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Cellularity changes in developing red and white fish muscle at different temperatures: simulating natural environmental conditions for a temperate freshwater cyprinid

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Summary

Muscle cellularity patterns in teleost fish have normally been investigated using animals reared under constant temperature conditions. In the present study, Danube bleak (*Chalcalburnus chalcoides mento*) were reared under two different rising temperature regimes (cold, 12–16 °C; warm, 18–20 °C) designed to mimic the natural conditions experienced by the fish in temperate freshwater environments. Samples were taken from both groups of animals at intervals during their development. Transverse sections at the level of the anal vent were examined using light and electron microscopy, histochemistry and immunohistochemistry techniques. Total cross-sectional area of red and white muscle, as well as fibre numbers and fibre cross-sectional areas of one epaxial quadrant per specimen, were measured. Analysis of fibre numbers and sizes indicated that white and red myotomal muscles each develop in a different manner. In white muscle, the initial growth phase is dominated by fibre hypertrophy, while the later larval growth phase also includes significant hyperplasia. Red muscle growth is mainly due to hypertrophy within the studied developmental period. The temperature regimes applied in the present study may modify the mechanisms of muscle growth in different ways. For white muscle, pre-hatching hyperplasia (i.e. proliferation of somitic white fibre precursor cells) is reduced under the cold regime whereas post-hatching hyperplasia is not. The inverse is true for white fibre hypertrophy. A similar situation is seen with red muscle except that post-hatching hyperplasia is low and refractory to temperature. Rates of increase in relative amount of red muscle appear to depend not only upon species and temperature but also upon whether the fish have been reared under changing or constant thermal regimes. These findings are discussed in relation to 'landmark' events of early ontogeny (hatching, onset of swimming, start of exogeneous feeding) and to their implications for future accurate interpretation of temperature effects on teleost developmental biology and functional ecology.

Key words: teleost fish, embryo, larvae, myogenesis, hyperplasia, hypertrophy, fibre size, Danube bleak, *Chalcalburnus chalcoides mento*.

Introduction

Muscle growth in fish, including early myotome expansion, is a plastic process (see Weatherley, 1990) that involves a combination of enlargement of the muscle fibres already existing (hypertrophy) and the recruitment of new fibres (hyperplasia) (Koumans and Akster, 1995; Rowlerson and Veggetti, 2001). The balance between these mechanisms determines the particular rate of growth and ultimate size of the species (Weatherley et al., 1988; Zimmerman and Lowery, 1999), but is dependent upon various internal and external factors. This is true for both 'white' (fast contracting, glycolytic) and 'red' (mainly slow contracting, oxidative) fibre types.

Previous work has shown that hyperplasia and hypertrophy of fish muscle each require distinct populations of myogenic stem cells (for a review, see Koumans and Akster, 1995). These cells may add new fibres to the myotome (hyperplasia) by apposition within proliferation zones peripherally ('stratified' type of growth) and/or by insertion between the fibres already existing ('mosaic' type of growth) (growth terminology after Rowlerson and Veggetti, 2001). They may also enlarge existing fibres (hypertrophy) by fusion, thus adding new nuclei (e.g. Johnston et al., 1998; Johnston, 2001). As hyperplasia and hypertrophy may both occur in different muscle types, the myogenic cells involved must be expected to have divergent developmental programmes.

One aspect of this divergence relates to muscle cell sizes and numbers, as reflected in fibre size distributions. These vary in relation to intrinsic factors, such as the degree of ploidy (Suresh and Sheehan, 1998; Johnston et al., 1999) and strain-dependent genomic differences (Valente et al., 1999; Johnston et al., 2000a–c). Perhaps more importantly, variations may arise as a result of extrinsic environmental factors, including availability of oxygen (Matschak et al., 1995), diet (Alami-Durante et al., 1997; Galloway et al., 1999a), exercise (Sänger, 1992), and ambient temperature (e.g. Johnston et al., 1998). The resulting patterns of fibre numbers and fibre size distributions are often referred to collectively as the 'cellularity' of the muscle. Cellularity is frequently used as an indicator of changes in the hypertrophy–hyperplasia balance, which is also an important determinant of fish flesh quality and is thus of applied, economic relevance (Johnston, 1999; Johnston et al., 2000d).

Among the external factors affecting fish muscle cellularity, ambient temperature is probably the most important. Its variation is known to evoke different patterns of response, which may be complex and may appear contradictory.

Temperature may affect the hypertrophy–hyperplasia balance of developing white muscle, although the intensity and direction of the response appears to vary not only between species, but also intraspecifically, depending upon developmental stage, brood stock source and season. Such heterogeneity of white muscle cellularity response to the thermal environment is broadly documented in studies of fish from diverse teleost taxa. These include clupeids (Vieira and Johnston, 1992; Johnston et al., 1995, 1998; Temple et al., 2000), cyprinids (Nathanailides et al., 1995a; Alami-Durante et al., 1997), salmonids (Weatherley et al., 1979; Stickland et al., 1988; Usher et al., 1994; Hanel et al., 1996; Matschak et al., 1998; Johnston et al., 2000a,c), gadids (Galloway et al., 1998) and pleuronectids (Brooks and Johnston, 1994; Galloway et al., 1999b). Many of the effects of temperature on white muscle are summarized by Galloway (1999).

Temperature also affects the hypertrophy–hyperplasia balance of red fibres, but here the reactions are more directional. In situations of red muscle increase under cooler conditions, such increase relies mainly upon fibre hypertrophy, although a hyperplastic component may also be present (e.g. striped bass *Morone saxatilis*, Egginton and Sidell, 1989; turbot *Scophthalmus maximus*, Calvo and Johnston, 1992; smelt *Retropinna retropinna*, Meyer-Rochow and Ingram, 1993; pearlfish *Rutilus frisii meidingeri*, Stoiber, 1996; sea bass *Dicentrarchus labrax*, Ayala et al., 2000). However, it must be noted that under colder conditions there are also examples where total red muscle amounts apparently decrease, despite the presence of a high density of fibres (e.g. in the Atlantic cod *Gadus morhua*, Galloway et al., 1998).

Initial temperature effects on muscle cellularity at the time of hatching may be partly compensated for by the time of first feeding (Johnston and McLay, 1997; Johnston et al., 2000b). A further complicating factor is that the thermal history of the teleost embryo may be 'imprinted' and continue to exert an influence during post-embryonic muscle growth. This has been suggested for the herring *Clupea harengus* (Johnston et al., 1998) and is also likely to occur in the Atlantic salmon *Salmo salar* (Nathanailides et al., 1995b; Johnston et al., 2000b,c), Atlantic cod *G. morhua* (Galloway et al., 1998) and sea bass *D. labrax* (Ayala et al., 2000).

Singly or in combination, all of the above may serve to alter the overall ratio of white to red muscle. Cooler conditions may raise the proportion of red muscle (goldfish *Carassius auratus*, Johnston and Lucking, 1978; striped bass, Egginton and Sidell, 1989; pearlfish, Stoiber, 1996), or reduce it (Atlantic cod, Galloway et al., 1998), or leave it largely unaffected (Atlantic salmon, Usher et al., 1994; herring, Johnston et al., 1995; adult scup *Stenotomus chrysops*, Zhang et al., 1996).

Whether temperature-induced changes in relative amounts of red muscle in fish of any age are reflected in sustained activity patterns, or whether the behaviour is there to avoid extensive thermal impact on locomotion, remains unclear.

Until now, investigations of thermal effects on muscle cellularity during early ontogeny have tended to use constant temperature regimes (but see, for example, Alami-Durante et al., 2000; Johnston et al., 2000b). This differs greatly from natural situations, where the animals are subjected to sometimes extremely variable environments within a single season.

In the present study, we make allowance for natural seasonal variations in environmental temperature. Danube bleak *Chalcalburnus chalcoides mento* spawn from the end of May to the beginning of June in the shallow waters of lake tributaries and off-runs. Eggs are deposited onto gravel beds or plants and may develop and hatch within a temperature range of 9–24 °C. Optimum hatching success (>90 %) is attained between 12 ° and 17 °C under constant laboratory conditions (Herzig and Winkler, 1985, 1986). Taking into account the intrinsic temperature tolerance limits of the species, we apply stepwise increases in temperature, in two single-season regimes with different starting temperatures. We compare our results with the presently available information on muscle development under constant temperature conditions, elucidated for a variety of cyprinid fish species (Stoiber, 1991, 1996). This potential limitation (i.e. a lack of direct control experiments at constant temperatures) has been taken into account in the interpretation of the results.

