Life history, trophic ecology, & prey handling by cownose ray, *Rhinoptera bonasus*, from Chesapeake Bay

(NA07NMF4570324)
Grant No. 713031

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November, 2010

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Executive Summary

Concerns over predation on commercial bivalve resources have been raised by fishery and aquaculture operations for many years and in several regions of the world. However, little evidence of actual predation on these resources has been documented and little is known about cownose ray biology and population that could be used to manage a fishery.

As a member of elasmobranchs fishes, cownose ray pose significant fishery management concerns: late age at sexual maturity, low fecundity, and long gestation. In addition to these biological constraints, demographic, social behavior, and trophic ecology characteristics of cownose ray subjected to a commercial fishery could impact management decisions.

This study aimed to document the age and growth and predation for cownose ray (Rhinoptera bonasus), the western Atlantic species of ray, focusing on the population that utilizes the Chesapeake Bay for pupping and mating during summer months.

Age and Growth

Male and female cowose rays were found to reach sexual maturity between ages six and eight, with females not contributing to recruitment until year eight or nine due to length of gestation.

Age estimates were obtained through vertebral centra analysis of 536 rays (217 males and 319 females). Rays sampled ranged from 30-110.5cm disc width (DW). The largest female ray recorded was 110.5cm DW (27.71 kg) at estimated age of 19. The largest male was 98cm DW (15.8 kg) and estimated at 16. The oldest ray observed was a female estimated at age 21 years with a disc width of 107cm.

Five growth models were fitted to length-at-age data; two forms of the three-parameter von Bertalanffy growth model were best-fitted, given males and females exhibited different growth patterns and indicating females grow larger than males.

Rate of Maturation

Mean DW at maturity for males and females was about 85cm, corresponding to age at maturity of 6-7 years old for males and 7-8 years old for females. Sexual maturity of female rays was largely determined if gravid or by these characteristics: (1) diameter of the largest ova, (2) uterus width (left uterus), (3) other characteristics, and (4) histological sampling of ovaries and left uterus. Sexual maturity of male cownose rays was determined using the following criteria: (1)vas deferens coiling (none, partial, complete; Neer & Caillliet, 2001); (2) milt (sperm-containing secretion) presence/absence from vas deferens and/or the urogenital papilla at the cloacal opening; (3) clasper calcification (not calcified, partially calcified, and calcified) and ratio of clasper length to disc width (Smith & Merriner, 1986), and (4) histological sampling. Cownose ray DW/weight relationship was described by the power curves $y = 1E\text{-}05x^{3.2596}$ ($R^2 = 0.9884$) for females and $y = 1E\text{-}05x^{3.2064}$ ($R^2 = 0.99$) for males.

Feeding Ecology

Oysters and clams were not found to make up a significant portion of the diet of cownose rays sampled from across the Cheseapeake Bay.

The stomachs of 781 cownose rays were sampled in the Chesapeake Bay from May 2006 to September 2009. Of these, 401 rays were obtained using fishery-dependent methods (haul seine and pound net), and 380 were obtained using fishery-independent methods, including a combination of a modified Dutch seine, long-line rigs, and bowfishing (bow and arrow). Dominant prey items of cownose ray observed in this study were thin-valved shellfish (shoft-shell, mocoma, and razor clams) and crustaceans with oysters and hard clams not observed as being a large part of the ray's natural diet.

However, dominant prey type was found to be site specific, and oysters and hard clams were represented in rays collected from sites associated with commercial shellfish aquaculture. Oysters comprised only 1% of bivalves from full stomachs sampled in fishery-dependent samples (only in pound net), and 7% of full stomachs sampled from fishery-independent samples (all from stomachs collected from commercial oyster grounds). Hard clams (*M. merceneria*), were not found in fishery-dependent samples and comprised 3% of bivalves from full stomachs of fishery-independent samples; 8% of bivalves from commercial oyster grounds (long line), 4% from shallow channels extending from Back River (Dutch seine), and 0% from various shoals (bow and arrow).

Prey Handling Trials

Cownose ray was found to experience a gape limitation that reduced the likelihood of predation on larger prey, such as oysters. Trials also indicated that rays seem to show preference for single, cultchless, oysters as opposed to aggregated, cultched, oysters, indicating that the spat on shell growout method may minimize cownose ray predation.

12 adult females rays, in groups of four, underwent prey handling trial at different times. Adult females (90-102cm DW, 12.7-20.0kg, 27-34mm jaw gape) and four young-of-year (YOY) rays (43-45cm DW, 2.1-2.6kg, 10-18mm jaw gape), participated in the trials. Bivalves shell height (SH), shell depth (SD), and shell width (SW) were measured.

Data suggest that rays select oysters of intermediate SH or SD. During comingled trials, three SH groups (30-40, 45-55, 60-70mm) had the highest probability of being eaten by adult rays while predation probability on smaller and larger oysters was significantly lower. Oysters with shell depth (SD) greater than 32mm also had the lowest predation success.

YOY rays demonstrated predation success on seed oysters, illustrating the durophagous feeding potential and trophic level positioning of cownose ray at an early life stage. In comingled trials with YOY rays, the smallest oysters (10-30mm SH) were most susceptible to predation. YOY rays attempted to feed on the largest oysters offered (30-40mm SH, 15-19mm SD), but were unsuccessful due to gape limitations.

Adult rays showed no preference between oyster species *C. virginica* and *C. ariakensis*. Rays showed preference for hard clams (*M. mercenaria*) over oysters (*C. virginica*). Initially, rays showed preference for soft clams (*M. arenaria*) compared to oysters (*C. virginica*), with selection becoming more equal toward the end of the 15min trial period.

Cultched oyster predation trials were run to compare the effectiveness of the "spat on shell" (SOS) technique used by oyster aquaculturists to that of the single, cultchless oyster growing method. Successful predation on SOS of 60-80mm SH was heavily dependent on cluster size and individual oyster orientation and degree of attachment within the cluster. An overall different strategy was observed in cownose ray predation on SOS oyster clusters; a cluster of oysters is methodically reduced to singles, which are then more easily preyed upon.

Purpose

Cownose rays (Rhinoptera bonasus) are the most common large batoids in Chesapeake Bay. Claims of dramatic increases in this population coupled with reports that oyster restoration and commercial grow-out efforts have experienced set-backs due to rays accessing and consuming deployed oysters on experimental reefs and commercial grounds have led to a debate over the potential for developing a commercial cownose ray fishery. The precautionary principal mandates an assessment of sustainability be conducted prior to development of a fishery. This study documents age and growth, rate of maturation, life history in the Chesapeake Bay, feeding ecology in the Chesapeake Bay, prey handling behaviors, and reproductive anomalie to address several needs for scientific research in the following areas:

Trophic Ecology

This study provides a contemporary diet study, assessing predation rates of cownose rays on oyster and clam resources and examines the stomach contents of cownose rays near and away from known oyster beds to assess the relative predation pressure of rays on oyster beds.

Concerns over predation on commercial bivalve resources have been raised by fishery and aquaculture operations for many years and in several regions of the world. However, little evidence of actual predation on these resources has been documented. Smith & Merriner (1985) investigated the diet of cownose rays with a very small sample size (N=40) found three dominant prey items: soft clams (*Mya arenaria*), Baltic macoma clams (*Macoma balthica)*, and stout razor clams (*Tagelus plebeus*). Commercial oyster species (*Crossostrea virginica*) were only found in one stomach and commercial hard clams species (*Mercenaria mercenaria*) were only identified in three stomachs.

Forage Abilities and Prey Handling

This study investigates prey manipulation experiments and bite force measures to determine whether a critical size to escape predation exists for *C. virginica* and *M. mercenaria*.

The ability of cownose rays to manipulate and crush adult oysters and hard clams has been questioned. Of the nine species of batoids that inhabit Chesapeake Bay during summer months, only two species, the cownose ray (*R. bonasus*) and the bullnose ray (*Mylipbatis fremenvillii*), possess grinding plates and jaw musculature capable of manipulating and crushing oysters and hard clams. Adult oysters and hard clams are rare in the stomach contents of cownose rays and their jaw morphology and musculature is less developed than the bullnose ray. While the bullnose ray may be capable of manipulating and crushing adult oysters and hard clams, they are relatively uncommon in Virginia waters and are unlikely to be major predators in bivalves in this region. Cownose rays, in contrast, are extremely abundant in Chesapeake Bay. Cownose rays are likely very capable of manipulating juvenile oysters and hard clams, however, there may be a critical size where these bivalves are no longer susceptible to predation by this species.

Age, Growth and Demographic Studies

This study produces accurate estimates of age at maturity that may be used to assess demographics and determine intrinsic rates of population increase.

There is growing interest in developing a commercial fishery for cownose rays as a means of lowering predation rates on oysters. Cownose rays in other parts of the world have been driven to endangered status by relatively small fisheries. Knowledge of age, growth and demographic parameters are essential to informed management of any species. Estimation of life history parameters including assessment of age and growth and maturity schedules is critical to determine sustainability of the population to exploitation prior to the development of a fishery. Smith & Merriner (1987) used aging of vertebral samples from cownose rays collected between 1976 and 1978 in Chesapeake Bay to estimate that males mature at 5-6 years of age and females mature at 7-8 years of age. This study was based on relatively few samples however (N=61 for males, N=54 for females) and was skewed toward younger age classes. Neer & Thompson (2005) estimated maturity occurred in 4-5 years for cownose rays in the northern Gulf of Mexico.

Reproductive Biology

This study provides insights into life history dynamics of cownose rays in Chesapeake Bay; information that is necessary prior to allowing exploitation.

Cownose rays are ovoviviparous and the embryos rely initially on a yolk sac for nourishment. Later development is supported by supplies of histotroph (uterine milk) provided to the embryo through trophonemata, highly specialized villi that extend from the uterine wall (Hamlett et al., 1985). Only the left reproductive tract is typically functional in cownose rays and only one pup is produced per reproductive cycle (Smith & Merriner 1986); however multiple births were observed in this study (Section 6). Gestation appears to be 11-12 months and ovulation occurs soon after parturition resulting in the annual production of typically one pup per female. These life history parameters suggest that intrinsic rates of increase are quite low. Indeed, Neer (2005) estimated that maximum rate of population change of for cownose rays in the Gulf of Mexico to only be 2.7% per year.

Sampling Approach

A total of 2,255 cownose rays were sampled from May 2006 through September 2009 in various locations in the Chesapeake Bay using fishery-dependent and fishery-independent methods for this study. Captured rays were processed for various biological assessment parameters. Of these 254 were used for age determination studies (Section 1), 1,422 were used to assess sexual maturity and 74 for histological review (Section 2), 781 were used to assess feeding ecology (Section 4), and 16 adults and 4 young-of-year were used to assess prey handling (Section 5). 194 embryos were collected from gravid females during sampling (Section 3). A total of 8 episodes of multiple births in cownose rays were documented in this study; two sets of twin live births from captive rays and 6 discovered during necropoy (Section 6).

Sampling was initially conducted solely by fishery-dependent methods, obtaining rays as bycatch of commercial haul seine and pound net operations. A distinct bias as to certain stomach content items was quickly realized relative to sampling method, specifically with amounts of teleost fish observed, indicating that natural prey items may not be accurately reflected in sampling protocol.

The bias of prey components observed within ray stomachs from fishery-dependent sampling resulted in the commencement of fishery independent sampling. A combination of a modified Dutch seine, long-line rigs, and bowfishing (bow and arrow), allowed the sampling of various habitats and generation of more diverse natural prey components for cownose ray. These fishery-independent methods were employed to remove rays from the water as soon as possible to minimize loss of stomach content, thus providing a more accurate assessment of cownose ray natural prey items.

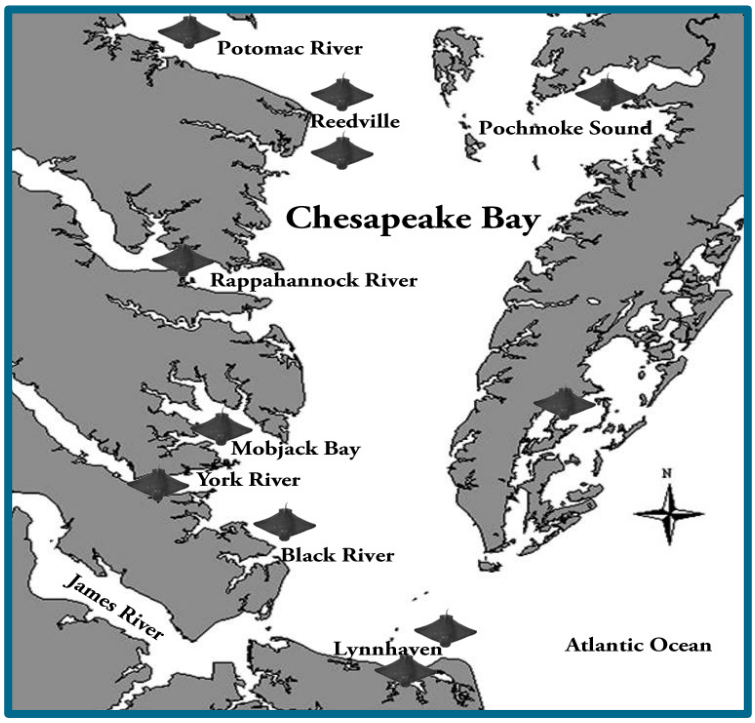

Figure 1. Sampling sites used in study.

These fishing methods were restricted to relatively shallow water habitats ranging from 0.6-3m. Modified Dutch seine was pulled for 20min each set by twin dead-rise boats in Back River channel along Plum Tree Bar (Poquoson, VA). Long-line sampling was conducted adjacent to commercial oyster grounds which were currently growing spat-on-shell (SOS) oysters, either wild SOS or cultured SOS with no cultured cultchless (single) oysters deployed (wild cultchless oysters are observed associated with commercial grounds, but intentional planting of cultchless oysters was not being practiced during this study). Long-line gear was tended three times per day to minimize time live rays were held hooked prior to landing. Bow and arrow sampling was conducted from boats by members of a local bowfishing organization and was conducted in Lynnhaven Inlet and Timber Creek (York River). Rays were immediately landed on boat after shot.

Management Considerations

Reports of cownose ray predation on commercial bivalves coupled with questionable claims of dramatic increases in the cownose ray population coast-wide (Myers et al., 2007) have spurred interest in developing a commercial fishery for cownose rays or at least identifying nonlethal deterrents for keeping cownose rays from commercial beds. Through this study of life history, trophic ecology, and prey handling, several management considerations were developed.

Life History Considerations

Cownose rays in the Chesapeake Bay are slow to reach reproductive maturity and have extremely low fecundity. Sexual maturity is reached in cownose ray from the Chesapeake Bay at 7-8yrs in females and 6-7yrs in males. Difference between ages at sexual maturity verse age at first reproduction needs to be observed for cownose ray which have an 11.5-12mo gestation. Female cownose rays who become sexually mature and mate for the first time at age 7 years do not complete gestation, and therefore do not contribute to recruitment, until age 8.

Though multiple births were observed in this study (Section 6), fecundity in cownose ray is considered low, remaining close to one pup per female per year. Gravid females are at three quarter-term gestation upon entering the bay in May, with parturition not occurring in Virginia waters until mid-June to early July.

Timing of parturition in cownose ray is an important consideration for fishery management. If fished when rays first become accessible to fishery in May and through mid-June (note that juveniles are not present in large quantities), for every mature female harvested, two rays will be removed from the population: mom and near-term embryo. If fished after parturition is completed (mid-July), offspring may be allowed to enter recruitment effort.

Mixing of the sexes is observed within the migrating cownose ray population as they reach the Chesapeake Bay extending through mating (early to mid-August), at which point sexual segregation occurs. Females are observed to remain in shallow water habitats throughout the summer and early fall, while it remains uncertain where male cownose ray inhabit when segregated due to lack of fishery-independent sampling of deep water habitats throughout the Bay and insufficient sampling of habitats along the eastern shore of Bay. Rays are easily accessible, and therefore more frequently observed, within near shore commercial haul seine and pound net fisheries. Throughout this study (2006-2010), landings of cownose ray as by-catch in these traditional fisheries (subsidy paid by state to fishermen for landing cownose ray) contained mixed sexes from May through July, but nearly 100% female from August through October. With possible commercial harvesting of cownose ray restricted to post-parturition, together with the aggregate foraging behavior within near-shore habitats, overexploitation of female rays can quickly occur with reliance on traditional fisheries. If a male-only fishery evolves for periods during the summer, alternative fishing methods may need to be explored to fish deeper water habitats.

Juvenile cownose rays 60-75cm DW age 1-4 are not highly represented in the Chesapeake Bay, and therefore not accessible to a fishery. Lack of juvenile rays subjected to harvesting can be viewed as passive exclusion conservation attribute. If juvenile rays do not largely participate in the reproductive event (migrating north in spring), that segment of the population

will not incur mortality associated with migration (natural predation) or mortality associated with a size-based fishery. However, as in most fish species where juveniles experience higher natural mortality than adults, juvenile rays likely experience natural mortality within nursery areas they occupy, but mortality is currently unknown for this segment of the ray population. Further, population estimates derived from aerial or tagging surveys within the northern most range of the cownose ray may under-estimate the overall population due to the absence of these year classes. Population studies need to incorporate means to include juvenile cownose rays that may not represent any given yearly migratory event.

Trophic Ecology Considerations

Results of feeding ecology experiments have found that although bivalves are important, the dominant prey type of cownose ray is site specific. This study found that, within each site sampled, various thin-shelled bivalves and crustaceans dominated diet, with oysters and hard clams only represented in rays collected from sites associated with commercial oyster grounds. Oysters comprised only 1% of bivalves from full stomachs sampled in fishery-dependent samples (only in pound net), and 7% of full stomachs sampled from fishery-independent samples (all from stomachs collected from commercial oyster grounds). Hard clams (*M. merceneria*), were not found in fishery dependent samples and comprised 3% of bivalves from full stomachs of fishery independent samples; 8% of bivalves from commercial oyster grounds (long line), 4% from shallow channels extending from Back River (Dutch seine), and 0% from various shoals (bow and arrow).

Fishery research investigating diet and prey assemblage routinely only examine stomach content; however, as seen in this study examination of spiral valves in durophagous elasmobranchs should be considered when investigating prey occurrence. Examination of spiral valves in conjunction with stomachs provided better enumeration of hard-bodied prey in cownose ray diet. Most prey flesh remnants found in the spiral valve were beyond recognition due to advanced digestion. Retention of non-digestable hard parts of certain prey in the spiral valve was largely identifiable to at least prey category and some to specie level. Spiral valves were not examined in fishery-dependent collected rays where commercially important oysters and clams were scarcely observed in stomach analyses. The possibility exists that more oysters and hard clams would have been observed if spiral valve examinations were performed throughout this study. However, the overall dominance of thin-shelled clams and crustacean prey, which also are found in the spiral valve when not present in the stomach, identified in cownose ray indicate a much higher ecological trophic role in cownose ray diet than oysters and hard clams.

Prey items found in rays captured adjacent to commercial oyster grounds were dominated by soft shell clams, mussels, and crabs, not available oysters. However, SOS oysters, not cultchless (single) oysters were deployed on the oyster grow out grounds, providing selectivity by the rays as to clustered oysters or other prey items associated with grow out areas. Oyster remnants identified in ray stomachs from these areas could not be classified as SOS or single oyster prey, and may have been wild single oysters which are naturally part of the habitat. Whether single or SOS origin, oysters remained less abundant prey observed. Soft-shell and hard clam prey from oyster grow-out sites represent natural infaunal populations, indicating oysters were deployed in areas where these bivalves pre-existed. Various crustaceans (crabs, barnacles, amphipods) and thin-shelled bivalves (mussels) are recruited to bottom structure, as deployed SOS oysters, thereby diversifying prey ecology.

Prey Handling Considerations

Results of this study suggest cownose rays are gape limited and unable to produce the force needed to crush large oysters. Therefore, oyster growers and those attempting to seed reefs with mature oysters (broodstock) should consider some measure of protection for shellfish until they reach a shell depth of at least 22-24mm and/or breed shellfish able to withstand forces above 1400N.

This study demonstrated that YOY rays can successfully prey on seed oysters up to 40mm SH. In most aquaculture settings, oyster seed is protected throughout growout by various containment methods (bags, floats, racks). However, cultchless oysters are produced for restoration efforts where small oysters are used to seed constructed reefs. In this application, thought should be given to habitat structure, with reefs providing refuge for small oysters to settle and be less susceptible to ray predation.

The results also indicate that oyster restoration efforts might not benefit from introducing different oyster species. Our data indicate cownose rays prey on *C. ariakensis* no differently than on *C. virginica*. Although the introduction of the fast-growing *C. ariakensis* has been suggested as a possible solution, the results of comparative predation trials indicate that rays do not discriminate between *C. ariakensis* and *C. virginica* and therefore the introduction of *C. ariakensis* to the Chesapeake Bay in order to restore oyster reefs or revitalize the commercial industry may not be an adequate solution.

This species was historically the dominant natural prey of cownose rays in Chesapeake Bay (Smith & Merriner 1985), however, natural disaster (Tropical Storm Agnes in 1972), disease, and overexploitation have led to the collapse of softshell clam stock in the estuary. Given the significant influence of SD on predation in the comingled trials of *C. virginica* and the similarity of SD in oyster-clam trials, higher predation on hard clams was unexpected. A ray must crush the clam at or near its deepest point (SD), whereas in oysters, rays can nibble the flattened, posterior edge of the shells. The ability to handle oysters and apply force along the edges of oysters negates some of the effects of the gape limitation. Further investigation into the amount of nutrition gained by clams over oysters or shell composition and structure could explain the preference.

Populating an area with un-protected single shellfish of size within jaw gape limit of cownose ray for restoration or commercial applications is extremely risky, with massive ray predation likely. The use of SOS as an alternative to single oysters for restoration and commercial extensive deployment to minimize cownose ray predation has promise. Observations of feeding trials suggest that the rays sense both SOS and single oysters equally as food; however fewer SOS oysters were preyed upon in all but one trial given a choice between SOS and single oysters. Cownose ray are opportunistic predators and will feed on prey which is available in abundance; however the energetic cost associated with predation on specific available prey type may influence ray predation strategies. Aggregated single shellfish lowers the cost per benefit for cownose ray.

Cownose ray were demonstrated to effectively prey on most SOS oysters; however, it was done so at a higher energetic cost compared to predation on single oysters and clams. Analysis of stomach content from rays feeding on commercial oyster grounds with only SOS deployed (see Section 5) showed dominance of non-bivalve prey (81% crustaceans, worms, etc..) which likely require less predation effort. Bivalve prey (19%) was dominated by thin-shelled mussels (23%) and burrowing clams (70%), with oysters only representing 7% of bivalve prey, indicating energy and physical cost of preying on SOS may be too high compared to other

available prey. Recruitment of prey types which require less energy expenditure than SOS (thin-shelled bivalves, crustaceans, gastropods, and polycheates) to areas of oyster deployment may provide some level of protection from ray predation.

Further Needs

Population Estimates

Knowledge of both, the ray population within the Bay as well as the total Atlantic population from which it stems is needed. Cownose ray which use the Chesapeake Bay for pupping and mating during summer months is part of an overall western Atlantic population along the U.S. East coast. In spring, they migrate north reaching the Chesapeake Bay in late-April to early-May. An unknown proportion of this Atlantic population of cownose ray enters the Bay while another segment of the population by-passes the Bay traveling to more northern coastal habitats in which to complete their breeding cycle.

With few known predators beyond various sharks, the decline in near-shore shark species inhabiting coastal waters along ray migratory route over the past decade would suggest a decline in predation on cownose ray, which in theory could result in a ray population increase. There have been many anecdotal reports of a massive cownose ray population increase over recent years, yet no formal research directly addressing cownose ray population size has been performed. With the number of failed predation attempts on cownose ray by large predators reported in this study, natural mortality by predation is evident though level of predation remains unknown. With no fishery in place, landings of cownose ray have not been recorded giving no estimate of fishing and discard mortality. Recent efforts by the state to promote cownose ray marketing through a subsidy program paid to industry has generated landings for cownose ray, but rays were retained as bycatch to targeted species and effort was not consistent. Ray mortality associated with culling practices of traditional fisheries has also not been recorded. Traditional tag and recapture studies performed to gain information on population size may be limited for this species due to the high potential for unequal recovery effort as a result of observed ray behavior (migratory and schooling). Aerial surveys may be useful for estimating ray population in the Bay during periods when rays aggregate together (May-June) in shallow water habitats, however, methods to accurately enumerate rays within the water column would need to be explored.

Sexual Dimorphism in Feeding and Prey Selectivity

Sex specific differences in food habits of cownose ray were observed in this study; however sample size was limited and a more thorough evaluation of sexual dimorphism is needed to draw better conclusions or hypotheses pertaining to feeding strategies between the sexes. Sexual dimorphism in cownose ray dentition or jaw gape was not observed, giving no indication that feeding ecology is different between the sexes. The majority of stomach samples collected in this study was from female rays, an artifact of sexual segregation and sampling methods employed. During May-July when sexes were mixed, stomach samples of adult male and female rays were obtained within the same sampling area, though limited in number.

Importance of Aggregate Feeding Behavior

Aggregate feeding behavior of cownose ray depicted in fishery-independent samples where

multiple rays captured in one location contained similar prey items and single dominant prey specie. These observations indicate that cownose ray forage in groups and selectively prey on species in high abundance. The highly opportunistic and aggregate feeding behavior of cownose ray as reported in this overall study, allows comprehension of devastation to oyster restoration efforts and commercial oyster grounds by ray predation where cultchless oysters (30-90mm shell height, SH) are used to seed reefs (Wesson in Fisher, 2009) or planted on grow-out grounds. Cultchless oysters 30-70mm SH are easily preyed upon by cownose rays (see Section 5), and when available in high density as in these practices, an aggregation of adult rays will maximize feeding potential by consuming as many oysters as possible. As observed in shellfish predation section of this study, ray predation is impeded by SOS oysters, an oyster growing technique which allows oyster spat to settle and grow to market size on a large empty oyster shell (cultch), creating a cluster of attached oysters. Though SOS oysters impeded ray predation, it was observed that given time predation success on SOS was attained. However, predation on SOS comes at a cost to the ray; energy expended manipulating oyster cluster to gain hold of a single oyster from cluster, and an increase in physical damage to mouth (lacerations by shells) and loss of teeth plates. Currently intensive culture of cultchless oysters provide for predator protection in grow out by use of cages, rack and bag, etc. On bottom extensive oyster culture using SOS may provide industry with an oyster grow-out method which can economically expand production; however predator protection will likely be pivotal to success.

Project Management

The project was managed by PI Robert A. Fisher (VIMS). Significant cooperation and intimate collaboration was collectively maintained between VIMS, VMRC, and the fishing industry throughout the study. Staff and faculty at VIMS, inclusive of Virginia Sea Grant staff, provided considerable assistance in data collection, statistical evaluation, and manuscript editing and preparation. Garrett Call was instrumental in data collection and analysis. Jim Kirkley, David Rudders, and Chip Cotton for statistical modeling assistance, Jill Dowdy for stomach analysis and reproductive histology, Cheryl Teagle for procurement support, and Janet Krenn for editing and manuscript preparation. Dean Grubbs of Florida State University assisted with ray biology and age and growth determinations and evaluations. Commercial fishermen assisting in this effort included; George Trice, John Dryden, Tommy Lewis, and Billy Lette. Seafood industry members who provided access to fishermen, commercial fishing vessels to collect ray samples, processing facilities for cownose ray processing, and oyster shellstock for predation studies included: Meade Amory (L D Amory Seafood), Ron Sopko (Seafarms), Dimitri Hionis (Bubbas), Lake Coward (Cowards Seafood), Ronny and Margaret (Ranson) Bevans (Bevans Oyster), Rufus Ruark (Shores and Ruark Seafood), Andy Drewer (Shore Seafod), John DeMaria (DeMaria's Seafood), Fishery independent sampling by bow and arrow was assisted by Chase Simmons of Whistling Dixie Bowfishing. Virginia state agencies assisting in this study include; Virginia Marine Resource Commission (Jim Wesson, Rick Robbins), Virginia Marine Products Board (Shirly Estes, Joe Cardwell, Mike Hutt). Collaboration with peak load testing (crush force) of cownose ray shellfish prey items (oysters, clams, mussels) was provided by Dr. Zia Razzaq of Old Dominion University Department of Engineering. Histological sampling and processing was provided by Rita Crockett, VIMS. Beth Firchau and the Virginia Aquarium (Virginia Beach, VA) provided access to their live cownose ray display for underwater photography.

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Section 1: Age and Growth

Determining the age at which cownose rays reach sexual maturity is important for regulating a fishery, if one is to be opened in the Chesapeake Bay. First and foremost, there must be a reliable means by which age can be estimated, and a way to correlate age to maturity.

Cailliet & Goldman (2004) found that light and dark bands are deposited annually on vertebrae, with narrow light bands indicating winter periods of slow growth and wider dark bands indicating quicker growth periods of summer. These bands could be counted, like tree rings, to determine ray age. However, this information must be correllated to some other size criteria for management purposes.

Most growth studies published on elasmobranch fishes only fit their data to forms of the von Bertalanffy Growth Function (Cailliet et al., 2006), though studies that employ multiple models often have shown that alternative models better fit the data (e.g. Neer & Thompson, 2005; Killam & Parsons, 1989; Zeiner & Wolf, 1993). This has been especially true of fishes such as batoids that grow relatively rapidly early in life but continue to grow in weight after growth in length or disk width have slowed considerably. For example, Neer & Thompson (2005) reported that the Gompertz growth model best fit the data for cownose rays in the Gulf of Mexico, and Xeiner & Wolf (1999) reported that the logistic growth form best growth using total length for the skate *Raja binoculata*.

This section assesses age of cownose rays and attempts to correlate age to weight based on growth models.

Methods

Sampling

Cownose rays were collected along the Virginia western shore of the Chesapeake Bay during summer months from 2006-2010 employing fishery-dependant and fishery-independent methods, including pound net, haul seine, long line, bowfishing, and an experimental modified Dutch seine.

A total of 536 rays were used for the age and growth assessments including 217 males and 319 females. Rays were sexed, weighed (kg), and measured for disc width (DW cm).

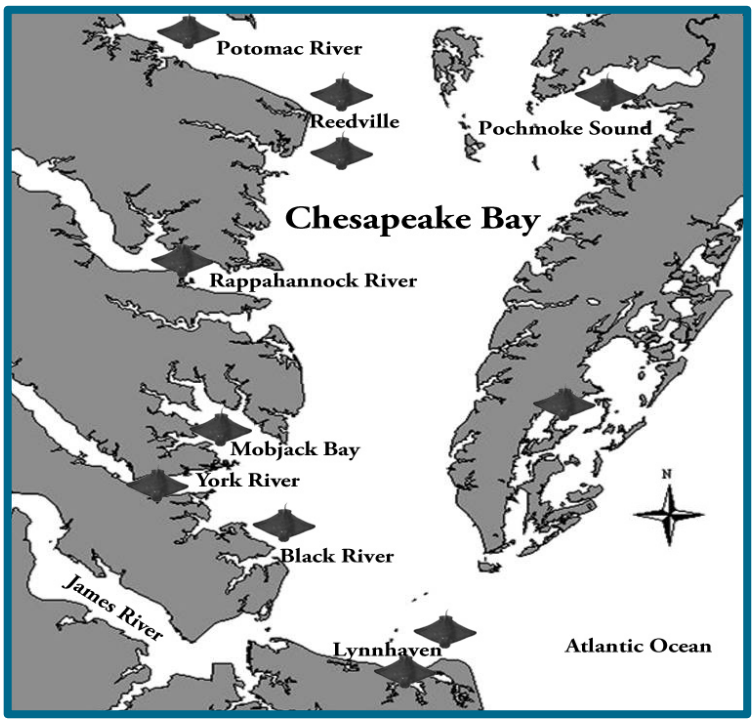


Figure 1. Sampling sites used in study.

Age Assessment

Starting from vertebrae furthest cranially

within abdominal cavity and then extending caudally, a section of the vertebral column consisting of 6-12 thoracic vertebral centra was removed and frozen for later age determination. Vertebral sections were thawed, cleaned of excess tissue in 75% ethanol, and then dried. Individual centra were removed from the vertebral section and mounted onto a cutting block for further sectioning. Using a Buhler Isomet low speed rotary diamond saw, vertebrae were sectioned sagittally through the focus of the centrum. Sections were mounted on a glass microscope slide via mounting medium. Samples were sanded and polished using wet fine grit sand paper in a series (grades 320, 400 and 600) until light was readily transmitted through the samples and annuli were distinguishable using a dissection microscope.

To assess age from vertebral sections, we assumed birth mark was associated with the change in angle in the intermedialia, the light and dark bands are deposited annually and represent a growth cycle (Cailliet & Goldman, 2004), and the light (narrow) bands represent winter periods of slow growth. Age was estimated by counting the number of light bands, but not including the birth mark. As seen in Figure 2, the birth mark is laid down prior to birth. Sampling period was taken into consideration when assessing age, with light (winter growth) bands frequently not highly differentiated at centrum distal edge until later into the summer sampling period where summer growth is subsequently laid down.

Two readers were used to independently assess age by counting winter bands without knowledge of animal disc width. When disagreement occurred between readers, both readers viewed vertebral sections together for consensus on a final age determination. If readers were still not in agreement on a section, the vertebra sample was eliminated from the study.

Growth Assessment

This study fitted five growth models to the observed size-at-age data using disk width (DW in cm). Age 0 consisted of at-term embryos collected within a 10-day period from end of June to first week of July when parturition was at its peak (half of females within samples had already pupped and the other half still carried at-term embryos). DW-age data was run through models twice, once including only whole-year age estimates and then using fractional age estimates for young-of-year (YOY).

Fractional ages were estimated at 0.125 and 0.3 years and defined as follows: age

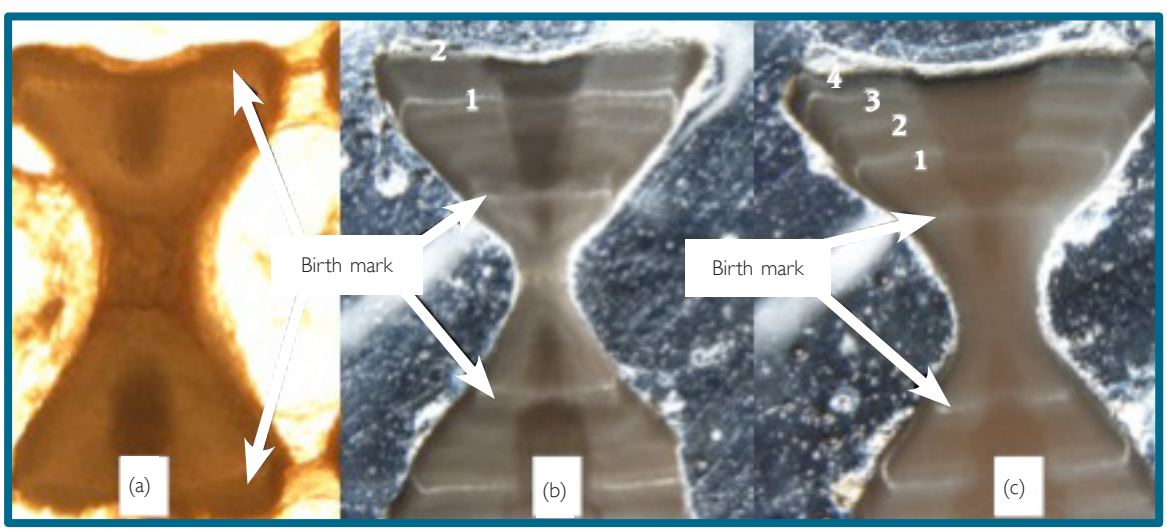


Figure 2. Sagitally sectioned vertebra from cownose rays showing birth mark and annuli (numbered); (a) section from near-term embryo (pre-birth); (b) 1-year-old ray; (c) 4-year-old ray.

0.125 are neonates collected from 2 week period in mid-late August and identifiable as they still tend to aggregate with adult females; age 0.3 were YOY collected during the second week of October and identifiable as they aggregate and begin exiting the Bay as a group for southern migration.

Model parameters were estimated using least squares estimation for the following models:

(1) modified (conventional) form of the VBGF using the estimated age at length zero (VBGFmod; Beverton & Holt, 1957);

(2) original form of the von Bertalanffy Growth Function using an empirically derived length at birth intercept rather than a theoretical age at length zero (VBGF; von Bertalanffy, 1938; Cailliet et al., 2006);

(3) 2-parameter form of the original VBGF, with fixed length at age zero;

(4) Gompertz model (Ricker, 1975); and

(5) logistic function (Ricker, 1975). We used the residual mean square error (MSE) and Akaike's Information criteria (AIC) as measures of goodness-of-fit for all models.

Equations for each of the models are as follows:

(1) VBGFmod:

$$DWt = DW\infty(1-e-k(t-to))$$

(2) VBGF:

$$DWt = DW\infty - (DW\infty - DW0)e-kt$$

(3) 2-parameter VBGF:

$$DWt = DW\infty - (DW\infty - 45)e-kt$$

$$DW0 = 45cm \text{ (mean length at birth)}$$

(4) Gompertz model:

$$DWt= DW0e[G(1-e-kt)]$$

(5) Logistic function:

$$DWt = DW\infty/(1+e-k(t-to))$$

Variables:

DWt = predicted length at age 't',
DW∞= theoretical maximum length,
DW0 = Length at birth,
k = the growth coefficient,
t = age,
t0 = age at length theoretically equals 0,
G = ln(DW∞ / DW0).

Results

A total of 536 rays were used for the age and growth assessments (Figure 3) with males ranging in size from 30-98cm DW (n=217) and females ranging from 30-110.5cm DW (n=319). The oldest ray observed was a fe-

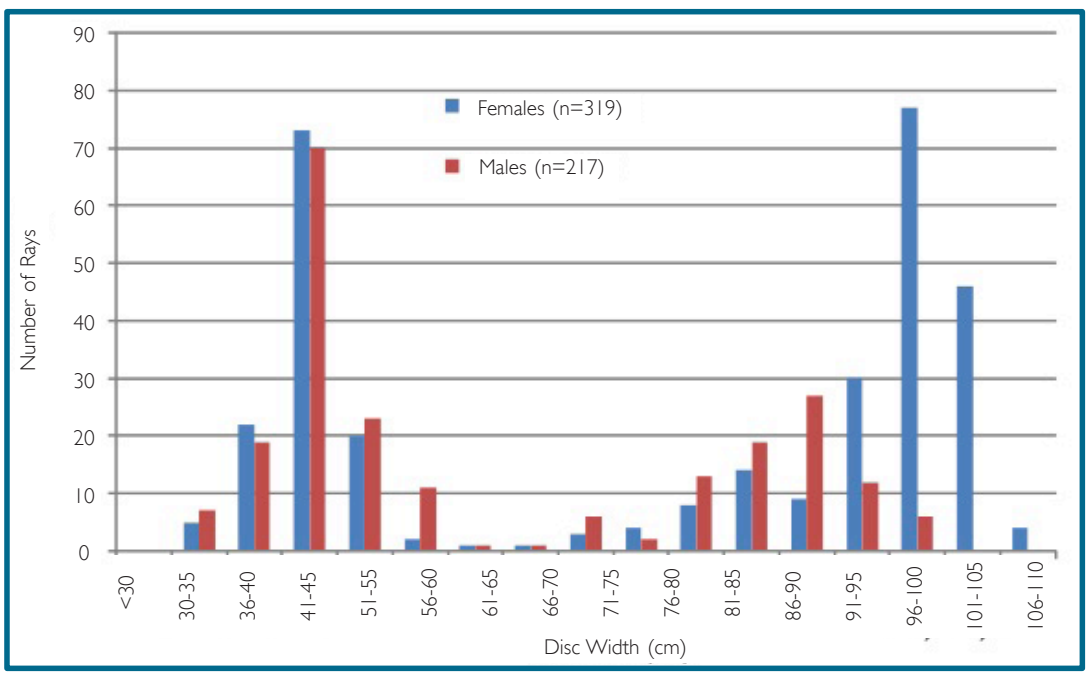


Figure 3. Number and size of cownose rays used for age and growth study.

male estimated at age 21 and 107cm DW. The largest ray was a female 110.5cm DW at estimated age of 19. The oldest male cownose ray was estimated at age 18 and 97cm DW. The largest male ray was 98cm DW at estimated age 16 years.

