Myosatellite cells in muscle of growing carp (*Cyprinus carpio*, L.)

Voor mijn ouders

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Myosatellite cells are small spindle shaped myogenic cells situated between the sarcolemma and the basal lamina that surrounds every muscle fibre. Based on information from mammals and birds, myosatellite cells are considered to play an important role in postlarval muscle growth in fish. Myosatellite cells are not only thought to provide the extra nuclei needed in the outgrowth of already existing muscle fibres (hypertrophy) but are also thought to provide new additional fibres (hyperplasia). The investigations described in this thesis showed that although the percentage of muscle nuclei that are myosatellite nuclei decreased with increasing length of the fish, the total number of myosatellite cells present in white axial muscle of carp remained about the same. However, the total number of myosatellite cells was so low, that it is very improbable that these cells are the only source of the additional muscle nuclei in hypertrophy. We developed a method to isolate and culture myosatellite cells from axial muscle of carp. Our *in vitro* studies showed that isolated carp myosatellite cells can differentiate into myotubes. These studies also showed that the population of myosatellite cells contains subpopulations that differ in their differentiational stage. The relative size of these experimentally defined subpopulations changed with increasing length of the fish. In young carp of about 5 cm standard length, muscle growth probably depends mainly on postmitotic myogenic cells that are formed in an earlier stage of growth. The correlation of the changes in the relative size of the subpopulations of myosatellite cells with the decrease in the occurrence of hyperplasia indicates that hyperplasia and hypertrophy are dependent on separate populations of myogenic cells. To prove this theory, markers that enable the identification of these cells will have to be found. We used isolated carp myosatellite cells as an antigen to produce monoclonal antibodies against markers specific for (subpopulations of) these cells. Such antibodies would also enable the (immuno-histochemical) lightmicroscopical identification of myosatellite cells *in situ*. The criteria for the *in situ* identification of these cells now still necessitates the use of transmission electron-microscopy. However, non of the antibodies we produced reacted with myosatellite cells only. In future investigations, the use of molecular biological techniques (eg. probes against genes of the MyoD family) probably will be of great use to increase our knowledge of the regulation of muscle growth in fish. This knowledge is not only important in comparative biology, but can also be of advantage in the field of fish-farming as justified optimisation of processes requires its thorough knowledge.

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STELLINGEN

1 Het is onwaarschijnlijk dat myosatellietcellen de enige bron zijn van extra spierkernen bij de groei van wit axiaal spierweefsel van de karper.
(Dit proefschrift).

2 Het aantal myosatellietcellen in wit axiaal spierweefsel van de karper neemt niet in belangrijke mate af tijdens de groei. De daling van het percentage spierkernen dat bestaat uit myosatellietcelkernen wordt veroorzaakt door de verdunning van de aanwezige myosatellietcellen over een toenemend aantal spierkernen.
(Dit proefschrift).

3 De populatie van myosatellietcellen in het spierweefsel van karpers bestaat uit subpopulaties die verschillen in hun mate van differentiatie. De verhouding tussen de omvang van deze subpopulaties verandert tijdens de groei.
(Dit proefschrift).

4 Aangezien hyperplasie bij de spiergroei van vissen tot ver in het adulte stadium een onderdeel kan zijn van het normale groeiproces, terwijl dit bij de spiergroei van vogels en zoogdieren slechts optreedt tot rond de geboorte, zijn vissen uitermate belangrijk voor vergelijkend onderzoek naar de regulatie van spiergroei.

5 There is "more to muscle than MyoD". M. Robertson (1990) Nature **344**,378-379.

6 De steeds verdere integratie van vakgebieden vereist dat bij het introduceren van afkortingen onderzocht wordt of deze niet reeds in gebruik zijn. Een voorbeeld: MHC wordt binnen de immunologie gebruikt als afkorting voor Major Histocompatibilty Complex, maar binnen het spieronderzoek als afkorting voor Myosin Heavy Chain.

7 Het verantwoordelijkheidsbesef van onderzoekers dient gelijke tred te houden met hun toenemende kennis, daar in het geval van toepassing van deze kennis de wetgeving noodgedwongen achter de feiten aanloopt.

8 Het verdient aanbeveling om de identiteit van de auteur(s) van een wetenschappelijk artikel onbekend te houden voor de referee's van het tijdschrift waarin beoogd wordt te publiceren.

9 Wetenschap is intrigerend, maar heeft slechts zelden baat bij intriges.

10 Het verkrijgen van een doctors-titel hoort niet het voornaamste doel van een AIO (OIO) onderzoek zijn.

11 "Puur natuur", is niet synoniem met "gezond".

12 De problematiek van bodemverontreinigingen laat zien dat een verantwoorde afvoer van afval te vaak werd afgedaan met een opmerking als "zand erover".

13 Een spreekwoord als: "Beter een vogel in de hand dan tien in de lucht", laat zien dat koopmanschap en milieubeleid al van oudsher slechts moeizaam tot elkaar te brengen zijn.

14 De schaalvergroting in de varkensmesterij maakt duidelijk dat men er helemaal niet van uitging dat vele varkens de spoeling dun maken.

15 Het niet lamineren van de omslag bevordert het "stuklezen" van een proefschrift.

J.T.M. Koumans

Myosatellite cells in muscle of growing carp (*Cyprinus carpio* L.).

Wageningen, 21 december 1992

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Muscle growth

Skeletal muscle fibres

Skeletal muscle consists of many muscle fibres. A muscle fibre, or muscle cell, is a syncytium formed by the fusion of many separate cells. It contains many nuclei, situated at the periphery of the fibre, and great numbers of contractile filaments called myofibrils. These myofibrils are formed by serially-repeated units called sarcomeres, which give the muscle cell its striated appearance. Skeletal muscle fibres can be divided into a number of categories or fibre types, each of which is characterized by a unique set of contractile, (imuno)histochemical, and morphological properties. For fish the main fibre types are white, pink, and red (van Raamsdonk *et al.*, 1982; Rowlerson *et al.*, 1985; Scapolo *et al.*, 1988; Akster and Osse, 1978; Akster, 1985)

Teleost muscle differs from that of terrestrial vertebrates in a number of aspects. For example: Most teleost muscle fibres are multiple (Hudson, 1969; Akster, 1983; Moss, 1988) and poly-neurally (Hudson, 1969; Westerfield *et al.*, 1986; Moss, 1988) innervated, (more than one end-plate originating from more than one axon) whereas the usual type of innervation in adult terrestrial vertebrates is with a single end-plate derived from a single axon (Bennet, 1983). In teleosts, the organisation of the axial muscle is in myotomes and the mature fibre types (white, red, and pink) are segregated or zoned to a much greater degree than they are in terrestrial vertebrates (Bone, 1978). Neuromuscular spindles appear to be absent in teleost muscle, where they occur in all terrestrial vertebrates (Bone, 1978).

Development of skeletal muscle fibres

In vertebrate muscle growth, the precursors of muscle fibres are the mono-nucleated embryonic mesenchymal cells (or presumptive myoblasts) which do not themselves fuse or synthesize muscle specific proteins (Dienstman *et al.*, 1975; Turner, 1978; Allen *et al.*, 1979). These cells proliferate and differentiate into mono-nucleated myoblasts which start to

accumulate muscle specific proteins. The myoblasts "align" and fuse end to end to form long, cylindrical, multi-nucleated cells called myotubes (for fish see: Nag and Nursall, 1972; Schattenberg, 1973). In these (primary) myotubes the production of muscle specific proteins continues and gradually definite sarcomeres emerge giving the cell a cross-striated appearance.

In mammals and birds the primary myotubes provide a framework along which a population of still persisting myoblasts become longitudinally oriented (Kelly and Zacks, 1969; Ontell and Dunn, 1978) and fuse to form multi-nucleated secondary myotubes. The biphasic nature of muscle fibre development leads to a rosette arrangement of muscle fibres whereby the centrally located larger fibres are the primary myotubes and the surrounding smaller fibres the secondary myotubes. In the adult, the two generations become indistinguishable in size.

During myogenesis, prior to the expression of the adult isoforms, myotubes express developmental myosin heavy chain (MHC) isoforms called 'embryonic' and 'neonatal' MHC (Stockdale and Boone Miller, 1987; Sanes, 1987). New observations suggest that this nomenclature of developmental isoforms of MHC is faulty because in adult animals some muscles can still contain fibres that express "embryonic" or "neonatal" MHC isoforms (d'Albis *et al.*, 1986; Stockdale and Boone Miller, 1987; Bredman *et al.*, 1991). The neural dependence of primary and secondary myogenesis and its relation to fibre-type differentiation still is an unsolved puzzle (Fredette and Landmesser, 1991). However, it is clear that different types of myoblasts exist that form myotubes which contain different isotypes of MHC (Stockdale and Boone Miller, 1987; Fredette and Landmesser, 1991). Innervation, hormones, and activity can subsequently modulate the isoform expression pattern of the "adult" fibre.

In fish, a biphasic development of primary and secondary myotubes is not observed, but newly formed small fibres also show (immuno)histochemical differences with mature (large) muscle fibres (van Raamsdonk *et al.*, 1982; Akster, 1983; Rowlerson *et al.*, 1985; Scapolo *et al.*, 1988)

Growth of muscle

Muscle growth not only occurs during embryogenesis and in the foetal/larval stage, but there is also a tremendous postnatal/juvenile gain in muscle mass. Muscle growth can occur by increase in the number of muscle fibres (= hyperplasia) and by outgrowth of already existing fibres (= hypertrophy). In most mammalian and avian muscles the number of fibres does not, or only for a short time, increase after birth (Goldspink, 1972; 1974; Campion, 1984). Postnatal growth in these animals therefore results mainly from hypertrophic growth of existing muscle fibres. The muscle fibres increase in length by the addition of new sarcomeres at the ends of the existing myofibrils and they increase in width by an increase in the number and diameter of myofibrils (Goldspink, 1970; 1980).

In mammals and birds, the number of fibres of a muscle is quite an important factor influencing growth-rate and maximum size of that muscle. Under equal circumstances, the larger the number of fibres in a muscle, the higher the growth-rate and the larger the muscle will become (Penney *et al.*, 1983; Stickland and Handel, 1986). This relationship even led to the question if, in animals for meat production, the number of fibres in an "indicator-muscle" (a small muscle that is relatively insignificant in terms of the total mass of the body) could be used to predict the growth of the total muscle mass (Timson *et al.*, 1989). The effect of fibre number on the growth-rate of the muscle may well be caused by the concomitant higher surface/volume ratio of the muscle fibres during growth. A high surface/volume ratio, causing a slower diffusion of nutrients and elements into the fibres, will slow down, and ultimately even stop muscle growth (Weatherley *et al.*, 1988).

In adult muscle, fibre width is much affected by use. It increases with exercise, due to hypertrophy (Walker, 1966; Greer Walker, 1971; Johnston and Moon, 1980; Sänger, 1992), and decreases with disuse (Darr and Schultz, 1989).

Fish muscle growth

In fish, muscular tissue forms a large part of the body mass: some 40-60% of the total body mass in most fish is locomotor muscle (Bone, 1978). Growth of fish muscle is different from that of mammals and birds. In growing fish, in addition to hypertrophy, hyperplasia can play an important role in muscle growth even after the juvenile stage (Greer-Walker, 1970; Stickland, 1983; Weatherley and Gill, 1984; Weatherley *et al.*, 1988; Weatherley, 1990). However, the relative importance of hyperplasia is maximal in small (young) fish and

decreases with increasing growth. Comparable to the number of muscle fibres present in mammalian and avian muscle, the ratio between hyperplasia and hypertrophy appears to be an important factor in defining individual growth-rate and maximum size of fish (Stickland, 1983; Weatherley and Gill, 1984; Weatherly *et al.*, 1988). In ten investigated freshwater species, Weatherley *et al.*, (1988) found a strong relationship between the length of the fish at which hyperplasia stops to occur and the maximum reported length for that species (also see Battram and Johnston, 1991).

Another special aspect of fish (muscle)growth is the large variation of growth-rate that can occur depending on circumstances as the availability of food and differences in temperature. Therefore, age is not a good parameter in the study of fish muscle growth. Growth related events as the cessation of hyperplasia and fibre diameter distribution show a much better relationship with fish length. Even under extreme artificial circumstances as an injection with bovine growth hormone and the subsequent large differences in growth-rate, fibre diameter distribution remains largely correlated to fish length (Weatherley, 1990).

Some studies employing heavy exercise training (Gonyea *et al.*, 1986; Appell *et al.*, 1988; McCormick and Schultz, 1992), indicate that, under such training circumstances, also in adult mammals and birds hyperplasia can occur. However, it might well be that under such conditions the increase in fibre number is caused by a form of regeneration (McCormick and Schultz, 1992; Winchester and Gonyea, 1992).

Regeneration

Regeneration is a special form of muscle growth after injury or transplantation. The extent and success of regeneration varies with the nature of the injury, but in all situations the process involves: revascularisation, cellular infiltration, phagocytosis of necrotic damaged muscle, proliferation of muscle precursor cells fusing either into multi-nucleated myotubes or with the ends of damaged muscle fibres (Hinterberger and Barald, 1990), and finally, re-innervation; see Carlson (1986) and Grounds (1991) for reviews. Like in embryonic muscle growth, the newly formed myotubes will differentiate into mature muscle fibres which will increase in volume. The muscle precursor cells used in regeneration probably persist throughout life (Schmalbruch and Hellhammer, 1976). They are thought to descent from embryonic myoblasts (Armand *et al.*, 1983; Armand and Kieny, 1984), and were called myosatellite cells (Mauro, 1961). Subsequent investigations showed that myosatellite cells

are not only involved in regeneration of muscle, but are involved in all types of postnatal muscle growth.

Myosatellite cells

Myosatellite cells

Myosatellite cells are small, more or less spindle shaped cells. As a rule, they contain a heterochromatic nucleus and except for free ribosomes and polysomes their small amount of cytoplasm contains only a small number of other organelles. They are situated between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibres (Mauro, 1961) and are present in all investigated vertebrate species, but never have been found in cardiac striated muscle or in smooth muscle (Allbrook, 1981)(for a review see Campion, 1984). Because of their intimate apposition with the myofibre surface and the lack of known specific markers, myosatellite cells can *in situ* only be identified with electronmicroscopy.

Myosatellite cells and muscle growth

Myosatellite cells are considered important in (re)generation of muscle.
An important aspect of muscle growth is the increase in the number of myonuclei accompanying the increase in muscle mass (Allen *et al.*, 1979; Cardasis and Cooper, 1975; Enesco & Puddy, 1964). Developing fibres have, compared to mature fibres, a high nuclear density (Harris *et al.*, 1989). During initial growth of the fibres the nuclear density decreases but, after a critical nucleus/sarcoplasm ratio has been reached, outgrowth of the fibres can only occur if additional myonuclei are added to the myofibres (Cardasis and Cooper, 1975; Enesco and Puddy, 1964; Winchester and Gonyea, 1992). However, as the myonuclei in the myofibres are considered incapable of undergoing mitosis, and embryonic myoblasts do not longer exist, another source of myonuclei must be present (Cardasis and Cooper, 1975; Enesco and Puddy, 1964). Myosatellite cells are considered to be that source of additional myonuclei. Indications that the myosatellite cells are important in normal muscle growth come from the labelling experiments of Moss and Leblond (1970; 1971). They demonstrated that, immediately after labelling nuclei with ^{3}H-thymidine, the label did, as expected, not appear in the myonuclei but did appear in myosatellite nuclei. Later, as the percentage of labelled myosatellite cells started to decrease, the label also appeared in the myonuclei.

Winchester *et al.*, (1991) found a rapid and large increase in proliferation of myosatellite cells in stretch-enlarged quail muscle. Further indications come from the strong correlation between the number of existing myonuclei and the rate of muscle growth (Penney *et al.*, 1983; Campion *et al.*, 1982) and between the proliferation rate of myosatellite cells and the rate of muscle growth (Campion *et al.*, 1982; Darr and Schultz, 1989; Mulvanney *et al.*, 1987; Joubert and Tobin, 1989).

In vitro both myosatellite cells and embryonic myoblasts proliferate and eventually (under permissive circumstances) fuse to form multi-nucleated myotubes in a way that resembles embryonic myogenesis (Konigsberg, 1963; Wakelam, 1985). Although, *in vitro*, it is possible to form myoblast-myosatellite hybrid cells (Cossu *et al.*, 1980), it is clear that myosatellite cells and embryonic myoblasts are not the same (Cossu *et al.*, 1983; Cossu *et al.*, 1985: Chevallier *et al.*, 1987; Hartley *et al.*, 1991).

Investigations

Almost all of the cited investigations, were on myosatellite cells of mammals and birds. Most earlier investigations on the role of myosatellite cells in muscle growth were about morphology, number, and location of myosatellite cells in different muscles of many species, including amphibia and fish. An important aspect of these investigations was the identification of myosatellite cells *in vivo* and *in vitro* (Ontell, 1974; Lipton, 1977); which still remains an important topic (Grounds *et al.*, 1992; this thesis). Within a muscle, red oxidative muscle fibres, contain more myosatellite cells than white glycolytic fibres (Kelly, 1978; Gibson and Schultz, 1982; Düsterhöft *et al.*, 1990). A slightly higher percentage of myosatellite cells can be found in the region of neuro-muscular junctions (Kelly, 1978).

Certainly after the generation of myogenic cell lines like the L6 and L8 lines of rat (Yaffe, 1968), DZ cells (Linkhart *et al.*, 1980) and C2 cells (Yaffe and Saxel, 1977) of mouse, and QM cells of quail (Antin and Ordahl, 1991), muscle cell culture became an important tool in animal growth research (Allen, 1987). Isolated myogenic cells are used as an *in vitro* differentiating model (Wakelam, 1985) in more fundamental research in the field of cell, developmental and molecular biology. Investigations include:

-The process of *(in vitro)* differentiation as can be observed with

(monoclonal)antibodies raised against developmentally regulated antigens (Lee and

Kaufman, 1981; Hurko and Walsh, 1983; Walsh *et al.*, 1984; Kaufman and Foster, 1985).

-The effect of the type of culture media (Linkhart *et al.*, 1981), and more recently, hormones and growth factors (Allen *et al.*, 1983; Florini *et al.*, 1984; 1991; Dodson *et al.*, 1985) on proliferation and differentiation. The best understood purified growth factors are fibroblast growth factor (FGF), the insulin-like growth factors (IGF-I and -II), and transforming growth factor-ß (TGF-ß). It is currently held that FGF and IGFs have mitogenic properties for myogenic cells, whereas TGF-ß either has no effect or suppresses cell proliferation. Furthermore FGF and TGF-ß are potent inhibitors in the early stages of myogenic differentiation (White and Esser, 1989; Florini and Magri, 1989). Also a soluble factor from injured muscle is known to exert a strong mitogenic activity on myogenic cells (Bischoff, 1990).

-The *in vitro* role of the substratum (Foster *et al.*, 1987; Öcalan *et al.*, 1988; Goodman *et al.*, 1989; Hartley and Yablonka Reuveni 1990), the *in vivo* role of the basal lamina, (Hughes and Blau, 1990; Caldwell *et al.*, 1990; Grounds, 1990) and, for instance, the role of the extracelluar matrix receptor, integrin, (Menko and Boettiger, 1987) on proliferation and differentiation of myosatellite cells.

-Physical factors that promote the in vitro formation and growth of muscle cells, like mechanical stimulation (passive stretch, Vandenburgh *et al.*, 1989; Vandenburgh & Karlish, 1989) and electric fields (Dover and McCaig, 1989).

-The existence of subpopulations of myogenic cells (Cossu and Molinaro, 1987; Stockdale & Boone Miller, 1987; Schafer *et al.*, 1987; Sanes, 1987; Feldman and Stockdale, 1991), and the presence of myosatellite cells in several stages of differentiation (Grounds and McGeachie, 1989).

-The role of recently discovered genes like MyoD1 and myogenin in muscle determination and differentiation (Braun *et al.*, 1989a; 1989b; Montarras *et al.*, 1989; Eftimie *et al.*, 1991; Ott *et al.*, 1991; Florini *et al.*, 1991). Probes against these genes are also used to identify myogenic cells *in vivo*. (Grounds *et al.*, 1992).

A large number of investigations on the importance of myogenic cells in muscle growth and regeneration was, and is, medically orientated. Investigations included the existence of differences between myosatellite cells of normal and dystrophic individuals (Ishimoto *et al.*, 1983; Ontell *et al.*, 1984; Wright, 1985; Terasawa, 1986; Cossu *et al.*, 1986; Grounds,

1990) and the role of myosatellite cells in regeneration (Snow, 1977; Allbrook, 1981; Schultz *et al.*, 1986). They even include the possible use of myosatellite cells in gen therapy; the curing of inherited diseases by injection of genetically altered myosatellite cells (Hoffman, 1991; Barr and Leiden, 1991; Dhawan *et al.*, 1991).

Myosatellite cells in fish

In investigations concerning fish muscle growth, (a.o. Kryvi, 1975; Stickland, 1983; Akster, 1983; Romanello *et al.*, 1987; Veggetti *et al.*, 1990; Battram and Johnston, 1991) myosatellite cells are also considered important. However, in these studies mainly the presence of the myosatellite cells, their morphology, or the percentage of myonuclei that are myosatellite cells is investigated. Therefore, the importance of myosatellite cells in fish muscle growth is largely based upon analogy with the mammalian and avian system of muscle growth.

Myosatellite cells and hyperplasia

Myosatellite cells are not only thought to play a role in hypertrophy of muscle tissue, but also in hyperplasia; both in exercise induced hyperplasia (McCormick and Shultz, 1992) in mammals and birds, and in hyperplasia in fish, (ao. Veggetti *et al.*, 1990).

In mammals and birds the myosin isotype of of young fibres developing in vivo is different form that of adult fibres and similar to that of myotubes formed by fused isolated myosatellite cells (Whalen *et al.*, 1978; Matsuda *et al.*, 1983; Yamada *et al.*, 1989; Düsterhöft *et al.*, 1990). In fish, newly formed small fibres also show (immuno)histochemical differences with mature (large) muscle fibres (van Raamsdonk *et al.*, 1982; Akster, 1983; Rowlerson *et al.*, 1985; Scapolo *et al.*, 1988). Myotubes formed by myosatellite cells that we isolated from carp of 5 cm standard length also contain an isotype of myosin different from that found in larval and adult muscle tissue (B. Fauconneau, INRA, Rennes, France, personal communication). This suggests that, in fish (carp), new fibres are *de novo* formed from myosatellite cells. The presence of so called myosatellite-fibres (myosatellite cells that

already contain a small myofibril, Koumans *et al*, 1990) gives an other indication that myosatellite cells play a role hyperplasia.
However, the process of formation of new fibres can be species depending, as in some fish species fibre-splitting (Scapolo *et al.*, 1984; Willemse & Liewma Noordanus, 1984; Romanello *et al.*, 1987) seems a more obvious way of formation of additional muscle fibres than *de novo* formation from myosatellite cells.

Design of the present study

Although extensive studies have been carried out on the role of myosatellite cells in muscle growth of mammals and birds, only very few studies on the role of myosatellite cells in fish muscle growth are known (Kryvi and Eide, 1977 (shark); Powell *et al.*, 1989 (rainbow trout)). The importance of myosatellite cells in fish muscle growth is therefore largely based upon analogy with the mammalian and avian system of muscle growth. The investigations described in this thesis were designed to elucidate the role of myosatellite cells in post hatching muscle growth of carp (*Cyprinus carpio* L.), as an example of teleost fishes. The following questions were addressed:

- *Can myosatellite cells of carp be isolated, and do they differentiate in vitro, forming multi-nucleated myotubes, in a way comparable to isolated myosatellite cells of mammals and birds?* (Chapter II).

As no method for the isolation of myosatellite cells from fish muscle existed we had to develop our own method (by modifying available techniques described for mammalian and avian muscle). Identification of isolated myosatellite cells was based on (ultrastructural)morphology, immunohistochemical demonstration of desmin (a muscle specific intermediate filament protein), and fusion of the isolated cells into myotubes (formation of myofibrils).

- Are carp myosatellite cells, like those of mammals and birds, responsible for hypertrophy of fish muscle?

To answer parts of this question we studied, in white axial muscle of carp with a standard length between 3 cm and 56 cm, the relation between the numbers of myosatellite cells and the (increase in) numbers of myonuclei per fish (Chapters III and VI). The number of myosatellite cells was electronmicroscopically (TEM) determined as a percentage of the myonuclei. The number of myonuclei per gram of tissue was calculated by combining TEM data on the percentage of total nuclei in muscle that were myonuclei with biochemical data on the amount of DNA per gram of tissue and per nucleus. The total number of myonuclei was calculated by multiplying the number of nuclei per gram of tissue with the estimated amount of white axial muscle per fish.

Two lightmicroscopical methods promising a relatively quick way to estimate the number of myosatellite cells present in a tissue sample were also developed and tested. We investigated whether the yield of the isolation procedure was proportional to the calculated number of myosatellite cells in the tissue, as this would present a relatively fast, more qualitative, method for determination of differences in myosatellite content of muscle samples. We also determined the percentage of total and of heterochromatic nuclei inside the muscle fibres' basal lamina (myosatellite nuclei) in tissue sections which were stained using an antibody against laminin, to estimated the percentage of myonuclei being myosatellite nuclei. The percentages of myosatellite nuclei obtained with this method were compared with those obtained by the TEM method (Chapter III).

As there appeared to be a relatively low number of myosatellite cells in white axial muscle of carp, we investigated the plausibility of the generation of all of the additional myonuclei from those few cells. To do this, we calculated the maximal allowable duration of the cell-cycle time of carp myosatellite cells to account for the observed growth. We compared this calculated cell-cycle time to known myosatellite cell-cycle times of other animals.

- Another important question addressed in the present study is: Do subpopulations of myosatellite cells exist and are they involved in different mechanisms of muscle growth?

An unexpected large part of the population of myosatellite cells isolated from young carp of 5 cm standard length appeared to be postmitotic (desmin positive)(Chapter II). In carp

smaller than about 15 cm standard length the DNA content of the white axial muscle tissue decreases, but in larger carp the DNA content slowly starts to increase again (Chapters III and V). Therefore, a large percentage of postmitotic myosatellite cells in larger carp seems highly unlikely. We investigated whether the percentage of *in vitro* proliferating (5-bromo-2'-deoxyuridine (BrdU), a thymidine analog, positive) myosatellite cells and postmitotic (desmin positive) myosatellite cells was correlated with the length of the 'donor' fish and with the changes in the DNA content of its tissue. An *in vitro* change in the percentage of the experimentally defined subpopulations of desmin positive and BrdU positive myosatellite cells strongly suggests an actual *in vivo* change in the population of myosatellite cells (Chapter IV).

If different subpopulations of myosatellite cells are involved in hyperplastic and in hypertrophic muscle growth the relative size of these subpopulations has to change during growth as the occurrence of hyperplasia is known to be related with fish length. Therefore, we investigated the relation between the *in vitro* change in the percentage of differentiated and proliferating myosatellite cells, and the relative importance of hyperplasia and hypertrophy during growth (Chapter V). To investigate the occurrence of hyperplasia we determined the diameter distribution of white axial muscle fibres of carp ranging from 3.4 cm - 56 cm standard length.

- Is it possible to develop a lightmicroscopical method that can be used for the unambiguous in situ identification of myosatellite cells.?

An obvious way to achieve such a goal is the production of antibodies against markers specific for (undifferentiated) myosatellite cells. To obtain such antibodies we employed the monoclonal antibody technique (Chapter VII), in which myosatellite cells, isolated from carp of about 5 cm standard length, were used as an antigen.

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CHAPTER II

MYOSATELLITE CELLS OF *Cyprinus carpio* (Teleostei) IN VITRO: ISOLATION, RECOGNITION AND DIFFERENTIATION

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Summary

We have developed a method for the dissociation and purification of myosatellite cells from white epaxial muscle of carp. The dissociated myosatellite cells were identified by their morphology, their ultrastructure, the formation of multinucleated myotubes containing myofibrils and the immunocytochemical demonstration of desmin. Desmin and 5'bromo-2'deoxyuridine (BrdU) were used to identify terminally differentiated and proliferating myosatellite cells respectively. The in vitro behavior of myosatellite cells dissociated from carp of 5 cm standard length differed from that described for myosatellite cells of mammals and birds. No substantial proliferation of the myosatellite cells could be observed. Most cells were differentiated (desmin positive, BrdU negative) 17 hours after plating, regardless of the medium used. This indicates that the investigated white epaxial muscle of carp of 5 cm standard length contains subpopulations of myosatellite cells, arrested at various stages of differentiation.

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Introduction

Myosatellite cells, first described by Mauro (1961), are small spindle-shaped cells having a heterochromatic nucleus. They are situated between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibers. During growth and regeneration of muscle fibers, these cells are the source of additional myonuclei. Myosatellite cells are present in the striated muscle of all investigated vertebrate species (for review, see Campion, 1984). Although the presence of myosatellite cells has been demonstrated in the skeletal muscle of some species of fish (shark, Kryvi, 1975; Kryvi and Eide, 1977; eel, Willemse and van den Berg, 1978; carp, Akster, 1983; trout, Powel *et al.*, 1989) our knowledge of the role of myosatellite cells in the growth of fish muscle is still limited.

The growth of fish muscle differs from that of mammals and birds. In fast growing fish species, hyperplasia (addition of new fibers) plays an important role in muscle growth even after the juvenile stage (Greer-Walker, 1970; Stickland, 1983; Weatherly and Gill, 1984). In mammals and birds, as a rule, the increase in the number of muscle fibers stops shortly after birth (Goldspink, 1972, 1974; Campion, 1984). Further muscle growth is mainly the result of hypertrophy (outgrowth of existing fibers). This difference in muscle growth may be reflected by differences in the myosatellite cell populations of these animals.

Myosatellite cells in situ have been studied by light microscopic methods (Ontell, 1974; Bischoff, 1986; Mulvaney *et al.*, 1988). But definitive proof wether a cell is a myosatellite cell can often only be provided by electron microscopy. This method, however, is time-consuming and presents difficulties when immunocytochemical techniques are used. The study of myosatellite cells in vitro has the important advantage that light microscopy can be used to recognize the myosatellite cells in culture on the basis of their morphology (Lipton, 1977), thus making combined morphological and immunocytochemical studies possible (Kaufman and Foster, 1985; Allen, 1987).

As a first step in studying the role of myosatellite cells in the growth of fish muscle, we developed a method for the dissociation of these cells from fish (carp) skeletal muscle and their subsequent culture in vitro. We used immunocytochemical techniques to localize a) desmin, a muscle-specific intermediate filament protein and marker of differentiated myosatellite cells and b) BrdU (5'bromo-2'deoxyuridine), a marker of proliferating cells (Kaufman and Foster, 1988).

Materials and methods

Animals

We used carp of 5 cm standard length that were reared at our institute at a temperature of 23°C. Preliminary transmission electron microscopic studies (J.T.M. Koumans and H.A. Akster, unpublished results) revealed that, in animals of this length, the percentage of myosatellite nuclei with respect to total muscle nuclei is high (± 10% in white epaxial muscle). The fish were anesthetized with MS-222 (Sandoz). If used for the isolation of myosatellite cells, the fish were immersed in 70% alcohol for 30 s to sterilize the external surfaces.

Isolation of myosatellite cells

White epaxial muscle was excised under sterile conditions and collected in complete medium (15% horse serum (HS; Flow) in 90% Dulbecco's modified Eagle medium (DMEM) (Gibco) plus PS (penicillin 50 U/ml, streptomycin 100 μg/ml) on ice. Subsequently, the tissue was minced, the fragments were centrifuged (300 g, 5 min) and washed twice in 90% DMEM to remove serum components.

The tissue fragments were treated with collagenase Type Ia (Sigma) in 90% DMEM at a final concentration of 0.2%, for 45 min at room temperature with gentle agitation; 5 ml collagenase solution was used per gram of tissue. The fragments were collected by centrifugation (300 g, 5 min), washed once with 90% DMEM, triturated through a 10 ml pipette five times and centrifuged again (300 g, 5 min).

