

**DEVELOPMENT OF DIGITAL TECHNIQUES FOR ANALYSIS OF
HORMONE REGULATION OF MELANOPHORE ACTIVITY IN
RED DRUM**

A Senior Scholars Thesis

by

WILLIAM ANDREW RUSSEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Zoology

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Approved by:

Research Advisor:
Associate Dean for Undergraduate Research:

Duncan MacKenzie
Robert C. Webb

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ABSTRACT

Development of Digital Techniques for Analysis of Hormone Regulation of Melanophore Activity in Red Drum
(April 2008)

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Melanophores are pigment bearing cells, which have been used as a practical method to demonstrate mechanisms of hormone interaction. A current teaching laboratory has been restructured in order to increase student participation, provide tangible results, and reduce subjectivity in procedures to more accurately analyze hormone interaction. To accomplish this, digital cameras were used to photograph scales of red drum (*Sciaenops ocellatus*), which were then analyzed using ImageJ software to determine melanosome area. From collected data, a region posterior to the pelvic fin and dorsal to the lateral line on the red drum was determined to yield stable basal melanophores to provide a standard starting state for student experiments. By measuring the area of basal melanophores, one can compare dispersed melanophore area after hormone treatment (α -MSH and norepinephrine) to quantify a hormone's effect. Hormone interaction can be illustrated by subjecting scales to different hormones in series. Other chemicals such as IBMX and a K^+ rich medium were also used to demonstrate melanophore regulation. Experiments showed that basal state of melanophores could not be manipulated by changing

illumination of environment or by removing scales from red drum at different times of day. Physiological doses of hormones to be used in the laboratory were determined by testing various doses' effect on melanophore activity over time. Once physiological doses were established, a sequence of serial chemical treatments was developed to best demonstrate melanophore activity by examining dispersing and aggregating effects in the presence of other chemicals. This method of melanophore analysis has proven useful as a teaching tool, but may also be used to analyze novel treatments on melanophore activity.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Duncan MacKenzie for giving me the opportunity to pursue my own research project under his guidance. I would also like to thank the other members of the lab, Dr. Bill Kohn, Thomas Miller, and Richard Jones.

I would also like to thank the National Institute of Health for providing their ImageJ software free to the public. Without their product I would not have been able to develop the laboratory as a digital analysis laboratory. Thanks to the Texas A&M BioAquatics staff who helped maintain our red drum.

Finally, thanks to my family for their unconditional love and support in all I do.

NOMENCLATURE

MSH	α -melanocyte stimulating hormone
IBMX	3-Isobutyl-1-methyl-xanthine
MS-222	Tricaine methanesulfonate
M	Molarity
VA	Ventral anterior location of red drum
VP	Ventral posterior location of red drum
DA	Dorsal anterior location of red drum
DP	Dorsal posterior location of red drum

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CHAPTER I

INTRODUCTION: MELANOPHORES, HORMONES, AND DIGITAL ANALYSIS

Melanophores are pigment cells located in the dermis or epidermis of all vertebrates. Each melanophore contains numerous organelles called melanosomes which contain the pigment melanin. Melanin can be brown or black in color, and is the most common pigment found in the skin of fish (Evans, 1993). Many species of fish, amphibians and reptiles are able to regulate melanophore activity to undergo rapid color change. This is achieved by movement of melanosomes throughout the melanophores on actin filaments and microtubules (Dinh *et al.*, 2007). When aggregated, melanosomes are contained within a central reservoir, appearing as just a microscopic dot, making the melanophore appear light (Dinh *et al.*, 2007). When dispersed throughout the cell on microfilaments, the melanophore will appear dark. The contrast in appearance between aggregated melanophores and dispersed melanophores is what we perceive as a lightening or darkening of the skin. A high percentage of dispersed melanophores results in a dark body coloration. Melanophores are thus capable of causing dramatic changes in coloration, which is critical in vertebrates as a mode of crypsis, a signal to mates, or a warning to predators (Evans, 1993). Because of the importance of these behaviors, numerous studies have examined how melanophores are controlled.

This thesis follows the style of *General and Comparative Endocrinology*.

Melanophore regulation

The regulation of melanophore activity is complex, with both neural and hormonal control regulating the intracellular levels of cyclic 3-adenosine monophosphate (cAMP), a common intracellular second messenger. The autonomic nervous system activates the secretion of neurohormones, such as norepinephrine from chromaffin tissue, which are delivered by the blood to dermal or epidermal melanophores (Hadley, 2007).

Norepinephrine binds to G-protein coupled receptors which then activate an intracellular signal cascade, ultimately releasing calcium stores inside the melanophore (Dinh et al., 2007). Calcium then binds to calmodulin and activates phosphodiesterase which breaks down and thus reduces the intracellular concentration of cAMP. Decreased levels of intracellular cAMP result in melanosome aggregation (Evans, 1993). A separate pathway has been described through which melanophores are directly innervated by nerves of the sympathetic nervous system (Hadley, 2007). In this case, norepinephrine is stored in the nerve axon terminal, and is secreted after depolarization (Burton and Vokey, 2000). Norepinephrine secretion through either pathway is generally the result of stress to the animal and will result in a rapid paling of the individual as melanophores aggregate (Masazumi, 2002).

Another route through which color change is regulated is via the pituitary gland.

Endocrine regulation of melanophores occurs primarily through the pituitary hormone melanocyte-stimulating hormone (MSH) (Hadley, 2007). MSH is secreted from the pituitary gland into the systemic circulation to reach receptors on the surface of

melanophores (Hadley, 2007). When MSH binds to a G-protein coupled receptor on melanophore membranes it will activate adenylate cyclase to produce cAMP. Increased levels of intracellular cAMP result in melanosome dispersal. It is important to note that MSH and a second pituitary hormone melanin-concentrating hormone (MCH) have antagonistic effects on melanophores (Hadley, 2007). The endocrine pathway of melanophore regulation therefore does not only control melanosome dispersal, but aggregation as well.

Importance of melanophores

Observing melanophore response to hormones under a microscope is a practical method for demonstrating principles of endocrinology as a teaching laboratory. Because melanophore activity is regulated by multiple hormones, it is possible to analyze hormone interactions by administering hormones to melanophores *in vitro* and observing melanophore response. Melanophores can then provide a system to examine integrated endocrine control of cellular function. For example, melanophore activity can be used to determine how a hormone influences cAMP levels, or to examine the effects of multiple hormones with agonistic effects on cAMP production. Other methods to examine endocrine function, such as extirpation/replacement experiments or injection studies, are invasive, difficult to perform, and potentially injurious to the subject. In contrast, melanophores on scales may be easily manipulated and observed, with minimal handling of the fish itself. Several previous studies have utilized melanophores as a useful tool for demonstrating hormone interactions in fish (Burton and Vokey, 2000; Logan *et al.*,

2006; Zhu and Thomas, 1997). It is because the method is so widely used that it has been developed as a teaching laboratory for Biology 388 in the Department of Biology at Texas A&M University. The standard method requires a trained eye to examine and score individual melanophores on each scale from a value of one to five, with one being completely aggregated and five being completely dispersed. While this method is effective for an experienced scorer, it has practical limitations in an undergraduate teaching laboratory. Each student must be taught how to score a melanophore correctly, but it is unlikely that any two students would score all the melanophores the same. Since scoring melanophores correctly is essential to determining hormonal effects, errors in scoring could entirely mask a significant response. Therefore, the overall objective of this project is to develop a new analysis method which will eliminate the need to score melanophores, and thus scoring error, in order to better convey the concepts of hormone interaction to students.

