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Bush, Stephanie Lynn

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Defensive Behaviors of Deep-sea Squids:
Ink Release, Body Patterning, and Arm Autotomy

by

Stephanie Lynn Bush

A 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 George K. Roderick

Dr. Bruce H. Robison

Fall, 2009

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by

Stephanie Lynn Bush

ABSTRACT

Defensive Behaviors of Deep-sea Squids:
Ink Release, Body Patterning, and Arm Autotomy

by

Stephanie Lynn Bush

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Roy L. Caldwell, Chair

The deep sea is the largest habitat on Earth and holds the majority of its' animal biomass. Due to the limitations of observing, capturing and studying these diverse and numerous organisms, little is known about them. The majority of deep-sea species are known only from net-caught specimens, therefore behavioral ecology and functional morphology were assumed. The advent of human operated vehicles (HOVs) and remotely operated vehicles (ROVs) have allowed scientists to make one-of-a-kind observations and test hypotheses about deep-sea organismal biology.

Cephalopods are large, soft-bodied molluscs whose defenses center on crypsis. Individuals can rapidly change coloration (for background matching, mimicry, and disruptive coloration), skin texture, body postures, locomotion, and release ink to avoid recognition as prey or escape when camouflage fails. Squids, octopuses, and cuttlefishes rely on these visual defenses in shallow-water environments, but deep-sea cephalopods were thought to perform only a limited number of these behaviors because of their extremely low light surroundings.

The Monterey Bay Aquarium Research Institutes' ROVs were used to determine whether or not deep-sea squids are limited in their behaviors compared to their shallow-water relatives. First, eighteen species of deep-sea squids were observed to release ink when encountered by an ROV (Chapter 2). Ink release was observed from the surface to below 1800 m and included six types of ink release, some of which have not been observed from shallow-water cephalopods.

Ink release could serve as a visual defense by blocking the cephalopods' silhouette or bioluminescence stimulated by the squids' movement, or causing predators to confuse ink pseudomorphs with potential prey. It could also, or instead, have a chemical function by having distasteful or noxious qualities, blocking predator olfactory senses, stimulating feeding behaviors, or functioning as an attractant so that predators attack the ink. To investigate potential chemical functions, ink collected from deep-sea and shallow-water cephalopods was analyzed using liquid chromatography – mass spectroscopy (Chapter 3). The goal was to compare ink chemical composition

between species to see if deep-sea species or a few particular species had vastly different chemical compositions that may warrant further investigations.

An ethogram – a catalogue of every behavior a species performs – of the deep-sea squid *Octopoteuthis deletron* Young 1972 was produced from over eight hours of ROV video footage of 76 individuals (Chapter 4). *Octopoteuthis deletron* is capable of numerous color, posture, and locomotor changes. Individuals can also change patterns of bioluminescence produced by their arm-tip photophores, leading to the first description of bioluminescence as a cephalopod behavioral component. Additional work was performed with *O. deletron* to determine if this species can autotomize arms (Chapter 5). ROV observations, laboratory and *in situ* experimentation, and histological sectioning were conducted. *Octopoteuthis deletron* can autotomize any of the eight arms at numerous points along the length. This species is the first squid described to autotomize arms, has the uncommon capability of economy of autotomy, and is one of very few animals that can perform attack autotomy.

Our understanding of cephalopod behavioral ecology has been greatly advanced by the research presented here. The hypotheses that deep-sea squids do not release ink and that their defensive behaviors are very limited were both falsified. Clearly there is much additional work to be done on these and other deep-sea animals to understand the ecology of the deep sea.

Dedicated to my grandmother,
E. Georgeann Benson,
the strongest woman I know...

Roar!!!

Table of Contents

List of Figures	ii
List of Tables	iii
Acknowledgements	v
Vita	viii
Chapter 1: Introduction	1
Chapter 2: Ink Utilization by Mesopelagic Squid	4
Chapter 3: Liquid Chromatography – Mass Spectroscopy Comparison of Cephalopod Inks	22
Chapter 4: Behaving in the Dark: Locomotor, Chromatic, Postural, and Bioluminescent Behaviors of the Deep-Sea Squid <i>Octopoteuthis deletron</i> Young 1972	29
Chapter 5: Economy of Autotomy in the Mesopelagic Squid <i>Octopoteuthis deletron</i> Young 1972	57
Chapter 6: Conclusions	71

List of Figures

Figure 2-1	20
Figure 2-2	21
Figure 3-1	28
Figure 4-1	49
Figure 4-2	50
Figure 4-3	51
Figure 4-4	52
Figure 4-5	53
Supplemental Figure 4-1	54
Supplemental Figure 4-2	55
Supplemental Figure 4-3	56
Figure 5-1	67
Figure 5-2	68
Figure 5-3	69
Figure 5-4	70

List of Tables

Table 2-1	18
Table 3-1	26
Table 5-1	65

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The smartest thing I ever did
Was decide to work on squid

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I'm indebted to the East/West Program for turning me into a thinking, analyzing, questioning field scientist. I'll never forget the experiences, the amazing friends, and the dedicated professors! A special thanks to Kathy Ann 'The Weed Queen' Miller for showing me how silly, fun, and enthusiastic one can be in science. A special thanks also to Jan Newton, who helped get my leg in the door at MBARI with a killer recommendation letter.

Hooray for George Matsumoto, MBARI summer intern coordinator extraordinaire, for letting me take part, not once, but twice, in this amazing program. Without it I would never have been introduced to the deep sea and all its denizens – my life truly would have taken an entirely different track. Thanks also for always thinking of me when opportunities arose: leading student tours of MBARI, teaching courses for the Osher Lifelong Learning Institute, and outside seminars. I continue to work with many of the people/organizations I met in this way.

I can't really thank Bruce Robison enough for letting me be a part of the Midwater Ecology Group, the hours of sea-time dedicated to my project, scientific advice, paper editing assistance....But one of best things I learned from Bruce was the excitement of discovery. If I am even a fraction as enthused about my career as he is about his I'll count myself among the truly lucky. I'll never forget the twinkling, excited eyes, or the laughter!

Thanks to Kim Reisenbichler, whose bark is worse than his bite, for answering endless silly questions, breaking out his humor at opportune moments, and keeping me in line.

Rob Sherlock, my chatter buddy for endless hours out at sea – thankfully I don't think we ever ran out of things to ramble about. Kudos to Rob for endless advice, stats assistance, equipment locating abilities, hardware/software help, and just being a great person and friend.

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Crissy 'Champus' Huffard. Oh squid sister, where to begin. Truly one of the funniest and silliest people I know, who brings the me out of me. So happy to have her as colleague and friend.

Steve Haddock took me on my first ever multi-day research cruise, mumbled strange new animal names as I frantically tried to keep up in VARS, provided Mac support, desk space, and humorous encouragement.

Annette Gough helped with the ins and outs of MBARI administration, which I gracefully avoided if at all possible. Thanks for your patience! I am also eternally grateful that she fought to keep me at a desk where I could spread out, breath, and get my work done.

The video lab staff was always ready to help find what I needed, search the database, make video clips, and all sorts of things for me. Extra special thanks to Susan Von Thun the Hired Gun for dealing with the same questions time after time (what's that jelly again?) and her biting tongue in the assistant scientist chair. Way to keep me on my toes, my friend!

Many thanks to the crew of the *R/V Pt. Lobos* and pilots of the *ROV Ventana*, including Craig Dawe, Knute 'Knutachteuthis' Brekke, D.J. Osborne, Mark 'Marko' Talkovic, and Mike Burczynski. Not only did they get us safely out to sea, keep an extremely complicated, vulnerable, and temperamental piece of equipment running, put up with half-crazed, last minute cable-tying of experimental equipment onto the ROV, and chase endless squids for me, they treated me like just another one of the guys (when often I was the only woman onboard), taught me to talk and joke like a sailor, provided endless hours of classic rock, and whose camaraderie generally kept me sane as we stared at the marine snow whizzing by while tossing about on the boat that will make even the strongest lose their lunch.

Mike Conway, Mike Burczynski, and Martine Suro procured much of the needed equipment-additions to the *ROV Ventana*.

The crew of the *Western Flyer* and pilots of the *ROV Tiburon/ROV Doc Ricketts*, including Randy Prickett, Bryan Schaefer, Dave French, Buck Reynolds, Mark Talkovic, Eric Nelson, and Bernard Roth, got us out there catching, inking, and tickling squids.

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It has been a pleasure being a grad student in the Caldwell lab, which brings together two of the most engaging, complex, and fascinating animal groups – stomatopods and cephalopods. If I was not truly enamored with the latter I surely would have worked on mantis shrimp. I did actually make forays into that world in my first few years at Cal, but alas, the cephs called me back...Thanks for sharing this world of animal behavior Roy! Thanks for letting me have the freedom to explore. Thanks for believing in me as a scientist.

Thanks to the many current and former Caldwell lab members including Crissy Huffard, Karen Osborn, Becky Williams, Liz McKinzie, Emily Griffen, Callie Martinez, Molly Wright, Joey Pakes, Jean Alupay, and Jennifer Hofmeister, who have been some of my best friends during grad school. Roy really knows how to pick a great group of people that work together, support each other, and laugh along the way.

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And now it's time for a banjo!

Vita

Doctoral Candidate, Department of Integrative Biology, University of California, Berkeley
Graduate Researcher/Collaborator, Midwater Ecology Group, Monterey Bay Aquarium
Research Institute

MBARI webpage: <http://www.mbari.org/staff/sbush/>

UC Berkeley webpage: <http://socrates.berkeley.edu/~sbush/>

UC Museum of Paleontology Field Notes - Squid Science:

http://www.ucmp.berkeley.edu/science/profiles/bush_0704index.php

EDUCATION

2004-present, University of California, Berkeley

Dissertation title: Defensive Behaviors of Deep-sea Squids: Ink Release, Body
Patterning, and Arm Autotomy

Advisor: Dr. Roy L. Caldwell

2002-2003, Three Seas Program, Northeastern University, Boston, MA

Intensive marine biology field- and course-work at 3 locations: The Marine
Science Center, Nahant, Massachusetts; Discovery Bay Marine Laboratory,
Discovery Bay, Jamaica; and Friday Harbor Laboratories, San Juan Island,
Washington

1998-2002, Illinois Wesleyan University, Bloomington, IL

Bachelors of Science, Biology, Magna Cum Laude, May 2002

2000-2001, James Cook University, Townsville, Queensland, Australia

Study-abroad concentrating on marine biology and statistics course-work

PUBLICATIONS

Bush, S. L., Robison, B. H., and R. L. Caldwell. 2009. Behaving in the dark: locomotor, chromatic, postural and bioluminescent behaviors of the deep-sea squid *Octopoteuthis deletron* Young, 1972. Biol. Bull. 216: 7-22.

Bush, S. L. and B. H. Robison. 2007. Ink utilization by mesopelagic squid. Marine Biology. 152(3):485-494.

LaJeunesse, T. C., Lee, S., Bush, S. L., and J. F. Bruno. 2004. Persistence of non-Caribbean algal symbionts in Indo-Pacific mushroom corals released to Jamaica 35 years ago. Coral Reefs. 24(1):157-159.

Bush, S. L., Precht, W. L., Woodley, J. D., and J. F. Bruno. 2004. Indo-Pacific mushroom corals found on Jamaican reefs. Coral Reefs. 23(2): 234.

FELLOWSHIPS, SCHOLARSHIPS, and HONORS

National Science Foundation, Graduate Research Fellowship, 2006-2009
Outstanding Graduate Student Instructor, UC Berkeley, 2007
UC Museum of Paleontology Remington-Kellogg Grant, 2008
Robert and Nancy Beim Field Research Fellowship, 2008
Society of Integrative and Comparative Biology, Student Support, 2007
Graduate Research Funds, UC Berkeley, 2007, 2008, 2009
Sigma Xi Grants-in-Aid of Research, 2006
Julius Thomas Hansen Travel Fellowship, UC Berkeley, 2006
Graduate Student Summer Fellowship, UC Berkeley, 2006, 2007, 2008
Ralph I. Smith Grant for Marine Research, UC Berkeley, 2005
Bermuda Biological Station for Research, Course Scholarship, 2005
Phi Beta Kappa, National Honor Society, inducted 2002
Beta Beta Beta, Biology Honors Society, inducted 2002
Dean's List, Illinois Wesleyan University, 1998-2000, 2001-2002
Golden Key International Honor Society, nominated 2001
Presidential Scholarship, Illinois Wesleyan University, 1998-2002

TEACHING EXPERIENCE

Graduate Student Instructor – Biology and Geomorphology of Tropical Islands,
University of California, Berkeley, 2009

Course Designer and Lecturer – Natural History and Human Interactions of Monterey
Bay, Osher Lifelong Learning Institute, California State University, Monterey Bay,
2009

Course Designer and Lecturer – The Deep Sea, Osher Lifelong Learning Institute,
California State University, Monterey Bay, 2008

Graduate Student Instructor – Invertebrate Zoology, University of California, Berkeley,
2006

Graduate Student Instructor – Animal Biology: A Behavioral View, University of
California, Berkeley, 2005

Graduate Student Instructor – General Biology, University of California, Berkeley, 2004,
2005

Teaching Assistant – Oceanography, Three Seas Program, Northeastern University,
2004

Teaching Assistant – Marine Conservation Biology, Three Seas Program, Northeastern
University, 2004

Teaching Assistant and Lab Instructor – Marine Biology, Harvard University, 2003

PRESENTATIONS

“Squids will be squids”

Biology and Geomorphology of Tropical Islands, Lecture Series, 2009

“Deep-sea squid covert activities...”

TONMOCON III, 2009

“Deep sea squid: What are they inking?”

STEM Colloquium, Hartnell College, 2009

“How to survive as a squid in the deep sea”

NOAA Ocean Explorer Teacher Enrichment Workshop, 2009

“Hunting and hiding in the dark: Predator-prey interactions in the deep sea”

Sea Creatures Rediscovered, Pacific Grove Natural History Museum, 2009

“Deep sea research: Understanding animal adaptations”

California State University, Channel Islands, Resource Management course, 2008

“Arm autotomy in a mesopelagic squid”

Micheli Lab, Hopkins Marine Station, Stanford University, 2008

“Deep sea research and squid defenses”

UC Berkeley Lawrence Hall of Science, MARE Summer Leadership Institute, 2008

“Defensive behavior of a deep-sea squid”

American Society of Limnology and Oceanography Conference, 2007

“Posing in the dark: Behavioral components of the deep-sea squid *Octopoteuthis deletron*” - World Congress of Malacology, 2007

“Autotomy as a deep-sea squid defense”

Society of Integrative and Comparative Biology Conference, 2007

“Animal adaptations to the deep sea”

Camp SEA Lab, 2007

“Ink release by deep-sea squid”

11th International Deep-sea Biology Symposium, 2006

“Why do deep-sea squid release ink in the dark?”

American Malacological Society Conference, 2006

“How does a midwater squid avoid becoming a sperm whale (or ROV) meal?”

MBARI Summer Intern Symposium, 2006

“A day in the life of a squid” - Bermuda Biological Station for Research Scientific Photography Course Symposium, 2004

“It’s pitch-black down here: Why are the squids still inking?”
MBARI Summer Intern Symposium, 2003

POSTERS

Robison, B. H., R. E. Sherlock, K. R. Reisenbichler, S. L. Bush. *In situ studies of the dark ocean: deep pelagic biology and ecology*. ASLO Aquatic Sciences Meeting, Nice, France, 2009

RESEARCH and FIELD EXPERIENCE

2004-present Researcher, deep-sea squid defensive behaviors. Ink release, ink chemical composition, marine organism reaction to cephalopod ink, deep-sea squid body patterning, deep-sea squid arm autotomy. Collaborator: Dr. Bruce Robison, Monterey Bay Aquarium Research Institute.

2007-present Researcher, liquid chromatography-mass spectroscopy of cephalopod ink. Collaborator: Dr. Ulla Anderson, University of California, Berkeley

2006 Assistant intern coordinator and researcher, deep-sea squid arm autotomy. Advisor: Dr. George Matsumoto, Monterey Bay Aquarium Research Institute.

2005 Researcher, stomatopod reaction to squid ink. Hawaii Institute of Marine Biology, Coconut Island.

2003 Intern, deep-sea squid ink release. Advisor: Dr. Bruce Robison, Monterey Bay Aquarium Research Institute.

2003 Researcher, courtship behavior in the Caribbean Reef Squid. Bonaire, Netherlands Antilles. Collaborator: Jennifer Mather, University of Lethbridge.

2003 Researcher, heat shock response in anemones. Independent study, Three Seas Program. Advisor: Dr. Michael Lesser, University of New Hampshire.

2003 Researcher, heterotrophy in corals. Discovery Bay, Jamaica. Independent study, Three Seas Program. Advisor: Dr. Pete Edmunds, California State University, Northridge.

2002 Researcher, primary productivity. Friday Harbor Laboratories, WA. Independent study, Three Seas Program. Advisor: Dr. Jan Newton, University of Washington.

2002 Researcher, bryozoan natural products. Advisor: Dr. Elizabeth Balser, Illinois Wesleyan University.

2001 Researcher, coral colonization. Orpheus Island Research Station, James Cook University, Australia. Advisor: Dr. Bette Willis, James Cook University.

2001 Station assistant. Orpheus Island Research Station, James Cook University, Australia.

RESEARCH CRUISES

R/V Nathaniel B. Palmer, Antarctica. MBARI, 2008, 2009

R/V Pt. Lobos, Monterey Bay. MBARI, 2003-present (64 cruises, incl. as chief scientist)

R/V Western Flyer, Monterey Bay; Oregon. MBARI, 2003-present (12 cruises)

R/V Derek M. Bayliss, Monterey Bay. MBARI, 2006

R/V New Horizon, Gulf of California, Mexico. University of Rhode Island, 2006

R/V Pt. Sur, Monterey Bay. Moss Landing Marine Labs, 2008

PROFESSIONAL SERVICE

Reviewer:

The Biological Bulletin

Marine Biology

Marine Biology Research

Journal of Experimental Marine Biology and Ecology

Oregon Sea Grant

EDUCATIONAL OUTREACH and VOLUNTEER WORK

Kayaking and Snorkeling Instructor, Young Women in Science, Monterey Bay Aquarium

Squid Science: Field Notes from Stephanie Bush, University of California Museum of Paleontology web feature

Elkhorn Slough Foundation and National Estuarine Research Reserve

California Coastal Cleanup Day, Asilomar Beach, Monterey, CA

Science Fair Judge, The Aptos Academy, Aptos, CA

PROFESSIONAL SOCIETIES and ORGANIZATIONS

American Academy of Underwater Sciences - Scientific Diver

American Malacological Society

American Microscopical Society

American Society of Limnology and Oceanography

Animal Behavior Society

Sigma Xi, Associate Member

Society for Integrative and Comparative Biology

Western Society of Naturalists

Women in Science, UC Berkeley

Chapter 1: Introduction

The oceans cover nearly 75% of the Earth's surface and have an average depth of 3800 m (Angel, 2003). The deep sea is comprised of everything below the photic, or epipelagic, zone (0 – 200 m; McFall-Ngai, 1990). The deep ocean is therefore the largest habitat on the planet, home to much of the planet's animal biomass and unexplored biodiversity (Robison, 2004; Warrant and Locket, 2004). Only a small fraction of it has been studied, however, due to the inherent difficulty in performing research in this vast and challenging ecosystem (Robison, 2009).

Low ambient light, high pressure and temperatures below 10°C are the most pertinent abiotic factors of the deep ocean (Robison, 2004). The light in the marine realm comes either from the sun or biological-derived illumination (bioluminescence). Much of the sunlight is reflected off the surface, and that which makes it into the water is absorbed and scattered by water molecules and particulate material, vastly reducing the light as depth increases (McFall-Ngai, 1990; Gál et al., 2001). Different wavelengths are absorbed unequally such that most red wavelengths fail to reach beyond 100 m depth, giving the ocean its blue color (Warrant and Locket, 2004). Nearly 90% of all light is removed by 100-200 m and no visible light gets beyond 1000 m (Tett, 1990).

Despite the low ambient light levels, midwater predators commonly have very sensitive vision (Schusterman, et al., 2000; Fristup and Harbison, 2002; Warrant and Locket, 2004). There are no safe havens in this three-dimensional habitat lacking structures and deep-sea animals must rely upon their own camouflage to remain undetected (McFall-NGai, 1990; Robison, 2009). Animals across numerous phyla have evolved similar mechanisms of crypsis (McFall-Ngai, 1990). In the epipelagic and upper mesopelagic, individuals commonly have reflective sides, counter-shading, or whole-body transparency (Denton, 1970; Johnsen, 2001). In the mesopelagic (200 – 1000 m), species have red, purple, brown or black body coloration, which appears black in the absence of red light (Johnsen, 2005). Light down-welling from the surface is many times greater than that reflected laterally or from below and predators looking upward may decipher the silhouette of potential prey therefore many deep-sea animals cancel this by possessing downward directed photophores that counter-illuminate their bodies (Young, 1977; Young, 1983).

Food in the deep ocean derives from epipelagic productivity (Robison, 2009). It makes its way through the surface waters uneaten or is consumed by the large numbers of diel vertical migrators that feed on epipelagic prey at night and return to depth at dusk (Barham, 1966). Food availability, and therefore animal densities, are low (Herring, 2002). Low light, cold surroundings, limited food availability, and the fact that animal movement may stimulate bioluminescence have selected for lowered animal metabolisms and activity (Childress, 1995; Seibel et al., 1997; Robison, 2004).

Shallow-water cephalopods have well-developed visual senses used to perceive their surroundings and rapidly modify their skin coloration and texture, body postures, and locomotion to blend in, resemble a non-edible object, break up their body outline (disruptive coloration), or appear larger than they are (Hanlon and Messenger, 1996). Deep-sea cephalopods, however, were assumed to have limited visual behaviors because of the abiotic features of the deep sea discussed above (Voss, 1967).

The advent of Human Operated Vehicles (HOVs) and Remotely Operated Vehicles (ROVs) have allowed observations of animals that have rarely been observed alive, let alone in their natural habitat. An ROV is a tethered submersible equipped with lights and cameras for locating and observing marine organisms, and may also have manipulators and collection devices (Robison, 1993). The Monterey Bay Aquarium Research Institute, founded by David Packard, is a consortium of scientists and engineers dedicated to studying the deep ocean and developing technologies for it. Over the last 20 years, MBARI has had three ROVs: ROV *Ventana*, ROV *Tiburon*, and ROV *Doc Ricketts*. Initial observations using these ROVs indicated that deep-ocean squid behaviors were not as limited as originally thought. We consequently used these ROVs to obtain additional observations, collect squids for laboratory examinations, and performed *in situ* experiments to explore the behaviors of deep-sea squids. Throughout this work we assume that observed behaviors are at least a subset of those naturally occurring in the species studied and that the most probable responses to encounter with an ROV are defensive.