The pellet was resuspended in a 0.1% trypsin (Dyfco; 1:250) solution in 90% DMEM (5 ml per gram original tissue), incubated for 15 min at room temperature with gentle agitation and centrifuged for 1 min at 300 g. The supernatant was aspirated and diluted with twice the volume of cold complete medium to block further trypsin activity. The collected tissue fragments (pellet) were given a second trypsin digestion step, similar to the first. After this second digestion, the suspension was diluted with twice the volume of cold complete medium. This diluted suspension and the diluted supernatant from the first digestion were centrifuged for 15 min at 300 g, and the resulting pellets were triturated in 5 ml complete medium, 5 times using a 10 ml pipette and 5 times using a 5 ml pipette. The resulting

suspensions were filtered twice through a 100 μm filter gauze, and the cells in the filtrate were washed once again in complete medium and plated at 10^6 cells/ml.

In experiments in which BrdU was used, this was added to the 90% DMEM, complete medium and the enzyme solutions at a final concentration of 10 μM. In one experiment, a solution of 10 mg/ml of BrdU in PBS was injected intraperitoneally, to give 10 g BrdU/kg body weight, 4 h before dissociation, this to ensure that every cell in S-phase just before and during the dissociation procedure would be labeled.

Purification of the isolated myosatellite cells

To purify the myosatellite cells from the major part of the "contaminating cells", two methods were used. In the first method, the "crude" cell suspension, produced by the dissociation, was preplated (Yaffe, 1968) for 15 min on a non-coated substratum with subsequent plating of the non-attached cells (depleted of fibroblast-like cells) on a gelatin-coated substratum. In the second method, the crude cell suspension was plated on a laminin-coated (Foster *et al.*, 1987, slightly modified) substratum and incubated for 15 min with no disturbance. The medium with the non-attached cells was aspirated and new complete medium was added to the attached cells (enriched in myosatellite cells). To apply the laminin coating, the substratum was precoated with a 100 μg/ml solution of poly-L-lysin (PLL) (Sigma, Mw > 300000) in distilled water at an amount of 16 μg/cm^2 for 2 h at 25°C. After this precoating, the PLL--solution was aspirated, the substratum was washed once with distilled water, air dried, covered with a solution of 20 μg/ml laminin (Sigma) in 90% DMEM at 2 μg/cm^2 and incubated for 24 h at 25°C. Immediately prior to the plating of the cell suspension, the laminin solution was removed by aspiration.

Culturing of the cells

Cells were cultured in complete medium at 25°C in a water-saturated atmosphere containing 5% CO_2 in air. Culture medium was changed every day. Besides the complete medium mentioned earlier (15% horse serum in 90% DMEM plus PS), the following media were also tested: 15% HS + 2% chicken embryo extract (CEE), 15% HS + 2% CEE + 5% pooled carp serum, 15% HS + 2% CEE + 10% fetal calf serum (FCS), or 15% FCS + 10% HS, all in 90% DMEM plus PS. In some experiments the complete medium was

replaced by medium with less serum (1% HS in 90% DMEM plus PS), after 7 days or after prolonged (3 weeks) culturing of the cells in complete medium.

Depending on the planned use of the cells, they were cultured in a 25 cm^2 culture flask, in a culture disk on glass coverslips, or in a 24-well culture plate on nucleopore filters. The first observations were routinely made 17 h after plating and subsequently every 24 h. The total amount of cells present in the culture flasks (surface area of the culture substratum was 2500 mm^2) was estimated by counting the number of cells present in 10 randomly chosen fields, each with an area of 0.744 mm^2.

Transmission electron microscopy (TEM) of cultured cells

Cells in culture flasks were used for TEM. The flasks were rinsed three times with 90% DMEM to remove serum components. The cells were fixed in situ with Karnovsky's (1965) fixative diluted 1:2 with 0.1 M Na cacodylate buffer (pH 7.3), postfixed with 1% OsO_4 on ice, dehydrated in graded alcohols, incubated in a 1:1 absolute alcohol:Epon mixture and embedded in Epon. The optical quality of the polymerized Epon permitted the recognition and marking of the cells using phase-contrast microscopy. Those parts of the flasks containing the marked cells were cut out. The application of pressure parallel to the plane of the flask caused separation of the part of the flask and the Epon containing the cell. Ultrathin sections were cut parallel or transverse to the plane of the flask on a Reichert-Jung Ultracut E; they were contrasted with uranyl acetate and lead citrate and examined in a Philips EM 201C electron microscope.

Scanning electron microscopy (SEM) of cultured cells

Cells cultured on glass coverslips or on nucleopore filters were used for SEM. Coverslips and filters were rinsed three times with 90% DMEM, fixed as described for TEM, rinsed with distilled water, dehydrated with graded alcohols, critical point dried (Anderson 1951), coated with gold in a vacuum evaporator and examined in a Philips 535 scanning electron microscope at 20 kV.

Electron microscopy of myosatellite cells in situ

Karnovsky's (1965) fixative was injected into the muscle. After 30 min fixation in situ, the muscle was excised, cut in small pieces and fixed for a further hour in Karnovsky's

fixative. Subsequently, the tissue was postfixed in 1% OsO_4, dehydrated in graded alcohols and propylene oxide and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Immunocytochemistry of cultured cells

Cells on glass coverslips were rinsed three times with 90% DMEM and fixed in cold (-20°C) acetone for 5 min.

Immunocytochemical demonstration of desmin As a first antiserum, we used a polyclonal rabbit antiserum against chicken gizzard desmin (Ramaekers *et al.*, 1983), kindly provided by Dr. F.C.S. Ramaekers of the University of Nijmegen, the Netherlands. The antiserum was diluted in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA). Immunohistochemical reactions with this antiserum on cryostat sections of carp tissue, showed that it only reacted specifically with desmin in the muscle tissue. Further controls were carried out by omission of steps of the immunohistochemical reaction and preabsorption of the primary antiserum with chicken gizzard desmin.

As a second antiserum, we used goat or swine anti rabbit-Ig conjugated to TRITC (Tetramethyl-rhodamine-iso-thiocyanate) or to HRP (horse radish peroxidase) (Dakopatts), diluted in PBS. After each incubation step the sections were rinsed twice for 5 min each in TRIS/NaCl/T-20 (0.05% TRIS, 1% NaCl and 0.05% Tween-20, pH 7.6) and 3 times for 5 min each in 0.05 M TRIS, pH 7.6 (TBS, TRIS buffered saline).

Immunocytochemical demonstration of incorporated BrdU After fixation, the cells were incubated in 2 N HCl for 20 min at room temperature to denaturate the DNA, neutralized by a wash in 0.1 M Na-tetraborate, pH 8.5 and washed twice in TBS. This procedure does not interfere with the immunocytochemical demonstration of desmin; this is very convenient in desmin/ BrdU double staining. As a first antiserum, we used a monoclonal anti-BrdU antibody (Eurodiagnostics) and as a second antiserum, rabbit or goat anti mouse-Ig conjugated to TRITC, FITC or HRP (Dakopatts). The sections were rinsed as described for the desmin staining.

Results

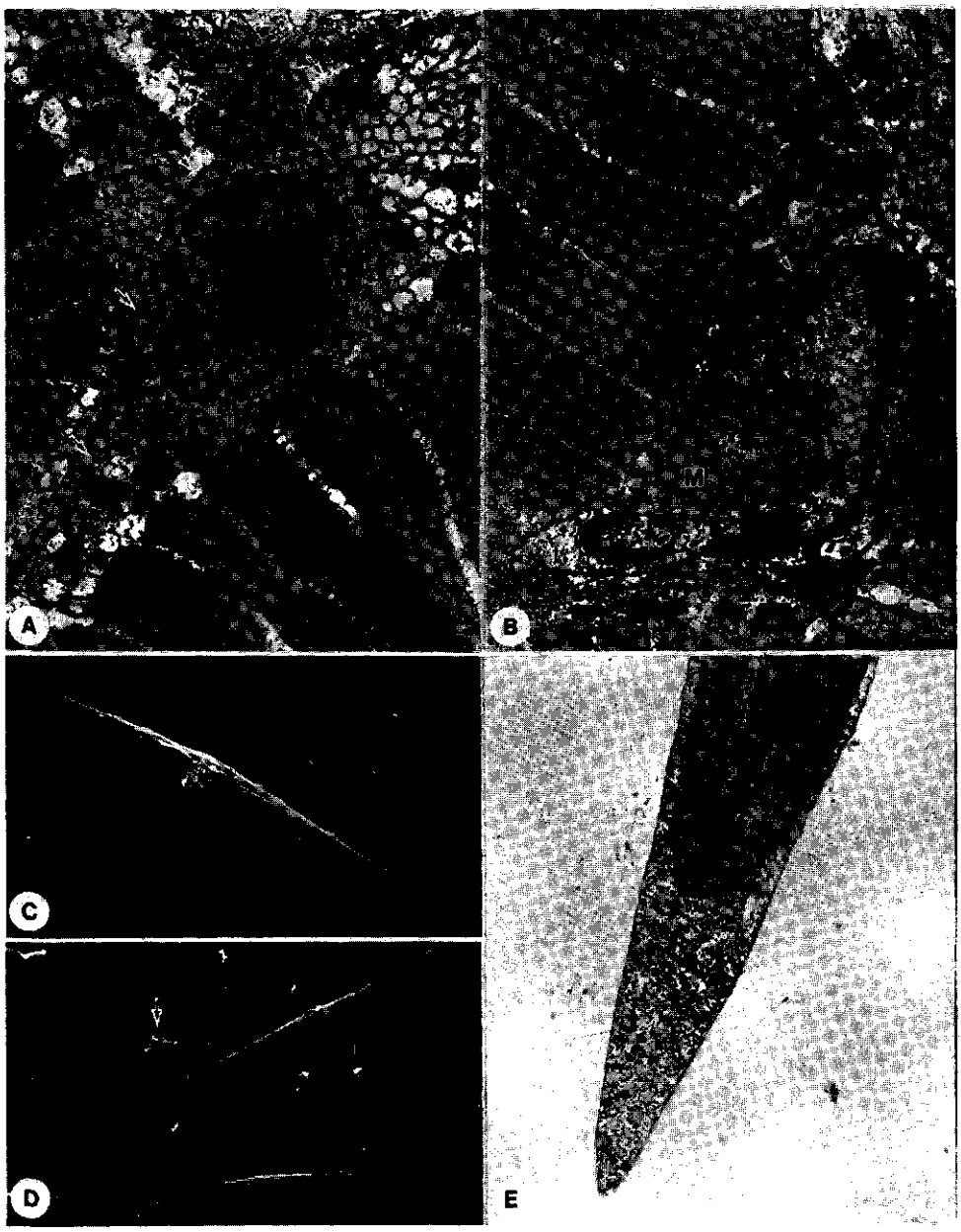
Morphology and ultrastructure of myosatellite cells

Myosatellite cells in situ Myosatellite cells of the carp are mononucleated cells. They have little cytoplasm which contains free ribosomes and polysomes, and a paucity of other organelles (Fig. 1A), with some variation in the amounts of perinuclear cytoplasm and polyribosomes. The myosatellite cells lie closely apposed to a myofiber. They are covered by the basal lamina (Fig. 1A) of the myofiber. As a rule, myosatellite cells have a heterochromatic nucleus, in contrast to the larger euchromatic nuclei of the myofibers.

We also found cells that resembled myosatellite cells in their position under the basal lamina of muscle fibers, but they contained small myofibrils. These so called myosatellite fibers have also been reported for other vertebrates (Takahama *et al.*, 1984) and have previously been described in carp (Akster, 1983). The myofibrils of these satellite fibers are often found close to a small indentation of their nucleus. Generaly basal lamina material occupies part of the space between the myosatellite fibers and the muscle fibers (Fig. 1B), suggesting an ingrowth of the basal lamina. This could mean that myosatellite fibers are a differentiation stage in the formation of new fibers from myosatellite cells.

Myosatellite cells in vitro Myosatellite cells of the carp in vitro are small (length 73.2 $\pm$ 15.4 μm, width 3.5 $\pm$ 0.7 μm, mean $\pm$ sd) spindle-shaped cells (Figs. 1C and 1D). They have little cytoplasm and often a small part of the membrane at one or both ends of the cell is a bit ruffled (Fig. 1D). The ultrastructure of these cells (Fig. 1E), resembles that of myosatellite cells in situ. They differ considerably from fibroblast-like cells, which have a more stellate appearance and more cytoplasm and are rich in rough endoplasmatic reticulum, as has also been described by Konigsberg (1963) and Lipton (1977).

Other cell types A few large multinucleated syncytia could be recognized, 17 h after isolation in addition to myosatellite cells and fibroblastlike cells. These cells exhibited cross striations that were visible light microscopically. Their nuclei were centrally arranged and not at the periphery of the cells as in "adult" myofibers. These myotube-like-structures must already have been present in the intact muscle, because, in view of the time course of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development of other myotubes, it seems unlikely that they were formed in culture in such a short time.

After three or more days in culture,"giant" cells (syncytia of monocytes or macrophages; Bayne, 1986) could be seen.

Dissociation and purification

The enzymatic digestion procedure that we have developed yielded about 10^6 cells per gram of epaxial white muscle when the cells were plated in a gelatin-coated culture flask after preplating in a non-coated culture flask. About 25% of these cells could be recognised as myosatellite cells on the basis of their typical spindle-shaped morphology.

When the "crude" cell suspension was plated directly into a laminin-coated culture flask, $3x10^5$ cells per gram original tissue attached to this specific coating within 15 min. On the basis of their morphology, 80%-90% of these cells could be recognised as myosatellite cells or small myotubes.

The constancy of the yield of myosatellite cells per gram white epaxial muscle in carp of 5 cm standard length i.e. 251400 $\pm$ 16284, mean $\pm$ sd (n = 10), was very remarkable in view of the complexity of the dissociation procedure. The yield of myosatellite cells declined noticeably when carp larger than 6 cm standard length were used.

Culturing of the cells

At the time of the first observation (17 h after plating), some of the myosatellite cells were engaged in the process of "alignment" (alignment of single myoblasts in long chains; Inestrosa, 1982), and the first fusions into small multinucleated myotubes had occurred. Three days after isolation, the first larger myotubes with more than 10 nuclei per cell had

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Fig. 1. **Ultrastructure and morphology of carp myosatellite cells. A. Ultrastructure of myosatellite cell in situ. Note the basal lamina (arrows) covering both the myosatellite cell and the myofiber, x 13600. B. Ultrastructure of myosatellite fiber in situ. Note the ingrowth of the basal lamina (arrow) between the myosatellite fiber and the myofiber, and the myofibril marked M, x 19500. C. Myosatellite cell in vitro (17 h after plating on a laminin-coated substrate). Scanning electron microscopy, x 1450. D. Scanning electron microscopical picture of myosatellite cells in vitro. Note the ruffled end (arrow), x 500. E. Ultrastructure of myosatellite cell in vitro. Note the abundancy of free ribosomes, the scarcity of other organelles. This is similar to the ultrastructure of the myosatellite cell in situ (Fig 1A), x 11500.**

Fig. 2. **Morphology and ultrastructure of carp myotubes formed in vitro on a laminin-coating.** A. **Large myotube formed 5 days after plating of the myosatellite cells. Note the amount of nuclei (arrows). Phase contrast microscopy, x 430. B. formation of myofibrils in a carp myotube in vitro. TEM, x 38000.**

formed (Fig. 2A). With TEM (Fig. 2B) formation of myofibrils in these myotubes could be demonstrated. After five days, the first myotubes showed vaguely visible cross striation under the light microscope. At this time almost all of the spindle-shaped non fused cells had disappeared as result of their fusing into myotubes.

During these five days of culturing, no significant increase in the number of nuclei in myogenic cells was found, indicating that proliferation of these cells was only limited. The number of fibroblast-like cells however increased rapidly.

The use of different types of complete medium or the changing to differentiation medium had no influence on the behavior of the cultured cells.

Immunocytochemistry

Reactivity with anti-desmin All myosatellite cells engaged in "alignment" or fused into myotubes at all stages of development, were desmin-positive (Fig 3A). At the time of the first observations (17 h after plating), up to 80% of the myosatellite cells were desmin-positive (Fig. 3B). This in contrast to fibroblast-like cells which showed no, or sometimes only a very faint, reaction with the anti-desmin serum (Fig. 3C).

A reaction carried out 1 h after plating showed that at that time 15.8 % of the total number of cells was desmin positive (that is about 20% of the myosatellite cells). Because many of the cells had not yet obtained their typical spindle-shaped morphology, an exact percentage of desmin-positive myosatellite cells could not be calculated.

Reactivity with anti-BrdU Only a few (10%) myosatellite cells were BrdU positive 17 h after plating (Figs. 4A and 4B). Very few myosatellite cells were both desmin- and BrdU-positive (< 1%). We found no effect of BrdU on the time course of development or the behavior of the myosatellite cells in culture, although incorporated BrdU interferes with the differentiation of myoblasts (Bischoff and Holzer, 1970). This is probably due to the paucety of BrdU labeled cells in our cultures. In the experiment where BrdU was injected into the carp 4 h before dissociation, cryosections of the muscle tissue revealed that in situ labeling occured.

The limited amount of proliferation, regardless of the type of medium used, the presence of many desmin positive myosatellite cells, the engagement of many cells in "alignment" and the fact that only very few cells were both BrdU and desmin positive, even

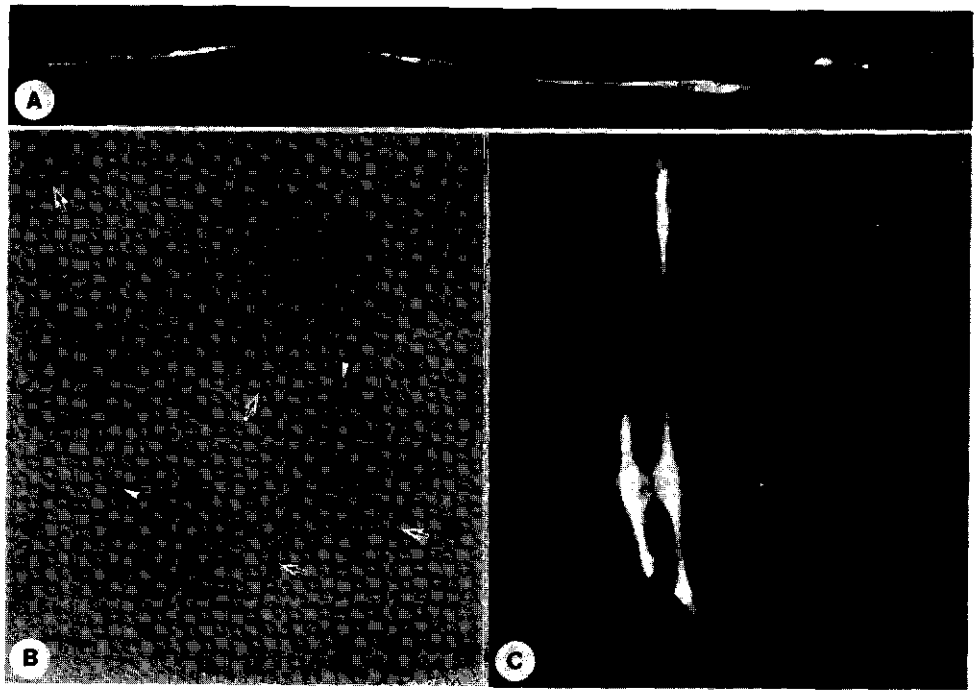

Fig. 3. **Immunocytochemical demonstration of desmin in carp myosatellite cells in vitro plated on a laminin-coating. A. Desmin-positive myosatellite cells in alignment or fused into a small myotube. Fluorescence microscopy, x 360. B. View of a culture 17 h after plating. Note the dark stained (arrows) desmin-positive myosatellite cells , and the lightly stained (arrow-heads) desmin-negative myosatellite- and fibroblastlike-cells. HRP/DAB staining, HE counterstaining, x 180. C. Two desmin-positive myosatellite cells and three scarcely visible desmin-negative fibroblastlike cells. Fluorescence microscopy, x 540.**

after 179 h in culture, all indicate that, 17 h after plating, many of the harvested cells were already differentiated.

Discussion

Recognition of myosatellite cells in vitro

Identification of myosatellite cells in vitro mainly depends upon morphological criteria, on the recognition of their ability to form muscle-specific proteins and to fuse into multinucleated myotubes (Lipton, 1977; Allen, 1987; Kaufmann and Foster, 1988). Although

antisera against markers specific for differentiation stages of myogenic cells have been developed (Wakshull *et al.*, 1983; Walsh *et al.*, 1984; Kaufman and Foster, 1985; 1988; Rong *et al.*, 1987), markers for myosatellite cells in general have not yet been obtained. The use of cell morphology to identify myosatellite cells in vitro presents problems because the morphology and ultrastructure of cultured myosatellite cells can be altered by culture conditions, such as the composition of the medium, or the use of a specific coating of the substrate (Lipton, 1977; Öcalan *et al.*, 1988). Recognition of myosatellite cells in mitosis is not possible, because these cells have lost their specific spindle shape (Darnell *et al.*, 1986). Thus the ultimate criterion for recognition of myosatellite cells in vitro is their ability to fuse and to develop into myotubes containing specific muscle proteins. In retrospect, these cells could be identified as myosatellite cells.

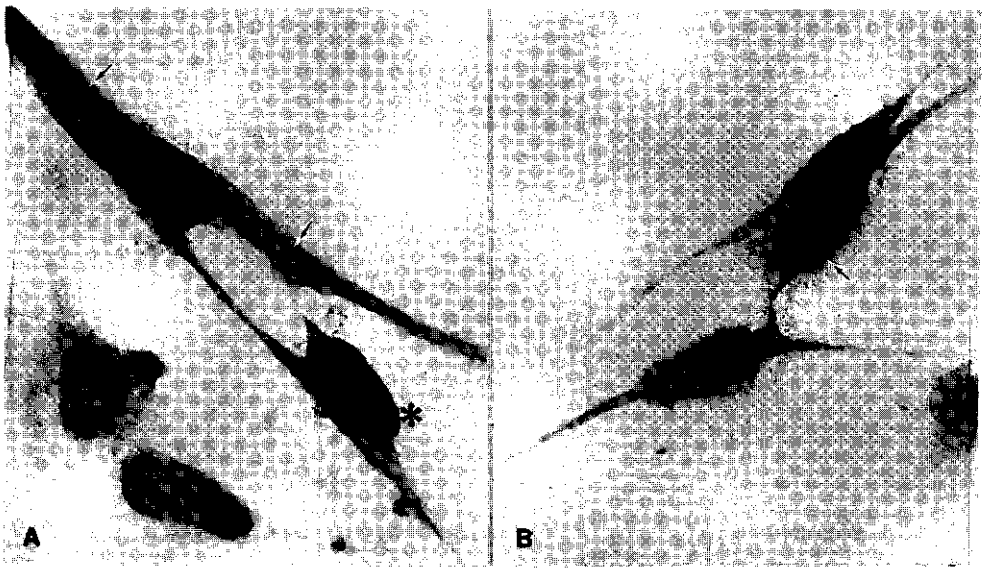

Fig. 4. **Double staining for desmin and BrdU, 17 h after plating on a laminin coating. HRP/DAB staining, HE counterstaining, x 440. A. Part of a desmin-postive (arrows), BrdU-negative small myotube together with a desmin-negative, BrdU-positive (asterik) myosatellite cell, and 2 desmin-negative, BrdU-negative fibroblastlike cells. B. Two desmin-negative, BrdU-negative myosatellite cells together with a desmin-negative, BrdU-positive (arrow) myosatellite cell.**

Our evidence for the identification of our cultured cells as myosatellite cells can be summarized as follows:

1) We found that, 17 h after plating 80-90% of the cells present on the laminin-coated substrate had a spindle shape as has been described for myosatellite cells of mammals and birds (Lipton, 1977; Campion, 1984). They also resemble the cells that Powel *et al.* (1989) isolated from trout skeletal muscle and that they believe to be myosatellite cells.

2) The ultrastructure of these cells resembled that of myosatellite cells in situ with respect to the paucity of organelles other than free ribosomes and polysomes.

3) The cells showed alignment and fused into myotubes developing cross striation.

4) Many of these spindle-shaped cells (up to 80%) also showed another muscle characteristic; they reacted with an antiserum to desmin, a muscle-specific intermediate filament protein (Lazarides, 1982; Kaufman and Foster, 1988).

In view of these observations we concluded that these cells are indeed myosatellite cells.

Purification

Methods, described in the literature, for the purification of myosatellite cells from the crude mixture of cells and debris obtained by enzymatic digestion of muscle tissue are: preplating (Yaffe, 1968), Percoll gradient centrifugation (Yablonka-Reuveni *et al.*, 1987), cell sorting techniques (Yablonka-Reuveni, 1988), and use of a laminin coating of the substratum (Kühl *et al.*, 1986; Foster *et al.*, 1987; Öcalan *et al.*, 1988). Also selective detachment of myosatellite cells from their substratum by cytochalasin B (Sanger, 1974) or by a specific monoclonal antibody (CSAT, Cell Substratum ATtachment, in chicken, Neff *et al.*, 1982) have been used.

Carp myosatellite cells did not survive Percoll gradient centrifugation or the use of cytochalasin B. It was impossible to recognise the myosatellite cells as a separate population, from the light scatter signals in a cell sorter.

Preplating can be used to purify a myosatellite cell population. However to achieve a purification level similar to that obtained with plating on a laminin coating, this procedure has to be repeated many times and results in a considerable loss of cells. The use of a laminin coating however gives a high purification without substantial loss of myosatellite cells. In addition to its effect on the attachment of the cells, laminin is also known to have a strong promotional effect on proliferating and on differentiating myosatellite cells of

mammals (Foster *et al.*, 1987; Öcalan *et al.*,1988). We therefore decided to use laminin coating of the substrate as a way to purify the myosatellite cells.

Differentiation

The earliest differentiation characteristic that we could discern was the presence of desmin. We found that the presence of desmin precedes fusion, as was also found for myosatellite cells of mammals and birds (Kaufman and Foster, 1988).

In mammals replicating myosatellite cells (as revealed by BrdU labeling) may already be desmin-positive, whereas in chicken probably only postmitotic myosatellite cells are desmin-positive (Kaufman and Foster, 1988). We have found that only a small fraction (< 1%) of the myosatellite cells are both desmin- and BrdU positive. This suggest that, in carp, desmin is also a characteristic of postmitotic myosatellite cells.

Proliferation of carp myosatellite cells in culture was only moderate, as was apparent from the lack of increase in myogenic cell nuclei and from the low number (10%) of myosatellite cells that were BrdU-positive 17 h after plating. We checked whether a slow diffusion rate of BrdU into the tissue fragments or a very long cell cycle could have caused an underestimate of the number of proliferating cells, by using continuous labeling, starting in vivo and continuing for 179 h in vitro. This procedure did not change the percentage of labeled nuclei. Attempts to enhance proliferation by changing the formula of the medium did not succeed. Proliferation remained moderate and the majority of the myosatellite cells rapidly differentiated and fused into myotubes. Therefore most of the myogenic cells in culture were probably postmitotic.

According to the "lineage model" of Quinn *et al.*, (1984; 1985), myogenic cells that differentiate from stem cell to postmitotic terminally differentiated muscle cell have to progress through a fixed number of divisions. The rate of progress through this lineage can be influenced by environmental factors. This means that muscle may contain several subpopulations of myosatellite cells arrested at various stages of differentiation (Quinn *et al.*, 1985; Grounds and McGeachie, 1989). The presence of a high percentage of BrdU-negative and desmin-positive myosatellite cells in culture 17 h after plating suggests that these cells have progressed through the entire, or almost the entire, differentiation lineage before dissociation. This would mean that white epaxial muscle of carp of 5 cm standard length

contains a high percentage of differentiated myosatellite cells and a relatively small percentage of "stem cells" and cells in the earlier stages of differentiation.

Carp of 5 cm standard length are young fish. We now think it likely that the comparative richness in myosatellite cells in carp of this size is the result of the presence of a large number of differentiated myosatellite cells. Quinn *et al.*, (1984) and Yablonka-Reuveni *et al.*, (1987) have shown that young chicken embryos have a higher percentage of myogenic cells in the later stages of differentiation than that is found in older embryos. Therefore the high percentage of postmitotic myosatellite cells in carp of 5 cm standard length could be an age-dependent phenomenon. However, the age dependence of myosatellite cell differentiation in carp and its function in muscle growth are matters for further investigation.

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CHAPTER III

NUMBERS OF MYOSATELLITE CELLS IN WHITE AXIAL MUSCLE OF GROWING FISH: *Cyprinus carpio* L. (Teleostei)

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Key words: *Myosatellite cells, in-situ, in-vitro, muscle growth, white axial muscle, Cyprinus carpio L. (Teleostei)*

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Summary

By means of transmission electron microscopy (TEM), the percentage of myosatellite cells was shown to decrease from about 6% in carp of 5 cm standard length (SL) to less than 1% in carp larger than 18 cm SL. The ratio between muscle nuclei and non muscle nuclei remained constant. These TEM data, combined with data on the amount of DNA per gram of muscle tissue and per nucleus, were used to calculate the numbers of myosatellite cells per gram of tissue (TEM-DNA method). Total numbers of myosatellite cells could be calculated from the TEM-DNA data and the calculated amounts of muscle tissue per fish. After a slight initial increase, the total number of myosatellite cells in the white axial muscle of a carp appears to be rather constant during the growth phase of the fish. But the myosatellite cells become more and more diluted over an increasing number of myonuclei with age. In addition, the reliability of two new light microscopic methods for the determination of numbers of myosatellite cells was examined. The percentages of myosatellite cells were

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determined by counting the numbers of total nuclei and of heterochromatic nuclei situated inside the muscle fibers' basal laminae which were stained using an antibody against laminin. These percentages were not significantly different from those determined with TEM. The yield of myosatellite cells per gram of muscle tissue, isolated with a previously developed dissociation method, showed a direct relation to the number of myosatellite cells calculated to be present in the tissue (TEM-DNA method), but at a 1% level. Both methods are alternative ways to determine numbers of myosatellite cells when it is impossible or difficult to use TEM (e.g., large sample sizes, combination with immuno-histochemical methods).

Introduction

Study of muscle growth in fish is not only interesting from a viewpoint of fish production, but it also confronts us with a dynamic system in which hyperplasia and hypertrophy play roles of changing importance, influenced by factors such as genetic background, age, and environment. This interplay continues even after the juvenile state, in contrast to the situation in mammals and birds where, as a rule, an increase in the number of fibers stops shortly after birth (Goldspink, 1972).

Myosatellite cells play an important role in muscle growth. They supply additional muscle nuclei to muscle fibers that increase in volume (hypertrophy) (Moss and Leblond, 1970; 1971; Allen *et al.*,, 1979; Campion, 1984). They also play a role in the formation of new fibers in regeneration (Allbrook, 1981; Ontell *et al.*,, 1984; Carlson, 1986) and probably as well in hyperplasia (Veggetti *et al.*,, 1990; White and Esser, 1990). Myosatellite cells are present in the striated muscle of all investigated vertebrate species (for review, see Campion 1984) including fish, (Nag and Nursall, 1972; Kryvi, 1975; Kryvi and Eide, 1977; Sandset and Korneliussen, 1978; Willemse and van den Berg, 1978; Akster, 1983; Koumans *et al.*,, 1990; Veggetti *et al.*,, 1990).

Many problems still have to be solved concerning the role of myosatellite cells in growth of fish muscle. The regulation of recruitment, proliferation, and differentiation of the myosatellite cells in relation to hyperplastic and/or hypertrophic muscle growth is largely unknown.

The central problem in this study is the variation in the total number of myosatellite cells in white axial muscle during growth of the carp. White axial muscle was used because the bulk of muscle in carp consists of this type.

The study of myosatellite cells in situ mainly depends on transmission electron microscopy (TEM) since satellite cells are small and are located between the muscle fiber's basal lamina and the sarcolemma (Mauro, 1961). They contain numerous ribosomes, but other organelles are few. However, the availability of a light microscopic (LM) method would have advantages. It is less time consuming and more suitable for combination with techniques such as autoradiography and immunohistochemistry. Therefore, two new LM methods for estimating the numbers of myosatellite cells in fish muscle were tested by comparing the results obtained by these methods with the results obtained by using TEM. Thus the amounts of myosatellite cells present in the white axial muscle of carp from 5 cm to 18 cm standard length (SL) were determined in three different ways:

a) The percentages of muscle nuclei that were myosatellite cell nuclei were determined by means of TEM. From these data and from the amount of DNA per gram of muscle and per nucleus, the number of myosatellite cells per gram of muscle was calculated (TEM-DNA method).
b) The basal laminae of the muscle fibers were stained using an antiserum against laminin. Then the percentage of myosatellite cells was obtained by determining the percentage of nuclei situated inside these basal laminae that were heterochromatic.
c) Myosatellite cells were isolated from the muscle using a dissociation procedure that already was known to give a surprisingly constant yield of myosatellite cells per gram of muscle (Koumans *et al.*,, 1990).

