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The University of Southern Mississippi

Investigating the Co-Regulatory Role of *midline* and *extramacrochaetae* In Regulating Eye Development and Vision in *Drosophila melanogaster*

by

Lillian Forstall

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2014

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Abstract

The Honors thesis research focused on the roles of *extramacrochaetae* and *midline* in regulating eye development and the vision of *Drosophila melanogaster*. It is known from previous studies that *extramacrochaetae* (*emc*) and *midline* (*mid*) independently regulate the formation of ommatidial units in the *Drosophila* compound eye. However, the thesis focuses on the interaction of these two genes and their co-dependent roles in regulating eye development. This study also attempts to explain the recovered formation of ommatidial units and interommatidial bristles when the expression of both of these genes is reduced and whether flies doubly mutant for these genes have recovered phototactic ability. Specific genotypes of flies were subjected to larval and adult phototaxis assays to assay their phototactic ability. A Western analysis was performed on *extramacrochaetae* mutants, *midline* mutants, and wild-type flies to determine whether the Emc and Mid proteins interacted in a co-regulatory fashion within developing larval tissues.

The larval phototaxis assays revealed a slight decrease in photoreception in the *mid-RNAi* larvae when compared to the wild-type larvae. However the data was not conclusive to definitively determine if the *mid-RNAi* mutants displayed a significant decrease in photoreceptive ability. The adult phototaxis assays were more definitive than the larval assays. The emc^{1} flies displayed a slight decrease in photoreceptive ability. Both the *mid-RNAi* and the flies doubly mutant for mid^{GA174} and emc^{1} displayed a significant decrease in photoreceptive ability. The Western blot and immunofluorescence studies revealed an interaction between *mid* and *emc*, and the future nature of this interaction will be resolved in greater detail

Key Terms: Fruit fly, compound eye, gene regulation, gene expression, Honors College.

Dedication

Barbara, Mark and John Forstall

Without whom I could not conquer my greatest hopes and fears.

You are the perfect support system.

Acknowledgements

I would like to thank my thesis advisor, Dr. Sandra Leal, for guiding me through the process of becoming a true scientific researcher by making genetics and developmental biology come alive. This thesis would not be possible had it not been for her creative spirit and willingness to pass on her knowledge to others. Thank you for everything you do.

I would also like to thank Ken Buford, without whom I would not have completed all of my research, and without whom I might have lost a finger.

Additionally, I would like to thank the Honors College for giving me the opportunity to better myself through research and challenging me with accelerated classes. Without the Honors College, I would not have achieved all the goals I set when I set foot on this campus.

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List of Abbreviations

Central nervous system ..... CNS

Double mutant ..... DM

Inhibitor of differentiation ..... ID

extramacrochaetae ..... *emc*

midline ..... *mid*

Morphogenetic furrow ..... MF

Sensory organ precursor ..... SOP

Transmission electron microscope ..... TEM

*w*emc^{1}P{neoFRT}80B/TM6B, Tb^{1}* ..... *emc^{1}*

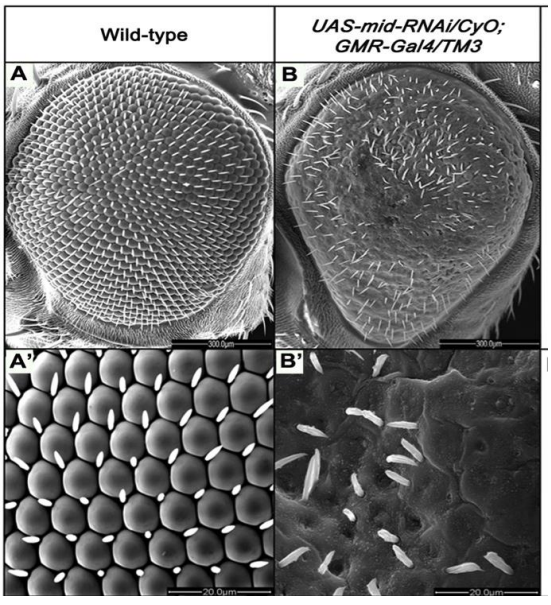
Wild-type ..... WT

Chapter 1: Introduction

The eye of *Drosophila melanogaster* is an assembly of 800 subunits referred to ommatidia. A single ommatidium is composed of specialized cells that are sequentially generated during third-instar larval stages of development. Each ommatidium is composed of eight photoreceptor neurons where two inner receptors are surrounded by six outer receptors. Once this core of photoreceptor neurons is assembled, it is surrounded by accessory cells, including pigment cells, and covered by four cone cells. The formation of each ommatidium is a highly regulated process and when completed, the compound eye of *Drosophila* is composed of roughly 800 ommatidial subunits.

Two transcription factor genes have been found to play a role in the development of the *Drosophila* eye: *midline* (*mid*) and *extramacrochaetae* (*emc*). There are several aims of the research proposal. Firstly, Das et al. (2013) indicated that *mid* mutants exhibited severe eye defects including ommatidial fusion, loss of pigmentation, and decreased bristle complex development. Figure 1, Panel B, illustrates the *mid* mutant phenotype. Panel A illustrates the wild-type adult compound eye (Das et al., 2013).

Figure 1



I hypothesized that these *mid* mutants were blind. In order to test this hypothesis, I subjected both larval and adult *Drosophila* mutants and wild-type flies to appropriate phototactic assays. Interestingly, flies doubly homozygous mutant for mid^{GA174} and emc^{1} recovered many of the defects observed in the homozygous *mid* mutant flies (Figure 2C) (Das et al., 2013). Thus, I hypothesized that *UAS-mid-RNAi/+;* emc^{1}*/GMR-Gal4* flies would regain phototactic activity due to their recovered ommatidia and bristle formation (Fig. 2C).

There is a significant co-expression of the *mid* paralog H15 and *emc* during early eye imaginal disc developmental stages (Figure 3). It appears that the expression of *emc* is changed when *mid* expression is reduced (Fig. 3) (Das et al., 2013). The Emc protein appears to shift to the anterior region of the eye imaginal disc. Therefore, I hypothesized that these proteins interacted with each other. To test this hypothesis, I performed a Western analysis of homogenates prepared from WT and *mid-RNAi* tissues of third-instar larvae to detect levels of Emc expression. Figure 2A illustrates a wild-type eye. Figure 2B illustrates the *mid* mutant phenotype. Figure 2C illustrates that the *UAS-mid-RNAi/+;* emc^{1}*/GMR-Gal4* compound eye exhibits a recovery of bristles.