DW-weight relationship for cownose rays in this study was similar between the sexes (Figure 3), and described by the power functions:

female, $y = 1E\text{-}05x^{3.2596}$ $R^2 = 0.9884$; and

male, $y = 1E\text{-}05x^{3.2064}$ $R^2 = 0.99$.

The raw length-at-age data indicated that male cownose rays grow faster and reach a smaller maximum size than females; therefore we analyzed data for each sex separately. All growth models fitted to observed length-at-age data were significant ($p<0.0001$), with results using fractional age estimates similar to those using only whole-number age. The two forms of the three-parameter von Bertalanffy growth model provided the best fit to the observed size-at-age data for male and female cownose rays (Tables 1, 2) with observed model parameters and growth rates further illustrating differences between the sexes (Figures 4 and 5). These models had the lowest residual mean square error (MSE) and the lowest Akaike's Information Criteria (AIC) values. The Gompertz model and the two-parameter von Bertalanffy model had the worst fit to our data for both males and females. The estimates for asymptotic maximum disc width (DW∞) were biologically reasonable for all models for males and females except the logistic growth model which underestimated this parameter for both sexes. The maximum observed disc width was 106cm for females and 97cm for males in all models except the logistic model produced DW∞ estimates of 95-97cm for males and 104-106cm for females. The two-parameter von Bertalanffy model produced

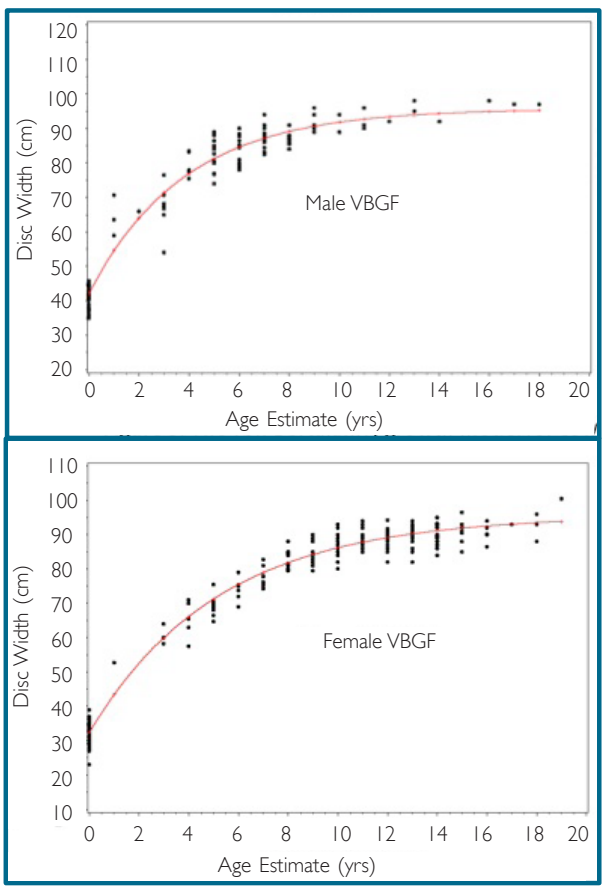


Figure 4. The von Bartalanffy growth model for cownose rays from the Chesapeake Bay not using fractional age 0 observations.

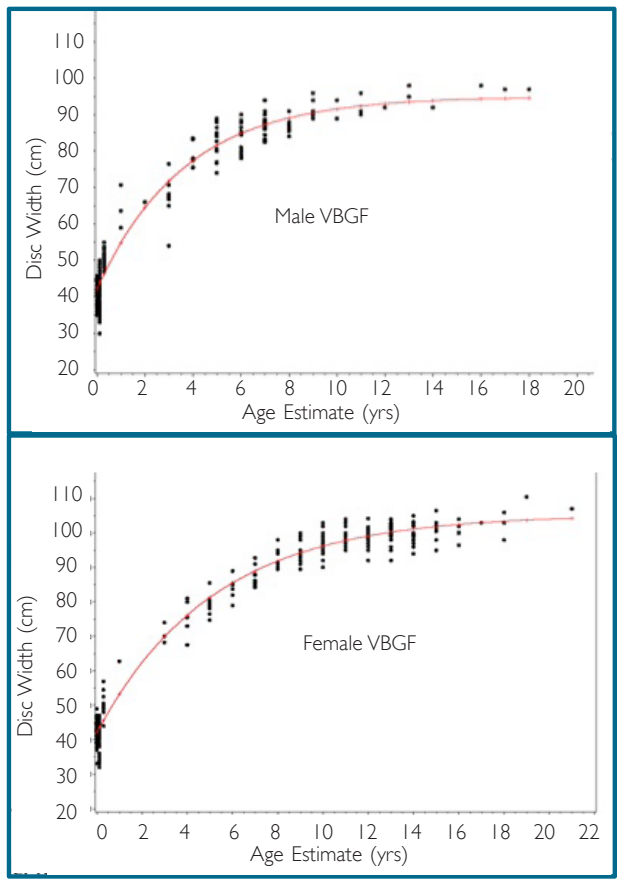


Figure 5. The von Bartalanffy growth model for cownose rays from the Chesapeake Bay using fractional age 0 observations.

	Model	L_∞	k(year^{-1})	t_0	L_0	AIC	MSE
Males	2ParmVB	97.095(±1.73)	0.2333(±0.019)	na	45	1295.6	21.704
	VBGF	**94.983(±1.40)**	**0.2741(±0.021)**	**na**	**42**	**1251.3**	**17.554**
	VBGFmod	94.983(±1.40)	0.2741(±0.021)	-2.14	na	1251.3	17.554
	Gompertz	95.224(±1.44)	0.3070(±0.021)	na		1295.9	21.740
	Logistic	92.713(±1.11)	0.4330(±0.025)	0.363	na	1269.2	19.061
Females	2ParmVB	106.34(±0.93)	0.1778(±0.008)	na	45	1775.0	14.995
	VBGF	**105.34(±0.76)**	**0.1931(±0.008)**	**na**	**42**	**1702.4**	**11.865**
	VBGFmod	105.34(±0.76)	0.1931(±0.008)	-2.64	na	1702.4	11.865
	Gompertz	104.26(±0.70)	0.2364(±0.008)	na		1766.6	14.605
	Logitic	102.30(±0.49)	0.3226(±0.009)	1.059	na	1707.5	12.056

Table 1. Five growth models used to evaluate cownose rays (without fractional age estimates for YOY rays). N=319 females, N-218 males

	Model	L_∞	k(year^{-1})	t_0	L_0	AIC	MSE
Males	2ParmVB	96.446(±1.57)	0.2422(±0.019)	na	45	808.5	17.072
	VBGF	**95.685(±1.34)**	**0.2622(±0.018)**	**na**	**42**	**785.6**	**15.122**
	VBGFmod	95.685(±1.33)	0.2622(±0.018)	-2.14	na	785.6	15.122
	Gompertz	94.920(±1.33)	0.3125(±0.020)	na		811.7	18.482
	Logistic	93.061(±1.04)	0.4253(±0.023)	0.411	na	798.5	16.585
Females	2ParmVB	105.99(±0.82)	0.1814(±0.007)	na	45	1388.2	11.921
	VBGF	**105.48(±0.71)**	**0.1911(±0.007)**	**na**	**42**	**1350.3**	**10.223**
	VBGFmod	105.48(±0.71)	0.1911(±0.007)	-2.69	na	1350.3	10.223
	Gompertz	104.09(±0.62)	0.2387(±0.007)	na		1383.7	10.223
	Logitic	102.36(±0.46)	0.3207(±0.008)	1.052	na	1351.4	10.269

Table 2. Five growth models used to evaluate cownose rays (with fractional age estimates for YOY rays). N=319 females, N-218 males

best estimates of DW∞ for both males (97.1cm) and females (106.3cm).

A previously published model of age and growth in cownose rays from Chesapeake Bay (Smith & Merriner, 1987) produced DW∞ for males (119cm) and females (125cm) that were far larger, but these estimates were also far larger than the largest observed specimens. Observed size-at-age of both sexes is given in Table 3.

Our data suggested that cownose rays grow considerably faster during the first few years than has been previously reported, justifying much higher estimates of the growth coefficient (k). The two-parameter von Bertalanffy model produced the lowest estimates of the growth coefficient (k) for males (0.2333 year-1) and females (0.1778

		Age (yrs)										
		0	0.12	0.3	1	2	3	4	5	6	7	8
Females	AvgDW (cm)	42.31	42.47	51.5	62.8		70.75	75.4	79.12	84.71	86.69	92.95
	SD	2.75	3.83	2.06	na		2.94	5.49	3.18	3.6	2.56	2.74
	N	63	51	20	1	0	3	5	8	7	13	10
Males	AvgDW (cm)	41.87	42.53	51.4	64.45	66	66.96	79.55	82.85	83.16	87.16	86.66
	SD	2.45	4.38	2.35	5.92	na	6.82	3.61	4.49	4.03	3.26	2.17
	N	51	58	32	3	1	7	5	16	14	14	9

Table 3. Mean size-at-age for male and femal cownose rays from Chesapeake Bay. DW=disc width, SD=standard deviation.

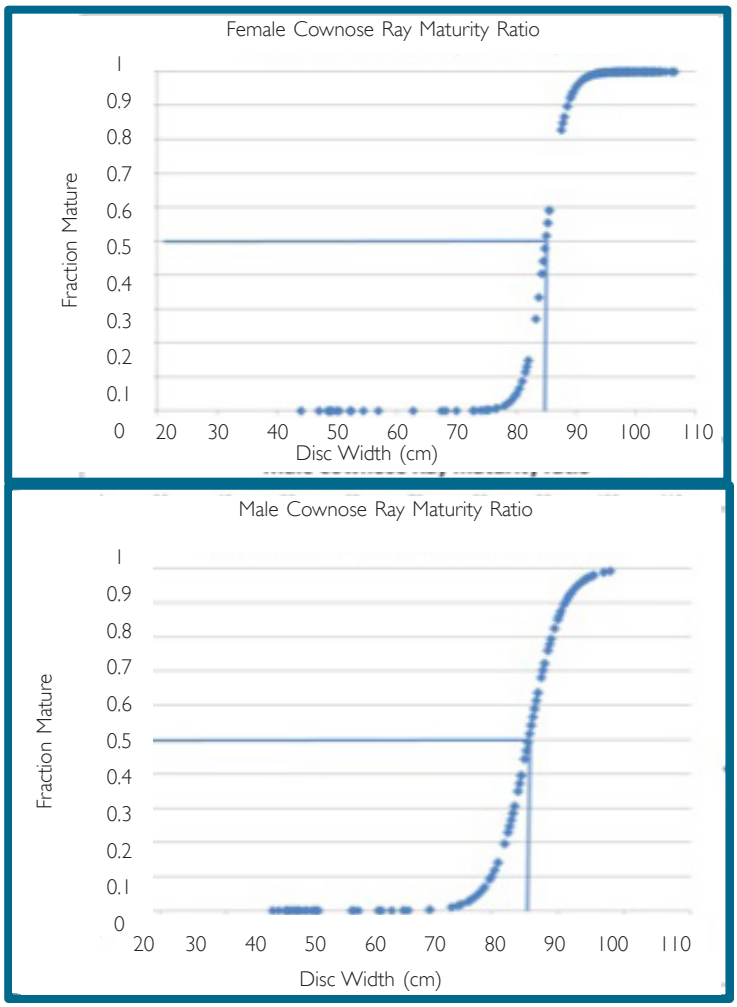


Figure 6. Maturity ogives for cownose rays from the Chesapeake Bay for females (top) and males (bottom).

year-1), and the logistic model produced the highest estimates (0.4333 year-1 for males and 0.3226 year-1 for females). As determined by this study, the best-fit models (three-parameter von Bertalanffy models) estimated growth coefficients of 0.2741 for males and 0.1931 for females and are much higher than previous estimates (e.g. 0.126 year-1 for males and 0.119 year-1 for females – Smith and Merriner, 1987).

The relationship between size and maturity is best indicated by maturity ogives for male (N=91) and female (N=307) cownose rays (Figure 6). The size (DW) at the point of the curve corresponding to 50% mature is used as an indicator of the size at which maturity is reached. Mean DW at maturity for mature males and females was 85 cm, corresponding to an age at maturity of 6-7,~6-7 and for males, 85.5cm DW, corresponding to age 7-8 and ~7 for females. Difference between age at sexual maturity verse age at first reproduction needs to be observed for female cownose ray which have an 11.5-12mo gestation. Female cownose rays who become sexually mature and mate for the first time at age 7 years do not complete gestation, and therefore do not contribute to recruitment, until age 8. The proportion of mature males increased more gradually than that observed for females.

Age (yrs)												
9	10	11	12	13	14	15	16	17	18	19	20	21
94.36	97.8	99.71	98.78	99.83	100.13	100.63	100.45	103	102.5	110.5		107
3.36	3.29	2.96	2.86	2.84	2.99	2.99	2.49	na	3.31	na		na
15	25	22	23	23	17	11	6	1	4	1	0	1
91.75	92.75	92.33	92	96.5	92		98	97	97			
2.67	2.5	3.21	na	2.12	na		na	na	na			
6	4	3	1	2	1	0	1	1	1	0	0	0

Discussion

Throughout sampling from various locations in the bay, 2-4 year old rays (60-80cm DW) were not represented, and since these age groups represent juveniles that have not reached sexual maturity, they may not participate in the spring northern migration (reproducing effort) and remain in more southern estuaries.

Since gear type largely employed for sampling (haul seine, pound net) targets fish of size well below that of juvenile rays, and neonate rays are routinely captured using these gear types, it is thought that juvenile cownose rays do not widely use the Chesapeake Bay during their juvenile life stage. YOY migrate out of the Chesapeake Bay, with large amounts of only young of year caught in pound nets at mouth of Bay shortly after adults had exited, indicating that YOY may not make the migration to southern wintering grounds with the reproducing mass, but over winter in other estuaries along the east coast south of the Bay where water temperatures are more favorable. Further, since these young rays, and previous 2-3 year classes (juveniles) have not reached sexual maturity, they likely to not participate in the spring northern migration (reproducing effort), and remain in more southern estuaries. Trawl surveys conducted in the Bay by CHESMMAP at VIMS from 2002 to 2010 collected 161 cownose ray ranging in size from 24.0-111.8cm, however, no rays between 54.7 and 71.9 were recorded.

Section 2: Rate of Maturation

Determining the age at which cownose rays reach sexual maturity is important. This section aims to correlate disc width (DW) and age with reproductive maturity in cownose rays.

Both male and female cownose rays have paired genital tracts running along the dorsal side of peritoneal cavity on each side of the vertebral column. In both sexes, the paired reproductive tracks are separated but terminate in the common cloacal opening.

In females, eggs are ovulated into the peritoneal cavity and collected by the funnel shaped ostium, which is the beginning of oviduct. The egg is moved caudally along the oviduct to the oviducal gland where fertilization takes place. The fertilized egg moves into the uterus for gestation. Both oviducts terminate into the common cloaca through vaginal openings in uteri.

In male cownose rays, sperm produced in the testis is transported sequentially along the genital tract through the epididymis, vas deferens (ductus deferens), and seminal vesicle, from which sperm ladened seminal fluid is discharged into the cloacal opening through a pore in the urogenital papilla. Seminal fluids collected from both genital tracts in the cloaca are transported to claspers during insemination.

Neonate and juvenile cownose ray possess rudimentary reproductive organs and genital tracts that are largely nondescript. Paired ovaries and testes begin differentiating by age two and continue to enlarge in pre-adult rays (age 3-5) with developing follicles and spermatocysts, respectively. Immature ovaries and testes deteriorate rapidly post-mortem, making recovery difficult if necropsy is delayed.

Methods

Sexual maturity of female rays was largely determined if gravid or through these means:

(1) diameter of the largest ova,

(2) uterus width (left uterus),

(3) other characteristics, and

(4) histological sampling of ovaries and left uterus.

Diameter of the largest three ova within the ovary was measured (mm) to obtain mean maximum ova diameter (MOD). Rays with ova greater than 10 mm were considered to be mature (Smith & Merriner, 1986). Histological sampling of ovaries was performed to document stage of vitillogenesis and ova development. Additional observations made during the course of this work were used to further assess sexual maturity and are provided in discussion.

Sexual maturity of male cownose rays was determined using the following criteria:

(1) clasper calcification (not calcified, partially calcified, and calcified) and ratio of clasper length to disc width (Smith & Merriner, 1986),

(2) histological sampling of testes and vas deferens for presence/absence of mature sperm in a select number of individuals,

(3) vas deferens coiling (none, partial, complete; Neer & Caillliet 2001);

(4) presence or absence of seminal fluid from vas deferens and/or the urogenital papilla at the cloacal opening;.

Testes, both lobes, were sub-sampled and weighed for comparison and maturity correlations. Claspers of immature rays are small (short) and flexible and not able to function during copulation. With maturity, claspers go through a calcification that stiffens them, while allowing articulation with the base of pelvic fin (to rotate clasper for insertion into female), both necessary for successful mating. Presence or absence of seminal fluid was determined by applying slight pressure inward then caudally along the terminal end of urogenital tract where the paired ducts (vas deferens) converge. Milt, if present, is expressed through genital papilla. Histological samples for both sexes were initially preserved in 10% neutral buffered formalin, later imbedded in paraffin, sectioned, and stained using hemotoxylin and eosin following standard histological procedures. For male testis tissue, homogeneity of developing tissue throughout the testis was performed by analysis of tissue from cranial, medial and caudal portions of testis lobe. No difference was found between lobe sections within a sample; therefore all sampling of testis occurred by sections removed from the medial-caudal region of testis lobes. Since the testis lobes in cownose rays vary in size (and with females having one of their paired ovaries functional the other non-functional), preliminary histological analysis was performed confirming functionality in both testis lobes.

Female Sexual Maturity

Ova Diameter

During late gestation (May-June) the functional ovary in mature female cownose rays simultaneously contain ova in several stages of development, ranging in size from microscopic to the largest ova observed of 46mm in diameter. Ovaries were routinely observed macroscopically to contain 3-4 ova significantly larger than the rest (Figure 1), with these largest ova routinely following a consistent size reduction from the largest.

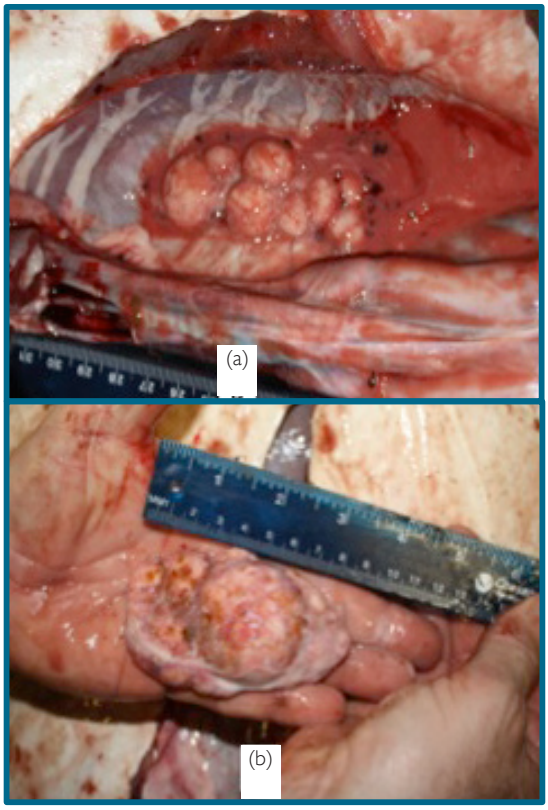


Figure 1. Left ovaries of cownose rays; (a) multiple ova in various stages of development; (b) single ova significantly larger than the rest.

Average diameters of the 3 largest ova in female rays >90cm DW collected from mid-June were 29.6, 22.2, and 16.8mm (n= 40).

Since fecundity is typically one young per female per breeding cycle for cownose rays, only the largest ova are likely sequentially released into the body cavity to enter the oviduct via the ostia (ovulation). Ova size is observed to increase as females mature reaching 12-18mm in diameter at 85cm DW (Figure 2), the estimated median size at maturity for cownose rays from the Chesapeake Bay (see Section 1). Tracking ova size in mature rays from May through September provided indication of ovulation. Diameter of the largest ova still attached to ovary, as well as the average diameter of the three

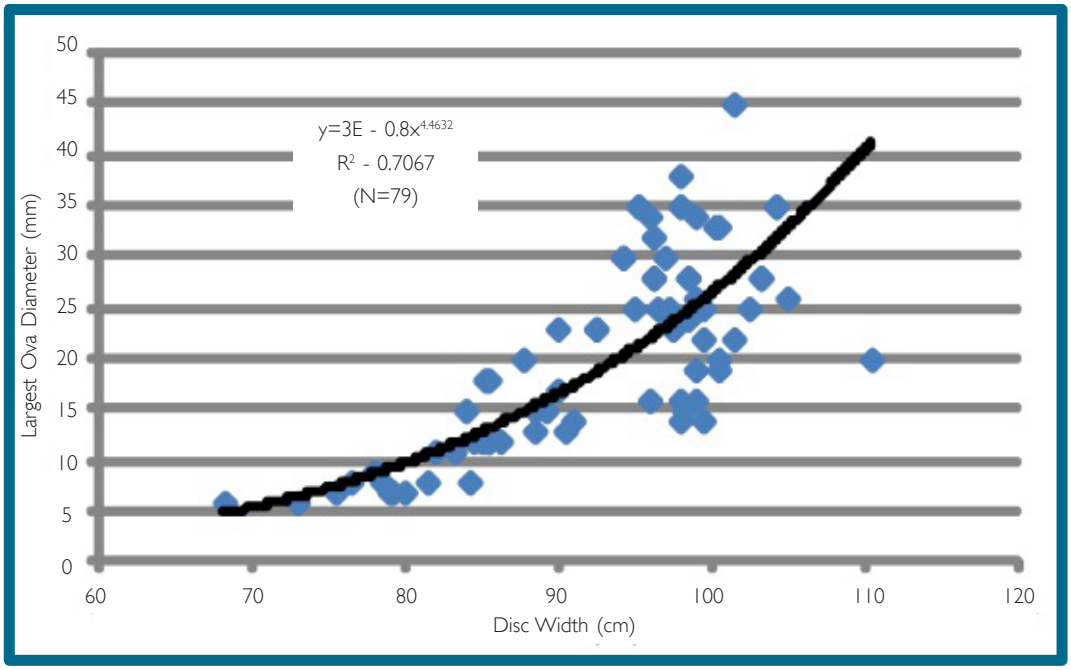


Figure 2. Relationship between disc width and largest ova in female cownose rays May-early July (pre-mating).

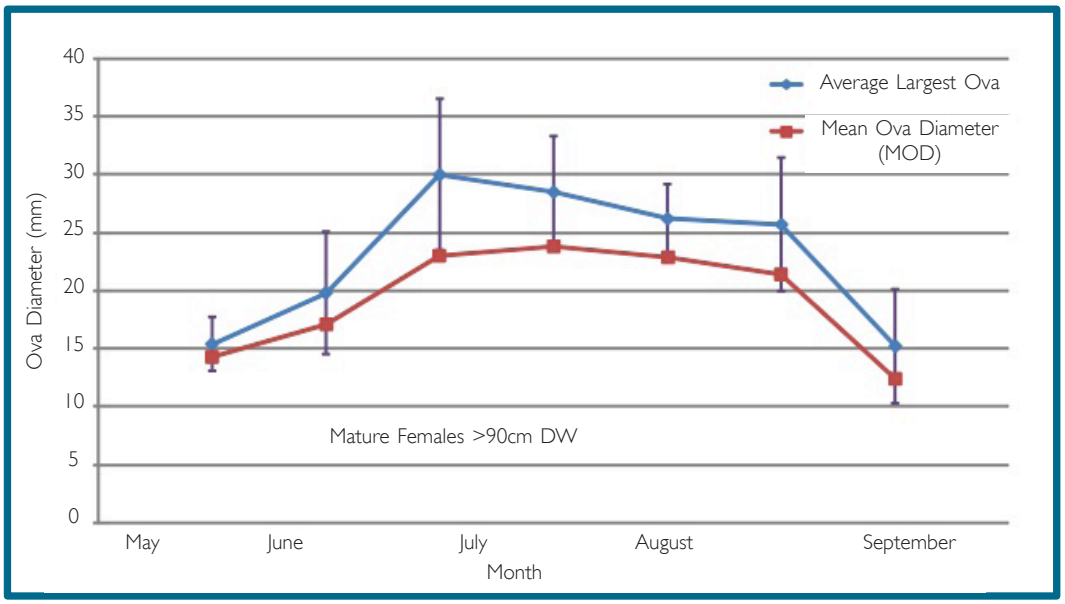


Figure 3. Change in ova size in mature (>90cm DW) female cownose ray during residency in the Chesapeake Bay. Reduction in ova size late June, indivating the beginning of ovulation.

within an ovary was averaged and plotted over time (Figure 3). During late gestation (May -June) ova were observed to continually enlarge with increasing amounts of yolk. Beginning late-June, size of largest ova and MOD began to decrease, indicating the beginning of ovulation. By mid-late August the average diameter of largest ovarian ova significantly decreased, indicating ovulation had occurred.

Width of Left Uterus

For female rays between DW 83.75 and 90.5cm with mature ova present (ova >10 mm) nine out of ten had active left uterus widths measuring less than 25mm. Above DW 90.5cm, the active uterus width was typically double that of rays below this size (Figure 4). The first occurrence of uterus width doubling was noted for an individual with a DW of 82cm. There were no signs of

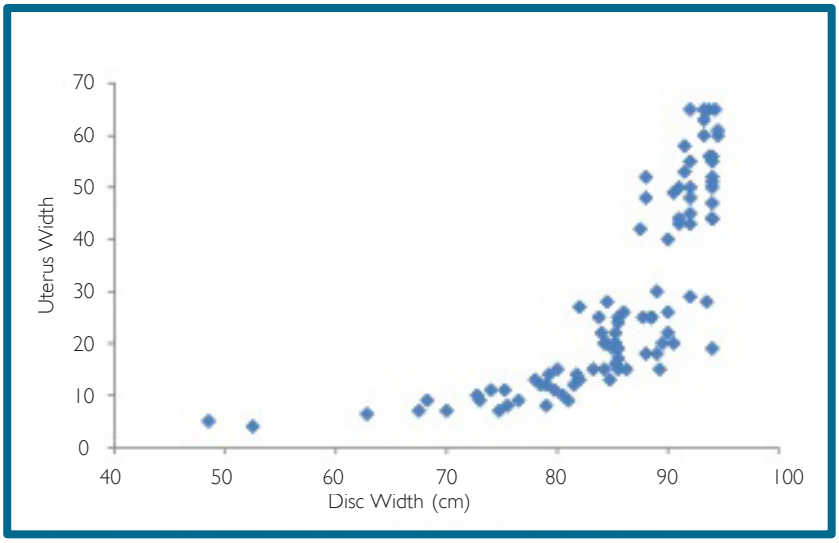


Figure 4. Left (functional) uterus width (n=91) in female cownose rays <95cm DW from pre-mating period (May June).

mating or recent gestation in this individual and no mature ova were found. The width of left uterus begins to increase as rays approach 80cm DW and a distinct change is observed beginning at 82-84cm DW.

Other Characteristics

In pre-adult females, both left and right ovaries are present, but only the left ovary continues to develop functionally while the right ovary does not, resulting in the right reproductive tract of female cownose rays being non-functional. The left ovary begins to visually differentiate from the right ovary during maturation approximately at disc width (DW) 48-50cm when the anterior-dorsal area appears granulated (Figure 5). As female rays mature, developing follicles in left ovary germinal epithelium accumulate vitellogen (yolk) and increase in diameter (Figure 6) while the oviduct begins to expand, providing 3-dimentional structure along the vertebral column and body wall.

Determination of females entering their first reproducing year was based on observed ova diameter, uterus width, and the presence of highly viscous, gelatinouse material inside the uterus (Figure 7). Females entering their first reproductive event (~84-86cm DW, age >6) had largest single ova ranging from 8-20mm in diameter and MOD of 9.0-17.6mm (late May). In preparation for first gestation, rapid expansion of the

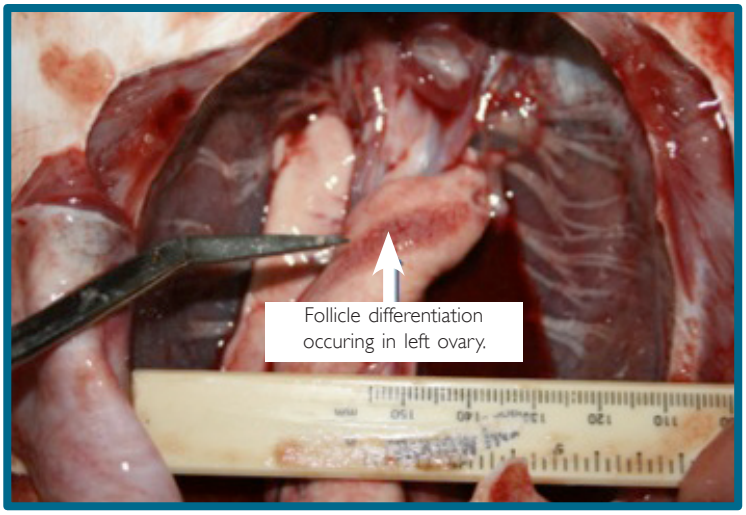


Figure 5. Left ovary embeded in epigonal gland showing follicle development in juvenile femaile cownose ray.

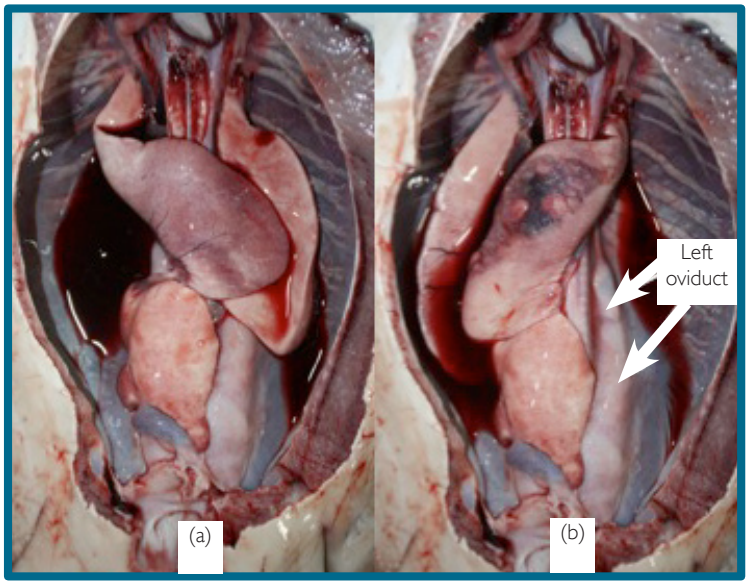


Figure 6. Ventral view of a 86.25cm disc width (DW) female cownose ray showing paired ovary/epigonal gland complexes; (1) right complex positioned to show lack of ovarian development; (b) left complex positioned to show location and developing ova. NOTE: Left oviduct is expanding from vertebral column, indicating the female is entering her first breeding cycle.

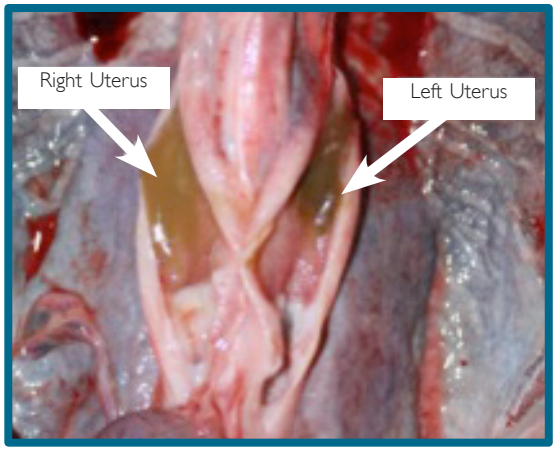
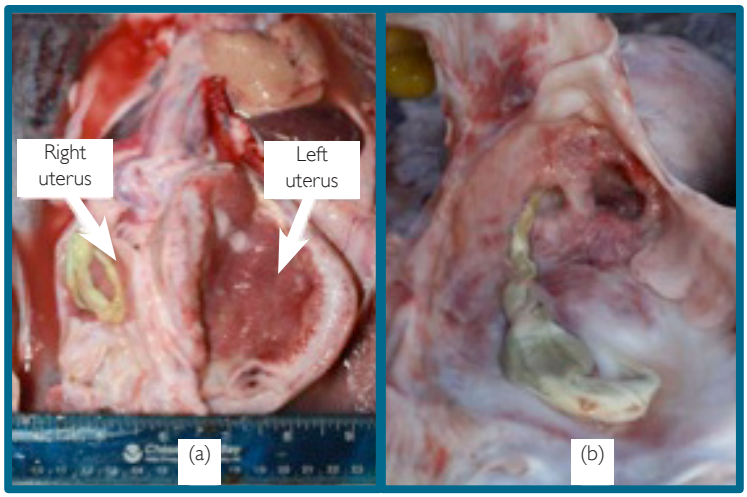
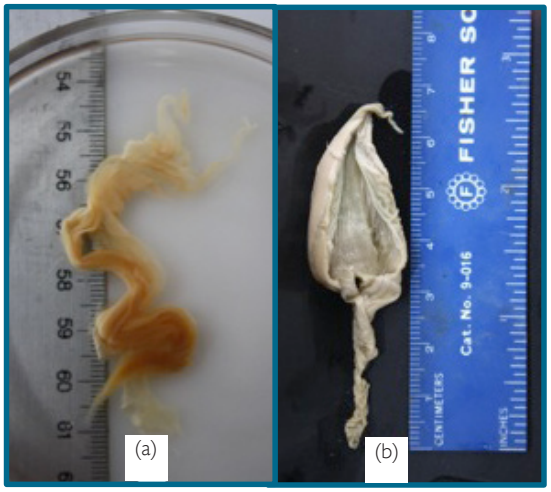


Figure 7. Gelatinous material ("goo") contained within uteri of immature (83cm DW) cownose ray .



Figrure 8. "Ribbons" observed in non-functional uterus of mature cownose rays; (a) ribbon in right uterus, embryo removed from left uterus; (b) ribbon slipping from right non-functional uterus.



Figrure 9. "Ribbons" from non-functional uterus of gravid female cownose rays; (a) ribbon from early gestation (September) fragile, tears easily; (b) ribbon from late gestation (May) spongy, more rigid, showing construction by strands of material).

left uterus in width and wall thickness and trophenemata elongating and darkening in color from pink to red occurs. The presence of a caramel colored, highly viscous gelatinous material ("goo") was observed inside the uteri of rays that were determined to have not yet gone through a pregnancy or parturition. This material is present within both uteri of immature rays and darkens in color as they reach maturity.

In females that have previously undergone gestation, goo is not observed in either uterus; however, an elongated ribbon-like material is observed only in the non-functional uterus. This ribbon appears is tapered with ragged ends and appears in sheets or strands (Figure 8). Ribbons vary in size, from 8-15mm wide to 40-90mm long, and color, from pale yellow to light green. In rays with developing embryos (August/September), ribbons in the non-functional uterus are fragile and break and tear easily upon handling but become more rigid and spongy in texture as gestation progresses (May-July) suggesting they are produced annually in conjunction with breeding (Figure 9).

Further evidence that ribbons are produced each breeding cycle was observed in two older females (98cm DW). It was observed that these females had well-developed left uteri, indicating that previous year(s) gestation had occurred in left uteri. However, these females were carrying an embryo in the right uterus for the first time, as evidenced by thin uterine wall and short, not highly developed trophenemata.In these cases, ribbons were present in the left, non-functioning uteri.

Preliminary biochemical properties testing using protein electrophoresis identifies the goo found in immature females and ribbons found in non-functioning uteri of mature females to be very similar. Both are characterized as a high molecular weight phosphoprotein which could be related to vitellin.

Histological Sampling

Histological sampling of ovaries was performed to document stage of vitillogenesis and ova development leading to sexual maturity. Left uterus width (widest point), and qualitative assessment of uteri wall thickness, and trophenemata development and color was also used in this study to correlate sexual maturity. Left uterus width is observed to rapidly increase when females enter their first reproductive event, and trophonemata enlarge and become highly vascularized.

All Myliobatoid rays exhibit uterine viviparity, formerly known as aplacental viviparity (Campagno, 1990; Conrath in FAO report, 2005). Common examples discussed in literature include the cownose ray (Rhinoptera bonasus in Smith & Merriner, 1986), southern stingray (Dasyatis Americana in Hamlett & Koob, 1999; Maruska et al., 1996) and the yellow spotted stingray (*Urobatis jamaicens* previously Urolophus in Fahy et al., 2007).

Uterine development in cownose rays was described by Hamlett & Koob (1999) and McMillan (2007). The entire internal epithelia surface of the uterus forms trophonemata (villous projections) to produce his-

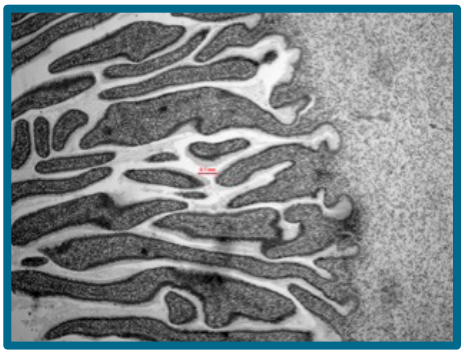


Figure 10. (40x) Light microscopy image of the trophonemata (villa) from the left uterus in a female cownose ray measuring 85.5 cm DW (caught in a haul seine near Poquoson, VA on 5/6/2010). The three largest ova measured 12.0, 9.0 and 6.5 mm in diameter. There was no embryo present in this female and the active (left) and false (right) uteri measured 19 and 16 mm respectively. This female is thought to be entering her first breeding cycle.

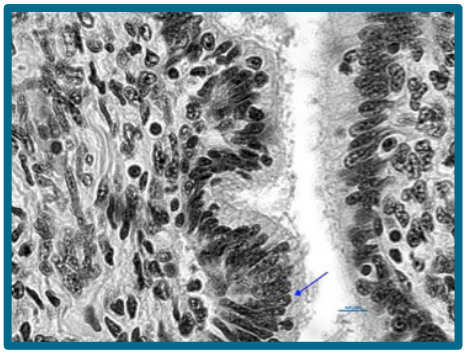


Figure 11. (600x) Light microscopy image at high magnification of the trophonemata (villa) from the left uterus in a female cownose ray measuring 85.5 cm DW (same individual in Figure 10). The arrow is indicating the simple columnar epithelium.

