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OBJECTIVES

Recent management changes in the Chesapeake Bay blue crab fishery have focused on increasing the proportion of females that reproduce during a first spawning season and survive to reproduce during a second spawning season (Miller et al. 2011). Survival of a significant number of females to a second spawning season has the potential to both increase total reproductive output of the spawning stock and increase population stability by buffering the impact of small year-classes (Miller et al. 2011). These benefits would not be realized if females are unable to fertilize broods in their second spawning season due to sperm limitation. The objectives of this study were to explore variation in sperm and seminal fluid quantity with respect to: 1) season, 2) location, 3) operational sex ratio (OSR), 4) relative time since mating, and 5) fertilization. This information was used to model the effects of observed variation in sperm stores on an individual female's lifetime brood production.

MATERIALS AND METHODS

Biological data

Mature female blue crabs were collected and frozen during a variety of targeted sampling efforts (described below) during the period 1996-2012 to explore variability in the quantity of sperm and seminal fluid transferred during mating and stored prior to spawning. Prior to dissection and processing, frozen crabs were placed in a bucket of cool, fresh water and allowed to thaw completely (approximately one hour). Carapace width (CW) was measured as the distance between lateral spine tips. Carapace condition was determined to be clean (C), partially dirty (PD), or dirty (D), and was used as a relative indicator of time since mating because mature female blue crabs will not mate again.

Mature females classified as C had clean carapaces with a bright white abdomen, indicating recent mating within the past several months. Crabs that were PD had some yellowing of the carapace and were assumed to be within their first year after mating. Crabs that were classified as D were brown in color with obvious darkening of the carapace and often had fouling organisms (barnacles, bryozoans, etc.) on the legs and carapace, and were assumed to be in their second year after mating.

Each crab was dissected by removing the abdomen and carapace. To determine whether spawning (and thus sperm use for fertilization) had occurred recently, the presence of external egg masses was noted and, if there was no visible egg mass, the pleopods were examined under a dissecting microscope for egg remnants. This method provided information on recent spawning but was likely not useful for determining whether females collected in May or June spawned the previous summer or fall because egg remnants could have been lost during the winter. Spermathecae were removed and weighed individually. The weight of spermathecae in recently-mated females was indicative of the quantity of seminal fluid transferred during mating (Kendall & Wolcott, 1999). Spermathecae were stored in 20 ml scintillation vials in 70% ethanol for sperm enumeration. We stored both spermathecae in the same vial for all crabs dissected prior to October 2010 and stored each spermatheca separately after that time (e.g. 2012 lower bay samples).

To remove sperm from spermathecae, the entire contents of a vial (storage ethanol plus all spermathecae stored in that vial) were poured into a petri dish. This ensured that loose spermatophores suspended in the storage ethanol were not lost. The spermathecal membranes were separated from the sperm plug (hardened seminal fluid plus spermatophores that was indicative of recent mating) or from the spermatophores if the seminal fluid had already been resorbed. The spermathecal membrane was rinsed thoroughly with 70% ethanol to ensure no spermatophores adhered to it and was discarded. Using a razor blade, the spermathecal contents were chopped into fine pieces (<1 mm). The spermathecal contents and liquid were then poured into a 16 mL glass PYREX© Dounce homogenizer. The sample was homogenized to break apart the spermatophores into individual sperm cells. This process took about 10 minutes per sample when no seminal fluid was present and at least 1.5 hours per sample when a hard sperm plug was present. After homogenization, the total volume of liquid plus spermathecal contents was recorded.

To enumerate the sperm, one drop of the sample was placed on a Petroff-Hausser Counting Chamber (with a 5x5 grid) and examined using a phase contrast microscope at 400x magnification. All 25 squares in the grid were counted and the number of sperm present was recorded. The total quantity of sperm per crab was calculated using the equation

Quantity of sperm =((Number of sperm cells counted)/(Volume of counting chamber (mL)))×Dilution (mL) (1)

where the volume of the counting chamber was 1x1x0.02 mm, or 0.00002 mL and

the dilution was the total volume of homogenized liquid and spermathecal contents. This process was repeated three times per sample and the three calculations were averaged to produce the final number of sperm per crab. Beginning in 2010, only one spermatheca was counted for an individual crab and the number of sperm was doubled to calculate the sperm quantity. The change from processing two to one spermathecae was made to minimize mechanical sample processing time in order to maximize sample sizes and because there is no theoretical or empirical reason for this sub-sampling to introduce bias (Duluc et al. 2005, Rodgers et al. 2011).

