Myofibrillar Gene Expression in Differentiating Lobster Claw Muscles

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ABSTRACT

Lobster claw muscles undergo a process of fiber switching during development, where isomorphic muscles containing a mixture of both fast and slow fibers, become specialized into predominantly fast, or exclusively slow, muscles. Although this process has been described using histochemical methods, we lack an understanding of the shifts in gene expression that take place. In this study, we used several complementary techniques to follow changes in the expression of a number of myofibrillar genes in differentiating juvenile lobster claw muscles. RNA probes complementary to fast and slow myosin heavy chain (MHC) mRNA were used to label sections of 7th stage (~3 months old) juvenile claw muscles from different stages of the molt cycle. Recently molted animals (1–5 days postmolt) had muscles with distinct regions of fast and slow gene expression, whereas muscles from later in the molt cycle (7–37 days postmolt) had regions of fast and slow MHC expression that were co-mingled and indistinct. Real-time PCR was used to quantify several myofibrillar genes in 9th and 10th stages (~6 months old) juvenile claws and showed that these genes were expressed at significantly higher levels in the postmolt claws, as compared with the intermolt and premolt claws. Finally, Western blot analyses of muscle fibers from juvenile lobsters ~3 to 30 months in age showed a shift in troponin-I (TnI) isoform expression as the fibers differentiated into the adult phenotypes, with expression of the adult fast fiber TnI pattern lagging behind the adult slow fiber TnI pattern. Collectively, these data show that juvenile and adult fibers differ both qualitatively and quantitative in the expression of myofibrillar proteins and it may take as much as 2 years for juvenile fibers to achieve the adult phenotype. J. Exp. Zool. 307A:281–295, 2007. © 2007 Wiley-Liss, Inc.

Lobster claws are dimorphic, with a large crusher claw and a slender cutter claw (Govind, '84, '92; Govind et al., '87). The muscles responsible for closing the claws show similar specialization, with the muscles of the crusher claw being composed of slow fibers, whereas those of the cutter are predominantly fast. This dichotomy is established during the juvenile stages of development, over a series of approximately 13 molt cycles (Govind, '84, '92; Govind et al., '87). Lobsters develop over a series of distinct stages, each stage being followed by a molt. The first three stages represent the larval stage of development and these lobsters live a planktonic existence. Following the larval stage, the developing lobsters settle to the benthic substrate and take on the general appearance of an adult lobster. Before the fifth molt stage, claw closer muscles are symmetrical, each being composed of a central core of fast fibers, surrounded by a region of slow fibers (Govind, '84, '92; Govind et al., '87). After this...
stage, the muscle fibers begin a gradual transformation into the predominantly fast fibers of the cutter claw, and the entirely slow fibers in the crusher. In adult snapping shrimp, claw transformation into the specialized snapping claw proceeds as fast fibers selectively degenerate to be replaced by slow fiber growth (Govind et al., '87). By contrast in the juvenile lobster claws, there has never been any observation of selective fiber degeneration, and so the fiber type transition has always been interpreted as an example of fibers switching from one type to another (Govind, '84, '92; Govind et al., '87). This process of muscle differentiation in lobster claws provides a unique model for determining the factors that influence muscle phenotype, since fast-to-slow and slow-to-fast transitions occur simultaneously in the same individual (Fig. 1).

When the process was initially described by Govind, Lang, and colleagues, several of the factors influencing claw differentiation were identified (Lang et al., '77a,b, '78, '79; Govind and Lang, '78; Costello and Lang, '79; Ogonowski and Lang, '79; Ogonowski et al., '80). In the early juvenile stages, the most used claw becomes destined to differentiate into the crusher claw and the other claw will become the cutter. In the absence of activity in either of the claws, neither claw becomes a crusher and the adult lobster will possess two similar cutter claws (Govind, '84, '92; Govind et al., '87). The precise factors that act as a switch during these early juvenile stages have not been identified, but exercise of the muscle is clearly not the cue. In fact, if both claws perform the same amount of activity during the developmental window when the dichotomy is established, neither of the claws will differentiate into a crusher claw (Lang et al., '78; Govind and Pearce, '86, '89). Therefore, an asymmetry in the amount of activity between the two claws is ultimately required to produce the crusher claw. These patterns suggest that neural input to the muscle fibers is central to differentiation and led Govind to suggest that the claw dichotomy stems from an asymmetry that is established at the level of the central nervous system (Govind, '84, '92).

In decapod crustaceans, the claw closer is controlled by two excitatory neurons and one inhibitory motor neuron. One of the excitatory neurons controls phasic contractions, whereas the other elicits more prolonged, tonic contractions. In adult, some of the closer fibers are controlled exclusively by the phasic motor neuron, some by the tonic motor neuron, and some of the fibers receive input from both neurons (Lang et al., '79; Costello and Govind, '83). The number of fibers controlled by the phasic and tonic neurons differ between claws, with more of the closer fibers receiving input from both the tonic and phasic neurons, whereas a larger proportion of the cutter fibers is controlled by the phasic neuron alone (Lang et al., '79; Costello and Govind, '83). However, no simple correlation with motor neuron type exists, as both slow and fast muscle fibers receive innervation from the phasic, tonic, or both motor neurons (Govind, '84, '92; Govind et al., '87).

