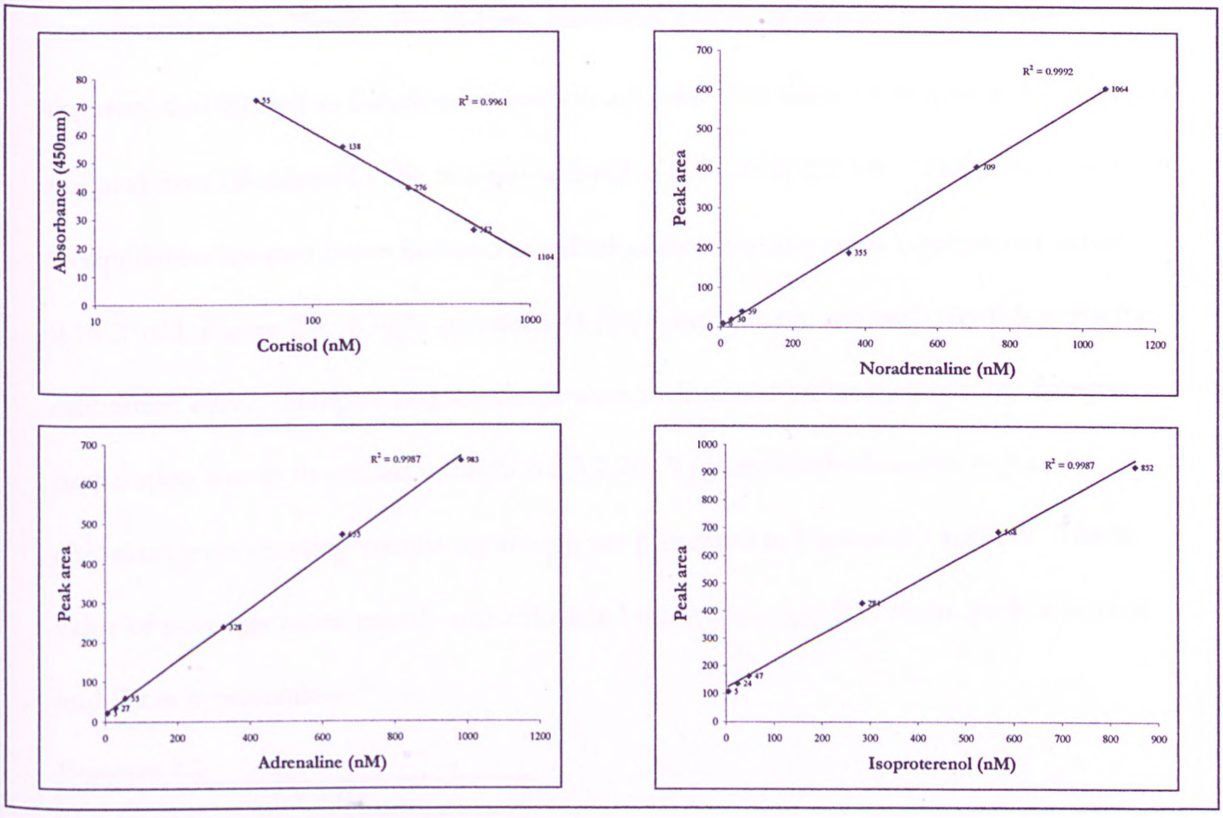


Figure 2.6 Plasma hormones standard curves
Cortisol standards were measured in duplicate in an ELISA assay, single determinations of catecholamines measured fluorometrically after separation by HPLC.

2.3.3.4c K value -Spectrophotometric detection

HPLC analysis with spectrophotometric detection was used to determine the levels of ATP and its breakdown products using method adapted from Ryder [198]. This method was selected as it allows the simultaneous quantitation of all six components of K value.

- Apparatus and chromatographic conditions

The HPLC apparatus was as described in Section 2.3.3.4a with the exception of the detector which was a Spectra series UV 100 (Thermo Separation Products Ltd., Hemel Hempstead, Hertfordshire, UK). Chromatographic separation was achieved at ambient temperature on the C18 column described in Section 2.3.3.4b. The mobile phase was 0.04M potassium dihydrogen orthophosphate and 0.06M dipotassium hydrogen orthophosphate at pH 6.9. The flow rate through the column was 2ml/min and the samples were injected manually using a 25µl Hamilton syringe *via* a 10µl loop. Run time was approximately 25min. The buffers, standards and samples were prepared daily, degassed and filtered as described in Section 2.3.3.4a. The detector was set at λ254nm, and the peak area calculated by the integrator detailed in Section 2.3.3.4a. The column was left to equilibrate for two hours before a standard calibration curve was constructed, range 0.16-27nM, Figure 2.8. Single injections of five samples were run each day following the calibration curve. Samples and standards were maintained on ice throughout. Sample preparation was as described in Section 2.3.2.2c. Typical standard curves and a chromatogram showing sample separation are presented in Figures 2.7 and 2.8. The K value of post *rigor mortis* muscle was calculated using Equation 2.2, where HxR is inosine and Hx is hypoxanthine.

Equation 2.2

$$K = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx}$$

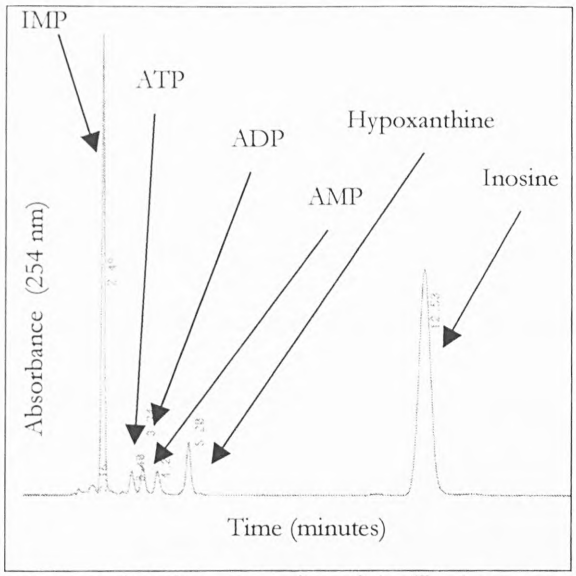


Figure 2.7 Typical chromatogram showing separation of the K value components
Separation of the six components of the K value in a trout muscle extract.

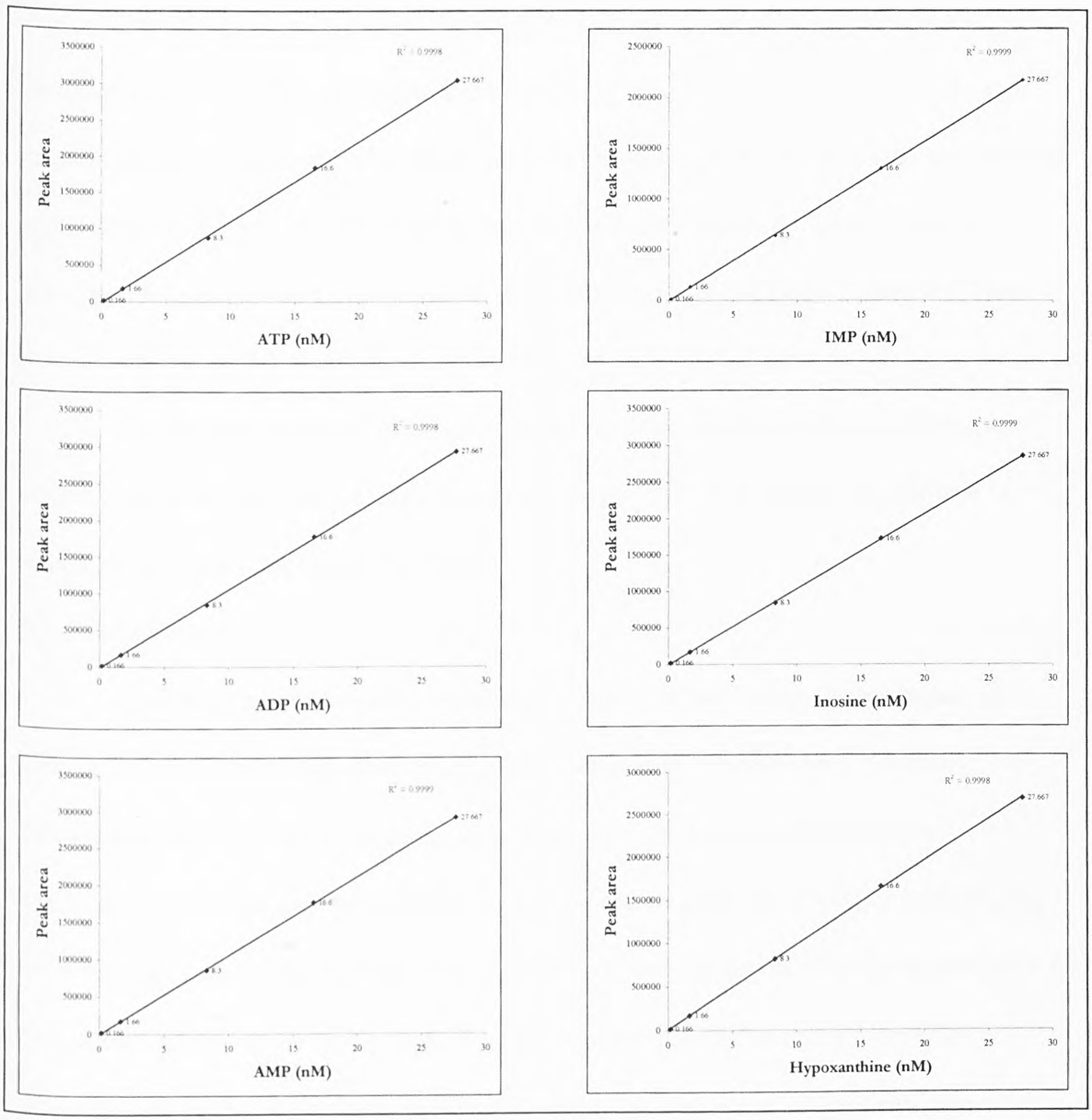


Figure 2.8 K value components standard curves
Results from single determinations

2.4 Fish quality assessment

All visual assessments were performed against a neutral grey background under D_{65} lighting conditions in a Judge II-S viewing booth (Gretag Macbeth, Altrincham, Cheshire, UK). One observer with 11 years experience of fish handling and three years training in goniometric assessment performed the sensory evaluations. At least two practise sessions were undertaken before data collection.

2.4.1 pH

The first muscle pH measurements were taken in the field so a penetration electrode (model FC230B) designed for use with meat was used. It was connected to a battery operated pH meter, model HI8424 (Hanna instruments, Leighton Buzzard, Bedfordshire, UK). This pH meter was used throughout to ensure consistency of measurement. Immediately after death, prior to blood sampling, the electrode was inserted approximately 0.75cm into the epiaxial muscle posterior to the head (pH_i). Other pH measurements were taken in the epiaxial muscle along the dorsal ridge, Figure 2.1. Fish were stored on ice in the chill room, 2.0±0.4°C, between measurements.

The pH was measured in the plasma directly after the aliquot for catecholamine analysis had been removed i.e. after one freeze thaw cycle. The plasma was allowed to acclimate to room temperature for 10min.

2.4.2 *Rigor mortis*

Rigor mortis was assessed by measuring 'angle of droop' using, depending on fish size, either an Orthotic Solutions tractograph (Canonbury Products Ltd., Brackley, Northamptonshire, UK), or a jointed carpenters rule with protractor (my Great Grandad's). The fish were laid laterally on a horizontal surface the posterior half hanging over the edge, see Section 1.3.4.3. The angle the base of the tail made to the horizontal was measured and converted to the angle from the horizontal by subtracting it from 90°. Regardless of salmonid species, the first measurements were taken in the field, Appendix 2, Figure 10. The fish were boxed and iced and transported to the laboratory where they

were laid on stainless steel benches in a chill room, 2.0±0.4°C, and covered with ice. They were left undisturbed between measurements to minimise the effects of handling. Triplicate measurements were made on each side of the trout and single measurements on the salmon, at several time intervals over three to five days.

Duration of *rigor mortis* was defined as the time from when the angle of droop measurements exceeded 30 degrees until they fell below 30 degrees, Figure 2.9. *Rigor mortis* contraction strength was the greatest angle measured, and time to peak *rigor mortis* was the time at which a fish was in maximum *rigor mortis*, Figure 2.9.

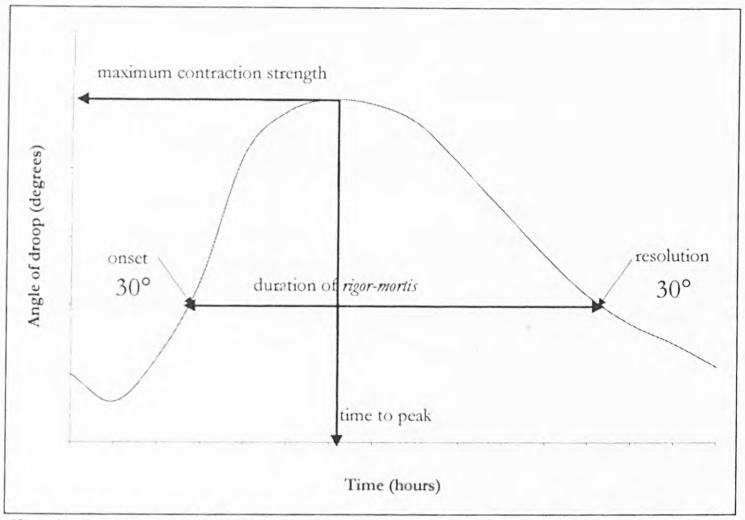


Figure 2.9 Generalised *rigor mortis* plot showing development of *rigor mortis* with time

2.4.3 Freshness

2.4.3.1 Instrumental measurement

Fish freshness was measured by using a Torrymeter (Distell Freshness Meter model 295, Distell Industries Ltd., Fauldhouse, West Lothian, UK), as this is an objective non-destructive technique. Triplicate measurements were taken daily on one side of the fish, to reduce handling between *rigor mortis* measurements. The meter was placed parallel to the lateral line on the epiaxial muscle marginally anterior of the dorsal fin, to avoid the underlying red muscle, which spoils faster.

2.4.3.2 Visual assessment

Additionally, in the starvation experiment when more time was available a subjective, visual assessment of fish freshness was performed. This assessment scored attributes between zero and two; lower scores signified greater loss of freshness. The scores were summed and expressed as a percentage of the total possible score. The characteristics evaluated are described in Table 2.1. The belly flap assessment commenced only after gutting, day two.

Table 2.1 Freshness assessment criteria.

Score	2	1	0
Characteristic			
Eye contour	convex	planar	concave
Eye brightness	clear and bright	slightly cloudy	opaque
Gill colour	bright red	pink/grey	grey
Skin appearance	predominantly shiny and smooth	partly shiny and smooth	dull and rough
Belly flap appearance	firm and bright	dull	pulling away from the flesh

2.4.4 Centrifugal drip

Centrifugal drip was selected over the press method for estimating the water-holding capacity, as multiple samples could be processed simultaneously. It was determined approximately 96h after death in the rainbow trout. Approximately 0.3g of muscle from the caudal-peduncle area was excised using the biopsy punch. The sample was placed in a Vecta spin™ 3 filter centrifuge tube (3ml, 10μm polypropylene mesh, Whatman, Maidstone, Kent, UK) and centrifuged at 1200*g* at 9°C for 90min (model 4237R, ALC®, Winchester, Virginia, USA). A low speed was selected to minimise microstructural damage to the muscle, the long run time to maximise 'water' loss and the temperature to avoid evaporative loss and condensation gain. The fluid released was weighed on an analytical balance and the percentage weight loss calculated.

2.4.5 Carcass characteristics and downgrading

2.4.5.1 Morphological measurements

Fish length and weight were recorded for calculation of Fulton's Condition Factor (K), Equation 2.3. Where weight is in g, and fork length (Figure 2.1) in cm.

Equation 2.3

$$K = 100 \times \frac{\text{weight}}{\text{fork length}^3}$$

The somatic indices were calculated from Equation 2.4, using the spleen for spleen somatic index (SSI), liver for hepato somatic index (HSI), and the intestinal tract with gall bladder and liver removed for digestive somatic index (DSI).

Equation 2.4

$$\text{somatic index} = \frac{\text{organ weight}}{\text{whole body weight}} \times 100$$

The dress-out percentage was calculated using Equation 2.5 where gutting consisted of evisceration and kidney removal, N.B. the gills were left in place.

Equation 2.5

$$\text{dress} - \text{out}\ \% = \frac{\text{ungutted weight}}{\text{gutted weight}} \times 100$$

2.4.5.2 Fillet fat content

The Distell fat meter was used to estimate the fat content of rainbow trout fillets (Distell Fat Meter model 692, Distell Industries Ltd., Fauldhouse, West Lothian, UK) as it is a fast, non-destructive method. The fillets were wiped to remove ice and slime and the dorsal fat trimmed away. The sensor head was applied to the skin side of the fillet the measurements were taken on the stainless steel bench in the chill room readings were taken in duplicate.

2.4.5.3 Downgrading

In the downgrading assessment, attributes were assessed visually and scored on a scale of zero to five where a lower score signified less damage, see Table 2.2. Post *rigor mortis* fish were evaluated for surface damage; e.g. scale loss, haemorrhages, bruises, and

skull damage. One fillet was removed and appraised for blood spotting (internal haemorrhages), gaping and colour. Spinal damage was assessed from the 'fish frame' by counting dislocations and haemorrhages. Before the gaping assessment fillets were drawn, skin down, over a 90° angle to simulate rough handling.

The sensory texture evaluation used in the starvation experiment was a finger compression test. This involved pressing the forefinger into the epiaxial muscle of the fillet posterior to the dorsal fin and scoring the impression left, none (0), < 2mm (1), > 2mm (2).

Flesh colour was evaluated subjectively using the *Salmo*Fan™ (Roche Products Ltd., Heanor, Derbyshire, UK). The observer was previously Ishihara tested for colour vision. All colour measurements were taken on the epiaxial muscle slightly posterior to the dorsal fin.

Table 2.2 Downgrading assessment criteria.

Score	0	1	5
Characteristic			
Scale loss	none	≤1% total surface area	≥5% total surface area
External haemorrhages	none	1 haemorrhage	5 or more haemorrhages
Bruises	none	1 bruise	5 or more bruises
Skull damage	none	small crack or indentation	shattered skull
Internal haemorrhages	none	1 haemorrhage	5 or more haemorrhages
Spinal damage	none	1 haemorrhage or dislocation	5 or more haemorrhages or dislocations
Gaping	none	total length of myotomal separation ≤20% body length	total length of myotomal separation ≥body length

2.4.6 Instrumental colour measurements

Instrumental colour measurements were taken with a Chroma II reflectance meter with d-0 incident light (Minolta UK Ltd., Milton Keynes, Bucks, UK). An average of three CIE 1976 $L^*a^*b^*$ values were taken under illuminant D_{65}. To prevent cushioning the measurements were taken through optical quality glass, 60mm diameter 2.5mm depth (Blacks Opticians, Aberdeen, Aberdeenshire, UK). The values of chroma and angle of hue were calculated using Equations 2.6 and 2.7.

Equation 2.6: Chroma

$$\sqrt{a^{*2} + b^{*2}}$$

Equation 2.7: Angle of Hue

$$(\tan^{-1} \frac{b^*}{a^*}) * 57.295$$

2.4.7 Instrumental texture measurements

Texture was measured using a Stevens Compression Response Analyser 200 (Stevens and Son Weighing Machines Ltd., Loughton, Essex, UK). The flesh was allowed to acclimate to room temperature, 20-21°C, for 1-2h before the measurements were taken.

2.4.7.1 Compression test

A double compression test was performed on the cut surface of the fillet using an 8mm-diameter cylindrical Perspex probe. The fillet was positioned skin side down so that the probe travelled medial to lateral. The probe was positioned over the epiaxial muscle inferior to the dorsal fin avoiding pin bones. Immediately before the measurement was taken the fillet was fully stretched and wiped dry with absorbent tissue. The machine cycled at 5mm/min and compressed 2mm into the flesh. The peak force in grams was recorded during each of three readings taken in adjacent areas. The depth of the fillet was measured using digital callipers and the probe was cleaned after each test. The peak force of the second compression was divided by the peak force of the first compression to give the ratio of second to first bite, Figure 2.10A. The adhesiveness of the sample was evaluated from the negative force values between compressions, Figure 2.10A.

2.4.7.2 Shear test

A Warner-Bratzler 'shear test' was performed on cylindrical samples cut from the epiaxial muscle inferior to the dorsal fin perpendicular to the plane of the fillet. Samples were cut with a 10mm-diameter apple corer, the pin bones were avoided and the underlying red muscle and skin were removed. The sample depth was measured using digital callipers and weight was recorded using an analytical balance. Evaporative losses were kept to a minimum by measuring the samples immediately after preparation. Samples

were positioned under the Warner-Bratzler blade medial side uppermost. The blade dimensions were 70mm wide, 4mm thick with a 60° V cut, 35 mm long. It was unsharpened and fitted loosely through a 7mm wide slit in the base plate. The peak force in g was recorded after a single cycle, Figure 2.10B. The blade travelled 30mm at 120mm/min, and was cleaned after each cut.

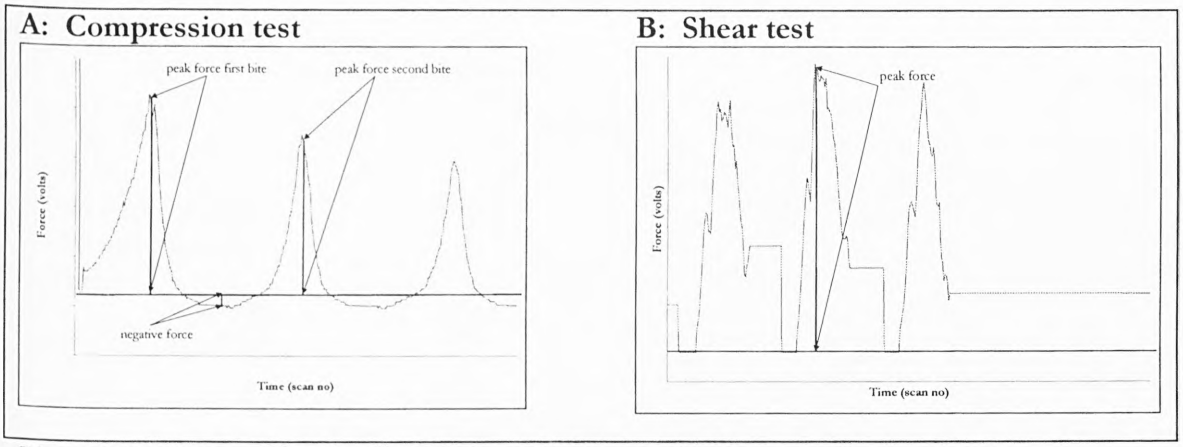


Figure 2.10A-B Generalised force time plots of texture measurements
These force time plots show the results of three consecutive tests on rainbow trout samples.

2.5 Statistical methods

2.5.1 Data treatment

Data were analysed using the statistical package Minitab for Windows Version 12.21 (Minitab Ltd., Coventry, UK). Supplementary analysis was performed using Graph Pad InStat version 3.05 for Windows 95/NT, Graph Pad 'Detecting outliers using Grubbs' test' (GraphPad Software, San Diego, California, U.S.A.) and SPSS for Windows releases 9.0 and 10.0.

Ordinal data was analysed using the Mann Whitney U test (two groups) or Kruskal Wallis test (multiple groups) followed by a Dunn *post hoc* test. Values were adjusted for ties. Cardinal data was tested for normality using the Kolmogorov and Smirnov test. Variances were tested for equality using F tests (two groups) or Bartlett (multiple groups). The selection process for the statistical and *post hoc* tests is shown in Figure 2.11.

The General Linear model was used to check for interactions between weeks, in the methods groups, in the trout killing experiments. Grubbs' test was used to remove outliers.

The software calculated the regression lines using the least squares method. T tests were used to test for parallelism and identical regression lines using Equations 2.8-2.10.

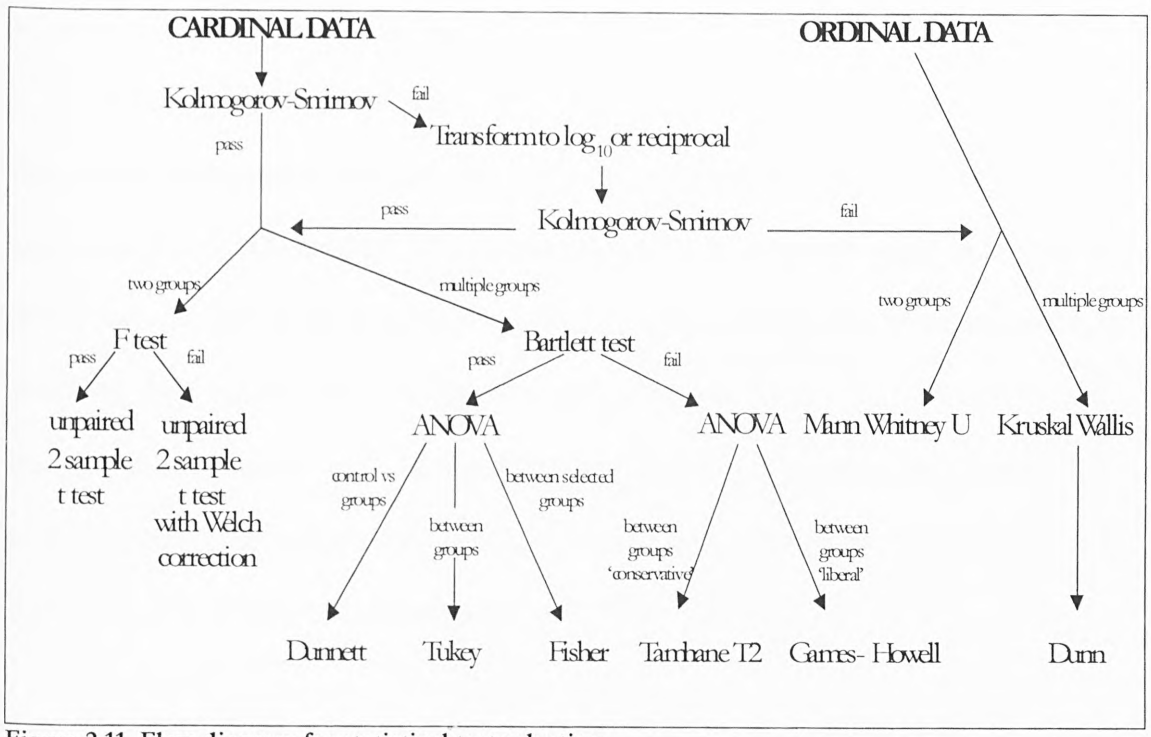


Figure 2.11 Flow diagram for statistical test selection

Equation 2.8: The pooled standard deviation, s.

$$s = \sqrt{\frac{(n_1 - 2)\sigma_1^2 + (n_2 - 2)\sigma_2^2}{(n_1 - n_2 - 4)}}$$

Equation 2.9: The slope t statistic, t_b.

$$t_b = \frac{b_1 - b_2}{s\sqrt{\frac{1}{\sum(x_{i1} - \bar{x}_1)^2} + \frac{1}{\sum(x_{i2} - \bar{x}_2)^2}}}$$

Equation 2.10: The intercept t statistic, t_a.

$$t_a = \frac{a_1 - a_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2} + \frac{\bar{x}_1}{\sum(x_{i1} - \bar{x}_1)^2} + \frac{\bar{x}_2}{\sum(x_{i2} - \bar{x}_2)^2}}}$$

2.5.2 Data presentation

Results were expressed as means (± one standard deviation) unless stated otherwise. Levels of significance are represented by: $P \geq 0.05$, ns, the difference is not significant; $P < 0.05$, *, the difference is significant; $P < 0.01$, **, the difference is highly significant; $P < 0.001$, ***, the difference is very highly significant.

In the test for parallelism and identical regression lines, where the significance level of the intercept is in parentheses i.e. (*), the difference was significant. This would, however, be expected as a difference had been detected between the slope of the lines.

Box and whisker plots were used to show the distribution of the data, Figure 2.12: they can be interpreted as follows. The dot in the box symbolises the mean value and the line across the box the median. The box represents the inter-quartile range or 'middle half' of the data. Therefore, the bottom of the box is the first quartile, Q1, while the top of the box is the third quartile, Q3. The lines extending from the box are the whiskers, showing the general extent of the data. They extend to the lowest and highest observations that are in an area defined by: a lower limit of Q1 - 1.5 (Q3 - Q1), and an upper limit of Q3 + 1.5 (Q3 - Q1). The asterisks are observations outside these limits.

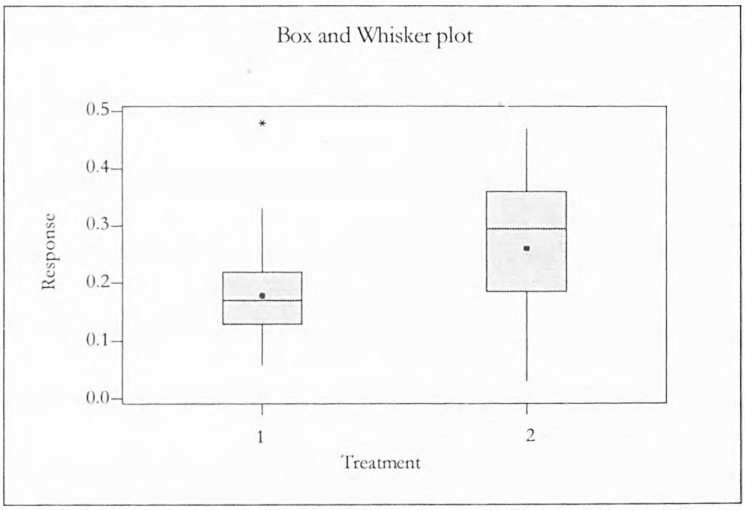


Figure 2.12 Generalised box and whisker plot
Box and whisker plots were used to show the distribution of the data.

3 UK FARMED FISH: HARVESTING TECHNIQUES

3.1 Introduction

An investigation into how harvesting methods effect quality and welfare of farmed fish requires information about the harvesting techniques used on farms. This information is not widely available due to the sensitive nature of the subject. It was therefore necessary to approach the industry to determine current practices.

3.2 Materials and methods

The British Trout Association was contacted but they declined to discuss the harvesting techniques used by their members on grounds of confidentiality. The Scottish Salmon Growers Association (SSGA) provided a report commissioned in 1995 entitled 'The Humane Slaughter of Atlantic Salmon' [9]. No pre-slaughter and only two slaughter procedures were mentioned in this report: more detailed material was required.

As a broad picture of farm practices was necessary and surveys and interviews were impractical, a questionnaire was used to cross-examine the industry. A distribution list of 250 UK fish farms was compiled from the internet, yellow pages and information received from Trouw Aquaculture. A questionnaire was designed and administered *via* the postal service in September and October 1999, Figure 3.1A-B. Confidentiality was assured by anonymity of reply. Additionally, data from the two highest production categories, which were potentially identifiable, were combined due to a low response from these farms.

Please cross all boxes that apply.

1. Is your farm predominantly Table ☐ Restocking ☐ Hatchery\fingerling ☐
   Freshwater ☐ Marine ☐?

2. Which species do you grow?
   Rainbow trout ☐ Brown trout ☐ Atlantic salmon ☐ Other ☐(Please specify).....

   If you grow more than one species which is the main one? .....

3. How many tonnes of your main species do you produce a year?
   <50 ☐ 51-150 ☐ 151-250 ☐ 251+ ☐

4. Do you starve your fish before harvesting? Yes ☐ No ☐

   If yes for how many days? .....

5. Which killing methods do you use?

	Portion sized fish	Larger fish	Runts & surplusfish
Blow to the head	☐	☐	☐
Spike into the brain	☐	☐	☐
Electrocution	☐	☐	☐
Left to die in the air	☐	☐	☐
Left to die on ice	☐	☐	☐
Left to die in ice slurry	☐	☐	☐
Die in ice slurry containing salt	☐	☐	☐
Die in carbon dioxide saturated water	☐	☐	☐
Die in buffered CO_2 saturated water	☐	☐	☐
Anaesthetic	☐	☐	☐
(Please specify).....			
Other	☐	☐	☐
(Please specify).....			

Figure 3.1A Questionnaire page 1

6. When you crowd your fish to facilitate netting/pumping during harvesting what is the maximum time they are left crowded before they are killed?

.....

7a. If you kill your fish individually (e.g. blow to the head) how many can you kill per hour? .....

How many people are in the killing team? .....

7b. If you kill your fish by a batch method (e.g. ice slurry) what is the maximum number & weight killed at a time? .....

What is the time taken from starting to fill the bin with live fish to the removal of the last dead fish in that batch? .....

How many people are in the killing team? .....

8. Are your fish processed on site?

No □ boxed and iced □ processed further □

If so, how long after death are they put on ice? .....

how long after death are they processed? .....

9. How do you rate the carcass quality of your fish?

excellent □ good □ average □ below average □ poor □

10. Do you have a specific problem with the carcass quality of your fish?

Yes □ No □

(Specify only if you wish to).....

.....

If yes in your opinion is it influenced by your harvesting procedure?

Yes □ No □

Please use the back of this questionnaire for any additional information you wish to provide and return it to:

Hazel Byrne, Food Science and Technology Research Centre,
The Robert Gordon University,
St Andrews Street, Aberdeen,
Scotland. AB25 1HG.
Tel: 01224 262866 Fax: 01224 262828 e-mail: h.byrne@rgu.ac.uk

Thank you.

Figure 3.1B Questionnaire page 2

3.3 Results

One hundred and eleven questionnaires were returned (44.4%), 37 (14.8%) were unanswered, 12 (4.8%) came from fish farms that did not admit to killing fish and 62 (24.8%) provided information on fish harvesting methods.

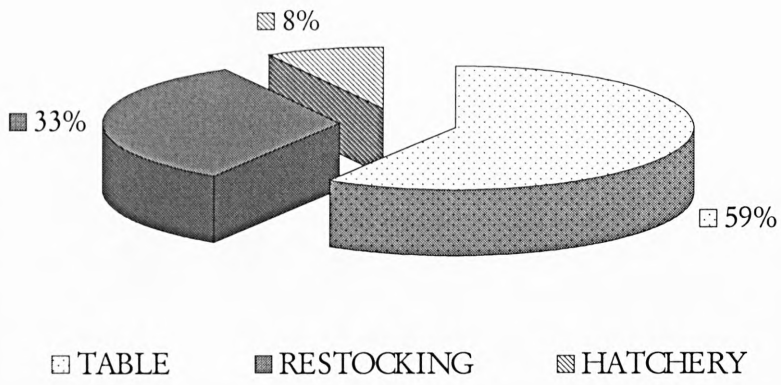
3.3.1 Business information

Harvesting techniques vary with species and quantity of fish to be killed, and therefore information about the type of business run, its annual production and the species cultivated was requested. The breakdown of farm type, size and species grown is shown in Figure 3.2A-C. Of the replies received 16 farms (26%) sold to multiple market types, and 46 (59%) grew at least part of their stock for the table. More than half of the replies (55%, 34) came from small farms producing less than 50 tonnes per year and just over a quarter (26%, 16) came from medium sized farms. Rainbow trout farms provided 69% (59) of the replies, brown trout 21% (18), and Atlantic salmon were grown on only 4% (3) farms. A proportion of the farms grew more than one species 37% (23).

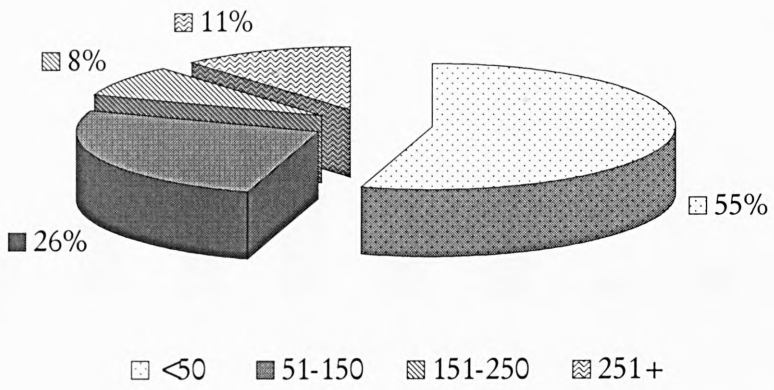
3.3.2 Pre-harvest husbandry techniques

Questions were asked about pre-harvest husbandry as the way fish are treated before harvest can have profound effects on their welfare and on end-product quality. The questionnaires returned showed that the majority of fish sold for restocking are starved for 10-20 degree-days, Figure 3.3A. Fish starved for the table market are fasted for a slightly longer time, 20-30 degree-days, Figure 3.3B. Only one farm starved fish for over a week. Most fish are crowded for up to 30 minutes to facilitate handling at harvest, Figure 3.3C. Figure 3.3A describes farms that sell only live fish, Figure 3.3B farms that sell at least part of their stock for the table; both assume a water temperature of 10°C.

A: Business type, n=78



B: Farm annual production in tonnes, n=62



C: Species cultivated, n=85

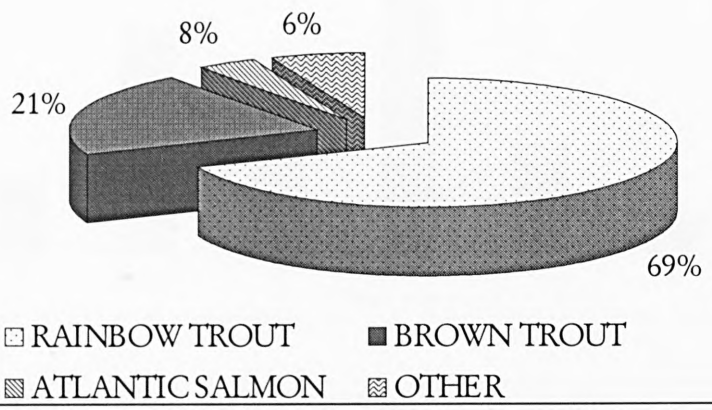
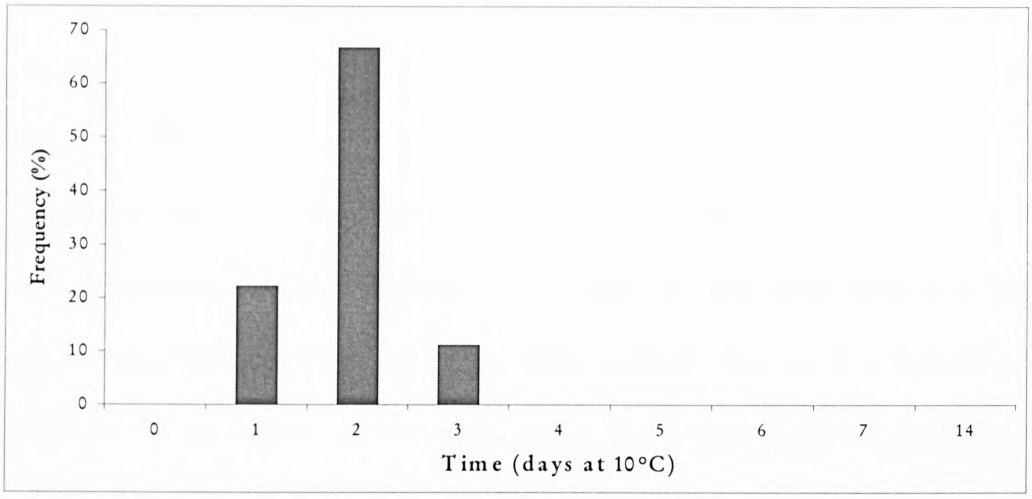
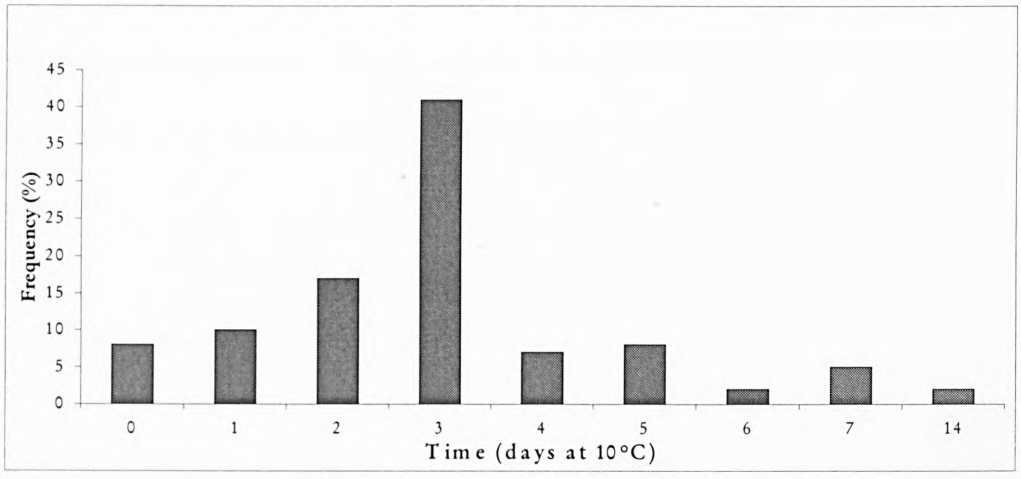


Figure 3.2A-C Business information
Breakdown of business information received from the questionnaire.

A: Starvation before sale for restocking, n=9



B: Starvation before sale to the table market, n=59



C: Maximum crowding time prior to harvest, n=51

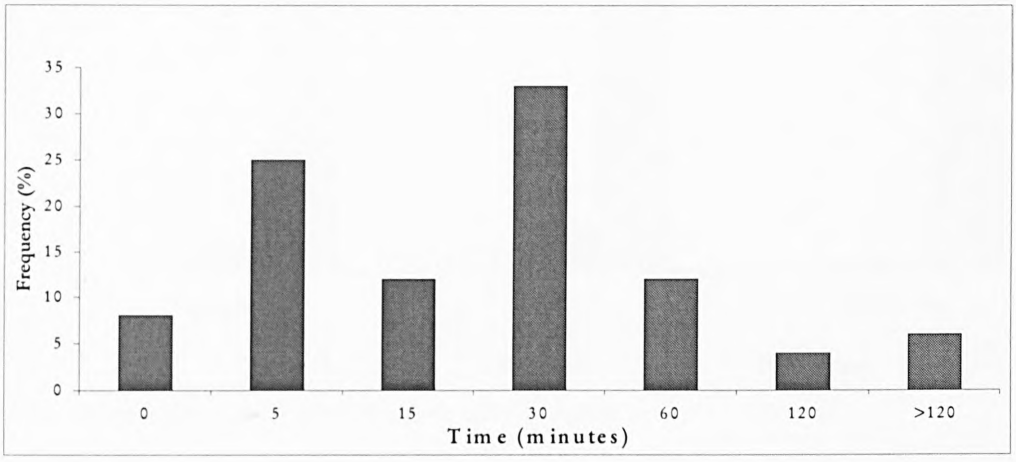


Figure 3.3A-C Pre-harvest husbandry techniques used on UK fish farms
Use of crowding and starving before fish harvesting.

3.3.3 Stunning and killing methods

Questions were asked about the stunning and killing methods used on farms although, to avoid confusion, the only terminology used was killing. Answers from farms producing 151-250 tonnes and 251+ tonnes per annum were combined. The majority of farms (90%) did not bleed their fish, Figure 3.4. When exsanguination was used, gill cut (7%) was applied preferentially to gill pull (3%). Larger fish were more likely to be bled than portion sized or surplus fish. The main killing methods were blow to the head (41%) or death in air (24%), although electrocution and ice slurry were widely used (14% each), Figure 3.5. The most common technique of killing fish independent of size was blow to the head. However when large numbers of portion sized fish were killed death in ice slurry was the predominant method, Figure 3.6A-C. Fish that were surplus to requirements were not killed in ice or ice slurry. Large fish were slightly less likely to be killed by electrocution.

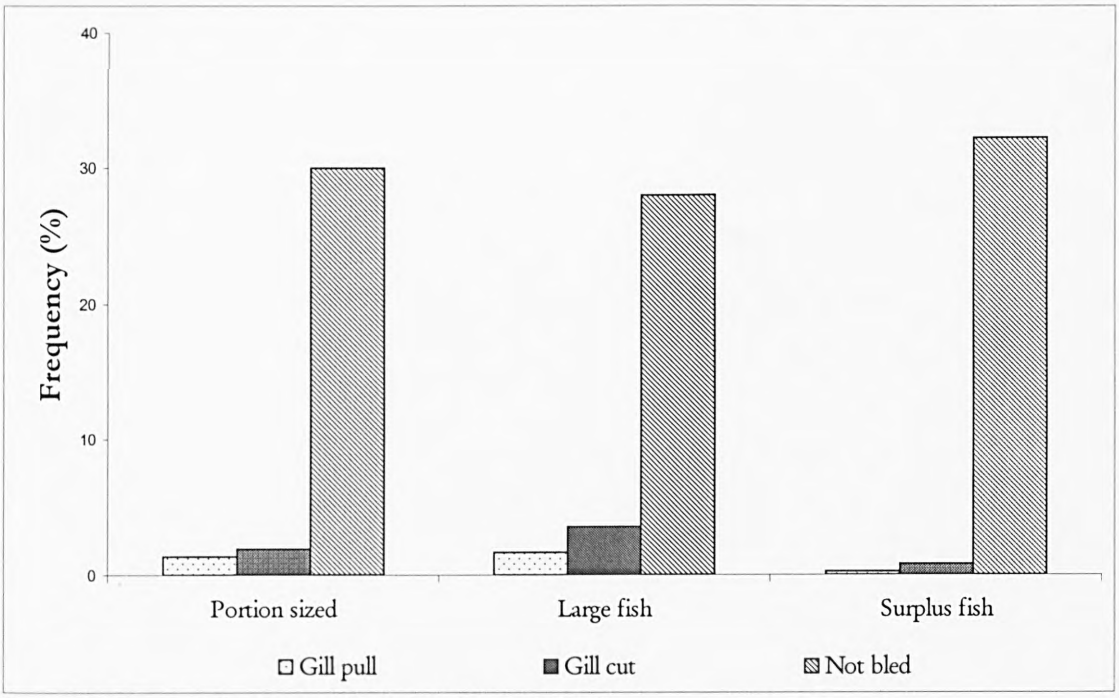


Figure 3.4 Exsanguination methods used on UK fish farms
n=360

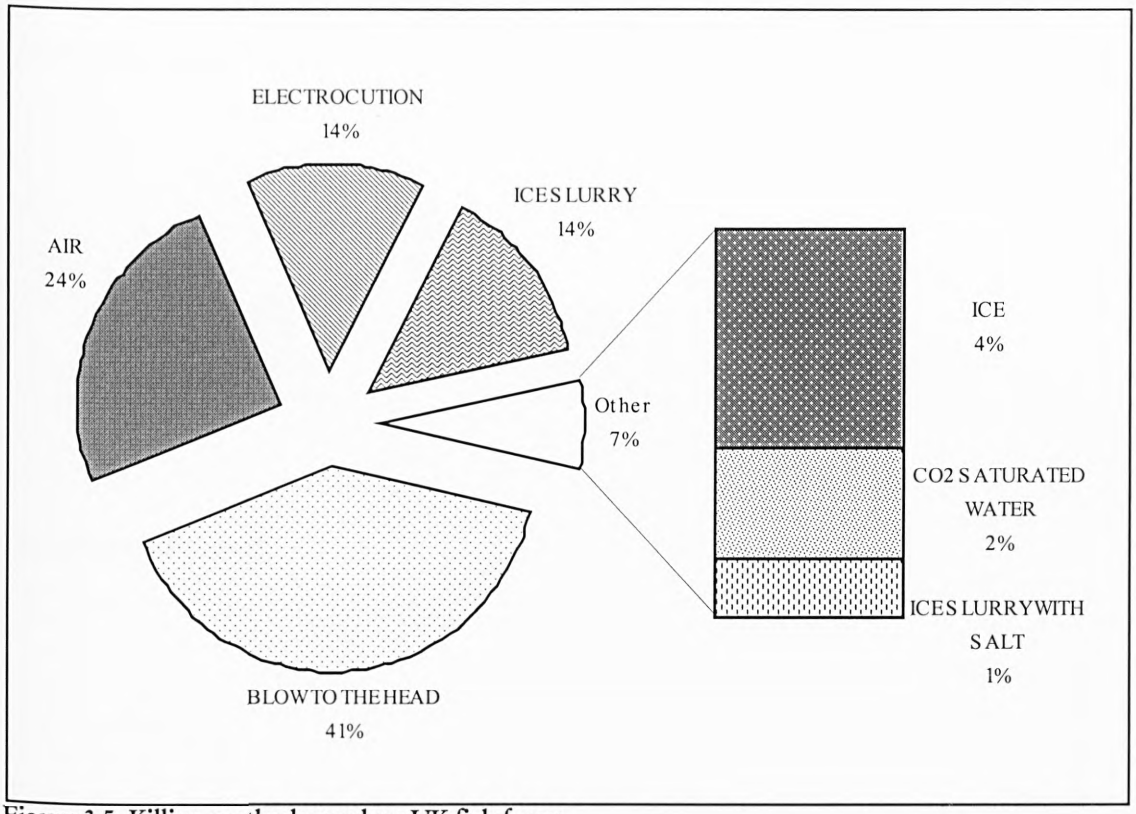


Figure 3.5 Killing methods used on UK fish farms
n=99

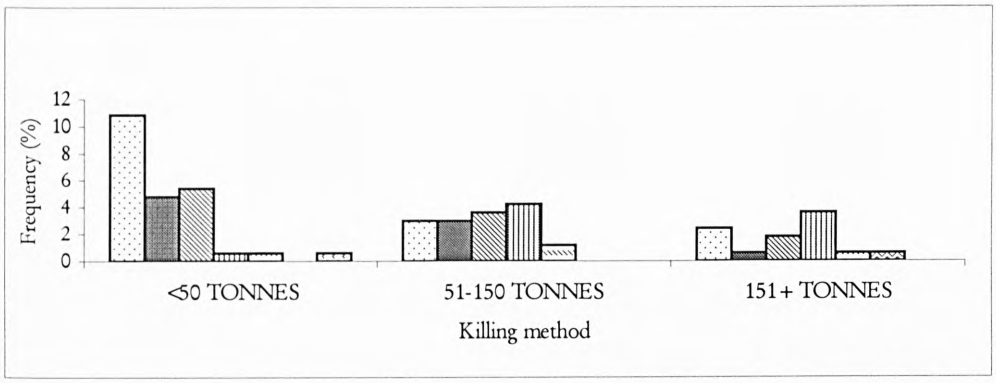
3.3.4 Harvesting rate

The maximum number of fish killed per man-hour where the fish were killed individually, e.g. blow to the head, was 750. The greatest amount of fish killed at one time using a batch method, e.g. electrocution, was six tonnes.

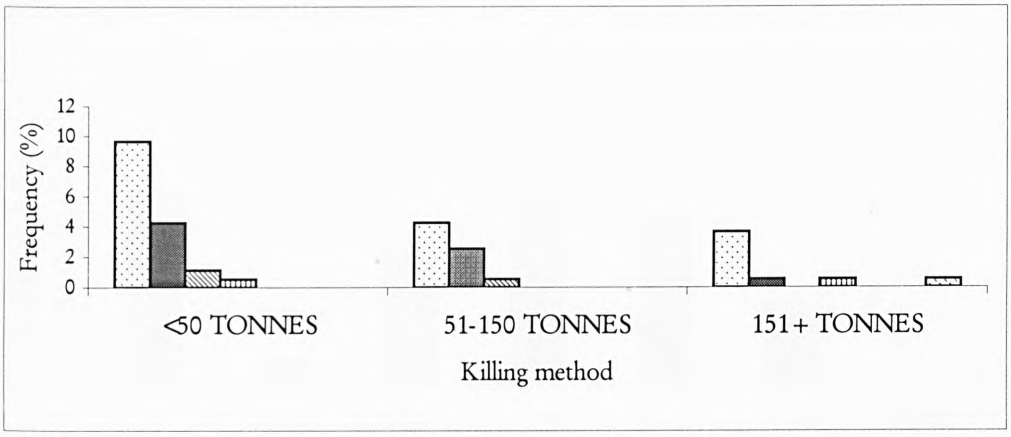
3.3.5 Fish processing

There were no major differences between the size of farms that processed, partially processed or did not attempt to process their fish on site, Figure 3.7A. Thirty seven percent of farms sold their fish unprocessed (27), 29% (21) boxed and iced their fish while 34% (25) processed their fish further. The time that elapsed before icing and or processing was highly variable, Figures 3.7B-C. There are two peaks in the data for the time elapsed before icing and processing. The first immediately after death and the second after 16-30 minutes, the majority of fish were dealt with within two hours of harvest. The longest time a batch of fish was left to die before processing or sale was 3.5 hours.

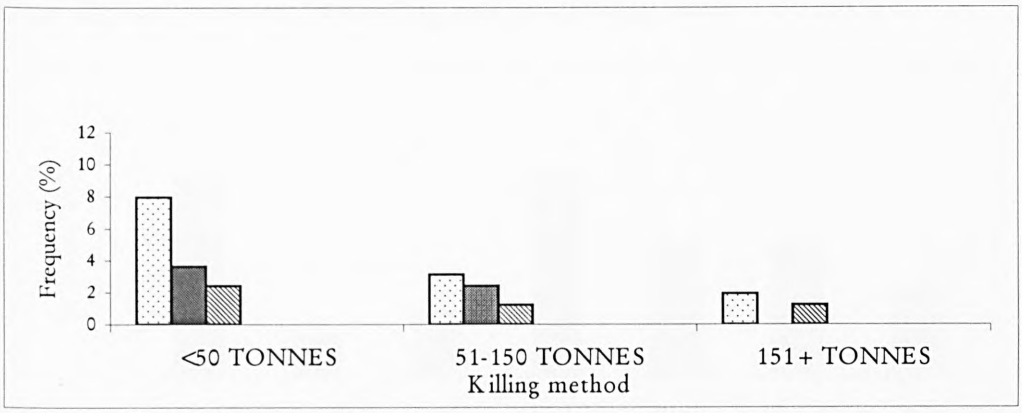
A: Portion sized fish, n=79



B: Large fish, n=47



C: Surplus fish, n=40



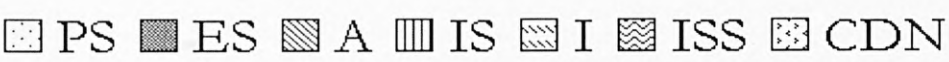
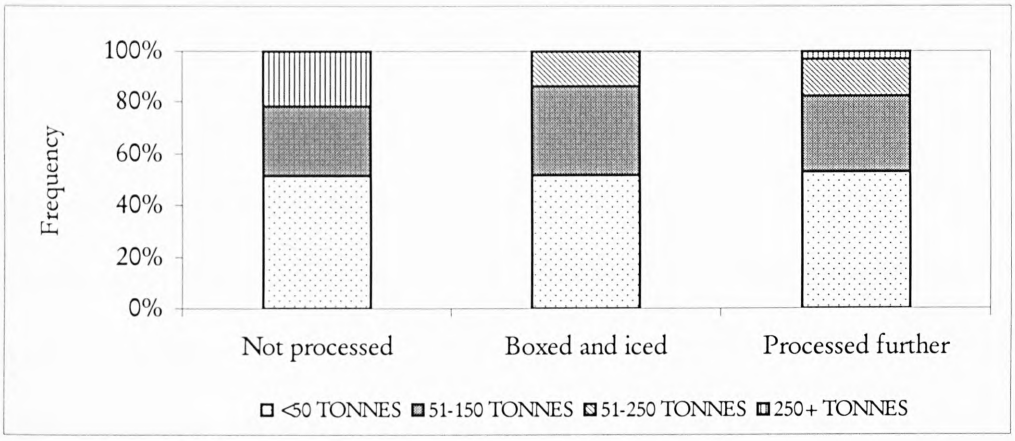
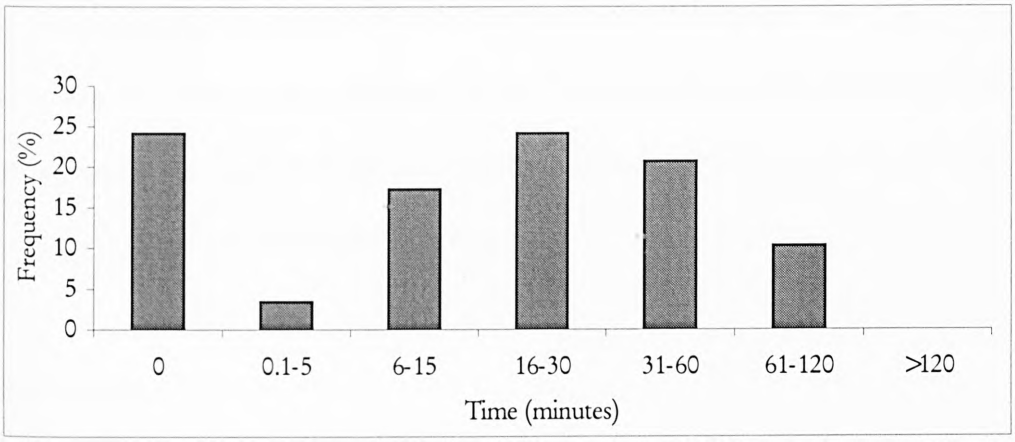


Figure 3.6A-C Breakdown of killing methods by fish size and farm production capacity
PS=blow to the head, ES=electrocution, A=air, IS=ice slurry, I=ice, ISS=ice slurry with salt, CDN=CO_2 saturated water

A: The proportion of farms processing fish on site, n=73



B: Time elapsed before icing, n=29



C: Time elapsed between harvesting and processing, n=39.

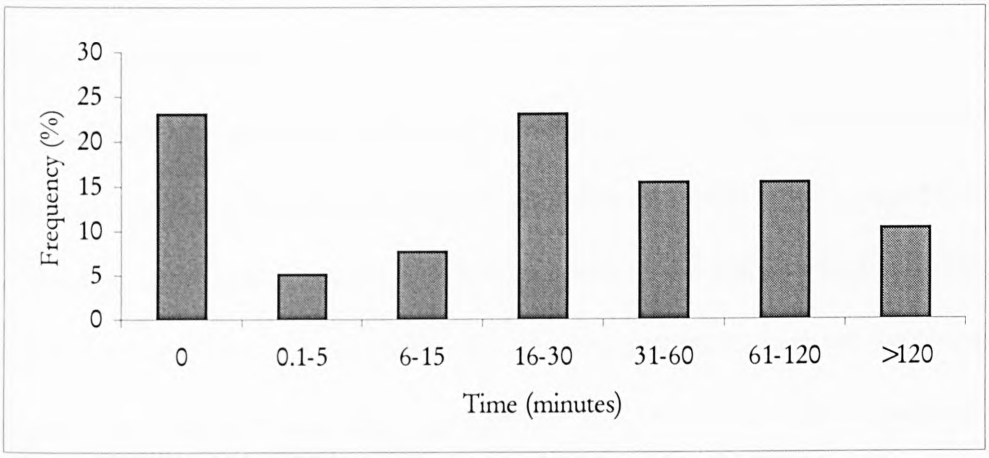


Figure 3.7A-C Processing ability of UK fish farms

3.3.6 Product quality

The fish producers that responded to the questionnaire were confident of the high quality of their fish. Only two responses were in the average category; the others (57, 96.6%) were in good or excellent. Only 13 farms (21%) offered an opinion as to whether harvesting procedures could effect product quality, and eight (61.5%) felt, that to some extent, it did. Only seven farms (11.3%) indicated the quality of their fish could be improved. Those farms that specified an occasional quality problem mentioned soft flesh.

3.3.7 Additional salmon information

The report commissioned by the SSGA recommended slaughtering salmon using a gill cut after stunning with either a blow to the head or immersion in carbon dioxide saturated water [9]. Interviews conducted on five Scottish Atlantic salmon farms confirmed this and provided the additional information that fish are starved for at least one week before harvest (Strachan unpublished data).

3.4 Discussion

Harvesting practices currently used on UK fish farms were ascertained to ensure the proposed work was industrially relevant.