Materials and methods

Animals and location of muscle

Carp 3 to 56 cm in standard length (SL) and reared at 23°C at our institute were used. The fish were over-anesthetized with MS-222 (Sandoz) and, when used for the isolation of myosatellite cells, immersed in alcohol 70% for 30 sec for external sterilization.

When using fish smaller than 15 cm SL, myosatellite cells were isolated from the entire strip of white epaxial muscle between the pectoral girdle and the anus. In larger fish,

myosatellite cells were isolated from muscle tissue that was excised from three regions along this strip: between the pectoral girdle and the first radius of the dorsal fin, between the 4th and the 7th radii of the dorsal fin (about the middle of this strip), and at the region of the anus.

For differential counting of nuclei (with TEM and with LM) white epaxial muscle was excised between the 4th and 7th radii of the dorsal fin of fish from 3 to 18 cm SL. Pilot experiments revealed that in fish smaller than 2.5 cm no unequivocal recognition of myosatellite cells could be made because a continuous muscle basal lamina was lacking. In fish larger than 18 cm, the percentage of myosatellite cells was so low (<1%) that large sample sizes would be necessary to obtain reliable data from the differential counts.

Fig. 1. Ultrastructure of a myosatellite cell in situ. Note the small amount of cytoplasm, the heterochromatic nucleus, the scarcity of organelles except for ribosomes, and the basal lamina covering both the muscle fiber and the myosatellite cell (arrows). x 20,000.

Transmission electron microscopy

After over-anesthesia of the fish, Karnovsky's (1965) fixative was injected directly into the muscle tissue. After 30 min fixation in situ, muscle was excised, cut in small pieces, fixed for another hour in Karnovsky's fixative, postfixed in 1% OsO_4, dehydrated in graded alcohol and propylene oxide, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate. From each tissue sample, nuclei were counted and distinguished as myosatellite cell nuclei (Fig. 1), muscle fiber nuclei, and nuclei outside the muscle fiber's basal lamina (fibroblast nuclei, endothelial nuclei, blood cell nuclei, etc.). Each sample counted included at least 100 muscle-fiber nuclei. Myosatellite fibers, which resemble myosatellite cells in their position under the muscle fiber basal lamina but contain a few myofibrils (Koumans *et al.*,, 1990), were also counted. For comparison with results obtained by LM, these numbers were added to those of the myosatellite cells.

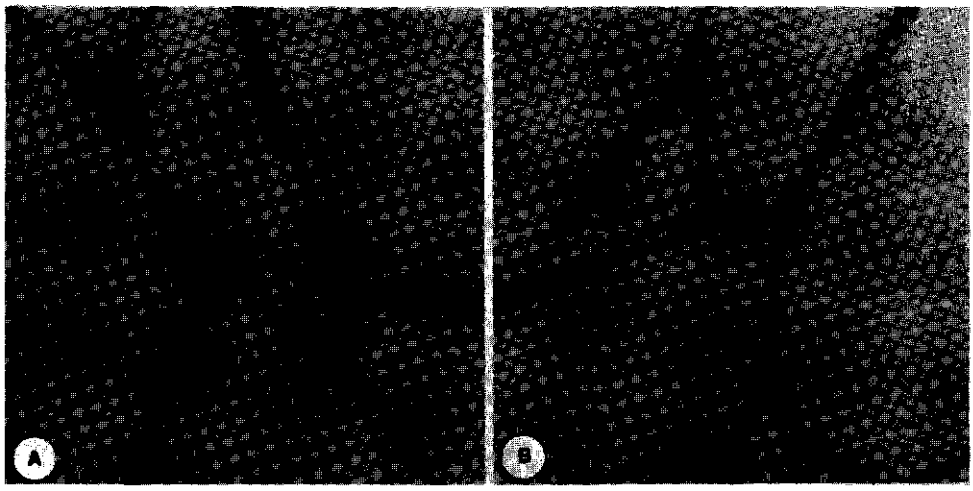

Fig. 2. **Staining of the basal lamina (rabbit anti-rat laminin, HRP/DAB, He counterstaining).**
A. Note the heterochromatic nuclei (myosatellite nuclei) inside the basal lamina, with a dark rim on one side only (arrowheads), and a heterochromatic nucleus outside of the basal lamina in the endomysium (arrow) enclosed by a dark rim. This rim is caused by the overlap of the nucleus with the basal lamina staining. B. Euchromatic myonuclei inside of the muscle's basal lamina. x 1,000.

Light microscopy

Muscle tissue from fish of 4-16 cm SL was fixed in 4% formol, and embedded in paraffin. Sections (4 μm thick) were applied to poly-L-lysine coated glass slides and, after deparaffination, were treated with a trypsin solution (0.1% trypsin; Dyfco 1:250; 0.1% $CaCl_2$; pH 7.8) for 15 min at 20°C. To stain the basal lamina a polyclonal rabbit antiserum against rat laminin was used (57E6, Cleutjens, 1989; a generous gift of Dr. J.P.M. Cleutjens, Department of Pathology, University of Limburg, The Netherlands). The second step was incubation with a horse-Radish peroxidase-coupled goat anti-rabbit immunoglobulin antiserum (GaR-Ig/HRP; Dakopatts). Subsequently the sections were incubated with DAB/H_2O_2 and counterstained with hemalum eosin (HE). From each sample, a minimum of 300 nuclei were differentiated in heterochromatic nuclei within the muscle fiber basal lamina (myosatellite cell nuclei), euchromatic nuclei within the muscle fiber basal lamina (muscle fiber nuclei), and nuclei outside the basal lamina (Fig. 2). This method does not distinguish between myosatellite fibers and myosatellite cells.

Most LM methods for the recognition of myosatellite cells described in the literature depend on the recognition of heterochromatic or proliferating (^{3}H-thymidine labeled) nuclei inside the muscle's basal lamina. One way to do this is to remove all nuclei outside the basal lamina, i.e., the single fiber technique as used by Cardasis and Cooper (1975), Bischoff (1986) and Mulvaney *et al.*, (1988). Staining of the basal lamina was chosen here because it permits the use of tissue sections. The advantage of this method is the maintenance of topographic relationships within the tissue.

Isolation of myosatellite cells

The dissociation procedure used for the isolation of myosatellite cells consisted of a combination of enzymatic (0.2% collagenase and 0.1% trypsin) and mechanical (trituration) treatments and is described in detail elsewhere (Koumans *et al.*,, 1990). From fish smaller than 10 cm SL, 4 gm of muscle, and from fish larger than 10 cm SL, about 6 gm of muscle was used. Larger amounts of muscle from larger fish were used because of the expected lower yield of myosatellite cells per gram of tissue in larger fish. The excised tissue was collected in complete medium: (15% horse serum (HS; Flow) in 90% Dulbecco's modified eagle medium (DMEM)(Gibco) plus PS (penicillin 50 U/ml, streptomycin, 100 μg/ml). It was placed on ice, weighed, and cut in small ($\pm$ 1 mm^3) pieces before dissociation. To

purify the myosatellite cells, the isolated cells were plated in a 25 cm^2 laminin coated culture flask. At 17 hr after plating, the myosatellite cells could be recognized by their specific spindle shape and by their behavior (alignment and fusion; Koumans *et al.*,, 1990).

The nuclei of single, aligned, and just fused myogenic cells were counted in 10 randomly chosen fields of 0.744 mm^2. Nuclei in larger myotubes, which did not show the outlines of freshly fused individual myosatellite cells, were excluded from the counts. It is very probable that these myotubes were not formed in vitro, but were already present in vivo (Koumans *et al.*,, 1990). The number of myogenic nuclei per culture flask was used to calculate the number of myosatellite cells isolated per gram of dissociated muscle tissue.

Estimation of total amounts of white axial muscle

Shansui and Hiroshi (1971) found that in carp larger than 20 mm, bodyweight (BW, in grams) is related to standard length (SL, in mm) as follows: log BW = 3.049 * log SL - 4.689. Oikawa and Itazawa (1984) have shown that in carp from 0.4 to 1900 gm BW the contribution of the (eviscerated) trunkweight (TW) to the bodyweight of the carp increases from 40% to 60%. (For BW between 0.4 and 5.2 gm: Log TW = log 0.434 + 1.100 * Log BW; for BW between 6.3 and 1,900 gm: Log TW = log 0.485 + 1.033 * log BW.) As the trunk of carp consists mainly of white muscle, the same values were used for the total amount of white axial muscle. For carp larger than 41 cm (> 1900 gm), these data were extrapolated.

DNA measurement

White muscle, excised from the 3 regions of the epaxial musculature as specified above, was homogenized in a phosphate-saline buffer (0.05M $NaPO_4$, 2.0M NaCl, 2 x 10^{-3} M EDTA). The DNA content of the homogenate was measured in an Aminco-Bowman spectrophotofluorimeter (excitation wavelength 346 nm, emission wavelength 458 nm) using the fluorochrome Hoechst 33258 (Sigma) as described by Labarca and Paigen (1980). Herring sperm DNA (Promega) was used as a DNA standard.

To obtain the amount of DNA per (haploid) nucleus, the number of cells/ml was counted in five dilution samples of carp sperm cells in a Bürker hemocytometer; and thereafter the DNA content of these samples was analyzed.

Statistics

Significance of the observed correlations and differences was tested with Spearman rank correlation tests, Mann-Whitney U tests, and a Friedman two way analysis of variance test (Siegel, 1956).

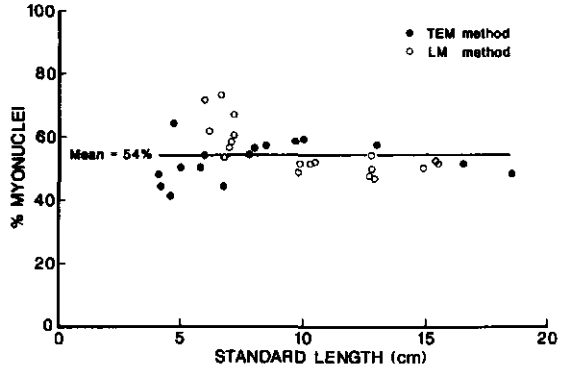

Fig. 3. **Percentages of total nuclei that are muscle nuclei, determined by TEM (solid circles) and LM (open circles). The line shows the mean percentage (54%) calculated from all data points.**

Results

Percentages of nuclei in the muscle tissue

Muscle nuclei The percentage of the total nuclei in muscle tissue that are myonuclei (nuclei present within the muscle fiber basal lamina) did not vary greatly with the length of the fish (Fig. 3). For the data obtained with TEM, no significant correlation between fish length and the percentage of muscle nuclei was found (two tailed Spearman rank correlation test: rs = 0.43, $.05 < P < .10$, n = 17). For the data obtained with LM, a slight negative correlation between these parameters was found (rs = -0.66, $.001 < P < .01$, n = 19). This is caused by a relatively high percentage of muscle nuclei found in carp of 6-7 cm SL

(Fig. 3). For carp between 7 and 16 cm length, no significant correlation was found (rs = -0.383, $.10 < P < .20$, N =15). The difference between the data obtained with TEM and those obtained by LM was not significant (two-tailed Mann-Whitney U test; U = 140, $P >> .10$, $n_1 = 17$, $n_2 = 19$). As the TEM data are more reliable than the LM data, because more criteria are available for the identification of the myosatellite cells, there probably is no significant correlation between fish length and the percentage of muscle nuclei. Therefore a mean (± SD) percentage of muscle nuclei, 54% ± 7%, was calculated from the combined data of both methods for carp between 5 and 18 cm SL (n = 36).

Myosatellite nuclei Both TEM and LM show that with increasing length of the fish the percentage of muscle nuclei that are myosatellite nuclei declines (Fig. 4). The TEM data show that, at the level of the 4th to 7th radii of the dorsal fin, the percentage of myosatellite cell nuclei declines from about 6% in carp of 4 cm to about 1% in carp of 18 cm SL. With the LM method, the percentage of myosatellite cells was found to decline from about 8% in fish of 4 cm to 1.5% in fish of 16 cm SL. (Fig. 4). The data from this LM method are not significantly different (higher) from those obtained by TEM (one-tailed Mann-Whitney U test, U = 157, $P >> .05$ (≈ 0.35), $n_1 = 17$, $n_2 = 20$).

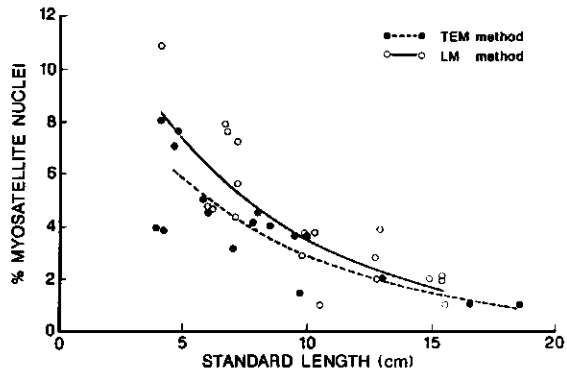

Fig. 4. Percentages of muscle nuclei that are myosatellite nuclei, determined by TEM (solid circles) and LM (open circles). The curves are calculated from a linear regression on the log transformed data. TEM: log % = -0.062 SL + 1.073 (R^2 = 0.83). LM: log % = -0.065 SL + 1.184 (R^2 = 0.67).

DNA measurements

Carp larger than 5-7 cm SL showed no difference in the amount of DNA per gram white epaxial muscle for the three regions along the strip of epaxial muscle (Friedman two-way analysis of variance, $Xr^2 = 2.667$, K = 3, r = 12, $.20 < P < .30$). With a Spearman rank correlation test, no significant correlation between the amount of DNA per gram of tissue and the standard length of the fish was found (two-tailed test, rs = -0.484, $.05 < P < .10$, N = 13). However, this test is not suitable to find a correlation of multiple functions. The shape of the curve, calculated by multiple regression from the mean value of the three body regions for carp larger than 5 cm SL (Fig. 5), indicates ($R^2 = 0.88$) that the amount of DNA/gm tissue decreases between 5 and 20 cm SL, to increase again between 20 and 48 cm. As the percentage of total nuclei that are muscle-fiber nuclei remains constant, the decrease in DNA per gram of tissue indicates a decrease in the nucleus/sarcoplasm ratio of the investigated muscle.

The amount of DNA per haploid (sperm) nucleus was 2.5 ± 0.25 pg (n = 5).

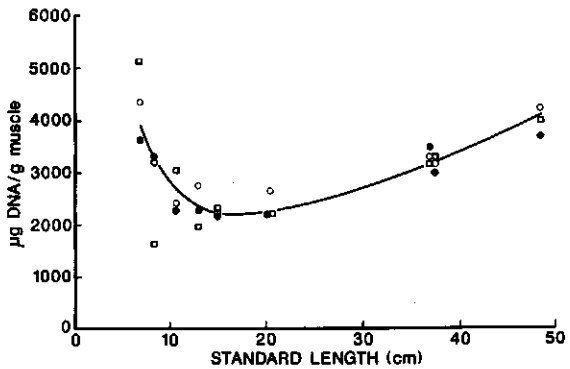

Fig. 5. **Amount of DNA per gram (wet weight) of white epaxial muscle. Muscle was excised from the region directly behind the pectoral girdle (solid circles), from the region between the 4th and 7th radii of the dorsal fin (open circles), and from the region near the anus (squares). The curve shows a multiple regression on the mean of the values for the three regions along the body axis. µg DNA = 100.43 SL (cm) + 32544.67/SL (cm) - 1486.82 ($R^2 = 0.88$).**

Number of myosatellite cells per gram white axial muscle

TEM-DNA method The number of myosatellite cells per gram of white epaxial muscle (Fig. 6) was calculated from the measured DNA content of the tissue, the amount of DNA per diploid nucleus, and the percentage of myosatellite cell nuclei present in the muscle tissue.

Dissociation method The results from the tissue dissociation procedure (Fig. 6) showed the same pattern as observed with the TEM-DNA method, but at a lower level. From linear regression after double log transformation of the data, it appears that the logarithm of the number of myosatellite cells/gram white epaxial muscle is correlated with the logarithm of the standard length to the power -2.5, both for the data from the TEM-DNA method (-2.54) and that from the isolation (-2.50) method.

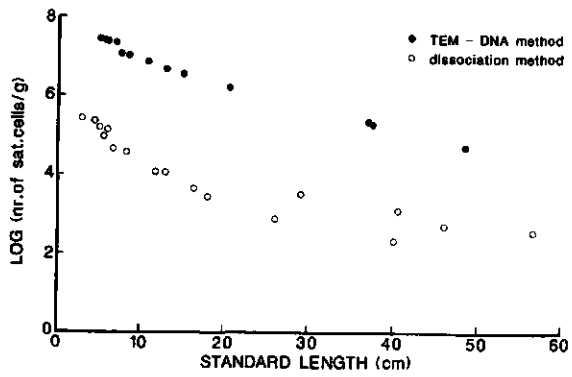

Fig. 6. **Numbers of myosatellite cells per gram (wet weight) of white epaxial muscle. The data points from the dissociation method (open circles) show the number of myosatellite cells isolated per gram of white epaxial muscle. Data from the TEM-DNA method (solid circles) are calculated from TEM data on the percentage of myosatellite cells and from the amount of DNA per gram muscle and per diploid nucleus. For carp larger than 18 cm SL, the TEM data have been extrapolated.**

The dissociation procedure also showed that the number of myotubes already formed in vivo (e.g., showing cross-striation at 17 hr in culture; Koumans *et al.*,, 1990), which were isolated from the muscle tissue, decreased with increasing standard length. Among the cells isolated from carp of 3 cm SL, this type of cell was not unusual; among cells isolated from carp of 5 cm SL they were still present, but among cells obtained from carp larger than 7 cm such myotubes were never found. This indicates that, at least up to a length of 7 cm, hyperplasia plays a role in muscle growth.

Total number of myosatellite cells

The relation of bodyweight (BW) and standard length (SL) of the carp used in this study indeed followed the relation (log BW (gm) = 3.046 * log SL (mm) - 4.689) found by Shansui and Hiroshi (1971). Combination of the amount of white axial muscle per fish, obtained from the fish weight by the formulae developed by Oikawa and Itazawa (1984), and the number of myosatellite cells per gram of white axial muscle resulted in the curves shown in Figure 7. The curve obtained with the data from the TEM-DNA method suggests that the total number of myosatellite cells per fish increases slightly between 5 cm and 20 cm SL and remains constant in larger fish, although a decrease in fish larger than 40 cm is possible. The data from the dissociation procedure are very similar (Fig. 7). Although the correlation coefficient of the linear regression line ($R^2 = 0.55$) is not high, the suggested increase in the number of myosatellite cells with increasing length is significant (Spearman rank correlation test, rs = 0.59, n = 15. $.01 < P < .02$, two tailed test).

Discussion

Although myosatellite cells are considered to be responsible for growth of skeletal muscle (Weatherley and Gill, 1987), this is the first study describing myosatellite cell numbers during growth of a teleost fish. The introduction and development of new methods in the study of myosatellite cells are necessary to allow for the use of immunohistochemistry, which enables the study of the process of differentiation. Therefore, data on percentages and numbers of myosatellite cells obtained by a new LM method and a new in vitro method were compared with those obtained by TEM to check the reliability of the new methods.

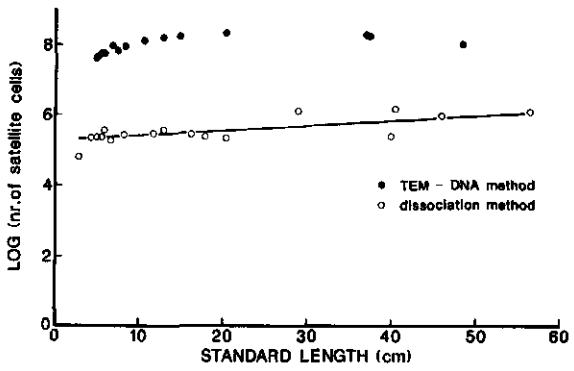

Fig. 7. **Total numbers of myosatellite cells in white axial muscle per fish, by the dissociation method (open circles) and TEM-DNA method (solid circles). A linear regression (log nr of nuclei = 0.0014 SL(cm) + 5.28:R^2 = 0.55) line has been drawn through the dissociation method data points. The graph shows that, with both methods, the absolute number of myosatellite cells does not decrease markedly with increasing length.**

Comparison of methods

Classification of nuclei; TEM versus LM All methods depending on heterochromatism of the nucleus to identify myosatellite cells are not absolutely reliable, because heterochromatism is not an absolute criterion to distinguish between myosatellite nuclei and "real" muscle nuclei. The TEM method is more reliable than LM methods, because better and more criteria are available for the recognition of myosatellite cells. However, the agreement between the two methods shows the reliability of the basal-lamina staining technique. Therefore, this method is acceptable for the estimation of myosatellite cell numbers when it is impossible or difficult to use TEM (e.g., large sample sizes or combination with immune histochemical methods).

Yield of the dissociation method The results of the dissociation procedure for myosatellite cells were compared with the number of cells calculated to be present in the tissue with the TEM-based method. This comparison shows that the efficiency (the percentage of myosatellite cells that are isolated in the total number present in the tissue) remains constant over the range of standard lengths sampled, although this efficiency is rather low. Recently, Düsterhöft *et al.*, (1990) found that, in muscles of rat, the numbers of myosatellite cells that could be isolated from the tissue were proportional to the myosatellite cell numbers found in these muscles by means of TEM (Gibson and Schultz, 1983). Therefore, the yield of myosatellite cells very probably is proportional to the number of cells actually present in the tissue. This means that, for comparative purposes, the yield of the isolation procedure can be used as a global estimate of the number of myosatellite cells present in the muscle tissue.

These two new LM based methods to determine the number (percentage) of myosatellite cells can be used in studies of muscle growth where the use of TEM-based methods is difficult or even impossible.

DNA measurements

In the present study the amount of DNA per gram of white axial muscle was about 1.5-2 times higher than that found by Bouche *et al.*, (1975). Because the amount of DNA per diploid nucleus (5 pg) was 1.5-2 times greater than the 3.4 pg found by Hinegardner and Rosen (1972), this difference probably is a systematic one. The main difference between the method used in this study and that of those authors is the use of herring sperm instead of sea-urchin sperm as a DNA standard. The difference cancels out, however, if these data are used to calculate the number of nuclei per gram of tissue. It certainly does not influence the comparison of the numbers of myonuclei between carp of different lengths.

Numbers of myosatellite cells in growing muscle

Both methods that were used, the TEM-DNA method and the dissociation method, show a slight increase in the total number of myosatellite cells up to a standard length of 20 cm. The plateau and the subsequent slight decrease found in the number of myosatellite cells present in larger fish by the TEM-DNA method were obtained by extrapolation of data from smaller fish and so are less reliable. The continuation of the slight increase shown by the dissociation method may be more reliable. However, the general impression obtained from

both curves is that in growing carp the total number of myosatellite cells is rather constant. A decrease in myosatellite number during growth as generally has been described in mammals and birds (Pearson and Young, 1989), was not found. But in mammals and birds, the total number of myosatellite cells in a muscle also may be constant or may even increase. Cardasis and Cooper (1975) found that the total number of myosatellite cells was "somewhat constant" for individual muscle fibers from gastrocnemius muscles of growing mice older than 14 days. Gibson and Schultz (1983) pointed out that although percentages of myosatellite cells decrease with increasing volume of the muscle tissue, absolute numbers of satellite cells may actually remain constant or even increase for a certain muscle in a given period of time.

As the total number of myosatellite cells remains constant or even increases during growth, the decrease in the percentage of myosatellite cell nuclei with increasing length of the carp appears to be caused by the dilution of the myosatellite cells by an increasing number of muscle-fiber nuclei. These additional muscle nuclei also descend from myosatellite cells and, therefore, the proliferation of the myosatellite cells must be an important factor correlated to growth rate. A correlation between myosatellite cell proliferation and hypertrophic muscle growth has indeed been described for pigs (Mulvaney *et al.*,, 1988) and rats (Joubert and Tobin, 1989).

The proposed need for proliferation of myosatellite cells in fast-growing young carp seems to be in contrast with the high percentage of postmitotic myosatellite cells isolated from white epaxial muscle of carp of 5 cm SL (Koumans *et al.*,, 1990). The present study, however, shows that the occurrence of a high percentage of postmitotic cells in small carp is accompanied by a decrease in the nucleus/sarcoplasm ratio. Thus in the size range between 5 and 15 cm SL the production of new myonuclei (supplied by myosatellite cells) is probably low compared with the increase in the amount of muscle tissue. The increase in the amount of DNA per gram of muscle tissue in carp larger than 15 cm SL indicates that the nucleus/sarcoplasm ratio is increasing again. This suggests that in these larger fish the proliferation rate of the myosatellite cells increases. Recent studies indicate that this indeed occurs (unpublished findings).

Hyperplasia and hypertrophy

A decrease in the nucleus/sarcoplasm ratio, as found in carp between 5 cm and 15 cm SL, is also described for rat muscle (Pearson and Young, 1989) and for chicken anterior and posterior latissimus dorsi muscle (Matthew and Moore, 1987) and, therefore, is not unique for fish. However, a decrease in the amount of DNA per gram of tissue is not necessarily caused by a changing nucleus/sarcoplasm ratio of the already existing muscle fibers. Hyperplasia also may play a role. If myosatellite cells, which have little cytoplasm, differentiate into new muscle fibers (probably via the myosatellite fiber stage), the amount of muscle cytoplasm increases without a corresponding increase in myonuclei. Therefore, the decreasing nucleus/sarcoplasm ratio in carp from 5 to 15 cm SL and the high percentage of postmitotic myosatellite cells in carp of 5 cm SL may be related to a predominance of hyperplasia in this stage.

The increasing myonucleus/cytoplasm ratio in carp larger than 15 cm SL could be related to a decreasing role of hyperplasia. Such a decrease in the role of hyperplasia in muscle growth of carp larger than about 15-20 cm SL would be in agreement with the work of Weatherley *et al.*, (1980), Stickland (1983), and Veggetti *et al.*, (1990). Those authors reported that in growing fish the role of hyperplasia decreases while that of hypertrophy increases with increasing length, till in large fish hypertrophy seems to be the sole process of muscle growth. Weatherley and Gill (1984) also pointed out that the growth rate of fish is largely determined by hyperplasia as the more important process. Fish with a high share of hyperplastic growth grow faster than fish with a high share of hypertrophic growth. Factors that influence the relative importance of hyperplastic and hypertrophic growth of muscle, therefore, can greatly influence the growth rate of fish.

Muscle growth in fish probably is determined by the number of myosatellite cells, their distribution over different subclasses and the behavior of these subclasses in relation to hyperplasia and hypertrophy. These factors deserve more attention in further studies.

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CHAPTER IV

INFLUENCE OF FISH SIZE ON PROLIFERATION AND DIFFERENTIATION OF CULTURED MYOSATELLITE CELLS OF WHITE AXIAL MUSCLE OF CARP.

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Key words: *Myosatellite cells, In-vitro, Desmin, 5'bromo-2'deoxyuridine, Cyprinus carpio L. (Teleostei)*

Submitted to: Differentiation

Summary

The in vitro proliferation (uptake of 5-bromo-2'-deoxyuridine (BrdU) and the degree of differentiation (presence of desmin) of myosatellite cells isolated from white axial muscle of carp between 3 cm and 27 cm standard length (SL) were examined 17 hours after isolation. The fraction of the myosatellite cells that were both desmin positive and BrdU positive never exceeded 2% of the total number of isolated myosatellite cells, irrespective of the standard length of the donor(s). This indicates that, for carp, the temporal relation between replication and desmin expression of myosatellite cells is different from that described for myogenic cells of mammals and birds. The percentage of BrdU positive myosatellite cells was significantly correlated with standard length; it increased from 10 % for carp of about 5 cm SL to 40 to 50 % for carp between 20 cm and 27 cm SL. The percentage of desmin positive myosatellite cells was about 50 to 60 %; it was not significantly correlated with standard length. The percentage of myosatellite cells that were both BrdU negative and desmin negative showed a step wise difference in this percentage

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with increasing length. Fish smaller than 10 cm SL, had more of these cells (10 to 40%), than larger fish (which had 0 to 12%). So, apparently the composition of the myosatellite cell population changes during growth. The low percentage of proliferating cells, and the relatively high percentage of differentiated (desmin positive) myosatellite cells obtained from 3 cm to 6 cm large carp, suggests that, in these small fish, muscle growth strongly depends on the use of a pool of myogenic cells that has been formed at an earlier stage of their development.

Introduction

Growing fish muscle provides a dynamic system in which hypertrophy and hyperplasia play a role of changing importance, depending on age, genetic background and environment (Greer-Walker *et al.*, 1972; Stickland, 1983; Weatherley and Gill, 1984). This in contrast to mammals and birds where, as a rule, hyperplasia stops shortly after birth and hypertrophy appears to become the only way of muscle growth (Campion, 1984; Goldspink, 1972; Goldspink, 1974), although hyperplasia can play a role in activity-induced muscle growth (Gonyea *et al.*,1986; White and Esser, 1990).

Myosatellite cells, first described by Mauro (1961), play an important role in muscle growth. They supply the additional myonuclei in hypertrophy (Allen *et al.*, 1979; Campion, 1984; Moss and Leblond, 1970; 1971) and a correlation between the proliferation rate of the myosatellite cells and muscle growth has been described for rats (Joubert and Tobin, 1989) and pigs (Mulvaney *et al.*, 1988). In regeneration of muscle (Allbrook, 1981; Carlson, 1986; Ontell *et al.*, 1984), new fibers arise from these cells. They probably also play a role in hyperplasia in growth of fish muscle (Koumans *et al.*, 1991; Romanello *et al.*, 1987; Rowlerson *et al.*, 1985; Veggetti *et al.*, 1990; Weatherley and Gill, 1984).

As recognition of myosatellite cells in vivo requires the use of transmission electronmicroscopy (TEM), which imposes its limits on the study of these cells (Koumans *et al.*, 1990; 1991), we developed a procedure to isolate myosatellite cells from white axial muscle of carp of 5 cm SL (Koumans *et al.*, 1990). This procedure, which involves purification of the cells on a laminin coated substrate, resulted in cultures that contained 80 to 90% myosatellite cells. The isolated myosatellite cells could be distinguished from the non myogenic cells by their typical spindle shaped morphology (Koumans *et al.*, 1990).

This earlier study showed that, in juvenile carp of 5 cm SL, 17 hr after isolation only few (10%) of the isolated myosatellite cells proliferated (as determined by the uptake of 5-bromo-2'-deoxyuridine (BrdU), a Thymidine analog). Most of the myosatellite cells (80 %) contained desmin, an early marker of differentiation. This low percentage of proliferating cells seemed to be in contrast with the expected high proliferation rate of myosatellite cells in growing muscle of young carp. The number of myosatellite cells that could be isolated from the tissue was proportional to the satellite cell content of the tissue calculated on the basis of ultrastructural (TEM) investigations (Koumans *et al.*, 1991). This indicates that changes in the occurrence of the different subtypes *in vitro* reflect changes in the composition of the myosatellite cells population *in vivo*.

The DNA concentration of the muscle tissue showed a decrease in carp between 5 cm and 15 cm, followed by an increase in larger fish (Koumans *et al.*, 1991). Therefore, we expected the proliferation rate of the myosatellite cells to increase in these larger carp.

In the present study we investigated the *in vitro* proliferation and the degree of differentiation of myosatellite cells isolated from carp from 3 cm to 27 cm SL, by adding BrdU to the culture medium and, at 17 hrs after isolation, determining the percentages of BrdU labeled satellite cells, and of desmin positive satellite cells by immuno-cytochemistry (Koumans *et al.*, 1990).

