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Programmed Cell Death in Flight Muscle Histolysis of the House Cricket

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Abstract

We have characterized the process of flight muscle histolysis in the female house cricket, *Acheta domesticus*, through analysis of alterations of tissue wet weight, total protein content, and percent shortening of the dorsal longitudinal flight muscles (DLMs). Our objectives were to (1) define the normal course of histolysis in the cricket, (2) analyze the effects of juvenile hormone (JH) removal and replacement, (3) determine the effects of cycloheximide treatment, and (4) examine patterns of protein expression during histolysis.

Our results suggest that flight muscle histolysis in the house cricket is an example of an active, developmentally regulated cell death program induced by an endocrine signal. Initial declines of total protein in DLMs indicated the JH signal that induced histolysis occurred by Day 2 and that histolysis was essentially complete by Day 3. Significant reductions in tissue weight and percent muscle shortening were observed in DLMs from Day 3 crickets. Cervical ligation of Day 1 crickets prevented histolysis but this inhibition could be reversed by continual topical treatments with methoprene (an active JH analog) although ligation of Day 2 crickets did not prevent histolysis. A requirement for active protein expression was demonstrated by analysis of synthesis block by cycloheximide and short-term incorporation of ³⁵S-methionine. Treatment with cycloheximide prevented histolysis. Autofluorographic imaging of DLM proteins separated by electrophoresis revealed apparent coordinated regulation of protein expression.

Keywords

Flight Muscle Histolysis; Programmed Cell Death; Juvenile Hormone; Dorsal Longitudinal Muscle; Cricket

1. Introduction

Flight muscle histolysis is a developmentally regulated event, which occurs within the first few days of adult emergence in the house cricket, *Acheta domesticus*. Histolysis results in degeneration of the flight musculature and accounts for loss of flight in winged adult crickets.

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Flight muscle histolysis is a crucial developmental event that affects reproductive capacity, dispersal potential, and individual survival characteristics of insects (Finlayson, 1975; Wyatt and Davey, 1996; Marden, 2000). Either direct or facultative relationships between histolysis and reproductive maturity are seen in many insects (Pener, 1985).

Initial demonstrations suggesting juvenile hormone (JH) acts as the endocrine signal for flight muscle histolysis in the house cricket were based on measurements of muscle working area (Bocharova-Messner et al., 1970). Allatectomy of last instar crickets was used to produce adults with no circulating JH. Allatectomy prevented histolysis and maintained working area of the muscle. Flight muscles of allatectomized controls implanted with active corpora allata (CA) underwent histolysis. A change from reddish-brown color to pale white was observed in dorsal longitudinal flight muscles (DLMs) during histolysis, parallel to loss of excitability and contractility.

Gutman et al. (1974) investigated neural control of flight muscle histolysis in the house cricket using electron microscopy. Some accumulation of synaptic vesicles at nerve endings of neuromuscular junctions in degenerated muscles was noted suggesting a possible involvement of neural signals in histolysis, but the primary control was determined to be endocrine, since nerves to degenerating muscles remained intact. Similar results have been more recently been reported in relation to neural and endocrine control of flight muscle degeneration in the cricket, *Gryllus bimaculatus*, which exhibits a more sustained flight ability, but also undergoes histolysis (Shiga et al., 2002). Using light microscopy and cytohistochemistry, Srihari et al. (1975) noted formation of vacuoles, presence of pycnotic nuclei, disorganization and loss of muscle fibers in DLMs on the fourth day following adult ecdysis in the house cricket. A JH analog, methyl, 3,7,11,11-tetramethyl-2-dodecanoate enhanced histolysis, whereas ecdysone administered to one-day-old adult crickets caused a temporary inhibition of histolysis in the DLMs. Two day and older crickets were unaffected by ecdysone.