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- Young, R. E. 1983. Oceanic bioluminescence: an overview of general functions. *Bulletin of Marine Science* **33**:829-845.

Chapter 2: Ink Utilization by Mesopelagic Squid

Abstract Remotely operated vehicle dive video recordings of deep-sea squid ink release were examined to determine species, ink release type, release depth, and accompanying behavior/s. Ink release was commonly observed between the surface and 1842 m in Monterey Bay, CA, and surrounding waters. Six ink release types were observed: pseudomorphs, pseudomorph series, ink ropes, clouds/smokescreens, diffuse puffs and mantle fills. Each species released ink throughout all or most of its depth range; inking was not limited to shallow, sunlit waters. Individuals of each species released one ink release type more commonly than other types, however, multiple ink types could be released by individuals of all species. Common behaviors preceded and/or followed each release type; pseudomorphs and pseudomorph series were generally associated with escape behaviors, while ink ropes, clouds, and puffs normally involved the animal remaining adjacent to or amid the ink. Deep-sea squids may use ink for defensive purposes similar to those of shallow-dwelling species when they release pseudomorphs, pseudomorph series, or large clouds, and may use ink puffs in intra-specific communication. The function of ink ropes and mantle fills is unknown.

Introduction

The coleoid cephalopods, squid, octopus, and cuttlefish, gained enhanced mobility by losing the protective external shell utilized by their ancestors (Wells, 1994). However, this evolutionary step also made them more vulnerable to predation by marine mammals, sharks, fish, seabirds, and other cephalopods (Clarke, 1977; Guerra et al., 1993; Dunning et al., 1993; Thompson, 1994). Perhaps in response to increased predation risk, coleoid cephalopods exhibit a complex repertoire of escape and predator avoidance behaviors. Well-known examples of these behaviors include elaborate skin color and texture modifications, frequent postural changes for crypsis and polyphensim, or rapidly changing of physical appearance, and the discharge of ink to distract predators, preventing attack, and allowing escape (MacGinitie and MacGinitie, 1968; Dilly and Herring, 1978; Moiseev, 1991; Vecchione and Roper, 1991; Hanlon and Messenger, 1996; Hunt, 1996; Forsythe and Hanlon, 1997).

For cephalopods, changes in skin coloration or body shape may be metabolically 'cheap' compared to ink release (Boletsky, 1987; Hanlon and Messenger, 1996). Skin chromatophores and erector muscles have direct neural connections to the brain (Florey, 1969; Dubas et al., 1986), and posture change requires moving the highly flexible appendages (Hanlon and Messenger, 1996), allowing for near instant, ever-changeable, neurally controlled defenses. Inking, however, requires the high-energy production and expulsion of melanin and mucus, which shunts calories from growth and reproduction (Russo et al., 2003). However, because inking is commonly observed, it is assumed to be a common and effective predator avoidance technique (Hanlon and Messenger, 1996; Nolen and Johnson, 2001).

Inking has been commonly categorized as one of two types: 'clouds' or 'smokescreens', which are large, diffuse releases of ink, and 'pseudomorphs', which are dense blobs of ink the approximate size and shape of the individual releasing them (MacGinitie and MacGinitie, 1968; Lucero et al., 1994; Hanlon and Messenger, 1996; Huffard and Caldwell, 2002; Caldwell, 2005). The former obscures the animal from view, presumably deceiving the predator as to the location of the potential prey while

the cephalopod remains amid the ink (Moynihan, 1985). The latter resembles the animal and may function as a decoy while the individual escapes (Moynihan and Rodaniche, 1982, Caldwell, 2005). We do not ignore the possibility that ink may contain chemical deterrents in addition to, or in lieu of, these visually deceptive uses (MacGinitie and MacGinitie, 1968; Lucero et al., 1994).

Both pseudomorphs and clouds seemingly rely on simple visual deception. Most observations have been made in shallow, sunlit waters, therefore, it has been assumed that the use of ink is largely a visual defense that would be useless at night and for deep-sea cephalopods (Wells, 1978; Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1996). It should be noted, however, that nocturnal, shallow-water cephalopods sometimes release ink (Anderson and Mather, 1996; Christine Huffard, personal communication, personal observation). More importantly, squid have been observed releasing ink at depth, even below 200 m, beyond which more than 99% of surface illumination has been attenuated (Tett, 1990; Hunt, 1996; Hunt and Seibel, 2000; Robison, 2004). Such observations call into question the hypothesis that ink release plays a primarily visual role and point to our currently limited understanding of deep-sea ecology.

Undersea vehicles are the principal means by which observations of mesopelagic cephalopods, fishes, and zooplankton have been made (Barham, 1963, 1966; Alldredge et al., 1984; Widder et al., 1989; Vecchione and Roper, 1991; Moiseev, 1991; Seibel et al., 2005). Characteristic of these observations are the lights and vibrations that accompany a vehicle's presence. While these disturbances undoubtedly affect the animals being observed, these effects have yet to be thoroughly quantified (Hunt et al., 2000; Widder et al., 2005). Anecdotal evidence (Robison et al., 2003) suggests that dark-adapted animals are temporarily blinded by vehicle lights and it is worth noting here that many squids remain in place as a vehicle approaches (Vecchione and Roper, 1991; Moiseev, 1991; Hunt et al., 2000; this paper).

The current study aimed to describe ink released by deep-sea squid so that hypotheses regarding its functionality could be refined. Additionally, we tested the hypothesis that ink release frequency would decrease with diminishing ambient light levels and therefore predicted that fewer ink releases would occur as depth increased.

Materials and Methods

Ink release observations were obtained from the dives of two remotely operated vehicles (ROVs), *ROV Ventana* and *ROV Tiburon*, operated by the Monterey Bay Aquarium Research Institute (MBARI). Each MBARI ROV dive is recorded on High Definition (HD) and/or digital-beta videocassettes. With over 2900 *Ventana* dives and over 1000 *Tiburon* dives, there are more than 14,000 hours of video observation recorded from the waters of the Monterey Bay, CA, and surrounding areas. The appearance and behavior of encountered biological entities and physical features are annotated during and after dives by scientists and video-lab staff using the MBARI-designed Video Annotation Reference System (VARS) software (Robison, 1993). Annotations are matched to depth, temperature, salinity, light transmission, and location, and the resulting database is searchable for observations of interest.

Each squid ink release sequence was viewed multiple times at full and slow speeds. The following information was noted: squid species, time at which the animal

or ink was first observed, characteristics of position, posture, coloration, and behavior of the squid, and the ink release type. Data were summarized in Microsoft Excel to visualize patterns. Ink releases were binned into 100 m depth intervals to examine changes in release frequency with depth.

Results

We observed ink release by 878 individuals of at least 18 species (Table 1). Our hypothesis that ink release frequency would decrease with increasing depth was not supported, as we observed an increase from 300 – 600 m followed by a decrease (Fig. 1). This decrease reflected the decrease in overall squid occurrence with depth (data not shown). The majority of ink releases occurred within the mesopelagic (200 – 1000m, n = 742); a smaller number occurred in the bathypelagic (1000 – 4000m, n = 59; Fig. 1). The one hundred thirty-seven releases observed within epipelagic waters (0 – 200m) were primarily of *Loligo opalescens* (96 of 130 ink releases). The shallowest depth at which we observed ink release was 10.56 m below the surface (*L. opalescens*); the deepest observed ink release occurred at 1842.1 m (*Octopoteuthis deletron*).

Six ink release types were observed: pseudomorphs, 'pseudomorph series' (2 or more consecutively released pseudomorphs), 'ropes', 'clouds', 'puffs', and 'mantle fills'. Pseudomorphs and clouds were categorized as described above. The other ink release types were categorized according to the following definitions. Ink ropes consisted of long, continuous releases of ink. Short ink releases, that dissipated rapidly, were categorized as puffs. Mantle fills were exhibited only by the cranchiid *Galiteuthis phyllura*, which held the mantle closed and released ink inside the transparent mantle cavity, thereby becoming opaque.

Pseudomorphs were the most common type of ink release observed (n = 326). Between the surface and 100 m pseudomorphs were released most commonly by *Loligo opalescens*; from 300 - 600 m pseudomorphs were released by all other species observed. Ropes and ink clouds were the second and third most frequent release types (n = 204 and 203, respectively). Ink ropes, most commonly released by adult *Gonatus onyx*, were frequent from 300 – 1100 m, and *G. onyx* released most of the clouds that occurred from 300 – 700 m. Pseudomorph series, the fourth most commonly observed ink releases (n = 134), were released mostly from 300 – 700 m and were predominantly released by *G. onyx* and *Chroteuthis calyx*. The majority of puffs released occurred from 300 – 500 m (n = 40), and were released by mostly by *L. opalescens*, but also *C. calyx* juveniles, *Dosidicus gigas*, *G. onyx* adults and juveniles, *Octopoteuthis deletron*, and Unknown sp. B. Fifteen *G. phyllura* utilized mantle fills from 400 – 1100 m.

Each species tended to release a particular ink type more frequently than any other. However, individuals of most species were capable of releasing up to five different ink release types. For example, *G. phyllura* utilized mantle fills more often than other ink types, but individuals were also observed to release pseudomorphs, pseudomorph series, clouds, and ink ropes. The release type coordinated with the individual's behavior. Typically, the release of a pseudomorph was followed by the animal's jet-escape (186 of 246 pseudomorph releases for which behavioral data were available), sometimes with a quick change in trajectory. Behavior following the release of a series of pseudomorphs depended upon species; *C. calyx* moved away without a jet-escape from released ink 17 of 31 times, whereas *G. onyx* jet-escaped in 24 of 28

instances. When we encountered a cloud, the squid that had emitted the ink was found either within or next to the ink cloud in 171 of 181 observations. A thick cloud in which the animal was only intermittently visible was characteristically produced by more active species such as *Dosidicus gigas* or *G. onyx*. A more diffuse cloud that surrounded the motionless individual was observed from less active species such as *C. calyx* or *Histioteuthis heteropsis*, or when individuals had inked prior to encounter with the ROV. Squids were usually visible in a position immediately next to or near ink ropes (93 of 132 occurrences).

Despite these patterns, however, ink release behavior was variable. For example, juvenile *C. calyx* released pseudomorph series then hid among the pseudomorphs in the same orientation (n = 11 of 25). Also, some individuals were observed to release more than one ink type; 71 individuals were observed releasing two ink release types and 10 individuals were observed releasing three ink types. For example, a few *G. onyx* released clouds in which they remained briefly, then jet-escaped while releasing a pseudomorph (n = 5).

Descriptions of each species' inking behavior are given below. The numbers presented comprise ink releases for which associated behaviors were determined. Short video sequences demonstrating some of the following descriptions can be found at www.mbari.org/midwater/squidink.

Species Accounts

Ancistrocheirus lesueurii

A light-colored individual was observed while swimming downward using its large fins, holding the arms straight and together. The squid stopped, turned red, and positioned the mantle horizontally with the arms curled down under the head. The tentacles were stretched down below the arms with the clubs clasped together. The animal contacted the ROV and released a large diffuse cloud, remaining within it (1554.9 m).

Bathyteuthis berryi

This species has only been encountered four times by MBARI ROVs; three of these sightings included the release of ink. Of the four, two individuals had already released ink prior to observation by the ROV. Of these, one individual (1342.1 m, Fig. 2A) was positioned vertically, arms pointed downward, next to four interconnected ink releases creating a structured cloud. We observed this individual for a few seconds before it made two mantle contractions and escaped from view. The second of these individuals (1350.75 m) was observed in the same vertical posture, surrounded by a series of five pseudomorphs and directly above a similarly structured cloud. An additional thin ink rope was released during direct observations of this second individual. The third observed inking individual (1424.4 m) was initially viewed without attendant ink, positioned vertically in the water. After a few minutes observation it rapidly jetted in a large loop, released a pseudomorph, and escaped from view.

Berryteuthis cf. anonychus

We observed a single individual (1483.84 m) for several minutes, during which time it changed posture from horizontal to vertical to J-pose, with mantle tip pointed

vertically upwards and arms pulled dorsally and back, all the while slowly moving deeper. After the individual came into contact with the ROV, it released a pseudomorph and downward jet-escaped.

Chiroteuthis calyx

Adult individuals were commonly encountered in the 'fishing' posture, with feeding tentacles held within grooves of the fourth arms and extending vertically below the body (Hunt 1996). The arms and proximal portion of the mantle remained horizontal while the distal portion tilted upward. Individuals were sometimes sighted in this posture within a diffuse cloud of ink (n = 8 of 16 clouds). *Chiroteuthis calyx* adults were relatively slow to react to the ROV presence, or even to retract their tentacles until they had been watched for some time and/or pursued. When approached closely, the individual commonly assumed a horizontal posture, retracted the tentacles, arrayed the arms in an inverted 'V' bowed outward but touched distally and the webs of the arms spread and turned red. The dorsal mantle reddened when the individual was disturbed, and a further disturbed *C. calyx* will turn red around and between the eyes. Adult animals either remained within or adjacent to released pseudomorphs or pseudomorph series (n = 4) or attempted escape (n = 5).

Juveniles of the family Chiroteuthidae, doratopsis paralarvae, have more elongate necks than adults, as well as an extension of the gladius, the 'tail', which supports ornamentation (Vecchione et al. 1992). *Chiroteuthis calyx* doratopsis paralarvae were encountered in either a horizontal or vertical position, but then typically assumed a vertical orientation, with the tail pointing downward. They occurred at shallower depths than adults and released ink more commonly, producing anywhere from one to 30 pseudomorphs of the same length and approximate thickness as the body (n = 54, Fig. 2B). We often observed individuals remaining motionless among several pseudomorphs they had released (n = 11). Although less common, juveniles also released clouds of ink in which they remained in a straight or bent horizontal position (n = 14).

Dosidicus gigas

Humboldt squid, *D. gigas*, commonly occur from Baja California to South America, and only intermittently in Monterey Bay, albeit with increased frequency in recent years. The most common types of ink released were huge clouds or ropes (n = 14 and 3, respectively, Fig. 2E). Individuals were observed in J-pose moving down and away from ink clouds released from their upward pointed funnel. Following ink release, the now non-inking funnel was directed downward propelling the individual up within the ink cloud. Individuals occasionally hid in another squid's cloud, sometimes adding their own ink to it. In other instances cloud ropes or large pseudomorphs were produced as the animal jetted by the ROV, the ink blocking the camera for several seconds.

Galiteuthis phyllura

This species frequently displayed the 'cockatoo' posture, in which the body is positioned horizontally with the arms held together above the eyes at 90° to the body axis (Moiseev 1991; Vecchione and Roper 1991). This was commonly accompanied by

a reddening of the arms and the skin around the eyes, as well as expansion of the sparse mantle chromatophores. When the animal is highly disturbed, the deep-red arms are pulled back around the body like an inverted umbrella, revealing the buccal mass and beak. Usually, such changes in posture and skin coloration were favored over ink release. On several occasions however, we observed *G. phyllura* releasing ink into its inflated mantle cavity (n = 15, Fig. 2D), then releasing it from the mantle cavity in short, consecutive puffs or continuously seeping the diffuse ink out either side of the siphon. Less frequently, individuals released a pseudomorph or pseudomorph series and either remained near it (n = 2), quickly moved a few meters away (n = 1), or jet-escaped (n = 5).

Gonatus onyx

We encountered the deeper-dwelling, solitary adults more frequently than shallow-living, congregating juveniles, and accordingly observed more of the former releasing ink (n = 195 adults, 45 juveniles). *Gonatus onyx* employed five inking strategies, each with a commonly associated behavior. Ink ropes were principally released by adult *G. onyx* (Fig. 4). An ink rope could consist of one continuous release of ink or multiple consecutive ones separated by small gaps. Ink was released in various configurations: straight or curved, V-shaped or J-shaped, or irregular, with blunt or tapered ends (Fig. 2F). These releases were common (n = 85) and typically, we came upon an extant ink rope with the animal located above, below, or at either end holding its position. As the ROV advanced, the individual turned a deep red and changed to a J-pose, the body vertical with arms curled upward toward the dorsal mantle (Moiseev 1991; Hunt and Seibel 2000). Upon further approach, the animal turned white and jet-escaped, mantle-tip downward, in a few cases releasing a pseudomorph (n = 5). Pseudomorphs or pseudomorph series were often the sole type of ink released (n = 69 and 19, respectively); in most cases the individual jetted past the ROV, during or after release (n = 72).

Adult *G. onyx* occasionally released clouds that generally blocked the animal from view as it hid inside. Clouds were observed less frequently than other releases (n = 39); they occurred when the ROV was relatively close to the individual, or in some cases when it bumped into the ROV as it was jetting past.

Juveniles of *G. onyx* released pseudomorph series, producing a linear or curving path of up to 8 small pseudomorphs as they jet-escaped downward (n = 15). Several individuals released a single pseudomorph and jet-escaped (n = 15). We also observed juveniles releasing clouds (n = 9) and ink ropes that were shorter than adult-released ropes (n = 8).

Grimalditeuthis bonplandi

The single individual observed (1014.93 m) was positioned almost horizontally, with the distal portion of the mantle and fins pointed upwards 45°. This posture was similar to a commonly observed *C. calyx* posture, a member of the same family. The *G. bonplandi* individual was next to two short, vertical, straight ink ropes. After a few minutes of observation, the individual inked a pseudomorph as it jetted downward, and inked another pseudomorph upon collection in the ROV's sampler bucket.

Helicocranchia cf. pfefferi

One individual of this cranchiid species was encountered. This individual was moving down when first encountered (951.2 m) and soon released a pseudomorph, later releasing three more while jetting between stationary periods. At rest, this individual oriented itself horizontally and held its' arms up vertically (cockatoo posture) while the distal portions of arms I and II drooped forward. Based upon arms I and II sucker configuration we determined the individual was a male (Young, 1972).

Histioteuthis heteropsis

Individuals were commonly encountered in the 'J-pose', with the distal portion of the arms tightly curled back toward the dorsal mantle or to the sides of the body. Individuals typically spinned and moved slowly away when approached. If followed, individuals commonly changed to a vertical posture with arms held together and pointed downwards, and began downward movement; in some cases we encountered individuals already in this vertical posture. The body was often bent such that the large, left eye was directed upward and the smaller eye slightly downward (see Young 1975a). *Histioteuthis heteropsis* sometimes released ink without prolonged disturbance, but usually inked only when approached closely or bumped. Ink clouds were released, often while in J-pose (Fig. 5, n = 4 of 5 clouds) and the individual always remained within the released cloud. Pseudomorphs were released, generally from a straightened or non-J-pose posture (n = 5 of 6 pseudomorphs). In four of six observations pseudomorph release was followed by jet-escape (Fig. 2C); individuals jetted after pseudomorph series in two of three occurrences.

Histioteuthis hoylei

Two *H. hoylei* individuals have been encountered during MBARI dives; one released ink (500 m). The individual's body was vertically oriented with arms held together and pointed straight downward. It used fins to spin on its axis, tending to face the ROV with the smaller, downward directed eye. Contact with the ROV resulted in the release of a pseudomorph as the animal jetted downward. A second pseudomorph was released several minutes later as the animal moved slowly upwards.

Loligo opalescens

These fast-moving, schooling squid were commonly encountered in shallow water as they utilized the bright lights of the ROV to hunt. At times, large groups jetted by the ROV and only one individual inked, whereas at other times multiple individuals remained around the front of the ROV and suddenly inked due to the activity of the ROV or other unknown disturbance. Ink releases were pseudomorphs (n = 89), pseudomorph series (n = 17), a cloud (n = 25), a short rope (n = 17) or a quickly diffusing puff (n = 18). The release of a pseudomorph or short rope was usually followed by quick jet-escape, while individuals commonly maintained position next to cloud releases or puffs.

Moroteuthis robusta

A single individual was observed posed vertically. There was a notable greyish coloration. We approached with a suction sampling device, pulling the arms into the

collection tube. The individual turned red and released a pseudomorph as it was pulled into the sampling container (440.3 m).

Octopoteuthis deletron

Individuals were usually encountered in a horizontal position, arms held apart either curled back over the dorsal mantle or held upward. We observed some individuals remaining in this posture for an extended time, however, other individuals immediately began to move downwards. When disturbed individuals sometimes changed color from light red to deep red, sometimes with alternating light and dark red bars or stripes (26 of 67 individuals changed color). In all cases in which clouds were released, the individuals remained within or near the ink release (n = 23). Less frequently, we observed individuals releasing pseudomorphs (n = 10) or pseudomorph series (n = 3), accompanied by attempted jet-escape, or ink ropes (n = 3), next to which they stationed themselves. Five individuals released very diffuse puffs; in one of these cases the squid then jet-escaped, otherwise individuals remained by these releases.

Planctoteuthis spp.

Seven of ten *Planctoteuthis spp.* individuals encountered released ink: three *P. oligobessa*, two *P. danae*, and two that could not be identified to species. Two *P. oligobessa* were encountered with straight vertical bodies and arms pointed down. One individual then took the J-pose posture and later released a pseudomorph upon collection (802 m). The other individual released a series of three pseudomorphs as it jetted away after contacting the ROV (1350.82 m). The third *P. oligobessa* released 5 pseudomorphs (802.5 m), then posed horizontally, pulled the arms up, then went into J-pose. One *P. danae* began in a horizontal posture with arms up (964 m), while the other was encountered in J-pose (1182.4 m). Both individuals released pseudomorphs upon collection. One *Planctoteuthis sp.* began in J-pose and released a pseudomorph (1169.2 m) as we attempted to collect it. The other *Planctoteuthis sp.* was positioned vertically with the arms down and released three pseudomorphs as it jetted upward (1835 m).

Taonius cf. borealis

We observed one individual of this species. Upon encounter the individual already displayed the cockatoo posture with red arms and eyes, with some open mantle chromatophores. The animal released a series of two pseudomorphs (638.5 m) as it jetted away then after pursuit released another series of two pseudomorphs and jet-escaped.

