THE BIOENERGETICS AND BEHAVIOUR OF THE RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* WHEN FEEDING ON A COPPER CONTAMINATED DIET
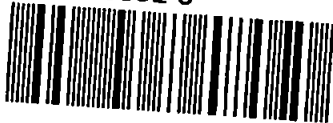
by

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ABSTRACT

The bioenergetics and behaviour of rainbow trout *(Oncorhynchus mykiss)* when feeding on a copper contaminated diet.

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The long-term effects of sub-lethal dietary Cu-exposure were investigated in relation to consequential energy shifts and resultant locomotory adaptation in *O. mykiss*. This study represents the first to attempt to quantify the physiological cost of dietary heavy-metal exposure, the timing and extent of daily behavioural adaptation and resultant significance, in terms of ecologically important behaviours to the fish. Two long-term (2 & 3 months) dietary exposures of 730 mg Cu kg^{-1} mg d.w. feed showed exposed fish to have a 3-fold increase in liver [Cu] and 10-fold increase in intestinal [Cu] compared to control fish. Consequently exposed fish elicited a detoxification response, and metallothionein production was also greatly increased in these tissues. Cu-uptake was regulated into the body and physiological homeostasis was maintained although a 2-fold increase in lipid peroxidation product was found in the liver of exposed fish. Simultaneous analysis of voluntary spontaneous swimming and $\dot{V}O_2$, showed exposed fish to have a 1.52 mmol O_2 kg^{-1} h^{-1} increase over controls, and it was also shown that the cost of routine metabolism became more critical for exposed fish at higher swimming speeds. Although, results further showed that the increase in both standard metabolism and routine metabolism over controls by Cu-exposed fish varied greatly between individuals. The increased energetic requirement to remain active when feeding on a Cu-contaminated diet was off-set by a reduction in swimming activity. Growth rates remained indifferent between treatments suggesting that the reduction in activity fully compensated for the increased cost of standard, and routine metabolism in exposed fish. Finite behavioural analyses over the 24-h cycle showed control fish to display periodicity in specific swimming speed, peaking in activity during the night-dawn period. Cu-exposed fish exhibited a different circadian behavioural profile, lacking distinct periodicity in specific swimming speed favouring low-level activity during the night-dawn period, and investing in higher cost swimming activity only during feeding periods. It was suggested that the high swimming activity of control fish during non-feeding periods was associated with inter-individual competition and development of the feeding hierarchy. Direct observation of trout social groups, showed a 50% reduction in the activity of the alpha fish leading to a similarly sized reduction in encounters with subordinates when the group was feeding on a Cu-contaminated diet. Consequently, a general reduction was observed in the strength of the feeding hierarchy, measured as a reduction in the size disparity between individuals within a discrete social group, and a reduction in social stress effects on subordinate fish estimated from lactate accumulation within the muscle. Feeding hierarchies are a result of initial paired interactions, and direct behavioural examination showed that fish feeding on a Cu-contaminated diet were less willing to compete in expensive escalated interactions, favouring withdrawal at a lower level of energetic investment. This lower withdrawal threshold in Cu-exposed fish, was suggested to be related to lower self-assessment of Resource Holding Power (RHP), due to the increased metabolic costs of routine metabolism in Cu-exposed fish, and a reduced resource value of a contaminated diet. The results of this investigation are discussed with regard to energetic decisions undertaken by the fish, associated with the relative costs and benefits of investing energy into growth or activity, to maximise net energy intake (food) when feeding in a social group. And how evaluation of strategies to maximise net energy gain may become more critical in rainbow trout when feeding on a Cu-contaminated diet

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AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Date.....19/10/2001........

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"It is literally the best of times and the worst of times. There has never been a more exciting time to be alive. When we are beginning to actually read the book of life itself, and we have the potential to apply that understanding for good stewardship and husbandry of this marvellous world that we are heir to. Or we can just thoughtlessly bend it to create more bits of garbage to amuse ourselves. Our activities are creating conditions that are driving the sixth great wave of extinction. The wave on who's breaking tip we stand."

Sir Robert May, State of the Planet

"Watch where the Huskies go
And don't eat the yellow snow."

Frank Zappa

CHAPTER ONE

GENERAL INTRODUCTION TO BIOENERGETICS
AND CU-TOXICOLOGY

"At the most fundamental of biological thinking an organism may be regarded as an entity which takes up energy, primarily in the form of nutrients from its environment and ultimately converts this energy into progeny. The law of natural selection asserts that those organisms which are most efficient in this process are the ones most likely to survive." (Townsend and Calow, 1981).

1.1 Introduction

Elevated levels of contaminants within the aquatic environment, can become deleterious to fish health and physiology (Sprague, 1971). The fish may invest energy into the detoxification and regulation of these contaminants, and it may be expected that increased expenditure into these compensatory mechanisms will ultimately detract energies from processes necessary for increased fitness success.

1.1.1   General principles of bioenergetics

Lotka (1924) first suggested the essential concept of a 'law of maximum energy limitations for biological systems'. This shaped the principle of biological energetics emphasising that the basic source of energetic fuel is food, and all activities necessary for survival must be allocated from this finite resource. The study of bioenergetics involves the examination of energy gains, losses and transfers within the whole organism, and may be expressed in the form:

$$R = F + U + M + P \quad \text{(Winberg, 1956)}$$

Where: R = energy gained as food

F = energy lost as faeces

U = energy lost as nitrogenous excretory products

M = energy expended in a range of bodily functions - Metabolism

P = energy storage or growth

Winberg (1956) further subdivides M into:

M_M = minimal costs for maintaining bodily function

M_A = minimal costs associated with activity

M_F = minimal costs related to digestion, absorption and processing of food.

And P into:

P_S = somatic (body) growth

P_R = production of gametes

A major advantage of the bioenergetics model is that all components of the energy budget must balance. Therefore, if one component is too difficult or expensive to measure directly, it can be estimated from easily obtained measurements or approximations of other components (Rice, 1990). The energy metabolism of fish has been extensively investigated within the past four decades and there exist many excellent reviews (Fry, 1971; Beamish, 1978; Brett and Groves, 1979; Calow, 1985; Jobling, 1985, 1994; Lucas, 1996), presenting a large body of data on how endogenous and exogenous factors may influence fish growth and reproduction. Such insights are used within the aquaculture industry to provide valuable predictions of ability to minimize costs and maximise production (Ross et al., 1988c: Jobling, 1994; Lucas, 1996), and may also aid in the development of effective fisheries management strategies through understanding patterns of energy flow that can regulate and limit populations (Li and Brocksen, 1977; Mortensen, 1977; Kooi et al., 1998; Carlisle, 2000).

Within the energetics budget there is a fundamental relationship between growth/production (P) and metabolism/respiration (M) (for review see Jobling, 1985), with

metabolism having preferential allocation of energy and thus determining accordingly the amount of energy available for investment in P (Calow, 1985; Priede, 1985). Energy losses due to metabolic demands are usually a large component of the bioenergetic budget of a fish and the costs of metabolism are partitioned into the components; standard metabolism (M_M), metabolic costs of activity (M_A), digestion and assimilation of food (M_F), (Jobling, 1994). The relationship between oxygen consumption and metabolism, relating to activity, are shown in Brett's (1964) model (fig. 1). The model shows that even at zero activity, there is still be a subsistence energy cost (M_M), due to the minimum metabolic rate accompanying the cost of maintenance, relating to the minimum function of circulation, respiration, excretion and muscle tone (Brett, 1962). Oxygen consumption increases exponentially with activity (M_A), and routine activity has been used to express the average oxygen consumption of fish, which are undergoing continuous oxygen recording during free activity. The maximum metabolic limit will be established by the amount of oxygen the fish can absorb across its gills from the water, and derive energy from food, via the aerobic conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) within mitochondria (Eckert and Randall, 1983). At this level of activity the fish must assimilate anaerobic energy reserves and oxygen debt will occur, it is said that at this level of activity the fish has reached its U_{crit}. The difference between the standard and maximum level of aerobic metabolism is considered to represent a measure of the amount of aerobic energy available to the fish under more or less natural conditions (Weiser, 1985).

Previous studies to determine standard metabolism have induced fish to swim against currents of known velocity in flow-through respirometers from which the metabolic cost of swimming and, by back extrapolation to zero velocity the cost of standard metabolism are calculated (Brett, 1964; Fry 1971). It has been realised that linear forward motion at constant velocity comprises only a fraction of the spontaneous activity of fish in nature, and analysis of

Figure 1. Brett's (1964) model showing the general relationship between oxygen consumption and swimming speed, for an 18 cm, 50g yearling sockeye performing in air saturated fresh water at 15^{0}C.

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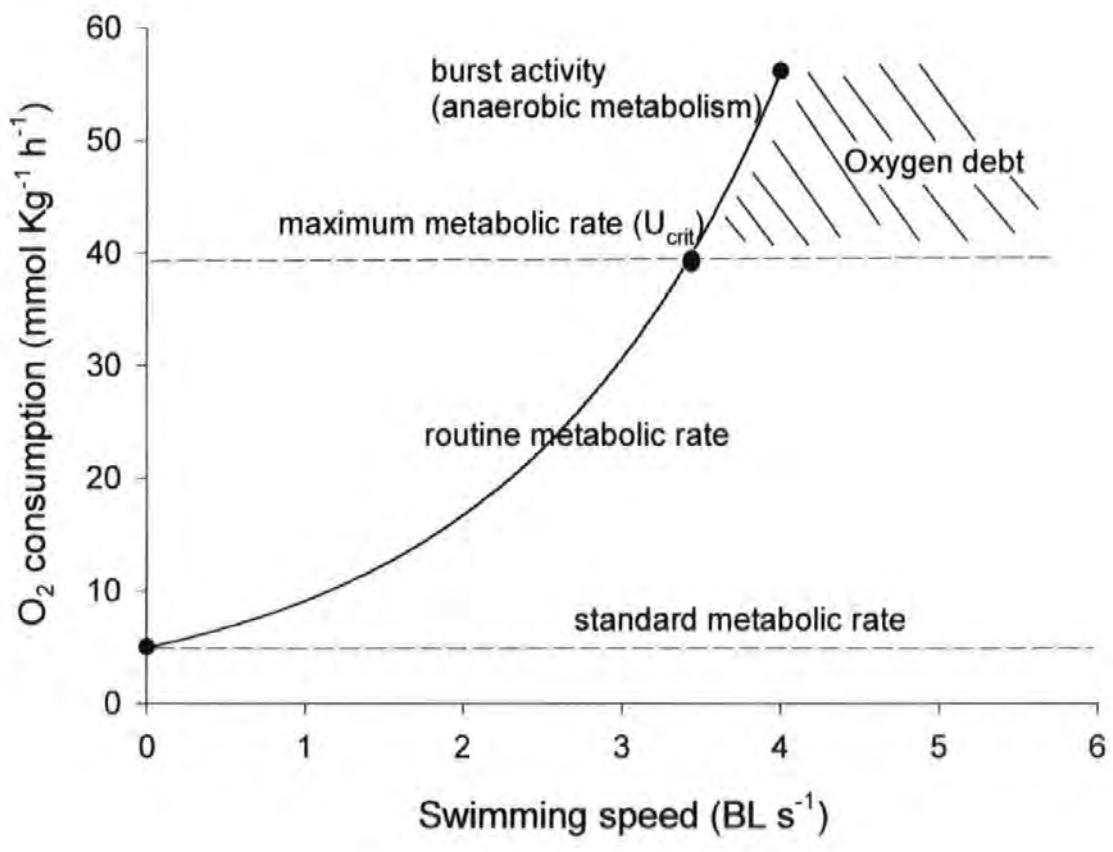
more realistic patterns of swimming may allow increased accuracy of bioenergetic models (Forstner and Weiser, 1990). Investigations to measure the spontaneous swimming activity of fish have been carried out by means of mechanical activity recorders (Spoor, 1946), heat loss sensors (Beamish, 1964), photocells (Smit, 1965; Scarfe, 1982) and more recently computer aided tracking devices (Winberg et al., 1993). Moreover, with the development of radio and acoustic transmitters, researchers have utilised correlates such as ventilation rate (Oswald, 1978), locomotor muscle electrograms, EMGs (Rogers and Weatherley, 1983), heart rate (Priede and Young, 1977; Lucas et al., 1993) and tailbeat frequency (Ross et al., 1981) to estimate metabolic rate in free-ranging fish. Research has shown that an exponential relationship exists between the rate of oxygen consumption and the degree of activity.

Once food has been consumed an organism must process and assimilate energy derived from it. The sum of all costs involved during handling and digestion of food has been termed ' apparent specific dynamic action (SDA)' and represents the rise in metabolic rate concerned with the digestion and assimilation of food (Krebs, 1964; Beamish, 1974; Jobling, 1981). SDA can be measured by direct calorimetry (Smith et al., 1978), but in the majority of studies SDA has been measured indirectly via oxygen consumption (e.g. Beamish, 1974; Jobling and Davies, 1980; Lucas and Priede, 1992), and more recently, to enable field estimates, by heart rate telemetry (Armstrong, 1986; Lucas and Armstrong, 1991). Increases in post-prandial metabolism attributable to SDA in some fish species have been demonstrated to equal or actually exceed the level of active metabolism measured during maximum sustained swimming (Soofiani and Priede, 1985). The high oxygen demand following feeding, has been suggested to reduce the 'scope for activity' in fish by up to 30-50% (Muir and Niimi, 1972; Beamish, 1970, 1974; Vahl and Davenport, 1979).

In summary, for investment in growth or reproduction (P) the energy intake from external sources (food; R) must exceed metabolic (M) requirements. This idea of how a limited

supply of energy may be distributed amongst several competing consumers has been termed "energy partitioning" (Townsend and Calow, 1981), and allocation is based on a flexible strategy responding to both internal and external conditions (Weiser and Medgesey, 1991). The optimisation of energy is crucial to the expression of life-history, too little investment in somatic maintenance and repair and the animal may die before it can reproduce, too great an investment the organism will mature late and reproduce slowly (Calow, 1977, 1979; Kirkwood, 1981).

It has been proposed that above a certain threshold environmental change, such as temperature, pH or salinity, may impede oxygen uptake and utilisation. This will invariably affect the flow and allocation of metabolic energy within an animal (Weiser and Medgesey, 1991). If alteration in the environment causes stress to the animal, the extra investment in tissue repair or other mechanisms of stress resistance might increase survival but decrease the resources available for reproduction and growth (Kirkwood, 1987; Sibly and Calow, 1989). Consequently, environmental factors increasing stress will place the organism at a disadvantage since it will require the expenditure of excess energy, which is ultimately incompatible with survival. Correspondingly, behavioural or physiological modification to environmental change by the organism leading to a reduction in expenditure will permit an animal to accumulate any surplus of energy that may enhance fitness. Thus, the analysis of energy budgets can be used to provide valuable information on how stressful a specific environmental situation might be for a given population of animals. Fish populations bioenergetics models have been used extensively to examine the effects of a wide range of environmental variables on stress. These may include direct energy sinks such as parasites (*e.g.* Kitchell and Breck, 1980), or indirect changes in behavioural repertoire owing to alterations in current velocity (Godin and Rongeley, 1989), environmental temperature (Bennett, 1979) and food availability (Boisclair, 1992). More recently due to the realisation of the deleterious

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effects of pollutants from anthropogenic sources into the aquatic environment, bioenergetics models have been used to study the fate of contaminates in aquatic animals and especially the accumulation and resultant consequences for fish (Webb and Brett, 1973; Lett *et al.*, 1976; Norstrom *et al.*, 1976; McKim *et al.*, 1987; Woodward *et al.*, 1995).

1.1.2. Heavy metal exposure routes in fish

Toxicity of heavy metals in fish has been an extensively researched field of ecotoxicology (for reviews see Mance, 1987; Handy, 1996). Studies have been made mainly from exposures under laboratory conditions, but a degree of effort has been made to contrast laboratory data with that derived from the field. Most heavy metal studies have focused on acute waterborne exposure and many of these used to predict LC_{50} values to establish environmental standards and laws to regulate emissions. As a consequence of this, and increasing efforts in wastewater management, there has been a decrease of aqueous ionic compounds in today's rivers and much of the heavy metals present are contained in particulate fractions, and in plants and benthic animals (McIntosh *et al.*, 1978; Tessier et al., 1984). Field studies by Dallinger and Kautsky (1985) on the Augraben and Leifer Gruben rivers in Italy, and by Woodward *et al.*, (1994) on the Clark Fork River Canada, both found that low metal contents of the water (Cu = 2.9 - 4.4 μg l^{-1}) were in contrast to elevated concentrations of metals within the sediments (26.7 - 35.9 μg g^{-1} dry weight), indicating a chronic rather than an acute contamination of the river system. It has been suggested that certain essential metals can be taken up from sediments and bioconcentrated into plants (Cu = 43.1 – 117.6 μg g^{-1} dry weight, Dallinger and Kautsky, 1985), and benthic organisms such as isopods and snails (Cu = 900 – 1 210 6 μg g^{-1} dry weight, Dallinger and Kautsky, 1985), reaching these high concentrations at the trophic level of prey organisms on which predatory fish feed. This type of chronic sub-lethal pollution may have implications more meaningful than acute toxicity, as it

has been suggested that ecologically the death of an individual is largely irrelevant, and what is more important is changes in competitive performance of coexisting groups of organisms and flow patterns of energy and matter at the ecosystem level (Kinne, 1980). Despite these observations, there has been relatively little research devoted to the study of chronic sub-lethal exposures on fish (McGeer *et al.*, 2000), or the consequent effects of dietary contaminants (Handy, 1996). However, existing field data have shown that the amount of metal transferred by food can be high enough to attain biologically harmful concentrations in fish (Dallinger *et al.*, 1987), and have been suggested as a plausible cause of the decreased survival, growth and health of brown and rainbow trout in contaminated rivers (Woodward *et al.*, 1995). Thus, it should be considered that dietary heavy metal contamination may present a hazard to the sustainability of fisheries in these rivers.

1.1.3 Cu toxicology in fish and acclimation to exposure

In this study the effects of dietary copper on rainbow trout were investigated. It was considered a suitable environmental variable to investigate concomitant shifts in energy allocation, as a previous study by Handy et al. (1999) found it to significantly reduce activity, without any obvious physiological disturbances. It is also an essential nutrient for all organisms, required for energy metabolism by the mitochondria and is a constituent part of enzymes (70) and proteins (*reviewed by* Linder, 1991). Hence, rainbow trout require a daily copper ration of 3 mg Cu Kg^{-1} food d.w. (Ogino and Yang, 1980), Therefore, fish have physiological mechanisms at the sites of uptake (gill and intestine) to either excrete or regulate its adsorption from the environment (Clearwater *et al.*, 2000). Dietary Cu-exposures used in this study (730 mg kg^{-1} d.w. feed) were of ecological relevance, as Cu at such levels have been found within the diets of predatory fish in the wild (Dallinger and Kautsky, 1985; Woodward *et al.*, 1995). There exists a large body of literature on the deleterious effects of Cu-exposure on fish (although

these consist mainly of aqueous exposures). These include alterations to tissue morphology (Baker, 1969), inhibition of Na, K ATPase (Lauren and McDonald, 1987a), tissue lipid peroxidation (Baker et al., 1998), as well as altered energy metabolism, loss of appetite, reduced growth, decreased aerobic scope, suppressed locomotor activity, and mortality (Drummond *et al.*, 1973; Waiwood and Beamish 1978; Scarfe *et al.*, 1982; Reid and McDonald, 1988; De Boeck *et al.*, 1995, 1997; Taylor *et al.*, 1996; Handy *et al.*, 1999; McGeer *et al.*, 2000).

When challenged with chronic sub-lethal Cu-exposure, fish show a general process of acclimation, which has been suggested to comprise three phases (McDonald and Wood, 1993). The initial 'shock' phase, corresponds to assorted disturbances to physiological homeostasis. This is usually short lived (a few days) after which 'compensation' starts comprising of mobilization of metal-binding proteins (metallothionein) in various tissues (Bremner, 1979; Cherian and Goyer, 1978), the sequestering of copper in membrane-bound cytoplasmic inclusions in the hepatic parenchyma (Lanno *et al.*, 1987), and up-regulation of Cu ion excretion mechanisms, coincident with increased biosynthetic processes to help repair damage and correct physiological disturbances (Bradley et al., 1985; Hogstrand and Wood, 1996). Ultimately, a 'recovery phase' is apparent, where the internal physiology of the animal either returns to the pre-exposure condition or, a new equilibrium is established (McGeer *et al.*, 2000).

Physiological compensation during will require the reallocation of energy from other parts of the energetic budget. Indeed, studies exposing fish to aqueous sub-lethal copper concentrations have shown reductions in the growth of exposed fish, which after a period of few weeks recover to initial pre-exposure levels (Lett *et al.*, 1976; Dixon and Sprague, 1981). Other studies have observed a reduction in swimming capacity, whilst growth rates remained unaffected (McGeer *et al.*, 2000). Differences in observations within the literature may be

accounted for by experimental methodology, as toxicity can be influenced by water chemistry, fish species, life stage and temperature (Harrison, 1986; Taylor *et al.*, 1996). However, all literature on aqueous Cu-exposures of fish, agree that damage occurs to the branchial epithelium, and this can damage the fish's ability to uptake oxygen and regulate ions (Lett *et al.*, 1976; Waiwood and Beamish, 1978; Dixon and Sprague, 1981; Beaumont *et al.*, 1995; Taylor *et al.*, 1996; McGeer *et al.*, 2000).

During dietary Cu-exposures, the gills remain intact (Handy, 1996). Thus the fish's ability to maintain oxygen uptake for metabolic procedures, and regulate ions could be assumed to remain unaffected. This may in turn influence the fish's acclimation response, as an increased ability to deal with physiological disturbance. Exposure via the diet has approximately a 1000 times more tolerable limit in fish compared to aqueous exposures (Handy, 1996). The reduced toxicity of dietary exposure is due to the low bioavailability of Cu when bound to organic material (Woodward *et al.*, 1994), and the buffering capacity of the intestinal mucosa, which provides a formidable barrier to toxicant uptake (Clearwater *et al.*, 2000). As the bioavailability of elements and subsequently their rate of accumulation is much lower in contaminated feed than the equivalent dose presented in aqueous forms (Miller *et al.*, 1993), acute toxicity is normally of little significance and more meaningful are the sub-lethal adverse effects resulting from toxic concentrations administered over longer periods of time. Thus, the majority of these dietary studies have been long-term (4-24 weeks), and fish have been repeatedly dosed with elevated levels of copper (Murai *et al.*, 1981; Knox *et al.*, 1982; Lanno *et al.*, 1985a,b, 1987; Julshamn *et al.*, 1988; Handy, 1992, 1993; Miller *et al.*, 1993; Mount *et al.*, 1994; Farag *et al.*, 1994; Woodward *et al.*, 1994; Berntessen *et al.*, 1999; Handy *et al.*, 1999; Kamunde *et al.*, 2001). From these studies it has been suggested that dietary Cu concentrations of above 730 mg Cu Kg^{-1} diet d.w. exceed the regulatory capacity of rainbow trout, reducing specific growth rate and causing haematological disturbances after 8 weeks

exposure (Lanno *et al.*, 1985). A study by Handy *et al.*, (1999) exposing rainbow trout to a dose of 500 mg Cu Kg^{-1} diet d.w. for 12 weeks, found an absence of physiological disturbances and unaffected specific growth rates. Although metallothionein levels were elevated in both the liver and intestine of exposed fish, showing a degree of investment in detoxification mechanisms that could lead to an elevation in standard metabolism, though this was not determined in the study. Observations from this exposure did however show that Cu-treated fish spent 35% less time swimming than control fish, and it was suggested that the reduction in locomotor activity was a metabolic 'sparing effect' to enable detoxification of Cu without concomitant reductions in feeding efficiency and growth rate parameters.

Comparison between the observations made by Handy *et al*, (1999), and that of the higher dietary Cu-exposure study by Lanno *et al.*, (1985) suggest that measurement of swimming activity in fish, provides a more sensitive indicator of energetic shifts due to dietary Cu-exposure than alterations of growth. The fact that energy required for detoxification procedures is firstly compensated for by reductions in activity and only at higher dose levels are growth rates reduced, implies that growth has preferential allocation of energy over metabolism for activity. However, energy allocation by the fish during exposure may not be merely influenced by Cu toxicity of the diet, and other environmental factors may also be influencing acquirement of food, assimilation and transfer within the fish. Amongst toxicology literature there is a general trend to overlook biotic factors, such as inter-individual competition and predation, which may induce stress and reduce acclimation potential (Pottinger and Pickering, 1992), and may in turn influence the degree of toxicity. It is suggested that, differential allocation of resources, according to status, can produce great variability in the growth and fitness of different members of the population (Metcalfe, 1986), and therefore seems plausible to suggest it may also influence an individual's ability to acclimate to toxicant exposure.

If no competition exists, the fish will feed independently of each other allowing energetic investment in routine metabolism, and storage of energy (P). However, under social circumstances, salmonids will function within a feeding hierarchy (Yamagashi, 1962), and individual fish must now invest substantially more energy in acquiring food (Li and Brocksen, 1977). The amount of food acquired by an individual will depende on its relative size in the group and excess energy available for aggressive encounters, with a positive correlation existing between metabolic expenditure on activity and food intake (Cutts et al., 1998). Behavioural strategies involved in energy maximisation will depend on immediate environmental conditions. Thus, the interplay between an individual's fitness, feeding regime, social group size, and relative aggressiveness may all modify a fish's ability to acclimate to sub-lethal toxicity. This may be even more relevant when the resource, requiring energetic expenditure to obtain is the source of contamination itself.

During this study Rainbow trout (*Onchorhynchus mykiss*) were exposed to a dietary copper dose of 730 mg Cu Kg^{-1} diet d.w. for a period of 2-3 months. Rainbow trout were used as they were the preferred fish of previous dietary exposures and as a member of the group *Salmonidae* they are economically the most important group of freshwater/anadromous fish, forming the basis of an aquaculture industry and are highly valued components of the recreational fisheries (Pottinger and Pickering, 1992). The concentration of the dietary load was carefully selected so that it fell at the suggested limit of the regulatory response for dietary Cu-toxicity in rainbow trout (Lanno et al., 1987), and we would presume to see sufficiently measurable adaptive or compensatory biological responses elicited by the animal. To qualify and quantify responses, the trout were examined at several levels of biological organisation (biochemical, physiological, behavioural) and bioenergetic concepts applied to the data providing a physiological framework for energetic efficiency assessment. As well as the

alteration of physiological components, ecologically important behaviours controlling food intake and utilization were also studied.

Importantly, the analysis of swimming capacity and activity in this study were analysed under voluntary swimming conditions where activity and VO_2 were monitored simultaneously, allowing evaluation of routine metabolism and voluntary activity. In previous toxicological studies, examination of swimming activity has been measured only at discrete periods throughout the day, with the assumption that the extent of alterations in activity will be similar at all periods of the diel cycle. In this study, fish swimming activity and finite behaviours were monitored using computer-imaging equipment throughout the complete diel cycle, as it may be expected that fish exhibit different behaviours at different periods of the diel cycle.

The study further explores relationships not yet considered in toxicology research by assessing how pollutants responsible for energetic shifts, may influence behavioural strategies, in terms of maximizing energy intake whilst minimizing expenditure. This was assessed in both individual and social fish, as the intake rate of foraging animals will arise from variation in two characteristics; their intrinsic ability to forage in the absence of competitors (foraging efficiency), and the detrimental effect of competitors on their intake rate (Stillman et al, 2000). Net energy gain by the animal will be a consequence of increasing food intake rate, whilst reducing the expenditure of energy to acquire the food. The balance between activity costs and food intake rate is suggested as a major contributor to inter-population growth in natural populations (Boisclair and Leggett, 1989; Metcalfe et al., 1995).

The hypothesis for fish feeding on a Cu-contaminated diet is that, although Cu at this level (730 mg Cu kg^{-1} d.w. feed) of exposure is not directly toxic, it will require the continued investment of energy that will be incompatible with the optimal homeostasis of the fish. Compensatory energetic shifts (most probably from activity, Handy et al., 1999), may in turn affect an individuals behaviour, and indeed how it interacts with conspecifics.

1.1.4 Objectives and aims of the present study

The primary objective of the study was to provide information on how long-term exposure to environmentally realistic levels of copper within the diet will affect physiological and behavioural performance of rainbow trout (*Oncorhynchus mykiss*).

The specific aim of the research was to measure biochemical and physiological variables within the fish that may be related to energetic efficiency, then to describe in a bioenergetics context that may explain the energetic trade-off between toxicity and adaptive responses by the organism, in terms of behavioural strategies involved in the procurement of energy itself (food).

The specific objectives of the study were:

1. To investigate the extent of physiological disturbance due to Cu dietary exposure (730 mg Cu kg^{-1} feed d.w.).

2. To quantify the energetic investment in detoxification processes of Cu-exposed fish indirectly using oxygen consumption and physical activity measurements.

3. To determine the extent of concomitant alterations in behavioural repertiore over the fish's circadian cycle.

4. To evaluate to what extent alterations in behaviour may influence energetic efficiency in terms of social factors controlling net rate of food intake.

5. And finally, assess the affects of perturbed energy allocation on ecologically important behaviours, such as foraging ability and inter-individual competition.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

The fish used for physiological and behavioural analyses in this study were derived from two separate Cu-exposure trials, these have been termed Trial A and Trial B and are described respectively below.

2.1 Experimental fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Hatchlands Fish Farm (Rattery, Devon) in the spring of 1999 (Trial A) and 2000 (Trial B). In 1999 the fish weighed 5g $\pm$ 0.2g and in 2000 they weighed 25g $\pm$ 0.5g (mean $\pm$ S.D., n = 200). The fish were held for 14 days in a stock aquaria supplied with recirculating fresh water (for water quality parameters see Table 1), and were fed a control diet supplemented with a prophylactic antibiotic (oxylinic acid, 2 mg kg^{-1} dry weight of feed) for the first 10 days.

2.2 Diet formulation

A diet was prepared so that the toxicity of copper via the oral route could be studied. A dietary concentration of 730 mg Cu Kg^{-1} feed was chosen because it approached the maximum tolerable Cu dietary load for trout (Lanno *et al.*, 1985). Two different methods of diet formulation were used between Trial A and Trial B and these are described below. The formulation of the control diet used in Trial A was prepared from raw ingredients according to Handy *et al.*, (1999), following the diet formulation described in Table 1. The expected Cu content of the control diet was 11.92 mg kg^{-1} d.w. feed based on the manufacturer's data of ingredients composition. The Cu- supplemented diet was prepared using the same formulation except that 2.9 g of wheat feed was omitted to compensate for the mass of copper sulphate

Table 1. Composition of control and Cu-supplemented diets used in Trial A.

	Control diet	Copper diet
Fishmeal (g. d.w.)	600	600
Maize gluten (g. d.w.)	85	85
Wheatfeed (g. d.w.)	195	192.1
Cod liver oil (g)	60	60
Trouw (U.K.) Vitamin and mineral premix (g d.w.)	40	40
$CuSO4.5H_2O$ (g)	0	2.9
Total Cu content (mg kg-1 d.w. feed)	23.3 ± 1.25	726 ± 1.6
Proximate composition		
Moisture (% d.w.)	12	10
Protein (% d.w.)	53	51
Lipid (% d.w.)	19	17
Ash (% d.w.)	10	12

Data for Total Cu content are mean ± S.E., n = 5. Proximate composition n = 8.

added to the food. The Cu-supplement of 2.9 g of CuSO4.5H$_2$O was dissolved in 300 ml of deionised water and gradually added to 1 kg of the dry ingredients to give an expected Cu content of 730 mg kg^{-1} feed. The resulting paste was extruded through a Hobart food mixer (model A-120, 3mm-extrusion plate) and air-dried at 40°C for 96 h. To prevent oxidation of diets by copper during storage (Baker *et al.*, 1998), pellets from both diets were stored at –20°C in airtight plastic containers and removed two hours before feeding to defrost. Copper content of the Cu-exposure and control diets (Table 1) was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy ICPAES (see section 2.2.7), and proximate composition of the diets was determined using methodology detailed in section 2.2.3.

For the Cu-exposure of fish used for Trial B, a different method of diet formulation was employed. Due to the nature of the experiments a much more uniform pellet size was desired, thus a commercial ready-made diet was used (TROUW, U.K, size 02). The proximate composition of this diet as stated by manufacturer was 13% moisture, 44% protein, 24% lipid and 8% ash. Cu concentration of the manufactured diet was 11 mg kg^{-1} d.w. feed. Additional Cu was supplemented by firstly drying 1 kg of control diet in the oven at 40°C for 24 h to increase water absorbency. This dried feed was continually agitated in a Hobart food mixer and Cu added as a fine spray over a 10 minute period from a 2.9g of CuSO$_4$.5H$_2$O in 500 ml solution. The mixture was dried in the oven at 40°C for 24h and then coated with a 2% gelatine solution using the same method of application as described for the Cu, and dried again at 40°C for 24 h. This coated individual pellets with the nominal Cu concentration of 730 mg kg^{-1} feed d.w. and sealed it within a gelatine coat to prevent Cu leaching out, when in contact with water. The control diet underwent an identical procedure as the Cu-supplemented diet, omitting the CuSO$_4$.5H$_2$O from the procedure. For determination of Cu concentration, pellets were grouped into samples of 8 pellets (considered a single fish's daily ration) and

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analysed by ICPAES. Cu concentrations of the control and Cu-supplemented diet were (mean ± S.E., n = 6) 10.8 ± 1 and 721 ± 2.4 mg Cu kg-1 feed d.w. respectively.

2.3 Exposure to dietary copper

The supply of Cu-exposed fish for experimental investigations was derived from two major Cu-exposure trials. The first was carried out from 1.2.99 to 19.4.99 (84 days), and examined the metabolic trade-off between activity and maintenance in trout during dietary Cu-exposure. The second exposure was carried out from 1.4.00 to 27.5.00 (56 days) and investigated the concomitant effects of metabolic and behavioural alterations observed during the 1999 exposure. Throughout the thesis these exposure trials will be termed Trial A and Trial B respectively. The exposure period of Trial B was shortened to 56 days instead of the 84 days used in Trial A, as results from Trial A showed no further change in behavioural and physiological responses after this time. All other physical variables (except diet formulation, see above) were identical for the two trials.

The main feeding aquaria was purpose built for the toxicological feeding trials (fig. 2), and designed to eliminate aqueous Cu leachate from contaminated faeces and feed. Before fish were introduced into the system, the biofilter was seeded with 2 l of commercially bought bacterial culture (J & K Aquatics, U.K.). This media contained cysts of *Nitrosomonas* and *Nitrobacter*, the *Nitrosomonas* utilised ammonia (NH_3) within the aquarium water converting it to nitrite (NO-N), and the *Nitrobacter* converted this to nitrate. To start the biological process the aquarium was spiked with ammonium chloride (10 mg l^{-1}), and the resulting decline in aqueous ammonia was monitored following the methods of Verdouw *et al.* (1978). Samples were read on a Phillips Spectrophotometer 200 at 660 nm wavelength. Standards were made up from ammonium chloride in deionised water (DI) water 2.5 – 20 µg ml^{-1}. The

Figure 2. Cu-exposure aquarium consisting of 6 x 185 L tanks, each received filtered aerated water from the same biofilter. Flow rate into each tank was 20 l min^{-1}, reflushing tanks with filtered water every 9.25 minutes, arrows show direction of water flow. The drainage water from all tanks passed into a large pre-filter containing filtrate of decreasing pore size, before filtering through a series of charcoal mesh bags (1 mm), and finally entering the main biofilter (1000l). Charcoal filters (1 mm) attached to the return pumps ensured no ammonia or possible leeched aqueous copper was returned to the holding tanks. These filters were replaced fortnightly.

Cu

Cont.

Cont.

Cont

Cu

Cont

Cu

Spray bar

Standpipe

Prefilter

Sponge -
Filter-wool -
Charcoal -

Biofilter (1000 l)

Pump

Charcaol filter

30
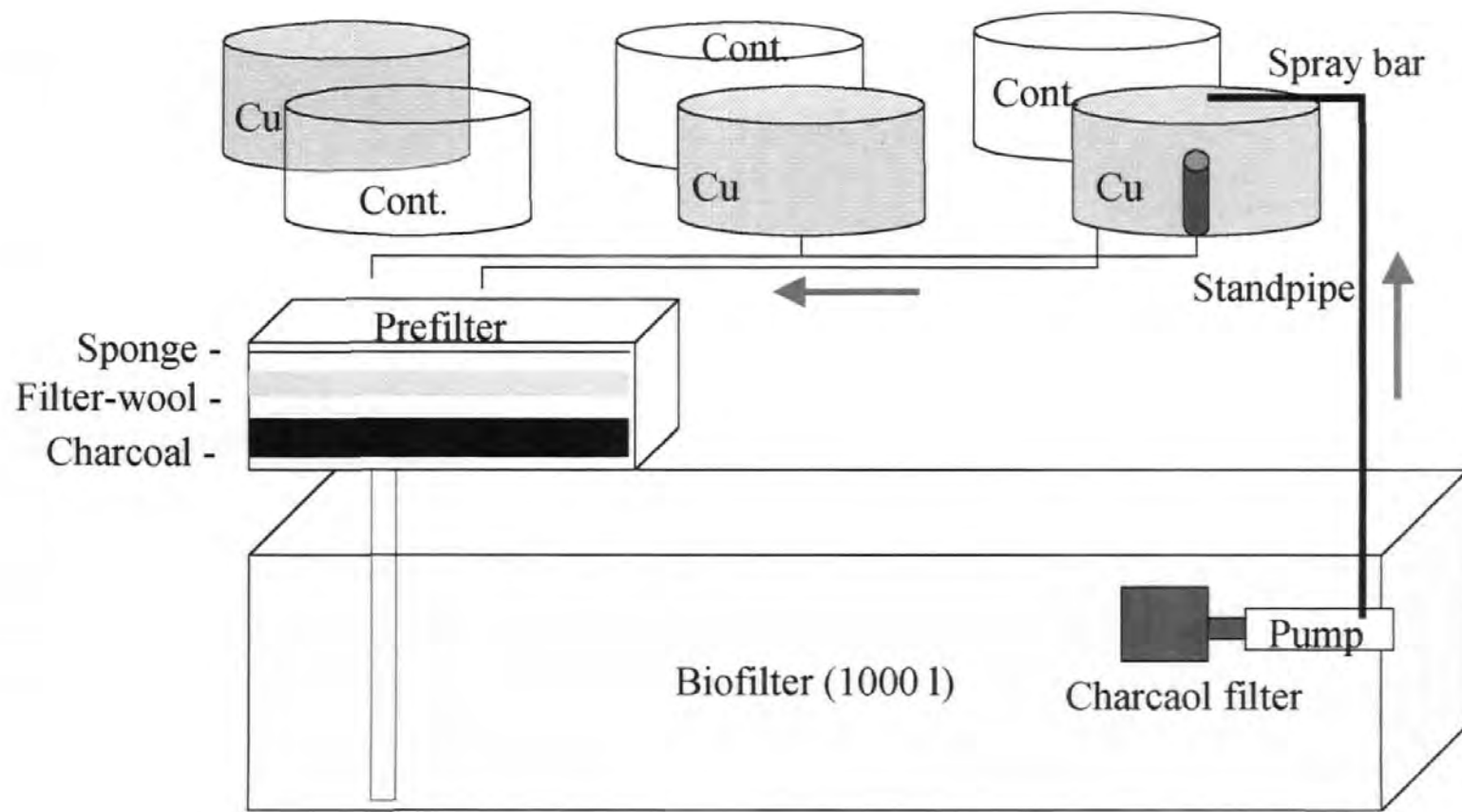
resulting rise and subsequent fall of nitrite was monitored following methods of Strickland and Parsons (1972), and read on a Phillips Spectrophotometer at 540 nm wavelength. Standards were made up from 1.064 g potassium nitrite in 249 ml of DI water and 1 ml of 5M NaOH. This solution contained 700 mg l^{-1} of NO-N, and was diluted with DI water to give standards within the range of 1-10 mg l^{-1}. Only when ammonia and nitrite levels had fallen below 0.5 mg l^{-1} were fish introduced to the exposure aquarium (after 8 weeks at $15^{0}C$).

Once in the exposure aquarium, fish were given 7 days acclimation before half of the tanks were chosen at random and switched to the Cu diet. Throughout the exposure period fish were sampled for respirometry and behavioural analyses from the tanks in weekly rotation. This ensured that fish had at least a 3-week rest period before the same tank was sampled again (probability of selecting same individual again <0.1%). The light regime throughout the exposure period and for all behavioural experiments was kept under a 12L:12D (0700:1900) photoperiod.

2.4. Water quality

Due to the nature of the study it was of vital importance that optimum water quality was maintained at all times to ensure gill integrity, growth rates and general physiology (Poxton 1990). Water samples were taken twice weekly before and after feeding for NH_3, NO-N, Cu and other ion analysis. Oxygen concentration, temperature and pH were monitored daily (Table 2). Total NH_3 levels were typically 0.2 ± 0.01 mg l^{-1} (mean $\pm$ S.E. n = 40), NO-N levels were consistently below detection limits <0.1 mg l^{-1}. Pre and post-feeding water quality analyses showed no rise in aqueous copper levels, which remained < 0.1 μmol l^{-1} throughout the experiment.

Table 2. Measured water quality parameters during both Cu-exposure trials

	Trial A Mean ± S.E. n = 30	Trial B Mean ± S.E. n = 20
Total NH$_3$ (mg l^{-1})	0.022 ± 0.015	0.021 ± 0.013
NO-N (mg l^{-1})	<0.001	<0.001
Temperature (^{0}C)	15	15
PH	6.8 ± 0.4	6.8 ± 0.8
Dissolved O$_2$ (%)	95 ± 1.2	93 ± 2.2
Ca (mmo l^{-1})	0.33 ±0.005	0.31 ±0.003
Cu (μmo l^{-1})	<0.01	<0.01
K (mmo l^{-1})	0.079 ± 0.003	0.068 ± 0.01
Mg (mmo l^{-1})	0.010 ± 0.003	0.011 ± 0.008
Na (mmo l^{-1})	0.32 ± 0.005	0.33 ± 0.007

2.5. Routine monitoring of nutritional performance

All the fish in each aquarium were weighed as a group, fortnightly, to calculate mean growth rates between treatments. A 2% body weight feed ration was size-calculated by taking the mean of all 6 tanks (control and exposed) and supplied to all tanks as a single ration at about 15:00 h daily. Fish were observed during feeding to ensure all food given was consumed.

The growth calculations used during both feeding trials were (Braaten, 1979),

Specific growth rate (SGR) = $\frac{\text{Ln (final weight)} - \text{Ln (initial weight)}}{\text{Number of days}}$ x 100

Food Conversion Ratio (FCR) = $\frac{\text{Amount eaten}}{\text{Weight change}}$

2.6 Tissue sampling and preparation for enzymology

The sampling of tissue for metabolite and other enzyme analysis followed the methods of Wang *et al.*, (1994), who evaluated different methods of tissue sampling and reported the following method as the most efficient for preserving enzyme activities in fish tissue, especially muscle.

Fish were removed quickly by netting from the appropriate tank and immersed in a blacked out 3 l tank containing 0.5g l^{-1} MS-222 (Sigma Ltd, Poole, Dorset). This anaesthetic was neutralised to pH 7 with NaOH to avoid acidification of the blood. When the fish lost balance and did not respond to touch, they were removed, pithed and a red/white muscle (red muscle, only accounted for 5% of the total muscle area, Goolish, 1989) sample (0.5g) was removed from the left flank between the dorsal fin and lateral line with a sharp scalpel. This sample was then freeze-clamped with liquid N_2 cooled aluminium tongs and stored at -80°C until analysed. After the muscle was excised, heart, liver, gill and intestine were then removed in

that order and frozen using the above procedure. The entire process, from removal of fish from water until freeze clamping of the muscle, took about 30-40 s, for heart 40-60 s, liver 60-80 s, gill 80-100 s and intestine 100-120 s. Tissue was stored at -80^{0}C for 1-2 weeks before enzyme analysis. After removal from the deep-freeze, tissue was homogenised (0.5 g) on ice in 2.5mls Tris buffer pH 7.2 (5-fold dilution of tissue). A Status x 120 homogeniser (Weston-Super-Mare, U.K.) with a 6 mm head was used, and each sample given four 5 second bursts at 30 000 rpm with a 10 second rest period in between to prevent warming of the tissue from friction generated by the blades. The crude homogenate was centrifuged at 3000g for 5 min to remove large cellular debris, and the supernatant containing the desired enzymes was decanted off in 200 µl aliquot sample tubes and replaced into the -80°C freezer. Samples were analysed within 1-2 days of the homogenisation procedure.

2.7 Trace metal analysis

All glassware and plastics, including pipette tips used in the ion analysis procedures were soaked in 5% (1.6M) Aristar nitric acid for 24 h to leach surface metals. They were then rinsed three times in DI water and air dried prior to use.

All reagents for trace metal analysis of tissues, diet and water samples were spectrosol grade or equivalent. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICPAES) was chosen as the method for ion analysis as it offers several benefits for the type of analysis required. Firstly, it is more sensitive than flame spectroscopy giving improved resolution in the determination of water, feed and tissue ion concentration s (i.e. Ca, Cu, K, Na, Mg). It also has a lower inter-element interference level, which was necessary to obtain accurate low level Cu concentrations against high levels of cellular ions (i.e. Na, K). Also a large number of elements can be obtained from a single set of excitation conditions, hence only a low sample volume is needed. The machine used was a Varian 200 ICPAES (Varian instruments,

Cambridge). Each sample was introduced through a peristaltic pump and nebulised by a flow of argon, and the resulting finely divided drops were carried into a plasma torch (8000K). The sample becomes atomised and the emission spectra generated as the sample cools returns to a lower energy state. Each element has a unique set of wavelengths at which it will emit energy. Immediately before running samples or standards, background noise was reduced and sensitivity optimised by trimming a search window around the desired emission peak, using a solution containing inorganic constituents at similar concentrations as those going to be analysed. The concentrations of elements within each sample were read at a rate of about 5 per minute and a sample volume of 5 ml was enough to obtain the concentration value of up to 6 elements per sample.

2.8 Computer aided behavioural tracking

Swimming activity and associated behaviours of Cu-exposed fish were closely monitored and analysed using the Ethovision Behavioural Tracking system (Tracksys Ltd, Nottingham, U.K.). Application and measured parameters for specific experiments are described in the relevant chapters, but details of basic hardware and technical information on tracking methodology are described below. A Sanyo CCD B/W (VCB-3327P, Japan) video camera was suspended about 1.5 m above or parallel to the aquarium in which fish behaviour was to be recorded. The camera was connected directly to a computer (Fujitsu Intel 80286), and the analogue image of the fish translated into digital information by a TARGA+ frame-grabbing card (Picolo, Angleur, Belgium). The contrast between the fish and the background was maximised by using either white tanks or a white background when using glass tanks, to optimise fish detection by the imaging software. The acquisition of tracking data was based on the following principle. The designated arena for each tracking experiment was defined on

Table 3. ICP detection limits (n = 12)

Element	Detection limit for F.W. (μmol l^{-1})	Detection limit for 0.5 g tissue sample (μmol d^{-1} d.w.)
Ca	0.5	0.05
Cu	0.96	0.096
K	35.5	3.5
Mg	0.5	0.05
Na	14.8	1.48
Zn	1.2	0.12

Standards (Ca, K, Mg, Na = 1mMol, 100 μM, 10 μM; Cu = 100 μM, 10 M, 1 μM) were acidified and kept in air tight plastic containers for up to 3 months.

the monitor, with the computer only detecting objects within this user-defined area. A picture of the empty test arena was digitised and subtracted from a digitised picture of the test arena containing the animal, leaving data for the animal and noise. The noise was removed by a threshold operation (determined before tracking) and the remaining signal transformed to a binary image. Irrelevant objects such as faeces were ignored because objects were tested against a criterion of size (Nilsson *et al.*, 1993). Before each trial the computer digitised image was calibrated into real position values by placing an object of known length into the camera field of view and it's actual length (cm) matched with the corresponding pixel defined computer image. The computer recorded the pixel position of the centre point of the fish and these were recorded as an X,Y co-ordinates and converted into real position values before analysis. A predetermined sampling rate of 4 frames s^{-1} was chosen, and a minimal distance moved threshold (non-moving filter) of <0.5 cm was set before data analysis (movement below that level between consecutive samples was therefore ignored). This was to prevent the recording of slight "apparent" movements, which could be, attributed to noise, drift or tail beats and not true locomotion (fig. 3). From these point positions scaled to real distances using the calibration, absolute movement of each fish between user defined time interval was determined. Analysis software (Noldus, Nottingham,U.K.) calculated behavioural parameters, which included: total distance moved (cumulative change in position of the fish between consecutive video images), and swimming speed (total distance moved per unit time). Each track was monitored visually during recording to ensure fish were tracked accurately. Tracks that showed image interference greater than 0.5% were discarded; overall 10% of tracks were not included in the analyses for this reason.

Analysed data was exported to Excel Version 6.0 for further analysis. Each behavioural track per animal contained a large volume of data, thus computer macros were created that would duplicate the analysis on individual tracks. Behaviours could also be scored by key

Figure 3. Relationship between video sampling rate and total distance moved determined by

Ethovision. The plateau shows optimum sample rate and true locomotion by the fish. Higher

sampling rates give false estimation of movement, by calculating background noise and body

wobble.
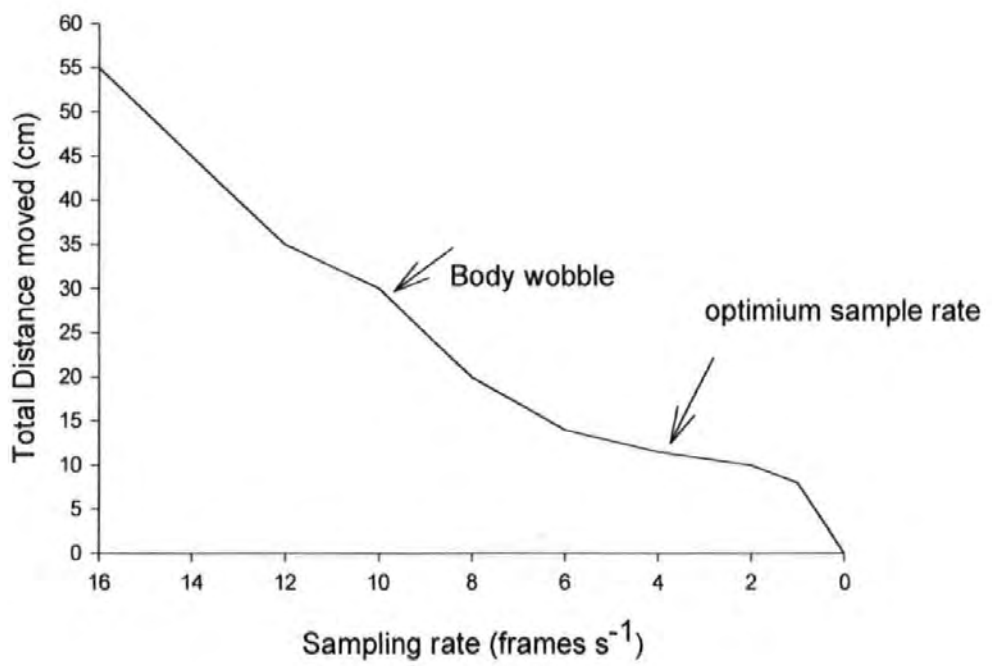
assignment during tracking and this aspect of the analysis will be dealt with in the appropriate chapters.

