Progress Report

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Project Title: Effects of changing population density on blue crab Reproduction

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The blue crab, *Callinectes sapidus*, supports a major commercial fishery in the Chesapeake Bay that has been subjected to intense fishing pressure. Mortality increased in this population in part due to overfishing (Miller & Houde 1998), which led to a decline in the number and biomass of the spawning stock (Lipcius & Stockhausen 2002). Annual fecundity of the blue crab population is dependent upon the number of females, size and age at maturity, and the number of eggs produced by individual females during the spawning season (Prager et al. 1990, Stearns, 1992). Changes in any of these parameters can significantly affect the reproductive output of the population. From the early 1990's through 2005, the average carapace width of males and females significantly declined (VIMS Trawl Survey). This suggests that females were maturing at smaller sizes (60-120 mm), and likely younger ages, than in years when abundance was greater. This had an important impact on the energy allocated to reproduction, effectively reducing the number of eggs produced (Wells 2009).

Preferential fishing pressure on large males also resulted in reduced male size. Smaller males transfer fewer sperm (Jivoff 1997a, 1997b) which may further impact the fecundity of the Chesapeake Bay population. Using microsatellite DNA analysis of sperm stored in the female spermathecae it has been determined that greater than 80% of females have mated with more than one male (Wells and McConaugha 2011). Whether this is in response to insufficient sperm deposition by single males or for other genetic reasons remains unclear at this time. To understand the genetic importance of multiple mating we need to understand how sperm from multiple males is utilized in female reproduction. Is there multiple paternity within a brood or are sperm from multiple males used in sequence? These questions can have an important impact on the population genetics of the Bay blue crab. In an exploited species with only one or two year classes in the spawning stock, modern management efforts must not only address abundance and spawning stock biomass, but must work to optimize reproductive output which may not be directly related to spawning biomass.

Our recent work on blue crab egg production suggests that population density strongly affects reproductive output in the blue crab. Data for egg number and size from 2002 through 2007 demonstrated that a previously determined allometric relationship between mature female carapace width and fecundity was no longer the controlling factor in determining egg production in this population (Prager et al. 1990; Wells 2009; Wells and McConaugha submitted). While there remains an allometrically determined upper limit on egg production, based on internal body space available for egg storage (Hines 1982, Prager et al. 1990), egg production during the study period was significantly below that upper limit. This suggested that egg production was controlled by other factors such as energy resources, sperm limitation or other stressors. Under

high fishing pressure (2002-2003) females produce fewer larger eggs resulting in egg number being well below the allometric expectation. As population density began to increase towards the end of the study (2006-2007) there was a significant increase in the number of eggs and energy allocated to egg production (Table 1; Prager et al. 1990; Wells and McConaugha submitted) supporting the concept that population abundance affects reproductive output of the Chesapeake Bay population of blue crabs.

In the presence of environmental stress or a limiting factor, females are more likely to allocate more maternal resources into individual eggs while maintaining the energy of the brood constant (Calado et al. 2005). This leads to fewer, larger eggs enhancing the likelihood that an individual larva will survive (Pernet et al. 2003; Calado et al. 2005). As conditions improve, greater numbers of smaller eggs are produced. Changes in maternal investment may be an indicator of resource availability or a sign of stress (Stearns 1992, Xu et al. 1993, Trippel 1995, Marteinsdottir & Begg 2002). Trade-offs between egg size and number have been reported for the Bay blue crab population during the initial study period (2002-2007) (Wells 2009). During a period of high fishing pressure and low abundance (2002-2003) females allocated high levels of energy to a smaller number of large eggs. Data reported for egg size and number for the Bay blue crab demonstrated that these trade-offs were evident both inter- and intra-annually (Tables 1 and 2, Wells 2009;). At times when resources are plentiful or stress is reduced, there should be no limitation on egg size or number beyond the allometric constraints of internal storage. The trade-offs between egg number and size was less evident in the Chesapeake Bay population at the close of the prior study when fishing pressure had been reduced. Egg size and egg number were increasing indicating that prior stressors, such as harvest pressure, had diminished resulting in greater egg production (Wells 2009) and a more optimal reproductive output for the population. More extensive fishing regulations were imposed during 2008-2009 that greatly reduced harvest pressure on the Bay crab population. Population abundance increased rapidly (Chesapeake Bay winter dredge survey). Results for egg number and size in 2010 suggest that both parameters have continued to increase (McConaugha and Wells, NOAA interim report 2010). Continued monitoring of the reproductive output of the population is needed because as population numbers reach target levels for three or more years, some of the new harvest regulations will begin to be phased out.