Seasonal variation and OSR

In 2010, blue crabs were collected from the Rhode River, Maryland, a sub-estuary of the middle Chesapeake Bay, in order to quantify seasonal variability of sperm stores and OSR. Crabs were purchased approximately every month from June-October from a commercial fisher working in the river. An initial determination of whether crabs were newly inseminated was made based on carapace condition and hardness. This was confirmed after dissection by the presence of seminal fluid or hardened sperm plugs within the spermathecae. A total of five to seven individuals per sample date were later processed except for the July sample when only two recently-mated females were available. Differences in sperm quantities and spermathecae weight by month were evaluated using the non-parametric Kruskal-Wallis One-way Analysis of Variance (ANOVA) by Ranks due to low sample sizes. A post-hoc Dunn's test was used to identify differences among groups. All statistical tests were performed using SigmaPlot version 12.3 and used $\alpha=0.05$ to assess statistical significance.

To compare sperm quantity with OSR, we analyzed additional crabs collected from the Rhode River during a long-term otter trawl survey conducted by the Smithsonian Environmental Research Center. The trawl survey has been conducted yearly since 1982 from April to December with three replicate sample days per month. Each day, three stations within the river were sampled for 10 min (approximately 900 m tow distance) with a 4.88 m otter trawl (3.81 cm stretch mesh size). In many years, portions of the samples were placed in plastic zip-top bags and stored frozen. Operational sex ratio within the Rhode River was calculated for each month in 2010 by dividing the total number of intermolt (stage C) males having CW > 107 mm, a size at which >50% are sexually competent (Van Engel 1990), by the number of prepubertal females. Prepubertal females were identified by the triangular shape and dark color of the abdomen (Smith & Chang 2007). It should be noted that the trawl survey did not sample in very shallow water often occupied by blue crabs (Ramach et al. 2009), but it provided the best available data for calculating OSR. Visual inspection of the data indicated clustering of sample dates around OSR values of 2-3 (n=8), 4-5 (n=15), and 15 (N=7). Because of this clustering, which made determination of the form of the relationship difficult, and low sample sizes, the three clusters of data were treated as groups and compared using Kruskal-Wallis One-way ANOVA on Ranks. A post-hoc Dunn's test was used to identify differences among groups.

To determine whether seasonal patterns were consistent across years, recently-mated females collected in 2005 and 2008 during August and September (the months with lowest and highest sperm quantities in 2010) were processed for sperm quantity and

spermathecae weight. Five individuals were processed for each sample period except September 2008, when only four recently-mated females were available from frozen samples. Values for 2005 and 2008 were compared to 2010 values using Kruskal-Wallis One-way ANOVA on Ranks, again due to low sample sizes. A post-hoc Dunn's test was used to identify differences among groups.

Spatial distribution

To examine spatial variation of sperm stores in the Chesapeake Bay, mature female crabs were originally purchased in September 2010 from commercial fishers in the Gunpowder, York, and James Rivers for comparison with Rhode River crabs. Again, only crabs that were recently-mated were selected for processing in order to control for differences in sperm quantity due to sperm loss over time (Wolcott et al. 2005) and sperm use during fertilization. The Gunpowder River, Maryland represented the upper bay (n=5), Rhode River crabs from the long-term trawl survey described above represented middle bay (n=6), and the York (n=3) and James Rivers (n=3) in Virginia represented the lower bay and were combined for analysis in order to have a comparable sample size (n=6). Statistical differences were determined using Kruskal-Wallis One-way ANOVA on Ranks due to low sample sizes. A post-hoc Dunn's test was used to identify differences among groups.