Unknown sp. B

Large individuals, most likely of the family Gonatidae, have been observed several times traveling in small groups. Individuals most commonly released pseudomorphs and jet-escaped (n = 11). Clouds were also released, with the individual remaining within or next to the release (5 of 6 clouds). One individual released a pseudomorph series and remained near the ink. Lastly, individuals released diffuse puffs (n = 5) while jetting, holding onto the ROV, or remaining near the ink.

Unidentified spp.

In 243 cases we were unable to identify the species of squid releasing ink because 1) the squid released the ink while jetting past too fast for a clear view or out of view, 2) we were unable to stop the ROV in time and passed by, or 3) the individual was too far away to observe definitively.

Discussion

Contrary to previous generalizations (Voss, 1967; Hanlon and Messenger, 1996), the squids observed in this study utilized ink throughout their depth ranges, to a maximum depth of 1842 m. The majority of our observations occurred at depths beyond which sunlight penetrates, where visual skills are limited to possibly discerning shadows from below or to detecting organisms via reflected bioluminescence (Young and Roper, 1976; Robison, 1999). If ink release was not an effective defense for these species, then the ability to either produce and/or release ink would likely be lost over time as has occurred for many deep-sea octopuses (Voight, 1997). Although the specific function of ink released by deep-sea squid is uncertain, these species may use ink as a decoy (pseudomorph) or smokescreen (cloud), similar to ink release behavior described for shallow-dwelling cephalopods (Hanlon and Messenger, 1996). A midwater predator may locate prey by observing from below and attempting an attack. If a squid releases a pseudomorph in its place while jetting away, then the predator may attack the ink and the potential ensuing confusion might allow the squid time to escape (Hanlon and Messenger, 1996, Caldwell, 2005). On the other hand, ink cloud release creates large shadows within or behind which a squid may hide, providing a refuge from bioluminescence from any direction or from predators searching for silhouettes. Cloud release may not require the high-energy jet-escape maneuvers normally associated with pseudomorph or pseudomorph series release.

The diffuse puffs released mainly by *L. opalescens*, *D. gigas* and Unknown sp. B, all of which travel in small to large groups, may be adapted for intra-specific communication. These ink releases appear to contain little mucous and diffuse rapidly into the surrounding water, where they may be detected by conspecifics. Gilly and Lucero (1992) demonstrated that individuals of *L. opalescens* responded to a 1:20 dilution of ink or L-Dopa, a precursor to the main ink component melanin, with increases in funnel contractions. In this species, the detection of ink or its constituents elicits an escape response. Instead of an individual using ink to deceive a predator, in these cases individuals may use ink to warn conspecifics or otherwise signal group members in a darkened environment where visual communication is not possible (Lucero et al., 1994).

Although pseudomorphs, pseudomorph series, puffs and clouds may fit into the functional descriptions presented for shallow-water cephalopods, we have described observations of two release types that have not been well-explored or explained. Some cranchiids fill their mantle cavity with ink, as discussed above for *G. phyllura*. Only the eyes, slender digestive gland, and ink sac of these otherwise transparent squid create shadows for predators searching from below (Young, 1975b; Robison, 1999), and they possess counter-illuminating photophores used to cancel out these shadows (Robison, 1995; Johnsen, 2001). However, ink release inside the mantle cavity may enhance the squids' ability to remain cryptic at various depths by making individuals opaque.

Johnsen (2001) discusses the prevalence of transparency at euphotic and dysphotic depths, and a shift to red or black coloration at aphotic depths. Red and black organisms absorb the blue-green wavelengths of most bioluminescence, whereas transparent organisms scatter this light and thereby compromise their crypticity (Johnsen, 2001; 2002). Additionally, some shallow-water cephalopods can see light polarization, allowing them to pick out transparent prey (Shashar et al., 1998). Deep-sea squid prey upon each other (personal observation), and if they also see polarized light they may be able to break the camouflage of transparency. Filling the mantle cavity with ink may protect against this search method by blocking the polarized light reflected from the transparent mantle tissue. We observed *G. phyllura* releasing ink into the mantle cavity at both dysphotic and aphotic depths. Individuals do not seem adversely affected by this behavior as we have observed individuals for up to 20 min *in situ* after ink release with no apparent ill-effect. Collected individuals maintained in the laboratory appeared undamaged up to several hours after ink was released into the mantle cavity.

The other novel ink release utilized in the deep sea was an ink rope. Adult *G. onyx*, for example, most commonly release long ropes of ink, which may resemble the form of an elongate, stinging siphonophore such as *Apolemia spp.* Shape mimicry is a very common defensive tactic used by deep-sea animals; there are no physical features to hide behind and thus many species disguise themselves (Robison, 1999).

A complementary use of ink by mesopelagic squids may be as a chemical deterrent to predation, whereby the ink causes confusion or is noxious to the predator. Previous authors have described animals becoming confused when they encounter the ink of shallow-water cephalopods (MacGinitie and MacGinitie, 1968; Moynihan and Rodaniche, 1982). Prota et al. (1981) suggested that the tyrosinase found in ink could be the cause of observed irritation to organisms, and Russo et al. (2003) found that tyrosinase in the ink of *Sepia officinalis* was toxic to a number of cell lines derived from rats and humans. A deterring or noxious chemical component in cephalopod ink may help explain its use at any depth. A cloud of ink provides a large volume of possible chemical deterrence, creating a refuge so the squid does not have to out-swim a potential predator. Pseudomorphs provide a more concentrated, possibly higher dose to a predator that mistakenly attacks it. Ink chemical deterrence suggests an extra metabolic expense to the already costly behavior of inking, in a habitat where animals already have low metabolic rates. Alternatively, we have observed that deep-sea squid mucus can be very glutinous and may hinder some predators that use water vibrations for prey detection. For example, pinniped detection of prey using vibrissae may be compromised by covering these delicate sensory structures in viscous material (Ahl, 1986; Watanabe et al., 2004).

Another possibility may be distraction or temporary blinding of a potential predator with a bioluminescent cloud. This tactic has been attributed to several deep-living fishes and crustaceans that release glowing clouds (Young 1983). However, among cephalopods, only species of the sepiolid genus *Heteroteuthis* are known to release luminous fluid with their ink (Herring, 1977; Dilly and Herring, 1978; P. Herring personal communication). The archaic, bathypelagic cephalopod *Vampyroteuthis infernalis* releases a cloud of dimly glowing particles from its arm tips when it is harassed (Robison et al., 2003), but it has no ink sac. While our laboratory investigations are not complete, we have never observed luminous ink discharged from

any of the squid species we have examined. Thus there is, to date, little or no evidence of a luminous component to the inking behavior we have reported.

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Table 2-1. Squid species, ink releases observed, and depth range of releases. Unidentified spp. were jetting or observed too briefly for identification. They likely represent a combination of the listed species and not undescribed spp.

Species	Individuals releasing ink	Depth range (m)
<i>Ancistrocheirus lesueurii</i>	1	1554.9
<i>Bathyteuthis berryi</i>	3	1342.1 - 1424.4
<i>Berryteuthis cf. anonychus</i>	1	1483.84
<i>Chiroteuthis calyx</i>	Adults = 27 Juveniles = 66	49.6 - 637.53 122.7 - 708.66
<i>Dosidicus gigas</i>	24	69.2 - 707.9
<i>Galiteuthis phyllura</i>	29	174.3 - 1172.1
<i>Gonatus onyx</i>	Adults = 195 Juveniles = 45	18 - 1725.8 154.22 - 1263.8
<i>Grimalditeuthis bonplandi</i>	1	1014.93
<i>Helicocranchia cf. pfefferi</i>	1	951.2
<i>Histioteuthis heteropsis</i>	12	373.9 - 722.5
<i>Histioteuthis hoylei</i>	1	500
<i>Loligo opalescens</i>	154	10.56 - 482
<i>Moroteuthis robusta</i>	1	440.3
<i>Octopoteuthis deletron</i>	41	347.7 - 1842.1
<i>Planctoteuthis danae</i>	2	964 - 1182.4
<i>Planctoteuthis oligobessa</i>	3	802 - 1350.82
<i>Planctoteuthis sp.</i>	2	1169.2 - 1835
<i>Taonius cf. borealis</i>	1	638.5
Unidentified gonatid	25	290 - 502.8
Unidentified spp.	243	23.6 - 1631
Total	878	10.56 - 1842.1

Figure Legends

Figure 2-1. Squid ink releases observed in 100 m depth intervals from the surface to 2000 m. Only releases for which depth data were available are included.

Figure 2-2. (A) *Bathyteuthis berryi* amid a diffuse ink cloud. (B) *Chiroteuthis calyx* juvenile below a pseudomorph. (C) *Histioteuthis heteropsis* jet-escaping after pseudomorph release. (D) *Galiteuthis phyllura* with ink-filled mantle cavity. (E) *Dosidicus gigas* with an ink cloud. (F) *Gonatus onyx* near an ink rope.

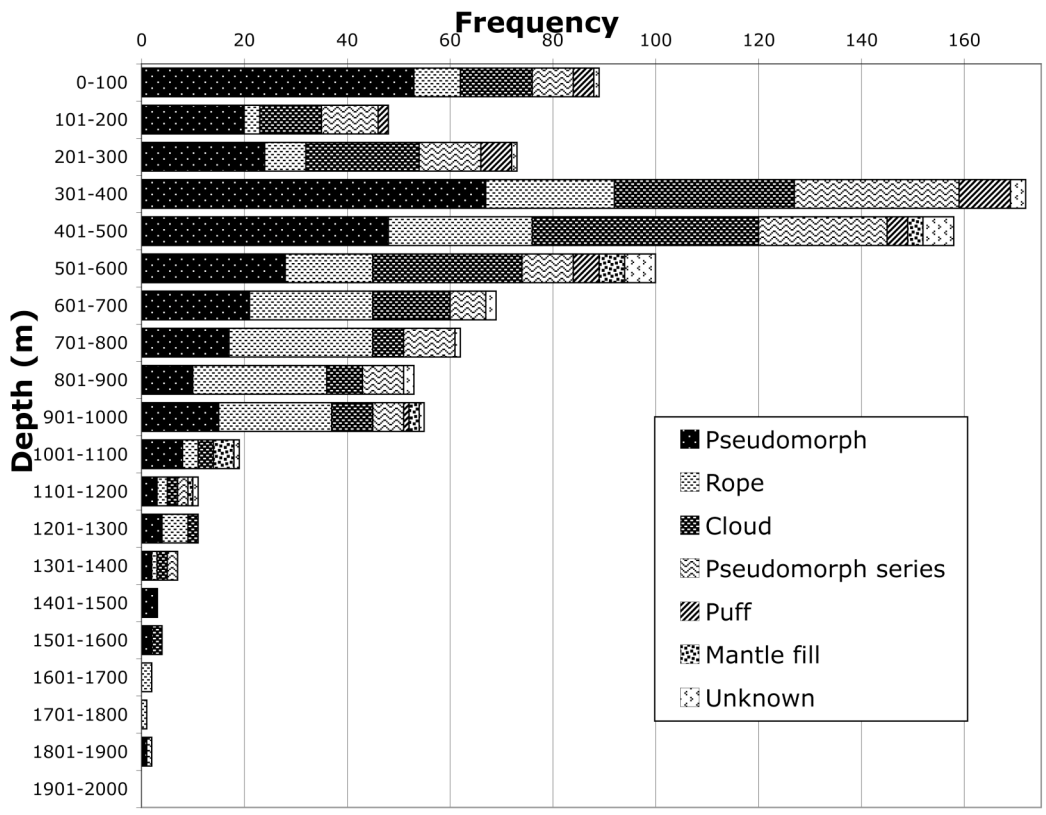


Figure 2-1

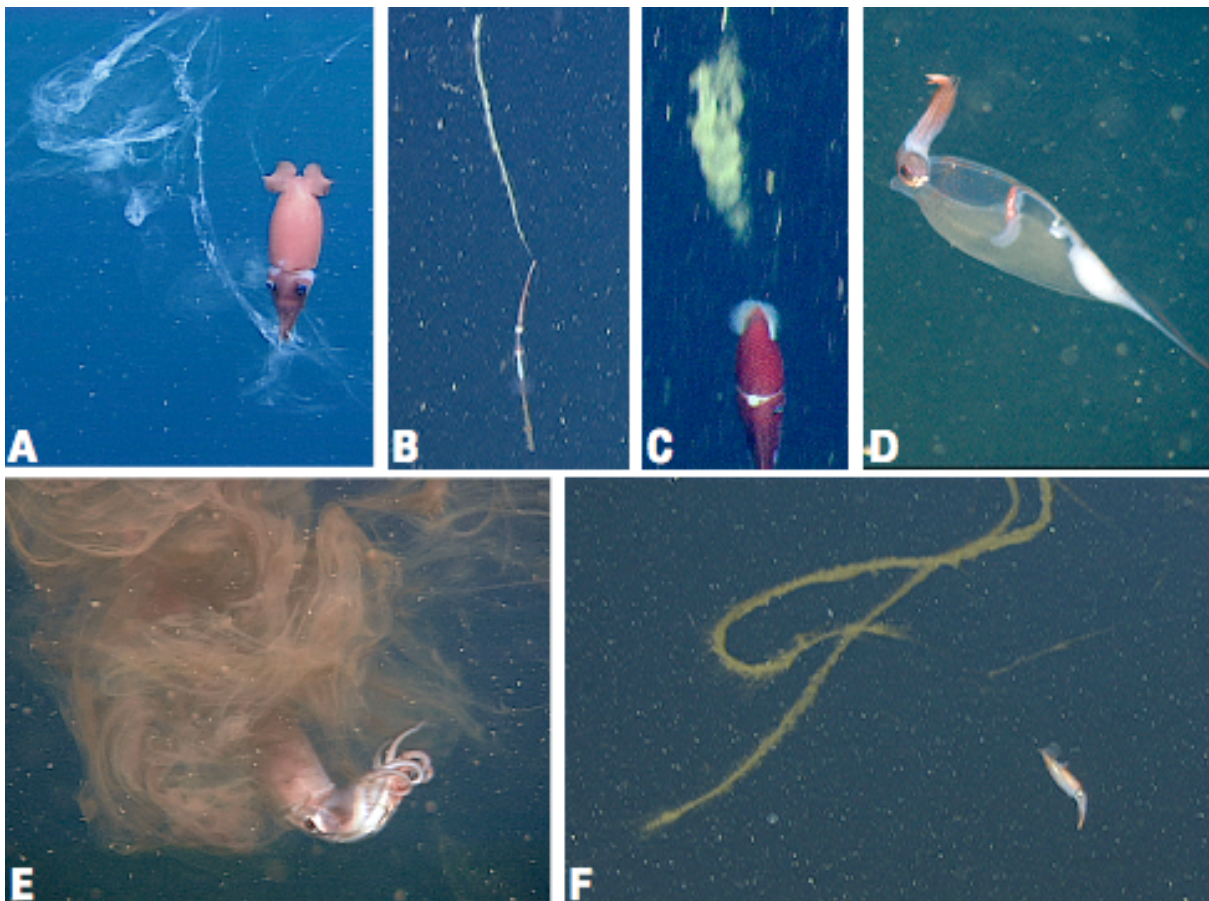


Figure 2-2

Chapter 3: Liquid Chromatography – Mass Spectroscopy Comparison of Cephalopod Inks

Abstract Cephalopod ink was compared using liquid chromatography – mass spectroscopy to look for compositional differences between deep-sea and shallow-water species. Differences were found between pure ink taken directly from the ink sac and released ink due to the presence of mucous in released ink. Only one analysis of a shallow-water cephalopod was successful. Ink from this shallow-water octopus, *Octopus bimaculoides*, did not differ greatly from deep-sea squid inks.

Introduction

Cephalopod ink release is a well-known but poorly studied secondary defense. The dark, melanin-based material released in the presence of a potential predator is assumed to visually confuse the pursuer, allowing the cephalopod to escape. There are two commonly discussed types of ink release: (1) copious ink forming a cloud or smokescreen, presumed to visually block the cephalopod from view, and (2) a pseudomorph ('false body') of the approximate size as the releaser, that is confused for the cephalopod and attacked (Boletzky, 1997). Among the few studies to look at cephalopod ink release, Caldwell (2005) found that green turtle (*Chelonia mydas*) hatchlings ceased attacks on adult pygmy octopuses (*Octopus bocki*) after biting into ink pseudomorphs. Huffard and Caldwell (2002) observed blue-ringed octopuses releasing ink toward predators, during aggressive interactions with conspecifics, and at rejected potential mates.

Ink release was presumed useless in the deep sea's low ambient light. However, Bush and Robison (2007) described several ink release types in deep-sea squids. Some of these (e.g. ink clouds) could function by blocking bioluminescence caused by the squid's movement from a predator's view. These observations may also lend evidence to hypotheses that cephalopod ink has a chemical defense function. Conspecific ink is an alarm cue in *Doryteuthis (Loligo) opalescens*, *Sepioteuthis sepioidea*, and *Sepia officinalis* (Gilly and Lucero, 1992; Wood et al., 2008; Boal and Golden, 1999). The moray eel *Gymnothorax mordax* was attracted to *Octopus bimaculoides* ink (Gruninger, 1997). Derby et al. (2007) found relatively high concentrations of free amino acids in ink from several cephalopod species and suggest that cephalopod ink could be used in phagomimicry, causing feeding behaviors in the absence of food, as it is in sea hares.

We hypothesize that a chemical function could explain ink release by some deep-sea squids. Some functional possibilities are: (1) ink could be an alarm cue, (2) distasteful, (3) block olfactory senses, (4) act as a phagomimic, or (5) serve as a predator attractant. To begin looking at these possibilities, we collected ink from numerous deep-sea cephalopod species and analyzed it chemically to contrast species ink compositions. Our goal was to direct attention to species with disparate ink compositions for further investigations into the above possibilities.

Materials and Methods

The Monterey Bay Aquarium Research Institute's Remotely Operated Vehicles (ROVs) *Ventana* and *Tiburon* were used to obtain deep-sea cephalopods from Monterey Bay,

CA. The deep-sea squid *Pterygioteuthis* sp. was collected via trawl net in the Gulf of California. Ink from the octopus *Octopus bimaculoides* (a shallow-water outgroup) was obtained from coastal Santa Barbara, CA. Two sample types (pure and released) were taken when possible; each was taken from different individual of a given species. Pure ink samples were collected directly from the specimens' ink sac, either by draining the ink sac contents or putting the intact ink sac into a 2.0 or 5.0 ml Nalgene Cryogenic Vial. Released ink was collected from sample buckets containing a recently collected specimen. A clean plastic pipette was used to retrieve the ink sample and then transferred directly to a 5.0 ml Nalgene Cryogenic Vial, 15 ml, or 50 ml BD Falcon Conical Tube. All samples were immediately frozen at -80°C or -40°C.

Samples were transferred to a 5.0 ml Centrex 0.2 µm Nylon Centrifuge Filter (Whatman Inc., Piscataway, NJ) and were filtered for 10 min at 1500 x g. The filtrate was transferred to an Agilent 2 ml screw-top glass vial with a 250 µl polypropylene insert and placed into the sample holder of an Agilent (Hewlett-Packard) 1100 Series HPLC. We used a 5µ C18-A30 x 3.0 mm C18 column (Varian Polaris) and the HPLC was interfaced to a Agilent (Hewlett Packard) G1946B MSD with orthogonal electrospray ionization source. The injection volume was 20 µl, and each sample was run for 30 min at a flow rate of 4 ml/min.

Solvent A of the mobile phase was water and 0.05% formic acid and Solvent B was acetonitrile with 0.05% formic acid. The gradient was as follows: 0 min, 95% A, 5% B; 1 min, 95% A, 5% B; 25 min, 10% A, 90% B. The post-run was 5 min., returning to initial conditions of 95% Solvent A.

For each LC spectrum, the ten main peaks, including the basepeak, between 5 and 10 min run time were identified and aligned with the other species' spectra. Agilent ChemStation software was used to obtain the MS spectra for each of these peaks. Presence/absence of constituents within the MS spectra for these ten peaks were used to compare the ink composition between species. A Non-metric Multi-Dimensional Scaling (NMDS) ordination calculated using a Bray-Curtis coefficient dissimilarity matrix with 100 restarts was performed in Primer-E 6.0 (Clarke and Warwick, 2001). The arrangement of samples in an NMDS ordination is based on the rank dissimilarity between two samples; samples that are more closely spaced in the ordination are more similar. We used a Kruskal 1 fit scheme to allow equal dissimilarities to be represented by unequal distances in the ordination (Clarke and Gorley, 2006). Shared presence of a chemical constituent between samples (cephalopod species' ink) increases the similarity between samples, however, shared absence between samples does not increase similarity.

Results

We ran 31 cephalopod ink samples from 20 cephalopod species, however, only 16 successful LC-MS spectra from 12 species were obtained (Table 1). The distance between points in the NMDS ordination indicate the degree of similarity between ink compositions (Figure 1). The stress of an MDS is a measure of how well the visual representation matches the raw data. Stress values from 0.10 – 0.20 are fair to poor indicators of the relationship between samples. The stress for the LC-MS MDS is 0.13 and may be used to point out species of interest (Kruskal, 1964).

The pure ink samples and released ink samples are loosely clustered with other pure ink and released ink, respectively; these groupings are likely caused by the presence of mucous in released ink. Interestingly, *V. infernalis* mucous composition is more similar to pure inks than released inks. The major outliers from the pure ink cluster are the deep-sea octopus *Japetella diaphana* and the deep-sea squid *Octopoteuthis deletron*. *Octopoteuthis deletron* is also an outlier from the released inks, along with *Onychoteuthis borealjaponica*. *Octopus bimaculoides* ink has a similar composition to the deep-sea cephalopod inks analyzed here.

Discussion

There are several shallow-water and deep-sea octopuses that have either greatly reduced or completely lost the ancestral ink sac, whereas there are no known species of deep-sea squid that have lost the ink sac (Voight, 1997; Norman and Finn, 2001). The latter may be a phylogenetic relict, though it probably indicates that ink release is a useful defense for deep-sea squids.

We have successfully used LC-MS to show differences in ink composition. That the pure and released inks are separated by this analysis is a proof of its viability - mucous present in released ink confers constituents not present in the pure inks. It is interesting that ink from the deep-sea octopus *Japetella diaphana* was more different in composition to deep-sea squid inks than that of the shallow-water octopus *Octopus bimaculoides*. *Octopoteuthis deletron* ink is an outlier from both the released and pure groupings. This may indicate some interesting chemistry in this species. Since *O. deletron* released ink is more similar to *V. infernalis* mucous than any of the other released ink samples, and they both fall more closely to the pure inks, there may be some constituents lacking from the mucous of these species that are present in the other species' released inks.