Over the last two decades there has been much discussion in the literature about the role of predation as a natural selection force in the evolution of exploited species (Stearns and Koella 1986; Law and Grey 1989; Reznick et al. 1990; Reznick et al. 1997; Law 2000 for a review; Witting 2002; Conover and Munch 2002; Olsen et al. 2004; Muthiga et al. 2009). For commercially exploited stocks where historically, harvests often exceed 50% of the annual stock recruits, such as the blue crab, fishing is a major source of mortality. Harvest mortality and harvest size limits could be expected to induce phenotypic adaptive responses, especially in size and age at maturation, fecundity per brood and lifetime fecundity (Stearns and Koella 1986; Ford and Seigal 1994; Abrams and Rowe 1996; Law 2000). High mortality drives these parameters, in both observational and modeling studies, towards early maturation at smaller size (Law and Grey 1989; Witting 2002; Roy et al. 2003). In species where fecundity increases with the size of females (i.e. increased body weight or length) these changes result in reduced per capita fecundity. Changes in size at maturity, egg number and size have been documented for the Bay

blue crab population (Wells, 2009). In addition, multiple mating has been observed in greater than 98% of the current population. This may be another sign of changing reproductive parameters in response to fishing pressure.

High fishing mortality and minimum size limits can provide strong selective forces that can result in evolutionary changes. Recent data for fisheries where harvest pressure has been greatly reduced or a sanctuary has been developed, shows that females recover quickly to the average sizes and reproductive output observed prior to overexploitation (Halpern and Warner 2002; Hawkins and Roberts 2004; Floeter et al. 2006). Regulations have been imposed on the Chesapeake Bay blue crab fishery in the last few years that have significantly reduced the harvest pressure on the population. It is likely that some of the recovery in female size and egg production observed can be attributed to the increasing population density associated with these regulations.

Because commercial fishing is not a controlled experiment, it is impossible to determine how and to what extent environmental factors affect phenotypic plastic changes in life history parameters including age and size at maturity. Environmental factors such as temperature and availability of food can have a major impact on life history parameters (Abrams and Rowe 1996; Ernande et al. 2004). Separating the impact of fishing mortality and environmental changes on life history parameters is extremely important if we want to manage fisheries for future generations. Numerical analysis has been the driving force for current fisheries management because of the pressure to manage short-term fluctuations (inter-annual to decadal time scales). However to manage for the long term, ecology and evolution need to become the driving forces for future management plans (Hutchings 2002).

Overall Objective

The overall objective of this study is to examine the effects of harvest pressure on blue crab reproductive effort. Previous data for this population showed that egg production and quality were lower at times of increased harvest pressure and decreased abundance (Wells 2009; Wells and McConaugha submitted and in prep). Size-specific harvesting of males in the population has resulted in reduced male size, and reduced reproductive output (Abbe 2002; Kendall et al 2002; Jivoff 2003). Females seem to have compensated for this by mating with multiple males, which can alleviate sperm limitation and increases the potential for genetic diversity (Wells and McConaugha 2011). In the last few years harvest pressure on the population has decreased and abundance has increased (Chesapeake Bay winter dredge survey). In 2006 and 2007, when the increase in abundance began, there was greater maternal reproductive investment with increased egg number, size and energy content per brood. Data from samples collected during the 2010 spawning season suggests that egg production has continued to increase with population abundance (McConaugha and Wells, NOAA Interim Report 2010). Microsatellite DNA analysis has shown that females have mated with multiple males in the last two seasons, but if the sperm from multiple mates is being utilized within single broods is unknown (Wells and McConaugha 2011). Determining if multiple paternity is occurring in conjunction with multiple mating will provide greater insight into the genetic diversity of this exploited population. It was hypothesized that collections from the 2011 spawning season would show that mature female size; number of eggs produced, and means egg