Figure 2

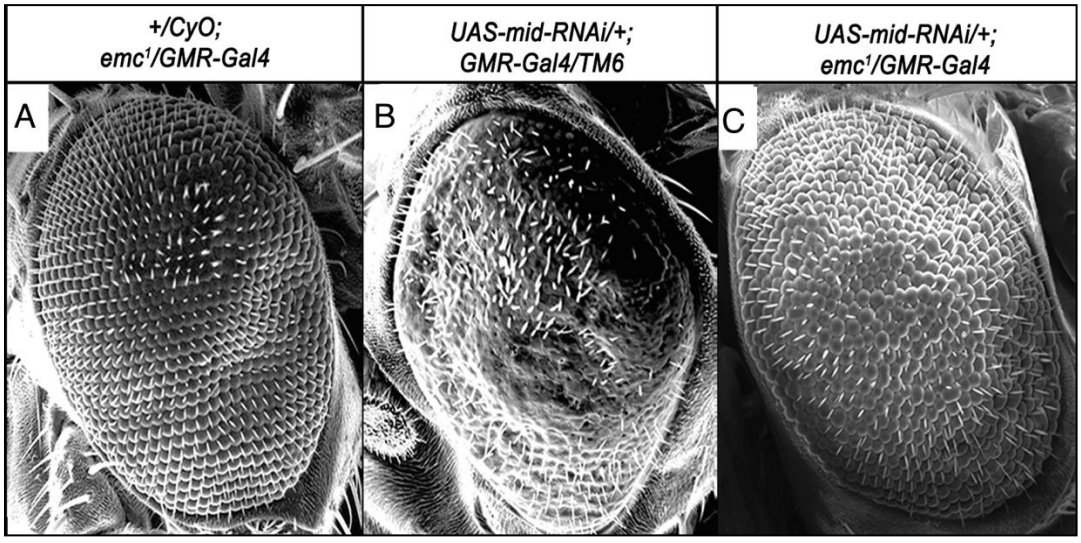
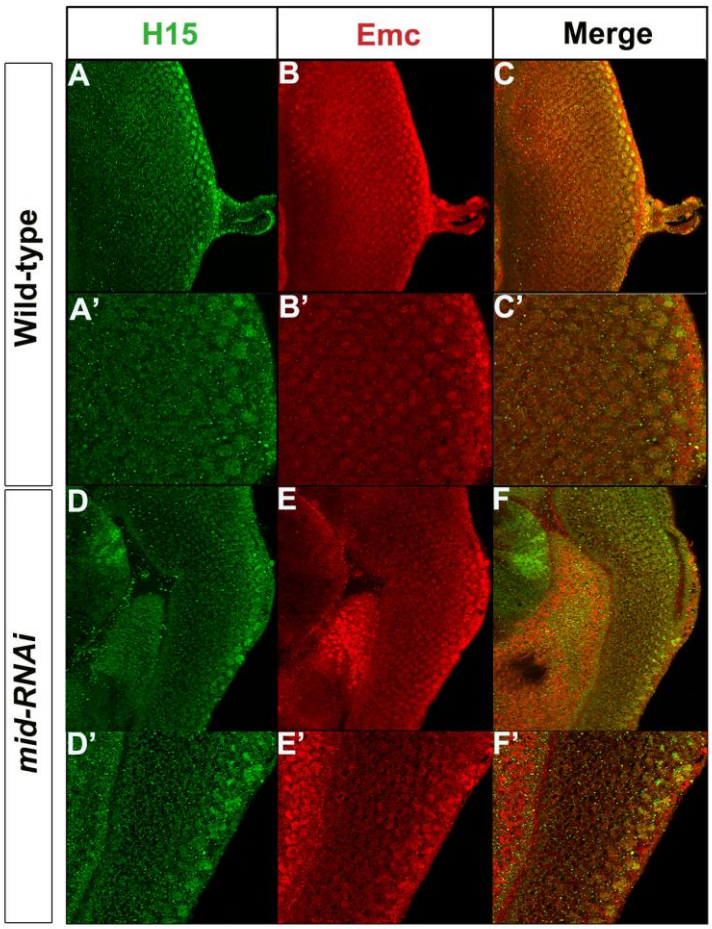


Figure 3



In summary, I am seeking to determine if the *mid-RNAi* mutant flies are blind. If they are blind, I will determine whether reducing *emc* expression in *mid-RNAi* flies recovers their vision. Since *mid* and *emc* seem to be collaborating to regulate eye development and vision, I will also assay for this interaction biochemically by performing Western analyses.

Chapter 2: Literature Review

Drosophila melanogaster

Drosophila is an important model organism in studying heritability and human diseases. Many of the genes within the *Drosophila* genome are homologous to the human genome. Understanding the *Drosophila* genome can give a better understanding of the developing central nervous system (CNS) and eye development in humans.

Drosophila melanogaster emerged as a model organism about 100 years ago and has since made strides in the field of genetics (Roberts, 2006). Reproducing and growing these organisms is very simple because of their ability to live on a simple diet of spoiled fruit, and they can easily survive in small vials. Their life cycle from embryo to adult is ten days, and the adults live for an average of four weeks. This total life span of three and half weeks allows for several genetic crosses to be completed in a short amount of time. The fruit fly genome has been sequenced and contains approximately 13,600 genes. The genome is contained in only four chromosomes (Adams MD et al. 2000). The small number of chromosomes allows for easy genetic manipulation and production of mutants for study. Humans have significantly more genetic information than fruit flies, but nearly all genes in the human genome have a homolog located within in the *Drosophila* genome (Twyman, 2002). According to Daniel St. Johnson, "197 of 287 known human disease genes have *Drosophila* homologs" (St. Johnson, 2002). This means that the function of mutant fly genes can be translated to the human genome to give us a better understanding of the mechanisms by which homologous genetic mutations work in humans.

extramacrochaetae and *midline* Genes

The *midline* (*mid*) gene is a transcription factor gene that is essential for the proper formation and development of the central nervous system (CNS). The *mid* gene regulates axonal projections that form synapses in the CNS. From previous studies done in the Leal lab at The University of Southern Mississippi, the reduction of *mid* expression in the fruit fly resulted in the loss of interommatidial bristles (Das et al 2013). However, when a reduction of expression of both *mid* and *emc* were combined, the formation of ommatidia was recovered and the eyes appeared almost normal. The phototactic ability of both the larvae and the adult mutant flies has yet to be determined.

