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## Plasticity of myosin heavy chain expression with temperature acclimation is gradually acquired during ontogeny in the common carp (*Cyprinus carpio* L.)

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**Abstract** Common carp (*Cyprinus carpio* L.) were reared from hatching until 61 mm total length (TL) at 21 °C. At 14 weeks and 20 weeks post-hatch, corresponding to initial lengths of 30 mm and 44 mm respectively, fish were acclimated to 10 °C using a rate of cooling of 1 °C per day. A statistical model was used to compare the time course in the change of white muscle myofibrillar ATPase activity with temperature acclimation. The myosin heavy chain (MHC) composition of white muscle myofibrils was investigated using peptide mapping. A significant increase in myofibrillar ATPase activity was observed after 2–3 weeks in the 44 mm group, but not until 4–5 weeks in the 30 mm group, when they had reached 37 mm TL. The MHC banding pattern of 120 mm TL fish acclimated to 10 °C or 21 °C for a minimum of 6 weeks were distinct from each other. The MHC peptide map characteristic of 10-°C-acclimated fish was not observed in individuals less than 37 mm length. We therefore conclude that the capacity to alter the composition and properties of myofibrils with cold acclimation is acquired in juvenile carp at around 37 mm TL.

**Keywords** Muscle · Temperature acclimation · Ontogeny · Myosin heavy chain · *Cyprinus carpio*

**Abbreviations** *EGTA* ethylene glycol-bis (b-aminoethyl ether)N,N,N',N'-tetraacetic acid · *MHC* myosin heavy chain · *SDS-PAGE* sodium dodecyl sulphate poly acrylamide gel electrophoresis · *TL* total length

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### Introduction

Adult carp (*Cyprinus carpio* L.) are able to tolerate a wide range of temperatures, ranging from near freezing in ice-covered ponds in the winter to 30 °C in summer (Michaels 1988). In Europe, during mid summer, sexually mature individuals require prolonged periods at temperatures of 22–28 °C in order to spawn and produce eggs (Michaels 1988). The minimum temperature for normal development of the fry and larvae is 15 °C (Rothbard and Yarron 1995). However, during their first year, juvenile stages become tolerant of lower temperatures and are able to over-winter at 0–5 °C (Rothbard and Yarron 1995). Therefore, cold tolerance in this species is acquired at some point before the onset of the first winter.

When environmental temperature changes, cyprinid fish exhibit acclimation responses. One important component of acclimation at the molecular level is a modification in the properties of white muscle myofibrillar ATPase activity (Johnston et al. 1975; Heap et al. 1985; Crockford and Johnston 1990; Hwang et al. 1990, 1991; Johnson and Bennet 1995). For example, the  $Mg^{2+}Ca^{2+}$  ATPase activity of fast-muscle myofibrils assayed at 1 °C, was 2.8-fold higher in 1 °C-acclimated goldfish than 26 °C-acclimated goldfish (Johnston et al. 1975). The ATPase of myofibrils was also more susceptible to thermal denaturation in cold-acclimated than warm-acclimated goldfish (Johnston et al. 1975). Similar effects of temperature acclimation on myofibrillar ATPase activity have been reported for the common carp (Crockford and Johnston 1990; Hwang et al. 1990). The mechanism is thought to involve changes in the expression patterns of myosin heavy chain (MHC) isoforms (Hwang et al. 1991; Watabe et al. 1992; Guo et al. 1994; Watabe et al. 1995). Three major isoforms of MHC have been found in the fast myotomal muscle of common carp and their expression patterns were shown to be dependent on acclimation temperature (Watabe et al. 1995; Imai et al. 1997). The entire coding sequence of

these genes has now been reported (Kikuchi et al. 1999). There is some evidence that myofibrillar ATPase activity (Heap et al. 1986; Crockford and Johnston 1990) and the relative proportions of MHC isoforms (Hwang et al. 1991; Watabe et al. 1995) vary continuously with acclimation temperature. However, there is no simple relationship between myosin composition and swimming performance (Wakeling et al. 2000). Acclimation was found to extend the temperature range for fast-start behaviour, but improvements in maximum swimming speed at intermediate temperatures were only observed for relatively large differences in acclimation temperature (Wakeling et al. 2000). Thus, although myosin-ATPase activity varies continuously with acclimation temperature there is a threshold for an observable effect on swimming speed at the whole animal level.