2.9 Routine Statistics

Sigmaplot Version 4.0 was used for graphical representation of data, line fits, and associated regression statistics including One-way ANOVA's for best-line fits. All other statistical analyses were carried out using Statgraphic 4.0. As standard data was tested for normality using the Summary Statistics function, this gave the Standard Skewness and Standard Kurtosis values for each data set under analysis. Abnormally distributed data were either transformed (1/X, log) or non-parametric statistics applied. Significant differences in mean/median values (Multi sample comparison) and distribution (F ratio) were determined between fish on the same diet but from different tanks. Data sets were grouped by treatment only if no significant differences were obtained in both means and distribution. Further detailed methods of specific statistics used are included in the relevant chapters.

CHAPTER THREE

THE BIOCHEMISTRY AND ENZYMOLOGY OF FISH FED ELEVATED

DIETARY COPPER

3.1 Introduction

It is well established that exposure of fish to excess copper via the water or diet will result in accumulation of the metal within the body tissues (McDonald and Wood, 1993; Handy, 1996). At higher internal concentrations, Cu will become deleterious to cellular physiology (Buckley, 1982; Collvin, 1985; Overnell and McIntosh, 1988; Carbonell and Tarazona, 1994; Pilgaard et $al.$, 1994), as Cu ions will compete for binding sites with essential metals, directly poisoning plasma membrane ion-transport Na^+/K^+ and Ca^{2+} ATPases (Dixon and Sprague, 1981; McDonald and Wood, 1993; Viarengo et $al.$, 1993). Trout sampled from field sites contaminated by both water and food-borne Cu have shown elevated whole body Cu and lower concentrations of Na^+ and Ca^{2+} within plasma, compared to fish from clean sites with low whole body Cu concentrations (Farag et $al.$, 1995). As well the ionic Cu^+ form competing for membrane transport with essential metals, Cu can also exist in the reduced valence state, creating free radicals, which can act as a catalyst for lipid peroxidation in biological systems (Wills, 1985). A Cu-induced free radical can bring about the abstraction of a hydrogen atom from a polyunsaturated fatty acid side chain (Yagi, 1999), forming peroxides which can react with proteins to modify the structure of membranes, altering the fluidity and structure of cells. Such changes in the structural integrity of cell membranes may ultimately result in tissue damage and cell death (Halliwell and Gutteridge, 1985). The reaction of peroxides with proteins will form fluorophores, and these can be measured directly using a fluorometer (Fletcher et $al.$, 1973; Yagi, 1999). Elevated lipid peroxidation products have been successfully measured in the liver, muscle

and gill of fish exposed to elevated (> 75 µg Cu l^{-1}) aqueous Cu (Radi and Matkovics, 1988), in the hepatic mitochondria of rats fed 1000 mg Cu kg^{-1} feed for 8 weeks (Sokol *et al.*, 1990), and the whole body tissue of mullet following dietary exposures of and 2400 mg Cu kg^{-1} feed for 10 weeks (Baker *et al.*, 1998).

Histological examination of tissues can provide details of consequential effects of toxicant exposure, detailing disturbance and damage, primarily at the site of toxicant uptake. The deleterious effects of aqueous Cu-exposure on fish gill morphology are well described and include the detachment and shortening of the secondary lamellae, necrosis of chloride cells, hyperplasia, and epithelial lifting (Baker, 1969; Bilinski and Jonas, 1973; Alazemi *et al.*, 1995; Sola *et al.*, 1995). During dietary Cu exposure, uptake occurs via the gastrointestinal tract (Handy, 1993). Histological examination of the intestine and pyloric caece in salmonids after long-term elevated dietary Cu-exposure has shown, gut impaction, swelling, ulceration, increased apoptosis, cell proliferation and epithelial lifting (Berntessen *et al.*, 1999; Kamunde *et al.*, 2001). Whereas examination of gills from dietary Cu- exposed fish shows an absence of pathology (Handy *et al.*, 1999).

Once Cu has entered the blood stream either via the branchial epithelium or the intestine it will be transported to the liver. The result will be an accumulation of Cu within the liver, which is considered to play a central role in the metabolism of copper (Bremner, 1987), removing newly absorbed copper from the circulation of the body (Grosell *et al.*, 1997). Histological examination of the liver has shown the accumulation of Cu within cytoplasmic inclusions, sequestering Cu within the cell and thus protecting cell components from the injurious effects of the free copper ion (Lanno *et al.*, 1987). It is realized that metallothionein (MT) is largely involved in hepatic detoxification responses (Bremner 1987), acting as a sink to sequester metal ions and has been associated with reducing oxidative stress and free radical scavenging (Schlenk and Rice, 1998). Increased MT

concentrations have been reported in the liver and gills of fish following controlled aqueous Cu-exposures (Dixon and Sprague, 1981; Roch *et al.*, 1992), and within the liver and intestine of dietary Cu-exposed fish (Woodward *et al.*, 1994; Berntssen *et al.*, 1999; Handy *et al.*, 1999), with the gills showing negligible induction of metallothionein.

Detoxification procedures elicited by fish in response to Cu-exposure via the diet may also protect cells from free radical damage. Indeed, Handy *et al.*, (1999) found that livers of dietary Cu-exposed fish lacked any necrosis. However, they did have an extreme reduction in lipid content, suggested to represent an increased energetic cost for hepatic detoxification. Other authors have reported similar findings, of increased glycolysis occurring in the tissues of copper intoxicated teleosts (Srivastava, 1982; Radhakrishnaia et al. 1992). The measurement of changes in the concentrations of enzymes along different metabolic pathways may provide an estimation of altered energy metabolism, as a sustained change in flow through a metabolic pathway will often be associated with changes in enzyme concentration (Driedzic and Hochachka, 1978). Tissues using anaerobic metabolism will contain high levels of lactate dehydrogenase (LDH), necessary to convert pyruvate into lactate and ensure a continues supply of oxidized NAD, thus determining the rate of glycolysis (Bostrom and Johansson, 1972). This is in contrast to the respiratory enzyme cytochrome C oxidase (CytoC) whose specific activity can be correlated to the degree of aerobic metabolism in the tissue (Lawrie, 1953). It has been demonstrated that fish will switch from aerobic to anaerobic metabolism under stressful situations (Johnston and Goldspink, 1973), and studies have shown that organisms will increase the degree of anaerobic metabolism due to Cu-exposure (Bilinski and Jonas, 1973; Arasu and Screenivasula Reddy, 1994; Sastry *et al.*, 1997). The anaerobic process is less efficient in terms of energy production then aerobic metabolism, and may even become fatal through

acidosis if tissue lactate concentrations are eventually allowed to upsurge in the blood stream (Black, 1958).

In attempts to assess overall physiological status and general health in fish, blood is one of the most frequently examined tissues (Houston, 1997). Haematological indices will provide a good measure of aerobic scope in fish, as oxygen delivery from the gills to the tissue will be a function of blood O_2 carrying capacity. This can be measured indirectly via assessing haemoglobin content, haematocrit indices, and red blood cell counts. Other haematological measurements can provide an indication of stress, such as plasma glucose and cortisol levels (Gregory and Wood, 1998). Also the nutritional state can be assessed from measuring plasma triglycerides and plasma protein content (McKim et al., 1970). Previous studies have shown that exposure of fish to certain metals can decrease blood heamaglobin, heamatocrit and plasma protein, and cause an increase in plasma glucose and cortisol (Gaudet et al., 1975). The degree of response appears to be dependant on metal type, species of fish, water quality, and length of exposure (McKim et al. 1970; Christensen et al., 1972; O'Neill, 1981; Cyriac et al., 1989; Munoz et al., 1991; Dethloff et al., 1999). Though most authors agree that haematological indices are transient with the animals showing a degree of acclimation after a relatively short period of exposure (1-2 weeks).

It has been suggested that Cu exposure will result in an integration of toxic effects and compensatory responses at the organism level (Hebel et al., 1997). During sub-lethal chronic exposures, after an initial acclimation period, and the initialisation of detoxification and compensatory responses, an increased state of tolerance to the metal will develop (Dixon and Sprague, 1981; Lauren and Mcdonald, 1987a, b; McDonald and Wood, 1993; Grosell et al., 1997, 1998). However, the process of acclimation will require energetic investment by the organism, Dixon and Sprague (1981) concluded that the reduced growth observed during aqueous Cu-exposure represented the increased metabolic costs associated

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with acclimation. For dietary Cu exposures in fish there is considerable disagreement as to the limit and extent of toxicological and compensatory responses. For example, the upper toxic limit for dietary Cu exposure, leading to a reduction in growth rates has been disputed in both channel catfish (Murai *et al.*, 1981) and rainbow trout (Lanno *et al*, 1985; Kamunde *et al.*, 2001). Certainly, toxicity of Cu will depend on its bioavailability to the organism. Previous studies experimenting with different diets have shown that the amount of copper bioavailable to the animal will be dependant on diet composition (Milner, 1979, 1982; Dallinger and Kautsky, 1985; Miller *et al.*, 1992).

Within this chapter I wished to further evaluate the toxic limit of dietary Cu-exposure and regulatory capacity of rainbow trout by exposing them to the suggested upper toxic dietary concentration of 730 mg Cu Kg^{-1} diet (Lanno *et al.*, 1985) for 12 and 8 weeks (a substantial part of the results are from analysis of fish after 12 weeks exposure, and only the lipid peroxidation data is from fish analysed after 8 weeks exposure). Biochemical and physiological measurements are made with associated tissue metal residue concentrations to help determine impact of dietary Cu-exposure on rainbow trout. Additionally nutritional parameters are examined for possible disturbances to nutrient absorption and/or metabolic costs associated with acclimation.

3.2 MATERIALS AND METHODS

3.2.1 Tissue ion analysis

Ion concentrations within individual organs were measured to confirm Cu-exposure and also to outline any major ionic disturbances in tissues collected from control and Cu-exposed fish after 12 weeks Cu-exposure (Trial A). Fish were terminally anaesthetised (MS-222, 0.5 g l^{-1}, buffered pH 7, 4M NaOH) and whole organs were sampled in the following order: muscle, gill, heart, intestine, spleen, brain, eyes, and dried to constant weight at 80°C for 48 h, weighed and then digested in 0.5 ml 4M HCl and 0.5 ml 4M HSO_4 (APHA, 1985). Acid-digested samples were diluted to 5 ml with deionised (DI) water before ion analysis. The same digest procedure was carried out for Cu determination of feed. Each sample was analysed simultaneously for Na, K, Ca, Mg, Cu and Zn by ICPAES (see section 2.2.7). At the end of Trial B it was not considered necessary to check for ion disturbances (due to lack of effect in Trial A), and only the liver was examined for Cu concentration to confirm exposure.

3.2.2 Measurement of metallothionein levels

Metallothionein levels were determined using a method adapted from Viarengo *et al.*, (1997) by Pedersen (1999), providing another indicator of exposure, as well as an estimation of energetic investment in the process of detoxification. Metallothionein levels were quantified by using a partially purified metalloprotein obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. Care was taken to prevent the oxidation of sulfhydryl groups, and to prevent the contamination of the sample by low molecular weight thiols and degradation caused by proteolytic enzymes. This was achieved

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with rapid extraction steps and the addition of reducing agents to the sample mixture, dithiothreitol (DTT), and a chemical that prevents the action of proteolytic enzymes, phenylmethylsulfonylfluoride (PMSF).

Approximately 1g of wet weight tissue was removed from the -80^0C freezer (dissection prior to storage see section 2.2.6) and was ground with a pestle and mortar in a small quantity (5 ml) of liquid nitrogen. The resultant pulverized tissue was weighed, and 3 ml of 1 mM DTT and 30 μl of 0.5 mM PMSF in ethanol (-20°C) were added. The mixture was sonicated (3 x 15 s) at 40% output power using a sonication probe (Sonicator-W-385, U.K.) and then a further 30 μl ice cold PMSF was added. The resulting mixture was placed in an ultra centrifuge (Beckman TL-100; California, U.S.A.) and centrifuged for 55 000 rpm at 4°C for 70 min to obtain the cytosolic fraction. The resulting supernatants were decanted into vials and snap frozen in $N_{2(l)}$ ready for ethanol / chloroform extraction of metallothionein.

The rest of the analysis followed the procedure of Viarengo et al., (1997). Absolute ethanol (500 μl, 95%) and chloroform (40 μl) kept at –20°C were added to 500 μl of thawed cytosolic fraction and mixed vigorously. The mixture was then centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was poured into a new tube and 3 times the volume of ethanol added and vortexed for 15 s and left for 1 hour at –20°C. The samples were then centrifuged at 7000 rpm for 10 min. The supernatant was decanted off and the pellet washed with 2 ml of washing buffer (87 % ethanol, 1 % chloroform and 12 % 20 mM Tris-HCl, 0.5 M sucrose, pH 8.0) and centrifuged again at 7000 rpm for 10 min at 4^0C. The pellet was placed on ice and dried in a fume cupboard under a continuous flow of nitrogen gas to prevent oxidation. The dried pellets were dissolved in 300 μl of 5 mM Tris-1mM EDTA buffer (pH 7) and mixed. A calibration curve was prepared from 0.5 to 16 mg ml^{-1} GSH (reduced glutathione) dissolved in Tris-EDTA buffer. Following this 4.2 ml of 0.43 mM

DTNB (5,5-dithiobis-2-nitrobenzoic acid) solution were added to 0.3 ml of each of the standards and the samples were incubated for 15 min to allow the colour to develop. They were then read with a AP1 UNICAM UV/VIS UV-4 spectrophotometer (England) at 412 nm wavelength.

Metallothionein levels were determined from the calibration curve and the following equation was used to back-calculate MT levels to the wet wt. of the initial sample,

Wet weight of $\qquad$ = $\qquad$ initial weight of the sample (g) x volume of sample purified (0.5ml)
(0.5ml)
tissue sample (g/ml) $\qquad$ initial volume of buffer added to ground sample (3ml)

3.2.3. Growth and nutritional characteristics

To establish whether exposure to oral copper influenced food intake, or energy utilisation (reflected by carcass composition), mean growth parameters per treatment were calculated (section 2.2.5.) as well as the proximate composition of fish (moisture, crude protein, crude fat, carbohydrate, mineral matter or ash, determined following standard methods in A.O.A.C. 1990). Carbohydrate concentration of fish was determined by subtraction of other compositional parameters from total dry weight.

Moisture content

The moisture content of feed and carcass was determined by drying samples in a flat aluminium dish in a fan assisted oven (Pickerstone E 70F, Thetford, Norfolk, U.K.) at 105°C until a constant weight was recorded, usually between 24-28hr.

Moisture content (%) = Weight of fresh sample- Weight dry sample
                                     Weight of fresh sample

Determination of whole body protein content

The protein content of feed and fish carcass was determined by the Kjeldahl method. A 500 mg sample of dried feed or carcass from each fish was weighed into a borosilicate digestion tube containing 20 ml of concentrated H_2SO_4 (4M) and 2 Kjeldahl catalyst tablets (2 x 3g K_2SO_4, 105 mg $CuSO_4.H_2O$ and 105 mg TiO_2 (Thompson and Capper Ltd., Runcorn, Cheshire, U.K.). Digestion was carried out in a Gerhardt Kjeldatherm digestion block (C. Gerhadt Laboratory Instruments, Bonn, Germany) for 30 min at 250^0C followed by a further 2 h at 380^0C with the acid fumes collected and neutralised by 15 % NaOH in a Gerhardt Turbosog unit. After cooling, the Gerhardt Vapodest 3S distillation unit diluted the sample with distilled water and neutralised with 40% NaOH. The ammonia in the sample was then collected into 50 ml of saturated orthoboroic acid (H_3BO_3) by steam distillation. Using '4.5' indicator, the distillate was titrated against 0.25M HCl and the percentage protein in the dry sample determined according to,

$$\% \text{ crude protein} = \frac{(\text{sample titre (ml)} - \text{blank titre (ml)}) \times 0.25 \times 14 \times 6.25}{\text{sample weight (mg)}} \times 100\%$$

Where,

      0.25 = [HCl] in moles,

      14   = relative atomic mass of nitrogen,

      6.25 = constant describing relationship between

              nitrogen and protein content of sample

Determination of total lipid content

Lipid determination was derived using the method of Barnes and Blackstock (1973) and was followed by a gravimetric determination of the lipid content of the solvent extraction. In this modification to the procedure, 500 mg of dry material was weighed into a 50 ml conical flask to which 10 ml of chloroform : methanol (2:1) was added. The flasks were sealed and left overnight at room temperature. At the end of this period the extract was suction-filtered through a Whatman No.2 filter into a test tube and the residue in the conical quantitatively removed using a further 10 ml of chloroform : methanol. Duplicate 5 ml aliquots were transferred to pre-weighed test tubes and the solvent evaporated at 60^0C using a water bath. The weight gained by the test tube was attributed to the lipid slick left behind by the solvent evaporation. This was proportional to the lipid content of the sample and hence the percentage of lipid in the dry material was calculated according to,

$$\text{lipid (\%)} = \frac{4 \times \text{weight gain of tube (g)}}{\text{sample weight (g)}} \times 100$$

Determination of ash content

The ash content of the dry material was determined in accordance with A.O.A.C. (1990). Dry samples of 500 mg were each accurately weighed into a pre-weighed crucible and heated for 8 h at 525°C in a Carbolite GLM 11/7 muffle-furnace (Carbolite furnaces Ltd., Bamford, Sheffield, U.K.). The residue in the crucible was the non-combustible, or ash, component of the sample. Re-weighing of the crucible plus contents, and comparison with the weights of each before combustion yielded the mass difference, which was then used to derive the value for sample ash content. The calculation of percentage ash in the sample was as follows,

$$ash \ (\%) = \frac{(weight \ of \ crucible \ + \ residue) - weight \ of \ crucible \ (g)}{sample \ weight \ (g)} \times 100$$

3.2.4 Blood sampling

At the end of trial A (12 weeks), 6 fish were removed from each treatment (2 per tank) and individually anaesthetised with buffered (pH 7) MS222 (0.2 g l⁻¹) and whole blood was taken via puncture of the caudal vein using a 500 μl (0.33 x 13 mm/ 29 G x ½") syringe. To prevent blood clotting, syringes were pre-heparinised by flushing twice with a heparin (lithium salt) solution containing 1000 units ml⁻¹. Before the addition of blood 2 drops of lithium heparin were also added to sample vials. Haematocrit and haemoglobin concentrations were determined immediately following methods given in Handy and Depledge (1999). For haemoglobin determination Sigma Diagnostics kit No. 525-A was used and samples read at 540 nm wavelength on a Philips 200 spectrophotometer (Taiwan). For each sample taken, 20 μl aliquot of blood was mixed with 0.98 ml of Dacie's fluid and red and white cells counted on a Neubauer haemocytometer following the method described by Houston (1990). The remaining blood was centrifuged (3000 g for 3 min, Micro Centaur MSE, California. U.S.A) and the serum frozen (-20°C) until required for further analysis. Fish were then sacrificed by terminal anaesthesia followed by pithing, and following this procedure, tissue samples for ion analysis were collected from fish.

Serum glucose

Blood serum was collected following methods described above and glucose levels determined the same day using a Sigma Diagnostics kit (No. 16-UV). A 1.0 ml aliquot of Glucose (HK) reagent (1.5 mmol l⁻¹ NAD, 1.0 mmol l⁻¹ ATP, 1000 U l⁻¹ hexokinase, 1000 U l⁻¹ G-6-PDH, 2.1 mmol l⁻¹ Mg, pH 7.5) and 10 μl of tissue reagent were added to a

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cuvette, mixed by gentle inversion and incubated at 30°C for 5 minutes. Cuvettes were read at 340 nm wavelength on a Phillips 200 spectrophotometer. Glucose concentration was calculated using Glucose Standard Cat No. 16-100 and the following equation,

$$\text{Glucose concentration (mmol l}^{-1}) = \frac{\text{A sample} - \text{A blank}}{\text{A standard} - \text{A blank}} \times \text{Concentration of standard}$$

Serum Triglycerides

Triglyceride concentration was determined using Sigma Diagnostics kit No. 339 on serum collected from control and Cu-exposed fish. A 1.0 ml aliquot of Triglyceride (GPO-Trinder) reagent (0.6 mmol l^{-1} ATP, 3.0 mmol l^{-1} Magnesium salt, 125 mmol l^{-1} 4-Aminoantipyrine, 1.69 mmol l^{-1} Sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine, 50 000 U l^{-1} Lipase, 1000 U l^{-1} Glycerol kinase, 2000 U l^{-1} Glycerol phosphate oxidase, 2000 U l^{-1} Peroxidase, 0.05% Sodium Azide, pH 7) was added to a 1 ml cuvette containing 10 µl of tissue homogenate, mixed by inversion and incubated at 25°C for 15 min and read at 540 nm wavelength on a Philips 200 spectrophotometer with water as blank. Triglyceride concentration was calculated using Sigma Diagnostics aqueous Glycerol Standard set, Cat. No. 339-11 and the following equation,

$$\text{Triglyceride conc. (mmol l}^{-1}) = \frac{\text{A sample} - \text{A blank}}{\text{A standard} - \text{A blank}} \times \text{Concentration of standard}$$

Serum Creatine Kinase

Damage to skeletal muscle, brain tissue or heart muscle may cause elevation in serum Creatine Kinase (CK). Serum was obtained as above, and CK determined using Sigma Diagnostics Cat. No. 47-UV. CK reagent, 1.0 ml (30 mmol l^{-1} Creatine Phosphate, 2 mmol l^{-1} ADP, 5 mmol l^{-1} Adenosine Monophosphate, 2 mmol l^{-1} NAD, 20 mmol l^{-1} N-Acetyl-l-Cysteine, 3000 U l^{-1} HK (yeast), 2000 U l^{-1} G-6-PDH, 10 mmol l^{-1} Magnesium ions, 20

mmol l^{-1} D-Glucose, 10 μmol l^{-1} Di(adenosine 5') pentaphosphate, 2 mmol l^{-1} EDTA, pH

6.7) was incubated at 30°C for 5 min then 20 μl of sample was added and mixed by

inversion, where upon each cuvette was placed in a constant temp (30°C) incubator (Grant,

Cambridge, U.K.) for 3 min. Incubation absorbance was recorded at 340 nm wavelength

with water as blank (Initial A). Each cuvette was incubated for a further period of 120 s

with absorbance readings taken every 30 s (to check the linearity of the reaction rate). The

absorbance of the sample at 120 s was termed Final A. Change in Absorbance per min (ΔA)

was calculated by (Initial A – final A) / 2, and CK was determined according to the

equation,

$$CK \ (Ul^{-1}) = \frac{\Delta A \ per \ min \ x \ TV \ x \ 1000}{6.22 \ x \ LP \ x \ SV}$$

Where ΔA per min is the change in absorbance per min at 340nm, TV denotes the total

volume (ml) of the assay, SV is the Sample volume, 6.22 is the Millimolar absorptivity of

NADH at 340nm, and LP is the lightpath distance through the cuvette (cm), and 1000 is the

conversion of units per ml to units per litre. The concentration determined by standard curve

was then calculated back to pyruvate concentration per gram of tissue, by multiplying by

dilution factor of homogenate.

Serum Cortisol

Serum was obtained as above and analysed for cortisol concentration by an immuno-

enzymatic assay (Beckman Access Immunoassay System, kit no.33600, detection limit

11nmol l^{-1}). This is a competitive binding immuno-enzymatic assay utilising paramagnetic

particles coated with a capture antibody. The detection system is a chemiluminescent

substrate activated by alkaline phosphatase. The resulting light generated was read by a

luminometer, and the unknown values of samples were determined from a standard curve provided.

3.2.5. Tissue protein determination

Tissue protein levels were examined for fluctuations that may arise from Cu-exposure, perhaps indicating alterations in general nutritional parameters and also provided an index for quantifying enzyme activity within tissue homogenates. The method was a micro-plate method based on the Hartree (1972) modification of the Lowry protein assay. Prior to assay, sample was diluted 100 fold to fall within assay range, and assay was carried out according to methods given in Hartree (1972). Samples were measured colourmetrically at 630 nm wavelength on a Dynatech MRX microplate reader (Guernsey, U.K.). Standards were prepared using Bovine serum albumin dissolved in distilled water 0, 40, 80, 120, 160, 200 μg ml^{-1}.

3.2.6. Cardiac muscle lactate dehydrogenase

The whole heart was excised using a scalpel to cut it free from the bulbous arterioles, and homogenised using methods in section 2.2.6 before lactate dehydrogenase (LDH) analysis (Sigma diagnostics kit DG1340-K detection limit 2 μmol min^{-1}). A 1.0 ml volume of pre-warmed 30°C lactate dehydrogenase reagent (50 mmol l^{-1} Lactate, 7 mmol l^{-1} NAD, pH 8.9) was pipetted into a cuvette with 50 μl of heart homogenate and mixed by gentle inversion before being placed into a temperature controlled (30°C) spectrophotometer (Helios β-Unicam, Birmingham, U.K.). This was incubated for 30 s and absorbance read at 340 nm wavelength (Initial A). Absorbance was then re-read at 30 and 60 seconds with the latter reading (Final A) used to calculate ΔA per min (Final A - Initial A). Change in Absorbance

per min (ΔA) was calculated by (Initial A – final A), and lactate was determined according to the equation,

$$LDH\ activity\ (Ul^{-1}) = \frac{\Delta A\ per\ min\ x\ TV\ x\ 1000}{6.22\ x\ SV\ x\ LP}$$

Where ΔA per min is the change in absorbance per min at 340nm, TV denotes the total volume (ml) of the assay, SV is the Sample volume, 6.22 is the Millimolar absorptivity of NADH at 340nm, and LP is the lightpath distance through the cuvette (cm), and 1000 is the conversion of units per ml to units per litre. The concentration determined by standard curve was then calculated back to pyruvate concentration per gram of tissue, by multiplying by dilution factor of homogenate.

3.2.7. Determination of Lipid Peroxidation

Lipid peroxidation of tissue can be measured indirectly by determination of its accumulative product, malonaldehyde, in the tissue. Here it was examined within the liver (main target organ of dietary copper, Lanno et al., 1985a), skeletal and cardiac muscle (main effector tissues associated with locomotor activity). The level of lipid peroxidation was determined using the method of Yagi (1999). Tissues were extracted from fish (see section 2.2.6) after 8 weeks exposure to dietary copper (Trial B). A 20 μl aliquot of each sample, 4 ml of 8.3% H_2SO_4 and 0.5 ml of 10 % phosphotungstic acid were mixed gently in a centrifuge tube and allowed to stand at room temperature for 5mins before centrifuging (Beckman T-J-6) at 1600 g for 10 min. The supernatant was discarded and sediment mixed with 2 ml H_2SO_4 and 0.3 ml 10% phosphotungstic acid and centrifuged at 1600 g for 10 min. The supernatant was discarded and the pellet was re-suspended in 4 ml distilled water and 1 ml TBA reagent, then heated in an oil bath at 95°C. Tubes were removed after 60 min and 5 ml of n-butanol added and tubes vortexed for 20 sec, then allowed to cool before centrifuging at

1600 g for 15 min. The n-butanol formed as a pink layer on the surface of the tubes and this was pipetted off onto a micro plate well and read on a Perseptive Biosystems Cytofluor II (New York, U.S.A.) at 553 nm with 515 nm excitation. A standard was made from reacting 0.5 nmol tetramethoxypropane in 4 ml of water with TBA reagent. This was heated at 95°C for 60 min, 5 ml of n-butanol added, vortexed for 20 sec and centrifuged at 1600 g for 15 min before reading on a Dynatech MRX microplate reader. Lipid peroxidation was calculated and expressed in terms of its accumulative product malonaldehyde.

Malondialdehyde conc.(nmol/ml homogenate^{-1})
$$= \frac{\text{Fluorescence intensity of sample} \times 25}{\text{Fluorescence intensity of standard solution}}$$

3.2.8. Statistical analysis

Two-tailed student's t-tests were used to determine any significant differences in most physiological parameters measured between control and Cu-exposed fish. The data for the accumulation of copper within tissue and other tissue ion concentrations on the whole were not distributed normally (determined using F-test) and a Mann-Whitney U-test was therefore used instead to determine any significant differences between the medians. Data was considered significant if $P<0.05$.

3.3 RESULTS

The biochemical and physiological analyses of tissue and blood parameters in this chapter were carried out on trout after 12 weeks exposure to dietary Cu (726 mg Cu Kg^{-1} d.w. food) during spring 1999 (Trial A). Apart from the analysis for lipid peroxidation products, which was carried out on tissues from fish exposed for 8 weeks to a similar Cu concentration (721 mg Cu Kg^{-1} d.w. food) during spring 2000 (Trial B). Physiological analyses results from the 2 separate exposure trials have been grouped into this chapter to provide an estimation of the extent of physiological damage due to dietary Cu-exposure.

3.3.1 Tissue ion and metallothionein levels

Copper exposure caused a marked increase in Cu concentration within the liver (Mann-Whitney U-test, $U = 58$, $P = 0.007$) and intestine ($U = 64$, $P = 0.0009$) of fish exposed to 726 mg Cu kg^{-1} feed for 12 weeks (Table 4). Similar liver Cu concentrations were also obtained from Trial B, exposing fish to 721 mg Cu kg^{-1} feed for 8 weeks. Fish from Trial A were further examined for Cu concentrations within other tissues, brain, spleen, heart, and striated red/white muscle which all remained below detection limits (<0.096 μmol g^{-1} d.w.) regardless of treatment. Cu-content of the gills was around 0.01 μmol g^{-1} d.w. for both treatments (Table 4). The elevation of Cu within tissues caused a significant increase in hepatic (8-fold; t-test, $t = -20.44$, $P < 0.0005$) and intestinal metallothionein (2-fold; $t = -6.04$, $P = 0.0005$) of exposed rainbow trout compared to controls (Table 5). However, metallothionein concentrations in the anterior gut (stomach plus oesophagus) did not change significantly, suggesting that the intestine is the primary site of Cu absorption in the gastrointestinal tract. Branchial metallothionein concentrations did not change, reflecting the absence of Cu accumulation in the gills.

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Table 4. Tissue ion concentrations of control and fish fed dietary Cu (726 mg Cu kg^{-1} feed) after 12 weeks exposure (Trial A). Data are mean ± S.E. (n = 10). * statistically significant from control (P<0.05, Mann-Whitney U-test). B.D. below detection limit of ICPAES 0.001 (μmol g^{-1} d.w.). In Trial B determination was only undertaken for liver [Cu], results were 2.4 ± 0.24 and 16.12 ± 1.33 μmol g^{-1} d.w. (mean ± S.E., n = 10) for control and Cu-exposed fish respectively.

		Ca	K	Mg	Na	Zn	Cu
Brain	Control	39.7 ± 30	36 ± 7.6	12 ± 6	293 ± 98	2.93 ± 1	B.D.
(μmol g^{-1} d.w.)	Cu	11.4 ± 15	38 ± 12	5 ± 5	283 ± 65	0.9 ± 1	B.D.
Gill	Control	353 ± 48	17 ± 8	21 ± 4	176 ± 21	6.56 ± 2	0.011 ± 0.004
(μmol g^{-1} d.w.)	Cu	360 ± 30	18 ± 4	22 ± 4	170 ± 11	4.72 ± 2	0.013 ± 0.008
Intestine	Control	41 ± 14	29 ± 6	27 ± 4	64 ± 14	7.7 ± 1.2	0.12 ± 0.04
(μmol g^{-1} d.w.)	Cu	60 ± 38	37 ± 15	30 ± 11	117 ± 25	10 ±3.4	1.72 ± 0.07*
Liver	Control	3.9 ± 1.3	46 ± 18	14 ± 4	140 ± 15	0.78 ± 0.8	4.68 ± 0.65
(μmol g^{-1} d.w.)	Cu	5.9 ± 2	41 ± 15	17 ± 2	151 ± 13	2.87 ± 1.2	17.37 ± 0.36*
Muscle	Control	49.5 ± 4	76 ± 43	28 ± 5	56 ± 16	0.40 ±1.02	B.D.
(μmol g^{-1} d.w.)	Cu	48.9 ± 5	98 ± 36	32 ± 3	72 ± 11	0.6 ± 0.8	B.D.
Spleen	Control	198 ± 32	-	92 ± 54	109 ± 143	7.4 ± 3.4	B.D.

Table.5 Tissue metallothionein (MT) after 12 weeks dietary Cu-exposure (Trial A).

	Control Diet (23 mg Cu kg^{-1} feed)	Copper diet (726 mg Cu kg^{-1} feed)
Hepatic MT (μg g^{-1} w.w.)	18.3 ± 1.8	143.9 ± 6.2*
Intestinal MT (μg g^{-1} w.w.)	15.3 ± 2.8	30.8 ± 2.1*
Ant. Gut MT (μg g^{-1} w.w.)	16.73 ± 2.1	20.15 ± 1.45
Gill MT (μg g^{-1} w.w.)	33.1 ± 1.1	27.2 ± 4.7

Data are mean and S.E. (n = 6). * statistically significant from control (P< 0.05, t-test).

Intestinal MT is analysed in lower part of gut including lower intestine and pyloric ceaca, and Ant. MT refers to anterior gut.

Table 4 summarises the concentration of key ions within the tissues of rainbow trout after 12 weeks Cu-exposure (726 mg Cu kg^{-1} feed). Ca, K, Mg, Na, Zn showed no significant (t-test, $P<0.05$) disturbances in any of the measured organs of Cu-exposed fish. For example, brain Na and K concentrations (mean ± S.D., n = 8) were 283 ± 65 and 37.9 ± 11.8 μmol g^{-1} d.w. in Cu-exposed fish compared to 293 ± 98 and 36.3 ± 7.6 μmol g^{-1} d.w. in controls respectively. In striated muscle Na and K concentrations were 71.6± 10.45 and 98 ± 36.4 μmol g^{-1} d.w. in Cu-exposed fish compared to 56.3 ± 15.9 and 76.36 ± 43.3 μmol g^{-1} d.w. in controls. Thus, Na:K ratios in both brain (Na:K, ~ 8) and muscle (Na:K, ~ 0.7) were normal. Similar observations on Na and K concentrations were also evident for the heart.

3.3.2 Growth and nutritional performance

Overall there was no change in the mean growth rate (Table 6) of the control and Cu-exposed fish (Mann Whitney, U-test, U = 1182.5, P = 0.68) after 12 weeks dietary Cu-exposure (Trial A). Food conversion ratios were similar between groups and the proximate composition of the carcass showed no significant differences between treatment groups. Although Cu-fed fish did have a slightly higher lipid and lower ash content than those fed a control diet, this was not significant.

3.3.3 Blood chemistry

No disturbance was observed to general haematology (haemoglobin, haematocrit, red and white cell counts, protein, triglycerides or glucose) at the end of a 12 week Cu exposure (726 mg Cu kg^{-1} feed) period (Table 7). The normal haematocrit values and red cell counts suggest that no osmotic disturbances or alteration to red cell volume occurred due to Cu-

Table 6. Growth and nutritional performance in fish fed dietary copper (726 mg Cu kg^{-1} feed) after 12 weeks exposure (Trial A)

	Control Diet (23 mg Cu Kg feed^{-1})	Copper diet (726 mg Cu Kg feed^{-1})
Initial mean weight (g)	5.0 ± 0.2 (100)	5.0 ± 0.2 (100)
Final mean weight (g)	21.3 ± 1.5 (55)	20.4 ± 1.5 (79)
Specific growth rate (% day)	1.72	1.67
Feed conversion ratio	0.94 ± 0.08 (55)	0.91 ± 0.07 (79)
Condition factor	1.42 ± 0.05 (55)	1.39 ± 0.08 (79)
Proximate carcass composition		
Moisture (%)	68.7 ± 1.1 (8)	65.9 ± 1.5 (8)
Protein (%)	15.4 ± 0.7 (8)	15.1 ± 1.8 (8)
Lipid (%)	11.2 ± 1.8 (8)	12.9 ± 1.2 (8)
Ash (%)	2.42 ± 0.1 (8)	1.91 ±0.09 (8)

Data are mean ± S.E. (n = 10). Data was tested for statistical differences using F-test and Two Tailed Students t-test ($P < 0.05$). No differences were observed.

Table 7. Blood chemistry in fish fed dietary copper (726 mg Cu kg^{-1} feed) after 12 weeks exposure (Trial A).

	Control Diet (23 mg Cu Kg feed^{-1})	Copper diet (726 mg Cu Kg feed^{-1})
Red blood cells (10^6 mm^3)	0.74 ± 0.04	0.75 ± 0.03
White blood cells (10^3 mm^3)	11.9 ± 2.0	14.9 ± 2.5
Haemoglobin (g dl^{-1})	7.19 ± 0.13	7.04 ± 0.10
Serum glucose (mmol l^{-1})	6.4 ± 0.6	7.4 ± 0.9
Serum protein (g l^{-1})	15.4 ± 2.4	13.0 ± 0.9
Serum triglcerides (mmol l^{-1})	3.17 ± 0.8	3.27 ± 0.7
Serum cortisol (nmol l^{-1})	200 ± 42	262 ± 55

Data are mean ± S.E. (n = 10). Data was tested for statistical differences using F-test and Two Tailed Students t-test, no differences between treatments were observed.

exposure. The normal haemoglobin content of the blood indicates that the O_2 carrying capacity was maintained in Cu-exposed fish. Circulating cortisol levels were slightly elevated within the Cu-exposed group but within-group variability probably masked any significant effects.

3.3.4 Tissue enzymology

Lactate dehydrogenase (LDH) levels were examined within the liver, skeletal and cardiac muscle of fish exposed to 726 mg Cu kg^{-1} feed for 12 weeks (Trial A). Exposed fish showed a 140% elevation in LDH concentration within the cardiac muscle compared to controls (Table 8). An opposite effect occurred within the skeletal muscle with Cu-fed fish showing a 47% reduction in LDH levels compared to controls. Liver LDH was unchanged by dietary Cu-exposure. Similarly, Cytochrome C oxidase levels showed no significant difference between control and Cu-exposed fish in liver, skeletal and cardiac muscle (Table 8).

Tissue malondialdehyde (MDA) concentration, used to indicate the degree of tissue lipid peroxidation (Fig. 4), showed a significant increase within the liver of fish exposed to 721 mg Cu kg^{-1} feed for 8 weeks (Trial B) compared to controls (t-test, $t = 3.6$, $P<0.05$). This increase confirmed that cellular damage had occurred within the liver, and this was attributable to the increase in Cu concentration within the organ. MDA levels for both treatments were 2-fold higher in cardiac and skeletal muscle compared to the liver, but did not show any significant difference between treatments.

Table. 8 Tissue biochemistry in fish fed dietary copper (726 mg Cu kg^{-1} feed) after 12 weeks exposure (Trial A).

	Control Diet (23 mg Cu Kg feed^{-1}	Copper diet (726 mg Cu Kg feed^{-1}
Liver LDH (μmol min^{-1} g^{-1} w.w.)	100.3 ± 26.9	87.7 ± 14.6
Cardiac muscle LDH (μmol min^{-1} g^{-1} w.w.)	215.3 ± 17.8	518.2 ± 115.6*
Skeletal muscle LDH (μmol min^{-1} g^{-1} w.w.)	87.5 ± 6.5	56.5 ± 5.0*
Liver Cyto C oxidase (μmol Cyt C oxi mg protein^{-1} min^{-1})	0.14 ± 0.089	0.147 ± 0.12
Cardiac Cyto C oxidase (μmol Cyt C oxi mg protein^{-1} min^{-1})	0.379 ± 0.07	0.6 ± 0.5
Skeletal muscle Cyto C oxidase (μmol Cyt C oxi mg protein^{-1} min^{-1})	0.167 ± 0.04	0.14 ± 0.12

Data are mean and S.E. (n = 10). * Statistically significant from control (P< 0.05, t-test).

Figure 4. Lipid peroxidation within the liver, skeletal and cardiac muscle of control (dark bars) and Cu-exposed (striped bars) fish. Tissue collected from fish exposed to 721 mg Cu kg^{-1} feed for 8 weeks (Trial B). * data that is statistically different from control (t-test, t= 3.6, P<0.05).
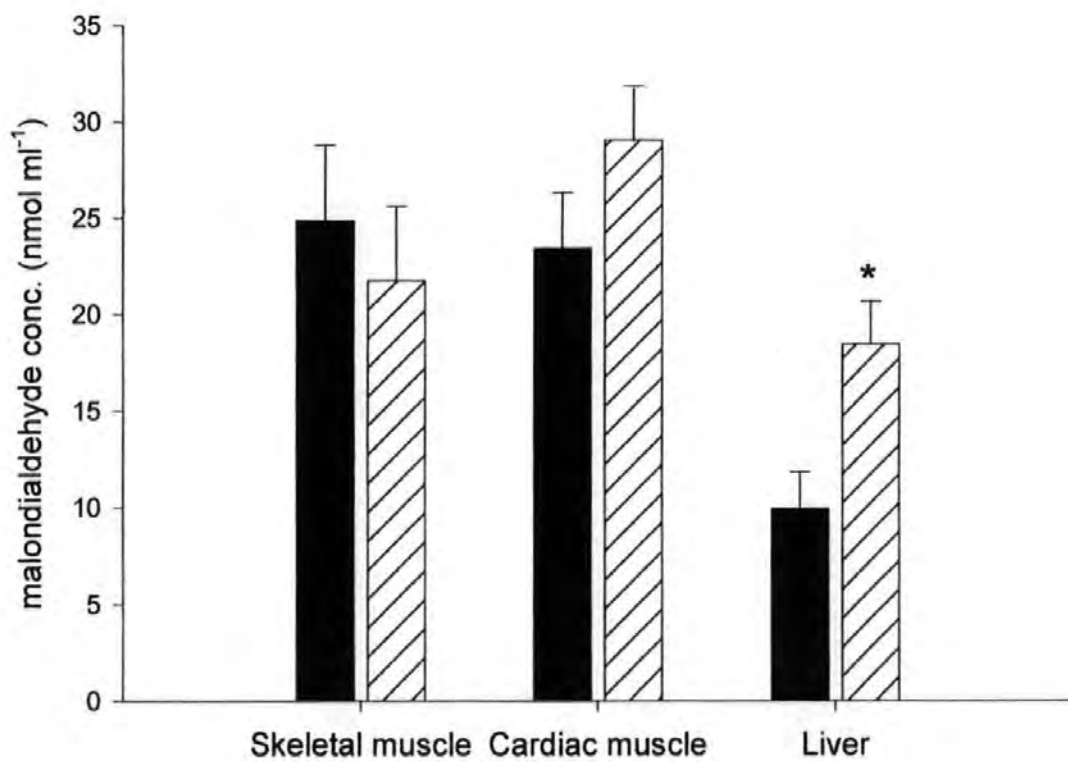
3.4 Discussion

This study as whole was concerned with the bioenergetics of trout exposed to elevated dietary Cu. The investigation of compensatory responses and energetic shifts elicited by the animal due to Cu effects will be dependant on the amount of copper entering the body of the animal, its distribution to other organs and resultant deleterious effects to cellular homeostasis. This of course will be modified by energy fluxes associated with energy uptake from the food, and degree of investment in detoxification procedures.

Cu accumulation and metallothionein

The exposure of rainbow trout to dietary Cu (730 mg Cu Kg^{-1} d.w. feed) resulted in a significant accumulation of Cu within the liver and intestine of the exposed fish, indicating an overloading of the Cu regulating capacity. However, there was no significant increase in mean liver Cu content between the 8 week (Trial B) and 12 week (Trial A) exposures, suggesting that sufficient excretory mechanisms had been initialised by at least 8 weeks exposure which checked further Cu-accumulation within the liver of exposed fish. Liver [Cu] in this study were similar to results by Kamunde et al. (2001) who exposed rainbow trout to an elevated copper dose of 1000 mg Cu kg^{-1} d.w. feed. for 4 weeks, further suggesting that an upper [Cu] limit is tolerated in the liver, after which excretory mechanisms are established and check further accumulation. The absence of any water Cu concentration and absence of Cu accumulation within the gills, indicated that there was no significant leakage from experimental pellets and the dietary route was the only means of exposure. ICPAES analysis of other tissues showed no significant accumulation of Cu (brain, muscle, spleen, plasma), and the pattern of tissue Cu accumulation observed within this study was typical of that reported in other chronic dietary Cu-exposures (Knox *et al.*, 1982; Lanno *et al.*, 1985; Gatlin *et al.*, 1989; Berntssen *et al.*, 1999; Handy *et al.*, 1999,

Kamunde *et al.*, 2001). This evidence and the fact that the accumulation of Cu within the intestine and liver was similar to levels reported by Handy *et al.*, (1999) and Benstssen *et al.*, (1999) despite the higher Cu dose used in this study, highlights the important role of the intestine in controlling the uptake of dietary copper. The induction of metallothionein (MT) is considered to be a homeostatic mechanism that responds to increases in the cellular content of potentially toxic metals (Roesijadi, 1992), reducing their bioavailability to metabolically important pathways in the cell. The 3-fold increase in MT within the intestine suggests that Cu is being actively chelated in the intestinal mucosa, whilst its absence in the anterior gut suggests that the major site of excess Cu entry is limited to the pyloric caecae and large intestine only. Contaminated intestinal mucosa may be sloughed as a possible route for excretion of the Cu, and maybe related to increased cell turnover observed in dietary heavy metal exposed fish (Greene and Moran, 1994; Berntssen *et al.*, 1999).

Once absorbed across the intestinal mucosa Cu is transported via the hepatic portal vain to the liver, which is suggested to be the main organ involved in detoxification and excretion of excess Cu (Bremner, 1987). Here it is sequestered by hepatic MT and locked into distinct membrane bound cytoplasmic inclusions in the hepatic parenchyma (Lanno *et al.*, 1987). Cu-exposed fish showed a 9 fold increase in hepatic MT, over controls and MT levels were similar to those reported by Handy *et al.* (1999) who exposed rainbow trout to a dietary Cu dose 33% less than that used in this study for an equal time period. Perhaps further indicating that the intestine deals with a major burden of the dietary Cu load and only a regulated portion, that can be sufficiently dealt with by hepatic MT is passed through to the liver. The absence of metallothionein production in the gills further indicates that the gills remained uncontaminated during the dietary exposure.

Effects of dietary Cu on fish physiology

The regulation of dietary copper by trout within this experiment seemed to be sufficient, and exposed fish had steady-state tissue Cu levels without significant physiological dysfunction, indicated by the lack of tissue ion disturbances in most organs. Except Mg concentrations within the spleen whose decreased Mg content in Cu-exposed fish might suggest splenic release of erythrocytes ([Mg] red cells around 7 mmol l^{-1}, Houston and Gray, 1988). During aqueous Cu-exposures disturbances to internal ion concentrations have been reported (Miller *et al.*, 1992; Pilgaard *et al.*, 1994), however, waterborne Cu-exposures will cause gill damage associated with ion-regulatory disturbances (Sola *et al.*, 1995). In this study the gills remained clear from Cu contamination and it could be expected that normal gill function was maintained, and ion transfer across the gills was not impeded. Handy *et al.*, (1999) observed a lack of ion disturbance in trout exposed to 500 mg Cu kg^{-1} d.w. feed. The authors concluded that physiological homeostasis was maintained, and similar findings could be assumed within this study exposing trout to the higher (730 mg Cu kg^{-1} d.w. feed) dietary Cu dose.

In this study, Cu-exposed fish elevated intestinal and hepatic MT to bind with free Cu in the liver. However, an accumulation of lipid peroxidation products resulting from Cu-induced free radical damage was found in the liver of trout after 8 weeks Cu-exposure (Trial B). The accumulative levels of malondialdehyde observed in Cu-exposed fish, indicate that a degree of cell death and possibly tissue damage occurred in the liver. Malonaldehyde levels recorded here were comparable to those observed by Radi and Matkovics (1988), in carp exposed to waterborne CuSO4, and by Farag *et al.*, (1995) in trout exposed to a mixture of heavy metals (As, Cd, Cu, Pb, Zn). The level of oxidative damage observed in this study agrees with observations of previous authors, suggesting that the degree of observed damage was not severe enough to cause liver dysfunction. The lack of lipid

peroxidation products or elevated Cu in other tissues (muscle, heart) suggests that, Cu absorbed across the intestinal wall was mostly sequestered by the liver and did not pass into the blood for transfer to other internal organs where it may have caused oxidative damage. This differs from aqueous Cu-exposure (e.g. waterborne exposure) where other organs become contaminated by Cu (Handy, 1992), and could therefore, be expected to undergo oxidative damage. Although lipid peroxidation products were not measured in the gut in this study, Farag et al. (1995) reported lipid peroxidation of the liver, pyloric caeca and large intestine of free ranging brown trout feeding on heavy metal contaminated invertebrates. Due to the elevated intestinal Cu and metallothionein levels found in Cu-exposed fish in the present study, it seems reasonable to assume that a degree of oxidative damage may have occurred. The lack of growth rate or nutritional effects would suggest that normal gut function was maintained and nutrient absorption was not particularly compromised. A histological study on midgut morphology of elevated dietary Cu (1000 mg Cu kg^{-1} d.w. feed) observed that dietary Cu modestly affected gut morphology, but agreed that Cu did not severely impact gut structure and function (Kamunde et al., 2001).

Haematological measurements are a good indicator of physiological condition and general health (Houston 1997), and circulating serum cortisol has been put forward as one of the most sensitive indicators in rainbow trout of stressful environmental conditions (Hane et al., 1966; Wedemeyer, 1969b; Grant and Mehrle, 1973; Barton et al., 1980; Hille 1981). My results showed no significant difference between treatments, and cortisol concentrations were typical of measurements from fish kept in social groups (Gregory and Wood, 1999). Moreover, heamatocrit values, triglycerides, glucose and proteins, all of which are used for the diagnosis of both dietary deficiencies and environmental stress (Blaxhall, 1972; Houston and De Wilde, 1972) were not different between treatments. The lack of Cu effects on erythrocyte and haemoglobin concentrations suggests that the O$_2$ delivery capacity of the

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blood was maintained. Indeed, leukocyte counts showed that immune response was neither over stimulated nor suppressed. If any change had occurred to general homeostasis due to dietary Cu-exposure the fish appeared to have acclimated fully by the 12th week of exposure.