Although the basic pattern of claw differentiation is well known, the precise events occurring within the muscles during the process have remained elusive. Using histochemical staining methods, fibers intermediate between the fast and slow phenotypes have been identified at the anatomical junction of the fast and slow fibers (Govind et al., '87). This observation suggests that fiber switching might occur in this region, with a fast-to-slow transition occurring in the developing

![Fig. 1. Overview of lobster development and the stages used for the different techniques used in this study. Each developmental stage is separated by a molt. The first three stages correspond to the larval phase, the 4th–13th represent the juvenile phase, and following the 13th phase a lobster is considered to be an adult. During approximately the first four stages, the claws are symmetrical and both possess a central core of fast fibers surrounded by slow fibers. Following the 4th stage, claws begin a process of differentiation that will continue until approximately the 13th juvenile stage. After that stage, the adult muscle phenotype is established and persists throughout the lifetime of the lobster. The 7th stage juveniles used in this study were approximately 3 months old and had a carapace length of about 10 mm. 9th–10th stage lobsters were approximately 6 months old and had a carapace length of about 21 mm. By 2.5 years, small adult lobsters have a carapace length of about 100 mm. Further details about the developmental process in lobsters are available in the review by Govind ('84).](image-url)
crusher claw and a slow-to-fast transition taking place in the developing cutter. Previous studies have found that protein synthesis increases in premolt and postmolt claw muscles (Skinner, '65, '66; El Haj et al., '96), although the data reporting concomitant increases in myofibrillar gene expression has been equivocal (Whiteley et al., '92; El Haj et al., '96; Mykles, '97; Whiteley and El Haj, '97). We recently reported subtle increases in MHC and actin in premolt and postmolt lobster muscles together as compared with intermolt muscles (Medler et al., 2005). In this study, we were interested in identifying the precise spatial and temporal patterns of myosin heavy chain gene expression in juvenile claw muscles from different stages of the molt cycle. In adults three fiber types (fast, slow-twitch or S1, and slow-tonic or S2) express different assemblages of myofibrillar protein isoforms of myosin heavy chain, paramyosin, troponin-T, troponin-I, troponin-C, tropomyosin, and P75 (Mykles, '85, '88; Medler and Mykles, 2003, 2004). The identity and function of P75 (75 kDa protein) is unknown, but it is only expressed in fast fibers (Mykles, '85; Medler and Mykles, 2003). Claw muscles from 7th stage juveniles were taken at different intervals following molting and were probed for fast and slow (S1) MHC mRNA using in situ hybridization. In complementary analyses, mRNA levels of several myofibrillar protein genes in claw muscles from 9th and 10th stage juveniles were quantified using real-time PCR. Finally, individual muscle fibers from the juveniles 3 months to 2.5 years in age were analyzed by SDS-PAGE and Western blotting to reveal the myofibrillar isoform assemblage present. We focused primarily on differences in TnI isoforms, five of which are expressed in various adult muscles (Mykles, '85, '88). Collectively, these analyses revealed marked fiber heterogeneity and significant differences in gene expression with respect to molt stage and age.

MATERIALS AND METHODS

Animals

Juvenile lobsters, Homarus americanus, were raised in the culture facility at the Bodega Marine Laboratory from larvae (Chang and Conklin, '93). The left claw of 4th stage larvae was autotomized to induce differentiation of the right claw into the crusher type; the regenerated left claw differentiates into the cutter type (Govind and Pearce, '89). Claws were autotomized from juveniles and either processed for in situ hybridization (see methods below) or quickly frozen in isopentane cooled in liquid N2 and then stored for later analysis (see real-time PCR and Western blotting methods below). Claws from 39 7th stage animals were processed for in situ hybridization. In addition, paired claws from 25 9th and 10th stage animals were used for real-time PCR analysis and for analysis of myofibrillar proteins in single muscle fibers.

Synthesis of RNA probes

Fast and slow (S1) RNA probes were made from plasmids (pcDNA II, Invitrogen, Inc., Carlsbad, CA, USA) containing the sequences of interest. The fast MHC cDNA is 1,529 bp in length encoding the C-terminal region of the molecule and has an open reading frame of 1239 bp (GenBank accession no. U03091) (Cotton and Mykles, '93). The slow MHC cDNA is 1,795 bp in length, encoding the same region of the MHC as the fast sequence, and has an open reading frame of 1,523 bp (GenBank accession no. U03091) (Cotton and Mykles, '93). The slow MHC cDNA is 1,795 bp in length, encoding the same region of the MHC as the fast sequence, and has an open reading frame of 1,523 bp (GenBank accession no. AY232598) (Medler and Mykles, 2003). The two sequences share 79% sequence identity within the open reading frame, but diverge significantly in the 3'-UTR (Medler and Mykles, 2003). Plasmids were linearized with either Spe or Xba restriction enzymes (Invitrogen) and isolated with a 1% agarose gel containing 0.1 mg/mL ethidium bromide in TAE buffer (40 mM Tris, 1 mM EDTA, and 0.1% acetic acid) and run at 100 V. Linearized plasmids were purified from the gel with Qiagen Gel Extraction Kit (Qiagen, Valencia, CA). DNA concentration was determined by measuring absorbance at 260 nm. Anti-sense and sense digoxigenin (Dig)-labeled RNA probes were synthesized using Dig-RNA Labeling Kit with either T7 or Sp6 promoters (Roche Molecular Biochemicals, Indianapolis, IN). Before ethanol precipitation, probes were hydrolyzed by alkaline hydrolysis for 10 min at 60 °C. In some cases, anti-sense probes were linearized with Nco restriction enzyme (Invitrogen) and alkaline hydrolysis was omitted. The Nco enzyme cuts in the fast and slow (S1) MHC open reading frame, omitting much of this highly conserved sequence, and reducing the potential for cross-hybridization.

