

Faculty of Science

**RESPONSE OF BODY MORPHOMETRICS AND EYE DEVELOPMENT OF THE  
CRAYFISH (*CAMBARELLUS PATZCUARENSIS*) TO DIFFERING LIGHT  
WAVELENGTHS EXPERIENCED DURING JUVENILE LIFE**

2017 | LAUREN MARIA ZINK

**B.Sc. Honours thesis - Biology**



**RESPONSE OF BODY MORPHOMETRICS AND EYE DEVELOPMENT OF THE  
CRAYFISH (CAMBARELLUS PATZCUARENSIS) TO DIFFERING LIGHT  
WAVELENGTHS EXPERIENCED DURING JUVENILE LIFE.**

by

LAUREN MARIA ZINK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE (HONS.)

in the

DEPARTMENT OF BIOLOGICAL SCIENCES

(Animal Biology)



**THOMPSON RIVERS UNIVERSITY**

This thesis has been accepted as conforming to the required standards by:

Louis Gosselin (PhD), Thesis Supervisor, Dept. Biological Sciences  
Cynthia Ross Friedman (PhD), Co-supervisor, Dept. Biological Sciences  
Mark Rakobowchuk (PhD), Co-supervisor, Dept. Biological Sciences  
Robert Higgins (PhD), Examining Committee member, Dept. Biological Sciences

Dated this 21 of April, 2017, in Kamloops, British Columbia, Canada

© Lauren Maria Zink, 2017

## ABSTRACT

Thesis Supervisor: Professor Louis Gosselin

The growth process in crustaceans is periodic and can be affected by a variety of environmental factors. Photoperiod has been hypothesized to indirectly affect growth by altering feeding behaviour; in addition, previous studies have found that light quality directly affects eye development in many crayfish species both externally through ommatidia development and internally by altered pigments present in the eye. However, it is not known at what stage in life these changes occur. A key characteristic in crayfish growth is allometric (proportional) growth exhibited between body size and sexually selected characteristics such as cheliped size. This study examined whether specific light wavelengths (blue, red, white, and no light) influence the growth and morphology of the orange dwarf crayfish, *Cambarellus patzcuarensis*, in terms of body size, eye size, and specific eye parameters of ommatidia size and density in both juveniles and adults. Crayfish were raised from one-week old juveniles to adult (16 weeks) or juvenile (7 weeks) life stages in controlled environments. Upon sampling, digital photographs of each individual were taken to assess body size and colouration and both eyes were removed. The left eyes were fixed and scanning electron micrographs taken to document surface features of the eye. Eyes were then embedded in resin and sectioned to measure the depth of ommatidia inside the eye. The right eyes were pooled by treatment, homogenized, and pigments suspended into solution before absorbance spectra over the visible light range were taken using a spectrophotometer. The light wavelengths experienced during juvenile growth did not significantly influence body growth or the development of external morphological characteristics of the eye such as eye size, ommatidia size or ommatidia density in individuals of either life stage ( $P > 0.05$ ). The absorbance spectra of homogenized eye tissues suggests light wavelengths did influence the production of pigments in the eyes. In particular, the eyes of individuals grown under blue light had lower absorbance in the 550-570 nm range and through to 750 nm, which is consistent with the absorption spectrum of ommin pigments. Eye size as well as ommatidia size and density developed proportionally with body size in juveniles ( $P < 0.05$ ); however, this allometry did not appear to be maintained into adult life. Ommatidia size and density were also correlated in juveniles in relation to eye size ( $P < 0.05$ ); however, only ommatidia density remained correlated with eye size in adults. External morphology was not found to differ between treatments, but absorbance spectra appeared to differ from 550-750 nm (yellow-orange).

## **ACKNOWLEDGEMENTS**

I would like to thank my team of supervisors for their guidance, support, and wisdom in their respective specialties through the development and carrying out of this project. I would also like to thank Dylan Ziegler and Dr. Jacob Omajali for their technical expertise in data collection. I would like to thank the Thompson Rivers University Undergraduate Experience Award Program for providing the funding to carry out this project as well as the NSERC Discovery grants held by Drs. Louis Gosselin and Cynthia Ross Friedman. Special thanks to Cody York and Darci Warkman for feeding the crayfish when I was unable to be in lab. Finally, I would like to express my sincere thanks to all those who have, either knowingly or not, lent a hand in this venture.

## **DEDICATION**

I dedicate this thesis to Teegan Chamberlain, who has been by my side during this venture providing both encouragement and “fresh eyes” throughout.

# TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iii
Dedication.....	iii
Table of Contents.....	iv
List of Figures.....	v
Introduction.....	1
Growth process and patterns in crayfish.....	1
Eye structure and development in crayfish.....	1
Methods and Materials.....	4
Rearing of crayfish: tank setup and maintenance.....	4
Imaging and eye removal at end of experiment.....	5
Exoskeleton colour brightness and external body measurements.....	6
Effects of controlled wavelength exposure on absorption spectra of the eye.....	7
Growth of eye parameters.....	8
Statistical analyses.....	10
Results.....	10
Effects of controlled wavelength exposure on growth and morphology.....	10
Effects of controlled wavelength exposure on absorption spectra of the eye.....	11
Exoskeleton colour brightness.....	12
Growth of eye parameters.....	13
Discussion.....	16
Effects of controlled wavelength exposure on growth and morphology.....	16
Effects of controlled wavelength exposure on absorption spectra of the eye.....	18
Exoskeleton colour brightness.....	19
Growth of eye parameters.....	19
Future directions.....	20
Literature Cited.....	21
Appendix A: Tank Setup.....	23
Appendix B: LED Light spectra.....	24

## LIST OF FIGURES

Figure 1. Longitudinal section of a crayfish eye showing layers within the eye. Figure reference: see literature cited Meyer-Rochow 2001.....	3
Figure 2. Exoskeleton brightness sampling areas for unpigmented (A) and pigmented (B) regions as well as carapace length measure (C). .....	6
Figure 3. Scanning electron micrograph of eye showing distances measured to calculate percent eye covered by ommatidia (A) and eye diameter (B). .....	9
Figure 4. Light micrograph of cross-section of eye showing diameter (A) and sampling points to measure ommatidia depth (B and C). .....	10
Figure 5. Body size (as measured by carapace length) of individuals at end of experiment as a function of colour treatment and life stage (Juvenile= Generation 2; Adult = Generation 1). Differences among treatments are not quite significant, possibly due to small sample size in the dark treatment (Blue = 9 crayfish, Red = 11 crayfish, White = 9 crayfish, Dark = 2 crayfish). Error bars represent standard error of the mean. ....	11
Figure 6. Absorbance of eyes (grouped by treatment) over the range of the visible spectrum. Inset shows the full range of tested wavelengths; main graph shows an expanded view of results over the 525 – 750 nm range. Point A represents peak characteristic of xanthommatin. Point B represents absorbance range of ommin pigment. Point C represents unidentified peak in red light treatment spectrum. Absorbance values have been standardized by eye volume. ....	12
Figure 7. Brightness values of pigmented and unpigment regions of the exoskeleton of juvenile and adult crayfish. A brightness value of 1 represents pure black, and a value of 140 represents pure white. ....	13
Figure 8. Eye size (diameter) as a function of body size (carapace length). Regression line is for juveniles only. ....	14
Figure 9. A: Ommatidia size as a function of body size (carapace length). Error bars show standard deviation. B: Ommatidia density as a function of body size (carapace length). In both graphs, the regression line is for juveniles only. ....	15
Figure 10. A: Ommatidia size as a function of eye size (diameter). Regression is for juveniles only. Error bars represent standard deviation. B: Ommatidia density as a function of eye size (diameter). ....	17

## **INTRODUCTION**

### ***Growth process and patterns in crayfish***

Growth is a key component in the existence of an organism. For crustaceans, growth occurs in periodic dimensional increases, rather than continuous growth as observed in many other taxa. This method of growth entails two phases: (1) a phase of cell division and protein synthesis and (2) a molting phase (ecdysis), which is the process of shedding the calcified exoskeleton to allow expansion of the soft tissue beneath, before a new exoskeleton reforms (Aiken and Waddy 2015). The molt stage of an individual can be estimated by hardness of the shell or by colouration of the shell, as it becomes progressively darker leading up to a molt (Needham and Brunet 1957, Ramalho et al. 2008; Stumpf et al. 2011). The growth of crayfish can show high variation in parameters such as molt frequency and size increase per molt; however, crayfish exhibit the characteristic of allometric or relative growth, which is the proportional growth of two changing dimensions (Lovett and Fedler 1989). Studies across many species of crayfish have revealed allometric growth between body size and sexually selected characteristics such as cheliped size and abdomen width (Boyd and Page 1978; Buřič et al. 2010; Mariappan et al. 2004).

An individual's molt cycle can be affected by the environmental conditions it experiences, such as temperature, seasonality, water movement cycles, density/crowding, habitat substrate, water quality, nutrition, and photoperiod (Aiken and Waddy 2015). In comparison to other environmental factors, less work has been done to investigate the effect of photoperiod on growth. Photoperiod is hypothesized to only indirectly affect growth through altering behaviour and feeding activity (Aiken and Waddy 2015); however, other properties of light, such as light quality, have directly affected eye structure and development, which has been well described (Culver 1987; Mejía-Ortíz and Hartnoll 2005; Cooper et al. 2001; Tokarski and Hafner 1984; Walcott 1974).

