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# The Evolutionary Biology of Vision in *Daphnia*

Christopher S. Brandon

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THE EVOLUTIONARY BIOLOGY OF VISION IN *DAPHNIA*

by

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Bachelor of Science  
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Submitted in Partial Fulfillment of the Requirements

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University of South Carolina

2015

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## DEDICATION

To my wife and son, Legna and Tiago, for providing the constant glow of hope and direction like a lighthouse burning brightly through the misty darkness.

## ACKNOWLEDGEMENTS

There are so many people who contributed to the success and completion of this work for which I would like to express my gratitude. To my advisor, Jeffry Dudycha, who provided valuable guidance and encouragement at all stages of these projects. To my committee members Tammi Richardson, Sally Woodin, Todd Oakley, and Dan Speiser who provided great insight and direction.

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## ABSTRACT

Eyes have attracted the attention of evolutionary biologist since the field's infancy. In *On the Origin of Species*, in fact, Darwin famously remarked on the proposition that natural selection could engineer the eye, saying “[it is] absurd in the highest possible degree.” Though, he goes on to explain, beautifully and simply, how his theory of evolution by natural selection could produce such an organ. Indeed, eyes are remarkable examples of complex information acquisition systems that have evolved from simple beginnings. Eyes allow animals to extract environmental information from light, which informs physiological and behavioral responses to resources, predation, and mates. The morphological and physiological features of eyes define the absolute bounds of visual capabilities. These characteristics of eyes highlight why they are particularly interesting from an evolutionary perspective: variation affects what and how environmental information can be collected and processed, thereby potentially altering many of the animal's ecological interactions. While a rich literature has documented myriad facets of eye evolution, there remain many areas that merit more investigation. The aim of my thesis is to broaden our understanding of the evolution of vision by exploring three related, yet different, aspects using the ecological model organism, *Daphnia*. I present a study that examines the ecological factors that potentially influence eye morphology. Second, I present a study that demonstrates fitness variation associated with eye diameter, and pair these observations with information on genetic variation of

eye diameter. Lastly, I present a study evaluating the evolution of opsins—the gene largely responsible for vision—in *Daphnia*.

## TABLE OF CONTENTS

DEDICATION .....	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT .....	v
LIST OF TABLES .....	ix
LIST OF FIGURES .....	xi
CHAPTER 1. INTRODUCTION.....	1
1.1 INTRODUCTORY REMARKS .....	1
1.2 <i>DAPHNIA</i> AND THEIR OPTICAL ENVIRONMENTS .....	1
1.3 EYES, VISION, AND <i>DAPHNIA</i> .....	4
1.4 BRIEF NOTE ON THE EVOLUTION OF VISION.....	9
1.5 THESIS OUTLINE .....	11
CHAPTER 2. ECOLOGICAL CONSTRAINTS ON SENSORY SYSTEMS: COMPOUND EYE SIZE IN <i>DAPHNIA</i> IS REDUCED BY RESOURCE LIMITATION .....	15
2.1 INTRODUCTION.....	16
2.2 METHODS .....	19
2.3 RESULTS.....	23
2.4 DISCUSSION .....	25
CHAPTER 3. SELECTION ON INCREMENTAL VARIATION OF EYE SIZE IN A WILD POPULATION OF <i>DAPHNIA</i> .....	39
3.1 INTRODUCTION.....	40



3.2 METHODS .....	42
3.3 RESULTS .....	46
3.2 DISCUSSION .....	49
CHAPTER 4. THE EVOLUTION OF THE OPSIN GENE FAMILY IN <i>DAPHNIA</i> .....	55
4.1 INTRODUCTION .....	56
4.2 METHODS .....	59
4.3 RESULTS .....	63
4.4 DISCUSSION .....	70
CHAPTER 5. SUMMARY .....	90
REFERENCES .....	93
APPENDIX A: CHAPTER 2 SUPPLEMENTARY INFORMATION .....	113
APPENDIX B: CHAPTER 4 SUPPLEMENTARY INFORMATION.....	118
APPENDIX C: PERMISSION TO REPRINT CHAPTER 2 .....	120

## LIST OF TABLES

Table 2.1 Results of ANOVA on the effects of different environmental treatments on <i>Daphnia</i> spp. absolute eye diameter, and the percent increase in the compound eye surface area (total light collection ability) in high resource and dim light levels, and significant differences between means are noted in bold .....	32
Table 2.2 Results of ANOVA on the effects of different environmental treatments on <i>Daphnia</i> spp. body length, and significant differences between means are noted in bold .....	33
Table 2.3 Results of an ANCOVA on the effects of different environmental treatments on <i>Daphnia</i> spp. eye size using body length as a covariate.....	34
Table A.1 Animal sample sizes, body length and absolute eye measurements for each factor level .....	114
Table A.2 Results of ANOVA on the effects of different environmental treatments on <i>Daphnia</i> spp. body length.....	115
Table 3.1 Selection differentials ( $s$ ) and selection gradients ( $\beta$ ) for the correlated phenotypic traits, natural logarithm transformed body length and eye diameter. ....	52
Table 4.1 Number of opsins in <i>Daphnia pulex</i> and <i>D. magna</i> .....	77
Table 4.2 <i>D. pulex</i> opsin genes scaffold locations, which are designated by the start and stop codons, and were identified from the most recent curation available at from the JGI (available at <a href="http://genome.jgi-psf.org/Dappu1">http://genome.jgi-psf.org/Dappu1</a> ).....	78
Table 4.3 <i>D. magna</i> opsin genes scaffold locations, which are designated by the start and stop codons.....	80
Table B.1 Genbank accession numbers of mRNA sequences used as the bases for the <i>Daphnia</i> opsin phylogenetic analyses.....	118

## LIST OF FIGURES

Figure 1.1 A diagram illustrating how light attenuates in the water column.....	12
Figure 1.2 A diagram illustrating the various external and internal factors that affect the amount of light in a waterbody .....	13
Figure 1.3 A diagram illustrating the basic features of vision in <i>Daphnia</i> .....	14
Figure 2.1 A photomicrograph collage of the <i>Daphnia</i> species used in this study. The white line represents eye diameter measurements, and the black line represents body length measurements.....	35
Figure 2.2 <i>Daphnia</i> facet width in relation to eye diameter. Ordinary least squares regression reveals a strong positive relationship (slope = 0.228, adj. $R^2 = 0.81$ , $p < .0001$ ) .....	36
Figure 2.3 The effect of resource environment on relative eye size in <i>Daphnia</i> . Relative eye size values are based on body size covariate adjusted means from ANCOVA where eye diameter was the response variable and body length set as the covariate (see methods for details) separately for each species and stage. To present data on the same scale, means were normalized to the high resource environment within each species and stage (i.e., high resource is always set to 1.0). Means were tested at $\alpha = 0.05$ . Significant differences between means are noted with a p-value in bold. N.S.= not significant. Error bars are $\pm 95\%$ confidence intervals .....	37
Figure 2.4 The effect of light environment on relative eye size in <i>Daphnia</i> . Relative eye size values are based on body size co-variate adjusted means from ANCOVA where eye diameter was the response variable and body length set as the covariate (see methods for details) separately for each species and stage. To present data on the same scale, means were normalized to the dim light environment within each species and stage (i.e., dim light is always set to 1.0). Means were tested at $\alpha = 0.05$ . Significant differences between means are noted with a p-value. N.S.= not significant. Error bars are $\pm 95\%$ confidence intervals.....	38
Figure A.1 Light intensity variation between the experimental chambers (Bright, Dim) and the two shelves (Top, Bottom) within each chamber. Light intensity was measured as photosynthetically active radiation (400 nm-700 nm). Three measurements per shelf per chamber were recorded. Error bars are standard deviation.....	116

Figure A.2 *Daphnia* illustration showing measurements for body length and eye diameter.....117

Figure 3.1 For reasons of simpler presentation, we illustrate how the univariate trait, relative eye size, relates to relative fitness as opposed to illustrating the multivariate space. Relative fitness (individual clutch size/mean clutch size) as a function of relative eye size in the Knobby Knees pond population of *D. obtusa* ( $N = 229$ ,  $\beta = 0.0087 \pm 0.0015$  SE, adj.  $R^2 = 0.119$ ,  $p < 0.0001$ ). Solid line shows the least-squares regression and red dashed lines show 95% confidence interval. Clutch size was counted as number of eggs in the brood chamber of *D. obtusa*. Relative eye size is defined as the vertical residual value from a regression of *D. obtusa* eye diameter on body length for each individual. ....53

Figure 3.2 The mean relative eye size is displayed for each clone isolated from a metapopulation of *D. obtusa*. Relative eye size is shown for each clone as the mean value of the vertical residuals obtained from a least-squares regression performed on the entire *D. obtusa* data set (see methods). Error bars are standard error of the mean. ....54

Figure 4.1 Phylogeny of *D. pulex* and *D. magna* r-opsins based on maximum likelihood (ML) analyses of protein-coding nucleotide sequences. ML analyses were run using GTR model of evolution as implemented in RAxML. The phylogenetic tree is rooted at vertebrate visual c-opsins. Values on branches indicate bootstrap support. Branch lengths are proportional to the substitution/site scale bar. Branches that lead to putative visual opsins of broadly defined (ultraviolet, blue, red, and green) wavelength classes have been colored by their respective wavelength sensitivity. The bracket tree on the right is the hypothesized family of opsins that existed in the most recent common ancestor of *D. pulex* and *D. magna*, approximately 200 mya. Black closed circles (•) identify *D. pulex* and open boxes (□) identify *D. magna*. Crosses (+) indicate opsin genes that have truncated sequences .....81

Figure 4.2 An illustration of exon-intron gene structure and genome location of *Daphnia* ultraviolet (UV) sensitive, blue (BL) sensitive, and unknown (UN) wavelength-sensitive opsins. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other.....83

Figure 4.3 An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* long wavelength A clade (LOPA; putative green-sensitive). Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is

identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* LOPA clade and their phylogenetic relationships, and the B) *D. pulex* LOPA clade and their phylogenetic relationships.....84

Figure 4.4 An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* long wavelength B clade (LOPB; putative red-sensitive). Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* LOPB clade and their phylogenetic relationships, and the B) *D. pulex* LOPB clade and their phylogenetic relationships .....85

Figure 4.5 An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* arthropsin clade. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* arthropsin clade and their phylogenetic relationships, and the B) *D. pulex* arthropsin clade and their phylogenetic relationships. Two arthropsin gene structures are not shown for *D. magna* because large regions of the genome were missing within the genes .....86

Figure 4.6 Phylogeny of *D. pulex*, *D. magna*, and other species c-opsins based on maximum likelihood (ML) analyses of protein-coding nucleotide sequences. ML analyses were run using GTR model of evolution as implemented in RAxML. The phylogenetic tree is rooted at vertebrate melanopsins (r-opsins). Values on branches indicate bootstrap support. Branch lengths are proportional to the substitution/site scale bar. The *C. teleta* 2 opn5 branch has been broken for illustration purposes; the gap represents 2 substitutions/site. The scale bar indicates the number of nucleotide substitutions per site. The bracket tree on the right is the hypothesized pteropsin family that existed in the most recent common ancestor of *D. pulex* and *D. magna*, approximately 200 mya. Black closed circles (•) identify *D. pulex* and open boxes (□) identify *D. magna*. Crosses (+) indicate opsin genes that have truncated sequences .....87

Figure 4.7 An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* pteropsin clade. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read

on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* pteropsin clade and their phylogenetic relationships, and the B) *D. pulex* arthropsin clade and their phylogenetic relationships .....88

Figure 4.8 An illustration of the exon-intron gene structure and the genomic locations of opsin-3 and opsin-5 in *D. pulex* and *D. magna*. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other .....89

Figure B.1 Phylogenetic analyses of all coding *Daphnia* opsins using coding nucleotide sequences. Analyses were run as described in the methods, section 4.2. Tree is rooted at two *D. pulex* allostatin genes that are members of the GPCR superfamily. Tree on the left is bootstrap consensus, with bootstrap values listed on the branches. The tree on the right is the genetic distances, and substitutions/site are proportional to scale bar .....119

# CHAPTER 1

## INTRODUCTION

### **1.1 Introductory remarks**

Sensory systems have attracted the attention of evolutionary biologist since the field's infancy. In fact, Darwin famously expressed bewilderment at just how evolution could generate the necessary complexities of a functioning eye, though he went on to beautifully and simply explain how his theory of evolution by natural selection could produce such an organ (Darwin, 1859). Sensory systems provide necessary environmental information that informs an animal's physiological and behavioral responses to resources, predation, and mates. Eyes, in particular, allow animals to extract environmental information from light. The morphological and physiological features of eyes define the absolute bounds of their capabilities (Land & Nilsson 2012), and variation of these features affect how an animal perceives its environment, potentially altering many ecological interactions. These characteristics of eyes (and indeed sensory systems) highlight why they are particularly interesting from an evolutionary perspective: variation affects information processing, which ultimately permeates most aspects of the animal's ecology. In this thesis, I examine various areas of the evolution of vision by exploiting the common ecological model organism, *Daphnia*.

## 1.2 *Daphnia and their optical environment*

*Daphnia* are freshwater crustaceans that inhabit waterbodies ranging from temporary ponds to large lakes (Wellborn *et al.*, 1996; Benzie, 2005). Their central importance in aquatic food webs and the ease of field and laboratory studies have led them to be one of the best known organisms in terms of ecology (Rudstam *et al.*, 1993; Sommer & Sommer, 2006). They are cyclic parthenogens (Innes & Hebert, 1988), allowing genetically identical individuals to be exposed to different conditions. They are also the subject of a large international genomics consortium and were the first crustacean to have their genome sequenced (Colbourne *et al.*, 2011).

Visual systems process environmental information encoded by light. To appreciate the context in which these systems are evolving it is necessary to understand how light acts in the environments in which *Daphnia* live. *Daphnia* typically inhabit either freshwater lakes or ponds. To some extent, *Daphnia* can be characterized as either being a lake species or a pond species, but there are many species that can inhabit both lakes and ponds (Benzie, 2005). Lakes are generally characterized as large waterbodies that thermally stratify forming distinct layers and are present year round (Wetzel, 2001). Ponds, conversely, are characterized as smaller, shallower waterbodies that do not typically stratify. Many ponds are also ephemeral and dry up seasonally.

Light behaves characteristically different in water than it does in air, and there are therefore several properties of the optical environment in water that are distinctly different than that on land (Wetzel, 2001). Light attenuates down the water column and can often leave the depths of a lake extremely dim or completely dark. *Daphnia* exploit this property of lakes and use the shadows of the deep to escape visual predators and



harmful ultraviolet light through diel vertical migration behaviors (Ringelberg, 1999; Leech & Williamson, 2001; Hansson *et al.*, 2007). The amount of particulate material, both living and nonliving, can affect how rapidly the light attenuates down the water column, and the type and amount of particulate matter can vary from waterbody to waterbody (Wetzel, 2001). A lake can also experience a rapid algae bloom therefore increasing the particulate matter and drastically affecting the light environment on a relatively short timescale (e.g., Abrantes *et al.*, 2006). In ponds attenuation of light can be so strong that, light can be nearly extinguished at the bottom even in shallow ponds (<1 m)(V.-Balogh *et al.*, 2009). Thus, in some ways, the light environment in ponds can mirror a deep lake in terms of a vertically graded light-field.

The color of the water environment can also change from lake to lake or from pond to pond. In clear waters, white light (all visible spectra) dominates the shallow depths of the water column. Pure water absorbs blue light least, so in clear waters blue light penetrates deepest (Hutchinson, 1967; Wetzel, 2001). Dissolved substances in the water column absorb light at specific wavelengths and shift the color of underwater light (Fig. 1.1). Different dissolved substances alter the light field differently. Waters with high concentrations of dissolved bicarbonate materials, or hard water, alter the underwater light-field to blue-green. The most common alteration to the underwater light field is due to colored dissolved organic matter (CDOM). In CDOM waters (usually resembling the color of tea) the shorter wavelengths are almost totally absorbed in the first few centimeters, leaving the underwater light-field dominated by longer (orange-red) wavelengths (Fig. 1.1)(Hutchinson, 1967; Wetzel, 2001).

The amount of light that reaches the water surface often experiences daily and/or seasonal shifts (Fig. 1.2). The total amount of light that penetrates the water column depends on a number of factors (Fig. 1.2). Some factors change daily and seasonally, but other external factors can determine if a light environment is perpetually dim or bright, no matter how clear and free of substances the water is. Meteorological conditions and canopy cover can drastically reduce the amount of light reaching the water surface, and subsequently the optical environment within the waterbody. Seasonal features—namely the amount of solar radiation reaching the surface of a given area on Earth due to the angle of incidence—directly affect the intensity of light reaching the water’s surface (Hutchinson, 1967; Wetzel, 2001). Variations in seasonal features are especially prevalent in higher latitudes where the angle of solar incidence is most affected by season. Waterbodies located in lower latitudes experience a much more evenly positioned sun and thus experience a more consistent amount of solar radiation through the seasons. For ponds, which are commonly found in forested habitats, seasonal changes in foliage can significantly affect the amount of light reaching a pond’s surface. The light intensity (photons/area/time) at the surface of a pond under a closed forest canopy can be almost 90% less than light intensity at the surface of a pond under open canopy (Cáceres *et al.*, 2008).

Overall, *Daphnia* inhabit a wide range of light environments. Their visual system may therefore experience varying differences in light-mediated selection pressures.

### 1.3 Eyes, vision, and *Daphnia*

#### 1.3.1 Eyes and visual system capabilities

Eyes allow animals to collect and process environmental information from light. There is a dizzying array of morphological diversity among eyes (Salvini-Plawen & Mayr, 1977), but they can be broadly defined into eight known functional classes: pigment cup eye, compound pigment pit eye, aquatic camera-type eye, terrestrial camera-type eye, apposition compound eye, refracting superposition compound eye, concave-mirror eye, and reflecting superposition compound eye (Land & Nilsson, 2012). Interestingly, seven of these eight functional classes can be found among the members of the phylum Arthropoda. Each of these classes of eyes function by specifically different—though broadly similarly—means. In general, the visual capabilities of eyes can be defined by three general components: resolution, light sensitivity, and wavelength discrimination.

Resolution, or visual acuity, refers to the precision of detail an eye can sample from the light environment, whereas sensitivity refers to the number of photons captured by an eye's receptors. Larger eyes enhance both resolution and sensitivity for two key reasons. First, the diameter of the lens will increase, which increases the surface area available to gather light (i.e., more photons are sampled) and hence sensitivity is improved. Resolution improves because a wider lens reduces the width of a diffraction spot, or the circular spot at the point of focus created by a light wave passing through a lens. In other words, a wider lens allows less blur between two points (Land and Nilsson,

2012). Second, larger eyes allow for longer focal lengths, which reduces the minimal sampling angle and allows for more detail to be sampled (Land and Nilsson, 2012).

The ability to discriminate light based on wavelength underlies color vision, or more rudimentary wavelength dependent behaviors. Photoreceptor cells capture photons via visual pigments, a molecule comprised of an opsin protein and a retinal chromophore, embedded in their cellular membranes (Nathans, 1987). Though the chromophore physically reacts with the photon, it is the opsin protein that allows fine spectral tuning of the visual pigment (Kochendoerfer *et al.*, 1999). Different classes of opsins allow for specific sensitivities to different wavelengths of light. However, the overall spectral sensitivity of the photoreceptor is defined by visual pigments, non-visual filtering pigments, and interactions among pigments within photoreceptors. Photoreceptors may absorb a broad spectrum of light, but they do so at diminishing efficiencies away from their peak wavelength sensitivity. To have color vision, an animal needs at least two photoreceptor classes that are maximally sensitive at distinct wavelengths. Many invertebrates can “see” different wavelengths and can react to differences in light color, but true color vision requires the ability to neurally process and behaviorally learn differences in hue, saturation, and brightness (Kelber & Osorio, 2010).

### 1.3.2 *The visual system and ecology of Daphnia*

*Daphnia* possess an embryonically-fused, apposition compound eye, and many possess a second simple, or nauplius eye (reviewed in Ringelberg, 1987). The apposition compound eye—a common eye-type found among diurnal arthropods—is the major visual organ in *Daphnia*. The function of the nauplius eye is not well understood. The

apposition eye contains an array of single light collecting units called ommatidia, where each individual ommatidium produces an optical image (Nilsson & Kelber, 2007). A *Daphnia* ommatidium contains a crystallin cone that focuses light onto a collection of eight photoreceptor cells called the rhabdom (Fig. 1.3). The crystallin cone is surrounded by shielding pigment cells, which act to prevent light that enters one ommatidium from bleeding into another.

The architecture of the *Daphnia* apposition compound eye seems to have evolved for visual tasks based on overall light sensitivity and coarse resolution (i.e., low visual acuity). The compound eye of *Daphnia* consists of twenty-two ommatidia. For the compound eye, the angle between adjacent ommatidia, or the inter-ommatidial angle, determines the eye's visual acuity (Horridge, 1978; Land, 1997). *Daphnia* have large inter-ommatidial angles due to their relatively bulbous ommatidia. Several studies have attempted to estimate the average inter-ommatidial angle in *Daphnia* and have proposed the angles were either 38° or 54°, though neither study empirically measured the angles (Frost, 1975; Young & Downing, 1976; Ringelberg, 1987). Nevertheless, either angle proposed is enormous. These enormous angles equate to very low visual acuity, which suggests that *Daphnia* likely lack the capability to resolve images of conspecifics at any appreciable distance. Furthermore, *Daphnia* have wide photoreceptor cells that enhance visual sensitivity, or photon catch, but at the expense of visual acuity (Young & Downing, 1976; Land, 1997).

A number of studies have characterized the neurophysiology and optical capabilities of the *Daphnia* compound eye. *Daphnia* are sensitive to four wavelengths including a wavelength in the ultraviolet (Smith & Macagno, 1990), they can discriminate

polarized light (Baylor & Smith, 1953; Baylor & Hazen, 1962), and their compound eye can “track” and “fixate” on a white light stimulus (Consi *et al.*, 1990). *Daphnia* have also displayed a range of light-induced behaviors, including the well-documented case of phototaxis and diel vertical migration (reviewed in Ringelberg, 1999). A few species of *Daphnia* have been shown to have differential behavioral response to the color of light. *D. pulex* displays different swimming patterns under constant red or blue light, and it has been suggested that this might be an adaptation for food location (Smith & Baylor, 1953), though this idea has been dismissed by others (Stearns, 1975; Ringelberg, 2010). However, other research has shown a positive feeding response in *Daphnia* to light filtered through an aquarium of yeast populated water (Young *et al.*, 1984), a positive feeding response under green light (Hamza & Ruggiu, 2000), and a preference for green and blue opaque colors (Hamza & Ruggiu, 2000).

What are *Daphnia* looking at, and why? The functional role of vision and its ecological relevance to *Daphnia* is unclear. Research done by Ringelberg *et al* (1974) showed that *D. magna* use a mechanism called contrast orientation, which is facilitated by the compound eye, to orient their body vertically in the water column. He proposed that *Daphnia* use the contrast border of Snell’s window to locate the surface of the water. He argues this orientation is key to efficiently engaging in diel vertical migration (DVM)—a well-studied mechanism to avoid visual predators (Zaret & Suffern, 1976), and ultraviolet light (Leech & Williamson, 2001). However, gravity sensing mechanisms were not known in *Daphnia* then, but have since been described (Meyers, 1985). Furthermore, diel vertical migration behavior of *Daphnia* is mediated by changes in light intensity (Ringelberg, 1995), which a simple nauplius eye can detect. The image-forming

compound eye is an unnecessarily complex tool to accomplish this behavior. In fact, *D.magna* (a species often studied for DVM) can also engage in DVM without a compound eye (Harris & Mason, 1956). Though the role of DVM (and its possible visual component) is clear in stratified lakes and large ponds, it is less clear how DVM in shallow ponds facilitates a need to have a compound eye. Another proposed ecological role of vision is “shore flight” whereby the compound eye can detect decreasing percent polarization originating from the shore and thus *Daphnia* can locate and avoid the shore, where there are often more predators (Schwind, 1999). In contrast, *Daphnia* have also been known to engage in diel horizontal migration where they migrate towards the littoral zones to seek refuge among plants (Burks *et al.*, 2002).

