Microscopic Evaluations of Differences Between Synchronous and Asynchronous Insect Flight Muscles

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ABSTRACT:

Projectin and mp20, protein components of insect muscle are under investigation for their role in muscle contraction. Many studies in *Drosophila melanogaster* flight muscles indicate that projectin is found between the Z and I band regions, whereas mp20 is totally absent. In this study we used locations reported in *D. melanogaster* as a model to investigate the location of the projectin epitopes and mp20 in *Danaus plexippus* (monarch butterfly), *Manduca sexta* (tobacco hornworm moth), *Apis mellifera* (bee) and *Acheta domesticus* (cricket). All insects listed apart from *A. mellifera* have flight muscles with properties different from *D. melanogaster*. Using indirect immunofluorescence microscopy (IMF), the protein locations were studied in each insect model, and confocal micrographs were obtained. Also, RT-PCR data were collected to determine the presence or absence of mp20 in the cricket and bee. Data suggest that the projectin location in the three insect models appears to agree with the predictions from *D. melanogaster*. Our data show that the location of mp20 depends on the muscle types, but not the physiological nature (synchronous or asynchronous).

INTRODUCTION:

Asynchronous flight muscles of derived insects use a delayed stretch activation/ shortening deactivation mechanism to produce oscillatory contraction for flight (Josephson et al, 2000). Unlike synchronous muscles, the muscle contractions of asynchronous muscles do not correlate 1:1 with nerve action potentials; i.e. more wing strokes are produced per action potential in asynchronous muscles. Asynchronous flight muscles have a higher resting stiffness in comparison to synchronous muscles, which could be due to how the thick filaments are connected to the Z-band. Connecting filaments (C-filaments) attach to the ends of the Z band and are one of the components responsible for the high resting stiffness of the

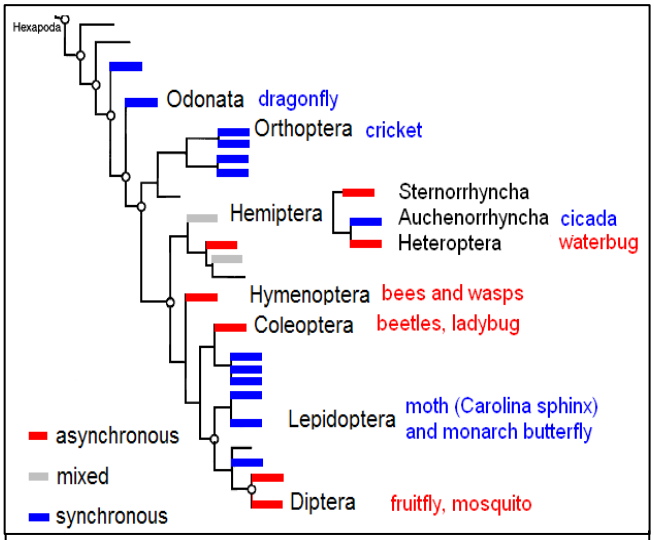

Figure 1: simplified insect phylogeny indicating the distribution of synchronous and asynchronous flight muscles

muscles (Ayme-Southgate et al, 2006). Studies indicate that most derived insects have asynchronous flight muscles, and basal insects have synchronous muscles (Josephson et al, 2000). Figure 1 shows a phylogeny of these insects and their different muscle types.