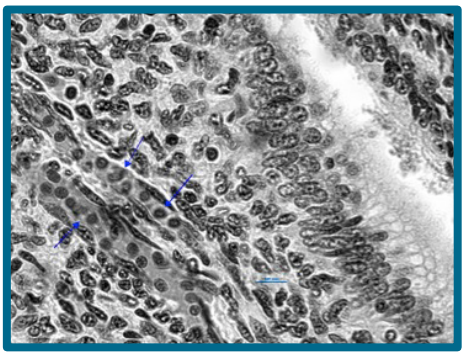


Figure 12. (600x) Light microscopy image at high magnification of the trophonemata (villa) from the left uterus in a female cownose ray measuring 85.5 cm DW (same individual in Figure 10 and 11). Arrows indicate branching capillaries showing the villa are highly vascularized.

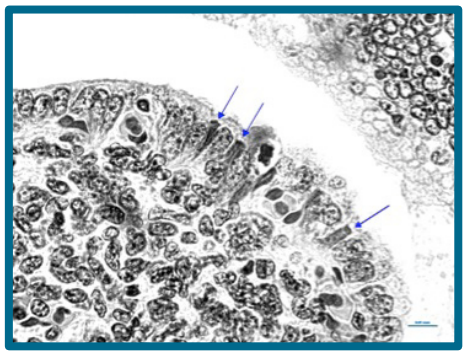


Figure 13. (600x) Light microscopy image at high magnification of the right uterine tissue in a female cownose ray measuring 86.0 cm DW (caught in a haul seine near Poquoson, VA on 5/26/2009). The arrows indicate simple columnar cells. The left and right uteri were 26.0 and 25.0 mm respectively.

Figure 14. (600x) Light microscopy image at high magnification of the right uterine tissue in a female cownose ray measuring 86.0 cm DW (caught in a haul seine near Poquoson, VA on 5/26/2009). The arrow indicates a highly viscous substance that is found inside both uteri of immature females.

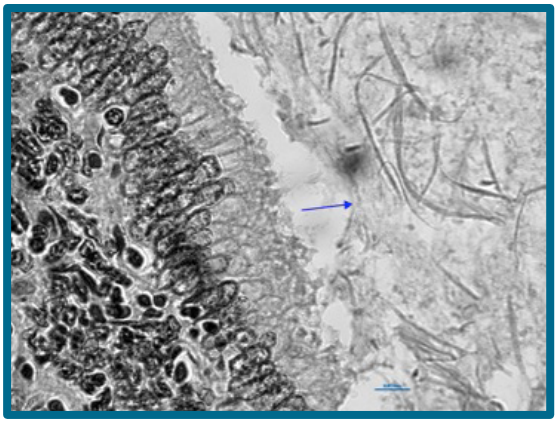


Figure 15. (200x) Light microscopy image of the right ovarian tissue in a non-gravid female cownose ray measuring 98.5 cm DW (caught in a haul seine near Poquoson, VA on 5/27/2010). No developing follicles are present in this tissue resulting in no ova production. The outer edge pictured here is the germinal epithelium made up of simple cuboidal cells. Just beneath this layer (lighter band) is a moderately dense connective tissue know as the tunica albuginea.

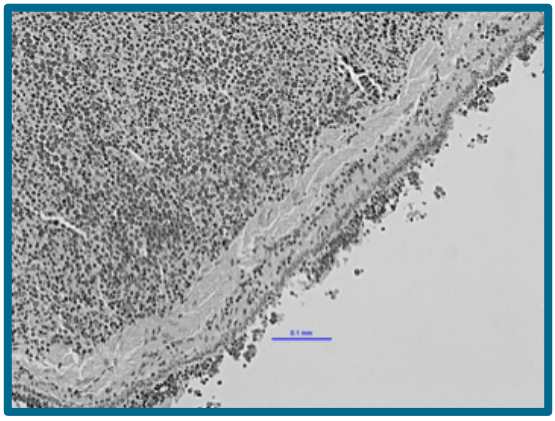
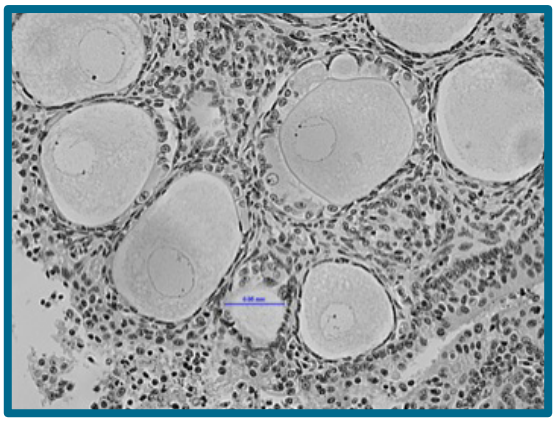


Figure 16. (200x) Light microscopy image of the left ovarian tissue in a non-gravid female cownose ray measuring 48.5 cm DW (caught in a pound net near Lynnhaven, VA on 5/20/2009). The average size of developing follicle was measured as 100 microns. All follicles for this individual were pre-vitellogenic. The active uterus measured 5 mm and the false uterus measured 4 mm. This female was considered immature.



totroph. In females with fertilized eggs the trophonemata epithelium is cuboidal. In the uterus of females with late term fetuses, the epithelium is simple squamous. Hamlett & Hysell (1998) documented the uterine tissues of a gravid *Urolophus jamaicensis* (yellow spotted stingray) showing highly vascularized trophonemata. Each villa has a core vessel that branches into capillaries. Simple columnar cells line secretory crypts with several apical secretory vesicles. This uterine development was also observed in the cownose ray (Figures 10-13). Macroscopically, trophenemata in maturing rays become more elongated and change from pink to red in color as vascularization increases.

Within both uteri of immature female cownose rays (females which have not gone through a gestation), a highly viscous substance is found (Figure 14). This substance is caramel in color and has a consistency of thick molasses. Mature female cownose rays, gravid females and those which had a prior pregnancy, do not possess this substance within either uteri. Histological evaluation of this substance in uterine provides speculation that it is secreted by the trophonema-

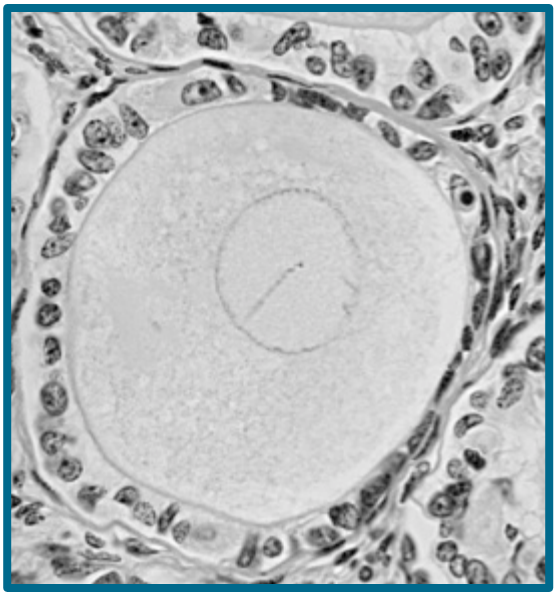


Figure 17. (400x) Light microscopy image shows one of several previtellogenic follicles found in ovarian tissue from a non-gravid female cownose ray measuring 48.5 cm DW (caught in a poundnet off Cape Henry, VA on 5/20/2009. The follicle cells of the outer rim are cuboidal and loosely packed. In this individual, there were only previtellogenic follicles found classifying her as an immature female. Note the cuboidal follicular cells surrounding the follicle.

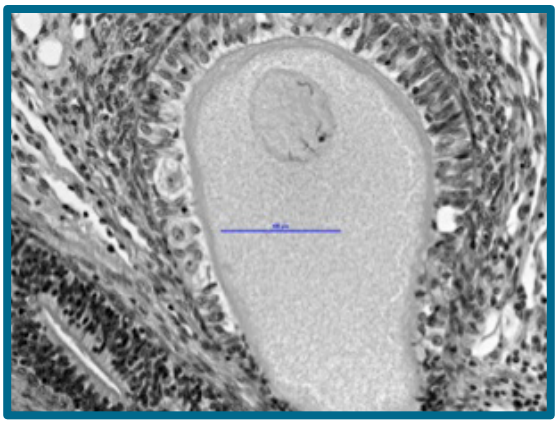


Figure 18. (200x) is a developing ova from a non-gravid female cownose ray (Rhinoptera bonasus) measuring 86.25 cm DW (caught off Poquoson, VA in May 13, 2009). Both uteri in this female were narrow and flaccid (15 mm) and the largest ovum recorded was 12 mm. The follicular wall cells are compacting and becoming columnar. Note the thickness of the zona pellucida, a non-cellular glycoprotein layer that is manufactured in part by the follicular cells.

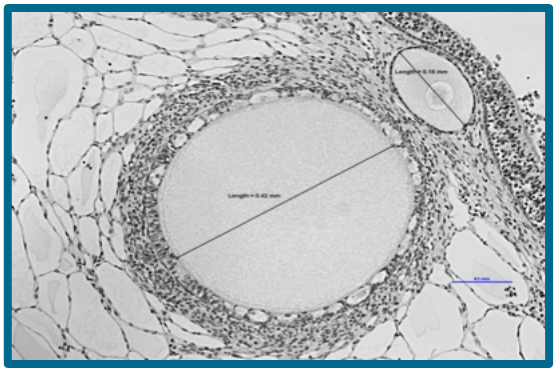
ta, but for what purpose is not understood at this time. Preliminary biochemical properties testing using protein electrophoresis characterizes the substance as a high molecular weight phosphoprotein which could be related to vitellin. Further biochemical testing is needed to further characterize this substance.

In elasmobranchs, the ovaries are paired organs embedded in the epigonal gland which are suspended from the peritoneal cavity. Although paired organs, only the left ovary is functional and produces ova. The right ovary fails to differentiate and does not produce ova (Figure 15). Development of ova in the ovaries follows three stages of vitellogenesis; previtellogenic follicle (Figure 16), vitellogenic developing follicle and term follicle.Stages of follicular vitellogenesis have been used to indicate state of sexual maturity in female winter skates (*Leucoraja ocellata*) (Sulikowski et al., 2005), female thorny skates (*Amblyraja radiata*) (Sulikowski et al., 2006) and Atlantic stingrays (*Dasyatis americana*) (Maruska et al., 1996) in combination with morphological parameters.

Vitellogen is a specific protein synthesized by the liver, released into the blood and transported to the ovary (McMillan, 2007). The zona pellucida surrounding the surface of the oocyte (follicle) begins to compact during vitellogenesis and allows selective transport of proteins (i.e. vitellogen) and metabolites (McMillan, 2007).

In previtellogenic follicles the cells are simple cuboidal with a modest number of transport organelles (Figure 17). These unilaminar follicles are surrounded by a single layer of simple cuboidal to simple squamous follicular cells. Hamlett et al. (1999) dis-

Figure 19 and Figure 20. (100x) show microscopic ovum (420 microns) sectioned from ovarian tissue of a non-gravid female cownose ray (Rhinoptera bonasus) measuring 73 cm DW (caught in a haul seine off Poquoson, VA in late May of 2010). Both left and right uteri were narrow (9 mm) and flaccid. The second largest ovum was 180 microns, pictured here. In Figure 11, the arrow is indicating the thickening zona pellucida. The three largest ova measured for this immature individual were 6.0, 4.0, and 4.0 mm.



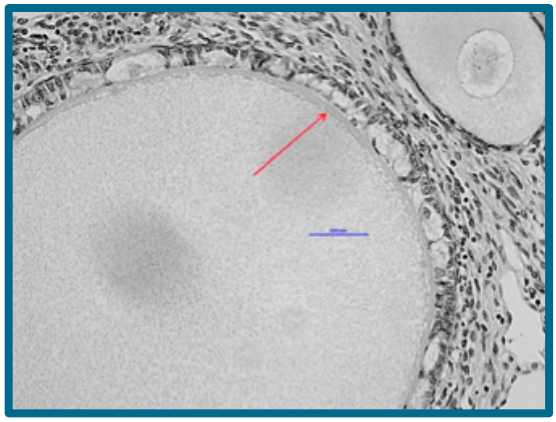
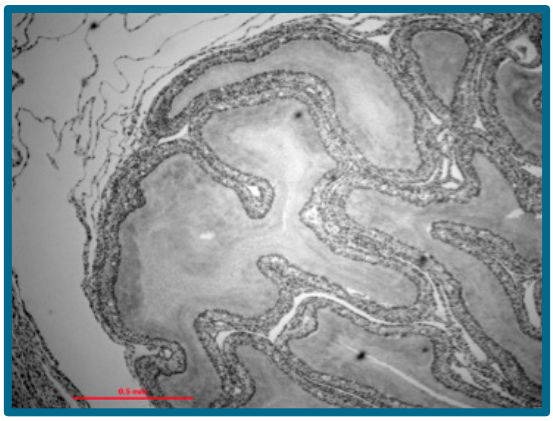


Figure 21. (40x) shows a vitollogenic follicle sectioned from a female cownose ray (Rhinoptera bonasus) measuring 98.5 cm DW (caught in a haul seine off Poquoson, VA on May 27, 2010). This female had an embryo in the right uterus, and the left uterus had gone through a previous gestation (47 mm). The three largest ova contained in the left ovary of this female measured 18, 18, and 13 mm.



cusses how the mitotic proliferation of follicular cells transforms the follicles changing the surrounding cell structure to columnar (Figure 18) and mutilaminar with elongate nuclei and apical transport vesicles visible. It is at this time in development that yolk precursors get transported to the oocyte (Hamlett et al, 1999b).

Figure 19 and Figure 20 (100x) show microscopic ovum (420 microns) sectioned from ovarian tissue of a non-gravid female cownose ray measuring 73cm DW (caught in a haul seine off Poquoson, VA in late May of 2010). Both left and right uteri were narrow (9mm) and flaccid. The second largest ovum was 180 microns, pictured here. In Figure 20, the arrow is indicating the thickening zona pellucida. The three largest ova measured for this immature individual were 6.0, 4.0, and 4.0 mm.

In vitollogenic follicles, the follicular wall will have extensive inward folding (Figure 25) but be tightly compact and intact throughout the entire follicle. The infolding generates greater surface area for transportation of yolk into the oocyte (Hamlett et al., 1999).

Follicular atresia within the ovary occurs in species with yolky eggs when developed follicles are not ovulated. The process begins with hormonal triggers and the follicular wall folds in and collapses down

on the oocyte as it disintegrates. Small cytoplasmic vacuoles begin to appear in the follicular cells and the yolk is deteriorated allowing for those nutrients to be recycled. When the yolk is completely removed the follicular and the cells will be broken down by phagocytosis and reabsorbed along with other cytoplasmic components that have deteriorated (McMillan, 2007). In atretic follicles, an inflammatory response may be common and is exhibited by the presence of eosinophilic granualar cells, lymphocytes, and white blood cells.

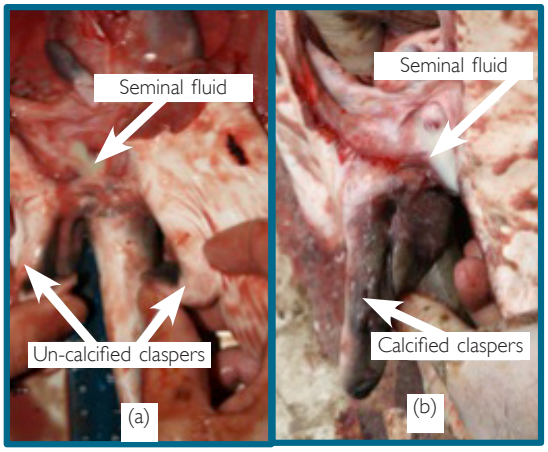


Figure 22. Ventral view of male cownose rays; (a) immature ray, seminal fluid present but claspers small and not calcified; (b) mature ray, claspers fully calcified.

Male Sexual Maturity

Clasper Calcification and Length

Maturity in male cownose rays is essentially based on the production of mature sperm and functionality of claspers, with success in reproducing contingent upon both. We routinely observed male rays 75-85cm DW having mature, coiled vas deferens and sperm present but were unlikely able to mate due to lack of clasper calcification, rigidity. Therefore, these animals were sexually immature (Figure 22).

Clasper size also contributes to maturity status (Figure 23). In comparing clasper length with DW, this study found that there is a more rapid increase in clasper length observed near 80cm DW, indicating onset of maturity (Figure 24), and supporting the earlier model of maturity occuring near 85cm DW (see Section 1).

Figure 23. Claspers of cownose rays varying in size. Claspers 1, 2, and 3 from immature males (DWs 76.5cm, 79cm, and 78.5cm). Claspers 4 and 5 immature, but calcifying/approaching maturity (DWs 83.5cm and 83cm).

Testis Correlation

Unlike ovaries in females, both paired testes lobes in cownose rays are functional; however the left lobe grows larger than the right. Histological examination of both testes and respective vas deferens confirmed sperm development and transport, with seminal fluids expressed equally from both sperm sacs at urogenital papilla also ob-

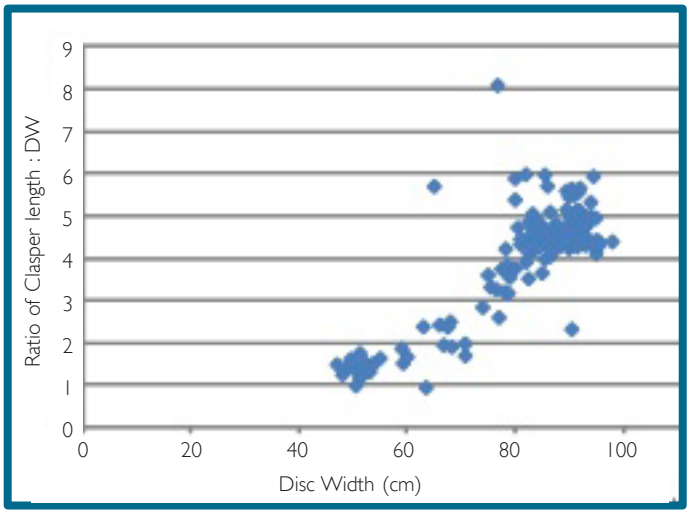


Figure 24. Relationship between outer clasper length and disc width for male cownose rays from the Chesapeake Bay (n=148).

Figure 25. Testes from male cownose ray showing size and shape differences between lobes.

Disc Width (cm)	Left Testis (g)	Right Testis (g)	Variance Left	Variance Right	N
75-80	63.5	27.2	0.008	0.003	6
81-85	86.2	54.4	0.014	0.007	19
86-90	163.3	122.5	0.042	0.019	12
91-95	158.8	117.9	0.02	0.014	13

Table 1. Weight difference in male cownose ray testes through maturation.

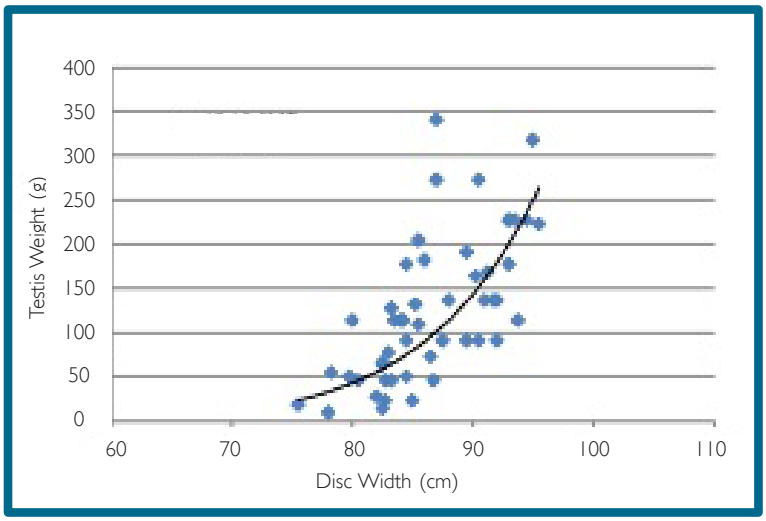


Figure 26. Comparison of weight of left testis to disc width of cownose ray.

served (Figure 22b). Left and right testes in male cownose ray differ in size and shape throughout growth and maturation (Figure 25), with the weight of left testis greater than the right (Table 1).

Weight of left testis was observed to grow rapidly as 80cm DW is attained and progressed through maturity (Figure 26).

Size, shape, and weight difference between mature testes lobes is likely due to available anatomical space within the body cavity in which they develop. The large liver prevents growth ventrally for both lobes; however, the right lobe also competes with the stomach and elongated spiral valve for space, resulting in a thinner lobe with less mass (Figure 27).

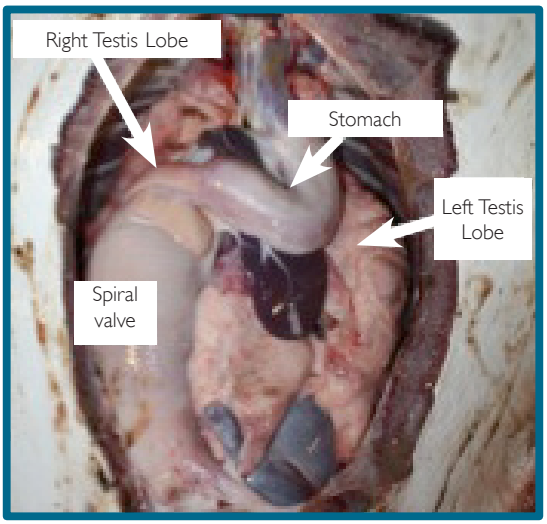


Figure 27. Ventral view of male cownose ray (liver removed) to show orientation of testes in abdominal cavity. Growth of right testis is restricted by digestive tract.

Male Histology

Seven stages in spermatogenesis (I, II, III, IV, V, VI, and VII) have been described in the literature by Conrath (2005) for elasmobranchs in general and were observed for cownose ray in this study. All seven stages of spermatogenesis can be observed in a single mature cownose rays.

Figure 28 shows stage I spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (87cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was coiled, milt was expressed, and the claspers were rated 3 (out of 3) for rigidity. All seven stages of spermatogenesis were documented for this individual.

Figure 29 shows stage I and II spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (82cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was beginning to coil, milt was not expressed, and the claspers were rated 1 (out of 3) for rigidity. This individual had stages I through V present in the testicular tissues sampled.

Figure 30 shows stage III spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (82cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was beginning to coil, milt was not expressed, and the claspers were rated 1 (out of 3) for rigidity. This individual

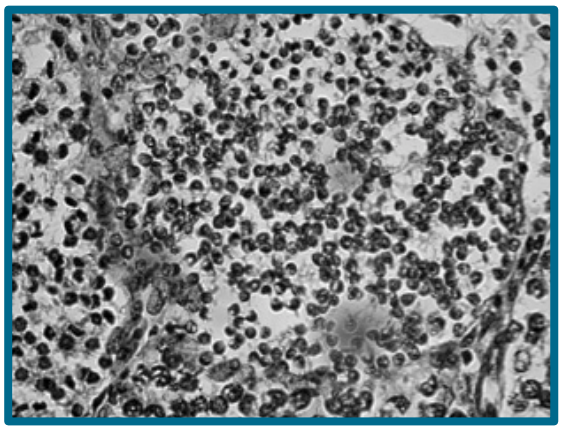


Figure 28. Stage I – Germ cells and loosely organized spermatogonia not yet bound by a basal membrane to form spermatocysts.

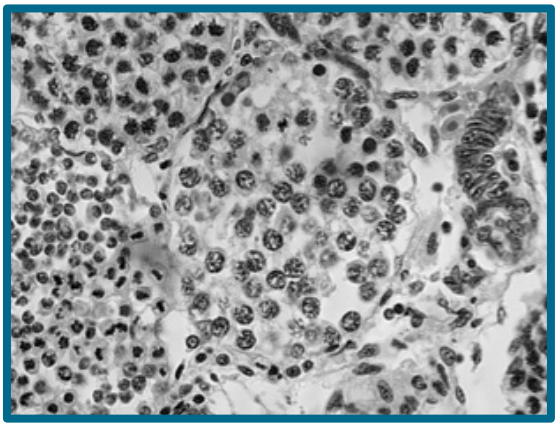


Figure 30. Stage III- The Sertoli cells begin to migrate to the periphery of the spermatocyst. The first meiotic division occurs during this stage.

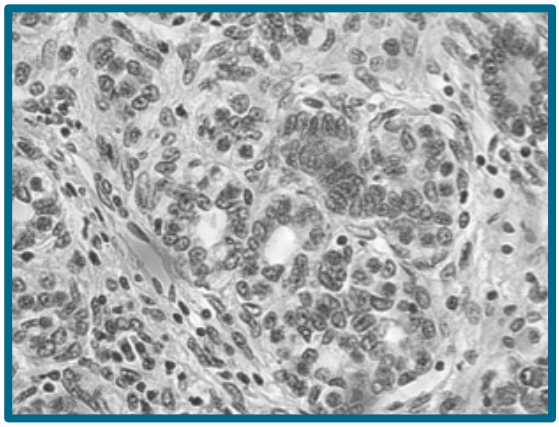


Figure 29. Stage II- These early spermatocysts contain an inner layer of Sertoli cells, a peripheral layer of spermatogonia and a hollow lumen at the center.

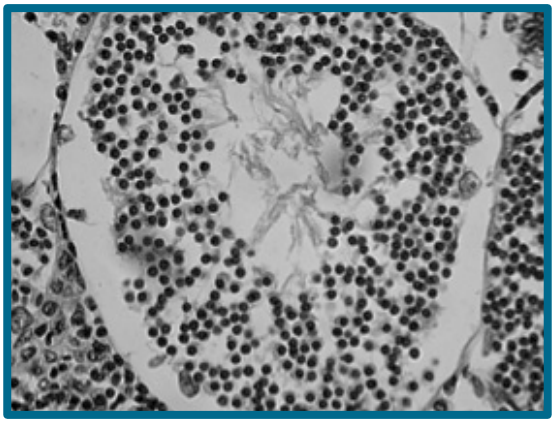


Figure 31. Stage IV- A second meiotic division of secondary spermotocytes forms spermatids in this stage.

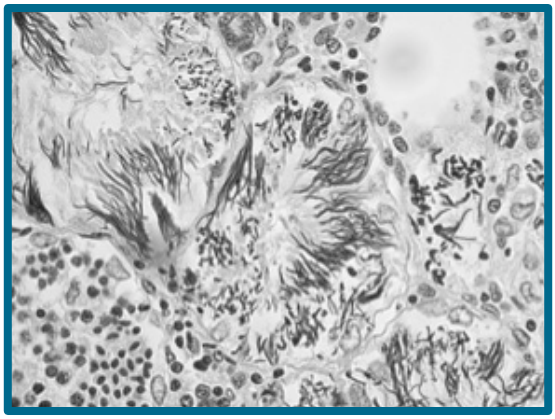


Figure 32. Stage V- Immature sperm are present at this stage and are radially oriented with tails in the lumen (center) of the spermatocysts but without being organized into clumps.

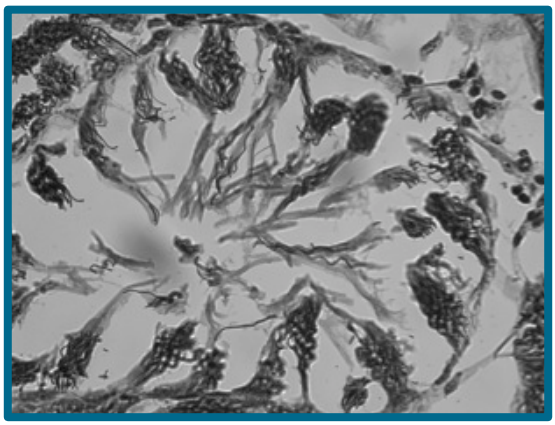


Figure 33. Stage VI- Mature sperm are present at this stage in spermatogenesis. They are packed in tight bundles and each bundle is associated with a Sertoli cell that will rupture and release the sperm.

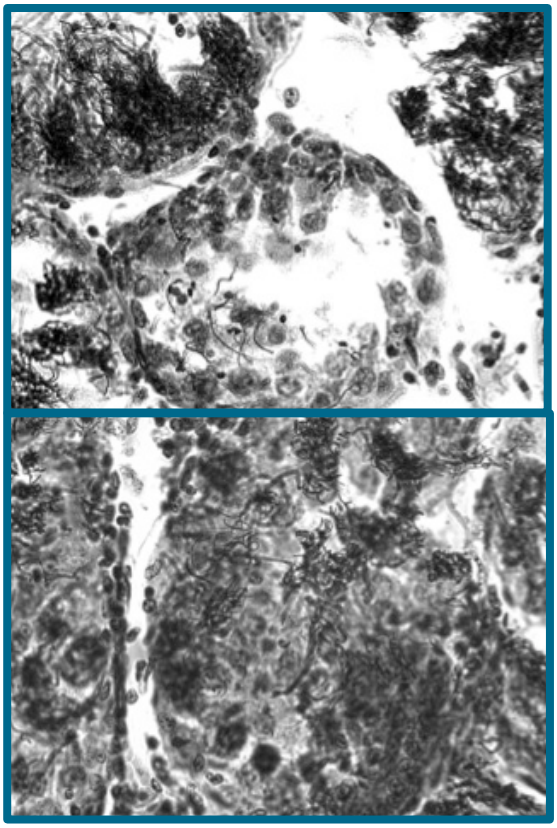


Figure 34. Stage VII – This stage is characterized by unreleased mature sperm. Free sperm is present and scattered among the ruptured spermatocysts.

had stages I through V present in the testicular tissues sampled.

Figure 31 shows stage IV spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (87 cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was coiled, milt was expressed, and the claspers were rated 3 (out of 3) for rigidity. All seven stages of spermatogenesis were documented for this individual.

Figure 32 shows stage V spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (82cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was beginning to coil, milt was not expressed, and the claspers were rated 1 (out of 3) for rigidity. This individual had stages I through V present in the testicular tissues sample.

Figure 33 shows stage VI spermatogenesis (400x) in testicular tissue collected from the left testis of a male cownose ray (87cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was coiled, milt was expressed, and the claspers were rated 3 (out of 3) for rigidity. All seven stages of spermatogenesis were documented for this mature individual.

Figure 34a and 34b show stage VII spermatogenesis (400x) in testicular tissue col-

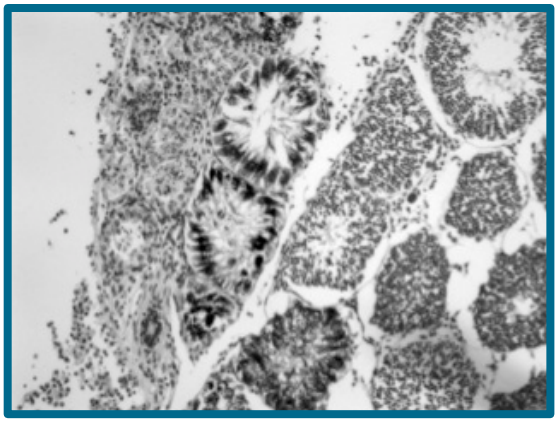


Figure 35.

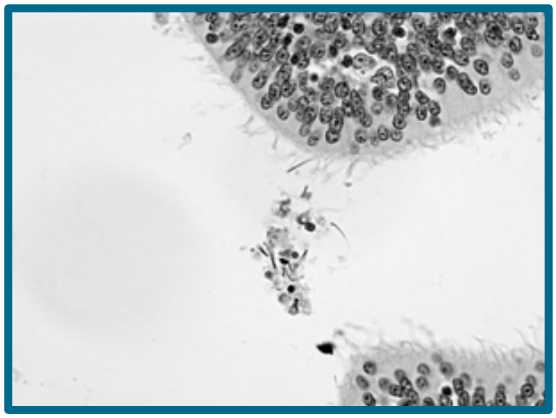


Figure 36.

lected from the left testis of a male cownose ray (87cm DW) caught in a haul seine off Poquoson, VA on May 26, 2009. The vas deferens of this individual was coiled, milt was expressed, and the claspers were rated 3 (out of 3) for rigidity. All seven stages of spermatogenesis were documented for this mature individual.

Since the paired testes in cownose rays vary in size (and with females typically having only one functional ovary), histological analysis was performed to confirm functional spermatogenesis in both testis lobes. Mature sperm (stage VI) were found in both the left and right testes of an individual measuring 87cm DW (Figure 35) (200x). The vas deferens from this same ray also contained spermatozoa (Figure 36) (400x) further confirming functional sperm development and transport. Homogeneity of developing tissue throughout the testis was performed by analysis of tissue from cranial, medial and caudal portions of testis lobes. No difference was found between lobe sections within a testis; therefore allowing consistent sampling from the medial-caudal region of testis lobes.

Discussion

Male maturity status was not as definitive as females due to many instances in which some reproductive structures were mature (production of sperm) but others were not (lack of clasper size and/or calcification). This period of partial maturity was observed in 75-86 cm DW rays, which spans multiple age classes. Of male rays observed between 75 and 86 cm DW (n=57), 47.3% expressed seminal fluids but only 8.8% possessed fully calcified claspers.

Section 3: Life History in the Chesapeake Bay

Cownose ray migrate into the Chesapeake Bay in the spring. While in the Bay, gestation of one year class ends and breeding for the next occurs. This section addresses mating and congregation observations between the sexes as well as embryo development, both of which have implications for management.

From 2006-2009, cownose ray were observed to reach the Chesapeake Bay within a 2-week period in the spring (last week in April to first week in May) and exit the Bay by the first week of October. Observed water temperatures during these periods of movement in and out of the Bay ranged from 14-17°C in spring and 20-24°C in the fall (Figure 1).

Sexual Segregation

Cownose rays are highly social animals, routinely observed aggregating in numbers which vary by size and sex depending on period within breeding cycle. Cownose rays appear to migrate north in the spring at a sex ratio close to 1:1. However, during residency in the Bay, sexual segregation occurs. This social behavior aspect of cownose ray life history while in the Chesapeake Bay will play an important role in fishery management if a commercial fishery is developed. Schooling behavior by size and sex and timing of parturition and subsequent mating are critical life history parameters that will impact fishery management.

Cownose ray collected throughout this study relied heavily on near-shore commercial haul seine and pound net fisheries with rays landed as by-catch. Sampling was augmented by

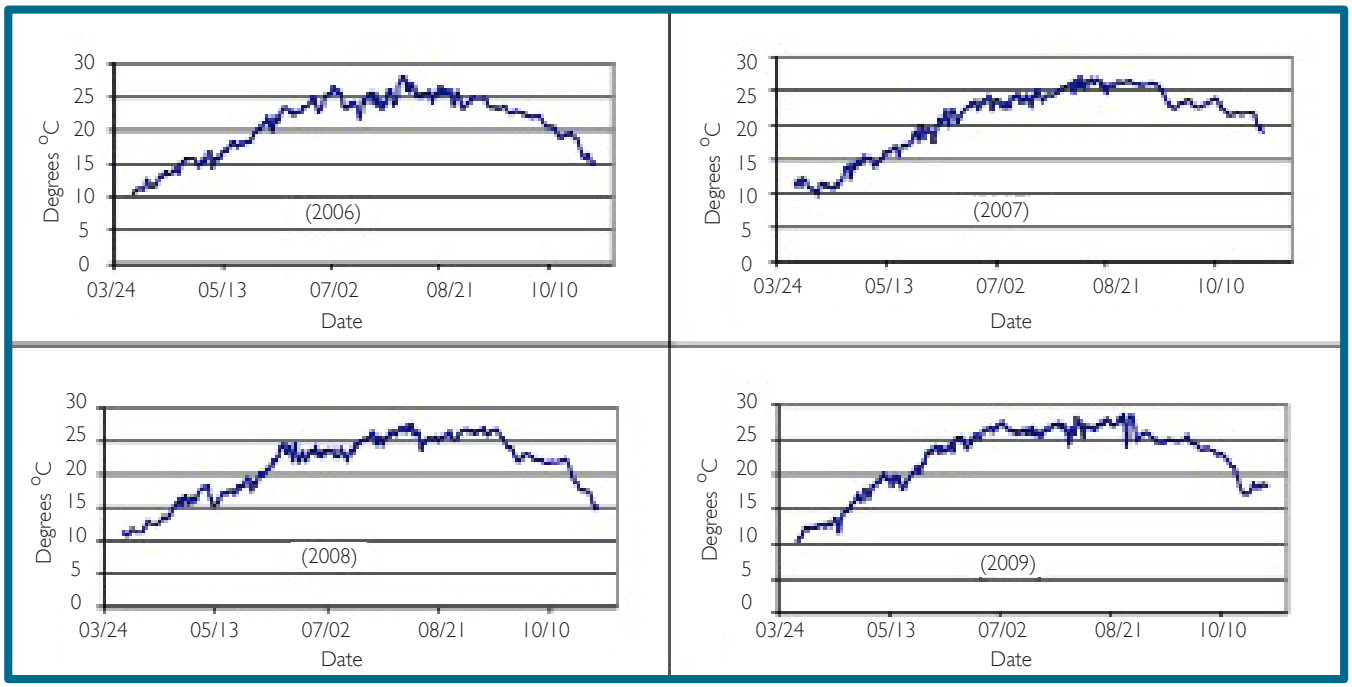


Figure 1. Average water temperature at the mouth of Chesapeake Bay (Chesapeake Bay Bridge Tunnel) May-October, 2006-2009.

fishery-independent methods; however they also fished near-shore habitats. Thus sexual segregation information for cownose ray in the Bay was limited to near-shore habitats in Virginia waters. Mixing of the sexes was observed in cownose ray as they enter the Bay in May (Figure 2) and was observed in all sampling sites in the Bay to continue through parturition in late June and early July (Figures 3, 4, 5) and subsequent mating through July. Segregation by sex was observed in cownose ray once mating concluded by early August with no mature males observed in samples collected from mid-August through September.

Females remain in near-shore environments throughout summer and are thus highly subjected to traditional fisheries. Post-mating, male cownose rays were not present in near-shore environments sampled, suggesting that males move into deeper waters or migrate to the eastern shore side of the Bay after mating. Sampling from deep water channels in the Bay and from the eastern shore shoal areas was limited in this study, resulting in a gap in ray sexual segregation information. In cownose rays collected in September (n=135) by the CHESMMAP trawl survey (2002-2010) which samples deeper water (15-75 ft) habitats in the Bay, mixing of the sexes was observed (62% female, 38% male); however, 88% of rays collected were young of year (< 54cm DW) and 93% were immature (<85cm DW) with the remaining mature rays mixed 58% female 42% male. Segregation of sexes to different habitats in the Bay may provide a feeding strategy within cownose rays in which competition for prey items is minimized,

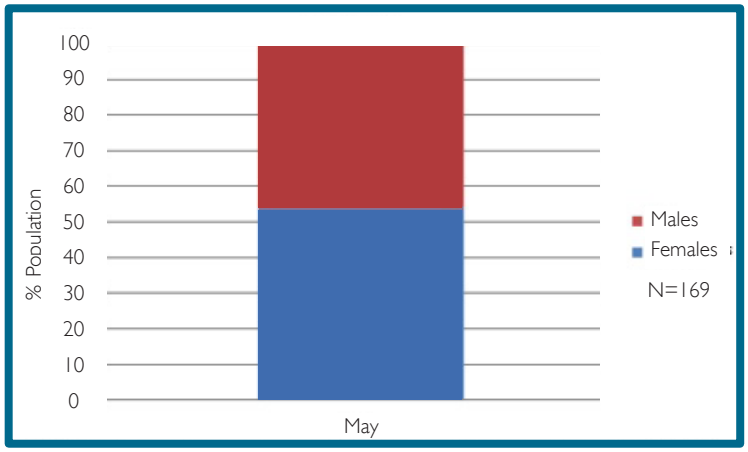


Figure 2. Sex ratio of cownose ray collected at the mouth of the Chesapekae Bay (Cape Henry, VA) in May as schooling rays enter the Bay.

