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Eyestalk Ablation Has Little Effect on Actin and Myosin Heavy Chain Gene Expression in Adult Lobster Skeletal Muscles

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Abstract. The organization of skeletal muscles in decapod crustaceans is significantly altered during molting and development. Prior to molting, the claw muscles atrophy dramatically, facilitating their removal from the base of the claw. During development, lobster claw muscles exhibit fiber switching over several molt cycles. Such processes may be influenced by the secretion of steroid molting hormones, known collectively as ecdysteroids. To assay the effects of these hormones, we used eyestalk ablation to trigger an elevation of circulating ecdysteroids and then quantified myofibrillar mRNA levels with real-time PCR and myofibrillar protein levels by SDS-PAGE. Levels of myosin heavy chain (MHC) and actin proteins and the mRNA encoding them were largely unaffected by eyestalk ablation, but in muscles from intact animals, myofibrillar gene expression was modestly elevated in premolt and postmolt animals. In contrast, polyubiquitin mRNA was significantly elevated (about 2-fold) in claw muscles from eyestalk-ablated animals with elevated circulating ecdysteroids. Moreover, patterns of MHC and actin gene expression are significantly different among slow and fast claw muscles. Consistent with these patterns, the three muscle types differed in the relative amounts of myosin heavy chain and actin proteins. All three muscles also co-expressed fast and slow myosin isoforms, even in fibers that are generally regarded as exclusively fast or slow. These results are consistent with other recent data demonstrating co-expression of myosin isoforms in lobster muscles.

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Introduction

Lobster muscles are complex in organization, with different muscles being composed of a distinct assortment of different fiber types. The factors responsible for establishing and maintaining these fiber types are unknown, but one possible influence is the group of molting hormones known as ecdysteroids. These steroid hormones control complex processes associated with molting and may affect muscle gene expression. Experimental removal of eyestalks causes an increase in circulating ecdysteroids and thus provides a means of manipulating hormone concentration. In this study, we examined the effects of eyestalk ablation and elevated ecdysteroids on skeletal muscle gene expression.

The muscles of the large claws of decapod crustaceans undergo a significant atrophy of up to 60%–75% of their mass prior to molting, so that the bulky muscle can be withdrawn from the narrow basi-ischial opening (Mykles and Skinner, 1990; Ismail and Mykles, 1992; Mykles, 1997; West, 1997). After the molt, muscle mass is quickly regained (Skinner, 1966; Mykles, 1997). Consistent with these findings, myofibrillar protein synthesis is elevated by about 3-fold to 10-fold immediately before and after molting (Skinner, 1965; El Haj *et al.,* 1996; El Haj, 1999). Intracellular proteinases, including calcium-activated proteinases and the ubiquitin-proteasome system, are also active at the time of molting and appear to be involved in controlling the molt-induced muscle atrophy (Mykles and Skinner, 1990; Mykles 1997, 1998). Levels of polyubiquitin protein and mRNA increase in crustacean claw muscles during premolt, when ecdysteroids are elevated (Shean and Mykles, 1995; Koenders *et al.,* 2002; Spees *et al.,* 2003). Although molting is a complex process influenced by multiple factors, one of the major factors known to regulate the

process is the synthesis and secretion of these ecdysteroids (Chang and Bruce, 1980; Landau *et al.,* 1997).

In crustaceans, ecdysteroids are synthesized in the Y-organ, which secretes the hormones as they are produced (Skinner, 1985). Ecdysteroid levels are controlled by molt-inhibiting hormone (MIH), a peptide produced by the X-organ/sinus gland complexes, one of which is located in the optic ganglion of each eyestalk (Van Herp and Kallen, 1991; Landau *et al.,* 1997). Circulating concentrations of the molting hormone, predominantly 20-hydroxyecdysone (20E), generally remain below 50 pg/μl until the animal enters the premolt period (Chang and Bruce, 1980; Snyder and Chang, 1991). At this time, hemolymph concentrations of 20E begin to rise, and remain elevated until after molting, when they return to basal levels (Chang and Bruce, 1980; Snyder and Chang, 1991). Ablation of the eyestalks removes the principal source of MIH and effectively disinhibits the production and secretion of ecdysteroids from the Y-organ (Chang and Bruce, 1980; Wheatly and Hart, 1995).

Among other operations constituting the complex molting process, ecdysteroids are thought to control muscle growth and differentiation, although the specific effects of 20E concentration on the skeletal muscles of crustaceans are ambiguous. Premolt conditions or direct treatment with 20E increase rates of muscle protein synthesis (Skinner, 1965, 1966; El Haj *et al.,* 1996; El Haj, 1999), and some studies have suggested that actin expression increases in response to 20E treatment (Whiteley *et al.,* 1992; Whiteley and El Haj, 1997). In fiddler crabs, the ecdysteroid receptor has been cloned, and receptor expression is up-regulated in the large claw muscle during premolt, a stage when muscle in the major claw atrophies dramatically (Ismail and Mykles, 1992; Chung *et al.,* 1998; Durica *et al.,* 2002). However, El Haj *et al.* (1996) found that ecdysteroid treatment did not affect total RNA synthesis and suggested that changes in muscle protein synthesis might largely be due to control of translation.

Crustaceans possess several different muscle types that have varying mechanical properties and levels of fatigue resistance. In lobsters, at least three identifiable muscle types are present: fast, slow twitch (S_1), and slow tonic (S_2) (Silverman *et al.,* 1987; Neil *et al.,* 1993; Mykles, 1997; West, 1997). Fast fibers have sarcomere lengths of about 4 μm; they also have high rates of ATP hydrolysis and are more susceptible to fatigue than slow fibers. Slow fibers can be further divided into at least two groups: S_1 fibers are characterized by intermediate sarcomere lengths (6–10 μm), intermediate ATPase activities and shortening velocities, and intermediate fatigue resistance; whereas S_2 fibers possess long sarcomeres (up to 12 μm), low ATPase activity and fiber shortening velocities, and the highest levels of fatigue resistance. S_2 fibers are generally thought to play a role in providing long-term postural support.

