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The Effect Of Exercise On Muscle Growth And Muscle-Specific Gene Expression In
The Common Carp (*Cyprinus carpio* L.) And Rainbow Trout (*Oncorhynchus mykiss*
Walbaum)

Christopher Ian Martin

Submitted to the University of St. Andrews for the degree of Doctor of Philosophy

Thesis supervisor

Professor Ian A. Johnston

Gatty Marine Laboratory

School of Biology

University of St Andrews

September 2003



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Abstract

Forced exercise training has long been known to be a powerful stimulus for muscle growth in several families of teleosts. The effect of exercise on muscle growth in the common carp (*Cyprinus carpio* L.) was studied in five experiments, at a range of swimming velocities between 1.7 and 3.0 body lengths per second (bls^{-1}). Muscle fibre cross-sectional area was measured in fast, slow and where identified, intermediate muscle fibres. Exercise was shown to affect the fast and slow musculature differently in a manner analogous to the teleost starvation response. In response to moderate endurance exercise training fast muscle fibres were recruited to the myotome, but this process was balanced by atrophy of larger existing fast fibres. In contrast, slow to moderate exercise training induced slow muscle fibre hypertrophy. In response to a hyperplastic stimulus, the number of myonuclei per myofibre increased, implying a single population of myogenic progenitor cells provides the cells for post-embryonic muscle growth. A comparative study was conducted on rainbow trout (*Oncorhynchus mykiss* Walbaum), a species associated with a high inherent swimming capacity. Exercise training at 0.8 and 1.6 bls^{-1} induced a 24 – 30% increase in mean fast muscle cross-sectional area through hypertrophy. To identify putative molecular signaling pathways underlying teleost muscle growth, the nuclear localisation and/or overall expression of myogenic regulatory factors (MRFs), calcineurin and associated substrate transcription factors and myostatin was compared between exercised and tank rested controls. The overall expression of MRFs was invariant in all experimental treatments. In common carp, increased nuclear localisation of the primary MRFs, especially MyoD, was positively correlated with larger mean fibre cross-sectional area. Increased nuclear localisation of the calcineurin protein was associated with hypertrophic growth of fast and slow muscle fibres in the common carp. Exercise-induced fast fibre hypertrophy was strongly correlated with increased calcineurin nuclear localisation in rainbow trout. However, a second experiment on common carp revealed no association between extensive fibre hypertrophy and nuclear localisation of the calcineurin protein. Furthermore, NFAT2 nuclear localisation did not follow a pattern that suggested NFAT2-mediated transcriptional activity was concurrent with muscle growth in the common carp or rainbow trout. In rainbow trout, myostatin active peptide was downregulated slightly (6 – 7%) in groups exhibiting exercise-induced hypertrophy of fast muscle fibres, consistent with its proposed role as a negative regulator of muscle growth. It was concluded that the molecular pathways examined might contribute to the regulation of muscle growth in teleosts but only as part of a more complex regulatory network.

Chapter 1: General Introduction

1.1 Introduction

Recently, the problem of overfishing and the collapse of capture fisheries has increased the importance of aquaculture as a source of dietary protein, as currently more than 1 billion people depend on fish as their primary source of animal protein (FAO, 2003). Historically, aquaculture is thought to have arisen in ancient China or Egypt, where evidence of this practice is provided in tomb engravings depicting the capture of tilapia (*Oreochromis mossambicus* Peters) from artificial pools (FAO, 1987). The domestication of common carp (*Cyprinus carpio* L.) for aquacultural purposes is thought to have occurred around the 1st century AD in Roman *picinae* at settlements on the banks of the River Danube (Balon, 1995) whereas aquaculture of salmonid species is a more recent phenomenon. In 2001, cyprinid and salmonid aquaculture accounted for almost one sixth of the world's fisheries production (20 million metric tonnes) and the global revenue from fish farming exceeded \$56 billion (FAO, 2003). Ironically, the practice of farming carnivorous species such as salmonids can accentuate the problem of declining fish stocks, as large inputs of wild fish are required for feed (Naylor *et al.*, 2000).

The common carp is a member of the Cyprinidae family and is originally thought to have occurred naturally throughout Western Europe, Eurasia, China, South-East Asia and India. This species is now considered to have a global distribution, as it was one of the first species to be introduced to other countries (Kottelat, 1997). The common carp is an omnivorous freshwater species, tolerant of a wide range of environmental conditions, thriving between temperatures of 3 and 32°C and capable of attaining a large body size over a relatively long life span. The

maximum-recorded age, standard length and body mass of a common carp is 47 years, 1.2m and 37.3kg respectively (Allen, 1991; IGFA, 2001).

The rainbow trout (*Oncorhynchus mykiss* Walbaum) is a member of the Salmonidae family that is thought to be native to the Kamchatka peninsula in the Eastern Pacific. However, in common with the common carp this species has been introduced widely for aquacultural purposes and now has a global distribution (Gall & Crandell, 1992). The rainbow trout is an anadromous temperate species, found in waters between the temperatures of 10 and 24°C, but the natural habitat for this species is freshwater reaching a temperature of 12°C in summer (Gall & Crandell, 1992). The maximum recorded age, total length and body mass for rainbow trout have been reported as 11 years, 1.2m and 25.4kg respectively (Robins & Ray, 1986; Bristow, 1992).

The dominant tissue in the majority of teleost species is the myotomal musculature that can account for as much as 65 – 70% of total body mass (Weatherley & Gill, 1985). Myotomal muscle normally comprises the edible portion of a fish, although in the case of some species, the entire anatomy can be exploited: Icelanders have been known to soak cod (*Gadus morhua* L.) bones in sour milk until they decompose to a point where they become edible, Newfoundland fisherman have a penchant for the cod “britches” or fried female gonads and the Japanese enjoy cod milt (Kurlansky, 1999). A great deal of aquaculture research concerns maximising growth of the myotomal musculature to increase the flesh yield from commercially valuable species. The studies of Weatherley and Gill (see review 1981) demonstrated that attainment of large ultimate body size in fish was inversely proportional to mean fibre diameter. Muscle comprises a large proportion of live weight in fish and in large teleost species such as common carp and rainbow trout, realisation of large body size

occurs through the continuous recruitment of new muscle fibres to the myotome and subsequent outgrowth of these fibres. Therefore somatic growth performance and growth of muscle tissue are intrinsically linked. Growth performance in teleosts can be influenced by the manipulation of several environmental and genetic components such as photoperiod (*e.g.* Bromage *et al.*, 1984; Johnston *et al.*, 2003c), temperature (*e.g.* Alami-Durante *et al.*, 2000; Johnston *et al.*, 1998; Johnston *et al.*, 2000), stocking density (*e.g.* Holm *et al.*, 1990; Irwin *et al.*, 1999), feeding interval (*e.g.* Boujard *et al.*, 1995; Biswas & Takeuchi, 2003), nutritional conditions (*e.g.* Kiessling *et al.* 1991; Galloway *et al.*, 1999), growth hormone administration (Weatherley *et al.*, 1980), genetic strain (Valente *et al.*, 1998, 1999) and polyploidy (Suresh & Sheehan, 1998; Johnston *et al.*, 1999). Subjecting teleosts to sustained periods of forced exercise activity is a further technique that has been utilised to augment body mass and muscle growth in farmed Atlantic salmon (*Salmo salar* L.) (Totland *et al.*, 1987). The potential of low intensity exercise to benefit somatic growth performance through increased muscle growth in commercially relevant species such as common carp and rainbow trout warrants further investigation.

In mammalian skeletal muscle, the calcineurin signaling pathway, myogenic regulatory transcription factors (MRFs) and growth/differentiation factor 8 (myostatin) have all been implicated as key regulatory factors controlling muscle growth and development. Orthologues of mammalian genes involved in these pathways have been cloned in numerous lower vertebrate and invertebrate species. Although this does not imply conservation of function between disparate evolutionary groups it does provide a key starting point to investigate potential signalling networks regulating muscle growth and development in lower vertebrates such as teleosts. Increased understanding of these regulatory pathways could provide a means of

improving aquaculture practices and contribute to guaranteeing sustenance for one fifth of the global population that depend on fish as the primary source of dietary protein.

1.2 Muscle growth

1.2.1 Mammalian muscle growth

Postnatal skeletal muscle growth in mammalian and avian species primarily involves expansion of existing fibres (hypertrophy), whereas muscle fibre recruitment (hyperplasia) is completed largely during foetal development (Goldspink, 1972). It is only under extreme circumstances, such as during regeneration after a muscular injury or in response to mechanical stress (*e.g.* heavy resistance training) that higher vertebrates can engage in a hyperplastic style of growth (reviewed by Kelley, 1996; Reggiani & te Kronnie, 1999). For example, an increase in the number of aerobic muscle fibres was observed in the deltoid muscles of professional swimmers relative to sedentary controls. Moreover, the extreme increase in muscle mass observed in elite bodybuilders was attributed to hyperplastic muscle growth when no difference in mean fibre area was observed between this group and weight-matched controls (reviewed by Antonio & Gonyea, 1993).

Mammalian and avian skeletal muscle tissue is comprised of highly specialised, terminally differentiated syncytial cells adapted for the purpose of force generation. During initial expansion of muscle fibres, the volume of cytoplasm per nucleus increases until a critical point is reached where further hypertrophy is not possible without the contribution of additional myonuclei to the fibre. DNA accretion precedes protein accretion and the myonuclei-to-fibre size ratio is maintained during

hypertrophic muscle growth (reviewed by Allen *et al.*, 1979; Campion, 1984; Schultz, 1996). Since muscle cells are functionally post-mitotic, an external source of nuclei (DNA) is required to meet the demands of growth, training and repair. Satellite cells are a pool of quiescent undifferentiated precursors that become activated, proliferate, express myogenic markers and differentiate to fulfil this function. Ultimately, differentiated satellite cell progeny are incorporated into existing cells or fuse together to form new muscle fibres (reviewed by Hawke & Garry 2001; Zammit & Beauchamp, 2001).

Mauro was the first to describe myosatellite cells in 1961 and these cells are thought to be present in the skeletal muscle of all vertebrates (Koumans *et al.*, 1991; reviewed by Antonio & Gonyea, 1993). Characteristically, satellite cells are small, quiescent, undifferentiated myogenic precursor cells, which are located wedged between the plasmalemma and basal lamina of a terminally differentiated muscle fibre of a previous generation (Quinn *et al.*, 1988; Schultz, 1996). Quiescent satellite cells can be identified by the expression of several markers, including myocyte nuclear factor (MNF), myf5, M-cadherin, c-Met and Pax7 (Garry *et al.*, 1997; reviewed by Zammit & Beauchamp, 2001). Prior to activation, satellite cells express skeletal muscle markers MNF, myf5 and M-cadherin, which suggests that these cells are already committed to a myogenic lineage. On activation of these cells in response to perturbation (growth, training or injury), the MyoD family of transcription factors are sequentially expressed in proliferating myoblasts and differentiating myotubes (reviewed by Zammit & Beauchamp, 2001). The role of the MyoD family in myogenesis will be considered further (section 1.6).

Moss and Le Blond (1971) confirmed Mauro's hypothesis that myosatellite cells were involved in growth and regeneration of muscle by observing the uptake of

tritiated [^3H] thymidine into proliferating satellite cells. One hour after administration, [^3H] thymidine was detected in satellite cell nuclei and subsequent transfer of label to myofibre nuclei after a time lapse demonstrated that muscle fibre nuclei do not proliferate but are the progeny of undifferentiated muscle precursor cells. The time lapse is sufficient for the completion of a cell cycle by the satellite cell and fusion of the daughter cell to an established muscle fibre (Moss & Le Blond, 1971).

The proportion of myonuclei that are satellite cells varies with species, age and type of muscle fibre: in mice, satellite cells comprise ~30% of all myonuclei during embryonic development, which fell to ~4% in adults and ~2% in senile individuals (Snow, 1977). The smaller proportion of satellite cells in adult rodents was due to an overall increase in myofibre number in oxidative and glycolytic myofibres and an accompanying reduction in the number of satellite cells in the glycolytic fibres (reviewed by Schultz & McCormick, 1994).

Resistance training or load-induced hypertrophy (stretch, tenotomy, ablation of synergists) has been shown to induce satellite cell activation, proliferation and fusion of progeny to existing myofibres to contribute to hypertrophic muscle growth (reviewed by Hawke & Garry, 2001). For example, strength training in females resulted in a simultaneous increase in satellite cell and myonuclei content in the trapezius muscle (Kadi & Thornell, 2000). Growth factors, developmental temperature and nutritional status are further factors known to affect activity of the satellite cell population. Work on poultry myogenic satellite cells has demonstrated that growth factors such as insulin-like growth factors (IGFs) and fibroblast growth factors (FGFs) are key mitogenic substances that have the potential to greatly enhance satellite cell proliferation, differentiation and fusion of embryonic myoblasts

(McFarland, 1999). Subjecting 3-day old chicks to heat shock caused a concomitant increase in IGF-1 induction and satellite cell DNA synthesis; this treatment was associated with maximal weight gain in body and breast muscle by the 42nd day of development (Halevy *et al.*, 2001). The number and activation state of satellite cells is also influenced greatly by nutrition; in malnourished children, satellite cells are generally inactive and fewer in number. Recovery from this state is marked by large increases in the number and proportion of activated satellite cells (Hansen-Smith *et al.*, 1978, 1979).

1.2.2 Teleost muscle growth

In teleosts, the swimming musculature comprises a greater proportion of the total body mass than in the majority of vertebrates, up to 70% of live weight in some species (Gill *et al.*, 1982; Weatherley & Gill, 1985). As well as the primary means of propulsion in fish, the myotomal muscle also functions as a protein store providing energy during starvation, migration and for the elaboration of reproductive products (Gill *et al.*, 1989; Weatherley & Gill, 1987). Functionally, vertebrate skeletal muscle is extremely diverse and is also a source of blood glucose to fuel muscle contraction and amino acids (*e.g.* glutamine) for neurotransmitter synthesis and acid base balance (reviewed by Goldspink, 1998).

Muscle growth and development in fish differs from that of higher vertebrates, in that postnatal muscle growth in fish occurs through a combination of hyperplasia and hypertrophy (Weatherley & Gill, 1985). The ability to recruit new muscle fibres is retained well into adulthood in species reaching more than 5 – 6 cm standard length, thus greatly influencing body mass accumulation (Stickland, 1983; Weatherley & Gill, 1985; Stoiber & Sanger, 1996). A remarkable feature of Atlantic salmon development is the continuous recruitment of muscle fibres, the number of white

muscle fibres per trunk cross-sectional area increasing from around 5000 post-hatch to over 1 million in a 2 sea-winter fish (reviewed by Johnston, 2001). The roles of fibre recruitment and fibre hypertrophy change in importance during the lifecycle of a fish and are influenced by environmental factors to a greater extent than in birds and mammals (Koumans *et al.*, 1991; Koumans & Akster, 1995).

Like higher vertebrates, fish muscle tissue is made up of highly specialised, post-mitotic and terminally differentiated muscle cells. Sustained hyperplastic growth by mitotic division of existing cells is unavailable to myocytes, in contrast to hepatocytes (Weatherley & Gill, 1987; Weatherley & Gill, 1989). Despite being post-mitotic and highly differentiated, the muscle tissue of teleosts must be capable of meeting the demands for increase that a growing fish makes of it (Weatherley & Gill, 1985). As in higher vertebrates, muscle fibre hypertrophy is mediated by satellite cell (or myogenic progenitor cell) activation, proliferation and fusion of additional differentiated cells to existing muscle fibres (Weatherley & Gill, 1989; Koumans & Akster, 1995). The activity of satellite cells is thought to extend the period of hyperplastic growth beyond the early stages of ontogeny in fish (Koumans *et al.*, 1991; Stoiber & Sanger, 1996). Fibre splitting has been proposed as the origin of new fast fibres in the eel (*Anguilla anguilla* L.) but this has yet to be corroborated using electron microscopy (Stoiber & Sanger, 1996).

In a mature adult vertebrate species that no longer exhibits muscle growth, satellite cells are normally held in reserve in a quiescent state, but are responsible for muscle regeneration when activated by muscular damage (Bischoff, 1974). In juvenile animals, proliferation of myosatellite cells is required for growth and a strong correlation between the rate of growth and the proliferation rate of these cells has been found to support this (Koumans & Akster, 1995). In teleost axial muscle,

different subclasses of myosatellite cells are believed to be responsible for the hyperplasia observed at different life stages. A pre-established pool of stem cells is thought to be involved in highly hyperplastic juvenile growth, whereas in the adult stages, hyperplasia results from a resumption of proliferation in the satellite cell population (Stoiber & Sanger, 1996). This is a parallel of the different myoblast subtypes observed in the embryonic, fetal, neonatal and adult stages of development in higher vertebrates (Stockdale, 1992). The behaviour of myosatellite cell subtypes in relation to the processes of hypertrophy and hyperplasia, as well as the total number of myosatellite cells and their division between the subclasses, is thought to determine muscle growth in fish (Koumans *et al.*, 1991). Direct measurements of myogenic progenitor cell cycle time in teleosts are now available; cell cycle duration was nearly double in the Antarctic Harpagifer (*Harpagifer bispinis* Forster) at 5°C compared to 10°C (Brodeur *et al.*, 2003a), which fits the hypothesis that reduced temperature may cause a longer myogenic cell cycle time in ectothermic vertebrates than that observed for mammals (Akster *et al.*, 1995).

An inverse relationship between standard length (5 – 18 cm) and the proportion of myonuclei that are satellite cells in red and white muscle fibres has been demonstrated in common carp (Koumans *et al.*, 1991). In this size range, the overall number of myonuclei increased but the total number of myogenic progenitors remained relatively constant. Differences in myosatellite cell number and distribution are caused by genetic and environmental factors, and are thought to contribute to the range of growth rates and ultimate body sizes seen in teleost species (Weatherley & Gill, 1985; Stoiber & Sanger, 1996).

1.2.3 The dynamics of teleost muscle growth

Weatherley & Gill (1985, 1987, 1989) extensively studied fast muscle fibre growth dynamics in a range of teleost species with widely differing ultimate growth sizes, and found that the point at which muscle fibre recruitment ceases during ontogeny is directly related to the growth potential and ultimate body size of a species. Hence, in a small, slow growing species such as the bluntnose minnow (*Pimephales notatus* Rafinesque), new fibre formation is arrested while they are still very small and subsequent growth occurs solely through hypertrophy of existing fibres. This type of increase is analogous to postnatal muscle growth in higher vertebrates (Weatherley *et al.*, 1987). As ultimate body size increases, cessation of fibre recruitment occurs later in development, as observed in the muskellunge (*Esox masquinongy* Mitchill), a very large teleost species. Sustained recruitment is characterised by the presence of small diameter ($< 20 \mu\text{m}$) fibres, which results in similar mean fibre diameters for a 70 cm muskellunge and a 7 cm bluntnose minnow (Weatherley & Gill, 1989). After the termination of fibre recruitment, muscle growth occurs through hypertrophy of existing fibres. A similar profile of muscle fibre growth dynamics to the muskellunge was observed recently in the white seabass (*Atractoscion nobilis* Ayres), a large marine teleost. Sustained fibre recruitment resulted in the impressive ultimate standard length of 133 cm in the largest specimen studied (Zimmerman & Lowery, 1999).

Weatherley & Gill (1989) postulated that adult teleosts reach an ultimate body size when fibre outgrowth is no longer possible due to a limiting average fibre diameter. The fibre surface area to unit fibre length ratio decreases during growth, and it is thought that there is a limiting fibre diameter (120 – 270 μm) beyond which the surface area to unit fibre length ratio becomes unfavourable for metabolic

exchanges related to fibre (and fish somatic) growth (Weatherley & Gill, 1989). This hypothesis arose from data that implied maximum fibre diameter was a species-specific characteristic, where teleost species with a small ultimate body size displayed a smaller maximum fibre diameter (approx. 120 μm) relative to larger species (approx. 270 μm).

Muscle fibre recruitment was observed in mature adults of a slow-growing medium-sized species (the grass pickerel, *Esox americanus vermiculatus* Lesueur) whose fibre diameter frequencies resembled those of species capable of much larger ultimate body sizes (Weatherley & Gill, 1987). Through bovine growth hormone administration (40 $\mu\text{g/g/2}$ weeks) the grass pickerel was able to express a latent growth potential and reach an enhanced ultimate size. Hence, Weatherley & Gill (1987) demonstrated that alternative factors (hormonal/ somatotrophic) apart from muscle fibre growth dynamics might be responsible for small ultimate size in fish. In mammals, growth factors such as IGF and FGF affect satellite cell activity and it is likely that a deficit in production of mitogenic growth factors would have a downstream effect upon satellite cell proliferation and muscle growth dynamics (McFarland, 1999). A further factor affecting muscle growth in teleosts is nutritional status. *In vitro* studies of satellite cells removed from young rainbow trout (1 – 3 cm) demonstrated the effect of fasting on satellite cell activity, as initial satellite cell proliferation was retarded by a period of fasting (Fauconneau & Paboeuf, 2000).

1.3 Muscle fibre types

1.3.1 Mammalian myosin heavy chain expression

Goldspink (1998) stated that adaptability is a prerequisite for the survival and success of an animal species; the inherent mutability of skeletal muscle tissue contributes in no small way to this process. The concomitant repression and expression of subsets of the panoply of myosin heavy chain (MHC) isogenes, facilitates reconstruction of the contractile apparatus in response to changing environmental conditions and/or physical stimuli (reviewed by Goldspink 1996, 1998). For example, in rodent fast muscle, the response to exercise induced overload shows a pattern of conversion from fastest to slowest isoform of MHC:

MHC IIb/ fast → IIx → IIa → I/ slow

In rats, changes in muscle fibre phenotype are the result of rebuilding myofibrils with more energy efficient contractile and calcium handling properties (reviewed by Talmadge, 2000). Muscle fibre type conversions are also observed in common carp, which are able to express different MHC isoforms in response to seasonal temperature fluctuations, allowing more economical force generation (Gerlach *et al.*, 1990).

A single gene from a highly conserved multigene family encodes each MHC isoform (Nguyen *et al.*, 1982). At least eight skeletal muscle MHC isoforms are present in mammals, 31 are thought to exist in chicken (Robbins *et al.*, 1986) and up to 28 separate MHC genes have been found in carp (Gerlach *et al.*, 1990). In any one muscle fibre, the predominantly expressed MHC isoform determines which type of molecular motor is formed, for example, expression of MHC-I induces muscle fibres of the slow type I phenotype (Donoghue & Sanes, 1994). The intrinsic velocity of contraction (V_{\max}) is set by the MHC isoform a muscle fibre contains. Different isoforms of myosin have contrasting rates of cross-bridge cycling and ATP energy

transduction, hence different rates of contraction. The same isoform of actin (α -skeletal) is present in all studied vertebrate striated muscle and does not contribute to intrinsic differences in V_{\max} between functionally distinct muscle fibre phenotypes (reviewed by Hoh, 1991).

Three main types of skeletal muscle have been shown to occur intermixed in the skeletal muscle of higher vertebrates: slow-twitch oxidative (type I), fast-twitch oxidative (type IIa) and fast-twitch glycolytic (type IIb) fibres (Burke *et al.* 1971). A further fast-twitch muscle fibre type was recently discovered, and termed type IIx due to contraction velocity characteristics intermediate to those of types IIa and IIb (Schiaffino *et al.*, 1989). A muscle fibre may at any one time express a combination of MHC isoforms, co-expression resulting in a whole spectrum of muscle fibres with intermediate properties and phenotypes (Goldspink, 1998).

It was previously thought that a homogenous population of myoblasts gave rise to uniform myotubes, which subsequently differentiated into different fibre types due to innervation (reviewed by Donoghue & Sanes, 1994). However, results from studies on aneural chick embryos suggest that myoblasts have inborn heterogeneities, represent distinct myogenic lineages and form myotubes with distinct phenotypes (see Stockdale, 1992). Neurogenic factors, thyroid hormone and exercise act upon the myofibres at a later stage during development and can influence fibre type (reviewed by Donoghue & Sanes, 1994; Sakuma *et al.*, 1999). Furthermore, the extreme specialisation of MHC isoforms observed in the jaw musculature of carnivores, provides compelling evidence for myoblast heterogeneity. The masseter muscles of the cat contain two unique MHC isoforms (jaw-specific slow and “superfast” myosin isoforms) which are so dissimilar to corresponding isoforms observed in the limb

musculature, to suggest control of muscle fibre type in the first instance is myogenic rather than neurogenic (reviewed by Hoh, 1991).

During development of muscle fibres in higher vertebrates (rats and humans), there is a complex expression pattern of MHC isoforms, such that different generations of myotubes express different subsets of MHC isoforms (reviewed by Ennion *et al.*, 1999). Primary myotubes initially incorporate the embryonic, neonatal and slow β cardiac isoforms of MHC, in contrast to the embryonic, neonatal and fast MHC isoforms observed in secondary myotubes. As the embryo develops, expression of embryological and neonatal MHC isoforms is downregulated, as muscle fibres begin to take on the adult phenotype expressing adult slow and fast MHC isotypes (Ennion *et al.*, 1999). MHC isoform transitions during vertebrate development are thought to be controlled at the transcriptional level. In the rabbit, changes in mRNA levels occurred with switches in the corresponding contractile proteins expressed in the embryonic muscle fibres (McCoy *et al.*, 1998). Developmentally regulated isoforms of MHC have also been described for avian (Hofmann *et al.*, 1988) and amphibian (Radice & Malacinski, 1989) species and recently two developmentally expressed MHC isoforms (*Eggs22* and *Eggs24*) have been described in a fish species (Ennion *et al.*, 1999).

1.3.2 Teleost muscle fibre types

A major difference in the development of fish muscle from that of higher vertebrates is the spatial and temporal segregation seen in the formation of different muscle fibre types (Koumans & Akster, 1995). In the myomeric musculature of fish, different fibre types occupy distinct myotomal zones. As in higher vertebrates, the axial muscle of adult fish contains three main fibre types; these can be distinguished macroscopically during dissection on the basis of their colour: red, pink and white

(Johnston *et al.*, 1977). As well as appearance, the following characteristics can be used to define muscle fibre types in fish: anatomical location, fibre diameter, blood supply, histochemical, biochemical and electrophysiological properties (Bone, 1978; Johnston, 1980).

Slow red (analogous to mammalian type I) muscle fibres tend to be found at the periphery of the myotome in a superficial monolayer and concentrated in a V-shaped notch at the lateral line. Slow fibres are rich in lipids, glycogen and myoglobin, to which they owe their red appearance. Slow muscle is typically composed of small diameter fibres, which have a slow rate of contraction. These fibres have a rich blood supply, many mitochondria and are equipped biochemically for an aerobic metabolism (high oxidative enzyme activity) (Boddeke *et al.*, 1959; Bone, 1978; Johnston, 1980).

The majority of the total myotomal cross-sectional area (> 90%) is a deeper muscle layer made up of large diameter fast white (analogous to mammalian type IIb) fibres, which have a low concentration of myoglobin. These fibres have a low concentration of capillaries, few mitochondria and function mainly anaerobically with high glycolytic enzyme activity (Bone, 1978; Weatherley & Gill, 1987). The boundary between fast and slow muscle tends not to be clearly defined. A zone of mosaic fibres of intermediate enzyme and metabolic characteristics separates the slow muscle from the bulk of the fast fibres: intermediate or pink muscle fibres, analogous to mammalian fast oxidative fibres (Boddeke *et al.*, 1959; Johnston *et al.*, 1977; Mosse & Hudson, 1977).

In common with higher vertebrates, the division of myotomal muscle into three fibre types is a gross over-simplification, because as many as seven different fibre varieties have been described for some species, *e.g.* in the trunk musculature of

the Atlantic cod (Korneliussen *et al.*, 1978). Furthermore, white muscle is not always a uniform structure consisting of similarly sized fibres. In many teleost species such as Atlantic salmon and common carp, new fast fibres form on the periphery of existing fibres rather than in zones of proliferation observed in other fish species, giving a mosaic appearance (Boddeke *et al.*, 1959; Stickland, 1983).

1.3.3 Fibre type functions

Goldspink postulated that the majority of vertebrate musculature co-expresses different isoforms of myosin heavy chain, thus representing a “two or three geared propulsive system”. This characteristic of muscle allows sufficient mechanical and thermodynamic efficiency in generating force over a range of contraction velocities (reviewed by Goldspink, 1998).

It is proposed that in any activity, the slow/type I fibres are recruited first and if excess force is required, the fast/type II fibres are then called upon to generate the necessary power and speed. Type I fibres have a relatively low ATPase activity and therefore hydrolyse ATP slowly and have a slow cross-bridge force generation cycle. These properties make slow fibres very economical for maintaining isometric forces and producing slow successive movements. Therefore, type I fibres are extremely important for postural adjustments in higher vertebrates and are typically most numerous in muscles such as the soleus, which is continuously recruited in routine activities such as standing, walking and running (Hnik, 1985). Type IIb fibres are adapted for short bursts of rapidly fatiguing high power activity, in contrast to type IIa fibres which can sustain high power output over a longer period, due to a more oxidative metabolism and a higher mitochondrial content (reviewed by Goldspink, 1996, 1998). Lifestyle differences of contrasting animal species can be related to basic differences in muscle fibre type composition. Breeds of dog such as huskies and

Border collies are virtually continuously active, which is reflected in the predominance in canine striated musculature of fast-oxidative type IIa muscle fibres and contributes to fatigue resistance. The behaviour of cats is characterised by short bursts of explosive activity; the principal muscle fibre type is the fast-glycolytic type IIb and due to the build up of waste metabolites, the animal becomes rapidly exhausted (Goldspink, 1996). In contrast, the prevalent muscle fibre type in humans is the intermediate fast-twitch type IIx fibre.

1.3.4 Fibre type roles in fish

In fish, multi-gearred systems have been proposed as higher levels of heterogeneity of the myotomal fibre population (greater than three fibre types) have been recognised (Korneliussen *et al.*, 1978). Boddeke and colleagues (1959) proposed that in teleosts, red muscle functions during slow speed continuous swimming (cruising), whereas white muscle is associated with rapidly-fatiguing activity, such as prey capture or predator evasion. However, a simple division of labour between fast and slow fibre types does not exist, as both fibre types are recruited at high sustainable cruising speeds (Hudson, 1973). In this scenario, white fibres are thought to avoid fatigue by the rotation of motor units and the periodic rest afforded by the glide phase during “flick-glide” swimming activity (Hudson, 1973).

The role of intermediate muscle is unclear, because many teleost species lack this fibre type and in those that possess pink muscle, it appears to perform only a minor function, *e.g.* in trout (Davison, 1983). However, Johnston and colleagues (1977) proposed that pink muscle functions at intermediate swimming speeds in the common carp, thus avoiding an oxygen debt by sparing the fast muscle. Intermediate fibres have also been associated with the ability to swim continuously for long periods, as sedentary or relatively inactive fish have much reduced or an absence of

these fibres (Mosse & Hudson, 1977). However, the semi-pelagic fish *Hemiramphus* species lacks intermediate muscle but has the capacity to swim continuously, hence Mosse & Hudson (1977) proposed that these fibres fulfil another purpose, as an undifferentiated stock of precursor fibres. The presence/absence of intermediate muscle fibres and the interspecific variation observed in the function of this fibre-type during routine swimming activity implies that the role of intermediate muscle fibres in teleosts cannot be generalised and must be considered on a species by species basis.

The relative proportion of the red muscle in a teleost myotome shows a direct correlation with the ability to maintain continuous swimming activity (Boddeke *et al.*, 1959; Bone, 1966; Mosse & Hudson, 1977; Gill *et al.*, 1989). Boddeke *et al.* (1959) described four groupings of fish species on the basis of red muscle complement, lifestyle and behaviour. Mosse & Hudson (1977) demonstrated that classification of fish into these categories is an over-simplification, as it is impossible to draw clear-cut distinctions between teleost groups based upon the above criteria. Nonetheless, various studies have demonstrated that teleost behavioural patterns may be correlated with myotomal fibre types (Gill *et al.*, 1982). In a histochemical study of the axial musculature of five fish species, Gill *et al.* (1989) found that the yellow perch (*Perca flavescens* Mitchill) had a greater proportion of red muscle than the other species studied. The propensity for this species to indulge in extensive free-cruising behaviour may account for the greater amount of red muscle observed in the perch than in the specialised “ambush predator” species studied. In another study, the stickleback (*Gasterosteus aculeatus* L.) was found to be the epitome of the “sprinter” classification, with none of the red fibres usually associated with teleosts (te Kronnie *et al.*, 1983).

Further research indicates that the proportion of different muscle fibre types can be influenced by the type of habitat occupied and water temperature (Gill *et al.*, 1989). For example, interspecific variation was observed in size matched roach (*Rutilus rutilus* L.) taken from running water and still water habitats. The running water habitat was associated with significantly higher levels of slow muscle in the myotome. All of the evidence indicates that the different fibre types fulfil different locomotory functions and are typically recruited at different swimming velocities. As well as the roles fulfilled by various muscle fibre types in swimming behaviour, the growth performance of different fibre types can also influence the growth potential of fish (Gill *et al.*, 1989).

1.4 The effect of exercise upon muscle growth

1.4.1 Exercise-induced muscle adaptation

Skeletal muscle is a dynamic tissue capable of undergoing profound morphological, biochemical and physiological changes in response to the functional demands placed upon it. The nature of the compensatory adaptation depends upon the stimulus: heavy resistance training augments muscle power output by increasing muscle volume through muscle fibre hypertrophy and, under certain conditions, muscle fibre recruitment. Endurance exercise training improves the aerobic capacity of muscle through increased production of oxidative enzymes and myoglobin (reviewed by Antonio & Gonyea, 1993; Kelley, 1996; Reggiani & te Kronnie, 1999). Exercise-induced muscle fibre type transitions are another facet of improved muscle performance. In humans, endurance training has long been recognised to increase the proportion of aerobic fibres, in contrast to 'all-out' sprint training that redirects fibre phenotype in the opposite direction (reviewed by Talmadge, 2000). The same suite of

adaptive responses can be seen in teleosts subjected to exercise training, including compensatory hypertrophy, enzymatic adaptations, improved exercise performance and alterations in relative fibre phenotype proportions.

1.4.2 Definitions of endurance- and sprint-training exercise in teleosts

The majority of teleost exercise training experiments have used sustainable swimming speeds over an extended time period (reviewed by Davison, 1997). For example, rainbow trout were exercised at 60% of their critical swimming velocity (U_{crit}) for 18 hours a day over a 28-day period (Farrell *et al.*, 1991). Activity of this type was defined as low intensity or endurance exercise training because although the fish were pushed hard the exercise was submaximal, sustainable and did not lead to rapid exhaustion (reviewed by Davison, 1997). The effect of endurance exercise training on somatic growth and muscle cellularity has been evaluated in numerous teleost species utilising a range of swimming velocities. The swimming speeds used (in body lengths per second, bls^{-1}), can be categorised as slow (0.5 – 1.5), moderate (1.6 – 3.0) and fast (3.1 – 4.5) endurance exercise (Davison & Goldspink, 1977; Davison & Goldspink, 1978; Young & Cech, 1994).

The effect of high intensity exercise or sprint training on teleosts has been less well studied (reviewed by Davison, 1997). High intensity short duration training protocols have involved vigorous chasing for a short time period daily (Gamperl *et al.*, 1988; Hernández *et al.*, 2002) or a U_{crit} swim test on alternate days (Gallaughier *et al.*, 2001). Chasing induced sprinting velocities of up to $11.4 bls^{-1}$ in small (9 cm) rainbow trout, which grew 81% less than tank rested controls (Gamperl *et al.*, 1988). In early classic swimming experiments, Bainbridge (1958, 1960) measured the burst swimming capacities of common carp and rainbow trout (13 and 15 cm total length) at 12.5 and $11.7 bls^{-1}$ respectively.

The definitions of slow, moderate and fast endurance exercise are appropriate for the small specimens (5 – 20 cm) used in the above swimming experiments (Davison & Goldspink, 1977; Davison & Goldspink, 1978; Young & Cech, 1994). However, the observation that U_{crit} tends to be inversely proportional to fork length (Wardle, 1975) means that large fish will fatigue at velocities small fish can readily sustain. For example, Farrell *et al.* (1991) observed that the U_{crit} of 35 cm rainbow trout was 2.3 bls^{-1} . Using the above definitions, 2.3 bls^{-1} would be classified as moderate endurance exercise rather than maximal sustainable swimming performance. Therefore for fish larger than 20 cm total body length, the definitions of endurance exercise training would have to be revised.

1.4.3 Fibre-specific response to exercise

Numerous swimming experiments have been performed on fish to evaluate the effect of exercise upon the myotomal musculature. The division of the myotome into distinct fibre-type zones has already been discussed, as has the proposed division of labour between different phenotypes at various swimming velocities. The participation of different fibre types at various intensities of swimming activity has been measured using electromyography and has been shown to vary between species. In brook trout (*Salvelinus fontinalis* Mitchill) it was demonstrated that aerobic red (slow) muscle fibres were active at all speeds measured below 5 bls^{-1} , in contrast to the anaerobic white (fast) fibres, which were not recruited until speeds above $1.5 - 1.8 \text{ bls}^{-1}$ (Johnston & Moon, 1980a). The threshold velocity below which fast fibres were inactive was lower in coalfish (*Pollachius virens* L.) (0.8 bls^{-1}) whereas slow fibres were active at all swimming speeds examined ($0.25 - 3.6 \text{ bls}^{-1}$) (Johnston & Moon, 1980b). This pattern was repeated in the mirror carp (*Cyprinus carpio* L.), and in addition, environmental temperature was shown to affect the swimming speed at

which fast fibres were first recruited to activity: at 10°C fast fibres were active at around 1.5 bls⁻¹, compared to 2.5 bls⁻¹ at 20°C (Rome *et al.*, 1984).

Interspecific variation observed in roach taken from still and running water habitats revealed a higher proportion of aerobic muscle and a higher swimming capacity in fish from the running water environment (Broughton *et al.*, 1978, 1981). In contrast to this example of ‘natural training’, sustained periods of forced swimming activity have been used routinely to evaluate the effects of exercise on muscle growth in fish. The effect of forced exercise on muscle growth will be considered in three families of teleost: the Gadidae, Salmonidae and Cyprinidae.

1.4.4 The effect of exercise on muscle growth in gadoid species

Greer-Walker and associates (1971, 1973) established that hypertrophy of fast and slow muscle fibres was induced by continuously exercising a marine gadoid species (coalfish) at a range of velocities for a 42 day period. At the lowest speed (1 bls⁻¹), extensive hypertrophy of the slow fibres occurred, whereas fast muscle hypertrophied to the greatest extent at the highest swimming speed used (3 bls⁻¹). This was most likely a consequence of the contrasting pattern of involvement of slow and fast muscle fibre types at different swimming velocities. Johnston and Moon (1980b) reported a similar result in coalfish exercised at 2.1 bls⁻¹ for three weeks. At increased swimming velocities (2 – 4.5 bls⁻¹) hypertrophy was observed in fast fibres, whereas slow fibres underwent hyperplasia as well as hypertrophy (Greer-Walker & Emerson, 1978). In a more recent study of another gadoid species, there was no effect of slow speed endurance exercise (0.5 – 1.0 bls⁻¹) on fast fibre size observed in Atlantic cod after a 9-month experimental period (Bjørnevik *et al.*, 2003).

1.4.5 The effect of exercise on muscle growth in salmonid species

Salmonids are the most common subjects in teleost exercise studies. The bias towards this group most likely reflects the ease of obtaining specimens from commercial hatcheries, the economic importance of salmonid species and the inherent swimming capacity of this group (Davison, 1989). Continuous low velocity (1 bls^{-1}) endurance training of rainbow trout resulted in a significant adaptive increase in slow muscle fibre mass (Nahhas *et al.*, 1982; Davie *et al.*, 1986). Brook trout exercised continuously for 21 days at 3 bls^{-1} displayed extensive hypertrophy of the aerobic musculature, mean slow fibre area was 70% larger relative to tank rested controls (Johnston & Moon, 1980a). Brown trout (*Salmo trutta* L.) were exercised at $1.5 - 3.0 \text{ bls}^{-1}$ for 28 days or 4.5 bls^{-1} for 14 days and compared with three equivalent tank rested groups (Davison & Goldspink, 1977). An increase in slow fibre number and diameter was observed at all swimming velocities, the highest increase in number observed at 1.5 bls^{-1} and the largest extent of hypertrophy found at 3 bls^{-1} . Pink muscle fibres were hypertrophied at the intermediate swimming speed (3.0 bls^{-1}) but atrophied at the highest velocity (4.5 bls^{-1}). Fast (white) muscle fibres increased in diameter at all swimming speeds, but hypertrophied to the greatest extent at 3.0 bls^{-1} .

Totland and colleagues (1987) conducted an industrial scale study (~5000 specimens) into the effect of sustained exercise activity upon muscle growth, by exercising Atlantic salmon in a 20 m raceway. For comparison, a reference group was confined in a sea cage at the same stocking density as the raceway specimens. The raceway group were exercised at a mean velocity of 20 cms^{-1} , equivalent to around $0.40 - 0.45 \text{ bls}^{-1}$. At the end of the eight-month experimental period, the weight gain in the exercised fish was 38% higher, primarily due to an increase in the mass of the lateral swimming musculature and the mean cross-sectional area of fast

fibres was 17% greater than in caged fish. In this experiment, a lower swimming speed induced hypertrophy in the fast muscle fibres than in previous experiments on salmon and trout (Davison & Goldspink, 1977; Nahhas *et al.*, 1982). As discussed previously, this was partly due to the larger specimens used in this study, as large body size affects swimming efficiency by lowering the maximal sustainable swimming speed (Wardle, 1975). Therefore fast muscle fibres will be recruited for activity at relatively lower swimming speeds than in smaller fish and the increased neuromuscular activity may stimulate hypertrophic growth of these fibres (Totland *et al.*, 1987).

1.4.6 The effect of exercise on muscle growth in cyprinid species

There are relatively few examples in the literature of exercise and training studies using cyprinids, which is probably a consequence of their difficulty to train (Sänger, 1992). Members of the carp family are not renowned swimmers and are typically found in still or slow moving water. In a prolonged swimming study, chub (*Leuciscus cephalus* L.) and nase (*Chondrostoma nasus* L.) were induced to exercise at 2 bls⁻¹ for a 17-week period (Sänger, 1992). The significant findings in this study were the increase in overall slow fibre area, hypertrophy of individual slow muscle fibres and an increase in the number of intermediate muscle fibres in both species. In a similar experiment, nase were subjected to endurance exercise training at 2.5 – 3.5 bls⁻¹ for 13 weeks, which resulted in hypertrophy of the slow and intermediate fibres by 13% and 41% respectively (Hinterleitner *et al.*, 1992). Danube bleak (*Chalcalburnus chalcoides mento* Guldenstadt) under the same experimental conditions displayed hypertrophic growth of the slow and fast fibres by around 15% relative to tank rested controls (Hinterleitner *et al.*, 1992). The difference in the observed hypertrophic responses was attributed to divergence in lifestyle and typical

swimming behaviour employed by these two species. However, the hydrodynamic consequences of the larger body size of the bleak used in the experiment may have caused fast muscle fibres to be more heavily utilised in this species than in the nase, thus stimulating fast fibre hypertrophy in the bleak.

Davison and Goldspink (1978) continuously exercised goldfish (*Carassius auratus* L.) at 1.5, 3 and 4.5 bls⁻¹ for 28 days to examine the effect of exercise on myotomal ultrastructural characteristics, relative to three groups of tank rested controls. Slow muscle fibre recruitment was only observed at the lowest swimming velocity (1.5 bls⁻¹) and only a small degree of hypertrophy was apparent at the two higher speeds. There was no significant change in intermediate fibre characteristics under any conditions, whereas there was a clear proportional relationship between swimming speed and fast fibre hypertrophy: at 4.5 bls⁻¹ the greatest increase in fast fibre diameter was observed, 22% larger relative to tank rested animals. The higher survival rate at all swimming velocities and the degree of hypertrophic growth observed in goldfish exercised at 4.5 bls⁻¹ when compared to the data for brown trout in an equivalent experiment (Davison & Goldspink, 1977) belies the 'sedentary' reputation of cyprinids. However a greater capacity to cope with exercise activity was observed in the salmonid species, as brown trout exercised at the lower two swimming speeds were still capable of increasing in body mass during the experiment, whereas growth retardation or loss of body mass was observed in all exercised goldfish (Davison & Goldspink, 1978).

In addition to causing hypertrophy of muscle fibres, prolonged exercise can produce alterations in fibre type composition and produce a greater resistance to fatigue (Nahhas *et al.*, 1982). Moderate endurance exercise conditioning of a perciforme species (striped bass, *Morone saxatilis* Walbaum) has also been used to enhance

swimming performance and overall condition of hatchery-reared fish before their release into natural environments (Young & Cech, 1994). Further benefits of swimming fish include a faster somatic growth rate, more resistance to disease, increased stamina and endurance, a better co-ordinated stress response and subsequent to exercise training, an increased survival rate in sea released smolts (coho salmon, *Oncorhynchus kisutch* Walbaum) (reviewed by Woodward & Smith, 1985). On the basis of these findings, endurance exercise training appears to have potential as a means of stimulating increased somatic growth in teleosts through muscle fibre recruitment and hypertrophy. Putative underlying molecular signaling pathways controlling muscle growth in teleosts will now be considered.

1.5 Calcineurin

Calcineurin, a heterodimeric protein phosphatase, has been implicated on the basis of *in vitro* (Musaro *et al.*, 1999; Semsarian *et al.*, 1999) and *in vivo* (Dunn *et al.*, 1999) studies in the regulation of hypertrophic growth in mammalian skeletal muscle. Calcineurin is a highly conserved enzyme, ubiquitously expressed in eukaryotes from yeast to humans (reviewed in Hemenway & Heitman 1999 and Rusnak & Mertz 2000). In higher vertebrates calcineurin-mediated signaling regulates key cellular processes such as T-cell activation and memory development as well as muscle function. In eukaryotic microorganisms calcineurin is known to regulate the cell cycle, ion homeostasis and glucan synthesis (reviewed by Sugiura *et al.*, 2001). There is a high level of evolutionary conservation in the calcineurin subunit (A and B) amino acid sequences between diverse evolutionary groups (reviewed by Guerini, 1997). It is also notable that in both yeast and humans key transcription factors fulfil pivotal roles in calcineurin-mediated regulation of gene expression in response to

calcium (Ca^{2+})-mobilising stimuli (reviewed by Sugiura *et al.*, 2001). Interestingly, conservation of calcineurin function has recently been demonstrated in the fruit fly, where calcineurin signaling is necessary for the formation of indirect flight muscles and neural development (Gajewski *et al.*, 2003). It was proposed that studies of the calcineurin signaling pathway in lower vertebrates would demonstrate that calcineurin signaling is a major regulatory pathway controlling skeletal muscle hypertrophy in all vertebrates.

1.5.1 Calcineurin – a brief history

Two research groups separately discovered calcineurin in 1976: Wang and Desai detected calcineurin as a column fraction that inhibited the calmodulin-dependent cyclic nucleotide phosphodiesterase; Watterson and Vanaman used calmodulin-affinity chromatography to obtain highly purified calcineurin fractions from bovine brain extract. The first purification of calcineurin is attributed to Klee and Krinks (1978). Klee *et al.* (1979) established the term “calcineurin” when subsequent studies had demonstrated the Ca^{2+} /calmodulin binding properties of calcineurin and the abundance and localisation of this protein in bovine neuronal tissue. The biochemical properties of calcineurin were revealed in studies of enzymatic extracts (protein phosphatase 2, PP2) capable of dephosphorylating the α and β -subunits of phosphorylase kinase (Stewart *et al.*, 1982). A purified fraction (PP2B) from rabbit skeletal muscle was shown to copurify with calcineurin in bovine brain extracts and based on subunit composition, it was concluded that PP2B and calcineurin were identical proteins (Stewart *et al.*, 1983). To summarise, calcineurin is a Ca^{2+} /calmodulin dependent protein phosphatase corresponding to protein phosphatase type 2B, which belongs to one of four classes of serine/threonine-specific protein phosphatases present in mammalian tissue (Cohen, 1989).

1.5.2 Calcineurin Structure

The heterodimeric structure of the calcineurin enzyme was recognised in early studies (Wang & Desai, 1977; Klee & Krinks, 1978). Mammalian purified calcineurin consists of calcineurin A, a 57 – 59 kD catalytic subunit and calcineurin B, a 19 – 20 kD regulatory subunit. The two subunits are tightly bound and can only be disassociated under denaturing conditions (Klee *et al.* 1988). The size and tissue localisation of the catalytic and regulatory subunits is dependent upon the isoform. In mammalian tissues calcineurin A α and A β have a ubiquitous distribution, the A γ isoform is specifically located in the testis. There are two isoforms of calcineurin B, B α is ubiquitously distributed whereas B β is testis specific (reviewed in Sugiura *et al.*, 2001).

Calcineurin has been extensively cloned in evolutionary divergent organisms, with sequences available for yeast, fungi, insect, mouse, rat and human (reviewed in Guerini 1997, Hemenway & Heitman 1999, Rusnak & Mertz 2000). The calcineurin B subunit is highly conserved between evolutionary distant organisms and varies from 19 to 20 kD in size. The predicted amino acid sequence of mammalian calcineurin B is 86% homologous with insect (*Drosophila melanogaster*) and 54% identical with yeast (*Saccharomyces cerevisiae*). The size of the catalytic subunit (calcineurin A) is more variable, from 57 – 59 kD in mammals to 57 – 71 kD in lower eukaryotes. The murine calcineurin A predicted amino acid sequence is 54% identical with yeast (reviewed in Hemenway & Heitman 1999). Despite sequence dissimilarities, the functional domains of the calcineurin A subunit are highly conserved throughout all eukaryotic species (reviewed in Rusnak & Mertz 2000).

1.5.3 Calcineurin Inhibition

Liu *et al.* (1991) identified calcineurin as the common target of two novel naturally occurring immunosuppressive ligands, cyclosporin A (CsA) and FK506 (or tacrolimus). Separate microorganisms produce CsA and FK506: *Hypocladium inflatum gams*, a Norwegian soil fungus, and *Streptomyces tsukubaensis*, a Japanese soil bacterium. Borel of Sandoz Pharmaceuticals (now Novartis) first described the immunosuppressive activity of CsA in 1976. The reduced toxicity and morbidity of CsA lead to approval for clinical applications in 1983 and subsequent widespread use this immunosuppressant in prevention of allograft rejection (reviewed in Kiani *et al.*, 2000). CsA prevents expression of cytokine genes including the interleukin-2 (IL2) and IL4 genes in activated T-lymphocytes (Kronke *et al.* 1984, Herold *et al.*, 1986). Kino *et al.* (1987) of Fujisawa demonstrated a similar immunosuppressive activity in FK506 to CsA, but 100 times more potent (reviewed in Allison, 2000).

Liu *et al.* (1991, 1992) established the mechanism of immunosuppression of CsA and FK506. These immunosuppressant ligands bind ubiquitous intracellular proteins, cyclophilin and FKBP12, known collectively as immunophilins (immunosuppressant binding proteins). The immunophilin-immunosuppressant complex binds to calcineurin with high affinity to potently inhibit calcineurin activity. CsA and FK506 also blocked calcium-dependent nuclear translocation of a transcription factor, nuclear factor of T-cells (NFAT), which is essential for an effective immune response (Flanagan *et al.* 1991). The connection between calcineurin and activation of T-lymphocytes was made when overexpression of calcineurin in Jurkat cells rendered them less susceptible to the immunosuppressive effects of CsA and FK506, activated the IL2 promoter and enhanced expression of the NFAT transcription factor (Clipstone & Crabtree, 1992; O'Keefe *et al.*, 1992). In

summary, the binding of the immunophilin-immunosuppressant complex to calcineurin blocks dephosphorylation of the cytosolic component of NFAT, thus inhibiting nuclear translocation and transcription of cytokine genes (reviewed in Rao *et al.*, 1997, Horsley & Pavlath 2002; Hogan *et al.* 2003). More recently, cytoplasmic calcineurin binding protein (cabin1; Sun *et al.*, 1998), calcineurin inhibitor (CAIN; Lai *et al.*, 1998) or myocyte-enriched calcineurin interacting proteins 1, 2 and 3 (MCIP1, 2 and 3; reviewed by Rothermel *et al.*, 2003), have been shown to inhibit calcineurin activity.

1.5.4 Calcineurin/NFAT signaling

Antigenic activation of T-cell receptors leads to fluctuations in intracellular levels of calcium. The amplitude and duration of calcium signals controls the response of pro-inflammatory transcriptional regulators NF- κ B, c-jun N-terminal kinase (JNK) and NFAT (Flanagan *et al.* 1991; Dolmetsch *et al.*, 1997). The former two transcription factors are exclusively activated by a large spike in free intracellular Ca^{2+} levels, in contrast to NFAT activation through a low, sustained Ca^{2+} plateau. NFAT nuclear translocation and activation is prevented through CsA and FK-506 inhibition of calcineurin (Clipstone & Crabtree, 1992; O'Keefe *et al.*, 1992). A sustained elevation of intracellular Ca^{2+} levels elicited by ionomycin (a Ca^{2+} ionophore), selectively activates calcineurin, a downstream target of intracellular Ca^{2+} signaling, which leads to the dephosphorylation and nuclear translocation of NFAT1 and increased DNA binding (Shaw *et al.*, 1995). This effect is extremely sensitive to calcineurin activity, as the addition of immunosuppressants CsA and FK506 or withdrawal of ionomycin reverses the trend. NFAT1 is rephosphorylated, DNA binding activity is reduced and NFAT1 is exported back across the nuclear envelope (Loh *et al.*, 1996). Nuclear translocation of calcineurin is also observed despite

calcineurin's lack of nuclear localisation signal; cytosolic calcineurin is thought to complex with NFAT before transfer to the nucleus (Bossler *et al.*, 1993; Shibasaki *et al.*, 1996).

The five members of the NFAT family of transcription factors are closely related to the Rel/ NF- κ B family. NFAT5 is the primeval family member, present in the *Drosophila* genome, but is constitutively nuclear and activated by osmotic stress rather than calcineurin (Lopez-Rodriguez *et al.*, 1999; reviewed in Hogan *et al.* 2003). The other four NFAT genes are classified as follows: NFAT1 (p, c2); NFAT2 (c, c1); NFAT3 (c4); NFAT4 (x, c3) (reviewed in Kiani *et al.*, 2000). In unstimulated T-cells, NFAT transcription factors are phosphorylated and remain within the cytoplasm (Flanagan *et al.* 1991). Activated calcineurin dephosphorylates a serine rich region in the amino terminus of NFAT, which unmasks the nuclear localisation signal and results in nuclear import (Beals *et al.*, 1997). Upon nuclear translocation, NFAT transcription factors bind cooperatively with another transcription factor, AP-1 (Fos/Jun), at NFAT: AP-1 composite elements. Alternatively, NFAT can bind as a dimer to DNA elements that resemble NF- κ B sites (reviewed in Hogan *et al.*, 2003). It was proposed that rephosphorylation of NFAT4 by a 'vigorous nuclear NF-AT kinase' is counteracted by the maintenance of a calcineurin/NFAT4 nuclear complex (Shibasaki *et al.*, 1996). Reduction in the level of intracellular Ca^{2+} results in rephosphorylation of NFAT proteins by glycogen synthase kinase-3 (GSK-3), nuclear export and cessation of NFAT mediated transcription (Beals *et al.*, 1997). Translocation of the NFAT protein to the cytoplasm is also the result of the exportin protein Crm1 binding to the nuclear export signal (NES) on NFAT. The maintenance of a non-catalytic association between calcineurin and the NFAT protein at binding sites clustered in close proximity to the Crm1 binding site, masks the NES and

prevents repeated cycling of NFAT across the nuclear membrane (Zhu & McKeon, 1999).

The ubiquity of calcineurin isoforms in all body tissues (reviewed by Sugiura *et al.*, 2001), the abundance of two NFAT isoforms in cardiac and skeletal muscle (NFAT2 and NFAT4; Hoey *et al.*, 1995), the inhibition of skeletal muscle cell differentiation through CsA administration (Hardiman *et al.*, 1993; Abbott *et al.*, 1998) and a connection between myopathy and cyclosporin treatment in transplant patients (reviewed by Briel & Chariot, 1999) would suggest that calcineurin/NFAT signaling is not restricted to B and T-lymphocytes. The subsequent discovery of calcineurin at the heart of cardiac and skeletal muscle hypertrophic growth processes has led to intense interest in the calcineurin signaling pathway in the field of muscle research (Molkentin *et al.*, 1998; Musaro *et al.*, 1999; Semserian *et al.*, 1999).

1.5.5 Calcineurin signaling in cardiac muscle

Coronary heart disease is the primary cause of morbidity and mortality in the Western hemisphere, accounting for over 120,000 individuals in the United Kingdom and 500,000 in the USA each year (British Heart Foundation, 2003; NHLBI, 2002). Cardiac hypertrophy is caused by intrinsic and extrinsic pathologic stimuli including hypertension, myocardial infarction, cardiac arrhythmias, valvular disease, endocrine disorders and congenital mutations in the sarcomeric proteins (reviewed in Olson & Molkentin, 1999). In the first instance, cardiac hypertrophy is a beneficial compensatory mechanism that enhances cardiac output, but sustained enlargement of the myocardium results in cardiomyopathy, heart failure, decompensation and sudden death. The typical response of cardiomyocytes to hypertrophic stimuli involves enlargement of cells, accumulation of sarcomeric proteins and myofibrillogenesis. 'Fetal' gene expression is recapitulated in the myocardium; developmental isoforms

of actin and myosin (α -skeletal actin and β -MHC) are expressed at the expense of the corresponding adult isoforms (α -cardiac actin and α -MHC) and there is re-expression of atrial and b-type natriuretic peptides, markers of cardiac hypertrophy (reviewed in Sugden, 1999; Olson & Molkentin 1999;, Olson & Williams 2000).

In 1882, Ringer demonstrated Ca^{2+} was a necessary component of the external solution to elicit contraction in an isolated frog heart; more recently, intracellular Ca^{2+} was identified as a potential second messenger in cardiac hypertrophy (reviewed in Schwartz & Mercadier, 1996). Agonists such as angiotensin II (AngII), phenylephrine (PE) and endothelin-1 (ET-1) stimulate the hypertrophic response in cardiomyocytes and also serve to increase the intracellular concentration of Ca^{2+} . In working heart preparations mechanical stimuli such as stretch and increased workload induce higher intracellular levels of Ca^{2+} (reviewed in Molkentin *et al.*, 1998). Abnormalities in myocardial calcium homeostasis are associated with heart failure (reviewed in Balke *et al.*, 1998). A nuclear association was identified between dephosphorylated NFAT3 and the zinc-finger transcription factor GATA4, which activated transcription of b-type natriuretic peptide, a marker of cardiac hypertrophy. The sustained plateau in intracellular Ca^{2+} levels and the involvement of a known calcineurin substrate (NFAT3), lead to speculation that the Ca^{2+} /calmodulin dependent phosphatase calcineurin was responsible for transduction of hypertrophic stimuli in the heart (Molkentin *et al.*, 1998).

This hypothesis seemed to be borne out by subsequent *in vitro* and *in vivo* studies. The typical suite of hypertrophic responses observed in cultured cardiomyocytes treated with humoral factors AngII and PE was prevented by treatment with CsA and FK506 (Molkentin *et al.*, 1998). In cultured neonatal rat cardiomyocytes, the same hypertrophic agonists (AngII and PE) and 1% fetal bovine

serum induced calcineurin enzymatic activity, through increased expression of calcineurin A β mRNA and protein (Taigen *et al.*, 2000). Transfection of cultured cells with an adenovirus expressing CAIN, a non-competitive peptide inhibitor of calcineurin (Lai *et al.*, 1998), or AKAP79, the calcineurin inhibitory domain, reduced calcineurin enzymatic activity and prevented cardiomyocyte hypertrophy (Taigen *et al.*, 2000).

In a murine model that paralleled human heart disease, CsA and FK506 prevented fatal cardiac hypertrophy in transgenic animals that expressed constitutively active forms of calcineurin or NFAT3 in the heart (Molkentin *et al.*, 1998). In an alternative model, hypertrophic cardiomyopathy (HCM) developed as the result of sarcomeric dysfunction induced by abnormal expression of tropomodulin, myosin light chain-2, or fetal β -tropomyosin in the hearts of transgenic mice. Administration of calcineurin inhibitors CsA and FK506 prevented development of HCM in this murine model (Sussman *et al.*, 1998). CsA treatment also attenuated some but not all aspects of cardiac hypertrophy in a transgenic mouse model of cardiomyopathy, induced by overexpression of constitutively active G α_q protein in cardiomyocytes (Mende *et al.*, 1998).

Molecular mechanisms that control left ventricular hypertrophy (LVH), an important adaptive response to pressure overload, are studied using conventional genetic and haemodynamic overload rodent models. LVH develops in the spontaneously hypertensive rat (SHR) after five weeks of age or can be induced in other strains by aortic banding (Zhang *et al.*, 1999). In several studies, CsA treatment was shown to inhibit or diminish the effects of pressure overload in rats and mice (Sussman *et al.*, 1998; Meguro *et al.*, 1999; Lim *et al.*, 2000; Øie *et al.*, 2000). Typically, aortic-banded rats showed an increase in heart-to-body weight ratio by as

much as 34% over a 14-day period; CsA administration significantly attenuated this increase (Lim *et al.*, 2000). Treatment with CsA after the initial 14 days for a further 14-day period demonstrated that pressure-overload hypertrophy of the left ventricle was reversible (Lim *et al.*, 2000). The inhibition of LVH by CsA was not always advantageous, as prevention of this adaptive response lead to an increased likelihood of decompensation and heart failure (Meguro *et al.*, 1999; Øie *et al.*, 2000). Endurance training can also induce beneficial non-pathological LVH and increased left ventricular mass is commonly observed in highly trained athletes (reviewed by Maron, 1986). Development of LVH in response to voluntary exercise training in rats (group mean 2.4 km.d⁻¹ for 10 weeks) was prevented by administration of CsA (Eto *et al.*, 2000).

Heart failure patients displayed elevated protein levels (up to 4-fold) of activated calcineurin A in left ventricular heart samples relative to normal patients (Lim & Molkenin, 1999). Increased calcineurin enzyme activity (over 3-fold) and association of the catalytic subunit (calcineurin A) with calmodulin was also displayed in pressure-overloaded hearts in aortic-banded rats (Lim *et al.*, 2000). These findings inspired intense speculation that coronary heart disease could be treated through pharmacological inhibition of the calcineurin pathway, using already well-characterised immunosuppressants CsA and FK506 (Barinaga, 1998; Izumo & Aoki, 1998). However a substantial body of literature provided evidence to the contrary.

In the late 1980s, post-transplant treatment with cyclosporin was associated with systemic hypertension and LVH in humans (McKoy *et al.*, 1988) and an increase in heart-to-body weight ratio in rats (Sadeghi *et al.*, 1987). In contrast to the other findings in the study, CsA treatment in mice did not prevent cardiomyopathy induced

by cardiospecific overexpression of a constitutively activate retinoic acid receptor (Sussman, *et al.*, 1998). Moreover, two preliminary studies (Luo *et al.*, 1998; Müller *et al.*, 1998) demonstrated intervention with CsA or FK506 had little effect in rodent models of LVH, in stark contrast to previously described studies utilising similar experimental designs. This result was repeated in subsequent experiments using aortic-banded rodents; mRNA expression of atrial natriuretic factor, a marker of cardiomyocyte hypertrophy, was elevated in CsA treated and untreated groups (Ding *et al.*, 1999; Zhang *et al.*, 1999). Furthermore, there was no difference in heart-to-body-weight ratio in CsA treated and non-treated groups in the spontaneously hypertensive rat model (Zhang *et al.*, 1999). Calcineurin enzymatic activity was diminished in aortic-banded animals relative to controls and further depressed in the CsA treated group (Ding *et al.*, 1999). Using a similar experimental approach, Tsao and co-workers (2000) followed up a previous study of calcineurin A (CnA) protein activity in left ventricle tissue samples from heart failure subjects (Lim & Molkenin, 1999). Transcripts of CnA α and CnA β were decreased and there was proportionally less full-length CnA protein present in failing human hearts (Tsao *et al.*, 2000).

Several counterarguments to all of these findings have been proposed, including alternative splicing of CnA α and CnA β transcripts (Lim & Molkenin, 2000), differences in the time course of experiments using rodent models, the use of different strains of laboratory animals as well as the positioning and severity of the aortic constriction (Molkenin, 1998; Walsh, 1999; reviewed by Molkenin 2000). There is also the question of the ability of systemically delivered CsA to inhibit the large quantities of calcineurin present in the myocardium (reviewed by Olson & Williams, 2000). Overall, however, these results suggest that a single pathway coordinating the adaptive hypertrophic response in the myocardium to diverse stimuli

is unlikely. Molkenin (1998) suggested that calcineurin signaling was a parallel pathway involved in the adaptive response to haemodynamic stress. In all likelihood “many central regulatory pathways interconnect or cross talk with one another in the orchestration of the hypertrophic response” (Molkenin 2000). Alternative signaling factors include mitogen-activated protein kinases (MAPKs) and calcium/calmodulin dependent kinases (CamK), AngII receptors and specific G- α proteins (Molkenin, 2000). Phenylephrine and serum induced hypertrophy of cardiomyocytes is associated with activation of myocyte enhancer factor-2 (MEF2) transcription factors. Maximal activation of MEF2 is facilitated by MAPK phosphorylation of the DNA binding domain, which can only occur after CaMK signaling dissociates inhibiting class II histone deacetylases from this region (Lu *et al.*, 2000a). Sugden (1999) first proposed that cross talk between calcineurin and MAPK pathways (p38, JNK, extracellular signal-regulated kinases – ERKs) regulated the hypertrophic response in cardiomyopathy. CsA has recently been found to inhibit p38 and JNK as well as calcineurin (Matsuda & Koyasu, 2003), which would seem to negate part of that argument, considering cardiac hypertrophy was still observed in rodent pressure overload models with CsA intervention. An alternative approach to pharmacological inhibition utilised doubly transgenic mice that were engineered with cardiospecific overexpression of calcineurin and human myocyte-enriched calcineurin-interacting protein-1 (hMCIP1) (Rothermel *et al.*, 2001). Expression of hMCIP1 prevented symptoms of cardiomyopathy previously linked with expression of a constitutively active form of calcineurin in the heart. Hypertrophic responses associated with β -adrenergic receptor stimulation or exercise training were also inhibited by expression of the hMCIP1 transgene (Rothermel *et al.*, 2000). In a recent review, Rothermel *et al.* (2003) suggest a modulatory role for MCIPs1-3, functioning as an endogenous

feedback regulator of calcineurin activity. The role of calcineurin in cardiomyopathy remains ambiguous, however it is unlikely that a single pathway regulates this process; further work is required to elucidate the function of this signal transduction pathway in association with a multitude of other regulatory pathways, in regulating hypertrophy of the human heart.

1.5.6 Calcineurin signaling in skeletal muscle hypertrophy

Administration of CsA to cardiac transplant patients prevents allograft rejection, but can cause well-documented side effects such as nephrotoxicity, hepatotoxicity and hypertension (Ventura *et al.*, 1997). CsA therapy has also been reported to cause muscle disorders in transplant patients; cessation of CsA treatment reverses the symptoms of myopathy and muscle weakness (Goy *et al.*, 1989; Fernandez-Sola *et al.*, 1990; Grezard *et al.*, 1990; Arellano & Krupp, 1991). In two of these case studies, muscle tissue biopsies revealed type-2 muscle fibre atrophy and ultrastructural abnormalities, implying CsA induced toxic or non-specific myopathy (Goy *et al.*, 1989; Fernandez-Sola, 1990). Subsequently, CsA was found to inhibit differentiation in human myoblast cell cultures (Hardiman *et al.*, 1993; Abbott *et al.*, 1998). Further *in vitro* and *in vivo* studies on rats demonstrated adverse effects of CsA treatment on skeletal muscle mitochondrial respiration, reduced endurance exercise capacity and reduced capillarity and oxidative capacity of limb musculature, which may account for impaired exercise performance in transplant patients (Hokanson *et al.*, 1995; Mercier *et al.*, 1995; Biring *et al.*, 1998; reviewed by Briel & Chariot 1999). These findings implied CsA treatment inhibited normal calcineurin function in skeletal muscle and potential conservation of hypertrophic growth regulatory pathways in cardiac and skeletal muscle. In addition to skeletal muscle hypertrophic signaling, calcineurin has been identified as a critical component in three

other processes that will be discussed subsequently: 1) nerve- and activity-stimulated muscle fibre phenotypic adaptation, 2) skeletal muscle differentiation and 3) determination of primary muscle fibre number during development.

An *in vitro* model of insulin-like growth factor 1 (IGF-1) mediated hypertrophic growth was utilised to illuminate the underlying molecular mechanisms in skeletal muscle hypertrophy. Semsarian *et al.* (1999) induced hypertrophy and transformation of myofibre phenotype to a glycolytic metabolism in IGF-1 treated post-mitotic C2C12 mouse myotubes. Administration of IGF-1 activated calcineurin through mobilisation of free intracellular Ca^{2+} , resulted in dephosphorylation and nuclear translocation of NFAT2. Calcineurin activation and glycolytic fibre-type transformation was also observed in rat latissimus dorsi muscle injected with a plasmid encoding stable localised IGF-1 expression. The hypertrophic response of myotubes in cell culture was prevented by administration of calcineurin inhibitors CsA and FK506, thus implicating calcineurin in skeletal muscle hypertrophy. Musaro *et al.* (1999) induced hypertrophy in differentiated rat L6E9 myocytes by forced expression of non-circulating muscle specific IGF-1. The calcineurin signal transduction cascade was initiated by an accumulation of calcineurin A transcripts in IGF-1 treated myocytes and predominantly nuclear localisation of calcineurin protein. Transfection of L6E9 cell culture with a constitutively active form of calcineurin paralleled the effect of IGF-1 treatment. In contrast, myocyte differentiation and the IGF-1 initiated hypertrophic responses were attenuated by treatment with CsA. Transcripts of the transcription factor GATA2 were detectable in IGF-1 treated myocytes but were not observed in CsA treated cohorts, suggesting activated calcineurin or IGF-1 mediated GATA2 expression. A subsequent *in vivo* study linked GATA2 expression with muscle fibre hypertrophy through muscle-restricted

expression of IGF-1, suggesting GATA2 as a marker of skeletal muscle hypertrophy (Musaro *et al.*, 2001). Calcineurin, GATA2 and NFAT2 accumulate and are found complexed in a proportion of muscle fibre nuclei. This combination synergistically initiated a programme of gene expression leading to hypertrophy. The association of GATA2, NFAT2 and calcineurin underscored the parallel between skeletal muscle hypertrophy and hypertrophic signaling in cardiomyopathic growth, involving transcription factors GATA4 and NFAT3 (Musaro *et al.*, 1999; Molkentin *et al.*, 1998).

Dunn *et al.* (1999) were the first to demonstrate *in vivo* a potential molecular signaling pathway linking increased muscle usage to skeletal muscle hypertrophy. In the hind limb musculature of the rat, the plantaris muscle was overloaded by surgical removal of the gastrocnemius and soleus muscles. Compensatory functional overload by ablation of synergists results in rapid hypertrophy of the plantaris muscle as a whole, outgrowth of individual muscle fibres and conversion of muscle fibre phenotype from fast-to-slow. Pharmacological blockade of calcineurin signaling through administration of powerful immunosuppressant drugs cyclosporin A and FK506 inhibited the adaptive hypertrophic response *in vivo*. The level of calcineurin in whole muscle extracts from vastus intermedius muscle tissue was highly correlated with muscle mass in rats (Spangenburg *et al.*, 2001). The effect of CsA treatment on maintenance of muscle mass and recovery from atrophy induced by hindlimb suspension, differs depending upon muscle phenotype (Mitchell *et al.*, 2002). In both situations, CsA administration more severely affected the fast-twitch plantaris than the slow-twitch soleus muscle. This implied fast phenotypic muscle growth is entirely calcineurin-dependent whereas slow phenotypic growth occurs through calcineurin-dependent and independent pathways.

In one of the first studies to imply a potential role for calcineurin signaling in the growth of skeletal muscle, distinct nuclear localisation of NFAT isoforms was observed at different stages of myogenesis: nuclear translocation of NFAT4 was only observed in myoblasts, NFAT1 and NFAT2 were present only in myotube nuclei (Abbott *et al.*, 1998). Horsley *et al.* (2001) investigated the role of NFAT4 in skeletal muscle growth using NFAT4 null mice. They demonstrated that during growth of multinucleate myocytes, outgrowth of myotubes and myonuclear accretion is under the control of an NFAT4-regulated pathway. Overall, these data implied a fundamental role for the calcineurin signal transduction pathway in mammalian skeletal muscle growth processes, however scepticism was rife among several research groups as to the role of calcineurin signaling in skeletal muscle hypertrophy.

Tissue-restricted constitutively active calcineurin was expressed in transgenic mice under the control of a muscle creatine kinase enhancer (Naya *et al.*, 2000). No evidence was found for transgene generated skeletal muscle hypertrophy compared to wild-type littermates, despite a 10-fold overexpression of activated calcineurin. Musaro *et al.* (2001) developed an *in vivo* model of IGF-1 induced hypertrophy in transgenic mice by coupling the muscle specific IGF-1 isoform to a myosin light chain promoter (MLC/mIGF-1). Transgene expression was limited to predominantly fast-twitch skeletal muscles such as the thigh triceps or gastrocnemius and increased wet mass of these muscles was observed in transgenic adults compared with wild-type counterparts due to hypertrophy of the type IIb fibres. Administration of calcineurin inhibitor CsA for three weeks did not attenuate MLC/mIGF-1 induced hypertrophic growth in transgenic mice. Musaro and co-workers (2001) proposed *in vivo* compensation through alternative multiple regulatory pathways, such as calmodulin

kinase or phosphatidylinositol 3-kinase (PI(3)K), rescued the hypertrophic response despite CsA treatment.

Two research groups repeated the original *in vitro* and *in vivo* experiments (Semsarian *et al.*, 1999; Musaro *et al.* 1999; Dunn *et al.* 1999) that first identified a key role for calcineurin in skeletal muscle hypertrophy. Rommel *et al.* (2001) observed that addition of IGF-1 to differentiated myotubes at a dose sufficient to cause hypertrophy did not result in nuclear translocation if NFAT2 and CsA treatment did not attenuate IGF-1 stimulated hypertrophy. Moreover, neither pharmacological stimulation by addition of a Ca²⁺ ionophore nor genetic activation through constitutive expression of activated calcineurin were adequate to induce hypertrophy in differentiated myotubes. Bodine *et al.* (2001), using the same compensatory muscle hypertrophy model as Dunn *et al.* (1999), demonstrated that calcineurin activity did not increase in response to functional overload of the rat hind limb musculature and intervention with CsA or FK506 could not prevent the adaptive hypertrophic response. Both papers dismissed the involvement of the calcineurin signaling pathway in regulating hypertrophy of differentiated myotubes and proposed an alternative pathway initiated by PI(3)K and PI(3)K-regulated protein kinase B (Akt). Downstream targets/effectors of PI(3)K/Akt signaling include mTOR (mammalian target of rapamycin), and glycogen synthase kinase 3 β (GSK3 β). Akt mediated phosphorylation of mTOR and GSK3 initiates molecular signaling cascades resulting in upregulation of protein synthesis.

The PI(3)K/Akt pathway was upregulated *in vitro* and *in vivo* by IGF-1 or increased weight bearing hypertrophic stimuli and downregulated during muscle atrophy. Pharmacological blockade of PI(3)K through LY294002 completely blocked IGF-1 induced hypertrophy of myotubes (Rommel *et al.*, 2001). Inhibition of the

more downstream mTOR through rapamycin intervention was less effective, but still diminished myotube hypertrophy (Rommel *et al.*, 2001). Rapamycin treatment almost completely attenuated adaptive hypertrophy in functionally overloaded plantaris muscle fibres and prevented recovery of fibre size during a reloading period after hindlimb suspension (Bodine *et al.*, 2001). Genetic deactivation through expression of kinase-inactive Akt or stimulation through a constitutively active Akt construct resulted in atrophied or hypertrophied myotubes respectively (Rommel *et al.*, 2001). Genetic activation through expression of constitutively active Akt induced muscle fibre hypertrophy *in vivo* and prevented atrophy after denervation; the hypertrophic response was prevented by rapamycin administration (Bodine *et al.*, 2001). Finally, genetic blockade of this pathway by overexpression of an Akt inhibitor (SHIP-2) also prevented *in vivo* adaptive hypertrophy.

Further evidence disassociating the calcineurin-signaling pathway from skeletal muscle growth was presented in more recent studies. CsA intervention did not prevent recovery of muscle mass associated with beneficial interventions (intermittent reloading or exercise) after hind limb suspension or spinal cord transection (Dupont-Versteegden *et al.*, 2002). Calcineurin inhibitors (CsA, FK506 and CAIN/cabin1) did not attenuate nerve activity dependent increases in fibre size in regenerating rat soleus muscle (Serrano *et al.*, 2001). Stimulation of hypertrophy and prevention of denervation atrophy through expression of constitutively active expression of Akt or by a Ras double mutant that selectively activated the PI(3)K/Akt pathway, was completely inhibited by rapamycin treatment (Pallafacchina *et al.*, 2002). Rapamycin treatment or transfection of innervated regenerating muscle fibres with a kinase-inactive mutant of Akt caused incomplete inhibition of nerve activity dependent growth. This finding implied that alternative nerve-activity reliant

PI(3)K/Akt independent signaling pathways mediate skeletal muscle growth (Pallafacchina *et al.*, 2002).

Dunn *et al.* (2002) refuted the claims of Bodine and colleagues (2001) and postulated that the lower drug (CsA) dosage and different treatment regime used by this research group was not sufficient to inhibit hypertrophy *in vivo*. Moreover, the extensive calcineurin-mediated dephosphorylation of NFAT2 and MEF2 observed during overload (Dunn *et al.*, 2001) suggested that it would be 'premature to rule out a function for calcineurin in skeletal muscle growth' (Dunn *et al.*, 2002). In response, Yancopoulos & Glass (2002) proposed that the inhibition of hypertrophy after four weeks in Dunn and co-workers' original study was more consistent with non-specific toxicity of the calcineurin inhibitors used and that immunosuppressant treatment was insufficient to prevent hypertrophy induced by two weeks of functional overload. The role of calcineurin in skeletal muscle hypertrophy remains ambiguous; the calcineurin and PI(3)K/Akt pathways may act in parallel to effect muscle fibre hypertrophy in different physiological contexts or may be distinct regulators of skeletal muscle phenotype and hypertrophy respectively.

1.5.7 Calcineurin signaling in skeletal muscle fibre-type specification

It is well established that there are fundamental differences in neuromuscular activity and intracellular Ca^{2+} levels in different muscle fibre phenotypes (reviewed by Pette & Vrbova, 1999; Talmadge, 2000). Low frequency tonic motor nerve activity (10 – 15 Hz) is associated with slow muscle gene expression, induction of slow fibre phenotype and prolonged elevation of intracellular calcium concentration to between 100 and 300 nM (Chin & Allen, 1996). Fast fibre phenotype results from high frequency phasic firing of the motor nerve, with extended periods of quiescence. Transient, high amplitude ($\sim 1 \mu\text{M}$) spikes in free intracellular Ca^{2+} concentration are

associated with bursts of neural activity and resting Ca^{2+} levels are generally low (50 nM) (Westerblad & Allen, 1991). Fast-to-slow switches in muscle fibre phenotype can be induced by chronic low frequency stimulation of the motor nerve (Williams *et al.*, 1986), treatment of myotubes in cell culture with a calcium ionophore to elevate the basal intracellular level of Ca^{2+} (Kubis *et al.*, 1997), cross-innervation or exercise training (reviewed by Talmadge, 2000). The differences observed in intracellular Ca^{2+} concentration between myofibre phenotypes and the ability of calcineurin to distinguish between transient and prolonged elevations of intracellular Ca^{2+} (Dolmetsch *et al.*, 1997), inspired Chin and co-workers (1998) to investigate a putative role of calcineurin in fibre-type specification.