Flight muscle histolysis has been extensively investigated with respect to fitness gain trade-offs in cricket species that exhibit wing polymorphism (Tanaka, 2001; Roff and Gelinas, 2003; Zhao and Zera, 2006). Application of JH analogs was observed to induce histolysis of flight muscles based on color changes of muscles in the wing dimorphic cricket, *Modicogryllus confirmatus* (Tanaka, 1995). Genetic population analysis suggests that traits for control of expression of JHE and juvenile hormone binding proteins (JHBPs) are inter-related in the wing dimorphic cricket, *G. assimilis* (Gu and Zera, 1996). JHE activity is elevated in geographically isolated *G. firmus* populations that exhibit higher proportions of macropterous (flight capable) morphs (Roff and Fairbairn, 1999). Analyses of interrelationships between endocrine control, muscle histolysis and the evolutionary aspects of flightlessness have been extended with the demonstration that rhythms of JH and levels of ecdysone display a heritable plasticity associated with wing morphs (Zera, 2006).

Programmed cell death (PCD) was originally defined as the physiologic death of cells and tissues associated with developmental remodeling (Lockshin and Zakeri, 2001). Early studies demonstrated that inhibition of *de novo* protein expression was able to block PCD (Lockshin, 1969; Tata, 1966). Although recognized as an example of PCD (Gutman et al., 1974; Lockshin, 1985; Wyatt and Davey, 1996), little attention has been directed toward cell death pathways as mechanisms of flight muscle histolysis. Active requirements for protein synthesis and ubiquitin proteolysis during degeneration of aphid flight muscles were investigated in relationship to PCD (Kobayashi and Ishikawa, 1994). The specific elimination of a nymphal component of a flight steering muscle in *Locusta migratoria* appears also to proceed through PCD (Meuser and Pflüger, 1998).

We have characterized the process of flight muscle histolysis in the female cricket through analysis of alterations of tissue wet weight; total protein content; and shortening of DLMs. Our specific experimental objectives were to (1) define the normal course of histolysis in the cricket, (2) analyze the effects of hormone removal and replacement, (3) determine the effects of cycloheximide treatment, and (4) examine potential alterations of protein expression during histolysis.

2. Materials and methods

2.1. Insects

Virgin female house crickets were obtained as adults of known age or instars from the colony maintained at the Department Biological Sciences, LSU, (Baton Rouge, LA) or provided by commercial cricket breeders (Fluker Farms, Port Allen, LA or Ghann's Cricket Farm, Augusta, GA). Crickets were reared at 30°C with a L:D 14:10 photoperiod. Adult crickets were isolated at emergence and designated age Day 0 (Clifford and Woodring, 1991). White imagoes were isolated prior to cuticular tanning.

2.2 Measurement of DLMs

Crickets were anesthetized by exposure to carbon dioxide gas prior to experimental manipulations. DLMs were excised from crickets by ventral dissection. Individual DLMs were rinsed with cricket ringer, removed intact, blotted to remove adherent fat body and frozen in liquid nitrogen and stored at -80°C. Tissue wet weight, total protein content and percent muscle shortening were measured for individual DLMs. Relative shortening of DLMs was determined as the change in length of the muscles after severance of the posterior connection to the cuticle of specimens pinned to dissecting trays. This variable is reported as $[1 - (\text{cut length}/\text{original length})] \times 100$. Measurements were made using an ocular micrometer mounted on a dissecting microscope. Tissue wet weight was found using an analytical balance (Sartorius). The determination of total protein for individual DLMs was performed by the bicinchoninic acid method (BCA Assay, Pierce, Rockport, IL). Homogenates (10 µl) from individual DLMs were assayed in triplicate.

2.3 Treatments

Cervical ligations were made using unwaxed dental floss. Crickets were ligated at age Day 1 or age Day 2. Day 1 crickets are able to live ≈ 8 days after ligation (Woodring and Sparks, 1987). Crickets in these experiments were sacrificed after no more than 3 days (at Day 4). Ligated crickets lose head functions but are mobile and respond to touch. Continual exposure to methoprene (Sandoz, Dallas, TX) was achieved by allowing crickets to walk on 7.5 mm filter paper disks spotted with 100 µl of 3% methoprene in mineral oil. Controls were mineral oil only. Crickets in one experimental group (CYX+) were given injections of cycloheximide (5 µl of 10 mg/ml in distilled water) beginning at Day 1 and continuing to Day 3 at 12 hr intervals. Controls (Day 3) were injected with distilled water beginning at Day 1. Another group (Recovery) was given the same schedule of cycloheximide but treatments were discontinued at Day 3 and the DLMs were not excised until Day 5. Stock solution of ^{35}S radiolabeled methionine/cysteine (NEN, Dupont) was prepared in Grace's complete insect media at a concentration of $\approx 0.2 \mu\text{Ci}/\mu\text{l}$. Crickets age Day 0, 1, and 2 were injected with $\approx 1 \mu\text{Ci}$ radiolabel mixture in 5 µl Grace's complete insect media with and without cycloheximide. DLMs were excised and homogenized after 12 hrs overnight incubations. Samples of homogenates (25 µg of total protein) were subjected to SDS-PAGE and fluorographic imaging.