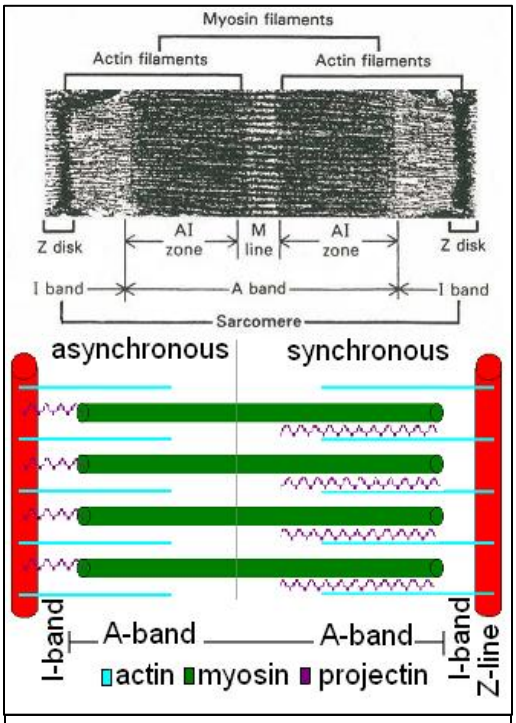

Figure 2: Sarcomere Structure

The two proteins under investigation are projectin and mp20. Projectin is proposed to modulate muscle stiffness while mp20 is proposed to regulate actin-myosin interactions. In asynchronous flight muscles, projectin is one of the C-filament proteins. The large size of projectin could allow for it to cover the entire C-filament, with one end embedded in the Z-band and the other in the thick filament producing the necessary elasticity and stiffness for asynchronous flight (Saide et al, 1989). In *D. melanogaster* projectin is present in synchronous and asynchronous muscles, but the position in the sarcomere is different for two muscles types. As seen in Figure 2 projectin is found over the A band for synchronous (leg, head etc.) muscles of *D. melanogaster,* but is found over the I-Z-I band in indirect flight muscles (IFMs) as anticipated for a component of the C-filament (Ayme-Southgate et al, 2006). The question is whether this difference in localization is because of differences between synchronous and asynchronous or differences between flight and non-flight. To address this question, projectin localization in other insects with synchronous and asynchronous flight muscles was tested.

mp20 is the other protein under investigation. While mp20 is not present in the asynchronous muscles of *D. melanogaster,* studies have shown that mp20 is in the synchronous muscles. Western blot technique of the head, flight and leg muscles from *D. melanogaster* stained with the mp20 antibody shows this correlation in Figure 3. mp20 is absent from the flight muscle (Lane 2 in Figure 3) confirming its absence from asynchronous muscle. According to available genome sequences the presence of the mp20 gene is confirmed in Hymenoptera, Lepidoptera, Coleoptera, and Hemiptera. We are investigating to see if the absence of mp20 is common among all asynchronous muscles or amongst all flight muscles.

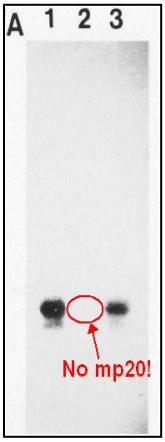

Figure 3: Western blot of D. melanogaster with mp20 antibody

MATERIALS AND METHODS:

Insects

The insects used in this study were *Danaus plexippus* (monarch butterfly), *Manduca sexta* (tobacco hornworm moth), *Acheta domesticus* (cricket) and *Apis mellifera* (bee). The butterfly and moth were purchased from Educational Science as cocoons and hatched in the lab. The crickets were purchased from PetSmart. The bees were a gift from Dr. Vance, a Biology professor at the College of Charleston.

Immunofluorescence

Myofibrils are extracted from the thorax of insects (from above) using dissecting techniques and placed on poly-L-lysine treated slides and allowed to sit for 10 minutes for attachment to the slide. The myofibrils are fixed on the slides using a solution of formaldehyde, 1X PBS (Phosphate Buffer Saline), and Triton. After washing in PBS, the slides are placed in a 5% goat serum in 1XPBS for blocking. The primary antibodies are then applied to the slides diluted 1:100. If two primary antibodies are used, they are added to the slides together in equal amounts. The samples are covered with cover slips, incubated in a humid chamber and washed after incubation in 1XPBS 2 times for 10 minutes each. The secondary fluorescent antibodies are applied in the dark (1:100 dilution), covered and incubated. Phalloidin can also be applied at this point along with a secondary antibody. The phalloidin and secondary are applied in equal amounts. After incubation, the slides are washed with 1XPBS in the dark. Excess liquid is removed and the slides are mounted using a mounting medium (Vectorshield, Inc). The slides are examined by epifluorescence confocal microscopy on an Olympus confocal microscope. The program used to capture and process images is Olympus Slidebook.

Antibodies

The antibodies used in this study are listed:

Anti-projectin: **mac150**, **Core-1P**, **3b11** and **P5**, **NT2** and **anti-FRAM**
Anti-kettin: **mac155**, anti α-actinin: **mac276** and **4g6**
The “mac” antibodies are rat monoclonal antibodies. 3b11, P5, and 4g6 are mouse monoclonal antibodies; Core-1P, NT2, and anti-FRAM are rabbit polyclonal antibodies.