Transfection of C2C12 skeletal muscle myotubes with a constitutively active form of calcineurin was shown to upregulate slow-fibre-specific gene promoters of myoglobin and troponin I slow (TnIs). CsA administration inhibited this effect. Calcineurin-mediated transactivation of slow-fibre-specific gene expression was further implied by the identification of NFAT binding motifs within the promoter regions of myoglobin and TnIs. Combinatorial interactions between NFAT and other transcription factor proteins, such as MEF2, were identified as a requirement for optimal calcineurin-stimulated myoglobin expression. CsA administration to intact animals blocked endogenous calcineurin activity and promoted fast-to-slow fibre-type conversions in the soleus of mature rats. This challenged the findings of Biring and colleagues (1998) that showed no alteration in MHC expression in response to clinically relevant doses of CsA. The molecular mechanism proposed by Chin *et al.* (1998) connected neuromuscular activity with fibre-type specific gene expression. Tonic motor nerve activity, typical of neurons innervating slow myofibres, causes a sustained increase of intracellular Ca^{2+} and activation of calcineurin.

Dephosphorylated NFAT is translocated to the myonuclei, where it combines with other downstream mediators to bind promoter regions controlling the cassette of slow fibre-specific expression. Neuromuscular activity in fast myofibre subtypes is not sufficient to activate calcineurin, thus NFAT remains phosphorylated and located within the cytoplasm and fast-fibre-specific gene transcription occurs in the absence of the slow-fibre-specific program. These findings attracted intense interest, not least because of the potential to use calcineurin-signaling as a therapeutic tool to manipulate patterns of gene expression in skeletal muscle and improve quality of life in heart failure patients and sufferers of Duchenne's muscular dystrophy (Chin *et al.*, 1998). However, direction of myofibres towards a slow phenotype can occur independently of DNA binding by NFAT. Elimination of NFAT DNA consensus elements in slow or oxidative fibre-specific promoter/enhancer regions did not completely abrogate calcineurin transcriptional regulation or fibre type-specific gene expression, suggesting an alternative means through which skeletal muscle can respond to calcineurin-dependent signaling (Chin *et al.*, 1998; Calvo *et al.*, 1999). MEF2 proteins have been shown to fulfil this transactivating function in skeletal myocytes through synergistic activation by calcineurin and CaMK (Wu *et al.*, 2000a). In transgenic mice, calcineurin-mediated dephosphorylation of MEF2 in response to neuromuscular stimulation or expression of constitutively active calcineurin was required for upregulation of a slow-fibre-specific enhancer (Wu *et al.*, 2000a). Therefore, MEF2 and NFAT are downstream effectors of the calcineurin-signaling pathway that determine skeletal myofibre phenotype in response to motor nerve stimulation.

Additional studies emphasised the role of calcineurin in determining muscle fibre phenotype. Expression of constitutively active calcineurin in transgenic mice

caused an increase the number of muscle fibres expressing slow MHC (MHC-I) and increased transcript number of slow fibre-specific genes in the gastrocnemius (myoglobin, TnIs and sarcomeric mitochondrial creatine kinase) (Naya *et al.*, 2000). Muscle fibre phenotype transition from slow MHC-I to fast oxidative MHC-IIa was observed in the soleus but not the plantaris muscle of CsA treated rats (Bigard *et al.*, 2000). Calcineurin-mediated myogenic differentiation was found to specify a slow muscle specific phenotype by slow MHC expression (Delling *et al.*, 2000).

Chronic low frequency electrostimulation or Ca^{2+} ionophore treatment of rabbit skeletal muscle myocytes stimulated fast-to-slow conversion of muscle fibre phenotype and nuclear import of NFAT2; CsA treatment attenuated slow MHC-I expression but did not reverse downregulation of MHC-II_{d/x} or affect expression of MHC-II_a or MHC-II_b, which implied calcineurin signaling regulates expression of slow but not fast isoforms of MHC (Meißner *et al.*, 2001). CsA-sensitive nuclear translocation of NFAT2 was observed in response to typical slow fibre patterns of electrical stimulation of cultured adult mouse myotubes; NFAT2 nuclear import was neither induced by an intermittent high frequency fast-twitch pattern of electrical activity nor continuous very low frequency (1 Hz) stimulation (Liu *et al.*, 2001). Continuous low frequency stimulation of rabbit myotubes (1 – 10 Hz) for ≥ 5 minutes in a 45-minute cycle was sufficient to induce nuclear translocation of NFAT2, upregulation of MHC-I mRNA and conversion of muscle fibre phenotype (Kubis *et al.*, 2002). The rest period of ≤ 40 minutes was not sufficient for complete nuclear export of NFAT2, leading to nuclear accretion of NFAT2 and transduction of the electrical stimulus to effect fibre-phenotype transition (Kubis *et al.*, 2002).

In vivo administration of calcineurin inhibitors (CsA/FK506/CAIN-cabin1) prevented upregulation of a MHC-I promoter in regenerating rat soleus muscle and

increase of MHC-I mRNA in response to continual low frequency electrostimulation (Serrano *et al.*, 2001). Furthermore, it was proposed that calcineurin maintains the slow muscle fibre gene expression program because CAIN intervention induced fast-to-slow MHC isoform transformation in adult rat soleus muscle. Recently, *in vitro* Ca^{2+} ionophore application has been shown to activate the MHC-IIa promoter to a greater extent than promoters of more glycolytic isoforms of MHC (MHC-IIb and MHC-IIc/x) (Allen & Leinwand, 2002). Treatment of myotubes with calcineurin or CaMK inhibitors, severely reduced activation of the MHC-IIa promoter, whereas overexpression of constitutively active calcineurin upregulated MHC-IIa protein expression. Consensus binding sites in the promoter region of MHC-IIa for downstream calcineurin effectors MEF2 and NFAT2 were required for Ca^{2+} induced activation of the MHC-IIa promoter. These findings identified calcineurin as a regulator of fast oxidative myosin heavy chain (MHC-IIa) expression in addition to initiating and maintaining the slow muscle fibre program (Allen & Leinwand, 2002; Serrano *et al.*, 2001).

Another potential target of calcineurin signaling in the determination of skeletal muscle phenotype is peroxisome-proliferator-activated receptor- γ co-activator-1 (PGC-1 α), which activates mitochondrial biogenesis and oxidative metabolism (Lin *et al.*, 2002). Transgene expression of PGC-1 α has been shown to induce fast-to-slow conversions of MHC-II fibres, raise expression of typical slow fibre proteins (myoglobin and TnIs), and increase resistance to fatigue. Cell culture experiments demonstrated that PGC-1 α and MEF2 cooperate to initiate transcription and PGC-1 α is a target of calcineurin signaling. These findings suggest that PGC-1 α is another important downstream effector of calcineurin-signaling in specialisation of muscle fibre type (Lin *et al.*, 2002).

In summary, the calcineurin-signaling pathway appears to be a convincing candidate for nerve or activity dependent specification of muscle fibre phenotype. The identification of the Ras/MAPK(ERK) signaling pathway in nerve-activity dependent differentiation of slow muscle fibres (Murgia *et al.*, 2000) suggests that fibre-type determination is under control of multiple regulatory pathways. Further investigation is required to identify potential cross talk between pathways and completely elucidate this process.

1.5.8 Calcineurin signaling in myogenesis

Mammalian skeletal muscle differentiation is characterised by commitment to a myogenic lineage, withdrawal from the cell cycle, phenotypic differentiation and cell fusion to form myotubes. Treatment of human myoblasts with CsA was shown to inhibit differentiation (Hardiman *et al.*, 1993). CsA exhibited a dose-dependent inhibition of human myoblast differentiation, indicated by abrogation of embryonic MHC expression, decreased creatine kinase activity and fewer multinucleated myotubes (Abbott *et al.*, 1998; Rommel *et al.*, 2001). Further observations strengthened the case for a pivotal role in myogenic differentiation for calcineurin: increased endogenous calcineurin activity and NFAT4 nuclear translocation were associated with myogenic differentiation, whilst transfection of cultured myocytes with a constitutively active form of calcineurin or addition of CsA or CAIN, potentiated or attenuated differentiation respectively (Delling *et al.*, 2000; Friday *et al.*, 2000). Calcineurin inhibitors arrested differentiation at the commitment stage but did not affect proliferation (Abbott *et al.*, 1998; Friday *et al.*, 2000).

Members of the bHLH MyoD family of transcription factors (MRFs) were identified as downstream effectors of calcineurin mediated-differentiation. Forced expression of constitutively active calcineurin strongly upregulated myogenin, a

process that is NFAT-independent (Friday *et al.*, 2000). Treatment of myotube cell cultures containing mononucleated 'reserve' cells with the Ca^{2+} ionophore ionomycin resulted in increased Myf5 transcripts and protein in the mononucleated cell fraction (Friday & Pavlath, 2000). Treatment of myoblast cultures with CsA or CAIN attenuated Myf5 mRNA expression, whereas overexpression of NFAT2 increased Myf5 transcripts. This led to the conclusion that calcineurin and its downstream target NFAT2 regulate Myf5 expression in satellite cell-like reserve cells (Friday & Pavlath, 2000). Further examination has uncovered an increasingly complex model of calcineurin-mediated differentiation in skeletal muscle, involving activation of MEF2 and MyoD (Friday *et al.*, 2003). The mechanism of activation by calcineurin has not been conclusively demonstrated, but may involve direct dephosphorylation of MEF2 (Wu *et al.*, 2000a) and indirect activation of MyoD through inhibition of Egr-1, an upstream regulator of MyoD-inhibitory protein Id (Friday *et al.*, 2003). MyoD and MEF2 bind essential elements in the myogenin promoter to facilitate differentiation. Therefore Friday and co-workers (2003) proposed calcineurin is a major regulator of skeletal muscle differentiation through the initiation of upstream transcription factors MEF2 and MyoD and subsequent induction of myogenin expression.

The NFAT4 transcription factor has been associated with the determination of primary fibre number during development; adult NFAT4 null (NFAT4^{-/-}) mice have an overall reduction in muscle mass and myofibre number, the losses not confined to a particular fibre type or related to alterations in phenotypic proportions (Kegley *et al.*, 2001). Secondary myofibres develop normally; therefore NFAT4 null mutants reveal a function for this transcription factor in early myogenesis.

To summarise, calcineurin is a highly conserved protein phosphatase enzyme whose functions in higher vertebrates include regulation of skeletal and cardiac

muscle hypertrophy, nerve- and activity-dependent muscle adaptation, regulation of skeletal muscle differentiation and determination of primary myofibre number. The role of calcineurin signaling in skeletal muscle hypertrophy is currently controversial but cannot be dismissed without further work.

1.6 Myogenic and MEF2 regulatory factors

1.6.1 Myogenic regulatory factors

During ontogeny, the pool of cells from which skeletal muscle develops is specified prior to somitogenesis. These cells can be identified by their expression of transcription factors belonging to the MyoD/basic helix-loop-helix (bHLH) and MADS (MCM1, agamous, deficiens, serum response factor)-box families (reviewed by Brand-Saberi & Christ, 1999). Four distinct but closely related members of the myogenic regulatory factor (MRFs) family of bHLH transcription factors have been found to play a major role in skeletal muscle formation and are highly conserved between humans (Braun *et al.*, 1990) birds (Pownall & Emerson, 1992) amphibians (Gurdon *et al.*, 1992) and fish (Rescan, 1997). The MRF family consists of four members that are specifically expressed in skeletal muscle: MyoD, myf5, myogenin and myf6 (Megeny & Rudnicki, 1995; Rudnicki & Jaenisch, 1995). The MRFs belong to a superfamily of transcription factors that share a common bHLH motif; the helix-loop-helix region interacts with ubiquitous gene products of the E2A family (such as E12 and E47) to form heterodimers and the basic domain facilitates binding of the heterodimeric complex to consensus E-box DNA binding elements (CANNTG) common to many muscle-specific promoters such as desmin, troponin I and muscle creatine kinase (Davis *et al.*, 1990; Olson 1992).

Ectopic expression of MRFs in non-muscle cells such as 10T1/2 fibroblasts demonstrated the striking ability of each MRF to initiate the myogenic program and produce myogenic derivatives from a variety of non-muscle cells (Weintraub *et al.* 1989; Choi *et al.*, 1990). A clearer definition of the roles of the individual MRFs has been provided by gene ablation experimentation. Double knockout mice embryos lacking the *myf5* and *MyoD* genes were devoid of myoblasts and differentiated skeletal muscle, no myogenic markers were evident and myogenin was not transcribed (Rudnicki *et al.*, 1993; reviewed by Weintraub, 1993). In myogenin null mice, formation of myoblasts occurs but differentiation does not occur *in vivo*, leading to severe muscle deficiency and perinatal death in homozygous mice (Hasty *et al.*, 1995). Mice carrying a homozygous mutation in the *myf6* gene displayed malformations in rib development and increased myogenin expression. It was proposed that *myf6*, as the predominant MRF in adult skeletal muscle, regulates skeletal muscle maturation and downregulates myogenin expression in postnatal muscle development (Zhang *et al.*, 1995).

Developmental expression of MRFs occurs before myotome formation, with *MyoD* and *myf5* the first members of this family expressed in avian and mammalian embryos respectively (Buckingham, 1994). *MyoD/myf5* are the first transcription factors expressed in all known skeletal muscle lineages, followed by myogenin and lastly *myf6* (reviewed by Yun & Wold, 1996). The temporospatial segregation of MRF expression suggests distinct roles for each MRF family member during myogenesis (Zhang *et al.*, 1995). The determination of proliferating muscle progenitor cells is controlled by the primary MRFs, *MyoD* and *myf-5*; subsequent differentiation, maturation and fusion of myoblasts to form multinucleated myotubes is controlled by the secondary MRFs, myogenin and *myf6* (Megenev & Rudnicki,

1995; Rudnicki & Jaenisch, 1995). Expression of myogenin is considered to be the earliest marker of differentiation in myocytes, MyoD/myf5 are markers of determination in skeletal muscle progenitors.

1.6.2 MEF2 transcription factors

For stable commitment of cells to a myogenic fate and for muscle differentiation to occur, MRF proteins must interact with a second important group of transcription factors, belonging to the MADS box family (Molkentin & Olson, 1996). In mammals, there are four distinct MEF2 proteins in this group (MEF2A – MEF2D), which contain the characteristic extreme N-terminus MADS-box DNA binding domain adjacent to the highly conserved MEF2-specific region (reviewed by Black & Olson, 1998). The MEF2 proteins form homo- or heterodimers and bind an A/T-rich element present in the promoter/enhancer regions of many muscle-specific genes (Gossett *et al.*, 1989). In contrast to MRFs, MEF2 factors alone are not sufficient to activate myogenesis, however combinatorial interactions between MEF2 and MRF proteins at the MADS box and bHLH domains have been shown to augment this process (Molkentin *et al.*, 1995; Molkentin & Olson, 1996). Co-expression of full-length MEF2C with the bHLH region of myogenin efficiently activates myogenesis in 10T1/2 fibroblasts, in contrast to a lack of myogenic activity observed with expression of the abbreviated myogenin alone (Molkentin *et al.*, 1995).

1.6.3 Regulation of skeletal muscle differentiation

Post-translational control of MRF activity occurs through numerous positive and negative regulatory pathways, involving interactions with factors such as bHLH inhibitory proteins Id and Twist, Notch/Delta cell-cell signaling family members, a family of cysteine-rich proteins (CRPs) characterised by the presence of a LIM double

zinc finger domain and pax-3 (reviewed by Yun & Wold, 1996; Rescan, 2001). Pax-3 is thought to initiate the induction of myogenesis in vertebrate limb muscle development. Results from targeted gene knockout experiments suggest pax-3 acts genetically upstream of bHLH genes, to confer cells in the developing embryo with muscle forming capabilities (reviewed by Ludolph & Konieczny, 1995). In higher vertebrates there are four Id proteins (Id1 – Id4) that regulate myogenesis by sequestration of bHLH transcription factors, preventing DNA binding and induction of differentiation (reviewed by Norton *et al.*, 1998). The interaction between bHLH transcription factors and members of the family of CRPs was identified as a crucial stage of muscle differentiation (Kong *et al.*, 1997; Krempler & Brenig, 1999).

Recently, MRFs and MEF2 factors have been implicated as downstream targets of numerous intracellular signaling pathways regulating differentiation, including PI(3)K/Akt, MAPK, CaMK and calcineurin-mediated pathways. The role of the calcineurin pathway in differentiation and specification of muscle fibre phenotype by direct activation of MEF2, indirect MyoD activation, induction of myf5 expression and myogenin transcription has been discussed in a previous section (Friday *et al.*, 2000; Friday & Pavlath, 2000; Wu *et al.*, 2000a; Friday *et al.*, 2003).

Proliferation and differentiation of cultured myoblasts can be induced by insulin-like growth factors (IGFs), which have the ability to positively manipulate underlying myogenic molecular mechanisms, including upregulation of myogenin mRNA (reviewed by Florini *et al.*, 1996). IGF-1 is also necessary for normal embryonic development in mice (Powell-Braxton *et al.*, 1993). The PI(3)K/Akt signaling pathway is stimulated by IGFs, which has the effect of increasing MyoD and MEF2 transcriptional activity and binding to the myogenin promoter region, leading to transcriptional induction of myogenin (Xu & Wu, 2000). Rapid activation

of p38, a MAPK, accompanies *in vitro* differentiation of myocytes induced by serum withdrawal. Inhibition of p38 prevents differentiation in myogenic cells lines and human primary myocytes, whereas enhanced endogenous p38 activity results in increased myotube formation and expression of myogenic markers (Wu *et al.*, 2000b). Furthermore, p38 enhances transcriptional activities of MyoD, MEF2A and MEF2C, by direct dephosphorylation of MEF2 factors and indirect activation of MyoD (Wu *et al.*, 2000b). As discussed previously, binding of class II histone deacetylases (HDACs) to MEF2 factors prevents their activation through MAPK-mediated phosphorylation and participation in cardiac hypertrophy (Lu *et al.*, 2000a). An interaction between the MEF2 DNA binding domain and HDACs has been identified as an inhibitor of skeletal muscle myogenesis (Lu *et al.*, 2000b). Execution of the skeletal muscle differentiation program requires disassociation of the inhibitory HDACs from MEF2 factors, which is effected by CaMKV signaling (McKinsey *et al.*, 2000). Recently, the p38 MAPK, CaMK and calcineurin intracellular signaling pathways have been identified as parallel but necessary participants in transcriptional regulation of myogenin during skeletal muscle myogenesis (Xu & Wu, 2000; Wu *et al.*, 2000b). Myogenin expression is attenuated or abolished by inhibition of any one of these signaling pathways.

To summarise, MyoD family and MEF2 transcription factors are fundamentally important in the regulation of skeletal muscle differentiation. Myogenic differentiation is an extremely complex process, and multiple non-redundant intracellular signaling pathways tightly control the activity and expression of these regulatory factors. In addition to fulfilling a key function in early muscle development, expression of myogenic regulatory factors persists into adulthood and

MRFs are thought to participate in fibre-type specification and muscle fibre hypertrophy in mature skeletal muscle tissue.

1.6.4 Fibre-type specification by myogenic regulatory factors

The differing levels of MRFs observed in phenotypically distinct fibre types provide evidence that MRFs potentially fulfil a role in maintaining fibre type diversity. Moreover, alterations in MHC expression observed during muscle fibre type transitions occur in tandem with shifts in MRF expression. In the rat hindlimb, MyoD transcripts preferentially accumulate in fast-twitch muscles with myogenin primarily observed in slow-twitch muscles; within a single muscle, MyoD and myogenin mRNA localisation correlates with fast glycolytic and slow oxidative fibres respectively (Hughes, *et al.*, 1993; Voytik *et al.*, 1993). The same pattern of expression was observed for MyoD and myogenin proteins, with myf5 preferentially accumulated in fast type musculature (Sakuma *et al.*, 1999). Manipulation of muscle fibre phenotype by cross-reinnervation or thyroid hormone administration caused parallel changes in MRF expression (Hughes, *et al.*, 1993). After denervation-induced phenotypic changes of fast-type muscles, a rapid upregulation of all four MRFs was observed; a 150 to 200-fold increase in myogenin transcripts within 7 days was most notable (Voytik *et al.*, 1993). In further studies, MyoD protein nuclear localisation and accumulation of MyoD transcripts was correlated with the fastest subclass of muscle fibre type (MHC Iib-type), which implied development and maintenance of fast Iib fibres is a function of MyoD (Hughes *et al.*, 1997; Kraus & Pette, 1997). Correlation does not equal causation however, as demonstrated by the subsequent failure of electrostimulation or hypothyroidism to induce corresponding changes in the pattern of MRF expression (Kraus & Pette, 1997). More recently, forced overexpression of myogenin in fast-type Iib fibres resulted in a subtle shift

towards an oxidative metabolism but no transition in the predominant MHC expressed (Hughes *et al.*, 1999). Furthermore, the post-exercise effects of a single bout of heavy-resistance training demonstrated a correlation between upregulation of myogenin transcripts and protein and increased levels of type I and type IIa MHC mRNA; a similar relationship was observed in the level of MyoD mRNA and protein and expression of type IIb MHC transcripts (Willoughby & Nelson, 2002). Overall these results suggest MRFs play some part in determining muscle fibre characteristics, but most likely as the downstream targets of multiple recently identified intracellular signaling pathways (Wu *et al.*, 2000a & 2000b).

1.6.5 MRFs and muscle growth through hypertrophy

The concept of a finite myonuclear domain and satellite cells as the external source of nuclei required for the outgrowth of terminally differentiated myofibres is well established (reviewed by Allen *et al.*, 1979; Campion, 1984). Proliferation and terminal differentiation of myogenic precursor cells in response to injury or hypertrophic stimuli is characterised and regulated by sequential expression of members of the MyoD family of transcription factors, which is used as an index of the myogenic response to perturbation (Rosenblatt, 1994; Smith *et al.*, 1994). Numerous studies describe changes in expression of MRFs in mature skeletal muscle in response to a diverse range of stimuli including forced exercise, surgically induced compensatory overload, stretch overload, electromyostimulation and denervation. For each stimulus, several case studies will be examined to evaluate the contribution of MRFs in mature skeletal muscle growth.

MyoD, myogenin and myf6 transcripts are all transiently upregulated in human adult skeletal muscle within 24 hours of a single episode of heavy resistance training (Psilander *et al.*, 2003). A similar response was observed in myogenin

mRNA expression in rat muscles exposed to resistance training, however a greater overall myogenic response was observed with a 48-hour rest period between training sessions (Haddad & Adams, 2002). MyoD and myogenin transcripts were significantly upregulated in the tibialis anterior and extensor digitorum longus muscles of the rat hindlimb, 3 hours after a bout of repeated eccentric contractions (Peters *et al.*, 2003). Furthermore, where downhill treadmill running was used to induce eccentric contractions of the rat hindlimb soleus muscle, increased numbers of mononuclear cells positive for transcripts of primary MRFs MyoD and myf5 were observed (Armand *et al.*, 2003). Results from these studies suggest unambiguously that MRFs are involved in the adaptive response of mature skeletal muscle to exercise activity.

Surgical removal of synergistic muscle groups in the rat hindlimb is a standardised laboratory model of functional overload used to induce compensatory hypertrophy in the remaining weight-bearing muscle (see Adams *et al.*, 1999; Dunn *et al.*, 1999; Sakuma *et al.*, 1999). Examination of the myogenic response to functional overload has given ambiguous indications regarding involvement of MRFs in this process. The onset of compensatory hypertrophy of the plantaris muscle was accompanied by a transient upregulation of MyoD and myogenin mRNA expression 12 to 24 hours post-surgery (Adams *et al.*, 1999). This is in contrast to results presented by Sakuma and co-workers (1999), who described an initial downregulation of MyoD, myogenin and myf5 proteins in the first 8 days of mechanical overloading, followed by a period of overcompensation up to 28 days post-surgery. In a third study, functional overload of the soleus muscle did not display any marked changes in expression of MyoD or myogenin transcripts (Mozdziak *et al.*, 1988). These results appear contradictory, but across all three studies there are differences in the

parameters measured or the functional overload model used. Overall however, MRFs may have a significant role to play in compensatory hypertrophy of fast muscles such as the plantaris, but have less effect in the adaptive response of slow muscles such as the soleus.

The effects of alternative stimuli are more straightforward: elevated myogenin mRNA levels are present in the chicken anterior latissimus dorsi (ALD) muscle across the entire experimental time course, peaking 6 days after initiation of stretch-induced hypertrophy (Carson & Booth, 1998). An upregulation of MyoD, myogenin and myf5 transcripts was observed in the same slow tonic ALD muscle of quail after three days of static stretch stimulation (Lowe & Alway, 1999). A combination of static stretch and chronic low frequency electrostimulation of the tibialis anterior muscle of adult rats for a 2-hour period resulted in a transient signal, where levels of myf5 and myf6 mRNA increased significantly and remained high for the following 30 minutes, before returning to basal levels by 20 hours post-activity; there was no noticeable induction of myogenin message within the experimental time course (Jacobs-El *et al.*, 1995). Using an experimental design first pioneered with rats, two 30-minute bouts of electromyostimulation separated by a 48-hour rest period were shown to increase the levels of myogenin transcripts in both able bodied and spinal cord-injured subjects (Bickel *et al.*, 2003). Motor denervation in rats had previously been shown to cause a gradual downregulation of MyoD and myf5 proteins, concurrent with atrophy of the denervated muscles (Sakuma *et al.*, 1999). Finally, recovery of muscle function after damage caused by femoral artery ligation in mice hind limbs is marked by induction of MyoD expression on the first day after removal of the obstruction (Paoni *et al.*, 2002). The findings of these experiments suggest that the MRFs play a pivotal role in adaptation of skeletal muscle to increased workload or recovery from an injurious

event, a characteristic that is retained into adulthood and can still be reactivated after extended periods of quiescence (Bickel *et al.*, 2003).

1.6.6 Myogenic and MEF2 regulatory factors in lower vertebrates

At the time of writing, a search of the online TrEMBL protein database (<http://us.expasy.org>) revealed numerous amino acid sequences available for teleost MRFs and MEF2 transcription factors, reflecting the recent explosion in sequencing technology and bioinformatics. In teleosts, the temporospatial expression of MRFs has largely been described in relation to early developmental regulation of myogenesis (Kobiyama *et al.*, 1998; Delande & Rescan, 1999). The relatively high levels of MyoD, myf5, myogenin and MEF2C observed in neonatal common carp declined after the first month, to give only a weak signal in 7 month old juveniles (Kobiyama *et al.*, 1998). The high level of expression is thought to be associated with a phase of sustained fibre recruitment in the juvenile carp. In rainbow trout two nonallelic forms of MyoD are observed (termed TMyoD and TMyoD2), which are thought to have arisen as the result of a genome duplication event in the evolutionary history of salmonids (Rescan & Gauvry, 1996). The two MyoD genes have evolved separate functions as shown by distinct temporospatial expression in the developing embryo and restriction of TMyoD/TMyoD2 expression to different muscle fibre types (Delande & Rescan, 1999). Multiple isoforms of MEF2 have been found in lower vertebrate species, for example MEF2A, MEF2C and MEF2D in zebrafish (*Danio rerio* Hamilton) (Ticho *et al.*, 1996). In common carp, juvenile expression of MEF2A coincides with downregulation of MEF2C and MRFs, implying that the comparative importance of these factors changes during development (Kobiyama *et al.*, 1998).

The expression of MRFs in mature teleost skeletal muscle is less well studied, however there are several examples of increased expression of myogenic factors in

response to alterations in feeding regime. A sub-antarctic notothenioid was acclimated to typical summer or winter photoperiod and temperature conditions and the myogenic response to a single feeding event was evaluated (Brodeur *et al.*, 2003b). The proportion of myogenic progenitor cells positive for MyoD protein and proliferating cell nuclear antigen (PCNA) was significantly increased in both groups. Co-expression of PCNA and MyoD in myogenic progenitor cells is a marker of myogenic cell activation (Yablonka-Reuveni & Rivera, 1994) and gives an indication of the level of nuclear proliferation in response to a given stimulus, in this case a recent feeding event (Brodeur *et al.*, 2003b). An increase in myogenin positive cells followed shortly thereafter in the group of notothenioids acclimated to simulated winter conditions. In rainbow trout subjected to a 10-week period of fasting, myogenin mRNA was upregulated in the fast myotomal muscle 12 days after a refeeding event (Chauvigné *et al.*, 2003). These findings imply there is a role for MRFs in mature myotomal muscle of fish, in the mobilization and differentiation of myogenic progenitor cells to recover muscle mass and function after a fasting period and also during normal muscle growth in response to a feeding event. Based on previous data on MRFs expression in mammalian overload models, one would also predict the MRFs to play a role in the compensatory muscle growth response observed in teleosts subjected to chronic exercise.

1.7 Myostatin

1.7.1 Myostatin – a negative regulator of muscle growth

Myostatin, a recently identified member of the transforming growth factor- β (TGF- β) superfamily, is also known as growth/differentiation factor 8 (GDF-8).

Expression of myostatin in mammals is muscle specific, restricted to the developing somites in the myotome during early development and expressed in numerous muscle groups in adults (McPherron *et al.*, 1997; Sharma *et al.*, 1999). In myostatin null mice, the weight of individual muscles is up to three fold greater than wild-type littermates, due to a combination of increased muscle fibre number and muscle fibre hypertrophy; these findings identified myostatin as a putative negative regulator of skeletal muscle mass (McPherron *et al.*, 1997).

Myostatin is initially synthesised as a 375 amino acid propeptide (unprocessed full-length protein, 52 kD) that in common with other members of the TGF- β family has an amino-terminal secretory signal and a bioactive domain at the conserved carboxy terminus. The propeptide is cleaved at a conserved RXXR region (amino acids 263 – 266) giving rise to the N-terminal latency associated peptide (LAP, 42 kD) and a 26 kD active processed peptide (Sharma *et al.*, 1999). The amino acid sequence of the myostatin active peptide is highly conserved across species, with an almost 100% similarity amongst higher vertebrates and an 88% similarity between the human and zebrafish peptides, suggesting an evolutionarily conserved function (McPherron & Lee, 1997). Three breeds of double-muscled cattle, Belgian Blue, Piedmontese and Asturiana de los Valles, have been shown to possess mutations in the active peptide region that result in the synthesis of an aberrant or truncated protein product; for instance the Belgian Blue myostatin sequence has an 11-base pair deletion that effects a translational frameshift and complete loss of function in the myostatin active peptide (Kambadur *et al.*, 1997; McPherron & Lee, 1997; reviewed by Kocamis & Killefer, 2002). The resulting double-muscled phenotype, first described at the start of the 19th century (Culley, 1807), occurs primarily as a consequence of increased fibre number. This phenotype is reminiscent of that

observed in a murine mutant, homozygous for a disrupted version of the myostatin active peptide (Kambadur *et al.*, 1997). However, it should be noted that mutations in the functional myostatin protein are found in three other breeds of cattle, in which the double-muscling phenotype is not observed; this suggests dysfunction of one major gene may not entirely account for double-muscling in cattle (reviewed by Kocamis & Killefer, 2002).

1.7.2 Myostatin function

In normal skeletal muscle, the myostatin gene is implicated in controlling the proliferation and differentiation of myoblasts. In cell culture experiments, the presence of myostatin prevents the transition of myoblasts from G1 to the S phase of the cell cycle. This is achieved through upregulation of p21 (an inhibitor of cyclin-dependent kinase, Cdk), subsequent loss of Cdk2 activity and accumulation of hypophosphorylated retinoblastoma protein, all of which are required for cell cycle withdrawal and differentiation (Thomas *et al.*, 2000; Joulia *et al.*, 2003).

Recently, an E-box motif in the myostatin promoter was identified that binds MyoD *in vitro* and *in vivo* (Spiller *et al.*, 2002). Activity in the myostatin promoter was highest in myoblasts during the G1 phase of the cell cycle at the peak of MyoD expression, however in quiescent reserve cells that lack MyoD, activity of the myostatin promoter was diminished (Spiller *et al.*, 2002). Overexpression of non-endogenous myostatin in myoblasts led to a decrease in MyoD protein (Joulia *et al.*, 2003). High levels of myostatin mRNA expression occur in parallel with increased MyoD transcripts during embryonic development in Belgian Blue cattle (Bass *et al.*, 1999). Overall, these findings suggest MyoD initiates withdrawal from the cell cycle by regulating myostatin gene expression (Spiller *et al.*, 2002). The “generalised muscular hyperplasia phenotype” is most likely a consequence of unconfined

proliferation of myogenic progenitors in animals lacking functional myostatin, leading to an increase in potential muscle mass in double-muscled cattle (Bass *et al.*, 1999; Thomas *et al.*, 2000).

Interestingly, the coding sequence for human myostatin contains five substitutions in conserved amino acid residues. Two of the substitutions are polymorphic in the general population, occurring in significantly different frequencies in Caucasians and African Americans (Ferrell *et al.*, 1999). Despite the higher frequency of these mutations correlating with higher muscle mass in African Americans, there was no significant racial difference in response to strength training (Ferrell *et al.*, 1999). In a follow-up study, the same group detected a trend in increased hypertrophic response to heavy resistance training in a limited number of female subjects with the less common myostatin allele (Ivey *et al.*, 2000). Several studies have sought to emphasise the inverse relationship between myostatin expression and muscle mass. Sexual dimorphism in mice was shown to be linked to differences in abundance of the mature processed myostatin peptide; body and muscle mass in male mice was 40% larger than female counterparts and was associated with a 40 – 60% lower level of active myostatin peptide (McMahon *et al.*, 2002). Increased serum levels of myostatin-immunoreactive peptide were correlated with muscle wasting as a consequence of HIV infection in adult men (Gonzalez-Cadavid *et al.*, 1998) and inversely correlated with lean mass in physically frail elderly women (Schulte & Yarasheski, 2001). In contrast, myostatin transcripts were downregulated by 37% in response to a 9-week heavy-resistance strength-training program (Roth *et al.*, 2003). However, attenuation of myostatin expression in this study was not correlated with increases in muscle volume or strength and suggested that further

work is required to confirm a causal link between myostatin expression and muscle hypertrophy/atrophy.

This viewpoint is further strengthened by observations regarding myostatin expression during hindlimb unloading and muscle atrophy in rodents. Wehling and co-workers (2000) reported 110% and 37% increases in levels of myostatin mRNA and protein respectively, in conjunction with atrophy of rat hindlimb musculature induced by a 10-day period of unloading. Daily 30-minute episodes of reloading during the unloading period were sufficient to ameliorate muscle atrophy usually associated with this treatment, however myostatin transcripts were still elevated by 55% through this treatment. Furthermore, hindlimb suspension in myostatin null mice resulted in a greater loss of muscle mass than in wild-type contemporaries. This was contrary to the expectation that the atrophic effect of hindlimb suspension would be ameliorated by a lack of a functional myostatin protein (McMahon *et al.*, 2003). Myostatin undoubtedly fulfils a function in negative regulation of skeletal muscle growth, yet the exact role remains ambiguous and further work will be required to fully elucidate myostatin function and upstream regulation of this growth/differentiation factor.

1.7.3 Myostatin in lower vertebrates

Recently, a substantial body of work has emerged concerning the functional role of myostatin in commercially important teleost species, suggesting there is optimism among aquaculturalists that identification of myostatin polymorphisms and subsequent genetic improvement could be utilised to increase flesh yield from farmed fish. The first non-mammalian orthologues to be identified were from the tilapia and white bass (*Morone chrysops* Rafinesque) and the predicted precursor protein sequences contained 376 and 377 amino acid residues respectively (Rodgers, *et al.*,

2001). In common with other members of the TGF- β family, teleost myostatin contains the consensus RXXR proteolytic site, nine conserved scattered cysteine residues and when processed gives rise to a conserved C-terminal 13 kD bioactive monomer (Rodgers, *et al.*, 2001; reviewed by Johnston *et al.*, 2003a).

Since the initial cloning and sequencing of myostatin in tilapia and white bass, orthologues have been described in numerous teleost species (reviewed by Johnston *et al.*, 2003a), with duplicate myostatin isoforms described in one perciforme and several salmonid species (Maccatrozzo *et al.*, 2001; Østbye *et al.*, 2001; Rescan *et al.*, 2001; Roberts & Goetz, 2001). Higher predicted amino acid identity was found in the salmonid orthologues (90 – 93%) than in the gilthead seabream (*Sparus aurata* L.) (68%). The two versions of myostatin are thought to have arisen in teleosts as the result of an ancient genome duplication event (Rescan *et al.*, 2001). Unlike mammals, teleost myostatin expression occurs in a wide range of tissue types, for example in the rainbow trout Tmyostatin I is ubiquitously expressed, while Tmyostatin II expression is restricted to the muscle and brain (Rescan *et al.*, 2001). Tissue-specific expression of duplicate teleost myostatin orthologues parallels expression of mammalian myostatin in muscle and GDF-11, a highly related factor expressed only in neural and eye tissue (see Maccatrozzo *et al.*, 2001; Østbye *et al.*, 2001). It was proposed that subsequent to genome duplication, myostatin isoforms evolved separately and adopted distinct functions, a possibility that has previously been considered in the case of TMyoD1 and 2 orthologues in rainbow trout (Delande & Rescan, 1999). Differential expression of duplicate myostatin orthologues implies that the functional role of myostatin may not be restricted to regulation of muscle growth in fish, but may fulfil multiple roles in diverse tissue types (Østbye *et al.*, 2001; Roberts & Goetz, 2001).

Recent studies have confirmed a role for myostatin during skeletal muscle development in zebrafish (Vianello *et al.*, 2003) and demonstrated myostatin mRNA expression is affected by fasting in larval and adult stages of tilapia (Rodgers *et al.*, 2003). These findings have been welcomed by the aquaculture industry in particular, as they seek to maximise fish somatic and muscle growth through manipulation of myostatin expression.

1.8 Summary of project aims and *a priori* hypotheses

The broad aim at the outset of this study was to develop a reproducible model of muscle fibre hypertrophy in teleosts. The model would then be used to elucidate putative molecular signaling pathways regulating muscle growth and to examine the effect of training on muscle fibre phenotype in common carp. A more detailed examination of each objective is outlined below:

- 1) The fast white musculature is the dominant tissue type in teleost fish. Endurance exercise training (slow, moderate and fast) has been shown to stimulate fast muscle fibre hypertrophy in numerous species from four separate families of teleosts (see section 1.4). Based on previous findings, hypertrophy of fast muscle fibres would be expected in response to endurance exercise (slow to moderate) in juvenile common carp and rainbow trout (5 to 15 cm fork length). The primary objective of the project was to develop a model of exercise-induced fast muscle fibre hypertrophy in common carp and rainbow trout, two commercially important teleost species that are commonly used in aquaculture research.

- 2) Recruitment and hypertrophy of slow and intermediate muscle fibres have been demonstrated for several cyprinid species in response to endurance exercise training (see section 1.4.6). Growth of slow and/ or intermediate muscle fibres through hypertrophy and/ or recruitment would be expected in common carp subjected to sustained periods of endurance exercise training. The second aim of the project was to examine the effect of slow to moderate endurance exercise on the growth of the slow/ red and where identified intermediate/ pink musculature in common carp.

- 3) Expression of myosin heavy chain (MHC) isoforms can be manipulated by endurance exercise in mammals (see section 1.3.1). Endurance exercise drives MHC isoform expression towards the type I (slow/ aerobic) fibre phenotype and switches in muscle fibre phenotype occur. Seasonal temperature fluctuations have been shown to affect MHC isoform expression in common carp. If the teleost response to endurance exercise parallels that of higher vertebrates, an increased fraction of aerobic muscle fibres (slow and intermediate) would be observed per myotomal cross-sectional area. The third aim was to evaluate the effect of moderate endurance exercise on muscle fibre phenotype in common carp.

- 4) Calcineurin has been implicated as a regulator of muscle growth and development in higher vertebrates and also in one invertebrate species (see section 1.5). Calcineurin is a ubiquitous highly conserved protein-phosphatase enzyme. In both yeast and humans, calcineurin regulates gene expression via dephosphorylation of key transcription factors in response to fluxes in

intracellular Ca^{2+} levels. The expected result of exercise-induced calcineurin activation would be increased nuclear localisation of calcineurin and NFAT2 proteins, and upregulation of MEF2 family transcription factors. The fourth objective of this study was to examine the potentially conserved role of calcineurin signaling in the regulation of muscle growth in common carp and rainbow trout.

- 5) Upregulation of MRFs is a known response to hypertrophic stimuli such as exercise in mammals (see Section 1.6). Manipulation of feeding regime has been shown to influence MRFs and PCNA expression in two teleost species. MRFs expression regulates the proliferation and differentiation of myogenic progenitor cells to provide the necessary nuclei for post-mitotic muscle growth. Therefore, an increase in MRFs and PCNA expression would be expected in the hypertrophic muscle growth response to endurance exercise in teleosts. MRFs expression has also been shown to control muscle fibre phenotype in mammals. The fifth objective of this project was to examine the potential roles of MRFs in the teleost response to endurance exercise and in fibre-type specification in non-exercised controls.
- 6) The role of myostatin (GDF-8) as a negative regulator of skeletal muscle mass is well known in higher vertebrates (see section 1.7). In mammals, down-regulation of myostatin expression has been demonstrated in response to a hypertrophic stimulus and an inverse relationship exists between myostatin expression and muscle mass. At the time of writing, myostatin has been cloned in numerous species of teleosts. If the inverse relationship holds true, then

myostatin expression will be downregulated as part of the adaptive response of exercise-induced fast fibre hypertrophy. The final objective of this project was to examine myostatin expression in response to endurance exercise in rainbow trout.

Chapter 2: Methodological development

2.1 Introduction

The broad aim of this project was to assess the impact of endurance exercise training on muscle growth, muscle fibre phenotype and muscle-specific gene expression in the common carp and rainbow trout. Before this could be examined in detail, it was first necessary to develop several experimental techniques. Muscle-specific gene expression was analysed using a standard western blotting protocol employing a range of commercial and custom-made antisera. The antibody screening section (section 2.2) provides a summary of the western blotting technique and details of the antisera used. Histological and immunohistological techniques were employed to identify different muscle fibre phenotypes in transverse cryosections of myotomal muscle tissue. The Mayer's haematoxylin, S58 and myofibrillar ATPase (mATPase) staining protocols are outlined in the histochemistry and immunohistochemistry section (section 2.3).

2.2 Antibody screening

2.2.1 Introduction

In higher animals, antibodies are produced by B-lymphocytes in response to an infection with foreign material such as a virus or bacteria. The antibody recognises and binds a particular site on the antigen, an antigenic determinant or epitope, and the immune response is initiated to neutralise the infection (reviewed by Harlow & Lane, 1988). An antibody is a complex of four polypeptides, two heavy chains and two

light chains, bound together to form a “Y” shaped molecule. The antibody structure comprises two distinct regions, the fragment antigen binding (Fab) component and the crystalline fragment (Fc). The Fab mediates antigen binding and includes the tips of the “Y”, whereas the Fc determines the nature of the immune mechanism used to destroy the foreign matter and is subdivided into 5 classes (IgM, IgG, IgA, IgD and IgE) (Harlow & Lane, 1988). Within the Fab region, there is a high level of sequence variability in the amino acids at the ends of the heavy and light chains at the tips of the “Y” and it is this variability that confers specificity to different antigens. The variable regions are made up of 4 framework (FR) regions and 3 hypervariable (HV) sites. The HV regions directly interact with the epitope of an antigen, the FR regions confer stability to the interaction. The epitope is a site on the antigen that the immune system responds to by making antibody and is frequently one unique structure on the antigen recognised by a particular HV site (reviewed by Harlow & Lane, 1988).

Manufacturing antibodies in laboratory animals is an important tool in life sciences research, and is used routinely for the detection of proteins, carbohydrates, complex lipids and nucleic acids (reviewed by Harlow & Lane, 1988). There are two categories of antibodies used in research that have various advantages and disadvantages: monoclonal and polyclonal antibodies. The immune response is characterised by the ability of an antibody to detect and bind to an antigen. The lymphocyte that originally synthesised the antibody is stimulated to grow and produce a line of clonal cells that have the capacity to produce more of that particular antibody. In the typical eukaryotic immune response, numerous antibodies detect distinct epitopes on a particular antigen, each antibody produced by a different group of cells. A polyclonal antibody is a heterogeneous mixture of antibodies produced by different groups of lymphocytes that target many different epitopes on the same

antigen. The major advantage of using polyclonal antibodies in research is that they are inexpensive to produce. However, the inherent variability in the immune response of laboratory animals results in different affinities to an antigen between different batches of a polyclonal antibody. As a consequence, immunological laboratory techniques require recalibration with every new batch of polyclonal antisera (reviewed by Maniatis *et al.*, 1989).

When an antibody is identified with desired specific binding properties, the cell (lymphocyte) it originated from can be cloned to allow production of large quantities of the antibody. A monoclonal antibody is produced by these means to give a homogeneous antiserum directed against one particular epitope of an antigen. The advantages of a monoclonal antibody are the high affinity for a particular target epitope and the associated increased reliability. However, screening for specific clones can be time consuming and production of monoclonal antisera is more costly (reviewed by Maniatis *et al.*, 1989).

Numerous companies specialise in the mass production of monoclonal and polyclonal antisera for biomedical research, such as Santa Cruz Biotechnology Inc. (CA, USA), a major supplier used as a source of antibodies in these experiments. However, commercial antibodies are mainly raised against antigens from higher vertebrate species and have a weak affinity for the same protein in a more distant evolutionary group such as teleosts. In this situation, often the best alternative approach is to use the available internet-based bioinformatics databases and tools to detect a short peptide region unique to the protein of interest and have a species-specific anti-peptide antibody manufactured. Numerous companies now offer a custom-made antibody service that includes the synthesis of the short amino acid sequence coupled to a highly immunogenic large carrier protein (bovine serum

albumin or keyhole limpet hemocyanin), administration of the peptide-carrier conjugate to a host mammal species (rabbit, donkey, goat etc.), monthly booster injections for a three month immunisation programme and affinity purification of the resulting antiserum using the original peptide. Although this approach may be preferable to using off the shelf anti-mammalian antisera, it is still prone to the inherent problems associated with the generation of antibodies, namely the variability of the immune response to the antigen in the host species.

The aim of the antibody screening process was to identify mammalian- and teleost-specific antibodies, which positively cross-react with key muscle-specific antigens that are potentially involved in the regulation of muscle growth in the common carp and rainbow trout. The commercial mammalian-specific antisera and teleost-specific anti-peptide antibodies were considered to be specific if the following criteria were satisfied: 1) the teleost protein detected was a similar molecular mass as the mammalian positive control; 2) the positive cross-reaction observed was in the predicted size range associated with use of that particular antiserum or similar to the molecular mass of the antigen entered in the ExPASy protein knowledgebase; and 3) there was some evidence of sequence homology between the antigen initially used to generate the antibody and the teleost protein of interest (or that of another teleost species, lower vertebrate species or invertebrate). This section will describe the affinity of ten mammalian-specific antibodies and three teleost-specific anti-peptide antisera for potential muscle-regulatory antigens from a range of teleost species.

2.2.2 Materials and methods

2.2.2.1 *Experimental animals*

Six species of fish and two mammalian species were used to screen several commercially available antibodies and three fish specific antipeptide antibodies. Two mice (*Mus musculus*) and two rats (*Rattus norvegicus*) were obtained from the School of Biology Animal House, University of St Andrews. Juvenile fish (range of fork lengths 7.5 – 15 cm) were obtained from the Gatty Marine Laboratory Main Aquarium, University of St Andrews. The species of fish sampled were common carp, rainbow trout, Atlantic salmon, Atlantic cod, Antarctic spiny plunderfish (*Harpagifer antarcticus* Forster) and yellowbelly rockcod (*Notothenia coriiceps* Richardson). The following abbreviations for experimental animals are used in this chapter as figure annotations: mouse (M), rat (R), common carp (C), rainbow trout (T), Atlantic salmon (S), Atlantic cod (G), Antarctic harpagifer (H) and sub-Antarctic notothenioid (N).

2.2.2.2 *Preparation of samples for protein analysis*

All specimens were sacrificed by the administration of an anaesthetic overdose (MS222, tricane methanesulphonate, SIGMA) and severing the spinal cord (Schedule 1 Killing, UK Home Office Regulations). Animals were packed on ice at the point of sacrifice and dissected in a 10°C cold room to reduce the chance of proteolytic breakdown. The time of sacrifice to the point of dissection was never longer than 1 hour. A new sterile scalpel was used for each dissection. In each fish species the skin was first removed, then small blocks (approximately 500mg) of muscle tissue were dissected from a region above the lateral line at the level of the dorsal fin. In the common carp distinct fast and slow tissue samples were also taken to screen common

carp specific antipeptide antibodies against both tissue types. These tissues were distinguished by colouration, as fast and slow fibre types contain different levels of myoglobin. In the common carp, fast muscle tissue was carefully dissected from a region distinct from the lateral line. Superficial layers of red muscle, probably mostly slow muscle tissue, were sampled by dissecting out the V-shaped notch of dark muscle tissue present at the major horizontal septum. The hind limb musculature of mice and rats was carefully dissected out, after first removing the skin. Tissue samples were placed in cryovials, snap frozen in liquid nitrogen and stored at -80°C until required.

2.2.2.3 Preparation of total cellular protein extracts

To extract total cellular proteins from muscle tissue samples, the RIPA protocol outlined in section 18.38 of 'Molecular Cloning: A Laboratory Manual' (Maniatis *et al.*, 1989) was followed. RIPA (**R**adioimmuno **p**rotection **a**ssay or **R**adioimmuno **p**recipitation **a**ssay) cell lysis buffer enables efficient solubilisation of proteins and most antigens are not adversely affected by the components of the buffer. Details of RIPA buffer solution and concentrations of protease inhibitors are found in Appendix I.

Approximately 1g of frozen muscle tissue was placed in a clean weigh boat, to which was added 3ml of ice-cold RIPA buffer containing protease inhibitors. The tissue was thoroughly minced using a clean razor blade, then transferred to a 14ml Falcon[®] tube (Becton Dickinson). To prevent excessive foaming, 100 μl of Antifoam A (SIGMA) was added to the tube. The tissue was then disrupted further using a Kinematica Polytron PT2100 homogeniser on medium speed (20,000 rpm) for 30 to 45 seconds (Kinematica AG, Switzerland). To prevent proteolytic degradation, the tube was held on ice throughout the homogenisation step to maintain a low

temperature (approx. 4°C). Each homogenate was incubated on ice for 30 minutes, divided equally between 2×1.5ml eppendorfs (1 – 1.5ml per tube), and centrifuged at 10,000×g for 10 minutes at 4°C (Sigma 3K15 centrifuge, fixed-angle rotor 12154, Sigma Laborzentrifugen, Germany). The supernatant was removed to a clean eppendorf and the centrifugation step was repeated to obtain a clarified cell lysate. The total cellular protein extract (clarified cell lysate) was stored at –80°C until required.

2.2.2.4 Preparation of nuclear protein extracts

The technique of Blough and co-workers (1999) was used to extract nuclear proteins from skeletal muscle tissue. Details of extraction buffers and protease inhibitor concentrations are given in Appendix I.

Frozen muscle tissue (approx. 500mg) was placed in a clean weight boat and 3ml of ice-cold Buffer 1 containing freshly prepared protease inhibitors was added. The frozen tissue was sliced thinly with a razor blade and transferred to a 14ml Falcon® tube (Becton Dickinson). 100µl Antifoam A (SIGMA) was added to prevent foaming and the tissue was disrupted using a Kinematica Polytron PT2100 homogeniser on medium speed (24,000 rpm) for 45 seconds (Kinematica AG, Switzerland). The homogenate was centrifuged at 3000×g for 8 minutes at 4°C (Sigma 3K15 centrifuge, fixed-angle rotor 12156, Sigma Laborzentrifugen, Germany). After discarding the supernatant, the pellet was resuspended in 1ml of Buffer 2 (containing protease inhibitors) and incubated on ice for 30 minutes with occasional mixing. The suspension was centrifuged as before. The resulting supernatant (500-1000µl) was transferred to a 4ml capacity Amicon Ultra® (Millipore) centrifugal filter device with a 10,000 nominal molecular weight limit

(MWCO). An equal volume of Binding buffer (containing protease inhibitors) was added and the filter device was centrifuged at 4500×g for 30 minutes at 4°C. After 30 minutes the volume was reduced by 30-50%. An equal volume of Binding buffer was again added and the centrifugation step repeated. The desalted and concentrated nuclear protein extract was removed from the upper chamber of the filter unit and stored at -80°C until needed.

2.2.2.5 The Lowry assay

The Lowry assay (Lowry *et al.*, 1951) is a general-purpose protein assay that was used to determine the protein concentration of total cell lysates and nuclear protein subcellular fractions. A standard curve was prepared using a 10mg/ml stock solution of Bovine Serine Albumen (BSA) in phosphate buffered saline (PBS). The BSA stock solution was diluted in the same buffer as the protein samples (RIPA buffer or Binding buffer) to prepare standards from 25 to 200µg. 10-20µl aliquots of total cellular protein extracts were diluted to 400µl with RIPA buffer, to ensure the protein concentration fell within the range of the standard curve. 50-100µl aliquots of nuclear protein extracts were diluted to 400µl with Binding buffer. Sample dilutions were prepared in duplicate. 400µl of the 2×Lowry concentrate was added to each sample, mixed thoroughly and incubated for 10 minutes at room temperature. 200µl of 0.2N Folin Ciocalteu's reagent was added, vortexed and incubated for 30 minutes at room temperature. Lowry Assay solutions are summarised in Appendix II. Each sample assay was transferred to a 1ml capacity plastic cuvette and the absorbance was measured at 750nm, first using the relevant buffer as a blank. Typically, total cellular protein extracts were in the range of 5-10µg/µl and nuclear subcellular fractions yielded a protein concentration an order of magnitude lower than total cell lysates, in

the 0.5-1 μ g/ μ l range. Total protein extracts were regarded as 'crude' extracts, whereas nuclear extracts were regarded as 'purified'.

2.2.2.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous Laemmli system (Laemmli, 1970) was used to resolve the complex mixtures of proteins in the total cellular and nuclear extracts. Basic technical instructions for preparing and pouring acrylamide gels were followed from 'Molecular Cloning: A Laboratory Manual' (Maniatis *et al.*, 1989). Resolving gel concentration was manipulated to resolve proteins of different molecular weights. 6% gels were used to separate proteins in the 100 – 200 kilodalton (kD) size range, 12% resolving gels were used to separate proteins in the 30 – 100 kD size range and 15% gels were used to resolve proteins below 30 kD. A stacking gel concentration of 5% was always used. 1mm thick gels were cast using Bio-Rad Mini-Protein[®] III apparatus. Details of solutions for preparing and running Tris-glycine SDS-PAGE gels are summarised in Appendices III and IV.

The concentration of protein loaded per lane for SDS-PAGE and subsequent immunoblotting techniques was 5 μ g of nuclear protein extracts and 20 μ g of total cell lysates. Each sample was diluted with an equal volume of 2 \times treatment (Laemmli) buffer, boiled at 95°C for 5 minutes and cooled for several minutes at room temperature before loading. A molecular weight standard was loaded in one lane of each gel for the antibody screening procedure, to ascertain the molecular weight of detected proteins. For proteins below 100 kD, Bio-Rad Low Range biotinylated SDS-PAGE Standards (161-0306) and Santa Cruz 'Cruz Marker' molecular weight standards (sc-2035) were used. The molecular weight markers are denoted as "Ma" where present in all figures in this chapter. The molecular weight of proteins in the

size range 100 – 200 kD was determined with Bio-Rad High Range biotinylated SDS-PAGE standards (161-0311).

Standardised gel electrophoresis conditions were used in each SDS-PAGE performed. 100V constant voltage (15mA per gel) was applied for 1.5 hours to resolve the proteins, or until the dye front was within 1cm of the end of the gel.

2.2.2.7 Coomassie brilliant blue G staining

The Coomassie Brilliant Blue staining technique (Fazekas de St Groth *et al.*, 1963; Mayer & Lamberts, 1965) was used to visualise the total cellular and nuclear protein extracts resolved on acrylamide gels. The technique was also used to ensure equal protein loading across all samples in semi-quantitative Western blotting techniques. Gels were extracted from the glass plates and the stacking portion of the gel was discarded. The resolving gel was stained with Coomassie Brilliant Blue G staining solution (0.025% (w/v) Coomassie Blue G, 40% (v/v) methanol, 7% (v/v) acetic acid) for 30 minutes at room temperature on a rocking platform. Excess background was removed by destaining and gels were washed for 30 minutes in destain solution 1 (40% (v/v) methanol, 7% (v/v) acetic acid), then soaked overnight in destain solution 2 (6% (v/v) methanol, 4% (v/v) acetic acid). After washing in several changes of distilled water, gels were photographed using the Bio-Rad Versadoc 3000 (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) system with a white light conversion screen. In the antibody screening process, this technique was used to assess the quality of total cellular and nuclear protein extracts.

2.2.2.8 Protein transfer

Proteins resolved by SDS-PAGE were transferred from the resolving gel to a PVDF transfer membrane (Hybond-P, Amersham Pharmacia Biotech) using a

standard laboratory technique (Towbin *et al.*, 1979). The resolving gel and pairs of scouring pads and thick filter papers were equilibrated for 10 minutes in pre-chilled transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH8.3, 4°C). Pre-wetting with methanol for 30 seconds activated the PVDF membrane. The membrane was washed several times in PBS to elute the methanol, and was then equilibrated in pre-chilled transfer buffer for 10 minutes. The membrane was carefully placed on the resolving gel and sandwiched between two thick filter papers, with the scouring pads on the outside. The sandwich was arranged with the membrane on the anode side of the gel. Proteins were transferred from one pair of gels at any one time using the Mini Trans-Blot Cell apparatus (Bio-Rad). 100V constant voltage was applied for 1 hour to transfer the proteins. Current was variable, rising from 0.2 to 0.3A during transfer. The bio-ice component of the transfer apparatus, constant gentle stirring and chilling the transfer buffer beforehand, maintained a low temperature throughout transfer. Staining the membrane with Coomassie Brilliant Blue G staining solution visualised protein bands and confirmed successful transfer of proteins. However, membranes for immunological analysis were immersed in PBS and stored overnight at 4°C.

2.2.2.9 Standard western blotting technique

All incubation and washing steps in this technique were performed on a rocking platform to agitate the membrane and solutions. Non-specific sites on the membrane were blocked by incubation in blotting grade blocking solution (5% milk powder in PBS, 0.1% Tween 20) for 1 hour at room temperature. After 2 quick washes in PBS-T, the primary antibody was applied to the membrane diluted in 10ml of blocking solution. The optimal concentration of primary antibody was determined empirically, starting at the manufacturer's recommended concentration (1:500-1:2000) in conjunction with a 1-hour period of incubation at room temperature.

Mammalian tissue extracts were used as positive controls to screen commercial mammalian specific antibodies. If no positive cross-reaction was observed in the protein extracts of teleost species, the incubation time and/or the primary antibody concentration were increased. If spurious cross-reactions were observed in addition to a positive result, a higher dilution of the primary antibody was used to eliminate the reaction with non-related proteins. Tables 2.1 and 2.2 summarise the antibodies screened and the optimal concentrations/incubation times for each antibody.

Following primary antibody incubation, the membrane was thoroughly washed in 5 changes of PBS-T for 30 minutes. The choice of secondary antibody was determined by which animal the primary antibody was raised in and the type of molecular weight standard used. Bio-Rad biotinylated molecular weight standards were detected with an ExtrAvidin[®] peroxidase conjugated secondary antibody (SIGMA, E2886). In this case, conventional Santa Cruz Goat anti-rabbit (sc-2004) and Goat anti-mouse (sc-2005) secondary antibodies were used. Where the Cruz Marker molecular weight standard was used, Santa Cruz Cruz Marker compatible Goat anti-rabbit IgG (sc-2030) and Goat anti-mouse IgG (sc-2061) secondary antibodies were used. The optimal concentration of horseradish peroxidase (HRP) conjugated secondary antibodies was 7.5-10 μ l (1:1000 to 1:1333) per 10ml of blocking solution. The incubation periods were 1 hour at 1:1000 and 1.5 hours at 1:1333. Thorough washing of the membrane was repeated with 5 changes of PBS-T in 30 minutes.

Proteins were visualised using the Immun-Star HRP Chemiluminescent kit (Bio-Rad, 170-540). After the final wash in PBS-T, 2.5ml of chemiluminescence working solution (50% luminol/ 50% enhancer solution) was applied for 5 minutes per membrane (5 \times 7.5cm).

Table 2.1. Summary of primary antibodies screened for three myogenic regulatory factors (MyoD, myogenin and myf-5), a cell cycle protein (PCNA) and a transforming growth factor (myostatin). Optimised primary antibody concentrations and incubation times apply to common carp fast and slow tissue extracts and rainbow trout fast tissue extracts. sc = Santa Cruz Biotechnology Inc.; kD = kilodaltons, P = precursor; LAP = latency associated peptide; M = mature peptide; $\alpha\alpha$ = amino acids.

Antibody	Catalogue Number	Antibody Description	Predicted Molecular Weight (kD) (ExPASy)	Species Cross-reactivity	Location of Epitope (if known/disclosed)	Primary Antibody Concentration (Total)	Incubation time (Hours)	Primary Antibody Concentration (Nuclear)	Incubation time (Hours)
MyoD	sc-760 (M-318)	Rabbit polyclonal	34 (mouse & rat)	Mouse, rat, human & carp	Full-length mouse MyoD	1:1000	1	1:1000	1
myogenin	sc-576 (M-225)	Rabbit polyclonal	32 (rat)	Mouse, rat, human & carp	Full-length rat myogenin	1:500	1	1:500	1
myogenin F5D clone	DAKO M3559	Mouse monoclonal	28 (carp)	Mouse, rat, human & cat	$\alpha\alpha$ 138-158, carboxy terminus rat myogenin	1:500	1	N/A	1
myf-5	sc-302 (C-20)	Rabbit polyclonal	28 (mouse & human)	Mouse, rat, human, carp & trout	carboxy-terminus of human myf-5	1:500	1	1:500	1
myf-5	N/A custom antibody	Rabbit polyclonal	26 (carp)	Carp & trout	$\alpha\alpha$ 154-166, carboxy-terminus, Common carp myf-5	1:2000	1	1:2000	1
myostatin	N/A custom antibody	Rabbit polyclonal	P-55 LAP-40 M-25 (salmon)	Salmon, carp & trout	$\alpha\alpha$ 347-362, carboxy-terminus, Atlantic salmon myostatin	1:10,000	2	N/A	2
PCNA	sc-56 (P10)	Mouse monoclonal	29 (mouse, rat & zebrafish)	Mouse, rat, human, insect, yeast, carp & trout	Proprietary Epitope, undisclosed region	1:500	4	N/A	4

Table 2.2. Summary of primary antibodies screened for calcineurin and associated transcription factors. Optimised primary antibody concentrations and incubation times apply to common carp fast and slow tissue extracts and Rainbow trout fast tissue extracts. sc = Santa Cruz Biotechnology Inc.; kD = kilodaltons; α . = amino acids

Antibody	Catalogue Number	Antibody Description	Expected Molecular Weight (kD) (ExPASy)	Species Cross-reactivity	Location of Epitope (if known/disclosed)	Primary Antibody Concentration (Total)	Incubation time (Hours)	Primary Antibody Concentration (Nuclear)	Incubation time (Hours)
Calcineurin A (PP2B-A α)	sc-9070 (H-209)	Rabbit polyclonal	59 (human, mouse, rat, xenopus and scallop)	Mouse, rat, human, carp & trout	α 312-521, carboxy terminus	1:500	2	1:500	2
				human calcineurin	human α isoform	1:1000	4	1:1000	4
Calcineurin B	PC-359 Calbiochem	Rabbit polyclonal	19 (human, mouse, rat and scallop)	Mouse, rat, human, bovine, carp & trout	Full-length recombinant mouse native and denatured calcineurin B	1:2000	2	1:2000	2
						1:4000	4	1:4000	4
NFAT-c1	sc-1149-R (K18-R)	Rabbit polyclonal	101 (human)	Mouse, rat, human, carp & trout	Internal region of human NFATc1	1:400	4	1:400	4
						1:500	4	1:500	4
GATA-2	sc-267 (CG2-96)	Mouse monoclonal	51 (mouse, rat & human)	Mouse, rat, human & chicken	Proprietary Epitope, undisclosed region	1:500	1	1:500	1
						1:1000	1	1:1000	1
MEF2	sc-313 (C-21)	Rabbit polyclonal	55 (human), 54 (mouse), 51 (carp)	Mouse, rat, human, carp & trout	carboxy-terminus, human MEF2	1:500	1	1:500	1
				Carp & trout	α 445-458, carboxy-terminus, Common carp MEF2A	N/A		1:2000	1

Excess liquid was drained off and the membrane sandwiched between two clear acetate sheets. Peroxidase conjugated secondary antibodies oxidise luminol contained in the chemiluminescence reagent, to produce an enhanced light signal proportional to the amount of antigen detected by the primary/secondary antibody complex. The light signal was captured with the Versadoc 3000 Imaging System (Bio-Rad). The optical density and molecular weight of proteins were calculated using Bio-Rad Quantity One (Version 4.4.1) image analysis software.

2.2.2.10 Teleost specific antipeptide antibodies

Common carp specific antipeptide antibodies to detect myf-5 and MEF2A were designed for use against teleost nuclear protein extracts. Protein sequences for common carp myf-5 (O93493, Primary accession number) and MEF2A (O93494) were acquired from the ExPASy (Expert Protein Analysis System) molecular biology knowledgebase (<http://us.expasy.org/>). All other relevant protein primary accession numbers can be viewed in Appendix V. A web-based bioinformatics tool was used to identify potential regions of high antigenicity in the common carp myf-5 and MEF2A protein sequences (Molecular Immunology Foundation, <http://mif.dfci.harvard.edu/Tools/antigenic.pl>). The procedure to predict antigenic determinants was based on the method of Kolaskar and Tongaonkar (1990). Potential peptides for use as immunogens were identified and 9 and 16 antigenic determinants were detected in the common carp protein sequences for myf-5 (Fig. 2.1A) and MEF2A (Fig. 2.1B) respectively. The other criteria used to select a 10-14 amino acid residue were the practicalities involved in synthesising the peptide and the specificity to the protein of interest. Specificity was checked by performing a protein-protein BLAST search on the NCBI website, (<http://www.ncbi.nlm.nih.gov/BLAST>) against short, nearly exact matches. Potential residues were rejected if members of the same

protein family or domains common to other proteins were detected in the BLAST search. One final consideration was the homology of the selected common carp amino acid residue with protein sequences available for other teleost species. Higher homology indicated an increased likelihood of successfully detecting myf-5 and MEF2A proteins in tissue extracts from other teleost species. DNAMAN (Version 5.2.0, Lynnon Biosoft) bioinformatics software was used to perform multiple alignments of the available sequences for myf-5 and MEF2A proteins in fish (Fig. 2.2). This software was also utilised in the analysis of homology of available teleost orthologues with mammalian and teleost antigens that commercial antibodies were raised against.

A 13 amino acid residue was selected in the common carp myf-5 sequence from the 6th antigenic determinant, located at the carboxy terminus (Fig. 2.1A): amino acids 154-166, CNSPVWPQMNPNF. This region was 62% and 92% homologous to the equivalent region of the myf-5 protein in the pufferfish (*Takifugu rubripes* Temminck & Schegel) and the zebrafish respectively (Fig. 2.2A).

A 14 amino acid short peptide sequence was selected in the common carp MEF2A sequence from the 16th antigenic determinant, located at the carboxy terminus (Fig. 2.1B): amino acids 445-458, LGLGRPRPPGQREG. This region was 57% homologous with the equivalent region in of the MEF2A protein (Fig. 2.2B) in zebrafish.

The short peptide amino acid sequences were synthesised by Graham Kemp (Biomolecular Sciences, University of St Andrews) and conjugated to keyhole limpet hemocyanin (KLH), a large immunogenic carrier protein. Diagnostics Scotland™ (Edinburgh, Scotland) immunised two rabbits with the peptide-KLH conjugate (1-2mg/ml), once a month for 4 months. One rabbit was used for each peptide.

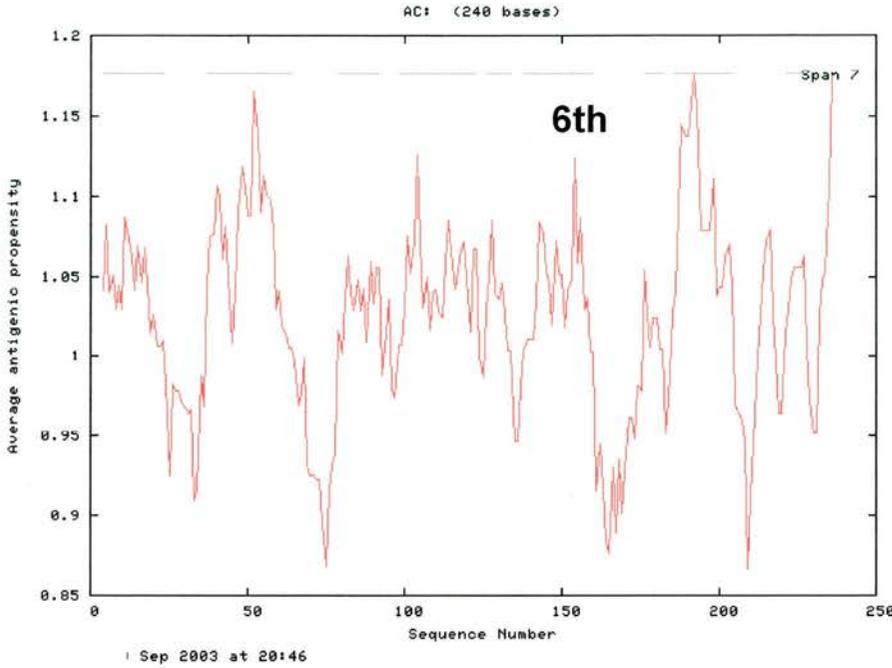
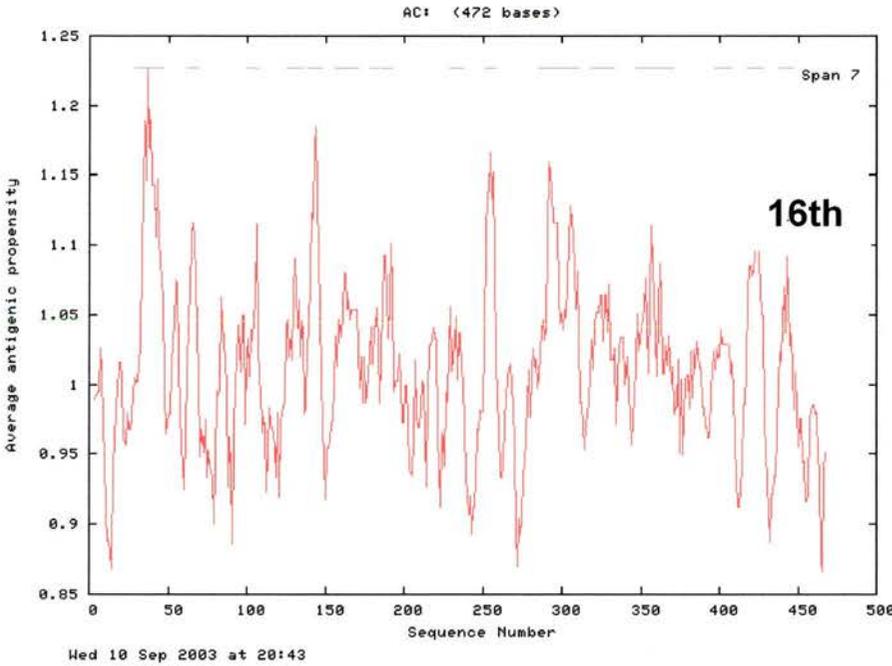
A**B**

Fig. 2.1. Plots of antigenicity for common carp myf-5 (A) and MEF2A (B) protein sequences. The peaks of the plot and dashed grey lines indicate regions of high antigenicity and potential epitopes in each sequence. The antigenic determinants (potential epitopes) selected for myf-5 and MEF2A antipeptide antibodies are shown on each plot (6th & 16th). The average antigenic propensity for the myf-5 and MEF2A plots was 1.03 and 1.01 respectively (Molecular Immunology Foundation, <http://mif.dfci.harvard.edu/Tools/antigenic.pl>).

A

CARP_myf5	C N S P V W P Q M N P N F	154-166
FUGU_myf5	S N S P V W Q Q M N A V Y	154-166
ZEBRA_myf5	C N S P V W P Q M N Q N Y	154-166

B

CARP_MEF2A	L G L G R P R P P G Q R E G	445-458
ZEBRA_MEF2A	L G L G R P P A G G A D E R	432-445

Fig. 2.2. Multiple alignments of the available protein sequences for myf-5 and MEF2A in teleost fish (DNAMAN, 5.2.0). Polyclonal antipeptide antibodies were raised against the regions of the common carp myf-5 (A, amino acids 154 - 166) and MEF2A (B, amino acids 445 - 458) protein sequences shown. A) The homology of the common carp myf-5 selected region with that of the pufferfish and zebrafish was 62% and 92%. B) The homology of the common carp MEF2A peptide with the same region of the zebrafish protein was 57%.

ExpASY primary accession identification numbers for the above proteins: common carp myf-5, O93493; pufferfish myf-5, Q90ZK9; zebrafish myf-5, Q9DDW0; common carp MEF2A, O93494; zebrafish MEF2A, Q98868 (<http://org.expasy.us>).

Freund's Complete Adjuvant (FCA) was administered with the initial injection, whereas booster injections were given with Freund's Incomplete Adjuvant (FIA). Test bleeds were taken 1 week after the 2nd, 3rd and 4th immunisations to provide polyclonal antisera. A pre-immune serum was provided as a negative control. The antisera were stored at -20°C until required.

An Atlantic salmon-specific myostatin antipeptide antibody, specific for a short peptide region of the mature peptide (amino acids 347 – 362) was kindly donated by Oivind Anderson (Norwegian University of Science and Technology, Norway). The antibody was provided as a lyophilised powder from 180µl of rabbit polyclonal antiserum. The powder was reconstituted with 180 µl of PBS (0.1% sodium azide as a preservative), split into 5µl aliquots and stored at -20°C until required. As with commercial antibodies, the optimal concentration of antipeptide antibodies for use in Western blotting applications was established empirically.

2.2.3 Results

2.2.3.1 Myogenic regulatory factors

The rabbit polyclonal MyoD antibody (sc-760, M-318) was raised against the full-length mouse MyoD amino acid sequence. The percentage homology between the mouse amino acid sequence and available sequences for MyoD orthologues in teleosts (see Appendix V) was 69% in rainbow trout and 62% in common carp. There was 87% similarity in the fish MyoD protein sequences and in a comparison of rat and mouse MyoD amino acid sequences there was 94% homology. The anti-mouse MyoD antibody cross-reacted with a 40 kD protein in the nuclear and total cellular protein extracts of all species tested, except the Atlantic salmon and rainbow trout (Fig. 2.3). In these two species, cross-reactions with higher molecular weight proteins

(approximately 80 kD) were detected in both nuclear and total protein extracts (Fig. 2.3B & C). The ExPASy molecular biology database described the molecular weights of the MyoD protein in mouse/rat and common carp/rainbow trout as 34 kD and 31 kD respectively, whereas the Santa Cruz anti-mouse MyoD commercial antibody was reported to detect an antigen of 40 kD in mouse, rat and human protein extracts (Santa Cruz Biotechnology, Inc.).

The rabbit polyclonal myogenin antibody (sc-576, M-225) was raised against the full-length rat myogenin protein. The percentage similarity between the rat amino acid sequence and available sequences for myogenin orthologues in teleosts was 52% in rainbow trout and 41% in common carp and there was 74% similarity in the fish myogenin protein sequences. The anti-rat myogenin antibody cross-reacted with a 28 kD antigen in the rat and common carp tissue extracts, but failed to detect an equivalent protein in the rainbow trout extracts (Fig. 2.4A & B). The weak cross-reaction observed for the common carp antigen in nuclear protein extracts with this antibody (Fig. 2.4A), was extremely difficult to reproduce and was very rarely detected without additional numerous spurious cross-reactions. The predicted molecular weight of the myogenin protein in rat and teleosts was 32 kD and 28 kD respectively (ExPASy) and the Santa Cruz anti-rat myogenin antibody was reported to cross-react with an antigen in the 25 – 40 kD size range in protein preparations from higher vertebrates (Santa Cruz Biotechnology Inc.). The monoclonal mouse anti-rat F5D myogenin antibody (DAKO, M3559) was raised against 21 amino acid residues (138 – 158) of the rat myogenin amino acid sequence. The equivalent regions of the common carp and rainbow trout amino acid sequences were 47% homologous, however there was a four amino acid insertion in this region in the common carp and a five amino acid insertion in the rainbow trout. This antibody detected a 28 kD

protein in the rat total protein extracts (Fig. 2.4C), which was not found in the common carp or rainbow trout total cellular protein extracts.

The rabbit polyclonal myf5 antibody (sc-302, C-20) was raised against an undisclosed region at the carboxy-terminus of the human myf5 protein. The percentage homology between the human myf5 amino acid sequence and available sequences for orthologues in teleosts was 56% in all cases (common carp, tiger pufferfish and zebrafish). The common carp and zebrafish myf5 sequences were 90% homologous, compared to 70% similarity observed in the comparisons of the myf5 sequences in the two Cyprinid species with the tiger pufferfish. The percentage similarity between the amino acid sequences for human myf5 and that of the mouse positive control was 89% but there was no available data for myf5 in rat. The anti-human myf5 antibody cross-reacted with weak affinity to a 56 kD protein in the nuclear protein extracts of the mammalian positive control (Fig. 2.5A). However, a similar result was not observed in common carp nuclear protein preparations. The same commercial antibody detected a 34 kD protein in total cellular protein extracts of all species tested (Fig. 2.5B), but this was accompanied by numerous spurious cross-reactions in all samples. A strong cross-reaction was observed in the mouse, rat and common carp samples, but the antibody reacted weakly with the trout total protein sample. The molecular weights of the myf5 proteins entered in the ExpASY knowledgebase for mouse and common carp were 28 kD and 26 kD respectively. This commercial anti-human myf5 antibody was predicted to cross-react with a protein in the size range of 25 – 55 kD in western blotting applications using protein extracts from human, rat and mouse tissue (Santa Cruz Biotechnology, Inc.).

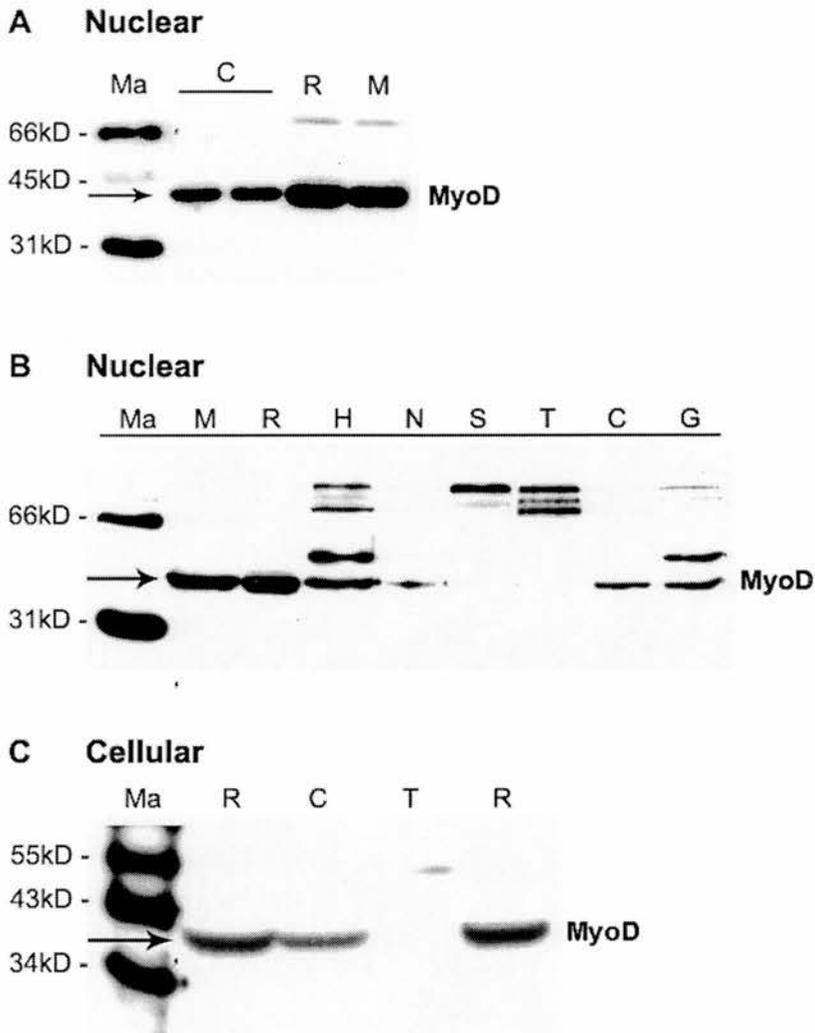


Fig. 2.3. The Santa Cruz mouse-specific MyoD antibody (sc-760, M-318) was screened against nuclear (A & B) and total cellular (C) protein extracts. Two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with several teleost species (H, Antarctic spiny plunderfish; N, yellowbelly rockcod; S, Atlantic salmon; T, rainbow trout; C, common carp; G, Atlantic cod). A & B) The MyoD antibody positively cross-reacted with a 40 kD protein in nuclear extracts from all species tested, except for Atlantic salmon and rainbow trout. C) The 40 kD protein was also detected in rat and carp total cellular protein preparations, but was absent from rainbow trout cellular extracts.