Lobster claw closer muscles are dimorphic: the large crusher claw contains slow fibers and the slim cutter claw contains, predominantly, fast fibers (Govind, 1992). In a previous study (Medler and Mykles, 2003), we used sequence-specific primers for fast and S_1 myosin heavy chain (MHC) with real-time PCR to measure expression patterns in different muscle types. We demonstrated that different muscles display unique patterns of myofibrillar gene expression, although many fibers co-express multiple MHC genes.

In the current study, we have employed these methods to measure the effects of elevated 20E on myofibrillar gene expression. The eyestalks of adult lobsters were amputated, causing the levels of ecdysteroids in the hemolymph to rise. At varying times after eyestalk ablation, muscle tissues from the claws and abdomen were harvested, and mRNA levels for actin, fast MHC, and S_1 MHC were measured. We also compared the expression of polyubiquitin in intact lobsters with that of the eyestalk-ablated animals with elevated ecdysteroids.

Materials and Methods

Animals and tissue preparation

Adult lobsters, *Homarus americanus,* were raised in the culture facility at the Bodega Marine Laboratory from larvae (Chang and Conklin, 1993). Both eyestalks were ablated at their bases with scissors, and the animals were maintained for 1 to 29 days before they were sacrificed and their muscle tissues were collected for analysis. Animals from the same group, but with the eyestalks left intact, served as controls. Hemolymph samples, taken at the time of sacrifice, were drawn from the base of the last pair of walking legs with a 1-ml syringe fitted with a 26-gauge needle. Muscles from the dimorphic cutter and crusher claws, and from the deep-abdominal flexor muscles, were quickly frozen in liquid N_2 and then stored at -80 °C until analysis (see below).

In independent analyses, muscles from lobsters at different phases of the natural molt cycle (intermolt, premolt, or postmolt) were collected for study. Premolt animals were identified by the synthesis of new exoskeleton and by pleopod setal development (Aiken, 1973), and postmolt animals (2–10 days postmolt) were identified on the basis of records of the last molt. Muscles from these animals were collected and quickly frozen as described above.

Measurement of hemolymph ecdysteroids

Hemolymph samples (100 μl) were mixed with 300 μl of methanol and centrifuged. Supernatants were then dried under vacuum and assayed for ecdysteroids by radioimmunoassay (Chang and O'Connor, 1979; Yu *et al.,* 2002). Values are reported as picograms of ecdysteroid per microliter of hemolymph.

Real-time PCR quantification of myofibrillar protein mRNAs

Total RNA from lobster muscles was isolated using TRIzol Reagent (Invitrogen). Tissues (50–200 mg) were placed in 1 ml of TRIzol Reagent for each 50–100 mg of tissue in a handheld glass homogenizer and were ground until completely homogenized. Insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min at 4 °C. After a 5-min incubation at room temperature, chloroform (0.2 ml per 1 ml TRIzol Reagent) was added to each supernatant. Samples were shaken by hand for 30 s, allowed to stand at room temperature for 5 min, and then centrifuged at 12,000 × *g* for 10 min at 4 °C. RNA in the aqueous phase was precipitated by the addition of isopropanol (0.5 ml per 1 ml TRIzol Reagent) and was then allowed to stand at room temperature for 10 min. The precipitated RNA was collected by centrifugation at 12,000 × *g* for 10 min at 4 °C and then washed with 75% ethanol. After air-drying, the RNA samples were dissolved in water and stored at −80 °C.

The RNA samples were treated with DNase (Invitrogen) for 15 min at room temperature to remove any genomic DNA contamination. First-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Invitrogen) and oligo dT primers. The reaction contained 2.5 μg of oligo(dT) 12–18, 2.5 m*M* dNTP, 1× first-strand buffer, 5 m*M* DTT, 2.5 units of RNase inhibitor, 1–2 μg of RNA, and 200 units of M-MLV reverse transcriptase.

cDNA synthesized from different tissues was used as a template for subsequent real-time PCR. Single cDNA samples were divided for use in separate reactions to measure the copy numbers of fast MHC, slow (S_1) MHC, and α-actin. Thus, three distinct transcripts were monitored for each sample and served as internal controls. The Light Cycler DNA Master SYBR Green I reaction mix for PCR (Roche Molecular Biochemicals) was used to amplify target cDNA with a Cepheid Smart Cycler instrument. PCR master mix was added to 25-μl sample tubes containing 5 μl of the first-strand cDNA reaction. The master mix consisted of 1× LightCycler-DNA Master Green I (contains dNTP mix, FastStart Taq DNA polymerase, SYBR Green I dye), 2.5 m*M* $MgCl_2$, and 0.5 μ*M* of each primer. PCR amplification consisted of denaturation of template and activation of the HS Taq (95 °C for 5 min), followed by amplification of the cDNA target (30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 6 s, and extension at 72 °C for 20 s). Primers for fast and slow (S_1) MHCs, as well as those for α-actin, were the same as those reported previously (Koenders *et al.,* 2002; Medler and Mykles, 2003). Plasmid DNA containing the specific cDNA sequences for each of the target sequences was used as the template for PCR to optimize reaction conditions. The fast MHC primers amplified a 315-bp product, while the S_1 MHC primers amplified a 453-bp product (Medler and Mykles, 2003). The actin primers were designed to amplify α-actin from lobster skeletal muscles as previously described, yielding a 401-bp product (Koenders *et al.,* 2002). Standard curves were constructed by using serially diluted purified plasmid DNA as the template and plotting the number of template copies as a function of the threshold cycle during which product began to accumulate exponentially. The melting temperature, which is a measure of the GC content and the length of the product, was used to identify the specificity of the PCR product. In addition, reaction products were usually separated on 1% agarose gels to verify product size. The cycle thresholds from reactions containing unknown amounts of cDNA were converted to number of copies with the standard curves (Medler and Mykles, 2003). The numbers of mRNA copies of the different sequences were standardized to micrograms of total RNA.