Objectives

My first objective will be to develop a new digital analysis method that maintains an acceptable correlation to the existing scale scoring method. I will be using the fish species *Sciaenops ocellatus*, commonly known as red drum, as the subjects for the development of a digital analysis of melanophores. Red drum are readily available and have already been used in scale studies (Zhu and Thomas, 1997). This revised method will be less subjective and provide a better visual demonstration of hormone interaction.

I will develop the new digital analysis method using ImageJ, a free software program created by the National Institute of Health that provides methods of analysis for digital photographs. ImageJ should allow for faster analysis of fish scales in contrast to the standard scoring method, while also providing a measure of the total area of scale occupied by melanosomes. The area of melanosomes is a more practical measurement than scale scoring because melanophores are not uniform in size or shape and will not necessarily distribute melanosomes evenly.

Once this digital analysis system is established, the second objective of this project will be to characterize basal melanophore activity in red drum. Defining basal melanophore state is important for a teaching laboratory to ensure that each student will be able to begin the laboratory with a similar scale preparation. To accomplish this, I will test if anesthetic, time of day, or dark background have any effect on basal state or sensitivity of melanophores. Once the basal state is established, I will then use digital analysis to evaluate hormone effects.

The third objective is to determine whether physiological doses of hormones, neurotransmitters, and second messengers stimulate cells in a predictable way. First I will test for physiologically-active doses of hormones and chemicals that will effectively demonstrate endocrine activity. My initial focus will be on MSH and norepinephrine, since both have been extensively studied and have highly conserved function in melanophore regulation across color-adaptive vertebrates (Hadley, 2007). The chemical

IBMX is a phosphodiesterase inhibitor which has been found to be effective in melanophore dispersal because it prevents the breakdown of intracellular cAMP (Rozdzial and Haimo, 1986). I plan to use IBMX as a dispersing treatment and norepinephrine as an aggregation treatment for comparison when testing other hormones.

Once hormone effects are determined, I will use them to accomplish my final objective. I will take what I have learned and create a procedure for a teaching laboratory to be used to analyze hormone interactions via *in vitro* evaluation of endocrine effects on melanophore activity. This laboratory will provide a non-invasive and relatively simple procedure to demonstrate hormone action in a hands-on teaching laboratory.

CHAPTER II

METHODOLOGY

Obtaining scales

Red drum were obtained from Texas Parks and Wildlife hatcheries and kept in a 2,000 gallon recirculating artificial seawater system at 25 °C on a 12 hr light, 12 hr dark photoperiod cycle. The fish were stocked into large 200 gallon tanks or 1000 gallon raceways beneath them. Both groups were fed a commercial diet daily. For each experiment, the red drum were caught with nets and transferred to a five gallon bucket filled with the same tank water. For some experiments, fish were anesthetized with 0.8g Finquel brand tricaine methane sulfonate (MS-222) added to the bucket. Due to the large number of scales required for some experiments, the red drum occasionally had to be humanely killed by an overdose of MS-222. Red drum were placed on a moist foam pad for scale removal. Six to eight scales were removed from each fish. The least destructive method of removing scales was to approach the scale, using forceps, from the posterior end at an angle towards either dorsal or ventral side. The purpose of angling the forceps was to avoid scraping or otherwise damaging the melanophores present on the surface of the scale. After removal, scales were placed into labeled plastic cups containing a physiological saline (Ringer's solution) for 30 min.

Digital image capture and analysis

The camera used in these experiments was a Scopetek DCM200 attached to a Nikon binocular dissecting microscope. To prepare a scale for photography, it was moved with forceps from the Ringer's solution to a plastic 24-well plate lid. The lid was then positioned underneath the microscope lens so that the scale occupied the center of the field. Two fiber optic lights were used to increase contrast between the melanophores and white background. Using fiber optic lights prevented heating the scale and medium. The scales are viewed at a magnification of 12X because higher magnifications began to crop out melanophores on large scales. Five drops of Ringer's Solution or treatment were then added to the scale resting on the lid. The scale was then ready to be photographed using the Scopetek Scopephoto program. Scopephoto has several functions I have used to help streamline the data collection process, as well as provide more informative pictures. Photographs were captured both manually and automatically. Manual photography allowed for pictures of basal melanophore state either before treatment or immediately following. Automatic photograph capture is used to take photographs at preset time intervals. With three cameras running, it was possible to photograph up to three scales simultaneously. This method allows for more precise analysis of changes in scales over time by eliminating user error (early, late, or missed photograph) at each time point.

Acquiring suitable contrast was the primary problem in scale photographic analysis. In order to improve contrast, I set the cameras to capture photographs in black and white.

ImageJ requires pictures to be in an 8-bit format in order to analyze melanophore areas. Setting the threshold of an image is the crucial first step to this process. The threshold is set by moving two sliders up and down on a gray scale range of 1 to 255. Any shades of gray between the threshold sliders appeared red. Upon clicking OK, the image is converted into a pure black and white image. The threshold region was analyzed as melanosome area. Capturing the photo in black and white allowed me to determine exactly what kind of contrast melanophores will have when analyzing. To achieve this properly, it was necessary to find a suitable vessel to hold the scales. Problems such as shadows and light artifacts encountered with traditional multi-well plates were minimized by placing scales in five drops of medium on the lids of 24-well plates. Well plate lids have low rings organized in the same configuration as their wells, providing minimal wall interference to affect lighting of the scale and contrast of melanophores.

Once scales were removed and photographs were taken, the area of the scales occupied by melanophores was calculated using ImageJ. After opening a photo in ImageJ, I outlined the desired region on the scale using the polygon tool, excluding light artifacts and epithelial tissue. Once the area had been selected, the area outside the selection was cleared. This melanophore region of the scale was converted into an 8-bit image for analysis. The next step was to set the threshold on an image, telling the program what to classify as melanosome area. The difficulty of setting a threshold varied with lighting and scale. Larger scales were more likely to experience slight lighting differences due to bending of light in solution. Finally, the threshold area was analyzed to give the area in

pixels. Pixel size was set at five to infinity to allow for small aggregated melanophores to be included while eliminating noise encountered during thresholding. This area is indicative of the total melanosome area occupied on the scale. I was interested in the area per melanophore because the total area of melanosomes on a scale may be low due to aggregated melanophores, or due to the scale having too few melanophores.