The acclimation of fish to sub-lethal heavy metal toxicity has been well established (for reviews see McDonald and Wood, 1993; Grosell et al., 1997), and within this study despite some oxidative damage to the liver, physiological homeostasis was also maintained in rainbow trout after 12 weeks Cu-exposure (726 mg Cu kg^{-1} d.w. food). However, the mechanisms of detoxification and acclimation must necessitate a degree of energetic investment. Enzymes concerned with aerobic and anaerobic metabolism may provide indications of alteration in energy utilisation between activities. In this experiment, after 12 weeks exposure, Lactate dehydrogenase (LDH), which catalyses the reversible reduction of lactate to pyruvate, the terminal step that characterises glycolysis in vertebrates (Hochachka, 1969), and the mitochondrial enzyme Cytochrome C oxidase, which is integral to the oxidation-reduction components of the electron transfer chain concerned with ATP production (Lawrie, 1953), were measured. Both enzymes were unaltered within the liver of Cu-exposed fish suggesting energy metabolism and associated pathways within the liver were unaffected by Cu-exposure despite a degree of lipid peroxidation occurring in the liver of Cu-exposed fish. The heart is an aerobic tissue and a steady supply of energy is required and maintained by the oxidation of pyruvate in mitochondria (Vessel, 1965 cited in Hoar and Randall, 1978). Cardiac LDH activities in whole hearts from control fish were around 215 μmol min^{-1} g^{-1} (as previous reports, e.g. Clark and Rodnick, 1998), with mean values around 2.5 fold higher in Cu-exposed fish. This may indicate that heart muscle of exposed fish in this study is increasing glycolytic capacity (Clark and Rodnick, 1998) in an inefficient attempt to maintain the high-energy phosphates needed for muscle contraction.

Unlike in the heart, the energy requirements of the skeletal muscle usually arise suddenly and are supplied by rapid glycolysis and pyruvate formation (Hoar and Randall, 1978). The reduction in LDH levels here is unclear but may reflect a reduction in the activity of the muscle. This may be assumed, as a reduction in swimming activity has been documented in dietary Cu-exposed trout (Handy *et al.*, 1999). The reason for differential responses of cardiac and skeletal muscle LDH to Cu is unclear, but probably relates to differences in Cu-susceptibility and substrate utilisation for energy metabolism (Powell *et al.*, 1999). No alteration of Cytochrome C oxidase was observed in both muscle types examined, which suggests that mitochondrial integrity and aerobic pathways associated with the energetic state of the cardiac and skeletal muscle remained unaffected by dietary copper.

It should be observed that due to sampling of fish for other experiments during the 12 week exposure period, only a small group of individuals were available for physiological analysis. Although no significant alterations were observed between control and Cu-exposed fish this may have been masked by within treatment intra-individual variability, and a larger sample size is recommended for future physiological analysis.

An energetics perspective

This study showed that growth and nutritional parameters were unaffected in rainbow trout exposed to 730 mg Cu Kg^{-1} d.w. feed for 12 weeks. These findings are in agreement with that of other authors who also observed no suppression of growth parameters in salmonids fed on a diet of 684 mg Cu kg^{-1} d.w. feed for 42 days, and 1042 mg Cu kg^{-1} d.w. feed for 28 days, (Miller *et al.*, 1993; Kamunde *et al.*, 2001, respectively). However, Lanno *et al.*, (1985) observed that exposing rainbow trout to 730 mg Cu Kg^{-1} feed d.w. resulted in a reduction in specific growth rates. The contrast in results may be attributed to differences in the valence state of Cu as this will alter bioavailability and significantly affect the amount

of Cu up taken into the body of the fish across the intestinal mucosa (Woodward *et al.*, 1994). Miller *et al.*, (1993), observed that diets containing a mixture of soya and animal protein had a more deleterious effect on growth rates then diets containing animal protein solely, and concluded that the contrast in growth effects under similar Cu concentration was due to Cu bioavailability. Lanno *et al.* (1985) used soybean meal as a protein substitute in the feed ingredients whereas in this study fishmeal was used solely.

A possible cause of growth reduction may be due to food refusal by the experimental fish. It has been suggested that the palatability and nutritional state of a diet is reduced in heavy metal exposure trials (Lanno *et al.*, 1985a, b; Woodward *et al.*, 1995), arising from the oxidation of lipids within the diet (Cowey *et al.*, 1984). Baker *et al.*, (1998) found that in a diet of 2396 mg Cu kg^{-1} d.w. feed there was a 304% rise in lipid peroxidation products and a 146% fall in α–tocopherol (Vit E) content, which acted to reduce food palatability and decreased energy content.

Diets in this study were frozen immediately after production and defrosted only a few hours before feeding, which most likely reduced Cu oxidising effects (Baker *et al.*, 1998). This appeared to have the desired effect and Cu-exposed fish did not have an observable suppression of appetite, and consumed all food presented. In Lanno *et al.*, (1985) study, appetite suppression and reduced growth rates did occur. However, fish were fed to satiation and not a fixed ration as in this study so a direct comparison on food intake rates could not be made.

After 12 weeks Cu-exposed fish had maintained physiological homeostasis, sustained growth rates and showed no indication of stress response. The role of the intestine played an efficient part in Cu sequestration and detoxification, limiting Cu uptake into the body of the fish. However, this would have taken an increased degree of energetic investment in maintenance, compared to that of fish digesting a control diet. Recent

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histological studies have shown an increase in intestinal mitochondria of dietary Cu-exposed (1000 mg Cu Kg^{-1} d.w feed) trout after 28 days exposure (Kamunde *et al.*, 2001). The authors suggested that this was probably due to an increased energy demand within the intestinal tissue to prevent Cu uptake. The Cu that did diffuse across the intestine into the blood stream was dealt with efficiently by the liver (MT induction, this study; intracellular compartmentalisation; Lanno *et al.*, 1987), preventing transport of Cu to other organs in the body. This would have required further energetic investment, and it seems probable that the cost of maintenance in Cu-exposed fish would have been greater than in controls. Bioenergetics theory predicts that an animal must first satisfy the energy demands of maintaining bodily functions (M$_S$) before energy is available for other processes such as activity, growth and reproduction (Calow, 1985). If the energy demands of M$_S$ are increased then it can be assumed that, if energy intake is constant, allocation to other components will be reduced. Within this experiment, fish were of a size too small for reproductive investment, and I observed no reduction in mean growth rates in Cu-exposed fish. Therefore, energy allocation must be redistributed from the remaining components of the metabolic equation. It is this redistribution of energy between components of the energetic equation for detoxification, and concomitant effects that will be investigated in further chapters.

CHAPTER FOUR

THE EFFECTS OF CU-EXPOSURE ON METABOLIC RATE AND
LOCOMOTORY BEHAVIOURS

4.1 Introduction

Within the confines of the energy equation (Chapter 1), energy gained by the animal will be that acquired from food after the processes of digestion and absorption, whilst energy expenditure will be that associated with somatic maintenance, growth, activity, reproduction and excretion (Elliot and Hurley, 1999; Kooijman *et al.*, 1999). In juvenile trout, reproductive costs are negligible, hence most of the energy acquired from food will be spent on growth, somatic maintenance, and metabolic activities such as swimming (Priede, 1985; Kooijman *et al.*, 1999).

Measurement of metabolism (M) in fish, and the relationship of M to the many biotic and abiotic environmental factors, is a basic requirement for the understanding of the processes involved within the energy budget equation. Direct calorimetry has been successfully applied to fish metabolism studies (Smith *et al.*, 1978; Van Ginneken et al., 1996). However, it is more usual to measure M through indirect calorimetry by measuring rates of oxygen consumption, and this technique has been used successfully for a number of years (Brett, 1964, Beamish 1964a, b. Webb, 1971, Jobling, 1982).

In studying metabolism via oxygen consumption a number of hazards can arise leading to large variability in the data. Firstly, variability itself may be inherent to the species, and differences in the activity of individual animals may alter O_2 consumption rates. Secondly, oxygen debt may occur at intense levels of muscular activity, leading to the anaerobic oxidation of pyruvic and lactic acid. If unknown debt is occurring, it means the rate of oxygen uptake would be less than demand, and consequently an error would be

introduced if performance was being related to O_2 consumption (Black *et al.*, 1962). Finally, environmental variables may influence oxygen consumption rates. Such as oxygen availability, which may be reduced by the interference of CO_2, NH_3 and pH, as a consequence of excretion, and temperature, which can increase the rate of metabolic reactions, and thus increase metabolism. It has been suggested that a 1^0C rise in temperature may produce an error of 20% in O_2 consumption values (*reviewed by* Fry, 1971).

The relationship between O_2 consumption rates and activity has been defined by Brett's (1964) model, which has been schematically shown in Chapter 1, fig 1. The difference between the rate of oxygen consumption at rest and at the stage of maximum (aerobic) activity has been termed the animal's *aerobic scope for activity* (Weiser, 1985). If the metabolic rate of a fish at rest was increased, and/or the Ucrit level was reduced then the animals aerobic scope would be reduced. For fish the maximum metabolic rate is primarily limited by the ability of the gills to extract O_2 from the water, and a general correlation exists between gill area and activity of different species (Gray, 1954). Fish must function within their aerobic scope (Fry, 1947), and during normal existence metabolism will fluctuate around an average level, termed 'routine metabolism' (Fry, 1957; Beamish 1964a). If metabolism is depressed below the basal metabolic rate it will impair physiological function, and life cannot be sustained for long. Similarly, if the fish is forced to work at or above the active or maximal metabolic rate, this cannot be sustained indefinitely and death will ensue (Priede, 1985).

In determining the sub-lethal effects of pollutants on fish, scope for activity has been recommended as a sensitive indicator (Brett, 1967; Sprague 1971), and both swimming capacity (maximum aerobic swimming ability) and swimming activity (voluntary spontaneous swimming) are commonly used to assess contaminant-related changes in locomotion, accurately reflecting the effects of pollutants on specific metabolic reactions

(Ellgaard *et al.*, 1977; Malizi *et al.*, 1984; Ellgaard and Guillot, 1988). Behaviour is the first compensatory modification by fish to environmental change occurring previous to alterations in growth (Scarfe *et al.*, 1982). It is also particularly important in terms of organism survival, indicating the fish's ability to feed, escape predators, maintain position in the current (Beamish, 1978), and is particularly important to fish such as salmonids that migrate upstream to spawn (Brett, 1965).

In general, aqueous heavy-metal pollution causes a mixture of ionoregulatory and respiratory impairments stemming from the disruption of gill epithelial tight junctions and ion transporting ATPases (Spear and Pierce, 1979; Spry *et al.*, 1981; Pilgaard et al., 1994). The swimming capacity of rainbow trout was reduced after exposure to copper at 12% of the LC_{50} (Waiwood and Beamish 1978), similar exposure concentrations have also been found to inhibit swimming activity (Drummond *et al.*, 1973). During chronic exposures Cu-exposure causes a degree of hypoactvity in rainbow trout in a concentration dependant manner (Ellgaard and Guillot, 1988). This is suggested to occur due to a reduction in O_2 uptake across the gills, reducing O_2 availability for oxidative phosphorolation during cellular ATP production (O'Hara, 1971), required for efficient muscle function. However, in Cu-exposure studies nearly all the literature has concentrated on waterborne exposures where Cu will enter the animal via the gills, causing the fore mentioned disturbances to branchial epithelium. In contrast to aqueous Cu-exposure, exposure via the diet will leave the gills intact (Handy, 1996), which suggests that respiration and branchial osmoregulation disturbance does not limit behavioural capacity under these conditions. Handy *et al.*, (1999) is the only study to date that measured the dietary effects of Cu on swimming activity. In this latter study chronic exposure of rainbow trout to dietary Cu (500 mg Cu kg^{-1} d.w. feed) resulted in a 35% reduction in the time spent swimming. This reduction in activity was suggested as a compensatory mechanism; energy was diverted from active metabolism (M_A) to aid in

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detoxification and excretion of the copper load via the gastrointestinal-biliary-hepatic system, whilst growth rates were maintained.

In this chapter these compensatory shifts in energy partitioning will be further explored. Firstly, rainbow trout were exposed to a higher dietary Cu load than that used by Handy *et al.*, (1999), to assess if reductions in activity were correlated with toxicant dose. Secondly, O_2 consumption during voluntary spontaneous activity was measured and back extrapolated to zero activity (Brett, 1964: Tytler, 1969) to see if reductions in locomotor activity by the fish met the extra metabolic cost of detoxification. I improved on previous attempts to relate VO_2 with activity (Waiwood and Beamish 1978; Weiser, 1985), by simultaneously recording these parameters in free-swimming fish that could voluntary adjust spontaneous swimming activity. Finally, to assess Cu-exposure effects on ecologically important behaviours, I examined the fish's locomotory activity associated with feeding where net energy gain was a function of energy expended in obtaining food and energy acquired from it.

4.2 MATERIALS AND METHODS

4.2.1 Simultaneous oxygen consumption and swimming speed measurements

Rainbow trout exposed to dietary Cu (730 mg Cu kg^{-1} d.w. feed) during Trial A and controls were measured for VO_2 using the indirect calorimetric method of rate of oxygen consumption whilst swimming under spontaneous voluntary conditions. The VO_2 of 3 trout per treatment was determined weekly between weeks 2 and 12 of exposure, during this period the fish ranged in size from 6 ± 0.2 g at week 2 to 25 ± 0.2 g at week 12.

Respirometer hardware

The chamber of the respirometer consisted of opaque Perspex walls with a transparent lid to enable video monitoring (Fig. 5, 6 & plate 1). The lid was sealed with 8mm neoprene ring held down by 6 butterfly nuts. The dimensions of the chamber were 40 L x 20 W x 20 H cm and allowed the fish (6-12 cm) to swim and turn freely. A recirculating system was constructed for each chamber with water pumped around the respirometer using a Maxi-Jet 500 pumphead (J & K Aquatics U.K.). A perspex baffle (bore size, 3mm) was set 2 cm back from the chamber water inlet and served to reduce eddies and aid laminar flow of the water within the respirometer chamber, required to increase accuracy of dissolved O_2 measurements by reducing dead space within the chamber. A flow rate of 2 cm s^{-1} across the chamber was considered sufficient to induce normal slow swimming (Wieser, 1985). This was determined using adjustable inlet and outlet taps, and calibrated using a 2-axis Electromagnetic Current Flow Meter Series 800 (Devon, U.K.). Each respirometer was fitted with an external 3L cartridge filter (Ehiem 2213) containing a chemical filtrate of powdered charcoal and Xeolite (NH_3 absorbent, J & K Aquatics Ltd.). After each trial the respirometer

Figure 5. Operational diagram of an individual closed respirometer system used to simultaneously monitor VO_2 and spontaneous swimming activity. Fish were monitored for swimming activity within the metabolic chamber (40L x 20 W x 20 H) under a flow rate of 2 cm s^{-1}. Blue arrows show direction of water flow, and 3-headed arrows represent 3-way taps. Water exiting the respirometry chamber was circulated through a filtration unit during acclimation, but this was bypassed during the 2 h analysis period. For water analysis discreet water samples were removed from the respirometery system and pulled through a flow cell containing an O_2/temp and a pH probe, using a peristaltic pump. After analysis, water was returned back to respirometer.

Pump

Venturi
Air inlet

Chemical
Filtration
tap

Metabolic chamber

baffle

Flow control
tap

Filter bypass
tap

Datalogger

3-way tap

Peristaltic
pump

pH

O_2

O_2

Flow cell containing O_2/temp & pH probe
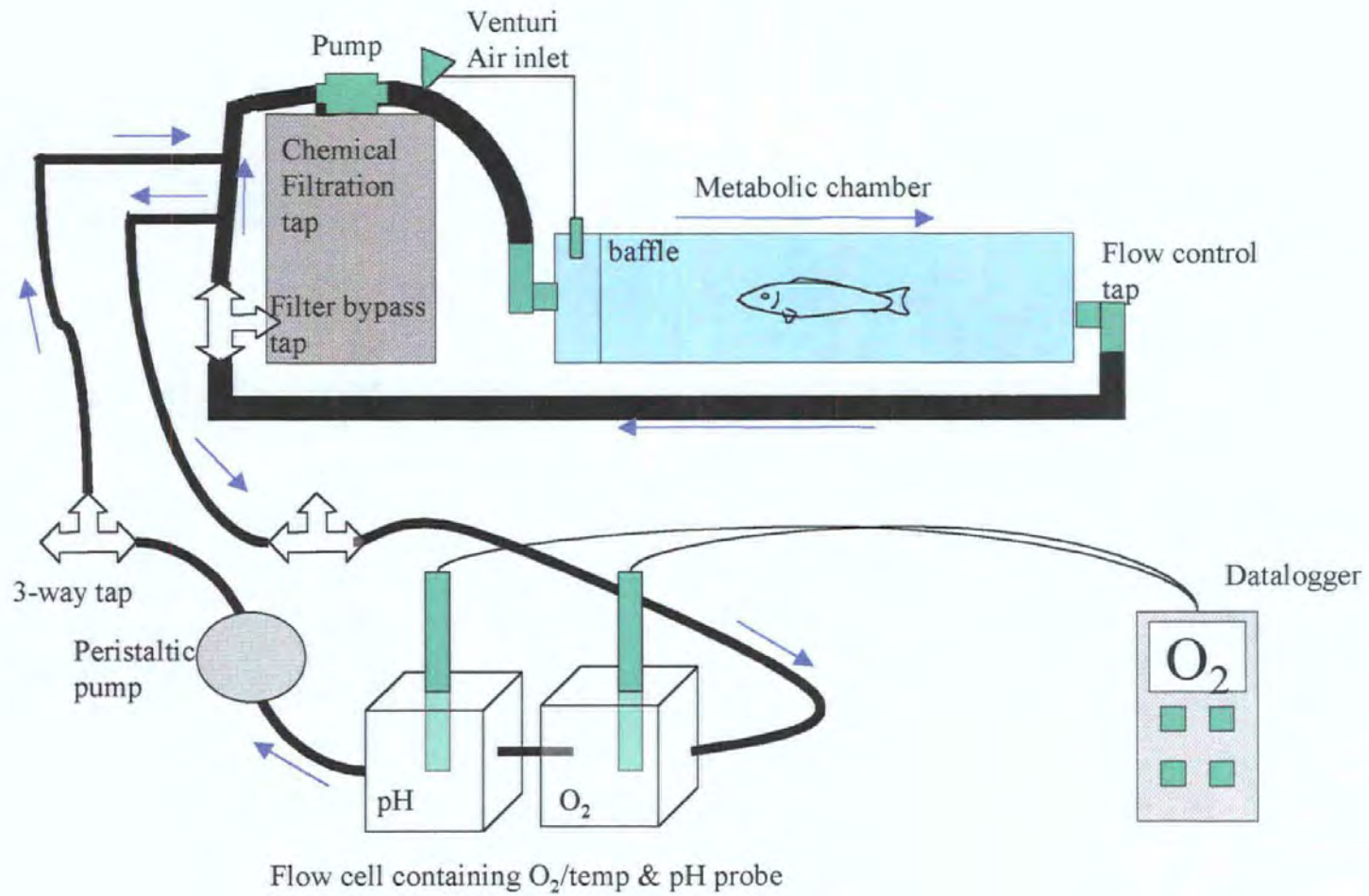
Figure 6. A schematic diagram showing the arrangement of a series of 7 respirometers used for measurement of VO_2 and spontaneous swimming activity. Each respirometer is a separate closed system, observed from above by a camera positioned 1.5m directly above chambers, and connected to the Ethovision tracking system. Blue arrows show the direction of water flow through each individual respirometer, and the direction of water flow through the flow cell containing O_2/temp and pH probes. For water analysis, discrete samples were taken from the desired respirometer via the 3-way tap batteries, and water was pulled through a flow cell containing O_2/temp and pH probes, using a peristaltic pump, after analysis the water sample was returned to relative respirometer using the return 3-way tap battery.

Chilled water bath

Flow reducer tap

Venturi

Respirometery Chamber

Baffle

Pump

Filter cartridge

3-way tap

Camera

Water sampler

Return, 3 way-tap battery

3-way tap battery

Water bath

Pump

pH

O₂

Ethovision and PC
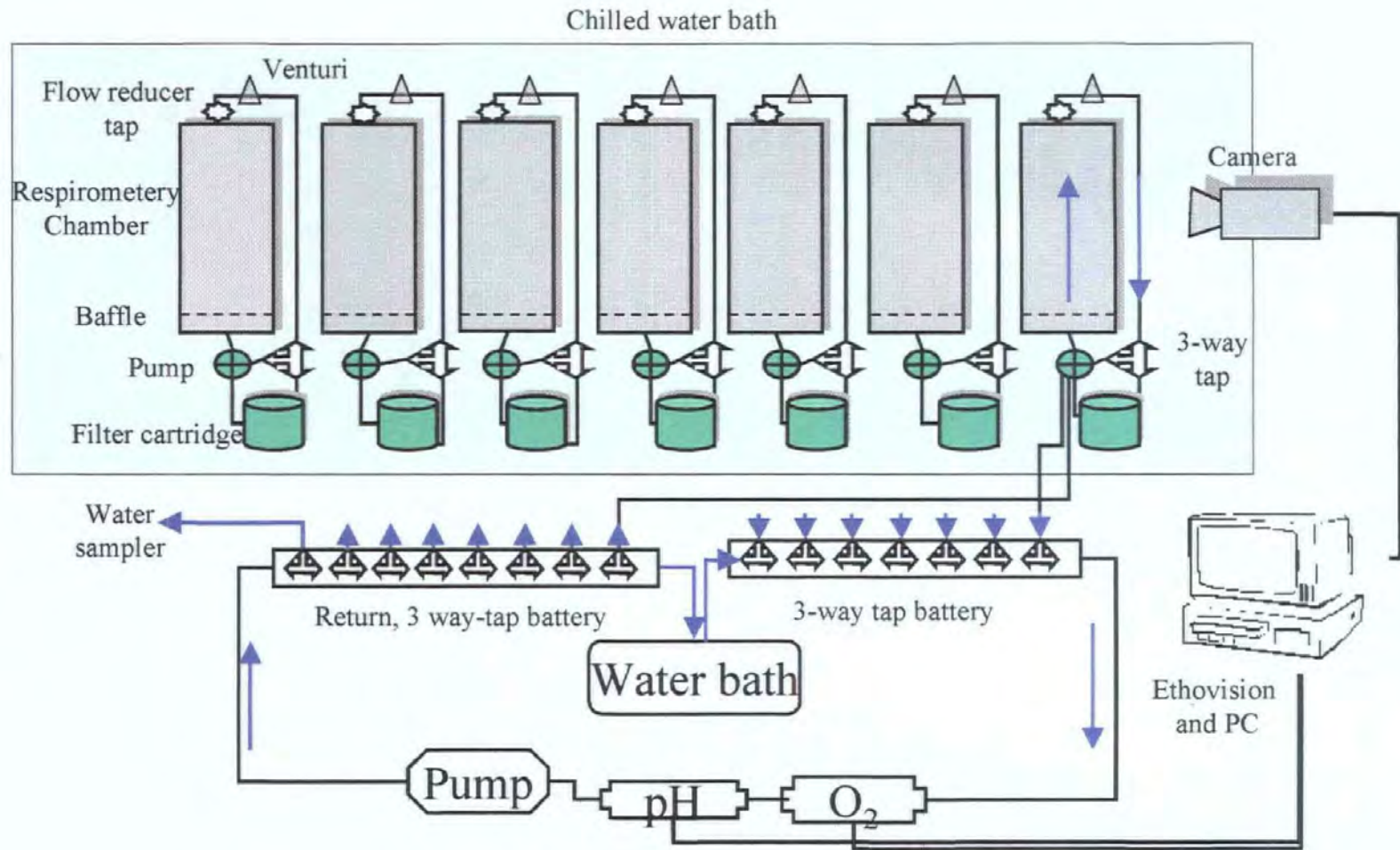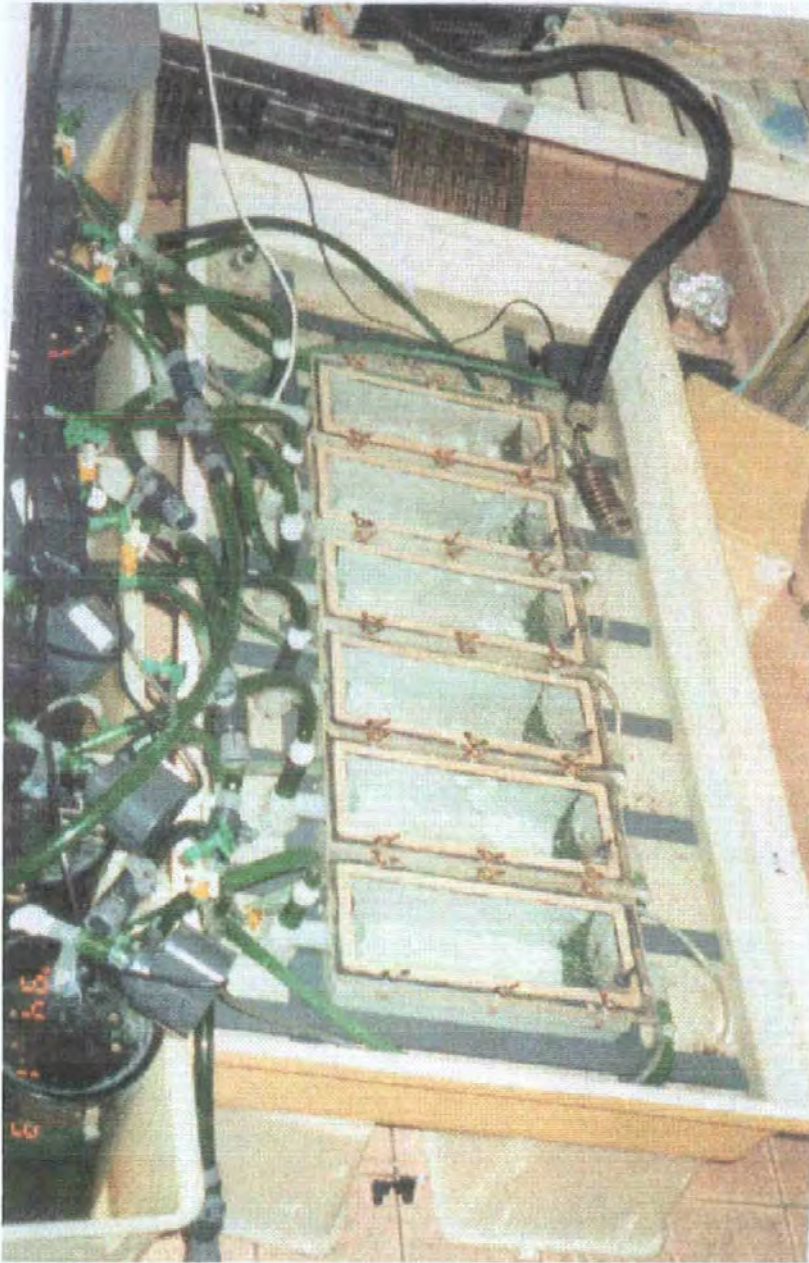
Plate 1. Series of 6 respirometers used for simultaneously measuring O_2 consumption whist simultaneously video tracking fish from above (the vacant slot at far end is position of empty chamber used for determining bacterial O_2 consumption rates). Individual respirometers were held within a chilled water bath at 15^0C by a rigid grid (grey) to prevent movement of chambers during video tracking. Separate filtration units for each respirometers (green) are also shown.

was emptied and refilled with fresh water from the same source as that used to supply the main exposure aquarium. Aeration of the respirometer was achieved using a venturi system. This was constructed from a T-piece inserted in the Eheim tubing carrying water from the pump head to the respirometry chamber (Fig. 4). The leg leading off of the T-piece was of a reduced size (3mm) and created sufficient suction to draw enough air into the system to keep the O_2 partial pressure within the chamber water at 100% $\pm$ 4% (mean $\pm$ S.D., n = 49). An airline tap attached to the T-piece could be closed preventing air intake during experimental recordings. Seven chambers were constructed as above and secured in a perspex frame in a water bath chilled to 15°C using a dip cooler (Spacecooling. U.K.). The cooled water was circulated around the chambers using a Maxijet 250 pumphead (Fig. 5).

Recording chamber O_2

To determine VO_2 a system was designed so that discrete water samples could be extracted from individual systems, analysed and returned to the same chamber without disturbing the fish (Fig 5 & 6). This was achieved by forcefully extracting water from respirometers using a peristaltic pump (SCHUCO, model 051-P, 850 ml min^{-1}) via asymmetric T-pieces (12mm bore cross piece with a 3mm leg) set in the Eheim tubing returning water from the chamber back to the pump head. Water could be extracted from each desired respirometer in turn using a battery of 3 way taps in series (one for each respirometer). Once extracted, test water was directed through 2 flow cells constructed from clear perspex blocks 5 x 5 x 10 cm attached to a 2 x 10 x 20 cm base and joined via 0.4 cm bore tubing. In the first block, an inlet point 3cm from base allowed water to flow into an internal compartment where a combined oxygen/temperature probe (WTW OX325) was situated. This block was air sealed using 3 silicon greased O-rings. The outlet port for the cell was on the opposite face from the inlet port and 2 cm higher to allow a standing pool of water to develop in the sampling cell.

Water flowed from this cell into the second cell for pH measurement using a pH probe (WTW SenTix 41-3). The water was then returned via the peristaltic pump back to its originating chamber. To return water after measurement to its respective respirometer, a battery of 3-way taps in reverse order to the inlet 3-way tap battery was used hence the internal space of the O_2 recording system was kept constant regardless of which respirometer was sampled. The 3-way tap system ensured that each chamber was a closed system, but water could be sampled from any desired chamber periodically. The use of the same probes and method of measurement for all respirometers reduced sampling variability. The exchange time for the flow cells containing the probes was evaluated using chambers filled with water of different pH's (4 & 7) and measuring the change in pH, within the flow cell. The 95% exchange time of the cell was 5 ± 0.25 sec (mean $\pm$ S.D. n = 10). To sample water from desired respirometer the appropriate inlet tap was opened and the pump switched on for a period of 10 sec. The return tap to this respirometer was opened 5 sec after the pump was switched on. This 5 sec delay kept cross contamination of water between individual respirometers to a minimum (<0.1% during trial). After 10 sec the pump was switched off and the water was allowed to stand within the flow cell for 60 sec allowing the probes to stabilise ($\pm$ 1% of O_2 reading after 5 min stabilisation). Using this method water could be sampled and accurately measured for O_2, pH and temperature from each respirometer every 75 sec. Both pH and O_2 electrodes were calibrated using WTW standard solutions. oxygen, temperature and pH measurements were recorded on a WTW Multiline P4 data logger which downloaded the data every 75 sec to a Compaq contra laptop, with Multi/Achat II software. This recorded data straight into a spreadsheet labelling appropriate measurements with time and chamber number. This spreadsheet was later exported to Excel 6.0 for detailed analysis.

Measuring oxygen consumption

Each week, 6 fish were randomly selected (3 control, 3 Cu-treated) from the appropriate treatments, and were individually placed (randomly) in one of the 6 respirometers arranged in parallel. Fish were acclimated for a 48-h period and remained unfed before measurements. During the acclimation period chemical filtration cartridges and aeration were present, However, when recordings were made the 3 way tap bypassed each filter and the venturi taps were closed. Ammonia analysis (Verdouw et al., 1977) was carried out on water samples taken both after the acclimation period, and post-experiment and these remained below detection limits in all experiments (total ammonia <0.1mg l^{-1}). Bacterial O_2 consumption was recorded in an empty chamber at the start and end of each experimental trial (0.3 ± 0.04% mean ± S.D. n = 30). This was deducted from final oxygen consumption values. The movements of 6 fish were tracked simultaneously for 2-hours using the Ethovision Behavioural Tracking System (see section 1.2.8.), and locomotory parameters (specific swimming speed) calculated, whilst water from each chamber was sampled for O_2, pH and temp every 8 minutes. From the combination of O_2 consumption and simultaneous activity measurements, the routine metabolic rate could be calculated, and by back extrapolation to zero activity standard metabolism estimated (Brett, 1964). From the camera observation position above the chambers, the fish's vertical position in the water column could not be determined. It is realised that flow in the chamber may have been less towards the walls and floor of the chamber than in the middle away from surfaces. Thus position of fish in tank may lead to error in actual activity values.

Fish were returned to the appropriate treatment tanks after experiments following the procedure given in section 2.2.3.
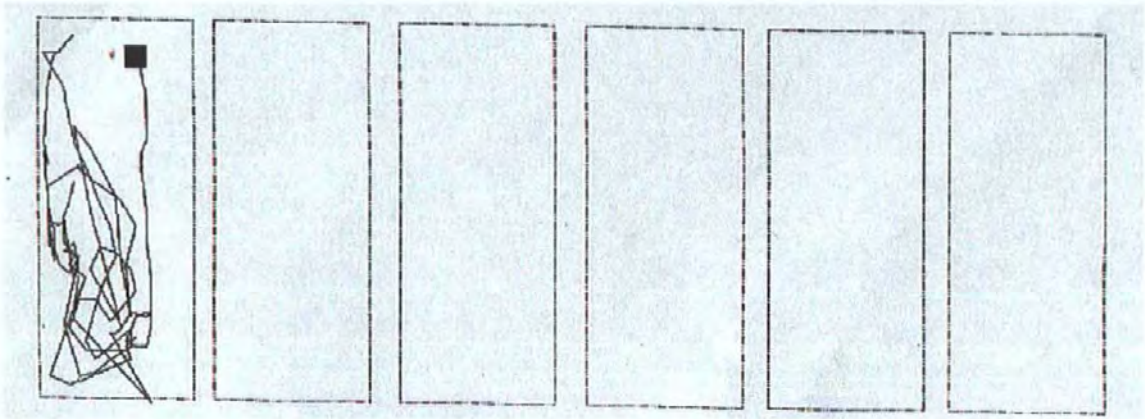
Figure 7. Example of a single Ethovision computer track of fish movements within a respirometer chamber (40L x 20 W x 20 Hcm). Square indicates start of track, which ran for 60 seconds at a sampling rate of 4 frames s^{-1}.

4.2.2. Weekly changes in specific swimming speed

To assess alterations in locomotion during the 12 week exposure period, and to give a judgement of similarities in typical fish activity during respirometry. A sample of 2 fish per treatment were removed at random from the holding tank every 7 days and placed separately into white rectangular arenas (45 W × 70 L × 30 H cm). The tanks were positioned side by side with the camera 1.5m above water level (Fig. 8). This enabled simultaneous activity recording of all arenas. Each tank contained 40 l of filtered (Eheim filter cartridge, see above), aerated water of identical quality to that provided in treatment. The fish were given 48 h to acclimate in arenas, where they were not fed. Following this settlement period, fish movements were tracked for a 2-h period using the Ethovision behavioural recognition system (see section 2.2.8). Gross distance moved by individuals was measured, and from the division of time increments specific swimming speeds, maximum attained speed, and the percentage of time spent swimming were calculated. Fish were returned to the appropriate treatment tanks after video tracking (see section 2.2.3.).

4.2.3 Effect of Cu-exposure on swimming activity during feeding

The motivation and efficiency of rainbow trout feeding on a Cu-contaminated diet were assessed using the Ethovision tracking system over a 4 week period to obtain an estimation of prey capture performance. Control and Cu dietary fish were removed from the holding tanks after 4 weeks exposure (Trial B) and placed individually into separate glass tanks (120L × 36W × 36 H cm). All tanks received water from the same biofilter (800 l), for water quality and husbandry parameters (see section 1.2.4). Each tank was shaded for 10 cm at one end of the tank, providing a refuge for the fish (fig. 10). Food (prepared as for Trial B, following methods 1.2.2) was added to the tank as individual pellets down an entry chute (ensuring feed entered each tank at the same location), to the end opposite the refuge. The

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Figure 8. Schematic diagram showing arrangement of large arenas used to simultaneously track 4 within arenas (45 W × 70 L × 30 H cm), containing filtered and aerated water. A camera positioned 1.5 m above the water surface, and connected to the Ethovision tracking system monitored fish activity.

Camera

Ethovision
and PC

Fish behaviour
arena

Venturi

Eheim pump & filter
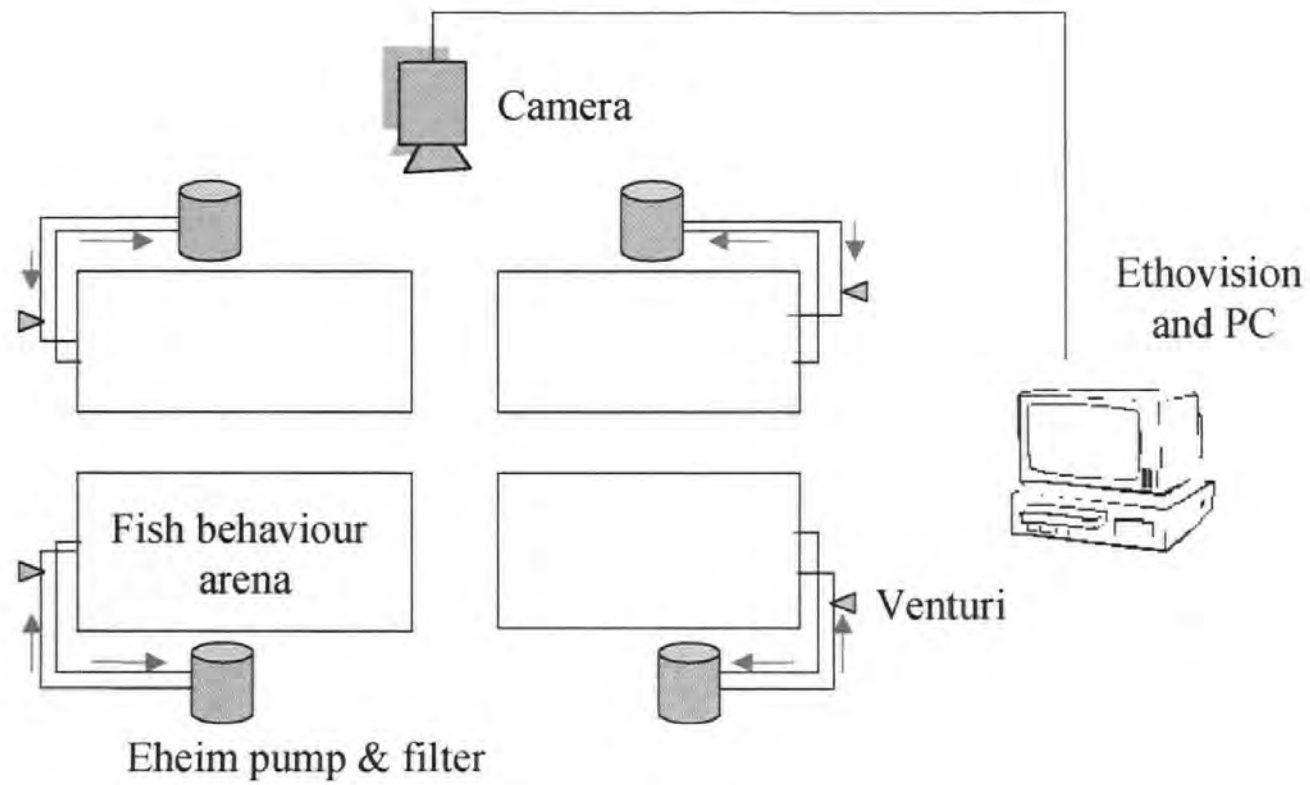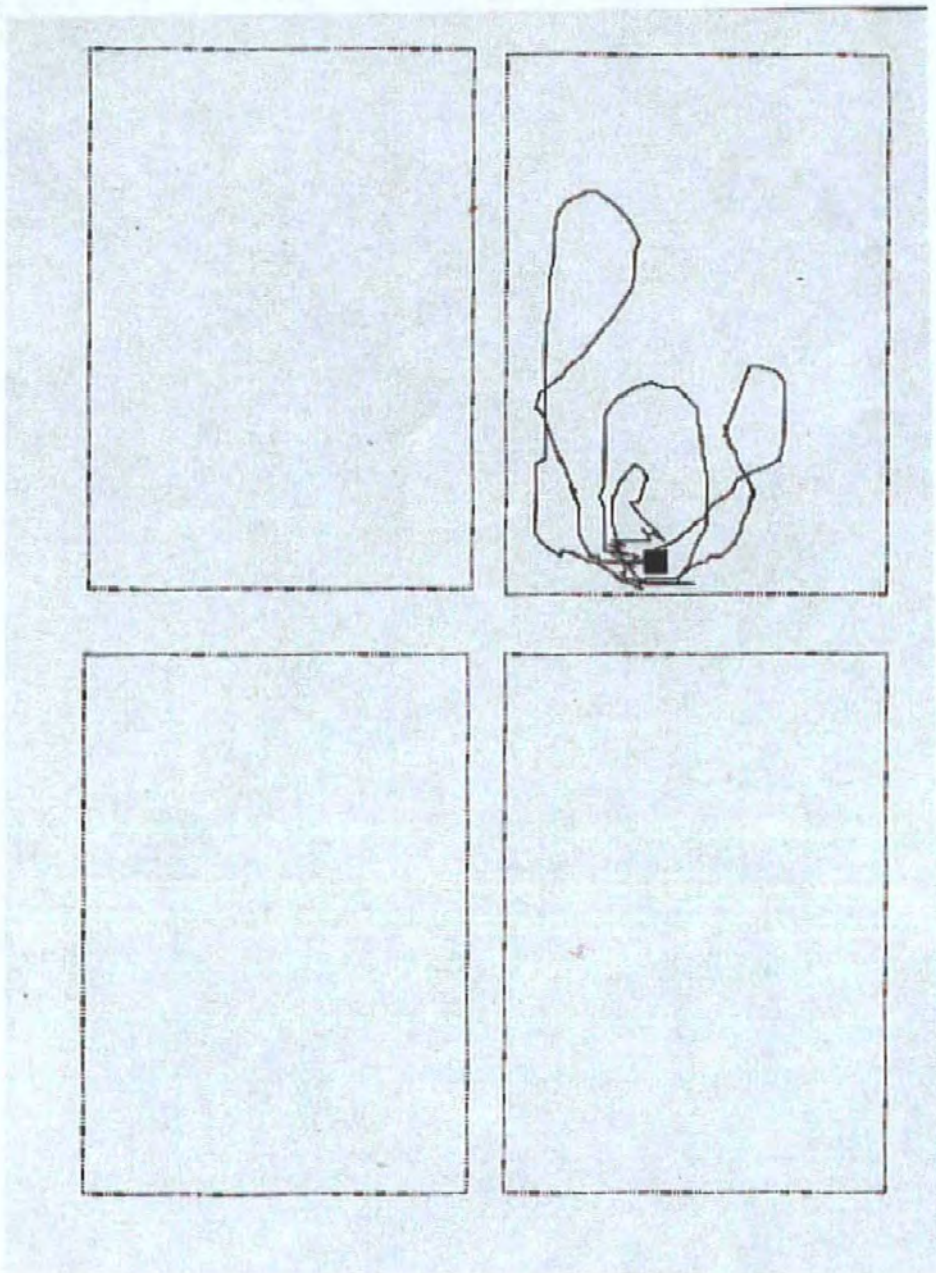
Figure 9. Example of a single Ethovision computer track of fish movements within a large arena. Square indicates start of track which ran for 20 seconds at a sampling rate of 4 frames s^{-1}.

water current generated by the external filtration and water aeration moved the pellets towards the refuge at the end of the tank at a rate of 2 ± 0.2 cm s^{-1} (mean $\pm$ S.E. n = 20). Each fish was presented with individual food pellets until fish refused 3 pellets consecutively. Fish were fed and monitored following this procedure twice daily. A single feeding period from first pellet retrieval until pellet refusal was termed a feeding bout. After the 7-day acclimation period, fish behaviour was observed both by direct observation and using the Ethovision tracking system (see section 2.2.8.). For each pellet retrieved, the orientated movements by the fish were video recorded. For detailed analysis of the feeding behaviour sequence the digitised image of the feeding arena was partitioned into 3 zones. These were, zone1 (sit and wait zone) which included the darkened refuge area (30 cm). Zone 2 (the sprint zone) covered the middle section of the tank (60 cm) and would allow determination of swimming speeds during pellet retrieval; and zone 3 (collection zone) where the pellet was collected by the fish (30 cm). After each pellet was collected the fish always returned immediately to the refuge shaded area. Once the fish had returned and orientated itself to face towards zone 3 another pellet was delivered (1-2 s). Each track was analysed to determine mean swimming speed of the fish within each zone during pellet retrieval, the percentage time spent within each zone, and the latency of time the fish entered into each zone after pellet delivery. Mean speed calculations are only determined in consecutive video frames when the fish is moving greater than 1 cm s^{-1} to prevent 'not true movement' (see section 2.2.8.) from affecting results. Only tracks where the pellet was retrieved by the fish were included in the analysis. Immediately, after pellet refusal, indicating fish had finished feeding, ventilation frequency was observed through an eyehole in the screen for a period of 60 s.

4.2.4. Statistical analysis

Linear regression was used to show trends between oxygen consumption and activity. These were compared where appropriate with Log transformation of oxygen consumption data (Brett, 1962), however no better line fit was observed so linear regression lines were used to calculate R_S .Trends between treatments were testeed using Analysis of Covariance (ANCOVA). F-ratio test was used to check for variance within data set and also to compare variance between treatments. Two-tailed Student's t-tests were used to compare mean data grouped by either time interval or treatment, and Mann-Whitney U-tests used when data was found to be non-parametric (maximum speed data). Non-linear regression was used to determine the line of best fit when straight lines failed to describe the trends adequately. Non-linear negative exponential line fits ($y = a * \exp (b / (x + c))$) were used to describe trends in percentage time spent swimming. For describing trends in feeding data, non-linear sigmoidal curve fits ($y = a / (1 + \exp (-(x - x0) / b)))$) were used. For all line fits probability of curve showing trends in data were analysed by One-way ANOVA, Normality test and Constant Variance Test. Only if curve was significant in all tests was line fit used to describe data. For all tests 95 % confidence limit was considered significant.

Figure 10. Operational diagram of equipment used to monitor swimming activity during feeding. Feed entered down a delivery chute, and swimming activity during collection was monitored by a camera positioned 1.5m away in parallel with the middle of the tank (120L x 36W x 36 H cm). The camera was connected to the Ethovision tracking system. Diagram shows computer-defined zones established to monitoring different aspects of food collection (Zone 1, sit and wait = 30 cm, Zone 2, sprint = 60 cm, Zone 3, pellet collection = 30 cm). Blue arrows show direction of water flow (2 cm s^{-1} within the tank), and brown dashed line indicates movement of food pellet (same as water flow) once entered water.

Screen

Ethovision tracking
system

Point of feed entry

Filtered and aerated water

Visual
observation

Zone 3   Zone 2   Zone 1

Chilled water
bath

Shaded
area

Eheim filter
cartridge

Camera
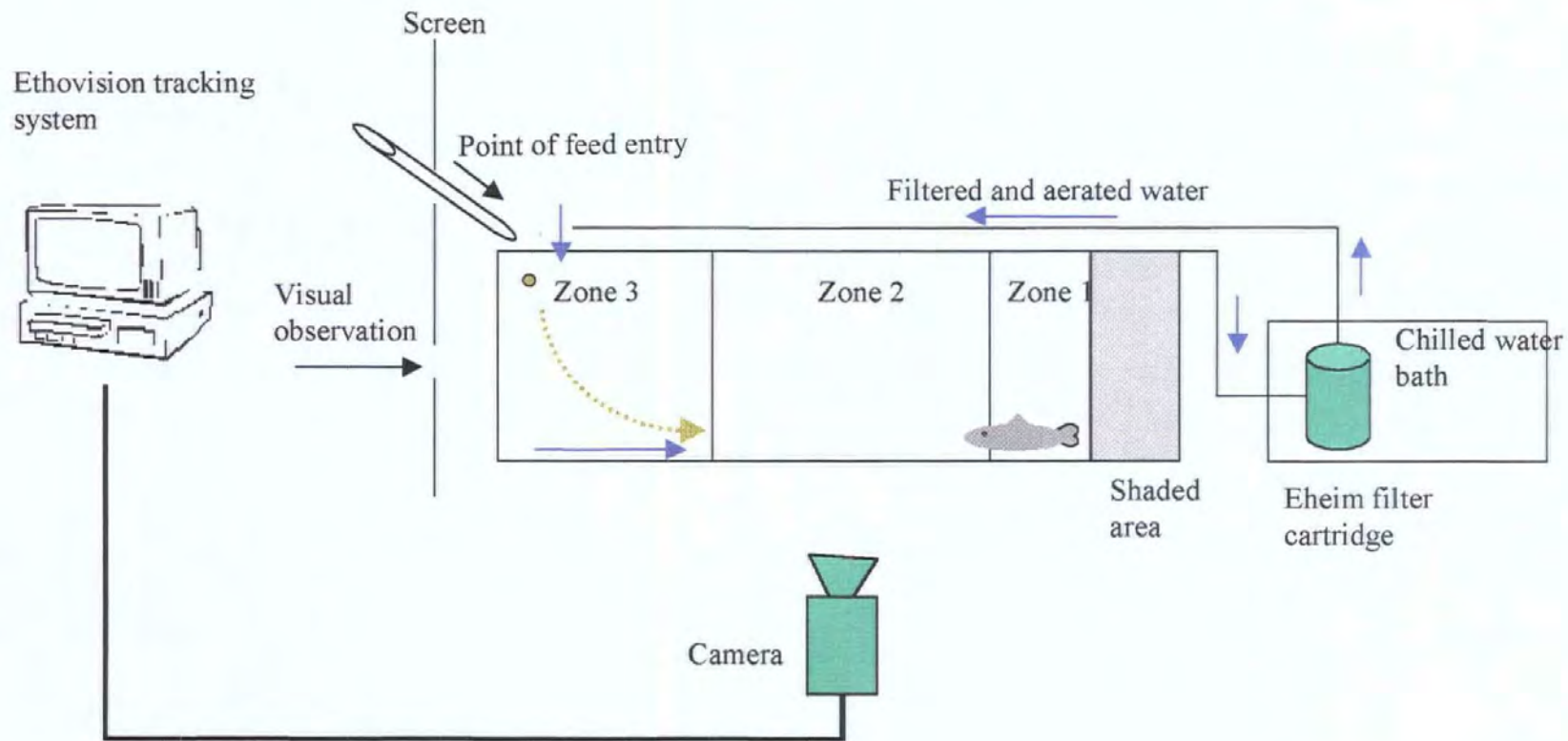
4.3 RESULTS

4.3.1. Oxygen consumption measurements

The relationship between voluntary spontaneous swimming speed and O_2 consumption was determined for both control (Fig. 11a) and Cu-exposed fish (Fig. 11b). As expected, fish from both treatments showed an increase in O_2 consumption with increasing swimming speed. Generally however, Cu-exposed fish required more oxygen than control fish to maintain a similar level of activity.