A & B) Ma, Bio-Rad low molecular weight markers (161-0306).
C) Ma, Santa Cruz markers' (sc-2035).

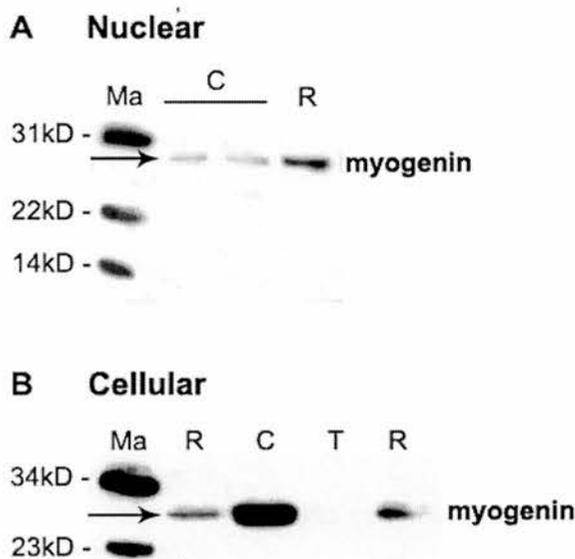


Fig. 2.4A & B. The Santa Cruz rat-specific myogenin antibody (sc-576, M225) was screened against nuclear (A) and total cellular (B) protein extracts. One mammalian species (R, rat) was used as a positive control for comparison with rainbow trout (T) and common carp (C) extracts. The myogenin antibody positively cross-reacted with a protein of 28 kD in rat and common carp nuclear and total protein extracts (A & B), but this protein was not detected in rainbow trout total cellular extracts (B).

A) Ma, Bio-Rad low molecular weight markers (161-0306).
 B) Ma, Santa Cruz markers (sc-2035).

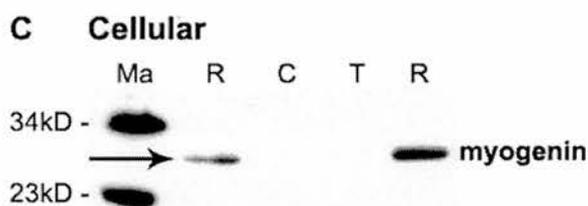


Fig. 2.4C. The DAKO rat-specific myogenin antibody (M3559, F5D) was screened against total cellular protein extracts. One mammalian species (R, rat) was used as a positive control for comparison with common carp (C) and rainbow trout (T) cellular extracts. The DAKO myogenin antibody positively cross-reacted with a 28kD protein in the rat samples, but no equivalent band was observed in the fish species tested.

C) Ma, Santa Cruz markers (sc-2035).

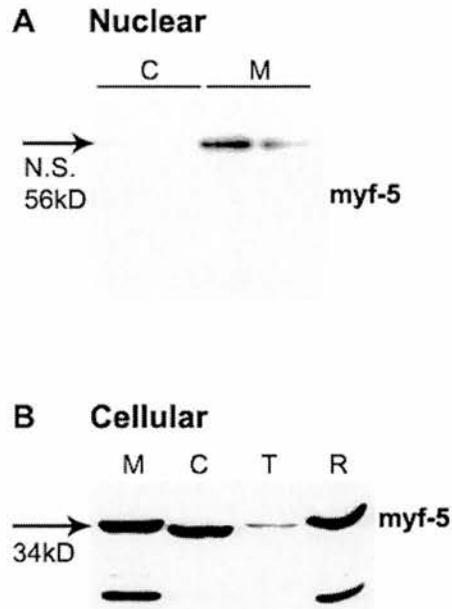


Fig. 2.5 A & B. The Santa Cruz human-specific myf-5 antibody (sc-302, C-20) was screened against nuclear (A) and total cellular (B) protein extracts. Two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with common carp (C) and rainbow trout (T) extracts. A) The Santa Cruz myf-5 antibody gave a non-specific (N.S.) cross-reaction with a 56 kD protein in mouse nuclear protein extracts, but in carp fast muscle nuclear extracts no cross-reaction was apparent. B) The Santa Cruz myf-5 antibody positively cross-reacted with a 34 kD protein in total protein extracts from all species tested.

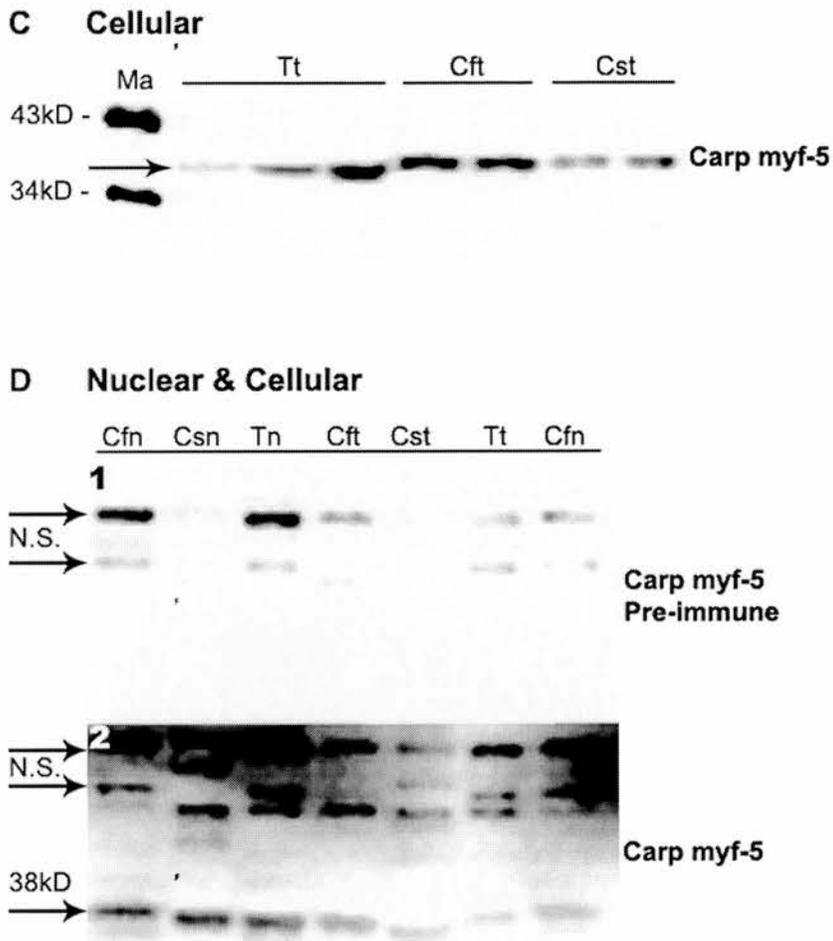


Fig. 2.5 C & D. The common carp-specific myf-5 antibody and pre-immune antisera were screened against a range of teleost tissue extracts: trout nuclear (Tn) and total cellular (Tt) extracts, carp fast (Cfn) and slow (Csn) muscle nuclear extracts, and carp fast (Cft) and slow (Cst) muscle total cellular extracts. Numerous non-specific (N.S.) cross-reactions were observed with both pre- and post-immunisation antisera (D1 & D2). However, a positive cross-reaction with a 37 – 38 kD protein was observed in all tissue types tested (C & D) using the carp-specific myf-5 antibody (3-month post-immune bleed).

C) Ma, Santa Cruz markers (sc-2035).

The common carp-specific rabbit polyclonal myf-5 antibody detected a 37 – 38 kD protein in the nuclear and total protein extracts from rainbow trout muscle tissue and common carp fast and slow muscle tissue (Fig. 2.5C & D2). A comparison of the results using the pre-immune bleed and the final antiserum demonstrated the specificity of the custom-made common-carp myf5 antibody (Fig. 2.5 D1 & D2).

2.2.3.2 Myostatin and PCNA

The rabbit polyclonal myostatin antibody was raised against a conserved short peptide sequence at the carboxy-terminus of the processed and precursor forms of the myostatin protein in Atlantic salmon. Currently, 12 full-length myostatin sequences from 11 species of teleosts belonging to 4 different orders (Salmonidae, Perciformes, Siluriformes and Cyprinidae) are available from the ExPASy knowledgebase (see Appendix V). The homology observed in the active peptide region (109 amino acid residues) of teleost myostatin was extremely high (99%); the 16 amino acid region (347 – 362) used to raise the anti-salmon antibody was identical in all teleost species except the zebrafish and channel catfish (*Ictalurus punctatus* Rafinesque), where a glycine was substituted for an arginine in the 7th position. The anti-salmon antibody cross-reacted with high affinity to several proteins in the rainbow trout total cellular protein extracts (Fig. 2.6A). The molecular weights of these proteins corresponded to the predicted mass of the myostatin precursor (PC, 53 – 55 kD), latency associated peptide (LAP, 40 – 41 kD) and mature active peptide (M, 17 kD) from previous work on myostatin and Atlantic salmon. The salmon-specific antibody cross-reacted with high affinity to two equivalent proteins in the common carp, but the lower molecular weight band of the active mature peptide was not observed in any of the repeated screenings of this antiserum against common carp total cellular protein extracts.

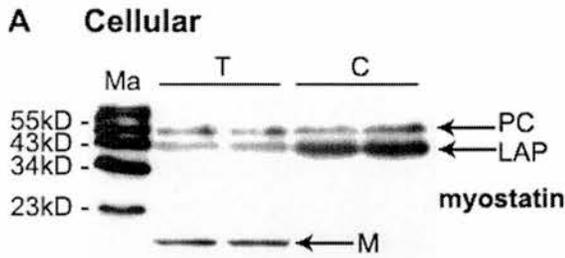


Fig. 2.6A. The Atlantic salmon-specific myostatin antibody was screened against rainbow trout (T) and common carp (C) total cellular protein extracts. The salmon-specific antiserum positively cross-reacted with the three different forms of myostatin expressed in trout total protein extracts, and the two types present in common carp preparations: the myostatin precursor protein (PC, 53 – 55 kD), latency associated peptide (LAP, 40 – 41 kD) and mature peptide (M, 16 kD). The mature peptide was not evident in common carp samples.

A) Ma, Santa Cruz markers (sc-2035).

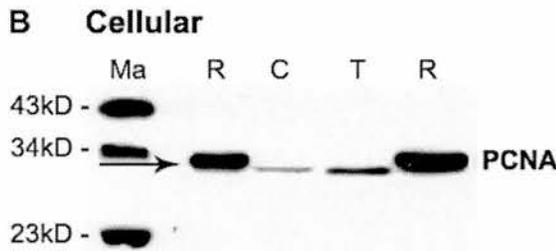


Fig. 2.6B. The Santa Cruz mouse-specific PCNA antibody (sc-56, PC10) was screened against total cellular protein extracts. One mammalian species (R, rat) was used as a positive control for comparison with rainbow trout (T) and common carp (C) extracts. The PCNA antibody positively cross-reacted with a 32 kD protein in the rat, carp and trout total protein preparations.

B) Ma, Santa Cruz markers (sc-2035).

The mouse monoclonal anti-PCNA (proliferating cell nuclear antigen, sc-56, P10) antibody was raised against an undisclosed region of the mouse PCNA amino acid sequence. The degree of homology between the mouse PCNA amino acid sequence and available orthologues of PCNA in zebrafish and the Japanese eel (*Anguilla japonica* Temminck & Schegel), was 84% and 77% respectively. The percentage similarity between the mouse PCNA sequence and that of the mammalian positive control (rat) was 92%. The anti-PCNA antisera cross-reacted with an antigen of 32 kD in total cellular extracts from all mammalian and fish species tested (Fig. 2.6B). The predicted molecular mass of this protein has been reported in the ExPASy protein knowledgebase as 29 kD in higher and lower vertebrates and the anti-mouse PCNA antibody was expected to specifically cross-react with a protein of 36 kD in higher vertebrates, insect (*D. melanogaster*) and yeast (*S. cerevisiae*) (Santa Cruz Biotechnology, Inc.). The overall level of homology of the mouse PCNA amino acid sequence with insect and yeast PCNA proteins was 67% and 31% respectively.

2.2.3.3 Calcineurin and associated substrate transcription factors

The rabbit polyclonal calcineurin A α (CnA α) antibody (sc-9070, H-209) was raised against a C-terminal 209 amino acid fragment of the human CnA α antigen. Overall, the CnA α protein was almost identical in higher vertebrates, and showed a high level of sequence homology with the African clawed frog (*Xenopus laevis*) (94%) and the giant scallop (*Patinopecten yessoensis*) (73%). The peptide (amino acids 312 – 521) used to raise the CnA α antibody was compared with the equivalent region in higher vertebrates, frog and scallop CnA α protein sequences and a high degree of similarity in this region was observed (65 – 99%). The anti-human calcineurin A α antibody cross-reacted with a 55 kD protein in the rat and mouse

nuclear protein positive controls, whereas a higher molecular mass protein was detected in common carp fast muscle nuclear extracts (Fig. 2.7A). In contrast, trout muscle nuclear and total protein extracts displayed a cross-reaction with an antigen 55 kD in mass, the same molecular weight as the protein detected in higher vertebrates (Fig. 2.7B). In the common carp slow nuclear, fast nuclear and fast total cellular protein extracts, the cross-reaction with the 75 kD protein was again evident and in addition, a lower molecular weight species (61 kD) was detected in carp slow muscle tissue total protein preparations (Fig. 2.7B). In the ExPASy protein database, the predicted molecular mass of the CnA α catalytic subunit was reported as 59 kD in higher vertebrate species, the African clawed frog and the Ezo giant scallop. Higher calcineurin A molecular weights were described in the fruit fly and various species of fungi and yeast (62 – 72 kD). The anti-human calcineurin A α antibody was expected to be immunoreactive with an antigen of 55 kD molecular mass in human, rat and mouse tissue extracts (Santa Cruz Biotechnology, Inc.).

A rabbit polyclonal calcineurin B α (CnB α) antibody (OncogeneTM, PC-359) was raised against the mouse full-length CnB α protein. A comparison of the mouse CnB α amino acid sequence with a higher vertebrate and several notable invertebrate species revealed a high level of sequence homology: human (99%), scallop (88%), blood fluke (*Schistosoma mansoni*, 84%), fruit fly (85%) and nematode worm (*Caenorhabditis elegans*, 78%). The anti-mouse CnB α antibody cross-reacted with an 18.7 kD antigen in the nuclear protein extracts of the mammalian positive controls (mouse and rat), the common carp fast and slow muscle tissue nuclear extracts and the rainbow trout nuclear protein preparations (Fig. 2.8A & B). The predicted molecular mass of the CnB α regulatory subunit was reported as 19 – 20 kD in the ExPASy protein database for numerous species that span the entire range of evolutionary

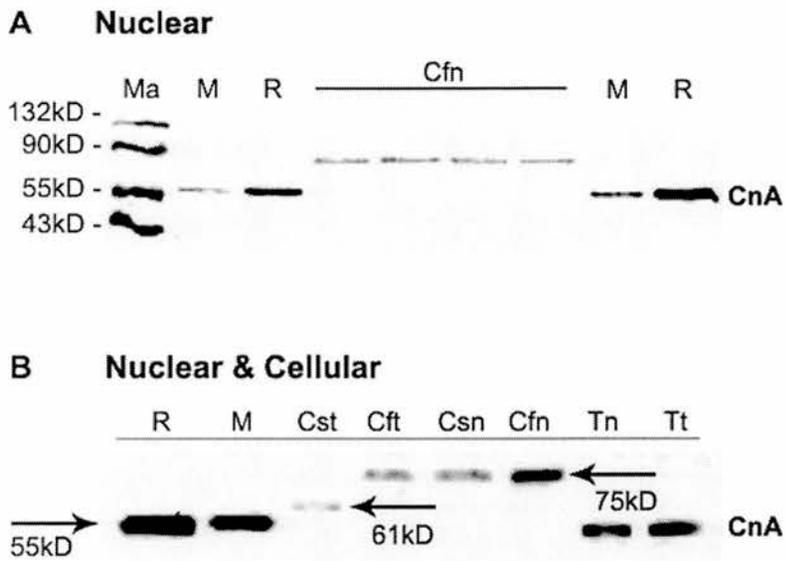


Fig. 2.7. The Santa Cruz human-specific calcineurin A antibody (sc-9070, H209, PP2B-A α , catalytic subunit specific) was screened against nuclear (A) and total cellular (B) protein extracts. Nuclear protein extracts from two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with a range of teleost protein extracts: trout nuclear (Tn) and total cellular (Tt) extracts, carp fast (Cfn) and slow (Csn) muscle nuclear extracts, and carp fast (Cft) and slow (Cst) muscle total cellular extracts. The Santa Cruz calcineurin A antibody positively cross-reacted with a 55 kD protein in mouse and rat nuclear extracts (A & B) and rainbow trout total and nuclear protein preparations (B). In common carp fast nuclear (Cfn), slow nuclear (Csn) and fast total cellular (Cft) protein extracts, this antibody cross-reacted with a 75 kD protein (A & B). In slow total muscle preparations (Cst), a 61 kD protein was detected (B).

A) Ma, Santa Cruz markers (sc-2035).

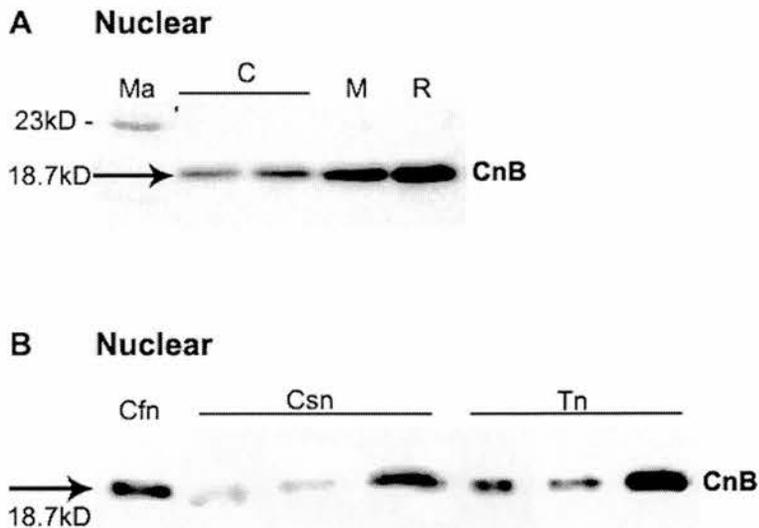


Fig. 2.8. The Oncogene mouse-specific calcineurin B antibody (PC-359, CnB Ab-1, regulatory subunit specific) was screened against a variety of nuclear protein tissue extracts. Two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with common carp fast muscle nuclear protein extracts (Cfn). A) The Oncogene calcineurin B antibody cross-reacted specifically with an 18.7 kD protein in the mouse, rat and carp nuclear protein preparations. B) The 18.7 kD protein was also detected with this antiserum in carp slow (Csn) and trout (Tn) nuclear protein extractions, in addition to carp fast (Cfn) muscle preparations.

A) Ma, Santa Cruz markers (sc-2035).

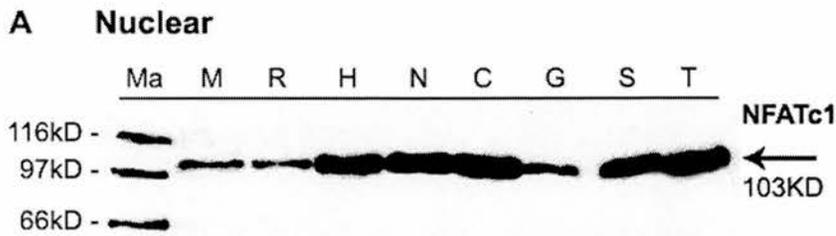


Fig. 2.9A. The Santa Cruz human-specific NFATc1 antibody (sc-1149-R, K18-R) was screened against nuclear protein extracts. Two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with six teleost species (H, Antarctic spiny plunderfish; N, yellowbelly rockcod; C, common carp; G, Atlantic cod; S, Atlantic salmon; T, rainbow trout). The Santa Cruz NFATc1 antibody positively cross-reacted with a 100 – 103 kD protein, in nuclear protein extracts from all species tested.

A) Ma, Bio-Rad high molecular weight markers (161-0311).

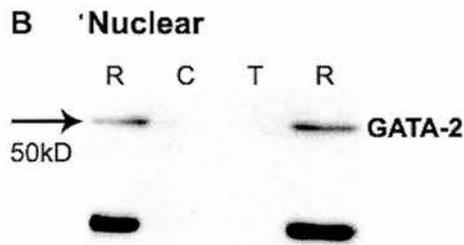


Fig. 2.9B. The Santa Cruz monoclonal GATA-2 antibody (sc-267, CG2-96) was screened against nuclear protein extracts. One mammalian species (R, rat) was used as a positive control for comparison with common carp (C) and rainbow trout (T) extracts. The Santa Cruz GATA-2 antibody positively cross-reacted with a 50 kD protein in rat nuclear protein extracts, whereas there was no cross-reaction evident with nuclear protein extracts from the two teleost species tested.

complexity (see Appendix V). This anti-mouse antibody was expected to detect a 19 kD antigen in western blotting applications using protein extracts from higher vertebrates (Oncogene™ Research Products, MA USA).

A rabbit polyclonal NFATc1 antibody (sc-1149-R, K18-R) was raised against an undisclosed internal region of the human NFATc1 protein. Data for this antigen are scarce, however a comparison of homology between the human NFATc1 amino acid sequence and the fragments of this protein available for mouse and pig demonstrated high similarity within the internal region and overall homology of 66% and 68% respectively. The anti-human NFATc1 antibody cross-reacted with a 101 kD antigen in nuclear protein extracts from mammalian positive controls (mouse and rat) and 6 species of teleosts (Fig. 2.9A). The predicted molecular mass of the human NFATc1 protein was 101 kD in the ExPASy knowledgebase, however there were no available sequences from lower evolutionary groups for comparison. This anti-human commercial antibody was reported to be broadly immunoreactive with all four members of the NFAT family (NFAT1 – 4) in human, rat and mouse tissue extracts (Santa Cruz Biotechnology, Inc.). A mouse monoclonal antibody against GATA2 cross-reacted with a 51 kD protein in the mammalian positive control (rat) but no equivalent reaction was determined in the nuclear protein extracts from common carp and rainbow trout (Fig. 2.9B). The reported molecular weight of the GATA2 protein was 51 kD in higher vertebrates (ExPASy), which corresponded with the expected molecular mass of an antigen detected with this antibody in mouse, rat or human protein extracts (Santa Cruz Biotechnology, Inc.).

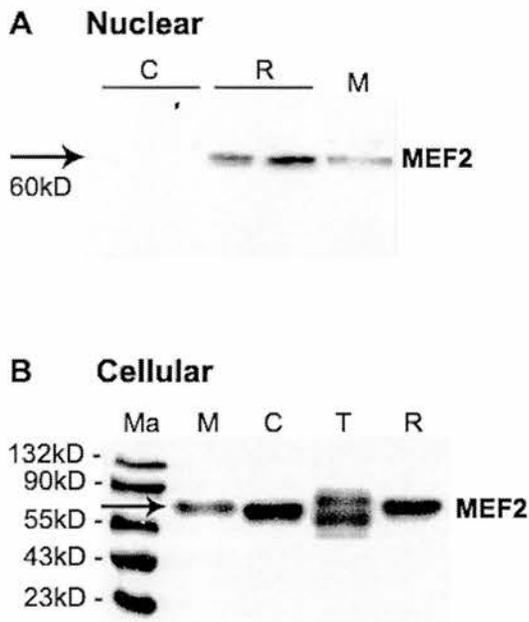


Fig. 2.10 A & B. The Santa Cruz human-specific MEF2 antibody (sc-313, C-21) was screened against nuclear (A) and total cellular (B) protein extracts. Two mammalian species (M, mouse & R, rat) were used as positive controls for comparison with common carp (C) and rainbow trout (T) extracts. A) The Santa Cruz MEF2 antibody positively cross-reacted with a 60 kD protein in mouse and rat nuclear protein extracts, but in carp fast muscle nuclear extracts no cross-reaction was evident. B) The MEF2 antibody cross-reacted with proteins in the 66 – 75 kD molecular weight size range in cellular protein extracts from the four species tested.

B) Ma, Santa Cruz markers (sc-2035).

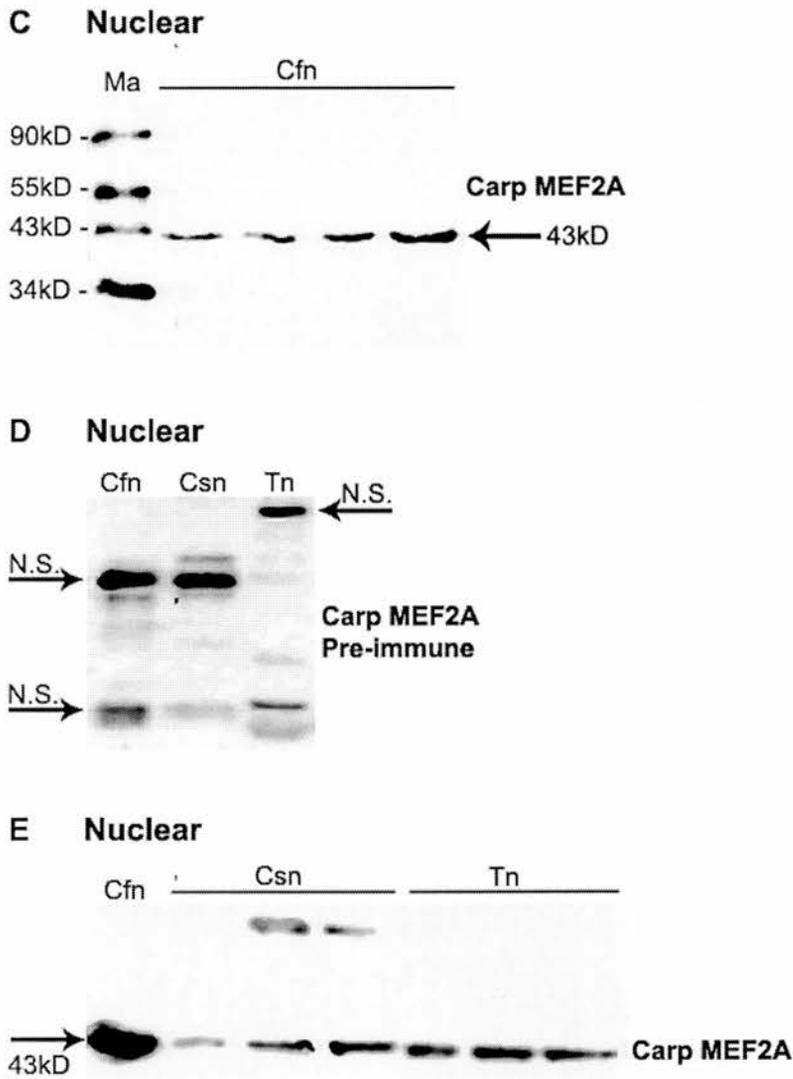


Fig. 2.10 C – E. The common carp-specific MEF2A antibody and pre-immune antisera were screened against a range of teleost tissue extracts: rainbow trout (Tn), carp fast-type (Cfn) and slow-type (Csn) muscle nuclear protein preparations. Numerous non-specific (N.S.) cross-reactions were observed with the pre-immune bleed (D). In contrast, a strong positive cross-reaction was observed with a 43 kD protein using the carp-specific MEF2A antibody (3-month post-immune bleed) in all tissue types tested (C & E).

C) Ma, Santa Cruz markers (sc-2035).

The rabbit polyclonal MEF2 (sc-313, C-21) antibody was raised against a proprietary region of the human MEF2 C-terminus. The percentage homology between the human amino acid sequence and available sequences for MEF2A orthologues in teleosts was 69% in zebrafish and 70% in common carp. There was 91% homology in the fish MEF2A protein sequences and the comparison of the human MEF2A amino acid sequence with that of the mammalian (mouse) positive control demonstrated an 86% similarity, but there was no available data for MEF2A in rat. The MEF2 anti-human antibody cross-reacted with an antigen of approximately 60 kD in nuclear protein extracts of the mammalian positive controls (mouse and rat), but a similar reaction was not observed in the common carp nuclear extracts (Fig. 2.10A). The MEF2 commercial antibody cross-reacted with proteins in the 66 – 75 kD size range, in the total protein extracts of common carp, rainbow trout and two mammalian positive controls (Fig. 2.10B). The molecular masses of MEF2A (the most reactive MEF2 family isoform to the MEF2 antibody) available from the ExPASy database were reported as 54 kD and 51 kD for mouse and common carp respectively. The predicted molecular mass of the MEF2 antigen detected by this antibody was approximately 60 kD (Santa Cruz Biotechnology, Inc.).

The common carp-specific antipeptide antibody raised against a short peptide sequence from the MEF2A isoform, cross-reacted strongly against a 43 kD antigen present in nuclear protein extracts from common carp fast, common carp slow and rainbow trout fast muscle tissue samples (Fig. 2.10C & E). A comparison of the results using the pre-immune bleed and the final antiserum demonstrated the specificity of the custom-made common-carp MEF2A antibody (Fig. 2.10C, D & E).

2.2.4 Discussion

The immunoreactivity and specificity of numerous commercial and custom-made antibodies has been described. Mouse and rat tissue extracts were used as positive controls and antisera were screened against nuclear and total protein extracts from a range of teleost species and muscle tissue types. The affinity of a particular antiserum raised against the antigen of one species (*e.g.* mouse MyoD) for an orthologue in another species (*e.g.* common carp MyoD) can be used as an indication of evolutionary relatedness. The objective of the antibody screening process was to identify suitable antisera to allow the discernment of molecular signaling pathways underlying growth of the axial musculature in fish. As a general rule, the higher the degree of homology observed in protein sequences from evolutionary disparate groups, the higher the probability that the commercial anti-mammalian antiserum will positively cross-react with the teleost antigen of interest. The commercial and teleost-specific antisera were considered to be specific if the criteria outlined in the introduction (section 2.2.1) were satisfied.

2.2.4.1 Myogenic regulatory factors

In the case of the anti-mouse MyoD antibody, a strong cross-reaction observed with a 40 kD antigen in four of the teleost species examined was paralleled in the two mammalian positive controls. There was a relatively high degree of homology (62 – 69%) in the teleost orthologous MyoD amino acid sequences compared with the mouse MyoD protein. The antigen detected was within the size range predicted for the use of this antibody, although this was several kilodaltons higher than the quoted molecular mass from the ExPASy database. It was therefore concluded that the anti-mouse MyoD antibody positively cross-reacted with the MyoD protein in the common carp, Atlantic cod, Antarctic Harpagifer and sub-Antarctic Notothenioid.

The cross-reaction of the MyoD antibody against the Atlantic salmon and rainbow trout protein extracts was far less conclusive, however it is possible that the MyoD antibody positively cross-reacted with an 80 kD homodimer of the MyoD protein in the salmonid extracts. This viewpoint is supported by the similarity of the two trout MyoD orthologues with the mouse MyoD protein (69%). Conversely, it is just as likely that it was a spurious cross-reaction of the anti-mouse MyoD antibody with an unknown 80 kD protein. It was concluded that without independent verification of the existence of an 80 kD MyoD homodimer in salmonid tissue extracts, perhaps through using a custom-made teleost-specific MyoD antibody, the Santa Cruz anti-mouse MyoD antibody was unsuitable for use with salmonid species.

The 29 kD protein observed in common carp and rat nuclear and total protein extracts using the Santa Cruz myogenin antibody was concluded to be the myogenin protein, due to the molecular weight of the antigen detected and the reasonable degree of homology in the amino acid sequences of these species (41%). The observation of this protein in common carp nuclear extracts was not reproducible, however. There was no positive cross-reaction of this antibody with any of the trout protein tissue extracts screened, despite the higher level of similarity found in the rainbow trout and rat myogenin protein sequences (52%) relative to the common carp. The F5D monoclonal antibody did not cross-react with the myogenin antigen in the teleost samples, which was most likely due to substantial differences in the mammalian and fish myogenin proteins at the epitope the antibody was raised against. It was surmised that the Santa Cruz myogenin antibody was only suitable to detect the myogenin protein in common carp total cellular protein tissue extracts.

The 34 kD antigen detected in the total protein extracts of two mammalian and two fish species with the commercial Santa Cruz anti-human myf5 antibody, was

concluded to be the myf5 protein as the detected antigen was in the correct size range and there was relatively substantial homology between the human and teleost myf5 amino acid sequences (56%). In contrast, this antibody cross-reacted weakly with a 56 kD molecular weight antigen in mouse nuclear protein extracts and did not cross-react with antigens in any of the common carp samples screened. Although there is a chance that the anti-human myf5 antibody cross-reacted with a myf5 homodimer in the mammalian extract, the lack of reaction with teleost nuclear protein samples precluded the use of this antibody.

The common carp-specific myf5 antipeptide antiserum detected a 37 – 38 kD antigen in nuclear and total protein extracts from common carp fast and slow muscle tissue and rainbow trout fast muscle tissue. The predicted molecular mass of the myf5 protein in teleost species was approximately 26 kD (ExPASy). However, the cross-reaction of the carp-specific antiserum was within the size range expected when using the anti-human myf5 antibody (25 – 55 kD) and only several kilodaltons higher than the positive cross-reactions observed when using the commercial myf5 antibody. Furthermore, numerous cross-reactions were observed with the pre-immune serum taken before the commencement of the immunisation program, but none were in the 37 – 38 kD size range. It is extremely unlikely that the rabbit used to raise the carp-specific myf-5 antibody would be producing any carp-specific antibodies prior to immunisation, therefore the numerous bands observed when using the pre-immune antiserum were most likely non-specific cross-reactions with highly expressed antigens in the teleost protein extractions. Therefore it was cautiously proposed that the antigen detected using the common carp-specific antiserum was indeed myf5 and this antibody was suitable to detect myf5 in nuclear and total cellular protein extracts from common carp and rainbow trout.

2.2.4.2 *Myostatin and PCNA*

The Atlantic salmon-specific custom-made antibody cross-reacted in common carp and rainbow trout total protein extracts with antigens of similar molecular mass to those previously reported in Atlantic salmon (Østbye *et al.*, 2001). The putative myostatin precursor and latency associated peptide were 53 – 55 kD and 40 – 41 kD respectively, however the active peptide molecular mass was 16 kD, lower than the one reported by Østbye (2001) and co-workers (25 kD). It is proposed that the active peptide detected in the Atlantic salmon was a dimer of the myostatin active peptide, whereas the rainbow trout myostatin peptide was detected as a monomer. A 15 kD myostatin active peptide species has previously been described in the literature, as an antigen that is detected under reducing conditions or occurred as a purified form of the mature peptide (Gonzalez-Cadavid *et al.*, 1998; Thomas *et al.*, 2000; reviewed by Kocamis & Killefer, 2002). The mature peptide was not detected in any of the screenings of common carp total cellular protein extracts in fast or slow muscle tissue, but since the same antibody detected the precursor and LAP, it is likely that the myostatin peptide is expressed in quantities too small to detect in this species. The high level of homology observed in the active peptide of teleost species strongly suggested that the myostatin anti-salmon specific antibody would be specific for myostatin in rainbow trout, and common carp. Therefore it was concluded that the anti-salmon antibody was specific for the precursor and processed forms of myostatin found in rainbow trout and common carp and suitable for use against total cellular protein extracts. However, in subsequent experimental work the myostatin precursor protein and latency associated peptide could not be detected in common carp total protein extracts. Hence myostatin expression in response to exercise training was only examined in rainbow trout (Chapter 5).

The PCNA antibody cross-reacted with a 29 kD protein in the mammalian positive controls and this result was paralleled in the teleost species. The expected molecular mass of the antigen detected using this antibody was 36 kD, however the 29 kD protein detected was in agreement with the predicted molecular weight from the ExPASy protein knowledgebase. A high level of homology was also observed between PCNA sequences in higher and lower vertebrates (77 – 84%), therefore it was concluded that the Santa Cruz PCNA commercial antibody positively identified the PCNA antigen in common carp and rainbow trout.

2.2.4.3 Calcineurin and associated substrate transcription factors

The CnA α anti-human antibody cross-reacted with an antigen of 55 kD in the two mammalian positive controls and the nuclear and total protein extracts of the rainbow trout. The antigen corresponded to the expected molecular mass of the calcineurin catalytic subunit detected using this antibody and was only several kilodaltons lower than that reported in the ExPASy protein database. Moreover, the high level of homology observed in CnA amino acid sequences comparing higher vertebrates with a lower vertebrate (88%) and an invertebrate (73%) species (reviewed in Hemenway & Heitman 1999, Rusnak & Mertz 2000) led to the conclusion that the CnA α antibody was suitable for detecting the calcineurin catalytic subunit in rainbow trout tissue extracts.

The CnA α antibody detected a higher molecular mass antigen (61 – 75 kD) in nuclear and total protein extracts from fast and slow muscle tissue in the common carp. The 75 kD cross-reaction is potentially the regulatory and catalytic subunits of calcineurin that have failed to separate under denaturing conditions and the 61 kD cross-reaction observed in common carp slow muscle tissue is potentially a higher molecular weight CnA isoform. However, due to the weak affinity of the CnA α

antibody with the higher molecular weight antigens in the common carp, these results were difficult to reproduce and this antiserum was not extensively used in expression analysis studies in the common carp.

The high level of homology observed in the regulatory subunit of calcineurin (CnB α) between the amino acid sequences of higher vertebrates and those of invertebrates such as yeast, nematode worm and giant scallop, suggested that there would not be significant sequence variation in this antigen between higher and lower vertebrates (reviewed in Guerini 1997, Hemenway & Heitman 1999, Rusnak & Mertz 2000). The anti-mouse CnB α antibody cross-reacted with a 19 kD antigen in the nuclear protein extracts of all experimental species. The molecular mass was identical to that expected using this antibody against mammalian tissue extracts and the predicted molecular mass of 19 kD from the ExPASy protein database. It was therefore concluded that the anti-mouse CnB α antibody detected the regulatory subunit of calcineurin in rainbow trout and common carp.

Although the primeval member of the NFAT family has been identified in the fruit fly genome (Lopez-Rodriguez *et al.*, 1999; reviewed in Hogan *et al.* 2003), the only available sequences for NFATc1 were mammalian in origin. Therefore it was concluded that the NFATc1 antibody should be screened against a wide range of teleost species, using mouse and rat nuclear protein extracts as positive controls. The anti-human NFATc1 antibody detected a 100 – 103 kD antigen in the mammalian positive controls and all species of teleosts tested. This corresponded to the expected molecular mass of the NFATc1 protein observed when using this commercial antibody against tissue extracts from higher vertebrates and was the also the molecular weight of the human NFATc1 antigen in the ExPASy protein database. The anti-human NFATc1 antibody was concluded to be suitable for detection of a

teleost NFATc1 orthologue in rainbow trout and common carp tissue extracts. The mouse monoclonal GATA2 antibody did not cross-react with any antigens in the teleost nuclear protein extracts, in contrast to the positive identification of this protein in rat nuclear extracts. GATA2 is a molecular marker of skeletal muscle hypertrophy in higher vertebrates (Musaro *et al.*, 2001), but there are currently no sequences available for GATA2 orthologues in lower evolutionary groups.

The approximately 60 kD protein detected in total protein extracts of two mammalian positive controls and two species of teleosts using the commercial anti-human MEF2 antibody was concluded to be MEF2A, because the antigen detected was of the correct molecular mass and there was relatively high similarity between the human and teleost MEF2A amino acid sequences (69 – 70%). A similar result was observed in the nuclear protein extracts of mammalian positive controls, however no cross-reaction was observed in common carp nuclear protein extracts in any screening protocol, which ruled out the use of this antibody in MEF2 expression analysis.

The common carp-specific MEF2A antipeptide antiserum detected a 43 kD antigen with high affinity in nuclear protein extracts from common carp fast and slow muscle tissue and rainbow trout fast muscle tissue. The predicted molecular mass of the MEF2A protein in teleost species was approximately 51 kD (ExpASy). In the screening of the pre-immune bleed numerous cross-reactions were evident, but none were apparent in the region the putative teleost MEF2A antigen was observed. MEF2A is known to exist in a hypophosphorylated form, as it is the target of numerous intracellular signaling pathways (Xu *et al.*, 2003; Friday *et al.*, 2003). Therefore it was guardedly proposed that the 43 kD molecular mass antigen detected using the common carp-specific antiserum was a hypophosphorylated form of

MEF2A and this antibody was suitable to detect MEF2A in nuclear and total cellular protein extracts from common carp and rainbow trout.

In conclusion, the objective of identifying antibodies suitable for examining expression of muscle-specific proteins in common carp and rainbow trout was achieved in principle. The following commercial and teleost-specific antibodies were designated as suitable for use against common carp and rainbow trout tissue extracts: anti-human calcineurin $A\alpha$, anti-mouse calcineurin $B\alpha$, anti-human NFATc1, monoclonal mouse PCNA, common carp-specific MEF2A and common carp-specific myf5. Further experimental work demonstrated that the Atlantic salmon-specific myostatin antipeptide antibody was only suitable for determining myostatin expression in rainbow trout, but not common carp. The Santa Cruz anti-mouse MyoD and anti-rat myogenin commercial antibodies were found to positively cross-react with the common carp orthologous antigens, but not the equivalent proteins in rainbow trout.

2.3 Histochemistry and immunohistochemistry

2.3.1 Introduction

As described previously (section 1.3.2), different muscle fibre phenotypes occupy distinct myotomal regions in the teleost axial musculature. In addition to anatomical location, histochemical and immunohistochemical staining techniques can be employed to differentiate between slow, intermediate and fast muscle fibre types in thin transverse cryosections of myotomal muscle.

This section will describe the development of histochemical and immunohistochemical techniques to distinguish between different muscle fibre

phenotypes in common carp and rainbow trout. Furthermore, the development of these methods will allow the accurate measurement of muscle fibre cross-sectional area. The use of muscle fibre size as an indication of the effect of exercise on muscle growth in common carp and rainbow trout will be described in subsequent chapters.

2.3.2 Materials and methods

2.3.2.1 Experimental animals

Juvenile common carp and rainbow trout (range of fork lengths 10 – 15 cm) were obtained from Humberside Fisheries (Cleaves Farm near Driffield, East Yorkshire, UK) and College Mill Trout Farm (Almondbank near Perth, UK) respectively. The fish were maintained in freshwater aquaria at 15°C in the Gatty Marine Laboratory Main Aquarium, University of St Andrews. Each species was fed to satiation once daily (approx. 3 – 5% body mass) with a species-specific commercially available brand of fish food (Trouw Aquaculture).

2.3.2.2 Preparation of samples for histochemistry and immunohistochemistry

Individual fish were sacrificed by administration of an overdose of anaesthetic (MS222, tricane methanesulphonate, SIGMA), before severing the spinal cord (Schedule 1 Killing, Home Office Regulations). Fish were then placed in individual polythene bags and packed in ice until dissection. A new razor blade was used to make a complete incision through the body of the fish at the level of the anal pore, approximately 0.75 body lengths from the snout (see Figs. 3.4A & 5.1A). Taking a cross-section from this region of the anatomy, posterior to the anal pore, allowed the cutting of intact cryosections that were not disrupted by the digestive tract. A second incision was made posterior to the first, giving a steak approximately 5mm thick.

Depending upon the size of the fish and hence the size of the cross-sectional area, the steak was then either frozen intact or further subdivided into blocks (approx. 5×8×8mm) before cryopreservation. The tissue block was adhered to a 1cm² cork tile with cryomatrix (Thermo Shandon, PA, USA), with the first cut surface orientated upwards. The tissue was then snap frozen by immersion for 45 seconds in isopentane cooled to -159°C using liquid nitrogen. Samples were wrapped in aluminium foil and stored in liquid nitrogen until required.

2.3.2.3 Tissue sectioning

Tissue blocks were removed from liquid nitrogen storage and left in the cryostat (Cryocut 1800, Reichert-Jung) refrigerator cabinet for 1 hour to reach -20°C. Cryomatrix (Thermo Shandon, PA, USA) was used to attach the cork tile to a cryostat chuck and the block was positioned to cut tissue sections at right angles to the long axis of the fish. Transverse serial sections (7µm) were taken and picked up on Poly-L-lysine coated slides, to which they adhered. Slides were then air dried for a minimum of 30 minutes before storage at -80°C.

2.3.2.4 Histochemistry: Mayer's haematoxylin staining

In all histological techniques, frozen sections were first removed from the freezer and allowed to defrost and air dry at room temperature for 30 minutes. Slides were immersed in Mayer's Haematoxylin reagent for 5 minutes, and then rinsed in running tap water for 10 – 15 minutes. After drying, coverslips were mounted using glycerol.

2.3.2.5 Immunohistochemistry: S58 antibody staining

This technique was a modified version of one utilised by Devoto *et al.* (1996), which demonstrated the cross-reactivity of the S58 mouse monoclonal antibody with slow muscle fibres in developing zebrafish. The S58 antibody primarily recognises avian slow myosin heavy chain isoforms SM2 and SM3 (Crow & Stockdale, 1986). For this experiment, Frank Stockdale kindly donated several aliquots of the S58 antibody in tissue supernatant form.

Frozen sections were defrosted at room temperature for 30 minutes. The tissue was then fixed in acetone for 10 minutes and air-dried for 10 minutes. Sections were circumscribed with an ImmEdge™ pen (Vector Labs, CA, USA), and then immersed in blocking solution (5% NGS, 1.5% BSA & 1% Triton-X100 in PBS) for 1 hour at room temperature to re-hydrate the tissue and prevent non-specific binding of the primary antibody. Sections were then washed for 5 minutes in 3 changes of PBS. The S58 antibody tissue supernatant was diluted to a concentration of 1:10 in blocking solution and 100µl was applied to each slide. Sections were incubated in a humidity chamber overnight at 4°C. Slides were washed 3×3 minutes in PBS and 100µl per slide of Peroxidase blocking reagent (DAKO Cytomation Ltd., Cambridgeshire, UK) was applied for 10 minutes to reduce non-specific background. Slides were then washed in PBS for 3×3 minutes. A biotin conjugated anti-mouse secondary antibody (SIGMA) was diluted to 1:400 concentration in blocking solution and applied to the slides in 150µl aliquots. Tissue sections were incubated in secondary antibody for 1 hour at room temperature in a humidity chamber, then washed in PBS for 3×3 minutes. The biotinylated secondary antibody was detected using an Extr-Avidin Peroxidase conjugate (SIGMA), diluted to 1:50 in blocking solution and applied to slides in 150µl aliquots. Sections were incubated for 1 hour at room temperature in a

humidity chamber, then washed for 3×3 minutes in PBS. The reaction of the S58 antibody and common carp slow muscle fibres was then visualised as a red insoluble end product using AEC (3-Amino-9-Ethylcarbazole) and hydrogen peroxide, in dimethylformamide and sodium acetate buffer (pH 5.0). The reaction was terminated by several washes of distilled water, before coverslips were mounted with glycerol.

2.3.2.6 Myofibrillar ATPase (mATPase) Protocol

This version of the mATPase protocol is a variation on the techniques of Padykula & Herman (1955), Brooke & Kaiser (1970), Guth & Samaha (1970) and Johnston *et al.* (1974), adapted for the common carp. The method distinguishes between different muscle fibre phenotypes, based on the different levels of mATPase enzyme activity in each fibre phenotype. Frozen tissue sections were defrosted and air-dried for 30 minutes in total. Sections were immersed in an acidic pre-incubation medium (0.1M sodium acetate, 10mM EDTA, pH 4.6) for 1 minute at room temperature, followed by 2×1-minute washes in distilled water. Sections were then placed in an ATP incubation medium (200mM Tris-Cl, 18mM CaCl, 2mM ATP, pH 9.5) for 25 minutes on a rocking platform. This was followed by a 10-minute washing step in 1% calcium chloride with 3 changes, then a 10-minute incubation in 2% cobalt chloride. After 5×1-minute washes in distilled water, sections were developed in 0.5% ammonium polysulphide for 30 seconds then washed again in distilled water for 5×1-minute steps. Sections were dehydrated in a series of ascending alcohols (50%, 70%, 80% 95%, 2×100%) then cleared in xylene, 2×2-minutes. Finally, slides were mounted in Canada balsam.

Transverse sections stained using the histological (Mayer's haematoxylin and mATPase) and immunohistological (S58) techniques were photographed with a frame

capture camera (JVC, TK-F7300U) connected to a light microscope with $\times 5$ and $\times 10$ objectives. The resulting photographs were annotated using CorelDraw (v. 9.0) software.

2.3.3 Results

Staining with Mayer's haematoxylin did not distinguish between muscle fibre types but allowed clear visualisation of muscle fibre cellularity for the purpose of morphometric analysis in common carp (Fig. 2.11C – F) and rainbow trout (Fig. 2.12). Despite the largely homogeneous haematoxylin staining profile (Table 2.3), slow and superficial fast muscle fibres stained slightly darker than deep fast fibres in the common carp (Fig. 2.11D – F).

The avian slow myosin heavy chain specific S58 antibody only gave a positive cross-reaction with slow muscle fibres present at the horizontal septum and in the superficial slow muscle layer beneath the skin. As a result, dark red staining of slow muscle fibres was observed in common carp (Fig. 2.11B) and rainbow trout (data not shown) transverse tissue sections. Background peroxidase activity gave a less intense non-specific light red colouration of intermediate and fast muscle fibres (Table 2.3).

Anatomical location and mATPase staining allowed the identification of three different phenotypes of fast muscle fibre (Fig. 2.13), in addition to the slow muscle fibre phenotype (slow fibre mATPase staining not shown). Intermediate muscle fibres, located between well-defined regions of fast and slow muscle, were divided into two phenotypes on the basis of mATPase staining results: intermediate type a (Ia) fibres stained very heavily, whereas intermediate type b (Ib) fibres stained very lightly.

Table 2.3. The histochemical/ immunohistochemical staining profile for four muscle fibre phenotypes identified in transverse cryosections from common carp myotomal muscle. 0 unstained; + lightly stained; ++ moderately stained; +++ heavily stained.

Muscle fibre phenotype	Mayer's haematoxylin staining profile	S58 staining profile	mATPase staining profile
Slow	+++	+++	+
Ia	++/+++	0/+	+++
Ib	++/+++	0/+	0/+
Fast	++/+++	0/+	++

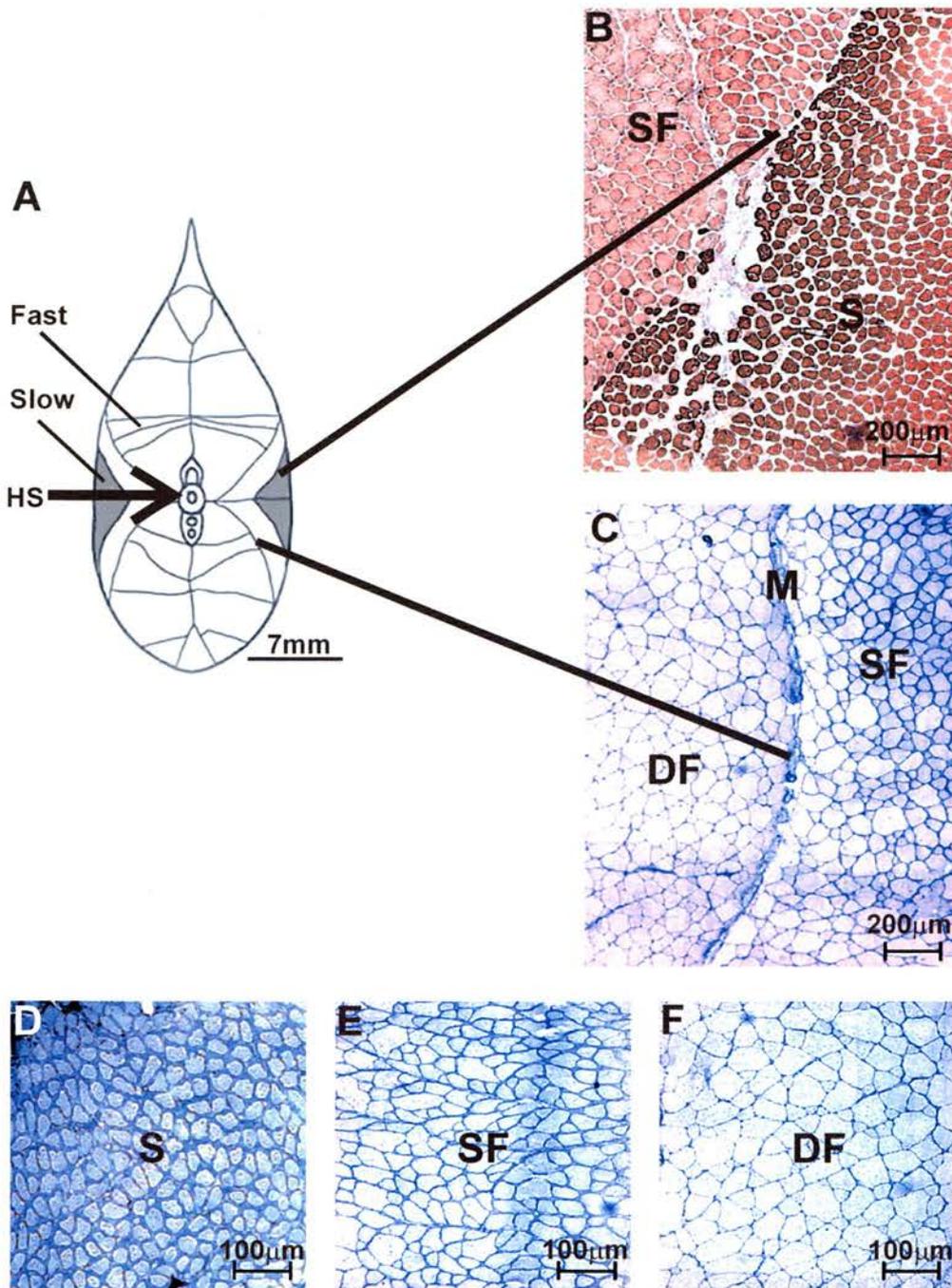


Fig. 2.11. Transverse sections of myotomal muscle from common carp showing the anatomical location of different muscle fibre phenotypes. A) Two major muscle fibre phenotypes were discernable during dissection, fast and slow. B) Slow muscle fibres (S) were identified as those that positively cross-reacted with the S58 antibody. C) Fast fibres were divided into two subclasses for swimming experiments 1 – 4: deep fast (DF) and superficial fast (SF). D – F) Along the axis of the horizontal septum (A, HS), three fibre types were found in the order slow (S), superficial fast (SF) and deep fast (DF) in haematoxylin stained sections. All images of sections are orientated with the dorsal edge upwards (B & C $\times 5$ objective; D – F $\times 10$ objective). M – myoseptum.

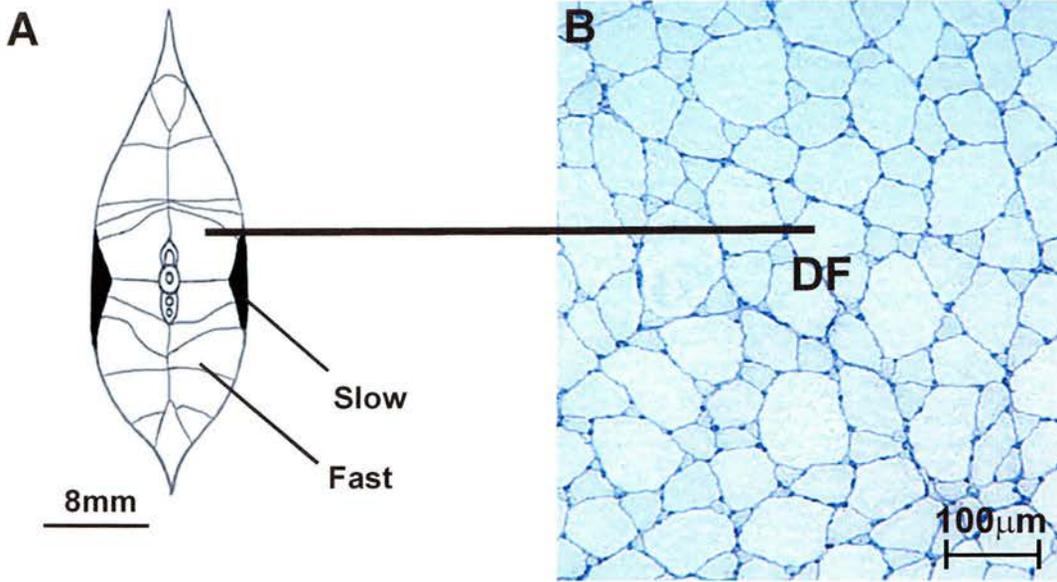


Fig. 2.12. Haematoxylin stained transverse myotomal muscle cryosection from rainbow trout. A) In the trout, two major muscle fibre types were discernable during dissection, fast and slow. B) Haematoxylin staining of $7\mu\text{m}$ thick tissue sections visualised fast muscle fibre cellularity. The image of the haematoxylin stained section is orientated with the dorsal edge upwards ($\times 10$ objective). DF indicates that the fast fibres photographed were deep fast fibres located towards the heart of the myotome.

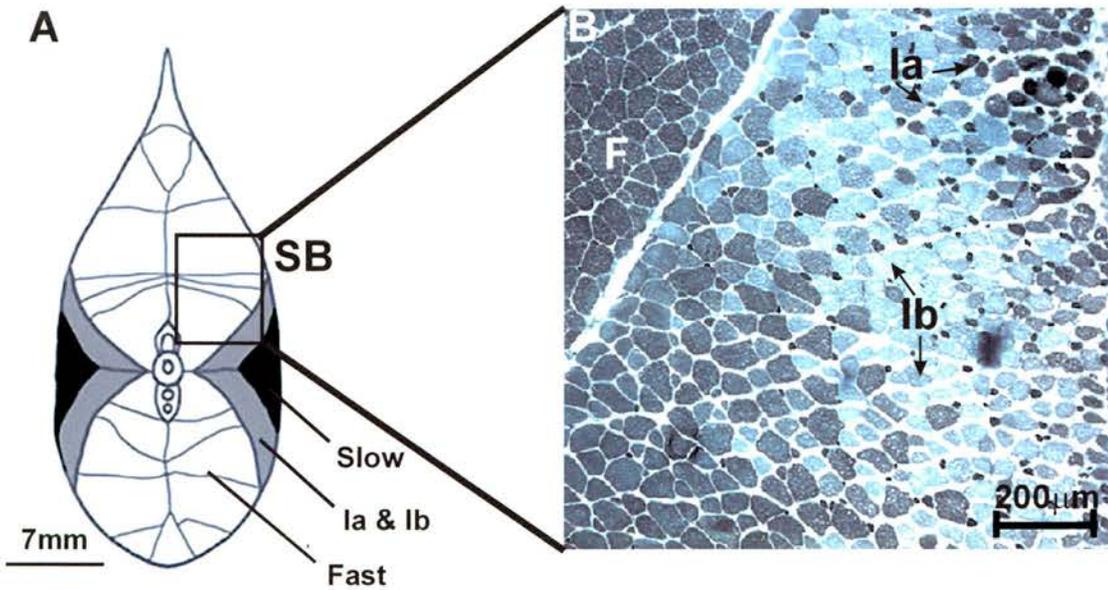


Fig. 2.13. mATPase stained transverse section of myotomal muscle from common carp. A) A block of muscle tissue was taken, immediately dorsal to the horizontal septum, in the right hand epaxial quadrant. Muscle fibre characteristics were examined in a scaled box area (SB) proportional to the total cross-sectional area and height of the myotome. B) A 7µm thick transverse section of carp muscle tissue stained for mATPase activity demonstrated that the fast muscle tissue comprised three broad categories of muscle fibre phenotype: Intermediate type a (la), Intermediate type b (lb) and Fast (F) fibres. In total, four muscle fibre types were defined in the Common carp (A) including the slow muscle fibre phenotype (not shown). The image of the transverse section is orientated with the dorsal edge upwards (**×5 objective**).

Slow muscle fibres appeared darker than Ib fibres but lighter than Ia fibres. Fast muscle fibres stained darker than both slow and Ib fibres, but lighter than Ia fibres. The mATPase histochemical staining profiles of each muscle fibre phenotype is summarised in table 2.3.

2.3.4 Discussion

The haematoxylin histochemical staining technique did not require a great deal of methodological development and was used in all swimming experiments to visualise muscle fibre cellularity. The S58 immunohistochemical protocol required rudimentary development, which involved optimisation of primary and secondary antibody concentrations and the inclusion of a peroxidase-blocking step. The S58 technique was used to identify slow muscle fibres in all swimming experiments except swimming experiment 1 (Chapter 3) and the time course swimming experiment (Chapter 4). The haematoxylin and S58 techniques were used to visualise and distinguish between fast and slow muscle fibres and allow the impact of physical training on muscle growth to be examined.

In contrast, the mATPase protocol involved a large amount of methodological development. Several steps were combined from four different protocols to adapt the mATPase technique for common carp (Padykula & Herman, 1955; Brooke & Kaiser, 1970; Guth & Samaha, 1970; Johnston *et al.*, 1974). The pre-incubation buffer was changed from an alkaline solution (pH 10.1 – 10.6) to an acidic (pH 4.6) sodium acetate/ EDTA buffer. The introduction of a dehydration step and a change of mounting medium from glycerol to Canada balsam allowed for better tissue preservation and visualisation of muscle fibre cellularity. A minor adaptation that should be noted was the continuous mixing of the ATP incubation medium to prevent

the ATP from precipitating out of solution. As a consequence of the time invested in developing this technique, the mATPase histochemical staining protocol was used in only the time course swimming experiment (Chapter 4). In swimming experiments 1 – 3, measurement of intermediate muscle fibres was not performed because there was no way of accurately identifying this fibre phenotype (Fig. 3.4B – D). In swimming experiment 4, the effect of exercise training on intermediate muscle fibres and the contribution of this fibre phenotype to the myotome were considered by dividing fast muscle into two categories: deep fast and superficial fast (Figs. 2.11C – F & 3.4E). This categorisation was made on the basis of anatomical location and haematoxylin staining profile (Table 2.3). Intermediate muscle fibres appeared to be absent from the rainbow trout myotomal musculature and therefore only the deep fast fibres were examined (Fig. 2.12). In conclusion, mATPase staining was used to visualise and distinguish four muscle fibre types in common carp and allow the impact of physical training on muscle fibre phenotype and muscle growth to be examined.

Chapter 3: The effect of exercise on muscle growth and muscle-specific gene expression in the common carp *Cyprinus carpio* L.

3.1 Introduction

The common carp is the world's foremost aquaculture species, accounting for almost 50% of the total output of farmed finfish annually (FAO, 2003). The dominant tissue in the majority of teleost species is the myotomal musculature that accounts for almost three quarters of total body mass (Weatherley & Gill, 1985). Growth of muscle tissue and somatic growth potential in teleosts are intrinsically linked. Skeletal muscle growth in teleost fish can be augmented through manipulation of various environmental and genetic factors. Continuous exercise is another factor capable of stimulating myotomal muscle growth in fish. 'Natural training' in a stream habitat induced an increased mass of aerobic muscle in roach, relative to individuals from a still water environment (Broughton *et al.*, 1978, 1981). Forced continuous low intensity exercise has been used to increase somatic growth in farmed fish. A long-term industrial scale study demonstrated that continuous low intensity exercise (0.4 – 0.45 bls⁻¹) increased muscle mass and somatic growth in Atlantic salmon, primarily through hypertrophic growth of fast muscle fibres (Totland *et al.*, 1987). A shorter period of continuous low intensity endurance exercise (28 days, 1.5 – 4.5 bls⁻¹) was also shown to induce hypertrophy in all muscle fibre types in salmonid species. Slow fibre hypertrophy and recruitment were maximised at a slow swimming velocity (1.5 bls⁻¹) (Davison & Goldspink, 1977; Johnston & Moon, 1980a; Davie *et al.*, 1986),

whereas hypertrophy of intermediate and fast fibres was optimised at a moderate swimming speed (3.0 bls^{-1}) (Davison & Goldspink, 1977).

In contrast to salmonids, there are relatively few examples of swimming studies involving cyprinid species, primarily because of their difficulty to train and also due to their preference for still or slow moving water (Sanger, 1992). However, chub, nase and Danube bleak demonstrated adaptations of the myotomal musculature including hypertrophy of different fibre types and increased production of slow and intermediate muscle fibres in response to prolonged endurance training (13 – 17 weeks) at moderate to fast swimming velocities ($2.0 - 3.5 \text{ bls}^{-1}$) (Hinterleitner *et al.*, 1992; Sanger, 1992). Differences in the adaptive response of each species were attributed to divergence in lifestyle and the swimming behaviour typically employed by these species. In a short-term experiment (28 days), another cyprinid species (goldfish) was subjected to continuous endurance exercise at slow, moderate or fast swimming speeds ($1.5, 3.0$ or 4.5 bls^{-1}). Slow muscle fibre recruitment occurred at the lowest speed and a clear proportional relationship between swimming speed and fast fibre hypertrophy was observed: the largest increase (22%) in fast fibre diameter was observed at the fastest swimming speed (4.5 bls^{-1}), relative to tank rested animals (Davison & Goldspink, 1978). The main objective of swimming experiments 1 to 4 was to develop a reproducible model of fast and/ or slow muscle fibre hypertrophy in the common carp, using forced exercise as the stimulus. Hypertrophy and recruitment of slow, intermediate and fast muscle fibres has been demonstrated in four other cyprinid species in response to endurance exercise. Based on these findings, fast and slow muscle fibre hypertrophy and/ or recruitment would be expected in response to slow to moderate endurance exercise training in common carp.

Vertebrate skeletal muscle is a highly specialised tissue, composed of post-mitotic terminally differentiated syncytial myocytes that are reliant on an external source of nuclei for growth. Satellite cells or myogenic progenitors are a pool of quiescent undifferentiated precursors that become activated, proliferate, express myogenic markers and differentiate to fulfil this function. Ultimately, differentiated satellite cell progeny are incorporated into existing cells (hypertrophy) or fuse together to form new muscle fibres (hyperplasia) (reviewed by Hawke & Garry, 2001; Zammit & Beauchamp, 2001). Activity of satellite cells is influenced by physical training, muscle growth or injury (reviewed by Antonio & Gonyea, 1993), nutritional status (Hansen-Smith *et al.*, 1978, 1979; Fauconneau & Paboeuf, 2000; Brodeur *et al.*, 2003b), mitogenic growth factors (McFarland, 1999), developmental temperature (Halevy *et al.*, 2001) and environmental temperature (Brodeur *et al.*, 2003a). Concomitant increases in the number of satellite cells and myonuclei can be induced by hypertrophic stimuli such as strength training (Kadi & Thornell, 2000). In swimming experiment 4 there was an additional objective to the development of the exercise overload model of muscle fibre hypertrophy. The effect of exercise on myonuclear density in isolated fast muscle fibres was also examined. If the teleost response to a chronic exercise overload stimulus paralleled that of higher vertebrates, an increase in the density of myonuclei per unit fibre length would be observed.

As in higher vertebrates, fish muscle fibre hypertrophy is mediated by myogenic progenitor cell activation, proliferation and fusion of differentiated cells to existing muscle fibres (Koumans & Akster, 1995). In large fast-growing teleost species, the activity of myogenic progenitors is thought to sustain the process of hyperplastic growth throughout much of adult life, which facilitates the attainment of a large ultimate body size (Weatherley & Gill, 1987; Koumans & Akster, 1995;

Stoiber & Sanger, 1996). The process of myogenic progenitor cell activation, proliferation and differentiation is characterised and regulated by the sequential expression of members of the family of muscle-specific myogenic regulatory transcription factors (MRFs) (reviewed by Watabe, 2001).

The MRF family of bHLH transcription factors includes MyoD, myf5, myogenin and myf6. MyoD and myf5 are the primary MRFs, involved in the determination of proliferating satellite cells, whereas myogenin and myf6 are the secondary MRFs that regulate differentiation and fusion of myoblasts to form multinucleated myotubes (Megeney & Rudnicki 1995; Rudnicki & Jaenisch, 1995). Transgenic and knockout mice have illustrated the central role of MRFs in myogenesis (Hasty *et al.*, 1993; Rudnicki *et al.*, 1993; Zhang *et al.*, 1995). Combinatorial protein-protein interactions between MRFs and members of the myocyte enhancer factor-2 (MEF2) family of MADS-box transcription factors are necessary for differentiation of skeletal muscle myocytes (reviewed by Black & Olson, 1998). The basic region of the MRFs and MADS-box domain of the MEF2 family mediate DNA binding with consensus elements (E-box and A/T rich domains respectively) present in close proximity in the promoter/enhancer regions of numerous muscle-specific genes, including desmin, troponin I and muscle creatine kinase (Gossett *et al.*, 1989; Davis *et al.*, 1990; Molkentin *et al.*, 1995).

In addition to fulfilling a key function in differentiation, there is also evidence that implicates MRFs in fibre-type specification and regulation of muscle fibre hypertrophy in mature skeletal muscle tissue. Differing levels of MRFs are observed in phenotypically distinct fibre types; protein and transcripts of MyoD and myf5 preferentially accumulate in fast-twitch fibres, with myogenin primarily expressed in slow-twitch fibres (Hughes *et al.*, 1993; Voytik *et al.*, 1993; Sakuma *et al.*, 1999). In

mammals, nuclear localisation of MyoD protein and transcript accumulation correlated strongly with type IIb glycolytic muscle fibres, suggesting a function of MyoD is the development and maintenance of fast IIb fibres (Hughes *et al.*, 1997; Kraus & Pette, 1997). Manipulation of fibre phenotype through cross-reinnervation, thyroid hormone administration or denervation caused parallel changes in MRFs expression (Hughes *et al.*, 1993; Voytik *et al.*, 1993). Upregulation of oxidative and glycolytic myosin heavy chain isoforms in response to exercise training in humans was associated with specific upregulation of myogenin and MyoD transcripts in respective fibre phenotypes (Willoughby & Nelson, 2002). Additional hypertrophic stimuli such as stretch (Lowe & Alway, 1999), surgically induced functional overload (Adams *et al.*, 1999; Sakuma *et al.*, 1999) and electromyostimulation (Bickel *et al.*, 2003) have been demonstrated to increase the expression of MRFs in existing myofibres, which implies a role for MRFs in the regulation of muscle fibre hypertrophy. In teleosts, an increase in the proportion of myogenic progenitor cells positive for MyoD and myogenin was shown to increase in response to a single feeding event in a sub-antarctic notothenioid (Brodeur *et al.*, 2003b). An upregulation of myogenin transcripts was observed in rainbow trout 12 days after a refeeding event when recovering from a 10-week period of fasting (Chauvigné *et al.*, 2003). These findings suggest a role for MRFs in the mature myotomal musculature of fish during normal growth. An important objective of swimming experiments 2 and 3 was to investigate the expression of MRFs during normal muscle growth and in response to exercise in common carp. Based on the observation that MRFs are upregulated in mammals and teleosts in response to overloading and feeding stimuli, an upregulation and/ or increased nuclear localisation of MRFs would be expected in response to an exercise stimulus in common carp. A secondary objective involved the examination

of MRFs expression in fast and slow muscle total cellular protein extracts from non-exercised fish, and hence the potential function of MRFs in fibre-type specification. If the teleost situation paralleled that of higher vertebrates, the primary MRFs (MyoD and myf-5) would be preferentially expressed in fast muscle tissue extracts and a secondary MRF (myogenin) would be more highly expressed in slow muscle tissue.

In higher vertebrates, the calcium/calmodulin dependent protein phosphatase calcineurin has been implicated in the regulation of hypertrophic growth in skeletal muscle on the basis of *in vitro* (Musaro *et al.*, 1999; Semsarian *et al.*, 1999) and *in vivo* (Dunn *et al.*, 1999) studies. The high level of conservation observed in the protein sequence and the ubiquitous expression of this enzyme implies that the function of this enzyme might be conserved between diverse evolutionary groups (reviewed by Guerini, 1997; reviewed by Sugiura *et al.*, 2001). Calcineurin is selectively activated by sustained increases in intracellular Ca^{2+} levels, which in skeletal muscle leads to dephosphorylation of nuclear factor of T-cells 2 (NFAT2) and nuclear localisation of the calcineurin/NFAT2 complex (Shaw *et al.*, 1995; Dolmetsch *et al.*, 1997). The nuclear association of calcineurin and NFAT2 synergistically initiates a programme of gene expression leading to hypertrophy (Musaro *et al.*, 1999; Semsarian *et al.*, 1999). In addition to its role in skeletal muscle hypertrophy, the calcineurin-signaling pathway is a putative regulator of several other processes in skeletal muscle: the regulation of myf5 gene expression in the reserve cell population of myotube cultures (Friday & Pavlath, 2000); the initiation of skeletal muscle differentiation through activation of MEF2 and MyoD (Friday *et al.*, 2003); nerve and activity-dependent specification of muscle fibre phenotype through dephosphorylation of NFAT and MEF2 transcription factors (Chin *et al.*, 1998; Bigard *et al.*, 2000; Delling *et al.*, 2000; Naya *et al.*, 2000; Allen & Leinwand, 2002, Serrano *et al.*, 2001);

and determination of primary myofibre number during development (Kegley *et al.*, 2001). The final objective of swimming experiments 2 and 3 was to examine the potentially conserved role of calcineurin signaling in the regulation of muscle growth in common carp. Increased nuclear localisation of calcineurin and NFAT2 proteins, and upregulation of MEF2 family transcription factors would be expected in association with exercise-induced hypertrophic muscle growth in common carp.

3.2 Materials and methods

3.2.1 Experimental animals

Common carp were purchased from Humberside Fisheries (Cleaves Farm near Driffield, East Yorkshire, UK) for all swimming experiments. The fish were stored in an outdoor holding pond at ambient temperature (swimming experiment 1) or maintained in freshwater aquaria at 20°C (swimming experiments 2 – 4) until required. Carp were fed to satiation once daily (approx. 3 – 5% body mass) with a species-specific commercially available brand of fish food (Trouw Aquaculture). Experimental groups had access to food for the same length of time each day. At the end of the feeding period, uneaten food was siphoned from the tanks to prevent food consumption in non-exercised fish whilst the exercise group was swimming. A brief summary of the exercise regime, water temperature, photoperiod and the time of year of each experiment was conducted is given in Table 3.1.

3.2.2 Swimming experiment 1

Twelve common carp were split into two equal groups, approximately size-matched for initial fork length. Starting body mass was not measured before swimming experiment 1. Prior to the experiment, the mean (\pm standard error, S.E.M.)

fork length of the non-exercise group was 3.8 cm (± 0.3), compared with 3.7 cm (± 0.2) in the exercise group. The tank rested group were placed in a solid-based cage suspended in the holding pond. The exercise group were introduced into the outdoor flume apparatus (Fig. 3.1). The outdoor flume was positioned above the ambient temperature holding pond, which in effect became the reservoir tank. After two days of acclimatisation to flume conditions at very low flow rate, the baffle was lowered to increase flow velocity to $8 \text{ cm}\cdot\text{s}^{-1}$, which corresponded to 2.1 – 2.3 body lengths per second (bls^{-1}). Flow velocity in this and subsequent experiments was measured using a Nixon Flowmeter probe and Novar Streamflo measuring device (Novonic Instruments, Gloucestershire, UK). Fish were exercised continuously for 23 hours a day and allowed to feed during the remaining hour. After 21 days both groups were sacrificed and samples were taken for analysis of muscle fibre cellularity. This experiment was conducted in November 1999.

3.2.3 Swimming experiment 2

Twelve common carp were divided into two equal groups on the basis of fork length, and moved from the aquarium to the heated (20°C) outdoor holding pond, where water temperature was maintained with a 5kW heater. Starting body mass was not measured before swimming experiment 2. Prior to the experiment, mean fork length of the non-exercise group was 5.3 cm (± 0.4), compared with 5.5 cm (± 0.3) in the exercise group. The apparatus from swimming experiment 1 (Fig. 3.1) was positioned over the heated holding pond. After two days of becoming accustomed to flume conditions at low flow levels, the flow speed was increased to $10 \text{ cm}\cdot\text{s}^{-1}$, equivalent to 1.7 – 1.9 bls^{-1} . Fish were trained for 16 hours per day and allowed to feed and rest in the remaining 8 hours.

Table 3.1. Summary of environmental conditions and experimental treatments in swimming experiments 1 – 4.

Experiment	Mean Water Temperature (°C)	Photoperiod (L, light; D, dark; hours)	Water Flow (cm.s ⁻¹)	Mean Group Swimming Speed (bls ⁻¹)	Daily Exercise (E)/Rest (R) Regime	Duration Of Experiment (days)	Total Distance Swum (km)	Time of experiment	N
Swimming Experiment 1	8.9	8L: 16D	8	2.1 – 2.3	23E: 1R	21	139	November 1999	6
Swimming Experiment 2	21.4	16L: 8D	10	1.7 – 1.9	16E: 8R	21	121	May 2000	6
Swimming Experiment 3	21.7	14L: 10D	30	2.6 – 2.7	14E: 8R	28	423	October 2000	9
Swimming Experiment 4	14.5	14L: 10D	30	2.6 – 2.7	14E: 8R	28	423	April 2002	9

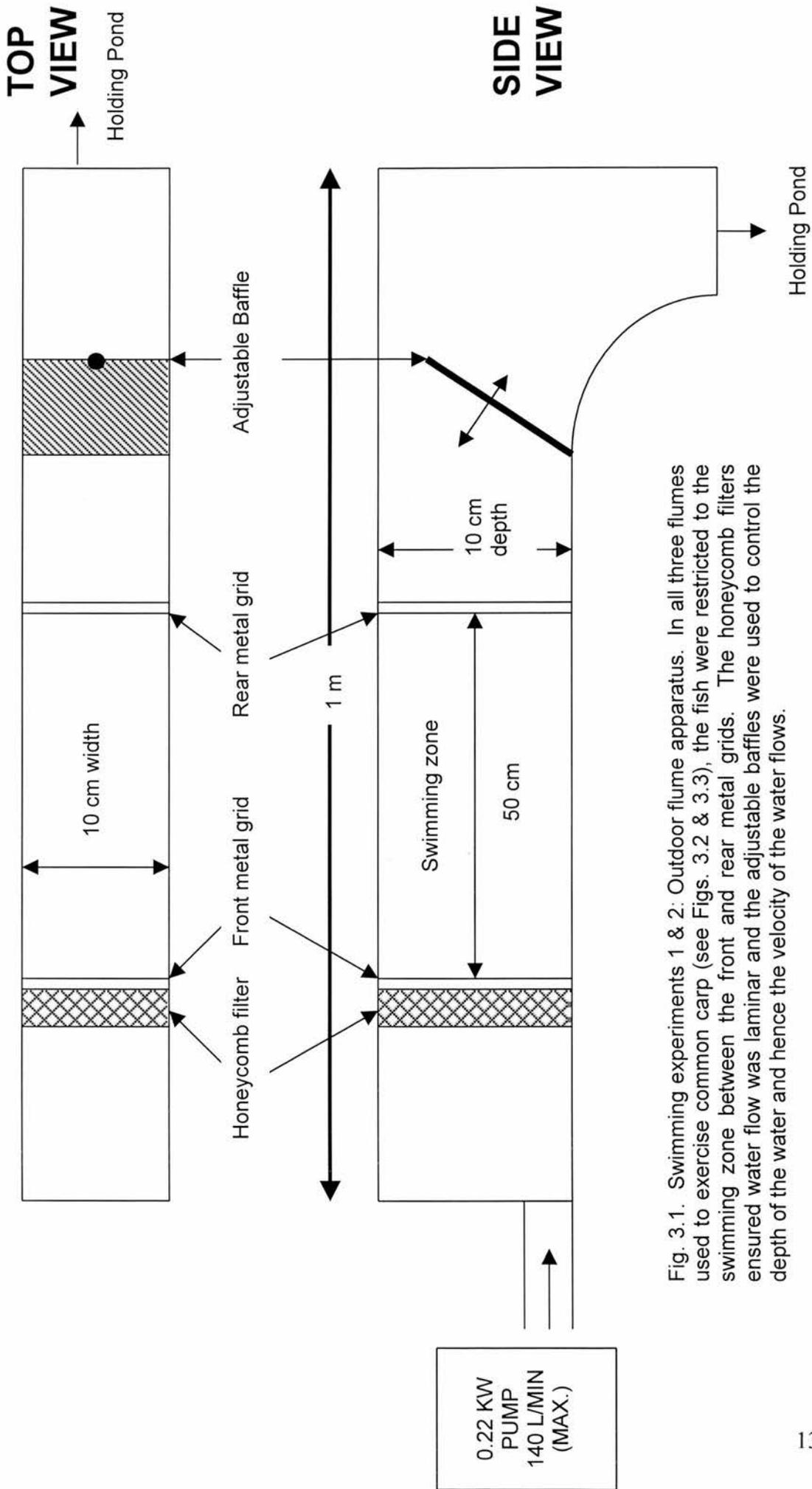


Fig. 3.1. Swimming experiments 1 & 2: Outdoor flume apparatus. In all three flumes used to exercise common carp (see Figs. 3.2 & 3.3), the fish were restricted to the swimming zone between the front and rear metal grids. The honeycomb filters ensured water flow was laminar and the adjustable baffles were used to control the depth of the water and hence the velocity of the water flows.

