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Flashing in the 'Disco' Clam *Ctenoides ales* (Finlay, 1927):

Mechanisms and Behavioral Function


By

Lindsey Dougherty


Dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley


Committee in charge:

Professor Roy L. Caldwell, Chair

Professor David R. Lindberg

Professor Damian O. Elias


Spring 2016

## Abstract

## Flashing in the 'Disco' Clam *Ctenoides ales* (Finlay, 1927): Mechanisms and Behavioral Function

### by

### Lindsey Dougherty

### Doctor of Philosophy in Integrative Biology

### University of California, Berkeley

### Professor Roy L. Caldwell, Chair

This dissertation investigated the 'disco' clam *Ctenoides ales* (Limidae), which is the only bivalve in the world that has a behaviorally-mediated flashing display. Topics covered include (i) mechanisms, ultrastructure and movement that produce the flashing, (ii) the fitness value (function) of the flashing, (iii) the clams' sensory abilities and vision, and (iv) the clams' ecology, distribution and habitat.

The flashing occurs on the clams' mantle lip. Electron microscopy revealed two distinct tissue sides; one highly scattering side that contains dense aggregations of spheres composed of silica (white), and one highly absorbing side that does not (red). High-speed video confirmed that the two sides alternate rapidly, creating the appearance of flashing. Optical modeling suggested that the sphere's diameter is nearly optimal for scattering light, especially at shorter wavelengths, which predominate in the ocean. This simple mechanism produces a striking optical effect.

Three potential hypotheses for the fitness value of the flashing were investigated; conspecific attraction, prey luring, and/or predator deterrence. The lack of movement toward other *C. ales* when given visual cues in behavioral trials, as well as the clams' inability to resolve flashing in other *C. ales* suggested conspecific attraction was not the function of the flashing. The lack of significant differences in prey abundance in experiments testing flashing versus non-flashing clams suggested prey luring was also not the function of the flashing. Predator deterrence is considered a possible function of the flashing due to (i) sulfur presence in the clam's tissues, suggesting a possible distasteful compound used in aposematism, (ii) behavioral responses by predators during feeding trials as well as tissue preferences that suggest probable distastefulness, and (iii) the clams' increase in flash rate when exposed to predators in the lab and the field, suggesting a potential warning signal.

The presence of photosensitive pigments (rhodopsin, tubulin and retinochrome) was suggested from immunohistochemistry results in the ~40 eyes of *C. ales* in collaboration with A. Nahm-Kingston (University of Maryland Baltimore County). Transmission electron microscopy done in collaboration with R. Dubielzig, L. Teixeira, and C. Schobert (University of Iowa Veterinary School) confirmed that the morphology of the eye was inconsistent with image-

formation or the ability to resolve flashing in conspecifics. Therefore, the visual capability of *C. ales* is most likely used for predator detection.

The clams' distribution, depth, main habitats, projected sex ratios, movement based on size (sex) and sphere properties with depth were identified through SCUBA research at four field sites. The clams are found throughout the Indo-Pacific, from depths 3m to ≥50m. Their movement doesn't vary with sex, and they exhibit a clumped distribution skewed towards males, which compounds their vulnerability in aquaria collections and highlights the need to determine their conservation status.

Studying biodiversity and evolution from an integrative, organismal approach requires a diverse arsenal of tools. Broad methodologies were utilized, including spectrometry, high-speed video, electron microscopy (scanning and transmission), energy-dispersive x-ray spectroscopy, mass-spectrometry, particle modeling, fluorescence microscopy, behavioral trials (feeding, looming, and aposematism), phototaxia trials (plankton), and multi-year underwater ecological studies. To achieve this multi-disciplinary approach, collaborations were fostered with Duke, UQ, UW (Madison) Veterinary School, UI, UMBC, and with the Chemistry, Earth and Planetary Sciences, Physics, and Environmental Science Policy and Management departments at Berkeley. Media coverage of this dissertation work appeared in Nature, Science, the New York Times, CNN, the BBC, National Geographic, the Washington Post, Science Friday, Science News and many more, which is evidence of the widespread interest in this fascinating organism.

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# DEDICATION

I dedicate this work to my mom.


Her unbridled spirit, creative mind, and adventurous heart made me adore the sea,

and her unequivocal love and support propelled me further than I ever imagined I could go.

# ACKNOWLEDGEMENTS

I would never have developed a life-altering love for the ocean had it not been for SCUBA diving. Getting certified at 14 years old in a frigid Colorado reservoir was not the most romantic introduction to the sport, but the first day I back-rolled into the ocean, things changed forever. My mom has been an ocean enthusiast my entire life. She, my aunt Carol, and my grandmom Furman instilled so much wonder in me for ocean creatures – all starting with the specimen bucket. I have an amazing and adventurous family who have accompanied me on so many dive trips – my mom, sister, Pat, Bobby, Mary, Sarah and Katherine. Working at my first dive shop in Boulder gave me an intense appreciation and adoration for people in the dive industry, evidenced most by my amazing bosses, Steve Weaver and Jane Steinbrecher. They also helped facilitate my undergraduate research at Lembeh Resort, through the generosity of Danny Charlton. I'm forever grateful to Paul Shepard, whom I somehow convinced (on a dive boat in Belize) to let me work for him as a SCUBA instructor in Zanzibar. My dive friends have irrevocably influenced my life as well as my research, and have solidified my love and respect for the ocean.

When my oceanic focus turned academic, Dr. Mel Cundiff at CU Boulder agreed to sponsor my research project on artificial reefs, and I'm grateful for his continued support and advice. While under his guidance doing undergraduate research in Indonesia, my family came to dive in Wakatobi, where I was first introduced to the 'disco' clam. I knew I wanted to focus my PhD on light and color, but it wasn't until I saw this amazing creature (which no one seemed to know anything about) that I had a focus, however far-fetched it might have seemed. Cue Dr. Roy Caldwell, who at the time of my application was not planning to take any more students. I decided to apply regardless, and unexpectedly, he decided that he would take 'just one more'.

Roy is one of those mentors who is incredibly humble about his achievements. It took years for me to learn more about his stories, his adventures, and his major contributions to the field of animal behavior. I still don't know what possessed Roy to agree to take me on as a PhD student, but I will be forever grateful that he took a chance on a student who was more accomplished in diving than in science; who had an MBA instead of a MS; and who had an unshakable desire to study a rare, flashing clam. The freedom and the faith Roy gave my research endeavors are undoubtedly a rare occurrence. I feel incredibly fortunate that I was able to accomplish as much as I did, and that I was given the opportunity to test wild ideas, to explore the furthest reaches of the underwater world, and to follow my curiosity with nothing but unbridled support.

Getting a PhD is hard. There is no way I would have survived it without the support of my family – my mom, dad, sister, John, and Pam. They endured countless hours of complaining, and were equally helpful in immersing themselves in my work in order to critique papers and offer ideas. My mom and uncle Pat even accompanied me on dive trips to scope out research sites. My mom's love of the ocean as well as color and light through her art definitely impacted my love of being underwater, and my mom and dad's support of my underwater photography fueled my creativity and supplemented my scientific endeavors. My aunt and uncle Carol and Clark offered unwavering moral support and encouragement from a very young age, and I am lucky to have them. I truly could not have asked for a more caring family, and I am forever grateful.

# Curriculum Vitae
**Lindsey Dougherty**
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## Education
| | |
|---|---|
| **University of California –** PhD in Integrative Biology | 8/11 – 5/16 |
| **University of Colorado** – Bachelor of Arts in Biology | 8/09 – 8/11 |
| **Colorado State University** – Master of Business Administration | 6/08 – 8/09 |
| **University of Colorado** – Bachelor of Science in Business Administration | 8/01 – 5/05 |

## Publications
Dougherty, LF, AK Niebergall, and KE Meyer. 2016, *in review*. Testing flashing as a prey lure in the 'disco' clam, *Ctenoides ales. American Malacological Bulletin.*

Dougherty LF. 2016, *in press*. Distribution, habitat, and ecology of the 'disco' clam, *Ctenoides ales*. In *Oysters and Clams: Cultivation, Habitat Threats and Ecological Impact.* RJ Hauppauge, NY: Nova Science Publishers.

Dougherty LF, S Johnsen, RL Caldwell, and NJ Marshall. 2014. A dynamic broadband reflector built from microscopic silica spheres in the 'disco' clam *Ctenoides ales. Journal of the Royal Socity Interface* **11**: 20140407.

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## Book Contribution
Dougherty, LF. 2016, *in press*. In *The Biology and Evolution of the Mollusca*. Ponder, WF and Lindberg, DR. Victoria, Australia: CSIRO Publishing.

## Magazine Contributions
Dougherty, LF. 2015. Disco clams' flashy discoveries. *Biosphere* **5**: 32-37.
Dougherty, LF. 2014-2015. Flashy Creatures – Asia. *Scuba Diver Ocean Planet* **8**: 32-37.

## Media Coverage of Research
**2015:** Science (AAAS), National Geographic, the Washington Post, Science News, LiveScience, Fox News, the Atlantic, Biosphere, Science Friday, Discovery Channel Daily Planet, New Scientist

**2014:** Nature, the New York Times (Article and Podcast), National Geographic, BBC Wildlife Magazine, Slate, Wired, ABC News, Biophotonics Magazine, Focus Magazine, Science Recorder, Science News

**2013:** CNN, Huffington Post, Popular Science, New Scientist, Daily Planet Discovery Channel, Thomson Reuters, Berkeley Science Review, Surface Optics

**Honors**
- Best Student Presentation (DIZ) – SICB Conference January 2016
- Invited Symposia Speaker: 'Young Researchers in Malacology' – AMS Conference 2015
- Finalist, Best Student Presentation (ABS) – SICB Conference January 2015
- Best Student Paper - AAUS Conference - September 2014
- SMART Program Awardee & Profiled Researcher - 2014
- Outstanding Graduate Student Instructor Award - Spring 2013
- Profiled Graduate Researcher – UC Integrative Biology Department – Spring 2013
- Profiled Undergraduate Researcher – University of Colorado Biology Department – 2011
- Phi Beta Kappa and Beta Gamma Sigma Honor Society – 2002, 2009
- International Business Certificate – 2005
- Presidents Leadership Class – 2001–2005

**Outreach**
- California Academy of Sciences: Earth Update Presenter (Nightlife)
- Bay Area Science Festival Presentations: Alcatraz, You're the Expert, Science…Sort Of
- East Bay Presentations: Nerd Nite East Bay, East Bay Science Cafe
- UC Berkeley Dive Control Board Graduate Student Member
- PADI Open Water SCUBA Instructor
- UC Musem of Paleontology Night at the Museum Presenter, Cal Day Presenter
- Local Outreach Program Participation: Dinner with a Scientist (Oakland Zoo), Pleasanton Middle School Science Night, Expanding Your Horizons Conference, Women in Science Group, UC Musem of Paleontology museum tours, Surfrider and Surfaid Foundations

**Grants**
- National Science Foundation EAPSI Fellowship, PADI Foundation Award, Animal Behavior Society Grant, Lerner Gray Memorial Fund, Conchologists of America Grant, SMART Grant, Sigma Xi Grant, UCMP Palmer Grant, Graduate Division Grants, Integrative Biology Department Grants

**Research**
Integrative Biology – University of California Berkeley: 8/11 – Present
- "Behavior and Mechanisms of flashing in *Ctenoides ales*: "disco clams"
Friday Harbor Labs – University of Washington, San Juan Islands, WA: 6/12 – 7/12
- "Community Fluorescence and Inducible Fluorescence in Two Polychaete Species"
CIRES – Cooperative Institute for Research in Environmental Sciences: 8/10 – 12/10
- "Trophic Effects of Anthropogenic Fish Introductions to High-Altitude Lakes"
Independent Study – Lembeh Resort, Sulawesi Indonesia: 5/10 – 6/10
- "Population, Species and Family Analysis of Artificial Reef Structures"
Independent Study – University of Colorado: 8/04 – 12/04
- "Sustainability in Colorado Mountain Resorts"

**<u>Employment</u>**

University of California Berkeley – Berkeley, CA: 8/11 – Present
- Graduate Student Instructor – Biology 1B (Fall 2011, Spring 2012)
- Animal Behavior (Fall 2012)
- Ecology and Evolution of Animal Behavior (Spring 2013, 2014)
- Biology and Geomorphology of Tropical Islands (Moorea, French Polynesia, Fall 2013)
- Teaching Colloquium (Fall 2014, 2015)

SMART Program – Berkeley, CA: 1/14 – 8/14
- Graduate Mentor for Undergraduate Mentee, Media Coverage

Aquatic Adventures Scuba & Swim - Pueblo, Colorado: 5/08 – 4/09
- Financial, Retail & Marketing Assistant Manager

Rising Sun Dive Centre PADI Open Water SCUBA Instructor: Zanzibar, Tanzania: 8/07 – 12/07
- Financial Assistant Manager, Instructor/Divemaster, Certified 30+ Divers, 500+ Logged Dives

Weaver's Dive and Travel Center - Boulder, CO: 6/06 – 8/07
- Financial/Retail/International Group Travel Planning

## Flashing display in *Ctenoides ales*: mechanisms (proximate analysis)

## Abstract

The 'disco' clam *Ctenoides ales* (Limidae) is the only species of bivalve known to have a behaviorally-mediated photic display. This display is so vivid that it has been repeatedly confused for bioluminescence, but it is actually the result of scattered light. The flashing occurs on the mantle lip, where electron microscopy revealed two distinct tissue sides; one highly scattering side that contains dense aggregations of spheres composed of silica, and one highly absorbing side that does not. High-speed video confirmed that the two sides act in concert to alternate between vivid broadband reflectance and strong absorption in the blue region of the spectrum. Optical modeling suggests that the diameter of the spheres is nearly optimal for scattering visible light, especially at shorter wavelengths which dominate their environment. This simple mechanism produces a striking optical effect.

## Introduction

Structural coloration is common in dynamic visual displays by terrestrial and marine animals (Mäthger *et al.* 2009; Meadows *et al.* 2009; Srinivasarao 1999; Vukusic and Sambles 2003; Hanlon and Messenger 1996). The spectral environment in which they live influences communication methods that use coloration (Mäthger *et al.* 2009; Osorioa and Vorobyev 2008; Sweeney *et. al.* 2003; Johnsen 2001; Cummings 2007; Seehausen *et al.* 2008; Johnsen 2012). In the ocean's euphotic zone, the "disco" or "electric" file clam *Ctenoides ales* (family Limidae) is found inside small crevices at depths of approximately 3-50m. At these depths, the majority of wavelengths available for visual displays are in the blue-green range (400-500nm) (Jerlov 1976). This is true even at shallow depths where long wavelengths have not yet attenuated (<15m), as the crevices in which *C. ales* are found are dominated by horizontal light composed of short wavelengths (Appendix 1).

The flashing display on the mantle lip of *C. ales* has been mischaracterized as bioluminescence (Okutani 1994; Mikkelsen and Bieler 2003), although it is actually mediated by light scattered from photonic nanostructures (Okubo *et al.* 1997). Structures of this sort typically use materials that have high refractive indices relative to the substrate, such as collagen, chitin, keratin and guanine (Welch and Vigneron 2007). Silica also has a high refractive index (n=1.43 at 589nm) (Welch and Vigneron 2007), but has only rarely been used as a biophotonic structure, such as in diatoms (Fuhrmann *et al.* 2004; Wilson 1966; Noyes 2008) and the weevil *Pachyrhynchus argus* (Parker *et al.* 2003). Photonic nanostructures of any substance however can enhance reflectance, such as the ultrathin, aperiodic filaments in scales of *Cyphochilus spp.* beetles (Vukusic *et al.* 2007) and the bead-studded scales in the wings of certain pierid butterflies (Stavenga *et al.* 2004).

Within bivalves, studies of light manipulation are limited to the bioluminescence of the marine clams *Pholas dactylus* and *Gastrochaena sp.* (Dubois 1877), and the iridophores of the giant clam *Tridacna*, which are thought to scatter light towards symbiotic zooxanthallae (Griffiths *et al.* 1992). *C. ales*, however, is the only known bivalve with a behaviorally-mediated

photic display. The fundamental characteristics of this display are described here to determine its potential as a signal. Ongoing studies of the function of the display are being conducted in the context of habitat-specific sensory ecology. In order to provide a preliminary comparative framework, we collected data from the morphologically and ecologically similar congener *Ctenoides scaber,* which does not flash.

## Materials and Methods

We used five techniques to investigate the display of *C. ales*; spectrometry, high speed video, transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDS) and optical modeling. Spectrometry, high-speed video, and TEM were used to provide a comparative framework on *C. ales* and *C. scaber*. Our prediction was that the display of *C. ales* would show differences in reflectance, ultrastructure, and mantle lip movement when compared to *C. scaber*. EDS and optical modeling were not appropriate for comparison, as *C. scaber* had no distinctive ultrastructure to warrant further analysis. Specimens were observed in the lab and *in situ* in Australia (Lizard Island, 14°38'S, 145°27'E) and Indonesia (Lembeh Straight, 1°27'N, 125°14'E and Kri Island, 0°34'S, 130°40'E). Lab work was conducted at ambient room temperature (23-26˚C).

### Spectrometry
Spectrometry was conducted using an Ocean Optics USB2000 (Ocean Optics, Dunedin, FL, USA) to measure reflection of the two distinct tissue sides of *C. ales*, and to look for any similar distinctions in *C. scaber*. An Olympus SZX9 microscope (Olympus, Waltham, MA) was used for magnification. A small portion of tissue ($<1cm^2$) was excised from the mantle edge and placed on a white reflectance standard (WS-2, Ocean Optics, Dunedin, FL, USA) 50mm away from the microscope objective. The standard and the tissue were submerged in salt water. The spectrometer, which used a Sony ILX511 linear silicon CCD array and fiber optic cable, was mounted in the microscope and aimed straight down at the tissue. The Olympus LG-PS2 light microsource (Olympus, Waltham, MA) was oriented at a 45° angle to the tissue outside of the seawater, and illuminated the tissue at 29.7° due to refraction (assuming a refractive index of seawater of 1.34). Results were analyzed using OOIBase32 software (Ocean Optics Inc.). The measured area was small and thus had to be imaged through a microscope. Therefore, due to the limitations of the microscope, the ultraviolet (UV) portion (300-400nm) of the reflectance was not measured.

### High-Speed Video
Black and white high-speed video was captured using the FASTCAM SA3 and analyzed with FASTCAM Viewer software in order to analyze the inner mantle fold movement of *C. ales*. (Photron, San Diego, CA, USA, Inc.). Images were taken at 1024 x 1024 pixel resolution at 1000 frames per second using a standard fluorescent bulb for illumination.

### Transmission Electron Microscopy
Tissues from three *C. ales* specimens and one *C. scaber* specimen were fixed in 2.5% gluteraldehyde to examine differences in ultrastructure between the two species. Six tissue fragments from *C. ales* and two tissue fragments from *C. scaber* were examined. Transmission Electron Microscopy (TEM) was conducted using the Philips/FEI Tecnai 12 TEM (Philips,

Hillsboro, OR, USA) at the Electron Microscopy Lab at the University of California at Berkeley. Tissue was fixed with osmium tetroxide and sections were stained with uranyl acetate and lead citrate.

## Energy Dispersive X-Ray Spectroscopy

A JEOL JEM2100 LaB$_6$ STEM analytical transmission electron microscope (JEOL, Peabody, MA, USA) fitted with a thin-window energy dispersive X-ray detector was used to conduct elemental analysis on tissue samples mounted on copper grids using spectral point acquisition. Analysis was done at the Centre for Microscopy and Microanalysis at the University of Queensland, Australia. Samples were analyzed at an accelerating voltage of 200kV in a bright field TEM at 600x magnification. The average diameter of the three-dimensional spheres was determined by fitting a histogram of the diameters of 176 circular sphere sections (measured from TEM images using Adobe Photoshop CS5.1) to a model that assumed the three-dimensional sphere diameters were normally distributed and that the spheres were randomly intersected by the section planes. The volume density of the spheres was determined by measuring the average area density from 25 square regions of interest (with average area of 9.26 $\mu m^2$) and then using standard stereological methods to convert this value to a volume density of 25±3 spheres per $\mu m^3$.

