

2018

Restoration of visual performance and opsin expression within the retina during eye regeneration in the Florida fighting conch (*Strombus alatus*)

Jamie M. Clark
University of South Carolina

Follow this and additional works at: <https://scholarcommons.sc.edu/etd>



Part of the [Marine Biology Commons](#)

Recommended Citation

Clark, J. M.(2018). *Restoration of visual performance and opsin expression within the retina during eye regeneration in the Florida fighting conch (Strombus alatus)*. (Master's thesis). Retrieved from <https://scholarcommons.sc.edu/etd/4910>

This Open Access Thesis is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact digres@mailbox.sc.edu.

Restoration of visual performance and opsin expression within the retina during eye
regeneration in the Florida fighting conch (*Strombus alatus*)

by

Jamie M. Clark

Bachelor of Science
University of South Carolina, 2017

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Marine Science

College of Arts and Sciences

University of South Carolina

2018

Accepted by:

Daniel I. Speiser, Director of Thesis

Tammi Richardson, Reader

Jay Pinckney, Reader

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

© Copyright by Jamie M. Clark, 2018
All Rights Reserved

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Daniel Speiser, for his help in designing this study and for his revisions on this thesis. I would also like to thank Dr. Jay Pinckney for his assistance with the statistical analyses used. I would like to thank Dr. Alex Kingston for her help with immunohistochemistry and with troubleshooting the experiments. Finally, I would like to thank Lauren Kunselman for her hard work taking screen shots of behavioral trials and measuring eyestalk angle.

ABSTRACT

Conch are slow-moving, herbivorous, marine gastropods that possess prominent camera-type eyes at the ends of long, flexible stalks. Compared to the eyes of other gastropods, those of conch are large (up to 1.5 mm in diameter) and have sophisticated optics that include a lens with a graded refractive index. Conch also have a remarkable ability to regenerate eye tissue: after an eye is lost, a new eye will develop to take its place within weeks. Eye regeneration in conch appears to occur rapidly compared to eye regeneration in other gastropods. Despite our knowledge of the complexity and regenerative abilities of the eyes of conch, we know little about the visual responses of these animals either when their eyes are intact or while they are regenerating. Therefore, we measured rates of eye regrowth and tested how visual performance changes during the process of eye regeneration in the Florida fighting conch, *Strombus alatus*. We found that rates of eye regrowth were greatest in *S. alatus* between 3-6 weeks following eye removal but began to slow down thereafter. We also found that conch with two intact eyes respond consistently to moving objects with angular sizes of 24° or greater and angular speeds between 27 and 82 °/s. We removed either one or both eyes from 24 conch and recorded the behavioral responses of these animals to visual stimuli once a week for twelve weeks. We found that the visual performance of conch with the left eye removed never declined to begin with, suggesting that they successfully compensated with their intact right eye. However, the visual performance of conch with the right eye and both

eyes removed declined immediately following eye removal but was regained four weeks and seven weeks thereafter, respectively. To learn more about the process of eye regeneration in conch, we used immunohistochemistry to visualize the expression of phototransduction proteins at different stages of eye regeneration. In the 4th week of eye regeneration, rhodopsin and Gq proteins were found to be present which suggests the return of visual performance. By studying the restoration of visual performance during eye regeneration in conch we will continue to gain more understanding of how a regenerating sensory system reconnects with an intact nervous system.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
ABSTRACT.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: METHODS.....	9
CHAPTER 3: RESULTS.....	20
CHAPTER 4: DISCUSSION.....	42
WORKS CITED	50

LIST OF TABLES

Table 2.1 Characterization of Behaviors	17
Table 2.2 Description of Visual Stimuli	18
Table 3.1 Comparisons of defensive responses with intact eyes	28
Table 3.2 Comparisons of eyestalk angle	29

LIST OF FIGURES

Figure 1.1 Feeding behavior of conch	7
Figure 1.2 <i>Strombus</i> eye diagram	8
Figure 2.1 Eye angle diagram	19
Figure 3.1 Time lapse of eye regeneration.....	30
Figure 3.2 Progression of eye regeneration	31
Figure 3.3 Observed defensive behaviors- week 0	32
Figure 3.4 Observed defensive behaviors- week 4	33
Figure 3.5 Observed defensive behaviors- week 6	34
Figure 3.6 Observed defensive behaviors- week 8	35
Figure 3.7 Visual performance throughout eye regeneration	36
Figure 3.8 Expression of $G\alpha_q$ within intact eyes	37
Figure 3.9 Expression of r-opsin within intact eyes	38
Figure 3.10 Expression of $G\alpha_q$ within regenerating eyes	39
Figure 3.11 Expression of r-opsin within regenerating eyes	40
Figure 3.12 Control labeling within regenerating eyes.....	41

CHAPTER 1

INTRODUCTION

The abilities of some animals to regenerate bodily tissue has long intrigued scientists. Regeneration is often defined as the regrowth or repair of cells, tissues, and organs (Alvarado & Tsonis, 2006). All animals possess some degree of reparative ability but only some are capable of regenerating complete body parts and organs. Regeneration is broadly but non-uniformly seen among animal phyla but the majority of these processes begin with the rearrangement of pre-existing tissue, then usage of adult somatic stem cells, and finally the dedifferentiation (when cells lose tissue-specific characteristics they become undifferentiated) and/or transdifferentiation (when permanently differentiated cells dedifferentiate and then re-differentiate into cells with different characteristics; Alvarado & Tsonis, 2006; Call *et al.*, 2005; Dinsmore, 1991). Most researchers subjectively divide invertebrate regeneration, typically thought to arise from a specialized cluster of cells, from vertebrate regeneration, typically thought to arise from differentiation of somatic cells (Slack, 2006).

Particularly, eye regeneration is interesting because of the highly specialized cells that make-up visual organs and the complex neural connections between these organs and the central nervous system. Differing strategies of eye regeneration have been detailed in a wide range of taxa. Various studies have reported regeneration of damaged retinas and lenses as an outcome of transdifferentiation in retinal pigment epithelium cells in the eyes

of vertebrates such as amphibians, fish, birds, and mammals during embryogenesis, in juvenile amphibians, and in adult newts (Call *et al.*, 2004; Mitashov, 1966; Mitashov, 1996). Among invertebrates, planarians have been shown to regenerate new tails or heads depending on which side of the flatworm is removed and a pair of eye spots develops following the regeneration of the head (Sakai *et al.*, 2000).

We propose gastropods (Gastropoda; Mollusca) as a promising group of animals in which to study eye regeneration because many slugs and snails have two accessible eyes on the ends of eyestalks and regeneration of these eyes seems to occur relatively quickly, within 2 weeks in *Ilyanassa* (Gibson, 1984) or up to a couple months in *Nassa* (Hanko, 1914). In the land snail, *Helix* (Eakin & Ferlette, 1973) as well as the marine gastropod *Ilyanassa* (Gibson, 1984) eye regeneration appears to strongly parallel organogenesis: the developing eye forms from an “eyecup” of invaginated epithelium (Eakin & Brandenburger, 1967; Bever & Borgens, 1988). Another advantage to studying eye regeneration in gastropods is because the layout of their visual system lets us study how a regenerating eye reconnects with the visual processing centers of the nervous system. Eakin and Ferlatte (1973) found that the presence of the optic nerve is essential for the formation of the new eye within *Helix* gastropods. In this study, it appeared that the original optic nerve degenerated prior to the axons of the regenerating eye developing down the old optic nerve pathway eventually fusing with the cerebral ganglion. Therefore, a regenerating eye will not become fully functional until these nerves have joined (Hughes, 1976; Eakin and Ferlatte, 1973). Once the optic nerve is reintegrated into the central nervous system, visual capabilities would presumably be restored. Considering how an entire eye forms from transdifferentiated epithelial cells then

reintegrates with the central nervous system relatively quickly is an intriguing question for biologists.

Within gastropods, the genus *Strombus* is of particular interest because they possess large conspicuous eyes, located at the ends of long stalks. The proboscis (an elongated, tubular feeding appendage) and eyestalks extend outside of the shell during foraging and movement (Figure 1.1). Previous studies have shown the similarities between the eyes of *Strombus* and the eyes of other molluscs. Like the eyes of cephalopods and many gastropods, the eyes of conch consist of a light-focusing lens, photoreceptors with specialized rhabdomeric structures, and innervation by an optic nerve that runs to the central nervous system (Gillary and Gillary, 1979; Charles, 1966). Seyer (1994) suggested that, similar to cephalopods, the eyes of *Strombus* have lenses with a graded refractive index, but that other gastropods tend to have homogeneous lenses (if they have lenses at all). In Figure 1.2, Gillary & Gillary (1979) outlined the anatomy of the camera-type eye of *Strombus luhuanus*. The retina is located at the back of the eye and is composed of rhabdomeric photoreceptors. These photoreceptors have outer and inner segments that are separated by a pigment layer. Between the eye capsule and the inner segments of photoreceptors is the neuropile. This layer contains a relatively complex system of connections between long neuronal cells (Gillary & Gillary, 1979).

The rhabdomeric photoreceptors in the retinas of conch have morphology similar to those found in the retinas of cephalopods. These similarities in morphology suggest that conch may detect light using molecular components similar to those expressed by the retinal photoreceptors of cephalopods and other gastropods (Katagiri *et al.*, 2001). These rhabdomeric photoreceptors in invertebrates tend to detect light using visual pigments

that involve r-opsin and Gq-type G proteins (Kingston *et al.*, 2015; Arendt, 2003). R-opsins are expressed in the membrane folds (rhabdoms) of the long cylindrical photoreceptor cells. When bound to a vitamin-A based chromophore and stimulated by a photon, r-opsins activate a heterotrimeric Gq-protein that initiates a signal cascade that leads to the opening of TRP ion channels and the depolarization of the photoreceptor cell.

The reliable light-influenced behaviors that are seen in *Strombus* also make them a good study subject. When threatened, conch exhibit either an escape response by rapid lunges (Field, 1977) or a defensive response in which they retract their eyestalks and proboscis into their shell as predator avoidance (Bouchet, 2015). These visually influenced defensive behaviors can help indicate the visual performance of its visual system because these dramatic, but reliable responses can be quantified on a spectrum of reaction to assess what the animal finds threatening. Another visually influenced response that Field (1977) reported in *Strombus maculatus* was the angling of eyestalks toward a predatory snail, *Conus pennaceus*, and the increasing of eyestalk “waving” (small arc-like movements of the eyestalks) directed toward this predator. This observed angling and fixation on a predator could help an animal escape predation and move in a better direction away from this predatory stimulus. Field (1977) also found a significant difference ($p < .01$) between escape responses of *S. maculatus* with intact eyes versus blinded animals. This study observed that the eyes were used to fixate on the predator and maintain an efficient escape course from the predator.

Even though the eyes of conch are large and complex relative to those of other gastropods, they have a remarkable ability to regenerate: after an eye is lost, a new eye will develop to take its place within weeks (Hughes, 1976; Gillary, 1983). The

regenerating eyes of *Strombus* have been reported to quickly gain the retina and cornea 8 days after eye removal then regenerating the lens days afterward. Hughes (1976) reported that the regenerating eyes of *Strombus luhuanus* exhibited epithelial invagination just 24 hours after removal and the eye grew to full size within one month at a temperature of 29°C. However, regenerating eyes in *Strombus luhuanus* became full size within several months at a temperature of 24°C (Gillary, 1971; Hughes, 1976). For comparison, a regenerating eye of the Mediterranean mud snail, *Nassa mutabilis*, become full size in about 75 days (Hanko, 1914); a regenerating eye of the garden snail, *Helix aspersa*, becomes visible after about 30 days at 20°C (Eakin & Ferlatte, 1973; Hughes 1976); while regenerating eyes in the slugs *Arion* and *Agriolimax* become fully developed in about 40 days (Chetail, 1963).

