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## A Descriptive Study of the Reproductive Biology of the Veined Rapa Whelk (*Rapana venosa*) in the Chesapeake Bay

Erica S. Westcott

*College of William and Mary - Virginia Institute of Marine Science*

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A DESCRIPTIVE STUDY OF THE REPRODUCTIVE BIOLOGY OF THE  
VEINED RAPA WHELK (*RAPANA VENOSA*) IN THE CHESAPEAKE BAY

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A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

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by

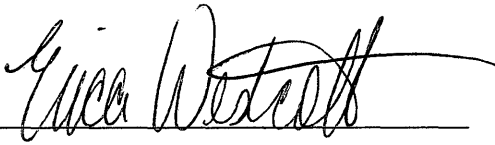
Erica S. Westcott

2001

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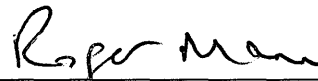
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Erica S. Westcott

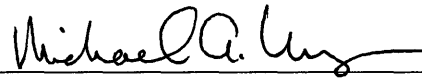
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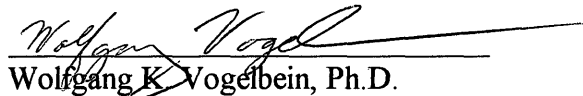
Roger L. Mann, Ph.D.  
Committee Chairman / Advisor



Gregory M. Ruiz, Ph.D.



Michael A. Unger, Ph.D.



Wolfgang K. Vogelbein, Ph.D.

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## ABSTRACT

Successful introduction of a species requires the establishment of a viable reproductive population in the receptor environment. In the event that animals become established post-introduction, there may be temporal and spatial variation within the population in the ability to successfully reproduce. The functional reproductive range of the invader may potentially be limited by the physical conditions of the receptor environment. This study describes various reproductive aspects of *Rapana venosa*, including gametogenesis, which is used as a surrogate for egg laying along an environmental and spatial gradient. Laboratory populations of animals collected from Chesapeake Bay and maintained at local temperatures and salinities have been observed mating from October through July. Field collections of adult rapa whelks (shell length >80 mm) were made year-round using opportunistic sampling based on commercial fisheries where this animal is bycatch. Representative individuals were sampled from the extreme ends of the environmental and spatial gradient of the observed population distribution. Individual animals were sacrificed and examined for gross external morphology as an indicator of sex ration and incidence of imposex. Histological analyses were used to describe progression of gametogenesis in individual animals. The observed relationship between gametogenesis and water temperatures in animals from the Chesapeake Bay is consistent with a.) Previously described seasonal reproductive activity in a native (Korean) population and b.) Laboratory observation of egg laying from mid-May through mid-August and field collections of egg masses in Chesapeake Bay. Collectively, these data sets indicate that the Chesapeake Bay population of Rapa whelks is successfully completing gametogenesis and egg laying throughout the range of collection.

A DESCRIPTIVE STUDY OF *RAPANA VENOSA*

## INTRODUCTION

### Non-Native Species Invasions

Establishment of an invasive, non-native species in a receptive location is dependent upon numerous physical and biological factors. Physical conditions, such as salinity and temperature, in marine and estuarine locations must be within the tolerance range of the organism. The invading organism must also be able to withstand challenge from local predators, parasites, and diseases. Reaching a size refuge from predation or having a cryptic habitat are possible ways of avoiding predation. The ability to reproduce and disperse once the organism arrives is critical to establishment in its new environment. Opportunistic, “r-selected” species which have short generation times may be favored in this respect (Pianka, 1970).

The introduction of non-native species (also termed exotic or non-indigenous) into an environment, either on land or in water, whether purposefully or accidentally, can have detrimental effects. Entire ecosystems can be affected if an introduced species reproduces successfully and becomes established. Extinction of a native species is possible through interspecific competition if resources are consumed by the invader, or if the invader preys upon native species. Non-native species invasions have been the cause of economic or ecological problems and health risks (Hamer et al., 1998). A few examples of invasions by non-native species which have caused notable changes in recipient ecosystems include zebra

mussels (*Dreissena polymorpha*) in the Great Lakes, brown tree snakes (*Boiga irregularis*) in Guam, and kudzu (*Pueraria lobata*) in the United States.

The case of the zebra mussel is one of the more comprehensively described non-native species invasions. Ballast water was the mechanism responsible for the initial introduction (Carlton, 1993). *D. polymorpha* was first discovered in the Great Lakes during 1988, and it is still spreading throughout North America (Kastner, 1997). The zebra mussel is indigenous to the Ponto-Caspian region of western Russia, and its spread through Europe was facilitated by man-made canals (Kastner, 1996). The zebra mussel is a great nuisance to aquaculturists, but much more so for engineers in municipal water supplies and power generator facilities, because it clogs pipes, filters, and other equipment. Dense zebra mussel populations have the ability to deplete the base of the food chain by consuming enormous quantities of plankton. The mussel is also an intermediate host for a parasitic trematode (*Bucephalus polymorphus*), and the trematode has been linked to fish kills in Europe (Kastner, 1997). *D. polymorpha* is known to compete with and exclude many freshwater Unionid mussels native to North America (Mackie, 1991; Baker and Hornbach, 1997). Zebra mussel population growth does not appear to be limited by predators in North America.

The brown tree snake is suspected of arriving in Guam in ship cargo from the New Guinea area. This snake is native to Indonesia, the Solomon Islands, and parts of Australia. Sightings in Guam began in the early 1950s, and by 1968 the snakes had almost completely dispersed throughout the island. There is no natural population control for the snake on Guam, and they are considered to be responsible for the extinction of 12 species of birds and the near extinction of others. Pigs and monitor lizards on the island do eat the brown tree

snake, but they only consume a small fraction of the total population present. In addition to being a general nuisance, and consuming domestic poultry and small pets, the snakes are also responsible for a significant number of power failures on Guam. As this reptile is arboreal, it is not uncommon for the snakes to be electrocuted when they climb on power lines. Brown tree snakes can reach lengths of up to eight feet (2.4 m) and can weigh up to five pounds (2.5 k). There is a risk that these snakes may spread to other nearby Pacific islands (Fritts, 2000).

Kudzu, my final example of a successful invading species, is a fast-growing perennial vine that was introduced to the United States from Japan in 1876. The vine was planted extensively across the southeastern U.S. to stabilize hillside erosion and has since become a serious weed problem. Kudzu invades forested areas and smothers trees. The vine spreads via stolons and rhizomes, thus it is very difficult to control unless the root system is eliminated. Use of kudzu as ground cover was ceased in 1953, when it became recognized as a pest weed (Plant Conservation Alliance website: <http://www.nps.gov/plants/alien/fact/pulo1.htm>).

#### Invasion of the Chesapeake Bay by *Rapana venosa*

The Rapa whelk (*Rapana venosa*), the focus of this study, is a non-native gastropod that has recently been found in the lower Chesapeake Bay (Harding and Mann, 1999). Its native range consists of the Sea of Japan and surrounding waters, including the Gulf of Bohai, Yellow Sea, and East China Sea (Tsi et al., 1983; Chung et al., 1993; Zolotarev, 1996). *R. venosa* was also introduced to the Black Sea, where it was first discovered in 1947. By 1972, the Rapa whelk had become established along almost the entire Black Sea coastline as well as the southern part of the Sea of Azov (Zolotarev, 1995). Prior to the Rapa whelk's

introduction in the Black Sea, predatory gastropods had only a modest role in structuring the molluscan community; however, the elevated predation pressure caused by *R. venosa* was associated with near local extinction of native *Ostrea edulis*, *Pecten ponticus*, and *Mytilus galloprovincialis* (Chukhchin, 1984). Zolotarev (1995) suggests that *R. venosa*, along with other species introduced to the Black Sea, were able to become dominant due to “their high adaptive capability and the instability of the Black Sea ecosystem, the latter being due to its few species, low level of competitive interactions, and constant growth of diverse anthropogenic loads.”

A recent classification by Kool (1993; Table 1) places *R. venosa* in the subfamily of Rapaninae (Gray, 1853), which precedes the subfamily Thaidinae given by Jousseaume (1888).

Phylum	Mollusca
Class	Gastropoda
Order	Neogastropoda
Superfamily	Muricoidea
Family	Muricidae
Subfamily	Rapaninae
Genus	<i>Rapana</i>
Species	<i>venosa</i>

Table 1: Classification of *Rapana venosa* according to precedence (Kool, 1993)



The Rapa whelk was first discovered in the Chesapeake Bay in 1998 during a VIMS trawl survey. It is not known if there has been only one introduction or multiple introductions of *R. venosa* to the Chesapeake Bay. Most likely, larvae were transported to the Chesapeake Bay via the ballast water of large commercial or military transoceanic ships that traveled from the Black Sea. There is concern that the Rapa whelk has or will become established in the Chesapeake Bay (Harding and Mann, 1999).

The complete distribution of *R. venosa* within the Chesapeake Bay is unknown at this time because most individuals are caught as bycatch of commercial fishing, which does not uniformly exploit the entire bay area. Some of the various gears which catch *R. venosa* include crab pots, crab dredges, and patent tongs used in the hard clam (*Mercenaria mercenaria*) fishery. The efficiency for catching *R. venosa* is unknown for these various gear types. Use of animals from bycatch in this manner is considered to be “opportunistic sampling” performed by the watermen, since a specific sampling scheme by time and region is not applicable. To encourage reporting of specimens collected as bycatch a bounty system is employed by VIMS researchers, but returns are probably not 100%. Other factors also contribute to the difficulty of designing a study with regular sampling, predetermined sample size, and application of typical statistical approaches. Adult whelks are difficult to locate because they frequently bury in bottom sediments. The potential use of SCUBA divers or underwater cameras to locate and count whelks directly is hampered by poor visibility, and in some locations, high shipping traffic. Sidescan sonar is one option under investigation to locate *R. venosa* within the Chesapeake Bay, although studies to date are limited.

Despite difficulties in locating the invader, the total number of Rapa whelks collected and reported from the Chesapeake Bay has risen from approximately 600 animals in mid 1999 to over 2300 animals by mid 2001. The documented range of *R. venosa* in the Chesapeake Bay is from the mouth of the Rappahannock River in the north, to the Chesapeake Bay Bridge Tunnel in the southeast, to just above the James River Bridge in the southwest, to the Lafayette River in the south. The Rappahannock and York River specimens were less abundant.

Collection methods were size selective because of the gear types used (crab dredge, patent tongs), so population demographics could not be viewed as evidence of lack of very small individuals. Individuals collected and used in this study vary in size from 86 mm to 168 mm SL (Table 2), with an average SL of 143 mm. The thick shell of *R. venosa* and probable rapid growth in its first and second year suggested this species might reach a size refuge from potential local crab and fish predators at a young age. The observed range of sizes suggested multiple cohorts, originating either from multiple introductions or active and successful breeding of the older cohorts - probably the latter given evidence of egg laying in the field and laboratory populations.

### A Review of Gastropod Reproduction

Several types of reproductive strategies are employed within the gastropods, all of which can be described by r- and K-selection criteria (Pianka, 1970). Organisms which are r-selected tend to have rapid development, reproduce early, bear many offspring, have a small body size, and have a short life span. They may also be semelparous, only spawning once

within their lifetime. K-selected organisms generally have slower development, reproduce later in life with fewer offspring, show iteroparity (i.e. spawning more than once in a lifetime), have a larger body size and a longer life span than those that are r-selected. Gastropods can either be monoecious (having both sexes within the same organism; hermaphroditic) or dioecious (having two separate sexes), and “most prosobranchs have separate sexes with little or no morphological distinction between males and females except for the reproductive organs” (Hughes, 1986).

Some gastropods are capable of reproduction at a very young age, while others must wait until several years of age before they are sexually mature. Age at first sexual maturity and life expectancy are important in estimating how many potential offspring an individual may produce in its lifetime. For example, the knobbed whelk (*Busycon carica*), a gastropod native to the Chesapeake Bay, does not become sexually mature until it has reached a relatively large size. Males reach sexual maturity at ~100mm, or 8 years of age, and females reach sexual maturity at ~150 mm, or 12 years of age (Kraeuter et al., 1989; Walker, 1988). This leaves the knobbed whelk comparatively little time for reproduction, based on an estimated average life span of 12-14 years. The waved whelk (*Buccinum undatum*), from the Gulf of St. Lawrence, Quebec, also becomes sexually mature later in life. Males reach sexual maturity within a size range of 49 to 76 mm, or approximately 5 to 6 years of age. Females attain sexual maturity at larger sizes of 60 to 81 mm in length, which is estimated to be 7 years of age (Gendron, 1992). Based upon the relationship between size and age, this potentially allows *B. undatum* 5 years for reproduction between attaining sexual maturity and death.

The observation that gastropod males are maturing before females is considered strong evidence for protandrous sexual development (i.e. sexually developing as a male first). Both Magalhaes (1948) and Walker (1988) have suggested that *B. carica* could be protandrous. *Crepidula fornicata* is a classic example of protandry; the adults are sessile and form semipermanent stacks in which copulation occurs. The top of the stacks are composed of younger males, which are in turn attached to larger females on the bottom of the stack. Sex change occurs in the bottom-most male (Collin, 1995).

#### Reproduction in *Rapana venosa*

*Rapana venosa* is an r-selected, dioecious gastropod (Chung and Kim, 1997), and it is not believed that these animals change sex (i.e. exhibit protandry/protogyny) at any point during their lifetime. The sex of the animal can usually be determined by the presence of a penis (Chung et al., 1993). All animals caught in the Chesapeake Bay since 1999 are apparently capable of reproduction (Harding and Mann, 1999). The size of *R. venosa* at first reproduction is between 35-78 mm (Chukhchin, 1984), and the largest specimen on record, 212 mm (Japan, 1968, shell collectors data), was also capable of reproducing. Chukhchin (1984) has described length-at-age relationships for *R. venosa* in the Black sea, but as of yet, not enough data have been collected to create an age-growth curve for *R. venosa* in the Chesapeake Bay (Roger Mann, personal communication). This information would be useful in determining age at first reproduction, and also fecundity-at-age relationships. Harding and Mann (1999) used *Busycon carica* age-length relationships as a comparison to conservatively estimate the age of *R. venosa* specimens in the Chesapeake Bay, and current length

measurements are being taken on *R. venosa* juveniles raised in the laboratory from the larval form to gain a better understanding of length-at-age relationships.

The reproductive cycle of *R. venosa* begins with gametogenesis. The gonads are located on the surface of the digestive gland in both males and females (Chung et al, 1993). *R. venosa* copulate and the female effects sperm storage. Chung et al. (1993) used histological methods to describe four male gonadal stages in *R. venosa* from the Korean population. These are: 1) the developing stage, 2) the mature stage, 3) the copulation or spent stage, and lastly, 4) the recovery stage. These stages are not distinct, but rather there is a continuum between the stages. Korean *R. venosa* females have been described using five gonadal stage definitions by Chung et al. (1993): they have both early and late developing stages, but they are otherwise similar to the male whelks. The early stage is characterized by smaller oocytes (60-70  $\mu\text{m}$ ), and the later stage by larger oocytes (120-150  $\mu\text{m}$ ) with yolk-rich cytoplasm. Spermatogenesis and oogenesis occur anywhere between September to March. Males reach the mature stage first, during the time frame of September through July. Females mature later during November through July, and the mature oocytes range from 190-240  $\mu\text{m}$ . At this time, spermatozoa and oocytes are fully formed.

Copulation in Korean *R. venosa* begins as early as February and can continue until June. *R. venosa* from the Chesapeake Bay population have been observed to form “mating piles” in laboratory holding tanks during this time period (personal observation). A male will insert his penis into the mantle cavity of the female and deposit a quantity of sperm. Females most likely mate with multiple males, but it is not known if she is selective as to which males’ sperm effects fertilization of her eggs, nor what her selection criteria might be. Female *R.*

*venosa* from the Korean population are found in the spent stage from late April to late July (Chung et al., 1993).

When female *R. venosa* spawn, they lay a mass of egg capsules, usually on a hard substrate and over a period of several hours to several days. When first laid, egg capsules are sickle-shaped and bright yellow in color, but they become pale yellow-white after a period of time. Larvae develop in the capsules: those capsules that contain well developed larvae are black, but those capsules which are dead are purple (Chung et al., 1993). In captivity, deposited egg capsules have been observed on tank walls, shells of other *R. venosa* in the tank, and shells of *Mercenaria mercenaria* which were provided to the whelks as potential prey items. Chung et al. (1993) counted the number of capsules in a mass (90-113) and also the number of eggs per capsule (average = 1096). Based on Chung et al.'s (1993) data from the Korean population, multiplying the number of capsules laid by the number of eggs per capsule provides a fecundity estimate of 88,560 to 140,233 total eggs for one mass. It is probable that a female whelk will lay considerably more than one egg mass per breeding season, and Chung et al. (1993) have estimated that a female could lay as many as 320,000 to 450,000 eggs per season!

Chung et al. (1993) observed males from the Korean *R. venosa* population entering the recovery stage first during the months of April to October. Any spermatozoa remaining from prior maturation have degenerated and new spermatogonia will form. After the females have completed their egg laying, they enter the recovery stage. Recovering individuals have been found from June through November. Those oocytes that were not discharged undergo cytolysis and new oogonia subsequently appear.

Once the egg cases have been deposited, the fertilized eggs immediately begin to develop. Actively swimming trochophore (Greek *trokhos*: wheel; Greek *-phoros*: bearing) larvae are visible after about two days post-fertilization. Shelled larvae are visible after six days, and veligers (Latin *velum*: veil; Latin *gerere*: to bear) are visible eight days after fertilization. Approximately 14 to 17 days after fertilization (at 18-25°C), the larvae are ready to hatch out of the egg cases. There is a small pore at the tip of each capsule that opens and allows larvae to escape (Chung et al., 1993). Most *R. venosa* larvae cultured at VIMS were assisted in hatching by carefully snipping the ends off of the egg capsule and allowing the larvae to swim out. Larval development times to metamorphosis were different from those reported by Chung et al. (1993). Larvae from Chesapeake Bay specimens required 14 to 28 days before they were ready to hatch. Development time appears to be closely linked with temperature.

After hatching, larvae are planktonic for 28 to 84 days depending on temperature and food availability. Such an extended period in the water column gives *R. venosa* larvae a very broad dispersal capability. This agrees with the proposed method of transit by ship ballast water from Black Sea waters to the Chesapeake Bay (Harding and Mann, 1999). *R. venosa* larvae swim and feed on algae and other organic particles with their 4-lobed velum during their planktonic phase. Larvae have been cultured in the laboratory on a mixed algal diet of *Pseudoisochrysis paradoxa*, *Chaetoceros gracilis*, and *Tetraselmis sp.* When the larvae are ready to metamorphose they preferentially settle onto a hard substrate, and after a few days, shed their velum.

At metamorphosis the velum is discarded, a foot develops to assist crawling, and the juvenile experiences a drastic change of living habits. Juveniles no longer rely on filtering particles out of the water, but graze on biofilm, bryozoa, and eventually larger prey as they mature (Chung et al., 1993; Mann and Harding, 2000). They have been observed in the laboratory to consume barnacles (*Balanus*), oyster spat (*Crassostrea virginica*), small mussels (*Mytilus edulis* and *Geukensia demissa*), and small clams (*Mya arenaria*). After the juvenile has reached adulthood and reproduced, the life cycle is complete.

#### Tributyltin (TBT) and Imposex in Other Gastropods

TBT-based marine antifouling paints were introduced in the 1960s and became popular in the 1970s when they were found to be extremely effective, especially when compared to the performance of older copper based paints (Huggett et al., 1992); however, TBT-based paints were also quite detrimental to non-target organisms. Some of the first noticeable effects were seen in Pacific oysters such as shell thickening and deformations of the shell (Huggett et al., 1992), and in various species of gastropods which exhibited signs of “imposex” (Ellis and Pattisina, 1990; Gibbs et al., 1988; Huggett et al., 1992). The term “imposex” was coined by Smith (1971) from observing the *imposition* of male *sexual* characters on females, such as the presence of a penis and vas deferens. Once such problems became evident, several countries placed bans or severe restrictions on the use of TBT based antifouling paints to reduce environmental contamination levels. TBT has been reviewed in a number of subject areas, including biological-chemical interactions, biochemistry and



metabolism of TBT, but only those references directly related to imposex and reproduction in gastropod populations have been cited in this study.

United Kingdom estuaries received a large amount of attention with respect to TBT pollution, the effects on various mollusc populations, and reduction of TBT contamination over time. Gibbs et al. (1988) performed an extensive two year study on the dog whelk (*Nucella lapillus*), an intertidal gastropod common to the UK, to determine the effects of TBT in the field as well as in the laboratory. They found that TBT concentrations of less than 1 ng Sn/L (in water) initiated a condition known as pseudohermaphroditism or imposex. Affected animals were mostly found near marinas and harbors where boat traffic was high. At higher concentration levels of TBT (>3-5 ng Sn/L), the effects of imposex were severe enough to sterilize female gastropods. This occurs when the vas deferens tissue blocks the release of egg capsules. In some cases, the capsule gland ruptures and can result in the death of the female. Populations of *N. lapillus* along the UK coast were drastically shrinking, and in some local regions disappeared completely (Bryan et al., 1986). Gibbs and Bryan (1986) noted in an earlier study that *N. lapillus* does not have a planktonic larval stage, so the capability for dispersion was very limited. Unaffected populations could not compensate very rapidly for the losses incurred by TBT. In essence, the dog whelk was threatened with local extinction by TBT contamination.