## Modeling Light Scattering from the Spheres

Modeling was used to determine the angle-weighted scattering of the spheres. Methods are described in electronic supplemental materials. Briefly, we followed the methods of Bettelheim and Siew (1983) to estimate the angle-weighted scattering from a dense collection of hard spheres as a function of the diameter of the spheres and their packing density.

## **Results**

## Morphology and Spectrometry

Both inner mantle folds of *C. ales* have a unique marginal edge with two distinct sides (Figure 1). The ventral side appears as a white band along the width of the tissue and is strongly scattering (>80% reflection over 400-550nm). The dorsal side of the tissue, however, is red and is weakly scattering (<5% reflectance over 400-550nm). This results in a roughly 16-fold difference in reflectance (Figure 1), so the furling and unfurling of the mantle creates a highly dynamic signal. Despite being thin (<25µm), the white ventral side is optically thick, and therefore opaque. Spectrometry of the mantle tissue of the congener *C. scaber* did not show any optical asymmetry.

## High Speed Video

Black-and-white high-speed video (1000 frames per second) confirmed that the marginal edge unfurls and then furls back up in a wave-like motion, similar to what was reported by Okubo *et. al.* (1997). The unfurling motion exposes the highly reflective ventral side, and the furling motion exposes the poorly reflective dorsal side. The rapid transition creates the flashing appearance. This pattern of movement occurs whenever the valves are open and infrared video shows that the movement also occurs in the dark. No equivalent movement was seen in the congener *C. scaber*.

## Transmission Electron Microscopy

TEM of *C. ales* showed that the tissue had two distinct sides; the ventral side of the mantle containing electron-dense spheres 0.30µm ± 0.4µm (mean± SD) in diameter, and the dorsal side of the mantle, which did not (Figure 2A). We measured $25 \pm 3$ spheres/$\mu m^3$, with a total volume fraction of $0.35 \pm 0.1$. *C. scaber* lacked any structures similar to those found in *C. ales* (Figure 2B).

## Energy Dispersive X-Ray Spectroscopy

The spheres of *C. ales* were composed of amorphous silica ($SiO_2$), based on the presence of silicon (1.70-1.80 keV) and oxygen (0.40-0.60keV) (Figure 3).

## Modeling Light Scattering from the Spheres

We modeled the angle-weighted scattering of the dense collections of spheres using methods developed by Bettelheim and Siew (1983) in order to determine how both sphere diameter and sphere packing influence angle-weighted scattering (Electronic Supplemental Materials File 4). The true three-dimensional sphere diameter was 0.30±0.04 µm (mean±SD) – smaller than the 0.5 – 0.6µm previously described by Okubo *et al.* (1997). The volume fraction of the spheres was 0.35±0.1. This showed that the diameters of the spheres were close to the optimal value for scattering visible light, especially at shorter wavelengths (400nm, 480nm) (Figure 4).

## Discussion

To our knowledge, *C. ales* is the first animal to use silica as a scattering structure via intracellular nanospheres. Indeed, it is unusual to see silica secreted by animals for any purpose. Exceptions include diatoms (Fuhrmann *et al.* 2004; Wilson 1966; Noyes 2008), sponges (Simpson and Volcani 1981) and the weevil *Pachyrhynchus argus* (Parker *et al.* 2003). Within mollusks, the only examples of silica secretion are in the radula in certain species of limpets and chitons (Hua and Lee 2007). This broadband scattering creates a dynamic display as the mantle is furled and unfurled. Similarly, many species of butterflies can create iridescent flashes as they fly using structural colors on one side of their wings. These flashes have been suggested to increase signal efficacy (Ghiradella *et al.* 1972; Kemp and Rutowski 2007; Vukusic *et al.* 2001; Rutowski *et al.* 2007). The signal created by the transition between the two sides of the mantle tissue is especially pronounced at shorter wavelengths, which predominate in the clams' 3-50m underwater crevice habitats (Jerlov 1976). The mantle movement of C. ales differs from typical mantle movement in bivalves, which often aids feeding and respiration by drawing seawater into the gills (Galtsoff 1964; Morton and Yonge 1964; Owen 1966). In *C. ales*, only the marginal edge of the inner mantle fold associated with the white band moves rapidly, suggesting feeding and respiration are not the primary function. This simple mechanism produces a striking optical effect that may function as a signal.

## Acknowledgements

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## Figure Captions

Figure 1. Spectrometry on Mantle and Lip Tissue. Top: *C. ales* and microscope photo of tissue (inset) showing points of measurement for spectrometry. Bottom: Percent Reflectance for points of measurement.

Figure 2. Transmission Electron Microscopy Species Comparison (A) TEM of *C. ales* inner mantle fold marginal edge showing electron-dense spheres (inset) in the white ventral side, and a lack thereof in the red dorsal side. (B) TEM of congener *C. scaber* lacks any similar electron-dense spheres.

Figure 3. Energy Dispersive X-Ray Spectroscopy (EDS). EDS elemental analysis shows the composition of the reflective spheres. Blue (Silicon) and red (Oxygen) combine to form the purple, amorphous silica spheres ($SiO_2$), while green (carbon) composes the underlying tissue. Both the outer shells (A) and the cores (B) of the spheres are composed of silica (silicon 1.70-1.80 keV, oxygen 0.40-0.60keV).

Figure 4. The Effect of Sphere Diameter and Density on the Total Amount of 400nm, 480nm, 550nm and 650nm Angle-Weighted Scattered Light from a Dense Collection of Spheres (arbitrary units). The mean values (dots) and error bars show the range of the parameters found in *C. ales* tissue at four different wavelengths. The size of the spheres found in *C. ales* is close to optimal for maximal light scattering at 400nm and 480nm. Units are normalized to one for the maximum angle weighted scattering for 400 nm light.

# Figures

## Figure 1.
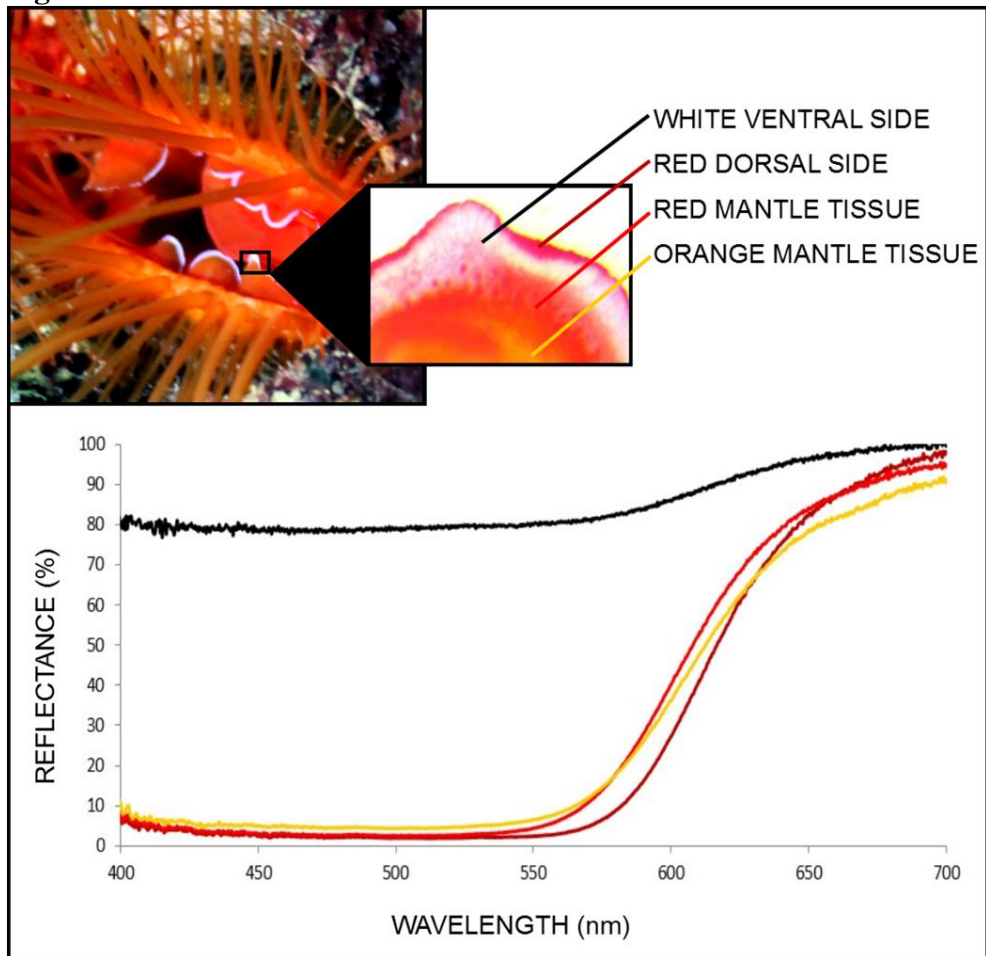


## Figure 2.
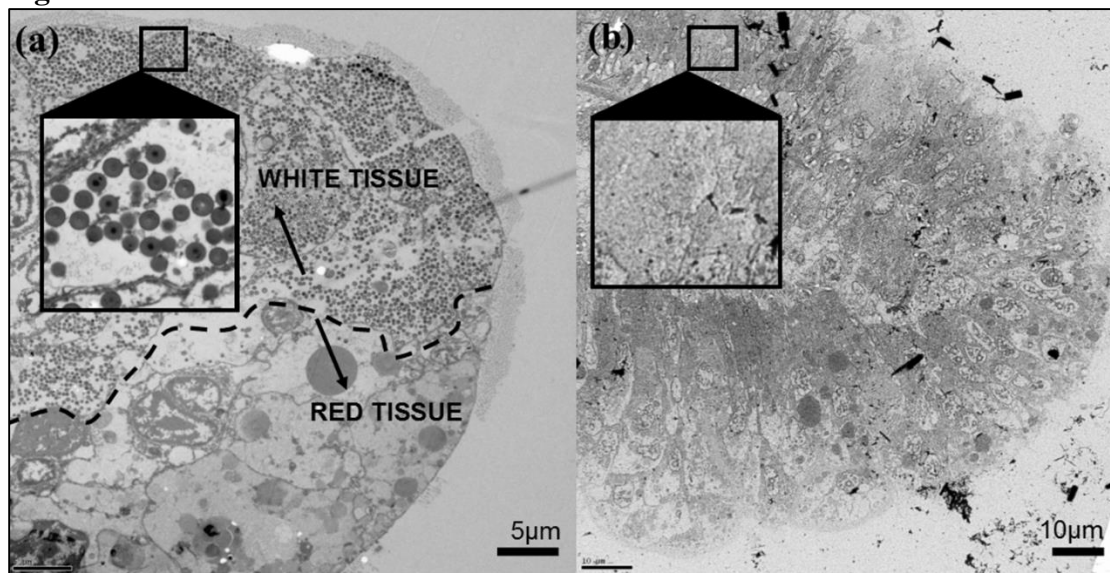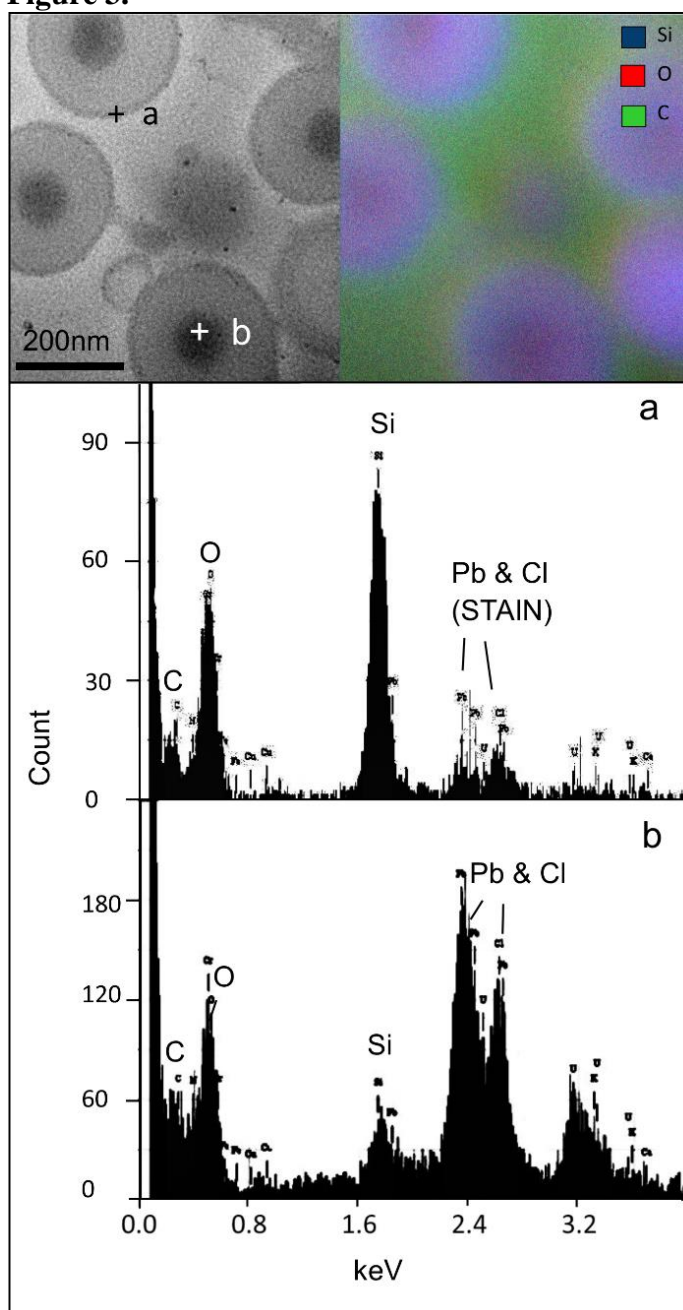
**Figure 3.**

**Figure 4.**
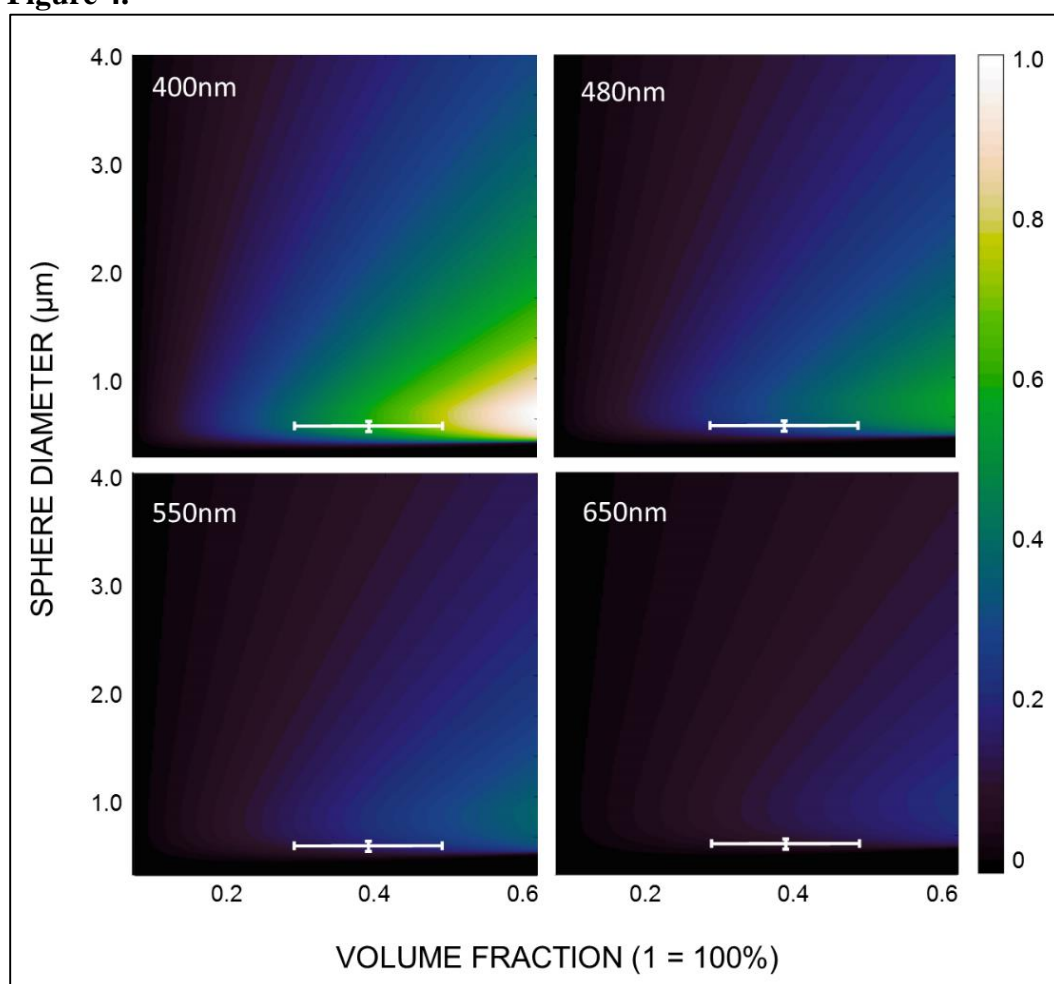
# Appendices

## Appendix 1.
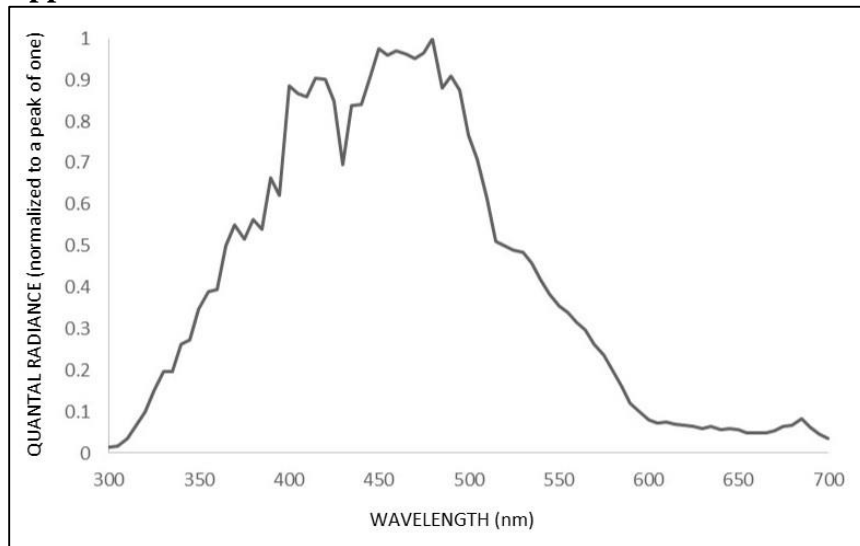


Appendix 1. Radiance Graph. This graph shows the radiance of water just below the surface and inside small crevices. This measurement of radiance was taken at the outer barrier of Yonge's Reef on Lizard Island, Australia, facing away from the reef at 2m depth (Lizard Island, 14°38'S, 145°27'E). Measurements were taken with at noon with an underwater spot spectrophotometer, the "Sub-Spec II". This is a custom housed Ocean Optics USB2000 and PDA palm-top computer. Measurements are limited to a 20˚ solid angle.

## Appendix 2.

Appendix 2. Optical Modeling. The following methods are based on those described in Bettleheim and Siew (1983), but modified so that scattering from an individual sphere is calculated using Mie Theory, rather than Rayleigh-Gans-Debye Theory, for increased accuracy. While the results for the collection of spheres (using the structure factor described in equation (4)) are not as accurate as those that would be obtained using finite element methods, they are computationally far less expensive. Since the goal here is not to determine the exact amount of scattering, but instead to ascertain which approximate combination of sphere size and density that scatters the most light, computational efficiency is crucial.