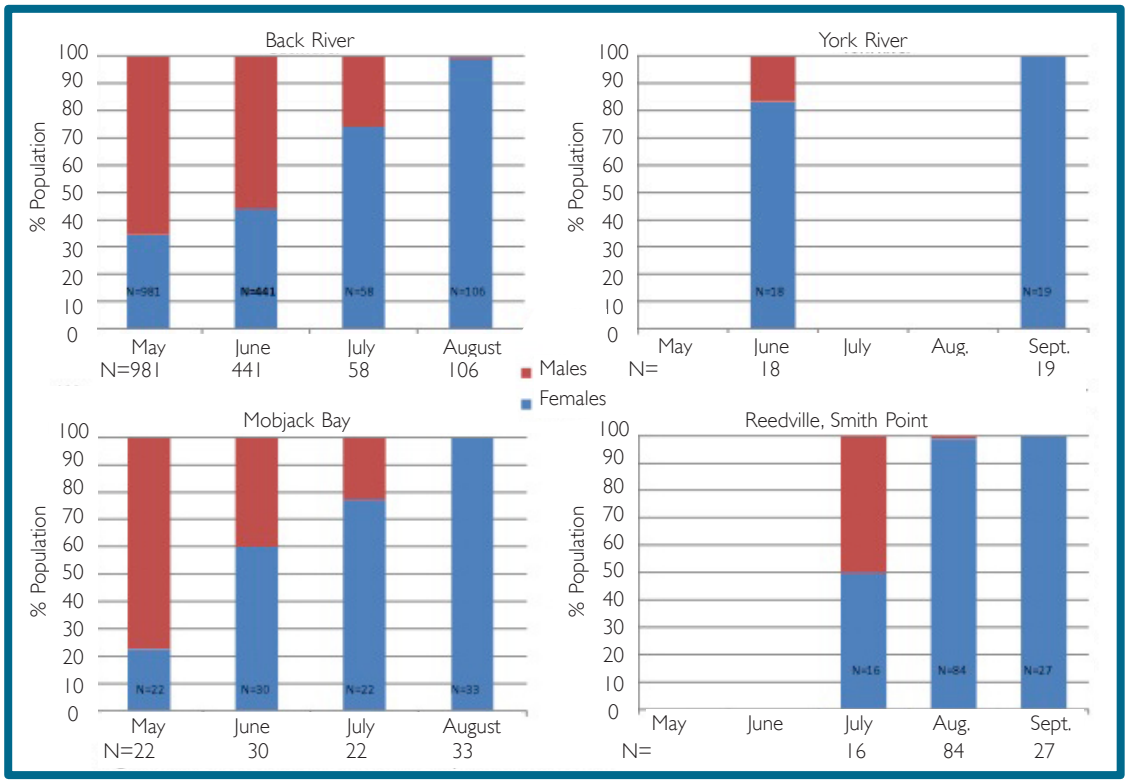


Figure 3. Sex ratio of cownose ray collected from various areas in the Chesapekae Bay.

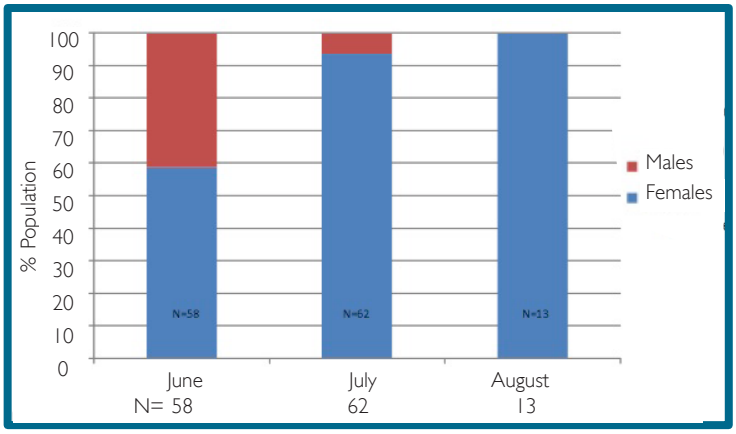


Figure 4. Sex ratio of adult cownose ray collected from Yocomico and Coan rivers of the Potomac.

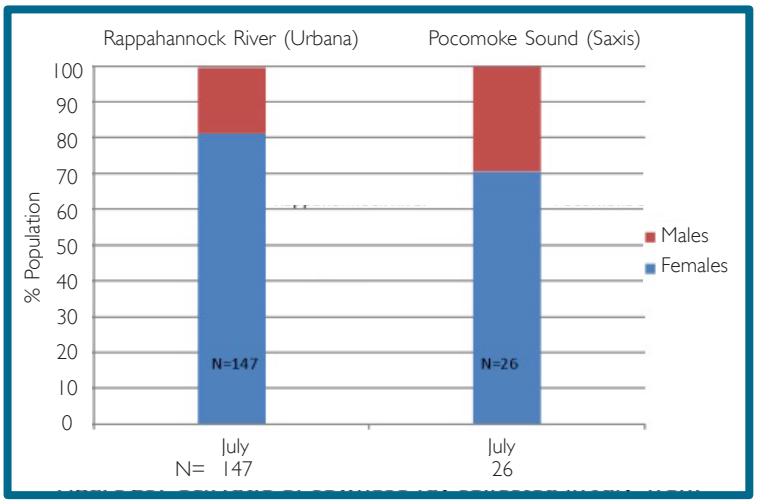


Figure 5. Sex ratio of adult cownose ray collected in July from Rappahannock River on western side of Chesapeake Bay and Pocomoke Sound on eastern side of Bay.

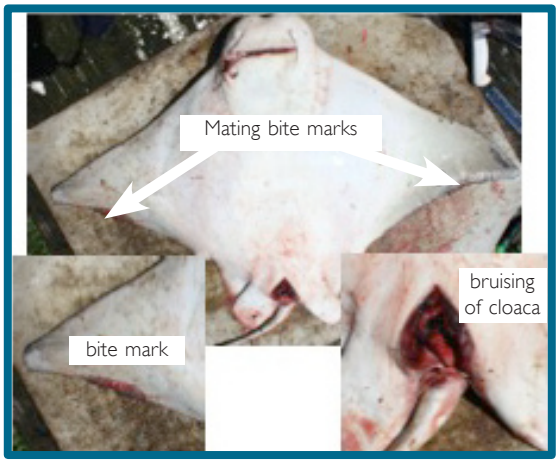


Figure 6. Female cownose ray showing mating (bite) marks on trailing edge of pectoral fins. Bruising of cloacal opening (insert right) is observed, suggesting repeaed and forceful mating occurs.

thereby allowing females with developing embryos more success in higher productive feeding habitats.

Mating

First signs of mating were observed in late June when parturition was still occurring and continued through early August. Evidence of mating was routinely observed in rays through this period, including mating bite marks (Figure 6) on female's pectoral fins and presence of seminal fluids within the female's uteri and cloacal opening. Males bite down on the trailing edge of female's pectoral fin when positioning and maintaining contact for copulation. The force exerted by this biting action is substantial, resulting in severe tissue abrasions, and frequent tissue loss from females pectoral fins. These mating marks are observed on both pectoral fins of females simultaneously, indicating multiple copulations occur and males use both claspers for mating. Frequent bruising (hematoma of tissue) in females cloacal opening during peak mating period (July) suggest forceful and repeated mating occurs. Within a single sample collection of 16 female rays from late June, half the females had recently given birth, the others still carrying term embryos, with those which completed parturition discharging seminal fluids from cloaca and, upon dissection, carrying seminal fluids within each uterus. New mating bite

Figure 7. Older mating mark in state of tissue repair.

marks, and presence of deposited seminal fluids in females, were observed to decline in late July and early-August (area dependent). Only healing (tissue repair) mating marks (Figure 7), and absence of seminal fluid inside females genital tract were observed from mid-August on, indicating that mating had concluded. This mating period coincides with period of ovulation described above. By early to mid August, mature females are gravid with developing embryos, resulting in gestation periods ranging from 11-12 months.

Embryonic Development

The smallest embryo collected was 66mm DW in early August. Embryos begin development initially through nourishment by the protein and lipid-rich external yolk from ovulated ova. The yolk is contained within a yolk-sac and is attached to the embryo by a yolk stalk (Figure 8). The yolk stalk attachment site is positioned medially on embryos ventral surface where esophageal-stomach section of the alimentary canal is located, with nourishment delivered directly to the digestive tract. During early development (August) the yolk sac averages 13.2% of the total embryo-yolk complex in weight (Figure 9). During yolk nourishing period, trophenemata (flattened, finger-like projections) lining the uterus continue to grow in length and begin producing histotroph (yellowish, lipid-rich uterine milk with distinctive whey aroma) which bathes the embryo (Figure 10) and is ingested and absorbed through the embryos spiracles and gills, thus nourishment is likely provided by both during this early embryonic period.

By late September, early October (Figures 8 and 9) the yolk sac is largely depleted (0.98% of total embryo weight) with further nourishment through parturition largely provided by trophenemata.

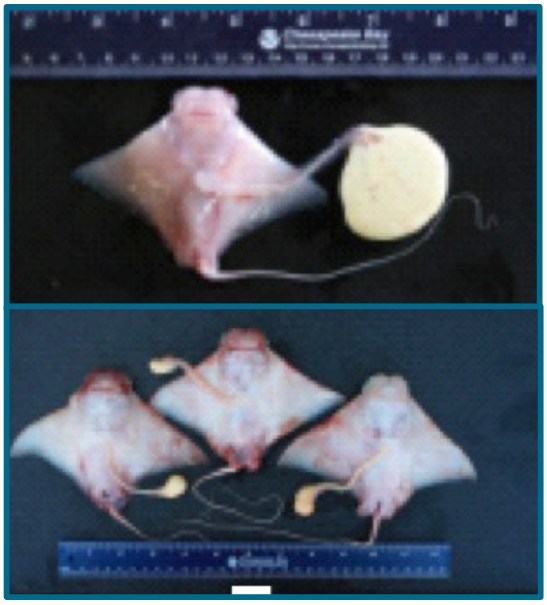


Figure 8. Cownose ray embryos with yolk sac attached; (a) large yolk reserve in August; (b) yolk sac nearly depleted in September.

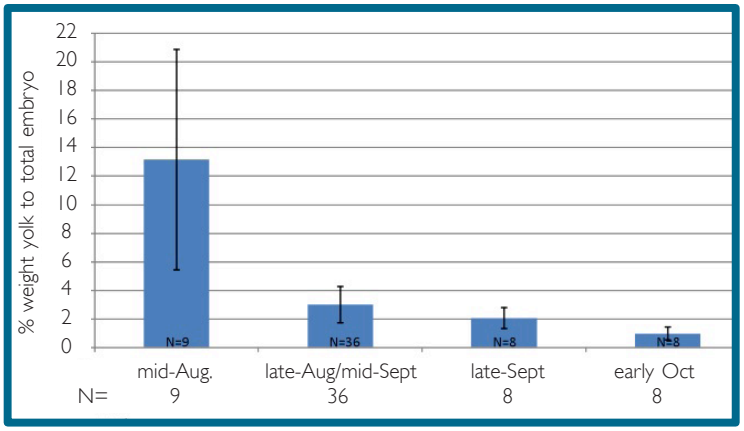


Figure 9. Percent yolk sac by weight of total embryo weight during early gestation in cownose ray.

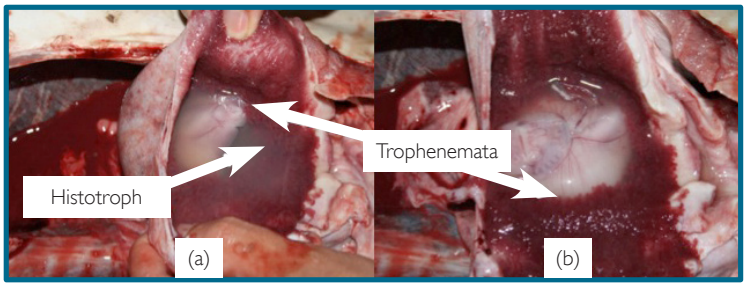


Figure 10. Cownose ray embryo in early gestation (Aug.); (a) bathed in histotroph inside uterus; (b) nore finger-like projectors (trophenemata) lining uterus.

	Early Gestation			Late Gestation			
	Aug.	Sept.	Oct.	Early May	Late May	June	July
Mean Embryo Total Wt (g)	61.5±25.5	86.3±36.3	171.9±18.4	362.8±109.2	572.5±106.8	1210.9±234.9	1325.4±412.1
Avg DW (cm)	9.8±1.29	15.9±1.7	21.8±0.9	28.3±3.2	31.9±1.9	41.7±2.8	42.3±2.69
N	9	22	8	19	30	89	17

Table 1. Size and weight of cownose ray embryo during early and late states of gestation.

Sex Ratio of Embryos

At time of migration to southern wintering grounds (late September, early October) gravid female cownose rays are carrying embryos averaging 21.4mm DW and 164.3 g (Table 1). Upon return to the Chesapeake Bay in early May (7 months), embryos average 28.3 cm DW and 362.8g. Relative to growth observed during first stage of gestation and size of embryos at time of southern migration (Oct), resulting size of three-quarter term embryos upon return (May), and growth to term (mid-June Early July) suggest developmental diapauses may occur during wintering period. Embryonic diapause has been reported in other elasmobranchs to allow birthing when water temperatures are optimal for juvenile growth (White, Hall & Potter, 2002; Simpfendorfer, 1992). Without knowledge of exact environmental conditions of over-wintering area for the Atlantic cownose ray, full understanding of embryonic growth during this period is not possible, but one can speculate that conditions are not as optimal during the winter compared to spring/summer conditions along the western Atlantic U.S. coast and growth diapause could be a strategy employed by the cownose ray to increase survivorship of newborn as well mother, increasing reproductive success by migrating north in spring with embryos still at three quarter term and when available food resources are more plentiful and allowing mother to obtain nourishment for completion of gestation (term) and subsequent mating. Young-of-year (YOY) are observed to migrate out of the Chesapeake Bay after adults had exited; only YOY rays caught in pound nets at mouth of Bay in October, indicating that YOY may not make the migration to southern wintering grounds with the reproducing mass, but over winter in other estuaries south of the Bay where water temperatures are more favorable.

In the spring, cownose rays migrate north along the Atlantic seaboard and reach the Chesapeake by early May. An unknown proportion of this Atlantic cownose ray population enters the bay, with another segment of the population continuing north to inhabit coastal estuarine systems of Virginias' eastern shore to New Jersey. Throughout sampling from various locations in the Bay for this study, 1-4 year old rays (60-75 cm DW) were scarce. Since gear type largely employed for sampling (haul seine, pound net) targets fish of size well below that of juvenile rays, and neonate rays are routinely captured using these gear types, it is thought that juvenile cownose rays do not widely use the Chesapeake Bay during their juvenile life stage. Trawl surveys conducted in the Bay by Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP) at VIMS from 2002 to 2010 collected 161 cownose rays ranging in size from 24.0-111.8cm DW, however, no rays between 54.7 and 71.9cm DW were recorded. Further, juvenile rays younger than at least three years of age may not participate in the spring northern migration and remain in estuaries to the south.

Gravid females are at three-quarter term gestation upon entering the Bay in May, with parturition not occurring until mid-June to early-July. Of females, 95.7% s >90cm DW

(N=70) sampled from mid-May to mid-June were gravid, with non-gravid females representing the smallest rays sampled (90-90.5cm DW) and having reproductive status similar to those entering first breeding cycle. Embryo growth during final quarter (late-May to early-July) of gestation is substantial with embryo weight more than doubling (Table 1).

At time of parturition, the term embryo completely extends (stretches) the uterus to the point uterine wall becomes transparent, with internal embryo visible through the uterine wall (Figure 11). Offspring birth size relative to maternal size in other myliobatiform rays has been characterized as larger females give birth to larger offspring (Devadoss, 1978; Raje, 2003). In this study, a weak positive correlation between female size and the size of term embryos was observed in cownose ray (Figure 12).

Cownose ray give birth to free-swimming young. At-term embryos (N=115) collected in late-June and early-July averaged 42.1 cm DW and 1.28 kg. Female term embryos averaged 42.3 cm DW (1.32 kg), and males averaged 41.9 cm DW (1.24 kg). Free-swimming neonates were first observed in late July but sampled in early August when aggregations of neonates with mature females were observed.

Figure 11. At-term embryo completely stretching uterine, allowing internal embryo to be viewed.

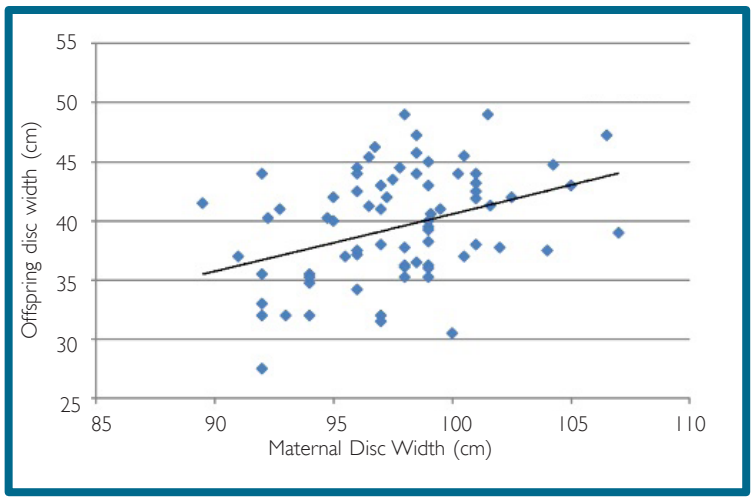


Figure 12. Maternal-offspring size relationship of at-term embryos (N=74).

Sexual Segregation: Embryos

A total of 109 neonates were collected the first week of August; 48% females averaging 42.47cm DW (1.06kg), and 52% males averaging 42.53cm DW (1.04kg). Neonate growth within the first 4-6 weeks post-parturition was negligible, with a nominal increase in DW but a considerable decrease in weight (17%) observed. Initial weight loss of 6.4% (n=5) was observed in the first 9 days after birth in captive cownose rays (see Section 6). At the time of migration south (late September-early October), young-of-year (YOY) rays were observed to aggregate together and leave the bay after the adults had already done so. Sampling pound nets at mouth of bay in early October resulted in the collection of only YOY rays (N=67); 38.5% were female and averaged 51.5cm DW (2.14kg), 61.5% were male and averaged 51.4cm DW (2.05kg).

Sex ratio of at-term embryos (N=115) was 55.6% female, 44.4% male. Neonate cownose ray were observed to school with mature segregated females (98% females) during August (Figure 30) at near equal sex ratio (48% female, 52% male). In Octo-

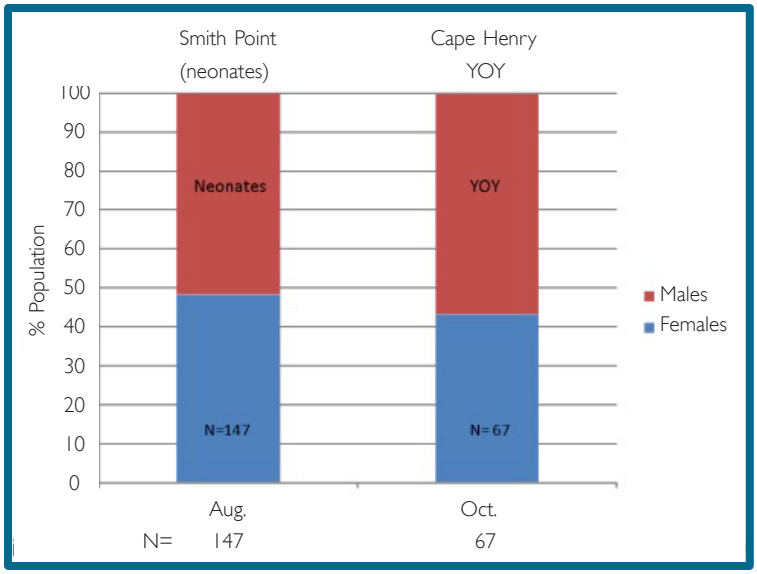


Figure 13. Sex ratio of free-swimming neonate cownose rays in August from Smith Point and YOY in September collected from pound nets at mouth of Bay (Cape Henry).

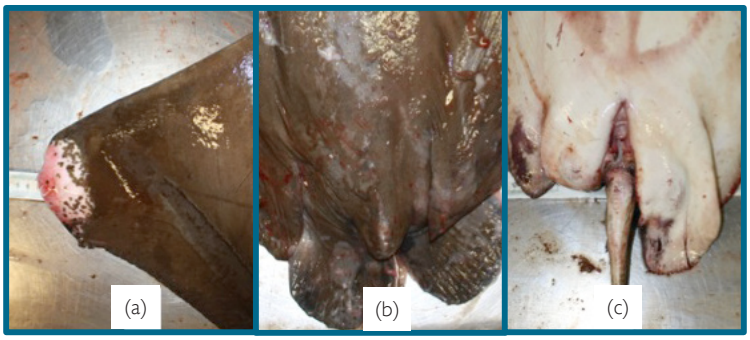


Figure 14. Results of predation attempts on cownose ray by large predators; (a) severed pectoral fin, (b) severed tail, and (c) severed anal fin.

ber (Figure 13), young of year rays were also observed with sexes highly mixed (38.5% female, 61.5% male), indicating that year 0 and juvenile cownose ray school in mixed sex groups while in the Bay.

Ray Mortality

Being an apex predator within the benthic community, cownose ray are thought to have few predators. Natural predation on cownose rays is largely thought to occur by sharks during ray's spring and fall coastal migrations between summer and winter grounds where near-shore shark species (*Carcharhinus obscures* and *C. plumbeus*) frequent (Castro, 1996; Ellis & Musick, 2007). Cobia has also been reported to feed on cownose ray (Arendt et al., 2001) and as near-shore shark species; predation should be expected since they are sympatric along ray migratory route. Once in the Bay, ray mortality is largely due to culling activities of fishermen who use picks (spike on a stick) to remove rays from nets to prevent escapement of targeted fish, or from boat deck overboard to ensure safe handling. Mortality associated with culling practices is not known. Currently there is no directed fishery for cownose ray in the Bay, with no fishing mortality estimates available.

Evidence of failed predation attempts on cownose ray were frequently observed on collected rays (Figure 14), all representing predation attempts from large predators attacking from behind (tail bit off at base, claspers bit off, large portions of anal fins removed) or, less frequent, from the side (tips of pectoral fins removed). Severed body parts were observed to be completely healed over at point of separation, suggesting efficient wound repair abilities of cownose ray. Signs of predation attempts were not consistently recorded for a large portion of this study; however, several specific examples of predatory attempts were recorded. In single hauls from commercial haul seine operations working different areas, 10 of 169 (16.9%), 7 of 80 (8.7%), and 12 of 153 (7.8%) rays were recorded with predatory wounds as described above. Five of 18 (27.7%) rays collected by long line over 2 days from same area also had predatory wounds. The relatively high percent of these predation scars observed suggests significant natural predation occurs on cownose ray.

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Section 4: Feeding Ecology in the Chesapeake Bay

Like most myliobatiform rays, cownose rays are durophagous upper-level carnivores which prey primarily on infaunal and epifaunal benthic invertebrates as mollusks, crustaceans, and polychaetes. The jaw of cownose ray is engineered to crush hard prey items (Figure 1) with re-enforced cartilage at point of prey crushing and highly mineralized teeth plates. Concerns over predation on commercial bivalve resources have been raised by fishery and aquaculture operations for many years and in several regions of the world. However, little evidence of actual predation on these resources has been documented. Smith & Merriner (1985) investigated the diet of cownose rays caught in Chesapeake Bay during the summers of 1976-1978. Most rays were captured over shallow sand and mud flats in the lower York River, and no samples were collected from known oyster beds. Sample sizes were very small (N=40) but the three dominant prey items were soft-shell clams (*Mya arenaria*), Baltic macoma clams (*Macoma balthica*), and stout razor clams (*Tagelus plebeus*). The remains of oysters (*Crossostrea virginica*) were only found in one stomach and hard clams (*Mercenaria mercenaria*) were only identified in three stomachs. Softshell clam populations are now depressed in Chesapeake Bay and there is concern that cownose rays have shifted to feeding on oysters and hard clams instead.

This trophic ecology research on cownose ray predation was performed to assess the relative importance of commercial bivalves in the diet of cownose rays and provide an evaluation of temporal dietary shifts that may have occurred since last studied in the 1970's. Further, results on prey item diversity and benthic trophic structure will assist an ecologically-based approach to manage cownose ray species upon initiation of a fishery.

NOTE: Data tables will be made available online at http://bit.ly/cZY1U0 .

Methods

Cownose rays were sampled for stomach analysis from May 2006 through September 2009 in various locations in the Chesapeake Bay using fishery-dependent and fishery-independent methods. Captured rays were processed for various biological assessment parameters. Stom-

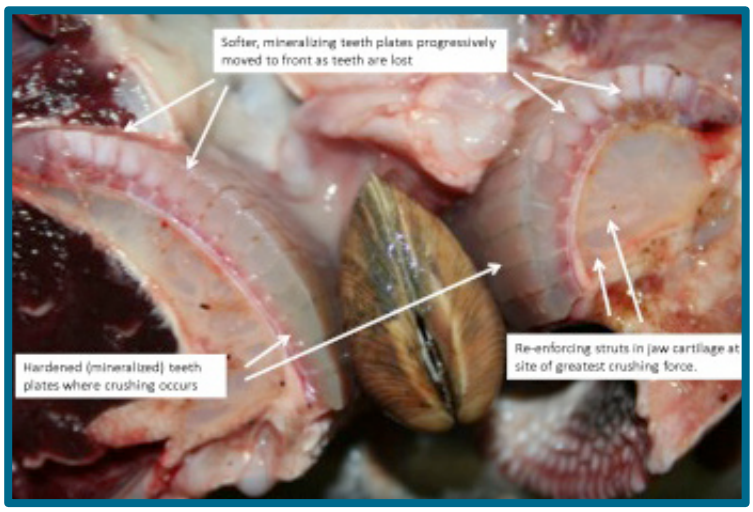


Figure 1. Jaw of cownose ray in cross-setion with hard clam plaed at point of prey crushing.

achs were removed by severing the esophagus as it entered the peritoneal cavity at the cranial side of digestive tract and were the stomach leads into the spiral intestine on the caudal side. Removed stomachs were placed in plastic whirlpack bags, frozen, and held in freezer cold storage until processed, from 4-6 weeks. Frozen stomachs were thawed in cool water within sealed sample bags for one to four hours depending on size. Once thawed, full stomach wet weights were recorded to nearest 0.000g on an electronic analytical balance. The stomach contents were then emptied into a petri dish for sorting and identification and the empty stomach was weighed. The overall stomach contents weight was then calculated by difference.

With the use of field guides and taxanomic keys, prey items were identified to the lowest possible taxon and sorted for collective weights for each food category. Some teleost remains were highly digested and species identification was dependent on locating and identifying the otoliths. Shell fragments of bivalves were identified to lowest possible taxon and sometimes to species if enough characteristic attributes were found (ie. hinges). Vegetation was identified as below ground (ie. seagrass rhizomes) or above ground. Decayed or rotten vegetation that was not recently living was described as detritus. Enumeration of prey items was not feasible due to the level of mastication of food items. Each food category was weighed to the nearest 0.000g. The total weight of each food category was expressed as a percentage of the overall weight of the stomach contents. Frequency of occurrence was recorded for each prey item identified.

Upon identifying vegetation components present in ray stomachs, further classified as above ground or below ground vegetation was made to provide additional information on ray feeding behavior. Above ground vegetation consisted of leaf, stem, and all dead, but not decomposed plant matter, presuming these plant parts would be associated with substrate surface or above. Plant rhizomes and root systems which typically embed vegetation into the bottom substrate were classified as below ground vegetation.

Sampling was initially conducted solely by fishery-dependent methods, obtaining rays as by-catch of commercial haul seine and pound net operations. A distinct bias as to certain stomach content items was quickly realized relative to sampling method, specifically with amounts of teleost fish observed, indicating that natural prey items may not be accurately reflected in sampling protocol. Cownose rays process food and evacuate waste quickly (personal observations during behavioral and feeding studies). Rays have relatively short upper digestive tracts constituting the esophagus and stomach, were food is secured and initial digestion occurs. Partially digested food in the stomach is moved along to the spiral valve where additional food breakdown and nutrient absorption occurs. Most soft-bodied prey items found in the spiral valve are beyond identification to specie, but hard, un-digestible parts of prey are retained longer and can be identified. By nature of commercial haul seine and pound net fishing practices, fish are initially entrapped within an enclosed area and held confined for periods ranging from 8 hours (haul seine working the tide) to 2 days (pound net) before they are landed. During confinement, rays are able to evacuate their stomachs of food preyed on prior to entrapment, and continue to feed on what is available within their confine area. Cownose rays are highly opportunistic feeders, and will actively feed on what is available. Fish become immobilized due to entanglement in gear and are actively fed upon by rays, which constitutes an observed prey item not considered a typical natural component in cownose ray diet. This observed behavior exemplifies the opportunistic feeding strategy of cownose ray which will actively consume food items which may not be preferred but are readily available and minimizes energy expenditure.

The bias of prey components observed within ray stomachs from fishery-dependent sampling resulted in the commencement of fishery independent sampling. A combination of a modified Dutch seine, long-line rigs, and bowfishing (bow and arrow), allowed the sampling of various habitats and generation of more diverse natural prey components for cownose ray. These fishery-independent methods were employed to remove rays from the water as soon as possible to minimize loss of stomach content, thus providing a more accurate assessment of cownose ray natural prey items. These fishing methods were restricted to relatively shallow water habitats ranging from 0.6-3m. Modified Dutch seine was pulled for 20min each set by twin dead-rise boats in Back River channel along Plum Tree Bar (Poquoson, VA). Long-line sampling was conducted adjacent to commercial oyster grounds which were currently growing spat-on-shell (SOS) oysters, either wild SOS or cultured SOS with no cultured cultchless oysters deployed (wild cultchless oysters are observed associated with commercial grounds, but intentional planting of cultchless oysters was not being practiced during this study). Long-line gear was tended three times per day to minimize time live rays were held hooked prior to landing. Bow and arrow sampling was conducted from boats by members of a local bowfishing organization and was conducted in Lynnhaven Inlet and Timber Creek (York River). Rays were immediately landed on boat after shot.

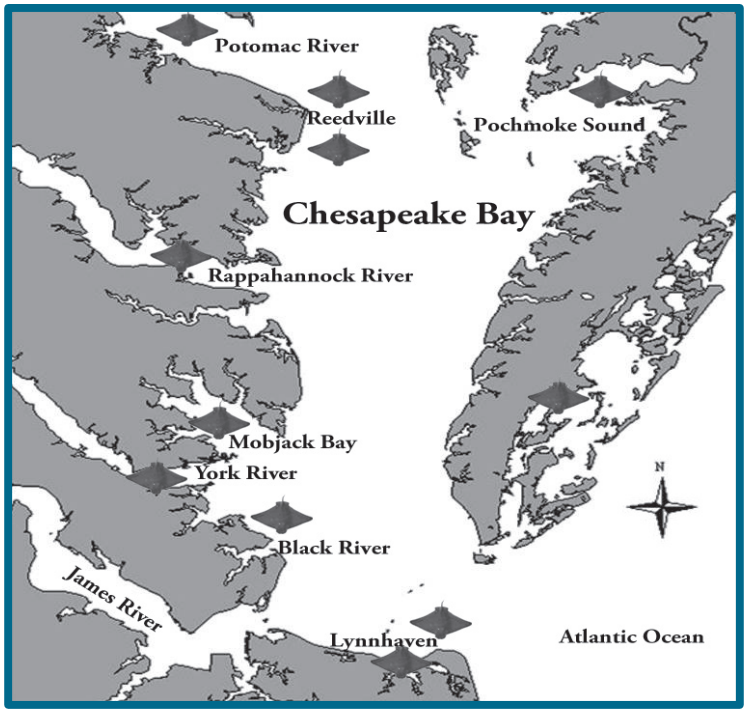


Figure 2. Cownose ray sampling sites in the Chesapeake Bay. Sampling sites are labeled with

Results

The stomachs of 781 cownose rays were sampled in the Chesapeake Bay from May 2006 to September 2009 (Figure 2). Fishery-dependent samples (n=401, 305 females and 96 males) were collected from haul seine operations fishing in Back River (Poquoson, VA), York River (Gloucester Pt., VA), and Mobjack Bay, and from pound net operations positioned off Lynnhaven, VA, Reedville, VA, and Smith Point, VA (mouth of Potomac River). Fishery-independent samples (n=380, 240 female, 140 male) were collected from Lynnhaven River, Back River (Poquoson, VA), Timberneck Creek (York River), Yeocomico River and Coan River (off Potomac River), Robinson Creek (off Rappahannock River, Urbanna, VA), and Pocomoke Sound (eastern shore side of Bay, Saxis, VA).

There are several different methods of reporting diet results. For this report, results of stomach content analysis are reported as number of occurrence of prey items and percent of observed prey item in stomachs with quantifiable contents. Because rays evacuate stomach content in a short time period after feeding, many rays were observed with empty stomachs. Twenty five percent of fishery-dependent stomach samples were empty and 36% of fishery-independent samples were empty. Fewer empty stomachs in fishery-dependent samples were expected due to the available food during period of confinement. Empty stomachs are represented in results with prey categories (bivalves, crustaceans,

fish, vegetation, other) as % of total rays collected per sampling method. Reporting empty stomachs initially as part of total rays sampled is thought to provide a more complete analysis of our findings and possibly prevent conclusions that inflate importance of any one prey item. Where empty stomachs are reported, % of any given prey item is relative to its occurrence in the total number of rays sampled. Within each sampling method, stomach content categories are further reported as % frequency of occurrence of total stomachs with at least one prey item (termed "full" as opposed to empty), not in-

Prey Items			% Frequency	
Group	**Latin name**	**Common name**	**Fishery Dependent**	**Fishery Independent**
Teleost Fishes	Anchoa mitchilli	Bay anchovy	1	
	Anchoa spp.	Anchovy	2	
	Brevoortia tyrannus	Atlantic menhaden	51	
	Cynoscion regalis	Weakfish	1	
	Dorosoma celedianum	Gizzard shad		29
	Leiostomus xanthurus	Spot	2	
	Micropogonias undulatus	Atlantic croaker	5	
	Peprilus spp.	Butterfish	3	
	Unid flatfish		3	
	Unid fish		3	71
Crustaceans	Ampithoe longimana	Amphipod	5	
	Barnacle spp.	Barnacle		8
	Callinectes sapidus	Blue crab	9	
	Callinectes spp.	Crab	4	
	Caprella penantis	Skeleton shrimp	13	
	Caprella spp.	Shrimp		8
	Corophium	Mud shrimp		9
	Crangon septemspinosa	Sand shrimp	18	
	Cumacean spp.	Hooded shrimp	4	
	Cymadusa compta	Smphipod		2
	Eurypanopeus depressus	Depressed mud crab	9	
	Gammarus spp.	Scud amphipod	4	
	Haustoriidae	Amphipod		8
	Leptocheirus spp.	Amphipod	<0.5	
	Monoculodes dewardsi	Red-eyed amphipod		8
	Xanthidae	Mud crab spp.		10
	Oedicerotidae	Amphipod		2
	Palaemonetes sp.	Ghost shrimp	4	
	Palaemonetes vulgaris	Marsh grass shrimp	4	
	Paracaprella spp.	Skeleton shrimp	4	
	Pinnixa spp.	Pea crab		2
	Rhithropanopeus harrisii	White-fingered mud crab		2
	Unid amphipods			2
	Unid crab parts		22	39

Table 1. Prey items identified in cownose ray stomachs by sampling method; "Unid"=unidentifiable. (Chart continued on next page.)

cluding empty stomachs. To provide relative importance of a given prey item, % frequency of prey item of total within each category is reported.

Stomach content analysis of cownose rays sampled in the Chesapeake Bay using fishery-dependent and fishery-independent methods provided quantitative information on feeding habits, composition of prey, and relative importance of prey items to

Prey Items			% Frequency	
Group	**Latin name**	**Common name**	**Fishery Dependent**	**Fishery Independent**
Bivalves	Anadara spp.	Blood ark	<0.5	
	Crassostrea virginica	Eastern oyster	1	2
	Gemma gemma	Gem clam	3	
	Macoma baltica	Baltic macoma	2	
	Macoma spp.	Macoma clam	3	
	Mercenaria mercenaria	Hard clam		3
	Modiolus demissus	Atlantic ribbed mussel		<0.5
	Mulinia lateralis	Dwarf surfclam	3	
	Mussel spp.			5
	Mya arenaria	Soft shell clam	2	9
	Rangia cuneata	Wedge clam	3	
	Razor clam spp.	Jackknife clam	2	
	Solenoidea	Purplish tagelus		2
	Tagelus divisus	Stout razor clam		2
	Tagelus plebeius	Razor clam		2
	Unid soft shell clams		70	40
Other	Ascidiacea	Sea squirt		(sv)
	Chironomus spp.	Midge fly		3
	Clymenella torquata	Bamboo worm		5
	Epitonium spp.	Bladed wentletrap	2	
	Eudendriums spp.	Hydrozoan	1	
	Cyathura polita	Isopod		(sv)
	Glycera spp.	Blood worm	2	
	Idotea balthica	Isopod	2	
	Livonica redmaii	Fish lice	18	
	Nassarius spp.	Mud snail		8
	Nereis spp.	Clam worm sp.	23	5
	Ovatella myosotis	Oval march snail		<0.5
	Pectinaria gouldi	Ice cream cone worm		3
	Polychaeate spp.		4	8
	Thais lapillus	Atlantic dogwinkle	2	
	Rock		6	5
	Sand			19
	Unid animal			3
	Unid material		35	19
	Unid molluscan meat		6	11
	Unid snail			11

CONTINUED Table 1. Prey items identified in cownose ray stomachs by sampling method; “Unid”=unidentifiable; “sv”= spiral valve. (Chart continued on next page.)

the cownose ray. The number of prey items identified within cownose ray stomachs was highly diverse and varied in occurrence within prey item categories (classification) and by sampling method (Table 1). Sixteen different species of bivalves, 25 different species of crustaceans, 4 species of gastropods, and 3 species of polycheate worms were observed in stomachs of cownose ray. Spatial patterns in diet were observed corresponding with collection site and sampling method. Other than fish prey items that biased fishery-dependent samples, various thin-shelled clams and crustaceans were the dominant prey items by % frequency of occurrence observed in all sampling methods.

Fishery-dependent sampling

The high prevalence of fish in fishery-dependent stomach samples exemplified the highly opportunistic nature of cownose rays, which actively consumed a prey item when available, such as immobilized fish, which in natural circumstances when fish are free swimming, would not be readily available.

Differences in ray stomach content observed between fishery-dependent methods indicate spatial differences in fishing habitats and corresponding prey associated with those habitats (Tables 2, Figure 3). Rays were collected from areas with differing environmental conditions as, salinity (ranging from 12-30ppt) and bottom type (silt/mud

	Pound Net	Haul Seine
Number of identifiable prey species	25	21
Number of different prey species	16	12
Number of unidentifiable prey species	9	9

Table 2. Stomach content based on fishery-dependent sampling method.

