THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT AND GROWTH OF TELEOST FISH

A thesis submitted to the University of St Andrews
for the degree of Doctor of Philosophy
by

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Declaration

I hereby declare that the research reported in this thesis was carried out by me, and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree. The research was conducted at the Gatty Marine Laboratory, School of Biological and medical Sciences, University of St Andrews, under the direction of Professor I. A. Johnston.

Certificate

I hereby certify that Mark Anthony Thomas has spent nine terms engaged in research under my direction, that he has fulfilled the conditions of General Ordinance No 12 (resolution of the University Courts No 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

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An introduction is given to the effects of temperature on fish development and growth.

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The effect of temperature on embryonic development was investigated in the shorthorn sculpin (*Myoxocephalus scorpius*) and the angelfish (*Pterophyllum scalare*), two teleosts with contrasting larval strategies. Newly hatched sculpin larvae were about 8 mm TL, and contained the full complement of somites (62), functional jaws and pigmented eyes. Newly hatched sculpin larvae also swam vigorously, and their myotomes contained a superficial monolayer of muscle fibres, which stained intensely for succinic dehydrogenase activity (SDHase), overlying an inner mass of fibres, which stained lightly for SDHase. In contrast, newly hatched angelfish were about 2.7 mm TL and were still in the process of adding somites. They lacked a mouth, had unpigmented eyes and were non-motile. The full complement of somites (38), functional jaws and pigmented eyes were only obtained by

angelfish at first swimming (4.5-4.8 mm TL). Muscle differentiation was not complete in all somites of newly hatched angelfish. Rostral somites contained muscle fibres with numerous myofibrils, whereas caudal somites contained only myoblasts with no myofibrils. Somites immediately posterior to the yolk sac contained myotubes with fewer myofibrils than the rostral muscle fibres. Two types of myotubes were present in angelfish: superficially situated myotubes were mononuclear while those deeper in the somite were multinuclear. All muscle fibres of newly hatched angelfish stained lightly for SDHase. By first swimming, all somites of angelfish larvae contained muscle fibres, and superficial (SDHase-positive) and inner (SDHase-negative) fibre types appeared. Muscle fibres of the angelfish were focally innervated with endplates being concentrated at myosepts. Functional endplates were not present in all somites of newly hatched angelfish, but the development of motor innervation was complete at first swimming.

Development was accelerated at higher temperatures. The rate of somitogenesis in sculpin larvae was positively correlated with temperature, with new somites being added at rates of about 2, 4 and 7 per d at 4, 8 and 12 °C, respectively. In the angelfish, the rate of somite formation at 31 °C (about 1.4 per h) was also greater than that at 24 °C (about 0.8 per h), and the time from fertilisation to hatching at 31 °C (1.88 d) was shorter than that at 24 °C (2.57 d). The time from fertilisation to first swimming in angelfish was also shorter at 31 °C (2.38 d) than at 24 °C (6.26 d). Using somite stage as a reference point, temperature was found to affect the relative timing of appearance of some morphological

features but not others. For example, 41-42-somite sculpin embryos at 12 °C contained dorsal aorta, heart and pronephros but these organs were absent in embryos of equivalent stage at 4 °C. The lens in eye and gut, however, appeared at the 37-38-somite stage at all temperatures. Development of motor innervation in angelfish was positively correlated with rearing temperature, with the last somite positive for HNK-1 and acetylcholinesterase activity being more caudal in 31 °C than in 24 °C embryos of equivalent somite stage. The relative timing of myofibrillargenesis, however, was not affected by development temperature. First swimming angelfish at 24 °C also contained more muscle fibres, of smaller sizes, than first swimming angelfish at 31 °C, but the total cross-sectional area of myotomal muscle was independent of development temperature. The formation of different morphological features was thus shown to have different thermal sensitivities.

Chapter 3

Muscle growth and myosatellite cell proliferation were investigated in shorthorn sculpin larvae (*Myoxocephalus scorpius*) using morphometric methods and immunocytochemical localisation of DNA-incorporated 5-bromo-2'-deoxyuridine. Newly hatched larvae (total length, TL, about 8 mm) were pelagic, continuous swimmers, but settlement rates increased with growth. By metamorphosis (about 16 mm TL), fish were completely

Chapter 4

Muscle growth was studied in angelfish (*Pterophyllum scalare*) incubated at 24 °C or 31 °C during embryonic development, then reared at 26 °C for up to 25 d. Growth performance was influenced by early thermal experience, with 24 °C fish being of greater total length (TL) than 31 °C fish after equivalent times at the common temperature. 24 °C fish also contained more muscle fibres, of a smaller cross-sectional area, than 31 °C fish of equivalent size. These differences were, however, only vestiges of an effect of development temperature which persisted for a short time after fish were transferred to the common temperature: somatic growth rates and the percentage contribution of hyperplasia to muscle growth were greater in 24 °C fish than in 31 °C fish only when TLs were less than 8 mm, and were not different thereafter. Myosatellite cell proliferation rates, determined by *in vivo* nuclear labelling with 5-bromo-2'-deoxyuridine, were also different between the groups only when TLs were less than 8 mm. Myosatellite proliferation was greater in the 31 °C fish, which used hypertrophy to a greater extent during muscle growth. This positive correlation between hypertrophy and myosatellite cell proliferation supports *in vitro* work which suggests that hypertrophy depends more on proliferating myosatellites while hyperplasia relies more on differentiating myosatellites. The TL at which myosatellite proliferation rates of 24 °C and 31 °C fish equalised (8 mm) was also the body size at which muscle fibre nuclear densities approached critical levels. This suggests that factors which triggered myosatellite cells to

divide might have arisen from the muscle fibres with which they are associated.

Chapter 5

General conclusions arising from the work are discussed, with some potentially profitable directions for future work.

Chapter 1

General introduction: temperature and the development and growth of fish

Introduction

During the Mesozoic era, which ended about 70 million years ago, three important vertebrate lineages evolved. The land saw the appearance of the endothermic birds and mammals, while the waters saw the rise of the teleost fish (Wootton, 1990). Teleost fish now account for nearly half of all vertebrate species (Moav, 1994), and have colonised virtually every aquatic habitat. For example, teleosts are found at altitudes in excess of 4000 m, on the Tibetan Plateau (Cao *et al*, 1981) and in the Andes (Payne, 1986), where the partial pressure of oxygen is only about 60 % of that at sea level (Wootton, 1990). Fish are also found in the blackwater rivers of the Amazon, which are close to distilled water (conductivity 20-30 μS cm^{-1}) (Lowe-McConnell, 1987), and in Lake Nakuru, Kenya, which is highly saline (conductivity 162500 μS cm^{-1}). Additionally, there are fish in water that reaches nearly 40 °C in lakes in East Africa (Lowe-McConnell, 1987) while species in the Antarctic Ocean live at temperatures consistently below 0 °C (DeVries, 1980).

Associated with this wide range of habitats is a profusion of fish body shapes (see Wootton, 1990). For example, the smallest vertebrate is a teleost, a goby that reaches sexual maturity at a length of 8 mm (Miller, 1984), while in the Amazon the arapaima, *Arapaima gigas* Cuvier, may reach lengths of 4.5 m (Moyle and Cech, 1982). Fish also demonstrate a variety of life-history patterns (Breder and Rosen, 1966), and use a variety of biological mechanisms to cope with their environment (Moav, 1994). These features, coupled with the importance of fish as a food source throughout human history (Weatherley and Gill, 1987), have resulted in fish being the subject of scientific study for at least three thousand years. For example, Aristotle (383-322 BC) recorded many facts concerning the structure and habits of the fish of Greece (Jordan, 1905), and ancient Hindu texts, particularly the work of Susrata (*circa* 600 BC), are known to contain theories correlating form and locomotion (Hora, 1935).

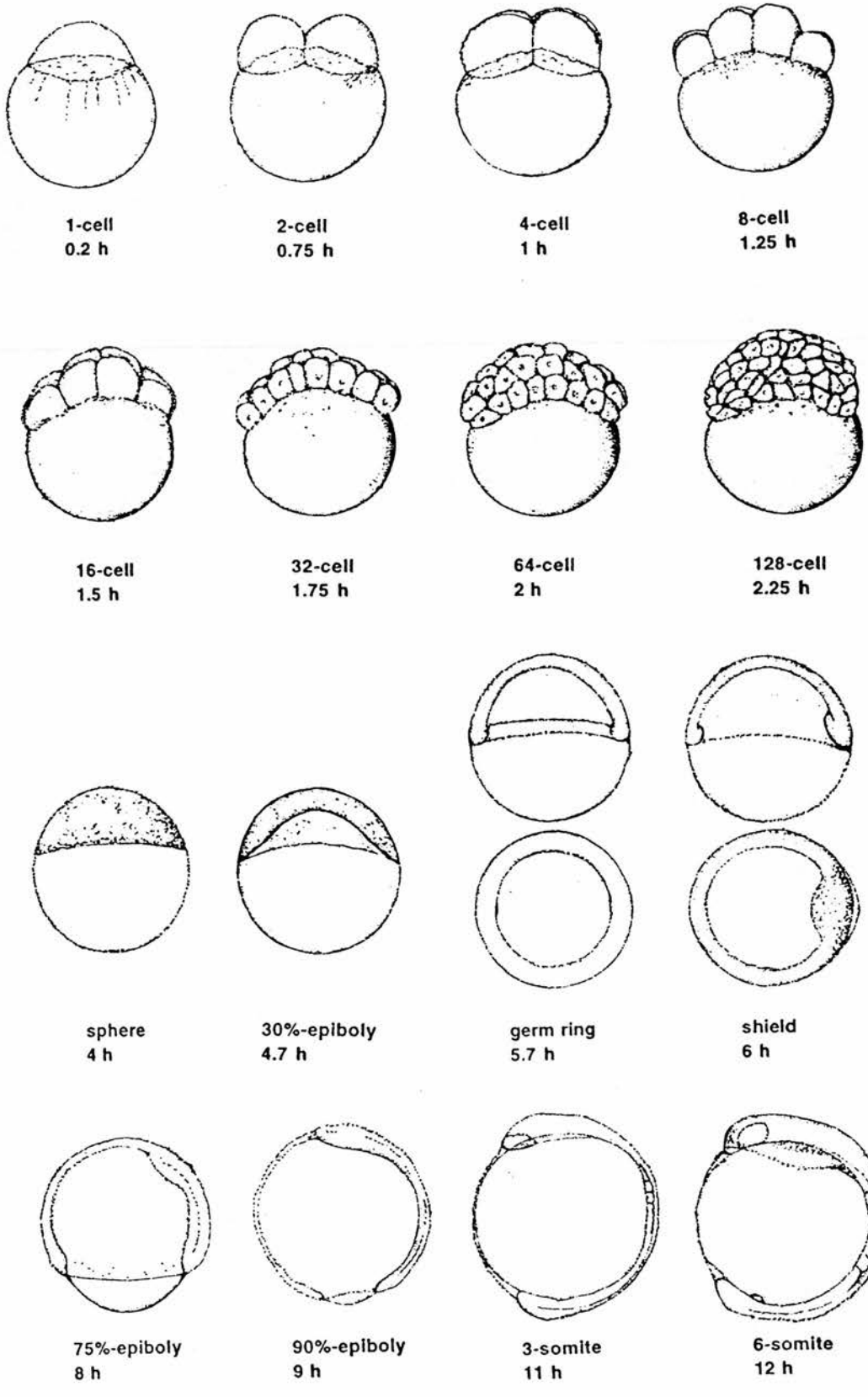
The greatest increase in our knowledge of fish biology has, however, come in the last few centuries with the advent of scientific method in the Western world. By the beginning of the present century, there was already a detailed understanding of many aspects of the morphology, anatomy, and mechanics of the skeleton and component parts of the swimming musculature (Arndt, 1873; Chevrel, 1913; Giacomini, 1898; Greene and Greene, 1913; Okamura, 1929; Retzuis, 1892; Schmalhausen, 1913; Supino, 1898; Trinchese, 1885). The development and growth of fish were also well understood. For example, meroblastic cleavage and embryonic development of the pipefish, *Siphostoma floridae*, were described by Gudger (1905), embryonic and larval morphology

were described for the angler, *Lophius piscatorius*, by Gill (1905), and growth rates of the herring, *Clupea harengus* L., were quantified by Williamson (1910*a*). Williamson (1910b) also showed that low temperature retarded embryonic development in herring, plaice (*Pleuronectes platessa* L.), and haddock (*Gadus æglefinus* L.). Studies of fish development and growth have, however, increased exponentially in the past 30 years, correlated with the increased interest in fisheries biology and intensive aquaculture systems.

In multicellular animals, development almost always starts with a single cell, the egg. Following its fertilisation by a spermatozoon, the egg becomes polar, with a vegetal region (containing yolky material) and an animal region (consisting of a cap of cytoplasm) (Fig 1). Cleavage then occurs, where the cytoplasm of the fertilised egg is partitioned to produce a multicellular embryo, the blastula, at the animal pole. In teleost fish cleavage is meroblastic (Gudger, 1905; Blaxter, 1988). The blastula which develops is thus disc-shaped, and is consequently referred to as a blastodisc (Fig 1).

Cell movements (such as epiboly, involution and convergence extension) then occur, reshaping the radially symmetric blastula into a bilaterally symmetrical gastrula (Warga and Kimmel, 1990). During epiboly, the cells of the blastodisc spread radially over the yolk, covering it with a layer of blastoderm, but leaving an opening, the blastopore (Fig 1) (Blaxter, 1988). Gastrulation follows (in zebrafish, *Brachydanio rerio* Hamilton-Buchanan, gastrulation begins at 50 % epiboly; Fig 1; Moav, 1994), where cells at the periphery of the blastoderm involute and migrate toward the animal pole (Fig 1). The blastoderm then consists of the noninvoluted epiblast (which gives rise to the ectoderm and mesoderm) and the involuted hypoblast (which forms the endoderm) (Kimmel *et al*, 1990). A thickened annulus, the germ ring, also forms at the site of involution (Fig 1).

Fig 1. Early stages in the embryogenesis of the zebrafish, *Brachydanio rerio*, at 28.5 °C. Diameter of each embryo illustrated is about 600 μm. (Reproduced from Kimmel *et al*, 1995.)


1-cell
0.2 h
2-cell
0.75 h
4-cell
1 h
8-cell
1.25 h
16-cell
1.5 h
32-cell
1.75 h
64-cell
2 h
128-cell
2.25 h
sphere
4 h
30%-epiboly
4.7 h
germ ring
5.7 h
shield
6 h
75%-epiboly
8 h
90%-epiboly
9 h
3-somite
11 h
6-somite
12 h

Involution is initially symmetric. However, soon after it begins, both epiblast and hypoblast cells converge toward one side of the embryo to produce a localised thickening of the germ ring. This structure, the embryonic shield (Fig 1), is the first marker of the dorsal side of the embryo. As gastrulation proceeds, the leading edge of the shield moves toward the animal pole, and the shield narrows and lengthens as converging cells intercalate to produce the longitudinal, or anteroposterior (AP), body axis (Fig 1). The development of the AP axis is believed to involve several genes, such as *axial*, which are expressed just before gastrulation (Moav, 1994). It is also an important milestone in development because it provides the major line along which later structures will develop (De Robertis *et al*, 1990).

By the end of gastrulation, differentiation of the neural tube has commenced, the head and the eye cups are identifiable, and somites begin to form in a rostral to caudal direction (Moav, 1994) (Fig 1). The tail bud also forms (Fig 1), extending posteriorly as development progresses (Blaxter, 1988). Additionally, a layer of ectodermal tissue is present, overlying a rod of axial mesoderm itself bounded by the lateral mesoderm (Moav, 1994).

Development of the vertebrate body plan is under the control of homeobox-containing genes (primarily the *hox* genes) (De Robertis *et al*, 1990), the expression patterns of which have been significantly conserved in vertebrates (Kimmel *et al*, 1991). *Hox* genes are clustered together on the chromosome and are expressed along the AP axis of the embryo in a region-specific pattern that not only matches their linear arrangement within the

gene cluster, but also divide the AP axis into bands with different developmental potentials (De Robertis *et al*, 1990). The boundaries of expression of these *hox* genes in fish are the segment boundaries, suggesting that these genes encode segment identity (Kimmel *et al*, 1991). Other homeobox-containing/*hox* genes specify the identities and fates of embryonic cells, thereby guiding the formation of organs (De Robertis *et al*, 1990). For example, *Hlx* and *Csx* play key roles in the development of the gut (Hentsch *et al*, 1996) and heart (Komuro and Izumo, 1993) of mice, respectively.

Before hatching many organ systems (e.g., heart) become functional, the embryo becomes very active, and the chorion (egg capsule) is weakened by the release of enzymes from the hatching glands (Blaxter, 1988). The degree of differentiation (i.e., complexity and organisation) of the newly hatched larva depends very much on the species. In many marine species, such as the jack mackerel (*Trachurus symmetricus* Nichols), the mouth and jaws are not formed, the eye is not pigmented, and a primordial fin-fold runs along the trunk in the median position (Kendall *et al*, 1984). Other species, however, hatch at a very advanced stage: in loricariids the dorsal and caudal fins are partly developed at hatching and, in flying fish (family Exocoetidae), notochord flexion (an event which precedes caudal fin formation) actually occurs before hatching (Blaxter, 1988). The degree of differentiation at hatching also varies latitudinally, with the larvae of many warm-water fish hatching at a much earlier stage of development than those of cold-water fish. For example, tambaqui (*Colossoma macropomum* Cuvier) (Johnston and Vieira, 1996) hatch before all

the somites are formed, and the larvae initially lack eye pigment, jaws, a gut and pectoral fins.

Many larvae also hatch with an endogenous supply of nutrition, the yolk sac. The duration of the yolk sac period depends not only on the species, but also on egg size (Blaxter, 1988). During this period the mouth and gut, become functional allowing the larva to transfer from endogenous to exogenous nutrition. The larval period which follows ranges from a few days to some months (and even 2-3 years in eels), again depending on species. During this time the larva is likely at least to double its length and to increase its weight by 10 to 100 times (Blaxter, 1988).

Allometric growth (where different structures grow at different rates) is important during larval development. In some species relative growth follows a U-shaped gradient along the body, with the fastest growth in the caudal region, linked to an increase in the propulsive area of the body. Growth is also fast in the head region where elaboration of feeding and respiratory functions may be taking place (Blaxter, 1988).

Progressive differentiation of adult characters (such as fin rays and skeleton) occurs. The larvae eventually pass through a process of metamorphosis to the juvenile stage. This process may be rather abrupt or it may be prolonged. Typically the blood becomes pigmented, scales and pigment appear on the body surface, the meristic characters such as the fin rays are complete, and the body shape becomes like the adult (Batty, 1984; Blaxter,

1988; Johnston and Horne, 1994). The juvenile thus appears as a small adult. In flatfish metamorphosis is a remarkable process, with the fish changing from a bilaterally symmetrical larva to an asymmetrical juvenile lying on one side. Changes take place to the skull and sense organs and, in particular, the eye of the side on which the fish comes to lie migrates across the skull to the other side (Blaxter, 1988).

Fish axial muscle

The axial muscle of fish is divided into segments (myotomes) by connective-tissue partitions (myosepta), and consists of many muscle fibres (Alexander, 1969). A muscle fibre is a syncitium 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 a striated appearance (Keynes and Aidley, 1981).

Skeletal muscle fibres can be divided into a number of categories, or fibre types, upon consideration of their contractile, (immuno)histochemical, morphological and mechanical properties (Bone, 1978). Fish contain two main muscle fibre types: red, or slow, muscle fibres and white, or fast, fibres (Arloing and Lavocat, 1875). A comparison between these fibre types is shown in Table 1. Red fibres are utilised by the fish for sustained, slow-speed swimming or cruising while the white fibres are used for bursts of higher speed. Several lines of evidence point to this conclusion. First, direct electromyographic recording from teleosts swimming freely, or in tunnel respirometers, has shown that electrical activity is found within the superficial red fibre zone when fish are swimming slowly, and within the deeper zone of white fibres during rapid swimming (Rayner and Keenan, 1967; Hudson, 1973).

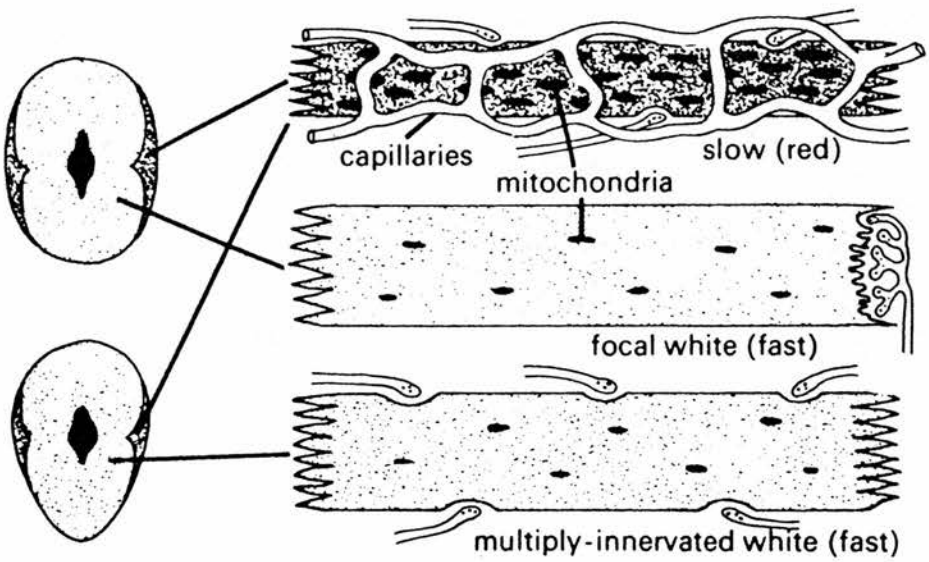
Table 1. A comparison between the fast glycolytic, or white, muscle fibres and the slow oxidative, or red, muscle fibres in fish myotomes. (Reproduced from Bone, 1978.)

Slow	*Fast*
Smaller diameter (20–50% of fast fibres)	Large diameter (may be more than 300 μm)
Well vascularized	Poorly vascularized
Usually abundant myoglobin, red	No myoglobin, usually white
Abundant large mitochondria	Few smaller mitochondria
Oxidative enzyme systems	Enzymes of anaerobic glycolysis
Lower activity of Ca^{2+}-activated myosin ATPase	High activity of enzyme
Little low molecular wt. Ca^{2+}-binding protein	Rich in low molecular wt. Ca^{2+}-binding protein
Lipid and glycogen stores	Glycogen store, usually little lipid
Sarcotubular system lower volume than in fast fibres	Relatively larger sarcotubular system
Distributed cholinergic innervation	Focal or distributed cholinergic innervation
No propagated muscle action potentials	Propagated action potentials; may not always occur in multiply-innervated fibres
Long-lasting contractions evoked by depolarizing agents	Brief contractions evoked by depolarizing agents

Second, biochemical and metabolic studies (reviewed in Bilinski, 1974) have shown that red fibres operate mainly by aerobic glycolysis or lipolysis while white fibres use anaerobic glycolysis. Examination of fish after exercise of different kinds has also shown the expected utilisation of metabolites by red and white fibres (for a review see Driedzic and Hochachka, 1978). Many fish also contain another muscle fibre type (i.e., pink, or fast oxidative glycolytic, fibres), the properties of which are intermediate between those of the red and white fibres (Brotchi, 1968; Flood and Storm-Mathisen, 1962; Johnston *et al*, 1977).

Fish axial muscle is innervated by anatomically discrete groupings of motoneurons, or motoneuron pools, that occur in characteristic locations within the spinal cord (Westerfield *et al*, 1986). The neurons of a given pool innervate a particular muscle in a precise manner that is highly reproducible in different individuals (Westerfield *et al*, 1986). In teleost fish, red muscle fibres are multiply innervated by motor axons that terminate in *en grappe* endings (Barets, 1961; Bone, 1964, 1966, 1970; Best and Bone, 1973) (Fig 2). Multiple innervation is also found in the white muscle fibres of higher teleosts (Fig 2), but in other fish white muscle fibres are focally innervated at their myoseptal ends (Bone, 1970). Focal innervation is sometimes found at both ends of the fibre but, more usually, is observed at one end of the fibre only (Best and Bone, 1973) (Fig 2).

Fig 2. Summary diagram showing the structure and innervation pattern of red and white myotomal muscle fibres in fish. Note that multiply-innervated white fibres are found only in higher teleosts. (Reproduced from Bone, 1978.)


capillaries
mitochondria
slow (red)
focal white (fast)
multiply-innervated white (fast)

As in other vertebrate embryos, the newly formed somites of fish consist of radially arranged epithelial balls of mesoderm (epithelial somites). The epithelial somites undergo a series of morphological changes that eventually result in their dissolution and differentiation into bone, cartilage, dermis and muscle (Keynes and Stern, 1988). During somite differentiation, cells in the ventromedial part of the somite form a loosely arranged mesenchyme that gives rise to the sclerotome cells from which the axial skeleton is constructed (Keynes and Stern, 1988).

The first cells in the maturing somite that can be identified as muscle precursors lie as a column of large cuboidal cells adjacent and lateral to the notochord. The cells elongate, giving rise to mononucleated myotubes which initially develop adjacent to the notochord and then migrate laterally through the somite to the lateral midline region, superficial to the rest of the somite (Raamsdonk *et al*, 1974; Johnston *et al*, 1995). These cells are fated to become at least a subset of the superficial/red muscle cells (Kimmel *et al*, 1991). Myotubes in the mid-somite, which gradually mature into the inner/white muscle fibres (Hanneman, 1992; Johnston *et al*, 1995), are formed by mononucleated embryonic mesenchymal cells (or presumptive myoblasts). These do not fuse, but proliferate and differentiate into mononucleated myoblasts.

By the end of embryonic development in fish, superficial (presumptive red) muscle fibres are usually present in a single

layer, while the remainder of the myotome contains inner (presumptive white) muscle fibres (Batty, 1984). As for adult muscle, larval inner/white fibres are of larger diameter, and have a higher content of myofibrils, than their superficial/red counterparts (Raamsdonk *et al*, 1978). Both larval fibre types are, however, relatively aerobic in character compared with adult muscle fibre types, containing numerous mitochondria and heart-type (H_4) lactate dehydrogenase subunits (El-Fiky *et al*, 1987). This suggests that the energy supply for larval muscle contraction is largely supplied by aerobic pathways (Johnston, 1994). This may be due to the scaling of aerobic metabolism in fish in proportion to body mass$^{0.70\text{-}0.82}$ (Goolish, 1991; Somero and Childress, 1980). The small size of larvae thus give them a very high mass-specific capacity for aerobic metabolism, and most of the oxygen required for metabolism is supplied by diffusion across the skin and intervening tissues (Batty, 1984). As body size increases, however, the capacity for aerobic metabolism decreases and anaerobic pathways (and consequently glycolytic fibres) become more important (Johnston, 1994).

Muscle development: molecular mechanisms

The recent characterisation of several transcriptional regulators in the myogenic pathway has led to an understanding of the molecular mechanisms which are responsible for the establishment of muscle specific gene expression (Molkentin and Olson, 1996). These muscle transcriptional factors belong to a family of basic helix-loop-helix (bHLH) DNA binding proteins, and

include myogenin, MyoD, myf-5 and MRF4 (for a review see Buckingham, 1994; for fish see Rescan *et al*, 1995). Muscle transcription factors act at multiple points in the skeletal muscle lineage to establish the skeletal muscle phenotype. For example, in the zebrafish myf-5 is the earliest expressed muscle transcription factor, appearing before any muscle differentiation. MyoD follows as myoblasts form, myogenin appears at the time of cell fusion, and MRF4 is expressed only in older myotubes (Grinnell, 1995). Knockout mutations in mice have also shown that myogenin is the most critical transcription factor for the final differentiation of muscle, with myogenin, plus either MyoD or myf-5, forming an apparently normal muscle phenotype (Metzger *et al*, 1995).

Muscle transcription factors are targets for the inductive signals that induce commitment of myogenic cells (Molkentin and Olson, 1996). It has been known for many years that signals from the neural tube and notochord of vertebrate embryos induce myogenesis in the somites. For example, Buffinger and Stockdale (1995) found that either neural tube or notochord could induce myogenesis in unspecified chick somites cultured *in vitro*. By using physical blocking techniques in explant cultures, Buffinger and Stockdale also showed the inducing activity was soluble and did not require cell-cell contact. Stern *et al* (1995) have also demonstrated that the neural tube and notochord isolated from chick embryos could induce somites to express muscle markers *in vitro*. The results of Stern *et al* (1995) additionally indicated that the dorsal and ventral regions of the neural tube contain factors that act cooperatively in the induction of myogenesis. The findings are also consistent with the experiments of Munsterberg and

Lassar (1995). By recombining somites and neural tubes from different axial levels of the embryo (which therefore represent different developmental stages) Munsterberg and Lassar (1995) found evidence for two signals from the axial organs that act in combination to induce muscle cell markers in the myotome: one signal originates from the floor plate/notochord, whereas the other originates from more ventral regions of the neural tube.

Previous studies have shown that members of the Wnt family of growth factors, which are expressed in the dorsal neural tube, and sonic hedgehog (Shh), which is expressed in the notochord and ventral neural tube, play important roles in the patterning of the somites (Takada *et al*, 1994; Johnson *et al*, 1994; Fan and Tassier-Lavigne, 1994). Co-culture of cells from developing somites with *Wnt-1*-expressing fibroblasts results in the weak induction of muscle markers, consistent with the notion that Wnt proteins may constitute the dorsal-inducing signal (Stern *et al*, 1995). Munsterberg *et al* (1995) have also shown that a subset of Wnt family members (Wnt-1, Wnt-3 and Wnt-4) cooperate with Shh to induce myogenesis in isolated somites *in vitro*. The source of Shh in the floor plate/notochord is relatively distal from the location of myogenic cells in the somites, and it has not yet been determined whether Shh acts directly on cells that adopt a myogenic fate or whether its effects are relayed through an intermediate regulator.

In addition to Wnt and Shh, differentiation of skeletal myoblasts has also been shown to be tightly controlled, through a repression-type mechanism, by serum and exogenous peptide

growth factors. These prevent entry into the differentiation pathway until their concentration is reduced below a critical threshold. Once this occurs, the muscle differentiation programme is activated, myotubes (mononucleated or multinucleated) are formed, and muscle-specific genes are transcriptionally activated (Florini *et al*, 1991).

Two of the most potent inhibitors of myoblast differentiation *in vitro* are fibroblast growth factor (FGF) and transforming growth factor type-ß (TGF-ß) (Clegg *et al*, 1987; Olson *et al*, 1986). Whether these growth factors regulate the timing of myogenesis *in vivo* remains to be determined, but the temporal and spatial expression of both FGF and TGF-ß is appropriate for a role in the regulation of myogenesis (Olson, 1992).

Repression of myoblast differentiation requires the continuous presence of growth factors. This suggests that intracellular signals produced when growth factors occupy their corresponding cell surface receptors, and which interfere with the mechanism for muscle-specific gene activation, are short-lived. It also implies that growth factor-inducible gene products are essential for the inhibition of muscle-specific gene activation. This has, indeed, been demonstrated *in vitro* for several protooncogenic signals that are rapidly induced by growth factors (Olson, 1992).

Following myotube formation, muscle cell nuclei lose the ability to reinitiate DNA synthesis, and muscle-specific genes become refractory to repression by exogenous growth factors. The molecular mechanism responsible for the loss in sensitivity to

growth factors is unknown, but it appears to be coupled to fusion because fusion-defective muscle fibre lines retain the ability to reenter the cell cycle in response to mitogenic stimulation (Nguyen *et al*, 1983; Spizz *et al*, 1986). A likely explanation for the loss in growth factor responsiveness in terminally differentiated muscle cells is the disappearance of one or more components of the signal transduction pathways (i.e., cytoplasmic relay proteins) that link the cell membrane with the nucleus (Olson, 1992).

The muscle differentiation programme is also intimately coupled to the cell cycle, such that muscle-specific transcription is initiated only when myoblasts are growth arrested in the G_0/G_1 phase. Arrest of myoblasts at other points of the cell cycle (sequentially, S, G_2 and M) does not result in muscle-specific gene activation. This suggests that the myogenic regulatory programme is dependent on gene products that are only expressed or functional in G_0/G_1, or inhibitory factors expressed at other phases of the cell cycle are incompatible with events required for activation of the myogenic programme (Olson *et al*, 1986; Spizz *et al*, 1986).

The cellular signals which are negatively downregulated by growth factors and activated oncogenes are the muscle transcription factors. Muscle transcription factors are also autoregulatory, and cross-activate expression of one another (Olson, 1992). It has been postulated that the autoregulatory interactions amplify the expression of these factors above the threshold necessary to activate the muscle differentiation programme when myoblasts become deprived of growth factors

(Olson, 1992). Myogenic HLH proteins also inhibit cell proliferation when expressed at high levels (Davis *et al*, 1987). This inhibition, which occurs in the G_1 phase of the cell cycle (Crescenzi *et al*, 1990), is not a secondary consequence of differentiation. For example, mutants of MyoD which possess its HLH motif, but which cannot activate myogenesis, inhibit proliferation (Olson, 1992).

Whether a myoblast divides or differentiates thus appears to be determined by a balance between growth and differentiation signals, with the overexpression of factors (e.g., hormones) that activate or inhibit either regulatory pathway tipping the balance in one direction or the other (Olson, 1992). Thyroid hormones, for example, result in increased MyoD gene transcription in myogenic cell lines and promote terminal differentiation (Muscat *et al*, 1994). On the other hand, the presence of insulin-like growth factors results in the production of cyclins D and E and the promotion of myosatellite proliferation (Schriever *et al*, 1996).

There have been few comparatively detailed studies on the dynamics of growth in fish muscle. This has been a significant deficiency in our knowledge because:
1) muscle comprises up to 80 % of the live weight of fish (Bone, 1978; Weatherley and Gill, 1987);
2) apart from its propulsive role, muscle is also important as an energy storage tissue in fish that they may utilise during starvation or gonad maturation (Weatherley and Gill, 1987);
3) muscle is the main edible tissue of fish, and is therefore of much value to man (Weatherley and Gill, 1987).

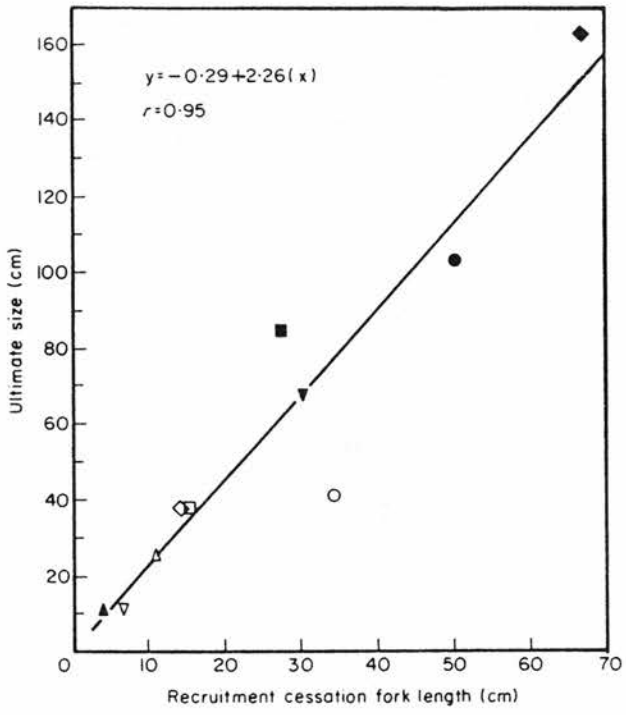
Muscle growth can occur by an increase in the number of muscle fibres (i.e., hyperplasia) and by the increase in size of already existing fibres (i.e., hypertrophy). In most mammalian and avian muscles, hyperplasia is restricted largely to the pre- and peri-natal period (Goldspink, 1972, 1974; Schultz, 1974; Stickland, 1981; Campion, 1984). Postnatal growth in these animals therefore results mainly from hypertrophic growth of existing muscle fibres. By contrast, hyperplasia continues in teleosts, together with hypertrophic growth, into the adult stage (Greer-Walker, 1970; Stickland, 1983; Weatherley *et al*, 1988; Weatherley, 1990). However, the relative importance of hyperplasia is maximal in small (young) fish and decreases with increasing growth (Weatherley, 1990). 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; Weatherley *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 and the maximum reported length for that species (see also Battram and Johnston, 1991) (Fig 3). Additionally, species or strains with a small ultimate body size were found to grow largely by fibre hypertrophy (Weatherley and Gill, 1987).

Another important aspect of muscle growth is the increase in the number of muscle nuclei (myonuclei) accompanying the increase in muscle mass (Allen *et al*, 1979; Cardasis and Cooper, 1975; Enesco and 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 myonucleus/sarcoplasm ratio has been reached, outgrowth of the fibres can only occur if additional myonuclei are added to the fibres (Cardasis and Cooper, 1975; Enesco and Puddy, 1964; Winchester and Gonyea, 1992). However, as the myonuclei in the fibres are post-mitotic (i.e., incapable of undergoing mitosis) (Campion, 1984), and embryonic myoblasts do not exist any more, another source of myonuclei must be present (Cardasis and Cooper, 1975; Enesco and Puddy, 1964). Myosatellite cells are that source of additional myonuclei (Campion, 1984).

Myosatellites are small, spindle-shaped cells which contain a heterochromatic nucleus and, except for free ribosomes and polysomes, their small amount of cytoplasm contains only a small number of other organelles (Campion, 1984). They are situated

Fig 3. The relationship between body size (fork length) at which the production of new muscle fibres (i.e., hyperplasia) ceases and the ultimate size for ten freshwater teleost species. Linear regression and correlation coefficient (r) are given. (open downward triangle: longnose dace, *Rhinichthys cataractae*; open upward triangle: pumpkinseed, *Lepomis gibbosus*; solid downward triangle, smallmouth bass, *Micropterus dolomieu*; solid upward triangle: bluntnose minnow, *Pimephales notatus*; open square: bluegill, *Lepomis macrochirus*; solid square: lake whitefish, *Coregonus clupeaformis*; open circle: grass pickerel, *Synbranchus marmratus*; solid circle: rainbow trout, *Salmo gairdneri*; open diamond: yellow perch, *Perca flavescens*; and solid diamond: muskellunge, *Esox masquinongy*.) (Reproduced from Weatherley *et al*, 1988.)


y = −0·29+2·26(x)
r = 0·95
Ultimate size (cm)
Recruitment cessation fork length (cm)
0
10
20
30
40
50
60
70
20
40
60
80
100
120
140
160

between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibres (Mauro, 1961) and are present in all investigated vertebrate species (for a review, see Campion, 1984). Indications that 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 a pulse of ^{3}H-thymidine (a compound which is incorporated into replicating DNA and which can later be localised by autoradiography) the label did not appear in the myonuclei but did appear in the myosatellite nuclei. However, as the percentage of labelled myosatellite cells decreased, labelled nuclei also appeared in muscle fibres. Strong correlations have also been found between the proliferation rate of myosatellite cells and the rate of muscle growth in several animals (Campion *et al*, 1982; Penney *et al*, 1983; Mulvanney *et al*, 1987; Darr and Schultz, 1989; Joubert and Tobin, 1989; Winchester *et al*, 1991).