The e*xtramacrochaetae* (*emc*) gene is currently being utilized in studies conducted on understanding CNS and wing development. However, only a few studies have been conducted to determine its role in *Drosophila* eye development. According to Bhattacharya and Baker of the Albert Einstein College of Medicine, *emc* mutants show that *emc* "is required for multiple aspects of eye development…including morphogenetic furrow progression, differentiation of R4, R7 and cone cell types, and rotation of ommatidial clusters" (Bhattacharya and Baker, 2009). The vertebrate homolog for *emc* is represented by Id, which encodes the Inhibitor of differentiation (Id) proteins while the vertebrate homolog for *mid* is *Tbx 20*.

Ommatidia and Eye Formation

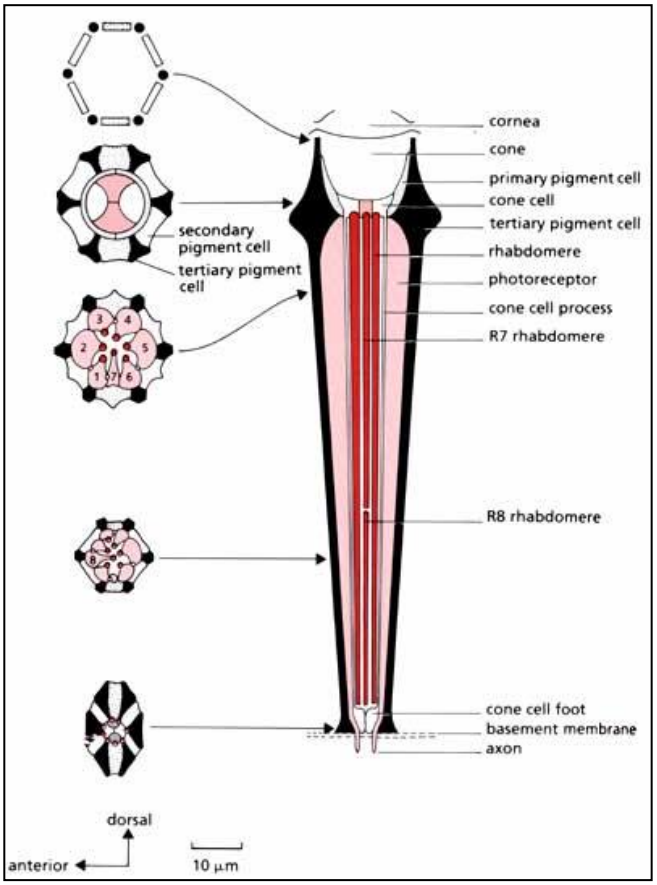
The construction of the compound eye in *Drosophila melanogaster* is a very complex and precise process. Eye development begins in the second-instar larval stage when the eye disc is no more than an undifferentiated sac of epithelial cells. During the third-instar larval stage, the morphogenetic furrow (MF), a wave of differentiation, advances from the

posterior of the disc to the anterior of the disc, and as the wave advances it transforms the eye disc into a highly organized field of ommatidia precursors (Ready et al., 1986). As the wave passes, photoreceptor neuronal cells are recruited to begin formation of the eye subunits called ommatidia. Eight types of photoreceptors make up one ommatidium.

The first photoreceptor to differentiate from the other epithelial cells in the eye-antennal disc is R8, which is followed by the other seven types of photoreceptors (R1-R7). According to Richard Carthew: "Each R8 neuron recruits one cell of each type, such that seven photoreceptors cluster around each R8 neuron." Each cluster of photoreceptors then recruits four non-neuronal cells to differentiate into cone cells, which are responsible for secreting the lens of each ommatidium (Carthew, 2007). Finally, primary (1˚), secondary (2˚), and tertiary (3˚) pigment cells surround the photoreceptor neurons.

The eye also contains many interommatidial bristles that aid in relaying sensory information to the brain. They are derived from a single sensory organ precursor cell (SOP). Eye epithelial disc SOPs are formed through a series of asymmetric divisions. The first division of the SOP produces the IIa and IIb daughter cells. The IIa cell divides to give rise to the socket and shaft cell, while the IIb cell gives rise to the IIIb daughter cell and terminal glial cell. The IIIb cell divides asymmetrically to produce the sheath and sensory neuron. Figure 3 illustrates the different aspects and components of a single ommatidium of the compound eye (Ready et al., 1986).

Figure 4



http://scotimages.me.uk/2011/11/03/seeing-inside-the-ommatidia-of-a-flys-eye/

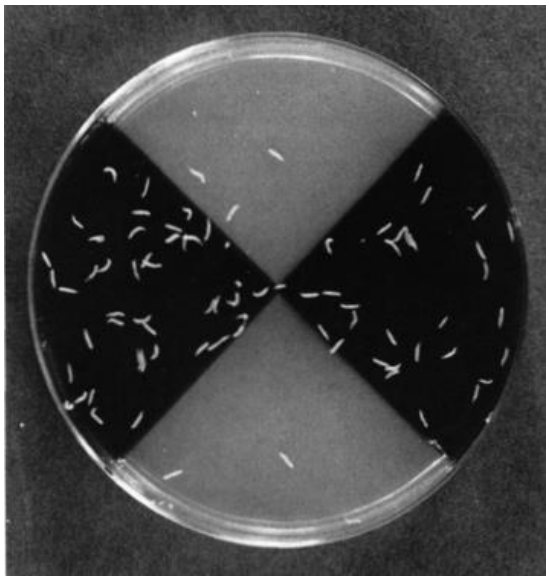
Chapter 3: Methodology

Larval Phototaxis

A Petri dish divided into four quadrants was used to create the surface for the larval phototaxis assay. Two opposite quadrants were lined with black construction paper and two quadrants were left blank. All four quadrants were filled with 1% clear agarose, enough to cover the walls of the quadrants to create a smooth surface. The Petri dishes were placed on a light box to create lighted and dark quadrants. 10 larvae were placed on the plate per each trial of the assay and given 5 minutes to migrate between light and dark quadrants (Connolly

and Tully, 1998). The larvae were cultivated from a population cage of specific genetic lines. The eggs were aged up to 4 days, when the third-instar larvae are developed. Third-instar larvae were subjected to the phototaxis assay with an appropriate response index calculated by subtracting the number of larvae in the light quadrants from the number of larvae in the dark quadrant and dividing that number by the total number of organisms on the plate.