#### **1.4 Brief note on the evolution of vision**

The evolution of eyes has been reviewed in near encyclopedic detail (reviewed in Land & Fernald, 1992; Arendt, 2003, 2009; Fernald, 2004; Gehring, 2004, 2005; Oakley & Pankey, 2008; Vopalensky & Kozmik, 2009; Lamb *et al.*, 2009; Nilsson, 2009; Land & Nilsson, 2012). The focus of this thesis is mostly on microevolution, and relatively—in terms of the grand span of evolutionary time—recent phenomena. However, a brief mention of the evolution of eyes is warranted.

The origin of eyes is somewhat controversial, and it is not surprising given the complex nature of the components necessary to make an eye a functional light receptive organ. Eyes probably evolved independently in at least a few instances (Land & Nilsson, 2012), although others argue, based on evidence from the master control gene Pax6, that the eye originated once and has since evolved into the numerous varieties we note today

(Gehring, 1996). The compound eye of *Daphnia*, however, likely evolved from a single ancestor (Nilsson & Kelber, 2007). There are indeed a number of homologous elements, especially at the molecular level, that eyes share in common. The protein responsible for the capture of photons (light), the opsin, evolved once and from a family of G-protein coupled receptors. The opsins likely diverged into their major subfamilies before the split of deuterostome and protostomes (Hering & Mayer, 2014). There are two major types of photoreceptor cells: the ciliary type with ciliary projections, and the rhabdomeric type with membranous projections. Typically, though not exclusively, ciliary cells are found in vertebrate visual systems whereas rhabdomeric cells are found in invertebrate visual systems. Salvini-Plawen and Mayr (1977) first detailed the diversity of these cell types across a broad taxonomic range, and were led to conclude that there were at least 40 independent origins of these cells. However, recent evidence has cast doubt on their independent origins (Arendt *et al.*, 2004). The eye is a complex organ with a complex evolutionary history of shared ancestry and independent origins, which make it a compelling system to better understand the intricacies of biological evolution.

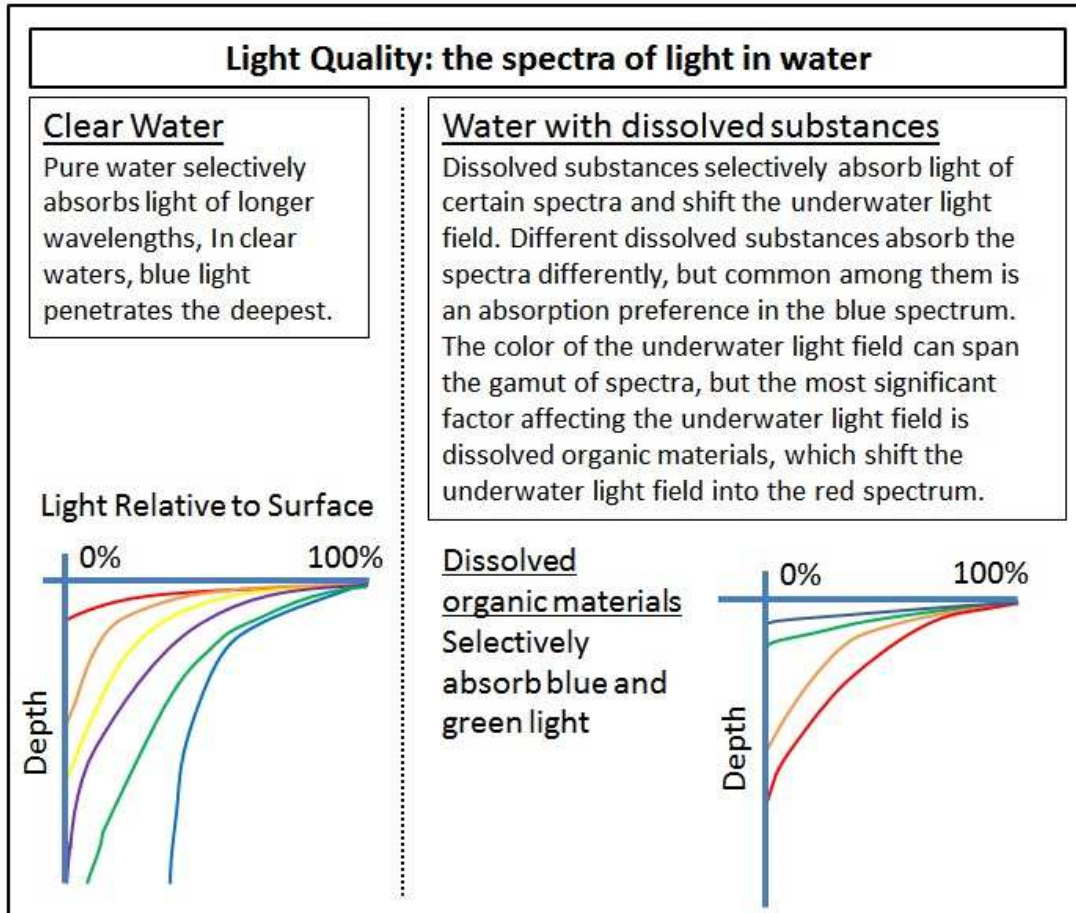
While the literature has detailed processes of macroevolution of the eye in voluminous record, studies of the eye in microevolutionary context are much less detailed. One of the interesting traits of eyes is that optical characteristics can give great insight into what the animal can see, and what it may be looking at. Many features of the eye co-vary with the environment and behavior in a predictable manner. For example, visually guided predators tend to have high visual acuity, while animals found in dim-light environments tend to have large eyes that are very sensitive to light. Additionally, many animals that possess the variants of color vision are more sensitive to wavelengths



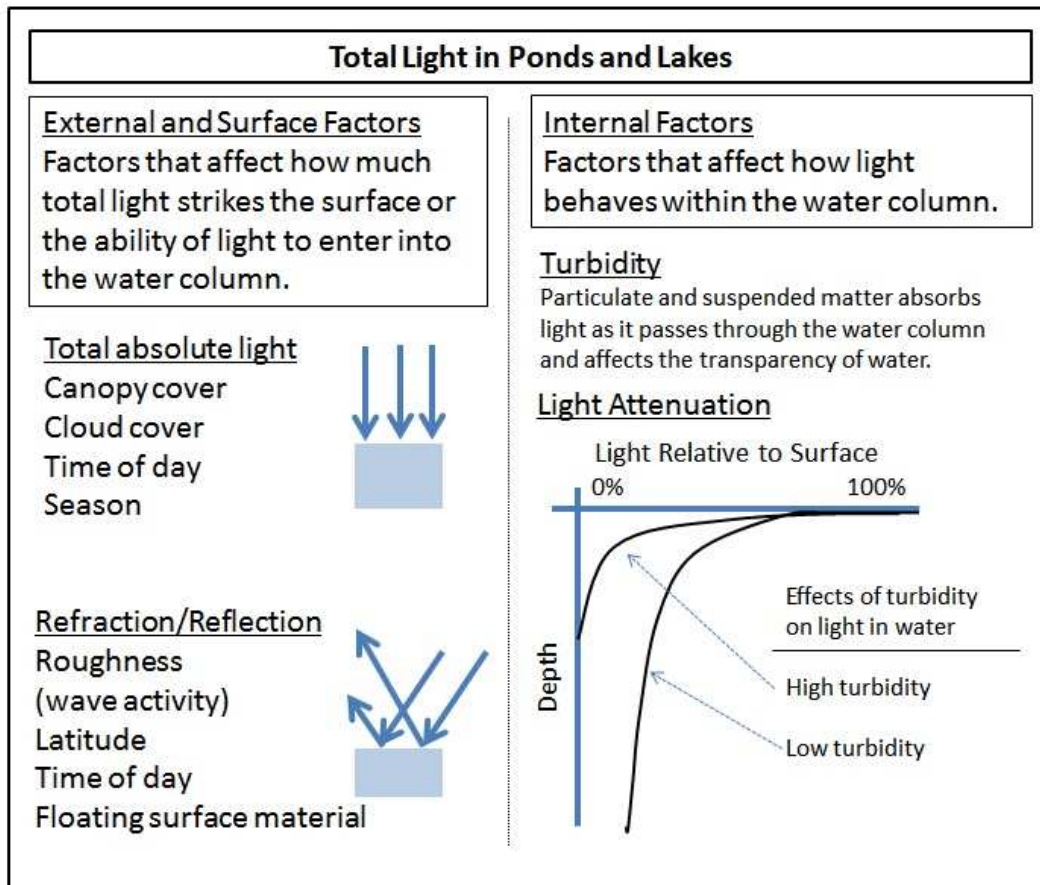
of light that are more dominant in their environment (Lythgoe & Partridge, 1989). Thus, there are clear ecological drivers of variation in different eye traits, but the patterns of variation have mostly been examined on macroevolutionary scales. To illuminate processes of evolution at macroevolutionary scales it is necessary to enhance our understanding of the contemporary forces of evolution that are shaping eyes between and among populations within species.

## **1.5 Thesis outline**

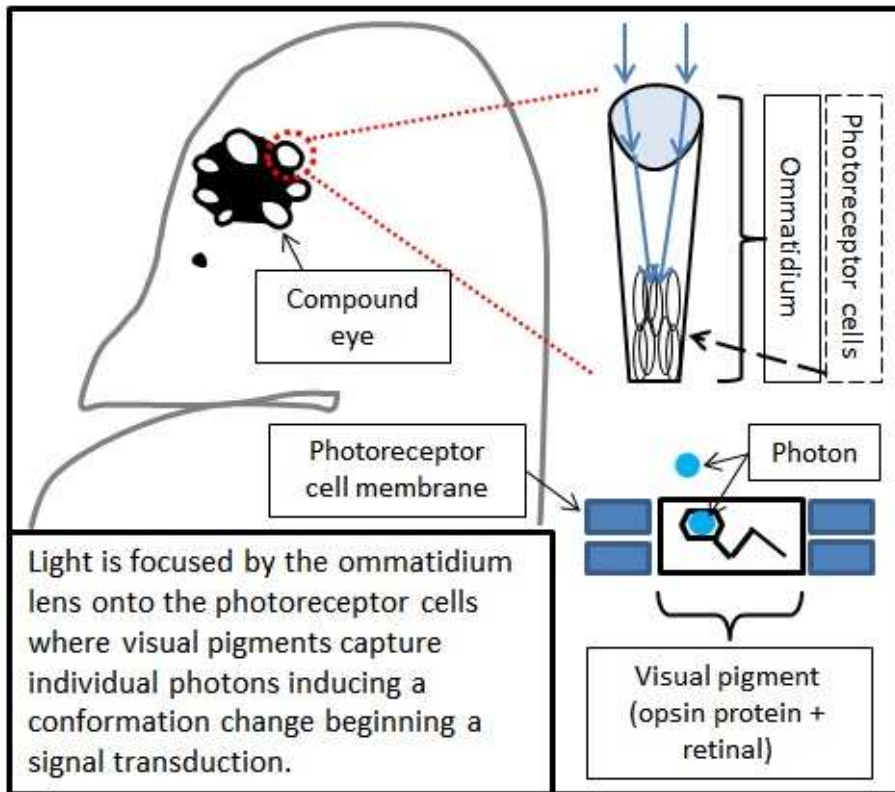
Darwin's initial intrigue of the complexity of the eye has spawned a remarkable library of information that has examined the evolution of eyes and visual systems in general. While the literature has documented myriad facets of eye evolution, there still remain many areas that merit more investigation. My thesis aims not on a single focal point within the study of the evolution of vision; rather, I sought to broaden our understanding by exploring related, yet different, aspects of the evolution of vision by exploiting the biology and ecology of the common water flea, *Daphnia*. Recently, commentators have urged researchers to examine sensory systems in the context of contemporary processes of evolution (Chittka, 2001; Dangles *et al.*, 2009). I present two studies that examine the variation of eye morphology in the context urged by the commentators (Chapter 2, Chapter 3). Additionally, I present a study on opsin evolution in *Daphnia*, which may yield insight on the evolution of vision in *Daphnia*, but also the broader scope of opsins in crustaceans (Chapter 4).



**Figure 1.1** A diagram illustrating how light attenuates in the water column.



**Figure 1.2** A diagram illustrating the various external and internal factors that affect the amount of light in a waterbody.



**Figure 1.3** A diagram illustrating the basic features of vision in *Daphnia*.

## CHAPTER 2

ECOLOGICAL CONSTRAINTS ON SENSORY SYSTEMS: COMPOUND EYE SIZE IN

*DAPHNIA* IS REDUCED BY RESOURCE LIMITATION<sup>1</sup>

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<sup>1</sup> Brandon, C.S., and J.L. Dudyca. Published in the *Journal of Comparative Physiology A*. 2014. **8**: 749-758

## 2.1 Introduction

Eye size is an important determinant of visual capabilities. In apposition-type compound eyes, differences in eye size are also reflected in the structural units that influence the principal elements of visual capabilities (Land, 1997; Land & Nilsson, 2012). One such element, sensitivity, which refers to the number of photons captured by an eye's receptor, can be enhanced by larger compound eye size. Apposition compound eyes are a composite of individual optical units called ommatidia, each of which are singularly capable of forming an image (Land & Nilsson, 2012). An ommatidium contains a facet that collects and focuses light onto a set of photoreceptor cells. A bigger compound eye can accommodate wider facets, thus increasing aperture size, a critical aspect of improving sensitivity (Land & Nilsson, 1990, 2012).

Comparative morphological studies across a broad range of taxa have demonstrated that the brightness of the light environment is a strong predictor of eye morphology. This evolutionary association is a robust pattern that has been demonstrated in mammals (Veilleux & Lewis, 2011), bony fish (Schmitz & Wainwright, 2011), sharks (Lisney & Collin, 2007), birds (Hall & Ross, 2007), lizards (Hall, 2008), beetles (Bauer *et al.*, 1998), bees (Somanathan *et al.*, 2009), and crustaceans (Hiller-Adams & Case, 1985). However, these studies focus on eye morphology as a fixed property of species, and ignore the potential for phenotypic plasticity of eye size.

Environmental factors that are directly tied to vision undoubtedly are key evolutionary drivers of visual systems (Nilsson, 2009). However, factors that are not tied directly to vision may also affect visual systems. We refer to these factors as the “non-sensory environment.” The resource environment, for example, may constrain the size of

eyes because eyes are energetically expensive (Niven *et al.*, 2007; Niven & Laughlin, 2008), and their costs place limits on the net benefit of large eyes. In cavefish, eyes have regressed to near uselessness, but their close relatives that live above-ground have maintained fully functional eyes (Jeffery, 2005; Borowsky, 2008). Caves are resource-limited environments, and the loss of eyes in cavefish may be driven to some degree by the relatively high energetic costs of the visual system coupled with minimal benefit of vision (Niven & Laughlin, 2008). The resource environment has also been implicated in variation of eye size in marine crustaceans (Hiller-Adams & Case, 1985, 1988). Hiller-Adams and Case (1985) found that in benthic decapods eye size increases with decreasing ambient light levels (i.e., with increasing depth), in line with the expectation that larger eye size enhances photon capture and improves vision in dimmer environments. In contrast, they found the opposite trend in pelagic crustaceans (Hiller-Adams & Case, 1984, 1988), and suggest the pattern is due to large eyes that become an energetic burden in the resource-limited pelagic zone. These correlative examples suggest that effects of light environment may depend on resource availability.

If the mechanism driving the macroevolutionary pattern reflects the balancing of costs and benefits of vision, we might expect to find a similar association when examining phenotypic variation within species. Larger eyes benefit an organism by increasing information acquisition, but at an energetic cost. Increasing the capacity to acquire information is only useful to an organism if it enhances some quality of fitness or survival. Developmental investment in eyes and the ability to acquire information beyond what is useful for an organism may needlessly siphon resources away from other somatic and reproductive tissue. Relevant data on fluctuating costs and benefits reflected in

phenotypic plasticity of eyes are limited. In a selection experiment, Nijhout and Emlen (1998) found that allocation to horn development in beetles was negatively genetically correlated with eye size. Merry *et al.* (2011) found evidence of phenotypically plastic eye size in butterflies in response to resource availability. However, we know of no experimental study that has manipulated the light environment, nor any that have examined the combined effects of both sensory and non-sensory environments on eye size.

Here, we test the hypothesis that resources and light jointly determine the plastic response of eye size in four species of *Daphnia*, a freshwater microcrustacean. *Daphnia* inhabit environments that vary in light and resource availability, and may therefore experience changes in the balance of costs and benefits of investment in vision. We consider the absolute eye size and eye size relative to body size to address both visual capabilities and energetic allocation. Changes in absolute eye size may affect *Daphnia* visual performance through both sensitivity and resolution. *Daphnia* have relatively crude resolving capabilities due to the low number of ommatidia (22) present in their eye (Young & Downing, 1976). We also measure facet lens width of ommatidia in conjunction with absolute eye size. Changes in relative eye size reflect shifts in the allocation of resources to the visual system, and thus provides an index of the energetic investment an individual makes in vision.

We exposed *Daphnia* to a dim/bright environmental contrast and tested the prediction that (i) in dim light compound eyes would be larger, on average, in absolute (more light collection) and relative size (more resources allocated) than compound eyes of animals reared in a bright environment. We also examined *Daphnia* eye response



under a high/low resource quantity contrast where we predicted that (ii) animals reared in a low resource environment would exhibit smaller eyes on average, both in absolute and relative scale, than those reared in a high resource environment.

## **2.2 Methods**

### *2.2.1 Experimental design*

We manipulated *Daphnia* rearing environments by experimentally crossing high and low resource levels with bright and dim light levels. We conducted experiments in four species, allowing us to test whether eye size responses are robust across species that inhabit different light and resource environments. Since *Daphnia* have indeterminate growth, allocation patterns may change as animals grow older (Dudycha & Lynch, 2005), and we therefore repeated the experiments at both early and late adulthood.

In the high resource treatments, animals were fed 20,000 cells/ml of the green alga *Ankistrodesmus falcatus* daily from birth, whereas in the low resource treatments animals were fed 5,000 cells/ml. Previous work has shown that this scale of resource availability induces substantial variation in *Daphnia* resource allocation (Tessier & Consolatti, 1991; Dudycha, 2003) and morphology (Lynch, 1989).

*Daphnia* species and intra-specific populations inhabit a wide range of light environments that can fluctuate widely in terms of absolute light levels. We sought to impose a consistent environmental contrast of a relative order that multiple species of *Daphnia* experience. We used two lake species where light environments are best defined by the vertical distribution within a lake, and two pond species where light environments are best characterized by the amount of canopy cover. We therefore categorized light environments as bright versus dim based on similar magnitude differences found between

light intensity in a lake epilimnion and hypolimnion (Wetzel, 2001), and ponds under sparse versus dense canopy (Cáceres *et al.*, 2008).

Two environmental chambers (Percival Scientific, Inc., Iowa, USA) were set to subject the animals either to dim ( $10 \mu\text{E m}^{-2} \text{s}^{-1}$ ) or bright light ( $110 \mu\text{E m}^{-2} \text{s}^{-1}$ ) conditions. Light levels were measured using a  $4\pi$  PAR radiometer (Biospherical Instruments Inc., California, USA). Each chamber had two shelves with two fluorescent lights above each shelf. We measured light on both shelves and found minimal differences (Fig. A.1). Light attenuation was also measured between high resource and low resource treatments, and we found a difference equal to  $\sim 6\%$  of the total difference between the dim and bright light treatments. Under the dim condition, lights were wrapped in three layers of neutral density screening (charcoal fiberglass screen wire; Phifer Inc. Alabama, USA), whereas the high light lamps were left unmanipulated. We randomized beaker locations and rotated them daily within chambers to control for minor variations of light within a chamber. To minimize chamber effects, the experimental lighting setup was switched between the two chambers on every third day during the experiment.

We assayed each ontogenetic stage in separate experimental cohorts (i.e., individual animals were only measured once). Early adulthood was defined as the instar after the release of the first clutch of offspring. Late adulthood was defined as the instar after the fourth clutch, where the animal is effectively past a point of adding to overall fitness (Taylor & Gabriel, 1992).

Two species were isolated from permanent lakes (*D. parvula* Fordyce and *D. pulicaria* Forbes) and two were isolated from temporary ponds (*D. pulex* Leydig and *D.*

*obtusa* Kurz). We conducted our experiment with a single clone from each of four species. *D. parvula* was isolated from McReynolds Lake (30° 54' 03" N, 87° 55' 47" W) in southern Alabama, USA. *D. pulicaria* was isolated from Lake Sixteen (42° 33' 52" N, 85° 36' 47" W), and *D. pulex* from Pond of the Village Idiot (42° 43' 10" N, 85° 23' 16" W) in southwestern Michigan, USA. *D. obtusa* was isolated from Powerlines Pond (33° 45' 49" N, 80° 38' 30" W) at Congaree National Park, South Carolina, USA.

Mothers of experimental animals were maintained at low density at 20°C on a 12:12 L:D photoperiod in filtered (1 µm) hypolimnetic lakewater. Mothers were fed vitamin-enriched *Ankistrodesmus falcatus* daily (Goulden *et al.*, 1982).

To start each experiment, neonates (< 15 hr old) were placed individually into 100 mL of filtered lake water, and randomly assigned a treatment. A light dusting of cetyl alcohol prevented surface film entrapment (Desmarais, 1997). We began each experiment with approximately 40 replicate individuals per treatment per ontogenetic stage (see Table A.1 for sample sizes).

Experimental animals were moved into fresh filtered lake water every other day. We performed feeding and water changes under dim red light during the dark cycle of the photoperiod to prevent disruptions to the brightness of light during the day phase.

### 2.2.2 Measurements

Animals were sacrificed in droplets of 0.25 M KCl and photographed within five minutes. Lateral photographs were taken through a Nikon 1500 SMZ dissecting scope at 30x magnification to include the entire body, and at 112.5x to maximize precision in measuring eye size, then analyzed in ImageJ (Schneider *et al.*, 2012). Body length was measured from the top of the head just above the eye to the base of the tail-spine (Fig.

2.1). Although *Daphnia* eyes are approximately spherical, most individuals deviate somewhat. Eye diameter was therefore taken at the widest diameter.

We measured the width of ommatidial facets to verify that the actual light collecting units varied in tandem with eye size. Measurements of ommatidia were taken at 112.5x magnification. *Daphnia* ommatidia are large and bulbous, but the pigmentation of the *Daphnia* compound eye makes it impossible to see all the facets clearly. For each individual, we therefore measured three ommatidial facets (of 22) based on the clarity of the facet, and not with regard to the regional position of the ommatidium within the eye.

### 2.2.3 Percent increase

We used mean values of eye diameter calculated for each treatment and stage level to calculate percent increase in eye area (Table 2.1). We calculated *Daphnia* eye area for each mean value of absolute eye diameter for each treatment level, developmental stage, and species (Table A.1). We used the surface area equation for a sphere to calculate eye area:

$$eye\ area = 4\pi\left(\frac{1}{2}\ mean\ eye\ diameter\right)^2$$

We present percent increase in eye area as the percent difference in eye area in the high food treatment versus low food treatment, and the difference in dim light versus high light.