RT PCR of polyubiquitin mRNA

In the current study, a semiquantitative method was used to measure the difference in polyubiquitin mRNA between intact animals and eyestalk-ablated animals with elevated ecdysteroid concentrations (> 200 pg/μl). PCR amplification of the polyubiquitin cDNA is incompatible with real-time PCR, because the head-to-tail orientation of the individual ubiquitin mRNA sequences leads to multiple ubiquitin PCR products of differing sizes (Koenders *et al.,* 2002). Therefore, we used a standard PCR and monitored the amount of product at the end of a different number of cycles. PCR conditions were those used previously by Koenders *et al.* (2002). We determined that the most prominent, 225-base-pair product in samples from intact and eyestalk-ablated animals could best be discriminated following 21 cycles of amplification. Ethidium-bromide-stained PCR products separated on 1% agarose gels were saved as digital images and quantified by densitometry (NIH Image 1.62).

Analysis of actin and MHC proteins

Muscle tissues from eyestalk-ablated and intact controls were collected as described. Samples used for protein analyses were processed according to the methods of Mykles (1985). Briefly, frozen muscles were glycerinated for 2–3 h, with stirring, in ice-cold buffer containing 20 m*M* Tris-HCl (pH 7.5), 50% glycerol, 100 m*M* KCl, 1 m*M* EDTA, and 0.1% Triton X-100. Single fibers or fiber bundles were removed from the muscle and solubilized in 250 μl of SDS sample buffer containing 62.5 m*M* Tris-HCl (pH 6.8), 12.5% glycerol, 1.25% SDS, and 1.25% β-mercaptoethanol. Muscle samples were left in this solution overnight at room temperature with occasional vortexing. To analyze myofibrillar isoform assemblages, SDS-PAGE was performed with a discontinuous gel system, as described in Mykles

(1985). Briefly, 10% separating gels (37.5:1 acrylamide: N,N-methylenebisacrylamide) were used to separate about 4–6 μg myofibrillar proteins, using a Bio-Rad Mini-Protean 3 gel system. The gels were stained with Coomassie blue or silver (Wray *et al.,* 1981), and the relative amounts of actin and MHC from the silver-stained gels were determined by scanning densitometry (NIH Image 1.62).

Statistical analyses

For regression analyses, the numbers of myofibrillar mRNA copies were log-transformed to reduce variance and regressed against ecdysteroid concentrations. In addition, we examined these values as a function of the number of days after eyestalk ablation. The number of myofibrillar mRNA copies and the MHC/actin ratios were compared among fiber types with a one-way ANOVA, followed by Bonferroni post-ANOVA tests (experiment-wise $\alpha = 0.05$).

For analysis of gene expression in muscles from intact animals at different molt stages, the numbers of myofibrillar mRNA copies were log-transformed and analyzed by ANOVA. A factorial ANOVA was used to analyze the number of copies of mRNA, with the factors being molt stage (intermolt, premolt, or postmolt), muscle type (deep-abdominal flexor, crusher, or cutter), and specific myofibrillar gene (actin, S_1 MHC, fast MHC). Between four and seven samples were taken for each unique treatment group (molt stage × muscle type × myofibrillar gene). Statview 5.0.1 (SAS Institute Inc.) was used for all statistical analyses.

Results

Effects of eyestalk ablation on ecdysteroid titers

Ecdysteroid concentrations in the hemolymph increased significantly as a function of the number of days after eyestalk ablation ($P = 0.001$; Fig. 1). Elevated levels of hormones were detected within the first few days after ablation, but these levels were quite variable. Overall, eyestalk-ablated animals, collectively, had significantly higher ecdysteroid concentrations than the intact controls, as determined by an unpaired Student's *t*-test ($P = 0.009$). The mean (± SD) ecdysteroid concentration was 13.4 ± 12.8 pg/μl for the intact control lobsters *versus* 156.4 ± 145 pg/μl for the eyestalk-ablated animals.

Effect of ecdysteroid concentration on myofibrillar mRNAs

Levels of mRNAs for actin, fast MHC, and S_1 MHC were quantified by real-time PCR for muscles from the crusher and cutter claws and for the deep abdominal flexors. When these levels were examined as a function of the time since eyestalk ablation, only actin message expressed in the deep-abdominal flexor muscles changed, decreasing significantly as a function of the number of days since ablation ($P = 0.002$; Fig. 2). About 30 days after eyestalk ablation, the amount of actin message was more than 10-fold lower than in intact animals. However, these genes encoding actin, fast MHC, and S_1 MHC showed no significant changes in expression as a function of ecdysteroid concentration in any of the muscles sampled (Fig. 3A–C, E–G, I–K).

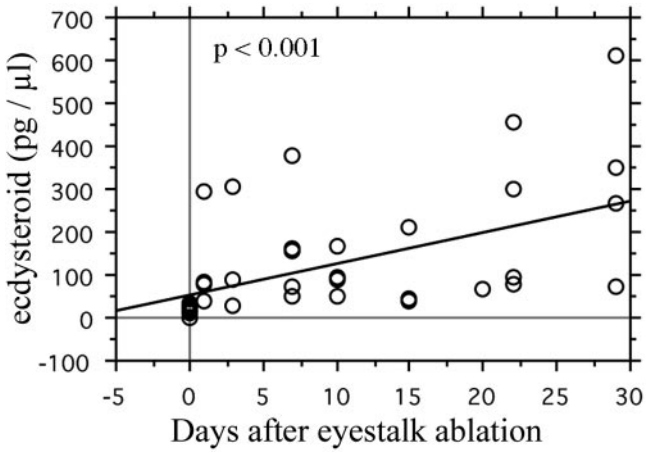

Figure 1. Effect of eyestalk ablation on hemolymph ecdysteroid concentration. Ecdysteroids significantly increased following eyestalk ablation ($P = 0.001$). Animals were eyestalk-ablated on day 0, and concentrations of ecdysteroids in the hemolymph were measured at the time of tissue collection at various times over the following weeks. Control animals with intact eyestalks are shown at day 0. Although the linear increase in ecdysteroid titer is significant, the values from different animals are highly variable.

Effect of muscle type on myofibrillar mRNAs

When intact and eyestalk-ablated animals were combined into a single group, significant differences in mRNA levels were detected among muscle types for all three myofibrillar genes (Fig. 3D, H, L). Actin expression was highest in slow muscles of the crusher claw, being almost 7 times higher than in the fast cutter claw, and more than 1000 times higher than in the fast deep flexors (Fig. 3D). Fast MHC expression was highest in the deep flexors, being almost 4 times higher than in the cutter claw muscle, and more than 3000 times higher than in the crusher claw muscle (Fig. 3H). Slow (S_1) MHC expression was highest in the crusher claw muscle, being almost 15 times higher than in the cutter claw muscle, and almost 3000 times higher than in the deep flexors (Fig. 3L).