Figure 5



Adult Phototaxis Assay

Adult flies doubly mutant for *mid*^{GA174} and *emc*^{1} exhibited a partial recovery of normal cell morphology in the compound eye. As such, I was interested in determining whether their sight was recovered. The ability of *Drosophila* larva to detect light was determined by a simple phototaxis assay. A petri dish was divided into four quadrants that were colored black and white. It is known that if the larvae have a sense of photoreception, the fly larvae will avoid light. Different strains of *Drosophila* adults were also subjected to a simple phototaxis assay. The flies were introduced to a T-tube apparatus where one branch

was lighted and the other branch was darkened. Wild-type *Drosophila* adults that are equipped with adequate photoreception are attracted to the lighted branch or exhibit positive phototaxis.

The adult phototaxis assay was completed using a slow phototaxis technique. An original apparatus, shown in Figure 6, was developed to determine the photoreceptive ability of multiple genetic strains. The apparatus consisted of a light and dark branch with the flies being introduced conjoined area of the two branches. The apparatus was placed next to a light to regulate thermal and photo levels. Adult *Drosophila* were given 3 minutes to migrate to their preferred area. A collection vial was placed at the end of each branch to collect individuals in the apparatus. Flies were introduced to the apparatus individually to eliminate behavioral overlap.

Figure 6

1.

Immunofluorescence

Larval Dissection

The dissection of *Drosophila* larvae was performed in Tri-PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.2% Triton X-100, pH 7.5) on ice. I placed a small puddle of PBS onto the center of the dissection plate. I then placed the larvae on the droplet of PBS and under the microscope; I used thicker-tipped forceps to grab the base of

the mouthparts. I grabbed about one-quarter of the way down the body from the head with the forceps. I gently pulled the forceps longitudinally in opposite directions. Ideally, the cuticle will break at the base of the mouthparts or neck and the internal organs will spill out. I located the central nervous system (CNS) and using fine-tipped forceps, I removed the tissues around the CNS. The brain and attached eye discs were then removed carefully. Since the brain is an accessory part of this experiment, it was detached from the eye discs but its removal is not necessary.

Antibody staining was completed after the removal of the PBS from the dissecting plate. To fix the eye imaginal discs, 270μL of PAXD and 30μL of formaldehyde were placed in each well of the dissecting plate. The discs were rinsed for 30 minutes in this solution on a shaker. The PAXD/formaldehyde mixture was drained from the wells and replaced with PAXD. The discs were rinsed 3 times with PAXD for 10 minutes each. After the PAXD rinses, the discs were rinsed with PTX 3 times for 10 minutes each. The discs were given a final rinse with PTX 3 times for 5 minutes each. The PTX was drained and a solution of 100μL of 1% goat serum and 300μL of PBT was added to each well. The plate was incubated in 4˚C overnight.

After overnight incubation, the eye discs were incubated with a cocktail of primary antibodies containing H15, Emc, CI, all with a concentration of 1:2000, for 4 hours at 25˚C. The discs were rinsed with PTX 3 times for 10 minutes each. They were then rinsed with 3 washes of PBT for 10 minutes each. The discs were incubated with secondary antibodies for 1 hour each at 25˚C. The secondary antibodies used were anti-rabbit Emc 488, anti-guinea pig 594, and anti-mouse 405 all with a concentration of 1:4000. After secondary antibody incubation, the discs were rinsed 3 times with PTX for 10 minutes each and then rinsed with

PBT 3 times for 10 minutes each. The discs were mounted to microscope slides using DAPCO and pictures were taken with a confocal microscope.

Adult Eye Dissection

To collect the eyes from the adult *Drosophila*, the flies must be frozen. The flies were collected in a container and placed on dry ice. Ethanol was poured into the dry ice container to flash-freeze the flies. The sieve that was used to separate the heads from the bodies was placed on dry ice. Once the flies were frozen, they were placed on a 0.0278-inch metal sieve and sifted through the mesh screen with a frozen paintbrush. The fly heads were collected on a 0.01 inch sieve that was also sitting on dry ice. The heads were collected, labeled and placed in the -70˚C freezer for storage.

Western Blot for Protein Interaction

Western Blot Technique

There were two gels involved in making the cassette for electrophoresis in the Western blot. The cassette was composed of two pieces of glass that were taped at the bottom. The first gel was the separating gel and Table 1 shows the different gel solutions that were prepared.

Table 1: Separating Gel (7 mL)

Stock Solution	8%	10%	12%	15%
40% Acrylamide	1.4 mL	1.75 mL	2.1 mL	2.625 mL
1.5 M Tris-HCl, pH 8.8	1.75 mL	1.75 mL	1.75 mL	1.75 mL
Water	3.7 mL	3.4 mL	3.0 mL	2.5 mL
10% SDS	70 µL	70 µL	70 µL	70 µL
10% APS	70 µL	70 µL	70 µL	70 µL
TEMED	4.7 µL	4.7 µL	4.7 µL	4.7 µL

The separating gel was added to the cassettes and a layer of isopropanol was added to the gel. The cassette was then allowed to polymerize for 30 minutes. The second gel, the stacking gel, was prepared with the proportions in Table 2.

Table 2: Stacking Gel

Stock Solution	4%
40% Acrylamide	0.20 mL
1.5 M Tris-HCl, pH 8.8	0.25 mL
Water	1.5 mL
10% SDS	20 µL
10% APS	20 µL
TEMED	2 µL

The isopropanol layer was poured off and the excess was removed with a piece of Whatman paper. 1mL of the stacking gel solution was added to each cassette and the comb was inserted, avoiding any air bubbles. The gels were then allowed to polymerize for 20 minutes. The tape was removed from the bottom of the gels and the comb was removed from the top. The gels were then inserted into the running box. The running buffer was made by combining 15.1g of Tris, 72.0 g Glycine, 5.0 g SDS and filled up to 1L with water. The 5X running buffer was then diluted to 1X and poured into the gel box.