Typically, the visual complexity seen in *Strombus* gastropods is associated with fast-moving predators which rely heavily on vision for prey capture (Land, 1981; Seyer, 1994). With this comparison in mind, it seems quite surprising to find this level of complexity in the eyes of a slow-moving herbivore (Seyer, 1994). Gillary and Gillary (1979) report that the eye of *Strombus luhuanus* “seems to mediate relatively complex visual behavior”, yet it is still unknown why conch possess such sophisticated eyes for presumably simple tasks. Seyer (1994) observed visually influenced behavior, such as the slow aiming of eyestalks to moving objects and fast defensive responses when objects approached the animal, in *Strombus* that hinted towards capabilities more advanced than discriminating light from dark. These observations from Seyer also suggested that *Strombus raninus* had the potential to resolve smaller objects within its environment. Studying the visual performance of the *Strombus* eye is valuable because of the unknown

abilities and spatial resolution this complex eye facilitates. Furthermore, when during eye regeneration these visual abilities return is unknown and could help understand the ecological role of these complex camera-type eyes for a slow-moving herbivore.

The Florida fighting conch (*Strombus alatus*) was chosen to be a representative of the genus *Strombus*. This species is native to the southern Atlantic Ocean, the Caribbean Sea, and the Gulf of Mexico and is a medium sized conch reaching a maximum size of 110 cm (Bouchet, 2015). This species of *Strombus* was selected because it is commercially available and abundant along the Florida coast.

The aim of our investigation is to 1) measure rates of eye regeneration under laboratory conditions using light microscopy and digital measurement techniques; 2) Evaluate when visual performance becomes restored during eye regeneration by utilizing behavioral trials and recording the defensive responses of 24 conch to visual stimuli; and 3) Detail where and when rhodopsin and Gq are present in an intact eyes and regenerating eyes.



Figure 1.1. Natural feeding behavior of the Florida fighting conch (*Strombus alatus*), with extended eyestalks and proboscis. Picture Credit: LA Dawson.

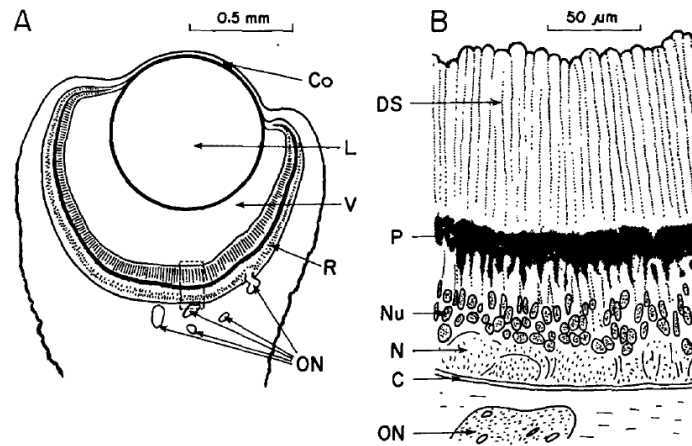


Figure 1.2. Figure adapted from Gillary & Gillary 1979. (A) Mid-sagittal section of a *Strombus* eyestalk with the camera-type eye shown inside the eyestalk. (B) Enlargement of the retinal layers. Abbreviations: capsule (C), cornea (Co), distal segments (DS), lens (L), neuropile (N), nuclear layer (Nu), optic nerve (ON), pigmented layer (P), retina (R), and vitreous body (V).

CHAPTER 2

METHODS

Specimen acquisition and care

We acquired twelve Florida fighting conch (*Strombus alatus*) from Gulf Specimen Marine Laboratories Inc. (Panacea, FL) in September 2016 and another twelve specimens in July 2017. At the University of South Carolina (Columbia, SC), we housed the conch in open-topped trays of live sand in a Living Stream System (Frigid Units, Toledo, OH) with recirculating natural seawater at a temperature of 19°C and a salinity of 35 ppt. We provided light to the aquaria with a Hydra FiftyTwo LED module (AquaIllumination, Ames, IA) set to a light/dark cycle of 12 hr: 12 hr that included simulated sunrise, sunset, and lunar cycle. We numbered the shells of each conch and fed them with sinking algae wafers (Hikari, Kansai, Japan) once a week.

Eye Removal and Tissue Preparation

We anaesthetized specimens in a saturated solution of 7.3% MgCl₂ and filtered seawater (dissolved Magnesium Chloride Hexahydrate [MgCl₂ • 6 H₂O; Fisher Scientific, Pittsburgh, PA, USA] directly into filtered seawater) between 10-45 minutes or until the eyestalk withdrawal reflex was absent (Seyer, 1994). The length of immersion time in the solution depended on the size of conch and water temperature. Larger animals and chilled MgCl₂ anesthesia resulted in longer immersion times. We removed the anterior part of

the eyestalk, just above the sensory appendages, with serrated dissecting scissors. We fixed the remaining eye capsule in a 4% formaldehyde and filtered seawater solution for 4 hours at room temperature. We then rinsed the tissue 3 times for 30 minutes each in 0.1 mol l⁻¹ phosphate buffered saline (PBS; 1 x PBS, diluted from a 10 x PBS stock; Corning, Corning, NY). Following fixation, we prepared the eyes for cryosectioning with overnight incubations at 4 °C in 10%, 20%, and 30% sucrose solutions in PBS. Using a Leica CM1850 cryostat set to -20 °C, eyes were sectioned at 15 µm, and sections were collected onto SuperFrost Plus slides (Fisher, St. Clair Shores, MI) then stored at -20 °C until use.

Eye Regeneration

To better understand the time scale of regeneration of the eyes of conch under laboratory conditions, we performed trials to measure the rate of eye regrowth. The time scale of eye regeneration in *S. alatus* is important to document because the size of an eye influences its optical properties and thus its function. We amputated the left eyes from eight conch using the aforementioned eye removal and tissue preparation protocols. We photographed each specimen every 7 days for 8 weeks. We placed the animals in a dish of filtered seawater underneath a Leica S6D (Leica Microsystems, Buffalo Grove, IL) dissecting microscope and photographed them with a Nikon D5000 camera (Nikon Corporation, Melville, NY). We then cropped, enhanced, and compiled all the pictures to show the progression of eye regeneration over 8 weeks. We used Adobe Photoshop to measure the eye capsule diameter and volume over the 8-week period.

Behavioral Trials: Visual Performance

We tested visual performance of *S. alatus* during the first 12 weeks of eye regeneration by presenting conch with various visual stimuli and recording their defensive responses. The eyestalk withdrawal reflex is a defensive behavior where the eyestalks and proboscis are retracted into the shell to presumably avoid predation. We placed the behavioral responses that we observed into 8 different categories to demonstrate a spectrum of reaction (Table 2.1). Prior to eye removal, we tested all conch as a control then tested them again each week after removing designated eyes (following the aforementioned eye removal protocol). We performed three different types of removals: left eye removal, right eye removal, and both eye removal. Therefore, with 24 experimental animals, there were 8 randomly chosen animals per eye removal type.

We set up a behavioral arena in an undisturbed, dark area to test the visual performance of conch during eye regeneration. This behavioral arena consisted of a central 2-gallon aquarium (with sand, room temperature filtered seawater, and bubbler), 2 orthogonally placed webcams (HD 1080p, Logitech, Lausanne, Switzerland), and a computer screen (E1913SF, Dell, Round Rock, TX, USA) placed on a metal frame so that it was 21.5 cm above the eyestalks of the conch tested. The screen was 38 cm wide, giving it an angular width of 82° from the perspective of the test animals. We combined the visual stimuli being shown and two webcam recordings into one video file per trial with XSplit Broadcasting Software (SplitmediaLabs, Kowloon, Hong Kong). We kept the conch stationary throughout each trial by using a cushioned clamp in the center of the aquarium. We placed an algae pellet below the conch to encourage foraging and natural

eyestalk extension. Without an algae pellet present, conch were more likely to exhibit lunging behavior around the aquarium which creates unpredictable eyestalk extension and would vary the relative positioning of visual stimuli between trials. Therefore, we used an algae pellet throughout the entirety of behavioral trials to keep the conch in a consistent position. Once the conch had both eyestalks extended, we began the trial recording.

For these trials, we presented the conch with three different PowerPoint (Microsoft, Redmond, WA) presentations containing white backgrounds and circular, black targets of varying size, speed, and direction that all moved across the screen in one direction then moved back across the screen in the opposite direction. The first presentation involved targets ranging in size between 3° and 35° (Table 2.2) that moved at a speed of $16^\circ/\text{sec}$ and moved from the animal's left to right side. The second presentation involved targets with an angular size of 29° traveling from the animal's left to right side but with different speeds, 1 second ($82^\circ/\text{sec}$), 3 second ($27^\circ/\text{sec}$), or 5 second ($16^\circ/\text{sec}$) to move all the way across the computer screen. The third presentation involved targets with an angular size of 29° that moved at a speed of $16^\circ/\text{sec}$ but with one of the four following directions: from the animal's right, left, back, or front. We measured the absolute irradiance (from 400-700 nm) of each target in the middle of the screen (presumably to be the greatest decrease in light levels experienced by the conch) using a spectrometer system (Table 2.2) from Ocean Optics (Dunedin, FL) that included a Flame-S-VIS-NIR-ES spectrometer, a QP400-1-UV-VIS optical fiber, and a CC-3 cosine-corrector. We calibrated the absolute spectral response of our system using a HL-3P-CAL

calibrated Vis-NIR light source and operated the spectrometer using Ocean View software.

We randomized the order of targets within each of the presentations for every trial to account for potential habituation. We measured visual performance by presenting visual stimuli on three consecutive days (size: Monday, speed: Tuesday, and direction: Wednesday) for 12 weeks after eye removal. We recorded the defensive responses digitally and the characterized behavior was recorded as shown in Table 2.1 for both the first and second passes of each target. For statistical analyses, we condensed the behavioral reactions into ‘no reaction’ or ‘positive reaction’ which gave a percentage of conch that displayed a positive response to each target during each week after eye removal. We ran a univariate ANOVA using SPSS (IBM Corp. Armonk, NY) for each eye removal type over the twelve weeks of eye regeneration. We also ran a univariate ANOVA using the defensive responses of conch with intact eyes to each type of target (varying size, speed, or direction) to test if some targets induced more defensive responses than others.

Measuring Eye Angle

During behavioral trials, we noticed that conch would angle their intact eyestalks towards the visual field of the missing eyestalk and that the angle of eyestalks in the double eye removal group did not change drastically. To quantify eyestalk angle, we took screenshots 3 seconds before the first target shown in all Wednesday (direction) trials of every conch. The lateral and anterior-posterior angle of each eyestalk was measured from two orthogonally placed cameras (Figure 2.1) using FIJI image analysis software

(Schindelin *et al.*, 2012). We then ran a one-way ANOVA using SPSS (IBM Corp. Armonk, NY) for each eye removal type over the twelve weeks of eye regeneration to compare the angle of each eyestalk during eye regeneration to the angle of that intact eyestalk.

Immunolabeling of retinas

The primary antibodies for all of our immunolabeling experiments were commercially available, polyclonal, and raised in rabbit. The first primary antibody we used was anti-G $\alpha_{q/11}$ (Millipore-Sigma, Billerica, MA) which was designed against peptide QLNLKEYNLV corresponding to amino acids 350-359 of human and mouse Gq protein. The second primary antibody we used was anti-octopus Rhodopsin (Cosmo Bio Co., LTD, Tokyo, Japan) that was designed against the full-length rhodopsin protein from octopus. The secondary antibody we used for all of our immunolabeling experiments was goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA) that bound to the primary antibody. We also used Phalloidin stain bound to TRITC (Tetramethylrhodamine Isothiocyanate; ThermoFisher Scientific, Waltham, MA) to stain F-actin within tissue sections and DAPI (4',6-diamidino-2-phenylindole; ThermoFisher Scientific, Waltham, MA) to stain the nuclei of the photoreceptors during the secondary antibody treatments.

We first detailed opsin expression within the retina of intact, 100% regenerated eyes to evaluate where opsin expression occurs within the retina. We used 15 single-eye removals to study the expression of opsin during regeneration. We amputated eyes from each conch that were determined to be fully intact by observing the relative size of the

eye and comparing to our initial eye regeneration measurements. To study $G\alpha_q$ and r-opsin expression throughout eye regeneration, we randomly chose 5 conch per regeneration stage. We removed eyes that were 25%, 50%, and 75% regenerated using eye removal and tissue preparation protocols prior to immunolabeling. Eye capsule diameter measurements from our initial eye regeneration studies determined that eyes were 25% regenerated in the 4th week after eye removal, 50% regenerated in the 6th week after eye removal, and ~75% regenerated in the 8th week.