Developmental-stage specific isoforms of myosin have been described in fish (Ennion et al. 1999; Wakeling et al. 2000; Johnston et al. 1997). For example, two developmentally regulated MHC gene transcripts (Eggs22 and Eggs24) have been identified in the fast myotomal muscle of common carp that are expressed between 22 h post-fertilisation and 2 weeks post-hatch (Ennion et al. 1999). Also, embryonic/larval protein isoforms of MHC have been identified in common carp (Wakeling et al. 2000), herring (Johnston et al. 1997, 1998), plaice (Johnston and Horne 1994), Arctic charr (Martinez et al. 1991; Martinez and Christiansen 1994) and sea bream (*Sparus aurata*; Mascarello et al. 1995).

Wakeling et al. (2000) investigated white muscle MHC and ATPase activity in carp larvae reared at temperatures decreasing from 21 °C at hatch to 8 °C over 29 weeks. They found that the myofibrils did not show the characteristic MHC isoforms expressed at low temperature or changes in myofibrillar ATPase activity until the fish had been at a constant temp of 8 °C for 10 weeks. In contrast, changes in myofibrillar ATPase activity of adult fish only takes 2–3 weeks (Heap et al. 1985). This suggests that some developmental competency is required to adjust myosin isoform composition to decreasing water temperatures. The aim of the present study was to test the hypothesis that the ability to modify MHC expression with temperature acclimation is gradually acquired during ontogeny and becomes evident at some specific stage of development. A statistical model was used to compare the time course of changes in myofibrillar ATPase activity starting at two stages of development and the results correlated with changes in MHC composition.

## Materials and methods

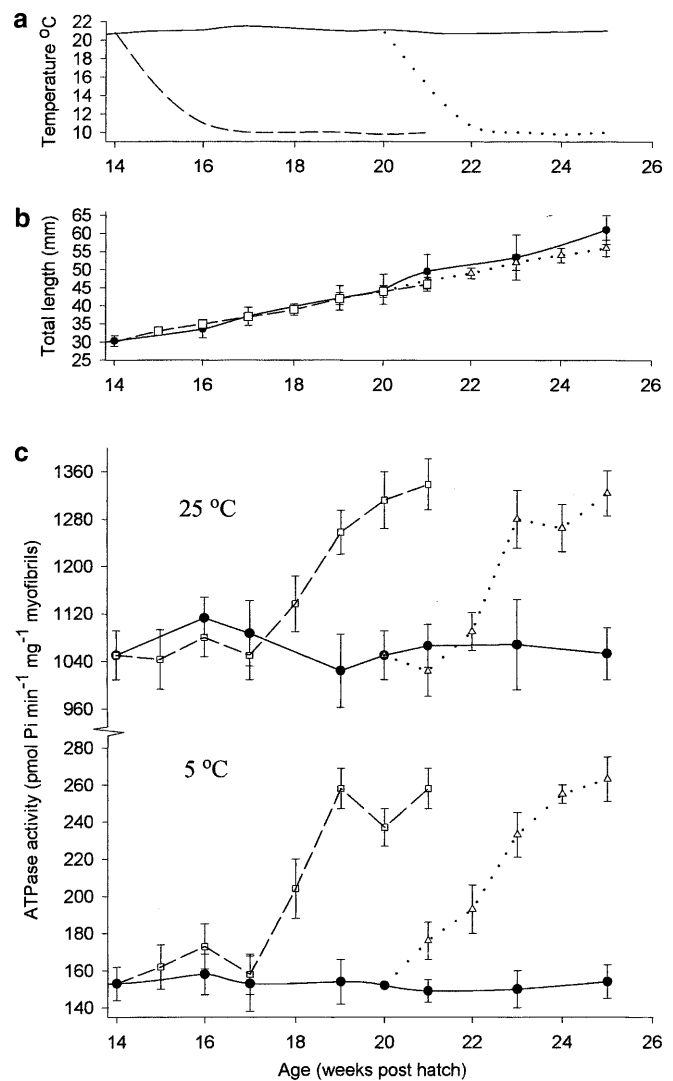
### Fish

Larvae of the common carp (*C. carpio* L.), representing the offspring of more than ten females spawned at 23 °C, were obtained 4 days post-hatch from a commercial supplier (Humberside Fisheries, Driffield, England). The larvae were transferred to the Gatty Marine Laboratory, University of St. Andrews and reared at a