### ***Eye structure and development in crayfish***

The compound eye allows animals like the crayfish to achieve a large field of view with a small spherical eye structure (Cao et al. 2015). The small size of the compound eye allows crayfish to not expend additional energy in the creation of a large eye, as well as allows for less total weight of the individual allowing for quicker movement and escape from predators (Aiken and Waddy 2015). The compound eyes in arthropods are divided into individual ommatidia, each

containing pigments, cone cells, or secreted lenses depending on the species (Callaerts et al. 2006). Crayfish have a type of compound eye referred to as superposition eyes, which are deemed complex because they can form complete images and contain large amounts of photoreceptor and pigment cells (Erclik et al. 2009). In the compound eye, an image is formed by integration of input from each ommatidia to form a single image at the retinal surface, allowing crayfish to see in greater detail from further distances than vertebrates (Land 1990, Erclik et al. 2009).

The compound eye observed in crayfish is composed of layers containing dioptric (light refracting and absorbing) structures and a rhabdomere, which is equivalent to a retina in vertebrates (Figure 1) (Meyer-Rochow 2001, Chen and Hua 2016). Within the dioptric zone of the compound eye, there is a pigment screen, which consists of cells that either absorb or reflect light, called pigments (Struwe et al. 1975). These pigments have been broadly classified into ommochromes (absorptive pigments), and pteridines (reflective and/or fluorescent pigments) (Struwe et al. 1975). Previous studies have found that reflecting pigments remain stationary, whereas the absorptive pigments are able to migrate between cells in the eye (Bryceson 1986). The structure and composition of this pigment screen has been found to alter the spectral sensitivity of the eye in arthropods (Struwe et al. 1975).

The superposition compound eye is able to morphologically adapt to allow for maximum optical information in the natural environment. Characters of the eye that have been observed to differ between habitats include ommatidial facet diameter, interommatidial angle, photoreceptor presence and arrangement, and the dimensions of the rhabdomere (Chen and Hua 2016). Changes in the structural shapes of cells as well as cell contents can occur to maximize the performance of the visual system and have been observed to change over the lifetime of a single individual and be triggered by ambient light conditions, stressing the need to develop a clear understanding of eye development in crayfish throughout life (Meyer-Rochow 2001). This adaptation occurs throughout the development of the ommatidia, which can take place in one of two ways: the "row by row" fixed development type in which ommatidia are all created of equal size and are unable to change beyond formation, or the "morphogenetic front" continuous development type in which ommatidia can change in size after being formed (Harzch and Hafner 2006). The morphogenetic type of development is typically exhibited in crustaceans (Harzch and Hafner 2006).

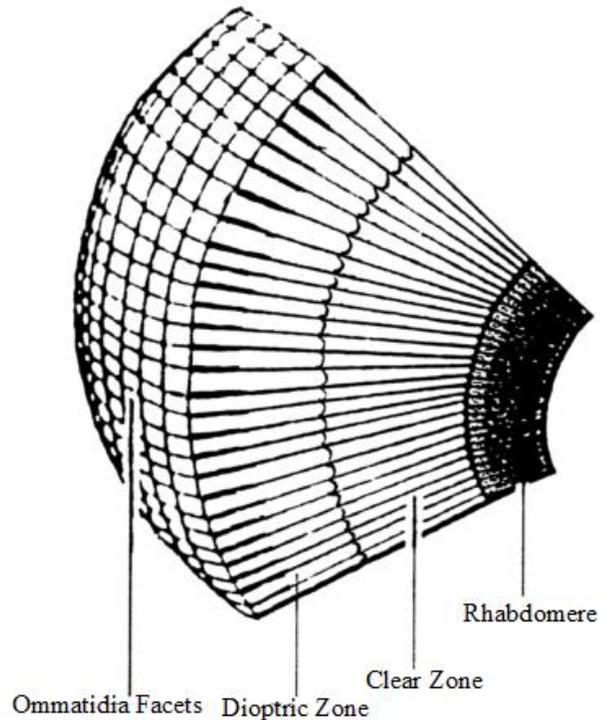


Figure 1. Longitudinal section of a crayfish eye showing layers within the eye.  
Source of figure: Meyer-Rochow (2001).

Many studies, such as those carried out by Culver (1987), Mejía-Ortíz and Hartnoll (2005), Cooper et al. (2001), Tokarski and Hafner (1984), and Walcott (1974), have found differences in eye structures between species of crayfish from habitats with different levels of light exposure, including facet size, cone length, pigmentation, and rhabdomere size. For example, crayfish that inhabit caves with little light exposure tend to have smaller eye size and ommatidia when compared to crayfish living in areas with high light exposure such as a shallow spring (Culver 1987). What these studies did not take into account was ontogeny as the eye continues to develop throughout life, as well as the influence of specific light wavelengths.

The present study aimed to determine the effects of exposure to certain, controlled wavelengths of light on the development and morphology of the eye in the orange dwarf crayfish, *Cambarellus patzcuarensis*, as well as relate the development of the eye to the allometric growth patterns observed in other quantitative features of crayfish. This species is commonly sold for aquarium use and therefore is subjected to a variety of light conditions depending on the tank in which they are housed. The specific objectives of this study were to determine: (1) if the development of eye parameters (eye size, ommatidia size, ommatidia

density, ommatidia depth, and pigments present in the eye) is influenced by the wavelengths of light to which individuals are exposed to during juvenile life; and (2) whether or not any of dimensional parameters of the eye (eye diameter, ommatidia size, ommatidia density, and ommatidia depth) exhibit allometry between each other or with body size of the individuals at both juvenile and adult life stages.

## **METHODS AND MATERIALS**

### ***Rearing of crayfish: tank setup and maintenance***

Nine large tanks (38 L) and two small tanks (7.5 L) were set up with all components needed to simulate a semi-natural crayfish habitat, including protective shelters and sediment in which they can burrow. Water chemistry (pH, nitrates, nitrites, ammonia) was monitored on a weekly basis. Each large tank contained 22 litres of water, and each small tank contained 2 litres of water at all times (see Appendix A for detailed tank setup).

Strings of light-emitting diode (LED) lights were embedded into the wire mesh of the tank lids and the intensity of the light at the surface of the sediment was standardized to  $0.6 \text{ W/m}^2$  by immersing a photometer in the tanks with the sensor at the surface of the sediment (see Appendix B for spectra of LED lights). Light intensity was recorded in seven locations (each of the four corners, and three evenly spaced samples down the center line of the tank) in each tank by moving the photometer's sensor around the tank to assess the average light intensity throughout each tank. Light bulbs were then added or removed from the string, as needed, and arranged in a way that the intensity of the light was even, both throughout each tank as well as among all colour treatments (red, blue, and white). The tanks were completely covered with opaque black rubber sheeting to prevent all external light from entering the tanks. The rubber sheeting did not affect the water temperature, which remained constant at  $23^\circ \text{ C}$  for the entirety of the experiment.

One week prior to the arrival of the crayfish, the tanks were set up and sinking fish food was introduced into each tank for the week to establish a bacterial bed to prevent New Tank Syndrome, a common aquarium disease in which high mortality is observed due to lack of established bacterial colonies. Once established, the food was removed and fifty-nine young crayfish, purchased from Shrimpkeepers Anonymous Ltd. (Calgary, AB, Canada), were introduced into the tanks and acclimatized using an established protocol (available online: [www.merlan.ca](http://www.merlan.ca)). Six crayfish were introduced to each of the blue, red and white tanks, and the

remaining five crayfish were placed into the two small tanks (two into one tank, three into the other) as a secondary control with zero light exposure (referred to as the “dark treatment”). The lights for the tanks ran on timers so that the tanks (other than the dark tanks) were exposed to 12 hours of light followed by 12 hours of darkness each day. The crayfish were fed crustacean food (Crab Cuisine brand) in excess every second day and any uneaten food was removed the next day from the tank. Tanks were cleaned using a gravel vacuum once per week, removing 20% of the total water volume at each cleaning and replacing it with an equal amount of de-chlorinated tap water at a pH of 7.6.

Juveniles aged approximately 1 week and measuring approximately 2.5 mm carapace length were placed in the experimental tanks on 15 June 2016. This cohort, referred to as generation 1, was held in the tanks until the end of the experiment on 5 October 2016, at which time they had reached the age of 16 weeks. Several crayfish in generation 1 reached maturity in August and produced a set of juveniles. A set of 20 of these juveniles, referred to as generation 2, were then also placed in the same four experimental tanks on 24 August 2016, with 5 juveniles of generation 2 per light treatment. At the time of assigning juveniles of generation 2 to the experimental tanks, they were aged approximately 8 days and were comparable in size to juveniles of generation 1 on June 15. Juveniles of generation 2 were reared in the experimental tanks up to 12 October 2016, at which time these juveniles had reached the age of 7 weeks.

### ***Imaging and eye removal at end of experiment***

All individuals remained in the light treatments until they were permanently removed from their tank and used for measurements. Crayfish were collected from the tanks and used for measurements after exposure to the light treatments for 16 weeks, and thus as adults (generation 1) or after only 7 weeks and thus still as juveniles (generation 2). At the end of each trial, individual crayfish were haphazardly selected, removed from the tanks, and put into individually-labelled dishes. All sampled individuals were in-between molts and had hardened exoskeletons. Prior to eye removal, a digital photograph of the individual was taken using a dark-field dissection microscope, and this image was later used to measure exoskeleton colouration and external body size. Both eyes were then removed at the eyestalk using dissection scissors, as carried out by Smith (1940) and Walcott (1974). The left eye was used in spectrophotometry analysis of eye pigments, while the right eye was used in microscopy analysis of the ommatidia.