Solutions for in situ hybridization

Water for all solutions used for in situ hybridization was treated with 500 μL/L of diethylpyrocarbonate with stirring overnight and then
autoclaved to remove any RNase activity. Sodium/sodium citrate (SSC) solutions were diluted from a 20X stock solution and contained 150 mM NaCl and 15 mM sodium citrate (pH 7.2). Phosphate buffered saline (PBS) contained 10 mM Na2HPO4, 3 mM KH2PO4, and 124 mM NaCl (pH 7.25). Hybridization buffer consisted of 50% deionized formamide, 4X SSC, 50 mM sodium phosphate buffer (pH 6.8), 1X Denhardt’s solution, 0.1% SDS, 0.25 mg/mL salmon sperm DNA, 5% dextran sulfate, and 0.25 mg/mL tRNA. Denhardt’s solution used in the hybridization buffer was composed of 0.2% each of Ficoll Type 400, Tween-20 (polyoxyethylene-sorbitan monolaurate), and bovine serum albumin. Blocking buffer for incubation of the anti-Dig-AP antibody contained 150 mM NaCl, 100 mM Tris-HCl (pH 7.4), 2% horse serum, 2% goat serum, and 5 mM levamisole.

**In situ hybridization**

The in situ procedures used in this study are essentially the same as those described in a previous study in our laboratory (Koenders et al., 2002), with the exception of different hybridization temperatures. Proximal and distal ends of the claw were severed and discarded and the remaining tissue was fixed overnight at 4°C in 3.7% formaldehyde, 0.5 M NaCl, 15 mM KCl, 10 mM EDTA, and 25 mM HEPES-NaOH (pH 7.5) and rinsed in buffer without formaldehyde. Claws were then dehydrated through a graded ethanol series, followed by a graded series of xylenes, embedded in paraffin, and sectioned (10 µm thick sections).

Sections on slides were heated for approximately 5 min at 60°C to melt the paraffin and then cleared in two washes with xylenes. Sections were then re-hydrated through a graded ethanol series (95% 5 min/70% 5 min/50% 5 min) and then into water. Sections were incubated in 20% acetic acid for 3 min at 4°C and then rinsed twice in water (2 min per rinse). Next, sections were washed in SSC for 2 min, immersed in 0.2 N HCl for 20 min, and then placed into fresh SSC for 2 min. The sections were then treated with Proteinase K (25 µg/mL) for 12 min at 37°C, followed by two washes in 0.1 M glycine in PBS (5 min per wash). Sections were next treated with 0.1 M triethanol amine/0.25% acetic anhydride with agitation for two incubations (5 min per wash). Following this, they were twice washed in 2X SSC (2 min per wash), incubated for 15 min in 0.1 M glycine in 0.1 M Tris (pH 7.4), and twice washed a second time in 2X SSC. Finally, the sections were allowed to equilibrate in hybridization buffer for 1 hr at 60°C in a sealed, humid container. After 1 hr, the hybridization solution was replaced with fresh solution containing RNA probes (250 ng/mL) and sections were incubated overnight (approximately 16 hr) at 60°C in the sealed, humid container. Coverslips, sealed around the edges with rubber cement, covered the sections during the incubation period.

Following incubation with RNA probes, sections were washed in 2X SSC/50% formamide for 5 min, and then incubated in fresh solution for 1 hr at 60°C. Sections were then twice washed in SSC at 60°C for 1 min per wash, and then treated with RNase A (0.135 units/mL) for 30 min at 37°C. After this, sections were again washed in 2X SSC/50% formamide for 5 min, followed by the 1 hr incubation at 60°C. Sections were then washed three times in 0.1 M Tris (pH 7.4) for 3 min per wash, and then incubated in blocking buffer for 1 hr at room temperature. Finally, sections were incubated in anti-Dig-AP antibody (Dig Nucleic Acid Detection Kit, Roche Biochemicals, Indianapolis, IN) (1:500) in blocking buffer for 1 hr at room temperature.

After incubation with the anti-Dig-AP antibody, sections were washed three times (5 min per wash) in 0.1 M Tris (pH 7.4) and then covered with detection buffer containing NBT/BCIP (Roche Molecular Biochemicals, Indianapolis, IN). Tissues were allowed to develop in the dark, with periodic visual monitoring, until color changes were evident (usually about 1 hr). Sections were then washed several times in water, dehydrated through a graded ethanol series, twice washed in xylenes, and then mounted with Permount and a coverslip.