constant temperature of 21 °C (Fig. 1a). At 14 weeks (30 ± 2.3 mm total length (TL), mean ± SEM,  $n=40$ ) and 20 weeks (44 ± 3.2 mm TL, mean ± SEM,  $n=40$ ) post-hatch, approximately 200 fish were transferred to separate tanks and the water temperature reduced, by 1 °C per day, from 21 °C to 10 °C (Fig. 1a). Fish transferred at 14 weeks and 20 weeks were reared until they reached average total lengths of 45 mm and 53 mm, respectively (Fig. 1b). In all experiments the photoperiod regime was 12 h light:12 h dark. Fish were fed ad libitum on *Artemia nauplii* and weaned onto commercial pellets (BOCM Paul, UK). Another batch of carp that had been reared at 21 °C (120 ± 7.3 mm TL, mean ± SEM,  $n=40$ ) were acclimated to either 10 °C or 21 °C for a minimum of 6 weeks.

### Muscle sampling and preparation

The myotomal muscle was dissected on a chilled microscope stage at 5 °C. The head, tail and gut of the fish were removed and the



**Fig. 1** Age of carp larvae/juveniles plotted against (a) experimental temperature regime, (b) total body length (mean ± SE;  $n=20$ ) and (c)  $Mg^{2+}Ca^{2+}$ ATPase activity of white muscle myofibrils (mean ± SE;  $n=6$ ) assayed at 5 °C and 25 °C. Fish were reared at constant 21 °C (solid line, filled circles), or cooled to 10 °C starting at 14 weeks post-hatch (dashed line, open squares), or 20 weeks post-hatch (dotted line, open triangles)

skin peeled off under a dissecting microscope to remove the majority of the red muscle fibres. Care was taken to sample only the inner white muscle fibres adjacent to the spinal chord, thereby avoiding contamination with pink and red fibres (Akster 1985). Myofibrils were prepared by homogenising the muscle in 20 volumes of preparation buffer containing (mmol l<sup>-1</sup>): Tris-HCl 10, NaCl 50, EDTA 1, pH 7.4 at 1 °C using the methods of Johnston et al. (1997).

#### Myofibrillar ATPase assay

Mg<sup>2+</sup> Ca<sup>2+</sup> myofibrillar ATPase activity was measured as described by Ball and Johnston (1996) with the following modifications. The protein concentration of the myofibrillar solution was initially measured using a total protein kit (Sigma Diagnostics, Sigma) followed by the microprotein-PRTM kit (Sigma Diagnostics, Sigma). The concentration of myofibrils was adjusted to 1 mg ml<sup>-1</sup>. ATPase activity was measured at 5, 15 and 25 °C in an assay medium which contained 0.1 mg myofibrils in 62.5 mmol<sup>-1</sup> Tris-HCl (pH 7.5 at 5 °C), 3.8 mmol<sup>-1</sup> MgCl<sub>2</sub>, 5 mmol l<sup>-1</sup> ATP and 0.2 mmol<sup>-1</sup> CaCl<sub>2</sub> or 2 mmol l<sup>-1</sup> ethylene glycol-bis (b-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA). The pH of the assay medium was allowed to vary with temperature. The reaction was stopped by the addition of an equal volume of 10% (m/v) trichloroacetic acid. The inorganic phosphate produced was measured using Sigma inorganic phosphorous measuring kit (Sigma diagnostics). Results were expressed as nanomoles of phosphate produced per milligrams myofibrils per minute.