The samples were then placed in the heat block at 100˚C for 5 minutes and spun at top speed for 5 minutes. The wells in the gel were loaded with the sample and allowed to settle. The gels were electrophoresed at 100 volts through the stacking gel and 150 volts through the separating gel.

A transfer buffer was prepared by mixing 2.4g of Tris, 11.3g of glycine, 200mL of 10% methanol and filled up to 1L with water. Ice-cold transfer buffer is used if doing a same-day transfer. The PVDF membrane was soaked in 100% methanol for 30 seconds and the membrane was transferred to the transfer buffer. The membrane was allowed to soak for

a few minutes before it became part of the "sandwich." Whatman paper and fiber pads were also soaked in the transfer buffer. In the larger side of the transfer holder, all materials were placed in this order: 2 fiber pads, Whatman paper, inverted gel, a nitrocellulose membrane, Whatman paper, and 2-3 more fiber pads. After adding the second Whatman paper, air bubbles were removed by rolling them out with a 5mL pipette. The "sandwich" was then inserted into the gel box and the transfer buffer was added to the chamber. It was transferred at 30 volts for 2 hours on the bench.

It was necessary to create a block so that the antibodies tagged only specific proteins. To create the block, TBST was made by diluting 10X TBS (100mL 1M Tris-Cl with a pH of 7.5, 88g NaCl, and filled to 1L with water) to 1X and adding Tween to 0.1% (1mL Tween in 1L of 1X TBS). 5% w/v Nonfat Dry Milk (NFDM) in TBST was made. 20mL of this solution was sufficient for one gel. The transfer apparatus was disassembled and the nitrocellulose membrane was moved to a pipette tip box that contains TBST. It was important to keep the membrane from drying out. The membrane was rinsed briefly and the TBST was discarded.

For the primary antibody incubation, 1% w/v NFDM in TBST was made. The antibody was diluted to the proper level in 10mL of 1% NFDM in TBST. This solution was incubated on a shaker at 50RPM for 1 hour.

A wash was performed, and the antibody solution was recycled. Sodium azide was added to the solution until it made up 0.05% of the solution and was stored in a cold room. The membranes were rinsed twice and then rinsed 3 times for 5 minutes each with TBST.

For the secondary antibody incubation, the antibody was diluted to the proper level in 10mL of TBST. This membrane was incubated on a shaker at 50RPM for 1 hour. The

secondary antibody solution was discarded, and the membrane was rinsed twice followed by 3 washes of 5 minutes each using TBST.

Finally the reagents from an Amersham ECL kit (1mL of Solution A and 25µL of Solution B) were mixed. 2mL of solution was sufficient for 1 gel/membrane. The membrane was drained (without letting it dry) and placed (protein side up) on a flat piece of cling film. ECL solution was pipetted directly onto the membrane. The membrane was incubated for 5 minutes and the solution was drained. The membrane was wrapped in cling film and taped into the exposure cassette. The membrane was then exposed to the film for the appropriate amount of time and was developed.

Chapter 4: Results

Larval Phototaxis Assay

Wild-type and *mid-RNAi* larvae were reared on an apple juice/agar solution that was plated in a 60mm Petri dish. Larvae were allowed to develop to the third-instar stage when eye discs are fully developed.

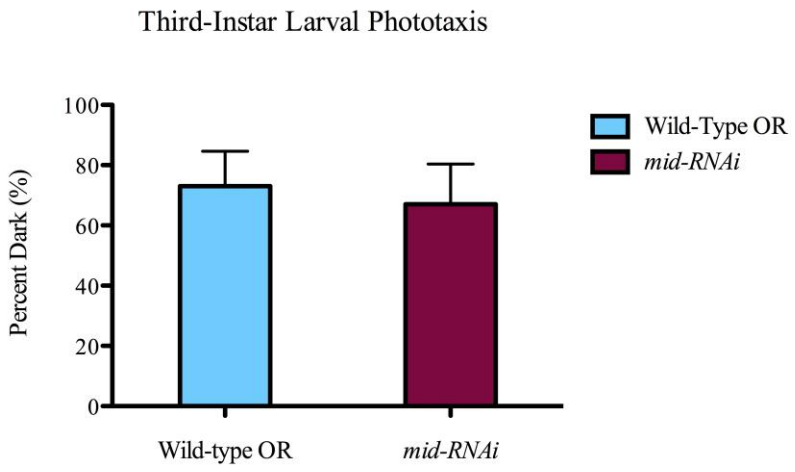


Figure 7: The bar graph depicts negative phototaxis. Approximately 74 +/- 10 of WT larvae migrated to the black quadrants (purple bar) while only 66 +/- of *mid-RNAi* larvae migrated to no black quadrants.

Figure 7: Wild-type and *mid-RNAi* larvae are negatively phototaxic. Ten third-instar larvae were placed in the center of an 87mm Petri dish containing 1% agarose and were allowed to migrate for 5 minutes. The Petri dish with two dark quadrants and two light quadrants was placed over an illuminated transparent box. The response index was obtained from 10 independent trials of each condition. Response indexes were calculated using the following formula: (organisms in dark - organisms in light) / (total number of organisms in the trial).

Wild-type larvae were used a control to compare the phototaxic response of *mid* mutant larvae. Wild-type larvae exhibited a greater negative phototaxic response when compared to the *mid* larvae. The *mid* larvae exhibited a decreased negative phototaxic response. The phototaxic response difference between wild-type and *mid* larvae was not significant enough to determine if phototaxic response differed in the third-instar stage.