Tissue culture experiments have shown that there are distinct classes of myosatellite cells for fast (white) and slow (red) muscles and at the embryonic, neonatal and adult stages in the life cycle (for a review, see Stockdale, 1992). Additionally, *in vitro* studies on the common carp, *Cyprinus carpio* L., suggest that hyperplasia and hypertrophy of fish muscle might depend on myosatellite cells which have entered different pathways (Koumans *et al*, 1993). According to Koumans *et al* (1993), hypertrophy of fish muscle is particularly dependent on myosatellites which have entered proliferation pathways, and which divide to produce the additional nuclei required by fibres

that are increasing in size. Hyperplasia on the other hand is believed to rely more on myosatellites which have entered differentiation pathways, and which fuse to produce new myotubes/immature muscle fibres (Koumans *et al*, 1993).

Fish are often exposed to diurnal and seasonal temperature variations, and move through thermoclines between layers of water that differ considerably in temperature (Wootton, 1990). Temperature is one of the most pervasive of abiotic environmental factors because of its effect on the rate of chemical reactions (Schmidt-Nielsen, 1975). While mammals and birds evade the effects of temperature changes by maintaining a constant core body temperature, endothermy is not found in most fishes (the exceptions being the tunas and their relatives) (Stevens and Neill, 1978). Thermal homeostasis has not been adopted by fish for several reasons. Maintenance of a constant body temperature, for example, involves the significant cost of a higher rate of metabolism (Schmidt-Nielsen, 1975). Additionally, the short distance separating the blood flowing through the gills and the water in which fish live provides a path for the dissipation of body heat to the external environment (Brett, 1956).

Considerations of fish development must take temperature into account since: 1) the thermal environment experienced by fish can change dramatically (e.g., due to seasonal/diurnal warming or cooling) as they develop (Johnston *et al*, 1996); 2) the early developmental stages of fish are much more sensitive to temperature change, with temperature tolerances of embryos and larvae being more restricted than those for adults (Blaxter, 1992); and 3) temperature change often results in plasticity of fish development (Johnston *et al*, 1996).

One way in which developmental plasticity can arise is through thermal activation of switches in the developmental programme. This leads either to the initiation of developmental arrest or to the production of alternative phenotypes differing in morphological features, behaviour and/or life-history characters (Scheiner, 1993). A particularly striking example of this type of plasticity is the determination of the sex of some fish, such as the pejerrey (*Odontesthes bonariensis*) (Strussmann *et al*, 1996) and the Atlantic silverside (*Menidia menidia*) (Lagomarsino and Conover, 1993), by the temperature during a critical period in development.

Developmental plasticity can also arise from temperature effects on the rates and degrees of expression of the developmental programme. In contrast to developmental switches, which produce discontinuous phenotypes, temperature effects on the rate of developmental processes produce a continuum of

phenotypes. Somitogenesis, for example, occurs at greater rates as temperature increases. In Atlantic herring from the Clyde region of Scotland, a new somite segregates from the paraxial mesoderm once every 176 min at 5 °C compared with once every 52 min at 15 °C (Q_{10}= 3.4 from the 5- to 55-somite stage) (Johnston *et al*, 1995). However, when expressed as a fraction of the time from fertilisation to hatching, somitogenesis occurs earlier at 5 °C than at 15 °C. For example, after 32 % of the development time there are around 42 somites at 5 °C but only 25 somites at 15 °C (Johnston *et al*, 1995).

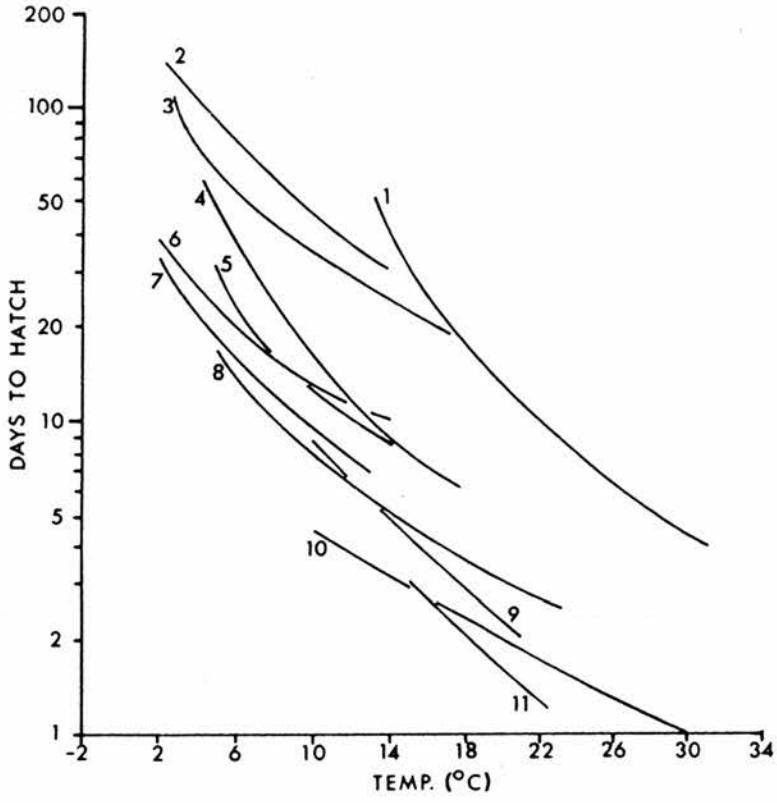
Rearing temperature also affects the relative timing of development of other organs in fish. Such effects of temperature on development arise since, at any given point during embryogenesis, different organs and tissues will have reached different points in their developmental programmes and may therefore exhibit somewhat different temperature sensitivities (Johnston *et al*, 1996). For example, in herring pectoral fin buds develop in the order 8 °C>12 °C> 5 °C, and pronephric tubules form at the 40-somite stage at 12 °C but not until after the 61-somite stage at 5 °C (Johnston, 1993). The order in which eye pigmentation, mouth opening and pectoral fin development occur in the Japanese flounder (*Paralichthys olivaceus*) also varies with rearing temperature (Fukuhara, 1990). The same is also true for the appearance of a loop in the gut and the swim bladder in the turbot (*Scophthalmus maximus*) (Gibson and Johnston, 1995).

In addition to effects on the appearance of different organs and tissues, temperature also influences the time at which certain

major events of development occur. For example the time from fertilisation to hatching decreases in most fish as temperature increases (Blaxter, 1988) (Fig 4), as does the time to metamorphosis (Johnston *et al*, 1996). Temperature, however, has been found to be inversely related to the body size of some fish (e.g., Atlantic salmon, *Salmo salar* L., the coral-reef fish, *Siganus randalli* Woodland, and the herring) at developmental milestones such as hatching and metamorphosis (Nathanailides *et al*, 1995; Collins and Nelson, 1993; Johnston *et al*, 1996). The negative correlation between development rate and body size suggested by these results implies that temperature also influences the efficiency at which food reserves (both endogenous and exogenous) are converted to body tissue in developing fish. This has been demonstrated in the Walleye pollock, *Theragra chalcogramma* (Pallas) using the relative levels of RNA and DNA as indicators of the feed to gain ratio (Canino, 1994).

Phenotypic plasticity observed during ontogeny is believed to involve some cost to the organisms, for example in terms of maintenance of regulatory genes and enzymes (Moran, 1992). Additionally, some effects of temperature on the developmental programme may influence mortality rates, as has been demonstrated in the Atlantic cod (*Gadus morhua* L.) where embryonic development temperature affects the formation of features essential to larval feeding, respiration and locomotion, and thus of importance to larval survival (Vonherbing *et al*, 1996). Since only very few of the larvae of fish eventually give rise to adults (for marine fish, values are typically less than 0.1 %), and because even small effects of temperature on mortality rates of

Fig 4. The time from fertilisation to hatching for 11 species of teleost related to temperature. 1: desert pupfish, *Cyprinodon macularius*; 2: brook trout, *Salvelinus fontinalis*; 3: rainbow trout, *Salmo gairdneri*; 4: smelt, *Osmerus eperlanus*; 5: Atlantic herring, *Clupea harengus*; 6: plaice, *Pleuronectes platessa*; 7: Pacific cod, *Gadus macrocephalus*; 8: rockling, *Enchelyopus cimbrius*; 9: mackerel, *Scomber scombrus*; 10: grey mullet, *Mugil cephalus*; and 11: striped bass, *Morone saxatilis*. (Reproduced from Blaxter, 1988.)


200
100
50
20
10
5
2
1
DAYS TO HATCH
-2
2
6
10
14
18
22
26
30
34
TEMP. (°C)
1
2
3
4
5
6
7
8
9
10
11

the early developmental stages of fish significantly affect recruitment to the adult population (Ferron and Leggett, 1994), further studies on developmental plasticity in fish are of considerable importance.

In the Clyde herring, myotube formation occurs at the same somite stage, regardless of temperature (Johnston *et al*, 1996). The formation of myofilaments, however, occurs at the 42-somite stage at 5 °C, the 38-somite stage at 8 °C, and as early as the 27-somite stage at 15 °C (Johnston *et al*, 1995). These results suggest that myotube formation might not be causally linked to myofibrillargenesis and that there are, in effect, at least two separate developmental processes involved in constructing a fully functional muscle unit. Support for this hypothesis has come from work with the mutation genotype *fub-1* of the zebrafish which allows the development of myotubes but not myofibrillar proteins and myofibrillar construction (Felsenfeld *et al*, 1990, 1991).

Staining Clyde herring embryos for acetylcholinesterase activity has revealed that functional muscle endplates form at progressively earlier somite stages as temperature is raised from 5 to 15 °C (Johnston *et al*, 1996). Antibody staining with antibodies against HNK-1 (a carbohydrate moiety involved in the NCAM/L2 cell adhesion reaction) and α-acetylated tubulin (which stains all neural processes) has also been used in Buchan herring, and the outgrowth of motor axons was found to be retarded at low temperatures, occurring at the 30-somite stage at 12 °C, but not until after the 40-somite stage at 5 °C (Johnston *et al*, 1996). As in the Clyde herring, however, there was no effect of temperature on the timing of myotube formation in Buchan herring (Johnston *et al*, 1996), suggesting that a change in temperature might uncouple

myogenesis and motor neuron development with respect to the formation of somites (Johnston *et al*, 1995).

Recent research has additionally shown that the temperature of embryonic development influences the number and cross-sectional areas of muscle fibres at hatching in a number of species, including herring (Vieira and Johnston, 1992), Atlantic salmon (Stickland *et al*, 1988; Nathanailides *et al*, 1995) and plaice (*Pleuronectes platessa*) (Brooks and Johnston, 1993). For example, in experiments with Clyde herring, the number of white muscle fibres increased with rearing temperature whereas the average cross-sectional area of the fibres at 5 °C was almost double that at 8 or 12 °C (Johnston *et al*, 1995). As a result the total cross-sectional area of myotomal muscle increased in the order 5 °C>12 °C> 8 °C (Johnston *et al*, 1995). It has been suggested that the influence of temperature on the numbers and sizes of muscle fibres in larval fish might involve differential effects of temperature on the rate of division of the somite stem cell population on the one hand, and the production of signals required for muscle differentiation and the fating of myoblast lineages on the other (Johnston *et al*, 1996).

In general, there is a positive correlation between rearing temperature and the growth rate of fish (Weatherley and Gill, 1987). The effect of rearing temperature on the mechanisms of muscle growth in fish has, however, only been investigated in a few species. For example, whitefish, *Coregonus lavaretus*, larvae reared at high temperatures used hypertrophy to a greater extent in muscle growth than those reared at low temperatures (Hanel *et al*, 1996). In contrast, an increase in the rearing temperature of rainbow trout (*Salmo gairdneri* L.) (Weatherley *et al*, 1979) and salmon juveniles (Higgins and Thorpe, 1990) results in increased hyperplasia.

In recent experiments with the Atlantic salmon, it has also been found that fish from eggs incubated at low temperature (5-10 °C) grow faster, and use hypertrophy to a greater extent in muscle growth, than those from high temperature (11 °C) eggs when both groups are reared at a common temperature (11 °C) (Nathanailides *et al*, 1995). One possible explanation for this finding is that development temperature could have influenced the numbers of differentiating and proliferating myosatellites and, in so doing, altered the balance between hyperplasia and hypertrophy in future muscle growth. This could have been achieved either directly (the transcriptional activities of different genes have different temperature sensitivities; Chen *et al*, 1993) or indirectly (temperature can affect the development of glands, such as the thyroid gland (Inui and Miwa, 1985; Seikai *et al*,

1986), which produce hormones that regulate the transcription of genes responsible for cellular signals).

My study thus investigates the effect of temperature on organogenesis and muscle development and growth in a tropical fish, the angelfish (*Pterophyllum scalare* Lichtenstein), and an arctic-boreal fish, the shorthorn sculpin (*Myoxocephalus scorpius* L.). Temperatures used were within the normal ranges for the animals (Soomai, 1994; Beddow *et al*, 1995). Embryos were staged by somite number since this has been shown, in zebrafish, to more accurately predict the timing of development of specific structures (Metcalfe *et al*, 1990; Eisen *et al*, 1986) than staging by elapsed time after fertilisation (Westerfield, 1994). Larvae from the different groups were compared using body length since it is the length of a fish which determines its Reynolds number, hydrodynamic regime, swimming speed, endurance and tail-beat frequency, and thus its swimming behaviour (Johnston, 1994).

Chapter 2

The effect of temperature on embryonic development in the teleost fish *Pterophyllum scalare* and *Myoxocephalus scorpius*

Introduction

The thermal environment experienced by fish can change dramatically as they develop (e.g., due to seasonal/diurnal warming or cooling). The early developmental stages of fish are particularly sensitive to temperature change, with normal development only occurring over a range of temperatures much narrower than the range for adult survival and growth (Blaxter, 1992). Outside this range abnormalities (such as twins conjoined in the abdominal region) and mortality increase considerably (Stockard, 1921). Considerations of fish development must therefore take temperature into account.

Numerous end products of differentiation, including pigmentation patterns (Schmidt, 1919) and meristic characters (Hubbs, 1922; Tåning, 1952), are influenced by development temperature. Several studies (Hayes *et al*, 1953; Fukuhara, 1990; Johnston, 1993) have even demonstrated that temperature can determine the order in which certain characters appear. For example, in Atlantic herring (*Clupea harengus* L.), the pectoral fin

buds and pronephric tubules form at later somite stages in embryos incubated at 5 °C than in those at 8 and 15 °C (Johnston, 1993). Rearing temperature also influences the differentiation of the swimming muscles and the outgrowth of motor axons in developing fish. In Clyde herring, the formation of myofibrils in rostral myotomes occurs at the 42-somite stage at 5 °C, the 38-somite stage at 8 °C, and as early as the 27-somite stage at 15 °C (Johnston *et al*, 1995). Differences in the cellularity of the myotomal muscle have also been reported for newly hatched Atlantic salmon (*Salmo salar* L.) (Stickland *et al*, 1988), herring (Vieira and Johnston, 1992), and plaice (*Pleuronectes platessa* L.) (Brooks and Johnston, 1993). Staining Clyde herring embryos for acetylcholinesterase activity has revealed that functional endplates form at progressively earlier somite stages as temperature is raised from 5 to 15 °C (Johnston *et al*, 1996). Staining herring embryos with antibodies against HNK-1 (a carbohydrate moiety involved in the NCAM/L2 cell adhesion reaction) has also shown that the outgrowth of motor axons is retarded at low temperature, occurring at the 30-somite stage at 12 °C but not until after the 40-somite stage at 5 °C (Johnston *et al*, 1996).

Studies on the influence of temperature on development are small in number compared with the great variety of developmental patterns which exist in fish (see Russell, 1976; Fahay, 1983; for a review see Blaxter, 1988). For example, many warm-water fish, such as the tambaqui (*Colossoma macropomum* Cuvier) and the curimatã-pacú (*Prochilodus maggravi* Walbaum), produce small larvae (2.5-3.5 mm) which hatch before all the

somites have formed, and which lack eye pigment, functional jaws and a gut (Johnston and Vieira, 1996). In contrast, many temperate species, such as the herring, produce large larvae (8-10 mm) which hatch with the full complement of somites, and which have pigmented eyes, functional jaws and a gut (Johnston *et al*, 1996). We therefore investigated the effect of temperature on embryogenesis in fish with contrasting larval strategies. The shorthorn sculpin (*Myoxocephalus scorpius* L.) is an arctic-boreal, marine fish which produces large (about 8 mm), relatively well-developed larvae which swim vigorously on hatching (King *et al*, 1983). The angelfish (*Pterophyllum scalare* Lichtenstein), on the other hand, is a tropical, freshwater fish which produces small (2-3 mm) larvae which hatch at a comparatively early stage of development, and which are initially non-motile (Axelrod and Schultz, 1955).

Fish

Embryonic development was studied in the angelfish, *Pterophyllum scalare* Lichtenstein, and the shorthorn sculpin, *Myoxocephalus scorpius* L.

Adult angelfish were obtained from a commercial supplier (WorldWide Pet Supplies, Glasgow) and held at 26 °C (temperature range ±1 °C) with a 12L:12D light cycle. The eggs of one female (total length, TL= 96 mm) were fertilised by two males (TL= 87 and 76 mm) during natural spawnings, which occurred between May and September 1995. Fertilised eggs were incubated in aerated freshwater at 24 and 31 °C (range ±1 °C).

Adult shorthorn sculpin were caught in St Andrews Bay, Scotland, during January 1995, then maintained under ambient conditions of temperature and photoperiod at the Gatty Marine Laboratory. Ripe individual were stripped, and the eggs of six females (155-293 g) were fertilised with the milt of two males (158 and 166 g). Fertilised eggs were transferred to filtered, aerated seawater (24 h artificial illumination) at 4, 8 and 12 °C (range ±1 °C).

Histology and electron microscopy

Embryos were fixed in Bouin's fluid (3-24 h) for light microscopy. In order to improve penetration of the fixative, the egg capsules were either punctured or, where practicable, the chorion was removed. Eggs and isolated embryos were embedded in wax, and transverse, sagittal and coronal sections were cut at 7 μm thickness and mounted on gelatin coated slides. Sections were stained with haematoxylin-eosin.

Dechorionated embryos were fixed for electron microscopy for 3-24 h in 2.5 % gluteraldehyde, 2.5 % paraformaldehyde, 1 % sucrose, 2 mmol l^{-1} $CaCl_2$, 100 mmol l^{-1} NaCl, 100 mmol l^{-1} sodium cacodylate, pH 7.4 (at 4 °C). Specimens were washed in the same solution minus the fixative and post-fixed for 2 h in a solution containing 2 % osmium tetroxide in 100 mmol l^{-1} sodium cacodylate, pH 7.4 (at 4 °C). Samples were subsequently washed in buffer, dehydrated through alcohol, stained *en bloc* with uranyl acetate in 70 % ethanol and embedded in araldite resin (47 % araldite epoxy resin, 47 % dodecyl succinic anhydride, 5 % benzyldimethylamine, 1 % dibutyl phthalate). Semi-thin sections (0.5-1.0 μm) were cut on a Reichert OMU2 ultramicrotome and stained with toluidine blue in order to determine the orientation of the specimens. Ultrathin sections (50-80 nm thick) were cut and mounted on pyroxyline-coated copper grids of 300 μm mesh size and stained with aqueous saturated uranyl acetate and Reynold's lead citrate. Sections were viewed with a Philips 301 transmission electron microscope at 60 KV.

HNK-1 localisation and acetylcholinesterase activity

Embryos were dechorionated and the yolk-sac removed. In order to improve penetration of the antibodies and substrates, the embryos were incubated in a solution of 1 % saponin in phosphate-buffered saline (PBS: 10 mmol l^{-1} phosphate buffer, 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, pH 7.4) for 12-24 h at 4 °C then washed thoroughly in PBS. HNK-1 was localised with a mouse anti-HNK-1 monoclonal antibody (Sigma Immunochemicals, Poole, Dorset), which was in turn detected with a goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC) (Sigma Immunochemicals). Specimens were observed using epifluorescence optics at a wavelength of 488 nm. Staining for acetylcholinesterase (AChE) was performed by the direct colouring method of Karnovsky and Roots (1964).

Histochemistry

Larvae were anaesthetised with an overdose (1:10000 w/v in water) of MS222 anaesthetic (3-aminobenzoic acid ethyl ester), and mounted on cryostat chucks in embedding medium (Cryomatrix; Shandon Products, Pittsburgh, USA). They were then rapidly frozen in 2-methyl butane cooled to near its freezing point (-159 °C) in liquid nitrogen. Blocks were equilibrated at -20 °C in a cryostat (Leica Instruments GmbH, Germany), and serial, 10 μm transverse sections were cut from the region immediately posterior to the anus. Sections were mounted on glass coverslips

and stained for succinic dehydrogenase activity (SDHase) (Nachlas *et al*, 1957).

Statistical analyses

Normal quantile plots revealed that all data were normally distributed (either originally or following logarithmic transformation). Results from the different temperatures were thus compared using a *t*-test. All data are presented as means ±SD.

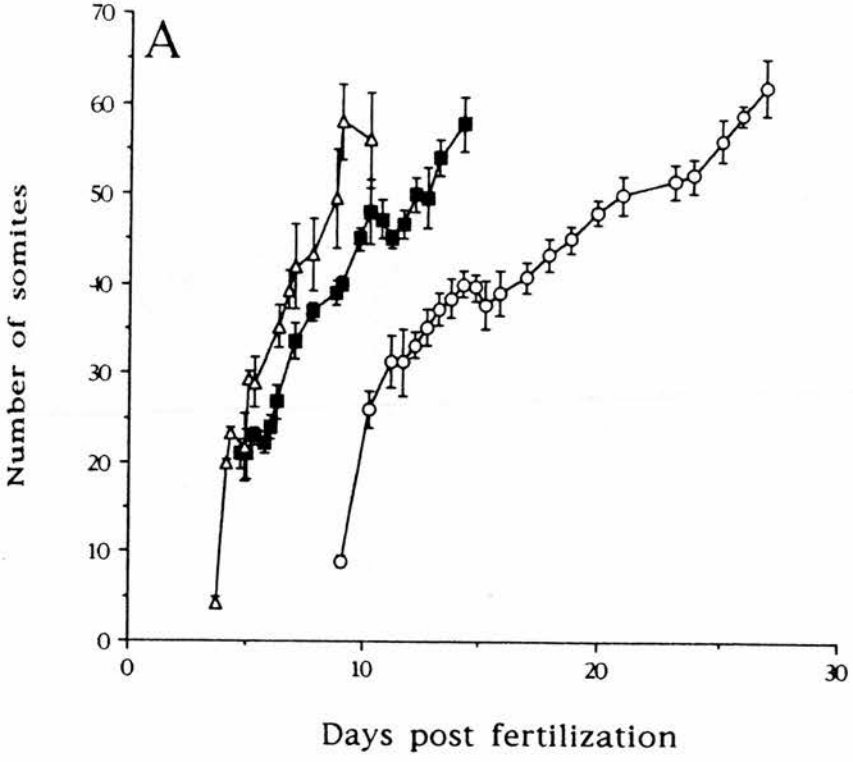
Somitogenesis and differentiation in M. scorpius

In shorthorn sculpin, only embryos at 4 °C survived to hatching. Embryos at 8 and 12 °C only survived to the point at which spontaneous movements were observed in the eggs. The rate of somitogenesis increased with temperature. Between the 20- and 50-somite stages, new somites were added at the rate of 2, 4 and 7 per day at 4, 8 and 12 °C, respectively (Fig 1A) (t=4.623-7.158, P<0.01).

The rate at which body length increased was greater at 4 °C than at 8 °C (t=7.250, P<0.01) (Fig 2), but the rate of body elongation in 8 °C embryos was not significantly different from that in 12 °C embryos (t=1.413, P>0.10) (Fig 2).

At 4 °C, shorthorn sculpin larvae hatched 48 d after fertilisation at a TL of 7.88±0.12 mm (n=10 fish). The trunk of newly hatched larvae contained about 62 somites (Fig 1A). No further somites were added after hatching. Newly hatched larvae had pigmented eyes, a small yolk sac (body depth at middle of the yolk sac was about 16 % of TL), and an open mouth (see Fig 12). They also swam continuously.

Fig 1. Rates of somitogenesis in, A, shorthorn sculpin embryos at 4 °C (open circles), 8 °C (closed squares) and 12 °C (open triangles) (n= 10-12 fish at each temperature), and, B, angelfish embryos reared at 24 °C (open squares) and 31 °C (closed triangles) (n= 6-10 fish at each temperature). Values are mean ±SD.


A
Number of somites
Days post fertilization
0
10
20
30
40
50
60
70

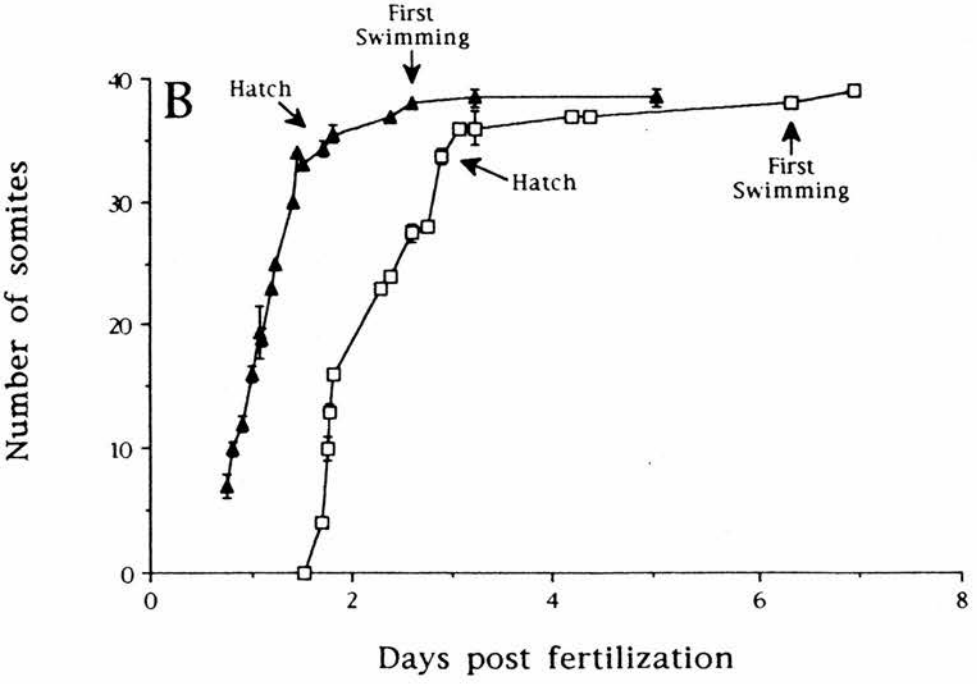
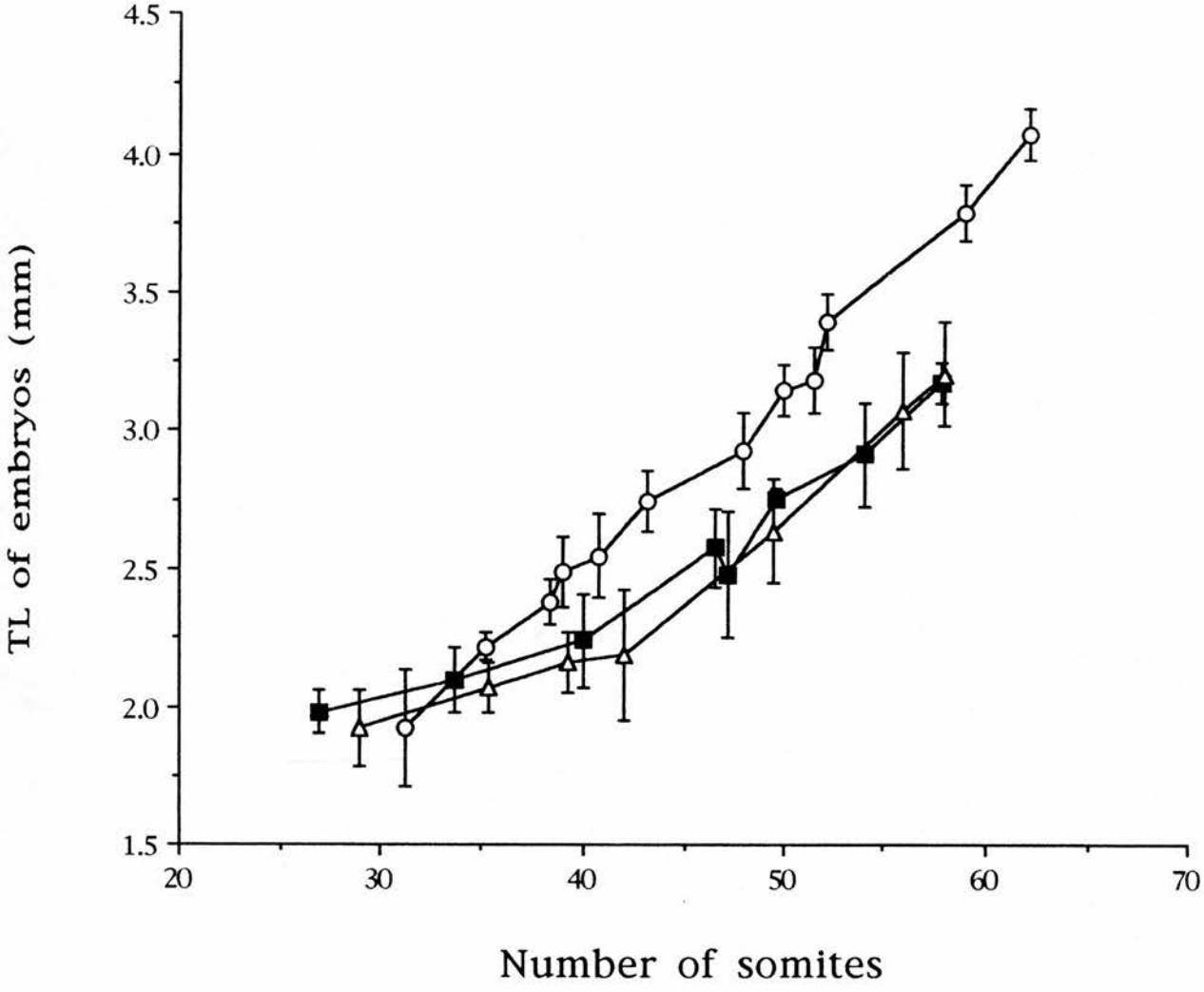

B
First Swimming
Hatch
Hatch
First Swimming
Number of somites
Days post fertilization
0
10
20
30
40
2
4
6
8

Fig 2. Total length (TL) in relation to somite stage for sculpin embryos reared at 4 °C (open circles), 8 °C (closed squares) and 12 °C (open triangles). Values are mean ±SD (n=10-12 fish at each temperature).


4.5
4.0
3.5
3.0
2.5
2.0
1.5
TL of embryos (mm)
20
30
40
50
60
70
Number of somites

In the angelfish, the rate of somite formation, between fertilisation and hatching, was greater at 31 °C (about 1.4 per h between the 10- and 30-somite stages) than at 24 °C (about 0.8 per h) (t=3.470, P<0.01) (Fig 1B). The time from fertilisation to about 70 % hatching was also shorter at 31 °C (1.88 d) than at 24 °C (2.57 d). Hatching occurred at about 34 somites at both temperatures (t=0.683, P>0.10) (Fig 2B). The TL at hatching at 24 °C (2.71±0.08 mm, n=10) was not significantly different from that at 31 °C (2.65±0.18 mm, n=10) (t=0.963, P>0.10). Newly hatched angelfish had unpigmented eyes and a large yolk sac (body depth at middle of yolk sac was about 31 % of TL), but lacked a mouth (see Fig 9). They were also incapable of swimming, and remained attached to the substratum on which eggs were laid (via secretions from glands at the top of the head). They did, however, twitch vigorously.

First swimming in the angelfish occurred 3.69 d and 0.50 d after hatching (or 6.26 d and 2.38 d after fertilisation) at 24 °C and 31 °C, respectively (see Fig 1B). The TL at first swimming was greater at 24 °C (4.79±0.13 mm, n=16) than at 31 °C (4.53±0.21 mm, n=16) (t=3.578, P<0.01). The eyes were fully pigmented by first swimming and an open mouth was present. The size of the yolk sac was also greatly reduced (body depth at middle of yolk sac was about 20 % of TL at both temperatures) (see Fig 10). First swimming occurred at the 38-somite stage at both temperatures (t=0.754, P>0.10, n=10) (Fig 1B).

Organogenesis in M. scorpius

In the shorthorn sculpin, the spinal cord, notochord and eye bulges were all present by the 30-somite stage at 4, 8 and 12 °C (Figs 3, 4, 5). The relative timing of development of some organ systems was found to be independent of development temperature. For example, the lens in eye and gut both appeared at the 37-38-somite stage at all temperatures (Figs 3, 4, 5). Several other organ systems, however, appeared at earlier somite stages at 12 °C than at 8 °C or 4 °C. For example, while 41-42-somite sculpin embryos at 4 °C lacked dorsal aorta, heart and pronephros, these organs were present in embryos of equivalent stage at 12 °C (Figs 3, 4, 5).

Motor innervation in P. scalare

The development of motor innervation occurred in a rostral to caudal direction, with the rostral somites being the first to express HNK-1 (Fig 8A) and AChE activity (Figs 8B, C).

HNK-1 was localised at the myosepts and at the roots of outgrowing motor axons (Fig 6). The motor roots grew toward the caudal myosepta of the somites in which they were observed (Fig 6). AChE activity was observed in motoneuron cell bodies. Each somite contained 3-4 primary motoneuron cell bodies within the spinal cord (Fig 7). AChE activity was also observed at the endplates, which were situated at the myosepts (Fig 7).

Fig 3. Transverse sections of shorthorn sculpin embryos at different stages of development, showing the appearance of the spinal cord (sc), notochord (nt), gut (g), dorsal aorta (da) and pronephros (p) at 4, 8 and 12 °C.

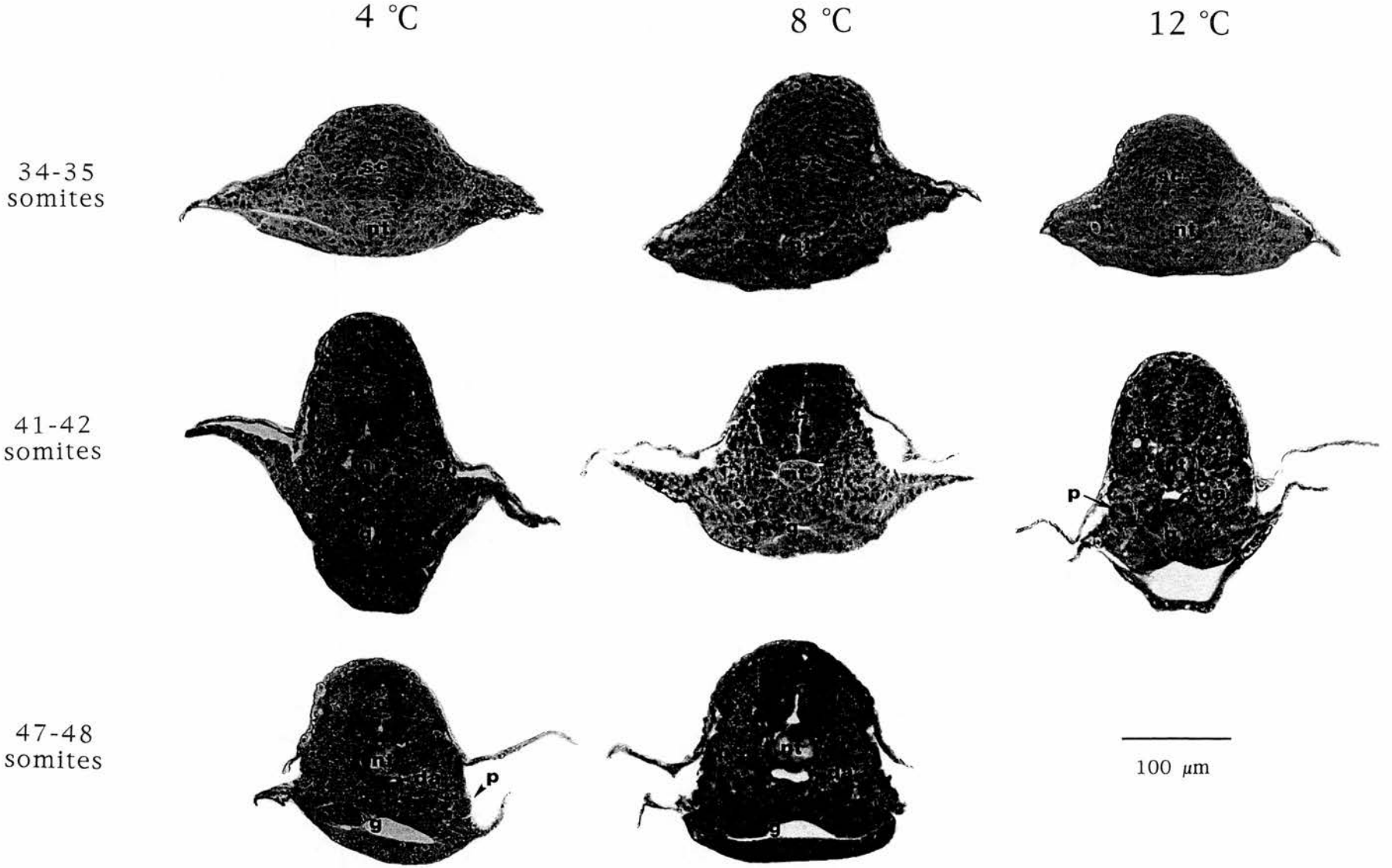

4 °C
8 °C
12 °C
34-35 somites
41-42 somites
47-48 somites
sc
nt
p
g
100 μm

Fig 4. Sagittal sections of shorthorn sculpin embryos, cut parallel to the dorsoventral axis and through the notochord (nt), showing the appearance of the eye (e) and heart (h) at 4, 8 and 12 °C.