We adapted immunohistochemical protocols from Kingston *et al.* 2015 to detail $G\alpha_q$ protein and r-opsin expression in *S. alatus*. We rehydrated sections at room temperature in three 10 minute washes of 1 x PBS with 0.3% Triton X-100 (PBS-TX) then blocked them in PBS-TX +10% normal goat serum (blocking buffer) for 1 hour at room temperature. We diluted anti-rhodopsin (primary antibody) at a concentration of 1:1000 and anti- $G\alpha_{q/11}$ (primary antibody) at a concentration of 1:500 in 300 μ L blocking buffer and applied this solution to each slide. We then covered the slides with strips of Parafilm (Bemis, Bellwood, IL) and stored them horizontally at 4°C overnight. The following day, we then washed the slides three times in PBS at room temperature. After washing the slides, we applied secondary antibodies to each slide at a diluted concentration of 1:400 with Phalloidin and DAPI at a diluted concentration of 1:1000 in 300 μ L blocking buffer. We covered the slides with Parafilm and stored them horizontally at 4°C overnight. Following the secondary antibody overnight incubation, we washed the slides in 1 x PBS three times for 30 minutes at room temperature, in the dark (using aluminum foil). After immunolabeling, we mounted the slides using Fluoromount-G (Southern Biotech, Birmingham, AL), covered with #1.5 thick coverslips

(Fisher Scientific, Pittsburgh, PA, USA), then sealed with clear nail polish. In all immunohistochemical images, blue represents DAPI staining of nuclei, green represents rhodopsin antibody labeling, cyan represents $G\alpha_q$ antibody labeling, and yellow represents Phalloidin staining of F-actin.

We used two types of controls to address the possible nonspecific binding of antibodies and the autofluorescence of tissue: (1) secondary-only controls that lacked primary antibodies and (2) untreated controls that lacked primary and secondary antibodies. Otherwise, we treated and imaged our control and experimental samples identically throughout our experiments.

Confocal Imaging

We accomplished confocal imaging using a Leica TCS SP8 X system (courtesy of the Twiss Lab, Columbia, SC; Leica Microsystems, Buffalo Grove, IL), with a water-immersion 40X objective (NA 1.10). We collected images at 1024 x 1024 pixels. For imaging, we used a 488-nm line of pulsed light (32%) from a white light laser (500 mW, 70% power) to excite the Alexa Fluor 488 (ThermoFisher Scientific) secondary antibody and a 555-nm line of pulsed light (32%) from a white light laser (500 mW, 70% power) to excite the TRITC (ThermoFisher Scientific). We also used a 405-nm diode laser (120 mW, 20% power) to excite DAPI. We utilized identical imaging parameters to those of the other treatment samples for the secondary-only and untreated controls. We processed our images using FIJI/ImageJ (Schindelin et al., 2012) and arranged them using Adobe Photoshop (Adobe, San Jose, CA). We applied z-stacks projected to a single plane using maximum pixel values for all of our images of eyes from *S. alatus*.

Table 2.1. Characterization of behaviors recorded during behavioral trials. We condensed behaviors into 4 categories: no reaction (red), positive reaction (blue), and secondary reaction (green).

Type of Behavior	Description of Behavior
No Reaction	Conch did not move; stalks remain at the same position they started in; behavior was not affected
Froze/stopped moving to watch	Conch was moving or pursuing a different behavior and stopped as soon as the target was noticed then immediately returned to the previous behavior
Double Eye Flinch	Both eyestalks quickly moved but returned to their original position
Right Eye Flinch	Only the right stalk quickly moved but returned to their original position
Left Eye Flinch	Only the left stalk quickly moved but returned to their original position
Partial Reflex (eyes outside shell)	Typically, both stalks are involved in this response; stalks are retracted and do not return to original position; stalks remain outside the shell after this reflex
Partial Reflex (eyes inside shell)	Both stalks are retracted into the shell; eyes and or sensory appendages are still seen by the cameras
Full Reflex into shell (eyes not seen by camera)	Stalks were retracted all the way into the shell; eyes and sensory appendages are not seen by the cameras
Second Reaction right after first reaction	A secondary reaction
Second reaction to second pass of target	A secondary reaction to the second pass of the target

Table 2.2. Description of visual stimuli used during behavioral trials to test visual performance in *Strombus alatus* over the course of eye regeneration.

Target Number	Description	Angular Size (deg)	Angular Speed (deg/sec)	Irradiance (photons/cm ² /sec)	Percent Decrease in Illumination
0	White Screen	--	--	1.37E+14	--
1	Size 1 (smallest)	3	16	1.37E+14	-0.3
2	Size 2	5	16	1.37E+14	-0.04
3	Size 3	12	16	1.36E+14	-1
4	Size 4	19	16	1.29E+14	-6
5	Size 5	24	16	1.18E+14	-14
6	Size 6	29	16	1.09E+14	-21
7	Size 7 (largest)	35	16	9.97E+13	-27
8	Speed 1 (slowest)	29	16	1.18E+14	-21
9	Speed 2 (medium speed)	29	27	1.18E+14	-21
10	Speed 3 (fastest)	29	82	1.18E+14	-21
11	Direction 1 (from the conch's right)	29	16	1.18E+14	-21
12	Direction 2 (from the conch's left)	29	16	1.18E+14	-21
13	Direction 3 (from the conch's behind)	29	16	1.18E+14	-21
14	Direction 4 (from the conch's front)	29	16	1.18E+14	-21

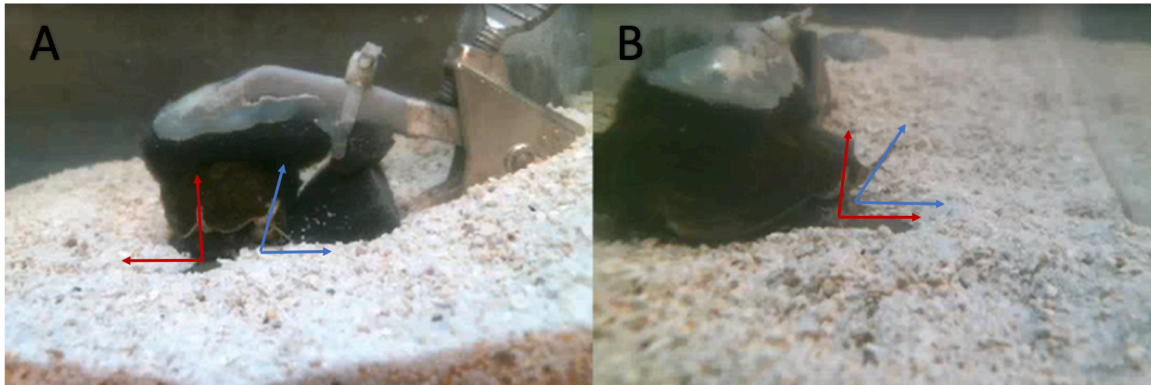


Figure 2.1. Representation of the behavioral arena set up with the conch held in the cushioned clamp within the middle of the aquaria. Eye angles were measured using ImageJ. The red angle is the measurement taken of the right stalk and the blue angle is the measurements taken of the left stalk. Angles were taken in reference to the sand substrate and the direction the eye capsule was pointed towards. A) Camera angle from the front of the conch to measure the lateral eyestalk angles. B) Camera angle from the side of the conch to measure the anterior-posterior eyestalk angles. Photo Credit: Jamie Clark

CHAPTER 3

RESULTS

Eye Regeneration

The presence of an eyespot occurred 2 weeks after eye removal and the eye capsule was mostly regenerated within 6 weeks after eye removal (Figure 3.1). The average eye capsule growth rate over 8 weeks after eye removal was 0.013 mm/week. Eye capsule growth rates were greatest between 3-6 weeks after eye removal but began to decrease once the diameter reached 0.51 mm and a volume of .0113 mm³ during the 6th week (Figure 3.2). At 8 weeks after eye removal, the eye capsule was only about 75% regenerated compared to the full-grown eye capsule diameter. This time scale of eye regeneration in *S. alatus* is important for comparisons of eye size with the restoration of visual abilities following the loss of an eye.

Behavioral Trials: Visual Performance

Before any eye removals, the visual performance of conch with intact eyes was tested to compare with the visual performance of conch with regenerating eyes (Table 3.1). With intact eyes, the smallest target that *S. alatus* responded to was target 4 with a size of 12°, a speed of 16 °/sec, and a 6% decrease in illumination compared to a blank white screen (Figure 3.3). The largest target *S. alatus* responded to was target 7 with a size of 35°, a speed of 16 °/sec, and a 27% decrease in illumination compared to a blank

white screen. However, target 6 with a size of 29°, a speed of 16 °/sec, and a 21% decrease in illumination compared to a blank white screen induced more defensive responses (12 out of 24 conch) in animals with intact eyes than the larger 35° target (10 out of 24 conch). Double eye flinches were the defensive response seen most often in conch with intact eyes between the varying sizes of stimuli. With intact eyes, conch exhibited significantly more defensive responses to targets 6 and 7, with angular sizes of 29° and 35° respectively, than the smaller targets ($p < .05$; Table 3.1).

With intact eyes, conch responded to the slowest target most frequently when shown targets with three different speeds in a randomized order (Figure 3.3). The slowest target, target 8, was 29° in size, 16 °/sec in speed, and had a 21% decrease in illumination compared to a blank white screen (16 out of 24 conch). The target with medium speed (target 9 with a size of 29°, a speed of 27 °/sec, and a 21% decrease in illumination compared to a blank white screen) did not induce significantly different defensive responses (13 out of 24 conch) than the slowest target ($p = .196$). Double eye flinches and partial reflexes into the shell were the defensive responses seen most often in conch with intact eyes between the varying speeds of stimuli.

With intact eyes, conch responded to the target coming from behind them (target 13 with a size of 29°, a speed of 16 °/sec, and a 21% decrease in illumination compared to a blank white screen; 11 out of 24 conch; Figure 3.3) significantly more often ($p = .035$; Table 3.1) than they responded to the target coming from in front of them (target 14 with a size of 29°, a speed of 16 °/sec, and a 21% decrease in illumination compared to a blank white screen; 4 out of 24 conch). Conch with intact eyes also responded to the targets coming from their right (target 11 with a size of 29°, a speed of 16 °/sec, and a

21% decrease in illumination compared to a blank white screen; 9 out of 24 conch) and from their left (target 12 with a size of 29° , a speed of $16^\circ/\text{sec}$, and a 21% decrease in illumination compared to a blank white screen; 9 out of 24 conch). However, conch with intact eyes did not significantly respond to the target coming from behind them (target 13) compared to the other targets. Double eye and left eye flinches were the defensive response seen most often in conch with intact eyes between the varying directions of stimuli.

During eye regeneration, *S. alatus* responded fairly consistently to the two largest targets, target 6 with with a size of 29° , a speed of $16^\circ/\text{sec}$, and a 21% decrease in illumination compared to a blank white screen and target 7 with a size of 35° , a speed of $16^\circ/\text{sec}$, and a 27% decrease in illumination compared to a blank white screen. The largest target of 35° was reacted to most frequently by conch during week 4 (10 out of 24 conch; Figure 3.4) and during week 8 (13 out of 24 conch; Figure 3.6). During week 6, target 6 (with an angular size of 29°) was the most reacted to by conch (11 out of 24 conch; Figure 3.5).

The slowest target (target 8, 29° in size, $16^\circ/\text{sec}$ in speed, and a 21% decrease in illumination) induced the most defensive responses during week 4 (8 out of 24 conch; Figure 3.4) and week 6 (13 out of 24 conch; Figure 3.5). The target with medium speed (target 9 with a size of 29° , a speed of $27^\circ/\text{sec}$, and a 21% decrease in illumination) induced slightly more defensive responses (15 out of 24 conch; Figure 3.6) than the slower target during week 8 post-eye removal (Figure 3.6).

During the 4th week of eye regeneration, conch responded more often to the target coming from its right-hand side, target 11 (6 out of 24 conch; Figure 3.4).

However, in weeks 6 and 8 of eye regeneration, conch responded to the target coming from its left-hand side, target 12, more often than any other direction (12 out of 24 conch; Figures 8 and 9).