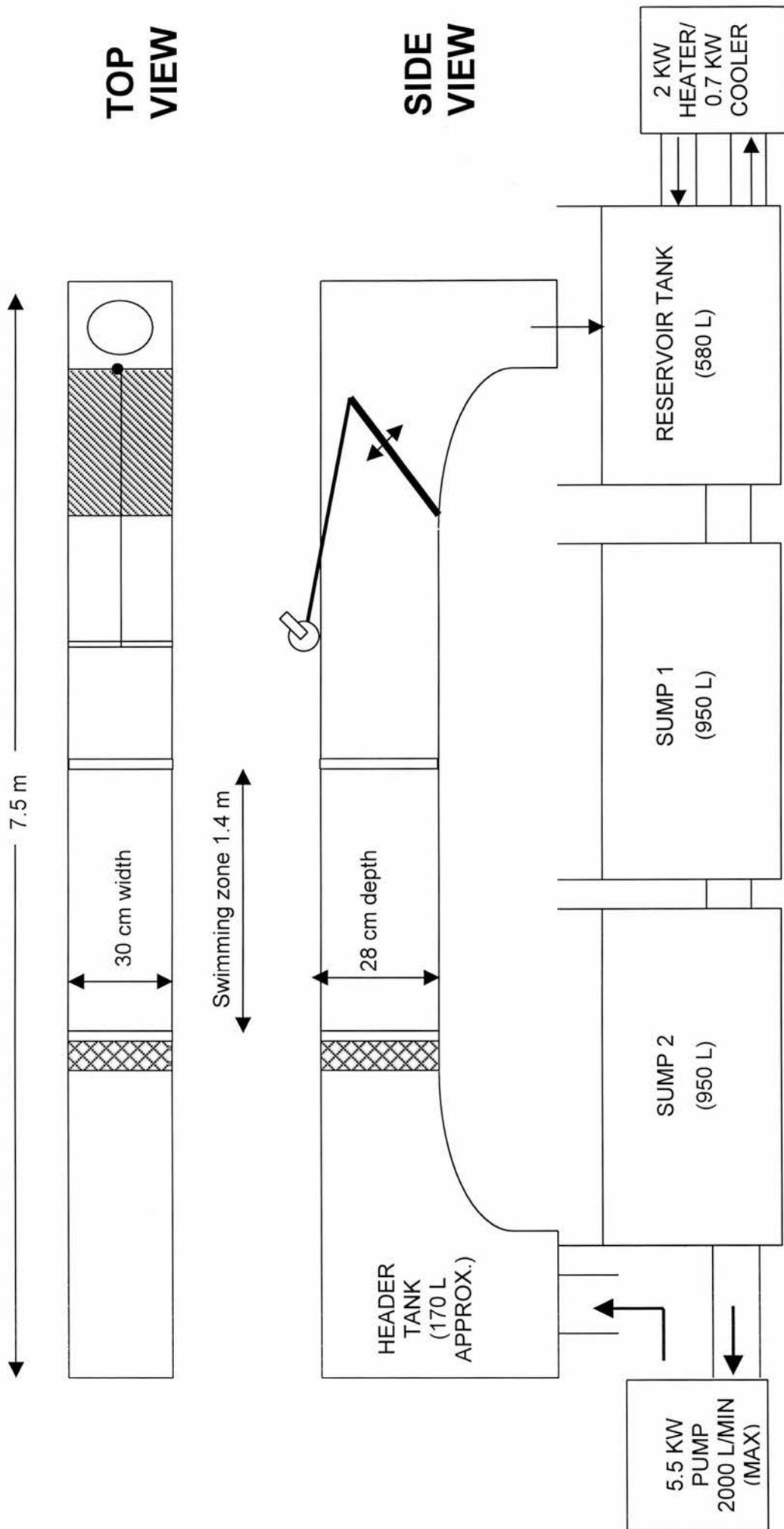


Fig. 3.2. Swimming experiment 3: 7.5 m Armfield tilting flume apparatus

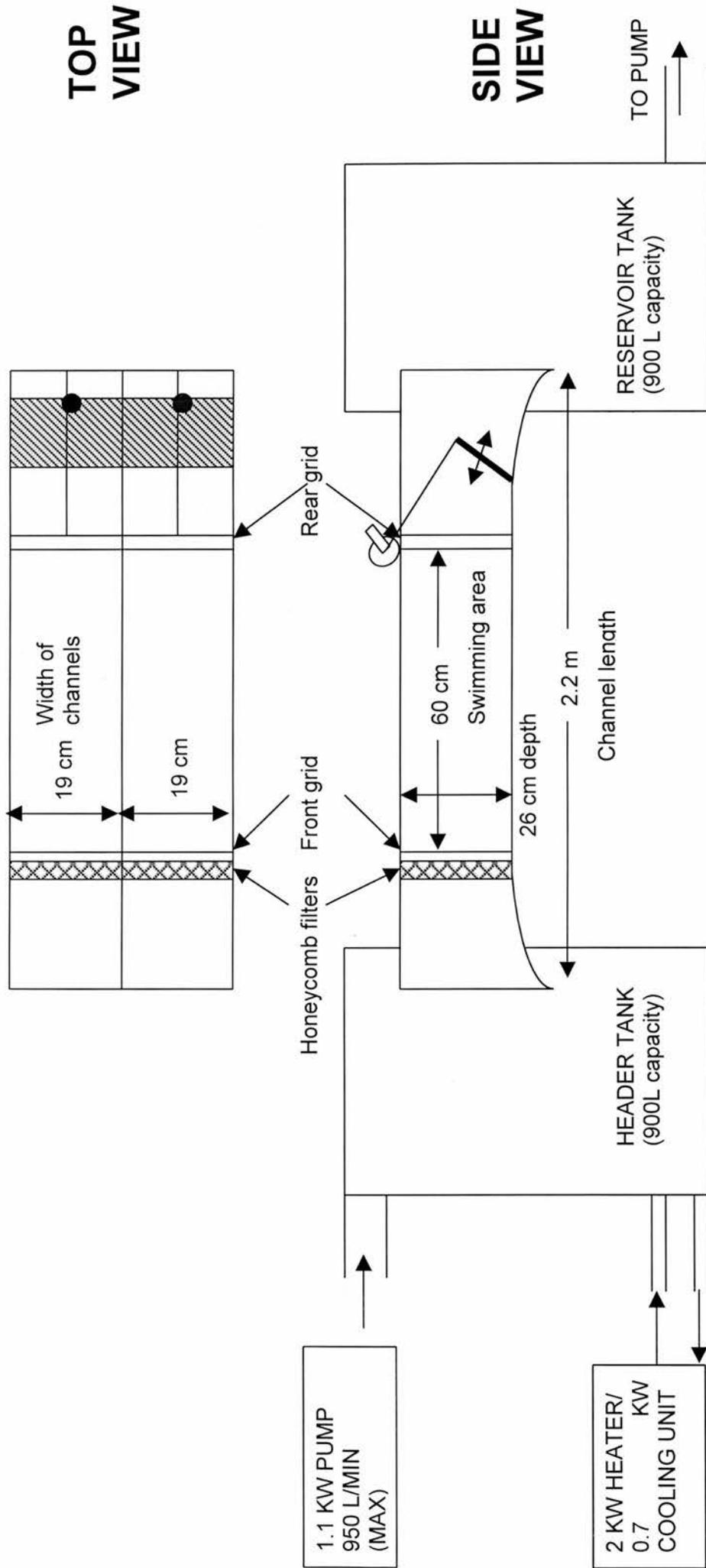


Fig. 3.3. Swimming Experiment 4: Custom-built double channelled aquarium flume

This regime lasted for 21 days, at the end of which all specimens were sacrificed and muscle samples taken for histological and protein analyses. This experiment was conducted in May 2000.

3.2.4 Swimming experiment 3

Eighteen common carp were anaesthetised with MS222 and a PIT-tag (AVID Inc., Norco, California) was injected into the intra-peritoneal cavity of each animal to allow identification of individual fish. Fork length and body mass were measured every four weeks to give an indication of growth rate in individual fish in the 8 weeks leading up to the experiment. These measurements were used as a means of dividing the fish into closely size-matched groups. Prior to the swimming experiment, mean fork length and body mass of the non-exercise group were 11.4 cm (± 0.2) and 44.9 g (± 1.8), compared with 11.4 cm (± 0.2) and 45.6 g (± 2.1) in the exercise group. The groups were then placed in two holding tanks on top of the sump tanks for the Armfield S6 tilting flume apparatus (Fig. 3.2). After acclimatising to the new conditions for several days, the exercise group was introduced into the swimming channel of the flume at a low flow velocity. The water speed was gradually increased to 30cm s^{-1} , (equivalent to $2.6 - 2.7\text{ bl s}^{-1}$). The forced exercise period was 14 hours in this experiment. At the end of this period, the carp were netted and placed back in the holding tank, where they fed and rested over the next 10 hours. The exercised group was trained in this manner for a period of 28 days, after which both groups were sacrificed and samples taken for muscle fibre and protein expression analyses. This experiment was conducted in October 2000.

3.2.5 Swimming experiment 4

Eighteen common carp were anaesthetised, PIT-tagged and measurements of fork length and body mass taken. The fish were divided into groups for tank rest and exercise treatments and separated into the two swimming channels of the aquarium flume (Fig. 3.3). Prior to the experiment, mean fork length and body mass of the non-exercise group were 11.4 cm (± 0.2) and 43.4 g (± 1.6), compared with 11.4 cm (± 0.2) and 43.8 g (± 2.0) in the exercise group. After several days, the water flow in one swimming channel was increased to 30cms⁻¹ (equivalent to 2.6 – 2.7 bls⁻¹), whilst the extremely low flow situation was maintained in the other channel. This was achieved by increasing the overall rate of pumping, lowering the baffle in the swimming channel and raising the baffle in the channel occupied by the tank rested group. Fish were exercised for 14 hours per day, then fed and rested in the remaining 10 hours. After 28 days of such treatment, all fish were sampled for muscle fibre analysis. This experiment was conducted in April 2002.

3.2.6 Fish Sampling

At the conclusion of each experiment, fish were immediately sacrificed by administration of an overdose of anaesthetic (MS222, tricane methanesulphonate, SIGMA), before severing the spinal cord (Schedule 1 Killing, Home Office Regulations). Each fish was given a unique sampling number before fork length and body mass was measured. Fish were then placed in individual polythene bags and packed in ice until dissection. Body mass (BM) was measured to the nearest tenth of a gram; fork length (FL) was measured to the nearest millimetre. The following equation was used to calculate the condition factor (CF) of each fish: $CF = 100BM/FL^3$.

A total cross-sectional area (steak) was dissected from each fish at the level of the anal pore (Fig. 3.4A) and samples were prepared for histochemistry and immunohistochemistry using a previously described protocol (sections 2.3.2.2 & 2.3.2.3). The outline of the steak was traced onto a clear acetate sheet, to give the total muscle cross-sectional area. In swimming experiment 4, digital photographs were taken of each steak to improve the accuracy of this measurement. In swimming experiments 1 and 2 the steak of muscle tissue was frozen as a whole block (Fig. 3.4B & C). In swimming experiment 3, the right hand side (RHS) of the steak was divided into 2 blocks and frozen separately (Fig. 3.4D, 1 & 2). In swimming experiment 4 the RHS of the myotome was cut into 4 blocks before cryopreservation (Fig. 3.4E, 1 – 4).

3.2.7 Muscle fibre morphometry

Morphometric analysis was performed for each experimental subject to gauge the effect of exercise or tank rest on muscle growth. In swimming experiments 1 to 4, three broad phenotypes of muscle fibre were recognised for the purposes of morphometric analysis. The previously described histochemical (Mayer's Haematoxylin, section 2.3.2.4) and immunohistochemical (S58, section 2.3.2.5) staining protocols were used to identify fast and slow muscle fibre phenotypes in common carp (Fig. 2.11). Slow muscle fibres were defined as those that positively cross-reacted with the S58 antibody (Fig. 2.11B). The rest of the myotome was made up of fast muscle fibres, which were divided into superficial fast (SF) and deep fast (DF) categories (Fig. 2.11C – F). The effect of exercise on the growth of deep fast fibres was examined in swimming experiments 1 – 4.

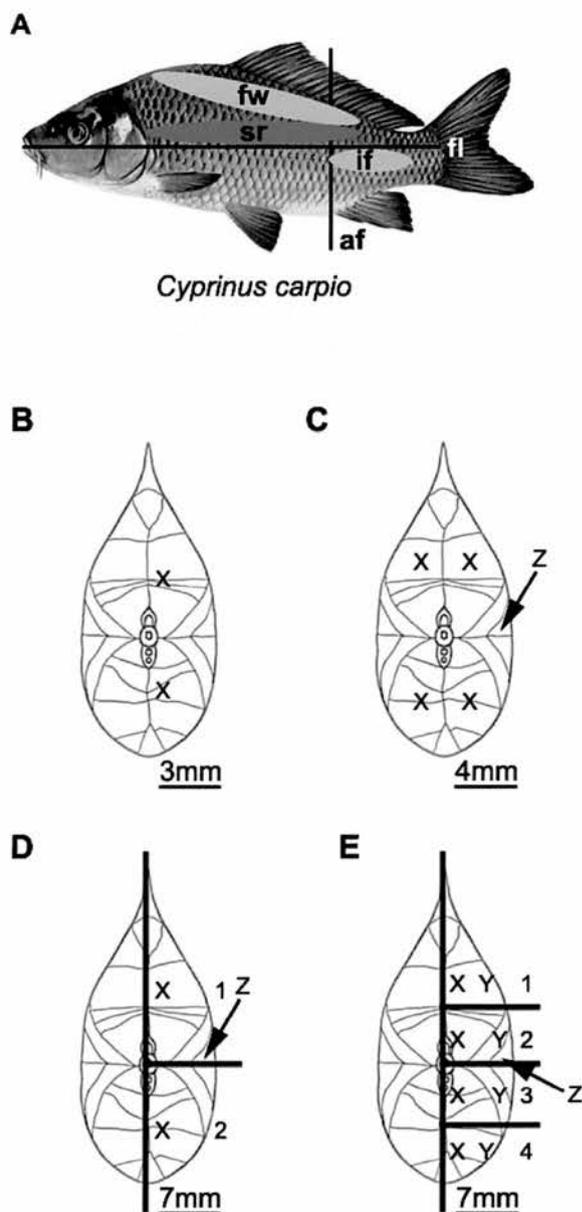


Fig. 3.4. Common carp were exercised or tank rested for various time periods depending upon the experiment. Fork length (A, **fl**) was measured from snout to the most posterior region of the body. Total cross-sectional area (T_{CSA}) was measured from a 5mm thick steak, cut immediately anterior to the anal fin (**af**). Fast white (**fw**) and slow red (**sr**) muscle tissue samples were taken from the anatomical regions indicated. In swimming experiments 1 to 4 (B – E), muscle fibre cross-sectional areas (F_{CSA}) were measured at the regions of the myotome marked X (deep fast fibres), Y (superficial fast fibres) and Z (slow fibres). Scale bars = half the width of the fish at the horizontal septum and indicate the different size of fish involved in swimming experiments 1 – 4. In swimming experiment 4, isolated fast muscle fibres were dissected from the region indicated (**if**). (Common carp illustration by N. Weaver, Migdalski & Fichter, 1977, p.143).

Superficial fast muscle fibres occupied a small proportion of the total fast muscle cross-sectional area (4 – 6%) and the effect of exercise on the growth of these fibres was only considered in swimming experiment 4. Growth of slow muscle fibres was measured in swimming experiments 2 to 4.

Transverse sections stained with Mayer's Haematoxylin reagent (Fig. 2.11C – F) were photographed with a frame capture camera (JVC, TK-F7300U) connected to a light microscope with a $\times 10$ objective. Images of random fields of view were taken of deep fast (X), superficial fast (Y) and slow (Z) muscle fibres at pre-determined areas of the myotome (Fig. 3.4B – E). The number of muscle fibres measured at each point (X, Y or Z) and the total number of each fibre type measured in the different experiments is summarised in Table 3.2. Muscle fibre cross-sectional areas were measured using image analysis software: in swimming experiment 1, Tema 99 software was used (Tema Scan Beam, v. 1.0); all subsequent experiments utilised Sigma Scan Pro software (v. 5.0.0, SPSS Inc.). Muscle fibre diameter (D) was expressed as the diameter of the equivalent circle from the measurement of muscle fibre cross-sectional area (A): $D = 2(A/\pi)^{0.5}$.

The total number of fast muscle fibres (N) was estimated from the measurements of total fast fibre cross-sectional area (T_{CSA}) and mean fast fibre area (F_{CSA}): $N = T_{CSA}/F_{CSA}$. T_{CSA} was measured from the acetate sheet tracing by scanning the image into Sigma Scan Pro image analysis software. In swimming experiment 2, total slow fibre number was estimated by the same method: $SN = ST_{CSA}/SF_{CSA}$. In swimming experiment 4, an improvement in the estimation of total fast fibre number was made by taking into account the contribution of superficial fast fibres and measuring the T_{CSA} from digital photographs. The mean area of the T_{CSA} that comprised superficial fast fibres was 6% in both experimental groups. In each

subject, area measurements of 72 randomly selected superficial fibres ($72/1200 = 6\%$) were combined with 1200 area measurements of deep fast fibres. The combined F_{CSA} was then used to estimate total fast fibre number.

3.2.8 Nuclear density of isolated fast muscle fibres

In swimming experiment 4, a thin strip of fast muscle tissue spanning several myotomes (approx. 1.5 cm in length) was dissected from a region ventral to the lateral line immediately posterior to where the 5mm thick steak was taken (Fig. 3.4A, **if** - isolated fibres). The tissue was secured to a block of silicone elastomer gel (RS Components, 409-5715) with 10mm insect pins and fixed overnight in 4% paraformaldehyde in PBS. After several washes in PBS, the tissue was stored at 4°C in PBS containing 0.5% sodium azide as a preservative.

The thin tissue slice was equilibrated in 2×SSC (300mM NaCl, 30mM sodium citrate, pH 7.4) for 5 minutes. Watchmaker's forceps were used to gently tease apart bundles of fibres, until 20-30 fibres were isolated. Fibres were placed on a glass slide and circumscribed using an ImmEdge™ pen (Vector Labs, CA, USA). SYTOX Green nucleic acid stain (150µM in DMSO, Molecular Probes, C-7590) was diluted to a 1:300 concentration in 2×SSC. Isolated fibres were incubated in the Sytox Green working solution for 5 minutes, rinsed 3 times in 2×SSC and excess buffer was drained from the slide. Fluorescent mounting medium (DAKO, S3023) was used to mount coverslips. A small amount of silicone grease (RS Components, 494-124) was placed under each corner of the coverslip to prevent flattening of the fibres.

Isolated fibres were examined using the Bio-Rad Radiance 2000 Laser Confocal microscope system and Bio-Rad Lasersharp 2000 software. From each experimental subject, 12 muscle fibres were selected which were cleanly dissected

and the fluorescent green nuclei clearly labelled. The Laserssharp 2000 application was used to take a z-series through each muscle fibre at 2 μ m intervals, to allow the visualisation of all myonuclei. A composite image of the z-series for each muscle fibre was examined using Bio-Rad Laserpix image analysis software (Version 4.0.0.13). The mean of three width measurements was taken as a measure of muscle fibre diameter and muscle fibre length was also measured. The number of myonuclei was measured per cm of muscle fibre length. The data for all muscle fibres in each experimental group was pooled to evaluate the overall effect of exercise on myonuclear density.

3.2.9 Protein expression analysis

In swimming experiment 2, nuclear protein extracts were prepared from fast muscle tissue using a previously described technique (section 2.2.2.4). In swimming experiment 3, nuclear and total protein extracts were prepared from fast and slow muscle tissue (sections 2.2.2.3 & 2.2.2.4). Calculating the protein concentration in each extract, resolving all extracts by SDS-PAGE, and staining with Coomassie Brilliant Blue G for comparison, was a prerequisite to equal gel loading of samples (sections 2.2.2.5 – 2.2.2.7). Optical density measurements were made of Coomassie stained gels for each sample to evaluate protein loading, using Bio-Rad Quantity One (version 4.4.1) image analysis software. Optimised Western blotting techniques were employed to assess the effect of exercise on muscle specific gene expression (section 2.2.2.9), in swimming experiments 2 and 3. The optimised concentrations of primary antisera are given in Tables 2.1 and 2.2.

In each protein analysed, every sample was loaded in triplicate to give a mean optical density for the expression of that protein in an individual fish. Tank rested and exercised samples were loaded in alternate lanes to prevent loading order from

influencing the final result. Replicating each fish three times involved multiple gels and membranes; to correct for inevitable differences in transfer efficiency, the mean optical density for all samples on a membrane was compared between membranes. A correction factor was then applied to optical density measurements to reduce variation between membranes due to transfer dissimilarities.

3.2.10 Statistical analysis

The Anderson-Darling test of normality was first used to assess the distribution of the data (Minitab v. 13.2). Analysis of covariance was used to compare morphological and muscle fibre characteristics between experimental groups, using fork length as the covariate (Zar, 1996). 1st order linear regressions were plotted for the above variables against fork length. The final condition factors in swimming experiments 1 and 2 were compared using a two-tailed T-test and a Mann-Whitney Rank Sum test respectively. In swimming experiments 3 and 4, a two-way mixed design analysis of variance was used to compare condition factor between and within subjects over the experimental period.

Non-parametric smoothing and bootstrapping techniques were employed to compare distributions of muscle fibre diameters between experimental groups. The methods of Johnston *et al.* (1999) based on those of Silverman (1986) and Bowman and Azzalini (1997) were applied; a smooth kernel function was used to obtain an estimated probability density function of muscle fibre diameters for each experimental group. 100 bootstrap estimates of the distribution of the combined population were made. Experimental groups were considered to be significantly different if regions of the smooth probability density functions lay outwith the area of 100 bootstrap estimates. An in-house statistical program written in the open source software R (Johnston *et al.*, 1999) was used to generate smooth probability density functions and

bootstrap estimates. A non-parametric Kolmogorov-Smirnov test was used to compare the distributions. The 5th, 10th, 50th, 95th and 99th percentiles of fibre diameter were calculated from the fitted curves. Percentiles were compared using the Mann-Whitney Rank Sum test if the distributions were found to be significantly different.

For protein expression, the group mean optical density \pm S.E.M. is reported as the percentage of the tank rested group. For western blots and Coomassie stained gels, comparisons of optical density between experimental groups were made using a two-tailed T-test or a Mann-Whitney Rank Sum test. The Spearman Rank Correlation Coefficient (r_s) was calculated to indicate correlation between the level of nuclear localisation of particular proteins and mean muscle cross-sectional area.

3.3 Results

3.3.1 Swimming experiment 1

Common carp were exercised for a 21 day period, at 2.1 – 2.3 bls⁻¹ for 23 hours daily, or confined to a tank for the duration of the experiment. At the end of the experimental period, mean fork length and body mass of the tank rested fish were marginally higher than those observed in the exercised group (Table 3.3), however the difference in body morphology was not significant (Fig. 3.5A). Mean fast fibre cross-sectional area in the tank rested group was 584 μm^2 , compared to 390 μm^2 observed in the exercised fish (Fig. 3.5B). The 33% smaller fast fibre cross-sectional area in the exercised group was significantly different from the tank rested controls ($F_{1,9} = 18.7$, $P < 0.01$, ANCOVA).

Table 3.2. Swimming experiments 1 - 4: summary of the number of each muscle fibre phenotype measured

Experiment	Number of deep fast fibres measured at each X	Total number of deep fast fibres measured	Number of superficial fast fibres measured at each Y	Total number of superficial fast fibres measured	Number of slow muscle fibres measured at each Z	Total number of slow fibres measured
Swimming Experiment 1	500	1000				
Swimming Experiment 2	400	1600			1000	1000
Swimming Experiment 3	750	1500			750	750
Swimming Experiment 4	300	1200	300	1200	1200	1200

Table 3.3. Swimming experiment 1: summary of muscle fibre data in the tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, figures in brackets show percentage difference between the groups, N=6.

Experimental Treatment	Initial Fork Length (cm)	Fork Length (cm)	Body Mass (g)	Condition Factor	F _{CSA} (μm^2)	Fibre Diameter (μm)
TR	3.8 \pm 0.3	3.9 \pm 0.2	1.7 \pm 0.2	2.8 \pm 0.1	584 \pm 40	24.7 \pm 0.8
E	3.7 \pm 0.2 (2.6)	3.7 \pm 0.3 (6.0)	1.5 \pm 0.3 (10.7)	2.9 \pm 0.1 (2.5)	390 \pm 14 (33.3)	19.8 \pm 0.4 (19.7)

Several areas of the average probability density functions of fast fibre diameter distribution for each experimental group were outside the grey shaded area of 100 bootstrap estimates of the total population (Fig. 3.6A), which suggested that there was a significant difference in the density distributions of the experimental groups ($P < 0.01$, Kolmogorov Smirnov). The average of the 5th, 10th, 50th, 95th and 99th percentiles of fibre diameter were significantly greater in the tank rested group relative to the exercised fish (Fig. 3.6B).

3.3.2 Swimming experiment 2

Common carp were exercised for a 21 day period, at 1.7 – 1.9 bls⁻¹ for 16 hours daily, or confined to a tank for the duration of the experiment. Mean fork length and body mass of the exercised group were 7% and 41% higher than those observed in tank rested controls at the end of the 21 day experimental period, however the difference in body composition was not statistically significant (Fig. 3.7A). The mean condition factor of the exercised group was significantly higher than the tank rested group (Table 3.4), indicating that for a given fork length exercised fish had a greater body mass ($P < 0.05$, Mann-Whitney Rank Sum Test). The total fast fibre cross-sectional area was 31% greater in the exercised carp ($F_{1,9} = 21.8$, $P < 0.01$, ANCOVA) and the number of fast muscle fibres was also 40% higher in this group ($F_{1,9} = 59.0$, $P < 0.01$, ANCOVA) compared to the tank rested controls (Fig. 3.7B & C). In common with swimming experiment 1, mean fast muscle fibre area (Fig. 3.7D) was larger in the tank rested controls, but in this case the difference was not statistically significant ($F_{1,9} = 4.3$, $P < 0.1$, ANCOVA).

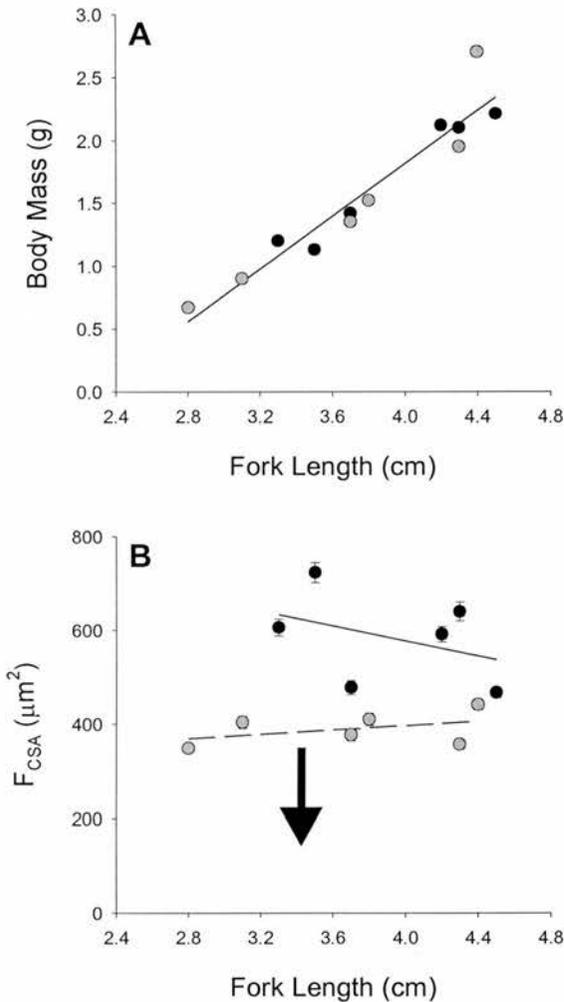


Fig. 3.5. Swimming experiment 1: the effect of tank rest or a three-week exercise-training regime ($2.1 - 2.3 \text{ bls}^{-1}$, 23 hours per day) on body mass (BM) and mean fast muscle fibre cross-sectional area (F_{CSA}) relative to fork length (FL). A) The body mass of exercised (E, grey symbols) and tank rested (TR, black symbols) groups was not significantly different post-training (NS, ANCOVA). B) Mean fast fibre cross-sectional area was significantly smaller in exercised fish compared to tank rested controls ($P < 0.01$, ANCOVA). NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: A) TR & E: $\text{BM} = -2.4 + 1.1 \text{ FL}$, $R^2 \text{ adj.} = 0.90$, $\text{df} = 10$, $P < 0.001$. B) TR: $F_{\text{CSA}} = 897 - 80 \text{ FL}$, $R^2 \text{ adj.} = 0.16$, $\text{df} = 4$, $P > 0.4$; E: $F_{\text{CSA}} = 304 + 23 \text{ FL}$, $R^2 \text{ adj.} = 0.18$, $\text{df} = 4$, $P > 0.4$. The arrow indicates the trend of a smaller mean F_{CSA} in the exercised group. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

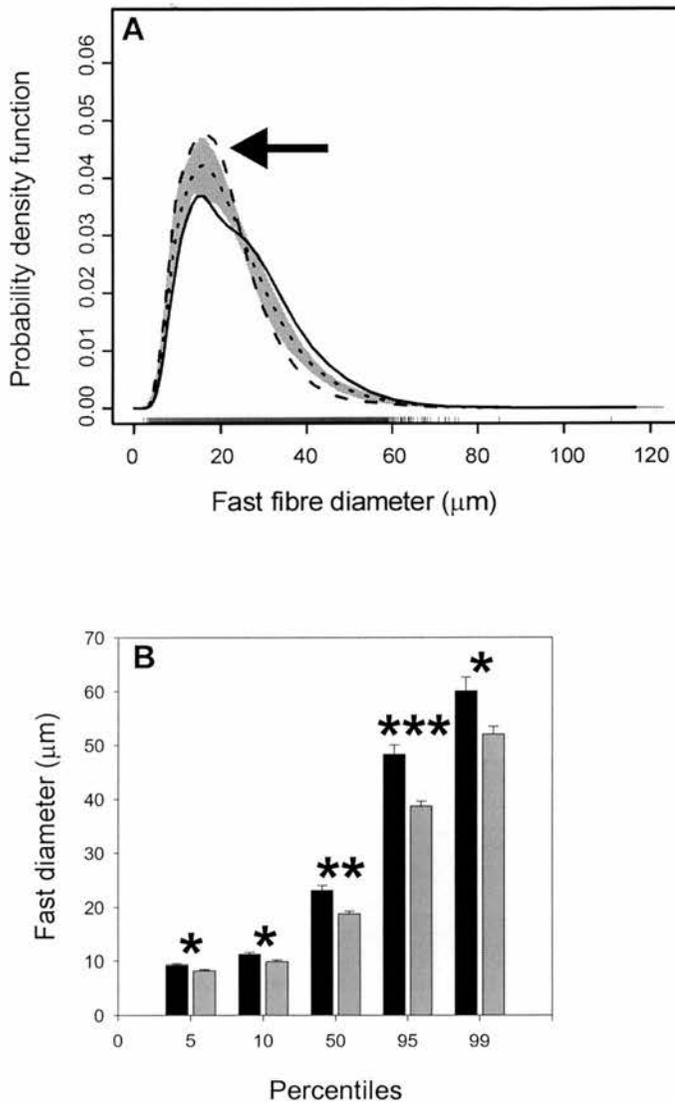


Fig. 3.6. Swimming experiment 1: the effect of tank rest or a three-week exercise-training regime ($2.1 - 2.3 \text{ bls}^{-1}$, 23 hours per day) on the distribution of fast muscle fibre diameters in tank rested (TR) and exercised (E) fish. A) The mean smooth probability density functions (pdf) of fast muscle fibre diameter distribution for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded area represents 100 bootstrap estimates of the fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups ($P < 0.01$, Kolmogorov Smirnov). The arrow represents the apparent right-to-left shift of the distribution of fast muscle fibre diameters in E relative to TR fish. B) A comparison of the percentiles for fast muscle fibre diameter in the TR (black fills) and E (grey fills) groups confirmed that the average of the 5th to 99th percentiles of fast fibre diameter were significantly greater in the TR group relative to E fish. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$: Mann-Whitney Rank Sum-test). Values represent group mean \pm SEM, $N = 6$.

Table 3.4. Swimming experiment 2: summary of overall morphological characteristics and fast muscle fibre data in the tank rested (TR) and exercised (E) groups of carp. Values represent group mean \pm SEM, figures in brackets show the percentage difference between experimental groups, N=6. CSA = cross-sectional area. Fast fibre number rounded up to the nearest 100.

Experimental Treatment	Initial Fork Length (cm)	Final Fork Length (cm)	Body Mass (g)	Condition Factor	Fast T_{CSA} (mm^2)	Fast Fibre Number	Fast F_{CSA} (μm^2)	Fast Fibre Diameter (μm)
TR	5.3 \pm 0.4	5.9 \pm 0.4	6.2 \pm 1.1	2.9 \pm 0.1	38.4 \pm 4.4	36000 \pm 2900	1053 \pm 54	33.1 \pm 0.7
E	5.5 \pm 0.3 (3.8)	6.3 \pm 0.3 (6.8)	8.7 \pm 1.3 (40.8)	3.5 \pm 0.2 (19.8)	50.3 \pm 4.0 (31.2)	50500 \pm 1500 (40.0)	993 \pm 62 (5.7)	32.1 \pm 0.9 (3.0)

Table 3.5. Swimming experiment 2: summary of slow muscle fibre data in the tank rested (TR) and exercised (E) groups of carp. Values represent group mean \pm SEM, figures in brackets show the percentage difference between experimental groups, N=6. CSA = cross-sectional area. Slow fibre number rounded up to the nearest 100.

Experimental Treatment	Slow T_{CSA} (mm^2)	Slow Fibre Number	Slow F_{CSA} (μm^2)	Slow Fibre Diameter (μm)
TR	1.9 \pm 0.4	5600 \pm 800	319 \pm 25	18.9 \pm 0.7
E	2.6 \pm 0.3 (38.9)	7200 \pm 800 (27.3)	360 \pm 15 (13.2)	20.1 \pm 0.4 (6.2)

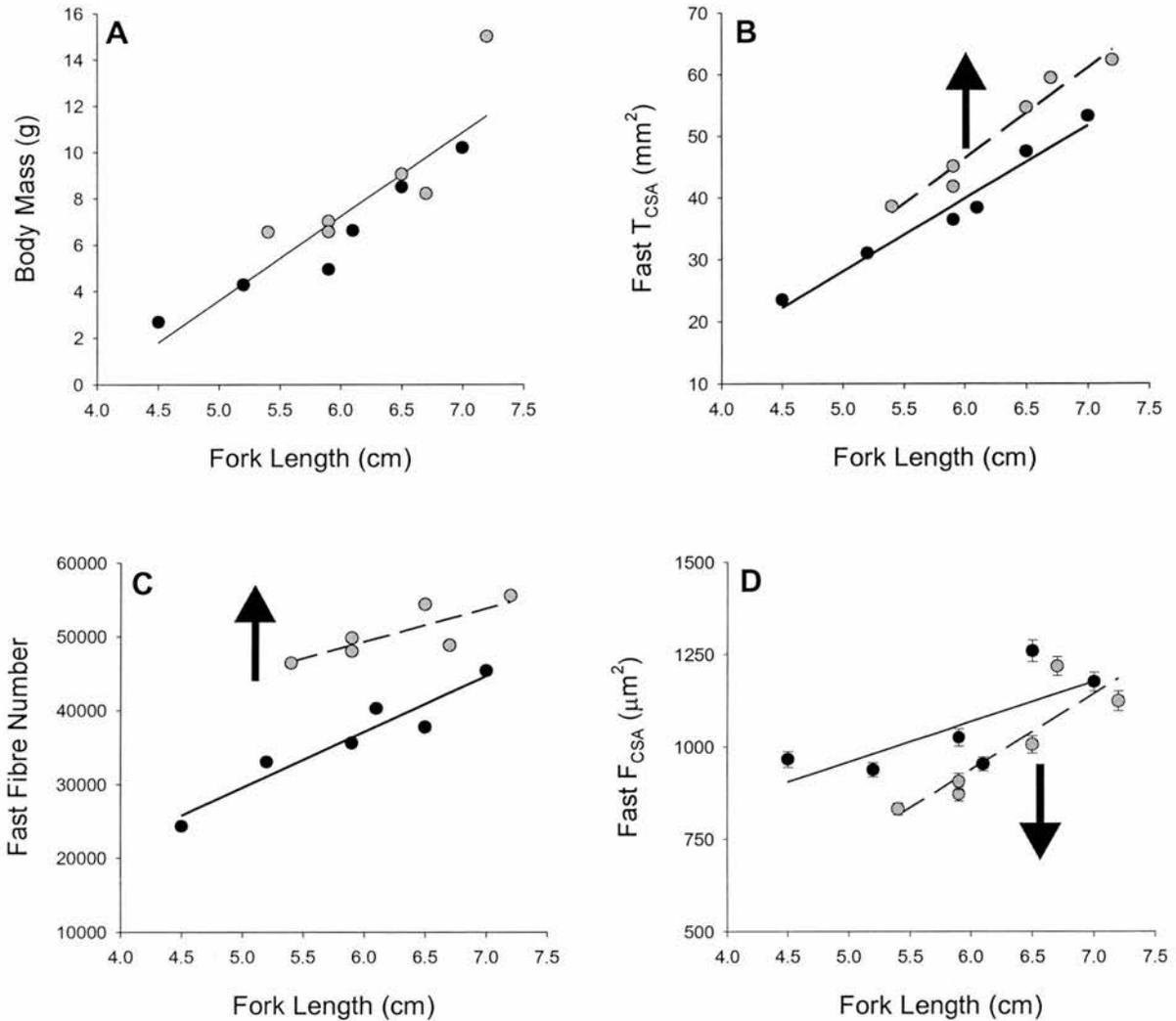


Fig. 3.7. Swimming experiment 2: the effect of tank rest or a three-week exercise-training regime ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours per day) on body mass (BM), total fast muscle fibre cross-sectional area (T_{CSA}), fast fibre number (FN) and mean fast muscle fibre cross-sectional area (F_{CSA}) relative to fork length. A) The body mass of exercised (E, grey symbols) and tank rested (TR, black symbols) fish was not significantly different post-training (NS, ANCOVA). B & C) Total fast muscle cross-sectional area and fast fibre number were significantly higher in exercised fish relative to tank rested controls ($P < 0.01$, ANCOVA). D) Mean fast fibre cross-sectional area was smaller in the exercised group, but this difference was not significant (NS, ANCOVA). Arrows indicate the trend of fast muscle fibre growth in the exercised group, relative to the tank rested controls. NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: A) TR & E: $BM = -14.5 + 3.6 FL$, $R^2 \text{ adj.} = 0.77$, $df = 10$, $P < 0.001$. B) TR: $FT_{CSA} = -31 + 12 FL$, $R^2 \text{ adj.} = 0.96$, $df = 4$, $P < 0.001$; E: $FT_{CSA} = -42 + 15 FL$, $R^2 \text{ adj.} = 0.94$, $df = 4$, $P < 0.001$. C) TR: $FN = -8282 + 7556 FL$, $R^2 \text{ adj.} = 0.88$, $df = 4$, $P < 0.01$; E: $FN = -22365 + 4485 FL$, $R^2 \text{ adj.} = 0.56$, $df = 4$, $P < 0.1$. D) TR: $F_{CSA} = -415 + 109 FL$, $R^2 \text{ adj.} = 0.42$, $df = 4$, $P < 0.1$; E: $F_{CSA} = -297 + 206 FL$, $R^2 \text{ adj.} = 0.72$, $df = 4$, $P < 0.05$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

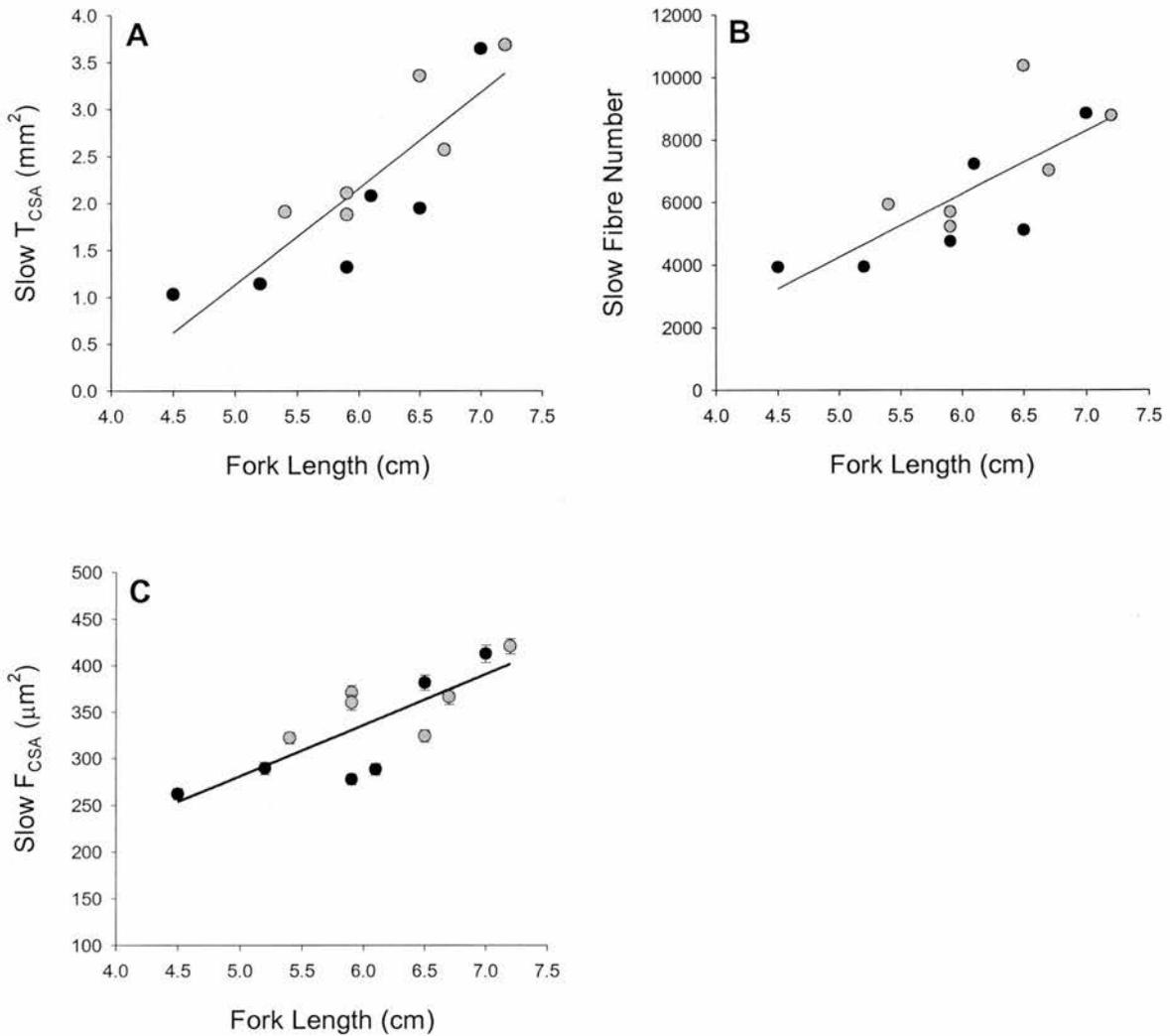


Fig. 3.8. Swimming experiment 2: the effect of tank rest or a three-week exercise-training regime ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours per day) on total slow muscle cross-sectional area (ST_{CSA}), total slow fibre number (SFN) and mean slow fibre cross-sectional area (SF_{CSA}), relative to fork length. A – C) The common regression line for the combined population of the exercised (E, grey fills) and non-exercised (TR, black fills) groups is shown, as there was no significant difference between experimental treatments in the slow fibre characteristics examined (NS, ANCOVA). NS, non-significant; ANCOVA, analysis of covariance.

Lines were plotted using the method of 1st order regression, giving the following equations: A) TR & E: $ST_{CSA} = -3.99 + 1.02 \text{ FL}$, $R^2 \text{ adj.} = 0.72$, $df = 10$, $P < 0.001$. B) TR & E: $SFN = -5848 + 2019 \text{ FL}$, $R^2 \text{ adj.} = 0.54$, $df = 10$, $P < 0.001$. C) TR & E: $SF_{CSA} = 7.7 + 54.7 \text{ FL}$, $R^2 \text{ adj.} = 0.60$, $df = 10$, $P < 0.01$.

$R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

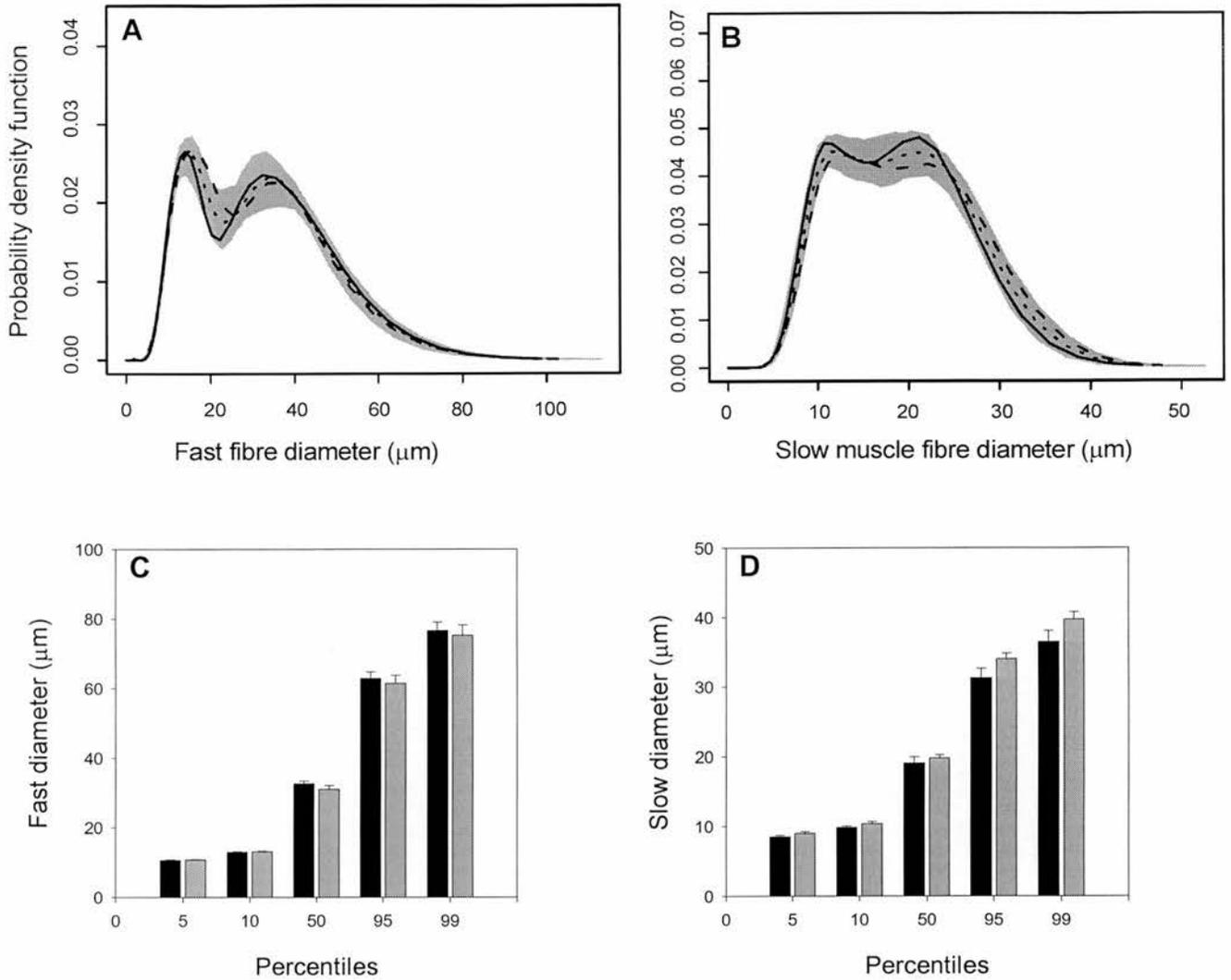


Fig. 3.9. Swimming experiment 2: the effect of tank rest or a three-week exercise-training regime ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours per day) on the distribution of fast and slow muscle fibre diameters in tank rested (TR) and exercised (E) fish. A & B) The mean smooth probability density functions (pdf) of fast (A) and slow (B) muscle fibre diameter distributions for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded areas represent 100 bootstrap estimates of the fast and slow muscle fibre diameter distributions for the two experimental groups combined. The dotted line shows the average fast and slow muscle fibre distributions of the combined groups. The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between the muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups for both fast and slow muscle fibre phenotypes (NS, Kolmogorov Smirnov). C & D) A comparison of the percentiles for fast (C) and slow (D) muscle fibre diameters in the TR (black fills) and E (grey fills) groups confirmed that there was no significant difference in the average of the 5th to 99th percentiles of fast and slow fibre diameter (NS, Mann-Whitney Rank Sum-test). Values represent group mean \pm SEM, N = 6. NS, non-significant.

In the exercised carp, there was an apparent increase in the total slow muscle fibre cross-sectional area, through a combination of slow muscle fibre recruitment and hypertrophy. There was a 39% increase in total slow muscle fibre cross-sectional area, 27% more slow muscle fibres and a 13% increase in slow muscle fibre cross-sectional area, relative to tank rested animals (Table 3.5). However, these increases were not significant when fork length was used as a covariate (Fig. 3.8A – C). The average probability density functions of fast and slow fibre diameter distributions for each experimental group were confined within the grey shaded areas of 100 bootstrap estimates of the total populations (Fig. 3.9A & B), which confirmed that there was no significant difference between the experimental groups in fast and slow muscle fibre density distributions.

3.3.3 Swimming experiment 3

Common carp were exercised for a 28 day period, at 2.6 – 2.7 bls⁻¹ for 14 hours daily, or confined to a tank for the duration of the experiment. Condition factor was measured in both experimental groups over a twelve-week period, for 8 weeks prior to commencing the swimming experiment, and at the beginning and end of the 4-week exercise or tank rest treatments (Table 3.6, Fig. 3.10A). There was a significant interaction between experimental group and time of measurement ($F_{3, 48} = 7.6$, $P < 0.001$, 2-way mixed ANOVA), which indicated that changes in condition factor over the 12-week period were not constant across the exercised and tank rested groups (Table 3.8). Analysis of between-subjects simple effects (Table 3.9) revealed that there was no significant difference in condition factor between the experimental groups in the 8 weeks prior to the start of the exercise training period.

Table 3.6. Swimming experiment 3: summary of growth characteristics for two groups of common carp over a 3-month period. Values represent group mean \pm SEM, N=9. FL = Fork Length (cm), BM = Body Mass (g) and CF = Condition Factor. Start and end refer to the beginning and end of the 4-week exercise or tank rest treatment. Growth rate indicates the daily rate of change from one month to the next.

Experimental treatment	FL		FL		FL		BM		BM		BM		CF		CF	
	August	September	October (START)	November (END)	August	September	October (START)	November (END)	August	September	October (START)	November (END)	September	October (START)	November (END)	November (END)
TR	9.5 ± 0.2	10.2 ± 0.2	11.4 ± 0.2	12.7 ± 0.2	22.2 ± 1.2	30.2 ± 1.3	44.9 ± 1.8	64.9 ± 2.6	2.6 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	3.2 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	3.2 ± 0.1
Growth rate ($\text{cm}\cdot\text{d}^{-1}$, $\text{g}\cdot\text{d}^{-1}$ or CF units. d^{-1})	0.03	0.04	0.05	0.06	0.28	0.71	0.53	0.71	0.008	0.007	0.007	0.005	0.008	0.007	0.007	0.005
E	9.6 ± 0.2	10.3 ± 0.2	11.4 ± 0.2	13.1 ± 0.2	22.8 ± 1.5	30.8 ± 1.8	45.6 ± 2.1	62.4 ± 3.1	2.6 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	2.8 ± 0.1
Growth rate ($\text{cm}\cdot\text{d}^{-1}$, $\text{g}\cdot\text{d}^{-1}$ or CF units. d^{-1})	0.03	0.04	0.06	0.06	0.29	0.60	0.53	0.60	0.007	0.007	0.009	-0.009	0.007	0.009	0.009	-0.009

Table 3.7. Summary of fast and slow muscle fibre data for the tank rested (TR) and exercised (E) groups in swimming experiment 3. Values represent group mean \pm SEM, figures in brackets show the percentage difference between the groups, N=9. CSA = cross-sectional area.

Experimental Treatment	Fast T_{CSA} (mm^2)	Fast Fibre Number	Fast F_{CSA} (μm^2)	Fast Fibre Diameter (μm)	Slow F_{CSA} (μm^2)	Slow Fibre Diameter (μm)
TR	296 \pm 14	138700	2143 \pm 36	48.6 \pm 0.4	924 \pm 52	33.1 \pm 0.8
E	291 \pm 13 (2.9)	176300 (27.2)	1653 \pm 56 (22.8)	42.0 \pm 0.7 (13.7)	754 \pm 41 (5.7)	30.1 \pm 0.8 (9.1)

Table 3.8. Swimming experiment 3: Two-way ANOVA (mixed design) summary table comparing condition factor in the tank rested and exercised groups from August to November 2000.

Source	SS	df	MS	F	P
A (Group)	0.172	1	0.172	5.9	P < 0.05
S/A	0.470	16	0.029		
B (Time of measurement)	2.17	3	0.722	34.5	P < 0.001
A × B (interaction)	0.477	48	0.159	7.6	P < 0.001
B × S/A	1.006	48	0.0021		
Total	4.29	71			

Table 3.9. Swimming experiment 3: Between-subjects simple effects, analysis of the simple effect of group on condition factor. S – start of swimming experiment, E – end of swimming experiment.

Time	SS	df	MS	F	MS _{w,cell}	df _{w,cell}
August	0.0002	1	0.0002	0.01	0.023	64
September	0.0066	1	0.0066	0.29		
October (S)	0.0043	1	0.0043	0.19		
November (E)	0.6377	1	0.6377	27.7 (P < 0.001)		

Table 3.10. Swimming experiment 3: Within-subjects simple effects, analysis of the simple effect of time on condition factor. TR – tank rested group, E – exercised group.

Group	SS	df _{numerator}	MS	F	MS _{B × S/A}	df _{B × S/A} (Denominator)
TR	1.67	3	0.56	26.5 (P < 0.001)	0.021	48
E	0.98	3	0.33	15.5 (P < 0.001)		

Table 3.11. Swimming experiment 3: Within-subjects simple comparisons, analysis of group mean condition factor at each 4-week time point. TR – tank rested group, E – exercised group.

Group/Time	SS	df _{numerator}	MS	MS _{B × S/A}	df _{B × S/A} (Denominator)	F	P
TR (Aug – Sept)	0.2204	1	0.2204	0.021	48	10.5	P < 0.01
TR (Sept – Oct)	0.1861		0.1861			8.9	P < 0.01
TR (Oct – Nov)	0.0966		0.0966			4.6	P < 0.05
E (Aug – Sept)	0.1626		0.1626			7.7	P < 0.01
E (Sept – Oct)	0.3346		0.3346			15.9	P < 0.001
E (Oct – Nov)	0.3060		0.3060			14.6	P < 0.001

Table 3.12. Swimming experiment 4: Two-way ANOVA (mixed design) summary table comparing initial and final condition factor in tank rested and exercised groups.

Source	SS	df	MS	F	P
A (Group)	0.005	1	0.005	0.002	NS
S/A	3.458	16	0.216		
B (Time of measurement)	0.078	1	0.078	16.0	P < 0.01
A × B (interaction)	0.067	1	0.067	13.7	P < 0.01
B × S/A	0.078	16	0.005		
Total	3.685	35			

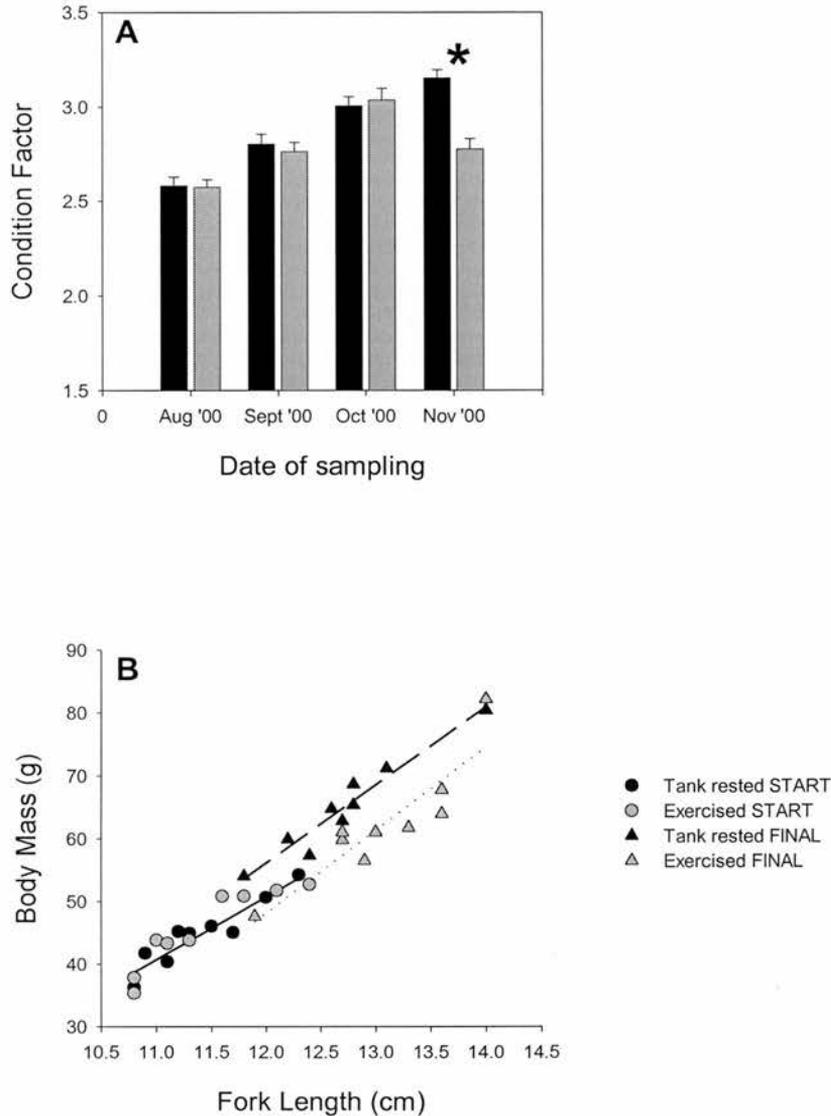


Fig. 3.10. Swimming experiment 3: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on condition factor and body mass (BM) relative to fork length (FL). A) Overall condition factor was invariant (NS, two-way mixed ANOVA) between tank rested (TR, black fills) and exercised (E, grey fills) treatments in the months prior to the experiment (Aug – Oct). Post-training (Nov), overall condition factor was significantly lower in the exercised group relative to tank rested fish ($P < 0.05$, two-way mixed ANOVA). B) There was no significant difference in body mass between experimental treatments at the start of the training programme (NS, ANCOVA). At the conclusion of the experiment, body mass relative to fork length was significantly higher in tank rested fish compared to the exercised group ($P < 0.001$, ANCOVA). Values represent group mean \pm SEM, $N = 6$. NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: A) starting body mass, TR & E: $\text{BM} = -69.8 + 10.1 \text{ FL}$, $R^2 \text{ adj.} = 0.83$, $\text{df} = 16$, $P < 0.001$. Final body mass, TR: $\text{BM} = -92.6 + 12.4 \text{ FL}$, $R^2 \text{ adj.} = 0.93$, $\text{df} = 7$, $P < 0.001$; E: $\text{BM} = -110.3 + 13.2 \text{ FL}$, $R^2 \text{ adj.} = 0.78$, $\text{df} = 7$, $P < 0.01$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

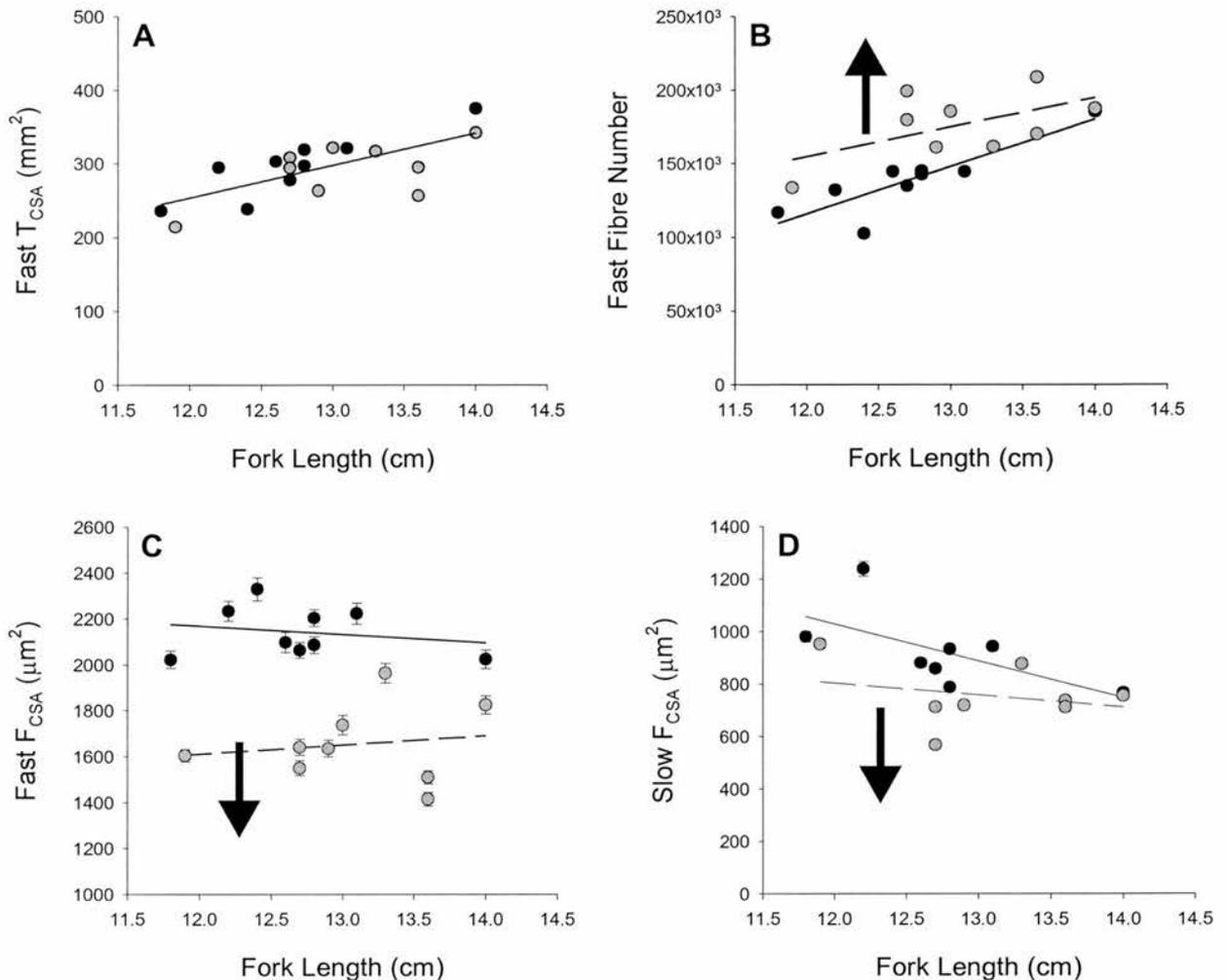


Fig. 3.11. Swimming experiment 3: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on total fast muscle cross-sectional area (FT_{CSA}), fast fibre number (FN), mean fast fibre cross-sectional area (F_{CSA}) and mean slow fibre cross-sectional area (F_{CSA}), relative to fork length (FL). A) Total fast muscle cross-sectional area of exercised (E, grey symbols) and tank rested (TR, black symbols) experimental treatments was not significantly different post-training (NS, ANCOVA). B) The number of fast muscle fibres was significantly higher in exercised carp ($P < 0.01$, ANCOVA). C & D) Mean fast F_{CSA} and slow F_{CSA} were significantly higher in tank rested controls relative to the exercised group ($P < 0.001$, ANCOVA). Arrows indicate the trend of muscle growth in the exercised group (increased fast fibre number, lower mean fast F_{CSA} and slow F_{CSA}) relative to the tank rested controls. NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: A) TR & E: $FT_{CSA} = -279 + 44FL$, $R^2 \text{ adj.} = 0.46$, $df = 16$, $P < 0.01$. B) TR: $FN = -272042 + 32311 FL$, $R^2 \text{ adj.} = 0.72$, $df = 7$, $P < 0.01$; E: $FN = -87440 + 20169 FL$, $R^2 \text{ adj.} = 0.21$, $df = 7$, $P > 0.1$. C) TR: $F_{CSA} = 2605 - 36FL$, $R^2 \text{ adj.} = -0.10$, $df = 7$, $P > 0.5$; E: $F_{CSA} = 1135 + 40 FL$, $R^2 \text{ adj.} = -0.10$, $df = 7$, $P > 0.5$. D) TR: $SF_{CSA} = 2723 - 141FL$, $R^2 \text{ adj.} = 0.28$, $df = 6$, $P > 0.1$; E: $SF_{CSA} = 1353 - 46FL$, $R^2 \text{ adj.} = -0.10$, $df = 6$, $P > 0.2$.

$R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

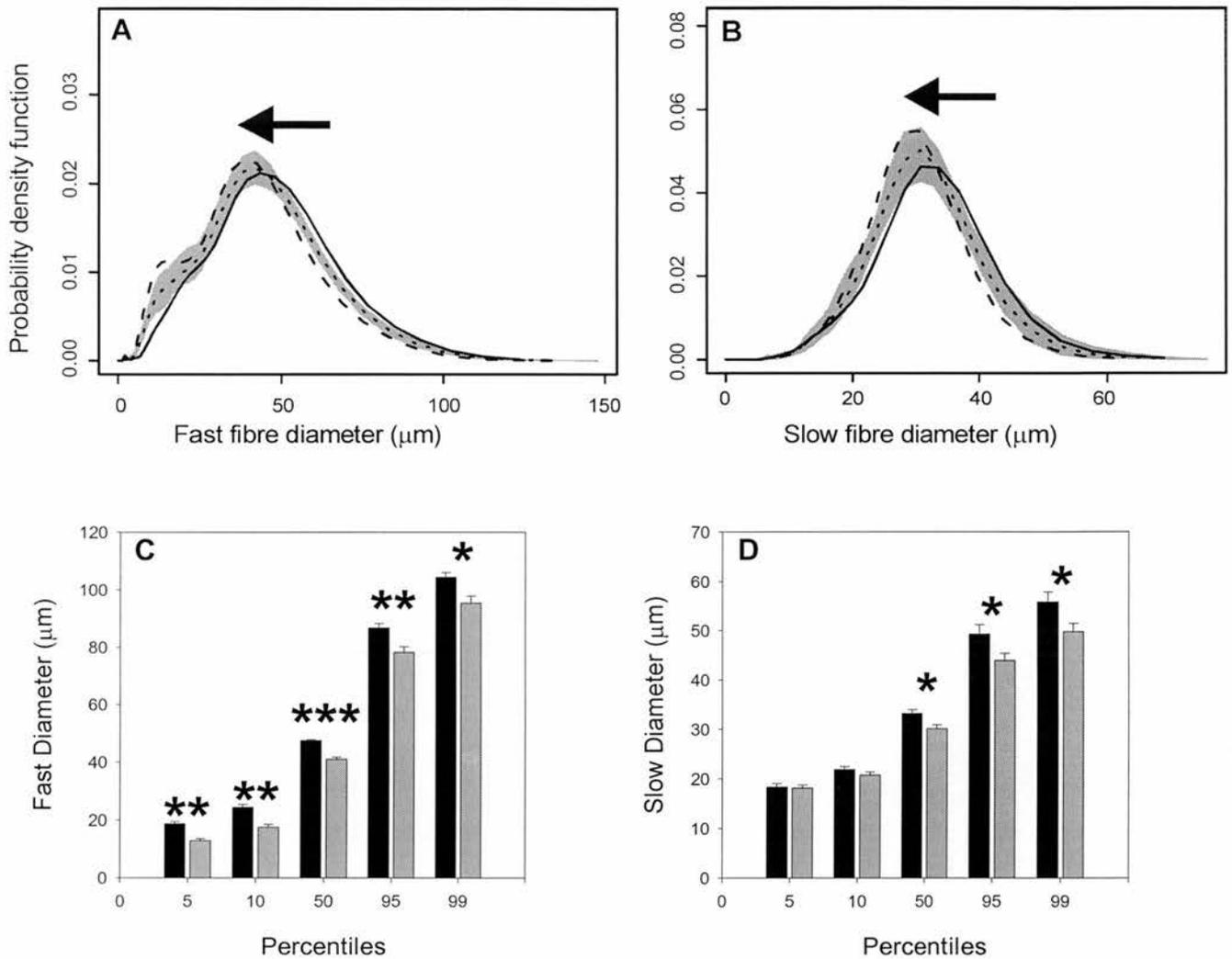


Fig. 3.12. Swimming experiment 3: the effect of tank rest or a four-week exercise-training regime ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on the distribution of fast and slow muscle fibre diameters in tank rested (TR) and exercised (E) fish. A & B) The mean smooth probability density functions (pdf) of fast (A) and slow (B) muscle fibre diameter distributions for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded areas represent 100 bootstrap estimates of the fast and slow muscle fibre diameter distributions for the two experimental groups combined. The dotted line shows the average fast and slow muscle fibre distributions of the combined groups. In both muscle fibre types, several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population. This suggested that there was a significant difference between the muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups for fast and slow muscle fibre phenotypes (Fast, $P < 0.01$; Slow, $P < 0.05$, Kolmogorov Smirnov). The arrows indicate a right-to-left shift of the distribution of fast and slow muscle fibre diameters in E relative to TR fish. C & D) A comparison of the percentiles for fast (C) and slow (D) muscle fibre diameters in the TR (black fills) and E (grey fills) groups confirmed the significant difference in fibre size. The average of the 5th to 99th percentiles of fast fibre diameter and 50th to 99th percentiles of slow fibre diameter were significantly greater in the TR group relative to E fish (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$, Mann-Whitney Rank Sum-test). Values represent group mean \pm SEM, $N = 9$.

However, the condition factor observed in the exercised carp at the end of the experiment was significantly lower than that of the tank rested controls ($F_{1,64} = 27.7$, $P < 0.001$), indicating that for a given fork length tank rested fish were heavier relative to the exercised group. Analysis of within-subject simple effects (Table 3.10) showed that condition factor changed significantly in both experimental groups between August and November 2000. Subsequent within-subjects simple comparisons (Table 3.11) demonstrated that the month-to-month increase in group mean condition factor observed in both groups prior to the exercise or tank rest treatments was significant; the reduction/increase in group mean condition factor observed in the exercised and tank rested groups respectively over the course of the training period was also statistically significant (data are summarised in Table 3.11). At the end of the 4-week experiment, final body mass was significantly higher in the tank rested controls relative to fork length (Fig. 3.10B), compared to the exercised group ($F_{1,14} = 19.4$, $P < 0.001$, ANCOVA). There was no significant difference in body mass relative to fork length in the two experimental groups prior to exercise or tank rest treatment.

The total fast muscle cross-sectional area (Fig. 3.11A) at the level of the anal pore (approx. 0.75 body lengths from the snout) was not significantly different between experimental groups relative to fork length. The number of fast muscle fibres within the total myotomal cross-sectional area (Fig. 3.11B) was 27% greater in the exercised group of carp compared to tank rested controls, an increase that was highly significantly different ($F_{1,14} = 11.8$, $P < 0.01$, ANCOVA). The mean cross-sectional area of fast and slow muscle fibres (Fig. 3.11C & D) was 23% and 6% lower in exercised fish relative to non-exercised controls and these dissimilarities were significant (Fast F_{CSA} : $F_{1,14} = 46.6$, $P < 0.001$; Slow F_{CSA} : $F_{1,14} = 4.8$, $P < 0.05$,

ANCOVA). Areas of the average probability density functions of fast and slow fibre diameter distributions for each experimental group lay beyond the grey shaded areas of 100 bootstrap estimates of the total populations (Fig. 3.12A & B), indicating that there was a significant difference between the experimental groups in fast and slow muscle fibre density distributions (Fast, $P < 0.01$; Slow, $P < 0.05$: Kolmogorov Smirnov). The average fast fibre diameter (Fig. 3.12C) was greater in the tank rested group at the 5th, 10th, 50th, 95th and 99th percentiles, which was significantly different from the exercised group of carp. The average of the 50th, 95th and 99th percentiles of slow fibre diameter (Fig. 3.12D) was significantly higher in tank rested controls compared with the exercised group.

3.3.4 Swimming experiment 4

Common carp were exercised for a 28 day period, at 2.6 – 2.7 bls⁻¹ for 14 hours daily and compared to the tank rested controls. Condition factor was measured in both experimental groups at the start and end of the 4-week experimental period (Table 3.13, Fig. 3.13A). There was a significant interaction between experimental group and time of measurement ($F_{1, 16} = 13.7$, $P < 0.01$, 2-way mixed ANOVA), which indicated that changes in condition factor during the short-term swimming experiment were not constant across experimental groups (Table 3.12). Post-hoc analysis revealed that there was no significant difference in the between-subjects simple effects. Therefore there was no difference in condition factor between experimental treatments before or after the 4-week swimming experiment. Within-subjects simple effects demonstrated that there was no significant change in mean condition factor over the 4-week experiment in the tank rested group. However there was a significant reduction in mean condition factor observed in the exercised group of carp after 4 weeks of exercise training ($F_{1, 32} = 28.9$, $P < 0.001$).

Table 3.13. Swimming experiment 4: summary of growth characteristics for two groups of common carp over the 4-week experimental period. Values represent group mean \pm SEM, N=9. FL = Fork Length (cm), BM = Body Mass (g) and CF = Condition Factor. Start and end refer to the beginning and end of the 4-week exercise or tank rest treatment. Growth rate indicates the daily rate of change from one month to the next.

Experimental treatment	FL		FL		BM		CF	
	START	END	START	END	START	END	START	END
TR	11.4 ± 0.2	12.2 ± 0.2	43.4 ± 1.6	54.9 ± 2.6	2.91 ± 0.05	3.02 ± 0.04		
Growth rate (cm.d ⁻¹ , g.d ⁻¹ or CF units.d ⁻¹)	0.03		0.4				0.004	
E	11.4 ± 0.2	12.6 ± 0.2	43.8 ± 2.0	53.4 ± 3.1	2.94 ± 0.06	2.68 ± 0.05		
Growth rate (cm.d ⁻¹ , g.d ⁻¹ or CF units.d ⁻¹)	0.04		0.3				-0.009	

Table 3.14. Summary of deep fast, fast superficial and slow muscle fibre data for the tank rested (TR) and exercised (E) groups in swimming experiment 4. Values represent group mean \pm SEM, figures in brackets show the percentage difference between the groups, N=9. CSA = cross-sectional area.

Experimental Treatment	Fast T _{CSA} (mm ²)	Total Fast Fibre Number	Deep Fast F _{CSA} (µm ²)	Deep Fast Fibre Diameter (µm)	Superficial Fast F _{CSA} (µm ²)	Superficial Fast Fibre Diameter (µm)	Slow F _{CSA} (µm ²)	Slow Fibre Diameter (µm)
TR	340 \pm 43	161500	2097 \pm 75	46.8 \pm 0.7	1703 \pm 113	44.4 \pm 1.4	668 \pm 16	27.5 \pm 0.3
E	332 \pm 20 (2.4)	161900 (1.8)	2050 \pm 54 (2.0)	46.0 \pm 0.7 (1.7)	1495 \pm 69 (12.2)	41.6 \pm 1.1 (6.3)	782 \pm 12 (17.1)	30.1 \pm 0.2 (9.4)

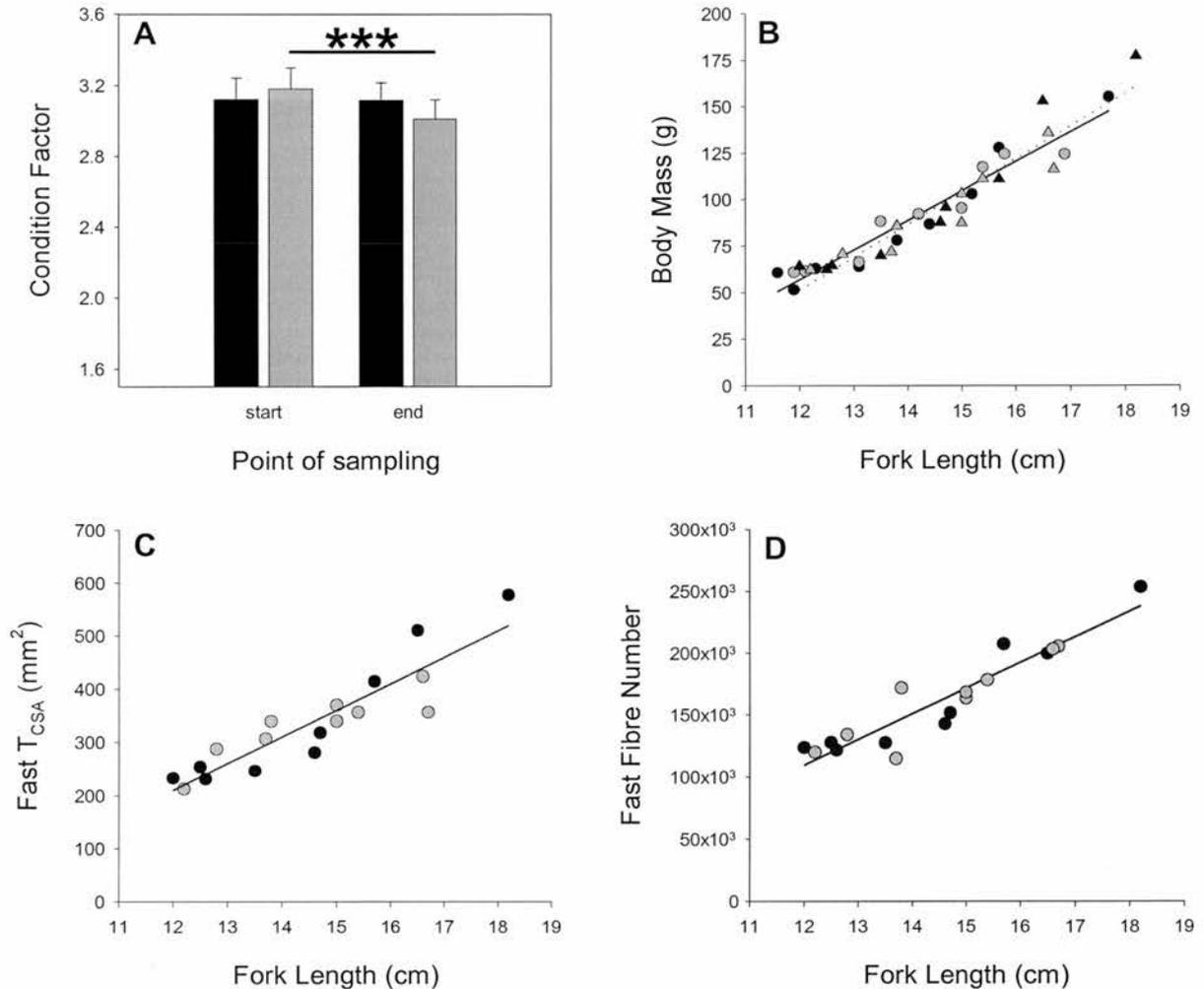


Fig. 3.13. Swimming experiment 4: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on condition factor, body mass (BM), total fast muscle cross-sectional area (FT_{CSA}) and total fast fibre (deep & superficial) number (FN) relative to fork length (FL). A) Condition factor was invariant between tank rested (TR, black fills) and exercised (E, grey fills) treatments before and after the experiment (NS, two-way mixed ANOVA). A significant reduction in mean condition factor in exercised fish was observed over the course of the experiment ($P < 0.001$, two-way mixed ANOVA). B) There was no significant difference in body mass relative to fork length between tank rested and exercised groups before (circles) or after (triangles) the experimental treatment (NS, ANCOVA). C & D) There was no significant difference in total fast-type muscle cross-sectional area or the total number of fast-type fibres (deep & superficial) in exercised and tank rested carp (NS, ANCOVA). Data are mean \pm SEM (where calculable), $N = 9$. NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: B) starting body mass, TR & E: $BM = -135 + 16 FL$, $R^2 \text{ adj.} = 0.93$, $P < 0.001$. Final body mass, TR & E: $BM = -161 + 18 FL$, $R^2 \text{ adj.} = 0.89$, $P < 0.001$. C) TR & E: $FT_{CSA} = -389 + 50 FL$, $R^2 \text{ adj.} = 0.81$, $P < 0.001$. D) TR & E: $FN = -138486 + 20664 FL$, $R^2 \text{ adj.} = 0.86$, $P < 0.001$.