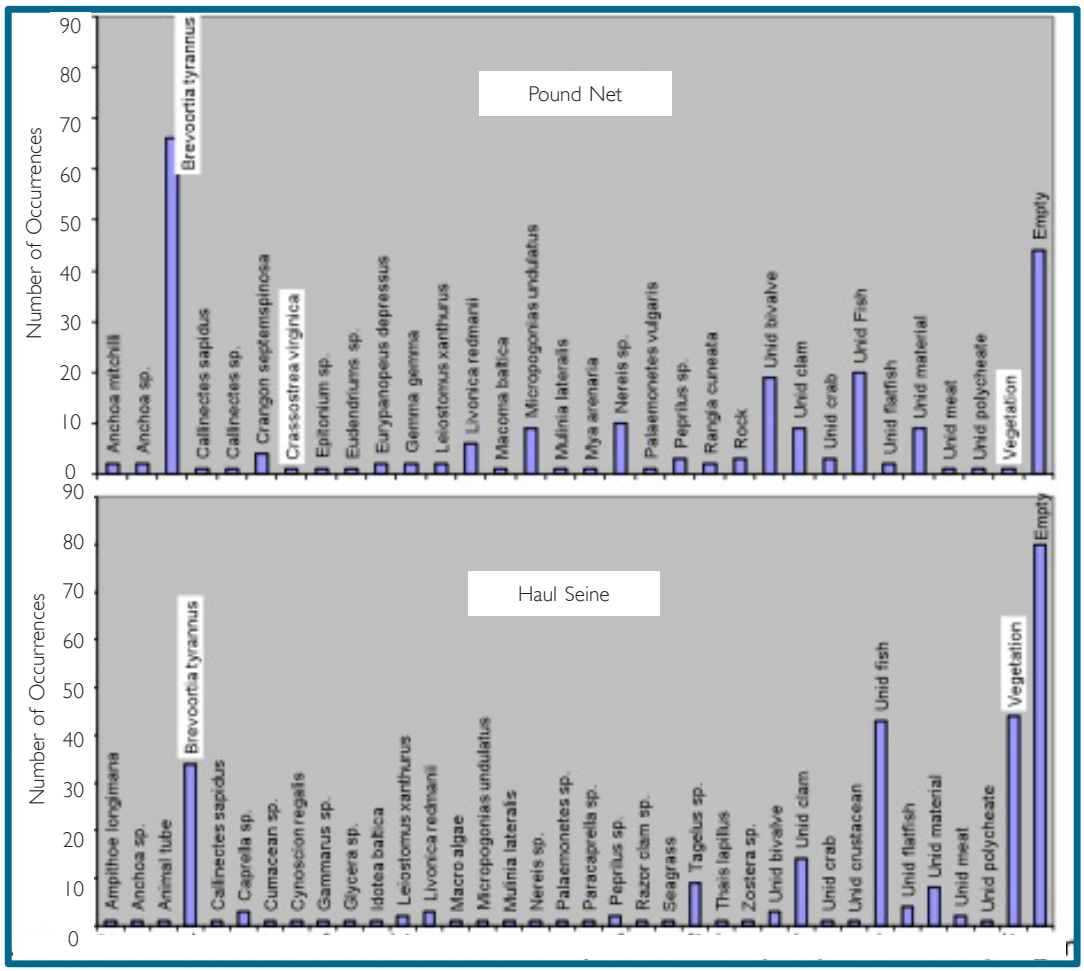


Figure 3. Stomach content based on fishery-dependent sampling method.

	York River	Mobjack Bay	Back River (Poquoson)
Number of identifiable prey species	5	4	19
Number of different prey species	3	1	17
Number of unidentifiable prey species	9	5	2

Table 3. Number of species of prey items observed from different haul seine sampling sites.

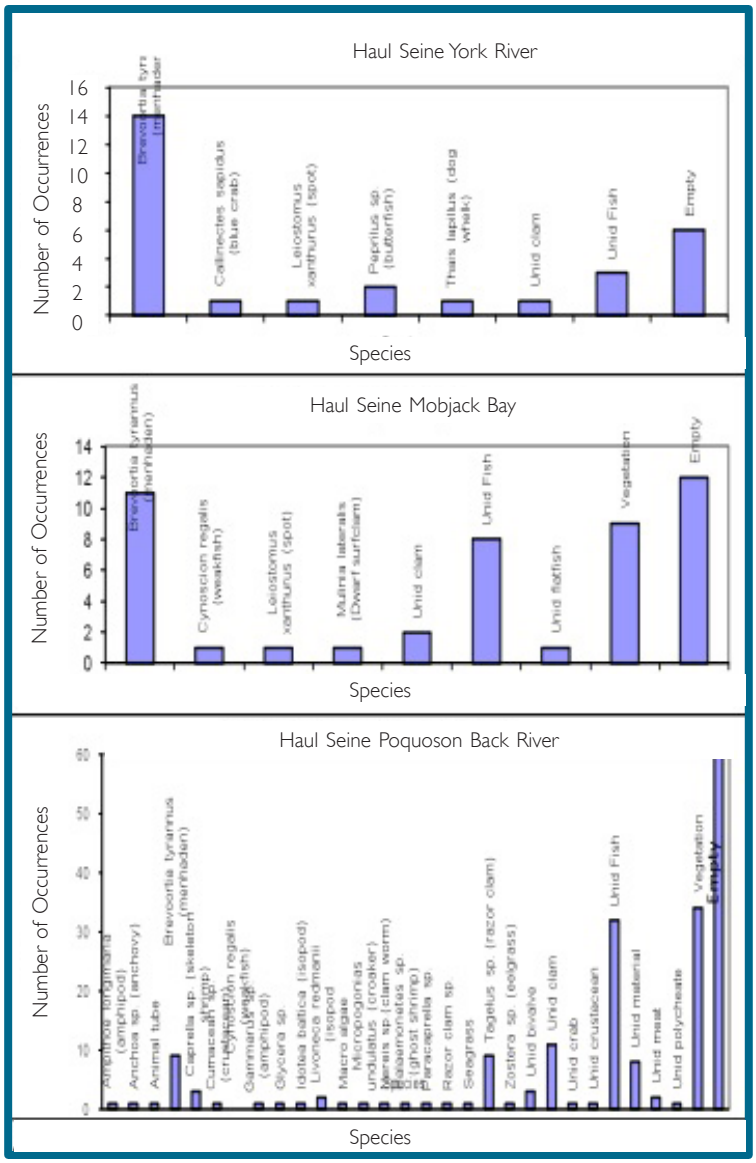


Figure 4. Prey items observed from different haul seine samping sites. Note prey diversity in Back River compared to other sites.

to sand, vegetated or not). Pound nets are stationary gear and therefore largely situated in a single habitat, and are placed in the bay, outside tributaries. Haul seines are mobile gear which can be fished in various locations, including the diverse tributaries, spanning several differing marine habitats which rays feed, therefore providing access to more diverse prey species. Haul seines are restricted to shallow water where fishermen work with the outgoing tide in water depths of ~3-5 feet. These shallow water areas are often supported with submerged aquatic vegetation (SAV), which provides nursery cover for many marine species, help stabilize adjacent shorelines, and often associated with infaunal and epifaunal prey of cownose ray. As a result, vegetation was more frequently observed in ray stomachs collected by haul seine method. Likewise, a higher frequency of prey items associated with SAV structure was observed in haul seine samples; various shrimp spp., and Callinectes crabs.

Spatial differences in prey diversity was observed within haul seine samples from various locations in the Bay (Tables 3 and Figure 4), with nearly three times the number of prey items recorded in Back River samples than that observed from York River or Mobjack Bay.

Excluding fish, bivalves followed by crustaceans dominated prey type in fishery-dependent samples combined (Figure 5) and within each gear type (Figures 6, empty stomach removed from chart). Five different fish prey species were recorded from pound net collected rays and seven different species from haul seine gear (Figure 7) with menhaden (*Brevoortia tyrannus*) most occurring. Seven different bivalve prey species were recorded in pound net sampled ray stomachs and three different species in haul seine caught rays, with razor and soft-shell clams (*Tagalus* and *Mya spp.*) the most abundant bivalve prey observed from both gear types (Figure 8). Twice as many crustaceans were observed in haul seined ray stomachs than in pound net samples (Figure 9). Smaller crustacean species (*Caprella, Gammerus, Ampithoe*) which commonly inhabit sea grass beds were

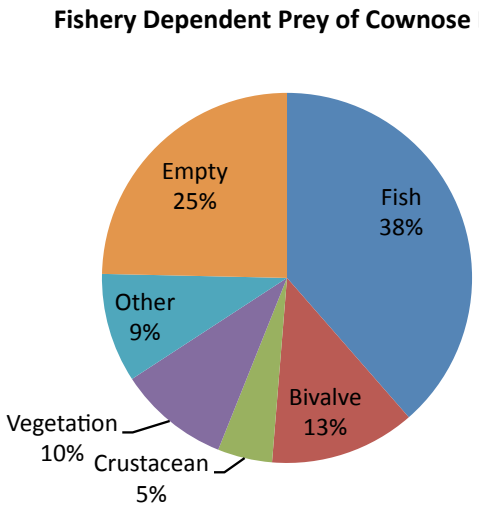


Figure 5. Stomach content of cownose rays sampled from fishery-dependent methods (haul seine and pound net).

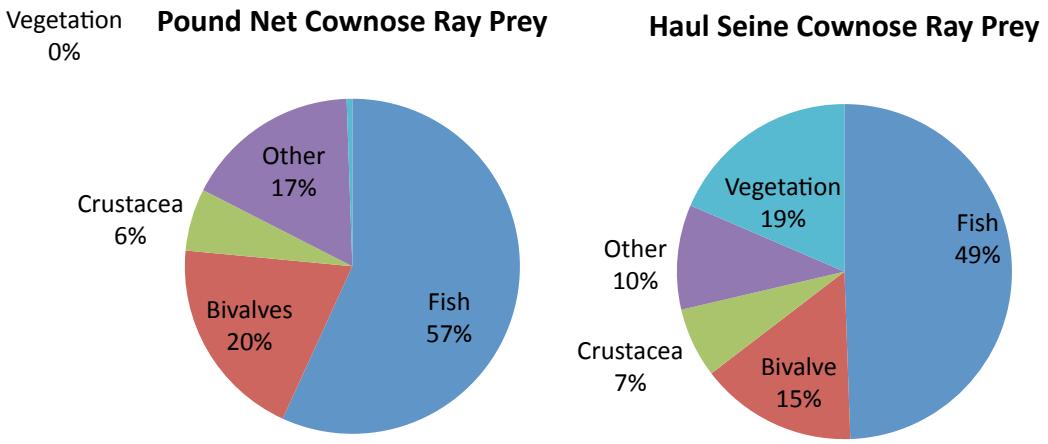


Figure 6. Stomach content of cownose rays by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)

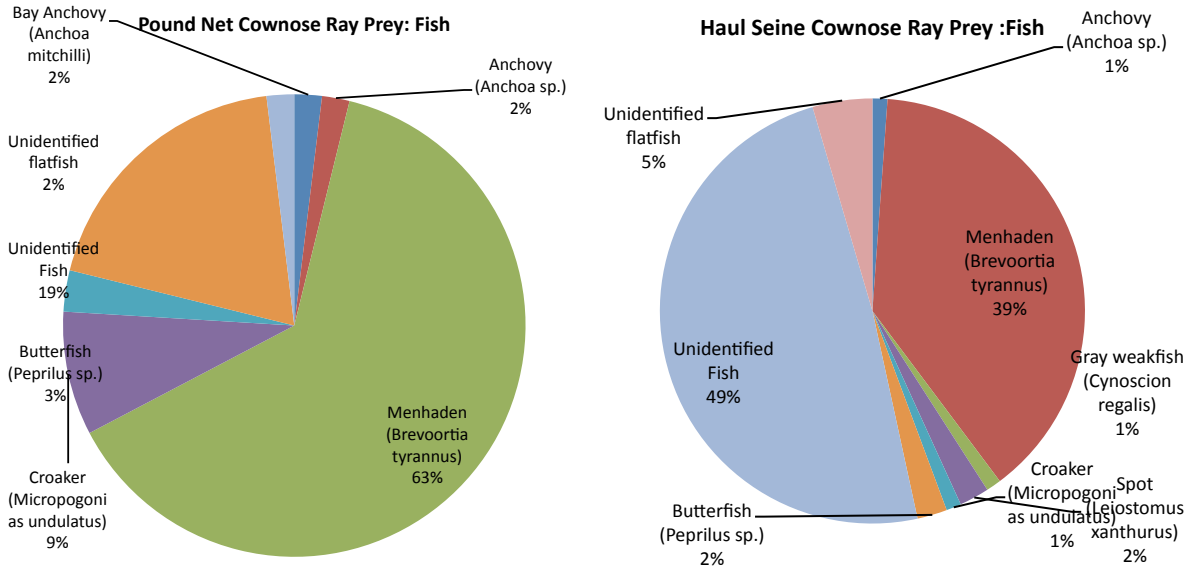
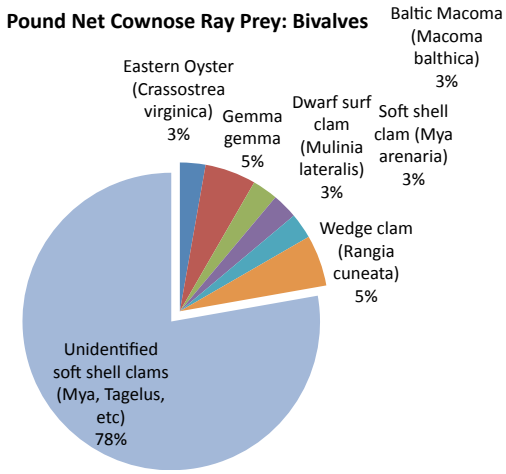


Figure 7. Fish prey items found in stomachs of cownose rays by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)



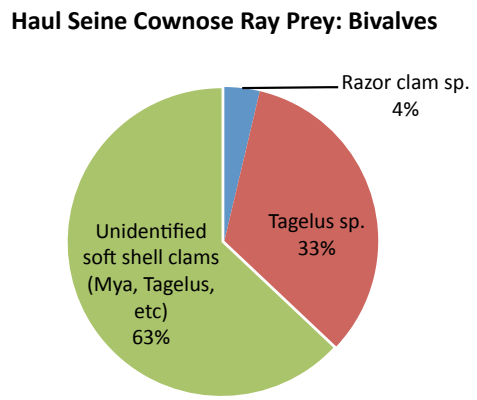
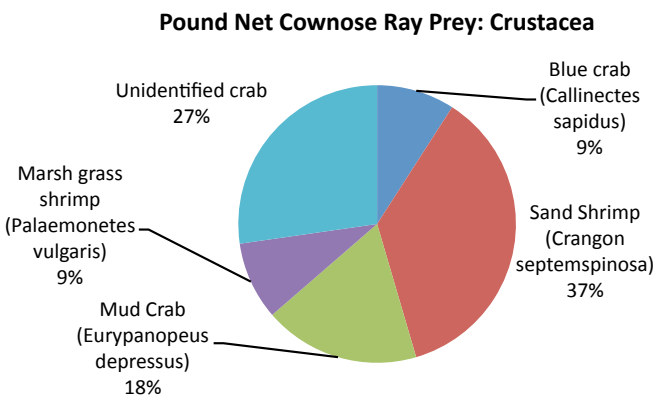


Figure 8. Bivalve prey items found in stomachs of cownose ray by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)



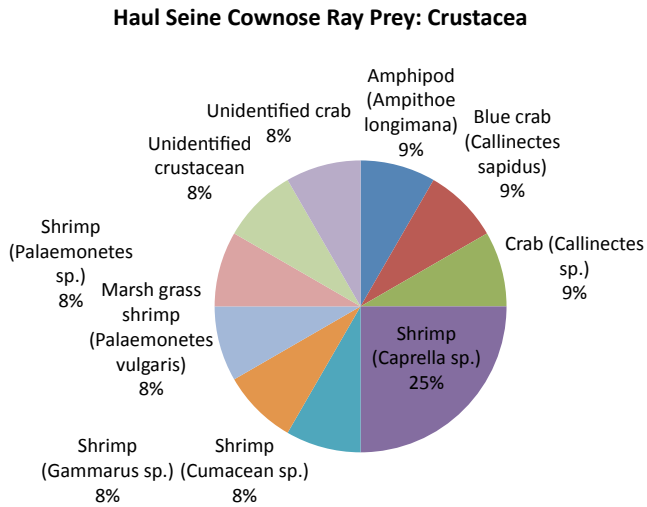
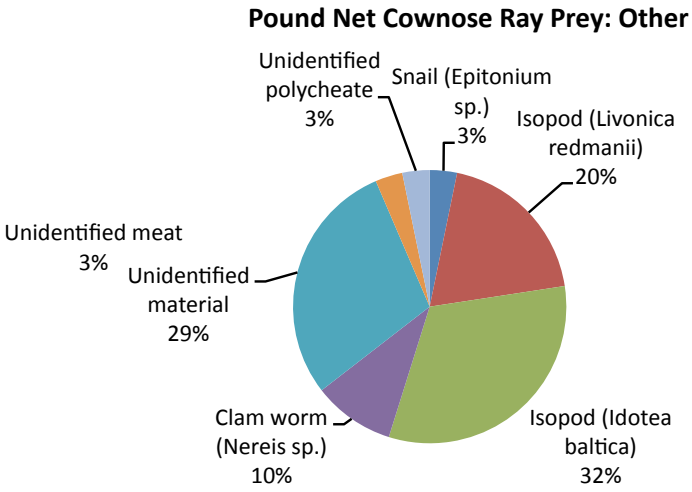


Figure 9. Crustacea prey items found in stomachs of cownose ray by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)



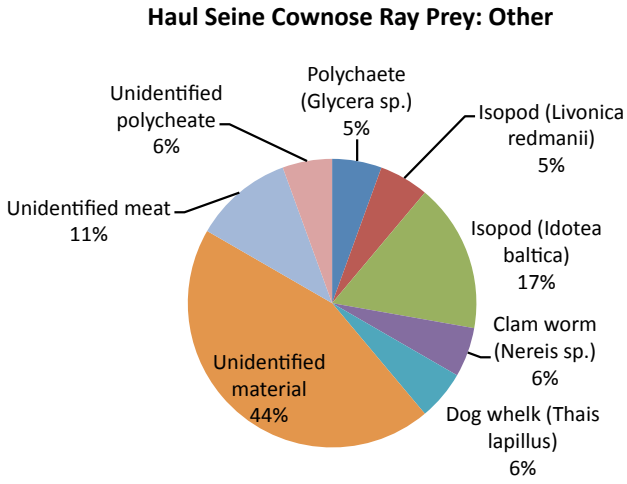
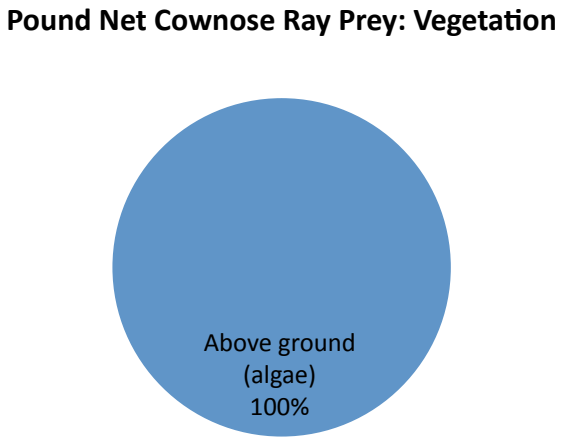


Figure 10. Other prey items found in stomachs of cownose ray by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)



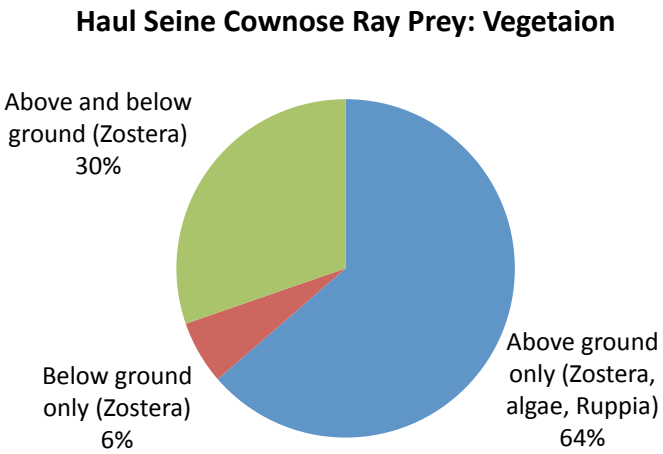


Figure 11. Vegetation prey items found in stomachs of cownose ray by sampling method; left: pound net; right: haul seine. (Empty stomachs excluded.)

only observed in haul seine sampled rays. *Crangon* and *Palaemonetes* shrimp and *Eurypanopeus* and *Callinectes* crab were most observed crustaceans in pound net sampled rays. The sand shrimp (*Crangon septemspinosa*) was only observed in pound net sampled rays (37% frequency), though they are reported to inhabit benthic environments common to both gear types (open sandy bottoms, eel grass beds). Other identifiable prey items found in ray stomachs included small isopods and worms, with *Idotea balthica* isopod most frequently observed (Figure 10). Vegetation was found in ray stomachs collected by both gear types: however, above and below ground vegetation was observed in haul seine samples but only above ground vegetation was observed in pound net caught rays (Figure 11). 36% percent of vegetation observed in ray stomachs from haul seine samples contained below ground Zostera remnants. Note high number of menhaden (*Brevoortia tyrannus*) and low number of oysters (*Crassostrea virginica*) within both gear types, and difference in amount of vegetation between gear types.

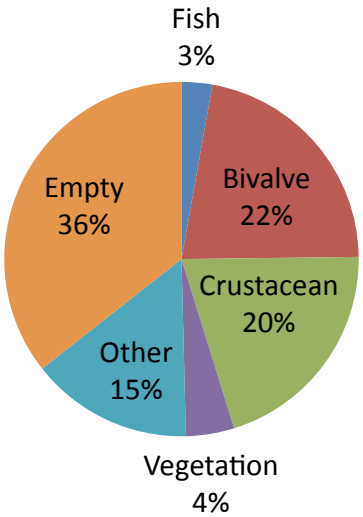


Figure 12. Stomach content of cownose rays sampled from fishery-independent methods (modified Dutch haul seine, long line, and bow fishing). Empty stomachs included.

Fishery-independent Samples

In an attempt to observe a more representative assemblage of natural prey items in cownose ray diet, fishery-independent sampling was performed using an experimental modified Dutch seine, long line rigs, and bow and arrow. The presence of fish and vegetation found in ray stomachs from fishery-independent samples (Figure 12) was considerably less than that found in haul seine and pound net caught rays with fish only represented in long-line samples (Figure 13) in which hooks were baited with fish (*Dorosoma cepedianum*). For this reason, fish observed in ray stomachs from fishery-independent samples was considered opportunistic food and not a natural prey component. Less vegetation observed in fishery-independent samples largely reflects

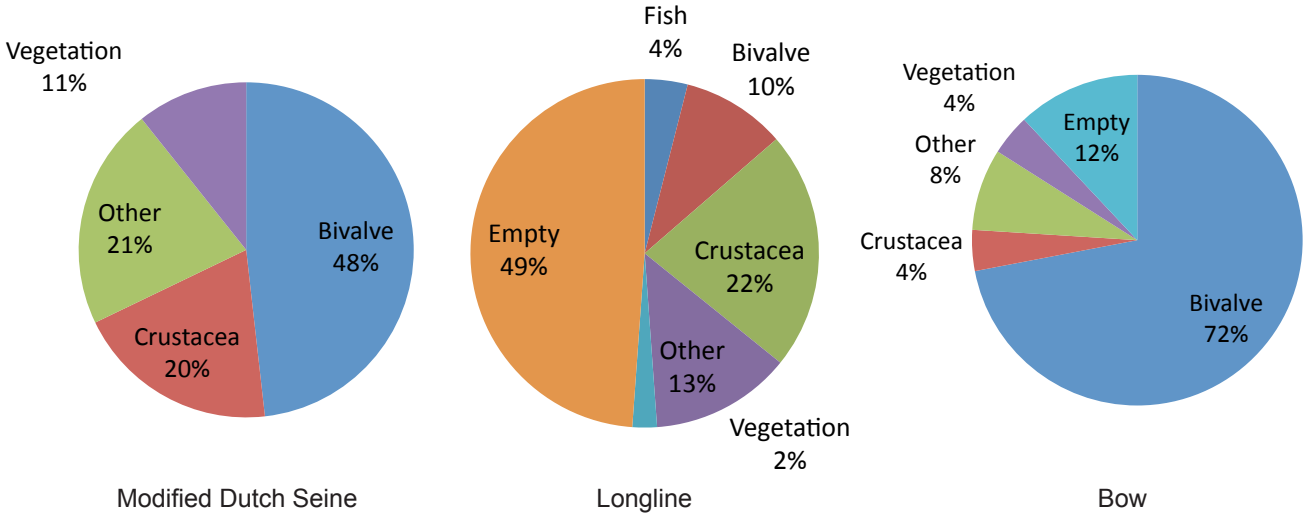


Figure 13. Stomach content of cownose rays sampled from fishery-independent methods (modified Dutch haul seine, long line, and bow fishing). Empty stomachs included.

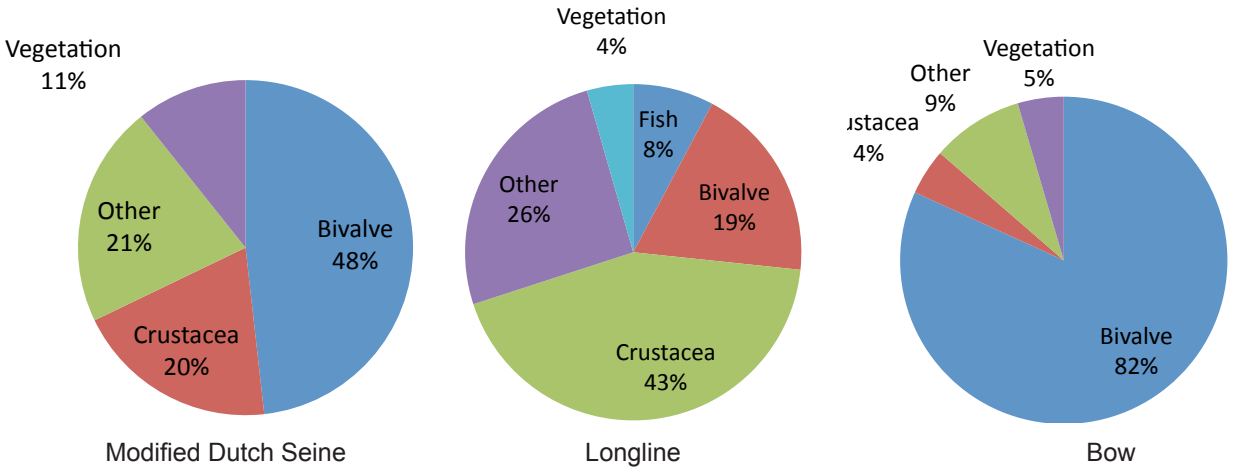


Figure 14. Stomach content of cownose rays sampled from fishery-independent methods (modified Dutch haul seine, long line, and bow fishing). Empty stomachs excluded.

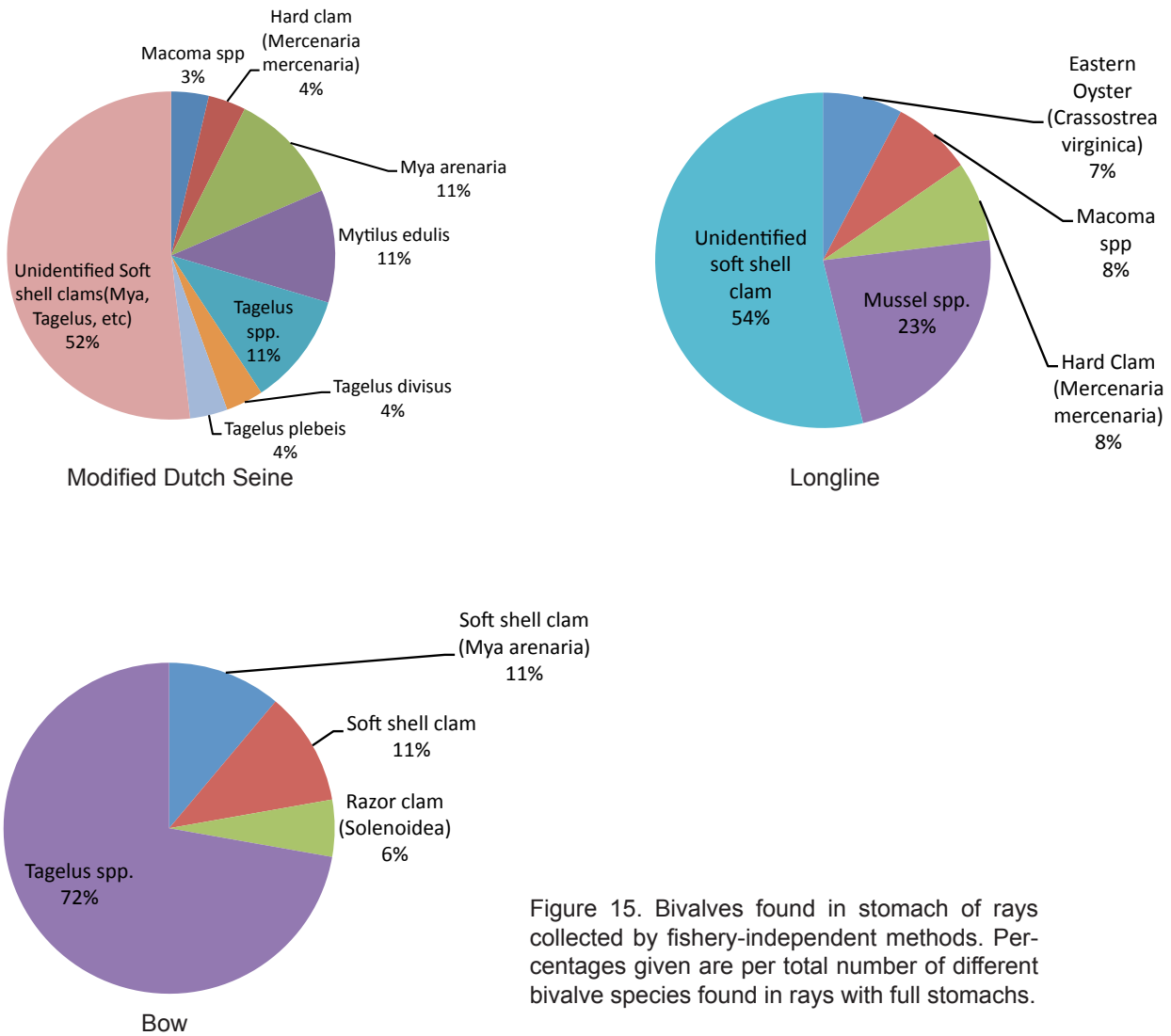


Figure 15. Bivalves found in stomach of rays collected by fishery-independent methods. Percentages given are per total number of different bivalve species found in rays with full stomachs.

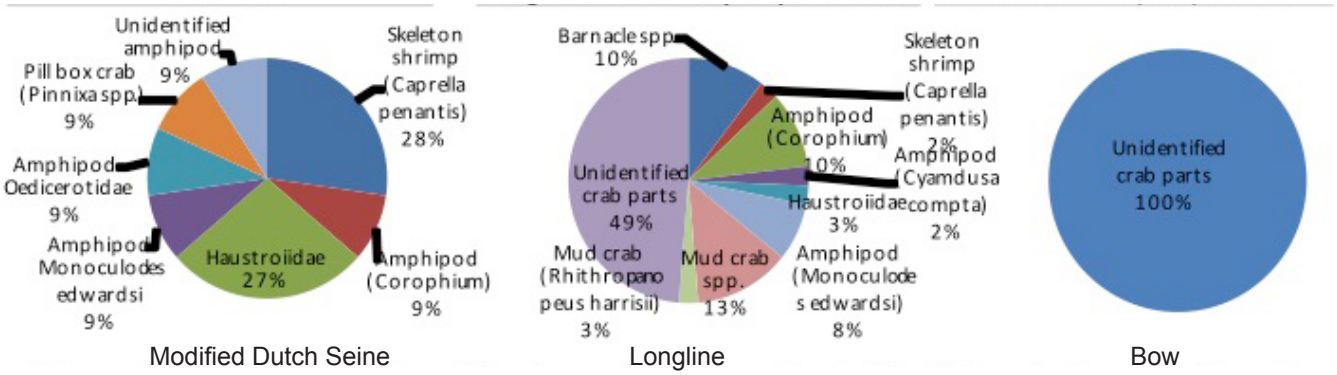


Figure 16. Crustaceans found in stomachs of rays collected by fishery-independent methods. Percentages given are per total number of different crustacean species found in rays with full stomachs.

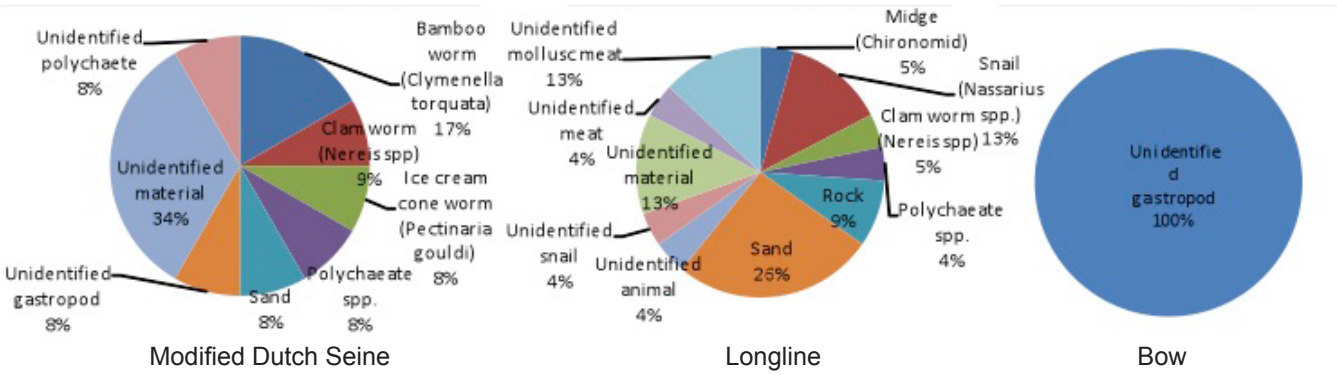


Figure 17. Other material found in stomachs of rays collected by fishery-independent methods. Percentages given are per total number of different crustacean species found in rays with full stomachs.

habitat differences from which sampling occurred.

The amount of empty ray stomachs observed varied by gear-type from no empty stomachs in Dutch seine samples, 12% in bow and arrow samples, to 49% of all rays sampled from long-line fishing (Figure 13). Both Dutch seine and bow and arrow methods extract rays from the water with little lag-time associated from point of capture/impalement to landing, providing no time for rays to evacuate stomach content through digestive tract. No regurgitated food was observed upon landing rays on boat. Empty ray stomachs observed in bow and arrow samples are likely from rays within the shoaling group which had not yet begun to feed. However, empty stomachs from long line samples is likely associated with the length of time between hooking and retrieval of long-line gear, which may allow rays to begin evacuating stomach content.

Bivalves and crustaceans were the dominant prey for all fishery-independent sampling methods (Figure 14). Thin-shelled species greatly dominated bivalve prey (Figure 15) in all gear types: 100% in bow and arrow gear (*Tagelus* and *Mya spp.*); 96% in Dutch seine samples (*Tagelus*, *Mya*, and *Macoma spp.*); and 85% in long line samples (*Mya*, *Macoma*, and Unidentified soft clam species). Hard clams (*Mercenaria mercenaria*) were found in long line samples (8%) and Dutch seine samples (4%), but not in bow and arrow caught rays. The eastern oyster (*Crassostrea virginica*) was only observed (7%) long line caught rays. Small, shallow-burrowing amphipods were dominant crus-

tacean prey items observed in Dutch seine (91%) samples and 36% of crustaceans from long line samples (Figure 16). The mud crab (*Rhithropanopeus harrisii*) was only observed in long line samples (16%), which, like barnacles (10%) are associated with benthic structure such as oyster reefs. Only unidentifiable crab parts were observed in bow and arrow samples.

Other prey items observed in cownose ray from fishery-independent sampling were various benthic worms and small gastropods (Figure 17) all of which are shallow burrowers or associate with substrate surface. Polycheate worms (*Clymenella torquata, Pectinaria gouldi, Nereis spp.*) were highly observed in Dutch seine and long line samples, but were absent in ray stomachs collected by bow and arrow. The relatively high frequency of sand observed in ray stomachs from long line and Dutch seined samples is likely attributed to foraging tactics related to the excavation of deep burrowing prey as softshelled clams.

Fishery-independent methods largely targeted shoaling rays caught in shallow waters affected by tidal exchange. Cownose ray shoaling behavior allows access to more diverse prey items. Diversity of prey in ray stomachs was highest in Dutch seine and long lined rays, with frequency of bivalve and crustacean prey similar. Low diversity of prey items was observed in bow and arrow sampled rays (Figures 14-17); however high dominance of a single prey type (*Tagelus* clams) was also observed indicating a foraging and feeding strategy where cownose ray forage in groups and selectively prey on species in high abundance.

All vegetation found in ray stomachs collected from fishery-independent sampling was classified as above-ground vegetation (*Zostera*, *Ruppia*).

Spiral Valves

During processing long lined rays, large amounts of shell fragments were noticed retained in the spiral intestine, or spiral valve, in rays with stomachs containing little to no prey items (Figure 19). Therefore, we sampled spiral valves along with stomachs from longlined rays to see if we were missing occurrences of prey items, es-

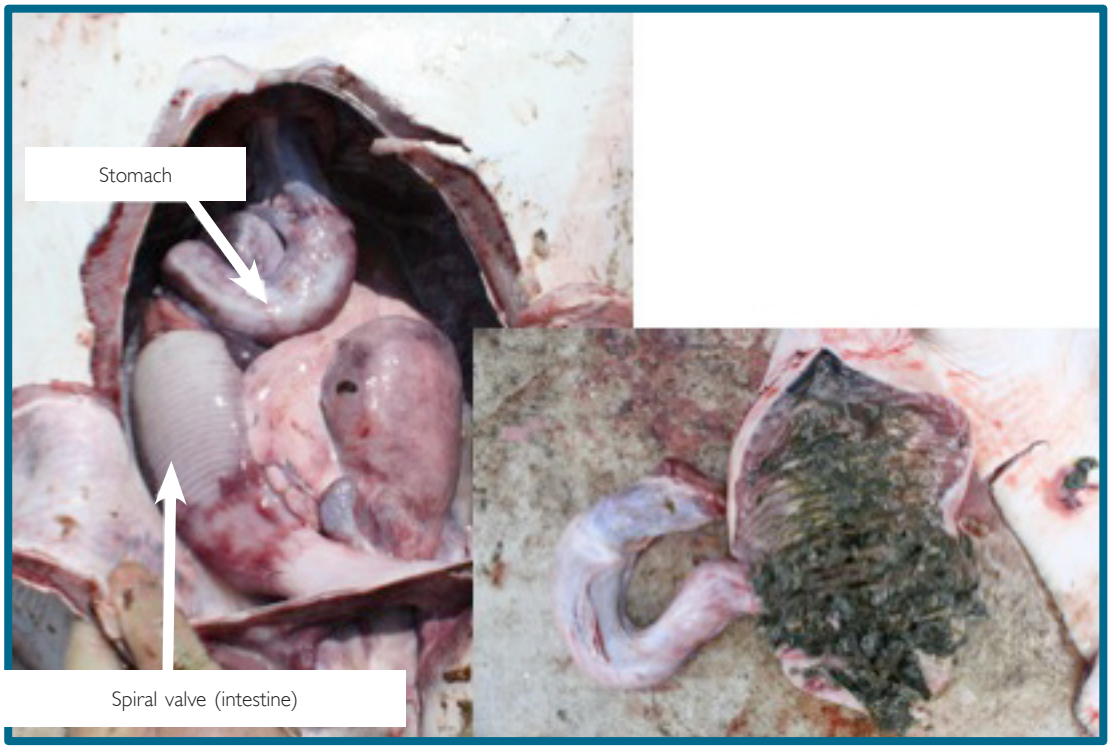


Figure 18. Ventral view of cownose ray abdominal organs (liver removed) showing orientation of stomach and spiral valve. Inset picture showing empty stomach but shell fragments from oysters, razor clams, softshell clams, and crabs retained in spiral valve.