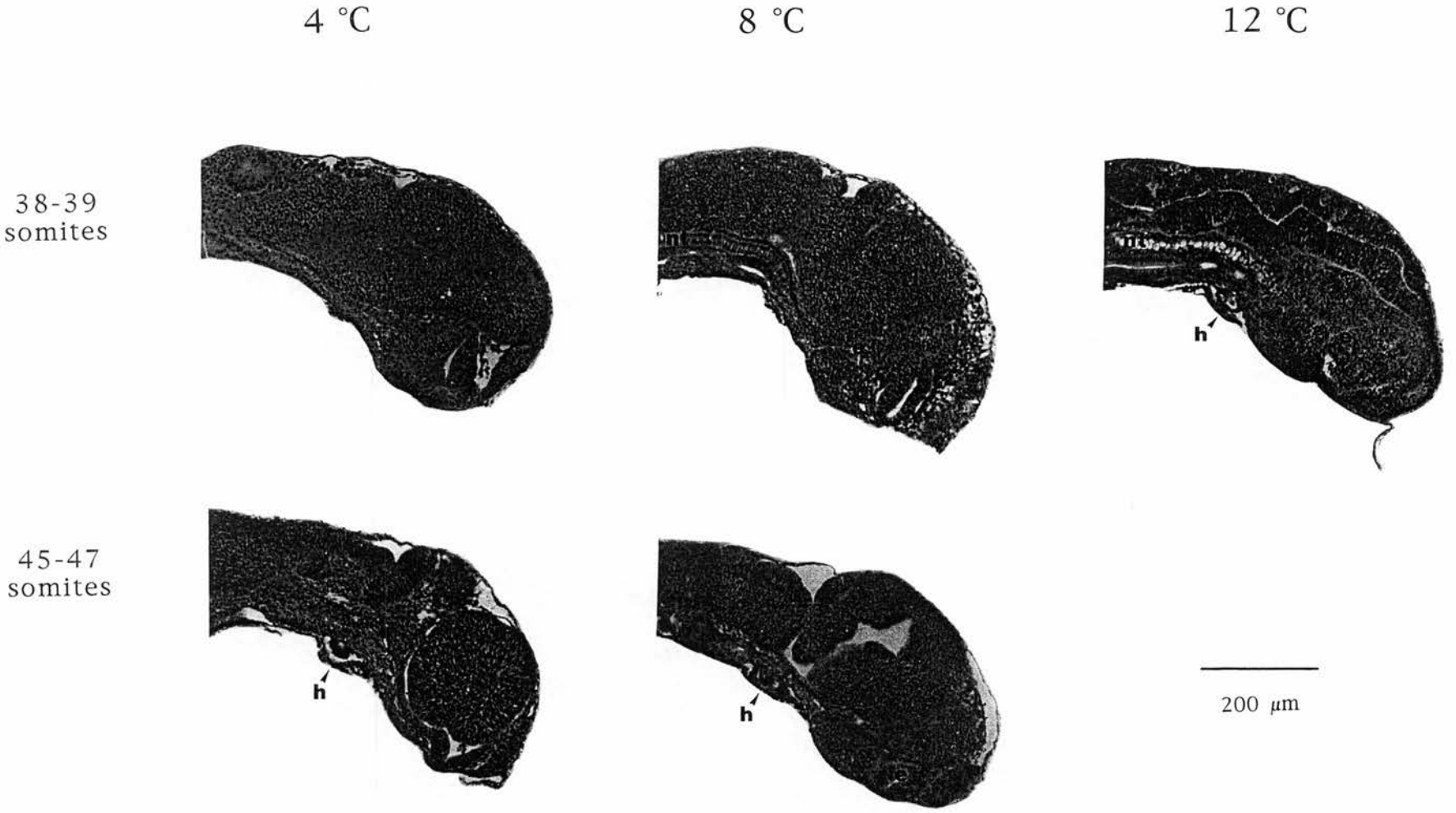

4 °C
8 °C
12 °C
38-39 somites
45-47 somites
h
h
h
200 μm

Fig 5. Relative timing of organogenesis in shorthorn sculpin embryos reared at 4 °C (blue), 8 °C (green) and 12 °C (red). Development was assessed by examining transverse and sagittal sections of embryos using light microscopy (see Figs 3 and 4). Values are mean ±SD, n=6 fish at each temperature, and $P<0.01$. Scale bars represent 0.5 mm.

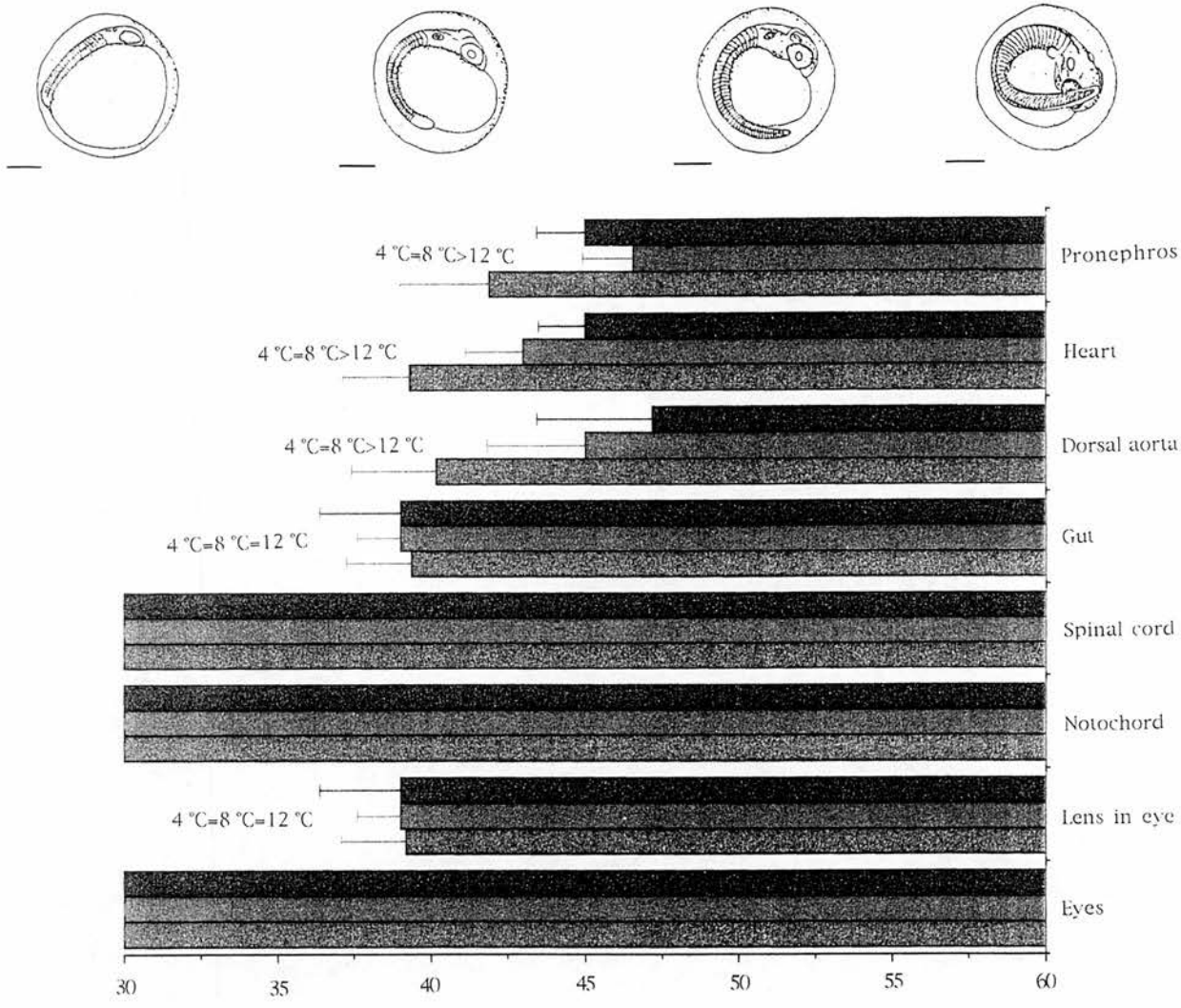

4 ℃=8 ℃>12 ℃
Pronephros
4 ℃=8 ℃>12 ℃
Heart
4 ℃=8 ℃>12 ℃
Dorsal aorta
4 ℃=8 ℃=12 ℃
Gut
Spinal cord
Notochord
4 ℃=8 ℃=12 ℃
Lens in eye
Eyes
30
35
40
45
50
55
60
Somite number at which organ first appears

Fig 6. Rostral somites of angelfish positive for HNK-1. HNK-1 was localised at the myosepts (ms) and at the motor roots (mr). Note that the motor root of each somite grows towards the caudal myoseptum. Scale bar represents 50 μm.

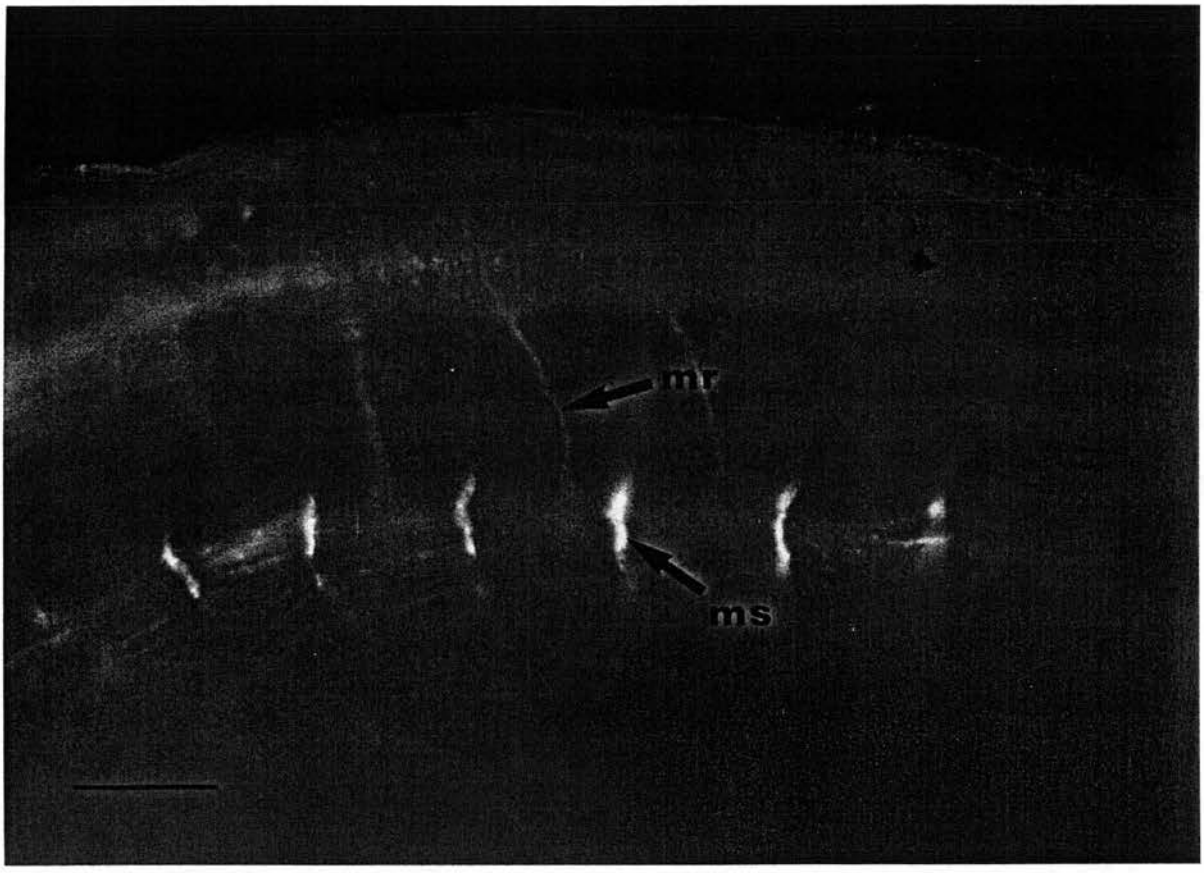

mr
ms

Fig 7. Rostral somites (with planes of focus at the spinal cord, A, and just beneath the skin, B) and caudal somites (C) of a newly hatched angelfish larva stained for AChE activity. cb: motoneuron cell body; myf: myofibrils; ms: myosept. Scale bars represent 200 μm.

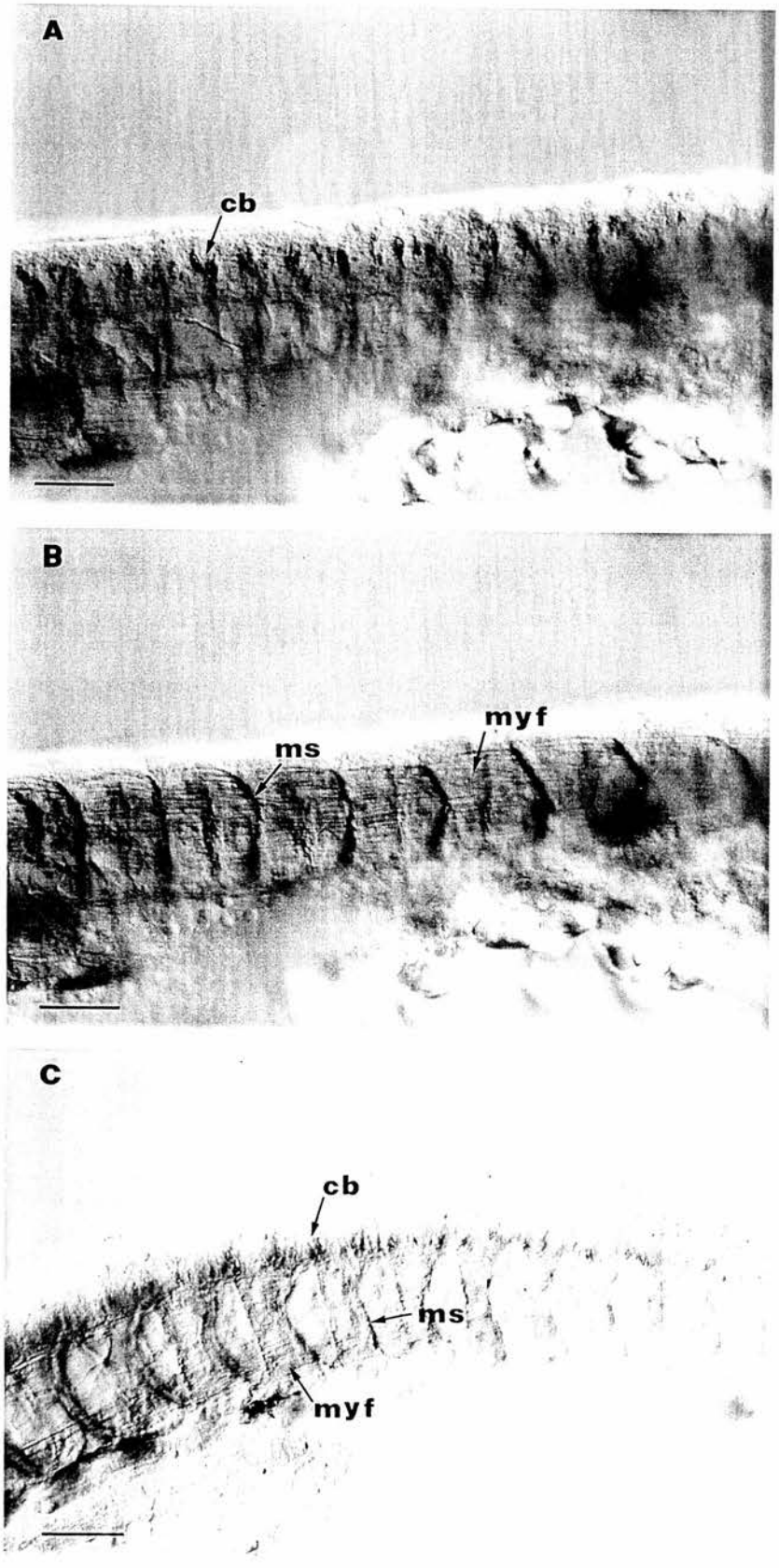
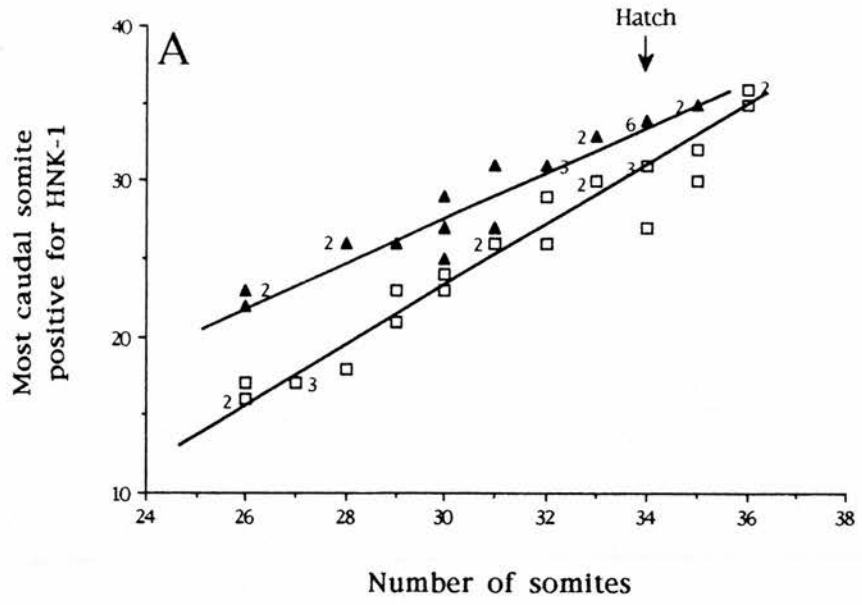
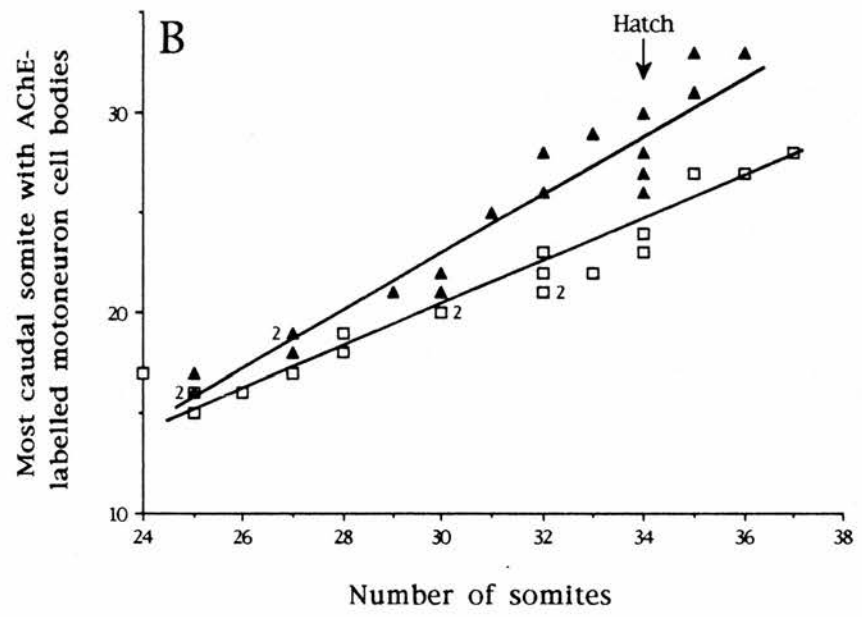
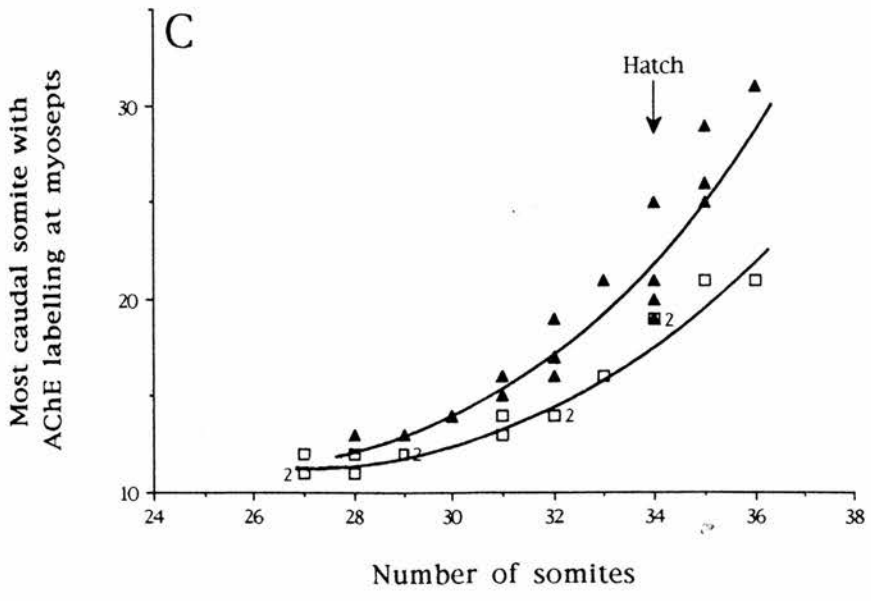

A
cb
B
myf
ms
C
cb
ms
myf

Fig 8. Expression of HNK-1 (A), and AChE activity in motoneuron cell bodies (B) and at myosepts (C), in angelfish embryos and larvae reared at 24 °C (open squares) or 31 °C (closed triangles). Each point gives the value for a single fish unless otherwise indicated.


A
Hatch
Most caudal somite positive for HNK-1
Number of somites
40
30
20
10
24
26
28
30
32
34
36
38


B
Hatch
Most caudal somite with AChE-labelled motoneuron cell bodies
Number of somites
30
20
10
24
26
28
30
32
34
36
38


C
Hatch
Most caudal somite with AChE labelling at myosepts
Number of somites
30
20
10
24
26
28
30
32
34
36
38

HNK-1 was the first marker of the development of axial muscle innervation to be expressed in the somites of angelfish embryos (Fig 8A). AChE activity was then observed in the cell bodies of motoneurons (Fig 8B), with AChE activity at the endplates occurring some time later (Fig 8C). For example, a 29-somite embryo at 31 °C contained about 25 somites positive for HNK-1 and about 21 somites with AChE activity in motoneuron cell bodies, but only about 13 somites with AChE activity at the endplates (Fig 8). Additionally, at hatching, 24 °C embryos contained about 28 somites positive for HNK-1 and about 23 somites with AChE activity in motoneuron cell bodies, but only about 18 somites with AChE activity at myosepts (Fig 8).

Development of motor innervation was positively correlated with rearing temperature in angelfish. The last somite positive for HNK-1 was more caudal in 31 °C angelfish than in 24 °C angelfish of equivalent stage (t=4.160, P<0.01) (Fig 8A). The same was also true for AChE activity in motoneuron cell bodies (t=4.866, P<0.01) (Fig 8B) and at endplates (t=2.089, P<0.02) (Fig 8C).

Muscle differentiation and myofibrillargenesis in P. scalare

Muscle differentiation and myofibrillargenesis occurred in a rostral to caudal direction in angelfish (Figs 9-11). At hatching, rostral somites contained muscle fibres with numerous myofibrils (Figs 9A, D), somites immediately posterior to the yolk sac contained myotubes with fewer myofibrils than rostral somites (Figs 9B, E), and caudal somites contained only myoblasts with no

Fig 9. Coronal sections of newly hatched angelfish larva, cut at the level of the notochord, and observed using light microscopy (A-C) or electron microscopy (D-F). A and D show rostral somites, B and E show somites immediately posterior to the yolk sac of newly hatched larva, and C and F show caudal somites. myf: myofibrils; mt1: mononucleated myotube; mt2: multinucleated myotube; mb: myoblast. Scale bars represent 50 μm on sections observed with light microscopy, 0.2 μm on electron micrographs, and 1 mm on drawing.

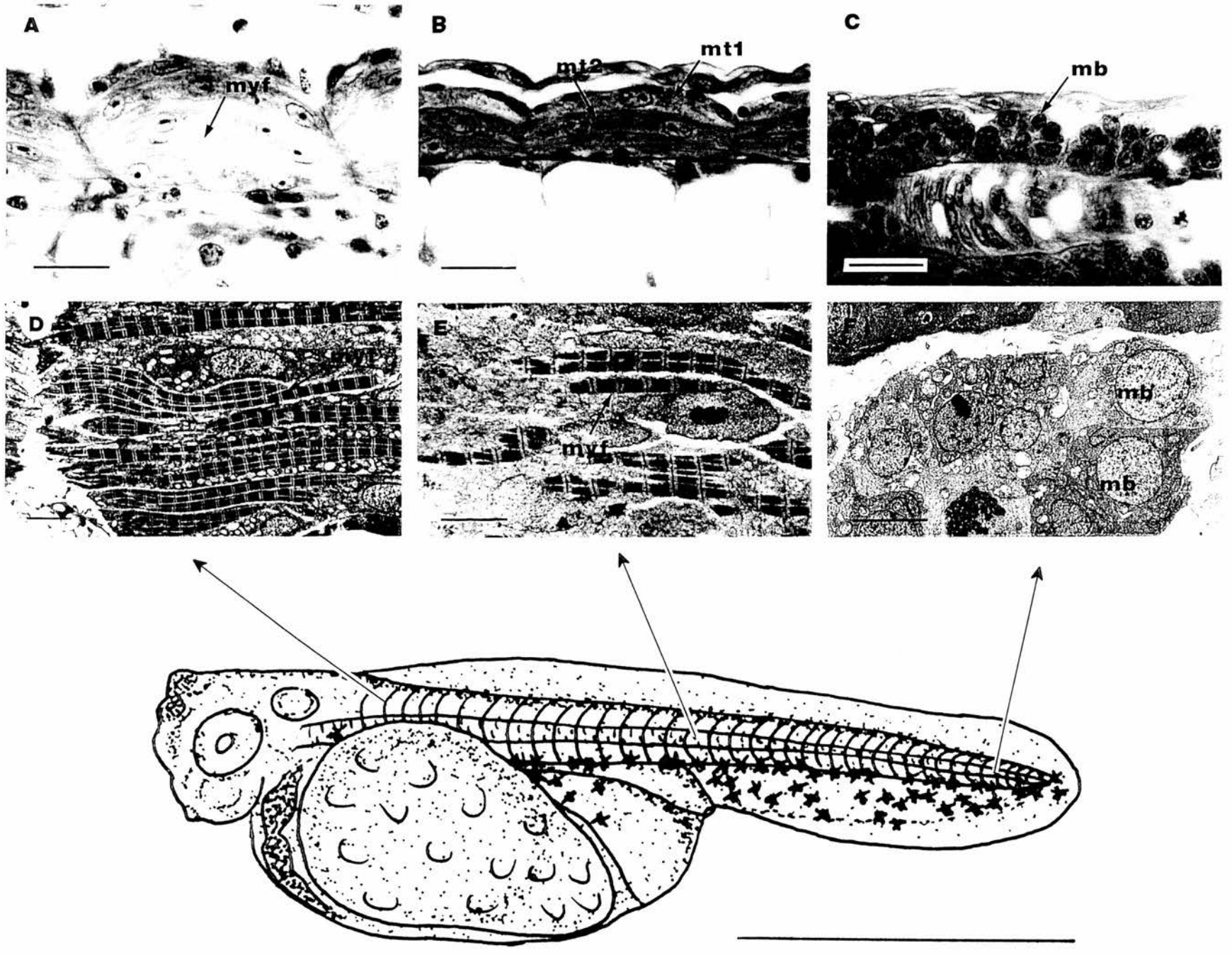

A
myf
B
mt2
mt1
C
mb
D
E
myf
F
mb
mb

Fig 10. Coronal sections of first swimming angelfish larva, cut at the level of the notochord, and observed using light microscopy. A: rostral somite; B: somite immediately posterior to the yolk sac; C: caudal somite. myf: myofibrils. Scale bars represent 50 μm on sections observed with light microscopy, and 1 mm on drawing.

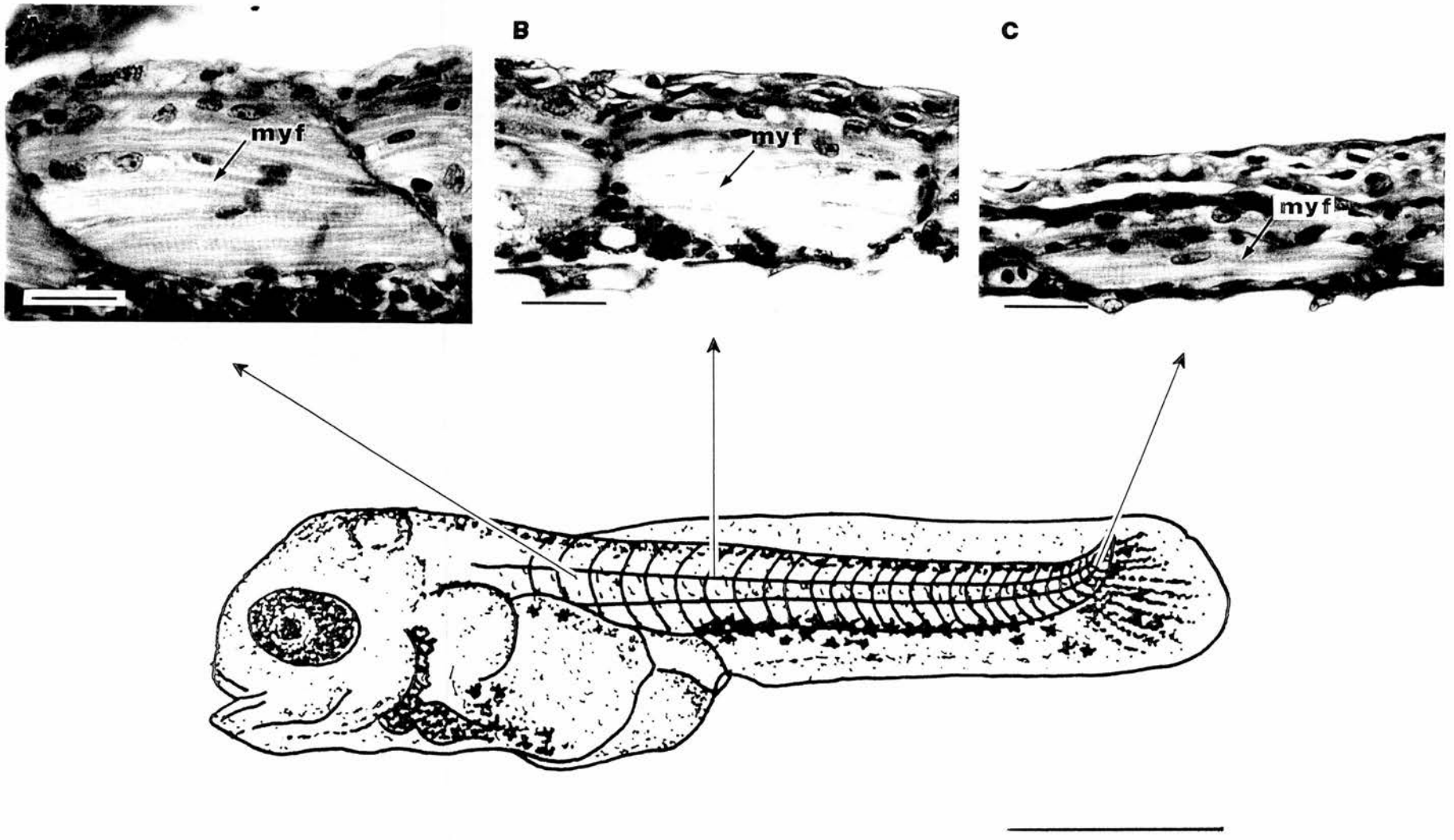
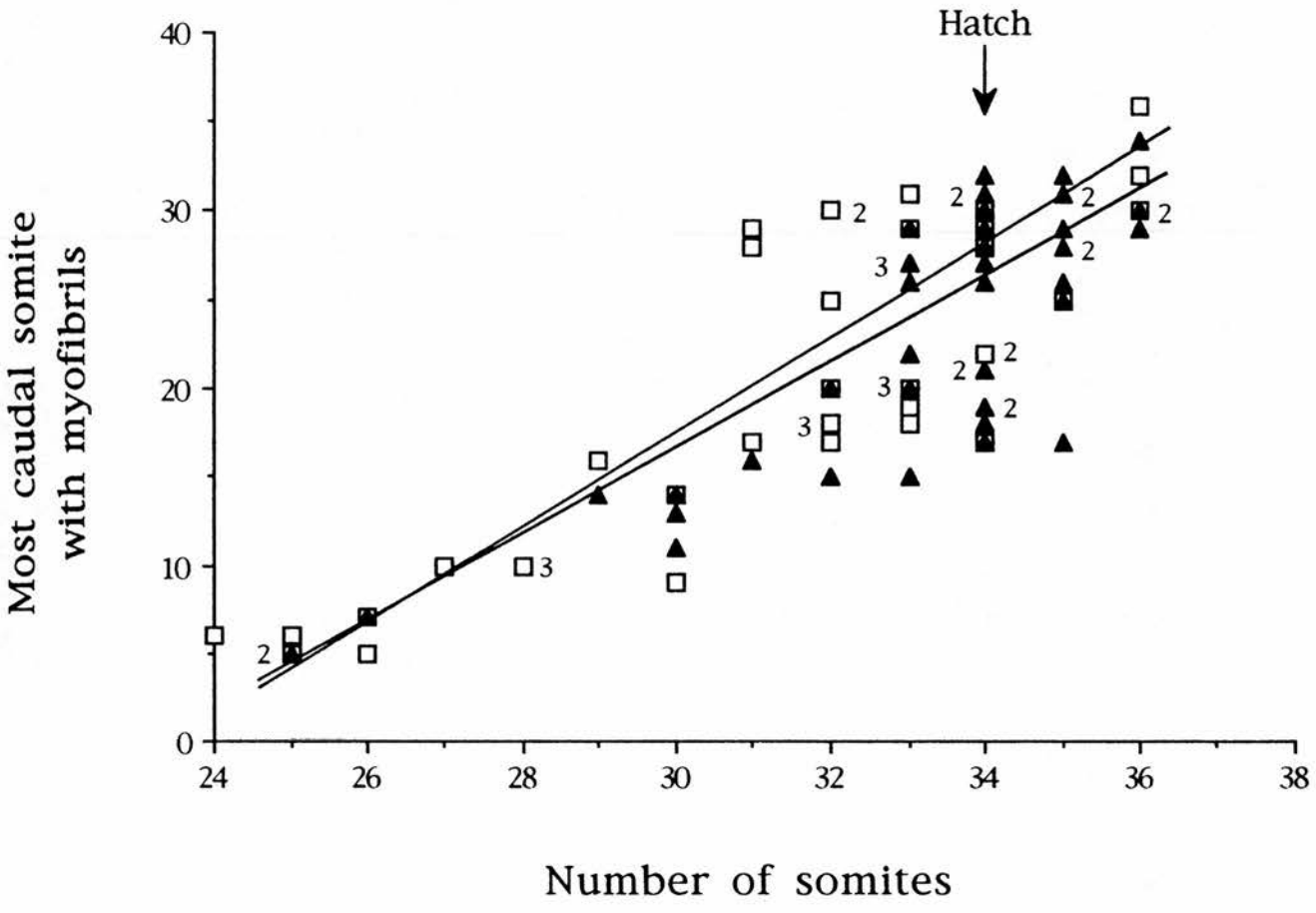

myf
B
myf
C
myf

Fig 11. Myofibrillargenesis in angelfish embryos and larvae reared at 24 °C (open squares) and 31 °C (closed triangles). The presence of myofibrils was using glycerol-cleared embryos/larvae and Nomarski differential interference contrast optics. Each point shows the value for a single fish unless otherwise indicated.


Hatch
Most caudal somite with myofibrils
Number of somites
0
10
20
30
40
24
26
28
30
32
34
36
38

myofibrils (Figs 9C, F). By first swimming, muscle fibres were present in all somites (Fig 10).

Myotubes which were superficially situated (i.e., just beneath the skin) were mononuclear (mt1 in Fig 9B) while those situated deeper in the somites were multinuclear (i.e., they contained 2 or more muscle nuclei) (mt2 in Fig 9B). Myofibrils were first evident at around the 24-somite stage (Fig 11). At hatch, 15-30 of the 34 somites contained well differentiated myofibrils (i.e., observable using Nomarski differential interference optics) (Fig 11).

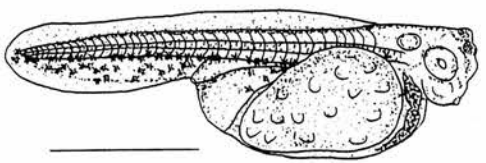
The relative timing of myofibrillargenesis in the angelfish was not affected by development temperature, with the most caudal somite containing myofibrils not being significantly different in 24 °C and 31 °C fish of equivalent somite stage (Fig 11) (t=0.850, P>0.10).

In newly hatched angelfish there were no distinct muscle fibre types, with all fibres remaining unstained for SDHase activity (Fig 12A). By first swimming, however, the somites immediately posterior to the yolk sac contained a single layer of superficial muscle fibres which stained moderately for SDHase activity overlying an inner mass of muscle fibres which remained unstained (Fig 12B).

First swimming angelfish at 24 °C contained more muscle fibres (697±23, n=16 fish) than those at 31 °C (651±22, n=16 fish) (t=5.740, P<0.01). Muscle fibres in first swimming, 31 °C angelfish

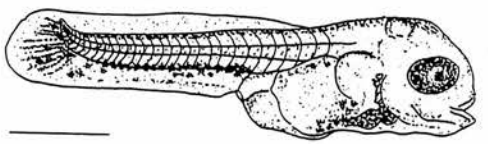
Fig 12. Transverse sections of newly hatched angelfish (A), first swimming angelfish (B), and newly hatched sculpin (C) stained for succinic dehydrogenase activity (SDHase). Sections were cut from the region immediately posterior to the yolk sac of fish. s: superficial muscle layer; i: inner muscle mass. Scale bars represent 50 μm on photomicrographs and 1 mm on drawings.