When these expression levels were grouped by muscle type, characteristic patterns were observed for each muscle (Fig. 4). In the crusher muscles, actin expression was almost 1000 times higher than S_1 MHC expression and more than 1500 times the level of fast MHC expression (Fig. 4A). Fast cutter muscles expressed more copies of fast MHC than the other genes, being more than 3 times higher than actin

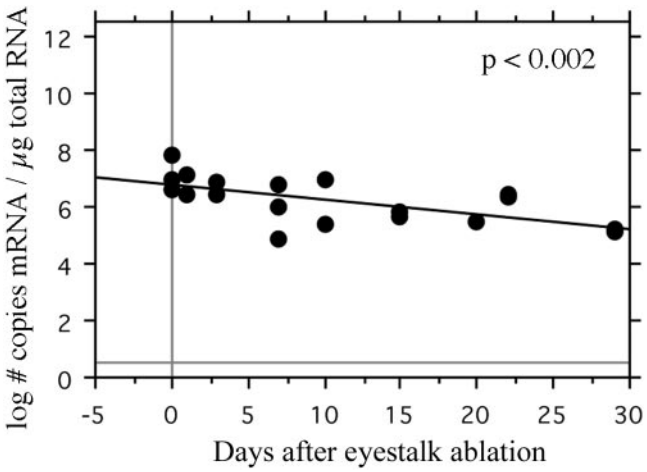

Figure 2. Level of actin mRNA in deep-abdominal flexor muscles as a function of the time following eyestalk ablation. After nearly 30 days without eyestalks, actin expression fell by about one order of magnitude ($P = 0.002$). Actin mRNA data are the same as those in Figure 3C.

expression and more than 7000 times higher than S_1 MHC expression (Fig. 4B). In the fast deep flexors, fast MHC expression was almost 2000 times greater than actin expression and more than 6 orders of magnitude higher than S_1 MHC expression (Fig. 4C). Each of the three muscle types expressed both the fast and S1 MHC isoforms. This co-expression was more prominent for the cutter and crusher claw muscles, but less dramatic for the deep-abdominal muscle (Figs. 3E–L, 4, 5D–I).

Effect of molt stage on myofibrillar mRNAs

Premolt and postmolt muscles consistently exhibited slightly higher levels of myofibrillar gene expression than intermolt muscles (Fig. 5). Although these differences were not dramatic, they were significant when molt-stage muscles (premolt and postmolt combined) were compared with intermolt muscles ($P = 0.0422$; Table 1). The effect of molt stage was not significant when pre- and postmolt muscles were treated as separate groups ($P = 0.13$; data not shown). The average myofibrillar gene expression in molt-stage animals (premolt and postmolt) was about 4 times greater than that observed in intermolt animals; but given the level of variability, this is not a precise value. As expected, significant differences were also detected with respect to the muscle type and the specific gene of interest ($P < 0.0001$; Table 1, "Muscle" and "Gene," respectively). In addition, the interaction between muscle type and gene expressed was significant ($P < 0.0001$; Table 1, "Muscle * Gene"). Each of these effects reflects the muscle-specific expression patterns described above—namely that each muscle type expresses different myofibrillar genes in unique proportions.

Effects of eyestalk ablation on myofibrillar protein composition

Relative concentrations of MHC and actin proteins were assessed by SDS-PAGE and densitometry. Significant differences in the myosin/actin ratio were observed among all three muscles, with the fast deep-abdominal muscle having the highest MHC/actin ratio, followed by the cutter muscles and finally the crusher muscles (Bonferroni post-ANOVA test; experiment-wise $\alpha = 0.05$) (Fig. 6). However, the myosin/actin ratio in none of the three muscle fiber types changed as a function of time after eyestalk ablation ($P = 0.30$; data not shown), indicating that a selective loss of thin filament proteins did not occur during the experiment.

Effects of elevated ecdysteroids on polyubiquitin expression

Previous studies have shown that polyubiquitin protein and mRNA increase in crustacean muscles during premolt, when ecdysteroids are elevated (Shean and Mykles, 1995; Koenders *et al.,* 2002; Spees *et al.,* 2003). As a positive control for the systemic effects of elevated ecdysteroids, we used a semiquantitative PCR method to compare polyubiquitin expression in claw muscles from intact control animals and eyestalk-ablated animals with elevated ecdysteroid titers (> 200 pg/μl). We detected a significant elevation (about 2-fold) in polyubiquitin expression in those animals with elevated ecdysteroids, when they were compared with intact control animals ($P < 0.0001$; Fig. 7).

Discussion

We have shown that experimentally elevated ecdysteroid concentrations triggered in lobsters by eyestalk ablation have little or no effect on myofibrillar gene expression. In contrast, animals undergoing natural molting exhibit slightly elevated myofibrillar gene expression. Overall, the data reported here are consistent with previous studies indicating that myofibrillar gene expression is elevated during molting, but these changes are not dramatic and are sometimes difficult to detect. These same data also confirm that patterns of gene expression among muscle fiber types differ, and that many fibers are polymorphic, expressing multiple myofibrillar isoforms.