Materials and methods

Rearing and sampling of fish

All Danube bleak *Chalcalburnus chalcoides mento* Agassiz used in this study were laboratory-reared from artificially inseminated eggs. Parent animals were caught in the 'Zeller Ache' river, a tributary of the Mondsee, a lake approximately 30 km east of Salzburg, Austria. Fish were kept under rising temperature schemes to simulate natural conditions. One group was reared at 12 °C with a rise in temperature to 14 °C at hatching and to 16 °C at the onset of free swimming. The second group was reared at 18 °C with a rise to 20 °C at hatching. These

Table 1. *Developmental stages of the two temperature regimes compared in the present study*

Stage	State of development	Age (d.p.f.)		Body length (mm)	
		Cold	Warm	Cold	Warm
1: 40-somite embryo	Spontaneous movements inside the eggshell, eyes unpigmented	8	3	4.6±0.2	4.5±0.2
2: Hatching (50–52 somites)	C-start responses to disturbances; eyes pigmented	11	5.5	5.4±0.1	6.2±0.2
3: Onset of free swimming	Free horizontal swimming, swimbladder filled with gas	29	10	7.6±0.1	7.6±0.1
4: First exogeneous feeding	Selective aiming of prey, gut contains food particles	38	16	7.4±0.3	8.0±0.2
5: Larva	Highly mobile, efficient shoaling, rapid body growth	77	35	9.0±0.5	8.9±0.8
6: Advanced larva	As stage 5, but larger	91	38/42	9.7±0.8	9.7±0.8

d.p.f., days post fertilization.
Body length values are means ± S.D. ($N \geqslant 6$).
Passage times through the developmental stages were much shorter under the warm temperature regime.

intervals were chosen to ensure the highest possible survival rates of fish fry while at the same time remaining within the confines of natural environmental variation. Maximum temperature variation was ±0.5 °C for both groups. The rate of water flow, recirculation, photoperiod and *ad libitum* supply of live plankton and commercial fish food of appropriate particle size were kept constant (all according to the standard procedures of the Scharfling fish breeding laboratory). Samples from both groups ($N \geqslant 6$) were taken at different developmental stages: (1) 40-somite embryo, (2) free embryo just after hatching, (3) free embryo at onset of free swimming, (4) larva at first exogeneous feeding, (5) larva, (6) advanced larva. See Balon (1985) for life history terminology. Details of time differentials of developmental stages under the two temperature regimes are provided in Table 1.

Note that in stages 1, 3, 5 and 6, fish of the two temperature groups are of equivalent average body length (size stages). By contrast, stages 2 (hatching) and 4 (first feeding) have been chosen irrespective of body size because of their probable relevance as 'thresholds' for muscle development. These stages compare animals that are assumed to have achieved similar degrees of muscle functionality (muscle sufficiently developed to shed the egg shells, stage 2, and to hunt for prey, stage 4). For these two stages, cold-bred fish are on average shorter than their warm-bred counterparts.

Sampled animals were killed by overdose of MS-222 (3-aminobenzoic acid ethyl ester, Sigma); the vitelline membranes of embryonic stages were removed with fine forceps under a stereomicroscope prior to further processing. For each stage, further sets of animals ($N \geqslant 6$) were weighed and measured to determine average body mass and length for that stage.

Within the present study, reference is also made to growth data of cyprinid fish reared at constant temperatures as a part of two previous studies. Detailed protocols are given in Stoiber (1991) (roach *Rutilus rutilus*, Danube bleak) and Stoiber (1996) (pearlfish).

Light microscopy and morphometry

Specimens to be used for morphometric measurements were immersion-fixed in Bouin's solution (Romeis, 1968). Six samples of each temperature group and stage were dehydrated in a graded series of ethanols and embedded into Technovit 7100 (Heraeus-Kultzer).

Transverse sections were cut on a Reichert-Jung 2030 microtome at the level of the anus. Semithin sections (3 μm) were mounted on glass slides and dried for 1 h on a hotplate. Sections were subsequently stained using a modified periodic acid methenamine silver (PAMS) procedure (technique modified after Hanel et al., 1996). This staining method shows good specificity for the basal lamina and thus provides contrast enhancement of the fibre outline.

To record total cross-sectional areas of red and white muscle, the silver-stained sections were photographed on standard black-and-white negative film at defined magnifications using a Reichert Polyvar microscope. Negatives were visualized on the monitor of a slide projection system previously checked for being free from distortion. The outlines of muscle areas were traced to transparent foils with an 0.35 mm ink pen. To measure the size of individual muscle fibres, fibre outlines of one epaxial quadrant per fish were traced onto paper using a drawing attachment. This method was found to be more accurate in detecting very small new fibres than tracing from photographs *via* the projection system described above.

Muscle total cross-sectional areas and fibre cross sections were measured using the digital 2D-morphometry software *MuscleMorph 1.0* (Minnich and Muska OEG, Salzburg, Austria) within the image analysis programme *Optimas*™. As red and white muscles are treated separately throughout the study, and fibre-type identification has a somewhat ambiguous history in the literature, we justify our fibre typing using immunocytochemistry to supplement standard histochemical techniques (see below).

Immunocytochemistry and histochemistry

All techniques applied used specimens that were cryofixed without pre-treatment. Freshly killed animals were oriented on small strips of aluminium foil, coated with a thin layer of Tissue-Tek O.C.T. compound (Miles, Elkhart, USA) and plunged into 2-methylbutane cooled close to its freezing point (−158 °C) by liquid nitrogen. Frozen specimens were stored in

liquid nitrogen until required for further processing. 7–15 μm transverse sections were cut on a Leitz 1720 cryostat, collected on poly-L-lysine coated slides and glass coverslips, and dried at room temperature (20 °C) for 1 h. Slides and coverslips were either used immediately or wrapped separately in aluminium foil and stored at −30 °C (slides, if briefly) or in liquid nitrogen (coverslips and slides).

To distinguish developing red fibres from prospective white fibres, sections were reacted after fixation in cold acetone (5 min, −20 °C) with the following antisera: (1) monoclonal mouse anti-chicken slow myosin heavy chain IgA (*S58*; tissue culture supernatant) diluted 1:10, and (2) polyclonal rabbit anti-teleost (sea bream *Sparus aurata*) slow muscle myosin (*4/96 3c*) diluted 1:250. The *S58* antibody was raised by F. Stockdale, Stanford University, USA and has been shown to discriminate reliably between developing red and white fibres in the zebrafish *Danio rerio* (Devoto et al., 1996; Du et al., 1997; Barresi et al., 2000). The *4/96 3c* serum is also non-commercial and was provided by A. Rowlerson, King's College, University of London, UK; it has also been tested in fish (A. Rowlerson, personal communication). To detect candidate markers of presumptive myosatellite cells and of activated myogenic cells committed to differentiation, antisera staining the tyrosine kinase receptor c-met and the myogenic regulatory factor MyoD, respectively, were employed in fish from the beginning of the larval growth phase onwards. For this purpose, cryosections were fixed after the method of Johnston et al. (1999) using 4 % paraformaldehyde followed by acetone (10 min each, at room temperature) and reacted with the following antibodies: (1) rabbit anti m-met (1:100, Santa Cruz, USA; for reactivity with c-met in mammals see Cornelison and Wold, 1997; in fish, see Johnston et al., 1999), and (2) rabbit anti MyoD (1:100, Santa Cruz, USA). Cy3-labelled rabbit anti-mouse IgG and goat anti-rabbit IgG (Jackson, West Grove, USA), diluted 1:100, were applied as secondary antibodies using indirect immunofluorescence staining (Coons et al., 1955); details of the technique are described by Stoiber (1996). Specificity examinations of antibodies in addition to the references above included negative and positive controls.

Photographs of the results were taken using a Reichert Polyvar microscope adapted for fluorescence photography. It should be noted that photographs involving the Cy3 marker (which normally appears reddish), have been digitally converted to yellow tones to increase optical contrast and aid interpretation.

Electron microscopy

Specimens for transmission electron microscopy (TEM; employed mainly for satellite cell identification) were immersion-fixed overnight at 4 °C using Karnovsky's paraformaldehyde-glutaraldehyde fixative (Karnovsky, 1963) diluted to half-concentration with cacodylate buffer (containing dimethylarsinic acid, 0.15 mol l^{-1}, pH 7.4). Specimens were post-fixed in 1 % osmium tetroxide (3 h, 4 °C), dehydrated in a graded series of ethanols and embedded into Epon 812 epoxy resin. Ultrathin (60–80 nm) cross sections were cut on a Reichert OmU 3 ultra-microtome at the level of the anus of the specimen. Sections were mounted on coated 75-mesh copper grids, contrasted with aqueous solutions of uranyl acetate and lead citrate, and viewed in a Philips EM 300 electron microscope at an acceleration voltage of 80 kV.