Imposex has been shown to be a graded response in some gastropods. One example is described by Gibbs et al. (1987), while using the dog whelk as an indicator in TBT contamination studies. In addition to using relative penis size ( $RPS = (\text{mean length of female penis}^3 / \text{mean length of male penis}^3) \times 100$ ) as a measure of imposex (Bryan et al., 1986), a vas

deferens sequence (VDS) index was also categorized in six stages (Gibbs et al., 1987): Normal females with no signs of a penis or vas deferens are equivalent to Stage 0. Commencement of vas deferens growth near the genital papilla constitutes Stage 1. In Stage 2 females, penis development is initiated, and vas deferens growth near the genital papilla continues. Stage 3 females have a small penis, and vas deferens growth is initiated at the base of the penis. The vas deferens fuses during Stage 4, and imposex female penis length is comparable to that of a male. Stage 5 females have vas deferens tissue overgrowing the genital papilla, and hyperplasia may also occur. A Stage 6 imposex female is considered to be “grossly affected,” where the capsule gland lumen contains aborted egg capsules, and the female is effectively sterilized.

A note should be made that not all species conform exactly to these 6 stages. Mensink et al. (1997) did not observe blockage of the female genital pore in *Buccinum undatum*, even in advanced stages of imposex. Oviduct blockage was also not observed by either Bryan et al. (1989) working with *Ilyanassa obsoleta* or Bryan et al. (1993) working with *Nassarius reticulatus*. One dog whelk population near the north Kent coast in the UK, appeared exempt from these losses due to the fact that the males had a genetic disorder known as Dumpton Syndrome (DS) (Gibbs, 1993). The males in this population either have an undeveloped or only partially developed genital system. Similarly, the females in the population did not appear to be fully affected by imposex as is usually the case in a TBT-contaminated environment. The few females who developed a vas deferens did not develop a penis. Female dog whelks with DS are able to mate with males which are not afflicted, but only this particular enclave has escaped extinction.

As concern about TBT in the environment grew, a number of scientists proposed that some gastropod species may serve as indicators of the presence and severity of TBT contamination. Ellis and Pattisina (1990) found imposex to be affecting several shoreline species of molluscs on the Pacific coast of Canada and in southeast Asia. They correlated imposex with the presence of TBT coming from boat and ship traffic, and supported the use of the six category coded system, developed by Gibbs et al. (1987), for distinguishing phases of vas deferens development. In this way, imposex in selected stenoglossan gastropod species could then be used to “rate” the levels of global TBT contamination. Bryan et al. (1993) examined *Nassarius reticulatus* as a suitable indicator of TBT pollution in southwest England before and after restrictions on TBT use had been put in place. They determined that *N. reticulatus* was a “useful alternative” to *N. lapillus*, though tissue analysis provided a better indication of TBT levels than observation of imposex within the population. In Australia, the use of *Thais orbita* as a bioindicator of TBT was studied (Foale, 1993; Wilson et al. 1993). Tester and Ellis (1995) proposed that a simple system for measuring imposex frequency in gastropod populations anywhere in the world could be used to monitor the effectiveness of TBT controls. They examined four different species found on Southern Vancouver Island – *Nucella emarginata*, *Nucella lamellosa*, *Nucella canaliculatis*, and *Searlesia dira* – and concluded that this method of measuring imposex frequency is “the simplest, least expensive, and broadest scale” method for monitoring the effectiveness of TBT controls. Approximately 118 species of gastropods are known to have been affected with imposex (Oberdorster et al., 1998).

### Imposex in *Rapana venosa*

One of the most prominent abnormalities observed in female *R. venosa* specimens taken from the Chesapeake Bay is imposex (Table 6); however, reproduction in imposex female *R. venosa* does not appear to be compromised. Whether or not TBT is the actual cause of imposex in these animals has yet to be determined, but the possibility exists. The lower Chesapeake Bay, where the majority of the introduced gastropods have been found, is subject to heavy commercial and military ship traffic as well as recreational boat traffic. TBT concentrations differ from 2-6 ng/L in areas where the Rapa whelk has been found (Unger, 2001). The effects of TBT exposure on *R. venosa*, including localized, widespread, and long term effects are unknown. Imposex could potentially play an important role in determining the success of reproduction, which in turn has an impact upon the overall invasion performance of *R. venosa*. Imposex has also been reported in both Korean and Black Sea *R. venosa* populations, but there are no data currently available on imposex frequency.

### Objectives

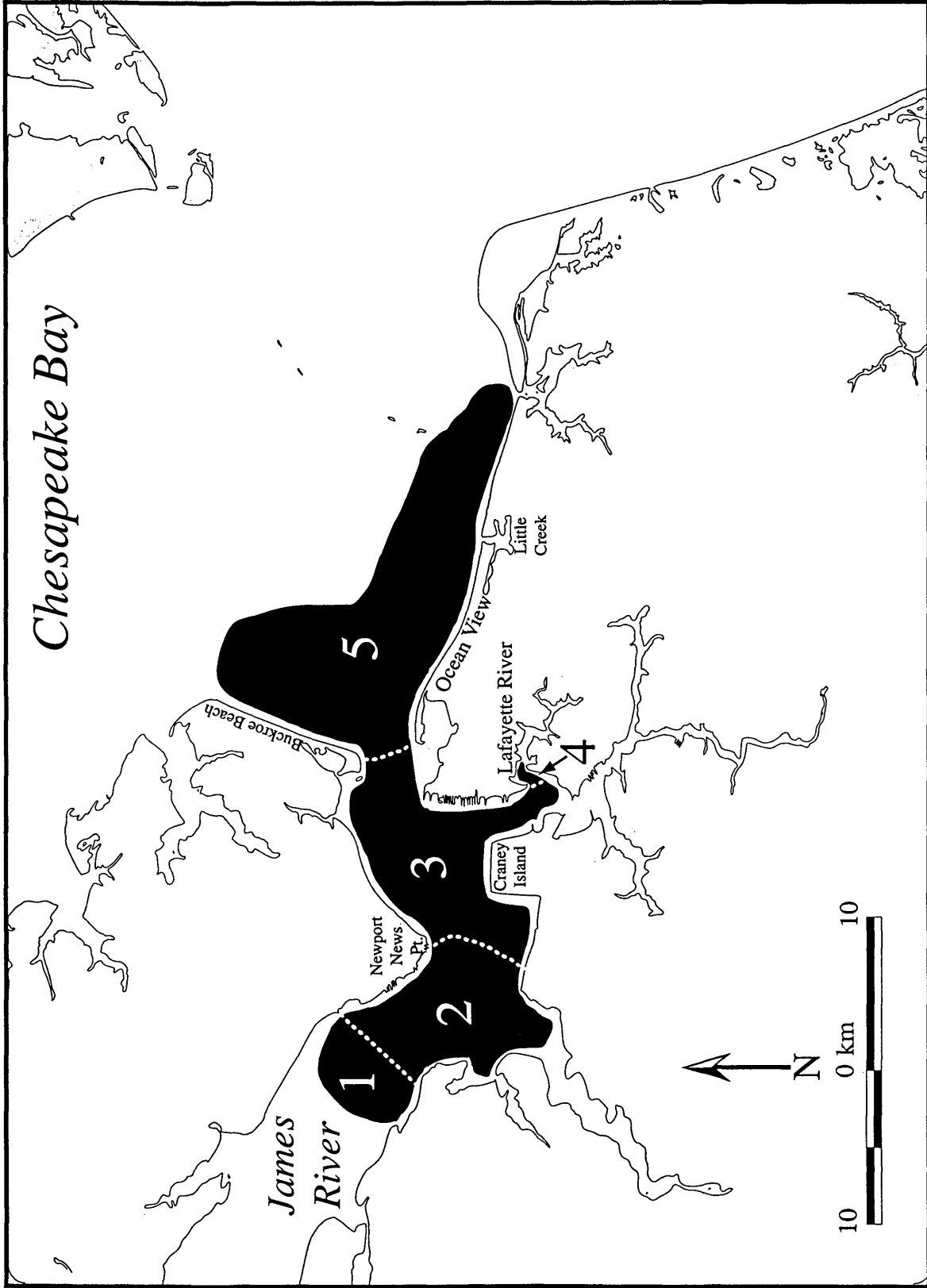
The topic of this investigation is to examine the spatial and temporal variation of reproduction (gametogenesis) in Chesapeake Bay *R. venosa*. The objectives of this study are to 1) stage and describe the reproductive state of gonads taken from Chesapeake Bay *R. venosa* specimens. 2) Elucidate the anatomy of the reproductive system for males, females and imposex females, and examine key reproductive characteristics such as sex ratio, gonadal synchrony, and imposex frequency. 3) Describe gonadal abnormalities encountered during the study.

## MATERIALS AND METHODS

### Animal collection

Animals were collected by commercial watermen from eight different locations within the lower Chesapeake Bay during the period of March 1999 to October 2000 and divided into holding tanks at the Gloucester Point VIMS laboratory according to origin (Figure 1): Upper James River (Area 1), James River (Area 2), Hampton Bar (Area 3), Lafayette (Area 4), Ocean View (Area 5), Lynnhaven Inlet/Little Creek (Area 5), York/Piankatank Rivers, and Eastern Shore (Chesapeake Bay side). For this study, only animals from James River (JR) and Ocean View (OV) were used because they represent end members of a geographic range.

Figure 1: A map of the distribution range of *Rapana venosa* within the Chesapeake Bay during 1999-2000.



### Tissue Extraction and Preparation

As part of a larger study, several animals from the eight different locations collected during different times of the year were sacrificed to effect the following whole body analysis. To examine temporal and spatial variability among the animals collected in the larger group, specimens from James River and Ocean View were selected as a sub-sample of this data set. Groups of animals were sacrificed on the following dates: 25 March 1999, 26 May 1999, 29 June 1999, 15 October 1999, 7 April 2000, 13 October 2000, and 9 May 2001 (Table 2). The samples from 25 Mar 1999 and 7 Apr 2000 were combined, as well as those from 15 Oct 1999 and 13 Oct 2000 because they were sacrificed during similar times of each respective year.

Each snail was measured for shell length (SL in mm, apex of shell to base of the columella) using a plastic measuring board and weighed to the nearest gram (TB = total body weight). Based on Chukchin's (1984) size range for size at first reproduction, all Chesapeake Bay specimens used in this experiment (SL = 86-168 mm) were larger than the minimum size at first reproduction and all should therefore be reproductively mature. The first method used to extract snails from their shells entailed each snail being briefly placed in the microwave (Berg and Adams, 1984); the body was then removed from the shell by hand, taking care to extract it in one piece, including all parts of the gonad. A later method (currently used) employed the use of a chisel, causing as little damage to the animal as possible, to remove the top few whorls of the spire to extract the snail from its shell. The shell and tissue were weighed separately (shell weight, tissue weight). Only tissue weight was recorded for snails which were extracted using the chisel method. An initial classification of sex based on external



morphology was then performed, after the soft body mass had been extracted from the shell. Males had a penis, approximately 30 to 40 mm in length. Imposex females had a small penis, which was usually less than 20 mm in length. Normal females did not have a penis (penis length = 0). Physical anomalies (i.e. presence of a double penis, gonad color, parasites, etc.), if present, were also noted.

The body was dissected into four main parts: gonad, proboscis, foot and viscera. The gonads were placed in glass jars with appropriate labels and preserved with an alcohol, formalin and acetic acid mixture (AFA - 5 liters: 1500 ml 95% ethyl alcohol, 1500 ml H<sub>2</sub>O, 1000 ml concentrated formalin, 500 ml glacial acetic acid, 500 ml glycerin) for later histological analysis.

For the 9 May 2001 sacrifice, the gonad sections were removed from freshly killed animals with a scalpel, placed in labeled cassettes, and then preserved in Bouin's fixative (Bouin-Duboscq solution: 150 ml 80% ethyl alcohol, 60 ml concentrated formalin, 15 ml glacial acetic acid, 1 g picric acid crystals). This second method of using fresh tissue and a different preservative was used so that a comparison between the two methods could be made.

Date	James River						Ocean View					
	Male		Imposex		Female		Male		Imposex		Female	
	SL	TB	SL	TB	SL	TB	SL	TB	SL	TB	SL	TB
	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)
25 Mar 99	146	582	143	503								
	148	566	141	470								
	157	639	136	460								
	139	466	122	373								
	136	505	133	476								
	146	544										
26 May 99	131	440	140	457			129	500	168	866		
	146	614					145	519	147	674		
	139	479					159	653				
	144	460					134	387				
	143	457					154	546				
	150	418					146	435				
29 Jun 99	137	365	131	291			133	353	151	482		
	153	568	147	451			142	485	138	480		
	144	528	135	500			138	530	143	529		
	138	500	143	518								
	145	501										
	151	521										
15 Oct 99	148	573	135	453			137	488	158	885	147	560
	155	579	151	513			152	555	151	732	144	572
	139	535	155	629			123	330	142	438		
	152	483	131	429			152	586	118	219		
	131	339					137	478	129	340		
	161	622					144	470	137	440		

Table 2: Shell lengths and total body weights of James River and Ocean View specimens, 1999-2001.

Date	James River						Ocean View					
	Male		Imposex		Female		Male		Imposex		Female	
	SL	TB	SL	TB	SL	TB	SL	TB	SL	TB	SL	TB
	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)	(mm)	(g)
7 Apr 00							157	790	144	825	151	750
							145	590	158	802	157	582
							146	711	155	105 0	160	849
							154	660	159	911	145	683
							143	648	149	738	158	930
							151	807			153	750
							140	535				
13 Oct 00											167	790
											155	970
9 May 01							147	640	150	620	105	180
							133	470			101	140
							143	680			86	120
							111	220			107	220
											135	570

Table 2 (cont.): Shell lengths and total body weights of James River and Ocean View specimens, 1999-2001.

### Histological Procedures

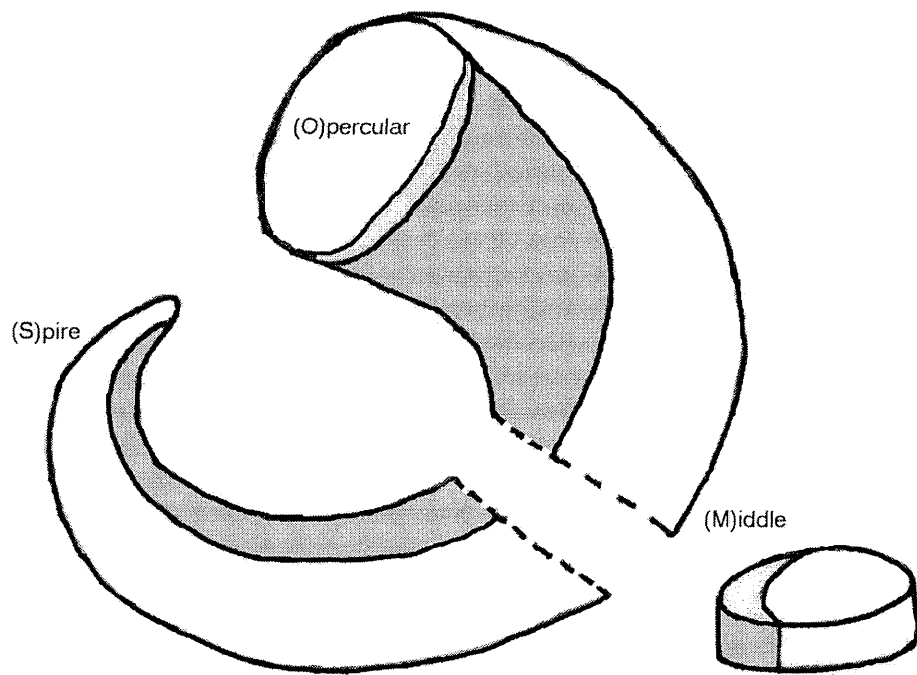
Gonads for histological examination were chosen, as mentioned earlier, from the OV and JR sites. Selection criteria required that gonads were complete and intact. Gonads that were broken or severely damaged were not used. In order to determine the developmental state, small sections of approximately 5mm thicknesses were taken from the middle portion

of each gonad using a razor blade (Figure 2). All sections were placed in plastic cassettes according to size (diameter of gonadal cross section), though in some instances gonads had such a large diameter they had to be cut into quarters and labeled accordingly. The cassettes were then rinsed in water and soaked in 70% ethyl alcohol overnight. Before samples were placed in the tissue processor (see Table 3 for schedule), they were transferred to 95% ethyl alcohol and soaked for approximately 45 minutes. The chemicals S-29 (tissue dehydrating agent), UC67 (Tissue Clear - xylene), and Tissue Prep (paraffin) are all Fisher Scientific Products. The tissue processor used was a Shandon Hypercenter XP. Once the tissue infiltration was complete, the samples were transferred to the embedding center (Miles Scientific, Tissue-Tek) and embedded in appropriate sized capsules. These were allowed to cool on a cold plate until hard, and they were stored in a plastic bag until ready to cut on the microtome.

	Step	Chemical	Time
<b>Dehydration</b>	1	S-29	3 hr.
	2	S-29	2hr.
	3	S-29	2hr.
	4	S-29	1hr.
	5	S-29	1hr.
	6	S-29	1hr.
	7	S-29	1hr.
	8	S-29	1hr.
<b>Clearing</b>	9	UC670	1hr.
	10	UC670	1hr.
<b>Infiltration</b>	11	Tissue Prep	1hr.
	12	Tissue Prep	1hr.

Table 3: VIMS tissue infiltration schedule.

Figure 2: Diagram of the histological sampling procedure, which consists of removing a cross section of tissue from the gonad.



Before sectioning, any blocks to be cut were chilled on ice for at least 30 minutes. All sections were cut on an AO Spencer 820 rotary microtome at a thickness of 6 $\mu$ m. Selected sections were floated on a warm water bath (~40°C) and transferred to the appropriate pre-labeled glass slide. The slides were allowed to dry briefly on a drying rack before being placed in an oven (42°C) overnight. Blocks that were difficult to cut due to the tissue being brittle were soaked in ice water, cold soapy water, or chilled glycerin for a few minutes before cutting.

All slides were stained with Harris Hematoxylin and Eosin Y according to the VIMS Routine Hand Staining Procedure (Table 4). All reagents were dispensed in 200 ml quantities.

	Step	Reagent	Time/Number of dips
<b>Deparaffinize</b>	1	xylene	3 minutes
	2	xylene	2 minutes
<b>Hydrate to water</b>	3	100% EtOH	15 dips
	4	100% EtOH	15 dips
	5	95% EtOH	15 dips
	6	95% EtOH	15 dips
	7	Running tap water	3 minutes
<b>Stain</b>	8	Harris Hematoxylin	5 minutes
	8a	Running tap water	2 minutes
<b>Differentiate Hematoxylin</b>	9	Acid Alcohol	1.5 minutes
	9a	Running tap water	5 minutes
<b>Blue</b>	10	NaHCO <sub>2</sub>	2 minutes
	10a	Running tap water	3 minutes
<b>Counter stain</b>	11	Eosin Y	3 minutes
<b>Differentiate Eosin &amp; Dehydrate</b>	12	95% EtOH	6 dips
	13	95% EtOH	6 dips
	14	100% EtOH	10 dips
	15	100% EtOH	10 dips
	16	xylene	3 minutes
	17	xylene	5 minutes
	18	xylene	5 minutes or until ready to coverslip

Table 4: VIMS Routine Hand Staining Procedure.



The addition of cover slips was performed under a fume hood. In preparation, the back of each slide was wiped with a Kimwipe to remove excess xylene. One or two drops of mounting fluid was placed on the tissue prior to placement of the cover slip. Care was taken not to trap bubbles under the cover slip. If bubbles were present, they were gently removed by pressing on the cover slip with a probe or blunt forceps and allowing the air to escape at the edge of the slide. Each slide was then blotted and placed on a slide tray. All completed slides were placed in an oven at 42°C for 24 to 48 hours to allow the mounting medium to harden. The slides were then examined under a light microscope and images captured using either a digital image analysis system or a 35 mm camera.

#### Gonadal Synchrony

It was not initially known if *R. venosa* gonads developed synchronously or asynchronously (i.e. having a single developmental stage present throughout the gonad), so a few select animals from the James River Shipyard site were chosen for further examination (Table 5). Protocols for examining gonadal synchrony were the same as above with the exception of the number of sections taken from each gonad. In this case, three sections were taken from each gonad and labeled with the appropriate abbreviation: one from the end nearest the operculum (O), one from the middle (M), and one from the tip nearest the spire (S). The finished slides were then examined under a light microscope and images captured using a digital image analysis system.