Materials and methods

Animals and sampling of muscle

We used carp of 3 cm - 27 cm standard length (SL: The distance between the snout tip and the base of the tail fin) reared at 23 °C at our institute. The fish were over-anaesthetized with MS-222 (Sandoz) and immersed in alcohol 70% during 30 seconds for external sterilization, before isolation of the myosatellite cells.

In fish smaller than 15 cm we isolated myosatellite cells from the entire strip of white epaxial muscle between the pectoral girdle and the anus. Care was taken to avoid excision of red or pink muscle. In larger fish, white muscle was excised from three regions along this strip: between the pectoral girdle and the first radius of the dorsal fin, between the 4th and the 7th radius of the dorsal fin, (about the middle of this strip), and at the region of the anus. We used about 3 grams of muscle from fish of 5 cm Sl and increased the amount of muscle tissue

in larger fish because of the expected lower yield of myosatellite cells per gram of tissue (Koumans *et al.*, 1991). The increasing amount of tissue needed to obtain a sufficient number of myosatellite cells (about 20 grams for fish of about 15 cm SL, and about 100 grams for fish larger than 25 cm SL) puts an upper limit to the studied size range. When using carp smaller than 10 cm SL, the muscle tissue from several fish was pooled. This was unavoidable because of the insufficient amount of muscle tissue that could be isolated from individual fish of these lengths.

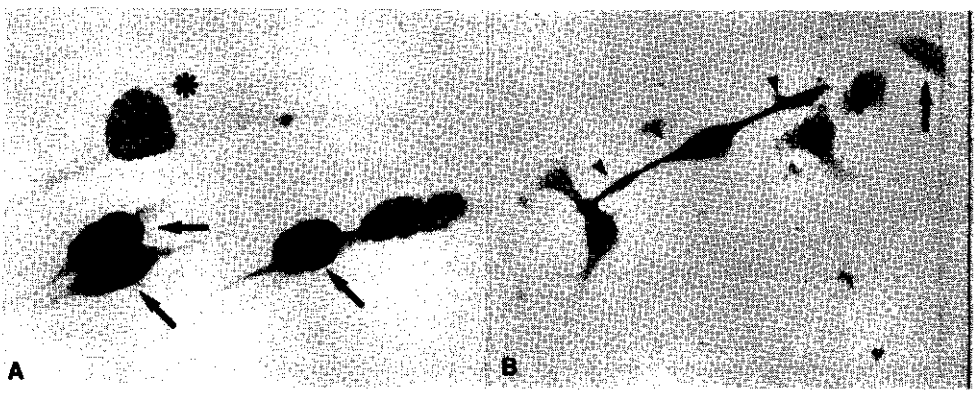

Fig. 1. **Double staining for desmin and BrdU, 17 h after isolation. HRP/DAB stain, HE counterstain. A.Two BrdU positive and desmin negative myosatellite cells (arrowheads) and a BrdU negative and desmin negative myosatellite cell, x 500. B. One BrdU positive and desmin negative myosatellite cell and part of a large BrdU negative and desmine positive (arrows) myosatellite cell, x 300.**

Isolation and culturing of the myosatellite cells

The excised tissue was collected in complete medium on ice, weighed, and cut in small (about 1 mm^3) pieces before dissociation; (Complete medium = 15% Horse Serum (HS)(Flow) in Dulbecco's Modified Eagle medium (DMEM)(Gibco) plus PS (Penicillin 50 U/ml, Streptomycin 100 μg/ml) + 10 μM BrdU). As an adaption to fish cell culture we lowered the osmolarity of the medium using DMEM diluted with *aqua bidest.* to 90% of its original concentration. All media and enzyme solutions contained 10 μM BrdU. The used dissociation procedure consisting of a combination of enzymatic (0.2% collagenase, 0.1%

trypsin) and mechanical (trituration) treatment, is described in detail elsewhere (Koumans *et al.*, 1990). The plating density was about 10^6 cells per ml cell suspension. 17 Hours after plating on a laminin coated substrate the isolated myosatellite cells could be recognized by their typical spindle shape. Tests for the presence of BrdU and Desmin were made 17 hrs after isolation of the cells, but in some instances a part of the cells was cultured longer, up to 179 hrs after isolation.

Immunocytochemistry of cultured cells

Cells on glass coverslips were rinsed three times with 90% DMEM and fixed in cold (-20° C) acetone for 5 min.

Immunocytochemical demonstration of BrdU After fixation, the cells were incubated in 2 N HCl for 20 min at room temperature to denature the DNA, neutralized by a wash in 0.1 M Na-tetraborate, pH 8.5 and washed twice in TBS. This procedure does not interfere with the immunocytochemical demonstration of desmin; which is very convenient in desmin/ BrdU double staining. As a first antiserum, we used a monoclonal anti-BrdU antibody (Eurodiagnostics) and as a second antiserum, rabbit or goat anti mouse-Ig conjugated to HRP (Dakopatts). After each incubation step the sections were rinsed twice for 5 min each in TRIS/NaCl/T-20 (0.05% TRIS, 1% NaCl and 0.05% Tween-20, pH 7.6) and 3 times for 5 min each in 0.05 M TRIS, pH 7.6 (TBS, TRIS buffered saline). After diaminobenzidine (DAB) incubation the cells were rinsed, counterstained with Heamalum Eosin (HE), dehydrated and embedded in Depex.

Immunocytochemical demonstration of desmin As a first antiserum, we used a polyclonal rabbit antiserum against chicken gizzard desmin (Ramaekers *et al.*, 1983), kindly provided by Dr. F.C.S. Ramaekers of the University of Nijmegen, the Netherlands. Immunohistochemical reactions with this antiserum on cryostat sections of carp tissue showed that it only reacted specifically with desmin (Koumans *et al.*, 1990). On immunoblots of carp muscle the antiserum only reacted with the desmin band (unpublished results).
As a second antiserum, we used goat or swine anti rabbit-Ig conjugated to HRP (horse radish peroxidase) (Dakopatts). The cells were rinsed and counterstained as described for the BrdU staining. Figure 1 shows a Desmin staining and a BrdU staining, with HRP-DAB, and HE counterstaining

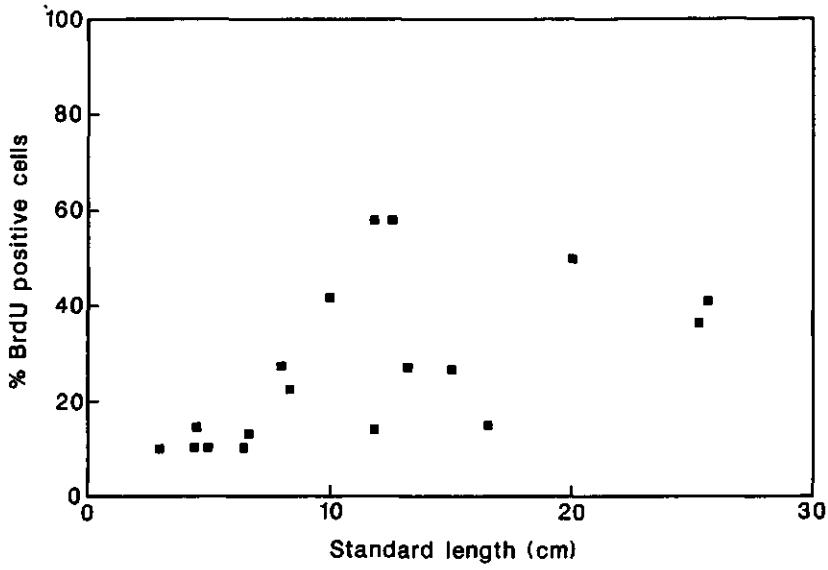

Fig. 2. **Percentage of myosatellite cells that are BrdU positive 17 h after isolation from white epaxial muscle of carp from 3 cm to 26 cm standard length.**

Counting Desmin and BrdU positive myosatellite cells

Earlier experiments with myosatellite cells isolated from carp of 5 cm SL (Koumans *et al.*, 1990) showed that 17 h after plating already some of the isolated myosatellite cells had aligned or even fused into small myotubes. Therefore the nuclei of single, aligned and just fused myosatellite cells were counted to determine the total number of myosatellite cells. Nuclei in larger myotubes, that did not show the spindle shaped outlines of freshly fused individual myosatellite cells, or even already showed cross striation were excluded from the counts. In view of the short time after isolation, it is very probable that these myotubes were not formed in vitro, but were already present in vivo (Koumans *et al.*, 1990). At least 300 nuclei per sample were counted to obtain the percentages of myosatellite cells that were BrdU positive and/or desmin positive. The percentages of BrdU positive myosatellite cells and of desmin positive myosatellite cells were determined in a separate BrdU and in a separate desmin staining. These percentages were verified using a BrdU-desmin double staining. This double staining was also used to determine the percentage of myosatellite cells that were both

BrdU positive and desmin positive. Data that could not be obtained from both the separate and the double staining methods, either because of large differences between the countings or because of one of the stainings being unsatisfactory, were not used (missing data in table 1).

Dilution experiment

To rule out the possibility that the observed results were affected by differences in plating density we also determined the percentage of desmin and BrdU positive myosatellite cells over a dilution series of myosatellite cells, isolated from carp of 4.5 cm SL.

Statistics

The Significance of the observed correlations between percentages of cells and standard length was tested with the Spearman rank correlation test (Siegel, 1956).

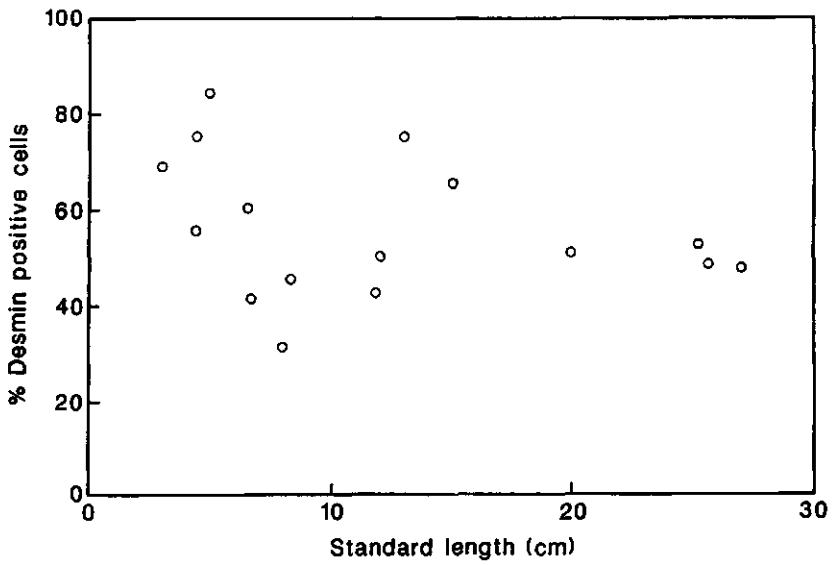

Fig. 3. **Percentage of myosatellite cells that are desmin positive 17 h after isolation from white epaxial muscle of carp from 3 cm to 26 cm standard length.**

Results

BrdU positive cells

The percentage of the isolated myosatellite cells (spindle shaped, and just fused, or aligned cells) that, 17 hrs after isolation, are BrdU positive increases from about 10% in carp of 3 to 5 cm Sl, as is consistent with earlier results (Koumans *et al.*, 1990), to 40-50% in the largest investigated carp (20-27 cm SL) (tab 1, fig 2). This increase in the percentage of BrdU positive cells is significantly correlated to standard length (Spearman rs = 0.69, $0.002 < p < 0.005$, two tailed test n=18), although for carp larger than 10 cm Sl the data showed a considerable amount of variation. All data from carp larger than 10 cm SL were obtained from muscle of individual fish while the data from carp smaller than 10 cm SL were obtained from muscle samples pooled from several fish. Therefore, the effect of individual variation was much more apparent in the muscle samples from the larger fish.

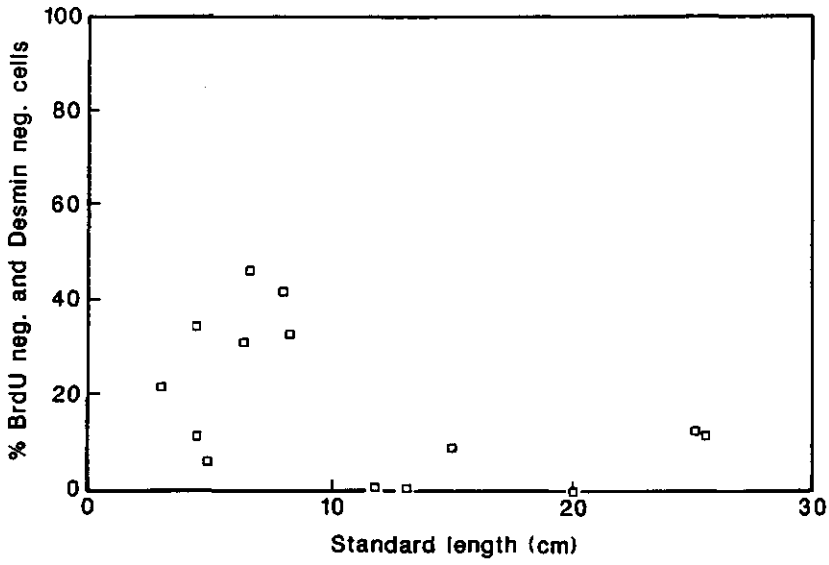

Fig. 4. **Percentage of myosatellite cells that are both BrdU negative and Desmin negative 17 h after isolation from white epaxial muscle of carp from 3 cm to 26 cm standard length.**

Desmin positive cells

The percentages of cells that were desmin positive fluctuated around 50-60% (table 1, fig 3). For the smallest investigated fish (3-5 cm SL) the percentage of cells that were desmin positive, was slightly higher (56-84%). This was consistent with previously reported data (Koumans *et al.*, 1991). There was no significant correlation between the percentage of cells that were desmin positive and standard length of the carp (Spearman rs = 0.32, $0.20 < p < 0.50$, two tailed test n = 16).

STANDARD LENGTH (CM)	PERCENTAGE			
	BrdU+	desmin+	BrdU+/ desmin+	BrdU-/ desmin-
3 ± 0.3 (n=16)	9,8	69	0	21,2
4.4 ± 0.2 (n=40)	10,3	55,7	0,4	34
4.5 ± 0.4 (n=13)	14,4	75	0	10,6
5 ± 0.4 (n=20)	10	84	0	6
6.5 ± 0.8 (n=7)	10	60	0,7	30
6.7 ± 0.4 (n=20)	12,9	41	0	46,1
8 (7.8 + 8.2)	27,5	31	0	41,5
8.3 ± 0.2 (n=3)	22,5	45	0	32,5
9.8 (9.7 + 10.0)	41,8	-	-	-
11.8	57,9	42,4	0,3	0
11.8	14	-	-	-
12 (12.2 + 11.7)	-	50	-	-
12.5	57,9	-	-	-
13.1	26,7	75	1,7	0
15	26,7	65	0	8,3
16.5	15	-	-	-
20	50	51	1	0
25.2	36	52	0	12
25.6	41	48	0	11
27	-	47	-	-

Table 1. Percentages of myosatellite cells that, 17 hrs after isolation from epaxial white muscle from carp of 3 cm to 27 cm standard length are:

- **BrdU positive (both BrdU pos. and desmin neg. , and BrdU pos. and desmin pos.).**
- **Desmin positive (both desmin pos. and BrdU neg. , and desmin pos. and BrdU pos.).**
- **Both BrdU positive and desmin positive.**
- **Both BrdU negative and desmin negative.**

(- : staining unsuitable to obtain reliable data).

Desmin positive, BrdU positive cells

The double staining showed that cells being both Desmin positive and BrdU positive occurred rarely (table 1). The percentage of these cells never exceeded 2%, irrespective of the time in culture (17 hrs - 179 hrs) or of the standard length of the donor(s).

Desmin negative, BrdU negative cells

The percentage of the isolated myosatellite cells that were both BrdU negative and desmin negative was higher for fish smaller than 10 cm SL (10-40%) than for larger fish (where it was 0-12%). The transition between the two groups was rather abrupt (table 1, fig 4).

Dilution effects

We found no effect of the plating density on the percentages of myosatellite cells that were Desmin positive and BrdU positive (Table 2). As the plating densities that we used in the experiments were well within the limits of this dilution experiment it is improbable that our results were influenced by differences in plating density.Desmin negative and BrdU negative cells

Cells/area (0.186 mm²) mean ± SD, (n=15)	Desmin positive cells (n=nr. of cells counted)	BrdU positive cells (n=nr. of cells counted)
75 ± 16	69% (n=449)	16% (n=257)
47 ± 13	77% (n=270)	12% (n=240)
35 ± 8	77% (n=293)	16% (n=243)
16 ± 6	76% (n=271)	13% (n=236)
10 ± 4	77% (n=261)	15% (n=226)
	(mean ± SD) (75 ± 3)%	(mean ± SD) (14 ± 2)%

Table 2. **Influence of plating density on the percentage of myosatellite cells that were BrdU positive and Desmin positive, 17 h after isolation from carp of 4.5 cm SL.**

Discussion

In this study on myosatellite cells isolated from fish muscle we could distinguish four types of cells:

1) **BrdU-/desmin+ cells**. The percentage of these cells fluctuates around 50-60% . From fish of 3-5 cm SL a slightly higher percentage (56-84%) of these cells was obtained than from larger fish. This means that the percentages of desmin positive cells in carp are higher than those found either for adult (37%) or for embryonic (3%) chicken (Yablonka-Reuveni and Nameroff, 1990), for fetal and newborn rat (28%) (Kaufman and Foster, 1989), or for young (5 to 10 weeks) bull calves (13%) (Allen *et al.*, 1991). It probably is also higher than the 40% found for fetal and newborn mouse (Kaufman and Foster, 1989).

2) **BrdU+/desmin- cells**. The percentage of these cells increased from about 10% in fish of 4-5 cm SL to about 40% in fish of 20-27 cm SL. The latter value is comparable to, or slightly lower than, the percentages of proliferating cells found for embryonic and adult chicken (about 50%) (Yablonka-Reuveni and Nameroff, 1990) and for fetal and newborn mouse and rat (Kaufman and Foster, 1989). But, the 10% of proliferating myosatellite cells found for young carp is very low compared to the values described for these other investigated animals.

3) **BrdU-/desmin- cells**. Fish smaller than 10 cm SL possessed more of these cells (10-40%) than larger fish (which had 0-12%).

4) **BrdU+/desmin+ cells**. Proliferating desmin positive cells. These cells were only seldom found ($\leq$ 2%).

BrdU positive cells

5-bromo-2'-deoxyuridine (BrdU), a Thymidine analog is incorporated in proliferating cells during the S-phase of the proliferation cycle. In a former study (Koumans *et al.*, 1990) we investigated the effect of the use of BrdU on the yield of the dissociation procedure, and on the composition of the population of myosatellite cells isolated from carp of about 5 cm SL and found that these effects were negligible.

Desmin positive cells

Desmin is an early marker of differentiation. In vitro it is among the first expressed muscle specific proteins (Dlugosz *et al.*, 1983). In the skeletal muscle lineage desmin expression has been thought to be specific for postmitotic cells (Bennet *et al.*, 1979; Capetanaki *et al.*, 1984; Gard and Lazarides, 1980). But recent studies (Allen *et al.*, 1991; Kaufman and Foster, 1988; Kaufman and Foster, 1989; Yablonka-Reuveni and Nameroff, 1990) showed that in cultures obtained from man, mouse, rat and chicken part or, in case of rat almost all (>94%) (Allen *et al.*, 1991), of the desmin positive myogenic cells are able to proliferate. This ability appears to be species dependent. Not only differences between birds and mammals (Yablonka-Reuveni and Nameroff, 1990), but also differences between different species of mammals (Allen *et al.*, 1991) exist. The propensity of myogenic cells to express desmin while replicating also appears to be influenced by the ontogenetic stage of the donor (Kaufman and Foster, 1989; Yablonka-Reuveni and Nameroff, 1990). This, probably is a difference between embryonic myoblasts and myosatellite cells. Other differences between these two types of myogenic cells are the inability of myosatellite cells to participate in embryonic muscle growth in birds (Chevallier *et al.*, 1987), the effect of a tumour promotor (TPA) on the differentiation of the cells (Cossu *et al.*, 1985), and the presence or absence of acetylcholine receptors (Cossu *et al.*, 1987).

In carp, we found that myosatellite cells that were both desmin positive and proliferating occurred only seldom. These cells never exceeded 2% of the number of isolated myosatellite cells, irrespective of the time in culture (17 to 179 hrs) or of the standard length of the donor(s) (tab 1). Further, 17 hrs after isolation a large part of the cells is already engaged in the proces of "alignment" and fusion, irrespective of the presence of BrdU (Koumans *et al.*, 1990), which is known to have a negative effect on the differentiation of cells (Bischoff and Holzer, 1970; Tapscott *et al.*, 1989). This indicates that in carp the desmin positive and BrdU negative myosatellite cells are postmitotic and not potentially proliferating cells.

Correlations with muscle growth

Until now, the relationship between *in vitro* myogenic cell proliferation and growth has been studied in muscle of mammals (Dodson and Allen, 1987; Schultz and Lipton, 1982) and birds (Doumit *et al.*, 1990; Wright, 1985), but not in fish muscle. In rats, proliferation of

myosatellite cells was found either to decrease with age increasing from 6 days to 30 month (Schultz and Lipton, 1982) or to remain constant from an age of 3 to an age of 24 month (Dodson and Allen, 1987). In Turkey from 3 to 15 weeks old the proliferation of isolated myosatellite cells increased with increasing age (Doumit *et al.*, 1990) and in chicken up to 20 month of age no effect on the proliferation of myogenic cells was found (Wright, 1985). In carp, we found an increase in the percentage of proliferating cells with increasing length.

The observed difference in the percentage of proliferating myosatellite cells *in vitro* can be caused by *in vitro* changes in the lag-time of cells entering the cell-cycle, by an *in vivo* increase of the percentage of cells that have entered the cell cycle, or by a combination of both mechanisms. Isolation and culturing are known to stimulate myosatellite cells in a regeneration type of reaction. Therefore it is impossible to translate *in vitro* data (for instance for lag-times) directly to *in vivo* data. However, the proportionality of the amounts of myosatellite cells isolated from the tissue and calculated to be present in the tissue (calculated from DNA measurements and ultrastructural investigation, Koumans *et al.*, 1991) suggests that the *in vitro* changes in the occurrence of the different subtypes of myosatellite cells reflect changes in the *in vivo* composition of the myosatellite cell population. Moreover, the unexpected low level of myosatellite cell proliferation in small fish is accompanied by a decrease in the DNA content of the muscle tissue (Koumans *et al.*, 1991). The observed increase in *in vitro* proliferation of myosatellite cells closely precedes or coincides with the termination of this decrease (at about 15 cm SL, Koumans *et al.*, 1991). This indicates that an in vivo increase of myosatellite cell proliferation indeed occurs.

The unexpected high amount of differentiated postmitotic myosatellite cells isolated from small fish could be related to the, compared to mammals and birds, prolonged occurrence of hyperplasia in fish. The question arises if, in fish, hyperplasia and hypertrophy are dependent upon different subclasses of myosatellite cells. This in analogy to the existence of different classes of myosatellite cells for growth and for repair as proposed by Cossu and Molinaro (1987): satellite cells for growth (slow division rate, fusing with growing muscle fibers, relatively abundant in young animals) and satellite cells for repair (capable of fast and repeated division, fusing mainly with other satellite cells, relatively abundant in older animals). The use of general markers as desmin (differentiation) and BrdU (proliferation) does not allow us to distinguish such subclasses. But our data show that (muscle) growth in carp is accompanied by changes in the composition of the myosatellite cell population. The

paucity of proliferating cells, and the presence of a high amount of differentiated (desmin positive) myosatellite cells in small (about 5 cm SL) carp in which hyperplasia is an important mechanism of muscle growth, suggests that, in this stage of muscle growth, a pool of earlier formed muscle precursor cells is incorporated into (new) muscle fibers.

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CHAPTER V

HYPERPLASIA AND HYPERTROPHY IN GROWTH OF CARP WHITE AXIAL MUSCLE

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Key words: *Fish, muscle growth, hyperplasia, hypertrophy, myonuclei, myosatellite cells.*

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Summary

In white axial muscle of carp addition of new fibres to the muscle mass (hyperplasia) decreased with increasing length of the fish. This was deducted from the decrease in the amount of small fibers. In carp larger than about 40 cm standard length (SL) hyperplasia no longer occurred (small fibres were absent), and muscle growth only occurred by means of hypertrophy (growth of existing fibers). The stage of growth in which many new fibres were added showed a relatively fast increase in trunkweight as was calculated from growth curves. During the stage of fast growth with a high occurrence of hyperplasia the DNA/protein ratio decreases. The high percentage of postmitotic myosatellite cells isolated from carp of 5 cm SL (Koumans *et al.*, 1990; Koumans *et al.*, in prep.) suggests that in hyperplasia a subpopulation of already differentiated myosatellite cells formed in an earlier stage of development is incorporated in new muscle fibres. The increase of the relative importance of hypertrophy appears to be correlated to an increase in the percentage of proliferating

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myosatellite cells 17 hrs after isolation in vitro. This suggests that in hyperplasia and in hypertropy different subpopulations of myosatellite cells are involved.

Introduction

The study of muscle growth in fish confronts us with a dynamic system in which, in addition to hypertrophy, hyperplasia continues to be important till after the juvenile state (Greer-Walker, 1970; Weatherley *et al.*, 1979; Stickland, 1983). This in contrast to the situation in mammals and in birds where, generally, increase in the number of muscle fibres stops shortly after birth (Goldspink, 1972) and hypertrophy alone is responsible for the postnatal growth of muscle.

In fish hyperplasia of the muscle tissue and growth rate are positively correlated. Fish that have a high share of hyperplasia, grow faster and reach a greater length than fish that grow mainly by hypertrophy (Weatherley & Gill, 1984; Weatherley, 1990). The mechanisms that regulate hyperplasia and hypertrophy in muscle growth of fish still are largely unknown. But, it is very likely that in both types of growth myosatellite cells are involved.

Myosatellite cells (Mauro, 1961) are known to supply nuclei to muscle fibres during hypertrophy in birds and mammals (Moss & Leblond, 1970; 1971; Allen *et al.*, 1979; Campion, 1984). They form new fibres in regeneration of damaged muscle (Allbrook, 1981; Ontell *et al.*, 1984; Carlson, 1986). Myotubes formed by isolated myosatellite cells of mammals and birds have an isotype of myosin similar to that of young fibres developing in vivo and different from that of adult fibres (Whalen *et al.*, 1978; Matsuda *et al.*, 1983; Yamada *et al.*, 1989; Düsterhöft *et al.*, 1990). In fish, myosatellite cells probably also play a role in hypertrophic growth as well as in hyperplastic growth. Myosin of newly formed small muscle fibres differs from that of older, larger fibres (Akster, 1983; Rowlerson *et al.*, 1985; Romanello *et al.*, 1987). So it is likely that in growing fish new fibres are formed by myosatellite cells, probably via the stage of myosatellite fibres (Koumans *et al.*, 1990). However, studies of white axial muscle of some Mugilidae suggests that, in these fish, increase in the numbers of fibres occurs by fibre splitting (Scapolo *et al.*, 1984; Romanello *et al.*, 1987). So this mechanism of fibre recruitment can not be ruled out. It may be that there are species dependent differences in the mechanism of hyperplasia.

Because of the probable function of myosatellite cells in hyperplasia and hypertrophy, study of their role in growth of fish muscle is of biological and also of economical interest.

In earlier studies (Koumans *et al.*, 1990; Koumans *et al.*, in prep.) we demonstrated the presence of different subclasses (differentiated and proliferating) of myosatellite cells in white axial muscle of carp. We determined the DNA contents of this tissue and, over a growth range of 5 cm to 20 cm standard length (SL), the ratio between myonuclei and other nuclei (fibroblasts, endothelium, etc.) (Koumans *et al.*, 1991). In the present study we determined the fiber diameter distribution, the DNA/protein ratio, and we extended the determination of the percentages of muscle and non muscle nuclei to carp from 3.4 cm to 56 cm SL. These data were used to investigate the relations between changes in the number and in the type of muscle nuclei and the occurence of hyperplasia and hypertrophy.

Materials and methods

Animals and location of muscle

We used carp of 3.4 - 56 cm standard length (SL) reared under standard conditions of light and food at 23 °C at our institute. The fish were over-anaesthetized with TMS (Tricaine Methanesulfonate, Crescent Research Chemicals) before excision of the muscle tissue. Three different sampling sites were used in the DNA and protein measurement experiments (fig. 1): between the pectoral girdle and the first radius of the dorsal fin (site A), between the 4th and the 7th radius of the dorsal fin (site B), and at the region of the anus (site C). In the fibre diameter experiments, samples were taken from site B only. The data for each standardlength are obtained from one animal.

Fibre diameters and percentages of nuclei

After over-anaesthesia of the fish, the muscle was injected with Karnovsky's (1965) fixative. After 30 minutes fixation in situ, muscle was excised between the 4 th and the 7 th radius of the dorsal fin (site B, fig. 1), cut in small pieces, fixed for another hour in Karnovsky's fixative, washed in cacodylate buffer, postfixed in 1% OsO_4, dehydrated in graded alcohol and propylene oxide and embedded in Epon. Semithin (1 μm) and ultra-thin sections were cut on a Reichert OMU II ultramicrotome. The semithin sections were stained

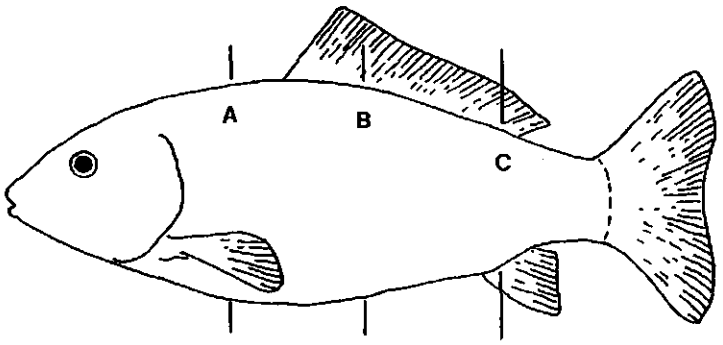

Fig. 1. **Sampling sites that were used in the DNA and protein mesurement experiments and in the fibre diameter distribution experiments. Site A is located between the pectoral girdle and the first radius of the dorsal fin. Site B is located between the 4 th and the 7 th radius of the dorsal fin (about in the middle of the fish). Site C is located at the region of the anus. In the DNA and protein measurement experiments, samples were taken from site A, B, and C, while in the fibre diameter experiments, samples were taken from position B.**

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with Toluidine blue and used for the examination of the fibre cross sectional areas (diameters). The sections were projected with a Olympus FHT projection microscope on a Calcomp 9100 digitizing tablet (resolution 40 lines/mm) connected to a Macintosh IIfx. The outlines of the projected fibres were traced with the cursor of the digitizing tablet. Per sample 100 to 125 fibres were measured. Care was taken to select only samples that showed "true cross sections" of the muscle fibres (as judged from the appearance of the myofibrils), to avoid overestimation of the areas. An object-micrometer was used to check if the projection was free from distortions (deviations from the known area were smaller than 1 %), and for calibration. The diameter of the fibres (uncorrected for shrinkage of the tissue) was calculated from the cross sectional area of the muscle fibres (the area was considered to be perfectly circular).

The ultrathin sections were contrasted with uranyl acetate and lead citrate. From each tissue sample, a number of nuclei, comprising at least 100 muscle fibre nuclei, was differentiated as myosatellite cell nuclei, muscle nuclei proper and nuclei outside the muscle fibre's basal lamina (fibroblast nuclei, endothelial nuclei, blood cell nuclei, etc.)

Protein measurements

Protein measurements were made with a Tectator Kjeltec auto analyzer 1030. Sample sizes were between 0.6 g and 2 g muscle wet weight (WW), (macro method). Smaller samples (20 mg to 40 mg WW) from fish smaller than 10 cm SL, were measured with a semi-micro method. To test the comparability of these two methods, different amounts of a 95% bovine serum albumin (BSA) preparation were measured. The results obtained by the semi-micro method (97.7 % $\pm$ 18.8, n=7) were not significantly different from those obtained by the macro method (95.0 % $\pm$ 1.6, n=13), although the semi-micro method showed a higher variation.