### 2.2.4 Statistical analysis

Our main objective was to examine the plasticity of eye size within species and developmental stages. We used ANOVA to examine the fixed effects of resource environment, light environment, and their interaction on absolute eye size and body size, running the analysis separately on each species at each ontogenetic stage. We were also

interested in the treatment effects on eye size relative to body size, since this reflects resource allocation trade-offs. We therefore ran an analysis of covariance (ANCOVA) on eye diameter (response) and body length (predictor) variables for each species at each ontogenetic stage, considering resource and light as fixed factors. These analyses were performed in SPSS v. 21 (IBM Corp., New York, USA).

To test the assumption that sensitivity increases with increasing eye size, we used ordinary least squares regression to analyze the relationship between ommatidial facet (the light collecting unit) width and eye diameter. For this analysis, we used R (R Team, 2013). We were interested in the global relationship, thus we performed our analysis on all experimental observations, pooling all measurements from all species, ages, and treatments. We measured three facets per individual eye, regressing mean facet width value against eye diameter.

## **2.3 Results**

### *2.3.1 Facet lens and eye size*

Regression analysis revealed a strong positive relationship between facet lens width and eye diameter (Fig. 2.2; slope = 0.228, adj.  $R^2 = 0.81$ ,  $P < .0001$ ), supporting the assumption that facet lens width increases with eye diameter.

### *2.3.2 Absolute eye size and body size*

High resources consistently led to larger absolute eye diameter than did low resources (Table 2.1). This reflected the pattern for body size, where individuals raised in a high resource environment were larger (Table 2.2). The only exception was *D. parvula* at early adulthood, where neither body size nor eye size was affected by resource level.

Depending on species and ontogenetic stage, high resources increased eye area, a strong determinant of light sensitivity, by 7% – 34% (Table 2.1).

Effects of light intensity were inconsistent across species and ontogenetic stage. Both *D. pulex* and *D. pulicaria* exhibited larger body sizes in bright light than in dim light by 3% – 5% (Table 2.2). However, absolute eye size was larger in bright light than in the dim light only in late adulthood for *D. pulex* (14% increase) and only in early adulthood for *D. pulicaria* (4% increase). Both observations directly contradict the predicted effect of light intensity. Light intensity did not affect body size or absolute eye size in *D. parvula*. In *D. obtusa*, the only significant difference was that absolute eye size was ~ 4% larger in dim light at early adulthood (Table 2.1).

In some cases, there were resource – light interactions, but the form of these interactions was not consistent across species. In *D. parvula* and *D. pulex*, there were interactive effects in body size (Table 2.2) and absolute eye size (Table 2.1) in early adulthood. In *D. obtusa*, there was a significant interaction in late adulthood in body size (Table 2.2) and absolute eye size (Table 2.1). The only resource – light interaction in *D. pulicaria* was in absolute eye size during early adulthood (Table 2.1).

### 2.3.3 Relative eye size

*Daphnia* generally showed significantly larger eyes relative to body size when raised in a high resource environment versus a low resource environment (Table 2.3, Fig 2.3). There were two exceptions in late adulthood. In *D. parvula*, the increase was only marginally significant, and in *D. obtusa* there was no effect.

The light environment generally had no effect on relative eye size in *Daphnia*, with exceptions in two cases. In *D. pulicaria*, relative eye size was slightly, but

significantly, larger in bright environments at late adulthood (Fig. 2.4). *D. parvula*, in contrast, had larger relative eye size in dim environments at early adulthood (Fig. 2.4).

The effects of treatment x body length interactions were few and inconsistent across species and ages. The light environment affected the relationship of eye size to body length in *D. pulex* at early adulthood, and in late adulthood in *D. parvula* (Table 2.3). In *D. obtusa*, an interaction of resource environment x body length was observed in early adulthood (Table 2.3).

## 2.4 Discussion

We found that resources have a more substantial influence on eye size than light intensity does. We consistently observed larger eyes in higher resource environments across species and ontogeny. In contrast, we observed few and inconsistent effects of light environments on eye size. This was a surprise because studies that examine eye size across species often find that dim environments are associated with the evolution of large eyes.

We also found a strong positive relationship between facet width and eye diameter in *Daphnia*. Facet width—or aperture size—is a prominent factor in determining a compound eye's sensitivity, where larger facets lead to increased sensitivity (Land & Nilsson, 1990). *Daphnia* have few ommatidia and limited resolving abilities (Young & Downing, 1976), and likely the most relevant visual capability affected by changes in eye size is sensitivity. Optical sensitivity in apposition compound eyes can be described by:

$$S = 0.62D^2 \Delta\rho^2 P_{abs}$$

where  $D$  is the facet diameter,  $\Delta\rho$  is the rhabdom acceptance angle, and  $P_{abs}$  is the proportion of photons absorbed (Land & Nilsson, 2012). All other things being equal, changes in facet diameter will change the values in  $S$ . We show that changes in facet diameter shows a strong correlation with changes in eye diameter, thus larger eye diameters increase facet diameters and ultimately enhance sensitivity. It seems unlikely that changes in the other parameters would change in an opposite fashion as to negate increases in sensitivity. Therefore, abundant resources allow for greater relative investment in eyes and lead to improved *Daphnia* visual capabilities.

Eye size scales positively with body size in *Daphnia*, and thus effects on body size may in part drive differences in absolute eye size. Nonetheless, absolute differences in eye size necessarily change optical characteristics. Body size constrains absolute eye size (Wehner, 1981; Rutowski, 2000), such that the optimal eye size in *Daphnia* may actually lie beyond what its body plan can accommodate. *Daphnia*, therefore, may benefit visually as a consequence of larger body size, where they exploit the added space to continue to grow the eye. Indeed, *Daphnia* grow indeterminately and continue to add size to the eye with no apparent plateau well after reproductive maturity (C. S. Brandon, unpublished).

Our results generally refute the hypothesis that phenotypic plasticity within species follows a pattern similar to the macroevolutionary pattern. Furthermore, our study highlights that a non-sensory factor can have strong effects on eye size, potentially large enough to have a major impact on visually-mediated ecological interactions. Together, these results indicate that the mechanisms driving within-species phenotypic variation in visual capability differ from those driving macroevolutionary divergence.



#### 2.4.1 Eye size and the light environment

We were surprised that our results showed no consistent response of compound eye size with respect to the light intensity. *Daphnia* possess an apposition type compound eye, which is common among diurnal arthropods. Many comparative studies have documented differences of apposition eye size in closely related taxa that have diurnal, nocturnal or crepuscular members, where they have shown that dim light environments tend to harbor animals with comparably larger eyes than their cousins in brighter environments (Bauer *et al.*, 1998; Land *et al.*, 1999; Greiner, 2006; Somanathan *et al.*, 2009). If plasticity is adaptive within generations, it should match adaptively evolved differences between generations. Thus, we predicted that *Daphnia* eye size would be larger in dim environments. That prediction failed in seven of our eight experiments. In fact, in two situations with a significant light effect, the direction was opposite to the prediction, with larger absolute eyes in bright light for late adult *D. pulex* and early adult *D. pulicaria*. Our prediction was supported only in early adult *D. obtusa*, and there it was merely a 4% increase of eye area in dim light.

There are other parameters that enhance a compound eye's sensitivity, which were not measured in this study, but could have changed in *Daphnia* as a consequence of the light environment. We focus on facet width in this study, a parameter that can be reasonably measured in an experiment at the scale presented here. Another prominent factor which affects sensitivity is the photoreceptor width, where an increase in photoreceptor width increases sensitivity (Land & Nilsson, 1990). This alternative strategy to enhance sensitivity comes with a cost to resolving abilities. It seems unusual that *Daphnia* would opt to increase the width of photoreceptors at the expense of

resolution, when they are capable of changing investment in eye size and facet width, which enhance sensitivity without sacrificing resolution. Increases in the time over which photoreceptors collect and process light signals (temporal summation) remains another option (Land & Nilsson, 2012), however longer sampling times can lead to blurring of the image especially in actively moving organisms such as *Daphnia*. Pigment migration is also a common strategy used in compound eyes (Bruin & Crisp, 1957), and possibly employed by *Daphnia* (Cellier-Michel *et al.*, 2000).

The canalization of the compound eye size and facet width with respect to the light environment may have arisen from the variant light environments that *Daphnia* inhabit. There is no systematic information on the light environment experienced by different species of *Daphnia*, but all of our species occupy a range of habitats that expose them to large differences in light environments. The light environment can vary from waterbody to waterbody (Wetzel, 2001). For example, ponds can vary in amount of canopy cover leading to a range of dim to bright ponds within a small geographic locale. The light environment also changes within a waterbody, especially in its vertical distribution. Even in shallow ponds, the dissolved and particulate matter can absorb light so rapidly as to practically extinguish light within the first half meter. In these environments, an individual may thus experience a large jump in available light within decimeters. Furthermore, spatial partitioning of lakes and ponds either through diel vertical migration and non-migration behaviors is highly variable within lakes and across water bodies (Weider, 1984; Tessier & Leibold, 1997), and among species (Tappa, 1965). *Daphnia* species may therefore experience highly divergent light environments on very

short timescales, and the compound eye may have evolved to operate in a broad range of light environments.

One limitation of our study is that in real lakes and ponds, changes in light availability are often accompanied by changes in spectrum (Hutchinson, 1975; Wetzel, 2001). For example, the hypolimnion of relatively clear waters is dominated by blue light, but waters containing calcium or dissolved organic substances shift the light field to the green or orange-red, respectively. *Daphnia* can inhabit the range of these environments, thus dim light in a white light field does not necessarily represent dim light conditions for all *Daphnia*. The change in environmental spectrum may elicit changes in other physiological features such as in the composition of visual pigments (Cronin & Caldwell, 2001; Fuller *et al.*, 2005). However, the strategy to deal with sustained differences in bright versus dim light across broad taxonomic scales has been to increase aperture and eye size.

#### 2.4.2 Eye size and resource environment

In general, *Daphnia* raised on high resources had larger eyes, both in absolute and relative dimensions, than those raised on low resources. This shows that a major aspect of the non-sensory environment can substantially influence visual capability and the investment organisms make in vision.

One important outcome of our data is that relative, and not simply absolute eye size, responds to resource environment. If *Daphnia* eyes were locked into a fixed allometric relationship with body size, only absolute eye size would have responded to resources. In contrast, our results demonstrate that these animals have the ability to modulate their allocation of resources to visual systems in response to a non-sensory

aspect of the environment. One previous report has also demonstrated resource-driven eye size plasticity, but the direction of eye response to low nutrition was opposite from our results. Merry et al. (2011) showed that the butterfly *Colias eurytheme* had relatively larger eyes when raised on a poor quality diet. The authors reasoned that animals raised on a poor quality diet invested relatively more in eye development to compensate for visual performance lost as a function of overall smaller size. This makes sense for an animal that requires high visual performance as an essential tool for foraging, oviposition, and mate detection. *Daphnia* are filter-feeding grazers, however, and the marginal gain from increasing investment in visual performance under poor resource environments may not offset the costs of resources re-allocated from other functions.

#### 2.4.3 Species differences

The response of eye size to resources was robust across species and ages, suggesting that it has deep evolutionary origins that may be maintained because it is generally adaptive for *Daphnia*. However, the consistent responses highlight that there were no obvious differences due to the environments in which these species evolved, i.e., lake versus pond. *D. pulex* and *D. pulicaria* had relative eye sizes that were larger in high resources at both ontogenetic stages. The parallel response may be explained by phylogeny as these are probably ecotypes of a single species (Pfrender *et al.*, 2000; Heier & Dudycha, 2009). The distantly related *D. parvula* also showed this pattern, although the differences between high and low resources were not as pronounced. *D. parvula* have the smallest absolute eye size and may be on the lower range of what is a functional eye for *Daphnia*, and small sacrifices in investment of the eye may severely hinder its relevant visual capabilities. Lastly, *D. obtusa* displayed a relative eye size response only

at early adulthood, showing that, at least for this species, investment in visual systems development can vary through ontogeny.

#### 2.4.4 Conclusion

We found that resources, an aspect of the environment not directly tied to vision, strongly influenced eye size in *Daphnia*, whereas light intensity, typically an important determinant of macroevolutionary divergence of eye morphology, had little effect. Our results show that environmental factors outside of those that directly mediate visually guided behaviors have likely influenced the evolution of visual systems in *Daphnia*. The sensory environment has certainly been a major driver of variation in eye size across multiple taxa, but our findings show that phenotypic variation in eye size cannot be understood solely in the context of the sensory environment.

**Table 2.1.** Results of ANOVA on the effects of different environmental treatments on *Daphnia* spp. absolute eye diameter, and the percent increase in the compound eye surface area (total light collection ability) in high resource and dim light levels, and significant differences between means are noted in bold.

Species	Stage	d.f.	Resource		Light		Resource x Light		Percent Increase	
			F	P	F	P	F	P	High Resource	Dim Light
<i>D. parvula</i>	Early	1, 100	3.84	0.0527	1.58	0.2121	<b>4.16</b>	<b>0.044</b>	5.9	3.6
	Late	1, 88	<b>11.98</b>	<b>0.0008</b>	1.23	0.2705	0.04	0.8507	13.1	-4.6
<i>D. obtusa</i>	Early	1, 76	<b>83.42</b>	<b>&lt;0.0001</b>	<b>4.38</b>	<b>0.0398</b>	0.31	0.5814	21.5	4.3
	Late	1, 76	<b>10.81</b>	<b>0.0015</b>	1.15	0.2879	<b>7.12</b>	<b>0.0093</b>	6.6	-2.8
<i>D. pulex</i>	Early	1, 140	<b>106.58</b>	<b>&lt;0.0001</b>	1.55	0.216	<b>12.44</b>	<b>0.0006</b>	20.2	-1.9
	Late	1, 50	<b>33.86</b>	<b>&lt;0.0001</b>	<b>6.25</b>	<b>0.0158</b>	0.01	0.9276	33.8	-12.4
<i>D. pulicaria</i>	Early	1, 144	<b>157.82</b>	<b>&lt;0.0001</b>	<b>11.46</b>	<b>0.0009</b>	<b>4.19</b>	<b>0.0424</b>	18.5	-4.3
	Late	1, 132	<b>55.92</b>	<b>&lt;0.0001</b>	1.36	0.2456	0.97	0.327	14.9	-1.9

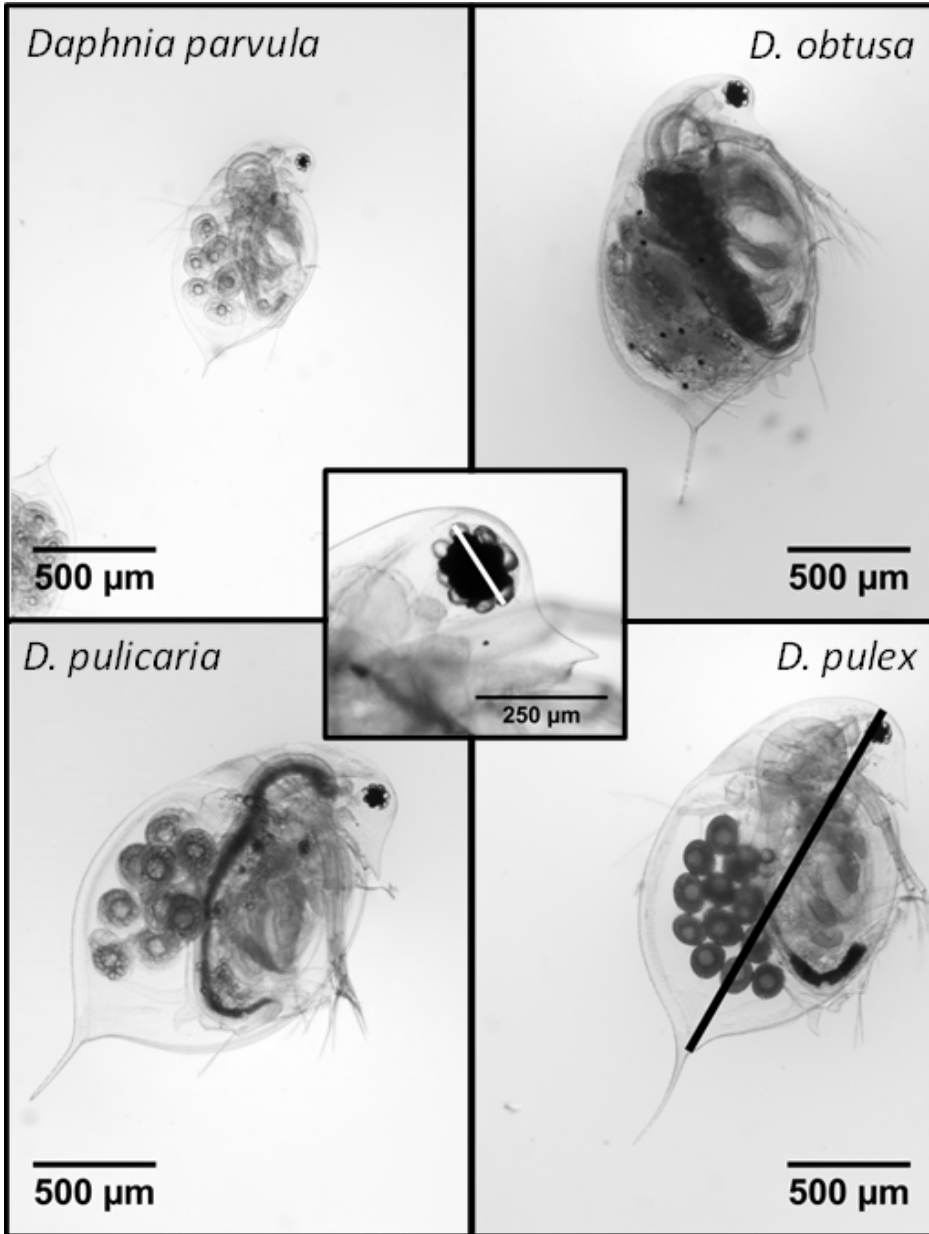
**Table 2.2** Results of ANOVA on the effects of different environmental treatments on *Daphnia* spp. body length, and significant differences between means are noted in bold.

Species	Stage	d.f.	Resource		Light		Resource x Light	
			F	P	F	P	F	P
<i>D. parvula</i>	Early	1, 100	0.702	0.404	0.141	0.708	<b>7.433</b>	<b>0.008</b>
	Late	1, 88	<b>8.898</b>	<b>0.004</b>	1.036	0.312	0.003	0.959
<i>D. obtusa</i>	Early	1, 76	<b>54.767</b>	<b>&lt;0.001</b>	3.569	0.063	0.062	0.804
	Late	1, 76	<b>31.766</b>	<b>&lt;0.001</b>	0.206	0.651	<b>4.314</b>	<b>0.041</b>
<i>D. pulex</i>	Early	1, 140	<b>181.345</b>	<b>&lt;0.001</b>	<b>12.06</b>	<b>0.001</b>	<b>8.123</b>	<b>0.005</b>
	Late	1, 50	<b>47.589</b>	<b>&lt;0.001</b>	<b>8.137</b>	<b>0.006</b>	0.32	0.574
<i>D. pulicaria</i>	Early	1, 144	<b>216.461</b>	<b>&lt;0.001</b>	<b>24.172</b>	<b>&lt;0.001</b>	3.131	0.079
	Later	1, 132	<b>45.287</b>	<b>&lt;0.001</b>	<b>20.598</b>	<b>&lt;0.001</b>	0.446	0.505

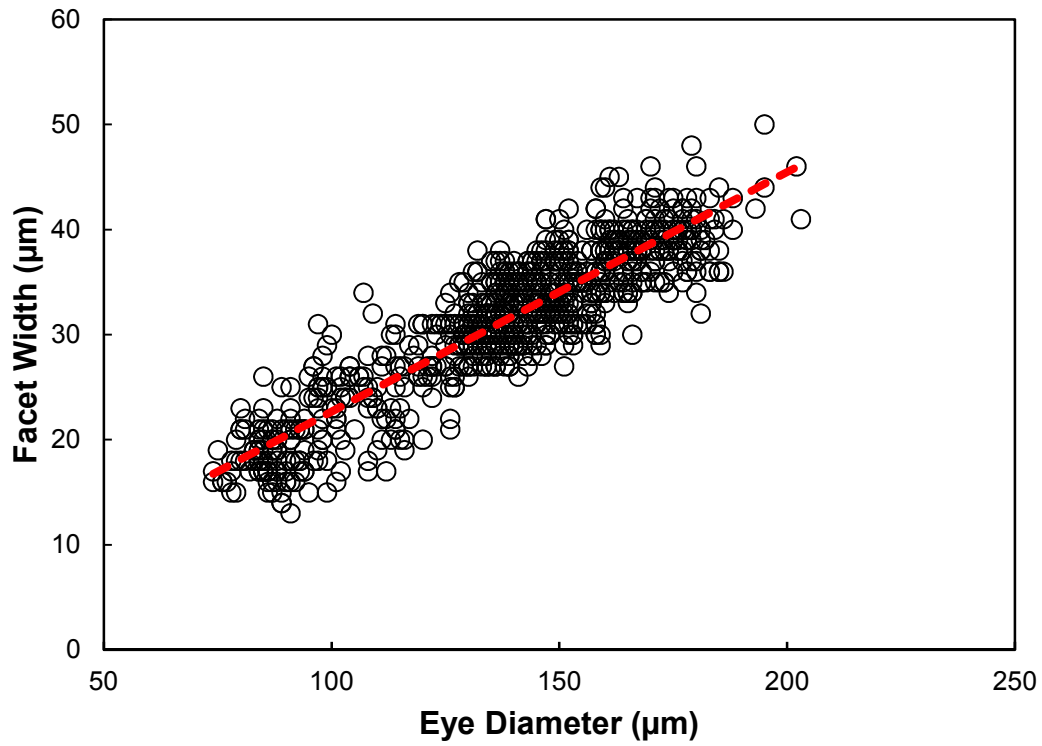
**Table 2.3** Results of an ANCOVA on the effects of different environmental treatments on *Daphnia* spp. eye size using body length as a covariate.