Date	James River			
	<i>Male</i>		<i>Imposex</i>	
	SL (mm)	TBW (g)	SL (mm)	TBW (g)
29 Jun 99	153	568	131	291
	144	528	135	500
	151	521	143	518

Table 5: Animal from which gonads were taken for examining gonadal synchrony.

### Gonadal Development

All slides were examined to determine the gonadal development stage. Males were categorized as either 1) developing, 2) mature, 3) spent, or 4) recovering. The developing stage was characterized by a lack of spermatozoa: only sperm precursors (spermatogonia, spermatocytes, spermatids) were present. A gonad in the mature stage contained a large quantity of spermatozoa.

Normal and imposex females were categorized as either 1) early developing, 2) late developing, 3) mature, 4) spent, or 5) recovering. Egg diameter was also measured in stages 1-3 to assist in more accurately staging female gonads. Ten eggs from each slide were measured and the average was taken. Egg diameters in the range of 60-70  $\mu\text{m}$  were considered to be in the early developing stage. The late developing stage was characterized by eggs in the range of 120-150  $\mu\text{m}$ , and mature eggs in the range of 190-240  $\mu\text{m}$ .

Male and female gonads in either the spent or recovering stages were similar, in that most of the gametes had been expelled, and any that remained were degenerated while new ones were formed. The gonadal development staging process has been followed as per Chung

et al. (1993). Any gonadal anomalies visible under the microscope were also recorded for each animal and its corresponding slide.

### Gross Anatomy of the Reproductive System

To elucidate the gross anatomy of the reproductive systems for male, female, and imposex *R. venosa*, a series of dissections were made on both previously preserved specimens from 1998-1999 and specimens caught in the wild during 2001. The male specimen used for the initial dissection was one of the first few animals caught within the Chesapeake Bay. The whole body was removed from the shell using the microwave method and preserved in 95% ethanol.

The second set of dissections used 8 animals which were anaesthetized in 7.5% w/v magnesium chloride solution for approximately 4 hours before being removed from their shells via the hammer and chisel method. Two male, 2 imposex female, and 4 normal female *R. venosa* were dissected to use as models for each respective sex. Imposex females were also dissected in the same manner to investigate the internal progression of imposex and determine its severity. Basic line drawings were made from these dissections to illustrate the distinguishing features of each respective sex (Figures 3a-3c). These features can be seen without the aid of a microscope. The asterisk of Figure 3b represents the probable path of the vas deferens within imposex female *R. venosa*. A more detailed illustration of the reproductive system of *Rapana venosa* can be found in Hou et al. (1990).

Figure 3a: Line drawing of male *Rapana venosa*. Key features include penis and vas deferens.

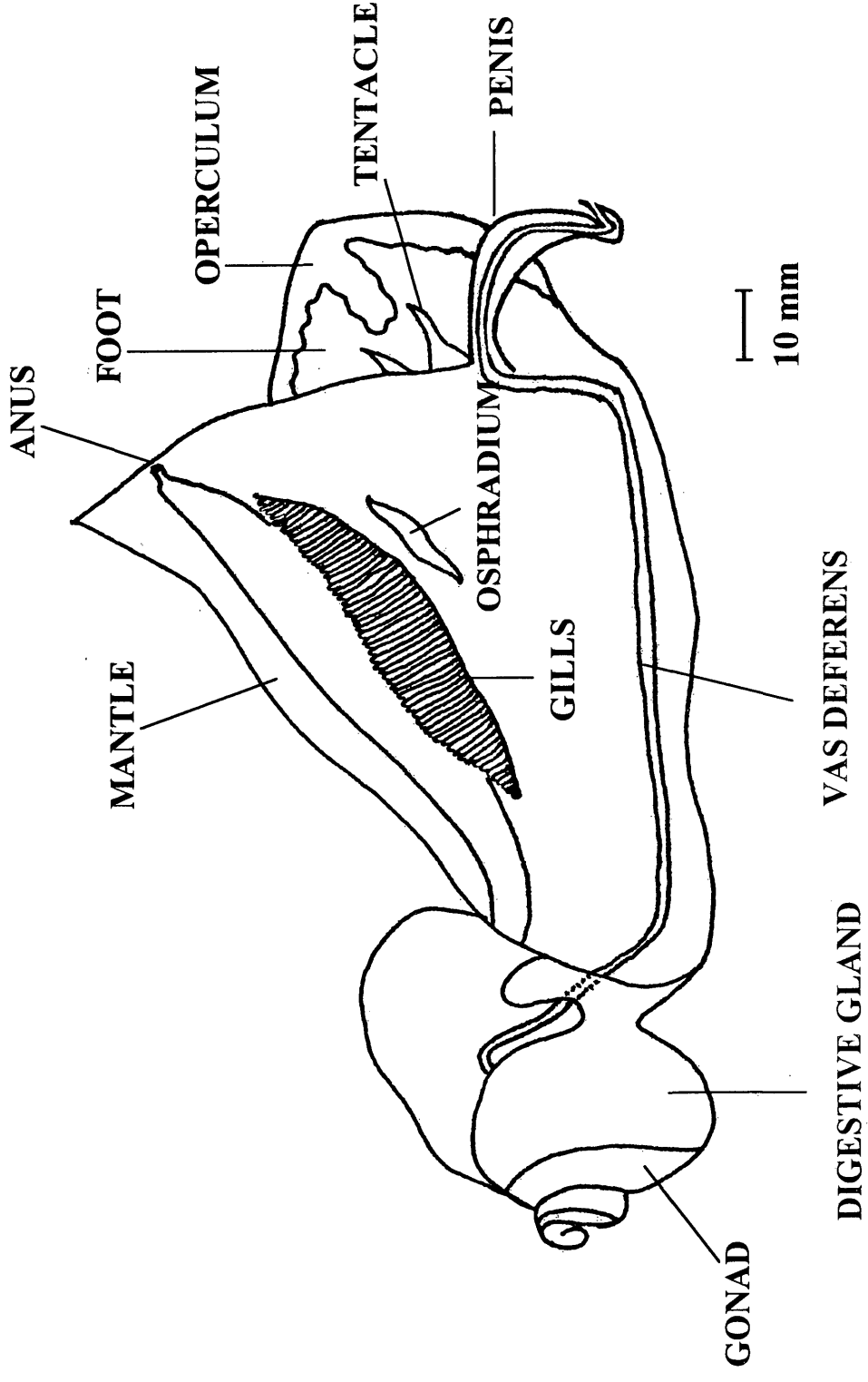


Figure 3b: Line drawing of imposex female *Rapana venosa*. Key features include female gonopore and penis. (\*Probable path of vas deferens)

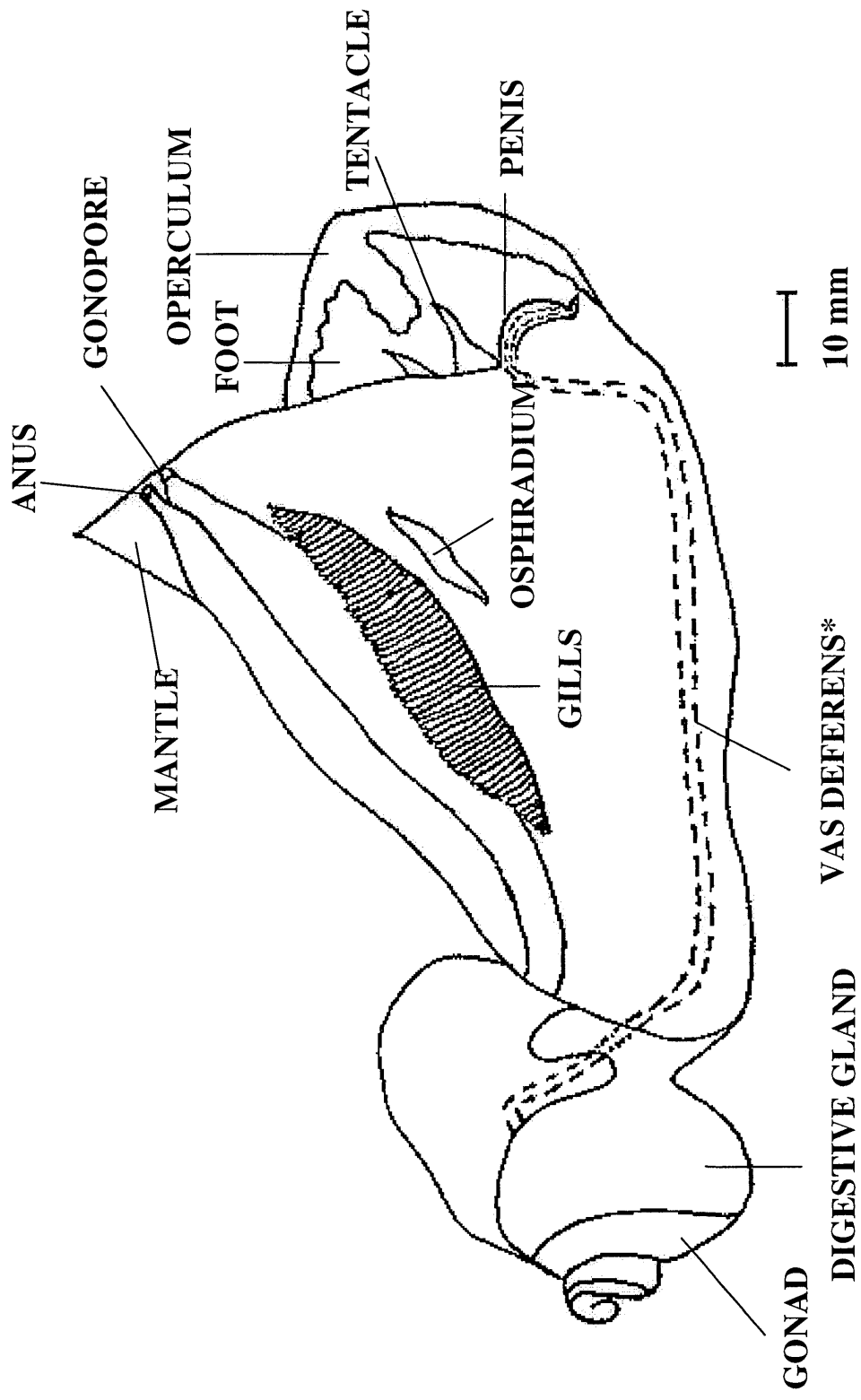
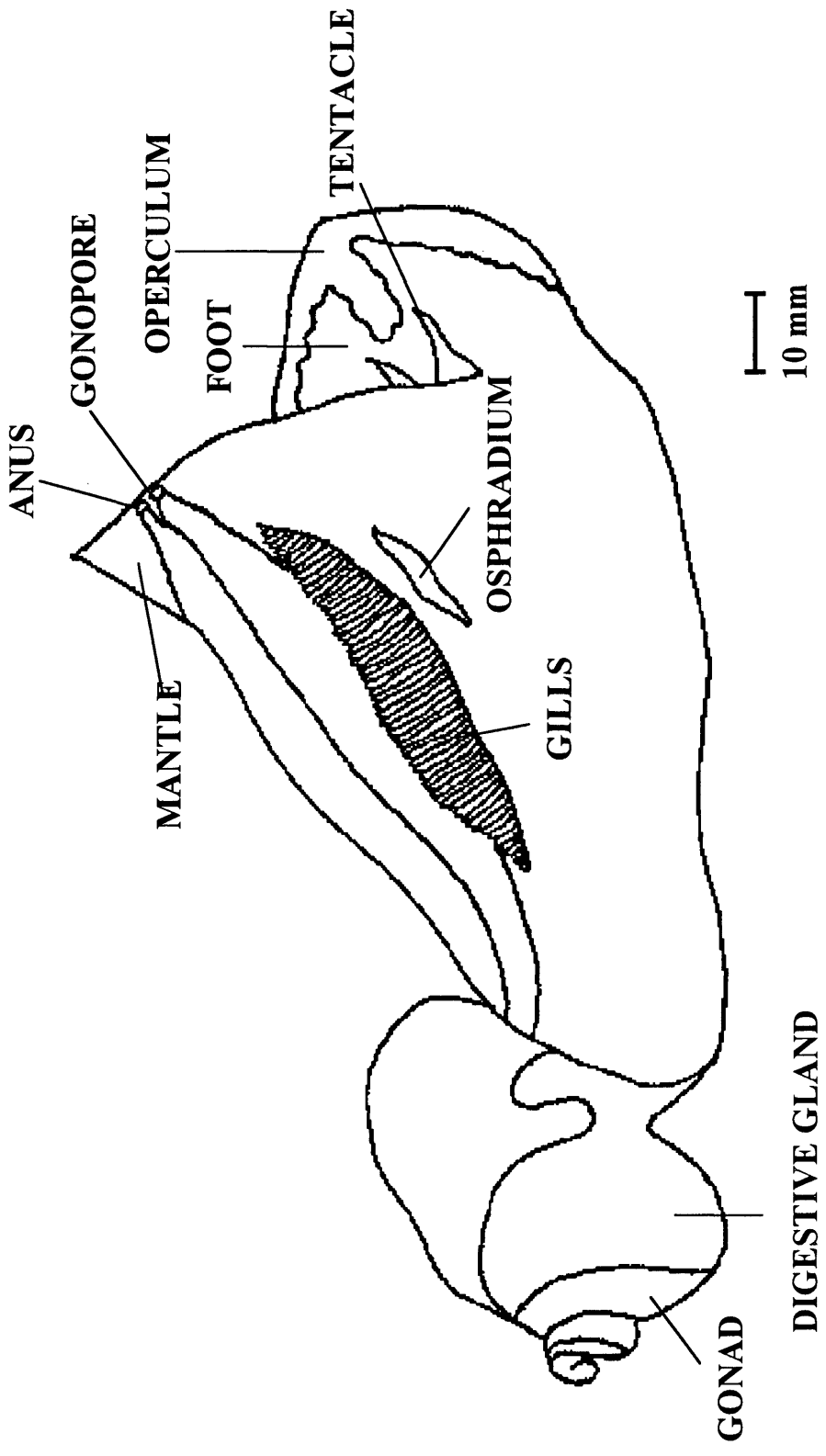


Figure 3c: Line drawing of female *Rapana venosa*. Key feature is the female gonopore.





## RESULTS

### Gonadal Synchrony

Histological slides from three males in the copulation stage and three imposex females in the mature stage were observed under the light microscope to determine if the gonad of *R. venosa* develops synchronously.

One James River male (29 Jun 99, 144 mm) appeared to have a completely synchronous gonad, while the other two James River males (29 Jun 99: 153 mm, 151 mm) were not entirely synchronous. The slides prepared from the spire section of gonad lagged behind only slightly in the stage of development. For the 153 mm male, the middle and opercular sections were at the state of late copulation, while the spire section was still in mid-copulation. The gonad of the 151 mm male was similar, as the middle and opercular sections were staged as mid-copulation and the spire section was staged as borderline mature/early copulation.

When examined, the gonads of the James River imposex females (29 Jun 99: 131 mm, 135 mm, 143 mm) were found to be in the mature stage in all three sections (spire, middle, opercular).

### Male Gonadal Development

Histological slides prepared from the gonads of male *R. venosa* were examined and all four stages of gonadal development were manifested (Figures 4-7). The percentages of males in each stage of gonadal development throughout the year are shown in Figures 8-10. Average male and imposex female penis sizes were correlated to shell length (Figure 22), and average penis size was also calculated by site (Figure 23). The male gonad (testis) appears as a purple crescent above the digestive gland, which stains pink or magenta. Seminal vesicles are present on the side of the gonad nearest the columella, and stain deeply with hematoxylin. The lumens of seminiferous tubules are visible under the light microscope, where spermatozoa production occurs.

The developing stage was characterized by the presence of numerous spermatogonia, spermatocytes, and spermatids (Figure 4). Despite the absence of spermatozoa in the lumens of seminiferous tubules, spermatozoa were present in the seminal vesicle. Another cell type, which is shaped like a spherical grape cluster, is plentiful and found in the seminal vesicle. These cells are believed to serve a secretory or nutritive function, and stain deeply with hematoxylin. Chesapeake Bay male *R. venosa* were found in the developing stage during April and May, when water temperatures were between 10-14 °C.

The seminiferous tubule lumens were filled with mature spermatozoa during the mature stage (Figure 5). Under the microscope, heads of the spermatozoa appear purple or dark blue as stained by hematoxylin, and the tails of spermatozoa are stained pink by eosin. Sperm production appears to be active at this time as there are still spermatozoa precursors present.

Figure 4: Histology of the male gonad in the developing stage. Note the absence of spermatozoa; only sperm precursors are present at this stage.  
(scale bar = 100 $\mu$ m)

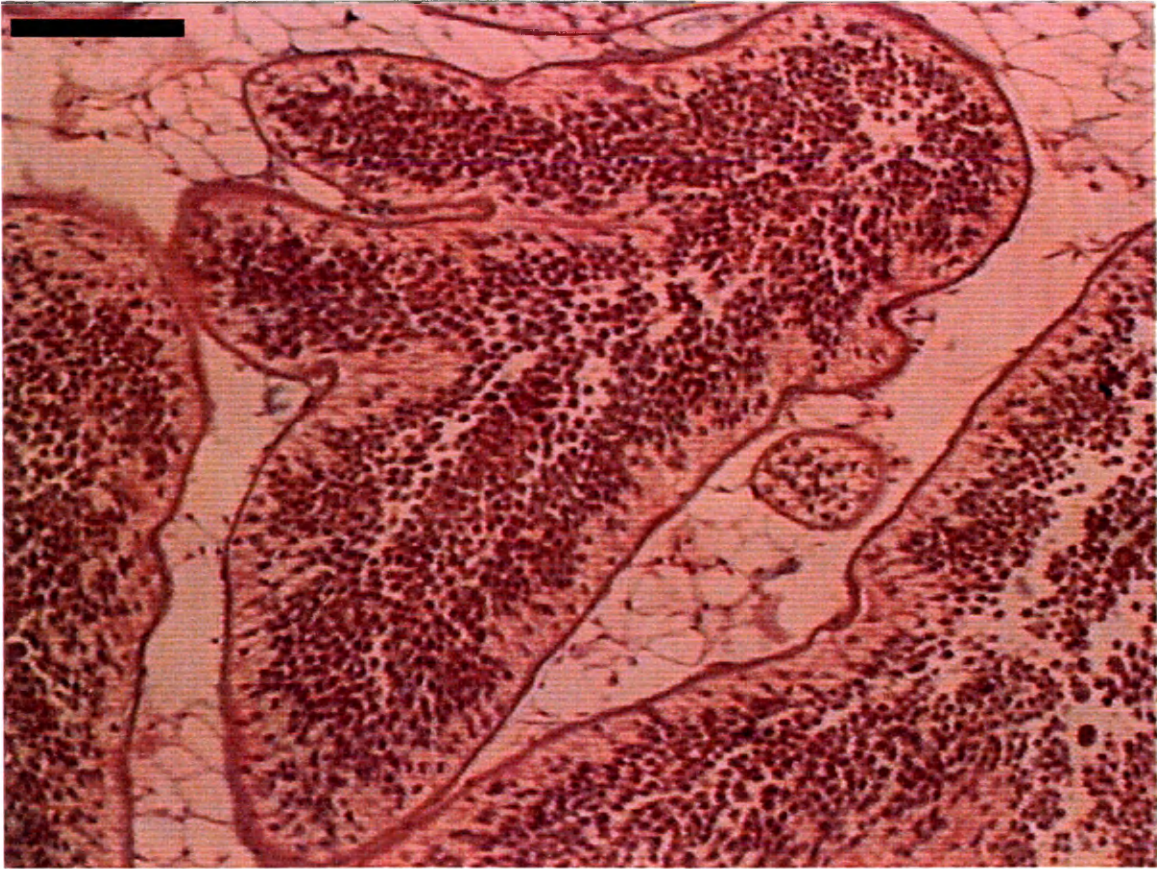


Figure 5: Histology of the male gonad in the mature stage. Many streaming sperm are present within the lumen. (scale bar = 100 $\mu$ m)