Over time, eyestalk ablation significantly elevated ecdysteroids to levels comparable to those observed in premolt lobsters experiencing a natural molt cycle (Fig. 1) (Snyder and Chang, 1991; Chang *et al.,* 2001). Although ecdysteroid concentrations were quite variable in eyestalk-ablated animals, this pattern is not surprising: ecdysteroid levels fluctuate significantly over time even in intact animals (Snyder and Chang, 1991). Ecdysteroids coordinate a number of physiological processes involved with molting in crustaceans, and several studies have suggested that these

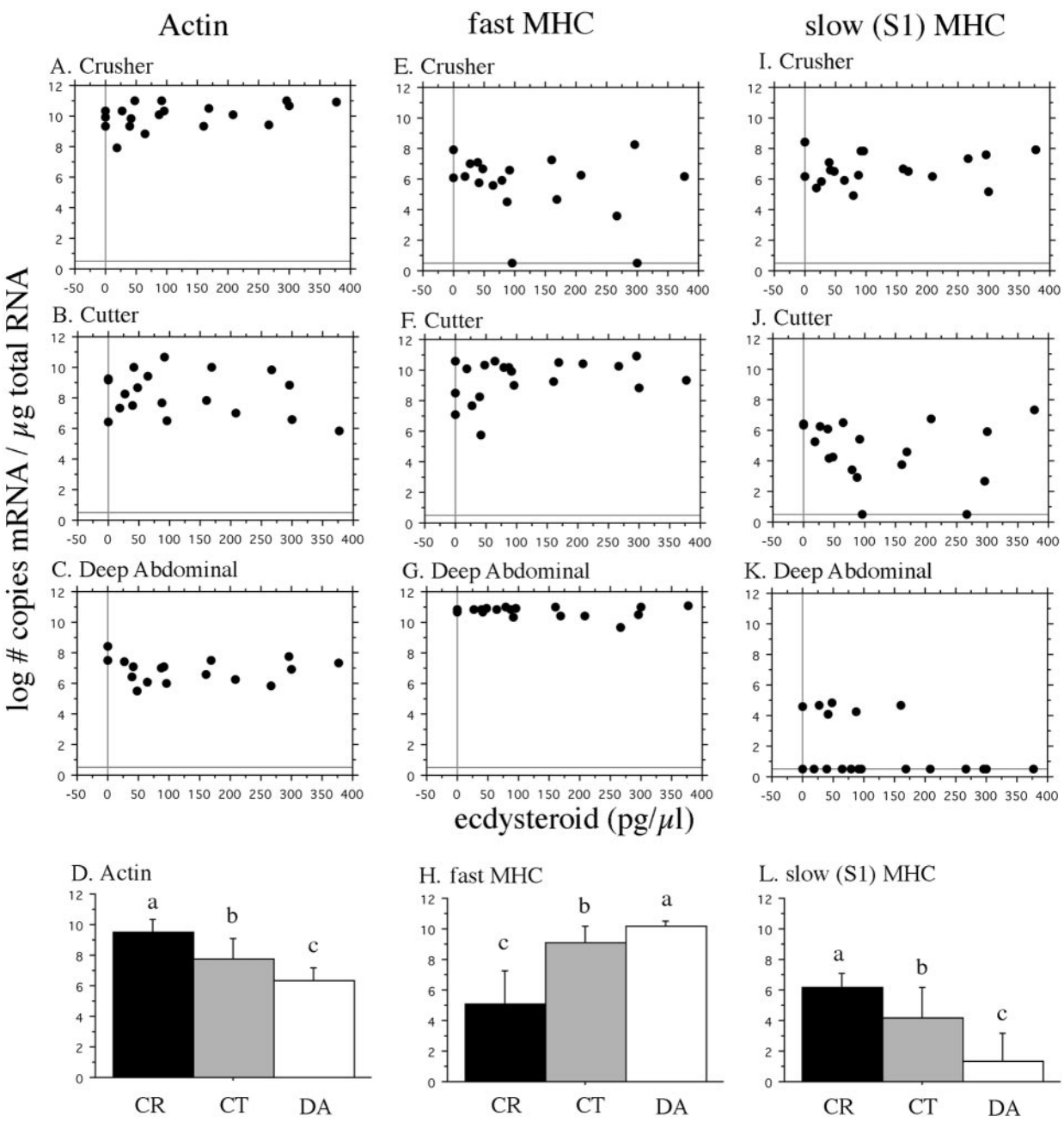

Figure 3. Myofibrillar gene expression as a function of ecdysteroid concentration. (A–C) Actin expression did not significantly change with increasing levels of ecdysteroids in any of the muscles examined. (D) But when the same data were grouped by muscle type, significant differences were found among the three muscle types: expression was highest in the slow crusher (CR) muscles, followed by the cutter (CT) and the deep-abdominal muscles (DA). (E–G) Fast MHC expression was not affected by ecdysteroid concentration in any of the three muscles. (H) But when grouped by muscle type, fast MHC expression was significantly higher in the deep-abdominal muscles than in the cutter or crusher claw muscles, and the fast cutter muscles expressed higher levels of fast MHC than the slow crusher claw muscles. (I–K) Slow (S_1) MHC expression was unaffected by ecdysteroids in the muscles examined. (L) When the data were grouped by muscle type, S_1 MHC expression was significantly highest in the slow crusher muscles, followed by the cutter muscles and the deep-abdominal muscles. Means with different letters in (D), (H), and (L) are significantly different from one another, as determined by a Bonferroni post-ANOVA test (experiment-wise $\alpha = 0.05$; $n = 16$–20; values are means $\pm$ SD).