Measuring the melanosome area per melanophore gave a more accurate representation of melanophore activity. Previously, scales had typically been analyzed by scoring the state of the melanophore on a 1 to 5 scale, which gave no indication of the size of melanophores (Burton and Vokey, 2000; Zhu and Thomas, 1997). Size is important for melanophore analysis because a large dispersed melanophore will cover a much broader area. Analyzing the area these melanosomes occupy better demonstrates of the overall effect these cells have on body pigmentation.

Experiments

Establishing basal state of melanophores

I first conducted several experiments to determine how variables such as body location, time of day, anesthetic exposure, and illumination would affect the basal state of scales. An understanding of these variables' effect on basal melanophore states could be used to increase consistency in melanophores for use in the teaching lab. A consistent basal state would also be helpful for future experiments if the basal state could be manipulated. For example, if trying to test the effect of a melanophore dispersing agent, it would be beneficial to use scales in a more aggregated state.

Location in red drum

To determine if melanophore density or stage varied by location on the red drum, I divided the fish up into four quadrants using the tip of the pectoral fin and the lateral line as landmarks: ventral anterior, ventral posterior, dorsal anterior, dorsal posterior (Fig. 1.). Six red drum were used for this experiment to determine if differences in melanophore distribution or basal activity varied with body size.

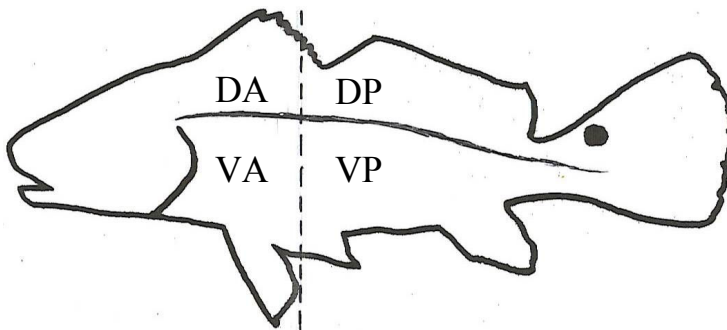


FIG. 1. 4 Quadrants of the red drum. VA: Ventral to lateral line and anterior to pectoral fin. VP: Ventral to lateral line and posterior to pectoral fin. DA: Dorsal to lateral line and anterior to pectoral fin. DP: Dorsal to lateral line and posterior to pectoral fin.

These six fish were divided into three groups; small (2 fish, mean weight 11.5g), medium (2 fish, mean weight 32.8 g), and large (2 fish, mean weight 63.2 g). For each fish, ten to twenty scales were removed from each quadrant and placed in Ringers Solution. Each scale was then photographed, and a random sample of 15 per quadrant was analyzed via ImageJ.

24 Hour sampling

The objective of this experiment was to determine if the basal melanophore state changed over a daily cycle. Scales were collected at different times of day in association with a separate concurrent experiment. Red drum were captured and scales collected at 6 p.m., 10 p.m., 2 a.m. and 6 a.m. Sufficient data had previously been taken characterizing melanophore states during the light phase of the photoperiod cycle, the 10 a.m. and 2 p.m. time points. Lights in the tank room went off at 7:45 pm, so the fish were captured in total darkness for the 10 p.m., 2 a.m. and 6 a.m. samplings. One scale was removed from five separate fish for each time period, photographed with the digital camera, and analyzed with ImageJ.

Illumination effect on basal melanophore state

In this experiment, I wanted to determine if tanks with less access to light would have more consistent basal melanophore states due to dimmer lighting. The raceways in our system were directly beneath large round tanks and were thus partially shaded. I hypothesized these shaded raceways may have more consistent melanophore distribution and/or higher ratio or dispersed melanophores. This experiment consisted of three groups: red drum held in a raceway underneath round tanks, a round tank above the raceway directly under fluorescent lights, and red drum held in a raceway underneath round tanks and partially covered with black plastic. These first two groups were the conditions already present in the lab. The black plastic was added to one raceway to further test if decreased illumination would increase the ratio and/or number of dispersed

melanophores. Five fish were selected from each group and two scales were collected and analyzed. Scales were held in medium for an additional 2 hours. A second set of photographs was taken of the scales of fish from the raceway with the black plastic to verify stability of melanophore states. This last step was necessary to verify that over the time course of an experiment, melanophores would remain near the basal state.

MS-222 effect on basal melanophore state

MS-222 is a common fish anesthetic, however it had not been determined if this anesthetic had any effect on melanophore basal state or sensitivity to treatment. Scales were removed from two anesthetized red drum and two which were not. Photographs of 6 scales for each treatment were taken at 30, 60, and 90 min. After the 90 min photograph, the scales were transferred to a fresh lid region where 5 drops of Sigma's L-(-)-norepinephrine bitartrate salt monohydrate (norepinephrine)[5.91×10^{-4} M] were applied to the scale to determine if MS-222 had altered melanophore sensitivity. Norepinephrine stimulates a fast and predictable melanosome aggregation response which made it ideal for measuring sensitivity of scales to stimulation. Another photograph was taken after 1 min of norepinephrine exposure.

Hormone and chemical dose responses

One objective of developing this procedure was to utilize it in a teaching laboratory to demonstrate the concept of dose response. It was therefore necessary to examine physiologically-active doses of hormones and synthetic drugs in red drum. The

chemicals I used for physiological dose response tests were: α -melanocyte stimulating hormone (MSH), norepinephrine, and 3-Isobutyl bitartrate salt monohydrate (IBMX). All were obtained from Sigma Chemical Co. (St. Louis). For each, I captured three fish and removed scales as described above. One scale from each fish was subjected to a control or one of four treatments which consisted of the chemical at a concentration of 1×10^{-5} M, 1×10^{-6} M, 1×10^{-7} M, and 1×10^{-8} M. Treatments were derived by serial dilutions from stock solutions (1×10^{-4} M for MSH and 1×10^{-5} M for norepinephrine). After treatment was applied to scales, a photograph was immediately taken to capture the basal state. Photographs were auto-captured at 5, 10, 15, and 20 min to quantify the melanophore response to the treatment.

Aggregating fluid

Aggregating fluid is a K^+ rich salt solution used by Burton and Vokey (Burton and Vokey, 2000) to aggregate flounder melanophores. The objective of examining the effects aggregating fluid was to develop a technique that could facilitate cell counting by providing an easily reversible aggregating agent, confirm cell innervation, and confirm cells are alive at the end of an experiment. To test this, four scales were subjected to control (Ringer's solution), aggregating fluid, or norepinephrine [5.91×10^{-4} M]. The aggregating fluid was compared to norepinephrine because norepinephrine is a predictable melanophore aggregating agent at this concentration. Each scale was photographed previous to treatment exposure, and then auto-captured at 2, 5, 10, 15, and 20 min.