**Immunohistochemistry with anti-P75 antibody**

Claw muscle sections used for in situ hybridization were also labeled with anti-P75 antibodies to differentiate fast and slow muscle regions at the protein level. The anti-P75 antibody is a polyclonal serum developed in rabbit injected with gel purified P75 protein and has been used previously for Western blotting (Sohn et al., 2000; Mykles et al., 2002; Medler and Mykles, 2003). Serial sections were processed as described above, to the point of being re-hydrated through the graded ethanol series and then into water. Next, sections were covered with a blocking solution containing...
2% bovine serum albumin in PBS for 1 hr at room temperature. The blocking solution was then replaced by the same solution containing the anti-P75 antibody (1:500) and covered the sections for 1 hr at room temperature. Next, the sections were washed three times (5 min each) in Tris-buffered saline (TBS) with 0.05% Tween (TTBS). Sections were then covered by blocking buffer containing a biotin conjugated anti-rabbit antibody (Vector Labs, Burlingame, CA; 1:500) for 1 hr at room temperature. After 1 hr, the sections were washed three times (5 min each) in TTBS and then covered by an avidin–peroxidase complex (ABC reagent, Vector Labs, Burlingame, CA) for 30 min. Finally, sections were washed three times (5 min each) in TTBS and then developed using diaminobenzidine as a substrate for the peroxidase enzyme. The development solution contained 200 μL diaminobenzidine (40 mg/mL), 50 μL NiCl2 (80 mg/mL), and 30 μL of H2O2 (30%) in 10 mL of 100 mM Tris buffer pH 7.4. Sections were allowed to develop until a visible color change was observed in the sections (approximately 5–10 min). Sections were then washed several times in water, dehydrated through a graded ethanol series, twice washed in xylenes, and then mounted with Permount and a coverslip.

**Real-time PCR**

Total RNA was isolated from whole, 9th and 10th stage juvenile claws by homogenizing the claws in 1 mL TRIZol reagent. After complete homogenization, samples were centrifuged for 10 min at 12,000 × g at 4°C to remove any insoluble material. Samples were then allowed to stand at room temperature for 5 min, before the addition of chloroform (0.2 mL per 1 mL TRIZol reagent). Samples were shaken vigorously by hand for 30 sec, allowed to stand at room temperature for 5 min, and then centrifuged at 12,000 × g for 10 min at 4°C. RNA was precipitated from the aqueous phase by the addition of isopropanol (0.5 mL per 1 mL TRIZol reagent). Samples were allowed to stand at room temperature for 10 min and then centrifuged at 12,000 × g for 10 min at 4°C to collect the precipitated RNA. Samples were washed with 75% ethanol and then allowed to air dry for about 10 min. RNA samples were dissolved in water and stored at −20°C.

RNA samples were treated with DNase (Invitrogen) for 15 min at room temperature to remove any genomic contamination, and then used as template for the synthesis of cDNA using MMLV reverse transcriptase (Invitrogen). Individual reactions contained 2.5 μg of oligo(dT) 12–18, 2.5 mM dNTP, 1X first-strand buffer, 5 mM dithiothreitol, 2.5 units of RNase inhibitor, 1–2 μg of RNA, and 200 units of MMLV reverse transcriptase. cDNA samples isolated were stored at −20°C until being used as template for real-time PCR.

The sequence-specific primers and conditions used for real-time PCR are the same as those described in previous studies (Medler and Mykles, 2003; Medler et al., 2004, 2005). Briefly, a master PCR reaction mix including SYBR green was prepared before adding either a known number of copies of cDNA of the gene of interest, or an unknown amount of cDNA from muscle samples. Known numbers of copies of cDNA in plasmids were serially diluted and used to generate standard curves consisting of cycle threshold as a function of copy number. These curves were used to determine the number of copies of a transcript in the juvenile muscles from cycle threshold. The master mix was the Light Cycler DNA Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN) and samples were run on a Cepheid (Sunnyvale, CA) Smart Cycle Instrument.

**Analysis of myofibrillar proteins in single muscle fibers**

Single muscle fibers were taken from juvenile claw muscles for analysis of the protein isoforms present in the fibers. Whole frozen claws were dried under vacuum (Speed Vac, Thermoscientific, Waltham, MA) and then stored in a sealed glass jar with desiccant. The exoskeleton was peeled back from dried claws and individual muscle fibers were carefully dissected out with fine forceps under a stereomicroscope. Individual fibers were collected in 1.5 mL microcentrifuge tubes and 20 μL of SDS sample buffer (31.25 mM Tris pH 6.8, 12.5% (v/v) glycerol, 5% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% β-mercaptoethanol) was added directly to the tubes. Fibers were then homogenized in the buffer using a small pestle that fitted directly into the microcentrifuge tubes. The entire homogenates from each of the fibers were loaded on 10% SDS-PAGE gels and separated under constant voltage (200 V) for approximately 45 min. Gels were then either processed for silver staining (Wray et al., '81) or for transfer of the proteins to PVDF membranes for Western blotting (Sohn et al., 2000; Mykles et al., 2002; Medler and Mykles,
The anti-troponin antibody was raised in rabbit against purified lobster troponin I and is a polyclonal serum that reacts with different troponin I isoforms in lobsters and crayfish (Sohn et al., 2000; Koenders et al., 2004; Medler et al., 2004). The relative amounts of specific proteins in bands from stained gels and blots were determined using scanning densitometry (NIH Image 1.62; Bethesda, MD).