### *Exoskeleton colour brightness and external body measurements*

Digital photographs, taken of each individual prior to eye removal, were used to measure body size and exoskeleton colour brightness. All images were analyzed using the software ImageJ version 1.51h (Rasband 1997-2016). To ensure a consistent imaging of body colouration among crayfish, a requirement for comparisons among individuals or among light treatments, the setup of the dissection microscope (light arm positioning, magnification, and top-mounted camera settings) remained constant throughout the imaging of all individuals from both generations. In addition, a white reference object as well as a ruler were present in each of the photographs to calibrate the white balance and distance measures, respectively, when analyzing the photograph with ImageJ. For each digital image, the white reference object was set to indicate the highest level of brightness (140) and the brightness of two areas of the carapace were then measured on a scale of 1 to 140, with 1 representing pure black and 140 representing pure white. The first area (label A in Figure 2), located along the center line of the posteriomedial edge of the carapace, appeared translucent and did not show significant variation between individuals; it is therefore said to be the “unpigmented region”. The brightness of the second area (label B in Figure 2) varied substantially among individuals, ranging from very lightly pigmented and almost as bright as the unpigmented region in some individuals to well-defined dark orange stripes in other individuals. This region is referred to as the “pigmented region”. The ImageJ software was then calibrated using the ruler before measuring the carapace length (from the tip of the rostrum to the posteriomedial edge of the carapace – label C in Figure 2).

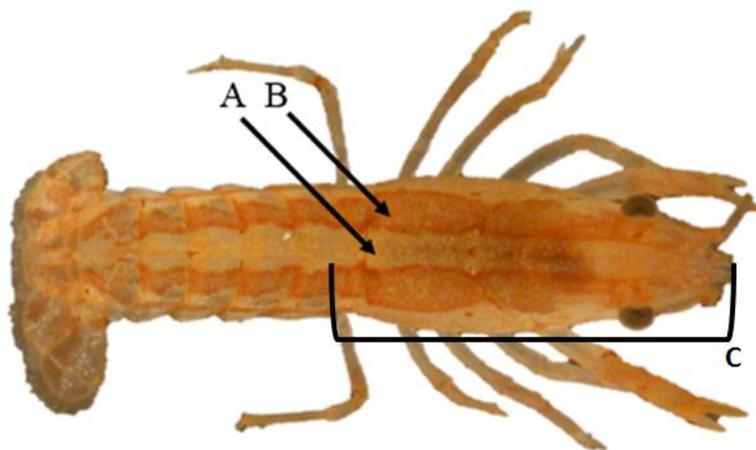


Figure 2. Exoskeleton brightness sampling areas for unpigmented (A) and pigmented (B) regions as well as carapace length measure (C).

### *Effects of controlled wavelength exposure on absorption spectra of the eye*

The left eye of each crayfish was immediately placed into individually-labelled Eppendorf tubes containing 50  $\mu\text{L}$  of 100% anhydrous ether alcohol and stored at  $-71^{\circ}\text{C}$  until processing. Due to their small sizes, the left eyes of all crayfish from a same light treatment, including both generations, were then pooled together to have enough eye material for spectroscopic analysis (i.e. minimum of 8 eyes per treatment). The dark treatment was not analyzed due to the small number of eyes (only 2) leading to an inadequate sample volume. The eyes were then transferred to 2 mL tubes containing 1 mL of 100% anhydrous ether alcohol. Two zirconia beads were then added to each of the three vials (blue, red, and white treatments) and each vial was shaken on a Biospec Mini Beadbeater for three 60 second intervals to break up the eye tissue, waiting two minutes between each interval to ensure that the solution did not reach a temperature that might cause protein denaturation. The zirconia beads were then removed from each vial and discarded. Vials were then sonicated for three 30 second intervals to lyse the cells and release pigments into solution, again waiting two minutes between intervals to prevent denaturation. The vials were then put onto an aliquot mixer overnight to allow the pigments to fully dissolve into the alcohol. Next, the vials were centrifuged for five minutes to create a dense pellet at the bottom of the vial and the supernatant was removed via needle into a 3 mL syringe. A 0.2  $\mu\text{m}$  syringe filter was then placed onto each syringe and the samples were run through the filter into a new Eppendorf tube. The vials were then vacuum centrifuged at  $30^{\circ}\text{C}$  for thirty minutes, at which point each vial contained approximately 250  $\mu\text{L}$  of solution. A portion of each sample was transferred to a quartz 200  $\mu\text{L}$  microcuvette and run through a scanning spectrophotometer to give absorbance readings for the range of the visible light spectrum (390 nm – 800 nm). The spectrophotometer was set to provide as many readings as possible for each sample; however, the spectrophotometer provided a different number of readings per sample and sampled at irregular wavelength intervals.

To account for differing total eye volumes between treatments, the volume of each eye that was included in a sample was calculated with the equation for the volume of a sphere, and using the diameter of the right eye as measured with the scanning electron micrographs, assuming that both left and right eyes of each individual were of equal size and that each eye is

spherical. The sums of the eye volumes for each treatment were used to calculate a standardized absorbance spectrum per unit eye volume.

### ***Growth of eye parameters***

The right eye of each crayfish was immediately placed into individually-labelled and sealed sample vials containing Karnovsky's fixative (25% glutaraldehyde, 4% paraformaldehyde, pH 6.8) and put onto an aliquot mixer for 48 hours at room temperature, after which the samples were removed from the mixer and put into a 4°C refrigerator. Once ready to process, the samples were washed with 0.025 M potassium phosphate buffer and returned to the refrigerator for 15 minutes. Two subsequent washes took place using the same potassium phosphate buffer at room temperature in the fume hood, waiting 15 minutes between washes. All samples were then washed three times with room temperature distilled deionized water, waiting 20-30 minutes between each wash. Samples were mounted on aluminum stubs using carbon tape and put into an environmental scanning electron microscope (Zeiss Life Sciences (LS) 15 EVO) and micrographs of the surface of each eye were taken under extended pressure (45 Pa) with as low voltage as possible (20.00 kV) to clearly see the ommatidia.

After taking micrographs, the eyes were returned to the vials and dehydrated through a series of successive alcohol washes using anhydrous ether alcohol of increasing concentrations (30%, 50%, 70%, 85%, and finally 90%), waiting 20 minutes between washes. Three washes were then performed, all with 100% anhydrous ether alcohol, and with 20 minute intervals between washes. The vials were then drained and filled with a 1:1 mixture of 100% anhydrous ether alcohol and LR White resin and put on an aliquot mixer in the fume hood for 24 hours. The solution was then replaced with a 3:1 mixture of 100% anhydrous ether alcohol and LR White resin and returned to the aliquot mixer for 24 hours. Three subsequent washes of 100% LR White resin were carried out on the aliquot mixture, each lasting 24 hours. The samples were then embedded in LR White using embedding capsule moulds with the eye oriented to be sectioned longitudinally. The LR White resin and accelerator were mixed in a 10:1 ratio and poured into the moulds. The moulds were then placed in a 60 °C oven to harden for 48 hours in Leica Ultracat incubator.

Once hardened, the samples were removed from the mould and sectioned in 2 µm increments. All sections were then mounted onto glass slides, using a slide warmer at 45° C to

ensure the sections adhered to the slide. The slides were then stained with Crystal Violet and viewed under the light microscope.

The scanning electron micrographs were used to calculate the surface properties of each eye using ImageJ. Eye diameter was determined for each individual by measuring the distance across the eye at the widest point (distance B in Figure 3). Ommatidia size was calculated by measuring the average and standard deviation of 10 ommatidia along the base of the eye lying perpendicular to the electron beam to ensure that the area reading was accurate. As was carried out by Culver (1987), ommatidia density was calculated by measuring the surface area of 16 ommatidia perpendicular to the electron beam. Density was calculated by dividing 16 by the measured area which was then converted to number of ommatidia per  $\text{mm}^2$ . Finally, the percentage of the eye covered with ommatidia was calculated by measuring the linear distance, from the tip of the eye towards the eyestalk, to the point at which the ommatidia stopped (distance A in Figure 3). This value was then divided by the previously measured eye diameter (distance B in Figure 3) under the assumption that the eyes were spherical.

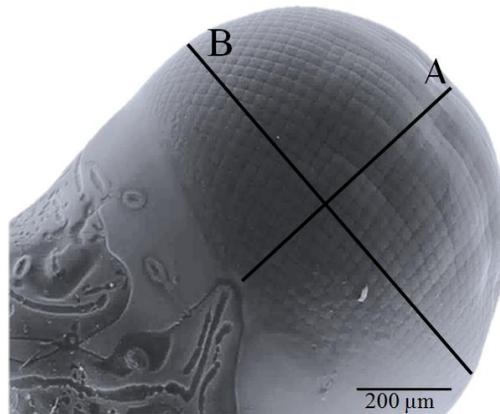


Figure 3. Scanning electron micrograph of eye showing distances measured to calculate percent eye covered by ommatidia (A) and eye diameter (B).

To calculate ommatidial depth, a microtome section from the center of the eye was selected, as confirmed by comparing the measure of the diameter of the section (distance A in Figure 4) compared to the eye diameter observed in the scanning electron micrograph (distance B in Figure 4). The depths of ommatidia in two locations (one on each side, corresponding to the base of the eye – B and C in Figure 4) were averaged to produce a single ommatidia depth measure.