diameter had further increased compared to previous data from a time of more intense harvest pressure. It was also hypothesized that despite improved female allocation to reproduction, seasonal differences in lipid, protein, and lipid fraction concentrations would be detected. It is also hypothesized that females will fertilize eggs within an individual brood with the sperm from multiple males, if they have multiply mated.

Specific Objectives

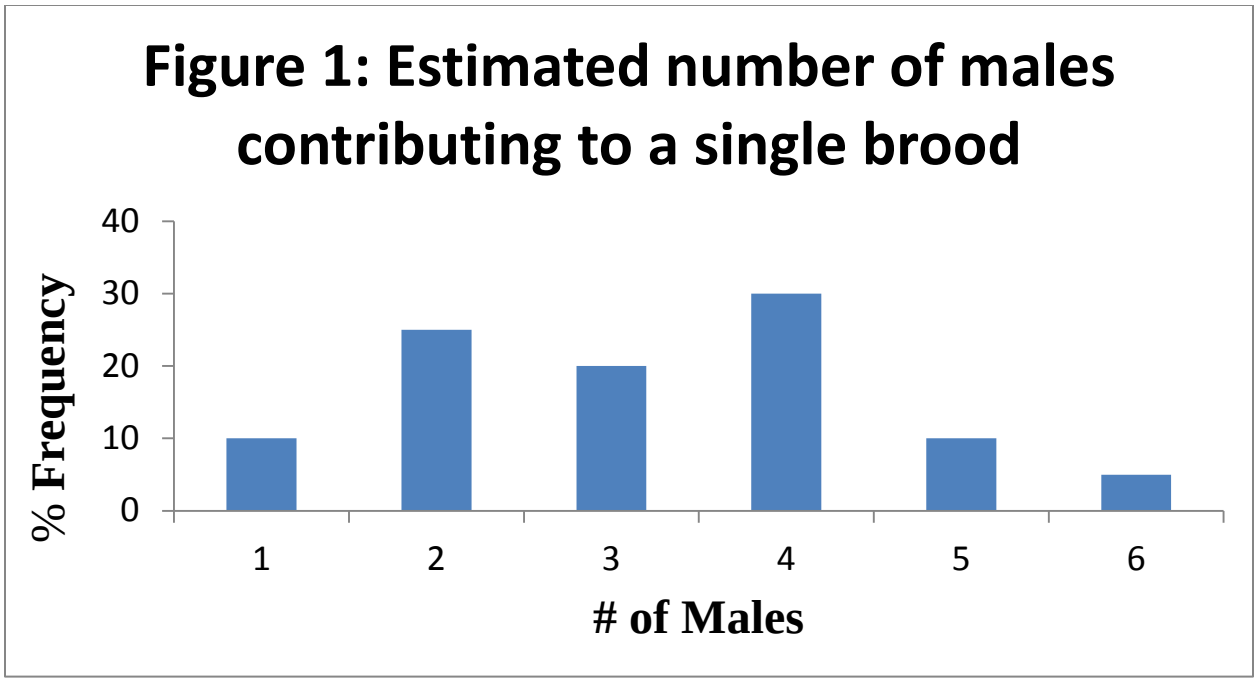
1. Determine changes in female size, fecundity and egg size throughout the 2011 spawning season.
2. Determine changes in bulk lipids, proteins and lipid fractions in eggs throughout the 2011 spawning season.
3. Determine if females are utilizing sperm from multiple male donors within a single brood.
4. As part of the no cost extension for this project we add the collection and analysis of female size, fecundity and egg size for 2012.