Data analysis

Statistical analyses of the data used commercial statistics software packages (Excel, Minitab). Means ± S.D. of fibre numbers and cross-sectional areas within one epaxial quadrant were calculated for each individual animal. Linear and logarithmic regressions were calculated and displayed. Overall temperature effects on individual red and white fibre numbers and mean fibre sizes were tested for significance using one-way analysis of covariance (ANCOVA) with temperature as a fixed factor and fish size as a covariate. Fibre size distributions of each fibre type (red, white) and developmental stage were plotted as histograms using 20 μm^2 size classes. To assess hypertrophy of the original ('first wave' somitic) fibre stocks without the complication of ongoing fibre recruitment (hyperplasia), the following rationale was applied. The numbers of red and white fibres present in one epaxial quadrant of the smallest stage 1 (40-somite embryo) specimen of Danube bleak in the present study and pearlfish from the database of Stoiber (1996) were taken as a reference to distinguish the populations of directly somite-derived (i.e. largest) red and white fibres in the subsequent stages 2–6. This was done under the premise that fibres once established grow continuously by increase in cross-sectional area and the first-established (oldest) fibres thus will always be the largest. One specimen of 18 °C-reared Danube bleak was excluded from all evaluations because of its atypical size (thus: N for 12 °C-reared fish = 36; N for 18 °C-reared fish = 35).

Results

Justification of fibre type classification

Morphometric work to compare the growth dynamics of different muscle fibre types requires reliable classification of all fibre types involved. The silver-staining procedure (PAMS) used in this study to trace red and white fibre cross-sectional contours in developing fish myotomes allows discrimination between these fibre types only by anatomical position and morphological criteria. For all developmental stages examined under both temperature regimes, the red fibres form a continuous monolayer that constitutes the entire lateral surface of the myotomes. This monolayer invaginates mid-laterally to project inwards toward the notochord, thereby sandwiching the horizontal septum (Fig. 1A). Red fibre cross-sectional profiles are rectangular, with myofibrils forming garland-like patterns (Fig. 1B). White fibre profiles are polygonal and develop a radial arrangement of myofibrils (Fig. 1B).

The results of the myofibrillar ATPase (mATPase) histochemistry and of the immunostaining with antibodies known to discriminate between red and white fibres support

the morphology-based fibre typing outlined above. The fibres of the superficial monolayer are mATPase-negative after alkaline pre-incubation, while under the same treatment the deep fibres remain positive (Fig. 1C). This is consistent with known mATPase staining properties for teleost red fibres and white fibres (e.g. Kilarski and Kozlowska, 1985; Scapolo et al., 1988; Kilarski, 1990). The superficial fibres react with the anti-slow myosin sera S58 (Fig. 1D) and 4/96 3c (not shown), which do not stain the deep fibres. The superficial fibres also react with the F59 antibody, which labels here, as in the zebrafish, red fibres more strongly than white (Fig. 1A) (for zebrafish results, see Du et al., 1997; Barresi et al., 2000). Together these findings permit classification of superficial fibres as slow contracting or 'red' and the deep fibres as fast contracting or 'white' (*sensu* Rome et al., 1988).

Intermediate muscle fibres (for fibre type characterization, see Rowlerson, 1994) are normally first detectable in cyprinids long after the onset of exogeneous feeding (Stoiber, 1996), and are certainly well beyond the developmental limits of the present study. Any possible progenitors of such fibres in the myotomes of the largest fish investigated (stage 6) are treated here as white fibres.

A few small 'tonic' type fibres (1–3 per myotome; criteria of identification given in Kilarski and Kozlowska, 1985, 1987; Sänger, 1997) were found in animals from the onset of free swimming onwards (Stoiber et al., 2002). For the purposes of the present study, however, these fibres were not treated separately but are included in the red fibre population.

Patterns of myogenic cell appearance common to both temperature regimes

From the 40-somite stage (stage 1) and even more from hatching (stage 2) onwards, small cells similar in appearance to myosatellite cells (Mauro, 1961; Campion, 1984) were found attaching to the dorsal and ventral apices of the myotomes, next to the terminations of the superficial red fibre layer. Some of these small cells are also attached to further cells within the mesenchyme layer surrounding the myotomes of that stage (Fig. 2A). By contrast, fish in the larval stages (stages 5, 6), myosatellite cells also occur inside the myotomes, together with very small fibres. These cells insert between the differentiated white fibres or at the medial boundary of the superficial red muscle layer (Fig. 2B). From the onset of free swimming, immunocytochemical staining using the m-met and MyoD antisera reveal small spots within the myotomes. The positions of these correspond to those of the above myosatellite cells and small fibres, thus appearing to confirm the TEM results (Fig. 2C,D).

Myogenic cells and very small fibres directly and unambiguously attached to the red muscle regions appear to be confined to the dorsal and ventral extremities of the red muscle layer up to and including the stage-5 larvae. By stage 6, hyperplastic growth of red muscle is apparent in the form of a few small fibres mixed with undifferentiated myosatellite-like cells close to the horizontal septum and lateral to the original red fibre layer next to the lateral line nerve (not shown).

Developmental changes of white/red muscle proportions and muscle cellularity under the two temperature regimes

The relative amounts of red muscle during early growth of the Danube bleak (as given by cross-sectional areas at the anus) together with other corresponding data of cyprinid species for comparison are shown in Fig. 3. Under rising temperature regimes there is little difference between the results for warm and cold-bred fish of the subject species (*t*-test; $P>0.05$). However, this is not the case for the same species under constant 20 °C conditions, and there is also considerable variation between results from different species (see Discussion).

Mean fibre sizes and numbers are summarized for each muscle type, developmental stage and temperature group in Table 2. It may be seen that the mean size of red fibres shows a trend towards larger values in cold-bred fish of stage 1 before hatching than in warm-bred fish of the same stage ($P=0.06$), but the reverse is true from the onset of exogeneous feeding (stage 4) onwards. Comparison of mean numbers of red fibres between temperature groups and within stages shows no significant difference at any developmental stage apart from stage 1, where the warm group has significantly more fibres than the cold ($P<0.05$) (Table 2).

Unlike red fibres, the mean sizes of white fibres are similar for the two temperature groups at stage 1 but are larger in the warm-bred animals in all following stages ($P<0.01$). This leads to 15–25 % larger mean fibre sizes in the warm-temperature stage-5 and -6 larvae (Table 2). Comparison of mean white fibre numbers between the thermal groups within each stage (Table 2) demonstrates that significantly smaller numbers are present in the cold group in stage 1. The cold group of fish has compensated for their apparent deficit of these fibres by the time of the onset of free swimming (stage 3). At the onset of exogeneous feeding (stage 4) there is an apparent transient recurrence of the cold fishes' deficit in white fibre numbers. This may be a direct consequence of the smaller body size of these animals at that stage (see Table 1) rather than a developmental trend.

To gain an overall impression of fibre development, covering all stages given in Table 2, one-way analysis of covariance (ANCOVA) was undertaken of fibre size and number, with temperature as a fixed effect and fish size (as given by total muscle cross-sectional area) as a covariate. The analysis was performed on red and white fibre data separately.

For the red fibres, the results are presented with and without the inclusion of stage 6, as it is clear that at this stage the temperature effects on cellularity have been altered. Thus, there is a small but significant difference in red fibre size, with larger fibres in the cold group in all fish up to stage 5 ($P=0.022$). This difference is no longer present if the stage-6 fish are included ($P=0.71$) (Fig. 4). In relation to overall red fibre numbers per epaxial quadrant, there is no significant temperature influence up to stage 5 ($P=0.25$). Inclusion of stage 6 does not alter the situation, although here a *P*-value of 0.075 may indicate a trend towards more red fibres in the warm-bred fish.