**Statistical analyses**

Paired *t*-tests were used to compare mRNA levels (fast MHC, P75, S1 MHC, and actin) in juvenile cutter and crusher claw muscles. Unpaired *t*-tests were used to compare the levels of mRNA (fast MHC, P75, S1 MHC, S2 MHC, and actin) for postmolt vs. premolt/intermolt claw muscles measured by real-time PCR. A factorial analysis of variance (ANOVA) was used to compare the number of copies of myofibrillar mRNA, with factors including myofibrillar gene (fast MHC, P75, S1 MHC, S2 MHC, and actin), claw type (cutter vs. crusher), and timing in molt cycle (postmolt vs. premolt/intermolt). A Bonferroni post-hoc test was used to make pairwise comparisons of individual means (experiment-wise error 0.05). Values were log-transformed because of a high level of variability among samples and to correct for the correlation between mean and variance in these data (Neter et al., ’90). Simple linear regression was used to examine the correlation between the number of copies of mRNA for different myofibrillar genes, including the correlation between levels of fast MHC and P75, and between S1 MHC and actin mRNAs, measured by real-time PCR. Relative levels of TnI isoforms were compared by ANOVA and pair-wise post-ANOVA comparisons were performed by Bonferroni test, with an experiment-wise error rate of 0.05. Since these levels were expressed as proportions, the values were arcsin transformed before the analyses (Neter et al., ’90). Statview 5.0.1 (SAS Institute Inc; Cary, NC.) was used for all statistical analyses.

**RESULTS**

**Spatial distribution of MHC transcripts**

Fast and slow (S1) mRNAs were identified in juvenile claw muscles with in situ hybridization using complementary, anti-sense RNA probes. In corresponding analyses, serial sections were labeled with an anti-P75 antibody to identify fast muscle regions at the protein level. Specificity of labeling with the RNA probes was confirmed by comparison with negative controls (sense RNA probes) and specificity of the anti-P75 antibody was verified by comparison with pre-immune serum (Fig. 2). The anti-P75 antibody showed some level of staining of slow muscle fibers as compared with the pre-immune control serum, although fast regions were consistently identified by greater staining intensity in those regions (Fig. 2C).

Distinct differences in staining patterns were identified between developing cutter and crusher claws (Fig. 3). Developing crusher claw muscles were composed of a central core of fast muscle fibers, surrounded by slow (S1) fibers (Fig. 3A). By contrast, developing cutter claws possessed a majority of fast muscles in 7th stage juvenile claws (Fig. 3B). The claw opener and the ventral portion of the cutter claws were composed of fibers expressing the slow (S1) MHC (Fig. 3B). These staining patterns were more distinct in 10th stage animals (Figs. 3C and D). The crusher muscles lagged behind the cutter claw in differentiation, as crusher claws still possessed a significant proportion of fast muscles at the 10th stage of development (Figs. 3C and D). These general staining patterns were frequently difficult to discern in 7th stage animals, as the apparent expression levels of P75 and fast and S1 MHCs overlapped in both the fast and slow fiber regions (Figs. 4B and C). There was some background staining with the fast sense probe (Fig. 3A). As shown previously using histochemistry (Ogonowski et al., ’80; Govind, ’84, ’87, ’92), fast fibers were located in the central region of the closer muscle and slow fibers were located dorsal and ventral regions of the closer muscle and in the opener muscle in both claw types (Figs. 2 and 3).

Differences in the spatial expression patterns of MHC were related to the time point within the molt cycle (Fig. 4). Within the first days following molting (1–5 days), distinct fast and slow (S1) regions were identified with RNA probes and by immunohistochemistry (Figs. 4A and B). At the mid to late stages of the molt cycle (7–37 days), these patterns were less distinct (Fig. 4C). In the late stages of the cycle, significant overlap in fast and slow (S1) MHC expression was observed and distinct fast and slow muscle regions were often difficult to identify (Fig. 4C). At 1 day postmolt, expansion of the new exoskeleton resulted in increased hemolymph space surrounding the
fibers (Fig. 4A). By 3 days postmolt the fibers were larger, indicating that there was significant fiber growth during this period (Fig. 4B). By 18 days postmolt, the space was largely filled by fibers (Fig. 4C).

Quantification of myofibrillar transcripts by real-time PCR

Whole 9th and 10th stage juvenile claws were homogenized and the isolated RNA was used as template for quantitative real-time PCR for several myofibrillar transcripts (fast MHC, slow-twitch [S$_1$] MHC, slow-tonic [S$_2$] MHC, P75, and actin). As expected, significant differences in expression were observed between the paired cutter and crusher claws from an individual (Fig. 5). Cutter claws had significantly greater expression of fast MHC and P75, whereas crusher claws had greater expression of slow (S$_1$) MHC and actin (Fig. 5). The mean expression levels of fast MHC and P75 were sixfold and 3.7-fold higher, respectively, in the cutter muscles than in the crushers. By contrast, slow (S$_1$) MHC expression was 12.7-fold higher, and actin expression was 19.8-fold higher on average in the crusher muscles. Expression of slow (S$_2$) MHC did not significantly differ between the claw types (data not shown), but had lower expression levels than the other myofibrillar genes (see below).

When expression levels were assessed as a function of the timing in the molt cycle, we found that within the first few days of molting (1–5 days), expression levels were consistently higher than the mid-to-late (7–37 days) stages of the molt cycle for each of the genes examined in the combined crusher and cutter claw muscles (Fig. 6). However, owing to the variability of the measurements, only the difference for the slow (S$_2$) MHC was statistically significant as compared by t-tests (Fig. 6). A more powerful factorial ANOVA revealed that collectively, myofibrillar gene expression was significantly different in several of the groupings (Table 1). The most important comparison of interest here is the time in the molt cycle (postmolt vs. intermolt/premolt), which showed a significant effect (Table 1, time in cycle:

![Image](image_url)