2.4 JH Biosynthesis rates

In vitro synthesis rates of JH from isolated CA were determined by means of a radiochemical assay (Feyereisen, 1985) as modified for the cricket (Woodring and Hoffman, 1994). Cycloheximide treatments were applied as previously described beginning at age Day 1 and continuing to Day 3 (CYX). Controls (D3 Control) received injections of distilled water only. The CA and *corpus cardiacum* (CC) complex were exposed by a sagittal cut through the head made between the antennae and compound eye. The CA/CC complex was removed, immersed in cricket Ringer's solution and the CA isolated. Isolated CA were immediately incubated for 90 min in 50 μ l medium 199 (Sigma, St. Louis, MO), buffered with 20 mM HEPES, pH 6.9, supplemented with 1% Ficoll 400 containing radiolabeled [methyl- 14 C]-methionine (final concentration 0.26 mM, sp. act 55 mCi/mmol). Glands were removed after incubations. Following extraction and thin layer chromatography, newly synthesized JH incorporating the radiolabeled methyl group was quantified by liquid scintillation counting (Beckman). CA from untreated Day 3 crickets were heat-killed by boiling at 94° C for 3 min and used as additional controls.

2.5 Radiolabel (35 S-Methionine) incorporation

Ligation and single topical application of methoprene (5 μ l of 3% methoprene in mineral oil, Sandoz, Dallas) were administered to crickets age Day 0, 1 and 2 in conjunction with radiolabel injections (5 μ l of $\approx$ 0.2 μ Ci/ μ l 35 S-methionine (ARCO, St. Louis, MO). Methoprene controls were treated with mineral oil. DLMs were homogenized following 12 hr overnight incubations, therefore, results are for the transition from Day 0 $\rightarrow$ 1, Day 1 $\rightarrow$ 2 and Day 2 $\rightarrow$ 3. Incorporation of 35 S-methionine was quantified by liquid scintillation counting of trichloroacetic acid (TCA) precipitated proteins from DLMs. DLM homogenates (2000 CPM/individual DLM) from Day 0 $\rightarrow$ 1, Day 1 $\rightarrow$ 2, and Day 2 $\rightarrow$ 3 crickets were subjected to SDS-PAGE and imaged by autofluorography.

3. Results

3.1 Overview of histolysis

The process of histolysis in flight muscles of the house cricket results in a marked degeneration of the DLMs in only a matter of days following adult emergence. When exposed by ventral dissections (Fig. 1), the DLMs display an elongated triangular shape with the apex attached by tonofibrils to a keel-like phragmatum (phr) projecting internally from the anterior margin of the pterothorax. Individual muscles attach separately to knob-like bifurcated processes of the phragma. The muscle insertion at the posterior dorsal margin of the thorax is broader than the anterior connection and no apodemal structures are obvious. *In vivo*, the medial faces of the DLMs are parallel and in close proximity to each other. Major tracheal trunks (tr) are located at the ventral margin of the DLMs. Other tracheal connections are made to the dorsal margin of the muscles. Smaller tracheal vessels branch from these trunks and eventually ramify to individual muscle fibers throughout the DLMs. DVMs originating at the terga extend to the coxa and serve in flight and in walking. DLMs of Day 0, and Day 1 crickets appear pinkish in color with firm smooth fibers (Fig. 1a and 1b). The pinkish cast to the DLMs begins to diminish and the muscles are less firm by Day 2 (Fig. 1c). The DLMs of Day 0, Day 1, and Day 2 crickets fibrillate spontaneously when exposed during dissection and shorten to approximately half their original length when cut. DLMs of Day 3 crickets are flaccid and white in color (Fig. 1d). When cut, these muscles shorten only slightly. Some differences in appearance of these muscles may be due to increased visibility of intramuscular tracheal fibers. DLMs from crickets older than Day 4 do not contract when cut but occasional fibrillations are seen. DLM remnants persist throughout the adult life of the house cricket but are indistinguishable from Day 3 DLMs by simple observation.