Standard metabolic rate (SMR) was calculated for individual fish by back extrapolation of the O_2 consumption data to zero swimming speed (Fig 11a, b). Regression lines fitted to the control data gave intercepts on the y-axis (R_S estimate) of 4.8 ± 0.2 μmols g^{-1} h^{-1} (mean ± S.E., n = 6), whilst the Cu-exposed fish gave an R_S of 4.1 ± 1.1 μmols g^{-1} h^{-1} (mean ± S.E., n = 6). The expression of voluntary swimming speeds and correlating VO_2 measurements between control and Cu-exposed fish produced a very different activity profile. As the spontaneous swimming activity of the fish increased to 0.3 BL s^{-1} the variance in VO_2 between Cu-exposed fish swimming when swimming at this speed is 4-fold greater than that of control fish (Table 9), despite the fact that twice as many measurements are made for Cu-exposed fish within this speed bin (which statistically should serve to reduce group variability). Between 0.3 and 0.4 BL s^{-1}, 50% more measurements were taken from control fish swimming at this speed than Cu-exposed fish. The within group variance for VO_2 was 11-fold lower in this specific swimming speed bin for controls than that measured in Cu-exposed fish (Table 9). Cu-exposed fish only achieved the higher speed bin (0.4 - 0.7 BL s^{-1}) throughout 4 VO_2 measurements, this was compared to 24 VO_2 measurements within this speed bin for control fish (Table 9). Multiple regression analysis (H_0 = assuming significant relationship between slopes) showed a significant relationship (F = 5.15, P = 0.04)

Figure 11 (a). The relationship between spontaneous swimming speed and O_2 consumption rate for 6 control fish. Data are plots for individual fish (Each set of symbols represents a separate fish's VO_2 measurements) fed a control diet for a period of 10-12 weeks. Each data point represents specific swimming speed and O_2 consumption taken every 8 minutes over a 2-hour period. Each data set per fish has been fitted with a separate linear regression line.

Figure 11 (b) This is the same as for (a) except VO_2 and activity measurements are from 6 Cu-exposed fish.
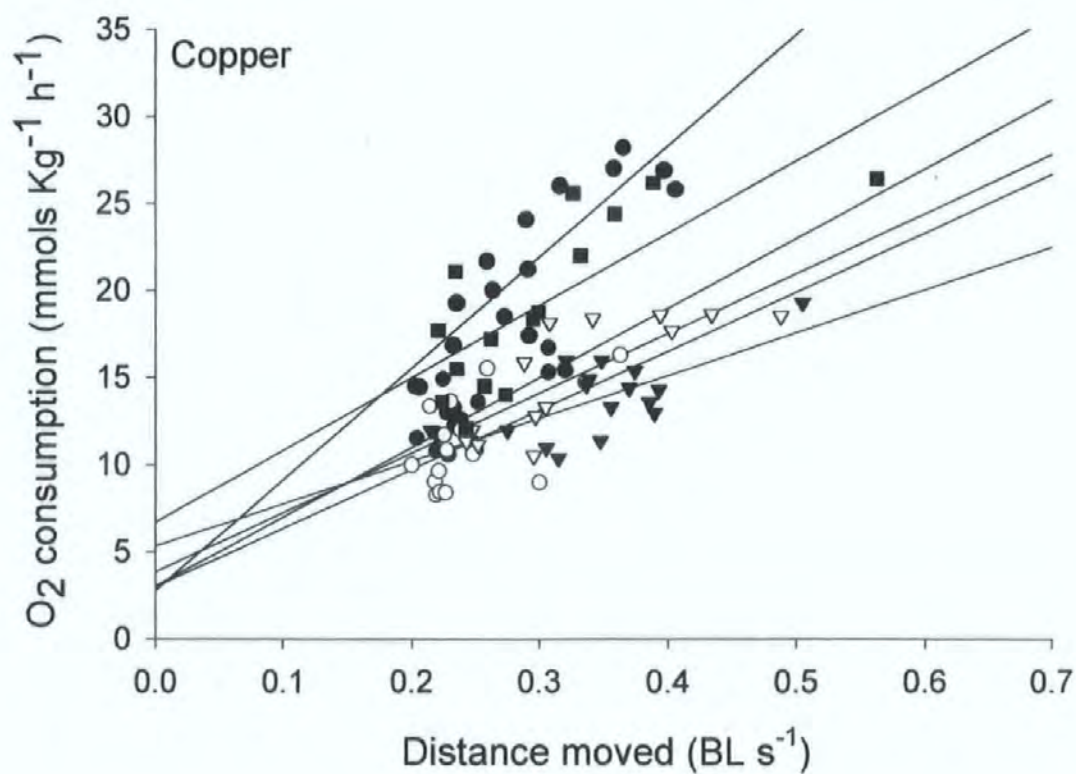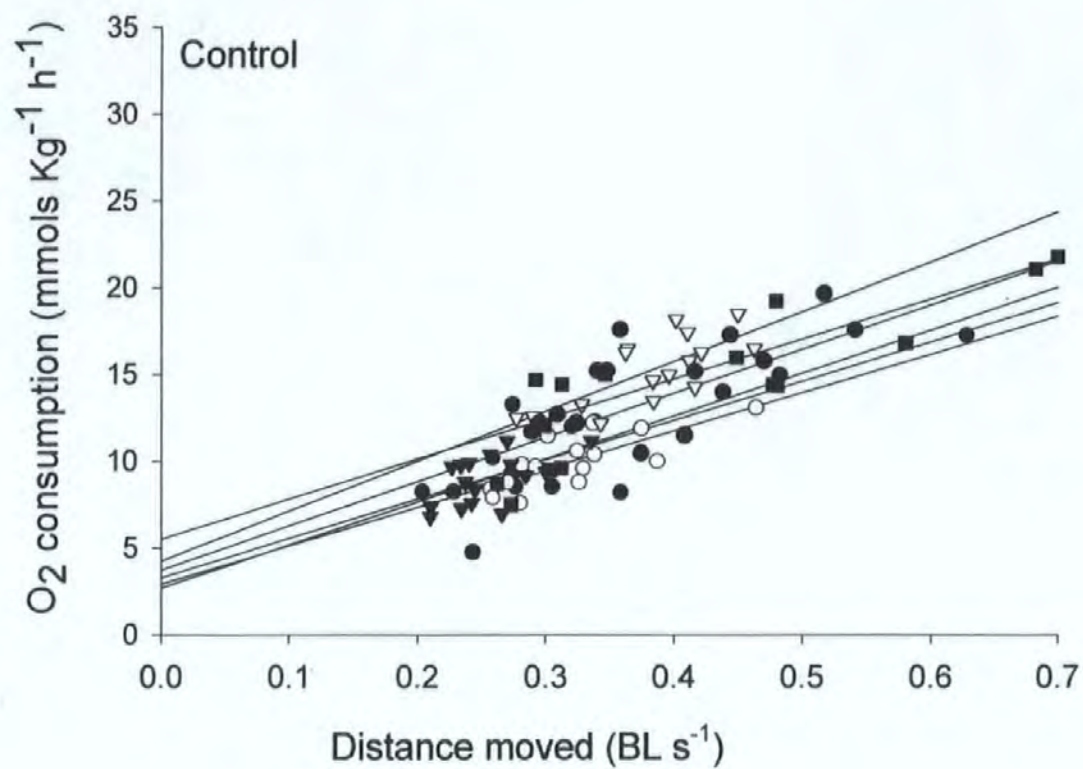
Table 9. Data shows number of VO_2 measurements taken for control and Cu-exposed fish voluntarily spontaneously swimming within 3 different specific speed-bins (n = 6 fish per treatment and 15 measurements per fish taken every 8 minutes over a 2-hour period), and the calculated variance within the VO_2 measurements determined within each specific speed bin for control and Cu-exposed fish.

Specific Swimming S_j		0.2 - 0.3 (BL s^{-1})		0.3 – 0.4 (BL s^{-1})		0.4 – 0.7 (BL s^{-1})
	n	**Variance**	**n**	**Variance**	**n**	**Variance**
Control	30	7.270471	34	6.266857	26	15.96603
Cu-exposed	60	26.72792	24	69.20542	6	1.656667

in the metabolic rate of control fish, but not for Cu-exposed fish $(F = 1.52, P = 0.19)$. To take account of large variations within an individuals swimming activity over the 2-hour period itself, and perhaps errors in measurement that occurred as a consequence of O_2 measurements lagging behind activity bouts, the O_2 consumption and voluntary spontaneous activity profiles for each fish measured at discrete 8 min intervals were also expressed as means for the 2-hour monitoring period and plotted graphically in fig. 12. This figure shows a similar general trend between dietary treatments as observed in fig 11a & b. That is, O_2 consumption increased with voluntary spontaneous swimming activity with Cu-exposed fish requiring more O_2 than controls to maintain a given level of activity. Using the same technique of back extrapolation R_S was calculated to be 4.2 and 3.8 mmol O_2 kg^{-1} h^{-1} for control and Cu-exposed fish respectively. These were not significantly different from each calculated using 95% confidence intervals. At higher levels of swimming activity Cu-exposed fish require proportionally more O_2 to maintain a given speed, producing a significant different aerobic scope between treatments, (ANCOVA, assuming unequal slopes, $F = 5.91, P = 0.019$). Routine oxygen consumption during spontaneous activity determined by mean O_2 consumption levels, irrespective of swimming speed, were significantly (Mann Whitney U test $P<0.05$) higher in the Cu-exposed fish (mean ± S.E. n = 30, control, 10.1 ± 0.4; exposed, 13.1± 0.7 mmol O_2 kg^{-1} h^{-1}). Log transformation of the oxygen consumption data did not produce a significantly better line fit for either control or Cu-exposed groups (control$_{log}$ $r^2 = 0.48$, Cu-exposed$_{log}$ $r^2 = 0.47$), thus for easier interpretation of data, regression lines were fitted to normal data.

Fig 13. shows that during respirometery analyses, Cu-exposed fish preferred to maintain lower swimming speeds (70 ± 6 % of their time swimming <0.3 BL s^{-1}), compared to only 55 .± 5 % by control fish. At the higher speed frequencies control fish spent proportionally

Figure 12. The relationship between voluntary spontaneous swimming speed and O_2 consumption rate. Data are mean and S.E. for individual fish (control = open circle; exposed = filled circle). The vertical error bar represents S.E. in O_2 consumption measurements made at 8-minute intervals over a 2-hour sampling period for a single fish, and horizontal bars is S.E. in specific swimming speed over the 2 hour period as determined by Noldus tracking software. Linear regression lines are fitted (Sigma-plot 4) for control ($y = 14.9x + 4.17$, $r^2 = 0.501$, P<0.001, n = 30) and Cu-exposed fish ($y = 32.7x + 2.78$, $r^2 = 0.486$, P<0.001, n =30) with 95% confidence intervals, (ANCOVA of intercepts , F = 46.7, P< 0.01, and slopes F = 5.91, P = 0.019). Data were collected between weeks 4 and 12 of the experiment. Data were corrected for weight specific changes in metabolic rate to the exponential of 0.8 (Cech 1990). 1 outlier was removed from the Cu-exposed data (0.48 BL s^{-1}, 5.8 mmol kg^{-1} h^{-1}), allowing a truer line fit to the data ($r^2 = 0.42$ before).
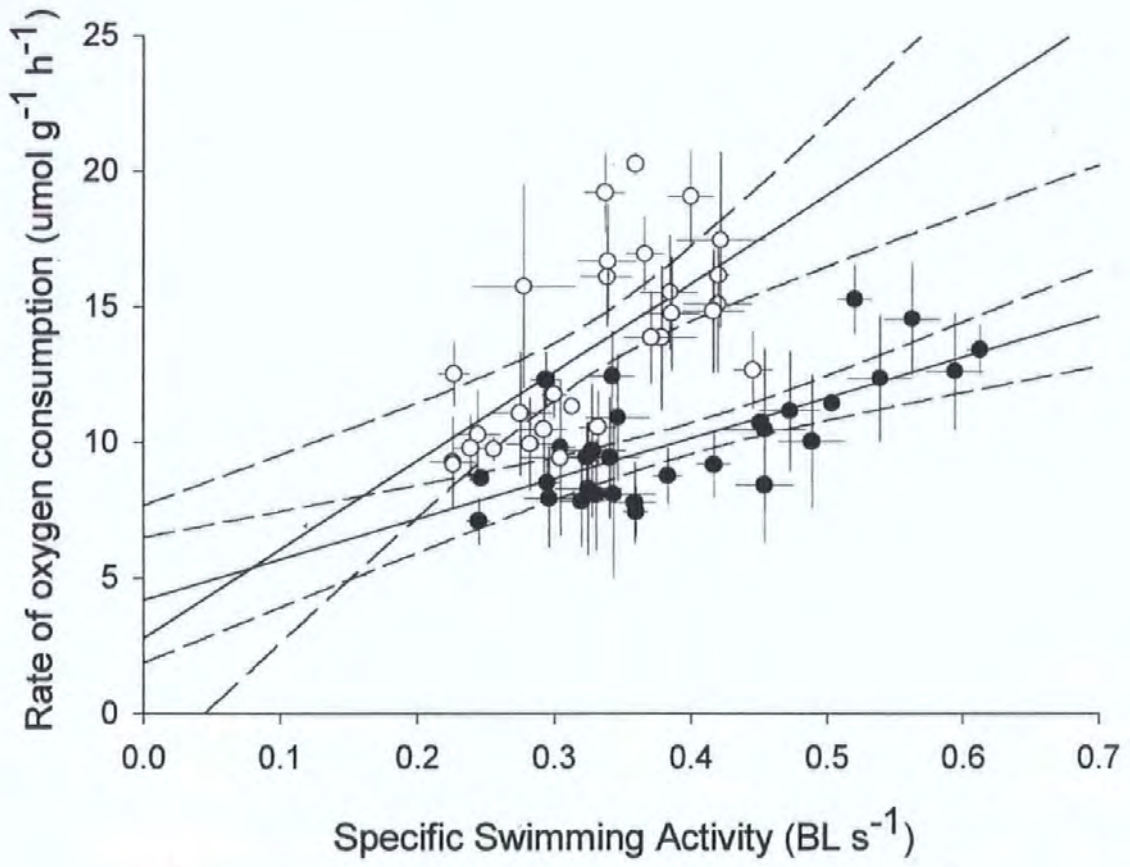
Figure 13. Mean (n = 30) changes in the percentage time spent swimming by control and Cu-exposed trout at 4 swimming speed bins. Analysis undertaken on swimming data obtained by Ethovision tracking system during 2 hours observation in respirometers.
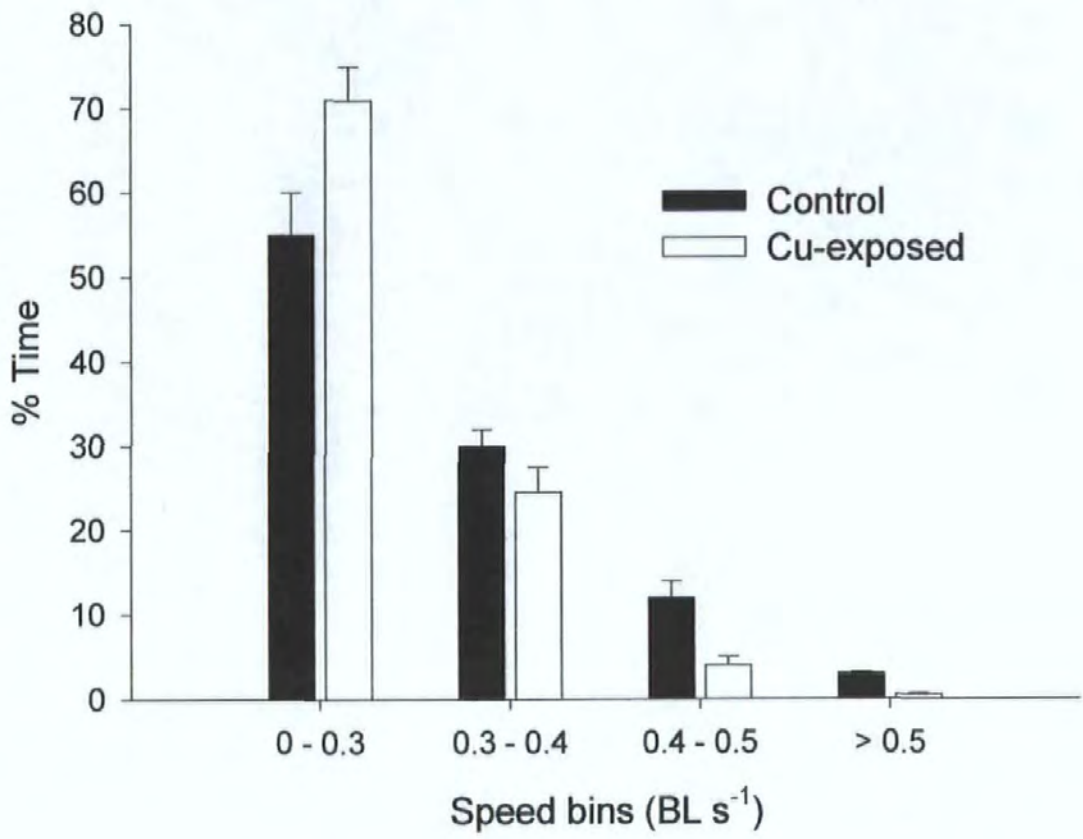
10-20 % more of their time compared to Cu-exposed fish, and Cu-exposed fish spent only 0.4 ± 0.2 % of their time swimming at speeds > 0.5 BL s-1, compared to 4.8 ± 0.2 % by control fish.

4.3.2. Weekly changes in spontaneous locomotory activity

The grand mean spontaneous swimming speed for control and Cu-exposed fish over the 12-week period was 0.31 ± 0.015 BL s^{-1} and 0.21 ± 0.015 BL s^{-1} (mean ± S.E., n = 96, significantly different, t-test, P<0.05) respectively. Specific swimming speed of Cu-exposed fish decreased progressively during the 12-week exposure. Specific swimming speed of controls remained relatively constant, during the 12-week experiment (Fig. 14). Cu-exposed fish initially displayed hyperactive behaviour, which meant they swam 20% further than control fish. During weeks 3 and 4 there was a switch in the behaviour of Cu-exposed fish from hyper to hypoactivity. The time lag of the switching response varied between individuals, which produced a large variance in the Cu fish data in the pooled data for weeks 3-4 (Fig. 14). By week 6 the specific swimming speed of Cu-exposed fish was significantly (t-test t = 1.24, P = 0.002) lower than controls (50%), and by the end of the experiment this difference had increased to 66%. ANCOVA showed that there was not a significant relationship (F = 24.3, P = 0.9) in the specific swimming activity between the two treatments over the 12 week exposure period.

The percentage time spent swimming by control and Cu-exposed fish was similar in the first fortnight of the experiment with both Cu-exposed and control groups spending around 40% of their time swimming (Fig. 15). As the experiment progressed, Cu-exposed fish gradually began to spend more time resting and less time swimming than control fish. Within treatment trends over time showed a significant decline (t-test, P = 0.002) in the amount of time-spent swimming by Cu-exposed fish, a trend not seen for control fish (t-test,

Figure 14. Weekly changes in spontaneous net swimming speed over 12 weeks exposure to dietary Cu. Data are mean and S.E., n = 8 (control = open circle; exposed = filled circle). Net swimming speed of individual fish was recorded in 2 hour sessions using the Ethovision tracking system every week. Data were pooled fortnightly for analyses. Note that net swimming speed is an average of all swimming speeds observed from "stationary" position holding to voluntary burst swimming within the recording period. * Cu-exposed fish significantly different from control at that time point (t-test P<0.05). # significantly different from data in first fortnight of the experiment (t-test P<0.05).
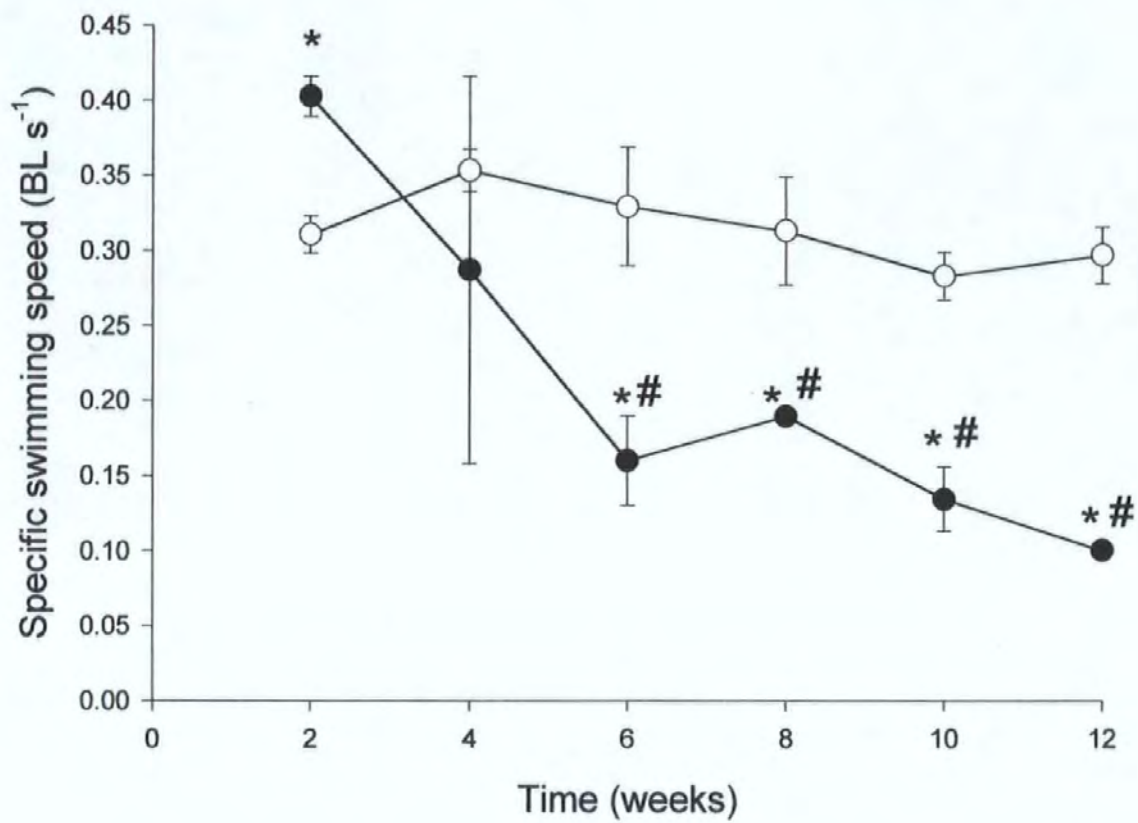
Figure 15. Weekly changes in the % time spent swimming over 12 weeks exposure to dietary Cu. Data are mean and S.E., n = 8 (control = open circle; exposed = filled circle) recorded over a 2 hour period weekly. Data were pooled fortnightly for analyses. Nonlinear regression lines have been fitted to both control ($25.5*\exp(3.1/(x + 5.3))$), $r2 = 0.83$, $P = 0.067$) and Cu-exposed data ($11.9*\exp(4.17/(x + 1.93))$), $r2 = 0.98$, $P = 0.002$). * Cu-exposed fish significantly different from control at that time point (t-test $P<0.05$). # significantly different from data in first fortnight of the experiment (t-test $P<0.05$).
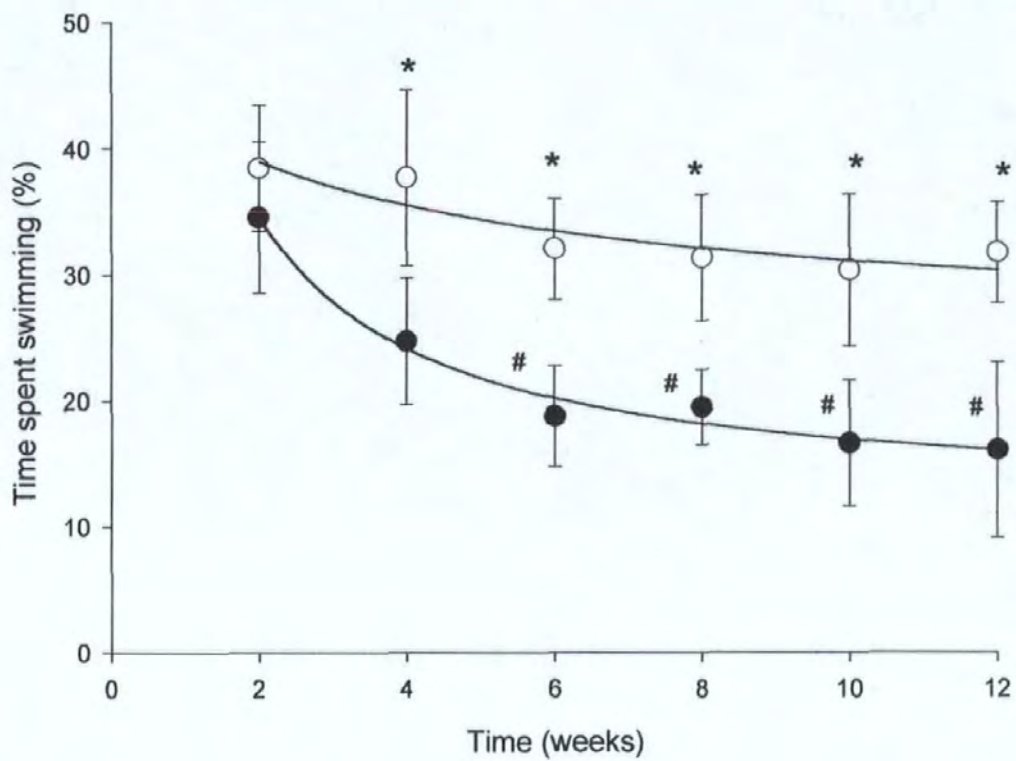
Figure 16. Weekly changes in the maximum speed attained over 12 weeks exposure to dietary Cu. Data are mean and S.E., n = 8 (control = open circle; exposed = filled circle) recorded over a 2-hour period weekly. Data were pooled fortnightly for analyses. Data were compared within treatments over time using multi-factor ANOVA and for treatment effects using Two Tailed Students t-test.
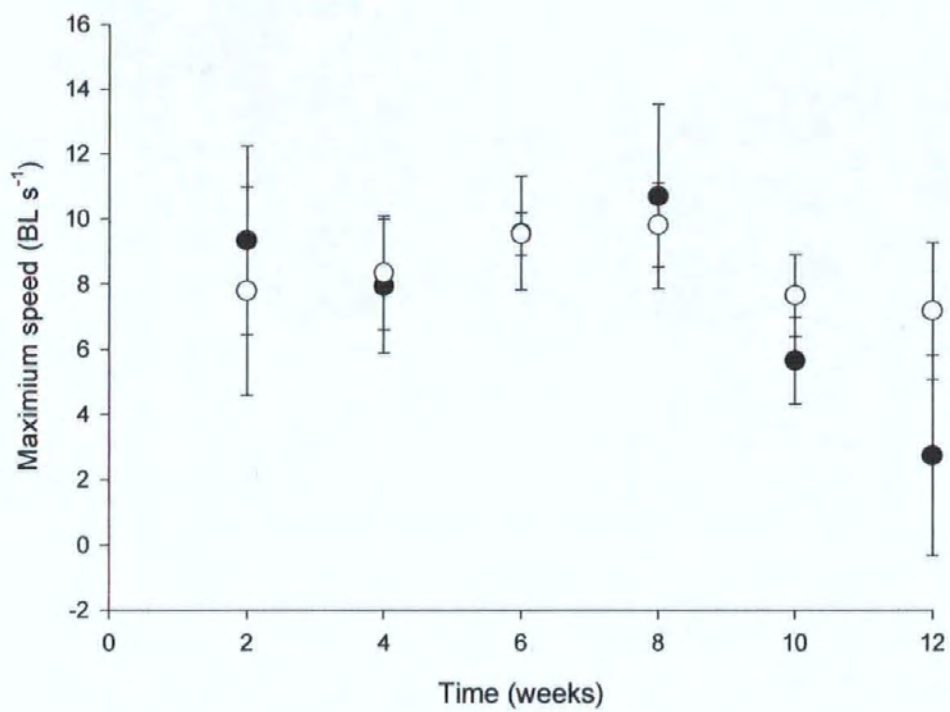
P = 0.07). By week 12, Cu-exposed fish only spent an average of 16% of their time swimming compared to 34 % in controls. Transformation of the data and analysis by ANCOVA also should that there was not a significant relationship (F = 23.2, P = 0.75) between the 2 treatments.

The maximum speed exhibited by both control and Cu-exposed fish over a 12-week exposure period showed no significant differences, over 12 weeks (fig. 16). Furthermore, there was no significant difference between control and Cu-exposed fish at any of the weekly sampling times. The highest maximum speed (10.1 BL s^{-1}) occurred in Cu-exposed fish at week 8 (n =8). After this time there was a significant (t-test, t = 12.4 P<0.05) decline in the maximum swimming speed achieved by Cu-exposed fish. By week 12 this gave the lowest recorded value of 2.75 BL s^{-1}. The average maximum speed for the entire 12 week period was 8.4 BL s^{-1} for control fish and 7.6 BL s^{-1} for Cu-exposed fish. Although the Cu-exposed fish gave a lower mean maximum speed than controls, this difference was not significant (t-test, t = 2.1 P = 0.33). However, there was a significant difference in variances between treatments (F = 5.5, P = 0.02) with Cu-exposed fish showing a greater variation in the maximum speeds attained.

4.3.3 Swimming activity during feeding

The swimming behaviour of control and Cu-exposed rainbow trout whilst retrieving food pellets was analysed by the Ethovision tracking system to explore any differences that may occur in feeding motivation and/or efficiency due to Cu effects on diet palatability and locomotion.

Figure 17 shows the mean speed of pellet retrieval by a Cu-exposed fish during its 1st, 5th and 9th feeding bout. On initial introduction of a food pellet fish were cautious and the mean speed of retrieval time was slow initially at just over 1.0 BL s^{-1}. With the introduction of

more pellets the mean speed of collection increased, to a maximum limit around 3.0 BL s^{-1}. By the 5th feeding bout the mean speed of initial pellet retrieval was higher than that of the 1st session but was still below the achievable maximum which was reached after the introduction of only 6 pellets compared to 14 pellets during the 1st session. By the 9th feeding session the first and consecutive pellets were retrieved around the maximum limit of 3 BL s^{-1}, and occasionally reaching 3.5 BL s^{-1}. It appears that the efficiency at which food pellets were retrieved reaches an upper limit around the 9th feeding session, after which pellets during consecutive feeding bouts were retrieved at this apparent maximum speed. Fish varied slightly (9.0 ± 0.8 mean ± S.E. n = 14) in the number of training sessions required to reach optimum feeding efficiency, but no significant difference (ANOVA F = 6.1 P = 0.94) occurred between treatments. Figure 17 showed the learning curve response of Cu-exposed fish and comparison of fish swimming performance between treatments was only examined on fish after they had reached the upper limit of feeding efficiency (after 9 feeding trials). After the 12th feeding bout fish from each treatment were analysed for pellet retrieval parameters within each computer-generated zone. Fig. 18a shows the mean swimming speed of fish from both treatments within these 3 computer defined zones. In zone 1 (reaction zone) there is no significant difference (ANOVA F = 7.2, P =0.83, n = 720 cumulative of 6 individuals each tracked 120 times) in mean swimming speed due to treatment. In zone 2 significantly (ANOVA, F = 2.4, P = 0.031) higher speeds are exhibited by control fish (5.77 ± 0.1 BL s^{-1}, mean ± S.E., n = 720) compared to Cu-exposed (4.58 ± 0.1 BL s^{-1}, mean ± S.E., n = 720), and though slightly elevated speeds are shown in zone 3 this is not significant.

Fig. 18b compares the percentage of the total time, from pellet entry to retrieval by the fish and returning to the stationary (anticipating next pellet) position in the refuge area, spent within each Zone. The graph shows that Cu-exposed fish spent significantly (t-test, t = 1.3, P < 0.05) more time in zone 1 (reaction zone) and zone 2 (sprint zone) than control fish,

but 10 % less time in zone 3 (collection zone). Overall, the time for a Cu-exposed fish to obtain a pellet and return to the refuge area was significantly greater than controls (15.2 ± 0.2 and 17.2 ± 0.4 s; mean $\pm$ S.E., n = 6 individuals, 120 tracks per fish; for control and Cu-exposed fish respectively). This increase in time was partly due to a reduced response time by Cu-exposed fish to the visual stimulus of the pellet, and the latency of time before entering zone 2 after pellet introduction was 2.3 ± 0.3 and 3.8 ± 1.2 (mean $\pm$ S.E., n = 6 individuals, 120 tracks per fish; t-test, t = 1.7, P < 0.05) for control and Cu-exposed fish respectively.

Due to the water current in the tank causing drift of the pellets ($2cm\ s^{-1}$) the initial delay in reaction (indicated by increased latency of entry into zone 2 and increased % of time in zone 1) to the visual stimulation of the pellet, and the lower mean speed overall of fish collecting it, resulted in the fish having to swim a shorter distance during feeding bouts. This strategy was highlighted by the reduced % of total time spent in zone 3 by Cu-exposed fish, although the mean speed when obtaining and ingesting the pellet was not significantly different between treatments.

Fish were fed until 3 consecutively delivered pellets were refused. There was no significant difference between treatments in the % body weight ingested daily (control, 2.1 ± 0.3 %, Mean $\pm$ S.E., n = 36; Cu-exposed, 1.9 ± 0.3 %, Mean $\pm$ S.E., n = 36; t-test, t =12.1, P = 0.42). The metabolic state of individuals estimated by counting opercular beats after feeding was not significantly different between treatments (control, 112 ± 6, Mean $\pm$ S.E., n = 36; Cu-exposed, 115 ± 11 , Mean $\pm$ S.E., n = 36, t-test, t = 4.5, P = 0.5).

Figure 17. The mean speed of a Cu-exposed fish retrieving Cu contaminated (726 mg Cu Kg^{-1} diet d.w.) pellets delivered consecutively in one feeding bout, to show the increase in retrieval swimming speeds with repeated feeding bouts. Fish were fed twice daily and every 4th feeding session is graphically represented. Filled circles (and dotted line fit) are from the initial feeding after 48 hrs acclimation within the tracking arena (r^2 = 0.89 P<0.0001). Grey circles (dashed line) represent the 5th feeding bout 48 hrs later (r^2 = 0.77 P<0.0001), and grey triangles (solid line) the 9th feeding bout again 48 hrs later (r^2 = 0.2 P< 0.064).
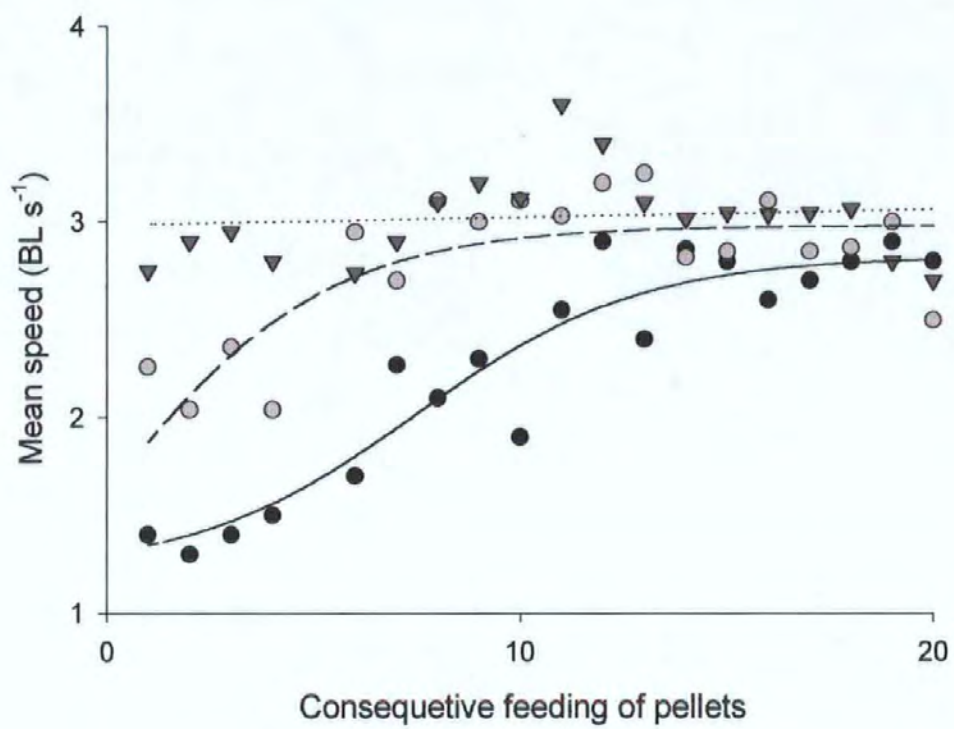
Figure 18a. Mean speed of fish in retrieving a single food pellet by crossing all zones (120 cm) and returning to resting position at refuge end (shaded area of tank). Arena is divided into 3 relative zones (Zone 1 = reaction ; Zone 2 = sprint; Zone 3 = collection) with food pellet delivered into zone 3. Data are mean and S.E. (n = 6 individuals, 120 tracks per fish, 680 per treatment), black bars indicate control fish retrieving control diet, and striped bars are Cu-exposed fish retrieving Cu-contaminated pellets. * Cu-exposed data that is significantly different from control data within same zone (ANOVA, F = 17.24, P = 0.0013).

Figure 18b. The % of the total time the fish spends in each zone within the arena from pellet introduction, retrieval to returning to resting position within Zone 1. Data are mean and S.E. (n = 6 individuals, 120 tracks per fish), black bars indicate control fish retrieving control diet, and striped bars are Cu-exposed fish retrieving Cu-contaminated pellets. * Cu-exposed data that is significantly different (ANOVA, P< 0.05) from control data within same zone.
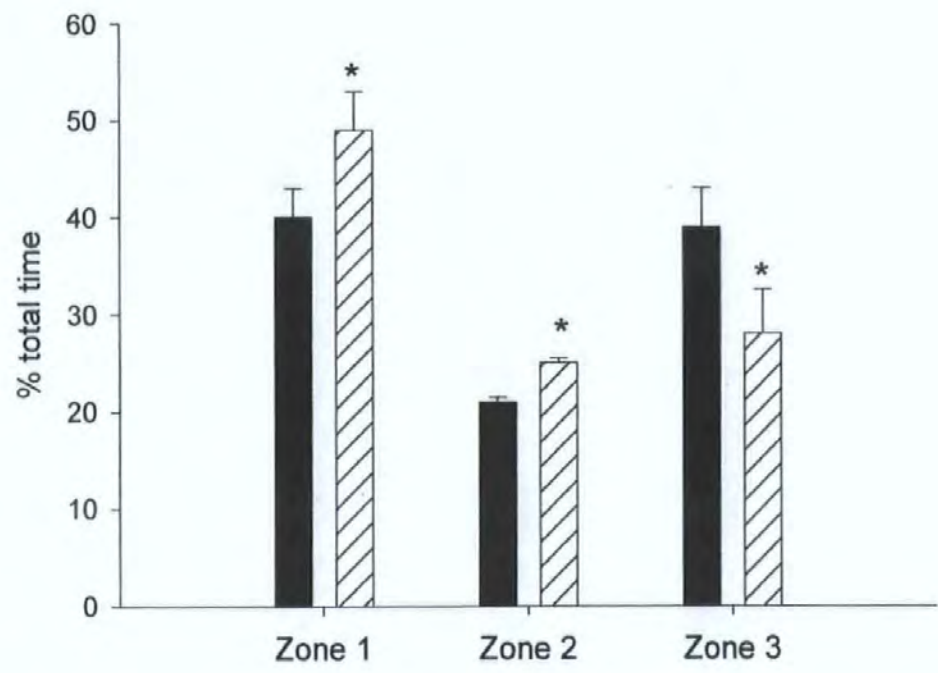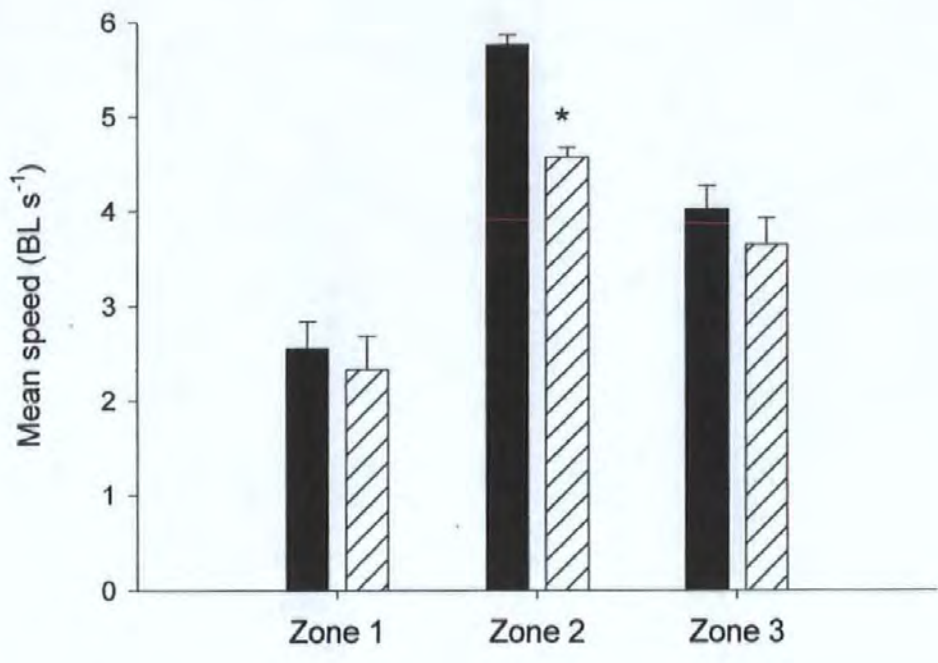
4.4 Discussion

Generally, the measurement of voluntary spontaneous swimming activity and VO_2 of control and Cu-exposed fish agreed with previous authors (Beamish, 1964a; Forstner and Weiser, 1990) and the VO_2 of fish increased with spontaneous swimming activity. However, rainbow trout fed on a diet of 730 mg Cu kg^{-1} d.w. feed, showed a greater requirement for oxygen to remain as active as trout fed a control diet. As a compensatory response Cu-exposed fish displayed a general lowering in swimming activity, and this occurred both in the presence and absence of food.

Effect of copper on standard (resting) metabolic rate

Measurements of R_S observed in this study for control and Cu-exposed fish (4.8 ± 0.3, 4.1 ± 1.3 mmol kg^{-1} h^{-1}) were not significantly different from each other, and values obtained were comparable to those obtained by both Webb (1971) and Skidmore and Tovell (1972) (4.75 mmol kg^{-1} h^{-1} and 5.6 mmol kg^{-1} h^{-1}, respectively) for rainbow trout at 15^0C. However, the R_S between individual fish was much more wide-ranging within the Cu-exposed group compared to the control group, suggesting that the exposed rainbow trout varied in their response and ability to acclimate to elevated dietary Cu. Inter-individual variability in metabolism due to the stress response has been previously documented in other fish species (Watenpaugh, 1985; Kolok, 1998), and has also been observed in crabs *(Carcinus maenus)* under sub-lethal aqueous Cu-exposure (Depledge, 1990). A possible cause of the observed variation in the toxic response of Cu- exposed fish, in this study, may have arisen due to disparity in the degree of exposure between individuals. Given that Cu was introduced to the animal via the diet, and the diet was introduced to the group of fish as a single meal from a point source. Under aquarium conditions a feeding hierarchy can arise within social rainbow trout (Yamagashi, 1962), resulting in an unequal distribution of the food ration. Fish liver Cu

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content was not correlated with R_S to confirm if any relationship existed, but the concept of individuals acquiring a disproportionate ration of the contaminated food supply will be dealt with in later chapters.

Although recent evidence has recognized that elevated levels of copper can occur naturally in the diet of natural fish populations (Dallinger and Kautsky, 1985; Dallinger et al., 1987; Farag et al., 1994, 1995, 1999; Woodward et al., 1994, 1995) this is the first study to examine the possible effects of this source of contamination on fish R_S. Previous literature documenting the effects of copper exposure on R_S have used waterborne exposures (Waiwood and Beamish, 1978; Beaumont et al., 1995). Unlike dietary Cu-exposure, aqueous exposure will damage the gills preventing them from functioning properly by increases diffusion distances and disrupting perfusion of blood through gills (Hughes, 1976; Tuurala and Soivio, 1982; Taylor et al, 1996). This authors found that unlike dietary exposures in this study, aqueous exposures caused a 10% elevation in SMR.

Effect of copper on routine metabolic rate

Dietary Cu-exposure did significantly increase the metabolic cost of routine activity (Fig. 12), (slope; Cu-exposed 8.36; controls, 4.16), particularly at speeds higher than 0.1 BL s^{-1}, and may result in a reduced Ucrit. This may be a reason as to why it was observed that Cu-exposed fish spent a greater percentage of their time swimming at lower speeds (fig. 13). This is in agreement with previous observations made by Handy et al., (1999) on dietary Cu-exposed trout. Similar to R_S observations, Cu-exposed fish had a larger inter-individual variation in routine metabolism than controls. A few Cu-exposed fish had R_A values comparable with controls, whilst others, had a VO_2 almost twice that of controls, when swimming at higher speeds. The higher requirement of oxygen to remain active in Cu-

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exposed fish indicates that Cu-exposed fish will reach the upper limit of metabolic scope (*defined by* Fry, 1947) at a lower level of activity than an uncontaminated fish.

The origin of these added locomotor costs for the Cu-exposed fish may relate to altered energy metabolism within skeletal muscle. Beaumont et al., (2000) observed an increase in ammonia ions in the muscle of aqueous exposed fish, suggesting that this could alter metabolic status of muscle and also cause electrophysiological disturbances. However, the normal ATPase activities observed by Handy et al (1999) and the normal Na^+ and K^+ levels in the skeletal muscle of fish studied here would suggest that this is not the case for oral Cu exposure.

The normal haematology observed in this study (Table 7) suggests that blood oxygen carrying capacity is unlikely to be a limiting factor in the swimming performance of Cu-exposed fish, but bulk delivery of blood (and oxygen) to tissues might be a problem given the abnormally high and variable LDH activities (Chapter 2) in the cardiac muscle of the Cu-exposed trout (Beamish, 1968; Asztalos, 1986).

Despite the obvious importance of aerobic scope to a fish's ecology concerning prey-capture, predator evasion, holding position in current and upstream migration, it seems somewhat surprising to discover that there have been only a few studies on the effects of this due to copper exposure. These consist of three aqueous exposure experiments by Waiwood and Beamish, (1978), Beaumont et al., (1995) and McGeer et al., (2000); and a Cu dietary study by Handy et al (1999). Comparisons of R_A results from this study with aqueous exposure experiments are relatively similar even though aqueous Cu will disrupt oxygen uptake capacity of the gills (Hughes, 1976; Tuurala and Soivio, 1982; Taylor et al, 1996). Beaumont et al., (1995) found aqueous Cu-exposure of 0.08 μmol l^{-1} Cu^{2+} at 15^0C, via gills, to cause a 38% increase in routine O_2 consumption of rainbow trout, and Waiwood and Beamish, (1978) observed a 26% increase in rainbow trout to the same exposure

concentration. This is compared to a 23% increase in O_2 consumption observed in dietary Cu-exposed fish from this study. The only previous Cu-dietary study to look at routine metabolic rate (Handy et al., 1999) observed no increase in routine oxygen uptake of Cu-exposed trout. However, dietary Cu levels were 35% less than concentrations used within this study. Moreover, in this study Cu-exposed fish generally lowered activity to reduce active metabolic rate, and as the study by Handy et al. (1999) did not correlate activity measurements with VO_2 measurement; reductions in swimming activity made by the fish, may have fully compensated for the increase in R_A oxygen demand. In addition, results presented here show large inter-individual variability in the routine metabolism of Cu-exposed fish, given that Handy et al., (1999) only analysed VO_2 in 4 fish per treatment, it is apparent that it would be difficult to conclude any significant results.

Do long term reductions in locomotor activity meet the extra cost of swimming in Cu-exposure fish?

On a daily basis fish adopt a very conservative power-budgeting strategy, with maximum power output in trout only occurring for less than 1% of the time (Soofani and Hawkins, 1982; Priede, 1985). The reduction in power requirement means that the fish can carry out its normal functions with a reduced probability of exceeding the limits of metabolic scope, as it is argued that this leads to a reduction in probability of mortality through an increased safety factor in metabolic functions (Priede, 1977). Results from this study show that the requirement for oxygen during elevated activity increases at a much greater rate in Cu-exposed fish compared to controls, and Cu-exposed fish respond behaviourally by down-regulating their metabolic requirements for oxygen. After an initial period of hyperactivity probably associated with avoidance behaviour (Scarfe et al., 1982), Cu-exposed trout decrease their specific swimming speed overall by 66% (from 0.29 in week 4 to 0.1 BL s^{-1} in

week 12) compared to no change in the controls (fig. 14). If this decrease in swimming speed is related to oxygen consumption using fig. 12, then this equates to a routine metabolic saving of about 5 mmol O_2 kg^{-1} h^{-1}. Even if the period of hyperactivity at the start of the experiment is included, this still equates to around a 30% saving in the observed routine metabolic scope of Cu-exposed fish over the entire experiment. In fact, by the end of the experiment the specific swimming speed of Cu-exposed fish decreased to around 0.1 BL s^{-1} (fig. 14), so that their metabolic demands were similar to that of the control fish operating at around 0.3 BL s^{-1} (figs. 12). Suggesting that Cu-exposed fish had almost fully compensated for the added cost of swimming by reducing swimming speed, and therefore matched their energy costs to the controls.

It has been suggested that naturally fish can switch between 'low cost' and 'high cost' spontaneous swimming activity, and alteration in the ratio of time spent undertaking each will adjust the cost of routine activity (Forstner and Weiser, 1990). This adaptive behavioural strategy to minimize energy loss has been observed in fish species in response to adverse environmental conditions such as temperature (Elliot, 1986; Taylor, 1988), current velocity (Godin and Rongeley, 1989); and biotic factors such as food availability (Cunjack and Power, 1987), and requirements of energy for gonadal development (Koch and Weiser, 1983). The concept of fish favouring a reduced behavioural strategy, as a metabolic sparing tactic, to aid in detoxification procedures whilst maintaining a reduced aerobic scope and growth rates, was introduced by Handy et al., (1999) in dietary Cu-exposed trout. This study agrees with this concept, and goes further to quantify the increased cost of maintenance and compensatory requirements in activity by Cu-exposed fish.

Feeding on a Cu-exposed diet; alterations in activity

Within the simplified environment of the tank, energy losses in the short-term were associated with R_A and R_S and energy intake resulted from food intake. Selection will favour the fish to maximize energy intake whilst reducing expenditure (Hewett and Johnson, 1987). In foraging for prey the theoretical optimum speed is that which maximizes differences between rate of energy intake and output (Ware, 1975). Control fish within this study, monitored whilst they were undertaking a sprint to retrieve food pellets (observations in Zone 2, Fig 18a), achieved a mean swimming speed of 5.8 ± 0.23 BL s^{-1}. This was below the maximum speed attained (9 ± 1.2 BL s^{-1}, $n = 24$) by control fish during tracking studies in the large tracking arena over the 12 weeks (Fig. 16). The fact that control trout were able to attain a higher maximum speed, than achieved when obtaining a food pellet, perhaps relates to an energy optimising strategy. As it is expected that endurance would decrease as the fish approached R_{max}, and fish may still need the flexibility in the aerobic scope for future pellet collections, and the large requirements of the metabolic scope for SDA once food acquired (Jobling, 1981).