$R^2 \text{ adj.}$, R^2 coefficient adjusted; df, degrees of freedom.

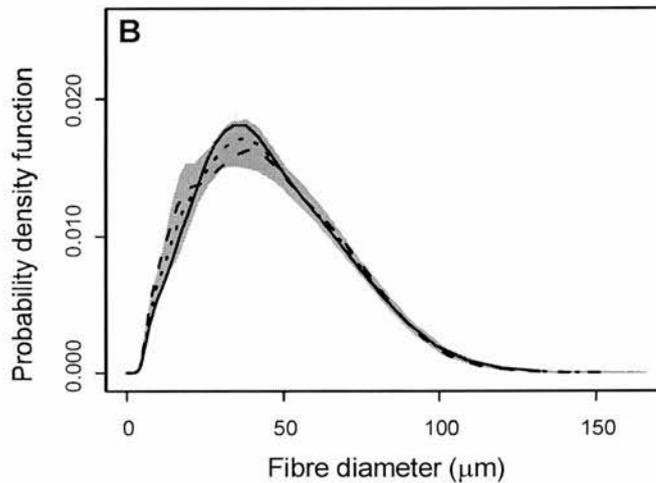
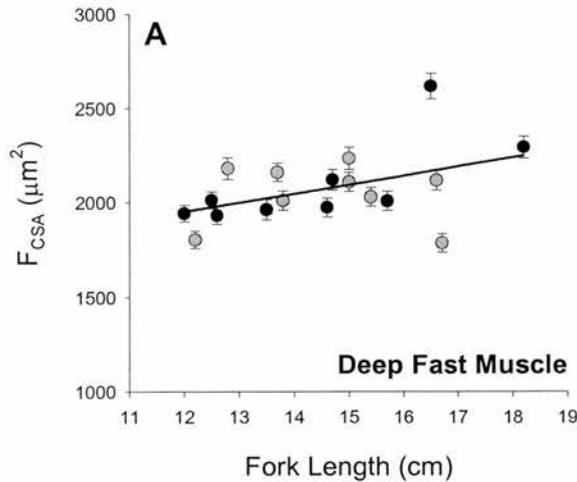


Fig. 3.14. Swimming experiment 4: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on deep fast muscle fibre growth. A) There was no significant difference in mean deep fast fibre cross-sectional area (F_{CSA}) between tank rested (TR, black fills) and exercised (E, grey fills) groups relative to fork length (FL) (NS, ANCOVA). B) The mean smooth probability density functions (pdf) of deep fast muscle fibre diameter distribution for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded area represents 100 bootstrap estimates of the deep fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between the deep fast muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups (NS, Kolmogorov Smirnov). NS, non-significant; ANCOVA, analysis of covariance.

Regression analysis data: A) TR & E: $F_{\text{CSA}} = 1388 + 48 \text{ FL}$, $R^2 \text{ adj.} = 0.15$, $\text{df} = 16$, $P < 0.1$. Data are mean \pm SEM, $N = 9$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

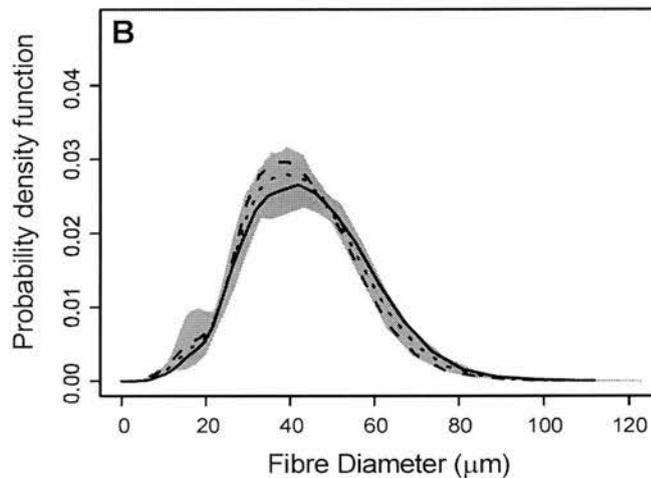
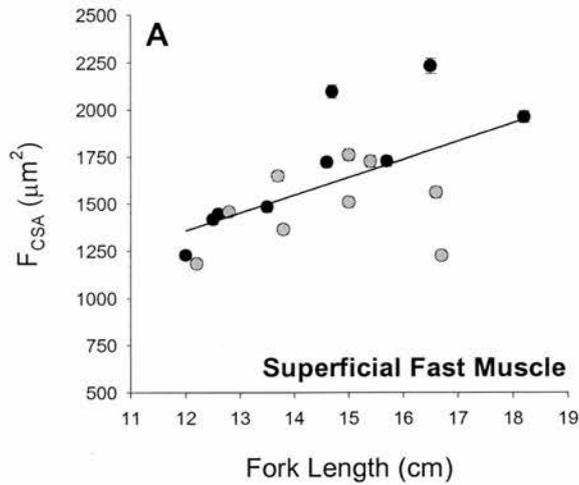


Fig. 3.15. Swimming experiment 4: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on superficial fast muscle fibre growth. A) There was no significant difference in mean superficial fast fibre cross-sectional area (F_{CSA}) between tank rested (TR, black fills) and exercised (E, grey fills) groups relative to fork length (FL) (NS, ANCOVA). B) The mean smooth probability density functions (pdf) of superficial fast muscle fibre diameter distribution for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded area represents 100 bootstrap estimates of the superficial fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between the superficial fast muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups (NS, Kolmogorov Smirnov). NS, non-significant; ANCOVA, analysis of covariance.

Regression analysis data: A) TR & E: $F_{\text{CSA}} = 212 + 95 \text{ FL}$, $R^2 \text{ adj.} = 0.29$, $\text{df} = 16$, $P < 0.05$.

Data are mean \pm SEM, $N = 9$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

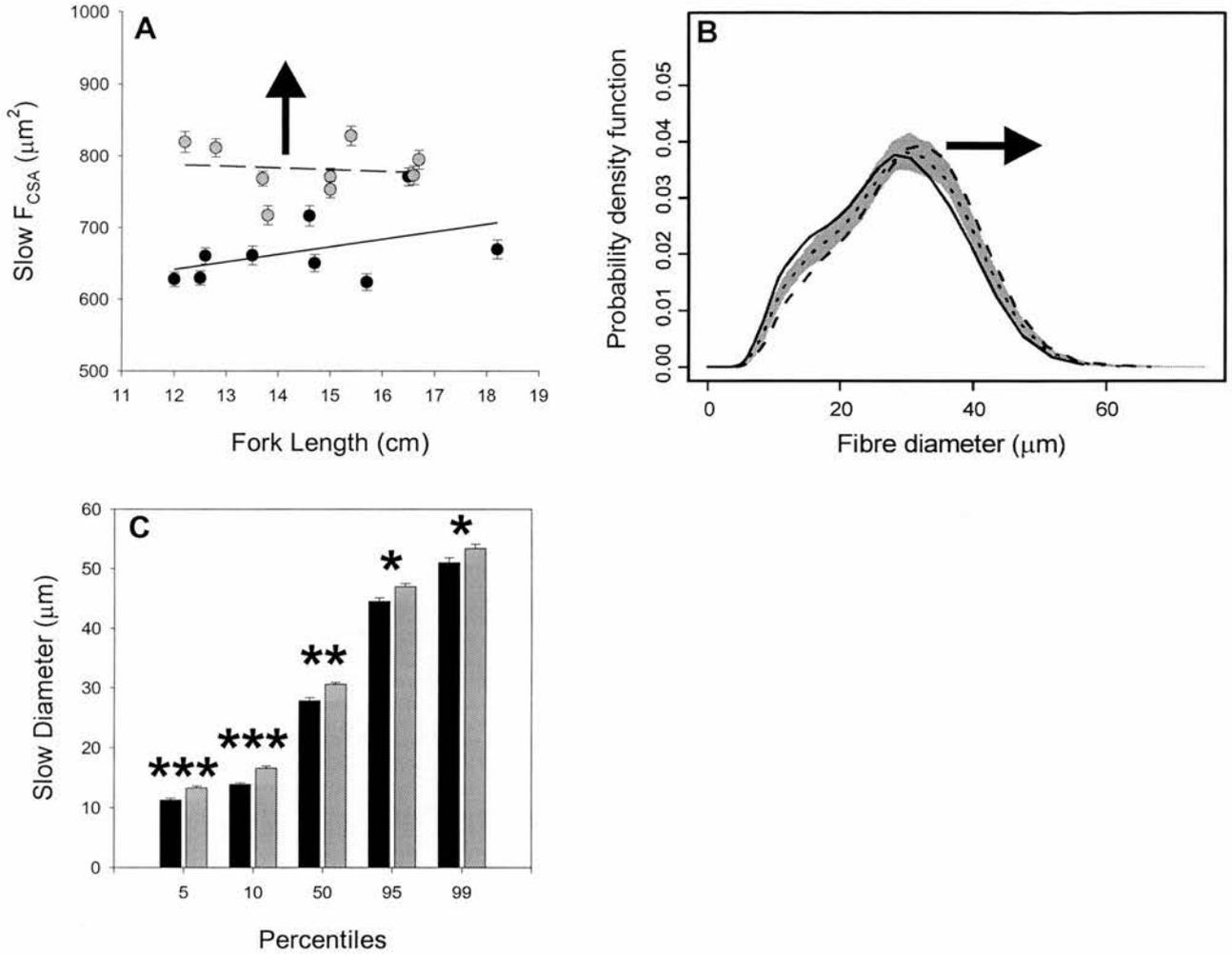


Fig. 3.16. Swimming experiment 4: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on slow muscle fibre growth. A) The mean slow fibre cross-sectional area (F_{CSA}) was significantly higher in the exercised (E, grey fills) group than the tank rested controls (TR, black fills), relative to fork length ($P < 0.001$, ANCOVA). B) The mean smooth probability density functions (pdf) of slow muscle fibre diameter distribution for each experimental group are represented by the solid (TR) and dashed (E) lines. The shaded area represents 100 bootstrap estimates of the slow muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the slow muscle fibre diameter distributions of the TR (solid line) and E (dashed line) experimental groups ($P < 0.01$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of slow muscle fibre diameters in E relative to TR fish. C) A comparison of the percentiles for slow muscle fibre diameter in the TR (black fills) and E (grey fills) groups confirmed that the average of the 5th to 99th percentiles of slow fibre diameter were significantly greater in the E group relative to TR fish. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Mann-Whitney Rank Sum-test). Data are mean \pm SEM, $N = 9$.

The linear regression line was plotted using the least squares method, giving the following equations: A) TR: $SF_{\text{CSA}} = 515 + 10.5 \text{ FL}$, $R^2 \text{ adj.} = 0.09$, $df = 7$, $P > 0.2$; E: $F_{\text{CSA}} = 818 - 2.5 \text{ FL}$, $R^2 \text{ adj.} = -0.13$, $df = 7$, $P > 0.5$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

Body mass relative to fork length was not significantly different before or after the 4-week experimental period (Fig. 3.13B), which emphasised that there was no difference in somatic growth between the groups in this experiment.

Total fast muscle cross-sectional area (Fig. 3.13C) and the total number of fast-type muscle fibres contributing to the total myotomal area (Fig. 3.13D) were not significantly different between exercised and non-exercised carp in swimming experiment 4 (Table 3.14). Subsequent morphological analysis showed that there was no significant difference in the mean cross-sectional area of deep fast and superficial fast muscle fibres between experimental treatments (Figs. 3.14A & 3.15A). There was also no significant difference in the density distributions of muscle fibre diameter observed in these two categories of fast muscle fibre. This was illustrated by the restriction of the average probability density functions of deep fast and superficial fast fibre diameter distributions for both experimental groups to within the grey shaded areas of 100 bootstrap estimates of the total populations (Figs. 3.14B & 3.15B). Mean cross-sectional area of slow muscle fibres (Fig. 3.16A) was significantly higher in the exercised group relative to fork length, when compared against the tank rested controls ($F_{1, 14} = 33.0$, $P < 0.001$, ANCOVA). Several regions of the average probability density function of slow fibre diameter distributions for each experimental group lay outside the grey shaded areas of 100 bootstrap estimates of the total population (Fig. 3.16B), which strongly suggested that there was a significant difference between the experimental groups in slow muscle fibre density distribution ($P < 0.01$: Kolmogorov Smirnov). A comparison of the 5th – 99th percentiles of group mean fibre diameter confirmed the density distributions were significantly different and a significantly greater mean slow fibre diameter was demonstrated in the exercised group relative to the tank rested controls (Fig. 3.16C).

3.3.5 Myonuclear density in isolated fast muscle fibres

The density of muscle fibre nuclei in isolated fast muscle fibres was measured using a DNA-specific green fluorescent stain (Fig. 3.17A & B). The number of myonuclei per cm was measured in 12 isolated fast muscle fibres dissected from each fish in swimming experiment 4 and the data was pooled to assess the effect of exercise on myonuclear density. The number of myonuclei per cm was positively correlated with fast muscle fibre diameter in both groups and the correlation was significant ($r_s = 0.61$, $P < 0.001$). The mean number of myonuclei cm^{-1} was 26% higher in the exercised group (Fig. 3.17B) relative to tank rested controls (Fig. 3.17A), corresponding to 1130 myonuclei cm^{-1} compared to 898 myonuclei cm^{-1} in a 100 μm diameter fast muscle fibre. The number of myonuclei per cm relative to fast muscle fibre diameter (Fig. 3.17C) was significantly higher in the exercised group when compared with tank rested controls ($F_{1,216} = 21.8$, $P < 0.001$, ANCOVA).

3.3.6 Myogenic regulatory factor expression

In swimming experiment 2, nuclear localisation of MyoD protein in fast muscle tissue was marginally higher in exercised fish relative to tank rested controls, but this difference was not statistically significant (Fig. 3.18A & B). In swimming experiment 3, MyoD nuclear localisation in fast muscle tissue extracts was 19% higher in the tank rested controls in comparison with exercised fish (Fig. 3.19A & B). The increased level of MyoD protein nuclear localisation in tank rested fish was statistically significant ($T = 2.7$, $DF = 15$, $P < 0.05$, two-tailed T-test). Mean fast fibre cross-sectional area was significantly correlated with nuclear localisation of the MyoD protein ($r_s = 0.49$, $P < 0.05$). There was no significant difference in the level of MyoD expression in total protein extracts from fast muscle tissue in the two experimental groups from the same experiment (Figs. 3.20A & B).

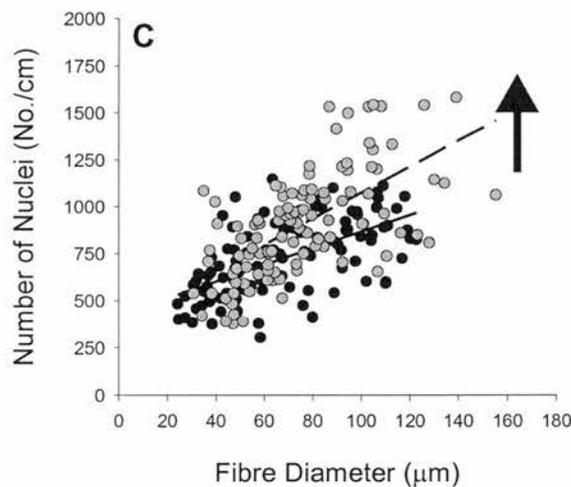
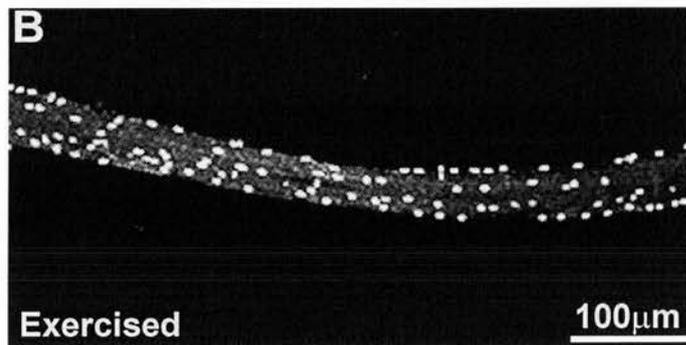
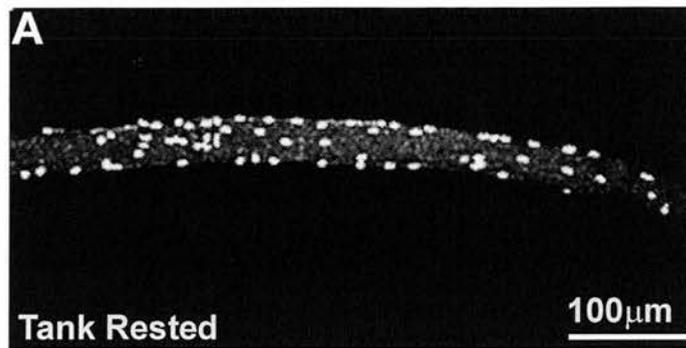


Fig. 3.17. Swimming experiment 4: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on fast muscle fibre myonuclear density. A & B) The number of myonuclei cm^{-1} was 26% lower in isolated fast fibres dissected from tank rested controls compared to exercised fish. C) The number of myonuclei cm^{-1} (N) relative to fast muscle fibre diameter (D) was significantly higher in the exercised group (E, grey fills) when compared with tank rested controls (TR, black fills) ($P < 0.001$, ANCOVA). The arrow indicates the trend of increased myonuclear density in the exercised group. ANCOVA, analysis of covariance.

Regression analysis data: lines were fitted using the method of 1st order linear regression resulting in the following equations: TR, $N = 418 + 4.6 D$, $R^2 \text{ adj.} = 0.36$, $df = 106$, $P < 0.001$; E, $N = 382 + 6.9 FD$, $R^2 \text{ adj.} = 0.39$, $df = 106$, $P < 0.001$. $R^2 \text{ adj.}$, R^2 coefficient adjusted; df , degrees of freedom.

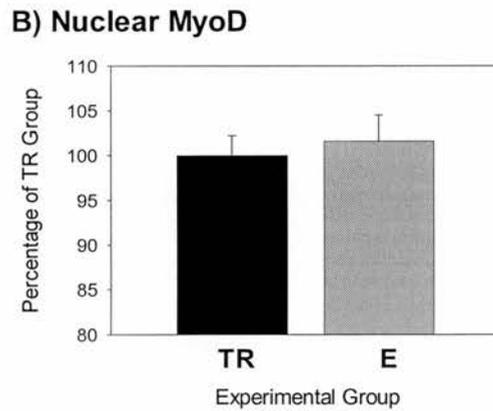
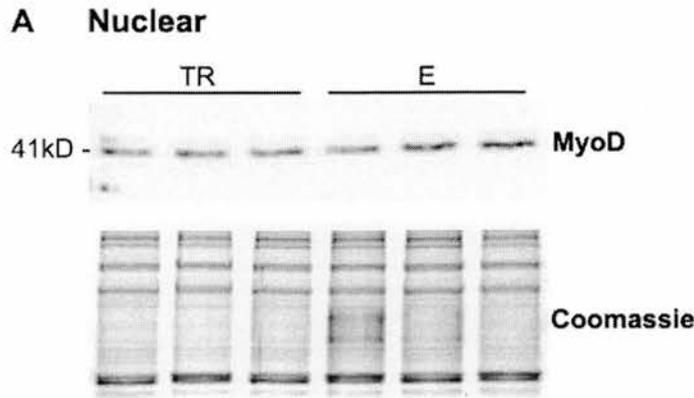


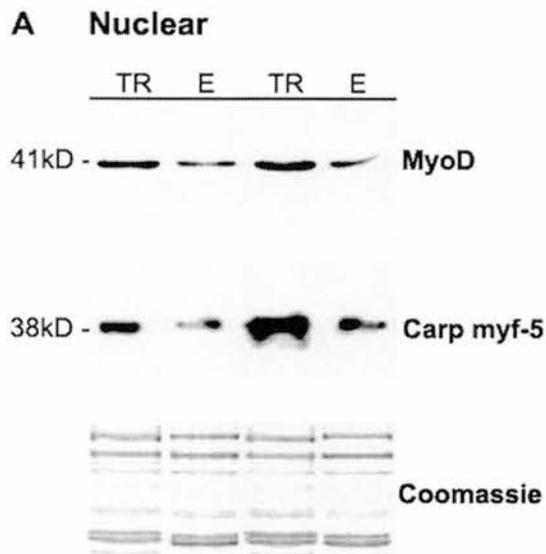
Fig. 3.18. Swimming experiment 2: the effect of tank rest or a three-week exercise-training regime ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours per day) on fast muscle tissue nuclear localisation of MyoD protein. A & B) There was no significant difference in MyoD nuclear localisation in fast muscle tissue extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Values represent group mean optical density expressed as a percentage of the tank rested controls \pm SEM, N = 6. NS, non-significant.

A similar pattern of expression of the other primary MRF, myf5, was observed in swimming experiment 3: no significant difference was observed in the expression of myf5 in total cellular protein extracts (Figs. 3.20A & C), but a 9% higher level of myf5 was observed in the nuclear protein extracts of tank rested controls relative to the exercised group (Fig. 3.19A & C). The higher degree of myf5 nuclear localisation in the tank rested group was significantly different to the exercised carp ($T = 3.6$, $DF = 15$, $P < 0.01$, two-tailed T-test). Increased nuclear localisation of myf5 in fast muscle fibres was significantly correlated with mean fast muscle fibre cross-sectional area ($r_s = 0.48$, $P < 0.05$) in this experiment. There was no significant difference between experimental treatments in the expression of myogenin in total cellular protein extracts (Fig. 3.20A & D).

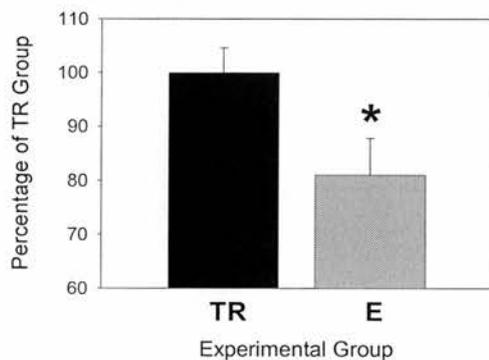
A comparison was made of the relative tissue localisation of the myogenic regulatory factors (MRFs) in total cellular protein extracts from different muscle fibre phenotypes (Fig. 3.21). All extracts analysed were from tank rested controls in swimming experiment 3. When results were corrected for a significant difference in total protein loading ($T = 5.5$, $DF = 15$, $P < 0.001$, two-tailed T-test) there was no significant difference in the level of MyoD, myf5 and myogenin expressed in the total protein extracts from fast and slow muscle tissue.

3.3.7 Calcineurin and substrate transcription factors

Nuclear localisation of the regulatory subunit of calcineurin (CnB) and calcineurin substrates NFAT2 and MEF2A was examined in fast muscle nuclear protein extracts in swimming experiment 2 (Fig. 3.22). There was no significant difference in the level of nuclear localisation of the CnB protein in exercised and non-exercised groups (Fig. 3.22A & B).



B) Nuclear MyoD



C) Nuclear myf-5

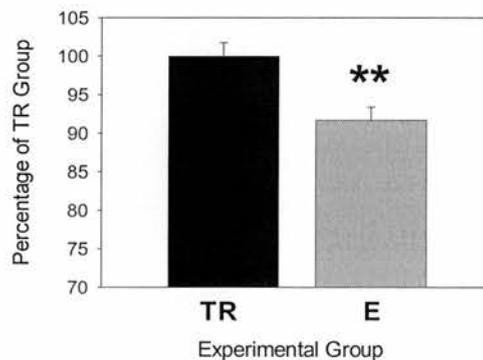
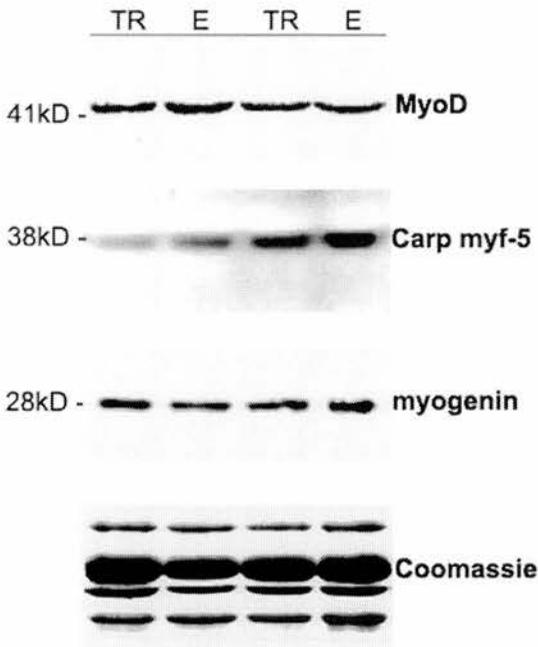
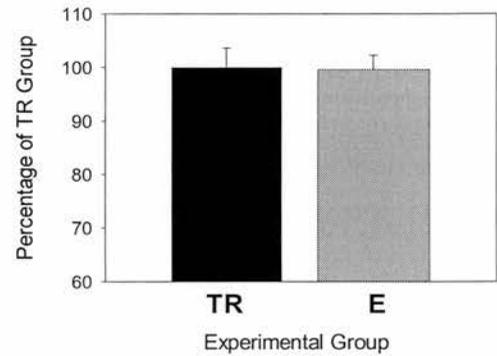


Fig. 3.19. Swimming experiment 3: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on fast muscle tissue nuclear localisation of MyoD and myf-5 proteins. A & B) MyoD nuclear localisation was 19% higher in the tank rested group (TR, black fills) relative to the exercised group (E, grey fills) in fast muscle tissue nuclear protein extracts ($P < 0.05$, two-tailed T-test). A & C) myf-5 nuclear localisation was 9% higher in the tank rested group relative to the exercised fish ($P < 0.01$, two-tailed T-test). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Values represent group mean optical density expressed as a percentage of the tank rested controls \pm SEM, $N = 9$. ** $P < 0.01$, * $P < 0.05$.

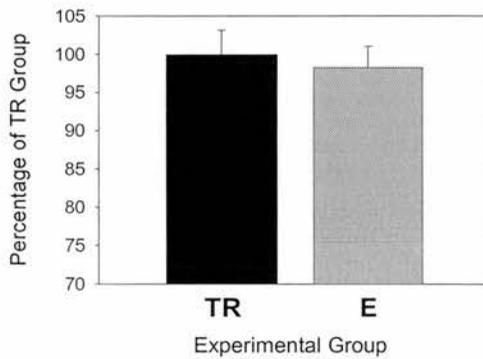
A Cellular



B) Cellular MyoD



C) Cellular myf-5



D) Cellular myogenin

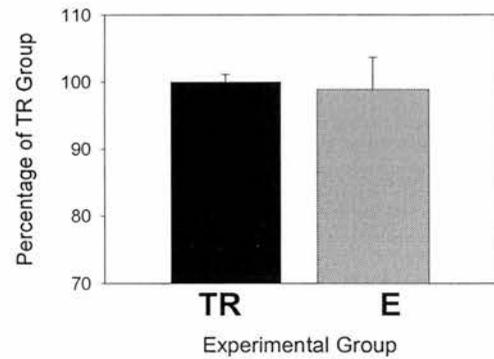


Fig. 3.20. Swimming experiment 3: the effect of tank rest or a four-week exercise-training regime ($2.6 - 2.7 \text{ bl s}^{-1}$, 14 hours per day) on total cellular expression levels of three myogenic regulatory factors (MRFs): MyoD, myf-5 and myogenin. A - D) There was no significant difference in MRFs expression in total fast muscle tissue cellular extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Values represent group mean optical density expressed as a percentage of the tank rested controls \pm SEM, N = 9. NS, non-significant.

A Cellular Extracts

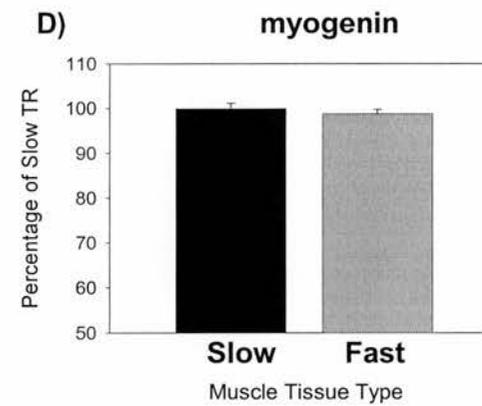
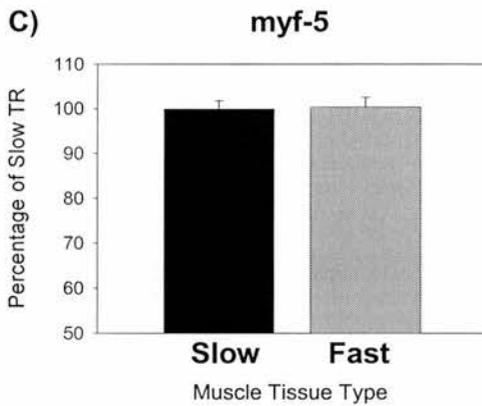
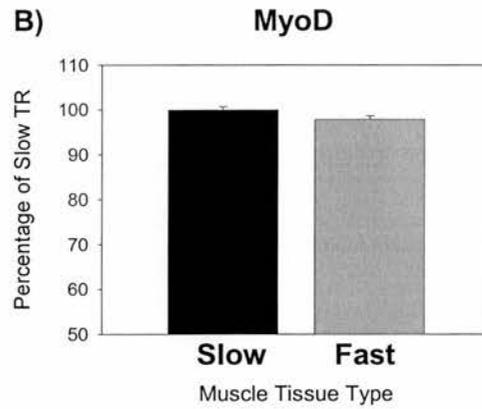
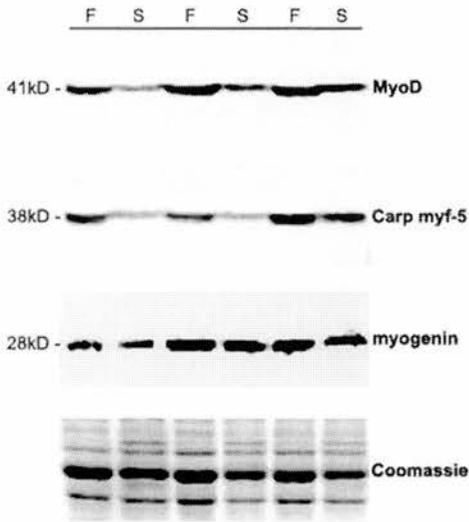
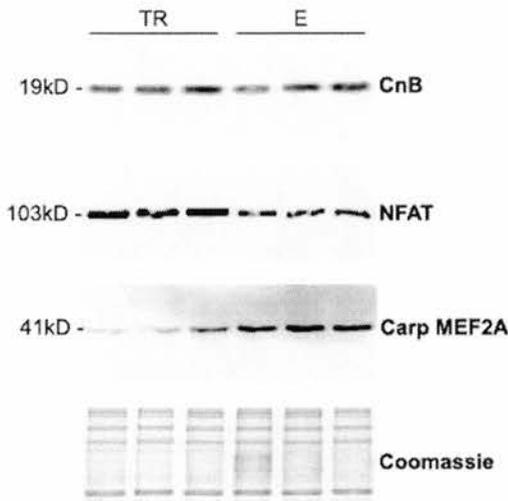


Fig. 3.21. Swimming experiment 3: expression of three myogenic regulatory factors (MRFs) in different muscle fibre phenotypes was examined in fast (F, black fills) and slow (S, grey fills) muscle total cellular protein extracts from tank rested common carp. A – D) There was no significant difference in MRFs expression in fast and slow muscle total cellular protein extractions (NS, two-tailed T-test). Data are group mean optical density \pm SEM, expressed as a percentage of the slow muscle results and corrected for a significant difference in protein loading ($P < 0.001$, two-tailed T-test). Optical density results from the Coomassie stained gels indicated a discrepancy in protein loading. $N = 9$ for fast and slow extracts. NS, non-significant.

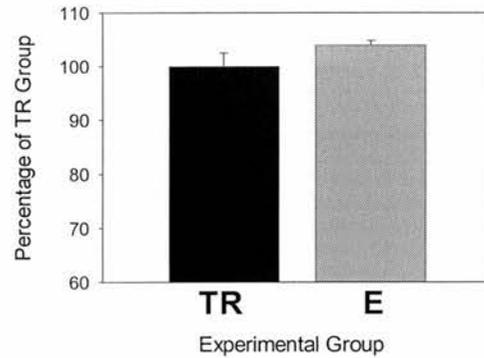
The nuclear localisation of the NFAT2 protein was 24% higher in tank rested controls, which was significantly different from the exercised group ($T = 5.5$, $DF = 6$, $P < 0.01$, two-tailed T-test) (Fig. 3.22A & C). A significant correlation did not exist between the increased extent of NFAT2 nuclear localisation and mean fast fibre cross-sectional area. There was a trend apparent in the nuclear localisation of the MEF2A protein, but the 9% higher level of MEF2A observed in the nuclear extracts of the exercised group was not statistically significant (Fig. 3.22A & D).

In fast muscle tissue extracts, nuclear localisation and overall expression of the catalytic (CnA) and regulatory (CnB) subunits of the calcineurin enzyme, was examined in the tank rested and exercised groups of carp in swimming experiment 3. Nuclear localisation of downstream calcineurin targets NFAT2 and MEF2 was also measured (Fig. 3.23). The nuclear localisation of the CnA subunit was 4% higher in the tank rested group relative to the exercised group, however this difference was not significant (Figs. 3.23A & B). In the tank rested group, a highly significant elevation (37%) in the level of CnB nuclear localisation was observed (Figs. 3.23A & C), relative to the exercised group ($T = 9.9$, $DF = 14$, $P < 0.001$, two-tailed T-test). CnB nuclear localisation was strongly correlated with mean fast muscle fibre cross-sectional area and this correlation was highly significant ($r_s = 0.79$, $P < 0.001$). There was no significant difference in the overall expression of either of the calcineurin subunits in total cellular protein extracts (Figs. 3.24A – C). A significantly higher (10%) level of NFAT2 protein was observed in the fast muscle tissue nuclear extracts of tank rested carp relative to the exercised group (Fig. 3.23A & D) ($T = 2.2$, $DF = 12$, $P < 0.05$, two-tailed T-test). There was no correlation between mean fast muscle fibre cross-sectional area and nuclear localisation of NFAT2.

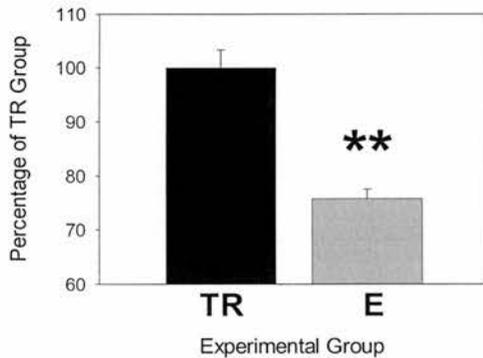
A Nuclear



B) Nuclear CnB



C) Nuclear NFAT



D) Nuclear MEF2A

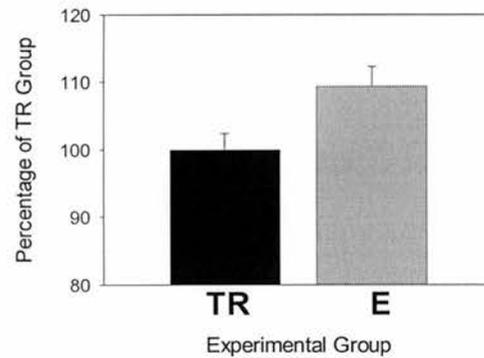
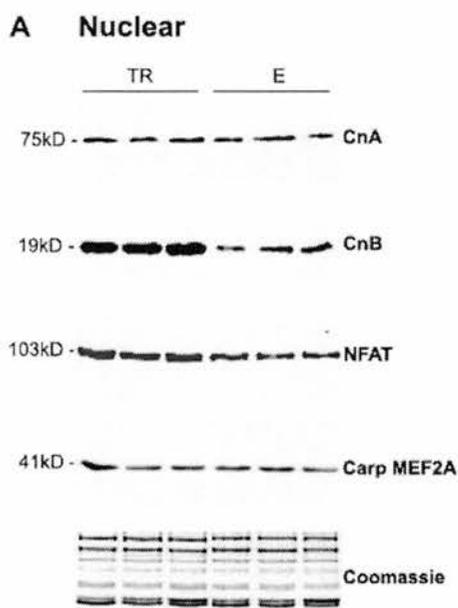
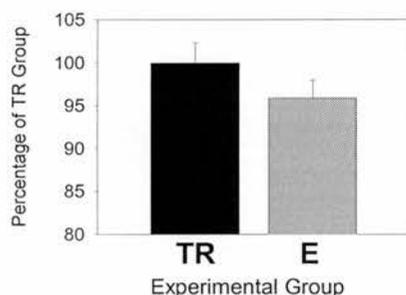


Fig. 3.22. Swimming experiment 2: the effect of tank rest or a three-week exercise-training regime ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours per day) on fast muscle tissue nuclear localisation of calcineurin B (CnB), and associated transcription factors NFAT and MEF2A. A & B) There was no significant difference in CnB nuclear localisation in fast muscle tissue nuclear protein extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). A & C) NFAT nuclear localisation was 24% higher in the tank rested group relative to the exercised fish ($P < 0.01$, two-tailed T-test). A & D) MEF2A nuclear localisation was 9% higher in the tank rested group relative to the exercised fish, but this increase was not significant (NS, two-tailed T-test). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Data are mean group optical density expressed as a percentage of the tank rested controls, \pm SEM, $N = 6$. NS, non-significant, ** $P < 0.01$.

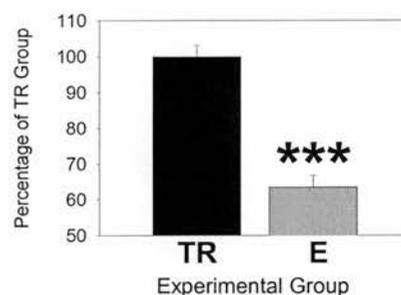
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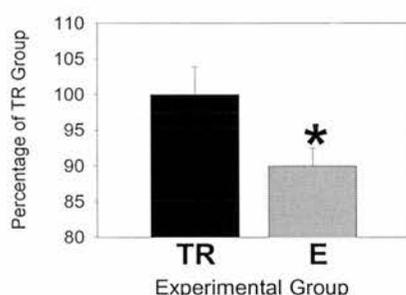
B) Nuclear CnA



C) Nuclear CnB



D) Nuclear NFAT



E) Nuclear MEF2A

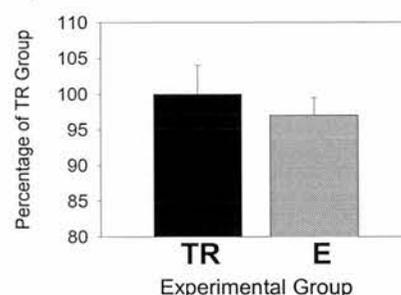
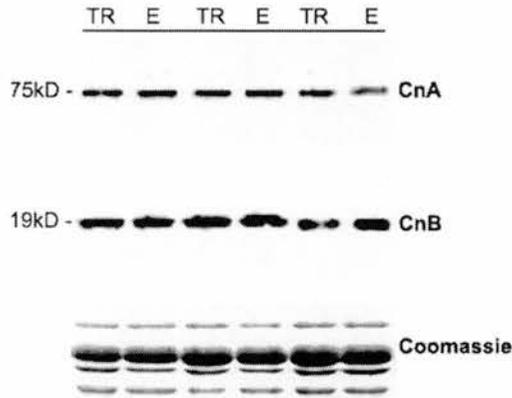


Fig. 3.23. Swimming experiment 3: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on fast muscle tissue nuclear localisation of calcineurin catalytic (CnA) and regulatory (CnB) subunits, and associated transcription factors NFAT and MEF2A. A, B & E) There was no significant difference in CnA nor MEF2A nuclear localisation in fast muscle tissue nuclear protein extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). A & C) CnB nuclear localisation was 37% higher in the tank rested group relative to the exercised fish ($P < 0.001$, two-tailed T-test). A & D) NFAT nuclear localisation was 10% higher in the tank rested group relative to the exercised fish ($P < 0.05$, two-tailed T-test). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Data are mean group optical density expressed as a percentage of the tank rested controls, \pm SEM, $N = 9$. NS, non-significant, * $P < 0.05$, *** $P < 0.001$.

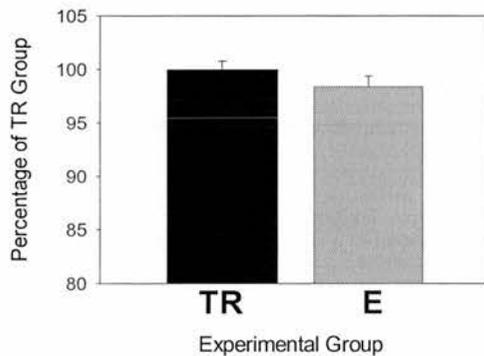
There was no significant difference in the nuclear localisation of the MEF2A protein (Figs. 3.23A & E), nor was there any difference in the molecular mass (41kD) of the MEF2A proteins detected in each experimental treatment that would indicate a calcineurin-mediated alteration in phosphorylation status.

A similar pattern of nuclear localisation and overall expression of calcineurin subunits and downstream transcription factor targets was found in slow muscle tissue extracts (Figs. 3.25). Significantly higher levels of the calcineurin subunits CnA and CnB were observed in the slow muscle nuclear protein extracts from the tank rested controls (Figs. 3.25A– C) which were 5% and 11% greater than the exercised group, respectively (CnA, $U_{9,9} = 12$, $P < 0.05$, Mann-Whitney Rank Sum test; CnB, $T = 3.9$, $DF = 10$, $P < 0.01$, two-tailed T-test). Mean slow fibre cross-sectional area was significantly correlated with nuclear localisation of the catalytic ($r_s = 0.60$, $P < 0.05$) and regulatory ($r_s = 0.61$, $P < 0.05$) subunits of calcineurin. There was no significant difference in the overall expression of the calcineurin subunits in slow muscle tissue total protein extracts (Figs. 3.26A– C). Nuclear localisation of the NFAT2 protein was significantly higher (10%) in the slow muscle nuclear extracts of the tank rested controls compared with the exercised carp (Fig. 3.25A & D) ($T = 4.4$, $DF = 10$, $P < 0.001$, two-tailed T-test). A significant correlation was not observed between mean slow fibre cross-sectional area and nuclear localisation of NFAT2. No significant difference was observed in the level of MEF2A protein in nuclear extracts or the molecular mass of the protein species detected (Figs. 3.25A & E).

A Cellular



B) Cellular CnA



C) Cellular CnB

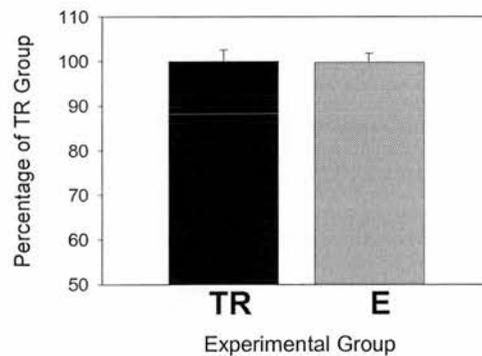
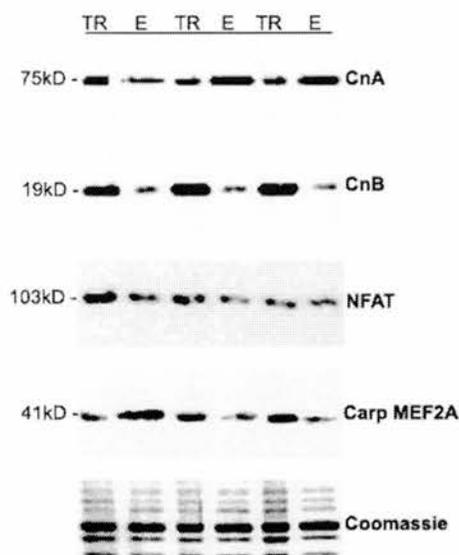
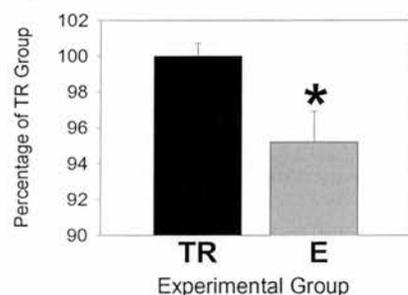


Fig. 3.24. Swimming experiment 3: the effect of tank rest or a four-week exercise-training regime ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on total cellular levels of the calcineurin catalytic (CnA) and regulatory (CnB) subunits in fast muscle tissue protein extractions. A - C) There was no significant difference in levels of the two calcineurin subunits in total fast muscle tissue cellular extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Values represent group mean optical density expressed as a percentage of the tank rested controls \pm SEM, N = 9. NS, non-significant.

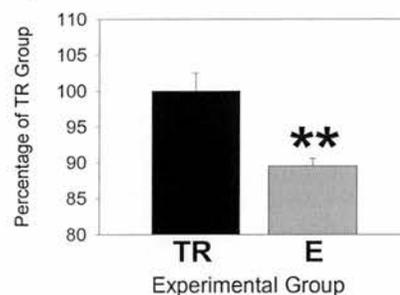
A Slow Nuclear



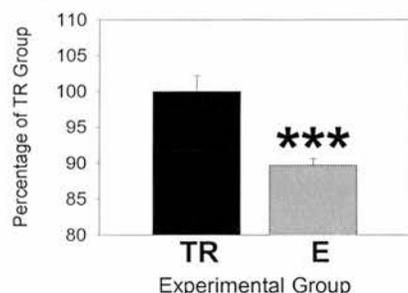
B) Slow Nuclear CnA



C) Slow Nuclear CnB



D) Slow Nuclear NFAT



E) Slow Nuclear MEF2A

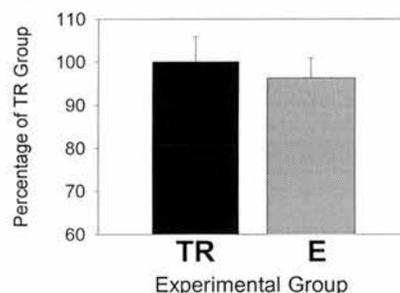
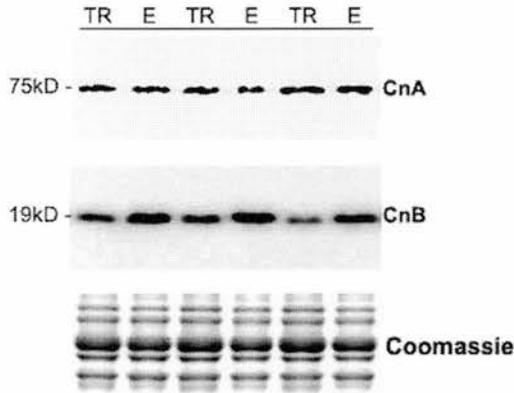
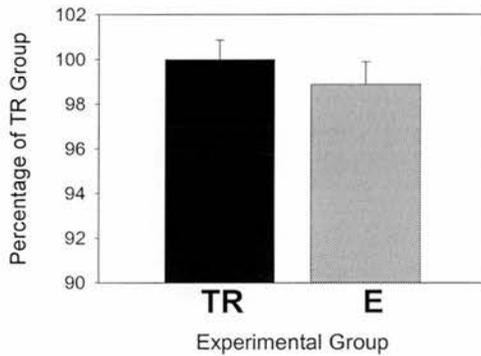


Fig. 3.25. Swimming experiment 3: the effect of tank rest or a four-week exercise-training programme ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on slow muscle tissue nuclear localisation of calcineurin catalytic (CnA) and regulatory (CnB) subunits, and associated transcription factors NFAT and MEF2A. A & B) CnA nuclear localisation in slow muscle tissue nuclear protein extracts was 5% higher in the tank rested controls (TR, black fills) relative to the exercised group (E, grey fills) ($P < 0.05$, Mann-Whitney Rank Sum test). A & C) CnB nuclear localisation was 11% higher in the tank rested group relative to the exercised fish ($P < 0.01$, two-tailed T-test). A & D) NFAT nuclear localisation was 10% higher in the tank rested group relative to the exercised fish ($P < 0.001$, two-tailed T-test). A & E) There was no significant difference in MEF2A nuclear localisation between the two experimental treatments (NS, two-tailed T-test). Slow muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Data are mean group optical density expressed as a percentage of the tank rested controls, \pm SEM, $N = 9$. NS, non-significant, * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$.

A Slow Cellular



B) Slow Cellular CnA



C) Slow Cellular CnB

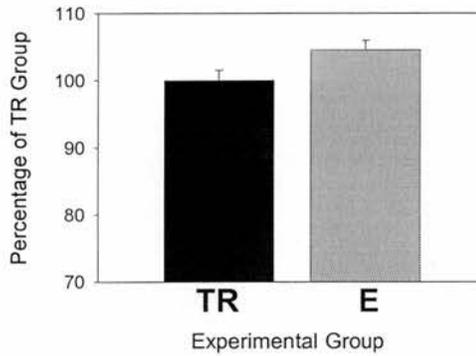


Fig. 3.26. Swimming experiment 3: the effect of tank rest or a four-week exercise-training regime ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours per day) on total cellular levels of the calcineurin catalytic (CnA) and regulatory (CnB) subunits in slow muscle tissue protein extractions. A - C) There was no significant difference in levels of the two calcineurin subunits in total slow muscle tissue cellular extracts between tank rested (TR, black fills) and exercised (E, grey fills) experimental groups (NS, two-tailed T-test). Slow muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from both experimental treatments. Values represent group mean optical density expressed as a percentage of the tank rested controls \pm SEM, N = 9. NS, non-significant.

3.4 Discussion

3.4.1 The effect of exercise on the somatic growth

Condition factor, a combined measure of fork length and body mass is an index of somatic growth. A higher measure of condition factor indicates greater body mass for a given fork length (see Totland *et al.*, 1987). The effect of exercise on somatic growth in fish has been extensively studied in aquaculture projects, with variable results. Intense, high-speed short duration bouts of exercise (5 minutes, twice daily) were shown to significantly reduce specific weight gain in rainbow trout (Hernández *et al.*, 2002), whereas prolonged low intensity exercise in Atlantic salmon significantly enhanced body mass (Totland *et al.*, 1987). In contrast, a recent industrial study of farmed Atlantic cod demonstrated no change in condition factor with prolonged continuous exercise at two low intensity swimming velocities (Bjørnevik *et al.*, 2003).

Swimming experiments utilising cyprinid species have also demonstrated unpredictable growth patterns related to long-term training at speeds between 2.0 and 3.5 bls⁻¹. Condition factor in chub and nase was indistinguishable in trained and untrained individuals (Hinterleitner *et al.*, 1992; Sanger, 1992), whereas a significantly higher condition factor was observed in exercised Danube bleak (Hinterleitner *et al.*, 1992). Condition factor in exercised and tank rested goldfish calculated from the group mean length and body weight data (Davison & Goldspink, 1978), demonstrated an inverse relationship between swimming speed and condition factor, where the greatest reduction in condition factor was observed in the group trained continuously at 4.5bls⁻¹ for a four week period. The effect of exercise training on condition factor in common carp was assessed in swimming experiments 1 to 4.

In swimming experiment 1, there was no significant difference in condition factor in response to tank rest or continuous swimming activity ($2.1 - 2.3 \text{ bls}^{-1}$) for a 21-day period (Table 3.2). Initial body mass measurements were not made in this pilot study and changes in condition factor over the course of the experiment could not be assessed. Mean fork length and body mass were greater in the tank rested group, which although not significantly different from the trained group (Fig. 3.5A) may be an indication of reduced somatic growth in response to exercise in this experiment. Data from swimming experiment 2 suggested that exercise could enhance somatic growth in common carp where a significantly higher condition factor was observed in trained fish in response to moderate velocity exercise training ($1.7 - 1.9 \text{ bls}^{-1}$) for 16 hours each day (Table 3.4). However at the outset of this experiment, common carp were approximately size matched for fork length, body mass measurements were not taken and the effect of exercise on condition factor over the course of the 21-day study period could not be examined.

To improve the experimental design, somatic growth characteristics were monitored for 8 weeks prior to exercise training in swimming experiment 3. In this experiment common carp were accurately size matched for fork length and body mass and assigned to two experimental groups; condition factor did not vary significantly between groups at the outset of the training regime, nor at the two previous time points prior to the swimming phase of the experiment. Under normal growth conditions, condition factor significantly increased in parallel in both experimental treatments during the initial 8-week period. Condition factor was significantly reduced in common carp exercised for fourteen hours each day at $2.6 - 2.7 \text{ bls}^{-1}$ for the four weeks of swimming experiment 3 (Fig. 3.10A). In swimming experiment 4, condition factor was also significantly reduced in the exercised group in response to the same exercise-training regime used in the previous experiment (Fig. 3.13A). The tank rested group in

swimming experiment 4 did not demonstrate a significant increase in condition factor over the course of this experiment, but this was most likely due to the different time of year and different mean water temperature conditions the experiment was conducted under.

In conclusion, the results from these swimming studies imply that under certain favourable conditions, slow to moderate speed endurance exercise can stimulate increased somatic growth in the common carp (swimming experiment 2). However at moderate exercise velocities in excess of 2 bls^{-1} , the effect of exercise training is detrimental to body mass accumulation, which is seen in the reduced condition factor of the exercised groups in swimming experiments 3 and 4.

3.4.2 The effect of exercise on the myotomal musculature

In salmonids the hypertrophic response of the fast and slow myotomal musculature to continuous exercise training is well established (Davison & Goldspink, 1977; Johnston & Moon, 1980a; Totland *et al.*, 1987). The adaptive response to prolonged continuous exercise activity varied between cyprinid species: in chub and nase slow muscle area fraction and fibre diameter were increased, as was the number and size of intermediate muscle fibres (Hinterleitner *et al.*, 1992; Sanger, 1992), whereas the Danube bleak displayed hypertrophic growth of the slow and fast muscle fibres in response to training (Hinterleitner *et al.*, 1992).

The mean fast fibre cross-sectional area was significantly reduced in the exercised group in swimming experiment 1, relative to tank rested controls (Fig. 3.5B). The peak density distribution of fast muscle fibre diameter in the exercised group appears to have been shifted to the left relative to the tank rested controls (Fig. 3.6A) and mean fast fibre diameter in the exercised group was significantly smaller for the 5th – 99th percentiles (Fig. 3.6B). The fast musculature of common carp has a mosaic appearance where new

small diameter fast muscle fibres form on the surface of existing muscle fibres (Boddeke *et al.*, 1959; Stickland, 1983). The lower mean fast fibre cross-sectional area in the exercised group could be the result of an input of new fast muscle fibres to the myotome stimulated by exercise activity. Slow and/ or intermediate muscle fibre recruitment has previously been demonstrated in brown trout nase and the Danube bleak in response to exercise training (Davison & Goldspink, 1977; Hinterleitner *et al.*, 1992). However, the total myotomal cross-sectional area was not measured in this pilot study and exercise induced fast fibre recruitment, which would cause a concomitant reduction in mean fast fibre cross-sectional area, could not be confirmed. Overall, the data in swimming experiment 1 indicate that exercise may have stimulated fast muscle fibre recruitment. Conversely, exercise may have restricted the growth of fast fibres relative to the tank rested controls. The experiment was conducted at ambient temperature and photoperiod in November and it is likely that the growth of all experimental animals was limited under these conditions. Hence, it is more likely that the smaller mean fast muscle fibre cross-sectional area observed in the exercised group was the result of protein depletion or atrophy. At lower environmental temperatures, fast muscle fibres are involved in swimming activity at relatively lower swimming velocities (Rome *et al.*, 1984). The reduced mean fast fibre cross-sectional area in the exercised group may have been caused by protein breakdown and tissue depletion to cope with elevated levels of lactic acid.

Common carp were exercised for a 21-day period ($1.7 - 1.9 \text{ bls}^{-1}$, 16 hours a day) in swimming experiment 2, which resulted in a significantly greater total fast muscle cross-sectional area relative to tank rested controls (Fig. 3.7B). The smaller mean fast fibre cross-sectional area and the significantly higher number of fast fibres in the exercised group (Fig. 3.7C & D) suggested that exercise-induced fast muscle fibre recruitment contributed to the significant increase in total myotomal cross-sectional area.

The fast fibre diameter density distributions of the experimental treatments were not significantly different however, which illustrated the intrinsic difficulties associated with measuring the dynamics of muscle fibre growth in fish, due to the different relative contributions of muscle fibre hypertrophy and recruitment (Fig. 3.9A). There was a noticeable trend of increased growth of the slow musculature in the exercised common carp in this experiment. Compared with tank rested controls, there was an increase in total slow muscle area fraction, fibre number and fibre cross-sectional area, but relative to fork length these adaptive responses were not significantly different (Fig. 3.8A – C). The density distribution of slow fibre diameter in the exercised group was shifted noticeably to the right relative to the tank rested group, which indicated that slow to moderate endurance exercise could stimulate slow muscle fibre hypertrophy in this species (Fig. 3.9B). In previous experiments on cyprinid species (Hinterleitner *et al.*, 1992; Sanger, 1992), the duration of the training period that enhanced slow muscle growth was several times longer than in swimming experiment 2.

In swimming experiment 3, there was no significant effect of a 28-day period of exercise ($2.6 - 2.7 \text{ bls}^{-1}$, 14 hours a day) on the total fast muscle cross-sectional area (Fig. 3.11A). Mean fast fibre cross-sectional area was significantly lower, whereas the total number of fast muscle fibres was significantly higher in the exercised group relative to tank rested controls (Fig. 3.11B & C). The peak density of the fast fibre distribution in the exercised group was shifted towards the left relative to tank rested controls, and the 5th – 99th percentiles of fibre diameter were significantly less (Fig. 3.12A & C). These results indicate that muscle fibre recruitment in the common carp was induced by a four-week period of endurance exercise training at a moderate swimming speed ($2.6 - 2.7 \text{ bls}^{-1}$). The similar distribution of total fast muscle cross-sectional areas observed in both experimental groups implied that there was no detrimental effect of exercise on overall

muscle mass. The lower mean fibre diameters in the exercised group at the 50th, 95th and 99th percentiles suggest that atrophy may be occurring in the larger fast muscle fibres in response to training. The smaller mean fibre diameters in the exercised group at the 5th and 10th percentiles suggest that there was an input of small diameter fibres to the myotome in response to training. The balance between fast muscle fibre recruitment and atrophy of the larger fast fibres led to similar total fast muscle cross-sectional areas in both experimental treatments. In this experiment, mean slow fibre cross-sectional area was significantly lower in the exercised common carp (Fig. 3.11D) and the peak density of the slow fibre distribution was displaced to the left relative to tank rested controls (Fig. 3.12B). The 50th, 95th and 99th percentiles of slow fibre diameter were significantly less in the exercised group (Fig. 3.12D). These results indicate that the exercise treatment had a detrimental effect on the slow musculature of exercised common carp in swimming experiment 3, causing atrophy and reduced cross-sectional area of the larger slow muscle fibres.

Swimming experiment 4 was conducted using the same experimental set-up as the previous experiment (2.6 – 2.7 bls⁻¹, 14 hours a day, 28 days). Critically however, different experimental apparatus (Fig. 3.3) was utilised and as a consequence water temperature could only be maintained at a mean of just 14.5°C during the experiment, compared to 21.7°C in swimming experiment 3. Perhaps as a consequence of lower water temperature or lower exercise intensity due to the smaller swim channel used in swimming experiment 4 (Fig. 3.3, aquarium flume apparatus), there was no significant effect of exercise on total fast muscle cross-sectional area (Fig. 3.13C). The total number of fast fibres (deep and superficial) contributing to the myotomal cross-sectional area was almost identical across experimental treatments (Fig. 3.13 D). There was also no significant variation between treatments in the morphometric characteristics of deep fast

and superficial fast muscle fibres when considered separately (Figs. 3.14 & 3.15). Exercise training did have a major effect on the slow musculature however, and mean slow fibre cross-sectional area relative to fork length was significantly greater in the exercised common carp (Fig. 3.16A). The slow fibre diameter density distribution of the exercised group was significantly transferred to the right relative to tank rested controls (Fig. 3.16B) and the 5th – 99th percentiles of mean slow fibre diameter were significantly higher in the exercised group (Fig. 3.16C). Therefore four weeks of exercise training in this experiment were sufficient to induce slow muscle fibre hypertrophy in common carp. The disparity of the results between swimming experiments 3 and 4 was most likely caused by the use of different experimental apparatus in these studies, which had a secondary effect on the water temperature. It is likely that the increased width of the swimming channel and higher flow capacity of the Armfield S8 flume utilised in swimming experiment 3 (Fig. 3.2) reduced the potential for ‘slip-streaming’ and led to a higher level of exercise training in this experiment relative to swimming experiment 4, despite the same flow rate ($2.6 - 2.7 \text{ bls}^{-1}$) being used in both experiments.

In conclusion, exercise training in the common carp induces fast muscle fibre recruitment or slow muscle fibre hypertrophy under different experimental conditions. Data from swimming experiments 2 and 3 suggest that moderate velocity ($2.6 - 2.7 \text{ bls}^{-1}$) endurance exercise training stimulates a higher degree of fast muscle fibre recruitment than slow to moderate ($1.7 - 1.9 \text{ bls}^{-1}$) exercise. Recruitment of slow and intermediate muscle fibres in response to exercise has been reported in salmonid and cyprinid species of fish (Davison & Goldspink, 1977; Hinterleitner *et al.*, 1992; Sanger, 1992). There was no evidence of fast muscle fibre hypertrophy in response to moderate endurance exercise training in any of the four swimming experiments ($1.7 - 2.7 \text{ bls}^{-1}$). Furthermore, results from swimming experiments 1 and 3 implied that exercise activity above 2 bls^{-1} might

inhibit fast muscle fibre growth or even result in fast muscle tissue depletion in common carp. Continuous endurance exercise training up to 4.5 bls^{-1} has been shown to induce fast muscle fibre hypertrophy in numerous teleost species and a proportional relationship between swimming speed and fast fibre hypertrophy has been demonstrated for goldfish (Davison & Goldspink, 1978), a cyprinid species with a similar lifestyle to common carp. However, the results of swimming experiments 1 to 4 suggest that unlike numerous other teleost species, exercise cannot be used as a stimulus for fast muscle fibre hypertrophy in common carp and therefore the first *a priori* hypothesis must be rejected.

Data from swimming experiments 2 and 4 suggest that slow muscle fibre hypertrophy is directly proportional to the level of endurance exercise experienced. Three weeks of slow to moderate speed endurance exercise training ($1.7 - 1.9 \text{ bls}^{-1}$) stimulated a small but non-significant increase in slow muscle fibre growth in swimming experiment 2. In swimming experiment 4, moderate velocity endurance exercise training ($2.6 - 2.7 \text{ bls}^{-1}$) for four weeks stimulated slow muscle fibre hypertrophy. Although atrophy of slow muscle fibres was observed at the same swimming speed in swimming experiment 3, the different experimental conditions in these studies makes comparison of the results problematical. It is likely that the exercised common carp in swimming experiment 3, due to contrasting properties of the swimming flumes used, experienced a higher intensity of exercise training. Therefore it can be concluded that moderate velocity endurance exercise training for four weeks is sufficient to stimulate hypertrophic growth in the slow/ aerobic muscle fibres of the common carp. Exercise-induced slow muscle fibre hypertrophy has also been demonstrated in several other species of cyprinids (Hinterleitner *et al.*, 1992; Sanger, 1992) and numerous other teleost species.

3.4.3 The effect of exercise training on myonuclear density

The *de novo* production of myonuclei through the activation and proliferation of myogenic progenitor cells, followed by withdrawal from the cell cycle and fusion to existing myofibres is well characterised in higher vertebrates (reviewed by Zammit & Beauchamp, 2001). Activation and proliferation of satellite cells can be induced by numerous stimuli in higher vertebrates including physical training and muscle injury (reviewed by Antonio & Gonyea, 1993). For example, a concomitant increase in the number of satellite cells and myonuclei in the trapezius muscle of females was induced by strength training (Kadi & Thornell, 2000).

Myogenic progenitor cell activity in teleosts has been shown to increase in response to a feeding event after a period of fasting (Fauconneau & Paboeuf, 2000; Brodeur *et al.*, 2003b) and cell cycle time is shorter at a higher environmental temperature (Brodeur *et al.*, 2003a). In Atlantic salmon, an increase in muscle fibre myonuclear density through manipulation of photoperiod or water temperature has been shown in association with muscle fibre recruitment (Johnston *et al.*, 2003b; Johnston *et al.*, 2003c). The effect of four weeks of moderate endurance exercise-training ($2.6 - 2.7 \text{ bls}^{-1}$) on the density of myonuclei in isolated fast fibres from trained and untrained experimental treatments was assessed in swimming experiment 4 (Fig. 3.17). A significantly higher density of myonuclei cm^{-1} was observed in the exercised group relative to tank rested controls.

Johnston *et al.* (2003c) proposed that separate genetic mechanisms regulate the processes of hypertrophy and hyperplasia in teleosts. A single population of myogenic precursor cells generate the nuclei required to meet the demands of growth and repair in mature teleost muscle and late signaling by local factors determines a hyperplastic or hypertrophic fate for these cells. In swimming experiment 3 it was proposed that

moderate endurance exercise training was a hyperplastic stimulus, however fast muscle fibre recruitment was not observed in swimming experiment 4 in response to the same intensity of training. In conclusion, increased myogenic progenitor cell activation and proliferation was stimulated by moderate intensity exercise in swimming experiment 4, which is evident in the higher density of myonuclei cm^{-1} observed in isolated fast muscle fibres from exercised fish. In this case it is proposed that late signaling by local factors determined a hypertrophic fate for these nuclei, as there is no evidence of fast muscle fibre hyperplasia in exercised fish in swimming experiment 4. This fits the model of DNA accretion preceding protein accretion observed in mammalian postnatal skeletal muscle growth through hypertrophy (reviewed by Schultz, 1996). The second *a priori* hypothesis can be accepted as the increased myonuclear density observed in mammals in response to an overload stimulus was also observed in common carp subjected to endurance exercise activity.

3.4.4 The effect of exercise on myogenic regulatory factor expression

Proliferation and terminal differentiation of myogenic progenitor cells in response to hypertrophic stimuli is regulated by sequential expression of MRFs (Rosenblatt *et al.*, 1994; Smith *et al.*, 1994). In higher vertebrates, numerous examples exist of exercise-induced upregulation of MRFs in response to heavy resistance training (Haddad & Adams, 2002; Psilander *et al.*, 2003) and exercise-induced eccentric muscular contractions (Armand *et al.*, 2003; Peters *et al.*, 2003). In fish, upregulation of MRFs transcripts has been demonstrated during recovery from a period of fasting (Chauvigné *et al.*, 2003) and a single feeding event stimulated an increased proportion of myogenic progenitor cells positive for MRFs (Brodeur *et al.*, 2003b).

The data from swimming experiment 3 indicates that increased nuclear localisation of MRFs protein is correlated with larger mean fast fibre cross-sectional area.

In swimming experiment 2, exercise treatment at 1.7 – 1.9 bls⁻¹ did not have a significant effect on mean fast fibre cross-sectional area and there was also no variance in MyoD nuclear localisation (Fig. 3.18). Exercise training at 2.6 – 2.7 bls⁻¹ led to a significantly lower mean fast fibre cross-sectional area relative to tank rested controls, which was significantly correlated with reduced nuclear localisation of the primary MRFs, MyoD and myf5 (Figs. 3.19). The overall expression of MRFs in total cellular protein extracts did not vary between experimental groups (Figs. 3.20).

For muscle fibre recruitment to occur, increased proliferation of myogenic progenitors is required to provide the nuclei that will subsequently differentiate and fuse to form new muscle fibres. Sequential expression of MRFs regulates proliferation and differentiation of myogenic progenitor cells. Exercised fish displayed relatively lower levels of MRFs nuclear localisation, which implied exercise did not provide a stimulus for increased proliferation of myogenic progenitors in swimming experiment 3. Yet significant exercised-induced fast muscle fibre recruitment was observed in this experiment. This may be explained by an initial transitory upregulation of MRFs that mediated increased proliferation of myogenic progenitors at the outset of the training period and by the end of the experiment this signal was diminished. Several studies have demonstrated the transient nature of MRFs expression in response to hypertrophic stimuli and the return to basal levels with the removal of the stimuli (Jacobs-El *et al.*, 1995; Carson & Booth, 1998; Lowe & Alway, 1999). The response to exercise in swimming experiment 3 involved a combination of muscle fibre recruitment balanced by atrophy of the existing fast muscle fibres. The lower level of MRFs nuclear localisation in common carp after 28 days of exercise training may be a reflection of exercise-induced atrophy. The significant somatic growth observed in the tank rested group most likely involved

outgrowth of fast muscle fibres through hypertrophy, which is reflected in the relatively higher nuclear localisation of primary MRFs MyoD and myf-5.

3.4.5 Fibre-type specification of myogenic regulatory factors (MRFs)

In higher vertebrates, a putative role of MRFs is the specification and maintenance of muscle fibre phenotype. Different members of the MRFs are preferentially expressed in phenotypically distinct muscle fibre types. Protein and transcripts of MyoD and myf5 accumulate in fast-twitch fibres, with myogenin primarily expressed in slow-twitch fibres (Hughes *et al.*, 1993; Voytik *et al.*, 1993; Sakuma *et al.*, 1999). However in the common carp, no significant difference was observed in the expression of MyoD, myf5 and myogenin in total cellular protein extracts from fast and slow muscle tissue extracts (Fig. 3.21).

In mammalian muscle, a strong correlation was observed between the nuclear localisation of MyoD protein and fast glycolytic type IIb fibres, suggesting a function of MyoD in the specification and maintenance of fast IIb fibres (Hughes *et al.*, 1997; Kraus & Pette, 1997). To elucidate a role for MRFs in muscle fibre type specification in common carp, a further study would examine the nuclear localisation of MRFs protein, rather than overall expression in total cellular protein extracts.

3.4.6 Exercise as a stimulus for the calcineurin signalling pathway

Sustained elevated levels of intracellular Ca^{2+} such as those observed with prolonged exercise activity, have been shown to selectively activate the protein phosphatase calcineurin (Dolmetsch *et al.*, 1997). In mammalian skeletal muscle activation of the calcineurin signalling pathway in response to functional overload or IGF-1 administration is associated with skeletal muscle hypertrophy (Dunn *et al.*, 1999; Musaro *et al.*, 1999; Semsarian *et al.*, 1999). Muscle specific gene expression is

mediated by nuclear translocation of dephosphorylated NFAT2 and calcineurin proteins (Musaro *et al.*, 1999; Semsarian *et al.*, 1999). Calcineurin mediated dephosphorylation of NFAT2 and MEF2 is also associated with nerve and activity dependent specification of muscle fibre type (Chin *et al.*, 1998). In addition, calcineurin mediates differentiation of myocytes by activating MyoD and MEF2 and regulates myf5 gene expression *in vitro* (Friday & Pavlath, 2000; Friday *et al.*, 2003).

In swimming experiment 2, there was no significant difference in nuclear localisation of the calcineurin regulatory subunit (CnB) (Fig. 3.22). In this experiment, exercise training at 1.7 – 1.9 bls⁻¹ did not have a significant effect on mean fast fibre cross-sectional area. In contrast, CnB nuclear localisation was strongly correlated with mean fibre cross-sectional area in swimming experiment 3; significantly larger mean fast and slow fibre cross-sectional areas were associated with increased nuclear localisation of the CnB protein in tank rested controls (Figs. 3.23 – 3.26). Additionally, a significantly higher level of the calcineurin catalytic subunit (CnA) was observed in slow muscle tissue nuclear extracts from tank rested controls, which was significantly correlated with mean slow fibre cross-sectional area. The overall expression of calcineurin subunits in total protein extracts did not vary between experimental treatments in swimming experiment 3.

A significantly higher level of NFAT2 protein in the nuclear fraction from tank rested animals was also observed in swimming experiments 2 and 3, but this was not significantly correlated with mean muscle fibre cross-sectional area. Increased nuclear localisation of NFAT2 has previously been shown to initiate the hypertrophic response in higher vertebrates in association with calcineurin (Musaro *et al.*, 1999; Semsarian *et al.*, 1999). Therefore the elevated levels of NFAT2 in the tank rested group potentially indicate that there is an additional function of NFAT2 in common carp that is yet to be

discovered. However in fast (Fig. 3.23) and slow (Fig. 3.25) subcellular (nuclear) extracts in the tank rested group in swimming experiment 3, the relatively higher level of nuclear localisation of calcineurin was associated with a similarly increased nuclear localisation of NFAT2. This result implies that during normal muscle growth in common carp calcineurin dephosphorylates NFAT2 and is translocated to the nucleus, where it may play a role in the regulation of normal muscle growth through hypertrophy.

Nuclear localisation of MEF2A was slightly elevated in the exercised group in swimming experiment 2 (Fig. 3.22), but this was not associated with any discernible change in muscle fibre morphological characteristics. Furthermore, where a significant difference existed in fast and slow muscle fibre cellularity between experimental groups (swimming experiment 3) nuclear localisation and the phosphorylation state of MEF2A was invariant in fast (Fig. 3.23) and slow (Fig. 3.25) muscle tissue subcellular extracts. These data suggest that the MEF2A transcription factor does not play a major role in growth of fast or slow muscle tissue in common carp, as nuclear localisation and phosphorylation do not vary significantly with muscle fibre cellularity in exercised and non-exercised experimental groups.

3.5 Conclusion and future directions

The main objective of the four swimming experiments was to develop a reproducible model of exercise-induced fast and/ or slow muscle fibre hypertrophy. This aim was only partially achieved, as the study species (common carp) did not respond to the exercise treatment as predicted. Lower intensity exercise (swimming experiments 2 & 4) did not elicit a noticeable change in fast muscle fibre cellularity, whereas higher intensity activity (swimming experiments 1 & 3) resulted in apparent fibre recruitment in conjunction with depletion of fast and slow muscle tissue. The development of the muscle hypertrophy model was hindered by enforced changes in experimental apparatus

between experiments. The swimming flumes used were of different sizes and, as a consequence, the water flow characteristics varied between experiments. For example, the increased width of the swimming channel and higher flow capacity of the Armfield S8 flume utilised in swimming experiment 3, probably reduced the potential for 'slipstreaming' and led to a higher level of exercise training in this experiment relative to swimming experiment 4, despite the same flow rate ($2.6 - 2.7 \text{ bls}^{-1}$) being used in both experiments. Hence, moderate endurance exercise training ($2.6 - 2.7 \text{ bls}^{-1}$) using the Armfield S8 flume (Fig. 3.2) caused fast fibre recruitment and atrophy of large fast and slow muscle fibres, whereas an identical level of exercise training in the smaller aquarium flume apparatus (Fig. 3.3) stimulated slow muscle fibre hypertrophy and accretion of myonuclei in fast muscle fibres. The disparity of these results was most likely caused by the use of different experimental apparatus in these studies, which had a secondary effect on the water temperature: water temperature could be maintained at 21.7°C using the Armfield S8 flume, compared with 14.5°C in the aquarium flume. In exercise studies water temperature is an extremely important factor, as it influences the swimming speeds at which different muscle fibre types are involved in swimming activity and hence can affect the subsequent growth of these fibres (Rome *et al.*, 1984). Despite the numerous inconsistencies in experimental design it is possible to conclude that in common carp, slow to moderate exercise stimulated slow muscle fibre hypertrophy, whereas moderate exercise stimulated fast fibre recruitment concurrent with fast and slow muscle tissue depletion.

The nuclear localisation and overall expression of MRFs, calcineurin and associated transcription factors NFAT2 and MEF2A were investigated in swimming experiments 2 and 3. These data strongly suggest that the calcineurin signaling pathway is involved in muscle fibre hypertrophy in the common carp under normal growth

conditions. The relative increase in nuclear localisation of calcineurin protein, MyoD and myf5 associated with hypertrophic muscle growth in the tank rested treatment in swimming experiment 3, also implies that the MRFs are potential downstream intracellular targets of calcineurin signaling in the common carp. Hypertrophic growth of fast and slow muscle fibres under normal growth conditions (tank rest) is significantly correlated with nuclear localisation of the calcineurin catalytic (CnA) and regulatory (CnB) subunits and the primary MRFs MyoD and myf5. These data suggest a role for the calcineurin signaling pathway and downstream intracellular targets such as the MRFs, in the regulation of axial muscle growth in the common carp.

Chapter 4: Time course of muscle responses to enforced exercise in common carp *Cyprinus carpio* L.

4.1 Introduction

The effect of exercise on the musculature of the common carp has previously been described in Chapter 3. Moderate intensity exercise training was shown to induce the recruitment of fast muscle fibres and atrophy of fast and slow muscle fibres. In contrast, lower intensity moderate exercise training stimulated slow muscle fibre hypertrophy and accretion of myonuclei in fast muscle fibres. The process of muscle fibre recruitment requires an input of nuclei, provided by the proliferation and subsequent differentiation and fusion of myogenic progenitor cells. Myogenic regulatory factors (MRFs) regulate the proliferation and differentiation of myogenic progenitor cells, yet overall expression of MRFs in the common carp was unaffected by 28-days of exercise training. It was proposed that the proliferative response of nuclei associated with muscle fibre recruitment was initiated by a transient upregulation of MRFs transcripts expression. The primary objective of the time course experiment was to elucidate the time course of MRFs expression in common carp in response to an exercise-training stimulus. A transient upregulation of MRFs transcripts is observed in higher vertebrates subjected to a range of hypertrophic stimuli. After the initial upregulation, expression of MRFs transcripts can remain elevated for up to 6 days or return to basal levels within 24 hours (Jacobs-EI *et al.*, 1995; Carson & Booth, 1998; Lowe & Alway, 1999). Based on these findings, an initial upregulation of MRFs would be expected in the common carp if

the teleost response to a chronic exercise overload stimulus paralleled that of higher vertebrates.