Results

Sampling for this project began in June 2011, at the beginning of the spawning season, in anticipation of receiving funds from the NOAA Chesapeake Bay Office. All of the samples necessary for fulfilling the objectives of this project were collected through the end of spawning in September, 2011. As part of the grant extension samples for 2012 were collected from May to the end of September, 2012. All samples for the microsatellite DNA analysis were collected during the spawning season in 2010.

Genetics

Microsatellite data demonstrated that the majority of females in this study mated with multiple males, and utilized the sperm from multiple males within a single brood. Only 10% (n = 2) of the females sampled had utilized sperm from only one male (Figure 1), with the other 90% (n=18) having two or more males accounted for. The highest percentage detected was four males within a single brood at 30% (n = 6). A small portion of the sampled population (5%, n = 1) had six sperm donors, which was two more male donors than was previously detected using stored sperm in the spermathecae (Wells et al, in revision). It is likely that there were more males contributing to each brood, but the assumption of heterozygosity requires conservancy in the estimate.



Fecundity, egg size and lipid content

The mean number of eggs produced per brood for 2011 was 7.9 x 10^5, and the mean carapace width was 141.7 mm. The mean egg diameter for the 2011 spawning season was 254.35 µm. The carapace width of mature females has increased since 2002, which would be expected of a population with reduced harvest pressure. The mean egg number produced per brood dropped precipitously from the highest values observed in 2010. The mean egg diameter for 2011 s also decreased from that observed in 2010 (Table 1). This suggests that the increases in reproductive output and female investment seen in blue crab population during the last 10 years, declined during the spawning season of 2011. Data from 2012 indicated that the size of females increased over that of 2011 but egg size decline significantly. The average number of eggs per brood in 2012 was 18.9 x 10^5 and the mean carapace width was 146.3 mm. The number of eggs per brood was a significant increase from the values observed in 2011. The number of eggs per brood was the highest in the 11 year history of observations and similar to the values from 2010. While the number of eggs/brood was the highest recorded, egg size was the smallest. The rapid increase in egg number but small egg size was similar to 2004. 2004 was the last time a near doubling of egg number over the previous year was recorded.

The intra-annual reproductive output data for 2011 (Table 2) demonstrates that the obvious trade-offs between average egg size and the number produced per brood observed in previous years did not hold in 2011. The mean number of eggs produced per brood was highest in June, which was also when the mean egg diameter was largest. In 2012 there were mixed results in the intra-annual reproductive output. Fecundity and egg size was up in June over the May samples. However higher egg numbers were seen in July and August but egg size was significantly reduced compared with the June numbers (Table 3).

Table 1: Mean carapace width, mean egg number per brood and mean egg diameter for 2002-2007 (Wells and McConaugha, submitted), 2010, 2011and 2012.

Year	Mean Carapace Width (mm)	SE	Mean Number of Eggs ($x10^5$)	SE ($x10^5$)	Mean Egg Diameter (μm)	SE
2002	**131.3**	**0.27**	**3.7**	**0.25**	**252.00**	**1.55**
2003	**134.1**	**0.14**	**4.3**	**0.19**	**224.84**	**1.58**
2004	**128.6**	**0.10**	**9.6**	**0.28**	**204.95**	**0.63**
2005	**133.8**	**0.07**	**9.1**	**0.24**	**205.63**	**1.10**
2006	**137.5**	**0.11**	**13.1**	**0.65**	**229.75**	**1.55**
2007	**136.0**	**0.12**	**11.1**	**1.07**	**258.71**	**1.42**
2010	**143.2**	**0.06**	**15.2**	**0.8**	**257.37**	**0.06**
2011	**141.7**	**0.05**	**7.9**	**0.01**	**254.35**	**0.05**
2012	**146.3**	**0.73**	**16.0**	**0.74**	**203.46**	**8.8**

Table 2: Intra-annual differences in reproductive output for 2011.