Newly hatched
angelfish larva

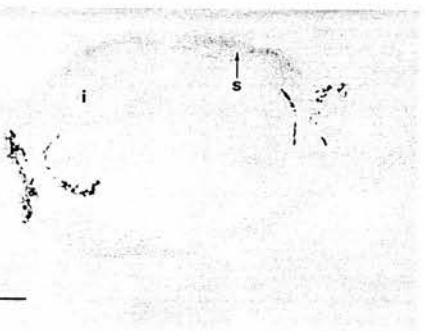


A

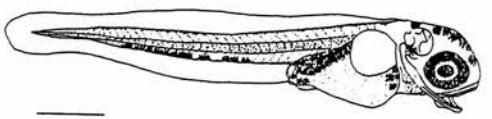
First swimming
angelfish larva



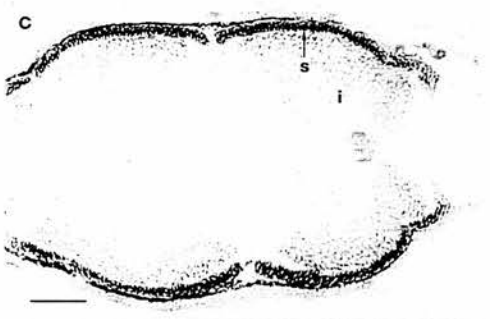
B



Newly hatched
sculpin larva



C



were, however, of greater mean cross-sectional area (43.1±3.2 μm^2, n=16) than those in their 24 °C counterparts (39.8±3.6 μm^2, n=16) (t=2.609, P<0.02). The total cross-sectional area of muscle was similar at both temperatures (about 0.028 mm^2) (t=0.283, P>0.10).

Muscle differentiation in M. scorpius

In newly hatched shorthorn sculpin, the somites immediately posterior to the anus contained 2 muscle fibre types. There was a single layer of fibres (superficial muscle), which stained intensely for SDHase activity, situated over an inner muscle mass which stained only lightly for this enzyme (Fig 12C).

Discussion

Organogenesis

An important finding of the present study was that changes in environmental temperature (of only a few degrees, and within the normal range of animals) can result in morphological variation in the early developmental stages of fish. Using somite stage as a reference point, temperature was found to affect the relative timing of the appearance of some morphological characters but not others. For example, the heart, dorsal aorta and pronephros were all absent in 41-42-somite sculpin embryos at 4 °C while being present in 12 °C embryos of equivalent somite stage. In contrast, the stage at which the gut and eye lens appeared (37-38 somites) was independent of temperature. In the angelfish, motor innervation occurred at earlier somite stages at 31 °C than at 24 °C, while the appearance of myofibrils was the same at both temperatures. The development of some organs thus proceeds in parallel within the natural temperature range of each species while the development of others become uncoupled with temperature change. These findings, and other cases of organogenesis being uncoupled with temperature (Hayes *et al*, 1953; Fukuhara, 1990, Johnston, 1993; Brooks and Johnston, 1993; Johnston *et al*, 1995), suggest that the formation of different organs have different thermal sensitivities.

The development of morphological structures is under the control of genes, particularly the homeobox-containing genes. For

example, in mice the homeobox-containing genes *Hlx* and *Csx* play key roles in the development of the gut (Hentsch *et al*, 1996) and heart (Komuro and Izumo, 1993), respectively. It is therefore tempting to speculate that differences in the relative timing of appearance of organs, at different temperatures, might have resulted from different effects of temperature on the transcription and/or translation of genes essential for their formation. Effects of temperature on gene transcription and translation have been demonstrated in fish. For example, the sequence of myofibrillar protein gene expression in herring embryos is influenced by temperature, with 1 d-old herring larvae reared at 5 °C containing a higher proportion of embryonic troponin T isoforms than 1 d-old larvae at 10 or 15 °C (Crockford and Johnston, 1993). Additionally, the expression of myosin heavy chain genes has been shown to be affected by acclimation temperature (Gauvry *et al*, 1996).

The genes responsible for the formation of different organs and tissues are also not expressed until the appropriate induction signals are received. The hedgehog family of proteins has been implicated as important signalling molecules in establishing cell positional information and tissue patterning (Stolow and Shi, 1995). For example, myogenesis in vertebrate embryos requires the production of sonic hedgehog (Shh) by the notochord and ventral regions of the neural tube (Molkentin and Olson, 1996). The production of Shh is itself regulated by thyroid hormones (Shi *et al*, 1996). Temperature could therefore have uncoupled the development of different organs by influencing embryonic thyroid hormone levels. An effect of temperature on endogenous thyroid hormone production has indeed been demonstrated in developing

fish (Inui and Miwa, 1985; Seikai *et al*, 1986). A given change in temperature could also have altered the levels of thyroid hormone receptors (in the nucleus), cytosolic thyroid hormone binding proteins (which transfer thyroid hormone molecules from the cell membranes to the receptors), and deiodinases (which transform thyroxine, the less active form of thyroid hormone, to triiodothyronine, the more active form) by different extents in different developing organs (see Shi *et al*, 1996).

Myogenesis

Myogenesis in the angelfish, as in all fish previously examined (see Johnston *et al*, 1996), occurred in a rostral to caudal direction: rostral somites of newly hatched fish contained muscle fibres with numerous myofibrils, the somites immediately posterior to the yolk sac contained myotubes with fewer myofibrils, and the most caudal somites contained only myoblasts with no myofibrils. In somites consisting solely of myotubes, the most superficial myotubes were mononuclear while inner myotubes were multinuclear (Fig 13C). This is similar to the situation in zebrafish (*Brachydanio rerio* Hamilton-Buchanan) (Raamsdonk *et al*, 1974), herring (Johnston *et al*, 1995) and tambaqui (Johnston and Vieira, 1996). It is, however, different from the situation in tadpoles of *Xenopus laevis* Daudin where all myotubes are mononuclear, with multinucleated myotubes occurring only after metamorphosis (Muntz, 1975).

In the zebrafish, herring and tambaqui, the first cells in the maturing somite that can be identified as muscle precursors lie as a column of large cuboidal cells adjacent and lateral to the notochord. These cells elongate, giving rise to the mononucleated myotubes which then migrate laterally through the somite to the midline region, superficial to the rest of the somite (Raamsdonk *et al*, 1974; Johnston *et al*, 1995). In zebrafish and herring these cells are fated to become at least a subset of the superficial muscle cells. This was probably also true for the angelfish: the mononucleated myotubes in newly hatched larvae was in the same position, with respect to the rest of the somite, as the SDHase-positive (presumptive red) muscle fibres of first swimming individuals. In the zebrafish, herring and tambaqui, the multinucleated myotubes, which gradually form the inner muscle fibres, are formed by the fusion of mononucleated myoblasts (Nag and Nursall, 1972; Schattenberg, 1973). These myoblasts were themselves formed by the proliferation of mononucleated embryonic mesenchymal cells (or presumptive myoblasts) (Hanneman, 1992; Johnston *et al*, 1995).

The myotubes of fish gradually mature into fully differentiated muscle fibres (Johnston *et al*, 1996; Johnston and Vieira, 1996). This contrasts with the situation in mammals in which myotubes formed early in development (primary myotubes) serve as a scaffold around which further myotubes (secondary myotubes) develop (Ontell and Kozeka, 1984). Shorthorn sculpin larvae at hatching contained two distinct muscle fibre types, with a monolayer of superficial (or presumptive red) muscle fibres overlying an inner (or presumptive white) muscle

mass. In fish, red fibres are used in sustained, slow speed swimming, while white fibres power bursts of higher speed (Bone, 1978). The presence of both fibre types in the myotomes of the sculpin was probably indicative of the use of axial muscle in both continuous and burst swimming in this species (Beddow *et al*, 1995). In contrast, the angelfish uses its pectoral fins during continuous swimming (i.e., diodontiform swimming), with axial muscle being used solely in burst swimming. This might have accounted for the poor development of distinct fibre types in this species, and the general anaerobic (SDHase-poor) nature of its axial muscle fibres.

Motor innervation

The timing of the development of motor innervation in fish has been shown to be influenced by temperature (see Johnston *et al*, 1996). In the angelfish, staining with antibodies against HNK-1 revealed, not only that the motor root of each somite runs along its caudal myoseptum (similar to the situation in herring; Johnston *et al*, 1996), but also that the outgrowth of these motor roots is retarded at low temperature. This suggests that at low temperature, the signal(s) which cause the motoneuron cell bodies to send out axons to the periphery (see Grinnell, 1995) are delayed compared to the high temperature.

Acetylcholinesterase activity consistent with functional endplates was concentrated at the myosepta indicating that axial muscle fibres of angelfish are focally, rather than multiply,

innervated (Bone, 1978). The formation of functional endplates was retarded at low temperature, occurring at later somite stages in 24 °C angelfish than in their 31 °C counterparts. Temperature, however, had no effect on myofibrillargenesis in the angelfish suggesting that temperature changes can uncouple motoneuron development and myogenesis with respect to the formation of the somites. The same was also found in the herring, where the formation of myotubes was independent of temperature while the outgrowth of motor axons was accelerated as temperature increased (Johnston *et al*, 1996).

Embryonic growth

At first swimming, angelfish reared at 24 °C were significantly longer than those reared at 31 °C. Shorthorn sculpin embryos at 4 °C were also of greater TL than equivalent embryos at 8 and 12 °C. These results imply that temperature can influence the efficiency at which the nutrient supplies of the yolk are converted to body tissue in embryonic fish. Support for this hypothesis comes from work in the Walleye pollock, *Theragra chalcogramma* Pallas in which embryos incubated at low temperature (6 °C) were found to have the same RNA/DNA ratios as those incubated at high temperature (12 °C) despite having lower rates of yolk utilisation (Canino, 1994).

Changes in the somatic growth of fish are also normally accompanied by changes in the mechanisms of muscle growth, with fast-growing fish usually depending more on hyperplasia

(the formation of new muscle fibres) than on hypertrophy (the increase in size of already existing muscle fibres) (Weatherley *et al*, 1988; Weatherley, 1990). Indeed, first swimming, 24 °C angelfish were found to have more muscle fibres, of a smaller average size, than first swimming angelfish at 31 °C. Such differences in the muscle cellularities of fish with different early thermal experiences may also reflect different thermal dependencies of myogenic cells to either enter proliferation or differentiation pathways. *In vitro* studies with the common carp (*Cyprinus carpio* L.) have shown that hypertrophy relies more on proliferating myogenic cells while hyperplasia is more dependent on differentiating myogenic cells (Koumans *et al*, 1993, 1994).

Concluding remarks

Morphological variation with temperature change, similar to that outlined above for the sculpin and the angelfish, has been shown to affect the survival of the early developmental stages of fish. For example, embryonic development temperature in the Atlantic cod (*Gadus morhua* L.) influences the formation of structures essential to feeding, respiration, locomotion, and thus the survival of the larval stage (Vonherbing *et al*, 1996). This might have also been true for the animals in our study. For example, between hatching and first swimming, angelfish at 24 °C had fewer somites with functional endplates/motor innervation than newly hatched individuals at 31 °C. This might have affected the twitching behaviour of pre-swimming larvae, a behaviour which enhances oxygen uptake by, and thus survival of, the

animals (Yoshida *et al*, 1996). The highest mortalities, during the life-history of fish, are found in the early developmental stages (as much as 20 % per d; Heath and MacLachlan, 1987; McGurk, 1984), and any factors that increase the survival rates of these stages would greatly enhance recruitment to the adult population. Our findings may therefore be important from the viewpoint of fisheries biology and in commercial fish production.

Chapter 3

Muscle growth in larval shorthorn sculpin, *Myoxocephalus scorpius*

Introduction

Skeletal muscle fibres can be divided into a number of categories, or fibre types, upon consideration of their contractile, (immuno)histochemical, morphological and mechanical properties (Bone, 1978). Fish contain two main muscle fibre types: superficially situated red, or slow oxidative, fibres and an inner mass of white, or fast glycolytic, fibres (Andersen *et al*, 1963; Greer-Walker and Pull, 1975; Johnston and Altringham, 1991). Many fish also contain a third muscle fibre type (i.e., pink, or fast oxidative glycolytic, fibres), the properties of which are intermediate between those of red and white fibres (Flood and Storm-Mathisen, 1962; Johnston et al, 1977; Korneliussen *et al*, 1978). The different muscle fibre types are used to power different modes of swimming. For example, red fibres are used by fish for sustained, slow-speed swimming, while white fibres are used for bursts of higher speed (Webb, 1975; Bone, 1978).

Muscle growth occurs by an increase in fibre numbers (hyperplasia) and by the increase in size of already existing fibres (hypertrophy). In most mammalian and avian muscles,

hyperplasia is restricted to the pre- and peri-natal period (Goldspink, 1972, 1974). Postnatal growth in these animals therefore results mainly from hypertrophic growth of existing fibres. In contrast, hyperplasia continues in teleosts, together with hypertrophic growth, well into the adult stage (Stickland, 1983; Weatherley *et al*, 1988). The relative importance of hyperplasia is, however, maximal in young fish and decreases with increasing body size (Weatherley, 1990). This decrease in hyperplasia is an important factor in defining the ultimate size of fish. For example, in ten investigated species, Weatherley *et al* (1988) found a strong relationship between the length of fish at which hyperplasia stops and the maximum reported length for that species (see also Battram and Johnston, 1991).

Investigations of muscle growth in fish must take temperature into account. Within a large part of the thermal tolerance polygon of a species (see Elliott, 1981, 1994), an increase in rearing temperature results in an increase in growth rate (Weatherley and Gill, 1987; Elliott, 1994). The effect of rearing temperature on the mechanisms of muscle growth in fish has, however, only been studied in a few species. These investigations have also produced varied results. For example, whitefish (*Coregonus lavaretus* L.) larvae reared at high temperatures were found to use hypertrophy to a greater extent in muscle growth than those reared at low temperatures (Hanel *et al*, 1996). In contrast, the contribution of hyperplasia to muscle growth in the rainbow trout (*Salmo gairdneri* L.) and Atlantic salmon (*Salmo salar* L.) was positively correlated to rearing temperature (Weatherley *et al*, 1979; Higgins and Thorpe, 1990).

Another important aspect of muscle growth is the behaviour of myosatellite cells. Myosatellites are situated between the sarcolemma and basal lamina of muscle fibres (Mauro, 1961), and either fuse to form new muscle fibres during hyperplasia or are incorporated into hypertrophying fibres (Campion, 1984). *In vitro* studies on the common carp (*Cyprinus carpio* L.) suggest that hyperplasia might depend more on differentiating myosatellites while and hypertrophy depends more on myosatellites that have entered proliferation pathways (Koumans *et al*, 1993). Koumans *et al* (1993) not only measured muscle fibre numbers and sizes in growing carp, but simultaneously isolated and cultured myosatellite cells from the fish. The decrease in hyperplasia and increase in hypertrophy, which occurred *in vivo* as the carp grew, was found to be correlated with *in vitro* decreases in the number of myosatellites which expressed desmin (a muscle-specific protein and marker of myosatellite differentiation) and an increase in the number of cultured myosatellites that incorporated 5-bromo-2'-deoxyuridine (BrdU, a marker of cell proliferation which is incorporated into replicating DNA) (Koumans *et al*, 1993). Direct translation of the *in vitro* results to the *in vivo* situation is not possible, however, since isolation and culturing are known to stimulate a regeneration type reaction in myosatellite cells (Koumans *et al*, 1993).

We therefore quantified hyperplasia to hypertrophy during growth in shorthorn sculpin (*Myoxocephalus scorpius* L.) larvae reared at several temperatures within the natural range of the species (see Beddow *et al*, 1995). Changes in the relative

contributions of the different growth mechanisms were also related to *in vivo* changes in myosatellite cell proliferation.

Fish

Adult shorthorn sculpin, *Myoxocephalus scorpius* L., were caught in St Andrews Bay during January 1994 and 1996, then maintained under ambient conditions of temperature and photoperiod at the Gatty Marine Laboratory. Eggs and milt were stripped from ripe individuals. In 1994 five females (122-444 g) and two males (158 and 166 g) were used, while six females (129-229 g) and one male (182 g) were stripped in 1996. Fertilised eggs were transferred to tanks of filtered, aerated seawater (24 h artificial illumination). Eggs were held at 5 °C and 8 °C until hatching during 1994 and 1996, respectively. Larvae were reared at 5 °C and 10 °C during 1994, and at 8 °C and 12 °C during 1996. All temperatures had a range of ±0.5 °C, and larvae were fed an *ad libitum* diet of 1-4 d old brine shrimp (*Artemia* sp) nauplii.

Morphological measurements and larval settlement

Total length (TL), snout to anus length, body depth at pectoral fin, and interorbital distance were measured using a Leica Wild M3C stereo microscope (Leica Instruments GmbH, Germany) and a combination of stage and eye-piece micrometers (Graticules Ltd, Kent).

During 1994, the settlement rate of 5 °C larvae was compared with that of 10 °C larvae. Settled larvae were defined as those which remained on the tank bottom for at least 5 s during a 5 min observation period (Gadomski and Caddell, 1991). Since larvae need not have been completely benthic, but only at the tank bottom for a certain period to be considered settled, the values obtained here were not absolute measures of larval settlement. They did, however, permit the abilities of different groups of larvae to maintain a pelagic existence, by continuous swimming, to be compared (Gadomski and Caddell, 1991).

Histochemistry

Larvae were killed in a 1:5000 (w/v) solution of bicarbonate-buffered MS222 anaesthetic (3-aminobenzoic acid ethyl ester) in seawater. Individuals were mounted on cryostat chucks in embedding medium (Cryomatrix; Shandon Products, USA) and rapidly frozen in 2-methyl butane cooled to near its freezing point (-159 °C) in liquid nitrogen. Blocks were equilibrated at -20 °C in a cryostat (Leica Instruments GmbH), and serial, 10 μm-thick, sections were cut from the somite immediately posterior to the anus of each fish. Sections were then stained for myofibrillar adenosine triphosphatase activity (mATPase) at pHs 10.2 and 10.6 (Johnston *et al*, 1975). Stained sections were traced with the aid of a microscope drawing arm and muscle fibre numbers and cross-sectional areas were determined using a Video-Plan image analysis system (Kontron Elektronik, Switzerland). In 1994, only fibres in the left, hypaxial

quadrant of the myotome were counted, but in 1996 all muscle fibres were measured.

Cell proliferation

Cell proliferation was investigated by the incorporation of BrdU into replicating DNA, and its subsequent localisation using mouse anti-BrdU monoclonal antibody contained in a commercial kit (Amersham International, Dorset). The cell labelling solution contained BrdU and 5-fluoro-2'-deoxyuridine in a 10:1 molar ratio. The latter compound inhibits thymidilate synthetase and increases BrdU incorporation by lowering competition from endogenous thymidine.

Larvae were incubated for 4, 8 or 24 h in the labelling solution (2 % v/v in seawater) and sacrificed by over-anaesthesia as above. BrdU-labelled fish and controls were fixed in Bouin's fluid and embedded in wax. Two serial sections, 7 μm-thick, were cut from the somite immediately posterior to the anus of each fish. The first section was stained with haematoxylin-eosin, and all muscle nuclei were counted. DNA-incorporated BrdU was detected in the second section with the mouse anti-BrdU primary antibody which was, in turn, detected with peroxidase conjugated goat anti-mouse secondary antibody. Peroxidase activity was revealed using diaminobenzidine in the presence of cobalt and nickel. This resulted in black staining at sites of BrdU incorporation.

Statistical analysis

Normal quantile plots revealed that all data were normally distributed (either originally or following logarithmic transformation). Results from the different temperatures were therefore compared using a *t*-test. All data are presented as means ±SD.

Morphological measurements and larval settlement

Growth rates of larvae reared, during 1994, at 10 °C were greater than those of larvae at 5 °C (t=16.042, P<0.01) (Fig 1). Growth rate was also positively correlated with rearing temperature during 1996, with 12 °C fish being of greater TL than 8 °C fish after equivalent times at the respective temperatures (t=3.957, P<0.01) (Fig 1). Growth rates of larvae reared at 12 °C during 1996 were not significantly different from those of larvae reared at 10 °C during 1994 (t=0.533, P>0.10) (Fig 1).

The shape of the body changed as larvae grew (see Fig 4), with the snout to anus length, body depth at pectoral fin and interorbital distance all increasing when expressed as a percentage of TL (Fig 2). Values for snout to anus length, body depth at pectoral fin and interorbital distance in 5 °C larvae were not significantly different from those in 10 °C larvae of equivalent TL (t=0.587-0.712, P>0.10) (Fig 2).

The relationship between settlement rate and TL is illustrated in Fig 3. The settlement rates of 5 °C fish were not significantly different from those of 10 °C fish of equivalent size (t=0.799, P>0.10) (Fig 3). Newly hatched larvae were highly active with low percentage settlement values (Fig 3). Percentage settlements increased with larval growth, reaching 100 % when 10 °C larvae were 15.2±0.6 mm TL (n=8 fish) (Fig 3). Fish became

Fig 1. Somatic growth of shorthorn sculpin larvae reared at 5 °C (closed squares) and 10 °C (open squares) during 1994, and at 8 °C (closed triangles) and 12 °C (open triangles). Values are mean ±SD (n=6-10 fish and 4-6 fish at each temperature during 1994 and 1996, respectively).

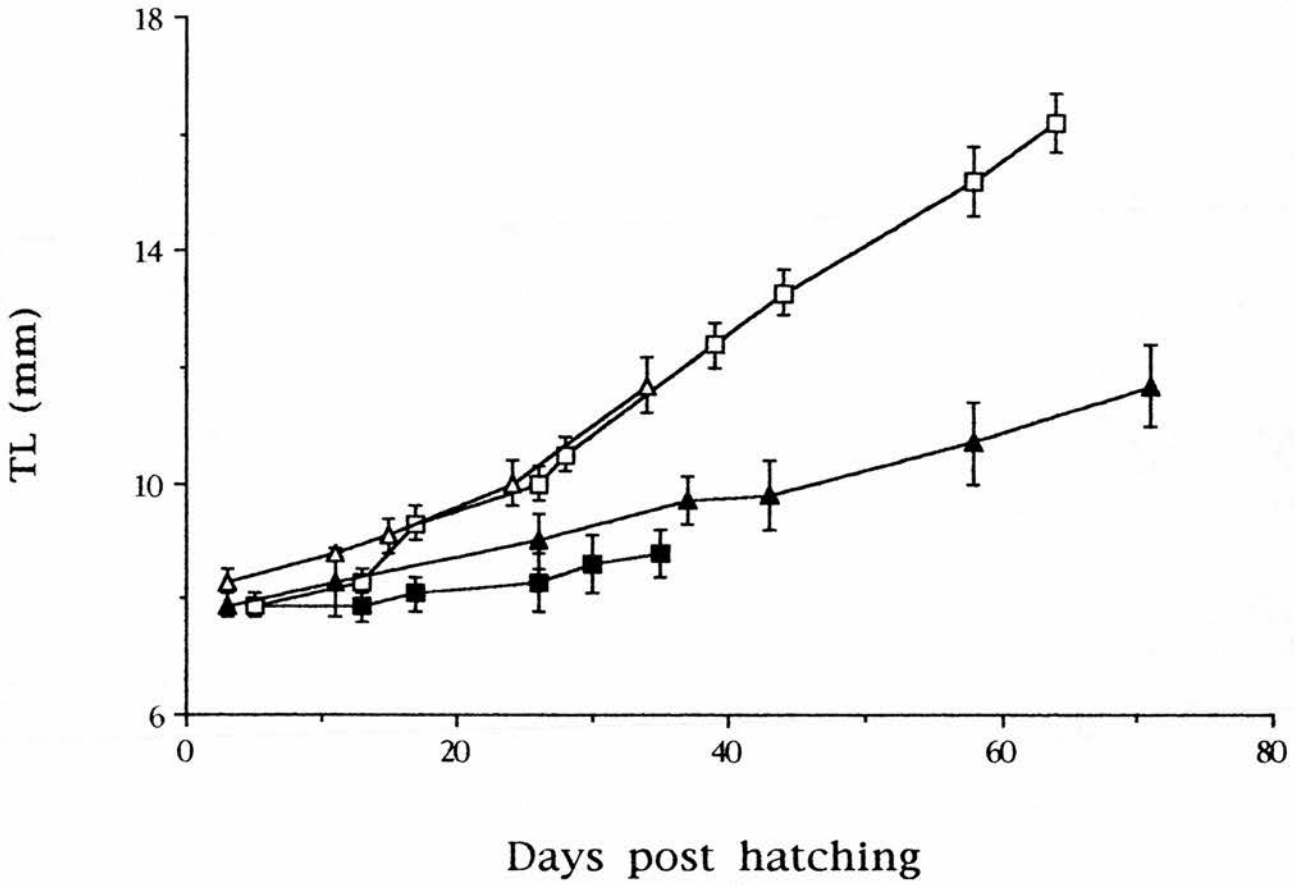
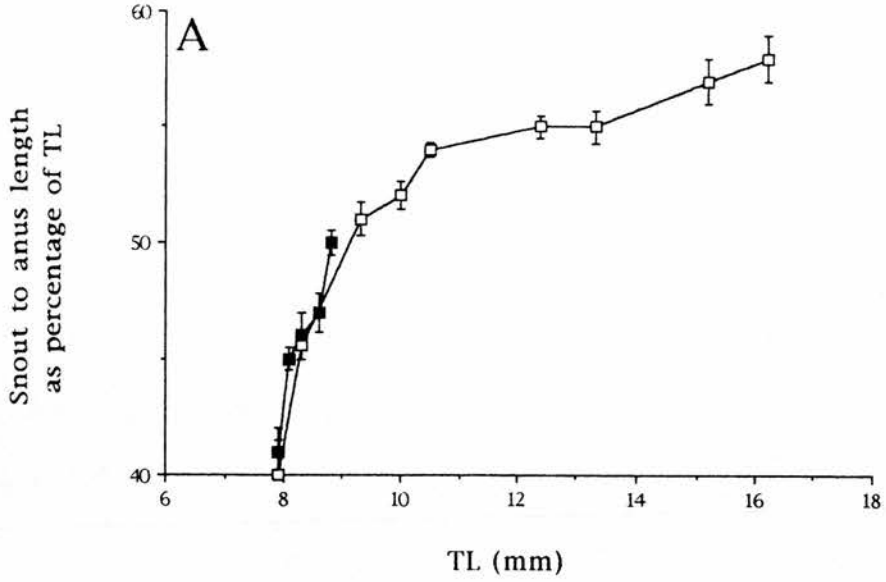
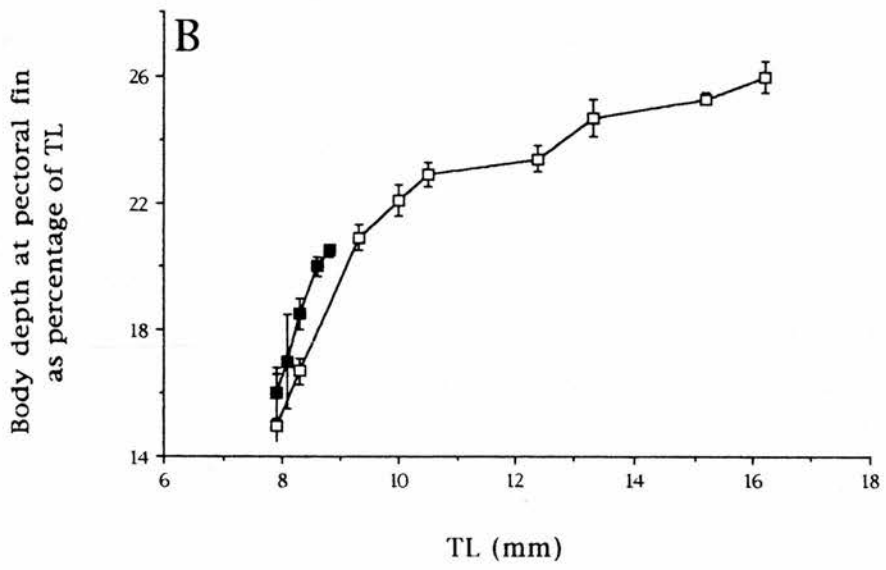

18
14
10
6
TL (mm)
0
20
40
60
80
Days post hatching

Fig 2. Growth of different regions of the body of larval shorthorn sculpin reared at 5 °C (closed squares) and 10 °C (open squares) during 1994, in relation to TL. Values are mean ±SD (n=6-10 fish at each temperature).


A
Snout to anus length as percentage of TL
60
50
40
6
8
10
12
14
16
18
TL (mm)


B
Body depth at pectoral fin as percentage of TL
26
22
18
14
6
8
10
12
14
16
18
TL (mm)

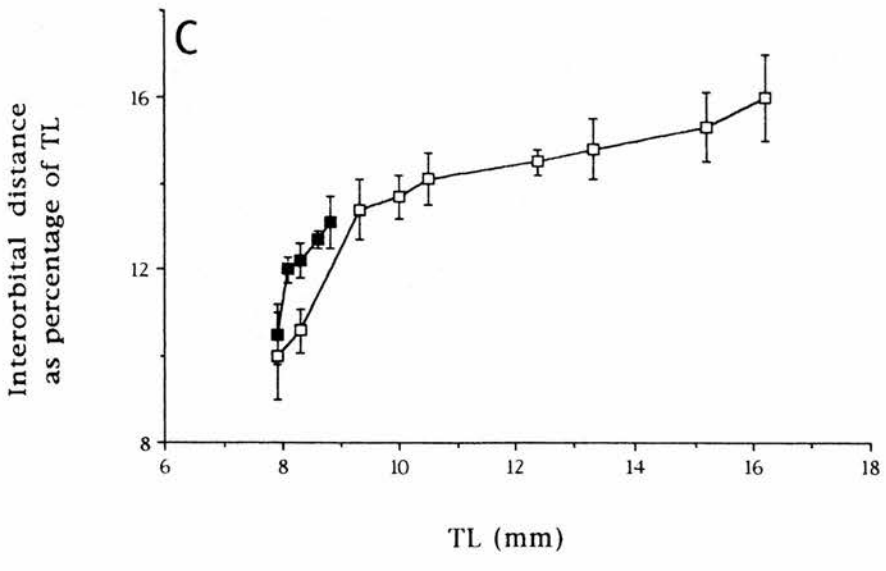
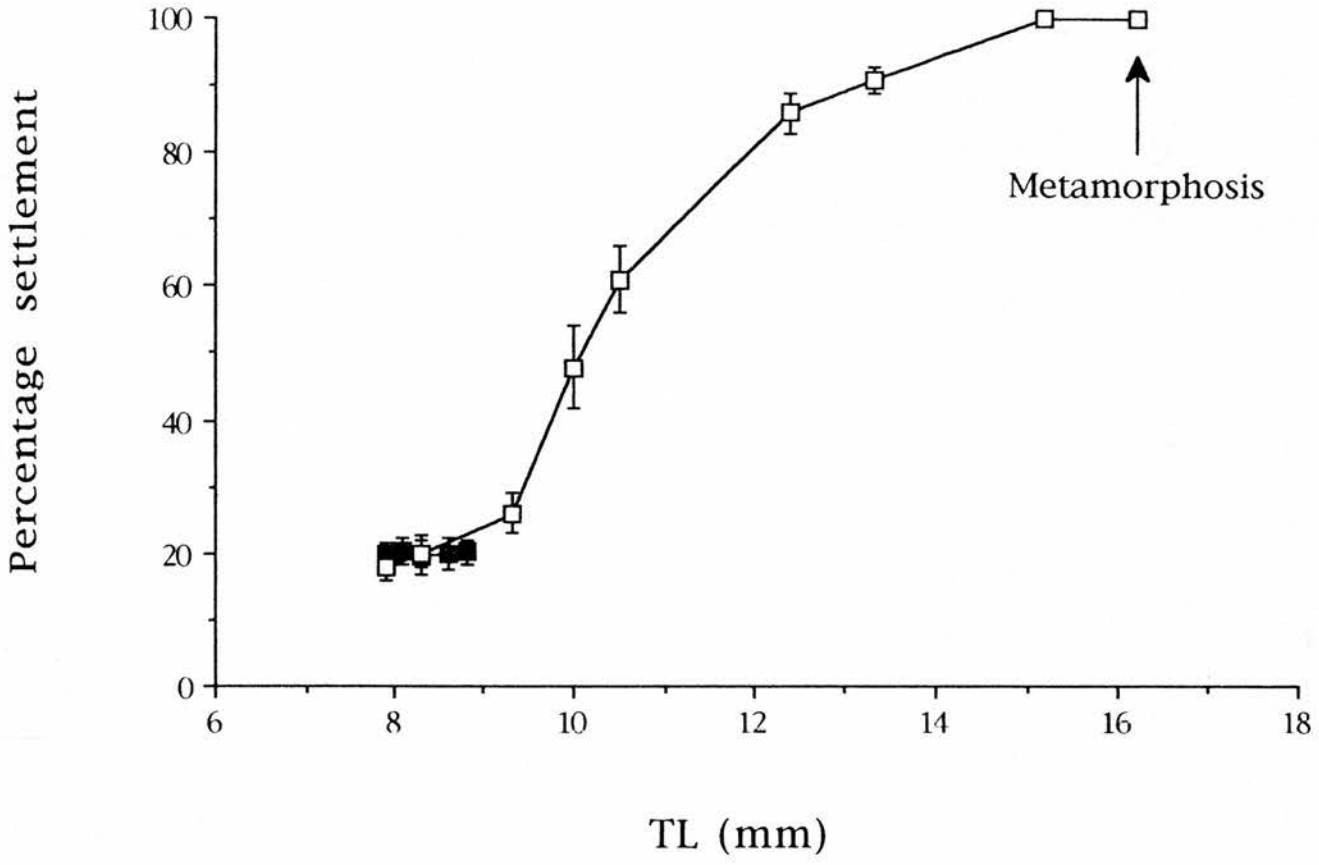

C
Interorbital distance as percentage of TL
16
12
8
6
8
10
12
14
16
18
TL (mm)

Fig 3. Settlement rates of larvae reared at 5 °C (closed squares) and 10 °C (open squares) during 1994. Observations were made on 8-15 randomly selected fish, from each temperature.


Percentage settlement
100
80
60
40
20
0
6
8
10
12
14
16
18
Metamorphosis
TL (mm)

truly benthic (i.e., at tank bottom for the entire observation period) at metamorphosis. Metamorphosis occurred 64 d after hatching, at 16.2±0.5 mm TL (n=10 fish), in fish reared at 10 °C during 1994 (Fig 4). The time to, and body size at, metamorphosis could not be determined for the 5 °C group of 1994 since all fish died shortly after yolk sac absorption (8.6-8.8 mm TL).

Fibre types and muscle growth

The histochemical properties of the muscle fibres of shorthorn sculpin larvae and juveniles are summarised in Table 1). The myotomes of newly hatched larvae contained a single layer of superficial muscle fibres which stained intensely for mATPase at pHs 10.2 and 10.6 (Figs 4A, B). Beneath these superficial fibres was an inner mass of fibres which stained intensely for mATPase at pH 10.2 (Fig 4A), but lightly at pH 10.6 (Fig 4B). The histochemical properties of inner muscle changed during larval development: at metamorphosis, inner fibres stained lightly for mATPase not only at pH 10.6 but also at pH 10.2 (Figs 4C, D). During metamorphosis a third muscle fibre type also appeared between the superficial muscle layer and the inner muscle mass (Figs 4C, D). These intermediate fibres stained intensely for mATPase at pH 10.2 (Fig 4C), but lightly at pH 10.6 (Fig 4D).

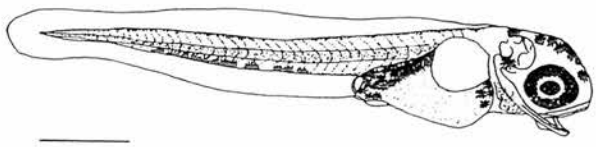
The muscle cellularities of larvae reared during 1994 were different at the two rearing temperatures. 5 °C larvae had larger

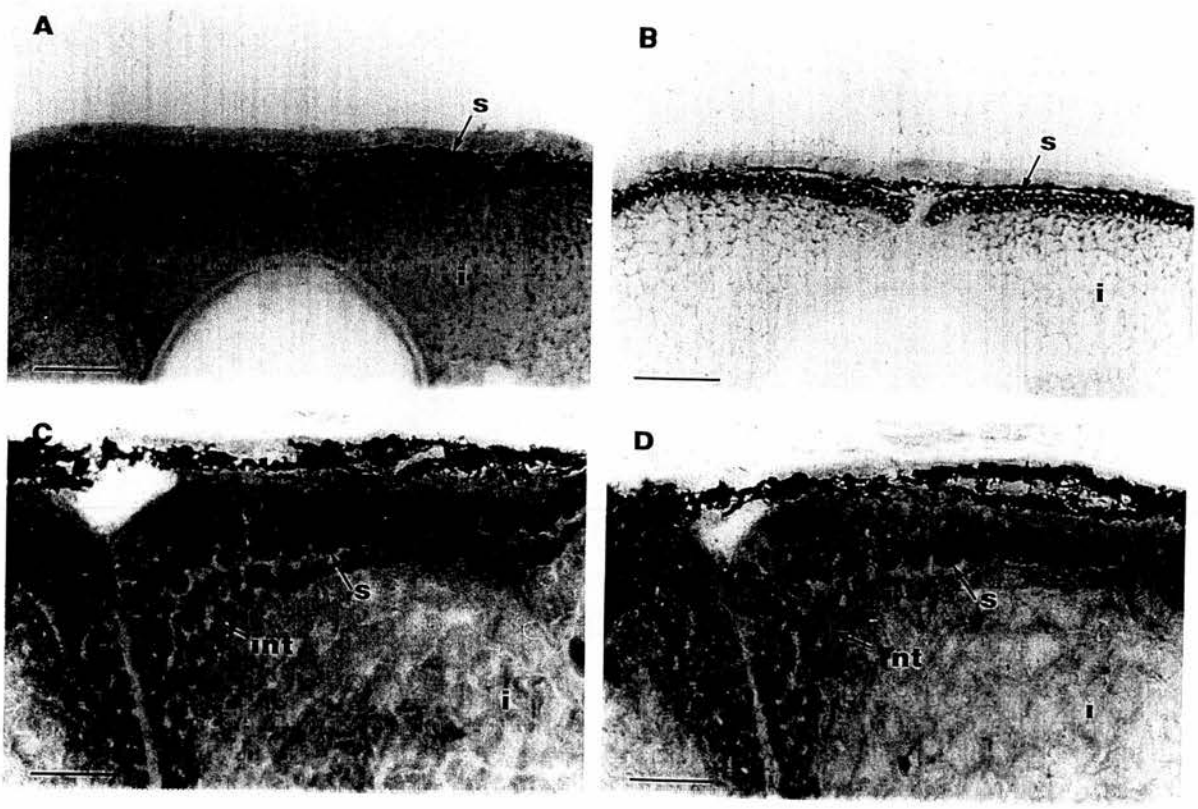
Table 1. Histochemical properties of superficial, inner and intermediate muscle fibres, for mATPase activity at pHs 10.2 and 10.6, in newly hatched and juvenile sculpin. NP: muscle fibre type not present.