Our goal was to compare shallow-water with deep-water species and look for major differences between ink samples. Unfortunately several of the shallow-water species analyses were unsuccessful or such that we could not compare them with current methods.

Ink composition of the one shallow-water octopus that could be included did not differ vastly from the deep-sea cephalopod ink samples. A previous study indicated that *Octopus bimaculoides* ink was attractive to one of its major predators, the California moray eel (Gruninger, 1997). Experiments with Northern Elephant Seals, *Mirounga angustirostris*, suggested a possible attraction to jumbo squid, *Dosidicus gigas*, ink (S. Bush, unpublished data). Perhaps deep-sea squid ink is also attractive to predators, allowing the squid to hide with a cloud, or escape as the predator seeks out a released pseudomorph.

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Table 3-1 Ink samples analyzed using liquid chromatography – mass spectroscopy.

Species	Analysis date	Type	Successful
<i>Bathyteuthis berryi</i>	5.7.08	pure	N
<i>Chiroteuthis calyx</i>	8.21.07	pure	Y
<i>C. calyx</i>	5.7.08	pure	N
<i>C. calyx</i>	8.21.07	released	Y
<i>Cranchia scabra</i>	5.7.08	pure	N
<i>Dosidicus gigas</i>	7.30.07	pure	N
<i>D. gigas</i>	7.30.07	pure	N
<i>Galiteuthis phyllura</i>	8.21.07	pure	N
<i>G. phyllura</i>	5.7.08	pure	N
<i>G. phyllura</i>	8.21.07	released	N
<i>Gonatopsis borealis</i>	5.7.08	pure	Y
<i>Gonatus berryi</i>	8.21.07	pure	Y
<i>Gonatus onyx</i>	7.30.07	released	N
<i>G. onyx</i>	8.21.07	released	Y
<i>Histioteuthis heteropsis</i>	8.21.07	pure	Y
<i>H. lunulata</i>	5.7.08	pure	N
<i>Japetella diaphana</i>	8.21.07	pure	Y
<i>Lolliguncula brevis</i>	5.7.08	pure	N
<i>Octopoteuthis deletron</i>	8.21.07	pure	Y
<i>O. deletron</i>	5.7.08	pure	N
<i>O. deletron</i>	8.21.07	released	Y
<i>Octopus bimaculoides</i>	8.21.07	pure	Y
<i>O. bimaculoides</i>	8.21.07	released	Y
<i>Onychoteuthis borealjaponica</i>	5.7.08	pure	N
<i>O. borealjaponica</i>	8.21.07	released	Y
<i>Planctoteuthis sp.</i>	8.21.07	pure	Y
<i>Planctoteuthis sp.</i>	8.21.07	released	Y
<i>Pterygioteuthis sp.</i>	8.21.07	pure	Y
<i>Sepia officinalis</i>	5.7.08	pure	N
<i>Taonius cf. borealis</i>	8.21.07	pure	Y
<i>Vampyroteuthis infernalis</i>	8.21.07	mucous	Y

Figure Legends

Figure 3-1. Multidimensional scaling ordination of cephalopod inks.

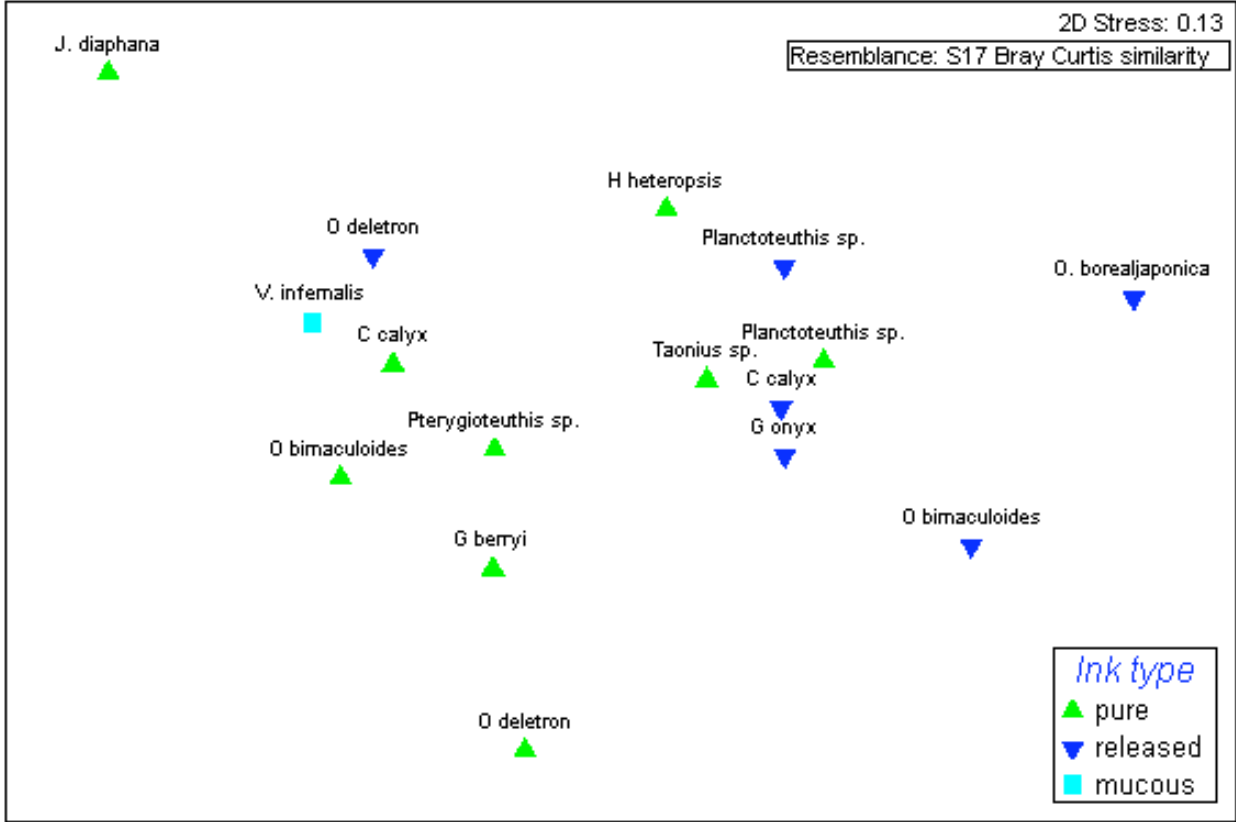


Figure 3-1

Chapter 4: Behaving in the Dark: Locomotor, Chromatic, Postural, and Bioluminescent Behaviors of the Deep-Sea Squid *Octopoteuthis deletron* Young 1972

Abstract Visual behaviors are prominent components of intra- and interspecific communication in shallow-water cephalopods. Meso- and bathypelagic cephalopods were believed to have limited visual communication, other than bioluminescence, due to the reduced illumination at depth. To explore potential visual behaviors in mesopelagic squid, we used undersea vehicles to observe seventy-six individual *Octopoteuthis deletron*. In contrast to predictions, we found this species capable of a variety of visually-linked behaviors not previously reported for a deep-ocean cephalopod. The resultant ethogram describes numerous chromatic, postural, locomotor, and bioluminescent behavioral components. A few common body patterns – the whole appearance of the individual involving multiple components – are characterized. The behaviors observed from individual squid were compared using a Non-metric Multi-Dimensional Scaling (NMDS) ordination, onto which hydrographic and observation parameters were mapped. Observation length, specimen collection, and contact with the vehicle affected which behaviors were performed. A separate NMDS, analyzing the body patterns, indicated that these sets of behavioral components could be visualized as groups within the NMDS ordination. While the functional roles of the behaviors described are not yet known, our findings of numerous behaviors in *O. deletron* clearly indicate that bioluminescence is not the sole method of visual communication by deep-sea squid.

Introduction

Shallow-water squids, octopuses, and cuttlefishes are renowned for their inter- and intraspecific visual communication (Packard and Sanders, 1971; Packard and Hochberg, 1977; Hanlon and Messenger, 1988, 1996). These cephalopods assess their surroundings with well developed vision, though in most species vision is monochromatic (Messenger, 1977; Kito *et al.*, 1992; Shashar *et al.*, 1998; Sweeney *et al.*, 2007). Individuals are capable of polyphenism consisting of near instantaneous changes in appearance through a broad range of camouflage and communication methods (Packard and Sanders, 1971; Packard and Hochberg, 1977; Hanlon and Messenger, 1988; Roper and Hochberg, 1988; Hanlon *et al.*, 1999; Barbato *et al.*, 2007). An individual's overall appearance, or body pattern, is comprised of the following component types: chromatic, textural, postural, and locomotor. Chromatic expression is achieved by pigmented chromatophores, underlying reflective leucophores and iridophores, and polarizing elements (Mirow, 1972a, b; Messenger, 1974; Packard and Hochberg, 1977; Cloney and Brocco, 1983; Packard, 1988; Mathger and Hanlon, 2007). Skin texturizing is accomplished with dermal muscles (Packard and Hochberg, 1977); this component type is present in octopuses and cuttlefishes but absent from squids. Postural components are the positions of muscular, flexible limbs and body (Packard and Sanders, 1971). Locomotor components and maneuvers are movements of the whole body or its constituents (Roper and Hochberg, 1988). Variations of bioluminescence, or biologically derived light, expressed by an individual can also be considered a type of body patterning component (Herring, 2000).

Body patterns used in inter-specific communication enable cephalopods to achieve search image impedance by using polyphenism to hinder recognition (Hanlon *et al.*, 1999), perform deimatic behavior to startle potential predators (Edmunds, 1974), and implement effective predation, for example *Sepia officinalis* may use the dynamic Passing Cloud body pattern to distract prey prior to attack (Hanlon and Messenger, 1988). There are multiple ways in which polyphenism is used to camouflage the individual. In background resemblance the individual approximates a random sample of the visual background (Endler, 1981). The juxtaposition of colored areas in disruptive coloration attract a predator's attention away from the animal's outline (Cott, 1940; Chiao *et al.*, 2007). Counter-shading is used to cancel shadows on a body surface caused by uneven illumination (Cott, 1940). Finally, in masquerade the animal resembles an inedible or non-prey entity (Endler, 1981; Hanlon *et al.*, 1999).

Body patterning may be used in intra-specific communication to attract and court mates, repel or deceive sexual competitors, coordinate movements within a shoal, and to warn conspecifics of predators (Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1996; Hunt *et al.*, 2000).

Species that experience diverse environmental encounters may benefit from a varied communication repertoire. Body patterning diversity in cephalopods is thought to be influenced by activity patterns (diel vs. nocturnal), social behavior, habitat complexity, predators, and reproductive mode (Hanlon and Messenger, 1988). Cephalopods that are either asocial, nocturnally active, use simple reproductive modes, or that dwell in homogeneous environments may display fewer body patterns than other cephalopods (Hanlon and Messenger, 1988). In the deep ocean, most species are asocial, the water is a nearly homogenous visual substrate, and both intra- and interspecific interactions are infrequent due to low animal densities (Childress, 1995; Herring, 2000). In addition, below 200 m, more than 99% of surface illumination has been attenuated and this 'twilight' environment makes visual communication less viable than in well-lit habitats (McFall-Ngai, 1990). Consequently, limited body patterning has been predicted for deep-ocean cephalopods (Hanlon and Messenger, 1996; Nixon and Young, 2003).

The characteristic distribution of light in the deep sea has led to convergence in camouflage tactics across taxa (Marshall, 1979; Johnsen, 2001, 2005; Warrant and Locket, 2004). In the relatively well-lit euphotic and upper mesopelagic zones, individuals are often counter-shaded, transparent, have highly reflective silvered sides, and/or have laterally flattened bodies to minimize their silhouette (Johnsen, 2001). Below 30 m depth, red wavelengths are completely absent from ambient light. At these depths, non-transparent individuals often have red, purple, brown or black body coloration, which appears black in the absence of red light (McFall-Ngai, 1990). In the mesopelagic (200 – 1000 m), light down-welling from the surface is many times greater than that reflected laterally or from below, and predators looking upward may decipher the silhouette of potential prey (Gleadall and Shashar, 2004). In an apparent response to this vulnerability, many deep-sea animals possess downward directed photophores that counter-illuminate their bodies, diminishing or canceling their silhouette (Young, 1977; Young, 1983).

Camouflage by deep-sea animals centers almost exclusively on the few successful tactics presented above: transparency, silvering, counter-illumination, and

red coloration. There has been no *a priori* reason to expect deep-sea cephalopods to be different, despite the multitude of capabilities known for shallow-dwelling cephalopods (Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1996). Consistent with this reasoning, most deep-sea cephalopods have been found to possess silvered eyes and digestive glands that are counter-illuminated, and bodies that are transparent or of dark, usually red, coloration (Chun, 1910; Voss, 1967; Herring, 1988).

Octopoteuthis deletron Young 1972 is a solitary mesopelagic squid that inhabits the eastern North Pacific from Alaska to Baja California, off Peru and perhaps Japan (Young, 1972). Individuals attain 17 cm mantle length, possess large, elliptical fins, and long arms with hooks in place of suckers along most of the length (Young, 1972). Members of the family Octopoteuthidae lose the tentacles at an early life-stage, leaving just the eight arms to function in prey capture (Young, 1972). Individual *O. deletron* possess a variety of photophores, the most conspicuous of which are single organs that occupy the terminus of each arm (Young, 1972; Vecchione *et al.*, 2002). This species also has a number of ventrally directed photophores: a series along the core of the four ventral arms, an arm base organ on arm pairs II, III, and IV, a pair of small medial head organs, a photophore posterior to each eye, a pair of visceral photophores situated ventral to the ink sac, and a single posterior 'tail' organ (Young, 1972). Counter-illumination by ventral photophores has been observed from the congener *Octopoteuthis neilseni* (Young and Vecchione, 2006).

Materials and Methods

Observations were made during dives of two remotely operated vehicles (ROVs) operated by the Monterey Bay Aquarium Research Institute (MBARI). The ROV *Ventana*, a 40 hp electro-hydraulic vehicle, ranges to 1500 m. Illumination is provided by six 400 W DSPL HID Daylight lamps. The ROV *Tiburon* is an electric vehicle that ranges to 4000 m; it has four 400 W DSPL HMI lights for illumination. Both of these arrays produce illumination in the daylight range (5500-5600 °K). One important difference between these vehicles is that hydraulic power produces more noise than electric power; the ROV *Tiburon* is quieter in the water. Each ROV dive is recorded on Panasonic D5 High Definition and/or Sony Digital Betacam standard definition videocassettes. Since 1988, MBARI has archived more than 15,000 hours of video footage from over 3200 ROV *Ventana* dives and over 1150 ROV *Tiburon* dives in the Monterey Submarine Canyon and surrounding waters. Organisms encountered are annotated by scientists and video-lab staff using MBARI-designed Video Annotation Reference System software and the annotations are synchronized with hydrographic parameters (Robison, 1993). Video sequences used in this study were viewed directly from ROV dive tapes on a Sony HR Trinitron monitor or a Sony MEU-WX2 Multiformat Engine and LMD-232W LCD monitor. Monitor settings maintained animal coloration according to ROV illumination produced *in situ*. All sequences were viewed multiple times at normal and slow playback speeds. Each unique behavior was defined, categorized as one of the following component types: chromatic, postural, locomotor, or bioluminescent, and recorded as present or absent from the observation sequence of each individual. A few of the common body patterns, or combinations of components, are also described.

The resulting presence/absence data matrix was used to explore (a) behavioral similarities between individuals and (b) behavioral components that were observed simultaneously (body patterns). For both analyses a Non-metric Multi-Dimensional Scaling (NMDS) ordination calculated using a Bray-Curtis coefficient dissimilarity matrix and 100 restarts was performed in Primer-E 6.0 (Clarke and Warwick, 1994). The placement of samples in an NMDS ordination is based on the rank dissimilarity between two samples; samples that are more similar are more closely spaced in the ordination. We used a Kruskal 1 fit scheme to allow equal dissimilarities to be represented by unequal distances in the ordination (Clarke and Gorley, 2006). Shared presence between samples increases the similarity between individuals, however, shared absence between samples does not increase similarity. Individual squid were treated as the samples, with behavioral components as the variables in the first NMDS analysis. This ordination indicates similarities between individuals with respect to the behaviors each performed. Each individual's encounter depth, temperature, salinity, oxygen, beam transmission, length of observation, year, month, observing vehicle, contact or no contact with vehicle, and whether or not the individual was collected, were mapped onto the ordination to assess potential correlations with observed behaviors. We were unable to quantify individual size and sex from the video and therefore could not map these factors onto the ordination. In the second NMDS analysis, behaviors were treated as the samples and individuals as the variables to explore which behavioral components were commonly observed together. This analysis could not be calculated using the full data set because the format of the data matrix cannot indicate which behaviors were performed simultaneously. We therefore performed the analysis with a subset of behaviors that constituted the body patterns we defined, including only those behaviors that were unique to one body pattern. Our goal was to validate the body patterns by visualizing them in the ordination.

Undersea vehicles have provided invaluable *in situ* observations of mesopelagic nekton and zooplankton (Barham, 1963, 1966; Alldredge *et al.*, 1984; Widder *et al.*, 1989; Moiseev, 1991; Vecchione and Roper, 1991; Seibel *et al.*, 2005, Bush and Robison, 2007). Disturbances caused by vehicle presence have yet to be quantified (Hunt *et al.*, 2000; Widder *et al.*, 2005). However, we assume that although encounter with an ROV is unfamiliar, it will not result in novel behaviors (Hunt, 1996). Furthermore, because animals likely enter defensive mode when confronted with unfamiliar stimuli, we believe that the behaviors observed represent, but are not limited to, defensive behaviors (Vecchione and Roper, 1991; Hunt, 1996; Hunt *et al.*, 2000, Bush and Robison, 2007).

Results

Seventy-six individuals of *O. deletron* were encountered during 64 ROV dives from 1992 to 2007. Observations were obtained between 07:11 and 21:29 Pacific Standard Time, and a total of 8.7 observation hours were accumulated. Individual observation length varied from a few seconds to over 37 minutes. Encounter depths were between 344 and 1841 m; most individuals (93%) were found between 344 and 787 m depth (Supplemental Fig. 1, <http://www.biolbull.org/supplemental/>). Our observations provided no evidence of diel vertical migration (Supplemental Fig. 2, <http://www.biolbull.org/supplemental/>). The range of hydrographic parameters where *O.*

deletron occurred was 2.3 – 7.8 °C, 0.14 – 1.36 ml/l O₂, 34.0 – 34.5 practical salinity units, and 76.4 – 92.4% beam transmission. Collected specimens ($n = 9$) measured 4 - 15 cm mantle length and represented immature and mature individuals of both sexes.

Ethogram

Octopoteuthis deletron has a broad repertoire of behavioral components with which it can respond to external stimuli. Here we describe all behavioral components that were performed by more than one individual. In most cases, the few components which were observed from only one individual can be classified as incomplete expressions of the components described here. The total number of components observed was 59 and they are organized into five categories. There are seven light chromatic components (Fig. 1, Table 1), 10 dark chromatic components (Fig. 1, Table 1), 17 postural components (Fig. 2, Table 1), 22 locomotor components and maneuvers (Table 1), and three bioluminescent components (Fig. 3, Table 1). We report the number of individuals observed performing each behavior rather than the total number of times it was observed, because individual observation times were not standardized. Components were not always completely expressed. Video sequences demonstrating some of the behavioral components, including the range of expression, can be viewed at <http://www.mbari.org/midwater/squidbehavior>. Similar components, described for previously studied squids are noted in the text as follows: components in single quotes are from published ethograms where each component has been explicitly defined, components in double quotes are from other published works. A brief description of four common body patterns is included.

Light chromatic components

Pale ($n = 12$) is a white or gray appearance of juveniles and adults. Very small individuals are translucent when expressing Pale. An individual may express Pale over the entire body, or any combination of the mantle, fins, head, funnel, and/or arms. This component is similar to the following: 'clear' of *Doryteuthis plei* (Hanlon, 1982), *Loligo forbesii* (Porteiro *et al.*, 1990), *Loligo reynaudii* (Hanlon *et al.*, 1994), *Doryteuthis pealeii* (Hanlon *et al.*, 1999), *Doryteuthis opalescens* (Hunt *et al.*, 2000), and *Sepioteuthis australis* (Jantzen and Havenhand, 2003), 'all clear' of *Alloteuthis subulata* (Lipinski, 1985), 'all light' of *Sepiola affinis* (Mauris, 1989), "clear" of *Gonatus onyx* (Hunt and Seibel, 2000), and "all white" of *Mastigoteuthis hjorti* (Vecchione *et al.*, 2002). **Pale Tail** ($n = 48$) consists of a small white postero-ventral mantle area. It may extend onto the posterior fins. On occasion the tail photophore can be seen within the Pale Tail. In a few cases, a small red spot covers the tail photophore making it conspicuous when surrounded by the Pale Tail. **Pale Fin Edge** ($n = 51$) is translucent or white along the entire edge of both fins. It is expressed in *S. affinis* during 'dark fins', (see Fig. 1 in Mauris, 1989), "colored fin centers" in *Chiroteuthis calyx* and *Galiteuthis phyllura* (Hunt, 1996), and "accentuated white fin edges" of *M. hjorti* (Vecchione *et al.*, 2002). Pale Fin Edge and Pale Tail are commonly expressed together. **Pale Lateral Fin Stripe** ($n = 15$) is an elongate pale area on the centro-lateral fin along the anterior-posterior axis. It may be expressed on the dorsal or ventral side. **Pale Fin Base Stripe** ($n = 11$) is a pale region along the mantle and/or fin where they meet. It is expressed either dorsally or ventrally, and is sometimes restricted to a central spot. **Pale Eye Circle** ($n = 12$) is a

white or gray area partially or entirely surrounding the eye. This is similar to ‘golden ocular epaulettes’, restricted to the dorsal eye, of *S. australis* (Jantzen and Havenhand, 2003). **Pale Arm Crown Band** ($n = 17$) is a transverse white or gray area at the base of the four ventral arms and/or four dorsal arms. The Pale Arm Crown Band can be restricted to four ventral spots, one at each arm base, located over the arm base photophore.