Proliferating cell nuclear antigen (PCNA) is a DNA polymerase δ associated peptide that is synthesised early in the G1 and S-phase of the cell cycle and whose expression is correlated with DNA synthesis (Bravo *et al.*, 1987; Baserga, 1991). Expression of PCNA in association with MyoD in myogenic cells is a marker of myogenic cell activation (Yablonka-Reuveni & Rivera, 1994). An increase in the number of PCNA positive myogenic progenitor cells has been shown in response to a single feeding event in the sub-Antarctic notothenioid (*Notothenia coriiceps* Richardson) (Brodeur *et al.*, 2003b). The second aim of this study was to quantify the time course of nuclear proliferation in response to exercise training, using PCNA expression as a marker. In chapter 3, slow to moderate intensity exercise training was shown to be a stimulus for accretion of myonuclei in fast muscle fibres, therefore an increase in PCNA expression over the duration of the time course experiment would be expected in response to an exercise training stimulus.

The effect of exercise on muscle fibre phenotype has been investigated extensively in humans as a means of adapting muscle for a particular purpose. It has long been recognised that endurance training increases the proportion and number of slow-twitch type I fibres (Gollnick *et al.*, 1972; Jansson & Kaijser, 1977; Jansson *et al.*, 1978). More recently, muscle fibre phenotype has been shown to change in the opposite direction; repeated 'all-out' 30-second sprints led to an increase in fast-twitch type IIa/b fibres (Jansson *et al.*, 1990; Esbjornsson *et al.*, 1993). Concomitant repression and expression of subsets of myosin heavy chain (MHC) isogenes, facilitates reconstruction of the contractile apparatus in response to changing environmental conditions and/or physical stimuli (reviewed by Goldspink 1996,

1998). Changes in muscle fibre phenotype are the result of rebuilding myofibrils with more energy efficient contractile and calcium handling properties (reviewed by Talmadge, 2000). In teleosts, muscle fibre type conversions have been described in common carp, which are able to express different MHC isoforms in response to seasonal temperature fluctuations, allowing more economical force generation (Gerlach *et al.*, 1990). Cold acclimation in goldfish has also been shown to increase the total number of slow and intermediate muscle fibres (Johnston & Lucking, 1978). An important objective of the time course experiment was to investigate the potential of endurance exercise to influence muscle fibre phenotype in the common carp. Two cyprinid species have previously demonstrated the ability to switch muscle fibre phenotype in response to changes in environmental temperature. Endurance exercise increases the proportion and number of slow-twitch type I and fast-twitch type IIa fibres in mammals. If the common carp response to endurance exercise training mirrors mammalian adaptations, an increase in the fraction of slow and intermediate fibre phenotypes would be expected.

Evidence for an involvement of the calcineurin-signaling pathway in muscle fibre hypertrophy under normal growth conditions was obtained in chapter 3. Increased nuclear localisation of the catalytic (CnA) and regulatory (CnB) subunits of calcineurin and downstream intracellular effectors MyoD and myf5, was significantly correlated with larger mean fast and slow muscle fibre area. The hypothesis that calcineurin is a regulator of axial muscle hypertrophy in the common carp was further investigated in this chapter. Increased nuclear localisation of calcineurin, MyoD and myf-5 proteins that is correlated with larger mean fast and slow muscle fibre size, would be expected in association with hypertrophic muscle growth in common carp.

4.2 Materials And Methods

4.2.1 Experimental Animals

Common carp were purchased from Humberside Fisheries (Cleaves Farm near Drifffield, East Yorkshire, UK). Fish were kept in a communal tank in the main aquarium of the Gatty Marine Laboratory at approximately 19°C for several months, before dividing into experimental groups. Carp were fed to satiation once daily (approx. 3 – 5% body mass) with a species-specific commercially available brand of fish food (Trouw Aquaculture). Experimental groups had access to food for the same length of time each day. At the end of the feeding period, uneaten food was siphoned from the tanks to prevent food consumption in non-exercised fish whilst the exercise group was swimming. A brief summary of experimental conditions is given in Table 4.1.

4.2.2 Experimental Design

Forty-eight common carp were divided into eight experimental groups on the basis of fork length, each group comprising six fish of a range of body lengths. The experimental set up is summarised in Table 4.2. The group mean fork length, body mass and condition factor (\pm S.E.M.) prior to the time course experiment are summarised in Table 4.3. There were two tank rested groups: TR₀ was sampled on day 1 and TR₁₆ was sampled after 16 days of tank rest. There were six exercised groups ranging from half a day (E_{0.5}) to 16 days of exercise (E₁₆). A digital photograph of each fish was taken before and after the experiment, to give an accurate record of growth over the duration of the experiment (Fujifilm Finepix 4900 Zoom). Fork length and body mass were measured in each fish before and after experimental treatment, and condition factor was calculated as before.

Table 4.1. Summary of environmental and experimental conditions for the time course experiment.

Mean Water Temperature (°C)	Photoperiod (L, light; D, dark; hours)	Water Flow (cm.s ⁻¹)	Mean Group Swimming Speed (bls ⁻¹)	Daily Exercise (E) /Rest (R) Regime (hours)	Duration Of Experiment (days)	Total Distance Swum (km)
18.6	18L: 6D	32	2.6 – 3.0	18E: 6R	0.5 - 16	0 – 332

Table 4.2. Description of experimental groups in the time course experiment.

Experimental Group	Duration Of Experiment (days)	Number Of Hours Exercised	Distance Swum (km)	Day Of Sampling
TR ₀	0	0	0	1
E _{0.5}	0.5	9	10	1
E ₁	1	18	21	2
E ₂	2	36	41	4
E ₄	4	72	83	8
E ₈	8	144	166	16
E ₁₆	16	288	332	16
TR ₁₆	16	0	0	16

After dividing into groups, fish were maintained in identical separate holding tanks positioned over the header tank on the Aquarium flume (see Fig. 3.3). As a method of distinguishing the individual fish in each group, small characteristic clippings were made in the pectoral or pelvic fins whilst the fish was anaesthetised with MS222. The 16-day exercise group occupied one swimming channel of the aquarium flume, whereas the other five exercise groups were trained consecutively in the other channel. The swimming speed used in both swimming channels for this experiment was 32cms^{-1} , which is the equivalent of $2.6 - 3.0\text{ bls}^{-1}$. The swimming speed used in the time course experiment was slightly higher than that used in any of the previous swimming experiments conducted using common carp ($1.7 - 2.7\text{ bls}^{-1}$). This was due to the higher variability in the size of fish used in this experiment compared to previous swimming experiments (see Table 4.3). A moderate swimming velocity ($2.6 - 3.0\text{ bls}^{-1}$) was used in this experiment because it was very similar to the experimental conditions that stimulated slow muscle fibre hypertrophy and accretion of myonuclei in fast muscle fibres in swimming experiment 4 ($2.6 - 2.7\text{ bls}^{-1}$).

At the end of the allotted period of exercise/tank rest, all carp were humanely sacrificed then processed for muscle fibre analysis. A total cross-sectional area (steak) was dissected at the level of the anal pore (Fig. 3.4A) from each fish. In this experiment, one block of muscle tissue was taken from the right hand side of the myotome at the horizontal septum and samples were prepared for histochemistry and immunohistochemistry as before (sections 2.3.2.2 & 2.3.2.3). Serial transverse sections ($7\mu\text{m}$) were cut and stained using the previously described histochemical (Mayer's Haematoxylin, section 2.3.2.4; mATPase, section 2.3.2.6) and immunohistochemical (S58, section 2.3.2.5) protocols to identify fast and slow muscle fibres in common carp (Figs. 2.11 & 2.13).

4.2.4 Muscle Fibre Morphometry

The mATPase histochemical protocol distinguished 3 different phenotypes of fast muscle fibre (Fig. 2.13). The mATPase histochemical staining profile of all muscle fibres is summarised in Table 2.3. To assess the effect of exercise on muscle fibre phenotype, an area scaled to the total fast muscle cross-sectional area (T_{CSA}) was positioned a scaled distance from the horizontal septum, on a mATPase stained transverse section from each fish. The distance from the horizontal septum was scaled to the vertical depth of the T_{CSA} in each fish. This was to ensure that the concentration of slow muscle fibres at the horizontal septum was avoided when positioning the scaled box area (Fig. 2.13A, SB). The outermost edge of the scaled box was aligned with the boundary between slow and fast muscle fibres, as the effect of exercise on intermediate and fast type fibres was the focus of this investigation. To assess the effect of exercise on the total number of slow and/or intermediate muscle fibres was not practical in this situation. Furthermore, due to time constraints, the effect of exercise on muscle fibre type and muscle fibre cellularity was only examined in 5 out of the 8 experimental groups. The number and size of each fast fibre phenotype within the scaled box was measured for experimental groups TR₀, E₄, E₈, E₁₆ & TR₁₆. The four exercised groups (E₄, E₈, E₁₆ & TR₁₆) studied were selected on the basis that these groups were the most likely to demonstrate an effect of exercise on muscle fibre characteristics. The TR₀ group was examined to provide a baseline measurement for muscle fibre size. The effect of exercise on the growth of slow and all-fast muscle phenotypes was examined in the same experimental groups. All muscle fibre cross-sectional areas were measured within the scaled box region using the same apparatus and software as before (section 3.2.7). Muscle fibre diameter was calculated from the area measurement as before.

4.2.5 Protein Expression Analysis

Fast and slow muscle tissue was dissected and preserved as previously described (section 2.2.2.2). Total cellular extracts were made from fast and slow muscle tissue in all 8 experimental groups (section 2.2.2.3). Nuclear protein extracts were made from fast and slow muscle tissue in 3 of the experimental groups: TR₀, E₁₆ and TR₁₆ (section 2.2.2.4). Standardised western blotting techniques (sections 2.2.2.9 & 3.2.9) were applied in this experiment, to observe the effect of different durations of exercise on muscle specific gene expression.

4.2.6 Statistical Analysis

The Anderson-Darling test of normality was first used to assess the distribution of the data (Minitab v. 13.2). A two-way mixed design analysis of variance was used to compare condition factor, fork length and body mass between and within subjects in all 8 experimental groups over the experimental period. Analysis of covariance with fork length as the covariate and post-hoc Tukey's tests were used to assess the overall effect of exercise on T_{CSA}. Muscle fibre cellularity was examined in 5 of the 8 experimental groups (TR₀, E₄, E₈, E₁₆ & TR₁₆). This characteristic was analysed using analysis of covariance and post-hoc Tukey's tests to assess the overall effect of exercise on F_{CSA}. Muscle fibre diameter density distributions were compared between the TR₀ group and the other four experimental groups (E₄, E₈, E₁₆ & TR₁₆), to determine the effect of experimental treatment upon muscle fibre diameter relative to the TR₀ baseline group. The E₁₆ and TR₁₆ groups were also directly compared to determine the effects of growth and exercise on muscle fibre diameter. The non-parametric statistics outlined in section 3.2.10 were used. The intermediate type a fibres were not considered because not enough of these fibres were found in all fish in the 5 experimental groups to enable objective analysis. The contribution of intermediate a, intermediate b and fast fibres to the scaled box area (total

number determined, proportion of total number and total area) was analysed using one-way analysis of variance and post-hoc Tukey's tests. Where data was found to be non-normal, the Kruskal-Wallis test and post-hoc multiple comparisons were performed.

For protein expression, the group mean optical density \pm SEM is reported as the percentage of the TR₀ group. For western blots and Coomassie stained gels, comparisons of optical density between experimental groups were made using one-way analysis of variance and post-hoc Tukey's tests. Where the distribution of data was found to be non-normal, the Kruskal-Wallis test and post-hoc multiple comparisons were performed.

4.3 Results

4.3.1 The effect of experimental treatment on somatic growth

Digital photographs of the largest specimens from the TR₁₆ and E₁₆ experimental groups taken at the start and end of the experimental period, gave a visual indication of marked changes in morphology between groups with greater somatic growth in the tank rested group (Fig. 4.1). Significant darkening of trained common carp was also observed in this experiment (Fig. 4.1D), however this reaction to the exercise treatment was not quantifiable. Darkening and increased pigmentation has also been observed in sprint-trained rainbow trout, which was attributed to stress induced by the training regime (Gamperl *et al.*, 1988). The total myotomal cross-sectional (Fig. 4.2A) area was significantly greater in the TR₁₆ group compared to three of the exercised groups (E_{0.5}, E₈ & E₁₆) with fork length as covariate ($F_{7, 39} = 2.6$, $P < 0.05$). Initial and final measurements of condition factor, fork length and body mass were compared across all experimental treatments to directly assess the effect of exercise on somatic growth (Fig. 4.2B – D, Table 4.3).

Table 4.3. Time course experiment: summary of gross morphology for eight groups of common carp. Values represent group mean \pm SEM, N=6. FL = Fork Length (cm), BM = Body Mass (g), CF = Condition Factor and FT_{CSA} = total fast muscle cross-sectional area. Start and end refer to measurements taken before and after the exercise or tank rested experimental treatment. Percentage change in fork length, body mass and condition factor over the experiment is shown in brackets.

Experimental treatment	FL		BM		BM		CF		CSA
	START	END	START	END	START	END	START	END	
TR ₀	11.1		47.6				3.41		153.5
One-off measurements	± 0.5 (0)		± 6.5 (0)				± 0.06 (0)		± 14.3
E _{0.5}	11.5	11.5	50.2	47.7 *	3.25	3.11 **			155.9
	± 0.4	± 0.5 (-0.2)	± 6.2	± 5.8 (-5.1)	± 0.08	± 0.08			± 15.4
E ₁	11.2	11.2	45.1	43.1	3.16	3.03 ** d			153.4
	± 0.4	± 0.4 (-0.3)	± 4.7	± 4.6 (-4.3)	± 0.09	± 0.08 (-4.0)			± 0.05
E ₂	11.9	11.7 *	59.7	52.5 ***	3.56 a	3.25 ***			173.7
	± 0.10	± 0.09 (-1.2)	± 2.1	± 1.8 (-11.9)	± 0.09	± 0.08 (-8.7)			± 3.8
E ₄	11.6	11.4 ***	52.9	50.9	3.32	3.37			163.5
	± 0.5	± 0.5 (-1.8)	± 5.9	± 5.9 (-3.8)	± 0.06	± 0.08 (1.6)			± 14.6
E ₈	11.6	11.8 *	50.9	48.8	3.15	2.94 ** c			168.7
	± 0.5	± 0.5 (1.1)	± 6.3	± 5.6 (-4.2)	± 0.04	± 0.03 (-6.6)			± 16.3
E ₁₆	11.3	11.6 ***	49.9	48.8	3.44	3.12 **			162.3
	± 0.4	± 0.3 (2.7)	± 4.7	± 4.0 (-2.2)	± 0.03	± 0.03 (-9.3)			± 9.3
TR ₁₆	11.1	11.9 ***	47.0	61.5 ***	3.30	3.45 *** b			202.3 *
	± 0.6	± 0.7 (7.3)	± 7.1	± 10.0 (30.8)	± 0.05	± 0.05 (4.6)			± 24.4

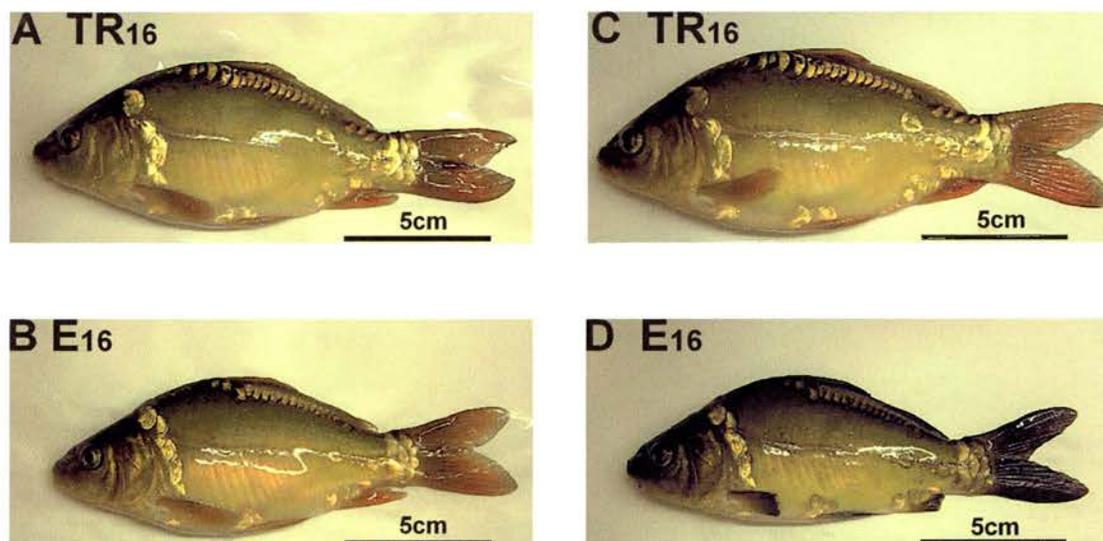


Fig. 4.1. The impact of experimental treatment on body morphology in the time course experiment. Digital photographs were taken of each individual at the start (A & B) and end (C & D) of the experiment, to give an accurate measure of fork length. The largest individuals from the 16-day tank rested (TR_{16}) and 16-day exercised (E_{16}) groups are shown, to give a visual indication of the marked changes in morphology between groups and the greater somatic growth observed in the tank rested animals. D) Significant darkening of exercised common carp was observed, however this reaction to the exercise treatment was not quantifiable.

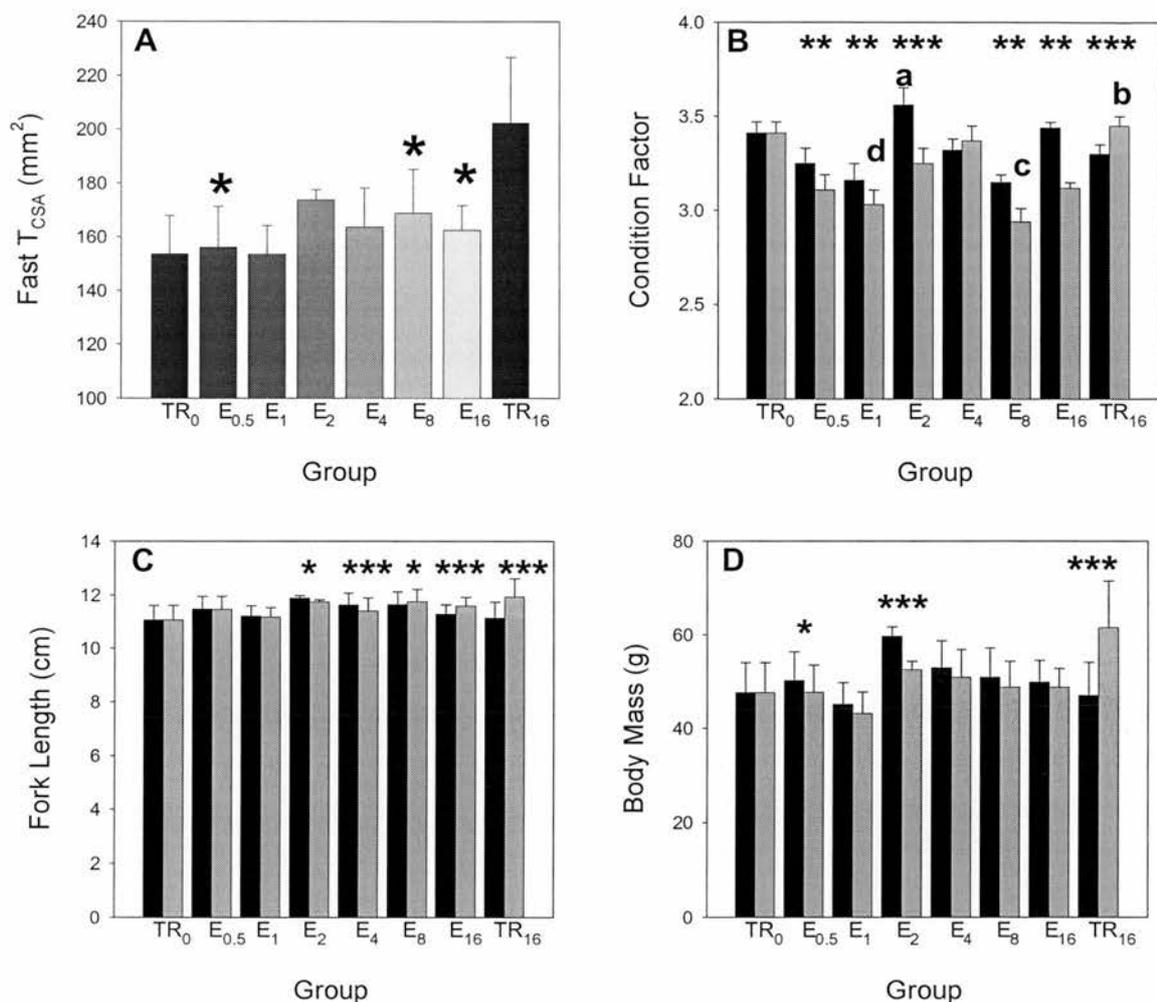


Fig. 4.2. Time course experiment: the effect of tank rest or exercise training on total fast muscle cross-sectional area (T_{CSA}), condition factor (CF), fork length (FL) and body mass (BM). Morphological characteristics (CF, FL & BM) were measured before (black fills) and after (grey fills) experimental treatment. A) Relative to FL, the TR₁₆ group mean T_{CSA} was significantly larger than that found in the E_{0.5}, E₈ and E₁₆ exercised groups post-training ($P < 0.05$, ANCOVA). B) CF was found to be significantly reduced in five of the six exercised groups (except E₄) after different durations of exercise treatment ($P < 0.01$, $P < 0.001$, 2-way mixed ANOVA). A significant increase in CF was observed in the TR₁₆ group over the time course experiment ($P < 0.001$, 2-way mixed ANOVA). Pre-training, the E₂ group had a significantly higher CF relative to the E_{0.5}, E₁ and E₈ groups ($P < 0.05$, 2-way mixed ANOVA). Post-training, the TR₁₆ group had a significantly higher CF relative to the E_{0.5}, E₁, E₈ and E₁₆ groups ($P < 0.05$, 2-way mixed ANOVA). Post-training, the E₈ group had a significantly lower CF relative to the TR₀, E₂ and E₄ groups ($P < 0.05$, 2-way mixed ANOVA). Post-training, the E₁ group had a significantly lower CF than the TR₀ and E₄ groups ($P < 0.05$, 2-way mixed ANOVA). C) Over the experimental period, FL decreased significantly in the E₂ and E₄ experimental groups, whereas significant growth in FL was observed in the E₈, E₁₆ and TR₁₆ groups ($P < 0.05$, $P < 0.001$, 2-way mixed ANOVA). D) Over the experimental period, BM decreased significantly in response to forced swimming activity in the E_{0.5} and E₂ groups, whereas significant increase in BM was found in the TR₁₆ group ($P < 0.05$, $P < 0.001$, 2-way mixed ANOVA). Values represent group mean \pm SEM, $N = 6$, a – d $P < 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistical analysis of condition factor revealed that there was a significant interaction between experimental group and the time course of exercise ($F_{7, 40} = 16.5$, $P < 0.001$, two-way mixed ANOVA), which indicated that changes in condition factor were not constant across all groups. Condition factor varied significantly between experimental groups both before ($F_{7, 95} = 4.5$, $P < 0.001$) and after the experimental treatment ($F_{7, 95} = 7.8$, $P < 0.001$). The E_2 group had a significantly higher condition factor relative to the $E_{0.5}$, E_1 and E_8 groups before the experimental treatment (Fig. 4.2B, **a**). The following significant differences in final condition factor existed between experimental treatments ($\alpha = 0.05$): the TR_{16} group had a significantly higher condition factor relative to the $E_{0.5}$, E_1 , E_8 and E_{16} groups (Fig. 4.2B, **b**); the E_8 group had a significantly lower condition factor relative to the TR_0 , E_2 and E_4 groups (Fig. 4.2B, **c**); the E_1 group had a significantly lower condition factor than the TR_0 and E_4 groups (Fig. 4.2B, **d**). A comparison of the initial and final condition factors for each group revealed that condition factor was significantly reduced in all of the exercised groups except E_4 , whereas condition factor increased significantly over the time course of the experiment in the TR_{16} group alone (Fig. 4.2B & Table 4.3).

Change in fork length did not vary consistently in all experimental groups over the time course of the experiment ($F_{7, 40} = 29.5$, $P < 0.001$, two-way mixed ANOVA). There was no significant difference in fork length between experimental groups before or after the experimental treatment. A significant increase in fork length was observed in the E_8 , E_{16} and TR_{16} groups, whereas a significant slight decrease in fork length was noticeable in the E_2 and E_4 groups (Fig. 4.2C & Table 4.3). The largest increase in fork length was observed in the TR_{16} group (7.3%) over the 16-day experiment, compared to a slight increase in length in the E_{16} group (2.7%).

Analysis of body mass showed that this parameter did not change consistently between experimental groups over the time course of the experiment ($F_{7, 40} = 26.7$, $P <$

0.001, two-way mixed ANOVA). Body mass did not vary significantly between experimental treatments before or after the experiment. In two of the exercised experimental groups ($E_{0.5}$ & E_2), body mass was significantly reduced over the time course of the experiment. The only significant increase in body mass was observed in the TR_{16} group, in which body mass increased by nearly 31% (Fig. 4.2D & Table 4.3).

4.3.2 The effect of exercise duration on muscle fibre morphometry

The overall effect of exercise or tank rest on muscle morphometry in 5 of the 8 experimental groups (TR_0 , E_4 , E_8 , E_{16} & TR_{16}) was analysed using analysis of covariance with fork length as the covariate and post-hoc Tukey's tests. Muscle fibre types were distinguished on the basis of ATPase activity and cross-reactivity with the S58 antibody (Figs. 2.11 & 2.13, Table 2.3) and five categories of fibre phenotype were considered: slow, intermediate type a (Ia), intermediate type b (Ib), fast and a combined category of fast and intermediate muscle fibre types ('all-fast').

Mean slow fibre cross-sectional area (Fig. 4.3A) was shown to be significantly higher in the E_{16} group relative to the TR_0 , E_4 and TR_{16} groups ($F_{4, 24} = 6.0$, $P < 0.01$, ANCOVA; * $P < 0.05$, **a** $P < 0.01$, Tukey's test). The largest increase in mean slow fibre area relative to the TR_0 group was 35% in the E_{16} group compared to 20% and 11% in the E_8 and TR_{16} groups respectively (Table 4.4). A comparison of the probability density functions for slow muscle (Fig. 4.4) showed that there was a significant difference between the fibre diameter distribution of the E_{16} group relative to the two tank rested groups, TR_0 and TR_{16} ($P < 0.05$, Kolmogorov Smirnov). The 5th, 10th and 50th percentiles of fibre diameter were significantly greater in the E_{16} group compared to the TR_0 group ($P < 0.01$, Mann Whitney Rank Sum test). Mean slow fibre diameter was significantly greater at the 50th percentile level in the E_{16} group relative to the TR_{16} group ($P < 0.005$, Mann Whitney Rank Sum test).

Table 4.4. Summary of the mean fibre cross sectional area (F_{CSA}) and diameter of 5 categories of muscle fibre phenotype in tank rested and exercised fish. Percentage difference relative to the TR_0 group is given in brackets. la=Intermediate type a; lb=Intermediate type b; All fast-type = la, lb & Fast. Values are group mean \pm SEM, N=6.

Experimental treatment	Slow F_{CSA} (μm^2)	la F_{CSA} (μm^2)	lb F_{CSA} (μm^2)	Fast F_{CSA} (μm^2)	All Fast-type F_{CSA} (μm^2)	Slow Fibre Diameter (μm)	Ia Fibre Diameter (μm)	Ib Fibre Diameter (μm)	Fast Fibre Diameter (μm)	All Fast-type Fibre Diameter (μm)
TR_0	388 ± 37	332 ± 35	1163 ± 113	1269 ± 95	1052 ± 69	21.0 ± 1.1	18.8 ± 1.0	36.9 ± 2.0	37.1 ± 1.5	33.1 ± 1.2
E_4	403 ± 19 (3.9)	250 ± 55 (-24.7)	1154 ± 64 (-0.7)	1131 ± 89 (-10.9)	994 ± 48	21.7 ± 0.5 (3.4)	16.4 ± 1.6 (-12.9)	37.0 ± 1.0 (0.3)	34.3 ± 1.2 (-7.5)	32.1 ± 0.6 (-3.1)
E_8	466 ± 11 (20.1)	196 ± 20 (-41.0)	995 ± 67 (-14.4)	1174 ± 93 (-7.5)	971 ± 66 (-7.7)	23.2 ± 0.3 (10.6)	14.8 ± 0.8 (-21.4)	33.9 ± 1.2 (-8.1)	35.1 ± 1.4 (-5.4)	31.6 ± 1.1 (-4.7)
E_{16}	523 ± 30 (34.8)	280 ± 30 (-15.6)	1282 ± 98 (10.2)	1498 ± 93 (18)	1236 ± 76 (17.5)	24.7 ± 0.7 (17.4)	17.0 ± 0.9 (-9.6)	38.9 ± 1.5 (5.5)	40.5 ± 1.3 (9.1)	36.2 ± 1.1 (9.4)
TR_{16}	431 ± 23 (11.1)	317 ± 22 (-4.6)	1532 ± 130 (31.8)	1728 ± 115 (36.1)	1408 ± 78 (33.8)	22.3 ± 0.6 (6.3)	18.4 ± 0.6 (-2.3)	42.8 ± 1.9 (16.2)	43.4 ± 1.5 (16.8)	38.5 ± 1.1 (16.3)

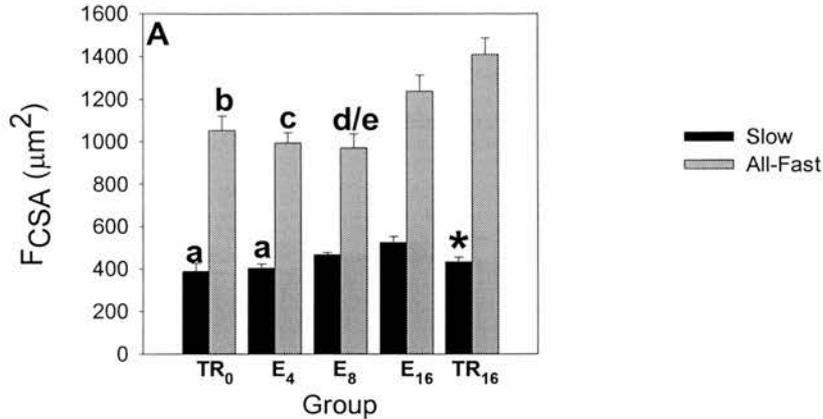


Fig. 4.3A. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₄, E₈ & E₁₆) on mean fibre cross-sectional area (F_{CSA}) for slow (black fills) and all-fast (grey fills) muscle fibre phenotypes. Slow F_{CSA} was significantly higher in the E₁₆ group relative to the TR₀, E₄ and TR₁₆ groups (P < 0.01, ANCOVA; * P < 0.05, a P < 0.01, Tukey's test). All-fast F_{CSA} was significantly higher in the TR₁₆ group relative to TR₀, E₄ and E₈ experimental treatments (P < 0.001, ANCOVA; b P < 0.05, c P < 0.01, d P < 0.001, Tukey's test). All-fast F_{CSA} was also significantly higher in the E₁₆ group relative to the E₈ experimental treatment (P < 0.001, ANCOVA; e P < 0.05, Tukey's test). Bars represent group mean F_{CSA} ± SEM, N = 6, ANCOVA, analysis of covariance.

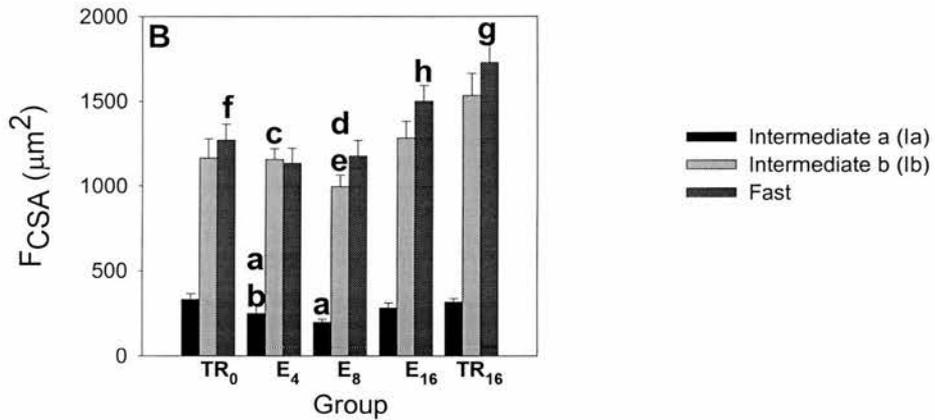


Fig. 4.3B. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₄, E₈ & E₁₆) on mean fibre cross-sectional area (F_{CSA}) for intermediate a (Ia, black fills), intermediate b (Ib, grey fills) and fast (dark grey fills) categories of muscle fibre phenotype. Ia F_{CSA} was significantly higher in the TR₀ group relative to the E₄ and E₈ experimental treatments (P < 0.01, ANCOVA; a P < 0.05, Tukey's test). Ia F_{CSA} was also significantly higher in the TR₁₆ group relative to the E₄ experimental treatment (P < 0.01, ANCOVA; b P < 0.01). Ib F_{CSA} was significantly higher in the TR₁₆ group relative to the E₄ and E₈ experimental groups (P < 0.01, ANCOVA; c P < 0.05, d P < 0.01, Tukey's test). Ib F_{CSA} was also significantly higher in the E₁₆ group relative to the E₈ experimental treatment (P < 0.01, ANCOVA; e P < 0.05). Fast F_{CSA} was significantly higher in the TR₁₆ group relative to the TR₀, E₄, and E₈ experimental treatments (P < 0.001, ANCOVA; f P < 0.05, g P < 0.001, Tukey's test). Fast F_{CSA} was also significantly higher in the E₁₆ group relative to the E₄ and E₈ experimental treatment (P < 0.001, ANCOVA; h P < 0.05). Bars represent group mean F_{CSA} ± SEM, N = 6, ANCOVA, analysis of covariance.

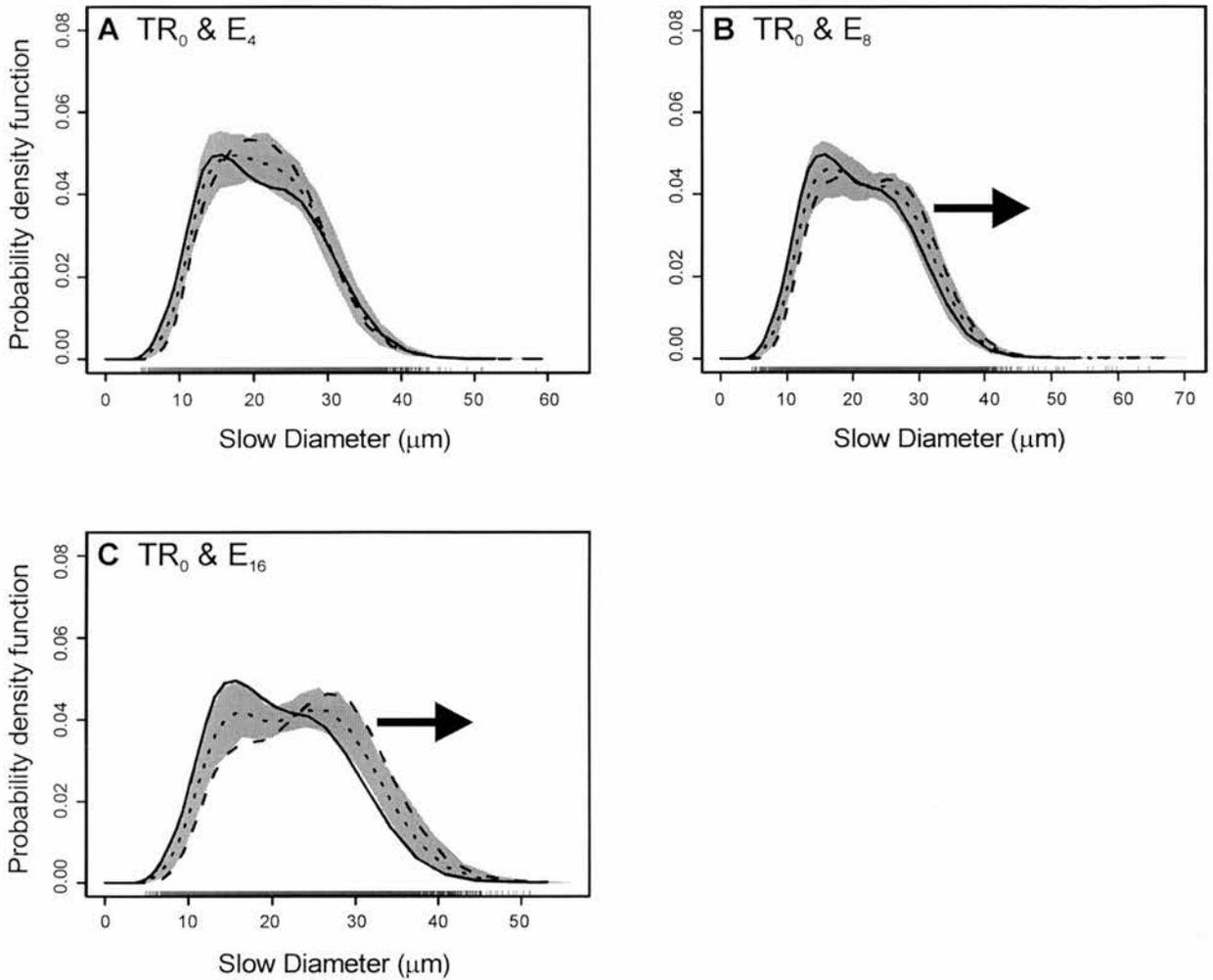


Fig. 4.4 A – C). Time course experiment: the effect of tank rest (TR_0) or exercise training (E_4 , E_8 & E_{16}) on the distribution of slow muscle fibre diameters. The mean smooth probability density functions (pdf) of slow fibre diameter distributions for each experimental group are represented by the solid (TR_0) and dashed (E_4 , E_8 & E_{16}) lines. The shaded area represents 100 bootstrap estimates of the slow muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. A & B) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between experimental groups (TR_0 & E_4 ; TR_0 & E_8) in the distribution of slow muscle fibre diameters (NS, Kolmogorov Smirnov). C) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the slow muscle fibre diameter distributions of the TR_0 and E_{16} experimental groups ($P < 0.05$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of slow muscle fibre diameters in E_{16} group relative to TR_0 fish. $N = 6$.

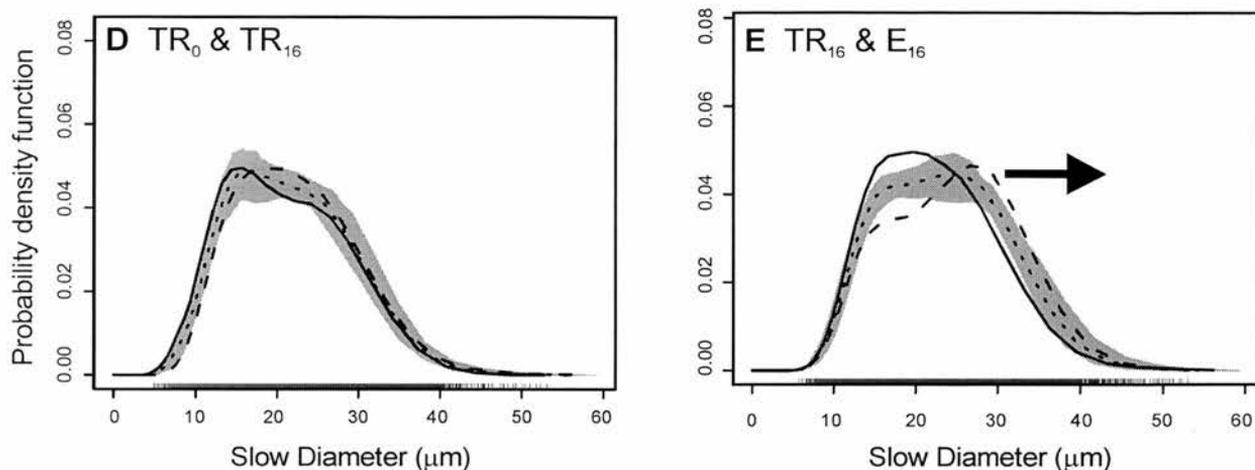


Fig. 4.4 D & E). Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₁₆) on the distribution of slow muscle fibre diameters. The mean smooth probability density functions (pdf) of slow fibre diameter distributions for each experimental group are represented by the solid (D, TR₀; E, TR₁₆) and dashed (D, TR₁₆ & E, E₁₆) lines. The shaded area represents 100 bootstrap estimates of the slow muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. D) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference in the distribution of slow muscle fibre diameters between tank rested groups (NS, Kolmogorov Smirnov). E) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the slow muscle fibre diameter distributions of the TR₁₆ and E₁₆ experimental groups ($P < 0.05$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of slow muscle fibre diameters in E₁₆ group relative to TR₁₆ fish. $N = 6$.

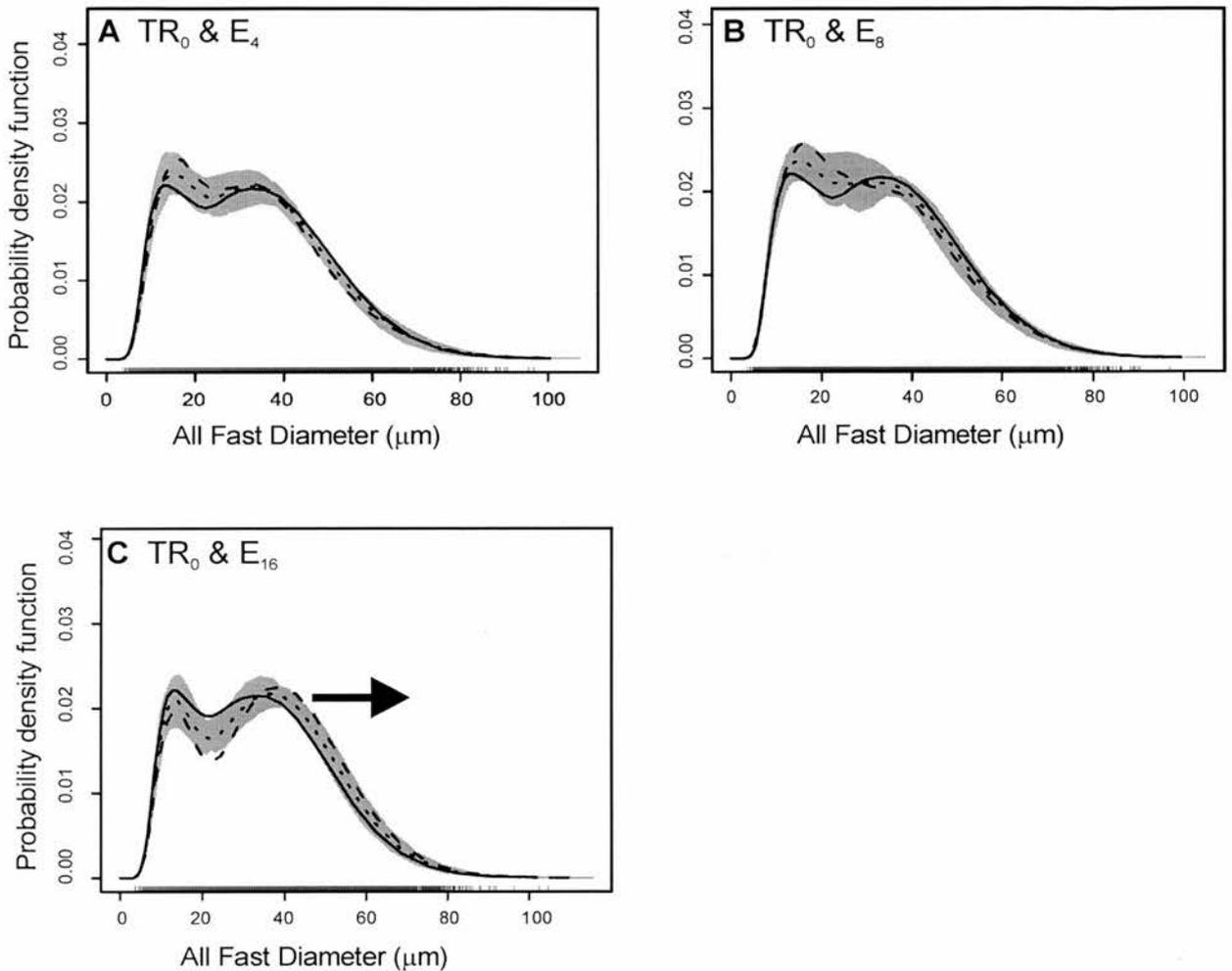


Fig. 4.5 A – C). Time course experiment: the effect of tank rest (TR_0) or exercise training (E_4 , E_8 & E_{16}) on the distribution of all-fast muscle fibre diameters. The mean smooth probability density functions (pdf) of all-fast fibre diameter distributions for each experimental group are represented by the solid (TR_0) and dashed (E_4 , E_8 & E_{16}) lines. The shaded area represents 100 bootstrap estimates of the all-fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. A & B) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between experimental groups (TR_0 & E_4 ; TR_0 & E_8) in the distribution of all-fast muscle fibre diameters (NS, Kolmogorov Smirnov). C) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the all-fast muscle fibre diameter distributions of the TR_0 and E_{16} experimental groups ($P < 0.1$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of all-fast muscle fibre diameters in E_{16} group relative to TR_0 fish. $N = 6$.

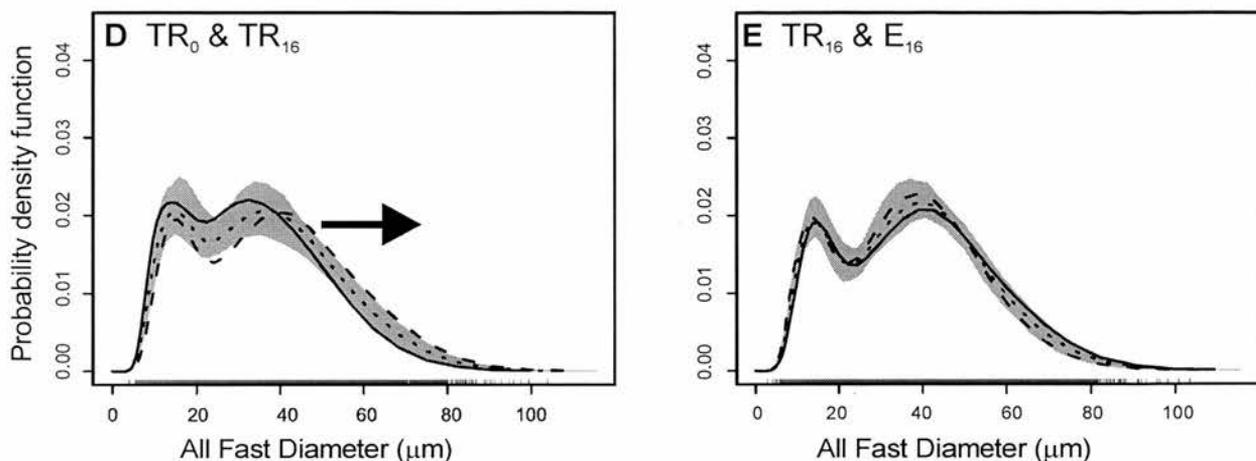


Fig. 4.5 D & E). Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₁₆) on the distribution of all-fast muscle fibre diameters. The mean smooth probability density functions (pdf) of all-fast fibre diameter distributions for each experimental group are represented by the solid (D, TR₀; E, TR₁₆) and dashed (D, TR₁₆ & E, E₁₆) lines. The shaded area represents 100 bootstrap estimates of the all-fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. D) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the all-fast muscle fibre diameter distributions of the TR₀ and TR₁₆ experimental groups ($P < 0.05$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of all-fast muscle fibre diameters in TR₁₆ group relative to TR₀ fish. E) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference in the distribution of all-fast muscle fibre diameters between the 16-day exercised (E₁₆) and non-exercised (TR₁₆) groups (NS, Kolmogorov Smirnov). $N = 6$.

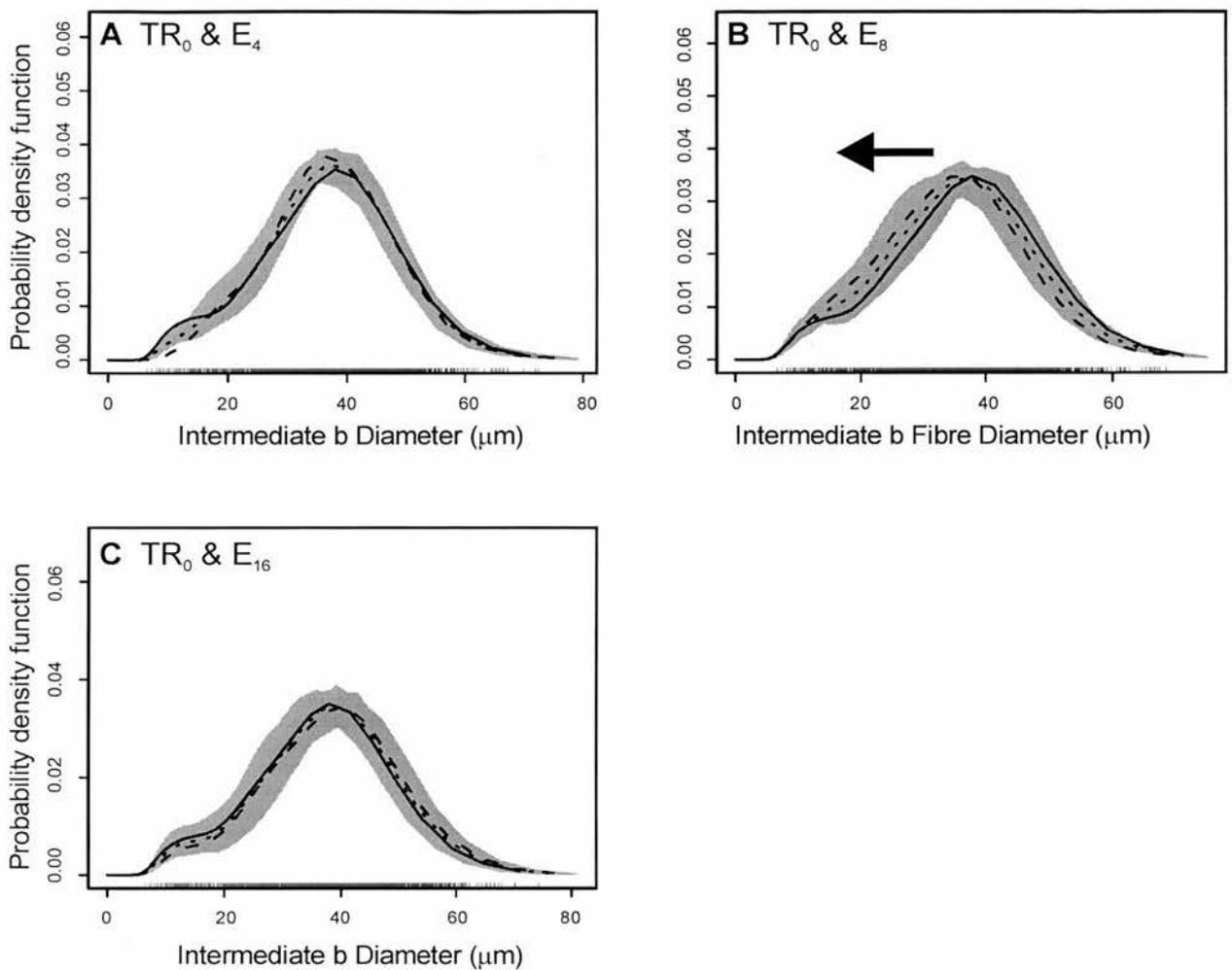


Fig. 4.6 A – C). Time course experiment: the effect of tank rest (TR_0) or exercise training (E_4 , E_8 & E_{16}) on the distribution of intermediate type b muscle fibre diameters. The mean smooth probability density functions (pdf) of intermediate type b fibre diameter distributions for each experimental group are represented by the solid (TR_0) and dashed (E_4 , E_8 & E_{16}) lines. The shaded area represents 100 bootstrap estimates of the intermediate type b muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. A – C) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference between tank rested (TR_0) or exercise trained (E_4 , E_8 & E_{16}) fish in the distribution of intermediate type b muscle fibre diameters (NS, Kolmogorov Smirnov). The arrow represents the apparent right-to-left shift of the distribution of intermediate type b muscle fibre diameters in E_8 group relative to TR_0 fish. $N = 6$.

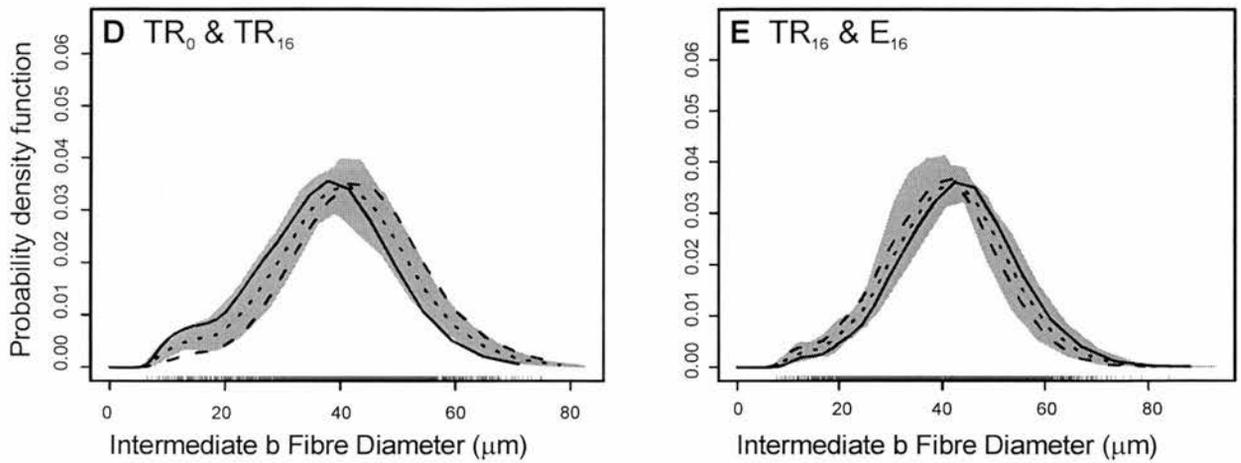


Fig. 4.6 D & E). Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₁₆) on the distribution of intermediate type b muscle fibre diameters. The mean smooth probability density functions (pdf) of intermediate type b fibre diameter distributions for each experimental group are represented by the solid (D, TR₀; E, TR₁₆) and dashed (D, TR₁₆ & E, E₁₆) lines. The shaded area represents 100 bootstrap estimates of the intermediate type b muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. D & E) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference in the distribution of intermediate type b muscle fibre diameters between the two tank rested groups (TR₀ & TR₁₆) and the two 16-day experimental treatments (TR₁₆ & E₁₆) (NS, Kolmogorov Smirnov). N = 6.

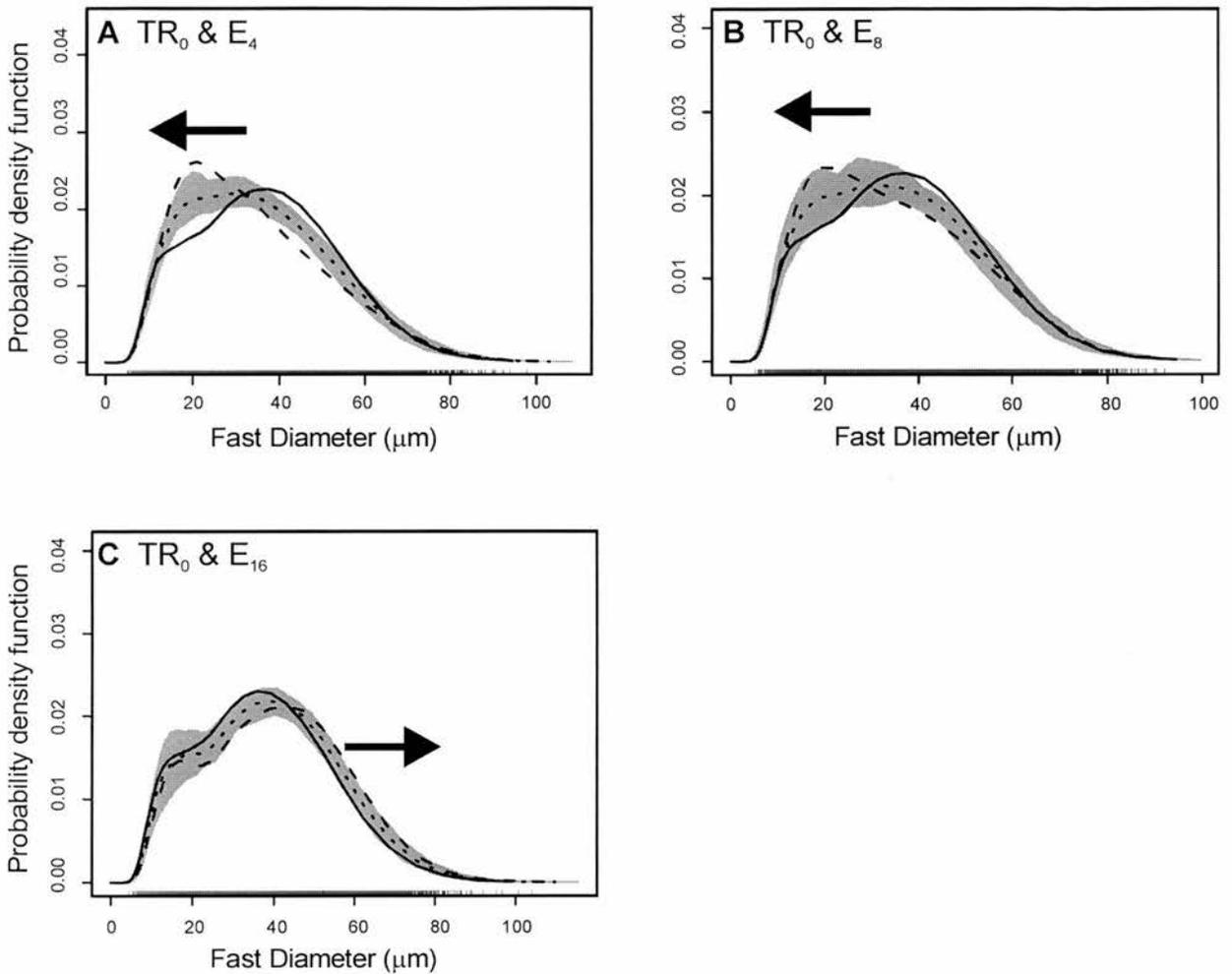


Fig. 4.7 A – C). Time course experiment: the effect of tank rest (TR₀) or exercise training (E₄, E₈ & E₁₆) on the distribution of fast muscle fibre diameters. The mean smooth probability density functions (pdf) of fast muscle fibre diameter distributions for each experimental group are represented by the solid (TR₀) and dashed (E₄, E₈ & E₁₆) lines. The shaded area represents 100 bootstrap estimates of the fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. A – C) The average probability density functions of each experimental group lay outside the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the tank rested (TR₀) and exercise trained (E₄, E₈ & E₁₆) fish in the distribution of fast muscle fibre diameters. However, the distributions were not significantly different (NS, Kolmogorov Smirnov). A) The arrow represents an apparent right-to-left shift of the distribution of fast muscle fibre diameters in the E₄ group relative to TR₀ fish. B) The arrow represents an apparent right-to-left shift of the distribution of fast muscle fibre diameters in the E₈ group relative to TR₀ fish. C) The arrow represents an apparent left-to-right shift of the distribution of fast muscle fibre diameters in the E₁₆ group relative to TR₀ fish. N = 6.

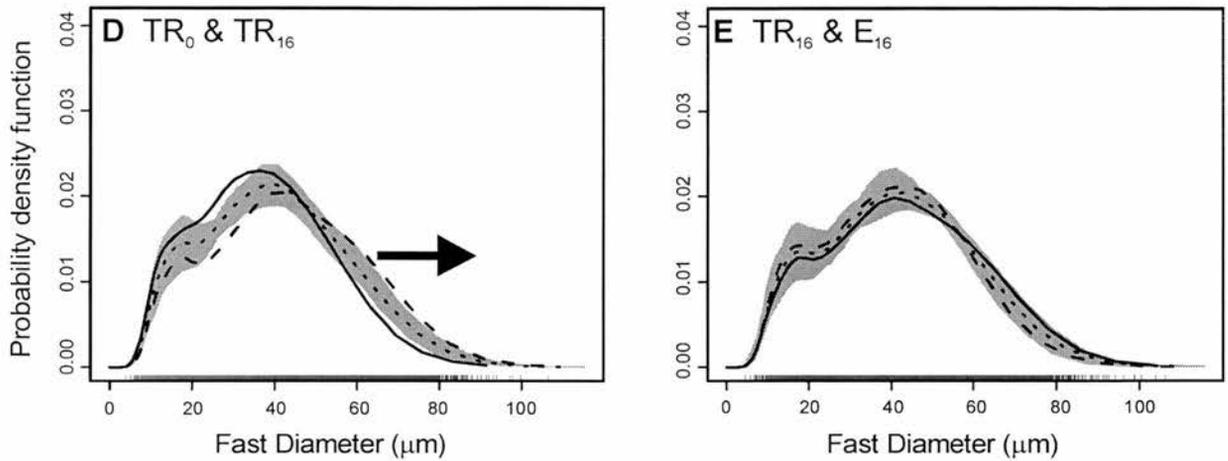


Fig. 4.7 D & E). Time course experiment: the effect of tank rest (TR_0 & TR_{16}) or exercise training (E_{16}) on the distribution of fast muscle fibre diameters. The mean smooth probability density functions (pdf) of fast fibre diameter distributions for each experimental group are represented by the solid (D, TR_0 ; E, TR_{16}) and dashed (D, TR_{16} & E, E_{16}) lines. The shaded area represents 100 bootstrap estimates of the fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. D) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population, which suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR_0 and TR_{16} experimental groups ($P < 0.05$, Kolmogorov Smirnov). The arrow represents the apparent left-to-right shift of the distribution of fast muscle fibre diameters in TR_{16} group relative to TR_0 fish. E) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population, which suggested that there was no significant difference in the distribution of fast muscle fibre diameters between the 16-day exercised (E_{16}) and non-exercised (TR_{16}) groups (NS, Kolmogorov Smirnov). $N = 6$.

The shift of the E_{16} density distribution towards the right implies that slow muscle fibres hypertrophied in response to 16 days of exercise training, relative to both tank rested groups. Slow fibre diameter density distributions were not significantly different in the comparison of the TR_0 group with E_4 , E_8 and TR_{16} groups.

The mean cross-sectional area of all-fast muscle fibres (Fig. 4.3A) was significantly higher in the TR_{16} group relative to the TR_0 , E_4 and E_8 experimental treatments ($F_{4, 24} = 8.8$, $P < 0.001$, ANCOVA; **b** $P < 0.05$, **c** $P < 0.01$, **d** $P < 0.001$, Tukey's test). The post-hoc Tukey's test also showed that the mean cross-sectional area of all-fast muscle fibres was significantly higher in the E_{16} group relative to the E_8 experimental treatment (**e** $P < 0.05$). Mean all-fast cross-sectional area was 34% and 18% larger relative to the TR_0 group, in the TR_{16} and E_{16} groups respectively (Table 4.4). Analysis of the density distributions for all-fast muscle fibres (Fig. 4.5) indicated that significant hypertrophy of these fibres occurred in the TR_{16} group relative to the TR_0 group ($P < 0.05$, Kolmogorov Smirnov). Comparisons of the percentiles in these groups showed that mean all-fast fibre diameter was significantly higher at each of the 5th – 99th percentile categories ($P < 0.05$, Mann Whitney Rank Sum test). A similar trend was apparent in the E_{16} group relative to the TR_0 group but this was not significant at the alpha level of 0.05 ($0.1 > P > 0.05$, Kolmogorov Smirnov). The E_4 and E_8 density distributions were not significantly different from the TR_0 group.

The mean cross-sectional area of the intermediate type a (Ia) muscle fibres (Fig. 4.3B) was significantly higher in the TR_0 group relative to the E_4 and E_8 experimental treatments ($F_{4, 20} = 6.2$, $P < 0.01$, ANCOVA; **a** $P < 0.05$, Tukey's test). The post-hoc Tukey's test also showed that the mean cross-sectional area of Ia muscle fibres was significantly higher in the TR_{16} group relative to the E_4 experimental treatment (**b** $P < 0.01$). The number of Ia muscle fibres distinguished in different fish was extremely variable, which made comparison of the fibre diameter density distributions impossible.

Relative to the TR₀ group, the three exercised groups examined showed a considerable reduction in mean Ia fibre cross-sectional area (Table 4.4). The largest decreases were observed in the E₈ (41%) and E₄ (25%) groups.

The mean cross-sectional area of the intermediate type b (Ib) muscle fibres (Fig. 4.3B) was significantly higher in the TR₁₆ group relative to the E₄ and E₈ experimental treatments ($F_{4, 24} = 7.9$, $P < 0.01$, ANCOVA; **c** $P < 0.05$, **d** $P < 0.01$, Tukey's test). The post-hoc Tukey's test also showed that the mean cross-sectional area of Ib muscle fibres was significantly higher in the E₁₆ group relative to the E₈ experimental treatment (**e** $P < 0.05$). An increase in mean Ib fibre cross-sectional area relative to the TR₀ group was observed in the TR₁₆ (32%) and E₁₆ (10%) groups, whereas a decline in mean area of these fibres was detected in the E₈ group (14%). However, comparison of the fibre diameter density distributions revealed no significant difference in Ib muscle fibre size as all distributions were confined within the grey shaded area of 100 bootstrap estimates of the combined populations (Fig. 4.6).

The mean cross-sectional area of the fast category of muscle fibre types (Fig. 4.3B) was significantly higher in the TR₁₆ group relative to the TR₀, E₄, and E₈ experimental treatments ($F_{4, 24} = 10.2$, $P < 0.001$, ANCOVA; **f** $P < 0.05$, **g** $P < 0.001$, Tukey's test). The post-hoc Tukey's test also showed that the mean cross-sectional area of fast muscle fibres was significantly higher in the E₁₆ group relative to the E₄ and E₈ experimental treatment (**h** $P < 0.05$). Relative to the TR₀ group, mean fast muscle fibre area was decreased in the E₄ and E₈ groups by 11% and 8% respectively (Table 4.4). Conversely, mean fast fibre area increased in the E₁₆ and TR₁₆ groups by 18% and 36% respectively. There was no significant difference in the distribution of fast muscle fibre diameter between the exercised and tank rested groups (Fig. 4.7). However the fast fibre diameter distributions for the two tank rested groups (Fig. 4.7D) lay outside that shaded area of bootstrap estimates and the

shift of the TR₁₆ distribution to the right was significant ($P < 0.05$, Kolmogorov Smirnov). The 50th, 95th and 99th percentiles of fibre diameter were significantly greater in the TR₁₆ group relative to the baseline TR₀ group, indicating significant fast fibre hypertrophy occurred in the tank rested group over the time course of the experiment.

4.3.3 The effect of exercise on muscle fibre phenotype

The effect of exercise or tank rest treatment on the proportions of fast muscle fibre phenotypes (Ia, Ib and fast) was determined for muscle fibres within a predefined area scaled to the total fast muscle cross-sectional area (Fig. 2.13, **SB**). A visual comparison of typical mATPase stained cryosections suggested that muscle fibre phenotype proportions altered over the course of the experiment in response to tank rest or exercise (Fig. 4.8). There was no significant difference in the total number of Ia fibres in the two tank rested and three exercised groups (Fig. 4.9A). The total number of Ib fibres was significantly higher in the three exercised groups relative to the TR₀ group ($F_{4, 29} = 6.9$, $P < 0.01$, one-way ANOVA; * $P < 0.05$, **a** $P < 0.001$, **b** $P < 0.01$, Tukey's test). The total number of fast muscle fibres within the scaled box area was significantly higher in the E₄ and E₈ groups relative to the E₁₆ and TR₁₆ experimental treatments ($F_{4, 29} = 9.5$, $P < 0.001$, one-way ANOVA; **c** $P < 0.01$, **d** $P < 0.001$, Tukey's test). The overall number of fibres measured within the defined area was significantly higher in the E₈ group relative to the E₁₆ treatment ($F_{4, 29} = 3.1$, $P < 0.05$, one-way ANOVA; **e** $P < 0.05$, Tukey's test).

There was no significant variation in the proportion of Ia fibres between experimental treatments (Fig. 4.9B). Analysis of the proportion of Ib fibres within the defined area revealed there was a significant difference between experimental treatments (KW = 19.3, DF = 4, $P < 0.01$, Kruskal-Wallis test). Post-hoc multiple comparisons demonstrated that the number of Ib fibres was significantly lower in the TR₀ group compared to the E₁₆ group (* $P < 0.05$). The proportion of Ib fibres in the E₁₆ group was 31% compared to 14% in the

TR₀ group. There was a significant difference in the proportion of fast muscle fibres (Fig. 4.9B) within the scaled box area ($F_{4, 29} = 2.9$, $P < 0.05$, one-way ANOVA) and post-hoc testing showed that the relatively lower proportion of fast muscle fibres in the E₁₆ group compared to the TR₀ group was significant (**a**, $P < 0.05$, Tukey's test). The proportion of fast fibres was 65% in the TR₀ group compared with only 53% in the E₁₆ group.

The total area of Ia muscle fibres (Fig. 4.9C) was significantly different between experimental treatments ($F_{4, 29} = 3.4$, $P < 0.05$, one-way ANOVA). The E₄ and E₈ groups have a significantly lower total Ia muscle fraction relative to the TR₀ group (* $P < 0.05$, Tukey's test). The percentage of the designated scaled box area occupied by Ia fibres was 3.2% and 3.4% in the E₄ and E₈ groups compared to 6.7% in the TR₀ experimental treatment. The total area of Ib type muscle fibres (Fig. 4.9C) varied significantly across experimental treatments (KW = 18.2, DF = 4, $P < 0.01$, Kruskal-Wallis test). Post-hoc multiple comparisons showed that the area of Ib fibres was significantly higher in the E₁₆ and TR₁₆ groups relative to the TR₀ group (**a**, $P < 0.05$). The proportion of the defined region that comprised Ib fibres was 26% and 32% in the TR₁₆ and E₁₆ treatments respectively, compared to 15% in the TR₀ group. The total area of fast fibres (Fig. 4.9C) was also significantly different between experimental groups ($F_{4, 29} = 7.9$, $P < 0.001$, one-way ANOVA). The area of the predefined region that comprised fast fibres was significantly higher in the TR₀ group relative to the E₁₆ group ($P < 0.001$, **b**) and was also higher than the E₄ and TR₁₆ groups ($P < 0.05$, **c**, post-hoc Tukey's test). The proportion of total area that was made up of fast fibres was 78% in the TR₀ group, compared to 71%, 70% and 65% in the E₄, TR₁₆ and E₁₆ groups in that order.

Table 4.5. The total fibre number, proportion of total fibre number (N) and proportion of total fibre area (A) of three phenotypes of fast muscle fibre in a defined region of the myotome. Values are group mean \pm SEM, N=6.

Experimental treatment	Ia total number	Ib total number	Fast-type total number	All Fast total number	Ia type proportion (N)	Ib type proportion (N)	Fast-type proportion (N)	Ia type proportion (A)	Ib type proportion (A)	Fast-type proportion (A)
TR ₀	391 \pm 47	247 \pm 22	1164 \pm 35	1802 \pm 80	21.4 \pm 1.6	13.7 \pm 1.0	64.9 \pm 2.2	6.7 \pm 0.8	15.3 \pm 1.7	77.8 \pm 1.6
E ₄	312 \pm 64	478 \pm 60	1319 \pm 71	2108 \pm 137	14.3 \pm 2.4	22.4 \pm 1.9	63.3 \pm 3.9	3.2* \pm 0.4	26.0 \pm 2.2	70.8 c \pm 2.2
E ₈	380 \pm 96	563 \pm 66	1357 \pm 86	2300 \pm 208	15.6 \pm 3.0	24.5 \pm 1.8	59.9 \pm 3.1	3.4* \pm 0.9	25.0 \pm 1.3	71.6 \pm 1.7
E ₁₆	274 \pm 37	525 \pm 35	903 \pm 47	1701 \pm 95	15.9 \pm 1.6	30.9* \pm 1.4	53.3 a \pm 1.8	3.6 \pm 0.5	31.9 a \pm 1.6	64.5 b \pm 1.8
TR ₁₆	349 \pm 94	425 \pm 36	992 \pm 71	1766 \pm 170	18.2 \pm 3.5	24.7 \pm 2.0	57.1 \pm 2.2	4.3 \pm 1.0	26.2 a \pm 1.4	69.5 c \pm 1.3

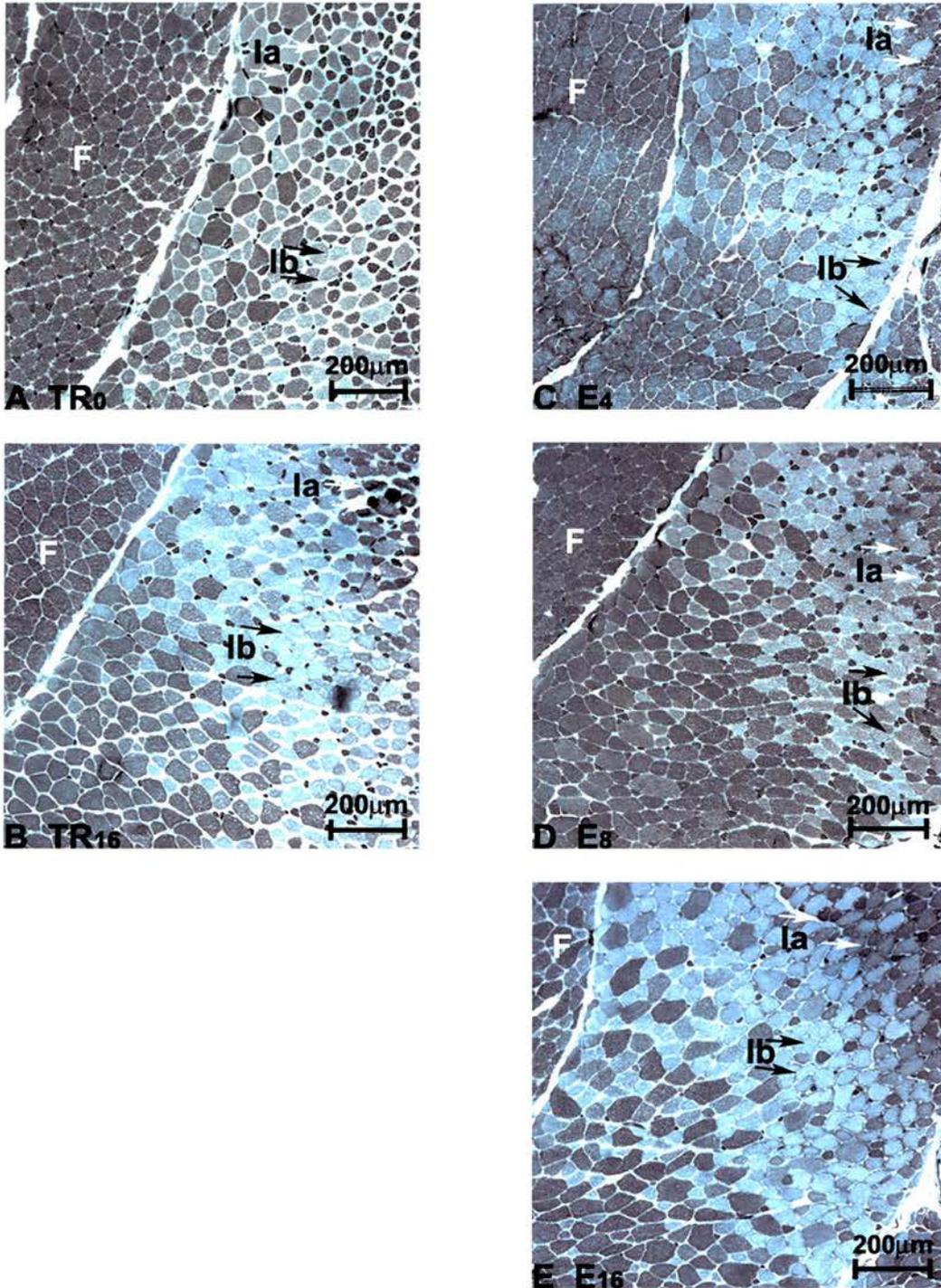


Fig. 4.8. Time course experiment: the effect of tank rest or exercise training on muscle fibre phenotype. Transverse sections of common carp muscle tissue stained for mATPase activity in the 0-day tank rested group (A, TR₀), 16-day tank rested group (B, TR₁₆), 4-day (C, E₄), 8-day (D, E₈) and 16-day exercised groups (E, E₁₆). Three phenotypes of fast muscle fibre types were described: intermediate a (Ia, white arrows), intermediate b (Ib, black arrows) and fast (F). A visual comparison of muscle fibre phenotypic characteristics within the scaled box area in mATPase stained cryosections, suggested that muscle fibre phenotype proportions were altered over the time course experiment in response to tank rest or exercise activity. All tissue sections are orientated with the dorsal edge upward, $\times 5$ magnifications.