There was a greater diversity of defensive responses prior to eye removal than during eye regeneration (Figures 3.3 – 3.6). It appears that conch performed partial responses with eyes still outside the shell and double eye flinches most often prior to eye removal (Figure 3.3). After eye removal in week 4, double eye and left eye flinches were occurring more often (Figure 3.4). During week 6 post-eye removal, partial reflexes and double eye flinches were the defensive responses being recorded most often. During week 8 post-eye removals, partial responses, right eye flinches, and double eye flinches were the defensive reflexes being recorded most often (Figure 3.6).

Our behavioral trials found that conch with the left eye removed experienced no decline in visual performance and presumably by successfully compensating for the loss of their left eye with their intact right eye. However, conch with the right and both eyes removed experienced declines in visual performance after eye removal but regained visual performance after 4 weeks and 6 weeks, respectively. Following the removal of a single eye, the intact stalk was the only stalk that would protrude outside the shell for 1-2 weeks. Following the removal of both eyes, the stalks did not protrude outside the shell very far but the sensory tentacles appeared to purposefully be sticking out. Conch did not once react to the smallest targets (3° , 5° , and 12° targets) throughout any of the trials and therefore these were excluded from the analyses.

For all eye removal types, there was a general increase in percentage of conch displaying positive responses within the 12 weeks of testing. The left eye removal group

demonstrated a steady increase in percentage of positive responses between weeks 1-4; however, the right and both eye removal groups decreased in percentage of positive responses between weeks 1-3 then began to increase. It is important to note that the both eye removal group still reacted to visual stimuli after eye removal, suggesting that conch have photoreceptors outside of their eyes.

A one-way analysis of variance showed significant differences between percent of positive responses between weeks for the left eye removal group ($p < .05$). However, there was no significant difference in percentage of conch showing a positive reaction prior to eye removal and most post-eye removal trials. The only trial post-eye removal that showed a significant difference from the responses with intact eyes was week 10 ($p = .019$). This group of conch still had an intact right eye for predator avoidance and defensive response that might have been used to compensate for the loss of the left eye (Figure 3.7).

A one-way analysis of variance showed significant differences between percent of positive responses per week for the right eye removal group ($p < .05$). There was found to be a significant difference between the responses with intact eyes to week 2 and 3 post-eye removal ($p < .05$). However, there was no significant difference between the responses with intact eyes and week 4 post-eye removal ($p > .05$) suggesting that visual performance was restored during the 4th week post-eye removal (Figure 3.7).

A one-way analysis of variance showed significant differences between percent of positive responses per week for the both eye removal group ($p < .05$). Trials within weeks 2-5 were found to be statistically different to the trials with intact eyes, however week 6 was not statistically different prior to eye removal. It appears that visual

performance was restored for the both eye removal group within 6 weeks after eye removal (Figure 3.7).

Measuring Eye Angle

By using one-way analysis of variance tests to compare the extension angles of each eyestalk between intact and regenerating eyes, we found that there were no significant differences regarding week or camera angle (Table 3.2). The intact and regenerating eyestalk extension angles did not differ from one another, signaling that there was no evidence of compensation in eyestalk extension angle with the loss of an eye. The only statistically significant difference seen was in the right eye removal group with the anterior-posterior angles of the left eyestalk. We observed statistically different eyestalk angles in weeks 11 and 12 from the angles of intact eyestalks ($p \leq .05$).

*Expression of $G\alpha_q$ protein and R-opsin in the fully intact and regenerating eyes of *S. alatus**

The camera-type eye of *Strombus alatus* exhibited similar morphology to the eyes of various species of conch from previous studies (Gillary & Gillary, 1979; Hughes, 1976; Seyer 1994). The eye of *S. alatus* consisted of a cornea, a graded refractive lens, as suggested by Seyer (1994), a gelatinous vitreous body, and a retina. The retina of *Strombus alatus* showed a distinct rhabdomeric layer also called the layer of outer segments. Underneath the outer segments is the pigment layer then cell nuclei and the neuropile. Our immunolabeling experiments of intact eyes indicate specific layers of the retina that vary in expression patterns of $G\alpha_q$ and r-opsin. The labeling of anti- $G\alpha_q$ was

limited to the back of eye in the neuropile layer but exhibited a lack of labeling in the outer segments of photoreceptors (Figure 3.8). Phalloidin labeled the outer segments of photoreceptors which show this layer is densely packed with photoreceptors and that the rhabdoms bend into each other through the shape of the eyecup. The back of the eye capsule exhibited labeling of r-opsin but there was also labeling in between cells within the nuclear layer and between the outer segments of photoreceptors (Figure 3.9).

In week 4 of eye regeneration, it appears that anti-G α_q was expressed in the back of the eye within the neuropile layer as well as in the outer segments of photoreceptors. However, we noted that the expression of anti-G α_q in the outer segments of photoreceptors is absent within week 6, week 8, and fully-intact eyes (Figure 3.10). We observed expression of r-opsin in the back of the eye within the neuropile and in the eye capsule of all regeneration stages including fully intact eyes (Figure 3.11). The outer segments of photoreceptors appear to increase in length and become denser within this layer as the eye regenerates.

For all immunolabeling experiments involving the eyes of *S. alatus*, secondary-only and untreated controls showed little nonspecific binding of secondary antibodies and low levels of autofluorescence. For all experiments involving labeling with primary antibodies (anti-G α_q and anti-r-opsin), we observed low levels of background fluorescence that were indistinguishable from the autofluorescence visible in the secondary-only and untreated controls (Figure 3.12).

Observations from Behavioral Trials

During behavioral trials, we noted differences between the angle, reflex, and activity of the the left and right eyestalks. When a conch emerged from its' shell, more often than not, the left eye was first to emerge. Both eyes were typically angled straight above the animal during all trials but the angle of the eyestalks varied between animal and trial. Depending on where the eyestalks were angled in the arena determined when the visual stimuli would come into view, which greatly affected the degree of reaction by animals and if there was any reaction at all. The right eyestalk seemed to move around and change its' angle more frequently than the left eyestalk most likely due to the anatomy and notches of the shell. With single eye removals, it appeared that the angle of the intact eyestalk would angle more towards the area of the missing visual field of the removed eyestalk. Animals with left eye removals seemed to be less responsive to visual stimuli than animals with right eye removals. During eye regeneration for conch with both eyes removed, the left eyespot at 2 weeks post eye removal appeared to be larger than the eyespot on the right eyestalk. Smaller conch were typically more energetic than larger animals by exhibiting more escape behavior and less foraging. During Monday trials (size targets), animals were energetic and less willing to feed because they still had food in their tanks from the Wednesday of the past feeding. However, during Wednesday trials (direction targets), animals were less energetic and fed more frequently with fully extended eyestalks because it had been a full week since feeding.

Table 3.1. Comparisons of the defensive responses of conch with intact eyes to different targets of the visual stimuli shown in behavioral trials. A univariate ANOVA was used with pairwise comparisons shown below. Bolded and italicized p-values are less than .05 and therefore show significance.

Target							
Sizes	1	2	3	4	5	6	7
1	---	1.000	1.000	.639	.160	.000	.000
2	1.000	---	1.000	.639	.160	.000	.000
3	1.000	1.000	---	.639	.160	.000	.000
4	.639	.639	.639	---	.348	.000	.000
5	.160	.160	.160	.348	---	.000	.005
6	.000	.000	.000	.000	.000	---	.348
7	.000	.000	.000	.000	.000	.348	---
Speeds	8	9	10				
8	---	.196	.000				
9	.196	---	.011				
10	.000	.011	---				
Directions	11	12	13	14			
11	---	1.000	.543	.130			
12	1.000	---	.543	.130			
13	.543	.543	---	.035			
14	.130	.130	.035	---			

Table 3.2. Comparisons of eyestalk angle between intact eyes and eye regeneration of the different eye removal types.

Eye Removal Type	Eyestalk and Camera	Resulting p-values from a univariate ANOVA
Left	Left eyestalk from front camera	.197
Left	Left eyestalk from side camera	.745
Left	Right eyestalk from front camera	.150
Left	Right eyestalk from side camera	.058
Right	Left eyestalk from front camera	.188
Right	Left eyestalk from side camera	.000 Weeks 11 (p= .05) and 12 (p= .02)
Right	Right eyestalk from front camera	.638
Right	Right eyestalk from side camera	.327
Both	Left eyestalk from front camera	.544
Both	Left eyestalk from side camera	.612
Both	Right eyestalk from front camera	.119
Both	Right eyestalk from side camera	.681

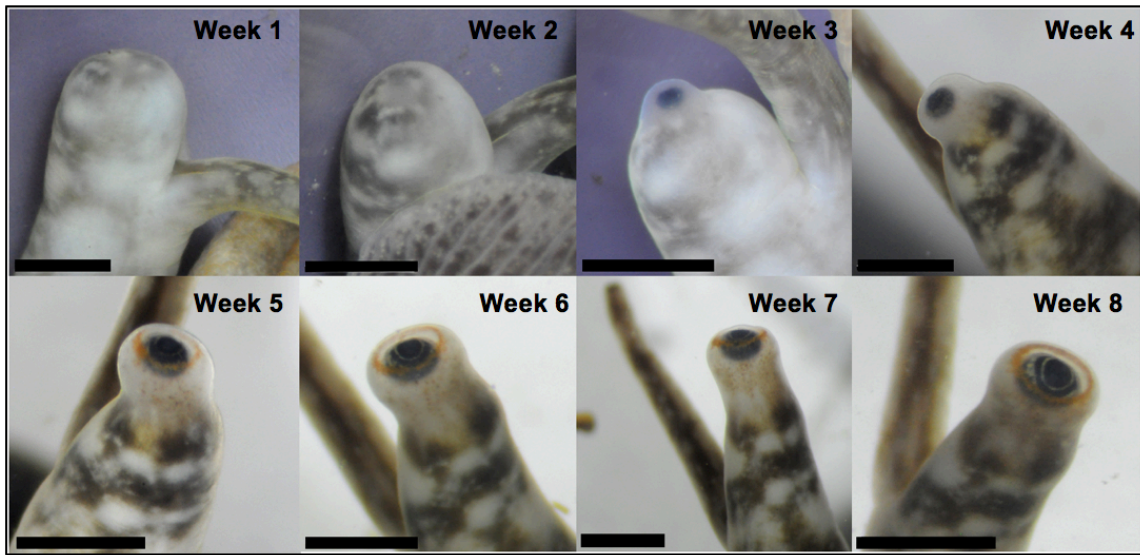


Figure 3.1. A time-lapse of eye regeneration in *S. alatus* over 8 weeks post-eye removal of the left eye. The eye capsule is regenerated first then the lost eyestalk epithelium is repaired. An eye spot is observed 2 weeks after eye removal and after 8 weeks of eye regeneration, the eye is about 75% the diameter of the original eye. Scale bar = 1mm. Photo Credit: Jamie Clark.