3.2 Time course of histolysis

Total protein content varied during the course of histolysis (Fig. 2a.). The highest mean total protein contents were found in DLMs from Day 0 (1.77 ± 0.105 mg/DLM; mean $\pm$ SEM), Day 1 (1.53 ± 0.075 mg/DLM) and white imagoes (1.75 ± 0.055 mg/DLM). A significant decline in total protein content of DLMs was seen in Day 2 crickets. Total protein content of Day 2 DLMs (1.28 ± 0.05 mg/DLM) was reduced $\approx 25\%$ from that of Day 0 and Day 1 muscles. This decline in total protein coincides with the color change observed in Day 2 DLMs in the cricket (Fig. 1c). A more dramatic decline of total protein content in DLMs was evident in Day 3 crickets. Total protein content in Day 3 DLMs (0.68 ± 0.032 mg/DLM) was less than 40% of the amount in Day 0 DLMs. No further significant declines in total protein content of DLMs of Day 4 (0.66 ± 0.049 mg/DLM) or Day 6 (0.55 ± 0.031 mg/DLM) crickets were seen. DLMs of white imagoes contained more total protein than larval muscles (0.86 ± 0.109 mg/DLM). Analysis of tissue weight wet (Fig. 2b) reveals a significant loss of muscle mass in DLMs by Day 3. No further loss of wet weight was evident in Day 4 or Day 6 DLMs. Wet weights of DLMs from Day 0 (6.22 ± 0.39 mg/DLM) differed significantly from those of Day 2 (4.07 ± 0.40 mg/DLM). No difference according to tissue wet weight was evident between DLMs from Day 2 and Day 1 (5.32 ± 0.53 mg/DLM). Muscles from crickets younger than Day 3 shortened approximately 30–50 % of initial resting length (Figure 2c). Muscles from crickets age Day 3 and Day 4 shortened only about 5% when cut. Day 6 DLMs did not shorten when cut. Muscle shortening of DLMs from white imagoes, though greater than those of Day 3 and older crickets, was significantly less than from Day 1 and Day 2 crickets.

Comparisons of relative hydration states of DLMs revealed that tissues from Day 6 crickets with a total protein to wet weight ratio of approximately 50% differed significantly from those of other age groups ($n = 6$, $\alpha = 0.05$, SNK). A tendency toward tissue condensation was evident from Day 1 throughout the course of histolysis in DLMs of the cricket (Linear Regression $p < .001$, $R^2 = 0.98$).

3.3 Effects of hormone removal and replacement

Ligation of Day 1 crickets reduced the loss of DLM total protein associated with histolysis, but this loss was restored by continual methoprene treatments (Figure 4a). Mean values for total protein (1.51 ± 0.15 mg/DLM) from DLMs of crickets ligated from Day 1 to Day 4 (Day 1–4 L) did not differ from total protein content of Day 1 or Day 2 controls. Mean total protein (0.59 ± 0.03 mg/DLM) of methoprene treated Day 1–4 ligated crickets (Day 1–4 L+M) was only approximately 40% of values for Day 1 and Day 1–4 ligated crickets and was not statistically different from Day 4 crickets. Loss of DLM total protein content was also apparent in crickets ligated at Day 2. Mean total protein content (0.711 ± 0.07 mg/DLM) of DLMs from crickets cervically ligated at age Day 2 was significantly reduced from those of Day 1, Day 2, and Day 1–4 ligated crickets. DLM total protein for these crickets (Day 2–4 L) did not differ from levels for Day 4 or methoprene treated Day 1–4 ligated crickets (Day 1–4 L+M). Methoprene treatments of crickets ligated at Day 2 did not result in further reductions in total protein content in DLMs. Tissue wet weights of DLMs from Day 1 to Day 4 ligated controls (Day 1–4 L) were not different from those of Day 1 crickets (Fig. 4b). A significant loss of muscle mass was observed in Day 1–4 ligated crickets treated with methoprene (Day 1–4 L+M). The mean values for methoprene treated ligated crickets were not different from those of Day 4 crickets. These results also indicate continuous treatments with methoprene replace JH and lead to induction of histolysis in DLMs.