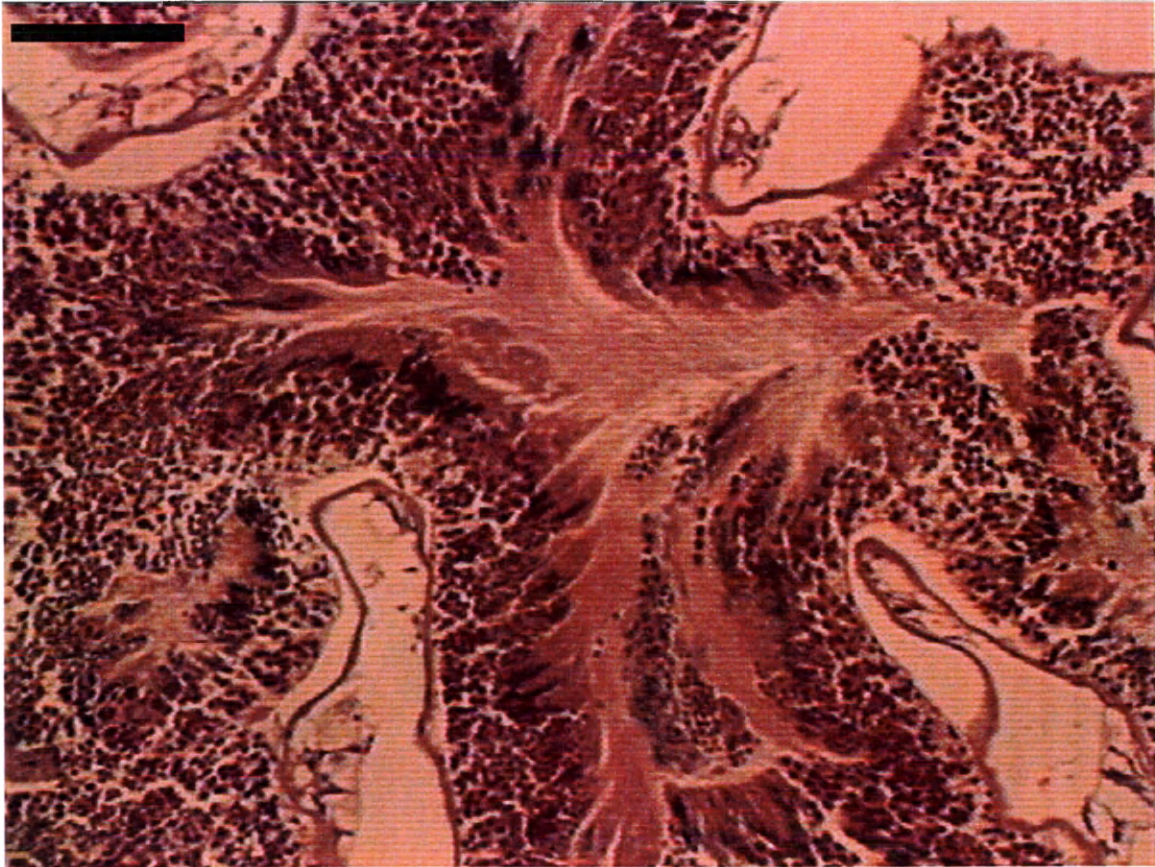


Figure 6: Histology of the male gonad in the spent stage. The sperm content of the lumen is greatly decreased from the mature stage. (scale bar = 100 $\mu$ m)



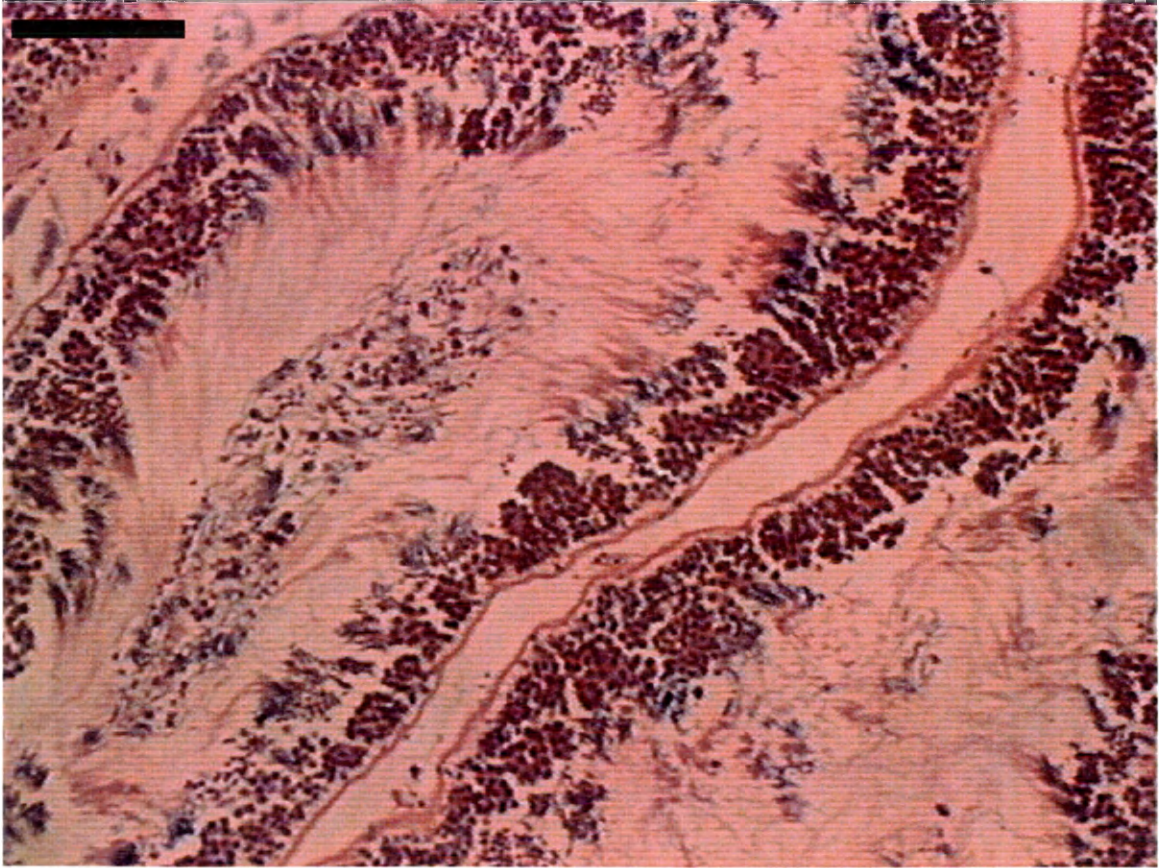


Figure 7: Histology of the male gonad in the recovering stage. Degradation of spermatozoa is occurring. (scale bar = 100 $\mu$ m)

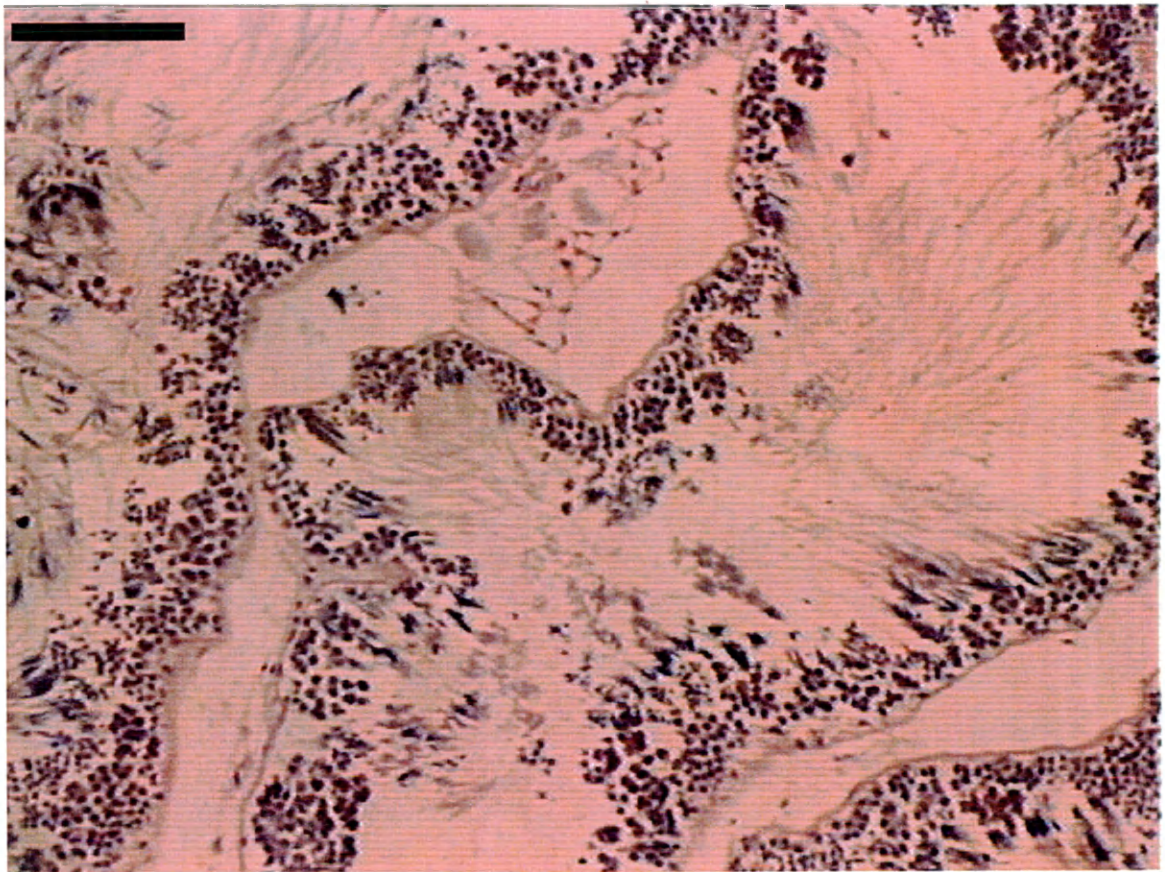


Figure 8: Gonadal development for James River males.

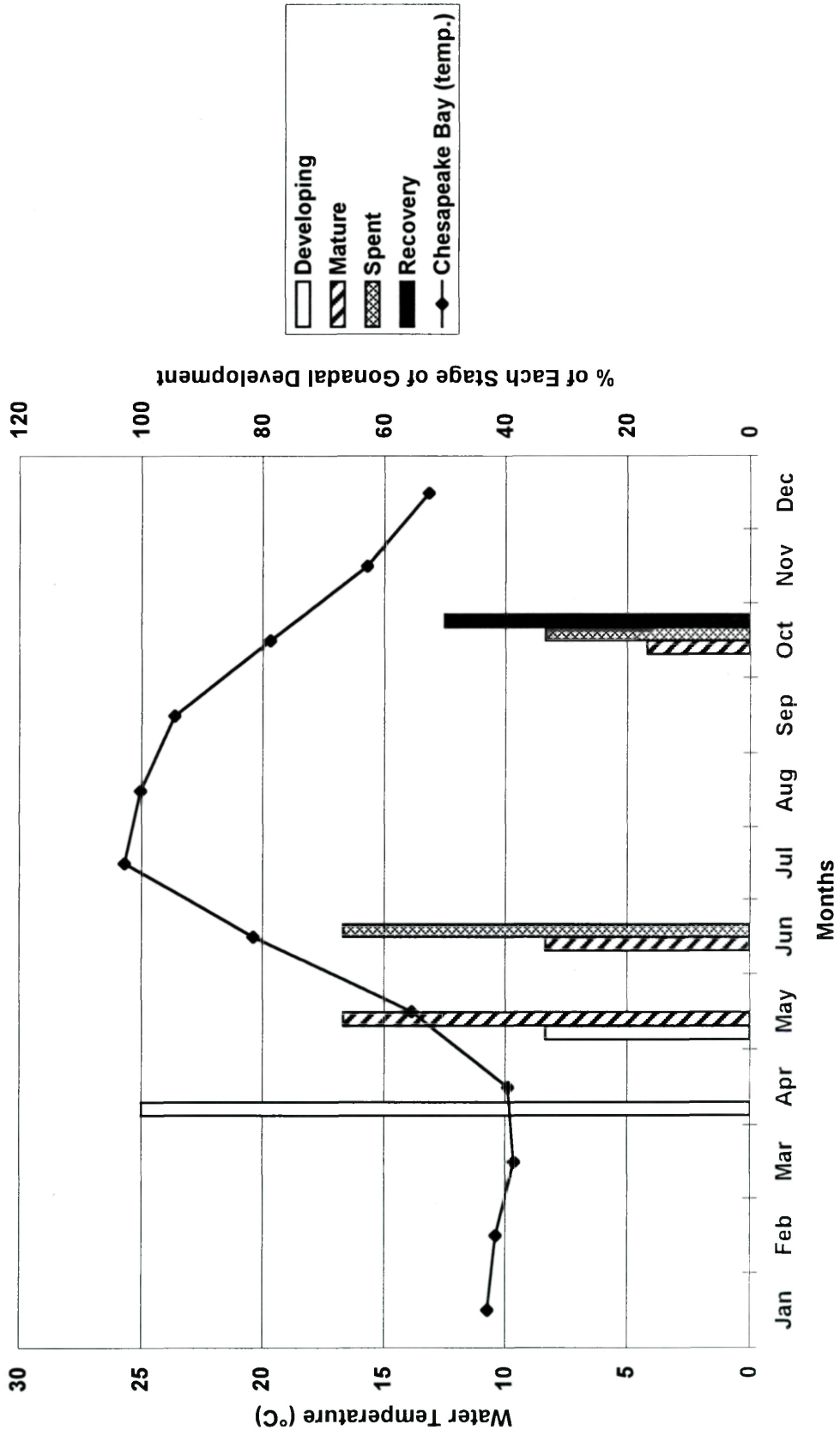


Figure 9: Gonadal development for Ocean View males.

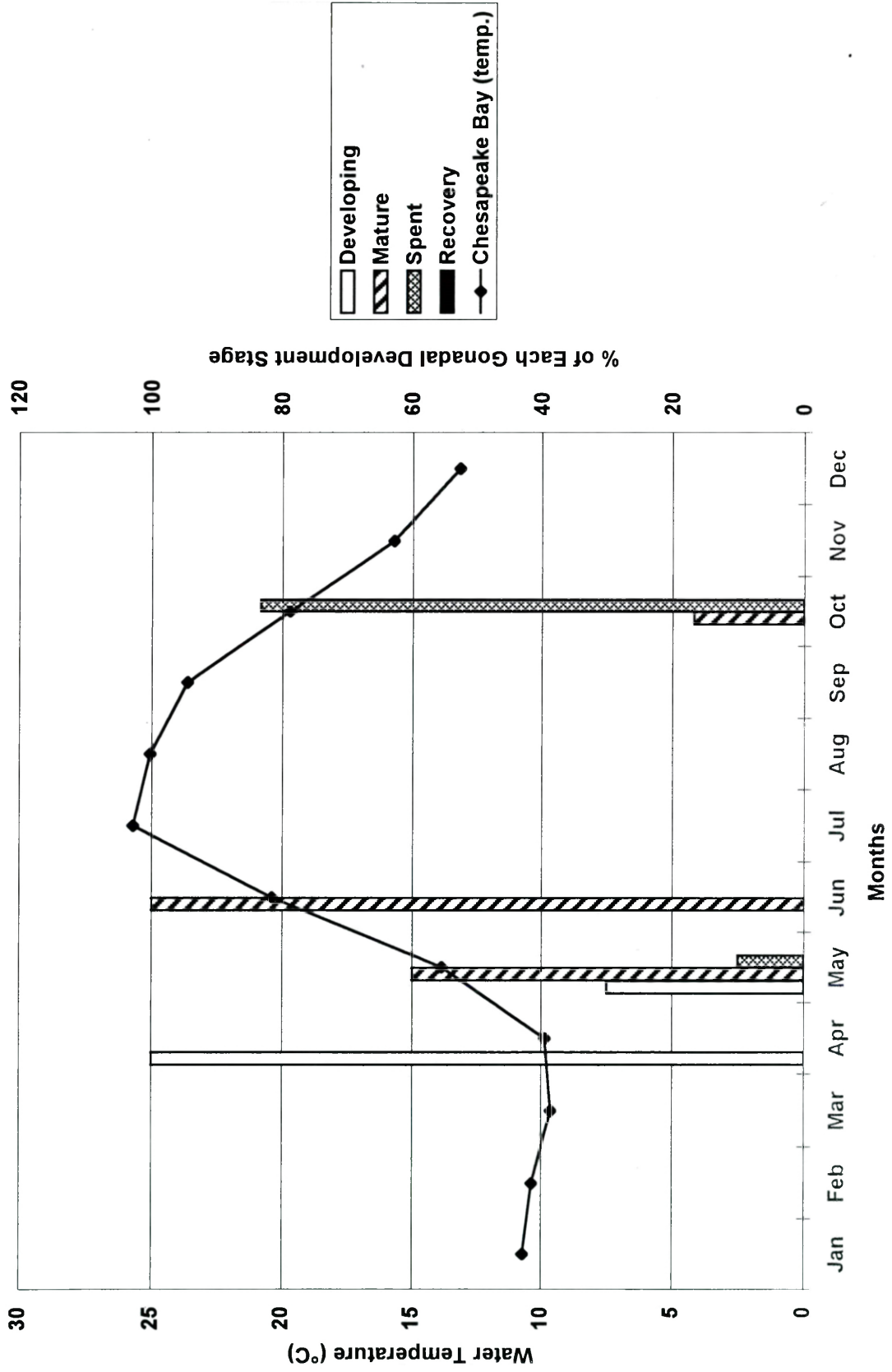
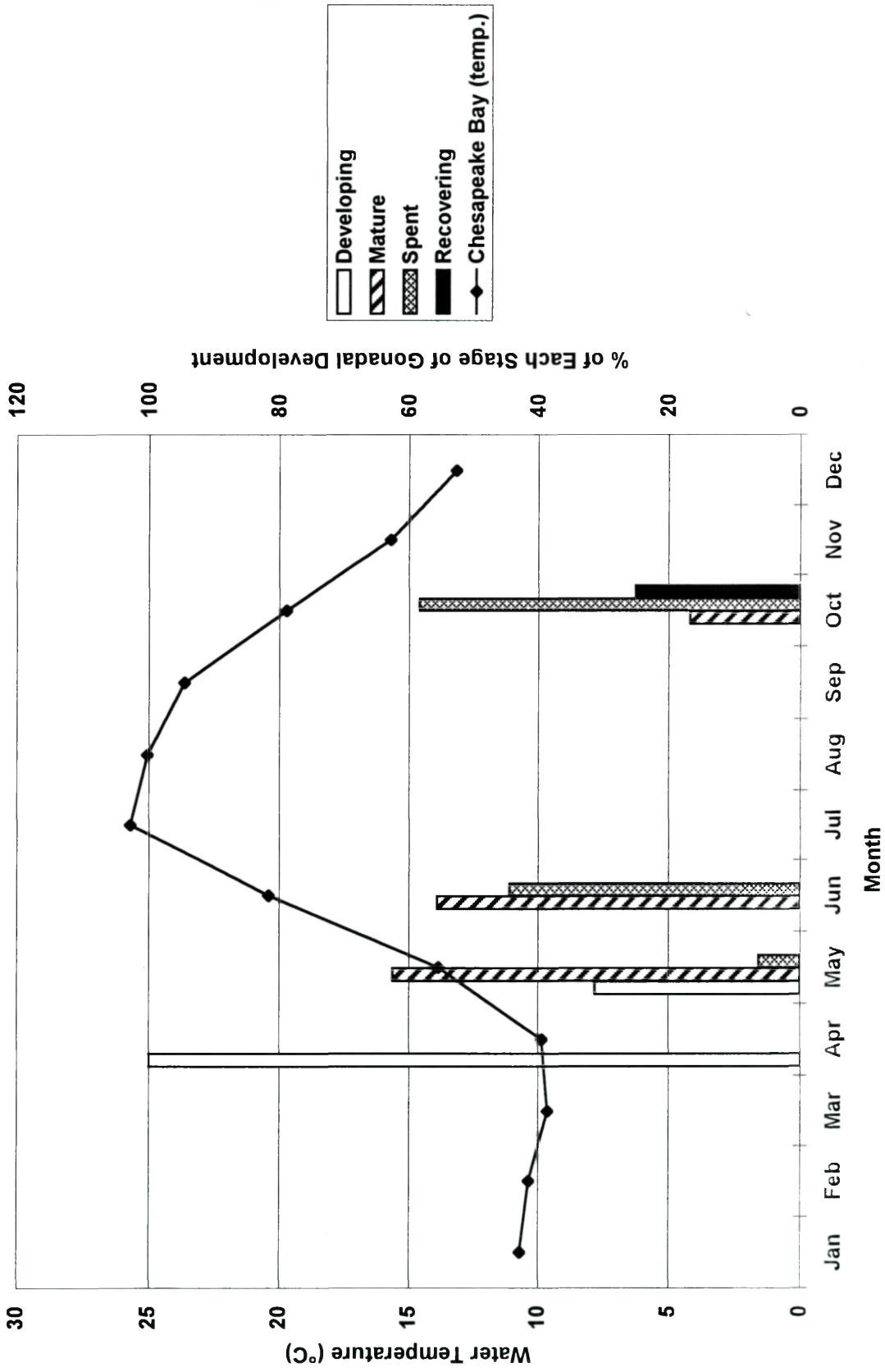


Figure 10: Gonadal development for all males, from James River and Ocean View.





Male whelks in the mature stage were found from May through October, when water temperatures were between 14-20 °C.