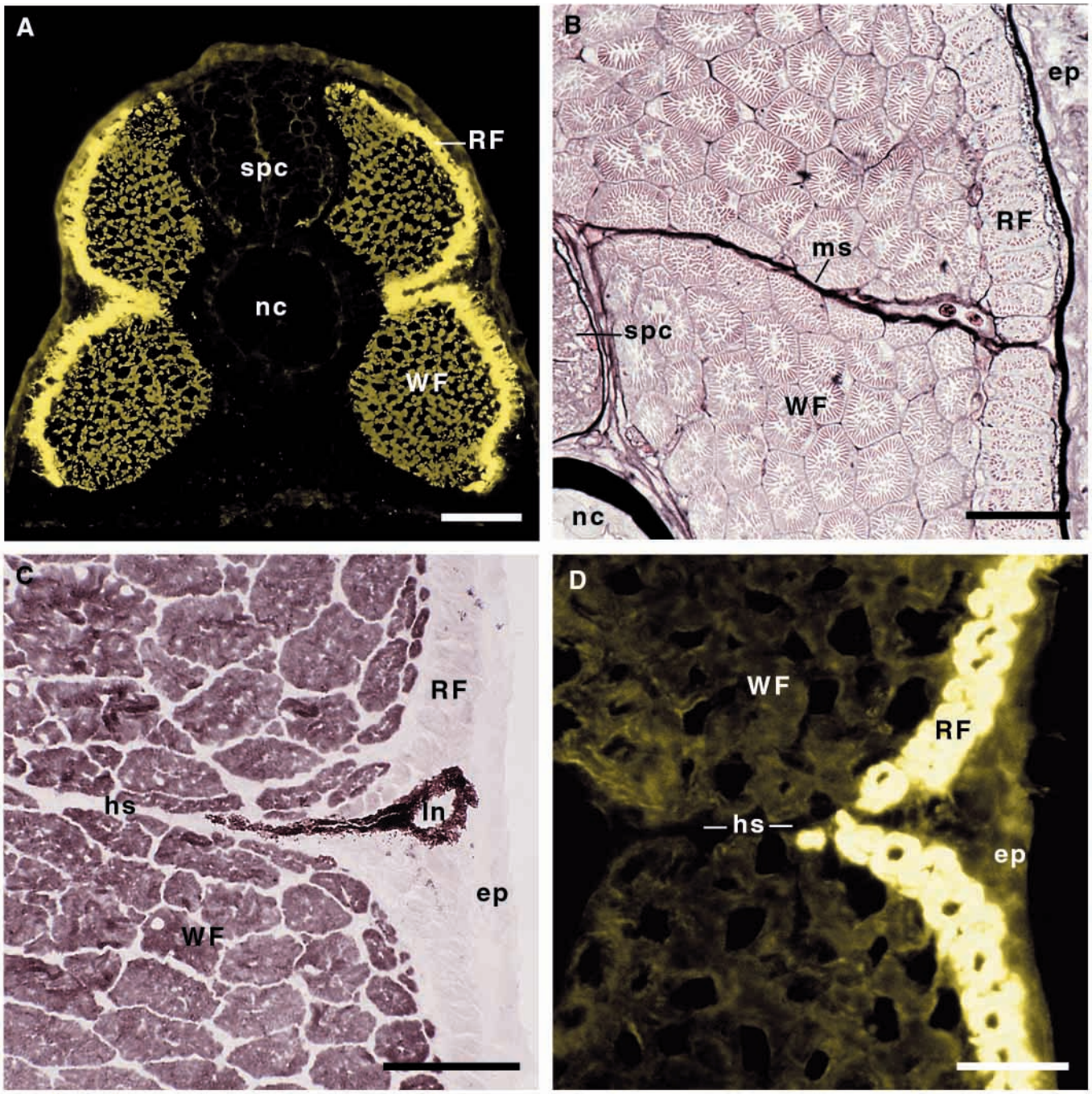


Fig. 1. Fibre typing and tracing of muscle fibre outlines in cross sections. (A) 40-somite (stage-1) embryo of the warm regime immunoreacted with the F59 antibody. The superficial monolayer of red fibres (RF) stains more intensively than the bulk of white fibres (WF) underneath. Scale bar, 100 µm. (B) Stage-5 larva of the cold regime. Detail of an epaxial quadrant with fibre contours and myofibrils as visualized by PAMS. Myofibrils form garland-like patterns in red fibres (RF) but radial patterns in white fibres (WF). Scale bar, 25 µm. (C,D) Discrimination between fibre types in the horizontal septum area. (C) Stage-6 larva (warm regime). Histochemical staining for myofibrillar ATPase after preincubation at pH 10.2 leaves white fibres (WF) active while red fibres (RF) are inactivated. Scale bar, 50 µm. (D) Swim-up larva (stage 3, cold regime). Immunostaining with the *S58* antibody highlights the red fibres (RF) monolayer, white fibres (WF) are unreactive. Scale bar, 25 µm. spc, spinal cord; nc notochord; ms, myoseptum; hs, horizontal septum; ln, lateral line nerve; ep, epidermis.

For the white fibres, ANCOVA of fibre sizes of the two thermal groups in relation to fish size shows no significant difference between temperature groups (P=0.94) (Fig. 4). However, the ANCOVA P-value for overall white fibre numbers (P=0.087) may indicate a trend towards more fibres in the warm-bred fish, a result similar to that of the red fibres.

To elaborate temperature differences further, fibre size distributions summed over all individuals measured, were

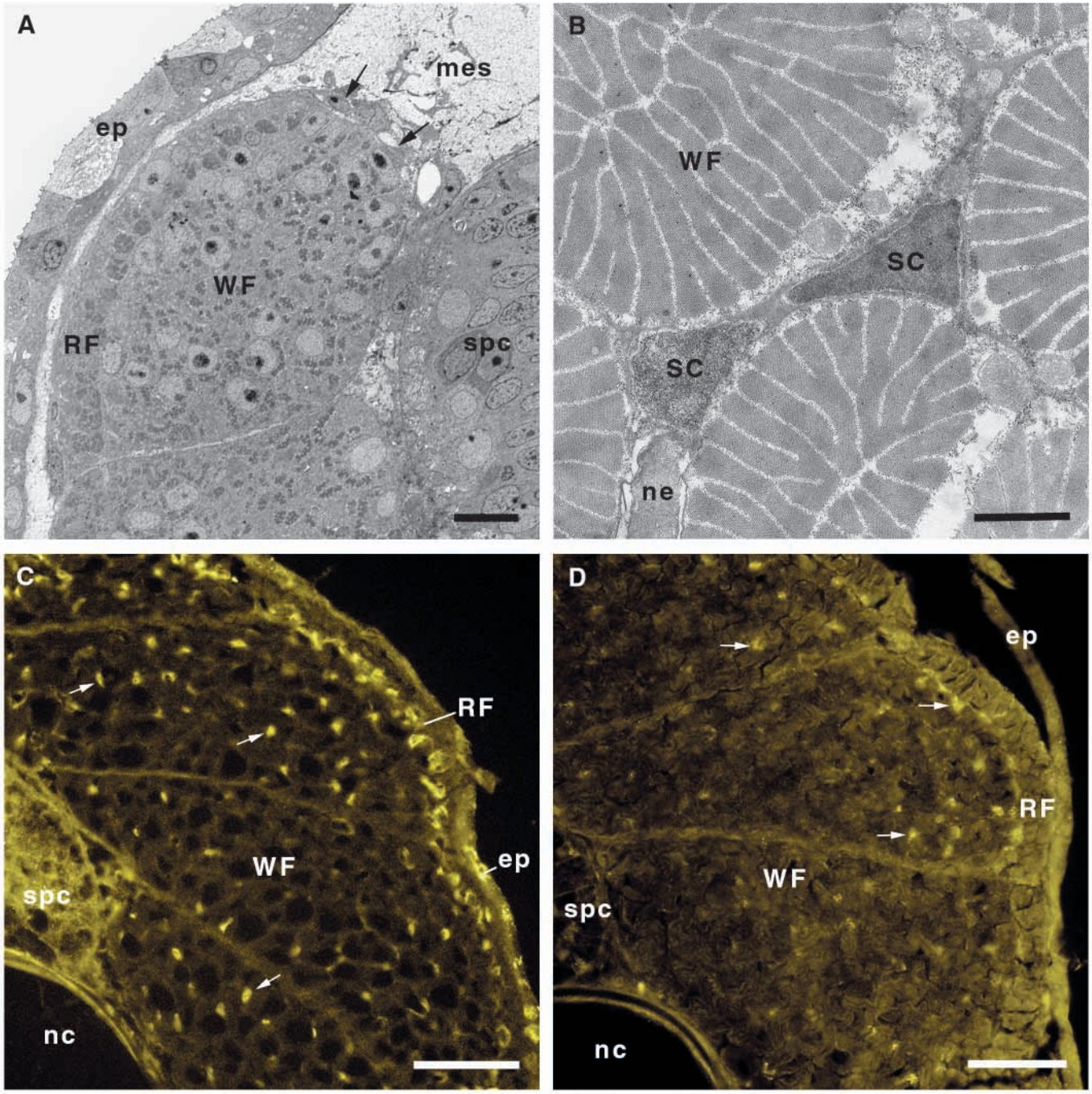


Fig. 2. Myogenic cells provide the basis of hyperplastic growth. All images are from cross sections. (A) Low magnification electron micrograph of a 40-somite embryo (stage 1, warm regime). Undifferentiated cells assumed to be myogenic are clustered within the mesenchyme (mes) next to the dorsal apex of the myotome and attach to the myotome's surface (arrows). Scale bar, 10 μm. (B) Electron micrograph of two myosatellite cells (SC) inserting between white fibres (WF) of a larva at first feeding (stage 4, warm regime). Scale bar, 2 μm. (C,D) Presumptive myosatellite cells and activated myosatellite cells within epaxial muscle of stage-5 larvae (warm regime), as labelled by anti m-met (C) and anti MyoD (D), respectively (arrows). Scale bars, 50 μm. spc, spinal cord; nc, notochord; ne, nerve; ep, epidermis; WF, white fibres; RF, red fibres.

plotted for each fibre type and developmental stage (Figs 5, 6). Modes were estimated where necessary as the most appropriate measure of the central tendencies of the distributions and in accordance with distribution shapes.

