**Title**
Sexual Reproduction and Early Development in the Estuarine Sea Anemone, Nematostella vectensis Stephenson, 1935



**Author**
Uhlinger, Kevin R.

**Publication Date**
1997-09-01

Peer reviewed

# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

**UMI**

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

# Sexual Reproduction and Early Development in the Estuarine Sea Anemone, *Nematostella vectensis* Stephenson, 1935

By

Kevin Robert Uhlinger

B.S. (University of Washington) 1982
M.A. (University of California, Davis) 1993

Dissertation

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Committee in Charge

1997

Kevin Robert Uhlinger

September, 1997

Zoology

Sexual Reproduction and Early Development in the Estuarine Sea Anemone, *Nematostella vectensis* Stephenson, 1935

## Abstract

This dissertation: 1) determines the factor(s) responsible for spawning induction in *Nematostella vectensis*; 2) isolates, describes, and documents the source of jelly from egg masses of *N. vectensis*; and 3) describes *N. vectensis*' early development.

*Nematostella vectensis* were maintained on a 7-day mussel feeding/water change regime over 159 days. Within 36 hours of mussel feeding/water change, 69.1% of females and 78.5% of males spawned reliably. Through manipulation of feeding, water change, oxygen and nitrogenous waste concentrations, spawning induction was found to be triggered by the oxygen concentration associated with water change, and not by feeding. Ammonia, anemones' major waste product, inhibited this induction in a concentration-dependent manner.

Female *N. vectensis* release eggs in a persistent jellied egg mass which is unique among the Actiniaria. The major component of this egg mass jelly was a positive periodic acid-Schiff's staining, 39.5-40.5 kD glycoprotein. Antibodies

developed in rabbits against this glycoprotein bound to jelly of intact egg masses and to granules (≤ 2.8 μm in diameter) present in female anemone mesenteries and their associated filaments. Antibodies did not label male tissues.

*Nematostella vectensis* embryos underwent first karyokinesis ~60 minutes following the addition of sperm to eggs. Second nuclear division took place, followed by first cleavage, 90-120 minutes later. Each of the 4 blastomeres that resulted from first cleavage contained a single nucleus. Arrangement of these blastomeres ranged from radial to pseudospiral. Embryonic development was both asynchronous and holoblastic. Following formation of the 4-cell stage, 71% of embryos proceeded to cleave again to form an 8-cell stage. In each of the remaining 29% of embryos, a fusion of from 2-4 blastomeres resulted in 4 possible patterns which had no affect on either cleavage interval timing or subsequent development. The fusion event was not due to ooplasmic segregation. Blastomeres isolated from 4-celled embryos were regulative and developed into normal planula larvae and juvenile anemones that were 1/4 the size of those that developed from intact 4-celled embryos. Embryos exhibiting the fusion phenomenon were examined at the fine structural level. The fusion phenomenon resulted in formation of a secondary syncytium and was not a mere compaction of blastomeres.

## Acknowledgements

I dedicate this thesis to my wife, Eleanor. Without her endless encouragement and the emotional and fiscal support she provided me, this thesis would not have come to fruition. I would also like to take this opportunity to thank the many people I have been associated with during my "graduate school experience". I thank Dr. Cadet Hand for sharing his *Nematostella vectensis* and its associated funding, as well as our experience of discovering new things about a relatively unknown animal. I would especially like to thank him for both his friendship and his invertebrate expertise which he so willingly shared, both have meant a great deal to me. I thank Dr. Wally Clark, my major professor, for first hiring me as a technician, and then encouraging me to pursue a Ph.D.; for encouraging me to work with Cadet on *Nematostella*; and for his suggestions on and funding of a portion of my research, including the cost of making my egg mass jelly antibody. I am greatly indebted to and wish to sincerely thank Dr. Fred Griffin for sharing his expertise and his time in guiding me through the use of various pieces of equipment, as well as for his suggestions in improving my scientific writing. My thanks also go to my other committee members, Drs. Jerry Hedrick and William Jeffery, for their critical reading of and feedback on the revision of this thesis. I thank Dr. Gary Cherr and Suzy Jackson for allowing me use of equipment and sharing their expertise with me. I thank Dr. Jim Clegg for allowing me space after my major professor left Bodega Marine Laboratory. I would like to acknowledge my appreciation for

the support afforded me through California Sea Grant Traineeships. Lastly, I would like to thank U.C. Davis's Department of Zoology. It has given me an opportunity to observe and decide upon what I do and do not want to become.

## Table of Contents

## Introduction

Sexual reproduction in the Actiniaria (sea anemones) has been described for many species (review in Shick, 1991). What is known about sexual reproduction and early development has been based on natural spawning events or manipulations of these events by various protocols, that while successful at the time, have not been dependably repeatable or predictable. The general inability to manipulate the reproductive biology of the Actiniaria has severely limited the kinds of research required to gain a better understanding of their developmental biology (Spaulding, 1974; Strathmann, 1987).

In 1992, Hand and Uhlinger reared *Nematostella vectensis*, an estuarine burrowing sea anemone, through successive sexual generations in the laboratory. This was the first instance in which sexually-mature sea anemones were raised, maintained, and routinely spawned in the laboratory throughout the year. *N. vectensis* was shown to not only spawn in a predictable manner but also to spawn throughout the year with no apparent seasonality. In addition to its sexual reproductive abilities, *N. vectensis* also asexually divided to form clones.

*Nematostella vectensis*' reproductive characteristics have proven very useful to me as a means to control experimental variability while exploring various aspects of the reproduction and early development of this species.

# Chapter 1

## Spawning Induction in the Estuarine Sea Anemone, *Nematostella vectensis*

## Introduction

Invertebrates have long been employed as model organisms for research in both developmental and reproductive biology. In great part these organisms have been members of the Phyla Nematoda, Annelida, Mollusca, Arthropoda, Echinodermata, or Urochordata, and have been used because of their availability, their ease of handling, ease in obtaining large numbers of gametes, ability to fertilize *in vitro*, and their capacity to be cultured through some, if not all, of their life stages. Research on these organisms has provided an understanding of basic animal development as well as the developmental diversity found amongst phylogenetic groups within the animal kingdom.

Anthozoan developmental biology, specifically regarding sea anemones, has eluded the scrutiny of scientific investigations. This lack of knowledge can be attributed to scientists' overall inability to reliably control the reproductive biology of these animals (see reviews by Stephenson, 1928; Mergner, 1971; Campbell, 1974; Spaulding, 1974; Strathmann, 1987; and Fautin *et al.*, 1989). Spawning in sea anemones, and the specific factors triggering induction of spawning, have not been studied in any great detail, although various factors responsible for spawning induction have been proposed, including: (1) light and lunar cycles; (2) water temperature; (3) exposure to air; and (4) cellular components derived from macerated reproductive tissues (Fautin *et al.*, 1989). Unquestionably, the most impressive example of lunar-induced spawning has been documented on the Great Barrier Reef where 112 species of anthozoans

spawned within a few nights of each other following the Spring full moon (Babcock *et al.*, 1986). In addition to lunar cycles, light manipulations have been reported to have an important influence on gamete release. For instance, Gemmill (1920) reported that spawning could be accelerated by placing *Adamsia palliata* under dark conditions. Other physical parameters, such as water temperature and exposure to air, have also been reported to be important factors for induction of spawning. Spaulding (1972) reported that an undocumented percentage of *Peachia quinquecapitata* spawned within 15 days of a treatment consisting of a dark cycle followed by increased water temperatures. Siebert (1974) reported spawning in *Anthopleura elegantissima* and *Anthopleura xanthogrammica* after a "desiccating" event that exposed anemones to air. Following this exposure, the anemones were resubmersed and subsequently released gametes. Another factor, reported several times in the literature, is exposure of an animal to reproductive products. Gemmill (1920) observed *Metridium dianthus* and *Adamsia palliata* spawning in tanks and suggested that spawning induction was "communicated by the sexual products." Following Gemmill's work several studies have implicated sperm as being an important factor in inducing female anemones to spawn. These studies included anthozoans: *Halcampa duodecimcirrata* (Nyholm, 1949); *Epiactis prolifera* (Uchida and Iwata, 1954, and Dunn, 1973; 1975); *Peachia quinquecapitata* (Spaulding, 1972); and *Metridium senile* (Clark and Dewel, 1974). Unfortunately, none of the above studies ascertained the specific factor(s) responsible for the induction of spawning.

In 1992, Hand and Uhlinger reported the first instance in which a sea anemone was successfully cultured through successive sexual generations in the laboratory. This anemone, *Nematostella vectensis*, a widely distributed, estuarine, burrowing sea anemone, was shown to reliably spawn year-round in a predictable manner when maintained on a specified feeding and water exchange regime. Hand and Uhlinger (1995) reported that this anemone also reproduced asexually and could be used to develop clones. In the present paper, using both *N. vectensis*'s asexual reproductive ability to form clones and its predictable spawning behavior, we present a series of experiments used to elucidate the factor(s) responsible for spawning induction in the laboratory.

## Materials and Methods

### Culture of *Nematostella vectensis* Clones

Multiple experiments were conducted involving the same basic culture techniques to which variable treatments were applied. Each experimental method is described below.

To reduce experimental variability, two clones of *Nematostella vectensis*, one female ($CH_2$♀) and one male ($CH_6$♂) derived from Chesapeake Bay animals (Hand and Uhlinger, 1992), were used. These two clones were derived from first generation siblings of a spawn that took place 6-7 April 1988.

Clones of each sex were reared and maintained in isolation from one another under a 12 hour light/12 hour dark light regime. Each clone was maintained at room temperature (20-22°C) either as individuals in 51x31 mm crystallizing dishes containing 25 ml of 1/3 natural seawater (1/3 NSW = 10 ppt salinity) or in groups of 20 individuals in 80x40 mm crystallizing dishes containing 100 ml of 1/3 NSW. These cultures received no aeration. Animals were maintained on a regular weekly feeding and water change schedule. Cultures were fed *Artemia* sp. nauplii (hatched from Sanders Brine Shrimp Co. - Premium Grade A cysts) every Monday, Wednesday, and Friday. In addition to this regular feeding, each anemone's diet was supplemented with ~1.0x1.0 mm pieces of *Mytilus californianus* ovary every Monday. Following Monday feedings, animals were transferred to a clean culture dish containing freshly prepared 1/3

NSW that had been aerated for a minimum of 3 hours. Anemones were maintained on this feeding/water change regime unless otherwise noted.

Effect of Starvation on Sexually-Mature Adults

Sixteen isolated anemones of each sex, which had been maintained on the 7-day feeding/water change cycle for 159 days, were used to determine the effect of starvation on reproductively-mature adults. These animals were not fed for a total of 87 days. Water was changed regularly every 2 weeks and any observed asexual divisions or spawning events were noted. Following this experiment, animals were returned to the normal 7-day feeding/water change regime.

Effect of Water Change vs. Feeding on Spawning

Forty female clonemates were isolated and each was maintained in an individual 51x31 mm crystallizing dish. Each anemone was fed 2-3 pieces of *M. californianus* ovary every Sunday followed by a transfer to a clean dish containing new 1/3 NSW. The anemones were subsequently fed *Artemia* sp. nauplii every Monday, Wednesday, and Friday. The 40 anemones were maintained on this regime until all were releasing egg masses dependably the day following mussel feeding and water change. Once animals were undergoing reliable reproductive cycling, they were separated into 4 groups of 10 individuals

each. Treatments were: 1) mussel feeding on Sunday and transfer to a clean dish containing new culture water on Sunday (S/S); 2) mussel feeding on Sunday and transfer to a clean dish containing new culture water on Monday (S/M); 3) mussel feeding on Sunday and transfer to a clean dish containing new culture water on Tuesday (S/T); or, 4) mussel feeding on Sunday and transfer to a clean dish containing new culture water on Wednesday (S/W). All spawning events were observed and recorded.

Effect of Oxygen Level of Water Change on Spawning

A total of 200 female clonemates maintained on the same feeding and water change regime were used for this experiment; however, 4 different treatments were used for the culture water: 1) aerated new 1/3 NSW (this served as the control); 2) aerated new 1/3 NSW saturated with compressed oxygen; 3) aerated new 1/3 NSW saturated with compressed nitrogen to replace all oxygen; and, 4) 7-day old used 1/3 NSW saturated with compressed oxygen.

Water for all treatments involving compressed gases was prepared by adding the specific treatment water to a clean Nalgene® graduated cylinder fitted with a 2-holed stopper in which a 1 ml disposable pipette was inserted to the bottom of the cylinder and a short exhaust tube was inserted to just above the water level. Oxygen levels were determined for experimental waters using an oxygen meter (Strathkelvin Instruments, Model 781 with a Microcathode oxygen electrode). Oxygen was bubbled through the water for Treatments 2 and 4 for

30 minutes. Treatment 4 water was obtained by pooling water from 4 cultures, each of which contained 20 clonemates that were maintained on the same treatment regime as the isolated animals of the experiment to be run. Following the pooling of the water, a volume sufficient for each run of the experiment was oxygenated. The oxygen level was equal to or greater than the level measured in the control treatment. Compressed nitrogen was bubbled through Treatment 3 water for 4 hours to remove all oxygen from the aerated new 1/3 NSW (determined by oxygen meter measurements).

Forty individual 24 ml screw-topped scintillation vials (10 vials for each of the 4 treatment waters) were filled and a single clonemate was added to each. The vials were promptly capped and sealed with a strip of Parafilm®. Each animal was monitored for spawning.

Effect of Ammonia Level of Water Change on Spawning

To determine low and high concentrations of ammonia to be used for this experiment, ammonia levels were measured in both the new and the 7-day old waters of the preceding experiment using the phenolhypochlorite method described by Parsons *et al.* (1984). A total of 160 clonemates were maintained on the basic feeding/water change regime. Ten isolated animals were placed individually into 51x31 mm crystallizing dishes containing 25 ml of one of 4 culture water treatments. Three treatments were made up using different quantities of a stock solution of 1000 mg/ml $NH_4Cl$ added to well-aerated, 0.45

μm filtered, 1/3 NSW. Following the addition of $NH_4Cl$ to the treatment waters, each was aerated for 15 minutes and dispensed into the culture dishes. The treatments were: 1) aerated new 1/3 NSW containing 0.0 mg/l ammonium (this served as the control); 2) aerated new 1/3 NSW containing 0.1 mg/l ammonium; 3) aerated new 1/3 NSW containing 0.2 mg/l ammonium; and, 4) aerated new 1/3 NSW containing 0.3 mg/l ammonium. All spawning events were noted for the duration of the experiment.

Statistical Procedures

SigmaStat™ Statistical Software (version 1.01, Jandel Corp.) was used for all statistical calculations. One-Way Analysis of Variance (ANOVA) was employed for analysis of spawning time and percentage of individuals that spawned relative to mussel feeding versus water change (Figures 3 and 4, respectively). In addition, the Student-Newman-Keuls Method of Pairwise Multicomparisons was applied *a posteriori* to determine the differences between the two treatments. Nonparametric analysis (Kruskal-Wallis ANOVA on Ranks) was performed on data from both the "effect of oxygen" and the "effect of ammonia/ammonium on spawning" experiments (Figures 5 and 6, respectively), since in both cases normality failed. Following the nonparametric analyses, Dunnett's Test was used to compare all treatments with the appropriate control.

## Results

### Sexual Reproduction on 7-Day Feeding/Water Change Regime

Sexually-mature animals maintained on a 7-day mussel feeding/water change regime spawned regularly over a 159-day period. During this period, the 16 female clonemates spawned a total of 379 times. Of this total, 262 (69.1%) of the spawns occurred within a 36-hour period following mussel feeding and water change. All egg masses averaged ≥ 1 cm. Diameter of the eggs within egg masses ranged between 170-210 μm. The 16 male clonemates spawned 149 times; 117 spawns (78.5%) took place within the same 36-hour period. Following the 159-day period these anemones were used for subsequent experiments.

### Effect of Starvation on Sexually-Mature Adults

To determine the effect of starvation on reproductively mature animals, the same animals that had been maintained for 159 days on a 7-day mussel feeding/water change regime were maintained for 87 days without feeding. During this period of starvation, both sexes continued to spawn.

Of the 233 times that starved females spawned (Figure 1), 101 of these spawns (43.3%) were within 36 hours after water changes (which occurred every 2 weeks). In addition, during non-water change weeks, 55 spawns (23.6%) took

place within 36 hours of the day that corresponded to what had been a regularly scheduled water change during the preceding 7-day feeding/water change regime. The remaining 77 spawns (~33%) did not fit into any time interval that could be related to water change. During the period of starvation, egg mass length decreased from an average of ≤10 mm at day 12 to an average of 2.5 mm at day 75. Egg diameter continued to range from 170-210 μm.

Starved males spawned a total of 97 times during the 87 day starvation period. Of that total, 76 spawns (78.3%) took place within 36 hours of the bi-weekly water change. Males did not show any tendency to regularly spawn during the periods between water changes (Figure 2).

Spawning data from *N. vectensis* maintained first on the 7-day feeding/water change regime, and then on the starvation/water change regime (for summary of spawning data see Table 1), indicated that water changes were playing a role in eliciting the induction of gamete release.

For the purpose of the experiments that followed, only females were used. By using females, multiple spawning events over the experimental period could be scored without subsequent water changes, whereas, to ensure male spawning events were separate, a water change had to be made after each spawn, thereby disrupting the intended experimental setup and procedure.

Effect of Mussel Feeding vs Water Change on Spawning

To further elucidate if water changes, rather than mussel feeding, played the major role in spawning induction, the two potential stimuli (feeding versus water change) were separated in time. All animals were fed mussel on the same day. Each treatment group's water was changed at progressively increasing 24 hour intervals following feeding (i.e., immediately following feeding, 1 day after feeding, 2 days after feeding, and 3 days after feeding). All spawnings were then noted and scored relative to both the mussel feeding and the water change.

During the 12 week experimental regime, the 40 females spawned a total of 367 times. Results of the 4 different treatments relative to both mussel feeding and water change are presented in Table 2. All treatments resulted in equivalent numbers of spawnings with the exception of Treatment 4 (fed Sunday/changed Wednesday). In this treatment the total number of spawns was approximately 10% less than in the other 3 treatments. Anemones in treatments 1, 2, and 3 that were fed mussel with a subsequent water change within 2 days remained expanded with tentacles extended. These animals also remained responsive to food (*Artemia* sp. nauplii) and tactile stimuli. Anemones in Treatment 4, in which water was changed 3 days after mussel feeding, tended to be more retracted (quite often with only a portion of one tentacle extended) and less responsive to both food and tactile stimuli.

Spawning times relative to mussel feeding as compared to water change were significantly different (ANOVA: $F_{1,6} = 8.94$; $P$=0.024). The average time to

spawning relative to mussel feeding over all treatments exhibited a general trend of increasing time, whereas the average time to spawning relative to water change exhibited a more constant time interval over the same treatments (Table 2, Figure 3). Calculation of the time to spawning over all treatments relative to mussel feeding yielded a mean time of 62.26 ± 9.46 hours (2.59 ± 0.394 days) as compared to 29.28 ± 5.64 hours (1.22 ± 0.235 days) when time was considered relative to water change .

In all treatments where mussel feeding was separated in time from water change, the percentage of individuals spawning within 36 hours of mussel feeding was significantly less ($P<0.05$) than the percentage of individuals spawning within 36 hours of water change (Figure 4). The average percentage of individuals spawning over all 4 treatments was 24.8 ± 12.23% (mean ± s.e.) when considering spawnings relative to mussel feeding as compared to 54.6 ± 8.57% when considering spawnings relative to water change.

Effect of Oxygen on Spawning

The water of cultures maintained for 7 days exhibited a 76.6% reduction in total oxygen concentration ($[O_2]$). In order to determine if $[O_2]$ actually played a major role in spawning induction, 4 treatment groups were tested. In Treatment 1 (control - aerated new 1/3 NSW) 72.5±11.8% (total ± s.e.) of the animals spawned, whereas in Treatment 2 (aerated new 1/3 saturated with compressed oxygen) 80.0±4.1% of the animals spawned. There was no significant difference

between the spawning results of Treatments 1 and 2. In Treatment 3 (aerated new 1/3 NSW that was deoxygenated); however, there were no spawns in response to the water change. Finally in Treatment 4 (7-day old 1/3 NSW from anemone cultures oxygenated with compressed oxygen), 2 anemones spawned during the 4 experimental runs resulting in a spawning percentage of 5.0±5.0% (Figure 5). All animals began each experimental run completely expanded with all their tentacles extended. By the second day of each experimental run, the animals under anoxic conditions (Treatment 3) withdrew all but a small portion of one tentacle and remained that way until they were returned to aerobic conditions. Once returned to normal control conditions, most of these anemones spawned within 36 hours (data not shown).