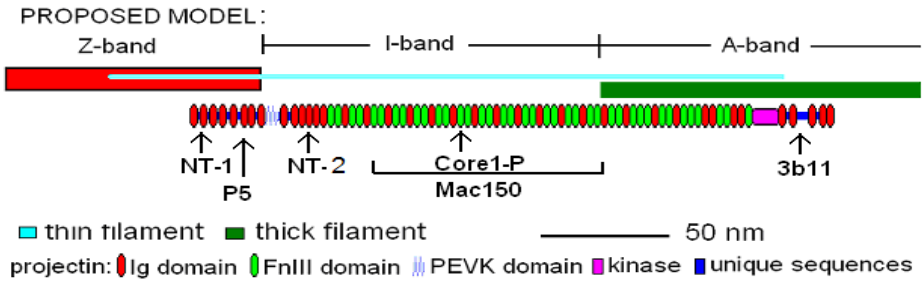

Figure 4: Location of antibodies in the sarcomere

The expected positions in the sarcomere for the antibodies used are shown in Figure 4.

RNA Extraction

The RNA extraction protocol followed in the lab is provided by Promega. The tissue sample and lysis buffer are added together into an autoclaved tube. The tissue is homogenized in the buffer using a pestle. This mixture is then transferred into the Spin Basket Assembly. Dilution buffer is then added to the tube and centrifuged for 10 minutes and the lysate is transferred to a clean tube. 95% Ethanol is then added to the lysate and this mixture is centrifuged again for 10 minutes. The elute is then discarded and RNA Wash Solution is added, centrifuged again for 1 minute and elute is discarded again. DNAase Incubation Mix is then added to the membrane and incubated for 15 minutes. DNAase Stop Solution is added after incubation and the tube is centrifuged for 1 minute. RNA Wash Solution is added, centrifuged for 1 minute and discarded. RNA Wash Solution is added again, centrifuged for 2 minutes and discarded. Nuclease Free Water is added to the membrane and centrifuged for 1 minute to elute the RNA.

RT-PCR for mp20

Once the muscle of the insects has been dissected, the RNA is extracted and purified from the legs, head and thorax. The RT-PCR reaction contains two primers based on the mp20 sequence. cDNA synthesis occurs and is amplified by the PCR cycles. The reaction is run on a TAE-agarose gel and the amplified products are cut from the gel. The DNA is purified from the gel and sent off for sequencing to an outside company (GEnWiz Inc).

DNA Extraction and Purification from Agarose Gel

DNA is extracted from the agarose gel and used for sequencing. Step 1 is the gel dissociation. The gel is sliced where the DNA is present and extra agarose is removed. DF Buffer is added to the sample and mixed by vortex, incubated for 10 minutes in 60°C water and then cooled to room temperature. Step 2 is the DNA binding step. The DF column is placed in a collection tube and the sample is transferred to the DF column. The sample is centrifuged for 30 seconds. Discard flow-through. Step 3 is the wash step. Wash buffer is added to the column, allowed to stand for 1 minute. Repeat centrifuge and discarding of flow-through. The step is repeated. The DF column is then centrifuged for 3 minutes to allow the column to dry. Step 4 is the DNA elution step. The dried column is transferred to a microcentrifuge tube and elution buffer is added. After 2 minutes, the column is centrifuged. The elute contains the purified DNA.

Protein Transfer to Nitrocellulose

Muscle tissue was extracted and homogenized using protein extraction buffer (Hodges composition) from the head, leg and thorax of the insect. The proteins were run on an SDS-polyacrylamide gel using 1x TGS buffer (Tris-Glycine-SDS). Allow the

gel to equilibrate in the transfer buffer (1x TGS +20% methanol). The apparatus for transfer to nitrocellulose is then assembled and placed set to run with the filter towards the positive electrode. Transfer is then run for 1 hour at 0.3 Amp.

Western Blot

TBST (Tris-Buffered Saline and Tween) with 2% bovine serum albumin (BSA) is used to block the filter after transfer is completed. A solution of antibody (1:1000 dilution) and TBST is used to bind the antibody for the filter. After incubation wash filter with TBST on a shaker. The second antibody (antiRabbit-HRP) is applied (1:7500 dilution) with TBST. Wash the filter in TBST 2 times for 20 minutes after incubation. The second antibody can be visualized using a chemiluminescent reaction.