Stage	Muscle fibre type	Staining for mATPase activity	
		at pH 10.2	at pH 10.6
Newly hatched	Superficial	Intense	Intense
	Inner	Intense	Light
	Intermediate	NP	NP
Metamorphosis	Superficial	Intense	Intense
	Inner	Light	Light
	Intermediate	Intense	Light

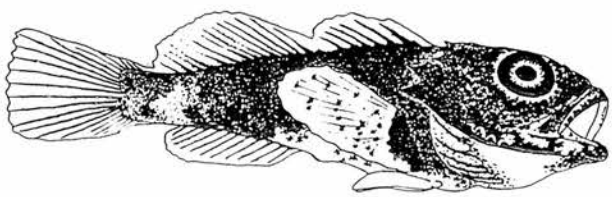
Fig 4. Appearance of fish at hatching and metamorphosis, and histochemical properties of the muscle at these stages. A: newly hatched larva stained for mATPase at pH 10.2. B: newly hatched larva stained for mATPase at pH 10.6. C: fish at metamorphosis stained for mATPase at pH 10.2. D: fish at metamorphosis stained for mATPase at pH 10.6. (s: superficial muscle fibres; i: inner muscle fibres; int: intermediate muscle fibres.) Scale bars represent 1 mm and 50 μm on drawings and photomicrographs, respectively.

Newly hatched larva





Juvenile (just after metamorphosis)

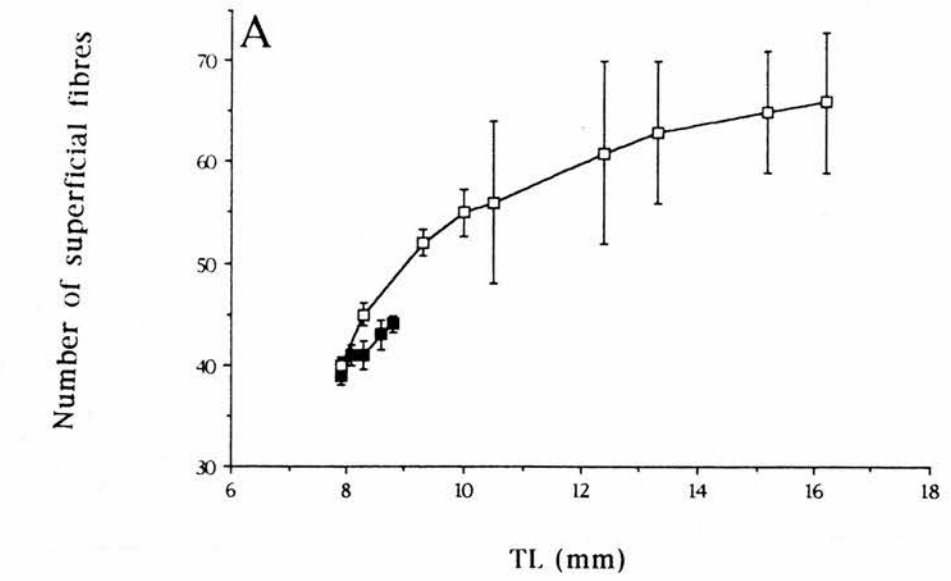


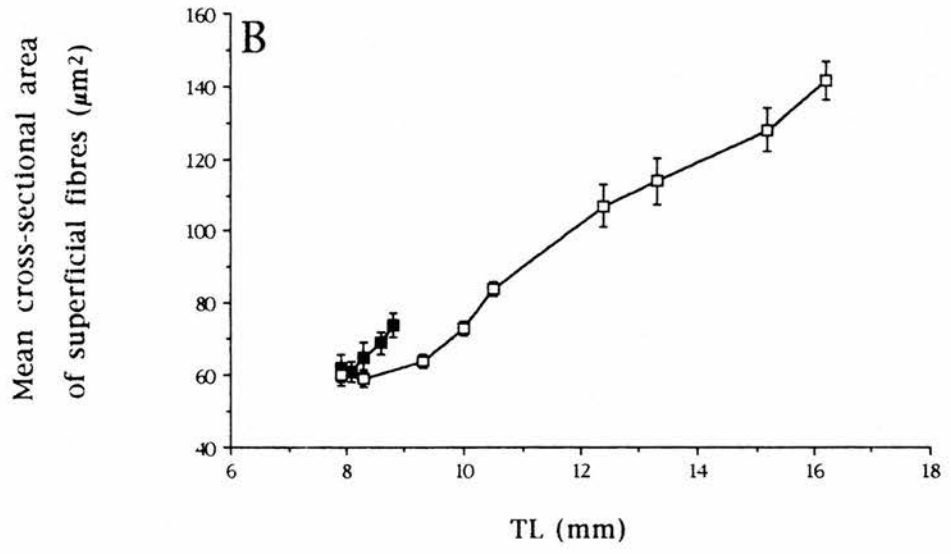
superficial fibres (t=5.706, P<0.01) (Fig 5), and more inner fibres (t=7.858, P<0.01) (Fig 6), than fish of equivalent size reared at 10 °C. In 1996, 12 °C larvae had the same number of superficial (t=0.215, P>0.10, Fig 7A) and inner (t=0.995, P>0.10, Fig 8A), muscle fibres, of the same mean size (for superficial fibres: t=0.764, P>0.10, Fig 7B; for inner fibres: t=0.556, P>0.10, Fig 8B), as 8 °C fish of equivalent size. Muscle cellularity data from the 1994 and 1996 experiments could not be compared since only a subset of the fibre populations was considered in 1994 while the entire populations were measured in 1996.

The percentage contributions of hyperplasia and hypertrophy to superficial and inner muscle were calculated using the data shown in Figs 7 and 8. Briefly, this was done by determining the orders of magnitude by which fibre numbers and fibre sizes increased over a given increase in TL, then expressing one order of magnitude as a percentage of the two combined (see also Stickland, 1983). The percentage contribution of hypertrophy to superficial muscle growth in fish reared at 8 °C during 1996 was not significantly different from that of fish reared at 12 °C (t=0.503, P>0.10, Fig 9A). The percentage contribution of hypertrophy to inner muscle growth was also the same at the two temperatures (t=2.179, P>0.05, Fig 9B). At each temperature, however, the contribution of hypertrophy to inner muscle growth was greater than that of superficial muscle (at 8 °C: t=3.197, P<0.01; at 12 °C: t=3.721, P<0.01) (Fig 9).

The percentage contribution of hypertrophy increased, in both fibre types, as fish grew (Fig 9). This increase was more

Fig 5. Numbers and cross-sectional areas of superficial muscle fibres in the left hypaxial quadrant of the somite immediately posterior to the anus of shorthorn sculpin larvae reared during 1994. Larvae were held at 5 °C (closed squares) and 10 °C (open squares). Values in C are the products of the mean values given in A and B, which are themselves expressed ±SD (n=6-10 fish at each temperature).


A
Number of superficial fibres
70
60
50
40
30
6
8
10
12
14
16
18
TL (mm)


B
Mean cross-sectional area of superficial fibres (μm²)
160
140
120
100
80
60
40
6
8
10
12
14
16
18
TL (mm)

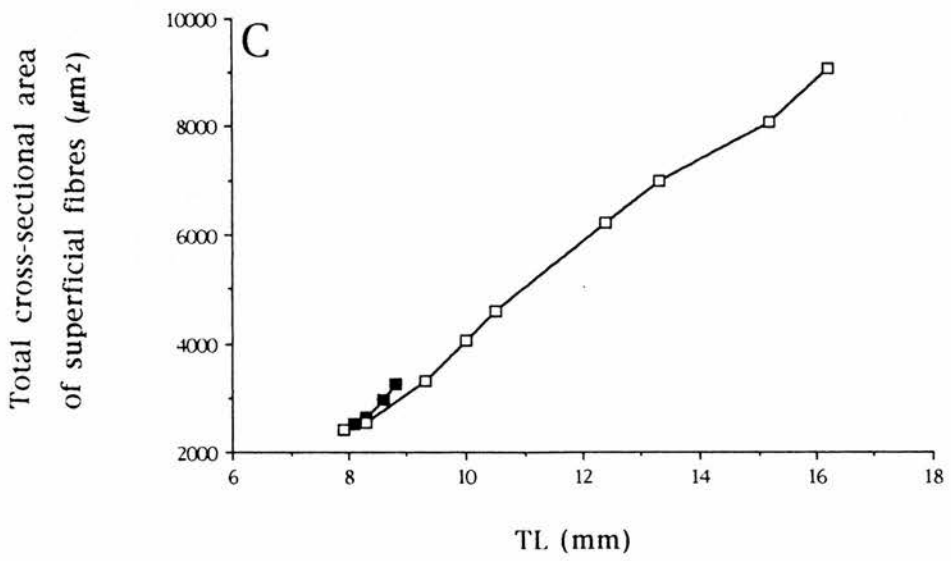
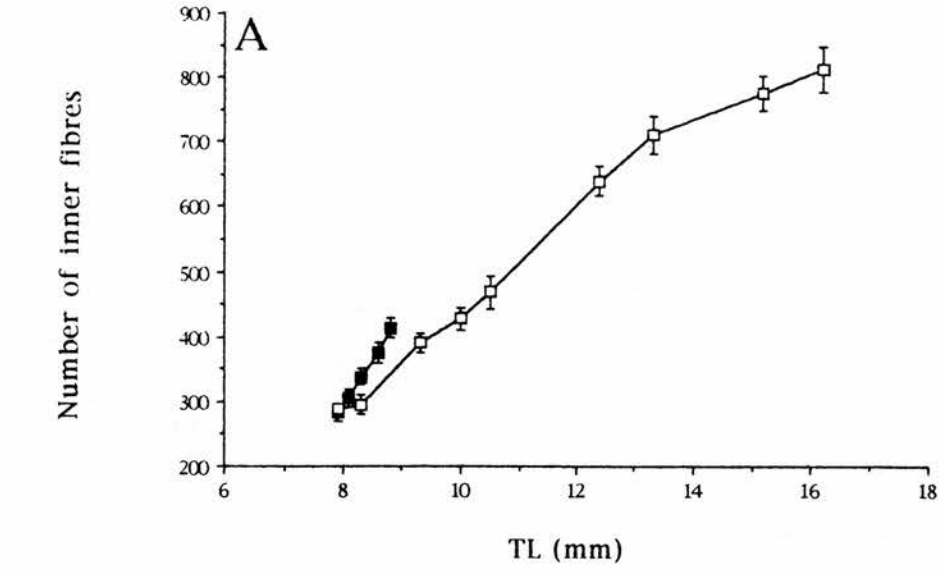
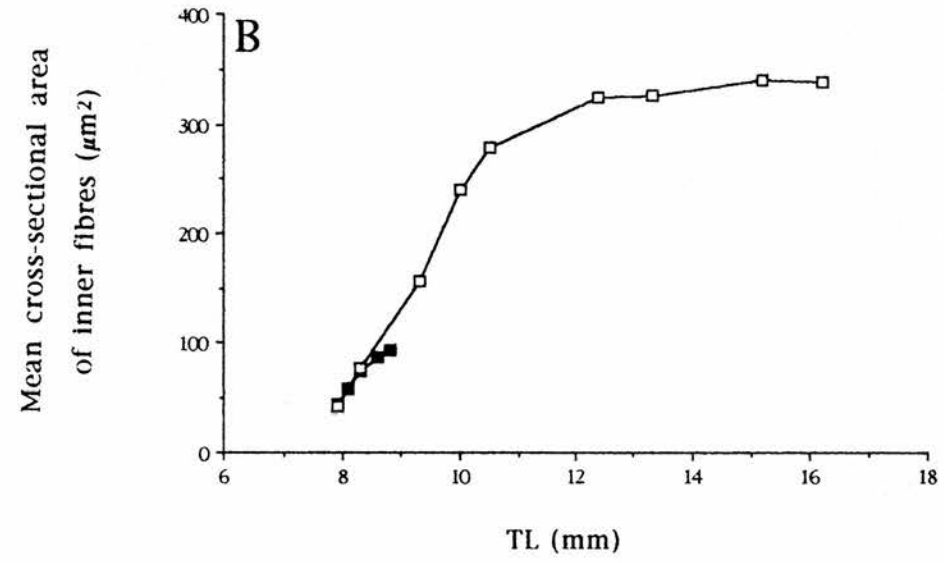

C
Total cross-sectional area of superficial fibres (μm²)
10000
8000
6000
4000
2000
6
8
10
12
14
16
18
TL (mm)

Fig 6. Numbers and cross-sectional areas of inner muscle fibres in the left hypaxial quadrant of shorthorn sculpin larvae reared during 1994. Larvae were held at 5 °C (closed squares) and 10 °C (open squares). Values in C are the products of the mean values given in A and B, which are themselves expressed ±SD (n=6-10 fish at each temperature).


A
Number of inner fibres
900
800
700
600
500
400
300
200
6
8
10
12
14
16
18
TL (mm)


B
Mean cross-sectional area
of inner fibres (μm²)
400
300
200
100
0
6
8
10
12
14
16
18
TL (mm)

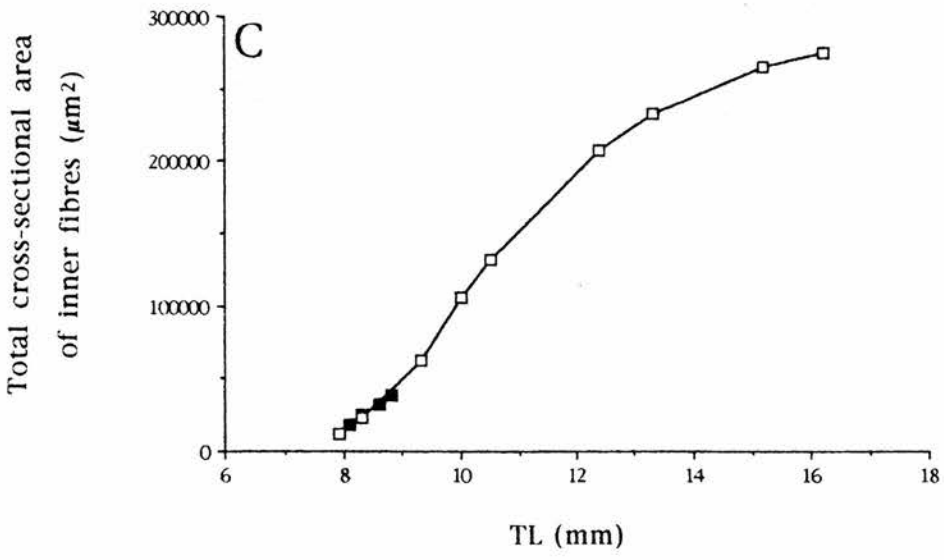
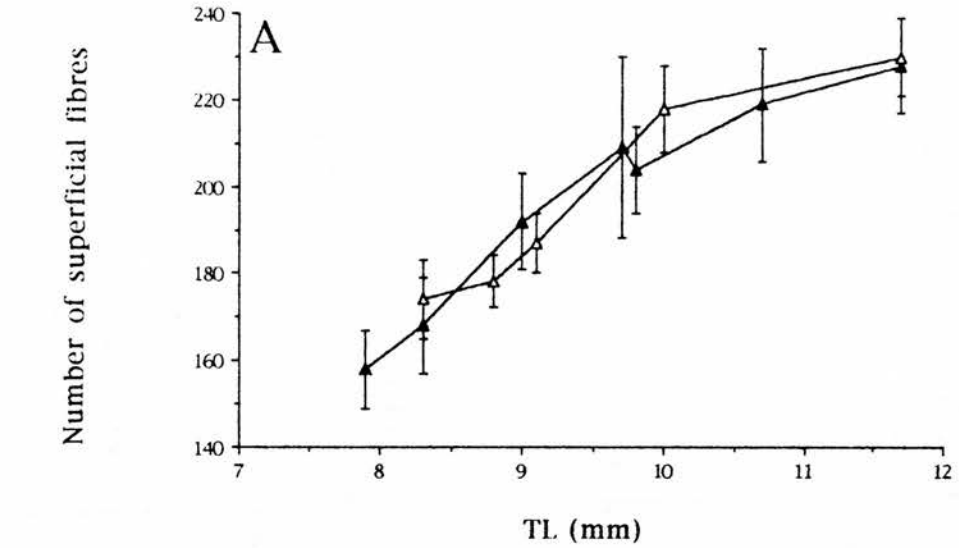
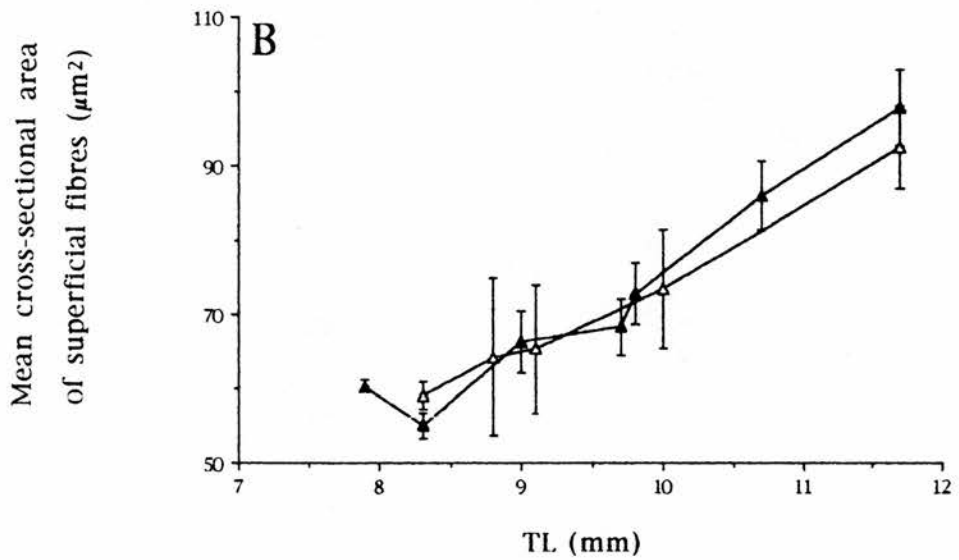

C
Total cross-sectional area
of inner fibres (μm²)
300000
200000
100000
0
6
8
10
12
14
16
18
TL (mm)

Fig 7. Numbers and cross-sectional areas of superficial muscle fibres in shorthorn sculpin larvae reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A and B are shown as means ±SD, while values in C are the products of these means (n=4-6 fish at each temperature).


A
Number of superficial fibres
240
220
200
180
160
140
7
8
9
10
11
12
TL (mm)


B
Mean cross-sectional area
of superficial fibres (μm²)
110
90
70
50
7
8
9
10
11
12
TL (mm)

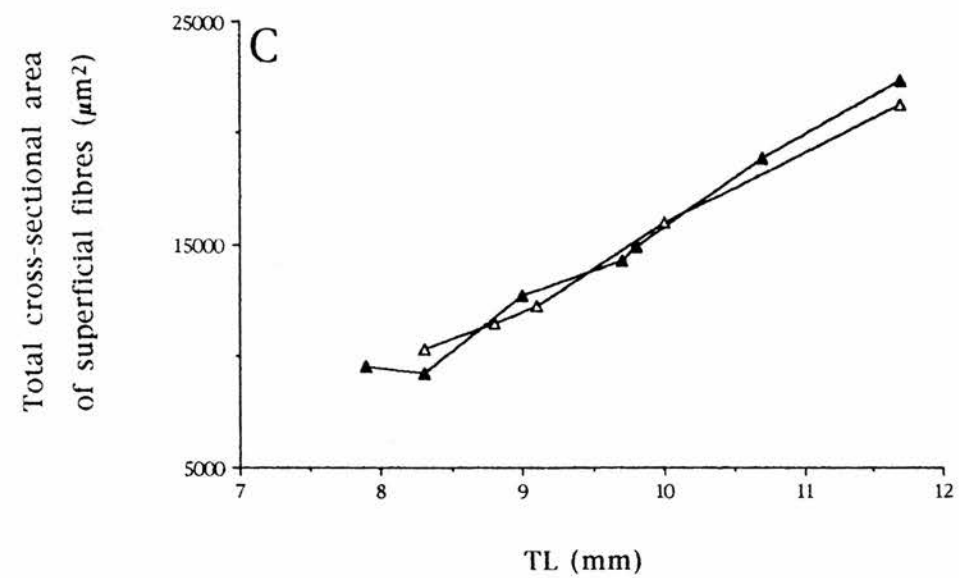
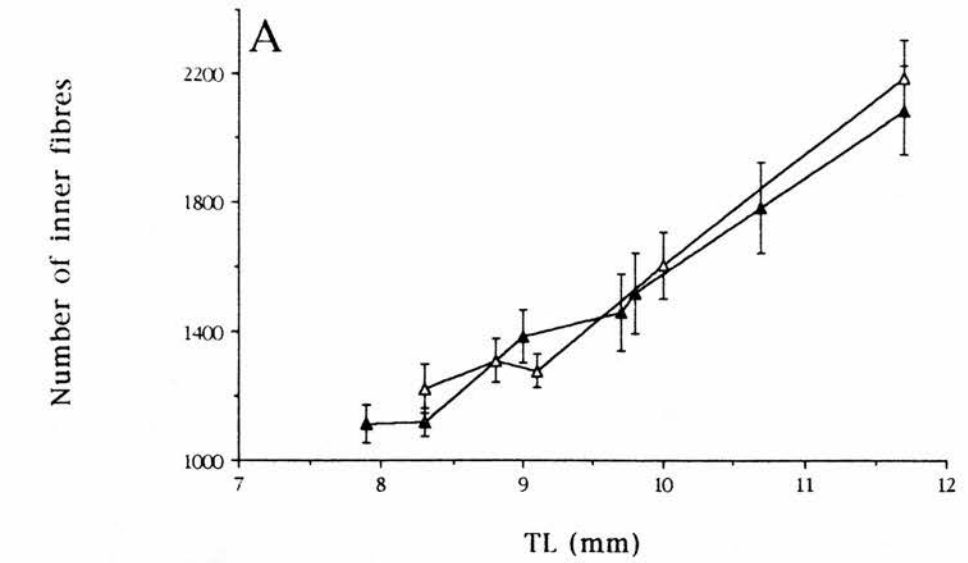
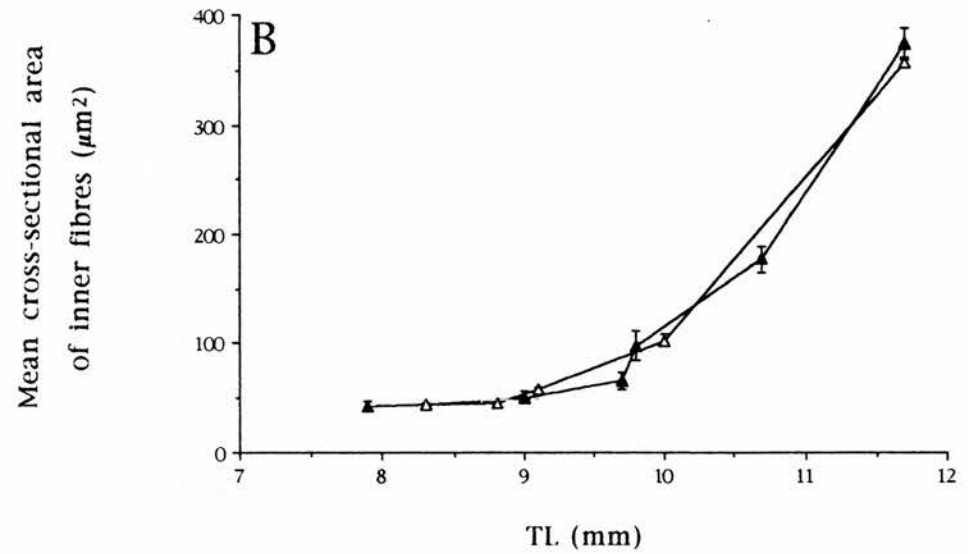

C
Total cross-sectional area
of superficial fibres (μm²)
25000
15000
5000
7
8
9
10
11
12
TL (mm)

Fig 8. Numbers and cross-sectional areas of inner muscle fibres in shorthorn sculpin larvae reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A and B are shown as means ±SD, while values in C are the products of these means (n=4-6 fish at each temperature).


A
Number of inner fibres
2200
1800
1400
1000
7
8
9
10
11
12
TL (mm)


B
Mean cross-sectional area
of inner fibres (μm²)
400
300
200
100
0
7
8
9
10
11
12
TL (mm)

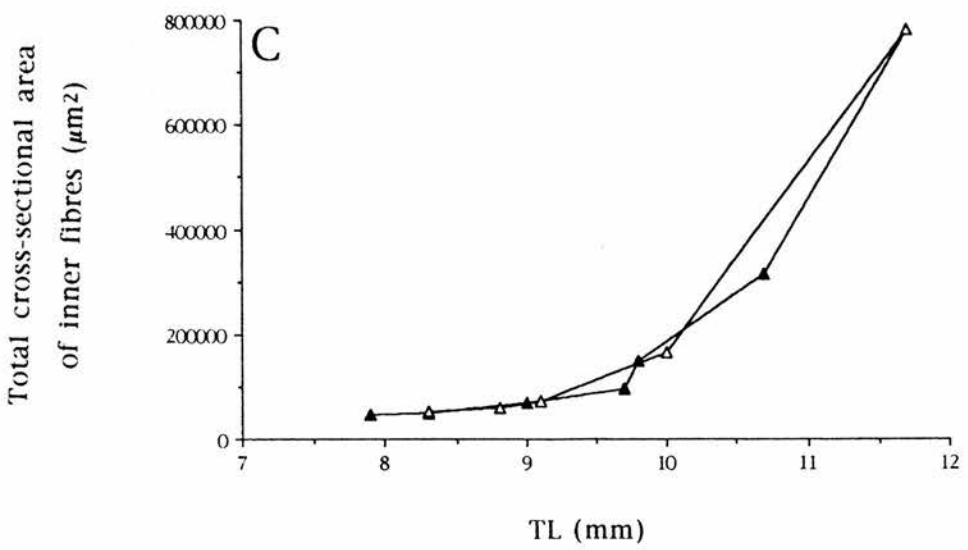
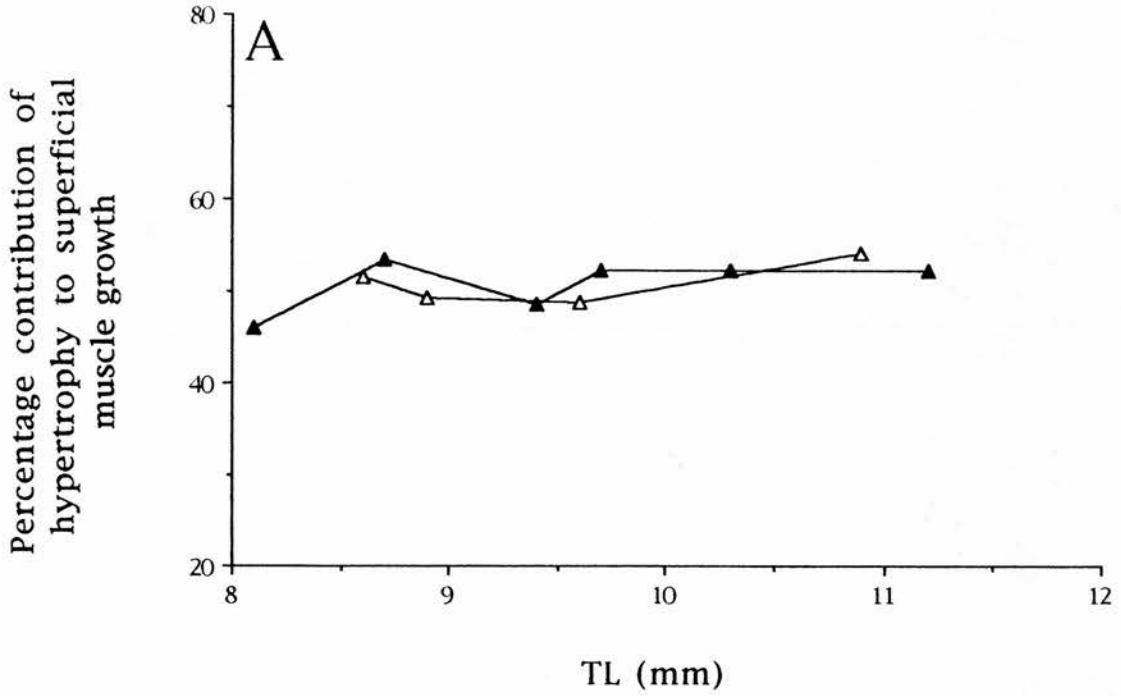
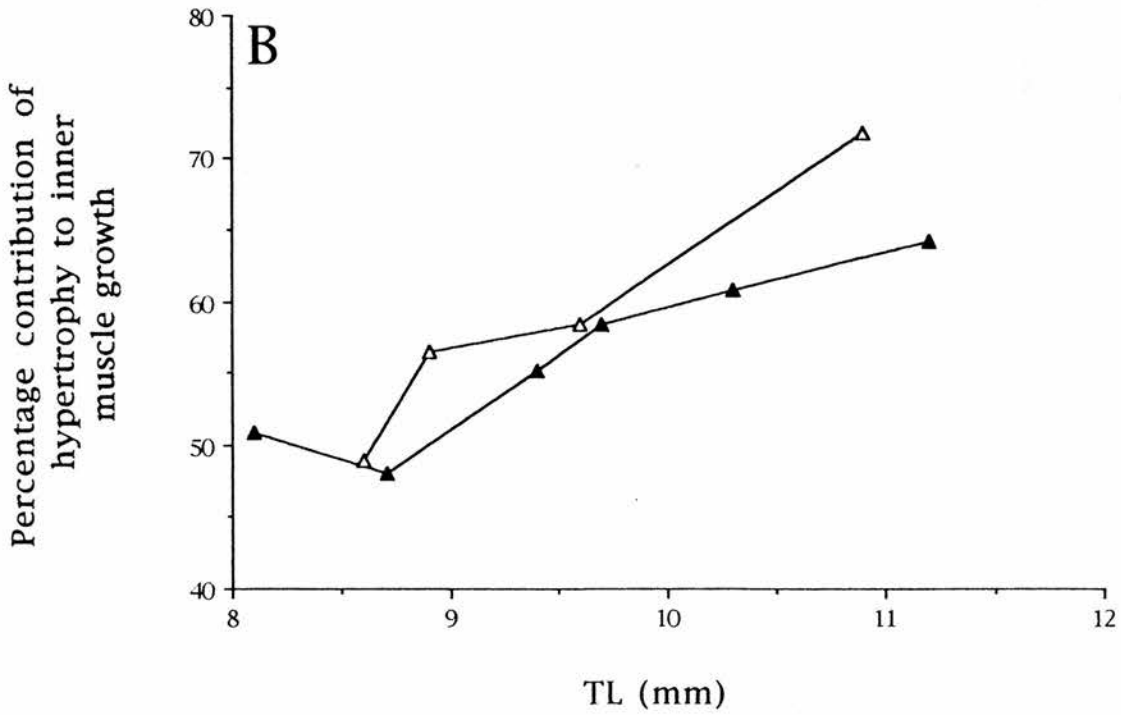

C
Total cross-sectional area
of inner fibres (μm²)
800000
600000
400000
200000
0
7
8
9
10
11
12
TL (mm)

Fig 9. Percentage contributions of hypertrophy to superficial (A) and inner (B) muscle growth in sculpin reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Each point gives the value obtained when data for all fish sampled over a 24 h period were combined. (The sum of the percentage contributions of hypertrophy and hyperplasia to muscle growth is 100 %)


A
Percentage contribution of hypertrophy to superficial muscle growth
80
60
40
20
8
9
10
11
12
TL (mm)


B
Percentage contribution of hypertrophy to inner muscle growth
80
70
60
50
40
8
9
10
11
12
TL (mm)

marked in inner muscle than in superficial muscle (t=5.894, P<0.01) (Fig 9). This result also suggests that the contribution of hyperplasia to growth remained high in superficial muscle, but decreased markedly in inner muscle (Fig 9).

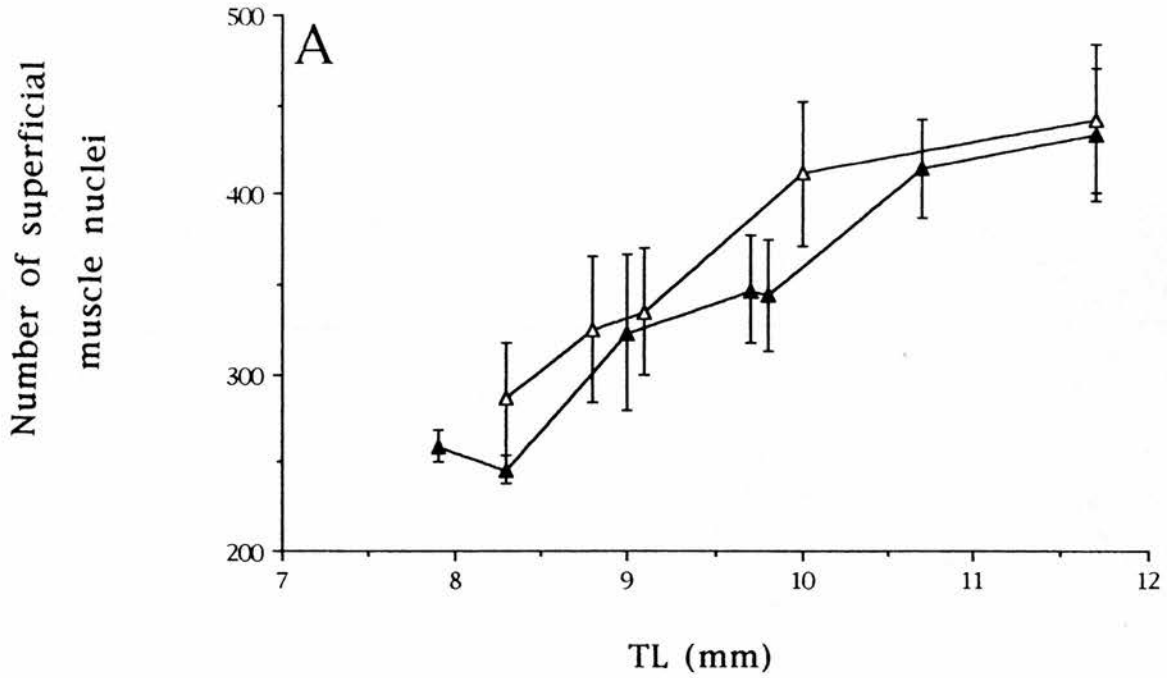
Muscle nuclei and nuclear proliferation

There was no difference between the number of superficial muscle nuclei per myotome in 12 °C larvae and 8 °C larvae of equivalent size (t=0.556, P>0.10) (Fig 10A). The number of inner muscle nuclei was also not significantly different between the 12 °C and 8 °C groups (t=0.874, P>0.10) (Fig 11A).

The nuclear density of superficial muscle in 12 °C larvae was not different from that in 8 °C larvae of equivalent size (t=0.874, P>0.10) (Fig 10B). The nuclear density of inner muscle was also not significantly different between equivalently sized larvae at the two temperatures (t=0.245, P>0.10) (Fig 11B). At each temperature, the nuclear density of superficial muscle was greater than that of inner muscle (at 8 °C: t=6.358, P<0.01; at 12 °C: t=5.798, P<0.01) (Figs 10B and 11B). The decrease in nuclear density with TL was, however, more pronounced in inner (Fig 11B) than in superficial (Fig 10B) muscle (at 8 °C: t=2.261, P<0.05; at 12 °C: t=2.340, P<0.05).

The relationships between the number of muscle nuclei labelled with BrdU and the time spent by fish in the labelling solution were all approximately linear (values of the product

Fig 10. Numbers and densities of muscle nuclei in superficial muscle of sculpin larvae reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A are shown as means ±SD, while values in B were obtained by dividing these means by values shown in Fig 6C (n=4-6 fish at each temperature).