In general, the absence of oxygen dramatically reduced spawning. There was also observed a significant decrease in spawning when oxygenated 7-day old water was used. This result, although potentially confounding, suggested that although oxygen is required for the induction of spawning while some other component present in the culture water was having an inhibitory effect on spawning.

Effect of Ammonium on Spawning

New 1/3 NSW contained no detectable ammonia, whereas the 7-day old culture water contained an average 0.25 mg/l. In order to eliminate other potentially confounding metabolites found in 7-day old culture water, a stock

solution of 1000 mg/ml $NH_4Cl$ was used to obtain the ammonia levels used in the treatment groups. The volumes of $NH_4Cl$ necessary to obtain experimental treatment levels increased salinity from 10 to 16‰ (refractive index from 1.3350 to 1.3358) and only slightly reduced pH from 8.07 to 7.91 at the highest treatment concentration (see Table 3 for all culture water data).

Anemones exposed to increasing concentrations of ammonia/ammonium showed significant decreases in their spawning percentage. Treatment 1 (0.0 mg/l) animals spawned 97.5±2.5% of the time while animals in Treatment 2 (0.1 mg/l) spawned 72.5±14.6% of the time. Animals in Treatment 3 (0.2 mg/l) and Treatment 4 (0.3 mg/l) spawned significantly less, 10.0±4.1% and 0.0% of the time, respectively (Figure 6). Results of this experiment correspond with results shown in Treatment 4 of the oxygen experiments (oxygenated, used 1/3 NSW containing ~0.25 mg/l ammonia), where the spawning percentage decreased to 5.0±5.0%.

## Discussion

To date, the inability to successfully spawn sea anemones has severely impacted research and the concomitant knowledge of the developmental and reproductive biology of these animals. *Nematostella vectensis*, however, has the potential of becoming an important model for this group. This anemone can be cultured in a wide range of salinities, ranging from 33-1/3 to 100% natural seawater. It can be conditioned in the laboratory to spawn in a predictable manner, producing large quantities of gametes year-round (Hand and Uhlinger, 1992). In addition to its dependable sexual reproduction, *N. vectensis* has the ability to asexually reproduce by transverse fission, which can occur naturally or be induced through artificial means (Hand and Uhlinger, 1995). Using these amenable characteristics, we have identified specific conditions for spawning induction.

The present study demonstrated that induction of spawning was elicited by feeding/water change protocols. To separate a feeding from a water change response, animals were starved and, although starvation resulted in diminished reproductive capacity, anemones continued to spawn in a predictable manner that corresponded to water change. Further experiments suggested that $[O_2]$ is necessary and has a positive influence on spawning; whereas, the presence of $NH_3/NH_3H+$, the principal waste product of anemones (Shick, 1991), inhibits spawning in a concentration dependent manner.

Animals that had been maintained on a 7-day mussel feeding/water change regime for 159 days responded in an interesting fashion when placed on a starvation regime with their water change extended from 7 to 14 days. The spawning that took place over the period of the starvation experiment appeared to be related to water change. Males tended to spawn 24-36 hours after water change, their total percentage of spawning decreasing over the length of the experiment (Figure 2). Similarly, females spawned 24-36 hours after water change. In addition to this spawning response, however, females also showed a tendency to spawn in a regular pattern during what would have been a feeding/water change cycle in the 159 day period preceding the initiation of starvation (Figure 1). The entrainment and maintenance of the 7-day spawning pattern in the face of the altered water change schedule is a unique and intriguing finding.

As discussed above, the percentage of animals spawning over the duration of the starvation period decreased (Figures 1 and 2). In addition to the decrease in spawning events, females also exhibited a reduction in their gamete output (we did not quantify gamete output for males). The average size of egg masses and accompanying number of eggs decreased over the duration of the period of starvation, while the apparent egg size remained unchanged (data not shown). This reduction in fecundity (number of eggs produced), without a reported change in egg diameter, agrees in part with other invertebrate reproductive strategies that have been summarized by Qian (1994).

Even though various parameters have been used to correlate food availability with reproductive output, invertebrates have been shown to follow one of several strategies. Qian (1994) stated that when food availability decreases organisms have been observed to: 1) decrease fecundity without changing egg quality (size and/or energetic content); 2) decrease egg quality without changing fecundity; 3) decrease both fecundity and egg quality; 4) decrease fecundity while increasing egg quality; or, 5) decrease egg quality while increasing fecundity. Decreased fecundity without changes in egg quality has been shown by Gremaré *et al.* (1988, 1989) in the polychaete annelid, *Capitella capitata.* Support for both the decrease in egg quality with fecundity change and the decrease in egg quality with an accompanying decrease in fecundity has been shown in the observations on bivalve molluscs, *Mytilus edulis* and *Placopecten magellanicus* (Bayne and Widdows, 1978; and, Bricelj *et al.*, 1987). Qian and Chia (1991) and Qian (1994) showed support for a decrease in fecundity while egg quality increased in a species of *Capitella*. Finally, McKillup and Butler (1979) found that the marine gastropod, *Nassarius pauperatus*, partially supported the prediction that egg quality decreased as fecundity increased when food availability became limited.

*N. vectensis* responded to starvation reproductively by reducing its gametic output. This decrease was reflected by smaller egg masses as well as a reduced number of eggs rather than a decreased egg size or apparent reduction in yolk content. Eggs produced during the first four weeks of starvation fertilized and developed into normal planulae and juvenile anemones (data not shown).

Egg masses produced after more than four weeks of starvation were not tested for viability or subsequent development. In addition to testing whether eggs produced beyond four weeks of starvation remain developmentally viable, the quantification of energy content of eggs over periods of both feeding and starvation needs to be addressed in order to complete our understanding of energetic apportionment in *N. vectensis*'s reproductive strategy.

Results from the experiments in which mussel feeding was separated from water change (see Table 2, Figures 3 and 4) showed that all treatments, except for one in which the water change was delayed for 3 days after mussel feeding (Treatment 4), resulted in equivalent numbers of spawns. In Treatment 4, a 10% reduction in spawning was observed. We hypothesized that this reduction was the result of decreased water quality. Animals in this treatment group tended to remain in a more retracted state than animals in the other experimental treatments. This is not surprising since mussel ovary and non-axenic brine shrimp additions to Treatment 4 cultures tended to increase populations of both bacteria and protozoans, which undoubtedly resulted in decreased $O_2$ and increased levels of metabolic wastes in culture dishes. In fact, it appears from our Treatment 4 data that the longer the water change was delayed following mussel feeding, the less time was required for animals to spawn following the water change (Figure 3). These results indicated that spawning was induced by the water change rather than mussel feeding.

Two water quality parameters were tested, $O_2$ and $NH_3H+$. Since $O_2$ concentrations were reduced an average of 76.6% in control cultures over a

period of 7 days, experimental culture conditions were designed to test the effect of varying [$O_2$] on spawning. Anoxic culture conditions unquestionably inhibited spawning; however, animals cultured in oxygenated 7-day old water exhibited a significantly reduced spawning. In this treatment, $NH_3H+$ appeared to have an overriding inhibitory effect, however, two questions need to be addressed concerning $NH_3H+$'s effect on spawning in *N. vectensis* before we can make this conclusion. First, in our experiments testing both effect of $O_2$ and the effect of $NH_3H+$, did the $NH_3H+$ disperse over the period of our experimental runs? Second, since we have stated that the there are both pH and salinity differences in our experimental culture waters (Table 3), could either of these parameters have caused the observed spawning inhibition?

In addressing the dispersal of $NH_3H+$ over the periods of our experimental runs we have to remember that in both the $O_2$ and $NH_3H+$ experiments, well-fed animals were introduced to the experimental media. We expect that in all cases the anemones continued to respire, producing both $NH_3$, as well as $CO_2$, over the 7 days they were observed during these trials. From our measurements of $NH_3$ in week-old culture water, initially containing only new 1/3 NSW, ammonia was not only produced by the anemones but it also remained in the culture media. In addition, all seawater used to make up our different treatment waters was filtered prior to use thus eliminating potential major consumers of $NH_3H+$ from our cultures (e.g., phytoplankton). Ammonia content could also fluctuate in the presence of bacteria (Harvey, 1966). Our anemones, in fact, were maintained in non-axenic cultures and bacteria was introduced to each of our treatments.

However, as Harvey points out, at 22°C, any ammonia/ ammonium consumed by bacteria would be cycled back to the culture water in its original form within the period of time that of each of our trials was maintained.

In considering the observed spawning inhibition in both the $O_2$ and $NH_3H$ experiments, we also have to address both changes in salinity and pH. New 1/3 NSW was ~10‰ salinity, pH 8.07, and contained no measurable $NH_3$. One week after the addition of well-fed anemones, this culture water's pH decreased to an average of 7.34 and its [$NH_3$] increased to an average 0.25 mg/l. There was no increase in salinity from 10‰ (refractive index = 1.3350). Since there was no change in salinity in the $O_2$ experiment, the two possible factors responsible for spawning inhibition in the week-old water treatment was either elevated ammonia/ammonium or decreased pH. Harvey (1966) states that as animals continue to respire (producing $CO_2$) pH will continue to fall. In order to sort out the responsible factor, increasing levels of $NH_3$ were tested in our ammonia experiment. As is evident in Table 3, as increasing quantities of $NH_3H$+ were added to new 1/3 NSW, salinity increased and pH decreased. We have observed that reproductively mature anemones will spawn following transfer from week old 1/3 NSW (10‰ salinity) to new ½ NSW (17‰ salinity) in a similar manner to when anemones are transferred from week old to new culture water of the same salinity (unpublished data). Consequently, we believe that the increased salinity (6‰ at the highest [$NH_3$ ] tested) due to addition of small quantities of 1 mg/l $NH_4Cl$ were insufficient to cause spawning inhibition. We also do not believe that the slight, initial pH decrease, caused by the addition of

$NH_4Cl$ to our experimental treatments (see Table 3), was sufficient to cause the spawning inhibition. As presented in our results, significantly fewer spawns took place within our 0.10 mg/l treatment than in our control treatment. In both cases the pH of both treatments was the same (pH 8.07). In addition, all anemones continued to respire (as is evident from each treatment's decreasing pH) and presumably continue to excrete $NH_3$. Specific effects of pH on spawning will be addressed in future experimentation.

Spawning in *Nematostella vectensis* has not been observed in nature to date. Frank and Bleakney (1976) found gonads present in 6 of 276 anemones collected in August and September in Nova Scotia, but not in any collected during other times of the year. From our own cultures, collected from different areas at different times of the year (Hand and Uhlinger, 1994), we found that animals did not spawn in the laboratory immediately following collection but required several weeks to attain spawning competence. Our laboratory-derived data does make sense if one considers the habitat of *N. vectensis*.

*Nematostella's* natural habitat is predominantly estuarine tidal marsh pools. The physical conditions in these pools can vary significantly in dissolved gases, organic and inorganic compounds, salinity, and temperature. These parameters are affected in no small part by the biota, ranging from bacteria through higher plants, invertebrates, and, in some cases, vertebrates. Depending upon the location of pools relative to the water sources and the overall weather conditions, they can approach anoxic conditions between periodic tidal flushes. Sexually-mature anemones residing in these pools would

be exposed to a fresh influx of sea water during a high tidal series. This influx of oxygenated seawater would replace at least some, if not all, of the water in the pools, lowering the ammonia concentrations that may have been increasing since the last high tidal series. This influx would provide an optimum opportunity for anemones to spawn, assuring appropriate conditions for larval development and dispersal.

The factors responsible for spawning induction in *N. vectensis* do not compromise the validity of published reports concerning spawning induction in other anemones (for review see Fautin *et al.*, 1989). Anemones are found in essentially every marine habitat. Therefore, it is not unreasonable to expect animals in habitats differing from those of *Nematostella* to respond to different cues.

**Table 1.** Percentage of animals spawning up to 36 hours following mussel feeding/water change or water change.

| | Male | Female | Egg Mass Length |
|---|---|---|---|
| 8-Day Cycle (234 days)[1] | 64 | 75 | ≥1 cm |
| 7-Day Cycle (159 days) | 78.5 | 69.1 | ≥1 cm |
| Starved 87 Days (water changed every 2 weeks) | 78.3 | 43.3 | ≤2 mm |

[1] Two different clones described in Hand and Uhlinger (1992)

**Table 2.** Results of the effect of mussel feeding versus water change on spawning.

| Treatment Group: (Feeding/Water Change)[1]: | Treatment 1 (S/S) | Treatment 2 (S/M) | Treatment 3 (S/T) | Treatment 4 (S/W) |
|---|---|---|---|---|
| Total Number of Spawns: | 95 | 92 | 95 | 85 |
| Average Time of Spawnings Relative to Feeding (Days)[2]: | 1.8 | 2.0 | 3.2 | 3.3 |
| Average Time of Spawnings Relative to Water Change (Days)[2]: | 1.8 | 1.1 | 1.3 | 0.7 |
| Percentage of Individuals Spawning w/in 36 hrs. Relative to Feeding: | 60.8 | 10.8 | 8.3 | 19.2 |
| Percentage of Individuals Spawning w/in 36 hrs. Relative to Water Change: | 60.8 | 66.7 | 61.7 | 29.2 |

[1] S/S = Sunday/Sunday; S/M = Sunday/Monday; S/T = Sunday/Tuesday; and S/W = Sunday/Wednesday.

[2] Average time of spawnings relative to feeding as compared to water change were significantly different (One Way ANOVA: $P = 0.024$).

**Table 3.** Ammonia, pH, and Salinity Measurements of Treatment Waters used in Oxygen and Ammonia Experiments.

| Treatment Water[1] | $[NH_3]$[2] | pH | Salinity (‰) | Refractive Index |
|---|---|---|---|---|
| $DH_20$ | 0.00 | 5.75 | 0 | 0 |
| NSW | 0.00 | 8.25 | 34 | 1.3392 |
| 1/3 NSW | | | | |
| New | 0.00 | 8.07 | 10 | 1.3352 |
| 1 week | 0.25 | 7.36 | 10 | 1.3352 |
| 1/3 NSW + 0.10 mg/l $NH_3$ | | | | |
| New | 0.10 | 8.07 | 15 | 1.3358 |
| 1 week | 0.25 | 7.34 | 15 | 1.3358 |
| 1/3 NSW + 0.20 mg/l $NH_3$ | | | | |
| New | 0.20 | 7.98 | 15.5 | 1.3359 |
| 1 week | 0.27 | 7.34 | 15.5 | 1.3359 |
| 1/3 NSW + 0.30 mg/l $NH_3$ | | | | |
| New | 0.30 | 7.91 | 16 | 1.3360 |
| 1 week | 0.33 | 7.32 | 16 | 1.3360 |

[1] $DH_20$ = Distilled Water; NSW = filtered, Natural Sea Water; New - refers to treatment water before the addition of sea anemones; and 1 week - refers to treatment water 1 week after the addition of sea anemones.

[2] mg/l total ammonia determined by phenolhypochlorite method (Parsons, 1984).

Figure 1. Spawning record of 16 *Nematostella vectensis* female clonemates over an 87-day starvation period. Animals were fed and water was changed every 7 days during the 159 days preceding starvation. Bold vertical line at day 88 represents resumption of feeding. Black fills represent the days the anemones spawned. (w) represents day the water would have been changed during the period prior to the starvation experiment. W represents day of actual water change during the starvation experiment. F represents the day feeding of anemones resumed.

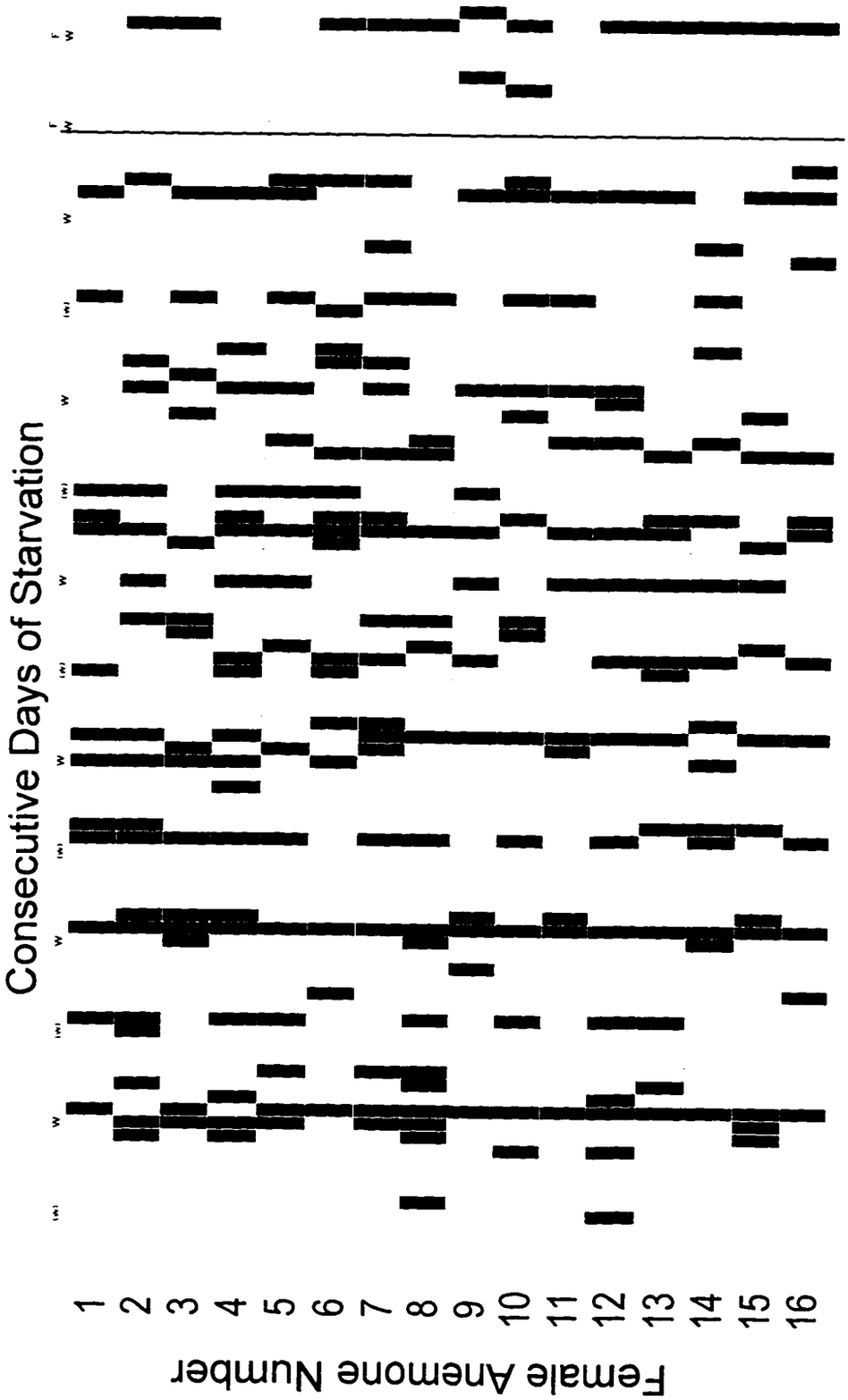


Figure 2. Spawning record of 16 *Nematostella vectensis* male clonemates over an 87-day starvation period. Animals were fed and water was changed every 7 days during the 159 days preceding starvation. Bold vertical line at day 88 represents resumption of feeding. Black fills represent the days the anemones spawned. (w) represents day the water would have been changed during the period prior to the starvation experiment. W represents day of actual water change during the starvation experiment. F represents the day feeding of anemones resumed.