3.4.1 Business information

Information on farm type and species cultivated was needed to determine the significance of the material gathered; the principal business of the farms contacted was not always distinguishable from their name. Developments in the UK fish farming industry have led to diversification from single species single market farms to those supplying multiple market types with more than one species. Some farms have also expanded their business to include hatchery operations to ensure continuity of supply. Of the questionnaires returned, 16 (26%) of farms produced fish for more than one market type and 23 (37%) grew multiple species. The majority of the replies were from rainbow trout farms selling to the table market. Annual production figures were requested because the

number and size of the fish to be harvested effects the harvesting methods used. Small farms killing only a few fish can afford the time to treat the fish individually, large farms killing thousands of small fish at a time cannot. Smaller farms responded more readily to the questionnaire, over half of the replies (55%, 34) came from farms that produce <50 tonnes per year. This may be a reflection of the composition of the industry. The majority of UK rainbow trout farms are small, and few companies produce more than 200 tonnes per year [65]. Whilst the majority of Scottish salmon farming companies produce over 200 tonnes per annum [226]. The predominance of replies from small farms may also have occurred, as the people dealing with the stock would have received the questionnaires. They would have been knowledgeable about the harvesting procedures used on the farm and may have had more time to complete the questionnaire. The business managers on the larger farms would have had less time to fill in and return the form. The low response 11% (7) from farms growing >250 tonnes per year reflects the low number of replies from salmon growers 4% (3).

The respondents to the questionnaire were predominantly small rainbow trout farms that supplied all or at least part of their stock for human consumption.

3.4.2 Pre-harvest husbandry techniques

Pre-harvest treatment may affect the quality of farmed fish. Starving effects the rate of autolytic spoilage, microbiological safety and the fishes energy reserves [207]. Crowding prior to slaughter may effect the fishes energy stores and osmotic balance [216].

The most frequent starvation period was 30degree-days (41%, 25). Only 2% of farms, (1) starved fish for longer than a week. This reflects the low returns from salmon farms; salmon are usually starved for longer than trout. Crowding times were similarly short indicating that the respondents were not harvesting vast quantities of fish at any one time. Less than a quarter of the fish producers crowded their fish for more than 30 minutes.

The responses to the pre-harvest husbandry questions reflect the material gathered on the business information giving the answers a degree of credibility.

3.4.3 Stunning and killing methods

Stunning and killing methods have the potential to affect product quality. Blow to the head was the most frequently used method (41%) followed by death in air (24%). Death on ice slurry and electrocution were also widely employed (14% each). The low proportion of farms using carbon-dioxide narcosis (2%) reflects the limited use of this method with rainbow trout and the low number of replies from salmon farms. The killing methods used on portion sized fish reflect 'trout only' methods as salmon are not sold at this size. The killing methods used by small farms on portion fish were predominantly blow to the head, whereas larger farms used ice slurry or air. Large fish and those surplus to requirements were killed most often by blow to the head independent of farm size.

Exsanguination, which can be used as a slaughter method, influences visual appeal to the consumer and storage qualities [170, 241]. The majority of fish were not bled; this again reflects the low response from salmon farms as salmon are often slaughtered. A gill cut was used in preference to a gill pull when fish were exsanguinated, as this is theoretically a more efficient method. Large fish were bled to a greater extent than portion fish, which is consistent with the fact that large fish used for the production of cold smoked sides are traditionally slaughtered.

The killing method results could not be analysed by species because the questionnaire design did not cater for farms growing multiple species.

There were signs that some respondents produced acquiescent responses i.e. they answered all the questions in the same way. For instance it is unlikely that excess fish or runts on small trout farms have their gills slit, as this would be additional work for no benefit.

3.4.4 Harvesting rate

Carcass quality can be affected by the harvesting rate and the number of fish killed per session. Physical damage can occur due to the close proximity of fish to each other and autolytic changes occur in fish that have died while subsequent fish are harvested. The question concerning harvest rate was poorly written. There was evidence that it was misread or misinterpreted; 750 fish killed individually per man-hour means killing a fish every 4.8 seconds. This may just be an artefact of the question which asked for numbers per hour, if only one fish was killed 4.8 seconds may be sufficient for the killing action although not the entire procedure. A maximum harvesting rate of six tonnes per man-hour was reported for batch killing. It can be easily imagined how physical damage can occur to fish when they are harvested in these quantities and at such speeds.

3.4.5 Fish processing

The principal factors that effect product quality are; the time that elapses between death and processing and storage conditions i.e. time and temperature [51]. Processing fish on site therefore has the potential to improve the quality of farmed fish. There were no particular patterns between the farms that did or did not process fish on site. The maximum time fish were left before icing and/ or processing was 3.5 hours. This extended time, particularly if un-chilled and in the summer, may have a more significant effect on carcass quality than any husbandry technique used at harvest.

3.4.6 Product quality

Perceived product quality could not be correlated with time elapsed before processing as 96.6%, (57/59) of the replies said their fish were either excellent or good. The majority of replies did not answer the question on whether harvesting procedures could effect fish quality. Of those farms that answered 61.5% (8) felt that it did to a certain extent. Only seven farms (11.3 %) indicated they had quality problems or that the quality of their fish could be improved, those that specified a problem mentioned soft texture.

3.4.7 General discussion

The replies to the questionnaire appear to have produced some coherent information, although acquiescent responses were in evidence in the answers to some questions. An attempt to eliminate evaluation apprehension, wanting answers to appear acceptable, was made by ensuring that the questionnaires remained anonymous. The honesty of some answers indicating that fish are often just left to die and that they can be crowded for more than two hours show that, to a certain extent, this succeeded. The low response rate to the question regarding the effects of harvesting on product quality indicates that demand characteristics were low i.e. the majority of the respondents were not responding to the intention of the questionnaire. However, whether the answers that were received to that question were the true opinion of farmers is uncertain.

3.5 Summary

The results of the questionnaire were slanted towards husbandry techniques used on small rainbow trout farms. Information on harvesting Atlantic salmon gathered from sources other than the questionnaire, indicates that the principal methods of killing salmon were slaughter by gill cut after stunning with either a blow to the head or carbon dioxide. In the UK salmon are routinely starved for at least one week. The questionnaire data suggested that the starvation period for rainbow trout sold to the table was between 20- and 30-degree days. Rainbow trout were not routinely crowded for more than 30minutes prior to harvest. The major killing methods used on rainbow trout farms were blow to the head, electrocution, death in air and ice slurry.

4 THE EFFECTS OF STUNNING AND KILLING TECHNIQUE ON THE WELFARE AND QUALITY OF FARMED RAINBOW TROUT

4.1 Introduction

Physiological stress and physical damage caused by stunning and killing techniques have the potential to affect the end-product quality of farmed fish, as outlined in Sections 1.3 and 1.4. It is not known which of the methods currently used by the UK trout farming industry has the greatest influence on stress physiology and carcass quality. This series of field studies investigated how the most commonly used stunning and killing methods influence the welfare and quality of farmed rainbow trout.

4.2 Materials and methods

4.2.1 Facilities

Field based experiments were performed on site at Mill of Elrick Fish Farm, Auchnagatt, Aberdeenshire, UK. The holding facility was a 2m-diameter fibreglass tank filled to a depth of 0.76m with river water flowing at 60l/min, Appendix 2, Figure 11. The water, as measured on sampling days was; temperature 10.1±2.2°C, dissolved oxygen 96±8% and pH 7.26±0.18, (dissolved oxygen meter model 9071, Jenway Ltd., Dunmow, Essex, UK). The air temperature was 13.3±4.4°C. Day length increased during the course of the study from 14h 7min with sunrise at 5.07am, to 17h 49min with sunrise at 3.19am.

4.2.2 Animals

Rainbow trout were obtained from a disease free farm. They were immature, all female, diploid fish. The eggs, which hatched over the same period, were sourced from Aquazure Hatcheries (South Africa). The fish were portion sized: 30.7±1.5cm; 391±55g; and had a Fulton's condition factor of 1.35±0.16.

4.2.3 Design

To reduce environmental and population variables the field studies were run consecutively over 12 weeks between April and July 2000. Base line data were obtained for comparison with the commercial methods by sampling minimally stressed fish. This 'control group' was anaesthetised *in situ* to prevent startle responses caused by the presence of people and netting actions. The use of anaesthetic in the tank precluded evaluating all methods simultaneously. The control samples were therefore collected on the first and last day of the field studies so that the data spanned the experimental period. Other experiments each tested all four of the industrial killing/ stunning methods chosen. A randomised block design was used on each occasion to negate effects of serial sampling, time, skill and environmental variables. A sample size of 20 was chosen due to logistical and financial restraints. In order to collect samples within minutes of death, three sets of fish were sampled for each killing/ stunning method, for blood samples (blood set), for pre *rigor mortis* muscle samples (muscle set), and *rigor mortis* and quality measurements (*rigor mortis* set). The muscle and blood sets of fish in each group were sampled concurrently and at random unless stated otherwise. The *rigor mortis* set were killed *en masse* at the end of each sampling session. If the sample size of the *rigor mortis* set of fish was not achieved due to 'missing fish', numbers were increased in the following session.

4.2.4 Conditions

The trout were stocked into the tank two weeks prior to sampling. They were fed a commercial diet (Crystale red 50, BOCM Pauls, Renfrew, Scotland, UK) for ten days and then starved for three days before sample collection. Sampling took place between 9.30hrs and 16.00hrs on the 14th day. The weather was variable during the sampling days and over the experimental period.

4.2.5 Killing and stunning methods

Four killing/ stunning methods commonly used on UK rainbow trout farms were selected, see Section 3.3.3. They were; death in ice slurry, death in air, blow to the head

(percussive stun) and 'electrocution' (electrical stun). Additionally fish were anaesthetised to act as a control. Ten fish were sampled for each set of fish in both of the control experiments and five in each of the four 'methods' experiments.

4.2.5.1 Control fish

The control fish were anaesthetised with benzocaine then killed by blow to the head prior to sampling. Benzocaine was chosen because the fish were left in the anaesthetic for an extended period, (up to six hours), and it was essential that they did not go into medullary collapse over the experimental period. Benzocaine (60g) was dissolved in 440ml of ethanol and diluted with 30l of river water. This solution was siphoned into the tank over a 20min period resulting in a final concentration of about 25ppm. During the procedure, the water supply to the tank was cut off. Oxygen levels were maintained by aeration supplied by an oil free compressor, (model FX95 205, FIAC, Marconi, Bologna, Italy), through a 'Leaky Pipe' diffuser, (model 19FFA, length 0.7 m, NCC supplies Ltd., Middlewich, Cheshire, UK). To minimise potential stress effects, the diffuser was placed in the tank two days prior to sampling. During sample collection, approximately every half-hour, a fish was revived to check that the fish in the tank were still alive. All fish were able to recover within 3 min. Once revived they were killed by percussion then discarded.

4.2.5.2 Death in ice slurry

The fish that died in ice slurry were processed together. They were netted into a bin which initially contained an ice: water mixture (3:1), and at temperature 0.9±0.1°C. The fish were left in the ice slurry for 10min, after which the water was drained and the fish were left in the ice for a further 60min. This protocol was followed, as it is similar to the industrial procedure (Steve Kestin, Bristol University, personal communication).

4.2.5.3 Death in air

The fish killed in air were netted individually into a 50l plastic bin and left for 15min to die.

4.2.5.4 Percussive stun

The percussive stun group were manually restrained and given at least two blows in quick succession on the dorsal surface of the skull, directly above the brain cavity.

4.2.5.5 Electrical stun

A commercial fish 'killer' (Wharton Controls, Warminster, Wiltshire, UK) was used to electrically stun the final group. They were netted into the water filled chamber, tank dimensions 1x0.5x0.3m, water depth 0.2m, with plate electrodes at either end. Electricity was applied for 6s at 24volts DC with full wave rectification, amperes varied with water conductivity and with the number of fish processed.

4.2.6 Analysis

4.2.6.1 Plasma biochemistry and related parameters

Haematocrit was measured within nine hours of sample collection by the method outlined in Section 2.3.2.1a. Plasma was stored at -80°C and analysed within 26 months of collection, as described in Section 2.3.3. Spleens were dissected from the fish four days *post mortem*, and the SSI calculated as detailed in Section 2.4.5.1.

4.2.6.2 Muscle biochemistry

4.2.6.2a Pre rigor mortis

Pre *rigor mortis* muscle samples were prepared and analysed as described in Sections 2.3.2.2b and 2.3.3.1. Creatine phosphate (CP), creatine (Cr), creatinine and ATP were analysed within ten days of collection and six hours of extraction. Lactate was analysed within six months from the same extract that had been stored at -80°C.

4.2.6.2b Post rigor mortis

Lactate and K value of post *rigor mortis* muscle were determined within 15 months of sample collection, from tissue taken four days *post mortem* using the techniques outlined in Sections 2.3.3.1 and 2.3.3.4c.

4.2.6.3 Muscle quality and related parameters

Rigor mortis development was measured over 72 hours as described in Section 2.4.2. Torrymeter readings were taken daily for four days on each fish, as outlined in Section 2.4.3.1. A downgrading assessment was performed on the *rigor mortis* set of fish after four (external assessments) and five (internal assessments) days ice storage, as detailed in Section 2.4.5.3. Colour measurements and compression tests were performed on fillets taken from the *rigor mortis* set of fish five days *post mortem* as described in Sections 2.4.6 and 2.4.7.1. The Warner–Bratzler 'shear' tests were performed on tissue taken from the blood set of fish after six days ice storage as described in Section 2.4.7.2.

4.2.7 Additional information

Sunrise and day length data were obtained from the U.S. Naval Observatory web site at http://aa.usno.navy.mil/data/docs/RS_OneDay.html. The longitude and latitude co-ordinates required to generate the data were obtained by using the postcodes of the fish farms sites at the web site http://www.streetmap.co.uk.

4.3 Results

4.3.1 Plasma biochemistry and related parameters

The extent of the primary stress response elicited by the killing/ stunning techniques was determined by analysing plasma for catecholamines and cortisol. Plasma glucose was measured as a secondary stress response indicator, as was haematocrit. Plasma protein and SSI were measured to relate to haematocrit. Anaerobic activity during death was evaluated from plasma pH and lactate measurements.

4.3.1.1 Plasma hormones

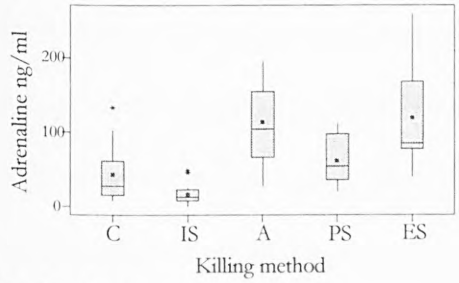
The effects of killing/ stunning method on plasma hormone levels are summarised in Figure 4.1A-B.

4.3.1.1a Catecholamines

The electrically stunned and death in air fish had the highest amounts of adrenaline, 277% and 264% of the control value. These levels were significantly higher than those of the other methods but did not differ significantly from each other. The adrenaline levels in the percussively stunned fish were not significantly higher than the controls although having 145% of their value. The fish that died in ice slurry had only 39% of the control adrenaline levels and were significantly lower than all the other groups, Figure 4.1A.

No significant differences were detected between the killing/ stunning methods in plasma noradrenaline levels, F=1.794, P=0.137, df=4, 92, power~0.41, n=20. The analysis measured; 9.05±5.61ng/ml of noradrenaline in the control group, 12.25±6.54ng/ml in the ice slurry fish, 15.42±10.52ng/ml in the death in air group, 16.10±15.60ng/ml in the percussively stunned fish, while the electrically stunned fish contained 12.00±4.65ng/ml.

A: Adrenaline

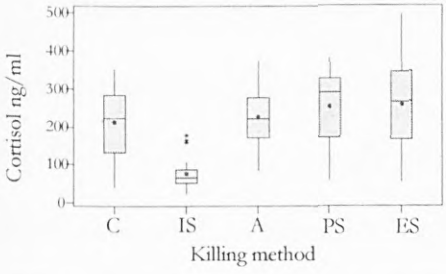


Inferential statistics
F=19.97, P=0.000, df=4, 94, power~0.99, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		*#	***#	ns#	**#
ice slurry	*$		***#	***#	***#
air	***$	***$		**#	ns#
percussive stun	ns$	***$	**$		*#
electrical stun	**$	***$	ns$	*$	

B: Cortisol



Inferential statistics
F=13.23, P=0.000, df=4, 95, power~0.99, n=20.

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		*#	ns#	ns#	ns#
ice slurry	***$		***#	***#	***#
air	ns$	***$		ns#	ns#
percussive stun	ns$	***$	ns$		ns#
electrical stun	ns$	***$	ns$	ns$	

Figure 4.1A-B Effect of 'killing' method on trout plasma stress hormones
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests, Tamhane$ (between groups, unequal variance, conservative), Games Howell# (between groups, unequal variance, liberal). Samples measured in duplicate, n=19-20. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

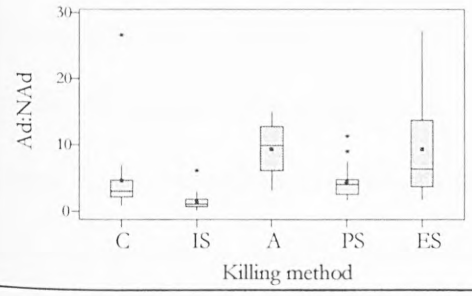
4.3.1.1b Cortisol

The cortisol concentrations measured in the trout plasma were found to differ between killing/ stunning methods, Figure 4.1B. There was significantly less cortisol in the ice slurry group compared with the other methods; no other differences were observed. Expressed as a percentage of the control values the cortisol levels were, 36%, 107%, 120%, and 122%, in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively.

In rainbow trout subjected to stress, significant amounts of catecholamines are released [176]. They are quantifiable in the plasma within seconds of stressor perception while cortisol levels take a few minutes to be synthesised and released in sufficient quantities to be detected [100]. The data therefore suggest that the electrically stunned and death in air fish had the highest stress levels while the percussively stunned fish were only slightly more stressed than the controls. The electrical stunning process might however have stimulated the adrenaline release and not 'stress' *per se*. The ice slurry fish showed little evidence of being stressed. However, as the half-life of adrenaline in the plasma is short i.e. a few minutes [100], these fish might initially have shown a response but were unable to sustain it due to their low body temperature. Body temperatures of the control, ice slurry, death in air, percussively and electrically stunned fish were; 9.6±1.9°C, 4.7±2.0°C, 13.2±2.4°C, 11.8±1.5°C and 12.1±1.8°C, respectively.

The level of noradrenaline in rainbow trout is generally thought to be lower than that of adrenaline, and its incremental increase in response to stress is similar to that of adrenaline [100]. Differences in the noradrenaline response might therefore have been too small to distinguish because of the high variability in the data, 14±11ng/ml, range 0-58ng/ml. The adrenaline: noradrenaline ratio of the fish shows the ice slurry fish had a significantly lower ratio compared with all the other groups, and the death in air fish had a significantly higher ratio compared with the control and percussively stunned fish, Figure 4.2. This reflects the very low levels of adrenaline in the ice slurry fish, and the high

adrenaline response with subordinate noradrenaline response in the death in air fish. The adrenaline: noradrenaline ratio expressed as a percentage of the control value were; 32%, 202%, 94%, and 201% in the ice slurry, death in air, percussively stunned and electrically stunned groups, respectively.



Inferential statistics
H=49.03, P=0.000, df=4, n=17-19

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	*§				
air	**§	***§			
percussive stun	ns§	**§	*§		
electrical stun	ns§	***§	ns§	ns§	

Figure 4.2 Effect of 'killing' method on adrenaline: noradrenaline ratio
Data presented as a box and whisker plot with significance levels of Kruskal Wallis *post hoc* test Dunn§ (between groups). Ratios calculated on an individual fish basis, outliers removed following a Grubbs test. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

With serial sampling, cortisol levels are expected to increase with time [82]. The design of this study was such as to negate the effects of serial sampling and environmental effects. The first and last cortisol measurements on each sampling session were 173±97ng/ml and 190±93ng/ml and were not found to differ significantly, t=0.54, df=34, P=0.59, n=18. This suggests that neither the increasing day length and earlier sunrise over the experimental period or the serial sampling regime, had a predominant effect on the fish. It took <30 degree-minutes for the control, percussively and electrically stunned fish to die, while for the ice slurry and death in air fish samples were collected after >330 and ~200 degree-minutes, respectively. The higher cortisol levels occurred in the fish that took the shortest time to die, where a cortisol response would have had insufficient time to build up to high levels. This might indicate that the lower than expected levels in the death in ice slurry and air fish were due to degradation/ clearance of the cortisol molecule and combined with cessation of synthesis rather than low levels of stress in these fish. The low levels in the ice slurry fish on the other hand might be due to a diminished response caused by the low body temperature.

4.3.1.2 Plasma chemistry

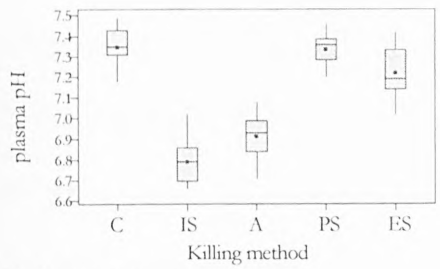
The effects of killing/ stunning method on plasma pH, lactate, glucose and protein are summarised in Figure 4.3A-D.

The control fish had the highest plasma pH at 7.35, Figure 4.3A. With the exception of the percussively stunned fish, the plasma pH values were significantly lower in the commercially stunned/ killed fish compared with the controls. Relative to control values the plasma pH readings were; 92%, 94%, 100%, 98% in the ice slurry, death in air, percussively and electrically stunned fish, respectively. There were significant differences between plasma pH in all the industrial methods, Figure 4.3A.

The plasma lactate levels mirrored the plasma pH values: the control fish had the lowest and the ice slurry fish the highest lactate concentrations. Compared with the controls, the plasma lactate levels were; 1193% in the ice slurry fish, 867% in the death in air fish, 152% in the percussively stunned fish and 320% in the electrically stunned fish. Similarly, there were significant differences between all the groups apart from the percussively stunned fish and the controls, Figure 4.3B.

The ice slurry fish had 124%, the death in air fish 95%, the percussively stunned fish 83%, and the electrically stunned fish 85%, of the plasma glucose levels detected in the control fish. The glucose level in the ice slurry group was significantly higher than that of all the other fish. There were no significant differences found between the other methods, Figure 4.3C.

A: pH

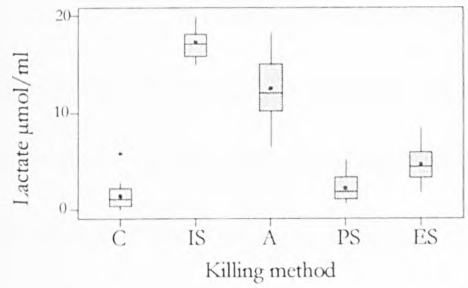


Inferential statistics
F=137.3, P=0.000, df=4, 93, power~1.00, n=20.

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***†	***†	ns†	***†
ice slurry	***‡				
air	***‡	**‡			
percussive stun	ns‡	***‡	***‡		
electrical stun	***‡	***‡	***‡	**‡	

B: Lactate

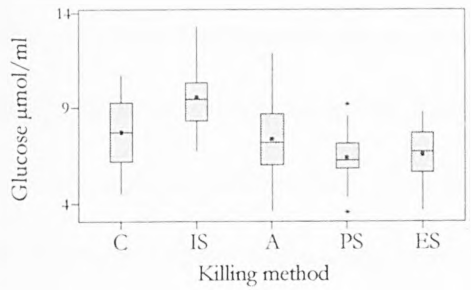


Inferential statistics
F=137.3, P=0.000, df=4, 94, power~1.00, n=20.

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	ns#	***#
ice slurry	***$		***#	***#	***#
air	***$	***$		***#	***#
percussive stun	ns$	***$	***$		***#
electrical stun	***$	***$	***$	***$	

C: Glucose

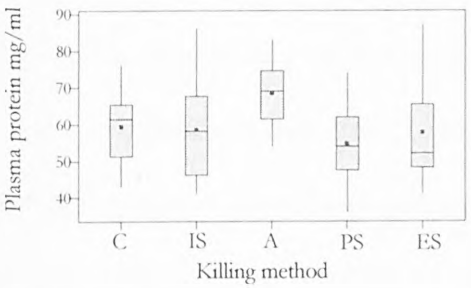


Inferential statistics
F=10.2, P=0.000, df=4, 94, power~0.98, n=20.

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		**†	ns†	ns†	ns†
ice slurry	*‡				
air	ns‡	**‡			
percussive stun	ns‡	***‡	ns‡		
electrical stun	ns‡	***‡	ns‡	ns‡	

D: Protein



Inferential statistics
F=3.92, P=0.006, df=4, 93, power~0.78, n=18-20.

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		ns†	*†	ns†	ns†
ice slurry	ns‡				
air	ns‡	ns‡			
percussive stun	ns‡	ns‡	**‡		
electrical stun	ns‡	ns‡	*‡	ns‡	

Figure 4.3A-D Effect of 'killing' method on trout plasma biochemistry
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests, Dunnett† (control vs. methods), Tukey ‡ (between groups, equal variance), Tamhane$ (between groups, unequal variance, conservative), Games Howell# (between groups, unequal variance, liberal). Samples measured in duplicate, n=19-20. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The plasma protein values were shown to vary between the killing/ stunning methods, Figure 4.3D. The death in air fish were found to have a significantly higher level of plasma protein compared with; the percussively and electrically stunned fish (Tukey test (between groups)), and the control fish (Dunnett test (control vs. methods)). The amount of plasma protein expressed as a percentage of the control value was; 98%, 115%, 92%, and 97%, in the ice slurry, death in air, percussively and electrically stunned fish, respectively.

Both the plasma lactate and pH values suggest that the control group sustained the least anaerobic activity of the fish studied, although plasma pH is subject to other influences e.g. the amount of carbon dioxide dissolved in the blood. The percussively stunned fish showed more evidence of activity than the controls but the difference failed to reach significance and could have been due to handling stress. The electrically stunned fish had slightly more plasma lactate than the percussively stunned fish, this might have been caused by electrical stimulation of the muscle, see Section 4.3.2.2a. Considerably more lactate had been produced in the death in air and ice slurry groups compared with the controls and the stunned fish. This might have reflected the time it took these fish to die, or increased activity while dying in attempting to maintain homeostasis. The ice slurry group showed the greatest amount of anaerobic activity, even though their body temperature was significantly lower than the other groups, $F=59.4$, $P=0.000$, $df=4, 95$, and the samples were taken after the longest time, >300 degree-minutes.

Adrenaline has the effect of lowering plasma glucose as it is used during the fight-or-flight response. The plasma adrenaline level in the ice slurry fish was exceedingly low; consequently, plasma glucose would be expected to be correspondingly high. Additionally, as glucose turnover does not increase during hypoxia in trout, unlike lactate [64], the ice slurry fish would have had decreased glucose utilisation due to their lower metabolic rate. Plasma glucose levels might also have risen due to leakage of glucose from liver cells. Glucose production although slower, would have continued at a faster rate than glycolysis

in the liver. Glucose would have been transported out of the cell down its concentration gradient, by the transporter which works independently of temperature and energy availability. Glycolysis would have been slower than gluconeogenesis as enzyme activities in the glycolytic chain are lower and feedback inhibition by build up of glucose-6-phosphate, (an early product of the glycolytic pathway), would have occurred. The plasma lactate levels were higher in the ice slurry fish as time had passed allowing levels to increase. When oxygen is unavailable, lactate is not utilised as a fuel resource and so levels would continue to rise [63, 64]. During hypoxia lactate production increases and it is therefore likely that there would have been increased movement of lactate from the muscle into the blood [244].

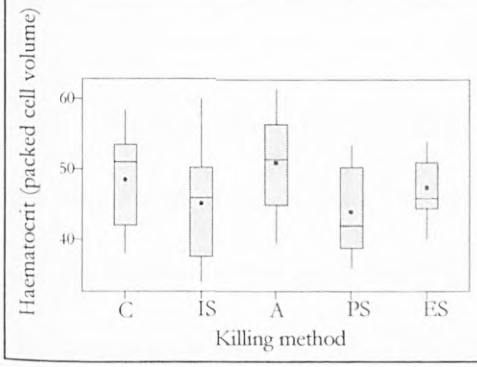
The plasma protein levels were seen to rise in the death in air fish relative to the control, percussively and electrically stunned fish. This suggests a reduction in plasma volume in the death in air fish, which would alter the haematocrit value, see Section 4.3.1.3. A possible cause might be the build up of muscle lactate, see Section 4.3.2.1.

4.3.1.3 Haematocrit

When animals prepare for increased activity as in the fight-or-flight response, the oxygen carrying capacity of the blood is enhanced. This is achieved by increasing numbers of RBC's in the circulation per unit volume of plasma and altering the affinity of haemoglobin for oxygen. Splenic contraction initiated by adrenergic stimulation will release additional RBC's into the blood [166]. The plasma volume might also decrease due to tissue fluid retention caused by the increased osmotic potential of the tissues to which lactate contributes [100]. Adrenergic stimulation of RBC's activates the membrane associated Na^+/H^+ pump, which extrudes protons in exchange for Na^+ [237]. Consequently, water enters the cell which swells, and the pH of the blood decreases [167, 168]. Hypoxia also induces erythrocyte swelling [168]. The combination of increased numbers of RBC's in the circulation, cell swelling and reduced plasma volume can be detected by measuring haematocrit.

The haematocrit values relative to the control fish were; 93%, 105%, 91%, 98% in the ice slurry, death in air, percussively and electrically stunned fish, respectively. The haematocrit of the fish that died in air was significantly higher than that of the percussively stunned fish, but there were no significant differences between the other groups, Figure 4.4.

The death in air fish had high plasma adrenaline levels, Figure 4.1A, and, as they were left for 15min to die, there was sufficient time for adrenergic stimulation of the RBC's to cause swelling, and thereby raising the haematocrit value. The haematocrit in the percussively stunned fish was significantly lower than the death in air fish as they had a low level of plasma adrenaline so RBC swelling did not occur in this group. Although the electrically stunned fish had high adrenaline levels, the samples were taken quickly, before RBC's swelling could occur. The ice slurry fish had very low adrenaline levels therefore, adrenergic stimulation of the erythrocytes did not occur in these fish. The higher level of plasma protein in the death in air fish suggests that the blood volume had decreased in this group.



Inferential statistics
F=2.90, P=0.029, df=4, 63, power=0.56, n=11-17

Significance levels

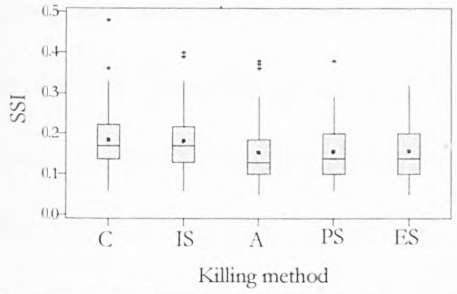
	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		ns†	ns†	ns†	ns†
ice slurry	ns‡				
air	ns‡	ns‡			
percussive stun	ns‡	ns‡	*‡		
electrical stun	ns‡	ns‡	ns‡	ns‡	

Figure 4.4 Effect of 'killing' method on haematocrit
Data presented as a box and whisker plot with significance levels of ANOVA *post hoc* tests, Dunnett† (control vs. methods) and Tukey‡ (between groups, equal variance). Data from duplicate measurements, P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

4.3.1.4 Spleen somatic index

The weight of the spleen relative to the weight of the fish, the SSI, can give a rough indication of the extent of splenic discharge.

The control fish had the greatest SSI, the ice slurry groups SSI was only slightly lower, 98% of the control value. The fish that died in the air had the lowest SSI, 84% of the control value. This was similar to those of the stunned fish with, 84% and 85% of the control value in the percussively and electrically stunned fish, respectively, Figure 4.5. The SSI of the death in air fish was just significantly lower than that of the control.



Inferential statistics
F=3.57, P=0.007, df=4, 297, power=0.68 n=55-66

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		ns†	*†	*†	*†
ice slurry	ns‡				
air	*‡	ns‡			
percussive stun	ns‡	ns‡	ns‡		
electrical stun	ns‡	ns‡	ns‡	ns‡	

Figure 4.5 Effect of 'killing' method on spleen somatic index
Data presented as a box and whisker plot with significance levels of ANOVA *post hoc* tests, Dunnett† (control vs. methods) and Tukey‡ (between groups, equal variance). P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***. Data from single measurement, an outlier was removed following a Grubbs test, the data were then transformed to $\log_{10}$ so that they conformed to normality.

It takes 3-5min for the spleen to release its contents [166]. The death in air fish were left for 15 min to die so there was sufficient time for the spleen to contract fully; their SSI was the lowest. It was significantly lower compared with the control group, which had not discharged their spleens. The RBC numbers in the circulation of the control fish would not therefore have been augmented, and so their haematocrit would not have increased. The electrically stunned fish might have been stimulated to discharge their spleen by the electrical current/ adrenaline discharge, and the percussively stunned fish by netting actions. However, there would have been insufficient time for the spleens in these groups to contract fully before the blood samples were taken. The ice slurry fish had time to

contract their spleens but show no evidence of having done so, possibly due to their low metabolic rate and adrenaline levels. Their low body temperature might have precluded splenic discharge, as it would have decreased the nerve impulse conduction rate and consequently their ability to initiate contraction.

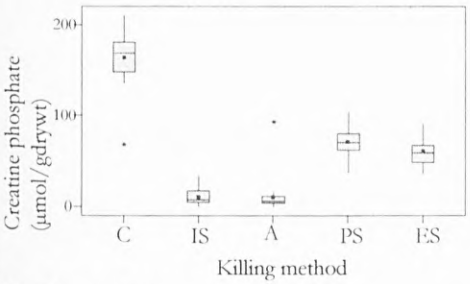
4.3.2 Muscle biochemistry

4.3.2.1 Pre rigor mortis

Metabolites in pre *rigor mortis* muscle were measured to give an indication of how well the animal was coping with its environment when it died by measuring its energy status. Three distinct groups can be seen in the data, Figure 4.6. The control group had the highest energy status and the least evidence of anaerobic glycolysis i.e. most creatine phosphate and ATP and least creatine and lactate. The fish that were left to die in air or on ice had the lowest energy status and showed most anaerobic activity i.e. they contained the lowest amount of creatine phosphate and ATP and the highest creatine and lactate levels. The fish that were percussively or electrically stunned fell between these two extremes.

Creatinine was not detected in any of the samples suggesting that creatine phosphate and creatine were not broken down any further.

A: Creatine phosphate

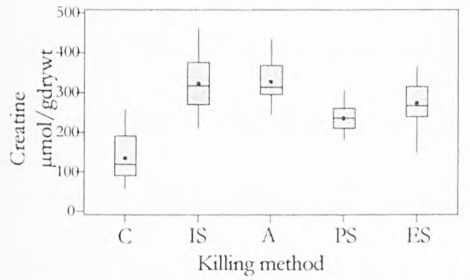


Inferential statistics
H=81.765, P=0.000, df=4, 95, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	***§				
air	***§	ns§			
percussive stun	ns§	***§	***§		
electrical stun	**§	**§	**§	ns§	

B: Creatine

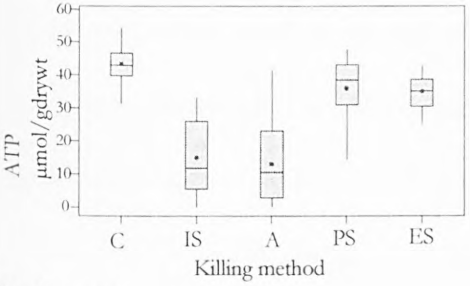


Inferential statistics
F=41.115, P=0.000, df=4, 95, power~1.00, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		**†	**†	**†	**†
ice slurry	***‡				
air	***‡	ns‡			
percussive stun	***‡	***‡	***‡		
electrical stun	***‡	*‡	*‡	ns‡	

C: Adenosine triphosphate

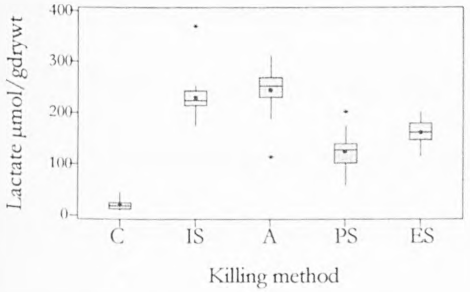


Inferential statistics
F=45.527, P=0.000, df=4, 95, power~1.00, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	*#	***#
ice slurry	***$		ns#	***#	***#
air	***$	ns$		***#	***#
percussive stun	*$	***$	***$		ns#
electrical stun	***$	***$	***$	ns$	

D: Lactate



Inferential statistics
F=45.527, P=0.000, df=4, 95, power~1.00, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	***#	***#
ice slurry	***$		ns#	***#	***#
air	***$	ns$		***#	**#
percussive stun	***$	***$	***$		**#
electrical stun	***$	***$	**$	**$	

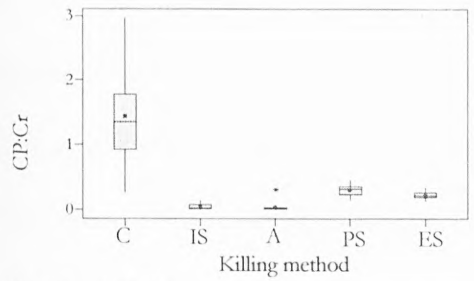
Figure 4.6A-D Effect of 'killing' method on pre *rigor mortis* muscle metabolites
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests, Dunnett† (control vs. methods), Tukey‡ (between groups, equal variance), Tamhane§ (between groups, unequal variance, conservative), Games Howell# (between groups, unequal variance, liberal) and Kruskal Wallis *post hoc* test, Dunn§ (between groups). N.B. Creatinine was not detected in any sample. Samples measured in duplicate. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The sum of the creatine phosphate and creatine measurements, the creatine pool values, did not differ significantly between the groups, F=1.958, P=0.1072, df=4, 95, power~0.35, n=20. The creatine pool values were; control 300±56μmol/gdry wt, ice slurry 333±64μmol/gdry wt, death in air 338±50μmol/gdry wt, percussive stun 308±37μmol/gdry wt and electrical stun 334±65μmol/gdry wt. This suggests that the creatine phosphate, creatine and creatinine measurements were accurate.

Metabolite ratios were calculated to provide a more precise value for comparison purposes. The CP: Cr ratio relates to the proportion of the creatine pool that is phosphorylated. The relative percentages of the CP: Cr ratio compared with the control value were; for ice slurry 2.8%, death in air 2.5%, percussive stun 21.5%, and electrical stun 15.8%. The CP: Cr ratio of the control fish was significantly higher than all groups apart from the percussively stunned fish, Figure 4.7A. The ice slurry fish did not differ significantly to the death in air fish, while the percussively stunned fish were not significantly different to the electrically stunned fish. However, the stunned fish had a significantly higher CP: Cr ratio than the ice slurry and death in air fish, Figure 4.7A.

The CP: ATP ratio gives an indication of the remaining high energy reserves in the fish, once the ratio falls below 1:1, creatine phosphate decline becomes rapid [259]. The death in air fish were the only group to have a CP: ATP ratio of less than one, 0.8:1, although the ice slurry fish ratio was only just larger 1.095:1, Figure 4.7B. The CP: ATP ratio of the control group was significantly higher than all the commercial killing methods. The ratio of the percussively stunned fish was significantly higher than the other commercial methods. The CP: ATP ratios expressed as a percentage of the control values were; for ice slurry 29%, death in air 21%, percussive stun 55%, and electrical stun 46%.

A: CP: Cr

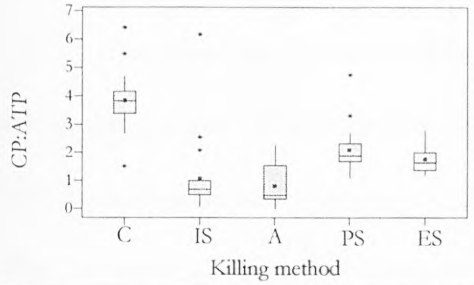


Inferential statistics
H=81.857, P<0.0001, df=4, 93, n=17-20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	***§				
air	***§	ns§			
percussive stun	ns§	***§	***§		
electrical stun	***§	**§	**§	ns§	

B: CP: ATP



Inferential statistics
H=57.591, P<0.0001, df=4, 91, n=19-20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	***§				
air	***§	ns§			
percussive stun	*§	**§	**§		
electrical stun	***§	ns§	ns§	ns§	

Figure 4.7A-B Effect of 'killing' method on metabolite ratios
Data presented as box and whisker plots with significance levels of Kruskal Wallis *post hoc* test, Dunn§ (between groups), n=17-20. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***. Ratios calculated on an individual fish basis.

The high-energy reserves remained intact in the control group because the anaesthetic blocked the motor nerves and so the muscle could not be prepared and/or used for activity. Correspondingly the muscle of the control group exhibited the least signs of anaerobic glycolysis, and contained the lowest levels of lactate. The fish that died in air or on ice slurry had the lowest CP: Cr and CP: ATP ratios and the highest lactate levels. The CP: Cr ratios were significantly lower than the control, percussively and electrically stunned groups; while the CP: ATP ratio was significantly lower than the control and percussively stunned groups. These fish had, in an attempt to maintain homeostasis, utilised the greatest amount of creatine phosphate and undergone the most anaerobic glycolysis. The CP: ATP ratio for the ice slurry fish were slightly higher than the death in air group, the difference was not, however, significant. A slower metabolic rate caused by their lower body temperature might have been responsible for this. The ratios of the stunned fish fell between the controls and the ice slurry and death in air fish, while the ratios of the percussive stun group were higher than those of the electrically stunned fish.

These observations suggest that the energy levels of the percussive stun group were closer to the controls than the electrically stunned group. The levels of creatine phosphate in the percussive stun group did not differ significantly to those of the control group, and the electrically stunned fish had significantly more lactate than the percussively stunned fish. The control fish therefore were the least metabolically active followed by the percussively then electrically stunned fish, the fish that were left to die without prior stunning in ice and air useing the most energy whilst dying.

Adrenaline levels, time, and temperature would have influenced the muscle metabolite pattern. The lowest CP: Cr and CP: ATP ratios and highest lactate levels were found in the death in air fish, where adrenaline levels were highest and there was sufficient time for them to influence the muscle. The ice slurry fish had very little adrenaline to influence muscle activity levels, their metabolic rate was low due to the body temperature and there was a long time in which energy depletion could occur. Their low high-energy status and high lactate level suggests that the time factor had a greater influence on muscle energy status than the adrenaline level. In the electrically stunned fish where there were high adrenaline levels but insufficient time for them to affect the muscle, the energy status was higher than in the ice slurry and death in air fish. The electrically stunned fish had only slightly less high-energy reserves and slightly more lactate than the percussively stunned fish that took a similar time to die but had lower adrenaline levels. For additional information on why the electrically stunned fish might have had higher lactate levels, see Section 4.3.2.2a. The control fish with a short dying time and low adrenaline levels had the most high-energy reserves remaining.

The high levels of lactate in the muscle of the ice slurry and death in air fish would have exerted an osmotic influence on the body fluids of these fish. The plasma volume would have decreased so raising their haematocrit. This, in addition to the other factors occurring in the death in air fish, caused their significantly higher haematocrit relative to the percussively stunned fish, Section 4.3.1.3 Figure 4.4. The fall in plasma volume would

not have been so severe in the ice slurry fish as the high plasma glucose would have exerted an osmotic potential and counterbalanced, to a certain degree, the influence of the muscle lactate.

The pattern of muscle and plasma lactate between the groups was similar although not identical. The ice slurry fish had a slightly higher amount of lactate in the plasma relative to the muscle. In live fish only 10-15% of muscle lactate enters the blood the rest remains in the muscle for conversion back to glycogen *in situ* [100]. The relatively higher plasma lactate in the ice slurry fish might be due to the increased time available for diffusion out of the muscle.

4.3.2.2 Post rigor mortis

4.3.2.2a Lactate

Post *rigor mortis* lactate levels give an indication of the amount of anaerobic glycolysis that has occurred in muscle during the early stages of storage. Lactate levels can be related to the 'quality' measurement, pH, although other factors such as storage temperature and microbial activity will also influence pH.

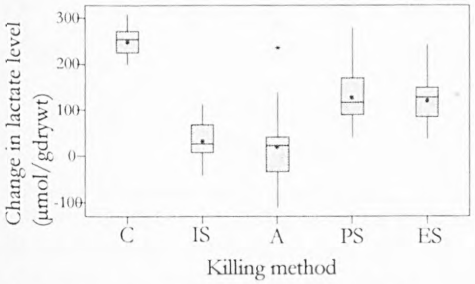
The highest post *rigor mortis* lactate levels were found in electrically stunned fish and the lowest in the percussively stunned group, although these failed to show statistical significance, $F=2.336$, $P=0.061$, $df=4, 95$, power~0.59, $n=20$. The lactate levels were control 269±31μmol/g dry wt, death in ice slurry 262±36μmol/g dry wt, death in air 252±44μmol/g dry wt, percussively stunned fish 247±49μmol/g dry wt, and electrically stunned fish 282±44μmol/g dry wt.

No differences were detected in lactate levels between the groups using a two-tailed test. The electrically stunned fish would have had a significantly higher level compared with the percussively stunned fish if a one-tailed test had been performed i.e. this group were expected to contain more lactate. Electrical stimulation is used by the meat industry to facilitate *post mortem* glycolysis and meat tenderisation [153]. Electrical impulses cause glycolytic enzymes to bind to cellular structures [165] and, by bringing the enzymes and/ or

substrates closer together accelerate the rate of glycolysis [153]. This offers a plausible explanation of why the muscle metabolite data in the electrically stunned fish were more akin to the death in air and ice slurry groups, while the percussively stunned fish were closer to the controls.

The change in lactate levels during four days ice storage, Figure 4.8A, shows that the control fish produced significantly more lactate than the commercial methods and the stunned fish produced significantly more lactate than the unstunned groups. This occurs because of the higher energy levels that remain in the muscle of the control fish, which fuel anaerobic glycolysis for a longer period *post mortem*.

A: Δ lactate over 4 days muscle fish

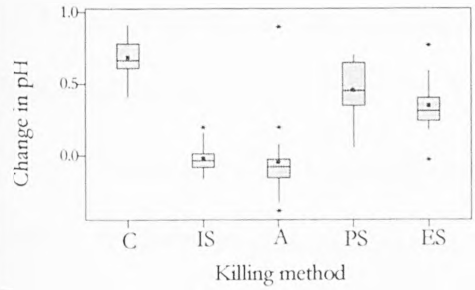


Inferential statistics
F=54.7, P=0.000, df=4, 95, power~1.00, n=20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	***#	***#
ice slurry	***$		ns#	***#	***#
air	***$	ns$		***#	***#
percussive stun	***$	***$	***$		ns#
electrical stun	***$	***$	***$	ns$	

B: Δ pH over 4 days blood fish



Inferential statistics
F=71.4, P=0.000, df=4, 95, power~0.73, n=20

Significance levels

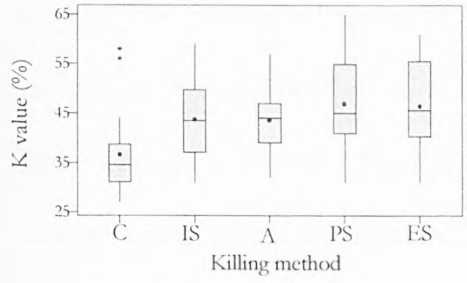
	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	***#	***#
ice slurry	***$		ns#	***#	***#
air	***$	ns$		***#	***#
percussive stun	***$	***$	***$		ns#
electrical stun	***$	***$	***$	ns$	

Figure 4.8A-B Effect of 'killing' method on Δ in muscle lactate and pH over four days
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests Tamhane$ and Games Howell# (between groups, unequal variance). Data from duplicate measurements (lactate) and single measurements outlier removed (pH), n=20. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***. Data calculated on individual fish.

4.3.2.2b K value

The ratio of nucleotide degradation products or K value, indicates how well a fish carcass maintains freshness; a lower value signifies fresher fish.

The control fish had a significantly lower K value than the fish killed using the industrial methods, Figure 4.9. The lower K value would be expected as the control fish had larger energy reserves immediately *post mortem* and so could continue to produce ATP thereby maintaining freshness. The greater accumulation of lactate during storage also suggests that more ATP was available *post mortem* in the control fish.



Inferential statistics
F=5.982, P=0.003, df=4, 95, power~0.92, n=19-20

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		*†	**†	**†	**†
ice slurry	*‡				
air	*‡	ns‡			
percussive stun	***‡	ns‡	ns‡		
electrical stun	***‡	ns‡	ns‡	ns‡	

Figure 4.9 Effect of 'killing' method on K value
Data presented as a box and whisker plot with significance levels of ANOVA *post hoc* tests, Dunnett† (control vs. methods) and Tukey‡ (between groups). Data from single measurements. Tissue was sampled four days *post mortem*. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***. The data was transformed to $\log_{10}$ and outliers were removed following a Grubbs test so that the data conformed to normality.

4.3.3 Muscle quality and related parameters

4.3.3.1 Muscle pH

Muscle pH alters the configuration of muscle proteins and so can influence meat quality. It relates, in part, to the amount of lactate produced by the muscle and so can give an indication of the rate/extent of anaerobic glycolysis.

4.3.3.1a The decline in initial pH

The slope of the regression line of the pH measurements taken in the first 60s after death was used to estimate the rate of decline in pH, Figure 4.10. The slope was significantly shallower in the fish that died in the ice slurry relative to the other groups, except the death in air fish, Table 4.1. Compared to the control fish the decline in pH was 20% in the ice slurry fish, 61% in the death in air fish, 111% in the percussively stunned

fish and 75% in the electrically stunned fish. This indicates the rate of anaerobic glycolysis was slower in the death in ice slurry fish, although the total amount of lactate in the tissue was higher. The lower pH, due to the higher lactate level would have diminished the activity of the glycolytic enzymes. The time elapsed before sampling i.e. the fish were left for 70min, allowed the lactate levels to build up even though rate of production was slow. Additionally the lower body temperature in these fish would have lowered metabolic activity and slowed the rate of decline in pH.

The intercept of the linear regression line might give a more representative value of the pH at death than an actual measurement in that the effects of small differences in the timing of the first measurement are decreased. The intercept of the control fish was significantly higher, 0.29-0.81 units, than those of fish killed by the commercial methods, Table 4.1 and Figure 4.10. The intercept of the ice slurry fish was significantly lower, 0.39-0.78 units, than all but the death in air fish, Table 4.1 and Figure 4.10. The intercept of the death in air fish was also significantly lower than the stunned fish, 0.42-0.52 units, Table 4.1 and Figure 4.10. The intercepts of the fish that had been stunned were significantly different to each other, 0.1 unit, Table 4.1 and Figure 4.10. This suggests that the control fish experienced less anaerobic activity than those subjected to the industrial killing/ stunning methods. The level of muscle activity increased in the following order, percussive stun, electrical stun and then the ice slurry and death in air groups.

Table 4.1 Parallelism and identical regression line test statistics for the effect of 'killing' method on muscle pH, t values and significance levels

intercept			**slope**		
method	control	ice slurry	air	percussion	electricity
control	----	4.408***	1.450 ns	0.631 ns	1.098 ns
ice slurry	25.857(***)	----	1.589 ns	4.467***	2.654**
air	18.378***	0.690 ns	----	1.823 ns	0.512 ns
percussion	8.152***	14.082(***)	10.979***	----	1.552 ns
electricity	10.840***	11.077(***)	8.811***	2.504**	----

Single pH measurements were made per fish, at 0, 30 and 60s *post mortem*, n=20.

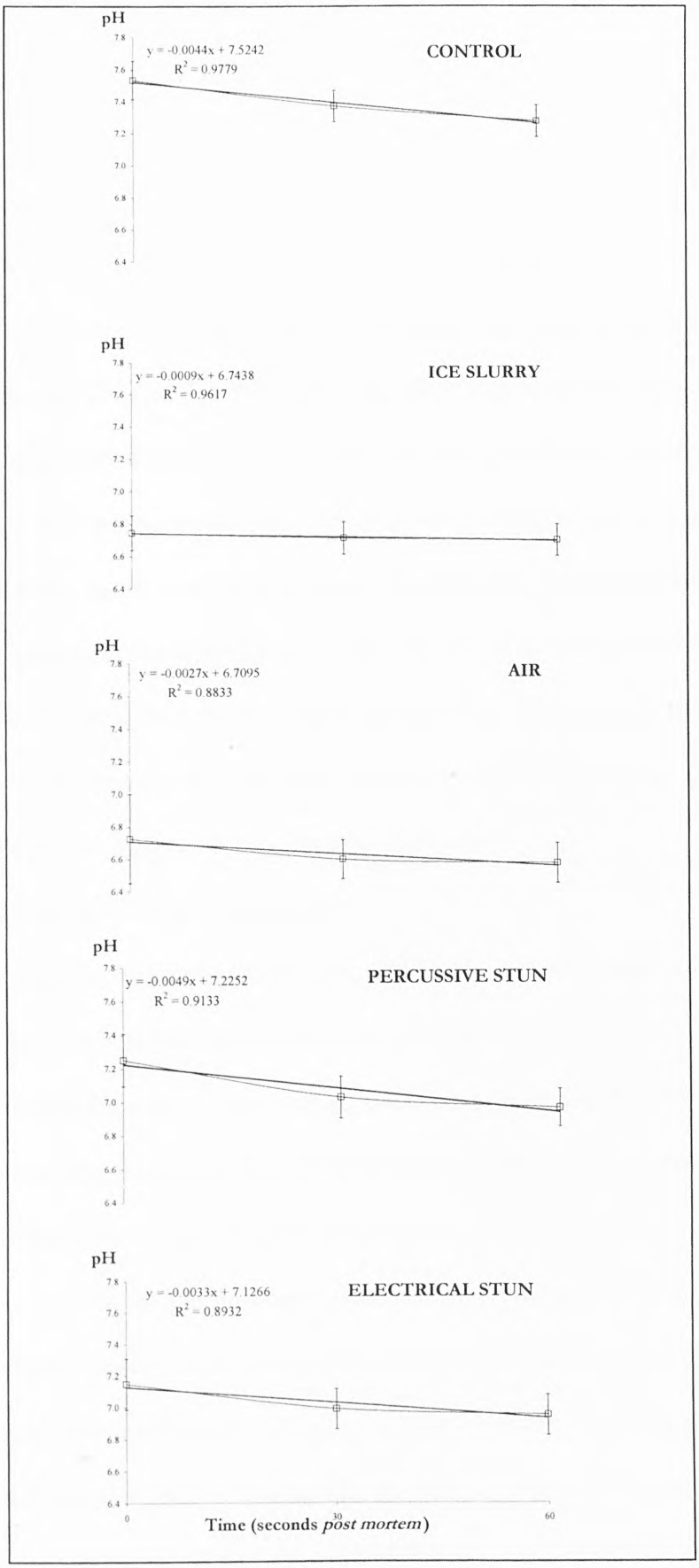


Figure 4.10 Effect of 'killing' method on muscle pH immediately *post mortem*
Results expressed as mean ± standard deviation, n=20 from single readings on the blood set of fish.

4.3.3.1b The pH taken at zero seconds

The pH taken at zero seconds, (pH_i), in the blood set of fish, shows the same groupings as the metabolite data from the muscle set of fish, Figures 4.6 and 4.11A. The pH_i values compared to the control were for the death in ice slurry fish, death in air fish, percussively stunned fish and electrically stunned fish; 90%, 89%, 96% and 95%, respectively. The control fish pH_i was significantly higher than those observed for the commercial methods. The fish that were left to die either in ice slurry or air had the lowest pH_i, were not significantly different to each other, but were significantly lower than the stunned fish. The fish that were electrically or percussively stunned had an intermediate pHi and did not differ significantly to each other. The pH_i data differs slightly from the intercept data described in Section 4.3.3.1a, in that, the pH_i of the percussive stun group was not significantly higher than the electrically stunned fish. This suggests that if the decline in pH over the first 60s was not linear, there was no difference in the level of anaerobic glycolysis occurring in the two stunning methods.

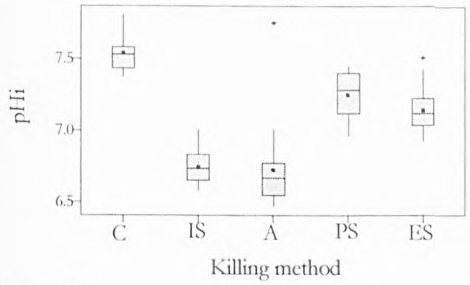
4.3.3.1c The pH taken ninety six hours post mortem

The pH taken 96 hours after death, pH_{96}, shows a significantly higher value in the control group relative to the fish that died in air and ice slurry, Figure 4.11B. The pH_{96} values compared with the control were for; the death in ice slurry, death in air, percussively stunned fish and electrically stunned fish; 99%, 99%, 100% and 99%, respectively.

The pH during ice storage can give an indication of the degree of spoilage occurring in fish. Initially the pH falls due to lactate formation then it rises due to TVB production. The pH_{96} of the control and the stunned fish had fallen relative to the pH_i due to greater amounts of lactate formed. The pH_{96}'s of the ice slurry and death in air fish were fractionally higher than their pH_i's. This implies that the ice slurry and death in air groups were less fresh than the other groups. The control fish pH_{96} was significantly higher than that of the ice slurry and air fish, but not to the stunned fish suggesting that, the control fish were the freshest. The lower post *rigor mortis* lactate levels in the control fish supports

this finding, although they failed the significance test. The K value data also showed the control fish to be the freshest, Section 4.3.2.2b.

A: pH_i

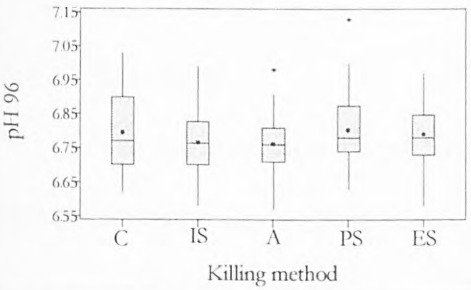


Inferential statistics
F=79.085, P=0.000, df=4, 95, power~1.00, n=20.

Significance level

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	***#	***#
ice slurry	***$		ns#	***#	***#
air	***$	ns$		***#	***#
percussive stun	***$	***$	***$		ns#
electrical stun	***$	***$	***$	ns$	

B: pH_{96}



Inferential statistics
F=5.293, P=0.000, df=4, 301, power~0.94, n=60-66.

Significance level

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		*#	**#	ns#	ns#
ice slurry	*$		ns#	ns#	ns#
air	**$	ns$		ns#	ns#
percussive stun	ns$	ns$	ns$		ns#
electrical stun	ns$	ns$	ns$	ns$	

Figure 4.11A-B Effect of 'killing' method on muscle pH
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests Tamhanes$ and GamesHowell# (between groups, unequal variance). Data from single measurements. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The pH of flesh declines *post mortem* due to cessation of ATP production from creatine phosphate and lactate accumulation. The pH measurements would therefore be expected to relate directly to the lactate and creatine phosphate data. The relationship between pH, lactate and creatine phosphate was verified by correlating ΔpH with Δlactate minus ΔCP. Creatine phosphate levels were assumed as zero four-days *post mortem*. Group averages for each week were used for the correlation as the measurements were taken on different sets of fish, Figure 4.12. A multiple regression of ΔpH, Δlactate and ΔCP gave the equation ΔpH=-0.05683 +0.001149Δlactate +0.003314ΔCP which explained 77.34% of the variance, with a correlation coefficient r= -0.95.

The change in pH of the fish over four days *post mortem* mirrors the change in the muscle lactate levels, Figure 4.8AB, indicating that the decline in pH over this period is predominantly influenced by lactate.

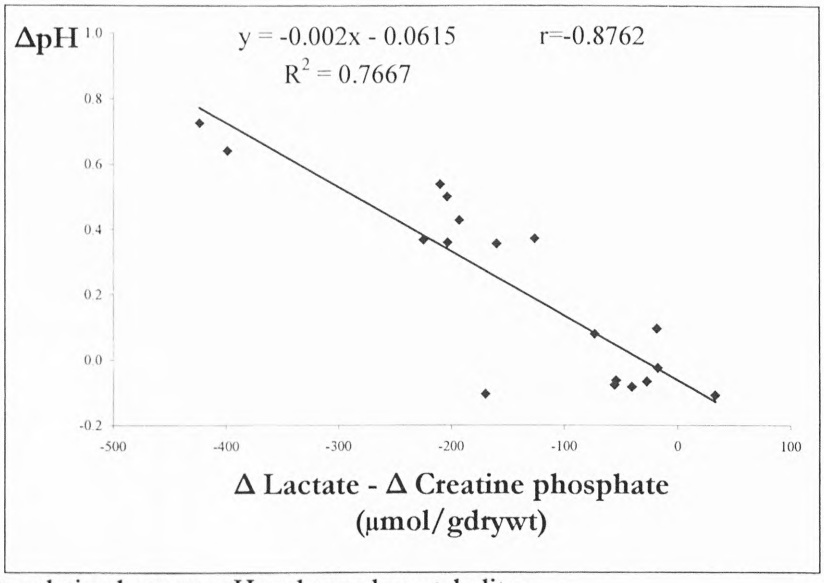


Figure 4.12 Correlation between pH and muscle metabolites
Group averages for each week were used for the correlation as the measurements were taken on different sets of fish, n=18, P<0.001.