RESULTS:

Projectin

The insects under investigation in my study were cricket, moth, bee and monarch butterfly. The localization of projectin in *D. melanogaster* was compared to the localization of the protein in these four insects. In synchronous muscle (leg, head, body) of D*. melanogaster* the location of projectin is over the A band along with myosin. In asynchronous muscle (used for flight) projectin covers the I-Z-I band of the C-filament (Lakey et al, 1990). The proposed model of the localization of projectin for *D. melanogaster* can be seen in Figure 4. The various epitopes (refer to Materials and Methods section) of projectin are seen in different regions of the I-Z-I band, verifying the localization in this region.

To compare the localization of projectin in the selected insects with the location in *D. melanogaster,* another protein with known position was used as co-stain. The second protein was either actin stained using phalloidin, kettin or α-actinin with specific primary antibodies followed by the secondary antibody specific to the primary species. The projectin region was visualized using projectin antibodies with a different fluorochrome for the secondary antibody.

Localization of Projectin In Flight Muscle Tissue of Cricket

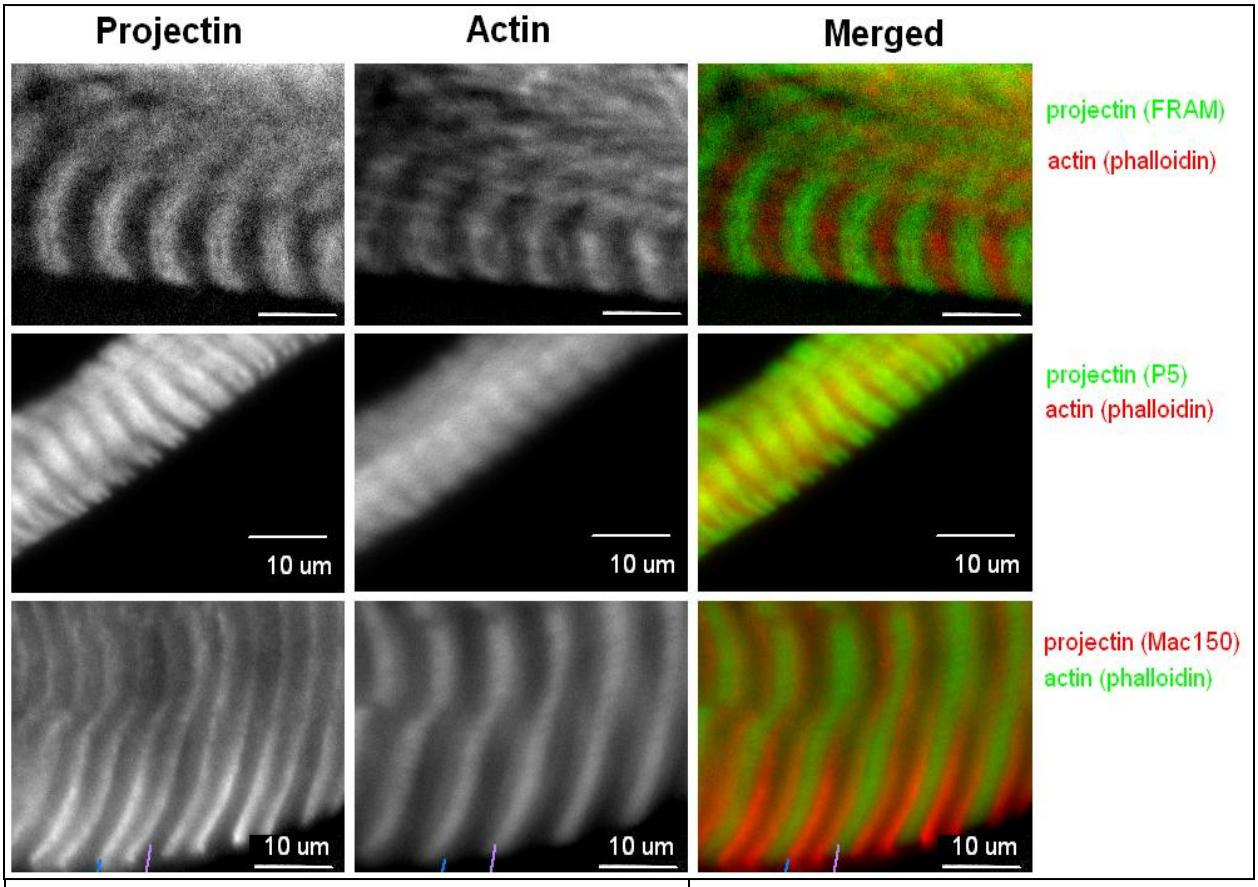

Figure 5: Projectin-actin combinations for cricket

The images in Figure 5 show the muscle patterns of actin and projectin for the cricket model. All images show that actin and projectin are in different locations. Three different epitopes (FRAM, P5 and Mac 150) of projectin were tested on the cricket flight muscle tissue and paired with phalloidin for the visualization of actin. Because projectin and actin are in different locations in this tissue, this shows that projectin is in the Z band which is consistent with the *D. melanogaster* model. In these images a split pattern can be seen in the projectin regions of the muscle, which has not been seen in the *D. melanogaster* model. The reason for the split pattern is hypothesized to be because of a longer sarcomere in the cricket than in other insects observed. The epitopes used are in different locations along the sarcomere showing an increase in the split the further away from the Z band the epitope is located.