Fig. 2. Specificity of labeling of MHC RNA probes and anti-P75 antibody. Sections of developing crusher claw muscles were labeled with (A, left) anti-sense fast MHC RNA probe or (A, right) sense fast probe; (B, left) anti-sense S$_1$ MHC RNA probe or (B, right) sense S$_1$ probe; (C, left) anti-P75 antibody or (C, right) pre-immune serum. Claws in A and C are from 7th stage lobster (3 days postmolt), whereas the claw in B is from a 10th stage lobster (molt stage unknown). The dorsal opener muscle and fast and slow muscle regions are indicated. Other characteristic structures include the apodeme (apo) of the closer muscle and fragments of the exoskeleton (exo).
$P = 0.0016$), with the higher expression being in the postmolt muscles. In addition, the overall level of expression was significantly different among genes (Table 1, gene: $P < 0.0001$; order of expression levels: actin $>$ [fast MHC $=$ P75] $>$ S1 MHC $>$ S2 MHC). Overall, the crusher claws had significantly higher expression than the cutter claws (Table 1, claw type: $P = 0.0308$). Finally, the interaction between claw type and gene represented a significant effect (Table 1, gene $\times$ claw type: $P = 0.0001$), as already demonstrated (Fig. 5).

Regression analyses revealed significant correlation among certain of the myofibrillar genes (Fig. 7). A significant correlation was found between the levels of fast MHC and P75 transcripts (Fig. 7A), whereas slow (S1) MHC and actin transcript levels were similarly correlated with one another (Fig. 7B). When levels from postmolt muscles were analyzed independently from the intermolt/premolt muscles, we observed a stronger correlation between both the fast MHC and P75, and between slow (S1) MHC and actin transcript levels, than in the muscles from the intermolt/premolt animals (Fig. 7). Specifically, the correlation between fast MHC and P75 expression was strong in the postmolt muscles ($P < 0.0001$; $r^2 = 0.73$), but was less pronounced later in the cycle ($P < 0.01$; $r^2 = 0.21$). Similarly, the correlation between expression of S1 MHC and actin was quite strong in postmolt muscles ($P < 0.0001$; $r^2 = 0.71$), but was less distinct in muscles from later in the intermolt cycle ($P < 0.015$; $r^2 = 0.19$).

**Myofibrillar proteins in single muscle fibers**

Individual fibers from the claw closer of the paired claws of 9th and 10th stage juvenile claws, as well as muscles from older juvenile and adult lobsters, were analyzed by SDS-PAGE and Western blotting. In this study, we were able to distinguish four of the five TnI isoforms, as the I3 and I4 were not resolved from one another using our gel system. Fast fibers from presumptive 

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Fig. 3. Differences between developing crusher claw muscles and cutter claw muscles. Serial cross sections of muscles were labeled with fast MHC RNA probe (left), S1 MHC RNA probe (middle), and anti-P75 antibody (7th stage only) (right). By the 7th molt stage, many of the developing crusher muscles still possess a distinct central region of fast fibers and distinct dorsal and ventral regions containing slow fibers (claw from 3 days postmolt animal). (B) Developing 7th stage cutter claws are primarily composed of fast fibers by this stage (claw from 7 days postmolt animal). The opener muscle and the ventral region of the claw closer are entirely slow. (C and D) 10th stage claw muscles possess similar staining patterns, but the patterns are often more distinct than the claws from 7th stage claw muscles (molt stage unknown in 10th stage claws).
cutter claws from 9th and 10th stage juveniles differed in TnI isoform expression from fast fibers from adult cutter claw (Fig. 8). At least four TnI isoforms were expressed at comparable levels in juvenile fast fibers, whereas TnI1 was the predominant isoform in adult fast fibers (Fig. 8A, compare lanes a and b). TnI5, which was expressed at very low levels in adults, was present at relatively high levels in juveniles. Similarly, many juvenile slow fibers expressed at least four TnI isoforms, whereas the differentiated fibers nearly exclusively expressed the TnI3/4 isoform. We assume that in the adult slow fibers, this was primarily TnI4, which is the predominant isoform in these fibers (Mykles, ’85, ’97). Differentiation of TnI isoforms took place earlier in the crusher claw muscles, as some of these muscles were fully differentiated in the earlier juvenile stages (Fig. 8). The transition from the juvenile fast phenotype to the adult fast phenotype occurred over a period of greater than 19 months, but less than 2.5 years (Fig. 8B). In contrast, the transition in TnI isoform composition in slow fibers took place earlier, with many of the juvenile crusher fibers, as early as 8th stage, possessing the same TnI isoforms as the adults (Fig. 8B, right claws). Undifferentiated cutter and crusher claws expressed similar levels of each of the TnI isoforms, although statistically significant differences were found (Fig. 8C). In clear contrast, fully differentiated cutter and crusher claw muscles expressed one predominant isoform to the near exclusion of others (Fig. 8D). We observed interindividual variability in the TnI composition of crusher claws of 10th-stage lobsters, as some lobsters had claws expressing only the adult isoforms, whereas others still possessed the juvenile isoform profile.