4.3.3.2 *Rigor mortis*

Rigor mortis measurements can be used as a measure of preharvest activity in fish. Fish processors require information on whether fish have passed through *rigor mortis* as it affects how the fish are handled.

Rigor mortis development was significantly different in the control fish compared with the fish killed by the industrial methods, Figures 4.13, and 4.14. The time to peak *rigor mortis* in the control fish was delayed, the maximal contraction strength was lower and the duration of *rigor mortis* was longer.

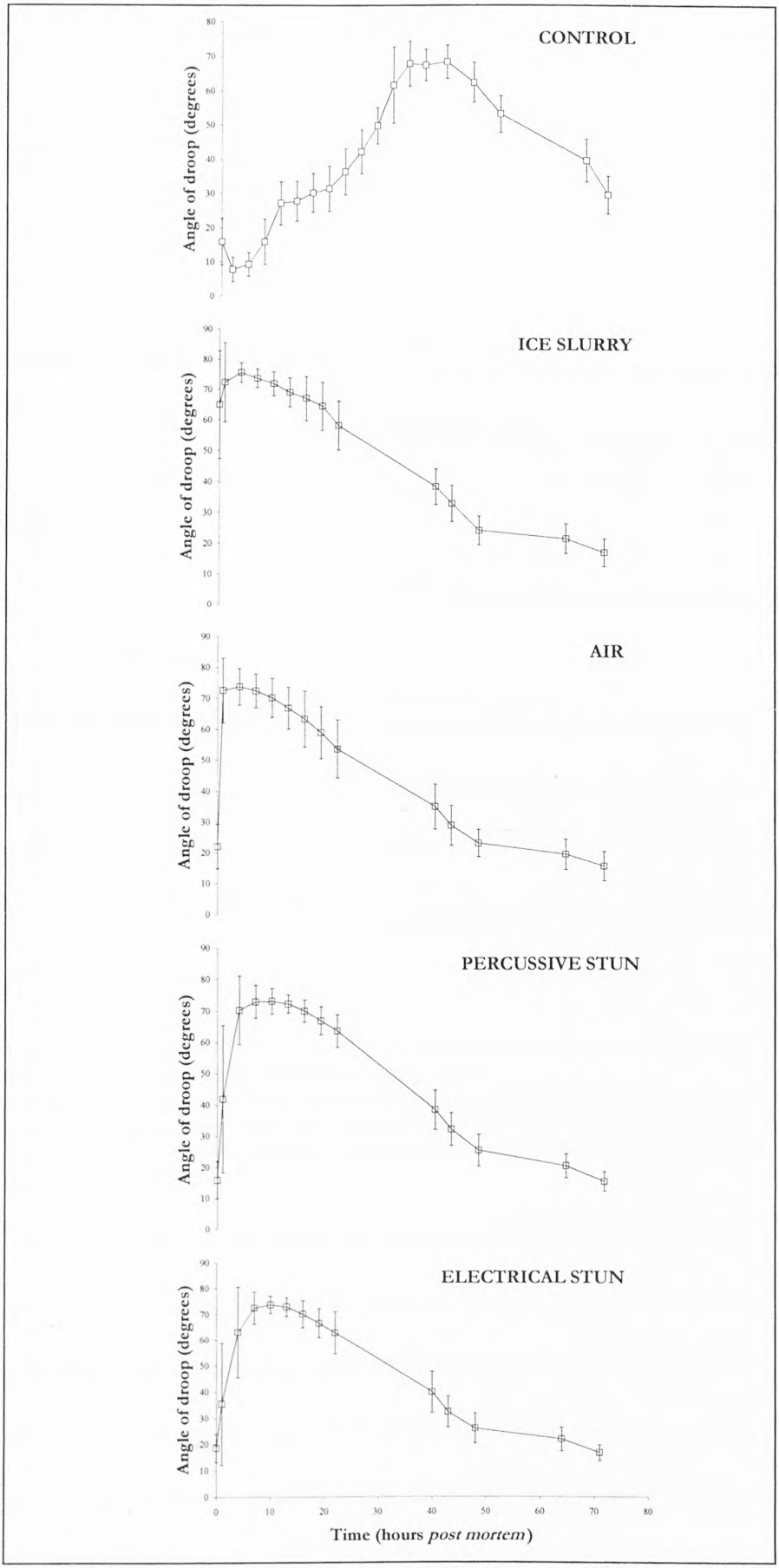
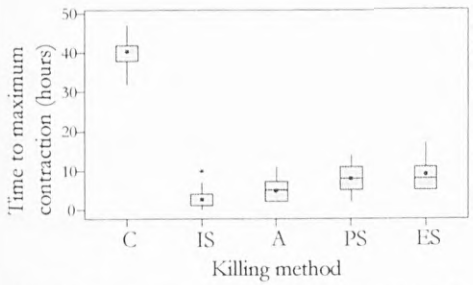


Figure 4.13 Effect of 'killing' method on *rigor mortis* development
Results expressed as mean ± standard deviation, n=20 or 26 (controls), average of triplicate readings on each side of the fish.

A: Time to maximum contraction

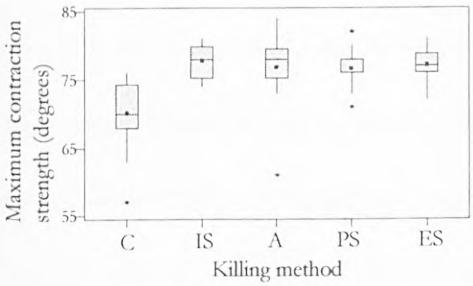


Inferential statistics
H=81.04, P=0.000, df=4, 101, n=20-26.

Significance level

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	***§				
air	***§	ns§			
percussive stun	***§	**§	ns§		
electrical stun	***§	***§	ns§	ns§	

B: Maximum contraction strength

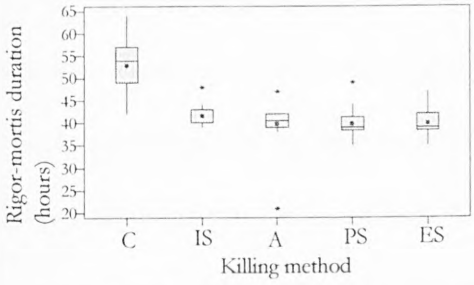


Inferential statistics
F=19.674, P=0.000, df=4, 101, power~0.99, n=20-26.

Significance level

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		***#	***#	***#	***#
ice slurry	***§		ns#	ns#	ns#
air	***§	ns§		ns#	ns#
percussive stun	***§	ns§	ns§		ns#
electrical stun	***§	ns§	ns§	ns§	

C: Duration of *rigor mortis*



Inferential statistics
H=57.94, P=0.000, df=4, 101, n=20-26.

Significance level

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry	***§				
air	***§	ns§			
percussive stun	***§	ns§	ns§		
electrical stun	***§	ns§	ns§	ns§	

Figure 4.14A-C Effect of 'killing' method on *rigor mortis*
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests Tamhane§ (between groups, unequal variance, conservative) and Games Howell# (between groups, unequal variance, liberal), Kruskal Wallis *post hoc* test Dunn§ (between groups). Data from triplicate measurements on each side of the fish. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

Time to maximum *rigor mortis* was earlier by 38h in the ice slurry fish, 36h in the death in air fish, 32h in the percussively stunned fish and, 31h in the electrically stunned fish. Compared with the controls, onset of *rigor mortis* was earlier by; 17h, 15h, 14h, 13h, in the ice slurry, death in air, percussively stunned, and electrically stunned fish, respectively. Resolution of *rigor mortis* was earlier by; 28h in the ice slurry fish, 29h in the death in air fish, 28h in the percussively stunned fish and, 26h in the electrically stunned fish.

The fish that died in ice slurry were the first to reach maximum *rigor mortis* and this occurred significantly earlier in fish stunned either by percussion or by electricity. The maximum contraction strength and duration of *rigor mortis* did not however differ between the commercial methods. When compared with the controls, maximum contraction strength was higher by 8° in the ice slurry fish, 7° in the death in air fish, 6° in the percussively stunned fish and, 7° in the electrically stunned fish.

The higher energy status of the control group as shown by the metabolite data would have caused the delay in *rigor mortis* development. The levels of ATP in the control fish were maintained for approximately 38 hours *post mortem*, until they were in full *rigor mortis*. The maximal contraction strength was less severe in the control group as individual muscle cells did not run out of ATP simultaneously. This indicates that the control fish exhibited the least signs of muscle activity at death.

The speed of *rigor mortis* development is dependent on the levels of energy in the muscle. Figure 4.15 shows the correlation between the data for time to maximum *rigor mortis* as measured in the *rigor mortis* set of fish with creatine phosphate plus ATP levels as measured in the muscle set of fish. The linear regression model explains 72% of the variance in the data, and has a correlation coefficient of 0.85.

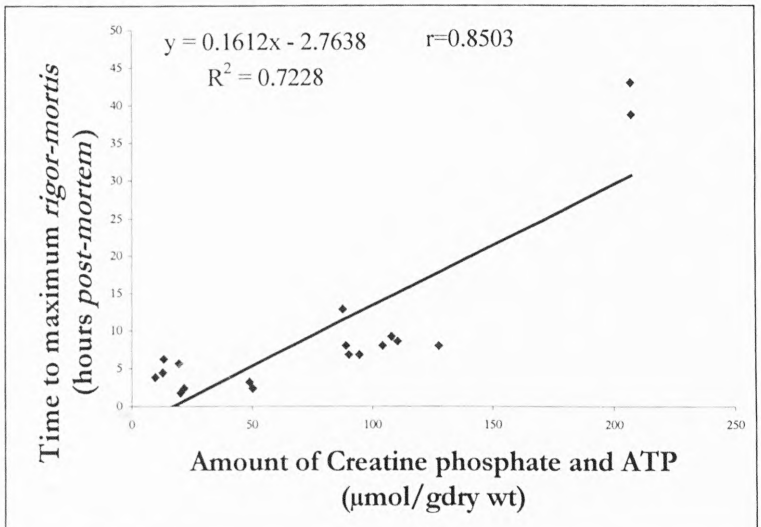


Figure 4.15 Correlation between time to maximum *rigor mortis* and muscle metabolites
Group averages for each week were used for the correlation as the measurements were taken on different sets of fish, n=18, P<0.001.

4.3.3.3 Freshness

Freshness was measured in the fish during four days ice storage using the Torrymeter, as freshness is an extremely important quality characteristic of fish.

The Torrymeter readings indicated that there was an overall decline in fish freshness in all but the control group, Figure 4.16 and Table 4.2. The decline in freshness in the control fish was significantly lower than that of all the commercial methods. Freshness declined at a significantly faster rate in the fish that died in the ice slurry compared with all the other groups, Table 4.2. The fish that died in ice slurry lost freshness at 6600% of the rate of the control fish, the death in air 4967%, the percussive and electrically stunned fish, 4400% and 4533%, respectively.

Table 4.2 Parallelism and identical regression line test statistics for the effect of 'killing' method on Torrymeter readings, t values and significance levels

intercept			**slope**		
method	control	ice slurry	air	percussion	electricity
control	----	-11.66***	-8.97***	-7.54***	-7.54***
ice slurry	-4.71(***)	----	5.94***	6.69***	5.75***
air	-2.90(**)	3.77(***)	----	1.88 ns	1.30 ns
percussion	-3.69(***)	1.57 ns	-1.72 ns	----	-0.351 ns
electricity	-3.58(***)	1.44 ns	-1.55 ns	0.00 ns	----

The slope represents the decline in fish freshness, n = 60 or 66 (controls), df = 116 or 122. Where the significance level of the intercept is in parentheses, it might be an artefact of the test. Triplicate measurements were taken on one side of the fish.

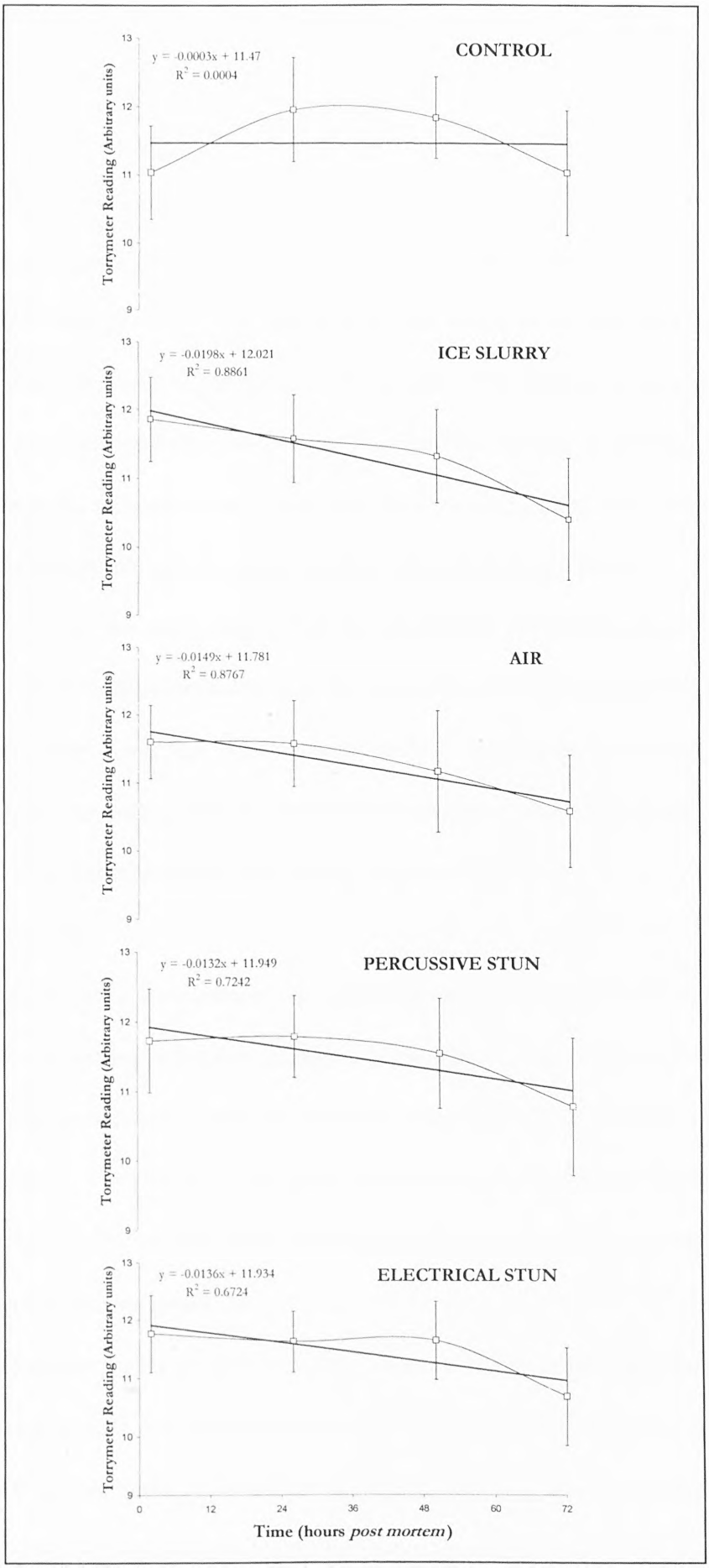


Figure 4.16 Effect of 'killing' method fish freshness

Results expressed as mean ± standard deviation, n=20 or 26 (controls), average of triplicate readings on each side of the fish.

Torrymeter readings increase in pre-*rigor mortis* muscle [160]. The control fish showed an apparent increase in freshness up to approximately 38h and then went into a steep decline. The maximum at 38h coincided with the peak *rigor mortis* measurement, Figure 4.13. The ionic balance in the cells was maintained up to that point, as there was sufficient ATP in the muscle to fuel the ion pumps. Post *rigor mortis,* as ATP levels fell, the ion pumps would have failed and Ca^{2+} diffused into the cell from the sarcoplasmic reticulum, altering the electrical properties of the muscle. The changes in ion sequestration *post mortem* are also affected by muscle pH, which affects ion binding to proteins as well as the rate of lactate and P_i production. These ions could also affect the cells' osmotic potential and consequently the compartmentalism of water and drip losses.

The data from this study suggest that the control fish were better at maintaining freshness over the four days measured than the industrial methods, although the decline in freshness once it started was fast. The death in ice slurry fish lost freshness first. The K value data support the finding that the control fish were the freshest, Figure 4.9. The post *rigor mortis* pH data also strengthens this finding, Figure 4.11.

4.3.3.4 Centrifugal drip

Centrifugal drip is a measure of water binding capacity and can be related to the stage of *rigor mortis*, texture and colour changes in fish flesh. It was evaluated on both the *rigor mortis* and the muscle sets of fish the data were combined. No differences were found between the groups, ($H=2.01$, $P=0.734$, $df=4$, $n=40$ or 46). Drip loss was 11±3%, 11±3%, 12±4%, 11±4%, 11±4%, in the control, ice slurry, death in air, percussive and electrically stunned fish, respectively.

As differences were not seen between the groups in centrifugal drip, the muscle metabolite data and pH_{96} were considered more sensitive indicators of muscle condition than drip. Had the test been performed while the fish were in *rigor mortis* differences might have been detected between groups due to the altered contractile state of the muscle proteins. The decision to measure water binding capacity four days *post mortem* was

consciously made to eliminate the effects of *rigor mortis* and to relate water binding capacity to the quality of fish reaching the consumer.

4.3.3.5 Downgrading

Fish carcasses that have been damaged during harvest might not be suitable for specific products. They are checked visually and sorted before sale, a process known as downgrading. Visual assessments were made of fish 'in the round', and on fillets, after four or five days ice storage, respectively.

Externally the fish were appraised for scale loss and haemorrhaging into the skin or eyes. The internal assessment evaluated fillet gaping, bloodspots in the flesh, and dislocations or haemorrhages into the spine. Attributes were scored on a scale of 0-5, the lower the score the less damage; scoring criteria are described in Section 2.4.5.3. The scores were summed and each killing/ stunning method expressed as a percentage of the total score, Figure 4.17.

As scale loss did not occur in the control group, the results were expressed as percentage of the percussively stunned fish. Scale loss was 44% of the value in the ice slurry and death in air fish and 43% in the electrically stunned fish. Compared with the control fish the level of external haemorrhages in the industrial methods were 175% in ice slurry, 200% in air, 375% in percussive stun and 150% in electrical stun. Internal haemorrhages were 133% in ice slurry and death in air fish, 67% in percussively stunned fish and 400% electrically stunned fish relative to the control. While spinal damage was absent in the ice slurry fish, the death in air fish had 133% of the control value, the percussive stun 33% and electrical stun 333%. Fillet gaping scores were, relative to the control, 103% ice slurry, 74% death in air, 87% percussive stun and 71% electrical stun.

Over 35% of the external damage observed occurred in the percussively stunned fish. The difference was not, however, statistically significant. These injuries can be explained by blows that missed the target area or were of excessive force [253]. The trout that were left to die in the air were unexpectedly robust against external damage. However,

as only small numbers of fish were killed at a time crush injuries as experienced by the industry did not occur.

In the internal downgrading assessment, the killing/ stunning methods exhibited similar gaping scores, Figures 4.17. In the other traits most of the damage observed occurred in the electrically stunned fish.

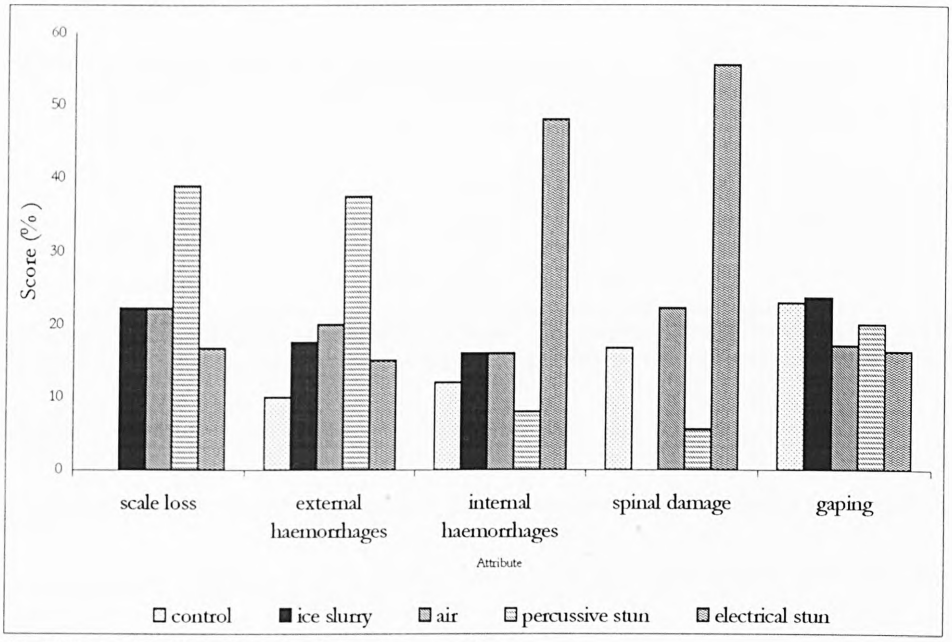


Figure 4.17 Effect of 'killing' method on downgrading
External evaluation four- and internal assessment five-days *post mortem*. Attributes were scored on a scale of zero to five, where zero signified no and five severe damage. The scores were summed and each 'killing' method expressed as a percentage of the total. Single evaluation, one observer, n=20 or 26 (controls).

The only statistically significant difference found between the methods was in the scores for spinal damage, Tables 4.3 and 4.4. Spinal damage was significantly higher, in the electrically stunned fish compared with the ice slurry and percussively stunned fish. Differences found in internal haemorrhaging (bloodspotting) were not significant. Electrical stimulation can cause strong uncoordinated contractions in skeletal muscle, which might cause spinal dislocations [192]. Sudden surges of adrenaline can increase arterial blood pressure and cause vessel rupture. The adrenaline levels in the electrically stunned group were high compared with the other killing methods apart from the death in air group and release would have been sudden caused by the electrical shock. The sudden adrenergic stimulation of muscle might have been the cause of the damage.

Table 4.3 Statistical data for the effect of 'killing' method on downgrading

attribute	Kruskal-Wallis (H)	P	df	n
scale loss	6.92	0.140	4	20-26
external haemorrhages	6.73	0.151	4	20-26
internal haemorrhages	9.13	0.058	4	20-26
spinal damage	15.86	0.003	4	20-26
gaping	4.66	0.324	4	20-26

The downgrading assessment was performed four (external) or five (internal) days *post mortem*, on the fish killed for *rigor mortis* measurements, one observer, single assessment.

Table 4.4 Effect of 'killing' method on spinal damage scores

method	control	ice slurry	air	percussion
ice slurry	ns			
air	ns	ns		
percussion	ns	ns	ns	
electricity	ns	**	ns	*

Results of Dunn *post hoc* test following a Kruskal Wallis test on the spinal damage score. Spinal damage was assessed five days *post mortem*, a single assessment was made per fish by one observer, n=20 or 26 (controls).

4.3.3.6 Colour

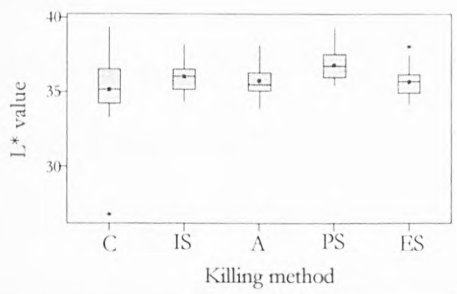
Flesh colour is an important quality characteristic of salmonids as consumers buy on visual appearance. Colour was measured on freshly prepared fillets from the *rigor mortis* set of fish five days *post mortem*.

4.3.3.6a Lightness

Significant differences were detected in lightness values, (L*), between the killing/ stunning methods. The percussively stunned fish had a significantly higher L* value than the controls and the electrically stunned fish as calculated by the Tamhane test, (conservative) Figure 4.18. The less stringent Games Howell test also showed the death in air fish to have a significantly lower L* value than the percussively stunned fish, Figure 4.18. The percussively stunned fish therefore had the palest fillets. Compared to the control fish the L* values in the other groups were 102% in the ice slurry, death in air, and electrically stunned fish and 105% in the percussively stunned fish. These data are therefore conflicting as lower L*'s were expected in less stressed fish. For the control group to have the lowest value agrees with the literature [186]. However, the other observations that the fish that died in the air or were electrically stunned had lower L*

values than the percussively stunned fish, are in direct contrast with that finding. The power of the test, 0.82, was sufficiently high for conclusions to be drawn from the data.

A: Lightness value



Inferential statistics
F=3.926, P=0.005, df=4, 101, power~0.82, n=20-26

Significance levels

	control (C)	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
control		ns#	ns#	*#	ns#
ice slurry	ns$		ns#	ns#	ns#
air	ns$	ns$		*#	ns#
percussive stun	*$	ns$	ns$		**#
electrical stun	ns$	ns$	ns$	**$	

Figure 4.18 Effect of 'killing' method on fillet colour
Data presented as box and whisker plots with ANOVA inferential statistic and *post hoc* tests, Tamhane$ (between groups, unequal variance, conservative) Games Howell# (between groups, unequal variance, liberal)). Data from triplicate readings taken on one fish fillet five days *post-mortem*, n=20 or 26 (controls). P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

4.3.3.6b Chroma

The chroma values, (C*), calculated from the CIE (1976) L*a*b* readings showed no differences between the experimental fish, F=1.644, P=0.1692, df=4, 101, power~0.43, n=20-26. The power of the test was low, so type two errors could not be discounted and conclusions of no difference could not be made. The values obtained were for the control fish 35.9±1.1, ice slurry fish 35.1±1.0, death in air fish 35.8±0.8, percussively stunned fish 35.1±0.9, and 36.2±0.9 electrically stunned fish.

4.3.3.6c Angle of Hue

Differences were not found in the angle of hue measurements in the fish, F=1.265, P=0.2886, df=4, 101, power~0.38, n=20-26. However, the test was also considered inconclusive as the power value was low. The values obtained were for the control fish 41.0±3.9°, ice slurry fish 40.6±3.4°, death in air fish 40.6±2.9°, percussively stunned fish 42.5±3.1°, and 40.2±3.8° electrically stunned fish.

4.3.3.6d Roche Salmofan™

Differences were not detected between the groups on the visual assessment using the Roche *Salmo*fan™, H=7.02, P=0.135, df=4, 101, n=20-26. The values obtained were for the control fish 29±2, ice slurry fish 28±2, death in air fish 28±2, percussively stunned

fish 27±2, and 28±2 electrically stunned fish.

Differences in lightness, hue and chroma are caused by changes in the solubility of muscle proteins. Enzyme binding in active muscle would increase the insoluble fraction, enhance anaerobic glycolysis and cause a rapid fall in pH. In muscle where the pH falls rapidly, the proteins denature and tend to fall out of solution. Fluid is lost from the tissue and the scatter of light from cut surfaces alters (Warriss and Brown 1987 and Warriss 1996 cited in [186]). As the degree of denaturation of proteins increases, more of the incident light will reflect diffusely, diffuse transmission will decrease, and consequently the translucency of the flesh declines. The difference in pH between the groups was resolving four days *post mortem*, the only statistically significant difference being between the controls and the ice slurry and death in air fish. There were no significant differences in centrifugal drip at four days *post mortem*, and *rigor mortis* had resolved. Therefore, at the time measured, there was little reason for colour differences to exist, and so the paler flesh in the percussively stunned fish might be due to the variable nature of the measurement or pigmentation in fish.

4.3.3.7 Texture

Fillet texture was evaluated, as it is critical during fish processing and an important organoleptic quality of trout.

4.3.3.7a Compression tests

Differences were not detected between the killing/ stunning methods in any of the compression tests. For peak force on first compression, F=0.7437, P=0.5644, df=4, 101, power~0.23, n=20-26. The values obtained were; control fish 95±19g, ice slurry fish 95±11g, death in air fish 98±21g, percussively stunned fish 102±19g, and, 93±18g electrically stunned fish. The ratio of second to first bite values obtained were for the; control fish 77±3, ice slurry fish 77±3, death in air fish 77±2, percussively stunned fish 77±2, and 78±2 electrically stunned fish. The inferential statistics for ratio of second to first bite were, F=0.1625, P=0.9569, df=4, 101, power~0.09, n=20-26. While the negative

force inferential statistics were, H=0.7437, P=0.1703, df=4, 101, n=20-26. The values obtained for negative force were for the control fish 8±1g, ice slurry fish 7±1g, death in air fish 7±1g, percussive stunned fish 7±0g, and 7±1g electrical stunned fish. All of the results were however considered inconclusive, as the power of the tests was very low.

4.3.3.7b Shear test

The fish that died in the ice slurry had a lower Warner-Bratzler peak force compared with the fish that were percussively or electrically stunned. The difference was only significant at the 6% level, F=2.293, P=0.065, df=4, 101, power~0.62, n=20-26. The values obtained were for the control fish 233±40g, ice slurry fish 212±37g, death in air fish 231±31g, percussively stunned fish 242±43g, and 242±27g electrically stunned fish. If a one-tailed test were performed, the data would indicate softer flesh in fish that died in the ice slurry relative to the fish that were percussively or electrically stunned. The justification for performing a one-tailed test can be found in the literature. A study by Gómez-Guillén *et al.* [83] performed a Warner-Bratzler test on Atlantic salmon tissue and found the shear strength of the connective tissue was lower in stressed fish although the shear strength of the myofibres was not affected. The power of the test in the present study was quite high, 0.62, so although no definite conclusions could be drawn from the data the phenomenon may warrant further investigation. The data from this study therefore weakly suggest a possible trend to softening in the ice slurry fish. Softening would occur because the rapid decline in pH would cause earlier leakage of proteases from lysosomes, which would be available to attack the collagen. The low pH would also make the collagen more susceptible to protease activity.

4.4 Discussion

Killing and stunning techniques most commonly used on UK trout farms were investigated for their effects on stress responses, muscle physiology and carcass quality in rainbow trout. The methods tested influenced these parameters to differing degrees. Their relationship to the literature and impact on fish welfare and end-product quality will now be considered.

4.4.1 Plasma biochemistry and related parameters

4.4.1.1 Plasma hormones

The stress response in fish is initiated by plasma catecholamines. Adrenaline and noradrenaline are secreted within milliseconds of stressor perception and so are ideal stress indicators in situations where stress perception is cut short by death [100]. Cortisol might not be such a useful stress indicator in these circumstances because it has to be synthesised before release, and so it takes longer for differences to become discernible and its effects apparent [100].

Plasma adrenaline in this study ranged from 1 to 259ng/ml depending on the killing/ stunning method used, noradrenaline levels were lower, 0-56ng/ml. Differences between the techniques tested were only detected in adrenaline levels. Extremely high levels of adrenaline were measured in some of the fish that were electrically stunned. In all probability, the stunning process caused this catecholamine release.

Catecholamine levels in rainbow trout after severe exercise, have been reported as 59ng/ml adrenaline, and 15ng/ml noradrenaline, both falling to the low nmol range after 2h [145]. While rainbow trout exposed to severe hypoxia and subsequently dying, showed maximal plasma concentrations of 47.44±12.57ng/ml adrenaline and 35.64±4.36ng/ml noradrenaline 3h after exposure to the stressor [176]. As the hypoxia experiment decreased the water oxygen content but did not expose the animals to air, the stressor might not have been as severe as in the present study; only 40% of the fish died. Correspondingly, these values were lower than the ones found in the present study where the fish had been

purposely killed. The catecholamine levels measured in the present study were therefore higher but in the same range as published work [145, 176]. Catecholamines were measured in rainbow trout plasma following stunning by Azam [13]. Adrenaline levels were reported to be, in fish stunned by AC electricity, DC electricity, carbon-dioxide narcosis, percussion and ice; 0.38, 0.22, 0.41, 0.16, 0.33pmol/ml, and the corresponding noradrenaline levels were; 0.21, 0.18, 0.56, 0.27 and 0.53pmol/ml [13]. These levels were considerably lower than those measured in the present work. This suggests that either these fish did not respond so severely to the stunning techniques as they did in the current study, or there were problems with sample deterioration and/ or analysis.

The adrenaline: noradrenaline ratios of the control and percussively stunned fish; 4.64:1 and 4.38:1 were close to the ratio reported for chromaffin tissue 5:1 [100], while the death in air and electrically stunned fish had higher ratios 9.36:1, and 9.31:1 suggesting a proportionately greater release of adrenaline in these fish. The lower ratio shown by the ice slurry fish 1.5:1 might intimate that the adrenaline had been metabolised faster than the noradrenaline.

The cortisol levels in the current study range from 10-320ng/ml. The ice slurry fish had a lower level than all the other methods, possibly due to greater metabolic breakdown of the molecule during the longer dying period, or reduced secretion/ production due to their lower body temperature. Milligan [145] reports cortisol levels in rainbow trout blood of 50-100ng/ml immediately following exhaustive exercise, which rose to 60-125ng/ml 2h later. In rainbow trout exposed to severe hypoxia, cortisol levels rose to 270±37ng/ml, 4.5h after the initial stressor [176]. The levels detected in this study were in general agreement with the literature values.

Levels of stress hormones are used to intimate the welfare of live fish. However, care must be taken in assuming high levels of stress hormones insinuate poor welfare. Hormones can continue to be released and/ or metabolised after the fish has lost consciousness and so might not always be valid welfare indicators. Their effects on muscle

and therefore flesh quality would still however be valid. The data from this study show the highest adrenaline levels in the electrically stunned and the death in air groups. The fish that were electrically stunned had been stunned within 30s of leaving their home tank, so any stress they experienced was short lived. Electrocuted trout lose sensibility immediately [118], and so high adrenaline levels might not indicate a welfare problem, just an automatic release of the hormone caused by the electrical stimulation. The death in air fish conversely were left for 15min to die and unless they lost consciousness immediately would have been aware of the air exposure. Fish have been shown to remain sensate for many minutes while dying out of water [119]. The environmental temperature influences how long fish remain conscious, 3min at 15°C and over 9min at 2°C [119]. This might have a bearing on fish that die in ice slurry. Their body temperature would be low and so they would remain sensate for longer than fish that die in the air at ambient temperatures. The fish that died in ice slurry in this study therefore would have been expected to be under more severe stress than the fish that died in the air due to the length of the dying period they experienced. However, the level of plasma adrenaline in the ice slurry fish was low, as was the cortisol concentration. Any adrenaline released during the netting procedure would have been metabolised early on during the dying process, as the half life of plasma adrenaline is a few minutes [100]. The low level of plasma adrenaline therefore suggests that these fish did not continue to produce adrenaline although they were probably conscious. The intensity of sensory information is conveyed to the brain by the frequency of nerve impulses and the numbers of fibres sending information [209]. The more important the information the more nerve fibres stimulated and the greater the frequency of impulses. Adrenaline might not have been released from the chromaffin cells in the ice slurry fish because even if large numbers of nerve fibres were responding to the stressor, impulse conduction would have been slow because of the low temperature [197]. If nerve conduction was very slow at the periphery of the fish, the frequency of action potentials in the nerve reaching the synapse might not have been sufficient to excite the post synaptic

neuron and so a response could not be elicited even though the core temperature of the fish was 4.7°C. Provided plasma adrenaline levels are a valid welfare indicator in this instance, then the welfare of the ice slurry fish does not appear to be compromised. The lower cortisol levels in these fish also strengthen this finding, as the fish were not stimulated to produce this chronic stress hormone, although sufficient time had elapsed for synthesis and release. Cortisol release is at the end of a cascade of events initiated by the brain and so if the brain were not stimulated then the cascade would not have started. The diminished primary response in the ice slurry fish found in the present study is in direct contrast to current thoughts on fish welfare at slaughter. Death in ice slurry and air are both thought to be inhumane methods for killing fish [118, 225]. The control fish had lower adrenaline levels compared with the electrically stunned and death in air fish, and similar, but slightly lower levels compared with the percussively stunned fish. The sensory and motor nerves in the control fish would have been blocked by the anaesthetic and so adrenaline levels were expected to be low in this group. The level of anaesthesia was kept at a low level, as the fish had to be sedated for an extended period. The occasional high adrenaline value in the control fish might indicate that once removed from the anaesthetic they started to rouse. The slightly higher adrenaline levels in the percussively stunned group compared with the control values would be due to the netting stresses and/ or incorrect stunning. Percussively stunned fish if hit correctly, lose consciousness instantaneously [118].

The data from this study suggest that percussive stun as a harvesting method could be recommended on welfare grounds, while death in air could not.

4.4.1.2 Plasma chemistry

Plasma glucose is often used as a secondary stress indicator as energy is mobilised to fuel activity to combat stress. Plasma glucose levels are generally acknowledged to rise with stress due to the influence of cortisol. In the current study the fish were sampled before the influence of cortisol was discernible; therefore, the primary influence on plasma

glucose levels was adrenaline. The effect of adrenaline is to lower plasma glucose [100]. The plasma glucose levels in this study ranged from 3.5-13.5μmol/ml, and the ice slurry fish were found to have significantly higher levels than the other killing/ stunning methods. Plasma glucose concentrations reported by Raaij *et al.* [176] were, before exposure to hypoxic water 2.93±0.15μmol/ml, and ~4.1μmol/ml 3.75h after exposure. The plasma glucose in the fish that survived the experiment then returned to pre-exposure levels while in the fish that died, glucose levels decreased to very low levels <1μmol/ml. The plasma glucose levels in the present study therefore support the finding of low adrenaline and cortisol levels in the ice slurry fish.

An increase in plasma protein levels can indicate lowered blood volume in fish. The values of plasma protein in this study ranged from 36-86mg/ml, with the highest amount in the death in air fish. This suggests that the plasma volume might have decreased in the death in air fish. Plasma protein in rainbow trout has been reported elsewhere at 48±0.1 mg/ml [232]. The reduced plasma volume would have been influenced by the osmotic potential of the muscle to which lactate contributes. The plasma lactate levels reflect muscle lactate content, and can give an indication of the level of anaerobic activity that has occurred in fish. Milligan [145] reports maximum blood lactate levels ranging from 12-20μmol/ml 0-2hrs following exhaustive exercise, in rainbow trout, while Raaij *et al.* [176] measured a maximum of 28μmol/ml 6h after exposure to prolonged hypoxic conditions in fish that subsequently died. Plasma lactate values in this study varied from 0-19.8μmol/ml and showed significant differences between all groups apart from the percussively stunned and the control fish. The highest lactate levels and therefore the greatest amount of energy expended in attempting to maintain homeostasis were in the ice slurry fish, then the death in air, electrically stunned and percussively stunned and control fish. Whether these results relate to welfare depends on when the fish lost consciousness. Lactate build up might just reflect the time it took the fish to die i.e. >330 degree-minutes ice slurry, 200 degree-minutes death in air and <30 degree-minutes in the other methods. The electrically

stunned fish might have shown slightly higher lactate levels due to enhanced glycolysis caused by electrical stimulation of the muscle.

Plasma pH values mirror plasma lactate values even though temperature and respiratory factors such as the amount of carbon dioxide dissolved in the plasma also effect pH. The plasma pH levels in the current study ranged from 6.66-7.49. These values were low due to the *post mortem* status of the fish. Plasma pH values in Milligan [145] ranged from 7.25-7.55 immediately after exercise, rising back to physiological levels of 7.9-8.0, depending on temperature, after 4h.

Plasma pH might therefore be a useful indicator of physiological activity in fish during harvest for the industry to use, as it is easily measured, and does not necessarily require the fish to be discarded.

4.4.1.3 Haematocrit and spleen somatic index

Other simple measurements that could be used by the fish farming industry to assess levels of physiological stress in fish at harvest are haematocrit and SSI. Stressed fish would be expected to have a higher haematocrit than unstressed fish as RBC's swell under adrenergic stimulation and hypoxia. They are also recruited into the circulation after adrenergic stimulation of the spleen causes it to contract. The blood volume might also decrease as extra-cellular fluids are taken into muscle cells due to increased osmotic potential.

The haematocrit results of this study range from 34-62% packed cell volume with the death in air fish having the highest value. In a recent study Benfey and Biron [24] report haematocrit values of 38.4±1.2%, rising to 45±2% in rainbow trout subjected to a confinement stress. Although Schreck *et al.* [205] reports a decrease in haematocrit following an electric shock 37.9±2.1% compared with a control value of 40.2±2.7% in rainbow trout. A previous study, Flos *et al.* [80] did not observe changes in haematocrit in rainbow trout that had been stressed through grading and transportation. Anaesthetics (including benzocaine) on the other hand, have been shown to increase haematocrit in rainbow trout

[57, 222]. The type and the extent of the stressor therefore influence whether differences are detectable because of the multiplicity of factors controlling haematocrit.

The SSI's in this study ranged from 0.06-0.4 with an average of 0.16. This value is low but within the range of rainbow trout that had been stressed by netting in Pearson and Stevens [166]. That work demonstrated splenic release of RBC's due to serial sampling. The work used small fish and did not report their condition factor, if the condition of the fish in the present study were higher then this would explain the lower SSI values. Pearson and Stevens [166] also demonstrated a measurable reduction of SSI in fish exposed to air exposure and with anaesthesia (MS222). With *in situ* anaesthesia the SSI remained high but when fish were netted into the anaesthetic the SSI declined, so netting rather than the anaesthetic *per se* was thought to cause splenic discharge.

The data collected for haematocrit and SSI therefore suggest that the well-being of the fish that died in air was the poorest, assuming they had not lost consciousness immediately after being removed from the water. If that were the case, the findings would be an artefact of the experimental timing.

4.4.2 Muscle biochemistry

The effect of stunning/killing methods on muscle biochemistry was investigated as the primary stress response prepares muscle for activity and muscle turns into meat. Muscle biochemistry therefore links stress to quality.

4.4.2.1 Pre rigor mortis

The severity of the homeostatic challenge presented to the fish by the killing/ stunning method was evaluated by measuring the pre *rigor mortis* muscle metabolites. The killing/ stunning method methods that decreased energy levels to the greatest extent were classified as the most severe. The control fish were found to have the highest energy levels and exhibited the least signs of anaerobic activity. The fish that were left to die in air or on ice had the lowest energy status and showed most evidence of anaerobic activity.

The CP: Cr and CP: ATP ratios can be used to compare the results of this study with literature values and evaluate the efficacy of the sampling/ analysis procedures as well as the effects of stress. In *in vivo* NMR experiments with rested common carp the CP: Cr ratio reported is high, in the order of 5:1 showing 80% phosphorylation of the total creatine pool [251]. However, with enzymatic analysis where creatine phosphate is measured in extracts of freeze clamped muscle, values are usually lower [91], although McFarlane *et al.* [144] report 73-80% phosphorylation of the creatine pool when freeze clamping whole trout fry.

In rainbow trout at rest the CP: Cr ratio has been measured at 3.62:1 (78% phosphorylated) [255] and 0.63:1 (39 % phosphorylated) [58]. In exercised rainbow trout a value of 0.03:1 (3 % phosphorylated) has been reported [58], showing that phosphorylation of the creatine pool decreases with exercise. The results from this study range from the control group; 1.44:1 (59 % phosphorylated), and the ice slurry and air groups; 0.03:1 (3% phosphorylated). The values therefore fall within the range of literature values.

The CP: ATP levels in enzymatic studies do not differ greatly from *in vivo* techniques as ATP is replenished by creatine phosphate during sample collection. In *vivo* techniques on carp gave a CP: ATP ratio of 4.56:1 [251], while freeze clamped rested rainbow trout have been recorded at 3.57:1 [78] and 3.33:1 [58]. In exercised trout, Dobson and Hochachka [58] reported a ratio of 3.09:1. The CP: ATP ratio of the control fish, 3.4:1 in this study is in accordance with published values. The considerably lower values seen in the 'methods groups', 0.8-2.1:1 reflects their *post mortem* status. The work of Erikson *et al.* [72] reports an even lower CP: ATP ratio in Atlantic salmon stressed at slaughter, 0.2:1.

To compare the muscle metabolite levels found in this study with published values they were recalculated as μmol/g wet weight ± standard deviation, Table 4.5. The lactate, creatine phosphate and ATP levels were within range of those in the literature. However, the total creatine pool is slightly higher. The levels in Dobson *et al.* [59] fall close to those of the control group, but these were the lowest found in this study. The high values measured

might reflect better preservation of the creatine phosphate in this study increasing the total pool values. Alternatively, they might be an artefact of the analytical procedure, as the assays were performed serially, with the creatine analysis following the creatine phosphate.

Table 4.5 Comparison of pre *rigor mortis* metabolite data with published values

metabolite	Wang 1994 rested	Dobson *et al.* 1987 rested	Dobson 1987 rested	Dobson 1987 exercised	Current study control	Current study air
lactate	2.00±0.16	5.76±0.49	1.56±0.95	23.38±0.51	4.1±1.70	44.3±13.93
CP	25.95±0.87	19.83±0.92	17.47±0.78	1.39±0.07	29.5±5.35	1.9±3.32
creatine	7.16±0.14	31.5±0.87	27.52±3.85	43.97±1.31	25.9±10.88	62.2±10.38
ATP	5.3±0.55	7.33±0.29	5.24±0.13	0.45±0.03	8.3±0.79	2.4±2.25

The reported levels of the metabolites measured in rested and exercised rainbow trout measured in μmol/g wet weight ± SEM, current study μmol/g wet weight ± SD [58, 59, 255].

The muscle metabolite data suggest that the fish that died in ice slurry and air had expended the most energy in attempting to maintain homeostasis whilst dying. They were therefore the most metabolically stressed, but whether this equates to poorer welfare, depends on how long they remained conscious. The lower energy status and consequent higher lactate levels could potentially cause a decline in flesh quality relative to less metabolically active fish. The electrically stunned fish showed less evidence of metabolic activity than the fish killed in ice slurry and air, but more than the percussively stunned fish. The difference between the electrically and percussively stunned fish could have been caused by the electrostimulation of the muscle and nerves. Providing the electrically stunned fish lost consciousness immediately they were shocked, the stunning method would not have had a negative influence on the well being of the animal, however the increased activity could have a detrimental impact on flesh quality. The control fish had both motor and sensory nerves blocked and was, for obvious reasons, the least metabolically active, theoretically they had the best muscle quality, the anaesthetic would however render it unfit for human consumption.

4.4.2.2 Post rigor mortis

4.4.2.2a Lactate

Post mortem lactate levels indicate the amount of anaerobic glycolysis that has occurred in muscle. The rate of lactate production will depend on the activity of the glycolytic enzymes and the energy supplies remaining in the muscle. The post *rigor mortis* lactate values of this study ranged from 138-379μmol/g dry wt. They increased at different rates over the four days ice storage reaching comparable levels irrespective of killing/ stunning method. This suggests that the potential of the tissue to produce lactate was similar in all the groups *ante mortem*.

Post mortem lactate production in fish muscle has been demonstrated in many species sardines: [259], cod [196] and Atlantic salmon [68]. Einen and Thomassen [68] showed that four days *post mortem* muscle lactate levels were higher than initial values, as in this study, but that the levels did not change over the next eight days.

4.4.2.2b K value

K value is a measure of fish freshness, it was measured on tissue taken after four days ice storage. The K value of the control group was significantly lower than the commercial killing methods, showing that these fish were fresher. The lower K value would be expected as the control fish had larger energy reserves immediately *post mortem* and so could maintain ATP levels for longer. The greater production of lactate in the control fish during ice storage also indicates that more ATP was produced in this group. This finding is supported by the *pre rigor mortis* metabolite data which, also indicates that the control fish had the highest energy levels.

Recalculating the data to K' values, for comparison with the literature, gave 40%, 49%, 50% 53%, 51% for the control, ice slurry, air, percussive stun and electrically stunned fish, respectively. A previous study, Rodríguez *et al.* [188], reported K' values of 49-58% in electrocuted rainbow trout three days *post mortem*. That K value increases with higher stress levels at death has been reported in Atlantic salmon by Erikson *et al.* [71]. The only

significant difference found between the stressed and unstressed salmon in that study was at two days *post mortem*. This would have been when *rigor mortis* in the unstressed salmon was less well developed and ATP levels would have been considerably higher. The results of this study show that rainbow trout tissue behaves in a similar fashion to the Atlantic salmon. The K value in the 'unstressed', (control), rainbow trout was significantly lower than the 'stressed' fish, (industrial methods), as *rigor mortis* had not quite resolved in the control while it had in the industrial methods groups.

These findings suggest that activity during harvest decreases freshness in rainbow trout flesh during subsequent storage, and so to obtain superior quality trout, activity should be minimised.

4.4.3 Muscle quality

4.4.3.1 Muscle pH

Measurements of muscle pH immediately after death can give an indication of the rate and extent of anaerobic activity in muscle. After prolonged ice storage, muscle pH can be used to signify the onset of spoilage. It influences the characteristics of constituent proteins and so affects meat quality.

The pH values reported in the literature for rainbow trout at death varies due to difficulties in obtaining stable readings and truly rested fish. Readings with stab electrodes are not always stable in the first few minutes *post mortem*, as they measure the rapid production of lactate that occurs and possibly mixing of intra- and extra-cellular fluids. The rate of decline in muscle pH is not generally presented in the literature. Muscle pH in anaesthetised rainbow trout has been reported as 7.21±0.02 [174] and pH 7.8±0.31 [186]. The extremely high pH values in Robb *et al.* [186] might reflect proton depletion during creatine phosphate resynthesis and mitochondrial oxidative phosphorylation, which occurs while tissue oxygen levels are high, or else a very low environmental temperature. Electro-stimulated fish in the same experiment had a pH of 6.7 ±0.03 [186]. Marx *et al.* [141]

investigated different slaughter procedures and measured pH's of 6.85 for percussive stun, 6.82 for electrical stun, and 6.75 for carbon-dioxide narcosis in rainbow trout. The pH of the control group in this study, 7.54±0.12, is therefore within the range of literature values in rested rainbow trout. The results of the commercial methods: percussive stun; 7.25±0.16, electrical stun; 7.15±0.16, ice slurry; 6.75±0.11, and air; 6.73±0.27 were also within the higher ranges of published values.

The pH during ice storage can give an indication of the degree of spoilage occurring in fish. Initially the pH falls and then it rises due to lactate and TVB formation, respectively. The pH_{96} values in the present study range from 6.57-7.13 and were similar to but slightly higher than those found for rainbow trout in the literature [14]. Differences in initial muscle glycogen levels and fish storage procedures mean direct comparisons of pH might not be reliable after this length of time. The pH_{96} of the control and the stunned fish had fallen relative to their pH_i values due to the increased lactate levels. The pH_{96} values of the ice slurry and death in air fish were fractionally higher than their pH_i's. This suggests that the ice slurry and death in air groups were less fresh than the other groups. The control fish pH_{96} was significantly higher than that of the ice slurry and air fish but not to the stunned fish suggesting that the control fish were the freshest. The lower post *rigor mortis* lactate levels in the control fish supports this finding. This data strengthens the finding that the control group was freshest as seen in the K value data.

The muscle pH data therefore suggests that the greater the energy expended in maintaining homeostasis whilst dying the greater potential for deterioration in muscle quality.

4.4.3.2 *Rigor mortis*

Development of *rigor mortis* is generally thought to indicate welfare of fish at harvest, fish that are 'stressed' enter *rigor mortis* earlier than 'unstressed' fish.

The peak in the *rigor mortis* contraction in this study occurred 38 h *post mortem* in the

control fish, and between 0-17h in the industrial methods. Data presented by Robb *et al.* [186] shows the maximum *rigor mortis* contraction in anaesthetised and electrostimulated rainbow trout at approximately 35 and 12 hours, respectively. The contraction strength was lower and the duration of *rigor mortis* was longer in the anaesthetised fish in Robb *et al.* [186], as shown in the current study.

Rigor mortis development is dependent on the reduction of muscle ATP levels, and can therefore be related to muscle activity during death. If the degree of muscle activity at harvest is a welfare issue, then the greater the delay in *rigor mortis* the better the well-being of the fish. *Rigor mortis* also relates to quality in that fish that are handled during *rigor mortis* are more subject to gaping, and processing procedures for some products need to be adapted to accommodate *rigor mortis* development. For sale 'in the round', a delay in *rigor mortis* onset might be thought beneficial as fish in *rigor mortis* are perceived to be fresh. However, for smoked products and fish fillets where *pre-rigor mortis* processing can lead to inferior quality products, early, although not immediate *rigor mortis* might be more desirable.

4.4.3.3 Freshness

Freshness is an extremely important factor when marketing fish. Torrymeter data collected in this study show that increased activity at harvest reduces fish freshness. The death in ice slurry fish lost freshness significantly faster then the other industrial methods. The control fish were significantly fresher than the commercial methods: a finding supported by the K value data. Published information on fish freshness using the Torrymeter however is not abundant. A study by Hattula *et al.* [87], found the Torrymeter was not sufficiently sensitive to detect differences caused by killing/ stunning method (trawl, gillnet and trapnet), in Baltic herring (*Clupea harengus*, Linnaeus 1758). A minimally stressed group was not possible in the work and the rate of decline in freshness as measured by the Torrymeter was not reported. In the first measurement of the current study the only difference detected was between the control group and the industrial methods, $H=50.73$, $P<0.0001$, $df=4$, $n=60\text{-}66$, this therefore supports Hattula *et al.'s* [87]

finding.

The data in this study therefore suggest that the quality of fish can be compromised at harvest through increased muscle activity leading to a reduction in freshness.

4.4.3.4 Centrifugal drip

Centrifugal drip was measured to determine if killing/ stunning method influenced drip losses in trout. Increased drip loss reduces the value of fish due to decreased yield and nutritional status.

Differences were not detected in centrifugal drip between the killing/ stunning methods tested on tissue taken after four days ice storage, values ranged from 7-19% loss. This finding agrees with Azam *et al.* [14] who did not find differences in drip loss between killing methods over ten days in rainbow trout, although drip loss increased with time. A previous study, Marx *et al.* [141], measured water binding capacity immediately after slaughter in a variety of species including rainbow trout. A consistently lower water binding capacity was detected in fish stunned by carbon-dioxide narcosis, although differences between stunning methods were not significantly different. This suggests that water binding capacity is affected more by the timing of *rigor mortis* (advanced in carbon-dioxide narcosis) than by killing/ stunning method *per se*. Direct comparison was not made with the literature values, as results are dependent on techniques used.

As this technique did not detect differences shown by other factors, it would not be a useful indicator of welfare or quality for the fish farming industry.

4.4.3.5 Downgrading

A visual assessment was performed to assess carcass damage caused by the killing and stunning methods.

Scale loss and external haemorrhages were greater in the percussively stunned fish, although this finding was not significant. The electrically stunned fish showed more evidence of blood spotting and spinal damage. Killing/ stunning technique did not appear to influence gaping.

The two harvesting methods recommended for fish on welfare grounds percussion and electrocution [118] have both been shown to be associated with quality defects. Percussive stunning is linked with increased levels of eye damage and bruising [253]. While electrical stunning increases the incidence of fillet bloodspotting and spinal fracture, Cary 1995 cited in [253, 258]. These findings were similar to the observations made in this study. However, Robb *et al.* [186] also report increased evidence of gaping in fish that have been electrostimulated compared with anaesthetised fish, a finding not seen in the current work. The methodology used by Robb *et al.* [186], 2min electrostimulation, (94v, pulsed DC at 14.3Hz), might have had a more marked effect on the fish flesh than the killing/ stunning methods used in the current study. The technique used to reveal gaping potential might also have been more severe: the results of the two studies might not therefore be directly comparable.

The results of the present study show that downgrading can be influenced by killing/ stunning technique. The quality defects associated with percussive and electrical stunning are readily visible and so might suggest to the fish farming industry that these techniques are less suitable for producing superior grade fish. The low muscle activity, which maintains freshness in fish killed by these methods, would not be apparent in a visual assessment and might therefore be overlooked. On a superficial level therefore, killing/ stunning methods that are recommended on welfare grounds appear to compromise quality.

4.4.3.6 Colour measurements

Flesh colour is an important quality trait in fish as up to 40% of a consumers decision to buy a particular fish product is determined by colour [178]. Both instrumental and visual colour measurements were made on fish fillets in the present study. The only colour reading to show a significant difference between the killing/ stunning methods tested was lightness. The percussively stunned fish were significantly paler than all but the ice slurry fish. The chroma and angle of hue values showed no differences although the

power of these tests was low, around 40%, so conclusions of no difference could not be made. Similarly, the visual assessment with the Roche *Salmo*fan™ was unable to detected differences between the methods.

For the lightness value to be higher in the percussively stunned fish compared with the controls would agree with the published literature. Fish that have experienced severe muscle activity show poorer colour characteristics [181, 182, 186]. However, for the percussively stunned fish to have inferior colour than the death in air and electrically stunned fish conflicts with this theory. Robb *et al.* [186] also report higher values for chroma and angle of hue and lower Roche colour card™ scores, in fish electrostimulated compared to anaesthetised fish; meaning that the flesh was more yellow and opaque in the electrostimulated fish. Dissimilarities in the *post mortem* handling of the fish and the timing of the measurements could influence the results. The instrumental colour measurements on the fillets in Robb *et al.* [186] were made over three days on the same fillets, the Roche colour card™ score was made on the same fillets 75 hours *post mortem*. Robb *et al.* [186] suggest that the differences observed in colour in the early measurements were real but that later ones might have been random.

No conclusions as to whether activity at slaughter influences flesh colour can be made from the data collected during the current study.

4.4.3.7 Texture

The texture of fish is important as a processing as well as an eating quality. It has been suggested that farmed fish have a softer texture and are therefore inferior quality to wild caught fish.

The results of this study show no significant differences in texture between the groups when tested using a two-tailed statistical test. If a one-tailed test was used, justified by published data demonstrating softening with stress in fish, the percussively and electrically stunned groups were significantly firmer than the death in ice slurry fish. The

work by Sigholt *et al.* [211] provides such a justification, where softening was detected in stressed Atlantic salmon relative to unstressed fish. However, other authors have failed to establish differences in texture with stress level. Ostenfeld *et al.* [163] did not see differences in hardness, elasticity or breakpoint in rainbow trout subjected to transportation stress. Faergemand *et al.* [75] found that fillet texture was more influenced by storage time than killing method (death in air or severed neck). Azam *et al.* [14] also did not detect differences in hardness or elasticity five days after slaughter in rainbow trout killed by electrical stun, carbon-dioxide narcosis or percussive stun. Textural properties of muscle are heavily dependent on fish size, sample size and location and test conditions and so direct comparisons with the literature could not made.

The data from the study therefore weakly suggests that softening of the flesh advances in fish that have experienced high levels of muscle activity during harvest.

4.5 Summary and conclusion

Killing/ stunning methods were shown to have an effect on the welfare and quality of farmed rainbow trout. The control fish contained low levels of adrenaline and the highest remaining energy reserves. They retained freshness longest as determined by both the K value and Torrymeter measurements. The fish that died in ice slurry and air, i.e. the fish that were probably sensate whilst dying, had the lowest energy status. Although, only the death in air fish had high adrenaline levels and so could be considered stressed. The ice slurry fish had very low adrenaline and cortisol levels but the decline in freshness in these fish was fastest as measured by the Torrymeter. This suggests that the primary stress response has a lesser influence than time on energy level reduction. *Rigor mortis* resolved earliest in the ice slurry group due to their lower energy status, and they showed a possible trend towards softening due to the rapid decline in pH caused by lactate formation. The percussively and electrically stunned fish were intermediate in terms of freshness. The percussively stunned fish had low levels of adrenaline while the electrically stunned fish had

released large amounts during the stunning procedure. The energy status of the percussively stunned fish was closer to that of the control group. The stunned fish sustained greater damage during killing, particularly the electrically stunned group.

The welfare of fish percussively stunned at harvest is good assuming they are struck correctly. Provided electrically stunned fish lose consciousness immediately, then this technique could also be recommended to the industry on welfare grounds. The well-being of fish left to die in the air is poor. For fish dying in ice slurry, the issue is less clear, the primary stress response appears to be diminished, but muscle activity is very high.

Lower activity levels in fish during death is advantageous for quality, as the higher the carcass energy status the longer freshness can be maintained. Percussive and electrical stunning therefore can enhance the freshness of fish relative to death in air and ice slurry. However, these stunning techniques can cause physical damage, which leads to downgrading of the fish.