A great deal of gonadal content is lost in the copulation stage (Figure 6). The center of each lumen gradually becomes empty, until only a few spermatozoa remain. Any precursors that are present are found mostly near the germinal epithelium, but a few are often found scattered in the lumen centers. Male whelks in the copulation stage were found from May through October, when water temperatures were between 14-20 °C. During this time, captive *R. venosa* were observed mating in the VIMS wet laboratory holding tanks.

Only a few males in the sample set had reached the early recovery stage by the month of October, when the water temperature was 20 °C. Degradation of remaining spermatozoa was observed (Figure 7). Some spermatozoa precursors were still visible near the germinal epithelium.

Prior to histological preparation, gonadal anomalies where present were noted. The majority of anomalies occurred in male specimens. The most frequently occurring abnormality, in 22% of all males, was the presence of blisters or pustules on the gonad. The blisters ranged in size from a few millimeters to a few centimeters. The second abnormality, in 10% of males, was the presence of tiny brown lesions found on and in the gonad. Only 4% of all males exhibited an unusual gonad color.

### Female Gonadal Development

No major differences were observed in gonadal development between normal and imposex females, so they will both be described in one group. The female gonad (ovary)

appeared as a purple crescent above the digestive gland, which stains pink or magenta. Ovarian development over time is shown in Figures 11-14. The percentages of normal and imposex females in each stage of gonadal development throughout the year are shown in Figures 15-20. Average egg diameter was also calculated for all females (normal and imposex) in early developing, late developing, or mature stages (Figure 21).

Female gonads in the early developing stage (Figure 11) contained a number of oogonia budding from the germinal epithelium and many small oocytes. Within the lumen, a number of free yolk granules were present, and these stained a bright pink or magenta. Smaller oocytes contained only a few small yolk granules, while larger oocytes had taken up a larger proportion of yolk granules over time. The nuclei, which stained purple, were clearly visible within the oogonia and oocytes. Chesapeake Bay female *R. venosa* in the early developing stage were found during the month of May only, when the water temperature was 14 °C.

Eggs in the late developing stage (Figure 12) were slightly larger than those in the early developing stage. At this time, the majority of the yolk granules had been taken up from the lumen, though oogonia were still budding from the germinal epithelium. Female whelks in the late developing stage were found during the months of April and May, when the water temperature was 10-14 °C.

In the mature stage, a significant increase in gonad area can be seen when viewing a slide with the naked eye. The usual thin crescent shape above the digestive gland bulges, and in some cases the gonad rivals the digestive gland in size. As viewed under the microscope, mature eggs completely fill the lumen (Figure 13), and a few oogonia are present along the

Figure 11: Histology of female gonadal development in the early developing stage. Note the few developing oogonia and copious amounts of yolk granules present.

(scale bar = 100 $\mu$ m)

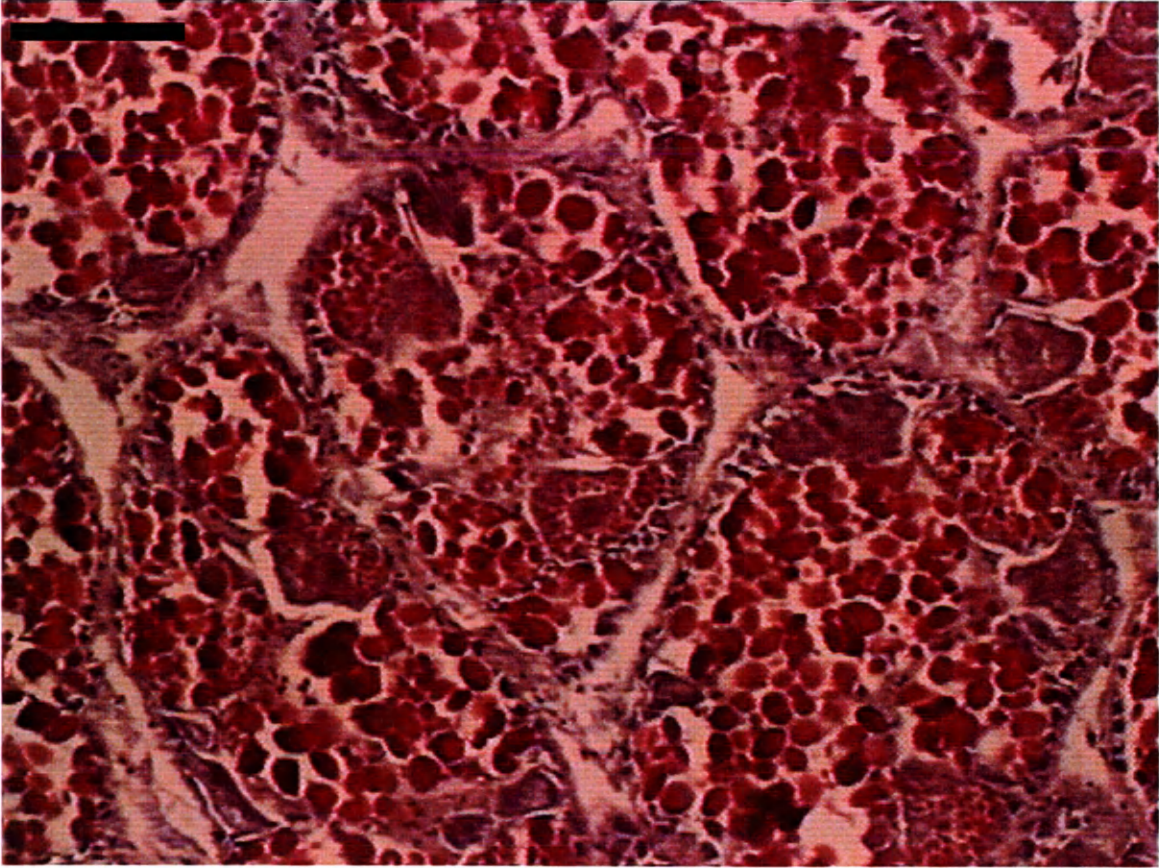


Figure 12: Histology of female gonadal development in the late developing stage. Increased numbers of small oocytes are present, and the amount of free yolk granules has decreased. (scale bar = 100 $\mu$ m)

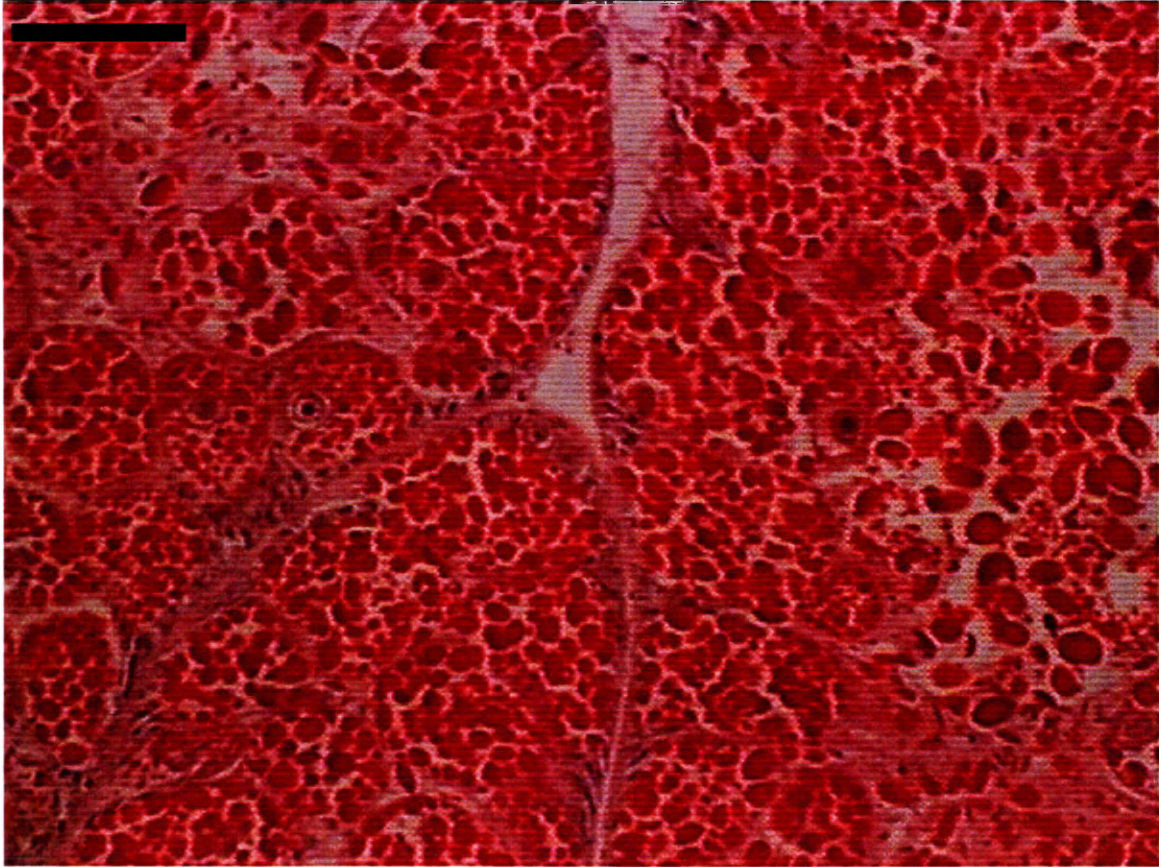


Figure 13: Histology of female gonadal development in the mature stage. Large oocytes fill the lumen, and nuclei are aligned along the center of the lumen.

(scale bar = 100 $\mu$ m)



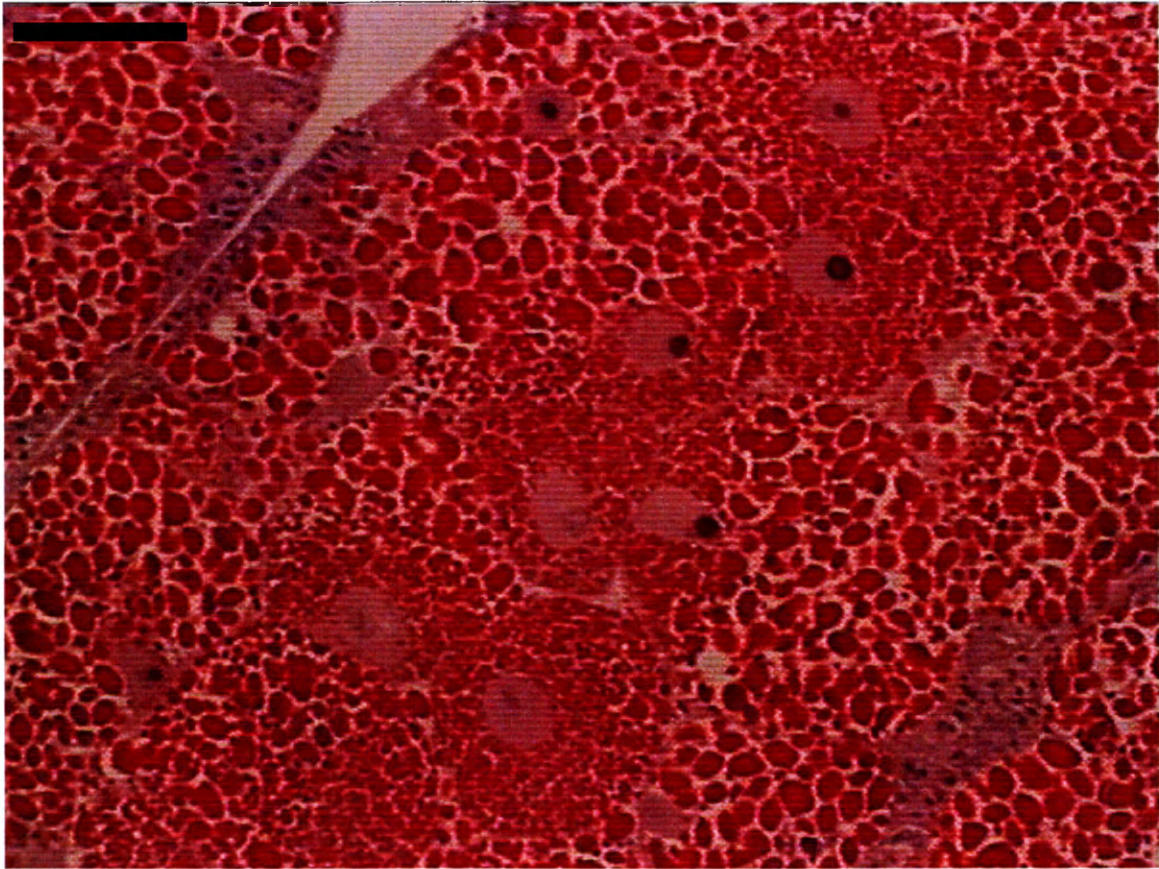


Figure 14: Histology of female gonadal development in the spent stage. Note the absence of mature oocytes; only small oocytes and oogonia remain. (scale bar = 100 $\mu$ m)

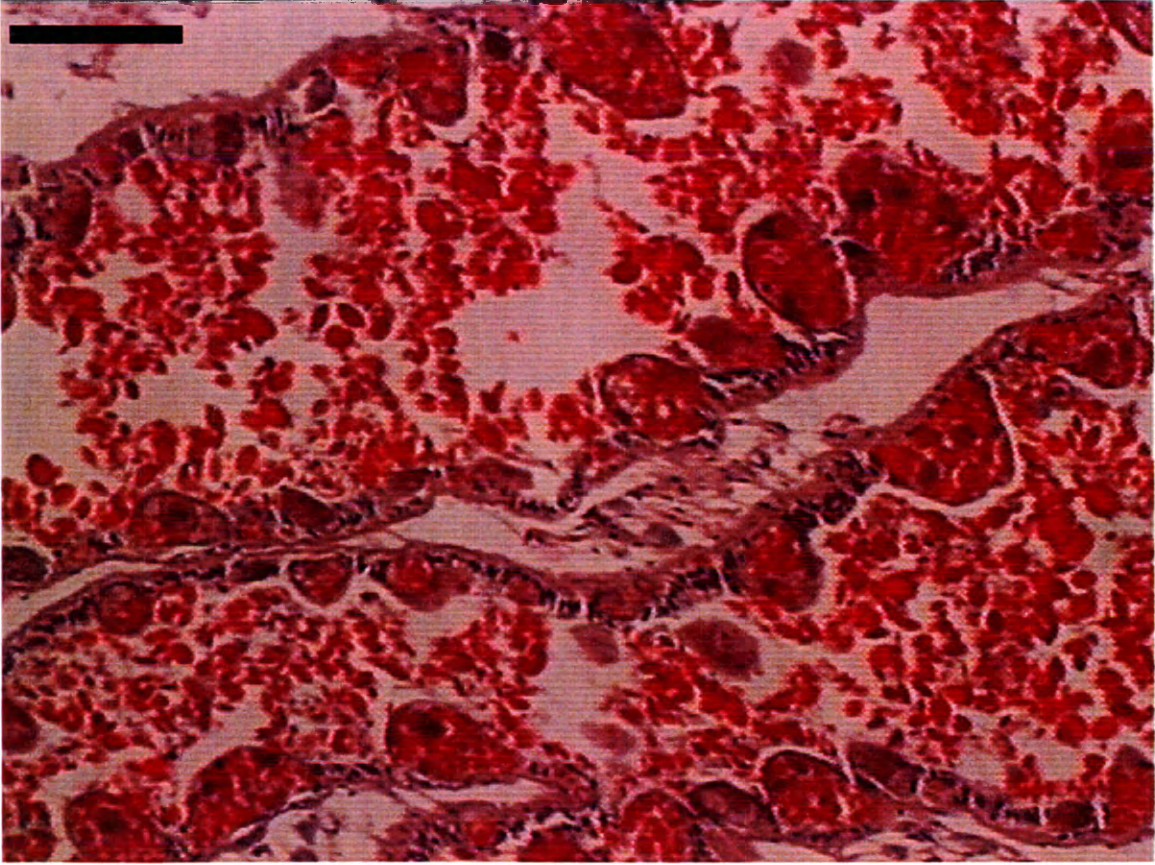


Figure 15: Gonadal development for James River imposex females.

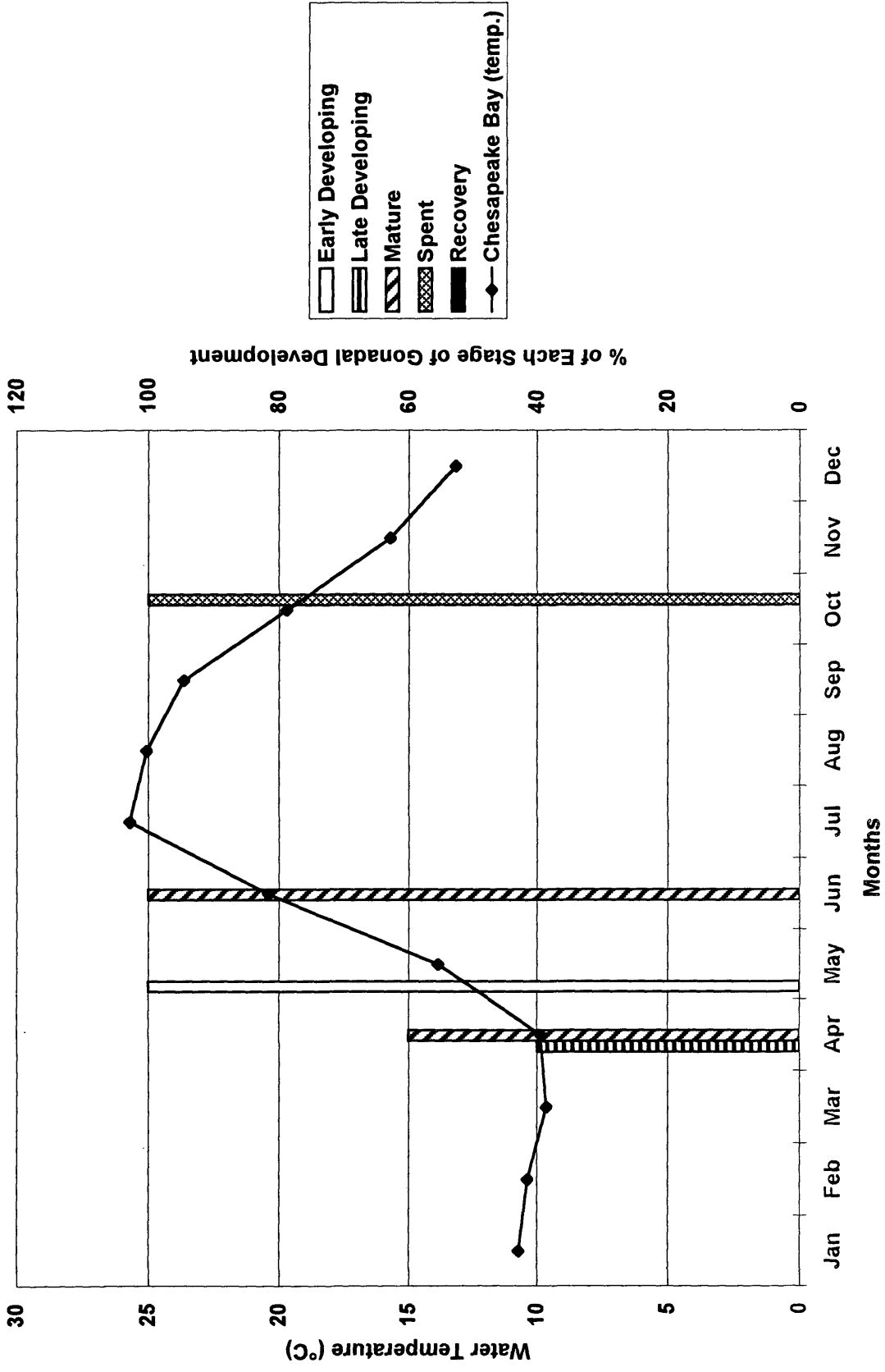


Figure 16: Gonadal development for Ocean View normal females.