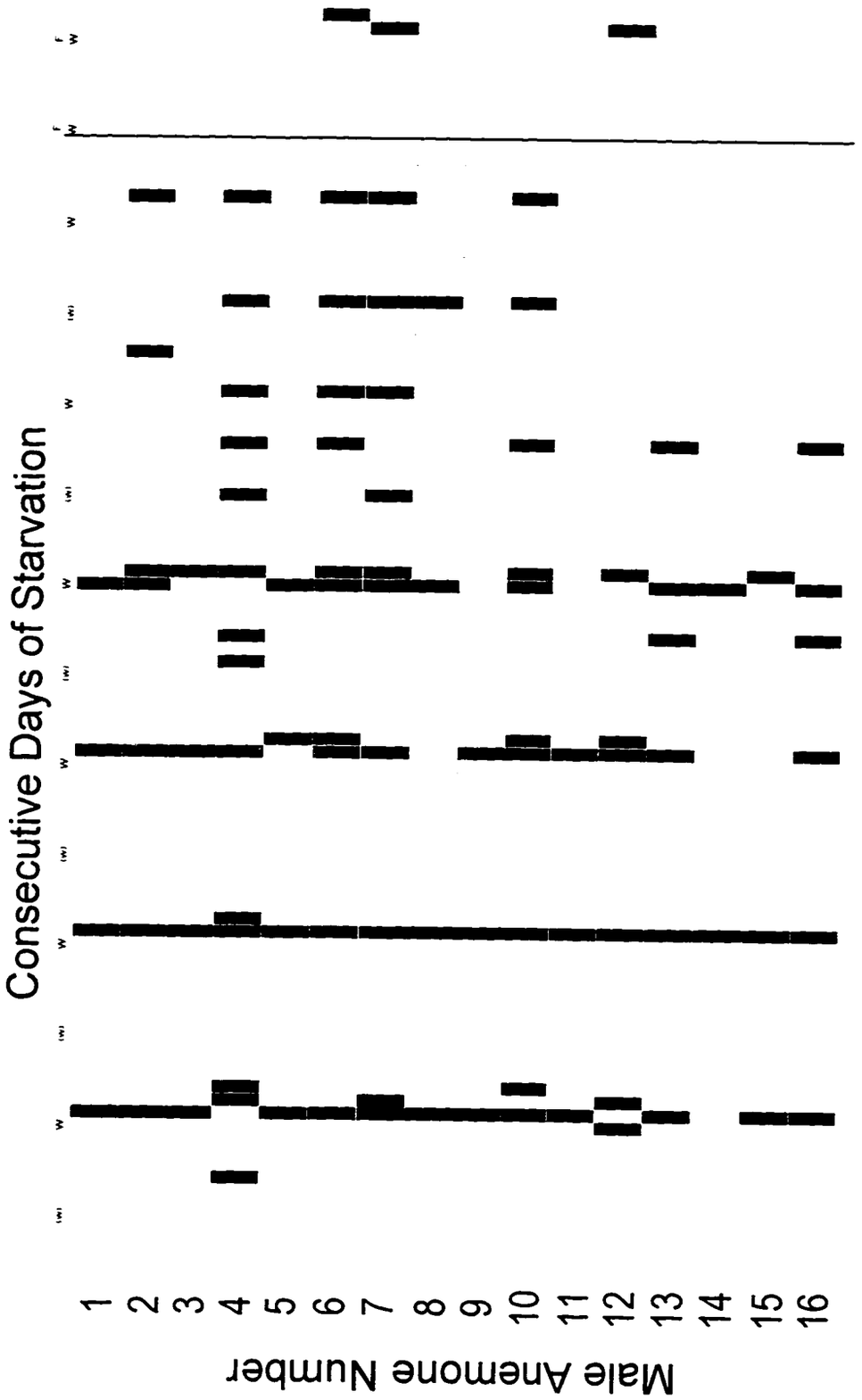


Figure 3. Mean period of time (+ s.e., in days) until spawning, relative to the feeding of mussel ovary (clear bar) and to the change of culture water (hatched bar). Total number of individuals for each treatment group was 40. Different treatment groups (left to right) represent a 24-hour delay in water change following mussel feeding. Each treatment group was maintained over a 12-week period. Treatment groups: S/S = Sunday Feeding/Sunday Water Change; S/M = Sunday Feeding/ Monday Water Change; S/T = Sunday Feeding/Tuesday Water Change; and S/W = Sunday Feeding/Wednesday Water Change. Spawning times relative to mussel feeding as compared to water change were significantly different (One Way ANOVA: $F_{1,6} = 8.94$; $P = 0.024$).

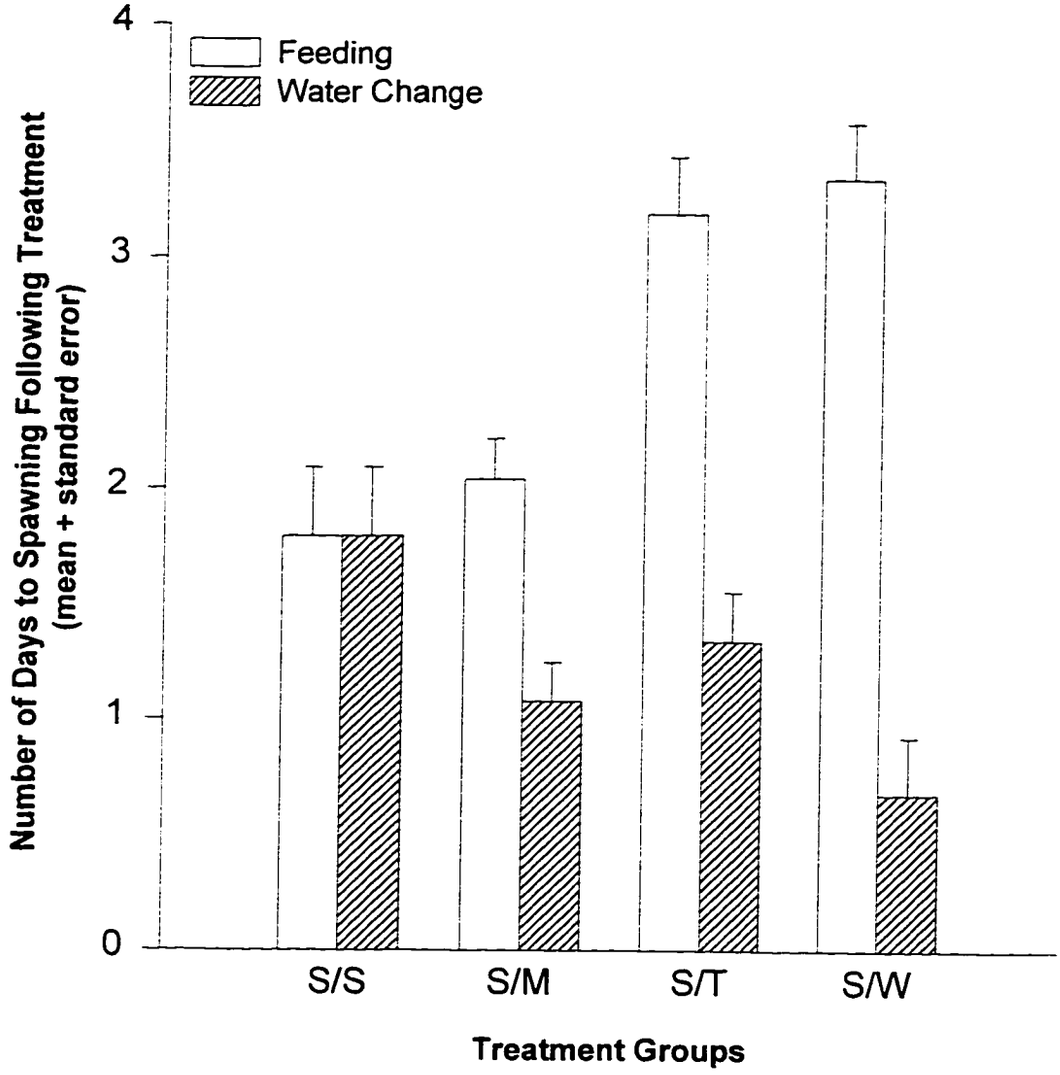


Figure 4. Mean percentage of individuals (+ s.e.) that spawned within 36 hours relative to either the feeding of mussel ovary (clear bar) or to the change of culture water (hatched bar). Percentages were based on the spawning record of all individuals in each treatment group. Total number of individuals for each treatment group was 40. Different treatment groups (left to right) represent a 24-hour delay in water change following mussel feeding. Each treatment group was maintained over a 12-week period. Treatment groups: S/S = Sunday Feeding/Sunday Water Change; S/M = Sunday Feeding/Monday Water Change; S/T = Sunday Feeding/Tuesday Water Change; and S/W = Sunday Feeding/Wednesday Water Change. Spawning times relative to mussel feeding as compared to water change were significantly different (One Way ANOVA: $F_{1,4} = 10.6$; $P = 0.031$).

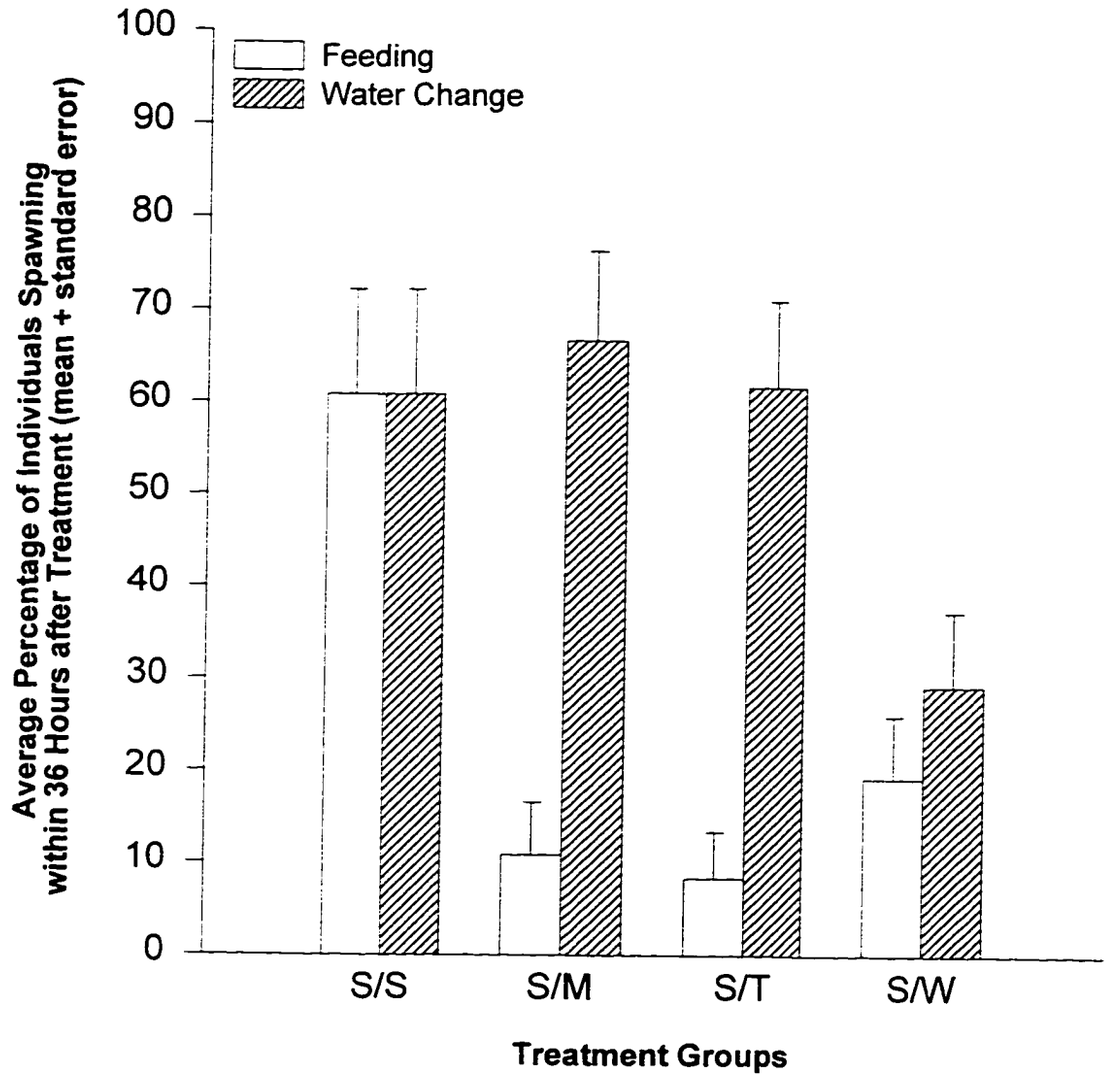


Figure 5. Effect of the presence of oxygen on the mean spawning percentage (+ s.e.) of 40 individual anemones in each of four water treatments. Differences in the median values among treatment groups were significantly different ($P$=0.006, Kruskal-Wallis One Way ANOVA on Ranks). De-oxygenated New 1/3 NSW and Oxygenated 7-Day Old 1/3 NSW treatment values varied significantly ($P$<0.05) from the Control treatment values; whereas, Oxygenated New 1/3 NSW treatment values did not (Dunnett's Method for All Pairwise Multiple Comparisons).

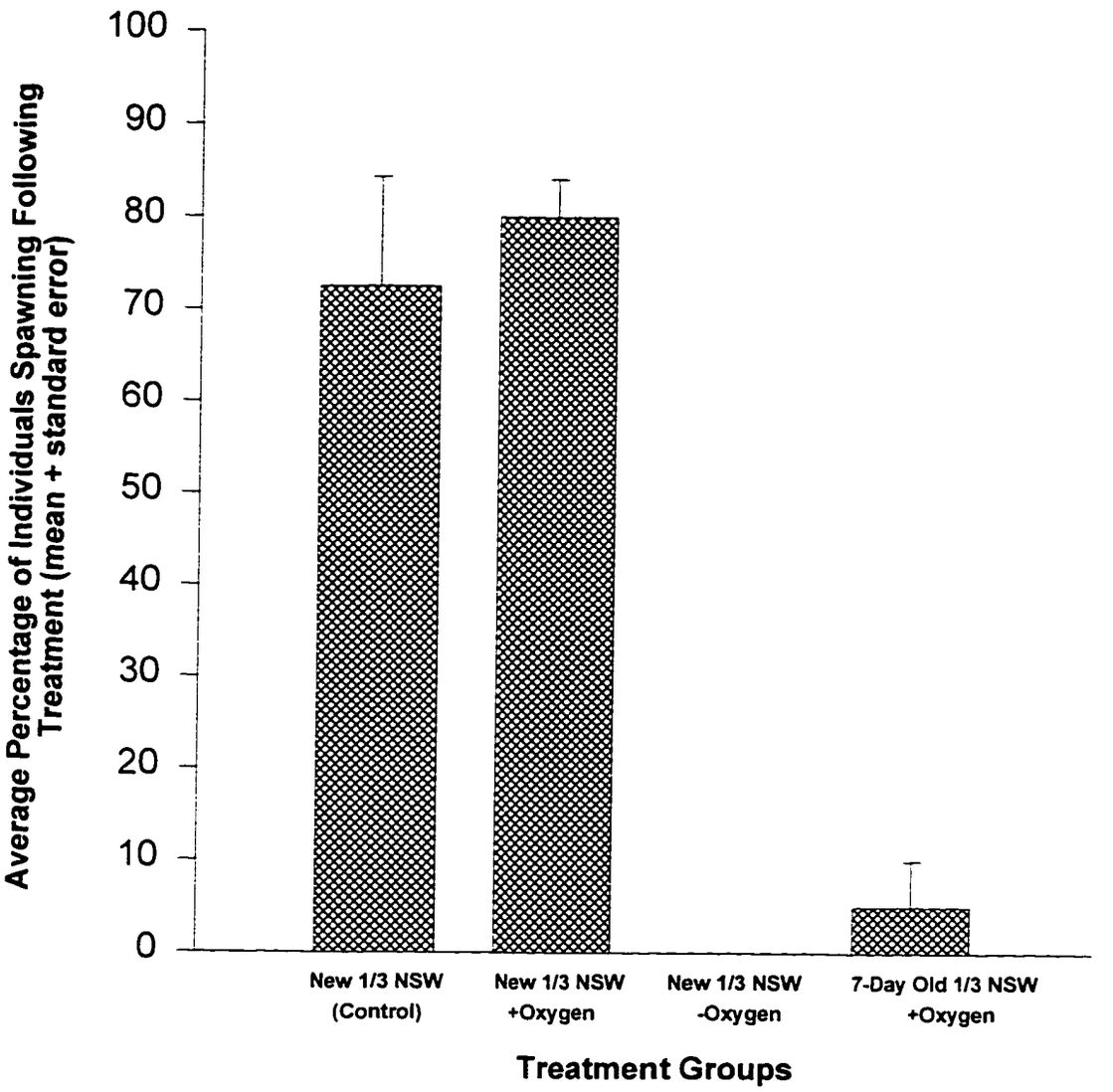
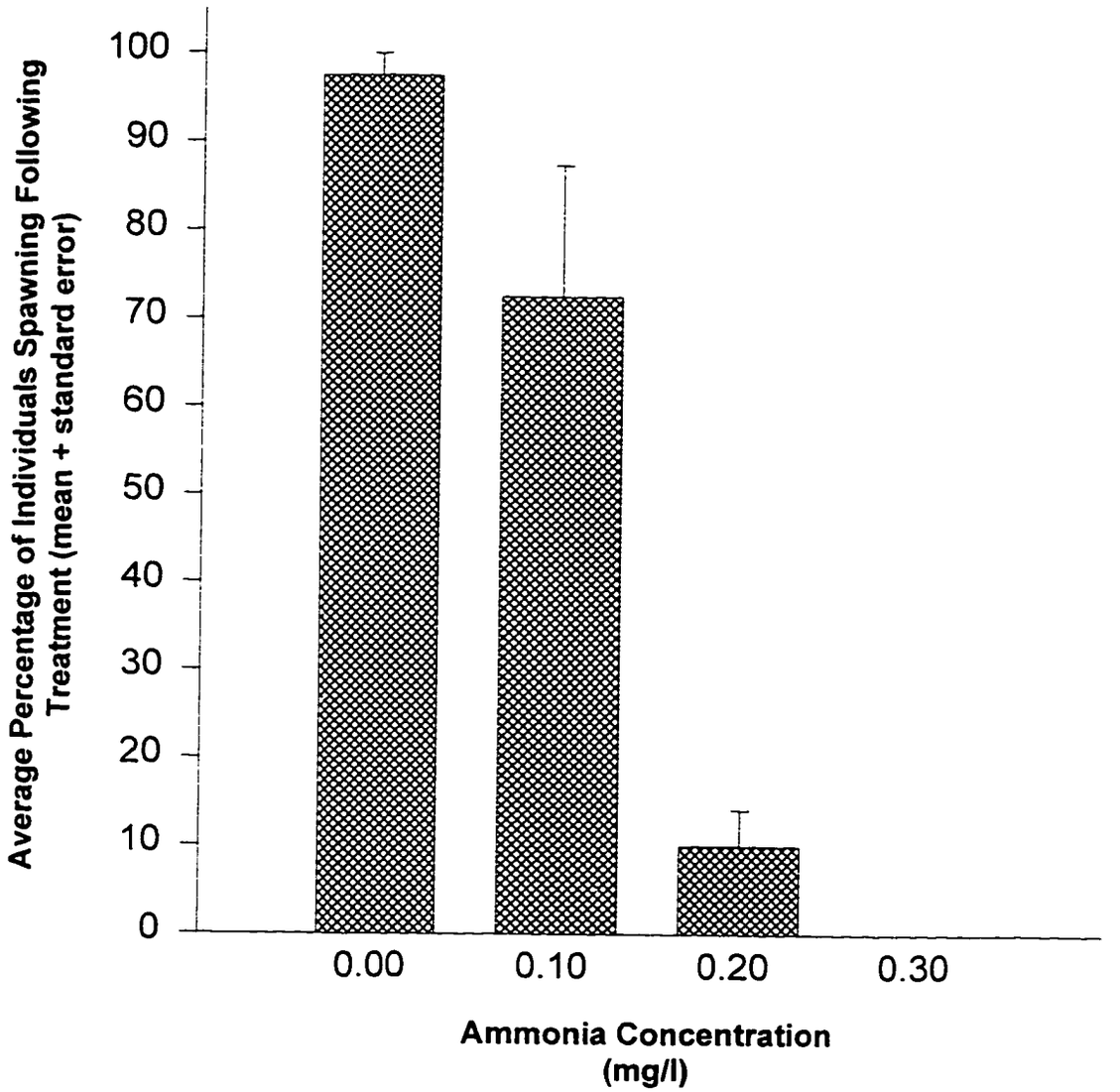


Figure 6. Effect of ammonia/ammonium concentration on the mean spawning percentage (+ s.e.) of 40 individual anemones in each of four different ammonia/ammonium concentration treatments. Differences in the median values among treatment groups were significantly different ($P<0.04$, Kruskal-Wallis One Way ANOVA on Ranks). 0.20 mg/l and 0.30 mg/l concentration treatments were significantly different ($P<0.05$) from 0.00 mg/l concentration treatment (Control); whereas, 0.10 mg/l concentration treatment was not (Dunnett's Method for All Pairwise MultipleComparisons).



# Chapter 2

## Origin of the Egg Mass Jelly in the Estuarine Sea Anemone, *Nematostella vectensis*

## Introduction

Eggs of most animals are surrounded by one or more investment coats which vary in chemical/physical characteristics. Egg coats can range from rigid, impermeable envelopes containing micropylar openings, such as those found in cephalopods, arthropods, and fish, to penetrable jelly coats that can either surround individual eggs, such as those found in crustaceans, echinoderms, hemichordates, ascidians, amphibians, and mammals, or surround groups of eggs as those found in annelids, molluscs, and a variety of amphibians.