5 THE EFFECTS OF KILLING AND STUNNING TECHNIQUE ON THE *POST MORTEM* BIOCHEMISTRY OF ANAESETHETISED RAINBOW TROUT

5.1 Introduction

Fish harvesting methods commonly used on UK fish farms cause a variable stress response in rainbow trout, as discussed in Chapter 4. To gain a better understanding of the effect of killing/stunning methods on fish welfare, further work was undertaken. The present study evaluated whether the biochemical changes observed during harvest were due to the killing/ stunning methods *per se*, or the primary stress response as mediated by the nervous system. This was achieved by killing anaesthetised fish using the harvesting methods tested previously.

5.2 Materials and methods

Immediately after the final sampling in the previous study, where fish had been anaesthetised *in situ*, an additional set of fish were sampled. These anaesthetised fish were killed/ stunned using ice slurry, air, or electricity as previously described in Section 4.2.5. Five fish were killed by each method for *rigor mortis* measurement, or for the provision of muscle or blood samples. The results are presented alongside the data from the other fish killed during that session, which act as the percussively stunned group, (n=10). The facilities and conditions are as described in Section 4.2. The fish were sized: 30.0±1.5cm; 380±50g; and had a Fulton's condition factor of 1.37±0.13. Air and water temperatures were 15°C and 11°C, respectively. Samples were analysed within the time scale detailed in Section 4.2.6.

5.3 Results

5.3.1 Plasma biochemistry and related parameters

The effect of killing/stunning technique on the blood of anaesthetised trout was evaluated to determine whether the stress response required initiation by the brain and or autonomic nervous system.

5.3.1.1 Plasma hormones

The effects of killing/stunning method on plasma stress hormone levels in anaesthetised fish are illustrated in Figure 5.1A-C.

5.3.1.1a Catecholamines

The death in air fish had the highest levels of adrenaline, 509% of that of the percussively stunned fish, while the levels in the electrically stunned and death in ice slurry fish were 146% and 17%, respectively. The adrenaline concentration in the death in air fish was significantly greater than that of all the other groups as indicated by the Games Howell *post hoc* test (liberal), Figure 5.1A. The more stringent Tamhane *post hoc* test narrowly failed to show a difference between the death in air and the percussively stunned fish, (P=0.051). Furthermore, the adrenaline levels in the ice slurry group were significantly lower than those of the percussively stunned fish, Figure 5.1A.

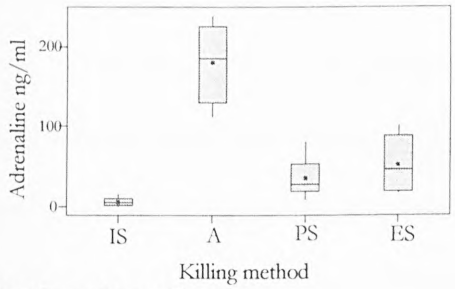
The noradrenaline data also showed differences between the groups. The death in air fish had significantly greater levels of noradrenaline than the ice slurry and percussively stunned fish, Figure 5.1B. However the power of the test was low and so no definite conclusions could be drawn from the data. Expressed as a percentage of the percussively stunned fish the noradrenaline levels were 141% in the ice slurry fish, 288% in the death in air fish, and 152% in the electrically stunned fish.

5.3.1.1b Cortisol

There were distinct differences between the killing/stunning method groups in plasma cortisol, Figure 5.1C. The ice slurry and death in air groups contained significantly less cortisol than the percussively stunned fish 33%, and 52%, respectively; while the

electrically stunned fish contained 87% of the percussively stunned fish cortisol levels. However, the power of the test was low and so no definite conclusions could be made from the data.

A: Adrenaline

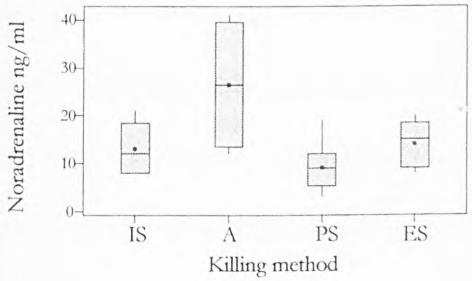


Inferential statistics
F=29.2, P=0.000, df=3, 20, power~0.90, n=4-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry		*#	*#	ns#
air	*$		*#	*#
percussive stun	*$	ns$		ns#
electrical stun	ns$	*$	ns$	

B: Noradrenaline

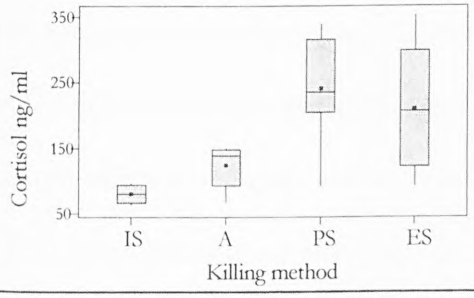


Inferential statistics
F=5.74, P=0.005, df=3, 20, power~0.28, n=4-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
air	*‡			
percussive stun	ns‡	**‡		
electrical stun	ns‡	ns‡	ns‡	

C: Cortisol



Inferential statistics
F=6.76, P=0.002, df=3, 19, power~0.53, n=4-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry		ns#	***#	ns#
air	ns$		**#	ns#
percussive stun	***$	**$		ns#
electrical stun	ns$	ns$	ns$	

Figure 5.1A-C Effect of 'killing' method on plasma stress hormones in anaesthetised trout
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests Tukey ‡ (between groups, equal variance) Tamhane $ (between groups, unequal variance, conservative), Games Howell # (between groups, unequal variance, liberal). Samples measured in duplicate, n=5 or 10 (PS). P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The plasma catecholamine levels indicate that the death in air technique elicited a greater stress response than the other methods, even though all the fish were anaesthetised. This suggested that, in this instance, catecholamine release might not only be mediated by the nervous system, but by factors such as low oxygen, high carbon dioxide or low pH

levels [115, 167, 168]. Another interpretation of the data would be that the anaesthetic lost effectiveness in the death in air fish as their body temperature was higher and the dying period longer allowing more clearance/ breakdown of the anaesthetic. If this were the case, then the death in air fish would have become aware of, and responded to, their circumstances and released adrenaline.

The death in ice slurry group contained the least plasma adrenaline although their noradrenaline levels were similar to those of the percussively and electrically stunned fish. The lower body temperature in these fish, 2.2±0.4°C, compared with the other groups, approximately 12°C, might have delayed or even prevented release of the hormone.

The adrenaline: noradrenaline ratio in the ice slurry fish was very low, 0.52:1, significantly lower than that of the death in air fish, 5.35:1, which was the highest. The high noradrenaline relative to the adrenaline values in the ice slurry group would have produced this effect. The adrenaline: noradrenaline ratios were also lower than expected in the percussively and electrically stunned fish, 3.37:1 and 3.93:1, respectively. The ratio in chromaffin tissue is reported to be 5:1 [100]. The lower than expected ratios might suggest that the fish were slightly hypoxic, as noradrenaline release is stimulated by hypoxia [30]. The prolonged anaesthesia might have caused hypoxia by decreasing the ventilation rate, which in turn would have reduced gaseous exchange. The adrenaline: noradrenaline ratios expressed as a percentage of the percussively stunned fish values were 15%, 159%, and 116% in the ice slurry, death in air, and electrically stunned fish, respectively, and are illustrated in Figure 5.2.

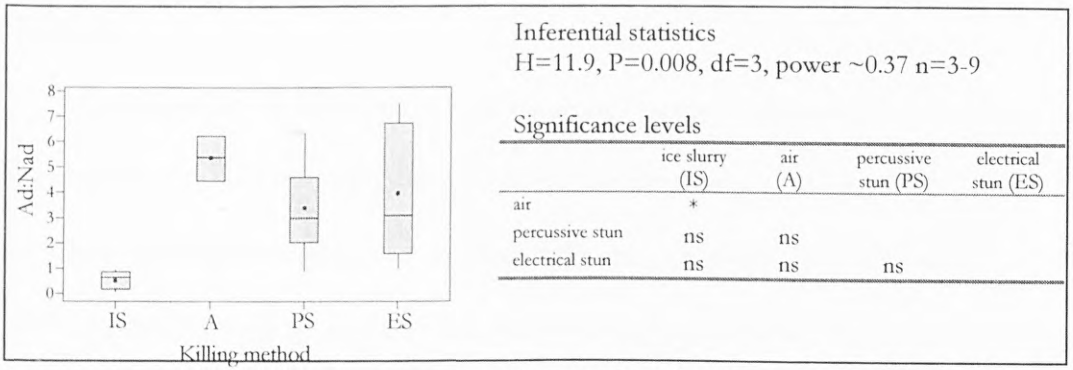


Figure 5.2 Effect of 'killing' method on adrenaline: noradrenaline ratio in anaesthetised trout
Data presented as a box and whisker plot with significance levels of Kruskal Wallis test with Dunn *post hoc* test. Ratios calculated on an individual fish basis, outliers removed. P≥0.05 ns, P<0.05 *.

The cortisol levels in the ice slurry and death in air fish were significantly lower than those in the percussively stunned fish: the ice slurry fish contained the least cortisol. There might have been a delay in cortisol production/release due to impaired nervous function due to the anaesthesia, and in the ice slurry fish, low body temperature. The percussively stunned fish were sampled earlier in the day and so would have been expected to contain slightly more cortisol than the other fish due to diurnal variation of cortisol. The difference due to daylight would however only be small, in the region of 0-25ng/ml [30]. The power of the test was not sufficiently high for firm conclusions to be made from this data.

5.3.1.2 Plasma chemistry

The effects of killing/ stunning method on plasma pH, lactate, glucose and protein in anaesthetised fish are summarised in Figure 5.3A-D.

Plasma pH values were similar in the percussively and electrically stunned fish and in the death in air and ice slurry fish. The plasma pH values of the latter group were about 5% lower than the former, Figure 5.3A.

Regardless of the killing methods used, plasma lactate concentrations had a high correlation with the plasma pH values, Figure 5.3A-B. The correlation coefficient for all the data was $r=-0.90$, $r^2=0.81$. The lactate levels in the electrically and percussively stunned groups were similar to each other, as were those in the death in air and ice slurry fish. The plasma lactate concentrations in the ice slurry and death in air fish expressed as a percentage of the percussively stunned fish were 521% and 762%, respectively.

Compared to all the other groups, there were significantly greater amounts of plasma glucose in the electrically stunned fish. The death in air fish also had significantly more plasma glucose compared with the percussively stunned fish. Relative to the percussively stunned fish, the plasma glucose values were; 145%, 148%, and 205% in the ice slurry, death in air and electrically stunned fish, respectively.

The percussively stunned fish had significantly greater levels of plasma protein than all the other groups. The ice slurry fish had 58%, death in air fish 69% and electrically stunned fish 72% of the plasma protein level in the percussively stunned fish. However, the power of the test was not sufficient for definitive conclusions to be drawn from the data.

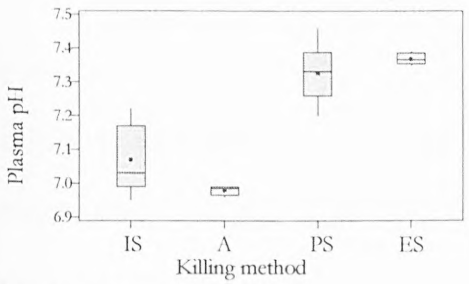
The plasma pH and lactate values suggest that the ice slurry and death in air fish underwent more anaerobic activity than the stunned fish even though they were anaesthetised: this might therefore reflect the time that had elapsed before sample collection.

The high glucose level in the electrically stunned fish and to a lesser extent the death in air fish are less easily explained. It could be an effect of the anaesthetic *per se*. The percussively stunned group would have been less affected, as they were anaesthetised for a shorter period and so the glucose levels would not have risen to such an extent. The prolonged anaesthesia in the death in air, ice slurry and electrically stunned groups might have made these fish more hypoxic, as discussed in Section 5.3.1.1. Hypoxia increases glucose production in the liver by altering enzymatic activity [269]. Therefore, these fish might have been expected to have higher glucose levels. Ionic disturbances have been reported in anaesthetised fish which might indicate that osmoregulation can be disturbed by anaesthesia [94, 222]. Thus, if the anaesthetic impaired osmoregulation, which is energetically expensive, then the plasma glucose levels might have risen as a consequence. The plasma glucose levels in the ice slurry fish would not have been influenced to the same extent because of their lower metabolic rate and the already elevated levels, discussed in Section 4.3.1.2. This still does not fully explain the very high glucose levels in the electrically stunned fish. The anaesthetic might have augmented any response these fish had to the electro-stimulation. Electricity is known to increase the permeability of cell membranes. The lipid molecules of the bilayer reorient to form hydrophilic pores in the membrane

through which cell metabolites can diffuse [88, 236, 261]. If the concentration of glucose in fish RBC's is greater than that of the plasma, as in mammals [230], then plasma glucose levels would rise as a result of electrostimulation. Resealing of the pores created by the electrical stimulation can be slower during anaesthesia [248]. Therefore, if electro-permeabilisation was occurring in the anaesthetised electrically stunned fish in present study, glucose diffusion from the RBC's into the plasma would be enhanced relative to the non-anaesthetised fish and glucose levels would rise commensurately.

The high plasma protein in the percussively stunned fish suggests either a reduction in plasma volume in these fish or an increase in plasma volume in the other groups. The percussively stunned fish were sampled first, so changes in plasma volume due to impaired osmoregulation would not have been evident. Other influences such as muscle lactate levels would not have reduced the plasma volume relative to the other groups, see Section 5.3.2. The lower plasma volume in the percussively stunned fish might have contributed to the relatively higher cortisol levels seen in this group. The ice slurry, death in air, and electrically stunned fish were anaesthetised for an extended period, 5.5h, so any reduction in osmoregulation efficiency had sufficient time to make detectable differences i.e. there was time for water to inflow across the gills.

A: pH

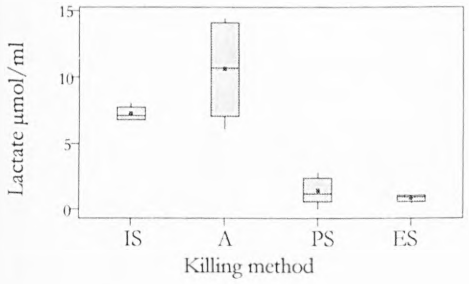


Inferential statistics
F=31.6, P=0.000, df=3, 19, power~0.76, n=4-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry		ns#	**#	**#
air	ns$		***#	***#
percussive stun	*$	***$		ns#
electrical stun	*$	***$	ns$	

B: Lactate



Inferential statistics
F=40.4, P=0.000, df=3, 20, power~0.94, n=4-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
ice slurry		ns#	***#	***#
air	ns$		*#	*#
percussive stun	***$	*$		ns#
electrical stun	***$	*$	ns$	

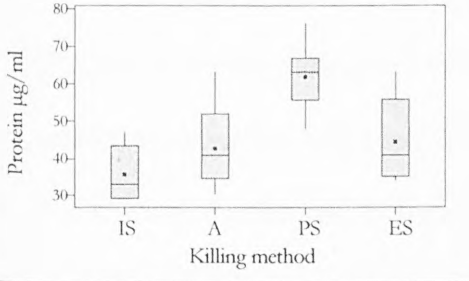
C: Glucose

Inferential statistics
F=13.3, P=0.000, df=3, 21, power~0.73, n=5-10.

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
air	ns‡			
percussive stun	ns‡	*‡		
electrical stun	*‡	*‡	***‡	

D: Protein



Inferential statistics
F=10.09, P=0.003, df=3, 21, power~0.57, n=5-10.

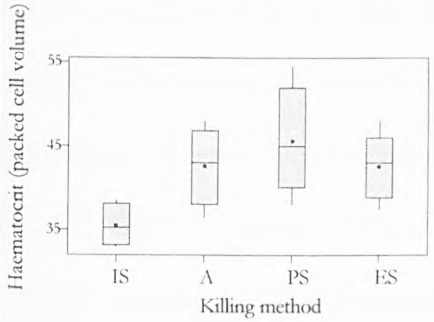
Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
air	ns‡			
percussive stun	***‡	**‡		
electrical stun	ns‡	ns‡	*‡	

Figure 5.3A-D Effect of 'killing' method on plasma biochemistry of anaesthetised trout
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* tests, Tukey ‡ (between groups, equal variance) Tamhane $ (between groups, unequal variance, conservative), Games Howell # (between groups, unequal variance, liberal). Samples measured in duplicate, n=5 or 10 (PS). P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

5.3.1.3 Haematocrit

The percussively stunned fish had the highest haematocrit values. Relative to this group, the values in the ice slurry, death in air and electrically stunned fish were; 78%, 93%, and 93%, respectively. The high haematocrit reading in the percussively stunned fish supports the finding of higher plasma protein levels. The lowest haematocrit, found in the ice slurry fish, might signify a reduction in RBC swelling due to low catecholamine levels, and/ or increased plasma volume as shown by the low protein levels.



Inferential statistics
F=3.69, P=0.034, df=3, 16, power=0.39, n=4-7

Significance levels

	ice slurry (IS)	air (A)	percussive stun (PS)	electrical stun (ES)
air	ns			
percussive stun	*	ns		
electrical stun	ns	ns	ns	

Figure 5.4 Effect of 'killing' method on haematocrit of anaesthetised trout
Data presented as a box and whisker plot with significance levels of ANOVA Tukey *post hoc* test. Data from duplicate measurements, P≥0.05 ns, P<0.05 *.

5.3.2 Muscle biochemistry

The level of muscle activity occurring in the fish was assessed by measuring pre *rigor mortis* muscle metabolites. No differences were detected between the killing/ stunning methods in the metabolites measured.

The creatine phosphate levels were; 163±5μmol/g dry wt in the ice slurry fish, 177±22μmol/g dry wt in the death in air group, and 162±19μmol/g dry wt in the percussively stunned fish, while the electrically stunned fish contained 155±18μmol/g dry wt. The inferential statistics for creatine phosphate were, F=1.445, P=0.259, df=3, 21, power~0.27, n=5-10.

Consequently, there were no differences between the harvesting methods in creatine, F=0.696, P=0.565, df=3, 21, power~0.15, n=5-10. The concentrations of creatine in the muscle were; 102±32μmol/g dry wt, 132±35μmol/g dry wt, 120±40μmol/g

dry wt, and 124±32μmol/g dry wt, in the ice slurry, death in air, percussively stunned, and the electrically stunned fish, respectively.

The inferential statistics for ATP were, F=0.789, P=0.514, df=3, 21, power~0.16, n=5-10. The levels of ATP in the muscle were; in the ice slurry, death in air, percussively stunned, and the electrically stunned fish; 44±6μmol/g dry wt, 49±7μmol/g dry wt, 47±5μmol/g dry wt, and 48±5μmol/g dry wt, respectively.

The muscle lactate levels were; 12±10μmol/g dry wt in the ice slurry fish, 32±14μmol/g dry wt in the death in air group, 24±11μmol/g dry wt in the percussively stunned fish, while the electrically stunned fish contained 32±21μmol/g dry wt. Differences were not found between the harvesting methods in muscle lactate, F=2.33, P=0.105, df=3, 21, power~0.33, n=5-10.

Creatinine was not detected in any of the samples suggesting that creatine phosphate and creatine were not degraded further.

The CP: Cr ratios were in the ice slurry, death in air, percussively and electrically stunned fish; 1.75:1, 1.39:1, 1.46:1 and 1.31:1, respectively. The killing/stunning method used did not give rise to differences in the CP: Cr ratio, F=0.97, P=0.42, df=3, 21, power~0.19, n=5-10.

The CP: ATP ratios were 3.77:1, 3.65:1, 3.48:1 and 3.23:1 in the ice slurry, death in air, percussively and electrically stunned fish, respectively and differences were not detected between the killing/ stunning methods, F=1.45, P=0.26, df=3, 21, power~0.27, n=5-10.

Killing/ stunning methods therefore did not appear to influence pre *rigor mortis* muscle metabolites in anaesthetised fish, although the percussively stunned fish had the highest and the electrically stunned fish the least energy reserves according to both ratios.

Muscle activity was minimal in all the fish probably because the anaesthetic prevented muscle stimulation either by blocking the motor nerves or by acting centrally on the brain.

5.3.3 Muscle quality

Muscle quality was measured in the anaesthetised fish killed/ stunned by the various methods to relate to the muscle biochemistry measurements as muscle activity can influence carcass quality.

5.3.3.1 Muscle pH

The pH of anaesthetised fish muscle was measured immediately *post mortem* for 60 seconds, as an estimation of the rate of anaerobic glycolysis occurring in the muscle.

The slope of the regression line of the pH measurements was used to estimate the rate of anaerobic activity, Figure 5.5 and Table 5.1. The slope was significantly shallower in the fish that died in the ice slurry relative to the percussively and electrically stunned groups, Tables 5.1 and 5.2. Compared with the percussively stunned fish, the decline in pH was; 19% in the ice slurry fish, 79% in the death in air fish, and 114% in the electrically stunned fish.

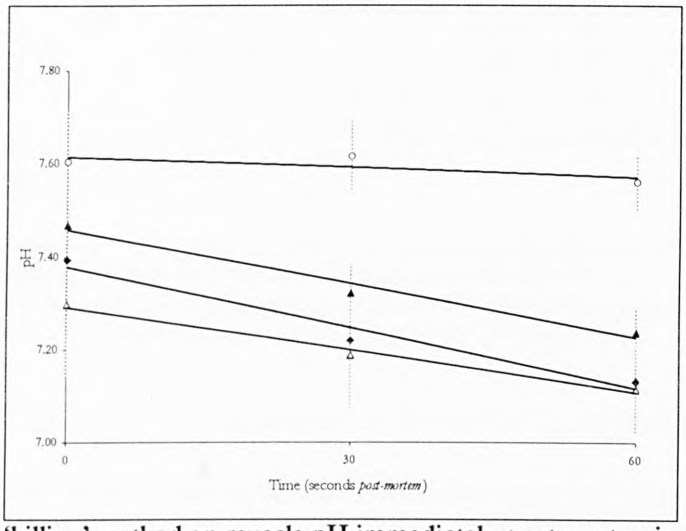


Figure 5.5 Effect of 'killing' method on muscle pH immediately *post mortem* in anaesthetised trout
Results expressed as mean ± standard deviation, n= 5 or 10 (PS) from single readings; ice slurry (○), air (△), percussive stun (▲), electrical stun (◆)

Table 5.1 Regression line data for the effect of 'killing' method on muscle pH in anaesthetised trout

method	intercept	slope	R^2	n
ice slurry	7.62	-0.00073	0.55	5
air	7.29	-0.00303	0.99	5
percussive stun	7.46	-0.00385	0.98	10
electrical stun	7.38	-0.00437	0.97	5

Single measurements per fish, (blood set).

Table 5.2 Parallelism and identical regression line test statistics for the effect of 'killing' method on the pH of anaesthetised muscle, t values and significance levels

intercept	slope			
method	ice slurry	air	percussion	electricity
ice slurry	----	-1.501 ns	-3.034 **	-2.897 **
air	5.567 ***	----	-0.648 ns	-0.859 ns
percussion	4.239(***)	-3.659***	----	-0.493 ns
electricity	4.935(***)	1.490 ns	2.064 *	----

Single measurements per fish, n=5 or 10 (PS).

The initial pH measurement (pH_i) was used to evaluate the amount of anaerobic glycolysis that had occurred in the muscle whilst the fish were dying. The data show a significantly higher pH in the ice slurry group compared with the death in air and electrically stunned fish. The muscle pH of the percussively stunned group was also significantly higher than the death in air fish, Figure 5.6.

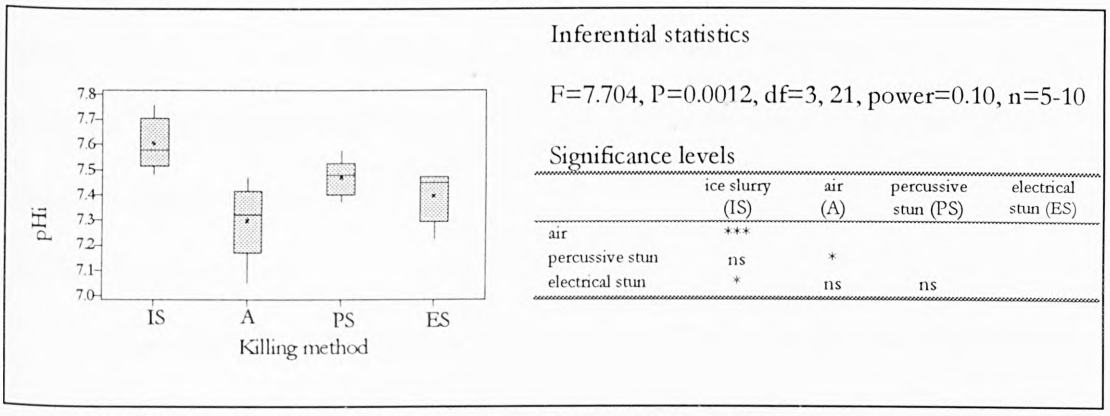


Figure 5.6 Effect of 'killing' method on muscle pH in anaesthetised trout
Data presented as box and whisker plots with significance levels of ANOVA Tukey *post hoc* test. Data from single measurements, n=5-10. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The rate of decline in pH was steepest in the electrically stunned group, which might suggest that the electrical stimulation increased the enzymatic activity in the muscle of these fish. The rate of decline in pH in the ice slurry group was the shallowest, which corresponds with the finding of the greatest muscle pH in this group. Their body temperature was the lowest and so, in the absence of nervous stimulation they would have undergone the least anaerobic glycolysis. The increased time elapsed between netting and sample collection did not appear to mask the effect of temperature in the ice slurry group. The muscle pH pattern relates to the metabolic activity of the fish as influenced by body

temperature whilst dying. The body temperatures of the fish were; 2.2±0.4°C in the ice slurry, 12.7±0.6°C in the death in air group, 11.5±0.2°C percussively stunned fish, and 12.3±0.3°C in the electrically stunned fish. The death in air fish spent 15min in the air dying, the air was 4°C warmer than the water, and consequently their body temperature rose. The water in the electrical stunning tank would have had time to equilibrate with the air temperature and so increased the body temperature of the electrically stunned fish.

The muscle lactate levels although not showing statistical significance, show a similar pattern to the pH data, this strengthens the supposition that body temperature influenced metabolic activity/ lactate levels. The lowest lactate levels were in the ice slurry fish 12μmol/g dry wt, then in the percussively stunned fish 24μmol/g dry wt. While the death in air and electrically stunned fish, both contained 32μmol/g dry wt. The death in air group probably contained higher levels of lactate because of time and temperature effects, while the electrically stunned fish contained more lactate because of temperature and electrical stimulation.

5.3.3.2 Rigor mortis

Rigor mortis development was measured over 72 hours in the anaesthetised fish killed by the various methods. Differences were not detected between the killing/ stunning methods in *rigor mortis*. The inferential statistics were: for time to maximum contraction $F=0.4695$, $P=0.7066$, $df=3, 22$, power~0.25; maximum contraction strength, $F=0.267$, $P=0.8487$, $df=3, 22$, power~0.08; and duration of *rigor mortis*, $F=2.585$, $P=0.0790$, $df=3, 22$, power~0.29. The power of the tests were however low and so conclusions of no difference might not be valid.

The fish reached maximum *rigor mortis* in; 39±6h, 38±2h, 37±5h, and 36±2h, in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. The maximum contraction strength was, in the ice slurry, death in air, percussively stunned and electrically stunned fish; 72±6°, 71±2°, 72±2°, and 72±1°, respectively. The duration of *rigor mortis* was; 45±8h in the ice slurry fish, 48±9h in the death in air group, 55±5h in the

percussively stunned fish and 53±7h in the electrically stunned group.

When considered in the light of the muscle metabolite data, which suggested that muscle activity was low in all the groups, the similarity in the *rigor mortis* measurements between all the killing/ stunning methods would be expected. The influence of temperature seen in the initial pH measurements would not have affected *rigor mortis,* as the fish were stored on ice for more than 35h before *rigor mortis* peaked. The *rigor mortis* data therefore suggests that the rate of ATP depletion was similar in all the groups and supports the finding of the muscle metabolite data.

5.4 Discussion

The effects of anaesthesia and killing/ stunning technique will now be discussed by presenting the present study alongside data from non-anaesthetised trout previously discussed in Chapter 4.

5.4.1 Plasma biochemistry and related parameters

5.4.1.1 Plasma hormones

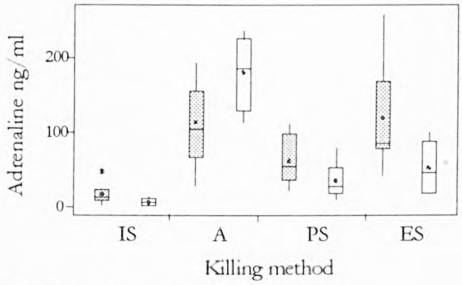
The effects of anaesthesia and killing/ stunning technique on the primary stress response was assessed by measuring plasma hormones and is illustrated in Figure 5.7A-C. Expressed as a percentage of the anaesthetised value, the adrenaline levels in the non-anaesthetised fish were; 283% in the ice slurry, 64% in the death in air fish, 177% in the percussively stunned fish and 232% in the electrically stunned group. A reduced adrenaline response would be expected in the anaesthetised fish, as they would have been unaware of the handling procedures and the nerves would have been less responsive. This was observed in all but the death in air fish, Figure 5.7A. Significant differences were shown between the anaesthetised and non-anaesthetised fish only in the death in air and electrically stunned fish. In the death in air fish, the anaesthetic treatment caused an apparent increase in adrenaline, while in the electrically stunned fish the effect of anaesthesia was to decrease the plasma adrenaline levels. The diminished adrenaline

response in the anaesthetised electrically stunned group suggests that the responsiveness of the nerves declined in these fish. The slightly higher, although not significant, adrenaline level in the electrically stunned fish compared with the percussively stunned fish suggests that, even when insensate and the nerves are responding poorly, electrical stimulation can produce a limited response. The higher levels of adrenaline in the anaesthetised death in air fish might suggest that there had been a delay in the primary stress response caused by the anaesthesia. A delay, due to the slower responsiveness of the nerves, would have left less time for adrenaline degradation/ clearance from the plasma before sample collection, and so produced an apparently greater response. Alternatively, the death in air fish might have roused, and produced an elevated response. A third and perhaps more plausible explanation would be enhanced catecholamine release from the chromaffin tissue due to hypercapnia in the anaesthetised fish. The carbon dioxide levels in the plasma could have risen due to the low ventilation rate caused by the anaesthesia. Work by Julio *et al.* [115] demonstrates that once the oxygen saturation of fish arterial haemoglobin falls below a threshold level of approximately 55-60% then there is an abrupt release of adrenaline. The amount of adrenaline released is greater in hypercapnic fish although the threshold level does not alter [115].

No differences were detected between the noradrenaline levels in the anaesthetised and non-anaesthetised fish, although the death in air anaesthetised fish showed a considerable increase in noradrenaline, which was concomitant with their higher adrenaline levels, Figure 5.7B. Expressed as a percentage of the anaesthetised value the noradrenaline levels in the non-anaesthetised fish were; 92%, 71%, 170%, and 87%, in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. The noradrenaline response is subordinate to that of adrenaline and so differences between anaesthetised and non-anaesthetised fish might not have been discernible. Alternatively, the high variability of the data might have precluded a statistically significant response.

In the cortisol measurements, the only difference detected between the anaesthetised and non-anaesthetised fish was in the death in air group, Figure 5.7C. There were significantly lower levels of cortisol in the anaesthetised death in air fish. This contrasts directly with the higher levels of the acute hormones in this group. It strengthens the hypothesis of a delayed primary response, in the anaesthetised fish. Expressed as a percentage of the anaesthetised value the cortisol levels were; 96%, 182%, 106%, and 124%, in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively.

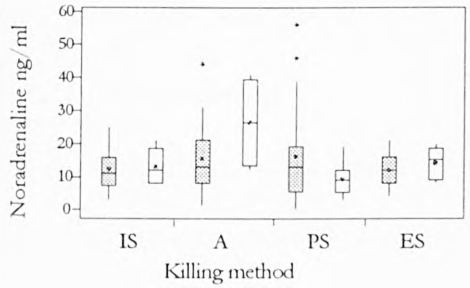
A: Adrenaline



Inferential statistics
F=17, P=0.000, df=7, 95.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	ns
air (A)	**
percussive stun (PS)	ns
electrical stun (ES)	**

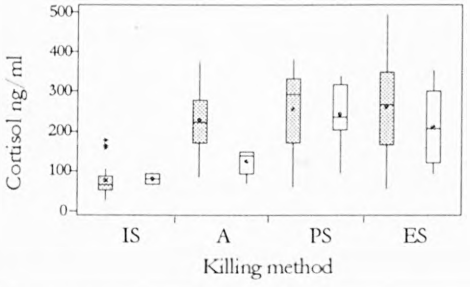
B: Noradrenaline



Inferential statistics
F=41.77, P=0.102, df=7, 94.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	ns
air (A)	ns
percussive stun (PS)	ns
electrical stun (ES)	ns

C: Cortisol



Inferential statistics
F=10.24, P=0.000, df=7, 96.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	ns
air (A)	*
percussive stun (PS)	ns
electrical stun (ES)	ns

Figure 5.7A-C Effect of anaesthesia and 'killing' method on trout plasma stress hormones
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fischer (between selected groups). Samples measured in duplicate, n=5 or 10 (PS) anaesthetised fish, and n=20 non-anaesthetised fish. P≥0.05 ns, P<0.05 *, P<0.01 **.

The ratio of adrenaline: noradrenaline did not change in the ice slurry, death in air, or percussively stunned fish, Figure 5.8. However, the adrenaline: noradrenaline ratio of the anaesthetised electrically stunned fish was significantly lower than in the non-anaesthetised group, Figure 5.8, reflecting the higher noradrenaline and lower adrenaline levels in the anaesthetised fish relative to the non-anaesthetised fish. The higher noradrenaline levels might suggest that the anaesthetised fish were more hypoxic, while the lower adrenaline levels reflects the lowered responsiveness of the nerves in the electrically stunned fish.

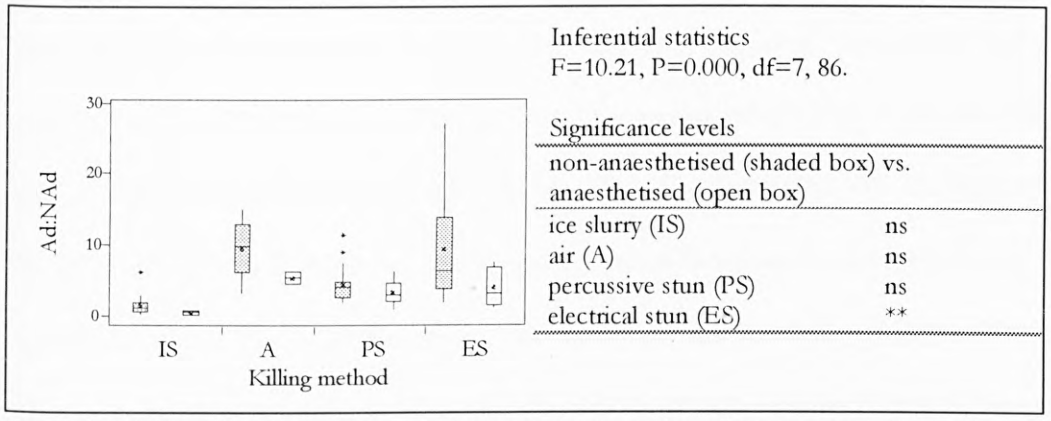


Figure 5.8 Effect of anaesthesia and 'killing' method on adrenaline: noradrenaline ratio
Data presented as a box and whisker plot with significance levels of ANOVA *post hoc* test Fischer (between selected groups). Data from duplicate measurements, calculated on an individual fish basis, $P \geq 0.05$ ns, $P < 0.05$ *, $P < 0.01$ **.

5.4.1.2 Plasma chemistry

The influence of killing/stunning method and anaesthesia on the secondary stress response and estimation of anaerobic activity in plasma is shown in Figure 5.9A-D.

Expressed as a percentage of the anaesthetised value the pH readings appeared similar in all the groups, however the pH's of the anaesthetised ice slurry and electrically stunned fish were significantly greater than their non-anaesthetised counterparts, Figure 5.9A.

Anaesthesia caused a similar although reversed pattern between the killing/ stunning methods in the plasma lactate levels, Figure 5.9B. Relative to the anaesthetised values the non-anaesthetised lactate concentrations were; 237%, 118%, 157%, and 554% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively.

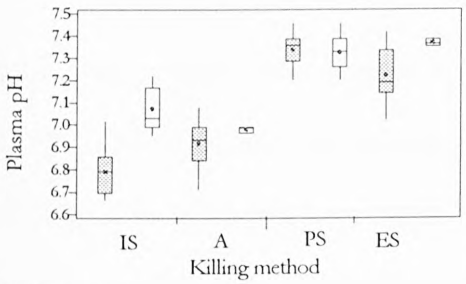
The higher plasma lactate in the non-anaesthetised ice slurry fish might indicate that there was more movement of lactate from the muscle to the plasma in these fish, as would be expected because the muscle lactate level was greater, see Section 5.4.2. The anaesthetised electrically stunned fish exhibited the least evidence of anaerobic activity. This would account for the significantly lower plasma lactate level, if the electrical stun increased metabolic activity in the muscle there would have been insufficient time for the lactate to enter the plasma before the samples were collected. There was no difference between the anaesthetised and non-anaesthetised percussively stunned fish as there was very little anaerobic activity in the non-anaesthetised group *per se*. There were high levels of plasma lactate in both the anaesthetised and non-anaesthetised death in air fish, although the anaesthetised fish level was slightly lower. This finding agreed with the high adrenaline levels in fish that died in the air, showing that even when anaesthetised, anaerobic glycolysis was enhanced in these fish. The higher plasma lactate levels in the ice slurry and death in air fish as a whole, indicate that the timing of sample collection was the major influence on plasma lactate.

The difference between the plasma glucose levels in the anaesthetised and non-anaesthetised fish did not follow the pattern seen with the pH and lactate data, Figure 5.9C. The anaesthetised fish had higher glucose levels than the non-anaesthetised fish although this was only significant in the death in air and electrically stunned groups. Presented as a percentage of the anaesthetised value the plasma glucose levels were 94%, 71%, 91%, and 46% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. A possible explanation for the higher glucose levels in anaesthetised trout would be reduced utilisation in these fish. This could occur provided the liver was still producing glucose at pre-anaesthesia levels, i.e. not affected by the anaesthetic. Possible causes for the decline in glucose utilisation might be: reduced uptake by muscle because of lower activity levels, impaired osmoregulation and, in all but the death in air fish, lower adrenaline levels. The less pronounced increase in plasma glucose in the ice slurry and

percussively stunned groups might have been caused by: the lower metabolic rate; and the already elevated level of plasma glucose in the ice slurry fish; and, insufficient time for the effect to develop in the percussively stunned fish. The high plasma glucose content in the anaesthetised electrically stunned fish might have occurred because of increased leakage of glucose from RBC's in these fish through electropermeabilisation in conjunction with another factor. For instance, the anaesthetic *per se,* lower adrenaline levels, hypoxia or impaired osmoregulation. The anaesthetic might have slowed down the resealing of pores in the RBC membrane [248]. Alternatively, other factors might have effected the extent of electropermeabilisation, such as the gradient of ionic strength across the charged membrane [158]. Hypoxia might influence the ionic composition of the RBC's as it causes extrusion of H^+ from the RBC's with a concomitant influx of Na^+.

The effect of anaesthesia on the plasma protein was to lower the level in all but the percussively stunned group, Figure 5.9D. Expressed as a percentage of the anaesthetised value the plasma protein levels were; 164%, 160%, 88%, and 129% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. This might suggest that osmoregulation was impaired as a result of the prolonged anaesthesia, and water was being absorbed across the gills, the net effect of which was beginning to have a detectable effect on plasma volume. As the samples from the percussively stunned fish were taken earliest, the difference had not had time to develop in these fish, or it was not sufficiently large to detect.

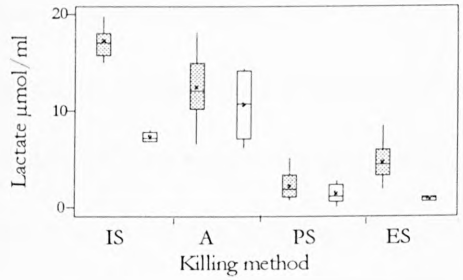
A: pH



Inferential statistics
F=78.32, P=0.000, df=7, 94.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	ns
percussive stun (PS)	ns
electrical stun (ES)	**

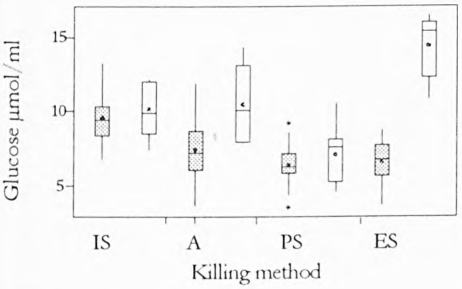
B: Lactate



Inferential statistics
F=127.2, P=0.000, df=7, 95.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	ns
percussive stun (PS)	ns
electrical stun (ES)	***

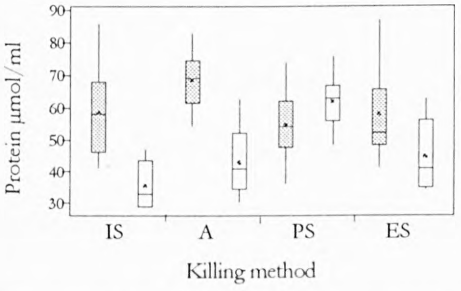
C: Glucose



Inferential statistics
F=18.33, P=0.000, df=7, 96.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	ns
air (A)	***
percussive stun (PS)	ns
electrical stun (ES)	***

D: Protein



Inferential statistics
F=7.59, P=0.000, df=7, 95.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	ns
electrical stun (ES)	*

Figure 5.9A-D Effect of anaesthesia and 'killing' method on trout plasma biochemistry
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fisher (between selected groups). Samples measured in duplicate, n=5 or 10 (PS) in the anaesthetised fish and n=20 in the non-anaesthetised fish. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

5.4.1.3 Haematocrit

Anaesthesia influenced the haematocrit in the ice slurry and death in air fish, Figure 5.10. Presented as a percentage of the anaesthetised value the haematocrit values were; 127%, 120%, 96%, and 112% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. The significantly lower haematocrit in the anaesthetised ice slurry and death in air fish might be due to an increased plasma volume, as suggested by the lower plasma protein levels. The effect of prolonged anaesthesia on osmoregulation might have caused the increase in plasma volume. The effect was not apparent in the percussively stunned group due to the shorter anaesthesia and consequently smaller plasma volume in these fish. The plasma volume of the electrically stunned fish was less affected than the ice slurry and death in air fish, which explains why the lower haematocrit was not so pronounced in these fish.

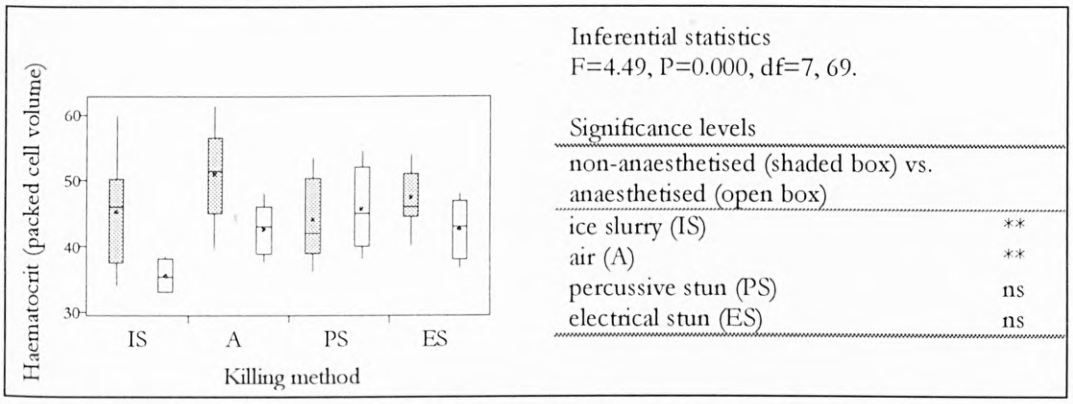


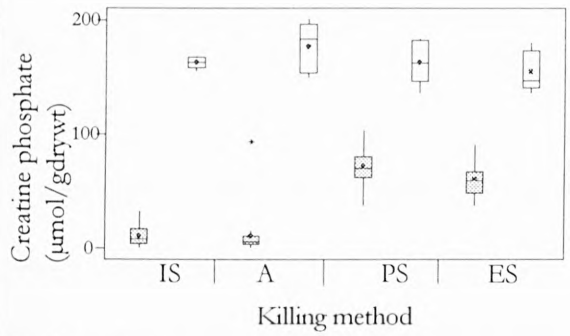
Figure 5.10 Effect of anaesthesia and 'killing' method on haematocrit
Data presented as a box and whisker plot with significance levels of ANOVA *post hoc* test Fischer (between selected groups). Data from duplicate measurements, $P \geq 0.05$ ns, $P < 0.05$ *, $P < 0.01$ **.

5.4.2 Muscle biochemistry

Muscle activity at harvest was determined by measuring pre *rigor mortis* muscle metabolites. Fish with the lowest energy status were deemed to have exhibited the most muscle activity whilst dying. The muscle of all the anaesthetised fish had a significantly higher energy status relative to their non-anaesthetised counterparts, Figure 5.11A-D. The anaesthesia prevented muscle stimulation and consequently the need for mobilisation of the energy stores.

Expressed as a percentage of the anaesthetised value the creatine phosphate content of the non-anaesthetised fish were; 7%, 6%, 44%, and 39% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively, Figure 5.11A. The non-anaesthetised values of creatine presented as a percentage of the anaesthetised amounts were; 317%, 247%, 196%, and 220% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively, Figure 5.11B. Expressed as a percentage of the anaesthetised value the ATP values in the non-anaesthetised fish were; 34%, 27%, 77%, and 72% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively, Figure 5.11C. Presented as a percentage of the anaesthetised value the lactate values in the non-anaesthetised fish were; 1900%, 754%, 526%, and 503% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively, Figure 5.11D.

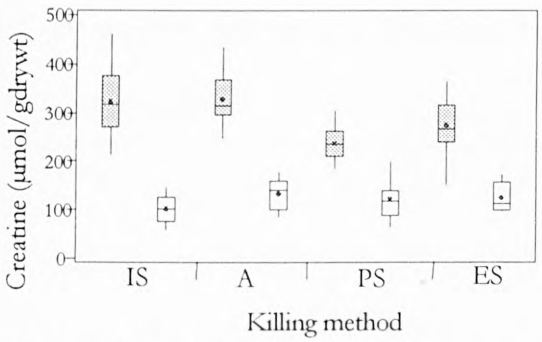
A: Creatine phosphate



Inferential statistics
F=205.48, P=0.000, df=7, 97

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

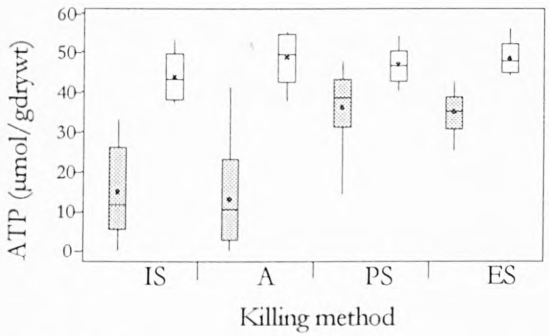
B: Creatine



Inferential statistics
F=36.76, P=0.000, df=7, 97

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

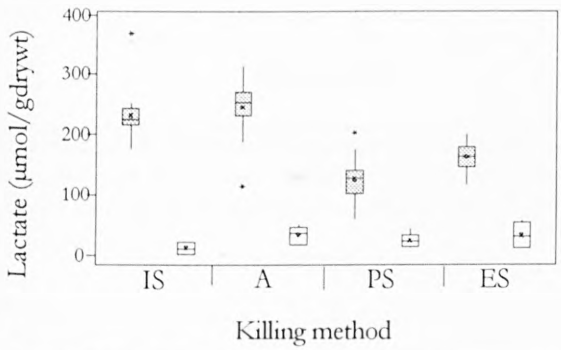
C: Adenosine triphosphate



Inferential statistics
F=32.96, P=0.000, df=7, 97

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

D: Lactate



Inferential statistics
F=97.13, P=0.000, df=7, 95

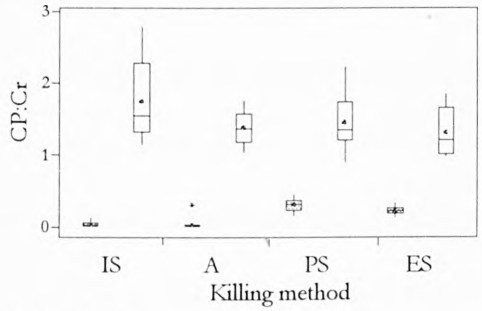
Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

Figure 5.11A-D Effect of anaesthesia and 'killing' method on muscle metabolites
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fisher (between selected groups). n=5 or 10 (PS) in the anaesthetised fish and n=20 in the non-anaesthetised fish P=0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

The CP: Cr and CP: ATP ratios also indicated that the anaesthetised fish had the higher energy levels, Figure 5.12A-B. Expressed as a percentage of the anaesthetised value the CP: Cr ratios in the non-anaesthetised fish were; 2%, 2%, 21%, and 17% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. Anaesthesia caused a similar pattern in the CP: ATP ratios. Relative to the anaesthetised values the non-anaesthetised CP: ATP ratios were; 29%, 22%, 61%, and 55% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively.

The higher energy status in the anaesthetised fish in all the killing/stunning methods suggests that nervous stimulation was required for mobilisation of the energy reserves in muscle.

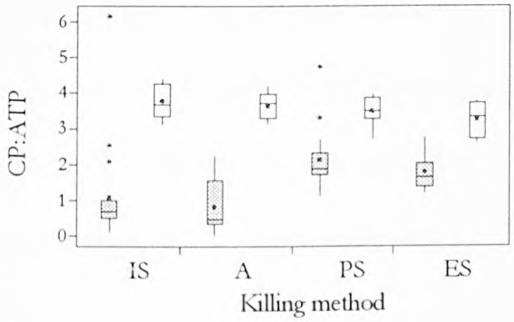
A: CP: Cr



Inferential statistics
F=111.4, P=0.000, df=7, 97.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

B: CP: ATP



Inferential statistics
F=20.98, P=0.000, df=7, 93.

Significance levels	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

Figure 5.12A-B Effect of anaesthesia and 'killing' method on metabolite ratios
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fisher (between selected groups). Data calculated on an individual fish basis P=0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

5.4.3 Muscle quality

5.4.3.1 Muscle pH

Anaesthesia affected muscle pH in all the killing/stunning methods tested. The decline in pH in the non-anaesthetised fish relative to the anaesthetised values were 123%, 89%, 127%, and 76% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. In both the anaesthetised and non-anaesthetised fish, the rate of decline in muscle pH was significantly lower in the ice slurry group compared with the percussively and electrically stunned fish, even though the actual pH of the anaesthetised fish was significantly higher than their non-anaesthetised counterparts. This suggests that the environmental temperature, which lowered the body temperature and therefore the metabolic rate of the fish was primarily responsible for the slower rate of decline in pH and not the low pH *per se* reducing enzyme activity, as suggested in Section 4.3.3.1a.

The pH_i in the anaesthetised ice slurry fish was significantly higher than the pH_i values of the other groups with the exception of the percussively stunned fish, Figure 5.13. This contrasts directly with the pH_i data of the non-anaesthetised fish, where the ice slurry and death in air fish had the lowest pH_i. These findings were supported by the muscle lactate data that was collected in a separate set of fish. The pattern in muscle pH between the killing/ stunning methods therefore changed depending on whether or not the ice slurry fish were anaesthetised. This implies that the effect of temperature on the formation of lactate/ decline in pH was masked by the effects of the primary stress response, neural stimulation and/or oxygen depletion in the non-anaesthetised fish.

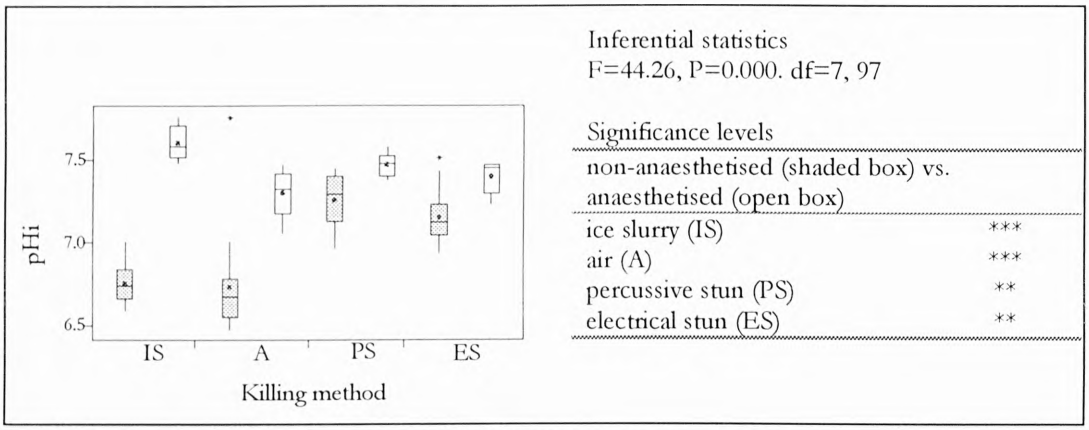
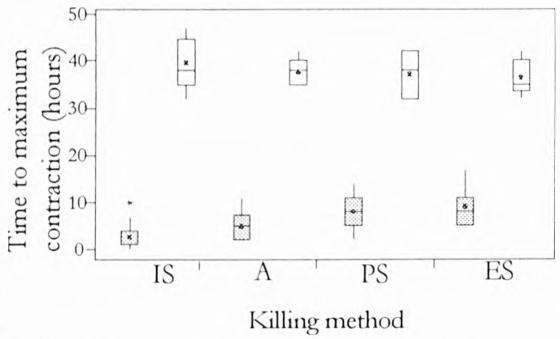


Figure 5.13 Effect of anaesthesia and 'killing' method on muscle pH
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fisher (between selected groups). Data from single measurements, pHi n=5 or 10 (PS) in the anaesthetised fish n=20 non-anaesthetised fish. P=0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

5.4.3.2 *Rigor mortis*

Anaesthesia had a significant effect on *rigor mortis* development. It delayed time to maximum *rigor mortis* in all the killing/ stunning methods, Figure 5.14A. The muscle in the anaesthetised fish had higher energy levels and so was able to sustain ATP production for longer, so delaying *rigor mortis*. The maximum contraction strength was significantly lower in the anaesthetised fish compared with the non-anaesthetised fish as the muscle cells did not all attain *rigor mortis* simultaneously, Figure 5.14B. While the effect of anaesthesia on the duration of *rigor mortis* was to increase it, although this finding was not quite significant in the ice slurry group, P=0.06, Figure 5.14B. The longer duration reflects the weaker contraction strength, as the tissues have a finite potential to produce ATP and it can be produced either quickly or slowly. Expressed as a percentage of the anaesthetised value, the time to maximum *rigor mortis* contraction in the non-anaesthetised fish was; in the ice slurry, death in air, percussively stunned and electrically stunned fish; 7%, 13%, 22%, and 25%, respectively. The maximum *rigor mortis* contraction strength in the non-anaesthetised fish expressed as a percentage of the anaesthetised values were similar in all the groups. Expressed as a percentage of the anaesthetised value the duration of *rigor mortis* was; 89%, 79%, 71%, and 73% in the ice slurry, death in air, percussively stunned and electrically stunned fish, respectively. The *rigor mortis* data therefore confirm the finding of the muscle metabolite data, which showed that anaesthesia reduced muscle activity during harvest.

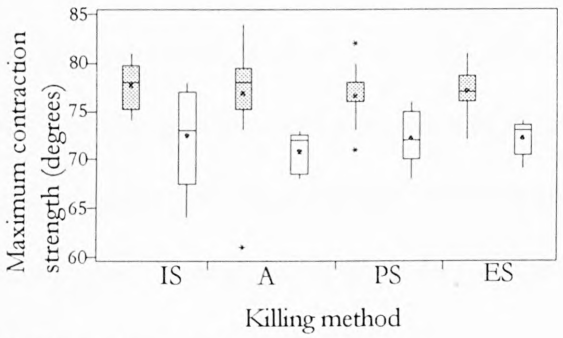
A: Time to maximum contraction



Inferential statistics
F=243.4, P=0.000, df=7, 98.

Significance	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	***
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

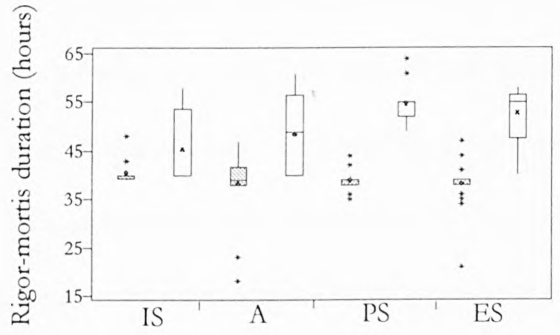
B: Maximum contraction strength



Inferential statistics
F=7.70, P=0.000, df=7, 98.

Significance	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	**
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	**

C: Duration of *rigor mortis*



Inferential statistics
F=19.24, P=0.000, df=7, 98.

Significance	
non-anaesthetised (shaded box) vs. anaesthetised (open box)	
ice slurry (IS)	ns
air (A)	***
percussive stun (PS)	***
electrical stun (ES)	***

Figure 5.14A-C Effect of anaesthesia and 'killing' method on *rigor mortis*
Data presented as box and whisker plots with significance levels of ANOVA *post hoc* test Fisher (between selected groups). n=5 or 10 (PS) in the anaesthetised fish and n=20 in the non-anaesthetised fish. P≥0.05 ns, P<0.05 *, P<0.01 **, P<0.001 ***.

5.5 Summary and conclusion

Anaesthesia was shown to have an effect on the results of killing technique. It diminished the adrenaline response in the electrically stunned fish, but in the death in air fish plasma adrenaline levels increased. The plasma cortisol level in the death in air group however declined with anaesthesia, leading to the suggestion that the primary stress

response in the death in air fish might have been delayed. The increased adrenaline response might suggest that the anaesthetised fish as a whole were hypoxic/ hypercapnic and so catecholamine release from the chromaffin tissue in the death in air fish was augmented. Furthermore, the adrenaline level might have been higher if the stress response was delayed as it would have been released later and so had less chance to be cleared from the plasma before sample collection. The adrenaline response in the electrically stunned fish, low in the anaesthetised and high in the non-anaesthetised fish, suggests that adrenaline release results from the electrical stimulation of nerves when they are less responsive, levels decline. The adrenaline: noradrenaline ratio in the electrically stunned fish altered as a consequence of the much reduced adrenaline response in the anaesthetised fish. Noradrenaline concentrations did not appear to be affected by anaesthesia although the death in air noradrenaline levels did increase considerably in line with the adrenaline levels.

Anaerobic activity, as deduced from the muscle and plasma, pH and lactate values, was influenced by the time elapsed before sample collection, and nervous stimulation. Anaesthesia reduced nervous stimulation and therefore lactate production and so the pH of the anaesthetised fish rose relative to their non-anaesthetised counterparts. A particularly pronounced reduction in lactate was observed in the anaesthetised ice slurry fish. Even though the primary stress response appears to be small in this group, and so the killing method could be mistaken as welfare friendly, on closer inspection of the evidence this study suggests that it is not. The degree of energy expended attempting to maintain homeostatic mechanisms was highest in this group. They showed the greatest increase in energy levels, CP: Cr ratio, when under anaesthesia. This suggests that killing fish in ice slurry might not be humane.

6 THE EFFECTS OF FASTING ON FLESH AND PROCESS QUALITY OF FARMED RAINBOW TROUT

6.1 Introduction

Rainbow trout sold to the table market in the UK are commonly 'starved' for approximately 30 degree-days before killing, as reported in Section 3.3.2. Starvation studies in trout have tended to focus on effects on metabolism, body composition, and eating characteristics [108-110, 120-122], often for periods of starvation which are longer than those commonly used at harvest by the fish farming industry [106, 179]. The effects of short-term 'starvation' as practised by the industry on process quality are less well documented. This laboratory-based study sought to redress the balance.

6.2 Materials and methods

6.2.1 Animals

The fish were obtained dead from the Mill of Elrick Fish Farm, Auchnagatt, Aberdeenshire, UK, in the autumn of 2001. They were diploid, mixed sex, rainbow trout 27.9±2.0cm, 313.0±55.1g, with a condition factor of 1.43±0.12.