Muscle shortening of Day 1–4 ligated specimens did not differ from Day 1 and Day 2 controls (Fig. 4c). Methoprene treatment of Day 1–4 ligated crickets resulted in significant and nearly complete loss of muscle shortening to levels not different from Day 4 controls undergoing normal histolysis. Muscle shortening of Day 2 ligated crickets was reduced from those of Day

1, Day 2, and Day 1–4 ligated controls, but this reduction was not significant. DLMs from Day 2–4 ligated crickets, either treated or not treated with methoprene appeared to retain ability to shorten.

3.4 Effects of cycloheximide

Fluorographic imaging of SDS-PAGE separated DLM proteins from crickets injected with ^{35}S Methionine/Cysteine revealed several bands in untreated (CYX-) controls but little or no incorporation of radiolabel in treated (CYX+) animals (Fig. 5.). Prominent bands were apparent at mobilities of ≈ 202 , 100, 42 and 29 kD in Day 0–1 samples (lane 1). Bands at 202 kD were less evident in Day 1–2 and Day 2–3 controls (lanes 3 and 5) controls. Bands at ≈ 143 kD were seen in Day 1–2 and Day 2–3 samples (lanes 3 and 5).

Cycloheximide treatments had significant effects on DLM parameters ($P = 0.001$). DLM total protein content (Figure 6a) following cycloheximide treatments (1.67 ± 0.18 mg/DLM,) did not differ from ligated (1.50 mg ± 0.13 /DLM) or Day 1 crickets (1.58 mg ± 0.10 /DLM) not undergoing histolysis. However, total protein levels from cycloheximide treatments were significantly greater than levels from degenerated Day 3 controls. DLMs from crickets in which cycloheximide treatments were discontinued (CYX+(R)) showed reductions in total protein comparable to those of Day 3 controls undergoing normal histolysis. Cycloheximide treatments resulted in maintenance of muscle shortening function to levels not different from non-degenerating DLMs (Fig. 6b). The protective effects of cycloheximide appeared to require continual presence of the antibiotic since loss of DLM total protein, mass and failure of muscle shortening occur when treatments are discontinued (Recovery).

An experiment was performed to analyze possible effects of cycloheximide treatments on JH synthesis (Fig. 7). No significant difference (ANOVA, $P = 0.0858$, $n=3$) was found in the *in vitro* rates of JH synthesis between isolated CA glands of crickets treated with cycloheximide and those not treated. CA of crickets treated with cycloheximide continued to synthesize and secrete JH.

3.5 Incorporation of Radiolabeled Methionine

Protein expression in DLMs during histolysis was quantified by assay of total protein in conjunction with short-term (12 hr) incorporation of ^{35}S -methionine. Consistent with previous experiments, total protein content of DLMs was altered during histolysis (Fig. 8). A significant reduction of total protein was evident at Day 3 for all treatments ($\alpha = 0.05$; SNK; $n=5$). Direct comparisons between controls for Day 0 $\rightarrow$ 1 and Day 1 $\rightarrow$ 2 show an initial decline of total protein occurred in DLMs at Day 2 ($P = 0.002$; LSM). Ligation, which interrupts the JH signal necessary for initiation of histolysis, results in maintenance of total protein in Day 1 $\rightarrow$ 2 ligated subjects.

Analysis of incorporation of ^{35}S -labeled methionine in TCA insoluble proteins of DLMs reveal significant reductions in protein expression occur after Day 1 (Fig 8). Effects of ligation by age ($P = 0.017$) indicate ligation may contribute to a decline in radiolabel incorporation in Day 0 $\rightarrow$ 1 ligated subjects ($P = 0.036$, LSM) as compared to Day 0 $\rightarrow$ 1 controls.