Source of variation	<i>D. parvula</i>		<i>D. obtusa</i>		<i>D. pulex</i>		<i>D. pulicaria</i>	
	F	p	F	p	F	p	F	p
Early Adulthood								
Resource (R)	<b>F<sub>(1,99)</sub>=4.14</b>	<b>0.0445</b>	<b>F<sub>(1,75)</sub>=19.15</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,143)</sub>=35.00</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,139)</sub>=8.03</b>	<b>0.0053</b>
Light (L)	<b>F<sub>(1,99)</sub>=5.71</b>	<b>0.0188</b>	F <sub>(1,75)</sub> =1.29	0.2601	<b>F<sub>(1,143)</sub>=4.16</b>	<b>0.0431</b>	F <sub>(1,139)</sub> =0.24	0.6239
R x L	F <sub>(1,99)</sub> =0.01	0.9418	F <sub>(1,75)</sub> =0.26	0.6141	F <sub>(1,143)</sub> =2.93	0.0900	<b>F<sub>(1,139)</sub>=5.86</b>	<b>0.0167</b>
Body length (bl)	<b>F<sub>(1,99)</sub>=140.77</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,75)</sub>=47.80</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,143)</sub>=8.18</b>	<b>0.0049</b>	<b>F<sub>(1,139)</sub>=43.25</b>	<b>&lt;0.0001</b>
R x bl	F <sub>(1,96)</sub> =3.04	0.0845	<b>F<sub>(1,72)</sub>=4.74</b>	<b>0.0327</b>	F <sub>(1,140)</sub> =0.01	0.9093	F <sub>(1,137)</sub> =0.08	0.7817
L x bl	F <sub>(1,96)</sub> =0.30	0.5842	F <sub>(1,72)</sub> =0.31	0.5786	<b>F<sub>(1,140)</sub>=4.87</b>	<b>0.0210</b>	F <sub>(1,137)</sub> =2.31	0.1308
R x L x bl	F <sub>(1,96)</sub> =1.57	0.2126	F <sub>(1,72)</sub> =2.92	0.0917	F <sub>(1,140)</sub> =0.32	0.8593	F <sub>(1,137)</sub> =1.13	0.2891
Late Adulthood								
Resource (R)	F <sub>(1,87)</sub> =2.73	0.1023	F <sub>(1,75)</sub> <0.01	0.9875	<b>F<sub>(1,131)</sub>=25.06</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,49)</sub>=5.37</b>	<b>0.0247</b>
Light (L)	F <sub>(1,87)</sub> =0.22	0.6408	F <sub>(1,75)</sub> =2.68	0.1060	F <sub>(1,131)</sub> =0.01	0.9177	F <sub>(1,49)</sub> =1.78	0.1880
R x L	F <sub>(1,87)</sub> =0.07	0.7953	F <sub>(1,75)</sub> =3.04	0.0854	F <sub>(1,131)</sub> =0.68	0.4108	F <sub>(1,49)</sub> =0.12	0.7271
Body length (bl)	<b>F<sub>(1,87)</sub>=192.94</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,75)</sub>=39.24</b>	<b>&lt;0.0001</b>	<b>F<sub>(1,131)</sub>=10.59</b>	<b>0.0014</b>	<b>F<sub>(1,49)</sub>=9.99</b>	<b>0.0027</b>
R x bl	F <sub>(1,84)</sub> =0.55	0.4621	F <sub>(1,72)</sub> <0.01	0.9888	F <sub>(1,129)</sub> =1.28	0.2594	F <sub>(1,47)</sub> =0.37	0.5439
L x bl	<b>F<sub>(1,84)</sub>=6.34</b>	<b>0.0137</b>	F <sub>(1,72)</sub> =0.49	0.4851	F <sub>(1,129)</sub> =0.59	0.4441	F <sub>(1,47)</sub> =0.18	0.6710
R x L x bl	F <sub>(1,84)</sub> =0.02	0.8981	F <sub>(1,72)</sub> =0.65	0.4220	<b>F<sub>(1,129)</sub>=4.40</b>	<b>0.0378</b>	F <sub>(1,47)</sub> =0.10	0.7518

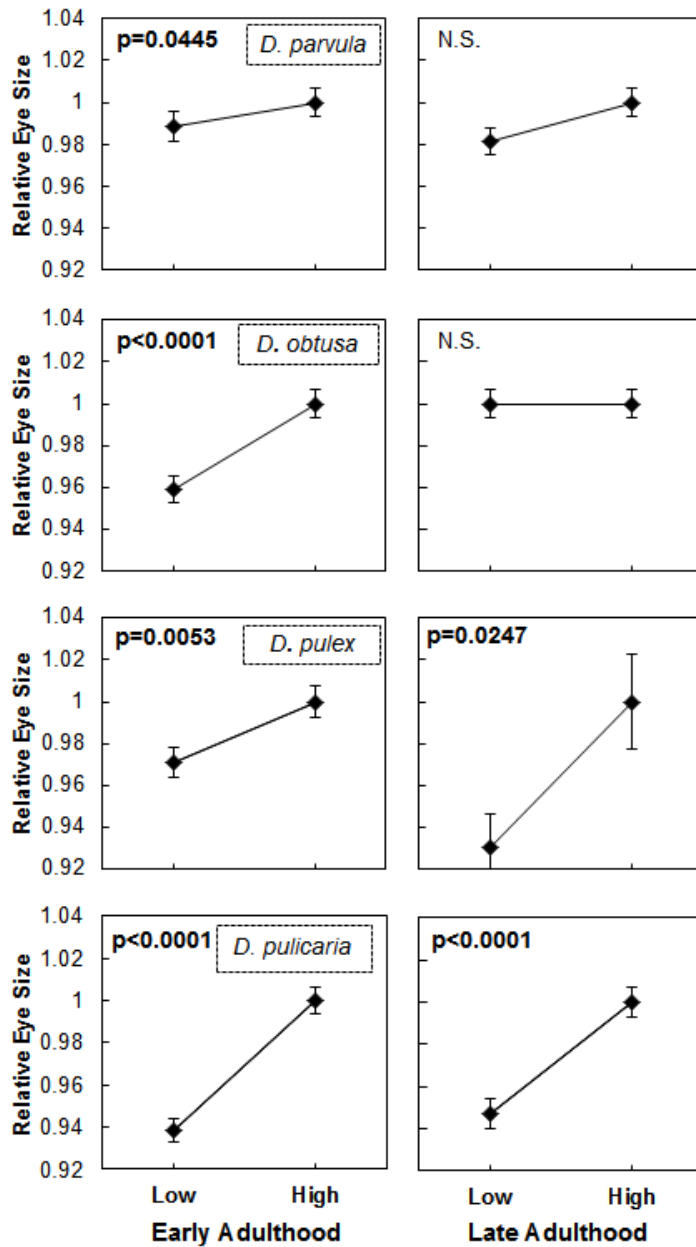




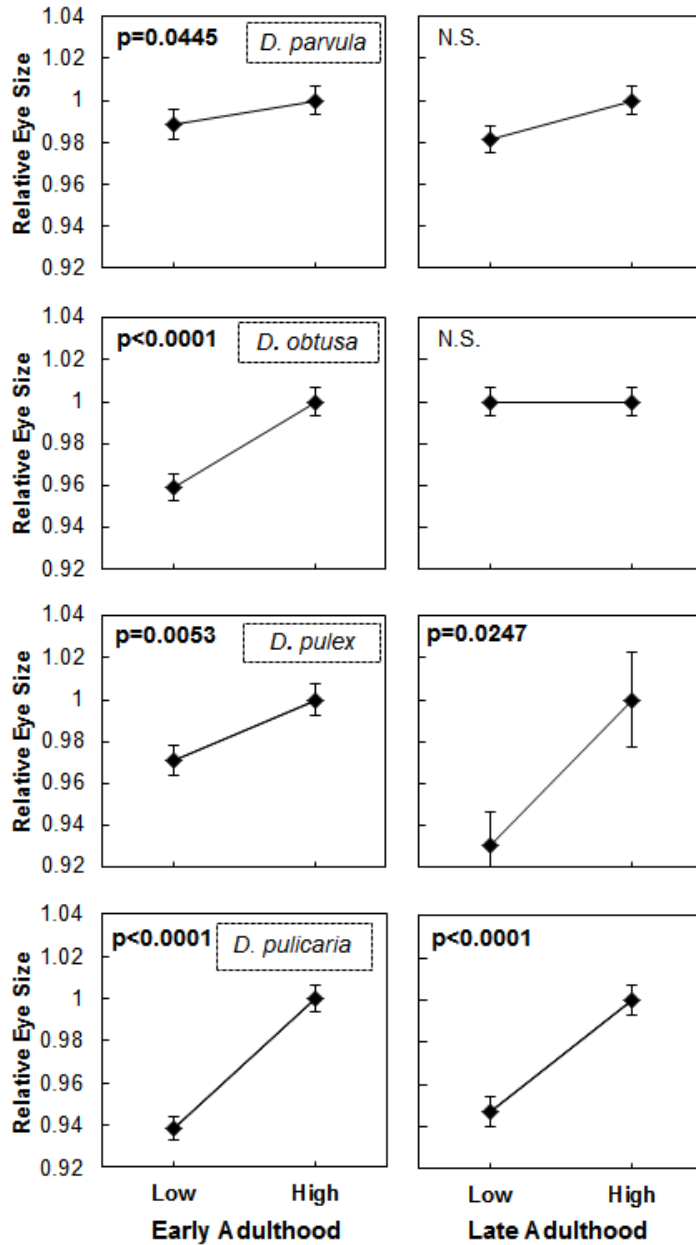
**Figure 2.1** A photomicrograph collage of the *Daphnia* species used in this study. The white line represents eye diameter measurements, and the black line represents body length measurements.



**Figure 2.2** *Daphnia* facet width in relation to eye diameter. Ordinary least squares regression reveals a strong positive relationship (slope = 0.228, adj.  $R^2 = 0.81$ ,  $P < .0001$ ).



**Figure 2.3** The effect of resource environment on relative eye size in *Daphnia*. Relative eye size values are based on body size covariate adjusted means from ANCOVA where eye diameter was the response variable and body length set as the covariate (see methods for details) separately for each species and stage. To present data on the same scale, means were normalized to the high resource environment within each species and stage (i.e., high resource is always set to 1.0). Means were tested at  $\alpha = 0.05$ . Significant differences between means are noted with a p-value in bold. N.S.= not significant. Error bars are  $\pm 95\%$  confidence intervals.



**Figure 2.4** The effect of light environment on relative eye size in *Daphnia*. Relative eye size values are based on body size co-variate adjusted means from ANCOVA where eye diameter was the response variable and body length set as the covariate (see methods for details) separately for each species and stage. To present data on the same scale, means were normalized to the dim light environment within each species and stage (i.e., dim light is always set to 1.0). Means were tested at  $\alpha = 0.05$ . Significant differences between means are noted with a p-value. N.S.= not significant. Error bars are  $\pm 95\%$  confidence intervals.

## CHAPTER 3

### SELECTION ON INCREMENTAL VARIATION OF EYE SIZE IN A WILD POPULATION OF *DAPHNIA*<sup>2</sup>

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<sup>2</sup> Brandon, C.S., James, T., and J.L. Dudycha. Submitted to the *Journal of Evolutionary Biology*

### 3.1 Introduction

Eyes are complex structures that historically have been used to call into question the entire theory of evolution, by arguing that the incremental process of adaptation by natural selection could not produce such structures. Nilsson and Pelger (1994) provided a striking counterpoint to this argument by showing theoretically that highly conservative models of natural selection could produce complex eyes from simple pigmented eye spots in only a few hundred thousand generations. Furthermore, the structural varieties of eyes that lie along this simple to complex continuum are all still functional in terms of obtaining light information, and, in fact, are represented by numerous forms that exist in nature (Salvini-Plawen & Mayr, 1977). Even so, evolutionary biologists have lacked empirical data to demonstrate directly the microevolutionary potential for adaptation in eye morphology.

Eyes provide environmental information that informs critical behaviors from finding food and mates to avoiding threats and predators. Their importance among animals is underscored by their near ubiquity in any environment where light is present. Morphological and physiological components of eyes define the bounds of an animal's visual capabilities (Land & Nilsson, 2012), and thus reveal a great deal about what aspects of the visual environment are important to an animal. An astonishing array of visual system diversity has been catalogued on broad taxonomic scales (Salvini-Plawen & Mayr, 1977). This variation is often argued to be driven by differences in selection by environmental differences (Garamszegi *et al.*, 2002; Ross & Kirk, 2007; Hall, 2008; Somanathan *et al.*, 2009; Veilleux & Lewis, 2011), or by differences in visually mediated behaviors (Nilsson, 2009; Møller & Erritzøe, 2010). This research has focused on

patterns at macroevolutionary scales, and thus is limited to indirect inferences about the selective value of small changes in visual structures.

To address this gap, we sought to examine the reproductive consequences of eye size variation in the freshwater crustacean *Daphnia obtusa* Kurz. Eye size is a general indicator of visual capability (Land, 1997; Land & Nilsson, 2012). Larger eyes typically enhance resolution and/or visual sensitivity, two key aspects of vision (Land & Nilsson, 2012). Indeed, studies have demonstrated that macroevolutionary-scale variation in eye size often co-varies with the light environment and/or behavior (Hiller-Adams & Case, 1988; Bauer *et al.*, 1998; Garamszegi *et al.*, 2002; Thomas *et al.*, 2002, 2006; Lisney & Collin, 2007; Somanathan *et al.*, 2009; Møller & Erritzøe, 2010; Schmitz & Wainwright, 2011; Veilleux & Lewis, 2011).

The optimal size of an eye for a given organism depends on its environment. In *Daphnia*, compound eyes likely benefit the animal by providing critical information for navigation (Schwind, 1999), orientation (Baylor & Smith, 1953; Ringelberg *et al.*, 1974; Novales Flamarique *et al.*, 2000), and resource location (Smith & Baylor, 1953; Young *et al.*, 1984; Hamza & Ruggiu, 2000). However, eyes come at a cost as well. Eyes are expensive in terms of building materials used during development, and they also demand a sizeable slice of an animal's energy budget (Niven & Laughlin, 2008). For example, Laughlin *et al.* (1988) showed that the retina of the blowfly *Calliphora vicina* accounted for 10% of its resting metabolic rate. Prolonged resource limitation has also been shown to reduce compound eye size disproportionately to body length in *Daphnia* (Brandon & Dudycha, 2014). *Daphnia* might also bear an ecological cost for its compound eye, because the darkly pigmented eye in an otherwise transparent body acts as a target for

visual predators (Zaret & Kerfoot, 1975; Branstrator & Holl, 2000). Selection can therefore potentially act from multiple angles on eye size in *Daphnia*.

In this report, we present an observational study where we measure a reproductive selection gradient on eye size from a wild population of *D. obtusa*. We estimate selection by measuring a fitness component in *D. obtusa* as the number of eggs present in its brood chamber (Vanni & Lampert, 1992). Eye size is positively correlated to body size in *Daphnia* (Brandon & Dudycha, 2014), we therefore analyzed both eye size and body size and considering the correlative effects in our analyses of selection (Lande & Arnold, 1983). We also considered the potential of eye size to evolve in response to selection by measuring genetic variation of relative compound eye size. *Daphnia* are cyclical parthenogens that can be maintained as clonal stocks in the laboratory. We can therefore estimate genetic variability among clones in a common garden experiment.

### **3.2 Study Site and Methods**

We measured selection on a wild population of *D. obtusa* in an ephemeral pond, Knobby Knees (KNB; 33°47'42" N, 80°45'18"), in Congaree National Park, an old-growth floodplain forest in South Carolina, USA. KNB is 20 meters from an intermittent creek with steep banks. Depth varies depending on rainfall and season, but has been measured as deep as 70 cm. Like most ponds in the *D. obtusa* metapopulation at Congaree, KNB is heavily shaded under forest canopy cover. Although *Gambusia* are present in the floodplain, we did not observe small fish that potentially prey on *Daphnia* in this pond at the time of sampling. We morphologically identified *Daphnia* in KNB



using the key in Hebert (1995), having previously verified that ponds at Congaree contain *D. obtusa* but no morphologically similar congeners via allozyme electrophoresis.

### 3.2.1 Selection on eye size

We sampled *D. obtusa* from KNB on 31 May 2013, a time when sexual reproduction and males were rare. Sampling was done according to procedures described in Dudycha (2004), generating a pooled sample drawn from throughout the pond. The sample was transported in a cooler with ice to the laboratory. We kept the sample of *Daphnia* at 4°C to arrest embryonic development and the molt cycle until ready for processing. We counted clutch size and measured morphology on a total of 229 individuals.

We counted eggs from living *Daphnia* within 36 hours of capture using a dissecting microscope. After counting, we preserved individuals in 100% ethanol and placed them into numbered wells on a 96-well plate for later imaging. The few females with resting eggs were excluded because an appropriate clutch size could not be determined. In addition, individuals carrying no eggs were excluded as this likely reflects a transition between reproductive modes. Exclusions accounted for less than 2% of the population, and thus have little effect on our analysis.

The compound eye of *Daphnia* is a composite of individual light collecting units called ommatidia. The facet lens diameter within an individual ommatidium significantly influences an animal's visual capabilities (reviewed in Land, 1997). We have previously demonstrated that facet diameter and eye diameter have a strong positive correlation in *Daphnia* (Brandon & Dudycha 2014). Beyond visual capabilities, total eye size

potentially impacts *Daphnia* in terms of energy and predatory visibility (see Introduction), thus we focused our study on total eye size. We used a Nikon 1500 SMZ dissecting scope to take lateral photographs of *Daphnia*, as illustrated in Brandon & Dudycha (2014). Photographs for body length were taken at 30X. Eyes were photographed at 112.5X magnification. We calibrated the dissecting scope with a stage micrometer to obtain pixel to length ratios, which we then used to obtain length measurements from the photographs. We measured *Daphnia* photographs using ImageJ freeware (Schneider *et al.*, 2012). We made body length measurements from the top of the head just above the eye to the base of the tail-spine. Although *Daphnia* eyes are approximately spherical, most individuals deviate somewhat. We therefore measured eye diameter at the widest diameter.

We estimated selection on the correlated phenotypes, eye diameter and body length, following Lande and Arnold (1983). To approximate a normal distribution we transformed each phenotype to natural logarithms. We analyzed the correlation between the transformed values of eye diameter and body length using Spearman's rank-order correlation test with the *Hmisc* package in R v3.0.2 (Harrell, 2015). We also transformed the fitness component to relative fitness by dividing an individual's clutch size by the mean clutch size. We estimated the total effects of indirect and direct selection on both eye diameter and body length by calculating the selection differential as the covariance between relative fitness and each respective phenotype. We standardized the selection differential to phenotypic standard deviation units. To measure the direct effect of selection on a set of correlated multivariate traits, we calculated the selection gradient as the partial regression coefficient from a multiple least squares regression analysis

following Lande and Arnold (1983). We also calculated the standardized selection gradient as the partial regression coefficients from a multiple regression on standardized phenotypic trait values (Lande and Arnold, 1983). All statistical analyses were performed using *R* v3.0.2 (R Team, 2013).

### 3.2.2 Genetic variation of eye size

We obtained samples from the Congaree metapopulation of *D. obtusa* for a common garden analysis of genetic variation in eye size from a total of nine ponds in the floodplain. These ponds are linked by periodic flooding (Conrads *et al.*, 2008), which is the likely cause of relatively low levels of microsatellite differentiation among ponds (Sebastian & Dudycha, unpubl. data). Most ponds are similar in general characteristics, although one (POW) has a substantially more open canopy.

We measured individuals from three size classes for each clone: small ( $\leq 900 \mu\text{m}$ ), medium (901  $\mu\text{m}$  -1399  $\mu\text{m}$ ), and adult ( $\geq 1400 \mu\text{m}$ ). These size classes reflect ontogenetic growth from juveniles to adults and were used to define a measure of eye size relative to body size for each clone. We measured 27-30 individuals from each of 41 clonal lineages that had been isolated from the field during several trips in May of 2010, 2011, and 2013. We initiated each clonal lineage by placing a single individual collected from the field into a culture medium of filtered (1  $\mu\text{m}$ ) hypolimnetic lake water. We maintained cultures in the laboratory at low density at 10°C in environmental chambers on a 12:12 light:dark photoperiod. We fed cultures a weekly diet of a vitamin-enriched green alga *Ankistrodesmus falcatus* (Corda) Ralfs. Experimental animals were generated from these laboratory stocks. To reduce effects due to maternal environment, we

separated animals from the laboratory stocks and carried animals through to at least the third generation before measuring. We began each generation from at least the third clutch of both the grand maternal and maternal generations. We reared the animals in common garden conditions at 20°C in an environmental chamber on a 12:12 light:dark photoperiod, with animals fed daily 20,000 cells/ml of *A. falcatus*. For imaging, we removed animals from culture media and sacrificed them in a solution of 0.25 M KCl.

We estimated broad-sense heritability ( $H^2$ ) as the ratio of genetic variance ( $V_G$ ; the variance of mean relative eye size among clones) to phenotypic variance ( $V_P$ ; the variance of relative eye size across all individuals), or  $H^2 = V_G / V_P$ . To generate mean values of eye size relative to body size we used residuals generated by an ordinary least-squares regression of eye diameter against body length for all individuals in all ontogenetic size classes ( $N = 1218$ ) using the linear model function in *R v3.0.2*. We used the residual values from the global regression analysis to then calculate the mean residual value for each clonal lineage and estimate  $H^2$ . Residual means were calculated using the *psych* package in *R v3.0.2* (Revelle, 2014). We tested the hypothesis that  $H^2 \neq 0$  using a Model II one way ANOVA where clone was treated as a random effect. We estimated  $H^2$  and employed a bootstrap approach to estimate standard error of the  $H^2$  ratio using the *H2boot* software package (Phillips, 2002), which uses ANOVA based estimates. We ran 1000 bootstrap replicates for each trait heritability estimate.

### 3.3 Results

Body length, eye diameter, and clutch size in adult female *D. obtusa* from Knobby Knees pond varied widely. Clutch sizes ranged from 2-13 and averaged  $5.8 \pm 0.14$  SE eggs per clutch. Like most fitness components, the distribution of clutch size was

not normal (Shapiro-Wilk test,  $W = 0.93$ ,  $P = 9.49 \times 10^{-9}$ ). Body length of adults ranged from 1080 to 1819  $\mu\text{m}$  (mean =  $1328.56 \pm 8.22$  SE). Absolute eye diameter had a mean of  $138.79 \pm 1.19$  SE  $\mu\text{m}$ , ranging from 99  $\mu\text{m}$  to 188  $\mu\text{m}$ . This is at least a four-fold difference in light collecting capacity. The sensitivity of the eye is defined as,

$$S = 0.62D^2\Delta\rho^2P_{abs}$$

where  $D$  is the diameter of the facet lens,  $\Delta\rho$  is the sampling angle (defines resolving ability), and  $P_{abs}$  is the proportion of photons absorbed (Land and Nilsson, 2012). An increase in  $D$ , with all the remaining components kept equal, will result in an increase in sensitivity that is proportional to the square. *Daphnia* facet lens diameter is positively and linearly correlated to changes in eye diameter (Brandon and Dudycha, 2014), such that a doubling in total eye diameter approximately equates to the same relative change in  $D$ .

. Body size is known to be a significant driver of clutch size in *Daphnia* (Gliwicz & Boavida, 1996), and regression analysis of our data confirms that clutch size increases with body length in *D. obtusa* ( $\beta = 0.0069 \pm 0.001$  SE,  $F_{(1,227)} = 48.2$ ,  $P = 4.93 \times 10^{-11}$ , adj.  $R^2 = 0.175$ ), though it accounts for only 17% of the variation in clutch size.

Unsurprisingly, eye diameter and body length have a strong positive correlation in *D. obtusa* ( $r_s = 0.58$ ,  $P = 4.8 \times 10^{-22}$ , adj.  $R^2 = 0.368$ ), however regression analysis reveals that nearly two-thirds of the variation ( $\beta = 0.0882 \pm 0.0076$  SE,  $F_{(1,227)} = 133.6$ ,  $P = 2.0 \times 10^{-16}$ ) in eye size is independent of body size. This is consistent with our previous work on phenotypic plasticity of eye size (Brandon & Dudycha, 2014), and allows for eye size to influence the fitness component independently of body size.

We observed that selection is acting on both body length and eye diameter, but that the strength of selection is stronger on eye diameter indicating that selection is operating on eye diameter independently of body length (Table 3.1, Fig. 3.1). Analyses of the selection differential, which accounts for all direct and indirect effects of selection, reveals that selection is stronger on eye diameter ( $s' = 0.19$ ), than body length ( $s' = 0.15$ ). This indicates that the expected change in mean eye diameter in phenotype is 20% of one standard deviation. We also measured the direct effects of selection on each trait by measuring the selection gradient, where our analysis indicated that the direct effects of selection were stronger on eye diameter ( $\beta' = 0.15 \pm 0.024$ ,  $F_{(2, 226)} = 47.8$ ,  $P = 2.0 \times 10^{-16}$ ) than body length ( $\beta' = 0.06 \pm 0.024$ ,  $F_{(2, 226)} = 47.8$ ,  $P = 0.015$ ). An increase of eye diameter of 19.9  $\mu\text{m}$  – slightly more than one standard deviation – is associated with an increase in clutch size of one egg, or an increase of nearly 20% of the mean clutch size.