A second experiment was then set up to determine if the effect of the aggregating fluid on melanophores was easily reversible. Three scales were removed from 3 different red drum. One scale was used as a control, and the other two were treated with the aggregating fluid. The treatment scales were subjected to the aggregating fluid for 5 min and then placed in Ringer's Solution. Photographs were then taken of the scales at 2 and 3 min intervals to determine when the aggregation effect had been "washed out". This experiment also provides the raw data for the correlation of digital analysis method with previous scoring method.

CHAPTER III

RESULTS

Development of the digital analysis system

Before any experiments could be run, it was necessary to establish procedures for Scopephoto, ImageJ, and the physical setup of the microscope and digital camera. Numerous adjustments were necessary to increase contrast of melanophores to background and improve image quality. For example, the medium used to bathe the scales after removal was changed from a red minimum essential medium to a colorless Ringer's Solution to increase contrast between melanophores and background. Twenty four-well plate lids were adopted to reduce shadows cast by 24 and 96-well plate wells. Switching to well plate lids also facilitated the handling of scales by removing the walls of wells which restricted forceps angles.

To validate the digital analysis system, aggregating fluid was used to generate melanophore samples across all stages of dispersion. The traditional scoring method was compared to digital analysis for total area (Fig. 2A) and for pixels per cell (Fig. 2B). A significant positive correlation was found between the previous method of average scored state and total area ($r^2 = .4571$, p value $< .001$, $N = 84$), as well as for the pixel area per cell ($r^2 = .6608$, p value $< .001$, $N = 84$). Pixels per cell was chosen as the standard measurement because it had a higher correlation with the average scored state

method. Also, a pixels per cell measurement accounts for the number of melanophores per scale as the average scored state method does.

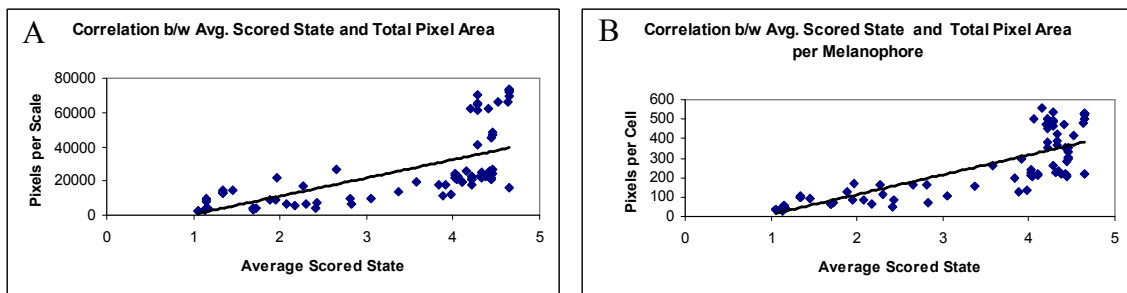


FIG. 2. Correlation between the previous method of melanophore staging and new digital analysis methods.

Establishing basal melanophore state

To establish an effective method to analyze melanophore activity it is necessary to have a predictable and consistent basal state to provide to students. Variables such as anatomical location, time of day, and light exposure were evaluated to determine if they influenced basal state.

Location of scales

The objective of examining different locations on various sizes of red drum was to find a location with at least 30 melanophores per scale and as consistent a pixel per cell count as possible. Melanophores on the perimeter of scales disperse differently than more central melanophores because those on the perimeter are constrained by the edge of the scale. Having at least 30 melanophores on a scale will increase the number of interior

melanophores. I found that for medium and large red drum, the dorsal scales had sufficient numbers of melanophores per scale (Fig. 3A). As fish grew in weight, they also exhibited an increased number of melanophores per scale. These data indicate that the dorsal side of larger red drum will provide the greatest number of melanophores for analysis.

The basal state of melanophores however, does not seem to be consistent by location (Fig. 3B, 3C). The basal average scored state of melanophores of the DA location on the red drum was significantly different between sizes. In both average scored state and pixel area measured by digital analysis, the DP quadrant contained less variability than the DA. The DA location on the red drum was found to be significantly different from the other three regions in medium sized fish in both methods. The DA was also significantly different in stage and pixel count from the DA's of the different sizes. Both the DA and DP provided sufficient number of melanophores, but the DA melanophore stages and pixel areas per cell were significantly different from the other three regions in medium sized red drum. The melanophores are also significantly different across size within the DA location. The DP quadrant also yielded the fewest scales lacking any melanophores. Ultimately, providing a consistent basal state to all students is more important than quantity of melanophores. As a result, the DP quadrant was chosen to be used for the teaching laboratory as well as for future experiments.