The main characteristics of the development of the fibre types under the two temperature regimes may be summarised as follows. In the 40-somite embryos (stage 1), prospective white and red fibres are of approximately the same size under both temperature regimes (Table 2). Fibre size distributions are single-peaked and narrow, with maxima within the 30 μm^2

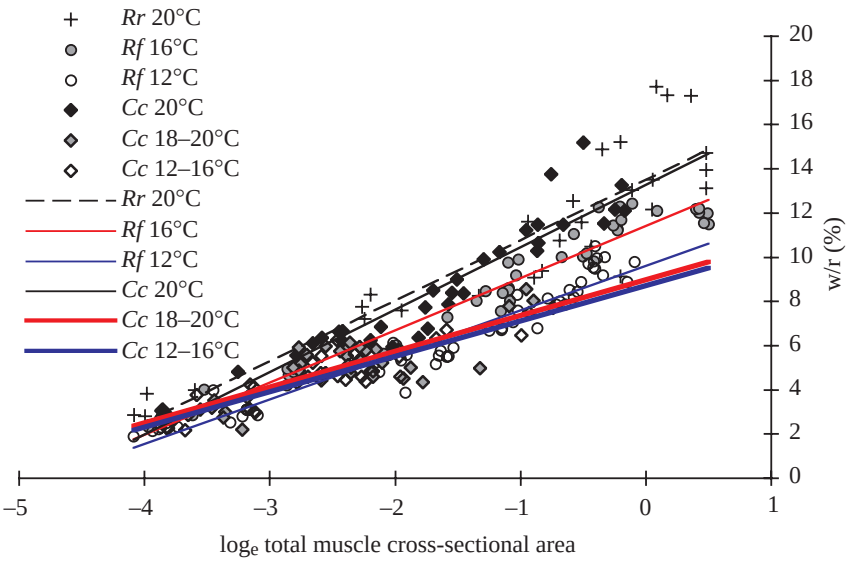


Fig. 3. Scatter plot with regression lines demonstrating the correlation of red muscle relative proportions (white-to-red ratio, w/r) with fish size as given by total muscle cross sectional area (c.s.a) in Danube bleak (*Cc*) reared under constant (thin black line) and changing (rising) thermal conditions (thick red and blue lines). Also shown, for comparison, are data for two further cyprinid species (roach, *Rr*; pearlfish, *Rf*) reared at various constant temperatures. Equations for Danube bleak: *Cc* 20 °C: $y=2.824x+13.284$ ($r^2=0.894$, $P<0.001$); *Cc* 18–20 °C: $y=1.6178x+8.9764$ ($r^2=0.686$, $P<0.001$); *Cc* 12–16 °C: $y=1.6032x+8.7289$ ($r^2=0.794$, $P<0.001$).

midpoint (i.e. 20–40 μm^2) classes (Figs 5A,B, 6A,B). Thus, there is no apparent temperature effect on the fibre size distributions, although there are slightly fewer fibres in the cold-bred fish. Of course, as also at all other stages, fibre numbers in the deep white part of the myotome exceed those of the superficial red fibre monolayer (see also Table 2).

At hatching (stage 2), white fibre size distributions of both temperature regimes have become positively skewed and each has a single peak that is now broader and formed by the 30 μm^2 and 50 μm^2 classes. A tendency towards fibre growth is particularly clear for the warm regime, where fibres larger than 80 μm^2 are much more frequent than with the cold regime (Fig. 5C,D). Red fibre distributions remain largely unchanged in shape apart from an increase in 30 μm^2 fibres at both temperatures (Fig. 6C,D). Note that from this stage onwards, the white fibre populations always contain numbers of fibres that are much larger than the largest red fibres.

In fish at the onset of free horizontal swimming (stage 3),

Table 2. *Mean sizes and numbers of white and red fibres for fish in each temperature group and stage*

A

	Fibre sizes (μm^2)					
	White fibres			Red fibres		
Stage	Cold	Warm	*P*	Cold	Warm	*P*
1	30.5±13.1	30.7±11.8	n.s.	32.2±14.2	29.7±12.3	0.06
2	46.3±22.5	**56.0**±34.3	<0.01	37.1±12.3	35.8±12.3	n.s.
3	105.8±72.4	**125.3**±82.2	<0.01	68.4±28.0	70.8±26.4	0.31
4	84.5±57.8	**111.9**±72.8	<0.01	59.4±25.1	**81.6**±33.0	<0.01
5	131.4±88.0	**175.8**±158.1	<0.01	99.5±55.0	**119.3**±71.1	<0.01
6	148.6±131.6	**171.8**±138.9	<0.01	93.3±71.5	**113.0**±65.1	<0.01

B

	Fibre numbers					
	White fibres			Red fibres		
Stage	Cold	Warm	*P*	Cold	Warm	*P*
1	88.3±7.0	**103.7**±11.7	0.03	31.5±3.6	**36.5**±2.9	0.02
2	105.8±5.6	**147.7**±16.8	<0.01	38.2±5.5	42.8±5.9	n.s.
3	137.5±17.4	137.5±8.9	n.s.	43.0±4.9	42.2±3.2	n.s.
4	143.0±14.8	**162.8**±8.0	0.02	42.2±4.7	44.5±4.9	n.s.
5	159.2±11.4	159.8±11.1	n.s.	41.7±3.1	44.5±3.8	n.s.
6	193.7±40.2	232.0±54.3	n.s.	43.8±4.8	49.2±5.9	n.s.

Values are means ± S.D. (N=6).

Values of samples that are significantly higher than those of their colder/warmer reared stage counterparts are given in bold; Student's *t*-test (significance taken at the P=0.05 level), n.s., not significant.

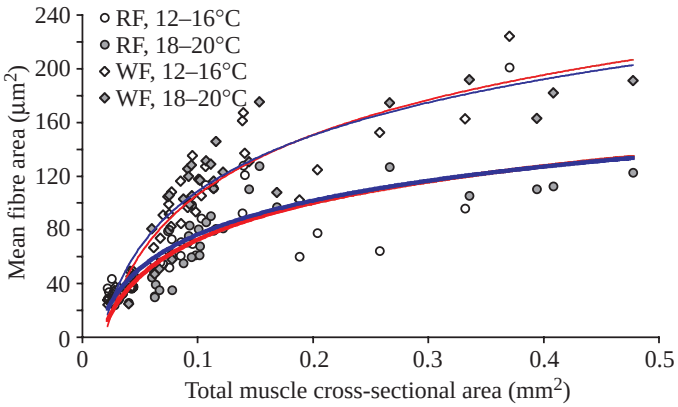


Fig. 4. Scatter plot showing the correlation of mean red (RF) and white fibre (WF) sizes with fish size as given by total muscle cross sectional area (c.s.a.) for Danube bleak reared under the two rising temperature regimes, 12–16 °C and 18–20 °C. Logarithmic regressions were calculated for red and white fibre data of each temperature regime: thick lines, red fibres; thin lines, white fibres; blue lines, cold regime; red lines, warm regime.

white fibres have broad size distributions which are still single-peaked with positive skew at both temperatures (Fig. 5E,F). The warm regime's distribution is more flattened with a peak formed by larger fibres than that of the cold regime (90 μm^2 *versus* 70 μm^2). Maximum fibre size clearly exceeds 300 μm^2 (some fibres >400 μm^2 may possibly be artifacts of the measurement technique employed). If compared to stage 2, the expansion towards larger fibre sizes has occurred at the expense of a decrease in numbers of small fibres (Fig. 5E). Red fibre distributions have also extended towards larger fibres, although at a much smaller scale than white fibre distributions and without skew. For both temperature regimes, the highest red fibre numbers are in the 70 μm^2 and the 90 μm^2 classes while numbers of fibres below that size have become greatly reduced (Fig. 6E,F).

When fish start exogeneous feeding (stage 4), their white fibre distributions resemble those of stage 3, except that they contain more small fibres below 60 μm^2 (Fig. 5G,H). This increase in the number of small fibres is particularly clear in specimens of the warm regime, where the size distribution has changed to a bimodal shape by addition of a small fibre peak at 30 μm^2 (Fig. 5G). By contrast, red fibre distributions stay close to a single-peaked form, with only a slight negative skew. This is more obvious in the cold-regime fish, where fibres >100 μm^2 are scarce (Fig. 6G,H).