Dark chromatic components

In **Dark** ($n = 61$), the mantle, fins, funnel, head and/or arms are red or pink. In general, smaller individuals produce a lighter shade of red or pink, however, the shade can vary within an individual. Dark in *O. deletron* is similar to ‘all dark’ of *D. plei*, (Hanlon, 1982), *A. subulata*, (Lipinski, 1985), *S. affinis*, (Mauris, 1989), *L. forbesii* (Porteiro *et al.*, 1990), *L. reynaudii* (Hanlon *et al.*, 1994), *D. pealeii* (Hanlon *et al.*, 1999), and *D. opalescens* (Hunt *et al.*, 2000), “solid orange” of *G. onyx* (Hunt and Seibel, 2000), “all-red” of *M. hjorti* (Vecchione *et al.*, 2002), ‘dark mantle’ and ‘all dark’ of *S. australis* (Jantzen and Havenhand, 2003), and “red coloration” of *Taningia danae* (Kubodera *et al.*, 2007). In **Mottle** ($n = 28$), the mantle, fins, funnel, head, and/or arms are covered in an irregular pattern of pale and red or pale and pink. **Irregular Dark Patches** ($n = 6$) are small, unevenly spaced red or pink patches on a pale body. This component is observed only in small individuals and is similar to the ‘irregular dark patches’ of *A. subulata* (Lipinski, 1985), ‘fin and mantle spots’ of *L. forbesii* (Porteiro *et al.*, 1990), and ‘fin spots’ of *L. reynaudii* (Hanlon *et al.*, 1994) and *D. pealeii* (Hanlon *et al.*, 1999). **Ventral Mantle Bands** ($n = 16$) consists of a variable number of pale, medium red, and/or deep red bands that span the breadth of the ventral mantle. They can occupy the entire mantle along the anterior-posterior axis or only part of its length and each band may vary in width. The ‘bands’ of *L. reynaudii* (Hanlon *et al.*, 1994) and *D. pealeii* (Hanlon *et al.*, 1999) are similar, except that in the shallow-water squids they are expressed both dorsally and ventrally. **Dark Fin Stripe** ($n = 6$) consists of red along the contours of the fin inside of the edge. Similar components in other species include: ‘stitchwork fins’ of *D. plei* (Hanlon, 1982), ‘dark fin line’ and ‘lateral mantle stripe’ of *L. forbesii* (Porteiro *et al.*, 1990), ‘fin stripe’ of *L. reynaudii* (Hanlon *et al.*, 1994), *D. opalescens* (Hunt *et al.*, 2000), and *S. australis* (Jantzen and Havenhand, 2003), ‘mantle margin stripe’ of *D. pealeii* (Hanlon *et al.*, 1999), “fin highlights” of *C. calyx* and *G. onyx* (Hunt, 1996; Hunt and Seibel, 2000), and “red fin edges” of *M. hjorti*, (Vecchione *et al.*, 2002). **Dark Eye Circle** ($n = 12$) is red or pink that surrounds the eye partially or entirely. This component is more extensive, but similar to, “eyebrow patches” of *C. calyx*, *G. phyllura*, and juvenile *G. onyx* (Hunt, 1996; Hunt and Seibel, 2000) and ‘shaded eye’ of *S. australis* (Jantzen and Havenhand, 2003). **Arm Bands** ($n = 13$) are regularly spaced rings along the arms consisting of alternating pale or pink with red. They occur on some or all arms, for example, arms IV could have Arm Bands while the others do not. One band may occur across either the ventral or dorsal four arms near the base, but distal to the arm crown. The **Dark Arm Crown Band** ($n = 21$) is a transverse band across the base of the four ventral and/or four dorsal arms over the arm base photophores. Dark Arm Crown Band sometimes manifests as four red spots. Some or all of the aboral arms in **Dark Aboral Arms** ($n = 3$) are colored red or pink while the oral arms remain pale. The similar components ‘dark arms’ of *L. forbesii* (Porteiro *et al.*, 1990) and ‘dark head and arms’ of

L. reynaudii (Hanlon *et al.*, 1994) do not include pale coloration of the oral arms. Some or all of the oral arms in **Dark Oral Arms** ($n = 16$) are colored red/pink while the aboral arms remain pale. This component is similar to 'white arms' of *D. pealeii* (Hanlon *et al.*, 1999), though the latter is without the pale aboral arms.

Postural components

In **Dorsal Mantle Up** ($n = 67$, Fig. 2A, D, F, H, L) the body is horizontal with dorsal mantle upward. This is common to many squids, for example the "horizontal" of *Octopoteuthis megaptera* (Vecchione *et al.*, 2002). In **Dorsal Mantle Down** ($n = 2$, Fig. 2G) the ventral mantle is upward, i.e. the individual is upside-down. In **Tail Up** ($n = 22$, Fig. 2C, J) the body is vertical, with the posterior mantle pointed upward. This component is equivalent to the mantle portion of the "head down posture" of mastigoteuthids (Roper and Vecchione, 1997; Young *et al.*, 1998; Vecchione *et al.*, 2002). In **Tail Down** ($n = 38$, Fig. 2B, E) the body is vertical with the posterior mantle downward. An equivalent "oblique body" occurs in *O. megaptera* (Vecchione *et al.*, 2002). In **Fins Out** ($n = 45$, Fig. 2A-D, H, K-L) one or both fins are held out to the side as in the "rigid fins" of *O. megaptera* (Vecchione *et al.*, 2002). The fins can be flapping while in Fins Out (see Locomotor components and maneuvers, below). **Fins Curved Ventrally** ($n = 23$, Fig. 2E-F) occurs when one or both fins bend toward the ventral mantle. It is equivalent to 'fins curved ventrally' of *D. opalescens* (Hunt *et al.*, 2000). In **Fins Curled Ventrally** ($n = 28$, Fig. 2J) one or both fins wrap closely around the body, one overlapping the other when both are curled. The fins can touch the mantle or not. The fins were never observed to pulse in this posture as *Mastigoteuthis magna* was observed to do, the latter creating a slow jet-propulsion (Roper and Vecchione, 1997). **Straight Arms** ($n = 28$, Fig. 2D, G, J) point away from the mantle and are either: (a) held together along their entire length (Fig. 2G) as in "aligned arms" of *Histioteuthis heteropsis* (Hunt, 1996) and 'rigid arms' of *S. australis* (Jantzen and Havenhand, 2003), (b) lined up next to one another in a plane (Fig. 2D) similar to 'arm plane' of *D. opalescens* (Hunt *et al.*, 2000), or (c) spaced slightly apart so arms do not touch distally (Fig. 2J). One or two arm-tips can stick out from the rest of the arms while in Straight Arms, similar to 'two raised arms' of *L. forbesii* (Porteiro *et al.*, 1990), and 'raised arms' of *D. opalescens* (Hunt *et al.*, 2000), and *S. australis* (Jantzen and Havenhand, 2003). **Dorsal Arm Curl** ($n = 63$, Fig. 2C, F, I) involves the arms curving toward the dorsal mantle. The variations of this posture include: (a) arms held together pointing above the head, i.e. perpendicular to the anterior-posterior axis, (b) arms curved back closely over the head (Fig. 2C), (c) arms curved over the dorsal mantle (Fig. 2F), (d) arms curved over the dorsal mantle, held side-to-side in a plane, (e) arms curved over the dorsal mantle, some or all arms distally curved back toward the head (Fig. 2I), and (f) arms spread from above the head to over the mantle, either straight or with curved tips. Equivalent components are the "cockatoo" posture of cranchiid squids (Vecchione and Roper, 1991), "J-curl" of *G. onyx* (Hunt and Seibel, 2000), 'J-curl' of *D. opalescens* (Hunt *et al.*, 2000), "arms flexed dorsally" of *O. megaptera* (Vecchione *et al.*, 2002), and 'upward curl' of *S. australis* (Jantzen and Havenhand, 2003). **Ventral Arm Curl** ($n = 3$, Fig. 2A) has the arms curved toward the ventral mantle. The 'downward curl' of *L. forbesii* (Porteiro *et al.*, 1990), 'drooping arms' of *D. pealeii* (Hanlon *et al.*, 1999), 'downward curling' of *D. opalescens* (Hunt *et al.*, 2000), and 'downward curl' and

'drooping arms' of *S. australis* (Jantzen and Havenhand, 2003) are all comparable. In **Splayed Arms** ($n = 9$, Fig. 2M) the arms are progressively further from the other arms along their length. This can occur while the arms are positioned in any direction relative to the mantle. Usually the arms spread from the base, however, the arms may be held together proximally and medially, with only the distal arms extended outward. The 'PI posture' of *S. affinis* (Mauris, 1989), 'splayed arms' of *L. forbesii* (Porteiro *et al.*, 1990), *L. reynaudii* (Hanlon *et al.*, 1994), and *D. opalescens* (Hunt *et al.*, 2000), and 'splayed arms' and 'flared arms' of *D. pealeii* (Hanlon *et al.*, 1999) are similar. **Arm Tips Inward** ($n = 18$, Fig. 2H) involves the arms held out from the head with the arm tips curving toward the oral region, sometimes crossing. The following modifications were observed: (a) one arm pair (pair I) was S-curved or C-curved above the other arms, or (b) one pair (pair IV) remained straight. In **Arms Curled** ($n = 10$) some or all of the distal arms are coiled to form a single loop or a few concentric whorls. One variation (Fig. 2E) consists of four or six arms curled and the resultant rings spread in a plane and lined up end to end, similar to the "Elk" posture of *G. onyx* (Hunt and Seibel, 2000). **Arm Keels Prominent** ($n = 10$) occurs when the skin flanges on the aboral arms are raised in one or more arm pairs. Keels on the third arm pair may function as airfoils for stabilization and lift modification during locomotion in *Lolliguncula brevis* (Bartol *et al.*, 2001). We observed Arm Keels Prominent while individuals were stationary. **Central Arm Space** ($n = 5$, Fig. 2B) occurs when: (a) the arms curve outward proximally then inward distally or (b) the arms are held together at the base, curved outward medially, then inward distally. In both cases, the tips curl orally and are surrounded by the arms. In **Lateral Arm Spread** ($n = 15$, Fig. 2D, K) the arms are spread to the sides in a few different conformations: (a) a 'V' in front of the head with arms straight or distally curved inward, (b) arms curve to either side then extend posteriorly along the mantle, (c) spread in a 180° arc away from the head, or (d) each dorsal arm meets with the corresponding ventral arm – i.e. LI with LIV, LII with LIII, RI with RIV, and RII with RIII – to form four pairs that spread away from the head. Similar postures are 'PB2' of *S. affinis* (Mauris, 1989), and 'downward V curl' of *L. reynaudii* (Hanlon *et al.*, 1994). In **Compact Arms** ($n = 7$, Fig. 2L) each arm is curved or curled and intertwined with the other arms, which commonly cover the head.

Locomotor components and maneuvers

While **Hovering** ($n = 51$), an individual maintains its position in the water column, either remaining motionless or using the fins for stabilization. Neutral buoyancy has been described for *O. megaptera* and many other deep-sea cephalopods (Vecchione *et al.*, 2002; Seibel *et al.*, 2004). **Fins Flapping** ($n = 59$) consists either of synchronized flapping of the edges or entire fins, or of sinusoidal waves along the length of the fins. An analogous movement is "fin undulations" of *M. magna* (Roper and Vecchione, 1997). In a **Full Fin Flap** ($n = 24$), the fins touch dorsally along the lateral edge, and then quickly swing all the way around the mantle to touch or overlap ventrally. This component may occur once or multiple times sequentially and generally accompanied Jetting or a Jolt (see below). Comparable locomotion occurs as "dorsoventral beat" of *M. magna* (Roper and Vecchione, 1997), "flapping of fins" in *O. megaptera* (Vecchione *et al.*, 2002), and "cycle of fin movement" in *T. danae* (Kubodera *et al.*, 2007). **Backward Gliding** ($n = 22$) *sensu* Hunt *et al.* (2000) is gradual posterior-first movement

using only the funnel, with fins held rigid. **Forward Gliding** ($n = 4$) *sensu* Hunt *et al.* (2000) is gradual anterior-first movement using only the funnel for propulsion, while the fins are held rigid. Speed for Backward and Forward Gliding is less than half a body length/sec. **Backward Swimming** ($n = 15$) is posterior-first movement using the funnel and Fins Flapping. **Forward Swimming** ($n = 10$) is anterior-first movement involving both the funnel and Fins Flapping. **Backward Jetting** ($n = 36$) is rapid posterior-first movement via funnel pulses. **Forward Jetting** ($n = 22$) is anterior-first movement involving rapid expulsion from the funnel. Speeds of two or three body lengths/sec are typical for Backward and Forward Jetting. Full Fin Flaps usually accompanied Forward and Backward Jetting. No matter the form of locomotion, whether gliding, swimming, or jetting, forward or backward, the movements we observed were typically downward (deeper) in the water column and only rarely upward. Movement toward greater depths has also been observed in *G. phyllura*, *G. onyx*, and *C. calyx* (Hunt, 1996; personal observations). **Rotating** ($n = 17$) is more than one body revolution around the vertical or horizontal axis. It can occur while the individual is stationary or moving. Rotation around the anterior-posterior body axis has been observed in *H. heteropsis* (Hunt, 1996). A **Jolt** ($n = 50$) is a sudden, quick change in posture or movement. Either just the arms are flung forward in a Jolt, once or a few times and then returned to their previous orientation, or the whole body moves. A Jolt may immediately follow a mechanical disturbance from the ROV ($n = 9$), without physical contact with the vehicle. **Arm Spread** ($n = 48$) involves all eight arms spreading into a wide circle, and then returning to the previous or a new posture. It can be a slow maneuver but is usually rapid. Most often the arms are straight while spread, but they can curve orally, clockwise, or counter-clockwise distally. Somewhat analogous maneuvers occur as 'peristaltic arm flare' in *S. australis* (Jantzen and Havenhand, 2003), "arms swept up" of *Vampyroteuthis infernalis* (Hunt, 1996; Robison *et al.*, 2003), and "spreading all arms" of *T. danae* (Kubodera *et al.*, 2007). A **Flip** ($n = 14$) involves the individual quickly rotating the body forward, end-over-end, $180^\circ - 360^\circ$ from its starting position. It is often preceded by a Jolt and can be concurrent with Arm Spread. It is comparable to the "somersault" of *T. danae* (Kubodera *et al.*, 2007). **Ink Release** is observed in several forms: (a) a cloud ($n = 25$), either diffuse or of interconnecting ink blobs, (b) a single pseudomorph ($n = 9$) or two or more consecutive pseudomorphs ($n = 2$), that hold their shape, (c) a diffuse puff ($n = 6$), (d) a long slender rope ($n = 2$), (e) a short, thin string ($n = 2$), and (f) alternating with defecation ($n = 1$). Seven individuals released more than one of the above ink types. Several squid ($n = 8$) inked within the collection device therefore the release type could not be determined. Commonly, as we approached a cloud we found the individual either within or adjacent to the ink. Pseudomorphs usually occurred as the squid jetted away from encounter. The ink is viscous and may remain intact for several minutes (Bush and Robison, 2007). **Arms Unroll** ($n = 6$) begins with the arms held together, straightening in unison along their length from a curved posture such as Dorsal Arm Curl. **Eye Blink** ($n = 18$) consists of the skin surrounding the left or right eye closing over the lens, then opening; it happens either once or a few consecutive times. **Writhing Arms** ($n = 9$) occurs as the arms wrap around or immediately in front of the head and weave in and out of each other. It was observed separately from the stationary Compact Arms postural component. During **Flicking Arm Tips** ($n = 9$), one or more distal arms are whipped back and forth. This sometimes

accompanies Writhing Arms. Individuals **Grasp** ($n = 23$) parts of the vehicle, usually with Arms Spread and followed by Forward or Backward Jetting. The similar component “attacking” occurs in *T. danae* (Kubodera *et al.*, 2007). **Bite** ($n = 4$) occurs when captured individuals are seen moving around in the clear collection apparatus opening and closing the beak against the container. Individuals can **Expose Arm Hooks** ($n = 2$) by pushing them out of the surrounding soft tissue. **Arm Autotomy** was observed in the laboratory; evidence of previous autotomy was seen as shortened arm stubs of individuals *in situ* ($n = 13$). This has been reported for other deep-sea cephalopods: “missing arm tips” in *O. megaptera* (Vecchione *et al.*, 2002) and in *O. neilseni* (Young and Vecchione, 2006), and “regenerated light organs” of *V. infernalis* (Robison *et al.*, 2003).

Bioluminescent components

Chromatophore layers surrounding the photogenic tissue control arm-tip photophore bioluminescent output. We assumed that arm-tip photophores were luminescent when we could see the pale, reflective photogenic tissue as the chromatophores contracted (Fig. 3). The number of bioluminescent components is probably under-estimated here because close observations in which we could clearly see the arm-tip photophores were limited. **Arm-tip Chromatophores Contracted/Expanded** ($n = 38$) is intermittent bioluminescence. The eight photophores are not synchronized and may flash at variable rates. This component is commonly coupled with the following postural components: Dorsal Arm Curl, Arm Tips Inward, Central Arm Space, Writhing Arms, Arms Curled, and/or Flicking Arm Tips. Similar behaviors have been reported as “responses 1 and 3” of *Pterygioteuthis microlampas* and *Pterygioteuthis giardi* (Young *et al.*, 1982), the “flashes” of *T. danae* (Roper and Vecchione, 1993), and the “flash” of *V. infernalis* (Robison *et al.*, 2003). **Chronic Arm-tip Chromatophores Contracted** ($n = 32$) signifies continuous bioluminescence of more than one minute and was observed with Arms Straight, Dorsal Arm Curl, Arm Tips Inward and/or Central Arm Space. **Acute Arm-tip Chromatophores Contracted** ($n = 27$) is brief, simultaneous bioluminescence of the eight arm-tip photophores, most commonly occurring during Arm Spread. The “bright flashes” and “short flash” of *T. danae* (Roper and Vecchione, 1993; Kubodera *et al.*, 2007), and “arm tips glowing” of *V. infernalis* (Robison *et al.*, 2003) are comparable. For many individuals, a combination of Arm-tip Chromatophores Contracted/Expanded and Chronic Arm-tip Chromatophores Contracted occurs. For example, arms I terminal photophores are in Chronic Arm-tip Chromatophores Contracted, whereas the other six photophores are in Arm-tip Chromatophores Contracted/Expanded.

Body patterns

We have not made an exhaustive list of the body patterns that *O. deletron* is capable of, but we present a few of the most commonly observed. The first pattern involves Dark with Pale Tail and/or Pale Fin Edge, and the postural components Dorsal Mantle Up, Dorsal Arm Curl, and Fins Out or Fins Curved Ventrally. In many cases Arm-tip Chromatophores Contracted/Expanded or Chronic Arm-tip Chromatophores Contracted were also performed. A second body pattern consists of Dark coloration with Jolt, Arm Spread, Forward or Backward Jetting, and Acute Arm-tip Chromatophores Contract; Flip is sometimes included. In the third pattern individuals paired Tail Down with

Backward Gliding or Backward Swimming, Rotating, Dark or Mottle on the dorsal surface, Ventral Mantle Bands, Dark Arm Crown Band, and Arms Curled. In a fourth body pattern, small individuals frequently demonstrated Pale (translucent) with Irregular Dark Patches over the mantle and fins and Arm Bands.

NMDS Ordination

The dataset for the NMDS analyses consisted of 76 individuals each with observed/not observed scores for the 59 behaviors described above. The first NMDS arranged individuals (data points) in two-dimensions based on the behaviors each squid performed in common with every other individual. Close data points indicate that the two individuals they represent performed more behaviors in common than individuals further apart in the ordination. The stress of an NMDS solution refers to the ease with which the data can be arranged into the given number of dimensions. Stress for our 2-dimensional solution is 0.19; stress for the 3-dimensional solution is 0.14. Kruskal (1964) indicates that stress values from 0.10-0.20 are fair to poor in terms of explaining variation of the data. Despite these high stress values, we present the 2-dimensional solution to show which of the factors affected the behaviors performed. Individuals encountered at similar depths do not cluster in our ordination (data not shown), indicating that depth is not correlated with observed behaviors. The same is true of salinity, temperature, oxygen concentration, beam transmission, observation year, month, and vehicle (data not shown). However, individuals that were observed for longer than ten minutes are clustered in the ordination and surrounded by individuals that were observed for ten minutes or less (Fig. 4A). Longer observation time led to more behaviors observed (Supplemental Fig. 3, <http://www.biolbull.org/supplemental/>) and therefore more behaviors in common. Individuals that contacted the ROV performed more behaviors in common than individuals that did not contact the ROV and individuals that were collected performed more of the same behaviors than individuals that were not collected (Fig. 4B-C).

The second NMDS, in which we used a subset of behaviors to test the validity of the four body patterns we defined (Table 1), is comprised of four clusters of behaviors (Fig. 5; 2D stress = 0.15; 3D stress = 0.09). All of the components included in this analysis are likely part of one or multiple body patterns other than the four described in the current study. This is likely the reason for the poor fit of the NMDS solution to the data. Nevertheless, each cluster corresponds to one of the body patterns, indicating that they are natural elements of *O. deletron*'s behavior.

Discussion

In contrast to predictions that deep-sea cephalopod body patterning is limited to transparency, silvering, red coloration, counter-illumination and bioluminescence (Hanlon and Messenger, 1988, 1996; Nixon and Young, 2003), we have seen that *O. deletron* is capable of numerous behavioral components. In fact, the total number of components now described for *O. deletron* (59) is comparable to that for other squids for which ethograms are available: 16 components (chromatic only) in *D. plei* (Hanlon, 1982), 59 mentioned for *S. affinis* (Mauris, 1989), 28 in *L. forbesii* (Porteiro *et al.*, 1990), 36 in *L. reynaudii* (Hanlon *et al.*, 1994), 56 in *D. pealeii* (Hanlon *et al.*, 1999), 39 in *D. opalescens* (Hunt *et al.*, 2000), and 48 in *S. australis* (Jantzen and Havenhand, 2003). It

is unclear what, if any, benefits are conferred by these numerous behavioral capabilities. However, possibilities include interspecific communication, such as attracting prey or deterring predation, and/or intraspecific communication, for example species recognition and mate attraction.