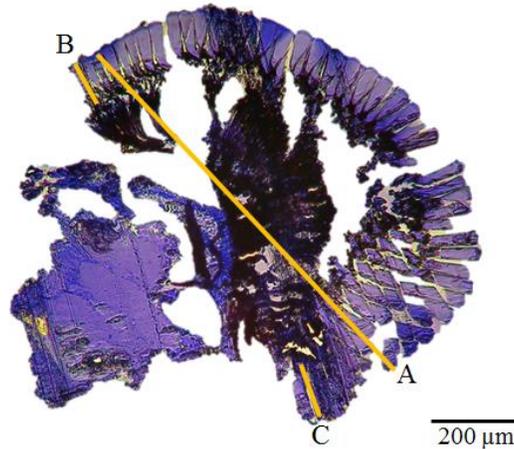


Figure 4. Light micrograph of cross-section of eye showing diameter (A) and sampling points to measure ommatidia depth (B and C).

### *Statistical analyses*

To determine the effect of the light colour treatments, One-Way Analysis of Variance (ANOVA) tests with Tukey Pairwise Comparisons were used to compare each of the eight morphology measures (carapace length, unpigmented area colouration, pigmented area colouration, eye diameter, ommatidia size, ommatidia density, ommatidia depth, and percent of eye covered with ommatidia) among the colour treatments. To analyze growth patterns in the individuals, Pearson Correlations were run between the same eight parameters.

## **RESULTS**

### *Effects of controlled wavelength exposure on growth and morphology*

Body size, as determined by carapace length, was not significantly affected by the light treatments during either the first 7 weeks of juvenile life (generation 2: ANOVA,  $F_{3,8}=1.70$ ,  $p=0.244$ ) or over a 16 week period (generation 1: ANOVA,  $F_{2,16}=0.54$ ,  $p=0.593$ ) (Figure 5). The five measured morphological traits associated with the eye were not significantly affected by the light treatments during the first 7 weeks of juvenile life or over a 16 week period: eye diameter (generation 1: ANOVA,  $F_{2,13}=0.68$ ,  $p=0.524$ ; generation 2: ANOVA,  $F_{3,8}=0.86$ ,  $p=0.500$ ), ommatidia size (generation 1: ANOVA,  $F_{2,13}=1.60$ ,  $p=0.420$ ; generation 2: ANOVA,  $F_{3,8}=3.45$ ,  $p=0.072$ ), ommatidia density (generation 1: ANOVA,  $F_{2,13}=0.11$ ,  $p=0.893$ ; generation 2: ANOVA,  $F_{3,8}=3.93$ ,  $p=0.54$ ), percent of the eye covered with ommatidia (generation 1: ANOVA,  $F_{2,13}=2.21$ ,  $p=0.149$ ; generation 2: ANOVA,  $F_{3,8}=0.50$ ,  $p=0.694$ ), and ommatidia depth (generation 1: ANOVA,  $F_{2,13}=0.49$ ,  $p=0.626$ ; generation 2: ANOVA,  $F_{3,8}=$ ,  $p=0.968$ ).

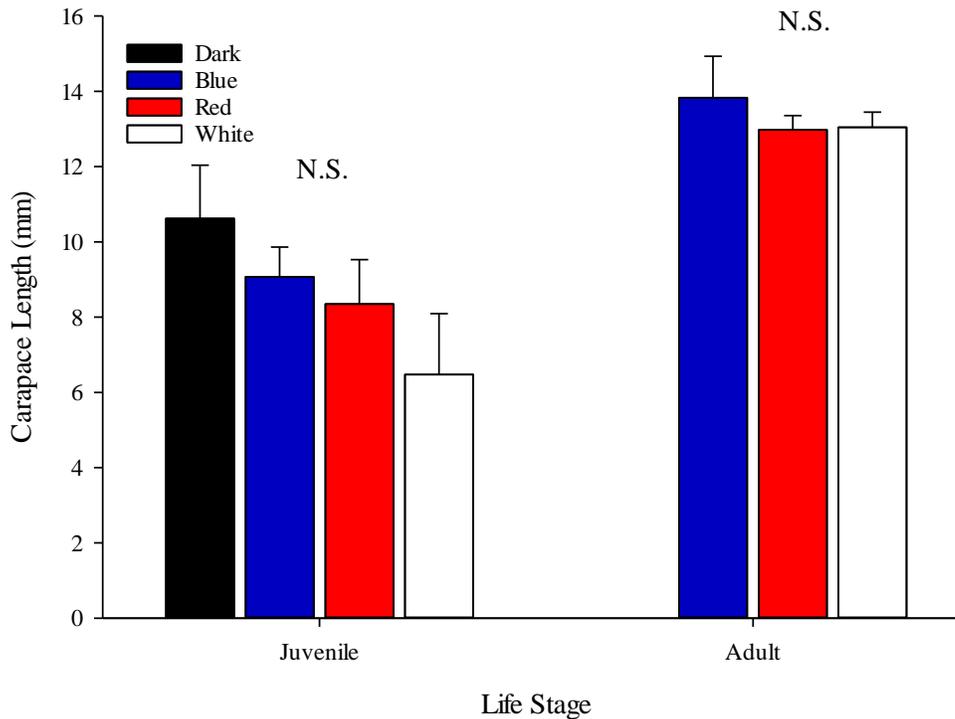


Figure 5. Body size (as measured by carapace length) of individuals at end of experiment as a function of colour treatment and life stage (Juvenile= Generation 2; Adult = Generation 1). Differences among treatments are not quite significant, possibly due to small sample size in the dark treatment (Blue = 9 crayfish, Red = 11 crayfish, White = 9 crayfish, Dark = 2 crayfish). Error bars represent standard error of the mean.

***Effects of controlled wavelength exposure on absorption spectra of the eye***

Differences in the absorption spectra of homogenized eyes among light treatments were observed (Figure 6). Eyes from all light treatments exhibited very similar absorption spectra at wavelengths ranging from 390 – 525 nm and from 750 – 800 nm, including a peak at 450 nm (label A in Figure 6). The absorbance spectra diverged among treatments, however, through the range of 550 – 750 nm. In this range, absorbance readings were generally highest for eyes from the white light treatment, intermediate for eyes from the red light treatment, and lowest for eyes from the blue treatment (Figure 6). In addition, absorbance declined across these wavelengths in eyes from all treatments, with the exception of a peak from 550- 570 nm (label B in Figure 6) and a modest absorbance peak at 670 nm for eyes from the red light treatment (label C in Figure 6). Due to equipment limitations, the spectrophotometer provided fewer absorbance readings for the blue light treatment than for the other treatments, precluding the ability to establish whether

there are absorbance peaks at wavelengths >550 nm for eyes from the blue light treatment; however, all readings for the blue treatment were below those of both the red and white treatments for the entire range of 550 nm-700 nm.