In larvae beyond the onset of exogeneous feeding (stage 5), the size distribution of warm regime white fibres maintains a bimodal shape. This is, however, somewhat masked by large numbers of fibres in the size classes between the two peaks (small fibre peak still at 30 μm^2, large fibre peak now at 130 μm^2) (Fig. 5I). Bimodality is clearer at this stage now for white muscle fibre sizes from the cold regime (small fibre peaks at 30 μm^2, large fibre peak at 150 μm^2) (Fig. 5J). Red fibre distributions of both temperature regimes are largely symmetrical and have spread out due to ongoing fibre growth without significant recruitment of new small fibres (Fig. 6I,J).

In advanced larvae (stage 6) white muscle, the warm regime's small fibre peak at 30 μm^2 is more prominent than in stage 5 while the large fibre peak has flattened. The distribution is thus transformed back to a single peak, this time with a strongly positive skew and many fibres larger than 250 μm^2. By contrast, the white fibres of the cold regime have maintained a bimodal distribution with peaks at 30 μm^2 and 150 μm^2 (Fig. 5K,L). The red fibre distribution of the warm regime fish exhibit further expansion towards larger fibre sizes with a flat-topped peak at 130 μm^2 due to the effect of hypertrophy (Fig. 6K). This is missing in fish reared under the cold regime (peak at 70 μm^2; Fig. 6L). The distributions of both regimes, however, have added a few more small fibres to their 30 μm^2 classes, reflecting the onset of red fibre recruitment at the lateral line nerve in the fish of this stage (Fig. 6K,L).

Fibre hypertrophy in original ('first wave' somitic) red and white fibre stocks (see Materials and methods) is elucidated in Figs 7 and 8.

Discussion

This study has provided new information on the development of fish muscles under rising temperature regimes. Analyses of fibre sizes and numbers indicate that white and red myotomal muscle of the Danube bleak each develop in a different manner. More importantly, some aspects of the observed developmental patterns appear to be independent of temperature, while others are clearly influenced by rising temperature regimes.

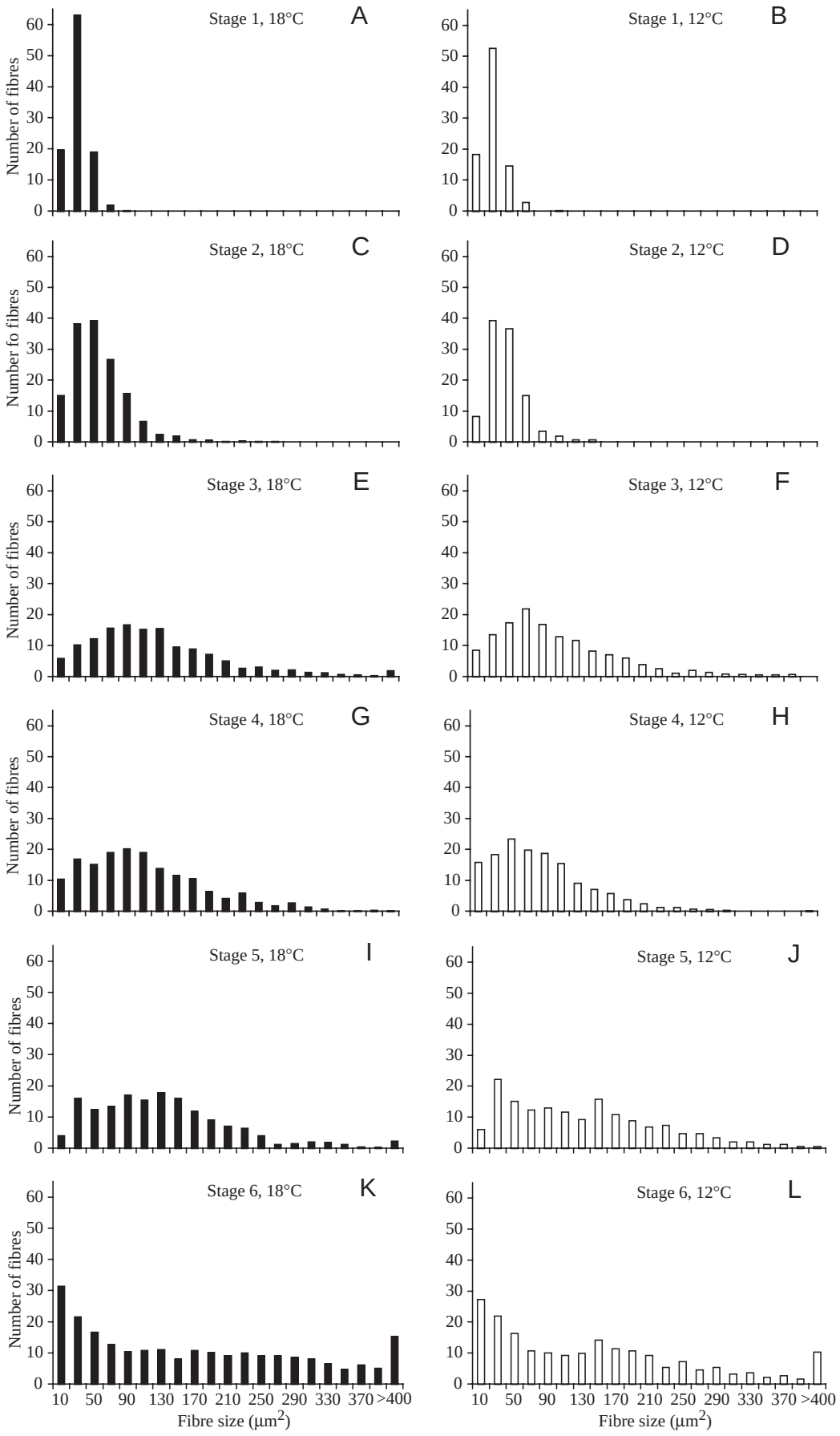
Notwithstanding the limitations imposed by lack of parallel constant temperature controls, as stated in the Introduction, it may be considered pertinent to discuss the results in the context of a direct comparison with constant acclimation temperature findings from other research, which used different (but closely related) fish species.

Temperature-independent features of muscle cellularity and growth

This study provides evidence that Danube bleak axial muscle, irrespective of different thermal rearing regimes, exhibits patterns of activation of hypertrophy and hyperplasia, that diverge between white and red muscle fibre types.

White muscle growth relies on both mechanisms, but with a clear shift in relative importance between them, which indicates that growth is to be regarded as a two-step process. The initial embryonic period (until after onset of free swimming, stage 3) is dominated by hypertrophy of the original (somitic) fibre stock. Recruitment of new fibres is low (see Fig. 5A–F) and largely confined to the dorsal and ventral peripheries of the white fibre bulks (see Fig. 2A). This is consistent with previous findings on myogenic cell commitment in the pearlfish (Stoiber and Sänger, 1996).

By contrast, in the later (larval) period (from first feeding,


Stage 1, 18°C
A
Stage 1, 12°C
B
Stage 2, 18°C
C
Stage 2, 12°C
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Stage 3, 18°C
E
Stage 3, 12°C
F
Stage 4, 18°C
G
Stage 4, 12°C
H
Stage 5, 18°C
I
Stage 5, 12°C
J
Stage 6, 18°C
K
Stage 6, 12°C
L
Number of fibres
Number fo fibres
0 10 20 30 40 50 60
10 50 90 130 170 210 250 290 330 370 >400
Fibre size (μm2)

Fig. 5. Frequency distributions of fibre sizes (cross-sectional areas) from white muscle of Danube bleak from the two temperature regimes. Data are grouped in 20 μm^2 size classes; histograms give mean fibre numbers per class for one epaxial quadrant per fish (*N*=6 for each stage and temperature).

stage 4 onwards), white muscle growth is supplemented by a surge of myosatellite-cell-based hyperplasia, thus creating bimodal fibre size distributions (Fig. 5G,I,J). Such a wave-like nature of white fibre proliferation has been described for other teleost larvae (e.g. Nathanailides et al., 1995a; Rowlerson et al., 1995; Stoiber, 1991; Ayala et al., 2000), and was found to persist in adult fish (e.g. Weatherley and Gill, 1985; Romanello et al., 1987; Johnston et al., 1999), where it may be subjected to seasonal variation (Johnston et al., 2000a,b).

A rather different pattern of early development is evident for red muscle of Danube bleak. Although structural maturation is more rapid in red fibres than in white fibres in the embryos, further red muscle growth at both temperatures remains largely confined to hypertrophy of the original monolayer fibre stock (see size distributions Fig. 6A–J). Any red fibre hyperplasia at the horizontal septum to form a mid-lateral wedge of red muscle, as known from teleost adults (Rome et al., 1988), is delayed up to and including stage 5, and thus begins long after the onset of white muscle hyperplasia. This is consistent with results from earlier work, also in the Danube bleak, using animals maintained at 20 °C (Stoiber, 1991).

Rather similar patterns of red muscle differentiation have been described for the species' cyprinid congener, the pearlfish (Stoiber, 1996; Stoiber et al., 1998), and the characid *Prochilodus marggravii* (Brooks et al., 1995).