6.2.2 Design and conditions

The two groups of ten fish were killed contemporaneously. They were percussively stunned then transported to the laboratory on ice, within 2h of 'death'. They were stored on ice, in a chill room, temperature 2.0±0.4°C, for six days. The 'starved' fish were fasted for three days at a water temperature of 11-13°C, i.e. between 30 and 40 degree-days. The fed group were killed approximately 13h after their last meal.

6.2.3 Analysis

6.2.3.1 Muscle chemistry

Muscle pH was measured daily for six days as described in Section 2.4.1. Lactate was determined in tissue taken after six days ice storage, which was subsequently stored for

less than four weeks, at -80°C, the methods used were described in Section 2.3.3.1.

6.2.3.2 Rigor mortis

Rigor mortis was measured at varying time intervals over 72 hours as described in Section 2.4.2.

6.2.3.3 Freshness

Torrymeter readings were taken daily for six days as described in Section 2.4.3.1. The rate of decline in freshness was defined as the slope of the regression line calculated from the day 2-6 data. K value was determined from tissue excised from the belly flap after six days ice storage using the method described in Section 2.3.3.4, the tissue was stored at -80°C for up to three weeks before analysis. Visual fish freshness assessments were undertaken daily for five days starting approximately 10h *post mortem*, as described in Section 2.4.3.2. The belly flap assessment commenced after *rigor mortis* had resolved, two days post-harvest, when the fish were eviscerated.

6.2.3.4 Carcass characteristics

Fulton's condition factor was calculated from length weight data taken on day one, while the dress-out percentage and somatic indices were estimated from data collected two days *post mortem*, using the formulae found in Section 2.4.5.1.

6.2.3.5 Fillet quality

After six days of ice storage, the fish were filleted and the colour of the freshly prepared fillets assessed as described in Sections 2.4.6 and 2.4.5.3. Distell fish fat-meter readings were also taken on the fillets and tissue water content was determined in muscle excised six days *post mortem* as described in Sections 2.4.5.2 and 2.3.2.2a. Texture was measured instrumentally on three samples excised from one fillet per fish using a Warner-Bratzler shear test, a finger compression test was also performed as detailed in Sections 2.4.7.2 and 2.4.5.3. The final test made on the fillets was a gaping assessment, described in Section 2.4.5.3.

6.3 Results

6.3.1 Muscle chemistry

The pH and lactate content of muscle were measured during storage, as they are associated with quality changes that occur in tissue, particularly spoilage. The muscle pH of the 'starved' fish taken 3h *post mortem* tended to be higher than that of the fed fish, however the difference was not significant, ($t=1.69$, $P=0.110$, $df=18$, power~0.333). The readings were 7.65±0.17 and 7.53±0.14 in the 'starved' and fed fish, respectively. The muscle pH declined rapidly during the first day after harvest in both groups, it then stabilised for the next five days, Figure 6.1. The pH of the 'starved' fish was equal to or higher than that of the fed fish on all but one occasion, day three. The difference between the two groups was significant only on day four, $t=3.385$, $P=0.0044$, $df=14$, power~0.73.

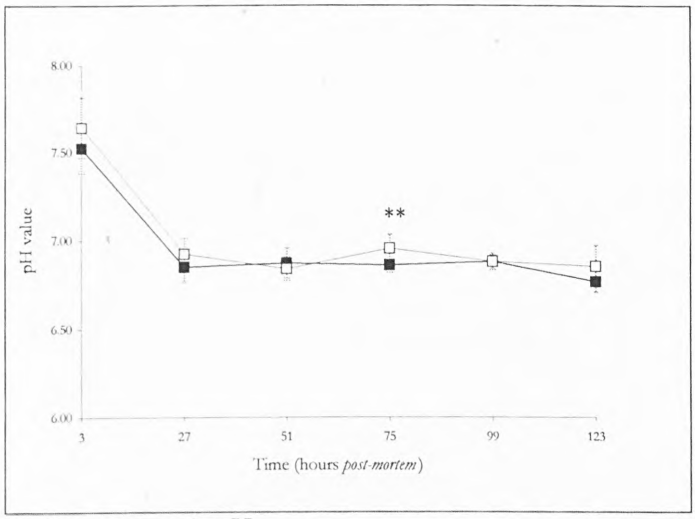


Figure 6.1 Effect of fasting on muscle pH
Fish were stored on ice in a chill room between readings. Results expressed as mean ± standard deviation, of single daily pH readings, fed (■), 'starved' (□), $P<0.01^{**}$, $n=10$.

Muscle lactate concentration was determined to relate to pH and freshness. After six days ice storage the lactate content of the fed fish was greater than that of the 'starved' fish, however the difference was not significant, $t=0.59$, $P=0.57$, $df=18$, power~0.09. The lactate levels were, 165±49μmol/g dry wt tissue and 154±35μmol/g dry wt tissue in the fed and 'starved' groups respectively. This corresponds with the lower, although not significant, pH in the fed fish on day 6 (123h), Figure 6.1. These observations suggest that

the fed fish muscle might have been more metabolically active compared with muscle from the 'starved' fish.

6.3.2 *Rigor mortis*

Rigor mortis was monitored over three days as a measure of *post mortem* energy status. The peak measurement in the fed fish occurred significantly earlier, 5h, than in the 'starved' fish, W=77.5, P=0.038, n=10, Figures 6.2 and 6.3. Differences were not however detected between the groups in duration of *rigor mortis* and maximal contraction strength, W=87.5, P=0.1911, n=10, and W=129.5, P=0.0685, n=10, respectively. Duration of *rigor mortis* was 34±2h and 39±9h, in the fed and 'starved' fish, respectively, while the maximum contraction strength (peak amplitude) was 76±6° in the fed fish and 73±4° in the 'starved' group.

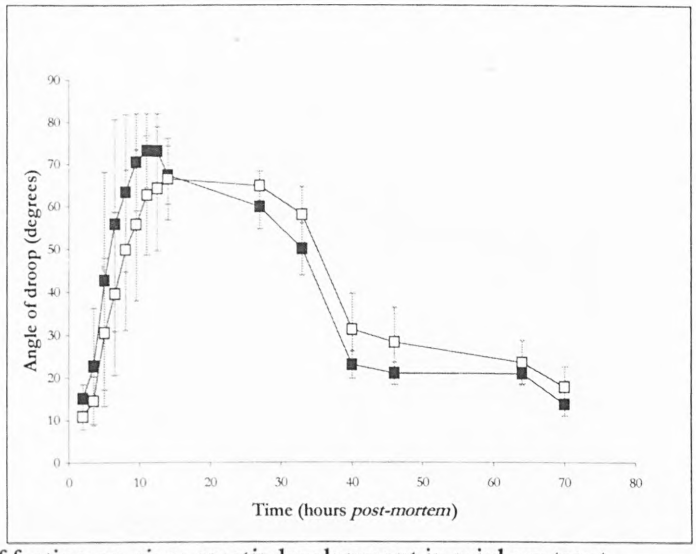


Figure 6.2 Effect of fasting on *rigor mortis* development in rainbow trout
Fish were stored on ice between measurements, results are expressed as mean ± standard deviation, triplicate measurements were taken on each side of the fish, fed fish (■), 'starved' fish (□) n=10.

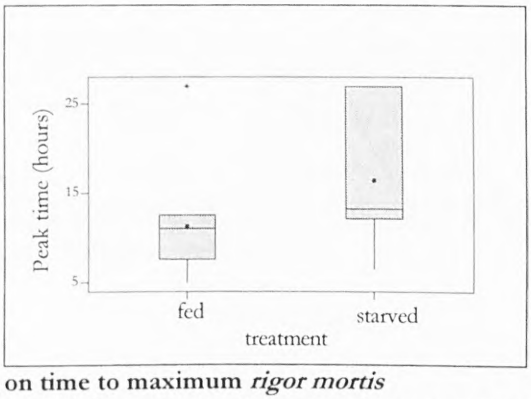


Figure 6.3 Effect of fasting on time to maximum *rigor mortis*
Effects of three days starvation in rainbow trout on time to maximum contraction strength in hours, n=10. Time to peak contraction was significantly earlier in the fed fish. P=0.038.

The earlier onset of *rigor mortis* in the fed group corresponds with the higher muscle lactate content and lower pH in this group, and strengthens the evidence for greater muscle activity in the fed fish.

6.3.3 Freshness

6.3.3.1 Torrymeter

Freshness is an important quality characteristic of fish, fish are starved before harvest to evacuate the gut, delay microbial spoilage and to increase freshness [51, 126].

The Torrymeter readings suggested that, from 30h onwards, (post peak *rigor mortis*), the decline in freshness in the fed fish was just significantly greater than in the 'starved' group, $t=2.481$, $P<0.05$, $df=6$, Figure 6.4, and Table 6.1. There was a significant difference between the two groups in the second reading, $W=134.5$, $P=0.020$, $n=10$.

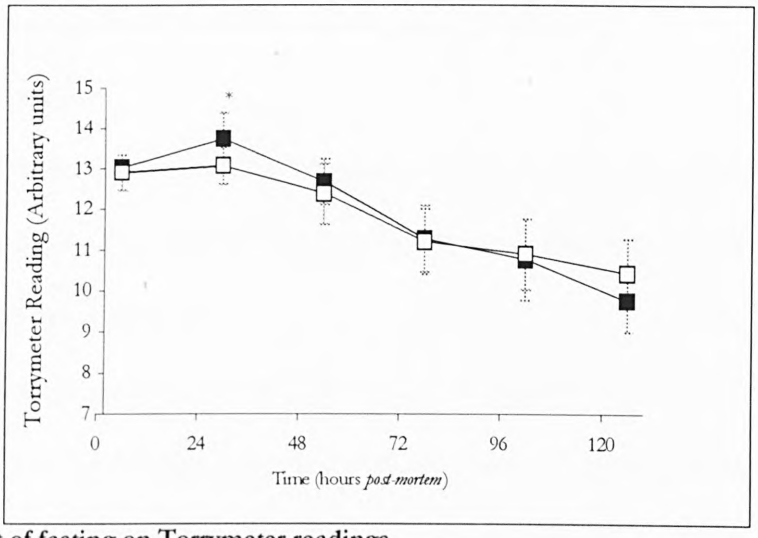


Figure 6.4 Effect of fasting on Torrymeter readings
Torrymeter readings were taken daily six times, between measurements fish were kept on ice in a chill room. Results expressed as mean ± standard deviation from triplicate measurements on one side of the fish, fed (■), 'starved' (□), $P<0.05$ *, $n=10$.

Table 6.1 Regression line data for the effect of fasting on Torrymeter readings

treatment	intercept	slope	R^2
fed	14.8	-0.041	0.9819
'starved'	13.7	-0.028	0.9545

Decline in freshness was classified as the slope of the regression line of the readings over the five days following peak *rigor mortis*, triplicate readings per fish, $n=10$.

6.3.3.2 K value

K value was measured on tissue taken six days *post mortem*. The fed fish had significantly higher K values compared with the 'starved' fish suggesting that the fed fish were less fresh, t=4.02, P=0.0008, df=18, power~0.81, Figure 6.5. The fed fish value expressed as a percentage of the starved group was 138%.

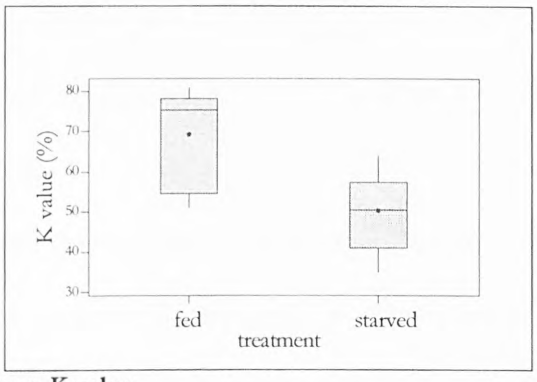


Figure 6.5 Effect of fasting on K value
K value was measured from tissue taken from the belly flap six days *post mortem*, single reading per fish, n=10. The 'starved' fish were significantly fresher than the fed group. P=0.0008

6.3.3.3 Visual assessment

A visual assessment of fish freshness showed declining scores (freshness) in both groups over five days. The 'starved' fish had higher scores than fed fish on each day, i.e. they appeared fresher, Figure 6.6.

The freshness assessment therefore further strengthens the evidence for increased metabolic activity in the fed fish compared with the 'starved' fish, as they show a faster decline in freshness in the fed fish.

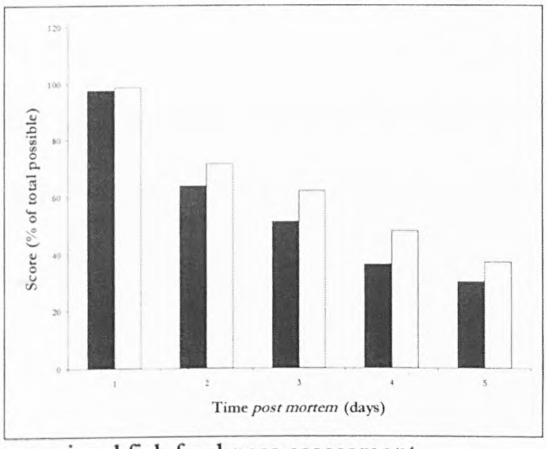


Figure 6.6 Effect of fasting on visual fish freshness assessment
Fish were scored between zero and two on multiple attributes, scores for each day were totalled. Lower scores indicate less fresh fish. The peritoneum score was omitted from the first assessment, single assessment each day, one observer, fed (■), 'starved' (□) n=10.

6.3.4 Carcass characteristics

Carcass quality was assessed to demonstrate the effects of fasting on the morphology of rainbow trout, as relative yields can alter with degree of starvation in fish.

6.3.4.1 Dress-out percentage

The dress-out percentage of the two groups was significantly different, W=66.0, P=0.003. There was a greater weight loss from gutting in the fed fish, Figure 6.7A. The dress-out percentage of the fed fish was 97% of the starved fish value.

6.3.4.2 Digestive somatic index

The DSI of the fed fish was significantly larger than that of the 'starved' fish, t=2.93, P=0.0089, df=18, power~0.65, Figure 6.7B. The digestive tracts of the fed fish contributed more to the total weight of the fish. The DSI of the fed fish was 119% of the starved fish value.

6.3.4.3 Hepato somatic index

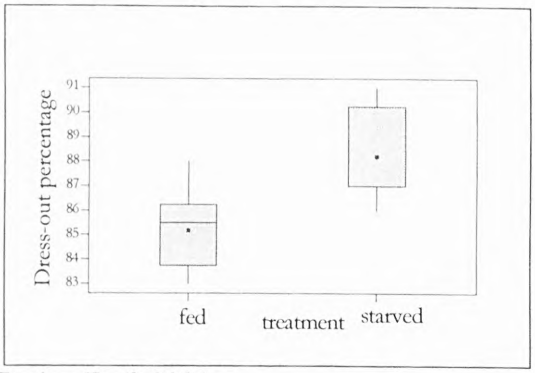
Differences were not detected between the HSI of the two groups, t=1.15, P=0.26, df=18, power~0.09, although the HSI of the fed fish was greater. The HSI values were in the fed and 'starved' fish 1.38±0.13 and 1.30±0.17, respectively.

6.3.4.4 Fulton's condition factor

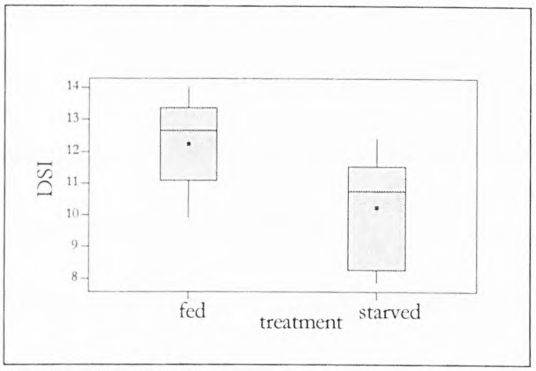
The fed fish had a higher condition factor than the 'starved' fish, t=3.15, P=0.0055, df=18, power~0.72, Figure 6.7C. The condition factor of the fed fish expressed as a percentage of the starved was 110%.

The carcass quality data therefore demonstrated that when 'in the round', the fed fish appeared fatter/ larger, but after gutting, the yield was smaller.

A: Dress-out percentage P=0.003



B: Digestive Somatic Index P=0.0089



C: Fulton's Condition Factor P=0.0055

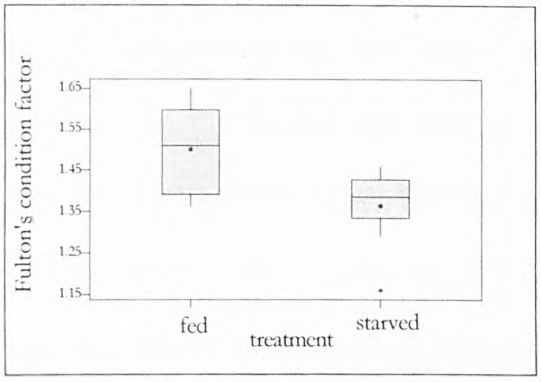


Figure 6.7A-C Effect of fasting on carcass characteristics
Fulton's condition factor was calculated on day one, dress-out percentage and somatic indices were calculated after gutting day two, n=10. Significant differences were found between the dress-out percentage, DSI and condition factor.

6.3.5 Fillet quality

The primary characteristics considered by consumers when deciding to buy fish are colour, perceived freshness and fat content: characteristics important for processing include fillet texture and gaping [240]. The fillets were therefore, assessed for colour, fat content, gaping, and texture.

6.3.5.1 Colour

The fillets of the fed fish had a significantly higher lightness reading, (L*), compared with the 'starved' fish, this indicates that they were paler, t=2.87, P=0.010, df=18, power~0.64, Figure 6.8A. The L* score of the fed fish was 106% of the starved fish value. The chroma value of the fed fish fillets was lower than that of the 'starved' fish, i.e. they were more translucent and the colour was less intense. The difference was not however significant, t=-0.76, P=0.45, df=18, power~0.11. The chroma values were for the fed and 'starved' fish 15±3, and 16±2, respectively. The angle of hue in the fed fish was higher, i.e. they were more yellow than the 'starved' fish, although again the difference was not significant, t=-0.69, P=0.505, df=18, power~0.10. The angle of hue values were 43±2°, and 42±3° in the fed and 'starved' fish, respectively. The fed fish had a lower Roche *Salmo*fan™ score than the 'starved' fish indicating a poorer colour in these fish. The difference was highly significant, W=77.5, P=0.0302, the fed fish value expressed as a percentage of the starved fish was 97%, Figure 6.8B.

These findings suggest that the colour of the flesh in the 'starved' group was superior to that of the fed fish.

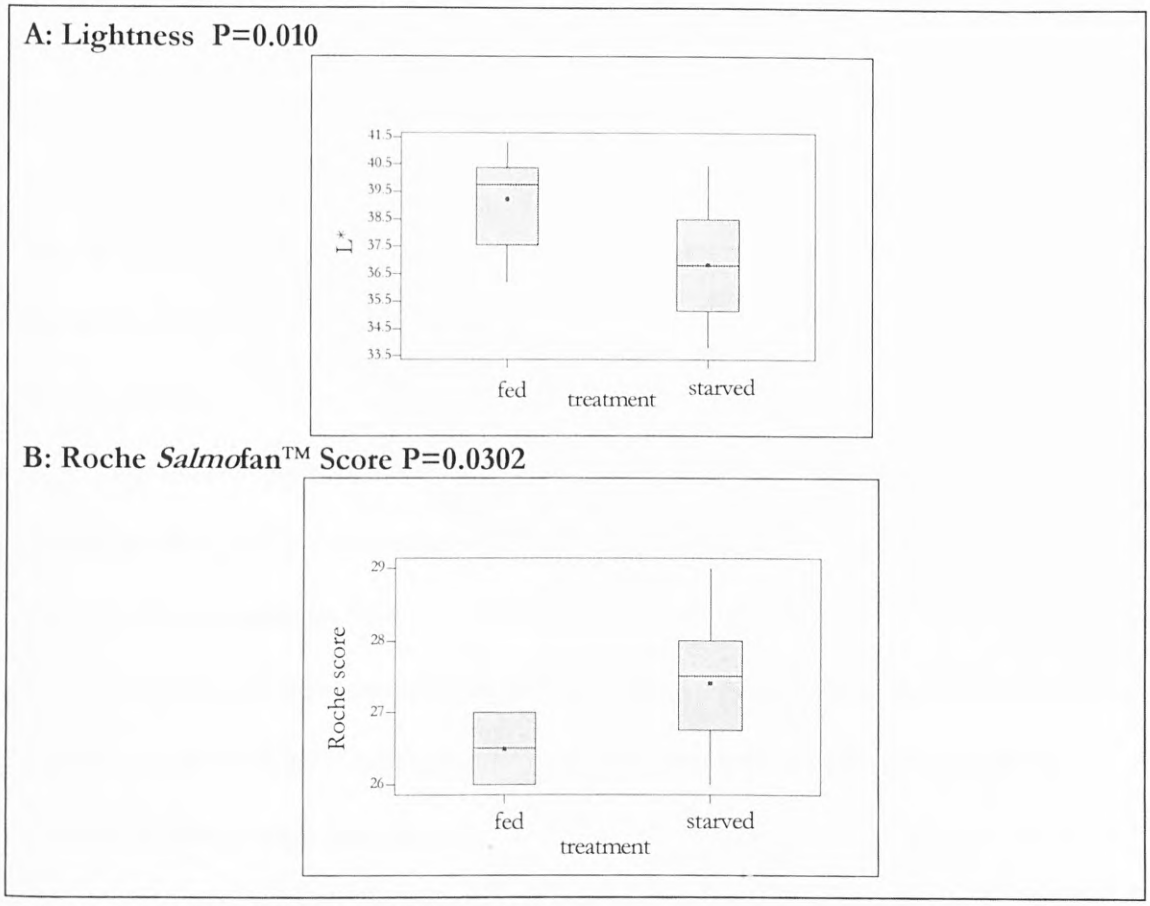


Figure 6.8A-B Effect of fasting on fillet colour
Instrumental colour measurements were made in triplicate on one freshly prepared fillet after six days ice storage. The *Salmo*fan™ assessment was made on the same fillet under D_{65} light, single reading per fish, one observer, n=10.

6.3.5.2 Fat and water content

The fillet fat and water content were determined to see if fasting affected muscle composition. There was no difference between the fat content of the two groups, t=-0.52, P=0.61, df=18, power~0.08. The fillets contained 2.2±0.8% and 2.4±0.9% fat, in the fed and 'starved' fish, respectively. The tissue water content was also similar in the two groups, W=117.5, P=0.3420. There was 80±1% water in the fed and 79±1% water in the 'starved' fish muscle. This suggested that withholding food for 30-40 degree-days did not alter the fat composition of rainbow trout muscle.

6.3.5.3 Gaping

Gaping in fish fillets decreases their process capability and so can reduce profitability in processing plants. The fillet gaping scores of the two groups did not differ significantly, W=122.0, P=0.1217. The incidence of gaping was low in both the groups, the scores were 1.3±0.5 in the fed and 0.9±0.6 in the 'starved' fish.

6.3.5.4 Texture

Texture is also an important process quality of fish, firm flesh is less easily damaged during handling and is therefore preferred by processors.

6.3.5.4a Finger compression Score

Differences were not detected between the two groups using the sensory texture assessment, W=105.0, P=1.000, as the scores were identical in each group, 0.3±0.5.

6.3.5.4b Warner-Bratzler shear test

The instrumental texture measurement also failed to show significance, t=-1.92, P=0.081, df=11, power~0.41. The peak force needed to cut the samples were, 186±39g and 252±101g, in the fed and 'starved' fish, respectively.

These results suggest that fillet quality declines only with respect to colour with fasting in rainbow trout.

6.4 Discussion

The effect of a three-day fast on the flesh and process quality of farmed rainbow trout was investigated. From the present study, it would appear that fasting influences meat quality characteristics.

6.4.1 Muscle chemistry

Muscle chemistry plays an important role in the configuration of muscle proteins, which in turn influence muscle quality, as discussed in Section 1.4. The majority of the muscle pH measurements were higher in the 'starved' fish, but only significantly so in one instance. The power of the tests were low on the other occasions, 0.15-0.45, and

consequently conclusions of no difference on the other days would be invalid.

The muscle lactate content six days *post mortem* was higher in the fed fish. This observation complements the lower (but not significant) pH reading. The difference in muscle lactate content between the groups also did not reach significance but the power of the test was again too low to state that a difference was not there, (power~0.09). If a one-tailed test were performed on the pH data then the differences become significant 50% of the time. The muscle data therefore might suggest that the fed fish had a lower pH possibly caused by higher lactate levels.

A possible justification for performing a one-tailed test would be that the lower pH and higher lactate levels would be expected in fed fish due to the effects of SDA and possibly the nutritional status. SDA manifests the physical and biochemical energy demands of digestion. When fish are fed, their metabolic activity for digestion and protein synthesis increases [107]. Protein synthesis has a high energy-demand [266] and so the muscle high-energy phosphates become depleted earlier in fed fish. Protein synthesis rates in rainbow trout muscle have been shown to decline over a 180degree-day starvation period from 0.38% to 0.09% [219]. If the nutritional status of the fed fish were higher, then more substrate would be available for energy production and so lactate levels might rise further than in 'starved' fish. The lower pH and higher lactate levels observed in the fed fish would therefore indicate that the muscle in these fish had been more active *post mortem*, exhausting energy reserves earlier. In the fed fish, SDA would increase energy demands of the tissue and so the muscle pH would decline faster; the pH of *post mortem* muscle in feeding fish would consequently be lower. Alternatively, if the muscle glycogen store in the 'starved' fish had become depleted there would be less substrate available for anaerobic glycolysis, and so the pH might not be able to fall so far.

Botta *et al.* [40] report a lower pH in Atlantic cod caught off Newfoundland in July and August - the feeding season. Rustad [196] also reports a lower pH in wild cod during the summer months while farmed cod had a low pH throughout the year.

Einen *et al.* [67] working with Atlantic salmon found no differences in pre *rigor mortis* muscle pH between starved and fed salmon and a decrease in pH with increasing ration in post *rigor mortis* flesh. The post *rigor mortis* muscle in the study by Einen *et al.* [68, 69] showed increasing lactate levels with increasing ration i.e. lower levels in starved fish.

The present study was therefore in general agreement with the literature. The slightly lower pH and higher lactate content of the fed fish although not significantly different, might suggest that the fed fish were more metabolically active than the fish that had been fasted for 30-40 degree-days.

6.4.2 *Rigor mortis*

Rigor mortis development was assessed in the fed and fasted fish as *rigor mortis* can give a rough indication of when supplies of high-energy phosphates in cells are exhausted.

Peak *rigor mortis* was reached significantly earlier in the fed fish. The duration of *rigor mortis* and the maximal contraction strength of the groups were however similar. If a one-tailed test were performed on the data then the fed fish would also have had a significantly stronger *rigor mortis* contraction, higher peak amplitude. This would correspond with the earlier time to peak *rigor mortis*.

A possible justification for performing a one-tailed test was that an advance in *rigor mortis* would be expected in fed fish, because of the effects of SDA. Earlier *rigor mortis* is also associated with increased contraction strength and shorter duration of *rigor mortis*. The later peak in *rigor mortis* suggests that the energy reserves in the 'starved' fish were maintained for longer, therefore individual muscle cells did not run out of high-energy phosphates simultaneously, and so the *rigor mortis* contraction strength was weaker.

The earlier onset of *rigor mortis* in the fed fish supports the finding of higher lactate and lower pH levels in these fish, i.e. more anaerobic activity had occurred in the fed fish, although the finding was not statistically significant.

6.4.3 Freshness

Maintaining fish freshness between harvest and sale is very important, starving farmed fish is thought to assist in maintaining freshness [34, 68]. The decline in freshness as measured by the Torrymeter over six days ice storage was just significantly faster in the fed fish. The daily Torrymeter readings did not detect differences between the two groups apart from on day two when the fed fish had the highest reading. This reading was taken when the fish were reaching peak *rigor mortis* suggesting that up until this point ATP production was sufficient to maintain homeostasis. If a one-tailed test were performed on the Torrymeter data, then the fed fish had a significantly lower Torrymeter reading on day six indicating that they were less fresh than the 'starved' group. The use of a one-tailed test can be justified as the fed fish were expected to lose freshness faster due to the effects of SDA, and there was further evidence to support a faster decline in freshness in the fed fish.

The fed fish had a significantly higher K value after six days ice storage i.e. they were less fresh than the 'starved' fish.

The visual assessment scores further strengthened the evidence of a faster decline in freshness in the fed fish. The freshness scores declined over five days and the 'starved' fish were considered fresher on each occasion.

The faster decline in freshness in fed fish can be explained by the effect of the last meal. The stores of high-energy phosphates would be depleted faster in fed fish due to the effects of SDA, and consequently the K value would be higher. The timing and the magnitude of the SDA response would be dependent on meal size, composition and the environmental temperature. The work of Beamish [21] provides evidence to suggest that the SDA response would have an effect beyond the 13 hours of fasting experienced by the fed group in the present study.

Blokhus [34], although stating that the Torrymeter was not well suited for freshness assessment in Atlantic salmon, presents evidence of a faster decline in readings in fed relative to seven day starved fish. The study by Blokhus [34] also showed faster production

of hypoxanthine in fed salmon and states that starving extended shelf-life. A decrease in freshness with feeding has also been shown in Atlantic salmon by Einen and Thomassen [68]. The K value in Einen and Thomassen's [68] study 12 days *post mortem* in fish 'starved' for three days or less was significantly higher than that of fish starved for two weeks.

The evidence from the current study therefore suggests that withholding food from rainbow trout prior to slaughter will ensure that freshness is maintained for a longer period post-harvest.

6.4.4 Carcass characteristics

The carcass characteristics of fish in the current study altered with feeding status. The fed fish had a significantly higher condition factor than the 'starved' fish, they were shorter for their weight. The amount of food in the gut would have produced this result, as a full intestinal tract would contribute a greater percentage to the whole body weight than an empty one. The dress-out percentage was consequently significantly smaller in the fed fish, and the DSI significantly larger. Although the HSI of the fed fish was greater, than that of the starved fish, the difference was not significant, (power~0.09). This suggests that the energy stores in the fish were not depleted to such an extent that a difference in liver weight could be detected.

A reduction in the HSI of fasting rainbow trout has been reported by others [150]. The HSI in Morata *et al.'s* [150] study was lower after five days starvation (first observation) but the difference was not significant until after ten days starvation (second observation), water temperature 14±1°C, initial fish size <100g. Work by Hilton [90] found an increased HSI approximately 24 hours post feeding in rainbow trout which declined to below initial levels after 72 hours starvation at 10°C and 15°C. Hilton [90] however suggests that the effects of diet composition on HSI and liver glycogen levels is of more significance than starvation time, the initial fish size was again small <100g. The low power of the HSI test in the current study might have produced a false negative result. However, the larger size of the fish, the short starvation period tested and the fact that salmonids naturally protect

hepatic glycogen stores during periods of fasting [208, 249], suggests there might not have been a difference to detect.

6.4.5 Fillet quality

6.4.5.1 Colour

The colour of salmonid fillets although predominantly influenced by pigment levels, is also subtly affected by the configuration of muscle proteins, and therefore might be influenced by *post mortem* metabolic activity, as detailed in Section 1.4.1.3.

The lightness, (L*), value of the fed fish was significantly higher than that of the 'starved' fish, indicating that the fed fish fillets were the palest. The power of the test, 0.64, was fairly high and so the chance of having a false positive result was not large. The chroma or colour intensity/ opacity of the flesh although lower in the fed fish did not differ significantly, (power~0.11). The calculated angle of hue was higher in the fed fish i.e. the flesh was more yellow and less red in this group. However, the difference was not significant and the power test was low 0.10.

The finding that the colour of the fed fish fillets was inferior to that of the 'starved' fish in the L* measurement was supported by the sensory evaluation. The Roche *Salmo*fan™ score was significantly lower in the fed fish. The evidence therefore supports the hypothesis that the flesh colour in the fed fish was inferior to that of the 'starved' group.

On close scrutiny of the data however, it was noted that although the weight of the two groups of fish were not significantly different, t=2.003, P=0.0604, df=18, the fed fish were significantly shorter, t=3.222, P=0.0073, df=12. The length and weight of the fed and starved fish were, 27±2.1cm and 29±0.9cm, and 290±61g and 336±40g, respectively. Trout are generally fed pigments only after they have reached 80-100g, smaller fish would therefore have consumed less pigment and so might be expected to be paler. An analysis of covariance, ANCOVA, was performed on the L* and treatment data, using body weight

as the covariate. The inferential statistics calculated were; F=8.91, P=0.008, df=1 for body weight and F=3.38, P=0.084, df=1 for the treatment variable. Body size therefore had a greater effect on the L* value than treatment. The treatment could only be said to have a significant effect on L* if a one-tailed test were performed, i.e. there was other evidence available to support the finding that fed fish were paler.

Such evidence is available in the literature. Regost *et al.* [179] found two months starvation intensified colour in cooked and raw flesh of brown trout. The work of Robb *et al.* [186] also indirectly support this finding in that colour (L*, chroma and angle of hue) improved in rainbow trout with a delay in *rigor mortis*. R*igor mortis* was not impeded by starvation in the work of Robb *et al.* [186] but by comparing electrically stimulated fish with those rested at harvest. However, Einen *et al.* [67] found decreasing ration increased lightness and decreased colour intensity in smoked salmon, although no difference was detected in hue.

The protein configuration of the fillets might not be the only factor influencing the colour of the fillets, the pigments might also be affected. Idoxanthin, a primary metabolite of astaxanthin, has a yellow hue while astaxanthin is more red [1]. A high idoxanthin to astaxanthin ratio might therefore make the flesh appear more yellow [260]. A possible explanation of the increased angle of hue in the fed fish fillets might be increased breakdown of astaxanthin to idoxanthin. Schiedt *et al.* [202] hypothesised that metabolic rate and or stressful rearing conditions effected the breakdown of astaxanthin, although idoxanthin can occur normally in *Salmo* species [1]. Idoxanthin has been shown to be less prevalent in large immature fish than small fish and maturing fish which would have faster metabolism [32].

The inferior colour observed in the fed fish although predominantly due to body size, might also have been influenced by faster metabolic rate in these fish caused by SDA.

6.4.5.2 Fat and water content

The consumer judges fish quality on; colour, fat content, assumed freshness, and texture: pale soft salmon are thought by processors to be fat [240]. The fat content of farmed fish can be manipulated in terms of quantity, distribution, and perceived quality[152].

The effect of the 30-40 degree-day fast on water content and the fat-meter readings between the fed and fasted fish showed no differences between the groups. The power of the water content test could not be calculated as the data were non-parametric, the fat content power test was low, 0.08. Consequently, conclusions as to whether three days starving lowered fat levels and increased water content of the muscle could not be inferred from the data. The fed fish had a higher water and a lower fat content than the 'starved' fish, if muscle fat was utilised during the fast, the reverse would be true. This was shown by [179] in brown trout after two months starvation. In rainbow trout a decrease in whole body fat was seen with starvation, but it only became significant 30 days after the last meal [150], the water content increased as the fat content fell. Jezierska *et al.* [106] report that the visceral fat in rainbow trout starved for 27 and 48 days was more mobile and contributed more to energy utilisation than muscle fat.

The fat and water content of rainbow trout muscle might not therefore be expected to alter after a three day fast in fish of this size.

6.4.5.3 Gaping

Strategies for reducing gaping in fish would be beneficial for the fish processor as gaping fillets fall apart when skinned and so are unsuitable for many products. Fat content and location have been implicated in gaping as well as muscle pH [240].

The gaping assessment in the present study did not detect differences between treatments. Theoretically, fed fish should exhibit more gaping. A fast decline in pH at slaughter is thought to make collagen more accessible to protease activity [211] and increase protease leakage from lysosomes [265]. Robb *et al.* [186] reported less gaping in rainbow trout with a higher muscle pH. Work by Einen *et al.* [67] found more gaping in well fed salmon

relative to starved fish or those on restricted rations. However, there wasn't a clear relationship between starvation and gaping in Einen's work and Andersen *et al.* found fillets without gaping had a lower pH than those that gaped [6, 67, 68]. The causes of gaping are multi-factorial and so other aspects might have masked any effects of fasting.

The gaping measurement in the present study did not show a difference between the two groups, even though *rigor mortis* was delayed and the pH of the 'starved' fish was higher. Gaping is a qualitative measurement and so the sample size might have been too small to detect differences if present or the length of the starvation time might not have been sufficient to produce any effect.

6.4.5.4 Texture

Texture is an important process trait in fish as soft flesh is difficult to handle.

The sensory finger compression test performed after six days storage gave identical scores for both groups. The Warner-Bratzler shear test in the fed and 'starved' fish also failed to show significance. The power of the Warner-Bratzler test was quite low, 0.41, so conclusions of no difference could not be drawn from the data. The difference became significant if a one-tailed test was applied to the data, and therefore this attribute might warrant further investigation. Justification for performing a one-tailed test, that fasting is thought to firm fish flesh can be found in the literature, although this usually involves a longer starvation period than that used in the current study. Regost *et al.* [179] report firmer flesh in unfed brown trout after two months starvation. While Gómez-Guillén *et al.*[83] found starving for seven days hardened the flesh of triploid Atlantic salmon. Einen *et al.* [67, 68] found firmer flesh in salmon starved for 58 days.

Textural changes can be influenced by the cellular proteins as well as the connective tissue that connects muscle blocks. Small textural changes following short-term starvation might be caused by the reduction of sarcoplasmic proteins [33]. The proteolytic enzymes contained in the sarcoplasm hydrolyse the structural proteins that contribute to flesh texture. Fed fish have greater amounts of sarcoplasmic proteins than starved fish [22].

Temperate and arctic fish have evolved strategies to cope with the limited food availability at certain times of the year. They use the structural body components most easily replaced once food becomes available again [33]. The glycogen stores and sarcoplasmic proteins are initially used in preference to the myofibrillar proteins. This reduces the quantity of muscle proteases available for degradation of structural components that causes flesh softening.

The contribution of collagen to fish texture might also be affected by fasting/ starvation, as collagen cross-linking continues while fish are not feeding. The collagen component of texture might therefore increase the firmness of the flesh during fasting. This however is thought to be of more significance during medium to long term starvation [83].

6.5 Summary and conclusions

A 30-40 degree-day fast was shown to have an effect on the process quality of farmed rainbow trout. The muscle pH of the 'starved' fish was generally higher than in the fed group although this was not always significant. The muscle lactate level confirmed the finding of a higher pH in the tissue, although the difference was not significant. The higher lactate level in the fed fish suggested that they might be more metabolically active, than the starved fish. This hypothesis was strengthened by the observation that the fed fish entered *rigor mortis* earlier than the 'starved' fish. The decline of freshness was faster in the fed fish, as shown by the Torrymeter data, visual assessment and K value. This confirms the supposition that the fed fish were the most metabolically active.

Fulton's condition factor was lower in the 'starved' fish indicating that they were slimmer. The fed fish had a lower dress-out percentage and consequently a higher DSI, showing that percentage yield was greater in the starved fish. The HSI, fillet fat and water contents however did not differ between the two groups, suggesting that the short fast did not influence meat composition. The sensory scores for gaping and texture did not differ between the two groups. The instrumental measurements for texture were significant only

at the 8% level with the 'starved' fish flesh being firmer. The fillet colour measurements indicated that the 'starved' fish had a superior colour on Roche *Salmo*fan™ and L* values. However this was affected more by body size than by treatment.

The data from this study therefore suggest that the short-term withdrawal of food from farmed fish before harvest can be beneficial for product quality. It delays the onset of *rigor mortis* and maintains fish freshness, and might also improve colour and texture of the flesh, although the evidence of this study was too weak to substantiate this.

Starving farmed trout for 30-40 degree-days does not appear to have severe effects on the nutritional status of the fish as the muscle fat content and HSI were not affected.

7 THE EFFECTS OF STUNNING AND KILLING TECHNIQUE ON THE WELFARE AND QUALITY OF FARMED ATLANTIC SALMON

7.1 Introduction

Physiological stress at harvest has been shown to influence end-product quality in farmed rainbow trout, as detailed in Chapter 4. To fulfil the title of this thesis, data was needed from another farmed salmonid species. Atlantic salmon were selected, as they are the main species in aquaculture production in the UK, (FEAP (http://dev.ibicenter.net/production/countries/uk_en.asp). A pilot study was performed to determine if methods used to evaluate harvesting techniques in rainbow trout were suitable for use in remote locations, i.e. salmon farms. Additionally, simple physical measurements were tested for their ability to detect differences in the carcass quality of salmon. Techniques that were found to be suitable were then used during a commercial fish harvest to evaluate whether stunning method influenced salmon welfare and quality at slaughter.

7.2 Pilot study

7.2.1 Materials and methods

7.2.1.1 Selection of parameters for use with Atlantic salmon in remote locations

The results of the study on the effects of stunning and killing technique on the welfare and quality of farmed rainbow trout, (Chapter 4), were examined with a view to selecting parameters for the study involving salmon. The parameters selected for assessing welfare were the plasma and muscle biomolecules associated with physiological stress: while carcass quality would be determined by measuring fish freshness, *rigor mortis* development, and the shear strength and colour of raw fillets.

7.2.1.2 Facilities

The pilot study was performed in October 2000 at Lighthouse of Scotland's Quarry Point site on Loch Fyne, Argyll. In the morning, prior to daily feeding, approximately 100

fish were gathered by seine net to the edge of the cage where they were allowed to rest for around three hours.

7.2.1.3 Animals

The Atlantic salmon were reared in 40m diameter cages in a sea water loch post smoltification, Appendix 2, Figure 12. They were stocked as $S0^+$ smolts in spring 1998 and were due to be harvested in early 2001. They were diploid, all-female, two sea winter fish. The salmon were 60.1±5.1cm, 3.2±0.7kg and had a condition factor of 1.4±0.2.

7.2.1.4 Design

The fish were sampled serially for practical reasons. They became visibly stressed due to the crowding and handling procedures, so the chosen sample size of 20 was reduced to ten. Blood and muscle samples were to be collected within two minutes of 'death', so two sets of fish were killed by each method. One set for pre *rigor mortis* muscle samples (M) and the other for blood samples, downgrading and *rigor mortis* assessments (B). The samples were collected in blocks of five in a MBBM pattern to reduce any bias caused by serial sampling.

7.2.1.5 Conditions

The samples were collected during daylight hours between 13:00h and 16:30h; daylength was 9h 41min, with sunrise at 7:14am. Air and water temperatures were 13°C and 12°C respectively. The weather was squally with occasional rain showers and wind speeds of 25-30mph.

7.2.1.6 Killing and stunning methods

The killing/ stunning methods tested were percussive stun and death in air as they had produced different levels of stress in rainbow trout. The percussive stun procedure involved netting fish individually from the cage onto the metal deck of the work-boat. The fish were manually restrained and multiple blows given to the head with a polypropylene club. After the percussively stunned group had been sampled, the death in air fish were netted *en masse* into a ~200l bin and left for 30min to die. The longer dying period

compared with the rainbow trout study, 15min, was due to the remote location of the cage.

7.2.1.7 Analysis

7.2.1.7a Collection

There was a four hour delay in icing the fish due to the remote location of the site.

7.2.1.7b Plasma biochemistry

The blood samples were centrifuged at ambient temperature within three hours of collection and the plasma frozen at -20°C for 24 hours before subsequent storage at –80°C. Plasma was analysed for adrenaline, noradrenaline, cortisol, pH, lactate, glucose and protein within fourteen months of sample collection, as outlined in Section 2.3.3.

7.2.1.7c Muscle biochemistry

Pre *rigor mortis* muscle was analysed for metabolites as described in Section 2.3.3.1. Muscle stored under vapour phase liquid nitrogen was analysed for high-energy phosphates within 10 days of sample collection. Lactate was measured within one month in the same extract, which had been stored at -80°C. Post *rigor mortis* muscle samples were taken from all fish five days after death and stored at –80°C for up to six months. Lactate and K value were determined using the methods described in Section 2.3.3.1 and 2.3.3.4.

7.2.1.7d Muscle quality

Muscle pH was measured immediately after death and at 98h *post mortem* as described in Section 2.4.1. The progression of *rigor mortis* was monitored over 90 hours using the technique described in Section 2.4.2. *Rigor mortis* was measured twice a day for logistical reasons. Torrymeter readings were taken twice daily to increase the number of data points in the regression analysis due to the reduced sample numbers, using the method outlined in Section 2.4.3.1. Downgrading was assessed after five days ice storage following which, the fillet colour and texture was measured. The Warner-Bratzler measurements were performed on five samples from each fish to increase the sensitivity of the test, the method used was as described in Section 2.4.7.2.

7.2.2 Results

7.2.2.1 Plasma biochemistry

Plasma was analysed to give an indication of primary and secondary stress responses occurring in the fish and the extent of metabolic activity during the harvesting process.

7.2.2.1a Hormones

The effect of killing/ stunning method on the primary stress response was determined by measuring plasma adrenaline, noradrenaline, and cortisol.

- *Catecholamines*

The fish that died following a percussive stun contained significantly less adrenaline than the death in air group, t=3.77, P=0.004, df=9, power~0.78, Figure 7.1A. The adrenaline level in the percussively stunned fish was 2.4% of the death in air value. Differences were not however detected in noradrenaline levels, t=0.90, P=0.378, df=18, power~0.13. The noradrenaline levels were 3±4ng/ml and 5±6ng/ml in the percussively stunned and death in air groups, respectively.

These data suggest that the death in air group were exhibiting greater signs of stress than the percussively stunned fish.

- *Cortisol*

The plasma of the percussively stunned fish contained significantly more cortisol, 146% than the death in air group, t=2.506, P=0.0233, df=16, power~0.54, Figure 7.1B.

These data suggest that either the percussively stunned fish were under more long-term stress than the death in air group, or there was a greater breakdown/ clearance of cortisol from the plasma in the death in air group.

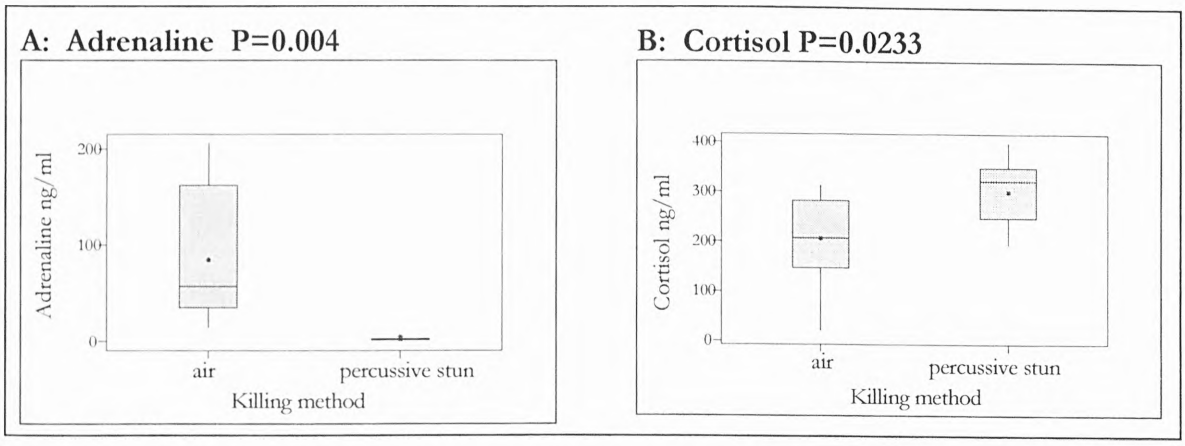


Figure 7.1A-B Effect of 'killing' method on salmon plasma stress hormones
Single determination of adrenaline n=10, duplicate determinations for cortisol n=9.

7.2.2.1b Chemistry

The secondary stress response and muscular activity, were assessed in the fish by measuring plasma pH, lactate, glucose and protein.

The plasma pH was significantly higher in the percussively stunned group, t=15.5, P=0.000, df=18, power~0.98, while the lactate levels were significantly lower, t=9.78, P=0.000, df=18, power~0.97, Figure 7.2A-B. The pH reading in the percussively stunned fish was similar to the death in air value, 108%, while the plasma lactate content was 34.5% of the death in air value in the percussively stunned fish.

Differences were not detected between the two groups in terms of plasma glucose, (t=0.356, P=0.726, df=17, power~0.06), or plasma protein, (t=0.7558, P=0.46, df=18, power~0.13). Glucose levels were, in the percussively stunned and death in air groups, 7±1.7μmol/ml and 7.2±1.1μmol/ml, respectively. Plasma protein levels were 62±7mg/ml and 65±9mg/ml in the percussively stunned and death in air groups, respectively.

The pH and lactate data suggest that the death in air fish were more metabolically active whilst dying than the percussively stunned fish. The secondary stress response, determined by a change in plasma glucose concentration, was not detectable. The plasma volume also did not alter significantly with killing/ stunning technique.

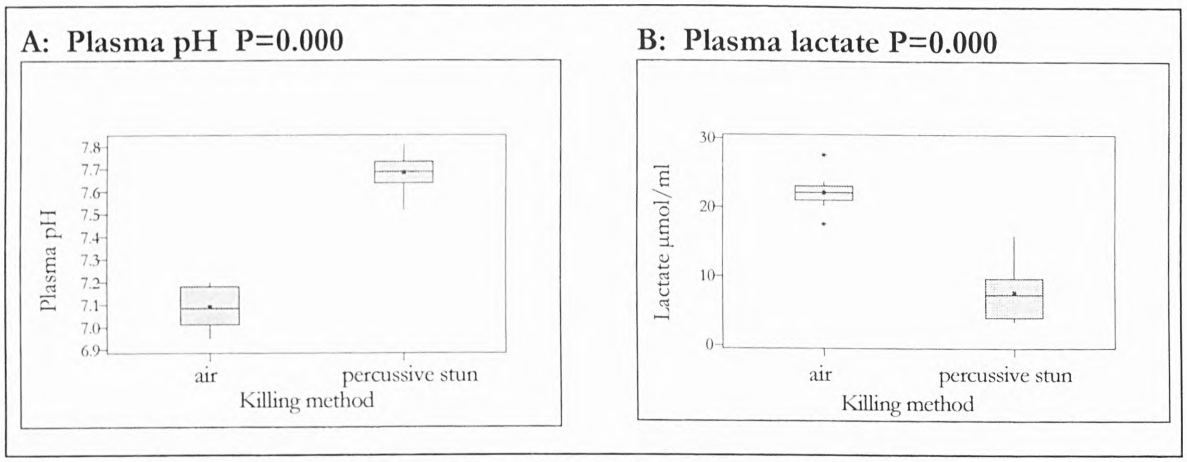


Figure 7.2A-B Effect of 'killing' method on salmon plasma biochemistry
Single reading pH, duplicate determinations for lactate, n=10.

7.2.2.2 Muscle biochemistry

Muscle metabolites were analysed to determine if killing/ stunning technique influenced muscle activity *peri-mortem.*

7.2.2.2a Pre rigor mortis

Killing/ stunning method made a distinct difference in pre *rigor mortis* metabolite content of salmon muscle, Figure 7.3A-D. The fish that were left to die in air had significantly lower levels of creatine phosphate, t=12.19, P=0.0000, df=11, power~0.97, and ATP, t=8.18, P=0.0000, df=18, power~0.96. They also contained significantly higher lactate, t=-7.77, P=0.0000, df=18, power~0.95 and creatine levels t=-3.11, P=0.0060, df=18, power~0.68. Creatinine was not detected in any sample, suggesting that there was no further breakdown of creatine and creatine phosphate. The metabolite levels in the percussively stunned fish expressed as a percentage of the death in air fish were for creatine phosphate, creatine, ATP and lactate, 754%, 77%, 53%, and 312%, respectively.

These observations suggest that the fish that died in air underwent greater amounts of muscle activity whilst dying compared with the percussively stunned fish, and confirmed the similar finding in the plasma data.

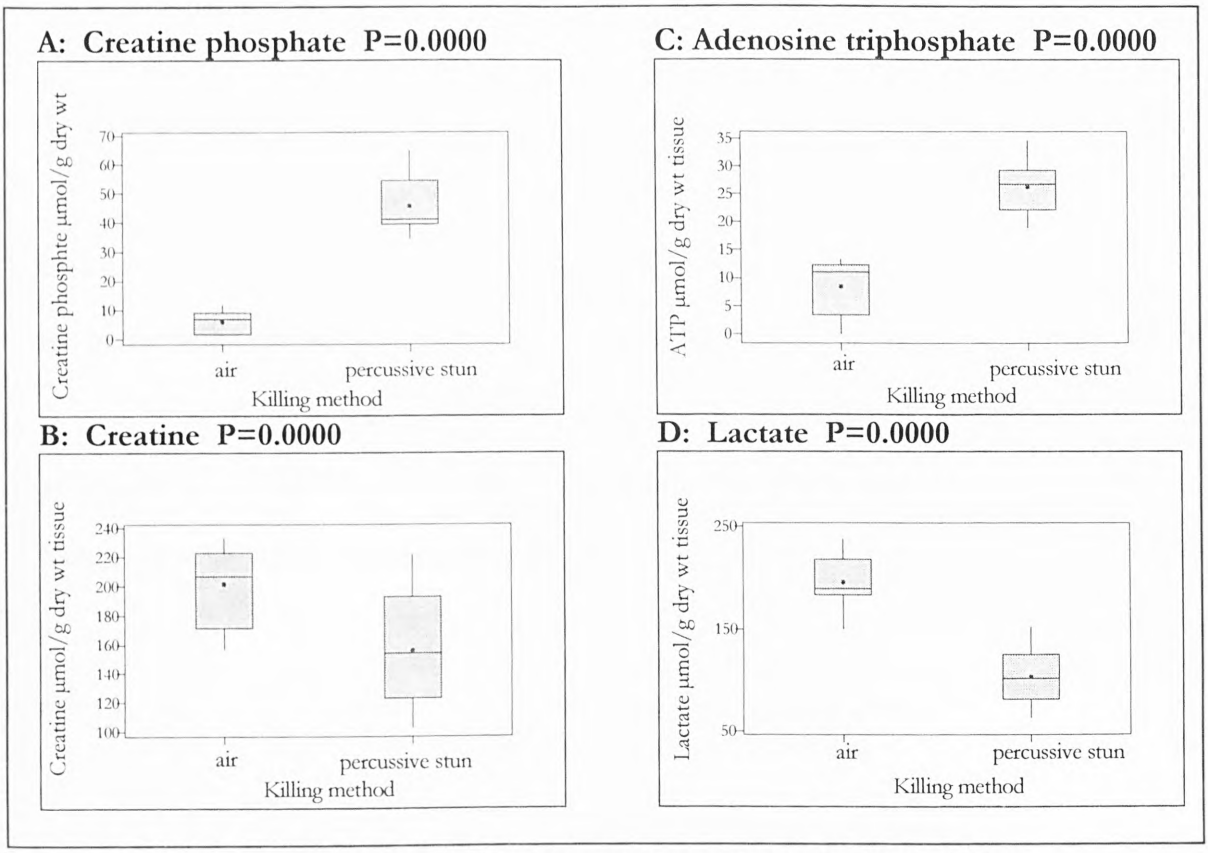


Figure 7.3A-D Effect of 'killing' method on salmon pre *rigor mortis* muscle metabolites
Samples were measured in duplicate, n=10

7.2.2.2b Post rigor mortis

Post *rigor mortis*, differences in lactate and K value observed between the killing/ stunning methods failed to reach significance, t=-1.19, P=0.24, df=38, power~0.21 and t=-1.01, P=0.32, df=33, power~0.1, respectively. The fish that died in air contained more lactate than those that died following a percussive stun, 243±42µmol/g dry wt tissue and 226±45 µmol/g dry wt tissue, respectively. The death in air group was less fresh than the percussively stunned fish with K values of 61±11%, and 58±7%, respectively.

7.2.2.3 Muscle quality

The effect of killing/ stunning method on carcass quality was assessed by measuring; pH, *rigor mortis*, freshness, downgrading, colour and texture.

7.2.2.3a Muscle pH

Muscle pH declines predominantly as a consequence of lactate production and so can be used as an indication of the rate of anaerobic glycolysis occurring in muscle.

The rate of decline in pH over the first minute *post mortem*, as measured by the slope of the regression line, did not show a difference between the groups, t=-0.544, P>0.05,

df=56, Figure 7.4. The intercept data show the percussively stunned fish had a significantly higher pH compared with the death in air fish, t=8.12, df=56, P<0.001, Figure 7.4.

The first pH reading, the initial pH, (pH_i), shows a very highly significant difference between the two groups, t=-8.11, P=0.0000, df=12, power~0.95, Figure 7.5A. The pH_i in the percussively stunned fish expressed as a percentage of the death in air group was 109%.

There was also a significant difference in the pH taken 96 hours *post mortem*, pH_{96}, t=3.51, P=0.0012, df=38, power~0.85, Figure 7.5B. The percussively stunned fish had the higher pH value, 101% of the death in air fish.

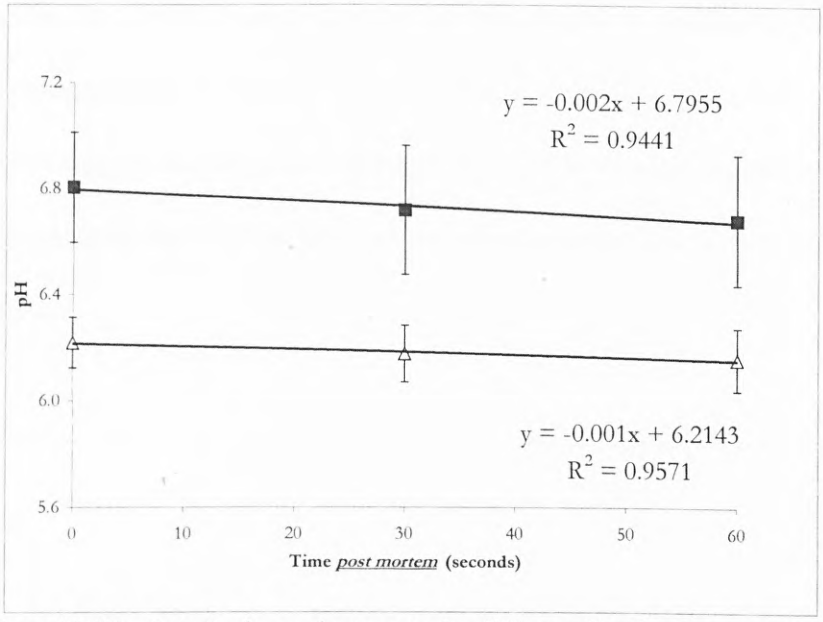


Figure 7.4 Effect of 'killing' method on salmon muscle pH immediately *post mortem*
Results expressed as mean ± standard deviation, single measurement per fish, air (△), percussive stun (■), n=10, P>0.05 in the rate of decline in pH.

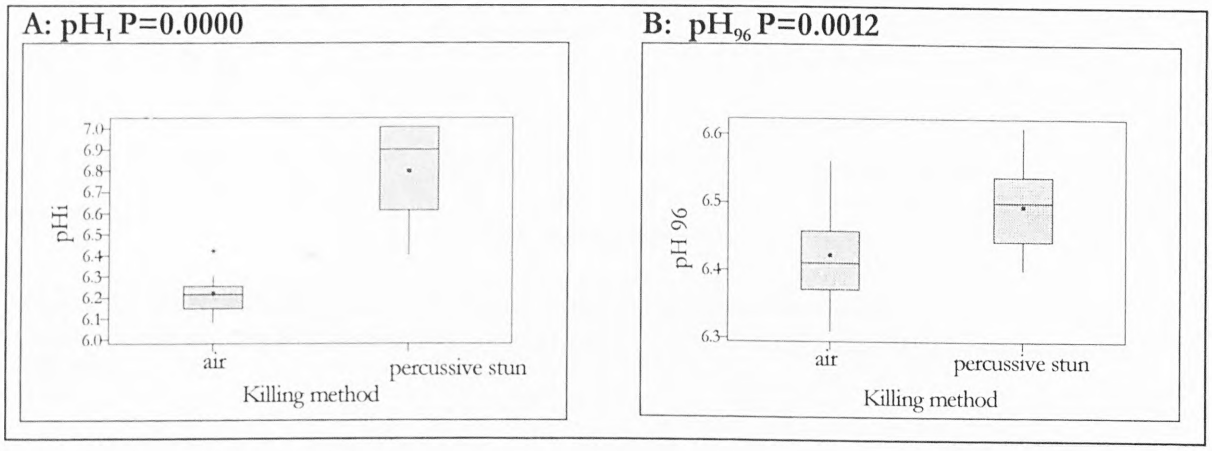


Figure 7.5A-B Effect of 'killing' method on salmon muscle pH
Single measurements per fish, n=10.