In 2013, a broader survey of spatial distribution was carried out at nine sites including: Bush River, Gunpowder River, Chester River, Rhode River, Little Choptank River, and Potomac River in Maryland and Rappahannock River, James River, and Cherrystone Inlet in Virginia. As in 2010, only crabs that were recently-mated were selected for processing to control differences in sperm counts or spermathecae weight due to time since mating. Statistical differences were determined using Kruskal-Wallis One-way ANOVA on Ranks due to low sample sizes. A post-hoc Dunn's test was used to identify differences among groups.

Spawning area

To evaluate the sperm stores of mature female blue crabs that have reached the spawning area in the lower Chesapeake Bay, crabs were collected near the boundary of the blue crab spawning sanctuary where harvest of females is restricted during summer. The crabs were purchased from commercial fishers using crab pots at Thimble Shoals, Virginia, within approximately 5 km of the spawning sanctuary. Batches of 30 crabs were purchased on 6 June, 9 July, 21 August, 27 September and 13 November 2012, approximately every six weeks during the mating and spawning season. All crabs in each collection were used to describe the composition of spawning area crabs with respect to carapace condition, evidence of spawning (presence of sponge or egg remnants), and presence of seminal fluid (sperm plug). A random subsample of 15 crabs from each collection date was later processed to quantify sperm quantity and spermathecae weight.

During winter 2012-2013, we obtained crabs from the Winter Dredge Survey in order to examine differences in sperm stores between "new" first-year spawners and "old" second-year spawners. Crabs were "aged" based on carapace condition, with clean white crabs considered "new" and dirty crabs considered "old." Crabs were collected by Virginia

Institute of Marine Science (VIMS) scientists in the lower bay (n=17 "new" and 15 "old") and north of the mouth of Rappahannock River (n=19 "new" and 20 "old"), and by Maryland Department of Natural Resources (DNR) in Maryland waters (n=16 "new" and 6 "old"). "Ages" were assigned by dredge survey biologists, with the exception of a third category with hardened seminal fluid still present "SF" comprised of five of the "new" crabs collected by Maryland DNR. No other crabs had remaining seminal fluid. Statistical differences were determined using Kruskal-Wallis One-way ANOVA on Ranks due to low and unequal sample sizes. A post-hoc Dunn's test was used to identify differences among groups.

Relationships between spermathecae weight and sperm quantity

The loss of sperm between mating and fertilization of the first brood was evaluated by exploring relationships between sperm quantity and spermathecae weight for two sets of crabs. The first comparison was for crabs collected near the lower bay spawning area in 2012, as described above, that did not have evidence of spawning (n=41). The second comparison was for crabs collected across a wide range of sampling locations (upper-lower bay), seasons, and years (1996-2012). For the second comparison, we pooled data for all 571 crabs analyzed for sperm quantity by our lab. This included the sampling efforts described above as well as crabs sampled by trap or dredge in the Rappahannock, York, and James Rivers in Virginia and Potomac River in Maryland, as well as by trotline in the Magothy, Patuxent, Manokin, and Little Choptank Rivers in Maryland. Crabs were processed following the methods described here and in Hines et al. (2003). The relationship between spermathecae weight and sperm quantity was determined by fitting a non-linear curve to the data using the Curve Fit tool. Goodness of fit was evaluated by examining residuals to ensure homoscedasticity.

Modeling

Lifetime brood production was modeled for mature females under different scenarios of initial numbers of sperm contributed at the time of mating followed by degradation or loss of sperm during storage. Initial contributions used were based on values observed in this study including $3x10^9$ sperm (Rhode and Gunpowder Rivers in September), $1x10^9$ (Rhode River in August), and $5x10^8$ (James and York Rivers in September). Three levels of sperm loss were used: 1) no loss, 2) 50% loss in the first three months (Wolcott et al. 2005) with no additional loss, and 3) 20% monthly loss (the constant rate of loss necessary for a 50% decline in sperm in three months). Brood production was also modeled using the average sperm quantity of females in the lower Chesapeake Bay spawning area that did not have evidence of spawning ($8x10^7$ sperm).