Adult Phototaxis Assay

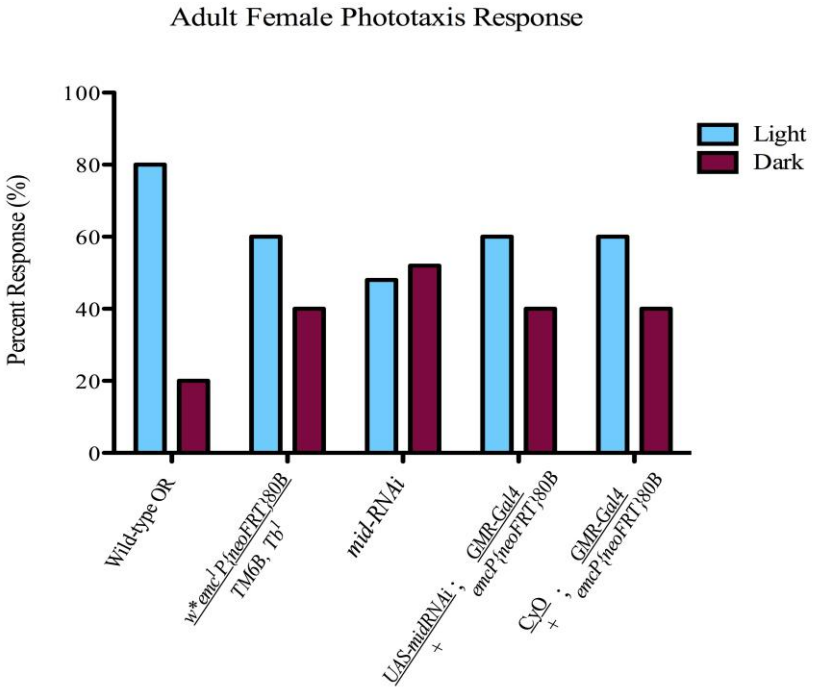


Figure 8: The bar graph depicts adult 4-day old female phototaxic responses. While WT flies exhibited normal phototaxis, all mutant flies were defective in navigating toward the light.

Figure 8: Wild-type adults are positively phototaxic. Flies were introduced to the phototaxis apparatus individually, allowed 3 minutes to respond, and their response recorded. The data were obtained from 25 independent trials for each genotype. The response index was created using the following formula: (number of organisms in light) / (total number of organisms per genotype in the study).

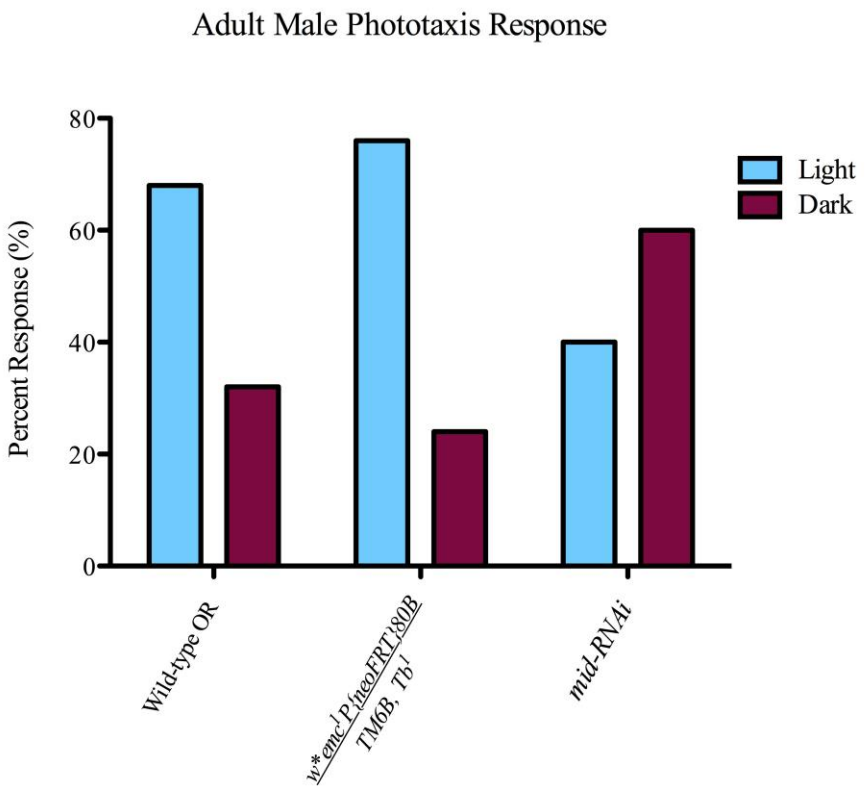


Figure 9: The bar graph depicts adult 4-day old male phototaxic responses. While WT flies exhibited normal phototaxis, all mutant flies were defective in navigating toward the light.

Figure 9: Wild-type flies are positively phototaxic. The data were obtained from 25 individual trials for each genotype. Phototaxis assay was not completed male flies doubly mutant for mid^{GA174} and emc^{1}.

Wild-type, emc^{1}, *mid-RNAi* and emc^{1}; mid^{GA174} adult flies were collected shortly after hatching and allowed to age for four days. They were aged to four days to allow a sufficient amount of time for receptors and ommatidial formation to be complete. Wild-type adult flies

were used as a control for the phototaxis assay to compare photoreceptive responses of other genotypes. The *emc*^{1} adult flies exhibited a 50% decrease of expression of *emc* in the eye. Therefore they were used as a control for the function of *emc* within the eye.

Male and female adult flies were subjected to the phototaxis assay separately to determine the difference of photoreception ability between sexes. Female *emc*^{1} adult flies exhibited decreased photoreceptive ability when compared to male *emc*^{1} adult flies. The *mid-RNAi* flies showed the greatest decrease in photoreceptive ability between all trials and both sexes. Both male and female *mid* adult flies showed a significant increase in phototaxic ability, creating a more random data assortment for that genotype.

Only female adult flies that are doubly mutant for *mid*^{GA174} and *emc*^{1} were subjected to the adult phototaxis assay. The *emc*^{1} and *mid*^{GA174} doubly mutant flies exhibited a decrease in photoreceptive ability. Their photoreceptive response paralleled that of *emc*^{1} adult flies, suggesting that the *emc* gene plays a significant role in photoreceptive ability. These results also suggest that the *mid*^{GA174} and *emc*^{1} doubly mutant adult flies cannot see as well as the wild-type adult flies.