These data suggest that more anaerobic glycolysis had occurred in the death in air group, although the speed of lactate production at the time measured was similar. The lower pH in the death in air fish suggests there was the potential for quality to be compromised in this group.

7.2.2.3b *Rigor mortis*

Rigor mortis was assessed, as it not only provides information on the degree of muscle activity that has occurred in the salmon, but it provides important information for fish processors. There was an earlier onset and resolution of *rigor mortis* in the fish that died in the air, Figure 7.6. The difference between the two groups in resolution of *rigor mortis*, 20h, was highly significant, t=-3.89, P=0.0037, df=9, power~0.8, Figure 7.7.

The data suggest that the death in air group underwent more muscle activity whilst dying than the percussively stunned fish and therefore supports the plasma and muscle biochemistry data.

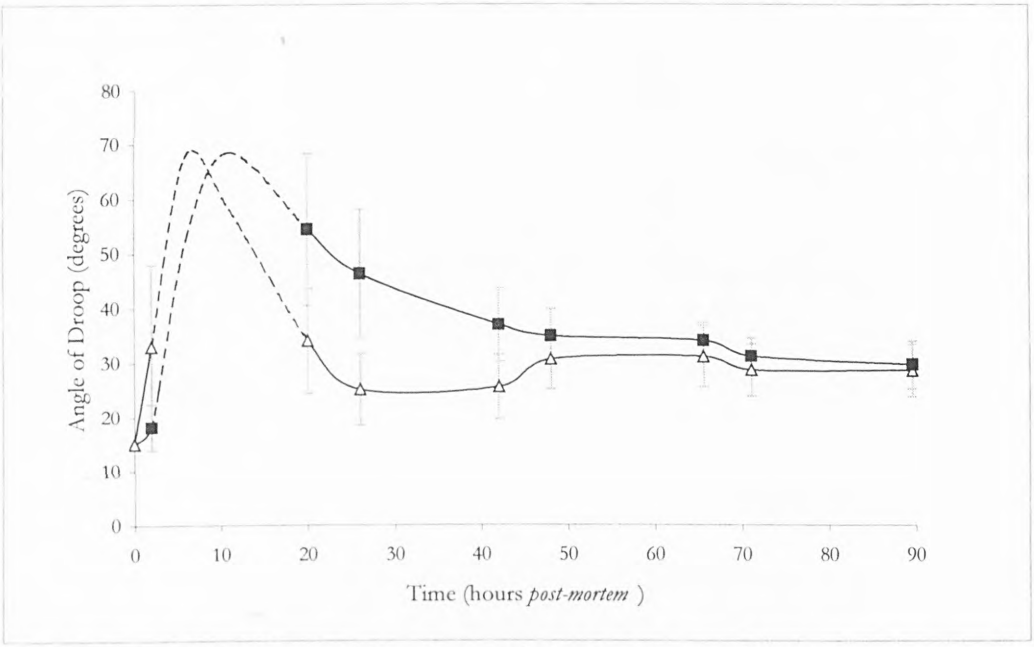


Figure 7.6 Effect of 'killing' method on *rigor mortis* development in salmon
Results expressed as mean ± standard deviation, duplicate measurements per fish, air (△), percussive stun (■), n=10.

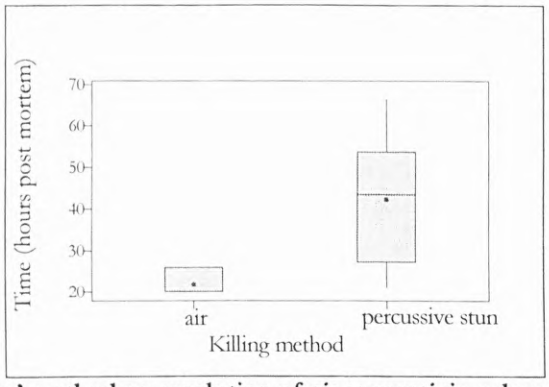


Figure 7.7 Effect of 'killing' method on resolution of *rigor mortis* in salmon
Duplicate measurements per fish, n=10, P=0.0037.

7.2.2.3c Freshness

The Torrymeter was used to assess freshness in the salmon, as freshness is an important characteristic of quality in fish.

The rate of decline in freshness as illustrated by the slope of a regression line, shows a significantly faster decline in the death in air fish, t=-2.856, P<0.05, df=12, Figure 7.8. The percussively stunned fish therefore maintained their freshness better than the death in air group.

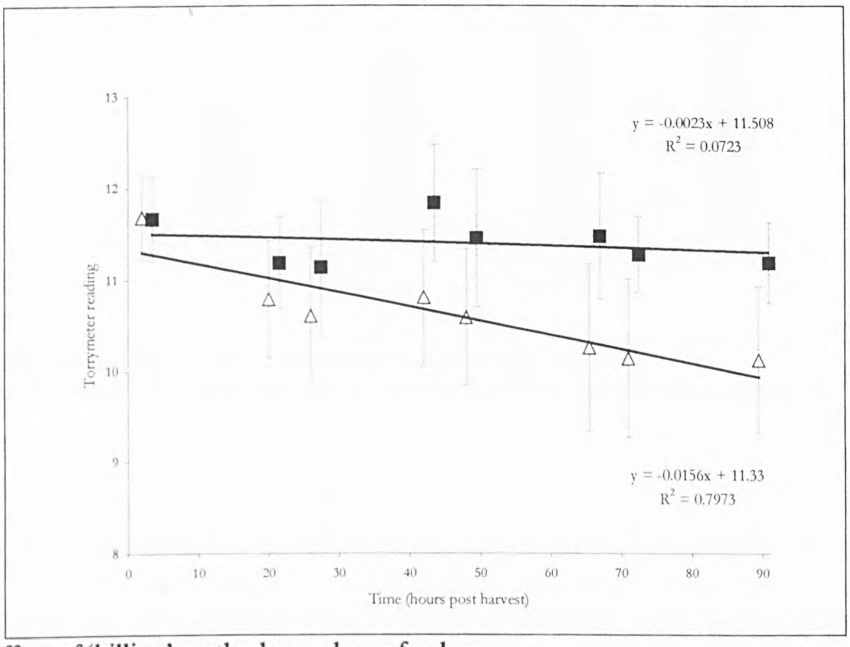


Figure 7.8 Effect of 'killing' method on salmon freshness
Results expressed as mean ± standard deviation, triplicate readings per fish, air (△), percussive stun (■), n=10. P<0.05

7.2.2.3d Downgrading

A downgrading assessment was performed five days *post mortem* on the fish killed for blood samples, to determine if carcass quality was compromised by killing/ stunning method.

Significantly higher scale loss was observed in the death in air fish, Figure 7.9, W=62.5, P=0.0012. There were no other significant differences between the two groups in the attributes assessed, although more damage was apparent in the fish that died in air in all apart from gaping, Figure 7.9. The inferential statistics were: external haemorrhages, W=84.0, P=0.0855; bloodspotting, W=93.5, P=0.3653; and gaping, W=103.5, P=0.9353. There was no bias in fish allocation to the sample groups as Fulton's condition factor was 1.38±0.10 and 1.41±0.25 in the percussion and death in air groups respectively: the difference not being significant, t=-0.56, P=0.58, df=24, power~0.09.

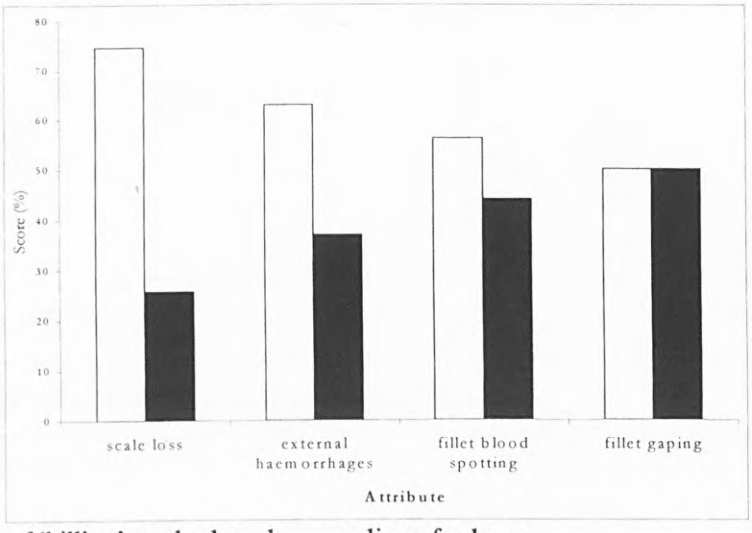


Figure 7.9 Effect of 'killing' method on downgrading of salmon
Single observer, one assessment after five days ice storage, air (□), percussive stun (■), n=10.

7.2.2.3e Colour

The colour of salmonid flesh is an important quality characteristic, as consumers consider flesh colour before purchase. Colour measurements were made on the blood set of fish five days *post mortem.*

There were no differences detected between the two groups in the lightness reading, L*, t=0.12, P=0.90, df=18, power~0.05, and Roche *Salmo*Fan™ scores, W=105.0,

P=1.000. The values measured in the L* and Roche *Salmo*Fan™ scores respectively were 35.2±1.4, 35.3±1.1, and 27±0.5, 27±0.8 in the percussively stunned and death in air groups, respectively. The angle of hue and chroma data showed significantly higher values in the fish that died following a percussive stun. The inferential statistics were for, angle of hue: t=-2.23; P=0.039; df=18; power~0.48, the values of the percussively stunned fish expressed as a percentage of the death in air group was 108%. For chroma the values of the percussively stunned fish expressed as a percentage of the death in air group was 111%, and the inferential statistics were t=-2.64; P=0.022; df=12; power~0.59, Figure 7.10.

The colour measurements suggest that the percussively stunned fish fillets were more yellow and opaque.

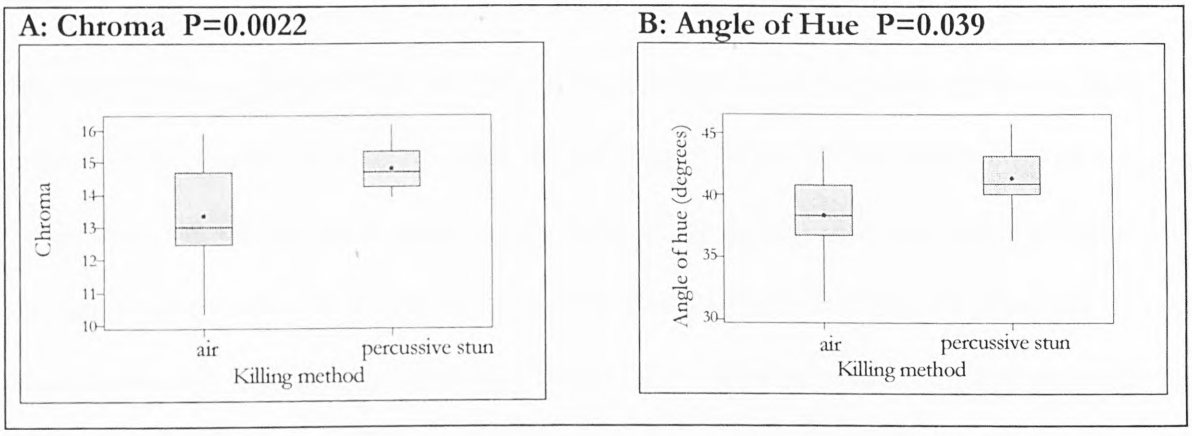


Figure 7.10A-B Effect of 'killing' method on salmon fillet colour
Instrumental colour measurements were taken after five days ice storage, triplicate measurements were taken on freshly prepared fillets, n=10.

7.2.2.3f Texture

Texture is an important organoleptic and process quality in salmon. It was assessed using a Warner–Bratzler shear test. The peak force required to cut the sample was in the percussively stunned and death in air fish 246±28g, and 240±46g, respectively. The difference between the two groups was not significant, t=-0.39, P=0.70, df=18, power~0.07.

7.2.3 Discussion

The suitability of various biochemical indicators of stress, and physical measurements of carcass quality, were assessed for use on farmed salmon in remote locations. The influence of physiological stress at harvest on salmon quality was also appraised.

7.2.3.1 Plasma biochemistry

Adrenaline, noradrenaline and cortisol were measured to assess the primary stress response of the fish. Plasma glucose was determined as a secondary stress response indicator, plasma pH and lactate measured anaerobic activity, while protein was assessed to detect changes in plasma volume.

The fish that were percussively stunned directly after netting showed little evidence of acute stress. Their adrenaline levels were very low unlike the fish that were left to die in air. The difference between the two groups was significant and the power of the test quite high, 0.78, so it could be concluded that the death in air fish exhibited greater signs of stress than the percussively stunned group. Catecholamine release occurs within seconds of perceiving a stressor so that even though the time that elapsed before sampling was short, it was still sufficient for adrenaline release. The higher adrenaline levels in the death in air fish suggest that these fish were stressed, while the percussively stunned fish were not. Published adrenaline values for Coho salmon (*Oncorhynchus kisutch*, Walbaum 1792) recalculated from [142] give 5.6±0.7ng/ml in unstressed fish, and 149.4±14ng/ml in stressed fish. The values in the current study; 2±1.5ng/ml in percussively stunned and 84±69ng/ml in death in air fish, are therefore similar to literature values.

Plasma noradrenaline levels did not differ significantly between the two killing/ stunning methods. The power of the test was however low, 0.13, and so conclusions of no difference were not valid. The noradrenaline levels in the present study, 2.7±4ng/ml and 4.8±6ng/ml, fall in the range of the literature values for unstressed 0.5±0.5ng/ml, and

stressed 33.8±3ng/ml Coho salmon [142]. As the values obtained in this study were slightly higher than the unstressed literature values, they might suggest that the fish were under moderate hypoxic stress possibly due to crowding, this would support the visual evidence of stress observed during sample collection, see Section 7.2.2.3d.

The plasma cortisol levels were significantly higher in the percussively stunned fish compared with the death in air group, the power of the test was only 0.54 so definitive conclusions cannot be drawn from the data. The cortisol values were 300±66ng/ml and 205±92ng/ml, in the percussively stunned and death in air fish, respectively. Literature cortisol values have been reported as 101±8ng/ml and 339±18ng/ml in Atlantic salmon kept at low and high stocking densities [216]. The high levels of cortisol in the percussively stunned fish contrasts directly with their lower adrenaline levels. The longer dying period in the death in air fish would, on initial inspection be expected to increase the cortisol levels, as there would have been more time for synthesis and release. A possible explanation of the apparent decrease in cortisol levels in the death in air group would be if cortisol clearance/ catabolism during this period was faster than cortisol synthesis and release.

The plasma glucose levels did not differ with killing/ stunning method, even though there was significantly more adrenaline in the death in air fish and cortisol in the percussively stunned fish. The effect of the two hormones on glucose levels would have been to lower glucose in the death in air fish and increase glucose in the percussively stunned fish. Therefore a visible difference would not have been unexpected. The power of the plasma glucose test was, however low, 0.06, and so a conclusion of no difference was not valid. The fish had not been starved before harvest and so plasma glucose levels might have been predominantly influenced by transport from the gut rather than by the hormones. The plasma glucose concentration in the current study ~7μmol/ml is similar to published values for juvenile salmon subjected to transport or confinement stress [98, 200].

There were significantly higher levels of lactate in the plasma of the death in air

group and a corresponding significantly lower pH value, the power of the tests were very high, ≥0.97. The higher level of plasma lactate in the death in air fish probably result from the longer dying period, allowing lactate levels to build up. Published plasma lactate levels in Atlantic salmon are 1μmol/ml to 3.5μmol/ml, the rise during transportation stress [98], and 0.9±0.1μmol/ml to 3.7±0.8μmol/ml, the rise caused by confinement stress [200]. The levels in the present study 7μmol/ml and 22μmol/ml were similar in concentration although slightly higher than the published values. The higher values would reflect the increased anaerobic activity in *post mortem* fish.

The plasma protein levels in the two groups did not differ significantly from each other. The power of the test was, however low, 0.13, and so conclusions of no difference might be invalid. The protein levels were 62±7mg/ml and 65±9mg/ml in the percussively stunned and death in air groups, respectively. These values are comparable to those quoted by Hemre and Hansen [89], 61-62mg/ml. The similarity of the protein levels suggests that the plasma volumes were not affected by killing/ stunning method.

7.2.3.2 Muscle biochemistry

7.2.3.2a Pre rigor mortis

Muscle metabolites were measured to determine the energy status of the fish at death. The two killing/ stunning methods showed distinct differences.

The values of creatine phosphate and ATP in Atlantic salmon measured in the present study are within the ranges of published data, summarised in Tables 7.1 and 7.2. Lactate and creatine levels have been recalculated into μmol/g wet weight to compare with literature values, Table 7.2. The muscle metabolite data for the percussively stunned group fall in the range of published values for rested and exercised salmon and the death in air group have slightly lower levels of high-energy phosphates. This was not unexpected, as the fish had been left to die for 30min before sampling.

The levels of creatine phosphate and ATP were significantly higher in the percussively stunned fish and the creatine and lactate levels significantly lower, indicating

that the percussively stunned fish expended less energy whilst dying than the death in air group.

Table 7.1 Comparison of muscle metabolite data with published values

Study	Erikson *et al.* 1997 Base line	Erikson *et al.* 1997 unstressed	Erikson *et al.* 1997 stressed	Erikson *et al.* 1999 unstressed	Erikson *et al.* 1999 stressed	Current study air	Current study percussion
Metabolite	n=6	n=12	n=12	n= 7	n=7	n=10	n=10
Creatine phosphate	71±48	49±20	8±5	84±26	12±3	6.1±3.9	45.6±9.5
ATP	21±5	22±6	10±6	30±3	4±1	8.4±5.1	26.3±4.6

Published levels of high-energy phosphates measured in base line, unstressed and stressed Atlantic salmon Erikson *et al.* [71, 72] compared with the current study. Expressed in μmol/g dry weight, mean ± standard deviation.

Table 7.2 Comparison of metabolite data with that derived from Wilkie *et al.* [267]

Study	Wilkie *et al* rest 12°C	Wilkie *et al* exercise 12°C	Current study air	Current study percussive stun
Metabolite	n=5-8	n=5-8	n=10	n=10
lactate	3±0.5	55±5	56.8±4.5	29.4±5.3
creatine phosphate	46±2	7±1	1.8±1.2	13.3±3.3
creatine	24	63	58.1±6.5	44.5±6.9
ATP	14.5±0.5	4.5±1	2.5±1.5	7.5±0.9

The reported levels of the metabolites measured in rested and exercised Atlantic salmon in μmol/g wet weight ± SEM Wilkie *et al.* [267], current study μmol/g wet weight ± standard deviation.

Fish that were percussively stunned had a higher energy status as shown by the CP: Cr and CP: ATP ratios. Percussively stunned fish also had lower lactate levels suggesting they showed less evidence of anaerobic glycolysis, than the fish that died in air.

The CP: Cr ratios in the present study were, 0.30:1 following a percussive stun and 0.03:1 for fish that died in air. For rested and exercised Atlantic salmon CP: Cr values calculated from the literature are 1.9:1 and 0.11:1, respectively [267]. The percussive stun group in the present work was therefore within the range of the literature, while the fish that died in air had a lower value than the exercised fish in the study by Wilkie *et al.* [267]. This probably reflects the fact that death is more stressful than exercise.

CP: ATP ratios in the present study were, for the fish that died following a percussive stun, 1.74:1 and in air 0.72:1. Literature values for Atlantic salmon are 0.95:1;

for fish anaesthetised in MS222 [174], 3.4:1 in rested fish, 2.2:1 in unstressed fish, and 0.85:1 in, stressed fish [71]. An even lower value, 0.2:1 has been reported in stressed Atlantic salmon [72], the results of the current study therefore are within the range of published values.

Death in air fish showed more evidence of muscular activity than the percussively stunned fish. Energy expended in attempting to maintain homeostasis whilst dying would have caused this increased 'activity'. The precise time of death of the death in air group could not be determined. As the fish were killed *en masse* the time from when the fish actually died to when the samples were collected might have influenced the results. The samples were taken within 1.5 hours of 'death' i.e. <2 hours from netting. The muscle samples were collected in two blocks interspersed by the blood samples. The metabolite data from the first and second block of samples did not differ significantly, indicating that the 'muscle activity' measured occurred during the first 30min. The inferential statistics were, for the two blocks of fish: creatine phosphate $t=0.1540$, $df=8$, $P=0.8814$; creatine $t=0.6431$, $df=8$, $P=0.5381$; ATP $t=0.5355$, $df=8$, $P=0.6068$; lactate $t=0.05723$, $df=8$, $P=0.9558$.

The mean values for the total creatine pool were 201.6μmol/g dry wt. and 207.3μmol/g dry wt. in the percussion and death in air groups respectively. These values did not differ significantly, $t=-0.43$, $P=0.67$, $df=18$, power~0.07. This confirms the validity of the creatine phosphate, creatine and creatinine measurements.

7.2.3.2b Post rigor mortis

Lactate levels were measured in post *rigor mortis* flesh to relate to pH measurements and, as an indication of anaerobic glycolysis occurring in the muscle during chilled storage. Post *rigor mortis* lactate levels in the two groups of fish were not statistically different, suggesting that they had the same *ante-mortem* energy status. The change in lactate levels over four days ice storage showed that the percussively stunned fish produced significantly more lactate than the death in air fish, ($t=6.51$, $P=0.000$, $df=16$), with a subsequent,

significant, fall in pH, (t=-6.48, P=0.000, df=12), Figure 7.11A-B. This suggests that the percussively stunned fish had higher energy reserves immediately *post mortem* due to their lower muscle activity whilst dying.

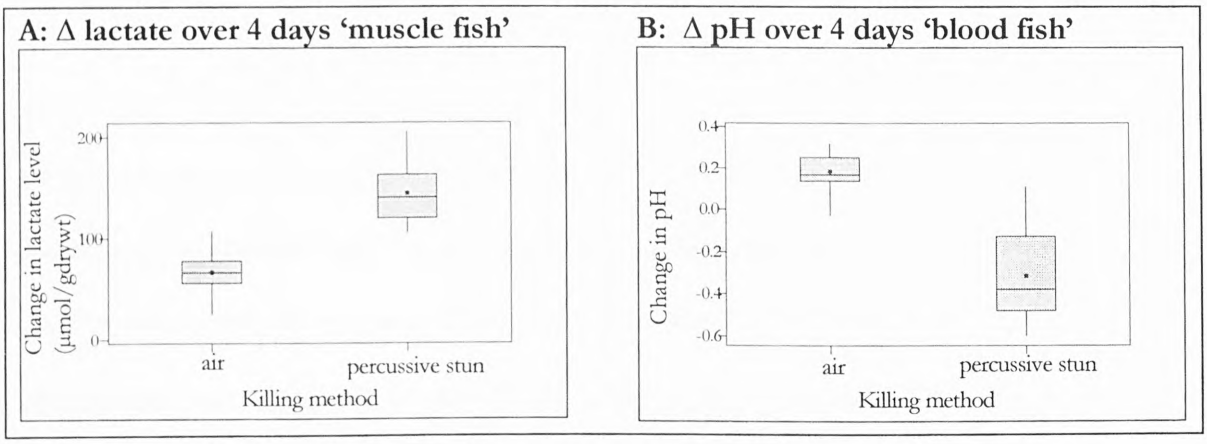


Figure 7.11A-B Effect of 'killing' method on change in muscle lactate and pH over four days
Data presented as box and whisker plot, data from duplicate (lactate) and single samples (pH), n=10. P=0.0000 for both A and B.

The rate of anaerobic glycolysis in the percussively stunned fish was therefore higher *post mortem*. This indicates that the muscle had a greater capacity to produce ATP than the death in air group; the lower K value measured after five days ice storage strengthens this finding. Autolysis was less advanced in these fish i.e. they were fresher, because the high-energy phosphates present were still capable of fuelling homeostasis mechanisms. The K values of the two groups did not however differ significantly. The power of the post *rigor mortis* muscle tests were low, 0.21 and 0.1 for lactate and K value respectively, and so conclusions of no difference might not be definitive.

Erikson *et al.* [71] report a higher K value in stressed Atlantic salmon relative to unstressed and 'baseline' fish two, four, and seven days *post mortem*, but not on days one or 13. The difference in K value in Erikson *et al.'s* [71] study was only found to be significant on day two, when *rigor mortis* had not fully resolved in the baseline fish. Erikson *et al.'s* [71] day seven K values are a few percentage points lower than those of the present study, 45% and 52%. The immediate icing and the seven days starvation of the fish in their study could account for this. The data from the present work is therefore in agreement with the literature, and suggests that K value may not be a sufficiently sensitive indicator for

distinguishing differences in fish freshness between killing/ stunning methods.

7.2.3.3 Muscle quality

7.2.3.3a Muscle pH

The pH was measured in pre *rigor mortis* muscle, as an early indicator of stress, and in post *rigor mortis* muscle, as a gauge of the onset of spoilage. The initial pH reading, pH_i, of the percussively stunned fish was significantly higher than the death in air group suggesting less anaerobic glycolysis had occurred in these fish, (power~0.95). These results confirm the higher pre *rigor mortis* lactate level in the death in air fish, (power~0.95), and corresponds with the plasma lactate and pH values. The pH_i values, 6.80±0.21 and 6.22±0.09 for the percussively stunned and the death in air fish, respectively, are within the range of the literature values for Atlantic salmon. Robb and Warriss [181] recorded pH_i values of 7.17, and 6.45 shortly after slaughter in Atlantic salmon stunned by percussion and carbon-dioxide narcosis, while Erikson *et al.* [71] report pH_i values of 7.4±0.1 in anaesthetised fish, 7.2±0.1 in unstressed fish, and 6.8±0.1 in stressed fish. The data from the present study therefore suggests that both groups were stressed when sampled, but the stress was less severe in the percussively stunned fish.

The rate of decline in pH during the first 60s after death, shows no difference between the two groups, suggesting the rate of anaerobic glycolysis occurring in the muscle did not differ at the time pH was measured. The pH_i of the death in air group was very low, possibly at its minimum as it was lower than its pH_{96}. Therefore, a rapid decline in pH caused by anaerobic glycolysis must have occurred during the 30min dying period. The consequent pH change and reduced substrate availability in the muscle might have limited enzymatic activity in the death in air group, so that the rates of anaerobic glycolysis were similar.

The pH_{96} relates to the decline in freshness of the fish. *Post mortem* muscle pH declines to a minimum that relates to *ante-mortem* glycogen levels. As fish begin to spoil, the

pH rises due to TVB production, once the pH has risen to 7.5, fish are considered spoilt [51]. The pH_{96} in the fish that died in air was higher than the pH_i indicating the start of spoilage, 6.22±0.09 rising to 6.42±0.07. The pH_{96} of the fish that were stunned was lower, (6.49±0.06), than the pH_i, (6.8±0.21), and may still have been falling, as there was more muscle lactate at 96h compared with pre *rigor mortis* levels. The Torrymeter data indicated that the percussively stunned group was fresher than the death in air fish, which supports this hypothesis. The work by Sigholt *et al.* [211] showed pH decreasing over two days, which stabilised or had possibly started to increase at eight days *post mortem*. The values in Sigholt *et al.* [211] were on days 0, 2 and 8 respectively, controls 6.95, 6.18, 6.22 and in the stressed group 6.6, 6.15, and 6.18. The pH values in the present study were therefore within the range of the published literature and they suggest that the death in air group were less fresh than the percussive stun group. The change in pH over four days was calculated for the blood set of fish, Figure 7.11B. It showed that the change in pH in the percussively stunned fish was greater than that in the death in air group. The change in lactate levels in the muscle set of fish confirmed this finding. The pH of the percussively stunned fish fell over the four days while there was a small increase in the pH of the death in air fish, suggesting the death in air fish were not as fresh.

7.2.3.3b <u>*Rigor mortis*</u>

Rigor mortis was measured as a welfare indicator of the fish at harvest. There was a significant difference between the two groups. The onset of *rigor mortis* was earlier in the fish that died in air. The fish were not accessible during the early stages of *rigor mortis* so the data was not robust enough for statistical comparison. Onset of *rigor mortis* was earlier than anticipated because the fish had been fed the previous day and icing of the carcasses was delayed. Resolution of *rigor mortis* was significantly earlier in the fish that died in air, which strengthens the evidence for increased activity as shown by the muscle metabolite data. The fish that died in air had a lower energy status at death and less potential to

produce ATP and so entered *rigor mortis* earlier. Percussively stunned Atlantic salmon have been reported to be in full *rigor mortis* between 15h and 45h, and in one instance, (carbon-dioxide narcosis), within 4h of slaughter [181]. These values are similar to the death in air fish in the present study. The work by Sigholt *et al.* [211] also demonstrated an earlier onset and resolution of *rigor mortis* in stressed Atlantic salmon compared with controls. Jerrett *et al.* [104] demonstrated how temperature affects the onset and resolution of *rigor mortis* in rested or electrically stimulated Chinook salmon muscle. The work of Jerrett *et al.* [104] shows exercised muscle enters *rigor mortis* in 4h independent of temperature, but rested muscle takes approximately 18h at 0°C or 6h at 12°C. Resolution of *rigor mortis* in the study by Jerrett *et al.* [104] was achieved in 9h in exercised muscle, but rested muscle took 27h at 0°C, and 10h at 12°C. The results of the present study are therefore similar to the literature and indicate that the death in air fish were more stressed than the percussively stunned group.

7.2.3.3c Freshness

The Torrymeter readings showed a decline in fish freshness over time in both groups. The deterioration was significantly faster in the fish that died in air. This finding is supported by the higher, although not significant, K value in the death in air group. The *rigor mortis* and muscle metabolite data also indicated a lower energy status in the death in air fish. Torrymeter readings similar to those taken in this study were shown in Atlantic salmon by Blokhus [34]. The slightly lower values, one unit, in the current study might be due to feeding status and the delayed icing of the fish.

7.2.3.3d Downgrading

Fulton's condition factor was >1 in both cases and did not differ significantly between the two groups. This shows there was no bias in fish selection and that the fish were in good condition.

A downgrading assessment was performed after five days chilled-storage to determine whether either killing/ stunning method had a detrimental effect on quality.

There was significantly greater scale loss in the death in air group. Visible signs of stress in farmed Atlantic salmon include lightening of the skin to pale blue and scale shedding. These responses were observed while sampling the percussive stun group. The objective evidence of falling pH_i in consecutive fish supported the observation. As the death in air fish were sampled after the percussive stun group, the greater scale loss in these fish might therefore be an artefact of the sampling regime. Although additional stress, as seen in the higher adrenaline levels and greater evidence of anaerobic activity caused by killing/ stunning method, or mechanical damage caused by crowding and 'flapping' as they died, might have produced the effect.

With the exception of gaping, the downgrading scores indicated greater damage in the fish that died in air. The differences were not, however, statistically significant. The higher stress levels and greater opportunity for physical damage in the death in air fish would explain this finding. High adrenaline levels would increase systemic blood pressure and therefore the potential for haemorrhaging. The adrenaline values were higher in the death in air group and so the case for performing a one-tailed test on the data would be justified. External haemorrhaging then becomes significant.

Gaping would be expected to be more prevalent in the death in air fish due to the lower muscle pH of this group [134]. The qualitative nature of the measurement and the small sample size might explain not finding a difference. Gaping is less pronounced in salmon than in species such as cod and so it might be more difficult to detect [127]. It also varies seasonally, being more prevalent in the spring and summer [151]. Since sampling occurred in October, the chance of detecting a difference might have been low. Conflicting results in relation to gaping and pH have been reported by Andersen *et al.* [6, 7], although Einen *et al.* [67] found increased gaping with decreasing post *rigor mortis* pH. Time in chilled storage has been shown to have a greater effect on gaping, in king salmon (*Oncorhynchus tschawytscha,* Walbaum 1792), than muscle activity at harvest [79].

The results of the current study therefore weakly suggest that stress at harvest may

increase the incidence of downgrading.

7.2.3.3e Colour

Colour measurements were taken on freshly cut fillets after five days ice storage. The visual colour assessment using the Roche *Salmo*Fan™ did not show a difference between the two groups. The instrumental colour measurements; angle of hue and chroma showed differences although, the lightness score, L*, did not. The higher values of chroma and angle of hue in the percussive stun group indicate that the fillets of these fish had a more intense colour, were more opaque, and were yellower than the death in air group. The increased colour intensity would make the fillets more appealing to the consumer but the reduced translucency and higher angle of hue would not. The power of the tests 0.48 for hue, 0.59 for chroma, were not so high that false positives could be discounted. The power of the L* test was very low, 0.05, and so no justified conclusions could be made from this data.

The readings obtained in this study were within the range found by Christiansen *et al.* [47] for chroma and at the lowest values for L*. The angle of hue measurement were considerably lower than Christiansen *et al.* [47], but Robb *et al.* [186] report similar values for angle of hue and L* but higher chroma values. Colour measurements vary with instrument, measurement technique, fat and pigment levels in the flesh, muscle *rigor mortis* status and cellularity so the results of this study are broadly in agreement with the literature.

The effect of increased muscular activity on colour is however in direct contrast with the literature. The study by Robb *et al.* [186] reports that 75 hours after slaughter, rainbow trout with high muscular activity have a lower Roche colour card™ score, higher L*, higher angle of hue, and higher chroma than anaesthetised fish. The study also states that the changes observed following resolution of *rigor mortis* might be random, as they were mostly smaller than the SEM. However improved colour with reduced stress had been reported previously 24 and 96 hours after death in rainbow trout [181, 258]. Although the

sensory panel in Sigholt *et al.* [211] did not detect significant differences in colour after 9 and 10 days storage. The measurements made in the present study might therefore be influenced more by the time the measurement was taken than by killing/ stunning method, and so no real conclusions could be made from the data.

7.2.3.3f Texture

Texture was measured, as a common criticism of farmed fish is that the flesh is soft and this causes difficulties during processing. A study by Gómez -Guillén *et al.* [83] found softening in 'stressed' salmon, exsanguinated without carbon dioxide narcosis, after seven days starvation, compared with fish anaesthetised with carbon dioxide, n=7. Sigholt *et al.* [211] also found muscle softening with increased stress levels in Atlantic salmon with a small sample size, (n=6), using sensory and instrumental tests, whereas Skjervold *et al.* [217] reported an *increase* in firmness with stress which was almost significant P=0.057 five days *post mortem*, (n=50). The fish in Skjervold *et al.'s* [217] study were starved for five days and subjected to a 24 hour crowding stress i.e. long term. Whereas in Sigholt *et al.* [211] the crowding stress was for 10min and the fish had been starved for 12 days. The different effects of stress on texture reflect the length of the *ante-mortem* stressor and the nutritional state of the fish. Long term stress depletes *ante-mortem* glycogen stores and only a small fall in *post mortem* pH occurs. This equates to DFD meat, see Section 1.4.3.1., whereas short-term stress is associated with a rapid decline in pH *post mortem* and muscle softening as seen in PSE meat, see Section 1.4.3.2. The fish in the current study underwent short to medium term stress but they had a high nutritional status as they had been fed the previous day. Their glycogen stores would not have become depleted during the confinement for sampling, so if any effect was to be expected it would have been softening in the death in air fish. The data however showed no differences in maximum shear force between the two groups six days *post mortem*. The sample size was small, as was the power of the test, (0.06), so a difference between the two groups could not be discounted.

7.2.4 Summary and conclusion

The biological indicators selected were suitable for use with Atlantic salmon in remote locations. The methods used were able to distinguish between high and low levels of stress. The fish that died in air were more stressed than those that died following a percussive stun, but whether this was due to the killing/ stunning method used or the sampling regime is indeterminable.

The Torrymeter was able to detect differences in carcass quality between the two groups provided readings were taken over an extended period. The percussively stunned fish were shown to maintain freshness for longer than the death in air group. The quality attributes of colour and texture require large sample sizes to determine whether they are influenced by stress at harvest. The timing of the measurements might have a more significant effect on the result than the handling stress *per se*.

7.3 Commercial fish harvest

The results of the pilot study suggested that the methods chosen were able to assess physiological stress at harvest and to a limited extent, its influence on carcass quality. They were therefore used during a commercial fish harvest to assess two harvesting techniques.

7.3.1 Materials and methods

7.3.1.1 Animals

The Atlantic salmon sampled during the commercial fish harvest had been reared in sea cages for 15 months post smoltification by Scottish Sea Farms Ltd. They were 76.1±4cm, 4.9±0.8kg, and had a condition factor of 1.10±0.13. They had been starved for seven days, at a water temperature of approximately 8-9°C, before 60tonnes were transferred to a well-boat. After overnight transportation, the fish were live-chilled in the well-boat to 5°C, at a rate of 1°C per hour. Transfer to the slaughter facility was by live pumping via a ~0.45x150m pipe, Appendix 2, Figure13.

7.3.1.2 Facilities and stunning methods

The salmon were dewatered and either carried *en masse* to a carbon dioxide bath or gravity fed into slurry ice and then stunned with MT4 fish stunners (Seafood Innovations, Australia), Appendix 2, Figure 14. The carbon-dioxide narcosis bath consisted of a 1.5x1.5x1m tank filled with seawater, 8.4°C, 98% dissolved oxygen. Carbon dioxide was dissolved in the water through a ceramic flat plate diffuser (Dryden Aquaculture Ltd., Edinburgh, Scotland, UK) until the pH reached ~5.03.

7.3.1.3 Sampling regime

Samples were collected in April 2002 between 9am and 12noon at a fish processing plant in South Shian, Argyll. Although the fish were sampled during a commercial harvest, samples were taken before the gills were severed. After stunning, the fish were brought to the sampling station dry, in batches to comply with the company's Health and Safety policy. Blood samples were taken from three fish removed from the dewatering pipe and killed by percussion (DP). These samples were taken to indicate stress levels in the fish prior to slaughter. Three fish stunned by the MT4 were also sampled for blood at this time. Ten fish were then immersed in the carbon-dioxide narcosis tank (CDN). They were sampled when all aversive behaviour had ceased and all fish had lost equilibrium. Blood samples were taken from the first five fish removed from the tank, 2-15min exposure. Muscle samples were taken from the remaining fish, 20-40min exposure. At the end of the harvesting run, a further four fish stunned by MT4 were sampled, two for blood and two for muscle.

7.3.1.4 Analysis

7.3.1.4a Plasma biochemistry

Blood samples were centrifuged within one hour of collection then transported on ice for nine hours before being frozen at -80°C. Plasma biochemistry was measured within eight weeks of sample collection.

7.3.1.4b Muscle biochemistry

Muscle metabolites were determined within two weeks of sample collection; samples were stored under vapour phase liquid nitrogen until analysed.

7.3.1.4c Muscle quality

The effect of stunning method on muscle pH was monitored immediately after stunning and then once daily for seven days on the fish sampled for blood. The effect of stunning method on the development of *rigor mortis* in the salmon killed for blood samples was monitored over 122h. Torrymeter measurements were taken over 170h. A downgrading assessment was performed seven days after harvesting on the fish taken for blood samples. Fish were stored on ice in a chill room at 2.0±0.4°C.

7.3.2 Results

7.3.2.1 Plasma biochemistry

Plasma biochemistry was measured to assess the stress response of the fish. The plasma was analysed for the hormones adrenaline, noradrenaline and cortisol. Plasma pH, glucose, lactate and protein were also measured to relate to fish activity during death.

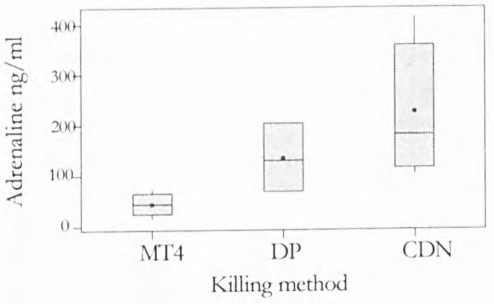
7.3.2.1a Hormones

The plasma adrenaline level in the fish killed by the MT4 stunner was shown to be significantly lower than that of the CDN fish, Figure 7.12. The adrenaline concentrations expressed as a percentage of the DP value were; 33% for the MT4 and 169% for the CDN groups.

The noradrenaline levels were at the limit of detection of the assay and were therefore not sufficiently reliable to be reported.

Cortisol levels were not found to differ between the stunning methods tested, P=0.167, df=2, H=3.65. The cortisol concentrations were 310±40ng/ml, 244±74ng/ml and 251±47ng/ml in the MT4, DP and CDN groups, respectively.

Where conclusions could be made from primary stress response data they suggest that the fish stunned by carbon-dioxide were under more acute stress than those stunned by the MT4.



Inferential statistics
P=0.018, df=2, H=8.08

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	*	ns	

Figure 7.12 Effect of commercial harvesting method on plasma adrenaline level
Single determination, sample size MT4 and CDN n=5, DP n=3. Kruskal Wallis test with Dunns *post hoc* test significance, ns not significant, * $P<0.05$.

7.3.2.1b Plasma chemistry

The plasma biochemistry was shown to differ between the two commercial harvesting techniques. Significant differences were detected between the fish killed by MT4 and CDN in, pH, lactate, and glucose, Figure 7.13A-C. The plasma glucose concentrations were 111% and 84% of the DP fish levels in the MT4 and CDN groups, respectively. Relative to the DP group the plasma pH values were; 101% in the MT4 fish and 95% in the CDN group while the plasma lactate concentrations were 84% and 203% in the MT4 and CDN fish, respectively.

The plasma protein levels were not found to differ significantly between the groups, (P=0.678, df=2, H=0.78), the values in the DP, MT4 and CDN fish were, 59±7mg/ml, 60±11mg/ml, and 61±3mg/ml, respectively.

A: Plasma pH

Inferential statistics
P=0.010, df=2, H=9.16

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	**	ns	

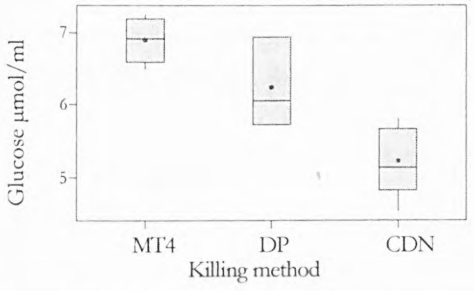
B: Lactate

Inferential statistics
P=0.024, df=2, H=7.48

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	*	ns	

C: Glucose



Inferential statistics
P=0.013, df=2, H=8.66

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	*	ns	

Figure 7.13A-C Effect of commercial harvesting method on plasma biochemistry
The pH measurements were from single, and glucose and lactate duplicate determinations. Sample size MT4 and CDN n=5, DP n=3. Kruskal Wallis test with Dunns *post hoc* test. Significance; ns not significant, * $P<0.05$, ** $P<0.01$.

Glucose is used by the fish in the fight-or-flight response to provide energy for muscle activity. Where adrenaline concentrations are high and cortisol levels stable, glucose levels would be expected to fall: this was seen in the CDN fish. The high plasma lactate level in the CDN group would have contributed to the fall in blood pH measured. The build up of carbon-dioxide, and H^+ released from the RBC in response to low oxygen levels, would also lower the plasma pH. The high plasma lactate concentration in the CDN group therefore corresponded with the low pH and glucose levels. This suggests that the CDN group expended more energy trying to maintain homeostasis whilst dying.

7.3.2.2 Muscle biochemistry

Muscle metabolites were determined to assess the energy status of the fish. Differences measured in muscle biochemistry between the fish stunned by the different methods were not statistically significant, probably due to the low sample numbers, Figure 7.14A-D. Creatinine was not detected in any sample suggesting that creatine phosphate and creatine had not been broken down further. The inferential statistics were for creatine phosphate, P=0.133, W=0; creatine, P=0.095, W=0; ATP, P=0.095, W=0; and lactate, P=0.095, W=0.

Figure 7.14 shows that the MT4 fish contained higher levels of creatine phosphate and ATP and lower levels of creatine and lactate. This suggests that they had used less energy while dying than the CDN group, as seen in the plasma biochemistry data. However as the sample size was very small no conclusions could really be made from the data.

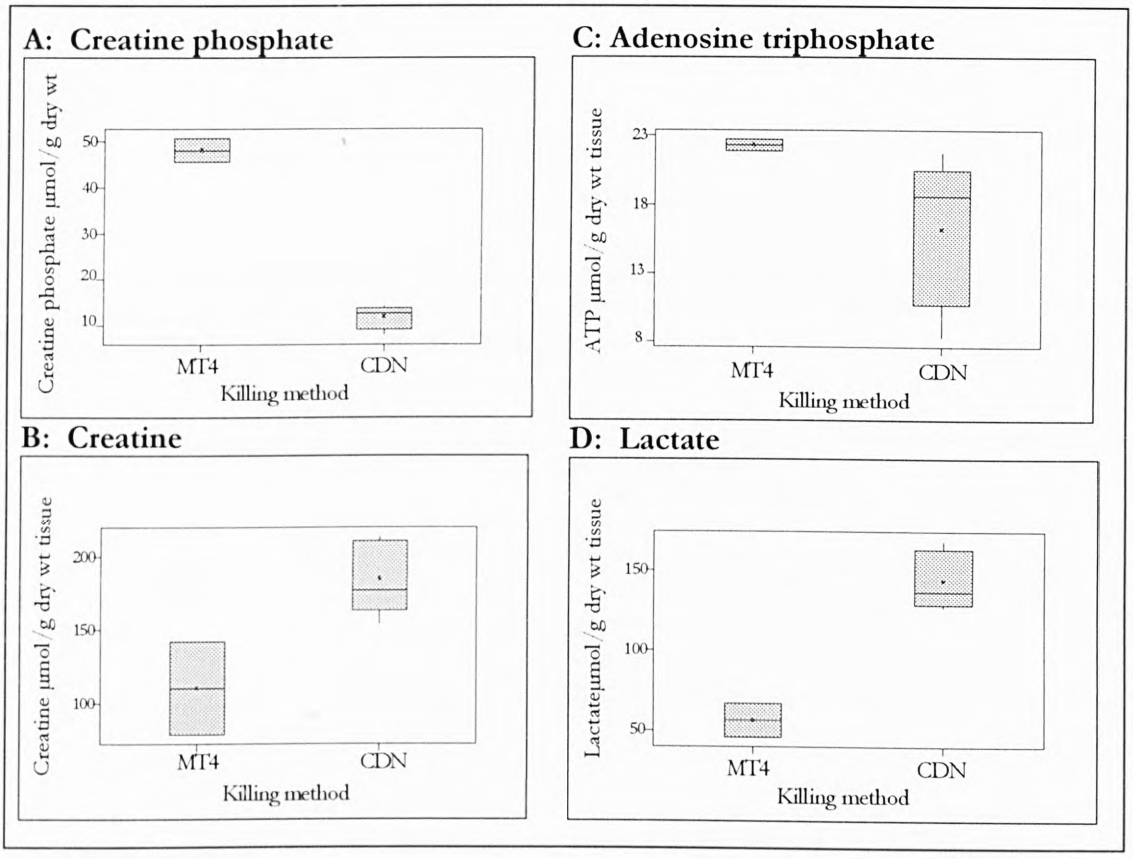


Figure 7.14A-D Effect of commercial harvesting method on pre *rigor mortis* muscle metabolites
Samples measured in duplicate, n=2 MT4, n=5 CDN.

7.3.2.3 Muscle quality

7.3.2.3a Muscle pH

The effect of stunning method on muscle pH was monitored immediately after stunning and then once daily for seven days, Figure 7.15. The initial pH of the DP fish was significantly higher, 0.82 units, than the CDN group, Figure 7.16, but this was no longer apparent 51 hours later, (P=0.117, H=4.29, df=2).

The muscle pH measurements taken immediately after stunning suggest that the CDN fish had expended the most energy whilst dying.

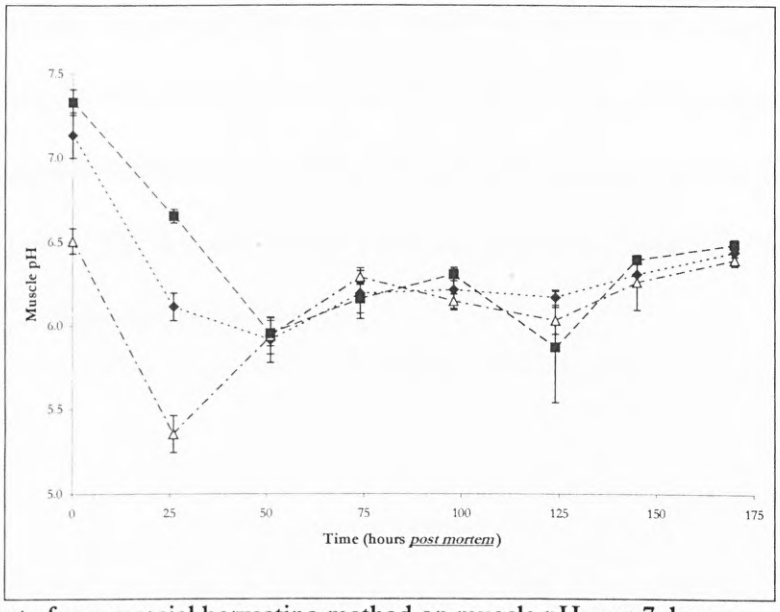


Figure 7.15 Effect of commercial harvesting method on muscle pH over 7 days
Data from a single daily measurement per fish. Results expressed as mean ± standard deviation, MT4 and CDN, n=5; DP, n =3 MT4 (◆), DP (■), CDN (△).

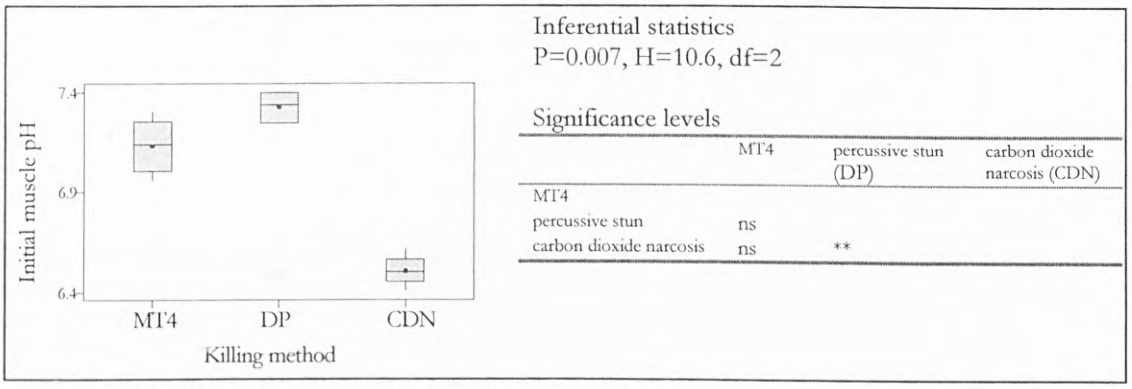


Figure 7.16 Effect of commercial harvesting method on muscle pH
Data from a single daily measurement, MT4 and CDN, n=5; DP, n =3. Kruskal Wallis test with Dunns *post hoc* test; ns not significant, *P<0.05, **P<0.01.

7.3.2.3b *Rigor mortis*

The effect of killing/ stunning method on the development of *rigor mortis* in the blood set of salmon was monitored over 122h, Figures 7.17 and 7.18A-C. There was a significantly earlier onset of *rigor mortis*, 14h, in the CDN group compared with the DP group. Resolution of *rigor mortis* in the CDN fish was significantly earlier, 18h, than in the MT4 fish. Time to maximum *rigor mortis* was also significantly earlier, 22h, in the CDN fish compared with the MT4 group. However, the maximum contraction strength and the duration of *rigor mortis* did not show statistically significant differences, possibly due to the low sample numbers. Inferential statistics for maximum contraction strength and duration of *rigor mortis* were, P=0.37, H=1.98, df=2, and P=0.09, H=4.86, df=2, respectively. The maximum contraction strength in the MT4, DP and CDN groups was; 60±12°, 53±3°, and 66±13°, respectively. The duration of *rigor mortis* was 34±27h, 29±0h, and 25±9h in the MT4, DP and CDN groups, respectively.

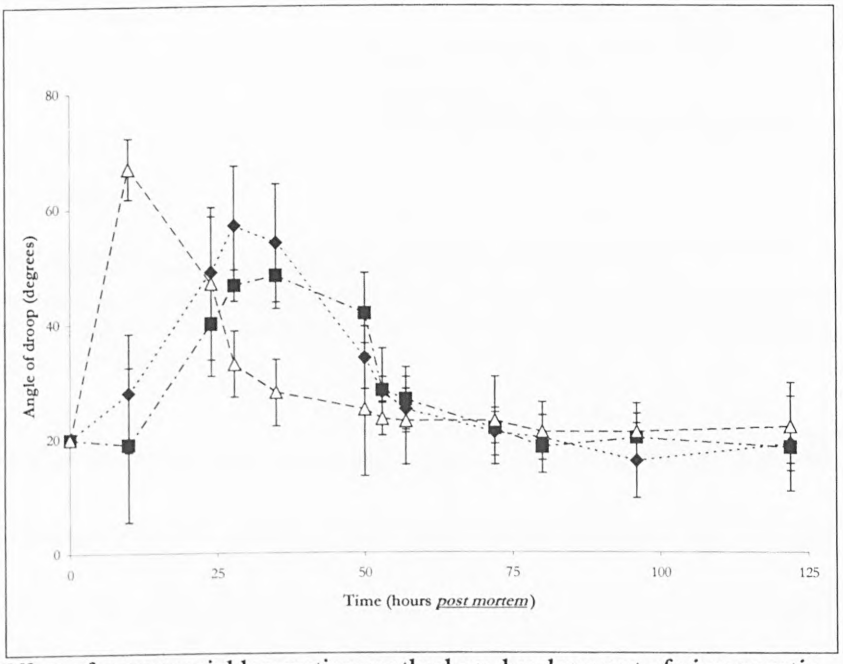
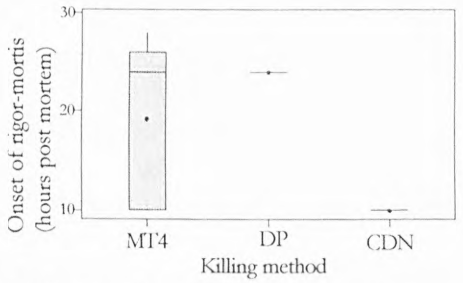


Figure 7.17 Effect of commercial harvesting method on development of *rigor mortis*
Average of single measurements on each side of the fish results expressed as mean ± standard deviation, MT4 and CDN n=5, DP n=3. MT4 (◆), DP (■), CDN (△).

A: Onset of *rigor mortis*

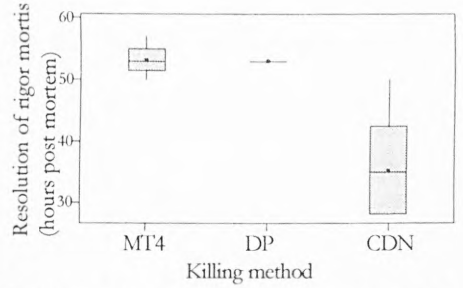


Inferential statistics
P=0.037, H=6.60, df=2

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	ns	*	

B: Resolution of *rigor mortis*

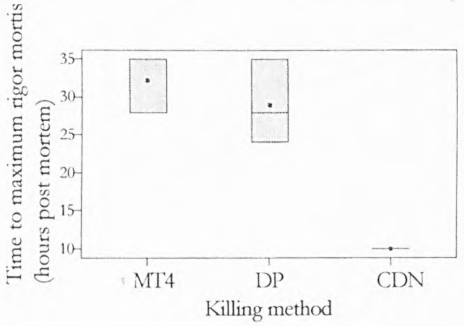


Inferential statistics
P=0.011, H=9.10, df=2

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	*	ns	

C: Time to maximum *rigor mortis*



Inferential statistics
P=0.007, H=9.80, df=2

Significance levels

	MT4	percussive stun (DP)	carbon dioxide narcosis (CDN)
MT4			
percussive stun	ns		
carbon dioxide narcosis	**	ns	

Figure 7.18A-C Effect of commercial harvesting method on *rigor mortis*
Average of single measurements on each side of the fish, onset, resolution measured between 30°, MT4 and CDN n=5, DP n=3. Kruskal Wallis test with Dunns *post hoc* test; ns not significantly different, *P<0.05, **P<0.01.

The CDN fish went into *rigor mortis* earliest of all the stunning methods, showing that they had expended most energy whilst dying. Higher contraction strength is generally associated with earlier onset and shorter duration of *rigor mortis*. The stronger contraction strength seen in the CDN group was not shown to be significantly higher than the other groups, possibly due to low sample numbers or the frequency of the measurements.

7.3.2.3c Freshness

Fish freshness was assessed on the fish taken for blood samples using the Torrymeter. The change in fish freshness with time is illustrated in Figure 7.19. The rate of decline in freshness as estimated by the slope of regression lines calculated from 24-170h post harvest is shown in Table 7.3. The rate of decline in fish freshness was significantly slower in the MT4 fish compared with the DP and CDN fish, Table 7.4. This suggests that these fish had the greatest remaining energy reserves, as tentatively suggested by the muscle biochemistry data.

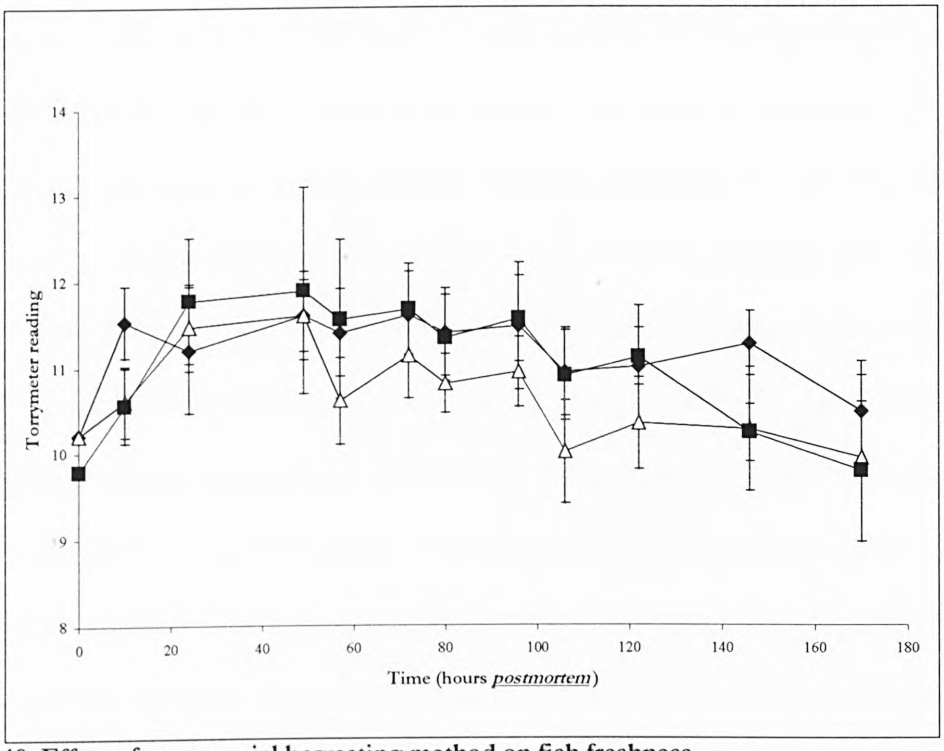


Figure 7.19 Effect of commercial harvesting method on fish freshness
Results expressed as mean ± standard deviation, MT4 and CDN n=5, DP n=3. MT4 (◆), DP (■), CDN (△).

Table 7.3 Regression line data for the effect of commercial harvesting method on Torrymeter readings

method	intercept	slope	R^2	n
MT4	11.7	-0.005	0.43	50
DP	12.4	-0.013	0.82	30
CDN	11.7	-0.011	0.72	50

The decline in fish freshness was defined as the slope of the regression line calculated from the average of the triplicate, twice daily readings from 24-170h *post mortem*.

Table 7.4 Parallelism and identical regression line test statistics for the effect of commercial harvesting method on Torrymeter readings, t values and significance levels

slope		intercept	
method	MT4	DP	CDN
MT4	----	-2.71(*)	-0 ns
DP	-3.26**	----	-2.51*
CDN	-2.29*	0.72ns	----

The slope represents the decline in fish freshness, n = 50 or 30 (DP), df=16. Where the significance level of the intercept is in parentheses, it might be an artefact of the test. Triplicate measurements were taken on one side of the fish.