Octopoteuthis deletron may use variable body patterning and positioning as a primary defense to cause search image impedance and avoid detection by a predator. Deep-sea cephalopods are known prey of teleosts, marine mammals, sharks, and other cephalopods (Hills and Fiscus, 1988; Clarke *et al.*, 1993; Antonelis *et al.*, 1994; Clarke, 1994; Thompson, 1994; Clarke, 1996; Klages, 1996; Smith and Whitehead, 2000; Markaida, 2005). Some of these predators forage visually, despite the limited light conditions of meso- and bathypelagic depths (Levenson and Schusterman, 1999; Fristup and Harbison, 2002; Southall *et al.*, 2002; Watwood *et al.*, 2006) and they probably form search images to increase hunting efficiency (Edmunds, 1974). Shallow-water cephalopods are capable of quickly changing their appearance to counteract visual search images (Hanlon and Messenger, 1996, 1999; Huffard, 2006), and it is likely that *O. deletron* uses complex postural, and bioluminescence body patterning toward this end. Locomotor components and maneuvers may serve to impede the hydrodynamic signature of squid. Many deep-sea fish have well-developed lateral lines that are capable of detecting even the smallest movements of their prey (Marshall, 1979). Pinniped vibrissae are sensitive to prey movements and can be used to track prey from hundreds of meters away (Dehnhardt *et al.*, 2001). *Octopoteuthis deletron's* extensive locomotor components and maneuvers may prevent detection by confusing such hydrodynamic search images.

Disruptive coloration and illumination may be used to obstruct the body outline and prevent detection. *Octopoteuthis deletron's* chromatic components that comprise interspersed white and red areas may serve a disruptive function. The red body coloration appears black while the white areas are discernible, breaking up the body outline. This effect was observed when encountered individuals were at the furthest limits of the vehicle's lights – the red blended with the dark background, but the white areas were conspicuous. Similarly, the body pattern consisting of a uniform Dark or Mottle dorsal side, with Ventral Mantle Bands and ventral Dark Arm Crown Band may cause predator confusion as the Rotating squid appears and disappears. Ventral photophores may produce disruptive illumination (Herring, 1977). The combination of disruptive illumination and disruptive coloration, whereby dark or light chromatic components and bioluminescence accentuate each other may be an effective cryptic strategy.

Secondary defenses are enacted once an individual is aware of a predator's presence or has been detected by a potential predator (Edmunds, 1974). Some components may be used to alert, blind, startle, or confuse predators once concealment has failed. Arm-tip photophore bioluminescence is conspicuous and may communicate to predators that the squid is alert and aware of its presence, thereby forestalling an attack (Young *et al.*, 1982). Young *et al.* (1982) predicted that bright bioluminescent displays might temporarily blind the sensitive eyes of predators, allowing an animal to elude a potential predator. The commonly observed Arm Spread, with all eight photophores simultaneously bioluminescent (Acute Arm-tip Chomatophores Contracted), was frequently followed by Forward or Backward Jetting. This sequence

may startle or bewilder a predator and allow escape. Downward movement is a common response of mesopelagic micronekton, including many squids, that serves to move an individual away from a disturbance and into darker water (personal observations).

The maneuvers Grasp, Bite, and Arm Autotomy probably constitute a last-resort defensive attempt to cause physical injury to a predator or to sacrifice a diversionary body part (Edmunds, 1974; Herring, 1977; R. Young in Hanlon and Messenger, 1996). The hooks and beaks likely irritate or injure predators, causing them to release the individual. An autotomized arm with bioluminescing arm-tip photophore may distract a predator while the squid retreats.

Body patterning could also be used for prey acquisition. Bioluminescent flashes, such as Arm-tip Chromatophores Contracted/Expanded, or Flicking Arm-tips during bioluminescence might attract crustaceans, fish, or cephalopods within reach by mimicking their prospective prey (Herring, 1977). Bioluminescent lures occur in anglerfishes and stomiatoid fishes, and photophores in some chiroteuthid, histioteuthid, cranchiid, and enoploteuthid squids, cirrate octopuses, and *V. infernalis* have been proposed as lures (Herring, 1977; Marshall, 1979; Hunt, 1996; Robison *et al.*, 2003).

The same chromatic components that effectively hide *O. deletron* from its predators could allow it to remain undetected as it hunts its own prey. In the deep sea the roles of predator and prey are sometimes suddenly interchangeable. Many species are capable of capturing and consuming individuals almost as large as themselves, so the effectiveness of remaining undetected may determine who obtains a meal and who becomes a meal.

Complex body patterning behaviors may also be important in intraspecific communication. Deep-sea animals are often widely dispersed and may have specific mechanisms to assist in species recognition, mate selection, and reproductive behavior (Herring, 2000). While long distance attraction probably involves chemosensory tactics, photophores are likely key species recognition tools at closer range (Herring, 1977, 2000). Photophore arrangement is often species-specific and some species also have sexually dimorphic photophore positions (Young, 1975; Herring, 1977, 2000). However, coloration, postures, locomotion and maneuvers may be important in these interactions as well, or as a means to orchestrate bioluminescent displays used to communicate with conspecifics (R. Young in Hanlon and Messenger, 1996). Cephalopods also have a lateral line analogue that could be used to detect and recognize potential mates by their particular locomotion or maneuvers (Budelmann and Bleckman, 1988).

Several non-communication possibilities exist to explain the behaviors of *O. deletron*, including physiology, locomotor efficiency, ontogeny, and phylogeny. Previous authors have predicted that physiological limitations during locomotion may cause certain color patterns, or may limit others due to connections between locomotor muscles and chromatophore muscles (Hunt, 1996; Huffard, 2006). However, in the current study we found no cases of chromatic components linked with locomotion. Hunt (1996) hypothesized that some postures were designed for maximum hydrodynamic efficiency during locomotion and therefore had no role in signaling. However, we observed many postures, maneuvers, and chromatic components in stationary individuals. Juvenile *O. deletron* occur in the upper few hundred meters of the water column (Okutani and McGowan, 1969). The behaviors we observed could be remnants

of these juvenile stages that are no longer useful at mesopelagic depths. While we observed only a few juveniles, they appeared to have a smaller behavioral repertoire than adult individuals (analyses not performed). Finally, *O. deletron*'s behaviors may reflect those of their ancestors, which recent phylogenies indicate were also deep-water species (Lindgren *et al.*, 2004; Strugnell *et al.*, 2006; Lindgren and Daly, 2007; Strugnell and Nishiguchi, 2007). It is possible that ancestral deep-sea cephalopods also had varied body patterning repertoires. We conclude that the behaviors we observed in *O. deletron* are unlikely to be vestigial given that they are metabolically costly and those used in intra-specific communication might attract predators. We assume that behaviors with no current utility will be lost over evolutionary time.

Non-metric Multi-Dimensional Scaling was successfully used to display similarities between individuals based on the behaviors observed from each and to assess observer-defined body patterns. While these analyses did not explain the majority of variation in our data sets, we did demonstrate that hydrographic data such as depth and oxygen did not appear to affect which behaviors an individual performed, but the length of our observations and the interactions of the squid with the ROV did affect an individual's behavior. This information is essential to obtaining further observations and interpreting the function of the behaviors defined in this study. Lastly, although the format of our data matrix did not allow determination of body patterns from the NMDS, we have shown that it may be used to support those previously defined.

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Figure Legends

Figure 4-1. Light and dark chromatic behavioral components of *Octopoteuthis deletron*.

Figure 4-2. Postural components of *Octopoteuthis deletron*. (A) Dorsal Mantle Up, Fins Out, Ventral Arm Curl. (B) Tail Down, Fins Out, Central Arm Space. (C) Tail Up, Fins Out, Dorsal Arm Curl. (D) Dorsal Mantle Up, Fins Out, Arm Keels Prominent, Straight Arms. (E) Tail Down, Fins Curved Ventrally, Arms Curled. (F) Dorsal Mantle Up, Fins Curved Ventrally, Dorsal Arm Curl. (G) Dorsal Mantle Down, Fins Curved Ventrally, Straight Arms. (H) Dorsal Mantle Up, Fins Out, Arm Tips Inward. (I) Tail Down, Fins Curved Ventrally, Dorsal Arm Curl. (J) Tail Up, Straight Arms, Fins Curled Ventrally. (K) Tail Down, Fins Out, Lateral Arm Spread. (L) Dorsal Mantle Up, Fins Out, Compact Arms. (M) Tail Down, Dorsal Arm Curl, Splayed Arms. All images are frame-grabs taken directly from ROV dive footage and are unmodified except for cropping. All images © MBARI.

Figure 4-3. Different stages of arm-tip photophore bioluminescence in *Octopoteuthis deletron*. (A) Chromatophores contracted. A frame-grab taken directly from ROV dive footage. Image © MBARI. (B) Chromatophores partially expanded. (C) Chromatophores fully expanded. (B) and (C) are laboratory images taken with a microscope. All images are unmodified except for cropping.

Figure 4-4. Non-metric Multi-Dimensional Scaling ordination of individual *Octopoteuthis deletron* behavior sets. Observation length (min), ROV contact, and collection correlated with behaviors observed. (A) Observation length (min). (B) ROV contact. (C) Collection.

Figure 4-5. Non-metric Multi-Dimensional Scaling ordination calculated from the subset of behavioral components that comprise the four body patterns (Table 1).

Supplemental Figure 4-1. *Octopoteuthis deletron* encounter depths.

Supplemental Figure 4-2. Encounter depth compared with time of day (Pacific Standard Time).

Supplemental Figure 4-3. Number of behaviors performed by individual *Octopoteuthis deletron* compared to the amount of time (min) each individual was observed.