Cu-exposed trout had a lower mean swimming speed than controls when retrieving prey items (Fig. 18a), and as a consequence took longer to retrieve a food pellet than controls (15.2 ± 0.2 and 17.2 ± 0.4 s; mean $\pm$ S.E., $n = 6$ individuals, 120 tracks per fish; for control and Cu-exposed fish respectively). Similar to control fish, Cu-exposed fish did not collect pellets at their apparent maximum speed. Which, when recorded (during non-feeding periods) was comparable to that recorded for control fish (fig. 16). Therefore, the mean swimming speed exhibited during pellet collection, was reduced below the apparent maximum swimming speed, to a greater extent by Cu-exposed fish compared to controls. If the theoretical optimum speed is that which maximizes differences between rate of energy

intake and output (Ware, 1975). Then it seems reasonable to assume that the increased cost of swimming in Cu-exposed fish (Fig. 12), and resultant reduced energetic benefit from the food pellet a possible cause for the reduced swimming speed in pellet collection of Cu-exposed fish.

Cu-exposed fish also displayed a delayed reaction response to delivery of the food pellets (fig. 18b). Impairment in ability to recognise pellet as food source was discounted as fish were observed frequently to re-orientate in direction of pellet when it entered the water, as control fish did. Delaying collection of the pellet reduced the distance between the fish and prey item. Hence, reducing the distance the fish needed to swim to collect it, and in the long-term would reduce the energy expenditure required to feed. However, increasing the total elapsed time between detection and consumption of prey will consequently entail a cost of reduced opportunity As a significant positive relationship exists between activity and feeding rates (Boisclair, 1992), and thus, over a limited time period control fish would collect more feed than Cu-exposed fish. The energetic strategy by Cu-exposed fish may be more one of cost minimizing rather than maximizing energy intake. This is reasonable to assume if the cost of obtaining the food is higher and the benefit derived from it less, as is the case for fish feeding on Cu-exposed diet due to increased metabolic requirements of maintenance and swimming. Estimation of metabolic rate, after the expensive task of food collection, was made by measuring ventilation frequency (Oswald, 1978) and was found to be similar between treatments. Suggesting that the reduced activity during pellet collection by Cu-exposed fish compensated for their increased R_S and R_A oxygen requirements. However, this was only a crude measure made over a short period of time, and a more detailed analysis of metabolic costs associated feeding and SDA would provide useful information in regards to the relationship between activity and feeding rates in dietary Cu-exposed fish.

Few studies have examined the effects of contaminates on ecologically important behaviours such as predation and predator success. Sandenheinrich and Atchison, (1989) observed an increase in handling time of prey in fish exposed to copper, and similar observations have been made from lead (Nyman, 1981) and zinc exposures (Cairns and Garton, 1982). Although these studies made no comparison between rates of activity and rates of feeding, and conclusions were made in regard to impairment of cognitive function, rather than in respect to metabolic strategies as in this study. Comparisons may be made between this study and a study examining the effects of parasites on sticklebacks (Milinski, 1984). Milinski observed that parasitised fish had an impaired metabolic scope, and therefore reduced swimming capacity, and when foraging they would choose less profitable prey items that did not incur such a high energy expenditure to obtain them. Similar to observations made here, an energy minimizing strategy may be more favourable for a fish when the cost : benefit ratio when foraging for food is reduced.

In summary, the exposure of rainbow trout to 730 mg Cu kg^{-1} d.w. feed resulted in an increase in the oxygen requirement for routine metabolism, and this resulted in an overall reduction in spontaneous activity, with Cu-exposed fish spending more time involved in low cost behaviours. Although Cu-exposed trout still possessed the swimming capacity to achieve higher speeds, they chose not to even when retrieving food items, favouring a strategy of minimizing expenditure over maximizing intake. Consequently, energy intake per unit time when feeding on a Cu-exposed diet was less than when feeding on a control diet. High energy cost swimming behaviours will be best expressed to coincide with high profitability from prey capture. In most fish species this if often synchronized by the light dark diel cycle (Young, 1999). This rhythmic activity over the diel period has been termed circadian behaviour, and will be analysed in response to dietary Cu-exposure in the following chapter.

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CHAPTER FIVE

BEHAVIOURAL AND PHYSIOLOGICAL CHANGES IN CIRCADIAN

ACTIVITY DUE TO CU-EXPOSURE

5.1 Introduction

Alteration in behavioural repertoire over the 24-h cycle determines what is termed the circadian cycle and indeed the expression of circadian rhythms is an integral part of behavioural adaptation to cyclical changes in the environment (Styrishave *et al*, 1995). Fish adaptation to repetitive events implies a physiological measurement of time that is expressed in rhythmic behaviour. A rhythm is considered exogenous when it is under direct control of an external periodic factor (e.g. day length), and on the other hand can be described as being endogenous when the biological activity persists oscillating under constant conditions (Cuenca and de la Higuera, 1985). The synchronizing effect of light cycles on the locomotor activity patterns of salmonids has been documented in both artificial and natural lighting conditions (*for reviews see* Thorpe, 1978; Boujard and Leatherland, 1992), with authors suggesting that circadian activity responds to exogenous light and dark cycles, but can also be maintained by fish for a limited period under a continuous light source (Adron *et al.*, 1973), implying an established endogenous rhythm.

Studies have shown a strong indication that the pineal organ is one component in a central neural system that constitutes the photoperiod-responding system of the animal (Falcon *et al.*, 1987, 1989; Zachmann *et al.*, 1992), i.e. the system that is responsible for the correct timing of daily and seasonal physiological and behavioural rhythms. Unlike mammals, knowledge of the fish circadian clock is still very limited, however, a few recent studies have shown that hormonal secretion within the body of the fish parallel those of

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activity and the regulation and expression of these hormones can be influenced on a diel basis by both exogenous and endogenous factors (Bjornsson *et al.*, 1989; Bry 1982; Winberg *et al.*, 1993a,b; Gregory and Wood, 1998; Porter et al.,, 1998). In all vertebrates the most pronounced and consistent circadian endocrine rhythm is that of melatonin production by the pineal gland. Melatonin secretion is synchronized with the light cycle, with levels remaining low during the day and elevated at night (Axelrod, 1974; Falcon *et al.*, 1987, 1989; Zachmann *et al.*, 1992). Studies in a few teleost species suggest the presence of a cellular circadian oscillator within the pineal organ controlling melatonin secretion (Zachmann *et al.*, 1992; Bolliett *et al.*, 1996). However, the pineal organ of rainbow and brown trout do not appear to contain a circadian oscillator, but melatonin synthesis is controlled directly by the pattern of illumination (Gern and Greenhouse, 1988; Zaunreiter *et al.*, 1998). Inter-cranial injections of melatonin have been observed to induce a sedative effect comparable with low dosages of anaesthetic tricaine in fish (Satake, 1979). Serotonin is suggested to stimulate dopamine release, and its elevation in fish has been observed to decrease spontaneous swimming activity, aggression and reduce feeding activity (Fenwick, 1970; Winberg *et al.*, 1993). As well as the daily synchronization with photoperiod, endogenous factors such as feeding motivation and appetite return can alter the secretion of hormones such as cortisol (Bry 1982), and alterations in circulating cortisol levels have been shown to alter aerobic swimming performance in salmonids and Arctic charr (Alsop and Wood, 1997; Gregory and Wood, 1998). Thus it appears that a number of environmental cues involving several endocrine systems may influence the diel rhythmicity in fish.

Many studies on patterns of diel activity in fish have relied upon indirect techniques. For example, Elliott, (1975a) relied upon stomach fullness to infer periods of feeding in brown trout *Salmo trutta*. In other investigations observations were confined to daylight hours (Jenkins, 1969a; Bachman, 1984) and nocturnal activity could not be observed.

Laboratory studies have utilised devices such as a demand-feeder, where fish were trained to obtain food by pressing a lever, providing observations on rhythmic patterns of feeding in rainbow trout (Adron *et al.*, 1973; Landless, 1976; Alanara and Brannas, 1993, Sanchez-Vazquez and Tabata 1998). Whilst diel activity around a demand feeding may by important in aquaculture studies, concerning feeding time preferences and resultant growth rate effects, it provides little support to wild studies where the diet will be limited in space and time. More recent investigations have used radio and acoustic telemetry to facilitate continuous observation of fish over 24 h cycle within their natural environment (Armstrong *et al.*, 1989; Clapp *et al.*, 1990; Young *et al.*, 1997). These studies have highlighted the complex relationship that exists between feeding (energy intake) and locomotor rhythms (energy expenditure associated with increased metabolism).

Energy losses associated with activity comprise a substantial portion of the daily energy budgets of fish (Boisclair and Sirios, 1993). An integral part of this overall activity is spent searching for food (Boujard and Leatherland, 1992). Thus, optimisation of activity associated with foraging behaviour can substantially reduce energy losses associated with swimming during non-feeding periods (Boisclair, 1992). Fish generally show rhythmic fluctuations in their locomotor and feeding activity (Muller, 1978; Helfman, 1993), which is suggested to coincide with food availability (Young, 1999). Typical visual feeders like salmonids, have been considered to be diurnal (Hoar, 1942), with peaks in activity around dawn and dusk (Eriksson, 1973; Landless, 1976; Boujard and Leatherland, 1992; Eriksson and Alanara, 1992). Although some studies have found a nocturnal peak in salmonid activity, with individuals remaining in refuges by day (Grove *et al.*, 1978; Fraser *et al.*, 1993, 1995; Eriksson, 1978; Heggenes *et al.*, 1993). These differences may relate to seasonal effects with nocturnal behaviour patterns being displayed by fish living at lower temperatures. Heggenes *et al.* (1993) suggested that the nocturnal behaviour observed in wintering fish was an

ecologically 'adaptive homeostatic behavioural response' with trout apparently allocating energy differently, adopting survival strategies that minimized energy expenditure.

Although many studies have shown sub-lethal concentrations of pollutants to have adverse effects on fish activity, few have considered the consequence of such exposure on diel activity. One study, by Steele (1989) did show that aqueous copper abolished the diel rhythm of sea catfish *(Arius felis)*, although the latter experiment only examined the effects of short-term exposures. Short-term exposure (72 h) to many pollutants induces hyperactivity in fish, which is concluded to arise as an avoidance reaction rather than an actual toxicant effect (Sprague *et al.*, 1965; Kleerkoper, 1976). In chronic sub-lethal exposure this hyperactivity is followed by a much longer, and perhaps more ecologically relevant, period of hypoactivty (Waiwood and Beamish, 1978; Scarfe *et al* 1982; Steele, 1985). Longer-term studies (24 days) have been conducted in decapod crustaceans under heavy-metal exposures. Authors observed a loss of both light and tidal rhythmicity in exposed *Carcinus maenas* and *Astacus astacus*, and concluded that if the role of the endogenous rhythm was to optimise energy utilisation, then disruption of this function would have deleterious effects (Depledge, 1984; Styrishave et al., 1995).

In the present study long-term dietary Cu-exposure was shown to reduce activity in Cu-exposed fish possibly as a energy sparing mechanism to aid detoxification. However, measurement of activity at discrete periods throughout the day may give unreliable estimates when extrapolated to account for total daily activity, as trout will express a variety of behaviours at particular times throughout the day when most appropriate. My first objective was to study the swimming activity of trout in the aquarium over the diel cycle using the Ethovision tracking system and infra-red lighting to observe finite behaviours both during the light and dark phases, and determine periodicity in measured parameters. A second objective was to examine possible alterations in diel activity patterns due to Cu-exposure, based on the

hypothesis that Cu-exposed fish suffer from an energy deficit, and adopt an energy minimizing strategy whilst maintaining food intake. Hormones important to circadian function (melatonin, serotonin, cortisol) were also measured during the light and dark phases to examine changes in circadian physiology due to Cu-exposure and correlated with behavioural observations.

5.2 MATERIALS AND METHODS

5.2.1. Circadian locomotory activity

After 12 weeks of Cu-exposure (Trial A), 8 fish were chosen at random from each treatment and their swimming activity monitored using the Ethovision behavioural tracking system over two consecutive diel periods (for tracking methodology see 2.2.8, hardware set up as 4.2.2.). Visible light remained on the 12:12 hr light:dark regime (identical to husbandary conditions) and tracking was undertaken during both photoperiods using infrared illumination. Four 40 Watt Infrared battery lighting units (Tracksys Ltd., Nottingham, U.K.) were used to illuminate the arenas, and an infrared filter lens was fitted to the video camera to enable fish recognition by the computer in both light and dark regimes. Fish tracks were recorded for 30 minutes each hour for the 48-h period. From these tracks of each fish, locomotory parameters (total distance moved, mean speed, maximum speed, turning frequency and meandering rate) were calculated (Noldus software, Rotterdam, Holland). Absolute Turning frequency (ATR) is the absolute amount of turning per unit time. It can range from 0 to 180 degrees s^{-1} and can be defined as the unsigned change in direction of movement of an object per unit time. Meandering rates can be defined as the unsigned change in direction of movement of an object relative to the distance moved by the object and is expressed in degrees cm^{-1}. It will be influenced by both the locomotory rate and the turning rate.

5.2.2. Circadian endocrinology

At the end of the 12 week exposure period (Trial A), 6 fish from each treatment were sampled for serum collection (see section 2.2.4) at 19:00h after the 12 hour light cycle, and another 6 fish from each treatment were sampled at 07:00h after the 12 hour dark cycle. Blood samples were centrifuged (3000 g for 3 mins, Micro Centaur MSE) and the serum collected was frozen

(-80°C) until analysis was undertaken 2 days later. Cortisol concentrations were determined using an immuno-enzymatic assay (Beckman Access Immunoassay System, kit no.33600, detection limit 11 nmol l^{-1}), and melatonin in serum by double antibody radio-immunoassay (ImmunoDiagnostic Systems,IDS, Tyne & Wear, kit No. RE293021, detection limit 2.5 pg ml^{-1}). Samples were first treated enzymatically to free the antigen from its binding proteins. The antibody bound fraction was then precipitated by the addition of a second antibody, and the resulting pellet counted with a gamma counter (Hertfordshire, U.K.). Sample solutions were read from a calibration curve. Serotonin was determined by ELISA (IDS, kit no.RE59121, detection limit 0.17 nmol l^{-1}). Detection was made via an anti-biotin-alkaline phosphatase conjugate, with p-nitrophenyl phosphate as the substrate. The resulting absorbance in each ELISA well was read photometrically on an ELISA plate reader using a calibration curve to calculate concentration of the sample solutions.

5.2.3 Statistical analysis

To determine if any difference occurred in activity over the circadian cycle, behavioural data from the 48hr tracks was analysed as follows. The first approach utilised periodogram analysis (see Enright, 1965; Williams and Naylor, 1978) to detect oscillations in time-series data. A computer program (Styrishave et al., 1995) was used to calculate the amplitude (standard deviation of the hourly means of activity) and confidence intervals (derived by randomising data and performing periodogram analysis) for the given behavioural parameter. When plotted graphically if true periodicity exists the peak statistic produced by the assumed period length (24 h) will exceed the confidence interval on the plot and hence significantly different at the 5% level. Fish activity records were tested for periodicity both as individuals and as a group mean for each treatment. Secondly, data from individual fish over the 48 h cycle were grouped into 4 time zones over the 24 h cycle to compare differences in activity between

control and Cu-exposed fish within those periods. The relative time and photo-periods of these data sets were 12:00-18:00, (day, 6 h light), 18:00-00:00 (dusk, 3 h light:3 h dark), 00:00-06:00 (night, 6 h dark), 06:00-12:00 (dawn, 3 h dark: 3 h light). In order to test the combined effects of copper and photoperiod on activity Analysis of Covariance (ANCOVA) was used. Enzymology data was grouped into both treatments and time of sampling cohorts for analysis. Mean differences were tested using Student t-test and distribution plots analysed by ANOVA. In each of the statistical analyses, differences at the < 5 % level were considered significant.

5.3 RESULTS

5.3.1 Circadain Behaviour of trout after 12 weeks Cu-exposure

There was a marked effect of Cu-exposure on the circadian behaviour of trout, substantially reducing the normally high nocturnal activity (figs. 19 & 20). Control fish showed circadian rhythmicity in all behavioural parameters (distance moved, mean swimming speed, maximum swimming speed, absolute turning frequency, meandering rate) measured over a continuous 48 h period (periodogram analysis, S = 24 h). However, Cu-exposed fish showed a general loss of periodicity in overall distance moved and mean speed parameters (fig 19a,b & 20), with only a statistically significant periodicity (S = 24h) remaining in the maximum spontaneous swimming speed attained (fig. 19c), and orientation parameters (fig. 21 & 22). Interestingly, whilst Cu-fish showed periodicity in maximum speed, it was not evident in the mean speed data (compare figs. 19b & c); indicating that scope for activity (metabolic scope) probably remained intact. Therefore, since these latter parameters are calculated components of total distance moved, they therefore show that Cu-exposed fish spent proportionally more time at low speeds (or resting) than control fish.

For further analyses of behavioural patterns the 24 h cycle was divided up into 4 equal time periods (fig 20). During the 6 hour continual daylight period (12:00 – 18:00) both control and copper fish exhibited similar behaviour patterns spending 55-60% of their time resting (<2cm/s), 35% at low swimming speeds (2-10 cm/s), 5-6% at medium speeds (10-20 cm/s), and less than 2% of the time at high speeds. However, in the following period (dusk) when only 3 hours of daylight occurred before dark, control fish alter their swimming patterns, resulting in an increase in overall activity. During this time resting was reduced to 36%, low swimming was increased to 50% and both medium and high swimming activity

Figure 19. Circadian swimming activity of control (filled circles) and Cu-exposed (grey triangles) fish, over a 48hr period (n=8) after 12 weeks Cu-exposure. Data are normalised for the inter-animal variability in basal activity by expressing each data point as a measured percentage increase in activity over that individual's lowest recorded level of activity. The data points are fitted with a waveform trend line, visually showing any rhythmicity within the data. (a) Total distance moved (control r^2 = 0.643; Cu r^2 = 0.307 P<0.001 for both curve fits), (b) Mean speed (control r^2 = 0.38; Cu r^2 = 0.249; P<0.001 for both curve fits), (c) Maximum speed attained (control r^2 = 0.626; Cu r^2 = 0.477; P<0.001 for both curve fits). Black horizontal bars indicate periods of darkness. Each measured parameter (Fig. 3a, b, c) has a graphical representation of the periogram statistic (S) performed on both control and copper data sets (Fig. insert in each panel). Dotted lines show confidence limits set by periogram analysis performed on randomisation of the data set. Where (S) peaks above the upper 95% confidence limit, periodicity is observed in the data. Duration of each period (hours) is determined by the highest point of the peak.

(a)
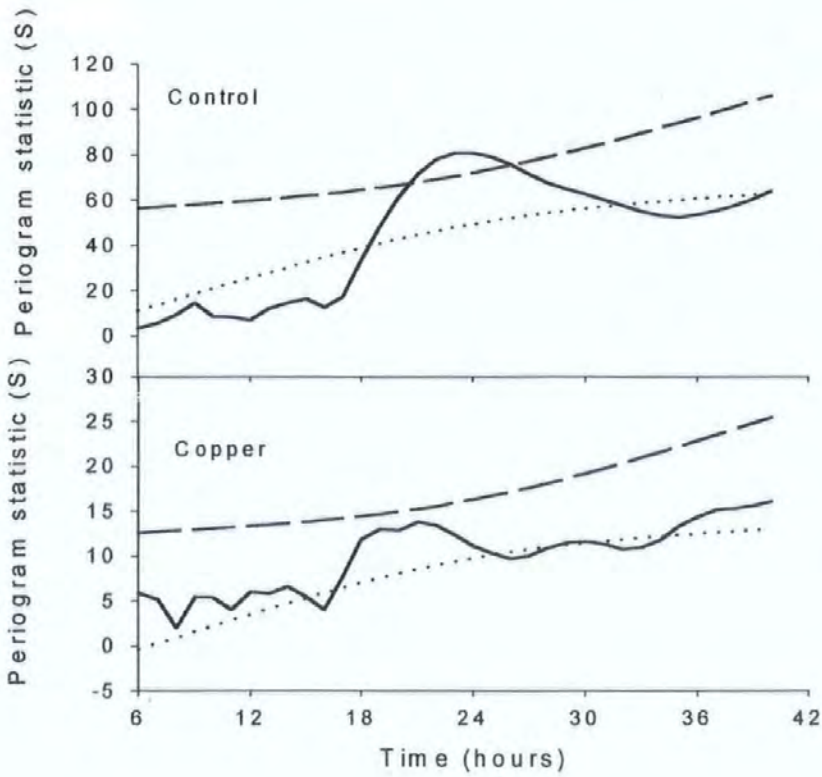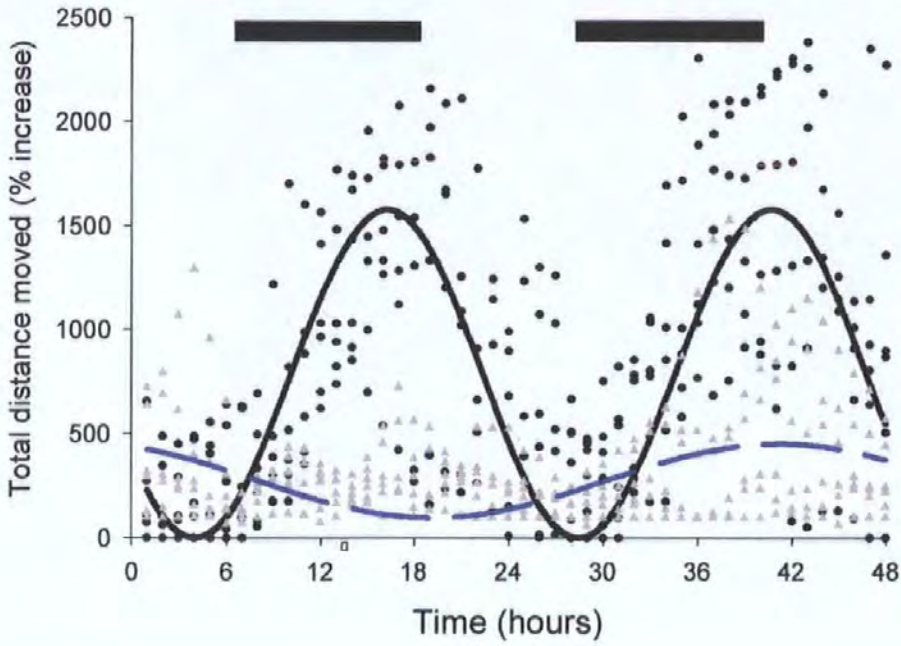
(b)
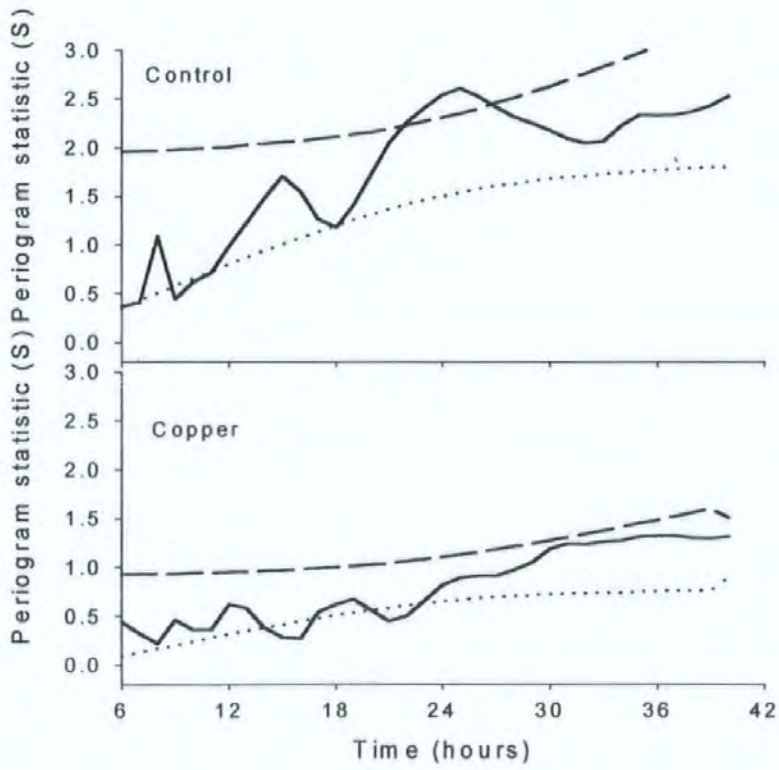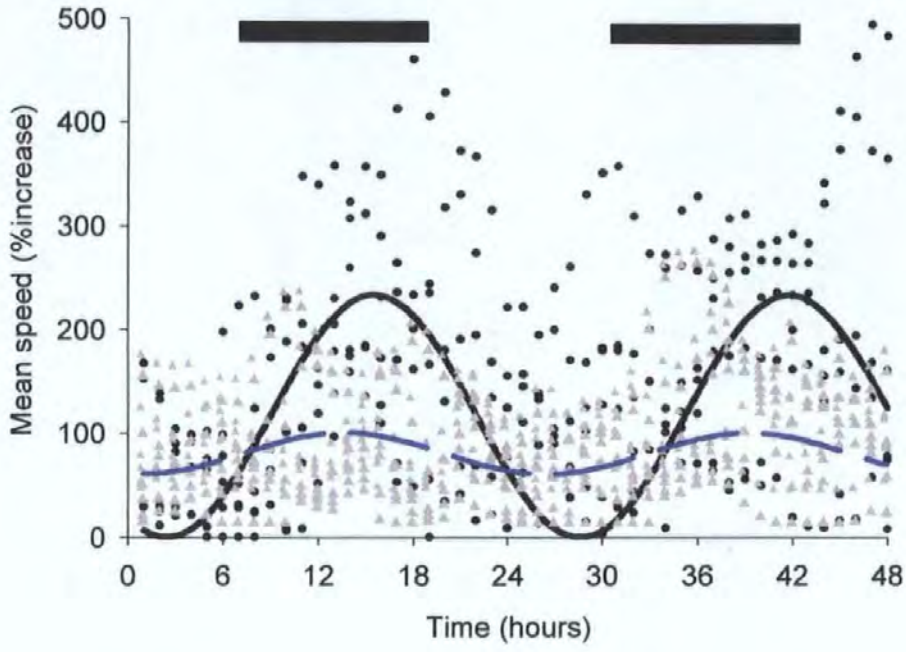
(c)
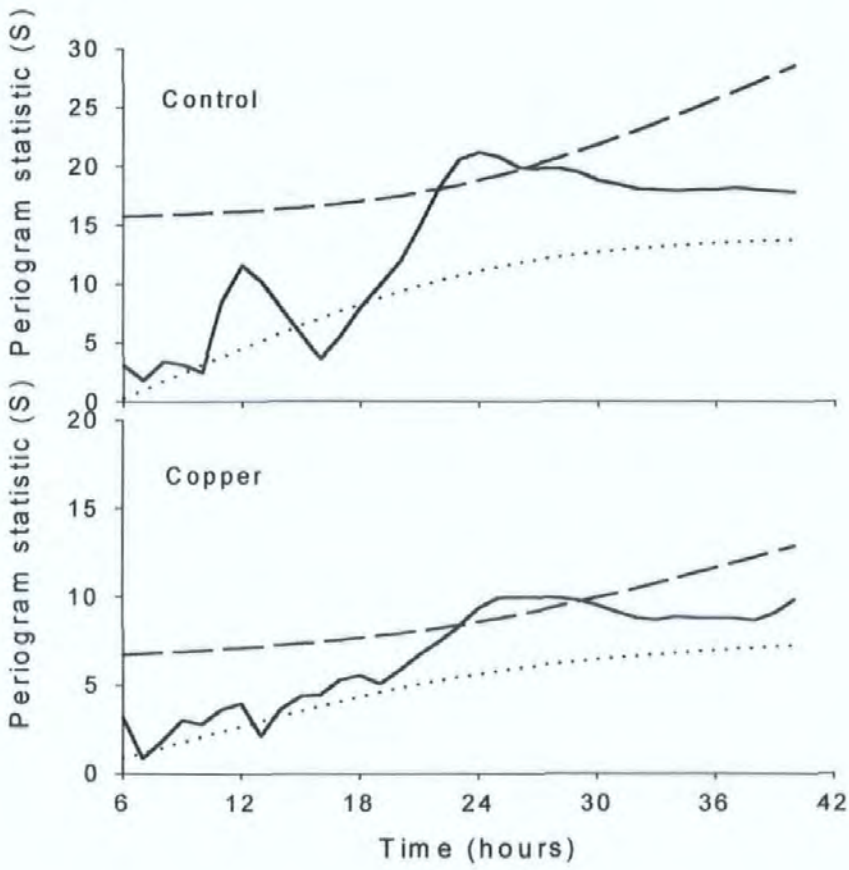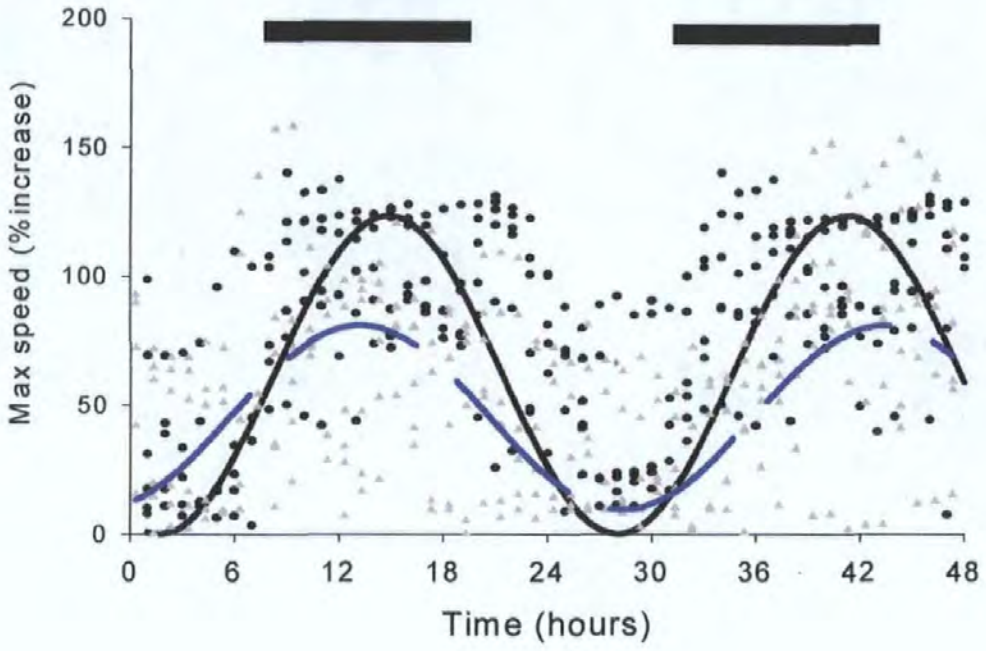
increased by 3 - 4%. Cu-exposed fish spent 10% more time swimming at slow speeds rather than resting, however, and spent significantly longer times resting in this period than controls (although their activity did increase slightly). Once into the period of full darkness (night) control fish continued to increase activity spending proportionally (30%) less time resting and at low speeds, and more time (15%) at high speeds. This increase in activity was carried over into dawn where control fish spent 22 % of their time swimming at a high speed and an equal amount of time resting. Cu-exposed fish on the other hand did not show such an increase in activity. Over the dark period they did not reduce their resting frequency but showed a reduction in low speed swimming to 33%, whilst the time spent swimming at medium speeds increased to 12%. Once into the period of dawn where highest activity was exhibited by controls, there was an alteration in the behaviour of Cu-exposed fish, which increased the time spent resting and decreased swimming time at low and medium speeds, but spent 5% more time at high speeds. This was however, still significantly less (22%) than spent high-speed swimming by control fish (Mann Whitney U test, U= 60, P = 0.0004).

The absolute turning rate (ATR) (fig.21) of both control and Cu-exposed fish showed significant periodicity of around 24 h. The ATR of control fish was around 75% greater than in the Cu-exposed fish, for both the highest (crest) and lowest (trough) wave amplitudes between treatments. However, there was a phase shift in the ATR between the two treatments, with control fish exhibiting the highest ATR during darkness whilst Cu-exposed fish exhibited a daily low during this time. In fig. 22 the ATR is expressed per distance moved by the fish, both treatments of fish show a very similar endogenous rhythm in this behavioural parameter turning more frequently as they swim during diel periods when food would normally have been presented to them during routine husbandry (fed at 15:00 h daily).

Figure 20. Mean changes in % time ($\pm$ S.E., n = 16) spent by (a) control and (b) Cu-exposed rainbow trout in 4 speed bins (<2, 2-10, 10-20, 20+ cm s^{-1}) during a 24 hr period after 12 weeks Cu exposure. In terms of BL s^{-1} these speed bins are <0.1, 0.1-0.5, 0.5-1.0, >1.0 respectively. The 24hr clock has been divided into 4 time zones (day, dusk, dark and dawn) each 6hrs in length, and total darkness is represented by black horizontal bar. * Copper data which is significantly ($P < 0.05$, t-test) different from the equivalent speed bin in the control data set, + indicates a speed bin that is significantly different ($P < 0.05$, t-test) from the speed bin in the previous time zone within treatment.
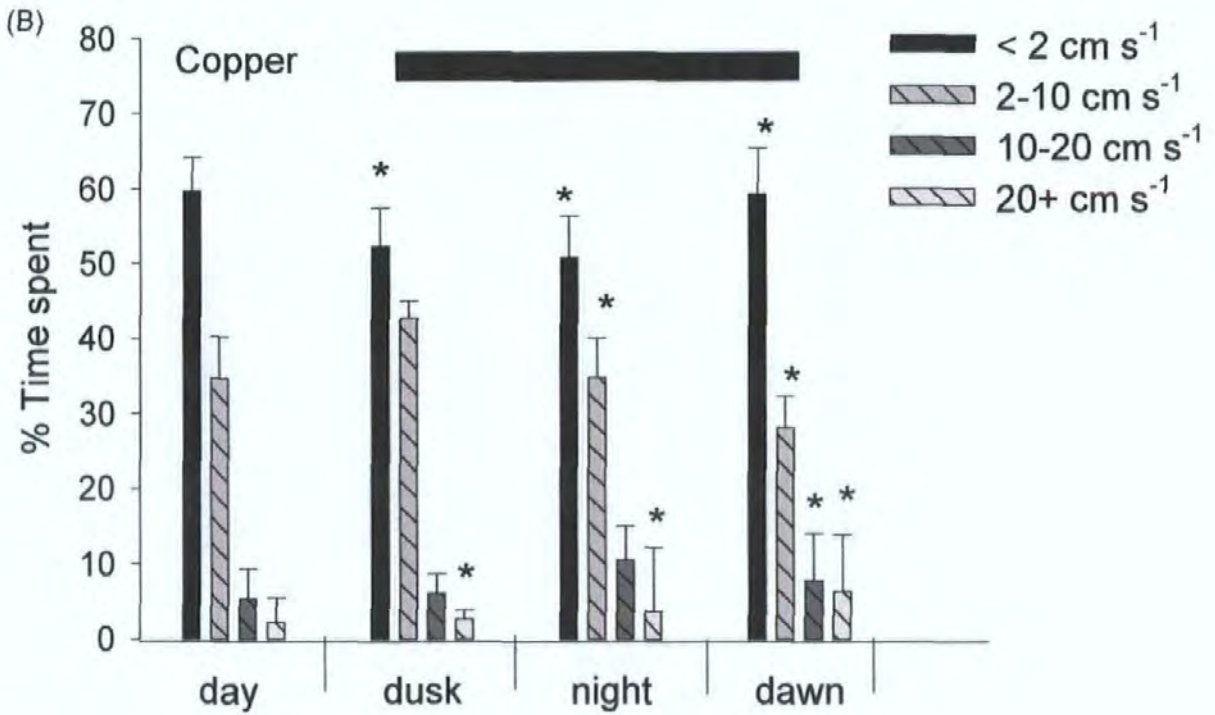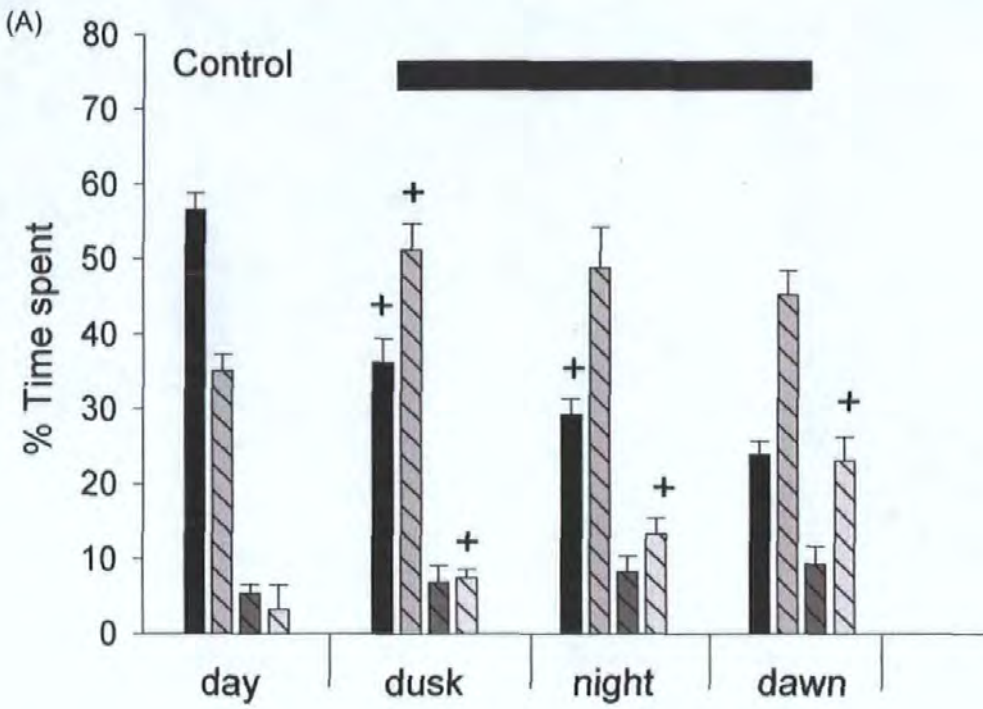
Figure 21. Absolute turning rate (degrees) of control (filled circles) and Cu-exposed (empty circles) fish, over a 48hr period, after 12 weeks Cu-exposure. Each data point represents a running average of 3 hourly measurements (1 hour previous and 1 hour after plotted time point). From these running averages ATR per fish (mean ± S.E., n = 8) are calculated per treatment. Dark bars represent periods of darkness. Non-linear regression lines (red) fitted to control (y = 311.1 + 46.9 x sin (2 x pi x 23.64 + 4.1), r2 = 0.79, P < 0.01) and Cu-exposed data (y = 154 + 47.6 x sin (2 x pi x 22.8 + 6.2), r2 = 0.78, P < 0.01). Graphical representation of the periogram statistic (S) performed on both control and copper data sets (Fig. insert in each panel). Dotted lines show confidence limits set by periogram analysis performed on randomisation of the data set. Where (S) peaks above the upper 95% confidence limit, periodicity is observed in the data. Duration of each period (hours) is determined by the highest point of the peak.
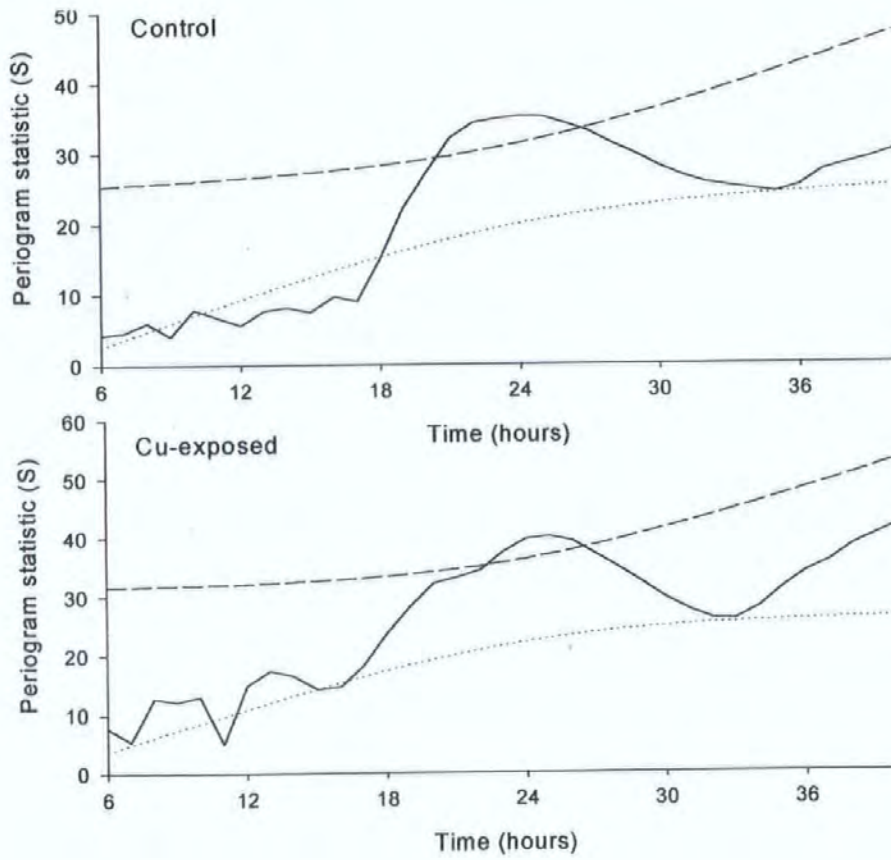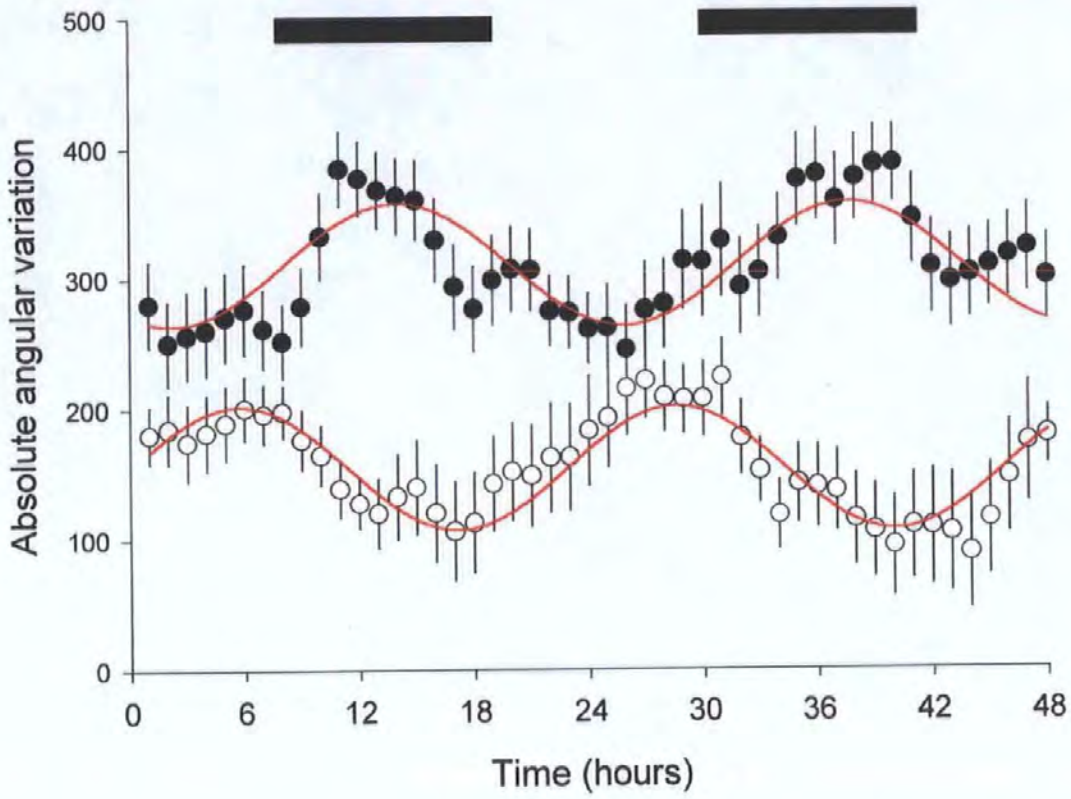
Figure 22. Meandering rate of control (filled circles) and Cu-exposed (empty circles) fish, over a 48hr period, after 12 weeks Cu-exposure. Calculated as the absolute degree of turning relative to the distance moved by the fish. Each data point represents a running average of 3 hourly measurements (1 hour previous and 1 hour after plotted time point). From these running averages meandering rate per fish mean ± S.E., n = 8 are calculated per treatment. Dark bars represent periods of darkness. Non-linear regression lines (red) fitted to control (y = 27.1 + 8 x sin (2 x pi x 23.4 + 6.3), r2 = 0.74, P < 0.01) and Cu-exposed data (y = 29 + 7.1 x sin (2 x pi x 25 + 0.27), r2 = 0.68, P < 0.01). Graphical representation of the periogram statistic (S) performed on both control and copper data sets (Fig. insert in each panel). Dotted lines show confidence limits set by periogram analysis performed on randomisation of the data set. Where (S) peaks above the upper 95% confidence limit, periodicity is observed in the data. Duration of each period (hours) is determined by the highest point of the peak.
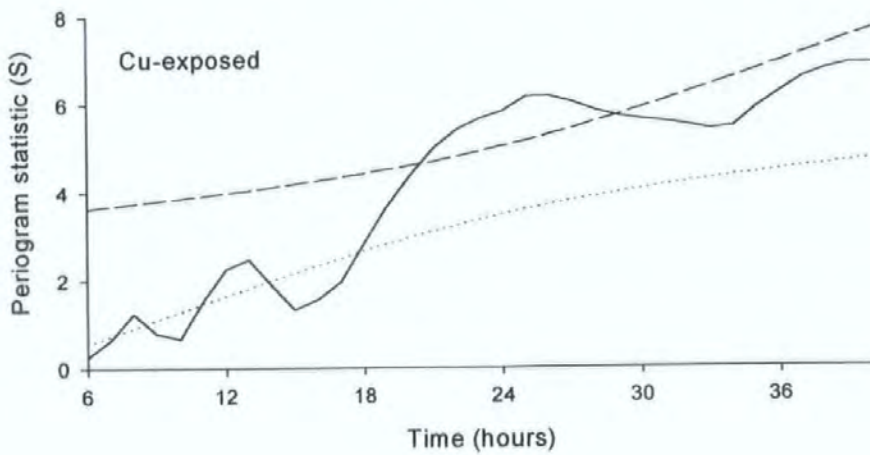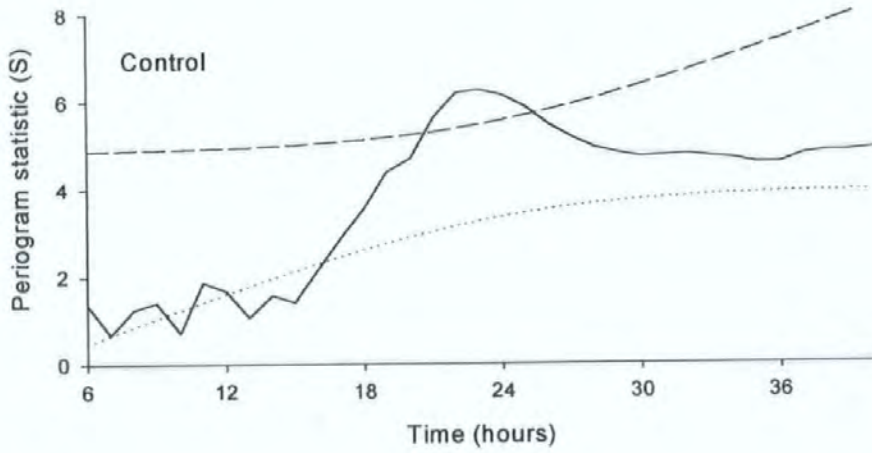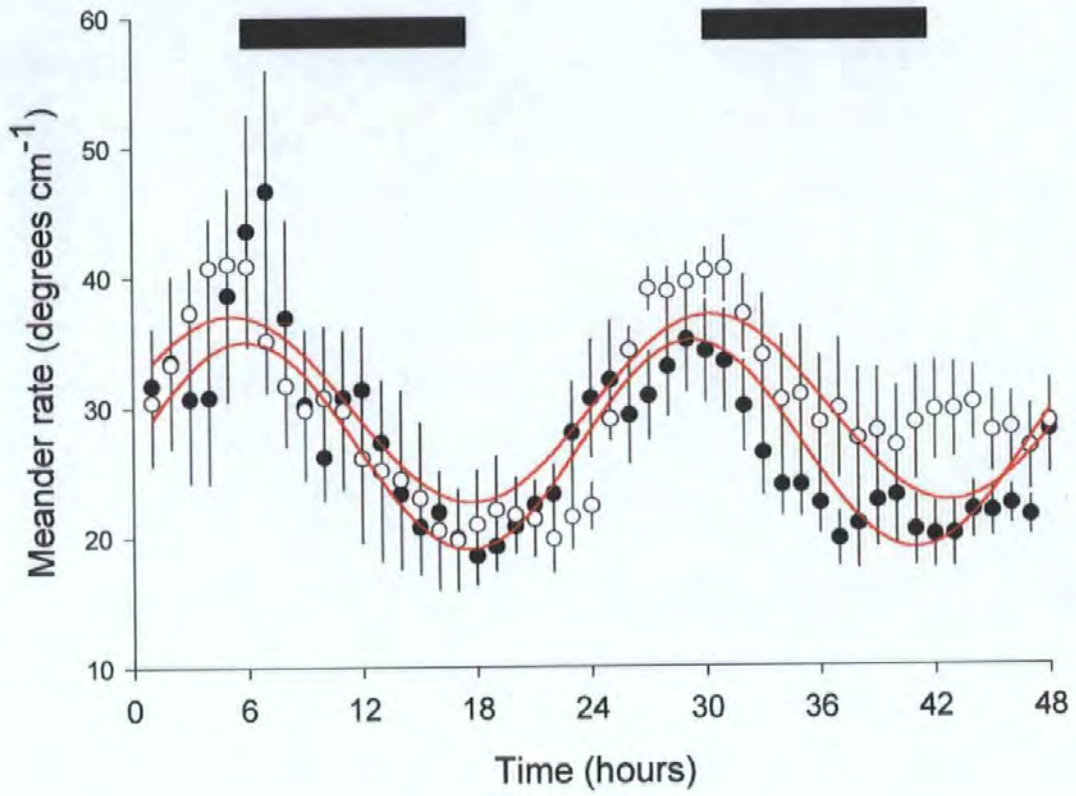
5.3.2 Circadian endocrinology

Circulating serum cortisol levels were elevated 2-fold in Cu-exposed fish compared to controls at the end of the light period (t-test, t = 2.07, P<0.05). However, cortisol levels were similar in both groups by dawn, and typical of the measurements from fish in social groups (Gregory and Wood, 1999). No significant difference (t-test, t = 1.65, P = 0.123) was observed in circulating melatonin between treatments although both treatments showed a 20% decline over the light period (Table 10). Serotonin levels, though similar between treatments at dawn, were significantly decreased (below the detection limit < 0.17 nmol l^{-1}) in Cu-exposed fish at dusk (Table 10). For all the hormone data there was no correlation between hormone levels and the order in which blood samples were collected. However, control fish showed an inverse exponential relationship between cortisol and serotonin levels (y = 26.903 Ln (x) + 184.76, r^2 = 0.89, n = 10, P<0.0001). This relationship was not evident for Cu-exposed fish (r^2 = 0.015, n=10, P>0.1).