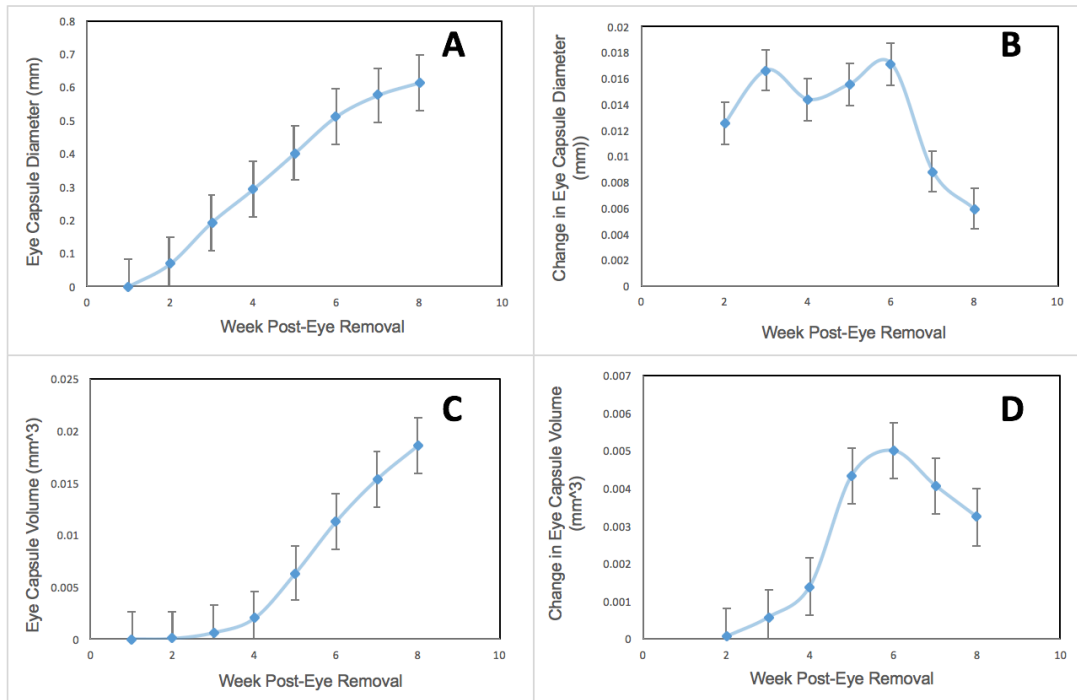


Figure 3.2. (A) The progression of eye capsule diameter (mm) over 8 weeks. Eye diameter consistently increases, but begins to plateau at 8 weeks post-eye removal. (B) The differences in eye capsule diameter were greatest between 3-6 weeks post-eye removal. After 6 weeks, changes in eye capsule diameter slowed down. (C) Eye capsule volume drastically began to speed up after 3 weeks post-eye removal. (D) Changes in eye capsule volume peaked at 5 weeks post-amputation, then began to decrease. Both eye capsule diameter and volume quickly sped up to a certain size within the 6th week post-eye removal, but then began to slowdown in growth.

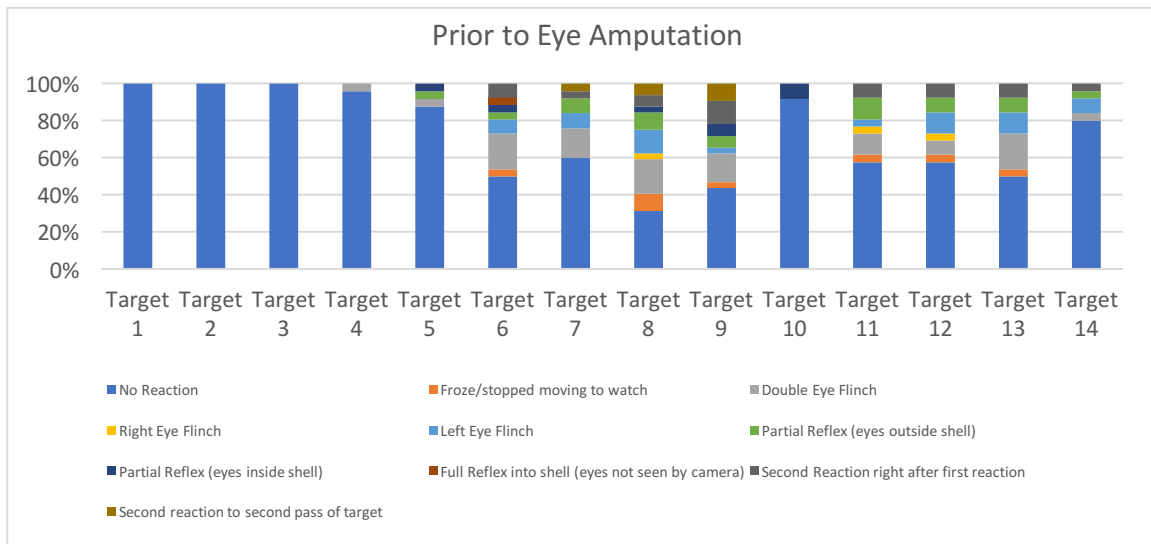


Figure 3.3. The defensive behaviors recorded as percentage of conch (out of 24) showing each behavior to each of the targets with intact eyes. Target 8 (29° in size and 16 °/sec in speed) induced the most defensive reflexes. Double eyestalk flinches were seen most often as the defensive behavior in conch with intact eyes.

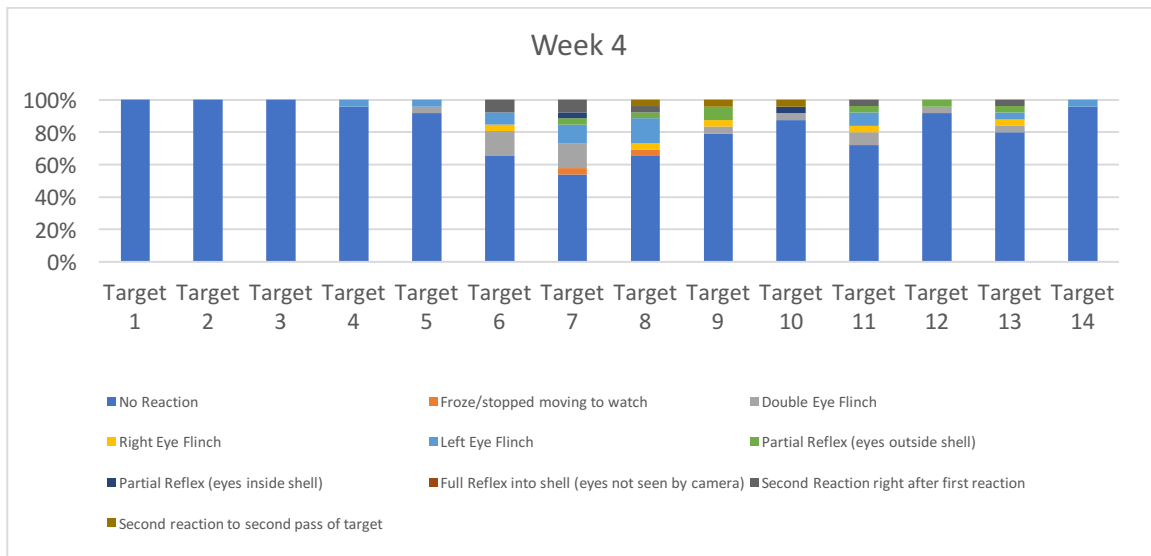


Figure 3.4. The defensive behaviors recorded as percentage of conch (out of 24) showing each behavior to each of the targets during the 4th week post-eye removal. The largest target (35° in size and 16 °/sec in speed) induced more defensive reflexes than other targets. Double eyestalk flinches and left eye flinches were seen most often as the defensive behavior in conch with regenerating eyes.

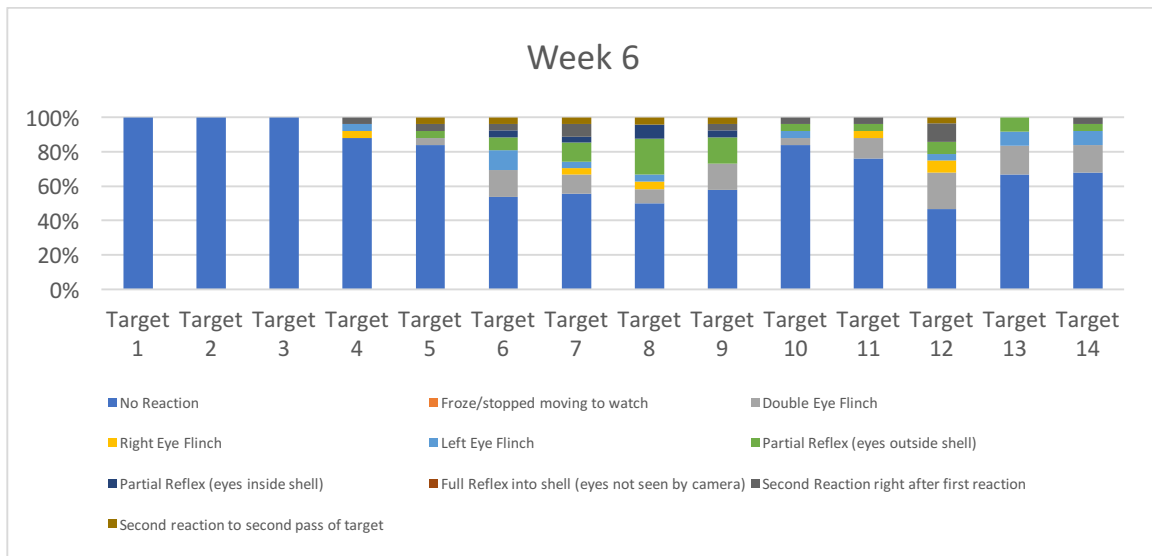


Figure 3.5. The defensive behaviors recorded as percentage of conch (out of 24) showing each behavior to each of the targets during the 6th week post-eye removal. The target coming from the left of the animal with a size of 29° and a speed of 16 °/sec induced the most defensive responses during week 6 post-eye removal. Double eyestalk flinches and partial reflexes were seen most often as the defensive behavior in conch with regenerating eyes.

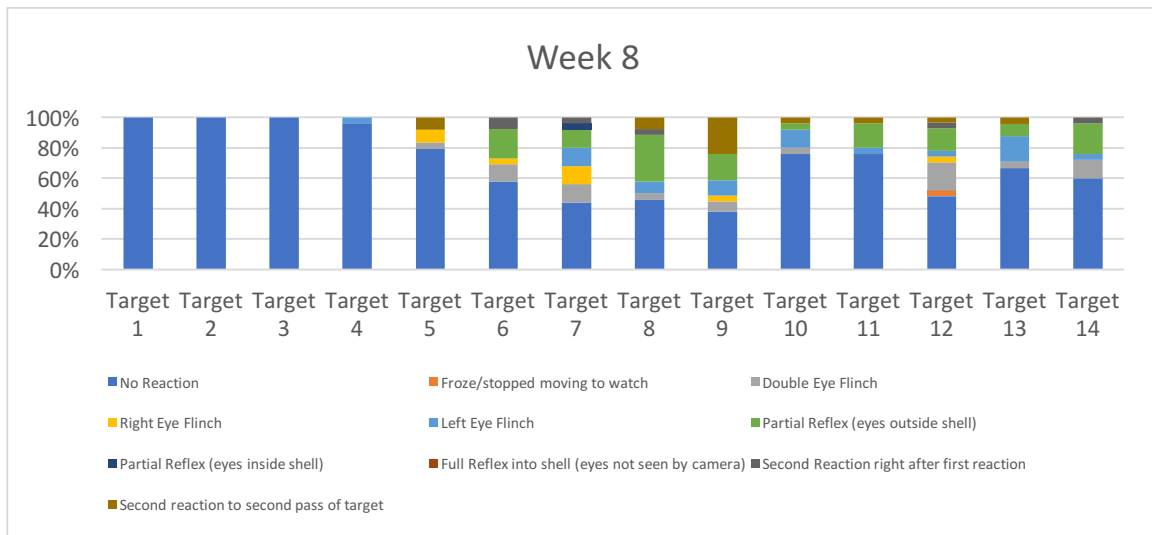


Figure 3.6. The defensive behaviors recorded as percentage of conch (out of 24) showing each behavior to each of the targets during the 8th week post-eye removal. The target with a size of 29° and a medium speed of 27 °/sec induced the most defensive response during week 8 post-eye removal. Partial reflexes were seen most often as the defensive behavior in conch with regenerating eyes.