Suppose one wishes to calculate how much light of wavelength $\lambda$ (*in vacuo*) traveling through a cytoplasmic medium with refractive index $n_{cyto}$ is scattered into an angle $\theta$ by a small

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but dense assembly of spheres that each have a radius $a$ and relative refractive index $m$ and together occupy a fraction $\phi$ of the total volume of the medium (*i.e.* the cell). Without loss of generality, one can assume that the total illuminated volume, the intensity of the incident light, and the distance at which the scattered light is measured are all unity. In this case, the scattered intensity at angle $\theta$ is then equal to the Rayleigh Ratio $R_\theta$, which is given by:

$$R_\theta = M(x,m,\theta)S(q)\frac{\phi}{\frac{4}{3}\pi a^3}. \tag{1}$$

The first term $M(x,m,\theta)$ is the intensity of light scattered at angle $\theta$ by a single sphere, calculated using Mie Theory, where $x$ is the size parameter and is given by:

$$x = \frac{2\pi a n_{cyto}}{\lambda}. \tag{2}$$

The second term is the structure factor $S$, which determines how much the light scattered from the various spheres interferes both constructively and destructively, which strongly affects how much scattered light is observed from the assemblage. If

$$q = 4x\sin\left(\frac{\theta}{2}\right), \tag{3}$$

then, using the Percus-Yevick approximation for hard spheres (see Ailawadi, 1980), this structure factor is given by:

$$S(q) = \frac{1}{1-\rho\tilde{c}(q)}, \tag{4}$$

where $\rho\tilde{c}(q)$ is the Fourier transform of the direct correlation function of the spheres and is given by:

$$\rho\tilde{c}(q) = -\frac{24\phi}{q^6}\left[\alpha q^3\left(\sin q - q\cos q\right) + \beta q^2\left(2q\sin q - \left(q^2-2\right)\cos q - 2\right) + \frac{\alpha\phi}{2}\left(\left(4q^3-24q\right)\sin q - \left(q^4-12q^2+24\right)\cos q + 24\right)\right], \tag{5}$$

in which the two coefficients $\alpha$ and $\beta$ depend only on $\phi$ and are:

$$\alpha = \frac{(1+2\phi)^2}{(1-\phi)^4}, \text{ and } \beta = -6\phi\frac{(1+\phi/2)^2}{(1-\phi)^4}. \tag{6}$$

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The last term in equation (1) is simply the number of spheres, given by the volume fraction divided by the volume of an individual sphere.

Now the degree to which a scattering substance reflects light back towards the source of the illumination (and thus appears highly reflective) is given by its angle-weighted scattering $H$. This equals:

$$H = S \cdot (1 - g),\tag{7}$$

where $S$ is the total amount of light scattered in all directions and $g$ is the asymmetry parameter, which equals the average cosine of the scattered light. Since the total amount of scattered light depends on the illuminated volume and here one is interested in determining the relative scattering for spheres of different sizes and packing densities, one only needs to know what $S$ is proportional to. Using standard integration over a sphere, this is:

$$S \propto \int_0^\pi R_\theta \sin\theta\, d\theta,\tag{8}$$

The asymmetry parameter equals:

$$g = \frac{\displaystyle\int_0^\pi R_\theta \sin\theta \cos\theta\, d\theta}{\displaystyle\int_0^\pi R_\theta \sin\theta\, d\theta}\tag{9}$$

Using (8) and (9), one can determine how angle-weighted scattering (*i.e.* reflectivity) $H$ depends on both the radii of the spheres $a$ and their volume density $\phi$ for light of a given wavelength $\lambda$.

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# CHAPTER 2

## Flashing display in *Ctenoides ales*: behavioral function (ultimate analysis)

## Abstract

The "disco" clam *Ctenoides ales* is a unique bivalve that has a vivid flashing display. The proximate question of *how* the photic display of *C. ales* functions has largely been answered (Chapter 1), but the ultimate question of *why* the photic display occurs had not been examined. This chapter explores three hypotheses regarding the function of the light display, including whether it acts as (i) a signal facilitating the recruitment of conspecifics, (ii) a phototaxic prey lure, and/or (iii) an aposematic anti-predator display. Conspecific recruitment was tested by giving *C. ales* visual and/or chemosensory cues of various stimuli, including other *C. ales* (n=82) and the congener *C. scaber* (n=39). Angle of orientation (°) and movement towards or away from the stimulus were tested over two hours. No stimulus significantly affected settlement. Prey luring was tested by measuring the flash rate (Hz) of *C. ales* when presented with a plankton (food) stimulus. The flash rate increased significantly. To test if the flashing in turn induced planktonic phototaxis, plankton density was tested in three experiments. Plankton were exposed to artificial clams with LED lights adhered to both valves (three treatments: LEDs off, on, or flashing) and a flashing playback trial utilizing a tablet display (two treatments: flashing and non-flashing). Finally, plankton density was tested *in situ* in live *C. ales* using SCUBA in Bali, Indonesia (-8'13", 114'65"). Samples were taken in high-light (flashing visible) and low-light (flashing not visible) settings. These three experiments did not show significant differences in plankton density between treatments. The results indicate that the flashing display of *C. ales* did not induce positive phototaxis in plankton. Predator deterrence was tested by identifying potential predators through *in situ* (SCUBA) and video observation. Crustaceans, cephalopods, stomatopods, fish, and echinoderms were all identified. The type of damage each predator caused was linked to the damage witnessed in dead *C. ales* valves that were collected while diving. In the lab, *C. ales* was tested for noxious chemicals including sulfuric compounds through acid tests, mass-spectrometry, and energy-dispersive x-ray spectroscopy. Sulfur was identified in all of the tissues examined, but the specific compound was unidentifiable. In preliminary trials, there was less sulfur in *C. ales* adductor tissue than *C. ales* gill tissue. When "inside" tissue (adductor) was compared against "outside" tissue (mantle, tentacle, and gills), there was significantly more sulfur in "outside" tissues, which are exposed to predators. Predator trials were conducted with the stomatopod *Odontodactylus scyllarus*, which was given choices of tissue types from *C. ales* or the congener *C. scaber*. *O. scyllarus* preferred adductor muscle (internal part of clam) over mantle tissue (external part of clam) in both species. These results suggest predator deterrence may be the function of the flashing behavior.

## Introduction

Potential functions of photic displays in marine species include, but are not limited to, conspecific recruitment, prey luring, and predator deterrence (aposematism). Conspecific recruitment, or attracting mates, occurs in species such as anglerfish (O'Day 1974; Shimazaki

and Nakaya 2003; Munk 1999) and flasher wrasse (Moyer and Shepard 1974). Prey luring is exhibited by several cephalopod species (Voss 1967; Hanlon and Messenger 1996; Johnsen *et al.* 1999; Randall 2005). Aposematism can be seen in marine gastropods (Rosenberg, 1989; Guilford, 1991), brittle stars (Grober 1988), and the blue-ringed octopus (Sheumack *et al.* 1978). These three hypotheses were examined as potential functions for the photic display of the 'disco' clam, *Ctenoides ales* (Finlay, 1927).

Conspecific recruitment was considered as a potential function of *C. ales* flashing, as many species of juvenile bivalves possess light-sensitive eyes during their peidiveliger stage, which precedes settlement (Carriker 1990). Juvenile bivalves are also capable of movement and will preferentially settle near other bivalves of the same species (Bertness and Grosholtz 1985). Field studies showed 60% of *C. ales* specimens were found in groups of 2-4 (n=106), with size differences suggesting that settlement is asynchronous (Dougherty *et al.* 2014).

Prey luring was considered as a potential function of *C. ales* flashing, as *C. ales* is a filter feeder (Gilmour 1964; Gilmour 1974), and some species of plankton exhibit a phototactic response to various light stimuli. Phototaxis to sunlight has been shown in crustacean larvae (McNaught and Hassler 1964) and many other species of early stage pelagic invertebrates. (Thorson 1964). Phototaxis can also be induced in the lab using red/yellow light (Martynova and Gordeeva 2010), green light (Barnes 1972), or blue light (Kim *et al.* 2005). It has been demonstrated that some filter feeding bivalves can preferentially ingest certain types of prey (Pace *et al.* 1998; Horsted *et al.* 1988), suggesting the possibility that other filter feeders such as *C. ales* could also be capable of preferential ingestion.

Aposematism was considered as a potential function of *C. ales* flashing, as many potential predators have the ability to detect the display of *C. ales*. Some crustaceans and fish have flicker fusion frequency thresholds from 13 – 75 Hz (Frank 2000; Frank 2003; Horodysky *et al.* 2008; Horodysky *et al.* 2010), which is well above the 2 – 4 Hz that *C. ales* flashes (Dougherty *et al.* 2014). If the 1mm-wide flashing mantle edge is viewed from a distance of 100 mm, it subtends an angle of approximately 0.5˚, which is visible to many reef fish, cephalopods, or sharp-eyed crustaceans (Land and Nilsson 2002). The flashing and movement, which widens the display beyond 1mm, may also allow animals with less acute vision to view the display, especially in close proximity. The most common forms of aposematism involve prey that harm the predator after ingestion through toxicity or unpalatability (Mappes 2005), and the warning signal prevents subsequent predation events (Guilford 1991; Tullrot 1994; Rosenberg 1989; Grober 1988). The visibility of the flashing to predators of *C. ales* suggest it could serve as a predator deterrent. These three hypotheses were tested in the laboratory and in the field in order to deduce the function of the photic display of *C. ales*.


## Materials and Methods

## Conspecific Recruitment

<u>Visual stimuli</u>
The conspecific recruitment hypothesis was tested by analyzing the clam's visual and chemosensory reaction to other *C. ales*. Ten 100L tanks were divided in half using barriers. The barriers were either transparent (allowing visual cues) or opaque (not allowing visual cues). They either permitted water flow through holes in the barrier (allowing chemosensory cues), or did not

permit water flow (not allowing chemosensory cues). On the first side of the tank ("stimulus side"), a stimulus was centered 1cm from the barrier and 8cm from each side of the tank, and enclosed to prevent it from moving (Figure 1). The stimuli included live *C. ales* (visual cues, chemosensory cues, or both), the non-flashing congener *C. scaber* (visual cues, chemosensory cues, or both), video playback of *C. ales* flashing (chemosensory cues), a rock, nothing, and water outflow (Table 1). The second half of each tank was the experimental half, which housed live *C. ales* (N=29). The live *C. ales* were centrally positioned 8cm vertically from the barrier and 8 cm horizontally from each side, directly facing the stimulus (0°). Each individual *C. ales* was used only once on the experimental side. Adult clams (>4cm shell width) were utilized for this study, as it was not possible to use juveniles due to both the inability to spawn the clams in a laboratory setting as well as the inability to collect juveniles from the field.

Each tank was surrounded with white cardboard to block external stimuli. Pumps were turned off for the duration of the trail to eliminate effects of water flow on the orientation or movement of the experimental clam (with the exception of tank 10). Each tank was checked once per hour for two hours, and the experimental clam's quadrat location, distance from the stimulus, angle from stimulus and change in angle was recorded. Whether clams were fully-open, half-open or closed was recorded. In order for a trial to be used, both clams could not be closed during either hour. Also, both clams could only be half-open for one hour. Experiments were conducted under fluorescent room lighting. The angle and distance (hypotenuse) of the experimental clam from the stimulus were analyzed using a one-way ANOVA.

## Prey Luring

Feeding trials
The prey luring hypothesis was first tested by analyzing whether the clam's flash rate changed in response to plankton (food). Changes in the flash rate (Hz) of *C. ales* were measured by providing two stimuli: water (control) and food (Reef Nutrition Phyto-Feast®). A 37.9L saltwater tank was covered in black boarding to exclude any other external stimuli. Two holes were fitted in the boarding; one for an Appollo Health GoLite P1© light to increase flash visibility, and one for a Sony Cybershot® DSC-W7 Digital Camera to record the flash rate. A 10mL syringe was fitted with 25cm of syringe tubing which was fed into the water ≤2cm from *C. ales*. The syringe delivered 1mL of water (n=9) or 1mL of food (n=18) to *C. ales.* The flash rate was recorded for a duration of 5 seconds before and after each stimulus. Pre and post stimulus flash rates were compared using a Mann-Whitney analysis.

Plankton and seawater collection
Plankton samples were collected using two Wildco Fieldmaster® 5" 80μm nets with 125mL collection bottles. Nets were attached to rope weighted with 4kg and towed 8m below the boat for 5 minutes at a speed of 20m/min. Seawater samples were collected in 2L plastic containers <1m below the surface at the position the nets were submerged. Collection was conducted 100 ±10m offshore in Pemuteran, Bali, Indonesia (-8'13", 114'65"). Plankton samples were used in lab trials <4 hours after collection, and were observed with a light microscope to confirm viability prior to experimental onset.

Artificial clam trials
*C. ales* valves were size matched and adhered so that the ventral opening of the shells was 2.0 ±
0.5cm. A 1.4m LED wire string with submersible flashing lights (SKU 2021001771, Sparkles
Make it Special®) was adhered to both valves along the interior pallial line. Six total artificial
clams (using 12 valves) were created. A 3L plastic bowl was filled with 300mL of collected
plankton and 1.5L of seawater (see plankton and seawater collection methods). The six artificial
clams were equally spaced around the circumference of the bowl with the ventral opening
aligned in the same angle towards the center. Two artificial clams had LEDs off, two had LEDs
on, and two had LEDs flashing (Figure 2). After one hour, 10mL samples were extracted from
inside each of the six artificial clams using 10mL syringes. Trials were conducted under Appollo
Health GoLite P1® lighting. The experiment was repeated four times (n=8 off, n=8 on, n=8
flashing). Results were analyzed using a correlated one-way ANOVA.

Flashing playback trials
A 1.26L transparent plastic rectangular container was filled with 500mL of seawater and 200mL
of collected plankton (see plankton and seawater collection methods). The container was placed
on top of a tablet that looped playback of a video developed by Y. Zeng at the University of
California, Berkeley, using a custom-made MatLab script (MATLAB 8.0 and Statistics Toolbox
8.1, The MathWorks, Inc., Natick, Massachusetts, United States). The video simulation was used
to test the plankton's response to flashing versus non-flashing patterns. The video had two white
parallel lines in contrast to a red background, which were sampled from images of *C. ales*. One
line flashed with a changing luminance profile based on sinusoidal curves at 1.65Hz, and the
other line had a consistent luminance. Two 100mm x 15mm petri dishes were placed in the
center of the container over each flashing line (Figure 3). The tablet was an Asus Nexus® 7
model NE370T Android version 5.0.2 with a 9.5cm x 15cm screen that used IPS TFT and LED
backlight with a resolution of 1920 x 1200 pixels. The video was looped using the Video
Looper® Version 2.7 Android Application. The two lines in the video were 6cm apart, and each
line was 0.1cm wide and 5.5cm long. After 1 hour, 10mL syringes were used to extract 10ml
samples from directly above each line inside the two petri dishes. Trials were conducted under
Appollo Health GoLite P1® lighting. Results were analyzed using paired t-tests.

In situ extractions
Study sites were established on two $5 \pm 1m^2$ coral formations at depths of 19-23m and
extractions were conducted using SCUBA equipment following the American Association of
Underwater Scientists (AAUS) guidelines. *C. ales* (N=22) were marked with numbered flagging
tape. Water extractions were taken using paired 10mL or 20mL syringes with 2cm plastic pipette
tip extensions. The paired syringes were attached 10cm apart from one another using flagging
tape. The paired syringes were numbered to correspond with the clam for which they were used.
Two 10mL water extractions were taken twice daily from each *C. ales* for four total daily
extractions per individual. The first set of samples was taken during a high-light setting (10AM
±30 minutes) - one sample from inside the open valves of the clam (high-light clam) and one
sample 10cm away from the clam (high-light control). The second set of samples was taken
during a low-light setting (5:30PM ±30 minutes) - one sample from inside the open valves of the
clam (low-light clam) and one sample 10cm away from the clam (low-light control). Samples
were taken from the same individual twice daily to compare plankton concentration during high
light, when the flashing is visible, to plankton concentration during low light, when the flashing

is not visible. Control samples were taken 10cm away from the clam to analyze whether plankton concentration varied as a result of mantle movement, which helps facilitate filter feeding (Galtsoff 1964; Morton and Yonge 1964; Owen 1966). Mantle movement occurs in both high and low light settings, ensuring flashing was the dependent variable in high-light clam vs. low-light clam sample comparisons. Control samples were taken in high-light and low-light to analyze whether plankton concentration varied as a result of diurnal plankton movement (McNaught and Hassler 1964; Thorson 1964; Kim *et al.* 2005). High-light and low-light samples from 17 individual *C. ales* (n=34) were compared to 13 high-light and low-light control samples (n=26) and analyzed using paired or unpaired t-tests.

Preservation and counting
Each 10mL plankton sample was preserved in 4% formalin within 6 hours of collection. Samples were stored in randomly numbered 25mL plastic bottles or double-sealed bags to avoid counting bias. Samples were counted within 2 weeks of preservation. Samples were filtered through 1cm$^2$ Elko© 6µm mesh (1% OA) using 20mL syringes. The mesh was placed on a glass slide with a coverslip and counted using a light microscope for ≤15 minutes per sample at 10x, 40x and/or 100x. Samples were counted independently by two researchers. Count numbers were compared and recorded cumulatively, excluding overlapping taxa. Counting was limited to plankton small enough to be ingested by *C. ales* (excluding organisms >500µm). This size limit was based on prey suitable for the similarly sized zebra mussel (Pace *et al.* 1998) and blue mussel (Horsted *et al.* 1988). Lower size limits excluded plankton <10µm due to microscope limitations and lower motility (Visser and Kiorboe 2006).

For the artificial clam trials, flashing playback trials, and *in situ* extractions, three analyses were conducted including different planktonic groups. Group (1) was "definite", including organisms that were confirmed to be both phototactic and motile. "Definite" organisms included those in the phylum Dinoflagellata (Jahn *et al.* 1963), subphylum Foraminifera (Zmiri *et al.* 1974), genus Nauplius (Forward 1974; Paffenhofer *et al.* 1996), genus Daphnia (Ebert 2005; McNaught and Hasler 1964), and subclass Copepoda (Alcaraz and Strickler 1988; Martynova 2010). Group (2) was "potential", including all organisms classified as definite as well as organisms that were confirmed to be motile but had varying levels of phototaxis across species. "Potential" organisms included those in the phylum Cyanobacteria (Nultsch 1973), division Chlorophyta (Hegemann 2008) and larvae from class Polychaete (Chia *et al.* 1984; Adandt *et al.* 2002) in addition to "definite" taxa. Group (3) was "total", including all planktonic groups present even if motility and phototaxis was not evident: "Total" included all "definite" and "potential" groups, as well as and subphylum Radiozoa, which has very limited motility (Matsuoka 2007), and class Diatomea (Cohn and Weitzell 1996) in which only pennate organisms exhibit motility, but require substratum, making motility unrealistic when considering a filter-feeding organism (Lind *et al.* 1997).

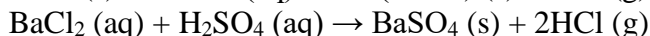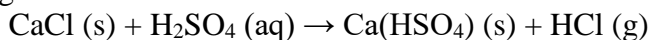## Predator Deterrence

Looming trials
The predator deterrence hypothesis was first tested by analyzing the clam's reaction to a potential predator. A looming stimulus (white 25cm x 25cm Styrofoam lid) was moved toward the clam and the flash rate was analyzed 5s before and after exposure. Experiments were conducted in a ten-gallon tank with black boards surrounding all sides to block external stimuli,

except the front which was used for the stimulus and video recording (Sony Cybershot® DSC-W7 Digital Camera). The looming stimulus was moved quickly (±2s) toward the clam 30s after video recording began. It was kept in place for 30s and then moved quickly back to its starting position. The flash rate (Hz) was calculated 5 seconds prior to and after the stimulus being moved toward the clam. Results were analyzed using a paired t-test.