Females were assumed to mate in September and begin spawning in June (the ninth month after fertilization). Spawning/brood production was assumed to occur monthly during summer (June-September) for two years after mating, for a maximum lifetime production of eight broods. Some crabs follow different maturity and brood production schedules including mating in spring and producing the first brood the same summer as has been observed for crabs in the lower bay (Aguilar et al. 2005) and North Carolina (Darnell et al. 2009). These alternative schedules were not addressed here in order to simplify comparisons between scenarios. An average brood size of $3x10^6$ eggs was used for

all calculations (Hines 1982, Prager et al. 1990). The range of sperm:egg ratios needed to achieve successful fertilization included 1:1, 10:1, 25:1, 80:1, and 100:1, covering a range of possible values based on prior studies (Bressac et al. 1994, Hines et al. 2003, Sainte-Marie & Lovrich 1994). This resulted in values of the number of sperm required to fertilize a single brood that ranged from $3x10^6$ to $3x10^8$. The total number of complete broods that could be produced was calculated for each combination of sperm:egg ratio, initial contribution of sperm, and level of sperm loss. Although other factors such as mortality and nutrition likely affect an individual's lifetime brood production, we chose a relatively simple model to focus on the effects of variation in the sperm:egg ratio required for fertilization, sperm stores, sperm use during fertilization, and other mechanisms of sperm loss.

RESULTS

Seasonal variation and OSR

The quantity of sperm stored in recently-mated female blue crabs in the Rhode River in 2010 varied seasonally, with the highest values in September and October and lowest in August (n=5-7 crabs per month except July when n=2). The mean sperm quantity for the entire season was $2.02x10^9 \pm 1.88x10^8$ (mean ± standard error of the mean reported here and for all subsequent values). Sperm numbers were moderate in June at 1.86x109±2.63x108 and lowest in August ($1.02x10^9 \pm 1.32x10^8$) (Fig. 1a), although the differences among months June-August were not significant. Sperm quantity was significantly higher during September ($2.83x10^9 \pm 5.06x10^8$) and October ($2.85x10^9 \pm 3.68x10^8$) than in August (p=0.001).

Spermathecae weight showed a similar seasonal pattern to sperm quantity, but there were no significant differences among months (Fig. 1b). The mean spermathecae weight for the season was 3.395±0.227 g. Spermathecae weight was moderate in June (3.680±0.589 g) and declined through August. In September, spermathecae weight increased to a high of 4.640±0.700 g.

The seasonal increase in sperm quantity from August to September 2010 persisted across multiple years. Sperm quantities in August were consistently lower than those in September in both 2005 and 2008, the two other years for which data were available (Fig. 2). However, the only significant difference between August and September was in 2010.

There was a positive, asymptotic relationship between OSR and sperm quantity. OSR was lowest in August (2.38 reproductively active males per prepubertal female), corresponding to the minimum number of sperm ($1.02x10^9 \pm 1.32x10^8$) and near minimum for spermathecae weight (2.556 g). Sperm quantity was significantly lower ($p<0.001$) when OSR was <3 (n=8, $1.1x10^9 \pm 1.0x10^8$) than when it was between 4-5 (n=16, $2.2x10^9 \pm 2.4x10^8$) or as high as 15 (n=7, $2.9x10^9 \pm 3.7\ x10^8$). Operational sex ratio was unknown at the time of sampling and by chance no samples were obtained when OSR was between 5 and 15. Significant differences were also detected for spermathecae weight (p=0.019) but these differences in spermathecae weight appeared to be due to four crabs with very high spermathecae weight (5-7 g) at moderate levels of OSR. There was no relationship between OSR and spermathecae weight when these data were removed.

Spatial distribution

Sperm quantity, but not spermathecae weight, differed by sampling location within Chesapeake Bay (n=5-6 crabs per location) in September 2010. Sperm quantity in the lower bay was significantly lower than both the middle and upper bay ($p=0.003$), however the middle and upper bay were not different from each other (Fig. 4a). Spermathecae weight was highest in the middle bay and lower in both the upper and lower bay, but there was no significant difference among the sites (Fig. 4b).

In August 2013, there were significant differences in sperm counts of recently-mated female crabs among sites, with upper Chesapeake Bay sites tending to have higher sperm counts than lower bay sites (Fig. 5). Bush, Gunpowder, Chester, and James Rivers had average sperm counts that exceeded $2x10^9$, whereas only Little Choptank River had average sperm counts less than $1x10^9$ ($8.8x10^8 \pm 5.2x10^8$). Sperm counts in Bush, Gunpowder, and Chester Rivers were significantly higher than Little Choptank River, Rappahannock River, and Cherrystone Inlet ($p<0.001$).