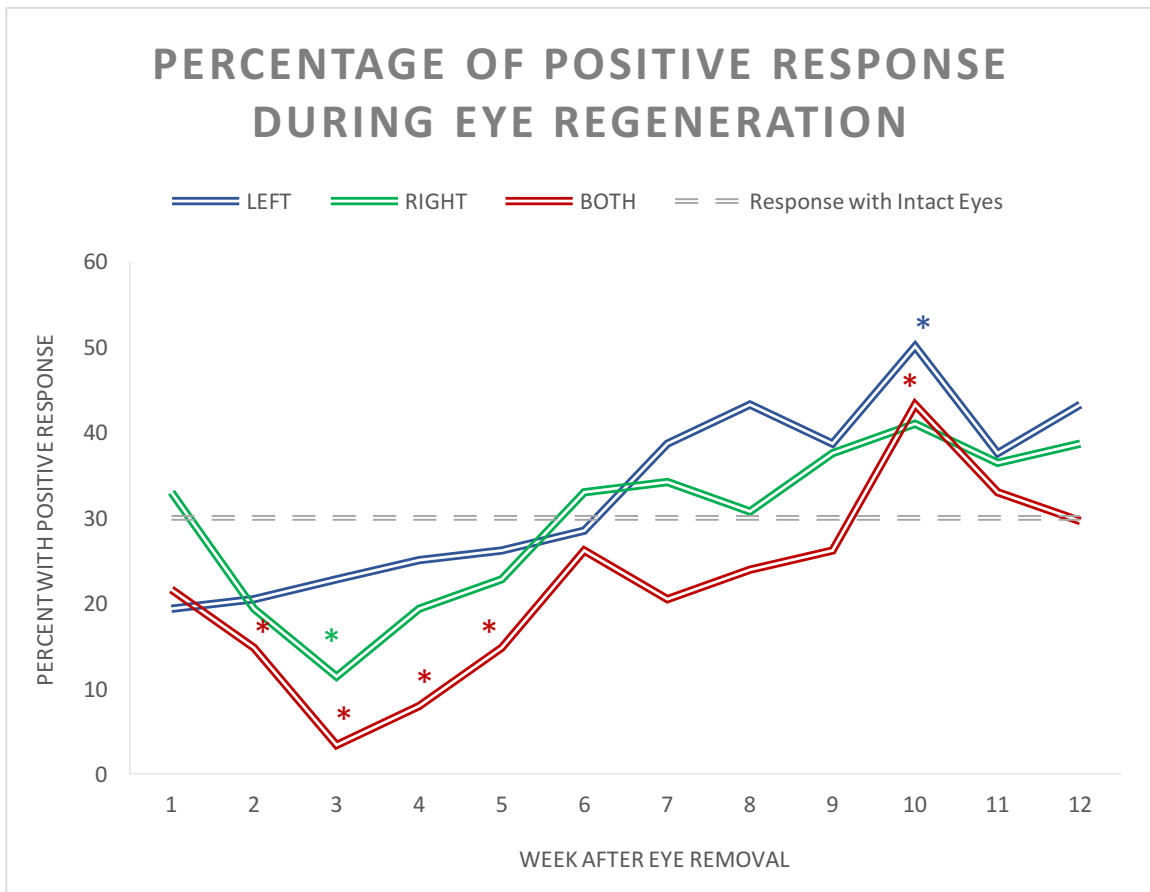


Figure 3.7. The percentage of conch showing a positive defensive response is shown during eye regeneration of the left, right, or both eyes. The dashed line shows the defensive responses of all 24 animals with intact eyes. Stars represent statistical significance between that week and the behavior before eye removals. There is a sudden decrease in conch showing defensive responses in the right and both eye removal groups. Visual performance appears to return in the right eye removal group during the 4th week after eye removal. In the both eye removal group, visual performance appears to return during the 6th week after eye removal. There were no statistically different weeks in the left eye removal group until the 10th week.

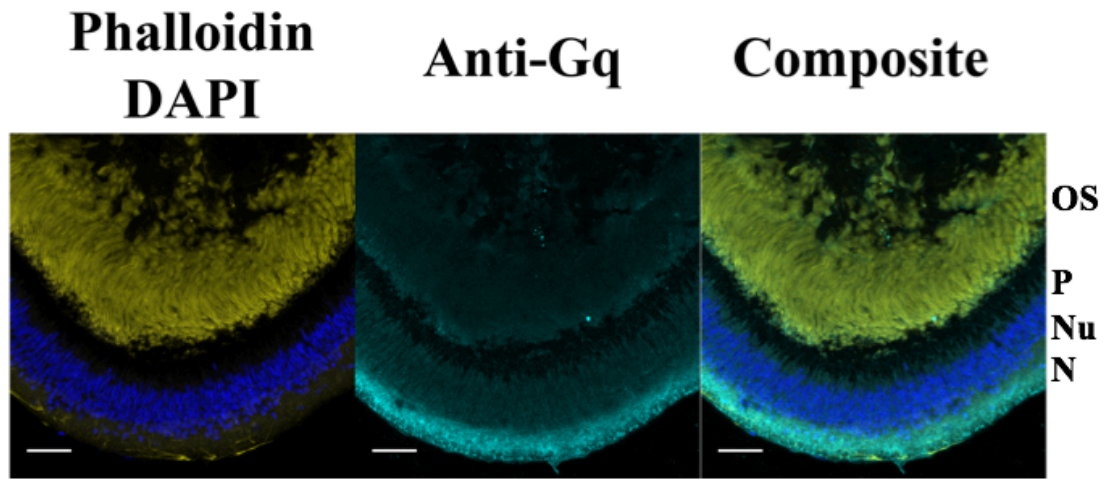


Figure 3.8. Expression of Gα_q protein in the fully intact retina of *Strombus alatus*. Gα_q (cyan) is present in the neuropile (N) within the back of the retina. Phalloidin (yellow) labeled the outer segments (OS) of photoreceptors which are shown to be curved and densely packed above the pigment (P) layer. The nuclei (Nu) of the inner segments of photoreceptors are seen DAPI stain (blue). Scale bar = 50μm.

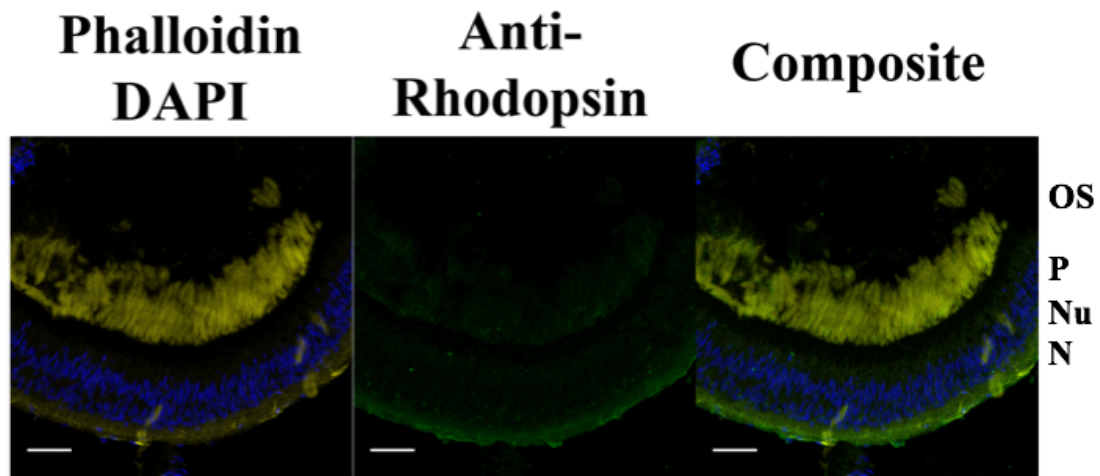


Figure 3.9. Expression of r-opsin protein in the fully intact retina of *Strombus alatus*. R-opsin (green) is present in the neuropile (N) within the back of the retina. Phalloidin (yellow) labeled the outer segments (OS) of photoreceptors which are shown cylindrical and densely packed above the pigment (P) layer. The nuclei (Nu) of the inner segments of photoreceptors are seen by DAPI stain (blue). Scale bar = 50 μ m.

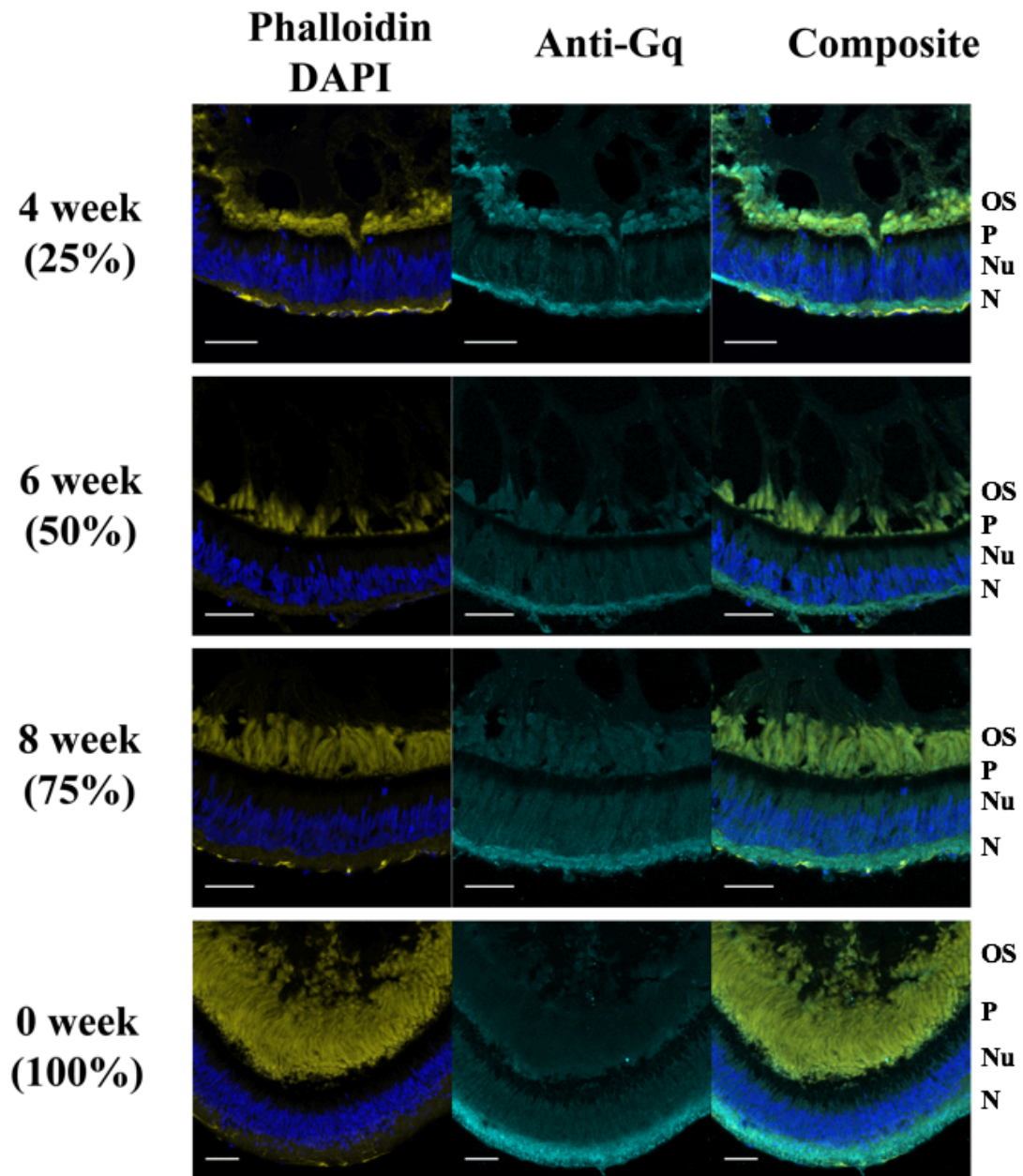


Figure 3.10. Expression of $G\alpha_q$ protein in the regenerating retinas of *Strombus alatus*. $G\alpha_q$ (turquoise) is present in the neuropile (N) within the back of the retina beginning within week 4 post-eye removal. The outer segments (OS) are above the pigment (P) layer. Phalloidin (yellow) labeled the outer segments of photoreceptors which become longer, wider, and more densely packed as the eye regenerates. The nuclei of the inner segments of photoreceptors are seen by DAPI stain (blue). Scale bar = 50 μ m.