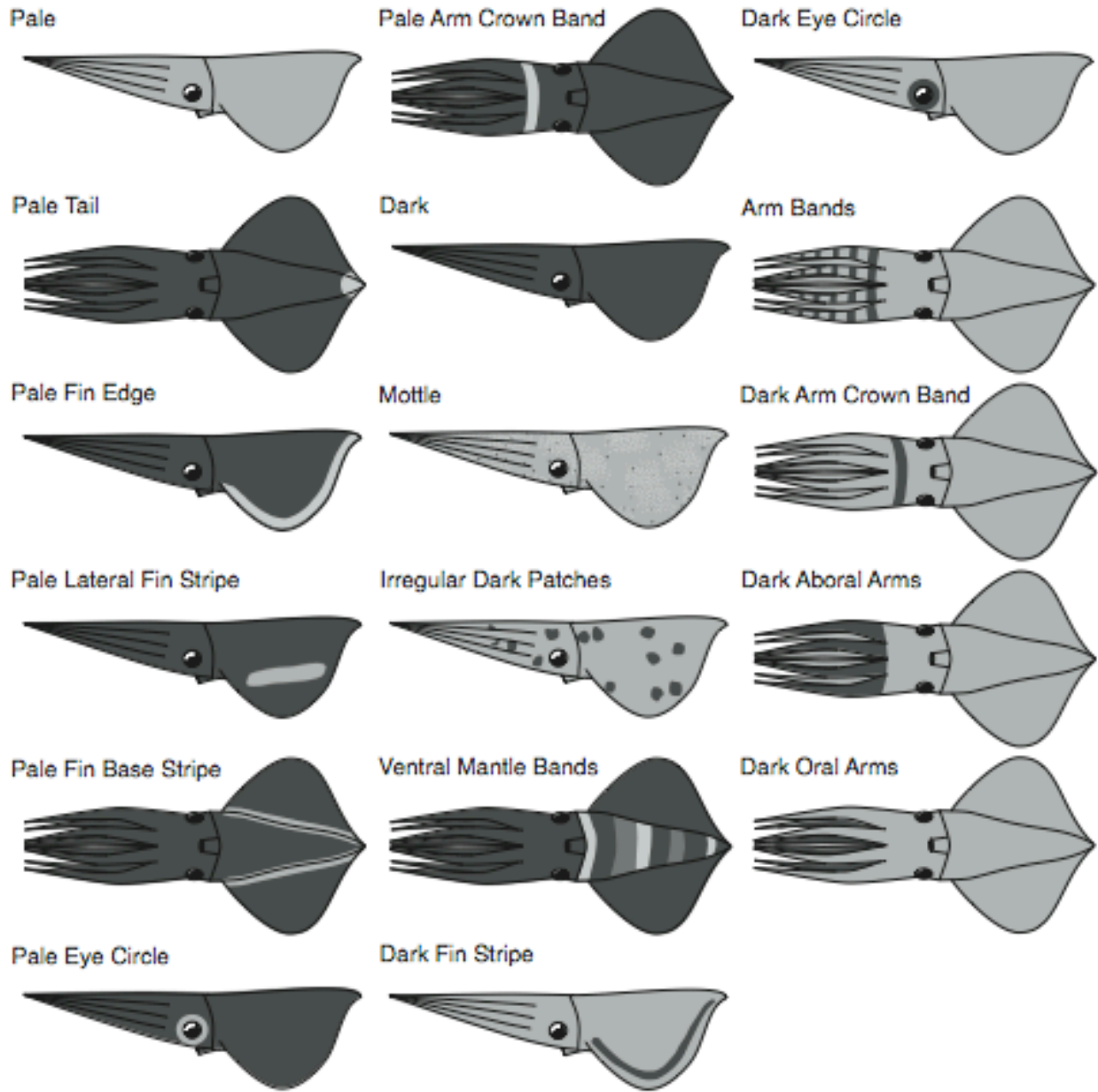


Figure 4-1

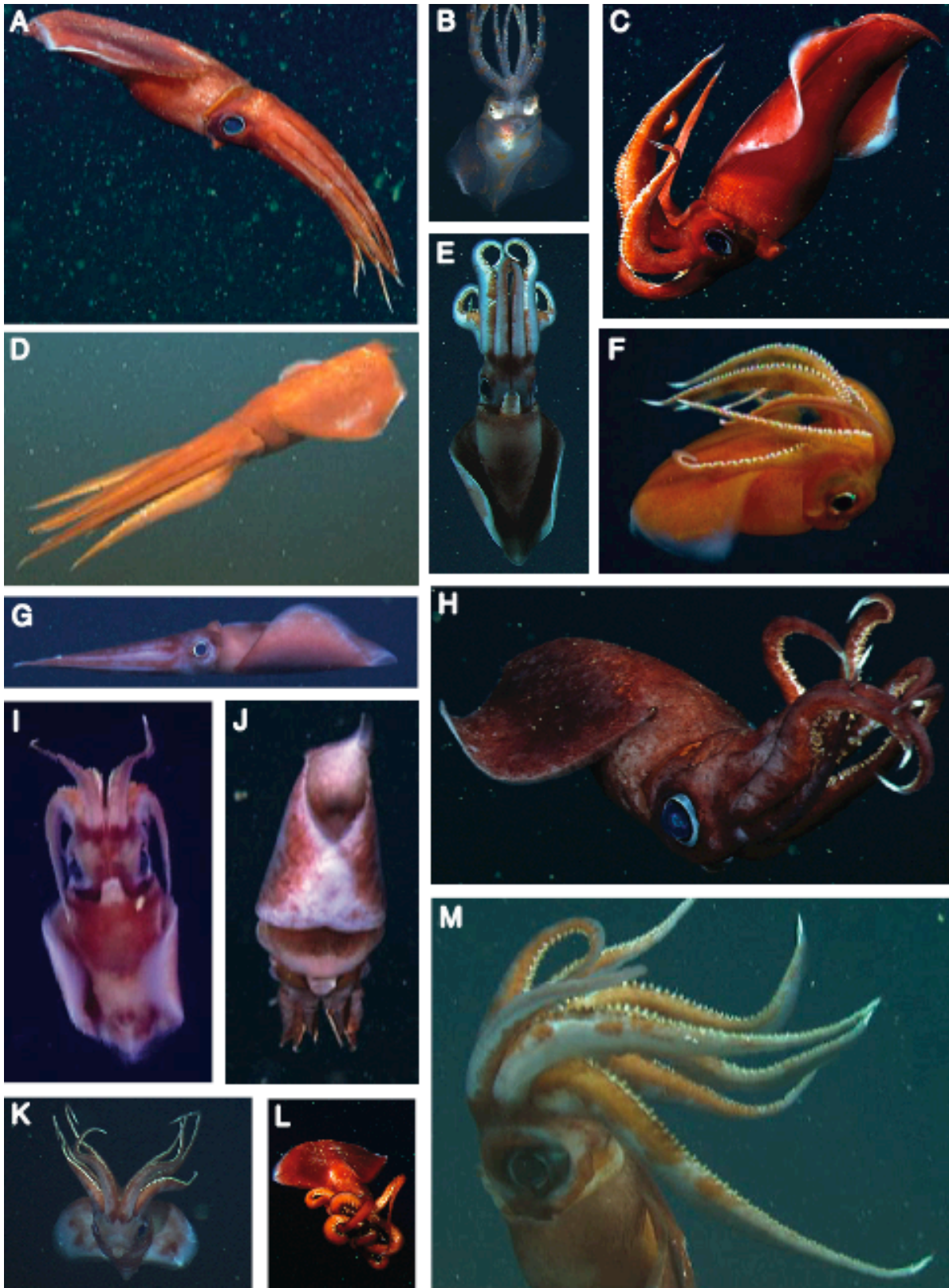


Figure 4-2

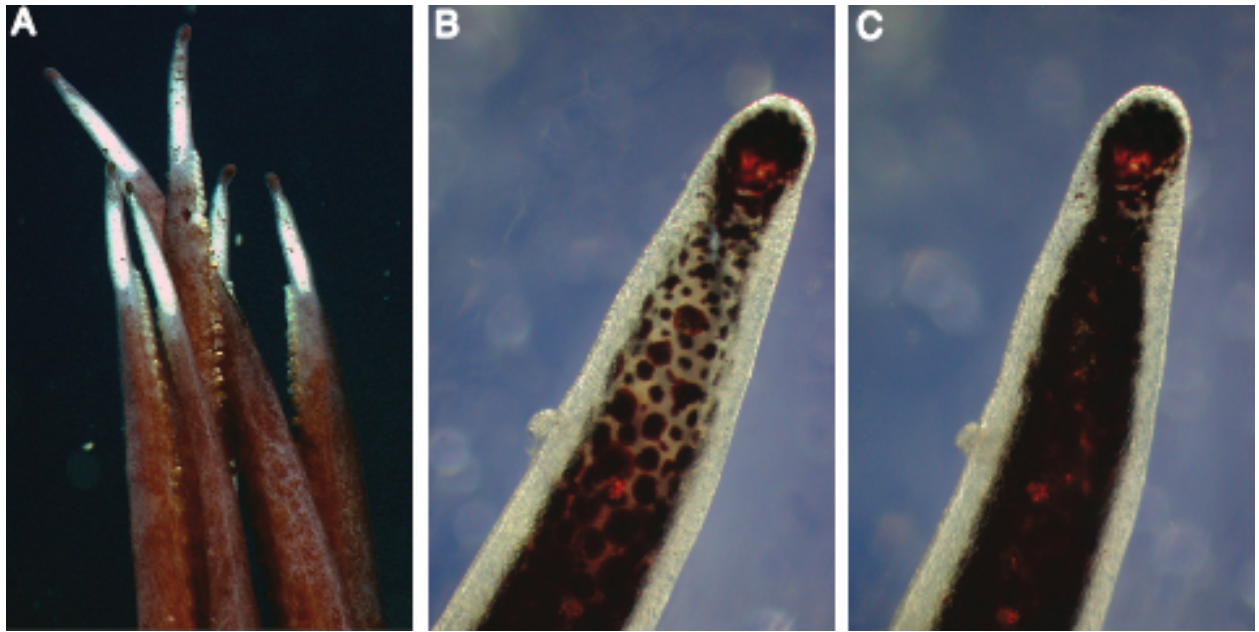


Figure 4-3

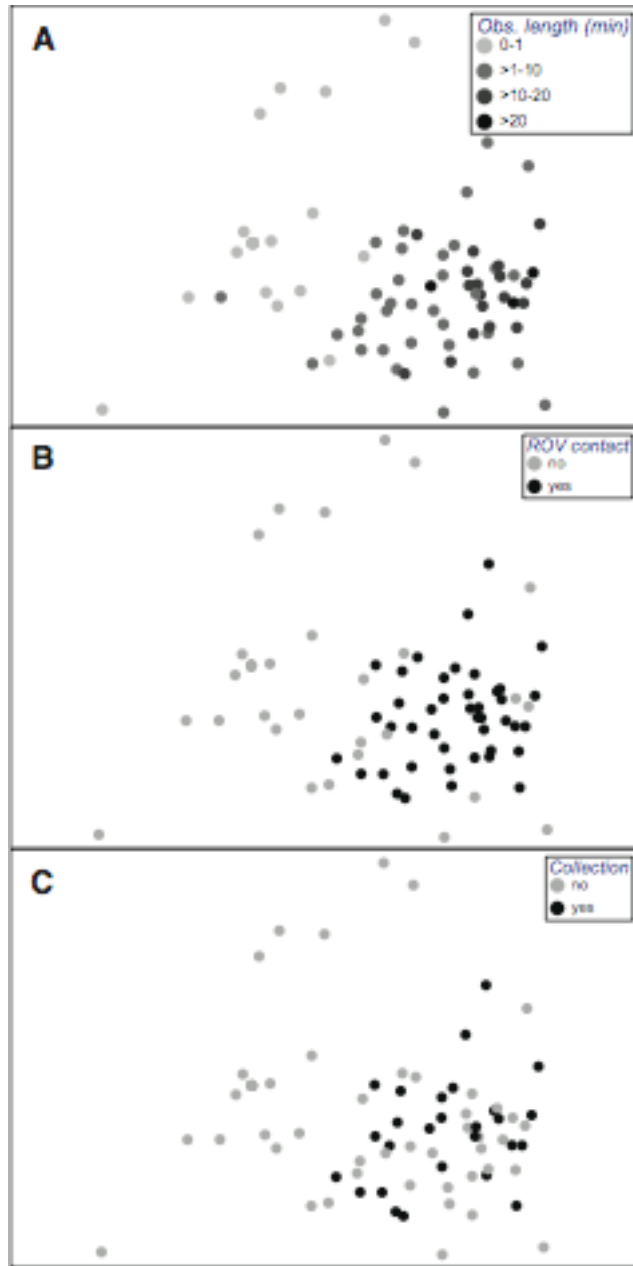


Figure 4-4

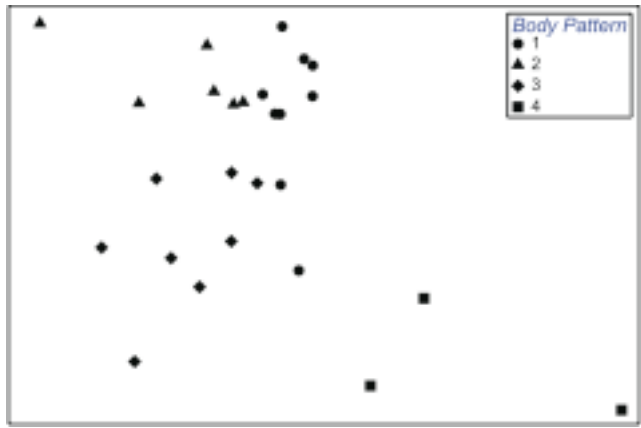
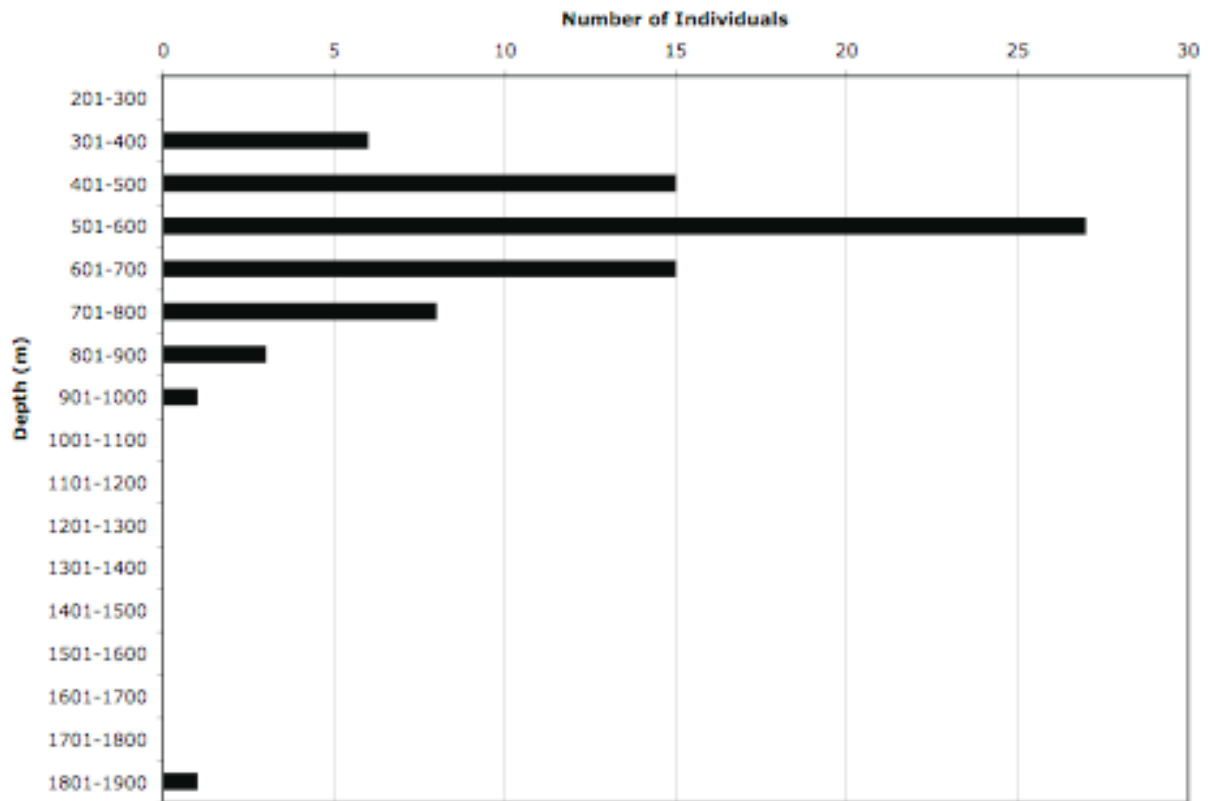
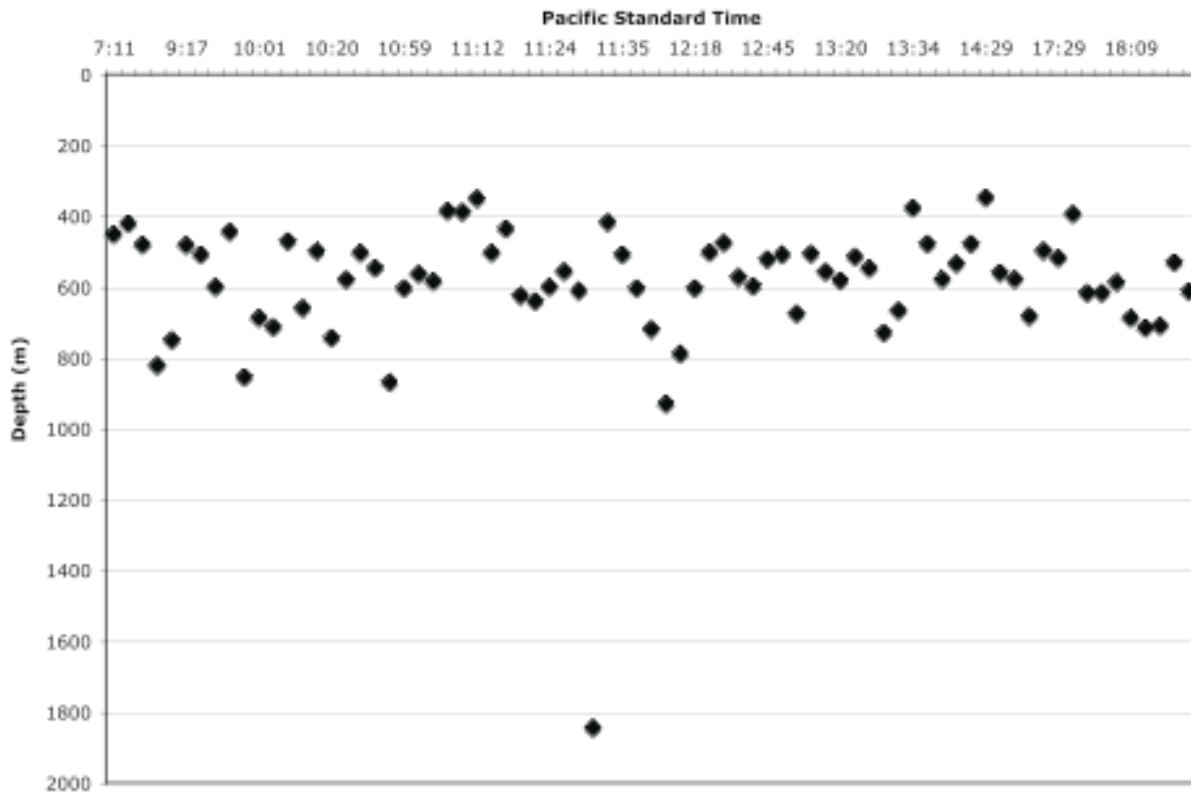


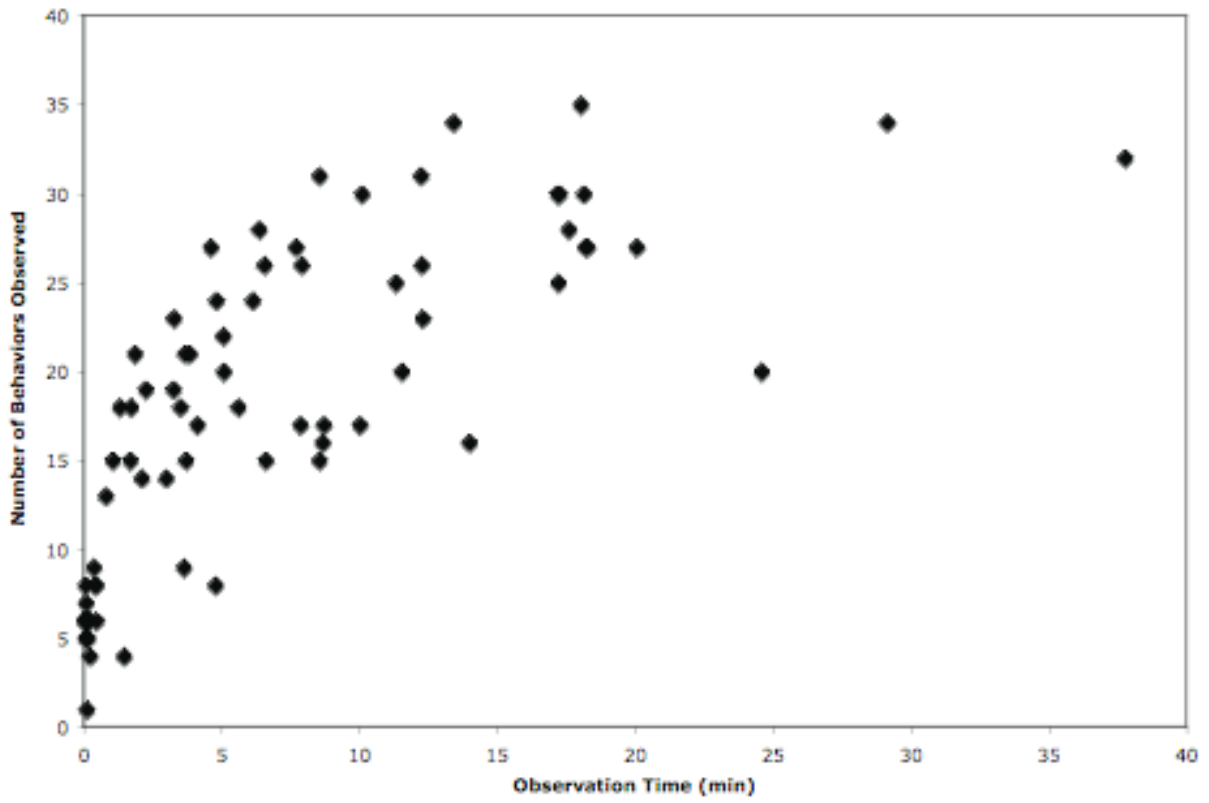
Figure 4-5



Supplemental Figure 4-1



Supplemental Figure 4-2



Supplemental Figure 4-3

Chapter 5: Economy of autotomy in the mesopelagic squid *Octopoteuthis deletron* Young 1972

Abstract Net collected individuals of the deep-sea squid genus *Octopoteuthis* sometimes have short, blunt-ended arms, indicating that these species may be capable of arm autotomy. *Octopoteuthis deletron* was studied to determine its' capacity for this defense. Remotely operated vehicles were used to examine individuals' arms for evidence of previous autotomy and subsequent regeneration. Arm morphology was also examined on live, preserved, and sectioned material. We induced autotomy both in the lab and in the field. *Octopoteuthis deletron* is the first squid reported to autotomize its' arms, the only cephalopod known to break an arm at the point of interaction (economy of autotomy), and is one of very few species that uses attack autotomy.

Introduction

A predator gains ground on its prey, makes a final lunge, and successfully grabs it. Or has it? In fact, the predator has actually been left with a writhing, severed appendage while the would-be victim escapes. This interaction, whereby an organism voluntarily severs a body part that distracts a predator, is the essence of defensive autotomy.

Anti-predator behaviors are categorized as primary or secondary defenses according to when they are enacted. Primary defenses operate regardless of predator presence and are often permanent features of the organisms' morphology or ecology — such as cryptic coloration or nocturnal activity — thereby decreasing the likelihood of detection (Edwards, 1974; Endler, 1981). Secondary defenses are performed after an organism has detected the presence of a potential predator (Edmunds, 1974). These defenses decrease the chance of a successful attack and include startle, counter-attack, protean (erratic) behavior, playing dead (thanatosis), fleeing, and autotomy. The latter, defined as the defensive loss of a body part, occurrence at a breakage plane, and its' nervous control, is typically the final defense in predator-prey interactions (Wilkie, 2001, MacGinnis 2006, Fleming et al 2007). Researchers have found reduced fitness in autotomized individuals caused by suboptimal mating, foraging, or both (Maginnis, 2006; Fleming et al., 2007; Naya et al., 2007; Wrinn and Uetz, 2008). Autotomy nonetheless occurs in a wide range of vertebrate and invertebrate taxa; these multiple independent evolutionary occurrences point to autotomys' defensive effectiveness (Maginnis, 2006; Fleming et al., 2007).

Voluntary detachment occurs at a breakage plane that is either a structurally weak anatomical fracture plane or a potential breakage site that experiences a loss of tensile strength during autotomy (Fleming et al 2007; Wilkie, 2001). These structures allow for a clean break that minimizes trauma and speeds healing (Wilkie, 2001). Most species require external resistance, usually the predator's grip, for autotomy to occur (Fleming et al 2007). Some organisms require a force equivalent to their own mass or more to autotomize; in some species autotomy only occurs if the predator shakes its head while gripping the prey (Fleming et al 2007). The threshold for breakage varies between species, individuals, and even limbs within an individual, indicating nervous regulation (Fleming et al 2007).

Some shallow-water octopuses autotomize arms (Norman and Finn, 2001; Ward 1998, Huffard, 2007, Norman & Hochberg 2005, Hochberg et al 2006). *Ameloctopus*

litalis autotomizes at a breakage plane near the base of the arm (Norman and Finn 2001). Other octopuses also have a specific area along the arm (e.g. between suckers 4 and 7 in *Octopus brachiotomus*) where autotomy occurs, but no breakage plane is evident (Ward 1998, Norman and Finn, 2001). An autotomized *Abdopus capricornicus* arm thrashed for more than an hour (Norman and Finn, 2001). Arm regeneration takes 10 weeks for *Octopus brachiotomus* and 2-3 months for members of the subgenus *Abdopus* (Ward 1998, Norman & Finn 2001). The octopuses that autotomize arms are not closely related, so this defense has probably evolved more than once within the octopuses. However, autotomy has not been previously reported in cuttlefishes, squids, or nautilus (Ward, 1998, Norman & Finn 2001).

Net-caught specimens of the deep-sea squid genus *Octopoteuthis* Ruppell 1884 commonly have broken, blunt-ended arms and uneven arm lengths (Young 1972; Young and Vecchione, 2008). These observations led scientists to suspect that these species are capable of arm autotomy (Vecchione and Young, 2008). However, arm loss due to physical damage from nets could not be ruled out and no cases of arm autotomy were observed. We tested the hypothesis that *Octopoteuthis deletron* Young 1972 is capable of arm autotomy by undertaking *in situ* observations and experimentation, laboratory manipulations, and histological sectioning.

Octopoteuthis deletron inhabits mesopelagic depths of the eastern North Pacific (Young 1972). Individuals grow to 17 cm mantle length, have fins that extend the length of the mantle, long arms and large eyes (Young 1972). Individuals lose the feeding tentacles at an early stage, using the eight arms for prey capture and handling (Young 1972). Each arm has a large terminal photophore and arm pairs II-IV have a series of embedded photophores along their length (Young 1972). The terminal photophores are approximately the same size on all the arms and produce intrinsic blue-green bioluminescence with a maximum output of 465 nm (S. Bush, unpublished data). The terminal photophores are believed to be used in prey attraction, mate recognition, or both (Bush et al., 2009). In addition, most of each arm is covered in oral hooks; proximal to the terminal photophore there are a few small suckers (Young, 1972).

Materials and methods

Submersible observations

We obtained directed *in situ* observations and reviewed footage from previous dives of three Remotely Operated Vehicles - ROVs *Ventana*, *Tiburón*, and *Doc Ricketts* - owned and operated by the Monterey Bay Aquarium Research Institute. *Octopoteuthis deletron* individuals were examined for breaks along each arm. Regeneration was observed as partial regrowth from a blunt end or photophores that were proportionally smaller than those of other arms, distal hooks that were smaller than proximal hooks, or both. For recording and reporting arm injuries, we follow the arm classification system of Roper et al. (1984): the dorsal arms are arms I, dorso-lateral are II, ventro-lateral are III, and ventral arms are IV. Cephalopod species descriptions include an arm formula describing the relative length of arm pairs; due to collection damage no arm formula was reported for *Octopoteuthis deletron*, therefore we cannot use relative arm length to determine whether autotomy, regeneration, or both occurred.

Laboratory observations

Seven individuals were collected for laboratory observations. We used arm touching, holding, pinching, pulling, brushing along arm hooks with Velcro, and electric shocks from a 6V battery to instigate autotomy. A freshly severed arm (#78) was dissected to study arm hook structure and function in arm autotomy.

Histology

One to eight arms from 15 *O. deletron* specimens were sectioned to look for fracture planes. Arms were also sectioned from one *Chiroteuthis calyx*, one *Gonatus* sp. and one *Vampyroteuthis infernalis*, none of which are thought to autotomize arms, as controls. All material was preserved in 10% formalin and arranged in cassettes for paraffin embedding. The Community Hospital of the Monterey Peninsula performed the embedding, staining, sectioning, and slide-making. Each slide was scanned under a dissecting or compound microscope for breakage planes and instances of regeneration not visible from gross morphological assessments.

In situ experiment

In previous studies, we observed several individuals grabbing (counter-attacking) the ROV though none resulted in arm autotomy (n = 23 of 76, Bush et. al, 2009). We hypothesized that autotomy requires arm hook attachment to provide resistance for the arm to separate and that the hooks were unable to attach to the ROV's metal components. We tested this hypothesis by mounting a bottle brush onto the swing arm of the ROV *Ventana* and ROV *Doc Ricketts*. The vehicle was manipulated to lightly touch an *O. deletron* individual on the arms or mantle to instigate counter-attack. Controls were performed with seven *Chiroteuthis calyx*, eight *Galiteuthis phyllura*, nine *Gonatus* spp., one *Histioteuthis heteropsis*, one *Japetella diaphana*, thirteen *Taonius cf. borealis*, and one *Vampyroteuthis infernalis*, none of which are thought to autotomize arms.

Results

Submersible observations

We observed 75 individuals during ROV dives from June 2003 – 2009 and reviewed recorded footage of 21 individuals from previous dives. Some observations were limited by viewing distance, therefore for 40 individuals we were unable to determine occurrences of arm autotomy or regeneration. There were 56 individuals for which we made determinations; 15 (27%) were missing portions from one to eight of their arms (Figure 5-1). These 15 individuals had a total of 37 autotomy injuries (Table 5-1). It was not possible to determine if specimens with multiple injuries obtained them during one or more interactions. Autotomy occurred at varying lengths along the arm; injuries occurred from just proximal to the terminal photophore to approximately $\frac{3}{4}$ of the arm. Loss of the distal tip, including the terminal photophore, was the most common type of injury (n = 22 of 37 injuries, 59%; Figure 5-2). In contrast, only one individual (#72) was missing $\frac{3}{4}$ of an arm. Some individuals were actively regenerating arms (n = 7 of 56, 12.5%). These arms had a disproportionately small distal arm and terminal photophore (Figure 5-3B-C).

Other observations included two arms accidentally severed from an individual upon collection that thrashed for 15 and 32 sec before going out of view. Also, we observed an autotomized arm slowly sinking through the water column (Figure 5-1B). It appeared to have a bite mark in the flesh immediately distal to the autotomy break.

Laboratory observations

We experimented with eleven individuals; seven (64%) of these autotomized one or more arms. While individual observations were unique, taken together it is clear that *O. deletron* arms are not inherently weak and instead require nervous control and voluntary autotomy by the squid. An *O. deletron* arm only partially autotomized or fully autotomized at the point where it was held, lifted, pinched, or electrically stimulated ($n = 7$). Other arms autotomized when they were accidentally dragged over the edge of a container. Arm autotomy therefore requires resistance, whether that be from the arm hooks grasping something or the arm being held extrinsically (for instance by a predator). Arm hooks from two individuals came off the arm when they were dragged over the loop side of Velcro, but autotomy did not occur.

One squid (#41) autotomized all of the arms immediately after transfer from a holding tank to an observation tank. The individual grasped the textured tank bottom with the arm hooks, somersaulted, and released ink while all eight arms autotomized. Each autotomized arm continued to thrash and the terminal photophores bioluminesced for approximately ten seconds. The bioluminescence was bright enough to be seen under full laboratory lighting. The autotomized sections measured 9 – 22 mm length ($n = 8$, mean = 15.9 mm). In addition to the detached arms, there were eight sections, one from the middle of each arm. These sections measured 2.1–4.0 mm length ($n = 8$, mean = 2.98 mm) and did not move. There were also 119 arm hooks either attached to or lying on the bottom.

An individual was kept in a kreisel – a circular aquarium designed to keep animals away from the sides – at 4–6° C in a dark room. A thawed piece of grass shrimp hooked onto a wooden feeding stick was fed to the individual. The individual attacked the food and feeding stick, autotomizing arm LIV midway along its length on the piece of food. LIII later autotomized onto the feeding stick. The terminal photophore glowed steadily for approximately 4 seconds then blinked on and off for an additional 3 seconds. I could not determine if these arms thrashed because observations were made in the dark. The stick was also covered in a large amount of very viscous mucus.

The arm hooks lie in two series within an undulating groove on the oral side of the arms (Figure 5-4A). The hook is surrounded by soft tissue sheath at rest and is comprised of one large hook and two accessory cusps (Figure 5-4B). The hooks advance out of the soft tissue sheath when used. The base of the hook attaches to the convex side of a cup-shaped ‘external mound’ (Figure 5-4D). This unit – including hook, soft tissue, and external mound – can bend to either side, forward, and backward, or rotate almost 360°. An ‘internal mound’ is situated within gaps of gelatinous arm tissue and its’ fibers radiate from the apex of the mound, connecting the mound to the arm musculature (Figure 5-4C, E-F). A fibrous cord emerges from this internal mound and inserts into the external mound (Figure 5-4C). The insertion point is offset toward the front of the hook, such that when pulled the hook will bend forward (Figure 5-4D). The external mound is not easily removed from the internal mound; it is easier to remove the

hook and sheath from the external mound or the internal mound from the arm musculature. A hook approximately half the size of the others was observed midway down the arm, indicating that it was lost and regenerated.

Histology

Evidence of regeneration was observed in 8 of 15 individuals (Figure 5-3A). In addition, several arms (n = 14) had one or more partial splits, usually from the oral (hook) side of the arm. These may have been the result of partial autotomies such as occurred in laboratory observations. Fracture planes were found in an arm from each of two individuals that had autotomized (Figure 5-3D). No regeneration, splits, or fracture planes were found in the control species.

In situ experiment

Four out of seven *O. deletron* tested with the bottle brush autotomized one or two arms (one arm, n = 3; two arms, n = 1). While one individual (#87) autotomized an arm after the first touch by the brush, the others did not autotomize arms until they had been touched from 10-16 times. In all cases the arms were autotomized when an individual counter-attacked and grabbed the brush. Again, one individual (#87) autotomized on the first counter-attack, whereas the others took from 3-11 counter-attacks before autotomy occurred. Most brush touches were on the arms resulting in the individual jetting backward a short distance, spreading the arms (in many cases with all terminal photophores bioluminescent at the widest arm spread, see Bush et al., 2009). In some cases this was followed by forward jetting to counter-attack. In the few cases where the squid was touched on the posterior mantle, the individual flipped around to orient the arms toward the brush before initiating the above sequence. None of the control individuals (n = 40) autotomized an arm.