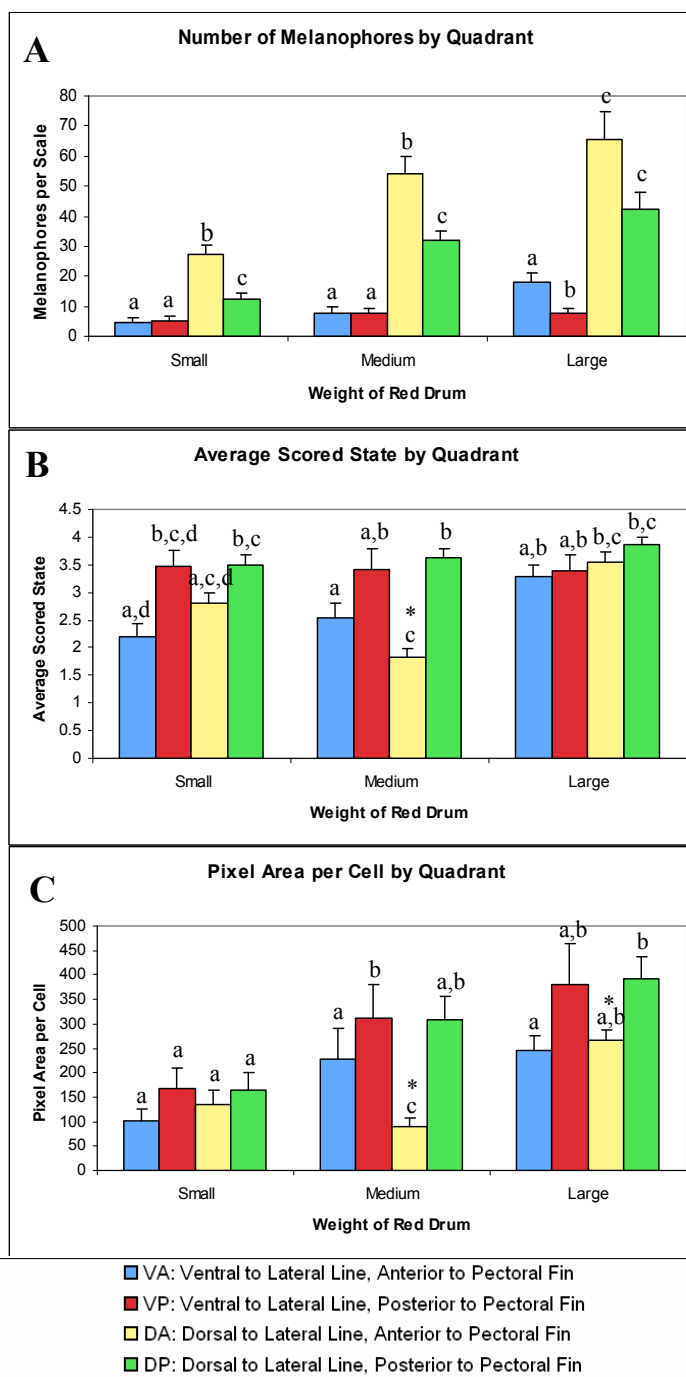


FIG. 3: Scale location data. (A) Average number of melanophores per scale in 4 different areas on the surface of red drum. Three sizes of red drum were used: small (mean = 11.5g), medium (mean = 32.8g), and large (mean = 63.2g) (B) Average scored state of 4 different areas on the surface of red drum (C) Pixel area per melanophore on 4 different areas on the red drum. Significance between locations within size categories indicated by letters a, b, and c. Groups sharing the same letter are not significantly different. * indicates significant difference from DP

24 Hour cycle

Melanophores were observed at 4 hour intervals of a 24 hr cycle to determine if there were significant differences in melanophore stage throughout a typical day. These data showed no evidence of a significant melanophore cycle in our laboratory-held red drum (Fig. 4).

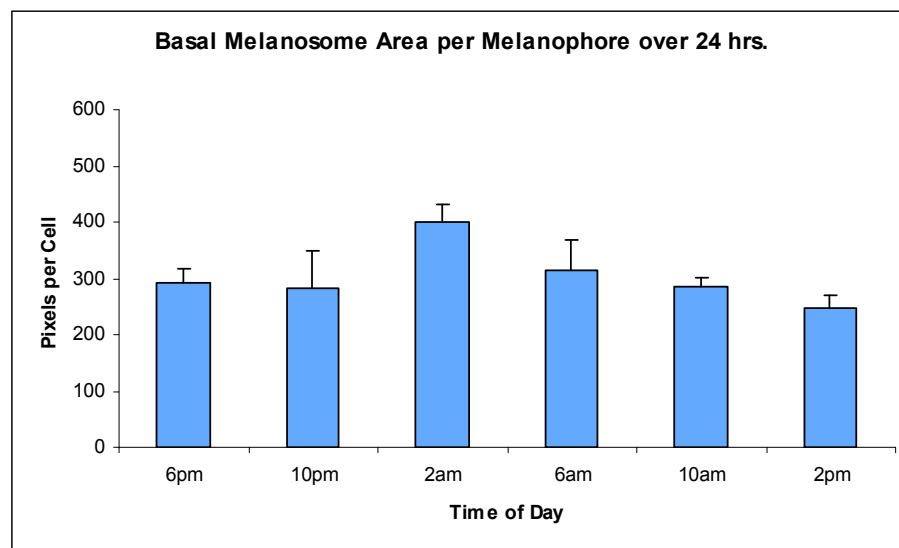


FIG. 4. Pixel area per melanophore from DP quadrant of red drum over 24 hr period. The 10 AM and 2 PM time points were taken from different days.

Illumination study

This experiment was performed to determine if illumination affected basal state of melanophores taken from animals held under 3 different lighting conditions. The various illuminations were not significantly different for number of pixels per cell. However, the dim illumination tank was significantly lower than the light and dark

illumination environments ($p < .01$ and $p < .05$ respectively) in number of melanophores per scale (Fig. 5).

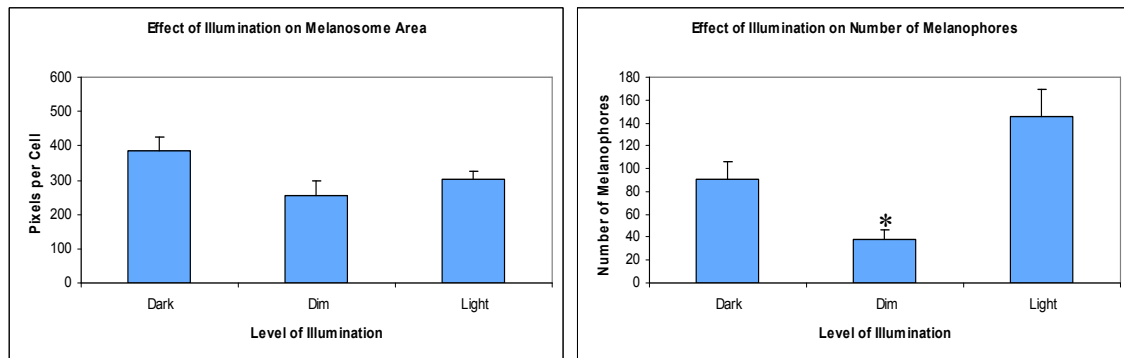


FIG. 5. Effects of 3 different stages of environmental illumination. (A) Pixel area per cell and (B) number of melanophores per scale.

* indicates significant difference in number of melanophores

MS-222 effect on basal melanophore state

The effects of MS-222 were tested to ensure that the anesthetic had no effect on melanophore activity. Pixel area per cell was examined in melanophores collected from anesthetized and non-anesthetized red drum. There was no significant difference found in basal melanophore state between anesthetized and non-anesthetized melanophores up to 90 min after scale collection (Fig. 6).