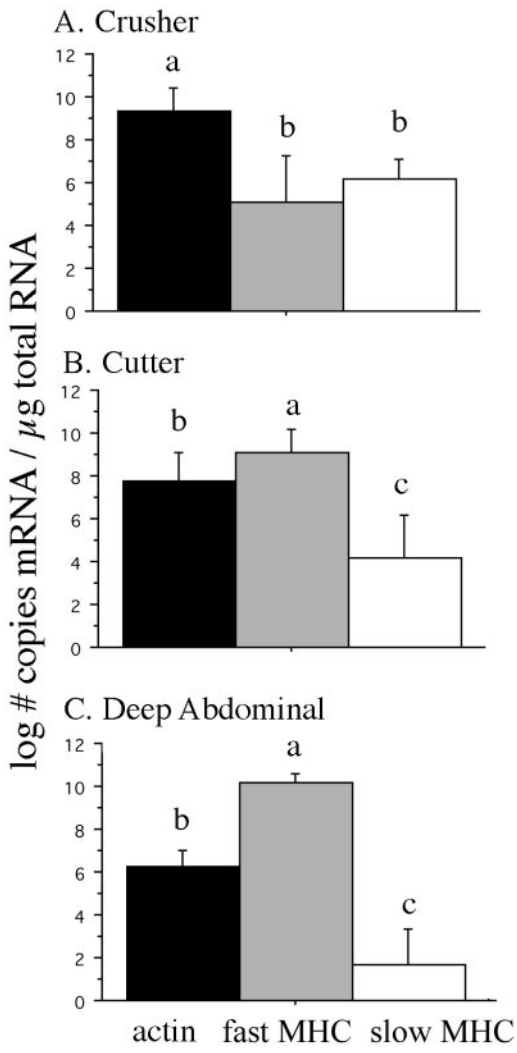

Figure 4. Different muscle types exhibit distinct myofibrillar protein expression patterns. (A) Slow muscles in the crusher claw expressed significantly higher levels of mRNA encoding actin than the mRNAs encoding either fast or slow MHC. Although slow (S_1) MHC expression was higher than fast MHC expression, the difference was not significant. In general, the expression of the fast MHC was much more variable than S_1 MHC in the slow muscles of the crusher claw. (B, C) Both fast muscle phenotypes exhibited significantly higher expression of fast MHC than of either actin or slow MHC, and actin expression fell between those of fast and slow MHC. In both muscle types, slow MHC expression was quite variable. Means with different letters are significantly different from one another as determined by a Bonferroni post-ANOVA test (experiment-wise $\alpha = 0.05$; $n = 16–20$; values are means ± SD). (Data are from the eyestalk-ablation experiment, which included both intact and ablated animals.)

hormones are responsible for such molt-related changes in skeletal muscles as the atrophy of the large cheliped muscles in some decapod crustaceans (El Haj and Houlihan, 1987; Ismail and Mykles, 1992; El Haj *et al.,* 1996; El Haj and Whiteley, 1997; Whiteley and El Haj, 1997). Moreover, an ecdysteroid receptor has been detected in lobster muscle (El Haj *et al.,* 1994). Other studies have reported increases in protein synthesis in crustacean muscles during premolt and postmolt periods (Skinner 1965, 1966; El Haj *et al.,* 1996), but whether these changes result from changes in myofibrillar gene expression is equivocal (Whiteley *et al.,* 1992; El Haj *et al.,* 1996; Mykles, 1997; Whiteley and El Haj, 1997). We detected no significant changes in myofibrillar gene expression in response to elevated ecdysteroid concentrations (Fig. 3). In contrast, muscles taken from intact lobsters demonstrated modest, but significant, increases in myofibrillar gene expression in premolt and postmolt animals when compared to intermolt animals (Fig. 5; Table 1). These results indicate that myofibrillar gene expression can increase before and after molting.

In this study, the expression patterns detected in intact lobsters experiencing a natural molt cycle provide a useful comparison for patterns observed in eyestalk-ablated animals. In both cases, myofibrillar gene expression is relatively constant, but the intact premolt and postmolt lobsters exhibited significantly higher expression when compared with the intermolt animals (Fig. 5; Table 1). This difference indicates that the physiological events following eyestalk ablation are not precisely equivalent to those occurring during the natural molt cycle. This conclusion is supported by diverse earlier findings. The X-organ/sinus gland complex located in the eyestalks is an important neuroendocrine center involved in several physiological processes, including molting, reproduction, osmoregulation, and energy metabolism (Van Herp and Kallen, 1991; Landau *et al.,* 1997). Indeed, eyestalk ablation is known to have significant effects on osmoregulatory processes (Jackson *et al.,* 1987; Charmantier-Daures *et al.,* 1994; Wheatly and Hart, 1995), energy metabolism (Rosas *et al.,* 1993; Chang, 2001), and reproductive processes (Khalaila *et al.,* 2002) in crustaceans. The peptide hormones released from the sinus gland probably have complex, broad physiological effects. We conclude, therefore, that eyestalk ablation is an experimental procedure with the potential to affect most important physiological systems in decapod crustaceans. As such, it is certainly not simply equivalent to increases in ecdysteroids during molting (Wheatly and Hart, 1995), and as an experimental tool, it should be used judiciously.

Our analysis of the relative amounts of MHC and actin revealed no changes at the protein level as a result of eyestalk ablation. In contrast, studies of molt-associated atrophy in crabs demonstrated that thin filament proteins were selectively lost during atrophy (Ismail and Mykles, 1992; Mykles, 1997, 1998; West, 1997). Consistent with established patterns in crustacean muscles (Jahromi and Atwood, 1969; Ismail and Mykles, 1992; West, 1997), we detected significant differences in the myosin/actin ratio among the different fiber types, with the fast muscles having proportionately more myosin (Fig. 6). But these ratios were