Sulfur detection
To test if *C. ales* contained or secreted sulfuric acid, such as certain marine gastropods (Fange and Lidman 1976), water samples were compared from "calm" and "disturbed" clams. *C. ales* specimens (N=20) were placed in individual 200mL jars with 100mL of sterile sea water. To collect the calm samples, clams were allowed to acclimate for 1 hour after which 10 mL water samples were collected. To collect the disturbed samples, the same 20 C. ales specimens were placed in new 200mL jars with 100mL of sterile sea water and a predatory event was simulated for 5 minutes (clams were pried open, squirted with water, hit or clamped with metal sticks). 10mL water samples were taken from each of the calm and disturbed clams. Calm and disturbed water samples were split into two 5mL samples and were reacted in 19mL test tubes with 5mL of 1M calcium chloride (CaCl) solution and then 5mL of 1M barium chloride (BaCl$_2$) solution, predicting the following reactions:

$$CaCl \text{ (s)} + H_2SO_4 \text{ (aq)} \rightarrow Ca(HSO_4) \text{ (s)} + HCl \text{ (g)}$$
$$BaCl_2 \text{ (aq)} + H_2SO_4 \text{ (aq)} \rightarrow BaSO_4 \text{ (s)} + 2HCl \text{ (g)}$$

All solutions were centrifuged for 10 minutes on speed 7 using an International Clinical Centrofuge. Reagent was pipetted out of solution and all precipitates were allowed to dry inverted for one minute. Precipitates were weighed and mass was compared using a t-test. The excess of calm and disturbed water samples were tested for pH using 5 drops of methyl orange ($C_{14}H_{14}N_3NaO_3S$).

Scanning electron microscopy and energy dispersive x-ray spectroscopy to determine sulfur level
Two pieces of $1mm^2 \pm 0.5mm$ tissue was taken from the adductor muscle, tentacles, gills, and mantle tissue of *C. ales, C. scaber,* and *C. mitis*. Tissues were preserved in 2.5% glutaraldehyde and frozen at -18°C. For microscopy, tissues were rinsed in a 0.1M sodium cacodylate buffer (pH 7.2) three times for 5 minutes each. Tissue was dehydrated using 10 minute rinses of 35%, 50%, 70%, 80%, 90%, and 100% (2x) ethanol, and dried using a critical point dryer. Samples were mounted on disks using carbon tape and sputter coated with carbon. Samples were analyzed using a Zeiss EVO Variable Vacuum Instrument -10 SEM with an energy-dispersive X-ray detector (EDS). An EDAX system for chemical analysis was used to analyze the chemical composition of clam tissue. Measurements were taken at six different locations on each tissue sample. Average net intensity of sulfur was compared across samples to determine sulfur level.

Predator identification for predator trials
Dead *C. ales* valves were collected while SCUBA diving. The damaged shells were classified into breakage types, photographed, and recorded. The type of shell damage was compared to the literature to determine the type of predator that caused the damage.

Predator trials
The predator deterrence hypothesis was further tested by analyzing the reaction of potential predators to the clam. The predator *Odontodactylus scyllarus* (peacock mantis shrimp, N=18)

was used, which has an overlapping habitat with *C. ales*. Each *O. scyllarus* was used in no more than 3 trials. In the first experiment, *O. scyllarus* was given a choice between *C. ales* mantle tissue and *C. ales* adductor tissue (n=12). The tissue was presented 10cm directly in front of the burrow, and 5cm from the base of the tank. The tissues were skewered on two sticks distanced 5cm apart. There were four possible outcomes; *O. scyllarus* would eat only the mantle tissue, only the adductor tissue, both tissues, or neither tissue. In 4/12 trials, *O. scyllarus* was not fed for an additional two days to assess preference when hungry. In the second experiment *O. scyllarus* was given a choice between the congener *C. scaber* mantle tissue and *C. scaber* adductor tissue (n=11). In 10/11 trials, *O. scyllarus* was not fed for an additional two days to assess preference when hungry. In the third experiment, *O. scyllarus* was given a choice between *C. ales* adductor tissue and *C. scaber* adductor tissue (n=9) to assess species preference. In the fourth experiment, *O. scyllarus* was given a choice between *C. ales* mantle tissue and *C. scaber* mantle tissue (n=5) to assess species preference and whether preference correlated to measured sulfur levels.


## Results

## Conspecific Recruitment

Visual stimuli
The distance each experimental clam moved toward or away from the stimulus (hypotenuse), and the angle of the experimental clam towards the stimulus were compared using a one-way ANOVA. There were no significant differences for the movement (hypotenuse) toward or away from the stimulus under any treatment (Figure 4): $F_{(8, 35)}= 1.2194$, p=0.2883. There were no significant differences under any treatment for the angle of orientation (Figure 5): $F_{(8, 35)}= 1.3099$, p=0.2395.

## Prey Luring

Feeding trials
There was no significant difference in the 5s before and after the water (control) stimulus (n=9, P=0.22, Mann-Whitney). There was a significant difference in the 5s after the food (Reef Nutrition Phyto-Feast®) stimulus, as the average flash rate increased from 1.6Hz ±0.6 to 2.2Hz ±0.6 (Figure 6a) (n=18, P=0.003, Mann-Whitney).

Artificial clam trials
The number of plankton collected after 1 hour from the artificial clams with lights turned off, on, or flashing were compared using a one-way ANOVA. There were no significant differences in the number of plankton found under any treatment; definite (n=8): $F_{(2, 21)}= 1.9060$, P=0.1735, potential (n=8): $F_{(2, 21)}= 1.6708$, P=0.2121, and total (n=8): $F_{(2, 21)}= 0.3784$, P=0.6836.

Flashing playback trials
The number of plankton collected after 1 hour from the petri dishes above the video playback of constant and flashing lines were compared using a paired t-test. There were no significant differences between the number of plankton collected from the flashing or non-flashing lines; definite (n=7), P=0.1437, potential (n=7), P=0.1942, and total (n=7), P=0.2325.

No significant differences were found when comparing high-light control samples to low-light control samples using a paired t-test; (n=13) definite P=0.5000, potential P=0.7556, and total P=0.2493. No significant differences were found in the high-light clam vs. high-light control samples using a paired t-test; (n=13 control/17 clam) P=0.05770, potential P=0.4682, and total P=0.5970. Two of the three low-light clam vs. low-light control samples showed no significant difference; potential P=0.8509, and total P=0.5137. The third showed significantly more plankton in the control sample; definite (n=13 control/17 clam), P=0.0429. There were no significant differences in the high-light clam samples vs. low-light clam samples using a paired t-test; (n=17) definite P=0.0869, potential P=0.3955, and total P=0.3162.

## Predator Deterrence

Looming trials
The mann-whitney analysis of the clam's reaction to a predator showed a significant increase in flash rate (Hz) in the 5 seconds before and after the stimulus (p = 0.0001, n=18) (Figure 6b).

Sulfur detection
Calm and disturbed water samples reacted with 5mL of 1M CaCl did not form a measurable precipitate. The mass of precipitates from calm (n=8) and disturbed (n=12) water samples reacted with 5mL of 1M BaCl were compared using a t-test. There was no significant difference in precipitate mass (P=0.8476). There were no measurable pH changes in the water samples treated with methyl orange.

Scanning electron microscopy and energy dispersive x-ray spectroscopy to determine sulfur level
Using ANOVAs and tukey HSD analysis, net intensity of sulfur was significantly lower in *C. ales* adductor muscle than *C. ales* gills (P=0.0093), *C. scaber* tentacle (P<0.0001), *C. scaber* mantle (P=0.0038), *C. mitis* tentacle(P=0.0001), *C. mitis* gills (P=0.0176). Net intensity of sulfur was significantly lower in *C. mitis* adductor muscle than *C. mitis* tentacle (P=0.0059), and C. scaber tentacle (P=0.0012). Net intensity of sulfur was significantly lower in *C. scaber* gills than *C. scaber* tentacle (P=0.0285) (Figure 7). The net intensity of sulfur was significantly lower in the "inside" tissues (adductor muscle and gills) than the "outside" tissues (mantle and tentacles) in *C. mitis* (P=0.0168) and *C. scaber* (P=0.0006) but not in *C. ales*. When gills were considered as "outside" tissues along with mantle and tentacles, the net intensity of sulfur was significantly lower in the "inside" tissues than the "outside" tissues in *C. ales* (P=0.0151) and *C. mitis* (0.0002) but not in *C. scaber*.

Predator identification for predator trials
Collection of dead *C. ales* valves underwater using SCUBA equipment resulted in four types of valve damage: ventral, dorsal, broad, or circular/oblong holes (Figure 8). Ventral damage can occur when crustaceans break away the margins of the valve with their claws and then pick out the flesh. Large crustaceans can also cause broad damage by crushing the valve with their claws (Carter 1968). Crustaceans identified during SCUBA surveys included the hermit crab *Dardanus pedunculatus*, the slipper lobster *Parribacus spp.*, and the crabs *Thalamita spp., Dromia dormia,* and *Carpillus convexus*. Dorsal damage could have been the result of stomatopod predation, as similar breakage patterns were observed in laboratory trials with *O. scyllarus*. Broad damage

resulted when the valve was crushed, most likely by the strong teeth of fish (Carter 1968). The fish identified during SCUBA surveys included the pufferfish *Arothron nigropunctatus*, the porcupinefish *Diodon liturosus*, and the triggerfish *Balistoides viridescens, Melichthys visua*, and *Odonus niger*. Holes were found in two forms – circular and oblong. Holes are the result of several marine invertebrates, including marine gastropods, which use both mechanical and chemical activity to bore through the valve with their radula (Carter 1968). Octopus can also bore holes through valves although the holes are smaller than gastropod holes, and oval rather than circular. Octopus can also pry apart the valves with their suckers (Carter 1968), which would result in undamaged valves. The octopus *Octopus cyanea* was found during SCUBA surveys, but no bivalve-feeding gastropods were recorded. Undamaged valves could also be the result of echinoderm feeding, in which extraoral feeding opens the valve without damage (Carter 1968).

Predator trials
In the first experiment, the predator *Odontodactylus scyllarus* (peacock mantis shrimp) was given a choice between *C. ales* mantle tissue and *C. ales* adductor tissue (n=12) assuming unequal sulfur levels between tissues. *O. scyllarus* ate both tissues in 6/12 trials, only adductor tissue in 6/12 trials. In 4/12 trials, *O. scyllarus* was not fed for an additional two days, and both tissues were eaten in 4/4 trials. In the second experiment, *O. scyllarus* was given a choice between the congener *C. scaber* mantle tissue and *C. scaber* adductor tissue (n=11). *O. scyllarus* ate both tissues in 7/11 trials, only adductor tissue in 4/11 trials, and never ate only mantle tissue. In 10/11 trials, the mantis shrimp was not fed for an additional two days, and in those trials, both tissues were eaten in 6/10 trials, only adductor was eaten in 4/10 trials. In the third experiment, *O. scyllarus* was given a choice between *C. ales* adductor tissue and *C. scaber* adductor tissue (n=9). Both adductor tissues were eaten in 9/9 trials. In the fourth experiment, *O. scyllarus* was given a choice between *C. ales* mantle tissue and *C. scaber* mantle tissue (n=5). *O. scyllarus* ate both tissues in 2/5 trials, only *C. ales* tissue in 1/5 trials, only *C. scaber* mantle tissue in 1/5 trials, and neither tissue in 1/5 trials. In all four experiments (n=37), *O. scyllarus* never ate only mantle tissue, regardless of species.

## Discussion

## Conspecific Recruitment
If the photic display of *C. ales* were a signal facilitating the recruitment of conspecifics, areas in which *C. ales* organisms were already present would recruit more *C. ales* than areas in which *C. ales* were absent, given the two habitats had similar biotic and abiotic conditions. *C. ales* would be expected to react more to conspecifics than congener species. The visual cues of a live *C. ales* or video playback of *C. ales* flashing did not alter the settlement position of the experimental *C. ales*, nor did it alter the angle the experimental *C. ales* oriented toward the stimulus. This suggests that visual cues do not influence settlement position in the adult *C. ales* used in this study.

## Prey Luring
If the photic display of *C. ales* were a lure for phototaxic prey, higher plankton concentrations would be found in water with flashing displays (visible in high light, not visible in

low light). The prey luring experiments were designed to test plankton phototaxis in three conditions; *in situ* plankton concentrations in natural ambient light where *C. ales* flashing was visible and not-visible (*in situ* extractions), controlled lab trials with highly concentrated plankton highlighting the morphology of *C. ales* (artificial clam trials), and a display designed to mimic the spectra and Hz of *C. ales* (flashing playback trials). Although the flash rate of *C. ales* increased in response to plankton, the flashing display did not result in significantly higher levels of plankton. Based on the results of these three experiments, we did not find any evidence of plankton phototaxis toward the light display of *C. ales*.

The flashing display may not be visible to plankton. There are no measurements of plankton contrast threshold or spatial acuity in the literature, and although the former may be fairly good, the latter is almost certainly very weak (S. Johnsen, pers. comm.). Absolute visual thresholds have been measured for plankton, although they are not the best gauge for object detection. Their values are higher than larger animals, suggesting the flashing is more visible to potential predators of *C. ales* than to prey. Absolute threshold for phototactic algae range from $5.0 \times 10^{13}$ to $1.8 \times 10^{17}$ photons $m^{-2} s^{-1}$ (Foster and Smyth 1980), foraminifera range from $1.0 \times 10^{15}$ to $1.0 \times 10^{19}$ photons $m^{-2} s^{-1}$ (Zmiri *et al.* 1974), copepods range from $1.4 \times 10^{11}$ to $1.0 \times 10^{14}$ photons $m^{-2} s^{-1}$ (Forward 1988, Cohen and Forward 2002), and the annelid larvae *Platynereis dumerilii* (Audouin & Milne Edwards, 1834) was measured at $5.0 \times 10^{14}$ photons $m^{-2} s^{-1} sr^{-1}$ (Randel and Jékely 2016). Larger animals such as fish, crustaceans and cephalopods have lower thresholds, ranging from $10^9$ to $10^{11}$ photons $m^{-2} s^{-1} sr^{-1}$ (Dusenbury 1992).

Flashing, especially at low frequencies, is not as effective in eliciting phototaxis in plankton as constant light (G. Jékely, pers. comm.). Plankton size may also be a limiting factor, as the experiment was constrained to plankton small enough to be ingested by *C. ales* (see plankton and seawater collection methods), but larger plankton are more likely to exhibit phototaxis (Clarke 1934; Jékely *et al.* 2008). Smaller sensory organs are also less powerful than larger ones, as size increases resolution through focal length, aperture, and light capture for increased sensitivity (Land and Nilsson 2012). The radiance of *C. ales* is unknown, but prior experiments confirming phototaxis in plankton utilized light sources that are larger and likely brighter than the 1mm display of *C. ales*, including sunlight (McNaught and Hassler 1964; Thorson 1964) which has a radiance of $10^{20}$ photons/$m^{-2} s^{-1} sr^{-1}$, or artificial lighting at various wavelengths (Barnes 1972; Zmiri *et al.* 1974; Martynova and Gordeeva 2010), which, if equivalent to "room light", has a radiance of $10^{17}$ photons/$m^{-2} s^{-1} sr^{-1}$ (Land and Nilsson 2012).

## Predator Deterrence

If the photic display of *C. ales* is an anti-predator display, then the presence of predators would influence the flash rate (Hz) and/or exposed display area, and the clam would contain some distasteful element. The presence of a fake predator significantly increased the flash rate, and sulfur was detected within the tissues. The type of sulfuric compound was unidentifiable using mass-spectrometry, and tests for sulfuric acid were negative. However, the varying levels of sulfur between tissues could be representative of their accessibility to predators. The adductor muscle of *C. ales*, which is internal and only accessible to predators when the shell is crushed or pried open, had significantly less sulfur than the gills, which are sometimes exposed, but it did not have significantly less sulfur than the mantle or tentacles, which are external and more easily accessible to predators. These results may be impacted by the low N value (N=1 for each tissue type) and from uneven surface measurements (n=6 measurements per tissue). Both of these issues will be remedied in future studies by using 10 organisms of each species to take the four

types of tissue samples (adductor, mantle, tentacles, and gills). Uneven surfaces will be remedied by embedding tissue samples in epoxy and using a microtome to cut level surfaces through the tissue.

Sulfur was also detected within the tissues of the congener *C. scaber*. Since both clams are red, the reason for *C. ales* flashing may be to augment its aposematism in a more complex environment. *C. ales* live in a much more cryptic environment than *C. scaber*, whose red coloration is more conspicuous among rocks and rubble (Jacobson, 1973). The 1mm-wide flashing display of *C. ales* is visible to many reef fish, cephalopods or sharp-eyed crustaceans (Land and Nilsson 2002), which were all confirmed as potential predators during field studies. Sulfur compounds are used as a chemical defense in certain organisms, including guava leaves (against insect predation) (Roussef, 2008), lucinid marine bivalves (Anderson, 1995), and marine gastropods (Rosenberg, 1989). The presence of sulfur in the tissues of *C. ales* could serve as a chemical defense, but may also be the result of sulfur-reducing bacteria, which will be explored in post-doctoral research. Of the three hypotheses, predator deterrence is the only function with supporting evidence.

## Acknowledgements

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## Table Captions

Table 1. Sensory cues, stimuli used, and tank number for recruitment trials testing visual attraction of conspecifics.

## Tables

**Table 1.**

| Sensory Cue | Stimulus | Visual | Chemo. | Tank # (n) |
|---|---|---|---|---|
| Visual Control | Rock | + | - | 3  (n=29) |
| Chemosensory Control | Water flow | - | + | 10 (n=28) |
| Visual/Chemosensory Control | Empty (control) | - | - | 4  (n=32) |
| | | | | |
| Visual Conspecific | Live *Ctenoides ales* | + | - | 1  (n=25) |
| Chemosensory Conspecific | Live *Ctenoides ales* | - | + | 5  (n=25) |
| Visual/Chemosensory Conspecific | Live *Ctenoides ales* | + | + | 2  (n=28) |
| | | | | |
| Visual Conspecific | Live *Ctenoides scaber* | + | - | 9  (n=11) |
| Chemosensory Conspecific | Live *Ctenoides scaber* | - | + | 8  (n=13) |
| Visual/Chemosensory Conspecific | Live *Ctenoides scaber* | + | + | 7  (n=15) |
| | | | | |
| Visual Enhanced Conspecific | Video *Ctenoides ales* | + | - | 6   (n=6) |

# Figure Captions

Figure 1. Tank setup for conspecific recruitment trials. Stimulus at top of tank, experimental *C. ales* at bottom of tank. Barriers and stimuli described in key.

Figure 2. (A) Artificial clam with attached battery-powered waterproof LED lights. (B) Artificial clams in 3L bowl; two flashing, two constant, and two off.

Figure 3. Flashing playback setup, with constant line (left) illuminated and flashing (right) in the dark phase of the on-off sequence. Petri dishes centered over lines.

Figure 4. *C. ales* movement (cm) towards (+) or away (-) from the stimulus (0) when presented with various stimuli (stimuli listed in Table 1).

Figure 5. *C. ales* angle of orientation (°) towards the stimulus (0°) when presented with various stimuli (stimuli listed in Table 1).

Figure 6. (A) Flash rate (Hz) of *C. ales* measured 5s before and after introducing a plankton (food) stimulus. Control (water) stimulus was not significant. (B) Flash rate (Hz) of *C. ales* measured for 5s before and after introducing a "looming" false predator ($25cm^2$ Styrofoam).

Figure 7. Mean sulfur content (net intensity) of the adductor, mantle, tentacle and gill tissues of *C. ales, C. mitis,* and *C. scaber*. Tissues not connected by the same letter are significantly different.