Temperature-influenced modifications of muscle cellularity and growth

Temperature effects on muscle cellularity are evident in the Danube bleak from the 40-somite stage onwards and affect fibre numbers and sizes. Similar to the temperature-independent characteristics of muscle growth discussed above, these effects also diverge between white and red muscle.

Comparing temperature effects within the different developmental stages 1–6 (Table 2) reveals that white fibre effects are characterized by a 'quality shift' – a marked change at the embryo/larva transition (i.e. at around hatching), which affects hyperplasia and hypertrophy in different ways. White fibre precursor proliferation (i.e. hyperplasia) during myotome formation is likely to be reduced under cold conditions (which leads to lower fibre numbers in the cold-group stage-1 and stage-2 embryos, Table 2), while post-hatching hyperplasia is largely unaffected by temperature, as shown by fibre counts (Table 2) and by TEM observations of myogenic (myosatellite) cell appearance (Fig. 2B), as well as by immunocytochemical staining of such cells with relevant antibodies (Fig. 2C,D) (comparison between temperature groups descriptive only). By contrast, white fibre hypertrophy as measured by mean fibre sizes is devoid of temperature effects in the embryos but appears to be strongly hampered in the cold after hatching (Table 2A). These effects are particularly clear for stages where temperature groups of similar body size are compared (1, 3, 5 and 6). Cellularity differences in stages 2 (hatching) and 4 (onset of external feeding) are likely to be also a consequence of the body size differences between the temperature groups (see Table 1).

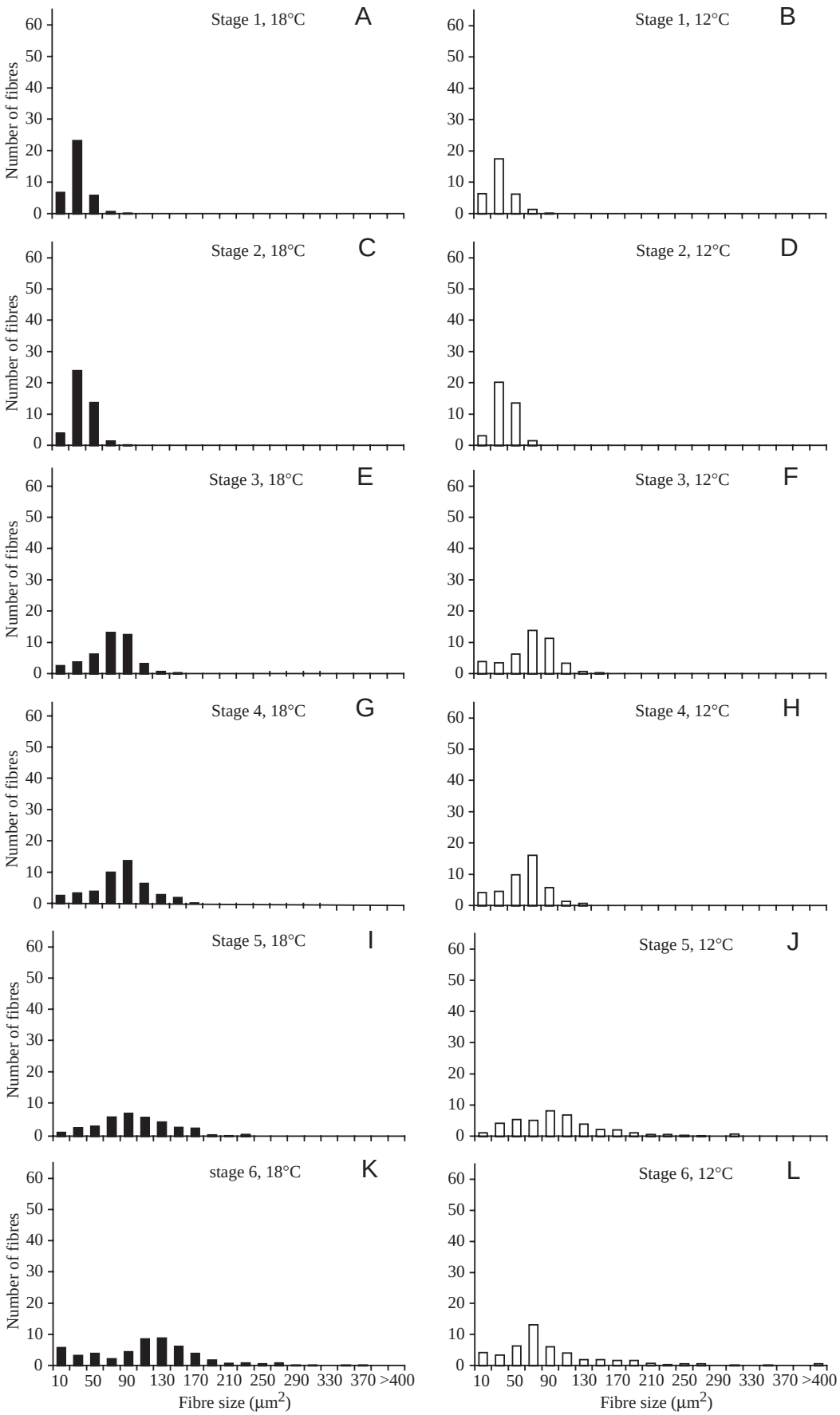
However, it should be noted that taking all post-hatching stages collectively, there is a much reduced (statistically insignificant) influence of low temperature on white fibre hypertrophy when the 'first wave' somitic fibres of the two thermal groups are compared alone (i.e. independent of bias in fibre size means through recruitment of new fibres). This is equally true for Danube bleak reared at rising temperature regimes and for pearlfish reared at constant temperature regimes (Fig. 8).

Although the overall dynamics of white fibre growth (within and between stages) are broadly similar under both temperature regimes, the growth pattern exhibits a time delay under cold conditions. This is demonstrated by the fact that the most distinct feature of the pattern (i.e. the onset of larval white fibre hyperplasia resulting in bimodal fibre size distributions), arises at a slightly less advanced stage of development (stage 4) in the warm-bred fish than in the cold-bred fish (stage 5) (see Fig. 5G,J).

The effects of temperature on red fibres are similar to the effects on white fibres, in that hyperplasia of somitic fibre precursors in the embryos is somewhat reduced under cold conditions. But in contrast to white fibres, any 'quality shift' of effects at the embryo/larva transition is missing. Correspondingly, within the period investigated for fibre size measurements, further red fibre hyperplasia (up to stage 5) is low and uninfluenced by temperature (Table 2).

It is well established that relative amounts of teleost red axial muscle may vary intraspecifically, dependent upon ambient temperature, and that increase of red muscle volume is a common response to a low temperature environment (see Introduction). However, the results of the present study of Danube bleak permit further elaboration, particularly when compared with findings from studies involving other cyprinid species and/or other rearing temperature strategies.

First, it is worth reiterating a result from the present study: that Danube bleak reared under changing temperature conditions show no overall difference in the relative proportion and rate of increase of red muscle, whether reared under warm or under cold conditions (Fig. 3, lower two lines). This is in contrast to the results obtained for some species reared under constant temperature regimes. Here, higher temperature shows a lower relative proportion and rate of increase of red muscle (Fig. 3, four upper lines). To make matters more complicated, cold bred fish appear to equate with higher amounts and rate of increase of red muscle, irrespective of whether they are reared in constant or in changing temperature environments. This appears not to be necessarily a species-specific reaction (cf. pearlfish, Danube bleak, Fig. 3). Finally, it may be noted that in the Danube bleak, warm bred fish in a changing temperature environment have a higher relative amount and


Stage 1, 18°C
A
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G
Stage 4, 12°C
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Stage 5, 18°C
I
Stage 5, 12°C
J
stage 6, 18°C
K
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L
Number of fibres
0 10 20 30 40 50 60
10 50 90 130 170 210 250 290 330 370 >400
Fibre size (µm2)

Fig. 6. Frequency distributions of fibre sizes (cross-sectional areas) from red muscle of Danube bleak from the two temperature regimes. Data are grouped in 20 μm^2 classes; histograms give mean fibre numbers per class for one epaxial quadrant per fish (*N*=6 for each stage and temperature).

rate of increase of red muscle than warm-bred specimens of the same species in a constant temperature environment (Fig. 3, upper of the two bottom lines, in comparison with the lower of the two top lines).

Although this may appear all rather complicated, the general conclusion that may be drawn is, in fact, quite simple: the relative amount of red muscle increases at rates depending not only upon species and overall temperature (see Introduction) but also upon whether the fish have been reared under changing or constant temperature regimes.