For each datapoint the measurements were made in triplicate and averaged.

DNA measurements

The excised muscle was homogenized in a phosphate-saline buffer (0.05 M $NaPO_4$, 2.0 M NaCl, 2 x 10^{-3} M EDTA). The DNA content of the homogenate was measured in a Aminco-Bowman spectophotofluorimeter (excitation wavelength 346 nm, emission wavelength 458 nm) using the fluorochrome Hoechst 33258 (Sigma) as described by Labarca and Paigen (1980). Herring sperm DNA (Promega) was used as an DNA standard (Koumans *et al.*, 1991).

Statistics

The Significance of observed correlations was tested with the Spearman rank correlation test (Siegel, 1956). The Friedman's two way analysis of variance test was used to test the significance of the differences between the DNA content and the protein content of the three regions of white axial muscle sampled along the length of the fish (Siegel, 1956).

Results

Fibre diameter frequency distribution

During growth, the frequency distribution of the diameters of white axial muscle fibres became broader and shifted to the right (fig. 2). In accordance with the literature (Weatherley *et al.*, 1988), we used a distribution with diameter classes of 20 μm (uncorrected for

shrinkage). Fibres with a diameter of 100 μm and larger were designated as large fibres, as fibres of this diameter were not present in the smallest fish. They first appeared in fish of about 8 cm SL.

The percentage of muscle fibres with a diameter smaller than 20 μm (the smallest diameter class) gradually decreased, while the percentage of fibres with a diameter larger than 100 μm increased (fig. 3). In fish smaller than 15-20 cm SL the percentage of fibres smaller than 20 μm was higher than the percentage of fibres larger than 100 μm, while the opposite was true for fish larger than 15-20 cm SL. The percentage of fibres smaller than 20 μm decreased to zero in fish between 34.5 cm and 43 cm SL.

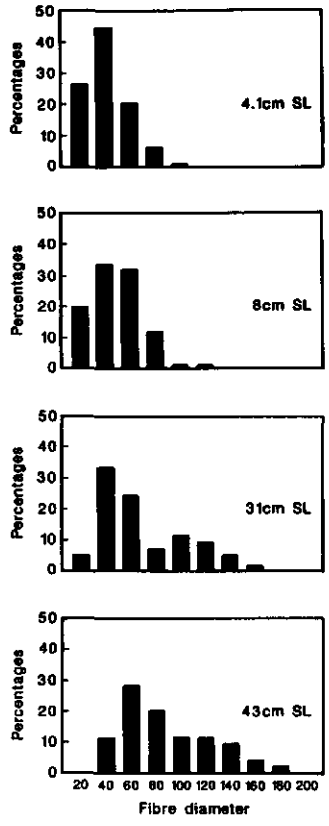

Fig. 2. Frequency distribution of muscle fibre diameters (uncorrected for shrinkage) for a carp of a) 4.1 cm SL, b) 7.8 cm SL, c) 31 cm SL, d) 43 cm SL. The maximum fiber diameter of each 20 μm size class is given at the x-axis.

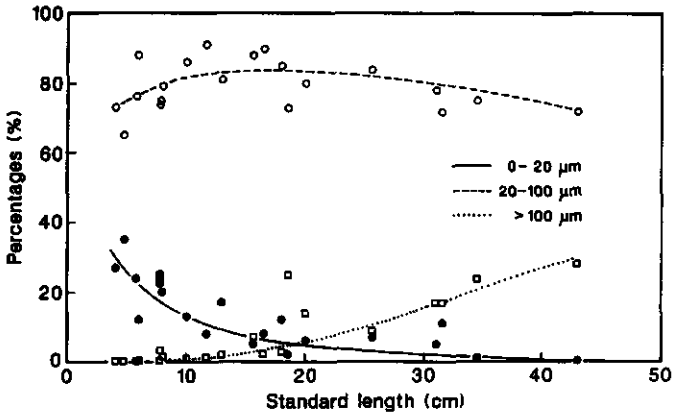

Fig. 3. Frequency distribution of small (0-20 µm), medium (20-100 µm) and large (≥ 100 µm) fibre diameters (uncorrected for shrinkage), as a function of standard length for carp between 4.1 and 43 cm SL, showing the data of all the sampled carp.

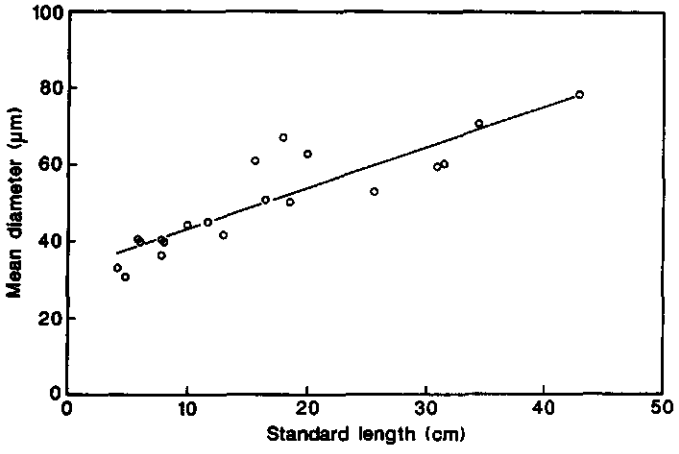

Fig. 4. Mean diameters (uncorrected for shrinkage) of muscle fibers from carp between 4.1 cm and 43 cm SL. Linear regression shows that mean fibre diameter = 32.51 + 1.06 x SL (cm) (N = 20, R^2 = 0.80).

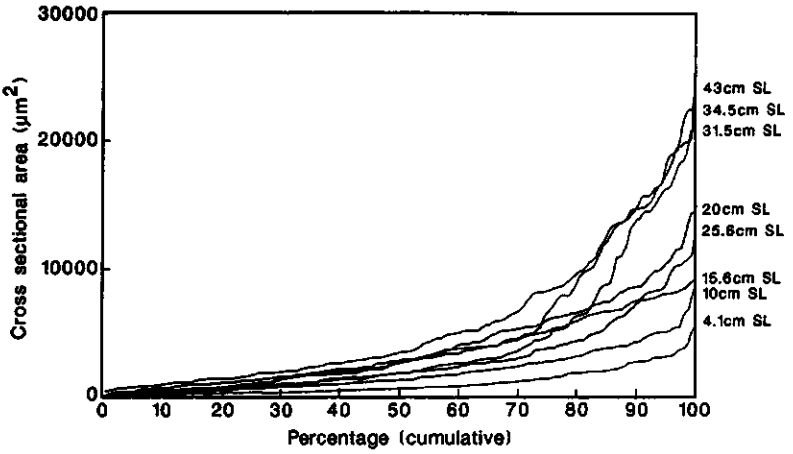

Fig. 5. Cumulations of the cross sectional areas (uncorrected for shrinkage; vertical axis, left) formed by fibers of increasing size (in percentage of the total fibre number) for carp of different standard lengths (vertical axis, right).

The percentage of fibres with a diameter between 20 μm and 100 μm (medium sized fibres) remained rather constant, about 80% of the total fibre number (Fig. 3). Fibres with a diameter between 160 μm and 180 μm (the largest class found) first appeared in fish of about 31 cm SL.

The mean diameter of the white axial muscle fibres increased linearly with the length of the fish (fig. 4; D (μm) = 1.06 x SL(cm) + 32.51, R^2=0.80, n=20)). In fish between 15 cm Sl and 25 cm SL the largest variation in the mean fibre diameter was found. The intermingling of lines in the cumulative graph of fibre cross sectional areas (fig. 5) also shows that in fish larger than 15 cm SL there is a considerable variation between individuals in the contribution that different size classes of fibres make to the total amount of muscle tissue. This indicates that there is an individual variation in the relative importance of hypertropy and hyperplasia. Figure 5 also shows that in larger fish (larger than 31.5 cm SL) the increase in the largest fibre diameter is much less than in smaller fish. This agrees with the negative relation between the size of a muscle fibre and its growth rate as described by Weatherley *et al.* (1988).

Nuclei

The percentage of the total amount of nuclei that were muscle fibre nuclei (including myosatellite cell nuclei) remained constant (52% ± 7, mean ± SD, n=23) over the entire size range. This means that, in white axial muscle, changes in the DNA/protein ratio of the muscle tissue reflect changes in the myonucleus/sarcoplasm ratio.

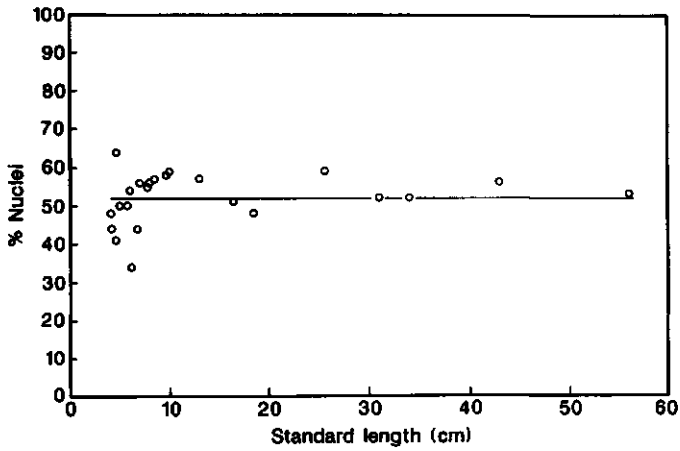

Fig. 6. The percentage of nuclei in white axial muscle that are muscle nuclei (muscle nuclei proper and satellite cell nuclei) is 52% (52 ± 7; Mean ± SD, N=23). This percentage is independend of the standard length of the fish.

DNA/protein ratio

The three regions of white axial muscle sampeled along the length of the fish (Table I) did not differ significantly in protein content (Friedman: $Chi^2 = 0.43$, n=15, $0.8 < p < 0.9$ two tailed). The amount of protein per gram tissue (Fig. 7) remained constant (19.4 % ± 1.1 % ; mean ± SD) during growth (Spearman, two tailed; rs = 0.30, $0.20 < p < 0.50$, N = 17). The differences in DNA content of the three individual sampling regions were significant (Friedman: $Chi^2 = 7.88$, n=17, $0.01 < p < 0.02$, two tailed test). The caudal muscle had a higher DNA content than more anteriorly situated muscle. This is mainly the case in carp smaller than 13 cm SL (Table I).

DNA and protein content of white axial muscle of carp.

	DNA (pg/g tissue)			Protein (g/gtissue)x100%		
SL (cm)	pos A	pos B	pos C	pos A	pos B	pos C
3.4	4847	4129	5298	22.32	19.91	19.85
3.6	4289	4217	5541	14.60	22.07	20.90
3.7	4453	4778	5176	19.45	19.03	18.79
4.9	3452	4109	4317	19.22	19.65	19.68
4.9	4104	4123	4501			
5.0	4320	3127	5559	19.37	20.44	18.91
5.6	3734	4115	4766	16.36	17.72	21.67
5.8	3844	3660	4385	19.69	19.02	19.34
6.9	3609	4328	5102	18.19	18.64	23.46
7.5				18.79	19.72	22.27
8.4	3244	3172	1617	19.42	19.47	18.24
10.7	2244	2394	3033		18.35	
13.0	2244	2732	1952	18.25	18.19	18.46
15.0	2212	2223	2282	18.78	18.81	18.69
20.5	2186	2628	2174	18.68	18.68	18.74
37.0	3441	3117	3148		18.79	
37.5	2957	3204	3251	19.57	18.92	19.45
48.5	3656	4188	3958	21.08	20.67	21.01

Table 1. **DNA (pg) per gram muscle tissue (wetweight) and amount of protein (gram protein per gram muscle tissue) x 100%. The samples were taken directly behind the pectoral girdle (pos A), in the region between the 4th an 7th radius of the dorsal fin (pos B) and in the region of the anus (pos C).**

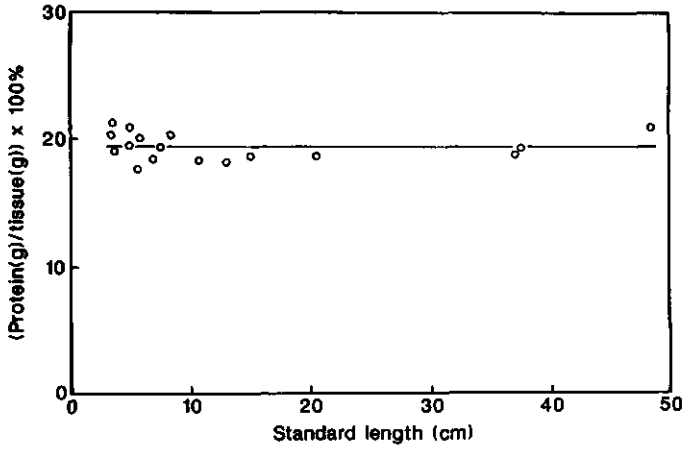

Fig. 7. Amounts of protein (gram protein per gram tissue x 100%) of white axial muscle. There is no correlation with standard length.

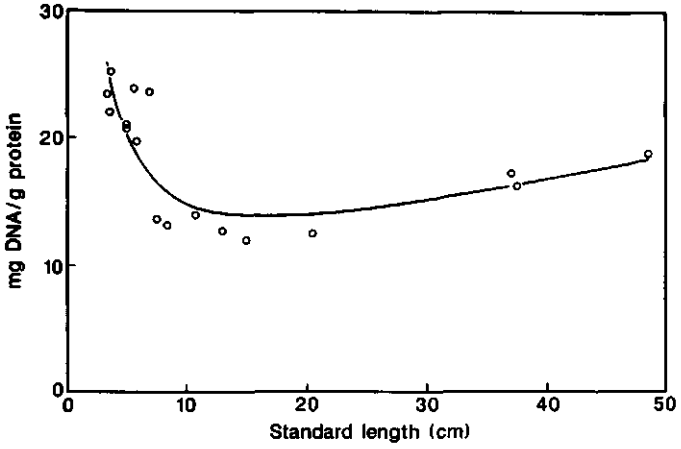

Fig. 8. DNA/protein ratios (pg DNA per gram protein) of white axial muscle. The curve was calculated from multiple regression. DNA/protein ratio = 3408.31 + 34312.96/SL (cm) + 119.50 x SL (cm) (N = 17, R^2 = 0.68). The curve shows a decrease in the DNA/protein ratio of carp from 3 cm to about 15 cm SL and an increase in the DNA/protein ratio of larger carp.

Although unsuspected from the data for DNA and protein content seperately, the DNA/protein ratio of the three regions was not significantly different ($Chi^2 = 1.08$, $n=13$, $0.50 < p < 0.70$, two tailed test). Therefore, and because of the close resemblance of the curves showing the relation between DNA/protein ratio and standard length for each individual sampling site, we averaged the data of the three regions to obtain one mean DNA/protein ratio for every fish (Fig. 8). As expected, the changes in DNA/protein ratio were similar to those in the amount of DNA per gram tissue. Between 5 cm Sl and 13 cm SL the DNA/protein ratio decreased to a level of about 50% of the ratio found in carp of 5 cm SL. In fish larger than 13 cm SL no clear rostro-caudal differences in DNA contents were found (Table 1). In these fish the DNA contents and the DNA/protein ratio slowly increased again; fish of about 50 cm SL had a similar DNA/protein ratio as fish of 5 cm SL. Although the increase in averaged DNA content was significant (Spearman, two tailed: $rs = 0.89$, $0.02 < p < 0.05$, $N=6$), the increase in DNA/protein ratio just failed significance (Spearman, one tailed: $rs = 0.77$, $0.05 < p < 0.10$, $N = 6$).

Discussion

Hyperplasia, growth rate and maximum size

Comparing the frequency distributions of fibre diameters of fish of different size (length) gives information about hypertrophy as well as about hyperplasia of muscle fibres. Information about hyperplasia can be gained from the presence or absence of small, new, muscle fibres. Although this is an indirect method to study hyperplasia it has been shown to be a reliable and important tool for the study of muscle growth (Weatherley *et al.*, 1979; Stickland 1983; Higgins & Thorpe, 1990; Weatherley, 1990).

The growth curve of optimally fed carp reared at 23 °C (adapted from Huisman, 1974) shows that, measured between hatching and an age of 32 weeks (= about 33 cm SL), bodyweight is a constant function of time (BW (g) = 0.033 x Age^3 (weeks); $R^2 = 0.998$, $N = 9$). The mass of the eviscerated trunk, which consist mainly of white axial muscle, is not a constant function of bodyweight. Therefore, changes in the relative mass of (eviscerated) trunkweight (as a percentage of bodyweight) describe changes in axial muscle growth more clearly than changes in bodyweight can.

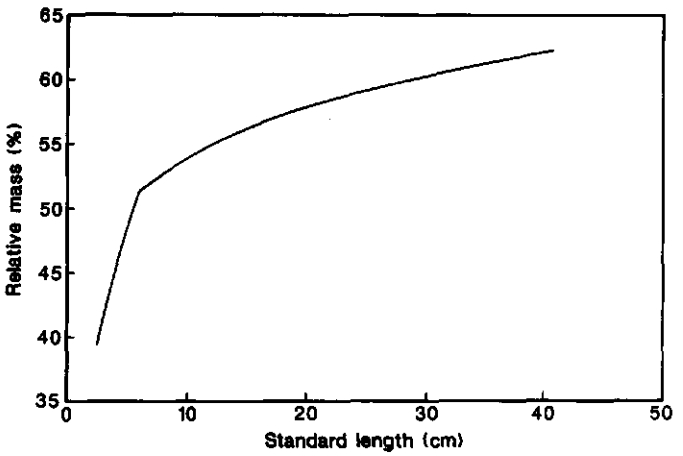

Fig. 9. The relation between the relative mass of the trunk (the percentage of the total weight formed by the weight of the trunk) and standard length. It was calculated from the formula: log Bw (bodyweight in grams) = 3.049 * log SL (mm) -4.689 in carp larger than 20 mm SL (Shansui and Hiroshi (1971), and the formulae: Log Tw = log 0.434 + 1.100 * Log Bw (for Bw between 0.4 and 5.2 gram), and Log Tw = log 0.485 + 1.033 * log Bw (for Bw between 6.3 and 1900 gram), describing the contribution of the (eviscerated) trunkweight (Tw) to the bodyweight (Bw) of the carp (Oikawa and Itazawa, 1984). The graph shows a very abrupt decrease in slope at a standardlength of about 6 cm SL followed by a more gradual decrease in larger fish.

The growth curve of the relative mass of eviscerated trunkweight (Fig. 9) was calculated from the combined data of Oikawa & Itazawa (1984) and Shansui & Hiroshi (1971), see Koumans *et al.*, (1991). The slope of this curve rather abruptly starts to decrease at 6 cm SL followed by a more gradual decrease in larger fish (Fig. 9). The decrease in the percentage of small muscle fibres (fig.2) which reflects the decrease in the occurrence of hyperplasia, follows a similar pattern. Thus in carp muscle a high share of hyperplasia in muscle growth appears to be accompanied by a fast growth rate of the muscle tissue, as has been found in other fish species (Weatherley & Gill, 1984; Veggetti *et al.*, 1990).

Although there can be considerable differences in growth rate of genetically related fish, caused by differences in: ration size, temperature, or (in extreme experimental situations) by injection with bovine growth hormone, the overall picture is that every length class of a species has a characteristic pattern of fiber diameter frequencies which is unaffected by even major differences in growth rate (Weatherley, 1990). This suggests a

large genetic influence on the determination of the maximum size of the fish under optimal conditions.

Weatherley *et al.* (1988) found strong indications that the length at which hyperplasia ceases, determines the maximum size of a fish. They found in 10 freshwater teleost species a constant relationship between the ultimate forklength described for these species of fish, and the fork-length at which hyperplasia in white axial muscle stops (about 44% of the ultimate forklength). This relation predicts a maximum length of about 1 meter for carp, which is in agreement with the actual reported maximum size for carp (Muus & Dahlstrøm, 1968).

Genetic determination of the amount of fibre recruitment (hyperplasia) is also suggested by the work of Greer Walker *et al.* (1972) who showed that in herring larger than 25 cm (when hyperplasia probably no longer occurs), the total fibre number (measured in cross section at a specific point along the length of the fish) is stock specific. Genetic determination of maximum size does not exclude individual variation within a species. In carp of 15-25 cm SL, a size range where addition of new muscle fibres has almost ceased, we found a considerable individual variation in fibre diameter distribution. Weatherley *et al.* (1980) found that a similar variation in fibre diameter distribution in rainbow trout was correlated to a variation in growth rate of these fish. As the amount of hyperplasia is clearly related to growth rate and maximum size of fish, investigating the mechanisms underlying the recruitment of new muscle fibers is also of agricultural interest.

Myonucleus/sarcoplasm ratio

In carp from 3-10 cm SL, a size class with a high recruitment of new fibers, we found a decrease in the myonucleus/sarcoplasm ratio. A similar decrease in DNA concentration with increasing length was found by Luquet and Durand (1970) in young rainbow trout, which also should have a high level of hyperplasia. In young mice a similar decrease in DNA/cytoplasm ratio (numbers of nuclei per muscle segment of 170 μm) occurs during hypertrophic growth (Cardasis & Cooper, 1975). An explanation for a decreasing DNA/protein ratio during a stage of growth when many new fibres are added is that hyperplasia is brought about by fusion of differentiated myosatellite cells, which have very little cytoplasm, followed by an increase in the amount of cytoplasm of the new fibres. Thus the decreasing DNA/cytoplasm ratio in fact is caused by the hypertrophy that accompanies hyperplasia. In carp larger than 20 cm SL, in which hypertrophy is the dominant mechanism

of muscle growth, an increase in DNA content was found. This was quite unexpected, and it is in contrast with the data of Luquet & Durand (1970) for rainbow trout, and of Cardasis & Cooper (1975) for mice. These authors found, after an initial decrease in small animals, a constant DNA (muscle nuclei) content during further growth. The cause and role of the increase in the DNA content of white axial muscle in larger carp remains unclear.

Subclasses of myosatellite cells

In vitro studies of myosatellite cells isolated from carp axial muscle (Koumans *et al.*, 1990; Koumans *et al.*, submitted) enabled us to recognize at least two subclasses of myosatellite cells; cells that already contained desmin (differentiated cells) and cells that incorporated 5-bromo-2-deoxyuridine (proliferating cells). Seventeen hours after isolation from small carp (3 to 8 cm SL) the cultures contained a high percentage of differentiated myosatellite cells and only few proliferating myosatellite cells. In larger carp the percentage of proliferating myosatellite cells in the cultures increased. In small carp hyperplasia has an important share in muscle growth. The high percentage of postmitotic cells present in these fish (Koumans *et al.*, 1990; Koumans *et al.*, submitted) and the decrease in DNA contents during this stage of growth suggests that in hyperplasia a subpopulation of already differentiated myosatellite cells formed in an earlier stage of development is used. The increase in the DNA contents and in the percentage of proliferating myosatellite cells coincide with the decrease of hyperplasia and the increase of the relative importance of hypertrophy. This suggests that in hyperplasia and hypertrophy different subpopulations of myosatellite cells may be involved.

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CHAPTER VI

NUMBERS OF MYONUCLEI AND OF MYOSATELLITE CELL NUCLEI IN RED AND WHITE AXIAL MUSCLE DURING GROWTH OF THE CARP (*Cyprinus carpio* L.).

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Key words: *Fish, muscle growth, hyperplasia, hypertrophy, myonuclei, myosatellite cells.*

Submitted to: J. Fish Biol.

Summary

We determined the percentages of myonuclei and satellite nuclei over a growth range of carp, as the increase in the number of myonuclei is an important aspect of the increase in muscle mass, and myosatellite cells are believed to be the source of new myonuclei. In white as well as in red axial muscle the percentage of the nuclei present in muscle that are myonuclei (muscle fibre nuclei + myosatellite nuclei) remained constant during growth (54 % and 32 % respectively). The difference in the percentage of non-muscle nuclei between white and red axial muscle is mainly caused by the higher content of endothelial nuclei in red axial muscle.

In white axial muscle the DNA/protein ratio (myonucleus/sarcoplasm ratio) decreased between 3 and 15 cm SL. In red axial muscle we found a continuous decrease in

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DNA/protein ratio over the entire investigated size range (3-50 cm SL). This may be related to a longer occurrence of hyperplasia in red than in white axial muscle.

In both fibre types the percentage of muscle nuclei being myosatellite nuclei decreased with increasing length. In white axial muscle it decreased from about 5% in carp of 5 cm SL to less than 1% in carp of 20 cm SL; for red muscle these values were 11% and 3% respectively.

For white axial muscle we calculated that, especially in larger fish, the myosatellite cells alone can not account for the increase in the number of myonuclei during growth. The percentage of proliferating nuclei in muscle tissue, measured by the uptake of 5-bromo-2'-deoxy-uridine (BrdU), is high enough to account for the total increase in nuclei. So indirect evidence is available that also another cell type present in the muscle tissue may be involved in the formation of additional myonuclei.

Introduction

Muscle growth is accompanied by an increase in muscle nuclei; the availability of nuclei even may be a limiting factor for muscle growth (Bishoff & Holtzer, 1969; Penney *et al*, 1983; Mulvaney *et al*, 1988; Joubert & Tobin, 1989). In postnatal growth of mammals and birds additional muscle nuclei are considered to be provided by myosatellite cells (Moss & Leblond 1970; Moss & Leblond 1971; Campion, 1984). Myosatellite cells, first described by Mauro (1961), are small spindle shaped-cells with a heterochromatic nucleus and few organelles other than free ribosomes and polysomes. They are situated between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibres. However, it is not entirely excluded that myonuclei also may originate from other than myosatellite cells (Grounds, 1991).

In muscle growth of fish, hyperplasia (formation of new muscle fibres) continues to be important till long after the juvenile state (Greer-Walker, 1970; Stickland, 1983; Weatherley *et al*, 1988). This in contrast to the situation in mammals and in birds where, generally, the increase in the number of muscle fibres stops shortly after birth, (Goldspink, 1972; 1974) and hypertrophy (outgrowth of existing muscle fibres) alone is responsible for the postnatal growth of muscle.

Studies on myosatellite cells in teleost fish muscle are scarce (Powell *et al*, 1989; Koumans *et al*, 1990; 1991), but also in fish these cells most probably contribute additional nuclei to growing fibres (hypertrophy). They may also give rise to new fibres in hyperplasia (Nag & Nursall, 1972; Rowlerson *et al*, 1985; Koumans *et al*, 1990; Veggetti *et al*, 1990), although this also has been attributed to fibre splitting (Scapolo *et al*, 1984; Willemse and Lieuwma Noordanus, 1984).

Red and white muscle of fishes differ in nucleus/sarcoplasm ratio (Luquet & Durand, 1970; Egginton & Johnston, 1982b) and probably also in the amounts of myosatellite cells as was found in mammals, birds, amphibia, sharks and hagfish (Düsterhöft *et al*, 1990; Matthew & Moore, 1987; Takahama *et al*, 1984; Kryvi & Eide, 1977; Sandset & Korneliussen, 1978). It has been suggested that hyperplasia continues longer in red than in white growing muscle (Stickland, 1983). So, comparison of the changes in the numbers of myonuclei and of myosatellite cell nuclei in red and in white axial muscle may give information about differences in growth between these fibre types. In earlier studies, we already determined these data for white axial muscle (Koumans *et al*, 1991; Koumans *et al*, 1992), In the present study a comparison with red axial muscle is made.

The quantitative comparison of both muscle types led to two other important questions. (a) Can the small number of myosatellite cells in white muscle account for the increase in myonuclei during growth?

(b) Are the observed percentages of proliferating nuclei large enough to account for the total increase in nuclei in carp white muscle tissue during growth? This to investigate the possible existence of myogenic cells other than myosatellite cells.

Materials and methods

Animals and location of muscle

We used carp of 3.4 - 56 cm standard length (SL) reared under standard conditions of light and food at 23 °C at our institute. The fish were over-anaesthetized with TMS (Tricaine Methanesulfonate, Crescent Research Chemicals) before excision of the muscle tissue. In the DNA and protein measurement experiments, red muscle samples and white muscle samples were obtained from three different sampling sites (Fig. 1): between the pectoral girdle and the first radius of the dorsal fin (site A), between the 4th and the 7th radius of the dorsal fin

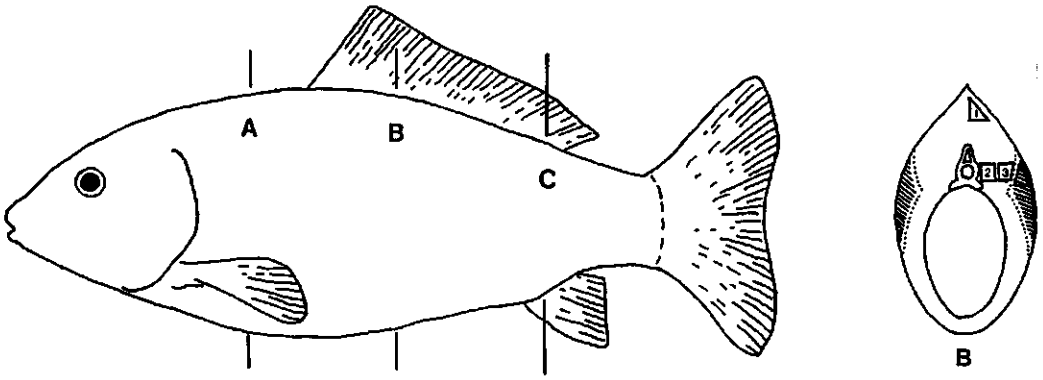

Fig. 1. **Sample sites. Red and in white axial muscle samples were taken from sites A, B, and C. In small carp (< 7 cm SL) the red muscle tissue was obtained from the entire strip of red axial muscle behind the pectoral girdle. The percentages of different nuclei, both in red and in white axial muscle, were taken from site B. White axial muscle was taken from position 2. Red axial muscle was taken from the marked position. To investigate the existence of growth zones in white axial muscle samples were taken from positions 1 (dorsal), 2 (medial) and 3 (lateral).**

(site B), and at the region of the anus (site C). In small carp (< 7 cm SL) the red muscle tissue was obtained from the entire strip of red axial muscle behind the pectoral girdle. Percentages of nuclei were determined from red and white muscle obtained from site B (Fig. 1) only.

Percentages of nuclei

After over-anaesthesia of the fish, the muscle was injected with Karnovsky's (1965) fixative. After 30 minutes fixation in situ, muscle was excised, cut in small pieces, fixed for another hour in Karnovsky's fixative, washed in cacodylate buffer, postfixed in 1% OsO_4, dehydrated in graded alcohol and propylene oxide and embedded in Epon. Semithin (1 μm) and ultra-thin sections were cut on a Reichert OMU II ultramicrotome. The ultrathin sections were contrasted with uranyl acetate and lead citrate. For muscle samples of carp from 3.4 cm - 18 cm SL, a number of nuclei, comprising at least 100 muscle fibre nuclei, was differentiated as myosatellite cell nuclei, muscle nuclei proper and nuclei outside the muscle fibre's basal lamina (fibroblast nuclei, endothelial nuclei, blood cell nuclei, etc.). For white

muscle of carp from 18-56 cm SL, myosatellite cells were not counted as a separate group as the percentage of these cells was so low that reliable data would have required large sample sizes. In carp smaller than 2.5 cm no unequivocal recognition of myosatellite cells could be made as a continuous muscle basal lamina was lacking.

Percentages of proliferating nuclei

To investigate the possible presence of growth zones we determined the percentage of proliferating nuclei in three different muscle zones. Carp of 5 cm, 10 cm and 15 cm SL were injected intraperitoneally with a 10mg/ml solution of 5-bromo-2'-deoxyuridine (BrdU) (Sigma B2506) in PBS. We used a dose of 250 μg/g bodyweight. Four hours after injection the carp were overaneasthetised with TMS and three samples of white muscle (dorsal, medial and lateral, see fig.1) were excised at the level of the 4^{th} -7^{th} radius of the dorsal fin (site B). Also a part of the gut was excised and used as a control for the labelling. The tissue was fixed in 4% formol, and embedded in paraffin. Sections (4 μm thick) were deparafinated, treated with a trypsin solution (0.1% Trypsin (Dyfco 1:250), 0.1% $CaCl_2$, pH 7.8) for 15 minutes at 37 °C, rinsed in TBS (0.05 M TRIS (pH 7.6)) and incubated in 2 N HCl for 20 min at room temperature. After a second rinse with TBS the section were incubated with a monoclonal anti-BrdU antibody (Eurodiagnostics) and, as a second antiserum, a rabbit or goat anti mouse-Ig conjugated to HRP (Dakopatts). After each incubation step the sections were rinsed twice for 5 min each in TRIS/NaCl/T-20 (0.05M TRIS, 1% NaCl and 0.05% Tween-20, pH 7.6) and 3 times for 5 min each in 0.05 M TRIS, pH 7.6 (TBS, TRIS buffered saline). After DAB incubation the sections were rinsed, counterstained with Heamalum Eosin (HE), dehydrated and embedded in Depex.