We observed wide genetic variation in terms of relative eye size in the metapopulation of *D. obtusa* at Congaree National Park ( $N = 41$ ,  $V_G = 10.74 \pm 2.77$  SE,  $H^2 = 0.21 \pm 0.04$  SE,  $P = 2.2 \times 10^{-16}$ ). We also observed a wide range of mean values across clones (Fig. 3.2). Broad-sense heritability measures are important in *Daphnia* because they undergo several generations of asexual reproduction in each population cycle, during which clonal selection can substantially alter the genetic composition of the population (Pfrender & Lynch, 2000; Haag & Ebert, 2007; Vanoverbeke & De Meester, 2010). Additionally, clones that are more reproductively successful, and hence more frequent when the population switches to sexual reproduction can contribute more sexual offspring.

### 3.4 Discussion

We found that small changes in eye morphology are under selection in a wild population of *D. obtusa*, observing a strong positive correlation between eye size and reproduction. The size of an eye is an important feature of its optical capability, such that increases in eye size can lead to enhancements in an eye's ability to resolve images, and/or capture more photons (Land, 1997; Land & Nilsson, 2012). In nature, there are many general examples across broad taxonomic scales of animals that perform tasks, such as flight or visual predation, for which excellent visual capabilities are needed where the observed pattern is that their eyes are larger relative to those species which do not perform such tasks (Garamszegi *et al.*, 2002; Møller & Erritzøe, 2010). A similar pattern exists in animals that inhabit dim light environments, which have larger eyes relative to those that inhabit light-rich environments (Bauer *et al.*, 1998; Thomas *et al.*, 2006; Hall, 2008; Somanathan *et al.*, 2009; Schmitz & Wainwright, 2011; Veilleux & Lewis, 2011). Eye size differences have also been documented between populations that may have different visual needs (Protas *et al.*, 2008; Glazier & Deptola, 2011), although these examples are far fewer than the differences documented across species. While eye size is not the only component that determines an animal's visual capabilities (Land & Nilsson, 2012), it is certainly an important trait which figures prominently into our understanding of how larger environmental differences and behavioral tasks affect variation of visual structures at macroevolutionary scales.

We use clutch size as an indicator of reproductive fitness in this study. Although reproduction provides an incomplete picture of fitness, clutch size drives short-term birth rates in *Daphnia*, and hence is a significant component determinant of  $r$ , the intrinsic rate

of population growth (Dudycha, 2001). Because *Daphnia* mature rapidly relative to their inter-clutch interval, only the first few clutches make substantial contributions to  $r$  (Dudycha & Tessier, 1999); consequently, the current reproductive investment is the most critical component of overall fitness in our population. At Congaree, *D. obtusa* inhabit shallow forest ponds that vary haphazardly with respect to their population demography and the duration in which they are filled with water. *D. obtusa* populations generally persist through clonal reproduction for weeks to months (~3-7 generations at field temperatures) before shifting into sexual, dormancy-based reproductive modes. Although larger eggs, and thus reduced clutch sizes, lead to larger offspring which perform better in low resource conditions, *D. obtusa* inhabit resource-rich ponds (Benzie, 2005). Thus, there should be little advantage to larger neonates (Guisande & Gliwicz, 1992), so it is unlikely that any offspring size-number tradeoff confounds our assessment of fitness.

We demonstrate that selection on eye size in our population has strong potential for evolutionary consequences, because there is substantial genetic variation of relative eye size within the metapopulation. When we returned to Knobby Knees in 2014, we were unable to determine whether there had been a response to selection, or whether the pattern of selection continued. This was because mosquitofish, *Gambusia sp.*, had invaded the pond (presumably during a flood event), and the population of *D. obtusa* had been replaced by *D. ambigua* Scourfield. Eye size is also variable across the *Daphnia* genus (Walterhouse & Dudycha, unpublished data), and *D. ambigua* has one of the smallest eye sizes (absolutely and relative to body size). Thus, the replacement of *D.*

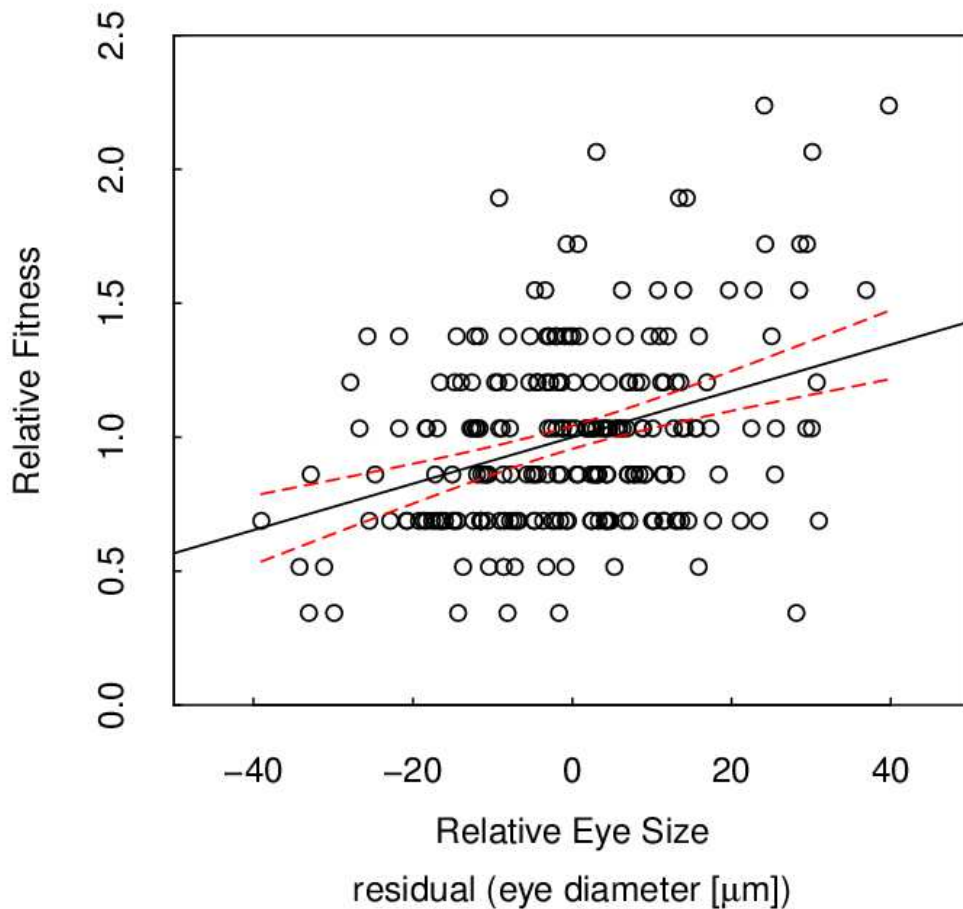


*obtusa* by *D. ambigua* is consistent with strong selection by zooplantivorous fish against large eye size (Zaret & Kerfoot, 1975).

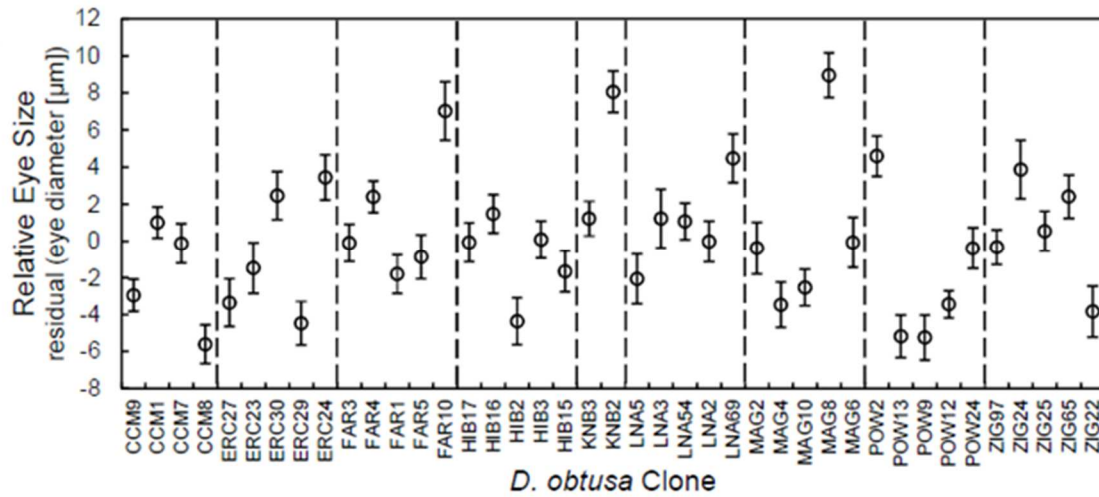
The literature documents a wide breadth of variation in eye morphology across species, and, to a far lesser extent, within species. Our data suggest that selection on incremental size variation may have led to the differences seen among species by demonstrating that there can be marked reproductive consequences to small differences in eye morphology. Future studies focusing on selection in the context of ecological and behavioral drivers defined from macroevolutionary studies may yield greater insights into the strength and tempo of these potential drivers within species.

**Table 3.1** Selection differentials ( $s$ ) and selection gradients ( $\beta$ ) for the correlated phenotypic traits, natural logarithm transformed body length and eye diameter.

Trait	Mean	Standard Deviation	$s$	$s'$	$\beta \pm SE$	P value	$\beta' \pm SE$	P value
Body length	7.1876	0.0911	0.014	0.15	$0.658 \pm 0.268$	$P = 0.015$	$0.060 \pm 0.024$	$P = 0.015$
Eye diameter	4.9246	0.1296	0.024	0.19	$1.168 \pm 0.188$	$P < 0.0001$	$0.151 \pm 0.024$	$P < 0.0001$



**Figure 3.1** For reasons of simpler presentation, we illustrate how the univariate trait, relative eye size, relates to relative fitness as opposed to illustrating the multivariate space. Relative fitness (individual clutch size/mean clutch size) as a function of relative eye size in the Knobby Knees pond population of *D. obtusa* ( $N = 229$ ,  $\beta = 0.0087 \pm 0.0015$  SE, adj.  $R^2 = 0.119$ ,  $p < 0.0001$ ). Solid line shows the least-squares regression and red dashed lines show 95% confidence interval. Clutch size was counted as number of eggs in the brood chamber of *D. obtusa*. Relative eye size is defined as the vertical residual value from a regression of *D. obtusa* eye diameter on body length for each individual.



**Figure 3.2** The mean relative eye size is displayed for each clone isolated from a metapopulation of *D. obtusa*. Relative eye size is shown for each clone as the mean value of the vertical residuals obtained from a least-squares regression performed on the entire *D. obtusa* data set (see methods). Error bars are standard error of the mean.

## CHAPTER 4

### THE EVOLUTION OF THE OPSIN GENE FAMILY IN *DAPHNIA*

## 4.1 Introduction

The sequenced genome of the freshwater microcrustacean, *Daphnia pulex*, revealed one of the largest catalogs of opsins—a family of genes primarily responsible for vision—of any known species (Colbourne *et al.*, 2011). The proliferation of this gene family in *Daphnia* belies the apparently unexceptional nature of its visual system. One possible explanation for the large opsin gene family is that it is a consequence of a genome-wide series of duplication events, which seems to be idiosyncratic of the *D. pulex* genome. In fact, there are a number of other gene families in *Daphnia* that are unusually large (Colbourne *et al.*, 2011). However, the opsin subfamilies seem to have expanded deep in *Daphnia* evolutionary history, and many of these opsins genes seem to code for fully functional proteins implying that they may continue to play functional roles in *Daphnia* vision (Colbourne *et al.*, 2011). Recently, a second species of *Daphnia*, *D. magna*, has had its genome sequenced. *D. pulex* and *D. magna* are members of separate subgenera and are distantly related with an estimated divergence time of approximately 200 million years (Colbourne & Hebert, 1996). Investigating the genomes of these two species can offer important insights into *Daphnia* opsin gene family evolution, but also provide insight into the potential functional importance of this unusually large gene set.

Opsins are members of a large and diverse class of G protein-coupled receptor (GPCR) genes that encode many of the proteins involved in sensory reception (reviewed in Terakita, 2005). Opsins function in photoreception, and are a necessary component for vision. Opsins absorb photons via a covalently bound chromophore, typically an 11-*cis* vitamin A<sub>1</sub> derivative. Upon absorption of the photon, the 11-*cis* vitamin A derivative alters conformation to 11-*trans*, and thus initiates a signal transduction cascade through

G-protein signaling (Nathans, 1987; Rosenbaum *et al.*, 2009). The opsin protein and its prosthetic group, the chromophore, together form the photoreceptive molecule generally referred to as a visual pigment or rhodopsin. The structure of the opsin protein is comprised of seven highly conserved transmembrane motifs, an N-terminus motif in the extracellular region, and a C-terminus motif located in the cytosol (Palczewski *et al.*, 2000). All functionally photoreceptive opsins contain a lysine residue in the seventh transmembrane domain that binds the retinal chromophore through a Schiff-base linkage (Lewis *et al.*, 1978).

The opsin gene family is loosely defined by three large clusters (Terakita, 2005; Shichida & Matsuyama, 2009) ciliary (c-) opsins, rhabdomeric (r-) opsins, and Group-4 opsins, a heterogeneous group of opsins including the photoisomerases (Porter *et al.*, 2012). *Daphnia* possess c-opsins, r-opsins, and a recently discovered member of the Group-4 opsins (Hering & Mayer, 2014).

The r-opsin cluster contains the opsins responsible for visual perception in *Daphnia*. The molecular structure of the opsin defines the basis of which wavelength the protein is most efficient at absorbing. Colbourne *et al.* (2011) discovered through phylogenetic analysis that *D. pulex* had four distinct putative wavelength-sensitive subgroups, ultraviolet, blue, and two long wavelength clades of green and red. This finding confirmed evidence from an electrophysiological study in *D. magna* that described spectral sensitivity in four peak wavelengths (Smith & Macagno, 1990). The *D. pulex* genome contains 25 long wavelength opsins, the largest subgroup of its opsin gene family. This is the largest number of visual opsins yet known. However, there are two other taxonomic groups that rival this number. The crustacean cousins of *Daphnia*, the

Stomatopods (mantis shrimp), contain a high number (6-15) of long wavelength opsins (Porter *et al.*, 2009), but they also have numerous photoreceptor spectral classes.

Odonata, or dragonflies, also contain up to 15 visual opsins, and mostly in the long wavelength class (Futahashi *et al.*, 2015).

The inexplicable diversity of opsins is not limited to visual opsins. Colbourne *et al.* (2011) described two other large clades of opsins within the *D. pulex* genome. The rhabdomeric type arthropods were first described in *Daphnia* (Colbourne *et al.*, 2011). Little is known about their function, but some evidence has shown that it is expressed in the central nervous tissue of the *Cupiennius salei* and the velvet worm *Euperipatodes kanangrensis* (Eriksson *et al.*, 2013). Additionally, *D. pulex* have nine pteropsins (c-opsin), which is the largest known in invertebrates (Hering and Mayer, 2014). Pteropsins likely function to mediate circadian rhythm in some capacity (Velarde *et al.*, 2005; Tierney *et al.*, 2015), but no empirical studies have as yet tested their biological role.

Here, we conduct a comparative evolutionary analysis of the opsin gene family of two *Daphnia* species, and genus that has provided interesting insights on contemporary processes of eye evolution (Brandon & Dudycha, 2014; Brandon *et al.*, 2015). We had two major aims of this study. First, the last common ancestor of these two species represents the basal *Daphnia* species, which therefore allows us to construct a hypothesis of the opsin gene family which existed in the basal *Daphnia* species. Second, we test the hypothesis that this inexplicable diversity of the opsin gene family in *Daphnia* serves some functional role.



## 4.2 Methods

### 4.2.1 *D. magna* opsin gene discovery

We searched for opsins in the *D. magna* genome in two stages. We first sought to build a preliminary catalog of *D. magna* opsins from an August 2012 predicted gene assembly, and we then used the gene hits from the initial set to search the full *D. magna* genome assembly. To build our preliminary set of opsins, we downloaded the database trall7set9rbest from wfleabase.org and used the NCBI standalone BLAST+ version 2.2.29+ software to conduct the search (Camacho *et al.*, 2009). We obtained protein sequences for 44 *D. pulex* opsins (LOPB10 and LOPA5 do not have protein sequences) described in Colbourne *et al.* (2011) from NCBI genbank, except for *D. pulex* pteropsin5, which was only listed on the Joint Genome Institute website (<http://genome.jgi-psf.org/Dappu1>). We used the 44 *D. pulex* opsin protein sequences as our bait for a blastp search of the *D. magna* gene prediction database. We retained hits with an e-value of  $5 \times 10^{-4}$  or lower. We then eliminated hits that blasted against the same gene identification tag. This search produced 30 unique gene hits, which we used as bait for a more extensive genome search.

We used the 30 identified *D. magna* opsin hits from our preliminary search as the basis of a gene-by-gene search. We searched the *D. magna* genome assembly 2.4 using the blast function available on wfleabase.org. For each *D. magna* protein sequence, we searched the genome using tblastn and recorded the scaffold location of the best hit. If the best hit for a gene hit a scaffold location already recorded from a previous gene's blast, we recorded the next best hit. When the total set had been completed once through, we then conducted an additional blast search for every gene and recorded each hit with a cut-

off e-value of  $5 \times 10^{-4}$ . We conducted an additional search using *D. pulex* opsins protein sequences to ensure that we had identified as many potential opsins as possible. To verify that genes were opsins, we performed reciprocal blast searches using blastp into the NCBI non-redundant protein sequence database.

We learned of a new opsin, neuropsin/opsin-5, recently identified by Hering & Mayer (2014) after we had conducted our search described above. Although our search of the *D. magna* genome did uncover opsin-5, we nonetheless obtained the *D. pulex* protein sequence for opsin-5 from NCBI genbank and performed a blast search of the *D. magna* genome using tblastn. This blast search uncovered another type of c-opsin, which had not been described in either Colbourne et al. (2011) or Hering & Mayer (2014). We searched for the *D. pulex* homolog of the c-opsin by blasting the *D. magna* c-opsin into the *D. pulex* genome (available at wfleabase.org).

#### 4.2.2 Phylogenetic analyses

All *Daphnia* opsins, except opsin-5, fall into two major clusters that diverged before the protostome-deuterostome split (Kojima *et al.*, 1997): the ciliary and the rhabdomeric opsins. Our aim was to examine the evolution of opsins in *Daphnia*, we therefore performed separate phylogenetic analyses for both clusters. We included opsin-5, which groups with the Group-4 opsins in the c-opsin analysis. For illustrative purposes, we also performed a phylogenetic analysis grouping all *Daphnia* opsins (Fig. B.1). Phylogenetic identities of *D. pulex* opsins were previously determined by Colbourne *et al.* (2011) and Hering & Mayer (2014). Our dataset included an opsin not previously reported by either study; we therefore used the phylogenetically-informed annotation

(PIA) tool developed by Speiser *et al.* (2014), which can place suspected opsins onto a pre-calculated phylogenetic tree.

We performed phylogenetic analyses on protein-coding DNA sequences rather than amino acid sequences because the DNA sequences provide better resolution for many of the recently duplicated genes. We aligned genomic DNA to the predicted amino acid sequence using Genewise to produce the protein coding sequences. For some arthropods, amino acid sequences were predicted only from cDNA sequences because large regions of their genomic DNA were missing from the assembly. Thus we used nucleotide sequences from cDNA and not gDNA for *D. magna* arthropsin 2 and 3. We aligned codon sequences with an open gap penalty of -2.9 using MUSCLE as available in the MEGA6 software package (Tamura *et al.*, 2013). We performed phylogenetic analyses using a maximum likelihood approach in the RAxML *version 8.1* software package (Stamatakis, 2014). The analyses were run using a general time reversal (GTR) substitution matrix and GAMMA plus proportion of invariable sites estimate. We set the RAxML software to automatically terminate bootstrap replication, which terminated at 400 replicates for both analyses. We performed the analyses without setting an outgroup to avoid constraining tree construction.

Our phylogenetic analysis of *Daphnia* rhabdomeric opsins included a set of vertebrate ciliary opsins from *Danio rerio* and *Bos taurus*, which we used to root the resulting tree. The mRNA and amino acid sequences for *D. rerio* and *B. taurus* were downloaded from NCBI (Table B.1). Only the full mRNA sequences were available on NCBI for some sequences, so we performed a pairwise alignment using the mRNA and corresponding amino acid sequence using Genewise

(<https://www.ebi.ac.uk/Tools/psa/genewise/>) to determine the protein coding sequence. For our analysis of *Daphnia* c-opsins we included a number of invertebrate and vertebrate ciliary opsins. Sequences were downloaded from NCBI and aligned as described above (accession numbers listed in Table B.1). We rooted the resulting c-opsin tree at the vertebrate melanopsins.

#### 4.2.3 Opsin gene and protein structures

We evaluated the exon-intron structures of *Daphnia* opsins to provide more clarity on the evolutionary relationships within each opsin subgroup. We retrieved exon-intron structures for *D. pulex* on JGI (<http://genome.jgi-psf.org/Dappu1>). We obtained the exon-intron structures for *D. magna* genes by pairwise sequence alignment using Genewise. *D. magna* protein sequences were aligned to their genomic DNA using the default parameters available on Genewise. Genomic regions were missing from two arthropods in *D. magna*, we thus could not deduce the gene structures of those opsins.

#### 4.2.4 *D. magna* opsin gene nomenclature

We named *D. magna* opsin genes following the naming convention already prescribed in Colbourne et al. (2011). For gene subgroups with multiple genes, we numbered the gene according to its homolog in *D. pulex*. To avoid confusion over homology, we numbered genes with a decimal number if there was no clear gene-to-gene homology between *D. pulex* and *D. magna* sequences.

## 4.3 Results

### 4.3.1 Opsin gene number and discovery

We found that the *D. magna* genome contained fewer opsin genes than the *D. pulex* genome. The *D. pulex* genome contains 48 opsin genes, whereas the *D. magna* genome contains 33 (Table 4.1). *D. magna* and *D. pulex* share the same number of opsins for ultraviolet, blue, unknown r-opsin, opsin-5, and the newly described c-opsin. The two genomes also share essentially the same number of arthropods (r-opsin) and pteropsins (c-opsin), with *D. pulex* containing one more of each opsin type than *D. magna*. The two long wavelength subgroups of r-opsins differ about two-fold between *D. pulex* and *D. magna*. The long wavelength A (putatively green-sensitive opsins) numbers 10 in *D. pulex* and 4 in *D. magna*, and the long wavelength B (putatively red-sensitive) numbers 15 in *D. pulex* and 8 in *D. magna*.

We also discovered an additional ciliary-type opsin not previously reported in *Daphnia*. Including a recently described opsin-5 (Hering and Mayer, 2014), the *D. pulex* genome contains a total of 48 opsin genes, which is two more than what was originally reported in Colbourne et al. (2011).