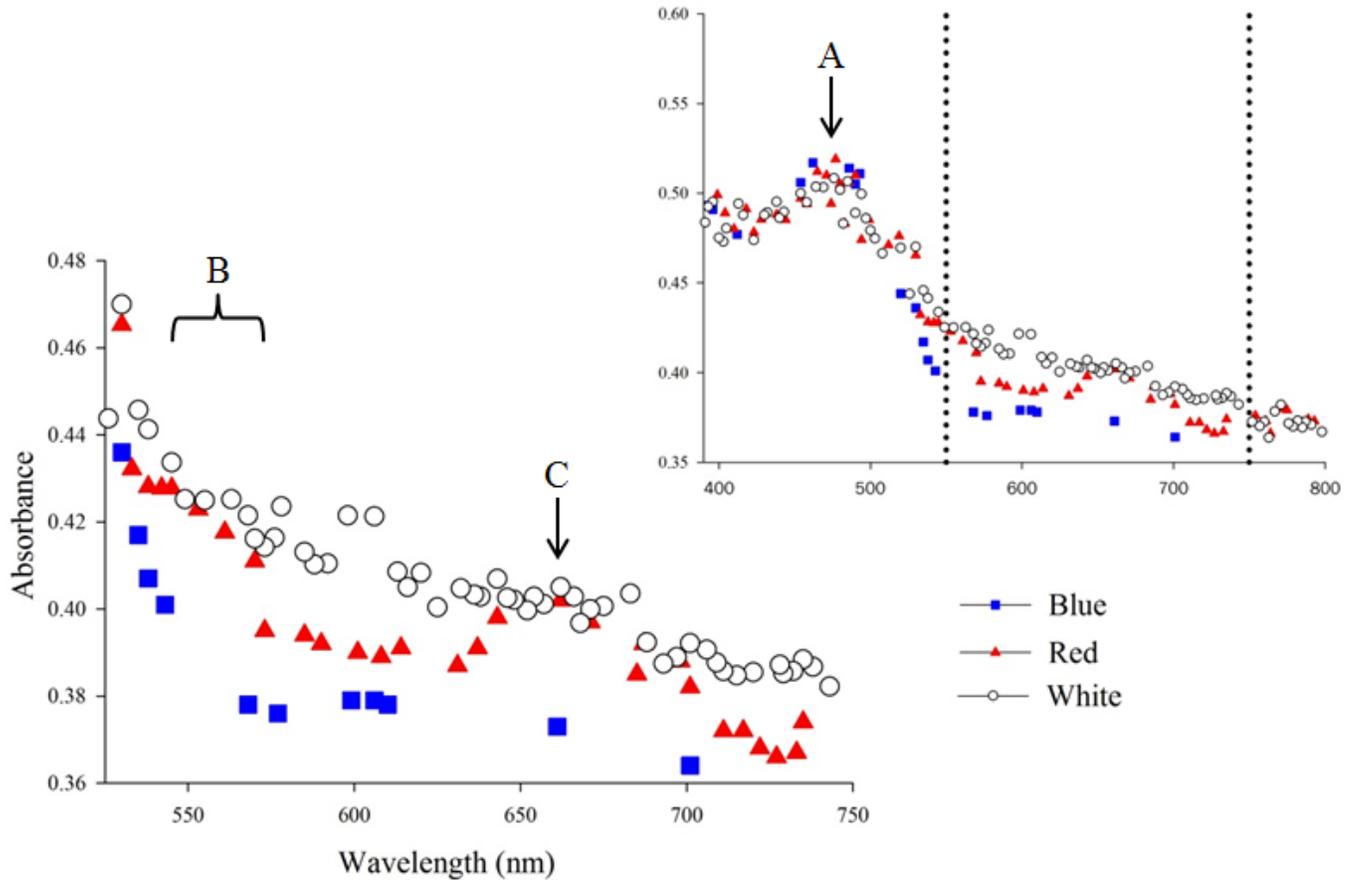


Figure 6. Absorbance of eyes (grouped by treatment) over the range of the visible spectrum. Inset shows the full range of tested wavelengths; main graph shows an expanded view of results over the 525 – 750 nm range. Point A represents peak characteristic of xanthommatin. Point B represents absorbance range of ommin pigment. Point C represents unidentified peak in red light treatment spectrum. Absorbance values have been standardized by eye volume.

### ***Exoskeleton colour brightness***

Exoskeleton colour brightness did not differ among light treatments in either the pigmented region (generation 1: ANOVA,  $F_{2,13}=0.98$ ,  $p=0.398$ ; generation 2: ANOVA,  $F_{3,8}=0.25$ ,  $p=0.862$ ) or the unpigmented region (generation 1: ANOVA,  $F_{2,13}=1.32$ ,  $p=0.295$ ; generation 2: ANOVA,  $F_{3,8}=0.05$ ,  $p=0.986$ ). However, colour brightness of the pigmented region of the carapace was significantly related to that of the unpigmented region in both juveniles

(n=12, p=0.002) and adults ( n=19, p<0.001), with brightness values showing a high degree of overlap between the two life stages (Figure 7).

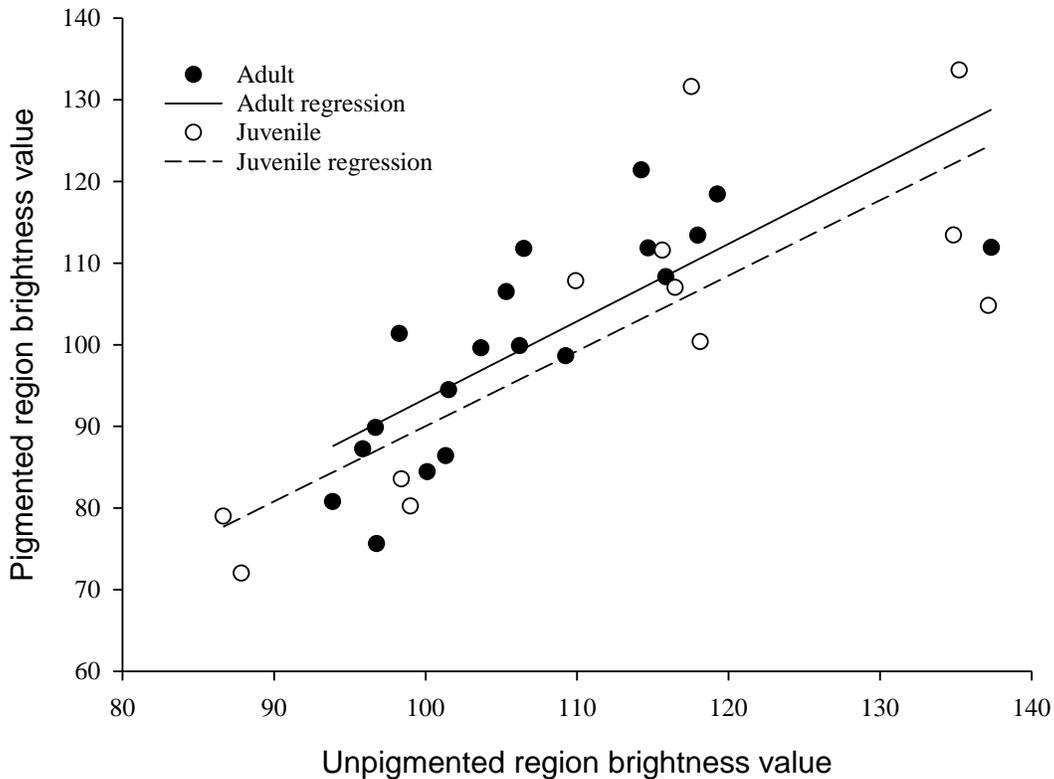


Figure 7. Brightness values of pigmented and unpigment regions of the exoskeleton of juvenile and adult crayfish. A brightness value of 1 represents pure black, and a value of 140 represents pure white.

### ***Growth of eye parameters***

The two life stages (juvenile and adult) did not exhibit the same relationship between eye parameters and carapace length. Overall eye size, as measured by eye diameter, was found to be correlated with carapace length in juveniles (Pearson correlation:  $r=0.901$ ,  $n=12$ ,  $p<0.001$ ) but not in adults (Pearson correlation:  $r=0.274$ ,  $n=19$ ,  $p=0.304$ ) (Figure 8). Average ommatidia size revealed the same pattern, with juveniles exhibiting proportionality with body size (Pearson correlation:  $r=0.883$ ,  $n=12$ ,  $p<0.001$ ) while adults showed no such relation (Pearson correlation:  $r=-0.099$ ,  $n=19$ ,  $p=0.716$ ) and had a much higher variation in ommatidia size per eye than juveniles (Figure 9A). Ommatidia density exhibited proportionality with body size in juveniles (Pearson correlation:  $r=-0.866$ ,  $n=12$ ,  $p<0.001$ ) but this relationship was not present in adults (Pearson correlation:  $r=-0.225$ ,  $n=19$ ,  $p=0.401$ ) (Figure 9B). Neither adults nor juveniles showed

a relationship between ommatidia depth and carapace length (Pearson correlation: adult crayfish,  $r=-0.187$ ,  $n=19$ ,  $p=0.522$ ; juvenile crayfish,  $r=-0.263$ ,  $n=12$ ,  $p=0.434$ ).

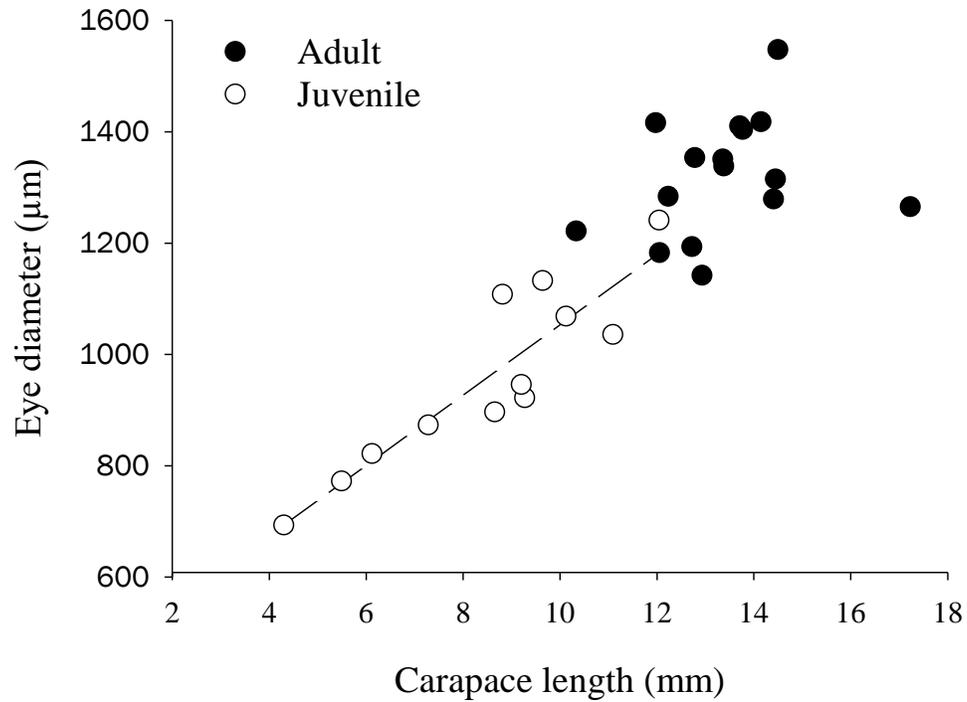


Figure 8. Eye size (diameter) as a function of body size (carapace length). Regression line is for juveniles only.