Some protein bands showing radiolabel incorporation are reduced (open arrows), increased (closed arrows) or relatively constant (gray arrows) during histolysis (Fig. 9). Distinct bands at approximately 47–49 kD (gray arrow) appear to be continually expressed at all ages and treatments. Prominent bands at approximately 143 kD (large closed arrow) are seen in Day 3 samples but not in Day 1 samples. A band migrating at apparent molecular weight greater than myosin also seems to increase at Day 2 (small arrows).

4. Discussion

Our results demonstrate significant losses of total protein content, tissue wet weight and muscle-shortening accompany histolysis in flight muscles of the cricket. Changes in total protein content, tissue wet weight, and muscle-shortening of DLMs suggest these measures are valid indices of the extent of degeneration associated with flight muscle histolysis in the cricket. Significant differences between Day 1 DLMs and Day 3 DLMs are observed in all variables investigated, but no significant alterations of DLMs are evident following Day 3. Cervical ligation to eliminate circulating JH and cycloheximide treatment to inhibit protein expression both block histolysis.

These results suggest that initial events of histolysis, including JH signal induction, occur by Day 2 and are essentially complete by Day 3. The onset of histolysis has previously been associated with presumed increases in JH titer that induce vitellogenesis in adult female crickets (Woodring and Sparks, 1987). Our results suggest an earlier JH signal (within 48 hr of adult emergence) that is sufficient to induce histolysis. Peaks of JH titer in cricket hemolymph have been shown to occur within this time frame (Renucci et al., 1990). Ligation at Day 1 blocked histolysis. Methoprene treatments to Day 1–4 ligated crickets restored histolysis and resulted in significant reduction in DLM total protein and muscle shortening. However, since DLMs from Day 2–4 ligated crickets still underwent histolysis, signals from JH or other possible cephalic factors must have been received before the time of ligation. The failure of methoprene treatments to affect histolysis of Day 2–4 ligated crickets is consistent with a requirement for a JH signal prior to Day 2 which is both necessary and sufficient to induce histolysis.

Myofibrillar systems are able to produce significant muscle shortening in DLMs from Day 2 crickets, but as histolysis proceeds this ability is lost. Alterations in contractile potential of involuting insect muscles have been reported in ISMs of the blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1956) and moth, *Manduca sexta* (Lockshin and Beaulaton, 1979). More recently, electrophysiologic studies have revealed dramatic declines in muscle contractility accompany PCD in insect muscle (Schwartz and Ruff, 2002). Changes in length of the DLMs of the cricket observed when muscles are cut as in our procedures probably do not represent normal *in vivo* contraction. Further studies are necessary to determine the extent to which observed alterations in muscle shortening of DLMs during histolysis are due to effects on contractile, elastic or excitation-coupling components of these muscles.

Cycloheximide prevents or delays DLM degeneration and PCD in the cricket. This suggests a requirement for specific protein expression both in the mechanism of PCD in this system and in the mode of action through which JH might mediate the events. We have addressed some concerns regarding the use of protein synthesis inhibitors to investigate PCD: (1.) The dosage level of cycloheximide was sufficient to block translation and incorporation of radiolabel into DLM proteins. However, crickets were able to survive the treatments. (2.) Cycloheximide treatments do not result in irreversible suppression of histolysis in DLMs of the cricket. Histolysis follows discontinuation of cycloheximide treatments. (3.) Cycloheximide does not appear to eliminate JH since JH was still synthesized and released from CA of cycloheximide treated crickets. These findings are consistent with reports that treatments with cycloheximide fail to significantly affect JH synthesis rates of the cyclic cockroach, *Diploptera punctata* (Feyereisen and Farnsworth, 1987). Taken together, these results support the likelihood that cycloheximide inhibits flight muscle histolysis in the cricket through suppression of protein expression essential to JH transduction pathways, cell death programs or proteolytic activation. Further studies, including direct measurements of hemolymph JH levels may be necessary to define threshold levels of hormone necessary for induction of histolysis and to assess possible involvement of cephalic factors other than JH, which may presumably be blocked by cervical ligation.