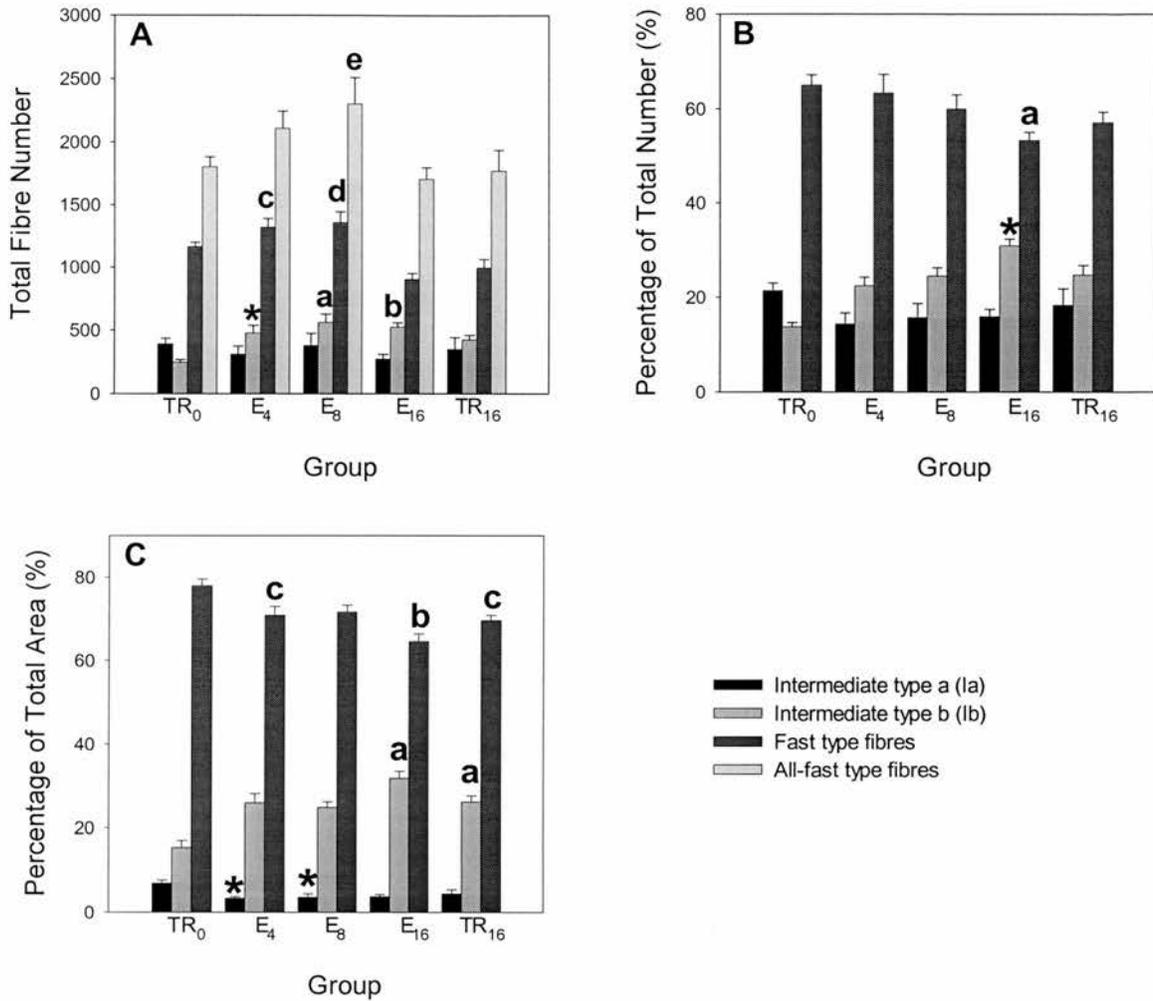
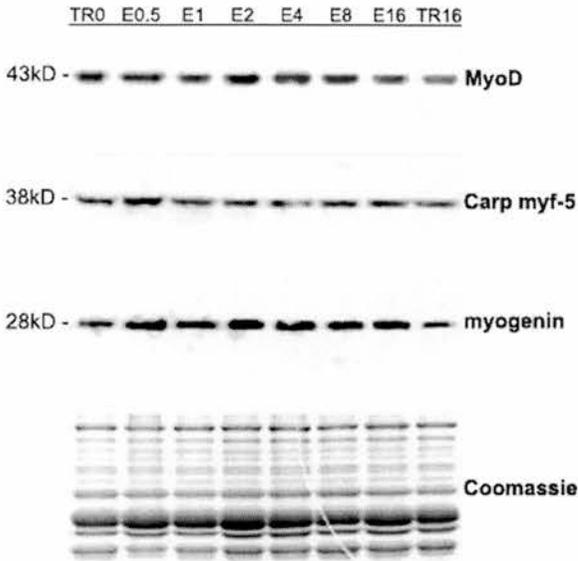


Fig. 4.9. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E₄, E₈ & E₁₆) on muscle fibre phenotype in a defined region of the myotome. The total number (A), proportion of total fibre number (B) and proportion of total fibre area (C) of each fast muscle fibre phenotype were examined. A) The total number of Ib fibres was significantly higher in the E₄, E₈ and E₁₆ groups relative to the TR₀ group ($P < 0.01$, one-way ANOVA; * $P < 0.05$, **a** $P < 0.001$, **b** $P < 0.01$, Tukey's test). The total number of fast fibres was significantly higher in the E₄ and E₈ groups relative to the E₁₆ and TR₁₆ experimental treatments ($P < 0.001$, one-way ANOVA; **c** $P < 0.01$, **d** $P < 0.001$, Tukey's test). The overall number of all-fast fibres was significantly higher in the E₈ group relative to the E₁₆ treatment ($P < 0.05$, one-way ANOVA; **e** $P < 0.05$, Tukey's test). B) The proportion of Ib fibres was significantly higher in the E₁₆ group relative to the TR₀ group ($P < 0.01$, KW, * $P < 0.05$ post-hoc multiple comparisons). The proportion of fast fibres was significantly lower in the E₁₆ group relative to the TR₀ group ($P < 0.05$, one-way ANOVA, **a** $P < 0.05$ Tukey's test). C) The E₄ and E₈ groups have a significantly lower total Ia muscle fraction relative to the TR₀ group ($P < 0.05$, one-way ANOVA; * $P < 0.05$, Tukey's test). The TR₀ group had a significantly lower total Ib muscle fractional area relative to the TR₁₆ and E₁₆ experimental treatments ($P < 0.01$, KW; **a** $P < 0.05$, post-hoc multiple comparisons). The fast muscle fractional area was significantly higher in the TR₀ group relative to the E₄, E₁₆ and TR₁₆ treatments ($P < 0.001$, one-way ANOVA; **c** $P < 0.05$, **b** $P < 0.001$, Tukey's test). Bars represent group mean \pm SEM, N = 6. ANOVA, analysis of variance; KW, Kruskal Wallis.

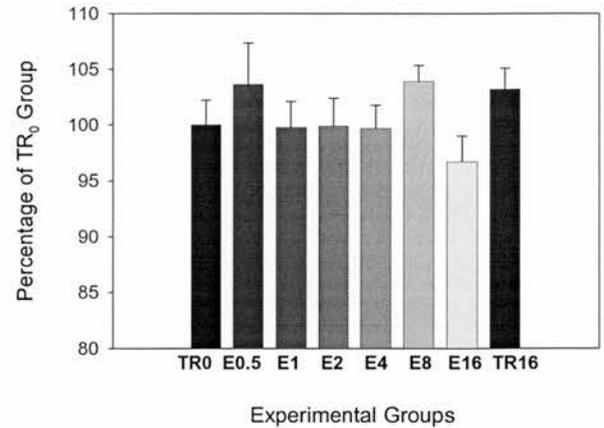
4.3.4 The effect of exercise on the expression of myogenic regulatory factors (MRFs) and PCNA

There was no significant difference in the total concentration of MRFs in total cellular protein extracts from fast muscle tissue with respect to experimental treatment (Figs. 4.10). Analysis of fast muscle tissue nuclear protein extracts from the two tank rested groups and the 16-day exercised group (Figs. 4.11A & B), demonstrated a significant difference in the localisation of MyoD protein ($F_{2, 17} = 9.6$, $P < 0.01$, one-way ANOVA). The increased level of MyoD protein in nuclear extracts from the E_{16} and TR_{16} groups was significantly higher than the baseline group TR_0 ($P < 0.01$, Tukey's test). MyoD protein levels were 9% and 10% increased in the E_{16} and TR_{16} groups relative to the TR_0 group. MyoD nuclear localisation was not significantly correlated with mean fast muscle fibre area, in contrast to previous findings. There was no significant difference in nuclear localisation of myf5 (Figs. 4.11A & C). There was no significant difference in the overall concentration of MRFs in total cellular protein extracts from slow muscle tissue with respect to experimental treatment (Figs. 4.12). Nuclear localisation of the MyoD protein in slow tissue nuclear extracts (Figs. 4.13A & B) was significantly different between the three experimental treatments compared ($F_{2, 17} = 4.9$, $P < 0.05$, one-way ANOVA). A minimal increase (5%) in MyoD nuclear localisation in the E_{16} group was significantly different from the TR_{16} group ($P < 0.05$, Tukey's test) and a significant correlation was observed between MyoD nuclear localisation in slow muscle tissue and mean slow muscle fibre cross-sectional area in the groups examined ($r_s = 0.50$, $P < 0.05$). There was no significant difference in myf5 nuclear localisation in slow muscle nuclear extracts (Figs. 4.13A & C).

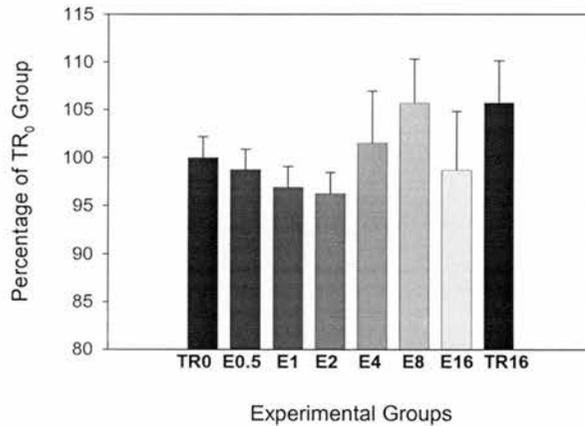
A Cellular Extracts



B) Cellular MyoD



C) Cellular myf-5



D) Cellular myogenin

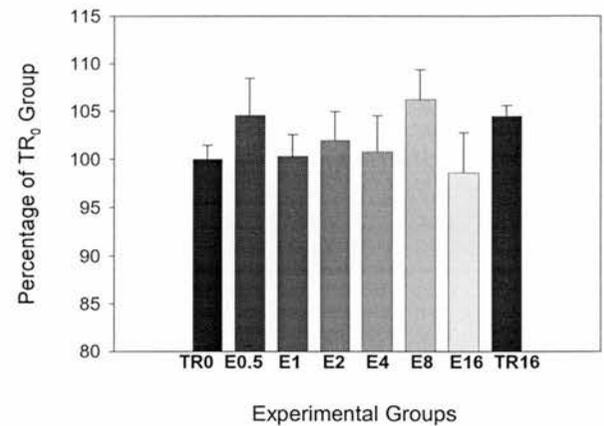
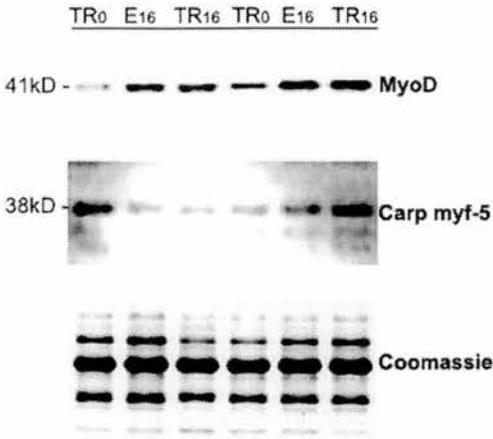
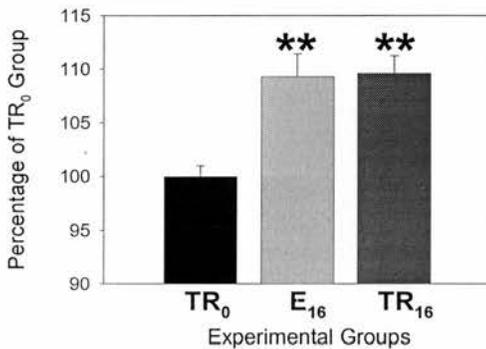


Fig. 4.10. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E_{0.5}, E₁, E₂, E₄, E₈ & E₁₆; 2.6 – 3.0 bls⁻¹, 18 hours per day) on total cellular expression levels of three myogenic regulatory factors (MRFs): MyoD, myf-5 and myogenin. A – D) There was no significant difference in MRFs expression in total fast muscle tissue cellular extracts between experimental treatments (NS, one-way ANOVA). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Values represent group mean optical density expressed as a percentage of the baseline tank rested group ± SEM, N = 6. NS, non-significant.

A Nuclear



B) Nuclear MyoD



C) Nuclear myf-5

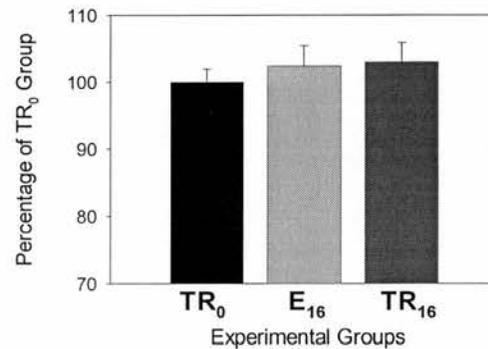
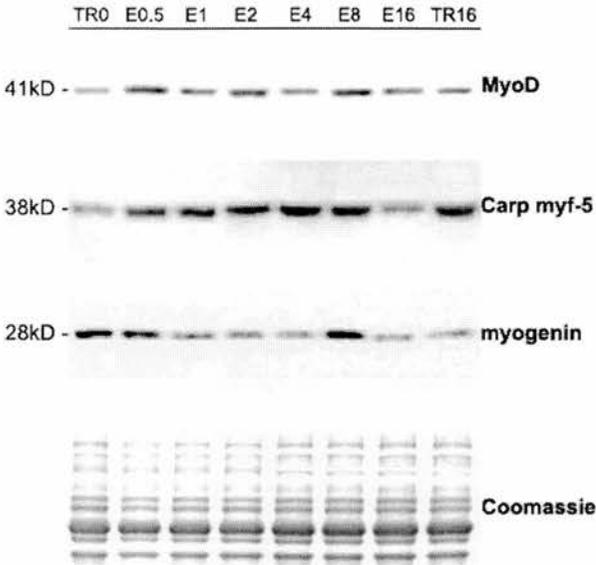
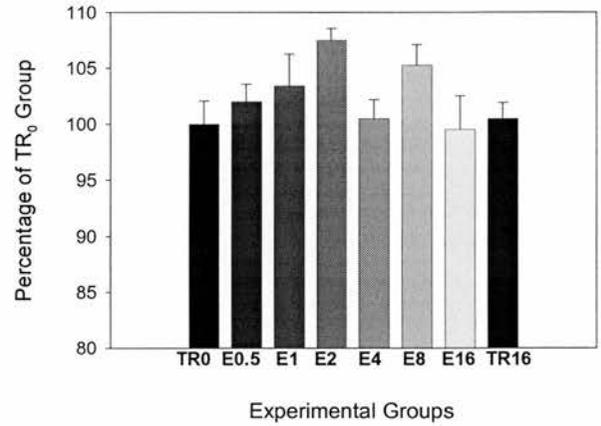


Fig. 4.11. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on fast muscle tissue nuclear localisation of MyoD and myf-5 proteins. A & B) MyoD nuclear localisation was 9% and 10% higher in the E₁₆ and TR₁₆ experimental treatments relative to the TR₀ group in fast muscle tissue nuclear protein extracts ($P < 0.01$, one-way ANOVA; $** P < 0.01$, Tukey's test). A & C) myf-5 nuclear localisation was not significantly different in the three experimental groups studied (NS, one-way ANOVA). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Values represent group mean optical density expressed as a percentage of the baseline tank rested controls \pm SEM, N = 6. NS, non-significant.

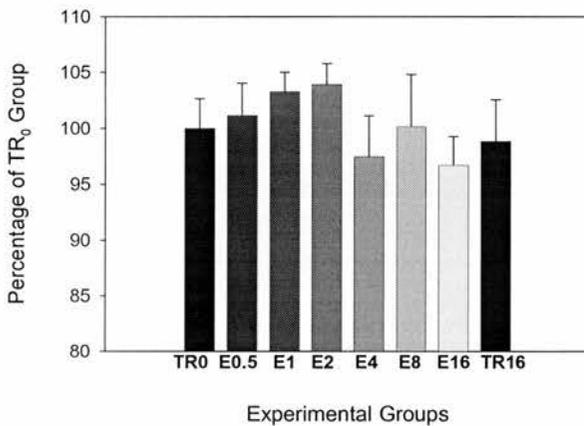
A Slow Cellular Extracts



B) Slow Cellular MyoD



C) Slow Cellular myf-5



D) Slow Cellular myogenin

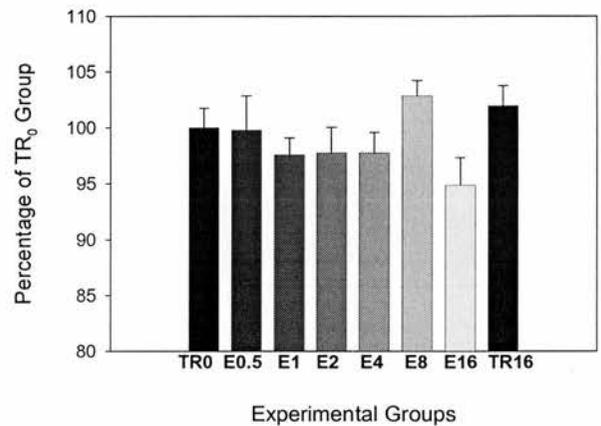
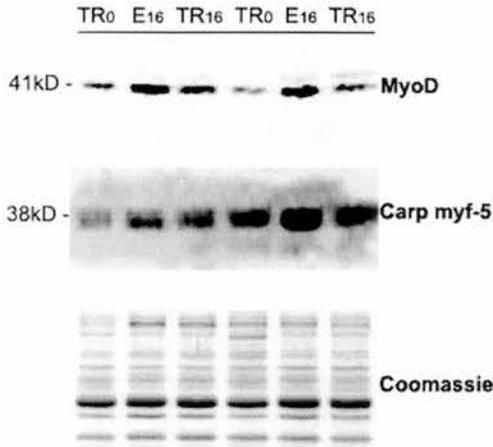
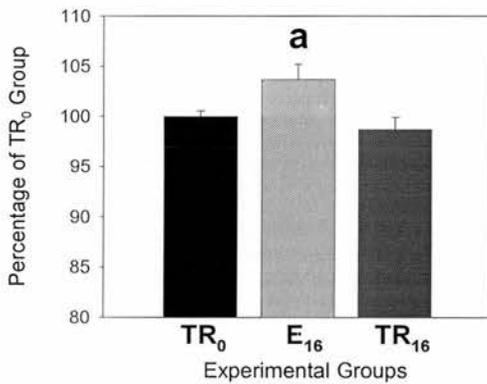


Fig. 4.12. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E_{0.5}, E₁, E₂, E₄, E₈ & E₁₆; 2.6 – 3.0 bls⁻¹, 18 hours per day) on total cellular expression levels of three myogenic regulatory factors (MRFs): MyoD, myf-5 and myogenin. A – D) There was no significant difference in MRFs expression in total slow muscle tissue cellular extracts between experimental treatments (NS, one-way ANOVA). Slow muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Values represent group mean optical density expressed as a percentage of the baseline tank rested group ± SEM, N = 6. NS, non-significant.

A Slow Nuclear



B) Slow Nuclear MyoD



C) Slow Nuclear myf-5

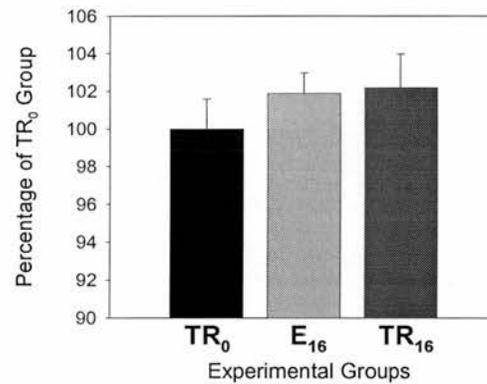


Fig. 4.13. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on slow muscle tissue nuclear localisation of MyoD and myf-5 proteins. A & B) MyoD nuclear localisation was 5% higher in the E₁₆ group relative to the TR₀ and TR₁₆ experimental treatments in slow muscle tissue nuclear protein extracts ($P < 0.05$, one-way ANOVA; * $P < 0.05$, Tukey's test). A & C) myf-5 nuclear localisation was not significantly different in the three experimental groups studied (NS, one-way ANOVA). Slow muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Values represent group mean optical density expressed as a percentage of the baseline tank rested controls \pm SEM, N = 6. NS, non-significant.

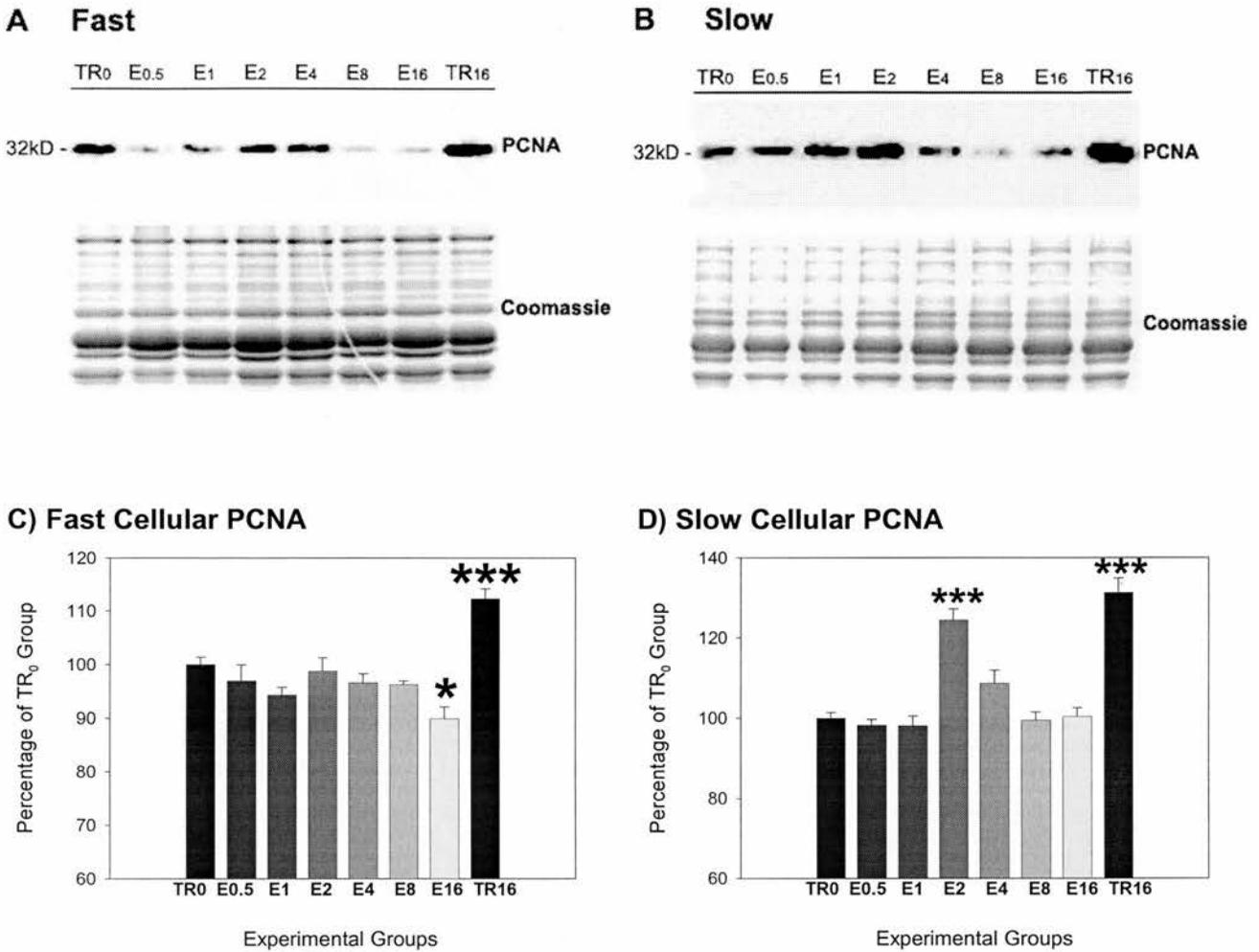


Fig. 4.14. Time course experiment: the effect of tank rest (TR₀ & TR₁₆) or exercise training (E_{0.5}, E₁, E₂, E₄, E₈ & E₁₆; 2.6 – 3.0 bls⁻¹, 18 hours per day) on total cellular expression of proliferating cell nuclear antigen (PCNA). A & C) In fast muscle tissue cellular extracts, PCNA expression was 12% higher in the TR₁₆ group, relative to the TR₀ group. PCNA expression was significantly higher in the TR₁₆ group compared with all other experimental treatments ($P < 0.001$, one-way ANOVA; *** $P < 0.001$, Tukey's test). PCNA expression was significantly lower in the E₁₆ group relative to the TR₀ and E₂ experimental treatments ($P < 0.001$, one-way ANOVA; * $P < 0.05$, Tukey's test). B & D) In slow muscle tissue cellular extracts, PCNA expression was upregulated by 24% and 31% in the E₂ and TR₁₆ groups, relative to the TR₀ group. PCNA expression in the E₂ and TR₁₆ groups was significantly different from all other experimental treatments ($P < 0.001$, one-way ANOVA, *** $P < 0.001$, Tukey's test). PCNA expression was invariant between the E₂ and TR₁₆ groups (NS, one-way ANOVA). Total cellular extracts were Coomassie stained to demonstrate equal gel loading of samples from all experimental treatments. Values represent group mean optical density expressed as a percentage of the TR₀ group \pm SEM, N = 6. NS, non-significant.

Proliferating cell nuclear antigen (PCNA) expression in total protein extracts from fast muscle tissue (Fig. 4.14A & C) was 12% higher in the TR₁₆ group which was significantly different to all other experimental groups ($F_{7, 47} = 11.4$, $P < 0.001$, one-way ANOVA, $P < 0.001$, Tukey's test). Relative to the TR₀ group PCNA expression was downregulated in each of the exercised groups and the largest decrease was observed in the E₁₆ group (10%). The E₁₆ level of PCNA expression was significantly lower than that of the TR₀ and E₂ experimental treatments ($P < 0.05$, Tukey's test). A significant correlation between fast muscle tissue PCNA expression and total fast muscle cross-sectional area was observed ($r_s = 0.37$, $P < 0.05$).

PCNA expression in total slow muscle tissue extracts (Fig. 4.14B & D) was upregulated by 24% and 31% in the E₂ and TR₁₆ groups, which were significantly different from all other experimental treatments ($F_{7, 47} = 17.4$, $P < 0.001$, one-way ANOVA, $P < 0.001$, Tukey's test). PCNA expression did not vary significantly between the E₂ and TR₁₆ groups. A significant correlation between slow muscle tissue PCNA expression and total muscle cross-sectional area was observed ($r_s = 0.40$, $P < 0.01$).

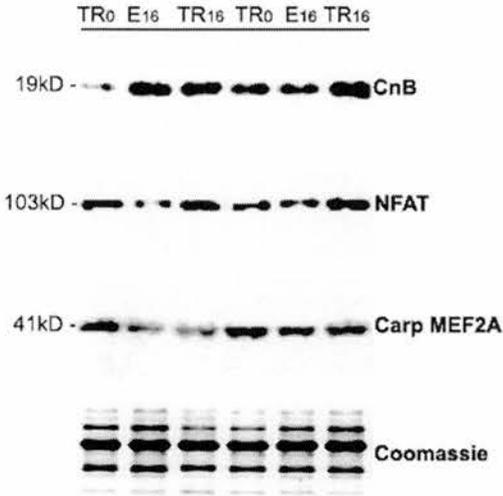
4.3.5 Calcineurin, NFAT2 and MEF2A localisation and expression in response to three experimental treatments

The nuclear localisation of the calcineurin regulatory subunit (CnB) in fast muscle nuclear fractions (Figs. 4.15A & B) was significantly different in tissue extracts from the two tank rested groups and the 16-day exercised group ($F_{2, 17} = 4.7$, $P < 0.05$, one-way ANOVA). The 6% increase in CnB nuclear localisation in the TR₁₆ group was significantly different from the TR₀ group ($P < 0.05$, Tukey's test). A significant correlation between CnB nuclear localisation in fast muscle and mean fast muscle fibre cross-sectional area was not observed. The overall expression of CnB in

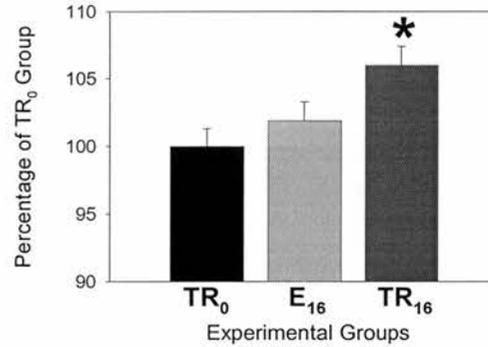
total cellular extracts was not significantly different in these TR₀, E₁₆ and TR₁₆ groups (Figs. 4.16A & B).

NFAT2 nuclear localisation (Figs. 4.15A & C) in fast muscle nuclear extracts was 6% and 8% higher in the TR₀ (NS) and TR₁₆ (P < 0.05) groups respectively, relative to the E₁₆ group. Nuclear localisation of NFAT2 in fast muscle nuclear protein extracts was not significantly correlated with mean fast muscle fibre cross-sectional area. The total concentration of NFAT2 in total cellular protein extracts (Figs. 4.16A & C) was 14% higher in both the E₁₆ and TR₁₆ groups relative to the TR₀ group (P < 0.05). A significant correlation was observed between mean fast muscle fibre cross-sectional area and NFAT2 expression in fast muscle total protein extracts ($r_s = 0.63$, P < 0.05). MEF2A nuclear localisation was invariant in the three experimental groups examined (Figs. 4.15A & D). Nuclear localisation and total expression of the calcineurin regulatory subunit (CnB) did not vary significantly in slow muscle tissue extracts from the TR₀, E₁₆ and TR₁₆ experimental groups (Figs. 4.17A & B; 4.18A & B). Nuclear localisation and overall concentration in slow muscle tissue extracts of NFAT2 was invariant in the TR₀, E₁₆ and TR₁₆ experimental treatments (Figs. 4.17A & C; 4.18A & C). In the slow muscle nuclear protein extracts, no significant difference was observed in MEF2A nuclear localisation in the TR₀, E₁₆ and TR₁₆ experimental groups (Figs. 4.17A & D).

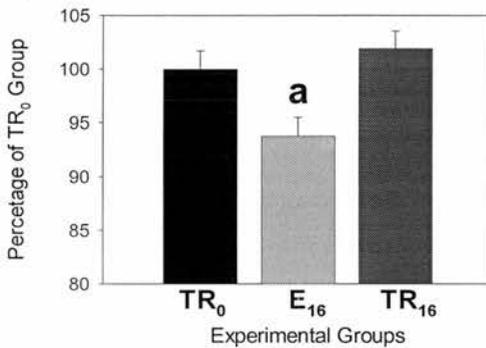
A Nuclear



B) Nuclear CnB



C) Nuclear NFAT



D) Nuclear MEF2A

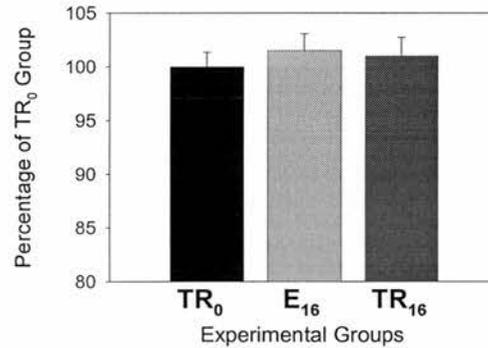
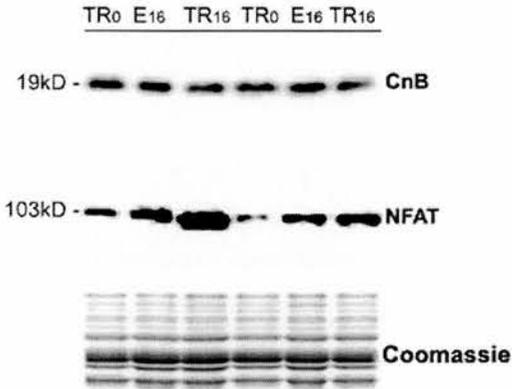
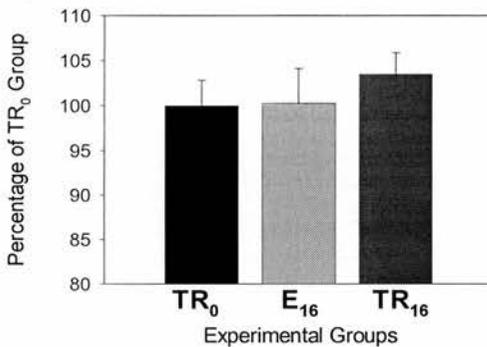


Fig. 4.15. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on fast muscle tissue nuclear localisation of the calcineurin regulatory (CnB) subunit, and associated transcription factors NFAT and MEF2A. A & B) A significant increase (6%) in CnB nuclear localisation in fast muscle tissue nuclear extracts was observed in the TR₁₆ group relative to the TR₀ experimental treatment ($P < 0.05$, one-way ANOVA; * $P < 0.05$ Tukey's test). A & C) NFAT nuclear localisation was 8% lower in the E₁₆ group relative to the TR₁₆ experimental treatment ($P < 0.05$, one-way ANOVA; **a** $P < 0.05$, Tukey's test). A & D) MEF2A nuclear localisation was invariant between the three experimental treatments examined (NS, one-way ANOVA). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, \pm SEM, N = 6. NS, non-significant.

A Cellular



B) Cellular CnB



C) Cellular NFAT

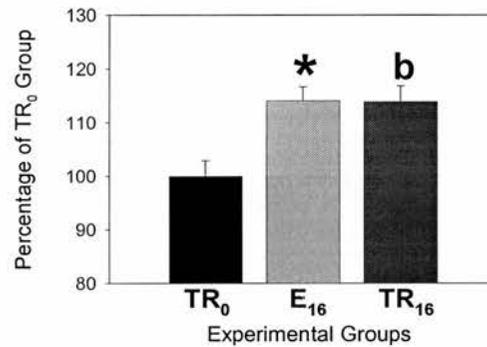
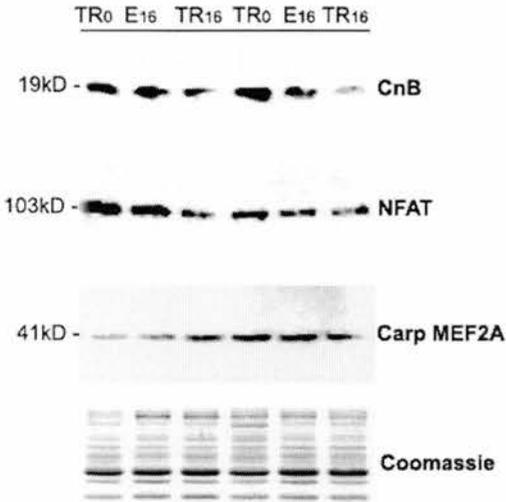
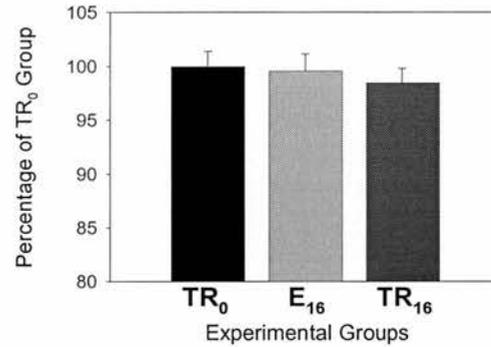


Fig. 4.16. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on total cellular expression of the calcineurin regulatory (CnB) subunit and NFAT2 transcription factor, in fast muscle tissue extracts. A & B) The overall expression of the CnB subunit in fast muscle tissue cellular extracts was invariant between the three experimental groups examined (NS, one-way ANOVA). A & C) NFAT2 expression was 14% higher in both the E₁₆ and TR₁₆ groups relative to the TR₀ experimental treatment ($P < 0.05$, one-way ANOVA; * $P < 0.05$, **b** $P < 0.1$, Tukey's test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, \pm SEM, $N = 6$. NS, non-significant.

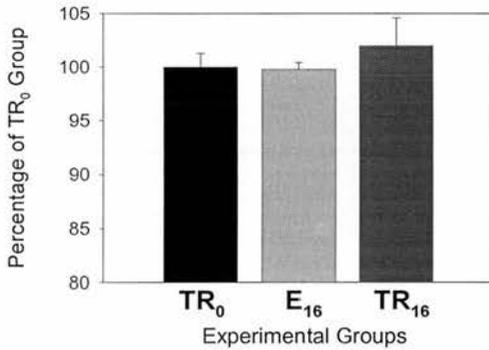
A Slow Nuclear



B) Slow Nuclear CnB



C) Slow Nuclear NFAT



D) Slow Nuclear MEF2A

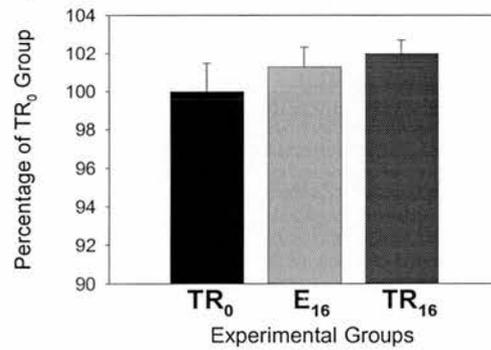
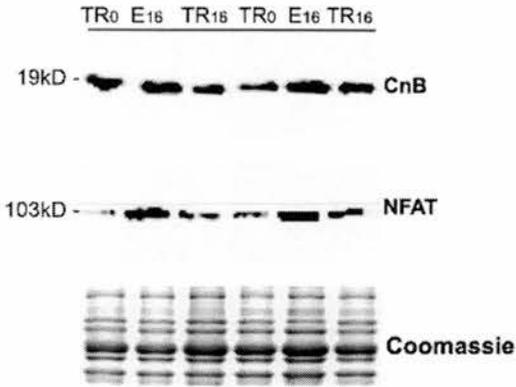
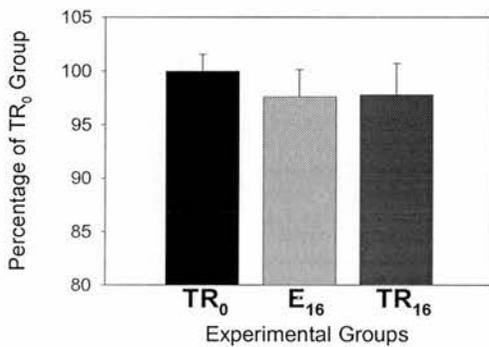


Fig. 4.17. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on slow muscle tissue nuclear localisation of the calcineurin regulatory (CnB) subunit, and associated transcription factors NFAT2 and MEF2A. A – D) In the three experimental groups examined, nuclear localisation of the calcineurin regulatory subunit, NFAT2 and MEF2A was not significantly different (NS, one-way ANOVA). Slow muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, ± SEM, N = 6. NS, non-significant.

A Slow Cellular



B) Slow Cellular CnB



C) Slow Cellular NFAT

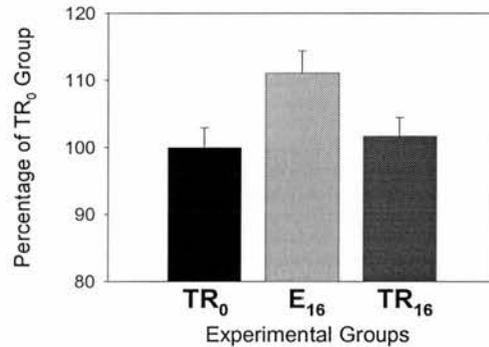


Fig. 4.18. Time course experiment: the effect of tank rest (TR₀, black fills; TR₁₆, dark grey fills) or exercise training (E₁₆, grey fills; 2.6 – 3.0 bls⁻¹, 18 hours per day) on total cellular expression of the calcineurin regulatory (CnB) subunit and NFAT2 transcription factor, in slow muscle tissue extracts. A – C) In the three experimental groups examined, total cellular expression of CnB and NFAT2 was not significantly different in slow muscle tissue extracts (NS, one-way ANOVA). Slow muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, \pm SEM, N = 6. NS, non-significant.

4.4 Discussion

4.4.1 Somatic growth

Data from this experiment unambiguously demonstrated the negative effect of exercise on somatic growth in the common carp. In previous experiments on cyprinid species, prolonged exercise training at 2.0 – 3.5 bls⁻¹ was shown to have no effect on condition factor in chub and nase (Hinterleitner *et al.*, 1992; Sanger, 1992), but condition factor of the Danube bleak was enhanced by exercise training. Exercise intensity was shown to have an inverse relationship with condition factor in goldfish (Davison & Goldspink, 1978) and a significant reduction in condition factor was demonstrated in common carp in response to sustained periods of exercise activity in excess of 2 bls⁻¹ (see Chapter 3). In this experiment the only group to display significant growth was the 16-day tank rested group (TR₁₆). The TR₁₆ group displayed significant concomitant increases in fork length, body mass, condition factor and total fast muscle cross-sectional area relative to all other experimental groups. Growth of the 16-day exercised group (E₁₆) was much reduced in comparison, illustrated by the 40mm² difference in mean total fast muscle cross-sectional area in favour of the TR₁₆ group. In this experiment, exercise at 2.6 – 3.0 bls⁻¹ has been shown to have a detrimental effect on somatic growth in the common carp, characterised by reduced condition factor, body mass and in some cases fork length. Furthermore, exercise training resulted in a loss of growth potential, illustrated by the significantly smaller mean total fast muscle cross-sectional areas. An industrial scale experiment on Atlantic salmon demonstrated a beneficial effect of prolonged low intensity exercise (0.40 – 0.45 bls⁻¹, continuous swimming activity over an 8 month period) on body mass accumulation (Totland *et al.*, 1987). In

swimming experiment 2 (see Chapter 3) lower intensity training ($1.7 - 1.9 \text{ bls}^{-1}$) may have stimulated the significantly increased somatic growth observed in exercised common carp. Therefore slow speed ($0.5 - 1.5 \text{ bls}^{-1}$) endurance exercise training should not be ruled out as a potential stimulus for augmenting muscle growth in the common carp, however short term (four weeks) moderate intensity endurance exercise training ($> 2.0 \text{ bls}^{-1}$) is detrimental to the growth potential of this species.

4.4.2 Muscle growth

Previous exercise studies involving common carp have demonstrated that moderate intensity exercise training initiated recruitment of fast muscle fibres and atrophy of existing fast and slow muscle fibres, whereas sustained slow to moderate intensity training stimulated slow muscle fibre hypertrophy (Chapter 3). Muscle fibre morphometry for the two tank rested groups (TR_0 & TR_{16}) and three of the exercised groups (E_4 , E_8 & E_{16}) was examined in this experiment. Significant exercise-induced slow muscle fibre hypertrophy was observed in the E_{16} group relative to the baseline group (TR_0) and 16 days of exercise training stimulated slow fibre hypertrophy that outstripped growth of these fibres in the TR_{16} group. This result is consistent with the slow fibre hypertrophy observed in the common carp in swimming experiment 4 (Chapter 3) and numerous previous studies that have demonstrated the connection between endurance training and hypertrophy of the aerobic musculature (Davison & Goldspink, 1978; Johnston & Moon, 1980a; Hinterleitner *et al.*, 1992; Sanger 1992).

The restriction or retardation of somatic growth in response to exercise training in this experiment was paralleled in the patterns of fast muscle fibre growth observed. The response of the fast musculature to exercise training at $2.6 - 3.0 \text{ bls}^{-1}$ appeared to involve a slight initial atrophy after 4 and 8 days (E_4 & E_8) followed by subsequent recovery and growth through hypertrophy by 16 days (E_{16}). Comparison

of the exercised groups relative to the tank rested group (TR_0) indicated that the initial atrophy followed by a resurgence of hypertrophic growth appeared to be repeated for each fast and intermediate fibre phenotypes distinguished by mATPase histochemistry. Hypertrophic growth of fast muscle fibres under tank rested conditions (TR_{16}) surpassed that of the E_{16} group and a greater mean fibre area was observed in each of the fast and intermediate fibre phenotypes described. The higher level of hypertrophic growth in the fast-type swimming musculature observed in the TR_{16} group relative to the E_{16} group is a further indication that moderate intensity exercise training is a stimulus that inhibits somatic growth in the common carp through diminution of growth of the fast musculature, a tissue that comprises 65 – 70% of total body mass. In response to high intensity exercise training (4.5 bls^{-1}) a salmonid species renowned for a high swimming capacity was shown to breakdown protein to produce amino acids to buffer the acidic effects of lactic acid (Davison & Goldspink, 1977). Therefore the reduced growth of the fast musculature in this experiment may have been caused by a short period (up to day 8) of protein breakdown and tissue depletion to cope with elevated levels of lactic acid, which subsequently recovered but caused the muscle growth of the exercised common carp to lag behind that observed in the tank rested group. In conclusion, the time course experiment has confirmed the findings of swimming experiments 1 to 4. Moderate intensity exercise ($2.6 - 3.0 \text{ bls}^{-1}$) is a stimulus for slow muscle fibre hypertrophy in the common carp, but reduces the potential for growth in fast and intermediate muscle fibres.

4.4.3 Muscle fibre phenotype

The ability to adapt muscle fibre phenotype to maintain efficient force production in the axial swimming musculature at different temperatures is one

attribute that allows cyprinid species such as the common carp and goldfish to inhabit environments with a wide thermal range (Johnston & Lucking, 1978; Gerlach, 1990). The plasticity of muscle fibre phenotype allows human athletes to develop muscle characteristics adapted for a particular function and is perhaps best illustrated by the extreme phenotypic adaptations observed in marathon runners and sprinters (reviewed by Goldspink 1996, 1998). The capacity of the fast musculature to adapt to increased functional use was examined in the common carp.

The total number and the proportion of Ia type muscle fibres did not vary significantly between experimental treatments. The proportion of the total area of Ia fibres was significantly reduced in the E₄ and E₈ groups in comparison with the TR₀ group. This result may be associated with an initial period of adaptation to exercise training (see section 4.4.2), where expenditure of energy exceeds food intake and nutrients are sequestered for exercise activity rather than somatic growth. Protein is mobilised to make up the energetic shortfall, resulting in tissue depletion and in this situation, atrophy of type Ia muscle fibres.

The total number of Ib type fibres in the exercised groups increased significantly in relation to the TR₀ group. The Ib proportion of the total fibre number within the scaled box region was significantly higher in the E₁₆ group relative to the TR₀ group. The proportion of the total area of the scaled box that comprised type Ib fibres was significantly higher in the E₁₆ and TR₁₆ groups in relation to the TR₀ group. The increase in volume of type Ib fibres in the E₁₆ group occurred in relation to the TR₀ group but not the TR₁₆ treatment, which suggests that over the duration of the experiment Ib fibre quantity was increasing as the result of an additional stimulus over and above exercise activity, such as fluctuations in environmental temperature over the course of the experiment.

The total number of fast fibres within the scaled box area was significantly different between the E₄ and E₈ groups and the E₁₆ and TR₁₆ experimental treatments. The alteration of fast fibre numbers within the scaled box area was most likely a consequence of changes in muscle fibre size due to the processes of tissue depletion and atrophy observed in some experimental groups (E₄ & E₈) and hypertrophic muscle fibre growth observed in others (E₁₆ & TR₁₆). This is a major limitation of the experimental design, which was encountered despite the careful positioning and measurement of the 'scaled box' area. In the E₄ and E₈ groups, smaller fast muscle fibres occurred as a result of tissue depletion or retarded growth relative to other experimental groups. Conversely, hypertrophic growth of fast fibres was observed in the E₁₆ and TR₁₆ experimental groups. As a consequence, an increase or decrease in fast muscle fibre number was observed within the scaled box area, making it impossible to accurately assess the effect of exercise on muscle fibre phenotype. An improved method of estimating the contribution of slow, intermediate and fast muscle fibre phenotypes to the total myotomal cross-sectional area, would involve the measurement of the total cross-sectional area of each fibre type. Muscle fibre cross-sectional area of a representative sample (approx. 1000 fibres) of each fibre phenotype would be measured. From these data, the total number of each muscle fibre phenotype could be calculated and the effect of exercise on muscle fibre phenotype could then be examined.

The decreased contribution of fast fibres to the total number and total area observed in three groups (E₄, E₁₆ & TR₁₆) relative to the TR₀ group, was most likely a consequence of concomitant increases in other fibre types that have been induced by an additional stimulus to exercise training, as discussed previously with regard to Ib fibres. The variation in water temperature over the course of the experiment is

another major limitation of the experimental design, because temperature is a known stimulus for alterations in the proportion of different muscle fibre phenotypes (Johnston & Lucking, 1978).

In teleosts, an increased proportion of slow muscle fibre fraction and elevated intermediate muscle fibre number occurred as a consequence of the life history of the animal and long-term prolonged exercise training (Broughton *et al.*, 1978, 1981; Hinterleitner *et al.*, 1992). An increase in the fraction of aerobic musculature has also been described in goldfish in response to a 3-month period of acclimation to cold environmental temperatures (Johnston & Lucking, 1978). The present study was carried out over a much shorted time period (16 days). Therefore it is likely that the timescale of the present experiment was too short to identify switches in muscle fibre phenotype in response to exercise in the common carp. A future experiment to assess the impact of exercise training on muscle fibre phenotype would have to use a much improved experimental design, incorporating a better method of estimating the proportion of different fibre phenotypes, a constant environmental temperature and a longer time scale (3 months).

4.4.4 MRFs and PCNA expression in response to training

An exercise-induced transitory increase in the expression of MRFs transcripts is a well-described characteristic of the myogenic response (Psilander *et al.*, 2003). Depending upon the duration and intensity of the stimulus, the time course of transcript upregulation can last for as little as 20 hours (Jacobs-El *et al.*, 1995) or peak after several days (Carson & Booth, 1998). In protein expression analysis, Sakuma (1999) and colleagues described an initial downregulation of MRFs protein in the first 8 days of functional overload, followed subsequently by recovery and overcompensation of expression by 28 days. In fish, feeding has been described as a

stimulus for increased myogenic activity and muscle growth, and was characterised by the increase of myogenic progenitor cells positive for MyoD and PCNA (Brodeur *et al.*, 2003b). The number of cells positive for MyoD increased by 20% and 44% 24 and 96 hours after feeding respectively. The number of cells expressing PCNA was increased by 60% at each of these time points. Co-expression of MyoD and PCNA is a marker of activation in myogenic progenitors (Yablonka-Reuveni & Rivera, 1994). The time course of MRFs and PCNA expression was examined in the present experiment.

The expression of MRFs in total cellular extracts from fast and slow muscle tissue was not significantly different at any of the time points between 9 and 288 hours of forced exercise activity. However nuclear localisation of the MyoD protein in fast tissue was significantly higher in the TR₁₆ and E₁₆ groups relative to the TR₀ group. The two 16-day experimental groups displayed an increase in mean fast fibre cross-sectional area and a hypertrophic shift in the fast muscle fibre diameter density distributions relative to the TR₀ group. The association between increased nuclear localisation of MyoD protein and larger muscle fibre diameter is consistent with previous results that demonstrated a positive correlation of fibre size and MyoD nuclear localisation in fast muscle tissue (see Chapter 3). A similar significant pattern of MyoD nuclear localisation was observed in the slow muscle tissue nuclear extracts, where a higher degree of MyoD nuclear localisation was associated with larger slow muscle fibre area. An increased level of MyoD protein was found in the E₁₆ group nuclear extractions relative to the TR₁₆ group and a significant correlation was demonstrated between nuclear levels of MyoD and mean slow muscle fibre cross-sectional area. The connection between increased nuclear localisation of MyoD and muscle growth through hypertrophy suggests that MyoD plays a key role in regulating

the activation and proliferation of myogenic progenitor cells to provide the necessary nuclei for fibre hypertrophy in the common carp.

PCNA expression was significantly upregulated in the TR₁₆ group in fast total cellular protein extracts relative to all other experimental treatments. The expression of PCNA in the E₁₆ group was significantly reduced in comparison to the TR₀ and E₂ groups. In slow muscle tissue extracts, PCNA expression was significantly higher in the TR₁₆ and E₂ groups in relation to all other experimental treatments. The expression pattern of PCNA in fast and slow muscle tissue suggests that the elevated PCNA levels in E₂ and TR₁₆ were associated with large body size. Significant correlations between PCNA expression in fast and slow tissue extracts and total myotomal cross-sectional area were observed. PCNA expression is a marker of DNA synthesis and these results have identified the two groups in which cell proliferation and growth has most likely occurred over the time course of this experiment. Due to imperfect size matching of experimental animals, the fish included in the E₂ group were the largest at the outset of the time course experiment. It is likely that two days of exercise training had little impact on the growth of the E₂ experimental group, which is reflected in the relatively high PCNA expression observed in slow muscle tissue extracts. Muscle fibre cellularity and morphological data demonstrated that the TR₁₆ group was the only experimental treatment to grow significantly over the duration of the experiment. The concomitant increase in PCNA expression in the TR₁₆ group was to be expected. A worthwhile future direction would be the examination of MyoD and PCNA nuclear localisation early in the myogenic response to a hypertrophic stimulus. The number of myogenic progenitor cells positive for MyoD and PCNA increased significantly after 24 and 96 hours after a feeding stimulus in a sub-Antarctic notothenioid (Brodeur *et al.*, 2003b) and in theory, increased nuclear

localisation of these proteins should occur in response to a hypertrophic stimulus such as forced swimming activity. In conclusion, it has been demonstrated that slow to moderate endurance exercise in common carp does not stimulate a transient upregulation of MRFs, unlike that observed in response to a range of hypertrophic stimuli in higher vertebrates. However, increased nuclear localisation of the MyoD protein was observed in association with fast and slow muscle fibre hypertrophy in the TR₁₆ and E₁₆ experimental treatments respectively. This implies that MyoD plays a role in regulating the activity of myogenic progenitor cells, to provide the nuclei necessary for hypertrophic muscle growth in the common carp. PCNA expression was not upregulated in response to moderate endurance exercise, which was unexpected because endurance exercise was previously shown to be a stimulus for myonuclear accretion. However, PCNA expression was elevated in the only experimental group to display significant growth over the duration of the experiment (TR₁₆), which suggests that PCNA expression can be used as an index of somatic and muscle growth in the common carp.

4.4.5 The calcineurin pathway and muscle fibre hypertrophy

The calcineurin pathway was implicated in the process of muscle fibre hypertrophy under normal growth conditions in a previous experiment involving common carp. Increased nuclear localisation of the calcineurin regulatory (CnB) and catalytic (CnA) subunits was observed in conjunction with increased nuclear localisation of the primary MRFs (MyoD and myf5), and was significantly correlated with muscle fibre size (see Chapter 3). On the basis of these findings it was proposed that in common carp, the calcineurin-signaling pathway is a regulator of muscle differentiation through activation of downstream transcription factors MyoD and myf5. Moreover it was proposed that calcineurin also regulates hypertrophic growth

of fast and slow muscle fibres in the normal non-exercised state. This hypothesis was tested in this experiment through examination of nuclear localisation and expression of CnB and substrate transcription factors in the three groups with the most disparate mean muscle fibre cross-sectional areas (TR₀, E₁₆ & TR₁₆).

A significantly higher level of CnB nuclear localisation was observed in the TR₁₆ group relative to the TR₀ group. Although the TR₁₆ group also possessed a larger mean fast fibre cross-sectional area relative to the TR₀ treatment, there was no discernible relationship between this characteristic and CnB nuclear localisation in fast muscle tissue. The levels of CnB in nuclear protein extractions from slow muscle tissue were not significantly different, despite significant slow muscle fibre hypertrophy occurring in the E₁₆ group relative to the two tank rested treatments. The overall levels of CnB in total extracts from fast and slow muscle tissue did not vary significantly. Overall, these data provide little support for the proposed model of muscle growth in the common carp, with calcineurin cast as a key central regulator of this process. The function of calcineurin as a mediator of skeletal muscle differentiation (Friday *et al.*, 2003) and muscle fibre phenotypic characteristics (Chin *et al.*, 1998) in higher vertebrates is unambiguous, however the role of calcineurin in skeletal muscle hypertrophy is less so. Several studies have called into question the role of calcineurin in skeletal muscle hypertrophy and demonstrated muscle fibre hypertrophy in the presence of calcineurin-specific inhibitors (Bodine *et al.*, 2001; Musaro *et al.*, 2001; Rommel *et al.*, 2001). Alternative intracellular signaling regulatory pathways, such as calmodulin kinase or phosphatidylinositol 3-kinase (PI(3)K) were proposed as the mediators of hypertrophic signaling (Musaro *et al.*, 2001). The role of calcineurin in skeletal muscle hypertrophy remains ambiguous but it has been suggested that the calcineurin and PI(3)K/Akt pathways may act in

parallel to affect muscle fibre hypertrophy in different physiological contexts or may be distinct regulators of skeletal muscle phenotype and hypertrophy respectively (Dunn *et al.*, 2002). The contrast in the data of this experiment to previous results that outlined the relationship between nuclear localisation of the calcineurin regulatory subunit and muscle fibre cross-sectional area may have been a consequence of the shorter time period this experiment (16 days, compared to 28 days in swimming experiment 3) and the reduced number of subjects in each experimental treatment. Further work is required to elucidate calcineurin function in lower vertebrates.

Increased dephosphorylation and nuclear localisation of NFAT2 and MEF2, consistent with a role for calcineurin in muscle fibre hypertrophy, was not observed in fast or slow nuclear protein extracts. Significantly higher overall expression of NFAT2 was observed in the E₁₆ and TR₁₆ total cellular protein extracts from fast muscle tissue, which was significantly correlated with mean fast muscle fibre cross-sectional area. Moreover, significantly increased expression of NFAT2 occurred in the slow muscle tissue total cellular protein extract of the E₁₆ group, an experimental treatment in which significant hypertrophy of slow muscle fibres was also observed. The raised expression of NFAT2 in total cellular extracts from experimental groups that displayed a concomitant increase in muscle fibre size implied that there was a potential role for NFAT2 in muscle fibre hypertrophy in teleosts. However, there was no obvious pattern to NFAT2 nuclear localisation in these tissues and transcriptional activity of NFAT2 depends upon dephosphorylation and subsequent nuclear translocation of the protein. In swimming experiment 3, increased nuclear localisation of NFAT2 was observed in the nuclear fraction from fast and slow muscle tissue in the tank rested group, which coincidentally had larger mean fibre cross-sectional areas. These initial findings are promising, but further investigation is

required to fully illustrate a potential role for the calcineurin signaling pathway and its substrate transcription factor NFAT2, in the process of muscle fibre hypertrophy in teleost fish.

In conclusion, the negative effect of exercise on somatic growth and fast muscle fibre volume in the common carp has been unambiguously established. Further evidence has been accrued that strongly suggests extensive slow muscle fibre hypertrophy is a consequence of moderate endurance exercise training in this species. Muscle fibre phenotype proportions were not altered by this design of short-term exercise training programme in the common carp. Any alterations in muscle fibre phenotype that did occur were not accurately measured due to the limitations of the experimental design. Improved designs for future experiments were discussed. Significant nuclear localisation of the primary MRF MyoD was associated with increased muscle fibre size in fast and slow fibre phenotypes, which implied a role for MyoD in activating myogenic progenitor cells for subsequent proliferation and provision of nuclei for muscle fibre hypertrophy. Finally, in contrast to previous data (Chapter 3), no evidence for the involvement of the calcineurin-signaling pathway in regulating fast and slow muscle fibre hypertrophy was found in the common carp.

Chapter 5: The effect of exercise on muscle growth and-muscle specific gene expression in the rainbow trout *Oncorhynchus mykiss* Walbaum

5.1 Introduction

For over 20 years it has been established that exercise is a powerful stimulus for muscle fibre hypertrophy in salmonids (Davison & Goldspink, 1977; Johnston & Moon, 1980a). The inherent swimming capacity and relative availability of salmonids makes this an attractive group for research (Davison, 1989). One study demonstrated that 8 months of continuous slow speed exercise activity enhanced body mass accumulation in Atlantic salmon by almost 40% (Totland *et al.*, 1987). Growth of the axial musculature that comprises the dominant tissue in this species contributed in no small way to this process and the overall increase in fast muscle fibres through hypertrophic growth was 17%, relative to controls maintained in sea cages. Almost continuous forced exercise in short duration swimming experiments (28 days) at velocities between 1.5 and 4.5 bls⁻¹ has been shown to be a hypertrophic stimulus in brown trout. The optimal swimming velocity for fast fibre growth was 3 bls⁻¹ (Davison & Goldspink, 1977).

Myostatin or growth/differentiation factor 8 (GDF8) is a recently identified member of the transforming growth factor- β (TGF- β) superfamily that has been shown to be a negative regulator of skeletal muscle mass in higher vertebrates (McPherron *et al.*, 1997). Myostatin is initially synthesised as a 375 amino acid

propeptide (52kD) that is cleaved at a conserved region to give rise to an N-terminal latency associated peptide (LAP, 42kD) and a 26kD C-terminus bioactive peptide (Sharma *et al.*, 1999). The doubled-muscled phenotype in three breeds of cattle (Belgian Blue, Piedmontese and Asturiana de los Valles) is perhaps the most dramatic visualisation of myostatin function in the negative regulation of skeletal muscle mass; these breeds possess a mutation in the coding region for the myostatin active peptide that results in a truncated protein and complete loss of myostatin function (Kambadur *et al.*, 1997; McPherron & Lee, 1997; reviewed by Kocamis & Killefer, 2002). The “generalised muscle hyperplasia phenotype” observed is most likely a consequence of unrestricted proliferation of myogenic progenitors in animals lacking functional myostatin and increased muscle mass occurs primarily through an increase in fibre number (Bass *et al.*, 1999; Thomas *et al.*, 2000). An E-box motif that binds MyoD has recently been identified in the promoter region of myostatin and it is proposed that MyoD regulates myostatin expression to initiate cell cycle withdrawal (Spiller *et al.*, 2002). The inverse relationship between myostatin expression and muscle mass has been demonstrated in frail elderly women and HIV positive men with muscle wasting (Gonzalez-Cadavid *et al.*, 1998; Schulte & Yarasheski, 2001). This relationship is consistent with a downregulation of myostatin transcripts observed in response to a hypertrophic stimulus (Roth *et al.*, 2003). Numerous myostatin orthologues have recently been described in teleosts, which is in part due to the current focus of aquaculture research on this growth/differentiation factor as a potential means of augmenting muscle growth in fish. Recent studies have confirmed a role for myostatin during skeletal muscle development in zebrafish (Vianello *et al.*, 2003) and demonstrated myostatin mRNA expression is affected by fasting in larval and adult stages of tilapia (Rodgers *et al.*, 2003).

The primary objective of this chapter was to develop an exercise-induced model of muscle fibre hypertrophy in the rainbow trout. Based on previous findings, extensive hypertrophy of the fast musculature would be expected in response to slow to moderate levels of almost continuous endurance exercise. Exercise-induced hypertrophy of the axial musculature in teleosts is a well-established model of muscle growth and could potentially be utilised to great effect in the elucidation of molecular signaling pathways underlying fast muscle growth in salmonid species. The final focus of this experiment was to examine the calcineurin-signaling pathway and myostatin expression in the rainbow trout in response to slow to moderate intensity endurance exercise training. If the inverse relationship holds true, then myostatin expression will be downregulated as part of the adaptive response of exercise-induced fast fibre hypertrophy. Contradictory evidence has been presented for the involvement of the calcineurin signaling pathway in hypertrophic muscle growth in the common carp (Chapters 3 & 4). Increased nuclear localisation of calcineurin and NFAT2 proteins, and upregulation of MEF2 family transcription factors would be expected in association with calcineurin-mediated hypertrophic muscle growth in rainbow trout.

5.2 Materials And Methods

5.2.1 Experimental design

Thirty rainbow trout were obtained from College Mill Trout Farm (Almondbank near Perth, UK) and maintained in freshwater aquaria at approximately 15°C. Trout were fed to satiation once daily (approx. 3 – 5% body mass) with a species-specific commercially available brand of fish food (Trouw Aquaculture). The fish were divided

into three equal groups on the basis of fork length. The experimental treatments were as follows: TR (tank rested), E₁₅ (slow endurance exercise group, at 15cms⁻¹ or 0.8 bls⁻¹) and E₃₀ (moderate endurance exercise group, at 30cms⁻¹ or 1.6 bls⁻¹). Prior to the experiment, the mean fork length (\pm standard error, S.E.M.) of the TR group was 18.4 cm (\pm 0.4), compared with 18.0 cm (\pm 0.3) and 18.2 cm (\pm 0.5) in the E₁₅ and E₃₀ experimental groups. The initial body mass was not measured to reduce the stress experienced by experimental animals through excessive handling. The exercise groups were placed into the two of the channels of the flume (Fig. 3.3) and the tank rest group was maintained in a holding tank situated above the header tank for comparison. After an initial period of several days, the velocity of flow was increased steadily in both swimming channels to 15 and 30cms⁻¹. The training regime consisted of 18 hours swimming per day, with the remaining time given over to feeding and recuperation. Before the conclusion of the experiment, four fish in the E₃₀ group, and one fish from each of the TR and E₁₅ groups had to be culled due to extremely poor condition. The fish were sacrificed after 30 days, fork length and body mass were measured and condition factor was calculated as before (section 3.2.6). A brief summary of the exercise regime, water temperature and photoperiod in each experiment is given in Table 5.1.

5.2.1 Muscle Fibre Morphometry

Samples were taken for histological analysis as previously described (sections 2.3.2.2 & 2.3.2.3). In this experiment, the right hand side of the myotome was divided into 4 blocks, two on either side of the horizontal septum. Serial transverse sections (7 μ m) were cut and stained using Mayer's Haematoxylin histochemical technique (section 2.3.2.4).

Table 5.1. Trout swimming study: summary of body morphology and muscle fibre characteristics in tank rested (TR), slow exercised (E_{15}) and fast exercised (E_{30}) groups of trout. Percentage difference relative to the TR group is shown in brackets. Values represent group mean \pm SEM. TR N=9, E_{15} N=9 and E_{30} N=6.

Experimental treatment	Fork Length (cm)	Swimming Speed (bls^{-1})	Distance Swum (km)	Body Mass (g)	Condition Factor	Fast Muscle T_{CSA} (mm^2)	Fast Muscle F_{CSA} (μm^2)	Mean Daily Water Temperature ($^{\circ}\text{C}$)	Photoperiod (L, light; D dark)
TR	19.7 ± 0.4	0	0	105.4 ± 4.4	1.38 ± 0.03	355 ± 11	2907 ± 64	15.6 for all experimental groups	18L: 6D for all experimental groups
E_{15}	19.4 ± 0.3 (-1.3)	0.77 ± 0.01	292	113.8 ± 4.8 (7.9)	1.55 ± 0.04 (12.3)	371 ± 11 (4.5)	3600 ± 74 (23.8)		
E_{30}	19.2 ± 0.4 (-2.6)	1.57 ± 0.03	584	117.2 ± 5.7 (11.2)	1.66 ± 0.05 (20.5)	377 ± 14 (6.2)	3769 ± 147 (29.6)		

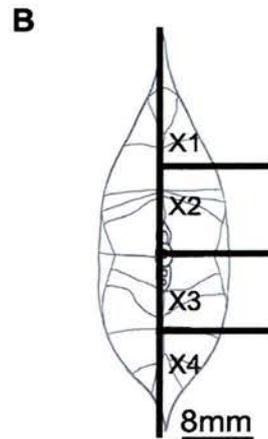
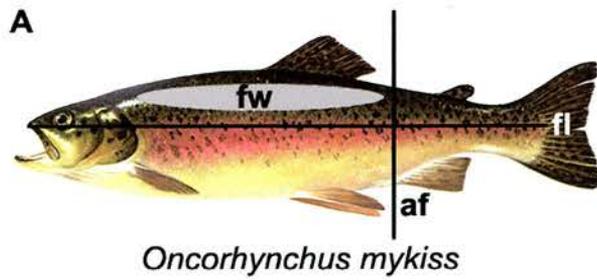


Fig. 5.1. Rainbow trout (*Oncorhynchus mykiss*) were subjected to exercise training or tank rest. A) Fork length (**fl**) was measured in each individual from snout to the most posterior region of the body. Fast white muscle tissue samples were taken for Western blot analysis dorsal to the lateral line (**fw**). A 5 mm thick total cross-sectional area (steak) was cut immediately anterior to the anal fin (**af**). B) Four blocks of muscle tissue were snap frozen for muscle fibre morphometric analysis. 300 fast muscle fibre area measurements were taken at each 'X' from random fields of view. (Rainbow trout illustration by N. Weaver, Migdalski & Fichter, 1977, p.119).

In each tissue block, 300 fast muscle cross-sectional areas (F_{CSA}) were measured from deep lying regions of the myotome using the same apparatus as previously described (section 3.2.7). Slow muscle fibres, identified using the S58 immunohistochemical technique (section 2.3.2.5), comprised a very small fraction of the total myotomal cross-sectional area and were therefore not considered in this experiment. Total cross-sectional areas (T_{CSA}) were measured from acetate tracings using Sigma Scan Pro software. Muscle fibre diameter was calculated from the area measurement as before (3.2.7). See Figure 5.1 for a summary of the sampling protocol in this experiment.

5.2.3 Protein Expression Analysis

Fast muscle tissue was dissected and preserved as previously described (section 2.2.2.2). Total cellular and nuclear protein extracts were made from fast muscle tissue in all experimental animals (section 2.2.2.3 and 2.2.2.4). Standardised western blotting techniques (sections 2.2.2.9 & 3.2.9) were applied in this experiment, to observe the effect of different swimming speed on muscle specific gene expression.

5.2.4 Statistical Analysis

The Anderson-Darling test of normality was used to assess the distribution of the data (Minitab v. 13.2). The overall effect of exercise on T_{CSA} and F_{CSA} in the three experimental groups was assessed by means of analysis of covariance and post-hoc Tukey's tests. Muscle fibre diameters were compared between the three experimental groups using the non-parametric statistics outlined in section 3.2.10. For protein expression, the group mean optical density \pm SEM is reported as the percentage of the TR group. For western blots and Coomassie stained gels, comparisons of optical density between experimental groups were made using one-way analysis of variance

and post-hoc Tukey's tests or the non-parametric equivalent (Kruskal-Wallis test and post-hoc multiple comparisons). The Spearman Rank Correlation Coefficient (r_s) was calculated to indicate correlation between the level of nuclear localisation or overall expression of a particular protein with mean fast muscle fibre cross-sectional area.

5.3 Results

5.3.1 Somatic growth

Condition factor significantly increased over the 30-day experiment in the two exercised groups (Table 5.1) relative to the tank rested controls ($F_{2, 23} = 11.0$, $P < 0.01$, one-way ANOVA). The exercised rainbow trout had a significantly greater body mass relative to fork length (Fig. 5.2A) in comparison with the tank rested controls ($F_{2, 20} = 12.3$, $P < 0.001$, ANCOVA). There was no significant difference in body mass or condition factor between the E_{15} and E_{30} exercised groups. The mean total fast muscle cross-sectional area of the exercised groups of rainbow trout was higher relative to the tank rested controls (Table 5.1), but this increase was not significantly different (Fig. 5.2B). Taken as a whole, these data indicate that body mass accumulation was significantly augmented by the exercise treatment, but the intensity of training did not have a significant effect.

5.3.2 Fast muscle fibre growth

Relative to the group of tank rested controls, the mean fast muscle fibre area of the E_{15} and E_{30} groups increased by 24% and 30% respectively (Table 5.1). The fast muscle fibre mean cross-sectional area (Fig. 5.2C) was significantly higher in the two exercised groups relative to fork length, when compared with the tank rested controls ($F_{2, 20} = 11.6$, $P < 0.001$, ANCOVA).

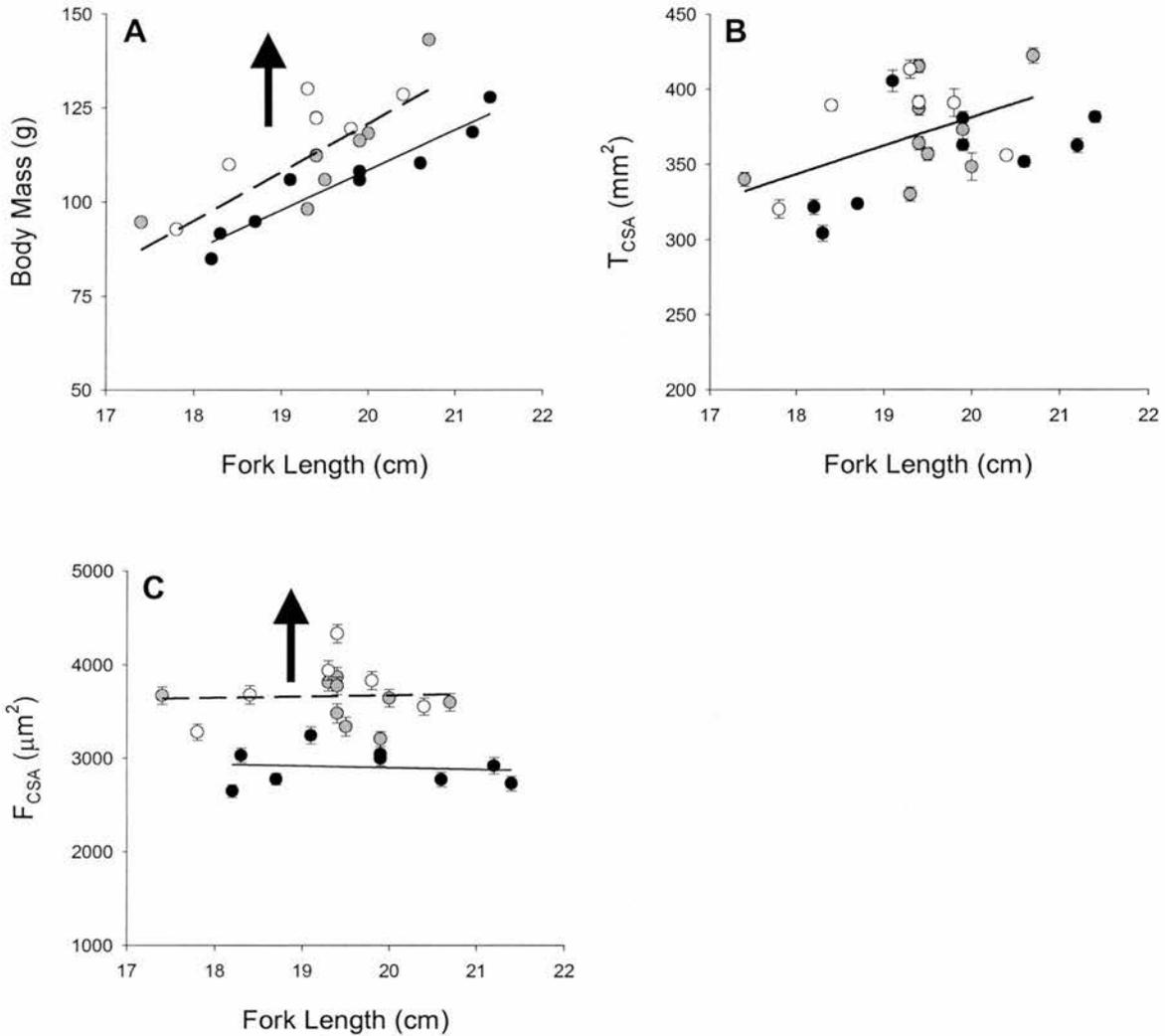


Fig. 5.2. Trout swimming experiment: the effect of tank rest (TR) or a 30-day exercise-training regime (E₁₅, 0.8 bls⁻¹; E₃₀, 1.6 bls⁻¹; 18 hours per day) on body mass (BM), total fast muscle fibre cross-sectional area (T_{CSA}), and mean fast muscle fibre cross-sectional area (F_{CSA}) relative to fork length (FL). A) The body mass of both exercised (E₁₅, open symbols; E₃₀, grey fills) groups was significantly higher compared to the tank rested group (TR, black symbols), relative to fork length ($P < 0.001$, ANCOVA). B) There was no significant difference in T_{CSA} between experimental treatments (NS, ANCOVA). C) Relative to fork length, F_{CSA} was significantly higher in the exercised groups than in the tank rested group ($P < 0.001$, ANCOVA). Arrows indicate the trend of increased somatic growth and fast muscle fibre hypertrophy in the exercised groups, relative to the tank rested controls. NS, non-significant; ANCOVA, analysis of covariance.

Lines were fitted using the method of 1st order linear regression, which gave the following equations: A) E₁₅ & E₃₀, combined regression equation: $FL = -122 + 12.3 BM$, $R^2 \text{ adj.} = 0.60$, $df = 13$, $P < 0.001$; TR regression analysis: $FL = -104 + 10.6 BM$, $R^2 \text{ adj.} = 0.90$, $df = 7$, $P < 0.001$. B) TR, E₁₅ & E₃₀, combined regression equation: $FL = 101 + 13.6 T_{CSA}$, $R^2 \text{ adj.} = 0.14$, $df = 22$, $P < 0.05$. C) E₁₅ & E₃₀, combined regression equation: $FL = 5235 - 0.4 F_{CSA}$, $R^2 \text{ adj.} = 0.22$, $df = 13$, $P < 0.05$; TR regression analysis: $FL = 3279 - 18.9 F_{CSA}$, $R^2 \text{ adj.} = -0.13$, $df = 7$, $P > 0.7$.

R² adj., R² coefficient adjusted; df, degrees of freedom.

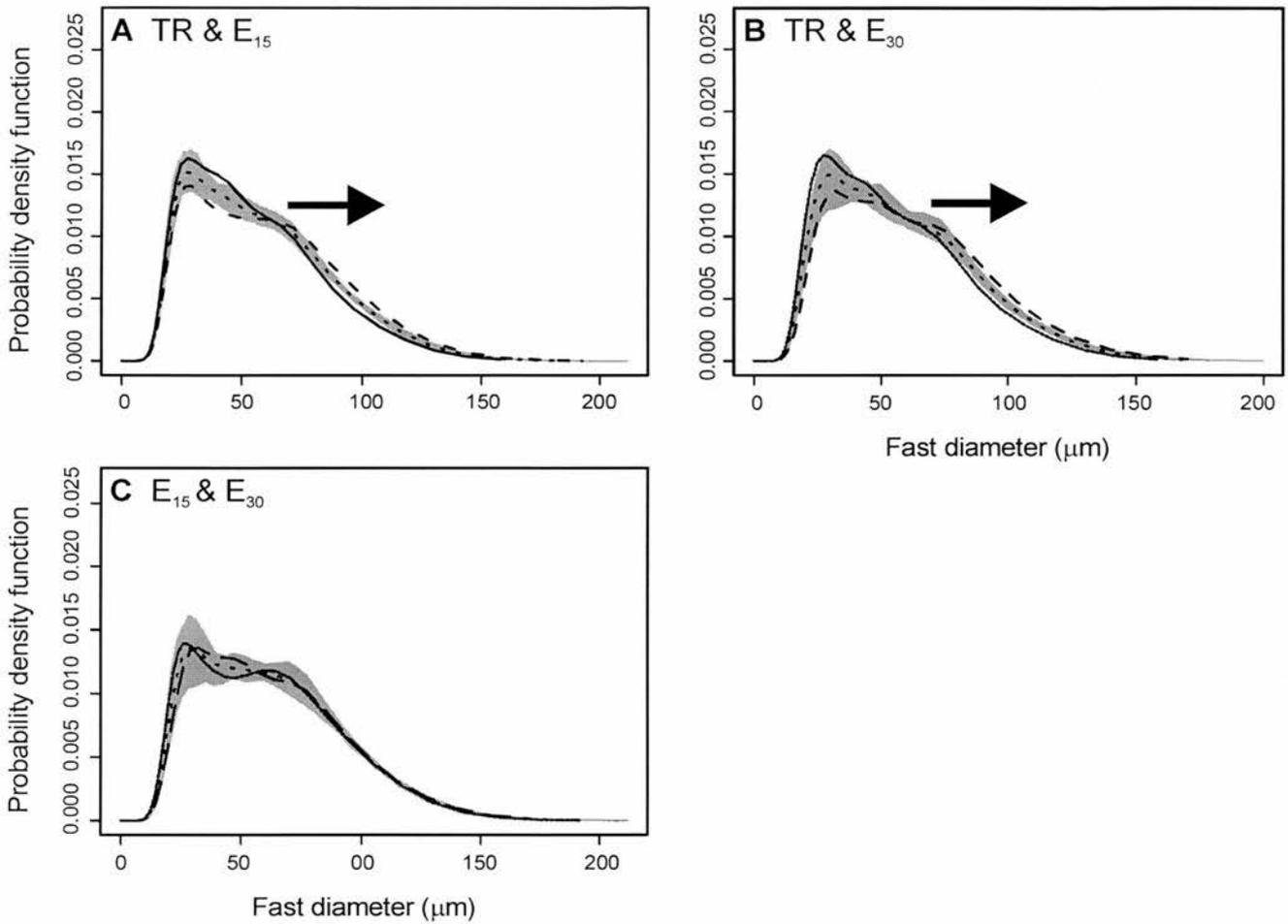


Fig. 5.3. Trout swimming experiment: the effect of tank rest (TR) or exercise training (E_{15} & E_{30}) on the distribution of fast muscle fibre diameters. The mean smooth probability density functions (pdf) of fast fibre diameter distributions for each experimental group are represented by the solid and dashed lines. In each statistical comparison, the shaded area represents 100 bootstrap estimates of the fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. A) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population. This suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR (solid line) and E_{15} (dashed line) experimental groups ($P < 0.05$, Kolmogorov Smirnov). B) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population. This suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR (solid line) and E_{30} (dashed line) experimental groups ($P < 0.05$, Kolmogorov Smirnov). C) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population. This suggested that there was no significant difference in the distribution of fast muscle fibre diameters between the exercised groups (E_{15} , solid line & E_{30} , dashed line) (NS, Kolmogorov Smirnov). The arrows represent the apparent left-to-right shift of the distribution of fast muscle fibre diameters in E_{15} and E_{30} groups relative to TR fish. TR & E_{15} , $N = 9$; E_{30} , $N = 6$. NS, non-significant.