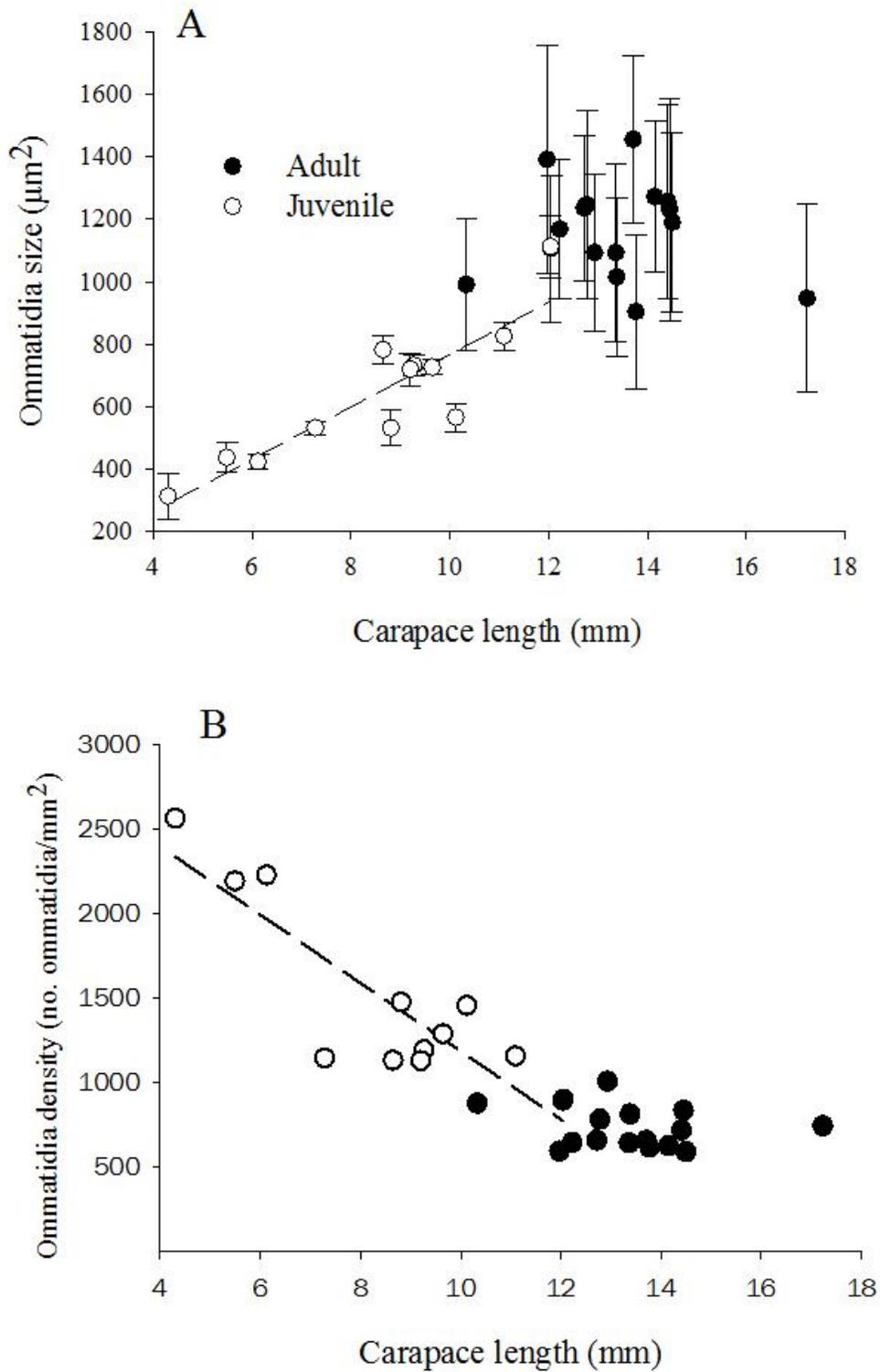


Figure 9. A: Ommatidia size as a function of body size (carapace length). Error bars show standard deviation. B: Ommatidia density as a function of body size (carapace length). In both graphs, the regression line is for juveniles only.

Ommatidia depth was not proportional with eye diameter in 7 week old juveniles (Pearson correlation:  $r=-0.242$ ,  $n=12$ ,  $p=0.474$ ) or in 16 week old adults (Pearson correlation:  $r=-0.454$ ,  $n=19$ ,  $p=0.103$ ). Ommatidia depth was not proportional to eye diameter in either juveniles (Pearson correlation:  $r=-0.719$ ,  $n=12$ ,  $p=0.694$ ) or adults (Pearson correlation:  $r=0.399$ ,  $n=19$ ,  $p=0.157$ ). Ommatidia size was proportional to eye diameter in juveniles (Pearson correlation:  $r=0.743$ ,  $n=12$ ,  $p=0.006$ ); however, this relationship was lost in adults (Pearson correlation:  $r=0.312$ ,  $n=19$ ,  $p=0.239$ ) (Figure 10A). Ommatidia density decreased with eye diameter in both the juveniles (Pearson correlation:  $r=-0.719$ ,  $n=12$ ,  $p=0.008$ ) and adults (Pearson correlation:  $r=-0.731$ ,  $n=19$ ,  $p=0.001$ ) (Figure 10B).

## DISCUSSION

### *Effects of controlled wavelength exposure on growth and morphology*

Under controlled environmental conditions (temperature, pH, food, substratum, photoperiod), exposure to differing light wavelengths did not affect the growth rate of *Cambarellus patzcuarensis*. The small sample size ( $n=2$ ) of juveniles in the dark treatment (generation 2) was likely responsible for the lack of significant difference between this treatment and the other light treatments. Casual observations suggest that juveniles reared in tanks with fewer adults may have grown faster than juveniles in tanks with several adults, accounting for some of the variation in their size at sampling; however, this was not formally quantified. The number of individuals in each tank were not standardized at all times across the different tanks and was often unequal due to mortality and the addition of generation 2 individuals to the tanks in week 10 of the experiment. This may account for the differing sizes at the time of sampling, as increased density and crowding have been found to have a direct effect on growth rate in crayfish (Aiken and Waddy 2015).

The present study revealed that eye morphology parameters (eye diameter, ommatidia size, ommatidia density, and ommatidia depth) did not differ among light treatments. This finding contrasts sharply with reports by Culver (1987) and Mejía-Ortíz and Hartnoll (2005) in which eye parameters appeared to vary in habitats with different levels of light exposure. This

difference may have occurred because those previous studies had sampled fully-developed adults directly from their natural habitat, whereas the present study used juvenile

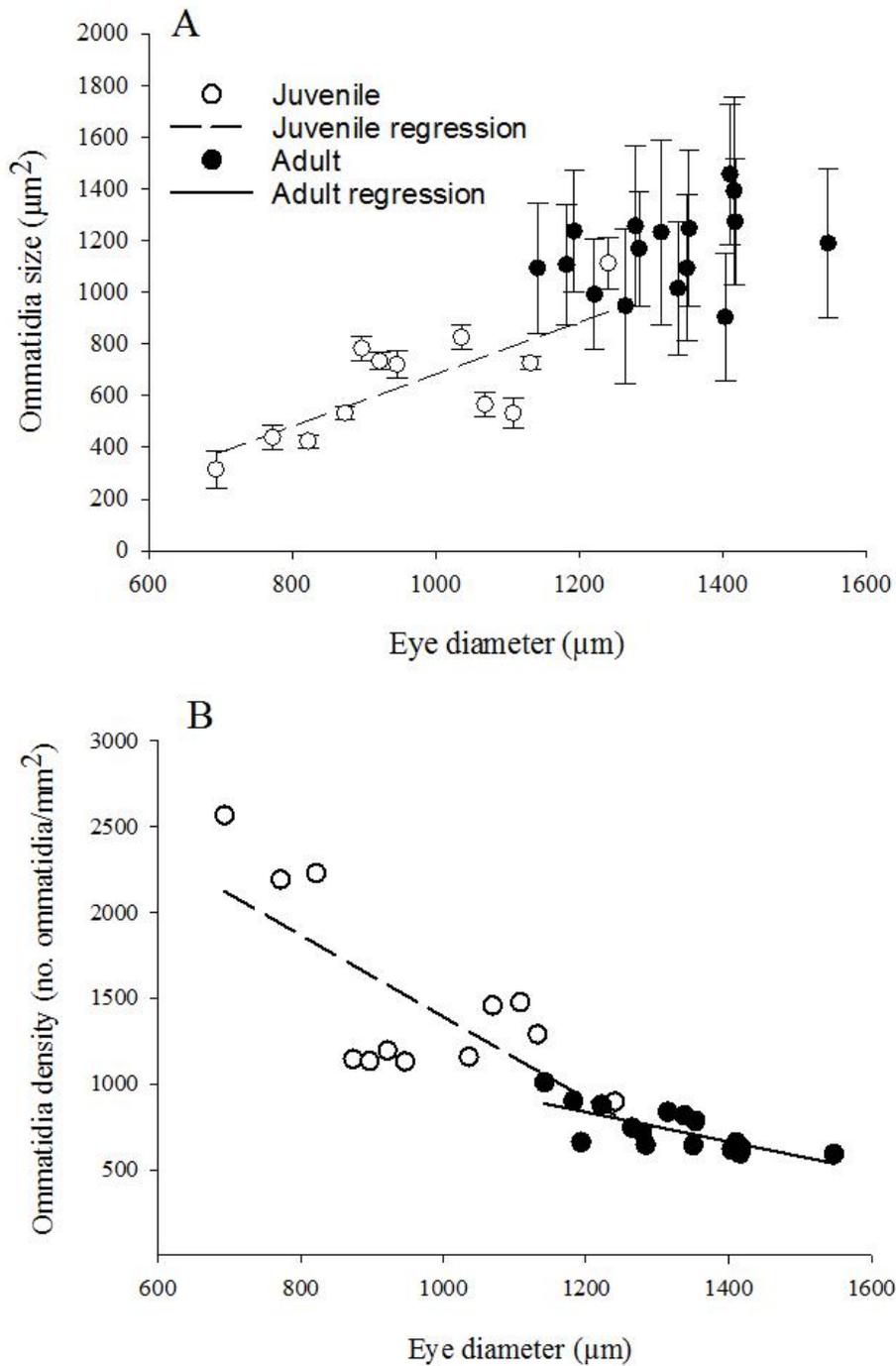


Figure 10. A: Ommatidia size as a function of eye size (diameter). Regression is for juveniles only. Error bars represent standard deviation. B: Ommatidia density as a function of eye size (diameter).