#### Gel electrophoresis

Peptide mapping of the MHCs was performed using the methods detailed in Johnston et al. (1998). Washed myofibrils were prepared from white muscle of the carp as previously described (Crockford and Johnston 1993). The total protein concentration was initially determined using a total protein kit 541-2 (Sigma Diagnostics, Sigma) followed by the microprotein-PRTM kit (Sigma Diagnostics, Sigma). Myofibrils were dissolved in the sample buffer to give a final total protein concentration of 2 mg ml<sup>-1</sup>.

The myofibrils were resolved in 0.75 mm thick, 8% (m/v) acrylamide gels following digestion with 5 µl of a 20-µg ml<sup>-1</sup> solution Endoprotease Glu-C protease (Sigma Chemicals, Dorset). Gels were then fixed and stained with PlusOne silver stain (Amersham Pharmacia Biotech, Uppsala, Sweden). Differences in the banding pattern between lanes was then detected using gel analysis software from Scion Image (NIH; <http://www.scionimage.com>).

#### Statistics

ATPase activity in experimental fish was expected to begin at a steady state, corresponding to the level of activity at 21 °C. As temperature decreased, after some delay corresponding to acclimation time, ATPase activity was expected to smoothly increase, eventually reaching a higher plateau corresponding to the temperature regime of 10 °C. This pattern can be modelled statistically by a logistic curve, which has an initial plateau and a smooth increase to a higher asymptote. A logistic curve was therefore fit to the ATPase activity data, with an additional parameter allowing for a difference in acclimation time between fish transferred at 14 weeks and those at 20 weeks. The statistical model used was:

$$ATPase_i = \beta_0 + \frac{\beta_1}{1 + \exp\left[-\frac{W_i - (\beta_2 + \beta_3 G_i)}{\beta_4}\right]} + \epsilon_i$$

where  $ATPase_i$  is the measured ATPase activity of the fish  $i$  ( $i = 1 \dots n$ ),  $\beta_0$  is the ATPase activity of fish in week 0,  $\beta_1$  is the asymptotic increase in ATPase activity,  $\beta_2$  is the time to 50% acclimation of fish acclimated at 20 weeks of age,  $\beta_3$  is the additional

time to 50% acclimation of fish acclimated at 14 weeks of age,  $\beta_4$  is the shape parameter,  $W_i$  is week,  $G_i$  is an indicator variable that is 0 for 20-week-old fish and 1 for 14-week-old fish, and  $\epsilon_i$  is the residual error, assumed to be normally distributed with 0 mean and constant variance. If the estimate of parameter  $\beta_3$  is not significantly different from 0 then there is no discernable difference in acclimation time between groups. We fitted this model by non-linear least-squares regression (nls function in SPlus 4.5 for Windows).

#### Results

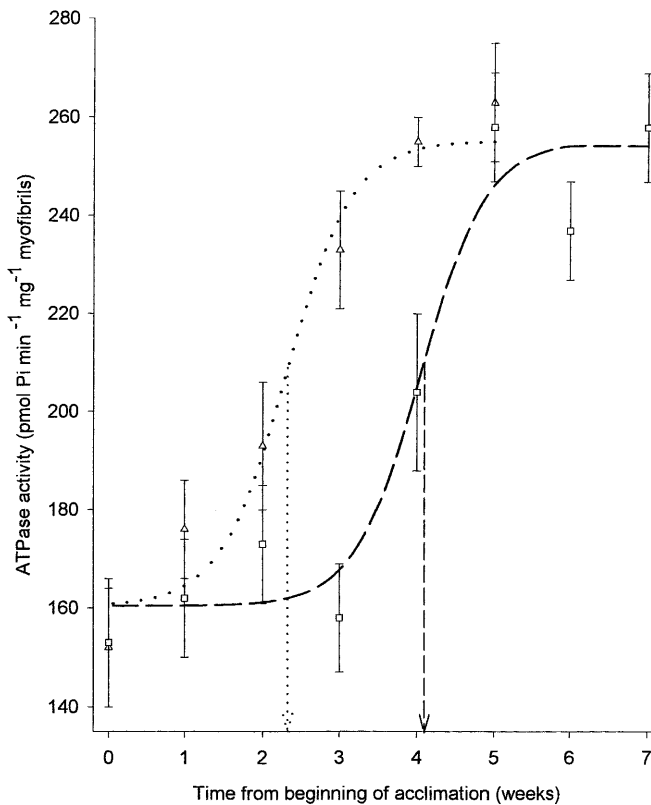
Fish reared at a constant 21 °C showed a steady level of ATPase activity (Fig. 1c; *filled circles, solid line*) over 25 weeks as TL increased from 30 mm to 60 mm (Fig. 1b). There was no significant difference between these values and those obtained from the myofibrils of 120-mm-TL fish acclimated to 21 °C (mean = 154.0 pmol Pi min<sup>-1</sup> mg<sup>-1</sup> myofibrils SEM = 5.06  $n = 40$ ).