Experiments based on short-term (12 hr) incorporation of ^{35}S -methionine more directly address regulation of protein expression since radiolabeled proteins must have acquired the label during incubation and are presumed to be newly synthesized. Results from this experiment demonstrate that protein expression is altered during the course of histolysis. Bands showing radiolabel incorporation seen in Day 2→3 samples (such as the distinct bands at ≈ 143 kD) potentially represent proteins up-regulated as a result of histolysis. However, the timing and level of expression of this band does not suggest it is a protein associated with the induction of histolysis. Instead, it seems more likely that proteins involved in the actual initiation of histolysis would be transiently expressed at levels below autofluorographic detection.

Radiolabel incorporation was significantly reduced at the onset of histolysis in the cricket. This may reflect a general cessation of protein synthesis characteristic of latter stages of rapid PCD (Zakeri et al., 1996), but could be coincidental to general reductions in protein synthesis due the end of the growth phase of flight muscles (Gomi et al., 1995). Reduced expression of myofibrillar proteins such as actin and myosin has been shown in other models of muscle degeneration (Schwartz et al., 1993); therefore, repression of specific proteins could be involved in DLM histolysis as well. The results from our analysis of ^{35}S -methionine incorporation experiments confirm previous indications of an initial significant decline in total protein at Day 2. DLMs from Day 1→2 controls have less total protein than Day 0→1 controls and were found to have the lowest level of radiolabel incorporation. Short-term ligation of Day 1→2 subjects inhibited initial declines of total protein suggesting that JH or other factors blocked by ligation may be responsible for induction of mechanisms to account for protein loss. A subtle but significant decline of radiolabel incorporation in short-term, ligated Day 0→1 crickets is evident. This supports the possibility that a sub-set of proteins induced by early JH signals might play a role in orchestrating the actual onset of histolysis and subsequent alterations in protein expression. Short-term methoprene treatments had no discernable effects consistent with an early and sufficient JH signal, but also possibly implicating mechanistic differences in the mode of action and responsiveness of the muscles to this analog. As previously noted, while our results support the conclusion that JH mediates flight muscle histolysis in the house cricket, other factors could be involved.

Flight muscle histolysis in the house cricket appears comparable to the process of involution of the intersegmental muscles (ISMs) of lepidopterans (Lockshin, 1985), an important and well-studied model of programmed cell death. The ISMs are abdominal muscles of larval origin, which persist through pupation and then actively function to aid in eclosion behavior. Following this essential role, declining levels of ecdysone trigger the rapid degeneration and eventual removal of the ISMs. The requirement for *de novo* protein synthesis in involution of the ISMs (Lockshin, 1969; Wadewitz and Lockshin, 1988) has been clearly linked to transcriptional regulation through molecular cloning of both up- and down-regulated genes (Schwartz et al., 1990). Genes for actin, myosin and the 20S proteasome unit are down-regulated (Schwartz et al., 1993; Jones et al., 1995), while those for polyubiquitin, the 26S proteasome units and other proteins are up-regulated at the onset of involution (Dawson et al., 1996; Kuelzer et al., 1999; Sun et al., 1996; Low et al., 2005).

Flight muscle histolysis in the house cricket appears to be an example of an active, developmentally regulated cell death program induced by an endocrine signal. It is most similar to models of PCD such as involution of lepidopteran ISMs in which hormonal signals control dissolution and restructuring of tissues during development. In the cricket, as in these examples, *de novo*, coordinately-regulated protein synthesis is required for the completion of the program of degeneration of the DLMs. Flight muscle histolysis, though sharing many characteristics with other models of PCD, is an example of endocrine regulated death of terminally differentiated adult tissues. In contrast to other models of PCD in insects, flight muscle

histolysis is an adult, post-metamorphic event that appears to be mediated by JH as opposed to the involution of larval tissues controlled by ecdysone.

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Fig 1. Ventral dissections of flight musculature of the house cricket. (A.) Day 0 (B.) Day 1 (C.) Day 2 (D.) Day 3. Dorsal longitudinal flight muscles (DLMs); Dorsal ventral flight muscles (DVMs); anterior phragmatum (Phr); Main ventral tracheal trunk (Tr).