Spawning area

There were strong seasonal differences in the composition of females in the spawning area of the lower Chesapeake Bay in 2012 (n=30 crabs per month). The proportion of female crabs with evidence of prior spawning in the lower bay was highest in June (80%) and August (100%) (Fig. 6a). In September and November, the samples were dominated by crabs without evidence of prior spawning (100% and 93% respectively). Samples in July were fairly evenly split between those with and without evidence of spawning. Nearly all crabs without evidence of spawning had clean or partially dirty carapaces in June and July, whereas a majority of crabs without evidence of spawning in September and November had partially dirty and dirty carapaces (Fig. 6b). Crabs with evidence of spawning were typically partially dirty through September, shifting to 100% dirty in November (Fig. 6c).

The number of sperm was highest for clean, recently-mated crabs with seminal fluid and decreased with increasing carapace dirtiness (Fig. 7a). Crabs without seminal fluid had low sperm numbers regardless of carapace condition. The weight of the spermathecae for crabs with seminal fluid was similar for clean and partially dirty crabs and decreased for dirty crabs (Fig. 7b). However, the latter difference could not be tested because only a single individual had both a dirty carapace and seminal fluid. Crabs without seminal fluid had low spermathecae weights regardless of carapace condition, as seminal fluid accounts for a large proportion of spermathecae weight.

Crabs from the lower bay without seminal fluid had significantly lower sperm quantities if they had spawned compared to crabs without evidence of spawning, irrespective of carapace condition (Fig. 8a; $P=0.010$). The range of sperm quantities was large for partially dirty and dirty crabs without seminal fluid and with evidence of spawning, with a minimum value of $4.27x10^6$ and maximum of $1.07x10^8$ (n=33). For comparison, crabs without evidence of spawning had sperm quantities ranging from $4.02x10^7$ to $1.32x10^8$ (n=14). Spermathecae weight for crabs without seminal fluid was

very low and not significantly different among carapace conditions or spawning evidence (Fig. 8b).

The quantity of sperm in mature female crabs collected during the 2012-2013 Winter Dredge Survey varied depending on their categorization as "new," "old," or "SF" (Fig. 9). Crabs with seminal fluid present were only collected in Maryland's portion of Chesapeake Bay and had an average of $1.2x10^9 \pm 2.7x10^8$ sperm (n=5). The remaining crabs did not exhibit consistent differences by site, but when grouped among sites did have higher sperm counts if categorized as "new" ($8.2x10^7 \pm 5.1x10^7$, n=47) or "old" ($5.2x10^7 \pm 4.8x10^7$, n=41). The groups "SF," "new," and "old" crab were each statistically significant from each other ($p<0.001$).

Relationships between spermathecae weight and sperm quantity

There was a positive relationship between spermathecae weight and sperm quantity. For crabs collected in the lower bay in 2012, the relationship was bimodal (Fig. 10a). Most individuals had either high sperm quantities ($1.31x10^9 \pm 1.69x10^8$) and spermathecae weights (2.516±0.123 g), or low quantities ($7.61x10^7 \pm 6.70x10^6$) and weights (0.248±0.033 g). When data for all 571 crabs collected between 1996 and 2012 were included, the relationship was asymptotic (Fig. 10b; $\log_{10}y=7.590+1.609*(1-e^{(-0.9659*x)})$; $r^2=0.536$). Sperm quantity reached an asymptote of $1.58x10^9$ although substantial variability in sperm quantity remained among individuals with high spermathecae weight.

Modeling

Modeled brood production for individual female blue crabs varied from a minimum of zero to a maximum of eight broods over a simulated two-year adult lifespan (Table 1). In simulations with no sperm loss except by fertilization, eight full broods were produced except at sperm:egg ratios of 80 or 100. In simulations with 50% reduction in sperm prior to spawning, declines in initial sperm load from $3x10^9$ to $5x10^8$ resulted in a dramatic decrease from eight to three broods at a sperm:egg ratio of 25. Smaller decreases in brood number were observed at other combinations of sperm:egg ratio and initial sperm load. When sperm load declined at a monthly rate of 20% throughout the adult lifespan, reductions in initial sperm load had the greatest effect at a sperm:egg ratio of 10, resulting in a reduction from four broods to one.