In contrast, following cold-acclimation, an increase in myofibrillar ATPase activity occurred in both the 14-week-acclimation group (Fig. 1c; *open squares, dashed line*) and 20-week-acclimation groups (Fig. 1c; *open triangles, dotted line*) at 5 °C, 15 °C and 25 °C (Fig. 1c; 15 °C not shown). The steady state values obtained from these groups following acclimation to 10 °C was not significantly different to the value obtained from myofibrils of 120-mm-TL fish acclimated to 10 °C (mean = 242.3 pmol Pi min<sup>-1</sup> mg<sup>-1</sup> myofibrils SEM = 5.47  $n = 40$ ).

The time course for changes in myofibrillar ATPase activity for fish initially reared at 21 °C and subsequently cooled to 10 °C at 14-weeks and 20-weeks post-hatch is also shown in Fig. 1c. The time course for the change in ATPase activity with acclimation differed for the two developmental stages studied. In the case of the 14-week post-hatch group (30 mm TL) acclimated to 10 °C, no significant change in ATPase activity was noted until 4–5 weeks from the onset of cooling (Fig. 1c.) At this point the fish had reached 37 mm TL (Fig. 1b.). In contrast, in the 20 weeks post-hatch group (42 mm TL), a significant increase in myofibrillar ATPase activity was observed after only 2–3 weeks (Fig. 1c). Following cold-acclimation myofibrillar ATPase activity increased at assay temperatures of 5 °C, 15 °C and 25 °C (Fig. 1c; 15 °C data not shown).

Figure 2 shows the logistic curve fitted to the myofibrillar ATPase activity assayed at 5 °C. The estimate of parameter  $\beta_3$  (the difference in 50% acclimation time between 14-week and 20-week fish) was 12.79 days (SE 1.89), with 95% profile confidence limits 8.25 to 16.13. This corresponds well with the difference of 2 weeks noted in the raw data at 5 °C. Time to 50% acclimation was estimated to be 29 days for the 14-week post-hatch group (Fig. 2, *filled square, fine dashed line*) and 16 days for the 20-week post-hatch group (Fig. 2, *filled triangle, fine dotted line*).

MHC composition was investigated by comparing peptide maps produced by digestion of the electrophoretically purified protein with Endoprotease Glu-C protease. The peptide map of MHC in juveniles