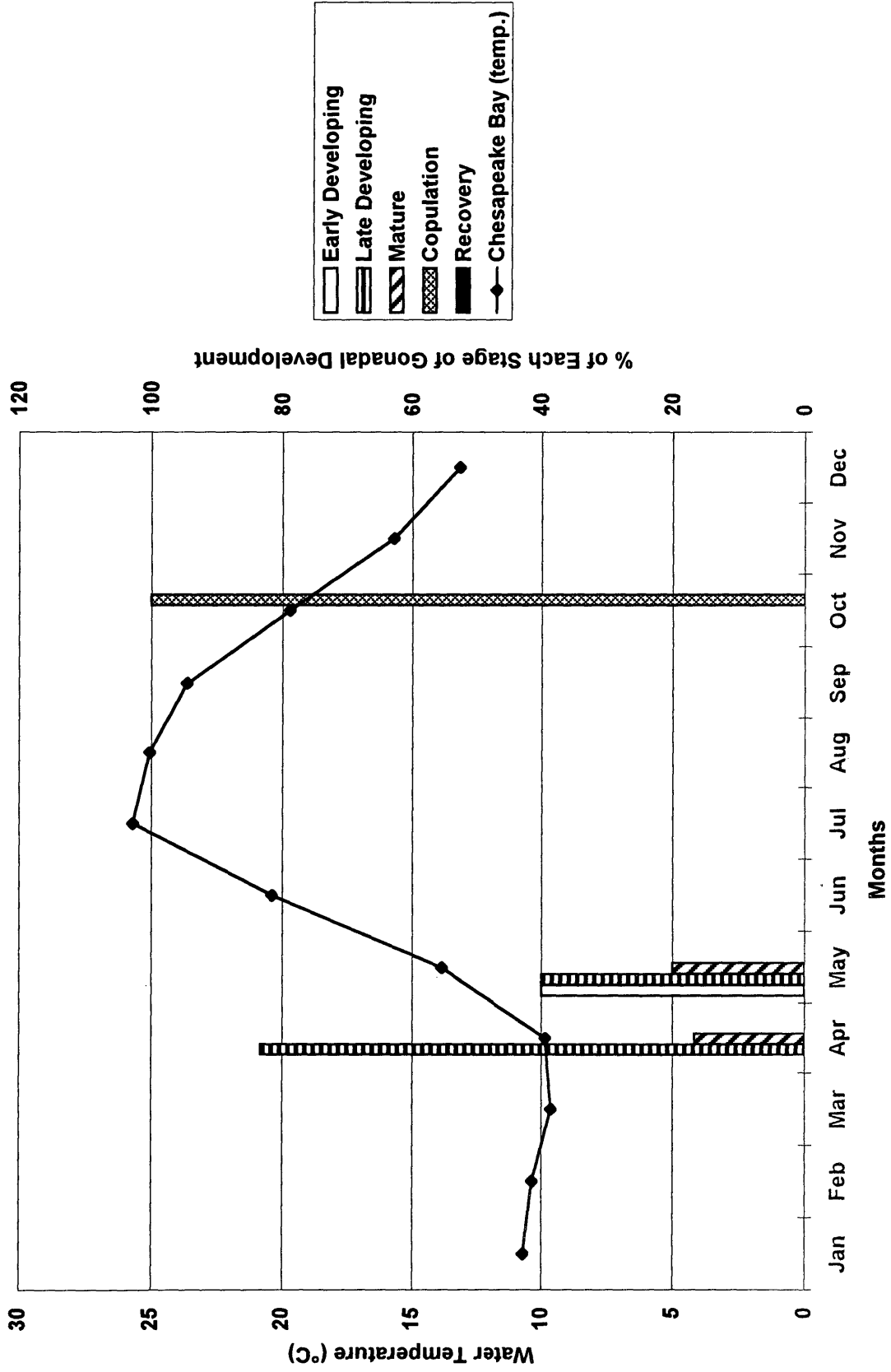


Figure 17: Gonadal development for Ocean View imposex females.



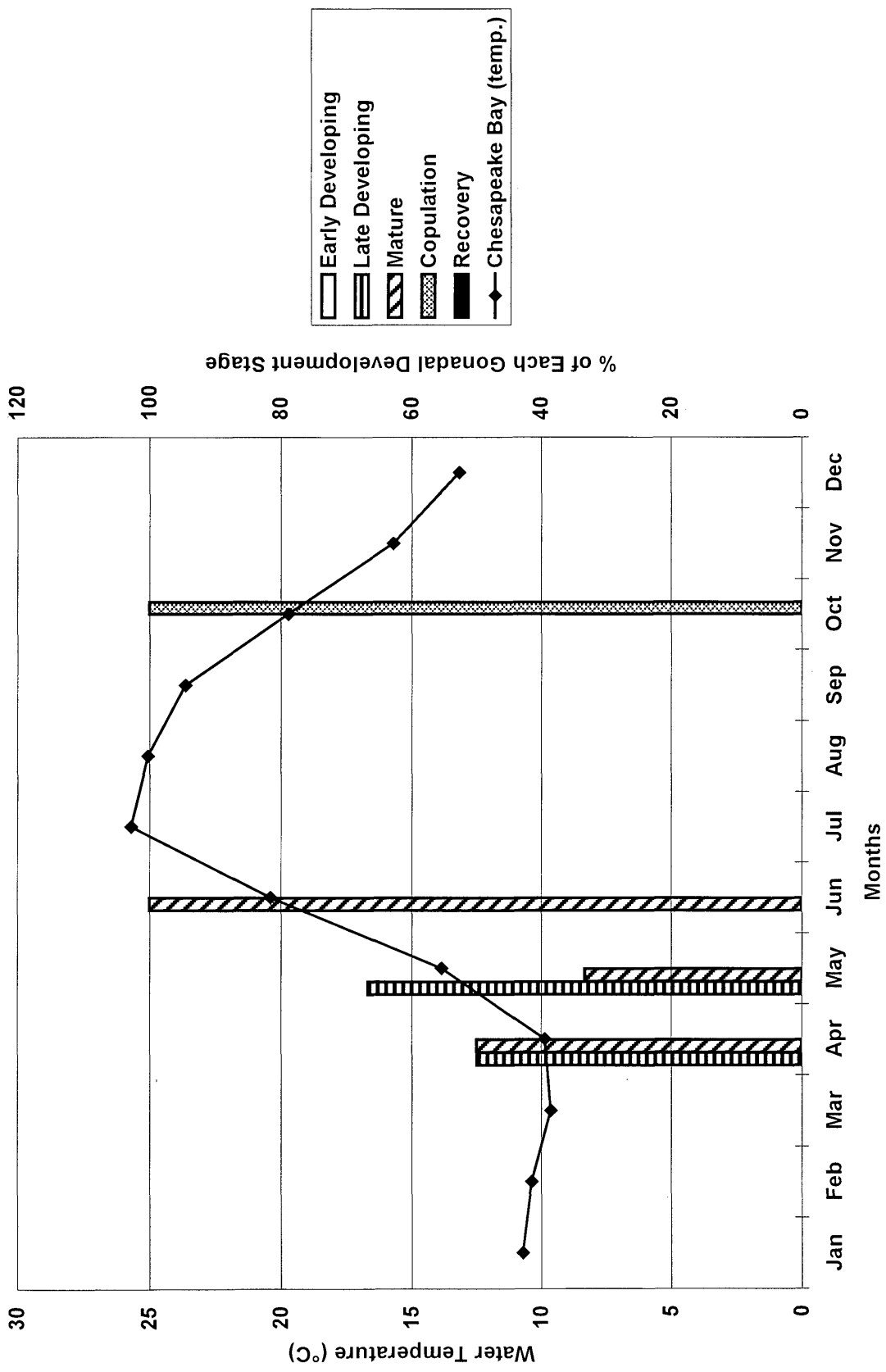


Figure 18: Gonadal development for all Ocean View females, both normal and imposex.

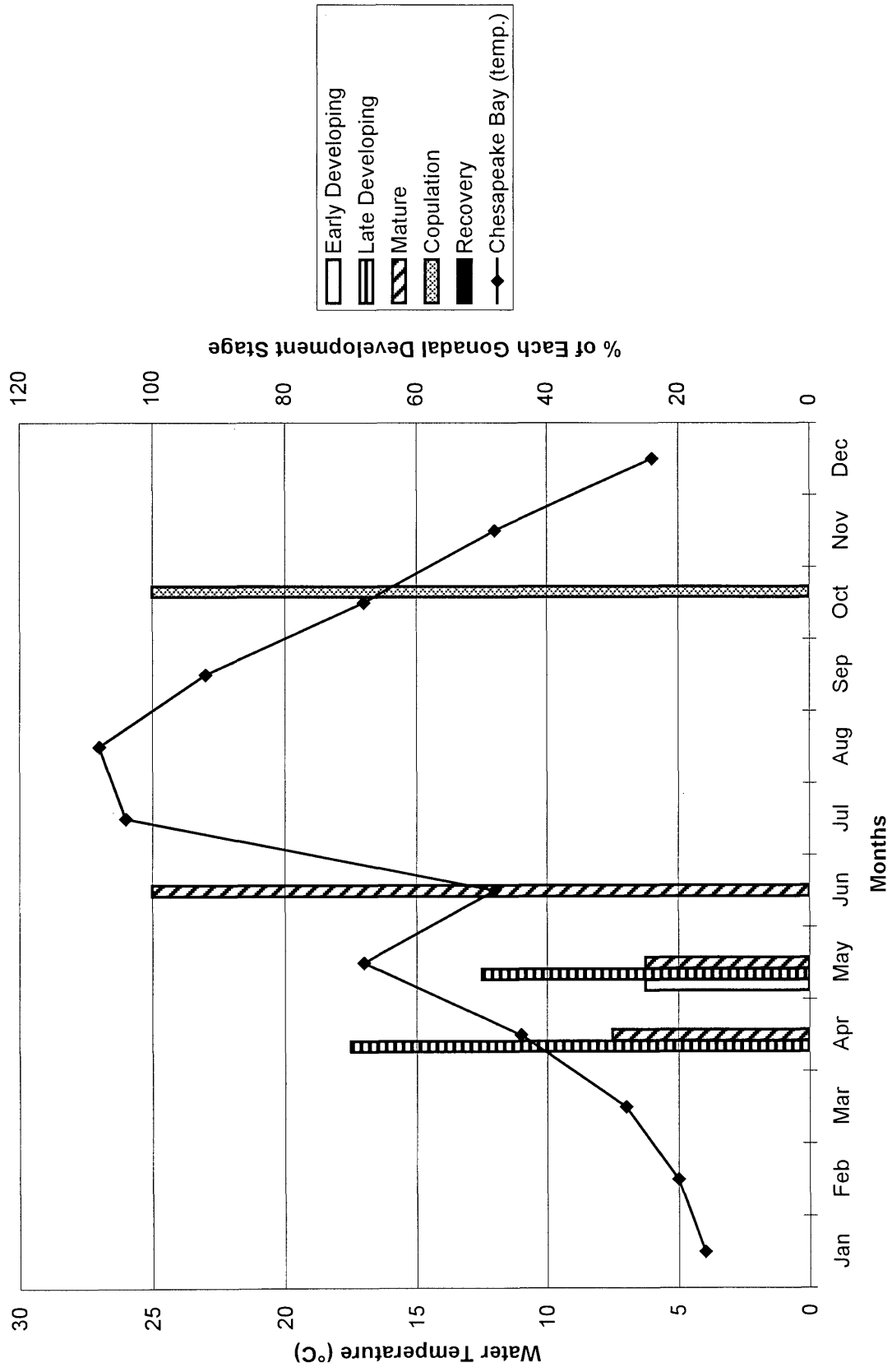


Figure 19: Gonadal development for all imposex females, from James River and Ocean View.

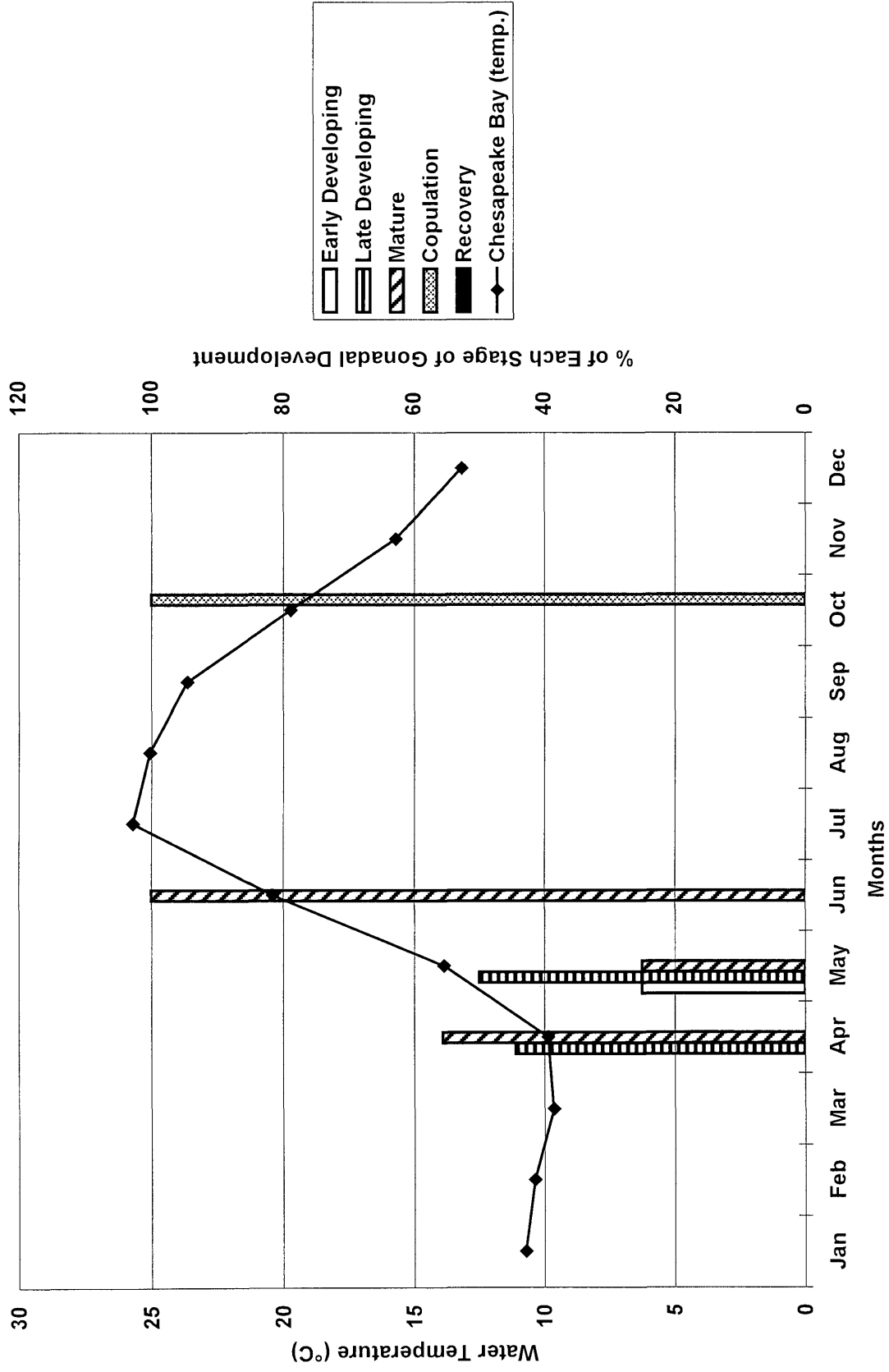


Figure 20: Gonadal development for all females, both imposex and normal, from James River and Ocean View.

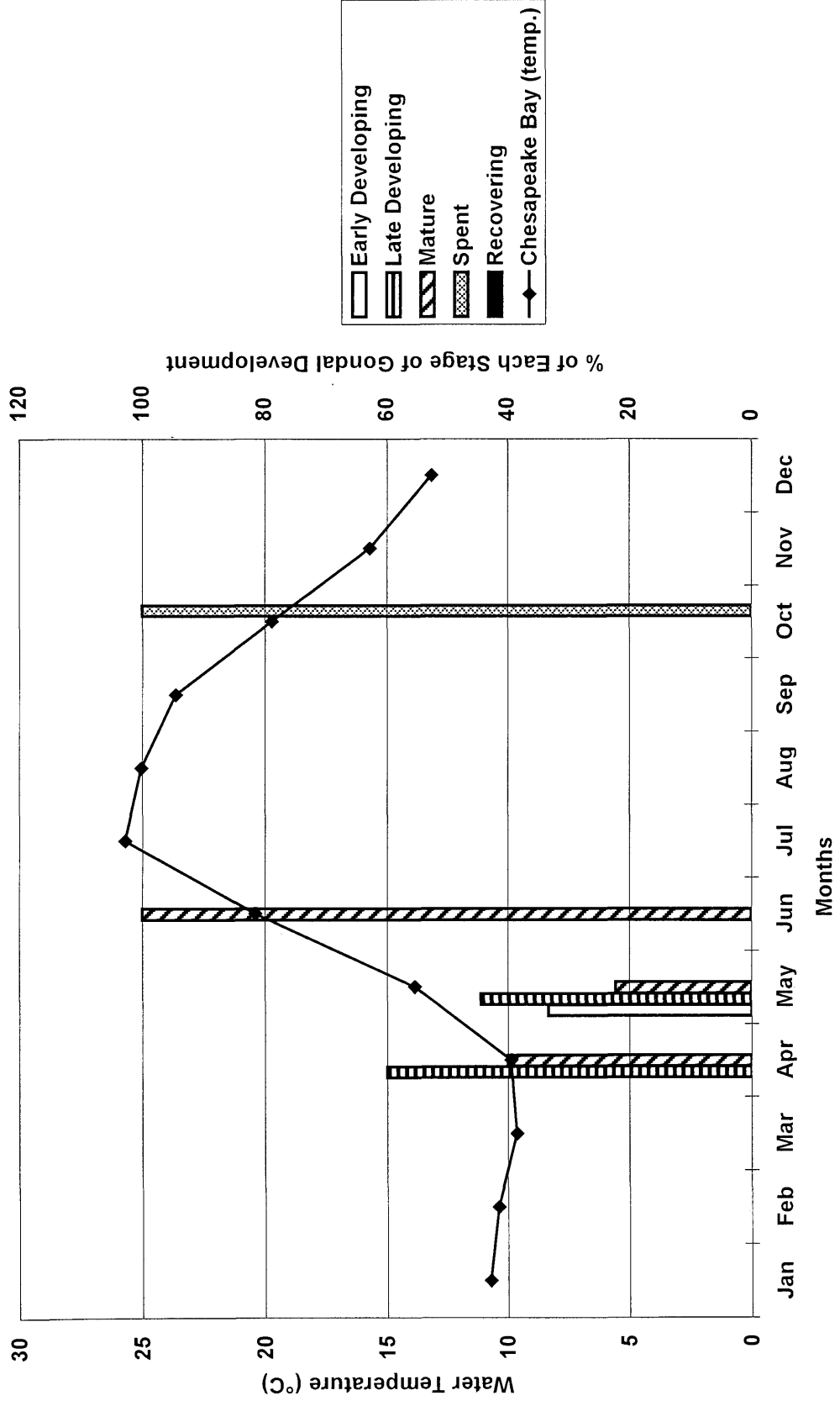
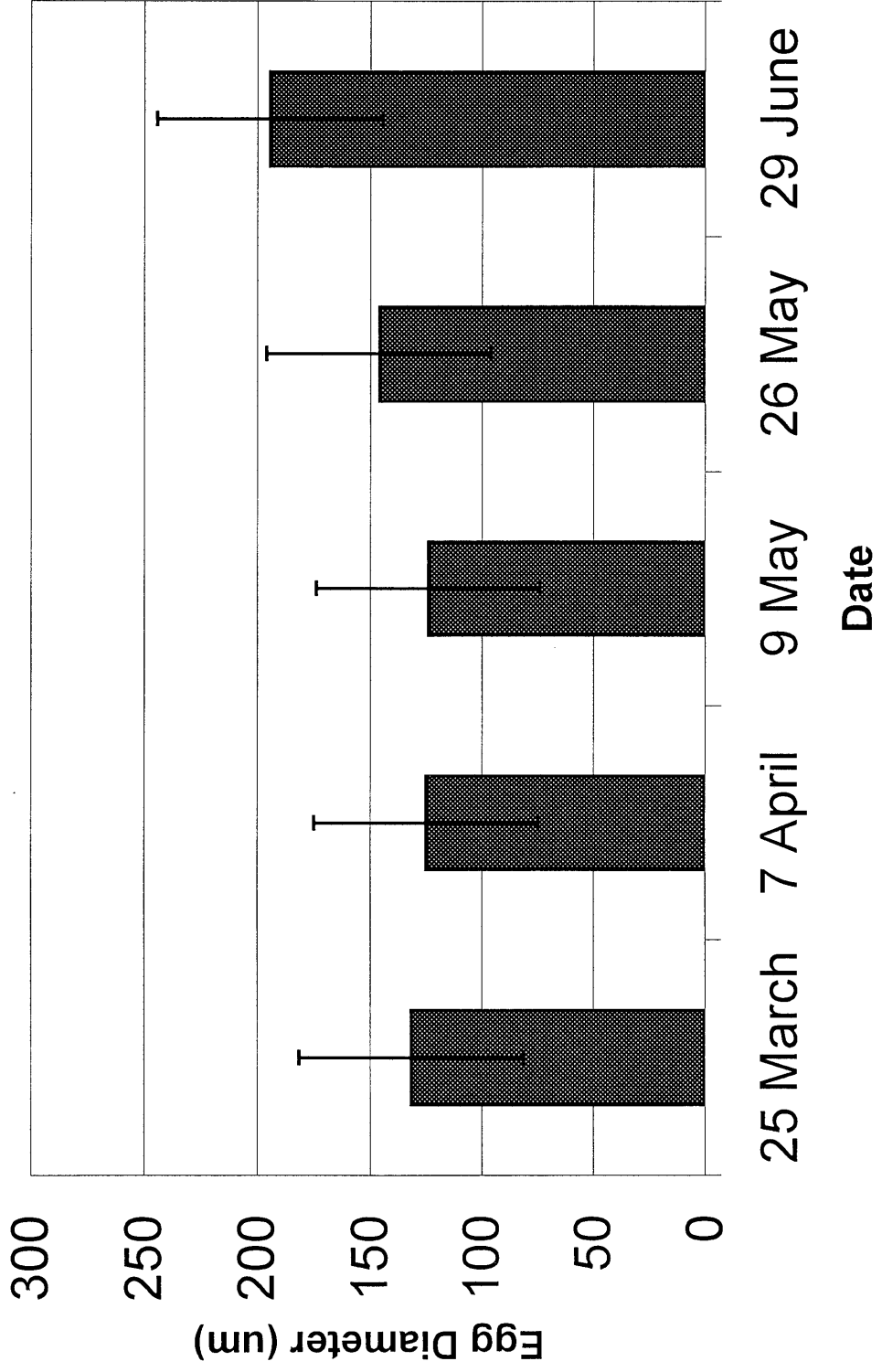


Figure 21: Average diameter of *Rapana venosa* eggs.





germinal epithelium. Mature eggs may appear polygonal in shape instead of being round or ovate because they are so large and are competing for space. The nuclei of mature eggs were surrounded by very fine yolk granules and aligned near the center of the lumen (Figure 13). Female whelks in the mature stage were found from April through June, when water temperatures rose from 10 to 20 °C.