Western Blot

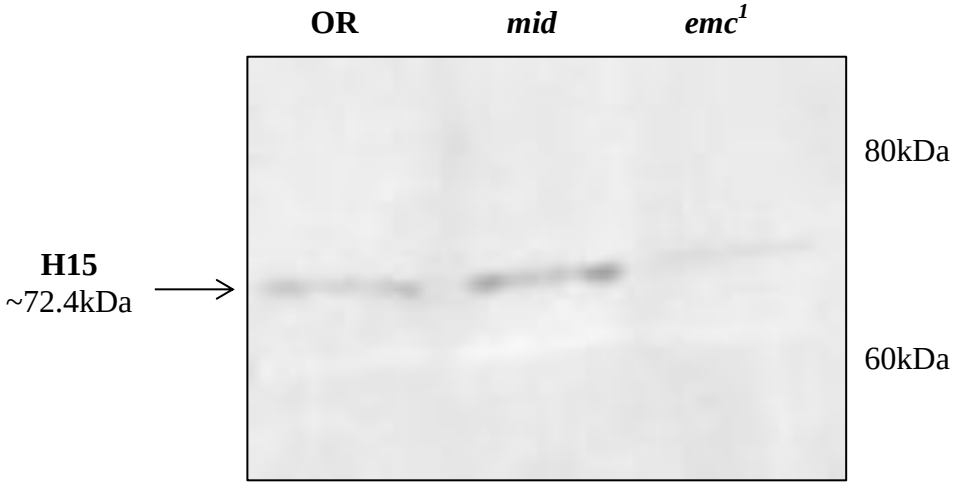


Figure 10: Protein levels of H15. The H15 protein is expressed in the tissues of developing larvae. Wild-type OR served as the control for H15 expression levels. *mid-RNAi* larvae expressed a greater amount of H15 protein than wild-type larvae and *emc*^{1} mutants expressed a decreased amount of H15 protein compared to wild-type larvae.

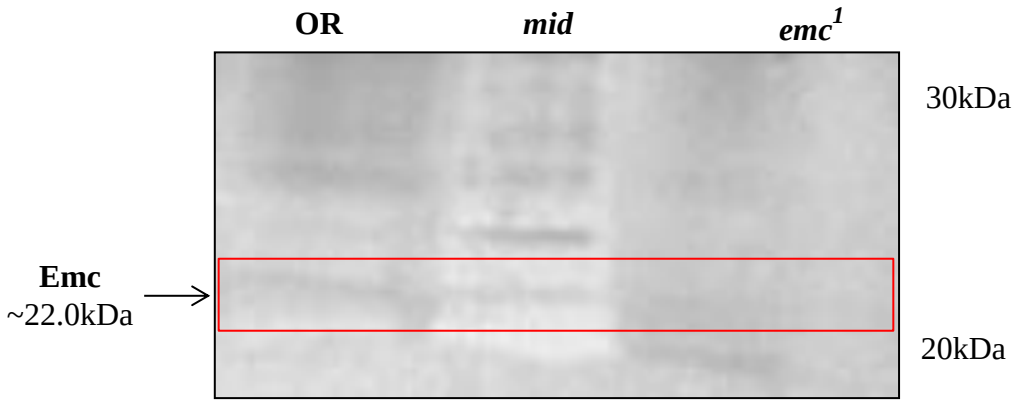


Figure 11: Protein levels of Emc. The Emc protein is expressed in the tissues of developing larvae. Wild-type OR served as the control for Emc expression levels. *mid-RNAi* larvae expressed a similar amount of Emc protein compared to wild-type larvae. The *emc*^{1} larvae expressed no Emc proteins.

The H15 protein was used as a paralog to stain for Mid proteins, meaning H15 was used to stain for the level of proteins produced by the *midline* gene in wild-type OR, *mid* mutant, and *emc*^{1} mutant larvae. Wild-type OR larvae were used as a control to stain for normal levels of H15 in developing larval tissues as seen in Figure 9. The *mid* mutant larvae were determined to have a higher concentration of H15 proteins in developing tissues. The *emc*^{1} mutants expressed the least amount of H15 protein in their developing tissues.

The Emc protein was used to stain for proteins produced by the *emc* gene in developing tissues of third-instar wild-type OR, *mid* mutant, and *emc*^{1} mutant larvae. The wild-type larvae served as a control for the levels of Emc protein expressed in developing tissues. The *mid* mutant larvae expressed a similar concentration of Emc protein while the *emc*^{1} mutants expressed no level of Emc protein in the developing tissues.

Immunofluorescence

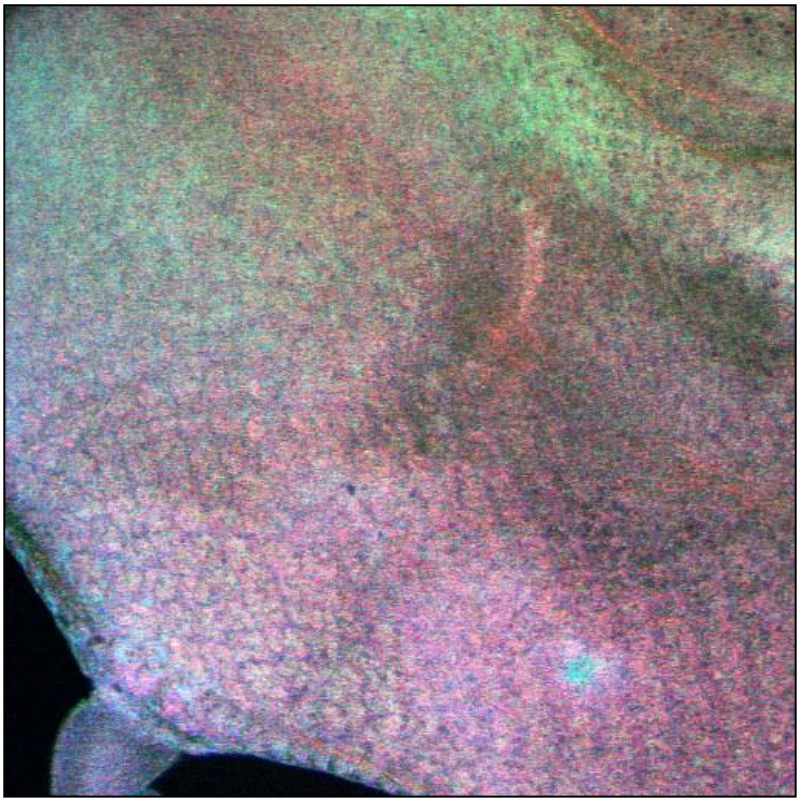


Figure 12: Photoreceptor cells in the eye imaginal disc were stained with H15 and Emc antibodies. Photoreceptor cells that contain *mid* are designated by the color red. Cells that contain *emc* are designated by the color green. The color yellow designates photoreceptor cells that contain *mid* and *emc*.