Figure 8. Valve damage types: (A) ventral, (B) dorsal, (C) broad, or (4) holes.
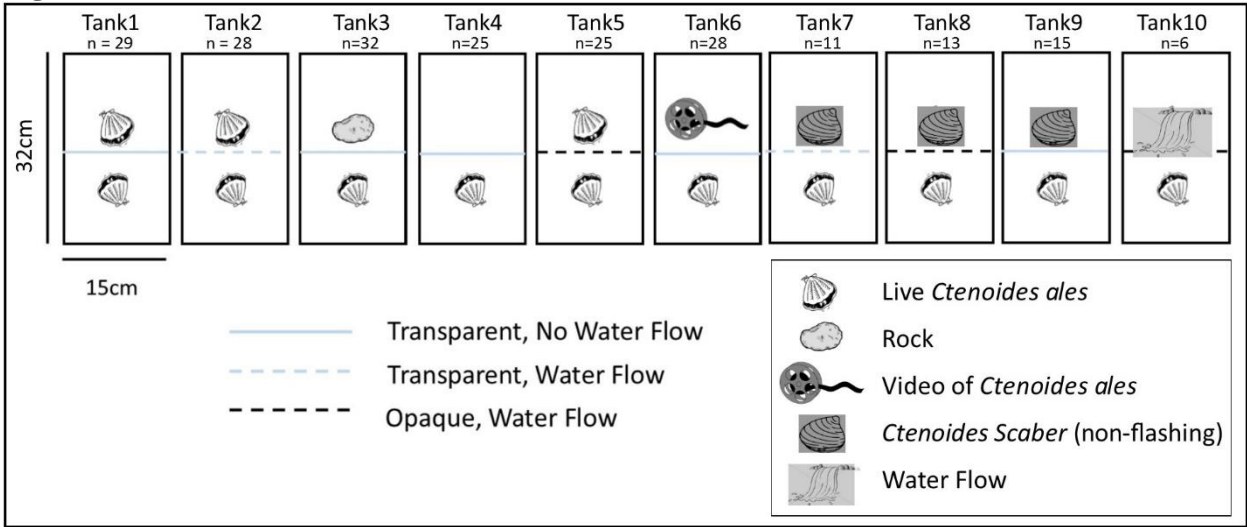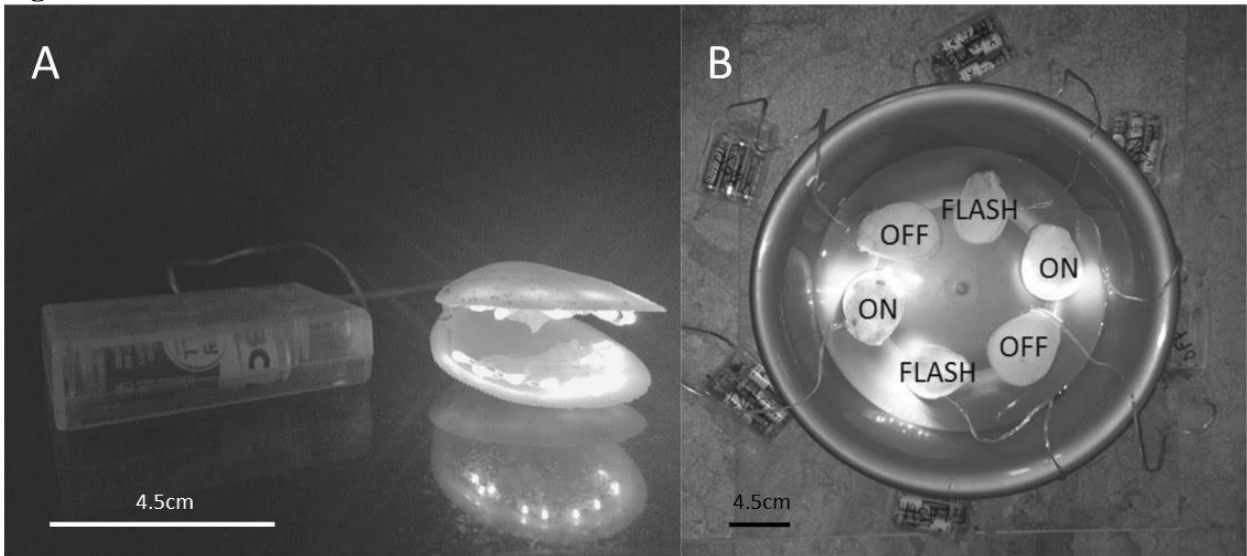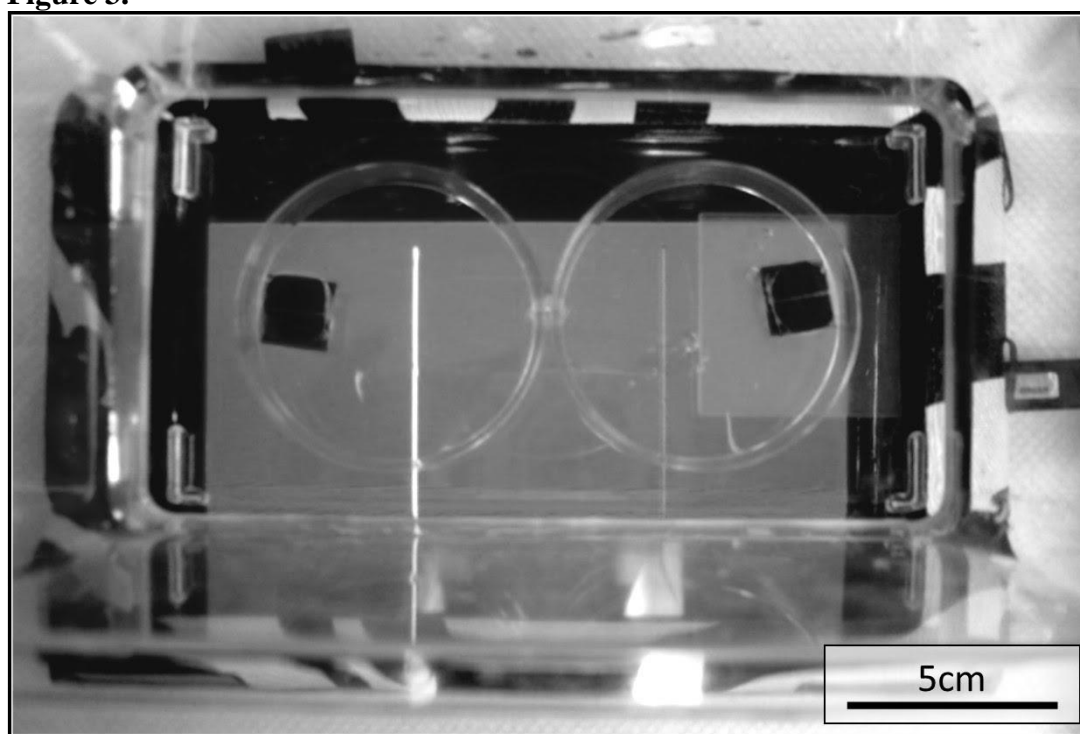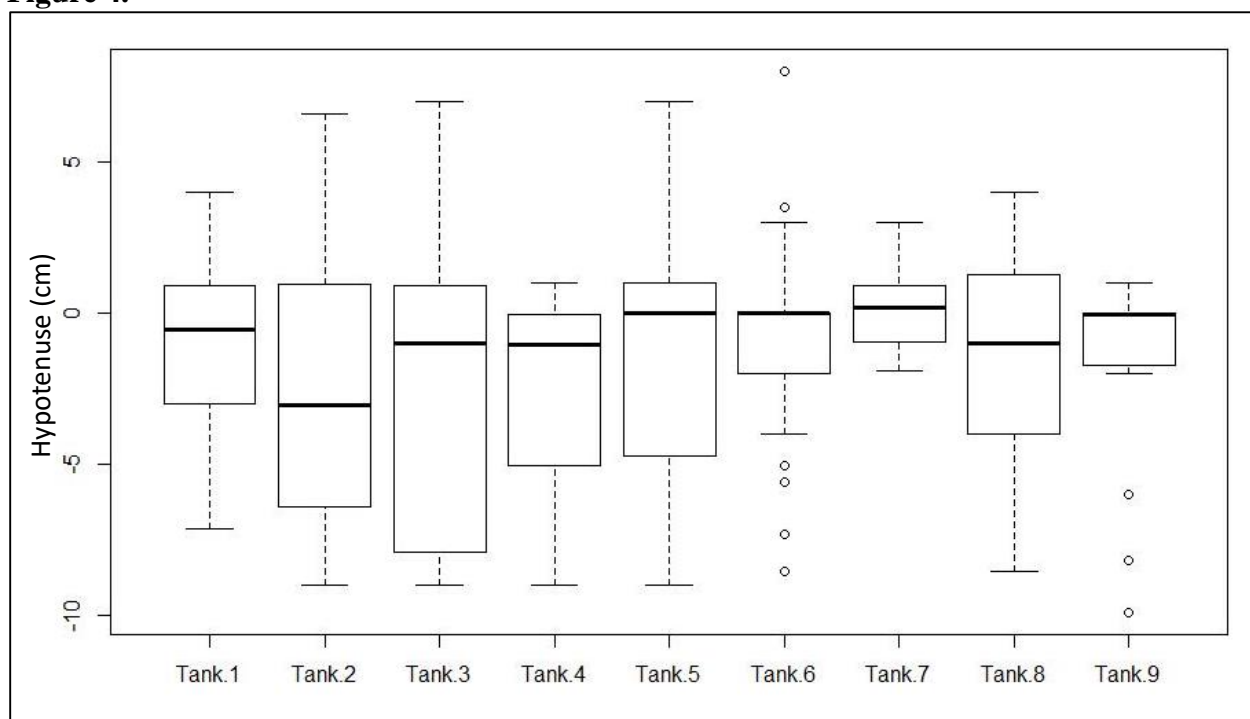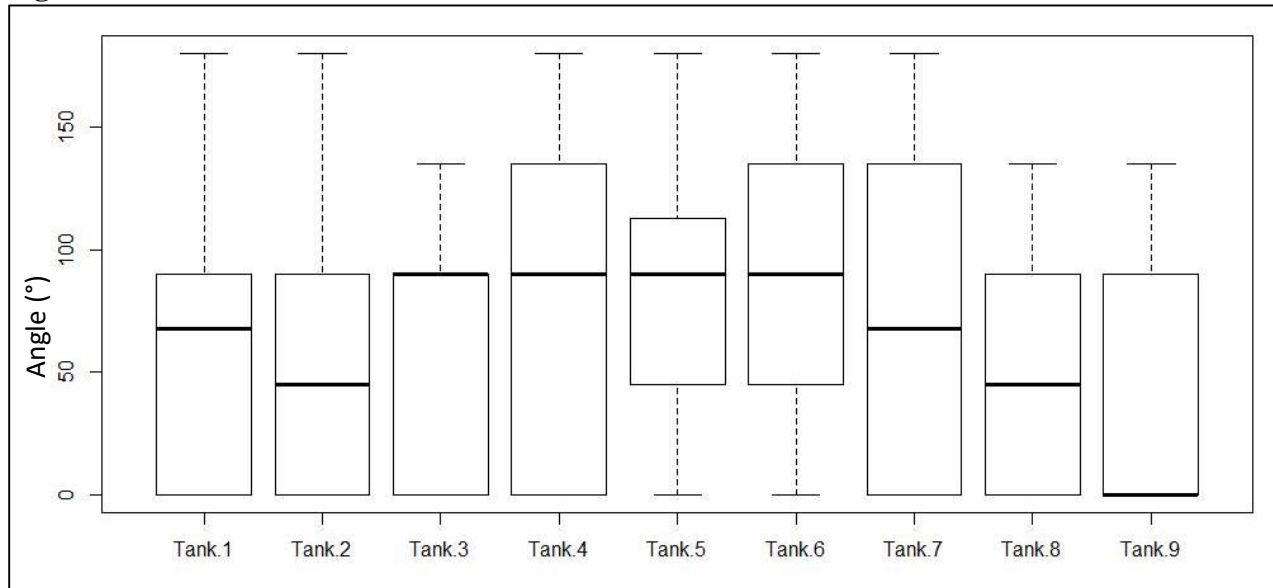
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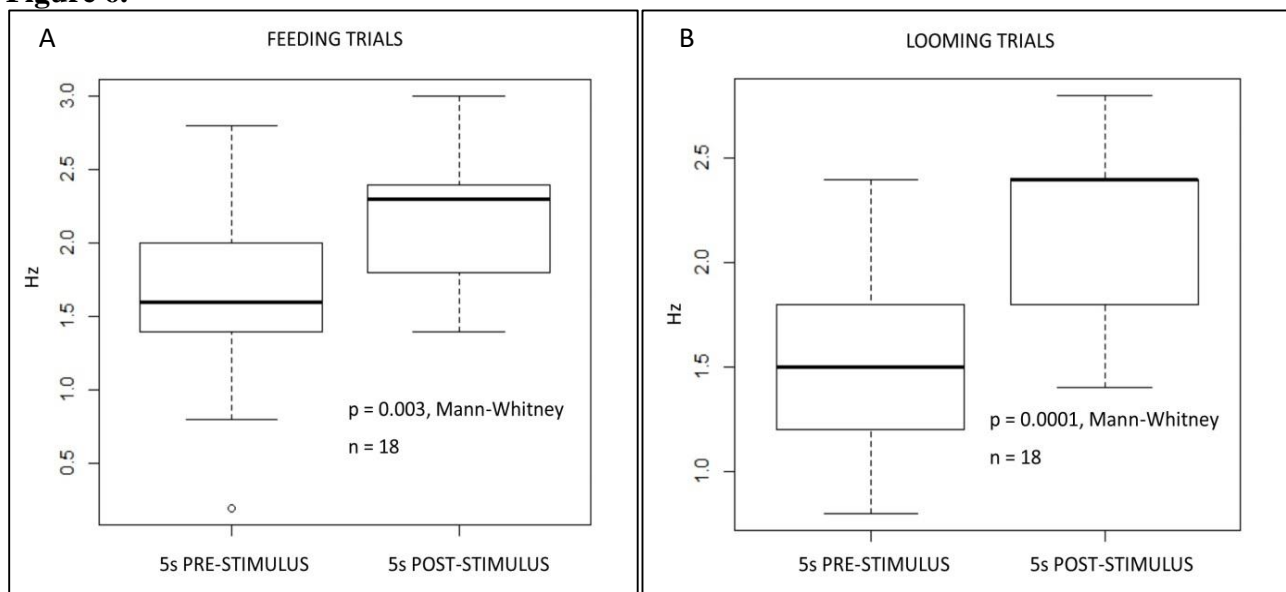
**Figure 1.**
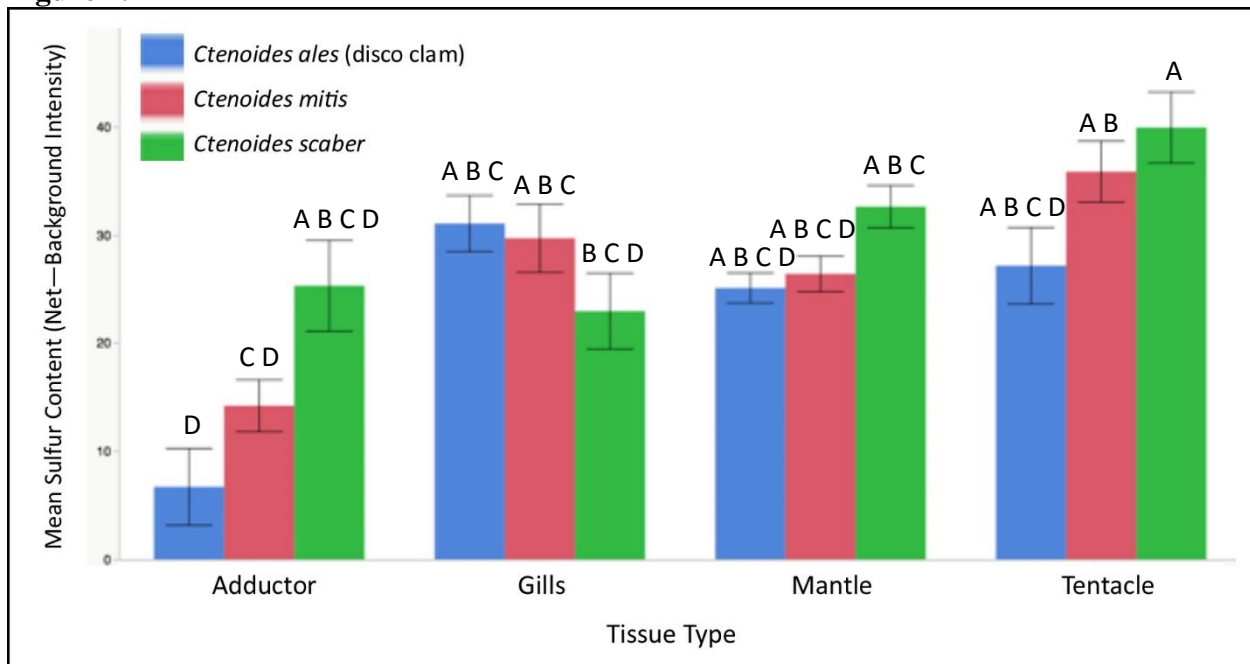


**Figure 2.**

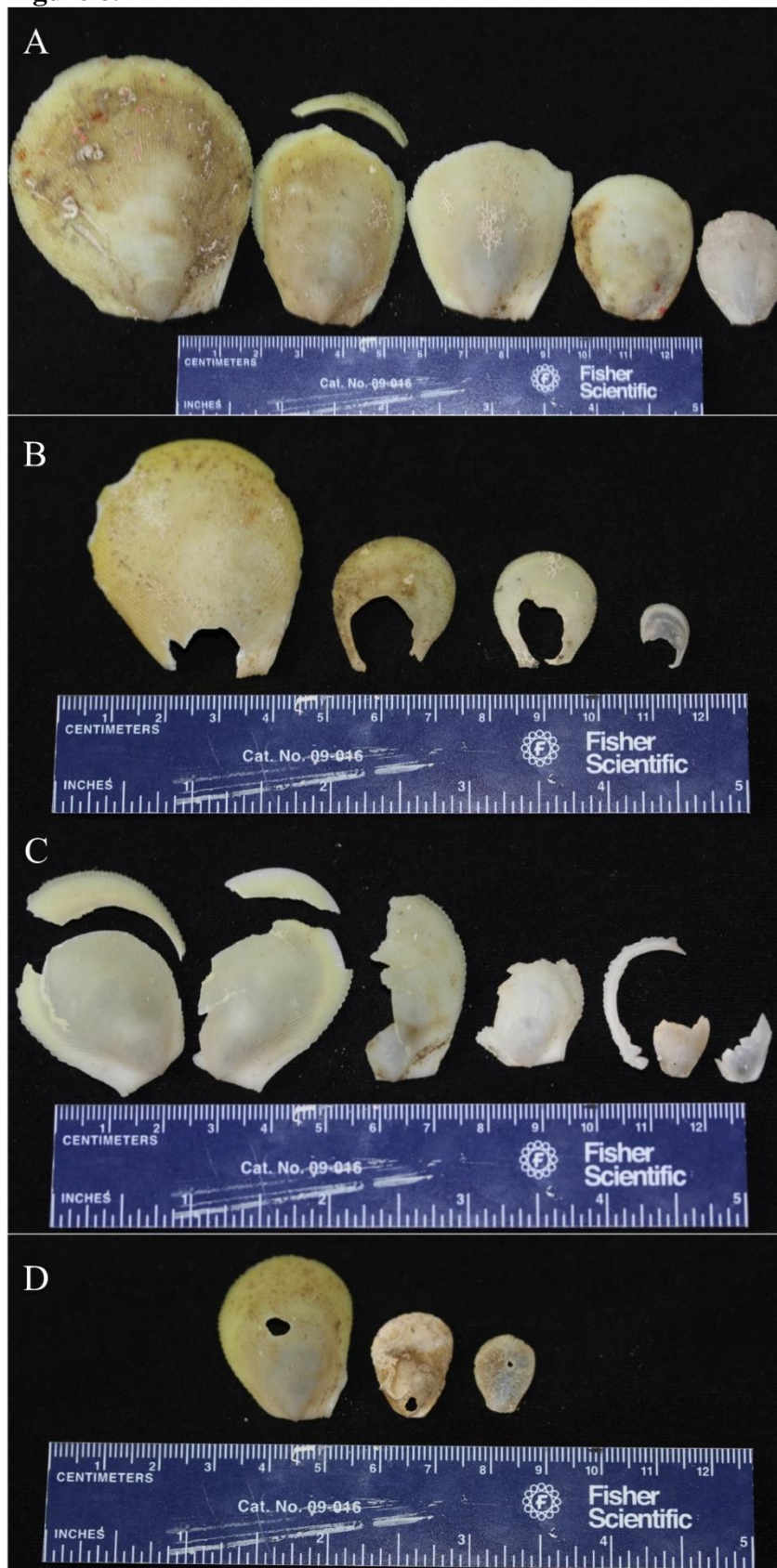**Figure 3.**



**Figure 4.**

**Figure 5.**



**Figure 6.**

**Figure 7.**

**Figure 8.**

# CHAPTER 3

## Optical structure and visual capability of *Ctenoides ales*

## Abstract

The type and number of eyes, as well as visual acuity, varies between taxa among bivalves. Bivalves are relatively immobile, so it is thought that their eyes are used primarily to detect predators. The eyes in the family Limidae have not been investigated as thoroughly as other families such as Pectinidae (scallops), likely because they are smaller, fewer, and hidden within the mantle tentacles. The eyes of *Ctenoides ales* were studied to help understand the role of vision in the function of their unique flashing display, and whether the display was visible to other *C. ales*. Eyes were investigated through flash photography to test for eyeshine. Behavioral experiments were used to test reactions to conspecific flashing or potential predators. Transmission electron microscopy (TEM) was used to determine eye morphology and structure. Fluorescence microscopy was used to look for variations in the location of musculature and A-T rich DNA regions between *C. ales* and the congener *C. scaber*. Immunohistochemistry was used to test for the presence, type, and location of photosensitive proteins. Photography revealed an obvious eyeshine, but the aggregates of yellow pigment behind the retina may be indicative of membrane breakdown and lipofuscin rather than a reflective layer. TEM revealed structure similar to other limid bivalves in the literature. Fluorescence did not reveal obvious differences between *C. ales* and the congener *C. scaber*. Immunohistochemistry suggests the presence of photosensitive proteins and a possible retinal stain. Behavioral experiments in conjunction with findings from microscopy confirm that *C. ales* is likely incapable of image formation or detection of conspecific flashing. The eyes of *C. ales* are more likely used in predator detection.