Investment coats of anthozoan eggs have received little attention. Of the species studied, however, there appear to be five types. These are: 1) eggs which lack a mucus/jelly-like coat, either before or after fertilization (Larkman and Carter, 1984); 2) eggs that are spawned within associated mucus/mucus strands which disperse rapidly post-spawning and do not exhibit any function related to fertilization (Dunn, 1972; Siebert, 1973 and 1974); 3) eggs that are spawned with no apparent coat, but produce an extracellular coat via a cortical reaction in response to fertilization (Gemmill, 1920; Clark and Dewel, 1974, Dewel and Clark, 1974); 4) eggs that are spawned with a mucus coat which is enhanced by cortical secretions post-fertilization (Nyholm, 1949; Strathmann, 1987); and 5) eggs that are spawned in a jelly egg mass, as originally described in *Nematostella vectensis* by Crowell (1946).

The egg masses of *N. vectensis* are unique in that they contain not only eggs, but also nematosomes which are round, motile, multicellular bodies

composed of flagellated cells and nematocytes (Williams, 1979). This egg mass jelly is readily penetrated by sperm and persists throughout development when swimming planula larvae swim out of the jelly mass approximately 36-48 hours postfertilization (Hand and Uhlinger, 1992). The production of the unique jellied egg mass of *N. vectensis* is addressed in the chapter which follows. The research presented investigates the source of jelly making up this egg mass, and describes the major component of this material.

## Materials and Methods

### Animal Maintenance and Spawning

Both female and male clones of *Nematostella vectensis* were used for the experimentation in this paper. Groups of 15 clonemates were placed in several 80x40 mm crystallizing dishes containing 100 ml 1/3 natural seawater (1/3 NSW). Animals were fed *Artemia* sp. nauplii (Sanders Brine Shrimp Co.) every Monday, Wednesday, and Friday. Each Monday, animals were fed pieces of *Mytilus californianus* ovary in addition to the *Artemia*. Following feeding, each group of sea anemones was transferred to a new clean dish containing aerated new 1/3 NSW. Within 24 to 36 hours following this water change, animals spawned. Each female released 1 to 2 egg masses (the largest containing well over 2000 eggs).

### Observation of Spawning Event

The ability to manipulate and predict spawning in *Nematostella vectensis* was used to determine the location of actual gamete release and the potential source of the egg mass jelly. After feeding and transfer to clean 1/3 NSW, animals were allowed to relax and expand. Once expanded, animals that were about to spawn, usually within 1-2 hours, showed little to no response to tactile stimulation until after spawning took place. Females that were expected to

spawn were removed to small Petri dish bottoms lined with dental wax and filled with aerated, 1/3 NSW. Following another period of relaxation and expansion, each animal was pinned down to the wax at both oral and physal ends using 00 insect pins, dissected lengthwise between a pair of mesenteries and pinned open. Pinning animals in this manner allowed all mesenteries to be observed in a single plane of view and allowed the entire spawning event to be observed and documented. Animals prepared in this manner were then either photographed, video-taped, and/or fixed at each stage of the spawning event.

Fixation and Preparation of Animal Tissue for Analysis

Whole or dissected animals of both sexes, as well as newly spawned egg masses, were fixed in either cold (0°C), 90% methanol containing 50mM EGTA, pH 6.0 (MeOH) or 0.22 µm-filtered 1/3 NSW containing 3% paraformaldehyde and 0.1% glutaraldehyde for ~1.5 hours. Samples fixed in MeOH were then transferred to cold (0°C) 100% ethanol (EtOH) for 30 minutes. Samples fixed in the paraformaldehyde mixture were rinsed for 15 minutes in filtered 1/3 NSW (3 times), once briefly in nanopure water, then dehydrated in a graded EtOH series, and were then infiltrated and embedded in Steedman's polyester wax (Norenburg and Barrett, 1987) for later sectioning and analysis.

Collection of Egg Mass Components and Mucus

Egg masses as well as entire female anemones were used to collect components of the egg mass to compare protein profiles.

Jelly: Freshly-spawned egg masses were used for both jelly and egg collection. Egg masses were collected and pooled. Egg masses were then transferred to 15 ml disposable centrifuge tubes with caps. 50mM Dithiothreitol (DTT) in 1/3 NSW, pH 8.0, was added to the egg mass volume to yield a final concentration of 10mM DTT. Centrifuge tubes were gently rotated until jelly was completely dissolved, after which free eggs were allowed to settle. Solubilized jelly was pipetted from each centrifuge tube and pooled at ~4°C. To remove disrupted eggs and nematosomes, pooled jelly was then centrifuged at 13.5 g for 10 minutes. Centrifuged jelly was repooled and dialyzed (1,000 molecular weight cut off = 1,000 MWCO) against nanopure water at 4°C for 12 hours. Following dialysis, jelly solution was aliquotted, lyophilized, and stored at -70°C until needed.

Eggs: Intact eggs, once free of jelly, were rinsed 3 times in filtered 1/3 NSW and aliquotted into 1.5 ml microfuge tubes. Once aliquotted, 1/3 NSW was removed, and the eggs were frozen at -80°C until needed.

Nematosomes: To obtain isolated nematosomes, females who were not expected to spawn were carefully removed from their culture dishes and gently laid on a piece of dental wax. Using small iris scissors, a ~ 2 mm incision was made between mesenterial insertions at the physal end of each anemone. Once

the animals were incised, the coelenteron fluid containing nematosomes drained from the anemones into discreet droplets on the wax which were pooled and centrifuged at 13.5 g for 15 minutes to pellet nematosomes. Incised anemones were returned to their culture dishes where they reinflated and healed.

Mucus: Mucus sheaths from the exterior of female anemones in culture were collected, rinsed in filtered 1/3 NSW, and centrifuged at 13.5 g for 15 minutes to form pellets.

Electrophoresis

Protein concentration of all samples was determined using the Micro BCA assay (Pierce - Rockland, IL). All egg mass components and mucus samples were solubilized in 62.5mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.001% bromophenol blue for SDS-PAGE (Laemmli, 1970). The 12% acrylamide gels, with equal amounts of protein loaded per well, were run on a Bio-Rad (Richmond, CA) Mini-Gel apparatus. After electrophoresis, gels were stained for proteins using Coomassie blue (Harlow and Lane, 1988) and silver stain (Merrill *et al.*, 1981), and for glycoproteins using periodic acid-Schiff reagent, PAS+, (Konat *et al.*, 1984). Gels were scanned using an Abaton Scan 300/GS scanner and scans were analyzed using NIH Image 1.60b software.

## Protein Isolation

The major jelly constituent was isolated in the following manner: Gels containing electrophoresed jelly were stained for 10 minutes using 0.05% Coomassie brilliant blue R250 prepared in nanopure water (Harlow and Lane, 1988) and destained with several nanopure water rinses over a one hour period. The major band was excised and the protein was electroeluted from the gel slices. Elutant was pooled and dialyzed (1,000 MWCO) against 4°C nanopure water for 12 hours. Following dialysis, jelly component isolate was pooled, lyophilized, and stored at -80°C until needed.

## Deglycosylation of Isolated Jelly Component

Lyophilized elutant was rehydrated with nanopure water and chemically acid-hydrolyzed using trifluoromethanesulfonic acid (TMFS) as per Karp *et al.* (1982). Approximately 3.0 mg of lyophilized isolated jelly component was resuspended in 900 µl of anhydrous TMFS and 100 µl of anisole under nitrogen and kept on ice for ~6 hours. This mixture was then added to 10 ml of pyridine:ether (1:9 v/v) placed in an acetone/dry ice bath. Following the precipitation of the protein with pyridinium salts, the mixture was centrifuged at 10,000 g for 10 minutes. The pellet was resuspended in 0.1 M $NH_4HCO_3$ and dialyzed against 0.1 M $NH_4HCO_3$ overnight at 4°C. The sample was then aliquotted and lyophilized for later use.

Antibody Production

Jelly component isolates (complete and deglycosylated) were delivered to Antibodies Inc. (Davis, CA), where they were combined and injected into New Zealand white rabbits to produce polyclonal antibodies to the anemone glycoprotein. When the antibody titer was determined to be maximized using standard published protocols (Harlow and Lane, 1988), animals were exsanguinated and serum was prepared.

Antibody Purification

The IgG fraction of the whole serum containing antibodies to the anemone jelly component was prepared using Protein A beads under low salt conditions (Harlow and Lane, 1988). Lyophilized Protein A-cross-linked agarose beads (Sigma Chemical Co.) were rehydrated in nanopure water to yield approximately 4 ml of hydrated beads. Beads were washed extensively with nanopure water followed by Tris-buffered saline, pH 8.0 (TBS = 10mM Tris, 0.9% NaCl) to remove lactose stabilizer. Following final TBS wash, the TBS was replaced with 2.0 ml of whole rabbit serum and the tube containing beads and serum was placed on a Nutator® and allowed to incubate at room temperature for ~4 hours. The tube was then hand-centrifuged to settle all beads. Serum was pipetted off and beads were washed in TBS, pH 8.0 for 5 minutes (5 times). Following TBS washes, IgG fraction of sera bound to beads was eluted off using 3 x 5 ml

washes of 100mM Glycine, pH 3.0. The IgG fractions were pooled, the pH was raised to ~8.0 using 1M Tris, pH 8.0, and the IgG was dialyzed (1,000 MWCO) against TBS, pH 8.0, at 4°C overnight. Dialyzed IgG fraction was concentrated using Centriprep 10® concentrators (Amicon). Protein concentration was determined using spectrophotometer absorbance readings at 280 nm. An approximation of IgG concentration was calculated using 1.35 absorbance units at 280 nm for IgG as an equivalent to 1mg/ml pure protein (see Harlow and Lane, 1988).

Histology and Light Microscopy

Egg masses and anemone tissues embedded in Steedman's polyester wax were sectioned (~10-14 μm in thickness) using razor blades on a rotary microtome. Sections were relaxed and mounted on gelatin-subbed microscope slides (Dr. Billie Swalla, personal communication) then prepared for either light or confocal microscopy. Sections to be viewed using light microscopy were de-waxed, stained using a modified Feulgen method employing de Tomasi's Schiff's reagent followed by a fast green counterstain (Pearse, 1968). Slides were destained and rinsed in 100% EtOH and then immediately cover slipped using Permount®.

Confocal Microscopy

Sectioned material mounted on gelatin-subbed slides was dewaxed and rehydrated by running slides down through a graded (95-30%) EtOH series. Following the 30% EtOH step, slides were rinsed 3 times in TBS containing 0.2% Tween-20 (TTBS), pH 8.0 (5 minutes/rinse), blocked in TTBS containing 1% bovine serum albumin (BSA), pH 7.4, and then incubated in primary antibody (1:50 IgG in TTBS + 1% BSA) for 1 hour. Following 1° antibody incubation, slides were rinsed 3 times in TTBS + 1% BSA (5 minutes each) then incubated in secondary antibody (Texas Red®-conjugated AffiniPure goat anti-rabbit IgG (L & H - Jackson ImmunoResearch Laboratories, Inc.) diluted 1:35 in TTBS + 1% BSA) for 1 hour, rinsed 3x (5 minutes each) in TTBS + 1% BSA, and once in TTBS. Coverslips were mounted on a drop of glycerine placed onto sections and sealed with nail polish. Sections were viewed on a BioRad MRC-600 confocal scanning microscope equipped with a 15 mW krypton/argon mixed gas laser.

## Results

### Egg Mass Formation

*Nematostella vectensis* females released eggs within a persistent jellied matrix. The presence of this egg mass prior to its expulsion from the female anemone's coelenteron was often visible in well-expanded animals (note in Figure 1 that a portion of the egg mass being released is visible within the anemone).

Direct observation of dissected, spawning female *Nematostella* revealed that the egg masses were made up of eggs and jelly released from all 8 mesenteries in regions central to their insertions in the body wall of the anemone. Eggs and jelly were released simultaneously and continually over a period of approximately 90 minutes following the initiation of spawning (Figures 2 and 3), however, the source of the egg mass jelly was not observable.

To begin determining the source of jelly, we first used conventional histological preparations of egg masses and spawning females' mesenteries. The egg mass jelly was Schiff's reagent positive (Figure 4). This positive staining proved fortuitous in that it could be used to locate the potential sites of storage/release of egg mass jelly in both spawning and non-spawning females. Eggs within egg masses showed no surface inclusions to be Schiff's+ (Figure 4). In contrast, mesenterial tissue of gravid females possessed Schiff's+ staining of granules that were present within the gastrodermis of the more oral regions of mesenteries underlaid by mature oocytes (Figure 5). The

Schiff's+ contents of gastrodermis began appearing outside of the mesenteries during egg release in spawning females (Figure 5).

Electrophoretic Analysis of Anemone/Egg Mass Components

Electrophoretic results showed egg mass jelly exhibited an overall protein profile different from the other anemone/egg mass components to which it was compared (Figure 6). The egg mass jelly contained a unique, major component with an molecular mass of between 39.5 and 40.5 kilodaltons (kD) that made up on average 36.5±4.4% (mean±standard error) of the total stained, soluble protein. This component appeared as a diffuse band in the egg mass jelly but was not present in isolated eggs, nematosomes, or epidermally-derived mucus. This egg mass jelly component was also the only band in the jelly isolate that stained PAS+, indicating that it was a glycoprotein (Figure 7).

Once the unique jelly component was identified, it was isolated by electroelution and prepared for use in antibody production. This preparation included extensive dialysis. When DTT was removed through dialysis, and the sample was not immediately lyophilized and stored at -80°C, a band of ~78 kD appeared following electrophoresis under non-reducing conditions in the presence of sodium dodecyl sulfate. This second band (~78 kD) disappeared upon addition of the reducing agent, β-mercaptoethanol (Figure 7). Deglycosylation of a portion of the jelly component was also performed to ensure an antigenic response in rabbits. Following deglycosylation,

the major jelly component migrated at a molecular weight of ~61 kD when electrophoresed (Figure 7).

Immunolocalization of Major Egg Mass Jelly Component

The IgG fraction of the serum obtained from rabbits injected with egg mass jelly component (a combination of both glycosylated and deglycosylated) was used to further localize the storage/release site of the egg mass jelly in female anemones. The IgG fraction recognized and bound to elements present in the jelly of the egg mass as well as that closely apposed to the eggs within the egg mass (Figure 8). The IgG fraction also recognized and bound to elements in sections of female anemone tissue. It specifically labeled the small (up to 2.8 μm), spherical granules present in both the mesenterial filaments and the gastrodermis overlying oocytes in mesenteries of gravid female *Nematostella* (Figures 9 and 10). The antibody did not recognize elements within eggs/oocytes of either egg masses or mesenteries. Control sections of female tissue and egg masses showed no signs of fluorescence (Figures 11 and 12). The antibody also did not label sections of sexually-mature males (Figure 13).

## Discussion

*Nematostella vectensis* is the only known sea anemone that releases its eggs within a persistent, jellied egg mass (Crowell, 1946). Work presented in this chapter has described the first direct observation of egg release (spawning) from the mesenteries of a living sea anemone. In addition, this work has been the first to investigate and localize the source of the egg/egg mass jelly of a sea anemone. Our investigations also have shown that the electrophoretic profile of egg mass jelly differs from profiles of the ectodermally-derived mucus as well as the other components making up the egg mass.

Few researchers have addressed the presence/absence of mucus/jelly-like substances associated with newly-released anthozoan eggs (Gemmill, 1920; Nyholm, 1949; Dunn, 1972; Siebert, 1973 and 1974; Clark and Dewel, 1974; Larkman and Carter, 1984), and only Nyholm (1949) and Clark and Dewel (1974) addressed the possible source/sources of these extracellular matrices. Nyholm (1949) observed that *Halcampa duodecimcirrata* eggs were secretion-covered when released. He speculated that these secretions were probably derived from gastrodermal gland cells located in the mesenteries from which the oocytes came. Following release, if the eggs were not fertilized, the secretion covering the eggs dispersed gradually. If, on the other hand, the eggs were fertilized, the eggs became "coated 5 to 15 minutes later with a gelatinous layer (derived from the eggs) that could be up to 1/4 of the diameter of the egg in thickness". Clark and Dewel (1974) reported that eggs of *Bunodosoma*

*cavernata* underwent a massive cortical reaction in response to fertilization. Following this reaction, material released by the cortical granules formed a persistent investment layer, speculated to participate in blocking polyspermy. *Nematostella vectensis* eggs within egg masses did label with the jelly antibody, indicating the presence of an extracellular coat. A possible explanation for this labeling could be the presence of the same sugar moeities on both the egg surface (glycocalyx) as well as in the egg jelly, however, we attribute this labeling to egg mass jelly that had become closely apposed to the egg surface membrane during the post-fixation dehydration. *N. vectensis* eggs do not undergo a dramatic cortical exocytosis following either spawning or addition of sperm to eggs/egg masses, nor is the formation or alteration of any coat seen (Chapter 3).

Frank and Bleakney (1976), in a histological study of *N. vectensis*, reported that there were at least 3 types of gland cells present in the mesenteries. It appears likely from our results that the cells which produce the egg mass jelly component correspond to Frank and Bleakney's Type C gland cell. Frank and Bleakney describe the Type C cells as being localized in the mesenterial filament, and it is unclear whether any were present in other regions of female mesenteries. We have found positive staining in both female mesenteries and mesenterial filaments.

The major component of the egg mass jelly is a 39.5-40.5 kD glycoprotein that is produced in the mesenterial gastrodermis. Upon spawning, this jelly component is released into the coelenteron with the eggs to form the egg mass.

Egg jelly/envelopes in other animal systems have been shown to be derived from various sources. In more evolutionarily-advanced animals, egg jelly/envelopes tend to be derived from specialized cells associated with developing eggs (i.e., follicle/nurse cells) and/or secreted by either regions/glands within female reproductive tracts (i.e., oviducts, uterine wall, or mucus/shell glands) or other secretory regions present in the exterior of an organism ( e.g., epidermis of platyhelminthes and annelids) (Hyman, 1967; Kumé and Dan, 1968; Duellman and Trueb, 1994). Anthozoans such as *N. vectensis*, as well as Cnidaria in general, are evolutionarily of tissue grade construction (Hyman, 1940). These lower metazoans have structurally simple ovaries which lack follicle/nurse cells (Eckelbarger and Larson, 1992). In addition to the lack of accessory cells in the ovary, these animals also exhibit diffuse gonadal tissues and do not have distinct organ systems. Consequently, analogous as well as homologous comparisons of egg jelly/envelop sources can not be easily made.

Egg jelly/envelopes in other animal systems also have been shown to serve several purposes. Functions include facilitating sperm-egg recognition and interaction, protecting the eggs/developing embryos, and participating in developmental metamorphosis and larval dispersal. Sperm interaction with specific egg jelly components occurs in a number of invertebrates; for example, molluscs (Mozingo *et al.*, 1995), polychaete annelids (Sato and Osani, 1996), shrimp (Griffin *et al.*, 1987), and echinoderms (Keller and Vacquier, 1994; Nishigaki *et al.*, 1996; Alves *et al.*,1997; Koyato *et al.*, 1997). Alterations of egg

investment coats by substances released from cortical granules have been shown to be involved in blocks to polyspermy; e.g., sea urchins (Foerder and Shapiro, 1977) and toads (Lindsay *et al.*, 1988). We can only speculate as to the potential roles this egg mass may play in the life history of *N. vectensis.*

Egg mass production is common in many animals yet little is known about the function they serve or the constraints they superimpose on developing embryos embedded in them. Some egg masses can be adherent and secure eggs/embryos to presumptive suitable habitats. In the opisthobranch mollusc, *Haminaea callidegenita*, the major component of the gelatinous egg mass matrix has been found to induce intracapsular metamorphosis in its larvae (Gibson and Chia, 1994). In addition to individual components of egg masses affecting developing embryos, physical constraints of embedment have been described. Booth (1995) has investigated oxygen diffusion in the large egg masses (37 mm in diameter) of the gastropod mollusc, *Polinices sordidus*, and has correlated this with embryonic development time. Those embryos at the periphery, exposed to higher oxygen levels, hatch first, while those embryos located centrally in the egg mass, which are exposed to low oxygen levels, arrest development. Only after the peripheral embryos hatch and leave the egg mass, do oxygen levels of the central egg mass increase. Concomitant with increased oxygen levels was the resumption of development in arrested embryos.