### 4.3.2 Blue, ultraviolet, and unknown r-opsins

*D. magna* and *D. pulex* have orthologous pairs of both the putative blue- (BLOP) and ultraviolet-sensitive (UVOP) opsins, along with a set of orthologous r-opsins (UNOP) with unknown wavelength-sensitivity (Fig. 4.1). The phylogenetic analysis indicates that these three subgroups of opsins diverged before the *D. magna-D. pulex* split. The blue and ultraviolet opsins cluster together with 97% bootstrap support and

form a group sister to the putative long wavelength-sensitive opsins (Fig. 4.1). The *D. pulex* blue opsin seems to have evolved at a faster rate than the blue opsin in *D. magna*. The ultraviolet opsins have evolved at a similar rate in both species, but marginally faster in *D. magna*. The phylogenetic analysis indicates that the unknown wavelength-sensitive r-opsins duplicated before the two *Daphnia* species split, and both orthologous sets form a clade with 100% boot strap support. Additionally, they cluster with, but sister to, the other putative visual r-opsins to form a group with strong bootstrap support.

The blue opsin exon-intron structure is highly conserved in *Daphnia*. The blue opsin has eight exons, where each exon is approximately equal in base pair length as its orthologous exon (Fig. 4.2). The intron sequences are also approximately equal in length in both blue opsins. The ultraviolet opsin structure differs in both species, but the majority of the gene is similar. The major distinction is that the 5th exon in the *D. pulex* UVOP is split in two in the *D. magna* UVOP (Fig. 4.2). Both pairs of unknown r-opsin orthologs share a conserved exon-intron structure, with a very distinct 1.5kb intron region shared among them all (Fig. 2). For both species, the unknown r-opsins are aligned in tandem.

#### 4.3.3 Long wavelength A opsins

The long wavelength A (LOPA; putative green-sensitive) opsins cluster with 100% bootstrap support for the monophyly of the clade. The LOPA clade forms two distinct groups that each cluster with strong bootstrap support. Group 1 (G1; Fig. 4.1) likely contained two ancestral LOPA genes that underwent further duplication events in each species separately. *D. pulex* LOPA1-4 and *D. magna* LOPA1.1 & 1.2 form

homologous groups and expanded independently within their respective lineages. *D. magna* LOPA1.3 does not have an ortholog in *D. pulex*, indicating a possible loss of an LOPA opsin in *D. pulex*.

Exon-intron gene structures provide further evidence of the clustering of the two distinct groups of the LOPA clade. In G1, a six-exon gene structure has been conserved in both species. The G1 LOPA opsins in *D. magna* are aligned in tandem on scaffold 2865 (Fig. 4.3A), whereas in *D. pulex* the G1 LOPA opsins are located across two scaffolds (Fig. 4.3B). *D. pulex* LOPA1-3 are located on scaffold 598 and are aligned in sequence, although the gap between LOPA1 and LOPA2 is twice as long as the distance between LOPA2 and LOPA3. Interestingly, *D. pulex* LOPA1-3 are located near the terminus of scaffold 598, and LOPA4 is also located near the terminus of scaffold 47. The location of these genes could indicate that scaffold 47 and scaffold 598 are linked.

Group 2 (G2) LOPA opsins phylogenetic relationships are also supported by conserved exon-intron gene structures (Fig. 4.3). *D. pulex* G2 LOPA opsins all contain eight exons (Fig. 4.3B), whereas *D. magna* contains nine (Fig. 4.3A). *D. pulex* G2 LOPA opsins have undergone a recent duplication event (Fig. 4.1). Gene scaffold information shows that they duplicated as pairs onto separate scaffolds (Fig. 4.3). Each pair contains an opsin with an incomplete sequence and an adjacent opsin with a full sequence (Fig. 4.3). *D. magna* LOPA6.1 is the only member of G2 LOPA and it is orthologous to the *D. pulex* G2 LOPA opsins (Fig. 4.1).

#### 4.3.4 Long wavelength B opsins

Similar to the LOPA clade, long wavelength B (LOPB; putative red-sensitive) clusters with 100% bootstrap support and forms two distinct groups, each with strong bootstrap support >75% (Fig. 4.1). The phylogenetic analysis shows that G1 had mostly expanded prior to the *D. pulex*-*D. magna* split. Many of the LOPB opsins have orthologous pairs between the two species. However, there were expansions in each lineage after their split. *D. pulex* LOPB3-5 and *D. magna* LOPB3.1&3.2 are the result of expansions that occurred in each respective lineage from a common LOPB ancestor (Fig. 4.1).

Two smaller clusters comprise G1, which are also supported by gene structural information (Fig. 4.4). *D. pulex* LOPB6 and LOPB3-5, and *D. magna* LOPB6 and LOPB3.1 & 3.2 share a conserved six-exon structure. *D. pulex* LOPB2,7 and 8, and *D. magna* have five exons. The exon-intron structure is similar within G1, except that in G1.1 the 5' end has two exons of approximately the same length as a single 5' exon in G1.2.

All of *D. magna* G1 LOPB opsins are located on scaffold 1877 (Fig. 4.4A). They are arrayed in tandem and separated by 1.5 kb-2.5kb between them. The phylogenetic analysis, along with gene structure information, reveals that these G1 LOPB opsins did not duplicate linearly along the scaffold, but instead have a more complex pattern (Fig. 4.4A).

*D. pulex* G1 LOPB genes are arrayed in tandem mostly on scaffold 40, except for LOPB8 which is located on scaffold 6 (Fig. 4.4B). The LOPB opsins are separated by



2.2kb-3.9kb between them. Similar with *D. magna* G1 LOPB opsins, the structural information and phylogenetic analysis reveal that the pattern of the G1 LOPB opsins is complex and did not occur linearly along the scaffold (Fig. 4.4B).

In G2, there were expansions in both *D. pulex* and *D. magna* arising from a single common LOPB ancestor (Fig. 4.1). The exon-intron structure of The G2 LOPB cluster is also supported by gene structural information, with all complete gene sequences sharing five exons of similar base pair length (Fig. 4.4). *D. magna* G2 LOPB opsins are located across three scaffolds (Fig. 4.4A). *D. magna* LOPB1.1 is located on scaffold 1877, 2.8kb upstream of the set of G1 opsins and on the opposite strand. *D. magna* LOPB1.2 and LOPB1.3 are located on scaffold 1899 and 3025 respectively (Fig. 4.4A).

*D. pulex* G2 LOPB have undergone a recent expansion as indicated by the phylogenetic analysis (Fig. 4.1). Most of the G2 LOPB opsins are located on scaffold 78, but one is located on scaffold 40 (Fig 4.4B). *D. pulex* LOPB1, 9, and 15 are full sequences but the rest of the G2 LOPB opsins, LOPB10-14, are incomplete sequences (Fig. 4.4B). They are arrayed in tandem along scaffold 78, but the duplication pattern is unclear (Fig. 4.4B).

#### 4.3.5 *Arthropins*

The arthropins cluster into two distinct groups, and most genes form orthologous pairs (Fig. 4.1). Interestingly, the clustering of these groups mirrors the scaffold locations of the arthropins (Fig. 4.5). In each species, the arthropsin family is located on two scaffolds. G1 arthropins are located on scaffold 13 in *D. pulex* (Fig. 4.5B), and scaffold

2452 in *D. magna* (Fig. 4.5A). G2 arthropins are located on scaffold 14 in *D. pulex* (Fig. 4.5B), and scaffold 1036 in *D. magna* (Fig. 4.5A).

In G1, both *D. pulex* and *D. magna* arthropins 7 and 6 group together. The phylogenetic analysis is unclear about the relationship of arthropin 8. However, scaffold and gene structural information suggest that both *D. magna* and *D. pulex* arthropin 8 are likely orthologs (Fig. 4.5). Both genes contain two exons that are both approximately equal in length, and the two exons are separated by a similar size intron region.

In G2, *D. pulex* and *D. magna* arthropins 2, 4, and 5 group into orthologous pairs with strong bootstrap support, except arthropin 2 which groups with 43% support (Fig. 4.1). *D. pulex* arthropin 1 does not have an apparent ortholog in *D. magna*. However, the genome assembly is missing information for *D. magna* scaffold 1036, where the ortholog of *D. pulex* arthropin 1 is likely located. In *D. pulex*, the arrangement of the five arthropins on scaffold 13 is similar to the arrangement of the four arthropins on scaffold 1036 in *D. magna* (Fig. 4.5). The region which is missing information on scaffold 1036 in *D. magna* matches the region where arthropin 1 is located in *D. pulex* (Fig. 4.5).

#### 4.3.6 Pteropsins

The *Daphnia* pteropsins form a monophyletic clade among other c-opsins (Fig. 4.6). Phylogenetic analysis shows that the pteropsin sub-family underwent an expansion before the *D. pulex*-*D. magna* split, but also underwent a subsequent expansion in each lineage. *D. pulex* pteropsins 5-8 are orthologous to *D. magna* pteropsins 7.1-7.5 (Fig. 4.6). The analysis indicates that the ortholog of pteropsin 1 has been lost in *D. magna* (Fig. 4.6).

The structure of pteropsins in both species reveals little about their phylogenetic relationship. Unlike the wide conservation of the exon-intron gene structures seen in the other opsin subgroups, there are no obvious similarities between orthologs. The *D. pulex* pteropsins are located on three separate scaffolds, whereas in *D. magna* the pteropsins are located across four (Fig. 4.7).

#### 4.3.7 Additional c-opsin

Both *Daphnia* genomes contain the additional c-opsin (Table 4.1), which form an orthologous pair in the c-opsin phylogeny (Fig. 4.6). Interestingly, they group distinctly separate from the *Daphnia* pteropsins.

The exon-intron structure is conserved for new *Daphnia* c-opsin. This c-opsin has six exons and the exon-intron basepair lengths are nearly identical in both species (Fig. 4.8).

#### 4.3.8 Opsin-5

Both *Daphnia* contain an opsin-5, which form an orthologous pair. *Daphnia* opsin-5 groups strongly with other invertebrate and vertebrate opsin-5 with 97% bootstrap support (Fig. 4.6).

The exon-intron structure is conserved for both opsin-5. In *D. magna*, opsin-5 is approximately 7kb in length with a number of large introns (Fig. 4.8). In *D. pulex*, opsin-5 is shorter with only ~5kb, but otherwise it has a similar structure as the *D. magna* opsin-5.

## 4.4 Discussion

Our analyses revealed that the expansive suite of opsins present in the *D. pulex* genome is not peculiar to that specific lineage, but also a characteristic of the *D. magna* genome. We found fewer opsins in *D. magna* (33) than the number of opsins in *D. pulex* (48). However, the opsin catalog contained in the *D. magna* genome is still one of the largest known. Additionally, *D. magna* and *D. pulex* have an estimated divergence time of 200 million years, and each lineage is a member of a separate subgenus: the *D. pulex* group within the *daphnia* subgenus, and *D. magna* are in the *cteno-daphnia* (Colbourne and Hebert, 1996). We have shown that despite millions years of evolution, both *Daphnia* lineages have maintained many complete opsin gene sequences across the different opsin clades, suggesting that the opsins have been maintained for some functional role in photoreception and vision.

### 4.4.1 Arthropopsins

The arthropopsins, which group sister to the visual r-opsins, likely have eight orthologous pairs in both species (Fig. 4.1). We could only locate seven arthropopsins in the *D. magna* genome compared to the eight present in *D. pulex*. However, the scaffold pattern and location of the arthropopsins in *D. magna* mirrors *D. pulex*. A single scaffold contains arthropopsins 1-5 in *D. pulex*, where a large intergenic region splits the arthropopsins into a tandem pair and a tandem triplet. We observe a similar scaffold pattern in *D. magna*, except that there is missing genome assembly information where there would be the third gene of the tandem triplet. Furthermore, the arthropopsins located on the orthologous scaffold regions also group together with strong node support. Our

phylogenetic analysis does not resolve the relationship of *D. magna* arthropsin 8 and *D. pulex* arthropsin 8. The exon-intron structure along with the scaffold information suggests that they are likely orthologs (Fig. 4.5). This would be a more parsimonious explanation as well, because otherwise the alternate scenario is that *D. magna* and *D. pulex* each maintained an arthropsin that was lost in the other species. The phylogenetic analysis coupled with the scaffold and gene structure information suggests that the last common ancestor of *D. pulex*-*D. magna* likely had eight arthropsin, which have been maintained in both lineages. The expansion of the arthropsin early in *Daphnia* evolution is intriguing because it hints that there may be multiple arthropsin in other cladocerans, and possibly even other crustaceans. Hering and Mayer (2014) have now identified phylogenetically several additional sequences of arthropsin in other taxa that were not previously recognized as arthropsin (Koyanagi *et al.*, 2005; Randel *et al.*, 2013). The discovery of these sequences suggest that arthropsin are an ancient clade that existed in the last common ancestor of pancrustacea, and potentially as deep as the last common bilaterian ancestor (Hering & Mayer, 2014).

#### 4.4.2 C-opsins

Pteropsins form a monophyletic group among *Daphnia*, suggesting that the expansion of this clade occurred after the arthropod-crustacean divergence (Fig. 4.6). However, the basal *Daphnia* species likely contained five pteropsins, raising questions about when this clade initially expanded and if it occurred much deeper in cladoceran evolutionary history. The pteropsins have undergone additional duplication events in both *D. magna* and *D. pulex* lineages. The pteropsin gene was first described in the honeybee *A. mellifera*, and shown to be expressed in its brain (Velarde *et al.*, 2005), and may play a

role in circadian rhythm entrainment. Further work by Koyanagi et al. (2013) has identified that these group of proteins are bi-stable (i.e., they do not lose their chromophore upon light absorption like other visual-based c-opsins), and are sensitive to blue and green wavelengths. From a *Daphnia*—and indeed a broader zooplankton—perspective, the potential role of pteropsins in circadian rhythm mediation is worth investigating further because the ecologically important diel vertical migration behaviors of *Daphnia* are partially influenced by the circadian clock (reviewed in Cohen et al. 2009).

We found an additional c-opsin in *Daphnia*, which to the best of our knowledge has yet to be described in the published literature. It clusters outside of the *Daphnia* pteropsins, but within the other invertebrate c-opsins (Fig. 4.6). However, the node is unstable and their phylogenetic relationship with the other invertebrate c-opsins is unclear.

#### 4.4.3 Opsin-5

Not unsurprisingly, we found the homolog of the newly discovered *D. pulex* neuropsin, opsin-5, in *D. magna*. Opsin-5 was until recently only known in vertebrates, but sequences have since been described in a few other invertebrates: a limpet, oyster, polychaete worm, and Tardigrades (Hering & Mayer, 2014). This opsin groups as part of the major Group 4 cluster (Hering & Mayer, 2014), which includes the photoisomerases (Porter et al. 2012). *Daphnia* thus contain representative opsins from the three major opsin clusters. In vertebrates, opsin-5 responds to ultraviolet light, and is expressed in several different non-visual tissues (Kojima *et al.*, 2011; Nakane *et al.*, 2014), but is also

interestingly expressed in the neural retina (Yamashita *et al.*, 2010). However, as with most of the non-visual opsins, little is known about its function in invertebrates.

#### 4.4.4 Visual r-opsins

The basal *Daphnia* species contained both a putative ultraviolet- and blue-sensitive opsin. It is notable that both lineages have only maintained a single copy of these two opsins, especially given the penchant for duplication across the other opsin clades (Fig. 4.1). There are quite a few species that inhabit lakes, environments where light towards the blue-green end of the visible spectrum dominates, but both *D. pulex* and *D. magna* inhabit long wavelength dominated pond environments. It would be interesting to investigate an ecological link between light environments and the duplication of visual opsins, i.e., whether lake species contained more copies of blue opsins than their pond inhabiting congeners.

The unknown-wavelength r-opsins duplicated before the *Daphnia* species radiation, and the two paralogs have been maintained in both *Daphnia* lineages. The unknown-wavelength opsins cluster among other arthropod ultraviolet and unknown wavelength opsins (Hering & Mayer, 2014), and we hypothesize that they are likely sensitive to ultraviolet light. However, no experimental work has as yet identified their wavelength sensitivity, or if they are indeed expressed in image-forming photoreceptive tissue.

The long wavelength clades are the most numerous of *Daphnia* opsins and contribute about one-half of the opsin gene catalog in *D. pulex* and about one-third in *D. magna*. The long wavelength A (LOPA), or putatively green-sensitive, clade has fewer

genes than the LOPB, or putatively red-sensitive, clade. Our phylogenetic analysis indicates that the basal *Daphnia* species had three LOPA opsins and six LOPB opsins. The exon-intron gene structures support the major sub-grouping of these genes. Exon-intron structures can be preserved for enormous time-spans—even across taxonomic kingdoms (Rogozin *et al.*, 2003), and the grouping of the gene structures of the *Daphnia* long wavelength opsins may hint that these two clades expanded deeper in cladoceran or even possibly crustacean evolutionary history. Intriguingly, research on opsins in stomatopods (mantis shrimp) has uncovered 6-15 middle/long wavelength opsins across a few species (Porter *et al.* 2009).

Many of the visual r-opsin sequences contain all the necessary components of a functioning opsin protein, with the exception of the recent expansion in LOPB G2 of *D. pulex*, which contains many truncated sequences. The opsin genes in these sub-families have persisted for millions of years, despite the fact that most gene duplicates erode from the genome in a few million years (Lynch & Conery, 2000). It is a curious fact, then, that *Daphnia* have maintained such a large repertoire of opsins. Both *Daphnia* species presented in this study are pond-dwelling species, which is likely the case for the ancestral *Daphnia* too as most extant species inhabit ponds (Benzie 2005; Colbourne *et al.* 1997). The pond environment is typically dominated by orange-red light, because many ponds are filled with color dissolved organic matter (CDOM), which preferentially absorbs shorter (green-blue-UV) wavelengths of light. While we are not advancing an ecological link *per se* between ponds and the expansion and maintenance of *Daphnia* long wavelength opsins, the correlation nevertheless stands-out as something worthwhile of further investigation. The four classes of wavelength-sensitive photoreceptors that



*Daphnia* possess provide enough information to decode color information in their environment (Barlow, 1982). An interesting question is whether *Daphnia* possess a color visual system similar to stomatopods (mantis shrimps), where the animal scans the environment and recognizes colors, as opposed to color discrimination by comparing the relative signals from different classes of photoreceptors (Thoen *et al.*, 2014). The neural processing power of *Daphnia* is limited, and this may be a viable option to exploit multiple visual pigments tuned to slightly different wavelengths.

Extracellular electrophysiological work has demonstrated that *D. magna* have four wavelength sensitive peaks in its compound eye (Smith & Macagno, 1990), and the animals respond behaviorally to light of different wavelengths (Smith and Baylor 1953; Young *et al.* 1984). Instances of opsin duplication has led to the evolution of different wavelength sensitivities in visual pigments (Frentiu *et al.*, 2007; Hofmann & Carleton, 2009), and the potential for differences in wavelength sensitivity of *Daphnia* visual pigments at least exists. Different opsins can be expressed within a single photoreceptor (Sakamoto *et al.*, 1996), thus one explanation may be that multiple long wavelength opsins with similar, but different, spectral sensitivities broaden the spectral sensitivity of the photoreceptor (Arikawa, 2003).

*Daphnia* possess both a compound eye and a simple nauplius eye. Oakley and Huber (2004) discovered a large number of duplicate opsins in two ostracod (Crustacea) species that expressed either in their median (simple) eye, or their compound eye. Opsins may act in a similar fashion in *Daphnia*.

#### 4.4.5 Conclusion

Both *D. pulex* and *D. magna* genomes contain massive catalogs of opsin sequences. Our phylogenetic analysis indicates the last common ancestor of *Daphnia* likely contained a large catalog of opsins, suggesting that a large opsin family is a general characteristic trait of *Daphnia*. Furthermore, our phylogenetic analysis is supported by exon-intron gene structure with the exception of the pteropsin clade. Exon-intron gene structure can be well preserved through long evolutionary timespans, which hints that the arthropods and long wavelength clades may have expanded much deeper in *Daphnia*—potentially crustacean—evolutionary history. In addition, both lineages have maintained large numbers of both pteropsins and arthropsin sequences, suggesting useful roles of these genes in some capacity. The finding that the large family of visual opsin contains sequences that are largely intact, suggests that the number of opsins might play some role in *Daphnia* visual systems. Future studies that investigate why there are so many opsins, and if they serve any utility, may yield better understanding of opsin evolution and vision in general.

**Table 4.1** Number of opsins in *Daphnia pulex* and *D. magna*.

Opsin type	number of genes	
	<i>D. pulex</i>	<i>D. magna</i>
UV	1	1
blue	1	1
unknown	2	2
Long wavelength A (green)	10	4
Long wavelength B (red)	15	8
Arthropsin	8	7
Pteropsin	9	8
Opsin-5	1	1
<i>new c-opsin</i>	1	1
total	48	33

*Total gene numbers include truncated and pseudogenes*

**Table 4.2** *D. pulex* opsin genes scaffold locations, which are designated by the start and stop codons, and were identified from the most recent curation available at from the JGI (available at <http://genome.jgi-psf.org/Dappu1>).

Gene name	protein ID	Genbank accession number	scaffold location	opsin class
BLOP	Dappu1_346936	EFX75461	scaffold_53:628972-627384	rhabdomic
UVOP	Dappu1_346937	EFX81332	scaffold_21:242254-243735	rhabdomic
UNOP1	Dappu1_346930	EFX70801	scaffold_95:373273-369266	rhabdomic
UNOP2	Dappu1_346935	EFX70796	scaffold_95:441206-436847	rhabdomic
LOPA1	Dappu1_307031	EFX63568	scaffold_598:26143-27650	rhabdomic
LOPA2	Dappu1_307030	EFX63569	scaffold_598:18146-19709	rhabdomic
LOPA3	Dappu1_67015	EFX63570	scaffold_598:14836-16355	rhabdomic
LOPA4	Dappu1_306275	EFX76309	scaffold_47:938824-940343	rhabdomic
LOPA5‡			scaffold_174:66413-66609	rhabdomic
LOPA6	Dappu1_302464	EFX66668	scaffold_174:68556-70214	rhabdomic
LOPA7	Dappu1_335676	EFX63276	scaffold_696:761-1320	rhabdomic
LOPA8	Dappu1_93838	EFX63277	scaffold_696:4555-6208	rhabdomic
LOPA9	Dappu1_346967	EFX63131	scaffold_776:5823-4190	rhabdomic
LOPA10	Dappu1_346968	EFX63132	scaffold_776:678-1905	rhabdomic
LOPB1	Dappu1_346974	EFX77537	scaffold_40:709794-707906	rhabdomic
LOPB2	Dappu1_305771	EFX77470	scaffold_40:716214-717823	rhabdomic
LOPB3	Dappu1_346975	EFX77471	scaffold_40:722123-723709	rhabdomic
LOPB4	Dappu1_305803	EFX77472	scaffold_40:728027-729621	rhabdomic
LOPB5	Dappu1_106095	EFX77473	scaffold_40:732744-734341	rhabdomic
LOPB6	Dappu1_305772	EFX77474	scaffold_40:737672-739175	rhabdomic
LOPB7	Dappu1_321382	EFX77475	scaffold_40:742430-743901	rhabdomic
LOPB8	Dappu1_346976	EFX87234	scaffold_6:1902006-1900546	rhabdomic
LOPB9	Dappu1_346977	EFX72327	scaffold_78:111257-112700	rhabdomic
LOPB10‡			scaffold_78:114113-114451	rhabdomic
LOPB11	Dappu1_346978	EFX72328	scaffold_78:119972-120349	rhabdomic
LOPB12	Dappu1_254506	EFX72329	scaffold_78:123959-124674	rhabdomic
LOPB13	Dappu1_346979	EFX72330	scaffold_78:126986-128226	rhabdomic
LOPB14	Dappu1_346980	EFX72331	scaffold_78:133375-134226	rhabdomic
LOPB15	Dappu1_346981	EFX72332	scaffold_78:142738-144181	rhabdomic
ARTHROPSIN1	Dappu1_346938	EFX83617	scaffold_14:758164-761748	rhabdomic
ARTHROPSIN2	Dappu1_346939	EFX83618	scaffold_14:766741-771298	rhabdomic
ARTHROPSIN3	Dappu1_346940	EFX83619	scaffold_14:779460-783216	rhabdomic
ARTHROPSIN4	Dappu1_346941	EFX83830	scaffold_14:847788-844292	rhabdomic
ARTHROPSIN5	Dappu1_346942	EFX83831	scaffold_14:839526-835973	rhabdomic
ARTHROPSIN6	Dappu1_346943	EFX84250	scaffold_13:689696-688112	rhabdomic
ARTHROPSIN7	Dappu1_223107	EFX84031	scaffold_13:961279-964481	rhabdomic
ARTHROPSIN8	Dappu1_346945	EFX84032	scaffold_13:1021380-1023187	rhabdomic

PTEROPSIN1	Dappu1_346957	EFX87345	scaffold_6:1015520-1013655	ciliary
PTEROPSIN2P			scaffold_6:1009166-1007372	ciliary
PTEROPSIN3	Dappu1_346958	EFX87346	scaffold_6:1006658-1004665	ciliary
PTEROPSIN4	Dappu1_346959	EFX86931	scaffold_6:767483-770451	ciliary
PTEROPSIN5P	Dappu1_51511		scaffold_25:431410-435620	ciliary
PTEROPSIN6	Dappu1_346964	EFX80365	scaffold_25:446162-452002	ciliary
PTEROPSIN7	Dappu1_346984	EFX80367	scaffold_25:460743-464047	ciliary
PTEROPSIN8	Dappu1_346985	EFX80369	scaffold_25:484111-488573	ciliary
PTEROPSIN9	Dappu1_346986	EFX89511	scaffold_2:3695086-3691118	ciliary
<i>new</i> C-OPSIN	Dappu1_106425	EFX77128	scaffold_42:899931-901421	ciliary
OPSIN-5	Dappu1_194423	EFX84680	scaffold_12:1001755-995927	group-4

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‡ Sequences were not used in phylogenetic analyses, because they are too short.