Localization of Projectin in Flight Muscle of Moth

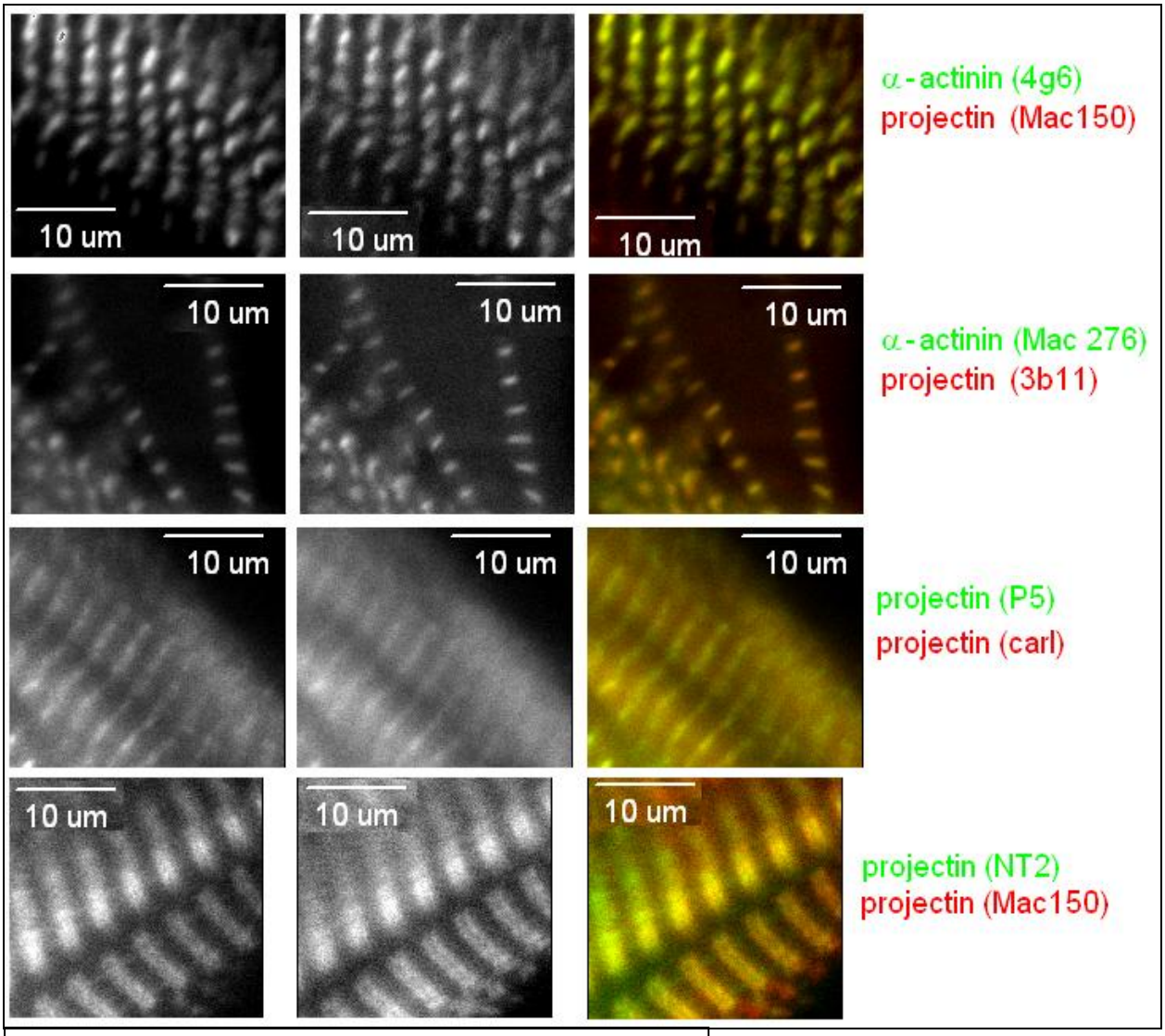

Figure 6: Projectin-combinations for moth

The images in Figure 6 show combinations of projectin with α-actin (Images 1 and 2) along with projectin with different epitopes of projectin (Images 3 and 4). Image 1 shows α-actinin and projectin in the same location which is not expected. Projectin should have been seen in the Z region which would have produced green and red florescence separately instead of the yellow color which indicates the two regions on top of each other. Image 2 shows the Mac 276 α-actinin epitope and 3b11 projectin epitope in the same location which is NOT expected because the location of 3b11 is predicted to be within the A band region (Refer to Figure 4). Image 3 and 4 show combinations of different projectin epitopes in the same region which is expected because they are both expected in the Z band region. A split pattern can be seen in the Image 4 and could

have occurred for many reasons. These include a different elasticity of the moth muscle or the muscle being overstretched during preparation.

mp20

In proposed models using *D. melanogaster* the protein mp20 is absent from flight muscles, unlike the projectin protein. Using immunofluorescence on monarch and cricket flight muscles the location of mp20 was determined and different from what was expected following the results from the *D. melanogaster* model.