## Introduction

The eyes of the 'disco' clam *Ctenoides ales*, which has a unique flashing display, were studied to help understand the role of vision and to determine whether the display was visible to other *C. ales* as a signal. The development of vision of numerous taxa, including bivalves, is largely attributed to the Cambrian explosion, in which many macrofauna evolved large eyes (Land and Nilsson 2012). Fossil records from the Burgess shale in Canada showed shallow-water marine fauna which were early representatives of modern animal phyla (Conway-Morris 1998) with compound eyes that superficially resembled those of modern arthropods (Land and Nilsson 2012). Vertebrate eyes clearly date back to a basic eye type in a common ancestor (vertebrate or chordate), but the origins of invertebrate eyes are less clear. Cephalic eyes may all stem from a common ancestor, but extracephalic eyes, such as the mantle eyes in bivalves, must have evolved separately using other pre-existing modes of light detection and neural signaling (Land and Nilsson 2012).

Molluscan eyes are often used as examples of convergent evolution with the complex lens eyes of cephalopods and vertebrates. However, it is more likely that they exhibit both convergent and parallel evolution on various levels, such as morphology, genetic regulatory networks, and photoreceptor cell types (Serb and Eernisse 2008). There is incredible eye diversity within Mollusca – various morphologies include simple eye cups, pit eyes, compound eyes, pinhole eyes, and mirrored eyes. The placement of eyes also varies, including cephalic eyes, mantle eyes, or eyes embedded in shells (Serb and Eernisse 2008). The range of eye size within Mollusca is vast: from <100µm in chitons (Speiser *et al.* 2014) to 25-40cm in giant squid (Roper and Boss 1982; Land 1981; Messenger 1981).

Bivalves are relatively immobile, so it is thought that their eyes are used primarily to detect predators and trigger a defensive response (Nilsson 1994). Most eyes are found nestled within the mantle lining the shell, which are called pallial eyes. The types of eyes vary between taxa. They can be open pit eyes or closed lens eyes, and some species possess multiple types of eyes along the mantle edge, such as ark clams (family Arcidae). Ark clams have both simple cup eyes as well as a compound eye (Patten 1887; Waller 1980; Nilsson 1994), which is similar to an arthropod compound eye but is evolutionarily independent (Nilsson and Kelber 2007). These clams can have as many as 200–300 compound eyes along the mantle edge, but they do not form images of the environment (Nilsson 1994). The number of eyes in bivalves varies widely, with some species having numbers in the tens, such as cockles, where eyes are associated with the siphon (Morton 2001). In contrast, giant clam eyes numbers in the thousands (Wilkens 1986), where eyes sense light (Wilkens 1988; Land 2003) and also direct light to symbiotic algae within the mantle (Fankboner and Reid 1990).

Scallops are one of the most well-known examples of mirror-eyes, which use guanine crystals and a concave mirror to redirect light to the double-retina and can form basic images (Land 1965, 1984). Scallop vision is quite advanced, as they can detect moving objects (von Buddenbrock and Moller-Racke 1953), direct their swimming accordingly (Hamilton and Koch 1996), and adjust the opening of their valves based on the size and speed of particles in the water (Speiser and Johnsen 2008).

The eyes in the family Limidae have not been investigated as thoroughly as other families such as Pectinidae (scallops), likely because they are much smaller, fewer, and more hidden within the mantle tentacles (Morton 2000). Of the ten genera in Limidae, only *Limaria* do not possess eyes (Morton 1979). Whether this is related to their unique defensive mechanism of tentacle autotomy and their inability to fully retract their tentacles to close their shell (Morton 1979), is unknown. Of the remaining genera, only some species possess pallial eyes (Dakin 1928), including *Lima* and *Ctenoides*. Species whose eyes have been studied include *Lima (Ctenoides) scabra* (Dakin 1928; Charles 1966; Bell and Mpitsos 1968), whose eyes are said to resemble those of the superfamilies Arcoida and Limopsoidea (Waller 1980). *Lima squamosal* (Hesse 1900; von Salvini-Plawen and Mayr 1977; von Salvini-Plawen 1982) was described as a pinhole type eye, with similar findings given for *Lima* (*Ctenoides*) *exavata* (Schreiner 1986) and *Ctenoides floridanus* (Morton 2000). *C. floridanus* was described as possessing ~18 eyes at the base of the pallial tentacles with a lens, collagen overlap, cornea, transverse fibers, pigmented cells, vacuolated cells, and an optic nerve (Morton 2000). In this chapter, the eyes of *C. ales* were studied using behavioral trials to assess whether *C. ales* could detect a predator movement

(looming trials), and whether they settled preferentially near one another using visual cues (recruitment trials). Fluorescence microscopy was used to detect differences in musculature and A-T rich regions of DNA between *C. ales* and the non-flashing congener *C. scaber*. Electron microscopy was used to compare the ultrastructure of *C. ales* eyes to other bivalves in the literature. Finally, immunohistochemistry was used to test for the presence and location of light-sensitive pigments. These methodologies aimed to gain a comprehensive view of the visual capability of *C. ales* and whether their visual capabilities relate to their unique flashing display.

## Materials and Methods

### Looming and Recruitment Trials
Live *C. ales* eyes were photographed with a Canon EOS® 60D 18.0 MP SLR - EF-S with a 60mm Canon lens before beginning trials. Photos were taken with and without flash to test for eyeshine, which could signify the presence of reflective tissue. To test the visual acuity of *C. ales*, various visual stimuli were presented and the behavioral response was measured. To test visual response to shadows and edges, changes in flash rate (Hz) was measured when *C. ales* were presented with a simulated predator. To test visual response to conspecifics, changes in settlement (hypotenuse from original placement and angle of orientation towards stimulus) were measured when *C. ales* were presented with another *C. ales* on the opposite side of the tank. Methods are described in Chapter 2.

### Fluorescence Microscopy
Eyes from both *C. ales* and the congener *C. scaber* were dissected using a dissecting scope and were fixed in 4% PFA (paraformaldehyde) in seawater for 20 minutes. Triton X100 (soap) was added to 0.1% for 10 minutes. Specimens were washed 4x for 5 min in 1x PBS (phos. buffered saline) and left overnight at 4°C. Samples were then diluted with 1:200 PA (phalloidin) at 0.555:PT (PT = PBS + 0.1% Triton) and left overnight at 4°C. Samples were then washed 3x quickly with PT, and washed every 1-2 hours after (5x). They were then left overnight at 4°C. Samples were then replaced with DAPI (50% glycerol, 0.1mg/mL MAS) and were allowed to sit for 30min – 1 hour, then the process was repeated. Samples were then left overnight (or over the weekend) at 4°C. The samples were then replaced with 70% glycerol, then mounted on slides for imaging. They were analyzed on a Zeiss® Axiophot microscope. Images were captured with a Diagnostic Instruments Spot Camera with assistance from N. Patel from the University of California, Berkeley. Figures were assembled using Adobe Photoshop®.

### Electron Microscopy
After physical examination and further photography under a dissecting microscope, 10 eyes (~1mm$^2$) were dissected from whole clams and fixed in 2.5% glutaraldehyde. Transmission electron microscopy (TEM) was conducted by R. R. Dubielzig, L. B. Teixeira, and C. S. Schobert at the Pathobiological Sciences Department School of Veterinary Medicine at the University of Wisconsin, Madison. Tissues were processed for paraffin sectioning, light microscopy, and plastic sectioning for TEM.

## Immunohistochemistry

Under a dissecting microscope, 10 eyes (~1mm$^2$) were dissected from whole clams and fixed in 4% paraformaldehyde in PBS (0.1M phosphate buffered saline, pH 7.4). Following dissection, eye tissue was fixed for four hours at room temperature. After fixation, tissues were stored in PBS at 4°C until use. The following immunohistochemistry methods were written and conducted by A. Nahm-Kingston at the University of Maryland, Baltimore County.

Two custom-made antibodies were designed against several cephalopod rhodopsins/retinochromes, along with a commercially available anti-acetylated alpha-tubulin (Sigma). Western blots and previous immunohistochemical labeling in thin sections show that these antibodies label the proteins against which they were designed (Kingston *et al*. 2015). Secondary antibodies included AlexaFluor 488 goat anti-rabbit, AlexaFluor 555 goat anti-chicken, and AlexaFluor 633 goat anti-mouse (LifeTechnologies).

Immunolabeling was performed by modifying a whole-mount immunolabeling protocol from Gonzalez-Bellido and Wardill (2012). All tissues were dehydrated in an ethanol series (ethanol in PBS) of 30, 50, 70, 90, and 100% for 20 minutes each, at room temperature, to remove lipids. Tissues were rehydrated in 90, 70, 50, and 30% ethanol series for 20 minutes each, at room temperature. Tissues were washed in PBS three times for ten minutes at room temperature, and blocked in PBS-TX (0.1M PBS+0.3% Triton-100; Sigma) plus 10% normal goat serum (NGS; Vector Labs) for two hours at room temperature. Primary antibodies were diluted at a concentration of 1:100 in 1ml PBS-TX+10% NGS and added to tissues contained in a 24-well plate. Primary antibody incubations lasted four days at 4°C. Tissues were washed in PBS-TX+10% NGS for one hour, three times, at room temperature. Secondary antibodies were diluted at a concentration of 1:50 in 1ml PBS-TX+10% NGS and applied to tissues. Secondary antibody incubations lasted three days at 4°C. Tissues were washed in PBS three times for thirty minutes at room temperature in the dark. Tissues were cleared in a thiodiethanol (TDE; 2'2'-thiodiethanol in PBS; Sigma) series of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 97% for one hour each, at room temperature, in the dark, and mounted in 97% TDE on 600μm-thick stainless steel slides with a cover slip on each side of a circular punch through the middle of the slide. Cover slips were sealed to the slide with nail polish. A Leica SP5 confocal microscope was used to image tissues. Images were taking using a 40x immersion objective. Images were processed using Fiji (Image J). Maximum-intensity images were created from z-stacks (Z-projection).

## Results

## Looming and Recruitment Trials

When the eyes are photographed with a flash there is a detectable eye-shine (Figure 1). TEM shows the presence of electron dense intracytoplasmic material behind the eye, which is suggestive of membrane breakdown and lipofuscin function rather than a reflective pigment. *C. ales* showed a significant response to the shadow or edges of a potential predator as tested in the looming trials (Chapter 2). *C. ales* did not show a significant response to the presence of conspecifics using visual cues (other live *C. ales* or video playback of flashing) (Chapter 2).

## Fluorescence Microscopy
Imaging of the eyes of *C. ales* and the congener *C. scaber* (Figure 2A, 2B) showed similar DNA patterning through fluorescent DAPI (Figure 2C, 2D) and muscular (actin) patterning through fluorescent phalloidin (Figure 2E, 2F).

## Transmission Electron Microscopy
Eye tissues had morphology which is typical of other members of the Limidae such as *C. floridanus* (Morton 2000), including a cornea, lens, pigmented cells and vacuolated cells (Figure 3).

## Immunohistochemistry
Tubulin labeling, which stains proteins in microtubules, may have labeled a retina within the *C. ales* eye (Figure 4A). The tubulin also labeled tufts of neural tissue, as seen in Figure 4D. The retinochrome labeling highlighted what are believed to be photosensitive pigments common in cephalopods (Figure 4B). The rhodopsin labeling, which is often found in rod photoreceptors, is somewhat inconclusive as there are no Western Blots to show that the antibodies labeled proteins of the proper molecular weight (Figure 4C). The composite image which depicts all three labels is shown in Figure 4E.

## Discussion

Although *C. ales* responded to a visual stimulus in looming trials by increasing flash rate, there was no response to the presence or flashing of conspecifics. From examining morphology through TEM results, the size and structural features of the eyes do not support the hypothesis that *C. ales* is capable of image perception or detection of the flashing displays of neighboring clams. This supports the negative findings testing recruitment to a visual stimuli of conspecifics. Immunohistochemistry suggests the presence of light sensitive pigments, which proposes that their eyes are capable of perceiving shadows or movement caused by potential predators. This explains the increase in flash rate during looming trials, which was also witnessed in field video taken without SCUBA divers present (Dougherty, unpublished data). Fluorescence microscopy did not reveal substantial differences in eye musculature or DNA between *C. ales* and *C. scaber*, so the vision of *C. ales* is likely similar to that of other Limid bivalves. *C. ales* vision is likely used for predator detection.

## Acknowledgements

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## Figure Captions

Figure 1. *C. ales* eyes (n=4) photographed with flash, revealing eyeshine. Photos taken without flash do not reveal eyeshine.

Figure 2. Fluorescence microscopy of *C. ales* (A) and *C. scaber* (B) eyes. Phalloidin stains actin musculature in *C. ales* (C) and *C. scaber* (D). DAPI stains genetic material in *C. ales* (E) and *C. scaber* (F).

Figure 3. Ultrastructure of the eye of *C. ales* showing parts of the visual morphology.

Figure 4. Immunohistochemistry (anti-opsin antibody labels) stains of a *C. ales* eye used to confirm the presence location, or type of opsin/photoreceptor. Tubulin (yellow), a protein, is shown as representing a possible retina (4A) and neural network (4D). Retinochrome (red), a photosensitive pigment found in cephalopods, is shown in 4B. Rhodopsin (green), found in photoreceptors, is shown in in 4C.

# Figures
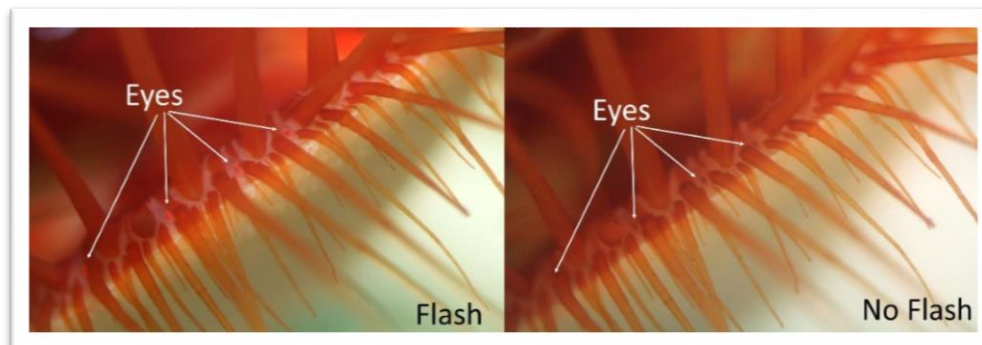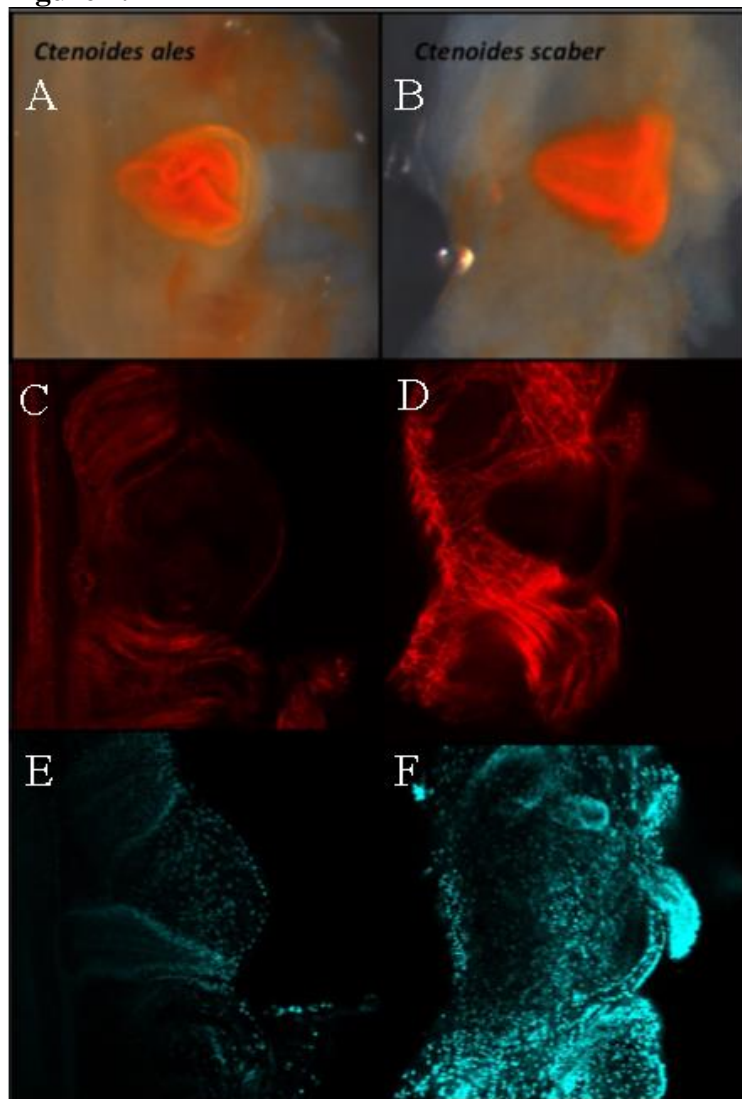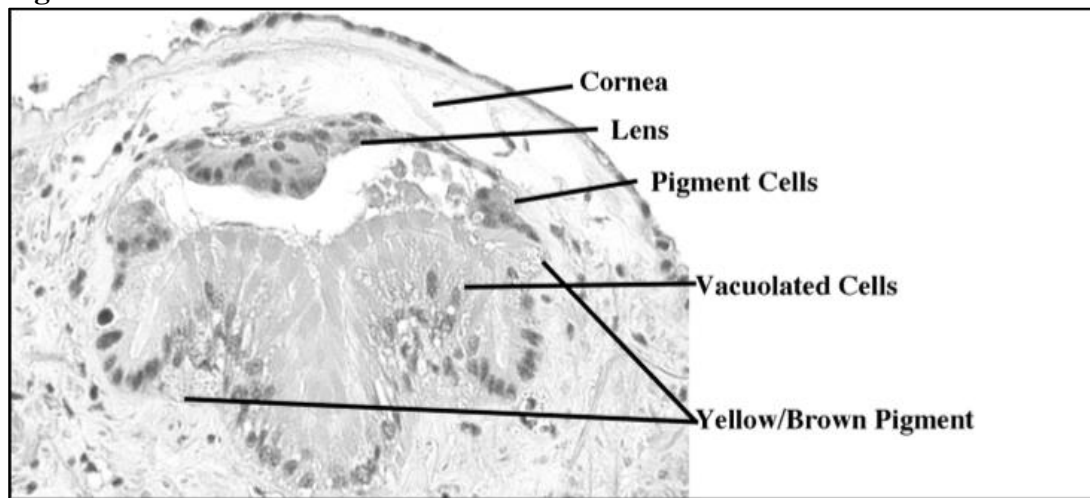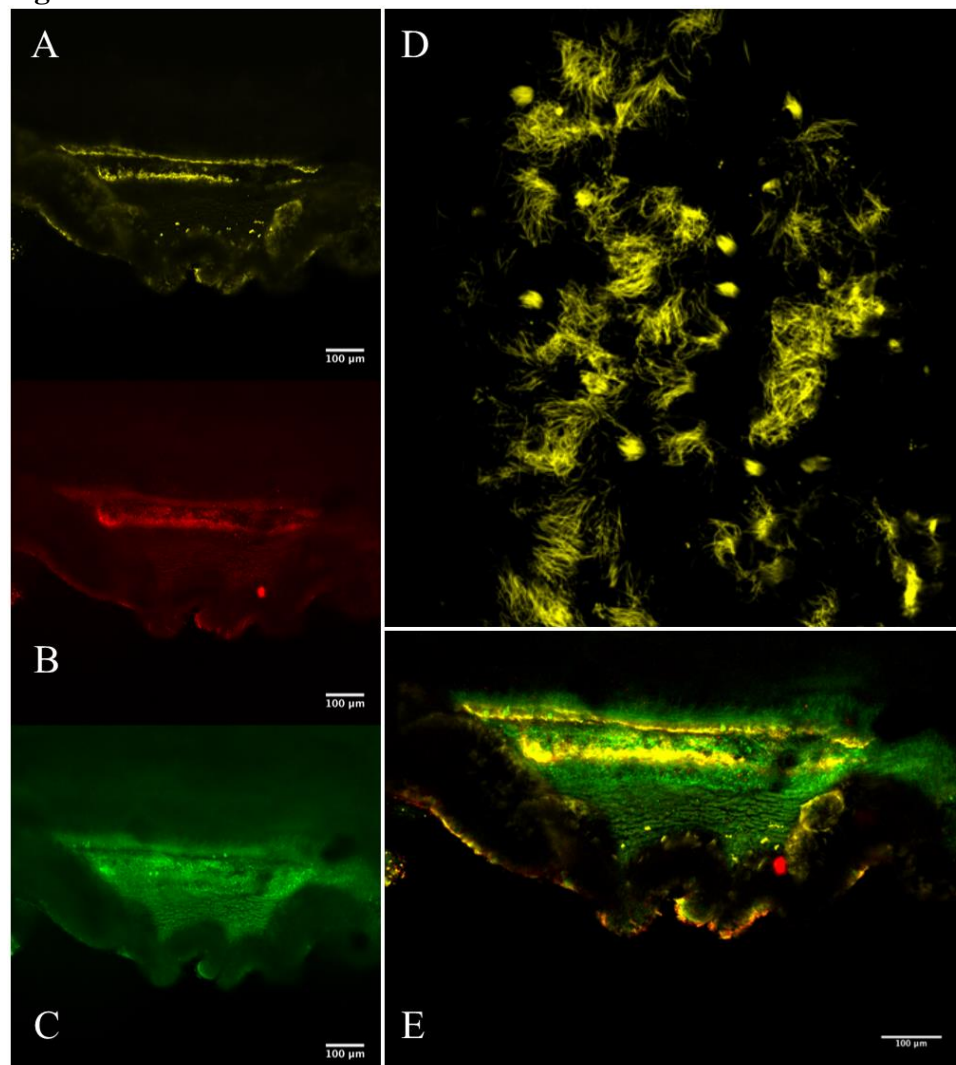
**Figure 1.**