*Nematostella vectensis* releases an adherent egg mass $\leq$3 mm in diameter. The egg mass jelly's adherent property may ensure successful survivorship of embryos by securing their egg mass within the more permanent

pools where the adult anemones exist. From the numerous fertilization runs performed in the laboratory, presence of jelly is not necessary for successful fertilization of eggs by sperm. Dejellied eggs, that had been washed several times, fertilized and developed normally without any apparent jelly requirement (Chapter 3). This suggested that the jelly is not required in sperm-egg interaction and that it does not play a role in this anemone's block to polyspermy. The egg mass in natural habitats may, however, enhance attraction of sperm. Miller (1985) has suggested that some evidence has been obtained that chemotaxis occurs in the Anthozoa. In addition to chemotaxis, egg masses/egg mass jelly may serve to protect eggs from digestive enzymes in the coelenteron. Bunde *et al.* (1978) have shown that glycoproteins containing 2-aminoethylphosphonic acid (AEP) are present in epidermal and gastrodermal cells as well as in mucus of sea anemones. AEP includes an unusual carbon-phosphorus bond which is resistant to hydrolysis and thus may protect tissues exposed to hydrolytic enzymes (Kittredge and Roberts,1969). Ottaway (1974) showed that juvenile *Actinia tenbrosa*, which are brooded in the coelenteron of adults, were resistant to digestive enzymes because of their mucus coatings. Although we have not proven AEP's presence in *N. vectensis* egg mass jelly, since *N. vectensis* eggs are released into the coelenteron up to 90 minutes prior to being spawned, the egg mass jelly could provide eggs with protection from digestive enzymes. Lastly, nematosomes are also contained within egg masses of *N. vectensis*. These bodies, which are engulfed by the jelly during egg mass formation, contain functional nematocysts. Although no predator to *N. vectensis*

egg masses has been identified, the quantity of jelly as well as the number of nematosomes present in this animal's egg mass may serve as a predation deterrent.

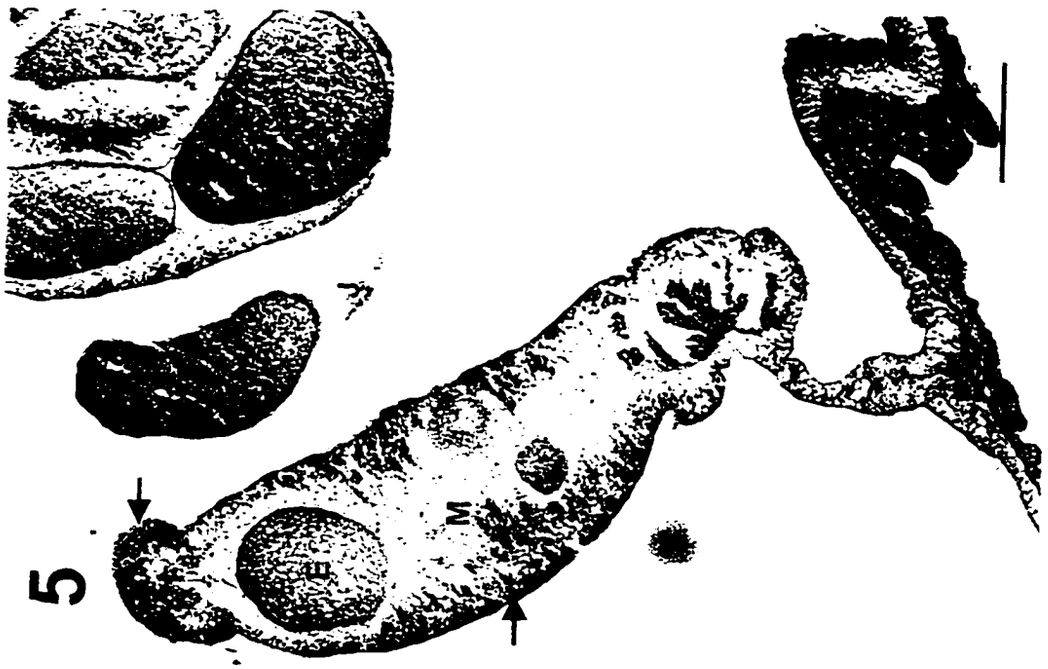
Figure 1. *Nematostella vectensis* female releasing eggs contained in a persistent jelly egg mass. (2x).

Figure 2. *Nematostella vectensis* female expected to spawn. Anemone was dissected and pinned open in order to view spawning event. (5x).

Figure 3. Same female anemone pictured in Figure 2, 45 minutes later. Note eggs have been released from all 8 mesenteries. Jelly was released at the same time as eggs, however, the immediate source of jelly could not be determined through this direct observation. (10x).

Figure 4. Light micrograph of a Feulgen-stained, fast green-counter-stained histological section of an intact egg mass. The egg mass is made up of eggs (E) and nematosomes (N) contained within a persistent jelly (J). Note jelly stains Schiff's reagent positive (Schiff's+). Eggs observed at this magnification exhibit neither investment coats nor surface inclusions containing Schiff's+ material. Scale bar = 100 µm.

Figure 5. Light micrograph of a Feulgen-stained, fast green-counter-stained histological cross section of spawning female anemone. Mesentery (M) of female anemone contains eggs (E). Note Schiff's+ material (arrows) is present in the gastrodermis of mesentery and mesenterial filament (F). Jelly (J) present in the coelenteron also stains Schiff's+. Scale bar = 100 µm.



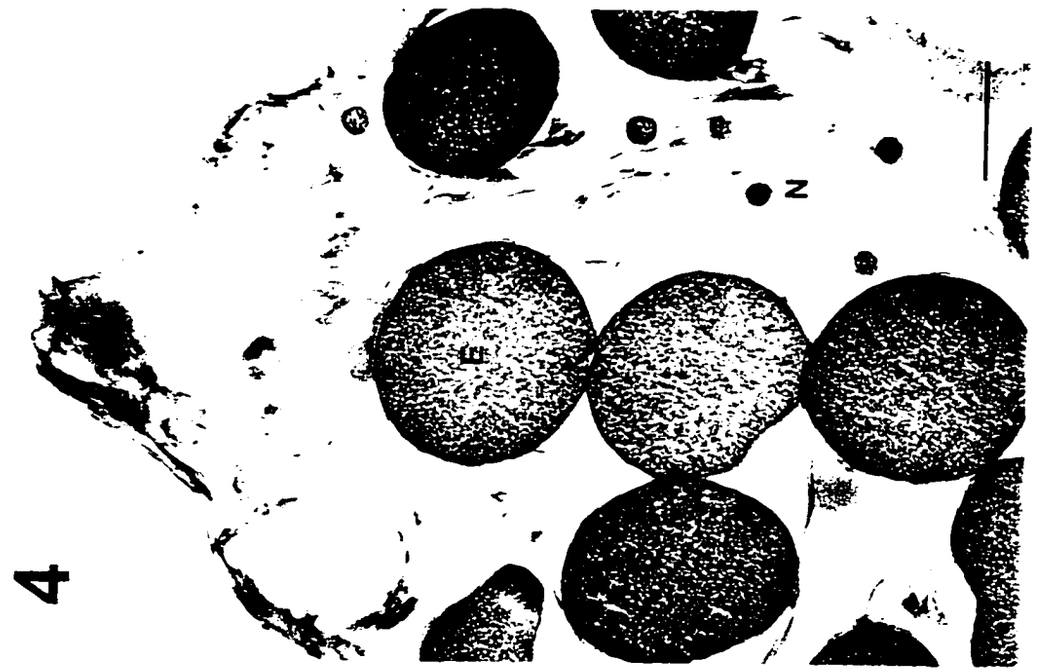
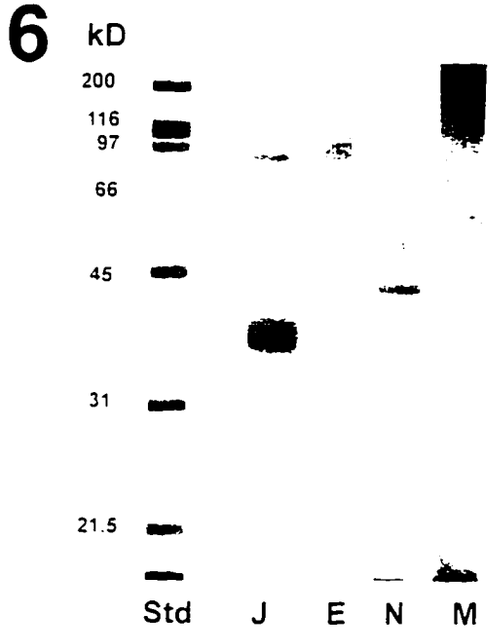



Figure 6. Electrophoretic characterization of *Nematostella vectensis* egg mass components and ectodermally-derived mucus. Protein standards (Std) of known molecular mass [in kiloDaltons (kD)], isolated sea anemone egg mass components [i.e., jelly (J), eggs (E), and nematosomes (N)], and ectodermally-derived mucus (M) were run on 12% SDS-PAGE gels. Following electrophoresis, gels were stained using Coomassie blue and/or silver stain. Note that the jelly contained a unique major band (ranging between 39.5 and 40.5 kD) when compared to all other sea anemone components.

Figure 7. Electrophoretic characterization of *Nematostella vectensis* egg mass jelly and the major protein component isolated from it. Lanes described below were taken from 12% SDS-PAGE gels stained using Coomassie blue and/or silver stain unless otherwise noted. Solubilized jelly (J) contained a major protein band ranging between 39.5 and 40.5 kD. This protein, making up ~36.5% of total stained jelly protein, was the only band recognized by periodic acid-Schiff's staining (PAS). Following electrophoretic separation, this jelly component was isolated using electroelution techniques. Freshly isolated elutant ($El_F$) was then deglycosylated ($El_D$). Freshly isolated elutant that was stored following dialysis ($El_S$) tended to dimerize over time and resulted in the formation of a second protein band (~78 kD). This second protein band disappeared following the addition of the reducing agent, β-mercaptoethanol, to the stored elutant ($El_{Sr}$).

6




7

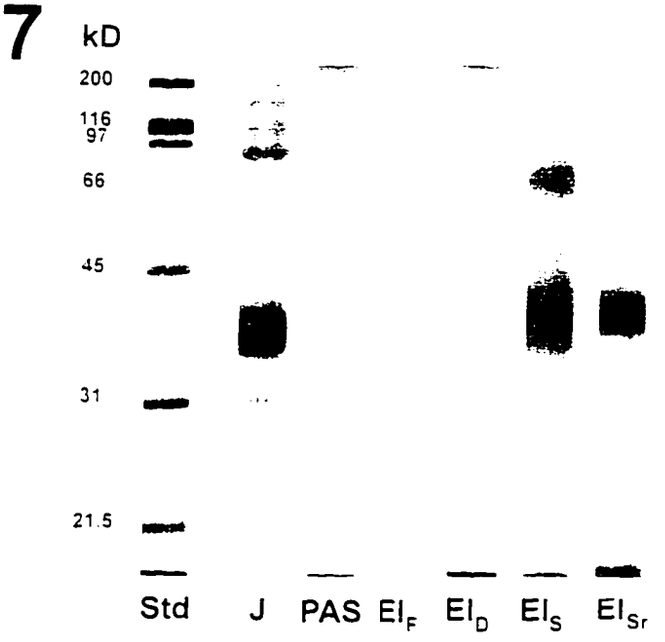


Figure 8. Confocal image of histological section of egg mass incubated with rabbit IgG developed against egg mass jelly component followed by Texas-Red®-conjugated goat anti-rabbit IgG. Note that egg mass jelly fluoresces and has collapsed around the egg. Scale bar = 100 μm.

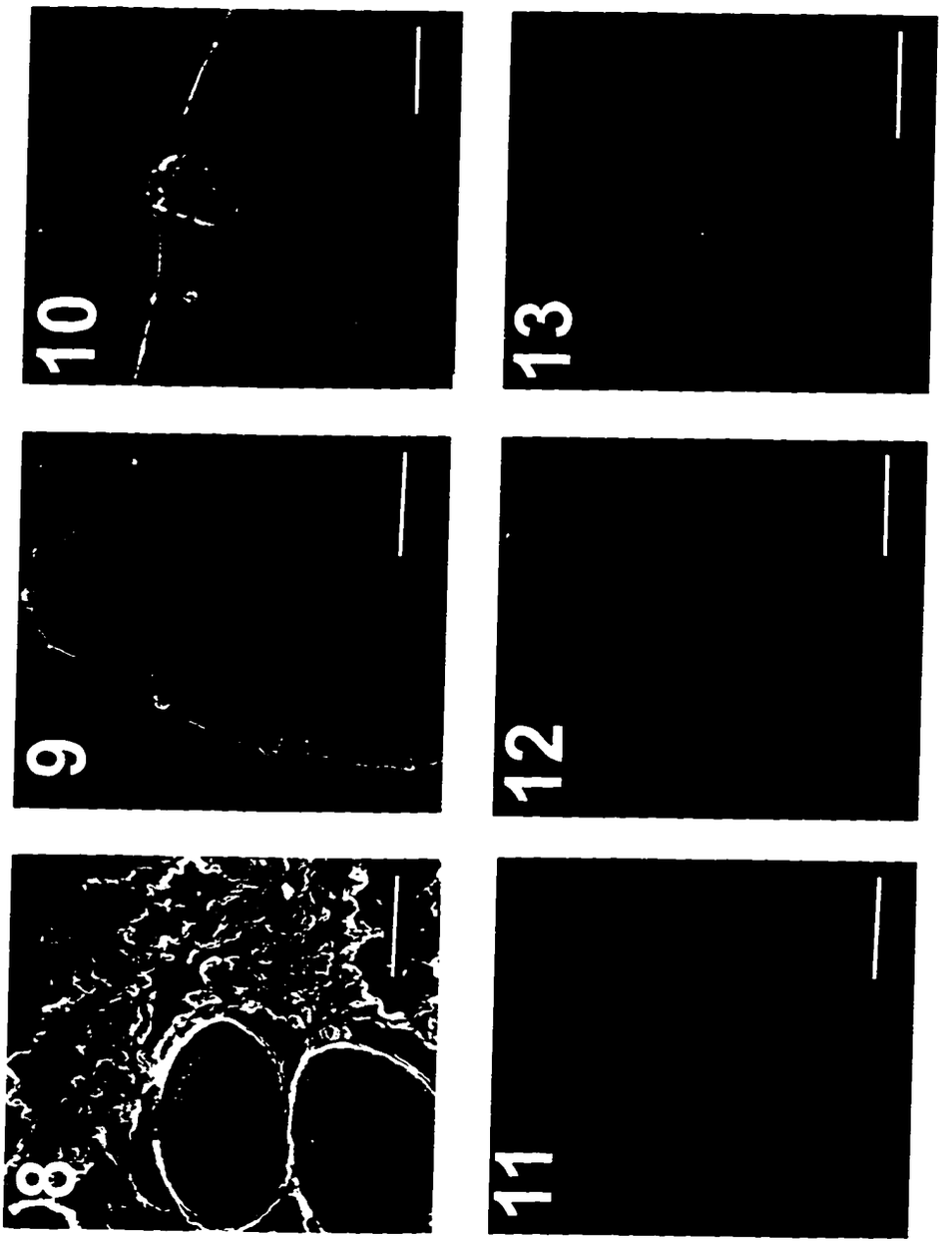
Figure 9. Confocal image of histological section of spawning female's mesentery incubated with rabbit IgG developed against egg mass jelly component, followed by Texas-Red®-conjugated goat anti-rabbit IgG. Note that fluorescence is localized to granules present in the gastrodermis of mesentery and mesenterial filament. Scale bar = 100 μm.

Figure 10. Enlargement of confocal image of spawning female's mesenterial gastrodermis showing closeup of granules. Scale bar = 25 μm.

Figure 11. Confocal image of control treatment of histological section of egg mass. Treatments of either primary rabbit anti-egg mass component IgG or secondary goat anti-rabbit IgG resulted in no fluorescence. Scale bar = 100 μm.

Figure 12. Confocal image of control treatment of histological section of spawning female's mesentery. Treatments of either primary rabbit anti-egg mass component IgG or secondary goat anti-rabbit IgG resulted in no fluorescence. Scale bar = 100 μm.

Figure 13. Confocal image of control treatment of histological section of male's mesentery with primary rabbit anti-egg mass component IgG followed by secondary Texas-Red®-conjugated goat anti-rabbit IgG resulted in no fluorescence. Scale bar = 100 μm.



# Chapter 3

## Variable Cytokinesis during Initial Developmental Stages in the Estuarine Sea Anemone, *Nematostella vectensis*

## Introduction

The Anthozoa is a very diverse group with respect to both egg characteristics and early development (Mergner, 1971; Spaulding, 1972; Campbell, 1974; Tardent, 1978; Beams and Kessel, 1983; and Fautin *et al.*, 1989). Early cleavages in most cnidarians are reported to be synchronized and radial (Hyman, 1940; Mergner, 1971; Tardent, 1978; Fautin *et al.*, 1989). Within the Actiniaria, two general patterns have been seen. Eggs with little or no yolk tend to undergo complete cleavages, whereas large yolky eggs generally cleave superficially with cytokinesis occurring only after multiple nuclear divisions (Fautin *et al.*, 1989). In sea anemones with small eggs (less than 150 μm), the first zygotic cleavage often results in the formation of a four-celled embryo. If an initial two-cell stage develops, it typically contains four nuclei, two in each blastomere. In larger, yolky eggs, cleavage tends to be meroblastic and is preceded by multiple nuclear divisions prior to first cytokinesis (Spaulding, 1974).

A phenomenon called "refusion" (McMurrich, 1891) occurs when blastomeres fuse to form a secondary syncytium following the first, or the first few, cleavage stages. This phenomenon was first observed in *Metridium marginatum* (= *M. senile*) (McMurrich, 1891). McMurrich (1891) noted that although this phenomenon was "well-marked", he could not conclude whether it was normal since he was unable to follow further development in these embryos. Wietrzykowski (1910,1914) observed a phenomenon that he termed "refusionnement" in *Edwardsia beautempsi* Quatr. In this anemone, which

belongs to the same family (Edwardsiidae) as *Nematostella vectensis*, the 2-hour old zygotes divide to form 4 blastomeres at first cleavage. These 4 blastomeres proceed to fuse to form a spherical, undivided, single cell that then cleaves into an 8-celled embryo. He observed this phenomenon in several thousand eggs and believed it was absolutely normal. Since Wietryzkowski (1914), the refusion phenomenon has only been reported not to occur in *Sargartia troglodytes* (Nyholm, 1943).

In this paper, we describe the early development in the edwardsiid sea anemone, *Nematostella vectensis*. Within this description, we detail the morphology as well as the incidence of "refusion" as described by McMurrich (1891) and Wietryzkowski (1910, 1914). We present an analysis of this phenomenon's effect on cleavage interval timing and viability. Finally, we present experimentation attempting to better understand what role this phenomenon plays in the early development of this sea anemone.

## Material and Methods

### Culture of *Nematostella vectensis* Clones

The two clones of *Nematostella vectensis*, one female and one male, that were used for research presented in this study are two of the clones originally described in Hand and Uhlinger (1992). These clones were derived from first generation siblings of a spawn that took place 6-7 April 1988. Clonal anemones were chosen for this set of experiments in order to reduce potential variability between individuals.

Clones of each sex were reared and maintained in isolation from each other. Each clone was maintained at room temperature (20-23°C) under a 12 hr light/12 hr dark cycle. Cultures of each clone were maintained in one third natural seawater (1/3 NSW, approximately 12 ppt) as described in Hand and Uhlinger (1992) with several differences. Several cultures of 15 animals of each sex, placed in 80x40 mm crystallizing dishes containing 100 ml 1/3 NSW, were fed 5-8 drops of concentrated *Artemia* sp. nauplii (Sanders Brine Shrimp Co.) every Monday, Wednesday, and Friday. All anemones were fed *Mytilus californianus* ovary following the replacement of each culture's water on Monday mornings. After the Monday *Mytilus* feeding, cultures were also fed *Artemia* nauplii. One hour after this *Artemia* feeding, each culture's animals were transferred into a clean culture dish containing aerated new 1/3 NSW. Animals began releasing gametes 24-36 hours following feeding and water change.

Gamete Collection and Handling

Once females had released gametes, the egg masses were collected in 2 ml aliquots, placed in disposable 15 ml centrifuge tubes, and 13 ml of 1/3 NSW containing 10mM Dithiolthreitol (pH 8.0) was added to each tube. Tubes were capped and gently rotated until eggs became completely free of jelly (~30 minutes). Eggs were then allowed to settle to the bottom of each centrifuge tube. Following settlement, eggs were washed 4 times with 1/3 NSW and then transferred into a 51x31 mm crystallizing dish containing 15 ml 1/3 NSW.