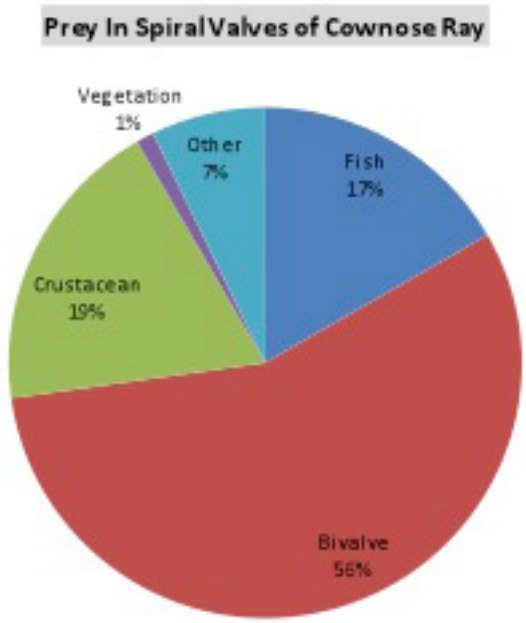


Figure 19. Prey items found in spiral valve of cownose ray collected by longline from commercial oyster grounds.

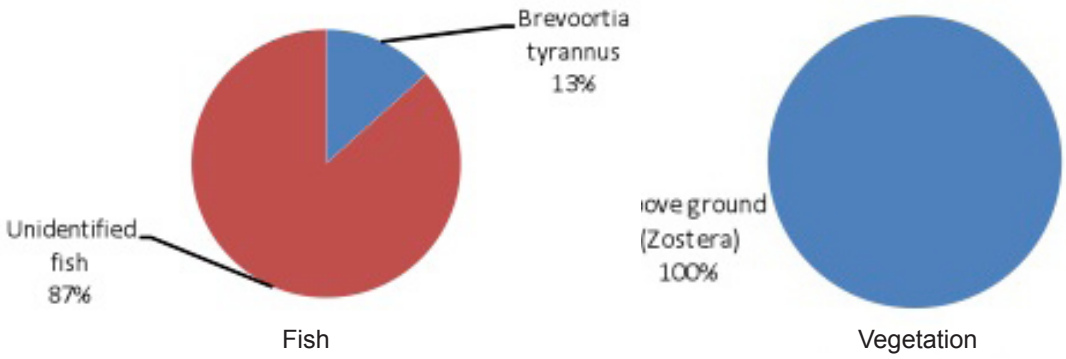


Figure 20. Fish and vegetation found in spiral valves collected by longline from commercial oyster grounds. Fish content is from bait used for capture.

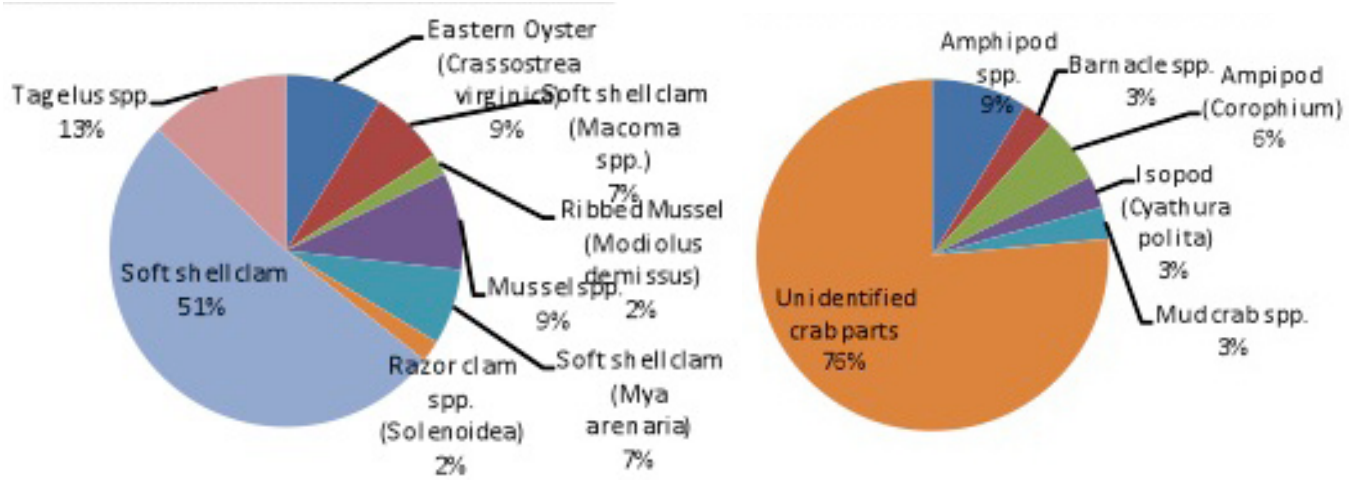


Figure 21. Bivalves and crustaceans found in spiral valves collected by longline from commercial oyster grounds.

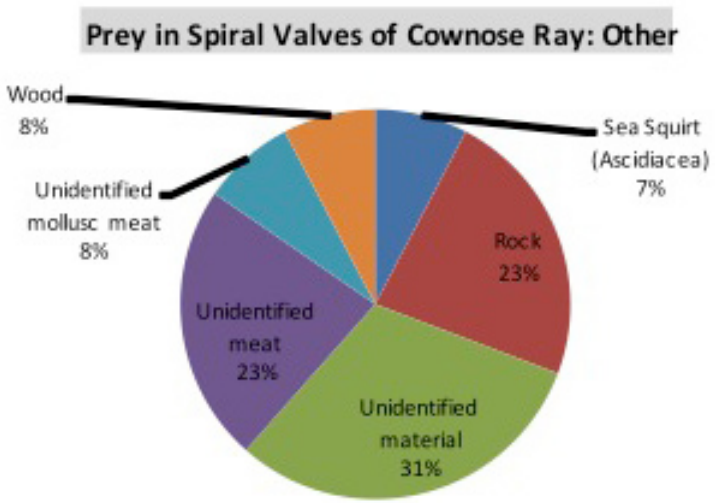


Figure 22. Other content found in spiral valves collected by longline from commercial oyster grounds.

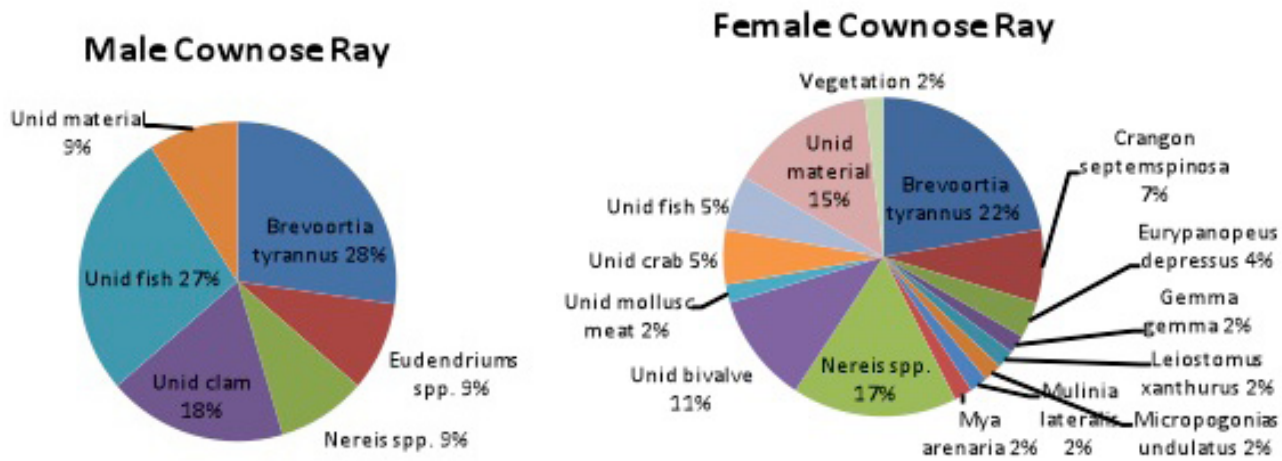


Figure 23. Prey items (%Frequency) from stomachs of female (N=18) and male (N=12) cownose rays from a single sample collected by pound net. Note prey diversity in females.

pecially hard prey items. In our evaluation of spiral valves, we did not include empty spiral intestines in our evaluation of prey items; therefore prey items found in spiral valves are reported as % of total spiral valves with quantifiable contents. Prey found in spiral valves is typically identified by hard body part remnants, with soft body tissue largely un-identifiable due to advanced digestion. Prey from spiral valves in long lined cownose ray were placed in five categories (Figure 20) and further identified to species, if possible, by category (Figures 21-22).

Bivalves (56%) dominated prey type found in ray spiral valves, followed by crustaceans (19%) then fish (17%). Fish observed in ray digestive tract is considered bait used to catch rays. Thin-shell bivalves (soft clams, mussels, razor clams) comprised 91% of bivalves found in ray spiral valves. There were nine cases in which the spiral valve contained *C. virginica* oysters, six of which had empty ray stomachs. Each spiral valve which contained *C. virginica* also contained soft shell clams or mussels of one or more species. Hard clams were not found in any spiral valves.

Prey Diversity by Sex

Prey diversity was observed between the

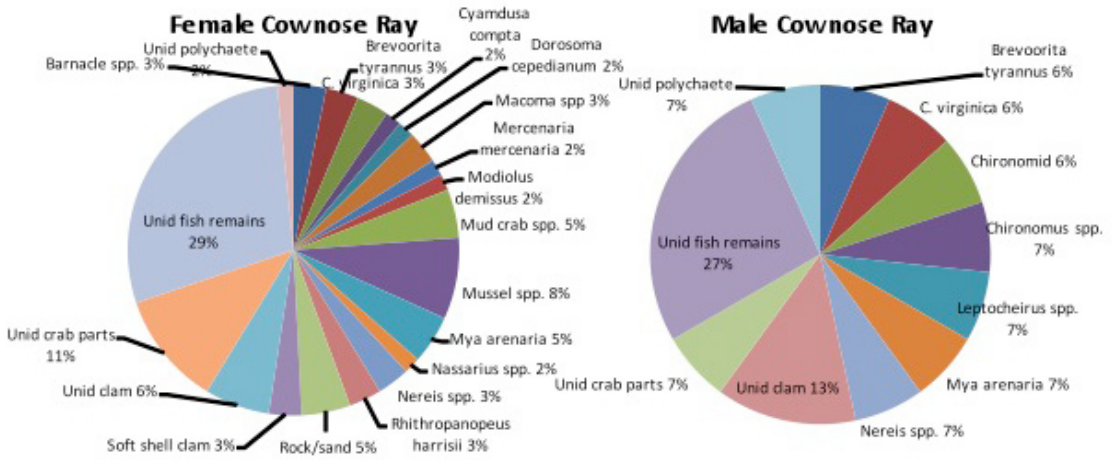


Figure 24. Prey items (% frequency) from full stomachs of female (N=74) and male (N=17) cownose rays collected by longline on commercial oyster grounds. Note prey diversity in females.

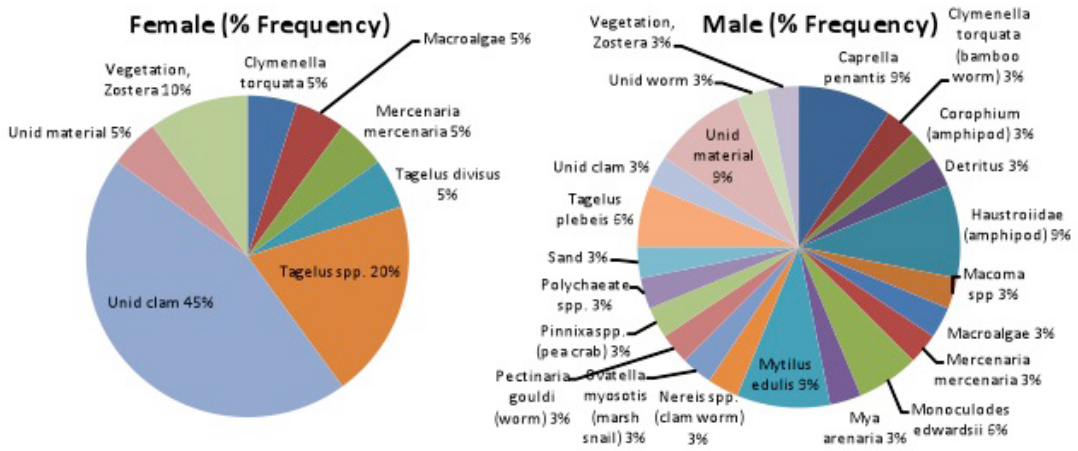


Figure 25. Prey items (% frequency) from full stomachs of female (N=74) and male (N=17) cownose rays collected by modified Dutch haul seinecommercial oyster grounds. Note prey diversity in females.

sexes in cownose ray collected during periods when mixing of sex occurred (May-July), however, sample size was limited. In haul seine and long line sampled rays (Figures 24), female cownose rays were observed to feed on a larger variety and more nutrient rich prey than males. Diversity observed between the sexes may be a result of unequal sample size; however, prey type indicates females may target prey which will provide more nutrient reserves. Excluding fish, prey identified in females was dominated by nutrient rich, high volume clams and crabs while prey from males was dominated by small worms and amphipods (*Nereis spp., Polycheate spp., Chironomus spp.*).

Diversity of prey between sexes was reversed in schooling rays collected by Dutch seine feeding along soft bottom, sandy river channel where it meets the Bay, at the mouth of Back River, with males observed preying on a much wider array of prey species (Figure Figure 26); however, a difference in foraging tactics between the sexes was observed. Within the group of foraging rays, prey identified in females once again consisted of larger individual prey items than those observed in males, but constituted deep-burrowing prey (*Tagelus spp.*) while males targeted epifaunal prey (mussels, worms). On percent weight basis (% of total weight from full stomachs), dominance of Tagelus clams in females and Mytilus mussels in males over other prey was further observed (Figure 26).

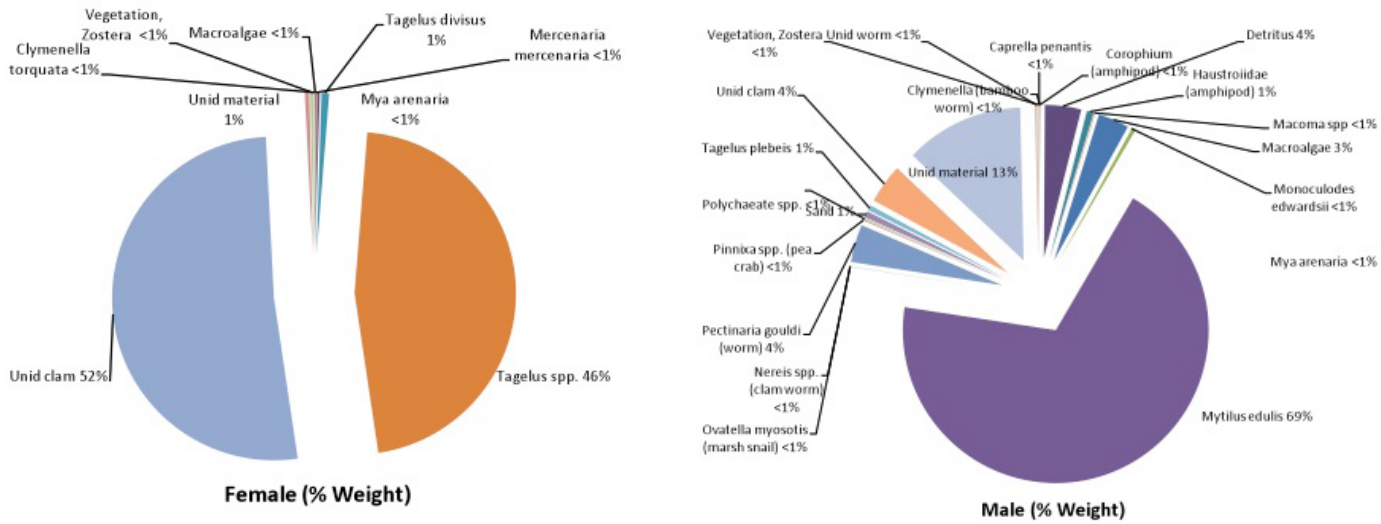


Figure 26. Prey items (% weight) from full stomachs of female (N=23) cownose rays foraging with males. Not dominance of deep-burrowing bivalves in female stomachs (left), and dominance of non-burrowing bivalves (mussels) and shallow-burrowing worms in males (right)

Discussion

Evaluation of prey items was completed for fishery-dependent and fishery-independent samples. Number of prey items identified within cownose ray stomachs was highly diverse and varied by prey item, sampling method, and spatial differences. Many items found within fishery-dependent stomach samples reflected fishing method employed and the highly opportunistic nature of cownose rays. Rays evacuate digestive tract content over a short period of time. Both haul seine and pound net fisheries employ a period of time where captured rays are held in the water within a confined area, which enables the ray to process and eliminate food consumed prior to or during entrapment. Further, food items occupying this confined area with entrapped rays are more susceptible to predation, especially fish species which become immobile due to entanglement in gear, which becomes evident comparing prey items by sampling method. An obvious bias exist within fishery-dependent samples of both prey items observed and the frequency of prey items found in ray stomachs; however, results remain valuable in evaluating cownose ray feeding behavior and prey diversity.

Excluding fish, a total of 52 different prey items were recorded (Table 1) with two major prey groups dominating the diet: bivalves and crustaceans. Major prey items most frequently observed included: thin-shelled bivalves (*Tagelus spp*. and Mya arenaria), crabs (mud crab spp.), amphipod shrimp (*Caprella spp*.), and benthic worms (*Nereis spp*.). Small prey items which were not observed in high frequency and not considered to be a substantial component of the ray diet included epifaunal crustateans *(Cymadusa compta* and *Oedicerotidae spp*.) and hydrozoans (*Eudendriums spp*).

Dominant prey items of cownose ray observed in this study were thin-valved shellfish (shoftshell, mocoma, and razor clams, and crustaceans) with oysters and hard clams not observed as being a large part of the ray's natural diet. These results parallel those of Smith & Merriner (1985). Even though the softshell clam population has been severely reduced in the Bay since the Smith & Merriner study (1976-78), cownose rays were observed to still target thin-valved bivalves and crab prey.

The variety of prey items found in cownose ray stomachs demonstrated the diversity of feeding ecology in cownose ray where both infaunal (clams, worms, and

small crustaceans) and epifaunal (crabs and mussels) prey were targeted. (Section 5 of this report demonstrates that cownose rays use a suction action to draw prey up from the benthos into their mouth, where manipulation and subsequent crushing of large prey occurs. This sucking method also results in small prey being ingested whole, which is routinely represented in stomach analyses of intact polycheates, isopods, and whole small clams (*Gemma spp.*). Deep burrowing prey, as *Tagelus* and *Mya* species, are excavated from the substrate largely through water movement in and out of the ray mouth; sucking and blowing action which liquefies the bottom substrate, exposing buried prey.)

As observed throughout all sampling methods, the dominant prey type is site specific. However, the common dominant prey type within each site sampled remained the various thin-shelled bivalves and crustaceans, with oysters and hard clams largely only represented in rays collected from sites associated with commercial oyster grounds. Oysters comprised only 1% of bivalves from full stomachs sampled in fishery dependent samples (only in pound net), and 7% of full stomachs sampled from fishery independent samples (all from stomachs collected from commercial oyster grounds). Hard clams (*M. merceneria*), were not found in fishery dependent samples and comprised 3% of bivalves from full stomachs of fishery independent samples; 8% of bivalves from commercial oyster grounds (long line), 4% from shallow channels extending from Back River (Dutch seine), and 0% from various shoals (bow and arrow).

Sex specific differences in food habits of cownose ray were observed in this study, however sample size was limited and a more thorough evaluation of sexual dimorphism is needed to draw better conclusions or hypotheses pertaining to feeding strategies between the sexes. The majority of stomach samples collected in this study was from female rays, an artifact of sexual segregation and sampling methods employed. During May-July when sexes were mixed, stomach samples of adult male and female rays were obtained within the same sampling area, though limited in number. In pound net and long line sampled rays, a difference in prey diversity between the sexes was observed, with females selecting a larger array and more nutrient rich prey. Physical size difference between the sexes of adult cownose rays may influence foraging behavior, with larger females better able to excavate deeper burrowing prey than smaller males. A difference in foraging tactics between the sexes was observed in schooling rays collected by Dutch seine where adult female (n=23) and male (n=33) cownose rays were observed to target different prey types; males targeted epifaunal mollusks (mussels) and shallow burrowing worms, while females targeted infaunal mollusks (*agelus spp.*). Nutrient gain received by females targeting razor clams (*Tagelus spp.*) over available mussels (*Mytilus*) is not clear since mussels contain larger amounts of many essential fatty acids, vitamins, and minerals than clams; however, *Tagelus* provide a higher yield per animal than *Mytilus*. Mixing of the sexes occurs prior to parturition and continues through mating, a period of time in which females require substantial food resources.

Cownose rays, in which adult males are smaller in size than females and not burdened with energetics of gestation, may employ a foraging strategy which ensures optimum nutrition to females by males selecting less nutrient rich prey when foraging with gravid and/or receptive females. After mating, sexual segregation occurs. Segregation by sex may further be a feeding strategy in cownose ray to reduce competition and allow females access to more prolific feeding areas. During segregated periods mature males were not observed in near-shore habitats were sampling occurred, resulting in no mature males collected from areas exclusive of females; therefore differences in feeding ecology by sex between areas during segregation was not achieved. Future research on cownose ray feeding ecology should include

investigation into sex specific differences, especially if a commercial fishery is established which would be highly selective of female rays given traditional harvesting methods and possible ray feeding strategies.

The foraging behavior of cownose rays seeking infaunal prey associated with SAV can result in the uprooting and ingestion (inadvertently or not) of vegetation while excavating the bottom in search of prey. Evidence of excavation foraging was observed in ray stomach samples which contained vegetation remnants composed of plant rhizomes and roots (below ground vegetation), parts which secure plants in the benthic substrate. Up-rooted SAV can be displaced by tidal action and river current, causing concern for stability in sensitive shorelines. However, displaced rhizomes, which are reproductive shoots, may also re-establish at another location and promote SAV dispersion. The largest portion of SAV identified in ray stomachs was classified as above ground vegetation (leaves and detritus) with below ground vegetation (rhizomes/roots) found in rays collected by haul seine, a gear type widely employed to fish near-shore habitats.

Fishery research investigating diet and prey assemblage routinely only examine stomach content; however, as seen in this study examination of spiral valves in durophagous elasmobranchs should be considered when investigating prey occurrence. Examination of spiral valves in conjunction with stomachs provided better enumeration of hard-bodied prey in cownose ray diet. Most prey flesh remnants found in the spiral valve were beyond recognition due to advanced digestion. Retention of non-digestable hard parts of certain prey in the spiral valve was largely identifiable to at least prey category and some to specie level. Spiral valves were not examined in fishery-dependent collected rays where commercially important oysters and clams were scarcely observed in stomach analyses. The possibility exists that more oysters and hard clams would have been observed if spiral valve examinations were performed throughout this study. However, the overall dominance of thin-shelled clams and crustacean prey (which also are found in the spiral valve when not present in the stomach) identified in cownose ray indicate a much higher ecological trophic role in cownose ray diet than oysters and hard clams.

Aggregate (group) feeding behavior of cownose ray depicted in fishery-independent samples where multiple rays captured in one location contained similar prey items and single dominant prey specie. These observations indicate that cownose ray forage in groups and selectively prey on species in high abundance.

Section 5: Prey Handling Behaviors

The ability of cownose ray to manipulate oysters and clams and test for relative prey preference and whether susceptibility to cownose ray predation changes with bivalve ontogeny was studied. This study investigated patterns of predation for captive adult and young of year (YOY) cownose rays on four species of bivalves including *Crassostrea virginica* Gmelin, *Crassostrea ariakensis* Fujita, *Mercenaria mercenaria* Linnaeus, and *Mya arenaria* Linnaeus and on spat-on-shell (SOS) *C. virginica*.

Cownose rays use several behaviors in feeding on benthic prey. Cownose rays are thought to excavate invertebrate prey from the substrate by using vigorous oscillations of the pectoral fins and by jetting water taken in by the spiracles during respiration from the mouth to further separate prey from sediment (Schwartz, 1967; Sasko, 2000). Inertial suction feeding moves prey from the sediment into the mouth. Anterior expansions of the pectoral fins form two mobile cephalic lobes in cownose rays. These lobes aid in prey capture by channeling prey towards the ray's mouth. The lobes may also serve in increasing suction strength by surrounding identified prey thereby creating a confined vacuum against the substrate (Fisher, personal observations). When not actively feeding, these lobes are retracted and held tight against the body, increasing hydrodynamic efficiency.

The jaws of cownose rays also are modified for durophagy. The jaws of sharks and rays consist of four primary cartilages, two in the upper jaw and two for the lower jaw. The symphyses that loosely connect the two sides of the mandible (lower jaw) and of the palatoquadrate (upper jaw) are fused in the rhinopterid and closely related myliobatid rays (Summers, 2000). Hyperdeveloped mandibular adductor and coracomandibular muscles in the jaws (González-Isáis 2003), highly calcified jaws, and hard pavement-like tooth plates enable cownose rays to feed on prey with hard shells. The tooth plates are interlocked distributing bite force across the whole jaw, rather than on a single point (Maschner, 2000). A 60cm cownose ray is capable of bite forces between 40 and 200N (Sasko and Maschner, in Sasko, 2000; Motta, 2004). Bishop and Peterson (2006) reported the force necessary to crush the shell of Eastern oysters (*Crassostrea virginica* Gmelin) is greater than 200N for any with a shell height greater than 30mm, suggesting that only very small oysters are susceptible to bite pressure that cownose rays can produce. Interestingly, the force required to crush the Suminoe oyster (*Crassostrea ariakensis* Fujita) is below 200N at all sizes (Bishop and Peterson, 2006), suggesting that at all life stages, this introduced species may be much more susceptible than native oysters to cownose ray predation.

We performed cownose ray predation experiments with captive rays to determine if a critical size or feature exists for *C. virginica* and *M. mercenaria* that can limit their susceptibility to predation and to examine patterns of ray predation on various bivalve species of commercial importance. Both cultchless (single) and cultched (SOS) oysters were tested in various oyster predation trials. Predation behavior was also investigated through video recordings of captive rays feeding on various shellfish species (videos available online: http://bit.ly/b6RKZc; search cownose ray).

Methods

Cownose rays are schooling fish (Smith and Merriner, 1985) that are strictly observed naturally foraging and feeding in groups. Therefore, each behavioral experiment comprised a group of four adult female rays ranging from 90cm disc width (DW) (12.7 kg) to 102cm DW (20.0 kg) and maximum jaw gape range of 27-34mm.

For subsequent trials we used cownose rays from the 2009 year class (~1.5 months old) measuring 43cm (2.1kg) to 45cm (2.6kg) DW and maximum jaw gape of 10-18mm referenced as young-of-year (YOY).

Jaw gape was measured on fresh whole dead rays at the maximum distance between teeth plates when simultaneously pulling the lower jaw ventrally and posteriorly (depressed state) and upper jaw ventrally and anteriorly (protruded state). Rays were caught by commercial fishermen using haul seine gear near Back River, Poquoson Flats, in the lower Chesapeake Bay and transported live to the Virginia Institute of Marine Science in Gloucester Point, Virginia.

Adult rays were held and predation trials were performed in an above–ground, oblong fiberglass tank (3m x 4.2m) with sand filter recirculation. Water depth was maintained at a depth of 0.6m.

YOY ray predation trials were held in 1.2m x 2.4m recirculation tanks with water depth of 0.6m.

Behavioral analysis

Feeding trials were conducted no more than once per day. Cownose rays were maintained in a less than satiated, but not starved, condition. Daily ration for elasmobranchs, including batoids, ranges from 0.3-4.3% of body weight per day (Wetherbee and Cortes, 2004). The state of hunger, or maintenance level, was achieved by feeding rays approximately 3% of their cumulative body weights per day in live oysters (average meat weights from various size oysters were calculated) and freshly killed and dismembered blue crabs. The total weight of bivalves (meats) consumed in most trials in this study did not exceed 3.0% of the total body weight of the cownose rays. Supplemental post-trial feeding occurred daily when estimated consumption by the rays was less than 3%.

When not feeding, rays schooled counter-clockwise around the holding tank while keeping close proximity to each other. Upon initiating each feeding trial, the rays typically made a single "investigatory" pass over the shellfish, and then routinely began preying on the shellfish upon their second pass, within 30-60 seconds of shellfish introduction.

At the completion of each trial, predation on shellfish was categorized as successful or unsuccessful. Handling time, or the overall effort, expended by rays mouthing, crushing, and successfully consuming various shellfish sizes/types was not quantified in this study though ray predation behavior was documented through video recordings (videos available online: http://bit.ly/b6RKZc; search cownose ray). Cases in which a bivalve was crushed by the rays and not consumed but death was certain, were recorded as successful predation due to the ecological effect in terms of ray-induced mortality on bivalve populations.

Predation Trials: cultchless oysters and clams

Oyster shell height (SH) was measured

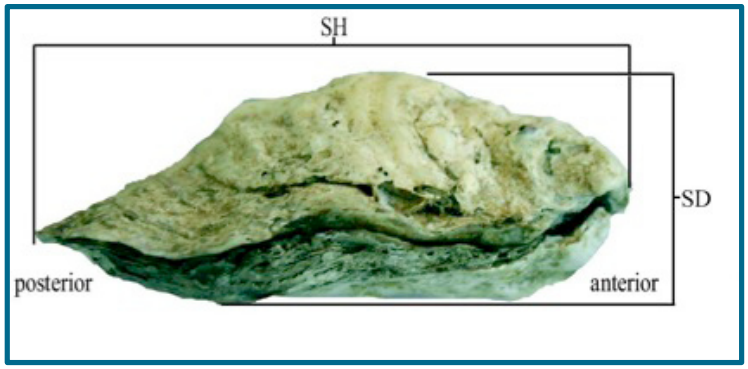


Figure 1. Side view of an oyster (*C. virginica*). SH= shell height, SD=shell depth

as the distance between an oyster's anterior (umbone) and posterior (bill) margin. Oyster width, or shell depth (SD) was also measured for each bivalve used in all trials (Figure 1), and represented the maximal distance between the outside surfaces of closed valves (left and right valves combined). Shell width (SW), was measured as the maximum distance across a valve perpendicular to shell height. Shell width was compared to SH in trials using *M. arenaria* due to its shell width being similar to SH in oysters.

Single (cultchless) oysters (*C. virginica* and *C. ariakensis*), single hard clams (*M. mercenaria*), and single soft clams (*M. arenaria*) were used for adult predation trials. Specimens of each species were divided into groups. *C. virginica* included the following shell height (SH) groups; 15-25mm (seed oysters), 30-40mm, 45-55mm, 60-70mm, 75-85mm, and 90-100mm. *C. ariakensis* included the following SH groups; 45-55mm, 60-70mm, and 75-85mm. M. mercenaria used in testing included; 30-35mm (little neck), 40-45mm, and 50-55mm (top necks) SH groups. *Mya arenaria* used in testing included the shell width (SW) group of 45-55 mm.

Trial duration (time allowed for predator-prey interaction) was randomly assigned each testing day. Timing of each trial commenced with the introduction of shellfish into the ray holding tank. Once trial time expired, rays were herded to the end of the holding tank opposite from where prey was introduced using a fence constructed of pvc that extended the width of the tank. The rays were corralled there until shellfish and crushed shell remnants were collected from tank bottom. Collection was performed by compiling the shell from the tank bottom using a one meter long rubber squeegee, followed by scooping shell from the pile with a two gallon capacity funnel attached with a one mm mesh filter bag, then finishing removal of small pieces using a six gallon wet-dry shop vacuum. Whole bivalves recovered after each trial were sorted from shell remnants, grouped to size or species classification, counted, and re-measured (SH or SW and SD).

Comingled oyster susceptibility trials

To evaluate size preferences, we comingled multiple shellfish size groups together and introduced them simultaneously to the rays. In comingled trials with adult cownose rays, 25 single oysters or clams per SH group (for a total of 150 oysters or 75 clams) were mixed and dumped into the holding tank approximately one meter from the tank's vertical end-wall, resulting in a mound of randomly mixed bivalves of various sizes covering approximately 0.5m^2. For *C. virginica*, feeding trials were conducted in triplicate for time periods of 7.5, 15, 30, and 45min, and duplicate for 60, 120, 240min periods.

For *C. ariakensis*, we only tested three SH groups (due to availability) in triplicate 30 min trials.

Preliminary investigations feeding rays *M. mercenaria* demonstrated that exceeding 15min was likely to exhaust the 25 clams in the 30-40mm SH size class, therefore clam selectivity trials were only conducted at 15min durations.

For comingled trials with YOY rays, 25 oysters per SH group (SH 10-20, 20-30, 30-40 mm) were comingled in a 2-gallon bucket, then dumped into the holding tank resulting in a mound ~20 cm^2. Triplicate 18hr feeding trials were conducted.

Data analyses were conducted using SPSS for Windows (16.0.0, SPSS Inc.). Adult comingled trials were initially evaluated using chi-square tests and G-tests in order to test the null hypothesis that predation success was equal for bivalves of all SH. In trials where predation success was unequal,

we used the Manly-Chesson alpha index of selectivity for variable prey abundance and normalized it to get electivity (-1 is complete avoidance and +1 is complete preference) in order to evaluate prey preferences. Actual count data were standardized to display the proportion of predation based on SH and SD measurements before and after comingled trials. The mortality data collected from these trials were also used to generate proportions of predation.

Binary logistic regression was used for both adult and YOY co-mingled trials to examine the effect of each SH group, SD, and time period (where appropriate) on predation probability where a binary response, alive (0)/dead (1), is related to one or more predictor variables. A logistic regression model predicted the probability of predation of three different bivalves in the comingled trials, *C. virginica, C. ariakensis,* and *M. mercenaria* by captive cownose rays.

The model can be expressed as:

$$\text{Logit}\{p(x)\} = \log\{p(x/1-(x)\} = b0 + b1x + b2x2$$

Where p(x) is the probability that a bivalve will be preyed upon as a function of a variable x and b0, b1, b2 are the regression parameters.

The equation can be rearranged to define estimated probability p(x) as:

$$p(x) = e\ (b0 + b1x + b2x^2)/\{1 + e(b0 + b1x + b2x^2)\}$$

Factors (x) contributing to the probability of predation p(x) included shell depth and SH groups and in one instance time period for *C. virginica*. For analysis of *C. virginica* the SH groups were: 15-25, 30-40, 45-55, 60-70, 75-85, 90-100mm. For C. ariakensis the SH groups were: 45-55, 60-70, 75-85mm. The groups for *M. mercenaria* were: 30-35, 40-45, and 50-55mm. We applied this model to each trial for time periods of 7.5, 15, 30, 45, 60, 120, 240min for *C. virginica*, 30min for *C. ariakensis*, and 15min for *M. mercenaria*. Time (x) was added as a factor to the model for *C. virginica* to generate a predicted probability across multiple time periods. Parameter estimates for each predictor variable were generated and evaluated for significance ($p < 0.05$). Model fit was evaluated using Hosmer and Lemeshow Tests.

Evaluation of Peak Load of *C. virginica* and *M. mercenaria*

Forty oysters (*C. virginica*) (SH 24-95, SD 12-35) and 36 hard clams *(M. mercenaria)* (SH 33-54, SD 21-31) were used to evaluate the force (load) required to crush each species. A 100 Kip Enerpac manual hydraulic pump and jack system was used, connected to a 5500lb (25kN) MTS Systems Corporation (Eden Prairie, MN, USA) load cell (Model 661.20B-01). The load cell was connected to a Voltmeter through an AC powered Bridge sensor (Model DMD 465WB) for taking load measurements. A standard "zero to 2-inch range" deflection dial gage (with a least count of 0.001 in) was used to record deformation/deflections of the shellfish specimen. Coupling the MTS load cell with Bridge sensor increased the resolution of the load readings greatly and the manual hydraulic pump gave precise control over the load increments/ intervals. The least applicable load was 0.7lbs or 3N with the above configuration.

The load cell was calibrated under MTS load frame system before testing shellfish. The calibration involved the application of a known load to the load cell assembly in increments and the corresponding voltage output recorded. This process establishes the voltage to load calibration relationship for the load cell. Bivalve samples were weighed and measured SH and SD. Specimens were placed on a solid steel platform under the load cell and load testing commenced. With all shellfish samples, the load cell was gently brought in contact with the specimen and the deflection dial gage was set to zero. A small increment of load was then applied using the hydraulic pump and corresponding deformation of specimen was recorded from

Trial Type	Species	SH (mm)	Mean SD	SD Range
oyster-oyster	*C. virginica*	75-85	27.1	21-32
	C. ariakensis	75-85	26.0	21-32
oyster-oyster	*C. virginica*	45-55	16.5	17-25
	C. ariakensis	45-55	17.0	14-20
oyster- hard clam	*C. virginica*	45-55	22.9	16-32
	M. mercenaria	35-40	24.9	21-32
oyster-soft clam	*C. virginica*	45-55	16.0	11-24
	M. arenaria	45-55 (SW)	16.0	11-20

Table 1. Shellfish used in adult cownose ray predation comparative trials. SH=shell height, SD=shell depth, SW=shell width

mechanical dial gage. This process continued until the specimen failed by crushing.

One of the two valves of specimens would fail first, at which point load readings were recorded indicting initial valve failure, or for the purpose of this study, mortality. Load readings were made at point of first failure (cracking of one valve) and again at point of second valve failure. Load was measured in kN (from the load cell) versus vertical deformation in mm (based on the dial gage readings). Compressive load readings were in pound-force (lbf) with 1 lbf = 4.4482 Newton.

Comparative predation trials

Adult predation trials were conducted comparing *C. virginica* and *C. ariakensis, C. virginica* and *M. mercenaria*, and *C. virginica* and *Mya arenaria*. In comparative trials, 25 specimens of both species from the same SH group with similar SD (Table 1) were comingled and simultaneously introduced into the holding tank with four adult rays. Trial time was held constant at 15min and performed in triplicate.

For oyster-soft shell clam trials; mortalities were counted at 3, 5, and 15min for triplicate trials. For comparative experiment testing preference χ^2 test or G-tests were performed and combined to test for significant (α =0.05) differences in the numbers of each species preyed upon. Independent tests of significance were combined using Fisher's (1954) method. We calculated Manly-Chesson alpha index of selectivity for variable prey abundance and normalized it to get electivity (-1 is complete avoidance and +1 is complete preference) (Chesson, 1978) to determine prey preference when appropriate.