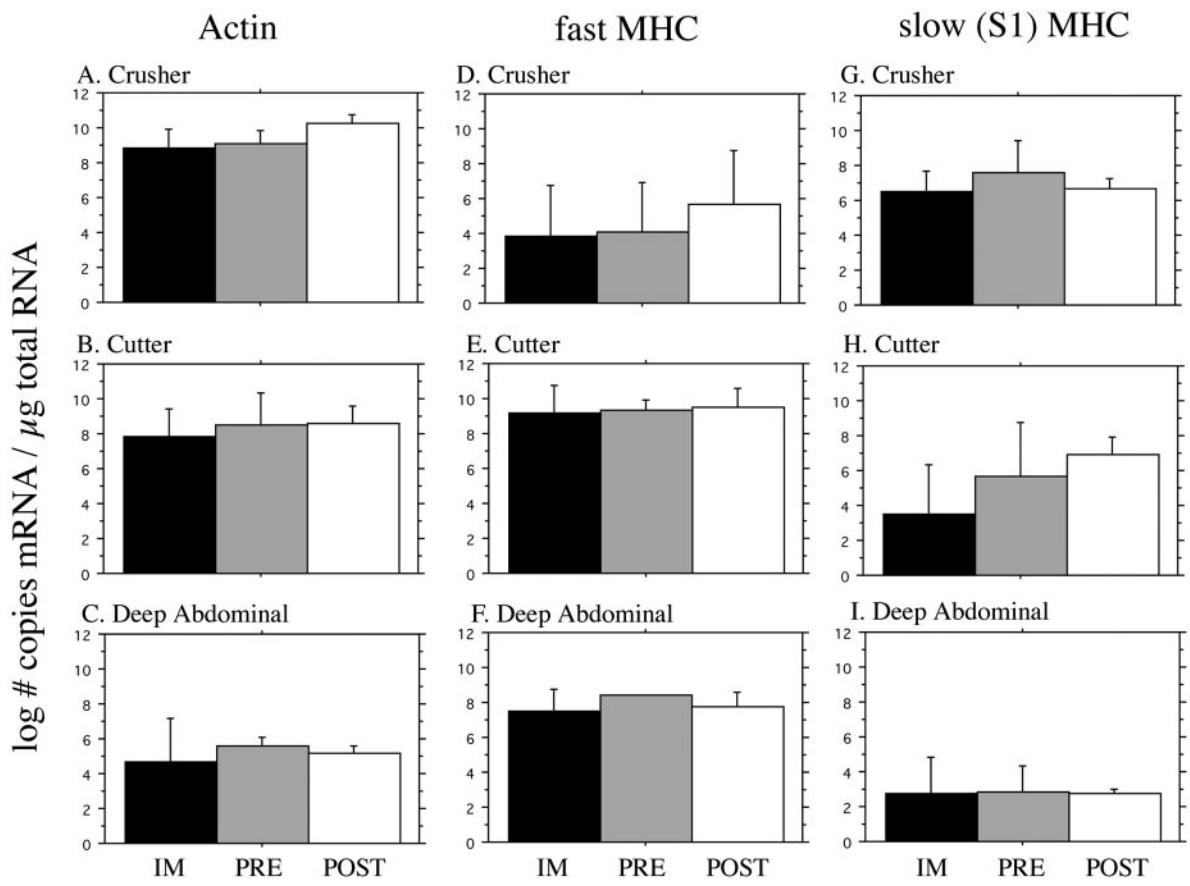

Figure 5. Myofibrillar gene expression in claw and deep-abdominal muscles as a function of molt stage in intact animals (intermolt: IM; premolt: PRE; postmolt: POST). (A–C) Actin expression, (D–F) fast MHC expression, and (G–I) slow (S_1) MHC expression. Expression of each gene was slightly, but not significantly, higher in the premolt and postmolt muscles. When the data were pooled and analyzed by factorial ANOVA, the premolt and postmolt muscles had significantly higher expression than muscles from intermolt animals (Table 1).

Table 1

ANOVA of intact lobsters from intermolt, premolt, and postmolt stages (premolt and postmolt combined as one group)

Source of Variation	DF	Sum of Squares	Mean Square	*F*-value	*P*-value
Stage	1	10.50	10.50	4.23	**0.0422**
Muscle	2	115.33	57.66	23.25	**<0.0001**
Gene	2	114.61	57.03	23.10	**<0.0001**
Stage*Muscle	2	3.09	1.55	0.62	0.5381
Stage*Gene	2	0.57	0.29	0.12	0.8916
Muscle*Gene	4	290.69	72.67	29.30	**<0.0001**
Stage*Muscle*Gene	4	3.45	0.86	0.35	0.8449
Residual	103	255.49	2.48		

Dependent variable: log # RNA copies/μg total RNA. Factors: Stage (intermolt and pre/postmolt); Muscle (slow (S_1) crusher claw, fast cutter claw, fast deep abdominal); Gene (actin, slow (S_1), fast MHC). **Bold** values indicate significant effects ($P < 0.05$).

affected neither by ecdysteroid concentration nor by the duration of eyestalk ablation (data not shown).

The failure of elevated ecdysteroids to affect myofibrillar protein levels was also evident when gene expression was examined. Lobsters with elevated ecdysteroids following eyestalk ablation did not show the increased gene expression that might have been expected from the slight elevation of myofibrillar gene expression in premolt and postmolt lobsters. In 1966, Skinner had already proposed that molt-induced atrophy is not under the direct control of ecdysteroids, suggesting instead that the complex process probably involves changes in motor neuron activity. Similarly, others have suggested that the control of skeletal muscle growth and remodeling is complex, and not under the direct control of ecdysteroids alone (El Haj and Houlihan, 1987; Whiteley *et al.,* 1992; El Haj and Whiteley, 1997; Whiteley and El Haj, 1997). Our analysis of muscle proteins, together with the mRNA data, indicates that lobster skeletal muscles are largely unaffected by the ecdysteroid elevation alone.

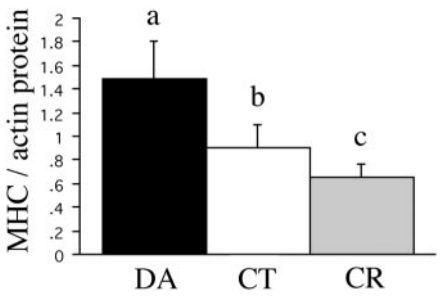

Figure 6. The ratio of MHC to actin proteins in fast and slow fibers from deep abdominal (DA), cutter (CT), and crusher (CR) claw muscles in eyestalk-ablated and intact control animals. Means with different letters are significantly different from one another as determined by a Bonferroni post-ANOVA test (experiment-wise $\alpha = 0.05$; $n = 16$; values are means $\pm$ SD). No changes in these ratios were detected for any of the three muscle types following eyestalk ablation (data not shown).

The natural molting process likely involves not only increased ecdysteroid titers, but also permissive events that prepare the tissues for molting. One such possibility is that ecdysteroid receptors in the muscles are not available under the conditions of experimental ecdysteroid elevation (El Haj *et al.,* 1994).

The different muscles were clearly distinguished by the patterns of MHC and actin expression, consistent with previously observed expression levels in adult lobsters (Medler and Mykles, 2003). Slow (S_1) MHC expression is highest in the slow crusher claw muscles, intermediate in the fast cutter claw muscles, and low to absent in the fast muscles of the deep abdominal flexors (Fig. 3L). A similar correlation was observed for actin expression, while fast MHC expression followed a reverse pattern (Fig. 3D, H). As we and others have previously suggested, differences in actin expression probably reflect differences in thin-to-thick filament ratios in the different fiber types, as slow muscles have significantly higher proportions of thin filaments (Jahromi and Atwood, 1969; Medler and Mykles, 2003). This pattern was confirmed in the present study: the significant differences detected in the relative amounts of MHC and actin in the different fiber types (Fig. 6) mirrored the differences in mRNA levels for the different fiber types (Figs. 3D, H, L; 4). Significant differences were found between the fast cutter muscles and fast abdominal muscles, both in terms of mRNA levels and the ratio of MHC and actin proteins (Figs. 3D, H, L; 6). A recent study of freshwater crayfish muscles demonstrated subtle differences in these fast muscles in terms of sarcomere length, troponin proteins, and Ca^{2+} activation kinetics (Koenders *et al.,* 2004). These findings suggest that, notwithstanding the nomenclature, the fast muscles of the claw and the abdomen are physiologically distinct.