**Table 4.3.** *D. magna* opsin genes scaffold locations, which are designated by the start and stop codons.

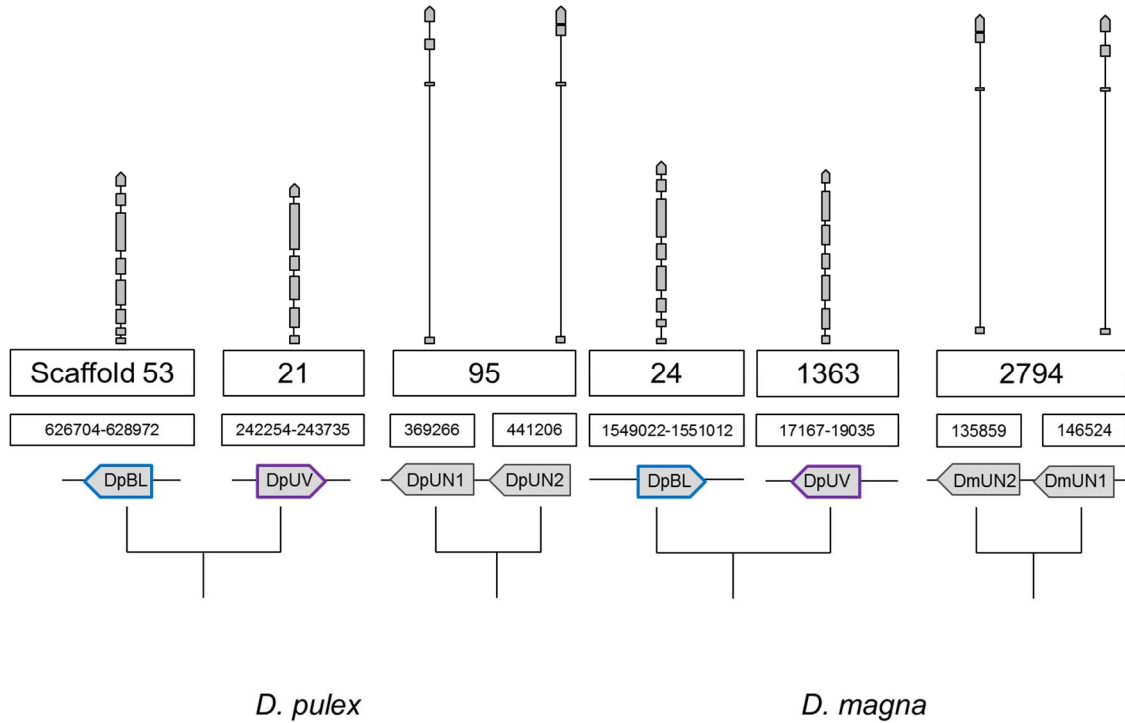
Gene name	scaffold location	opsin subfamily
BLOP	scaffold_24:1549022-1551012	rhabdomic
UVOP	scaffold_1363:19035-17167	rhabdomic
UNOP2	scaffold_2794:135859-140266	rhabdomic
UNOP1	scaffold_2794:141964-146524	rhabdomic
LOPA1.1	scaffold_2865:102066-104554	rhabdomic
LOPA1.2	scaffold_2865:106106-108228	rhabdomic
LOPA1.3	scaffold_2865:109496-111447	rhabdomic
LOPA6.1	scaffold_2861:307462-310310	rhabdomic
LOPB1.1	scaffold_1877:189524-187527	rhabdomic
LOPB2	scaffold_1877:192389-194427	rhabdomic
LOPB3.1	scaffold_1877:196076-198506	rhabdomic
LOPB3.2	scaffold_1877:200979-203793	rhabdomic
LOPB6	scaffold_1877:205251-207085	rhabdomic
LOPB7	scaffold_1877:209605-211562	rhabdomic
LOPB1.2	scaffold_1899:79603-78125	rhabdomic
LOPB1.3	scaffold_3025:597271-594888	rhabdomic
ARTHROPSIN2†	scaffold_1036:862580-855492	ciliary
ARTHROPSIN3†	scaffold_1036:859892-844966	ciliary
ARTHROPSIN4	scaffold_1036:713616-709063	ciliary
ARTHROPSIN5	scaffold_1036:704678-699669	ciliary
ARTHROPSIN6	scaffold_2452:181886-183497	ciliary
ARTHROPSIN7	scaffold_2452:532215-529688	ciliary
ARTHROPSIN8	scaffold_2452:525261-523506	ciliary
PTEROPSIN3	scaffold_1581:1466169-1462331	ciliary
PTEROPSIN4	scaffold_1581:1069888-1073838	ciliary
PTEROPSIN7.1‡	scaffold_1253:272678-271439	ciliary
PTEROPSIN7.2	scaffold_1253:285277-281687	ciliary
PTEROPSIN7.3	scaffold_1253:293375-289126	ciliary
PTEROPSIN7.4‡	scaffold_1253:301734-297593	ciliary
PTEROPSIN7.5	scaffold_1097:2625-713	ciliary
PTEROPSIN9	scaffold_1764:116752-120365	ciliary
<i>new</i> C-OPSIN	scaffold_868:1372594-1370567	ciliary
OPSIN-5	scaffold_1019:137315-129180	ciliary

†Sequences used for nucleotide based tree analysis were based on cDNA sequences, because large regions of the genome were missing from protein location. ‡ identifies a short incomplete protein that was not used for phylogenetic analyses.



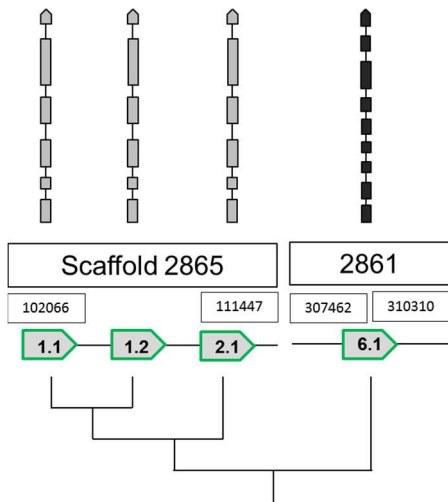
**Figure 4.1** Phylogeny of *D. pulex* and *D. magna* r-opsins based on maximum likelihood (ML) analyses of protein-coding nucleotide sequences. ML analyses were run using GTR model of evolution as implemented in RAxML. The phylogenetic tree is rooted at vertebrate visual c-opsins. Values on branches indicate bootstrap support. Branch lengths are proportional to the substitution/site scale bar. Branches that lead to putative visual opsins of broadly defined (ultraviolet, blue, red, and green) wavelength classes have been colored by their respective wavelength sensitivity. The bracket tree on the right is the hypothesized family of opsins that existed in the most recent common ancestor of *D. pulex* and *D. magna*, approximately 200 mya. Black closed circles (•) identify *D. pulex* and open boxes (□) identify *D. magna*. Crosses (+) indicate opsin genes that have truncated sequences.



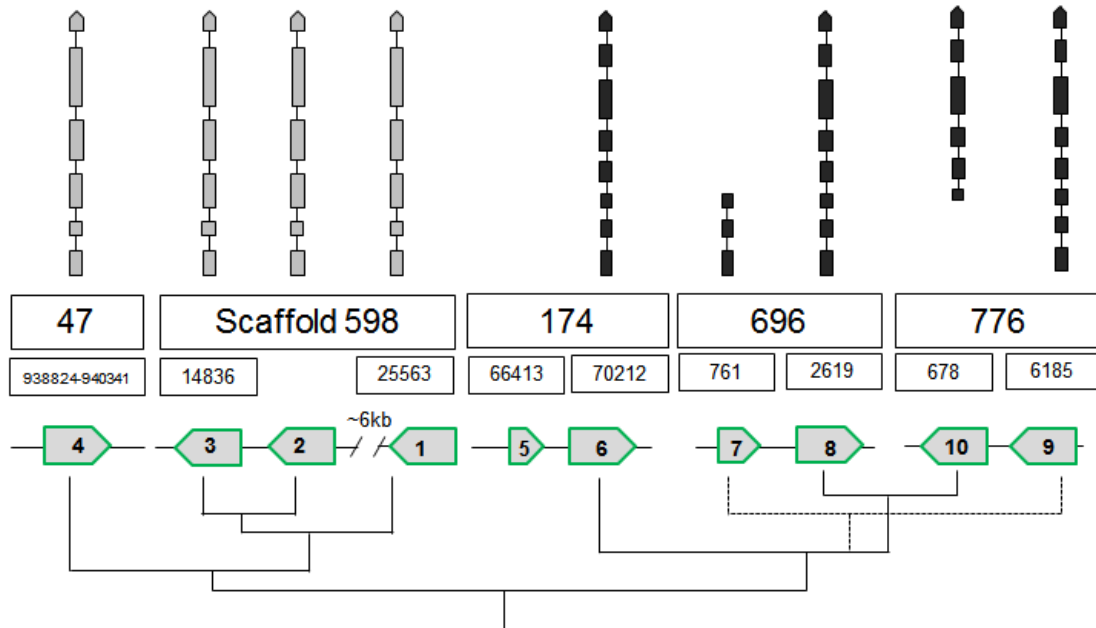


**Figure 4.2** An illustration of exon-intron gene structure and genome location of *Daphnia* ultraviolet (UV) sensitive, blue (BL) sensitive, and unknown (UN) wavelength-sensitive opsins. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other.

A)

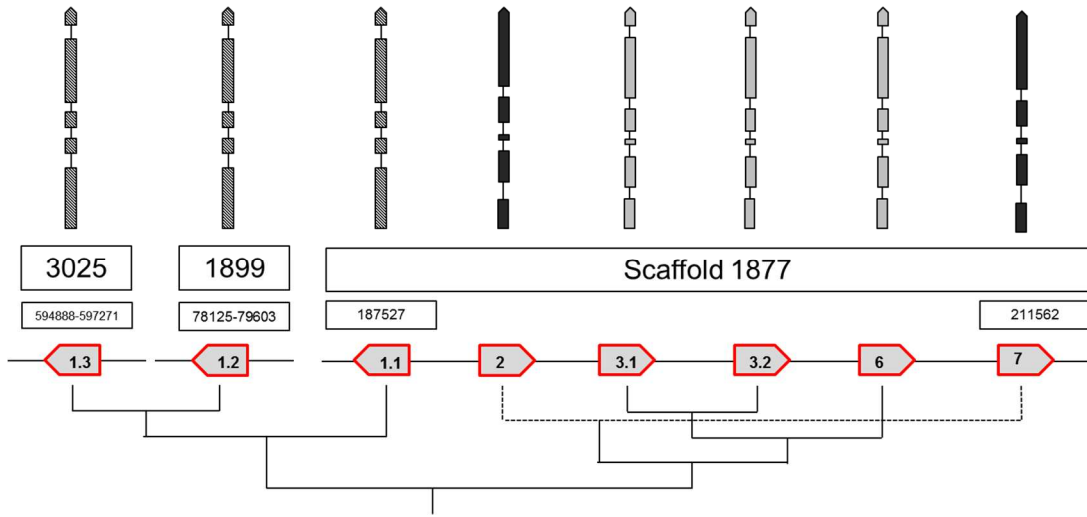


B)

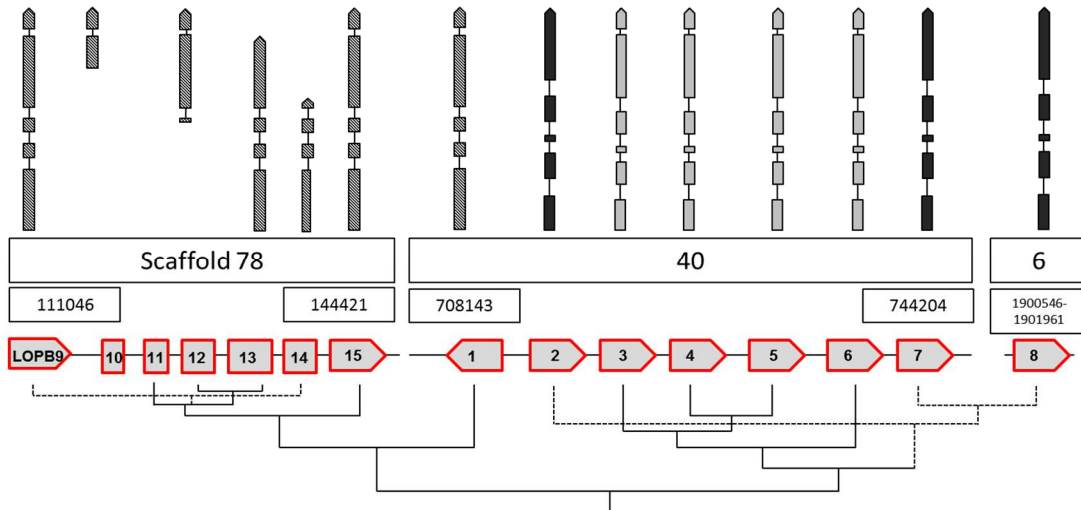


**Figure 4.3.** An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* long wavelength A clade (LOPA; putative green-sensitive). Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* LOPA clade and their phylogenetic relationships, and the B) *D. pulex* LOPA clade and their phylogenetic relationships.

A)

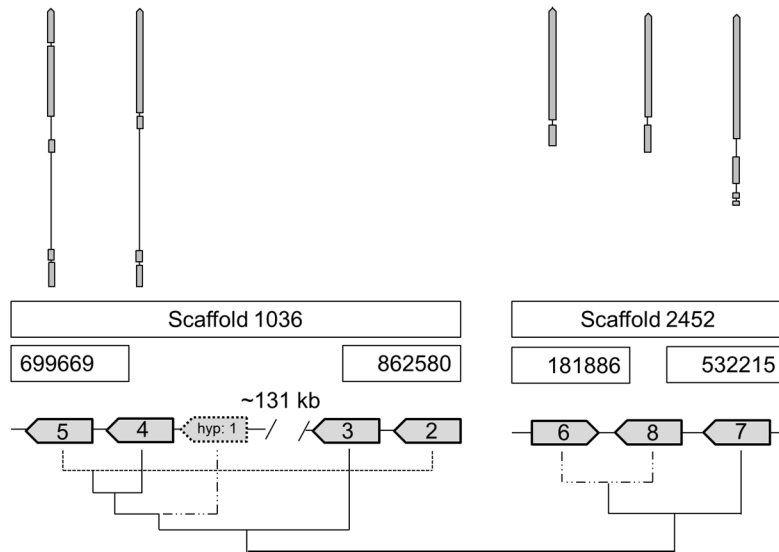


B)

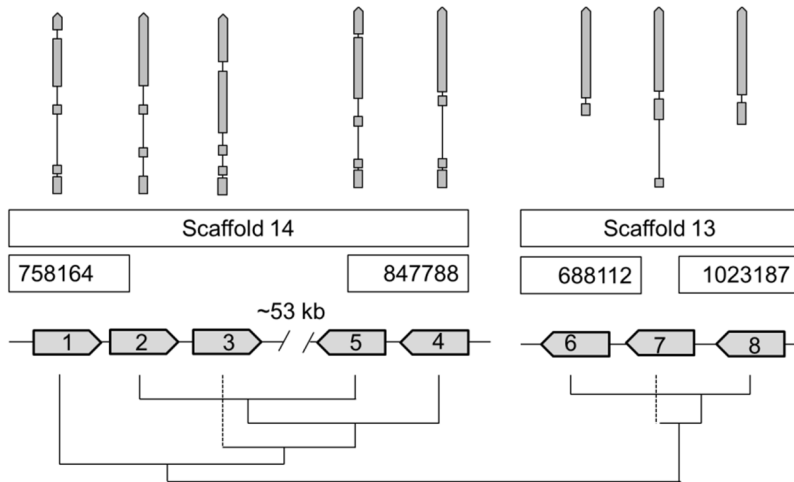


**Figure 4.4.** An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* long wavelength B clade (LOPB; putative red-sensitive). Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* LOPB clade and their phylogenetic relationships, and the B) *D. pulex* LOPB clade and their phylogenetic relationships.

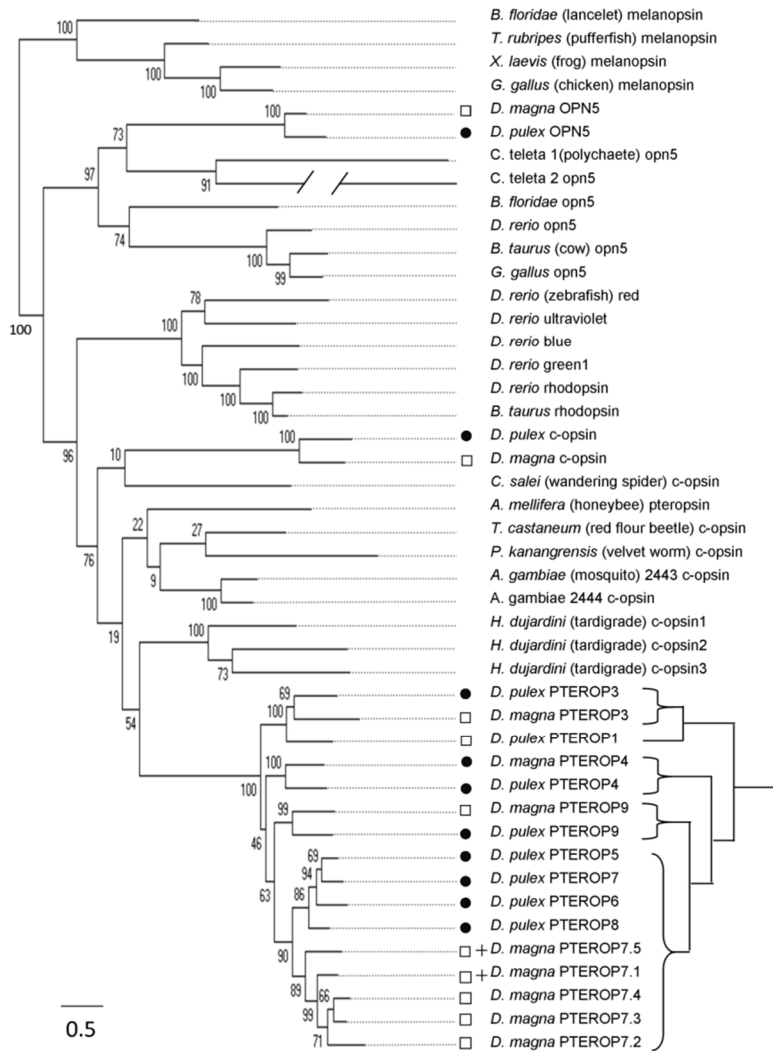
A)



B)

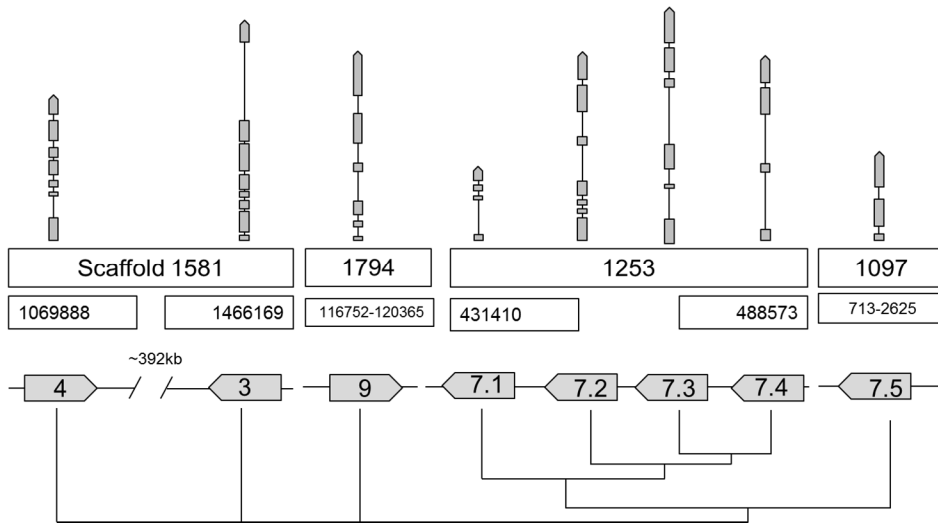


**Figure 4.5.** An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* arthropsin clade. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* arthropsin clade and their phylogenetic relationships, and the B) *D. pulex* arthropsin clade and their phylogenetic relationships. Two arthropsin gene structures are not shown for *D. magna* because large regions of the genome were missing within the genes.