Rate trials

We evaluated size-mediated predation rates by adult rays through predation trials grouping one hundred *C. virginica* oysters from a given SH size over a 15 min period. Rate is defined as the mean number of oyster mortalities per minute per ray within each individual time trial. Duplicate trials were performed for oyster SH: 30-40, 45-55, 60-70 and 75-85mm. Rates of predation were standardized to account for differences in oyster abundance in order to compare rates of predation to comingled feeding trials where rays were introduced to oysters of varying sizes.

Predation Trials: Cultched Oysters (Spat-On-Shell)

Deployment of cultchless (single) oysters on experimental reefs and commercial grounds experienced significant set-backs due to cownose ray predation in 2004 and 2006 and have led to discussions of alternative strategies to combat ray predation. One alternative strategy is the deployment of "spat

on shell" (SOS), in which oyster larvae metamorphose onto oyster shells (cultch) and grow as a cluster of oysters. SOS naturally occurs in the wild but can also be cultured, thereby increasing production capabilities. Production of SOS using aquaculture techniques is underway through a collaborative program between Virginia's oyster industry, the Virginia Marine Resources Commission, Chesapeake Bay Foundation, and the Virginia Institute of Marine Science. The spat on shell product is being tested at oyster restoration and commercial sites throughout the Virginia portion of the Chesapeake Bay. The Virginia oyster industry provides the infrastructure and labor behind this effort.

For the purpose of oyster restoration, advantages of SOS relative to the culture of single oysters include reduced predation from cownose rays, the ability to plant cultured oysters at a smaller size, and the reef building quality of spat on shell. Further, SOS may potentially allow commercial growers to expand production through on bottom extensive growout. Cownose ray predation trials were conducted using SOS to investigate how rays interact with clustered oysters and if refuge from predation is observed

Comparative predation trials were conducted comparing wild grown cultchless and SOS *C. virginica* with 4 adult cownose ray (92-100cm DW). Three time trials per four different time periods (7.5, 15, 30, 60min) were conducted with 60-70mm SH single and SOS oysters (size of individual oysters in cluster). Limited trials (two 15min, and single 30 and 60min) were also conducted with 75-85mm SH oysters. A total of 50 oysters were used per trial: 25 cultchless, and 25 SOS (5-6 clusters with 3-6 oysters per cluster).

To observe impact of YOY cownose ray on SOS seed, triplicate 18 hr predation trials were conducted with 25-30 SH single and SOS C. virginica. A total of 50 oysters were used per trial: 25 cultchless, and 25 SOS (4-5 clusters with 6-9 oysters per cluster).

Predation behavior

Prior to conducting predation trials, prey capture behavior and mechanics was documented through filming repeated predation attempts by captive rays on single shellfish (oysters, clams) and SOS oyster clusters. Oyster number, size, and angle of attachment (extension) from cultch in each SOS

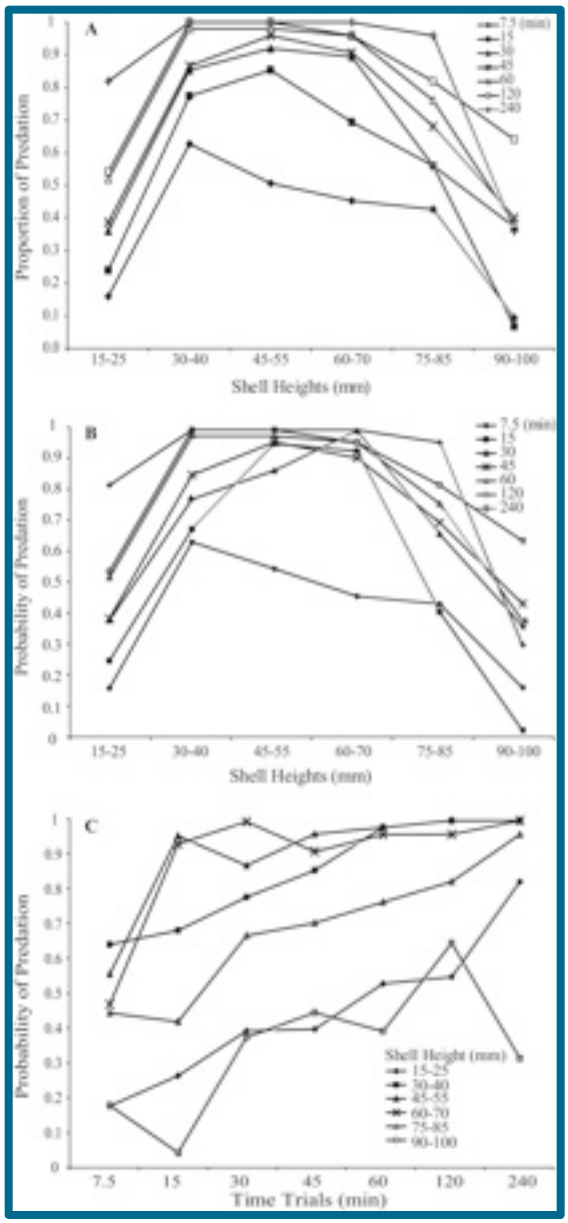


Figure 2. Results from comingled oyster trials with adult cownose rays. (a) Propostion of oysters (*C. virginica)* preyed upon for SH groups over various time trials based on count data. (B) Mean predicted probabilities of oyster (*C. virginica*) predation for SH groups from logistic regression model over various time trials. (C) Mean predicted probabilities of predation for oyster(*C. virginica*) SH groups over time.

Time Trial	Shell Heights	Success	Failure	α		Electivity	
7.5 min	15-25 mm	12	63	0.0504	±0.0002	-0.5835	±0.0097
N=3	**30-40**	47	27	0.3193	±0.0057	**0.3887**	±0.0232
	45-55	38	35	0.2272	±0.0008	**0.1871**	±0.0064
	60-70	34	40	0.1853	±0.0008	**0.0605**	±0.0078
χ^2 =73.42	**75-85**	32	44	0.1945	±0.0156	**0.0139**	±0.1880
p < 0.05	90-100	7	67	0.0233	±0.0016	-0.8180	±0.0994
15 min	15-25	18	57	0.0463	±0.0001	-0.6109	±0.0044
N=3	**30-40**	58	17	0.2637	±0.0072	**0.2584**	±0.0513
	45-55	64	11	0.3373	±0.0065	**0.4217**	±0.0239
	60-70	52	24	0.1944	±0.0007	**0.0904**	±0.0071
χ^2 =146.7	75-85	42	33	0.1461	±0.0039	-0.1072	±0.0781
p < 0.05	90-100	5	70	0.0121	±0.0004	-0.8945	±0.0534
30 min	15-25	27	48	0.0526	±0.0008	-0.5770	±0.0345
N=3	**30-40**	64	11	0.2283	±0.0048	**0.1751**	±0.0361
	45-55	69	6	0.2853	±0.0078	**0.3117**	±0.0356
	60-70	67	8	0.2741	±0.0076	**0.2852**	±0.0404
χ^2 =113.8	75-85	42	32	0.0997	±0.0017	-0.3038	±0.0422
p < 0.05	90-100	28	47	0.0616	±0.0034	-0.5559	±0.1637
45 min	15-25	29	46	0.0498	±0.0005	-0.5915	±0.0208
N=3	**30-40**	65	10	0.2188	±0.0038	**0.1524**	±0.0270
	45-55	72	4	0.3076	±0.0093	**0.3576**	±0.0350
	60-70	68	7	0.2374	±0.0013	**0.2130**	±0.0087
G =111.9	75-85	51	22	0.1213	±0.0005	-0.1875	±0.0103
p < 0.05	90-100	30	39	0.0652	±0.0035	-0.5313	±0.1522
60 min	15-25	26	24	0.0595	±0.0001	-0.5199	±0.0008
N=2	**30-40**	49	1	0.2607	±0.0003	**0.2755**	±0.0020
	45-55	49	1	0.2607	±0.0003	**0.2755**	±0.0020
	60-70	48	2	0.2313	±0.0005	**0.1998**	±0.0039
G =101.1	75-85	38	12	0.1361	±0.0068	-0.1562	±0.1245
p < 0.05	90-100	19	31	0.0517	±0.0041	-0.6194	±0.2023
120 min	15-25	27	23	0.0615	±0.0005	-0.5117	±0.0204
N=2	**30-40**	50	0	0.2482	±0.0010	**0.2430**	±0.0065
	45-55	50	0	0.2482	±0.0010	**0.2430**	±0.0065
	60-70	48	2	0.2191	±0.0001	**0.1673**	±0.0007
G =78.40	75-85	41	9	0.1344	±0.0004	-0.1284	±0.0073
p < 0.05	90-100	32	18	0.0886	±0.0032	-0.3716	±0.0970
240 min	15-25	41	9	0.1281	±0.0035	-0.1734	±0.0679
N=2	**30-40**	25	0	0.2174	±0.0001	**0.1629**	±0.0001
	45-55	50	0	0.2174	±0.0001	**0.1629**	±0.0001
	60-70	50	0	0.2174	±0.0001	**0.1629**	±0.0001
G =123.3	**75-85**	48	2	0.1941	±0.0120	**0.0885**	±0.0120
p < 0.05	90-100	15	35	0.0255	±0.0260	-0.7740	±0.0260

Table 2. Combined predation (success or failure) on oysters (*C. virginica*) for adult cownos ray comingled predation trials. SH in bold indicate preferred prey items.

cluster were varied to provide differing levels of difficulty to elicit possible alternative predation strategies. SOS tested included clusters of 1-6 oysters (per cultch). Limited ray foraging behavior was also investigated on shell substrate. Two bushels of oyster cultch shell (70-95mm SH) was spread within a square meter area in holding tank resulting in 6 inch thick shell bed and seeded with hard clams *M. mercenaria* (35-40mm SH) and blur mussels *Mytilus edulis* (40-50mm SH).

Results

Comingled Trials

In comingled trials with adult cownose rays, the proportion of oysters successfully eaten increased for all SH tested as time increased except for the largest SH class (90-100mm) (Figure 2A). SH of 30-40, 45-55, and 60-70mm were the most heavily selected for all time trial periods (Table 2). Lowest predation success was observed on 15-25, 75-85, and 90-100 SH oysters.

The probability of predation increased for all SH tested as time increased except for the 75-85 and 90-100mm oysters in the 15min time period and 90-100mm oysters in the 240min time period (Figure 2B, 2C).

Shell Height (mm)	Range of Shell Depths (mm)	Mean Shell Depth Before Feeding (mm)	Mean Shell Depth After Feeding (mm)
15-25	4-9	6	6
30-40	8-18	12	12
45-55	11-22	16	16
60-70	13-33	21	24
75-85	18-35	27	29
90-100	18-40	30	32

Table 3. Range of SD and mean SH of oysters (*C. virginica*) before and after adult cownose ray comingled predation trials.

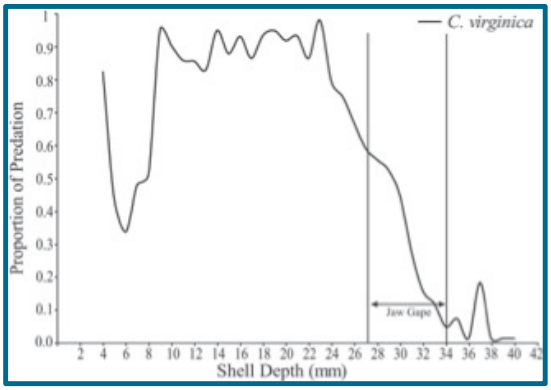


Figure 3. Proportion of oysters (*C. virginica*) eaten by cownose ray as a function of SD in comingled predation trials. Vertical lines represent the range of maximum jaw gape for adult rays used in predation trials.

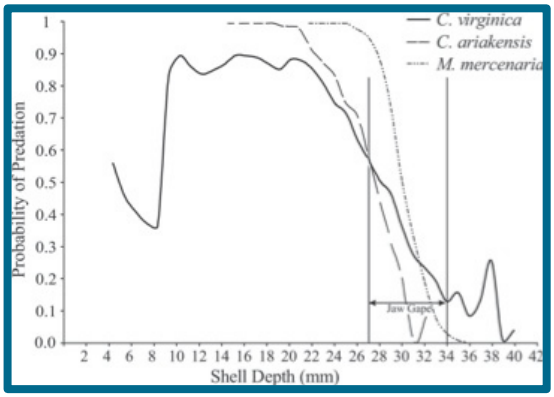


Figure 4. Mean predicted probability of adult cownose ray predation from logistic regression models for *C. virginica, C. ariakensis,* and *M. mercenaria* as functions of SD. Vertical lines represent the range of maximum jaw gape for adult rays used in predation trials.

Overall, oysters in the smallest and largest SH categories had the lowest selectivity.

Mean SD of oysters within each SH group increased 2-3mm between pre-trial and post-trial in the 60-70, 75-85, and 90-100mm oysters suggesting selection for those oysters with smaller SD in larger oysters (Table 3). No difference in mean SD was found in 15-25, 30-40, and 45-55mm SH oysters. Predation declined with increasing SD. The highest proportion of predation was observed in oysters with SD between 8 mm and 22mm while the lowest predation success recorded in oysters with SD greater than 32mm (Figure 3).

The highest probability of predation among bivalves tested was for *C. virginica* in the 8-22mm SD range, with predation declining with increasing SD (Figure 4). Probability of predation on *C. ariakensis* was highest for shell depths of 14-20mm. Similarly, predation declined as SD increased above 22mm. The highest probability of predation in *M. mercenaria* was observed in on shell depths between 21-26mm. A steep decline in predation was observed as SD increased above 26mm. A logistic regression equation predicted the probabilities of predation for *C. virginica* based on the eight variables tested (see Figure 4).

$$P(x) = 1/1+e-(7.260+ 0.013x1 + -0.302x2 + -6.590x3 + -2.575x4 + -0.896x5 + 0.457x6 + 0.906x7)$$

Where

P(x)= Prob (0, 1);

x1 = Time,

x2 = Shell Depth,

x3 = Shell Height 15-25,

x4 = Shell Height 30-40,

x5 = Shell Height 45-55,

x6 = Shell Height 60-70,

x7 = Shell Height 75-85.

All variables were significant at the 0.05 level and the Hosmer and Lemeshow Test (HL) was nonsignificant ($p > 0.108$) suggesting the model adequately fit the data. Individual analysis of each time trial period resulted in non-significant HL tests for all time periods except for the 15min period. Between three to five of seven parameter

Table 4. Parameter estimates $ß_0 ... ß_6$ correspond to the intercept, SD, five SH categories (SH 15-25, 30-40, 45-5, 60-70, 75-85mm) are presented for individual adult ray comingled predation trials. P-values of each variable are shown below parameter estimates and significance level for the Hosmer and Lemeshow tests (HL) of model fit are displayed.

Time Period	Intercept (β_0)	β_1/p SD	β_2/p 15-25	β_3/p 30-40	β_4/p 45-55	β_5/p 60-70	β_6/p 75-85	HL
7.5	4.608	-0.206	-5.004	-1.653	-1.112	-0.205	0.723	0.107
	0.001	***<0.05***	***<0.05***	0.076	0.139	0.705	0.088	
15	5.731	-0.279	-5.162	-1.085	0.690	1.069	1.990	***0.048***
	<0.05	***<0.05***	***<0.05***	0.287	0.415	0.103	***<0.05***	
30	9.302	-0.340	-7.744	-3.644	-1.292	1.208	0.217	0.230
	<0.05	***<0.05***	***<0.05***	***<0.05***	0.121	***0.016***	0.574	
45	9.144	-0.325	-7.546	-3.531	-0.458	0.594	0.492	0.202
	<0.05	***<0.05***	***<0.05***	***0.001***	0.614	0.288	0.225	
60	10.653	-0.384	-8.106	-2.329	-0.337	0.573	0.830	0.104
	<0.05	***<0.05***	***<0.05***	0.163	0.807	0.551	0.119	
120	10.234	-0.328	-7.982	14.704	16.174	0.295	0.518	0.981
	<0.05	***<0.05***	***<0.05***	0.998	0.998	0.751	0.320	
240	16.638	-0.554	-11.569	11.780	14.131	18.154	1.927	0.997
	<0.05	***<0.05***	***0.002***	0.999	0.998	0.997	***0.031***	

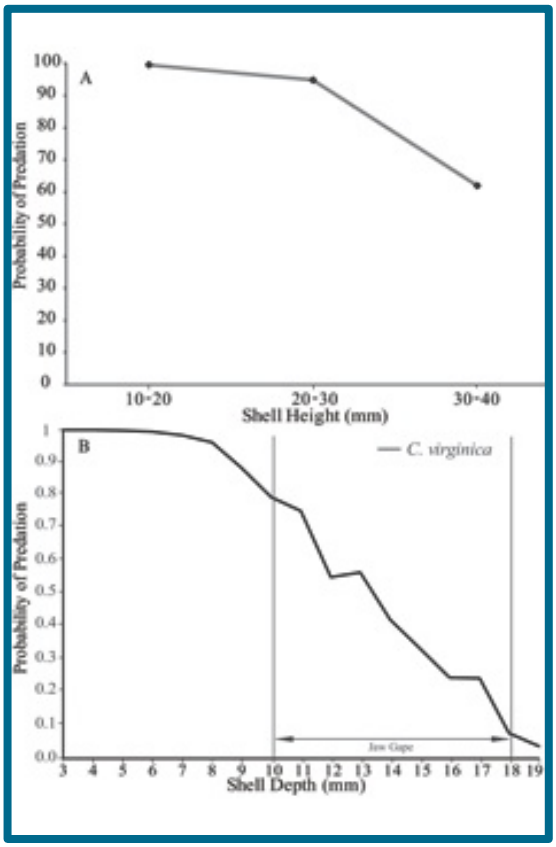


Figure 5. (A) Mean predicted probability of YOY cownose ray predation from logistic regression model for C. virginica as related to SH. (B) Mean predicted probability of YOY predation from logistic regression model of C. virginica as related to SD. Vertical lines represent maximum jaw gape frange for YOY rays used in predation trials.

estimates were significant for each period, but the parameter estimates for SD and the smallest SH group (15-25mm) were significant for all time trials (Table 4).

A logistic regression equation for *C. arikensis* was generated,

P(x) = 1/1+e-(15.329+ -0.556x1 + 16.421x2 + -0.819x3)

where the intercept and SD parameter estimates were significant ($p > 0.01$) and the SH parameter estimates were nonsignificant ($p > 0.998$, $p > 0.472$, respectively). However, the HLtest was significant ($p < 0.026$) suggesting the model did not adequately fit these data.

For hard clams (*M. mercenaria*), the logistic regression equation is,

P(x) = 1/1+e-(30.355+ -0.993x1 + 13.944x2 + -0.934x3)

The HL test was non-significant ($p > 0.394$) suggesting a better model fit and additionally two, intercept and SD ($p < 0.12$, $p < 0.05$, respectively) of four parameter estimates were significant. Predicted probabilities from the model are shown (Figure 4).

In co-mingled trials with YOY rays, the probability of predation declined as SH and SD increased (Figure 5). The equation generated for YOY predation is

P(x) = 1/1+e-(21.027+ 2.964x1 + -0.370x2 + 0.270x3)

Parameter estimates for intercept, and shell heights were nonsignificant ($p > 0.997$, $p > 0.850$, and 0285) whereas the estimate for SD was significant ($p < 0.05$). The HL test suggested the model did not adequately fit the data ($p < 0.049$).

Figure 6. Results of YOY cownose ray feeding trial investigating influence of SD on predation success on seed *C. virginica* oysters. Pre-and post trial views (left top and bottom) of 20-30mm SH, 7-11mm SD oysters showing 100% predation. Pre-and post trial views (right top and bottom) of 30-40mm SH, 8-20mm SD oysters, showing some escaped predation.

To further investigate influence of SD on YOY predation success, three additional feeding trials were conducted. Single trials were performed for oyster SH(SD): 10-20mm (4-9mm SD); 20-30mm (7-11mm SD); and 30-40mm (8-20mm SD). Fifty *C. virginica* oysters per SH groups were fed to YOY rays in 16hr time trials. Oysters 10-30mm SH with SD <10mm were easily preyed on by YOY rays, coinciding with the minimal jaw gape in YOY of 10mm. Predation failure was first observed in 30-40mm SH oysters with SD > 10mm (Figure 6). Oysters which escaped predation ranged from 29mm SH (12mm SD) to 40mm SH (13mm SD). SD of surviving oysters ranged from 10-20mm with average of 13.6mm.

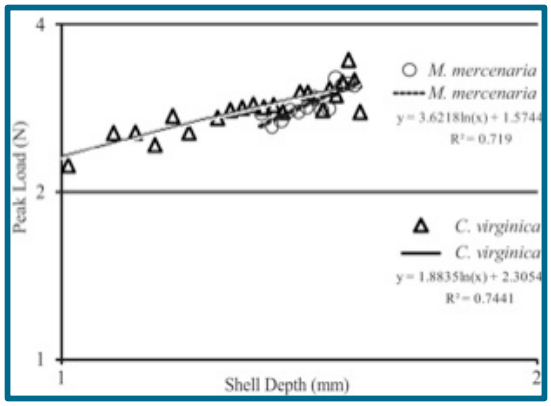


Figure 7. Peak load of *C. virginica* and *M. mercenaria* as related to SD, plotted on logarithmic axes.

Peak Load Trials

The force needed to cause failure in one or both valves in *C. virginica* and *M. mercenaria* increased as shell depth increased (Figure 7, 8). The plot of the log transformed SD and peak load displays that the load scales isometrically with shell depth.

For *M. mercenaria,* linear peak load is lowest at 21mm a SD and increases to nearly 1400N at 33mm SD (Figure 8A). Adult probability of predation and peak load in-

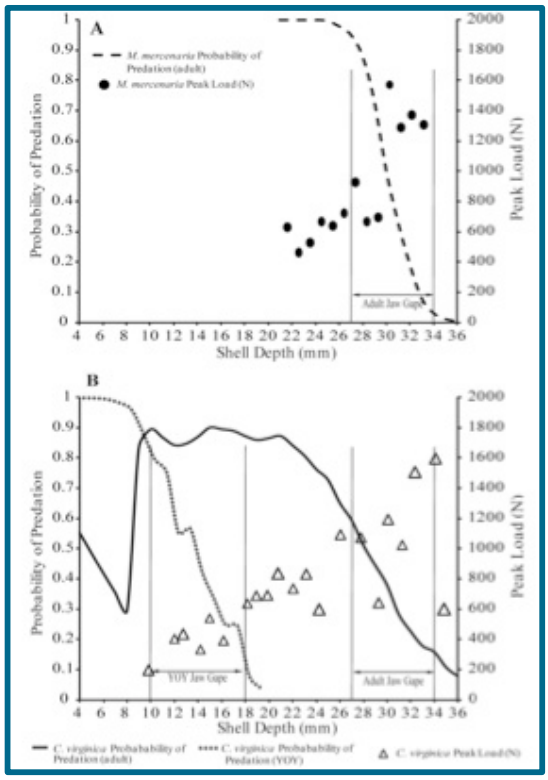


Figure 8. (A) Mean predicted probability, adult cownose ray predation and non-transformed peak load of *M. mercenaria* as related to SD. (B) Mean predicted probability of YOY and adult cownose ray predation and non-transformed peak load of *C. virginica* as related to SD..

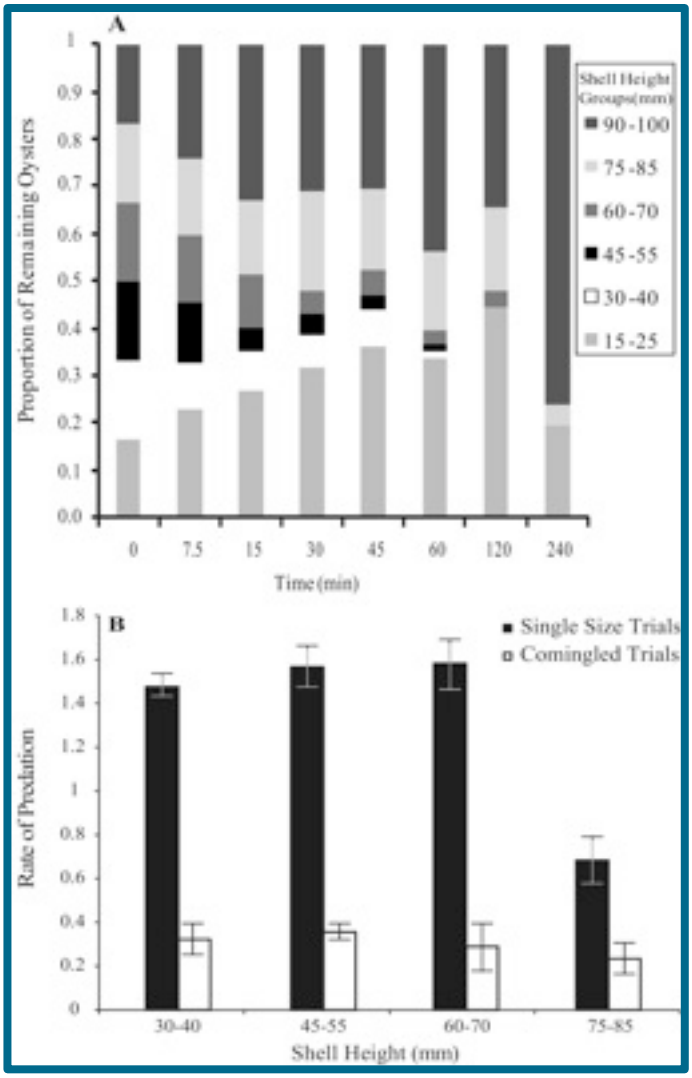


Figure 9. (A) Proportion of remaining oysters after each time period (*C. virginica*) for comingled predation trial periods. (B) Mean number of oysters (*C. virginica*) consumed per minute per adult ray for single-size rate trials compared to comingled trials. Comingled trial times of 15min were used for comparison.

tersect at 30mm for *M. mercenaria*.

Peak load for *C. virginica* is lowest at SG of 10 mm and increases to above 1500 N at 35 mm a SD (Figure 8B). Adult probability of predation and peak load intersects at 29 mm SD for *C. virginica*. YOY predation and linear peak load (*C. virginica*) intersect at 17 mm SD.

Rate Trials

The rate of predation for all oyster SH groups decreased with increasing trial time. In 7.5 min time trials, 30-40 mm SH oysters were preyed upon quickest, followed by the 45-55, 60-70, then 75-85 mm SH oysters (Figure 9A). Cownose ray predation rates on oysters were only a slightly higher on same size oysters compared with comingled oysters of varying sizes, except in the 75-85 mm SH (Figure 9B).

Comparative Trials Between Bivalve Species

No significant difference in predation was observed between *C. virginica* and *C. ariakensis* in both SH groups (SH 45-55, SH 75-85; $p > 0.222$, 0.186, respectively) tested. Predation success was highest (90-96% eaten) in 45-55mm oysters of both species. Predation success was significantly higher ($p < 0.0001$) and the rays selected hard clams (*M. mercenaria*, $\alpha = 0.736 \pm 0.002$, electivity = 0.473 ± 0.007), over oysters, (*C. virginica*, $\alpha = 0.263 \pm 0.002$, electivity = -0.473 ± 0.007).

Rays also selected soft clams, *M. arenaria* at 5min into a 15min trial ($\alpha = 0.742 \pm 0.003$, electivity = 0.485 ± 0.013) over oysters (*C. virginica* $\alpha = 0.257 \pm 0.003$, electivity = -0.485 ± 0.003) initially, then selection was more equal at the end of 15 min trial (*M. arenaria*, $\alpha = 0.570 \pm 0.014$, electivity = 0.141 ± 0.059; *C. virginica* $\alpha = 0.429 \pm 0.014$, electivity = -0.141 ± 0.059). Though SH was greater for *C. virginica* in oyster-hard clam trials, mean SD was similar for both species (Mean SD clams = 24.9 mm, Mean SD oysters = 22.9 mm).

Comparative Oyster Trials: SOS vs singles

Predation attempts were made on all oysters within each trial. Each failed attempt resulted in oysters, single or SOS, being re-distributed around the tank. As time increased, multiple attempts on both single oysters and SOS were observed, with predation on SOS

oysters generally increasing. In 60-70mm SH oyster trials with adult rays, significant differences were observed by Chi-square testing in 11 of 12 trials between cultchless and SOS oysters (Table 5). In one 60min trial the predation success was the same, with no difference in mortality observed. In one 7min 30sec trial the number of spat-on-shell mortalities exceeded the single oyster mortalities.

In all trials in which at least one oyster was preyed on from SOS cluster, oyster(s) with greatest extension from cultch were observed consumed. Results from limited trials with larger oysters (75-85mm SH) were similar to 60-70mm trials for respective times (Table 6). Predation success on SOS was only observed to exceed single oysters in the 30min trial.

In YOY trials, no SOS was successfully preyed upon (Table 10) while nearly all cultchless oysters were consumed. As with adult rays, YOY continued to attempt predation on SOS each time encountered regard-

Trial No.	Time (min)	N	SOS Consumed	Single Oysters Consumed	χ^2	P-value
1	7.5	50	4	20	21.120	<0.001
2	7.5	50	0	25	8.310	0.004
3	7.5	50	12	6	4.160	0.041
1	15	50	4	22	25.962	<0.001
2	15	50	2	18	21.333	<0.001
3	15	50	8	19	9.742	0.002
1	30	50	8	21	13.875	<0.001
2	30	50	5	21	20.513	<0.001
3	30	50	7	21	15.033	<0.001
1	60	50	12	23	11.524	0.001
2	60	50	3	25	39.286	<0.001
3	60	50	24	24	0.000	1

Table 5. Results of χ2 tests between groups of SOS oysters and single oysters (SH 60-70 mm) consumed in adult cownose ray predation trials.

Trial No.	Time (min)	N	SOS Consumed	Single Oysters Consumed	χ^2	P-value
1	15	50	15	25	12.500	0
2	15	50	3	15	12.500	0
3	30	50	16	15	0.085	0.771
4	60	50	10	22	12.500	0

Table 6. Results of χ2 tests of SOS and single oysters consumed (SH 75-85mm) in predation trials.

Figure 10. Young-of-year (YOY) cownose ray predation on single and SOS seed oysters (25-30mm SH); pre-trail (top left) and post-trial (bottom left). All single oysters were successfully preyed upon, with no predation success on SOS. Note seed oyster attachment to cultch with no oysters extending from cultch.

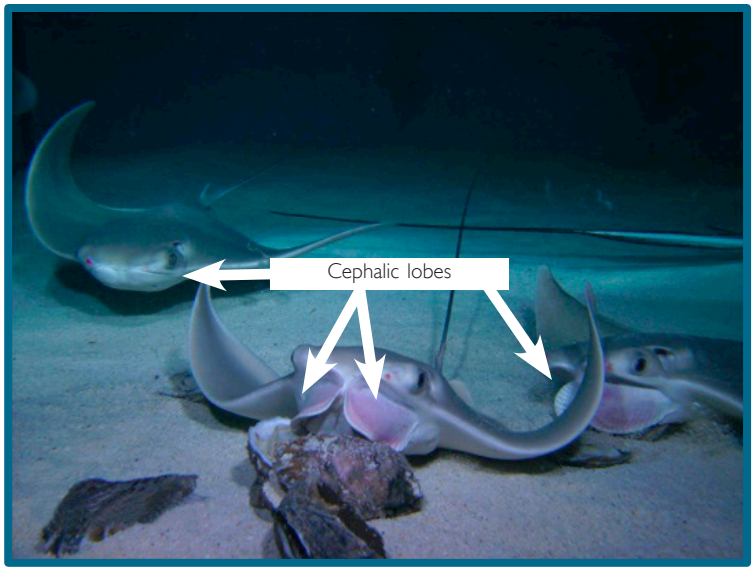


Figure 11. Cownose ray feeding on oysters. Ray at left with paired cephalic lobes retracted during swimming. Rays using cephalic lobes to channel oysters towards mouth (center) and to enclose the area around oyster (right) to increase suction feeding efficiency.

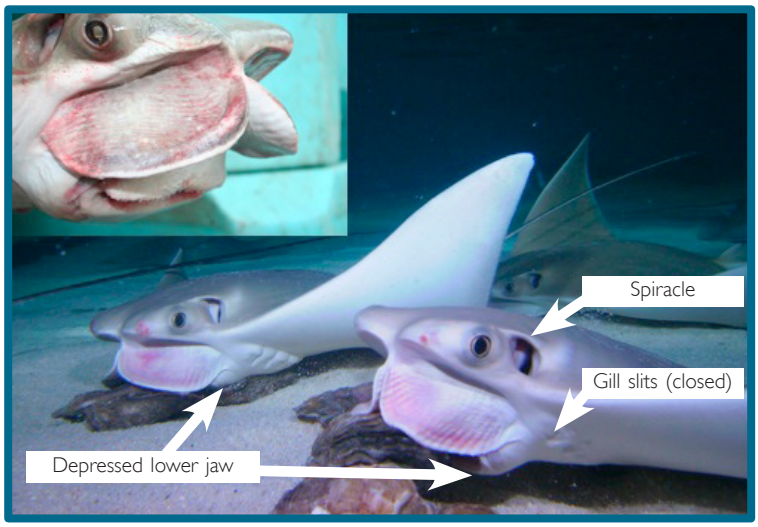


Figure 12. Cownose ray predation on oysters using suction mechanics. With cephalic lobes surrounding oyster(s), lower jaw depressed (inset), and upper jaw protruded, oysters are drawn up towards ray mouth by suction generated by rhythmic opening and closing of rays gill slits and spiracles (video avaoiable online http://bit.ly/b6RKZc ; search cownose ray).

less of past failure. Predation failure on SOS oysters was directly related to seed attachment to cultch, where most of one valve of seed was attached to cultch, providing little to no extension of seed oyster from cultch thereby providing nothing for YOY ray to grasp (Figure 10).

YOY mimicked adult predation behavior by attempting predation on prey as first encountered. After repeated failures on SOS, and upon depletion of single oysters, YOY were observed to continually try to prey on SOS oysters.

Predation behavior

Cownose ray approached shellfish prey in a consistent manner, showing no preference as to prey type. As rays approach exposed shellfish, paired cephalic lobes, extensions of pectoral fins, become extended towards bottom making contact and subsequently surrounding shellfish (Figure 11). These lobes aid in prey capture by physically channeling prey towards mouth and by facilitating affect of suction mechanics on shellfish by enclosing area around prey, thus increasing suction potential which draws shellfish towards ray mouth. Mechanics employed by cownose ray for securing both single (cultchless) and SOS oysters into mouth for manipulation and subsequent crushing involved repeated suction generated by water brought into the orobranchial cavity through the mouth by rhythmic opening and closing of gill slits and spiracles (videos available online: http://bit.ly/b6RKZc; search cownose ray). Gill slits and spiracles close while water is brought in through the mouth generating sucking action.

At the completion of each sucking episode, gills open expelling water from orobranchial cavity, and then close again, to repeat this suction cycle if needed for prey capture. In prey capture, this suction behavior is combined with the protrusion of upper jaw and depression of lower jaw which maximizes jaw gape and aids in securing shellfish between jaws (Figure 12).

The repeated suction cycles were observed in shellfish predation trials to function primarily in re-positioning shellfish that did not fit between protruding jaws in previous suction cycle. In high densities of cultchless shellfish, rays were observed to plow through multiple shellfish using rapid suction cycling to tumble prey, quickly re-positioning oysters and increasing likelihood of predation success.

Prey mass and shape was also observed to affect predation selection between co-mingled shellfish species. Lighter soft-shelled *Mya* clams were more susceptive to suction force than heavier *C. Virginia* oysters of similar size therefore drawn quicker to ray mouth during suction cycling (videos available online: http://bit.ly/b6RKZc; search cownose ray).

Uniformity in shape of the hard clam *M. mercenaria* was also observed to facilitate capture over *C. virginica* oysters during suction feeding with hard clams requiring less repositioning each suction cycle to fit between ray jaws than oysters which are more elongated and flattened along their width and often require repeated re-alignment attempts for capture.

Small prey items (as 25mm seed oysters in this study and polycheate worms in the wild) which are in close proximity to larger, heavier prey are drawn straight into the orobranchial cavity and likely ingested whole. Once prey was captured between jaws, rays would typically swim away to process prey. In predation soft clam trials with numerous highly susceptible prey available, rays were observed to initiate prey manipulation and crushing with multiple clams captured in mouth while still hovering above additional prey on bottom. Suction mechanics were employed throughout prey capture activities, including prey manipulation observed while swimming. Repeated suction cycles were observed to be sustained during swimming while manipulating large or heavy prey until prey was either captured or was dropped.

During prey processing prior to crushing, rays were observed to further manipulate shellfish within mouth by slight jaw movements. After initial crushing of prey, ray jaws were observed to further aid in re-positioning prey for additional crushing and to expel crushed shell fragments back through the mouth (videos available online: http://bit.ly/b6RKZc; search cownose ray). During prey processing, normal respiratory ventilation was observed, with large shell fragments removed through the mouth and small fragments expelled through gill slits and/or ingested to digestive tract. Though sand and debris are routinely observed being transported in through the mouth and out through the gill slits during prey capture and processing, only small shell fragments were observed to pass through the gill slits and no shell fragments were observed passing through the spiracles.

Shell fragments from thin-shell *Mya* clams were often observed to be fractured into uniformly small pieces, possibly indicating extensive mastication may be required to separate flesh from shell. This additional processing of *Mya* clams in the rays buccal cavity was further observed by rays repeatedly spitting partially crushed clams out of mouth then quickly drawing them back in for further processing.

Figure 13. Example of oyster shell fragments (left) remaining after cownose ray predation on various size oysters (*C. virginica*).

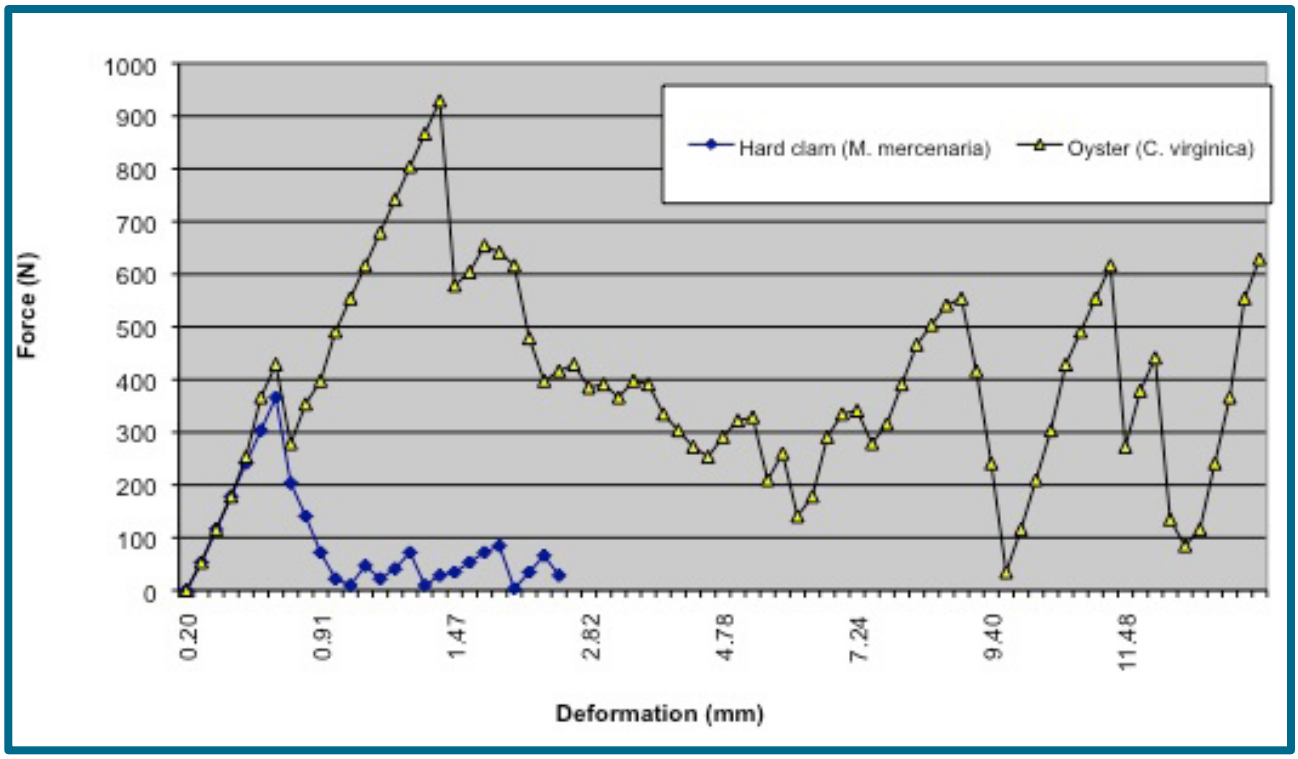


Figure 14. Comparison of compression force in Newtons (N) and deformation (mm) on hard clam (35mmSH, 21mm SD) and oyster (65mm SH, 26mm SD). Initial cracking (valve failure) occurred at 366 N in clams and 929 N in oysters.