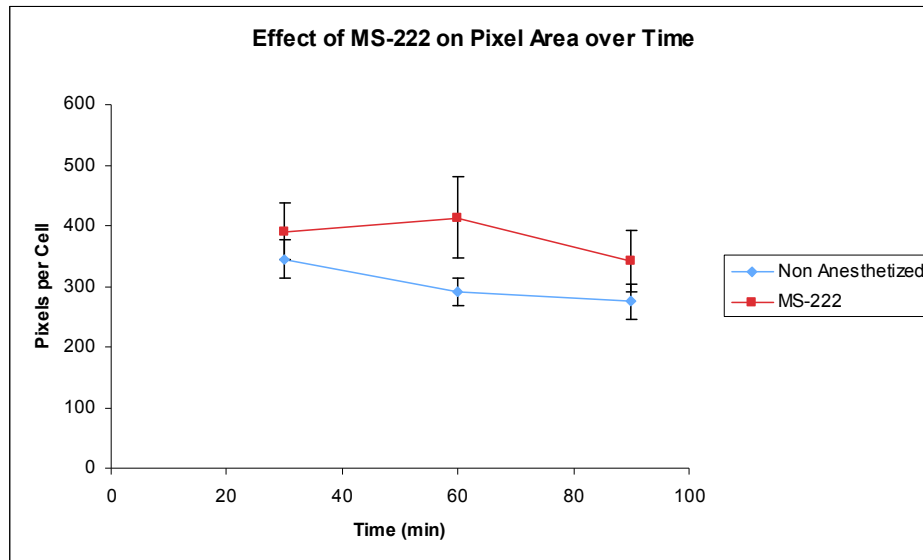


FIG. 6. MS-222 effect on basal melanophore state over time after removal from fish. Pixel area per cell determined at 30 min, 60 min, and 90 min.

Norepinephrine [10^{-5} M] was added to the scales after 90 min to determine if MS-222 affected sensitivity of melanophores to stimulus. The melanophores of both anesthetized and non-anesthetized red drum reacted in a similar manner to 1 min of norepinephrine exposure and were significantly aggregated (Fig. 7). These results showed that anesthetizing red drum before scale removal did not affect sensitivity to norepinephrine treatment after 90 minutes. MS-222 was therefore adopted as a standard protocol to anesthetize red drum for scale removal prior to hormone challenge experiments.

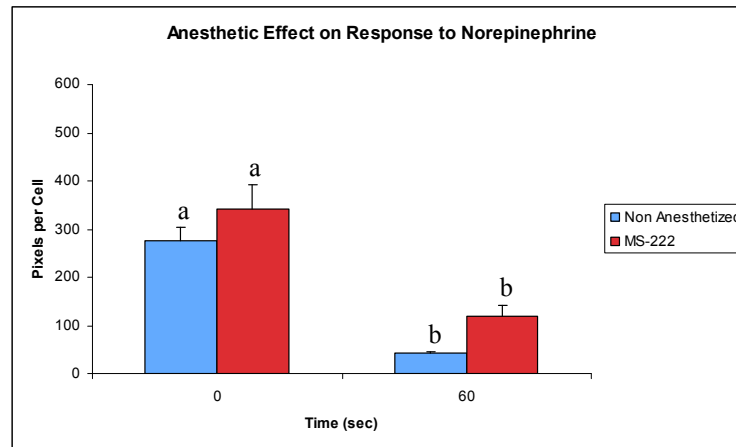


FIG. 7. The effect of MS-222 on norepinephrine response of both anesthetized and non anesthetized red drum scales. Groups sharing same letter are not significantly different.

Hormone and chemical dose responses

The norepinephrine time course/dose response experiment demonstrated the strongest aggregation response to be at a concentration of 10^{-5} M (Fig. 8). Norepinephrine at 10^{-6} M demonstrated a slower aggregation response. The norepinephrine concentration adopted for the teaching laboratory needs to elicit significant aggregation within 10 min. As such, the 10^{-5} M concentration was chosen to demonstrate neurotransmitter regulation of melanophore activity.

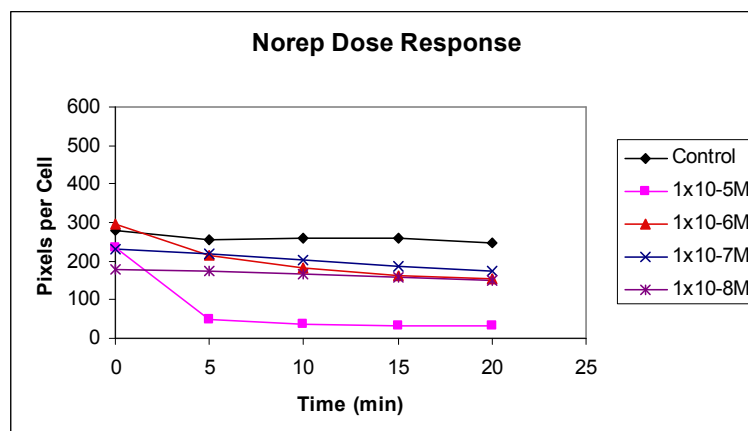


FIG. 8. Time course of different doses of norepinephrine on red drum aggregation.

For each chemical treatment, a time course/dose response experiment was performed. The MSH and IBMX experiments showed no induced change in melanophore state at dose concentrations of 10^{-5} M, 10^{-6} M, 10^{-7} M, and 10^{-8} M over 20 min.

Aggregating fluid

Observing hormone and drug-mediated melanophore dispersion proved difficult due to the dispersed basal melanophore state. A K^+ -rich balanced salt solution, termed aggregating fluid (Burton and Vokey, 2000), was used to aggregate scales so that dispersing chemical actions could be observed. Aggregating fluid proved to be as effective as 10^{-5} M norepinephrine at aggregating melanophores (Fig. 9A). These scales were then allowed to rest in Ringer's Solution for 30 min before being treated with 10^{-5} M MSH. A second experiment demonstrated the aggregating fluid's effect on melanophore state during 5 min of exposure was reversed in 10 min of placement in

Ringer's Solution (Fig. 9B). In contrast, norepinephrine's aggregation effect proved difficult to reverse (Fig. 9A).