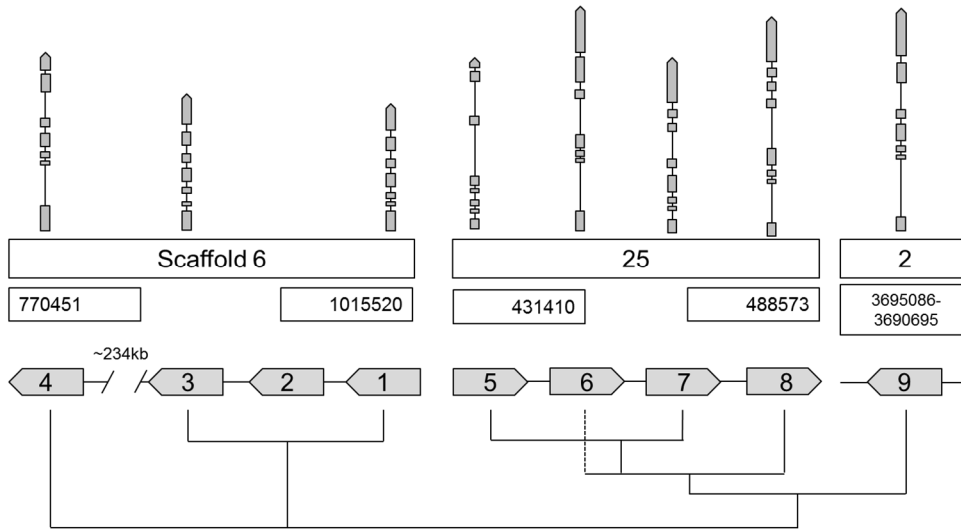


**Figure 4.6** Phylogeny of *D. pulex*, *D. magna*, and other species c-opsins based on maximum likelihood (ML) analyses of protein-coding nucleotide sequences. ML analyses were run using GTR model of evolution as implemented in RAxML. The phylogenetic tree is rooted at vertebrate melanopsins (r-opsins). Values on branches indicate bootstrap support. Branch lengths are proportional to the substitution/site scale bar. The *C. teleta* 2 opn5 branch has been broken for illustration purposes; the gap represents 2 substitutions/site. The scale bar indicates the number of nucleotide substitutions per site. The bracket tree on the right is the hypothesized pteropsin family that existed in the most recent common ancestor of *D. pulex* and *D. magna*, approximately 200 mya. Black closed circles (●) identify *D. pulex* and open boxes (□) identify *D. magna*. Crosses (+) indicate opsin genes that have truncated sequences.

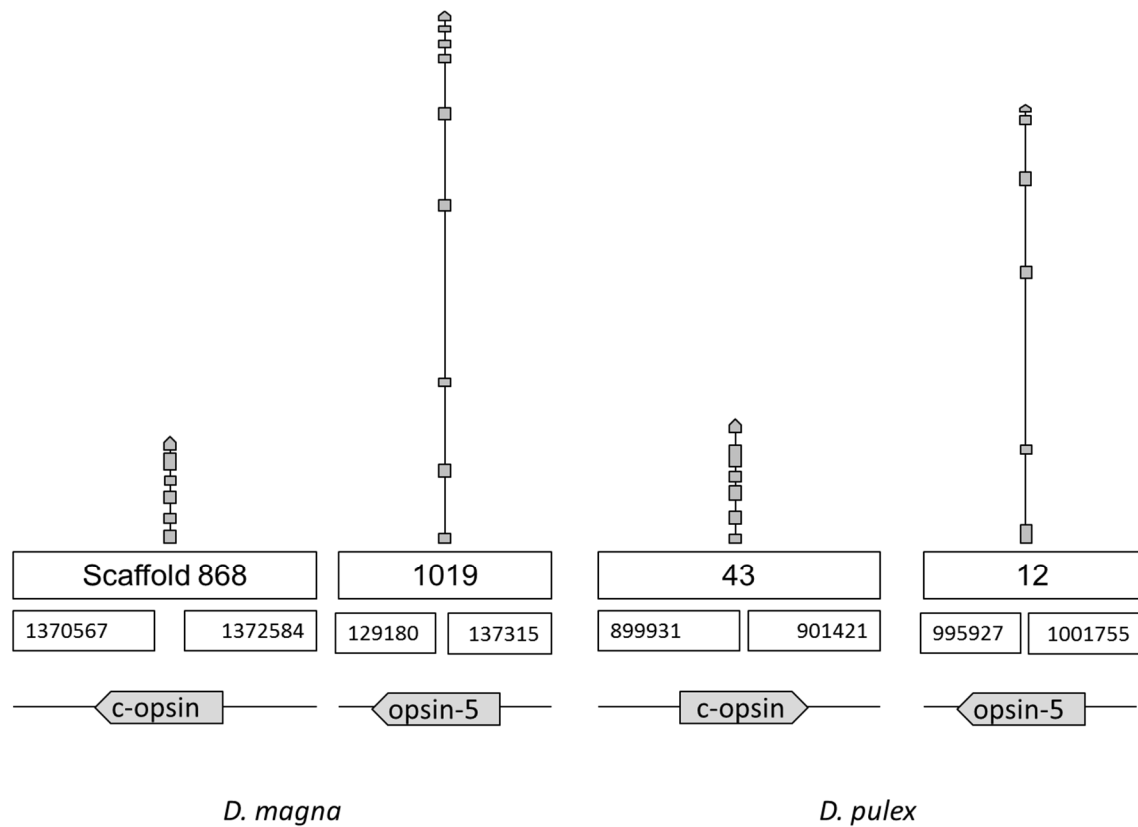
A)



B)



**Figure 4.7.** An illustration of the exon-intron gene structure and the genomic locations of the *Daphnia* pteropsin clade. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other. Illustrations show the A) *D. magna* pteropsin clade and their phylogenetic relationships, and the B) *D. pulex* arthropsin clade and their phylogenetic relationships.



**Figure 4.8** An illustration of the exon-intron gene structure and the genomic locations of opsin-3 and opsin-5 in *D. pulex* and *D. magna*. Scaffold numbers are identified in the top row of text boxes, and the genomic region shown is identified in the boxes immediately below. Genes read on the positive strand point right, and genes read on the negative strand point left. Exons are illustrated by gray boxes and introns by a line. Basepair distances of exons and introns are approximately proportional to each other.

## CHAPTER 5

### SUMMARY

Overall, these studies reveal multi-faceted insights on the evolution of vision in *Daphnia*, but also provide insights into the evolutionary mechanisms shaping variation of eyes in general. Furthermore, these works demonstrate the strengths of using *Daphnia* as a model to apply evolutionary thinking to the growing field of sensory ecology.

In Chapter 2, we show that eye size in *Daphnia* is influenced by the resource environment, but not by the light environment. This is contrary to the well-documented macroevolutionary pattern where larger eyes are typically associated with dim environments and smaller eyes are associated with bright environments. Eyes likely evolve to maximize visual information that fulfills the requirements for a certain visual task. However, eyes are energetically expensive tissue, and therefore are subject to resource allocation trade-offs. The light environment is undoubtedly a strong driver of patterns of variation seen at macroevolutionary scales, and probably at inter-population scales as well. Our data suggest that at least in terms of environmental-induced variation, these drivers are not influential. In addition, data from other studies suggest that this is not isolated to *Daphnia*. We conclude that non-sensory environmental factors, or factors not directly linked with visual tasks, can influence sensory systems, and in particular, that resource availability may be an important constraint on visual capability.



We also demonstrate that there is a selective advantage of compound eye size (Chapter 4). This result is significant for two reasons. First, most work has focused on variation at the level of species differences (as mentioned above). Authors often assume a selective advantage leads to the changes seen among these taxa because non-adaptive evolutionary mechanisms seem unlikely given how apparently optimized eyes can be to their environment and to the animal's ecology. Our work shows that there is variation in eye size among a meta-population of *Daphnia* species, and that there can be reproductive consequences to this variation. In other words, the potential for evolution in eye size exists within populations. Second, in public discourse, the complexity of the eye is often held up as a prime example of the implausibility of biological evolution, and ultimately of evidence for intelligent design. Of course, a colossal body of evidence documented in the literature suggests otherwise. Yet this idea is persistently touted by advocates of intelligent design (ID), or its variants. Our work does not deal a death blow to the position of ID advocates, but it serves as an example that eyes are subject to the same incremental evolutionary forces as “simple” traits.

Lastly, we show that the last common ancestor of *Daphnia* contained a large repertoire of opsin genes (Chapter 5). When the genome of *D. pulex* was first sequenced and analyzed, a total of 46 opsin sequences were discovered. At the time, and still presently, this was the largest set from any known animal genome. The genome of *D. magna* was recently sequenced, and according to current phylogenies the last common ancestor of *D. magna* and *D. pulex* is the ancestral *Daphnia*. Our results show that most of the opsin duplications occurred before the radiation of *Daphnia* species, suggesting that the large suite of opsin genes are characteristic of *Daphnia* in general. Though the

functional utility of such a large set are still unknown, genome and opsin sequencing efforts of other arthropods are showing that many species might have relatively sizable opsin sets. Clearly in *Daphnia*, and arthropods in general, further investigation into why so many opsin genes are being maintained and how they are used warrants more investigation.

We argue that *Daphnia* can provide useful insights into the evolution of vision, and serves as a particular strong model for asking questions about the contemporary processes of visual system evolution. *Daphnia* continue to provide scientific worth beyond their humble position among animals, and their utility for studies on eye evolution is no exception.

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APPENDIX A – CHAPTER 2 SUPPLEMENTARY INFORMATION

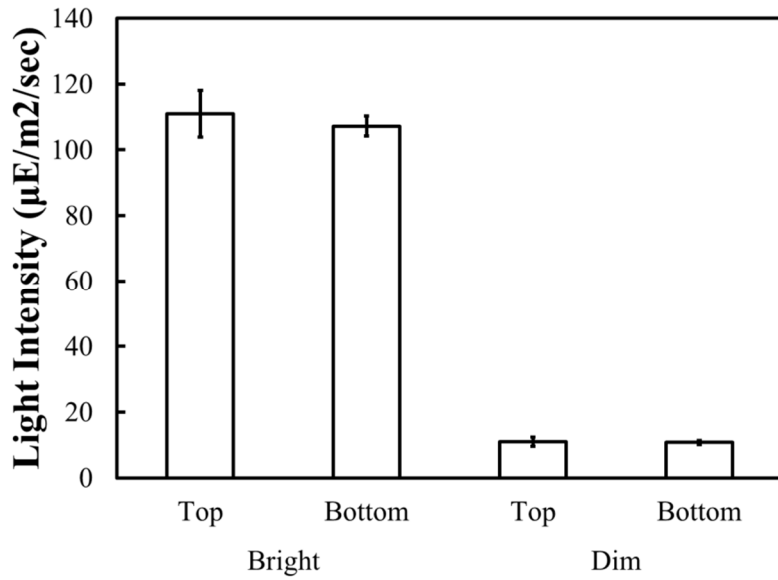
**Table A.1** Animal sample sizes, body length and absolute eye measurements for each factor level

Species	Stage	Factor		N	Body Length ( $\mu\text{m}$ ) $\pm$ s.e.m.	Absolute Eye Diameter ( $\mu\text{m}$ ) $\pm$ s.e.m.
		Resource	Light			
<i>D. parvula</i>	Early	20k	Bright	21	1,099 $\pm$ 15	87 $\pm$ 1
			Dim	27	1,138 $\pm$ 21	91 $\pm$ 1
		5 k	Bright	28	1,130 $\pm$ 12	87 $\pm$ 1
			Dim	28	1,079 $\pm$ 19	86 $\pm$ 1
	Late	20k	Bright	33	1,389 $\pm$ 33	110 $\pm$ 2
			Dim	15	1,364 $\pm$ 25	108 $\pm$ 2
		5 k	Bright	19	1,313 $\pm$ 14	104 $\pm$ 1
			Dim	25	1,285 $\pm$ 26	101 $\pm$ 2
<i>D. obtusa</i>	Early	20k	Bright	20	1,733 $\pm$ 35	149 $\pm$ 2
			Dim	20	1,793 $\pm$ 23	153 $\pm$ 1
		5 k	Bright	20	1,532 $\pm$ 23	136 $\pm$ 2
			Dim	20	1,578 $\pm$ 29	138 $\pm$ 1
	Late	20k	Bright	20	1,971 $\pm$ 27	174 $\pm$ 2
			Dim	20	2,028 $\pm$ 20	176 $\pm$ 2
		5 k	Bright	20	1,891 $\pm$ 19	173 $\pm$ 1
			Dim	20	1,855 $\pm$ 23	166 $\pm$ 2
<i>D. pulex</i>	Early	20k	Bright	38	1,968 $\pm$ 18	141 $\pm$ 1
			Dim	37	1,958 $\pm$ 15	143 $\pm$ 1
		5 k	Bright	35	1,801 $\pm$ 15	132 $\pm$ 1
			Dim	34	1,702 $\pm$ 14	127 $\pm$ 1
	Late	20k	Bright	15	2,161 $\pm$ 41	168 $\pm$ 4
			Dim	17	2,081 $\pm$ 33	157 $\pm$ 3
		5 k	Bright	10	1,938 $\pm$ 21	145 $\pm$ 4
			Dim	13	1,817 $\pm$ 26	136 $\pm$ 4
<i>D. pulicaria</i>	Early	20k	Bright	40	2,024 $\pm$ 14	150 $\pm$ 1
			Dim	39	1,944 $\pm$ 10	145 $\pm$ 1
		5 k	Bright	35	1,827 $\pm$ 11	136 $\pm$ 1
			Dim	34	1,789 $\pm$ 12	135 $\pm$ 1
	Late	20k	Bright	31	2,023 $\pm$ 15	173 $\pm$ 2
			Dim	30	1,937 $\pm$ 23	170 $\pm$ 2
		5 k	Bright	37	1,900 $\pm$ 16	160 $\pm$ 1
			Dim	36	1,836 $\pm$ 13	160 $\pm$ 1

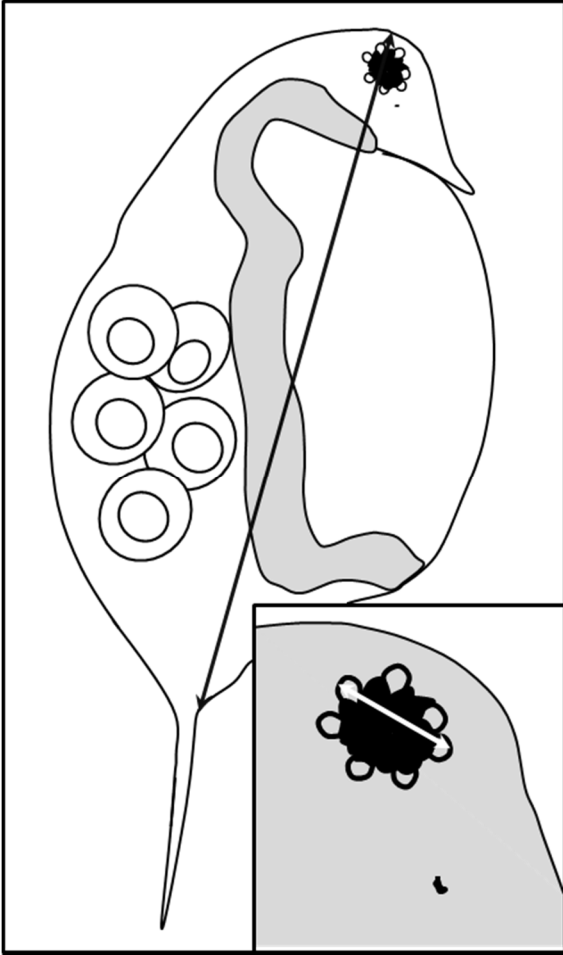


**Table A.2** Results of ANOVA on the effects of different environmental treatments on *Daphnia* spp. body length.

Species	Stage	d.f.	Resource		Light		Resource x Light	
			F-value	P-value	F-value	P-value	F-value	P-value
<i>D. parvula</i>	Early	1, 100	0.702	0.404	0.141	0.708	<b>7.433</b>	<b>0.008</b>
	Late	1, 88	<b>8.898</b>	<b>0.004</b>	1.036	0.312	0.003	0.959
<i>D. obtusa</i>	Early	1, 76	<b>54.767</b>	<b>&lt;0.001</b>	3.569	0.063	0.062	0.804
	Late	1, 76	<b>31.766</b>	<b>&lt;0.001</b>	0.206	0.651	<b>4.314</b>	<b>0.041</b>
<i>D. pulex</i>	Early	1, 140	<b>181.345</b>	<b>&lt;0.001</b>	<b>12.06</b>	<b>0.001</b>	<b>8.123</b>	<b>0.005</b>
	Late	1, 50	<b>47.589</b>	<b>&lt;0.001</b>	<b>8.137</b>	<b>0.006</b>	0.32	0.574
<i>D. pulicaria</i>	Early	1, 144	<b>216.461</b>	<b>&lt;0.001</b>	<b>24.172</b>	<b>&lt;0.001</b>	3.131	0.079
	Later	1, 132	<b>45.287</b>	<b>&lt;0.001</b>	<b>20.598</b>	<b>&lt;0.001</b>	0.446	0.505



**Figure A.1** Light intensity variation between the experimental chambers (Bright, Dim) and the two shelves (Top, Bottom) within each chamber. Light intensity was measured as photosynthetically active radiation (400 nm-700 nm). Three measurements per shelf per chamber were recorded. Error bars are standard deviation.



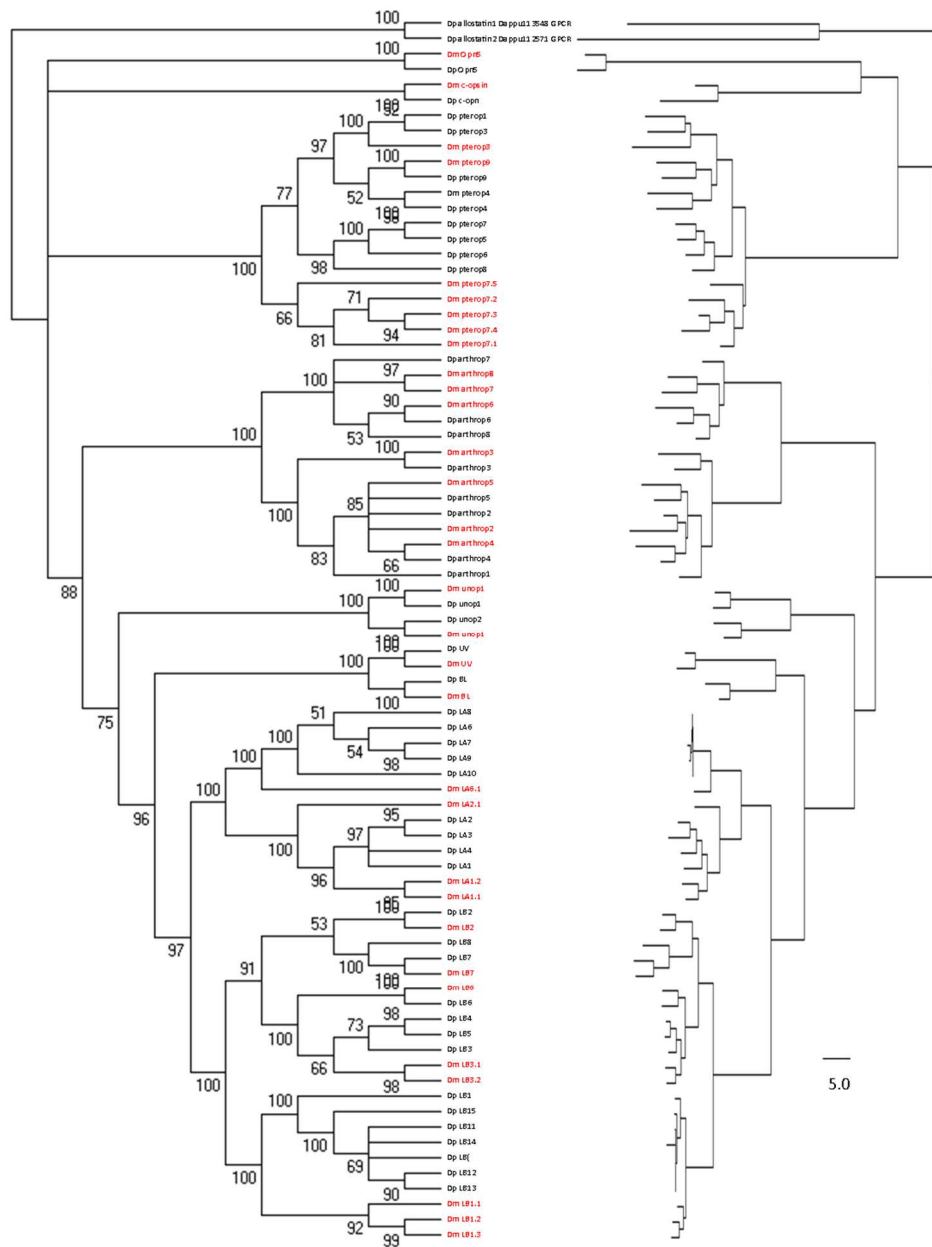
**Figure A.2** *Daphnia* illustration showing measurements for body length and eye diameter.

APPENDIX B – CHAPTER 4 SUPPLEMENTARY INFORMATION

**Table B.1** Genbank accession numbers of mRNA sequences used as the bases for the *Daphnia* opsin phylogenetic analyses.

Figure	Name in Figure	Full species name	Genbank accession number
4.1, 4.6	Danio rerio red	<i>Danio rerio</i>	AF104904.1
4.1, 4.6	<i>D. rerio</i> ultraviolet	<i>Danio rerio</i>	NM_131319.1
4.1, 4.6	<i>D. rerio</i> blue	<i>Danio rerio</i>	BC062277.1
4.1, 4.6	<i>D. rerio</i> green1	<i>Danio rerio</i>	AF109369.1
4.1, 4.6	<i>D. rerio</i> rhodopsin	<i>Danio rerio</i>	HM367063.1
4.1, 4.6	<i>Bos taurus</i> rhodopsin	<i>Bos taurus</i>	NM_001014890.1
4.6	<i>B. floridae</i> melanopsin	<i>Branchiostoma floridae</i>	XM_002596237.1
4.6	<i>T. rubripes</i> melanopsin	<i>Takifugu rubripes</i>	XM_003965548.1
4.6	<i>X. laevis</i> melanopsin	<i>Xenopus laevis</i>	NM_001085674.1
4.6	<i>G. gallus</i> melanopsin	Gallus gallus	AY036061.1
4.6	<i>C. teleta</i> 1 opn5	<i>capitella teleta</i>	ELU02401*
4.6	<i>C. teleta</i> 2 opn5	<i>capitella teleta</i>	ELT87227*
4.6	<i>B. floridae</i> opn5	<i>Branchiostoma floridae</i>	XM_002587782.1
4.6	<i>D. rerio</i> opn5	<i>Danio rerio</i>	NM_001200046.1
4.6	<i>B. taurus</i> opn5	<i>Bos taurus</i>	AB368182.1
4.6	<i>C. salei</i> c-opsin	<i>Cupiennius salei</i>	HF566407.1
4.6	<i>A. mellifera</i> pteropsin	<i>Apis mellifera</i>	NM_001039968.1
4.6	<i>T. castaneum</i> c-opsin	<i>Tribolium castaneum</i>	NM_001145478.1
4.6	<i>E. kanangrensis</i> c-opsin	<i>Euperipatoides kanangrensis</i>	HF566404.1
4.6	<i>A. gambiae</i> 2443 c-opsin	<i>Anopheles gambiae</i>	XM_312503.4
4.6	<i>A. gambiae</i> 2444 c-opsin	<i>Anopheles gambiae</i>	XM_312502.2
4.6	<i>H. dujardini</i> c-opsin1	<i>Hypsibius dujardini</i>	KM086336.1
4.6	<i>H. dujardini</i> c-opsin2	<i>Hypsibius dujardini</i>	KM086337.1
4.6	<i>H. dujardini</i> c-opsin3	<i>Hypsibius dujardini</i>	KM086338.1

\*Protein accession number. CDS sequences were retrieved from <http://genome.jgi-psf.org/Capca1/Capca1.home.html>



**Figure B.1** Phylogenetic analyses of all coding *Daphnia* opsins using coding nucleotide sequences. Analyses were run as described in the methods, section 4.2. Tree is rooted at two *D. pulex* allostatin genes that are members of the GPCR superfamily. Tree on the left is bootstrap consensus, with bootstrap values listed on the branches. The tree on the right is the genetic distances, and substitutions/site are proportional to scale bar.

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