Protein and dna measurements

Protein measurements were made with a Tectator Kjeltec auto analyzer 1030 as described by Koumans *et al* (1992)

For DNA measurements the excised muscle was homogenized in a phosphate-saline buffer (0.05 M $NaPO_4$, 2.0 M NaCl, 2 x 10^{-3} M EDTA). The DNA content of the homogenate was measured in a Aminco-Bowman spectrophotofluorimeter (excitation wavelength 346 nm, emission wavelength 458 nm) using the fluorochrome Hoechst 33258

(Sigma) as described by Labarca and Paigen (1980). Herring sperm DNA (Promega) was used as an DNA standard (Koumans *et al*, 1991).

Statistics

Significance of the observed differences was tested with Mann-Whitney U tests and Friedman two way analysis of variance tests. Significance of correlation was tested with Spearman rank correlation tests (Siegel & Castellan, 1988).

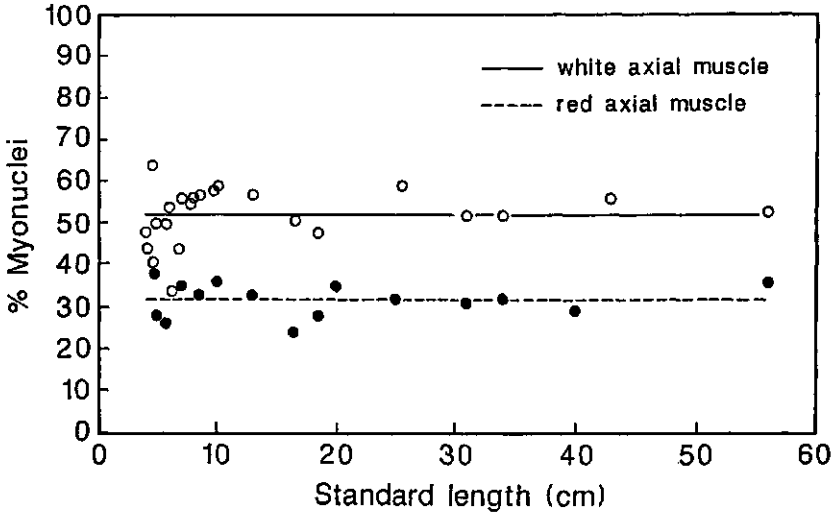

Fig. 2. Percentages of total nuclei that are myonuclei in white and in red axial muscle tissue of carp with a standard length between 3.4 cm and 56 cm. In white axial muscle the percentage of myonuclei is about 54% (53.9 ± 7.1; Mean ± SD, N = 23), while in red axial muscle the percentage of myonuclei is about 32% (32.0 ± 4.5, N = 15).

Results

Percentages of nuclei

Both in red and in white axial muscle the percentage of muscle nuclei (myonuclei + myosatellite nuclei) did not change during growth as was also found by Enesco & Puddy (1964) for rats. In white axial muscle the percentage of muscle nuclei is about 54% (53.9 ± 7.1; Mean ± SD, N = 23), while in red axial muscle the percentage of muscle nuclei is about 32% (32.0 ± 4.5, N = 15) (Fig. 2). The difference between red and white muscle is primarily caused by a difference in endothelial nuclei (24 ± 5% in red muscle and 8 ± 2% in white muscle). This is in accordance with the higher vascularisation of red muscle as described by Mosse (1979), Egginton & Johnston (1982a) and Sänger (1992). The differences in erythrocyte nuclei (10 ± 3% in red and 6 ± 3% in white muscle) and in other nuclei (fibroblasts, Schwann cells, etc; 33 ± 6% in red muscle and 32 ± 6% in white muscle) were smaller.

Red axial muscle had a higher percentage of myosatellite cells than white axial muscle. In both muscle types this percentages decreased with increasing length (fig.3).

Percentages of proliferating nuclei

The control (gut) tissue showed that, four hours after injection of 250 μg BrdU/g bodyweight, 14 ± 7% (mean ± sd; N = 5) of the epithelial cell nuclei in the intestinal crypts were labelled. A tenfold increase of the dose up to 2500 μg/g bodyweight did not increase the percentage of labelled nuclei. No signs of endogenous peroxidase reactions could be found in the control sections although erythrocytes and granulocytes were clearly present. In fish from 5 cm, 10 cm and 15 cm SL, four hours after injection about 3% of the nuclei in the muscle tissue were labelled (Table 1). The three different sample regions (dorsal, medial, and lateral) did not show significant differences in the percentages of proliferating nuclei.

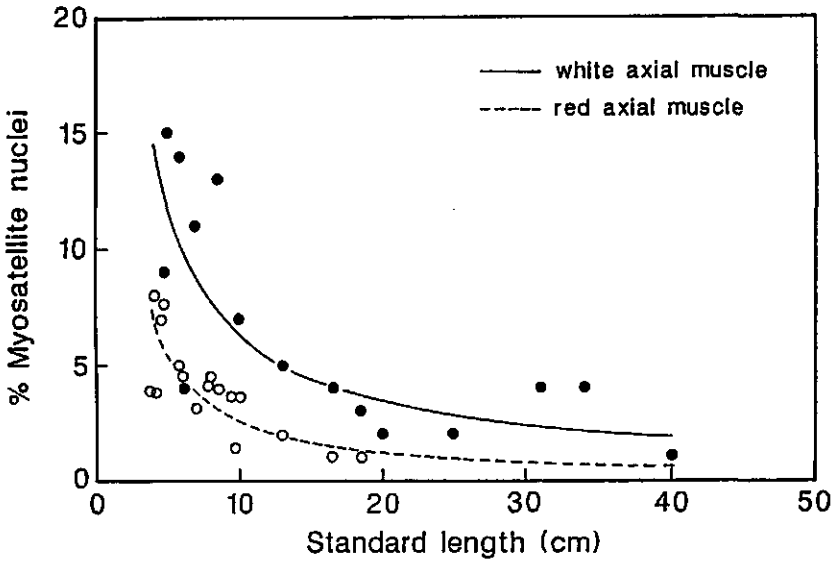

Fig. 3. Percentages of muscle nuclei that are myosatellite nuclei in red and white axial muscle tissue of carp with a standard length between 3.4 cm and 40 cm (red axial muscle) and between 3.4 cm and 20 cm (white axial muscle). Curve fitting resulted in the following equations: White axial muscle; % myosat. nuclei = 31.8 * SL (cm) $^{-1.41}$ (R^2 = 0.71). Red axial muscle: % myosat. nuclei = 49.7 * SL (cm) $^{-0.90}$ (R^2 = 0.66).

SAMPLE	STANDARD LENGTH 5 cm	10 cm	15 cm
DORSAL	2.7 %	3.7 %	2.8 %
MEDIAL	3.3 %	3.3 %	2.2 %
LATERAL	3.2 %	3.5 %	2.2 %

Table 1. Percentages of BrdU labelled nuclei at different cross sectional locations in white epaxial muscle at the 4th - 7th radius of the dorsal fin for carp of 5, 10 and 15 cm SL. Per sample more than 500 nuclei were counted.

DNA and protein

In white muscle no significant changes in the amount of protein per gram tissue (average protein content is: 19.4 % (wet weight) ± 1.1 % (mean ± SD), N = 17) were observed during growth (Fig. 4). No significant differences in protein content between the three different samples sites were found (Friedman two tailed: $Chi^2 = 0.43$, N = 15, $0.8 < p < 0.9$) (Table 2). White axial muscle did show a significant rostro-caudal difference in DNA content (Friedman two tailed: $Chi^2 = 7.88$, N = 17, $0.01 < p < 0.02$). However, the differences in the DNA/protein ratio of the three sample sites were not statistically significant (Friedman two tailed: $Chi^2 = 1.08$, N = 13, $0.50 < p < 0.70$).

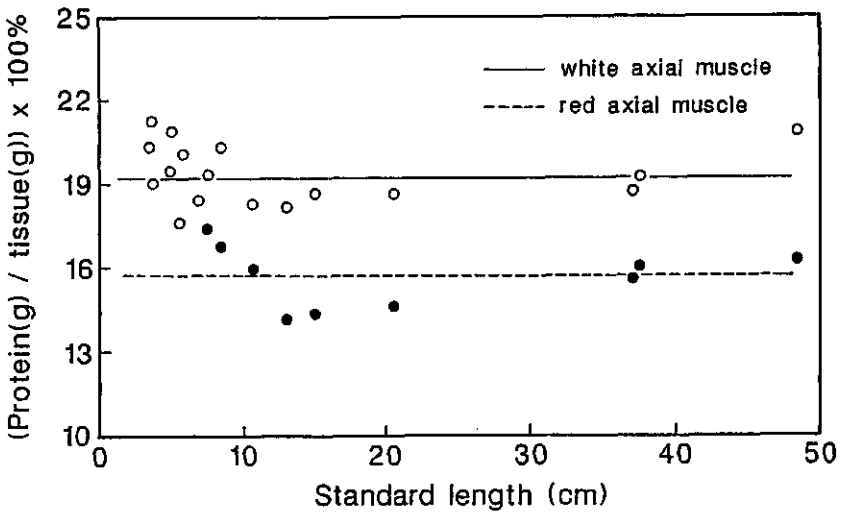

Fig. 4. Protein content, (gram protein/gram tissue (wet weight)) x 100%, of white and red axial muscle of carp with a standard length between 3.4 cm and 48.5 cm. The averaged protein content of white axial muscle is 19.4% ± 1.1% (mean ± SD, N = 17). The averaged protein content of red axial muscle is 15.7% ± 1.1% (mean ± SD, N = 9).

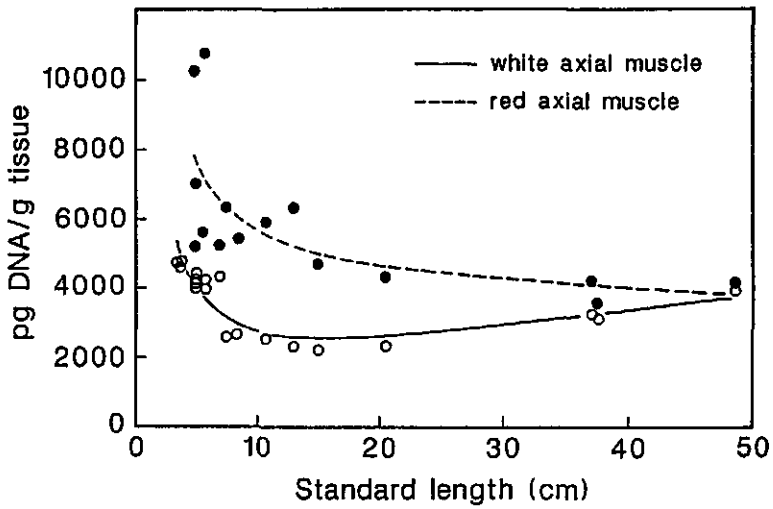

Fig. 5. DNA content (pg DNA / gram tissue) of white and red axial muscle of carp with a standard length between 3.4 cm and 48.5 cm. The fitted curves are calculated from multiple regression. White axial muscle: DNA content (pg/g tissue) = 785 + 14501/SL (cm) + 54.22 * SL (cm), (R^2 = 0.80). Red axial muscle: DNA content (pg/g tissue) = 3928 + 18883/SL (cm) - 10.12 * SL (cm), (R^2 = 0.46).

In red axial muscle the average protein content was: 15.7 % (wet weight) ± 1.1 % (mean ± SD, N = 9). Red muscle showed a significant rostro-caudal difference both in protein content (Friedman two tailed: $Chi^2 = 12.28$, N = 7, $0.001 < p < 0.01$) and in DNA content (Friedman two tailed: $Chi^2 = 7.22$, N = 9, $0.02 < p < 0.05$) between the three sample sites (Table 3). The differences in DNA/protein ratio of the three sample sites were not statistically significant (Friedman two tailed: $Chi^2 = 1$, N = 6, $0.50 < p < 0.70$).

Both for red and for white muscle, significant differences in DNA content of the individual sample sites existed. But, for red as well as for white muscle, the curves showing the DNA content as a function of standard length for each sample site were very similar. Therefore, we averaged the data from the three sample sites to obtain the curves for the DNA contents as shown in Fig. 5.

For each tissue the changes in DNA/protein ratio (Fig. 6) were similar to the changes in the amount of DNA per gram tissue (Fig. 5), as was expected from the small changes in protein content during growth.

In red axial muscle, the DNA content (Spearman two tailed: $r = 0.729$, $0.002 < p < 0.005$, $N = 15$) and the DNA/protein ratio (Spearman one tailed: $r = 0.783$, $0.01 < p < 0.025$, $N = 9$) curves showed a continuous decrease over the entire investigated size range. For white axial muscle the amount of DNA per gram (wet weight) tissue decreased by 50% from 5 - 15 cm SL. In fish larger than 13 cm SL an increase in DNA content was found. This increase was significant (Spearman, two tailed: $r = 0.89$, $0.02 < p < 0.05$, $N = 6$), but the resulting increase in DNA/protein ratio (Fig. 6) just failed significance (Spearman, one tailed: $r = 0.77$, $0.05 < p < 0.10$, $N = 6$).

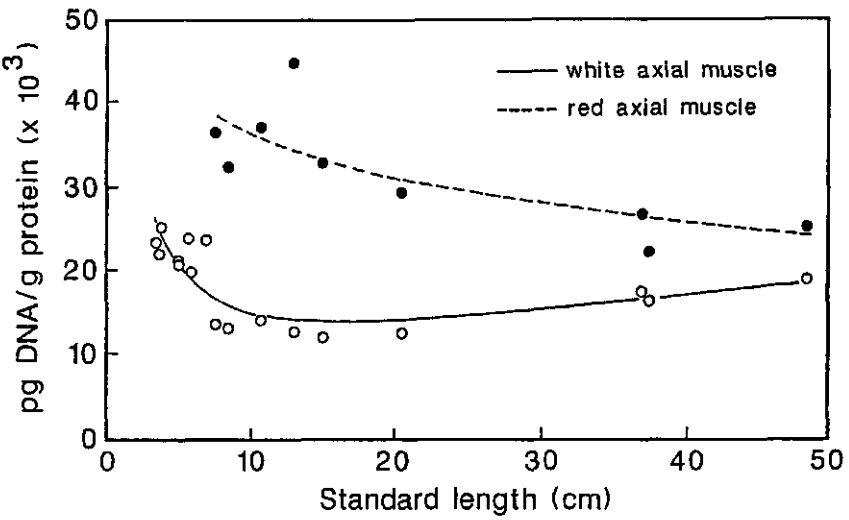

Fig. 6. DNA/protein ratio (pg DNA / gram protein) of white and red axial muscle of carp with a standard length between 3.4 cm and 48.5 cm. The fitted curves are calculated from (multiple) regression. White axial muscle: DNA/protein ratio (pg DNA/g protein) = 6312 + 63542/SL (cm) + 221.3 * SL (cm), (R^2 = 0.68). Red axial muscle: DNA/protein ratio (pg DNA/g protein) = 53817 - 17583 * log SL (cm), (R^2 = 0.58).

Protein (g/g tissue) x 100% of white and red axial muscle of carp

Standard length	white axial muscle			red axial muscle		
SL (cm)	site A	site B	site C	site A	site B	site C
3.4	22.32	19.91	19.85			
3.6	14.60	22.07	20.90			
3.7	19.45	19.03	18.79			
4.9	19.22	19.65	19.68			
5.0	19.37	20.44	18.91			
5.6	16.36	17.72	21.67			
5.8	19.69	19.02	19.34			
6.9	18.19	18.64	23.46	15.78		
7.5	18.79	19.72	22.27	16.31	17.61	18.43
8.4	19.42	19.47	18.24	16.81	20.81	22.55
10.7		18.35			16.02	
13.0	18.25	18.19	18.46		14.20	
15.0	18.78	18.81	18.69	13.58	14.40	15.09
20.5	18.68	18.68	18.74	13.68	14.30	16.14
37.0		18.79		15.16	15.67	16.17
37.5	19.57	18.92	19.45	15.16	15.92	17.20
48.5	21.08	20.67	21.01	15.62	15.15	18.32

Table 2. **Protein content, (gram protein / gram, wet weight, tissue) x 100% of white and red axial muscle. See figure 1 for the location of the sites A, B and C.**

White axial muscle had a higher protein content than red axial muscle (Fig. 4), as was also found by Love (1980). In small carp (about 5 cm SL), red axial muscle contained more DNA than white muscle did. This is mainly due to a difference in nuclei as the difference in mitochondrial DNA between the fibre types (red fibres have about 25 % volume mitochondria and white fibres about 3% volume, Akster, 1981; 1985) is unlikely to account for more than 2% of the measured difference (see Alberts *et al*, 1989). But at about 48 cm SL we found similar amounts of DNA in both muscle types (Fig. 5).

DNA (pg/g tissue) content of white and red axial muscle of carp

Standard length	white axial muscle			red axial muscle		
SL (cm)	site A	site B	site C	site A	site B	site C
3.4	4847	4129	5298			
3.6	4289	4217	5541			
3.7	4453	4778	5176			
4.9	3452	4109	4317		(10243)	
4.9	4104	4123	4501		(5212)	
5.0	4320	3127	5559		(7004)	
5.6	3734	4115	4766		(5606)	
5.8	3844	3660	4385		(10754)	
6.9	3609	4328	5102	4839	5244	5672
7.5				7783	5068	6219
8.4	3244	3172	1617	4860	5130	6341
10.7	2244	2394	3033	5796	6240	5721
13.0	2244	2732	1952	7075	5864	6066
15.0	2212	2223	2282	4478	4913	4706
20.5	2186	2628	2174	4087	4913	4706
37.0	3441	3117	3148	3718	4117	4734
37.5	2957	3204	3251	3285	3618	3771
48.5	3656	4188	3958		4126	

Table 3. **DNA (pg) per gram muscle tissue (wet weight). The data between brackets ( ) for red muscle tissue are obtained from the total strip of red axial muscle tissue behind the shoulder girdle. See figure 1 for the location of the sites A, B and C.**

As in white muscle 54% of the nuclei are myonuclei and in red muscle 32% a carp of 48 cm SL may have a higher amount of "muscle DNA" per gram muscle in white than in red axial muscle.

Discussion

DNA and protein contents of muscle

In red axial muscle caudal samples had a higher protein content than more anterior samples. This may be caused by a higher fat contents of anterior red muscle as was found by Frontier-Abou (1969).

In carp we found a decrease in DNA content during growth, both in white and in red axial muscle. A decreasing DNA content of the muscle tissue is usually found during a stage of growth when many new fibres are present. In young mice (Cardasis & Cooper, 1975) and in chicken (Matthew & Moore, 1987) a decrease in DNA/cytoplasm ratio was found during early growth when there are many small muscle fibres. A similar temporary decrease in DNA concentration with increasing length of young fish was found by Luquet & Durand (1970) in young rainbow trout. An explanation for this phenomenon is that hyperplasia is brought about by fusion of differentiated myogenic cells, which have very little cytoplasm, followed by an increase in the amount of cytoplasm of the new fibres until their minimum myonucleus/sarcoplasm ratio is reached. Thus the decreasing DNA/cytoplasm ratio in fact is caused by the hypertrophy that accompanies or closely follows hyperplasia. In young fish, where hyperplasia is an important mode of growth, the continuing addition of new small fibres may account for a relatively long period of decrease in DNA content.

In white muscle the decrease in DNA content stopped at a length of about 15 cm SL, indicating an important decrease in the occurrence of hyperplasia in fish of greater length. This is in agreement with our data on the occurrence of hyperplasia inferred from the presence of small muscle fibres (0 - 20 μm in diameter) in the tissue (Koumans et al., 1992). The continuing decrease in DNA content of red axial muscle suggests a continuing presence of very small fibres in this muscle type and therefore, a continuing occurrence of hyperplasia over the entire investigated growth range (see also Stickland, 1983). A prolonged occurrence of hyperplasia in red axial muscle probably serves to maintain a relatively small fibre size with a high surface/volume ratio that is more suitable to aerobic metabolism than the larger fibre size found in white muscle.

Hyperplasia and myosatellite cells

There are several indications for the origin of new fibres from myosatellite cells. In addition to myosatellite cells also myosatellite fibres, myosatellite cells containing myofibrils, are found in fish muscle (Akster, 1983; Koumans *et al*, 1990). Myosin of newly formed small muscle fibres of fish differs from that of older, larger fibres (Rowlerson *et al*, 1985; Romanello *et al*, 1987). In mammals and birds the myosin isotype of myotubes formed by isolated myosatellite cells is similar to that of young fibres developing in vivo, and different from that of adult fibres (Whalen *et al*, 1978; Matsuda *et al*, 1983; Yamada *et al*, 1989; Düsterhöft *et al*, 1990). Myotubes formed by myosatellite cells that we isolated from carp of 5 cm SL also contain an isotype of myosin different from that found in larval and adult muscle tissue (B. Fauconneau, INRA, Rennes, France, personal communication). For carp, this suggests an origin of new muscle fibres from myosatellite cells. Whether the higher percentage of myosatellite cells found in red axial muscle is related to a prolonged hyperplasia in this fibre type is questionable. Also in birds and in mammals oxidative muscle contains more myosatellite cells and more myonuclei than glycolytic muscle (Gibson & Schultz, 1982; Matthew & Moore, 1987; Düsterhöft *et al*, 1990). This difference has been related to differences in innervation, in metabolic rate, in protein turnover and in activity of the muscle fibres (Schultz, 1984; Düsterhöft *et al*, 1990). At the moment it is not yet possible to attribute a specific functional significance to the difference in satellite cells between red and white muscle of fish.

In some *Mugilidae* the new small fibres appearing between the large white fibres are histochemically and electrophoretically identical to the latter. As these fish also show a paucity (or even absence) of myosatellite cells, fibre splitting may be the mechanism of hyperplasia in the white muscle of these fish (Romanello *et al*, 1987). However, even if fibre splitting is the mechanism of formation of new muscle fibres in some fish species, it provides no solution for the problem of the origin of the additional myonuclei in hypertrophy.

The origin of myonuclei by proliferation of myosatellite cells.

We calculated if the myonuclei added during muscle growth could originate from the myosatellite cells. Therefore we made the following assumptions:

1. All nuclei contain the same amount of DNA (Enesco & Puddy, 1964).
2. Nuclei in terminally differentiated muscle cells are unable to proliferate (Cardasis & Cooper, 1975; Campion, 1984; Moss & Leblond, 1971).
3. Only myosatellite cells, cells situated underneath a muscle fibres basal membrane, form the additional muscle nuclei required in muscle growth (Moss & Leblond, 1970; Moss & Leblond, 1971; Campion, 1984).

Transition of our data from a length scale (cm) to a time scale (weeks) was based on a growth curve (fig. 7) of optimally fed carp reared at 23 °C (adapted from Huisman, 1974). This growth curve gives the relation between bodyweight (grams) and age (weeks). As we also know the relation between bodyweight (BW) and standard length: Log BW (grams) = 3.046 * SL (mm) - 4.689 for fish larger than 2 cm SL (Shansui & Hiroshi, 1971) it is possible to calculate the relation between age and standard length:

Age (weeks) = 0.25 + 1.05 * Sl (cm). (I)

We obtained an estimation of the total numbers of muscle nuclei present in white axial muscle, as a function of age from a combination of the data on: the amount of DNA per gram tissue, the amount of DNA per nucleus (Koumans *et al*, 1991), the percentage of muscle nuclei that are myonuclei and the increase in trunk weight as adapted (see Koumans *et al*, 1991; 1992) from Oikawa & Itazawa (1984) and Shansui & Hiroshi (1975). This resulted in:

Number of myonuclei = $3.1 * 10^9 + 191054 * age^4$ (weeks) (II)

(Fig. 8, $R^2 = 0.999$)

From the curve in figure 8 it is possible to calculate the increase in myonuclei as: $(4 * 191054) * age^3$ (weeks). (III)

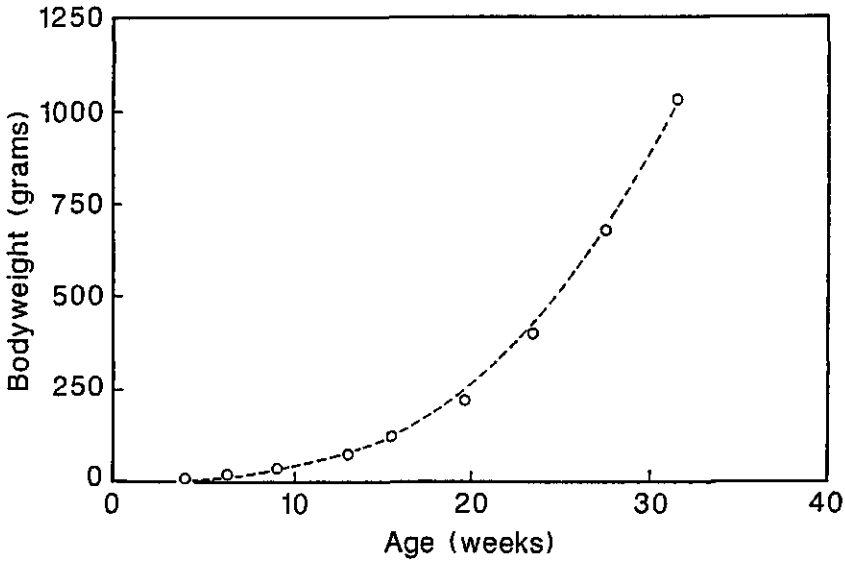

Fig. 7. Growth curve of carp showing the relation between bodyweight (grams) and standard length (adapted from Huisman, 1974): Bodyweight (grams) = 0.0326 * age^3 (weeks) (R^2 = 0.998).

To estimate the increase in the number of myonuclei per hour we divided equation III by (7 * 24). The increase in myonuclei per hour can also be expressed as a percentage of the myonuclei present (Fig. 9). As we know the percentage of myonuclei that are myosatellite nuclei, the increase in myonuclei per hour can also be expressed as a percentage of the myosatellite nuclei present (Fig. 10). This can be used to calculate the maximum cell cycle time of the myosatellite cells if they are held responsible for the total increase in myonuclei (Fig. 11). For carp of 5 cm SL this maximum cell cycle time is about 120 hrs. But with increasing length of the fish it decreases to less than 10 hrs in fish larger than 20 cm SL. As myosatellite cells of rat at 37 °C have cycle times of about 12 h (Bishoff, 1989) when exposed to growth factor from damaged muscle, one would expect in fish at 23 °C a cell-cycle time of about 36 hrs ($Q10^{2.8}$). Even this may be optimistic as Aamiri & Franquinet (1985) found that myosatellite cells of another heterotherm animal, Xenopus, have at 25 °C an *in vitro* cell-cycle time of 72 hrs. Therefore a cell-cycle time of 10 hrs is definitely too short.

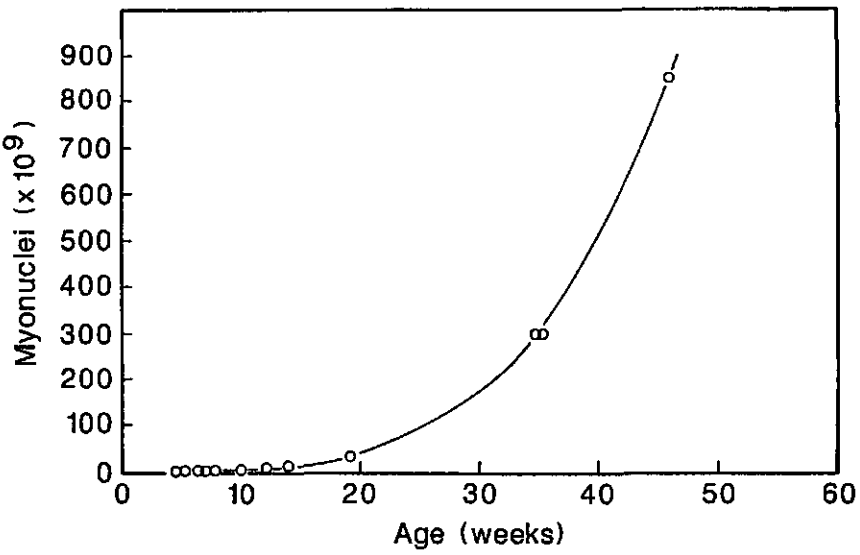

Fig. 8. Increase in the number of myonuclei as a function of age (weeks). The curve is calculated by regression analysis. Number of myonuclei = 3.1 * 10^9 + 191054 * age^4 (weeks) (R^2 = 0.999).

Moreover, not all of the myosatellite cells are able to proliferate. More than half of the myosatellite cells in carp white axial muscle probably are postmitotic (Koumans *et al*, 1990; Koumans *et al*, submitted). This means that myosatellite cells in carp white axial muscle can not account for the entire increase in myonuclei during growth. Therefore, (a) either the calculations we made to describe the increase in nuclei are inaccurate, (b) we underestimated the number of myosatellite cells or (c), one or more of the assumptions we made are not valid.

Calculations

We calculated that the critical length of carp, above which myosatellite cells are unable to account for the total increase in myonuclei is between 10 cm and 20 cm SL. In this length range also the highest reliability of our calculations is reached: (1) Here the curve of the percentage of muscle nuclei that are myosatellite nuclei is based on observation, not on extrapolation, and the correlation coefficient of the curve is reasonably high (R^2 = 0.71).

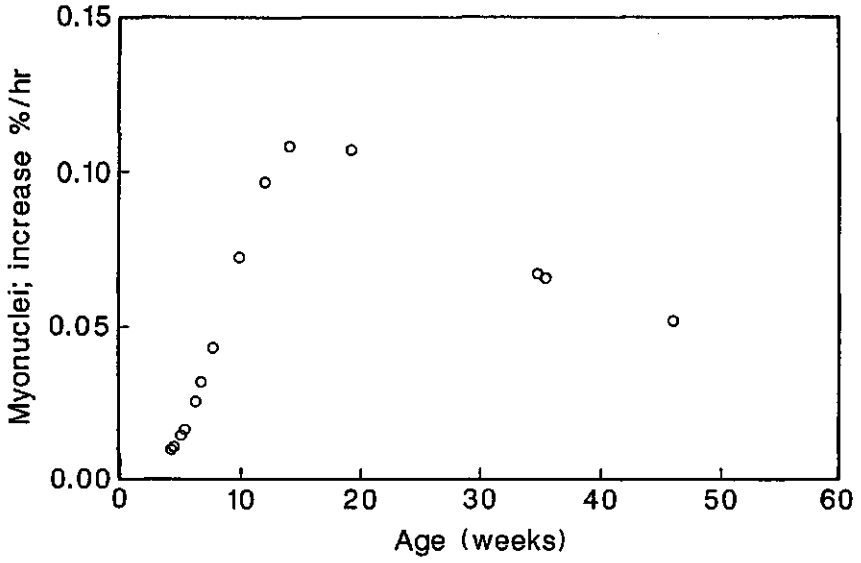

Fig. 9. Increase in myonuclei/hr as a percentage of the myonuclei that are present.

(2) The variation in the percentage of nuclei in the muscle tissue that are myonuclei (Fig. 2) in carp larger than 10 cm SL seems smaller than in carp of about 5 cm SL. (3) The curve of the weight of the body (Fig. 7), used to calculate the total number of myonuclei, accurately describes the data (R^2 = 0.998), as does the curve of the total number of myonuclei (Fig. 8), used to calculate the increase in myonuclei as a function of time (R^2 = 0.999). Since the number of myosatellite cells is calculated as a percentage of the number of myonuclei, possible systematic errors in the determination of the total number of myonuclei does not greatly influence our calculations. Therefore, we think that our calculations give a reasonably accurate view of the occurring increase in myonuclei.