Sperm release was detected as a cloudiness present in culture dishes. Once eggs had been dejellied and placed in crystallizing dishes, a 1-1.5ml aliquot of sperm solution (sperm and culture water) was added to each dish. As a control for each fertilization, an intact egg mass was placed in 15 ml 1/3 NSW and fertilized with 1-1.5 ml of the same sperm solution. An aliquot of sperm solution was also sampled in order to determine final concentration for each experiment. All fertilizations and subsequent experiments were performed at room temperature (21-25°C) in the laboratory.

After one hour, aliquots of the fertilized, dejellied eggs were transferred to viewing chambers, made of a microscope slide with a vacuum grease ring. The chambers were viewed with compound light microscopes equipped with a video camera connected to a video recorder. Developing embryos were monitored and VHS video recordings were made to aid in subsequent analysis of both

frequency and patternation of blastomeric fusion. Fourteen separate fertilization runs were performed.

Viability Testing

Dishes containing fertilized embryos were placed under a dissecting microscope and observed during first cleavage. Because development was asynchronous, embryos undergoing first cleavages were moved into the center field of view where their progress could be monitored.

Following the four-cell stage any embryos exhibiting fusion were transferred with a mouth pipette to a new, labeled culture dish containing 1/3 NSW. Embryos developing directly from four to eight cells were also transferred to a new, labeled culture dish containing 1/3 NSW to serve as a control. Embryos were then allowed to develop into spawning adults as described in Hand and Uhlinger (1992). Dishes were monitored regularly.

Blastomere Isolations

Dishes containing fertilized embryos were placed under a dissecting microscope and observed. Embryos that began to cleave were transferred to ~3 ml aerated 1/3 NSW contained in 100x20 mm disposable Petri dishes coated with agarose (Sigma, Type III, 1% in 1/3 NSW). Individuals exhibiting a radial 4-celled pattern were moved to the center of the Petri dish where each was teased

apart with glass needles, into 4 isolated blastomeres. Isolated blastomeres were then transferred to labeled 24-well tissue culture plates (Falcon®) containing 0.22 µm-filtered 1/3 NSW, at a concentration of 1 blastomere per well. Isolated blastomeres were maintained in these wells and subsequent development noted.

Histology

Isolated eggs and egg masses were collected and fixed in 1/3 NSW containing 3% paraformaldehyde and 0.1% glutaraldehyde for ~1.5 hours. Following fixation, samples were washed 3 times for 15 minutes each with 1/3 NSW, once with nanopure water, then dehydrated in a graded ethanol series. Following dehydration, samples were infiltrated and embedded in Steedman's polyester wax (Norenburg and Barrett, 1987). Embedded samples were serially sectioned using razor blades on a rotary microtome. Sections were then mounted on gelatin-subbed microscope slides (Dr. Billie Swalla, personal communication) and stained using a modified Feulgen method employing de Tomasi's Schiff's reagent, followed by a fast green counterstain (Pearse, 1968). Slides were de-stained and rinsed in 100% ethanol (EtOH) and immediately cover slipped using Permount®.

Confocal Microscopy

Embryos were fixed using either cold (0°C), 90% methanol (MeOH) containing 50mM EGTA, pH 6.0 or 0.22 µm-filtered 1/3 NSW containing 3% paraformaldehyde and 0.1% glutaraldehyde. Samples fixed in MeOH were rehydrated through a graded EtOH series, rinsed with Tris-buffered saline+Tween-20, pH 8.0 [TTBS = 10mM Tris (hydroxymethyl) aminomethane, 0.9% Sodium chloride, 0.2% Tween-20], and blocked in TTBS + 1% bovine serum albumin (BSA), pH 7.4. Samples were then incubated in a primary antibody (Ab) [either mouse monoclonal antibody to double stranded DNA (MAB3032 -Chemicon) diluted 1:100 or a monoclonal antibody which reacted to sea urchin β-tubulin (a gift from Dr. Roger Leslie to Dr. Philip Hertzler who graciously shared it with me) diluted 1:500 in TTBS + 1% BSA for 1 hr]. Following 3 TTBS + 1% BSA washes, samples were incubated for 1 hr in secondary Ab [Rhodamine-conjugated goat affinity purified Ab to mouse IgG (whole molecule) - Cappel] diluted 1:50 in TTBS + 1% BSA. Samples were then rinsed 3 times using TTBS, dehydrated through a graded EtOH series, and cleared in methyl salicylate. Once cleared, samples were placed in depression slides, cover slipped, then viewed using a Biorad MRC-600 confocal scanning microscope equipped with a 15mW krypton/argon mixed gas multi-line laser. The Y filter set was used to collect the rhodamine fluorescence with the 568 nm laser line. Images were collected using Kalman-averaging and down-loaded to a computer. Samples fixed in the paraformaldehyde-based fixative were rinsed

in TTBS and processed similarly to those fixed in MeOH, without having to be rehydrated first.

Electron Microscopy

Samples were fixed in 0.22 µm-filtered 1/3 NSW containing 2.5% glutaraldehyde and 1% paraformaldehyde for 1 hour. Following a rinse in 0.22 µm-filtered 1/3 NSW, samples were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.3, for 30 minutes. Specimens were then rinsed in distilled water and dehydrated using either a graded EtOH series for scanning electron microscopy (SEM) or a graded acetone series for transmission electron microscopy (TEM). Each dehydration was done at 15 minutes per step until reaching the 100% concentration. Following 3 changes at the 100% concentration (30 minutes each), SEM samples were critical point dried. Dried samples were mounted on aluminum stubs and were coated with approximately 200Å of gold. All specimens were viewed on a Hitachi S570 SEM. Pictures were recorded on Polaroid Type 55 4x5 land film.

Following the final 100% acetone dehydration step, TEM specimens were infiltrated with increasing concentrations of low viscosity epoxy resin (Spurr, 1969). After infiltration in two changes of 100% Spurr's (~8 hours), samples were pipetted into Beem capsules and subsequently placed in a polymerization oven set at 70°C for 8 hours. Polymerized samples were thick-sectioned (500 nm) and thin-sectioned (90 nm) using diamond or glass knives on an

ultramicrotome. Sections were collected on copper grids and stained with 70% MeOH containing ~7% uranyl acetate followed by lead citrate (Reynolds, 1963). Sections were observed using a Zeiss 902 TEM and pictures were recorded using Kodak Electron Microscope Film 4489.

Statistical Procedures

SYSTAT® 6.0.1 for Windows (SPSS Inc.) was used for statistical analysis of the effect fusion on cleavage interval timing. Comparisons of the two developmental pathways for each of 6 timed experiments employed independent t-tests with all time intervals data log-transformed to meet the assumptions of parametric statistics.

## Results

Female *Nematostella vectensis* were induced to release egg masses every 7 days. The relative size of these egg masses varied with some containing in excess of 2000 eggs. Egg masses consisted of a gelatinous matrix (jelly) containing both eggs and nematosomes (spherical, flagellated aggregations containing nematocytes). The nematosomes were observed to freely rotate within this jelly.

### Egg Morphology

*N. vectensis* eggs ranged in diameter from 170 to 210 μm (Figure 1). These isolecithal eggs were spawned after completion of meiotic maturation ( Figure 3). Although surrounded by a jelly mass, eggs did not appear to possess investment coats. The egg surface possessed ~4.1 μm long microvilli with a diffuse glycocalyx. An approximately 2.7 μm thick region underlies the egg plasma membrane where electron dense granules were arranged 3-5 deep (Figure 2). These round to ovate granules ranged in diameter/long axis from 0.4-1.1 μm. The female pronucleus (approximately 10.2 μm in diameter) was eccentrically located within the egg below the layer of cortical granules and was surrounded by a small region of clear cytoplasm (Figure 3). The remaining central region of the egg was heavily laden with yolk.

Early Development

Eggs were fertilized either within intact egg masses or after being separated from the egg mass matrix. Development proceeded identically under both conditions, showing no discernible differences when observed at the light microscope level. There was no perceivable activation response upon the addition of sperm to the eggs. First karyokinesis took place approximately 60 minutes after the introduction of sperm into egg suspensions and was not accompanied or directly followed by first cleavage (Figure 4). Instead, it was followed by second karyokinesis 90-120 minutes later (Figure 5). First cleavage (cytokinesis) then began with the unilateral progression of 2 perpendicular, meridional cleavage furrows that resulted in a 4-celled embryo in which each blastomere contained a single nucleus (Figure 6). Blastomere sizes at the 4-cell stage varied (from equal to unequal) as did cleavage type (from radial to pseudospiral) with no consistent pattern being observed (Figures 7 and 8). Development of embryos appeared to be holoblastic. An initial 2-cell cleavage stage was never observed.

Fertilizations between several different female and male *N. vectensis* clones led to the observation of variation in cleavage pattern between the 4- and 8-cell stage embryo. This variation was of two basic types. Either embryos cleaved directly from a 4-celled embryo to an 8-celled embryo, or a 4-celled embryo exhibited a fusion between 2 or more of its blastomeres prior to progressing through its next cleavage. In order to reduce potential variability due

to clonal variation and to gain a better perspective of the total number of embryos undergoing this fusion phenomenon, we selected one male clone and one female clone to use in all our fertilization runs. Each fertilization run entailed pooling eggs from 45 individuals of the same female clone and fertilizing them with sperm from 15 individuals of the same male clone. A total of 1032 embryos were observed during 14 separate fertilizations. Each fertilization entailed pooling eggs from up to 45 female clonemates and fertilizing them with sperm pooled from 15 male clonemates.

Refusion Patterns

Following the formation of the 4-cell stage, 71% of 1032 embryos proceeded to the 8-cell stage directly (Figures 9 and 10), whereas 29% of embryos (297) underwent a fusion of between 2 and 4 of their blastomeres before going on to their next cleavage stage. These fusion events were characterized as follows: 1) all four blastomeres fused, (Figure 11); 2) two of four blastomeres fused (Figure 12); 3) three of four blastomeres fused (Figure 13 ); or, 4) two pairs of blastomeres fused (Figure 14).

In 8 of 14 separate fertilizations, aliquots of embryos were observed for specific fusion patterns (Table 1). A total of 530 embryos in those 8 runs were used to quantify the frequencies of the different fusion patterns. Of the 530 observed embryos, 135 underwent a fusion event. Of those 135 embryos, 74.1% were of the 4 blastomere fusion type, 14.8% were of the 2 of 4

blastomere fusion type, 9.6% were of the 3 of 4 blastomere fusion type, and 1.5% were of the 2 pairs of blastomere fusion type.

One hundred embryos that underwent blastomere fusion were followed through subsequent development. Of these, 86 were successfully reared to spawning adults. Both sexes were present with the ratio of females to males being 42:44. Of 100 embryos that progressed directly from the 4-cell to the 8-cell stage, a total of 83 were successfully reared to spawning adults. Both sexes were present in a ratio of 43 females:40 males (Table 2).

Blastomere Isolations

To determine whether development in *N. vectensis* was mosaic or regulative, we conducted blastomere isolation experiments. A total of 33, 4-celled, radially-arranged embryos were used for this experiment. Table 3 presents the developmental outcome of each embryo's isolated blastomeres. These embryos were selected randomly from embryos derived from a fertilization of eggs collected from 16 female clonemates and sperm from a pool of 15 male clonemates. In 14 of 33 embryos, all four isolated blastomeres developed to the planula stage, and in 8 of the 19 remaining embryos, 3 of 4 blastomeres developed to the planula stage. There were 11 embryos in which at least 3 blastomeres did not develop to the planula stage. Of the total 44 blastomeres representing these 11 embryos, 18 developed into planulae, 11 never cleaved and degenerated, 1 began dividing but never reached the blastula stage, 9

reached the blastula stage but did not gastrulate, and 5, reaching the blastula stage, did not successfully complete gastrulation. In all cases where isolated blastomeres developed into what appeared to be normal planulae, they were ~1/4 size of planulae developing from a normal-sized embryo. Of the 98 planulae larvae that developed from isolated blastomeres, 60% (59 individuals) metamorphosed into juvenile anemones. Of those juvenile anemones, 25% (15 individuals) exhibited normal numbers of tentacles (4) and a well-developed 1° mesenteric couple.

Fusion Effect on Cleavage Interval Timing

In order to determine whether embryos that underwent blastomere fusion exhibited a developmental delay, we followed the timing of the first 2 cleavages, between the 4-cell stage and the next cleavage, to determine if an intervening fusion prolonged the onset of the second cytokinesis.

A total of 51 embryos underwent a fusion event in the 6 timed fertilization runs. The time interval between first and second cleavage for those 51 embryos was 40.1±13.3 (mean ± s.d.) minutes. The remaining 211 embryos underwent their second cytokinesis without a fusion event, but with a similar time interval, 40.1±12.3 minutes. There was no significant difference in time between the 2 developmental pathways over all fertilization runs ($t$=-0.308, DF=252, p>0.70). We also analyzed each individual run and found that all but one (Run 5) showed no significant difference in the time interval between first and second cleavages

(Table 4, Graph 1). In the run that did show a significant difference (Run 5), the time intervals of those embryos undergoing fusion (n=3) fell within the range of values observed in embryos that did not undergo fusion (n=101). Significant results in Run 5 are suspect because of the huge difference in sample size.

Was Fusion of Blastomeres Merely Compaction?

Embryos were examined at the fine structural level to determine whether the fusion event was formation of a secondary syncytium or a mere compaction of blastomeres. We compared samples of 4-cell embryos (Figures 15 and 16) to fused embryos (Figures 17 and 18). In embryos that underwent the fusion phenomenon, cell partitions/septa between fused blastomeres disappeared. In regions where membrane previously existed, little to no visible vesiculation or cytoplasmic/yolk patterning was observed other than the displacement of cortical granules at the surface where cleavage furrows had not completely disappeared. Microvilli near the site of previously existing cleavage furrows did not appear to change in length.

## Discussion

Both sexes of *Nematostella vectensis*, unlike other anthozoans, can be conditioned to release gametes in a predictable, synchronous manner every seven days throughout the year without any apparent seasonality. *N. vectensis* eggs at spawning are meiotically-mature, isolecithal, and have a characteristic cortical region which, unlike *Halcampa* (Nyholm, 1949), *Metridium* and *Bunodosoma* (Clark and Dewel, 1974), persists through the early developmental stages following fertilization without undergoing a dramatic exocytosis. Following fertilization, zygotes undergo two nuclear divisions prior to undergoing their first cytokinesis. This is not an uncommon occurrence in the Cnidaria where the first cleavage is characterized by the unilateral progression of two perpendicular cleavage furrows which begin in the same region of the embryo's surface. This cleavage is normally complete and ranges from radial to pseudospiral (Mergner, 1971) or radial to adradial to subspiral (Siebert, 1974). Infrequently, embryos with apparent radial cleavage have been observed to change to a more pseudospiral arrangement. This could be attributed to a blastomeric shift such as that noted by Wietryzkowski (1914) in *Edwardsia* and Kumé and Dan (1968) in the hydromedusae *Spirocodon* sp.

*N. vectensis* embryos exhibited fusion after reaching the 4-cell stage in approximately 29% of all individuals observed. Variations in the fusion patterns were similar to those of other anemones described by both McMurrich (1891) and Wietryzkowski (1910, 1914). Blastomeres of 4-cell stage embryos

sometimes fused in pairs, forming an apparent 2-cell stage, which in some embryos was an intermediate stage. In these embryos, this intermediate stage underwent a further fusion to form a 1-celled embryo, while in others the "2-cell stage" appearance was maintained until the next cleavage took place. Embryos that underwent fusion to form any of the 4 patterns mentioned in our observations (all 4 blastomeres fused, 2 of 4 blastomeres fused, 3 of 4 blastomeres fused, or 2 pairs of blastomeres fused) sometimes continued to reflect this fusion in their next cleavage stage, which resulted in an embryo with an odd number of blastomeres rather than 8. These variations undoubtedly correspond to Wietryzkowski's (1914) interesting variations. We are not sure that nuclei are evenly distributed when this occurs; however, cytokinesis apparently "catches up" with nuclei number during the later cleavage events. Embryos that underwent fusion continued to develop into normal planulae, juveniles, and adults.

We tested a hypothesis that would explain the reason for fusion of blastomeres in developing embryos. If development was mosaic and proper ooplasmic segregation (i.e., resulting in one or more of the blastomeres sharing important "determinants") had not occurred prior to the completion of the initial cleavage stage, fusion would provide a mechanism by which the segregation could still take place. The results of the blastomere isolations, however, suggest that *N. vectensis* embryos were capable of regulating. In 42% of the embryos, all 4 blastomeres developed into planulae, a percentage which does not correlate with the fusion frequencies. If the blastomeres which did not resume

cleavage (damaged in isolation) are eliminated from the data, ~67% of all blastomeres developed into planulae. From these data we conclude *N. vectensis* embryos regulate at the 4-cell stage.

The question of whether embryos separate into 4 complete blastomeres prior to fusion was addressed by examining serial sections of 4-celled embryos. Although we failed to observe cytoplasmic connections between blastomeres, we could not rule out the possibility that they existed. However, the fact that isolated blastomeres developed into complete organisms suggests that if they did exist, cutting them during the isolation procedure did not significantly affect development. Interestingly, there was no apparent difference in time to second cleavage between embryos undergoing either developmental pathway. Neither timing nor end results of development (i.e., mature adult anemones) appears to be affected by blastomere fusion.

Fusion of blastomeres, as observed in *Nematostella*, is not the same as the compaction events observed in many animals and probably best studied in mice (Ducibella and Anderson, 1975). During compaction in mice embryos, the initial rounded blastomeres increase the extent of apposition and flatten on each other, exhibiting blastomeric membranes that closely adjoin each other between cells. In *Nematostella*, blastomeres fused with no signs of membranes remaining. There were no apparent external differences, at the light microscope level, in the surface of unfertilized eggs compared to embryos that had undergone fusion. The only differences observed were at the electron microscope level. Remnants of the cleavage furrows were obvious on the

outside surface of fused embryos while the only intracellular variance was the interruption of the cortical region central to the furrows. These interruptions probably correspond to Wietryzkowski's(1910) less-stained regions.

The mechanism driving the fusion of blastomeres remains unknown. Nematostella undergoes the same phenomenon described by Wietryzkowski 80 years ago, yet there are no models to date that can explain the event. Many elements associated with the process of cytokinesis are well understood both biochemically and biomechanically. Schroeder (1973) first demonstated that the contractile ring, which is closely apposed to the newly formed cleavage furrow, contains actin and myosin with the contractile filaments aligned along the furrow. This contractile ring has been implicated as one of the major forces driving cytokinesis, but is probably not the sole major force driving cytokinesis. Kitanishi-Yumura and Fukui (1989) showed that actin and myosin dissociates in the cortex at prophase and reassembles to form unique cortical structures (including the contractile ring) at anaphase in a cyclic manner. Hiramoto (1990) reviewed physical changes in the cortex during cleavage and has given multiple examples of changes in cell stiffness before and during cleavage. He concludes that cell stiffness is mainly represented by the stiffness of the cell surface. Applying the elements of cytokinesis mentioned above, we can hypothesize one possible explanation for the fusion phenomenon observed between the first and second cleavages observed in embryos of *N. vectensis*.

As embryos enter their second anaphase transition, the cleavage furrows should form through reorganization of the actin and myosin in the cortex, and

begin to contract, initiating cleavage. If the contractile ring progresses far enough through the egg (before the forming cells entered prophase) to surpass the cell surface tension, then the embryo would remain in a four-blastomere state as the contractile ring dissociated. On the other hand, if the cells were to enter prophase prior to the contractile ring surpassing the cell surface tension, the actin and myosin of the contractile ring would reassociate with the cortex, cell surface tension would increase, and the blastomeres would appear to fuse, returning the cell membrane to its pre-cleavage condition. This hypothesis would suggest that the first cleavage stage was, in fact, not complete. If this line of reasoning is extended, the variation in blastomere fusions observed in *Nematostella vectensis* (Figure 9A-9D) could be explained. If the initial cleavage furrows progress at slightly different rates through the embryo, those surpassing the surface tension would lead to completed blastomeres whereas those that did not surpass the surface tension would lead to blastomeres which appear to fuse.

In addition to the actual mechanism responsible for the fusion phenomenon, the possible functions for this phenomenon remain unknown. Is the developmental program of *N. vectensis* simply "flexible" enough to tolerate such changes? Clearly, fusion does not hamper nor prolong subsequent developmental events in this anemone. Is this fusion event due to inadequate stores of cellular components required to successfully complete the initial cleavages, requiring several cell cycles to synthesize sufficient quantities? Or, is the fusion event a safety feature that ensures the proper allocation of nuclear and/or cellular components, or the proper synchronization of each blastomere of

the developing embryo? These hypotheses can be addressed by future research.