Month	Mean Number of Eggs ($x10^5$)	SE ($x10^5$)	Mean Egg Diameter (μm)	SE
June	**9.8**	**0.04**	**260.36**	**0.17**
July	**8.0**	**0.05**	**251.08**	**0.15**
August	**6.6**	**0.04**	**248.86**	**0.14**
September	**5.1**	**0.05**	**256.83**	**0.26**

Table 3 Intra-annual differences in reproductive output for 2012

Month	Number of Eggs ($x10^5$)	SE ($x10^5$)	Egg Diameter (µm)	SE
May	**8.6**	**1.1**	**204.12**	**3.4**
June	**19.3**	**1.6**	**208.07**	**1.43**
July	**24.4**	**1.6**	**201.23**	**0.99**
August	**20.4**	**1.2**	**202.54**	**0.91**
September	**11.4**	**0.56**	**201.47**	**0.92**

The average bulk lipid concentration for the 2011 spawning season was 8.38 µg/egg. This was higher than the average for the 2010 spawning season. The trend in lipid concentrations throughout the 2011 spawning season was similar to previous years, but the absolute concentrations were higher (Table 4). Lipid concentrations are normally higher in the second month of spawning than in the first. This was observed in the 2011 data with the highest concentrations detected in July followed by a decrease in August.

Table 4: Intra-annual differences in lipid concentrations of eggs for the 2011 reproductive season.

Month	Lipid Concentration per egg (µg)	SE
June	7.13	0.29
July	12.80	0.67
August	8.04	0.40
September	3.39	0.18

Discussion:

Previous microsatellite data demonstrated that females mated with multiple males based upon the stored sperm in the spermathecae (Wells et al, submitted). Data from this study has shown that the majority of females sampled utilize sperm from multiple males based upon the number of male microsatellites detected within a single brood. This indicates that females are continuing to mate with multiple partners which reinforces that there has been a shift in reproductive behaviors previously observed (Wells et al, submitted). Multiple mating by females

within the Chesapeake Bay population suggests that mate guarding by males is occurring on a very limited basis, which is quite different from the historically accepted reproductive norm.

There is also the possibility for increased genetic variation within the population due to the increase in the number of mates. Many females are harvested within the Chesapeake Bay before they have the chance to fulfill their lifetime reproductive potential. Usage of the sperm from multiple males within a single brood suggests that there is mixing of sperm in the spermathecae, and that females do not have the ability to preferentially utilize sperm from a mate. Utilization of sperm from multiple males will likely increase both the effective population size and the genetic variance within the population, which can improve the sustainability of the population in general (Sugg and Chesser, 1994; Zane et. al., 1999; Martinez et. al., 2000). This can also potentially make the population more resilient to inbreeding depression, disease and other population-level catastrophes. At a time when there are many questions surrounding the state and sustainability of this particular population, changes in reproduction as evidenced by this study strongly suggests encouraging adaptations on the part of mature females.

Female size, fecundity and egg size have previously been associated with changes in blue crab reproduction and health of the population (Wells, 2009). During periods of low population abundance, female size, and egg number were significantly reduced while egg size was significantly larger (2002-2003). The next 2 years showed increase in egg number but reduced egg size. Data from 2007 and 2010 show female size close to historical highs, fecundity approaching previous highs and egg size the largest recorded suggesting a healthy reproductive output.