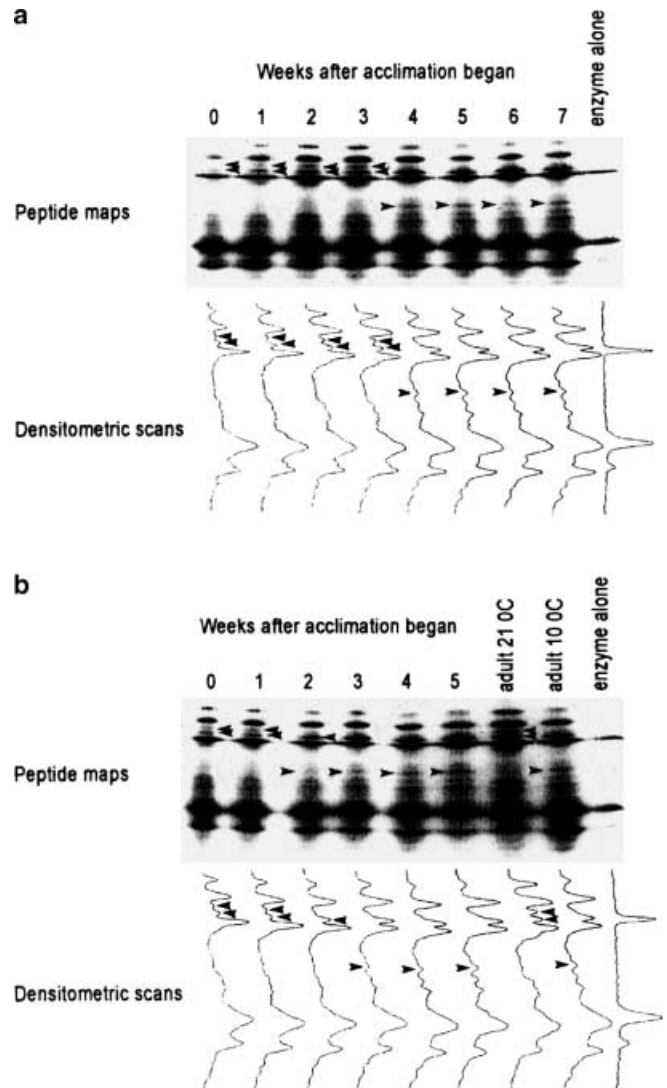


**Fig. 2**  $Mg^{2+}Ca^{2+}$  ATPase activity of white muscle myofibrils from fish acclimated to 10 °C at 14 weeks post-hatch (*open squares*) and 20 weeks post-hatch (*open triangles*) as illustrated in Fig. 1a. Standard logistic curves fitted to the data are shown: *dashed line* represents fish acclimated to 10 °C at 14 weeks and the *dotted line* denotes fish acclimated to 10 °C at 20 weeks post-hatch. The time to 50% change in myofibrillar ATPase activity for carp acclimated to 10 °C at 14 weeks of age (*fine dashed arrow*) and 20 weeks (*fine dotted arrow*) is shown

14 weeks post-hatch was identical to that of 21-°C-acclimated 120-mm-TL fish (Fig. 3a, *lane 0*; Fig. 3b). Fish of 120 mm TL had distinct MHC digest banding patterns when acclimated to 10 °C and 21 °C (Fig. 3b). On acclimation to 10 °C, the MHC characteristic of 10-°C-acclimated fish was evident after 4 weeks in the 14-week post-hatch group (Fig. 3a, compare *lanes 3* and *4*) and after 2 weeks in the 20-week post-hatch (Fig. 3b, compare *lanes 1* and *2*). Thus, the time course for the appearance of the MHC isoform(s) characteristic of cold-acclimated fish (Fig. 3a, b) was similar to that of changes in myofibrillar ATPase activity (Fig. 1c). The minimum total body length for the expression of the cold characteristic MHC isoform(s) and myofibrillar ATPase activity was approximately 37 mm, which in the present experiments occurred at 18 weeks post-hatch.

## Discussion

In this study, cold acclimation led to a significant increase in myofibrillar ATPase activity and an altered



**Fig. 3** Peptide maps and densitometric scans of myosin heavy chains isolated from carp white muscle myofibrils during acclimation from 21 °C to 10 °C at (a) 14 weeks post-hatch group and (b) 20 weeks post-hatch group, as illustrated in Fig. 1a. *Numbered lanes* represent the number of weeks after the acclimation began. The *enzyme-alone lane* is a lane loaded with just the enzyme used for the digest. Adult 21 °C and adult 10 °C is digest of MHC from 120 mm TL fish acclimated to 21 °C and 10 °C, respectively. Bands characteristic of 21-°C-acclimated, and 10-°C-acclimated fish are indicated by *left-facing arrows* and *right-facing arrows*, respectively

MHC expression, in both sizes of fish examined. However, the fish acclimated starting at 20 weeks post-hatch showed a response after 2–3 weeks compared with 4–5 weeks for the 14-week post-hatch group. The time course for changes in myofibrillar ATPase in the 20-week post-hatch group was similar to that previously reported for adult stages. For example, for adult carp, a change in ATPase activity was apparent within 2 weeks, complete within 4–5 weeks and was completely reversible (Heap et al. 1985).