Table 10. Circulating serum hormone levels after 12 h daylight (19:00) and 12 h darkness (07:00) in control and Cu-exposed (12 weeks) rainbow trout.

	Control (23 mg Cu Kg feed^{-1})		Copper (726 mg Cu Kg feed^{-1})	
Time	07:00	19:00	07:00	19:00
Serum cortisol (nmol l^{-1})	270 ± 54	130 ± 30	260 ± 56	$302 \pm 51*$
Serum melatonin (pg ml^{-1})	105 ± 15	83 ± 10	110 ± 21	89 ± 9
Serum serotonin (nmol l^{-1})	62 ± 14	44 ± 6	92 ± 14	$< 0.17* +$

Data are mean and S.E. (n = 5). * indicates Cu-exposed data which is significantly different from controls (t-test, P<0.05). + below detection limit of assay.

5.4 DISCUSSION

Effects of Cu-exposure on circadian swimming activity

It is well documented that most species of fish exhibit a diel pattern in activity (Boujard and Leatherland, 1992; Young, 1999). Species can adopt a nocturnal, diurnal or crepuscular rhythm in peak activity, and these rhythms are responsive to variations in environmental temperature, light intensity, predation risk and food availability (Clark and Levy, 1988; Heggenes *et al.*, 1993; Alanara and Brannas, 1998; Valdimarsson *et al.*, 1997; Metcalfe *et al.*, 1999).

In this laboratory study, the diel rhythm of the control rainbow trout exhibited a circadian rhythm in activity, measured as the total distance moved, with a periodicity of approximately 24 h. The mean swimming speed of a fish will determine its total distance moved, and therefore it exhibits a similar circadian profile, increasing during the dark phase and peaking around the start of the light phase (dawn). The effects of 12 weeks chronic dietary exposure appeared to have abolished much of this rhythm in activity with exposed fish swimming less distance (at a lower mean speed) compared to controls during darkness. To my knowledge this is the first report of an aquatic pollutant disrupting the circadian locomotor rhythm of fish.

The daily pattern of specific swimming speed is explored in more detail in fig. 20. Similar to a previous study by Handy *et al.*, (1999), who recorded swimming activity during day-light hours (11:00 - 13:00) using a lower Cu dose of 500 mg Cu kg^{-1} d.w. feed, Cu-exposed trout spent less time active, and proportionally more time at low swimming speeds than controls (figs. 19 and 20). This reduction in activity, when compared to control fish, was much more prominent during the night, particularly in the period leading up to dawn when the swimming activity of control fish peaked. This

explains the overall loss of periodicity in the total distance moved by Cu-exposed trout. Nevertheless, during this pre-lights on period, Cu-exposed fish did increase the proportion of time they spent at higher speeds over 20 cms^{-1}, and periodicity was observed when examined in the maximum swimming speed attained, peaking around the dawn period. This produced a similar circadian profile for maximum swimming speeds as observed in control fish, and may represent remnant behaviours exhibited by the Cu-exposed fish. Although, during this period of high-speed swimming, Cu-exposed fish also increased the percentage of time they spent at very low swimming speeds or resting. Consequently, mean swimming speed did not significantly increase at these times and periodicity was not apparent.

The apparent shift observed in finite swimming repertoires over the circadian cycle may be explained by previous findings on the metabolism of Cu-exposed fish obtained in this study (Chapter 3), showing that Cu-exposed trout had a higher requirement for oxygen to remain as active as control fish. Under normal conditions it is expected that endurance will decrease as the fish approaches R_{max} (Fry 1971). Thus, I suggest that Cu-exposed trout, to compensate for their increased oxygen requirements Cu-exposed trout spend more time at rest or at low swimming speeds than control fish. Therefore, the mean swimming speed of Cu-exposed trout does not exhibit a measurable diel rhythm in mean swimming speed. If mean swimming speeds are calculated for the 24 h period they are 0.46 ± 0.11 and 0.21 ± 0.07 BL s^{-1} (mean $\pm$ S.E., n = 8) for control and copper fish respectively. If these speeds are used to calculate routine metabolic rate (using fig.12), both treatments have very similar oxygen consumption rates of around 14 mmol kg^{-1} h^{-1}. This suggests that the observed reduction in activity during the night/dawn period was a metabolic saving strategy initiated by the Cu-exposed fish, and served to reduce total daily energy expenditure, to a level similar to control fish.

In this study, control fish steadily increased their activity during the dark period so that maximum activity coincided with dawn. Similar observations have been made of wild rainbow trout (Boujard and Leatherland, 1992), and the diurnal and crepuscular pattern of activity, has been suggested to be a behavioural factor relating to them being visual predators with low light conditions reducing their ability to catch prey items (Henderson and Northcote, 1985; Fraser and Metcalfe, 1997). As well as serving to synchronise activity with dawn food availability, whilst avoiding day-time predators (Metcalfe *et al.*, 1999). However, it may be somewhat erroneous to compare diel activity patterns of wild salmonids with laboratory observations made here, as it is expected for salmonids to show rapid changes in their daily activity response to alterations in the environment (Fraser *et al.*, 1993).

During this study fish were fed in the late afternoon (15:00), and therefore, peaks in activity did not coincide with food availability. The method of food presentation may also have differed from a wild situation, being presented to the group as a single daily ration. As a result, within the simplified environment of the tank individuals did not have to chase prey items, but instead may have had to compete with other individuals within the tank, for this short-period when food was present. To maximise food intake the captive fish would have needed to forage efficiently. The best strategy for a captive fish to maximise food intake may not have been to swim at a high speeds to catch prey, but rather favour a swimming pattern of a low speed and a high turning rate strategy. This may serve to maximise the number of pellets eaten off the bottom of the tank, and not waste foraging time by swimming between areas within the tank. This type of foraging is called area restricted searching and is observed in many animals when food is distributed in a temporal nature, allowing the maximisation of food uptake (Krebs and Davies, 1997). Indeed, by observation of the absolute turning rate per distance moved (fig. 21),

both control and Cu-exposed fish increased their meandering rate (degrees cm^{-1}) to peak around the time of day when food would have normally been presented to them during the previous 12 weeks exposure. Although fish were not fed during the tracking procedure the endogenous rhythm of fish can be sustained for a number of days even if food is not presented (Boujard and Leatherland 1992). Interestingly, at this period of the diel cycle when food would normally have been presented the activity profiles of control and Cu-exposed fish were least dissimilar in all behavioural parameters measured (mean speed, maximum speed, absolute turning and meandering rate). It seems logical to assume that the behaviours exhibited, were the most efficient in terms of strategy to maximize intake when feeding in a social group within the confines of the aquarium.

Complex swimming patterns, such as frequently turning, are metabolically expensive requiring a higher cost for activity than linear forward motion (Smit, 1965; Weatherley et al., 1982; Webb, 1990; Boisclair and Tang, 1993). This implies that for Cu-exposed trout, that do not show large nocturnal increases in mean speeds, the most metabolically expensive period of swimming undertaken during the diel cycle, was during feeding periods when they increased their frequency of turning per distance moved. This observation further suggests that Cu-exposed trout exhibited a cost minimizing strategy at non-feeding times during the diel cycle, and make up for losses associated with increased energy requirement for maintenance and activity due to Cu-exposure, by investing primarily in activity at times of day only when food was present. This is in contrast to control fish, which showed the highest period of activity during the dark/dawn period when food was not present. The rise in absolute turning rate (ATR) of control fish during nocturnal periods was not apparent when ATR was expressed per distance moved (meandering rate). This indicated that during nocturnal periods control fish spend a much greater proportion of time swimming linearly than they did during the

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apparent feeding times. I suggest that it was due to the large distance moved (1500% increase over day-time periods) and the confines of the tank that give the 50% increase in ATR during night/dawn periods. The metabolic cost of the relative activities displayed by control and Cu-exposed fish at different periods of the diel cycle was not determined, but such studies would provide valuable information relating to circadian metabolic strategies of both healthy and metabolically impaired fish.

Cu-exposure effects on hormones associated with diel activity

As in other vertebrates, the pineal gland in salmonids is thought to use photoperiodic information to synchronize daily behavioural and physiological events. Although, it is not clear whether this is achieved mainly through neural or endocrine pathways (Porter *et al*, 1996). The production of melatonin by the pineal exhibits a distinct diel rhythm with elevated levels during the hours of darkness (Randall *et al.*, 1995). Periods of high activity in animals are generally associated with high melatonin and low serotonin concentrations in the brain, and elevated plasma cortisol levels (Fenwick, 1970; Winberg *et al.*, 1993; Nelson, 1995). In control fish this appears to be the case with a trend of higher serum melatonin (reflecting secretion from the pineal gland (Porter *et al.*, 1996), and cortisol levels at dawn (Table 10). The slightly higher dawn serotonin levels in the blood may reflect serotonin secretion from the gut (Hansen and Skadhauge, 1997) during its own tissue-specific endogenous rhythm (Clements and Rees, 1998).

Cu-exposed fish manifest a pathophysiological endocrine response because they:- (i) fail to behaviourally respond to high melatonin at dawn, (ii) have relatively high cortisol levels during the day indicating a stress response (Sumpter, 1997), and (iii) cannot maintain circulating serotonin during the day (Table 10). The lack of a behavioural response to melatonin may be explained by circulating melatonin acting as a

Cu chelating agent, reducing oxidative damage as it has been suggested to have antioxidant properties (Abuja *et al.*, 1997). Alternatively, disruption of pineal night-specific Cu-ATPase (Borjigin *et al.*, 1999) might alter melatonin production due to serotonin inhibition. Cortisol elevation during the day may be related to mucosal cell turnover in the gut during tissue repair (Bury *et al.*, 1998). The mechanism causing failure of circulating serotonin remains unclear, and this serotonin block may not affect melatonin synthesis in trout (unlike other organisms, Coon *et al.*, 1998). Results from this study represent a preliminary investigation into hormone secretion levels in rainbow trout and how they may be affected by Cu-exposure. More detailed investigation into hypothalamus and brain stem concentrations may provide more quantitative data to explain associated changes in behavioural circadian rhythms in Cu-exposed trout.

An energetics perspective for alterations in the circadian profile

Trout have been cited as a clear example of an energy maximiser (Griffiths 1975, Bres, 1986). Thus the apparent increase in activity costs during non-feeding periods by control fish must have had longer-term energetic advantage, as investment in activity for day or night behaviours will depend on the relative costs and benefits of these activities at these times. I suggest that this nocturnal rise in control fish activity is due to competitive behaviours. I make this assumption, firstly because, wild and hatchery trout have been previously documented to have excessive energy expenditure in daily activities associated with inter-specific interactions (Newman, 1956; Mesa, 1991; Pottinger and Pickering, 1992), and hatchery fish kept within a confined space are much more aggressive than their wild counterparts (Yamagashi, 1962). Secondly, during the 12 weeks exposure, mortalities were mainly found in the control tanks (fig. 26); displayed body damage associated with intra-specific aggression (Turnbull et al., 1998), and were

always found at initial daily observation (08:30), immediately after the apparent peak in control fish activity. And finally, within the simplified environment of the tank the best strategy for a control fish would be, to simply, obtain more food than its competitors. This may be best achieved by agonistic encounters with conspecifics that serve to increase stress and suppress the appetite of subordinate fish (Li and Brocksen, 1977; Noakes and Leatherland, 1977;Berejikian *et al.*, 2000).

In summary, Cu-exposed fish do not periodically increase activity during non-feeding periods, to express behaviours that may be associated with inter-individual aggression, favouring to expend the largest proportion of their daily activity budget during feeding periods. Comparisons of observations made in this study may be drawn with over-wintering fish, who switch from a day-time foraging strategy in summer that maximises energy intake, to a cost-minimising shelter and move-strategy in winter, due to low food availability (Heggenes *et al.*, 1993; Valdimarsson *et al.*, 1997; Metcalfe *et al.*, 1998). This dual phasing capacity of salmonids to adverse changes in the environment has been simulated in salmonids under laboratory conditions and has been suggested to confer ecological flexibility by the fish to optimise survival (Eriksson, 1978). Thus, it appears that the flexibility of the circadian activity pattern is an important survival strategy for Cu-exposed fish, concerned with minimising energy expenditure whilst maintaining energy intake from feeding.

If this hypothesis is correct, and the elevation in activity of control fish during the dark period was associated with inter-individual interactions and dominance behaviours. Then it could be assumed that whilst the reduction in activity of Cu-exposed fish at these times aided in maintaining daily metabolic homeostasis, it may have further reaching implications relating to group interactions and social status of the individual. This

possible concomitant effect of a voluntary reduction in daily activity of fish feeding on a

Cu-contaminated diet will be examined in the following chapter.

CHAPTER SIX

THE EFFECTS OF CU-EXPOSURE AND INFLUENCE OF COMPETITION ON GROWTH AND ENERGETIC STATE OF RAINBOW TROUT

6.1 Introduction

The energy available to an animal will be determined by the quantity of food ingested, its nutrient content, and the proportion of energy assimilated from digested food. In theory, the energy utilisation strategy adopted by animals will be a cost minimising one, because energy allocation should be governed by the need to increase Darwinian fitness and minimise the cost of survival (Heggens *et al.*, 1993). In reality, strategies of energy utilisation will be mediated by environmental factors, such as temperature, food availability, competitors and predation (Ludwig and Rowe, 1990; Valdimarsson *et al.*, 1997; Metcalfe *et al.*, 1999), which will influence metabolism and food consumption through both physiological and behavioural means.

For an individual fish, growth rate will depend upon surplus energy acquired from the daily food ration (R) after costs associated with routine metabolism are satisfied, according to the equation,

$$R = F + U + M + P \text{ (Winberg, 1956)}$$

Where: R = energy gained as food

F = energy lost as faeces

U = energy lost as nitrogenous excretory products

M = energy expended in a range of bodily functions - Metabolism

P = energy storage or growth

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If the costs associated with acquiring food are high, such as high swimming speeds to catch prey, then the requirement of R will increase and little surplus energy will be available for growth. Likewise, if there is little energy cost associated with an energy rich diet, then growth rates can increase.

A solitary fish, without competitors, may only need to balance energy costs associated with obtaining prey and resultant benefit derived from them. However, wild or captive salmonids will normally function within a social hierarchy, and require an investment of energy to compete for food. For example, in salmonid populations as much as 80% of the daily energy budget, may be invested in competitive interactions (Li and Brocksen, 1977). The intensity of competition will be largely dependant on population density (McNicol and Noakes, 1981; Christiansen et al., 1992), food abundance (Jobling and Waardvick, 1983), and food distribution (Ryer and Olla, 1995). It may therefore be expected that increased investment in routine metabolism (T), needed to acquire food (R), will result in decreased growth (G), and consequently the degree of growth depensation may reflect the amount of energy used for competitive interactions.

Selection theory predicts that a fish should optimise growth rates, as this will have a positive influence on the age of maturation and survival rates (Huntingford and Turner, 1987). For a fish competing within a social group for a limited resource, growth rate may depend more on competitive ability than physiological efficiency. Under these conditions, selection for faster growing fish will favour more aggressive and competitive fish, rather than those that maximize the efficiency of growth (Weatherly, 1976; Doyle and Talbot, 1986; Swain and Ridell, 1990). Therefore, social interactions are likely to be an important determinant of the growth rate of fish living in social groups. This may develop from individual fish having short bouts of aggressive encounters, resulting in the reduced food intake of certain individuals and allowing a small number of aggressive individuals to

monopolise the food supply (Chapman, 1962, Noakes, 1980; Fausch, 1984). These more dominant fish will consequently have a better growth rate. Hence, under this scenario the variation in size-frequency distribution of the population would be expected to increase over time.

Results from the current study have demonstrated that rainbow trout consuming a Cu-contaminated diet exhibited a 30% increase in routine metabolic rate over controls; the amount of energy required to maintain the same level of activity for a Cu-exposed fish was greater than that expended by control fish. Subsequently, it was suggested that Cu-exposed fish reduced their swimming activity as a compensatory response to maintain overall metabolic expenditure equal to that of controls. Because the costs of aggressive encounters, to compete for resources are metabolically expensive (Hack, 1997), they require a significant degree of investment in activity and therefore will constitute a major component of the energy budget. In Chapter 4, it was shown that Cu-exposed fish require more oxygen to remain as active as control fish. Thus I hypothesis, that for a Cu-exposed fish to maintain the same level of competitiveness as a control fish, they will require a greater oxygen demand, thus a reduction in competitive ability may be observed.

In this chapter my aim was to ascertain the energetic cost of competition for food within a social group, and to determine what the consequence of the increased cost of activity due to Cu-exposure may have on social competition, and feeding hierarchy. To accomplish this objective, fish stocking density was manipulated, as this is a major factor influencing strength of feeding hierarchy (Yamagashi, 1962), and the growth parameters of individuals examined within these populations, to estimate the degrees of growth depensation due to varying strengths of competition. This was examined in both control and Cu-exposed fish to determine treatment effects. This was supported by observational analyses of fin damage, which results from aggressive interactions in rainbow trout (Abbott

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and Dill, 1985). Individuals from all groups at different stocking densities were analysed for muscle and liver carbohydrate metabolites, as aggressive encounters are costly and burst type activities will be supported mainly by anaerobic metabolism (Wood 1991; Milligan, 1996). This process will occur in the white muscle, where conversion of glycogen into lactic acid (Johnston and Goldspink, 1973), will reflect work intensity and competitive capacity of both control and Cu-exposed fish. Social groups were also observed directly for alteration in the intensity of behavioural interactions that may occur due to Cu-exposure.

6.2 MATERIALS AND METHODS

6.2.1 Cu-exposure of fish held within differently sized social groups

During spring 1999 (Trial A) rainbow trout in groups of 40 individuals (in triplicate) were exposed to dietary copper (730 mg Cu Kg^{-1} feed d.w.). Due to sampling for fish analysis and some low levels of mortality, approximately 20 individuals were present in each tank by the end of the 12th week of the exposure period (for methodology of Cu-exposure see section 2.2.2 - 2.2.5). Twice during the exposure period (week 6 & 12) all fish within tanks were individually removed, lightly anaesthetised (0.1g l^{-1} MS222), weighed and their lengths determined. Results obtained during this experiment (fig. 26), illustrated differences in weight distribution of Cu-exposed populations compared to controls. These results lead to the development of experiments carried out during the spring of 2000 (Trial B), in which rainbow trout were exposed to dietary copper (730 mg Cu Kg^{-1} feed d.w.) at three different levels of fish stocking density. A flow chart detailing the relative Cu-exposure trials and respective analysis with regard to this chapter is presented in fig. 23.

The 3 differently sized groups of fish used during Trial B, consisted of a large group in which the food was scattered diffusely over the surface of the water in the tank (DL); a reduced sized social group consisting of 10 individuals in which food was delivered from a point source (PS); each social group was duplicated per dietary treatment. Also 6 fish per treatment were kept isolated (ISO) during the exposure period.

Fish exposed within the DL group were kept under conditions identical to those described in section 2.2.3 (fig. 2), and were fed a 1% bodyweight ration twice daily by scatter feeding across the surface of the water (for diet formulation and ration calculation see sections 2.2.2. & 2.2.5 respectively).

Figure. 23 Flow chart illustrating the different Cu-exposure trials, and follow-on analyses, used in determination of results presented within this chapter.

Trial A (Spring 1999)
fish kept in groups 40 individuals exposed to
730 mg Cu kg^{-1} d.w. feed
for 86 days, (triplicate).

Trial B (Spring 2000)
fish in different sized social groups
exposed to 730 mg Cu kg^{1} d.w. feed.

Fish kept in social groups of
40 individuals (DL) exposed
for 56 days (duplicate).

Fish held individually (ISO),
6 per treatment
exposed for 28 days.

Fish in social groups, 10
individuals (PS) exposed
for 21 days (duplicate).

Behavioural observation during exposure

After 43 and 86 days exposure, fish
were individually weighed and the
length determined.

After each exposure period, fish within each
group were weighed separately and lengths
determined. Ion and enzyme analysis
was undertaken on internal organs.

Fish in each (PS) group (n = 10 per group) were placed in each of four glass tanks (120L x 36W x 36 H cm) to enable behavioural visual observation (fig. 24). All tanks received identical water (for water quality and husbandry parameters, see section 2.2.4.). Food was delivered via a digital automatic feeder at 1 % body ration (Nutrimatic 500), twice daily at 09.30 h and 17.30 h (commercial feed, Trouw, size 02). It was important that size of pellets was consistent so an equal mass of food ration was delivered to all tanks consistently (for Cu application to feed preparation see section 2.2.2.). This type of point source feeding and reduced number of individuals within the group (n = 10) served to increase effects of social hierarchy by amplifying the defensibility of the food resource (Kalleberg, 1958; McCarthy *et al.*, 1999). To further increase the defensibility of the food resource and to also enable an observational period of aggression associated with feeding, the fish were trained to respond to a red light (100W) source (Gee et al., 1994; Dr. Gee personal communication). This was switched on by a digital timer (Quantum, Cambridgeshire, U.K.) 1 minute prior to feeding and remained on for 1 minute once food was delivered. This was suspended above point of feed entry (fig. 24 & plate 2).

Because it is impractical to introduce the fish to a new environment and feeding regime and expect them to perform normal competitive interactions immediately, fish were trained before the experiment using the method outlined below. Initially, 80 (50 ± 5g) individuals were taken from the stock tank (see section 2.2) and 20 fish were placed into each of 4 glass tanks. These fish were fed using the above method for 7 days. After this time 10 individuals were selected for the experiment and the others removed. Individuals selected for the experiment each satisfied 3 criteria: they were of a similar size, they could respond to the red light as a food stimulus, and they were not overtly dominant or subordinate. This selection, was therefore, not a random procedure but it served to reduce hierarchical stress effects during the

Figure 24. Operational diagram of equipment used for small social group (n = 10 per tank) Cu-exposure feeding experiments. Blue arrows indicate direction of water flow. Digital automatic feeders delivered 1% feed ration twice daily at precisely 09.30 h and 17.30 h. A 100-Watt red light above feeders was illuminated for exactly 1 minute before and after feed delivery. To enable clearing of faeces and uneaten food from within the tank a current of 5 ± 0.3 cm s^{-1} was generated by positioning the inflow pipe and by using a drainage system consisting of a standpipe with an over-sleeve containing air-stone to increase upward movement of water within the sleeve (insert). A charcoal filter in the pre-filter and one on the return pump ensured any leached aqueous copper was not circulated back into the system. One tank remained on the control diet for the duration of the experiment (42 days) whilst the other was switched to a Cu-exposed diet after 21 days. The system was duplicated and biofilters connected by Eheim pump (1L s^{-1}) to ensure identical water parameters for both systems.

Timer

Red light

Digital Automatic feeders

Pre-filter
+ charcoal

Biofilter

Charcoal filter

pump

Standpipe
with sleeve
+ air-stone
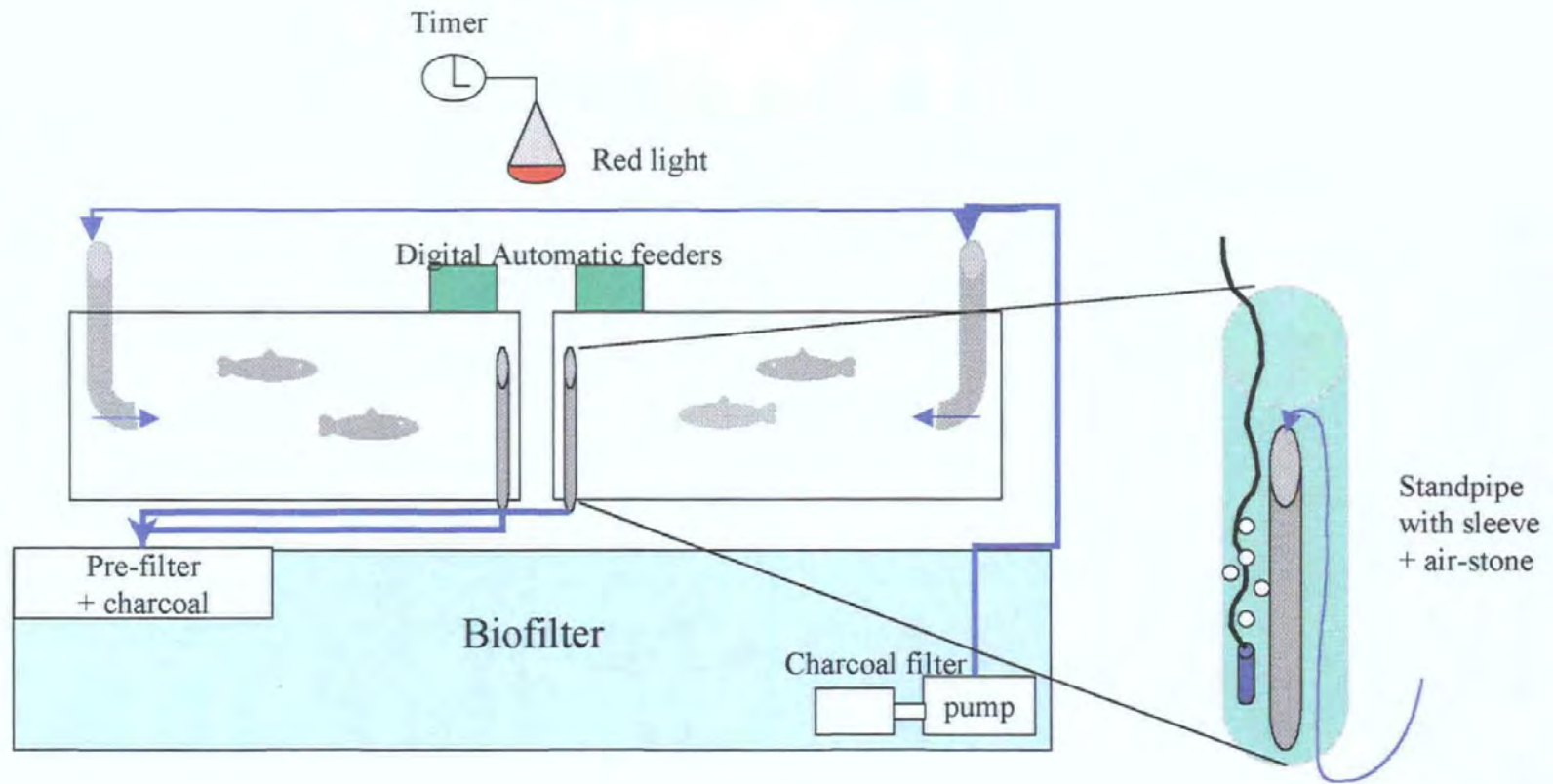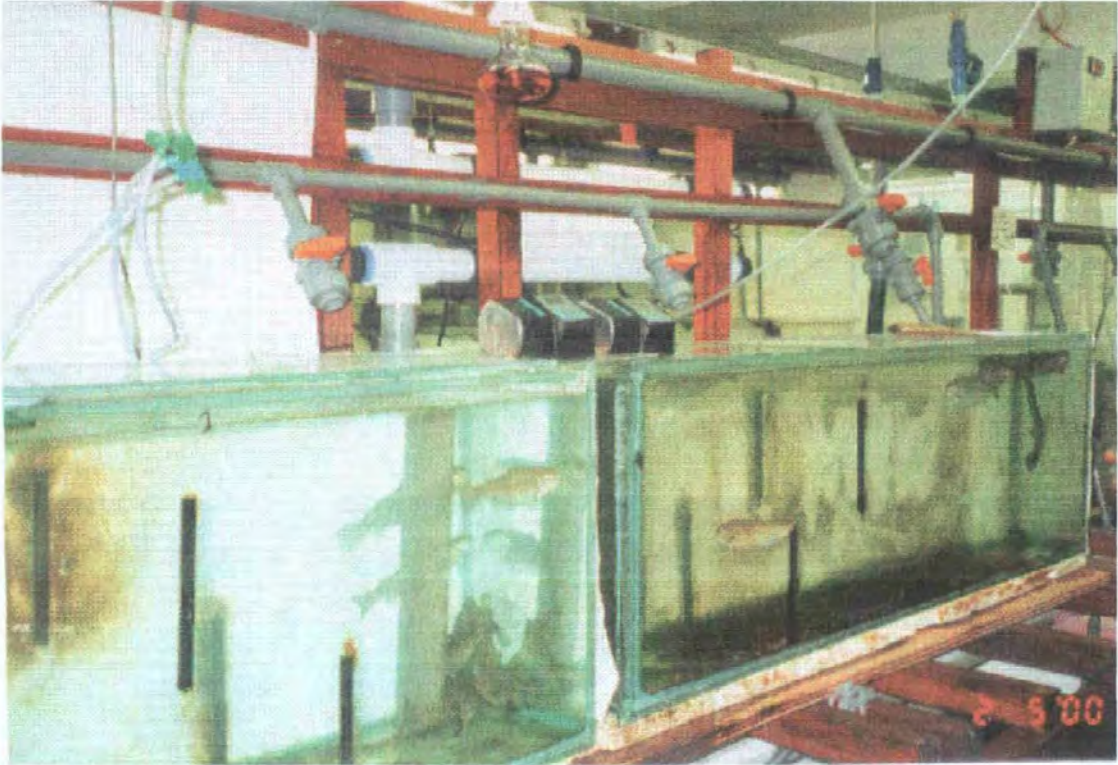
Plate 2. Aquarium system used to monitor effects of Cu-exposure on a point source feeding hierarchy (PS group, n = 10). Plate shows automatic feeders (Nutramatic 500) and red light source, used as visual stimulus for food delivery (switched on 1 min prior to feeding). Fish in near tank fed Control diet and far tank a Cu-contaminated diet (730 mg Cu kg-1 feed d.w.). The system above was duplicated, and both systems received water from same biofilter.

acclimation period, and also generated four social groups of equally sized fish for the comparative study. Following selection all fish were fed for 21 days on the control diet, when after this period 2 groups were selected at random and fed the Cu-exposed diet (730 mg Cu Kg^{-1} feed d.w.) for a further 21 days, whilst the other 2 fish groups remained on the control diet.

Isolated fish (n = 6 per treatment) were each kept in a glass tank (120L x 36W x 36Wcm), and all fish received water from the same biofilter (see section 1.2.4.). Each fish was fed twice daily at 1% bodyweight ration (see above). After a 7-day acclimation period 6 tanks at random were switched to the Cu diet the other 6 remained on the control diet.

6.2.2. Nutritional and biochemical effects of competition and Cu-exposure

Growth

To enable observation of interaction rates, hierarchy, and growth changes in individual fish, it was necessary to identify each fish throughout the exposure period. This was achieved by tagging anaesthetised (0.1g l^{-1} MS222) fish with a series of dots applied to both left and right flanks (fig. 25 & Plate 3) 48hrs before the commencement of each feeding trial. The combination of marks gave a unique identifier to each fish. Within the DL group, (n = 40) a sub-sample of 10 individuals were randomly selected and tagged. In the PS group all the fish within each tank were tagged (n = 10). It was not necessary to tag fish from the ISO group.

To observe alterations that may have occurred in growth rates due to dietary treatment or size of social group, tagged fish and all the ISO fish were lightly anaesthetised (0.1g l^{-1} MS222), and weight and length determined before the commencement of each experiment. In the DL group tagged fish were measured again after 28 and 56 days Cu- exposure, whilst in the PS group after 21 and 42 days, and after 28 days in the ISO group. From these weight
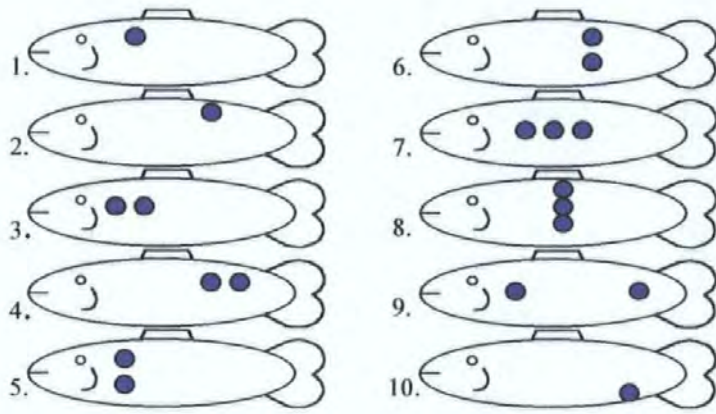
Figure 25. Illustration of fish markings and corresponding i.d. number. Fish were tagged on both flanks using a 2% Alcian blue solution applied using a Pan-jet (U.K.) 48hrs before commencement of exposure experiments. Fish recovered from this procedure within a few minutes, and it appeared to have no adverse effects on fish physiology or behaviour. Marks were observable on the fish for the 8-week exposure period.
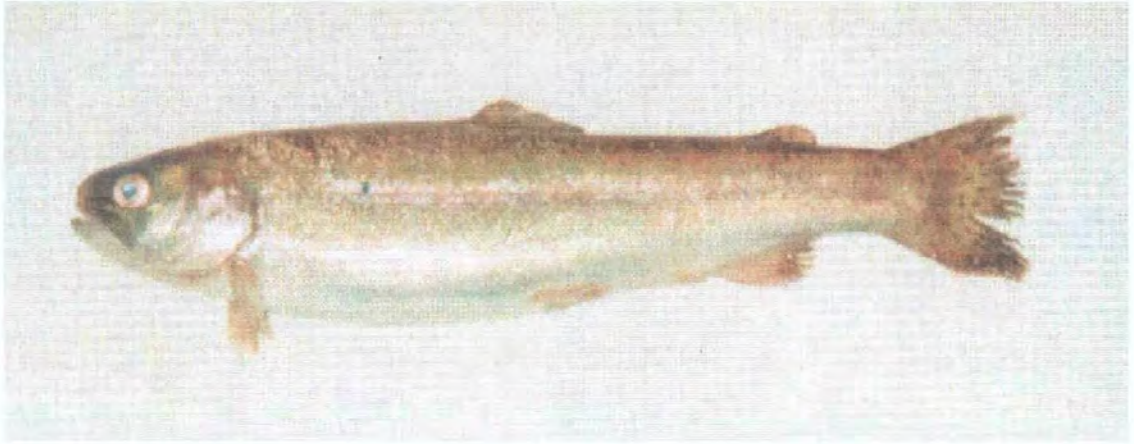
Plate 3. Rainbow trout *(Onchorhynchus mykiss)* used for all Cu-exposure experiments. Plate shows Alcine blue mark, applied using a pan-jet. Fish also displays signs of inter-individual aggression, with fraying and splitting of dorsal, caudal and anal fins.

measurements Specific Growth Rate (SGR) and Food Conversion Rate (FCR) were determined (See section 2.2.5.)

Tissue biochemistry

Muscle biochemistry was performed to assess the energy cost of competition. Fish were killed by terminal anaesthesia (0.5g, MS 222) and the collection of tissue and preparation procedure followed methods as in section 2.2.6. Cu analysis on excised livers was done by ICPAES (see section 2.2.7). Enzyme analysis was carried out on tissues from fish after they had been exposed to Cu at the three levels of stocking density described above (DL group = 56 days, PS group = 21 days, ISO group = 28 days).

Protein

Protein concentration within the liver and combined red and white skeletal muscle of fish was determined from each of the social groups using methodologies given in section 3.2.5.

Lactate

Lactate concentration was determined in the F1 fraction from liver and muscle crude homogenates (see section 1.2.5.) in fish from all 3 social groups. The assay was performed in triplicate in a microplate and read on a Dynatech MRX microplate reader. Reagent mixture, 300µl (0.43M glycine, 0.34M hydrazine, 3.1 mM β-NAD$^+$, 19 U ml^{-1} LDH) was added to 30 µl sample in each microplate well before being shaken on the Dynatech (shake program 3) for 30 seconds and incubated for 30 min at 37°C, then read at 340nm on Dynatech MRX. Standards were prepared from a 2 mmol l^{-1} stock solution diluted with deionised water in serial dilution. 0.0625, 0.125, 0.25, 0.5, 1, 2 mMol l^{-1} ($r^2 = 0.98$ n = 120). Concentration determined by standard curve was then calculated back to lactate

concentration per gram of tissue, by multiplying by the dilution factor of homogenate, used.

Glycogen

Glycogen concentration was determined in the liver and muscle of fish from the ISO, PS and DL groups. The assay was based on the Anthrone method of breaking glycogen into glucose for determination by colourimetric assay (Chun and Yin, 1998). An aliquot of 50 μl of muscle homogenate or 20 μl of liver homogenate (see section 2.2.5) was added separately in triplicate to 0.5 ml 30% KOH, and heated in an oil bath at 100°C for 20 min. Tubes were allowed to cool before the addition of 1.5 ml of anhydrous ethanol (Sigma) and then centrifuged at 4000 g for 15min. The supernatant was discarded and 0.5 ml of deionised water plus 0.2% Anthrone in 98% 4M HSO_4 was added and the tubes reheated in the oil bath at 100°C for 20 min. The tubes were removed, cooled and 330 μl of each sample pipetted into a microplate and read at 620 nm on a Dynatech MRX platereader. Glucose standards (1.6 μg of glucose is equivalent to 1.44 μg of glycogen, Chun and Yin, 1998) were made up in deionised water at concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.6 μg ml^{-1} (r^2 = 0.98, n =120). Glucose concentration was determined from a standard curve, and was then calculated back to glycogen concentration per gram of tissue, by multiplying by the dilution factor of the homogenate.

Pyruvate

Pyruvate concentration in fish skeletal muscle was measured using Sigma diagnostics kit Cat. No. 726-UV. 2 ml of supernatant from centrifuged tissue homogenates and 0.5 ml of Trizma base solution (1.5 mol l^{-1} Tris(hydroxymethyl)aminomethane) was added to a 1 cm

light-path cuvette and mixed by inversion (to bring reaction mixture to pH 7.4). 0.5 ml of solution of Trizma base solution containing 40% nicotinamide adenine dinucleotide (NADH) was then added and the cuvette inverted several times to mix. The sample was read immediately on a Phillips spectrophotometer 200 at 340 nm (Initial A), using water as a blank. 50 µl Lactate Dehydrogenase was then quickly added to the cuvette and inverted several times to mix. After 3 minutes the absorbance was reread (Final A). A further reading was taken one minute later to ensure reaction had reached completion. Change in Absorbance per min (ΔA) was calculated by (Initial A − final A) /3, and pyruvate was determined according to the equation,

$$\text{Pyruvate conc. (mmol } l^{-1}) = \frac{\Delta A \text{ per min x TV x 1000}}{6.22 \text{ x SV x LP}}$$

Where ΔA per min is the change in absorbance per min at 340nm, TV denotes the total volume (ml) of the assay, SV is the Sample volume, 6.22 is the Millimolar absorptivity of NADH at 340nm, and LP is the lightpath distance through the cuvette (cm), and 1000 is the conversion of units per ml to units per litre. The concentration determined from the standard curve was then calculated back to pyruvate concentration per gram of tissue, by multiplying by dilution factor of homogenate.

6.2.3 Behavioural observation of competition

Each tank within the PS group (fig. 24) was observed directly (and also video recorded to allow for detailed analysis) for a total of 12 h (20 min x 36 days) during non-feeding hours and for 72 min (2 min x 36 days) during feeding periods (when the red light was on). For each observation period the dominant fish within the tank was identified (using skin colour and posture; Abbott *et al.*, 1985), and its activity within the tank (estimated by counting the number

of lengths it swam in the tank – 'patrols') was recorded using a hand held tally-counter. An interaction between two fish that caused the subordinate to flee, or resulted in actual contact (Huntingford, 1976) was scored for both the dominant and subordinate fish. Whilst anaesthetised for growth measurements (see section 6.2.2) fish were also examined for damage to fins and skin, which may have indicated signs of inter-individual aggression (Turnbull *et al*, 1998). Details of body condition were recorded and scored in the following way: a score of 1, for no body damage; 2, slight fraying to dorsal and tail; 3, severe fraying with 1-3 splits to caudal/dorsal fins and damage to anal fin; 4, more than 3 splits to caudal and dorsal fins with spines showing and anal and pectoral fin damage; 5, as 4 but including scale loss or skin lesions; 6 was given if mortality occurred.

6.2.4 Statistical analysis

For comparison of median weights between tanks in Trial A (Fig 24) the Mann Whitney U-test was used, and the Kolmogorov-Smirnov test was also employed to examine alterations in weight distribution. For each physiological parameter measured during Trial B, replicates were compared using one-way ANOVA. If there was no significant difference ($P > 0.05$) between replicates, they were then pooled by treatment. F-test was used to examine difference in variance between samples. To test for significant differences between measured parameters within the social groups, and that of isolated fish ($n = 1$, 6 per treatment), individuals from social groups (if pooled then 6 from 20 individuals) were randomly selected for analysis using a random number generator and the mean from these individuals calculated and tested against that of the isolated fish (One–way ANOVA). To test for significant differences due to dietary treatment the Two-tailed Students t-test was employed. To test the significance of fitted regression lines One-way ANOVA was used. Multifactor ANOVA was used to determine relationship between SGR, liver Cu burden and size of social group.

6.3 RESULTS

6.3.1. Effects of competition and Cu-exposure on metabolites

The breakdown of glycogen to lactate and its precursor pyruvate within the muscle and liver characterize the energy invested in burst-type locomotory activity (Beamish, 1968). Within this experiment (Table 11) muscle glycogen was depleted 2-3 fold in social fish compared to fish kept in isolation regardless of dietary treatment. Cu-exposed ISO fish had a 28% decrease (t-test, $t = 3.2$, $P = 0.007$) in muscle glycogen compared to controls, but there was no significant difference due to dietary treatment. Mean liver glycogen was decreased by 31% (t-test, $t = 2.7$, $P = 0.04$) in ISO fish due to Cu-exposure. Fish exposed to copper within both the differently sized social groups showed approximately a 20% decrease in liver glycogen, though this was not significant possibly due to the large within group variance, which significantly increases with increasing competitive intensity (F-test, $P<0.05$). As the products of glycolysis, lactate and pyruvate levels were generally increased within both the liver and muscle due to social stress (Table 11), with muscle lactate levels being elevated 7-8 fold in social fish compared to solitary fish. Liver lactate levels were only significantly different in the PS group (ANOVA, $P < 0.05$). The PS group was the only group of fish that had an increase in muscle (t-test, $t = 2.7$, $P = 0.008$) and liver (t-test, $t = 2.9$, $P = 0.006$) lactate due to Cu-exposure. Pyruvate concentrations were elevated 2-fold (ANOVA, $F = 2.4$, $P = 0.03$) in both social groups compared to solitary fish, though were not significantly different from each other (Table 11).

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	Treatment	ISO group (n = 1)	DL group (n = 40)	PS group (n = 10)
Muscle glycogen	Control	12.2 ± 17.2 *	5.8 ± 7.6	5.8 ±10.8
(μmol g^{-1} w.w.)	Cu-exposed	8.8 ± 11.8 *#	5.9 ± 8.1	4.5 ± 6.8
Liver glycogen	Control	160.8 ± 9.3 * +	128.2 ± 12 +	106.7 ± 21 +
(μmol g^{-1} w.w.)	Cu-exposed	112.3 ± 11.1 # +	104.0 ± 14 +	92.1 ± 22 +
Muscle lactate	Control	3.6 ± 0.4 *	8.6 ± 1.8	10.7 ± 1.2
(μmol g^{-1} w.w.)	Cu-exposed	2.2 ± 0.2 *	8.7 ± 2.5 *	14.4 ± 1.3 *#
Liver lactate	Control	2.5 ± 0.5	2.8 ± 0.8	4.0 ± 1.0 *
(μmol g^{-1} w.w.)	Cu-exposed	2.4 ± 0.3	3.1 ± 0.6	6.1 ± 1.1 *#
Muscle Pyruvate	Control	0.12 ± 0.01 *	0.24 ± 0.02	0.21 ± 0.02
(μmol g^{-1} w.w.)	Cu-exposed	0.12 ± 0.01 *	0.2 ± 0.02	0.23 ± 0.03

Table 11. Carbohydrate metabolites measured within muscle and liver homogenates from control and Cu-exposed rainbow trout. Assume that stocking densities are in order of increasing competitiveness due to the influence of group size and food distribution, which was delivered from a point source in the PS groups and from a diffuse source in the DL groups. Data are mean ± S.E., n = 6 ISO (isolated fish) group, n = 20 for PS (small social) group and n = 20 for DL (large social) group. * significant difference (ANOVA, $P < 0.05$) between all other stocking densities within the same dietary treatment. # significant (t-test, $P < 0.05$) difference between dietary treatments within same stocking density. + significant difference in variance (F-ratio, $P < 0.05$) between other stocking densities within the same dietary treatment.

6.3.2. Effects of competition and Cu-exposure on growth parameters

There was no significant difference in the mean weights between control and Cu-exposed fish (Mann Whitney, U-test, U = 1182.5, P =0.68) after 12 weeks exposure (fig. 26). Mean body weights (not significantly different, t-test, P > 0.05) at the end of the experiment were 21.7 ± 9.0 g (n = 44) and 18.4 ± 5.1 g (n = 47) for control and Cu-exposed fish respectively. However the distribution of body weights within each tank differed between fish fed a control and Cu contaminated diet (fig. 26). These differences were not evident after 6 weeks of Cu-exposure (data not shown), however at week 12 (end of the experiment), the variability of individual fish weight fed a control diet was significantly greater (variance, 79.7, n = 45 fish) than in fish fed a Cu diet (variance, 32.7, n = 45 fish). In control fed tanks, fish weight distribution became skewed towards larger body size as the number of outliers in the lower quartile were reduced and the upper quartile was extended due to a small percentage of increasingly larger individuals. Control fish showed the normal aggressive behaviour of trout, and as expected, some mortalities occurred as the fish increased in size concomitant with a declining stocking density due to experimental sampling. The percentages of mortalities within the total population were 9.6% and 4.0 % for control and copper treatments respectively (fig. 26). All mortalities in control tanks were of small fish below the group quartile size range for that treatment group (mean weights shown in fig. 26). These small fish showed signs of social subordination with severe fraying of the dorsal and caudal fins, a condition typical of lower ranked individuals (Turnbull *et al.*, 1998). In comparison, fewer mortalities occurred in the Cu-exposed fish with none occurring in the latter half of the experimental period. The dead fish were not of a size below the group median and did not show signs of physical damage, appearing to have died from causes other than intra-individual aggression.

Figure 26. Distribution plots of individually weighed fish from control (empty boxes) and Cu-exposed (filled boxes) fish from each of 3 tanks per treatment (n = 14-16 fish in each tank at week 12), and pooled data within treatment. Median value represented by vertical black line through box. Box show the25th to the75th percentile. Tails show 95% percentile. * significantly different distribution of body size in each tank of copper treated fish compared to all control tanks (P<0.05, Kolmogorov-Smirnov) from all control data. # tank 1 in the Cu treatment was different from control tanks 2 and 3 only. Initial fish weight was 5g. At the end of experiment fish were similar mean weights of around 20g. Mortality rates for each tank are indicated, expressed as a % of the total cumulative mortality in the tank for the entire experiment. The mortality rate for pooled data are for all fish within treatment. Cont. = control; Cu = copper.
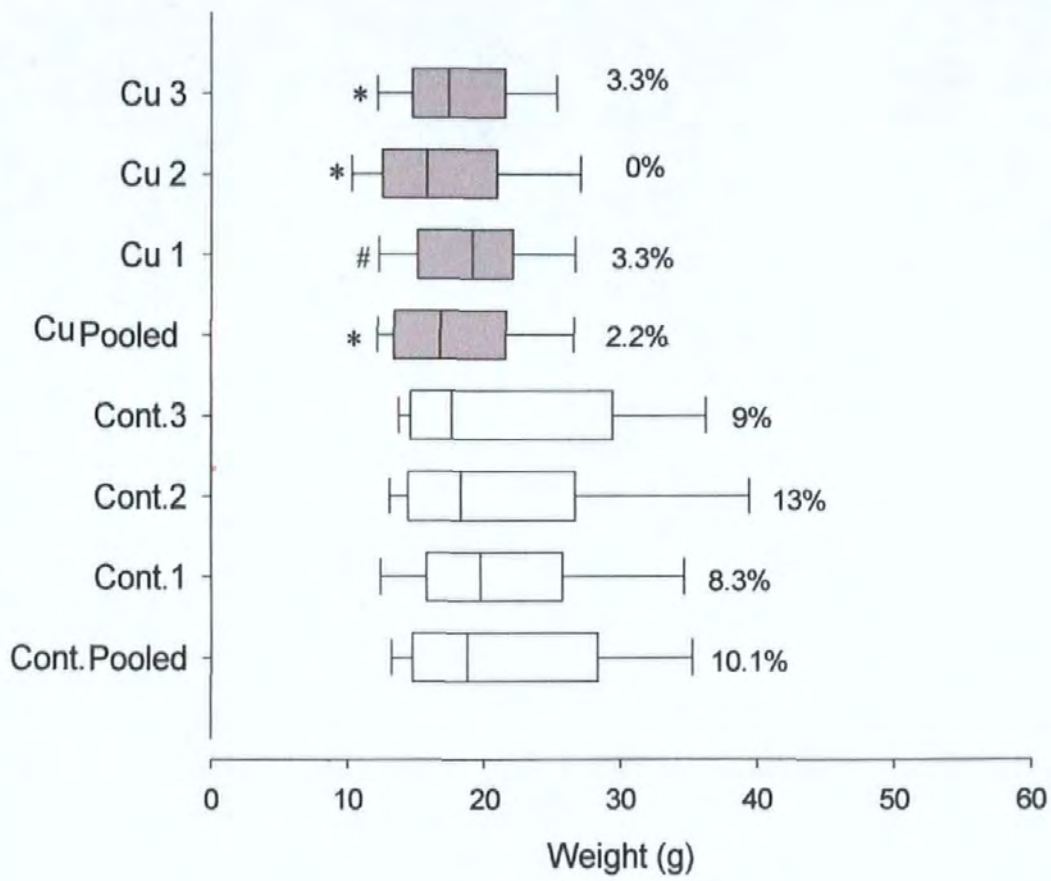
Table. 12 shows the Specific Growth Rates (SGR) and Food Conversion Ratios (FCR) of fish exposed for 8 weeks at 3 different stocking densities (these being ISO, n = 1; PS, n = 10; DL, n = 40). These results are in agreement with those from the previous 12 week exposure trial (Fig.24) with dietary Cu-exposure having no significant adverse effects on the mean growth rate of fish held within both sizes of social group (ANOVA, DL, F = 2.09, P = 0.15; PS, F = 3.1, P = 0.08) or in isolation (ANOVA, F = 1.1, P = 4.5). There was a significant difference in growth parameters due to stocking density however, with the DL group having a 2-fold reduction in SGR and FCR compared to solitary fish (ANOVA; control, F = 21, P = 0.009; Cu-exposed, F = 19, P = 0.01). There was also a further 20% reduction in SGR in the PS group (ANOVA, control, F = 1.6, P = 0.64; Copper, F = 0.56, P = 4.5) compared to the DL group (Table 12). The lack of any statistical significance in mean SGR and FCR between the 2 different sized social groups was probably due to the large within group variation (Table 12). This was significantly greater (f-test, control, F = 15.9, P = 0.0001; copper, F = 3.7, P = 0.003) for the PS group (n = 10) compared to the larger (n = 40) DL group.