Our research has described the early development and associated blastomere fusion phenomenon in *Nematostella vectensis*. This is the first mention of the fusion phenomenon in over 50 years and the first time since Wietryzkowski (1914) that experiments have attempted to gain a better understanding of the phenomenon. Based on our results, we conclude that this phenomenon is not a mere compaction of blastomeres but is a fusion of blastomeres that results in the formation of a secondary syncytium. This fusion event can result in 1 of 4 possible embryonic patterns, each of which continues to undergo subsequent divisions to eventually form a viable planula, then juvenile and finally adult anemone.

**Table 1.** Data and parameter summary for all fertilizations.

| Fertilization Run[1] | Total Embryos Observed | Total Number Refused | Percent Refused | Fertilization Run Temp. (°C) | Sperm Concentration (Sperm/mm³) | Timed Fertilization (Run #) | Number of Embryos Timed |
|---|---|---|---|---|---|---|---|
| A | 34 | 4 | 11.7 | - | - | - | - |
| B* | 165 | 85 | 51.5 | 25 | 79 | - | - |
| C | 12 | 7 | 58.5 | 24 | 430 | - | - |
| D | 83 | 22 | 26.5 | 23 | 172 | - | - |
| E | 85 | 50 | 58.8 | 23 | 484 | +(1) | 32 |
| F | 136 | 44 | 32.4 | 23 | 35 | - | - |
| G* | 79 | 4 | 5.1 | 23 | 77 | +(2) | 79 |
| H* | 64 | 10 | 15.6 | 23 | 72 | +(3) | 16 |
| I* | 50 | 6 | 12.0 | 21 | 388 | - | - |
| J* | 41 | 7 | 17.1 | - | - | - | - |
| K | 104 | 27 | 26.0 | 21.5 | 47 | +(4) | 46 |
| L* | 109 | 3 | 2.7 | 24 | 110 | +(5) | 109 |
| M* | 38 | 7 | 18.4 | 23 | 301 | +(6) | 38 |
| N* | 32 | 21 | 65.6 | 24 | 737 | - | - |
| Totals: | | | | | | | |
| 14 runs | 1032 | 297 | 28.8 | | | 6 runs | 320 |

[1] *refers to runs in which specific fusion patterns were noted.

**Table 2.** Summary of Viability Experiment.

| Developmental Pathway | Number Isolated | Number Survived to Sexual Maturity | Sex Ratio (♀/♂) |
|---|---|---|---|
| Fused | 100 | 86 | 42/44 |
| Direct | 100 | 83 | 43/40 |

**Table 3.** Blastomere isolation and development. Blastomere numbers do not represent any particular blastomere across embryos, but denote individual blastomeres from each embryo.

(Isolate formed key: + = blastomere developed to this stage; - = blastomere did not develop to this stage. Juvenile key: tentacle no. = number of tentacles; Ab = abnormal juvenile; 1 = first mesenteric couple made up of 2 mesenteries; ½ = only one half of the first mesenteric couple [1 mesentery] present; - = no mesenteries present.)

| | | | Isolate formed | | | | Juvenile | |
|---|---|---|---|---|---|---|---|---|
| Embryo | Blastomere | Cleaved | Blastula | Gastrula | Planula | Juvenile | Tentacle No. | 1° Mesenteric Couple |
| 1 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 4 | 1 |
| | 3 | + | + | + | + | + | 2 | ½ |
| | 4 | + | + | + | + | - | | |
| 2 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | - | | | | |
| | 4 | - | | | | | | |
| 3 | 1 | + | + | - | | | | |
| | 2 | + | + | - | | | | |
| | 3 | + | + | - | | | | |
| | 4 | - | | | | | | |
| 4 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 3 | 1 |
| | 3 | + | + | + | + | + | 3 | ½ |
| | 4 | + | + | - | | | | |
| 5 | 1 | + | + | + | + | + | 3 | 1 |
| | 2 | + | + | + | + | + | 2 | ½ |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | + | - | | |
| 6 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | + | - | | |
| | 3 | - | | | | | | |
| | 4 | - | | | | | | |
| 7 | 1 | + | + | + | + | + | 2 | ½ |
| | 2 | + | + | + | + | + | 2 | ½ |
| | 3 | + | + | + | + | - | | |
| | 4 | - | | | | | | |
| 8 | 1 | + | + | + | + | + | 3 | 1 |
| | 2 | + | + | + | + | + | 2 | - |
| | 3 | + | + | + | + | + | 1 | ½ |
| | 4 | + | + | + | + | - | | |
| 9 | 1 | + | + | + | + | + | 3 | 1 |
| | 2 | + | + | + | + | + | 2 | - |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | - | | | |
| 10 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | - | | | |
| | 4 | - | | | | | | |
| 11 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | - | | | |
| | 3 | + | + | + | - | | | |
| | 4 | - | | | | | | |
| 12 | 1 | + | + | + | + | + | 2 | ½ |
| | 2 | + | + | + | + | + | 2 | ½ |
| | 3 | + | + | + | + | + | Ab | |
| | 4 | + | + | + | + | - | | |
| 13 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | + | Ab | |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | + | - | | |

Table 3. (Cont.)

| | | | Isolate formed | | | | Juvenile | |
|---|---|---|---|---|---|---|---|---|
| Embryo | Blastomere | Cleaved | Blastula | Gastrula | Planula | Juvenile | Tentacle No. | 1° Mesenteric Couple |
| 14 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 2 | 1 |
| | 3 | + | + | + | + | + | 2 | 1 |
| | 4 | - | | | | | | |
| 15 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | + | - | | |
| 16 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | + | - | | |
| 17 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | + | + | + | + | - | | |
| 18 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | Ab | |
| | 3 | + | + | + | + | + | Ab | |
| | 4 | - | | | | | | |
| 19 | 1 | + | + | + | + | + | 3 | 1 |
| | 2 | + | + | + | + | + | 2 | ½ |
| | 3 | + | + | + | + | - | | |
| | 4 | - | | | | | | |
| 20 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | - | | | | | | |
| 21 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | - | | | | | | |
| 22 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 4 | 1 |
| | 3 | + | + | + | + | + | 4 | 1 |
| | 4 | + | + | + | + | + | Ab | |
| 23 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 4 | 1 |
| | 3 | + | + | + | + | + | 3 | 1 |
| | 4 | + | + | + | + | - | | |
| 24 | 1 | + | + | + | + | + | Ab | |
| | 2 | + | + | + | + | - | | |
| | 3 | + | + | + | + | - | | |
| | 4 | - | | | | | | |
| 25 | 1 | + | + | + | + | + | 3 | ½ |
| | 2 | + | + | + | + | + | 2 | ½ |
| | 3 | + | + | + | + | + | 2 | ½ |
| | 4 | + | + | + | + | + | 2 | ½ |
| 26 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 4 | 1 |
| | 3 | + | + | + | + | + | Ab | |
| | 4 | + | + | + | + | - | | |
| 27 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 3 | 1 |
| | 3 | + | + | + | + | + | Ab | |
| | 4 | - | | | | | | |
| 28 | 1 | + | + | + | + | - | | |
| | 2 | + | + | - | | | | |
| | 3 | + | + | - | | | | |
| | 4 | + | + | - | | | | |
| 29 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 3 | ½ |
| | 3 | + | + | + | + | + | 3 | ½ |
| | 4 | + | + | + | + | + | 2 | ½ |

Table 3. (Cont.)

| Embryo | Blastomere | Cleaved | Isolate formed | | | | Juvenile | |
|---|---|---|---|---|---|---|---|---|
| | | | Blastula | Gastrula | Planula | Juvenile | Tentacle No. | 1° Mesenteric Couple |
| 30 | 1 | + | + | + | + | - | | |
| | 2 | - | | | | | | |
| | 3 | - | | | | | | |
| | 4 | - | | | | | | |
| 31 | 1 | + | + | + | + | + | 4 | 1 |
| | 2 | + | + | + | + | + | 3 | 1 |
| | 3 | + | + | + | + | + | 2 | 1 |
| | 4 | + | + | + | + | + | Ab | |
| 32 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | - | | | |
| | 3 | + | - | | | | | |
| | 4 | - | | | | | | |
| 33 | 1 | + | + | + | + | - | | |
| | 2 | + | + | + | + | - | | |
| | 3 | - | | | | | | |
| | 4 | - | | | | | | |

**Table 4.** Results of independent *t*-tests on all time interval data log-transformed to meet the assumptions of parametric statistics.

| Fertilization Run | *t*-Value | df | p |
|---|---|---|---|
| 1 | 0.260 | 19 | >0.70 |
| 2 | -0.293 | 38 | >0.70 |
| 3 | 0.448 | 12 | >0.60 |
| 4 | 0.056 | 39 | >0.90 |
| 5 | -2.665 | 102 | <0.05* |
| 6 | 0.496 | 32 | >0.60 |
| All | -0.308 | 252 | >0.70 |

* Time intervals of embryos undergoing fusion of blastomeres (n=3) fell within the range of time intervals observed in embryos not undergoing fusion (n= 101).

Graph 1. Comparison of mean time intervals between first and second cleavages of embryos that either progressed directly or underwent fusion prior to their second cleavage. Timing of the onset of the first two cleavages in 320 individual embryos over 6 fertilization runs was monitored. There was no significant difference in time between the 2 developmental pathways over all fertilization runs ($t$=-0.308, DF=252, p>0.70). Numbers in parentheses represent the total number of individual embryos for each developmental pathway in each of 6 fertilization runs.

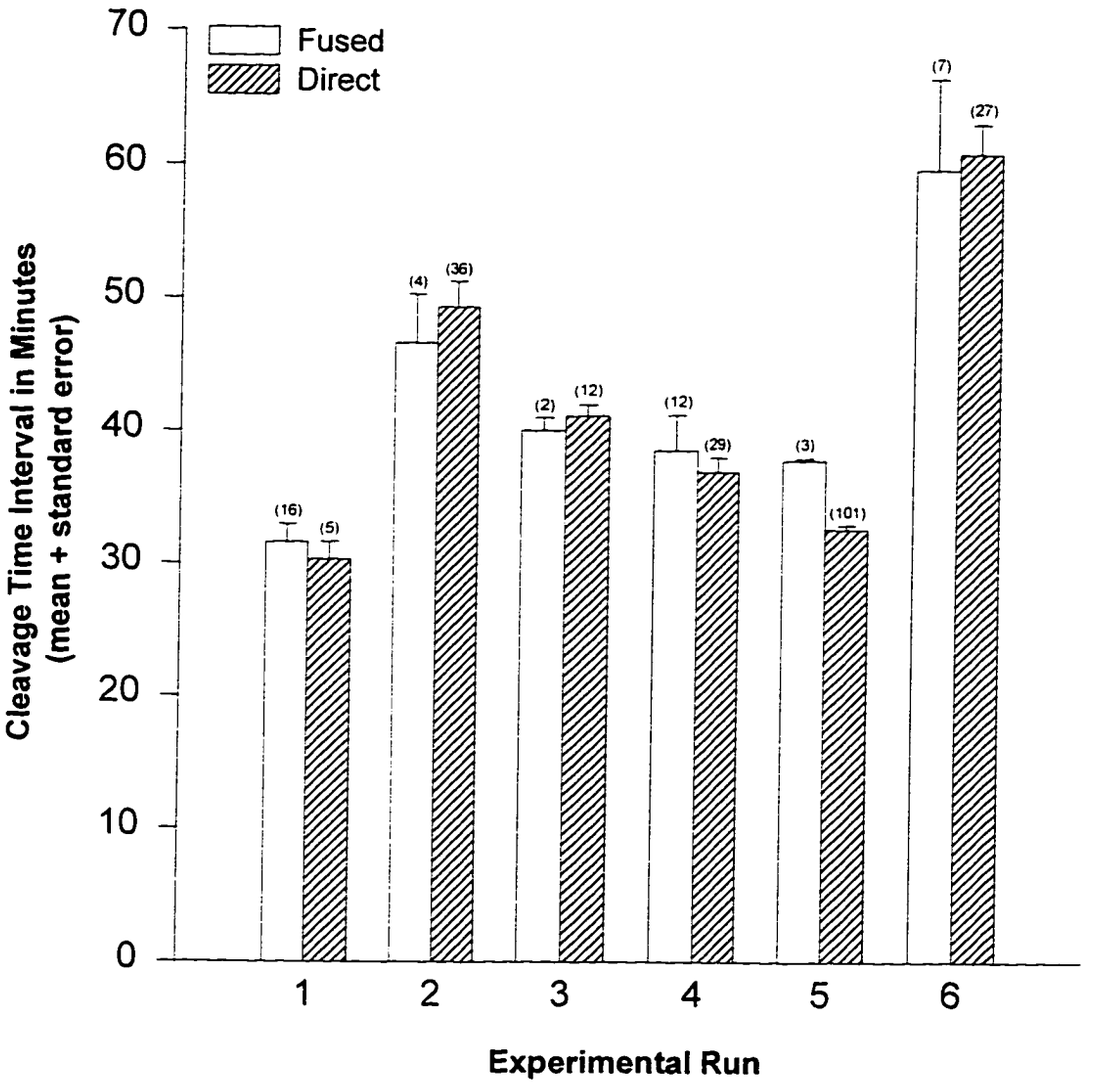


Figure 1. Scanning electron micrograph of an unfertilized *Nematostella vectensis* egg isolated from an egg mass. Scale bar = 40 µm.

Figure 2. Transmission electron micrograph of the surface of an unfertilized egg isolated from an egg mass. The egg surface is characterized by ~4.1 µm long microvilli (M). An approximately 2.7 µm layer of electron-dense cortical granules (CG) underlies the egg's plasma membrane. These cortical granules, ranging in diameter/long axis from 0.4 to 1.1 µm, are arranged 3-5 deep. Scale bar = 4 µm.

Figure 3. Light micrograph of a cross section of an egg in a newly spawned egg mass. Note that the pronucleus (arrow), surrounded by a small region of clear cytoplasm, is eccentrically located. Scale bar = 40 µm.

Figure 4. Confocal image of spindle of first karyokinesis in a developing embryo labeled with anti-β tubulin. Time is approximately 60 minutes after the addition of sperm to egg suspension.

Figure 5. Confocal image of late anaphase of second karyokinesis in a developing embryo labeled with anti-double stranded DNA antibody. Time is approximately 90 minutes after the addition of sperm to egg suspension.

Figure 6. Scanning electron micrograph of an embryo undergoing first cytokinesis. Note the simultaneous progression of two perpendicular, meridional cleavage furrows.

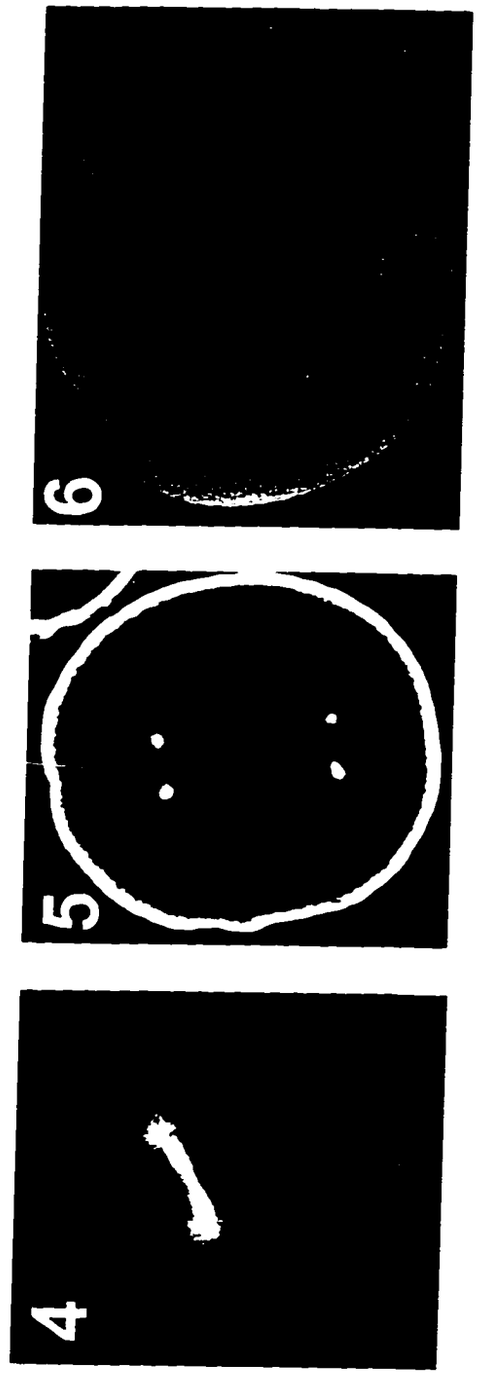
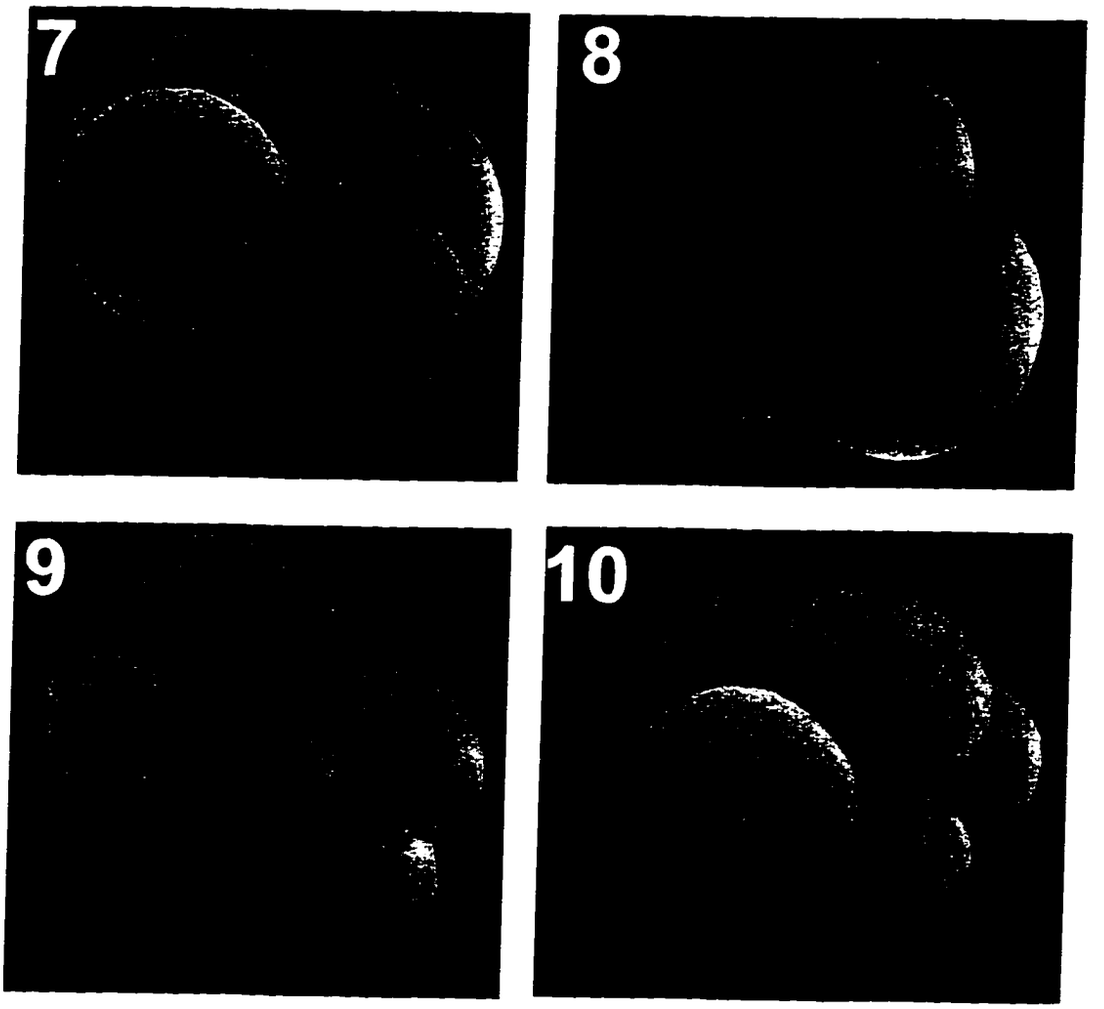


Figure 7. Scanning electron micrograph of a radially-arranged 4-celled embryo.

Figure 8. Scanning electron micrograph of a pseudospirally-arranged 4-celled embryo.

Figure 9. Scanning electron micrograph of the eight-cell stage of a radially-arranged embryo.

Figure 10. Scanning electron micrograph of the eight-cell stage of a pseudospirally-arranged embryo.



Of 1032 embryos observed, 297 exhibited either a complete or a partial fusion of their blastomeres following the 4-celled embryo stage. This fusion event preceded their next cleavage. These fusion events can be characterized as one of the following patterns:

Figure 11. Light micrograph of an embryo in which all 4 blastomeres fused prior to its next cleavage.