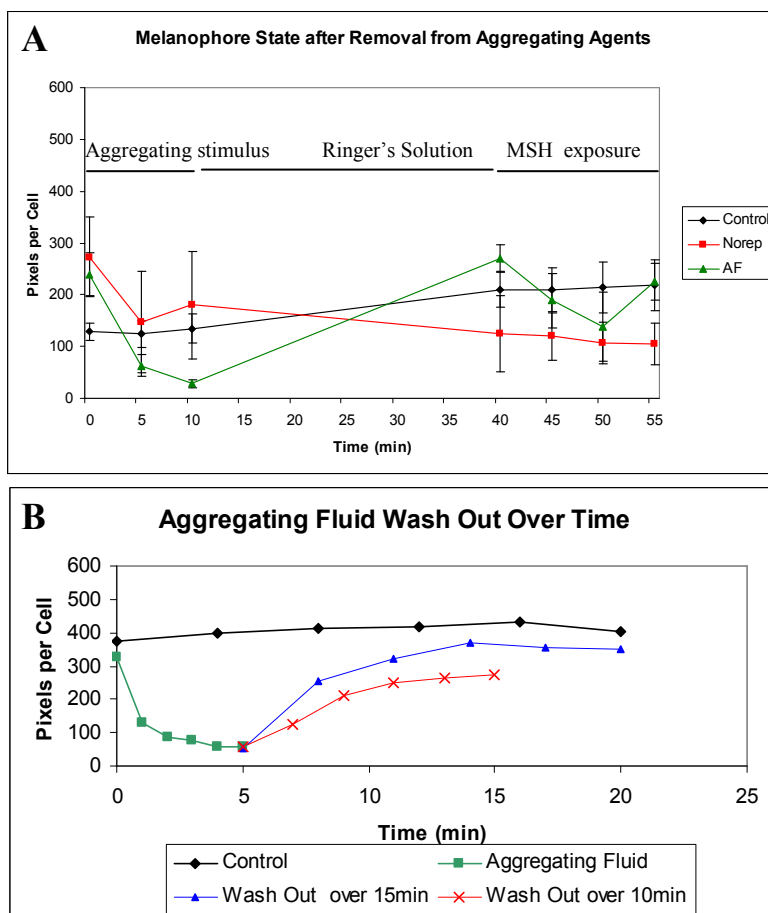


FIG. 9. Time courses for aggregating agents. (A) Effects of aggregating fluid and norepinephrine on pixels per cell for first 10 min, followed by a rest in Ringer's Solution, followed by exposure to MSH for 15 min. (B) Effects of removal of aggregating fluid for 10-15 minutes on melanophore dispersal.

CHAPTER IV

CONCLUSIONS AND DISCUSSION

The objective of my study was to develop a digital analysis procedure to observe hormone action by measuring melanophore activity. A new approach was taken, utilizing photographs which could be analyzed to quantify the area of melanophores, rather than a subjective scoring system. Although no new hormone experiments were developed for the laboratory, existing laboratory experiments were updated to utilize the new analysis method. Initially, it seemed the only change in laboratory protocol necessary was to incorporate hardware and software for capturing and analyzing photographs. The new hardware used for the lab was the Scopetek DCM200 digital camera and 24-well plate lids. The camera takes high quality photographs while being relatively inexpensive, which is important because five are necessary for the laboratory. However, the camera auto-adjusts lighting which sometimes results in poor contrast. The well plate lids provided easier scale handling as well as reduced shadowing from the 24-well plate walls. The software used to analyze the photographs was Scopephoto, which came packaged with the camera, and ImageJ photo analysis software. The Scopephoto program has a very useful auto-capture tool which allows for photographs to be taken in sequence without any change in scale position or lighting. The ImageJ program accurately measures the area provided by the photographs and has a tool which can be used to count number of melanophores.

Once the equipment setup was settled, it was then necessary to make several changes to provide greater contrast between melanophores and background. This included switching from a red minimum essential medium solution to clear Ringer's Solution. The red tint of the minimum essential medium would decrease contrast between melanophores and background. The photographs were converted to an 8-bit black and white format for ImageJ to analyze photographs. The camera was set to capture photographs in black and white so that I knew exactly how the picture would appear when it was analyzed in ImageJ.

Melanophore state could be compared easily with the scale staging method. This was useful in comparing hormone effects across several experiments. In order to improve on the previous method, I needed to ensure the results of the digital analysis method could be compared across experiments as well. To accomplish this, a standard magnification was established to observe melanophores. Size of melanophores was not considered in the previous scoring method, which resulted in scoring confusion. One melanophore may be at stage 5 on the scoring scale, but the size of the cell may make it appear to be a 3. Although scoring individual melanophores did not require consistent magnification to keep pixel area per cell measurements consistent, it was important to keep a consistent magnification so each scale from each experiment could have its pixels per cell compared. Observing a melanophore at a higher magnification would enlarge the melanophore in the image display. When analyzed, this would result in a larger pixel per cell count due to magnification, not physiology.

In the final procedure, scales were placed in 5 drops of solution on 24-well plate lids. These scales were then observed underneath a Scopetek DCM200 digital camera attached to a trinocular microscope at 12X magnification. The camera displayed to the computer screen in the Scopephoto program, which captured pictures in black and white. These photographs were then opened in ImageJ, converted to an 8-bit image, and thresholded for area. This area was divided by the cell count of the scale for the final value of pixels per scale. The cell count was acquired by placing scales after treatment into aggregating fluid for 10 min to aggregate to facilitate cell counting. It is much easier to count aggregated cells than dispersed, overlapping cells.

Once the procedure was established for capturing and analyzing photographs, it was necessary to validate the new system by comparing it with the previous scale scoring method (Burton and Vokey, 2000; Logan *et al.*, 2006; Zhu and Thomas, 1997). A significant positive correlation was found between the previous scoring method and the digital analysis of pixels per cell. This positive correlation indicates that the new digital analysis method yields results similar to those which were found with the previous scoring method. A significant correlation was also found between previous scoring method and total pixel area per scale. However, the pixels per cell measurement was chosen due to its higher correlation while also accounting for number of cells.

Establishing a standard basal state of melanophores is important to ensure a consistent starting point for each experiment. This included determining if basal condition of melanophores could be manipulated by environmental factors, handling procedure, or time in medium. I performed several experiments to determine if the basal state of melanophores was consistent across various anatomical locations of the red drum. The DA and DP locations on the red drum were found to provide an average of more than 30 melanophores per cell. The DA location had a significantly lower basal state from all other locations in mid-sized red drum while being highly variable across red drum sizes in both the previous and new method. This may suggest anterior melanophores are regulated differently than the rest of the body. The DP location was chosen because it was not significantly variable across size while containing sufficient numbers of melanophores. However, the DA location may be preferable for MSH and IBMX treatments which require an aggregated state in order to demonstrate activity. Interestingly, the average size of the red drum by the time dose responses were being run was twice that of animals used for scale location studies. When scales were removed from the DA region for the teaching laboratory, they were now similar in basal pixel per cell state to that of the DP region. It is possible that the DA region of these largest red drum may no longer contain a significantly more aggregated basal state from the DP location.

A second experiment sought to determine if basal melanophore state varied by time of day, but found no cycle. A previous observational study found that red drum

melanophores became more aggregated during the night, presumably due to elevated systemic circulation of aggregating hormones (Zhu and Thomas, 1998), although data was not presented. My study did not yield a significant difference in basal pixel per cell state. The range of the pixels per cell during the day was not broad enough to warrant replication. For teaching laboratories, scales are removed in the morning or afternoon, both of which provide a similar basal state to students. This simplifies the laboratory procedures by allowing each student to begin with similarly-staged melanophores regardless of the time of the laboratory.

The objective of the illumination experiment was to determine if red drum kept in a well-shaded environment would show a significantly different melanophore state from animals in illuminated tanks. A previous study found that tilapia kept in black tanks had significantly higher melanophore area per scale than those held in white tanks (van der Salm, 2005). In my study, however, there was no significant difference in pixels per melanophore across different illuminations, indicating that the amount of available light from the environment alone does not alter the state of melanophores. However, the dim lighting environment demonstrated a significantly lower number of melanophores per scale for reasons unknown. This may suggest the dim light is a stress. Norepinephrine is commonly released in response to stress and has shown to decrease the number of melanophores in medaka (Masazumi, 2002). I did not have enough time to replicate this experiment, but it suggests a physiological regulation of melanophore numbers.