crayfish originating from rearing facilities and then further reared under controlled light conditions. The present study suggested that ommatidia are able to develop beyond maturity based on the higher degree of variation in ommatidia size in adults compared to juveniles, so it is possible that the adults sampled in previous studies (likely at an age greater than 16 weeks as crayfish can live for many years) had further developed ommatidia than our 16 week old adult population. In addition, previous studies have collected crayfish in natural habitats with either bright (streams) or dark (caves) light conditions. The light conditions in those habitats therefore were likely very different from the light conditions in the present experiment and it is possible that the individuals are unable to adapt their eye morphology to the same degree within the wavelength range used in the present study. With the shallow depth of the water used in this experiment, it is unlikely that any wavelengths of light would be filtered out of the water before reaching the crayfish.

#### ***Effects of controlled wavelength exposure on absorption spectra of the eye***

While the exposure to different light wavelengths during juvenile growth did not affect the external morphology of the eye, the chemical composition of the eye did appear to differ between light treatments. In this study, pigments were identified by characteristic peaks rather than identification through chemical analysis of the homogenized solution, introducing an inherent possibility of error. The peak absorbance of homogenized eye tissues observed in all light treatments at 450 nm is common amongst crustaceans, and is consistent with the peak absorbance of the xanthommatin pigment. Xanthommatin exists in the pigment screen of the eye, within the dioptric zone, and functions to absorb light (Struwe et al. 1975). The white and blue lights used in this study produced wavelengths that peaked at 450 nm and 460 nm, respectively; however, individuals reared in red light exhibited an equal peak to those in blue or white which suggests that the production of this pigment may be irrespective of the environmental light conditions. In addition, the slight peak at 515 nm recorded here, and also in other Crustacea, is associated with metarhodopsin, a photoreceptor located in the rhabdomere (Cronin and Goldsmith 1982). The absorbance of eye tissues differed among treatments at wavelengths of 550 - 570 nm, a range that is known to contain the ommin pigment (Evans et al. 2015). Since ommin is known to exist within the pigment screen and functions in blocking out or reflecting incident rays of light to avoid over-stimulation of receptors, the decreased concentration of this pigment in crayfish that were reared under short (blue) wavelengths suggests this pigment may

reflect longer wavelengths of light (Evans et al. 2015). In addition, the production of this pigment may be influenced by the light wavelengths experienced during juvenile life. The peak observed from individuals in the red light treatment, although unidentified, may be related to the wavelength of light to which they were exposed to as the red lights emitted wavelengths that peaked at 640 nm. Homogenizing the entire eye and suspending the pigments in solution allowed us to demonstrate total pigment content of the eye, considering both the pigment screen as well as the rhabdomere, whereas previous studies had separated the different regions of the eye prior to analysis, providing the presence as well as location of each pigment (Struwe et al. 1975).

### ***Exoskeleton colour brightness***

Pigments found in the eyes, such as xanthommatin, have also been observed in the exoskeletons of crustaceans (Needham and Brunet 1957). These pigments, along with others, are responsible for the colour brightness observed in the exoskeleton of the crayfish. The brightness of the exoskeleton can vary with physiological changes such as nutrition and stage in the molt cycle (Aiken and Waddy 2015, Needham and Brunet 1957). The longitudinal striping along the exoskeleton of *Cambarellus patzcuarensis* allowed for a comparative analysis within individuals to determine if both the pigmented (stripe) and unpigmented areas are related. The positive correlation between these areas suggests that, since individuals were unlikely to be all at the exact same point in the inter-molt stage, that both the pigmented and unpigmented areas respond the same within the molt cycle. The overlap between the colouration of juveniles and adults suggests the pigmentation of the exoskeleton is fully developed by the age of 7 weeks, and that exoskeleton brightness does not develop progressively through to maturity. It has been hypothesized that vibrant exoskeleton colouration is sexually selected for in some species of crayfish (Aiken and Waddy 2015). Since generation 1 individuals reached maturity within approximately 11 weeks of age, it is possible that the colouration observed in the 7-week old generation 2 individuals is a result of those individuals approaching the point of maturity.

### ***Growth of eye parameters***

Juvenile *C. patzcuarensis* exhibited gradual changes in eye diameter, ommatidia size, and ommatidia density in relation to body size (carapace length). These allometric changes in eye size and structure suggest the development of the eye takes place proportionally with body size up to maturity. Once at maturity, eye size did not increase at the same rate as in juvenile life, likely due to the limitations of eye growth in crayfish. Crayfish are able to have small eyes due to

the ability of the compound eye allowing crayfish to take in adequate visual information while not expending additional energy in the creation of a large eye, as well as allows for less total weight of the individual to be able to move more quickly (Aiken and Waddy 2015). The observed increase in ommatidia size relative to eye size (diameter) suggests that individual ommatidia become enlarged to occupy more of the eye surface rather than new ommatidia being created to fill the space between existing ommatidia as the eye grows. In addition, the observed decrease in ommatidia density with an increase in eye size is consistent with the ommatidia becoming larger. The loss of these trends in adults and the increased variation of ommatidia size of adults in the present study suggests that this species exhibits the morphogenic front type of development. In this method of development, ommatidia equal in size are produced early in life but are then able to continue to develop both in size and contents after initially being formed. The proliferation (creation) and adaptation (further development) of the ommatidia are two defined steps in this mode of eye development, being defined both temporally and spatially (i.e. ommatidia must be completely formed before being able to adapt and new ommatidia are always formed toward the eyestalk) (Harzch and Hafner 2006).

### ***Future directions***

This study was the first to explore the ontogeny of crayfish eye parameters. However, the small sample sizes of crayfish used in these experiments likely limited my ability to detect trends that might have been revealed by a larger sample size. While previous literature demonstrated interspecific variation of ommatidia parameters (size, density, and depth) in habitats with extremely different light exposures, this study did not reveal the same trends when the wavelength of light was manipulated. With regards to the production of eye pigmentation, future work using liquid chromatography-mass spectrometry (LCMS) would aid in furthering the findings of this study. Liquid chromatography could be used to separate the pigments in solution based on both size and polarity, while mass spectrometry would identify atoms present in each of the pigments. This would allow for the derivation of possible molecular structures for each pigments to confirm the identities of the pigments present in the eye that are affected by light wavelength, which could then be related to their function through comparison to known pigments of which we know both structure and functions.