Numbers of myosatellite cells

We found no difference in the percentage of proliferating nuclei at different cross sectional locations (Table 1). Therefore, underestimation of the number of myosatellite cells by missing a growth zone, as described by Veggetti *et al* (1990) in larval fish, is unlikely.

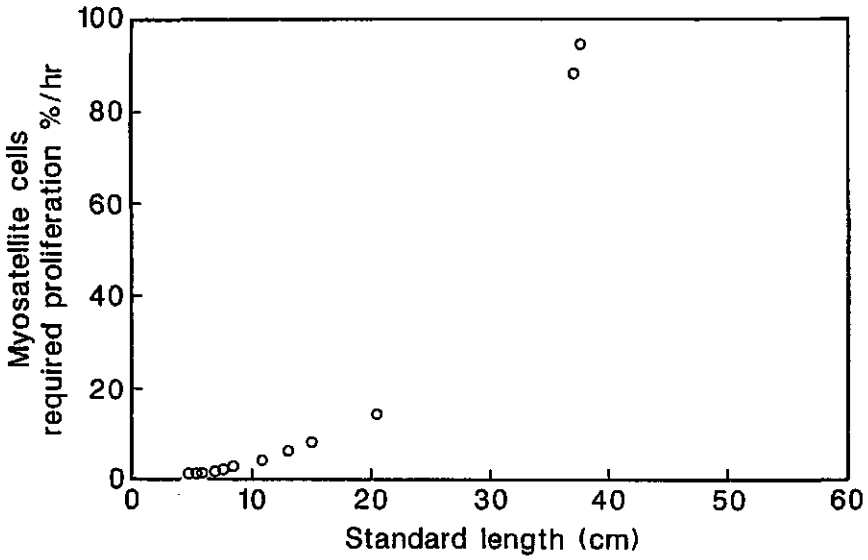

Fig. 10. **Increase in myonuclei/hr as a percentage of the myosatellite nuclei that are present.**

Underestimation of the number of myogenic cells by strict adherence to the classical criterion of the myosatellite cell (cells situated under a muscle fibres basal lamina) is possible; this concerns assumption 3, the definition of myosatellite cells.

Validity of the assumptions

1. Unequal DNA contents of nuclei would allow increase of nuclei by amitotic division as has been suggested by Boudjelida & Muntz (1987) in myogenesis of Xenopus laevis. This should be checked by densitometry of individual nuclei, but we do not think it a very likely mechanism. In rat and mouse muscle growth, polyploidy could not be observed (Enesco & Puddy, 1964)

2. The possibility that, in fish, differentiated muscle nuclei are still able to proliferate is not very likely. Although muscle precursor cells of some mammalian and bird species that already contain desmin (a muscle specific protein) show *in vitro* proliferation (Kaufman & Foster, 1988; Yablonka-Reuveni & Nameroff, 1990; Allen *et al*, 1991), terminally

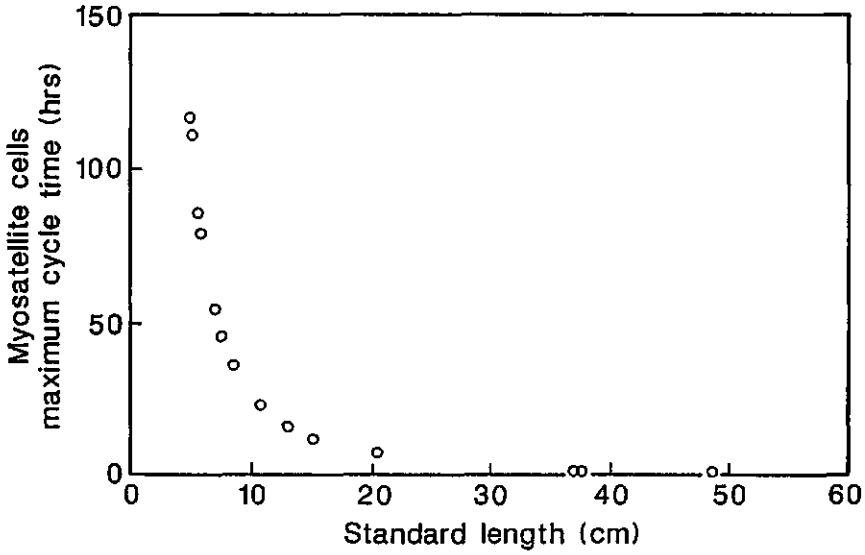

Fig. 11. Maximum cell cycle time of the myosatellite cells if these cells have to account for the total increase in myonuclei during growth.

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differentiated muscle nuclei of mammals and birds are considered unable to proliferate. Fish myonuclei may be different. However, *in vitro* experiments show that, in carp, even myosatellite cells that contain desmin, are already unable to proliferate (Koumans *et al*, submitted). So there is no apparent reason to assume that terminally differentiated myonuclei of carp are able to proliferate.

3. The assumption that only myosatellite cells, situated under a muscle fibres basal membrane, produce additional nuclei is not universally valid. In post-metamorphic amphibia muscle precursor cells are present outside the basal lamina as pericytes (Popiela, 1976), although amphibian larvae have many myosatellite cells (Popiela, 1976; Takahama *et al*, 1984). The position under the muscle fibres basal lamina is the main ultrastructural criterion for recognition of a myosatellite cell. Additional ultrastructural characteristics are paucity of cytoplasm and, in more differentiated cells, the presence of many polyribosomes. In carp, cells resembling myosatellite cells in ultrastructure, are found outside the basal lamina (Fig. 12 a).

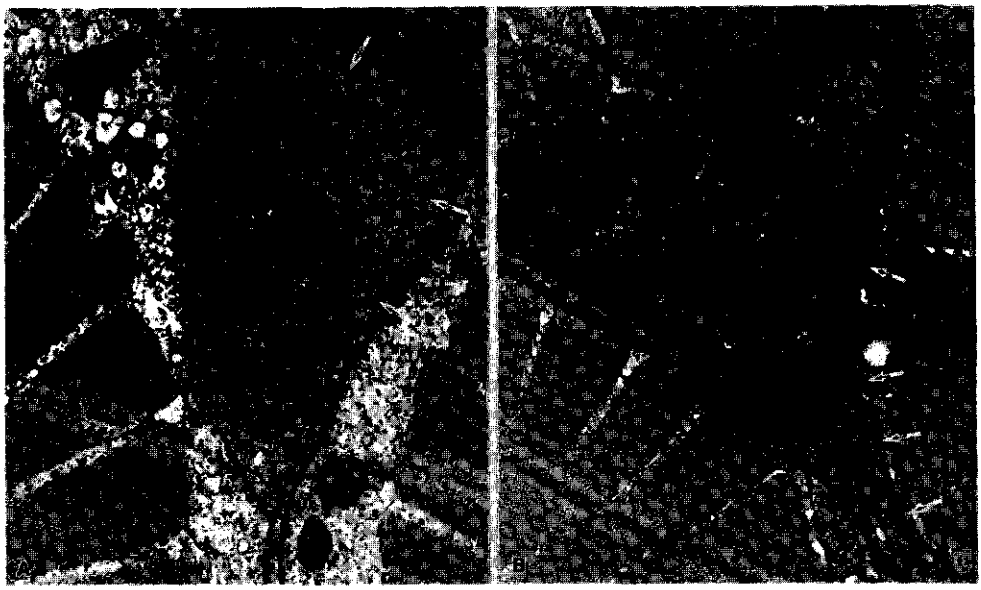

Fig. 12. TEM micrographs of carp muscle tissue showing: A. Myosatellite cell and three anuclear profiles (arrows) that resemble myosatellite cells in ultrastructure, but that are positioned outside the basal lamina of the myofibres (x 3000). B. Patches of basal lamina (arrows) between a myosatellite cell and myofibre (x 3000).

Furthermore, discerning myosatellite cells is often complicated by the presence of patches of basal lamina between myosatellite cell and muscle fibre (Fig. 12b). These observations suggest that, also in fish, myogenic cells may occur outside the muscle fibre's basal lamina. The lack of ultrastructural markers to distinguish undifferentiated myogenic cells from ditto fibroblast makes ultrastructural quantification of these cells unrealistic.

If myogenic cells, other than the classical myosatellite cells, are involved in muscle growth, the muscle tissue has to contain enough proliferating cells to account for the total addition of nuclei. About 3 % of the nuclei we found in the tissue of carp of 5 cm, 10 cm and 15 cm SL, were proliferating, which is not incompatible with the percentage of proliferating nuclei calculated to be necessary (fig 9).

We think it likely that other than myosatellite cells contribute to the increase of myonuclei in carp muscle growth. But proving this will require the availability of specific markers for myogenic cells. Presently, such markers are not available. However, ultrastructural identification of cells labelled with probes for muscle regulation genes, homologous to MyoD1 and myogenin in mammals, see Grounds *et al.* (1992), looks promising for solving the question of the presence of myogenic cells other than myosatellite cells.

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CHAPTER VII

THE PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST CARP (*Cyprinus carpio* L.) MYOSATELLITE CELLS.

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Key words: *Fish, muscle growth, myosatellite cells, monoclonal antibodies, Cyprinus carpio L. (Teleostei)*

Summary

Myosatellite cells play a very important role in muscle growth. Therefore, the availability of antibodies which enable *in situ* and/or *in vitro* identification of myosatellite cells would be of great advantage. As, to date, no specific myosatellite cell markers are known we decided to use myosatellite cells as an antigen to produce the desired antibodies with the monoclonal antibody technique. Induction of neonatal tolerance, in some experiments combined with passive immunisation with antibodies against immune-dominant non-specific antigens, was used to raise the production of myosatellite cell specific antibodies. Although some changes in the bias of the immune response could be observed after application of these techniques, none of the more than 1500 clones tested, produced antibodies reacting specifically with myosatellite cells. Our fusion experiments and data from the literature indicate that markers specific for myogenic cells, do not belong to the class of immune-dominant antigens.

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Introduction

Myosatellite cells (Mauro, 1961) play an important role in muscle growth. These cells deliver the additional nuclei in muscle cell hypertrophy (Moss and Leblond, 1970; 1971; Allen *et al.*, 1979; Campion, 1984), they form new fibres in regeneration of damaged muscle (Allbrook, 1981; Ontell *et al.*, 1984; Carlson, 1986) and, in fish, they probably also form new muscle fibres in hyperplasia (Weatherley *et al.*, 1979; Rowlerson *et al.*, 1985; Romanello *et al.*, 1987; Koumans *et al.*, 1990; Veggetti *et al.*, 1990).

Earlier studies (Koumans *et al.*, 1990; 1991) have demonstrated that the role of myosatellite cells in carp muscle growth can be studied both *in situ* and *in vitro*. In *in vitro* studies, myogenic (myosatellite) cells can be unambiguously identified when the cells contain muscle specific proteins recognisable with appropriate antisera. Undifferentiated myosatellite cells, however, can only be recognized by their specific spindle shaped morphology (Koumans *et al.*, 1990), which is not a 100% reliable criterium. *In situ* studies require the use of transmission electron microscopy (TEM) for the recognition of myosatellite cells (Koumans *et al.*, 1990). In larger carp, the percentage of muscle nuclei that belong to myosatellite cells is rather small; less than 1% in carp larger than 18 cm standard length (Koumans *et al.*, 1991). Therefore, TEM studies in large carp require large sample sizes and thus involve a large amount of work and time. Another limitation is that TEM can not be easily combined with immune-histochemical techniques. The strict requirement of preservation of cell morphology necessary for the identification of the myosatellite cells mostly results in loss of reactivity of the antigenic sites.

Immune-histochemical (light-microscopical) *in situ* recognition of myosatellite cells more easily would permit the use of large sample sizes, and immune-histochemical double staining techniques. Therefore, the study of carp (fish) muscle growth would very much benefit from the availability of antibodies enabling the *in vitro* and *in situ* recognition of undifferentiated myosatellite cells and/or of antisera enabling the *in vitro* recognition of several stages of differentiation of myosatellite cells. As we did not know of a specific myosatellite cell antigen that could be isolated and purified, we decided to start immunisation procedures for the production of specific immune reagents with the use of whole purified myosatellite cells as the immunogen.

Whole myosatellite cells constitute a complex mixture of antigens, giving rise to hundreds or thousands of different antibody-secreting plasma cell clones the products of which make up a conventional antiserum. Many of the clonal products in such an antiserum may be directed against undesired antigens. The monoclonal antibody technique (Köhler and Millstein, 1975) offers the opportunity for individual selection and culturing of those clones that produce relevant antibodies.

However, after immunization with a complex mixture of antigens a considerable bias exists towards obtaining antibodies specific for immune-dominant determinants (Thalhamer and Freund, 1985; Matthew and Sandrock, 1987). Our first fusion experiments showed that markers specific for myosatellite cells probably do not belong to the class of these immune-dominant determinants. In order to manipulate the bias of the normal immune response, and to increase the number of clones that produced antibodies against other, less dominant, antigens we used induction of neonatal tolerance (Roitt *et al.*, 1985) against non-myosatellite cell-specific immune-dominant antigens, in combination with or without passive immunisation (Thalhamer and Freund, 1985).

Materials and methods

Antigen

Immunisations were performed with myosatellite cell preparations isolated from epaxial white muscle of carp of about 5 cm SL, that had been cultured as described previously (Koumans *et al.*, 1990).

Before harvesting, the muscle cell culture monolayers were washed three times with culture medium (DMEM diluted 9:1 with H_2O) without serum, three times in PBS, and incubated for 1 minute in an EDTA solution in PBS (74.4 mg EDTA per 100 ml). The cells were brought into suspension by scraping them from the bottom of the culture flask with a rubber policeman and washed three times in PBS (centrifugation each time for 5 min at 350 g and 4 °C). For use as an immunogen, the cells were resuspended to a concentration of 10^6 cells in 250 μl PBS (mixed 1:1 with complete Freund's adjuvance for the first immunization, and mixed 1:1 with incomplete Freund's adjuvance for the booster injection), or 2 x 10^6 cells in 500 μl PBS for the final booster three days before fusion.

Tolerogen

As a tolerogen, a liver/brain cell suspension from the same fish as were employed for the preparations of the myosatellite cells was used. The liver/brain cell suspension was prepared by squeezing minced pieces of the organs through a nylon gauze filter (100 μm). After three wash steps in PBS with centrifugation for 5 min at 350 g and 4 °C, the cell suspension was resuspended in 10 times the volume of PBS and frozen in small aliquots (250 μl) at -80 °C prior to use. To induce tolerance, mice were, 1 day after birth, injected intra-peritoneally (IP) with 10 μl of this tolerogen. Every week thereafter, they were injected with increasing doses of the tolerogen, 25 μl, 30 μl, 250 μl, 250 μl and 250 μl respectively, until receiving their first IP injection with the myosatellite cell antigen preparation, at an age of 6 weeks (fig. 1). After the last injection with tolerogen and before the first injection with antigen, the reaction patterns of the sera of the mice were tested immune-histochemically on cryosections of axial muscle of carp.

Passive immunisation

For passive immunisation, the antigen preparation (myosatellite cells) was combined with hybridoma supernatants containing monoclonal antibodies produced from previous fusion experiments, or with antiserum from prior immunizations. Also in these experiments mice that had been treated with tolerogen to reduce the immune response against immune-dominant determinants were used. In the original work of Thalhamer and Freund (1985) antibodies directed against undesirable antigenic determinants were injected immediately prior to the immunisation with the antigen. Since, in our case, purified antibodies were not available, and injection with hybridoma supernatants would also involve co-injection of several proteins from the culture medium, we decided to let antigen-antibody complexes form *in vitro*, and to inject the washed immune complexes for immunization. After thawing the harvested myosatellite cell preparations and combining them with the antibody mix or the serum, the resulting immune complex was washed three times in PBS followed by centrifugation for 5 min, 350 g at 4° C, and the final suspension divided in small aliquots (the precipitate equivalent of 10^6 sat. cells in 250 μl PBS) that were frozen at -80° C until use.

In experiment A, the mice were injected with the antigen preparation combined with a mixture of supernatants from 7 hybridoma clones strongly reactive with other than myosatellite cells. Of the supernatant of each clone, 4 to 8 ml was combined (giving a total

of 36 ml) which was then reduced to 8 ml in an Amicon concentrating device using a low protein binding membrane (Millipore).

In experiment B, the mice were injected with a combination of the antigen preparation and serum of mouse 4 (fusion 1) diluted 1:10 in PBS. (Serum of mouse 4 could be diluted 10,000 times before loss of immune-histochemical reactivity was observed in cryosections of carp muscle tissue.)

Fusions

Fusions were carried out at the Laboratory of Monoclonal Antibodies (LMA) in Wageningen using the standard protocol of this laboratory. Sp 2/0-Ag-14 myeloma cells and nucleated spleen were washed in DMEM (centrifuged for 10 min. at 1000 rpm at room temperature (RT)), combined in a 1:4 relation and centrifuged to a combined pellet (5 min. at 1000 rpm at RT). The combined pellet was resuspended very gentle by adding, drop by drop, 1 ml of a solution of 40% (w/v) Polyethyleenglycol (PEG) 4000 (Merck no. 9727) in 5% Dimethylsulfoxide (DMSO) in MiliQ water in a total time of 1 minute and incubated for another minute. After incubation, the suspension was diluted with 13 ml DMEM, added drop by drop in a total time of 7 minutes (the first ml in 2 min, then 2 ml in 2 min, 5 ml in 2 min, and 5 ml in 1 min.) After centrifugation (10 min at 800 rpm at RT) the pellet was resuspended in selection medium (DMEM + 10% foetal calve serum (FCS) + 5% inactivated horse serum (HS) + 1μg Azaserine/ml + 0.1 mM hypoxanthine/ml + 50 IU penicillin/ml + 50 μg streptomycin/ml) in a final concentration of 10^6 spleen cells/ml. After incubation for 2 hrs in a large tissue culture flask, the non attached cells (depleted of macrophages) were plated in a 96 wells culture plate in a volume of 100 μl per well. The technique has been briefly depicted in figure 1.

Cells were cultured for 2 weeks in selection medium and thereafter in culture medium (without azaserine). Clones of interest were subcloned by limiting dilution immediately after the first screening (fig. 2). The latter step is meant to reduce the chance of their being overgrown by irrelevant (non-producing) cells.

Only part of the isolated spleen cells were used for the fusion experiments, since we could not handle more than 500 culture wells through the subsequent screening procedure. Spleen cells that were not used in the fusion, were frozen in liquid nitrogen and stored for potential future use.

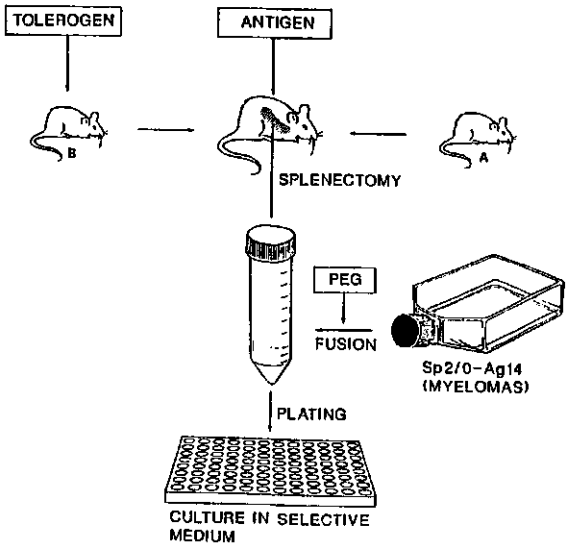

Fig. 1. Scheme of the fusion procedures. In fusions 1, 2 and 3, mice received their first injection with antigen at an age of 6 weeks (A = no tolerogen). In fusions 4 and 5 mice received a weekly injection with tolerogen, starting 1 day after birth, till at an age of 6 weeks they received their first injection with antigen (B = with tolerogen).

Screening

In a first screening we used cryosections of axial muscle of carp of 5 cm Sl. In these sections, a relative high percentage of myosatellite cells can be observed, but white-, pink-, and red muscle fibres, nerve tissue, skin, connective tissue, pigment cells, blood vessels and blood cells are also present (fig. 2). In a second screening, hybridoma supernatants that showed an interesting staining pattern (based on TEM information) were tested on cultured myosatellite cells that had been isolated from carps of 5 cm SL.

Both the cryosections and the cultured cells were fixed in cold (-20 °C) absolute acetone for 10 min. and 5 min. respectively. After fixation, the acetone was allowed to evaporate and the sections, or the coverslips with the cells, were incubated with the undiluted hybridoma supernatants for 1 hour at room temperature. To demonstrate the presence of bound antibodies, rabbit anti-mouse immunoglobulin conjugated to tetramethyl-rhodamine-iso-thiocyanate (RaM-Ig/TRITC) (Dakopatts) was used as a second antiserum. Between each

incubation step, the sections were rinsed twice for 5 min in TRIS/NaCl/T-20 (0.05 M TRIS, 1% NaCl and 0.05% Tween-20, pH 7.6), and 3 times for 5 min in 0.05 M TRIS, pH 7.6 (TBS, TRIS-buffered saline). After the last wash step the sections were mounted in a solution consisting of 90 ml Glycerol, 10 ml PBS and 100 mg Paraphenyleendiamine. The edges of the coverslips were sealed with nailpolish to prevent dehydration. The slides were examined within 24 hours after staining.

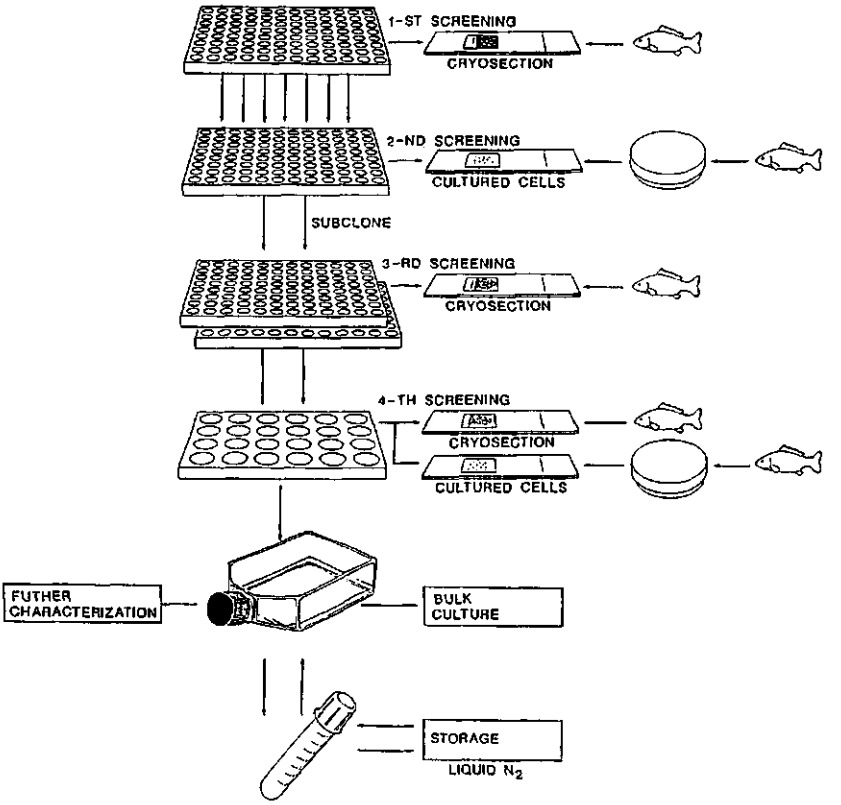

Fig. 2. Scheme of the screening procedure. Hybridoma supernatants were both tested on cryosections of carp muscle and on isolated myosatellite cells.

The effect of tolerance induction

To obtain information on the effect of tolerance induction on the production of antibodies with increased specificity, we divided the reaction patterns of the hybridoma supernatants into 5 different groups: a) Reactivity with connective tissue, b) Reactivity with muscle tissue, c) Reactivity with nerve tissue, d) reactivity with epithelia, and e) Reactivity with small cells (blood cells, but potentially also myosatellite cells).

Results

A total of 5 fusion experiments was carried out, the first three of which (fusion 1, 2, 3) were performed without additional techniques. In fusion nr 4, tolerance induction against immune- dominant antigens was used. In fusion nr 5 (A and B), this tolerance induction was combined with the passive immunisation technique.

Tolerance

Judged from tests for immune-reactivity against the tolerogen, both methods used to induce tolerance in the mice destined to serve as a source of antigen specific B cells for hybridoma fusion experiments appeared to have been successful. The immune response of mice that had been immunised with the tolerogen only, was either indistinguishable from that of the control mice, or showed only a very weak immune-response, mainly against connective tissue antigens. At a 1:10 dilution the sera from these mice showed a reactivity comparable to that from the control mice.

However, after immunisation with the antigen, both in fusion 4 and in fusions 5A and 5B, the activity of the sera collected on the day of the fusion showed no noticeable difference with those of the mice that were immunised the normal way (no tolerogen), both in reactivity pattern and in titre (positive reaction visible up to a dilution of 1:10,000).

Neither with, nor without passive immunisation, did the tolerance induction result in the production of myosatellite cell-specific antibody producing hybridoma clones. In the fusions 4 and 5, we observed a small reduction in the percentage of hybridoma's producing antibodies with reactivity for connective tissue as compared to fusions 1, 2 and 3 (fig. 3). Because of the small number of experiments however, it was impossible to prove that this effect is statistically significant.

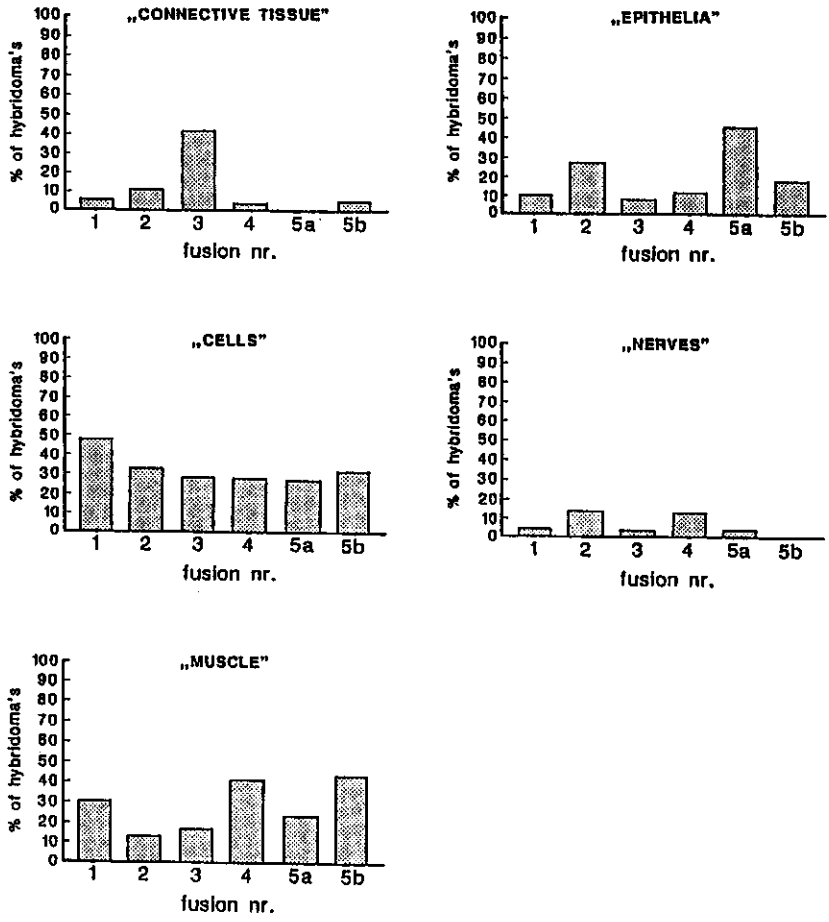

Fig. 3. **Percentages of hybridoma's reacting with the various subclasses (connective tissue, epithelia, cells, nerves, muscle). Fusions 1, 2 and 3 are without tolerance induction. Fusions 4 and 5 are with tolerance induction.**

Produced MoAbs

In fusions 4 and 5 (tolerance induction) many of the obtained monoclonal antibodies reacting with muscle tissue showed a reaction pattern similar to that of the K5 polyclonal anti-desmin antiserum (Koumans *et al.*, 1990). The vast majority (94%) of the 50 clones tested, produced immunoglobulins of the IgM subclass.

Although part of the more than 1594 clones tested, produced antibodies that bind to myosatellite cells, none of these antibody reactivities turned out to be specific. However,

some of the antibodies produced, may be of use in further investigations related to muscle development. The specificities of these antibodies are listed below.

Moabs reacting with muscle tissue

2B8 (IgM). This antibody demonstrates a reactivity similar to that of a polyclonal anti-desmin antiserum K5 which was produced at the Institute of Pathological Anatomy Nijmegen University (Ramaekers *et al.*, 1983), both on cryosections (***in situ***) and on isolated cells (***in vitro***). An antibody against desmin can be of use in structural studies of muscle cells but it can also serve as a differentiation marker of ***in vitro*** developing myosatellite cells (Koumans *et al.*, 1990). Western blotting revealed that this antibody indeed reacts with desmin but it also shows a weak cross reaction with vimentin.

S1E3B2 (IgM). The reaction pattern of this antibody on cryosections resembles that of 2B8. When analyzed on myosatellite cells cultures it again shows a reaction pattern comparable to 2B8. The antibody mainly reacts with large myosatellite cells and with cells containing myofibrillar material (cross striation pattern). However, it also reacts with clusters of fibroblast-like cells. Further, more detailed characterization of this antibody with respect to the antigen molecules it recognizes have to be carried out to obtain information about it's possible use as a differentiation marker.

1F2 (IgG1). This antibody shows a very specific reactivity with white axial muscle in carp, seabass and seabream. Western blotting experiments showed that it reacts with myosin heavy chain of white axial muscle of carp. In Seabass and in Seabream the antibody reacts in a mosaic-like way. Depending on the size of the muscle fibres, it shows no, a weak, or a strong reaction which suggests a reaction with a developmentally- regulated isoform of

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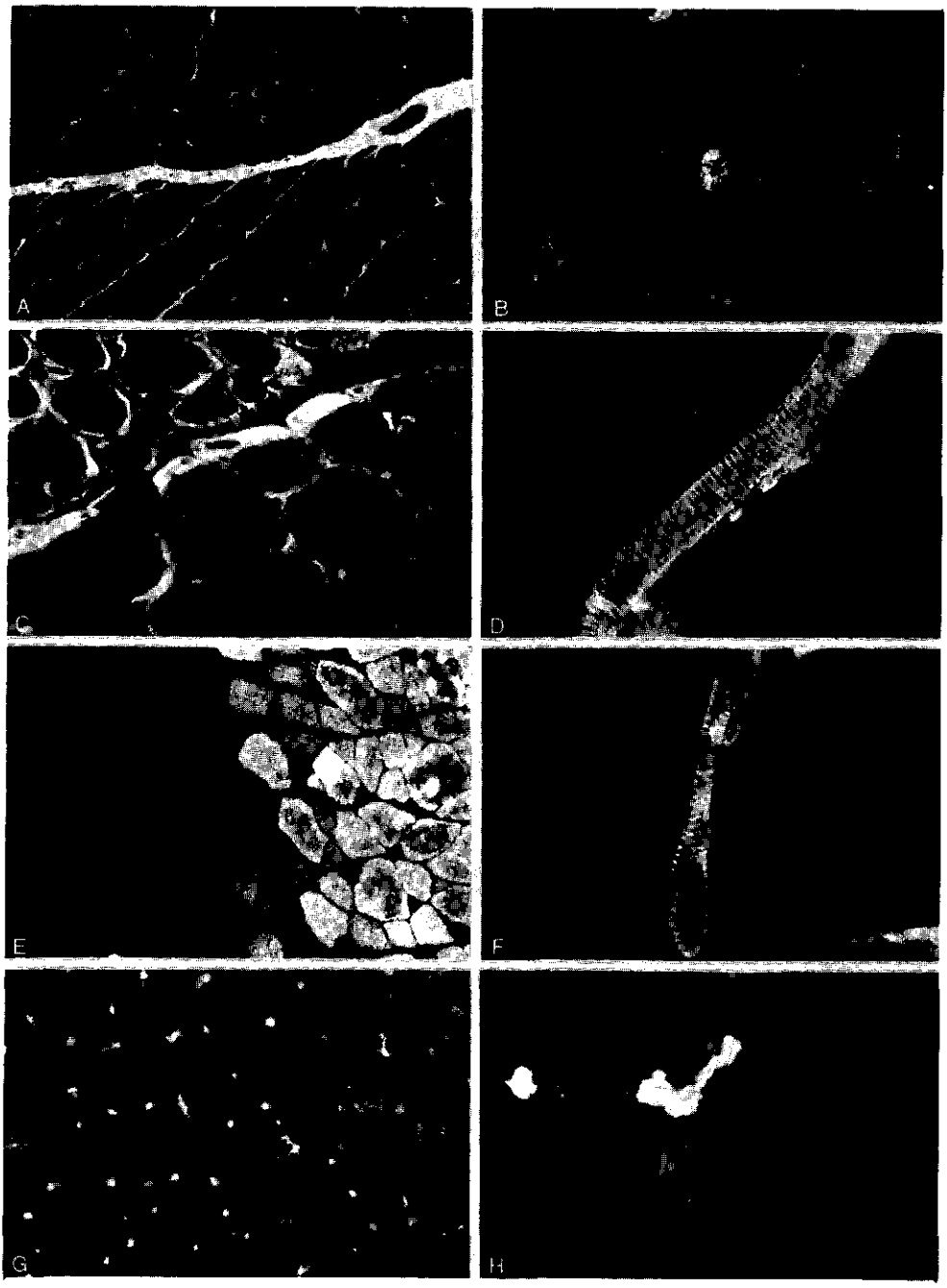
Fig. 4. Immuno-fluorescence micrographs (TRITC-staining) showing the reaction pattern of the monoclonals 5F12 (A and B), 2B8 (C and D), 1F2 (E and F) and 2A10 (G and H). The pictures A (x 100), C (x 400), E (x100) and G (x 400) show the reaction on cryosections of carp axial muscle. The pictures B, D, F, H, (x200) show the reaction on a one and a half month old myosatellite cell culture (large myotubes formed). 5F12 reacts with connective tissue; picture A shows a large positive myosept and the endomysia enveloping each fibre. 2B8 reacts with desmin; picture C shows the reaction with red axial muscle. 1F2 reacts with myosin heavy chain of white axial muscle; notice the absence of reactivity at the left side of picture E (red and pink axial muscle). It also reacts with *in vitro* formed myotubes. 2A10 reacts with epithelia and endothelium; picture G shows the reaction with capillaries in red axial muscle.