Figure 12. Light micrograph of an embryo in which 2 of 4 blastomeres fused prior to its next cleavage.

Figure 13. Light micrograph of an embryo in which 3 of 4 blastomeres fused prior to its next cleavage.

Figure 14. Light micrograph of an embryo in which 2 pairs of blastomeres fused prior to its next cleavage.

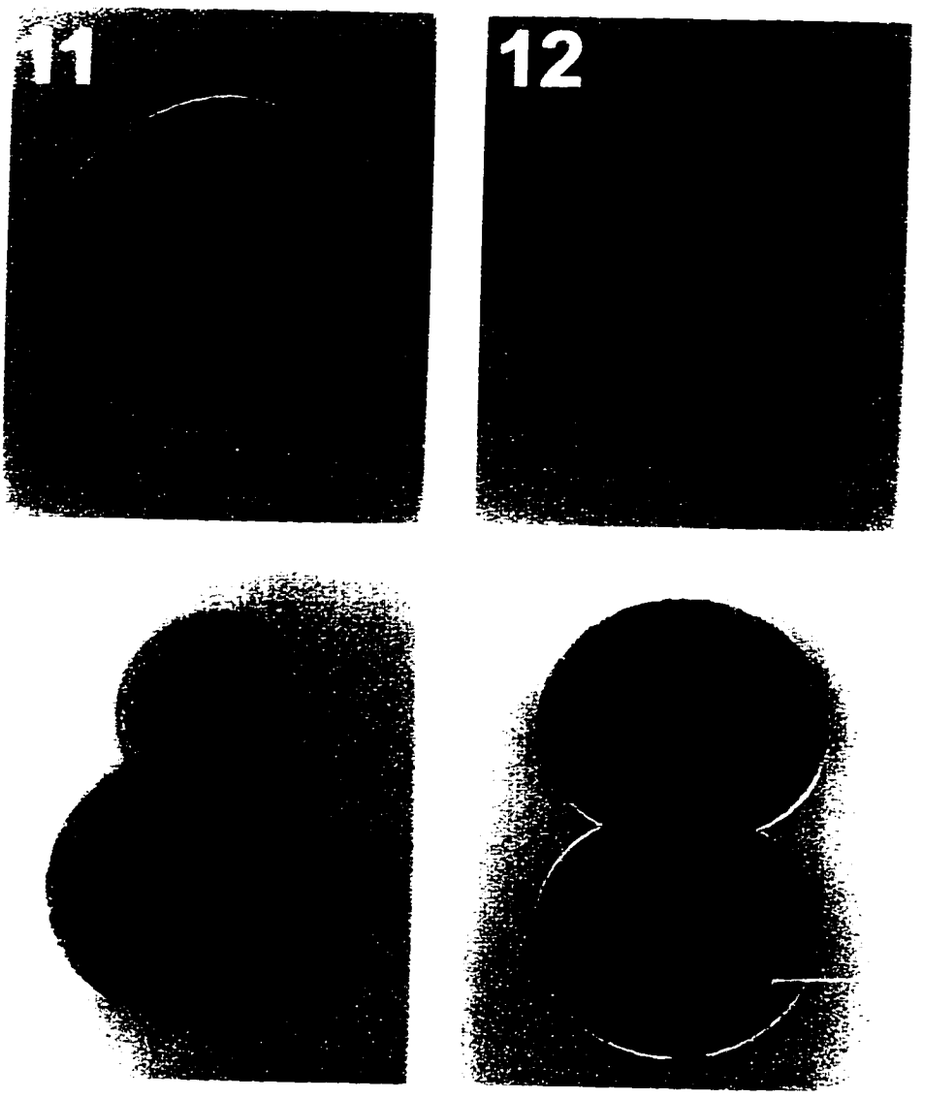
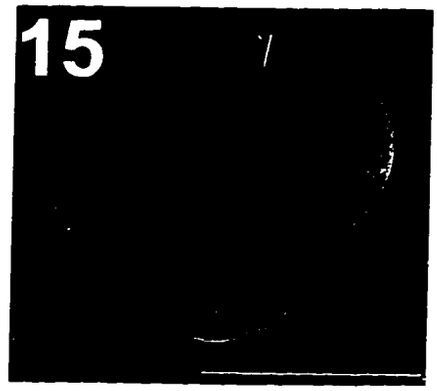


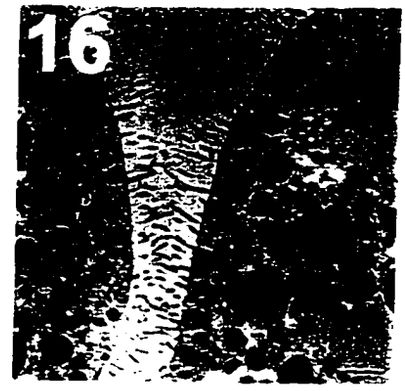
Figure 15. Scanning electron micrograph of a radial 4-cell stage embryo. Arrow marks one of the cleavage furrows. Scale bar = 100 µm.

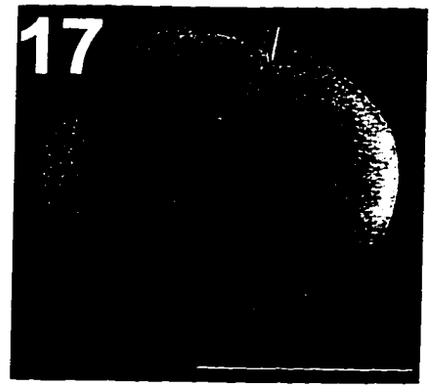
Figure 16. Transmission electron micrograph of a section through a cleavage furrow (arrow) between blastomeres of a 4-celled embryo. Scale bar = 3.0 µm.

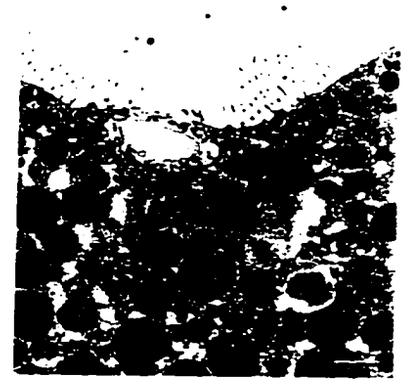
Figure 17. Scanning electron micrograph of a radial 4-cell stage embryo that was fixed undergoing complete fusion of blastomeres [notice the remnants of a cleavage furrow (arrow)]. Scale bar = 100 µm.

Figure 18. Transmission electron micrograph of a section through a cleavage furrow remnant in a 4-cell stage embryo undergoing fusion (notice the disruption/ displacement of the cortical granules and no evidence of membranes that were separating individual blastomeres prior to the fusion event. Scale bar = 3.0 µm.



## Literature Cited

**Alves, A.-P., B. Mulloy, J.A. Diniz, and P.A.S. Mourao. 1997.** Sulfated polysaccharides from the egg jelly layer are species-specific inducers of acrosomal reaction in sperms of sea urchins. *J. Biol. Chem.* **272(11):**6965-6971.

**Babcock, R.C., G.D. Bull, P.L. Harrison, A.J. Heyward, J.K. Oliver, C.C. Wallace, and B.L. Willis. 1986.** Synchronous spawnings of 105 scleractinian coral species on the Great Barrier Reef. *Mar. Biol.* **90:**379-394.

**Bayne, B.L., and J. Widdows. 1978.** The physiological ecology of two populations of *Mytilus edulis* L. *Oecologia (Berl.)* **37:**137-162.

**Beams, H.W., and R.G. Kessel. 1983.** 2. Cnidaria. Pp. 31-66 in *Reproductive Biology of Invertebrates, Vol. 1: Oogenesis, Oviposition, and Oosorption*, K.G. Adiyodi and R.G. Adiyodi, eds. John Wiley & Sons Ltd.,The Pitman Press, Bath.

**Booth, D.T. 1995.** Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. *J. Exp. Biol.* **198(1):**241-247.

**Bricelj, V.M., J. Epp, and R.E. Malouf. 1987.** Intraspecific variation in reproductive and somatic growth cycles of bay scallops *Argopecten irradians*. *Mar. Ecol. Prog. Ser.* **36:**123-137.

**Bunde, T.A., G.E. Dearlove, and S.H. Bishop. 1978.** Aminoethylphosphonic acid-containing glycoproteins: The acid mucopolysaccharide-like components in mucus from *Metridium senile* (L.). *J. Exp. Zool.* **206:**215-222.

**Campbell, R.D. 1974.** Cnidaria. Pp. 133-199 in *Reproduction of Marine Invertebrates, Vol. 1, Acoelomate and Pseudocoelomate Metazoans*, A.C. Giese and J.S. Pearse, eds. Academic Press, New York.

**Clark, W.H., Jr., and W.C. Dewel. 1974.** The structure of the gonads, gametogenesis, and sperm-egg interactions in the Anthozoa. *Am. Zool.* **14:**495-510.

**Crowell, S. 1946.** A new sea anemone from Woods Hole, Massachusetts. *J. Wash. Acad. Sci.* **36:**57-60.

**Dewel, W.C., and W.H. Clark, Jr. 1974.** A fine structural investigation of surface specializations and the cortical reaction in eggs of the cnidarian *Bunodosoma cavernata*. *J. Cell Biol.* **60:**78-91.

**Ducibella, T. And E. Anderson. 1975.** Cell shape and membrane changes in the eight-cell mouse embryo: Prerequisites for morphogenesis of the blastocyst. *Dev. Biol.* **47:**45-58.

**Duellman, W.E., and L. Trueb. 1994.** *Biology of Amphibians.* The Johns Hopkins University Press, Baltimore and London. 670 pp.

**Dunn, D.F. 1972.** Natural history of the sea anemone *Epiactis prolifera* Verrill, 1869, with special reference to its reproductive biology. Ph.D. Diss., Univ. of California, Berkeley. 187 pp.

**Dunn, D.F. 1975.** Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill, 1869. *Biol. Bull.* **148:**199-218.

**Eckelbarger, K.J., and R. Larson. 1992.** Ultrastructure of the ovary and oogenesis in the jellyfish *Linuche unguiculata* and *Stomolophus meleagris* with a review of ovarian structure in the Scyphozoa. *Mar. Biol.* **114(4):**633-643.

**Fautin, D.G., J.G. Spaulding, and F.-S. Chia. 1989.** Cnidaria. Pp. 43-62 in *Reproductive Biology of Invertebrates, Vol. IV, Part A,* K.A. Adiyodi and R.G. Adiyodi, eds. John Wiley & Sons, Chichester.

**Foerder, C.A., and B.M. Shapiro. 1977.** Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. *Proc. Natl. Acad. Sci. USA* **74:**4214-4218.

**Frank, P., and J.S. Bleakney. 1976.** Histology and sexual reproduction of the anemone *Nematostella vectensis* Stephenson 1935. *J. Nat. Hist.* **10:**441-449.

**Gemmill, J.F. 1920.** The development of the sea-anemones *Metridium dianthus* (Ellis) and *Adamsia palliata* (Bohad). *Phil. Trans. Roy. Soc. Lond.* **209B:**351-375.

**Gibson, G.D., and F.-S. Chia. 1994.** A metamorphic inducer in the opisthobranch *Haminaea callidegenita*: Partial purification and biological activity. *Biol. Bull.* **187:**133-142.

**Grémare, A., A.G. Marsh, and K.R. Tenore. 1988.** Short-term reproductive responses of *Capitella* sp. I (Annelida:Polychaeta) fed on different diets. *J. Exp. Mar. Biol. Ecol.* **123:**147-162.

**Grémare, A., A.G. Marsh, and K.R. Tenore. 1989.** Fecundity and energy partitioning in *Capitella capitata* type I (Annelida:Polychaeta). *Mar. Biol.* **100:**365-371.

**Griffin, F.G., W.H. Clark, Jr., J.H. Crowe, and L.M. Crowe. 1987.** Intracellular pH decreases during the *in vitro* induction of the acrosome reaction in the sperm of *Sicyonia ingentis. Biol. Bull.* **173:**311-323.

**Hand, C., and K.R. Uhlinger. 1992.** The culture, sexual and asexual reproduction, and growth of the sea anemone *Nematostella vectensis. Biol. Bull.* **182:**169-176.

**Hand, C., and K.R. Uhlinger. 1994.** The unique, widely distributed, estuarine sea anemone, *Nematostella vectensis* Stephenson: A review, new facts, and questions. *Estuaries* **17(2):**501-508.

**Hand, C., and K.R. Uhlinger. 1995.** Asexual reproduction by transverse fission and some anomalies in the sea anemone *Nematostella vectensis. Invertebr. Biol.* **114(1):**9-18.

**Harlow, E., and D. Lane. 1988.** *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor. 726 pp.

**Harvey, H.W. 1966.** *The Chemistry and Fertility of Sea Waters.* Cambridge University Press, London. 240 pp.

**Hiramoto, Y. 1990.** Mechanical properties of the cortex before and during cleavage. Pp. 22-30 in *Cytokinesis: Mechanisms of Furrow Formation during Cell Division,* G.W. Conrad and T.E. Schroeder, eds. *Ann. New York Acad. Sci.*, Vol. 582.

**Hyman, L.H. 1940.** *The Invertebrates: Protozoa through Ctenophora.* McGraw-Hill Book Co., New York and London. 726 pp.

**Hyman, L.H. 1967.** *The Invertebrates: Mollusca I.* McGraw-Hill Book Co., New York. 792 pp.

**Karp, D.R., J.P. Atkinson, and D.C. Shreffler. 1982.** Genetic variation in glycosylation of the fourth component of murine complement. *J. Biol. Chem.* **257:**7330-7335.

**Keller, S.H., and V.D. Vacquier. 1994.** The isolation of acrosome-reaction-inducing glycoproteins from sea urchin egg jelly. *Dev. Biol.* **162(1):**304-312.

**Kitanishi-Yumura, T., and Y. Fukui. 1989.** Actomyosin organization during cytokinesis: Reversible translocation and differential redistribution in *Dictyostelium. Cell Motil. & Cytoskel.* **12:**78-89.

**Kittredge, J.S., and E. Roberts. 1969.** A carbon-phosphorus bond in nature. *Science* **164:**37-42.

**Konat, G., H. Offner, and J. Mellah. 1984.** Improved sensitivity for detection and quantitation of glycoproteins on polyacrylamide gels. *Experientia* **40:**303-304.

**Koyato, S., K.M.S. Wimalasir, and M. Hoshi. 1997.** Structure of main saccharide chain in the acrosome reaction-inducing substance of the starfish, *Asterias amurensis*. *J. Biol. Chem.* **272(16):**10372-10376.

**Kumé, M., and Dan, K. 1968.** *Invertebrate Embryology*. Garland Publishing, Inc., New York and London. 605 pp.

**Laemmli, U.K. 1970.** Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* (Lond.) **227:**680-683.

**Larkman, A.U., and M.A. Carter. 1984.** The apparent absence of a cortical reaction after fertilization in a sea anemone. *Tissue & Cell* **16:**125-130.

**Lindsay, L.L., H. Yamasaki, J.L. Hedrick, and C. Katagiri. 1988.** Egg envelope conversion following fertilization in *Bufo japonicus*. *Dev. Biol.* **130(1):**37-44.

**McKillup, S.C., and A.J. Butler. 1979.** Modification of egg production and packaging in response to food availability by *Nassarius pauperatus*. *Oecologia (Berl.)* **43:**221-231.

**McMurrich, J.P. 1891.** Contributions on the morphology of the actinozoa. II. On the development of the hexactiniae. *J. Morph.* **4:**303-330.

**Mergner, H. 1971.** Cnidaria. Pp. 1-84 in *Experimental Embryology of Marine and Fresh-water Invertebrates*, G. Reverberi, ed. North-Holland Publishing Company, Amsterdam and London.

**Merrill, C.R., D. Goldman, S.A. Sedman, and M.H. Ebert. 1981.** Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211:**1437-1438.

**Miller, R.L. 1985.** Sperm Chemo-Orientation in the Metazoa. Pp.275-337 in *Biology of Fertilization, Vol. 2*, C.B. Metz and A. Monroy, eds. Academic Press, Inc., Orlando.

**Mozingo, N.M., V.D. Vacquier, and D.E. Chandler. 1995.** Structural features of the abalone egg extracellular matrix and its role in gamete interaction during fertilization. *Molec. Reprod. Dev.* **41(4):**493-502.

**Nishigaki, T., K. Chiba, W. Miki, and M. Hoshi. 1996.** Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. *Zygote* **4(3):**237-245.

**Norenburg, J.L., and J.M. Barrett. 1987.** Steedman's polyester wax embedment and de-embedment for combined light and scanning electron microscopy. *J. Electron Micro. Techn.* **6:**35-41.

**Nyholm, K.G. 1943.** Zur Entwicklung und Entwicklungsbiologie der Ceritharien und Aktinien. *Zool. Bidr. Uppsala* **22:**87-249.

**Nyholm, K.G. 1949.** On the development and dispersal of *Athenaria actinia* with special reference to *Halcampa duodecimcirrata* M. Sars. *Zool. Bidr. Uppsala.* **27:**465-506.

**Ottaway, J.R. 1974.** Resistance of juvenile *Actinia tenebrosa* (Cnidaria:Anthozoa) to digestive enzymes. *Mauri Ora* **2:**73-83.

**Parsons, T.R., Y. Maita, and C.M. Lalli. 1984.** Determination of Ammonia (Alternative Method). Pp. 14-17 in *A Manual of Chemical and Biological Methods for Seawater Analysis.* Pergamon Press, Oxford.

**Pearse, A.G.E. 1968.** *Histochemistry, Theoretical and Applied.* Little, Brown & Company, Boston. 759 pp.

**Qian, P-Y. 1994.** Effect of food quantity on growth and reproductive characteristics of *Capitella* sp. (Annelida: Polychaeta). *Invertebr. Reprod. Dev.* **26(3):**175-185.

**Qian, P-Y, and F.S. Chia. 1991.** Fecundity and egg size are mediated by food quality in the polychaete worm *Capitella* sp. *J. Exp. Mar. Biol. Ecol.* **148:**11-25.

**Reynolds, E.S. 1963.** The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17:**208-212.

**Sato, M., and K. Osanai. 1996.** Role of jelly matrix of egg masses in fertilization of the polychaete *Lumbrineris latreilli. Inverte. Repro. Dev.* **29(3):**185-191.

**Schroeder, T.C. 1973.** Cell constriction: Contractile role of microfilaments in division and development. *Am. Zool.* **13:**687-696.

**Shick, J.M. 1991.** *A Functional Biology of Sea Anemones.* Chapman and Hall, London. 395 pp.

**Siebert, A.E., Jr. 1973.** A description of the sea anemone *Stomphia didemon* sp. nov. and its development. *Pac. Sci.* **27**:363-376.

**Siebert, A.E., Jr. 1974.** A description of the embryology, larval development, and feeding of the sea anemones *Anthopleura elegantissima* and *A. xanthogrammica*. *Can. J. Zool.* **52**:1383-1388.

**Spaulding, J.G. 1972.** The life cycle of *Peachia quinquecapitata*, an anemone parasitic on medusae during its larval development. *Biol. Bull.* **143**:440-453.

**Spaulding, J.G. 1974.** Embryonic and larval development in sea anemones (Anthozoa: Actiniaria). *Am. Zool.* **14**:511-520.

**Spurr, A.R., 1969.** A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.

**Stephenson, T.A. 1928.** *The British Sea Anemones, Vol. 1.* The Ray Society, London. 148 pp. (+ plates).

**Strathmann, M.F. 1987.** Phylum Cnidaria, Class Anthozoa. Pp. 83-104 in *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast, Data and Methods for the Study of Eggs, Embryos, and Larvae.* University of Washington Press, Seattle.

**Tardent, P. 1978.** Coelenterata, Cnidaria. Pp. 69-415 in *Einleitung zum Gesamtwerk Morphogenetische Arbeitsmethoden un Begriffssysteme,* Reihe 1, Lieferung 1: A-I, F. Siedel ed. Gustav Fischer, Jena.

**Uchida, T. and F. Iwata. 1954.** On the development of a brood-caring actinian. *J. Fac. Sci. Hokkaido Univ.*, Ser. VI (Zool.). **12**:220-224.

**Wietryzkowski, W. 1910.** Quelques observations sur le developpement d'*Edwardsia beautempsii. Quatr. Bull. Soc. Zool. de France* (Paris). **35**:206-208.

**Wietryzkowski, W. 1914.** Recherches sur le developpement de l'*Edwardsia beautempsii. Bull. Acad. Sci.* Cracovie 1914, Ser.B., pp 782-816.

**Williams, R.B. 1979.** Studies on the nematosomes of *Nematostella vectensis* Stephenson (Coelenterata,Actiniaria). *J. Nat. Hist.* **13**:69-80.