Table 5.2. Trout swimming study: percentiles of mean muscle fibre diameter for tank rested (TR), slow exercised (E₁₅) and fast exercised (E₃₀) groups. Values represent group mean ± SEM, figures in brackets show percentage difference between the groups. TR N = 9, E₁₅ N = 9 and E₃₀ N = 6. ** P < 0.01, *P < 0.05, Mann-Whitney Rank Sum Test.

Experimental Treatment	5 th percentile	10 th percentile	50 th percentile	95 th percentile	99 th percentile
TR	20.2±0.4	23.8±0.5	50.6±0.9	109.2±1.6	133.3±2.3
E ₁₅	21.5±0.6 (6.5)	25.5±0.7 (7.1)	57.7±1.1 (14.1) **	120.5±1.2 (10.4) **	144.9±2.7 (8.7) *
TR	20.6±0.5	24.1±0.4	51.1±0.7	109.7±1.8	134.7±2.5
E ₃₀	23.8±1.0 (15.8) **	28.4±1.5 (17.9) **	59.2±1.8 (15.7) *	121.3±1.1 (10.6) **	146.5±2.7 (8.7) *
E ₁₅	21.5±0.8	25.6±1.0	58.3±1.3	119.7±1.8	143.2±3.7
E ₃₀	23.8±1.0 (10.5)	28.4±1.5 (11.1)	59.2±1.8 (1.6)	120.8±1.9 (0.9)	147.9±2.7 (3.3)

The E₁₅ and E₃₀ groups could not be distinguished on the basis of mean fast fibre area, suggesting there was no direct effect of exercise intensity on fast fibre hypertrophy. Regions of the fast fibre diameter density distributions of the E₁₅ and E₃₀ experimental treatments lay outside the grey shaded area of 100 bootstrap estimates when compared with the TR group (Fig. 5.3A & B). The exercised groups' distributions were shifted to the right relative to the tank rested controls, indicating that a significant increase in fast fibre diameter occurred as a consequence of exercise training ($P < 0.05$, Kolmogorov-Smirnov), whereas again there was no significant difference between the exercised groups (Fig. 5.3C). The 50th, 95th and 99th percentiles of fast fibre diameter were significantly greater in the E₁₅ group compared to the TR group (Table 5.2). The 5th – 99th percentiles of fast fibre diameter were significantly greater in the E₃₀ group compared to the TR group. These data provide strong evidence for exercise-induced fast muscle fibre hypertrophy in the rainbow trout relative to tank rested controls.

5.3.3 The Calcineurin-signaling pathway in relation to exercise treatment

The nuclear localisation of CnA, the catalytic subunit of calcineurin (Figs. 5.4A & B), was significantly higher in the two exercised groups relative to the tank rested controls ($F_{2, 17} = 16.1$, $P < 0.001$, one-way ANOVA). The elevated level of CnA nuclear localisation observed in the E₁₅ (5%) and E₃₀ (7%) groups was significantly correlated with mean fast muscle fibre cross-sectional area ($r_s = 0.76$, $P < 0.001$). No significant variation was observed in CnA nuclear localisation between the two exercised groups. The pattern of nuclear localisation of CnB, the regulatory calcineurin subunit (Figs. 5.4A & B), mirrored that of the catalytic subunit (CnA), where a higher degree of CnB was observed in the nuclear fraction of the exercised rainbow trout ($KW = 7.9$, $DF = 2$, $P < 0.01$, Kruskal-Wallis test).

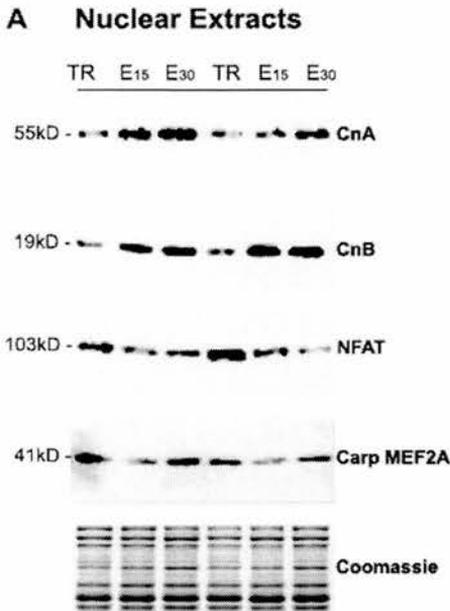
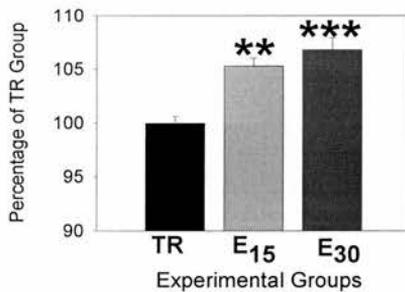
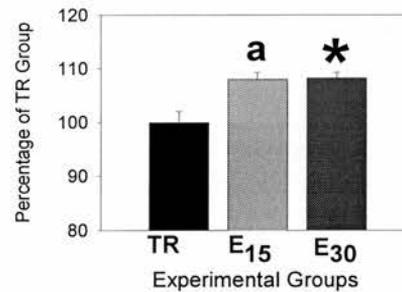


Fig. 5.4A - C. Trout swimming experiment: the effect of tank rest (TR, black fills) or exercise training (E₁₅, grey fills; E₃₀ dark grey fills) on fast muscle tissue nuclear localisation of the calcineurin catalytic (CnA) and regulatory (CnB) subunits. A & B) CnA nuclear localisation was significantly higher in the E₁₅ (5%) and E₃₀ (7%) groups relative to the TR experimental treatment ($P < 0.001$, one-way ANOVA; ** $P < 0.01$, *** $P < 0.001$, Tukey's test). A & C) CnB nuclear localisation was significantly higher in the E₁₅ (8%) and E₃₀ (8%) groups relative to the TR experimental treatment ($P < 0.01$, Kruskal Wallis; * $P < 0.05$, a $P < 0.1$, post-hoc multiple comparisons).

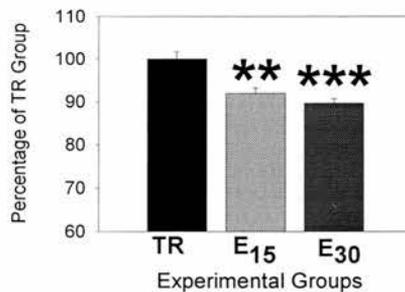
B) Nuclear CnA



C) Nuclear CnB



D) Nuclear NFAT



E) Nuclear MEF2A

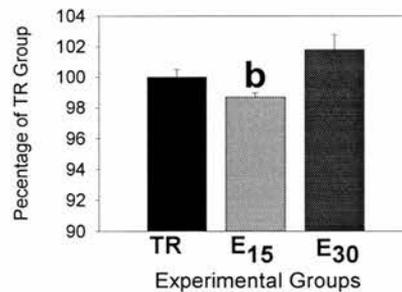
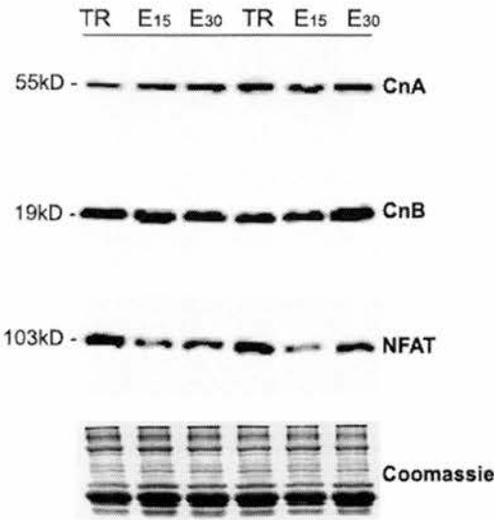
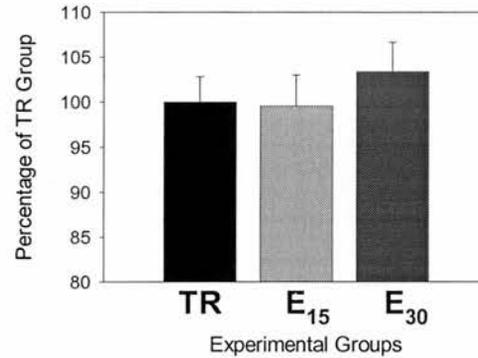


Fig. 5.4A, D & E. Trout swimming experiment: the effect of tank rest (TR, black fills) or exercise training (E₁₅, grey fills; E₃₀ dark grey fills) on fast muscle tissue nuclear localisation of the calcineurin associated transcription factors NFAT and MEF2A. A & D) NFAT nuclear localisation was significantly reduced in the E₁₅ (8%) and E₃₀ (10%) groups relative to the TR experimental treatment ($P < 0.001$, one-way ANOVA; ** $P < 0.01$, *** $P < 0.001$, Tukey's test). A & E) MEF2A nuclear localisation was significantly higher in the E₃₀ group relative to the E₁₅ treatment ($P < 0.05$, one-way ANOVA; b $P < 0.05$, Tukey's test). Fast muscle tissue nuclear protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR group, \pm SEM. TR & E₁₅, N = 9; E₃₀, N = 6.

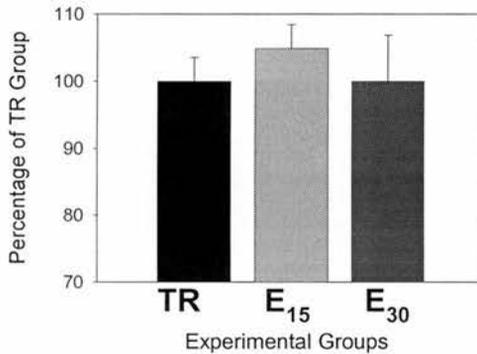
B Cellular Extracts



B) Cellular CnA



C) Cellular CnB



D) Cellular NFAT

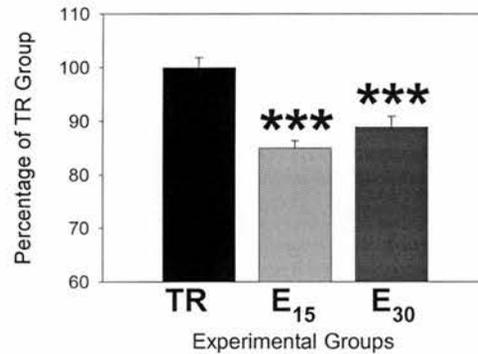


Fig. 5.5. Trout swimming experiment: the effect of tank rest (TR, black fills) or exercise training (E₁₅, grey fills; E₃₀ dark grey fills) on total cellular expression of the calcineurin catalytic (CnA) and regulatory (CnB) subunits, and NFAT2 transcription factor, in fast muscle tissue extracts. A – C) The overall expression of the calcineurin subunits in fast muscle tissue cellular extracts was invariant between the three experimental groups examined (NS, one-way ANOVA). A & D) NFAT2 expression was significantly lower in the E₁₅ (15%) and E₃₀ (11%) groups relative to the TR experimental treatment ($P < 0.001$, one-way ANOVA; *** $P < 0.001$, Tukey's test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, \pm SEM. TR & E₁₅, N = 9; E₃₀, N = 6. NS, non-significant.

Increased CnB nuclear localisation in the E₁₅ (8%) and E₃₀ (8%) groups was significantly correlated with mean fast fibre cross-sectional area ($r_s = 0.78$, $P < 0.001$). There was no significant difference in CnB nuclear localisation as a consequence of different intensities of exercise training. No significant variation of overall expression of both calcineurin subunits was apparent between experimental treatments (Figs. 5.5A – D).

The nuclear localisation of the NFAT2 protein was significantly reduced in both of the exercised groups of rainbow trout relative to the tank rested controls ($F_{2, 17} = 16.9$, $P < 0.001$, one-way ANOVA). The level of NFAT2 nuclear localisation was 8% and 10% higher in the tank rested controls relative to the E₁₅ and E₃₀ groups respectively (Figs. 5.4A & D). The nuclear localisation of NFAT2 protein was significantly inversely correlated with mean fast muscle cross-sectional area ($r_s = -0.52$, $P < 0.05$). No significant variation in NFAT2 nuclear localisation was observed due to the different intensities of exercise training. The lower NFAT2 nuclear localisation in the E₁₅ and E₃₀ groups may have been a consequence of a significantly lower overall expression of this protein in these groups ($F_{2, 17} = 23.7$, $P < 0.001$, one-way ANOVA). The level of NFAT2 protein expression was 15% and 11% lower in the exercised groups relative to the tank rested group (Figs. 5.5A & D). The total expression values of NFAT2 were significantly negatively correlated with mean fast muscle fibre cross-sectional area ($r_s = -0.68$, $P < 0.01$). Overall NFAT2 expression did not vary significantly with different intensities of exercise.

A slight but significant increase in MEF2A nuclear localisation was observed between experimental treatments. The E₃₀ experimental treatment displayed a 3% higher level of MEF2A nuclear localisation relative to the other exercised group (Figs. 5.4A & E).

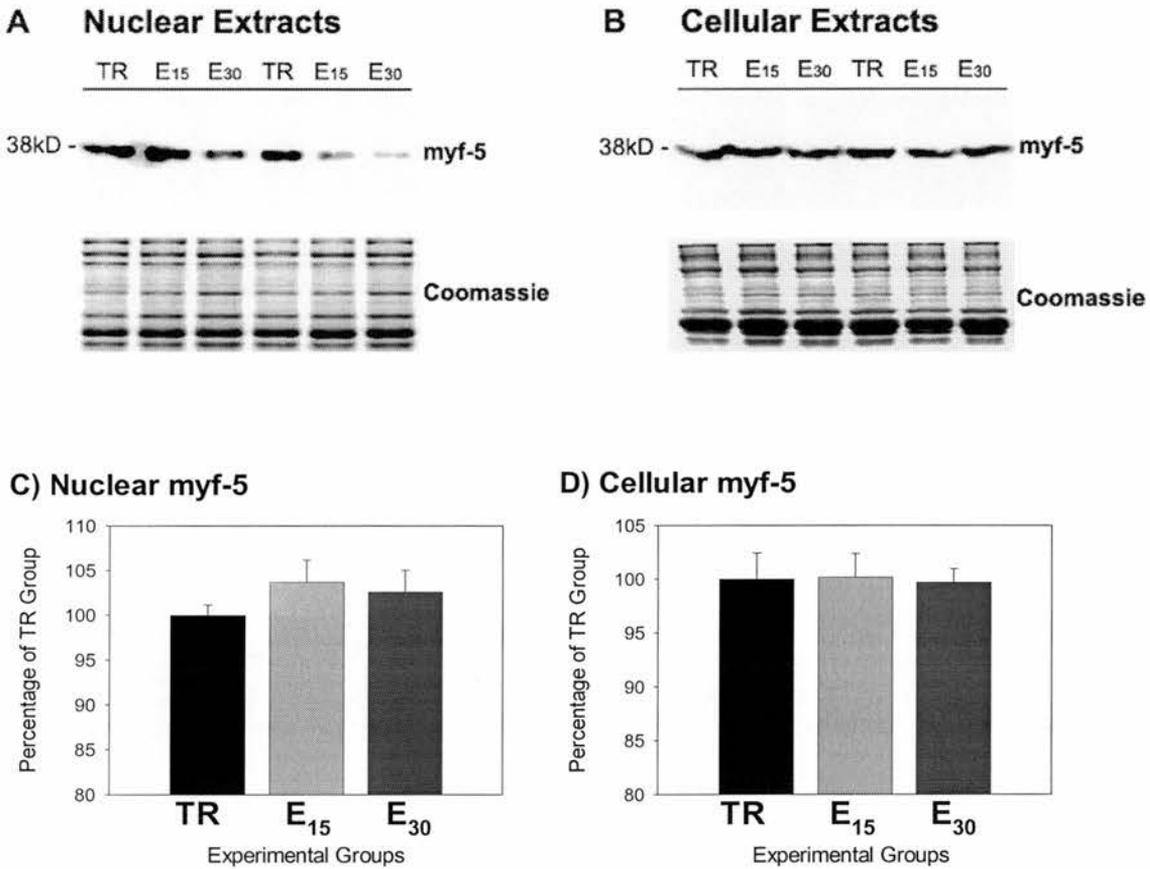


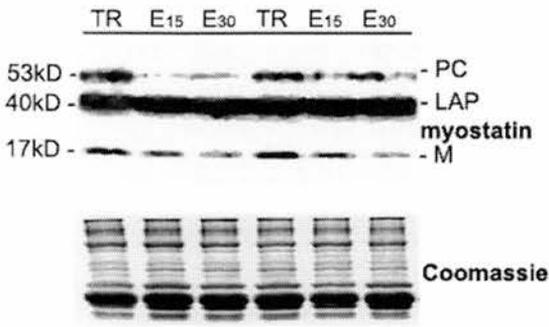
Fig. 5.6. Trout swimming experiment: the effect of tank rest (TR, black fills) or exercise training (E₁₅, grey fills; E₃₀ dark grey fills) on nuclear localisation and total cellular expression of the primary myogenic regulatory factor myf-5. A – D) The nuclear localisation and overall expression of the myf-5 protein in fast muscle tissue extracts was invariant between the three experimental groups examined (NS, one-way ANOVA). Fast muscle tissue subcellular and total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, ± SEM. TR & E₁₅, N = 9; E₃₀, N = 6. NS, non-significant.

Overall expression and nuclear localisation of myf5 protein, a downstream target of calcineurin-signaling, did not vary significantly between experimental treatments (Fig. 5.6).

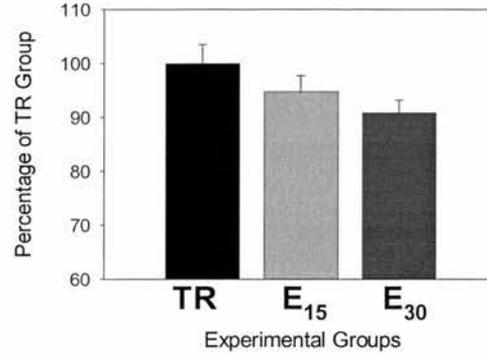
5.3.4 Myostatin expression in relation to exercise treatment

The expression of the 53kD myostatin precursor protein (PC) and the 40kD latency-associated peptide (LAP) did not vary significantly between experimental treatments (Fig. 5.7A – C). Expression of the myostatin precursor protein was downregulated in the two exercised groups by 5% (E₁₅) and 9% (E₃₀) relative to the tank rested controls, however this trend of reduced expression was not statistically significant. In rainbow trout the myostatin mature (active) peptide was detected as a 17kD monomer (Fig. 5.7A, **M**). Expression of the active peptide was significantly downregulated in the two exercised groups relative to the non-exercised controls ($F_{2, 17} = 4.4$, $P < 0.05$, one-way ANOVA). The overall expression of the active peptide was reduced by 6 – 7% in the E₁₅ and E₃₀ groups relative to the tank rested group (Fig. 5.7D). The expression of the myostatin active peptide in rainbow trout fast muscle tissue was inversely correlated with mean fast muscle cross-sectional area ($r_s = -0.50$, $P < 0.05$). No significant difference in expression of the active peptide was observed between the exercised groups.

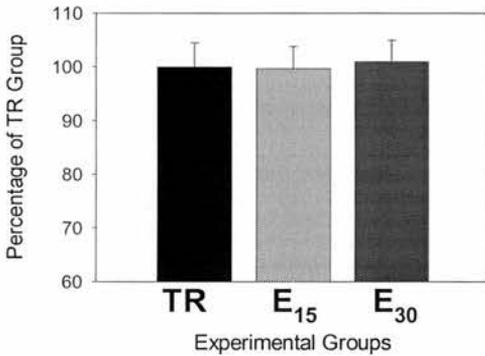
A Cellular Extracts



B) Pre-cursor



C) LAP



D) Mature Peptide

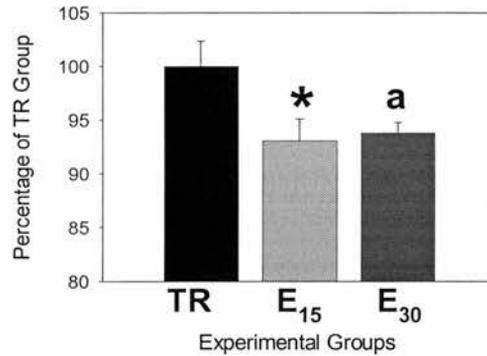


Fig. 5.7. Trout swimming experiment: the effect of tank rest (TR, black fills) or exercise training (E₁₅, grey fills; E₃₀ dark grey fills) on total cellular expression of myostatin in fast muscle tissue extracts. A) Three forms of the myostatin protein were detected in total fast muscle tissue cellular extracts: the pre-cursor protein (PC), latency-associated peptide (LAP) and the mature peptide (M). A & B) Expression of the myostatin PC was lower in the E₁₅ (5%) and E₃₀ (9%) groups relative to the TR experimental treatment, but this down-regulation was not significant (NS, one-way ANOVA). A & C) LAP expression was invariant between the three experimental groups examined (NS, one-way ANOVA). A & D) Expression of the myostatin mature peptide was significantly lower in the E₁₅ (6%) and E₃₀ (7%) groups relative to the TR experimental treatment ($P < 0.05$, one-way ANOVA; * $P < 0.05$, **a** $P < 0.1$, Tukey's test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal gel loading of samples from each experimental treatment. Data are mean group optical density expressed as a percentage of the TR₀ group, \pm SEM. TR & E₁₅, N = 9; E₃₀, N = 6. NS, non-significant.

5.4 Discussion

5.4.1 Muscle fibre hypertrophy

Concurrent with data from previous swimming experiments on teleosts from the Salmonidae family (Davison & Goldspink, 1977; Johnston & Moon, 1980a), endurance exercise training was shown to be a means of stimulating somatic growth and body mass accumulation through muscle fibre hypertrophy in the rainbow trout. In this case, a 30-day period of endurance exercise training for 18 hours a day at 0.8 – 1.6 bls⁻¹ induced a 24 – 30% increase in mean fast fibre cross-sectional area. Although a slightly higher level of muscle fibre hypertrophy was observed at 1.6 bls⁻¹, the increase was not significant. This model of exercise-induced muscle fibre hypertrophy was used to examine potential molecular signaling pathways regulating muscle growth in the rainbow trout. Increased muscle activity has long been known to increase intracellular Ca²⁺ levels for prolonged periods and in higher vertebrates just such a stimulus is known to preferentially activate the Ca²⁺-sensitive protein phosphatase enzyme calcineurin (Dolmetsch *et al.*, 1997).

5.4.2 Calcineurin – a regulator of muscle fibre hypertrophy in teleosts?

The calcineurin signaling pathway is thought to be one of many intracellular pathways in higher vertebrates that regulate skeletal muscle hypertrophy (Musaro *et al.*, 2001). Co-localisation of the calcineurin and NFAT2 proteins has been demonstrated in subsets of myonuclei in mammalian myofibres and this association is thought to synergistically initiate muscle-specific gene expression leading to muscle fibre hypertrophy (Musaro *et al.*, 1999; Semsarian *et al.*, 1999). In a previous experiment involving common carp, a significant correlation between calcineurin nuclear localisation and mean muscle fibre cross-sectional area was observed (see

Chapter 3), however no such relationship was established in a subsequent study (see Chapter 4). In several instances, increased nuclear localisation of the NFAT2 protein was associated with significantly higher levels of calcineurin nuclear localisation. In exercised rainbow trout, a significantly higher level of calcineurin catalytic and regulatory subunits was observed in the nuclear fraction from fast muscle tissue. A significant positive correlation between increased calcineurin nuclear localisation and mean fast muscle fibre cross-section area was also described. These data suggest calcineurin might have a regulatory role in exercise-induced muscle fibre hypertrophy in the rainbow trout.

A significant downregulation of overall NFAT2 protein expression and nuclear localisation was observed in response to exercise in this species. The lower overall expression of this protein is perhaps the underlying cause of a reduced level of NFAT2 detected in the nuclear fraction, but this does not explain the relatively lower levels of NFAT2 nuclear localisation in the experimental groups that displayed the largest increases in fast muscle fibre hypertrophy. The persistence of a non-catalytic association between calcineurin and NFAT is required to maintain NFAT-mediated transcription and the presence of NFAT in the myonuclei (Zhu & McKeon, 1999). Uncoupling of the calcineurin/NFAT complex or rephosphorylation of NFAT by vigorous NFAT kinases such as glycogen synthase kinase-3 (GSK-3) results in nuclear export and cessation of NFAT mediated transcription (Beals *et al.*, 1997). These data suggest that after the initial dephosphorylation and nuclear translocation of the calcineurin/NFAT2 complex, the association between these proteins is disrupted, leading to rephosphorylation and nuclear export of NFAT2 and increased nuclear localisation of the calcineurin protein. Nuclear localisation of the NFAT2 protein is required to mediate transcription of muscle-specific genes, therefore these results

imply that muscle fibre hypertrophy in the rainbow trout is potentially calcineurin dependent, but NFAT2 independent. Interestingly, the downregulation of NFAT2 observed in response to exercise implies that this transcription factor may fulfil an alternative role in teleost muscle.

Calcineurin activity has been shown to regulate Myf5 gene expression and initiate skeletal muscle differentiation by activating MEF2 in higher vertebrates (Friday & Pavlath, 2000; Friday *et al.*, 2003). In rainbow trout, activation of calcineurin through forced exercise activity had no significant effect on myf5 expression or nuclear localisation. Additionally, no change in MEF2A nuclear localisation was discernible in relation to exercise treatment.

Considerable further work is required to move beyond these correlative studies that implicate calcineurin as a key regulator of muscle fibre growth in fish. Pharmacological blocking of calcineurin activity through administration of specific calcineurin inhibitors cyclosporin A or FK506, or knockdown experiments targeting a calcineurin substrate such as NFAT2 are two techniques that would provide causal evidence for control of muscle growth in teleosts by calcineurin. Until that point however, these experiments provide an interesting insight into potential regulatory pathways.

5.4.3 Myostatin – a negative regulator of muscle growth in teleosts?

A significant inverse relationship was found between the expression of the myostatin active peptide in fast muscle tissue and mean fast muscle fibre cross-sectional area in rainbow trout. Expression of the myostatin active peptide was downregulated in the exercised groups of rainbow trout, in which significant fast muscle fibre hypertrophy was observed. The expression of the myostatin precursor protein was also downregulated in these groups, however the reductions observed

were not significant. In mammalian studies, an inverse relationship between muscle mass and the overall circulating levels of myostatin active peptide has been demonstrated in several cases (Gonzalez-Cadavid *et al.*, 1998; Schulte & Yarasheski, 2001; McMahon *et al.*, 2002). In experiments involving hypertrophic or atrophic stimuli, myostatin transcripts were upregulated/downregulated by 110% and 37% respectively (Wehling *et al.*, 2000; Roth *et al.*, 2003). A 37% increase was observed in myostatin protein levels in response to an atrophic stimulus in the same study (Wehling *et al.*, 2000).

In exercised rainbow trout, the downregulation of myostatin active peptide was 6 – 7% in the exercised groups, which displayed a 24 – 30% increase in mean muscle fibre area. Myostatin is a negative regulator of muscle mass through regulation of the proliferation of myogenic progenitor cells (Thomas *et al.*, 2000; Joulia *et al.*, 2003). Although the relatively slight reduction in myostatin active peptide was significant, it does not appear to be of a magnitude sufficient to allow enough myogenic progenitor cell proliferation to realise the degree of muscle fibre hypertrophy observed. There are six breeds of cattle that possess a functional mutation in the myostatin protein, but only three of which display the double-muscling phenotype. These data suggest that dysfunction of one major gene may not entirely account for double muscling in cattle (reviewed by Kocamis & Killefer, 2002). Similarly, a slight downregulation of the myostatin active peptide may not entirely account for the exercise-induced increase in muscle mass observed in rainbow trout. It is more likely that myostatin down-regulation is only a minor part of the entire intracellular signaling repertoire regulating muscle fibre growth in rainbow trout and further work is required to fully describe myostatin function in teleosts.

Chapter 6: General discussion

The effect of exercise on the muscle growth of teleosts and the investigation of potential underlying molecular signaling mechanisms involved in growth regulation were the main foci of this thesis. Specifically, six main objectives were outlined at the outset of this thesis: 1) to develop a model of exercise-induced fast muscle fibre hypertrophy in the common carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum); 2) to develop a model of exercise-induced slow muscle fibre hypertrophy in the common carp; 3) to examine the effect of exercise on muscle fibre phenotype in the common carp; 4) to examine the potential role of the calcineurin signaling pathway in regulating muscle growth in the common carp and rainbow trout; 5) to examine the potential role of myogenic regulatory factors (MRFs) in the regulation of muscle growth in common carp (MyoD, myf-5 and myogenin); and 6) to examine the potential role of myostatin in the regulation of muscle fibre hypertrophy in rainbow trout.

Chapter 2 described the preliminary work and technique development necessary for the identification of suitable antisera that provided a means of identifying proteins involved in putative pathways governing growth of teleost axial musculature. The vast majority of commercially produced antibodies are raised against mammalian species and the detection of teleost antigens with mammalian-specific antisera depends greatly upon the degree of homology between the mammalian antigen and the teleost orthologue in question. The affinity of a particular antibody for the same antigen in another species is often used as an indication of evolutionary relatedness. The alternative approach is to take advantage of the burgeoning volume of available bioinformatics data (ExpASY protein knowledgebase,

fugu and zebrafish genome projects) to determine a suitable short peptide sequence to raise a teleost-specific antiserum against. This is a relatively recent development that will increase in significance during the post-genomics era, which is reflected in the increasing number of companies that now offer custom antibody services. Generation of teleost-specific polyclonal antibodies is still subject to the same constraints inherent to all antibody production however, namely the unpredictable antigenic response of the host animal that gives rise to batches of antisera with dissimilar antigenic affinities. Chapter 2 outlined the suitability of six commercial mammalian-specific and three teleost-specific antisera to detect important muscle-specific antigens in the common carp, rainbow trout and several other species of teleost fish. The antibody screening data may in future prove to be a valuable resource for researchers wishing to study muscle-specific gene expression in teleosts.

Chapter 2 also described the development of histochemical and immunohistochemical staining techniques to distinguish different muscle fibre phenotypes in common carp and rainbow trout. The Mayer's haematoxylin histochemical staining protocol was used to visualise muscle fibre cellularity in both teleost species studied. In common carp and rainbow trout, slow muscle fibres were identified as those that positively cross-reacted with the S58 antibody. The mATPase protocol identified two intermediate phenotypes of muscle fibre in the common carp, in addition to the fast and slow musculature. Using these protocols, the effect of exercise on muscle fibre phenotype and growth of different muscle fibre types was examined.

In chapters 3 and 4, the main objective was to analyse the effect of exercise intensity and duration on muscle fibre cellularity in the common carp. The experimental design was based on swimming experiments conducted on teleosts over

twenty years ago. In salmonid and cyprinid species such as brook trout (*Salvelinus fontinalis* Mitchell) and goldfish (*Carassius aurata* L.), hypertrophy of slow, intermediate and fast fibre types was induced by continuous swimming at speeds between 1.5 and 4.5 bls⁻¹ for four weeks (Johnston & Moon, 1980a; Davison & Goldspink, 1978). More recently, long-term continuous swimming studies on salmonid (*Salmo salar* L.) and cyprinid species (*Chalcalburnus chalcoides mento* Gldenstdt) have also demonstrated considerable exercise-induced hypertrophy of the fast/white musculature (Totland, *et al.*, 1987; Hinterleitner *et al.*, 1992). From a mammalian perspective, it seems curious that endurance exercise activity is capable of stimulating muscle fibre hypertrophy (in teleosts). In humans, endurance exercise training results in switches in muscle fibre phenotype and increases the proportion of fibres with slow-twitch characteristics (Jansson *et al.*, 1978). Conversely, strength or heavy resistance training increases the number of fast-twitch fibres, but also augments muscle power output through muscle fibre hypertrophy (Jansson *et al.*, 1990). In comparison, rainbow trout subjected to the teleost equivalent of heavy resistance training (sprint-training) demonstrated reduced muscle growth in relation to non-trained controls (Gamperl, *et al.*, 1988).

Over the course of five experiments, exercise was found to have different effects on the fast and slow musculature in common carp. In contrast to salmonids and other cyprinid species, adaptation to moderate intensity exercise in the common carp involved significant recruitment of fast muscle fibres that balanced the atrophy of larger fibres (swimming experiment 3). Slow to moderate intensity exercise stimulated hypertrophy of the slow fibres and caused initial atrophy of the fast musculature that was partially recovered from 16 days after the initiation of the stimulus (swimming experiment 4 and time course experiment). Although similar

swimming speeds were used in the time course experiment and swimming experiments 3 and 4 ($2.6 - 3.0 \text{ bls}^{-1}$), it is likely that the experimental animals experienced different swimming intensities due to the different experimental apparatus used. The enforced change in experimental apparatus hindered the development of a definitive model of muscle fibre hypertrophy in the common carp, however it was still possible to draw several conclusions: moderate exercise did not stimulate fast muscle fibre growth through hypertrophy in the common carp; evidence for exercise-induced fast muscle fibre recruitment was provided by one of the experiments (swimming experiment 3) but this result was not repeated in subsequent studies using different experimental apparatus; and slow to moderate endurance exercise stimulated increased growth of the slow musculature through hypertrophy after 16 and 28 days of exercise. Exercise also inhibited somatic growth in the common carp, which is in accordance with a previous finding that condition factor was significantly reduced in another cyprinid species (goldfish) in response to a similar training regime (Davison & Goldspink, 1978). The growth retardation observed in exercised common carp is most likely a consequence of energy expenditure that exceeds food intake and sequestration of resources for exercise activity at the expense of somatic growth. Protein is mobilised from fast muscle tissue to make up the energy deficit. Members of the carp family tend to inhabit still or slow moving water and are reputed to be difficult to train as a consequence (Sänger, 1992). It could be argued that a model of fast muscle fibre hypertrophy would be an unlikely outcome using a sedentary cyprinid species such as the common carp as the subject of an exercise experiment. However, several previous studies using reputedly sluggish cyprinid species resulted in marked hypertrophy of the fast musculature and suggested a similar response to exercise would be observed in the

common carp. In conclusion, a model of muscle fibre hypertrophy was successfully developed in the slow-twitch/red muscle fibres, but not in the fast-twitch/white fibres in common carp.

In a situation parallel to exercise-induced atrophy, the preferential wasting of fast/white muscle and preservation of slow/red muscle is also observed in the starvation response of teleosts (Greer Walker, 1971; Johnston, 1981). Slow muscle is required to sustain swimming activity, while fast is required to change position in the flume and in behavioural interactions with other fish. In common carp starved for a 15-month period, the fast/white muscle was entirely depleted and disrupted yet the ability to swim was still retained, inferring that slow muscle was spared for this purpose (reviewed by Love, 1980). Starvation has been shown to affect the metabolic properties of fast muscle to a greater extent than slow muscle and as a consequence swimming capacity was diminished (Martinez *et al.*, 2003). Atlantic salmon (*Salmo salar* L.) demonstrated an increase in muscle fibre recruitment but not fibre size through manipulation of photoperiod and freshwater temperature regime, suggesting recruitment and hypertrophy are regulated by independent genetic mechanisms (Johnston *et al.*, 2003b; Johnston *et al.*, 2003c). Similarly, in common carp subject to moderate intensity exercise, fibre recruitment was stimulated concurrent with a decrease in the diameter of mature fibres. In contrast to cyprinid species, salmonids are renowned for their inherent swimming capacity. Chapter 5 describes a comparative exercise experiment on rainbow trout that demonstrated significant somatic growth, and hypertrophy of the fast musculature in response to two intensities (slow, 0.8 bls^{-1} and moderate, 1.6 bls^{-1}) of exercise training. A model of fast muscle fibre hypertrophy was successfully developed in the rainbow trout.

From an aquaculture perspective, the results from the common carp swimming experiments are not encouraging. Exercise appears to inhibit fast/white muscle fibre growth and stimulate hypertrophic growth of the slow/red musculature. In contrast, the increased somatic growth and fast muscle fibre hypertrophy observed in trained rainbow trout suggest exercise could be used to promote growth of this species in an aquaculture situation. In teleosts, the fast-twitch/white fibre type comprises the majority of the myotomal cross-sectional area (~ 90%), whereas the slow-twitch red fibre fraction seldom exceeds 10%. Therefore the fast/white musculature comprises the main edible portion of a fish and it is the growth of this tissue that aquaculturalists would seek to maximise (Johnston, 1999). Numerous extrinsic (environmental) and intrinsic (genetic) factors have been exploited to increase muscle growth and flesh yield from farmed teleost species, yet studies involving exercise as an extrinsic stimulus for muscle growth are few in number. Two industrial scale studies have provided conflicting data regarding the use of exercise to promote somatic and muscle growth in a fish-farming situation. The potential overheads involved in training thousands of fish on an industrial scale most likely preclude the use of exercise in aquaculture. More commercially viable techniques of stimulating growth in farmed fish include the use of continuous lighting to delay the onset of sexual maturation, the manipulation of developmental temperature or altering the nutrient content of feed.

Chapter 3 also described the effects of exercise on muscle fibre nuclear content, a parameter that has been shown to increase with strength training in humans (Kadi & Thornell, 2000). In two previous studies, stimuli (temperature and light) that induce muscle fibre recruitment have also been shown to result in an increased myofibre myonuclei content (Johnston *et al.*, 2003b; Johnston *et al.*, 2003c). The results from this study indicate that a hyperplastic stimulus induced a significantly

increased concentration of muscle fibre myonuclei in common carp. This suggests that the fate of cells involved in recruitment and hypertrophy is decided late in the response to local signaling. This model was first proposed by Johnston *et al.* (2003c), however the nature of the local signaling was not defined. A simple model would involve a single population of myogenic progenitor cells that proliferate to provide the cells to meet the demands of growth and repair in mature muscle in teleosts (Johnston *et al.*, 2003c).

The ability to switch muscle fibre phenotype in response to changes in environmental temperature has previously been demonstrated in two cyprinid species. In chapter 4, the effect of exercise on muscle fibre phenotype in the common carp was analysed, but due to several limitations in the experimental design it was impossible to draw any reliable conclusions. In this experiment the proportion of different muscle fibre phenotypes was examined within a defined region of the myotome. However, the proportions of each fibre type within this region were affected by changes in muscle fibre area caused by the exercise treatment, therefore it was not possible to examine putative fibre type conversions in response to exercise activity. An improved experimental method to analyse the effect of exercise on muscle fibre phenotype in fish was outlined in the conclusion to this chapter. Myogenic regulatory factors (MRFs) have been described as “master regulators” of muscle growth and development. Sequential expression of MRFs is known to regulate muscle growth through the activation, proliferation and differentiation of myogenic progenitor cells (Zammit & Beauchamp, 2001). In mature mammalian muscle, upregulation of MRFs transcripts and protein is a well-characterised response to diverse stimuli, including forced exercise, surgically induced compensatory overload, stretch overload, electromyostimulation and denervation. The expression and nuclear localisation of

MRFs was extensively examined in common carp in response to exercise. The overall expression of MRFs MyoD, myf5 and myogenin was invariant between experimental treatments and different muscle fibre phenotypes in all experiments. However increased nuclear localisation of the primary MRFs, particularly MyoD, was significantly correlated with larger muscle fibre cross-sectional area in common carp. These data imply a role for MyoD in regulating the myogenic precursor cell population during normal muscle growth in the common carp. Increased numbers of myogenic progenitor cells positive for MyoD were observed in the response to feeding in a sub-Antarctic notothenioid species (*Harpagifer bispinis* Forster) suggesting that as in higher vertebrates, MRFs fulfil a key function in the myogenic response to numerous stimuli (Brodeur *et al.*, 2003b).

The role of the calcineurin-signaling pathway in skeletal muscle hypertrophy is a controversial one. *In vivo* and *in vitro* studies have been performed and subsequently repeated which validate or refute the involvement of this protein phosphatase enzyme in mammalian muscle growth (Dunn *et al.* 1999; Musaro *et al.* 1999; Semsarian *et al.*, 1999; Bodine *et al.* 2001; Rommel *et al.* 2001). Nevertheless, calcineurin has been shown to play a pivotal role in skeletal muscle differentiation, regulation of Myf5 gene expression and regulating nerve and activity dependent specification of muscle fibre phenotype. The potential regulation of teleost muscle growth through the calcineurin-signaling pathway was examined in common carp and rainbow trout. In some respects the contradictory teleost data reflects the current impasse in mammalian calcineurin research. In two separate experiments involving different species (common carp and rainbow trout), increased nuclear localisation of the calcineurin protein was significantly correlated with hypertrophic muscle growth (chapters 3 and 5). In common carp the hypertrophy of fast and slow fibres occurred

under tank rested conditions, whereas the rainbow trout exhibited exercise-induced fast fibre hypertrophy. Conversely, a subsequent experiment on common carp revealed significant muscle growth through hypertrophy was not associated with an elevated level of calcineurin nuclear localisation (chapter 4). Moreover, the nuclear localisation of NFAT2, a downstream target of activated calcineurin, did not assume a pattern that suggested NFAT2-mediated transcriptional activity was concurrent with muscle growth in the common carp or rainbow trout. Therefore the involvement of the calcineurin-signaling pathway in the regulation of hypertrophic muscle fibre growth in the common carp and rainbow trout is uncertain. Substantial further work would be required to determine such a role for calcineurin in teleosts. Several different approaches would need to be taken to delineate the role of calcineurin in common carp and rainbow trout. Significant growth of fast muscle fibres was observed in common carp in the tank rested situation and also in trained rainbow trout. To test the hypothesis that calcineurin regulates muscle fibre hypertrophy in teleosts, tank rested common carp and exercised rainbow trout could be treated with the calcineurin inhibitor cyclosporin A (CsA) or just the drug vehicle alone (Cremophor EL; Dunn *et al.*, 1999). Inhibition of fast muscle fibre hypertrophy in the CsA treatment groups relative to the control (drug vehicle) groups would provide strong evidence of calcineurin regulation of muscle growth in these two species of teleost fish. A further approach would investigate the potential role of calcineurin signaling in fibre type specification in another cyprinid species, the goldfish. It has previously been shown that an increase in slow-twitch/red muscle fibre fractional area occurs as a consequence of cold acclimation (Johnston & Lucking, 1978). Inhibition of the cold acclimation response (increased slow muscle fraction) through treatment with CsA, relative to drug vehicle treated cohorts, would provide strong evidence for

the role of calcineurin in fibre-type specification in goldfish. The situation regarding calcineurin-signaling is as yet unresolved in higher vertebrates. A more complex picture of intracellular signaling-pathways regulating hypertrophic muscle growth undoubtedly exists, and alternative signaling pathways such as the calmodulin kinase and phosphatidylinositol 3-kinase have been proposed to interact with the calcineurin-signaling pathway to this end (Musaro *et al.*, 2001). However in conclusion, these data do not rule out a role for calcineurin in fish muscle growth and development, but merit further investigation considering the recent finding that calcineurin is required for the formation of the indirect flight muscles of an invertebrate species (Gajewski *et al.*, 2003).

Under normal circumstances, the N-terminal myostatin prodomain has been shown to bind to the cleaved C-terminal mature form of the myostatin protein, the active peptide. In this way, the prodomain is able to regulate the activity of the active myostatin protein (Hill *et al.*, 2002). Overexpression of the myostatin prodomain (precursor) in a transgenic line of zebrafish (*Danio rerio* Hamilton) has recently been shown to increase myofibre number, demonstrating that myostatin inhibits muscle fibre recruitment in zebrafish (Xu *et al.*, 2003). This result is reminiscent of the generalised muscle hyperplasia phenotype observed in double-muscled breeds of cattle (reviewed by Kocamis & Killefer, 2002). Myostatin expression was examined in rainbow trout and a slight but significant downregulation of the active peptide was observed in the groups exhibiting exercise-induced hypertrophy of fast muscle fibres (chapter 5). Myostatin expression was not evaluated in the common carp experiments due to an unreliable cross-reaction of the Atlantic salmon-specific antibody with common carp total cellular protein extracts. Although the difference was statistically significant, was the level of downregulation “biologically relevant?” It is unlikely

that the slight downregulation of a single gene is sufficient to cause the level of hypertrophy observed and the possibility that other factors interact with myostatin should not be ignored. Recently, the myostatin propeptide and the Follistatin-related gene have been shown to be inhibitory binding proteins of myostatin (Hill *et al.*, 2002). Overexpression of Follistatin in the skeletal muscle of transgenic mice resulted in a more severe phenotype than the myostatin mouse knockout. The impressive levels of hyperplasia and hypertrophy observed suggested that Follistatin might have a function other than inhibition of myostatin in skeletal muscle (Lee & McPherron, 2001). These data suggest that myostatin expression is inversely proportional to muscle fibre hypertrophy in the rainbow trout, implying that myostatin is a negative regulator of muscle growth in this species. However, further exercise experiments need to be conducted to confirm this result, as the level of down-regulation observed was relatively small.

The identification of potential regulatory pathways underlying muscle growth in common carp and rainbow trout has been limited in this case by single gene expression analysis. To investigate the potential involvement of a molecular signaling pathway such as calcineurin in the regulation of teleost muscle growth, the selection of a model species such as zebrafish would be advantageous, as the whole genome has been sequenced. The availability of zebrafish cDNA microarrays or oligonucleotide arrays would allow the analysis of global gene expression and the identification of clusters of genes whose expression alters in parallel. Recently, juvenile zebrafish have proved to be amenable to endurance training and have shown increased capillarisation and mitochondrial density in response to continuous exercise (Pelster *et al.*, 2003). In such a situation, zebrafish DNA gene chips would provide a fascinating insight into the molecular signaling pathways regulating the adaptive response to

exercise. One problem of using the zebrafish to study potential molecular signaling pathways regulating muscle growth in teleosts is that this species has a small ultimate body size and ceases to recruit muscle fibres at a relatively early life stage. Although this model species can provide valuable insights into potential underlying molecular signaling pathways, it is arguable whether these findings will be applicable to larger teleost species that are of commercial importance to the aquaculture industry. Several genome projects involving commercially relevant aquaculture species such as salmon and tilapia have been started recently to overcome this problem. The Salmon Genome Project (SGP, www.salmongenome.no) is a collaboration between 7 Norwegian research groups that has so far submitted 27,559 ESTs (expressed sequence tags) to GenBank (May 1, 2004). The objective of the SGP is to increase knowledge of the salmon genome through the generation of genomic data such as genetic and physical maps and interpretation of this data using bioinformatics. The ultimate goal of the SGP is to identify genes that will allow for the improvement of strains through selective breeding, by selecting for commercially important traits such as growth rate and flesh quality.

A final approach that allows the direct assignment of function to information mined from the zebrafish genome is the use of morpholino-modified oligonucleotides (morpholinos) (reviewed by Corey & Abrams, 2001). Microinjection of morpholino oligonucleotides into sphere-stage zebrafish embryos allows effective *in vivo* targeted translational inhibition, to 'knockdown' a single or multiple genes (Nasevicius & Ekker, 2000; Ekker & Larson, 2001). For example, a morpholino targeting the zebrafish orthologue of myostatin, an inhibitor of skeletal muscle growth in mammals, could provide an insight into muscle fibre recruitment during ontogeny of the zebrafish and test the hypothesis of Xu and colleagues (2003). Designing

morpholinos against the calcineurin substrate transcription factor NFAT2 would provide a means of disrupting calcineurin signaling in developing zebrafish embryos. The resulting phenotype of the developing zebrafish would perhaps give an indication of the true function of calcineurin signaling in muscle growth. If the zebrafish embryo developed relatively normally, but lacked the usual complement of superficial slow fibres, this would suggest that calcineurin specifies muscle fibre phenotype, but does not regulate muscle fibre hypertrophy (Bodine *et al.*, 2001; Rommel *et al.*, 2001). If the resulting zebrafish phenotype demonstrated abnormalities in the growth of the axial musculature and no obvious fibre outgrowth, this would provide support for the hypothesis that calcineurin is a regulator of muscle fibre hypertrophy in vertebrates (Dunn *et al.*, 1999); Musaro *et al.*, 1999; Semsarian *et al.*, 1999). Knockdown experiments in zebrafish are routinely conducted using morpholinos directed against members of the MyoD family. Thus far, redundancy of function in this family of transcription factors has been demonstrated in zebrafish, similar to that found in the equivalent mice knockout mutants (Yaniv Hinitz, pers. comm.). Therefore morphant technology represents a powerful tool for functional genomics applications. Careful use of such technology, in conjunction with increased knowledge of genetic and physical maps of teleost species provided by the various genome projects, will lead to the delineation of molecular signaling pathways underlying teleost muscle growth in the relatively near future.

Appendix I

Summary of protein extraction buffers and protease inhibitors

1) Total Cellular Protein Extraction Buffers

RIPA Buffer

50mM Tris-Cl (pH 7.5)
150mM NaCl
1% Nonidet P-40 (NP-40)
0.5% Sodium deoxycholate
0.1% SDS

Protease Inhibitors

a) PMSF: 10mM stock solution of Phenylmethylsulfonylfluoride (PMSF)
10 μ l of PMSF stock in 1ml of RIPA
0.1mM working concentration of PMSF

b) DTT: 100mM stock solution of Dithiothreitol (DTT)
10 μ l of DTT stock in 1ml of RIPA
1mM working concentration of DTT

Appendix I (continued)

2) Nuclear Protein Extraction Buffers

Buffer 1

10mM HEPES (pH 7.5)
10mM MgCl₂
5mM KCl
0.1mM EDTA
0.1% Triton X-100
Final pH adjusted to 8.0

Buffer 2

20mM HEPES (pH 7.9)
25% glycerol
500mM NaCl
1.5mM MgCl₂
0.2mM EDTA
Final pH adjusted to 8.0

Buffer 3

20mM HEPES (pH 7.9)
40mM KCl
2mM MgCl₂
10% glycerol

Protease Inhibitors

a) PMSF: 10mM stock solution of Phenylmethylsulfonylfluoride (PMSF)
10μl of PMSF stock in 1ml of Buffer 1 (0.1mM working concentration)
20μl of PMSF stock in 1ml of Buffer 2 (0.2mM working concentration)
20μl of PMSF stock in 1ml of Binding buffer (0.2mM working concentration)

b) DTT: 100mM stock solution of Dithiothreitol (DTT)
10μl of DTT stock in 1ml of Buffer 1 (1mM working concentration)
5μl of DTT stock in 1ml of Buffer 2 (0.5mM working concentration)
5μl of DTT stock in 1ml of Binding buffer (0.5mM working concentration)

c) Leupeptin: 4mg/ml stock solution stable at -20°C for 6 months
Stock solution diluted after defrosting to make 1mg/ml working solution
2μl of Leupeptin working solution in 1ml of Buffer 1, Buffer 2 or Binding buffer (2μg/ml working concentration)

d) Aprotinin: 10mg/ml stock solution stable at 2-8°C for 1 year
Stock solution diluted after defrosting to make 1mg/ml working solution
2μl of Leupeptin working solution in 1ml of Buffer 1, Buffer 2 or Binding buffer (2μg/ml working concentration)

Appendix II

Summary of Modified Lowry Technique Solutions

Copper Reagent

630mM Sodium carbonate
5mM Cupric sulphate (5×hydrated)
2mM Sodium potassium tartrate

2×Lowry Concentrate

60% copper reagent
20% SDS (1%)
20% 1M NaOH
A fresh batch was prepared before each assay

0.2N Folin Ciocalteu's Reagent

10ml 2N Folin Ciocalteu's reagent made up to 100ml with ddH₂O
Stable for several months stored in an amber bottle at room temperature

Appendix III

Summary of solutions used to prepare resolving and stacking gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

1) Acrylamide solution (Bio-Rad, 161-0156)

30% Acrylamide/Bis Solution (29:1)

2) Resolving Gel Buffer

1.5M Tris-Cl, pH 8.8

36.3g Trizma base, diluted with 150ml ddH₂O, pH adjusted to 8.8, made up to 200ml

Stored at 4°C for up to 3 months

3) Stacking Gel Buffer

0.5M Tris-Cl, pH 6.8

3.0g Trizma base, diluted with 40ml ddH₂O, pH adjusted to 6.8, made up to 50ml

Stored at 4°C for up to 3 months

4) 10% SDS

10g SDS made up to 100ml with ddH₂O

5) 10% Ammonium persulphate

100mg made up to 1ml with ddH₂O, prepared just prior to use

6) 2×treatment (Laemmli) buffer

5ml stacking gel buffer

8ml 10% SDS

4ml glycerol (20% v/v)

4mg Bromophenol blue

0.62g Dithiotreitol (0.2M)

Made up to 20ml with ddH₂O, frozen in 1ml aliquots and defrosted just prior to use

7) 10×Tank Buffer

0.25M Tris, 1.92M glycine, 1% SDS, pH8.3

30.3g Trizma base

144.1g Glycine

10g SDS

Made up to 1L with ddH₂O, stored at room temperature for up to 1 month

100ml diluted to working concentration of 25mM Tris/ 192mM glycine with ddH₂O

8) TEMED (SIGMA, T8133)

9) n-Butanol (BDH, Prod 275075R)

Appendix IV

Quantities of solutions used to prepare the resolving and stacking portions of 2 mini-gels using the Bio-Rad Mini-Protean[®] III apparatus

1) 5% Stacking Gel	3ml
H ₂ O	2.1
30% Acrylamide/ Bis	0.5
0.5M Tris-Cl (pH 6.8)	0.38
10% SDS	0.03
10% Ammonium persulphate	0.03
TEMED	0.003

2) 6% Resolving Gel (100-200kD proteins)	10ml
H ₂ O	5.3
30% Acrylamide/ Bis	2.0
1.5M Tris-Cl (pH 8.8)	2.5
10% SDS	0.1
10% Ammonium persulphate	0.1
TEMED	0.008

3) 12% Resolving Gel (30-200kD proteins)	10ml
H ₂ O	3.3
30% Acrylamide/ Bis	4.0
1.5M Tris-Cl (pH 8.8)	2.5
10% SDS	0.1
10% Ammonium persulphate	0.1
TEMED	0.004

4) 15% Resolving Gel (10-50kD proteins)	10ml
H ₂ O	2.3
30% Acrylamide/ Bis	5.0
1.5M Tris-Cl (pH 8.8)	2.5
10% SDS	0.1
10% Ammonium persulphate	0.1
TEMED	0.004

Appendix V

Relevant primary accession numbers from the Expert Protein Analysis Database (ExPASy, <http://org.expasy.us>)

Mouse MyoD	P10085
Rat MyoD	Q02346
Common carp MyoD	O93492
Rainbow trout MyoD1	Q91205
Rainbow trout MyoD2	Q91206
Rat myogenin	P20428
Common carp myogenin	O93491
Rainbow trout myogenin	Q91207
Human myf5	P13349
Mouse myf5	P24699
Common carp myf5	O93493
Pufferfish myf5	Q90ZK9
Zebrafish myf5	Q9DDW0
Atlantic salmon myostatin	Q90W17
Rainbow trout myostatin1	Q90ZC1
Rainbow trout myostatin2	Q90ZD1
Brook trout myostatin	Q98UB3
White Perch myostatin	Q90WC8
Striped Seabass myostatin	Q90WC9
White Bass myostatin	Q98TB3
Tilapia myostatin	Q98TB4
Seabream myostatin	Q90W05
Shi Drum myostatin	Q90W06
Channel catfish myostatin	Q90YY0
Zebrafish myostatin	O42222
Mouse PCNA	P17918
Rat PCNA	P04961
Zebrafish PCNA	Q9PTP1
Japanese eel PCNA	Q9W644
<i>Drosophila melanogaster</i> PCNA	P17917
<i>Saccharomyces cerevisiae</i> PCNA	P15873

Appendix V (continued)

Further relevant primary accession numbers from the Expert Protein Analysis Database (ExPASy, <http://org.expasy.us>)

Human CnA α	Q08209
Mouse CnA α	P20652
Bovine CnA α	P48452
Rabbit CnA α	Q8HZN0
Frog (<i>Xenopus laevis</i>) CnA α	O57438
Scallop (<i>Patinopecten yessoensis</i>) CnA α	Q9NKW8
<i>Drosophila melanogaster</i> CnA1	P48456
<i>Drosophila melanogaster</i> CnA2	Q27889
Blood fluke (<i>Schistosoma mansoni</i>) CnA	Q9NFN2
<i>Neurospora crassa</i> CnA	Q05681
<i>Aspergillus nidulans</i> CnA	P48457
Baker's yeast (<i>Saccharomyces cerevisiae</i>) CnA1	P23287
Baker's yeast (<i>Saccharomyces cerevisiae</i>) CnA2	P14747
Fission yeast (<i>Schizosaccharomyces pombe</i>) CnA	Q12705
<i>Cryptococcus neoformans</i> CnA1	O42773
Human CnB α	P06705
Mouse CnB α	Q63810
Scallop (<i>Patinopecten yessoensis</i>) CnB α	Q9NKW7
Blood fluke (<i>Schistosoma mansoni</i>) CnB α	Q9NFN1
<i>Drosophila melanogaster</i> CnB1	P48451
<i>Drosophila melanogaster</i> CnB2	Q24214
Nematode (<i>Caenorhabditis elegans</i>)	Q20804
<i>Neurospora crassa</i> CnB	P87072
<i>Naegleria gruberi</i> CnB	P42322
Baker's yeast (<i>Saccharomyces cerevisiae</i>) CnB	P25296
Fission yeast (<i>Schizosaccharomyces pombe</i>) CnB	Q94498
Human NFATc1	O95644
Mouse NFATc1	O88942
Pig NFATc1	O77638
Mouse GATA2	O09100
Rat GATA2	Q924Y4
Human MEF2A	Q02078
Mouse MEF2A	Q60929
Common carp MEF2A	O93494
Zebrafish MEF2A	Q98868

Appendix VI

Summary of western blotting data for swimming experiments 1-4

Table 1: Swimming experiment 2, data summary for MyoD levels in nuclear protein extracts (N) from **fast** muscle tissue; tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, N = 6.

Condition	Mean Density (INT)	Optical Density percentage of TR group	as TR	Percentage difference	Statistical significance
TR	288 \pm 8	100 \pm 2.33			
E	293 \pm 10	101.6 \pm 2.90		1.6%	NS

Table 2: Swimming experiment 3, summary of data for expression of MRFs in nuclear (N) and total cellular (T) protein extracts from **fast** muscle tissue; tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, N = 9.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density percentage of TR group	as TR	Percentage difference	Statistical significance
MyoD (N)	TR	964 \pm 44	100 \pm 4.57		18.9	P<0.05
	E	782 \pm 53	81.1 \pm 6.73			
MyoD (T)	TR	355 \pm 13	100 \pm 3.64		0.4	NS
	E	354 \pm 9	99.6 \pm 2.60			
myf-5 (N)	TR	892 \pm 15	100 \pm 1.73		9.0	P<0.01
	E	818 \pm 14	91.7 \pm 1.66			
myf-5 (T)	TR	542 \pm 17	100 \pm 3.15		1.7	NS
	E	533 \pm 15	98.3 \pm 2.74			
myogenin (T)	TR	317 \pm 4	100 \pm 1.13		1.1	NS
	E	313 \pm 5	98.9 \pm 4.74			

Table 3: Swimming experiment 3, summary of comparison of MRFs distribution in total cellular extracts from slow (S) and fast (F) muscle tissue, dissected from tank rested controls. Values represent group mean \pm SEM, N = 9.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density percentage of TR group	as TR	Percentage difference	Statistical significance
MyoD	S	888 \pm 6	100 \pm 0.7		2.1	NS
	F	870 \pm 6	97.9 \pm 0.7			
myf-5	S	670 \pm 12	100 \pm 1.75		0.4	NS
	F	672 \pm 14	100.4 \pm 2.13			
Myogenin	S	808 \pm 9	100 \pm 1.1		1.2	NS
	F	799 \pm 8	98.8 \pm 1.0			

Appendix VI (continued)

Summary of western blotting data for swimming experiments 1-4

Table 4: Swimming experiment 2, data summary for nuclear localisation of calcineurin B, NFAT and MEF2A in **fast** muscle tissue nuclear extracts; tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, N = 6.

Antigen	Condition	Mean Optical Density (INT)	Density percentage of TR group	Percentage difference	Statistical significance
Calcineurin B	TR	561 \pm 17	100 \pm 2.53	4.0	NS
	E	584 \pm 6	104.0 \pm 0.81		
NFAT	TR	258 \pm 11	100 \pm 3.35	24.2	P<0.01
	E	196 \pm 4	75.8 \pm 1.77		
MEF2A	TR	1451 \pm 52	100 \pm 2.4	9.4	P<0.1
	E	1586 \pm 47	109.4 \pm 2.92		

Table 5: Swimming experiment 3, summary of nuclear localisation (N) and/or overall expression (T), of calcineurin subunits A&B, NFAT and MEF2A; **fast** muscle tissue samples from tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, N = 9.

Protein	Condition	Mean Optical Density (INT)	Density percentage of TR group	Percentage difference	Statistical significance
Calcineurin (N)	A TR	411 \pm 10	100 \pm 2.34	4.1	NS
	E	394 \pm 8	95.9 \pm 2.08		
Calcineurin (T)	A TR	649 \pm 5	100 \pm 0.77	1.6	NS
	E	638 \pm 6	98.4 \pm 0.95		
Calcineurin (N)	B TR	393 \pm 12	100 \pm 3.06	36.5	P<0.001
	E	249 \pm 8	63.5 \pm 3.27		
Calcineurin (T)	B TR	326 \pm 8	100 \pm 2.6	0.2	NS
	E	326 \pm 6	99.8 \pm 2.0		
NFAT (N)	TR	103 \pm 4	100 \pm 3.87	10.0	P<0.05
	E	93 \pm 2	90.0 \pm 2.51		
MEF2A (N)	TR	336 \pm 13	100 \pm 3.96	3.0	NS
	E	326 \pm 8	97.0 \pm 2.47		

Appendix VI (continued)

Summary of western blotting data for swimming experiments 1-4

Table 6: Swimming experiment 3, results summary of nuclear localisation (N) and/or overall expression (T), of calcineurin subunits A&B, NFAT and MEF2A; **slow** muscle tissue samples from tank rested (TR) and exercised (E) groups. Values represent group mean \pm SEM, N = 9.

Protein	Condition		Mean Optical Density (INT)	Density as percentage of TR group	Percentage of difference	Statistical significance
Calcineurin (N)	A	TR	245 \pm 12	100 \pm 0.73	4.8	NS
		E	233 \pm 4	95.2 \pm 1.71		
Calcineurin (T)	A	TR	737 \pm 6	100 \pm 0.87	1.1	NS
		E	729 \pm 7	98.9 \pm 0.99		
Calcineurin (N)	B	TR	1262 \pm 31	100 \pm 2.48	10.5	P<0.01
		E	1130 \pm 13	89.5 \pm 1.11		
Calcineurin (T)	B	TR	454 \pm 9	100 \pm 1.51	4.6	NS
		E	473 \pm 11	104.6 \pm 1.34		
NFAT (N)		TR	337 \pm 7	100 \pm 2.18	10.3	P<0.001
		E	302 \pm 3	89.7 \pm 0.90		
MEF2A (N)		TR	1714 \pm 101	100 \pm 5.91	3.7	NS
		E	1651 \pm 230	96.3 \pm 4.62		

Appendix VII

Summary of western blotting data for the time course experiment

Table 1: Time course experiment, results summary for expression of MRFs in nuclear (N) and total cellular (T) protein extracts from **fast** muscle tissue; tank rested groups (TR₀ and TR₁₆) and 16-day exercised group (E₁₆). Values represent group mean ± SEM, N = 6.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance To TR ₀ group
MyoD (N)	TR ₀	577±6	100±1.00		
	E ₁₆	630±13	109.3±2.12	9.3	P<0.01
	TR ₁₆	634±10	109.6±1.65	9.6	P<0.01
MyoD (T)	TR ₀	884±20	100±2.21		
	E ₁₆	855±20	96.7±2.31	-3.3	NS
	TR ₁₆	912±17	103.2±1.86	3.2	NS
myf-5 (N)	TR ₀	1201±23	100±1.93		
	E ₁₆	1230±37	102.4±2.98	2.4	NS
	TR ₁₆	1237±35	103.0±2.81	3.0	NS
myf-5 (T)	TR ₀	351±8	100±2.15		
	E ₁₆	346±21	98.7±6.09	-1.3	NS
	TR ₁₆	371±17	105.7±4.46	5.7	NS

Table 2: Time course experiment, summary of data for MRFs expression in **fast** muscle tissue total cellular protein extracts in 8 experimental treatments. Values represent group mean ± SEM, N = 6.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance
MyoD	TR ₀	884±20	100±2.21		
	E _{0.5}	915±34	103.6±3.75	3.6	All NS
	E ₁	882±20	99.8±2.31	-0.2	
	E ₂	883±22	99.9±2.49	-0.1	
	E ₄	882±18	99.7±2.05	-0.3	
	E ₈	919±13	103.9±1.43	3.9	
	E ₁₆	855±20	96.7±2.31	-3.3	
	TR ₁₆	912±17	103.2±1.86	3.2	
myf-5	TR ₀	351±8	100±2.15		
	E _{0.5}	347±7	98.8±2.09	-1.24	All NS
	E ₁	340±7	96.9±2.18	-3.10	
	E ₂	338±10	96.3±2.19	-3.73	
	E ₄	356±19	101.5±5.45	1.50	
	E ₈	371±17	105.7±4.63	5.68	
	E ₁₆	346±21	98.7±6.09	-1.30	
	TR ₁₆	371±17	105.7±4.46	5.69	
myogenin	TR ₀	340±5	100±1.43		
	E _{0.5}	355±14	104.6±3.89	4.58	All NS
	E ₁	341±8	100.3±2.24	0.29	
	E ₂	347±10	102.0±2.98	1.95	
	E ₄	343±13	100.8±3.73	0.78	
	E ₈	361±11	106.2±3.15	6.22	
	E ₁₆	335±14	98.6±4.16	-1.40	
	TR ₁₆	355±4	104.5±1.10	4.48	

Appendix VII (continued)

Summary of western blotting data for the time course experiment

Table 3: Time course experiment, results summary for expression of MRFs in nuclear (N) and total cellular (T) protein extracts from **slow** muscle tissue; tank rested controls (TR₀ and TR₁₆) and 16-day exercised group (E₁₆). Values represent group mean ± SEM, N = 6.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density percentage of TR ₀ group	as of	Percentage difference To TR ₀ group	Statistical significance
MyoD (N)	TR ₀	699±4	100±0.55			
	E ₁₆	725±11	103.7±1.50		3.7	NS
	TR ₁₆	690±8	98.7±1.65		-1.3	NS
						E ₁₆ ≠TR ₁₆ (P<0.05)
MyoD (T)	TR ₀	834±17	100±2.06			
	E ₁₆	830±25	99.5±3.02		-0.5	NS
	TR ₁₆	838±12	100.5±1.39		0.5	NS
myf-5 (N)	TR ₀	1353±53	100±1.60			
	E ₁₆	1378±36	101.9±1.08		1.9	NS
	TR ₁₆	1383±60	102.2±1.78		2.2	NS
myf-5 (T)	TR ₀	992±26	100±2.66			
	E ₁₆	960±25	96.7±2.56		-3.3	NS
	TR ₁₆	981±36	98.8±3.72		-1.2	NS

Table 4: Time course experiment, summary of data for MRFs expression in slow muscle tissue total cellular protein extracts from 8 experimental treatments. Values represent group mean ± SEM, N = 6.

Myogenic regulatory factor	Condition	Mean Optical Density (INT)	Density percentage of TR ₀ group	as of	Percentage difference To TR ₀ group	Statistical significance
MyoD	TR ₀	834±17	100±2.06			
	E _{0.5}	851±13	102.0±1.55		2.0	All NS
	E ₁	8630±25	103.4±2.85		3.4	
	E ₂	897±10	107.5±1.07		7.5	
	E ₄	838±14	100.5±1.67		0.5	
	E ₈	878±16	105.3±1.86		5.25	
	E ₁₆	830±25	99.5±3.02		-0.5	
	TR ₁₆	838±12	100.5±1.39		0.5	
myf-5	TR ₀	992±26	100±2.66			
	E _{0.5}	1004±29	101.2±2.88		1.16	All NS
	E ₁	1025±17	103.3±1.69		3.30	
	E ₂	1031±19	103.9±1.86		3.92	
	E ₄	967±35	97.5±3.65		-2.53	
	E ₈	994±46	100.1±4.67		0.14	
	E ₁₆	960±25	96.7±2.56		-3.31	
	TR ₁₆	981±36	98.8±3.72		-1.17	
myogenin	TR ₀	427±7	100±1.74			
	E _{0.5}	426±13	99.8±3.08		-0.23	All NS
	E ₁	417±6	97.6±1.53		-2.43	
	E ₂	418±10	97.7±2.29		-2.26	
	E ₄	418±8	97.7±1.85		-2.27	
	E ₈	439±6	102.9±1.40		2.85	
	E ₁₆	405±10	94.8±2.46		-5.16	
	TR ₁₆	436±8	102.0±1.79		1.95	

Appendix VII (cont.)

Summary of western blotting data for the time course experiment

Table 5: Time course experiment, summary of data for PCNA expression in **fast** and **slow** muscle tissue total cellular protein extracts from 8 experimental treatments. Values represent group mean \pm SEM, N = 6.

PCNA	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance
Fast	TR ₀	917 \pm 13	100 \pm 1.37		TR ₁₆ \neq all other groups (P<0.001), EXCEPT TR ₁₆ \neq TR ₀ (P<0.01) B \neq E ₂ , E ₁₆ (P<0.05)
	E _{0.5}	890 \pm 26	97.0 \pm 2.94	-3.0	
	E ₁	865 \pm 13	94.3 \pm 1.49	-5.7	
	E ₂	906 \pm 22	98.8 \pm 2.46	-1.2	
	E ₄	886 \pm 15	96.6 \pm 1.68	-3.4	
	E ₈	883 \pm 6	96.3 \pm 0.65	-3.7	
	E ₁₆	825 \pm 18	89.9 \pm 2.19	-10.1	
	TR ₁₆	1030 \pm 20	112.3 \pm 1.92	12.3	
Slow	TR ₀	595 \pm 8	100 \pm 1.33		E ₂ , TR ₁₆ \neq all other groups (P<0.001), EXCEPT E ₂ \neq E ₄ (P<0.05).
	E _{0.5}	585 \pm 8	98.3 \pm 1.41	-1.7	
	E ₁	584 \pm 14	98.1 \pm 2.41	-1.9	
	E ₂	741 \pm 20	124.4 \pm 2.74	24.4	
	E ₄	647 \pm 21	108.7 \pm 3.24	8.7	
	E ₈	592 \pm 12	99.5 \pm 2.03	-0.5	
	E ₁₆	598 \pm 13	100.5 \pm 2.11	0.5	
	TR ₁₆	781 \pm 36	131.3 \pm 4.61	31.3	

Table 6: Time course experiment, summary of data for myostatin latency associated peptide expression in **fast** and **slow** muscle tissue total cellular protein extracts from 8 experimental treatments. Group mean \pm SEM, N = 6.

Myostatin LAP	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance
Fast	TR ₀	894 \pm 176	100 \pm 8.04		All NS
	E _{0.5}	911 \pm 227	102.0 \pm 10.17	2.0	
	E ₁	934 \pm 133	104.6 \pm 5.79	4.6	
	E ₂	925 \pm 157	103.5 \pm 6.95	3.5	
	E ₄	918 \pm 118	102.8 \pm 5.25	2.8	
	E ₈	960 \pm 125	107.4 \pm 5.33	7.4	
	E ₁₆	944 \pm 157	105.6 \pm 6.78	5.6	
	TR ₁₆	961 \pm 212	107.6 \pm 9.03	7.6	
Slow	TR ₀	570 \pm 116	100 \pm 8.34		All NS
	E _{0.5}	579 \pm 139	101.5 \pm 9.81	1.5	
	E ₁	623 \pm 119	109.4 \pm 7.76	9.4	
	E ₂	635 \pm 131	111.4 \pm 8.41	11.4	
	E ₄	535 \pm 90	93.9 \pm 6.84	-6.1	
	E ₈	546 \pm 109	95.8 \pm 8.12	-4.2	
	E ₁₆	533 \pm 80	93.5 \pm 6.14	-6.5	
	TR ₁₆	557 \pm 110	97.7 \pm 7.97	-2.3	

Appendix VII (cont.)

Summary of western blotting data for the time course experiment

Table 7: Time Course experiment, summary of nuclear localisation (N) and overall expression in total extracts (T), of calcineurin B, NFAT and MEF2A in **fast** muscle tissue samples; tank rested groups (TR₀ and TR₁₆) and 16-day exercised group (E₁₆). Values represent group mean ± SEM, N = 6.

Protein	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance
Calcineurin (N)	B TR ₀	933±12	100±1.31		
	E ₁₆	951±13	101.9±1.38	1.9	NS
	TR ₁₆	989±14	106.0±1.41	6.0	P<0.05
Calcineurin (T)	B TR ₀	637±18	100±2.82		
	E ₁₆	639±24	100.3±3.81	0.3	NS
	TR ₁₆	660±16	103.5±2.35	3.5	NS
NFAT (N)	TR ₀	354±6	100±1.69		
	E ₁₆	332±6	93.8±1.69	-6.2	NS
	TR ₁₆	360±6	101.9±1.61	1.9	NS TR≠E16 (P<0.05)
NFAT (T)	TR ₀	632±19	100±2.95		
	E ₁₆	721±19	114.1±2.58	14.1	P<0.05
	TR ₁₆	720±21	113.9±2.97	13.9	P<0.1
MEF2A (N)	TR ₀	722±10	100±1.34		
	E ₁₆	733±11	101.5±1.56	1.5	NS
	TR ₁₆	726±13	101.0±1.73	1.0	NS

Table 8: Time course experiment, summary of nuclear localisation (N) and overall expression in total protein extracts (T), of calcineurin B, NFAT and MEF2A in **slow** muscle tissue samples; tank rested groups (TR₀ and TR₁₆) and 16-day exercised group (E₁₆). Values represent group mean ± SEM, N = 6.

Protein	Condition	Mean Optical Density (INT)	Density as percentage of TR ₀ group	Percentage difference To TR ₀ group	Statistical significance
Calcineurin (N)	B TR ₀	907±13	100±1.40		
	E ₁₆	904±14	99.6±1.56	-0.4	NS
	TR ₁₆	893±12	98.5±1.34	-1.5	NS
Calcineurin (T)	B TR ₀	558±9	100±1.55		
	E ₁₆	544±14	97.6±2.51	2.4	NS
	TR ₁₆	557±16	97.8±2.88	2.2	NS
NFAT (N)	TR ₀	1648±21	100±1.27		
	E ₁₆	1645±10	99.8±0.63	-0.2	NS
	TR ₁₆	1681±43	102.0±2.57	2.0	NS
NFAT (T)	TR ₀	1697±50	100±2.93		
	E ₁₆	1887±62	111.1±3.28	11.1	P<0.1
	TR ₁₆	1726±48	101.7±2.76	1.7	NS
MEF2A (N)	TR ₀	1234±45	100±1.48		
	E ₁₆	1249±32	101.3±1.03	1.3	NS
	TR ₁₆	1259±22	102.0±0.70	2.0	NS

Appendix VIII

Summary of western blotting data for the trout swimming experiment

Table 1: Summary of data for nuclear subcellular localisation and total protein expression of calcineurin subunits and transcription factors NFAT and MEF2A; **fast** muscle tissue, slow exercised (E_{15}) fast exercised (E_{30}) and tank rested (TR) group, comparative trout swimming study. Values represent group mean \pm SEM, N = 6.

Protein	Condition	Mean Optical Density (INT)	Density as percentage of TR group	Percentage difference cf TR group	Statistical significance cf TR group
Calcineurin (N)	TR	1210 \pm 7	100 \pm 0.59		
	E_{15}	1274 \pm 10	105.3 \pm 0.75	5.3	P<0.01
	E_{30}	1293 \pm 15	106.8 \pm 1.13	6.8	P<0.001
Calcineurin (T)	TR	987 \pm 28	100 \pm 2.82		
	E_{15}	983 \pm 34	99.6 \pm 3.42	-0.4	NS
	E_{30}	1021 \pm 33	103.4 \pm 3.24	3.4	NS
Calcineurin (N)	TR	1100 \pm 22	100 \pm 2.02		
	E_{15}	1188 \pm 15	108.0 \pm 1.28	8.0	P<0.1
	E_{30}	1191 \pm 13	108.2 \pm 1.10	8.2	P<0.05
Calcineurin (T)	TR	863 \pm 42	100 \pm 3.50		
	E_{15}	905 \pm 48	104.9 \pm 3.48	4.9	NS
	E_{30}	863 \pm 56	100.0 \pm 6.84	0	NS
NFAT (N)	TR	1336 \pm 23	100 \pm 1.70		
	E_{15}	1229 \pm 16	92.0 \pm 1.26	8.0	P<0.01
	E_{30}	1200 \pm 13	89.7 \pm 1.10	10.3	P<0.001
NFAT (T)	TR	530 \pm 10	100 \pm 1.80		
	E_{15}	451 \pm 6	85.1 \pm 1.29	14.9	P<0.001
	E_{30}	471 \pm 9	88.9 \pm 2.01	11.1	P<0.001
MEF2A (N)	TR	1046 \pm 5	100 \pm 0.50		
	E_{15}	1033 \pm 3	98.7 \pm 0.25	-1.3	NS
	E_{30}	1066 \pm 10	101.8 \pm 0.98	1.8	NS
					S \neq F (P<0.05)

Appendix VIII (continued)

Summary of western blotting data for the trout swimming experiment

Table 2: Summary of data for myostatin expression in total protein extracts (T) from **fast** muscle tissue; slow exercised (E₁₅) fast exercised (E₃₀) and tank rested (TR) group, trout exercise experiment. Values represent group mean \pm SEM, N = 6.

Myostatin Form	Condition	Mean Optical Density (INT)	Density as percentage of TR group	Percentage of difference cf TR group	Statistical significance cf TR group
Pre-cursor protein (T)	TR	1152 \pm 40	100 \pm 3.49		
	E ₁₅	1092 \pm 32	94.8 \pm 2.90	-5.2	NS
	E ₃₀	1047 \pm 24	90.9 \pm 2.30	-9.1	P<0.1
Latency-associated peptide (T)	TR	1734 \pm 76	100 \pm 4.41		
	E ₁₅	1729 \pm 70	99.7 \pm 4.04	-0.3	NS
	E ₃₀	1752 \pm 68	101.0 \pm 3.91	1.0	NS
Active peptide (T)	TR	1240 \pm 30	100 \pm 2.37		
	E ₁₅	1154 \pm 23	93.1 \pm 1.99	-6.9	P<0.05
	E ₃₀	1162 \pm 12	93.8 \pm 1.00	-6.2	P<0.1

Table 3: Summary of results for myf-5 nuclear localisation (N) and level of expression in total cellular protein preparations (T) from **fast** muscle tissue extracts. Data for cell cycle protein PCNA expression in total protein extracts are also included; slow exercised (E₁₅) fast exercised (E₃₀) and tank rested (TR) group, trout swimming study. Values represent group mean \pm SEM, N = 6.

Protein	Condition	Mean Optical Density (INT)	Density as percentage of TR group	Percentage of difference	Statistical significance
myf-5 (N)	TR	776 \pm 9	100 \pm 1.12		
	E ₁₅	804 \pm 20	103.7 \pm 2.46	3.7	NS
	E ₃₀	796 \pm 19	102.6 \pm 2.42	2.6	NS
myf-5 (T)	TR	952 \pm 23	100 \pm 2.45		
	E ₁₅	954 \pm 21	100.2 \pm 2.18	0.2	NS
	E ₃₀	949 \pm 12	99.7 \pm 1.24	-0.3	NS
PCNA (T)	TR	1177 \pm 11	100 \pm 0.90		
	E ₁₅	1174 \pm 29	99.8 \pm 2.49	-0.2	NS
	E ₃₀	1211 \pm 45	103.0 \pm 3.75	3.0	NS

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Lao-tzu, 604 – 563 BC.

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