Crab abundance data from the winter dredge survey of 2011 suggested that there was a decrease in abundance, attributed to overwintering mortality (VIMS Winter Dredge Survey, VMRC). The mean reproductive output for 2011 was drastically reduced when compared to 2007-2010. Egg numbers were significantly lower than 2010 and 2007 while egg size was not significantly different. Fewer large eggs were being produced. This is similar to the years 2002-2005 when populations were recovering from the low levels of the early 2000's. Reproductive output in 2012 show increased female size and increased number of eggs per brood but highly significantly reduced egg size. This was similar to transitional years 2004-2005). In all years when fecundity data has been collected there have been intra-annual changes in egg production throughout the spawning season. The small egg size throughout the spawning season may have contributed to the reported low harvest in Virginia waters during the 2013 season.

The intra-annual reproductive output data for 2011 and 2012 (Table 2and 3) demonstrates that the obvious trade-offs between average egg size and the number produced per brood observed in previous years was no longer apparent. The mean number of eggs produced per brood was highest in June, 2011 which was also when the mean egg diameter was largest. Bulk lipid values in 2011 further support the concept that maximum reproductive output occurred in June 2011. This does support previous findings that greater reproductive effort was expended by females at the beginning of the spawning season (Wells 2009). As the average number of eggs produced per brood got smaller, so did the average egg size which is unusual for this population. In 2012 egg number and size were greatest in June as had been seen in previous years. However egg number significantly increased in July and August while egg size remain similar to that of June. This taken with the mean egg number and size from Table 1 suggests that the populations for 2011 and 2012 were under stress. Samples from 2013 have been collected and are being

analyzed as part of an undergraduate research program in my lab. The fecundity data available from 2002 through 2012 shows strong correlation with population data and represents an additional index for measuring the health of the Chesapeake Bay population of blue crab.

Our genetic results strongly support the concept of sperm limitation and possibly genetic diversity in the population due to multiple mating. The results of our fecundity work suggest that the Bay population continues under stress. The relaxation of the fishing regulations implemented in the early 2000's appears to have caused a regression in the population recovery.

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Appendix 1

METHODOLOGY

Egg Enumeration

Mature gravid females with orange sponges was collected weekly from a local crab processing house throughout the 2011 spawning season (May-September)... Collection from a local source allowed us to obtain a broad sample of females from across the spawning ground of the lower Bay. Female carapace width was measured to the nearest mm, and the abdomens removed, fixed in 10% buffered formalin and stored for later egg enumeration. Sub-samples of eggs from each sponge were frozen separately for paternity and biochemical analyses. Enumeration followed the dry-weight method outlined in Prager et al. (1990). Eggs were removed from the pleopods, and three subsamples of two hundred eggs counted from each sponge. The sponge and three replicate samples were dried to a constant weight at 60° C. The average of the three subsamples was used to calculate the total number of eggs in each sponge based upon the total dry weight of the sponge.

The mean egg diameter for each sponge was determined using an Olympus microscope equipped with an Optronics DEI-750 camera, and Image Pro imaging software. Images of ten randomly chosen eggs per sponge were digitized, providing sufficient data based on power analysis from preliminary data. The diameters of individual eggs were determined from the images using the linear measuring tool in the Image Pro software calibrated with an image of an ocular micrometer obtained at the same magnification used for the egg images.

The mean carapace width, mean egg number, and mean egg diameter for 2011 were used in comparisons with other years when these parameters were measured. These comparisons allowed us to determine if the trend toward larger female sizes, increased egg number per brood, and increased egg diameter continued. The data was also examined to determine if there were within season differences in egg production.

Biochemical Analysis

Eggs were analyzed for total lipids using the Bligh and Dyer (1959) method of extraction and gravimetric analysis. Analysis was performed in triplicate with 100 eggs. All samples were replicated three times to determine an average concentration. A chloroform, methanol, and water extraction (2:2:1) was used with sonication three times to allow for the complete lyses of cells and greatest amount of lipid recovery. At the end of the third round the chloroform layer was removed and placed into a pre-weighed test tube. The chloroform layer was evaporated under a steady stream of nitrogen and, once dry, the test tube was reweighed. The total amount of lipid in each sample was the difference in weight.