**DISCUSSION**

Earlier studies of developing lobster claws relying on ATPase histochemistry seemed to reveal a clear dichotomy between fast and slow regions of the juvenile claw muscles (Ogonowski et al., ’80; Govind, ’84; Govind et al., ’87). Indeed, as we began this study we anticipated finding fast fibers, slow fibers, and a limited number of “hybrid” fibers that expressed both isoforms of MHC. This is consistent with the established view of skeletal muscle plasticity, where these “hybrid” fibers are in the process of making an orderly transition from one “pure” muscle type to another (Pette and Staron, 2000, 2001; Pette, 2001). The results of the current study show that this traditional view of muscle phenotype and plasticity does not accurately fit the complex process of muscle differentiation in the lobster claw muscles. Although at certain stages significant differences
between fiber types are distinct, the more common pattern is one in which the differences between fast and slow fibers are blurred. Within the first few days of molting, myofibrillar gene expression is elevated and the anatomical distinction between fast and slow fiber populations is clear (Figs. 4 and 6). During this time period, not only are the fast and slow (S₁) MHC isoforms expressed at higher levels, but also there appears to be coordination between the individual myofibrillar genes that make up a particular fiber type (Fig. 7). After approximately 5 days, this gene expression decreases and the expression of different myofibrillar genes becomes less coordinated (Figs. 4 and 6). During the remainder of the molt cycle, distinct regions of fast and slow MHC expression are difficult to distinguish (Fig. 4). Our Western blot analyses of TnI isoforms similarly show that juvenile muscle fibers experience a period of development when multiple isoforms are expressed at nearly equal levels, before making the transition to adult phenotypes in which isoforms are expressed in a fiber-specific pattern (Fig. 8). Collectively, these data are consistent with our recent findings of co-expression of myofibrillar isoforms in adult lobster muscle fiber types (Medler and Mykles, 2003; Medler et al., 2004, 2005).

Over the past few years, we have identified a significant level of muscle fiber polymorphism in adult lobster muscles at both the protein and mRNA levels (Medler and Mykles, 2003; Medler et al., 2004, 2005). In slow fibers of the lobster legs and of the abdomen, fibers exist in a continuum between slow twitch (S₁) and slow tonic (S₂) phenotypes (Mykles et al., 2002; Medler et al., 2004). In this context, the co-expression of both fast and slow (S₁) MHC isoforms and multiple isoforms of TnI in the developing juvenile claw muscles is not surprising. Consistent with the idea of a continuum between fiber phenotypes, previous studies of sarcomere length and mechanical properties demonstrated that while two populations of fibers were present in the adult lobster claw muscles, fibers within the two populations

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**Fig. 5.** Differences in expression of myofibrillar genes in developing crusher (open bars) and cutter (filled bars) claw muscles assessed by quantitative real-time PCR in 9th and 10th stage lobsters. Values are averages of muscles from all molt stages. (A) Developing crusher claw muscles had significantly higher levels of slow (S₁) MHC, whereas (B and C) developing cutter claw muscles expressed significantly higher levels of fast MHC and P75. (D) Developing crusher muscles expressed significantly higher levels of actin than the cutter muscles. *t*-tests were performed on log-transformed data. Values are means ± SEM.
exist as a continuum (Lang et al., '77a,b, '78; Costello and Govind, '83). The results reported here show that the fibers in juveniles are not the same as those in adults. The process of fiber differentiation in the juvenile claw muscles may represent a gradual change in phenotype from one end of a continuum to another, rather than a discrete process of switching from one fiber type to another. Moreover, the TnI isoform analysis indicates that it may take 2 years for juvenile fibers to achieve the adult fast fiber phenotype.

The co-expression of multiple myofibrillar proteins in single juvenile claw fibers is most clearly demonstrated in the data from our Western blot analyses. Here, we focused our attention on isoforms of TnI, because we recently found that these proteins exist as a continuum in certain adult fiber types (Medler et al., 2004) and the isoforms can be identified through SDS-PAGE and Western blotting. In contrast, the fast and slow (S1) MHC isoforms analyzed by in situ hybridization and real-time PCR exhibit the same electrophoretic mobility and are not resolved using SDS-PAGE (Medler and Mykles, 2003). We found that single juvenile fibers had multiple isoforms of TnI, whereas adult fibers exhibit more restricted expression patterns (Fig. 8). Specifically, juvenile fibers in both types of differentiating claws expressed nearly equal levels of TnI1–I5, whereas the differentiated crusher claws almost exclusively expressed the TnI3/4 isoform. Fully differentiated cutter muscles expressed primarily TnI1, with low levels of other TnI isoforms. The differences between juvenile and adult myofibrillar isoforms underscore the fact that developing muscles are not strictly equivalent to the fully differentiated adult muscles. Differences between juvenile and adult claw muscle fibers were previously noted by Costello and Govind ('84), although the identity of the proteins was not known at that time. Crustacean muscles are similar to the muscles of other animals, including mammals and birds, as the fibers undergo a sequential change in protein isoforms during the transition from the juvenile to adult stages. Likewise, developing muscles in mammals and birds exhibit significant shifts in myofibrillar proteins as they mature (Schiaffino and Reggiani, '96; Bandman and Rosser, 2000).