A
Number of superficial muscle nuclei
500
400
300
200
7
8
9
10
11
12
TL (mm)

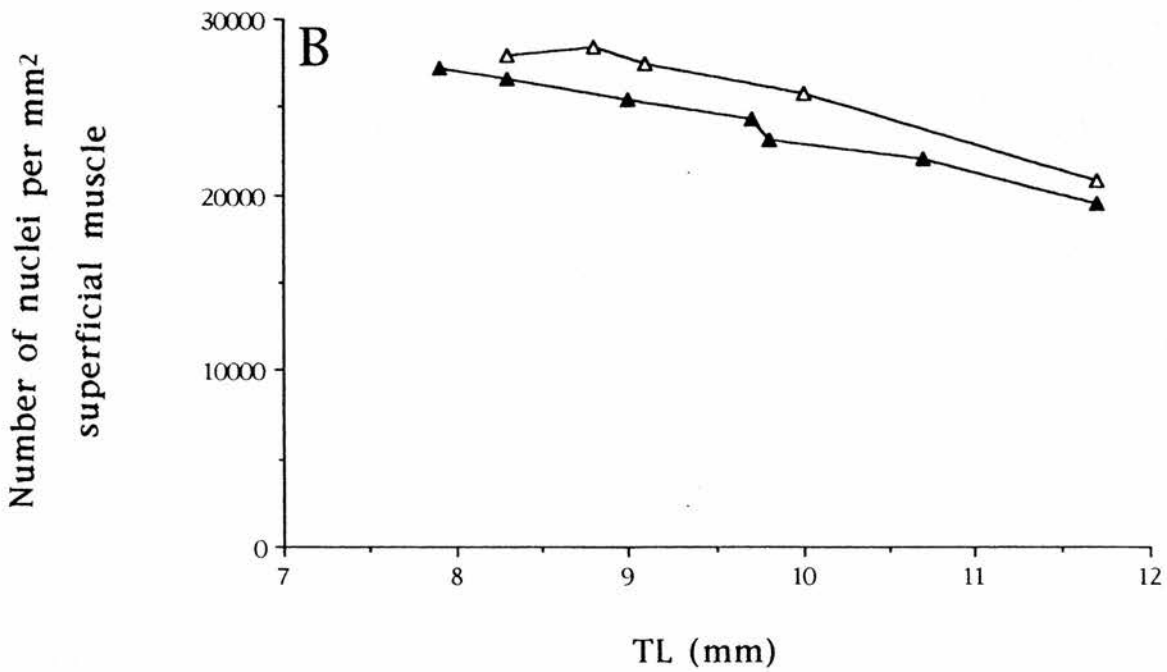
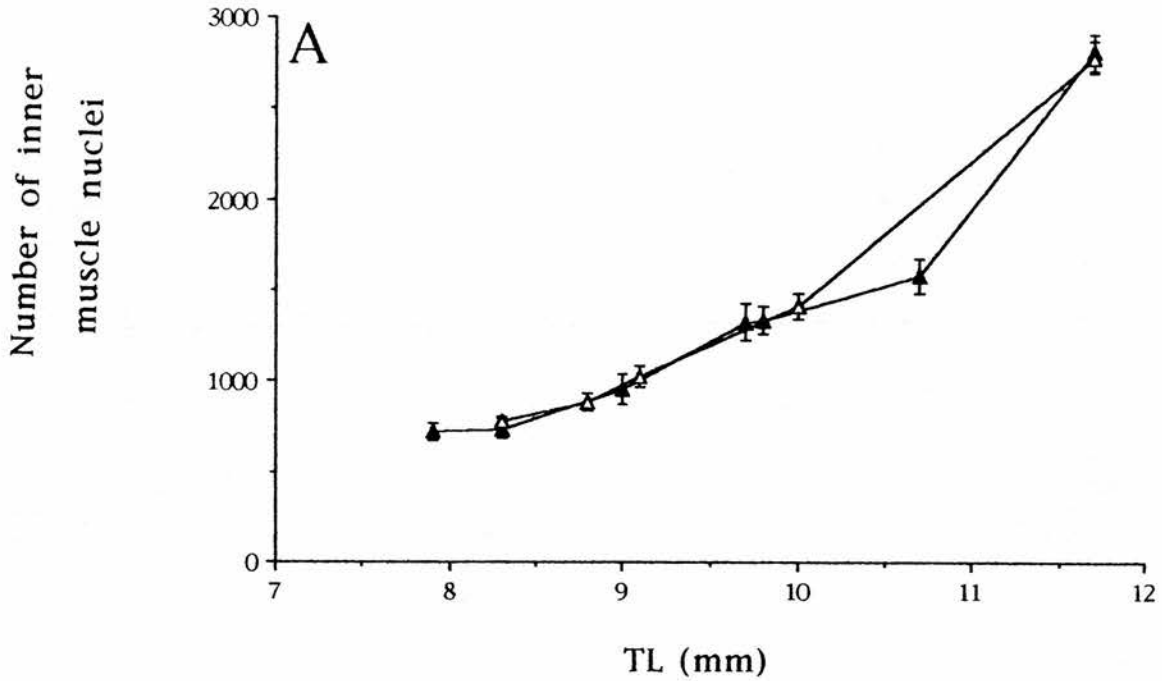
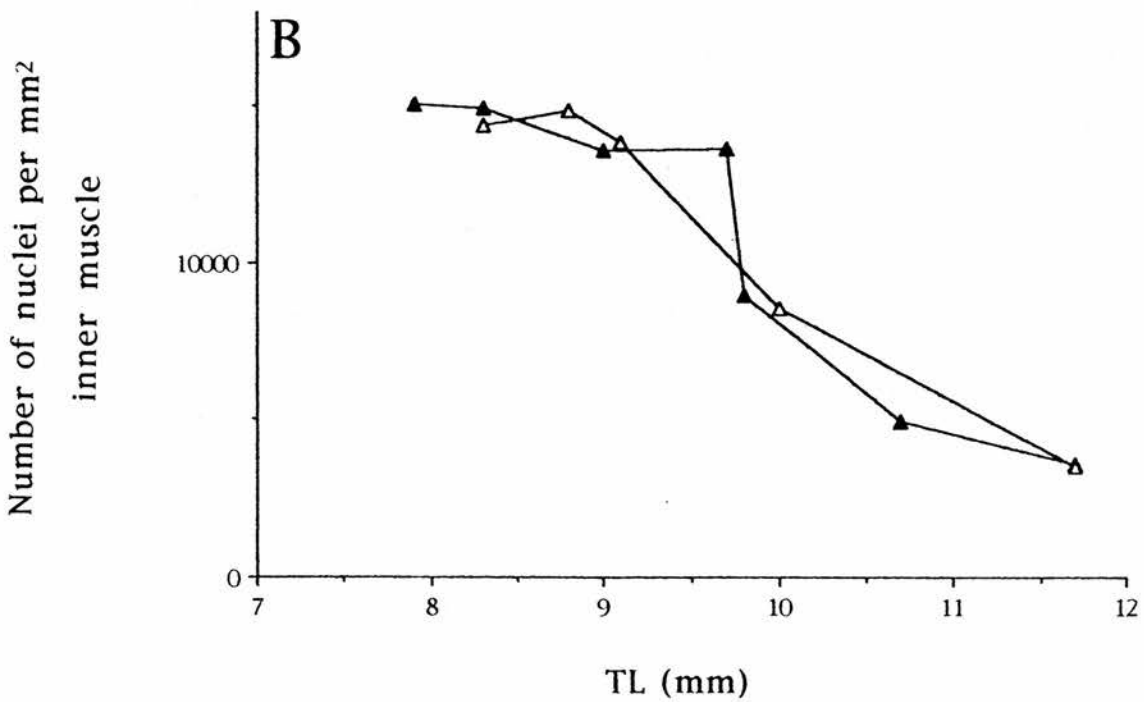

B
Number of nuclei per mm² superficial muscle
30000
20000
10000
0
7
8
9
10
11
12
TL (mm)

Fig 11. Numbers and densities of muscle nuclei in inner muscle of sculpin larvae reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A are shown as means ±SD, while values in B were obtained by dividing these means by values shown in Fig 7C (n=4-6 fish at each temperature).


A
Number of inner muscle nuclei
3000
2000
1000
0
7
8
9
10
11
12
TL (mm)


B
Number of nuclei per mm² inner muscle
10000
0
7
8
9
10
11
12
TL (mm)

moment correlation coefficient, r, were all greater than 0.917; $P<0.01$) (representative data are shown in Fig 12). The gradients of these lines were thus taken as the rates at which muscle nuclei were labelled with BrdU. Rates calculated in this manner are plotted as a function of TL in Figs 13 and 14. The number of superficial muscle nuclei labelled with BrdU per day in 12 °C fish was the same as in 8 °C fish of equivalent size (Fig 13A) (t=0.765, $P>0.10$). The number of inner muscle nuclei labelled with BrdU per day in 12 °C fish was also not significantly different from that in their 8 °C counterparts (Fig 14A) (t=0.912, $P>0.10$). The percentages of muscle nuclei labelled with BrdU per day were independent of rearing temperature (for superficial muscle: t=0.969, $P>0.10$, Fig 13B; for inner muscle: t=0.316, $P>0.10$, Fig 14B). At any given temperature, however, the percentage of nuclei labelled with BrdU per day was greater in superficial than in inner muscle (at 8 °C: t=9.031, $P<0.01$; at 12 °C: t=7.796, $P<0.01$) (Figs 13B and 14B). The increase in the rate of nuclear labelling with BrdU with TL was also more pronounced in inner than in superficial muscle (at 8 °C: t=5.164, $P<0.01$; at 12 °C: t=6.716, $P<0.01$) (Figs 13B and 14B).

Fig 12. Number and percentage of superficial muscle nuclei labelled with BrdU in relation to time spent by 8 °C (A and C; closed triangles) and 12 °C (B and D; open triangles) fish in the BrdU labelling solution. For clarity, only data for fish with TLs of about 8.3, 9.0 and 11.7 mm are shown. Each point shows the value for a single fish.

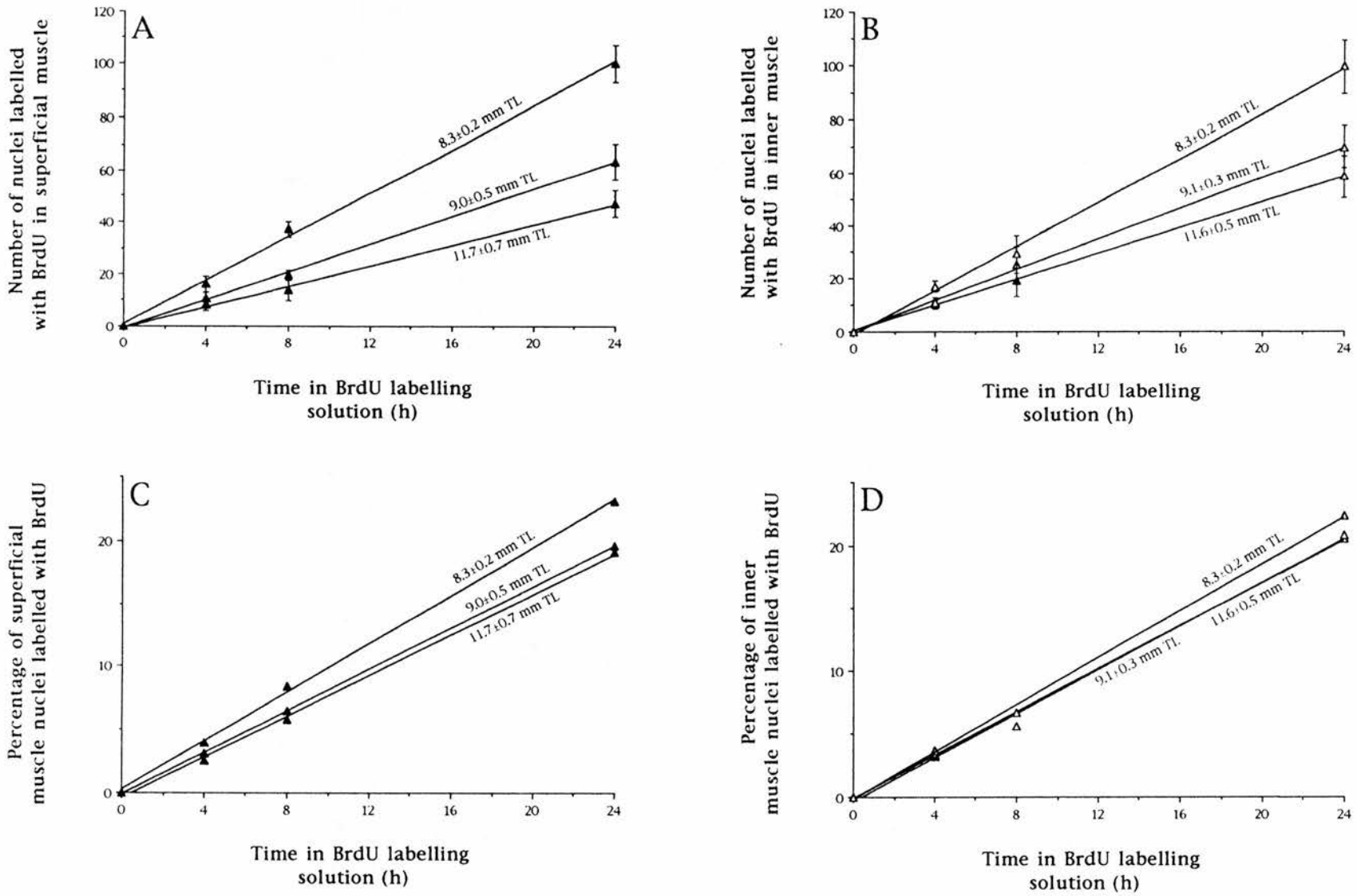
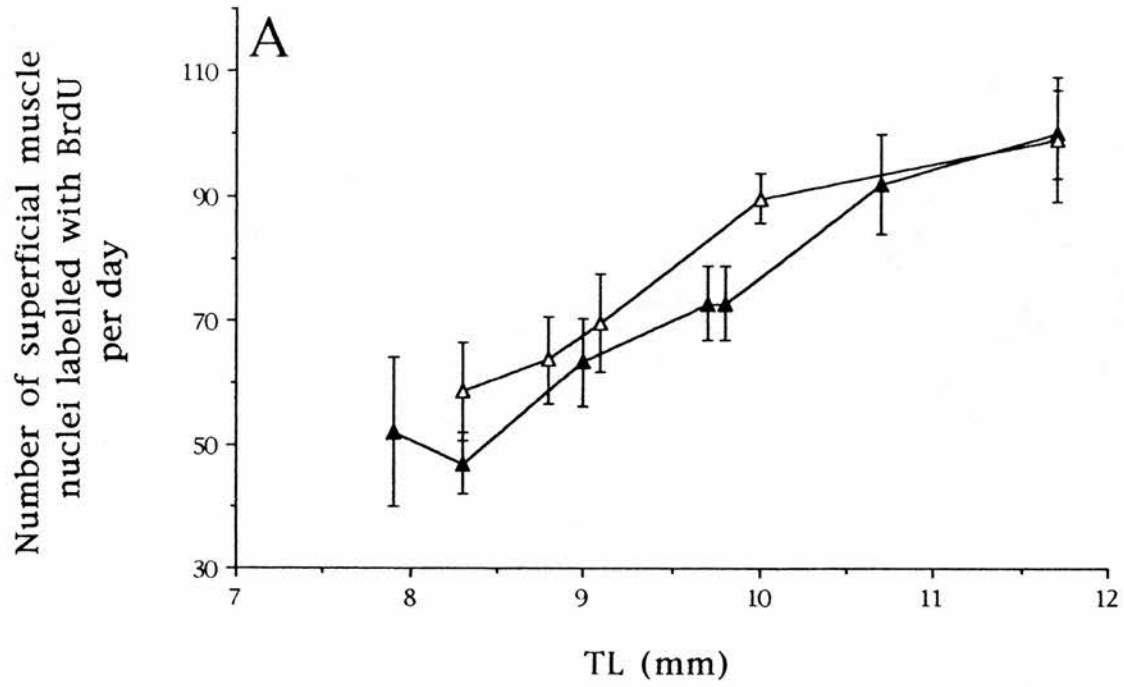

A
Number of nuclei labelled with BrdU in superficial muscle
120
100
80
60
40
20
0
0
4
8
12
16
20
24
8.3±0.2 mm TL
9.0±0.5 mm TL
11.7±0.7 mm TL
Time in BrdU labelling solution (h)
B
Number of nuclei labelled with BrdU in inner muscle
8.3±0.2 mm TL
9.1±0.3 mm TL
11.6±0.5 mm TL
Time in BrdU labelling solution (h)
C
Percentage of superficial muscle nuclei labelled with BrdU
20
10
0
8.3±0.2 mm TL
9.0±0.5 mm TL
11.7±0.7 mm TL
Time in BrdU labelling solution (h)
D
Percentage of inner muscle nuclei labelled with BrdU
8.3±0.2 mm TL
9.1±0.3 mm TL
11.6±0.5 mm TL
Time in BrdU labelling solution (h)

Fig 13. Absolute and relative values for the rate at which superficial muscle nuclei were labelled with BrdU in sculpin reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A are shown as means ±SD, while values in B were obtained by dividing these means by values shown in Fig 9A (n=4-6 fish at each temperature).


A
Number of superficial muscle nuclei labelled with BrdU per day
110
90
70
50
30
7
8
9
10
11
12
TL (mm)

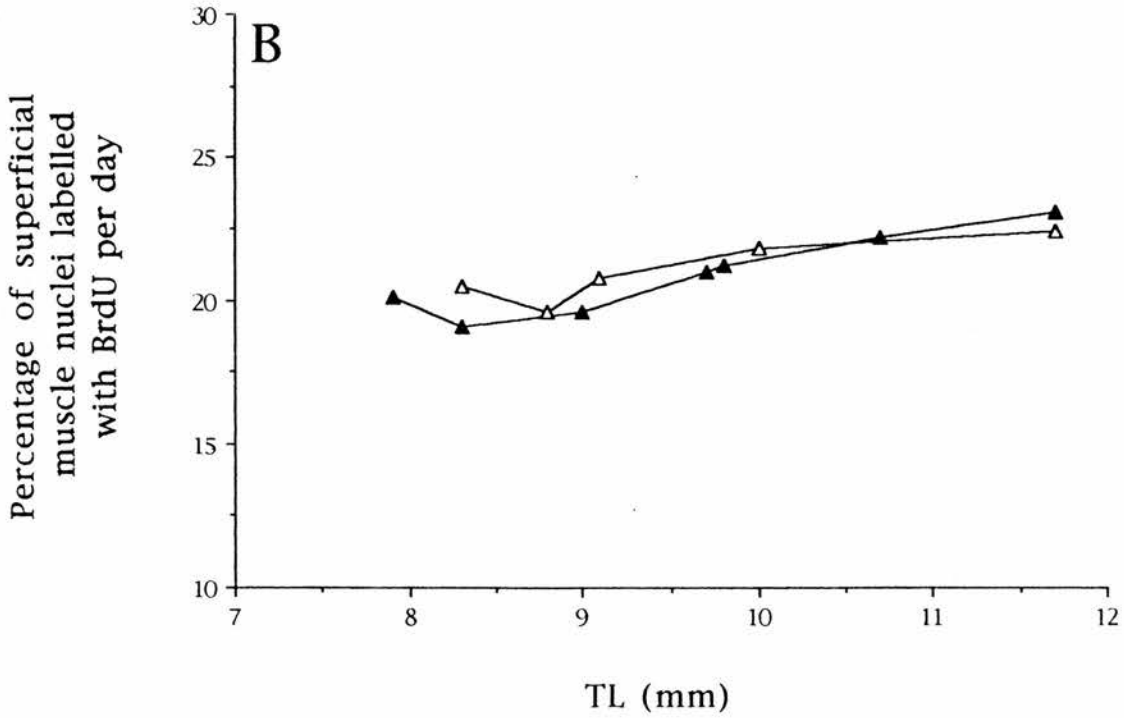
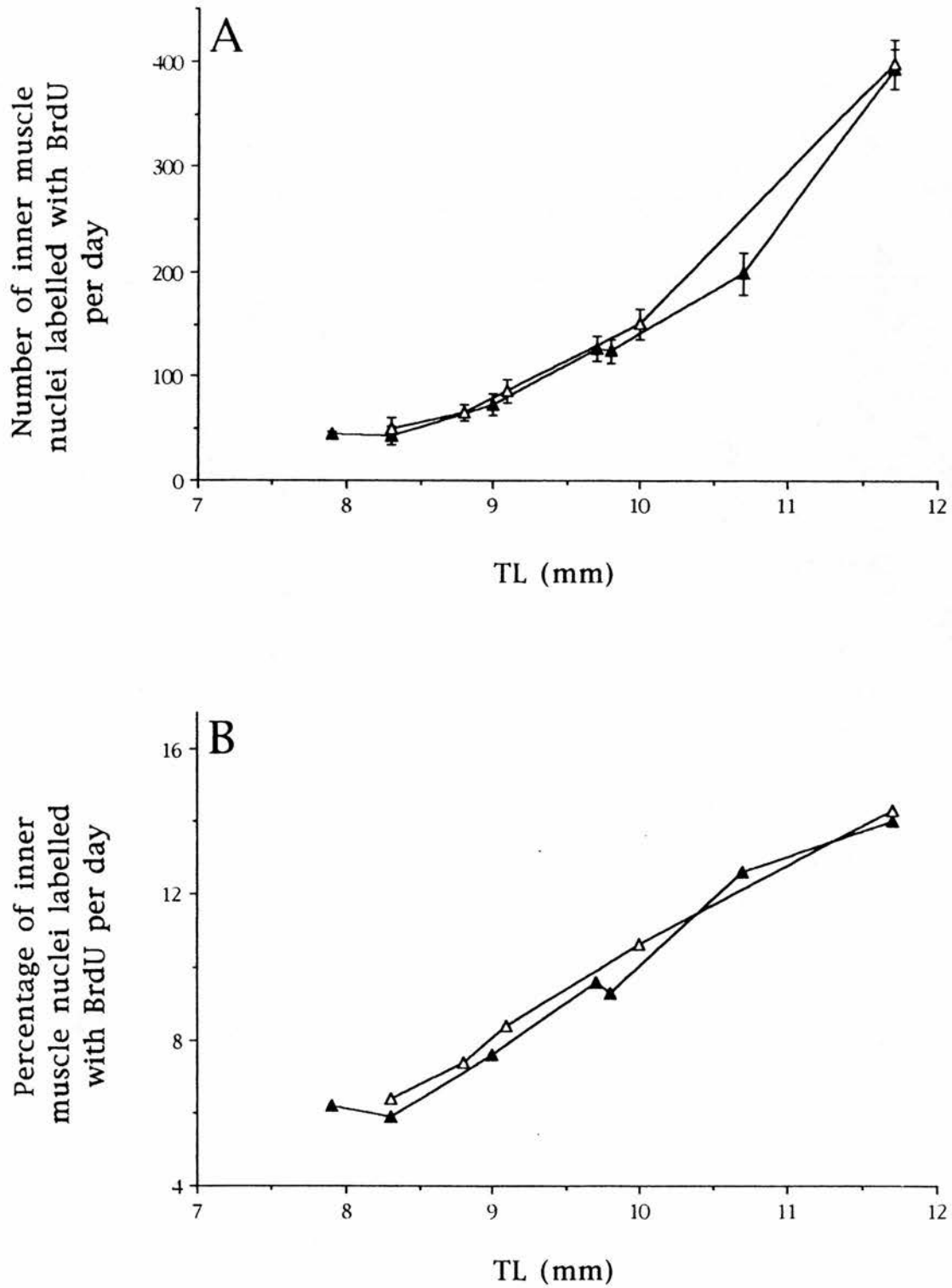

B
Percentage of superficial muscle nuclei labelled with BrdU per day
30
25
20
15
10
7
8
9
10
11
12
TL (mm)

Fig 14. Absolute and relative values for the rate at which inner muscle nuclei were labelled with BrdU in sculpin reared at 8 °C (closed triangles) and 12 °C (open triangles) during 1996. Values in A are shown as means ±SD, while values in B were obtained by dividing these means by values shown in Fig 10A (n=4-6 fish at each temperature).


A
Number of inner muscle nuclei labelled with BrdU per day
400
300
200
100
0
7
8
9
10
11
12
TL (mm)
B
Percentage of inner muscle nuclei labelled with BrdU per day
16
12
8
4
7
8
9
10
11
12
TL (mm)

Larval development and muscle fibre types

The distances between certain structures on the body changed with respect to one another as larvae grew (Fig 2), reflecting a dramatic change in body shape between hatching and metamorphosis (Fig 4). This allometry was probably necessary since larvae changed from being pelagic, continuous swimmers at hatching, to a benthic existence where swimming occurred only intermittently, by metamorphosis (Fig 3). Changes in the swimming mode of fish were accompanied by changes in myotomal muscle composition. At hatching, larvae possessed a monolayer of superficial (presumptive red) fibres surrounding an inner (presumptive white) muscle mass (Fig 4). By the end of larval life, however, intermediate (or fast red) muscle fibres appeared. The histochemical properties of the inner muscle fibres also changed between hatching and metamorphosis (Table 1 and Fig 4): at hatching inner fibres stained intensely for mATPase activity at pH 10.2 but lightly at pH 10.6; at metamorphosis, inner fibres stained lightly for mATPase at both pHs (Fig 4). Changes in the histochemical properties of muscle fibres between hatching and metamorphosis suggest that changes in myofibrillar protein expression occurred as larvae developed. The sequential expression of different myofibrillar protein isoforms during fish ontogeny has previously been demonstrated using histochemical (Scapolo *et al*, 1988; Veggetti *et al*, 1993) and electrophoretic (Brooks and Johnston, 1993; Crockford and Johnston, 1993)

methods. For example, in the plaice (*Pleuronectes platessa* L.), larval isoforms of myosin light chain 2 (LC2) are replaced by adult LC2 isoforms at metamorphosis (Brooks and Johnston, 1993). The different myofibrillar protein isoforms are postulated to have different functional properties which may be related to different modes of swimming: in the plaice, as in the shorthorn sculpin, metamorphosis involves a change from a pelagic to a benthic existence (Brooks and Johnston, 1993).

Somatic growth and muscle cellularity

In both 1994 and 1996, an increase in rearing temperature resulted in an increase in somatic growth rates (Fig 1). The rates of somatic growth in fish reared at 10 °C during 1994 were, however, not significantly different from those in fish reared at 12 °C during 1996 (Fig 1). additionally, an increase in rearing temperature of 1994 fish resulted in increased hyperplasia during superficial muscle growth (Fig 5), but plasticity in the mechanisms of muscle growth was not observed in the 1996 fish (Figs 7 and 8). These differences between the 1994 and 1996 year classes might have been due to the effects of temperature, on the growth performance of fish, varying from year to year. This was also found when the growth of herring larvae (*Clupea harengus* L.) at 5, 10 and 15 °C during 1991 (Vieira and Johnston, 1992) was compared with that of larvae reared at the same temperatures during 1993 and 1994 (Johnston *et al*, 1995). These differences might have been the result of different year classes having different genetic potentials with regard to growth performance

(Weatherley, 1990; Johnston, 1994), but variability in the development and growth of a single clonal strain of zebrafish (Streisinger *et al*, 1981) suggest that genetic variation alone can not account for differences in the growth phenotype of fish. Another cause of the observed differences in growth phenotype of fish from 1994 and 1996 sculpin, and between 1993/1994 herring, might have been differences between the eggs of fish from different year classes. For example, larval growth rate in numerous fish species has been shown to be positively correlated to egg size and inversely correlated to the time after ovulation at which eggs are fertilised (for a review see Blaxter, 1988).

As in most other fish (Weatherley and Gill, 1987; Weatherley *et al*, 1988; Weatherley, 1990), the contribution of hypertrophy to muscle growth increased as fish increased in TL (Fig 9). The increase in hypertrophy with TL was, however, greater for inner muscle, while the contribution of hyperplasia remained high in superficial muscle (Fig 9). This was also found in the Atlantic salmon (Stickland, 1983). The prolonged occurrence of hyperplasia in red/superficial muscle probably serves to maintain a relatively small fibre size, with a high surface to volume ratio that is more suitable to aerobic metabolism than the larger fibre sizes found in white muscle (Koumans *et al*, 1994).

Nuclear densities

The myonuclear density of superficial muscle (Fig 10B) was significantly greater than that of inner muscle (Fig 11B). The

myonucleus to sarcoplasm ratio of red muscle was also found to be greater than that of white muscle in the common carp (Koumans *et al*, 1994) and the eel, *Anguilla anguilla* L. (Egginton and Johnston, 1982). High myonuclear densities are usually found during fibre formation since this process involves the fusion of myogenic cells which have very little cytoplasm (Koumans *et al*, 1993). High nuclear densities are also a reflection of the active synthesis of contractile proteins which accompanies new fibre formation (Koumans *et al*, 1994).

The correlation between hyperplasia and high myonuclear densities, coupled with increases in hypertrophy with growth, suggest that the myonucleus to sarcoplasm ratio should have decreased with increase in body size. This was, indeed, found in the superficial (Fig 10B) and inner muscle (Fig 11B) of the sculpin, and has previously been observed in rainbow trout (Luquet and Durand, 1970) and in common carp (Koumans *et al*, 1994). Decreases in myonuclear density with increases in hypertrophy might also explain the finding that the nuclear densities of inner fibres decreased more rapidly with increase in TL than those of superficial fibres.

Myosatellite cell turnover kinetics

Following the formation of a muscle fibre, the volume of its cytoplasm increases. When a critical myonucleus to cytoplasm ratio is reached, however, continued increase in muscle fibre size can occur only if additional nuclei are provided by myosatellite

cells (Cardasis and Cooper, 1975; Enesco and Puddy, 1964; Campion, 1984). BrdU labels myosatellite cells during the S phase of the cell cycle (Schultz, 1996), but at certain concentrations it can affect the ability of myosatellite cells to undergo mitotic divisions (Bischoff and Holtzer, 1970; Labrecque *et al*, 1991). It was not possible to determine the levels of BrdU available to cells in our study, but the finding that the number of BrdU-labelled nuclei, in both the superficial and inner muscle of fish, was positively correlated with labelling time (Fig 12) suggested that the concentrations of BrdU used were at a level that did not alter myosatellite cell mitotic behaviour. This result also suggests that not all proliferating myosatellites were at the same phase of the cell cycle (i.e., the divisions of the myosatellite cells were asynchronous) (Wimber and Quastler, 1963).

A change in the rate of nuclear labelling with BrdU can mean either a change in the number and/or cell cycle time of proliferating myosatellites (Schultz, 1996). The absolute numbers of myosatellites present in the muscle of fish were not found, and so the values of these variables could not be determined. An increase in the rate of nuclear labelling with BrdU does, however, signify an increase in the rate of myosatellite cell proliferation regardless of whether changes are to myosatellite cell numbers and/or generation times (Rowlerson *et al*, 1995; Schultz, 1996).

The percentage of muscle nuclei labelled with BrdU (Figs 13B and 14B) and the percentage contribution of hypertrophy to muscle growth (Fig 9) both increased as TL increased. The magnitude of the increase in percentage BrdU-labelled nuclei with

TL was also similar to the magnitude of the increase in hypertrophy. For example, in superficial muscle, both variables increased only slightly with increase in TL (Figs 9A and 13B), while in inner muscle the percentage of BrdU-labelled nuclei and the percentage contribution of hypertrophy both increased markedly as larvae grew (Figs 9B and 14B). In the common carp, myosatellite proliferation rates (Alfei *et al*, 1994) and cell cycle times (Koumans *et al*, 1994) were also found to increase and decrease, respectively, as hypertrophy became more important to the growth of their muscle. These findings imply a positive correlation between hypertrophy and myosatellite cell proliferation. A possible explanation for this is provided by *in vitro* studies which suggest that hypertrophy depends more on proliferating myosatellites while hyperplasia relies more on myosatellite cells that are involved in differentiation pathways (Koumans *et al*, 1993). According to Koumans *et al* (1993), proliferating myosatellites produce daughter nuclei which become incorporated into muscle fibres that are increasing in size, while differentiated myosatellites fuse to form new myotubes/immature myofibres. The behaviour of a myosatellite cell is determined by the balance of factors that activate the proliferation and differentiation pathways (Olson, 1992). For example, thyroid hormones result in increased MyoD gene transcription in myosatellites and thus the promotion of differentiation (Muscat *et al*, 1994; Marchal *et al*, 1995). In contrast, insulin-like growth factors promote the production of cyclins D and E and, consequently, increase myosatellite proliferation (Schriever *et al*, 1996). It is therefore tempting to speculate that changes in the balance between hypertrophy and hyperplasia observed at

different temperatures, and in fish as they increased in size, were the result of thermal effects on the balance between proliferation and differentiation signals, and age-related changes in the levels of these signals, respectively. Indeed, temperature has been shown to influence the levels of thyroid hormones in fish (Inui and Miwa, 1985; Seikai *et al*, 1986), while the production of insulin-like growth factor I has been shown to change with age in the barramundi (*Lates calcarifer* Bloch) (Richardson *et al*, 1995). Differences in the ratios of hyperplasia to hypertrophy between superficial and inner muscle could also be due to different fibre types having different sensitivities to the respective signals. Differences in the sensitivities of red and white muscle fibre types to thyroid hormones (Kubista *et al*, 1971) and insulin-like growth factor I (Richardson *et al*, 1995) have previously been demonstrated. Different fibre types have also been shown to depend on different subclasses of myosatellite cells (Stockdale, 1992). This latter finding might explain the greater proliferation rates of myosatellites in superficial than in inner muscle, even though the latter fibre type used hypertrophy to a greater extent during growth.

The data also suggest an inverse relationship between the percentage of muscle nuclei labelled with BrdU and the nuclear density of muscle fibres (compare Fig 10B with Fig 13B, and Fig 11B with Fig 14B). This suggests that factors which trigger myosatellite cells to proliferate might arise from the muscle fibres with which they are associated. This has recently been supported by the finding that fibroblast growth factors, which promote the

mitotic activity of myosatellites (Olwin *et al*, 1994), are expressed in skeletal muscle fibres (Hannon *et al*, 1996).

Concluding remarks

Our results suggest that the balance between hyperplasia and hypertrophy in fish muscle is different in different fibre types. They also show that, in any given fibre type, the ratio of hyperplasia to hypertrophy decreases as animals increased in size. These phenomena are linked to differences in the turnover kinetics of myosatellite cells between fibre types, and to growth related changes in myosatellite cell proliferation, respectively. In general, myosatellite cell proliferation rates were positively related to the contribution of hypertrophy to muscle growth, supporting *in vitro* findings that hypertrophy might depend on myosatellites that have entered proliferation pathways, and that the contributions of hyperplasia and hypertrophy in fish are determined by the balance between factors that determine whether myosatellite cells proliferate or differentiate. This is of considerable importance to aquaculture since an increase in the number of myosatellites involved in hyperplastic processes, and thus an increase in hyperplasia itself, would result in enhancement of the growth performance of fish (Weatherley, 1990; Rowlerson *et al*, 1995).

Chapter 4

Early thermal experience influences future muscle growth in the angelfish, *Pterophyllum scalare*

Introduction

Studies of fish muscle growth are of particular interest because hyperplasia (i.e., the production of new muscle fibres) continues throughout much of the adult life of teleosts (Stickland, 1983; Weatherley and Gill, 1987; Weatherley, 1990; Rowlerson *et al*, 1995). This is in contrast to mammals and birds in which hyperplasia usually stops shortly after birth and hypertrophy (i.e., the increase in muscle fibre size) alone is responsible for postnatal muscle growth (Goldspink, 1972). The growth dynamics of muscle are also major factors in determining the somatic growth rate and ultimate size of teleosts (Weatherley and Gill, 1987; Weatherley *et al*, 1988; Weatherley 1990), with fish that rely more on hyperplasia growing at faster rates and to greater sizes than those in which hypertrophy is more important (Weatherley *et al*, 1988; Higgins and Thorpe; 1990; Weatherley, 1990).

Investigations of muscle growth in fish must take temperature into account since the balance between hyperplasia and hypertrophy in fish muscle is influenced by the temperature at which the animals are reared. For example, whitefish

(*Coregonus lavaretus* L.) larvae reared at high temperatures were found to use hypertrophy to a greater extent in muscle growth than those reared at low temperatures (Hanel *et al*, 1996). In contrast, the contribution of hyperplasia to muscle growth was positively correlated to rearing temperature in the rainbow trout (*Salmo gairdneri* L.) and Atlantic salmon (*Salmo salar* L.) (Weatherley *et al*, 1988; Higgins and Thorpe, 1990).

There is emerging evidence that the growth performance of fish might depend, not only on their immediate environmental conditions, but also on their early thermal experience. For example, Atlantic salmon from eggs incubated at low temperature (5-10 °C) grow faster, and use hypertrophy to a greater extent in muscle growth, than those from high temperature eggs (11 °C) when both groups are reared at a common temperature (11 °C) (Nathanailides *et al*, 1995). The temperature at which development occurs has also been found to affect the number of myosatellite cells (which divide to produce the additional nuclei required in muscle growth) in newly hatched fish (Johnston, 1993). The effect of development temperature on the muscle growth of fish might therefore be mediated through its influence on the size and/or turnover kinetics of the myosatellite cell population (Johnston, 1994). Here we investigate this hypothesis using the tropical cichlid, the angelfish, and 5-bromo-2'-deoxyuridine (BrdU, a thymidine analogue which is incorporated into replicating DNA) to monitor the mitotic activity of myosatellite cells *in vivo* (Schultz, 1996).

Fish

Adult angelfish (*Pterophyllum scalare* Lichtenstein) were obtained from a commercial supplier (World-wide Pet Supplies Ltd, UK), and held in freshwater at 26 °C (temperature range ±1 °C). The eggs of one female (total length, TL= 96 mm) were fertilised by two males (87 and 76 mm TL) during four natural spawnings between March 1995 and March 1996, then incubated at either 24 °C or 31 °C (range ±2 °C). Larvae were held at the respective temperatures until they were free swimming, then acclimated to 26 °C (range ±1 °C) for up to 25 d. During this period fish were held in perforated 2 l containers (2 fish per container) which were randomly arranged in a tank containing aerated freshwater at the acclimation temperature. This design ensured that the holding conditions were identical for fish from the different groups. All fish were exposed to a 12L:12D light cycle and fed *ad libitum* diets of either *Chironomus* sp larvae (adult fish) or *Artemia* sp nauplii (larvae and juveniles). The water in which fish were held was also changed by one third of its volume per week.

Cell proliferation and muscle cellularity

Cell proliferation was investigated by the incorporation of BrdU. BrdU was subsequently localised with a mouse anti-BrdU

monoclonal antibody using a commercial kit (Amersham International, UK). The cell labelling solution contained BrdU and 5-fluoro-2'-deoxyuridine in a 10:1 molar ratio. The latter compound inhibits thymidilate synthetase and increases BrdU incorporation by lowering competition from endogenous thymidine.

Fish were incubated for 4, 8 or 24 h in the labelling solution (60 μg BrdU per ml in freshwater), then killed in a 1:5000 (w/v) solution of MS222 anaesthetic (3-aminobenzoic acid ethyl ester) in freshwater. BrdU labelled fish and controls were fixed in Bouin's fluid and embedded in wax. Two serial sections, 6 μm thick, were cut from the somite immediately posterior to the anus of each fish. The first section was stained with haematoxylin-eosin, and all muscle nuclei were counted. The section was also traced with the aid of a microscope drawing arm, and muscle fibre number and cross-sectional areas were determined using a Video-Plan image analysis system (Kontron Elektronik GmbH, Switzerland).

DNA-incorporated BrdU was detected in the second section with the mouse anti-BrdU primary antibody which was, in turn, detected with peroxidase-conjugated goat anti-mouse secondary antibody. Peroxidase activity was revealed using diaminobenzidine in the presence of cobalt and nickel, which resulted in black staining at the sites of BrdU incorporation.

Statistical analyses

Normal quantile plots were used to determine the distribution of the data. Data are expressed as means ±SE and were compared with a *t*-test if normally distributed (either originally or after logarithmic transformation) and, if not, with a Mann-Whitney *U*-test. All curves were fitted using Cricket Graph Version 1.3.2 (Cricket Software, USA), and the correlation coefficient, *r*, was determined for normally distributed data.

Fish which initially developed at 24 °C and 31 °C will, henceforth, be referred to as 24 °C fish and 31 °C fish, respectively.

Larvae at first swimming

Results obtained for development time, body size, muscle cellularity and muscle nuclei in first swimming larvae are summarised in Table 1. The time between fertilisation and first swimming was greater in 24 °C fish than in 31 °C fish (Table 1). 24 °C fish were, however, of greater size at first swimming than their 31 °C counterparts (Table 1). Development temperature did not affect the total cross-sectional area of muscle in larvae at first swimming, but 24 °C fish possessed more muscle fibres, of a smaller size, than 31 °C fish at this stage of development (Table 1). The total number of muscle nuclei in larvae at first swimming was also greater for the 24 °C fish, but the rates at which muscle nuclei were labelled with BrdU were greater at first swimming in 31 °C fish (Table 1).