**Figure 2.**

**Figure 3.**



**Figure 4.**

# CHAPTER 4

## Distribution, habitat, and ecology of *Ctenoides ales*

## Abstract

The distribution, habitat and ecology of the 'disco' clam *Ctenoides ales* (Limidae) is described herein. Three locations in Indonesia and one location in Australia were explored and 210 individual clams were located at depths of 3-50m. The properties of the reflective silica spheres did not change with the depth at which the clam was collected. There were four main habitats identified, including coral reef walls, coral reef bommies, caves, and karst islands. These habitats shared characteristics of low water flow, sedimentation, and numerous holes or crevices. The clams were often found in clustered populations at these sites. *C. ales* is thought to be a protandrous hermaphrodite, and based on shell height at the time of change in the congener *C. scaber*, the clam size distribution indicated a sex ratio favoring males in *C. ales*. Clam movement over a 2-hour period did not differ based on sex. Clams were found in conspecific groupings (≥2 clams) in more than half of *in situ* observations (n=109/210), and the average difference in shell height between organisms was 10.2mm ± 7.2 (n=36). Issues associated with *C. ales* cultivation and habitat threats are also discussed.

## Introduction

File clams (family Limidae) are a cosmopolitan group of bivalves that possess mantle tentacles, are capable of swimming, and attach to substrates. Their habitat, morphology and ecology differ drastically from the more well-known burying clams that are harvested commercially as a food source. File clams are popular in aquaria, and similar to most ornamental aquarium species, are collected and imported from the wild (Chapman *et al.* 1997). Many, such as *Ctenoides scaber*, are known for their vivid red coloration, which is due to carotenoids (Lin and Pompa 1977). There are few studies of file clam aquaculture (Gomez *et al.* 1990), but there is no large-scale commercial harvesting of file clams, and therefore there is limited information on cultivation compared to the more commercially-relevant bivalves such as oysters, scallops and mussels. Little is known about the distribution, habitat, ecology, or life history of many species of file clam. For those same species, there is also no documentation of growth rates and sex ratios, which influence population composition and spawning.

One of the best studied file clams, which is also very popular in aquaria, is *C. scaber*, which a Western-Atlantic file clam that ranges from North Carolina to Brazil (Abbott 1974). It is a congener to *C. ales*, but does not have the characteristic flashing display of *C. ales* (Dougherty *et al.* 2014). *C. scaber*, along with several other species of file clams, are protandrous hermaphrodites, maturing from small males to large females as they grow (Gomez *et al.* 1990; Gomez *et al.* 1995; Järnegren *et al.* 2006; Lodeiros and Himmelman 1999). One reason for protandrous hermaphroditism in marine file clams is the permission of rapid somatic growth due to lower energetic costs of spermatozoa compared to ovules, but in *C. scaber*, there were similar slopes for male and female regressions of somatic tissues to shell height, suggesting the costs of producing male or female gametes were similar (Lodeiros and Himmelman 1999). In *C. scaber*,

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the switch from male to female occurs around 40mm shell height (Dukeman *et al.* 2005), and clams starting at 25mm shell height were found to be reproductively active (Lodeiros and Himmelman 1999). Although shell growth rate has not been measured in many file clams, including *C. ales*, some species of scallops can grow up to 10mm per month (Lodieros and Himmelman 1994; Lodieros *et al.* 1998). The growth rate of file clams is essential to understanding sex ratios in populations, and to predicting how changes in environmental conditions may affect growth and reproduction.

Many file clams are filter feeders, so their growth and reproduction are linked to plankton production (Barber and Blake 1991; Giese and Kanatani 1987; Sastry 1979). However, reproductive strategy is not dependent solely on feeding method, as the deep-sea dwelling file clam *Acesta sp. nov.*, which feeds on tube worm eggs, has a similar reproductive strategy to its ecologically similar congener *Acesta excavata*, which is a filter feeder (Järnegren *et al.* 2006). Filter feeders such as *C. ales* and *C. scaber* obtain all of their nutritional requirements by filter feeding, as they do not contain any photosynthetic symbionts. The habitat in which they live must therefore provide a continuous supply of plankton. Filter feeding occurs in the gills of file clams, which are also used for respiration. Bivalves exclude particles of particular sizes and compositions when they feed, and continually filter particles of a specific size range (Beninger and Veniot 1999; Hawkins *et al.* 1998; Shimeta and Koehl 1997; Tamburri and Zimmer-Faust 1996; Ward *et al.* 1998). When plankton concentrations are high, even after filtering, more than 70% of captured particles were rejected as pseudofeces prior to ingestion in mussels and oysters (Hawkins *et al.* 1998). Sedimentation can also affect filter-feeding bivalves, such as the hard clam *Mercenaria mercenaria*, which exhibited a decline in algal ingestion rate with increasing sediment loads (Bricelj and Malouf 1984). Habitat and feeding are linked, especially in coastal areas that are prone to sedimentation when undergoing development.

The size of the particles that bivalves ingest is important. The oyster *Crassostrea virginica* was found to reject particles >100μm, while ingesting particles of <40μm, or equivalent to the size of phytoplankton (Tamburri and Zimmer-Faust 1996). However, taste or odor is important as well, as chemical feeding stimulants were used to induce the oysters to ingest larger particles (Tamburri and Zimmer-Faust 1996). It is therefore not only the size, but the taste, that affect preferential ingestion. Because of their complicated feeding preferences, file clams are difficult to maintain in aquaria without extensive care and individual feeding, and are vulnerable to changes in sedimentation levels *in situ*. Some bivalves are known to consume conspecific larvae, which also has implications for successful distribution of larvae after spawning events (Tamburri and Zimmer-Faust 1996).

*C. ales* is a filter feeder, and is found throughout the Indo-Pacific, where recent coral cover loss has been greater than expected (Bruno and Selig 2007) due to a variety of factors but including sedimentation and storms, which can influence feeding. *C. ales* is thought to be a protandrous hermaphrodite, lives in small crevices, and has been found as shallow as 3m and as deep as 50m. Its photic environment changes as depth increases, as long wavelengths attenuate rapidly in the first 50m of sea water, leaving the majority of wavelengths in the blue-green range (400-500nm) (Jerlov 1976). Even at shallow depths, most illuminating light inside the small crevices where *C. ales* are found is from the horizontal direction, and therefore also dominated by blue-green wavelengths (Dougherty *et al.* 2014). *C. ales* is the only bivalve known to have a flashing light display, and the ultrastructure of the tissue and its rapid movement, which are unique to *C. ales*, suggest it may serve as a signaling function (Dougherty *et al.* 2014). Little is known about the life history or behavior of *C. ales*, and an obvious question is why the display

occurs. Possible functions of the flashing display currently being tested include a signal facilitating the recruitment of conspecifics, prey luring, or predator deterrence. *C. ales* are often found clustered in high density in relatively small areas. Additionally, more than one clam is often found inside the same crevice within the reef. The geographical, depth, and spatial distribution of *C. ales* were unstudied. In this article, ecological findings from 72 SCUBA dives in the habitats of *C. ales* are discussed, as well as what can be learned from their size distribution, and hence, their sex ratios and associated movement. The nature of their grouping in the wild is examined, including size differentials between individuals grouped together. Finally, habitat threats and cultivation issues are discussed.

## Materials and Methods

### Field observations

*C. ales* were located, observed, photographed and filmed over the course of 72 SCUBA dives on coral reefs in three locations in Indonesia; Lembeh, Sulawesi (1˚27'N, 125˚14'E), Pemuteran, Bali (8˚8'S, 114˚39'E) and Kri Island, Raja Ampat (0˚34'S, 130˚40'E), and one location in Australia; Lizard Island (14˚38'S, 145˚27'E). *C. ales* were located through exploratory dives in areas whose coral reef habitat was deemed suitable for occupation. Characteristics defining a suitable habitat were identified. Observations included measurements of clam depth, clam size, crevice size and distance to neighboring clams. Depth was determined using an Aeris Atmos© dive computer, and clam and crevice size were measured by placing a metal pointer with an attached ruler into the crevice as close to the clam as possible and taking a photograph. Shell width was measured, as shell height is not measurable *in situ* without removing the clam from its crevice. Shell width was then converted to shell height by measuring 55 single valves of dead shells found while SCUBA diving at the field site in Pemuteran, Indonesia, and calculating a simple linear regression value. Shell width was measured using the Adobe Photoshop© ruler function. Shell area was analyzed using ImageJ© software. The distance to nearest clam was measured using transect tape. Photographs were taken using a Canon 60D DSLR in Ikelite Housing or a GoPro Hero 3© camera. Video was taken using three GoPro Hero 3© cameras. Cameras were attached to 2kg weights and were positioned to film the clam(s), the larger crevice, or the coral formation in which the clam lived. Depth of video setup varied from 3m to 22m. Light and Motion© underwater lights (SolaDive 1200 and Gobe) were left with the cameras when filming at night (n=3) or in caves where low ambient light made camera visibility impossible to analyze (n=2). Cameras were set up on one dive and recorded until the battery died or the memory card was filled. Cameras were retrieved on the next consecutive dive. When necessary, cameras and lights were left in place overnight. Dive sites were chosen for video setup based on reef topography and feasibility of camera positioning. Sites were also chosen based on the ability to leave the video setup unattended for prolonged periods of time. The 30 videos captured ranged in length from less than one minute to over one hundred minutes. Total video footage time was 16 hours and 57 minutes. Film was reviewed at 4x speed using MovieMaker© software and film clips were made of significant events.

### Laboratory Experiments

*C. ales* were purchased through BlueZoo© Aquariums and housed in a 100-gallon aquarium with Fluval© filters in laboratory tanks at the University of California, Berkeley. Two *C. ales* were

purchased through CairnsMarine© in Cairns, Australia, which were collected from approximately 50m depth. Clam movement was measured by placing individual clams in 20 gallon tanks for 2 hours and photographing their position at 0hr, 1hr, and 2hr. The silica spheres found inside the reflective portion of the clam tissue were measured using the measurement tool in Adobe Photoshop©. The photographs showing the spheres were obtained through tissue prepared as outlined in Dougherty *et al.* (2014).

## Results

### Geographical, depth and spatial distribution

Three locations in Indonesia were explored to determine the number and density of *C. ales* present; Lembeh, Sulawesi (2 dives sites, n=3 clams), Pemuteran, Bali (9 dive sites, n=83 clams) and Kri Island, Raja Ampat (4 dive sites, n=117 clams). One location in northeast Australia was explored; Lizard Island (2 dive sites, n=7 clams). Clams were found between depths of 3m – 50m (collected by Cairns Marine). The diving for this study was done at ≤24m due to scientific diving regulations through the American Association of Underwater Scientists (AAUS), but *C. ales* has been found as deep as 50m, and could possibly live deeper. Photographs of tissue from clams collected from three different depths (10m, 50m, and unknown) were analyzed to determine differences in sphere size. Results indicated that the sphere sizes were significantly different, but that the difference was optically negligible because the variation did not result in a change in the light-scattering properties of the spheres (Figure 1). *C. ales* can be found along a single depth gradient, and they are often in dense groupings on coral reefs. In Australia, the clams were found an average of 3.3m±1.9 apart from one another (n=7). The clams are distributed throughout the Indo-Pacific region, as indicated by the GBIF (Global Biodiversity Information Facility), which has 47 specimen listings in 11 countries, including Indonesia, the Philippines, Australia (and Christmas Island), Japan, Fiji, Papua New Guinea, New Caldeonia, the Solomon Islands, Samoa, Timor-Leste, and Palau.

### Habitat Characteristics

Four main habitats were identified that were suitable for *C. ales* colonization; coral reef walls, coral reef bommies, caves, and karst islands. Small crevices were the primary requirement for occupation. These crevices generally ranged from 5-20cm (n=7). In addition to small crevices, holes made by boring clams were often found occupied by *C. ales*. Areas with heavy sedimentation, caves with little to no water flow, and areas of reef wall with limited competition from other corals, sponges and sea fans were the areas in which *C. ales* were found in the highest densities.

### Size, associated sex ratios and movement

The regression analysis of shell width and shell height gave a linear equation of y=1.1992x + 0.3169 with an $R^2$ value of 0.9809, suggesting 98.09% of shell height can be explained by shell width and indicating that the line is a good fit (Figure 2). All measured shell widths were converted to shell height using this equation, and the resulting clam shell heights ranged from 13.3mm to 80.2mm, with an average height of 37.4mm±14.3 (Figure 3). Two recent papers have

examined sex determinations based on shell height in the protandrous hermaphrodite *C. scaber*. Lodieros and Himmelman (1999) found the following proportions to be female; 21% of clams 15-30mm, 22% of clams 30-40mm, 55% of clams 40-50mm, 59% of clams 50-60mm, 70% 60-70mm and 78% of clams 70-80mm. Dukeman *et al.* (2005) found 17% of clams <40mm and 71% of clams ≥40mm to be female. Applying the ratios used by Lodieros and Himmelman (1999) to *C. ales*, with the implied caveat that there may be a differential in shell size at the time of sex change between these two species, approximately 34% of the live clams measured in Raja Ampat in 2013 were female (n=52), 31% of the live clams measured in the same area in Raja Ampat in 2014 were female (n=37), and 37% of the dead shells found while diving in Bali in 2015 were female (n=55). The same three groups were 36%, 31% and 37% female when applying the ratios used by Dukeman *et al.* (2005), respectively. The sex ratio of these three populations of *C. ales* would lie somewhere between 1M:0.5F and 1M:0.6F, which varies from the 0.6M:1F reported by Dukeman *et al* (2005) and the 1M:0.84F reported by Gomez *et al.* (1990). In the 2013 measurement, the average live clam shell height was 28.4mm±9.8 (n=52). In the 2014 measurement, the average live clam shell height was 28.0mm±7.3 (n=37). In the 2015 measurement of dead shells collected while diving, the average clam shell height was 38.1mm±19.0 (n=55). *C. scaber* may be slightly larger than *C. ales*, so *C. ales* may transition to female at smaller shell heights, which would increase the number of observed specimens that were female. Given these approximate size ratios in relationship to sex, movement over a 2-hour period was monitored based on clam size to determine if males or females were more active, but there was no relationship between the sex of the clam and distance moved (Figure 4). Although specimens kept in the laboratory were likely mixed-sex, spawning was never witnessed in any of the groups of ≤20 *C. ales* during the ≤3 months they were kept in aquaria. Spawning was also never seen in clams exposed to stressors which could induce spawning, such as movement between tanks or exposure to potential predators.

## Conspecific grouping and size variation

In field observations, 210 clams were found throughout four geographic locations (Figure 5). Observations indicated 48% (n=101) of the clams were alone, while 52% were found co-inhabiting crevices (approximately ≤25cm$^2$) in groups of ≥2 (n=109). Of the clams that were in groups, 49% of clams were in pairs of two (n=54), 25% were in groups of three (n=27), 15% were in groups of four (n=16), and 11% were in groups of six (n=12). The average difference in shell height of clam pairs photographed together was 10.2mm±7.2 (n=36). When comparing the sizes of the two (or more) clams inside a given crevice, the smaller clam was an average of 22.9mm±8.4 (n=18), and the larger clam was an average of 33.1mm±9.6 (n=18), suggesting a <20% chance that the two clams were different sexes according to sex determination based on size in *C. scaber* (Lodieros and Himmelmann 1999) and a <17% chance based on Dukeman *et al.* (2005). When the pairs are examined individually, in 13 of the 18 pairs, both clams had a shell height of <40mm, suggesting an 83% chance they were both male based on Dukeman *et al.* (2005). In 1 of the 18 pairs, both clams had a shell height of >40mm, suggesting a 71% chance they were both female. In the remaining 4 of the 18 pairs, one clam had a shell height of <40mm and one clam had a shell height of >40mm, with an average difference of 15.4mm±8.