Under the rising thermal regimes applied to Danube bleak in the present study, there is no clear trend towards larger red fibres in the cold, as reported previously in pearlfish reared under constant temperatures (Stoiber, 1996) (see ANCOVA results). However, rising temperature regimes do seem to have an overall effect on red fibre numbers in Danube bleak, with a clear trend towards more red fibres in the warm regime. This trend is just as apparent for the white fibres of the same species. Interestingly, here the results are partly contrary to, and partly in agreement with, the findings of Alami-Durante et al. *(*2000) using young carp *Cyprinus carpio*. In that study, white fibre numbers and sizes were found to vary clearly between fish hatched and maintained at constant temperatures (18 °C, 28 °C) and fish transferred from 18 °C to 28 °C at hatching. More white muscle mass resulted from more and larger fibres in the transferred group than in both the colder and the warmer constant groups.

If such results are found to be general, the implications are clear – it will be necessary to considerably revise present ideas on cyprinid red muscle development that have been based only on constant water temperature investigations (goldfish, Johnston and Lucking, 1978; pearlfish, Stoiber, 1996). Such revision would bring our understanding closer into line with the natural (changing) conditions experienced by the fish at temperate latitudes.

There appears to be a wide variety of developmental responses of red and white muscles of cyprinids to variations in temperature and temperature regimes. The overall effect on muscle cellularity is a net result derived from different growth mechanisms (hypertrophy and hyperplasia in varying proportions), which may be confined to distinct subpopulations of fibres arising at different fish ages. For example, we have demonstrated here that hypertrophy of 'first wave' somitic red and white fibres is apparently uninfluenced by ambient temperature even though temperature certainly has an overall influence (see above). This result is regardless of whether fluctuating (Danube bleak) or constant thermal regimes (pearlfish) are applied (Figs 7, 8).

The importance of a simulation of the natural thermal environment in fish muscle developmental studies is further elucidated by an investigation of Johnston et al. *(*2000b) using fluctuating (although smoothed) temperature regimes to investigate muscle differentiation in Atlantic salmon offspring from two thermally different (warm lowland, cold upland) river tributaries. Comparing fry of each origin exposed to each temperature regime, these authors showed that many variables of muscle growth (cellularity-related and others) are strongly influenced by the temperatures experienced by the fish fry during critical periods of early development.

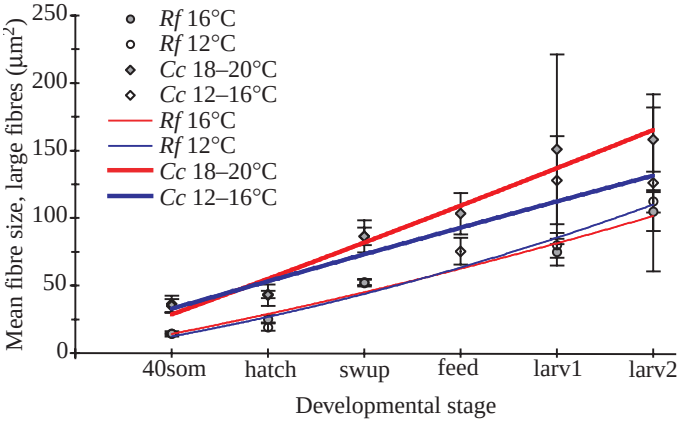

Fig. 7. Hypertrophy of 'first wave' somitic red fibres in Danube bleak (*Cc*) reared at rising temperatures (12–16 °C *versus* 18–20 °C) and pearlfish (*Rf*) reared at constant temperatures (12 °C *versus* 16 °C). Size (cross-sectional area) means of defined numbers of largest fibres per epaxial quadrant (*Cc*: *N*=25, *Rf*: *N*=40; for rationale see Materials and methods, *Data analysis*) within developmental stages as defined in Table 1 (40som, 40 somite embryo; hatch, newly hatched free embryo; swup, free embryo at onset of free swimming; feed, larva after first uptake of exogeneous food; larv1, *Cc* larvae of stage 5, *Rf* larvae at 11 mm body length; larv2, *Cc* larvae of stage 6, *Rf* larvae at 12.5 mm body length). Values are means ± 1 S.D.

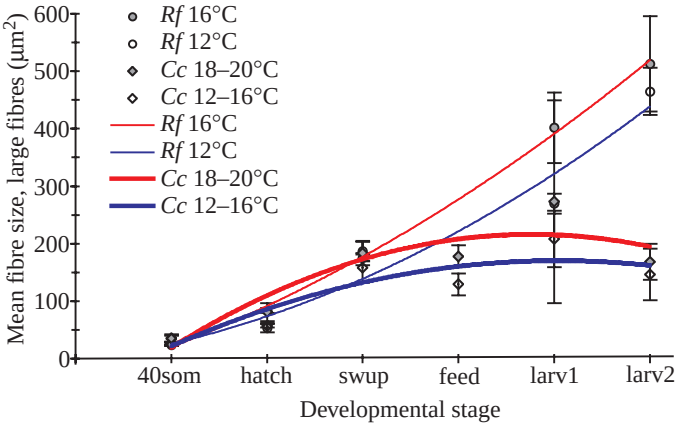

Fig. 8. Hypertrophy of 'first wave' somitic white fibres in Danube bleak (*Cc*) reared at rising temperatures and pearlfish (*Rf*) reared at constant temperatures. Size (cross-sectional area) means of defined numbers of largest fibres per epaxial quadrant (*Cc*, *N*=70; *Rf*, *N*=80) within developmental stages as defined in Table 1 (stage terminology and other explanations as in Fig. 7). Values are means ± 1 S.D.

Cellularity change in relation to the 'landmark' events of early ontogeny

In discussing patterns of muscle cellularity change in young fish, it is certainly worth also considering how these patterns are entwined with the three consecutive 'landmark' events of early ontogeny, namely (1) hatching (involves getting rid of the egg shell), (2) onset of free swimming (involves filling the swimbladder and attaining a capability of sustained locomotion) and (3) start of exogeneous feeding (involves the ability to manoeuvre precisely in three dimensions to hunt for prey).

In the light of the Danube bleak results, hatching must not be taken as a 'strong' developmental threshold in muscle formation. At both temperatures, comparison of fibre size frequency distributions between stages 2 and 1 fails to provide an indication of any significant cellularity change in the period before hatching. This is exceptionally clear for red fibres (Fig. 6A–D), but might require further explanation for white fibres, which show a trend towards larger fibre sizes in the hatchlings (Table 2A, Fig. 5C–D). One possible explanation for this difference comes from the relative delay of space-demanding myofibrillogenesis in the white fibres which, in contrast with the faster matured red fibres, certainly expands the initial phase of cell growth until shortly before hatching. Even if it requires functional white fibres to release the fry from their egg shells at both temperatures (which is suggested by the fine structural findings), a minor importance of hatching as a myogenetic threshold is further underpinned by the observation that cold-bred Danube bleak manage to hatch despite having less muscle fibres of each type in their myotomes than their warm bred counterparts (Table 2). As the cold-bred hatchlings are also on average shorter (Table 1), this suggests that cold incubation, in a general developmental context, promotes hatching at a less developed stage. Precocious hatching after cold incubation has also been described for other cyprinids (bream *Abramis brama*, Herzig and Winkler, 1986; pearlfish, Stoiber, 1996) and was thought to be a result of cold-induced hypoxia rather than a temperature effect *per se* in salmoniforms (*Coregonus albula*, Luczynski and Kirklewska, 1984; Atlantic salmon, Matschak et al., 1995, 1997; rainbow trout, Matschak et al., 1998).

Unlike hatching, considerable weight is attached to 'landmark' events 2 and 3 with reference to their possible function as myogenetic 'thresholds'. Indeed, the embryonic muscle system becomes subject to extensive reorganization when the fish switch from bottom rest to free swimming (event 2) and, perhaps more importantly, when they make first attempts to catch living prey (event 3). The latter involves a sudden upregulation of white muscle hyperplasia as reflected by the appearance of numerous m-met- and MyoD-positive cells (Fig. 2C,D) and bimodal fibre size distributions (Fig. 5G,I,J). Additionally, it involves the recruitment of a new fibre type – tonic fibres – a topic beyond the scope of the present study. Enhancement of hyperplasia to add to lasting hypertrophy at that stage of development is certainly an effective strategy to supply the fish with white muscle mass required for efficient prey strikes. This is also understandable from an energetic point of view. Hyperplastic growth on a large scale is thought to be more energy-consuming than hypertrophy (Matschak et al., 1995, 1997, 1998) and can be facilitated once nutrients are available from external sources.

In the end, however, as noted in the introduction, many of the 'cause and effect' relationships between temperature-induced patterns of muscle development and the behavioural pattern of the fish remain unclear. This, together with the new finding that changing temperature regimes may have strong effects on muscle development patterns that were not appreciated during constant temperature studies, opens the gate to a whole new avenue of research in the developmental biology and functional ecology of teleost fish.

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