When the observed value of sperm quantity for females in lower Chesapeake Bay without evidence of spawning was used ($8x10^7$ sperm), predicted lifetime brood production of an individual female was lower than for the scenarios described above. Only two full broods were predicted at a sperm:egg ratio of 10:1 without additional sperm loss and one full brood with 20% loss per month (Table 1). At a sperm:egg ratio of 1:1, five to eight full broods could be produced depending on whether additional sperm were lost. No more than a single full brood would be possible at a sperm:egg ratio of 25:1 or higher.

Table 1. Model simulations of brood production under various levels of sperm:egg ratio, initial sperm quantity, and sperm loss. Data reported are number of full broods produced assuming a brood size of $3x10^6$ eggs. The initial sperm quantity (italics) is either for different contributions of sperm at the time of mating (Scenarios 1-3) or for females that have already migrated to the spawning area (Scenario 4). In Scenario 4, sperm loss prior to brood 1 is not applicable (N/A) as this loss would be accounted for in the initial sperm quantity.

	Sperm:Egg Ratio				
Scenario	**1**	**10**	**25**	**80**	**100**
1) 3x10^9 at the time of mating					
No loss	8	8	8	8	8
50% loss prior to brood 1	8	8	8	6	5
20% monthly loss	8	4	3	1	0
2) 1x10^9 at the time of mating					
No loss	8	8	8	4	3
50% loss prior to brood 1	8	8	6	2	1
20% monthly loss	6	3	1	0	0
3) 5x10^8 at the time of mating					
No loss	8	8	6	2	1
50% loss prior to brood 1	8	8	3	1	0
20% monthly loss	5	1	0	0	0
4) 8x10^7 in spawning area					
No loss	8	2	1	0	0
50% loss prior to brood 1	N/A	N/A	N/A	N/A	N/A
20% monthly loss	5	1	0	0	0

Figure Captions

Fig. 1. Variation in (a) sperm quantity and (b) spermathecae weight in recently-mated female blue crabs *Callinectes sapidus* during the mating season in the Rhode River, Maryland in 2010. Error bars indicate standard error of the mean.

Fig. 2. Interannual variation in sperm quantity of recently-mated female blue crabs *Callinectes sapidus* in August and September in the Rhode River, Maryland. Data shown are for 2005, 2008, and 2010. Error bars indicate standard error of the mean.

Fig. 3. Relationships between operational sex ratio (OSR) and (a) sperm quantity and (b) spermathecae weight of recently-mated female blue crabs *Callinectes sapidus* collected in the Rhode River, Maryland in 2010. Each data point represents an individual crab. Data for OSR were calculated for the month in which each crab was collected, such that all crabs collected in the same month have the same value for OSR.

Fig. 4. Spatial variation in (a) quantity of sperm and (b) spermathecae weight for recently-mated mature female blue crabs *Callinectes sapidus* collected in 2010 from upper, middle, and lower Chesapeake Bay. Error bars indicate standard error of the mean. Letters at the bottom of vertical bars indicate statistical differences (α=0.05).

Fig. 5. Spatial variation in quantity of sperm for recently-mated mature female blue crabs *Callinectes sapidus* collected from nine locations in Chesapeake Bay. Error bars indicate standard error of the mean. Sperm quantity was compared among sites using One-Way Analysis of Variance. Statistical differences among sites were identified using the Holm-Sidak post hoc test and are indicated by the letters at right.

Fig. 6. Percent composition of samples of mature female blue crabs *Callinectes sapidus* collected in the spawning area of the lower Chesapeake Bay in 2012 with respect to (a) proportion of crabs with (white) or without (black) evidence of spawning (n=30 crabs each month), (b) carapace condition of crabs without evidence of spawning, and (c) carapace condition of crabs with evidence of spawning. Carapace conditions are clean (white), partially dirty (gray), and dirty (black).