A significant decrease in gonad area occurs during the latter half of the copulation stage. As the lumen is emptied of mature eggs, the central areas often contain only a few scattered yolk granules (Figure 14). Oogonia and small oocytes remain attached to the germinal epithelium. Female whelks in the copulation stage were found in the month of October only, when the water temperature was 20 °C.

No female gonad in the recovery stage was observed in this sample set.

Very few females exhibited gonadal abnormalities. One imposex female from James River (26 May 1999, 140mm) had an almost orange-colored gonad, and the digestive gland was dark purple. Another imposex female from James River (15 Oct 1999, 135 mm) had a ~2 cm blister on the gonad, similar to those reported in males.

#### Imposex Frequency and Penis Size

The total number of animals included in the sample set was 96 (Table 6). Two animals were excluded from the data sets used for calculations because of the nature of their anomalies. The 111 mm male whelk from James River (9 May 2001) was excluded because it had a double penis. One animal from the 7 Apr 2000 sacrifice (158 mm, 802g) was excluded

due to the fact that its true sex remains unknown. This animal exhibited signs of being an imposex female (i.e. large SL, relatively small penis size) that was producing sperm. For classification purposes only, this specimen is considered to be imposex.

	<b>Male</b>	<b>Imposex</b>	<b>Female</b>
<b>James River (JR)</b>	24	14	0
<b>Ocean View (OV)</b>	26	17	15

Table 6: Number of animals sampled from each site, listed by sex.

At the James River site, 100% of females were imposex, and 51.6% of Ocean View females were imposex. The imposex frequency of both populations combined is 66.7%. Female whelks from the James River site have an RPS value of 3.45%, and those from Ocean View have an RPS value of 0.429%. The RPS index for both populations combined is 0.956%.

Two tailed t-tests were performed using Microsoft Excel to determine if there were any significant differences between male penis size at each site, and female penis size at each site, respectively. James River males had an average penis size of 38.1 mm, and Ocean View males had an average penis size of 33.8 mm. There was a significant difference (0.0018) between the two sites for males. James River imposex females had an average penis size of 12.4 mm, and Ocean View imposex females had an average penis size of 10.6 mm. There was no significant difference (0.1790) between the two sites for imposex females.

Figure 22 depicts penis length as it is related to the shell length and sex of each animal sampled (with the exceptions as noted above). All normal females were recorded as having a penis length of 0 mm. Imposex females had penis lengths within the range of 5-20 mm, and males had penis lengths between 20-50 mm. No vas deferens was present in any imposex female *R. venosa* that was dissected for the purpose of elucidating gross reproductive anatomy.

Figure 22: Penis size as related to shell length and sex.

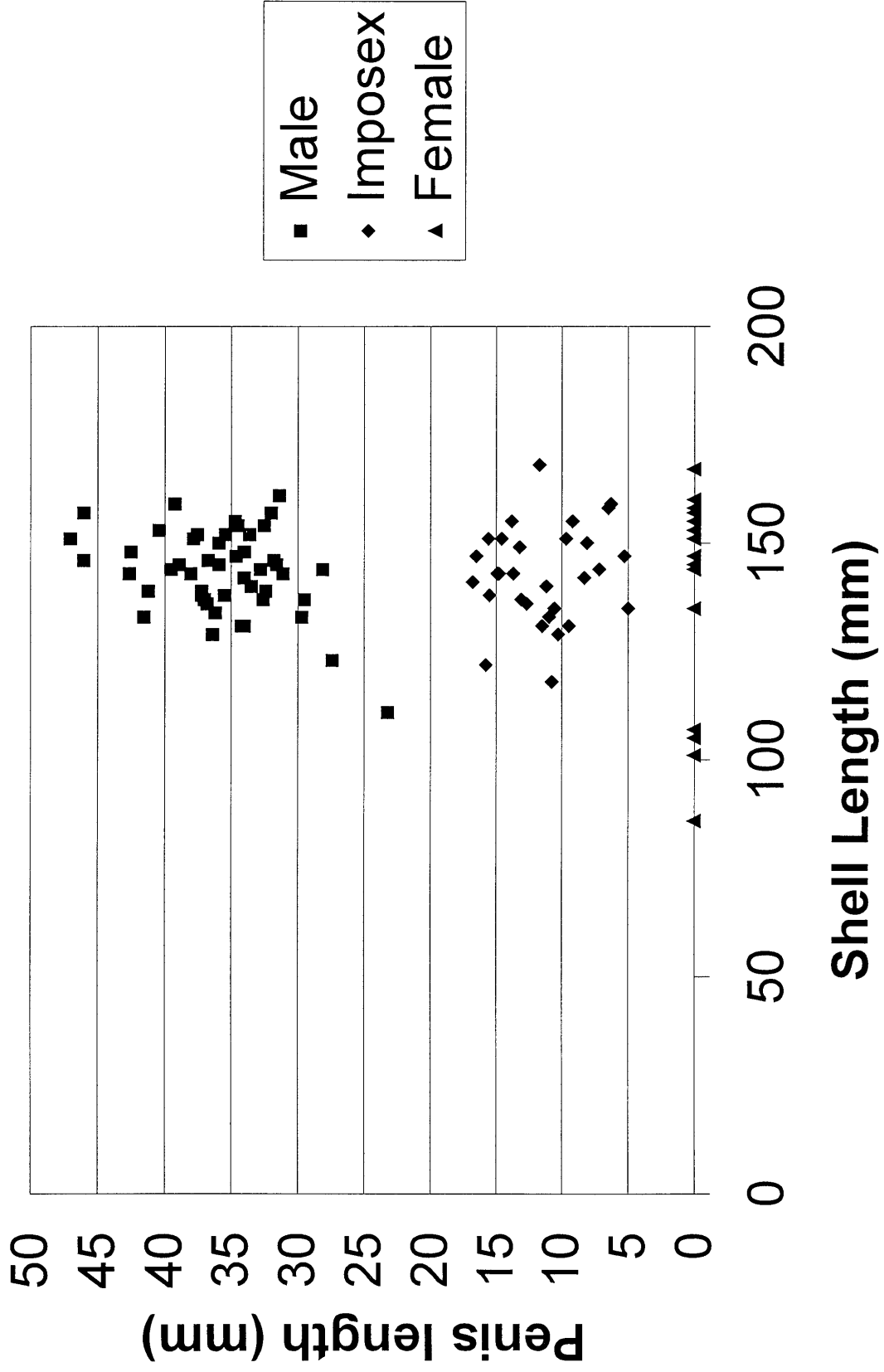
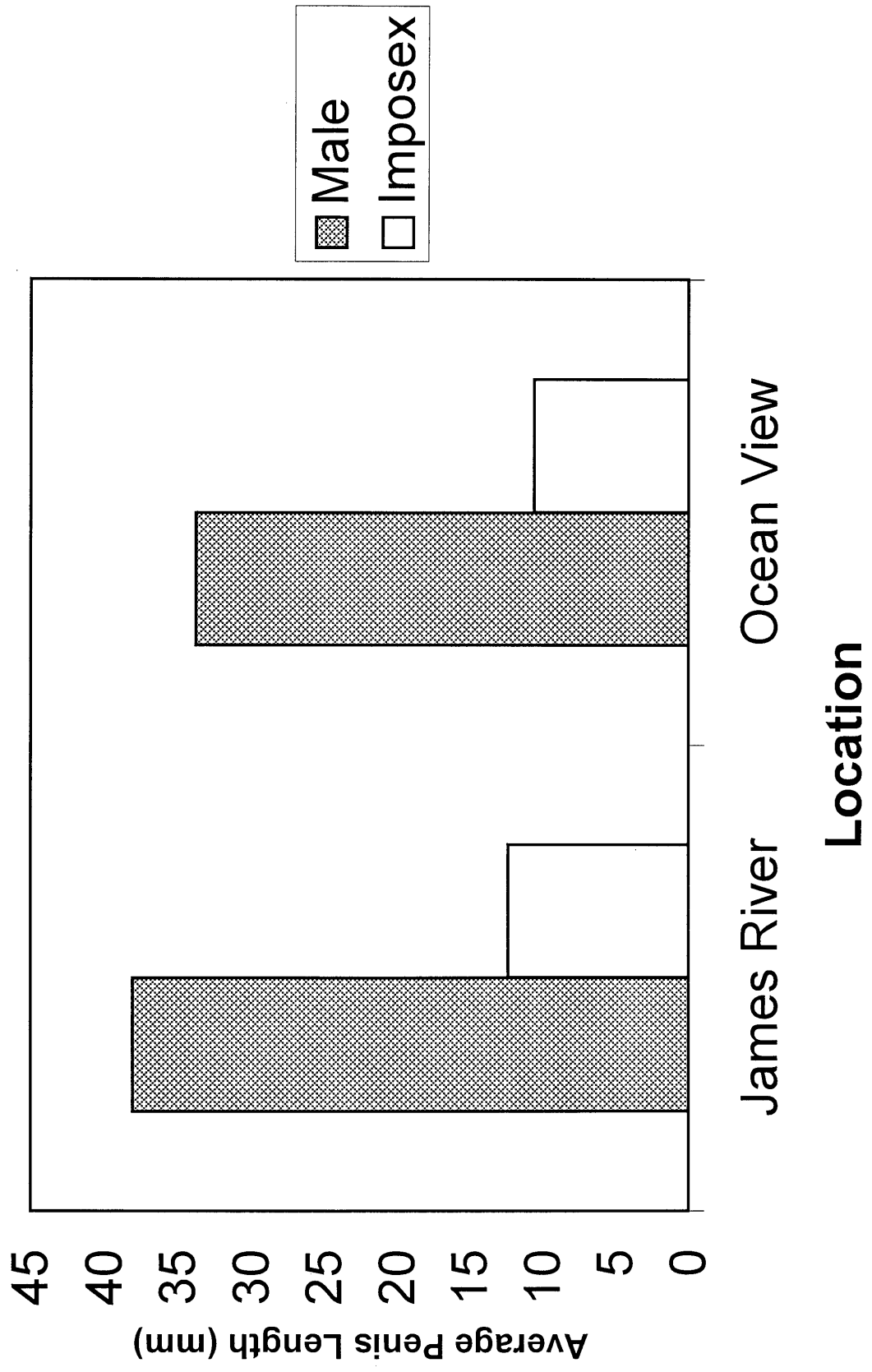


Figure 23: Average penis size of all sexes.





## DISCUSSION

### Successful Reproduction of *R. venosa* in the Chesapeake Bay

Based on observations of specimens collected from both study sites (JR and OV), both of these geographical end members contain functionally reproductive populations. There do not appear to be any individual reproductive foci (i.e. only one or a few specific sites containing reproductive animals) within the documented distribution range. Also, egg laying in captive animals occurs after the snails leave mating aggregations, so it is logical that members of the wild population would follow suit.

There do not appear to be any environmental constraints on *R. venosa* in the Chesapeake Bay when compared to those of its native Korean habitat. Adult *R. venosa* are able to tolerate a wide range of salinities. In Asian waters, the majority of *R. venosa* live in full salinity locations. Salinities for the lower Chesapeake Bay can range from 28 ppt near the bay mouth to 15 ppt near the James River Bridge. During normal tidal fluxes, and especially in times of drought or extreme rainfall, salinities in the upper James River are subject to frequent change, yet *R. venosa* is found living and reproducing near the James River Bridge.

Water temperatures of both native and non-native distribution ranges are similar throughout the year, with Korea having a colder water temperature during the winter than the Chesapeake Bay (Figure 24). Data for gametogenesis in Korean animals (after Figure 4 in Chung et al., 1993) is compared with collected and extrapolated data for Chesapeake Bay

specimens in Figure 24. Korean *R. venosa* are described as having an earlier recovery period, peaking in August, compared to specimens within the Chesapeake Bay, which probably reach their peak recovery period in December. The developing stage of both sexes also follows the trend of occurring earlier in Korean animals than in those from the Chesapeake Bay. The frequency of mature Korean *R. venosa* peaks in January for males and April for females. Spent males are first observed in February in Korea, though spent females are not observed until April. The timing delay between the two sexes is indicative of females effecting sperm storage.

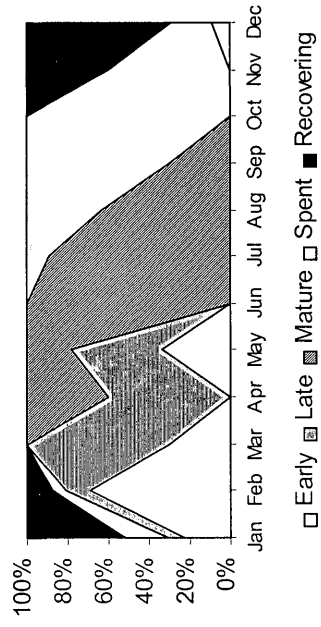
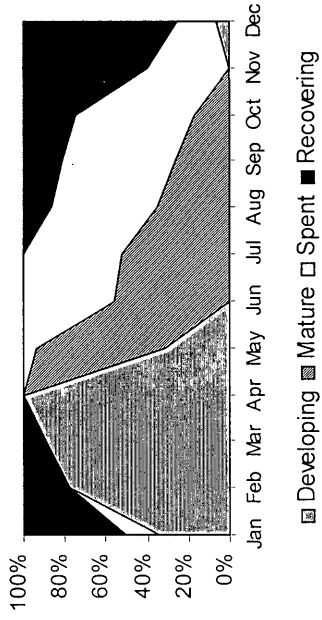
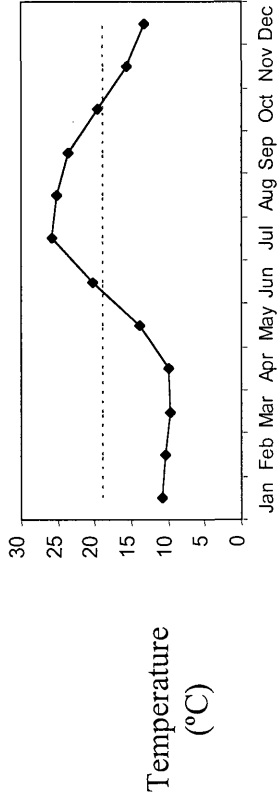
The critical point in gametogenesis, as related to temperature, occurs when mature females are ready to lay their eggs. Temperatures necessary for *R. venosa* larval development of 18°C or greater have been observed (Chesapeake Bay: 18-20°C, personal obs.; Korea: 18-20.5°C, Chung et al., 1993; Black Sea: 20-22°C Chukhchin, 1984). Assuming a critical temperature of 19°C, as represented by the dashed line in Figure 24, the optimal time for larval development is from June to October in both Korean and Chesapeake Bay waters. Korean egg masses deposited in April are subjected to a water temperature of 11°C (Chung et al., 1993), which is not yet warm enough to support developing larvae. Many of these early egg cases probably do not yield viable larvae.

The data from Figure 4 in Chung et al. (1993) suggest that egg laying for Korean *R. venosa* begins in April. However, Figure 5 (from Chung et al., 1993), which represents egg masses collected from the field, shows no egg laying activity in April. A close agreement exists between when egg masses were collected from Korean field sites, from May through

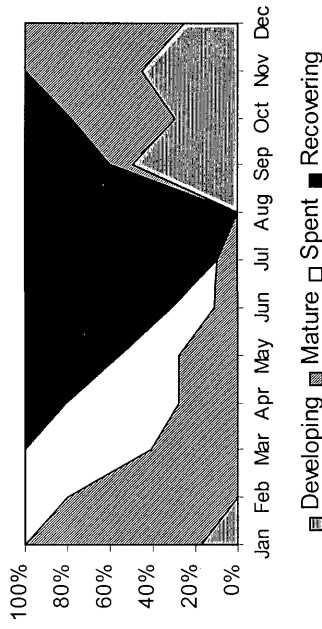
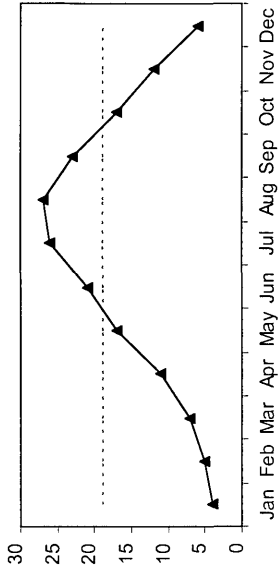
August, which is when Chesapeake Bay *R. venosa* were observed laying eggs in the laboratory.

Figure 24: Gonadal development: Chesapeake Bay vs. Korea.

# Chesapeake Bay



# Korea



Food sources for *R. venosa* within the Bay are plentiful and adult *R. venosa* happen to prefer the same habitat of one of its main prey items, the hard clam, *Mercenaria mercenaria*. Although oysters have been reported as a popular prey item in the Black Sea (Chukchin, 1984), adult Rapa whelks have not yet become a nuisance to existing oyster beds in the Chesapeake Bay. Young *R. venosa* raised in the wet laboratory prefer small oysters (*Crassostrea virginica*) to small hard clams (personal observation), most likely due to the thin shell of the prey which provides ease of penetration. The threat of adult Rapa whelks turning to oyster beds as a food source in the Chesapeake Bay is serious, both in ecological and economic respects. Currently, oyster restoration programs are underway in an attempt to repopulate the Bay with this important cornerstone species, and large amounts of both money and manpower have already been invested in this goal (Luckenbach et al., 1999; Mann, 2000). Overfishing, environmental degradation, and disease were factors responsible for the rapid decline of *C. virginica*, and the recent invasion of the Rapa whelk to the Chesapeake Bay is yet another negative component of this equation. During a 20 year period *R. venosa* decimated four dominant bivalve species of the Gudaytskii oyster bank in the Black Sea (Chukchin, 1984). Measures to control or reduce the *R. venosa* population within the Chesapeake Bay should strongly be considered. Creating an export fishery for the invasive species is one option, as there is a very small local market for native gastropod products (“conchs”).

As an invader of the Chesapeake Bay, *R. venosa* has not met much resistance in the way of predators, parasites, or disease. In the native habitat of *R. venosa*, octopods have been known to prey upon adult snails. Within the Chesapeake Bay, adult Rapa whelks (>100 mm)

are subject to low predation due in part to their thick shell and large, boxy shape. Small Rapa whelks may be subject to predation by sea turtles, crabs, and local gastropods (Harding and Mann, 1999).

After the sacrifice of over 400 *R. venosa* to date, only a few cases of parasitism by an unidentified roundworm were observed. Interestingly, young adult Rapa whelks do tend to accumulate the boring worm *Polydora websteri* on the outside of their shells (Mann and Harding 2000). In cases of extreme fouling the result can be numerous “mud blisters,” places where the worms have bored through most of the gastropod’s shell layers and the waste products of *P. websteri* accumulate. These blisters are visible in empty snail shells. Accumulation of *P. websteri* most likely occurs during the epifaunal early life history period when living on hard substrate and prior to a transition to a burrowing infaunal lifestyle. Infaunal adult Rapa whelks are often collected with little or no fouling of this type. At this time, no disease has been observed in *R. venosa*. The Rapa whelk has managed to successfully navigate the previously mentioned obstacles in its new habitat, and their invasion of the Chesapeake Bay can be termed a success.

The relationship between gonadal development in Chesapeake Bay specimens and water temperature is consistent with 1) the previously described reproductive activity in Korean populations (Chung et al., 1993); 2) observations of egg laying in the VIMS wet laboratory during the summers of 1999-2001; and 3) field collections of egg masses and reports by watermen of egg laying during the summers of 1999-2001.