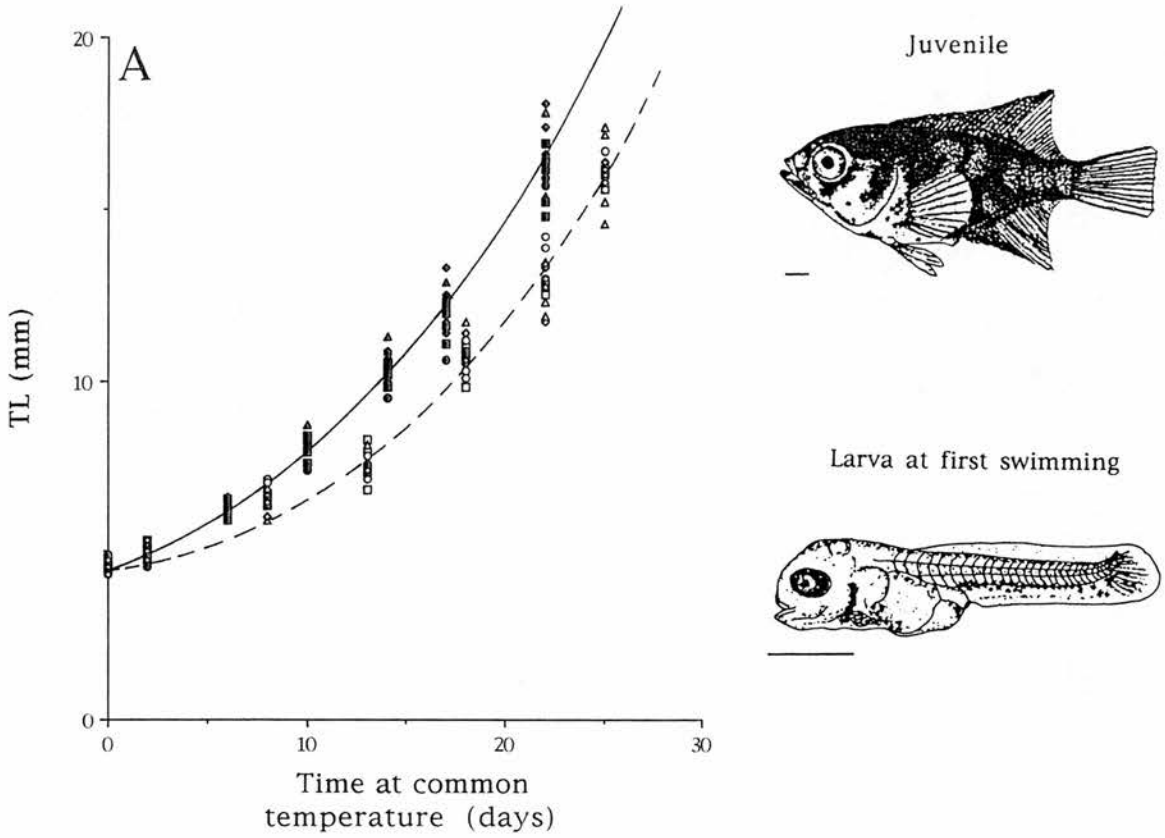
Somatic and muscle growth

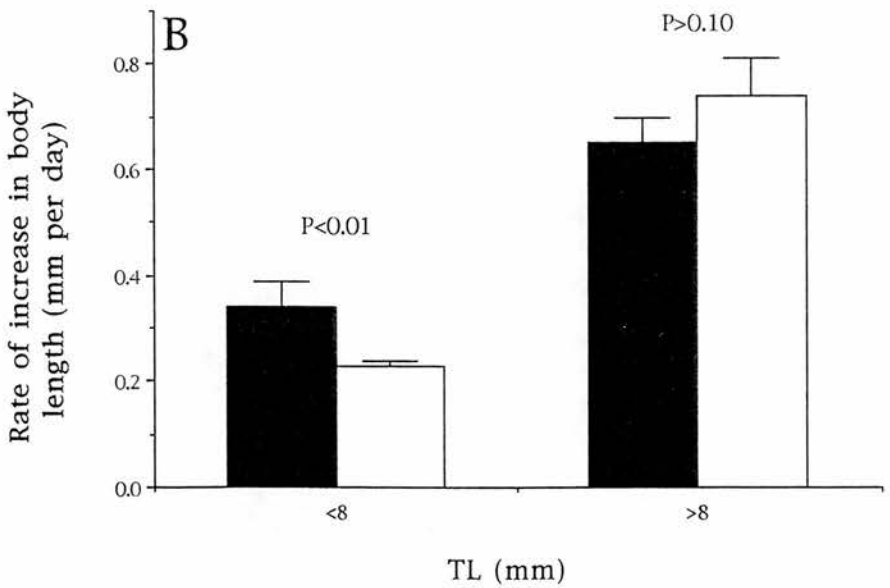
After equivalent times at the common temperature, 24 °C fish were of greater TL than 31 °C fish (Fig 1A) (t=9.459; P<0.01). For example, after 22 d the TLs of 24 °C and 31 °C fish were 16.4±0.2 mm and 12.9±0.2 mm, respectively. Growth rates were greater in 24 °C fish than in 31 °C fish up to 8 mm TL (U=24; P<0.05), but were not significantly different thereafter (U=66; P>0.10) (Fig 1B). Development temperature had no effect on the

Table 1. Summary of results obtained for angelfish larvae at first swimming. Data for fish from all four spawnings were combined. (* no difference between the groups; ** value for fish which initially developed at 24 °C is greater than that for fish which initially developed at 31 °C; *** value for fish which initially developed at 31 °C is greater than that for fish which initially developed at 24 °C.)

Variable	Early thermal experience of fish			
	24 °C	31 °C	*t*	*P*
Time after fertilization for 70 % of larvae (d)	6.26±0.17 (n=4)	2.38±0.11 (n=4)	19.162	<0.01**
TL (mm)	4.69±0.03 (n=16)	4.53±0.05 (n=16)	3.578	<0.01**
Total cross-sectional area of muscle (mm^2)	0.0278±0.0008 (n=16)	0.0281±0.0007 (n=16)	0.283	>0.10*
Number of muscle fibres present	696.6±5.8 (n=16)	650.7±5.5 (n=16)	5.740	<0.01**
Mean cross-sectional area of fibres (μm^2)	39.8±0.9 (n=16)	43.1±0.8 (n=16)	2.609	<0.02***
Number of muscle nuclei	495.8±1.2 (n=16)	460.7±1.6 (n=16)	17.353	<0.01**
Number of nuclei per mm^2 muscle	18084±521 (n=16)	16574±429 (n=16)	2.237	>0.02*
Number of muscle nuclei labelled with BrdU per hour	3.7±0.3 (n=12)	5.9±0.4 (n=12)	15.118	<0.01***
Number of nuclei labelled with BrdU per mm^2 muscle per hour	129.3±5.4 (n=12)	214.3±9.2 (n=12)	7.978	<0.01***
Percentage of muscle nuclei labelled with BrdU per hour	0.74±0.02 (n=12)	1.28±0.03 (n=12)	16.875	<0.01***

Fig 1. A: Somatic growth of 24 °C and 31 °C angelfish at 26 °C, and appearance of fish at the beginning (first swimming larvae) and end (22-25 d-old juveniles) of the sampling regime. Each point represents the value for a single fish. Data for fish from spawnings 1 (March 1995), 2 (August 1995), 3 (November 1995) and 4 (March 1996) are illustrated by squares, circles, triangles and diamonds, respectively, with closed and open points indicating whether the early thermal experience was at 24 °C or at 31 °C, respectively. Scale bars represent 1 mm. B: Rates at which body lengths increased in 24 °C (closed columns) and 31 °C (open columns) angelfish. Mean values of fish, from all four spawnings, are shown ±SE.

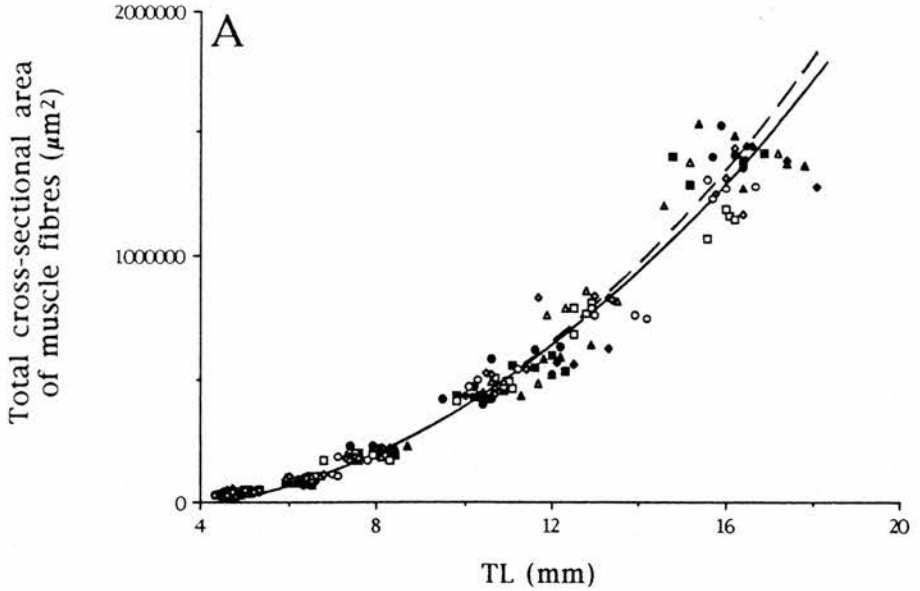

A
20
10
0
TL (mm)
0
10
20
30
Time at common
temperature (days)
Juvenile
Larva at first swimming

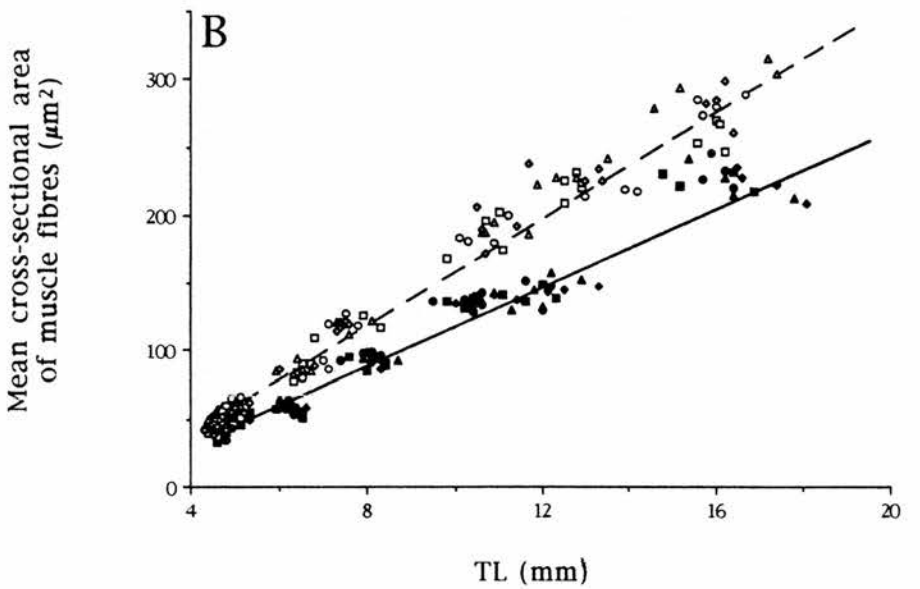

B
P>0.10
P<0.01
0.8
0.6
0.4
0.2
0.0
Rate of increase in body
length (mm per day)
<8
>8
TL (mm)

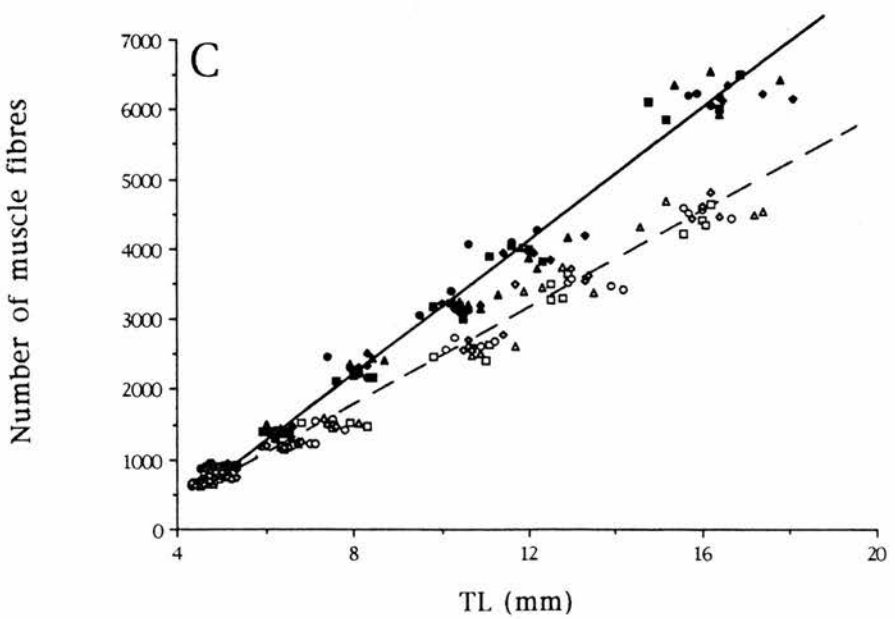
total cross-sectional area of myotomal muscle in fish of a given size (Fig 2A) (t=0.875; P>0.10). There was an effect, however, on muscle cellularity: 24 °C fish had more fibres (Fig 2C) (t=18.778; P<0.01), of a smaller mean size (Fig 2B) (t=14.773; P<0.01), than equivalently sized 31 °C fish. For example, the mean numbers of muscle fibres in angelfish of 16-17 mm TL were 6239±89 (n=8 fish) for 24 °C fish and 4540±54 (n=8 fish) for 31 °C fish. However, the mean cross-sectional areas of fibres were 226.1±2.7 μm^2 (n=8 fish) and 274.0±5.9 μm^2 (n=8 fish) for 16-17 mm angelfish initially incubated at 24 °C and 31 °C, respectively.

The percentage contributions of hypertrophy and hyperplasia to muscle growth were also calculated. Briefly this was done by determining the orders of magnitude by which fibre numbers and fibre sizes increased over a given increase in TL, then expressing one order of magnitude as a percentage of the two combined. For example, when 31 °C fish, from the fourth spawning, grew from 4.5±0.1 mm TL to 6.4±0.2 mm TL, fibre numbers increased by 1.84 times (from 657±14 to 1206±14) while fibre cross-sectional areas increased by 1.98 times (from 43.2±1.9 μm^2 to 85.7±1.3 μm^2). The percentage contribution of hyperplasia during this phase in the growth of the animals was thus 48.1 % (i.e., [1.84/(1.84+1.98)] x 100 %) while the percentage contribution of hypertrophy was 51.9 % (i.e., [1.98/(1.84+1.98)] x 100 %). Between first swimming and 8 mm TL, the percentage contribution of hypertrophy to muscle growth was greater for 31 °C fish than for 24 °C fish (U=0; P<0.01) (Fig 3). However, beyond 8 mm TL, the contribution of hypertrophy to muscle growth in 24 °C

Fig 2. Cross-sectional area and number of muscle fibres in 24 °C and 31 °C angelfish reared at 26 °C. Each point shows the value for a single fish. Data for fish from spawnings 1, 2, 3 and 4 are illustrated by squares, circles, triangles and diamonds, respectively, with closed and open points indicating whether the early thermal experience was at 24 °C or at 31 °C, respectively.


A
Total cross-sectional area of muscle fibres (μm²)
2000000
1000000
0
4
8
12
16
20
TL (mm)


B
Mean cross-sectional area of muscle fibres (μm²)
300
200
100
0
4
8
12
16
20
TL (mm)


C
Number of muscle fibres
7000
6000
5000
4000
3000
2000
1000
0
4
8
12
16
20
TL (mm)

fish was not significantly different from that in 31 °C fish (U=54; P>0.10) (Fig 3).

Muscle nuclei and nuclear proliferation

There was no difference between the number of muscle nuclei per myotome in 24 °C fish and 31 °C fish of equivalent size (Fig 4A) (t=0.527; P>0.10). The nuclear density (i.e., the number of nuclei per mm^2 muscle) was also the same for 24 °C and 31 °C fish (Fig 4B) (t=0.367; P>0.10). Nuclear density was initially high but decreased markedly during the initial growth of the animals (Fig 4B). After fish reached a TL of about 8 mm, however, nuclear density values began levelling off at about 7000-8000 nuclei per mm^2 muscle cross-sectional area (Fig 4B).

The relationships between the number of muscle nuclei labelled with BrdU and the time spent by fish in the labelling solution were all approximately linear (r=0.946-0.993; P<0.01) (representative data are shown in Fig 5). The gradients of these lines were thus taken as the rates at which nuclei were labelled with BrdU. Rates calculated in this manner are plotted as a function of TL in Fig 6. The number of muscle nuclei labelled with BrdU per hour was greater in 31 °C fish than in 24 °C fish of equivalent TL (t=6.427; P<0.01) (Fig 6A). For example, the mean numbers of muscle nuclei (in cross-section) labelled with BrdU per hour in 31 °C fish were about 11 % greater than those in equivalently sized 24 °C fish. The percentage of muscle nuclei labelled with BrdU per hour in 31 °C fish was also greater than

Fig 3. Percentage contribution of hypertrophy to muscle growth in angelfish reared at 26 °C but with different early thermal experiences. Each point gives the value obtained when data for all fish sampled over a 24 h period were combined. Data for fish from spawnings 1, 2, 3 and 4 are illustrated by squares, circles, triangles and diamonds, respectively, with closed and open points indicating whether the early thermal experience was at 24 °C or at 31 °C, respectively. (The sum of the percentage contributions of hyperplasia and hypertrophy to muscle growth is 100 %.)

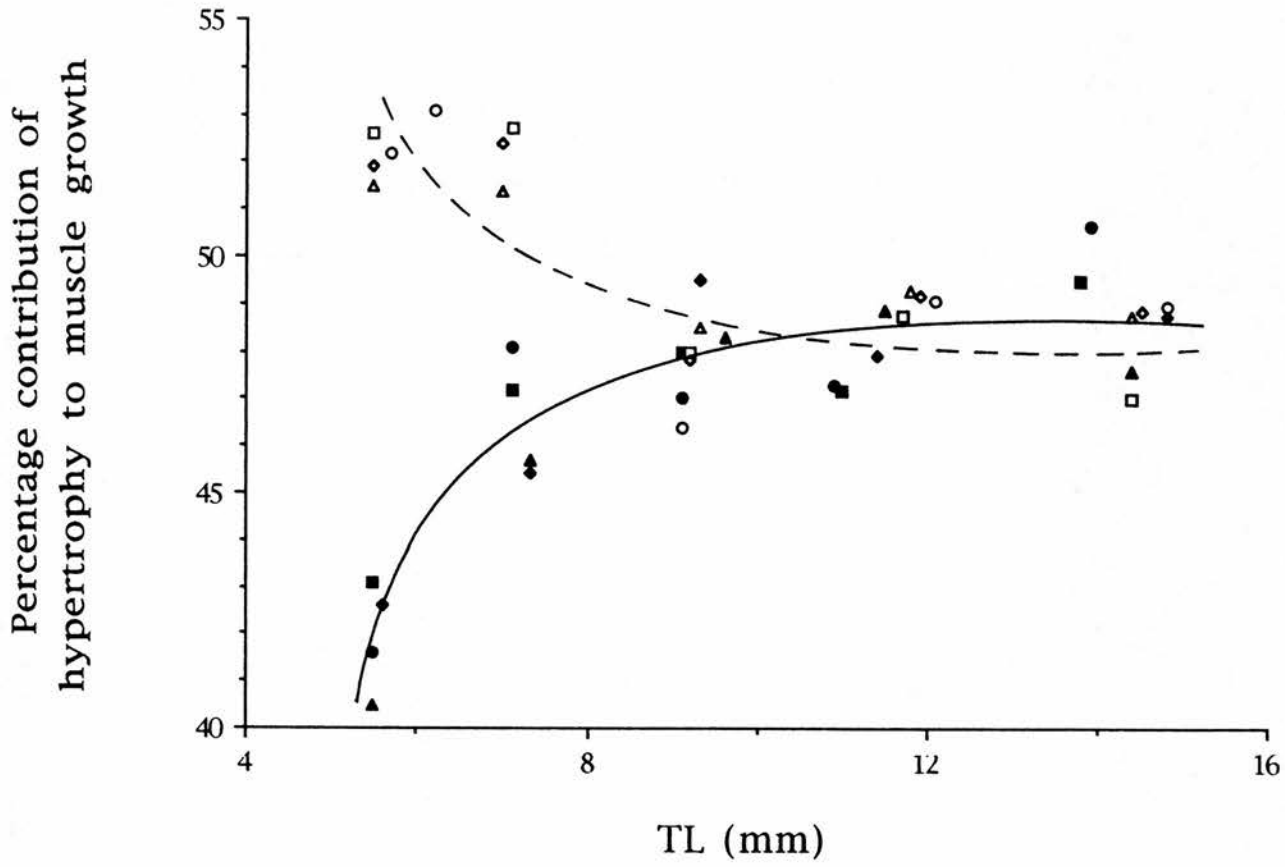
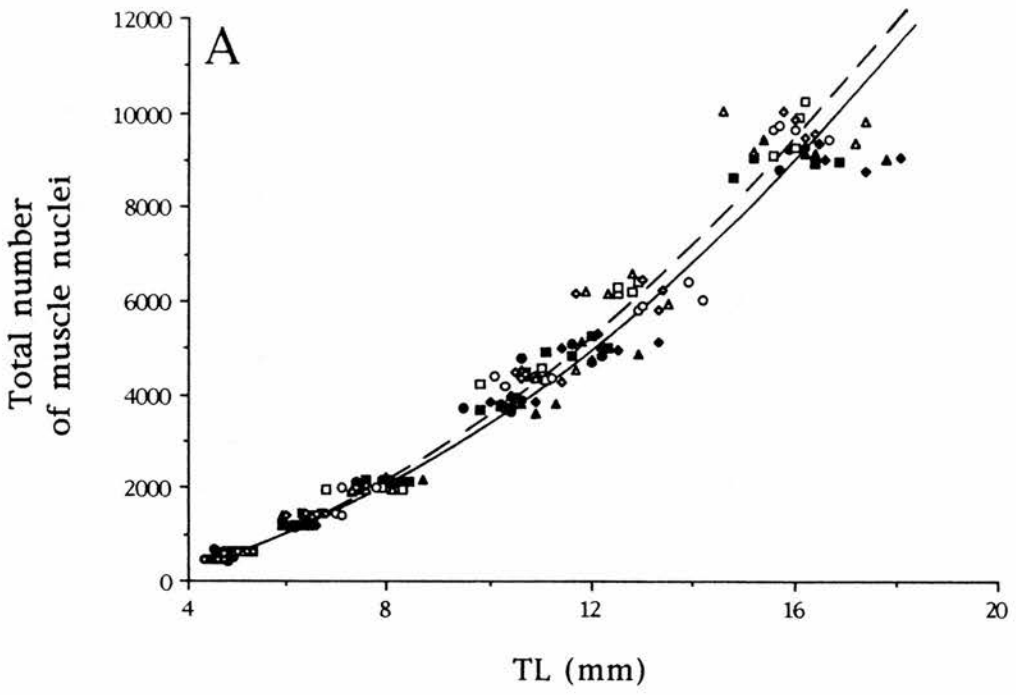

Percentage contribution of hypertrophy to muscle growth
55
50
45
40
4
8
12
16
TL (mm)

Fig 4. Number (A) and density (B) of muscle nuclei in angelfish reared at 26 °C but with different early thermal experiences. Each point shows the value for a single fish. Data for fish from spawnings 1, 2, 3 and 4 are illustrated by squares, circles, triangles and diamonds, respectively, with closed and open points indicating whether the early thermal experience was at 24 °C or at 31 °C, respectively.


A
Total number of muscle nuclei
12000
10000
8000
6000
4000
2000
0
4
8
12
16
20
TL (mm)

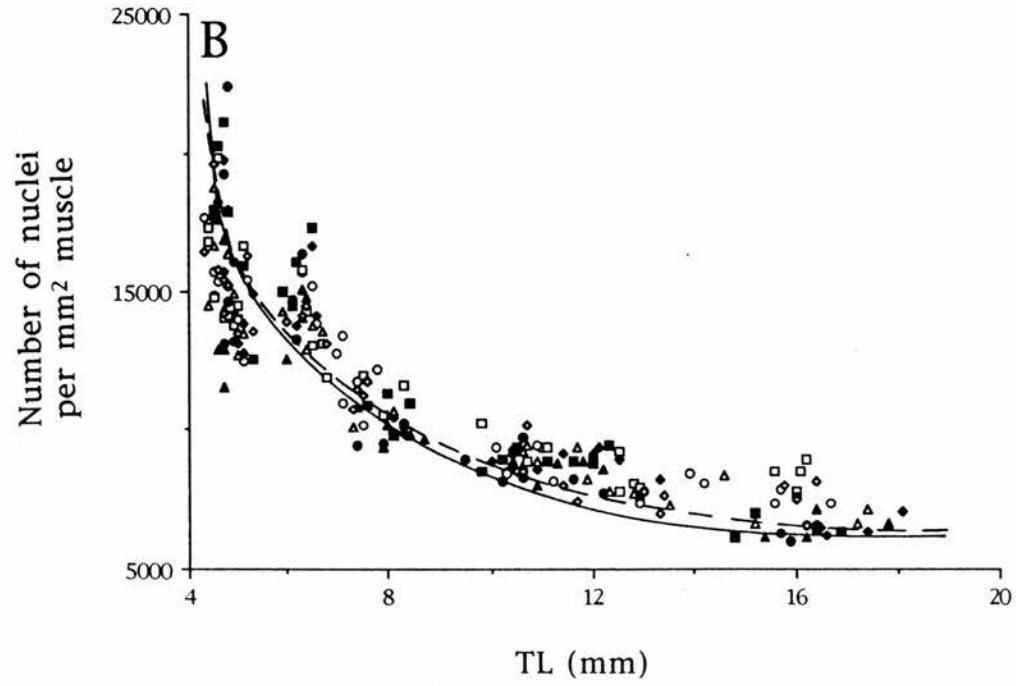
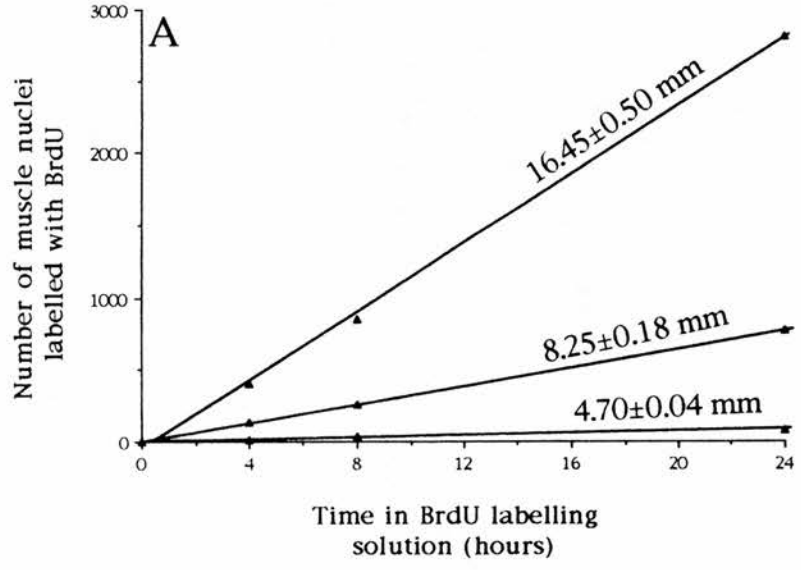
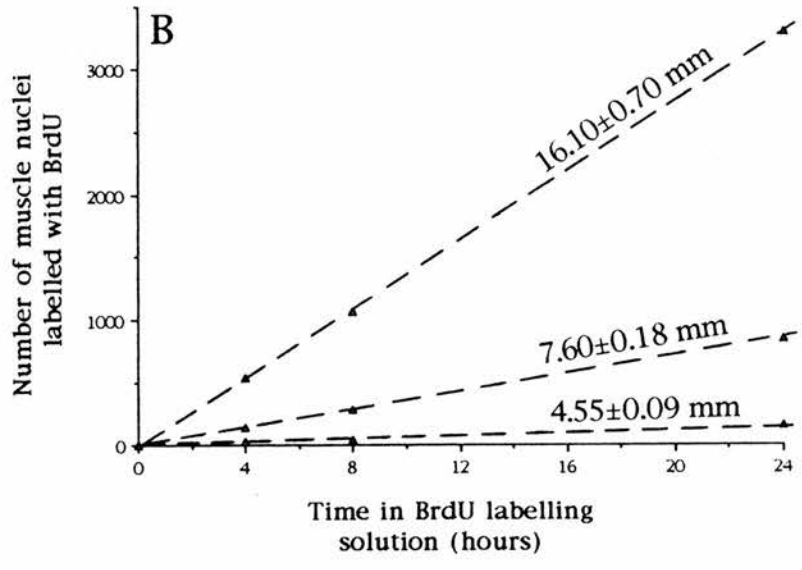
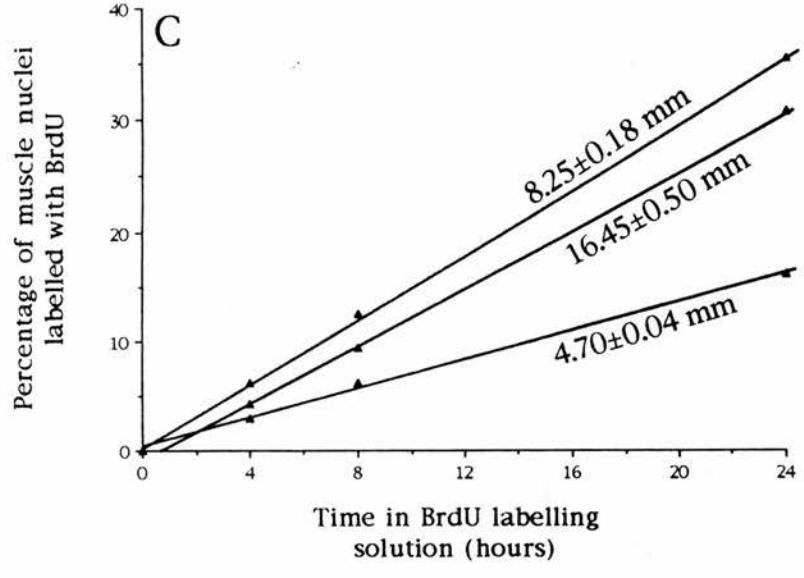

B
Number of nuclei per mm² muscle
25000
15000
5000
4
8
12
16
20
TL (mm)

Fig 5. Number and percentage of muscle nuclei labelled with BrdU in relation to time spent by 24 °C (A and C) and 31 °C (B and D) fish in the BrdU labelling solution. For clarity only data for fish from the third spawning, with TLs of about 4, 8 and 16 mm, are shown. Each point shows the value for a single fish.


A
Number of muscle nuclei labelled with BrdU
3000
2000
1000
0
16.45±0.50 mm
8.25±0.18 mm
4.70±0.04 mm
0
4
8
12
16
20
24
Time in BrdU labelling solution (hours)


B
Number of muscle nuclei labelled with BrdU
3000
2000
1000
0
16.10±0.70 mm
7.60±0.18 mm
4.55±0.09 mm
0
4
8
12
16
20
24
Time in BrdU labelling solution (hours)


C
Percentage of muscle nuclei labelled with BrdU
40
30
20
10
0
8.25±0.18 mm
16.45±0.50 mm
4.70±0.04 mm
0
4
8
12
16
20
24
Time in BrdU labelling solution (hours)

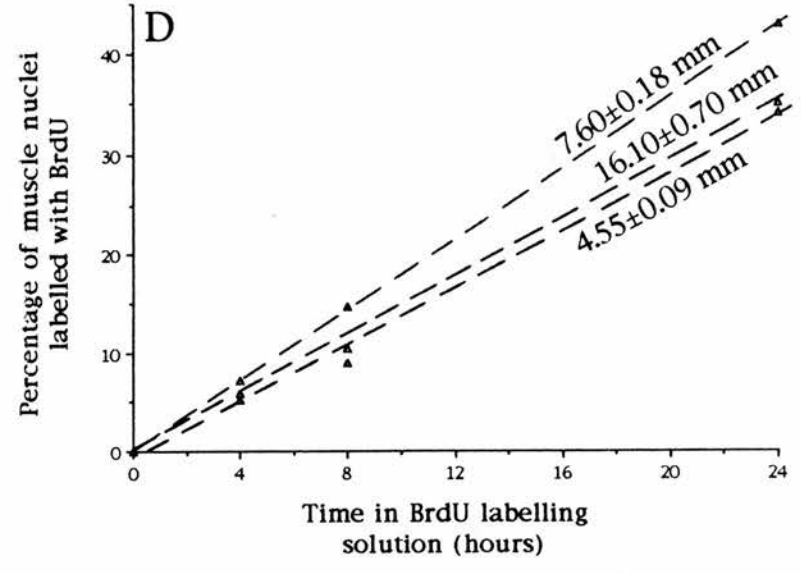
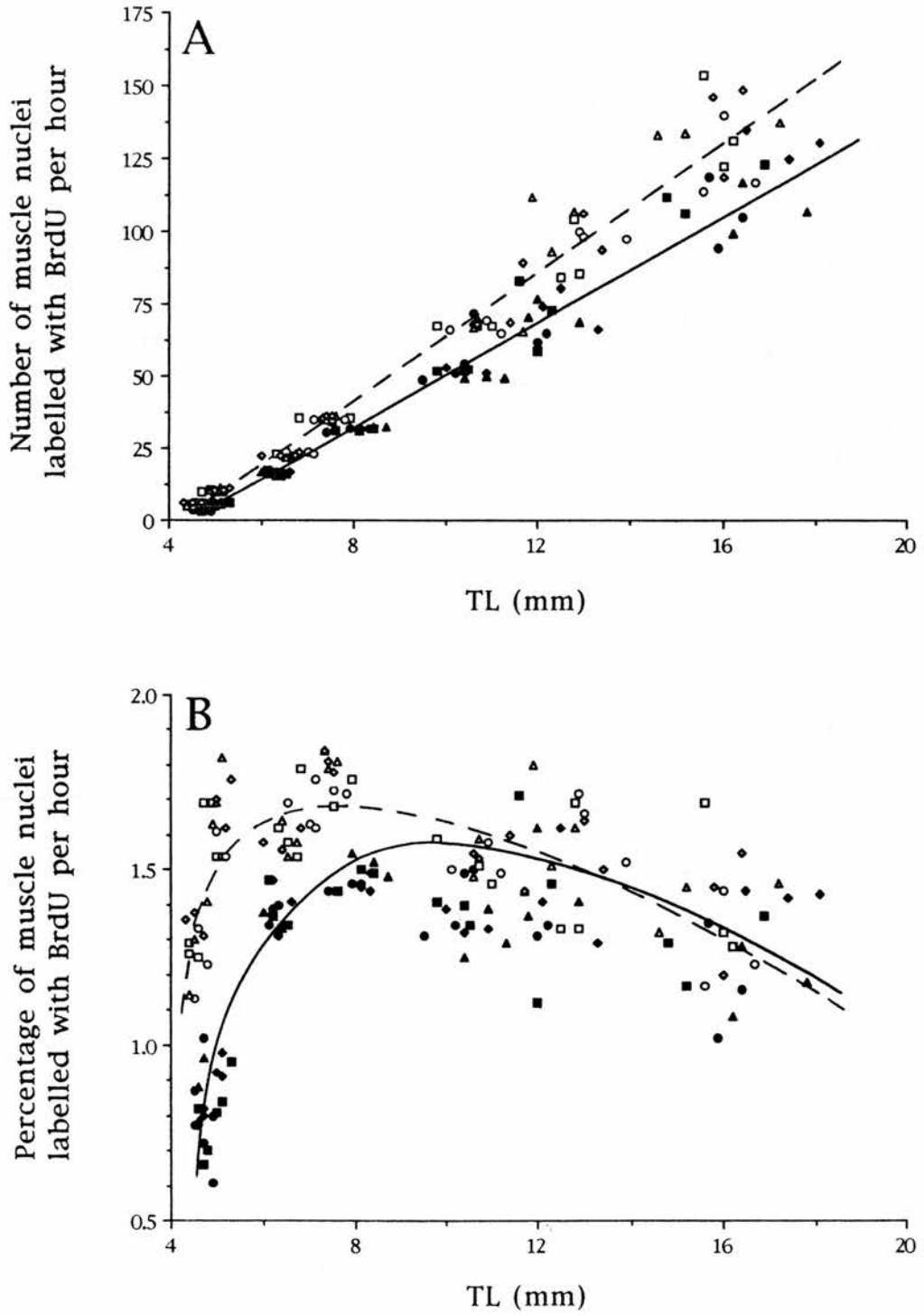

D
Percentage of muscle nuclei labelled with BrdU
40
30
20
10
0
7.60±0.18 mm
16.10±0.70 mm
4.55±0.09 mm
0
4
8
12
16
20
24
Time in BrdU labelling solution (hours)

Fig 6. Absolute and relative values for the rate at which muscle nuclei were labelled with BrdU in angelfish reared at 26 °C but with different early thermal experiences. Each point shows the value for a single fish. Data for fish from spawnings 1, 2, 3 and 4 are illustrated by squares, circles, triangles and diamonds, respectively, with closed and open points indicating whether the early thermal experience was at 24 °C or at 31 °C, respectively.


A
Number of muscle nuclei labelled with BrdU per hour
175
150
125
100
75
50
25
0
4
8
12
16
20
TL (mm)
B
Percentage of muscle nuclei labelled with BrdU per hour
2.0
1.5
1.0
0.5
4
8
12
16
20
TL (mm)

that of 24 °C fish (U=0; P<0.01) when TLs were less than 8 mm (Fig 6B). For fish greater than 8 mm TL, however, the percentage of nuclei labelled with BrdU in unit time was independent of early thermal experience (U=426.5; P>0.10) (Fig 6B).