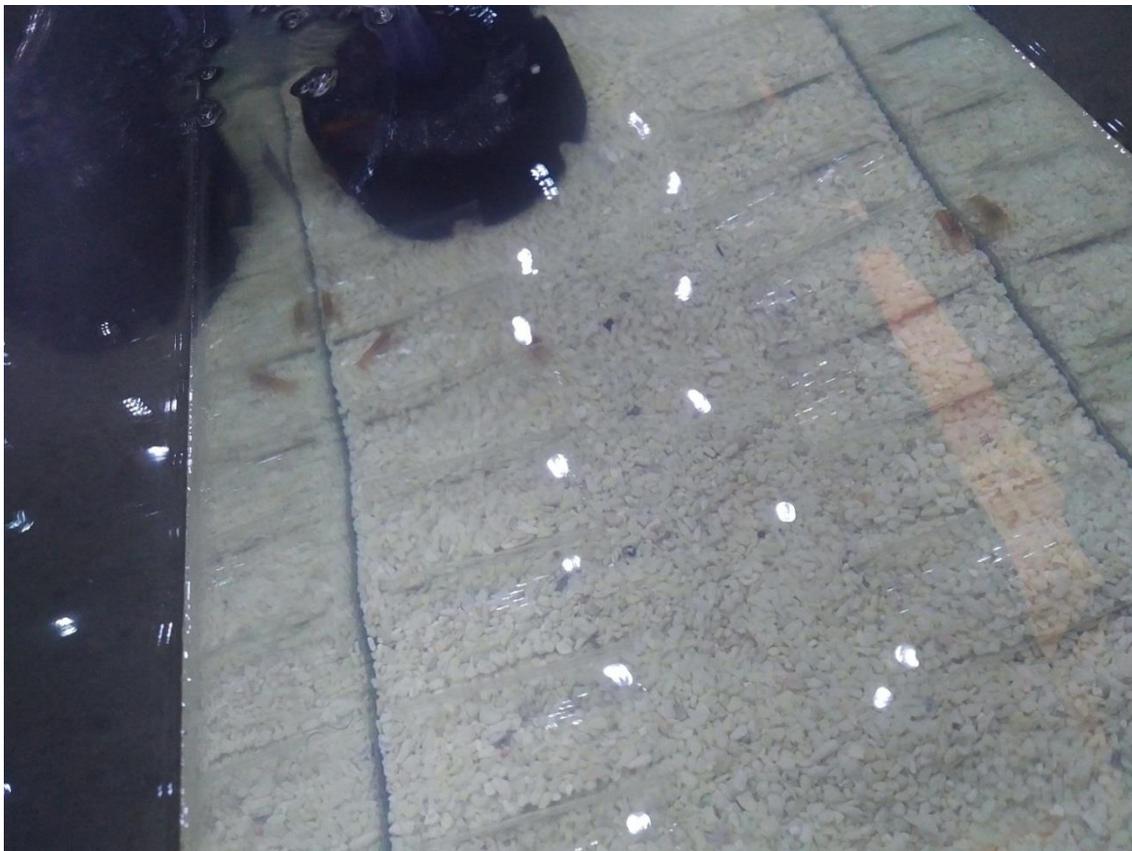
## LITERATURE CITED

- Aiken DE, Waddy S. 1992. The growth process in crayfish. *Rev. Aquat. Sci.* 6:335–381.
- Boyd JA, Page LM. 1978. The life history of the crayfish *Orconectes kentuckiensis* in Big Creek, Illinois. *Am. Midl. Nat.* 99:398–414.
- Bryceson KP. 1986. The effect of screening pigment migration on spectral sensitivity in a crayfish reflecting superposition eye. *J. Exp. Biol.* 125:401–404.
- Buřič M, Kouba A, Kozák P. 2010. Intra-sex dimorphism in crayfish females. *Zoology* 113:301–307.
- Callaerts P, Clements J, Francis C, Hens K. 2006. Pax6 and eye development in Arthropoda. *Arthropod Struct. Dev.* 35:379–391.
- Cao A, Shi L, Deng Q, Hui P, Zhang M, Du C. 2015. Structural design and image processing of a spherical artificial compound eye. *Optik* 126(2015):3099-3103.
- Chen QX, Hua BZ. 2016. Ultrastructure and morphology of compound eyes of the scorpionfly *Panorpa dubia* (Insecta: Mecoptera: Panorpidae). *PLoS One* 11:1–13.
- Cooper RL, Li H, Long LY, Cole JL, Hopper HL. 2016. Anatomical comparisons of neural systems in sighted epigeal and troglobitic crayfish species. *The Crustacean Society* 21:360–374.
- Cronin TW, Goldsmith TH. 1982. Photosensitivity spectrum of crayfish rhodopsin measured using fluorescence of metarhodopsin. *J. Gen. Physiol.* 79:313–32.
- Culver DC. 1987. Eye morphometrics of cave and spring populations of *Gammarus minus* (Amphipoda: Gammaridae). *J. Crustac. Biol.* 7:136–147.
- Erclik T, Hartenstein V, McInnes RR, Lipshitz HD. 2009. Eye evolution at high resolution: the neuron as a unit of homology. *Dev. Biol.* 332(2009): 70-79.
- Evans AB, Acosta ML, Bolstad KS. 2015. Retinal development and ommin pigment in the cranchiid squid *Teuthowenia pellucida* (Cephalopoda: Oegopsida). *PLoS One* 10:1–11.
- Harzsch S, Hafner G. 2006. Evolution of eye development in arthropods: phylogenetic aspects. *Arthropod Struct. Dev.* 35(2006): 319-340.
- Land MF. 1990. Direct observation of receptors and images in simple and compound eyes. *Vision Res.* 30(11): 1721-1734.
- Lovett DL, Felder DL. 1989. Application of regression techniques to studies of relative growth in crustaceans. *J. Crustac. Biol.* 9:529–539.
- Mariappan P, Balasundaram C. 2004. Studies on the morphometry of *Macrobrachium nobilii* (Decapoda, Palaemonidae). *Brazilian Arch. Biol. Technol.* 47(3): 441-449.
- Meyer-Rochow VB. 2001. The crustacean eye: dark/light adaptation, polarization sensitivity,

- flicker fusion frequency, and photoreceptor damage. *Zoolog. Sci.* 18:1175–1197.
- Needham AE, Brunet PCJ. 1957. The integumental pigment of *Asellus*. *Experientia* 13:207–208.
- Rasband WS (1997-2016) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>
- Ramalho RO, Correia AM, Anastácio PM. 2008. Effects of density on growth and survival of juvenile red swamp crayfish, *Procambarus clarkii* (Girard), reared under laboratory conditions. *Aquac. Res.* 9:577-586.
- Stein RA, Murphy ML, Magnuson JJ. 1977. External morphological changes associated with sexual maturity in the crayfish. *Source Am. Midl. Nat.* 97:495–502.
- Stumpf L, Calvo NS, Díaz FC, Valenti WC, Greco LSL. 2011. Effect of intermittent feeding on growth in early juveniles of the crayfish *Cherax quadricarinatus*. *Aquaculture* 319:98–104.
- Tokarski TR, Hafner GS. 1984. Regional morphological variations within the crayfish eye. *Cell Tissue Res.* 235:387–392.
- Walcott B. 1974. Unit studies on light-adaptation in the retina of the crayfish, *Cherax destructor*. *J. Comp. Physiol.* 94:207–218.

## APPENDIX A: TANK SETUP

The setup of all 38 L tanks was identical, as shown below. For the dark treatment in which 7.5 L tanks were used. The two small tanks were used for the dark treatment due to equipment shortage and being unable to embed lights into the lid design of the smaller tanks. The same amount of sediment (2 cm thick) was used in the small tanks but fewer shelters were placed in the tanks as well as a smaller filter (Aquatop CAF-10 as compared to the larger Aquatop CAF-40) to account for the lesser water volume. It was assumed that since fewer individuals were in these tanks compared to the blue, red, and white light treatment tanks that fewer shelters did not affect the crayfish. In all tanks, there were always more shelters than crayfish, allowing for all individuals to be in hiding at once. The shelters (as shown below) were transparent plastic tubes; this ensured that even if an individual was in a shelter, they were still exposed to the full 12-hours of light in the photoperiod.

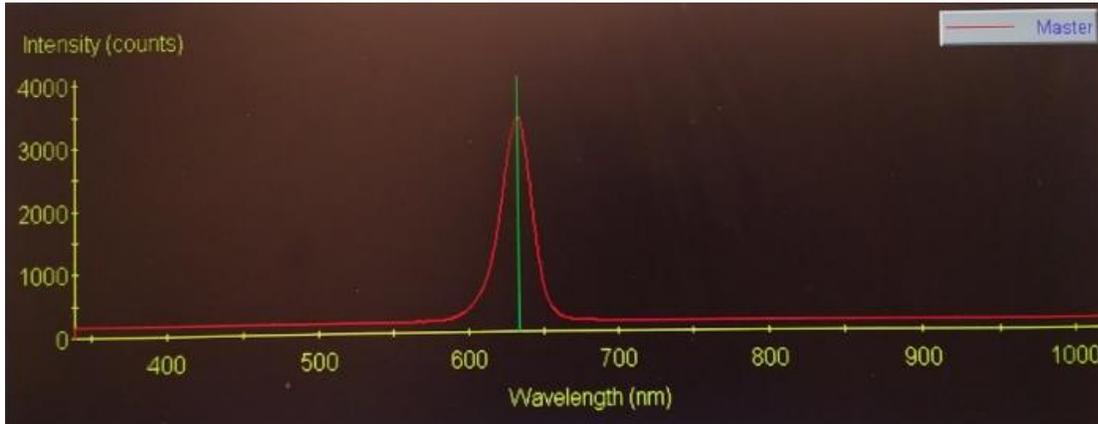


(note: the white spots in the above photo are reflections on the water's surface of the lights embedded in the lid of the tank)

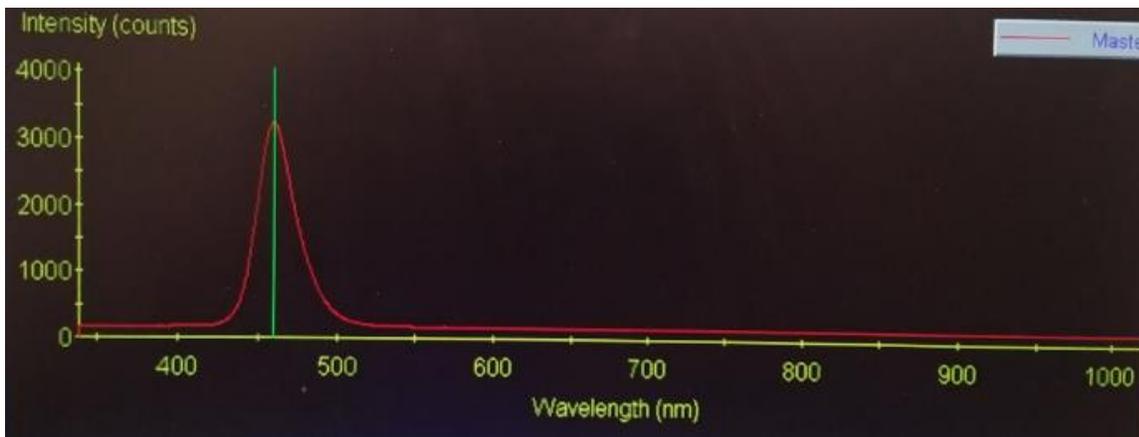
## APPENDIX B: LED LIGHT SPECTRA

An Ocean Optics USB 2000 Spectrometer was used to measure the wavelengths of light emitted by each of the colours (blue, red, white) of lights (see below).

Red light spectrum:



Blue light spectrum:



White light spectrum