A
B
C
D
E
F
G
H

myosin heavy chain. However, in carp such a mosaic like pattern could only be found in the transition zone between pink and white muscle, but not in the deep white zone. Also the fact that the antibody reacts with isolated carp myosatellite cells and myotubes is not in agreement with a reaction with only an "adult" isoform of myosin heavy chain. Experiments with sections of embryonal and larval muscle tissue will be used for further characterisation of the monoclonal.

4H1 (IgM) shows an ***in situ*** reaction with all (white, pink and red) axial muscle, but pink muscle gives the strongest reaction. ***In vitro*** it reacts strongly with large myosatellite cells and myotubes, but no cross-striation is visible. S1H11C11 (IgM) shows an ***in situ*** reactivity with pink muscle and also a very weak reactivity with white muscle, but not with red muscle. ***In vitro*** no clear reaction can be found with the isolated cells.
Further characterisation of these antibodies with respect to the antigen molecules they recognize have to show their possible use in the identification of white muscle (1F2) and their possible use as a differentiation marker of ***in vitro*** developing myosatellite cells.

Moabs reacting with non-muscle tissue

2A10 (IgA) The ***in situ*** reaction shows a staining of the epithelia (skin) and endothelia (blood vessels and capillaries). The staining of our test tissue readily shows the more intense vascularisation of red axial muscle compared to white axial muscle. ***In vitro*** the monoclonal shows an intense staining of small clumps of cells. The antibody still has to be characterised with respect to its specificity and the antigen molecules it recognizes and can possibly be of use in vascularisation studies.

5F12 (IgG1) The ***in situ*** reaction shows a staining of the connective tissue: subcutaneous connective tissue, myosepta, endomysia and endoneuria are all stained. ***In vitro*** the monoclonal shows a strong reaction with vacuoles in large fibroblast-like cells. The antibody still has to be characterised with respect to the antigen molecules it recognizes and can be of future use in image analyzing studies on numbers and area of muscle fibres in tissue sections.
Figure 4 gives an example of the reaction pattern of some of these antibodies.

Discussion

Antigen

In the absence of a myosatellite cell specific antigen, we decided to try and use whole myosatellite cells to produce the desired antibody reagents. To obtain a pure preparation of myosatellite cells, cell suspensions isolated from the muscle tissue were plated on a laminin-coated substrate (Koumans *et al.*, 1990). This procedure resulted in cultures in which about 80% of the cells were myosatellite cells and with only small amounts of muscle cell debris present. Because alterations of membrane proteins can be expected from the use of trypsin in the isolation procedure, the cells were not injected immediately after isolation, but cultured for 17 hrs. Within this short period of culture already the first signs of cell differentiation, i.e. their fusion into myotubes, became visible. Since differentiation of the myosatellite cells is accompanied by remodelling of the myoblast membrane antigens, both qualitatively and quantitatively (Lee and Kaufman, 1981;Kaufman and Foster, 1985), prolonged culturing may result into antibodies that react with myosatellite cells in a later stage of differentiation than present in the original tissue.

We chose carp of 5 cm SL for the isolation of the myosatellite cells because of the high numbers of myosatellite cells that can be obtained from their axial muscle. In an earlier study (Koumans *et al.*, 1990) it was shown that a large percentage (about 80%) of these myosatellite cells are already differentiated (desmin positive), and thus may share many determinants with outgrown muscle tissue. Correspondingly, many of the monoclonal antibodies with reactivity for muscle tissue, obtained from fusion experiments 4 and 5 (tolerance induction), show a reaction pattern similar to that of the K5 polyclonal antiserum against desmin (Koumans *et al.*, 1990). Although, in fish larger than 20 cm SL, the percentage of desmin positive isolated myosatellite cells probably is lower (Koumans *et al.*, submitted), this coincides with a considerable reduction of myosatellite cells (Koumans *et al.*, 1991), which makes it difficult to obtain suitable numbers for immunisation.

Screening

It cannot be emphasised enough that the screening procedure is the key to success in hybridoma production. The screening procedure should be fast, and easy to apply, as the number of clones to be tested can increase enormously in a very short time.

Since the purpose of our study was to characterise monoclonal antibodies (MoAbs) useful for immune-histochemical recognition of myosatellite cells *in situ* and *in vitro*, we decided to apply the same technique to the initial screening of the hybridoma clones. Furthermore, it is a convenient and profitable assay, which allows one to test, simultaneously, for reactivity with unrelated cells or tissues, thereby providing information on the specificity of the Moabs. In a first screening we used cryosections of axial muscle of carp of 5 cm Sl. Cryosections were chosen in order to avoid, as much as possible, the risk of loosing monoclonal antibody reactivity due to damage of antigenic sites in the test tissue when using fixatives. Since the preservation of cell morphology in these sections is sub-optimal, the hybridoma supernatants that showed an interesting staining pattern (based on TEM information) were screened in a second test on cultures of myosatellite cells isolated from carps of 5 cm SL. The use of cultured cells not only allows for the identification of (cross-)reactivity with cell types other than myosatellite cells, but also for possible reactions with only a subpopulation of myosatellite cells. The cryosections were used first in the screening procedure because, in contrast to coverslips with cultures of myosatellite cells, cryosections could be produced in larger quantities. Both the cryosections and the cultured cells were fixed in acetone, as this fixation offers a good compromise between the maintenance of cell antigenicity, and the preservation of morphological detail (Hancock and Atkins, 1986)

Tolerance

Immunological tolerance is the acquisition of non-reactivity towards particular antigens (called tolerogens) that would otherwise elicit an immune-response, and as such is the converse of immunity.

Induction of tolerance in neonatal animals (neonatal tolerance) is a well-known method to achieve such a state of non-reactivity, but the exact mechanism of the induction of neonatal tolerance is still not known. Immature B-cells are particularly susceptible to tolerization by contact with antigen, but later stages of B-cell development like mature B-cells and antibody-forming cells, appear to become increasingly resistant. T-cell ontogeny, by contrast, is not characterised by such a marked variability in the extent of antigen-susceptibility. Since during foetal live and in the first weeks of neonatal life, non of the cells of the immune system have reached maturity, the animal at this stage of development is particularly susceptible to

tolerance induction. It is thought that, during this period, individuals may also be acquiring tolerance to self antigens (Roitt *et al.*, 1985), for review see Schwartz (1989).

Tolerance can also be induced in adult animals, for instance with the help of immunosuppressive drugs such as cyclophosphamide. The immunosuppressive effects of cyclophosphamide are the result of selective killing of antigen-stimulated lymphocytes if the cytotoxic drug is administered in combination with an antigen. Using human lymphocytes it was determined that cyclophosphamide has its greatest effect on B-cells. T-helper cells were reported to be relatively resistant (Matthew and Sandrock, 1987). However, we decided for neonatal induction of tolerance because of the long lasting effects, and the easier application of this technique.

The (small) decrease of the percentage of hybridomas producing antibodies that react with connective tissue in experiments 5 and 4 (with or without the use of passive immunisation) indicates that the methods used in these experiments indeed may induce selective changes in the population of antibody-forming cells. However, the formation of a different set of antibodies still does not guarantee the generation of the desired antibodies.

Myogenic cell markers

Although biochemical analyses (2D-Gel electrophoresis) of myoblasts and fibroblast-like cells of chicken embryo's have shown that there are several polypeptides present in the first cell type which lack in the second, and vice versa (Yablonka-Reuveni *et al.*, 1988), the data do not indicate wether such polypeptides are unique to the developmental stage of the particular cell or also constitute part of other cells like myotubes and muscle cells too. The latter has been described in a number of studies with muscle-cell specific antibody reagents: Hurko and Walsh (1983) reported that 5.1H11 , a monoclonal antibody directed against human fetal muscle cells, and myogenic cells in tissue culture, allowed unambiguous identification of regenerating myofibres in biopsy specimens . This antibody also reacts with rhabdomyosarcoma tumour cells (Clayton *et al.*, 1986). However, later investigations revealed that 5.1H11 reacts with the neural cell adhesion molecule (NCAM). This molecule is also present on brain cells, peripheral nerve cells, and ganglionic elements (Strother *et al.*, 1990). A monoclonal antibody 24.1D5 which has been identified for its reactivity with an antigen present only on mononucleated cells specific to the muscle cell lineage, was later shown to react also with a small sub-population of flat cells in human brain cultures (Walsh

et al., 1984). The CSAT monoclonal antibody that induces chick skeletal myoblasts and myotubes to round up and detach from extracelluar substrata, also reacts with embryonic fibroblasts (Neff *et al.*, 1982). It was shown later that this antibody reacts with the ß subunit of a family of integrins that bind fibronectin, laminin and collagen (Engel and White, 1990). Kaufman and Foster (1985) prepared a collection of hybridomas with reactivity for E63 rat myoblasts surface antigens. One of these monoclonal antibodies, H36, reacts with differentiating newborn rat skeletal myoblasts and myogenic lines. The antigen is present at a basal level on replicating myoblasts. Its level increases on prefusion myoblasts but the molecule also persists on myotubes (Kaufman *et al.*, 1985). An antibody against myosatellite cells isolated from adult chicken muscle, called SAT 2 HIO, reacts with mononucleated cells closely associated with the basement membrane of myofibres *in vivo* (possibly myosatellite cells). However, this antibody also reacts with smooth muscle cells in blood vessels and also with cells in capillary walls (Yablonka-Reuveni and Nameroff, 1989; Grounds, 1991).

These studies, in combination with the present one, demonstrate that it is not easy to produce antibodies to markers exclusively present on myogenic cells (myoblasts, myosatellite cells). Most of the produced antibodies that react with a myogenic cell, detect developmentally regulated antigens which are not specific for myoblasts. These antibodies also react with various other tissue types (especially nerve cells), or they also react with the myotubes and muscle fibres formed by the myogenic cells (Walsh *et al.*, 1984; Kaufman *et al.*, 1985; Griffin *et al.*, 1987).

To date, there are no markers which can readily identify the source of undifferentiated myogenic precursor cells in vivo (Grounds *et al.*, 1992). This poses the question if a cell surface molecule unique to myoblasts really exists. There may be molecules common or shared among different cell types assuming critical roles in myogenesis either by virtue of their association with other components or by virtue of unique topographic distributions (Lee and Kaufmann, 1981).

In the present study, additional problems in the production of myosatellite cell-specific antibodies may be the "phylogenetic distance" of carp with the Balb/c mice used for the production of the antibodies. The amount of common immune-dominant determinants different for both species may be so high that the vast majority of the produced monoclonal antibodies will react with these common determinants. Another possibility is that specific antibodies may not have been recognised by in the first screening, as their antigens may only

be present in very limited amounts (scored as a negative reaction pattern), or because of their cross-reactivity with (an) other antigen(s) (non specific reaction pattern). Cross reactivity often has been recorded for antibodies of the IgM class, in combination with antigens disposing a high epitope density (eg. DNA, actin, myosin, vimentin, desmin) (Ghosh and Campbell, 1986). IgM antibodies in general have low affinity but high avidity due to their multivalence, making unexpected cross-reactions more common. Most of the antibodies we produced were of the IgM class and, as mentioned earlier, in fusion 4 and 5 reaction patterns similar to that of an anti-desmin antiserum were frequently noticed. Probably, the presence of a high percentage of desmin positive myosatellite cells in cultures obtained from carp of 5 cm SL gives a good explanation for this phenomenon. However, some of these reaction patterns may have been caused by cross-reaction of the antibodies with desmin.

Conclusions

None of the MoAbs produced in the present investigation are specific for myosatellite cells. However, within a defined system such as isolated myosatellite cells, the MoAbs may still be used to study the process of differentiation of these cells. This can involve studies on the effect of hormones, growth factors, and of age of the donor upon the differentiation process. In *in situ* studies the use of these MoAbs is much more restricted. In these studies the use of molecular-biological techniques such as *in situ* hybridisation with probes against muscle regulation genes (Ott *et al.*, 1991; Grounds *et al.*, 1992) is another possibility to obtain information on the activity of myogenic cells. When combined with the production of new monoclonal antibodies, the adaption of this new technique for use in the study of fish muscle growth certainly will be of great value for the progress in this field.

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CHAPTER VIII

SUMMARY, PERSPECTIVE AND FUTURE DIRECTIONS

Investigations at the department of Experimental Animal Morphology and Cell Biology of the Agricultural University of Wageningen, The Netherlands, (Akster, 1981; 1985; Akster and Osse, 1978; Akster *et al.*, 1985; 1989; Hoogenboezem *et al.*, 1991; Muller and Osse, 1984; Muller, 1987; Osse, 1990; Osse *et al.*, 1989; Sibbing, 1982; Sibbing *et al.*, 1986; van Leeuwen and Muller, 1985; van Leeuwen *et al.*, 1990) produced substantial knowledge on the structure, function, and use of muscle in fish. However, a fundamental knowledge on postnatal fish muscle growth was lacking. Fundamental knowledge of muscle growth is important not only because it is the essence of all fish-farming activities but, it also addresses the question of structure function relations throughout ontogeny. Fundamental knowledge of fish muscle growth is important in comparative studies, as fish provides a dynamic system in which both hypertrophy and hyperplasia play a role of changing importance depending on age, genetic background and environment. This in contrast to mammals and birds where, as a rule, only hypertrophy of muscle fibres contributes to the postnatal increase in muscle mass.

Extensive studies have demonstrated the importance of myosatellite cells in muscle growth of mammals and birds. Studies on the mechanisms underlying growth of fish muscle however, are very scarce. As, at the start of our investigations, no records of isolation and culture of fish myosatellite cells existed, we developed, tested and checked our own method (based on the methods used for mammals and birds) to isolate, identify, and purify myosatellite cells from carp white axial muscle (**Chapter II**). As expected from the behaviour of such cells in mammalian and avian systems, the isolated carp myosatellite cells aligned and fused to form multi-nucleated myotubes. Recent investigations (Fauconneau, INRA, Rennes, France, pers.comm.) showed that our method also can be used in other fish species. However, as teleost fish form a very heterogenic group, adapted to a wide range of habitats (Zijlstra & Osse, 1988; Powers, 1989), it is unjustified to extrapolate our findings in carp directly to other fish species, as these can differ strongly in the occurrence of hyperplasia (Weatherley and Gill, 1984) and the number of myosatellite cells in the tissue (Romanello *et al.*, 1987).

Electronmicroscopical (TEM) studies on the percentage of myosatellite cells in the tissue in combination with biochemical studies on the amount of DNA in the tissue, enabled us to estimate the total number of myosatellite cells in the white axial muscle tissue during growth (**Chapter III**). The total number of myosatellite cells remained about the same over an investigated length range of 3 cm to 56 cm standard length (SL). The observed decrease in the percentage of myonuclei that were myosatellite nuclei from about 6% in carp of 4 cm standard length to less than 1% in carp larger than 18 cm standard length, and in the yield of myosatellite cells from the isolation procedure, therefore appears to be caused by the dilution of a more or less constant population of myosatellite cells over an increasing volume of muscle (number of myonuclei) during growth. As the yield of the dissociation procedure was proportional to the number of myosatellite cells in the tissue based on TEM studies, it offers a relatively fast qualitative way to compare numbers of myosatellite cells in different muscle samples (compare Düsterhöft *et al.*, 1990). The percentage of myonuclei that were myosatellite nuclei was also determined by counting the numbers of total nuclei and of heterochromatic (myosatellite) nuclei inside the muscle fibres' basal lamina in tissue sections which were stained using an antibody against laminin. As the results obtained with this new method were statistically comparable to the results obtained with TEM, this new method offers a quantitative lightmicroscopical way of determining percentages of myosatellite cells in the tissue. However, as heterochromatism of the nucleus is not an absolute criterium for myosatellite cells, it does not allow the unambiguous *in situ* identification of these cells.

Combination of data on the increase in the number of myonuclei during growth, recalculated from a length scale to a time scale, with data on the number of myosatellite cells present in the tissue, enabled us to calculate the required maximal duration of the cell-cycle time of the myosatellite cells. This maximal required cell-cycle time appears to be so short, in carp larger than about 10 cm standard length, that serious doubts arouse about the assumption of myosatellite cells being the sole source of myonuclei in carp post-larval growth. Such an assumption is implicitly accepted in studies on postnatal growth of mammalian and avian muscle. Preliminary experiments involving *in vivo* labelling of proliferating myosatellite cells and TEM observations suggest the presence of myogenic cells, other than myosatellite cells, although amitotic division of myosatellite nuclei forms an alternative mechanism of addition of myonuclei (**Chapter VI**).

Our *in vitro* investigations (**Chapters II, IV**) revealed the presence of subpopulations of myosatellite cells in the tissue. The *in vitro* proliferative behaviour of the myosatellite cells, was studied with 5-bromo-2'-deoxyuridine (BrdU) labelling of proliferating cells in combination with a immunohistochemical staining for desmin (a muscle specific intermediate filament protein). The percentage of proliferating myosatellite cells increased with increasing length (3 cm - 27 cm standard length) of the fish from which they were isolated. In carp, desmin positive myosatellite cells probably are postmitotic. We could not find a correlation between the percentage of desmin positive myosatellite cells and length of the fish from which they were isolated. However, the large amount of individual variation of the data could mask such a correlation. The use of gynogenetically inbred carp strains (Komen, 1990) in future experiments offers a way to decrease the effect of individual variation and additionally will reduce the number of experiments (carp) needed to obtain statistically significant results.

In small carp, hyperplasia is a relative important mechanism in white axial muscle growth, as was deduced from the diameter distribution of the muscle fibres over size classes of carp (**Chapter V**). The occurrence of hyperplasia markedly decreased in white axial muscle of carp larger than about 15 cm standard length. In carp larger than 43 cm SI hyperplasia was no longer observed. The increase in the relative importance of hypertrophy, in carp larger than about 15 cm standard length, appeared to be correlated to an *in vitro* increase in the percentage of proliferating myosatellite cells and an *in vivo* increase in DNA content of the tissue. The low percentage of *in vitro* proliferating cells and the high percentage of differentiated (desmin positive) myosatellite cells obtained from about 5 cm large carp, together with the *in vivo* decrease in DNA content of the muscle tissue, suggests that, in these small fish with a high occurrence of hyperplasia, muscle growth strongly depends on a pool of previously formed myogenic cells. This suggests that in hypertrophy and hyperplasia different subpopulation of myosatellite cells are involved (**Chapter V**). However, the use of such general markers as BrdU (proliferation) and desmin (differentiation) does not allow the identification of such subpopulations of myosatellite cells.

To facilitate the identification of (subpopulations of) myosatellite cells and to demonstrate the existence of myogenic cells other than myosatellite cells in juvenile and adult carp, we need to develop markers that enable an unambiguous identification of all myogenic cells. Until now, the morphological criterium of being situated under a muscle fibres' basal lamina, together with other ultrastructural criteria still offer the best (although known to be

a not totally reliable) way of *in vivo* identification of myosatellite cells. But these criteria can not be used in the identification of myogenic cells outside the muscle fibres' basal lamina. This not only hampers the recognition of myogenic cells other than myosatellite cells in adult and juvenile carp. In carp, smaller than about 2.5 cm standard length, not all of the muscle fibres are completely surrounded by basal lamina. Therefore, recognition of undifferentiated myogenic cells in these small carp is not very reliable. Also the *in vitro* criterium of having an spindle shape is not an absolute reliable criterium for the identification of isolated myosatellite (myogenic) cells. An antibody against a specific 'myogenic' marker is more suited for the identification of such cells. We used isolated carp myosatellite cells as an antigen in an attempt to produce a monoclonal antibody that reacts with (a subpopulation of) myosatellite cells, as these are known myogenic cells (**Chapter VII**). Screening of the generated monoclonal antibodies showed that none of them reacted specifically with myosatellite cells. Also other investigators, as far as such studies with negative results reach publicity, have not been successful in producing a (monoclonal)antibody that can be used for the unambiguous *in situ* identification of undifferentiated myosatellite (myogenic) cells. Therefore, and in view of the great efforts in this part of the project, we have to consider the possibility that such specific, immunohistochemically detectable, myogenic markers are lacking. However, recently, another technique has become available. *In situ* hybridisation with probes against muscle regulation genes like MyoD1 and myogenin offers the opportunity of recognizing early myogenic activity in the muscle tissue (Grounds *et al.*, 1992). Probes, reacting with regulation genes like MyoD1 and myogenin in fish, would present a lightmicroscopical way of recognizing very early myogenic activity in the tissue. In combination with TEM they would also present a means of identifying myogenic cells other than myosatellite cells.

Nearly all fish pass through metamorphosis after hatching. Forms and functions undergo a fast transformation until the end of the larval stage. During this period, but also thereafter, allometric growth, also of the body muscles, is quit common. How such growth is caused, but also why it happens, are interesting questions; not only from the viewpoint of a scientist but also from the viewpoint of a fish culturer. Experience has shown that optimisation of processes requires its thorough knowledge.

The availability of markers that enable the identification of subpopulations, and

differentiational stages of fish myogenic cells is a point germane in the increase of our knowledge concerning the mechanisms underlying fish muscle growth. The developed method to isolate and culture myosatellite cells not only offers a useful tool for the production of such markers, but also allows the direct study of the influence of a variety of factors (donor age, hormones, growth factors, etc.) on the proliferation and differentiation of subpopulations of these myogenic cells.

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SAMENVATTING

Doel van dit proefschrift is om een beter inzicht te verkrijgen in de rol die myosatellietcellen spelen bij de post-larvale spiergroei van vissen, met als model de karper (*Cyprinus carpio* L.).

De regelmechanismen die een rol spelen bij de spiergroei van vissen zijn niet alleen interessant uit het oogpunt van van vergelijkende biologisch onderzoek. De kennis die uit dit soort onderzoek wordt verkregen kan mogelijk ook worden toegepast binnen de visteelt. Ervaring wijst uit dat voor een verantwoord beïnvloeden van processen een grondige fundamentele kennis van deze processen noodzakelijk is.

In **hoofdstuk I** wordt een algemene inleiding gegeven over spiergroei, myosatelliet-cellen en de rol van myosatellietcellen bij de spiergroei. Spiergroei kan plaatsvinden door middel van de uitgroei van reeds bestaande spiervezels (= hypertrofie) en door de aanmaak van nieuwe spiervezels (= hyperplasie). In het algemeen vindt bij zoogdieren en vogels de post-natale spiergroei plaats via het proces van hypertrofie. Bij vissen daarentegen kan, in combinatie met hypertrofie, hyperplasie een rol spelen tot ver in het adulte stadium. Een belangrijk aspect van de spiergroei is de toename van het aantal spierkernen. Uit onderzoek dat voornamelijk is uitgevoerd aan zoogdieren en vogels blijkt dat myosatellietcellen deze extra spierkernen leveren. Daarnaast spelen deze cellen ook een rol bij de regeneratie van beschadigd spierweefsel.

Myosatellietcellen zijn kleine spoelvormige cellen met weinig cytoplasma. Behalve vrije ribosomen bevat het cytoplasma weinig andere organellen. De kern is over het algemeen heterochromatisch. Het belangrijkste identificatie criterium is echter het feit dat ze gelegen zijn tussen de cytoplasma membraan van de spiervezels en de basaalmembraan die iedere spiervezel omgeeft.

Ook vissen bezitten myosatellietcellen. Algemeen wordt aangenomen dat de rol van deze cellen bij de spiergroei van vissen vergelijkbaar is met die bij zoogdieren en vogels, maar hierover is slechts zeer weinig bekend.

In **hoofdstuk II** wordt onderzocht of het mogelijk is om karper myosatellietcellen te isoleren en *in vitro* te laten differentieren tot myotuben. Dit om het myogene aspect van deze cellen ook bij vissen aan te tonen. Aangezien deze techniek nog niet eerder beschikbaar was, wordt in dit hoofdstuk veel aandacht besteed aan de identificatie van de geïsoleerde myosatellietcellen. Tevens werd een methode ontwikkeld om de myosatellietcellen zo zuiver mogelijk in handen te krijgen, voor verder gebruik in *in vitro* onderzoek en als antigeen bij de produktie van monoclonale antilichamen. Uit het eerste *in vitro* onderzoek blijkt dat er subpopulaties myosatellietcellen bestaan die verschillen in hun mate van differentiatie.

In **hoofdstuk III** worden methodes onderzocht om het aantal myosatellietcellen in het spierweefsel te onderzoeken. Gezien het ultrastructurele karakter van de kenmerken die gebruikt worden voor het herkennen van myosatellietcellen in het spierweefsel speelt electronenmicroscopie, met alle daar bij horende beperkingen, een belangrijke rol. Er wordt dan ook aandacht besteed aan de ontwikkeling en het testen van de betrouwbaarheid van twee alternatieve lichtmicroscopische methodes. Uit het onderzoek blijkt dat, bij de onderzochte groeireeks van vissen, geen grote verschuivingen plaatsvinden in het totale aantal myosatellietcellen. De aanwezige myosatellietcellen worden echter steeds verder verdund over het toenemende volume spier en het toenemende aantal spierkernen.

In **hoofdstuk IV** wordt onderzocht of de lengte van de vis van invloed is op de eigenschappen van de uit het dier geïsoleerde myosatellietcellen. Dit in verband met het mogelijk bestaan van subpopulaties myosatellietcellen met een verschillende rol (hyperplasie/hypertrofie) bij de spiergroei. Het met toenemende lengte van de vis toenemende percentage *in vitro* prolifererende myosatellietcellen wordt in verband gebracht met het *in vivo* toenemende DNA gehalte van het weefsel. Het hoge gehalte aan postmitotische cellen in karpers van zo'n 5 cm standaardlengte suggereert een grote bijdrage van een reeds in een eerder stadium van ontwikkeling gevormde pool van myosatellietcellen in deze kleine vissen.

In **hoofdstuk V** wordt het optreden van hyperplasie bij de spiergroei van de karper onderzocht. Uit het onderzoek blijkt dat het relatieve belang van hyperplasie afneemt met toenemende lengte van de vis. In karpers van van meer dan 43 cm standaardlengte kon hyperplasie niet meer worden waargenomen. Besproken wordt of de in hoofdstuk IV onderzochte verschuivingen in de grootte van subpopulaties van myosatellietcellen en de in het weefsel optredende veranderingen in het DNA gehalte, in verband kunnen worden

gebracht met het bestaan van verschillende, bij hyperplasie en hypertrofie betrokken subpopulaties van myosatellietcellen.

In **hoofdstuk VI** wordt onderzocht of het relatief kleine aantal myosatellietcellen in het weefsel in staat is om de totale toename in de hoeveelheid spierkernen tijdens de groei te verklaren. De verkregen gegevens, betreffende de maximale duur van de cel-cyclus bij karpers groter dan zo'n 15 cm standaardlengte, geven aan dat een scenario waarbij myosatellietcellen de enige leveranciers van spierkernen zijn zeer onwaarschijnlijk is. Het bestaan van nog een andere populatie van myogene cellen lijkt waarschijnlijk.

In **hoofdstuk VII** wordt onderzocht of het mogelijk is om monoclonale antilichamen te ontwikkelen gericht tegen markers specifiek voor (subpopulaties van) myosatellietcellen. Dergelijke antilichamen zouden niet alleen de lichtmicroscopische herkenning van myosatellietcellen in het weefsel mogelijk maken, maar ook het bestaan van diverse populaties van myosatellietcellen kunnen aantonen. Deze populaties kunnen verschillen in mate van differentiatie en/of functioneel verschillend zijn (hyperplasie/hypertrofie). Daarnaast kunnen met dergelijke antilichamen mogelijk ook myogene cellen anders dan myosatelietcellen aangetoond worden. Onze pogingen tot de productie van specifiek tegen myosatellietcellen gerichte monoclonalen zijn tot op heden niet gelukt. Wel zijn er enkele monoclonalen ontwikkeld waarmee het *in vitro* differentiatie proces van de myosatellietcellen bestudeerd kan worden.

In **hoofdstuk VIII** wordt een wat uitgebreidere samenvatting van het onderzoek gegeven. Hierbij worden ook mogelijkheden voor vervolgonderzoek besproken. In dit vervolgonderzoek zullen moleculair biologische methodieken een belangrijke rol spelen. Hierbij wordt vooral gedacht aan het gebruik van genprobes gericht tegen spier-regulatiegenen als MyoD1 en myogenin. Deze probes kunnen niet alleen gebruikt worden voor het herkennen van myogene activiteit in het weefsel, maar mogelijk ook voor het herkennen van subpopulaties van myogene cellen.

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CURRICULUM VITAE

Joseph (Sjo) Thomas Maria Koumans werd op 19 april 1959 geboren in Stein (Limb.).
In 1977 behaalde hij het diploma Atheneum B aan de Albert Schweitzer scholengemeenschap te Geleen.
In dat zelfde jaar werd gestart met een studie Biologie aan de, toenmalige, Landbouwhogeschool te Wageningen. Deze studie werd in 1986 afgesloten, met als afstudeervakken: Celbiologie (waarvoor ook een stage werd verricht bij de afdeling Kindergeneeskunde van het Academisch Ziekenhuis van de Vrije Universiteit van Amsterdam), Dierfysiologie en Ethologie. Gedurende de studie werden een groot aantal studentenassistentschappen verricht voor de vakgroep Experimentele Diermorfologie en Celbiologie (EDC). Bij deze vakgroep werd, aan het einde van de studie, ook de functie vervuld van amanuensis. Tevens werd nog gedurende een periode van 7 maanden gewerkt al laborant bij de vakgroep Fysiologie van mens en dier.
In 1987 werd hij aangesteld bij de sectie Functionele Morfologie van de vakgroep EDC op het AIO project zoals beschreven in dit proefschrift. Deze aanstelling werd verlengd met een periode van een half jaar tengevolge van ernstige vertraging in de voortgang van het project, opgelopen in het eerste jaar van de aanstelling.
Sinds maart 1992 werkt hij in deeltijdfunctie bij de sectie Ontwikkelingsbiologie van de vakgroep EDC als toegevoegd onderzoeker