Many studies of muscle phenotype and plasticity focus exclusively on the MHC isoform to identify muscle fiber types. While the MHC molecule is arguably the most important protein involved in producing a particular type of contractile response, we should not overlook the fact that myofibrillar proteins operate as complex assemblages of different proteins. During the process of muscle fiber differentiation or during fiber type transitions, a whole number of genes must be expressed in a coordinated fashion. In a previous study (Medler and Mykles, 2003), we reported that in adult lobster muscles, actin and S1 MHC mRNA levels were positively correlated with one another, as were fast MHC and P75. Actin is expressed at higher levels in lobster slow muscles, which probably reflects the higher actin to myosin ratio in these muscles (Medler and Mykles, 2003; Medler et al., 2005). In this study, we observed the same patterns of expression, with the following qualification: the correlation between gene

### Table 1. Factorial ANOVA of myofibrillar gene expression in 9th and 10th stage lobster claw muscles (log10 copies mRNA/total RNA) with factors: time in intermolt cycle (early vs. mid); myofibrillar gene (fast MHC S1, MHC, S2 MHC, actin, and P75); and claw type (cutter vs. crusher)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>4</td>
<td>194.454</td>
<td>48.614</td>
<td>44.537</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Claw type</td>
<td>1</td>
<td>5.187</td>
<td>5.187</td>
<td>4.752</td>
<td>0.0308</td>
</tr>
<tr>
<td>Time in cycle</td>
<td>1</td>
<td>11.300</td>
<td>11.30</td>
<td>10.353</td>
<td>0.0016</td>
</tr>
<tr>
<td>Gene × claw type</td>
<td>4</td>
<td>26.433</td>
<td>6.608</td>
<td>6.054</td>
<td>0.0001</td>
</tr>
<tr>
<td>Gene × time in cycle</td>
<td>4</td>
<td>8.141</td>
<td>2.035</td>
<td>1.864</td>
<td>0.1193</td>
</tr>
<tr>
<td>Claw type × time in cycle</td>
<td>1</td>
<td>0.985</td>
<td>0.985</td>
<td>0.902</td>
<td>0.3437</td>
</tr>
<tr>
<td>Gene × claw type × time in cycle</td>
<td>4</td>
<td>3.252</td>
<td>0.813</td>
<td>0.745</td>
<td>0.5628</td>
</tr>
<tr>
<td>Residual</td>
<td>157</td>
<td>171.369</td>
<td>1.092</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DF, degrees of freedom; SS, sum of squares; MS, mean square.
Dependent variable: log10 RNA copies/µg total RNA.
Bold values indicate significant effects (P < 0.05).
Significant differences in expression were observed for time in intermolt cycle (P = 0.0016), myofibrillar gene (P < 0.0001), claw type (P = 0.0308), and the interaction between gene and claw type (P = 0.0001).
expression was quite strong within the first few days of molting, whereas the correlation was much weaker in claw muscles later in the molt cycle (Fig. 7). These patterns were evident for both the correlation between P75 and fast MHC, and the correlation between S₁ MHC and actin.

Fig. 6. Differences in expression of myofibrillar genes between postmolt (1–5 days postmolt; open bars) and intermolt/premolt (7–32 days postmolt; filled bars) claw muscles assessed by quantitative real-time PCR in combined crusher and cutter claw muscles of 9th and 10th stage lobsters. For each of the genes examined, postmolt muscles exhibited higher levels of expression than intermolt/premolt, but except for the S₂ MHC, these differences were not significant when compared by t-tests. A more powerful analysis by ANOVA indicated that the postmolt muscles had significantly higher expression than the intermolt/premolt muscles (Table 1).

Fig. 7. Correlation among myofibrillar gene expression assessed by quantitative real-time PCR in the claws of 9th and 10th stage lobsters. (A) Fast myofibrillar genes (fast MHC and P75) were significantly correlated in their expression, as were (B) actin and S₁ genes. However, this correlation was much tighter in postmolt muscles (1–5 days postmolt; filled circles) than in intermolt/premolt muscles (7–32 days postmolt; open circles). The correlation between fast MHC and P75 expression (A) in postmolt muscles was significant ($P < 0.0001$; $r^2 = 0.73$; filled circles), but was less pronounced later in the intermolt/premolt muscles ($P < 0.01$; $r^2 = 0.21$; open circles). The same pattern was observed for the correlation between S₁ MHC and actin (B). In postmolt muscles the correlation was significant ($P < 0.0001$; $r^2 = 0.71$; filled circles), but was less distinct in intermolt/premolt muscles ($P < 0.015$; $r^2 = 0.19$; open circles).
Collectively, the data from the studies using in situ hybridization and from real-time PCR provide clues into the patterns of myofibrillar gene expression in the developing lobster claw muscles. Under the conditions of this study, a typical molt cycle for 7th–10th stage juvenile lobsters lasts approximately 30–35 days. Within the first few days of completing a molt, myofibrillar gene expression is mostly restricted to the established fiber types: fast fibers express fast MHC and slow fibers express S1 MHC. During this early period,
gene expression appears to be well coordinated for the different fiber types, as indicated by the correlation between fast MHC and P75 expression and between actin and S1 MHC, in fast and slow muscles, respectively. This coordination is significant, because fiber types are defined by whole assemblages of myofibrillar proteins that must interact effectively during muscle contraction. Later in the cycle, muscle gene expression is less well coordinated, as both fiber types often co-express fast and S1 MHC and the correlation between fiber-specific genes is much weaker. Throughout the molt cycle, the claw muscles experience a significant increase in muscle mass, as the exoskeleton fills with new muscle growth. The connection between muscle growth and the establishment of distinct fiber types is not clear, but is likely important in the development of the populations of fast and slow fibers in the claw. Differential growth of a population of fast and slow fibers, for example, could produce the final distribution observed in the cutter and crusher claw muscles. How differential fiber growth might be coordinated with changes in gene expression should be a focus of future research.

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LITERATURE CITED


Mykles DL. 1985. Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, para-