Embryonic development

As for most other fish (for a review, see Blaxter, 1988), an increase in temperature had an accelerating effect on the rate of angelfish development, with the time from fertilisation to first swimming being 2.6 times longer at 24 °C than at 31 °C (Table 1). 24 °C fish were, however, of greater TL at first swimming than 31 °C fish (Table 1). Development time was also found to be inversely related to body size when embryos of the Atlantic salmon (Nathanailides *et al*, 1995) and the coral-reef fish *Siganus randalli* Woodland (Collins and Nelson, 1993) were incubated at different temperatures. These results suggest that temperature can influence the efficiency at which nutrient supplies (all endogenous for pre-swimming angelfish, as feeding commences only after first swimming) are converted to body tissue in developing fish. This phenomenon has been demonstrated in the Walleye pollock, *Theragra chalcogramma* Pallas in which embryos incubated at low temperature (6 °C) have the same RNA/DNA ratios as those incubated at high temperature (12 °C) despite having lower rates of yolk utilisation (Canino, 1994).

Muscle cellularity and somatic growth

Development temperature also affected the muscle cellularity of fish and, thus, had an effect on the balance between

hypertrophy and hyperplasia during the growth of their axial muscle. Similar results were found in a study on the Atlantic salmon (Nathanailides *et al*, 1995). When salmon larvae with different early thermal experiences were reared at a common temperature (11 °C), larvae from embryos incubated at low temperature (5-10 °C) were found to use hypertrophy to a greater extent than those from embryos incubated at high temperature (11 °C) (Nathanailides *et al*, 1995). Differences between the muscle cellularities of low and high temperature salmon were eliminated, however, after about 3 weeks at the common temperature (Nathanailides *et al*, 1995). Differences between the muscle cellularities of 24 °C and 31 °C angelfish, observed at first swimming (Table 1), were still present after about 25 d at the common temperature. At both first swimming and the end of the sampling regime, 24 °C fish had more muscle fibres, of a smaller average size, than 31 °C fish (Table 1 and Fig 2). This suggested that 24 °C fish might have been more dependent on hyperplastic processes for muscle growth while 31 °C fish might have relied more on hypertrophy. However, when the contributions of hyperplasia and hypertrophy were calculated, such differences were found only when values for fish with TLs less than 8 mm were compared (Fig 3). Beyond 8 mm TL the ratios of the contribution of hyperplasia to that of hypertrophy in 31 °C fish were the same as those in their 24 °C counterparts (Fig 3). Our calculations also showed that, while 24 °C fish were of greater TL than 31 °C fish after equivalent times at 26 °C (Fig 1A), the growth rates of 24 °C fish were greater than those of 31 °C fish only when TLs were below 8 mm, and not significantly different thereafter (Fig 1B). These findings indicate that, as in most other fish

(Weatherley and Gill, 1987; Weatherley *et al*, 1988; Weatherley, 1990), rapid growth in the angelfish is positively correlated with the contribution of hyperplasia to muscle growth. The findings also suggest that the differences between the body sizes/muscle cellularities of 24 °C and 31 °C fish observed throughout the experiment were vestiges of an effect of development temperature which acted for only a brief period (first swimming to about 8 mm TL) after fish were transferred to the common temperature.

Nuclear numbers and myosatellite cell turnover kinetics

There was no difference between the numbers and densities of muscle nuclei in equivalently sized 24 °C and 31 °C fish (Fig 4). However, while the number of muscle nuclei increased as TL, and thus muscle volume, increased (Fig 4A), the nuclear density decreased between first swimming and 8 mm TL and began to plateau off thereafter (Fig 4B). Since all nuclei usually contain the same amount of DNA (Enesco and Puddy, 1964), our results suggest a decrease in the DNA content of muscle during initial growth. A decreasing DNA content of muscle tissue is usually found at a stage of growth when many new fibres are present. In young mice (Cardasis and Cooper, 1975) and in chicken (Matthew and Moore, 1987) a decrease in DNA/cytoplasm ratio was found during early growth when there are many small fibres. A similar decrease in DNA concentration with increasing length was found by Luquet and Durand (1970) in young rainbow trout. An explanation for this phenomenon is that hyperplasia is brought

about by fusion of myogenic cells (myoblasts or myosatellites) with very little cytoplasm. This is followed by an increase in the amount of cytoplasm of the new fibres until a critical myonucleus/sarcoplasm ratio is reached. Thus the decreasing DNA/cytoplasm ratio is caused by the hypertrophy that accompanies or closely follows hyperplasia.

After the critical myonucleus/sarcoplasm ratio has been reached, increase in muscle fibre size can occur only if additional muscle nuclei are provided by myosatellite cells (Cardasis and Cooper, 1975; Enesco and Puddy, 1964; Campion, 1984). BrdU labels myosatellite cells during the S phase of the cell cycle (Schultz, 1996), but at certain concentrations it can affect the ability of myosatellite cells to undergo mitotic divisions (Bischoff and Holtzer, 1970; Labrecque *et al*, 1991). It was not possible to determine the levels of BrdU available to cells in our study, but the finding that the number of BrdU-labelled nuclei in the muscle of fish was positively correlated with labelling time (Fig 5) suggested that the concentrations of BrdU used were at a level that did not alter myosatellite cell mitotic behaviour. This result also suggested that not all proliferating myosatellites were at the same phase of the cell cycle (i.e., the divisions of the myosatellite cells were asynchronous) (Wimber and Quastler, 1963).

Absolute values of the rates at which muscle nuclei were labelled with BrdU were greater in 31 °C fish than in equivalently sized 24 °C fish (Fig 6A). However, when the rates were expressed as a percentage of the number of muscle nuclei present they were, like the ratio of hyperplasia to hypertrophy, found to be different

only when 24 °C and 31 °C fish were smaller than 8 mm TL and were not different thereafter (Fig 6B). Thus, fish which used hypertrophic processes to a greater extent (i.e., 31 °C fish with TLs less than 8 mm) had a greater percentage of their muscle nuclei labelled with BrdU in unit time, suggesting that myosatellite cell proliferation rates are positively correlated to the contribution of hypertrophy to muscle growth. This has also been found in the common carp, *Cyprinus carpio* L., where the cell cycle time of myosatellites decreases as animals increase in size and hypertrophic processes become more important to muscle growth (Koumans *et al*, 1994).

A possible explanation for this situation is provided by *in vitro* studies on the common carp which suggest that hypertrophy depends more on myosatellite cell proliferation while hyperplasia is more reliant on differentiating myosatellites (Koumans *et al*, 1993). According to Koumans *et al* (1993), proliferating myosatellites produce daughter nuclei which become incorporated into muscle fibres that are increasing in size, while differentiated myosatellites fuse to form new myotubes/immature muscle fibres. Whether a myosatellite cell proliferates or differentiates is determined by the balance between factors that activate or inhibit the different pathways (Olson, 1992). Thyroid hormones, for example, result in increased MyoD gene transcription in myosatellites and the promotion of differentiation (Muscat *et al*, 1994; Marchal *et al*, 1995). In contrast, insulin-like growth factors promote the production of cyclins D and E and, thus, increased myosatellite proliferation (Schriever *et al*, 1996). It is therefore tempting to speculate that development temperature probably

exerted its effect on future muscle growth by influencing the balance between proliferation and differentiation signals. Indeed, temperature has been shown, for example, to influence the development of the thyroid gland in fish (Inui and Miwa, 1985; Seikai *et al*, 1986).

The finding that the proliferation rates of myosatellites in 24 °C and 31 °C fish equalised when animals were at about 8 mm TL (Fig 6B), the size at which the nuclear densities of the muscle fibres were approaching their critical levels (Fig 4B), also suggests that factors which trigger myosatellite cell proliferation might have arisen from the muscle fibres with which they associated. This is supported by recent experiments which have demonstrated the expression of fibroblast growth factors, which promote myosatellite cell proliferation, in skeletal muscle fibres (Olwin *et al*, 1994; Hannon *et al*, 1996).

Conclusions and suggestions for future work

The results of this study thus show that development temperature not only affects the rate and nature of fish development, but also influences the future growth characteristics of fish through effects on the turnover kinetics of their myosatellite cells. The growth performance of fish might thus be governed, not only by their genetic potential and their immediate environmental conditions, but also by their early thermal experience.

We postulate that the growth mechanisms used by fish were initially determined by the levels of systemic factors that promote myosatellite cell proliferation. As muscle fibres increased in size, however, the production of proliferation factors (such as fibroblast growth factors) by muscle fibres increased. With the approach of the critical myonuclear density, these muscle fibre-produced factors probably became the dominant stimulus for myosatellite cells to proliferate. The ratio of hyperplasia to hypertrophy then became the same for fish with different early thermal experiences. We therefore suggest experiments on the effects of temperature on the development of organs and tissues which produce systemic factors (such as the thyroid gland), and on the relationship between myonuclear density and the levels of muscle-produced proliferation factors. Also, as differentiating myosatellites were not considered in our study, we suggest investigations into the relationship between fibre hyperplasia/hypertrophy and the expression of myogenic regulatory factors in myosatellites.

Chapter 5

General discussion and future prospects

Within the last decade research conducted by the Fish Muscle Research Group at the Gatty Marine Laboratory has led to an increased understanding of developmental and growth plasticity in fish (for comprehensive reviews see Johnston, 1994; Johnston *et al*, 1996). For example the temperature at which fish develop has been shown to influence the relative timing of development at both the morphological (Vieira and Johnston, 1992; Johnston, 1993; Johnston *et al*, 1995; Johnston and Vieira, 1996) and molecular (Brooks and Johnston, 1993; Crockford and Johnston, 1993) levels. The balance between hyperplasia and hypertrophy, during muscle growth in fish larvae, was also found to be influenced by rearing temperature (Johnston, 1994; Johnston *et al*, 1996).

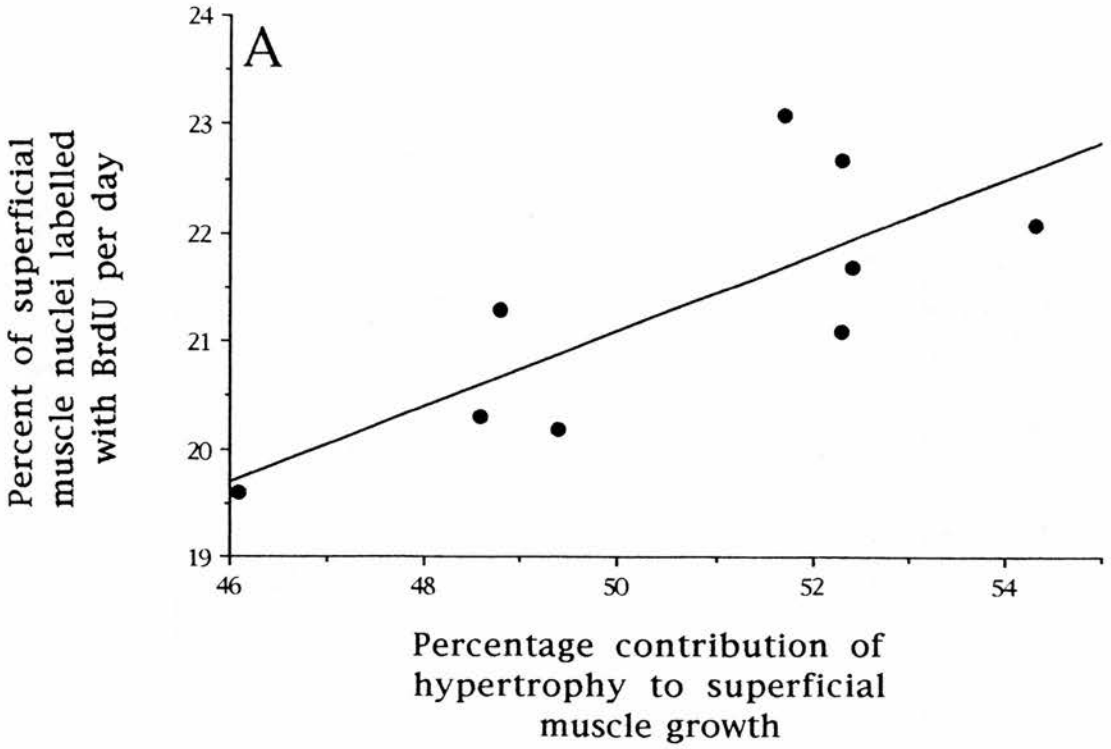
My study has added to the knowledge by investigating the effect of temperature on morphogenesis in the angelfish and the shorthorn sculpin, two species with contrasting larval strategies. It has also shown that the growth performance of fish is determined, not only by their genetic potential and their immediate environmental conditions, but also by their early thermal experience, and that the balance between hyperplasia and

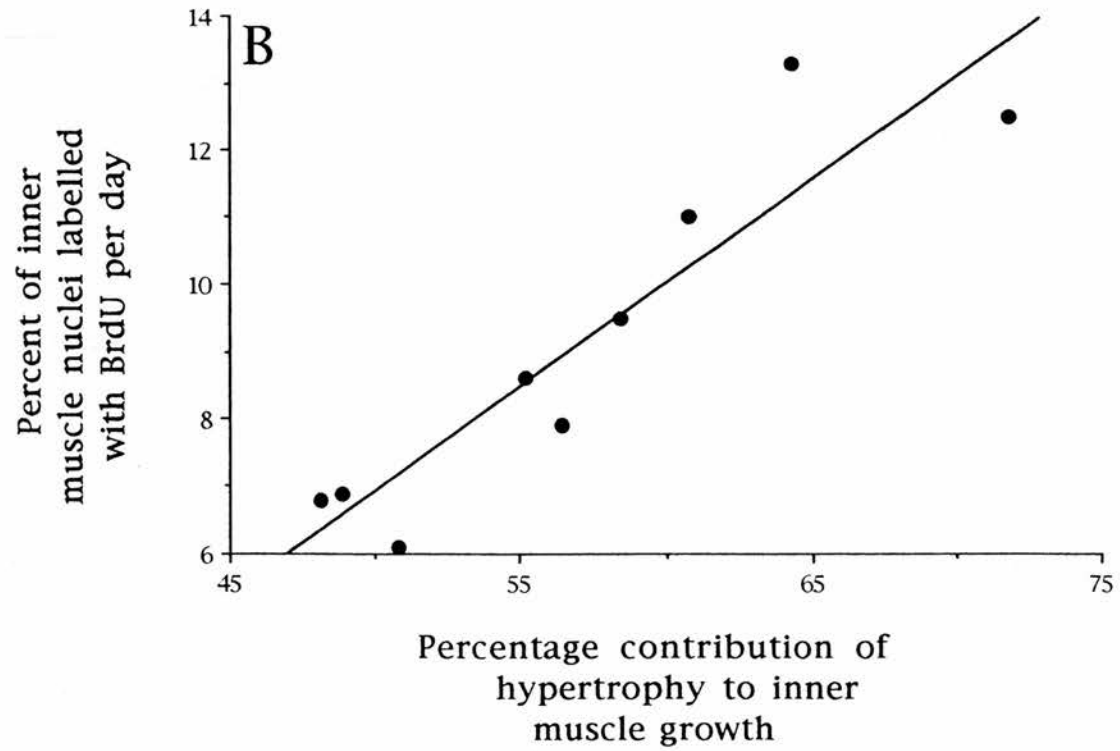
hypertrophy during fish muscle growth is dependent on the behaviour of the myosatellite cell population.

Muscle growth

A major finding of the present study is that hypertrophy requires more myosatellite cell proliferation than hyperplasia, with myosatellite proliferation rates being positively correlated with the contribution of hypertrophy to muscle growth (see Fig 1). This result, coupled with the finding that hyperplasia in fish muscle is positively related to the rate of desmin expression in myosatellite cells (Koumans *et al*, 1993; Alfei *et al*, 1994), suggest that hypertrophy relies more on myosatellite cell proliferation while hyperplasia is more dependent on differentiating myosatellites (Koumans *et al*, 1993, 1994). By controlling the balance between myosatellite cell proliferation and differentiation one might thus influence the balance between hyperplastic and hypertrophic processes during fish muscle growth. This finding has led to several new experiments aimed at determining the relationships between the mechanisms of muscle growth and myosatellite numbers, cell cycle parameters and differentiation in growing fish. Results from these studies, when coupled with the results from this thesis, would be of interest to commercial fish production since an increase in the ratio of hyperplasia to hypertrophy usually results in increases in the growth rate and ultimate size of fish (Weatherley *et al*, 1988; Weatherley, 1990;

Fig 1. Relationship between percentage contribution of hypertrophy to muscle growth and the proliferation rate of myosatellite cells in the myotomal muscle of shorhorn sculpin larvae reared at 8 and 12 °C during 1996. Data for fish from the two temperatures were combined. Each point gives the value for 4-6 fish.


A
Percent of superficial muscle nuclei labelled with BrdU per day
24
23
22
21
20
19
46
48
50
52
54
Percentage contribution of hypertrophy to superficial muscle growth

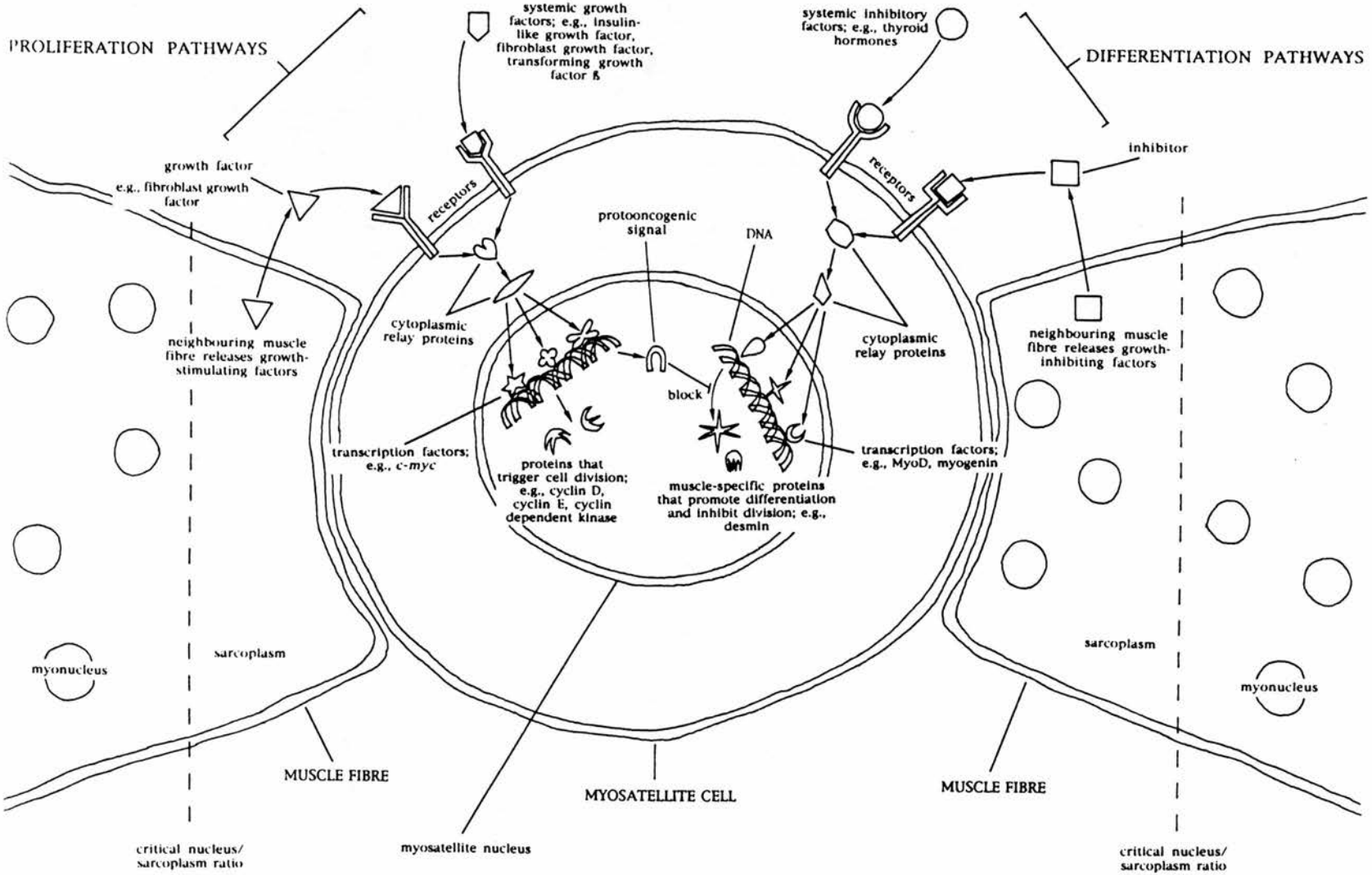

B
Percent of inner muscle nuclei labelled with BrdU per day
14
12
10
8
6
45
55
65
75
Percentage contribution of hypertrophy to inner muscle growth

Higgins and Thorpe, 1990). The results would also of interest since the cellularity of muscle has been shown to be important in determining the flesh quality, and thus marketability, of fish (Fauconneau *et al*, 1993).

Whether a myosatellite cell proliferates or differentiates is determined by the balance of opposing autocrine and paracrine cues (Olson, 1992). For example, insulin-like growth factor and fibroblast growth factor stimulate the expression of cyclins D and E, and thus promote the division of myosatellites (Schriever *et al*, 1996). In contrast, thyroid hormone increases the expression of muscle transcription factors, and thus promotes myosatellite cell differentiation (Muscat *et al*, 1994; Marchal *et al*, 1995). Effects of temperature on muscle growth may thus be mediated through an effect of temperature on the balance between proliferating and differentiating signals, and thus the balance between hypertrophic and hyperplastic processes. Indeed, temperature has been shown to influence the levels of thyroid hormones in fish (Inui and Miwa, 1985; Seikai *et al*, 1986). The increase in hypertrophy and decrease in hyperplasia which are observed during the growth of most fish (Weatherley *et al*, 1988; Weatherley, 1990), might also be explained by changes in the signals that promote myosatellite cell differentiation and proliferation as body size increases. The levels of insulin-like growth factors, for example, change in fish as they grow (Richardson *et al*, 1995).

The pathways involved in myosatellite cell proliferation and differentiation are summarised in Fig 2. Our finding that the proliferation rate of myosatellites increases as nuclear density

Fig 2. Stylised representation of the pathways via which a myosatellite cell is stimulated to either proliferate (stimulatory pathways) or differentiate (inhibitory pathways). For simplicity, the myosatellite cell is shown to be associated with two muscle fibres, but *in situ* a myosatellite cell is usually only associated with one. (Information summarised from preceding chapters; Schriever *et al*, 1996; Olson, 1992; Weinberg, 1996; Buckingham, 1994; Koumans *et al*, 1993; Hannon *et al*, 1996.)

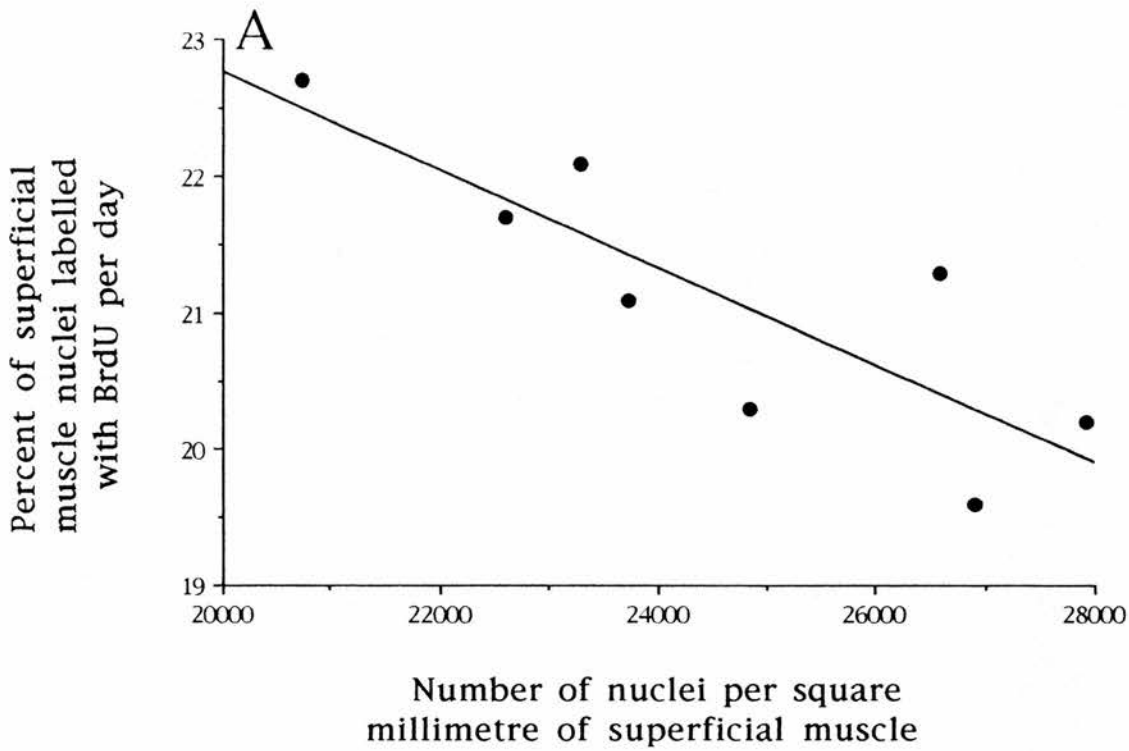

PROLIFERATION PATHWAYS
systemic growth factors; e.g., insulin-like growth factor, fibroblast growth factor, transforming growth factor ß
systemic inhibitory factors; e.g., thyroid hormones
DIFFERENTIATION PATHWAYS
growth factor
e.g., fibroblast growth factor
receptors
receptors
inhibitor
protooncogenic signal
DNA
cytoplasmic relay proteins
cytoplasmic relay proteins
neighbouring muscle fibre releases growth-stimulating factors
neighbouring muscle fibre releases growth-inhibiting factors
block
transcription factors; e.g., c-myc
transcription factors; e.g., MyoD, myogenin
proteins that trigger cell division; e.g., cyclin D, cyclin E, cyclin dependent kinase
muscle-specific proteins that promote differentiation and inhibit division; e.g., desmin
sarcoplasm
sarcoplasm
myonucleus
myonucleus
MUSCLE FIBRE
MYOSATELLITE CELL
MUSCLE FIBRE
critical nucleus/ sarcoplasm ratio
myosatellite nucleus
critical nucleus/ sarcoplasm ratio

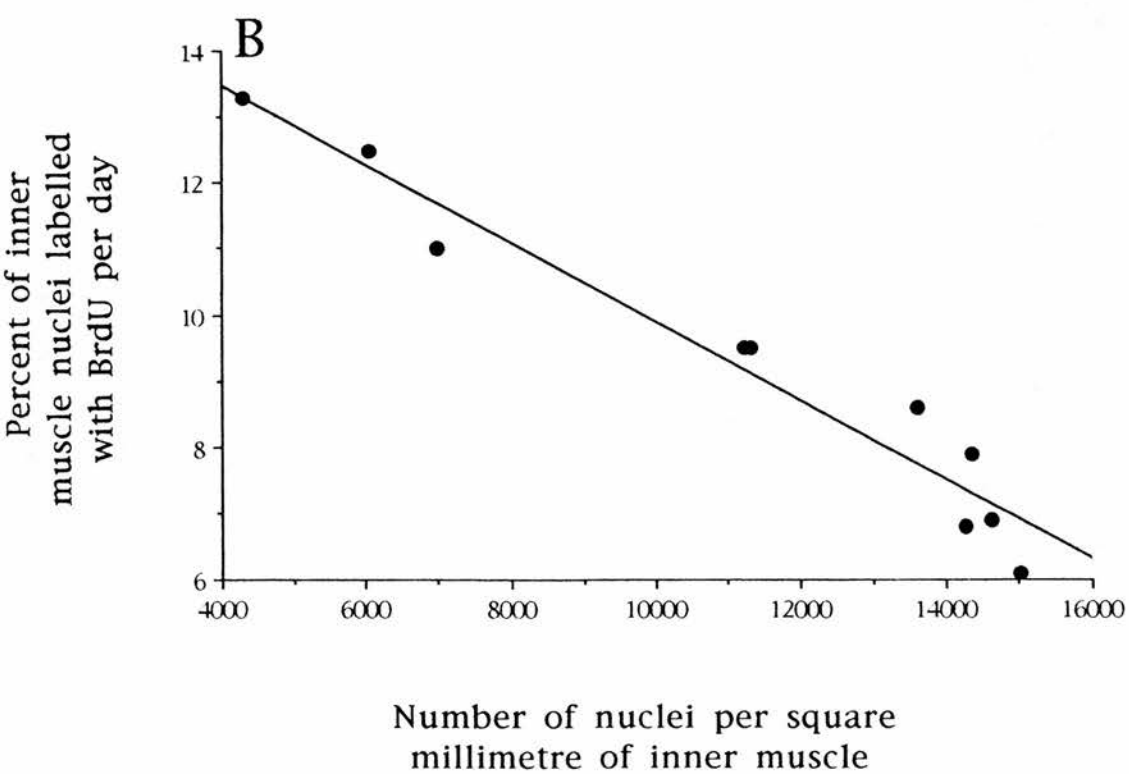
decreases (Fig 3) also suggests that signals which stimulate myosatellite cells to divide may be produced by the muscle fibres with which the myosatellites are associated (Fig 2). Support for this hypothesis comes from experiments by Olson *et al* (1994) and Hannon *et al* (1996) which have demonstrated the production of fibroblast growth factor (FGF) by muscle fibres. Further work is required, however, to determine the relationships between myonuclear density and the expression of FGF within muscle fibres, and between muscle FGF levels and myosatellite cell proliferation. Future experiments should also consider the production of differentiation signals (e.g., sonic hedgehog; Molkentin and Olson, 1996) by muscle fibres, and relate their expression to myonuclear density (see Fig 2).

Embryogenesis

Morphogenesis in embryos of the angelfish (*Pterophyllum scalare* Lichtenstein) and the shorthorn sculpin (*Myoxocephalus scorpius* L.) was influenced by incubation temperature: the appearance of certain structures, with respect to somite stage, proceeded in parallel within the natural temperature range of the species, while the appearance of others was uncoupled by temperature change of only a few degrees. For example, the formation of the pronephros, heart and dorsal aorta in sculpin embryos, and the development of motor innervation in the angelfish, all occurred at different somite stages at different

Fig 3. Relationship between myonuclear density and proliferation rate of myosatellite cells in the myotomal muscle of shorthorn sculpin larvae reared at 8 and 12 °C during 1996. Data for fish from the two temperatures were combined. Each point gives the value for 4-6 fish.


A
Percent of superficial muscle nuclei labelled with BrdU per day
23
22
21
20
19
20000
22000
24000
26000
28000
Number of nuclei per square millimetre of superficial muscle


B
Percent of inner muscle nuclei labelled with BrdU per day
14
12
10
8
6
4000
6000
8000
10000
12000
14000
16000
Number of nuclei per square millimetre of inner muscle

temperatures. There are other reports of changes in the relative timing of development in fish with temperature change (Fukuhara, 1990; Vieira and Johnston, 1992; Crockford and Johnston, 1993; Johnston *et al*, 1995). For example the sequence of pectoral fin formation, mouth opening and pigmentation of the eye has been shown to vary with environmental temperature in several tropical fish species (Fukuhara, 1990). Additionally, in the Atlantic herring (*Clupea harengus* L.) pronephric tubules form at the 40-somite stage at 12 °C but not until after the 61-somite stage at 5 °C (Johnston, 1993).

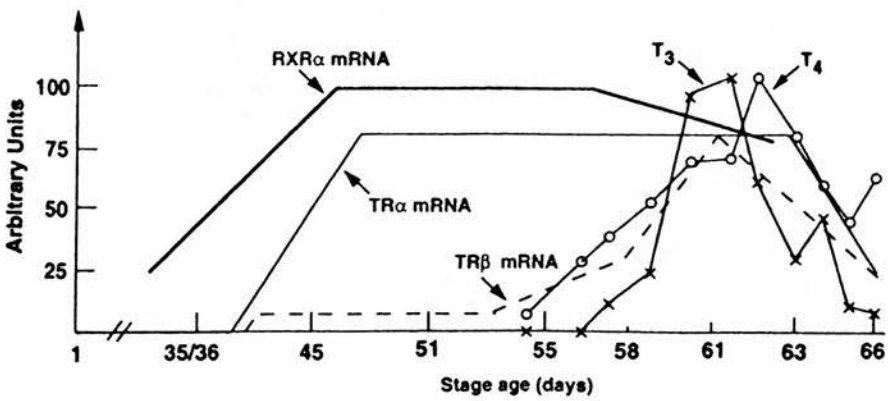
Developmental plasticity can arise from the activation of gene expression by temperature or through thermal effects on the rates and degrees of gene expression (Johnston *et al*, 1996). Gene expression during the development of morphological structures is known to be dependent on induction signals which establish cell positional information (Stolow and Shi, 1995). The production of induction signals, such as sonic hedgehog, is in turn regulated by paracrine cues (Shi *et al*, 1996). The most studied of these cues is thyroid hormone, which plays important roles in the development of muscle (Muscat *et al*, 1995; Marchal *et al*, 1995), motor innervation (Cuppini *et al*, 1994), the gut (Stolow and Shi, 1995) and paired appendages (Shi *et al*, 1996) in vertebrates.

The developmental role of thyroid hormone has been studied most extensively in the amphibian, *Xenopus laevis* (Daudin). Morphogenesis in *Xenopus* was found to be dependent not only on thyroid hormone levels, but also on levels of cytosolic thyroid hormone binding proteins, thyroid hormone receptors,

retinoic acid receptors and thyroid hormone deiodinases (see Fig 4). Collectively, these factors give the thyroid status of the animals. Thyroid status changes during development (Fig 4) and, in *Xenopus*, it has been shown that the appearance of a given morphological structure occurs only when thyroid status is of a certain value: by altering thyroid hormone levels, or the expression of genes responsible for thyroid hormone and retinoic acid receptors, one can change the developmental stage at which any given structure appears (Shi *et al*, 1996).

Temperature has been shown to influence the thyroid status of developing fish (Inui and Miwa, 1985; Seikai *et al*, 1986). If the situation in fish is similar to that in amphibians, then the alteration of thyroid hormone status by temperature might explain the appearance of certain features at different developmental stages in fish embryos subjected to different thermal regimes (see Fig 5). Studies relating organogenesis in developing fish to their thyroid status, and relating thyroid status to the expression of induction signals and organ-specific genes, are therefore suggested as avenues for future work. The results of these studies will be of immense interest to fisheries biologists and fish breeders since morphological variation during development has been shown to have a profound impact on the survival of the early developmental stages of fish (Vonherbing *et al*, 1996).

Fig 4. Correlation of the levels of endogenous thyroid hormone (TH) and the mRNAs of thyroid hormone receptor α (*TRα*), thyroid hormone receptor β (*TRβ*) and retinoic acid receptor α (*RXRα*) genes with developmental stage in *Xenopus laevis* (RXR forms heterodimers with thyroid hormone receptors, thereby increasing their affinity for thyroid hormone). The formation of the hindlimb and the intestine was found to occur only when these variables were at certain levels (Shi *et al*, 1996). C: conective tissue; E: intestinal epithelium; M: intestinal smooth muscle.



Developmemt of hindlimb

Development of intestine

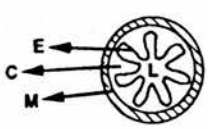
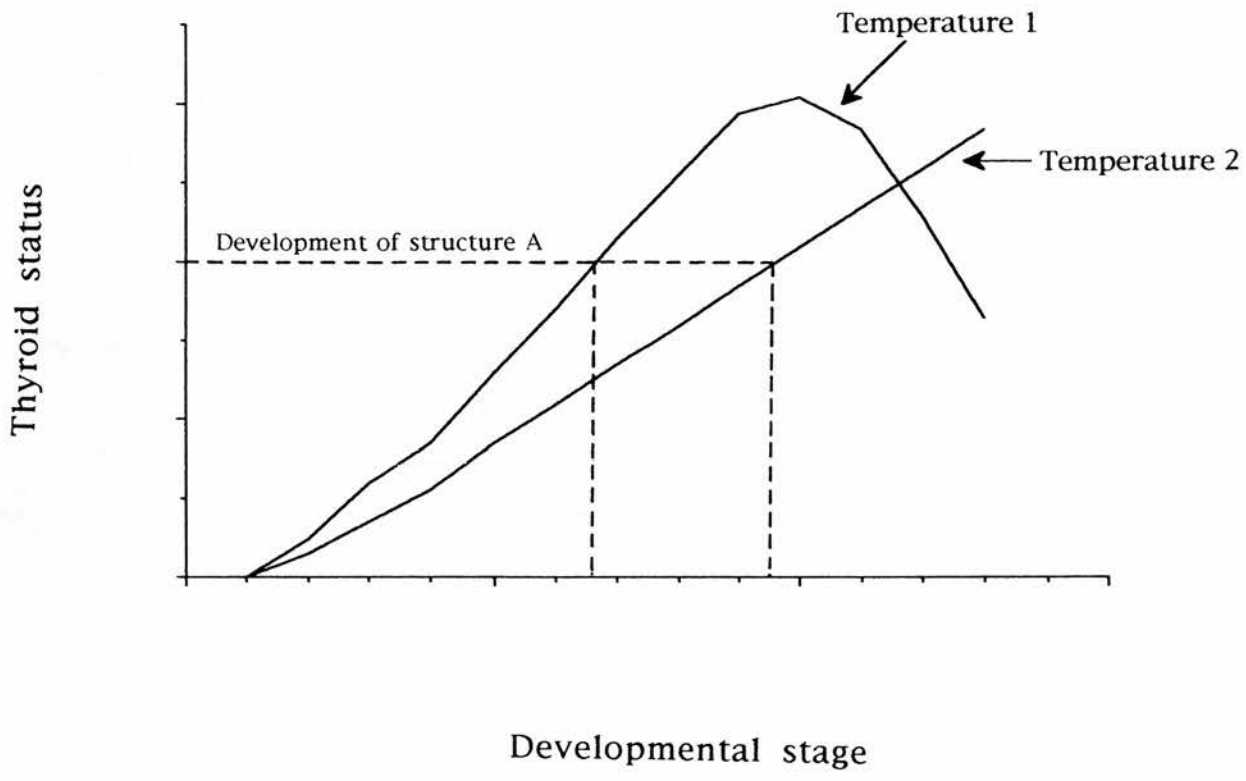


Fig 5. Thyroid status (arbitrary units) of developing vertebrate correlated with developmental stage. Thyroid status depends on the levels of thyroid hormones, thyroid hormone receptors, cytosolic thyroid hormone binding proteins, deiodinases that convert thyroxine to triiodothyronine, and retinoic acid receptors (see Shi *et al*, 1996). The development of a given organ may only occur at a given thyroid status, and by influencing the variables on which thyroid status is dependent, temperature might uncouple the development of certain organs.


Temperature 1
Temperature 2
Development of structure A
Thyroid status
Developmental stage

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