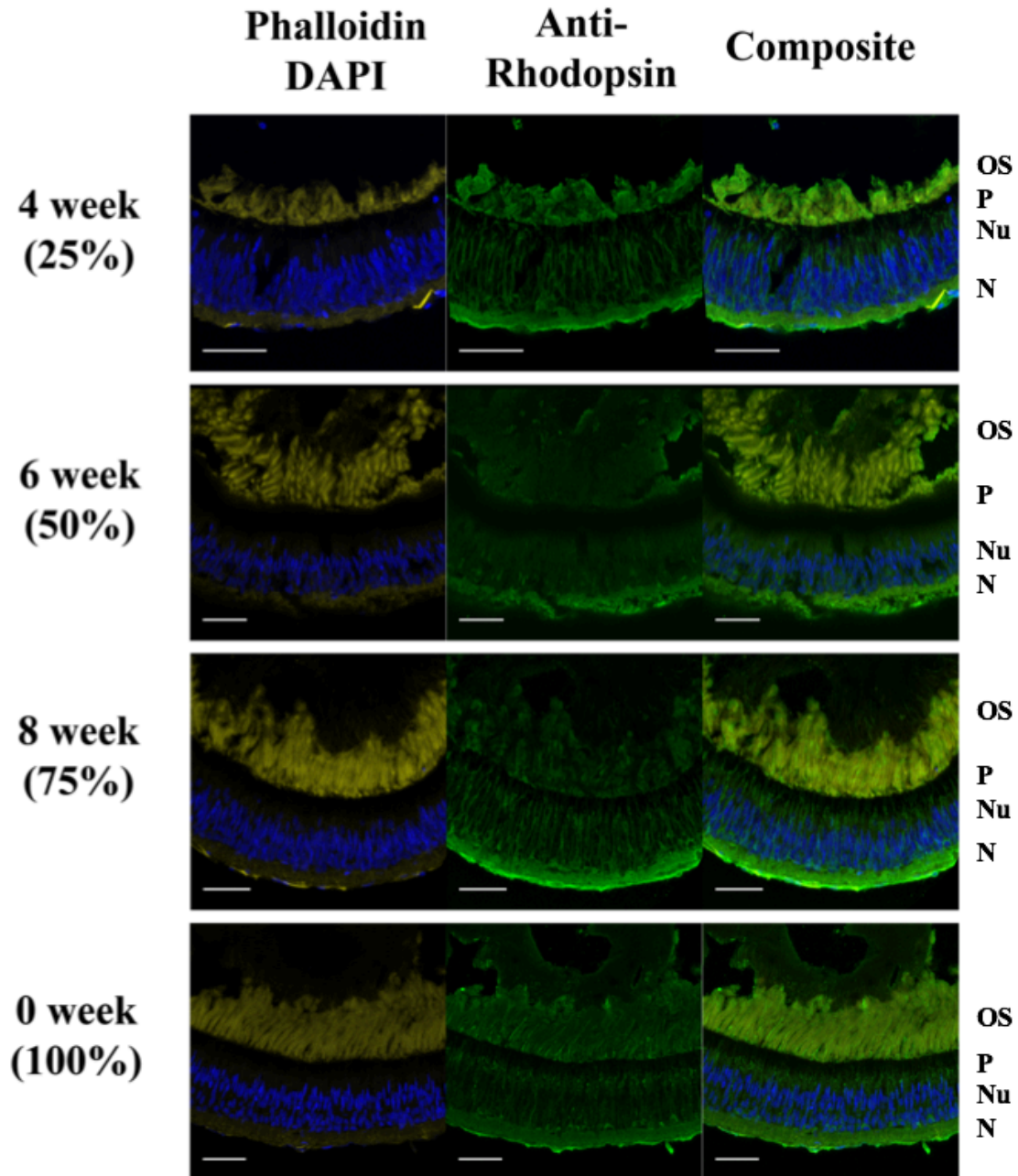


Figure 3.11. Expression of r-opsin in the regenerating retinas of *Strombus alatus*. R-opsin (green) is present in the neuropile (N) and between the outer segments (OS) of photoreceptors within the retina 4 and 8 weeks post-eye removal and in the intact eye. However, r-opsin does not appear to be present in the outer segments during week 6 post-eye removal. Phalloidin (yellow) labeled the outer segments of photoreceptors which become longer, wider, and more densely packed as the eye regenerates. The nuclei (Nu) of the inner segments of photoreceptors are seen by DAPI stain (blue) below the pigment (P) layer. Scale bar = 50 μ m.

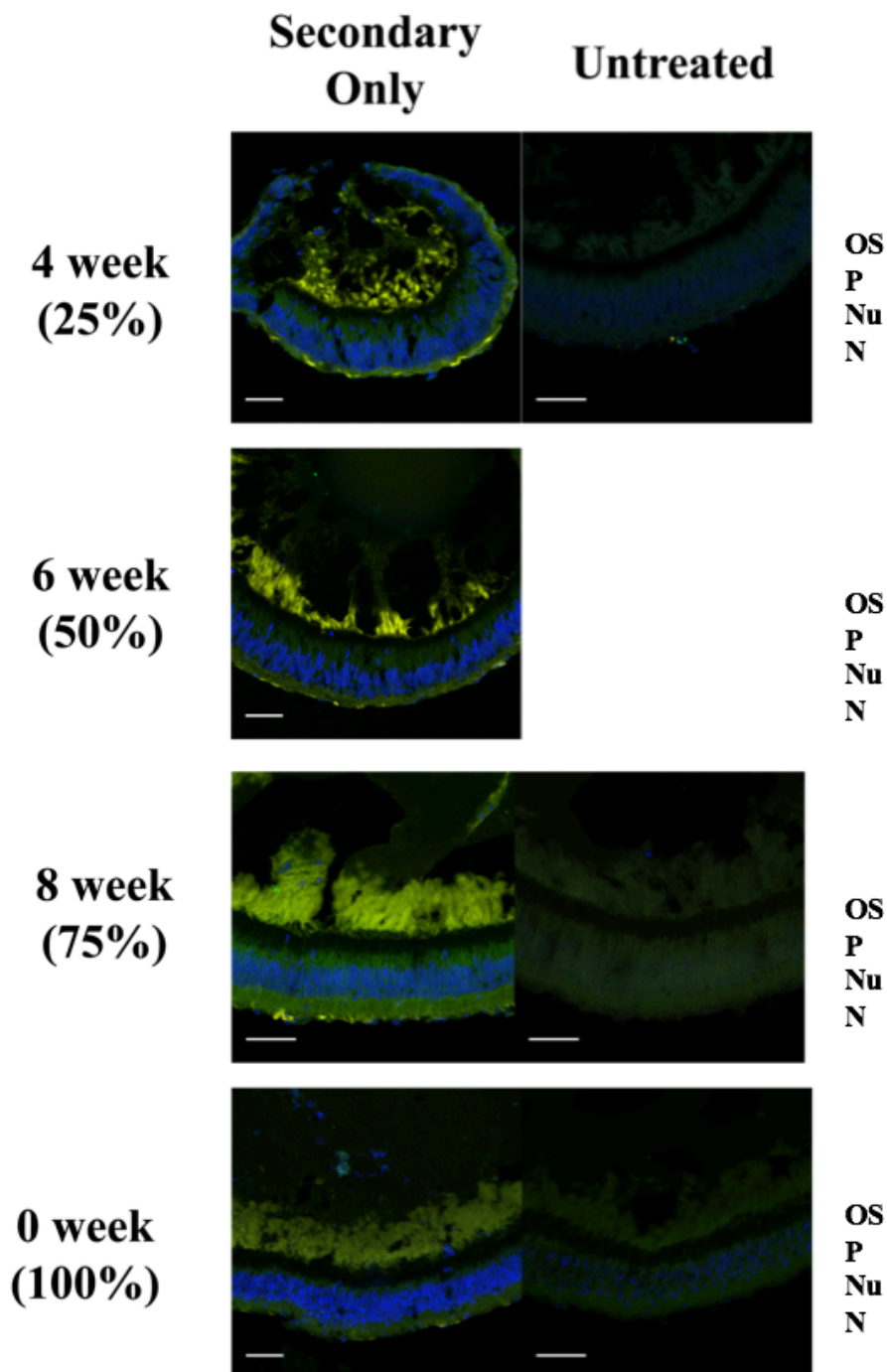


Figure 3.12. Control labeling in the retinas of *Strombus alatus*: Secondary antibody only controls and untreated controls for each week post-eye removal. However, all of the untreated sections fell off the slide during week 6 experiments. Phalloidin (yellow) labeled the outer segments (OS) above the pigment (P) layer and DAPI stained the nuclei (Nu) of the inner segments of photoreceptors above the neuropile (N). The secondary-only controls show minimal fluorescence and the untreated controls show minimal autofluorescence. Scale bar = 50 μ m.

CHAPTER 4

DISCUSSION

Our study of restoration of visual performance and opsin expression during eye regeneration of the Florida fighting conch (*Strombus alatus*) had several objectives. First, we sought to document eye regeneration of the Florida fighting conch (*Strombus alatus*) under laboratory conditions that might differ from other studies since eye regeneration is temperature dependent (Bever & Borgens, 1988). Second, previous studies have anatomically documented eye regeneration (Hughes, 1976; Bever & Borgens, 1988; Gillary & Gillary, 1979), however, here we explored visual performance during eye regeneration and when visual capabilities are restored during this process. Third, the layers of the *Strombus* retina have been well documented (Gillary & Gillary, 1979; Seyer, 1994), but the photoreceptive proteins expressed in the retina allowing for visual capabilities are poorly understood; thus, our goal was to detail $G\alpha_q$ and r-opsin expression within the retina and how expression changes during eye regeneration.

Eye regeneration

We have recorded the visual capabilities of *S. alatus* and how these capabilities return to the eye during eye regeneration. Eye regeneration rates documented in our study could theoretically be slower than other studies due to the temperatures our animals were

kept at (Hughes, 1976; Eaking & Ferlatte, 1973). Our animals were kept in a recirculating saltwater aquarium at a consistent 19°C. In previous studies, water was maintained at warmer temperatures such as 30°C (Hughes, 1976) and room temperature (Bever & Borgens, 1988). These warmer temperatures may lead to faster rates of eyespot appearance which were about 14 days in conch of our study (Figure 3.1), but occurring on average around 8.54 days in three different species of *Strombus* (Hughes, 1976) and just before 10 days in the mystery snail (family *Ampullariidae*; Bever & Borgens, 1988). Eye regrowth occurred the fastest between 3-6 weeks after eye removal but decreased after week 6 which perhaps signals that the eye is functional and energy can be allocated elsewhere. The energetic effects on eye regeneration between varying temperatures is not well understood but could be useful in targeting how eye regeneration would naturally occur in the wild and how it could vary with seasons.

Visual performance

We have reported for the first time the visual performance and the return of those abilities during eye regeneration in *S. alatus*. Similar to previous researchers, we observed that *S. alatus* demonstrates relatively complex visual behavior, such as sudden defensive reflexes with the sudden approach of objects (Gillary & Gillary 1979; Seyer, 1994) and increasing the “waving” of eyestalks toward a predator (Field, 1977). The defensive responses demonstrated by *S. alatus* varied between eye flinches to eyestalk withdrawals to full reflexes into the shell (Table 2.1). These behaviors were seen as defensive responses presumably to protect the animal from potential predators.

Prior to eye removal, conch responded to the slowest target (16°/sec) most often with a diversity of defensive responses (Figure 3.3). However, after eye removal in week 4, conch reacted to the largest target, target 7, most often with a lower diversity of reactions, exhibiting double eye flinches and left eye flinches more often (Figure 3.4). During weeks 6 and 8 post-eye removal, conch reacted to several targets indistinguishably but with a greater occurrence of partial responses than previous weeks (Figures 8 and 9).

Notably, in the behavioral studies of *S. alatus*, substantial differences in visually influenced behavior were seen between the left and right eyes. For the left eye removals, there was not a significant difference between behavior prior to eye removal and after eye removal until the 10th week. The lack of differing behavior could have resulted from these conch still having an intact right eye that could potentially compensate for this loss in its visual field. For the right eye removal group, visual performance declined but appeared to be restored in the 4th week after eye removal. This difference in the right eye removal group suggests that the left intact eye was not able to compensate for the loss in the right eye visual field.

When both of their eyes were removed, conch still exhibited defensive responses to visual stimuli alluding to the presence of photoreceptors elsewhere within tissue. However, these photoreceptors most likely cannot distinguish between shadows and objects leaving more sophisticated tasks for the complex camera-type eyes these animals possess. The visual performance of the both eye removal group still declined after eye removal, but appeared to be restored 6 weeks after eye removal which was later than the single eye removal groups. The longer time observed in the both eye removal group is

most likely due to the energetics of having to regenerate two eyes rather than just one. It would be interesting to see if there are differences in eye regeneration rates between the right and left eyes of *S. alatus*.