**Discussion**

Geographical, depth and spatial distribution
*C. ales* are broadly distributed throughout the Indo-Pacific region and can range as deep as 50m. The depth at which they are located does not affect the optical properties of silica spheres in their tissue, which produce their distinctive flashing display. The spheres are statistically different in size, but still scatter the same wavelengths, resulting in the broadband display of white light. In observations of depth along a vertical gradient, as well as observations of width along a horizontal gradient, clams were found in high densities within relatively small (≤15m) patches of habitat. Of the 16 total dive sites explored throughout Indonesia and Australia, which had 210 clams total, 6 sites had ≤2 clams. The other 10 sites had an average of 26 clams each, suggesting clustered population densities in small patches of habitat. These concentrated population densities could be a byproduct of currents or some other physical force, as *C. ales* are thought to be broadcast spawners. There may be some form of conspecific chemosensory attraction, such as in larvae from the oyster *Crassostrea virginica* which show attraction to cues from both live adult oysters and bacterial films growing on shell surfaces. The larvae respond by swimming vertically down in the water column, slowing horizontal swimming and then contacting the bottom and attaching with their foot, indicating settlement (Tamburi *et al.* 1992). It is unknown what cues affect settlement in *C. ales*.

Habitat Characteristics
The habitats in which high densities of *C. ales* were found shared common characteristics; relatively weak currents, high sedimentation, and numerous crevices or holes. Caves also consistently housed large numbers of clams in all three Indonesian locations. The number of clams found on a given reef did not change noticeably with depth, although the exploration of deeper habitats was limited by scientific diving restrictions. Dive sites with higher numbers of clams generally seemed to have less biodiversity of marine plants and animals, perhaps resulting in higher clam numbers due to decreased competition for space or fewer predation threats. The lack of biodiversity in addition to high sedimentation meant that the sites where the most clams were found were rarely used for SCUBA tourism.

Size and associated sex ratios and movement
If the size at which *C. ales* changes from male to female is similar to the congener *C. scaber*, approximately one-third of the specimens measured in the field were female (sex ratio between 1M:0.5F and 1M:0.6F). This varies significantly from the 0.6M:1F in *C. scaber* reported by Dukeman *et al.* (2005) but was closer to the 1M:0.84F reported in *C. scaber* by Gomez *et al.* (1990). Differences in this sex ratio are difficult to explain using sampling bias, as larger clams are easier to find while SCUBA diving. This sex ratio may be affected by the collection of organisms for sale in aquaria, depending on what size specimens are targeted. There was a broad size range of both living and dead (based on discarded valves) *C. ales*, suggesting predators target various sizes of individual. These observations may be similar to previous findings of juvenile *C. scaber* being predated upon by crabs (Dukeman *et al.* 2005) and parrotfish (Gomez *et al.* 1995). There was no trend between clam size and how much they moved in the laboratory, suggesting that male clams do not move more than female clams. It was hypothesized that males may move more than females in order to settle near females for spawning, as males are smaller and less likely to be permanently settled on the reef. However, it is unknown at what size the

clams become sessile, or permanently attached in their reef crevice habitats using byssal threads. The clams may not move at all after they have grown to a certain size, which could explain the lack of difference in movement based on shell height between males and females. Spawning was never witnessed either *in situ* or in the laboratory while studying *C. ales*. The congener *C. scaber* only spawns twice a year (Lodieros and Himmelmann 1999), once in June/July, and once in November/December. It is possible that spawning in captive *C. ales* either did not occur due to the time period kept in the laboratory being too short, the absence of cues that would occur *in situ*, such as temperature of plankton levels, or that spawning did occur but was not observed.

## Conspecific grouping and size variation

In field observations, 52% of specimens were found co-inhabiting holes in pairs of ≥2 (n=109). The average difference in shell height between 18 measured pairs was 10.2mm±7.2 (n=36). Depending on growth rate, which has not been studied in *C. ales*, clams may have settled in the same crevice after the same spawning event based on this average size difference. Of the 18 pairs, 9 pairs had a size difference of less than 10mm, 7 pairs had a size difference of 10-20mm, and 2 pairs had a size difference of 20-30mm. Without knowledge of the growth rate of *C. ales*, it is difficult to determine whether these pairs had asynchronous settlement, but a size difference nearing 30mm would suggest it is possible for some of the pairs. As many species of bivalves possess light-sensitive eyes in the pediveliger stage, during which settlement occurs (Carriker 1990) it was originally thought that the flashing display might act as a settlement cue for conspecifics. Specifically, perhaps small males were attracted to the flashing of large females in order to settle nearby. However, given this analysis of size similarities of clams in the field, it is possible that the groupings are instead a result of currents, some other physical force, or perhaps a chemosensory attraction between conspecifics, such as in *Crassostrea virginica* (Tamburi *et al.* 1992). Furthermore, examination of *C. ales* eyes using transmission electron microscopy revealed that the size and structural features of the eye tissues do not support the hypothesis that *C. ales* are able to detect the flashing displays of neighboring conspecifics. The analysis also determined that the eyes are not capable of image formation (Dougherty, unpublished data).

## Cultivation and Habitat Threats

To my knowledge, there have been no studies attempting laboratory cultivation of file clams. Organisms which have a pelagic larval stage possess many complex needs in terms of temperature, salinity and food (Thorson 1949), and are therefore difficult to cultivate in aquaria. Aquarists report that survivorship of adult file clams is often only a matter of months, making captive spawning unlikely in species such as *C. scaber*, which only has two major spawning events per year (Lodieros and Himmelmann 1999). There are no studies which have assessed the feasibility of farming file clams, but such studies have been conducted on other bivalves such as scallops, mussels, and oysters, which are of a greater commercial value. Collecting wild file clams for cultivation or for study is problematic due to their fragile coral reef habitats. Additionally, many file clams such as *C. ales* use byssal threads to attach themselves inside of small crevices within the coral (Dougherty *et al.* 2014), which makes it difficult to collect them without causing damage to the surrounding reef. If file clams are successfully collected and moved to aquaria, there remain challenges associated with their feeding, growth rate, and reproduction. Many file clams are broadcast spawners, and according to field findings based on the sizes of the congener *C. scaber*, only 31-37% of the *C. ales* seen were female. It is unknown whether collection for aquaria has influenced these numbers. Spawning was never witnessed in

captive clams during this three-year study. Like many organisms, file clams are threatened by changes in water temperature, which is often a catalyst for spawning. Some bivalves spawn when temperatures increase (Sastry 1979; Giese and Kanatami 1987; Lodeiros and Himmelman 1994; Velez *et al.* 1993), some bivalves spawn when temperatures decrease (Velez and Epifanio 1981; Lodieros and Himmelmann 1999), and some bivalves have no correlation between temperature and spawning (Velez *et al.* 1987; Marquez 1996; Lodeiros *et al.* 1997). In addition to feeding, spawning and reproductive challenges, file clams are considered by many to be ill-suited for commercial exploitation due to both the difficulty associated with their collection as well as the potential damage that harvesting would cause to surrounding coral communities (Lodeiros and Himmelman 1999). These complications, combined with the clams' sporadic distribution on reefs, suggest that further study is needed to understand their distribution, life cycle and other threats to their habitat.

## Acknowledgements

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## Figure Captions

Figure 1: Sphere size (µm) based on depth (m) at which clam was collected (n=150 spheres/clam).

Figure 2: Shell width (mm) measured against shell height (mm) to obtain a simple linear regression equation.
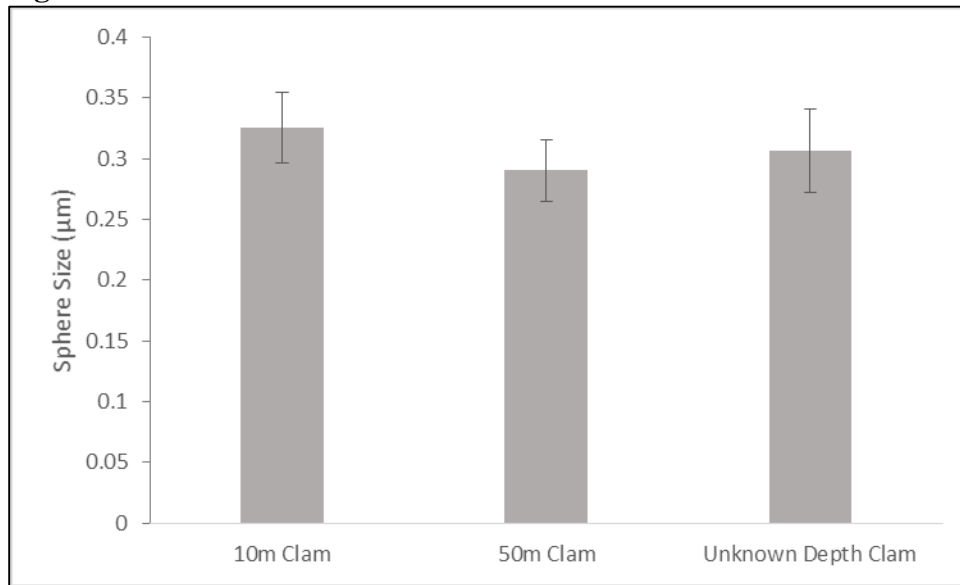
Figure 3: Shell height (mm) based on live clams photographed in 2013 and 2014, and valves collected in 2015.

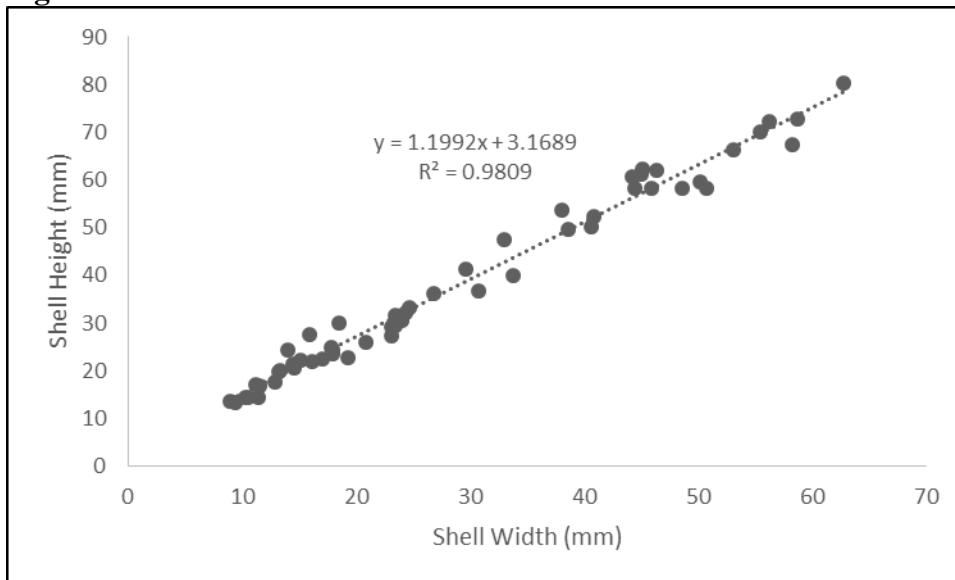Figure 4: Clam shell area ($cm^2$) and associated movement (cm) during a 2-hour trial.

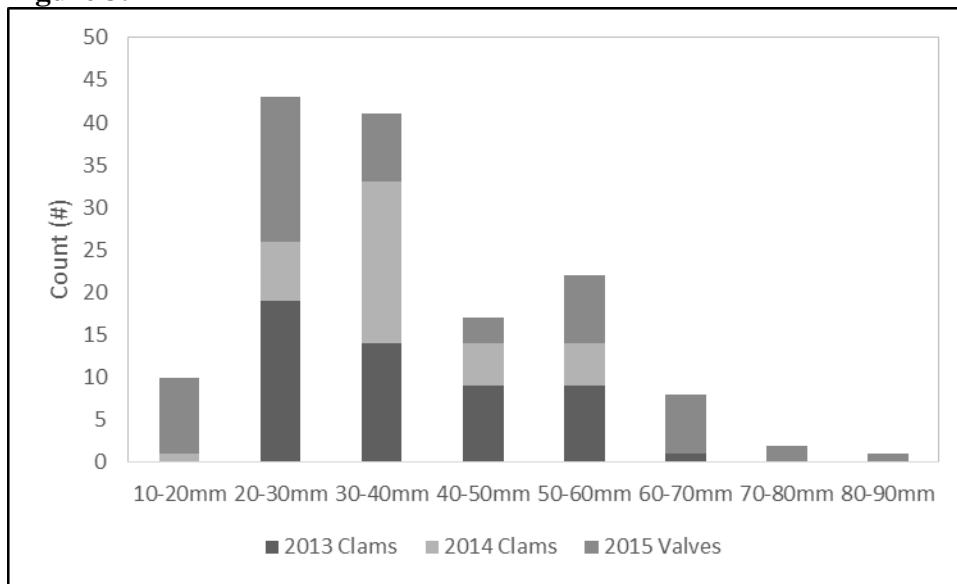Figure 5: Clam groupings (1-6 individuals) based on location.
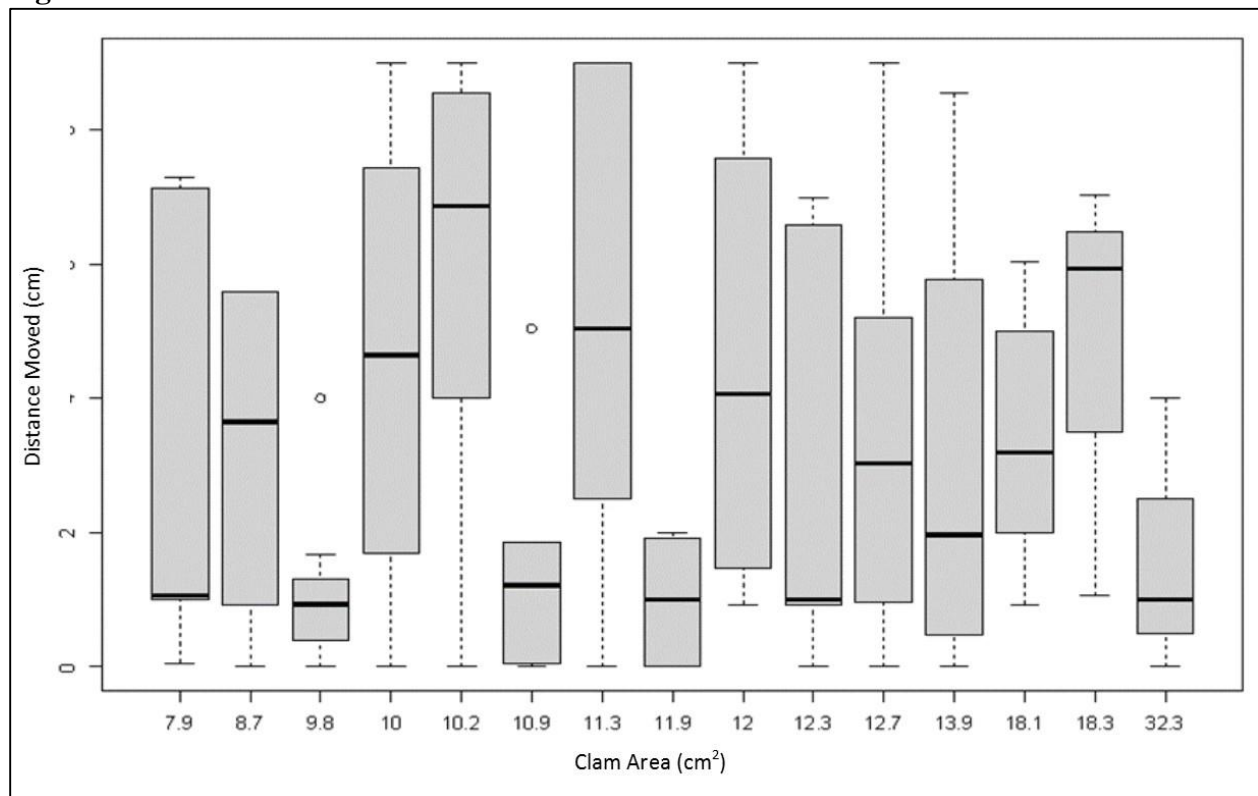
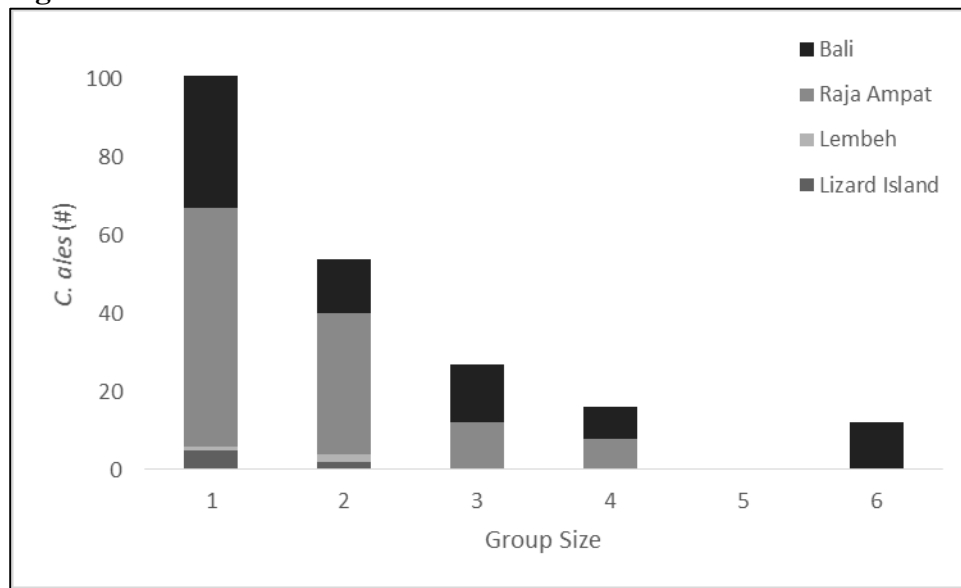# Figures

**Figure 1.**



**Figure 2.**

**Figure 3.**



**Figure 4.**

**Figure 5.**

# CONCLUSION

The oddities of the natural world are often the most captivating, and the driving forces through which they evolved are often the most interesting. The ocean offers a plethora of animals which fit the bill, including the subject of this PhD research - *Ctenoides ales* - the only clam in the world that flashes light. These complex adaptations require multiple mechanisms to function, are phylogenetically unique, and can provide a fitness value. They're a platform through which we can elucidate the powerful mechanisms that propel species, ecological, and genetic biodiversity.

This dissertation research allowed a comprehensive understanding of this distinctive animal through structural, mechanistic, physiological, behavioral, visual, ecological, and life history analyses. It explored how the flashing of *C. ales* works, why the flashing occurs, the clams' sensory abilities, and the clams' distribution, habitat, and ecology. Studying biodiversity and evolution from an integrative, organismal approach requires a diverse arsenal of tools. Future research placing this species in a phylogenetic context will take a comprehensive approach to investigating biodiversity, whether it is a derivation of behavior, life history, morphology, or resistance to the inevitable changes our ocean faces.

Remaining questions concerning *C. ales* include the classification of any symbionts, the source of the silica utilized in the flashing display, and the physiological controls of the mantle furling. The potential of *C. ales* flashing as a defense mechanism also brings into question other defensive techniques within the Limidae family, which will be investigated in postdoctoral research. A phylogenetic context will be established for morphological analysis, histological work, and evolutionary-developmental techniques concerning the family Limidae, as well as examining comparative bivalve genomics using assemblages to identify candidate genomic adaptations in *C. ales*.

Biomimetics is the extraction of good design from nature. In short; anything we can do, nature does better. The vision behind the scientific goals pursued throughout this dissertation was to highlight the inherent complexity and valuable insight that unknown and understudied organisms provide, especially when they have evolved in a manner that suggests novelty and diversity from closely-related species. The vision which was the basis for communicating these scientific goals was that the only way to truly succeed in science is to share with and educate the general public in order to further our understanding of the fascinating natural world, and to protect it in the process.