Protein analysis was done with a FLX800 fluorescent microplate reader equipped with a PC running KC Junior software, and the reactive chemical fluorescamine (Undenfriend et al., 1972; Lorenzen and Kennedy, 1993). Analysis was performed in triplicate with 100 eggs. Bovine serum albumin was used to generate a standard curve for each plate. Each sample was

sonicated for the greatest amount of protein recovery. The samples along with blanks, and standards were pipetted in 200 μL quantities onto a 96-well microplate and read with an excitation of 400 nm and an emission of 460 nm. Average protein concentration for each sample was calculated from the replicates.

Lipid fractions will be analyzed using separate quantification kits with appropriate standards for each fraction (Wako Chemicals USA, Inc.; Ohkubo and Matsubara, 2002). Triglyceride will be analyzed with the triglyceride quantification kit, phospholipid with the phospholipid C quantification kit, and cholesterol with the cholesterol E kit. The procedures provided with the kits have been modified for microplate analysis so that eggs can be analyzed in small quantities. All samples will be analyzed in triplicate to determine an average concentration. Samples will be extracted and analyzed with appropriate reagents from the kits. Each sample will be read on a Bio-Tek EL-800 spectrophotometric microplate reader at the appropriate wavelength for the lipid fraction. Concentrations for each lipid fraction will be determined from the standard curve generated by the program KC Junior that operates the microplate reader.

Concentrations of lipids, proteins, and lipid fractions will be analyzed inter- and intra-annually in conjunction with previously determined data from this blue crab population (Wells 2009, Wells and McConaugha in prep). This data will also be used to determine the average amount of energy allocated to each egg, which previously differed within and between spawning seasons indicating significant differences in reproductive investment.

Paternity Analysis

To determine the number of males contributing to paternity of a brood, eggs collected from twenty-five randomly chosen gravid females will be analyzed. Walking legs were also removed from each female sampled, so that the alleles at each loci for the mother can be determined. Based upon the prevalence of multiple mating detected previously (>95%), and the high heterozygosity of the loci chosen, twenty-five females will be a sufficient representative of the population for genetic analysis. Three sets of microsatellite loci primers (CSC-001, CSC-004, CSA-035) will be used for the detection of distinct PCR bands as previously identified by Steven et al. (2005) for blue crabs. Each of these loci was selected because all blue crab individuals (male, female, and offspring) previously evaluated were heterozygous for them, which makes them most useful for determining paternity (Bilodeau et al., 2005; Gosselin et al., 2005; Steven et al 2005). These same primers have been used in multiple mating analysis of sperm with success (Wells and McConaugha 2011). With only eggs available for paternity analysis (in-lieu of diploid males) male heterozygosity is then assumed as there is no way to be certain whether any putative sires might actually be homozygous. This results in a conservative estimate of actual paternity. Analysis of the eggs will be completed by PCR amplification of the microsatellites followed by genotyping. DNA will be extracted from the eggs and walking legs using the QIAGEN DNeasy kit then amplified via PCR (using the exact conditions of Steven et al., 2005; denature 2 minutes at 95° C, 30 cycles @ 1 minute at 95° C, 30 sec annealing, 70° C for 1 minute, and 30 minutes elongation at 70° C), and analyzed with an ABI 3700 automated sequencer. Genescan 3.1, and GenoTyper v2.1 software will be used on the raw data to determine the number of alleles from the potential fathers, while subtracting the maternal alleles

based upon the analysis of the walking legs. Broods will be scored as having multiple paternity if more than two different alleles on more than one loci are detected (Bilodeau et al., 2005; Gosselin et al. 2005). The number of males contributing to paternity will be determined by dividing the number of alleles that are different from the mother by two (Bilodeau et al., 2005).