7.3.2.3d Downgrading

The downgrading assessment was performed seven days after harvesting on the blood set of fish, Figure 7.20. There was very little evidence for downgrading in any of the fish and blood spots were absent from all the fillets. There were no significant differences detected in the low levels of damage observed between the groups in: scale loss, (H=1.81, df=2, P=0.41); external haemorrhages (H=0.48, df=2, P=0.79); or gaping (H=1.60, df=2, P=0.45). The CDN group had 125% of the scale loss of the DP group and the MT4 fish 75%. The external haemorrhages relative to the DP group were 200% and 250% in the CDN and MT4 groups, respectively. Fillet gaping showed 50% of the DP value in the MT4 fish and 200% in the CDN group. Fulton's condition factor did not differ significantly between the three groups of fish, (P=0.39, W=1.88), demonstrating there was no bias in sample selection. The values for the condition factor were in the DP, MT4 and CDN fish 1.16±0.17, 1.06±0.04, and 1.07±0.10, respectively.

Downgrading assessments require large sample numbers to produce reliable data, especially where levels of damage are low. Consequently no significant differences were detected among stunning methods. Anecdotal evidence suggests that Atlantic salmon shed scales when stressed. The CDN group shows slightly greater scale loss than the other groups, the evidence of higher adrenaline levels in these fish supports this. The external haemorrhages consisted mainly of bleeding into the eyes, there was no bruising. The MT4 group showed more evidence of eye damage, a result possibly of a sudden increase of inter-

cranial pressure caused by the stun or perhaps physical damage caused during transfer from the dewatering pipe to the stunning machine. There was no evidence for increased systemic blood pressure causing blood vessel rupture, caused by a sudden surge in adrenaline levels, as blood spotting was not observed in the fillets. Gaping was predominantly seen in the CDN group although again the extent of gaping was very low. Higher levels of activity at slaughter are thought to increase gaping and the biochemistry data showed evidence of increased activity in the CDN group.

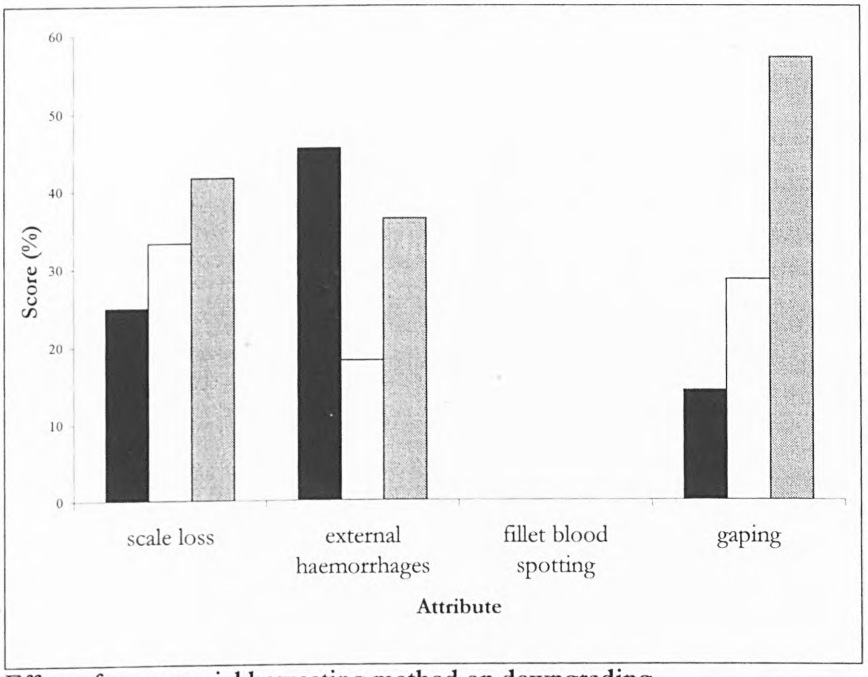


Figure 7.20 Effect of commercial harvesting method on downgrading
Single assessment made seven days post harvest, n=5 MT4 ■, n=3 DP□ and CDN▨ .

7.3.2.3e Colour

Fillets from the muscle set of fish were assessed for colour daily over five days, starting the day after harvest, Figure 7.21. Colour was also measured in the blood set of fish on one occasion, seven days *post mortem*, differences were not detected among the groups at this time. The inferential statistics were for: lightness, P=0.417, H=1.75, df=2; chroma, P=0.755, H=0.56 df=2; hue, P=0.595, H=1.045, df=2; and Roche *Salmo*fan™, P=0.34, H=2.13, df=2. In the MT4, DP and CDN groups respectively, the lightness, hue and chroma values were: 43.9±1.6, 42.7±2.6, and 42.1±1.1; 36.5±6.5°, 38.7±1.8°, and 36.0±5.6°; and 15.4±1.7, 15.7±0.6, and 16.3±2.0. The Roche *Salmo*fan™ scores were in

the MT4, DP, and CDN; 29.6±0.5, 29.3±0.6, and 29.0±0.7, respectively.

The colour of the fillets changed with time *post mortem*, Figure 7.21. The first colour measurement was taken while the fish were still in *rigor mortis* i.e. the muscle proteins were still contracting. As time passed and *rigor mortis* resolved, the values increased i.e. the flesh became paler, less translucent and more yellow. These changes were subtle as the Roche *Salmo*fan™ scores did not detect them. The timing of the measurement may therefore have a greater influence than killing/ stunning method on fillet colour.

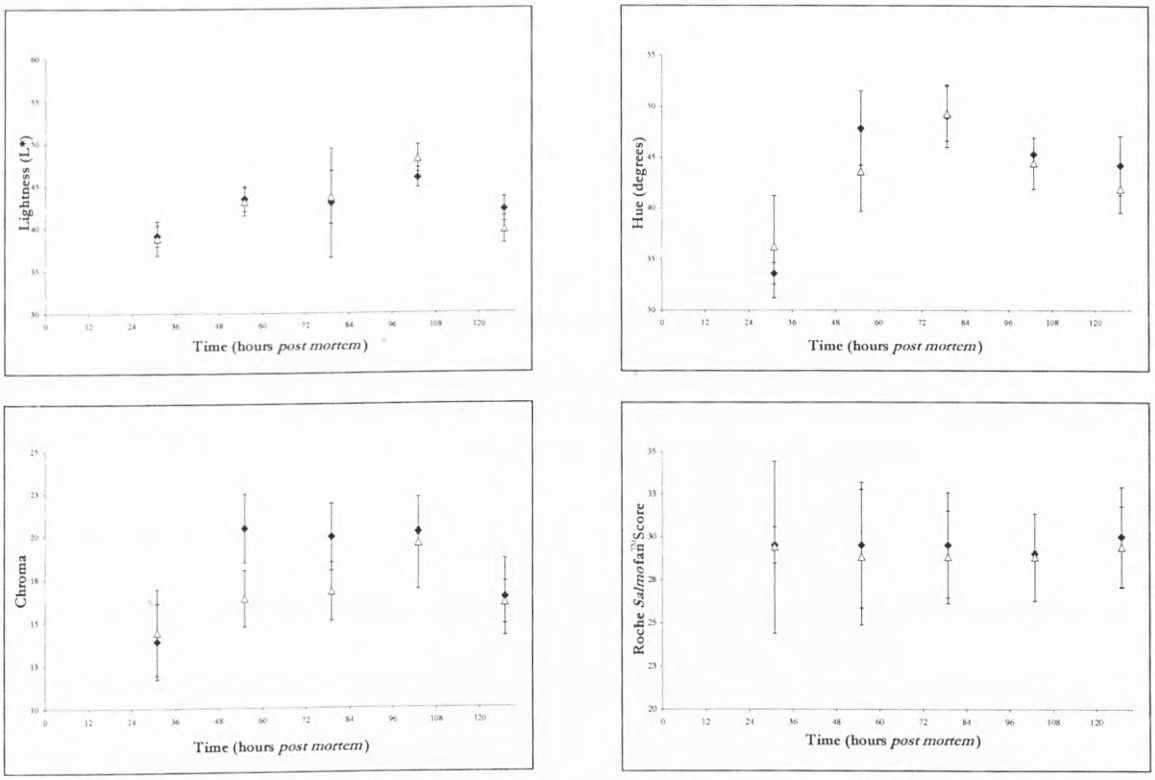


Figure7.21 Effect of commercial harvesting method on flesh colour over five days
Results expressed as mean ± standard deviation, data from triplicate measurements for L*, chroma and Hue, single assessment with Roche *Salmo*fan™, MT4 n=2, CDN n=5. MT4 (◆), CDN (△).

7.3.2.3f Texture

Texture was assessed using Warner-Bratzler shear tests on three sections of muscle cut from the fillets of the muscle set of fish on five days, starting the day after harvesting, Figure 7.22. Figure 7.22 shows the softening that occurs in fish as a result of *rigor mortis* resolution: a greater force was required to cut the tissue of the MT4 fish in the earlier measurements. This confirms the finding of later resolution of *rigor mortis* in the MT4 fish.

Texture was also measured on the blood set of fish on day seven *post mortem*, differences were not detected between the stunning methods in this set of measurements, P=0.59, H=1.05, df=2. The shear strengths measured were, 353±34g, 353±43g, and 322±57g in the MT4, DP, and CDN groups, respectively.

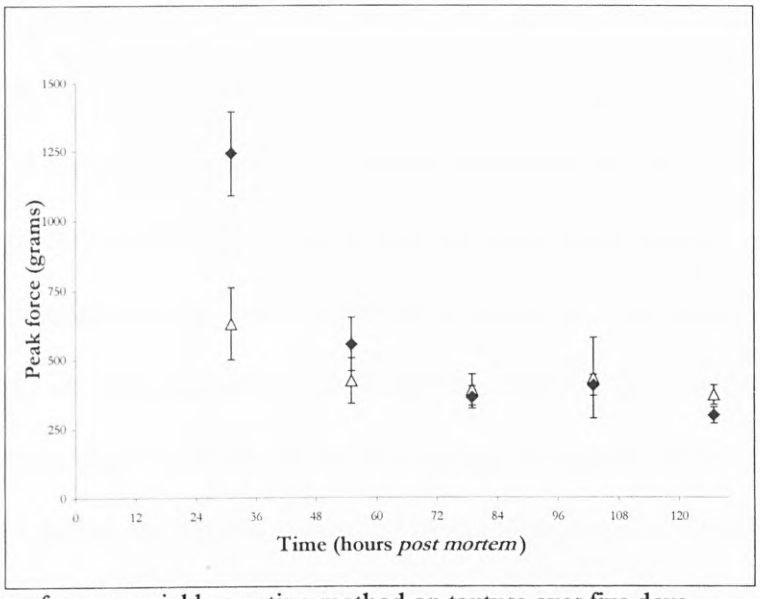


Figure 7.22 Effect of commercial harvesting method on texture over five days
Results expressed as mean ± standard deviation, MT4 (◆), CDN (△). Data from triplicate measurements, MT4 n=2, CDN n=5.

7.3.3 Discussion

During a commercial fish-harvest, two killing/ stunning methods were tested for their influence on the welfare and quality of farmed salmon. The results are discussed in the light of the pilot study and the literature.

7.3.3.1 Plasma biochemistry

The primary stress response was measured as an indicator of welfare. Significant differences were detected between the MT4 and CDN fish in plasma adrenaline, pH, lactate and glucose, although levels of cortisol and protein did not differ. The DP group fell between the two extremes.

Adrenaline levels in the CDN group were significantly higher than those in the MT4 but not the DP fish. The adrenaline levels were 45±22ng/ml in the MT4 fish, 134±67ng/ml in the DP group, and 225±131ng/ml in the CDN group. The slightly higher adrenaline levels in the DP group compared with the MT4 fish might have been

caused by more severe handling stresses in these fish. They had to be removed from the pipe before being percussively stunned, and since transfer to the sampling station took more time, they were out of water for longer before sample collection; hence more time was available for catecholamine release. The DP and CDN groups were not subject to additional chilling in the slurry ice before stunning, so their nerve conduction rates and consequent catecholamine release might therefore have been greater than the MT4 fish.

The pilot study fish contained less plasma adrenaline than the current study. The fish percussively stunned beside the rearing cage showed a much smaller adrenaline response, 2±1.5ng/ml, and the level in the death in air fish was also lower than in the CDN group, 84±68ng/ml. This suggests that both groups of fish in the commercial harvest study were showing signs of stress, but the MT4 group to a lesser extent. The additional stresses incurred during the transfer procedure from the well-boat or the live-chilling process, could have caused this effect. Alternatively, it might be an artefact of the experimental procedure as the pilot study fish were sampled faster and were from a different stock.

The cortisol levels measured in the present study were quite high; they ranged from 244-310ng/ml, slightly higher than those measured in the pilot study, death in air 205±92ng/ml, and percussively stunned 300±66ng/ml. The stocking density in the well-boat and the live-chilling process might have contributed to these higher values. However, the data might not be directly comparable due to differences in photoperiod and stock. Live-chilling has been shown to increase cortisol levels but to a lesser extent than crowding stress [216]. Cortisol values in Atlantic salmon recalculated from Skjervold *et al.* [216] were reported as 101±8ng/ml in fish kept at a low stocking density and 339±18ng/ml in fish kept at high density before live-chilling. After live-chilling the cortisol was measured at 184±23ng/ml and 311±28ng/ml, in the low and high stocking densities, respectively.

The plasma pH and lactate data in the commercially stunned fish indicate significantly greater metabolic stress in the CDN group relative to the MT4 fish. The CDN

fish had higher lactate levels and consequently lower pH than the MT4 fish. The DP fish fell between the two commercial stunning methods and were not significantly different from either. They were, however, closer to the MT4 group. The plasma pH of the percussively stunned fish in the pilot study was slightly higher than that of the MT4 and DP in the commercial methods. A possible explanation of this would be the speed of sample collection after the fish had been removed from the water. The acidification of the blood due to the release of H^+ from the RBC would be less apparent in these fish due to the lower adrenaline levels. The lactate levels in the commercially killed fish were however considerably lower than those measured in the pilot study, 7μmol/ml and 22μmol/ml in the percussively stunned and death in air fish, respectively. Plasma lactate levels were 5.8±0.5μmol/ml in the CDN, 2.4±1.6μmol/ml in the MT4 fish and 2.8±1.0μmol/ml in the DP group, with corresponding pH values of 7.02±0.12, 7.49±0.05, and 7.42±0.08. The lower metabolic rate from the live-chill process and the longer starvation period would have decreased lactate production. Published values of plasma lactate levels in live-chilled salmon have been measured at 6±0.8μmol/ml in fish kept at a low stocking density and 12±2μmol/ml in fish kept at high density before live-chilling [216]. After live-chilling the lactate levels were 5±1μmol/ml and 13±3μmol/ml, respectively [216]. The live-chilling process did not influence the plasma lactate in Skjervold *et al.'s* [216] study whereas the crowding did, therefore in the present study the starvation period might have influenced the lactate levels to a greater extent than the live-chilling process.

Plasma glucose levels were significantly higher in the MT4 group, 6.9±0.3μmol/ml, than in the CDN fish, 5.2±0.5μmol/ml, the DP group had 6.2±0.6μmol/ml glucose. The plasma glucose levels mirrored the pattern of the adrenaline data, which was not unexpected as adrenaline can have the effect of lowering plasma glucose concentration [100]. The higher levels of glucose in the MT4 fish suggests that they were expending less energy whilst dying than the CDN group. Plasma glucose levels in the live-chilled fish were similar to those of the pilot study, 7μmol/ml, even though the commercially stunned fish

had been starved. The lowering of plasma glucose levels in the CDN fish but not in the death in air fish in the pilot study might be a reflection of the effect of starvation and/or a lower rate of glucose production in live-chilled fish. The live-chilling process *per se* has been shown to be less influential than crowding stress in lowering plasma glucose levels [216].

Plasma protein levels did not differ between the groups in the present study, suggesting that the plasma volume did not change, even though the fish died in sea water and the muscle in the CDN group had higher, although not significant, lactate levels. Osmoregulation was therefore either not impaired during the CDN process, or in the time it took the fish to die, it did not have time to affect the plasma volume. The plasma protein levels were similar in both studies, 59mg/ml, 60mg/ml and 61mg/ml in the MT4, DP and CDN fish, respectively. While the pilot study figures were 62mg/ml and 65mg/ml in the percussively stunned and death in air fish.

7.3.3.2 Muscle biochemistry

Metabolites were measured in pre-*rigor mortis* muscle to evaluate the degree of muscle activity occurring during the dying process. Statistical differences were not detected between the two methods investigated using a two-tailed test. The small sample size of the MT4 group, (n=2), decreased the chance of finding a statistically significant result. A one-tailed test could be justified on the assumption that MT4, as a blow to the head technique, would cause less muscle activity in the fish than CDN. In this case, statistically significant differences were seen in creatine, ATP and lactate levels, P=0.0476 in all cases. The CDN fish showed greater signs of muscle activity, with higher creatine and lactate and lower ATP levels. In the creatine phosphate data an outlier was removed from CDN group, this reduced the sample size and precluded a statistically significant result even with a one-tailed test, (P=0.0667). The data therefore only weakly suggests that the CDN fish had expended more energy whilst dying than the MT4 group. This supposition is, however, strengthened by the plasma biochemistry data, which showed that the CDN fish were significantly more

metabolically active than the MT4 group.

The CP: Cr ratios were 0.06:1 and 0.48:1 in the CDN and MT4 fish, respectively indicating that the CDN fish had lower energy levels. These ratios are comparable with the pilot study results of 0.03:1 for the death in air fish and 0.30:1 in the percussively stunned fish. The slightly higher energy status in the MT4 fish compared with the percussively stunned pilot study fish might be due to the commercial pre-harvesting handling techniques. The seven-day starvation period and the live-chilling process would both lower the metabolic rate of the commercially killed fish, and so preserve the high-energy stores in the muscle.

The CP: ATP ratios were 0.65:1 and 2.15:1 in the CDN and MT4 groups, respectively. This indicates that the MT4 fish had greater energy reserves remaining than the CDN fish. Again, the results are comparable with the fish killed in the pilot study, 0.72:1 and 1.74:1 in the death in air and percussively stunned fish, respectively. The percussively stunned pilot study fish again had a lower energy status, probably due to the effects of temperature and SDA.

The creatine pool data for the CDN and MT4 fish, were 204μmol/g dry wt and 158μmol/g dry wt. They were not significantly different to each other, ($W=1.00$, $P=0.19$). The average creatine pool of the commercially stunned fish was not significantly different from those of the pilot study fish, ($P=0.30$, $t=1.005$, $df=25$), thus confirming the validity of the creatine phosphate, creatine and creatinine measurements.

The creatine phosphate, creatine and ATP levels in the present study are similar to those found in the pilot study. In the pilot study the levels of creatine phosphate were 6μmol/g dry wt and 46μmol/g dry wt. While creatine levels were 201μmol/g dry wt and 156μmol/g dry wt and ATP levels were 8μmol/g dry wt and 26μmol/g dry wt, in the death in air and percussively stunned fish, respectively. Creatine phosphate levels in the commercial killing methods study were, in the MT4 and CDN group, 48±4μmol/g dry wt, and 12±3μmol/g dry wt, respectively. Creatine levels were in the MT4 and CDN group,

110±45μmol/g dry wt and 185±26μmol/g dry wt, respectively, while ATP levels were, in the MT4 and CDN group, 22±1μmol/g dry wt and 16±5μmol/g dry wt, respectively.

These results suggest that the muscle in the MT4 fish had high levels of energy remaining, and even the CDN group which had been physically active during stunning had relatively high ATP levels and so were not completely exhausted. This might be a result of the live-chilling process lowering the metabolic rate of the fish. Chilling was not used in the slaughter of fish in a study by Erikson *et al.* [73] which assessed stress in Atlantic salmon by measuring creatine phosphate and ATP levels. Samples were taken in fish killed at the rearing cage edge, in the well-boat following 90min transportation and 4h rest and after slaughter following carbon-dioxide narcosis [73]. Creatine phosphate and ATP levels in fish killed at the cage were 23.4±10.2μmol/g dry wt and 11.8±4.7μmol/g dry wt, respectively. In the well-boat, 40.4±14.7μmol/g dry wt and 14.7±3.3μmol/g dry wt and following slaughter 18.6±10.8μmol/g dry wt and ATP 12.4±3.8μmol/g dry wt. When compared with the results of the present study, the fish sampled at the well-boat were comparable to the MT4 fish, while those following carbon-dioxide narcosis and slaughter were more akin to the CDN fish. This suggests that the MT4 process is less stressful to the fish than the CDN.

Muscle lactate levels in the commercial harvesting study were CDN, 144±18μmol/g dry wt, and MT4, 56±15μmol/g dry wt. The MT4 value was considerably lower than that found in the pilot study, which was similar to published values for exercised fish [267]. Lactate levels in the pilot study were in the death in air and percussively stunned fish 196μmol/g dry wt and 103μmol/g dry wt, respectively. The live-chill process would have slowed down the rate of lactate production. However, in the CDN group in the present study, this was masked by the higher muscle activity caused by the severity of the stunning method.

7.3.3.3 Muscle quality

Muscle quality parameters measured were; pH, *rigor mortis*, freshness, downgrading, colour and texture.

7.3.3.3a Muscle pH

Muscle pH was measured in the fish while blood was being collected and then daily for seven days. The CDN group had a lower pH compared with the MT4 and DP fish, suggesting greater muscle activity in the CDN fish, however the only significant difference was between the CDN and DP groups. The average pH of the first three MT4 fish was 0.22 units higher than the last two, which were among the final fish pumped from the well-boat. This might indicate that the fish were slightly more stressed at the end of harvesting than at the beginning. Had the sample sizes been larger or all the MT4 fish been sampled at the beginning of harvest they might also have shown a significantly higher pH compared with the CDN fish. At 26h *post mortem*, the statistical difference in pH between the DP and CDN groups was still apparent. The pH of the CDN group fell to an extremely low 5.4, which could possibly influence the collagen connecting the myotomes and so increase the susceptibility of these fish to gaping. *Rigor mortis* was resolving in the CDN fish at 26h while in the MT4 and DP groups it was still developing. By 51h *post mortem*, the differences in muscle pH had resolved, all the groups were coming out of *rigor mortis*, and by 74h, the muscle pH had begun to rise in all fish.

The muscle pH measured in the commercial harvest study were, 7.13±0.14 in the MT4 group, 7.33±0.08 in the DP group, and 6.51±0.08 in the CDN fish. This was slightly higher than the pilot study fish percussively stunned fish, probably due to the lower lactate levels, 6.80±0.21, and 6.22±0.09 in the percussive stun and death in air groups, respectively.

The rate of lactate production would have been higher in the pilot study fish compared with the commercially stunned fish because of the effects of SDA and the higher water temperature. Data published by Robb and Warriss [181] report pH values of 7.17, and

6.45 shortly after slaughter in Atlantic salmon stunned by percussion and carbon-dioxide narcosis respectively. While Erikson *et al.* [73] measured muscle pH in Atlantic salmon as 7.4±0.1 at cage edge, 7.4±0.1 4h after transportation, and 7.02±0.2 following carbon-dioxide narcosis. The results of the current study therefore are similar to published values.

7.3.3.3b Rigor mortis

Rigor mortis is an easily measured indicator of muscle activity at slaughter. *Rigor mortis* developed earlier in the CDN group indicating increased muscle activity in these fish. The time to maximum contraction was significantly earlier, 22h, in the CDN group compared with the MT4 fish. The contraction strength and duration of *rigor mortis* was not found to differ significantly between the two groups, possibly because of the small sample size and the infrequency of the measurements. Earlier *rigor mortis* reflected the lower energy status of the CDN fish muscle, and so strengthens the tentative supposition made from the muscle biochemistry data. When resolution of *rigor mortis* of the commercially stunned fish was compared with the pilot study data, a delay was seen, although it failed to show significance. *Rigor mortis* resolved at approximately 53h in the percussively stunned commercial methods fish, while in the pilot study percussively stunned fish, *rigor mortis* resolved at 42h. The delay in resolution of *rigor mortis* in the commercially killed fish would be due to the effects of starvation and live-chilling. *Rigor mortis* development in the present study was similar to published values. The work by Skjervold *et al.* [216] shows peak *rigor mortis* development 14-30h *post mortem* in live-chilled salmon, which was delayed relative to salmon that were not live-chilled.

7.3.3.3c Freshness

The Torrymeter data show the initial rise in readings that occurs in newly killed fish as they lose muscle tone. They then show a very gradual decline in freshness, with the CDN fish having lower values than the MT4 and DP fish. The later readings show a more rapid decline in freshness in both of the percussively stunned groups. The overall decline in freshness from 24 hours onwards was significantly faster in the CDN and DP fish

compared with the MT4 group. The freshness measurements therefore support the muscle metabolite data, and strengthen the evidence that the energy levels in the MT4 fish were the highest. It is interesting to note that the freshness readings at 170h *post mortem* in the three groups were not significantly different, (P=0.34, W=2.15), which might indicate that the shelf-life of fish killed by different methods does not necessarily alter, although the degree of freshness up until the later stages does. The fish would be expected to have the same energy levels before death, and so if stored so that microbiological activity did not unduly influence shelf-life, all the groups would be expected to spoil simultaneously. The decline in freshness in the fish in this study was slower than that in the pilot study as the pilot study fish had not been starved or chilled before killing/ stunning.

7.3.3.3d Downgrading

Fulton's condition factor did not differ significantly between the three groups of fish, indicating no bias in the selection of fish used in the experiment. It was >1 in all cases, indicating the fish were in good condition although they had been starved.

The downgrading assessment was performed after seven days chilled-storage. The CDN fish showed the most evidence of scale loss and fillet gaping and the MT4 fish showed the least. The MT4 fish had the highest incidence of external haemorrhages although not greatly different to the CDN, the DP had the lowest number of external haemorrhages. However, the differences in downgrading were not statistically significant. The existence of false negative results cannot, however, be discounted due to the low numbers of fish. The greater scale losses and fillet gaping observed in the CDN group would have resulted from the higher levels of stress in these fish. The greater number of external haemorrhages observed in the MT4 fish might be due to physical damage caused by the stunning process, or handling procedures prior to it. The absence of bloodspotting in all the commercially killed fish might have been influenced by the live-chill process. The low temperature might have decreased cardiac output and therefore lowered systemic blood pressure, thereby reducing the likelihood of haemorrhaging.

Compared with the pilot study fish, the increased gaping in the CDN fish is noteworthy, since it might be linked to the lower pH the muscle attained: the pH_{96} in the commercially killed fish was 0.31 units lower than the pH_{96} in pilot study death in air fish. As there is reputedly an increased incidence of gaping in salmon the springtime [146], the time of year may have influenced the gaping scores: April (commercially killed fish) *cf* October (pilot study fish). Scale losses were higher in both studies in the most stressed groups supporting anecdotal evidence of increased scale loss with stress in salmon. The low levels of external haemorrhages in the manually percussively stunned fish of both studies correspond, while the MT4 fish received greater amounts of external damage possibly while being transferred from the pipe to the machine.

7.3.3.3e Colour

Differences were not detected between the groups in the colour of the flesh after seven days ice storage in the blood set of fish. In the muscle set of fish, colour changed with time, these changes were however subtle as they were only detected by the instrumental measurements. The first measurements were made before the resolution of *rigor mortis* and were generally different to the subsequent measurements. Initially values increased with time so as *rigor mortis* resolved the fillets became; paler, yellower and more opaque. The pilot study data weakly suggested that the fish that were leasr stressed were more yellow and opaque which is contrary to published values.

7.3.3.3f Texture

There were no significant differences in fillet texture after seven days ice storage in the blood set of salmon. The texture of the muscle fish followed over days 2-6 showed a much greater force was needed to cut the sample in the MT4 fish compared with the CDN group, in the first measurement, 31h post harvest, Figure 7.22. The following day the values of both groups were lower and there was less of a difference between them. When these data are compared with the *rigor mortis* graph Figure 7.13, it can be seen that the first texture measurement was taken when *rigor mortis* was further advanced in the CDN fish -

rigor mortis was actually resolving. The MT4 fish were, however, nearing their maximum *rigor mortis* contraction, and so they would be expected to have a firmer texture. The texture results therefore support the *rigor mortis* data showing an advance in *rigor mortis* in the CDN group. The sample size in this study was small and so although a significant difference was not observed, a difference in texture between the two groups could not be discounted. Textural differences were also not detected in the pilot study.

7.3.4 Summary and conclusions

The CDN method of stunning salmon appears to increase muscle activity and promote adrenaline release relative to the MT4 method. This leads to an advance in *rigor mortis* and a faster decline in fish freshness. CDN reduces muscle pH to low levels, which could compromise carcass quality.

8 COMPARISON OF SPECIES AND THE EFFECTS OF HARVESTING TECHNIQUE

8.1 Introduction

Two killing/ stunning techniques that produced differing responses were used in both the rainbow trout work and the salmon pilot study; death in air and percussive stun, as detailed in Chapters 4 and 7. Data common to both species were compared to determine if killing/ stunning method influenced the two salmonids in a similar fashion.

8.2 Species comparison

8.2.1 Plasma Biochemistry

8.2.1.1 Hormones

The primary stress response in the two species killed by equivalent methods showed similar patterns, Figure 8.1.

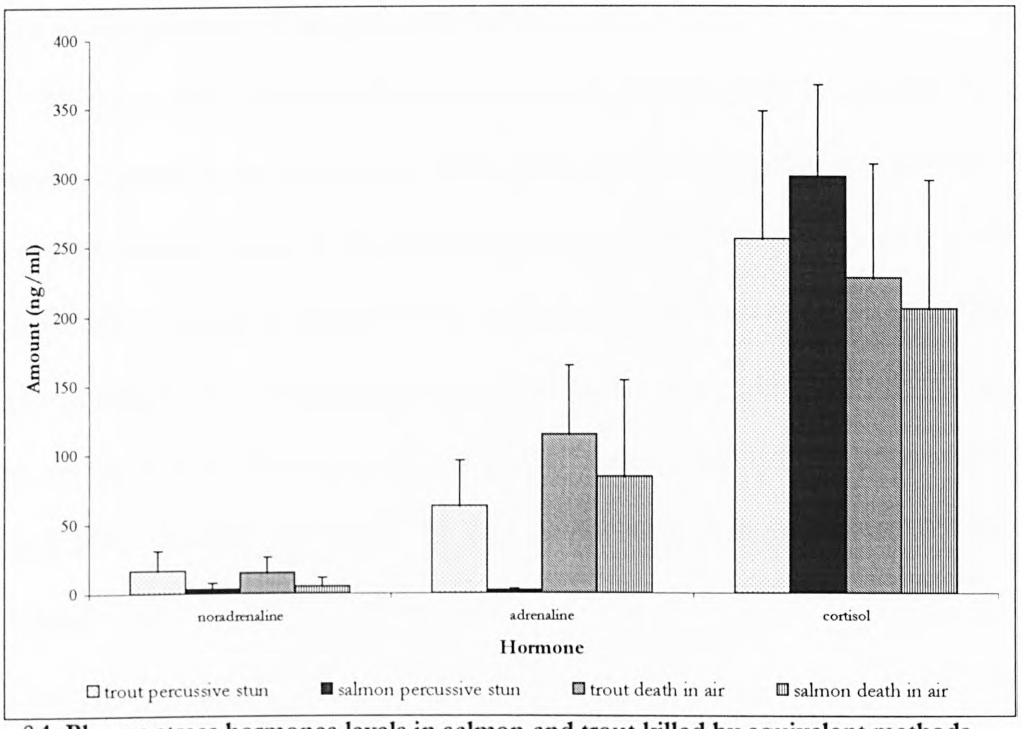


Figure 8.1 Plasma stress hormones levels in salmon and trout killed by equivalent methods
Results expressed as mean ± standard deviation, single determinations for adrenaline and noradrenaline, duplicates for cortisol, trout n=20, salmon n=10 (catecholamines), n=9 (cortisol).

8.2.1.1a Catecholamines

There were very low levels of noradrenaline detected in all the fish. The trout had significantly higher noradrenaline levels than the salmon, $P=0.002$, $t=3.436$, $df=26$ in the death in air fish, and $P=0.0011$, $t=3.714$, $df=23$, in the percussively stunned fish. The salmon noradrenaline concentrations expressed as a percentage of the trout values were for death in air and percussively stunned fish, 31% and 17%, respectively.

The adrenaline levels were greater than the noradrenaline levels in both species and the death in air groups of both species demonstrated a larger response compared with fish that were percussively stunned. The percussively stunned trout had significantly greater levels of adrenaline than the salmon killed by the equivalent method, $P<0.0001$, $t=8.544$, $df=19$. Although the adrenaline levels in the death in air trout were higher than in the salmon, the difference was not significant, $P=0.184$, $t=1.363$, $df=27$. The adrenaline levels in the salmon expressed as a percentage of the trout values were, 74% and 3%, for the death in air and percussively stunned fish, respectively.

The adrenaline: noradrenaline ratios were calculated in order to compare the adrenergic response in the two species while at the same time negating the effect of the higher catecholamine values in the trout compared with the salmon. The ratios of the two species killed by equivalent methods were fairly similar; the death in air killing method produced a higher value indicating a greater adrenergic response than the percussion technique, Figure 8.2. The ratios were for the percussively stunned fish; trout 5.3 ± 4.7, salmon 2.4 ± 2.4 ($P=0.25$, $t=1.17$, $df=20$), and for the death in air groups, trout 10.6 ± 7.5 and salmon 12.8 ± 9.9 ($P=0.59$, $t=0.55$, $df=22$).

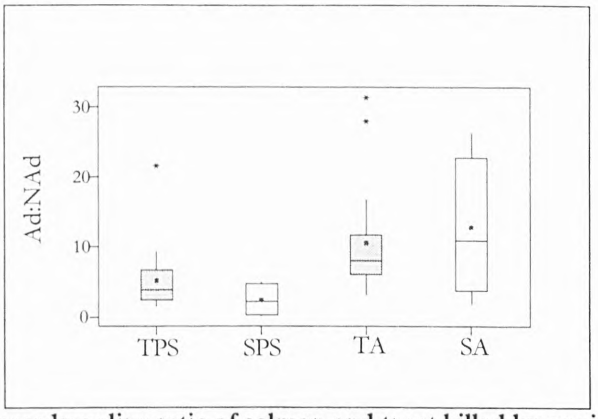


Figure 8.2 Adrenaline: noradrenaline ratio of salmon and trout killed by equivalent methods
Ratios calculated on an individual fish basis. TPS, trout percussive stun, n=18; SPS, salmon percussive stun, n=4; TA, trout death in air, n=19; SA, salmon death in air, n=5.

8.2.1.1b Cortisol

The plasma cortisol levels were lower in the death in air groups of both species compared with the percussively stunned fish, Figure 8.1. The actual cortisol values did not differ between species. The inferential statistics for cortisol were, P=0.53, t=0.63, df=27 and P=0.20, t=1.312, df=27 in the death in air and percussively stunned fish, respectively. The cortisol levels in the salmon expressed as a percentage of the trout values were for the death in air and percussively stunned fish 90% and 117%, respectively.

The primary stress response data therefore suggest that the two species responded in a similar fashion to harvesting technique although they exhibited differing levels of the plasma stress hormones. The salmon appeared to have a reduced stress response compared with the trout in all but one data set. This was unexpected as rainbow trout are thought to be more domesticated than salmon [235], and domestication results in an attenuated stress response [128, 264]. The response in the salmon might have appeared lower if it was delayed relative to that of the trout, i.e. if the salmon responded more slowly to stress. Otherwise, body size or species differences might have influenced the relative plasma hormone levels.

8.2.1.2 Chemistry

The other variables measured in the plasma showed less defined patterns, Figure 8.3.

The trout plasma pH values were significantly lower than those of the salmon, in the percussively stunned fish, $P<0.0001$, $t=11.396$, $df=28$, and in the death in air fish $P<0.0001$, $t=5.088$, $df=27$. The plasma lactate values were also significantly lower in the trout, $P=0.0001$, $t=4.248$, $df=10$, and $P<0.0001$, $t=8.506$, $df=28$, in the percussively stunned and death in air fish, respectively. The plasma pH values of the death in air and percussively stunned salmon expressed as a percentage of the trout values were, 103% and, 105%, respectively. While the plasma lactate content values of the death in air and percussively stunned salmon expressed as a percentage of the trout values were, 177% and, 349%, respectively.

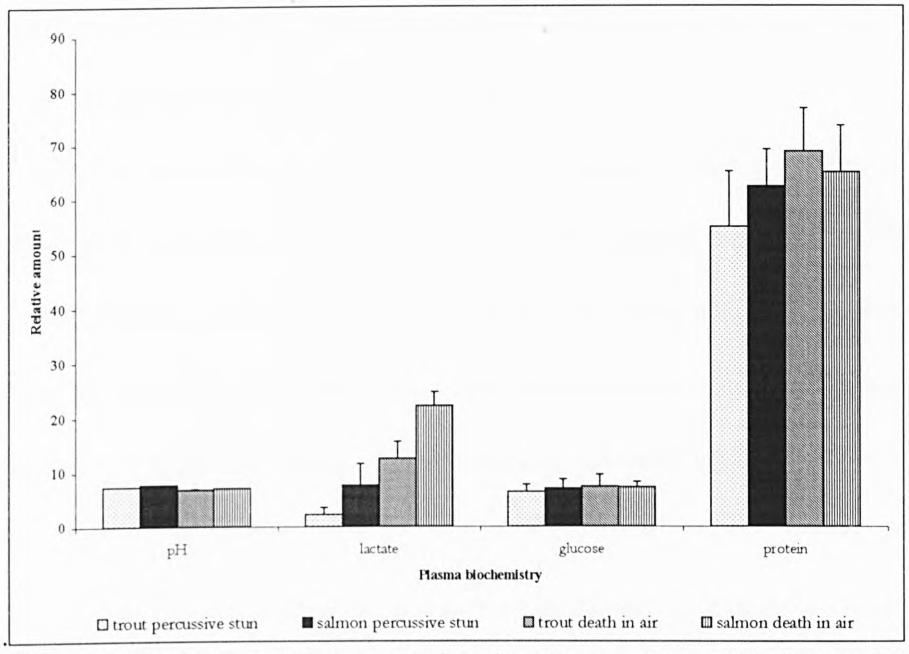


Figure 8.3 Plasma pH and metabolite concentrations in salmon and trout killed by equivalent methods

Results expressed as mean ± standard deviation, single determinations for pH, duplicates for lactate, glucose and protein, n=20 trout, n=10 salmon.

As the plasma lactate measurements would be expected to mirror pH, these data suggest that factors other than lactate were influencing plasma pH. Adrenaline stimulates the Na^+/ H^+ exchanger in the RBC membranes to extrude protons, to increase the oxygen

affinity of the blood [92], causing the pH of the plasma to fall. The adrenaline levels were higher in the trout plasma and so the pH in these fish might be expected to be lower.

The effects body size or scaling might also have influenced plasma lactate levels in the two groups of fish. The average size of the salmon, 3.2±0.7kg, was larger than that of the trout 0.391±0.055kg. As smaller fish have a higher metabolic rate than larger fish [48], the effect of body mass scaling would therefore be to lower the metabolic rate of the salmon relative to that of the trout. However, the anaerobic capacity of large fish is greater than that of small individuals, and so they can produce greater levels of lactate, albeit at a slower rate. The salmon would therefore have had a lower metabolic rate and would rely more heavily on anaerobic metabolism. They might therefore be expected to contain more plasma lactate compared with the rainbow trout, as observed in the data. The percussively stunned salmon with their lower oxygen demand might not have been deficient in oxygen at the time of sampling, therefore adrenaline would not have been released into the plasma and so the pH would not have declined as quickly.

The plasma glucose levels were similar in both species and did not alter with killing method, ($P=0.3133$, $t=1.027$, $df=28$) in the percussively stunned fish and ($P=0.8195$, $t=0.2305$, $df=26$) in the death in air fish. The death in air salmon value expressed as a percentage of the trout value was 98% and in the percussively stunned fish it was 109%. This suggests that the primary stress response did not influence plasma glucose levels in these fish.

The level of plasma protein did not differ significantly between species in the death in air groups, ($P=0.2685$, $t=1.131$, $df=26$). However, the percussively stunned trout had had significantly less plasma protein than the salmon killed by the equivalent method, ($P=0.0440$, $t=2.109$, $df=28$). The plasma protein levels in the salmon were 95% and 114% of the death in air and percussively stunned trout values. The plasma protein might have been influenced by the lower muscle lactate levels in the percussively stunned trout,

exerting less of an osmotic force on the plasma fluids, smaller fish metabolising more aerobically than the larger fish see Section 8.2.2. Furthermore, if osmoregulation had shut down in the salmon due to crowding stress, (they were noticeably stressed as shown by the physical signs of turning blue and shedding scales), they would have lost fluids to the environment as they were in seawater.

8.2.2 Muscle biochemistry

8.2.2.1 Muscle metabolite levels

The muscle metabolite data in the two species killed by equivalent methods shows similar patterns, Figure 8.4. In general, the salmon appear to have lower levels of all the metabolites than the trout.

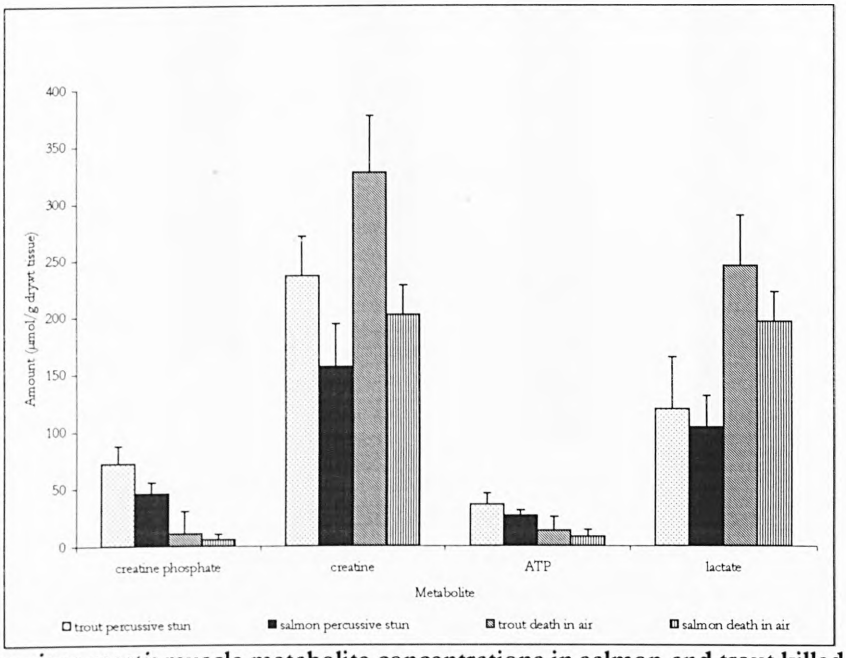


Figure 8.4 Pre r*igor mortis* muscle metabolite concentrations in salmon and trout killed by equivalent methods
Trout n=20, salmon n=10, results expressed as mean ± standard deviation from triplicate measurements.

The percussively stunned trout had significantly higher levels of creatine phosphate and ATP than the salmon, whereas levels in the death in air fish did not differ. The inferential statistics for creatine phosphate were, $P<0.0001$, $t=4.764$, $df=28$ in the percussively stunned fish and, $P=0.3245$, $t=1.009$, $df=27$, in the death in air fish. For ATP they were, $P=0.0006$, $t=3.891$, $df=27$ in the percussively stunned fish and, $P=0.1579$, $t=1.453$, $df=27$, in the death in air fish. The creatine levels were significantly higher in the

trout in both killing method groups, in the percussively stunned fish ($P<0.0001$, $t=5.774$, $df=28$) and in the death in air fish ($P<0.0001$, $t=9.119$, $df=27$). Whereas in the lactate concentrations the death in air trout had significantly higher levels than the salmon but the percussively stunned fish did not, $P=0.0034$, $t=3.207$ $df=27$, in the death in air fish and $P=0.1010$, $t=1.698$, $df=27$ in the percussively stunned fish.

Initial inspection of the pre *rigor mortis* muscle metabolite data from fish killed by equivalent methods showed the salmon contained lower amounts of the metabolites measured, Figure 8.4. The proportions of creatine phosphate, creatine and ATP in the tissue were however comparable, Figure 8.5.

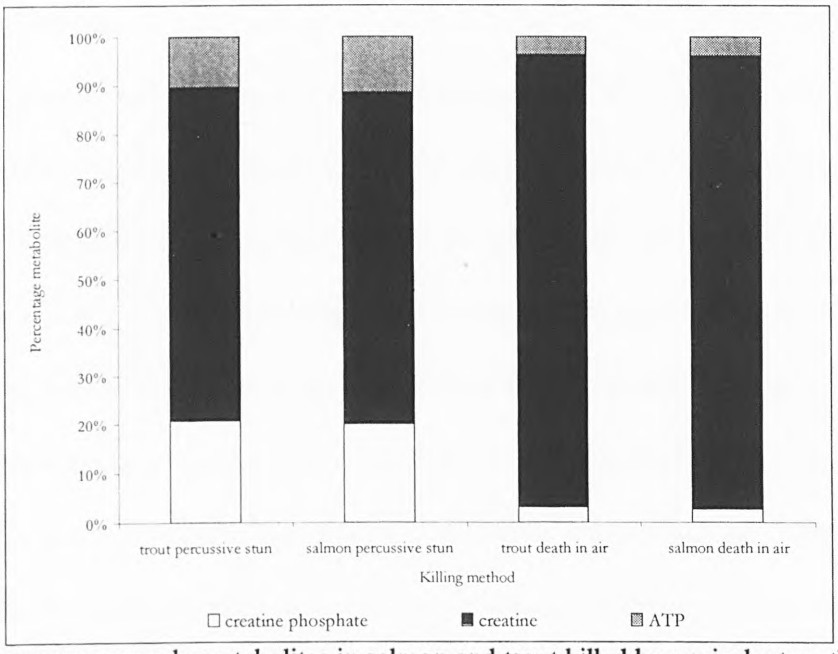


Figure 8.5 Percentage muscle metabolites in salmon and trout killed by equivalent methods
Trout n=20, salmon n=10, results expressed as mean ± standard deviation from triplicate measurements.

Species, size and starvation period were the major influences that could have caused the disparity in tissue metabolite levels. Minor influences were the 2°C higher water temperature in the salmon experiment and the extra 15 minutes dying time of the 'death in air' salmon.

The effects of SDA and temperature would have been to increase the metabolic rate of the salmon. However, as the metabolic rate/ oxygen consumption of fish decreases with increasing body size, the effects of body mass scaling on the salmon would have been to reduce the metabolic rate. The anaerobic capacity of fish increases with size, as levels of

enzymes associated with anaerobic metabolism LDH, CK and PK increase with body size, while citrate synthase associated with aerobic metabolism decreases [43]. Higher lactate levels would therefore be expected in the salmon relative to the trout. Work by Ferguson *et al.* [78] reports that following exhaustive exercise, lactate and ATP levels were higher in larger rainbow trout compared with smaller fish, although creatine phosphate levels were independent of fish size.

Fish have adapted to living in an environment where food availability fluctuates. They regulate their body composition in times of feast and famine mainly by varying levels of tissue fat and water. In adult salmonids the percentage whole body weight of protein remains relatively constant [206] at approximately 20% of the tissue. The water content of the fish in this study was measured at 66% for salmon and 78% for trout, values similar to those found in the literature. Therefore, the fat content of the two species was markedly different, i.e. ~14% in the salmon and ~2% in the trout. The compounds measured were expressed as μmol/g dry wt, so the difference in tissue water content was taken into consideration, but the difference in fat content was not. As creatine phosphate, creatine, ATP and lactate are only present in protein, they were under-represented in the salmon relative to the trout. When the higher fat content of the salmon was taken into consideration the values of the two species for creatine phosphate, creatine and ATP are similar, Table 8.1. When adjusted for fat levels the muscle lactate concentrations in the salmon are higher compared to those of the trout. This would be because of the effects of body size on anaerobic metabolism, the increased metabolic rate due to the effects of SDA, and the higher water temperature. The slightly lower fat-adjusted creatine phosphate levels in the death in air salmon might be due to the longer dying period they experienced. The creatine pool values did not differ significantly between the two species when fat content was taken into consideration, ($P=0.5126$, $t=0.6589$, $df=58$).

The muscle metabolite data therefore support the hypothesis of anaerobic scaling in salmonids. They also suggest that the size of the creatine pool in adult salmonids is

independent of species and size of fish.

Table 8.1 Comparison of pre *rigor mortis* muscle metabolites of the two species adjusted for fat content

	Trout	Salmon adjusted for fat	% difference
Creatine phosphate A	10.7	9.4	-14
Creatine phosphate PS	71.4	70.5	-1
Creatine A	327.4	311.1	-5
Creatine PS	236.1	241.1	+2
ATP A	13.0	13.0	0
ATP PS	36.1	40.6	+11
Lactate A	244.8	298.3	+18
Lactate PS	119.0	159.2	+25

Muscle metabolites expressed as μmol/g dry wt, from duplicate measurements, n=20 trout, n=10 salmon. A = death in air, PS= percussive stun.

8.2.2.2 Muscle energy status

To compare the energy status of the two species CP: Cr and CP: ATP ratios were calculated so as to negate the effects of the lower metabolite levels in the salmon. The percussively stunned fish of both species had a higher energy status than the death in air fish, and the ratios for fish killed by equivalent methods were remarkably similar, Figures 8.6 and 8.7.

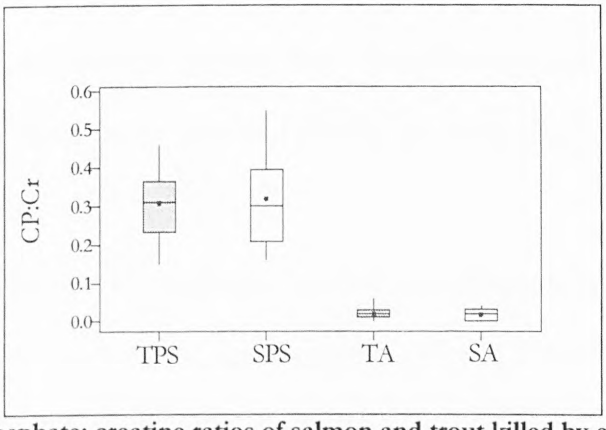


Figure 8.6 Creatine phosphate: creatine ratios of salmon and trout killed by equivalent methods
Ratios calculated on an individual fish basis. TPS, trout percussive stun, n=20; SPS, salmon percussive stun, n=10; TA, trout death in air, n=19; SA, salmon death in air, n=10.

The CP: Cr ratios were for the percussively stunned fish; trout 0.31±0.08, salmon 0.32±0.13, (P=0.8546, t=0.1873, df=12): and for the death in air groups, trout 0.02±0.01 and salmon 0.02±0.01, (P=0.679, t=0.4182, df=27), Figure 8.6. The CP: ATP ratios were for the percussively stunned fish; trout 1.98±0.51, salmon 1.80±0.54 (P=0.3773, t=0.8977, df=27), and for the death in air groups, trout 0.82±0.75 and salmon 0.74±0.56 (P=0.7970,

t=0.2602, df=23), Figure 8.7.

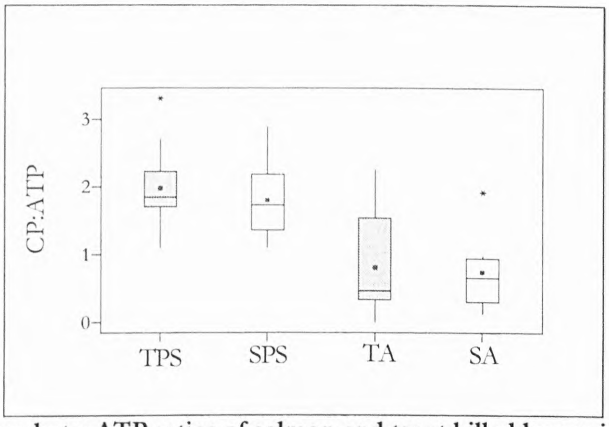


Figure 8.7 Creatine phosphate: ATP ratios of salmon and trout killed by equivalent methods
Ratios calculated on an individual fish basis. TPS, trout percussive stun n=19; SPS, salmon percussive stun, n=10; TA, trout death in air, n=17; SA, salmon death in air, n=8.

These data therefore suggest that the energy status of the two salmonid species when killed by equivalent methods was comparable.

8.2.3 Muscle quality

8.2.3.1 pH

The pH of the rainbow trout muscle was higher than that of the Atlantic salmon. The initial pH of the trout was significantly higher than that of the salmon killed by the equivalent method; $P<0.0001$, $t=7.458$, $df=26$ in the death in air fish, and, $P<0.0001$, $t=6.6608$, $df=28$ in the percussively stunned fish. The salmon muscle pH expressed as a percentage of the trout values were, 92% and 94% in the death in air and percussively stunned fish, respectively.

The relative difference in muscle pH between the killing methods in both species was however comparable. In the trout the muscle the pH was, 7.25 ± 0.16 in the percussion group, and 6.73 ± 0.27 in the death in air fish, the difference between the readings being 0.52 pH units. The salmon muscle pH readings were, percussion 6.80 ± 0.21, death in air 6.22 ± 0.09, with a difference of 0.58 pH units.

There was a faster rate of decline in the pH of the trout muscle immediately after death, percussion 0.30units/min and death in air 0.16units/min. The rate of decline in the salmon was in the percussively stunned group 0.12units/min and the death in air group

0.06units/min. The relative rate of decline therefore was similar in both species i.e. the pH in the percussively stunned fish declined approximately twice as fast as the death in air group.

The longer time it took to process the salmon from the cage might have caused the lower pH in the salmon relative to the trout. The higher temperature and the effects of SDA during the pilot salmon experiment would also have increased the metabolic rate and potentially the decline in pH. In the commercial harvesting methods study, muscle pH in the live-chilled percussively stunned salmon were similar to the percussively stunned trout study, 7.33±0.08 in the DP and 7.13±0.14 in the MT4 fish, indicating that scaling effects if any were minimal.

The pH_{96} of the pilot study salmon was however also lower than that of the trout. The inferential statistics were $P<0.001$, $t=16.604$, $df=77$ in the death in air group, and $P<0.001$, $t=16.778$, $df=52$ in the percussively stunned fish. Death in air salmon 6.42±0.07, percussively stunned salmon 6.49±0.06, death in air trout 6.76±0.08, percussively stunned trout 6.80±0.10. The pH_{96} in the salmon expressed as a percentage of the trout values were 95% in both sets of fish. The muscle pH in the commercial DP and MT4 fish was also quite low, 6.31±0.04 and 6.21±0.12. This suggests that the difference in muscle pH_{96} might be due to scaling or species effects rather than the timing of the measurement. The effects of body size on the decline in pH would be a faster decline with decreasing body size. The aerobic metabolism of small fish is faster, therefore the tissue oxygen levels would be utilised faster and cause an earlier mobilisation of creatine phosphate stores and faster switching to the anaerobic glycolysis mechanism of ATP production. This was shown in the faster decline in pH in the trout.

Larger fish have a greater anaerobic capacity and consequently produce greater amounts of lactate. The post *rigor mortis* salmon muscle lactate content adjusted for lipid content were; 375μmol/g dry wt tissue death in air salmon, 350μmol/g dry wt tissue percussively stunned salmon. The trout muscle lactate content was 252μmol/g dry wt

tissue death in air and 247μmol/g dry wt tissue percussion. This confirms the lower pH values observed in the salmon and suggests that the pH_{96} values might be attributable to scaling effects. The temperature would also have influenced the pH of the fish, the pilot salmon were sampled from water that was 2°C warmer than the trout which would increase metabolic rate and therefore lower the pH relative to the trout, while in the commercial study the fish were live-chilled.

8.2.3.2 Fish freshness

The K value data measured on tissue taken after 4 days (trout) and 5 days (salmon) ice storage showed no significant difference between killing methods in either species. There were significant differences between the species in K value showing that the salmon were less fresh than the trout, but this was to be expected as the tissue was one day older in the salmon. However, the rate of decline in freshness over four days as measured by the Torrymeter was shown to be faster in the death in air compared with the percussively stunned salmon. This was not observed in the trout killed by the same methods. The trout would be expected to have a lower metabolic rate even though they were smaller than the salmon as they had been starved for three days. The effects of SDA would not therefore have been apparent and the water temperature was also lower. The salmon on the other hand although larger had been fed the previous day. The faster decline in freshness in the salmon caused by the effects of SDA is supported by the data from the trout starving experiment, as described in Section 6.3.3.2. The fed trout showed a faster decline in Torrymeter readings than the starved fish over days 2-6. The fed trout also had a higher K value on day 6.

Harvesting procedures can therefore influence the keeping quality of farmed salmonids.

8.3 Conclusion

Generally, the response of the two salmonid species to the different killing/ stunning methods was comparable, although factors such as body mass scaling, specific dynamic action and environmental temperature caused differences.

Even though the catecholamine data was highly variable, the adrenaline: noradrenaline ratios were alike. The muscle metabolite data was more consistent and the CP: Cr ratios of the two species were almost identical, while the CP: ATP ratios were highly comparable. The total creatine pool values did not differ between the two species, which suggests that the creatine pool of adult salmonids is independent of fish species and size.

9 GENERAL CONCLUSION

9.1 Overview

The aim of this project was to determine whether existing harvesting practices used on salmonid farms could be tailored to minimise stress in stock while maintaining or improving carcass quality. The current harvesting practices used on UK fish farms were determined *via* a questionnaire. The most frequently used stunning and killing methods were then assessed for their effects on stress physiology and carcass quality in rainbow trout and Atlantic salmon. The effect of a short-term fast on the flesh quality of farmed rainbow trout was also investigated.

9.2 Principal findings

Routine starvation periods on UK fish farms were at least one week for Atlantic salmon, and 20-30 degree-days for rainbow trout. Salmon are generally slaughtered following percussive stun or carbon-dioxide narcosis, while the principal killing/ stunning methods used in rainbow trout production are percussive stun, electrical stun, death in air, and ice slurry.

In the present study, the killing/ stunning methods used for rainbow trout had profound effects on stress physiology and carcass quality. The methods that decreased muscle stimulation/ activity during harvest improved flesh quality by prolonging the period of optimum freshness. Fish that were stunned by percussion or electricity or were anaesthetised before death, showed decreased muscle activity immediately *post mortem* compared with fish that were left for extended periods to die in air or ice slurry. The electrical stunning process increased muscle activity, but to a much lesser degree than the ice slurry or death in air methods. Stunning fish prior to killing can however cause physical damage that leads to downgrading.

The effects of killing/ stunning methods on the welfare of farmed fish are less clear. Plasma adrenaline levels were high in both the death in air and electrically stunned

fish, which would seem to indicate that they were stressed and their welfare was poor. The relatively low level of muscle activity in the electrically stunned fish however, brought this into question. In anaesthetised electrically stunned fish, where the nerves were less responsive, and the fish were insensate, the adrenaline response was diminished, but to a lesser extent than in the percussively stunned fish. It was conjectured that the electrical stimulation induced adrenaline release in addition to increasing muscle activity in these fish.

In the anaesthetised death in air fish, plasma adrenaline levels were higher relative to their non-anaesthetised counterparts. It is possible that this augmented response occurred either as the result of adrenaline release caused by mechanisms other than those initiated by the brain, or by a delayed response due to lower responsivity of the nerves.

The adrenaline data for the ice slurry fish was even less clear-cut. The adrenaline levels were low in both the anaesthetised and non-anaesthetised fish suggesting that this killing method was humane. This, however, was in direct contrast with the evidence from the muscle activity data, which showed a massive reduction in activity with anaesthesia in this group. It was surmised that the lower body temperature reduced nerve impulse conduction so lessening the acute stress response. Whether impaired nerve conduction reduces nociception and thereby increases welfare in fish is however uncertain.

The response to harvesting methods in Atlantic salmon was also examined. Greater levels of muscle activity were observed in salmon that were left to die in air or in carbon dioxide saturated water, compared with fish that were percussively stunned. The acute primary stress response was also diminished in the percussively stunned fish relative to the death in air or carbon-dioxide narcosis groups of fish.

The effect of withholding food for 30-40 degree-days on the carcass quality of rainbow trout was also investigated. Freshness declined faster in fed fish compared with 'starved' fish. It was conjectured that muscle activity in fed fish was faster due to the effects of specific dynamic action.

9.3 Conclusions, implications, and recommendations

9.3.1 Conclusions

Killing and stunning techniques can cause a variable stress response in farmed salmonids. The methods that render fish insensate quickly, e.g. percussive and electrical stun, reduce muscle activity *peri-mortem*, which increases fish freshness. Methods used to stun fish can however physically damage the carcass and increase the primary stress response. Killing method should therefore be selected for the quality attributes desired in the final product. Carcass quality might, but not necessarily, have to be compromised to ensure ethical quality.