Another interesting pattern is the co-expression of multiple MHC isoforms in the same muscles. Although this may be due in part to the presence of multiple fiber types within the intact muscle, we have observed co-expression in single fibers (Medler and Mykles, 2003; Medler *et al.,* 2004). This type of co-expression has also been noted in a number of other animals and seems to represent the rule rather than the exception with respect to profiles of isoform expression in muscle (Peuker and Pette, 1997; Lutz *et al.,* 1998, 2001; Stevens *et al.,* 1999; Stephenson, 2001; Caiozzo *et al.,* 2003). A newly emerging challenge for skeletal muscle biologists is to define the extent and significance of single-fiber polymorphism (Stephenson, 2001; Caiozzo *et al.,* 2003). The role of motor nerves on factors that affect the expression of specific myofibrillar isoforms is suggested by the developmental changes in lobster claw muscles (Govind, 1992).

In contrast to the lack of effect on myofibrillar gene expression, elevated ecdysteroid levels were associated with higher levels of polyubiquitin mRNA. Polyubiquitin expression increases in premolt claw muscles when these muscles experience atrophy (Shean and Mykles, 1995; Koenders *et*

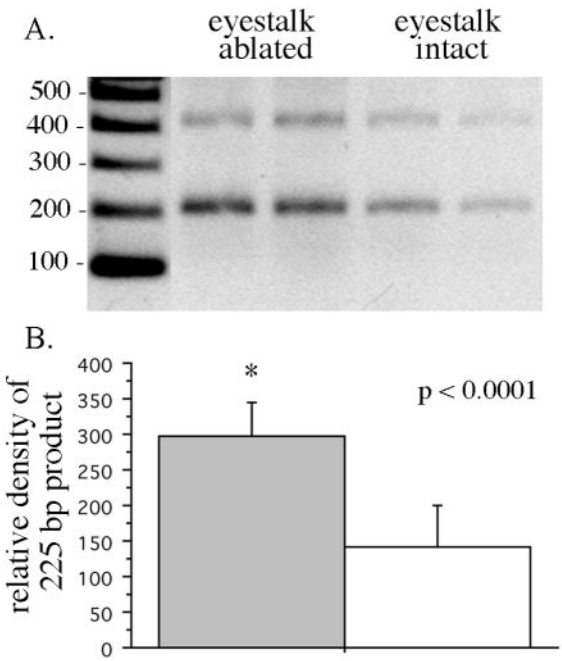

Figure 7. Polyubiquitin expression in animals with elevated ecdysteroid concentrations after eyestalk ablation. (A) PCR products following 21 cycles of eyestalk-ablated (left two lanes) compared with intact control (right two lanes) muscles (inverse image of ethidium-bromide-stained gel). The most prominent band was the smallest, a 225-bp product, which was subsequently quantified by densitometry (lane on far left consists of standards). (B) Quantification of the relative density of the most prominent (225-bp) PCR product from the muscles from eyestalk-ablated (left) and intact control (right) animals ($n = 8$; values are means $\pm$ SD). Cutter and crusher muscles from animals with elevated ecdysteroids (above 200 pg/μl) had significantly (*) ($P < 0.0001$) higher expression of polyubiquitin as compared with intact control animals.

al., 2002; Spees *et al.,* 2003), and this expression was therefore used in the current study as an alternative measure of the effects of elevated ecdysteroids. Ubiquitin is a highly conserved protein that becomes covalently bound to other proteins by an ATP-dependent conjugating system. Further addition of ubiquitin monomers (polyubiquitin) targets the bound protein for degradation by the proteasome (Mykles, 1998). The ubiquitin/proteasome system is clearly involved in atrophic processes in mammalian skeletal muscle (Mitch and Goldberg, 1996) and appears to be involved in crustacean muscle atrophy as well (Shean and Mykles, 1995; Mykles, 1998; Koenders *et al.,* 2002). In the present study, comparison of eyestalk-intact animals and eyestalk-ablated animals with high ecdysteroid levels confirms significantly higher polyubiquitin expression in the eyestalk-ablated group. These results indicate that the ablation—and possibly the ensuing ecdysteroid exposure—was affecting select cellular processes, although myofibrillar gene expression was not one of these.

Another exception to the general lack of effect of the elevated ecdysteroids was the reduced actin expression in the deep flexors as a function of the duration of eyestalk ablation. In these muscles, actin expression fell by about an order of magnitude 30 days post-ablation ($P = 0.002$; Fig. 2). We might dismiss this finding as a statistical artifact but for the level of statistical significance and because it is consistent in certain respects with previously reported data. Molting crabs and crayfish claw muscles selectively lose actin filaments as they atrophy, but it is not known whether this also occurs in the abdominal muscles (Mykles and Skinner, 1990; Ismail and Mykles, 1992; West, 1997). Previous studies have shown that synaptic efficacy in these muscles is significantly reduced by acute 20E application, and that escape responses in postmolt lobsters are less forceful than in non-molting animals (Cooper and Ruffner, 1998; Cromarty and Kass-Simon, 1998). These results were muscle-specific, as the effect of 20E in the claw opener muscles was opposite to that in the abdominal muscle, namely to enhance synaptic efficacy (Cromarty *et al.,* 1991). Further work is needed to establish whether the decline in actin expression in the phasic abdominal muscles is a general response to eyestalk ablation in lobsters.

Overall, the data reported here are consistent with previous studies indicating that myofibrillar gene expression is elevated during molting, but these changes are not dramatic and sometimes difficult to detect (Whiteley *et al.,* 1992; El Haj *et al.,* 1996; Whiteley and El Haj, 1997; El Haj, 1999). Molting is a complex process that involves not only increases in ecdysteroid concentrations, but other processes as well. Further study is needed to understand the complex factors involved in molt-associated changes in skeletal muscle growth and differentiation.

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