Some authors have suggested that 'real growth' is better measured by protein accretion (McCarthy et al., 1993; Carter et al., 1993a) and relates to overall energy content within the carcass of the fish (Van Weerd and Komen 1998). Comparison of protein content within the muscle and liver of control and Cu-exposed fish (Table 12) held at different stocking densities shows the fish from the ISO group have a significantly higher muscle (ANOVA, F = 86.3, P = 0.0001) and liver protein (ANOVA, F = 68, P = 0.0001) composition than either of the social groups. The stronger the competition within the group the more reduced the protein deposition within both the muscle (ANOVA, F = 155, P = 0.0001) and liver (ANOVA, F = 19.5, P = 0.003), and the increased within group variance (F-ratio, P < 0.05).

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	Treatment	ISO group (n = 1)	DL group (n = 40)	PS group (n = 10)
Specific growth rate (SGR)	Control	1.93 ± 0.05* +	0.96 ±0.1 +	0.8 ± 0.3 +
	Cu-exposed	1.67 ± 0.04* +	0.78 ± 0.1 +	0.65 ± 0.2 +
Food Conversion ration (FCR)	Control	2.06 ± 0.08* +	0.68 ± 0.1 +	0.58 ± 0.4 +
	Cu-exposed	1.72 ± 0.06* +	0.51 ± 0.2 +	0.38 ± 0.4 +
Muscle protein (μmol g^{-1} w.w.)	Control	149 ± 12.25*+	122.2 ± 21*+	91 ± 42.2*+
	Cu-exposed	152.1 ± 13.2*+	128.3 ± 22*+	85 ± 43.3*+
Liver protein (μmol g^{-1} w.w.)	Control	120.5 ± 13.8*+	79.6 ± 13.4*+	51.5 ± 24.1*+
	Cu-exposed	132 ± 11.2*+	78 ± 12.8*+	48.2 ± 21.4*+

Table 12. Nutritional performance of control and Cu-exposed fish held at 3 differently stocked densities. Assume that stocking densities are in order of increasing competitiveness due to the influence of group size and food distribution, which was delivered from a point source in the PS groups and from a diffuse source in the DL groups. Data are mean ± S.E., n =6 for ISO (isolated fish) group, n = 20 for PS (small social) group and n = 20 for DL (large social). * significant difference in mean (ANOVA, $P<0.05$) from other stocking densities within same dietary treatment. + significant difference in variance (F-ratio, $P<0.05$) between stocking densities within the same dietary treatment.

The relationship between Cu-exposure and weight distribution within a social group of fish is explored further in fig. 27a. This shows that the Cu content of the liver is elevated in Cu exposed fish and this Cu-burden is proportionate to the SGR of that fish (PS group, $y = 0.06x + 0.16$, $r^2 = 0.64$; ANOVA, $F = 27.2$, $P = 0.008$; DL group, $y = 0.149x + 0.06$, $r^2 = 0.4$; ANOVA, $F = 13.44$, $P = 0.02$). Control groups from all 3 stocking densities have a similar liver Cu burden of around 0.05 μmol g^{-1} w.w. day^{-1}, with no relationship occurring between SGR and liver Cu content.

The relationship between SGR and liver Cu content of social Cu-exposed fish suggests that the fish with the higher SGR are obtaining more of the food supply and as a consequence are also ingesting a larger Cu load, which is accumulating in the liver. In the PS group were SGR is more disproportionate within the group, there is greater correlation between SGR and liver Cu-burden ($0.06x$, $r^2 = 0.64$) than in the lesser competitive DL group ($0.15x$, $r^2 = 0.4$). ISO fish also show a proportional relationship between liver Cu burden and SGR (not enough data points for regression fit, correlation $r = 0.57$). However, the Cu-content of the liver is approximately 2-fold lower (fig. 27b) than the expected value derived from regression fits through the SGR and liver Cu burden of fish exposed within social groups.

6.3.3. Alteration in competitive hierarchy due to Cu-exposure

Separate groups of fish each consisting of individuals of a similar size can be reared under apparently identical conditions and yet differ in the frequency and intensity of aggression within the group (Andries and Nelissen, 1990; Oliveria and Almada 1996) or in the strength of the group feeding hierarchy and the degree of growth depensation subsequently observed (Winberg *et al.*, 1993; Jobling and Baardvik, 1994). Thus, to evaluate alterations in individual aggression and group feeding

Figure 27a. The relationship between liver Cu content and SGR of control and Cu-exposed fish held at 3 different stocking densities. Fish kept in isolation (ISO) indicated by crosses (black = control, red = Cu-exposed, n = 6). Fish kept within groups of 10 individuals (PS) marked by circles (light blue = Cu-exposed, dark blue = control, n = 20). Linear regressions are fitted for control (y = -6.6x + 0.05, r^2 = 0.15) and Cu-exposed fish (y = 0.06x + 0.16, r^2 = 0.64; ANOVA, F = 27.2, P = 0.008). Fish held within groups of 40 individuals (DL) indicated by squares (closed square= control, open square = Cu-exposed, n = 20). Linear regressions are fitted for control (y = -1.2x + 0.05, r^2 = 0.13) and Cu-exposed fish (y = 0.149x + 0.06, r^2 = 0.4; ANOVA, F = 13.44, P = 0.02).
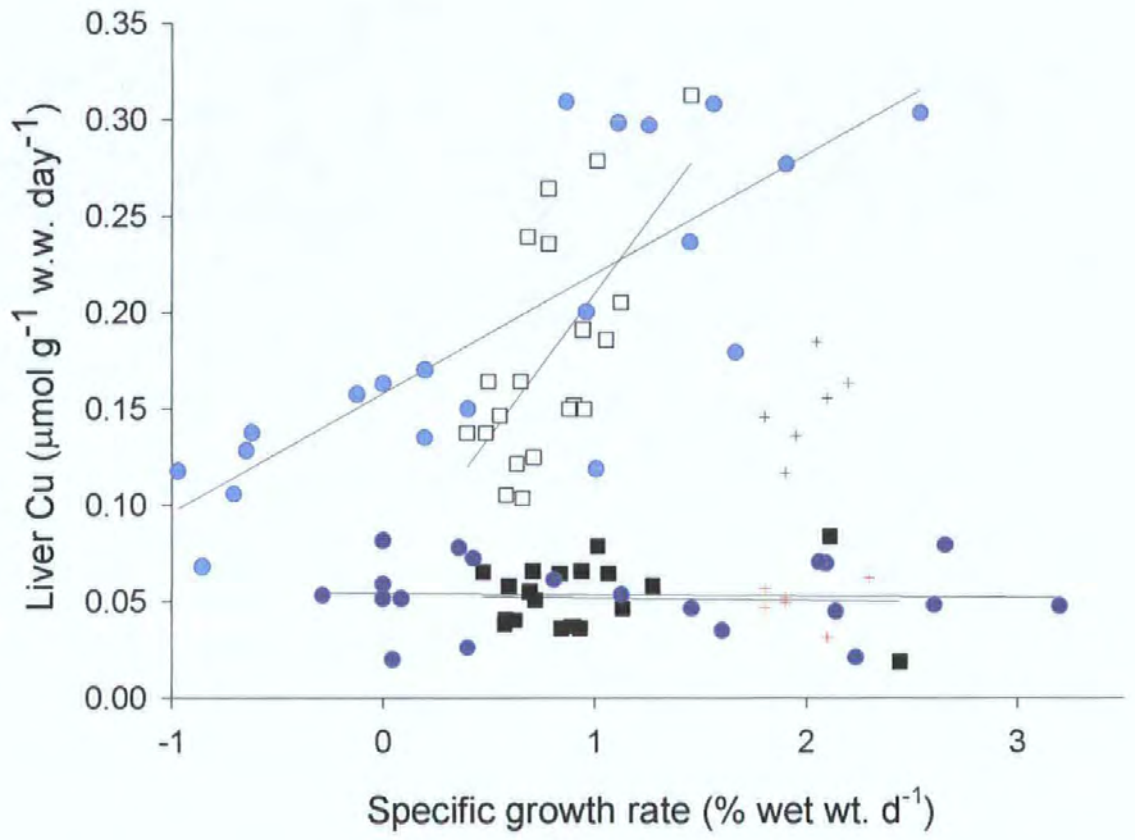
Figure 27b. The relationship between liver Cu content and SGR of rainbow trout exposed to dietary Cu within 3 differently sized stocked densities. Regression line derived from the data displayed in figure 25. Dashed black line represents the DL group (n = 40) solid line represents PS group (n =10). Grey circle shows the distribution of isolated Cu-exposed fish. Red dotted lines and arrows highlight the variation in liver Cu content with reference to SGR of isolated fish compared to fish exposed within social groups.
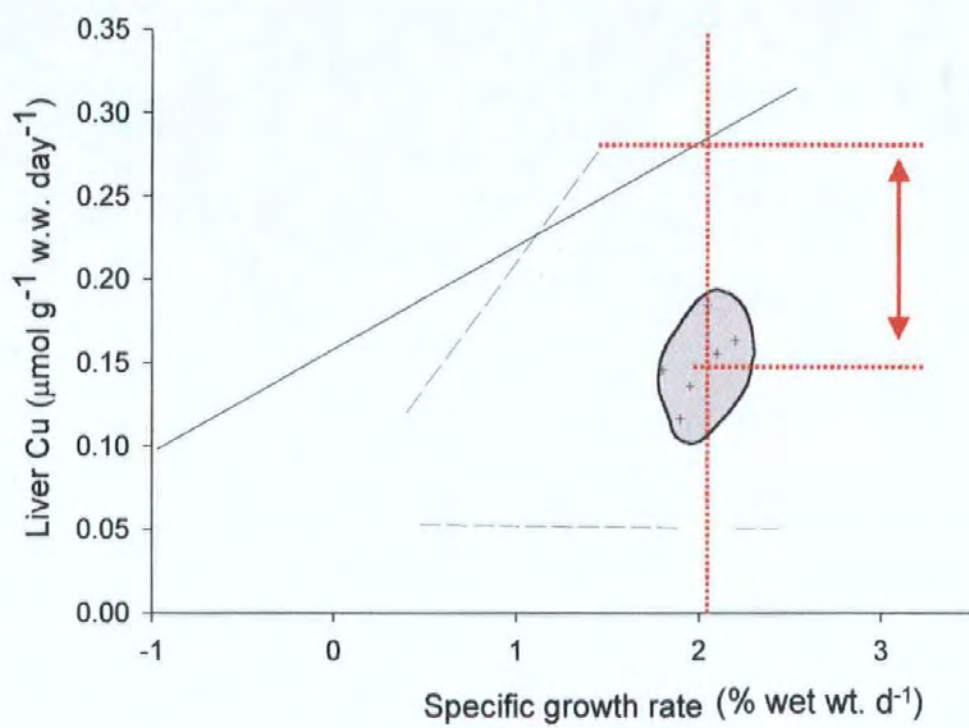
hierarchy due to Cu-exposure, comparisons were drawn between 2 consecutive time periods. During the first period (0-21 days, $t1$) all groups were fed a control diet. For the second period (22-42 days, $t2$) 2 groups were switched to the Cu-elevated diet (groups E1 and E2) whilst 2 groups remained on the control diet (groups C1 and C2). Between $t1$ and $t2$ the activity of the alpha fish within groups C1 and C2 was unchanged (Table 13) whilst in Groups E1 and E2 it was reduced by 20% (t-test, X, $t = 6.74$, $P = 0.0005$; Y, $t = 7.87$, $P = 0.001$).

In calculating levels of aggression, fish were only scored for the more energy expensive encounters of nips and chases. The encounter rate between control individuals (95% of these being made by the alpha fish) during non-feeding times showed a 50% (t-test, $P<0.05$) increase between $t1$ and $t2$ (Table 13), whilst Cu-exposed fish showed a 50% reduction in encounters between $t1$ and $t2$. During feeding times the encounter rate was around 10-fold higher in all groups compared to when food was not present (Table 13). This encounter rate during feeding times did not significantly alter (t-test, $P< 0.05$) in any groups between t1 and t2, and each social group was significantly different from each other regardless of treatment (ANOVA, $F = 4.84$, $P = 0.03$).

Within each PS group the main function of locomotory activity by the alpha fish appeared to be associated with dominance behaviours, and the alpha fish would traverse the tank as if patrolling a territory, and occasionally lunge at other fish, which generally remained within one corner of the tank. In Table 13 these dominance-subordinate encounters are calculated as a percentage of the total number of patrols of the tank that resulted in an encounter. The groups of fish that remained on the control diet increased this encounter rate by around 10%, although this was only significant in group C1 (t-test, $t = 2.19$, $P = 0.034$). In the groups switched to a Cu-exposed diet the % of patrols that result in an attack remained unchanged. Thus, results suggest that the observed reduction in aggression of the

	Period	Control		Cu-exposed	
		Group C1	Group C2	Group E1	Group E2
Activity of dominant fish, non-feeding (BL s⁻¹)	$t1$	0.31 ± 0.02	0.26 ± 0.04	0.25 ± 0.07	0.29 ± 0.03
	$t2$	0.33 ± 0.02	0.29 ± 0.06	$0.15 \pm 0.08*$	$0.16 \pm 0.01*$
No. of encounters, non–feeding (min⁻¹)	$t1$	1.09 ± 0.17	0.95 ± 0.14	1.03 ± 0.24	1.07 ± 0.15
	$t2$	$1.55 \pm 0.17*$	$1.49 \pm 0.15*$	$0.36 \pm 0.17*$	$0.47 \pm 0.14*$
No. of encounters during feeding (min⁻¹)	$t1$	10.7 ± 1.1	16.1 ± 1.2	17.1 ± 1.5	9.1 ± 0.86
	$t2$	12.6 ± 1.1	16.2 ± 1.3	18.4 ± 0.8	10.1 ± 0.9
% Patrols resulting in encounter, non- feeding	$t1$	33.3 ± 8.9	31.7 ± 6.4	25.5 ± 4.6	35.7 ± 8
	$t2$	$46.2 \pm 7.6*$	42.7 ± 8.2	25.6 ± 10.2	37.4 ± 9.1

Table 13. Activity and encounter rate of the alpha fish observed directly and by video recording for 20 minutes during non-feeding times and 2 minutes during feeding times. Data is mean $\pm$ S.E., n = 36. During $t1$ (0-21 days) fish from all groups were fed a control diet, during $t2$ (22-42 days) groups E1 & E2 were fed on the Cu-exposed diet, whilst groups C1 & C2 remained on the control diet. Patrol of tank is one complete traverse of tank and back made by the alpha fish. * significant difference (t-test, $P<0.05$) within same groups between 0-21 days and 22-42 days.

alpha fish due to Cu-exposure appear to be due to a general lowering in the level of activity. Behavioural interaction will effect the distribution of food within a discrete group of fish (Ryer and Olla, 1995, 1996). Differences in growth manifest themselves quickly and size disparity increases over time (Olla *et al.*, 1992). Thus coefficients of variation in growth rate are valuable indicators of competitive effects (Purdon, 1974; Rubenstein, 1981; Jobling and Wandsvik, 1983). The variance in size frequency distribution of the 4 (n = 10) social groups described above over consecutive time periods was compared (Table 14). The mean growth rate of a fish in all 4 groups was not significantly different, increasing by around 0.2 g day^{-1}. However, significant differences were observed in the size distribution of fish held within groups fed different dietary treatments. Between $t0$ and $t1$, the S.D. and variance of all groups increased (Group E2 increased S.D. 6-fold, Group C1 3-fold, whilst groups E1 and C2 only increase S.D. by around 2-fold). There was a significant increase (F-test, P <0.05) in within group variation in all groups except group C2, suggesting that social hierarchy wasn't fully developed within this group of fish at this time. Between $t1$ and $t2$ (Group E1 and E2 switched to Cu-exposed diet) both the control groups had significantly increased (F-test, P <0.05) within group variation in weight. Although variance and S.D. were increased between $t1$ and $t2$ in the 2 groups switched to the Cu-diet, this was not significant (F-test, P <0.05).

The degree of aggressive activity within a social group of fish is perhaps reflected by the degree of body damage sustained by individuals within the group (Turnbull *et al.*, 1998). The summed scores of individuals within each group were calculated at the end of $t1$ and $t2$ (Table 15). At the end of $t1$ (all groups on control diet) there was a significant difference (t-test, P<0.05) in the total score between all the 4 groups. By the end of $t2$ (groups E1 and E2 switched to control diet) both the control groups had significantly increased their scores,

		Control		Cu-exposed	
		Group C1	Group C2	Group E1	Group E2
$t0$	Mean	56.27	49.2	56.9	49.4
	S.D.	4.4	3.6	4.36	2.1
	Variance	19.7	14.9	19.5	14.6
$t1$	Mean	54.9	45.9	53.9	54.2
	S.D.	12.5	6.2	9.4	12.5
	Variance	156.9	61.2	156.2	88.11
	F-test (F, P)	0.29, 0.05	0.39, 0.08	0.21, 0.016	0.029, 0.006
$t2$	Mean	65.5	62.8	65.6	59.8
	S.D.	21.8	18	10.1	16.8
	Variance	411.2	255	286	129
	F-test (F, P)	0.129, 0.01	0.199, 0.01	0.86, 0.4	0.55, 0.19

Table 14. Mean body mass, S.D. and variance (n = 10) of fish within 4 social groups (PS groups) at 3 consecutive time periods each 21 days apart. F-test (two samples for variances) statistics used to compare weight frequency distribution within each social group between consecutive time periods. During $t1$ (0–21 days) all tanks were fed the control diet and during $t2$ (22- 42 days) groups E1 and E2 were switched to the Cu-exposed diet whilst groups C1 and C2 remained on the control diet. $P < 0.05$ considered significant difference in weight distribution between consecutive time periods.

whilst group E1 only slightly increased the summed score, and group E2 showed a decrease in summed scores. When scores t2 are subtracted from t1 then groups kept on a control diet have a significantly elevate level of body damage of individuals within the group over groups that had been switched to the Cu-exposed diet.

		End of t1 (0-21 days)	End of t2 (22-42 days)	t2 - t1
	Group C1	21	36	15
Control	Group C2	17	29	12
	Group E1	28	31	3
Cu-exposed	Group E2	29	25	-4

Table 15. Summed score of body damage for all individual fish (n = 10) within each social group (PS groups), reflecting the degree of inter-individual aggression. Groups C1 and C2 were fed the control diet during t1 & t2. Groups E1 & E2 were fed the control diet during t1 and Cu-exposed diet during t2.

6.4 DISCUSSION

Effects of Competition and Cu-exposure on metabolites

The ability of an individual to compete within a social group will be dependent on the energy resources available for energetically costly encounters (Pickering, 1992). Consequently, a positive linear relationship usually exists between the metabolic state and social status of an individual (Hogstad 1987). Glycogen deposits within the white muscle will reflect energy stores available to the fish, during social interactions, requiring burst performance swimming, these will become depleted mainly due to anaerobic degradation (Beamish 1968). The liver and red muscle have been suggested to act as energy stores for the white muscle (Robinson and Mead, 1973; *for review see* Bilinski, 1974), with a delay in glycogen depletion and lactate accumulation during burst activity, relative to the white muscle (Pritchard *et al.*, 1971; Milligan and Wood, 1986).

Measurement of the substrates for glycolysis (glycogen, lactate and pyruvate) in the combined red/white muscle and liver in 3 differently sized groups of fish (ISO, n = 1 per group; PS, n = 10 per group; DL, n = 40 per group) showed a 2-3 fold higher degree of anaerobic metabolism occurring in social fish compared to isolated fish (Table 11); and this increased with the rise in competitiveness of the social group. Glycogen levels recorded within the skeletal red/white muscle of the DL social fish (5.8 ± 7.6 and 5.9 ± 8.1 μmol g^{-1} w.w. for control and Cu-expossed fish respectively) were similar to results reported by other authors (e.g. 5-7 μmol g^{-1} w.w., Goolish, 1989; Pagnotta and Milligan, 1991; Wang *et al*, 1994) for rainbow trout *(Onchorhynchus.mykiss)*. The fish kept in isolation (ISO) had about a 30% increase in both liver and muscle glycogen, in contrast the PS group (where competitiveness was increased) showed around a 20% decrease. These results show the influence of stocking density on the energetic state of the fish, namely increasing activity

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and mobilizing liver glucose due to the increase in social stress (Pickering, 1994). Stocking density history in the literature is often not reported, but may account for the large variation that is often reported in fish metabolite studies (Adcock and Dando, 1983; Wang *et al.*, 1994).

Lactate and pyruvate measurements have been used extensively to reflect performance in exercised salmonids. Pyruvate is produced as a result of the breakdown of glycogen in the presence of oxygen to generate energy in the form of ATP. If oxygen is not present then the pyruvate breaks down to lactate. Thus, lactate and pyruvate ratios can provide a measure of aerobic and anaerobic metabolism occurring within the animal (Black, 1957; Dando, 1969; Johnston and Goldspink, 1973; Wokoma and Johnston, 1981; Blier and Guderley, 1993; Kieffer *et al.*, 1998).

In this study, isolated fish had around a 3-4 fold lower levels of lactate compared to fish held in social groups. This suggests fish in social groups had an additional anaerobic metabolic stress which was absent in isolated fish. Red/white skeletal muscle lactate levels reported here for isolated fish were similar to those reported within the muscle of resting trout (3-5 μmol g^{-1} w.w., Goolish, 1979; Adcock and Dando, 1983), with those of social fish being closer to that of rainbow trout after 30 seconds strenuous exercise (10-17 μmol g^{-1} w.w., Black *et al.*, 1962; Goolish, 1979). Hence, the muscle and liver metabolite levels for social fish in this study were about 1/3 of the mechanical limitation for maximum power output in rainbow trout as set by Goolish (1979). These results further show that social group history significantly affects the energetic state of individual fish.

The effects of Cu-exposure on carbohydrate metabolism was only significantly different from that of control fish in the PS group, where Cu-exposed fish had significantly greater muscle and liver lactate levels over controls. The higher lactate, but lack of effect of Cu-exposure on pyruvate levels, would serve to increase the lactate : pyruvate ratio. This

suggests that Cu-exposed fish had a reduced aerobic capacity to metabolise the lactate and resynthesize glycogen. This may become critical at higher levels of activity as high lactate levels (184% above normal) in the muscle can cause acidosis and result in mortality (Black, 1958). Previous studies exposing salmonids to aqueous copper (Bilinski and Jonas, 1973; De Boeck et al., 1995) have observed a rise in plasma lactate (50%) of Cu-exposed fish and suggested that it reflected a reduction in lactate oxidation due to respiratory disturbances. This study is the first to show a dietary contaminant having an apparent deleterious effect on glycolysis. Furthermore it suggests that the rate of depletion of energy reserves is higher for Cu-exposed fish resulting in less stamina at higher speeds. Respirometery analysis of Cu-exposed fish (in Chapter 3, fig. 13) showed that Cu-exposed fish did have a reduced aerobic capacity compared to control fish, and this was exacerbated at higher swimming speeds. Lactate Dehydrogenase (LDH) acts as a catalyst for the conversion of lactate to pyruvate. The reduction in muscle LDH recorded (in Chapter 2, Table 8) in Cu-exposed fish, further suggests that they have a reduced capacity to convert lactate back to pyruvate. The toxic mechanism of Cu on LDH is not known and requires further research.

Effects of competition and Cu-exposure on Growth

A clear relationship exists between the variation in physiology within a social group of fish, and its consequences on individual growth, mediated by feeding behaviour (Metcalfe et al., 1995, Cutts et al., 1998). This feeding hierarchy develops with more dominant individuals receiving a larger portion of the food supply, and having a better growth rate than subordinates (Metcalfe, 1989; Huntingford et al., 1990; Mesa, 1991 ; Olla et al., 1992). This is often exaggerated in the captive situation (Yamagashi, 1962) and the social group as a whole suffers from growth depensation, as individuals invest more energy into activity (Koebele, 1985), to increase competitiveness. Results presented here (Table 12) are in

agreement with previous authors (*reviewed in* Thorpe and Huntingford, 1992), with fish incurring a significant reduction in mean SGR and FCR due to social effects. The detrimental effects of group competition on mean growth rates are inversely related, and in the PS group where competition is exaggerated; SGR, FCR and protein accretion are all decreased to a greater extent compared to the less competitive social group (DL). Similar to previous results presented in Chapter 3, Cu-exposure had little effect on mean growth parameters though at higher levels of competitive stress (PS group) Cu-exposure did seem to bring about a degree of growth depensation (Table 11). However, due to the very nature of social competition, within group error prevented any significant changes in mean growth parameters due to Cu-exposure.

The results from SGR and liver Cu burden of individual fish under different degrees of competitive stress are correlated and compared in fig, 27a & b . Observations from these figures show that fish with the higher SGR's also had a higher liver Cu burden, suggesting that the faster growing fish were consuming more of the Cu-contaminated diet, than the slower growing fish. Interestingly, the ratio between SGR and liver Cu burden for individual fish was influenced by the competitiveness of the social group, with solitary fish (ISO) having a liver Cu burden 2-fold (14 μmol g^{-1} w.w. day^{-1}) lower than a fish feeding within the highly competitive PS group, with a similar SGR. From these results, it is assumed that the FCR in solitary (ISO) fish was more efficient than in social (PS, DL) fish. The ISO fish by having reduced activity expenditure, either consumed less food to maintain an equal growth rate, and/or were able to invest more in detoxification procedures reducing liver Cu burden. In order to confirm these assumptions, it would be necessary to determine how much daily food ration each of the fish within a discrete social fish consumed. Hence, FCR could be calculated for each individual fish within a social group, and relevant energy allocation between maintenance and activity determined. This could be achieved utilising

radiography techniques as described by Metcalfe *et al.*, (1992) and McCarthy *et al.*, (1992), and provides areas for further research. These results further show that social competitiveness can not only influence the energetic state of individuals but also has the capacity to alter the accumulation of pollutants within fish and may play a large part in resultant toxicity of the animal.

Size-frequency distribution plots of control and Cu-exposed social groups show that there was a decreased range of body weight distribution in Cu-exposed fish compared to controls by the end of the experiment (fig. 26). This latter observation was not an artefact of tank position because no differences were observed between tanks (populations) within treatment. Importantly, the smallest fish in control treatments showed severe fraying of fins typical of subordinate fish (Turnbull *et al.*, 1998) with a background level of associated mortality. These observations are consistent with the normal aggressive social behaviour of trout (McCarthy *et al.*, 1992; Winberg *et al.*, 1993b) and which leads to a domination-subordination hierarchy and increased range of body mass. Cu-exposed fish showed only slight fin fraying and minimal mortality by the end of the experiment, and coupled with a relatively uniform distribution of body weights in each Cu-fed treatment, suggests that these fish had suppressed normal hierarchical behaviours.

Reduction in hierarchy probably occurs due to a loss of aggressiveness of individuals, which will in turn be linked to the energy invested in activity for such behaviours. From behavioural examination of 4 (PS) social groups, it was apparent that individuals investing in high levels of activity and aggression were correlated with high food intake and growth efficiency. Both control and Cu-exposed subordinate fish did not invest in activity, obtained a low percentage of the food ration and had a much more reduced growth rate. Our results confirm those of Symons (1968) that the salmonids social hierarchy consists of aggressive dominant individuals, sub-dominants, and less aggressive subordinate fish.

Both control and Cu-exposed groups appeared to lack a linear social hierarchy with aggressive behaviour being highly polarised by one or two fish (Table 13), with the alpha fish making a large proportion (> 90%) of all the aggressive acts during non–feeding periods. The alpha fish was displaced in both Cu-exposed groups by the beta fish, after only 2 days Cu-exposure, group E1, and after 9 days Cu-exposure in group E2. The alpha fish was also displaced in the control group C1 after 36 days. Rank reversals have been observed in other hierarchy studies (Frey and Miller, 1972; Francis, 1988, 1990; Oliveira and Almada, 1996), but suggested to be more likely in subordinate fish. Observation here suggest that the extra energy invested by the alpha fish in aggressive acts during non-feeding periods did not serve to exclusively dominate food supply, and the alternative strategy by the sub-dominant to only compete when food was present was advantageous. Other authors, measuring food intake and growth have reported sub-dominants to have an equal if not better FCR than dominant fish (Metcalfe *et al.*, 1992; Adams *et al.*, 1998).

The dominance behaviour expressed by the alpha fish became reduced when they started to feed on a diet contaminated by copper. This may be due to them ingesting a greater dose of dietary copper with an increased food ration, resulting in a higher energy investment in detoxification procedures. Due to this CU-exposed dominant fish had less energy available for activity, and consequently, the number of encounters with con-specifics was reduced (Table 13). This in turn led to a reduction in competitive stress, and actual body damage of sub-dominants and subordinate individuals within the group.

Interestingly, during feeding periods all groups of fish regardless of dietary treatment maintained encounter rates at an apparent maximum. These encounters were made by roughly 5-6 of the fish within the tanks and were relatively irrespective of body size, suggesting that the expensive cost of competition for sub-dominant and subordinate fish, for this short period, was outweighed by the benefit derived by obtaining a larger

portion of the group's food supply. This was apparent even for fish feeding on a Cu-diet, where the energy gains will be less, and the cost of competitive behaviours more than that for control fish.

Although Cu fish maintained aggression rates during feeding, the reduction in activity and thus interaction rate during non-feeding times appeared to have the consequent effect of reducing the feeding hierarchy within the social group. The increase in body mass disparity of the group, which is a consequent factor of the feeding hierarchy, was reduced in social hierarchies when switched onto a Cu-exposed diet (Table 14). It has been suggested that in salmonids larger size is a consequence and not a cause of high status (Abbott *et al.*, 1985; Huntingford et al., 1990), and growth rates are more dependant on competitive ability than physiological efficiency (Doyle and Talbot, 1986; Swain and Ridell, 1990). When feeding on a Cu-contaminated diet, which will have relatively low value and be costly to obtain, investing large portions of energy into activity to obtain additional food, may not be the best strategy to maximize growth. A strategy of cost minimization may be favoured, even if this results in a decreased food ration. It remains unclear if the more even weight distribution of Cu-exposed fish is due to a more even food distribution, or due to a reduction of energy investment in competitive interactions and consequently reduction in subordinate stress effects. As previously mentioned, to confirm this hypothesis, radiography techniques (Metcalfe *et al.*, 1992; McCarthy *et al.*, 1992) are required to examine directly the association between competition, food intake and growth.

Extrapolations of these laboratory observations to wild fish suggest that the social competitive ability of fish in the wild could decline during chronic, sub-lethal pollution scenarios. This is due to the cost:benefit ratio of competing with conspecifics for food, being diminished for fish when competing for a contaminated food source. Intra-specific competitiveness is central to the natural selection processes of survival and reproduction

(Dawkins, 1995). In fish populations authors have suggested it an important factor regulating the growth of individuals, population density, and even responsible for down stream migration (Hoar 1951; Chapman 1962; Mortensen 1977; Noakes 1978; Elliot 1984, 1990; Metcalfe, 1991). Therefore, sub-lethal Cu-exposure having no apparent effect on salmonid growth rates may have ecological implications not yet fully realised.

CHAPTER SEVEN

THE INFLUENCE OF CU-EXPOSURE ON THE DOMINANT-SUBORDINATE

RELATIONSHIP IN RAINBOW TROUT

7.1 Introduction

Aggressive behaviour has been found to be a significant mechanism of intra-specific competition and is regarded as a factor important in the ecology and sociology of natural animal populations (Collias, 1944; Elliott, 1990). Contests between salmonids over the ownership of food resources have been documented in both wild (Dunbrack et al.,1996) and captive (Noakes and Leatherland, 1977; Huntingford et al., 1990; McCarthy et al., 1992) populations. This type of competition between conspecifics generates a feeding hierarchy with better competing individuals excluding more subordinate fish from the food supply (Cutts et al., 1998). This results in the more dominant fish within the group usually having better growth rates (Abbott and Dill, 1989), and it is suggested that this results in a better chance of survival and successful reproduction (Metcalfe et al., 1995; Johnsson et al., 1999).

The complex pattern of a feeding hierarchy will be organized by initial relationships between contact pairs (Braddock, 1945). The outcome from these disputes will be decided by each individual's fitness budget (surplus energy) available for expenditure during the interaction, and will be related to surplus energy available to the fish after the components of standard metabolism have been satisfied. Rather than competing in energetically expensive interactions for each dispute, selection will favour a strategy of assessment on the basis of which opponent is likely to expend it's fitness budget first (Parker, 1974). Behavioural decisions made during an interaction will be affected by motivation, which is dependent on the animal's perception of the external (e.g. food) and its internal environment (e.g. presence/absence of O_2 debt). Parker (1974) termed this the animals Resource Holding

Power (RHP). Decisions to escalate a fight or give up will be made in relation to proximate costs of the interaction, and motivational state. Game Theory predicts that the cost of fighting will increase when resource value increases, and probability of victory for an animal will increase when resource value increases only for that animal (Enquist and Leimar, 1987).

Escalation of an interaction to decide the dominant-subordinate relationship between individuals will incur a significant metabolic cost (Haller, 1995; Hack, 1997), due to the increased energetic requirement for rapid and powerful responses sustained by anaerobic glycolysis, necessary as the interaction escalates (Neat *et al.*, 1998). Under this sort of escalated activity muscle glycogen reserves will be rapidly depleted, resulting in the accumulation of lactate. This type of anaerobic activity can only persist finitely, as the high lactate levels will leak from the muscle into the blood stream, causing a decrease in blood pH that disrupts fluid and electrolyte balance (Wood, 1991), and ultimately can cause death (Black, 1958). Conversion of lactate back into glycogen occupies a major role of post-exercise metabolism taking hours for it to return to pre-exercise levels (25% conversion after 4hours; Gleeson 1996). The fishes ability to resynthesize lactate back to glycogen through the Embden-Meyerhof chain (Black, 1958), will probably be a key factor influencing RHP.

Due to the increasing cost of interaction escalation, individuals will need to make an assessment of their probability of winning or withdrawing from competition at the appropriate time and not waste energy unnecessarily. This can be explained using Parker's (1974) simple model; where each combatant assesses relative RHP; this correlates to an absolute probability of winning the interaction (C_{abs}). The stake played for is infliction of loss of RHP and is determined by the fitness budget of its opponent (each individual plays for the withdrawal of its opponent.). This defines a critical probability of winning (C_{crit}) for each combatant, above which escalation is the favourable strategy ($C_{abs} > C_{crit}$) and below which withdrawal is favourable ($C_{abs} < C_{crit}$). Escalation should occur only where $C_{abs} - C_{crit}$ is

positive for both combatants. Relative body size will be the first cue of an opponent's RHP, after which the interaction will consist of a sequence of repetitive behaviours and at each interaction in the sequence opponents will acquire some information on true fighting abilities of the opponent (Enquist and Leimar, 1987). Once cost of continuing an interaction outweighs the benefit derived from the resource withdrawal will be favourable.

In this chapter, the hypothesis that Cu-exposure impairs the ability of trout to compete as successfully with conspecifics in comparison to fish fed a control diet was addressed. Previous results in this thesis have shown that chronic sub-lethal dietary Cu-exposure (730 mg Kg^{-1} Cu feed d.w.) of rainbow trout *(Onchorhynchus mykiss)* caused a general lowering of activity, due to increased energy invested into detoxification and excretion of the copper load. Results further showed that Cu-exposure increased the cost of locomotor activity, and this became critical at higher levels of activity. As a compensatory response Cu-exposed fish generally lowered swimming activity, and on a daily basis swam 20-30 fold less in distance than control fish. Consequently, this resulted in less exclusion of the subordinate fish from the food resource by, and a more uniform growth rate within the population.

Escalated interactions between paired individuals require high swimming performance, and it is probable that the associated costs of aggression are greater for a Cu-exposed fish than a control fish. This would be expected to reduce the net energy benefit obtained when competing for a Cu-exposed diet, and may alter the fish's decision to invest energy in competitive behaviours. Although the effects of pollutants have not been directly examined previously in relation to competitive ability, previous studies examining the influence of environmental parameters such as temperature and water current velocity on competitive ability in salmonids have shown a general decrease in inter-individual competition, with the rising cost of swimming (McNicol and Noakes, 1981; Noakes and Grant, 1992)

The energy requirements of a fish will be supplied from a limited resource, and it can be expected that energies invested in agonistic interactions will leave less energy available for other activities such as growth and gonadal production. As previously suggested the increased cost of activity in a Cu-exposed fish and reduced resource benefit from the diet, may alter self-assessment of RHP and thus influence strategic decision making associated with aggressive disputes. To investigate this; Control versus Control, Cu-exposed versus Cu-exposed, Control versus Cu-exposed interactions were carried out under simplified environmental conditions. To assess the influence that competing for, and feeding on a Cu-contaminated diet may have on relative RHP, and sequentially the dominant-subordinate relationship.

Interactive behaviours between paired individuals were observed directly and recorded via the Ethovision tracking system, to gain information on interaction escalation and intensity. The advantage of increased body mass on relative RHP and probability of winning were assessed in terms of their influences on interaction escalation, and interaction outcome. These were compared with response thresholds, for alternative strategies in fish feeding on a Cu-contaminated diet. Finally glycogen and lactate muscle enzyme levels were taken from both control and Cu-exposed fish immediately after encounters as an indicator of the metabolic consequence of participating in escalated interaction, requiring elevated anaerobic respiration (Pough and Andrews, 1985). These were used to determine the metabolic cost of a competitive interaction on dominant and subordinate fish, and to what extent this may have been influenced by chronic Cu-exposure.

7.2 MATERIALS AND METHODS

7.2.1. Experimental animals

 Control and dietary Cu-exposed rainbow trout were required to determine what influence alteration of the energy allocation due to Cu-exposure, would have on the animal's ability to compete directly in a paired interaction. Thus, rainbow trout *(O. mykiss)* were kept in a holding aquarium for a period of 8 weeks (see section 2.2.3.). Three tanks of fish were selected at random and fed a Cu-exposed diet (730 mg Cu kg^{-1} feed d.w.) whilst the other three were fed a control diet. For details on aquarium design, water quality and diet see sections 2.2.1.-2.2.4.). After 8 weeks dietary exposure, healthy individuals were selected for behavioural analysis. Individual fish paired for behavioural interaction analysis were always collected from different tanks, and tanks sampled in rotation. The probability of a fish encountering an opponent it had encountered before was <0.001%.

7.2.2 Behavioural tracking

The arena design was the same as described in section 4.2.2., with the exception that only two tanks were monitored simultaneously. For tracking behavioural movements the Ethovision Behaviour tracking system was employed (see section 2.2.8). Each tank (45W x 70L x 30H cm) was divided in half by a Perspex screen drilled with 4mm holes every 1cm^2 to enable water flow between arenas. This was clipped onto the tank lip during the 24-hr acclimation period, making arena dimensions of 45W x 35L x 30H cm. Tracking was always initiated at the same time each day (11 a.m.). The Perspex divide was removed just before tracking was undertaken, and appeared to cause minimal disturbance to either fish. Fish were tracked for dominance-subordinate encounters in the following pairs, Control versus Control, Cu-exposed versus Cu-exposed and Control versus Cu. Interactions where paired fish were

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both previously fed on the same diet were removed from the exposure aquarium and paired irrespective of size, to estimate the effect of size disparity on competitive ability. Where as fish used for the control versus Cu-exposed interactions were selected so that fish were of a similar body size, to remove this variable and compare only Cu-exposure effects.

The two fish within each arena were tracked simultaneously and differentiation between each fish was on relative size where one of the objects tracked had to exceed a certain predetermined size threshold. In experiments where fish were of similar size the computer could not distinguish between the 2 fish. If the fish came together for an encounter it was not certain that the computer would correctly identify each fish with it's relative track once they parted. Therefore, when analysing data, behavioural parameters recorded for the paired fish, were either analysed as a total, or a mean measurement, for the duration of the encounter. Behavioural measurements determined by Ethovision software included, total distance moved by both fish and the combined mean velocity of both fish. Other behaviours recorded included the percentage time of the total interaction spent by the fish involved in displays. Fish were monitored from above making observation of frontal and lateral displays difficult; instead the Ethovision software was used to score the percentage time during the interaction that opponents spent within one body length (BL) of each other (calculated as mean BL of the pair), and these close range interactions without the opponents touching were scored as displays. Also escalated behaviours such as actual encounters (contact) were scored by key assignment through the keyboard.

7.2.3 Physiological measurements

After each behavioural interaction fish were removed individually lightly anaesthetised (0.1g MS222) and weight and length determined. To determine any alterations in energy reserves due to Cu-exposure, that may have affected competitive performance, fish from the Cu-

exposed versus control trials were killed by terminal anaesthetic (0.5g MS222), and tissue sampled (for methodology see section 2.2.6) for muscle and liver glycogen, and lactate (for enzyme methodology see 6.2.2.).

7.2.4. Ethical Note

These staged interactions would begin with a display period lasting a few minutes, with aggression levels escalating to actual body contact between opponents. In the few experiments where mouth grasping occurred this never lasted longer than one minute. Interactions were stopped as soon as loser fish made the decision to quit, retreating to a corner of the tank, subordinate fish were removed preventing any further interaction. Each fish was given 24hrs in isolation to observe health before returning to the stock tank, fish always made a full recovery, and staged interactions did not seem to cause any degree of abnormal stress of injury, or lasting harm.

7.2.5. Statistical analysis

Fitting of non-linear regression lines and One-way ANOVA highlighted any significant trends in measured interaction parameters within dietary treatment. To test for significant differences in measured parameters between Control Vs Control and Cu-exposed Vs Cu-exposed interactions data was normalised by log transformation and significant differences tested by ANCOVA. Significant differences between mean enzyme levels between winners and losers was determined using the Students Two tailed t-test.

7.3 RESULTS

For all interactions analysed, independent of dietary treatment, the duration and intensity of the interaction before dominance was established increased the least dissimilar the size between competitors. For many behavioural parameters measured this relationship was exponential. Cu-exposure did have a mitigating effect on the extent of each behavioural interaction, and significantly reduced the (ANCOVA, F = 43.5, P = 0.0001) duration of an interaction (fig. 28), compared to controls., The duration required to settle a dominance-subordinate relationship between two equally sized control fish, as determined by back extrapolation, was 678 seconds compared to 323 seconds for the Cu-exposed fish. The difference in interaction duration between the two dietary treatments, decreased with increasing size disparity. The total distance moved by both fish during the interaction (fig. 29) was probably a feature dependant on the duration of an interaction, with control fish swimming a much greater distance to settle the dominance-subordinate relationship compared to Cu-exposed fish (ANCOVA, F = 34.16, P < 0.001). However, the mean swimming speed determined as the grand mean for both fish during an interaction (fig. 30) was not different due to dietary treatment (ANCOVA, F = 0.89, P = 0.35), and both groups increased their mean speed exponentially at a similar rate as size disparity decreased.

The intensity of each interaction was firstly estimated, by scoring the percentage of time during the interaction spent involved by individuals in displays (fig. 31), and secondly by the actual encounter rate either fish inflicted on the other (fig. 32). Both aggressive behaviours increased with decreasing size disparity. The percentage of time within each interaction involved in displaying was similar for both control and Cu-exposed fish, with similar sized fish (< 20% body mass difference) spending 60-80% of the time during an interaction involved in displays within 1 body length of each other, this decreased to around

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Figure 28. Relationship between % body mass difference of 2 competitors and duration of the interaction to decide the dominance-subordinate relationship. Open circles represent interactions where both competing individuals had been fed a control diet for 8 weeks (n =29), filled circles fish had been fed Cu-exposed diet for 8 weeks (n =26). Regression lines fitted for control (y = 678 *exp (0.05 x), r^2 = 0.98; ANOVA, F = 674, P<0.001) and Cu-exposed fish (y = 323 *exp (-0.04 x), r^2 = 0.89; ANOVA F = 223.8, P<0.001).
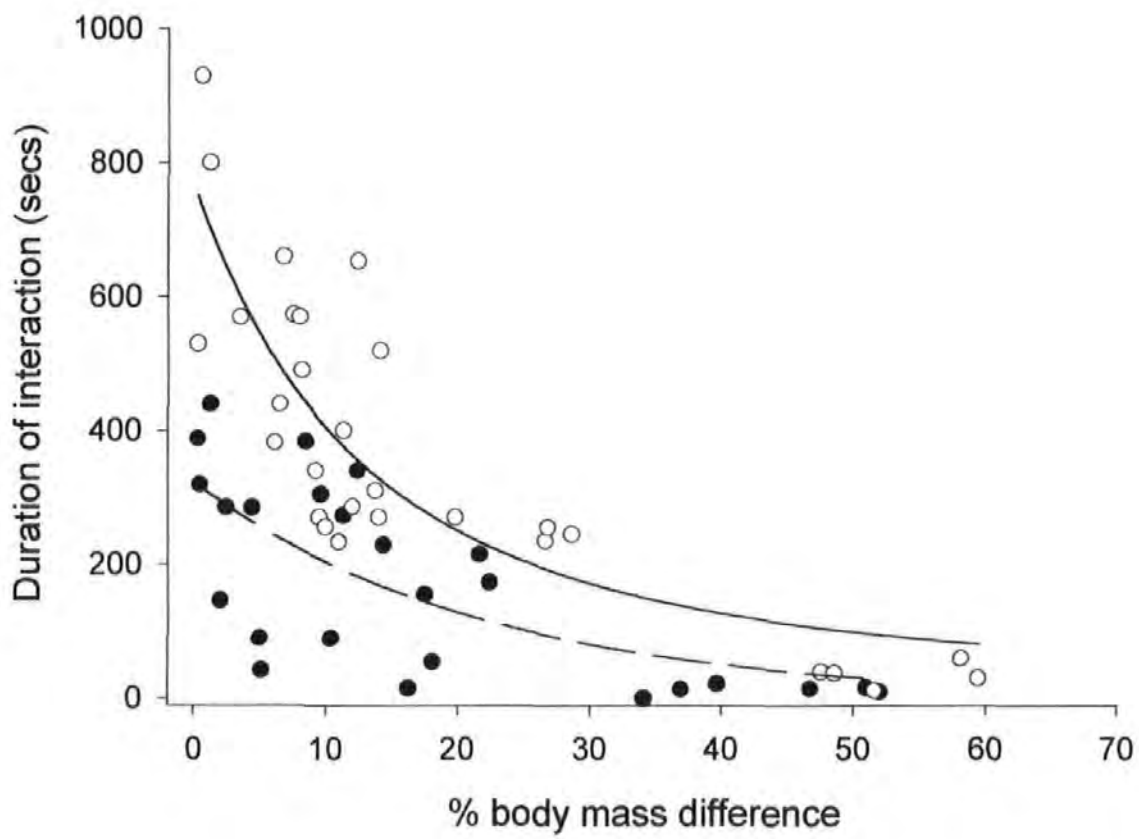
Figure 29. Relationship between % body mass difference and total distance moved by both competitors during interaction to decide the dominance-subordinate relationship. Open circles represent interactions where both competing individuals had been fed a control diet for 8 weeks (n = 29), filled circles fish had been fed Cu-exposed diet for 8 weeks (n = 26). Regression lines are fitted for control ($y = 51.7^*$ exp (0.057 x), $r^2 = 0.61$; ANOVA, F = 42.33, P<0.01) and Cu-exposed fish ($y = 10.77$ exp (-0.086 x), $r^2 = 0.38$; ANOVA, F = 15.2, P<0.01).
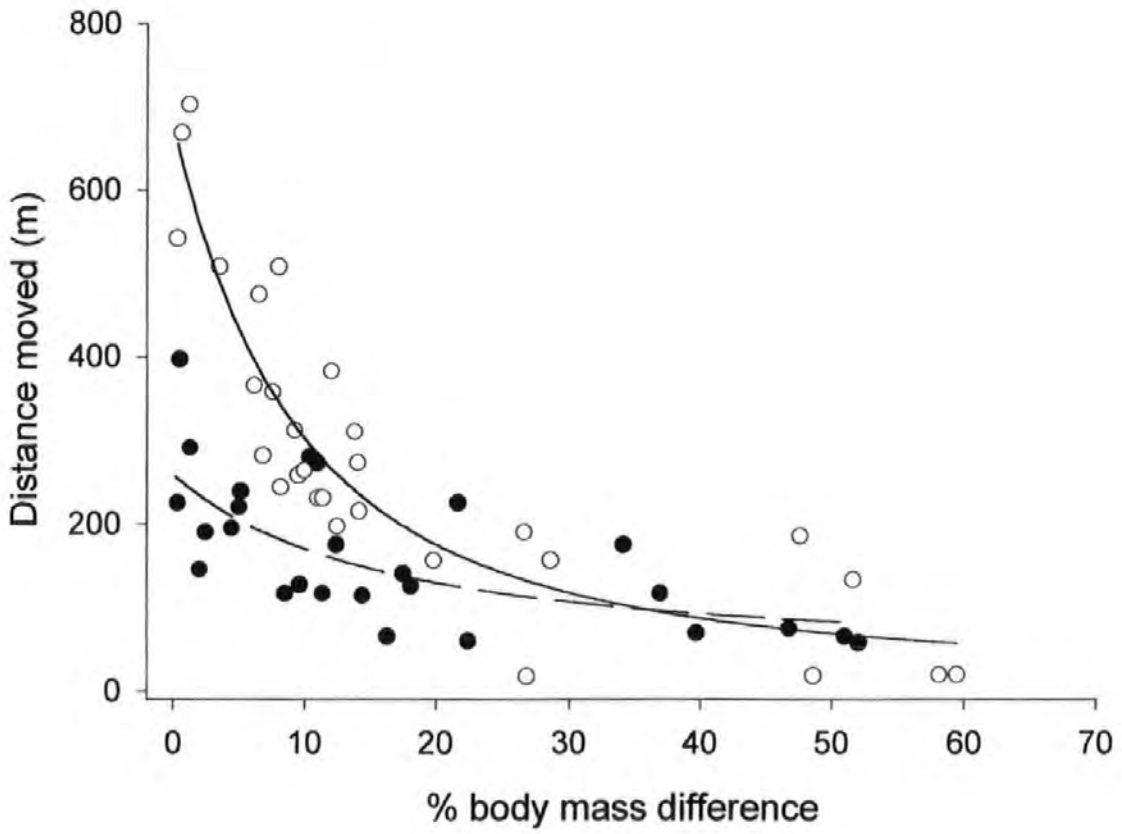
Figure 30. Relationship between % body mass difference and mean speed of both competitors during an interaction to decide the dominance-subordinate relationship. Open circles are bouts where both competing individuals had been fed a control diet for 8 weeks (n = 29), filled circles are fish had been fed Cu-exposed diet for 8 weeks (n = 26). Regression lines are fitted for control (y = 8.33* exp (-58.17 x), r^2 = 0.35; ANOVA, F = 6.5, P<0.01) and Cu-exposed fish (y = 4.9 exp (-105.6 x), r^2 = 0.51; ANOVA, F = 13.99, P<0.01).
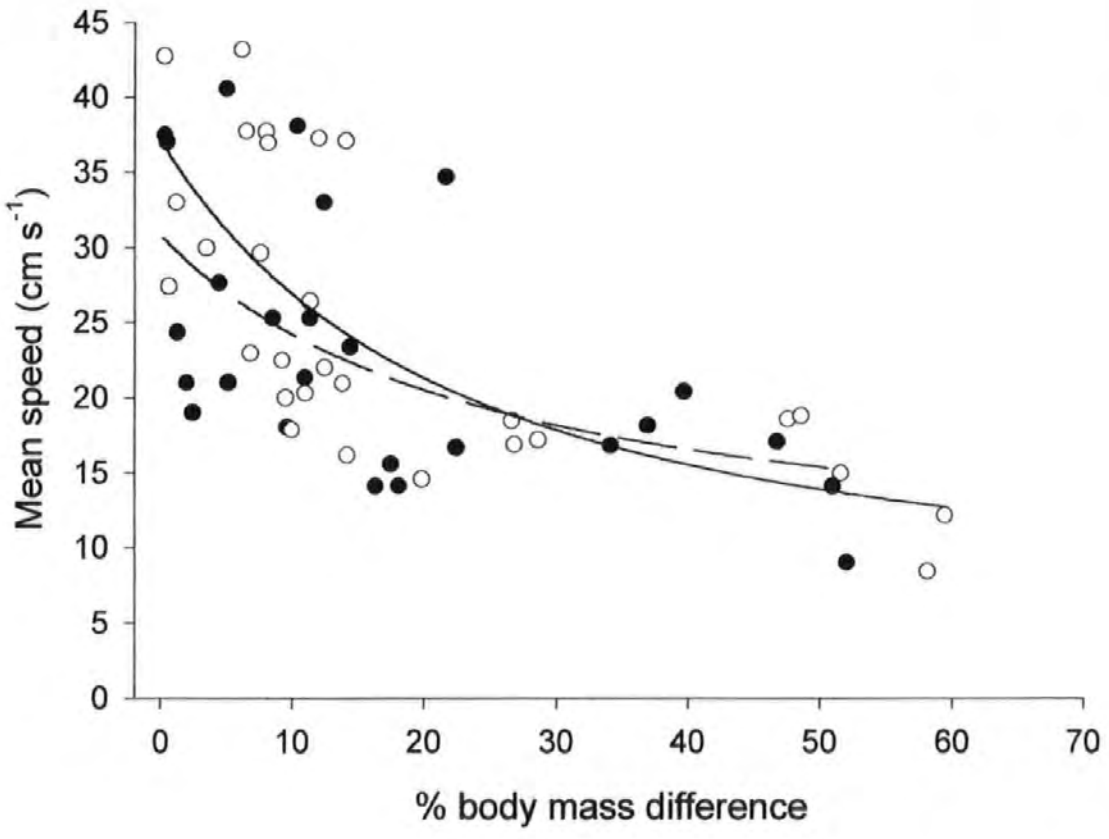
40-50% as size disparity increased to around 50% body mass difference. The total number of encounters inflicted by each competitor during an interaction, was related to the length of the bout, but equally reflected the degree of interaction escalation (ANCOVA, $F = 43.5$, $P < 0.001$). For equally sized control fish around 51 nips were required to settle a dominance-subordinate relationship whilst in the Cu-exposed fish this was reduced to only 11 nips (ANCOVA, $F = 51.23$, $P < 0.001$).