Oyster and hard clam processing by cownose ray typically results in fragmented shell pieces of random size, from whole valves to minute pieces (Figure 13). Rays seem to crush hard shelled bivalves only to the point of which soft-body parts can be separated from shell, which seemed to involve less processing than that observed with soft-shell clams, especially compared to the hard clam *M. mercenaria*.

Many occasions were observed of discarded oyster and hard clam shells/fragments with adductor muscle still attached. Resulting shell fragment size in oysters was also observed to be related to shell integrity, with oysters heavily infested with shell-boring *Polydora spp* worms tending to crumble and fracture into smaller and thinner pieces. During analysis of stomach and spiral valve content (Section 4) shellfish valve fragment ingested rarely exceeded 5mm in size. Further, significantly more ingested pieces of thin-shelled bivalve species were observed than thick-shelled bivalve species when both were identified as prey items within the same ray sample.

Tissue-shell separation mechanics in buccopharyngeal cavity prior to ingestion was not investigated in this study; however, it is reasonable to suspect that ingestion of shell is related to extent of prey mastication and density of resulting shell fragments, with more dense fragments removed more efficiently

Once engaged in prey capture, ray behavior was observed to differ by prey type and prey density. Single shellfish (hard clams, soft clams, oysters) of size not limited by ray jaw gape and physically separated from others was observed to be preyed upon the quickest, with least amount of effort expended by the ray.

Predation success for this prey type typically followed; single pass over prey with little to no stoppage, cephalic lobes channeling prey towards mouth, and with 1-3 suction cycles prey was captured. Upon capture, an

Figure 15. Cownose ray predation trial result showing SOS clusters of 1-6 (left to right) oysters pre- and post-trial.

increase in predation effort on oysters over clams was further observed, with oysters frequently taking longer to process.

Whole live oysters are considerably more elastic than hard clams (Figure 14), resulting in greater deformation in oysters occurring under compression prior to initial valve failure. Hard clams are brittle in comparison, resulting in valve failure under considerably less pressure. Once structural integrity of the whole clam is lost, minimal force is required for further crushing. After initial valve failure in whole oysters, remaining intact valve coupled with strong valve attachments (elastic hinge ligament and adductor muscle) require considerable force for further processing. Effort was observed to increase when prey density of same size prey increased, and continued to increase when single shellfish was comingled in various sizes.

When single shellfish were aggregated together (50-100 shellfish per 0.5m^2) rays would slowly plow through shellfish with pectoral fins contacting bottom to help maintain position and using cephalic lobes to channel multiple prey towards mouth. Rays would then continue to suck shellfish toward mouth by repeating suction cycles until sufficient prey was captured to initiate prey processing, at which time rays would routinely leave the bottom and continue to process captured prey while swimming. Increased effort was largely the result of sustained suction cycling while attempting to get multiple prey into mouth before processing, which was observed to occur on many occasions (videos available online: http://bit.ly/b6RKZc; search cownose ray).

The additional effort was likely cost efficient since multiple prey of substantial size was able to be processed within a relatively short time period. Dense populations of single shellfish of size within ray gape limit (as deployed in past restoration and commercial growout efforts) are extremely vulnerable to ray predation, especially when large number of rays aggregate at one site. When single shellfish prey of various sizes was comingled together (prey size ranging from well within to beyond ray jaw gape limit) effort was further increased largely due to passive selection occurring; rays would attempt prey capture on first shellfish encountered regardless of size. When approaching comingled shellfish, plowing behavior commenced, with successful prey capture random, largely reliant on persistence of ray to continue sustained suction mechanics until appropriate size prey, or multiple prey is captured.

Large prey at or extending beyond ray

Figure 16. Cownose ray attempting predation on large oyster. Repeated suction cycles are used to hold and re-position oyster at mouth while ray tries to capture oyster between jaws. This prey manipulation process frequently occurs as rays swim away with oyster(s) after initial failed attempts on bottom. Pectoral fins are used to help maintain position on bottom when feeding on large and/or aggregated prey .

Figure 17. Cownose ray predation on SOS oysters. Ray uses the bottom to push against trying to grasp a single oyster from cluster. If SOS cluster is not too heavy, ray will swim away with cluster while continuing to manipulate until either an oyster is secured between jaws and separated from cluster or ray losses hold and cluster is dropped.

jaw gape limit were observed to be viewed by rays as suitable prey, eliciting repeated capture attempts even though more suitable prey was positioned alongside and typically resulting in unsuccessful predation and expenditure of time and energy.

Oysters were observed to escape predation, typically a result of SD (32-34mm) exceeding ray gapes regardless of shell height. Long (75-95mm SH), shallow-cupped oysters were easily preyed on by rays which simply manipulated oysters between jaws with anterior or posterior first. Depth of the posterior margin in larger oysters was observed to contribute significantly to predation success/failure. Oysters largely maintain a tapering in SD along the anterior-posterior axis, with posterior margin shallower than the hinge margin. As rays manipulate larger oysters along anterior-posterior orientation, the posterior margin typically fits further within the ray mouth (Figure 15) and further between ray jaws, allowing rays to "nibble" this margin down until valve failure provides access to oysters soft body tissue which can be sucked by the ray from partially crushed oysters.

Greatest required predation effort by cownose rays was observed with SOS with multiple oysters on cultch. Since SOS oyster clusters maintain 3-dimential relief off bottom, rays approach SOS clusters with rostra pointing slightly upward enabling sub-rostral positioned mouth better access to prey. An overall different strategy was observed in cownose ray predation on SOS oyster clusters; where a cluster of oysters is methodically reduced to singles, which are then more easily preyed upon.

Successful predation on SOS (referencing grow-out oysters 60-80mm SH) was heavily dependent on cluster size, and individual oyster orientation and degree of attachment within the cluster. In trials (N=6) conducted to observe ray predation on SOS clusters of 1-6 oysters per cultch, all oysters were successfully prey on (Figure 15). Predation on small SOS clusters (1-3 oysters) was

similar to that of single large (>75mm SH) oysters (Figure 16), in which the ray initially brings prey to mouth through suction mechanics then carries it off, head slightly upward, while manipulating to secure between jaws.

This behavior involved repeated suctioning cycles to maintain prey at mouth, which routinely resulted in ray dropping SOS without initial success (Figure 17); however, in trials with more than 2 oysters per cultch SOS were successfully preyed on largely due to repeated predation attempts. In the wild, intact large oysters or small clusters of SOS that are dropped by swimming rays would likely have a greater change of escaping predation since repetitive attempts would not likely occur as in captive conditions. In larger clusters, rays struggled trying to lift cluster from bottom and were resigned to alter predation strategy. Rays would position themselves above large oyster clusters and use the bottom to push prey against while trying to get a hold of an individual oyster. This strategy was coupled with suction cycling and proved successful.

If rays where able to grasp an individual oyster between jaws, breaking it lose from the cluster typically resulted, at which point access to adjacent oysters in cluster became easier. However, regardless of oyster num-

Figure 18. Cownose ray predation on SOS. Ray has successfully grasped an individual oyster extending from cultch, which was easily broken free from cluster and consumed.

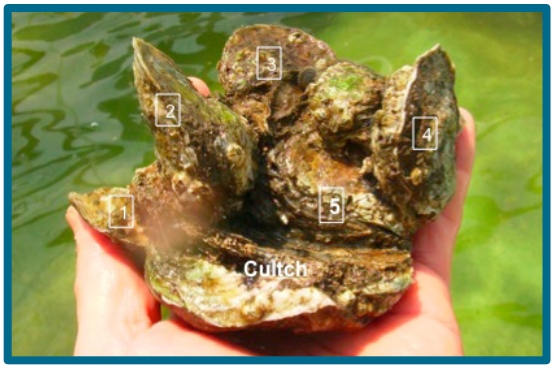


Figure 19. SOS oyster cluster with 5 oysters (1-5) attached to cultch. Cownose ray predation success was observed on oysters 1-4, with oyster 5 escaping predation. Rays removed oyster 1 first followed by 2, 4 then 3. Oyster 5 survived due to greater attachment to cultch giving ray no access point to grasp.

Figure 20. Abrasions and lacerations around mouth of cownose ray resulting from predation attempts on large SOS oyster clusters.

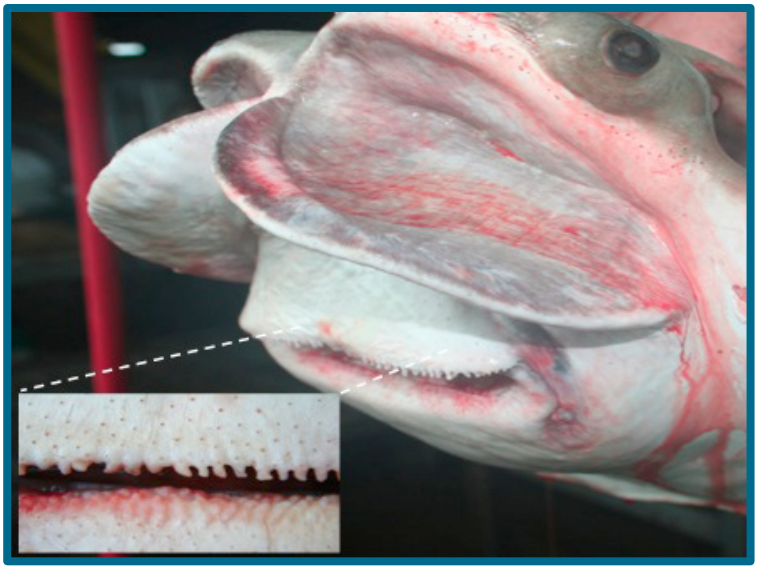


Figure 21. Numerous sensory pores are associated with the mouth region in cownose ray. These pores are connected by canals as part of the Ampullae of Lorenzini sensory system which detect weak electrical fields given off by prey.

ber per cluster, extent of oyster-to-cultch attachment significantly affected predation success/failure.

Oysters attached to cultch by minimal surface attachment area results in greater extension away from cultch and weaker connection to cultch, providing access for ray to grasp between jaws and requiring less leverage to break oyster loose from cluster (Figure 18). Likewise, wide spacing between oysters within cluster enables ray to grasp individual oysters. These relationships were also observed in SOS comparison trials where SOS oysters which escaped predation were largely oysters with a low profile (large portion of one valve attached to cultch resulting in minimal extension from cultch), or oysters densely grouped together with minimal spacing between (Figure 19).

Cownose ray predation strategies employed on SOS were more energy intensive than that observed with predation on single oysters and resulted in increased physical damage to the ray. The mouth region becomes inflicted with numerous abrasions and lacerations (Figure 20) from adjacent oysters as the ray works to secure a hold on an oyster within the cluster (videos available online: http://bit.ly/b6RKZc; search cownose ray) and proceed to break it lose. This trauma can be severe and may lead to infection or at best involve wound repair.

Sensory impairment may also result from these abrasions. Sensory pores are spread out over the head region in cownose ray and are densely populated around the mouth area (Figure 21). These pores are connected by canals to form an extensive sensory system in elasmobranchs, the Ampullae of Lorenzini. Weak electrical fields are detected within short distances by this sensory system and used to detect prey. Loss of teeth plates were also observed to increase as a result of predation on SOS. A greater number of teeth plates were consistently recovered from tank after SOS predation trials than single oyster trials, indicating predation on SOS may involve crushing mechanics not typically used

or ideally suited by cownose ray. Injuries around mouth and increased teeth loss contribute to an overall energetic cost involved with SOS predation over predation on other prey types.

Ray foraging behavior over prey-seeded shell bed involved slow, gradual movements across bed surface with shell contact made with both pectoral fins and extended cephalic lobes (Figure 22). Movement was more "sweeping" left and right as ray progressed forward (possibly using Ampullae of Lorenzini to locate prey), unlike that observed in un-covered prey which was mainly forward movement into prey. The cephalic lobes kept contact with shell substrate and performed light sorting of lose shell, however, no heavy excavation by cephalic lobes was observed. Prey was uncovered by the combination of depressed lower jaws forward motion into shell substrate and repeated suction and exhaling mechanics. Uncovered prey was captured by suction mechanics. Numerous sensory pores are associated with the peripheral edge and ventral surface of cephalic lobes when extended. Viewing foraging behavior over shell substrate it appears unlikely that rays would use cephalic lobes for excavating prey risking damage to sensory receptors.

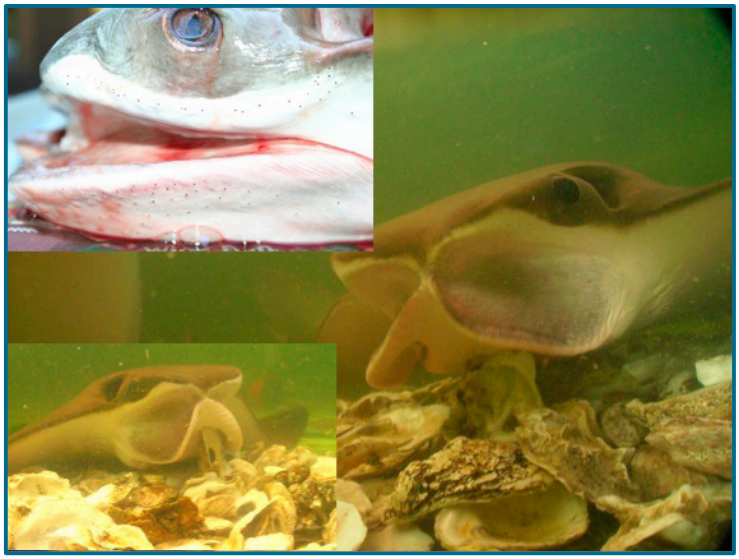


Figure 23. Cownose ray foraging over shell bed seeded with hard clams and blue mussels. Rays use cephalic lobes to aid in prey location and capture by light sorting of shell but not for excavation, possibly to avoid damage to sensory pores which cover lobes (inset).

Discussion

Observations of cownose rays feeding throughout this study showed that bivalves were viewed as a general food source and initial selection of potential prey was not based on a prey size. Cownose rays would indiscriminately suck shellfish toward their mouth, and if the shellfish fit between the ray's jaws and adequate crushing force was applied, the shellfish was eaten. If the prey was too large to fit between the crushing plates, it was discarded, and escaped predation, at least initially.

Shellfish mortality caused by cownose ray predation of particular SH and SD supports the idea that cownose ray jaw morphology has a quantitative gape limitation related to prey size. In general, adult cownose rays in this study were unable to consume bivalves above 31-32mm SD regardless of SH and YOY rays were not able to consume those above 15-16 mm SD.

Data suggest that rays select oysters of intermediate SH or SD. During comingled trials, three SH groups (30-40, 45-55, 60-70mm) had the highest probability of being eaten by adult rays while predation probability on smaller and larger oysters was significantly lower. Adult rays appeared unable to detect shorter (15-25mm SH) oysters and ingestion of those sizes was a result of collateral feeding only on smaller oysters in close proximity to larger target oysters. The tallest oysters (>75mm SH) were eaten in fewer numbers because they were too big (SH and SD) to be easily manipulated and required more handling time to consume than oysters of smaller SH and typically shallower SD. Thus, mid-sized oysters (30-70mm SH) fit more easily between the rays' jaws, resulting in higher predation. Given longer time to forage; however, successful predation on larger oysters increased. However, predation

rates of the largest two size classes remained lower than the three intermediate size classes regardless of time allowed, further indicating physical constraints, such as jaw gape, limited predation success.

YOY rays at age 1.5 months demonstrated predation success on seed oysters, illustrating the durophagous feeding potential and trophic level positioning of cownose ray at an early life stage. In comingled trials with YOY rays, the smallest oysters (10-30 mm SH) were most susceptible to predation. YOY rays attempted to feed on the largest oysters offered (30-40 mm SH, 15-19 mm SD), but were unsuccessful due to gape limitations.

The logistic regression model was used to determine the effect of SH and SD on predation. Although direct application of this model might not reflect predation in a natural setting with unlimited time, the model does support the generalization that adult cownose rays do not primarily prey upon very small or very large, deeper bivalves.

At nearly all SD, there was a direct relationship between trial duration and mortality for *C. virginica*. Given more time, rays would continue to manipulate larger oysters which had been attempted earlier in the trial by one or more rays without success. This aggregate crushing effect, combined with increases in feeding time, contributed to the higher amount of predation.

Regardless of time, greatest predation success in comingled trials were on oysters 30-70mm SH and 14-20mm SD. This suggests the rays actively selected oysters of this size range because they are within ray gape limitations. The Manly-Chesson Index further supports the preference for the aforementioned oysters SH. These preferences may be further explained by force requirements. The force required to crush bivalves was positively correlated with SD and scales isometrically. The rise in force needed to crush a bivalve at increased SD along with jaw gape and bite force limits may work in concert to lower ontogenetically the susceptibility of bivalves to predation.

Comparing results from comingled versus single size trials, slightly higher rates of predation were observed in single size trials except for the 75-85 mm SH oysters. The difference in the rate of predation may be due to the greater time required to sort through oysters of various sizes, including large oysters that cannot be successfully preyed upon at first attempt (75-85mm).

However, the differences in predation rates between trial types may be explained by passive foraging. Adult rays were observed manipulating and preying upon shellfish as they were encountered, regardless of the proximity of more susceptible prey. This passive foraging on oysters was also observed in YOY rays that indiscriminately initiated prey manipulation on the first oyster encountered regardless of oyster size.

Oyster predation rate in comingled trials declined for each SH category as time increased (Figure 8A). Rays initially depleted more susceptible prey resulting in fewer available prey as time progressed. Less available prey, a larger proportion of prey approaching or exceeding the gape or bite force limitations and satiation resulted in decreasing rates of predation over time.

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Section 6: Reproductive Anomalies

Over the course of this study, several reproductive anomalies were observed, including cases of multiple birth/embryos in both captive and live rays, as well as evidence of right uterus functionality, and albino embryos. This section documents and describes these cases.

Multiple Births/Embryos

Cownose rays have been reported to have only one embryo produced per reproductive cycle. However, a total of 8 episodes of multiple births in cownose rays were documented in this study. Two sets of twin live births from captive rays, and 6 separate in uterine multiple embryos discovered during necropsy of fishery dependent and independent acquired samples were observed. Maternal confirmation of live birth twins was performed by direct sequencing a portion of the mitochondrial DNA from the newborn pups and putative mothers of captive rays.

Captive Live Rays: Multiple Births

Cownose rays were caught in the Back River, Poquoson, VA using a modified Dutch seine June 24, 2008. The seine was towed for 30 minutes and a total of 26 rays were caught, 16 females and 10 males. From these, seven adult cownose rays (four males, three females) were transported live to a holding tank at the Virginia Institute of Marine Science (VIMS), College of William and Mary, for subsequent captive ray behavioral and predation studies. Three females were chosen from the six females that were notably pregnant with near-term embryos (abdominal bulging in ventral area associated with left uterus) to determine whether successful parturition would occur under captive conditions. Four males were chosen randomly. The remaining 19 rays were processed, and it was determined that all, except one female, was as sexually mature Seven females were determined to have recently pupped, and all of these postpartum females had recently mated, as evidenced by seminal fluids widely distributed within each uterus and around cloacal area.

Upon transfer to holding the tank, rays were tagged (spaghetti tags) in order to identify individuals for concurrent studies. Rays were held in a partial recirculating 4.3m x 6.4m holding tank system and maintained on oysters and hard clams and freshly dismembered blue crab. On the morning of July 8, 2008 two pups were observed free swimming in holding tank, with births occurring during the previous night. The next morning (July 9) three more pups were observed swimming in holding tank (total of five pups), with birthing again occurring during the night. Pups were sexed, weighed, and disc width (DW) measured same day of birth. Because multiple birthing to one or more captive females was obvious (five pups from three females), maternity testing was initiated. Pups were tagged with small T-tags and fin clips from were taken from posterior edge of right pectoral fin of adult females and pups and stored in 95% ethanol for mitochondrial DNA analysis.

Total genomic DNA was extracted from each tissue sample using a Qiagen DNeasy® Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. To assess the maternity of the captive pups, mitochondrial DNA sequences from the NADH dehydrogenase 4 (ND2), Cytochrome oxidase 1 (COI), ctyochrome b (Cytb) and 12S ribosomal RNA gene regions

Adult Female			Neonates			
Tag #	Weight (kg)	Disc Width (cm)	Tag #	Sex	Weight (g)	Disc Width (cm)
93	13.5	98	001	F	628	33.5
95	17.0	98.5	002	F	805	26.25
96	19.6	98	003	M	460	30.5
			004	F	807	35.75
			005	M	1560	43.75

Table 1. Adult female and newborn (neonate) cownose rays which resulted in multiple births in captivity.

were obtained for each individual via PCR amplification (Table 1). PCR reactions were performed using the Taq PCR Core Kit (Qiagen, Valencia, CA). Reactions included 10 – 20ng gDNA, 1 µM of each primer, 200 µM each dNTP, 0.025 units Taq polymerase and 1X Taq buffer with 1.5mM MgCl2 in a 25µl reaciton. PCR conditions consisted of an initial denaturation of 5min at 94° C, followed by 35 cycles of 1min at 94°C, 1min at 52-58°C, 1min at 72°C, and a final extension step for 7min at 72°C. Amplification products were cleaned with the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and the concentration was measured with a BioMateTM 3 series UV Sspectrophotometer (Thermo Spectronic, Madison, WI). Both the forward and reverse strands were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Sequencing reactions were electrophoresed on an ABI Prism 3130xI genetic analyzer (Applied Biosystems, Warrington, UK). The resulting chromatographic curves were analyzed using the Sequencing Analysis v. 5.2 software (Applied Biosystems, Warrington, UK) and exported for further analysis. Standard chromatogram format (SCF) curves from forward and reverse reactions were used to create a consensus sequence for each individual at each using Sequencher 4.10.1 (Gene Codes Corp., Ann Arbor, MI). All individual sequences were aligned in Macvector version 8.1.2 (MacVector, Inc., California, USA) using the ClustalW multiple alignment algorithm (Thompson et al. 1994) and pups were assigned to potential mothers based on DNA alignments.

Of the 15 sexually mature female rays collected during the sampling haul, two had multiple births in captivity. Five pups were born to three females, giving rise to birthing possibilities of two sets of twins and one single pup, or one set of triplets and two single pups. Newborn pups ranged in size from 30.5-43.75cm DW and weighed from 460 to 1560g at birth (Table 1).

All four mitochondrial gene regions (ND2, COI Cytb and 12S ribosomal RNA) were successfully amplified and sequences were obtained from three of the four regions. Overall 1865 bp were sequenced across the three loci that successfully amplified. There was no variation across an 850 bp region of the ND4 locus; all samples were identical. Amplification of a 575 bp region of the COI region resulted in alignment with two variable positions, both of which were conservative third position transitions. These two variable positions resulted in 2 haplotypes. One adult female, Rb7995 shared a haplotype with pups Rb1 and Rb4, while the other two females, Rb7993 and Rb7996 shared a haplotype with each other and with the pups Rb2, Rb3, and Rb5. Amplification of a portion of the Cytb region resulted in a 440 bp alignment. There were two variable positions in the alignment, both of which were transitions. These two variable positions resulted in 2 haplotypes. As with COI, one adult female, Rb7995 shared a haplotype

with pups Rb1 and Rb4, while the other two females, Rb7993 and Rb7996 shared a haplotype with each other and with the pups Rb2, Rb3, and Rb5.

Analysis of three pregnant cownose rays and five resulting pups based on the mitochondrial COI and Cytb regions demonstrated that female Rb7995 gave birth to two of the pups (001 and 004). The remaining two females had identical haplotypes based on both variable markers, thus the remaining three pups were identical to both female Rb7993 and female Rb7996. However, results indicate that either female Rb7993 or Rb7996 gave birth to at least two pups. The low level of variation based on mtDNA analysis is typical of elasmobranchs and has been attributed to the demographic characteristics of sharks (Heist, 1999).

Field Sampled Rays: multiple embryos

To acquire fishery independent samples for ray trophic ecology research objectives, long-line sampling was employed. Rays were fished at three different sites, each adjacent to commercial oyster grow-out areas. Lines 100 meters in length with 30 hooks per line baited with either menhaden or peeler crabs were fished three times per day targeting cownose rays. Upon capture, rays were iced, boxed and delivered to the (VIMS) for necropsy.

The first cownose ray multiple embryos observed in field sampling was a single set of twins from one of 10 female rays randomly sub-sampled from total of 156 females landed by haul seine fishermen on May 26, 2009. In subsequent sampling, seventy-three percent of 492 mature females sampled from May-July 2009 nearing parturition were found with a single pup in the left uterus, 25% had already delivered, and 2.3% (5 individuals) were found with two embryos in the left uterus. A single set of twins were found in three different haul seine samples: one set of twins in 60 adult females (1.6%); one set of twins in 77 adult females (1.3%); and one set of twins in 42 adult females (2.4%). Two sets of twins were found in a long line sample of 16 adult females (12.5%). Typically one embryo was larger in size; however, disparity in embryo size and weight within multiple embryo siblings was observed to decrease as gestation period reached term (see Section 3).

Sex ratio of twins was 1:1 in five of six sets with sex of largest sibling not consistent.

Date	Adult Female		Twin Embryos		
	Weight. (kg)	DW (cm)	Sex	Weight. (g)	DW (cm)
May 26, 2009 (not at term)	19.55	103	F	249.5	24.7
			M	340.2	27.5
May 28, 2009 (not at term)	17.01	98	F	476.3	30.1
			M	272.2	25.5
May 29, 2009 (not at term)	19.64	98	F	453.6	31.0
			M	294.8	26.0
June 23, 2009 (at term)	18.55	98	F	1,043.3	40.0
			M	839.2	39.0
July 2, 2009 (at term)	16.47	96.5	M	771.1	36.0
			M	907.2	38.0
July 2, 2009 (at term)	19.01	102	F	1,542.2	43.5
			M	1,451.5	43.5

Table 2. Adult female cownose rays and corresponding multiple embryos (twins) sampled from May and June 2009 as gestation of embryos reach term.

All embryos were cranial-caudally positioned within the left uterus and oriented back-to-back, dorsal surfaces contacting each other.

In three sets of twins, embryo heads faced cranially relative to mother, typical tail-first orientation of single embryo parturition, but slightly off-set with pectoral fins embracing the other (Figure 1). The other three sets of twins had embryos with heads facing in opposite directions of each other, one cranially, one caudally, and wings of one folded between the two (Figure 2). One of these three sets had ventrally positioned embryo with head facing caudally, the other two set with dorsally positioned embryo head facing caudally.

Cownose rays are ovoviviparous, a reproductive strategy that broods the young to a comparatively large size before birth, thus increasing survivability of neonates. As noted in twins from May samples, which are nearing completion of gestation but are still not at-term, difference in embryo size is apparent, with one larger than the other. Size of embryos at birth could impact survivorship, with smaller pups more vulnerable to stress and predation. However, as gestation nears completion (late June, early July), size parity between embryos was observed, though in each case reported here of at-term embryos, except in the July 2, 2009 longline sample, size at birth was still below calculated mean size of single embryos (1,270g and 42.57cm DW) for cownose rays in the Chesapeake Bay (concurrent study).

In multiple birth live rays, each pup experienced a decrease in weight over the first 9 days of life, with an average 6.4% (sd 1.6) loss from birth weight though suitable food was available (cut oyster, clam, crab, and shrimps meat) daily on the bottom of holding tank. Newborn rays were observed to struggle with diving to any depth for the first several days, mostly remaining on the water surface with head bobbing in and out of water and traveling in a random fashion. After several days, all newborn rays were swimming as adults and starting to investigate the tank bottom; however, consumption of food was not observed until day 6 when small pieces of cut clam were consumed by 3 of the 5 rays. All rays were observed to feed by day 9. Weight loss experienced during this period may have been influenced by captivity, however, observations of ray behavior during the first week of life suggest that newborn rays may not actively feed on their own for a period of time post-partum and loss of weight from birth is likely.

This is the first report of multiple pups from a single female cownose ray, *Rhinoptera*

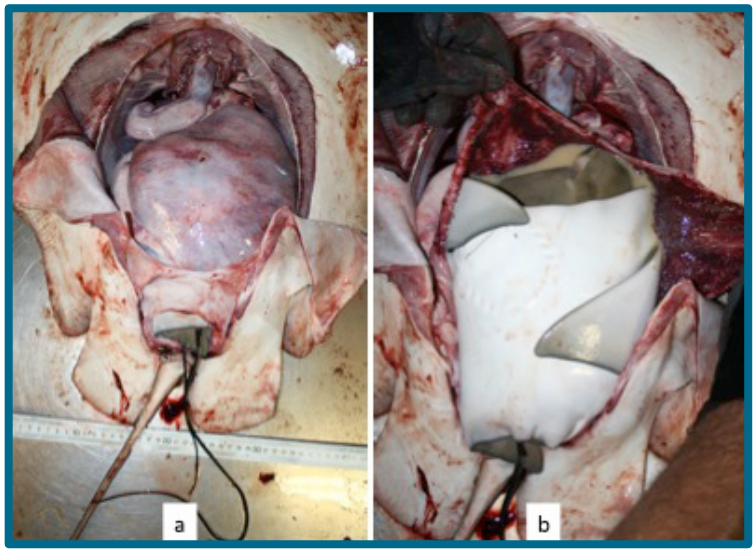

Figure 1. Twin embryo cownose rays; (a) in uterus with tails extending out of female; (b) twins positioned in uterus with heads facing same direction (cranially). Note histotroph in uterus near embryo heads.

Figure 2. Twin embryo cownose ray embryos positioned back-to-back in uterus with heads facing in opposite directions and pectoral fins folded between.

bonasus. Hundreds of necropsies previously performed by lead author on female cownose rays prior to the multiple births documented here from July 2008 identified a single embryo developing within the left uteri, providing significant testimony of fecundity being 1 young per female per year as reported by all others researching this species. Subsequent occurrences of multiple embryos observed (6 additional to date) within limited sampling during summer 2009 questions the extent of multiple births within this specie. Further, rays are routinely observed to abort embryos, especially near-term embryos, just upon death, and embryos readily "slip" from rays uteri during subsequent handling postmortem. These phenomena may prevent higher numbers of multiple embryos from being observed; however, removal of at-term embryos during necropsy throughout this study has provided no indication that additional embryos were present and had slipped (extension of uterus, positioning and orientation of embryo). Though multiple embryos and births have been documented in this report, the occurrence of multiple births in cownose rays in the Chesapeake Bay is considered low.

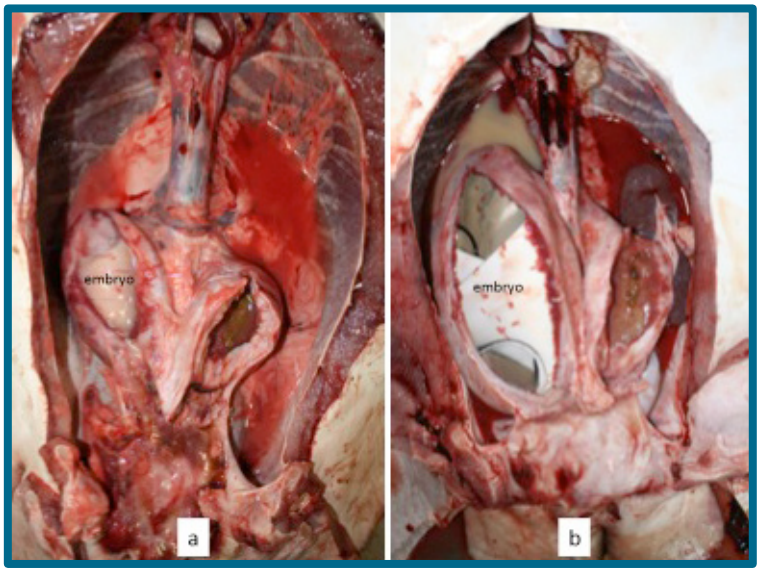

Figure 3. Ventral views of female cownose rays with embryo development in the right uterus; (a) early gestation (Sept. 1, 2010); (b) late gestation (May 26, 2010).

Embryo Development in the Right Uterus

This is the first report documenting functionality of the right uterus in cownose ray with an early and late term embryo removed from the right uterus of a 98.5 and 98cm DW females, respectively (Figure 3). Though functionality of right uterus was observed, functionality of the right ovary was not observed (Figure 4). In both cases, the left ovary remained functional (developing ova present) and the right ovary remained

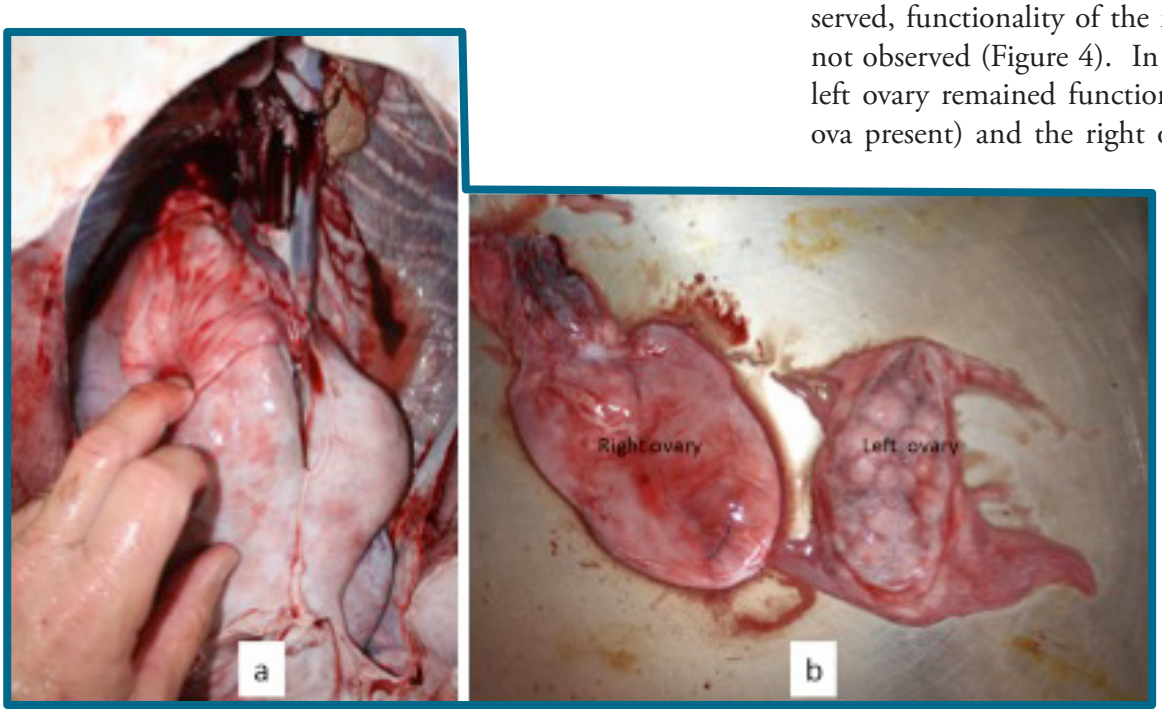

Figure4. Ventral view of female cownose ray with late embryo development in the right uterus; (a) nonfunctional right ovary/epigonal gland still attached; (b) left and right ovaries removed from same ray positioned to reference as removed. Left ovary showing developing ova.

non-functional, showing no follicular differentiation. Mode of ova transport from left ovary to ostium of right oviduct is puzzling noting the anatomical positioning of ovaries in cownose rays and physical obstructions within the peritoneal cavity between the two oviducts. Further, the non-functional left uterus in both cases appears anatomically to have gestated in prior breeding cycles: wide width, thick walled, with developed trophenemata (Figure 5). The presence of a "ribbon" (see Section 2) found in the non-functional uterus of adult rays remains consistent, with the "ribbon" occupying the left uterus in these cases.

Albino embryo

A male albinistic three-quarter term cownose ray embryo (DW, 158.7g) was removed from a normal pigmented female (94cm DW, 16.1kg) on May 28, 2009. The embryo was void of pigmentation with the exception of some darkening of tail and possessed developmental abnormalities (Figures 6).

The embryo was without eyes and mouth, had deformed cephalic lobes (bulbous instead of stream-lined), dwarfed left pectoral (shorter and narrower than right pectoral), spiracles not proportional in size and more medially positioned than normal, and a tail severely coiled. Pelvic fins, claspers, spine, and gill openings all appeared normal. A DNA sample was taken via fin clip and embryo preserved for later necropsy to evaluate internal anatomy.

Albinism or partial albinism within chondrichthyian fishes is based on integumentary and retinal melanin produced pigmentation, with albinism defined as those animals devoid of both derived pigmentations (Clark, 2002), and partial albinism, or leucism, originating from both. Schwartz (1959) reported a white cownose ray but albinism or leucism was not determined, but was later determined to be leucism by Clark (2002). Since the eyes of the cownose ray embryo reported in this current report were not present to express pigmentation, definitive classification of albinism has not be determined.

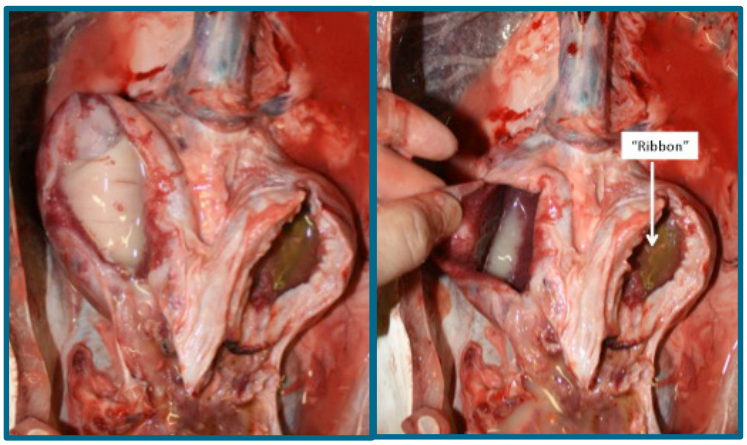

Figure 5. Ventral view of female cownose rays with early embryo development in the right uterus. Note size and wall thickness of left uterus and pressence of a ribbon.

Figure 6. Albinism in cownose ray embryo; (a) dorsal view; (b) ventral view.

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