9.3.2 Implications

Killing fish in ice slurry might appear humane to the fish producer as the fish cease to struggle relatively quickly. On initial inspection the low levels of primary stress hormones appear to support these observations. However, although their body temperature was low, fish killed in ice slurry fish exhibited high levels of muscle activity. This did not occur when the fish were anaesthetised. These fish might therefore be severely stressed. There are no obvious advantages to killing fish in ice compared with air, so the practise should be avoided, and fish iced after death. Death in ice slurry causes faster loss of freshness and might even cause softening of the flesh. Fish that are stunned before killing exhibit fewer signs of muscle activity than those that are left to die naturally and so maintain optimum freshness for a longer period. Percussive stun where practical and performed correctly will give good quality carcasses. Electrical stunning has the potential to slightly reduce quality but it is effective in stunning fish instantaneously.

It must be noted however that, pre-slaughter handling stresses might mask the variable stress response to killing/ stunning method in fish.

9.3.3 Recommendations

Salmonids should be killed as quickly as possible or stunned before killing to increase freshness and improve ethical quality. Ice slurry should not be used for killing salmonids as although it is uncertain whether it affects welfare, carcass quality declines. If stunning is impractical the fish should be allowed to die before being iced. The practise of a short-term fast before killing increases fish quality through freshness.

REFERENCES

1. Aas GH, Bjerkeng B, Hatlen B, Storebakken T (1997) Idoxanthin, a major carotenoid in the flesh of Arctic charr (*Salvelinus alpinus*) fed diets containing astaxanthin. Aquaculture **150**: 135-142
2. Aitken A, Mackie IM, Merritt JH, Windsor ML (1982) Fish Handling and Processing, 2nd Edn. HMSO, Edinburgh
3. Alderson R (2001) The Potential Impact of Genetic change on Harvest and Eating Quality of Atlantic Salmon. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 137-144
4. Allen C (2002) Animal Consciousness. In: Zalta EN (Ed) The Stanford Encyclopaedia of Philosophy. The Metaphysics Research Lab, Center for the Study of Language and Information, Stanford University, Stanford, URL=http:/plato.stanford.edu/archives/sum2002/entries/consciousness-animal/
5. Amlacher E (1961) *Rigor mortis* in Fish. In: Borgstrom G (Ed) Fish as Food I: Production Biochemistry and Microbiology. Academic Press, New York, vol 1, pp 385-409
6. Andersen UB, Strømsnes AN, Steinsholt K, Thomassen MS (1994) Fillet gaping in farmed Atlantic salmon (*Salmo salar*). Norwegian Journal of Agricultural Sciences **8**: 165-179
7. Andersen UB, Thomassen MS, Rørå AMB (1997) Texture Properties of Farmed Rainbow Trout (*Oncorhynchus mykiss*): Effects of Diet, Muscle Fat Content and Time of Storage on Ice. Journal of the Science of Food and Agriculture **74**: 347-353
8. Angus L, Malcolmson L, Nicolson A, Inkster A (1995) Improving the Quality of Farmed Salmon by *Iki Jime* Harvesting. North Atlantic Fisheries College, Fisheries Development Note **No. 1**,
9. Anon. (1995) The Humane Slaughter of Farmed Atlantic Salmon. Scottish Salmon Growers Association, pp 44
10. Anon. (1995) The Welfare of Animals (Slaughter or Killing) Regulations 1995. Statutory Instrument, Animals: Prevention of Cruelty. HMSO, UK, pp 2610-2644
11. Anon. (1998) 20-a-minute salmon stab from Baader Fish Farming International, vol **25**, pp 47
12. Aske L, Midling K (1997) Live capture and starvation of capelin cod (*Gadus morhua* L.) in order to improve the quality. In: Luten JB, Børresen T and Oehlenschläger J (Eds) Seafood from the Producer to the Consumer, An Integrated Approach to Quality. Elsevier Science, Amsterdam, pp 47-58

13. Azam K (1992) Factors affecting the *post mortem* quality of farmed fish with particular reference to methods of stunning. PhD Thesis University of Strathclyde, Glasgow.

14. Azam K, Mackie IM, Smith J (1989) The effect of slaughter method on the quality of rainbow trout (*Salmo gairdneri*) during storage on ice. International Journal of Food Science and Technology **24**: 69-79

15. Azam K, Mackie IM, Smith J (1990) Effect of Stunning Method on the Time of Onset, Duration and Resolution of Rigor in Rainbow Trout (*Salmo gairdneri*) as Measured by Visual Observation and Analyses for Lactic acid, Nucleotide-Degradation Products and Glycogen. In: Chilling and Freezing of New Fish Products, Aberdeen. International Institute of Refrigeration, pp 249-254

16. Azam K, Strachan NJC, Mackie IM, Smith J, Nesvadba P (1990) Effect of slaughter method on the progress of rigor of rainbow trout (*Salmo gairdneri*) as measured by an image processing system. International Journal of Food Science and Technology **25**: 477-482

17. Baker RTM, Pfeiffer A-M, Schöner F-J, Smith-Lemmon L (2002) Pigmenting efficacy of astaxanthin and canthaxanthin in fresh-water reared Atlantic salmon, *Salmo salar*. Animal Feed Science and Technology **99**: 97-106

18. Barton BA, Dwyer WP (1997) Physiological stress: effects of continuous- and pulsed-DC electroshock on juvenile bull trout. Journal of Fish Biology **51**: 998-1008

19. Barton BA, Grosh RS (1996) Effect of AC electroshock on blood features in juvenile rainbow trout. Journal of Fish Biology **49**: 1330-1333

20. Barton BA, Schreck CB (1988) Fasting and Diet Content Affect Stress Induced Changes in Plasma glucose and Cortisol in Juvenile Chinnook Salmon. The Progressive Fish Culturist **50**: 16-22

21. Beamish FWH (1964) Influence of Starvation on Standard and Routine Oxygen Consumption. Transactions of the American Fisheries Society **93**: 103-107

22. Beaulieu MA, Guderley H (1998) Changes in qualitative composition of white muscle with nutritional status of Atlantic cod, *Gadus morhua*. Comparative Biochemistry and Physiology **121A**: 135-141

23. Bellardi S, Bianchini ML, Formeris G, Palmegianio B (1999) Hypertrophic and hyperplasic responses of adipocytes as an effect on different feeding protocols in Rainbow trout. In: Aquaculture Europe '99 Towards predictable Aquaculture, Trondheim, Norway. European Aquaculture Society, 278pp

24. Benfey TJ, Biron M (2000) Acute stress response in triploid rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). Aquaculture **184**: 167-176

25. Bennett RM (1996) Willingness-to-pay measures of public support for farm animal welfare legislation. Veterinary Record **139**: 320-321

26. Berg T, Erikson U, Nordtvedt TS (1997) *Rigor mortis* Assessment of Atlantic Salmon (*Salmo salar*) and Effects of Stress. Journal of Food Science **62**: 439-446

27. Bergmeyer HU (1986) Methods of Enzymatic Analysis., 3rd Edn. VCH Verlagsgesellschaft, Germany

28. Bernier NJ, Randall DJ (1998) Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. Journal of Fish Biology **52**: 621-637

29. Bertelsen U, Børresen T, Purslow PP (2000) Mechanism of Gaping. Danish Institute for Fishery Research, Poster presentation

30. Biron M, Benfey TJ (1994) Cortisol, glucose and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis* Mitchill). Fish Physiology and Biochemistry **13**: 153-160

31. Bito M, Yamada K, Mikumo Y, Amano K (1983) Studies on *Rigor Mortis* of Fish-1. Difference in the Mode of *Rigor Mortis* among Some Varieties of Fish by Modified Cutting's Method. Bulletin of the Tokai Regional Fisheries Research Laboratory **109**: 89-96

32. Bjerkeng B, Hatlen B, Jobling M (2000) Astaxanthin and its metabolites idoxanthin and crustaxanthin in flesh, skin, and gonads of sexually immature and maturing Arctic charr (*Salvelinus alpinus* (L.)). Comparative Biochemistry and Physiology **125B**: 395-404

33. Black D, Love RM (1986) The sequential mobilization and restoration of energy reserves in tissues of Atlantic cod during starvation and refeeding. Journal of Comparative Physiology **156B**: 469-479

34. Blokhus H (1986) Aspects Related to Quality of Farmed Norwegian Salmon (*Salmo salar).* In: Proceedings of an International Symposium Co-ordinated by the Sea Grant College Program, Anchorage, Alaska. Elsevier Science, pp 615-628

35. Boggess Jr. TS, Heaton EK, Shewfelt AL, Parvin DW (1973) Techniques for Stunning Channel Catfish and their Effects on Product Quality. Journal of Food Science **38**: 1190-1193

36. Bond CE (1996) Biology of Fishes, 2nd Edn. Sanders College Publishing, New York

37. Bone Q, Marshall NB, Blaxter JHS (1995) Biology of Fishes, 2nd Edn. Chapman and Hall, London

38. Bonnet S, Fauconneau B, David K, Faure A, Laroche M (1996) Effect of Fasting and Stress on *Post Mortem* changes in Brown Trout Reared in Sea Water. In: Refrigeration and Aquaculture, Bordeaux, France. IIF-IIR Commission C2, pp 137-144

39. Botta JR (1995) Evaluation of Seafood Freshness Quality, 1st Edn. VCH, Cambridge

40. Botta JR, Bonnell G, Squires BE (1987) Effect of Method of Catching and Time of Season on Sensory Quality of Fresh Raw Atlantic Cod (*Gadus morhua*). Journal of Food Science **52**: 928-931+938

41. Bourne MC (1978) Texture Profile Analysis. Food Technology **32**: 62-66+72

42. Broom DM (1988) The Scientific Assessment of Animal Welfare. Applied Animal Behaviour Science **20**: 5-19

43. Burness GP, Leary SC, Hochachka PW, Moyes CD (1999) Allometric scaling of RNA, DNA, and enzyme levels: an intraspecific study. American Physiological Society **277**: R1164-R1170

44. Cappeln G, Jessen F (2001) Nucleotide Variation in cod (*Gadus morhua*). In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, Poster Presentation, pp 430

45. Chervova LS (1997) Pain Sensitivity and Behaviour of Fishes. Journal of Ichthyology **37**: 106-111

46. Cheyne A (1975) How the GR Torrymeter aids quality control in the fishing industry. Fishing News International **December**: 71-76

47. Christiansen R, Struksnaes G, Estermann R, Torrissen OJ (1995) Assessment of flesh colour in Atlantic salmon, *Salmo salar* L. Aquaculture Research **26**: 311-321

48. Clarke A, Johnston NM (1999) Scaling of metabolic rate with body mass and temperature in teleost fish. Journal of Animal Ecology **68**: 893-905

49. Clay P, Revell BJ (1995) The changing market position of farmed salmon in the U.K. is salmon losing its luxury image. In: Quality in Aquaculture, Trondheim, Norway. European Aquaculture Society, pp 372-373

50. Close B, Banister K, Baumans V, Bernoth E, Bromage N, Bunyan J, Erhardt D, Flecknell P, Gregory N, Hackbarth H, Morton D, Warwick C (1996) Recommendations for euthanasia of experimental animals: Part 1. Laboratory Animals **30**: 293-316

51. Connell JJ (1995) Control of Fish Quality, 4th Edn. Fishing News Books, Oxford

52. Cook JT, Sutterlin AM, McNiven MA (2000) Effect of food deprivation on oxygen consumption and body composition of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture **188**: 47-63

53. Cooke M (2001) Ethical Considerations for the Production of Farmed Fish - The Retailer's point of View. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 116-119

54. Curran CA, Poulten RG, Brueton A, Jones NSD (1986) Cold shock reactions in iced tropical fish. Journal of Food Technology **21**: 289-299

55. Curran CA, Poulter RG, Brueton A, Jones NR, Jones NSD (1986) Effect of handling treatment on fillet yields and quality of tropical fish. Journal of Food Technology **21**: 301-310

56. Damsgård B (1999) Small efforts prevent stress and aggression. Fiskeriforskning Info. Norwegian Institute of Fisheries and Aquaculture Ltd., Tromso, vol **6**,

57. Davidson GW, Davie PS, Young G, Fowler RT (2000) Physiological Responses of Rainbow Trout *Oncorhynchus mykiss* to Crowding and Anaesthesia with AQUI-S. Journal of The World Aquaculture Society **31**: 105-114

58. Dobson GP, Hochachka PW (1987) Role of Glycolysis in Adenylate Depletion and Repletion During Work and Recovery in Teleost White Muscle. Journal of Experimental Biology **129**: 125-140

59. Dobson GP, Parkhouse WS, Hochachka PW (1987) Regulation of anaerobic ATP - generating pathways in trout fast-twitch skeletal muscle. American Journal of Physiology **253**: R186-R194

60. Dunajski E (1979) Texture of Fish Muscle. Journal of Texture Studies **10**: 301-318

61. Duncan IJH (1986) Assessment of pain using conditioning procedures. In: Duncan IJH and Molony V (Eds) Assessing Pain in Farm Animals. Commission of the European Communities, Roslin, Scotland, pp 71-78

62. Duncan IJH, Molony V (1986) Assessing Pain in Farm Animals. Roslin Institute, Edinburgh, pp 92

63. Dunn JF, Hochachka PW (1986) Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. Journal of Experimental Biology **123**: 229-242

64. Dunn JF, Hochachka PW (1986) Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. Canadian Journal of Zoology **65**: 1144-1148

65. Dunn P (2000) 1999 Survey of Trout Production in England and Wales. Trout News **30**: 5-8

66. Eakins F, AL-Khayat HA, Kensler RW, Morris EP, Squire JM (2002) 3D Structure of Fish Muscle Myosin Filaments. Journal of Structural Biology **137**: 154-163

67. Einen O, Mørkøre T, Rørå AMB, Thomassen MS (1999) Feed ration prior to slaughter-a potential tool for managing product quality of Atlantic salmon (*Salmo salar*). Aquaculture **178**: 149-169

68. Einen O, Thomassen MS (1998) Starvation prior to slaughter in Atlantic salmon (*Salmo salar*) II. White muscle composition and evaluation of freshness, texture and colour characteristics in raw and cooked fillets. Aquaculture **169**: 37-53

69. Einen O, Waagan B, Thomassen MS (1998) Starvation prior to slaughter in Atlantic salmon (*Salmo salar*) I. Effects on weight loss, body shape, slaughter- and fillet-yield, proximate and fatty acid composition. Aquaculture **166**: 85-104

70. Erikson U (2001) Rigor Measurements. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 283-297

71. Erikson U, Beyer AR, Sigholt T (1997) Muscle High-Energy Phosphates and Stress Affect K-Values during Ice Storage of Atlantic Salmon (*Salmo salar*). Journal of Food Science **62**: 43-47

72. Erikson U, Sigholt T, Rustad T, Einarsdottir IE, Jørgensen L (1999) Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. Aquaculture International **7**: 101-115

73. Erikson U, Sigholt T, Selend A (1997) Handling stress and water quality during live transportation and slaughter of Atlantic salmon (*Salmo salar*). Aquaculture **149**: 243-252

74. Eskin NAM (1990) Biochemistry of Foods, 2nd Edn. Academic Press, London

75. Faergemand J, Rønsholdt B, Alsted N, Børresen T (1995) Fillet Texture of Rainbow Trout as affected by Feeding Strategy, Slaughtering Procedure and Storage *Post Mortem*. Water Science and Technology **31**: 225-231

76. Fauconneau B, Andre S, Chmaitilly J, Le Bail P-Y, Krieg F, Kaushik SJ (1997) Control of skeletal muscle fibres and adipose cells size in the flesh of rainbow trout. Journal of Fish Biology **50**: 296-314

77. FEAP (2000) FEAP 2000 European farmed fish production 1998-1999. Federation of European Aquaculture Producers, pp 2

78. Ferguson RA, Kieffer JD, Tufts BL (1993) The Effects of Body Size on the Acid-Base and Metabolite Status in the White Muscle of Rainbow Trout Before and After Exhaustive Exercise. Journal of Experimental Biology **180**: 195-207

79. Fletcher GC, Hallett IC, Jerrett AR, Holland AJ (1997) Changes in the Fine Structure of the Myocommata-Muscle Fibre Junction Related to Gaping in Rested and Exercised Muscle from King Salmon (*Oncorhynchus tschawytscha*). Food Science and Technology **30**: 246-252

80. Flos R, Reig L, Torres P, Tort L (1988) Primary and Secondary Stress Responses to Grading and Hauling in Rainbow Trout, *Salmo gairdneri*. Aquaculture **71**: 99-106

81. Garforth D (1997) BIM/ISGA hold series of salmon quality workshops. Aquaculture Ireland, pp 21-24

82. Gerwick L, Demers NE, Bayne CJ (1999) Modulation of Stress hormones in Rainbow trout by means of anaesthesia, sensory deprivation and receptor blockade. Comparative Biochemistry and Physiology **124A**: 329-334

83. Gómez-Guillén MC, Montero P, Hurtado O, Borderías AJ (2000) Biological Characteristics Affect the Quality of Farmed Atlantic Salmon and Smoked Muscle. Journal of Food Science **65**: 53-60

84. Gregory NG (1998) Animal Welfare and Meat Science, 1st Edn. CABI, Wallingford

85. Harris ELV, Angal S (1989) Protein Purification Methods, 1st Edn. Oxford University Press, Oxford

86. Hatae K, Yoshimatsu F, Matsumoto JJ (1990) Role of Muscle Fibers in Contributing Firmness of Cooked Fish. Journal of Food Science **55**: 693-696

87. Hattula T, Louoma T, Kostiainen R, Poutanen J, Kallio M, Suuronen P (1995) Effects of catching method on different quality parameters of Baltic herring (*Clupea harengus* L.). Fisheries Research **23**: 209-221

88. Heller R, Gilbert R, Jaroszeski MJ (1999) Clinical applications of electrochemotherapy. Advanced Drug Delivery Reviews **35**: 119-129

89. Hemre G, Hansen T (1998) Utilisation of different dietary starch sources and tolerance to glucose loading in Atlantic salmon (*Salmo salar*), during parr smolt transformation. Aquaculture **161**: 145-157

90. Hilton JW (1982) The effect of pre-fasting diet and water temperature on liver glycogen and liver weight in rainbow trout, *Salmo gairdneri* Richardson, during fasting. Journal of Fish Biology **20**: 69-78

91. Hochachka PW, Mommsen TP (1995) Biochemistry and molecular biology of fishes. Elsevier Science B.V.

92. Holk K, Lykkeboe G (1995) Catecholamine-induced changes in oxygen affinity of carp and trout blood. Respiration Physiology **100**: 55-62

93. Horrex H (1998) Quick Humane Slaughter. Fish Farmer **21**: 47

94. Houston AH, Madden JA, Woods RJ, Miles HM (1971) Variations in the Blood and Tissue Chemistry of Brook Trout, *Salvelinus fontinalis*, Subsequent to Handling, Anesthesia, and Surgery. Journal of the Fisheries Research Board of Canada **28**: 635-642

95. Hunter RS (1975) The Measurement of Appearance, 1st Edn. John Wiley & Sons, London

96. Hurling R, Rodell JB, Hunt HD (1996) Fiber Diameter and Fish Texture. Journal of Texture Studies **27**: 679-685

97. Huss HH (1988) Fresh Fish Quality and Quality Changes: A Training Manual. FAO, Rome

98. Iversen M, Finstad B, Nilssen KJ (1998) Recovery from loading and transport stress in Atlantic Salmon (*Salmo salar* L.) smolts. Aquaculture **168**: 387-394

99. Iwama GK, Morgan JD, Barton BA (1995) Simple field method for monitoring stress and general condition of fish. Aquaculture Research **26**: 273-282

100. Iwama GK, Pickering AD, Sumpter JP, Schreck CB (1997) Fish Stress and Health in Aquaculture, 1st Edn. Cambridge University Press, Cambridge

101. Jason AC, Richards JCS (1975) The development of an electronic fish freshness meter. Journal of Physics E: Scientific Instruments **8**: 826-830

102. Jeney G, Galeotti M, Volpatti D, Jeney Z, Anderson DP (1997) Prevention of Stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. Aquaculture **154**: 1-15

103. Jerrett AR, Holland AJ (1998) Rigor Tension Development in Excised 'Rested', 'Partially Exercised' and 'Exhausted' Chinook Salmon White Muscle. Journal of Food Science **63**: 48-52

104. Jerrett AR, Holland AJ, Cleaver SE (1998) Rigor Contractions in 'Rested' and 'Partially Exercised' Chinook Salmon White Muscle as Affected by Temperature. Journal of Food Science **63**: 53-56

105. Jerrett AR, Stevens J, Holland AJ (1996) Tensile Properties of White Muscle in Rested and Exhausted Chinook Salmon (*Oncorhynchus tschawytscha*). Journal of Food Science **61**: 527-532

106. Jezierska B, Hazel JR, Gerking SG (1982) Lipid mobilization during starvation in the rainbow trout, *Salmo gairdneri* Richardson, with attention to fatty acids. Journal of Fish Biology **21**: 681-692

107. Jobling M (1983) Towards an explanation of specific dynamic action (SDA). Journal of Fish Biology **23**: 549-555

108. Johansson L, Kiessling A (1991) Effects of Starvation on Rainbow Trout II Eating and storage Qualities of Iced and Frozen Fish. Acta Agriculturae Scandanavica **41**: 207-216

109. Johansson L, Kiessling A, Åsgård T, Bergland L (1995) Effects of ration level in rainbow trout , *Oncorhynchus mykiss* (Walbaum), on sensory characteristics, lipid content and fatty acid composition. Aquaculture Nutrition **1**: 59-6

110. Johansson L, Kiessling A, Kiessling K-H, Berglund L (2000) Effects of altered ration levels on sensory characteristics, lipid content and fatty acid composition of rainbow trout (*Oncorhynchus mykiss*). Food Quality and Preference **11**: 247-254

111. Johnston IA (1999) Muscle development and growth: potential implications for flesh quality in fish. Aquaculture **177**: 99-115

112. Johnston IA, Alderson R, Sandham C, Dingwall A, Mitchell D, Selkirk C, Nickell D, Baker R, Robertson B, Whyte D, Springate J (2000) Muscle fibre density in relation to the colour and texture of smoked Atlantic salmon (*Salmo salar* L.). Aquaculture **189**: 335-349

113. Johnston IA, Manthri S, Alderson R, Campbell P, Mitchell D, Whyte D, Dingwall A, Nickell D, Selkirk C, Robertson R (2002) Effects of dietary protein level on muscle cellularity and flesh quality in Atlantic salmon with particular reference to gaping. Aquaculture **210**: 259-283

114. Jones NR, Murray J (1964) Rapid Methods of Nucleotide Dephosphorylation in Iced Fish Muscle. Their Value as Indices of Freshness and of Inosine 5'-Monophosphate concentration. Journal of the Science of Food and Agriculture **15**: 684-689

115. Julio AE, Montpetit CJ, Perry SF (1998) Does Blood acid-base status modulate catecholamine secretion in the Rainbow trout (*Oncorhynchus mykiss*)? Journal of Experimental Biology **201**: 3085-3095

116. Kartevoll E (1995) Patterns of productivity and Growth in the Norwegian Salmon Farming Industry. In: Quality in Aquaculture, Trondheim, Norway. European Aquaculture Society, pp 323

117. Kennish JM, Kramer DE (1986) A Review of High Pressure Liquid Chromatographic Methods for Measuring Nucleotide Degradation in Fish Muscle. In: Proceedings of an International Symposium Co-ordinated by the Sea Grant College Program, Anchorage, Alaska. Elsevier Science, pp 209-220

118. Kestin SC (1994) Pain and Stress in fish. Royal Society for the Prevention of Cruelty to Animals, Horsham, pp 36

119. Kestin SC, Wotton SB, Gregory NG (1991) Effect of slaughter by removal from water on visual evoked activity in the brain and reflex movement of rainbow trout (*Oncorhynchus mykiss*). The Veterinary Record **128**: 443-446

120. Kiessling A, Åsgård T, Storebakken T, Johansson L, Kiessling K-H (1991) Changes in the structure and function of the epiaxial muscle of Rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age III. Chemical composition. Aquaculture **93**: 373-387

121. Kiessling A, Kiessling K-H, Storebakken T, Åsgård T (1991) Changes in the structure and function of the epiaxial muscle of Rainbow trout (*Oncorhynchus mykiss*) in relation to Ration and age II. Activity of key enzymes in energy metabolism. Aquaculture **93**: 357-372

122. Kiessling A, Storebakken T, Åsgård T, Kiessling K-H (1991) Changes in the structure and function of the epiaxial muscle of Rainbow trout (*Oncorhynchus mykiss*) in relation to Ration and age I. Growth dynamics. Aquaculture **93**: 335-356

123. Korhonen RW, Lanier TC, Giesbrecht F (1990) An Evaluation of Simple Methods for Following Rigor Development in Fish. Journal of Food Science **55**: 346-348+368

124. Laird LM (1999) Impact of the Quality Movement on Salmon Farming in Scotland and the developments of standards for Organic Aquaculture. In: Aquaculture Europe '99 Towards predictable Aquaculture, Trondheim, Norway. European Aquaculture Society, pp 278

125. Laird LM, Needham T (1988) Salmon and Trout Farming. Horwood

126. Lavéty J (1993) Post Harvest Conditions for Maximising Quality. Trout News **17**: 23-28

127. Lavéty J, Afolabi OA, Love RM (1988) The connective tissues of fish. IX. Gaping in farmed species. International Journal of Food Science and Technology **23**: 23-30

128. Lepage O, Overli O, Petersson E, Järvi T, Winberg S (2000) Differential stress coping in wild and domesticated sea trout. Brain, Behavior and Evolution **56**: 259-268

129. Lincoln R (1999) Growth rates of triploid rainbow trout over the spawning season. Trout News **28**: 20-22

130. Lindsay RC (1991) Chemical Basis of the Quality of Seafood Flavours and Aromas. Marine Technology Society Journal **25**: 16-22

131. Lines J (2001) Automated Humane Slaughter of Trout. Trout News **31**: 30

132. Lines J (2001) Automated Humane Slaughter of Trout. Trout News **32**: 32

133. Little AC, Martinsen C, Sceurman L (1979) Color Assessment of Experimentally Pigmented Rainbow Trout. Color Research and Application **4**: 92-95

134. Love RM, Haq MA (1970) The connective tissues of fish. III. The Effect of pH on gaping in cod entering *rigor mortis* at different temperatures. Journal of Food Technology **5**: 241-248

135. Love RM, Lavéty J, Steel PJ (1969) The connective tissues of fish. II. Gaping in commercial species of frozen fish in relation to *rigor mortis*. Journal of Food Technology **4**: 39-44

136. Luong JHT, Male KB, Masson C, Nguyen AL (1992) Hypoxanthine Ratio Determination in Fish Extract Using Capillary Electrophoresis and Immobilized Enzymes. Journal of Food Science **57**: 77-81

137. Lymbery P (2002) In too Deep: The Welfare of intensively farmed fish. Compassion in World Farming Trust, Petersfield, pp 65

138. MAFF (1989) Non Sensory Assessment of Fish Quality. Torry Research Station, Aberdeen, pp 6

139. MAFF (2000) Future Strategies for the English Farmed Trout Industry. MAFF, London, pp 16

140. Magnussen OM, Johansen S (1990) Influence of Handling and Slaughtering on Quality of Farmed Fish. In: Chilling and Freezing of New Fish Products, Aberdeen. International Institute of Refrigeration, pp 317-322

141. Marx H, Brunner B, Weinzierl W, Hoffmann R, Stolle A (1977) Methods of stunning freshwater fish: impact on meat quality and aspects of animal welfare. Zeitschrift Lebensm-Untersachung Forschung A **204**: 282-286

142. Mazeaud MM, Mazeaud F, Donaldson EM (1977) Primary and Secondary Effects of Stress in Fish: Some New Data with a General Review. Transactions of the American Fisheries Society **106**: 201-212

143. McDonald I, Jones NR (1976) Control of Thaw Rigor by Manipulation of Temperature in Cold Store. Journal of Food Technology **11**: 69-71

144. McFarlane WJ, Heigenhauser GJF, McDonald DG (2001) Creatine supplementation affects sprint endurance in juvenile rainbow trout. Comparative Biochemistry and Physiology **130A**: 857-866

145. Milligan CL (1996) Metabolic Recovery from Exhaustive Exercise in Rainbow Trout. Comparative Biochemistry and Physiology **113A**: 51-60

146. Mitche I (2001) Causes of Downgrading in the Salmon Farming Industry. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 129-136

147. Mitsui A, Nohta H, Ohkura Y (1985) High performance liquid chromatography of plasma catecholamines using 1,2-diphenyletheylenediamine as a precolumn fluorescence derivatization reagent. Journal of Chromatography **344**: 61-70

148. Montgomery C (1999) MAFF Review of Fish and Shellfish Cultivation and Health. Trout News **28**: 27-29

149. Moon TW (2001) Glucose intolerance in teleost fish: fact or fiction? Comparative Biochemistry and Physiology **129B**: 243-249

150. Morata P, Vargas AM, Sanchez-Medina F, Garcia M, Cardenete G, Zamora S (1982) Evolution of Gluconeogenic Enzyme Activities During Starvation in Liver and Kidney of the Rainbow Trout (*Salmo gairdneri*). Comparative Biochemistry and Physiology **71B**: 65-70

151. Mørkøre T, Rørvik K-A (2001) Seasonal variations in growth, feed utilization and product quality of farmed Atlantic salmon (*Salmo salar*) transferred to seawater as 0+ or 1+ smolts. Aquaculture **199**: 145-157

152. Morris PC (2001) The effects of nutrition on the composition of farmed fish. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 161-179

153. Morton DJ, Weidemann JF, Clarke FM (1988) Enzyme Binding in Muscle. In: Lawrie R (Ed) Developments Series. Elsevier Science, Barking, Developments in Meat Science, vol 4, pp 37-61

154. Nakano T, Tomlinson N (1967) Catecholamine and Carbohydrate Concentrations in Rainbow Trout (*Salmo gairdneri*) in Relation to Physical Disturbance. Journal of the Fisheries Research Board of Canada **24**: 1701-1715

155. Nakayama T (1996) Delay in *Rigor Mortis* of Red Sea-bream by Spinal Cord Destruction. Fisheries Science **62**: 478-482

156. Nakayama T, Goto E, Ooi A (1996) Observation of Characteristic Muscle Structure Related to Delay in Red Sea-Bream *Rigor Mortis* by Spinal Cord Destruction. Fisheries Science **62**: 977-984

157. Nakayama T, Liu DJ, Ooi A (1992) Tension Change of Stressed and Unstressed Carp Muscles in Isometric Rigor Contraction and Resolution. Nippon Suisan Gakkaishi **58**: 1517-1522

158. Neumann E, Kakorin S, Tnsing K (1999) Fundamentals of electroporative delivery of drugs and genes. Bioelectrochemistry **48**: 3-16

159. Nickell DC, Springate JRC (2001) Pigmentation of Farmed Salmonids. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 58-75

160. Oehlenschläger J (1992) Evaluation of some well established and some underrated indices for the determination of freshness and/or spoilage of ice stored wet fish. In: Huss HH (Ed) Quality Assurance in the fish Industry. Elsevier Science, pp 339-350

161. Offer G, Knight P (1988) The structural basis of Water-holding of Meat I: General Principles and Water Uptake in meat processing. In: Lawrie R (Ed) Developments Series. Elsevier Applied Science, Barking, Developments in Meat Science, vol 4, pp 63-172

162. Offer G, Knight P (1988) The structural basis of Water-holding of Meat II: Drip losses. In: Lawrie R (Ed) Developments Series. Elsevier Applied Science, Barking, Developments in Meat Science, vol 4, pp 173-243

163. Ostenfeld T, Thomsen S, Ingólfdóttir S, Rønsholdt B, McLean E (1995) Evaluation of the Effect of Live Haulage on Metabolites and Fillet Texture of Rainbow Trout (*Oncorhynchus mykiss*). Water Science and Technology **31**: 233-237

164. Overmier JB, Papini MR (1986) Factors modulating the effects of teleost telencephalon ablation on retention, relearning, and extinction of instrumental avoidance behaviour. Behavioral Neuroscience **100**: 190-199 Abstract only

165. Parra J, Pette D (1995) Effects of low-frequency stimulation on soluble and structure-bound activites of hexokinase and phosphofructokinase in rat fast-twitch muscle. Biochimica et Biophysica Acta **1251**: 154-160

166. Pearson MP, Stevens ED (1991) Size and haematological impact of the splenic erythrocyte reservoir in rainbow trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry **9**: 39-50

167. Perry SF, Bernier NJ (1999) The acute humoral adrenergic stress response in fish: facts and fiction. Aquaculture **177**: 285-295

168. Perry SF, Reid SG, Salama A (1996) The Effects of Repeated Physical Stress on the ß-Adrenergic Response of the rainbow trout red blood cell. The Journal of Experimental Biology **199**: 549-562

169. Pitcher TJ, Hart PJB (1982) Fisheries Ecology, 1st Edn. Croom Helm Ltd., London

170. Porter PJ, Kennish JM, Kramer DE (1992) The effects of exsanguination of sockeye salmon on the changes in lipid composition during frozen storage. In: Bligh EG (Ed) Seafood Science and Technology. Fishing News Books, Oxford, pp 76-83

171. Pottinger TG (2001) Effects of Husbandry Stress on Flesh Quality Indicators in Fish. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 145-160

172. Powell MD, Perry SF (1996) Respiratory and acid-base disturbances in rainbow trout (*Oncorhynchus mykiss*) blood during exposure to chloramine T, paratoluenesulphonamide, and hypochlorite. Canadian Journal of Fisheries and Aquatic Science **53**: 701-708

173. Price D (2000) The Multiple Retailers Perspective. In: Future Strategies for the English Farmed Trout Industry, UK. MAFF, pp 4

174. Proctor MRM, Dorgan M, McLoughlin JV (1992) The Concentrations of Adenosine Triphosphate, Creatine phosphate, Glucose-6-Phosphate, Lactate, and Glycogen in Skeletal Muscle of Marine and Freshwater Fish Species Anaesthetised with MS222. Proceedings of the Royal Irish Academy **92B**: 45-51

175. Proctor MRM, McLoughlin JV (1992) The Effects of Anaesthesia and Electrical Stunning on Chemical Changes in the myotomal Muscle of *Salmo salar Post-Mortem*. Proceedings of the Royal Irish Academy **92B**: 53-59

176. Raaij van MTM, Pit DSS, Balm PHM, Steffens AB, Thillart van den GEEJM (1996) Behavioral Strategy and the Physiological Stress Response in Rainbow Trout Exposed to Severe Hypoxia. Hormones and Behaviour **30**: 85-92

177. Raggi MA, Sabbioni C, Casamenti G, Gerra G, Calongi N, Masotti L (1999) Determination of catecholamines in human plasma by high-performance liquid chromatography with electrochemical detection. Journal of Chromatography **730**: 201-211

178. Rasekh J, Kramer A, Finch R (1970) Objective evaluation of canned tuna sensory quality. Journal of Food Science **35**: 417-423

179. Regost C, Arzel J, Cardinal M, Laroche M, Kaushik SJ (2001) Fat deposition and flesh quality in sea reared, triploid brown trout (*Salmo trutta*) as affected by dietary fat levels and starvation. Aquaculture **193**: 325-345

180. Reinert RE, Hohreiter DW (1984) Adenlyate Energy Charge as a Measure of Stress in Fish. In: Cairns VW, Hodson PV and Nriagu JO (Eds) Contaminant Effects on Fishes. John Wiley, London, pp 151-161

181. Robb D, Warriss P (1997) How Killing Methods Affect Salmonid Quality. Fish Farmer **November/December**: 48-49

182. Robb DH (1998) Some factors affecting the Flesh Quality of Salmonids. PhD Thesis, University of Bristol, Bristol.

183. Robb DHF (2001) Measurement of Fish Flesh Colour. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 298-306

184. Robb DHF (2001) The relationship between Killing Method and Quality. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 220-233

185. Robb DHF, Callaghan MO, Lines JA, Kestin SC (2002) Electrical stunning of rainbow trout (*Oncorhynchus mykiss*): factors that affect stun duration. Aquaculture **205**: 359-371

186. Robb DHF, Kestin SC, Warriss PD (2000) Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. Aquaculture **182**: 261-269

187. Robb DHF, Wotton SB, McKinstry JL, Sørensen NK, Kestin SC (2000) Commercial slaughter methods used on Atlantic salmon: determination of the onset of brain failure by electroencephalography. Veterinary Record **147**: 298-303

188. Rodríguez CJ, Besteiro I, Pascual C (1999) Biochemical changes in freshwater rainbow trout (*Oncorhynchus mykiss*) during chilled storage. Journal of the Science of Food and Agriculture **79**: 1473-1480

189. Rørå AMB, Mørkøre T, Einen O (2001) Primary Processing (Evisceration and Filleting). In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 249-260

190. Rose JD (2002) The Neurobehavioral Nature of Fishes and the Question of Awareness and Pain. Reviews in Fisheries Science **10**: 1-38

191. Ross LG, Ross B (1999) Anaesthetic and Sedative Techniques for Aquatic Animals, 2nd Edn. Blackwell Science, Oxford

192. Roth B, Moeller D (1999) Use of electronarcosis in Aquaculture. In: Aquaculture Europe '99 Towards predictable Aquaculture, Trondheim, Norway. European Aquaculture Society, pp 203-204

193. Roth B, Torrissen OJ, Slinde E (2001) The Effect of Slaughtering Procedures on Bleeding in Atlantic salmon (*Salmo salar*) and Rainbow trout (*Oncorhynchus mykiss*). In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 405-406 Poster Presentation

194. Ruane NM, Huisman EA, Komen J (2001) Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology **59**: 1-12 Abstract only

195. Ruane NM, Huisman EA, Komen J (2002) The influence of feeding history on the acute stress response of common carp (*Cyprinus carpio*). Aquaculture **210**: 245-257

196. Rustad T (1992) Muscle chemistry and the quality of wild and farmed cod. In: Huss HH (Ed) Quality Assurance in the Fish Industry. Elsevier Science, pp 19-27

197. Rutkove SB (2001) Effects of temperature on neuromuscular electrophysiology. Muscle & Nerve **24**: 867-882

198. Ryder JM (1985) Determination of Adenosine Triphosphate and Its Breakdown Products in Fish Muscle by High-Performance Liquid Chromatography. Journal of Agriculture and Food Chemistry **33**: 678-680

199. Sadler J, Pankhurst PM, King HR (2001) High prevalence of skeletal deformity and reduced gill surface area in triploid Atlantic salmon (*Salmo salar* L.). Aquaculture **198**: 369-386

200. Sadler J, Wells RMG, Pankhurst PM, Pankhurst NW (2000) Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. Aquaculture **184**: 349-361

201. Saito T, Arai K, Matsuyoshi M (1959) A New Method for Estimating the Freshness of Fish. Bulletin of the Japanese Society of Scientific Fisheries **24**: 749-750

202. Schiedt K, Foss P, Storebakken T, Liaaen-Jensen S (1989) Metabolism of carotenoids in Salmonids-I. Idoxanthin, a metabolite of astaxanthin in the flesh of Atlantic salmon (*Salmo salar*, L.) under varying external conditions. Comparative Biochemistry and Physiology **92B**: 277-281

203. Schiedt K, Leuenberger FJ, Vecchi M, Glinz E (1985) Absorption, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. Pure and Applied Chemistry **57**: 685-692

204. Schmidt GR, Cassens RG, Briskey EJ (1968) Development of an Isotonic-Isometric Rigorometer. Journal of Food Science **33**: 239-241

205. Schreck CB, Whaley RA, Bass ML, Maughan OE, Solazzi M (1976) Physiological Responses of Rainbow Trout (*Salmo gairdneri*) to Electroshock. Journal of the Fisheries Research Board of Canada **33**: 76-84

206. Shearer KD (1994) Factors affecting the proximate composition of cultured fishes with emphasis on salmonids. Aquaculture **119**: 63-88

207. Shepherd J, Bromage N (1988) Intensive Fish Farming, 1st Edn. Blackwell Scientific, Oxford

208. Sheridan MA, Mommsen TP (1991) Effects of Nutritional State on *in vivo* Lipid and Carbohydrate Metabolism of Coho Salmon, *Oncorhynchus kisutch*. General and Comparative Endocrinology **81**: 473-483

209. Sherwood L (1993) Human Physiology: From Cells to Systems, 2nd Edn. West Publishing Company, Minneapolis

210. Sigholt T, Erikson U, Rustad T, Johansen S (1995) The effect of immobilization with blow to the head, CO_2 or chilled water on High energy phosphates on *rigor mortis* in Atlantic salmon (*Salmo salar*). In: Aquaculture Europe '95, Trondheim, Norway. European Aquaculture Society, pp 403-404

211. Sigholt T, Erikson U, Rustad T, Johansen S, Nordtvedt TS, Seland A (1997) Handling Stress and Storage Temperature Affect Meat Quality of Farmed-raised Atlantic Salmon (*Salmo salar*). Journal of Food Science **62**: 898-905

212. Sigurgisladottir S, Hafsteinsson H, Jonsson A, Lie Ø, Nortvedt R, Thomassen M, Torrissen O (1999) Textural Properties of Raw Salmon Fillets as Related to Sampling Method. Journal of Food Science **64**: 99-104

213. Sinnott R (2000) Aspects of Carcass Quality and its monitoring in Rainbow Trout. Trout News **29**: 16-21

214. Sinnott R (2001) Carcass Quality Monitoring at the Farm and Factory. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 318-334

215. Sinnott R (2001) New Pigmentation Recommendations for Farmed Rainbow Trout. Trout News **32**: 18-19

216. Skjervold PO, Fjæra SO, Østby PB (1999) Rigor in Atlantic salmon as affected by crowding stress prior to chilling before slaughter. Aquaculture **175**: 93-101

217. Skjervold PO, Fjæra SO, Østby PB, Einen O (2001) Live chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture **192**: 265-280

218. Skrede G, Storebakken T, Næs T (1989) Color Evaluation in Raw, Baked and Smoked Flesh of Rainbow Trout (*Oncorhynchus mykiss*) Fed Astaxanthin or Canthaxanthin. Journal of Food Science **55**: 1574-1578

219. Smith MAK (1981) Estimation of growth potential by measurement of tissue protein synthetic rates in feeding and fasting rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Biology **19**: 213-220

220. Soengas JL, Strong EF, Fuentes J, Veira JAR, Andrés MD (1996) Food deprivation and refeeding in Atlantic salmon, *Salmo salar*: effects on brain and liver carbohydrate and ketone bodies metabolism. Fish Physiology and Biochemistry **15**: 491-511

221. Solomon MB (1987) Comparison of Methods Used for Measuring pH in Muscle Tissue. Journal of Food Science **52**: 1428-1429

222. Solvio A, Nyholm K, Huhti M (1977) Effects of anaesthesia with MS222, neutralised MS222 and benzocaine on the blood constituents of rainbow trout, *Salmo gairdneri*. Journal of Fish Biology **10**: 91-101

223. Sørensen NK, Brataas R, Nyvold TE, Lauritzen K (1997) Influence of Early Processing (Pre-Rigor) on Fish Quality. In: Luten JB, Børresen T and Oehlenschläger J (Eds) Seafood from Producer to Consumer, Integrated Approach to Quality. Elsevier Science B.V., pp 253-263

224. Sørensen NK, Tobbiassen T, Joensen S, Midling K, Akse L (2001) A Mechanical Compression Test to Assess Development of *Rigor Mortis* in Fish; Atlantic Salmon, Atlantic Halibut and Plaice. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 409 Poster Presentation

225. Spedding C (1996) Report on the Welfare of Farmed Fish. Farm Animal Welfare Council, Surbiton, pp 51

226. Stagg RM, Allan CET (2000) Scottish Fish Farms Annual Production Survey 1999. SERAD Marine Laboratory, Aberdeen, pp 30

227. Stanley DW (1991) Biological Membrane Deterioration and Associated Quality Losses in Food Tissues. Critical Reviews in Food Science and Nutrition **30**: 487-553

228. Storebakken T, No HK (1992) Pigmentation in Trout. Aquaculture **100**: 209-229

229. Struksnaes G, Talbot C, Roem A, Berg-Lea T (1995) Improving Carcass Quality of Atlantic Salmon. In: Quality in Aquaculture, Trondheim, Norway. European Aquaculture Society, pp 188

230. Stryer L (1988) Biochemistry, 3rd Edn. Freeman, New York

231. Sumpter JP, Dye HM, Benfey TJ (1986) The Effects of Stress on Plasma ACTH, a-MSH, and Cortisol levels in Salmonid Fishes. General and Comparative Endocrinology **62**: 377-385

232. Szebedinszky C, Gilmour KM (2002) The buffering power of plasma in brown bullhead (*Ameiurus nebulosus*). Comparative Biochemistry and Physiology **131B**: 171-183

233. Tachibana K, Doi T, Tsuchimoto M, Misima T, Ogura M, Matsukiyo K, Yasuda M (1988) The effect of swimming exercise on flesh texture of cultured red sea-bream. Nippon Suisan Gakkaishi **54**: 677-681

234. Takahashi K (1996) Structural weakening of skeletal muscle tissue during *post-mortem* ageing of meat: the non-enzymatic mechanism of meat tenderization. Meat Science **43**: S67-S80

235. Tave D (1993) Genetics for Fish Hatchery Managers, 2nd Edn. Kluwer Academic Publishers, Boston

236. Teissié J, Eynard N, Gabriel B, Rols MP (1999) Electropermeabilization of cell membranes. Advanced Drug Delivery Reviews **35**: 3-19

237. Tetens V, Lykkeboe G, Christensen NJ (1988) Potency of Adrenaline and Noradrenaline for ß- Adrenergic proton extrusion from red blood cells of Rainbow Trout, *Salmo gairdneri*. Journal of Experimental Biology **134**: 267-280

238. Thomas PM, Pankhurst NW, Bremner HA (1999) The effect of stress and exercise on *post-mortem* biochemistry of Atlantic salmon and rainbow trout. Journal of Fish Biology **54**: 1177-1196

239. Torrissen OJ, Sigurgisladottir D, Slind E (2001) Texture and Technological Properties of fish. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 412 Poster presentation

240. Torrissen OJ, Sigurgisladottir S, Slinde E (2001) Texture and Technological Properties of Fish. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 42-57

241. Tretsven WI, Patten BG (1981) Effect of Arterial Incisions on the Amount of Bleeding and Flesh Quality of Rainbow Trout. Marine Fisheries Review **43**: 16-18

242. Trout GR (1988) Techniques for Measuring Water-Binding Capacity in Muscle Foods-A Review of Methodology. Meat Science **23**: 235-252

243. Tucker CS (2000) Off -flavour problems in aquaculture. Reviews in Fisheries Science **8**: 45-88 Abstract only

244. Turner JD, Wood CM, Clark D (1983) Lactate and Proton Dynamics in the Rainbow Trout (*Salmo gairdneri*). Journal of Experimental Biology **104**: 247-268

245. Vallé M, Malle P, Bouquelet S (1998) Evaluation of fish decomposition by liquid chromatographic Assay of ATP degradation products. Journal of the Association of Analytical Chemists **81**: 571-575

246. Vazzana M, Cammarata M, Cooper EL, Parrinello N (2002) Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. Aquaculture **210**: 231-243

247. Veland JO, Torrissen OJ (1999) The texture of Atlantic salmon (*Salmo salar*) muscle as measured instrumentally using TPA and Warner-Bratzler shear test. Journal of the Science of Food and Agriculture **79**: 1737-1746

248. Velizarov S, Reitz M, Glück B, Berg H (1998) Electropermeabilization and electrofusion of human cells modified by anaesthetic agents. Bioelectrochemistry and Bioenergetics **47**: 89-96

249. Vijayan MM, Maule AG, Schreck CB, Moon TW (1993) Hormonal Control of Hepatic Glycogen Metabolism in Food-deprived, Continuously Swimming Coho Salmon (*Oncorhynchus kisutch*). Canadian Journal of Fisheries and Aquatic Sciences **50**: 1676-1682

250. Vijayan MM, Moon TW (1992) Acute handling stress alters hepatic Glycogen Metabolism in food deprived rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Science **49**: 2260-2266

251. Waarde van A, Thillart van den G, Erkelens C, Addink A, Lugtenburg J (1990) Functional Coupling of Glycolysis and Phosphocreatine Utilization in anoxic Fish Muscle. Journal of Biological Chemistry **265**: 914-923

252. Wall AE, Richards RH (1992) Occurrence of cataracts in triploid Atlantic salmon (*Salmo salar*) on four farms in Scotland. The Veterinary Record **131**: 553-557 Abstract only

253. Wall AJ (2001) Ethical Considerations in the handling and Slaughter of Farmed Fish. In: Kestin SC and Warriss PD (Eds) Farmed Fish Quality. Fishing News Books, Oxford, pp 108-115

254. Wang D, Tang J, Correia LR, Gill TA (1998) *Post mortem* Changes of Cultivated Atlantic Salmon and Their Effects on Salt Uptake. Journal of Food Science **63**: 634-637

255. Wang Y, Heigenhauser GJF, Wood CM (1994) Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: Acid-base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. Journal of Experimental Biology **195**: 227-258

256. Wang Y, Wilkie MP, Heigenhauser GJF, Wood CM (1994) The analysis of metabolites in rainbow trout white muscle: a comparison of different sampling and processing methods. Journal of Fish Biology **45**: 855-873

257. Warriss PD (1984) Exsanguination of animals at slaughter and the residual blood content of meat. The Veterinary Record **115**: 292-295

258. Warriss PD, Robb D (1997) Methods of Handling and Killing are Crucial to Quality. Fish Farmer **May/June**: 12-13

259. Watabe S, Kamal M, Hashimoto K (1991) *Postmortem* Changes in ATP, Creatine Phosphate, and Lactate in Sardine Muscle. Journal of Food Science **56**: 151-153+171

260. Wathne E, Bjerkeng B, Storebakken T, Vassvik V, Odland AB (1998) Pigmentation of Atlantic salmon (*Salmo salar*) fed astaxanthin in all meals or in alternating meals. Aquaculture **159**: 217-231

261. Weaver JC, Chizmadzhev YA (1996) Theory of electroporation: A review. Bioelectrochemistry and Bioenergetics **41**: 135-160

262. Wedemeyer G (1970) Stress of Anaesthesia with MS222 and Benzocaine in Rainbow Trout (*Salmo gairdeneri*). Journal of the Research Board of Canada **27**: 909-914

263. Wedemeyer G (1996) Physiology of fish in intensive culture systems, 1st Edn. Chapman and Hall, New York

264. Weerd JHV, Komen J (1998) The effects of chronic stress on growth in fish: A critical appraisal. Comparative Biochemistry and Physiology **120A**: 107-112

265. Whitting RC, Montgomery MW, Anglemeir AF (1975) Stability of rainbow trout (*Salmo gairdneri*) muscle lysosomes and their relationship to *rigor mortis*. Journal of Food Science **40**: 854-857

266. Wieser W, Krumschnabel G (2001) Hierarchies of ATP- consuming processes: direct compared with indirect measurements and comparative aspects. The Biochemical Journal **355**: 389-395 Abstract only

267. Wilkie MP, Brobbel MA, Davidson K, Forsyth L, Tufts BL (1997) Influences of temperature upon the postexercise physiology of Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences **54**: 503-511

268. Wills CC (1999) Fish skeletal muscle: adenosine triphosphate and adenine nucleotide metabolites in relation to the texture and quality of fish PhD Thesis, Trinity College. University of Dublin, Dublin, Abstract only.

269. Wright PA, Perry SF, Moon TW (1989) Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. Journal of Experimental Biology **147**: 169-188

270. Yoshikawa H, Kawai F, Kanamori M (1994) The Relationship between the EEG and brain pH in carp, *Cyprinus carpio*, subjected to environmental hypercapnia at an anaesthetic level. Comparative Biochemistry and Physiology **107A**: 307-312

271. Zayan R (1986) Assessment of pain in animals: Epistemological comments. In: Duncan IJH and Molony V (Eds) Assessing Pain in Farm Animals. Commission of the European Communities, Roslin, Scotland, pp 1-15

272. Zhou S, Ackman RG, Morrison C (1996) Adipocytes and lipid distribution in the muscle tissue of Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Science **53**: 326-332

273. Zimmerman M (1986) Behavioural Investigations of pain in Animals. In: Duncan IJH and Molony V (Eds) Assessing Pain in Farm Animals. Commission of the European Communities, Roslin, Scotland, pp 16-29

APPENDIX 1

HOME OFFICE

Animals (Scientific Procedures) Inspectorate
PO Box 6779, Dundee DD1 9WN
Switchboard: 01382 223189 Fax: 01382 221571

Our reference:

Your reference:

Date: 23 August, 1999

Dr N. Emmison
School of Applied Sciences
Robert Gordon University
St Andrews Street
Aberdeen
AB25 1HG

Dear Neil

Following our conversations recently it has come to my attention that under section 2(7) of the Animals (Scientific Procedures) Act that the killing of a protected animal is only regulated if all three of the following conditions are met:

The animal is killed for scientific purpose; and

The place where itt is killed is designated under the 1986 Act as a scientific procedure establishment; and

The method employed is not one appropriate to the animal under schedule 1 to the Act. (Schedule 1 lists humane killing methods that can be applied without any Home Office Licence authority.)

Thus, as it is proposed that you study the effects of different killing methods on stress levels and flesh quality in fish at a non-designated site, and that no regulated procedure will occur before these methods of killing are used, this work does not fall under the Act and therefore cannot be regulated.

I wish you every success in the project and would welcome information on the outcomes of the study. I understand that some similar work has previously been commissioned by MAFF (and published) which it might be worth trying to view if you have not already seen it.

Yours Sincerely

Dr Kathryn ~~Ryder~~ MB, BS, BSc, MRCP, DPhil.
Animals (Scientific Procedures) Inspector

APPENDIX 2

Figures 1-11, photography by Peter Reid.

FREEZE CLAMPING TECHNIQUE

Figure 1
Immediately the fish had been killed or stunned it was filleted to expose the white muscle.

Figure 2
An 8 mm biopsy punch was used to cut the muscle samples.

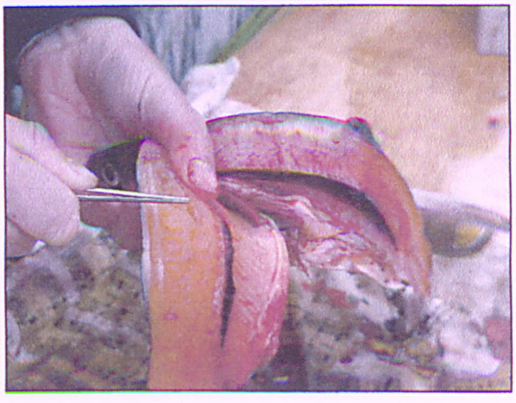


Figure 3
The samples were taken from the epiaxial muscle, avoiding the dorsal fat depot, pin bones, underlying red muscle and skin.

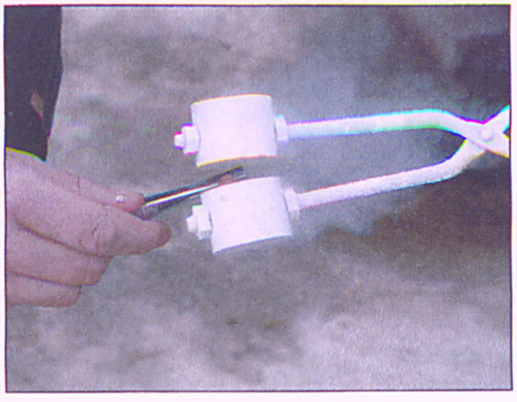


Figure 4
Single samples were transferred to the aluminium tongs, which had been pre-cooled in liquid nitrogen. Samples were simultaneously compressed and frozen.

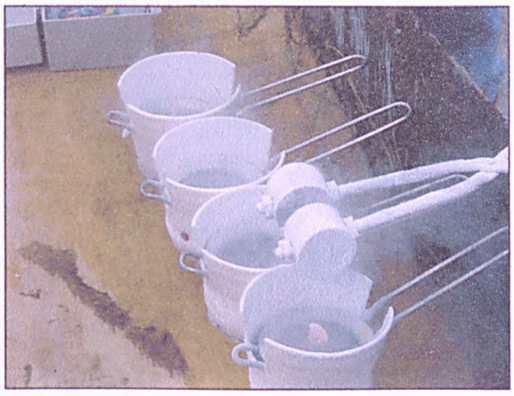


Figure 5
Frozen samples were dropped into liquid nitrogen so that they remained frozen while the other samples were taken.

Figure 6
When all the samples from a single fish had been collected they were wrapped in labelled aluminium foil.

Figure 7
The samples were transported and stored until analysed under vapour phase liquid nitrogen in a 'dry shipper'.

Figure 8
The muscle samples were frozen within in 2.5 minutes of 'death' to preserve the unstable metabolites.

BLOOD SAMPLING TECHNIQUE

Figure 9
The needle was inserted at an angle of 45° through the ventral surface of the caudal peduncle, so that the blood could be taken from the caudal vein.

RIGOR MORTIS MEASUREMENT

Figure 10
Rigor mortis was assessed by measuring the 'angle of droop', i.e. the angle the tail half of the fish made while hanging over a horizontal surface.

EXPERIMENTAL FACILITIES

Figure 11 Trout sampling facilities
The experimental animals were held in a 2m-fibreglass tank with a constant flow of river water. The fish were netted out individually.

Figure 12 Salmon pilot study sampling site
The salmon were reared in a cage in a seawater loch. A seine net was used to gather the fish to the edge of the cage the floatline of which is visible on the right hand side of the cage.

Figure 13 Commercial salmon harvesting: fish delivery system
The salmon were brought to the processing plant in a well-boat and piped into the killing station.

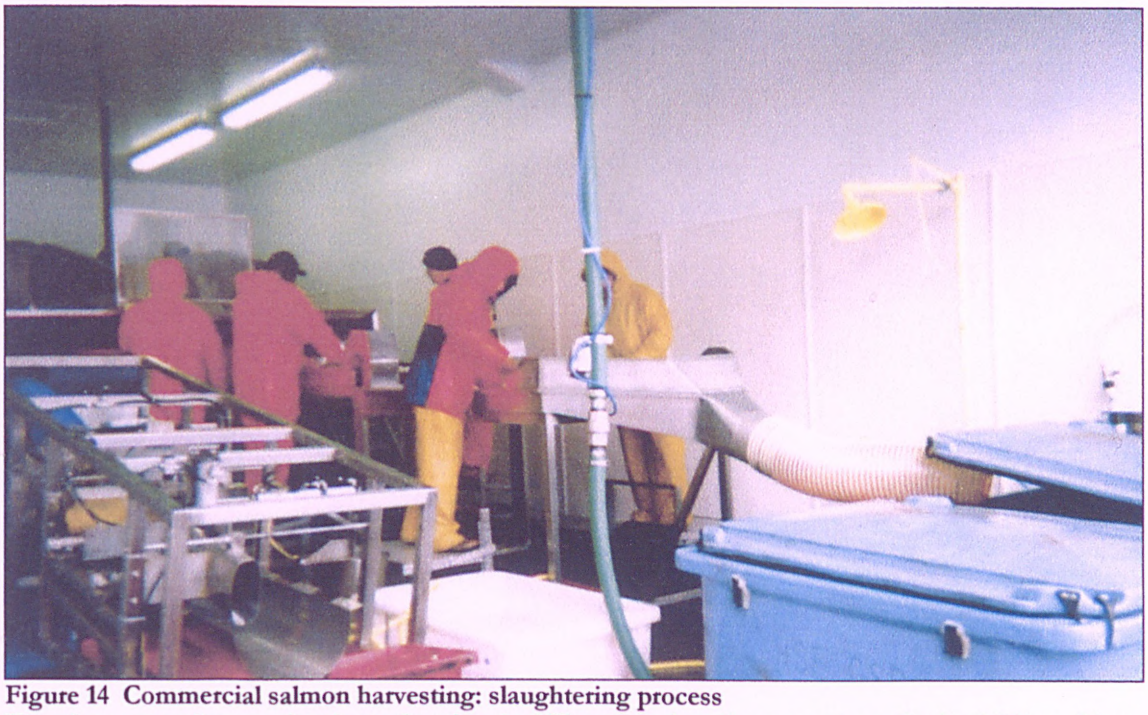


Figure 14 Commercial salmon harvesting: slaughtering process
The salmon were dewatered and transferred to the killing area where they were stunned using MT4 machine (Seafood Innovations Ltd), gill arches were cut manually and the fish left to die in slurry ice.