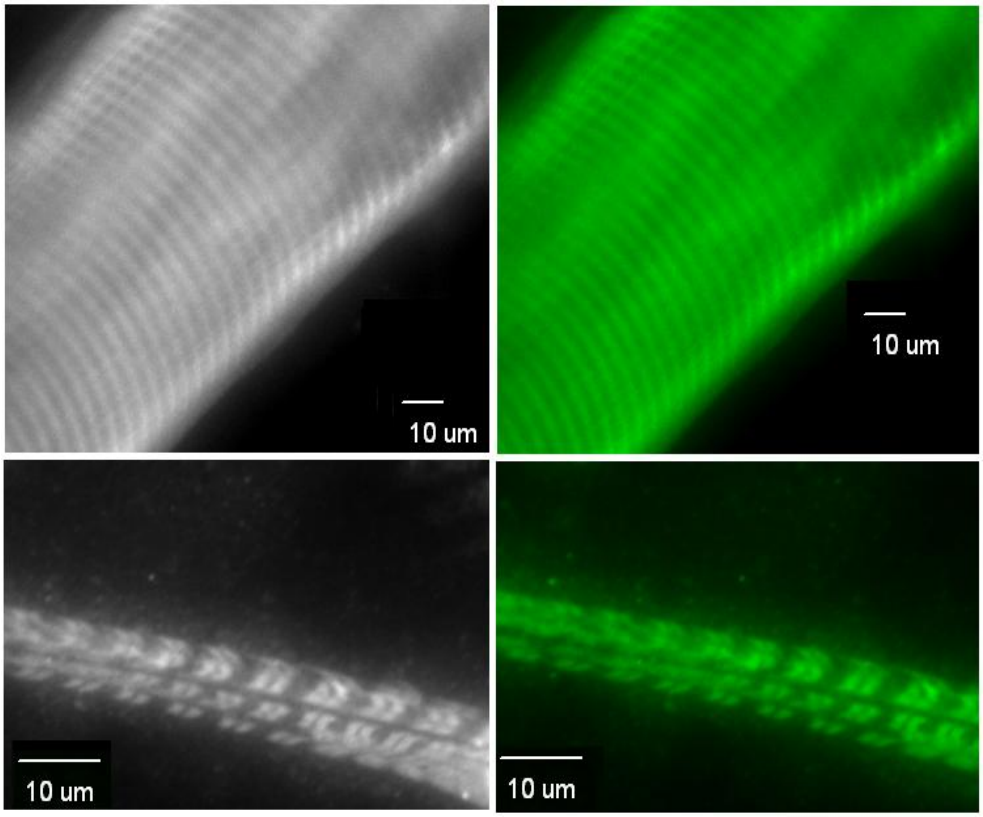

Figure 7: mp20 antibody on monarch flight muscles

The images in Figure 7 show monarch flight muscles stained with mp20 antibody. The visualization of the antibody on the muscle tissue shows that mp20 is present in the synchronous flight muscle of the butterfly. This is different from the results seen in *D. melanogaster (*Figure 3). Images of cricket flight muscle stained with mp20 antibody were also observed with similar results, showing the presence of mp20.

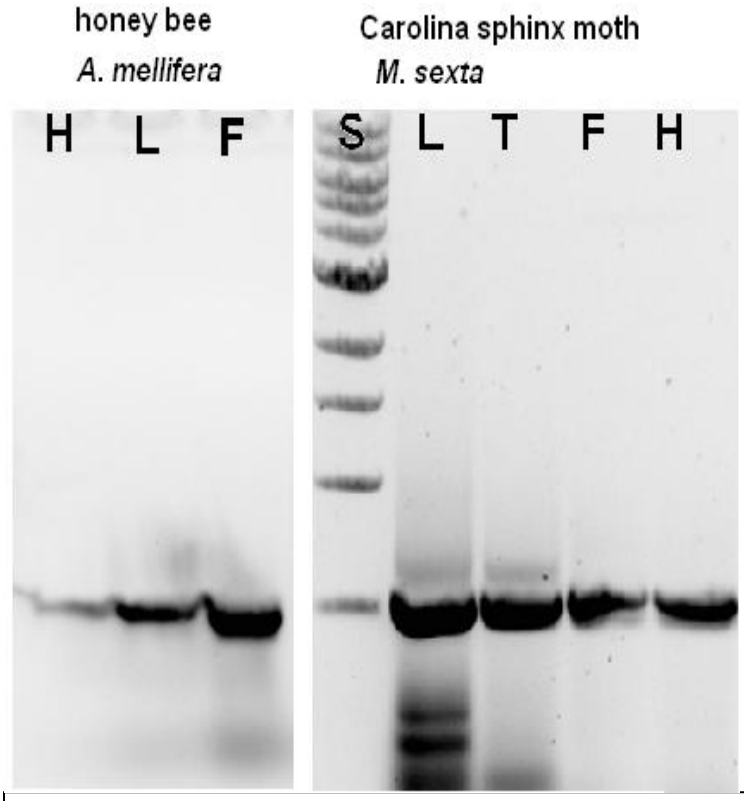

Figure 8: RT-PCR reaction gel analysis or the mp20 gene in all muscle types in Manduca sexta and Apis mellifera S: DNA ladder for size standard; L: leg; F: flight; T thorax; and H:head

RT-PCR reactions were performed for the bee and the moth to look for the expression of the mp20 gene in all muscle types. Figure 8 shows the presence of mp20 mRNA in all synchronous muscles of the honey bee, and moth, but also in the bee asynchronous flight muscles which are not expected based on results from *D. melanogaster.* Based on the results from the immunofluorescence microscopy and gel analysis it can be concluded that mp20 is present in ALL muscle types whether flight versus non-flight and synchronous versus asynchronous. *D. melanogaster* is an exception because mp20 is not present in the asynchronous muscle type.

DISCUSSION:

The projectin protein is proposed to be very large in size, covering the I band and anchored in the Z band. This large size is what aids in the stretch activation/deactivation of the indirect flight muscles and gives the higher stiffness to produce more beats of the wings per action potential. Looking at the immunofluorescence images of the various insects in our study, it appears that the location of projectin in the sarcomere is due to the muscle type being flight muscle and not asynchronous or synchronous muscle. To

further the study of this other insects could be used from the phylogeny and tested using the same techniques.

The mp20 protein is present in all muscles in our study except for the asynchronous muscles of *D. melanogaster.* This indicates that whatever the function of the mp20 protein (which is still unknown), it is compatible with the demands of flight muscles even if they are asynchronous. A wider study looking at more insects with both physiological type of flight muscles is required to clarify the relationship between the mp20 protein and flight muscles.

ACKNOLEDGEMENTS

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