Therefore, the red drum used for the teaching laboratory and dose responses should be

sampled from the light-exposed tanks since they provide a high quantity of melanophores, consistent melanosome area per scale, and are easier to capture and maintain. Scales should be removed from the DP location of red drum held in well lit tanks. Collection time during the day should have minimal effect on melanophore stage.

The anesthetic MS-222 was tested to ensure our results were not affected by the anesthesia process. This was important because anesthesia is necessary for the safety of the red drum when removing scales and should reduce handling stress. No significant change in basal state was observed, nor was a significant difference in norepinephrine sensitivity. This data gave me confidence that MS-222 was not affecting my data and should therefore remain part of the laboratory procedure. This experiment also demonstrated stability of melanophore pixels per scale for up to 90 min after removal from fish. This melanophore stability was consistent with untreated controls measured over the course of each experiment. Although I did not have time, it would be interesting to test MS-222's affect on the aggregating fluid response. MS-222 is a sodium channel blocker, which may hinder the nerve depolarization suggested as the mechanism of action of the excess potassium in aggregating fluid (Burton and Vokey, 2000). The aggregating fluid did work on red drum melanophores after anesthesia, but it is possible that if tested simultaneously, or without rest in Ringer's Solution, MS-222 may inhibit the melanophore response to high potassium medium.

My third objective was to examine time course/dose response curves for standard chemical treatments to ensure the new system could accurately display melanophore responses to chemical stimuli. Other laboratories have demonstrated MSH effect in nanomolar doses in winter flounder (Burton and Vokey, 2000) and red drum (Zhu and Thomas, 1997). Dose responses have also been established for norepinephrine and IBMX in zebrafish (Logan *et al.*, 2006) and red drum (Zhu and Thomas, 1997). In my norepinephrine dose response, norepinephrine demonstrated a strong aggregation response at 10^{-5} M, but not at a physiological range of 10^{-6} to 10^{-9} M which had been demonstrated in previous experiments (Zhu and Thomas, 1997). The dose responses for both MSH and IBMX were inconclusive; no significant change was observed even at highly concentrated doses. MSH and IBMX are both dispersing agents, but the melanophores may have been at or near their dispersal limits (average starting state for MSH = 312.8 pixels per cell). However, in previous studies, IBMX and MSH at similar concentrations have been shown to work at 30 minutes (Logan *et al.*, 2006). Because the teaching laboratory will not have 30 minutes to devote to each treatment, I tested for responses at 10 minutes, which may have not been sufficient to see full effects on dispersed melanophores. A second set of dose responses should be run out to 30 minutes for MSH and IBMX to determine if in fact I was simply sampling data too early.

To enhance effects of MSH, previous studies have performed MSH dose responses on previously-aggregated melanophores (Burton and Vokey, 2000). The aggregating fluid used to aggregate flounder melanophores before MSH treatment (Burton and Vokey,

2000) was completely reversible in a saline solution with red drum. Any dispersal effect seen in these aggregated melanophores would therefore not necessarily be due to MSH treatment (Fig. 9B). Norepinephrine at 10^{-5} M proved to be too strong of an aggregating agent to reverse effects. Melanin-concentrating hormone has also been used to pre-aggregate cells (Burton and Vokey, 2000), but this hormone was unavailable in our laboratory. In the future, I would be of interest to determine the effect of MCH on digitally analyzed red drum melanophores.

Ultimately, the scales did not react to the hormone MSH or IBMX regardless of the method of analysis. It is reasonable to assume if data was scored using previous method the results would have been similar due to the significant correlation found between the two methods. I believe that more work needs to be done to determine if doses of hormone can be found which demonstrate effects in 10 min. Alternatively, the lab could be restructured to allow more time for each treatment, which may allow results at concentrations closer to those shown in other laboratories.

My final objective was to take all the information gained from my experiments and redesign an existing teaching laboratory to demonstrate hormone actions using digital analysis of melanophores. The basal state data proved important because the teaching assistant could remove scales from the DP location of the red drum with confidence that regardless of time of day, illumination, or anesthetic, scales would provide a similar basal melanophore state for each student. This data coupled with melanophore state

stability in Ringer's Solution allows the teaching assistants flexibility in preparing the laboratory. The methods detailed above were implemented into a classroom teaching laboratory for spring semester 2008. For the teaching laboratory, students ran four separate experiments using the digital analysis setup. The teaching assistant removed scales from several red drum, and then groups of students collected four scales for experiments. The students placed scales on the 24-well plate lids, and viewed them under the digital camera using the Scopephoto software using preset camera configuration for black and white photographs. The photographs were captured individually and analyzed in ImageJ using the same thresholding procedure described above and demonstrated by the teaching assistants. Their first experiment tested MSH followed by K^+ -BSS. The second experiment was norepinephrine followed by IBMX. The third and fourth experiment tested an unknown antagonist or agonist, respectively, of an α or β adrenergic receptor. During the teaching laboratory, it was found that students had difficulty obtaining results in the analysis which confirmed the apparent melanophore states visually evident on the computer screen. I attribute this to two problems. First, photographs demonstrating proper lighting should be presented to the class and compared to improperly lit photographs. This would eliminate many of the problems students experienced when thresholding photographs. Secondly, the students' procedure should minimize moving scales for photography to maintain consistent lighting. Changing the angle which light is striking the treatment solution will alter the lighting of the scale. Due to time restrictions, students could not use the auto-capture feature and instead had to photograph separate experiments in parallel.

The new digital method of melanophore analysis is an improved system over the previous scoring method. It removes subjective scoring of melanophores, which can potentially mask hormone activity. Setting a threshold for the melanophores can still be somewhat subjective, but the teaching assistant can now easily evaluate student thresholding on the computer monitor. Becoming experienced at setting a threshold is much the same as becoming an experienced scorer. However, analyzing the pixel area per melanophore is much quicker than scoring each individual melanophore on a scale. This new digital analysis system is a faster and simpler method for observing hormone interactions on melanophores, while providing high quality images displayed on the monitor for all group members and the teaching assistant to view. This method will provide better data in the sense that the students can go back and forth between the photographs and match the change in pixel area per cell with the visual change in overall melanophore state. This method also greatly improved the teaching assistants' ability to assist students with data collection and analysis. I suggest that future studies be done to try to aspirate solution off of a scale and then treat scales with second solution to minimize scale handling after removal from red drum. This technique could be aided by the use of a thin layer of agar to hold the scale in place. Further work on dose responses and time courses could provide time efficient and physiologically relevant concentrations of hormones. If more work is done on refining the technique, I am confident the digital analysis method will provide more data, and faster analysis of the data for the teaching laboratory.

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