A total of 16 interactions were undertaken where control and Cu-exposed fish interacted with each other to determine the dominant-subordinate relationship. Control fish concluded to be the dominant fish in all interactions (Table 16) until Cu-exposed fish had a size advantage of >15%. After this Cu-exposed fish emerged the dominant fish in 4 out of 5. Overall, the duration and intensity (number of encounters) of control versus Cu-exposed fish interactions decreased with increasing size disparity between the two competitors. However, in interactions where Cu-exposed fish resulted in being the dominant fish, the duration and intensity of the interaction was greater than expected, from observations of previous Cu-exposed versus Cu-exposed paired interactions.

As the interaction duration increased so did the degree of anaerobic metabolism in the muscle, reflected by the depletion of muscle glycogen (Fig 33a) and associated accumulation of lactate (Fig. 33b). Subordinate fish had a greater level of muscle fatigue, measured as glycogen depletion and lactate accumulation, after an interaction compared to the dominant fish (Table 17), and the disparity between the competitors increased the longer the dominant-subordinate relationship took to be established (Fig 33b). Cu-exposed fish lost most of the interactions and accordingly had higher lactate and lower glycogen levels within the muscle. In the interactions that the Cu-exposed fish was the dominant fish, muscle metabolite levels were similar to that of dominant control fish.

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Figure 31. Relationship between % body mass difference of both competitors and % of time during an interaction spent within 1 body length of each other (involved in displays). Open circles are bouts where both competing individuals had been fed a control diet for 8 weeks (n = 29), filled circles are fish had been fed Cu-exposed diet for 8 weeks (n = 26). Linear regression lines are fitted for control (y = -1.02 x + 94.3, r^2 = 0.84, P<0.05) and Cu-exposed fish (y = -0.33 x + 67.12, r^2 = 0.24, P<0.05).
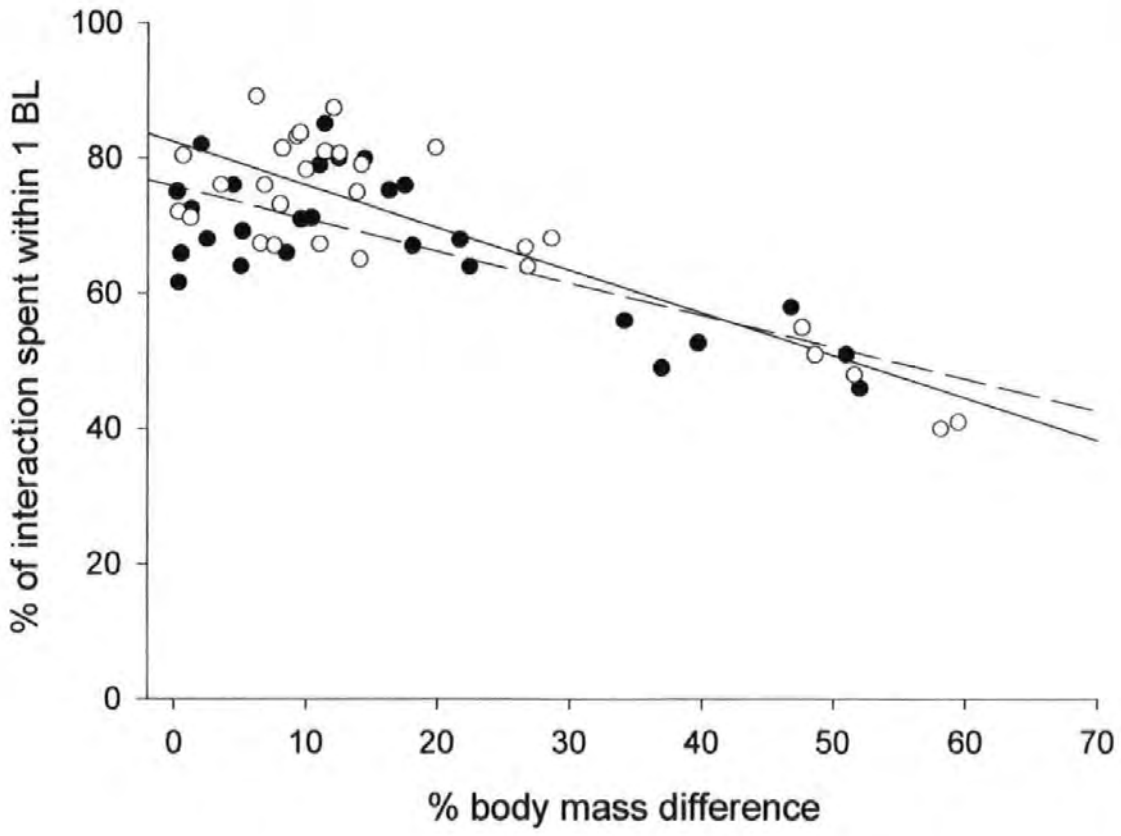
Figure 32. Relationship between % body mass difference of both competitors and number of encounters during an interaction to decide a dominance-subordinate relationship. Open circles are bouts where both competing individuals had been fed a control diet for 8 weeks (n = 29), filled circles are fish had been fed Cu-exposed diet for 8 weeks (n = 26). Regression lines are fitted for control (y = 51.7* exp (0.057 x), r^2 = 0.61; ANOVA, F = 42.33, P<0.01) and Cu-exposed fish (y = 10.77 exp (-0.086 x), r^2 = 0.38; ANOVA, F = 15.2, P<0.01).
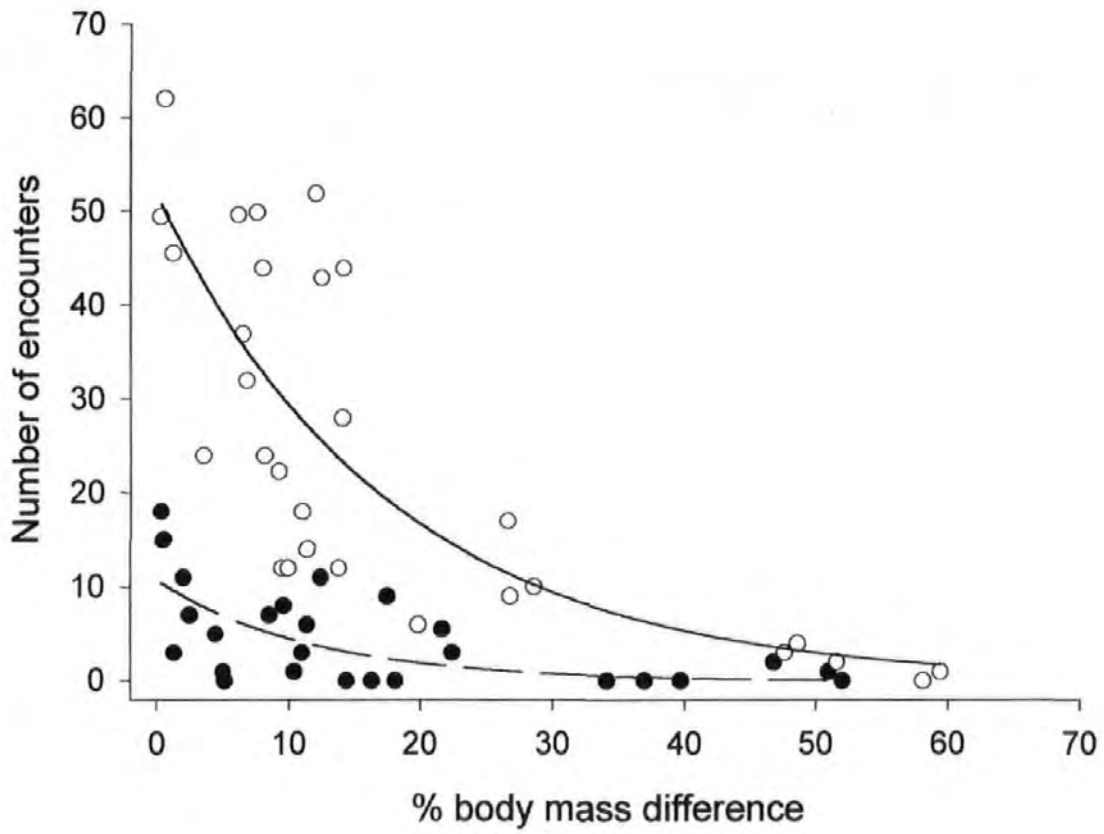
Figure 33. Relationship between length of interaction and concentration of metabolites (a) Glycogen and (b) Lactate within muscle of the dominant and subordinate fish. Open circles represent the subordinate and filled circles the dominant fish metabolite levels after interaction. Red crosses indicate data points that are from Cu-exposed fish. (a) Linear regression lines are fitted to dominant ($y = -9.4x + 7.39$, $r^2 = 0.6$, P<0.05) and subordinate fish ($y = -6.9x + 5.4$, $r^2 = 0.79$, P<0.05). (b) Non-linear regression lines have been fitted to dominant ($y = 48 * x / (1 + 507 * x)$, $r^2 = 0.67$; ANOVA, F = 9.45, P = 0.011), and subordinate fish ($y = 23* x / (1 + 373 * x)$, $r^2 = 0.56$; ANOVA, F = 7.27, P = 0.022).
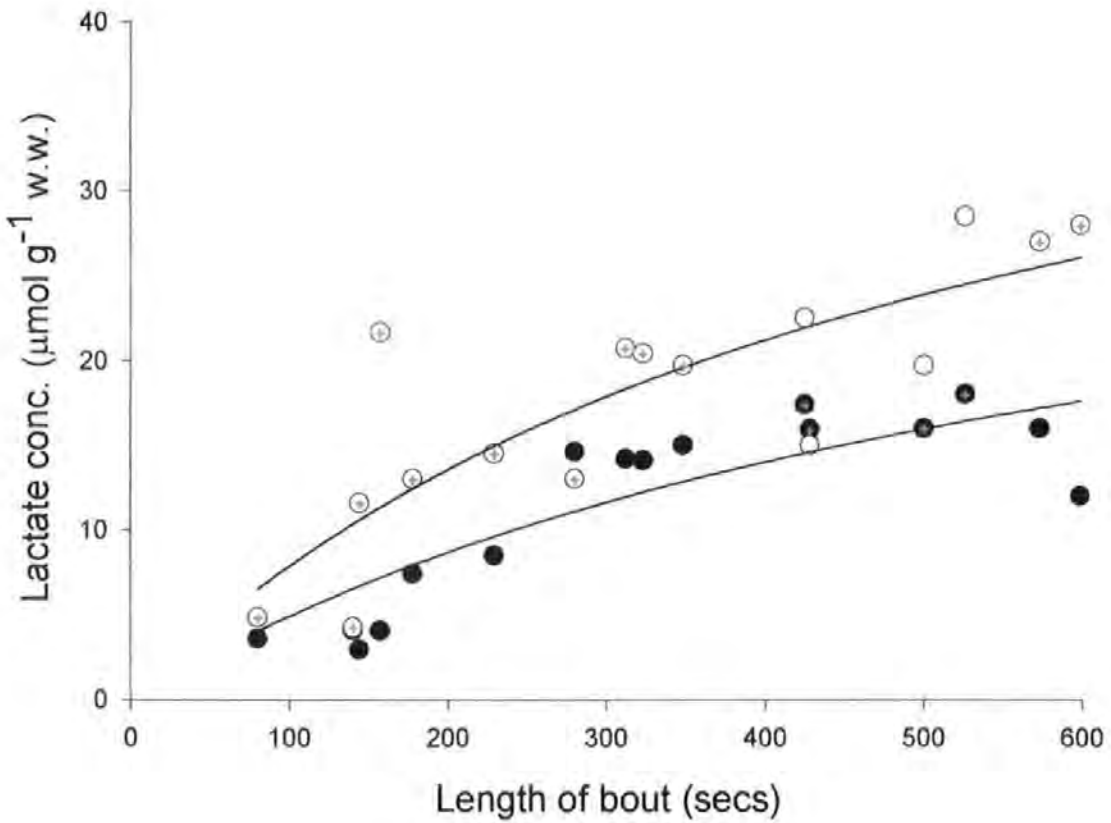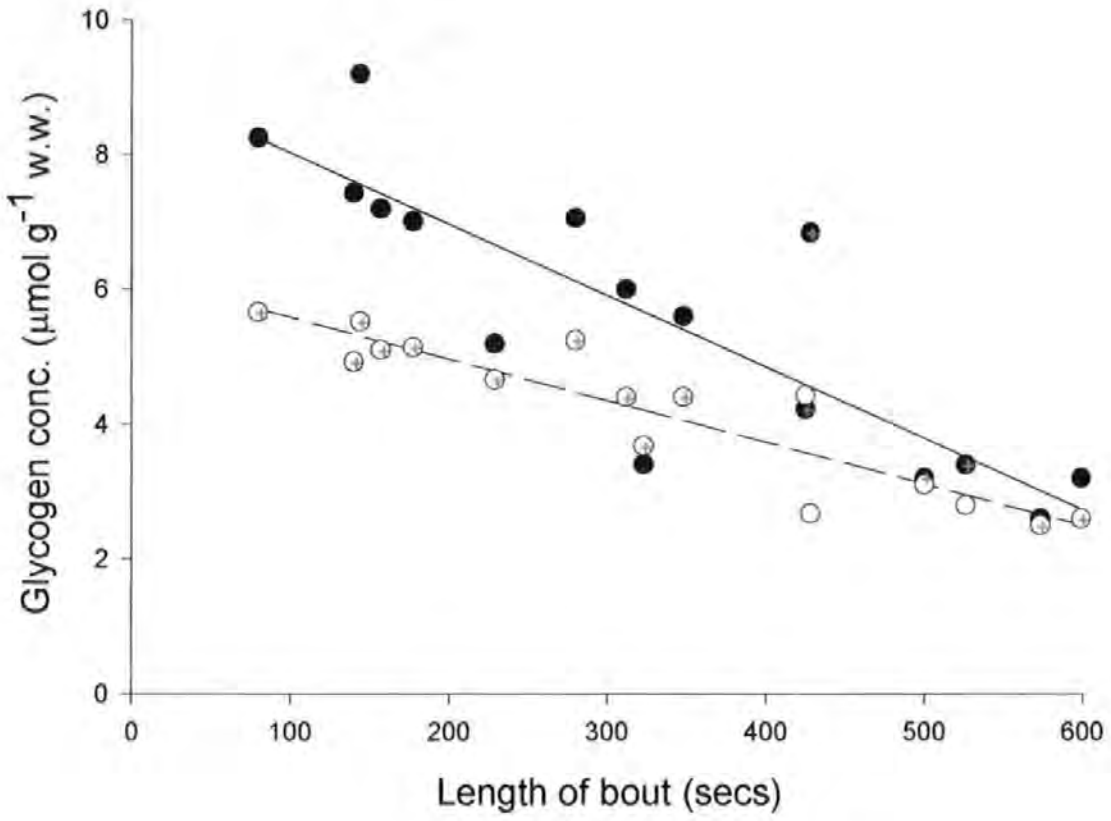
% body mass advantage of Cu-exposed fish	Duration of interaction (s)	Number of encounters	Diet of Dominant fish	Diet of Subordinate fish
-7.49	80	12	Control	Cu- exposed
-4.28	157.6	0	Control	Cu- exposed
-3.24	280	15	Control	Cu- exposed
-2.11	312	9	Control	Cu- exposed
-0.48	140	18	Control	Cu-exposed
1.94	177	18	Control	Cu- exposed
3.21	229	20	Control	Cu- exposed
3.69	348	0	Control	Cu- exposed
4.73	144	8	Control	Cu- exposed
6.05	323	9	Control	Cu- exposed
10.98	573	3	Control	Cu- exposed
14.44	526	33	Cu- exposed	Control
14.79	599	8	Control	Cu- exposed
18.6	500	25	Cu- exposed	Control
19.1	428	18	Cu- exposed	Control
19.6	425	28	Cu-exposed	Control

Table 16. Details from 16 interactions involving Control Vs Cu-exposed fish, data in order of escalating % body mass increase of the Cu-exposed fish.

	Dominant fish	Subordinate fish
Liver lactate (μmol g^{-1} w.w.)	2.784 ± 0.24	2.26 ± 0.14
Skeletal muscle lactate (μmol g^{-1} w.w.)	10.04 ± 1.56	23.16 ± 3.7 *
Skeletal muscle pyruvate (μmol g^{-1} w.w.)	0.164 ± 0.014	0.139 ± 0.02
Liver glycogen (μg g-1 w.w.)	540 ± 66.4	617.6 ± 69.6
Skeletal muscle glycogen (μg g-1 w.w.)	64.18 ± 4.7	45 ± 3 *

Table. 17 Muscle metabolites measured in the liver and skeletal muscle of fish, which was immediately excised and snap frozen after the dominance-subordinate relationship had been decided. Data are Mean ± S.E. (n = 16). * significantly different between winner and loser. (t-test $P<0.05$).

7.4 Discussion

Does feeding on a Cu-contaminated diet reduce the competitive ability of rainbow trout?

In general our results were in an agreement with those of other authors, with relative body size generally determining the outcome of a paired interaction, with the larger of the two fish being the dominant fish after a period of dispute (Newman, 1956; Jakobsson *et al.*, 1979; Abbott *et al.*, 1985; Johnsson *et al*, 1999). The intensity and duration of each interaction increased with decreasing size disparity between competitors. Maynard-Smith and Parker, (1976) suggest that the initial cue for fighting ability is size, after which the individuals will interact to gain more information to assess their probability of winning by assessing the others relative Resource Holding Power (RHP), suggested as a measure of absolute fighting ability (Parker, 1974).

In this study, 91% of contests when both contestants were previously feeding on the same diet resulted in the smaller fish retiring from the interaction. In the 9% where the larger fish conceded first, size disparity between competitors was < 4%. It would be more favourable for the subordinate fish to withdraw at the correct threshold of RHP prediction, incurring less cost during the interaction. For both control and Cu-exposed paired interactions, relative RHP assessment appeared to dictate fight escalation, but in Cu-exposed fish the dominant-subordinate relationship was decided within a shorter time period than in interactions of paired control fish (fig. 28). This required the control fish to swim a greater distance during a paired interaction to make assessment of each others relative RHP before the subordinate fish conceded (fig 29), and presumably expended more energy in this process than Cu-exposed fish.

The early withdrawal of the dietary Cu subordinate fish, suggests that the associated cost of escalated fighting to determine the withdrawal threshold, was reached at a lower level of the contest than in control fish. This may have been due to the reduced aerobic scope observed in the Cu-exposed fish (Chapter 3), and/or a reduced value placed on the Cu-exposed fishes resource (presumed to be the Cu-contaminated diet). Both factors would serve to reduce an individual's RHP, favouring early withdrawal and incurring fewer costs.

During an interaction an equal proportion of time was spend by both Control and Cu-exposed fish involved in displays (fig. 31), which have been suggested to be a cue for fighting ability (Johnsson and Akerman, 1998). The Cu-exposed fish were less willing to escalate the contest participating in 50% less contact encounters than would be expected from the reduced duration of the interaction alone (fig. 32). Escalated encounters are expensive compared to displays (Neat *et al* 1998). Therefore, results suggest that competing over a Cu-contaminated diet not only makes early withdrawal a better option for the subordinate fish, but also neither competitor wishes to invest energy in expensive elevated contact interactions, opting for lower cost display behaviours. This difference in RHP self-assessment between control and Cu-exposed fish is highlighted during Control versus Cu-exposed interactions. In these interactions control fish emerged the dominant fish when body mass is equal (Table 16). In agreement with previous Cu-exposed versus Cu-exposed interactions, the contest is less escalated than would be expected from two equally sized control fish, with the Cu-exposed fish retiring at a relatively low level of interaction intensity. Control fish continue to emerge the dominant fish from paired interactions until the body size difference is >15% in favour of the Cu-exposed fish (after which Cu-exposed fish win 4 out of 5 interactions). Interestingly, these interactions become increasingly escalated in duration and intensity, and are similar if not slightly higher in duration and intensity than Control versus Control interactions, when there is a >15% body size disparity between competitors.

An explanation for this observed escalation of the interaction to a degree that was not observed when Cu-exposed fish were competing against each other or when they did not have a >15% size advantage, may be derived from Parkers (1974) proposal that an escalation of a contest should only occur when probability of winning is positive for both combatants. Thus, the >15% size advantage was enough to raise the RHP for the Cu-exposed fish to a level where the probability of winning is equal to that of the smaller control fish. The control fish gains information during the interaction and evaluates the lower RHP of the larger Cu-exposed fish and competes at an equal if not higher degree than expected if relative RHP was affected by size disparity alone. Therefore, it seems plausible to assume that the fish with the lower RHP not only decided when withdrawal from then interaction was favourable, but also the level of escalation in intensity of the interaction.

Figure 30 shows that Cu-exposed fish can maintain a mean swimming velocity during an interaction equal to that of control fish. Results (Table 16) have further suggested that they do have the capacity to increase duration and escalate the intensity of a contest. Although, this is only done when the decreased RHP, due to feeding on a Cu-contaminated diet, is offset by an increased probability of winning. It seems plausible to conclude that the threshold for the increased probability of winning for a Cu-exposed fish is comparable to approximately a 15-20 % advantage in body mass. This figure is calculated from the 15% size advantage, required for a Cu-exposed fish to compete within an escalated interaction with a control fish (Table 16), and from the increase in size disparity that reduces the duration of a Control Vs Control interaction to equal the duration of a Cu-exposed Vs Cu-exposed interaction (fig. 28).

There was a significant metabolic consequence of escalated fighting in this study with a decrease in muscle glycogen and associated increase in muscle lactate (fig. 33 a, b), in both dominant and subordinate fish regardless of dietary treatment. As interactions escalated into

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actual body encounters the fish required predominantly expensive anaerobic metabolism (glycolysis), to provide rapid and powerful white muscle responses (Johnstone and Goldspink, 1973). There was not a significant difference in muscle glycogen between the dominant and subordinate fish after an interaction but muscle lactate became significantly elevated in subordinates. The disparity in muscle lactate concentration between dominant and subordinate fish increased with interaction duration. The lowest muscle glycogen concentration, in interactions >500 seconds, was 1-2 μmol of glycogen higher than those obtained by Pagnotta and Milligan (1991) in rainbow trout after 5 minutes exhaustive exercise. A similar comparison was observed in muscle lactate with maximum muscle lactate levels, attained in subordinate fish similar to those obtained in trout after 5 minutes exhaustive exercise (Pagnotta and Milligan, 1991), though the dominant fish had approximately 40% lower muscle lactate levels than the subordinate fish from the same interaction, suggesting that losers would approach physiological limit for muscular activity before dominant fish.

Results show that energetically costly escalated interactions are not totally exhaustive, with glycogen levels not fully depleted and muscle lactate concentrations within subordinates levelling off around 5 –10 μmols g^{-1} w.w. below the maximum values for mechanical limitations for rainbow trout as suggested by Goolish, (1979). To my knowledge this is the first study to compare metabolite levels of rainbow trout after dominant-subordinate interactions, but comparisons may be drawn to a study in cichlids *(Tilapia zillii)* by Neat *et al.* (1998). Results presented from this study, for rainbow trout, are broadly similar to the cichlid study, with significant depletion of muscle glycogen in both competitors and elevation in muscle lactate being significantly greater in subordinate fish. Intuitively it would be disadvantageous for a subordinate fish to continue competing within an interaction

until reserves completely depleted, and selection would favour assessment and conceding at a defined physiological maximum, perhaps leaving enough reserves to enable it to flee.

The overlying effects of interaction duration and the dominance-subordinate relationship masked any significant effects due solely to Cu-exposure on fish skeletal muscle metabolite levels during the control versus Cu-exposed fish interactions. Although, I suggest that due to the defined limits of anaerobic deposition and the reduced aerobic scope observed in Cu-exposed fish (Chapter 3), physiologically, Cu-exposed fish would endure a higher cost during an escalated interaction than a control fish. And presumably may take a longer time for recovery after an interaction. Post-exercise metabolism can be between 75% and 100% as metabolically expensive as the actual exercise itself (Reidy *et al.*, 1995). As a direct result of the increase in routine metabolism of Cu-exposed fish, I suggest that the cost of losing an interaction was physiologically more expensive for a Cu-exposed fish and consequently lowered its RHP, and favoured early withdrawal.

The social and ecological implication of the observed effects of Cu-exposure on fighting strategy may be explained using Maynard-Smith's (1982) Hawk-Dove model, which hypothesised that an animal can choose between an aggressive (Hawk) or passive (Dove) behavioural strategy. A hawk strategist will always compete, benefiting over competitors if it wins, but sustaining a large cost if it loses. Alternatively, the Dove strategist will share resources with its competitors, which will reduce benefits but incur no cost from escalated competition. In the rainbow trout social groups, used in this study (Chapter 5), when feeding on a control diet it appeared to be beneficial for individuals to exclude mates and gain preferential access to food supply. This occurred for individuals even when probability of winning not high, such as by sub-dominants. However, when feeding on Cu-contaminated diet, obtaining a larger proportion of the food supply, did not necessarily counterbalance the higher metabolic cost of competing for it, due to increased maintenance and reduced aerobic

scope. Thus, the probability of winning needed to be higher than in a control fish, to offset the large costs associated with losing the interaction (i.e. high lactate accumulation and possible body damage requiring repair from an already overburdened maintenance budget). Therefore, a strategy closer to the Dove model was favoured.

Previous social experience has been suggested to affect the competitive ability of individual fish (Frey and Miller, 1972; Adams and Huntingford, 1996). In this study, fish were previously kept in social groups, and it seems reasonable to assume that previous feeding hierarchal strategies may have influenced a fishes strategy when observed competing in a paired interaction. Consequently, in interactions with an increased probability of dominating opponent, RHP may still have fallen below the threshold, between cost of escalated interaction, and relative benefit derived from a Cu-exposed diet. Thus, it should be realised that competitive interactions observed here, may actually be due to stable strategies evolved during previous social experience, and not solely an explanation for the direct effects of Cu-exposure on feeding hierarchy.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

This study was undertaken with the aim of determining the long-term effects of sub-lethal dietary copper on rainbow trout *(Onchorynchus mykiss)*. The results of this study generally showed, that rainbow trout exposed to a dietary concentration of 730 mg Kg^{-1} Cu d.w. feed initiated a detoxification response, whilst maintaining physiological homeostasis and growth rates. This result disagrees with early literature, which suggested that 730 mg Cu Kg^{-1} d.w. feed exceeds the maximal tolerance limit in rainbow trout (Lanno *et al.*, 1985a,b), and adds further evidence to the hypothesis, that diet constituents can alter the proportion of bound copper influencing bioavailability, and in turn toxicity to the exposed fish (Miller *et al.*, 1993; Woodward *et al.*, 1994). Observed physiological effects were similar to those reported at lower dietary Cu-exposure concentrations for rainbow trout (500 mg Kg^{-1} Cu d.w. feed; Handy *et al.*, 1999) and Atlantic salmon (700 mg Cu Kg^{-1} d.w. feed; Berntssen *et al.*, 1999), and more recently to a study by Kamunde *et al.*, (2001) exposing rainbow trout to a higher dietary dose of 1000 mg Cu Kg^{-1} d.w. feed. That is, exposed animals reduced Cu uptake into the body and maintained Cu homeostasis, by the induction of intestinal and hepatic metallothionein (MT) levels, as well as an increased cell turnover within the intestine (Berntssen *et al.*, 1999; Kamunde *et al.*, 2001). Whilst these processes served to aid in the sequestering and excretion of the copper load, they also required an increased energetic investment, and raised the routine metabolic rate of Cu-exposed fish, and this increased demand was amplified at higher swimming speeds. In response exposed fish were observed to reduce overall swimming activity, and estimation of the cost of routine metabolism at this lower level of activity showed that exposed fish had fully compensated for the increased metabolic requirements, due to Cu-exposure.

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From a bioenergetics perspective, the observed behavioural adaptations provided valuable insight into daily energy decisions made by a fish when confronted with a more critical assessment of net energy gain, and this study represents one of the first to attempt to examine behavioural adaptations by fish. In terms of energy maximization, when challenged with increased routine metabolic costs, caused by an environmental pollutant. Nevertheless, the concept of behavioural adaptation by fish to changes in the environment is not a new one, and experiments have demonstrated salmonids to show rapid changes in daily activity patterns in response to alterations in environmental temperature, current velocity, light, food availability, inter-specific competition, and predation (Metcalfe *et al.*, 1991; Fraser and Metcalfe, 1997; Clark and Levy, 1988; Fraser, 1993; Heggenes *et al.*, 1993; Metcalfe et a., 1998). Indeed, the deleterious effects of Cu-exposure on fish swimming performance and activity have been well documented (Drummond *et al.*, 1973; Lett *et al.*, 1976; Waiwood and Beamish, 1978; Scarfe *et al.*, 1982; Wilson and Wood, 1982; Beaumont *et al.*, 1995), and authors have suggested that there are 'trade-offs' between the metabolic cost of detoxification and other processes vital to the survival of the organism (e.g. respiration, growth, reproduction; Maltby and Naylor, 1990; Handy *et al.*, 1999). However, few studies in the toxicology literature have attempted to examine the ecological consequence of aberrant swimming behaviours, that will be implied mainly through impairment of adaptive behaviours such as predation and predator success. A few studies have addressed the effects of contaminants on foraging behaviour, but have focused on mechanistic measures of prey capture (Sandheinrich and Atchison, 1985; Kislalioglu *et al.*, 1996). A more important question maybe: How is the net energy acquisition strategy of the fish affected by pollutant exposure? As pollutant effects on energy fluxes through the food chain will be more significant ecologically than the death of an individual (Kooijman, 1999).

The net energy gain by a fish will be a dynamic relationship relative to food intake, and energy expenditure to acquire food, and it has been suggested that as high as 88% of their activity costs are spent foraging for food (Boisclair, 1992). In both wild and captive salmonids food intake will be influenced greatly by the activity of other individuals, and gross growth efficiency can be substantially reduced due to the level of agonistic interactions with other individuals (Chapter 5). This inter-individual competition will lead to the development of a feeding hierarchy with the most dominant fish obtaining the best food supply or feeding stations in terms of energetic profitability (Jenkins, 1969; Fausch, 1984; Metcalfe, 1986). In determining dominance, size will often be the first cue (Parker, 1974), after which fish will interact to determine fighting ability of opponent (Enquist and Leimar, 1987), as a general rule in neutral territory, it can be expected that the competitor with least surplus energy to invest in competitive interactions will exhaust its fitness budget first and withdraw from the interaction. Therefore, energy decisions involving allocation between activity and growth will contribute in determining an individual's competitive ability, procurement of food and ultimately influence growth rates of competing fish (Abbott and Dill, 1985; Oliveira and Almada 1996; McCarthy et al., 1999). I have used this general theory of increased investment for increased return, in terms of activity costs and energy intake, and further developed it to apply to fish competing within a social group for a Cu-contaminated diet. Where, as established in previous chapters the net energetic cost:benefit ratio of feeding on a Cu-contaminated diet is less than that for fish feeding on a control diet.

Figure 34. illustrates my hypothesis for the development of the feeding hierarchy in social rainbow trout. In the control situation, increased investment by the individual in competitive ability, increases food intake, permitting the fish more energy for SGR and excess energy to expend on increased activity. Theoretically, this will create a positive feedback loop and enable the fish to out compete conspecifics and move further up in status

in the feeding hierarchy. Under these conditions, selection for a faster growing fish will favour more aggressive and competitive fish, rather than those that maximize the efficiency of growth (Weatherly, 1976; Doyle and Talbot, 1986; Swain and Ridell, 1990).

A different scenario may exist when fish are feeding on a Cu-contaminated diet (fig. 34), where an increased food intake will also lead to an increased intake of copper. This concept is supported by figs. 27 a & b (Chapter 5), where it may be assumed that the fish with the higher SGR were consuming more of the food supply and as a consequence had a higher liver Cu burden. This increased Cu burden within the intestine and liver (Table 4a, b) required energetic investment by the animal in metallothionein production, bile secretion, increased cell turnover and ion secretion mechanisms to facilitate detoxification (Handy, 1992, Handy *et al.*, 1999; Kamunde *et al.*, 2001). Moreover, Cu-induce oxidative damage was found in the liver of exposed fish and probably required a level of energetic investment in repair. Although, not correlated in this study it may be assumed that a relationship existed between liver Cu burden and oxidative damage, as similar findings were observed by Farag *et al.*, (1995), during a field study on heavy metal exposed brown trout.

Therefore, it seems logical to assume that the increased benefit of obtaining a large portion of the Cu-contaminated diet over other individuals in the group was markedly reduced, by the cost of detoxification. Although, back extrapolation to zero activity of VO_2 measurements from trout under spontaneous swimming activity showed no significant difference between control and Cu-exposed fish, Cu-exposed fish did have a greater requirement of O_2 to remain at the same level of activity as a control fish, suggesting a degree of physiological impairment. Also an increased level of anaerobic metabolism product (lactate) was found in the muscle of Cu-exposed fish when feeding under higher hierarchical

Figure 34. An hypothesis illustrating how the mechanisms shaping the normal feeding hierarchy in social rainbow trout *(Onchorhynchus mykiss)*, may be altered when the social group is feeding on a Cu contaminated diet. Grey arrow indicates the social rank of the fish within a discrete group, fish may become more or less dominant over conspecifics in relation to the food supply. Solid black arrows indicate normal social mechanisms responsible for forming feeding hierarchy, with the amount of energy available to the fish for encounters, being a determinant of rank relative to conspecifics. Dashed black arrows represent influential effect of Cu-toxicity on hierarchy, due to fish feeding on a Cu-contaminated diet, which will provide less energy return for increasing rank position.

Energetic investment in growth and activity

Increased competitive
ability

Increased food intake
from competition

Position in feeding hierarchy

Dominant ⟷ Subordinate

High Cu intake

Low Cu intake

Decreased competitive
ability

Reduced food intake
reduced exposure

Energetic investment in toxic response
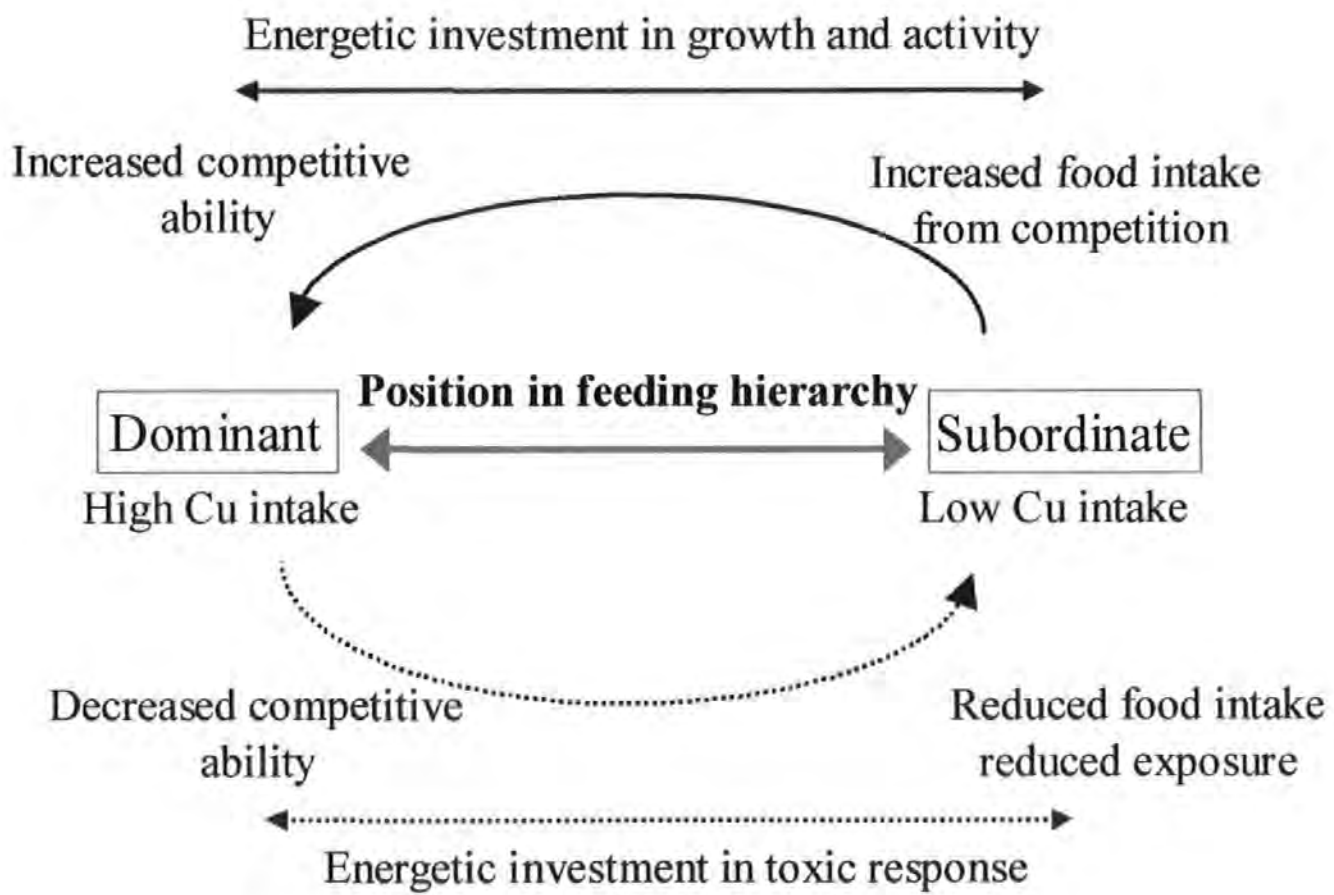
stress levels (Table 10), implying that endurance, as activity approaches R_{max} was also reduced in Cu-exposed fish.

Consequently, when feeding on a Cu-contaminated diet rainbow trout, in contrast to investing energy in activity to compete with conspecifics, alternatively, favoured a strategy of reducing aerobic scope and maintaining growth, leading to a general reduction in feeding hierarchal effects. Comparisons of the minimizing energy expenditure strategy of Cu-exposed fish may be drawn with sub-dominant salmonids who though have a reduced food intake to the more dominant fish, can maintain a similar SGR due to reduced feeding costs (Metcalfe, 1986).

Direct observation of control rainbow trout social hierarchies (Chapter 5) showed a general agreement with the hypothesis (fig. 34) with the more aggressive fish having a better growth rate than subordinates, and over time the size disparity of the group increased. Strength of the hierarchy was density dependent, with the degree of aggression, and concomitant effects on growth rates, being greater in social groups (PS) containing only a low number (10) of individuals. In further agreement with the hypothesis Cu-contaminated fish were less inclined to invest in activity than controls, and this led to a reduced inter-individual interaction rate between the dominant and subordinate fish. This reduced the degree of body damage sustained by subordinates and, I suggested, this subsequently lowered the stress levels of conspecifics. Serving to reduce the degree of size-disparity within a discrete group, compared to that observed when a social group was feeding on a control diet.

The study went on to investigate directly the dominant-subordinate relationship between individuals as it is suggested that the complex pattern of a feeding hierarchy will be organized by initial relationships between contact pairs (Braddock, 1945). In agreement with the hypothesis presented in fig. 34, Cu-exposed fish were less probable to invest energy in an escalated interaction compared to control fish, favouring withdrawal at a lower level of

energetic investment. Interestingly, Cu-exposed fish would invest in escalated interactions if the probability of winning were increased only for it. This was suggested to be equal to approximately a 15-20% body size advantage, and further highlights that Cu-exposed fish do maintain the energetic capacity for high levels of activity. Although, due to the increased energetic cost of routine metabolism, and consequently, reduced benefit from the food supply, they choose not to invest in high cost competitive activities to obtain a Cu-contaminated diet.

Bioenergetics models are based on the assumption that fish constitute thermodynamically closed systems (Boisclair, 1992) in which surplus energy (growth plus gonad production; Ware, 1975) represents a trade-off between energy intake and expenditures (Winberg, 1956; Kitchell and Breck, 1980; Hewett and Johnson, 1987). Therefore, investment of energy by the fish in behaviour, to maximize energy intake whilst reducing costs will by the temporal nature of food availability, be expressed at different times during the diurnal cycle where most advantageous in term of fitness gain. Interestingly, during periods when food would normally have been presented both control and Cu-exposed fish showed similar behaviour profiles. Exhibiting low activity in terms of distance moved but increased complexity of the swimming pattern turning more frequently per distance moved, suggested to serve in maximizing intake of food distributed in a tank through area restricted searching. Thus, Cu-exposed fish invest the highest portion of daily energy in activity, during times when food presented, directly concerned with foraging efficiently for food. This is in contrast to the preferred strategy of control fish, of investing high surplus energy in competitive behaviours that may result in obtaining a larger portion of the food supply by suppressing appetite of conspecifics.

Further work

Results from this study have clearly shown that chronic sub-lethal Cu-exposure via the diet reduces the aerobic scope of rainbow trout compared to controls. Further work is needed to qualify the effects of Cu-exposure on muscle metabolism and determine the exact cause of increased O_2 requirement when swimming at higher speeds. Results further showed that activity was altered between control and Cu-exposed fish, and suggestions were made as to the relative costs of these different types of finite behaviour, but actual costs of relative activities remained unqualified. Thus, in terms of suggesting a daily energetic cost for routine metabolism, it would be of benefit to extend the VO_2 and spontaneous activity observations, to all periods throughout the diel cycle. As well as examination after food intake to determine if the energetic cost of digestion and assimilation (M_F) of rainbow trout was increased due to Cu-exposure. The gut damage and increased mitochondrial presence observed by previous authors during histological examination (Bentssen *et al.*, 1999; Kamunde *et al.*, 2001) of dietary Cu-exposed salmonids, suggest that this may be the case.

The techniques developed in this study for determining routine metabolic rate were found to be accurate and provided a good non-invasive measure of voluntary spontaneous swimming behaviour in rainbow trout. It may be utilised for further respirometry analysis work, as well as in the field of toxicology, providing rapid determination of the cost of spontaneous swimming activity in fish. It also benefits from its multi-analysis ability, being able to examine up to 6 individuals concurrently. Additionally, it may provide suitable methodologies for developing 24 h toxicological monitoring programmes, due to the ability (with the use of infra-red lights) to monitor metabolic activities over both the light and dark cycle. The relevance of observations made within this study with previous studies on routine metabolism, and perhaps, previous literature on the deleterious effects of toxicants on swimming behaviour, showed that fish altered activity patterns greatly throughout the

light:dark cycle, and the compensatory behavioural responses of trout in this study to Cu-exposure were expressed temporally during the diel cycle. Observation of activity during feeding times showed a relative indifference in activity patterns, although during night/dawn periods when the control fish exhibited high specific swimming speeds, Cu-exposed fish remained relatively inactive. Thus, I suggest that discrepancies observed in the literature concerning behavioural responses to toxicants, may be partially accountable by the time of the diel cycle when the observations were made. Also, in observing swimming activities and behavioural strategies, results in this thesis suggest that social history has a large influence on the performance of individuals. Therefore, observations in toxicological studies may be modified by previous group size, method of food presentation and social status of individual, modifying actual uptake of toxicant and its comparative effects. I suggest that this may be accountable for the large variation observed when comparing toxicity studies both within and between experiments and authors.

Alteration in the circadian profile of Cu-exposed fish provided novel information on possible effects of toxicant exposure, which remains a relatively unstudied area of toxicology. This is in spite of endogenous rhythms being behavioural adaptation by the organism to promote the optimisation of energy utilization (DePledge, 1984). To date knowledge of the fish circadian system is still very limited (Sanchez-vasquez and Tabata, 1998), and its disruption by toxicants in the environment should be seen as an important area for further research. This study only briefly examined the alteration in plasma hormones (melatonin, serotonin, cortisol) concerned with the expression of the circadian rhythm. But results showed that Cu-exposure did have some degree of effect at the physiological level of circadian rhythms. These results, and the behavioural observations were very interesting, as serotonin is the precursor for melatonin, which is linked to the expression of circadian activity in vertebrates (Axelrod, 1974). Studies have also demonstrated it as a neurochemical

marker for social rank in salmonids, increasing with subordinate rank (Winberg, 1993), and having an inhibitory effect on both activity and food intake (Fernstrom, 1981; Samanin, 1989). Its apparent decline in circulating daytime levels in Cu-exposed fish along with the suppression of circadian activity and aggression of Cu-exposed fish, suggests a complex interaction, which requires much further research.

In studying aggression levels, and social rank effects of Cu-exposure, growth was measured as a surrogate for food intake, and equally energy could have been saved from reducing activity and invested in growth. Also the reduction in self-assessment of Resource Holding Power (RHP, dependant on individuals fitness budget and value of resource) by Cu-exposed fish remained unclear. I suggested that it may have been a combination of a reduction in energy resources available for competition, increased post-interaction cost due to increased anaerobic metabolism, and lowered assessment of resource value (Cu-contaminated food). As previously suggested radiography study as utilised by Metcalfe *et al.*, (1992) and McCarthy *et al.*, (1992), would measure, food intake directly. This would facilitate development of energy budgets to compare control and Cu-exposed fish. To study these and related issues further, it would be interesting to present more than one dietary treatment simultaneously, to determine if there is a preference between diets, feed fish within arenas for different time periods (a factor that can compound RHP, Enquist and Leimar, 1987), and lastly, alternate diets between control and Cu-exposed fish to see if relative RHP of fish is affected

From an ecological perspective, dietary Cu-exposure may have the potential to be detrimental to fish populations. Firstly, a reduced aerobic scope may hinder a fishes ability to catch prey; secondly, the disruption of circadian rhythms may hinder ability to coordinate timing of activity rhythms with food availability in the wild, and lastly, and perhaps most importantly, the reduction of competitive ability and therefore strength of social hierarchy

may have further reaching ecological implications. Intra-specific competitiveness is central to the natural selection processes of survival and reproduction (Dawkins, 1995), and authors have suggested it an important factor regulating the growth of individuals, population density, and even responsible for down stream migration (Hoar 1951; Chapman 1962; Mortensen 1977; Noakes 1978; Elliot 1984, 1990; Metcalfe, 1991).

As a final note, observations made in this study, may provide some explanation as to how Cu-contamination in the diet of predatory fish, may be deleterious to survival strategies that may influence the timing of life-history strategies, and a fish's ability to deal with rapid changes in the environment. Indeed, observations made in this laboratory study concerning energy allocation may be more critical in the natural environment, where food is spatially and temporally distributed, and optimum foraging strategies maybe made more imperative due to environmental factors such as predation risk. Although, results and observations within this thesis have been made in respect to chronic dietary copper exposure, I suggest that Cu-exposure was merely an additional stressor on the fish's energetic budget, and interpretations made here, were of an innate flexible adaptive strategy that permits fish to cope with adverse environmental change. It must however be observed, that this innate strategy whilst efficient in reducing the detrimental effects of pollutant exposure, will demand the continued expenditure of excess energy, which will ultimately be incompatible with the survival of the animal, and after all the study we may still arise at the same conclusion of don't pollute.

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To my family: *past and present*