Imposex females appear capable of producing viable eggs. Viable larvae have been produced by both normal and imposex females and have been cultured successfully in the

VIMS wet laboratory from the summer of 1999 to present (unpublished data). Histologically, eggs from normal females do not appear to be any different than eggs from imposex females. At present, a vas deferens has not been observed in any of the dissected Chesapeake Bay *R. venosa* specimens which exhibit imposex. No size-fecundity relationships have been rendered at this time, but data collection is in progress by other researchers. Research performed during the course of this thesis should provide support for related reproductive studies.

### Imposex and Tributyltin

TBT has not been confirmed as the cause of imposex in Chesapeake Bay *R. venosa*. Assuming that TBT is the culprit, several questions still remain unanswered at this time.

1) What level of TBT is necessary to induce imposex in *R. venosa*? Imposex can be induced in *N. lapillus* when TBT water concentrations are less than 1 ng/L (Gibbs, 1993). Given that TBT water concentrations are slightly less than 2 ng/L near the James River Bridge (Unger, 2001), adult Rapa whelks in this area are probably exposed to a somewhat higher concentration while burrowed. Bioaccumulation of TBT by hard clams (and other bivalves which are potential prey items) should also be taken into consideration when looking at methods of exposure. No other research has been published to date on the topic of imposex in *R. venosa*.

2) Once imposex has been initiated, what levels of TBT would be needed to cause more than Stage 3 imposex in Chesapeake Bay *R. venosa*? There appears to be a range of penis lengths for imposex animals at Stage 3 (Figure 22), so would lengths of subsequent imposex stages fall within such clearly defined size ranges?



3) Once imposex has been initiated, what levels of TBT would be needed to cause a complete sex change (female to male) with the ability to produce (viable) sperm? All but one of the imposex female specimens that have been collected fall into the category of Stage 3 imposex as defined by Gibbs et al. (1987), with the exception that no vas deferens formation has been observed. This will be discussed further in another section.

4) Is TBT passed on from the parent to the offspring (i.e. nutritive lipid transfer) or is TBT somehow included in egg case material as part of a detoxification strategy? *R. venosa* egg cases are composed of a protein-based material. Analyses of *R. venosa* foot tissue and egg capsules for TBT are underway by other researchers. Data from these analyses will be useful in attempting to answer many of these questions.

If TBT is not the cause of imposex in *R. venosa*, there are alternative contaminants which may be at fault, or perhaps it is a combination of more than one contaminant. Members of the organotin family, such as tri-n-propyltin (TPrT) have been shown to induce imposex in *N. lapillus* (Bryan et al., 1988). Laboratory experiments conducted by Nias et al. (1993) on *Lepsiella vinosa* suggest that copper may also induce imposex in these and other gastropods.

Conversely, there have also been reports of gastropods that seem unaffected by TBT-induced imposex. Gibbs et al. (1997) performed a series of experiments on five different gastropod species to determine the differential sensitivity of each to TBT. Four of the species developed imposex to varying degrees, while females of the fifth, *Columbella rustica*, did not exhibit any signs of masculinization (Gibbs et al., 1997). Ide et al. (1997) compared the accumulation of organotin compounds in two gastropods species, one of which was *Neptunea*

*antiqua*. Adult females of this species did not show signs of imposex, despite high body concentrations of TBT. Juveniles were still affected by imposex, however. This exception is significant in that the timing of TBT exposure during the life history of the animal may result in drastically different outcomes, or may regulate the severity of the imposex response.

Sex change and reproductive anomalies in invertebrate species other than gastropods, as well as some vertebrates, have been observed in response to endocrine disrupting contaminants. Nonylphenol, a non-ionic surfactant found commonly in detergents and plastics, has reportedly affected reproduction and development in the marine copepod *Tisbe battagliai* (Bechmann et al., 1999), larval development of *Crassostrea gigas* (Nice et al., 2000), and induced egg production in male fish (Sumpter, 2000). Nonylphenol has anti-androgenic properties. Other anti-androgens include some DDT metabolites (Metcalf et al., 2000; Semenza et al., 1997; Sumpter, 2000), methoxychlor (Sumpter, 2000), polychlorinated biphenyls (PCBs) (Olsson, P.-E. et al., 1999) and  $\beta$ -sitosterol (Tremblay and Van Der Kraak, 1998). Many polycyclic aromatic hydrocarbons (PAHs) have been reported to exhibit anti-estrogenic qualities (Sumpter, 2000).

There is a difference in imposex frequency between James River and Ocean View, but the cause of this difference is uncertain, though it may be spatial. The bottom type at each site is different: James River is typically a mix of mud and sand, while Ocean View is characterized by hard sand bottom. The difference in bottom types may affect the ability of contaminants, such as TBT, to concentrate within the sediment. There is also a greater amount of water movement to aid in flushing out of contaminants in the water column at the Ocean View site.

### Histological Methods

Many of the samples used for histological analysis were preserved in AFA for as long as two years. The length of time spent in preservative was considered to be the probable cause of brittle tissue (in female gonads only, usually mature) encountered while cutting blocks on the microtome. A comparison was effected with fresh gonad samples which were preserved in AFA or Bouin's for the recommended 48 hours. Neither the reduction of time spent in preservative, nor the use of a different preservative made the tissue any less brittle. The use of a brief ice water or chilled glycerin soak made most of the brittle samples easier to cut on the microtome, but they would often dissociate abruptly upon contact with the water bath. This reaction suggests that the eggs were so tightly packed in the gonad that the paraffin was unable to completely infiltrate the sample. Even though there was no noticeable difference between the samples preserved in AFA versus Bouin's, the samples that were preserved with Bouin's stained much more brilliantly than those preserved in AFA.

Notes were made of any gonadal abnormalities, either during the sacrifice of the animal or the preparation of gonad samples for the tissue processor. None of these abnormalities reflected negatively on the physical state of the gonad sections examined, nor did they appear to affect egg-laying females who produced egg masses prior to being sacrificed. *R. venosa* specimens with a gonad of unusual color may have been in poor health. Those specimens with blisters or pustules may have simply been reacting to the intrusion of *P. websteri* boring through the shell.

Minor difficulties were encountered while measuring egg size under the microscope. First, not all eggs are round. When choosing eggs to be measured, those that were round, evenly polygonal, or slightly ovate were preferred. In the case that the egg was slightly ovate, the longer dimension of the egg was measured. Second, because gonadal development is a continuum, not all egg diameter measurements fell exactly within the size range defined for each category. The stage of gonadal development was ultimately determined by the slide reader to be a combination of visual inspection of the slide and egg diameter measurements. In some cases, only small eggs were observed (and measured) because larger eggs had fallen out of the section while it was floating on the water bath. Thus, a gonad which was mature could easily be mistaken for one in the early or late developing stage if the process was based on measurements alone. When the eggs are mature, very small yolk granules tend to cluster around each nucleus, while larger yolk granules remained in the rest of the egg. Even if only fragments of large, mature eggs remained, this telling signature was an indication of maturity. Slides containing intact eggs, yet having measurements that fell between egg size categories, (i.e. borderline between early and late developing) were decided by the reader based on the average egg diameter and the overall appearance of the gonad.

As reported in the results, the gonadal development of the six James River specimens appeared to be, for the most part, synchronous. Some degree of gamete regeneration (i.e. sperm precursors, and oogonia arising from the germinal epithelium) was observed through the copulation stage. If this is the case, why do *R. venosa* females lay several egg masses over a given time period instead of laying one continuous egg mass all at once? Perhaps the females are not so much limited by egg production as by the production of egg case material,

which appears to be a tanned protein. The examination of a larger sample size, in various stages of development, would be helpful in answering the questions concerning gonadal synchrony.

### Male Gonadal Development

In the developing stage, spermatozoa were present in the seminal vesicles even when none were present in the gonad proper. West (1978) reports a similar observation for the buccinid gastropod *Colus stimpsoni*. Do sperm from the previous breeding season get used, and are they viable?

During the copulation stage of both sexes, gonad content is lost. Many slides representative of this stage gave poor histological preparations. This is probably a result of a lack of tissue content within the section rather than a paraffin infiltration problem. When the ribbon is placed on the water bath, there is little or no cohesive tissue remaining to hold the section together, and it abruptly dissociates as the paraffin melts.

Male *R. venosa* in the developing stage most likely occur before April within the Chesapeake Bay, but this is not evident in the graphs (Figures 12-14). Similarly, there are no mature or spent males represented on the graphs during the late summer, nor are there any recovering whelks during the winter months.

The cause of external gonadal blisters is unknown, as is the cause of the lesions.

### Female Gonadal Development

Two common features of fully mature eggs involve the nucleus. Visual observation with a light microscope reveals several tiny yolk granules surrounding the nucleus, while the more typical large yolk granules remain in the other half of the egg. This is most likely the division of animal and vegetal poles, as described in Taylor and Anderson's (1972) work on *I. obsoleta*. Perhaps the presence of other compounds, such as lipids, may be revealed with different staining techniques. The second feature involves the migration of nuclei to the lumen centers when the eggs are mature (Figure 10). Raven (1961) reported a similar event occurring in some molluscs.

During the course of reproduction, females lag behind males slightly in gonadal development.

No true female *R. venosa* were obtained from the James River site during any time of year, nor were there any true females obtained from the Ocean View site during the month of June. Female whelks were observed to be in the early developing stage during the month of May, but not April. These results are the product of animals from one site only (Ocean View), and the results might be different if larger numbers of females from both sites were sampled. No mature or spent females were represented on the graphs during the late summer, nor were there any recovering females, as was previously noted for the males.

The 145 mm female from 7 Apr 2000 exhibited clumps of tissue near the digestive gland that stained a brownish color, possibly adipose tissue. The 155 mm imposex female from 7 Apr 2000 had clusters of what appeared to be undifferentiated cells in the gonadal

lumen. Again, different staining regimes would be valuable in determining unknown cell types and contents.

#### Gross Anatomy Dissection Methods

One of the objects of the gross anatomical dissections, as mentioned in the methods, was to examine the VDS progression in *R. venosa* imposex females. The vas deferens in males was easily visible with the naked eye, and it should also have been equally visible if present in imposex specimens. Thus, histology to determine the location of the vas deferens was not performed because it was deemed unnecessary.

The vas deferens does not appear to be present in *R. venosa* equivalent late Stage 2 or early Stage 3 imposex females. Due to the absence of a vas deferens, no oviduct blockage occurred as described for *Nucella lapillus* (Gibbs et al., 1987). Another TBT-induced imposex question arises from this observation: In advanced imposex (VDS Stages 4-6), would the vas deferens grow from two directions as in *N. lapillus*, or would it proceed in a unidirectional fashion? It would have been very helpful to have these data (VDS Stage, presence or absence of vas deferens) collected from all of the previously sacrificed specimens, and not just from a select few chosen specifically for the purpose of gross anatomical dissection. Even a perfunctory dissection of removing the mantle to reveal the presence or absence of a female gonopore to confirm male or imposex female status during sacrifices would have been of greater assistance.

It is strongly suspected that at least one animal from Ocean View (9 May 2001), originally thought to be an imposex female, has been misclassified due to the use of external examination only. The gonad contained sperm, and the small penis size (23.2 mm) in addition

to a small body size (SL = 111 mm) suggests this animal is actually a young male, and it was treated accordingly in the data sets. Conversely, there was another animal from Ocean View (25 Mar 1999), which appeared to be a legitimate imposex female (penis length = 26 mm; SL = 158 mm), though the penis size is considered to be somewhat large. Sperm was present in the testis duct, which transports sperm from the gonad to the vas deferens, and sperm precursors were present in the gonad.

Despite the fact that such an occurrence may be entirely plausible, one example is not enough to justify imposex females becoming reproductively functional males in *R. venosa*. Another possibility is that this whelk was a male with an exceptionally small penis for its given shell length. Because there was no strong supportive evidence for the whelk being classified as one particular sex, it was not included in any calculation where sex determination was a critical factor. The use of a perfunctory dissection in the case of these two animals would probably have identified their true genders.

### Opportunistic Sampling

With the use of an opportunistic sampling scheme, temporal, spatial, and size differences all play a role in determining the rate of sample returns. The watermen pursue certain fisheries at different times of the year. The patent tong fishery for hard clams (*M. mercenaria*) in the James River (Newport News Shellfish Management Area) is from December 1 to March 15. The dredge fishery for blue crabs (*Callinectes sapidus*) is from December 1 to March 31. The crab pot fishery for blue crabs runs from April 1 to November 30 (Virginia Marine Resources Commission website:



<http://www.state.va.us/mrc/page4a.htm>). Most of the Rapa whelks received from watermen are turned in during this time, especially while clams are still being used for bait. Rapa whelk activity increases at this time as a result of warmer water temperatures. The whelks are in search of prey items in order to prepare for the summer reproductive period, during which they will form mating aggregations. During mid-May, most watermen switch to using menhaden (“bunker”) as bait because it is cheaper. Rapa whelk returns from the bounty program tend to decrease after the change in preferred bait (Roger Mann, personal communication).

The spatial aspect of opportunistic sampling is influenced by where the watermen fish for their target species. This may be determined by the fishery itself, regulations pertaining to the fishery, or where the watermen prefer to fish within stated bounds. Patent tongs are typically employed in depths of 20-40 ft of water, crab dredges in 10-30 ft, and crab pots between 10-20 ft. All of the gear types are typically used on sediment or shell bottom types. Juvenile Rapa whelks are rarely caught by these gear types because they often prefer hard substrate to crawl on, such as rip rap. Some adult whelks may also be excluded from a given fishery if they burrow in depths not reached by the gear. Size selectivity by the gear also needs to be taken into consideration. Even if small Rapa whelks are within the boundaries of a given fishery, they may slip through the teeth of patent tongs, or through the rings of a crab dredge or crab pots. (It should be noted, however, that most snails caught in the crab pot fishery are found clinging to the outside of the pots.)

So how can the invader be sampled throughout the bay without destroying the natural habitat? Even if it were possible, dredging large sections of the Chesapeake Bay is not an

ecologically sound method, nor would it be cost effective. At present, employing a bounty system with Chesapeake Bay watermen seems to be the most successful scheme, despite gaps in resulting sample sets for certain times of the year. There are numerous commercial fishing boats in operation throughout the year, and various methods of capture (though unintentional) are employed. The limitations of this approach are that watermen are probably not sampling all of the possible habitats where *R. venosa* may occur. Given that the complete distribution range of *R. venosa* within the Chesapeake Bay is not known, there may well be snails thriving in areas which are not subject to active fishery exploration. Further broad scale exploratory sampling, such as with side scan sonar, may eventually yield a more complete picture.

The effects of opportunistic sampling are evident when site demographics are examined. The lack of male and female samples for November through March is a product of opportunistic sampling, whereas the absence of data during the late summer months is a result of experimental conflict. Breeding experiments performed by other researchers were taking place at this time, so specimens were not available for sacrifice. Despite this fact, there have been observations of mating and egg laying in captive whelks during the summer months, even when the recorded data do not indicate such activity.

### Ecology of Invasions

*R. venosa* does not appear to have any biological or physical limitations in regards to survival and reproduction in the Chesapeake Bay. If this non-native species is allowed to thrive unchecked in the Chesapeake Bay, a situation similar to that which now occurs in the Black Sea may result. According to Zolotarev (1996), the low species diversity, low levels

of competitive interactions, and high anthropogenic input to the Black Sea have played a role in the ability of *R. venosa*, and other invasive species, to spread so quickly. The greatest impact *R. venosa* had in the Black Sea was on the populations of large bivalve molluscs (Zolotarev, 1996). Greatly reduced (native) species abundance in a location with already low biodiversity serve to destabilize the native ecosystem. Overfishing, disease, and anthropogenic input have already weakened the ecosystem in the Chesapeake Bay, and the growing presence of *R. venosa* puts that ecosystem in increased jeopardy.

Continued spread of Rapa whelks both within and beyond the Chesapeake Bay will rely heavily on the dispersal of the planktonic larval form, which may be transported via water currents and ship ballast water. Based on temperature and salinity tolerance data, *R. venosa* from the lower Chesapeake Bay have the potential to invade as far north as Cape Cod, Massachusetts, and as far south as Charleston, South Carolina (Mann and Harding, 2000).

Reproduction in Chesapeake Bay *R. venosa* females does not appear to be hampered by the condition of imposex. If imposex had a serious negative impact on Rapa whelk reproduction to the degree that *Nucella lapillus* is affected, this invasion would pose a less severe threat to the Chesapeake Bay ecosystem. Instead, *R. venosa* imposex females appear to be capable of reproducing as normal females, so reproduction can occur even at a site such as James River where all the females are affected by imposex. The continued spread of *R. venosa* within the Chesapeake Bay could potentially negatively impact the niches of local gastropods, such as the Atlantic Oyster Drill (*Urosalpinx cinerea*), and affect local bivalve populations (prey items - *Mercenaria mercenaria*, *Crassostrea virginica*).

## CONCLUSIONS

1. The current distribution range of *Rapana venosa* is equivalent to the functional reproductive range.
2. There does not appear to be any significant temporal or spatial reproductive variation between the James River and Ocean View sites.
3. The observed relationship between gametogenesis and water temperatures in animals from the Chesapeake Bay is consistent with previously described seasonal reproductive activity in a native (Korean) population, as well as observations of egg laying during mid-May through mid-August and field collections of egg masses in the Chesapeake Bay.
4. There do not appear to be any reproductive differences between imposex and normal females, despite the presence of male sexual structures on imposex females.
5. Both James River and Ocean View populations are successfully reproducing.

## APPENDIX

### Rapa Whelk with Linguine

Chef C. Meredith Nicolls, Jr. 1999, Café Rosso, Norfolk VA

#### *Ingredients:*

- 4 tablespoons olive oil
- 4 Rapana, very thinly sliced
- 4 cloves of garlic
- 4 tablespoons chopped parsley
- dry white wine
- crushed red pepper to taste
- salt and pepper
- ½ pound of linguine, pre-cooked

#### *Cooking directions:*

In a thick-bottomed saute pan, heat olive oil (be careful not to burn!). Add garlic, red pepper, and whelk. Saute briskly for 3 to 4 minutes. De-glaze with dry white wine. Add parsley. As wine is reducing, add pasta and stir vigorously. Once hot, divide equally on four plates and enjoy!

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### Rapa Fritters with Garlic Sauce

David T. Gauthier, 2000

#### *Fritter Ingredients:*

- 1 cup all purpose flour
- 2 teaspoons salt
- 1 scant cup milk
- 4 tablespoons ouzo or brandy
- 1 egg, lightly beaten
- 2 tablespoons chopped fresh cilantro
- small bowl of additional flour
- Rapa whelk meat

*Batter Preparation and Cooking Directions:*

Sift flour and salt into a large bowl, and make a well in the center. Gradually beat in the milk, ouzo or brandy, and egg to form a smooth-pouring batter. Stir in cilantro and let rest for 30 minutes.

To fry: Cut whelk meat into bite-sized chunks. Heat vegetable or peanut oil in a deep pan to 350 degrees or until a cube of bread brown in 30 seconds. Dust the whelk with seasoned flour, dip in batter, and deep fry in batches for 4 to 5 minutes or until crisp and golden. Drain on paper towels and serve with garlic sauce.

*Garlic Sauce Ingredients:*

- 2-3 slices of stale white bread
- 4 tablespoons water
- 2 garlic cloves
- 1 tablespoon lemon juice
- 1-2 pinches of cayenne pepper
- 6 tablespoons olive oil
- 4 tablespoons all purpose flour

*Garlic Sauce Preparation:*

Pour water over the bread and soak 5 minutes. Squeeze out the water, place the bread, garlic, lemon juice, and cayenne pepper in a blender and puree to form a thick paste. Gradually work in the oil a little at a time to form a thick sauce, thinning with 2 to 3 tablespoons boiling water if the sauce is too thick. Transfer to a bowl, cover and set aside until ready to use.

Rapa Chowder (New England Style)

Erica Westcott, 2000

*Ingredients:*

- 1 quart of rapa whelk meat
- 1/4 cup salt pork, diced
- 3/4 cup onion, sliced
- 3 cups potatoes, cubed
- 2 cups cold water
- 2 ½ cups milk, scalded
- ½ cup cream
- 2 tablespoons butter

*Cooking directions:*

Rinse and chop whelk meat into small chunks. Cook salt pork in kettle or deep pot over low heat until almost crisp. Add onions and simmer for 5 minutes or until soft, but not browned. Add potatoes and water, cover and simmer for 10 minutes. Add chopped whelk, scalded milk, and cream. Cook gently for 15-20 minutes or until potatoes are tender. Add butter, salt, and pepper to taste.

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## VITA

### **ERICA S. WESTCOTT**

Born in Westlake Village, California, 15 April 1977. Graduated from Reno High School in May, 1994. Received B.S. in Biology (*cum laude*) from Clark Atlanta University in May, 1998. Entered Master's Degree program at Virginia Institute of Marine Science/College of William and Mary in August, 1998.