Turay et al. (1991) used northern analysis to establish the time course for the expression of MHC gene(s) in the

white muscle of carp following warm acclimation. MHC mRNA concentrations started to increase after 2 days and reached a new steady state after 2 weeks, consistent with regulation of myosin expression at the transcriptional level (Turay et al. 1991). Imai et al. (1997) isolated three cDNA clones encoding fast muscle MHCs in carp which were differentially expressed in 10-°C, 20-°C and 30-°C acclimated individuals. Northern blot analysis using RNA probes to the three kinds of DNA fragments from the 3' untranslated region, confirmed that the expression of the corresponding mRNA was controlled at the transcriptional level in an acclimation-temperature dependent fashion (Imai et al. 1997).

Alterations in MHC isoforms are responsible for myofibrillar ATPase changes in carp fast skeletal muscle (Hwang et al. 1991; Watabe et al. 1992; Guo et al. 1994; Watabe et al. 1995). The MHCs each contain a globular S1 head region. This part of the molecule contains the actin-binding site and the ATPase-binding pocket, which are 4 nm apart (Rayment et al. 1993). Thus, MHC composition and ATPase activity are intrinsically linked. Temperature acclimation most probably induces a structural change in the myosin molecule, affecting the function of the ATPase active site and the interaction of actin with myosin. The time taken for ATPase activity to alter as a result of temperature acclimation is thought to reflect the period needed for changes in the protein structure of the myosin to occur, which is limited by the rate of protein turnover. Dreyfus et al. (1960) concluded that the myofibrils of rabbit muscle have a life-span of 30 days and Clarke (1993) stated that MHC in isolated adult cardiac myocytes has a half life of 15.6 days. Heap et al. (1986) found that when carp were starved, the ability of the myofibrillar ATPase to show thermal compensation disappeared, since in starved individuals protein turnover rate was greatly reduced or ceased. Upon re-feeding, however, the ability to alter myofibrillar ATPase in response to a change in acclimation temperature re-appeared (Heap et al. 1986).

The expression of MHC isoforms is controlled in a tissue- and developmental-stage specific-manner with each isoform optimised for the specific requirements of the different muscles (Emersen and Bernstein 1987). MHCs are encoded by a highly conserved multigene family (Nguyen et al. 1982) in which selective switching on/off facilitates reconstruction of the contractile apparatus in response to changing environmental conditions and/or physical stimuli (reviewed by Goldspink 1996, 1998). The thermal acclimatory ability of carp has been attributed to a polyploid gene structure (Goldspink et al. 1992). Chromosome duplication, which enables multiple gene copies to exist, is thought to have allowed the evolution of alleles suitable for the production of protein isoforms which suit different thermal environments (as discussed by Baldwin and Hochachka 1970; Somero 1975). A large proportion of MHC gene loci have remained as functional duplicates for over 20 million years (Ferris and Whitt 1977; Zang et al. 1995). The increased multiplicity may have allowed some carp

MHCs to diverge to function at different temperatures enabling the expression of multiple fast skeletal MHC isoforms (Kikuchi et al. 1999).

Seasonal temperature changes have been shown to affect the thermal optimum of several physiological processes during development. For example, an increase in the thermal optimum for growth during ontogeny was observed in larval plaice *Pleuronectes platessa* (Hovenkamp and Witte 1991) and winter flounder (*Pseudopleuronectes americanus*) (Buckley 1982). During maturation, flight muscles in the dragonfly (*Libellula pulchella*) undergo striking changes in thermal sensitivity of locomotor performance paralleling changing thoracic temperature during ontogeny (Marden 1995; Marden et al. 1996). Similarly, thermoregulatory competence improves with age in altricial nestling red winged blackbirds (*Agelaius Phoeniceus*), where nestlings undergo an ontogenetic shift in both body temperature and thermal sensitivity of neuromuscular performance (Choi and Bakken 1990).

In conclusion, changes in myofibrillar ATPase activity and MHC composition in the common carp were not observed before a certain stage of maturity (ca. 37 mm TL), suggesting some form of developmental competence is required. This length is usually reached prior to the onset of the first winter and appears to occur independently of environmental cues of temperature and/or photoperiod.

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