Fig 2.

Time course of alterations in DLMs during histolysis. (A.) Effects on DLM total protein content. (B.) Effects on DLM tissue wet weight. (C.) Effects on DLM muscle shortening. Larvae = mixed penultimate and last instar larvae; W. imago. $\approx$ 2 hrs post-emergence; Day 0 = day of emergence; Day 1 = 24–47 hrs; Day 2 = 48–71 hrs; Day 3 = 72–95 hrs; Day 4 = 96–119 hrs; Day 6 = 144–167 hrs. Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation. Means with the same letter are not significantly different (SNK, $\alpha = 0.05$, $n = 6$).

Fig 3. Alterations in DLM tissue hydration states (total protein content/tissue wet weight) during histolysis. The dashed line is the linear regression through the means ($R^2 = 0.98$). Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation. Means with the same letter are not significantly different (SNK, $\alpha = 0.05$, $n = 6$).

Fig 4.

Effects of ligation and methoprene treatments on DLMs. (A.) Effects on DLM total protein content. (B.) Effects on DLM tissue wet weight. (C.) Effects on DLM muscle shortening. Day 1 = 24–47 hrs; Day 2 = 48–71 hrs; Day 4 = 96–119 hrs; Day 1–4 L = ligated at Day 1 then excised Day 4; Day 1–4 L+M = ligated at Day 1 and treated with methoprene then excised at Day 4; D2–4 L = ligated at Day 2 then excised at Day 4; D2–4 L+M = ligated at Day 2 and treated with methoprene then excised at Day 4. Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation, means with the same letter are not significantly different (SNK, $\alpha = 0.05$, $n = 6$).

Fig 5. Cycloheximide inhibits radiolabel incorporation in DLM proteins. Fluorographic imaging of SDS-PAGE gel of proteins (25 µg/lane) isolated from the house cricket 12 hrs after injection with ^{35}S methionine/cysteine in Grace's insect medium with (+) or without (-) cycloheximide. Migrations of molecular weight markers are as indicated (kD).

Fig 6. Effects of cycloheximide treatment on DLMs. (A.) Effects on DLM total protein content. (B.) Effects on DLM tissue wet weight. (C.) Effects on DLM muscle shortening. Day 1 and Day 3 are normal controls, Day 3 controls were injected with distilled water, Day 3L were ligated at Day 1 and examined at Day 3, Day 3 CYX+ received twice daily treatments with cycloheximide from Day 1 until Day 3, Day 5 CYX+ (R) = Recovery after cycloheximide treatments were discontinued at Day 3. Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation, means with the same letter are not significantly different (SNK, $\alpha = 0.05$, $n = 5$).

Fig 7.

Effects of cycloheximide on *in vitro* JH synthesis rates. Day 1 crickets were treated (CYX +) or not treated (CYX -) with cycloheximide as described and then at Day 3, CA were assayed for JH biosynthesis. JH synthesis rates from heat-killed Day 3 CA were subtracted as background. Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation, means with the same letter are not significantly different (SNK, $\alpha = 0.05$, $n = 3$).

Fig 8.

Total protein content and radiolabel incorporation in DLM proteins during histolysis. (A.) Total protein (B) Radiolabel incorporation (TCA precipitated counts). Treatments are for the transition (12 hr) between the ages indicated. L= ligation, M=methoprene, L+M = ligation + methoprene. Error bars are mean $\pm$ SEM. Letters above error bars represent ranges for significant mean separation, means with the same letter are not significantly different (LSM, $\alpha = 0.05$, $n=5$).

Fig 9.

Fluorographic imaging of ^{35}S -methionine (2000 CPM/lane) in DLM proteins separated by SDS-PAGE. Treatments are for the transition (12 hrs) between the ages indicated. L= ligation, M=methoprene, L+M = ligation + methoprene. Closed arrows indicate proteins with increased incorporation; open arrows, decreased incorporation and gray arrows, relatively unchanged incorporation of radiolabel as a result of age or treatment. Migrations of molecular weight markers are as indicated (kD).