After autotomy, two of the four individuals jet-escaped, releasing an ink pseudomorph. The other individuals remained still, one within its own ink cloud. Terminal photophores on all the autotomized arms were bioluminescent. Two arms from an individual remained attached to the bottle brush. One of these was attached at the autotomized end while the distal part thrashed for 3 min 53 sec. The other arm was not visible at first but was then observed to moderately thrash for 48 sec. Thrashing resumed when the arm fell off the brush and lasted for 30 sec. One arm from another squid remained attached to the brush and did not move much.

Discussion

Octopoteuthis deletron is the first squid reported to autotomize arms. Each of *O. deletron*'s eight arms can autotomize and regenerate, therefore multiple arms can be autotomized at once and autotomy can be used as a defense several times. We propose two autotomy mechanisms: (1) an arm is grabbed by a potential predator, providing the resistance for autotomy and (2) the arm hooks grasp a potential predator and their attachment provides the resistance for autotomy. The thrashing, bioluminescent arms must attract the predator's attention in the darkness and allow the squid to retreat. The use of bioluminescent appendages or secretions as a defensive distraction has been observed in many deep-sea animals, including jellies, polychaetes,

and crustaceans (Osborn et al., 2009; Herring, 2002). Likely there are many more species, including squids, that use similar tactics.

Despite the obvious benefits, autotomy has numerous costs including reduced growth, decreased fecundity, or both, as the autotomized tissue is regenerated (Maginnis, 2006; Fleming et al., 2007). Unfortunately, we do not know typical growth rates, fecundity, or longevity for this, or any, deep-sea squid species. *Octopoteuthis deletron* has been proposed to use the terminal arm photophores as lures, so the loss of photophores could make prey attraction less effective (Bush et al., 2009). Similarly, subduing and handling prey may be less effective without the full use of all arms. Lastly, mate attraction, which probably involves the arms and terminal photophores, may be altered by autotomy.

However, the jettisoning of an arm (or arms) must confer an increased likelihood of escape and survival because instances of autotomy were relatively common in *O. deletron* (27%). This is comparable to other species: 63% male and 44% female *A. russatus* mice; 12% male and 25% female *A. cahirinus* mice (Shargal et al. 1999); 7.5-16.5% of spiders, 50% of harvestmen, and 90% of damselfly *Lestes sponsa* larvae (Bateman & Fleming, 2005); 38.4% female and 32.2% male *Carcinus maenas* crabs (Mathews et al., 1999); 42% of the crab *Hemigrapsus sanguineus* (Davis et al., 2005).

Octopoteuthis deletron is the first cephalopod that demonstrates economy of autotomy. Fracture planes were observed within a few mm of each other in histological section and autotomy always occurred where the arm was held, no matter where along the length. The ability to sever an appendage at one of numerous places along an appendage is not common among animals that autotomize, though it occurs in some clams, seastars, brittlestars, crinoids, and lizards (Delage and Herouard, 1903; Gilmour, 1962; Bustard, 1968; McVean, 1975). Economy of autotomy minimizes tissue loss while still allowing escape (Delage & Herouard, 1903, Fleming et al., 2007). *Octopoteuthis deletron* arms autotomize at the point of interaction. The autotomizable body parts of organisms that demonstrate economy of autotomy obtain injuries in proportion to exposure to injury (Fleming et al., 2007), and not surprisingly more injuries were observed on the distal portions of *O. deletron* arms compared to the proximal. The benefits of minimizing tissue loss off-set some of the fitness costs of autotomy (Fleming et al., 2007).

Octopoteuthis deletron is among the few species known to perform attack autotomy. Counter-attack has not been reported in cephalopods, although biting has (Hanlon and Messenger, 1996; Bush et al., 2009). In attack autotomy, as documented in some crabs, the would-be prey grips the predator with the chelae, then autotomizes it (Robison et al., 1970, McVean, 1975). *Octopoteuthis deletron*'s retractable, rotating arm hooks can potentially grab onto an attacker and provide resistance to the attached hooks for autotomy.

This squid has a series of defenses that escalate from primary to secondary, finally leading to attack and autotomy (Edwards, 1974; Bush et al., 2009). Primary defenses include crypsis and polyphenism, and the secondary defenses of jet-escape, ink release, and startling with bioluminescence come into play when primary defenses fail. *Octopoteuthis deletron* will counter-attack and autotomize one or more arms after repeated provocation. After autotomization, individuals sometimes move rapidly away. However, escape is both energetically expensive and may stimulate bioluminescence

allowing a predator to follow the squid (Seibel et al., 2000; Robison, 2004). Perhaps for these reasons, *Octopoteuthis deletron* has been observed to remain motionless, sometimes within an ink cloud, while the arm distracts the potential predator.

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Table 5-1. The number of arm injuries and arms affected for *O. deletron* individuals.

Individual (#)	Injuries (#)	Arms affected
6	4	LII, LIII, LIV
9	3	LI, LII, RI
11	1	LIV
16	1	RIII
21	1	RIII
26	1	RII
31	5	LI, LII, LIV, RI, RIV
33	1	RII
34	8	LI, LII, LIII, LIV, RI, RII, RIII, RIV
45	1	LII
66	1	RIII
67	1	RII
72	4	RIII, RIV, LII, LIV
75	3	LI, RI, RII
91	2	LI, RIII

Figure Legends

Figure 5-1. (A) An individual *O. deletron* missing the terminal photophore of arm LII (arrow). (B) Autotomized arm found sinking through the water column. Arrow points to autotomy break. (C) An individual with three arms autotomized at different lengths (arrows).

Figure 5-2. Number of arm injuries by proportion of arm lost.

Figure 5-3. (A) Two fracture planes (arrows) in a histological section of an *O. deletron* arm. The fracture plane on the left is where the arm autotomized, the fracture plane on the right is a point where the arm began, but did not autotomize. (B) A previously autotomized arm beginning to regenerate. No terminal photophore is yet present, chromatophores have developed only on the aboral side, and hooks have just begun to form at the proximal end. (C) A regenerating photophore (left) next to a fully formed photophore (right). (D) An example of regenerated arm damage (arrow) in a histological section.

Figure 5-4. (A) Oral side of an *O. deletron* arm showing alternating hooks. (B) Close-up of a single arm hook showing the arm hook unit embedded in the surrounding arm tissue. (C) Arm hook unit removed to show the mound of arm tissue on which the arm hook unit sits and the fibrous cord that connects the arm with the hook unit. (D) Underside of the arm hook unit showing the off-set insertion point (arrow) of the fibrous cord in (C). (E) Histological section of an *O. deletron* arm indicating the placement of the arm hook units within and between arm tissue. (F) Histological section of an arm hook unit showing the insertion of the fibrous cord (arrow) from the underside of the arm hook to the arm musculature.

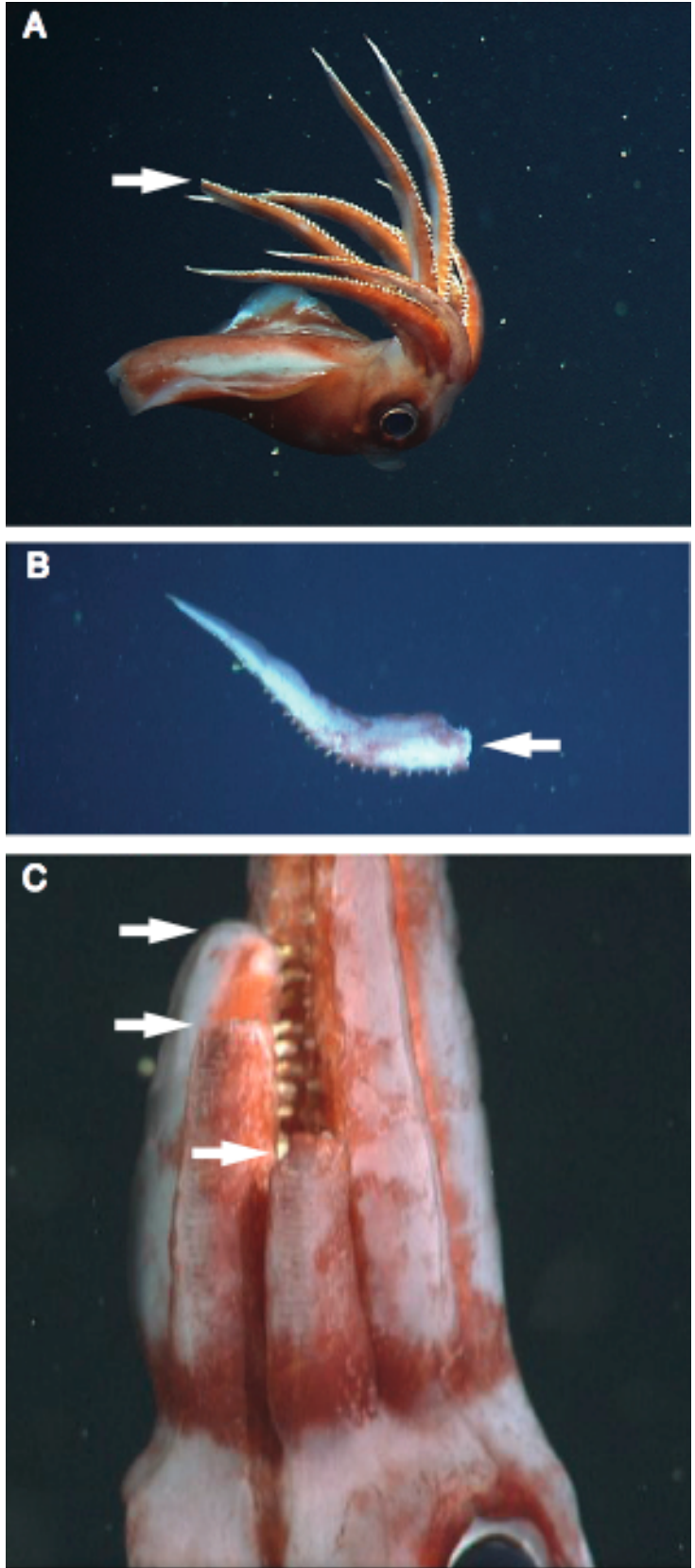


Figure 5-1

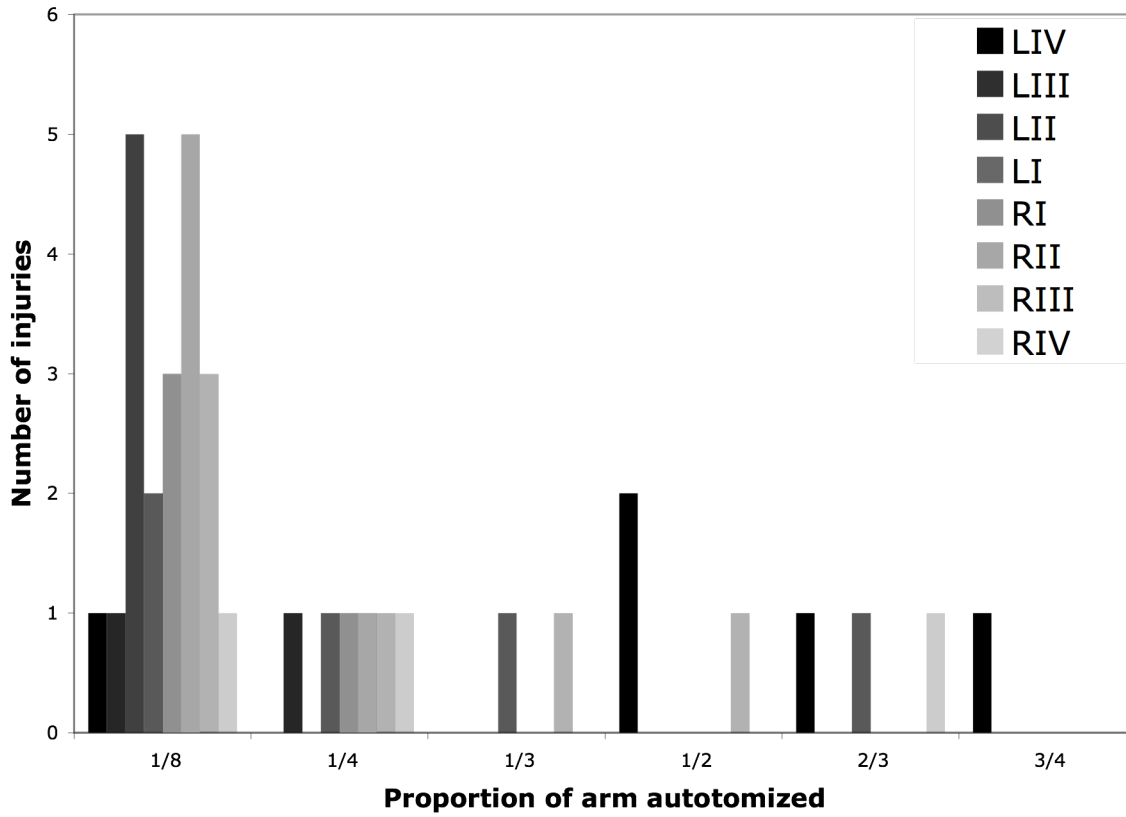


Figure 5-2

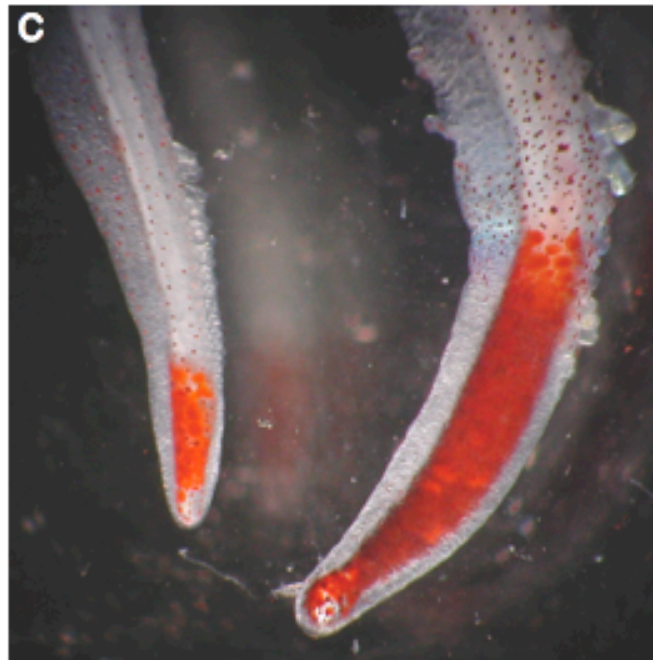
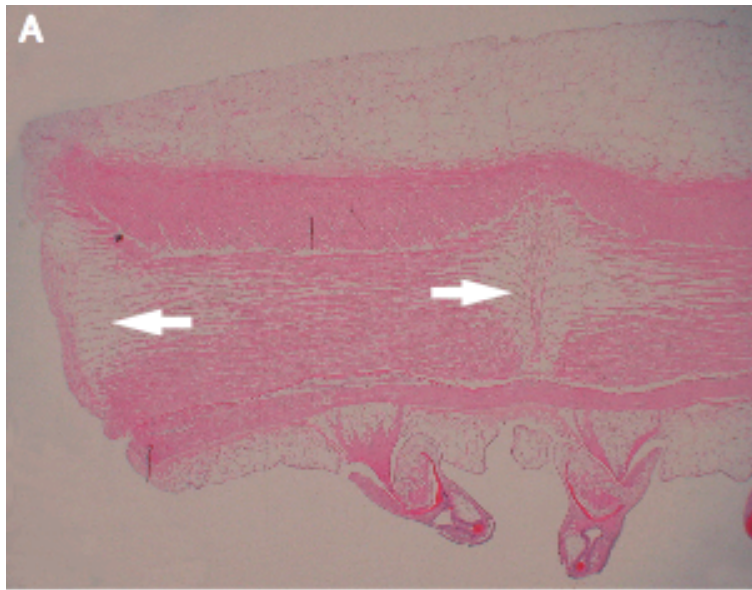


Figure 5-3

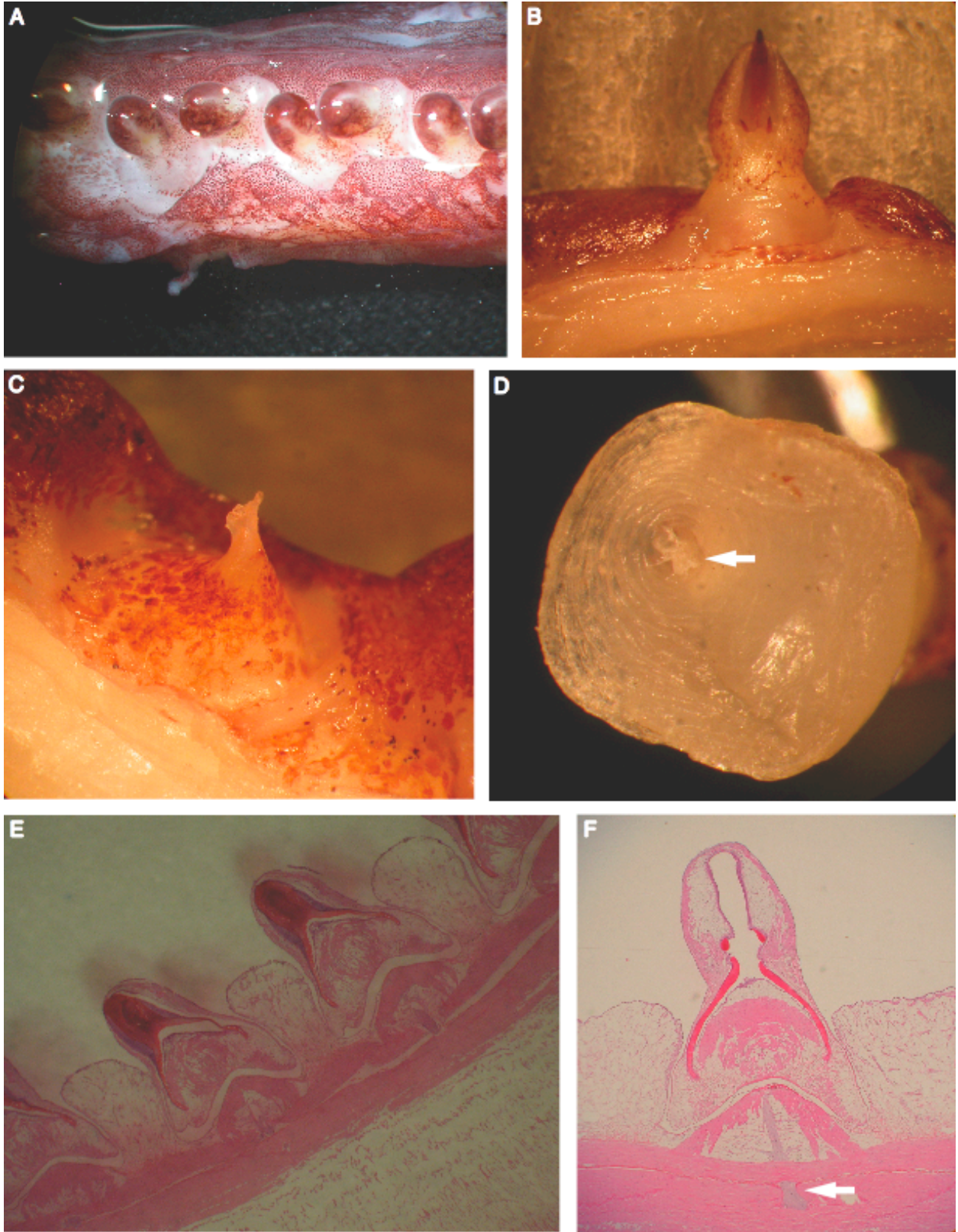


Figure 5-4

Chapter 6: Conclusions

Remotely operated vehicles (ROVs) were successfully used to obtain *in situ* observations of deep-sea squids and collect specimens for experimental work. The hypotheses that deep-sea squids do not release ink, have few color, posture, and locomotor behaviors, and have limited defenses were tested. A survey of deep-sea squid ink release showed that nearly all species inhabiting Monterey Bay and surrounding waters release ink (Chapter 2). Most of the ink releases observed were well below the photic zone and the deepest ink release was at least 800 m deeper than sunlight can penetrate (Robison, 2009). Six ink release types were described, some of which have not been observed in shallow-water cephalopods. Comparison of cephalopod inks showed that liquid chromatography – mass spectroscopy can be used to indicate differences in composition and highlight species for further investigation (Chapter 3). The ethogram of *Octopoteuthis deletron* indicated that this species has a much larger behavioral repertoire than predicted for a deep-sea squid (Chapter 4). Behavioral components observed included all of the categories used for descriptions of shallow-water cephalopod behavior: chromatic, postural, and locomotor. Additionally, this is the first species for which bioluminescent behavioral components have been described. Lastly, *O. deletron* can autotomize one or more of the eight arms at any of numerous places, minimizing tissue loss while remaining an effective defense (Chapter 5). This species is also one of very few known to attack potential predators and autotomize arms onto it.

These findings clearly indicate that deep-sea squids are much more behaviorally diverse than expected. In some ways this is not surprising since deep-sea cephalopods are thought to derive from shallow-water ancestors that presumably had the suite of behaviors that extant shallow-water cephalopods do (Jacobs and Lindberg, 1998). For this reason, some may argue that the behaviors described in the current work are phylogenetic relicts from this evolutionary history. However, there has been enough time for deep-sea octopuses to lose their ink sacs and change coloration (Voight, 1997). The fact that deep-sea squids have not done the same points to their current functionality.

While ROVs have been used to greatly improve our knowledge of the deep ocean, there continue to be limitations in observing this habitat that cannot currently be circumvented. These submersibles are large, loud, and bright and therefore likely reveal what deep-sea organisms do in a state of heightened awareness (Johnsen). We can at least assume that what we observe are defenses. Also, unfortunately for some of the research presented above, we cannot both watch the organism and observe its bioluminescence, making bioluminescent patterns unknown in many cases and uninterpretable in others.

It is difficult to wrap our heads around the workings of a habitat that is so unlike the one in which we reside. There are many questions still to answer about deep-sea ecology and only with expanded use of current technologies and support for development of new ones will we begin to understand this largest of the planet's habitats.

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