The immunofluorescence assay shows the interaction of *mid* and *emc* in the eye imaginal disc of OR third-instar larvae. The interaction of *mid* and *emc* is apparent and localized to the posterior of the morphogenetic furrow.

Chapter 5: Discussion

The larval phototaxis assay revealed a decreased phototaxic response in the *mid* mutant larvae. It is understood that *Drosophila* larvae are negatively phototaxic. However, the larval phototaxis assay is not conclusive because the eyes are still developing. In third-

instar larvae, the photoreceptors have not yet fully developed. Therefore, using larval phototaxis is meant to be qualitative rather than qualitative.

In the adult phototaxis assays, there was a more significant difference between genotypes according to the response index. Adult *Drosophila* are positively phototaxic. OR adult flies served as a control for the adult phototaxis assay. The emc^{1} mutant adult flies exhibited a similar response to the OR flies. The *mid* adult flies showed a significant decrease in phototaxic ability. The adult flies doubly mutant for mid^{GA174} and emc^{1} exhibited a phototaxic response similar to the mid^{GA174} single homozygous mutants. This difference in phototaxic response can be the result of a number of factors.

As seen in Figure 2, ommatidial and bristle formation was recovered in the flies doubly mutant for mid^{GA174} and emc^{1}. Therefore, it was assumed that photoreceptive ability would also be recovered. However, photoreceptive ability was only partially recovered. This suggests that receptor formation inside the ommatidia was incomplete or misconstrued. Transmission electron microscopy (TEM) will be used to inspect the interior of the compound eyes from flies doubly mutant for emc^{1} and mid^{GA174} to determine if photoreceptor structure is damaged resulting in reduced phototaxic function. A retinogram can also be used to measure the action potentials within the neurons of the eye (Zhu, 2013).

There was also an observed difference in photoreceptive ability between males and females of $w^{*}emc^{1}P\{neoFRT\}80B/TM6B, Tb^{1}$ genotype. It is suspected that *emc* is a sex-linked gene, meaning it will have different effects in males and females. In a study performed at Brandeis University, researchers examined the circuitry of male and female *Drosophila* brains and their response to light in terms of sleep and arousal (Shang et al., 2008). It could

also be true that *emc* plays a role in the formation of brain circuitry and that circuitry formation differs between males and females.

This study also suggests that *emc* is regulated by *mid*. When the expression of *emc* and *mid* were both reduced, ommatidial formation, pigmentation, and bristle complexes were recovered. This result would suggest that *mid* is necessary for the regulation of *emc* expression. The *mid* gene may even repress the expression of *emc* in wild-type flies, keeping ommatidial formation, pigmentation, and bristle complex formation normal.

Wild-type OR third-instar larvae were used as a control for the Western blot analysis. The Western blot revealed a decreased concentration of H15 protein in emc^{1}mutants when compared to the concentration of H15 protein in *mid* mutants. The *mid* mutants expressed a higher concentration of H15 protein when compared to the wild-type larvae. The lower concentration of H15 in emc^{1} mutants suggests that *midline* expression is being regulated by *emc*, or rather, *emc* and *mid* regulate each other through unknown pathways.

In the Western analysis of the Emc protein, the wild-type OR larvae also served as a control. The *mid* mutant flies expressed a similar concentration of Emc protein compared to the OR larvae suggesting that *emc* is present in the developing tissues of the third-instar larvae. The emc^{1} mutants expressed little to no Emc protein in the developing tissues. This observation is appropriate because the expression of *emc* in the emc^{1} mutants has been reduced.

The immunofluorescence assay revealed an interaction of *mid* and *emc* in a localized area of the posterior eye imaginal disc. It is known that *emc* is an important regulator for the differentiation of photoreceptors 8, 7, 4 and 3 (Baker and Bhattacharya, 2009). However, the

function of *mid* in the posterior of the eye disc is unknown but may play a role in the recovery of sight. The interaction of *emc* and *mid* is unknown but this lab does hypothesize that *mid* regulates photoreceptor formation. However, it is still not understood why *emc* is present in photoreceptors that have developed because it has only previously been known in be found in front of the MF. Why and how *emc* is interacting with *mid* after the MF is still in question.

Chapter 6: Conclusion

The aim of this study was to determine if the genes *emc* and *mid* were interacting with each other in the developing eyes of *Drosophila melanogaster* and if photoreception of the doubly mutant flies for emc^{1} and mid^{GA174} was recovered. The larval phototaxis revealed only a slight difference in photoreceptive ability between wild-type OR and *mid* mutants which suggests that photoreception may not have been lost in the third-instar stage, but may have been lost during the pupal or developing adult stages.

The adult phototaxis assay revealed a dramatic decrease in photoreceptive ability in the *mid* mutants. Photoreceptive ability was slightly recovered in the doubly mutant mid^{GA174} and emc^{1} adult flies suggesting there is non-superficial damage to the photoreceptor formation that cannot be seen with a light microscope. Statistical analysis of the data from the phototaxis study showed no significant increase in photoreceptive ability of the doubly mutant mid^{GA174} and emc^{1} flies. However, this lack of significance could be due to a small sample size and therefore, a greater sample size is required to determine true significance. Further testing is also required to understand the structural defects caused by the interaction of *emc* and *mid* in developing eye tissues.

The Western blot and immunofluorescence assays supported the interaction of *emc* and *mid* in developing eye imaginal discs. It was curious that *emc* expression was diminished in the tissue homogenates from tissue homogenates prepared from *mid* mutant flies. This observation suggests *mid* is required for Emc expression. The extent and purpose of a Mid and Emc interaction has yet to be determined and the lab is excited to continue working toward finding explanations for these interactions. In addition, the lab will also perform transmission electron microscopy to determine why photoreceptive ability was not recovered in the emc^{1} and mid^{GA174} doubly mutant flies although on the outside surface, they appeared quite normal. It is that either the photoreceptors have failed to differentiate or the axons that reach the optic lobe of the brain are damaged.

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