Previous studies have documented that Strombid gastropods respond to a mixture of chemical, visual, and tactile stimuli and that a chemical stimulus is sufficient enough to produce defensive responses such as lunging movements (Berg, 1974). It has also been suggested that molluscs have a well-defined chemosensory system where the visual system should be, hypothetically, less developed than it is (Emery, 1992). What this study and previous studies have found is the visual system of *Strombus* is complex and exhibits a variety of defensive responses that allude to a more accurate predator response. This selective defensive response would allow a wild animal to reserve energy for other tasks rather than wasting energy on false responses to non-threatening stimuli. It is probable that Strombid gastropods would be using a combination of visual and chemosensory cues to develop such a selective defensive response, but having both complex systems would allow for even more precise responses than originally hypothesized. Further studies could investigate the accuracy of the *Strombus* defensive response by using a variety of combinations of visual and chemical cues of predators and non-threatening animals to see what *S. alatus* responds to and to what degree of defensive reflex they exhibit.

It has also been documented that *Strombus gigas* and that other species of conch are nocturnal and are more active during dusk and night time than during the daytime (Randall, 1964; Sandt & Stoner, 1993; Seyer, 1994). Considering the nocturnal behavior of conch, another idea for the complexity seen in the eye of *Strombus* is that visual

performance is built for higher sensitivity for night-time activity. Seyer (1994) also suggests a possibility in *Strombus raninus* that the output from the rhabdoms may be neurally pooled together at night-time which would increase sensitivity at the cost of resolution. Behavioral trials could help clarify differences in visual performance between day and night activity. Also, measuring the spatial receptive field of a photoreceptor using intracellular electrophysiology could determine the spatial resolution of the *Strombus* eye.

Surprisingly, we did not find any differences between eyestalk angle before and after eye removal nor any evidence suggesting compensation in the intact eyestalk angle after one eye is removed (Table 3.2). As observed in our behavioral trials and those from Field (1977), eyestalk angle appeared to change after eye removal but these differences were not statistically different from the intact eyestalk angles. Field (1977) observed the angling of eyestalks toward *Conus* predators and an increase in eyestalk “waving” toward the predator. A possible error in our quantification of eyestalk angles could be taking one angle measurement as a snapshot in time per week and per animal, but not measuring the “waving” activity of each eyestalk, like Field (1977) had previously observed. Future studies could include analyzing the movement of eyestalks as visual stimuli are presented and to track the directions in which the eyestalks are angling towards.

Immunolabeling of Eyes

Our immunolabeling experiments yielded odd yet interesting results: 1) the photoreceptors in the retinas of *S. alatus* express molecular components similar to those

expressed by the photoreceptors of cephalopod and other gastropod retinas and 2) the photoreceptors in conch retinas begin expressing the molecular components of phototransduction early in the regenerative process, during week 4 after eye removal. The outer segments (rhabdoms) of the retina in *S. alatus* were found to be less densely packed, shorter and curved unlike other *Strombus* studies (Gillary & Gillary, 1979; Gillary, 1983; Seyer, 1994) or other mollusc camera-type eyes suggest (Katagiri *et al.*, 2001; Kingston *et al.*, 2015a; Kingston *et al.*, 2015b).

G α_q labeled the back of the eye in *S. alatus* within the neuropile and the eye capsule (Figure 3.8). This protein is widely used and transported around the eye but could theoretically be present anywhere in the retina. Kingston *et al.* (2015a) saw G α_q antibody binding throughout the inner (nuclear layer) and outer segments (rhabdomeric layer) of the retina in *D. pealeii* which is why we expected to see G α_q throughout these layers in *S. alatus* as well. It is unknown where G α_q is produced but it would be surprising if it was in the neuropile because this layer is densely packed with neurons and used for other tasks. It would be less surprising to see r-opsin and G α_q within the inner segments than the back of the eye. In order to clarify the makeup of these different layers and immunolabeling variation from expected results, a logical continuation would be to immunolabel more eyes from multiple species of *Strombus* with more antibodies (specifically anti-G proteins) and perform more histological studies on the layers of the retina.

R-opsin was present in the back of the eye within the neuropile and near the back of the eye capsule as well (Figure 3.9). This was unexpected because r-opsin should be expressed in the outer segments as suggested by previous studies on the immunolabeling

in similar camera-type eyes (Katagiri *et al.*, 2001; Kingston *et al.*, 2015a; Kingston *et al.*, 2015b) not in the back of the eye. R-opsins were found throughout the entirety of the outer segments within the retina of a sea slug (*Onchidium*; Katagiri *et al.*, 2001) and the Longfin Squid (*Doryteuthis pealeii*; Kingston *et al.*, 2015a). However, in Kingston *et al.* 2015b, r-opsin antibody labeled the top and bottoms of the outer segments due to a high density of rhabdoms, which was also a difference seen in the retinas of *S. alatus*. R-opsin is made in the inner segments but shuttled to the outer segments by vesicle transportation where we would be expected to see it present. There is no evidence that r-opsin is expressed anywhere else in the retina so why the anti-r-opsin labeled in the back of the eye is unknown.

Unexpectedly, the outer segments were labeled with phalloidin meaning there was presence of actin within this layer of the retina (Figure 3.8 & 3.9). Structurally, there could be actin present but actin labeling in gastropod retinas has not been reported. It would be less surprising to see phalloidin label the inner segments of photoreceptors more than the outer segments because there should be transportation proteins and microtubule tracks throughout this layer. There should be microtubule tracks in the outer segments but why phalloidin did not label these in the inner segments is unknown.

During the stages of eye regeneration, the outer segments of photoreceptors became wider, longer, and more densely packed. The retinal layers appeared to become more organized, separated, and thicker as the eye regenerated as expected (Figure 3.10 & 3.11). R-opsin and $G\alpha_q$ were both present in the 4 week (25% regenerated) eyes suggesting that visual capabilities could be restored at this regeneration stage which is consistent with the results from the behavioral analyses for single eye removals. To

continue looking at the presence of phototransductive proteins in the retina during eye regeneration, it would be of interest to look at how these proteins are expressed in regenerating eyes after both eyes were removed to see if their presence is in agreement with visual performance becoming restored 6 weeks after both eye removal.

WORKS CITED

- Alvarado, A. S., and Tsonis, P. A.** (2006). Bridging the regeneration gap: genetic insights from diverse animal models. *Nature Reviews Genetics*. 7(11), 873.
- Arendt, D.** 2003. Evolution of eyes and photoreceptor cell types. *International Journal of Developmental Biology*. 47(7-8), 563-571.
- Berg, C. J.** (1974). A comparative ethological study of Strombid gastropods. *Behaviour*. 51(3), 274-321.
- Bever, M. M. and Borgens, R. B.** (1988). Eye Regeneration in the Mystery Snail. *The Journal of Experimental Zoology*. 245, 33-42.
- Bouchet, P.** (2015). *Strombus alatus*. *MolluscaBase*. World Register of Marine Species <http://www.marinespecies.org/aphia.php?p=taxdetails&id=419694> on 2017-02-16
- Burphy, R. W.** (2011). Controls for Immunocytochemistry: An Update. *Journal of Histochemistry & Cytochemistry*. 59(1), 6-12.
- Call, M. K., Grogg, M. W., and Tsonis, P. A.** (2005). An Eye on Regeneration. *Anatomical Record. Part B, New Anatomist*. 287(1), 42-48.
- Call, M. K., Grogg, M. W., Del Rio-Tsonis, K., and Tsonis, P. A.** (2004). Lens regeneration in mice: implications in cataracts. *Experimental eye research*. 78(2), 297-299.
- Charles, G. H.** (1966). Sense organs (less cephalopods). In *Physiology of Mollusca* (ed. K. M. Wilbur and C. M. Yonge), pp. 455-521. London: Academic Press.
- Chétail, M.** (1963). Etude de la regeneration du tentacule oculaire chez un Arionidae (*Arion rufus* L.) et un Limacidae (*Agriolimax agrestis* L.). *Archives de Anatomie Microscopique et de Morphologie Experimentale*. 52(1), 129.
- Dinsmore C. E.** (Ed.). (1991). *A history of regeneration research*. Cambridge University Press: New York, NY.
- Eakin, R. M. and Ferlatte, M. M.** (1973). Studies of eye regeneration in a snail, *Helix aspersa*. *Journal of Experimental Zoology*. 184 (1), 81-96.
- Eakin, R. M. and Brandenburger, J. L.** (1967). Differentiation in the eye of a pulmonate snail, *Helix aspersa*. *Journal of Ultrastructure Research*. 18, 391-421.
- Emery, D. G.** (1992). Fine structure of olfactory epithelia of gastropod molluscs. *Microscopy research and technique*. 22 (4), 307-324.
- Field, L. H.** (1977). An Experimental Analysis of the Escape Response of the Gastropod *Strombus maculatus*. *Pacific Science*. 31 (1), 1-11.
- Gibson, B. L.** (1984). Cellular and ultrastructural features of the regenerating adult eye in the marine gastropod *Lymnaea stagnalis*. *Journal of Morphology*. 180 (2), 145-157.
- Gillary, H. L.** (1983). Electrical potentials from the regenerating eye of *Strombus*. *Journal of experimental biology*, 107 (1), 293-310.

- Gillary, H. L. and Gillary, E. W.** (1979). Ultrastructural Features of the Retina and Optic Nerve of *Strombus luhuanus*, a Marine Gastropod. *Journal of Morphology*. **159** (1), 89-115.
- Gillary, H. L.** (1971). Electrical responses from the mature and regenerating eye of *Strombus*, a marine gastropod. *American Zoology*. **2**, 672.
- Hankó, B.** (1914). Über das Regenerationsvermögen und die Regeneration verschiedener Organe von *Nassa mutabilis* (L.). *Archiv für Entwicklungsmechanik der Organismen*. **38** (3), 447-507.
- Hughes, H. P.** (1976). Structure and regeneration of the eyes of strombid gastropods. *Cell and tissue research*. **171** (2), 259-271.
- Katagiri, N., Terakita, A., Shichida, Y., and Katagiri, Y.** (2001). Demonstration of a rhodopsin-retinochrome system in the stalk eye of a marine gastropod, *Onchidium*, by immunohistochemistry. *Journal of Comparative Neurology*. **433** (3), 380-389.
- Kingston, A. C. N., Kuzirian, A. M., Hanlon, R. T., and Cronin, T. W.** (2015a). Visual phototransduction components in cephalopod chromatophores suggest dermal photoreception. *Journal of Experimental Biology*. **218** (10), 1596-1602.
- Kingston, A. C. N., Wardill, T. J., Hanlon, R. T., and Cronin, T. W.** (2015b). An Unexpected Diversity of Photoreceptor Classes in the Longfin Squid, *Doryteuthis pealeii*. *Plos one* **10** (9), e0135381.
- Land, M. F.** (1981). Optics and vision in invertebrates. *Handbook of Sensory Physiology, Vision in Invertebrates*. (ed. H.J. Autrum), pp. 471-592. Berlin: Springer.
- Mitashov, V. I.** (1996). Cell sources, regulatory factors and gene expression in the regeneration of the crystalline lens and retina in vertebrate animals. *Izvestiia Akademii nauk. Seriya biologicheskaya*. **3**, 298-318.
- Mitashov, V. I.** (1966). Comparative study of lens regeneration in cobitid fishes (*Misgurnus fossilis*, *Nemachilus barbatulus*, *Nemachilus dorsalis*). *Doklady Akademii Nauk SSSR*. **170** (6), 1439-1442.
- Randall, J. E.** (1964). Contributions to the biology of the queen conch, *Strombus gigas*. *Bulletin of Marine Science*. **14** (2), 246-295.
- Sakai, F., Agata, K., Orii, H., and Watanabe, K.** (2000). Organization and Regeneration Ability of Spontaneous Supernumerary Eyes in Planarians- fEye Regeneration Field and Pathway Selection by Optic Nerves. *Zoological science*. **17** (3), 375-381.
- Sandt, V. J. and Stoner, A. W.** (1993). Ontogenetic shift in habitat by early juvenile queen conch, *Strombus gigas*: patterns and potential mechanisms. *Fishery Bulletin*. **91**, 516-525.
- Seyer, J. O.** (1994). Structure and optics of the eye of the hawk-wing conch, *Strombus raninus* (L.). *Journal of Experimental Zoology*. **268** (3), 200-207.
- Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid et al.** (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9**, 676-682.
- Slack, J. M. W.** (2006). *Essential Developmental Biology*. Malden, MA: Blackwell Publishing company.