Fig. 7. Variation in (a) sperm quantity and (b) spermathecae weight for mature female blue crabs *Callinectes sapidus* collected in the spawning area of the lower Chesapeake Bay in 2012 with respect to carapace condition (clean [C], partially-dirty [PD], dirty [D]) and presence/absence of seminal fluid (hardened sperm plug). Error bars indicate standard error of the mean.

Fig. 8. Variation in (a) sperm quantity and (b) spermathecae weight for mature female blue crabs *Callinectes sapidus* collected in the spawning area of the lower Chesapeake Bay in 2012 for which no seminal fluid was present (i.e. the sperm plug was resorbed). Crabs were classified by carapace condition (partially dirty [PD] or dirty [D]) and presence (yes) or absence (no) of evidence of spawning (egg remnants on pleopods). Letters at the bottom of vertical bars indicate statistical differences (α=0.05). Error bars indicate standard error of the mean.

Fig. 9. Variation in sperm quantity of mature female blue crabs *Callinectes sapidus* collected during the 2012-2013 Winter Dredge Survey and categorized as “new” or “old” by dredge survey biologists. Sample sites were the lower bay (Lower), middle bay near the Rappahannock River mouth (Middle), and middle to upper bay in Maryland waters (MD). Only five crabs had sperm plugs (hardened seminal fluid) remaining in spermathecae, all of which were collected at the MD site. These crabs were all categorized as “new” and had much higher sperm quantities. They are indicated in the figure as crabs with seminal fluid (SF), which is indicative of recent mating. Note the break in the y-axis. Error bars indicated standard error of the mean.

Fig. 10. Relationships between spermathecae weight and sperm quantity for (a) mature female blue crabs *Callinectes sapidus* collected in the spawning area of the lower Chesapeake Bay in 2012 that did not have evidence of prior spawning (n=41) and (b) for all mature female blue crabs collected throughout Chesapeake Bay and analyzed for sperm quantity by our lab (n=571). A regression line and squared correlation coefficient are shown for the sigmoid relationship in (b).

Figure 1.

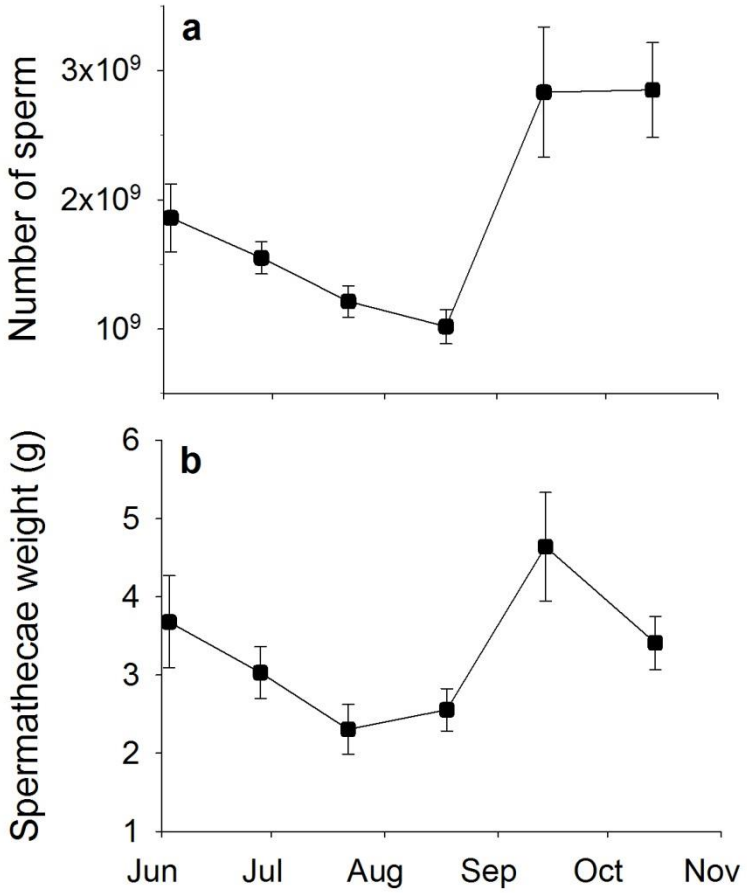
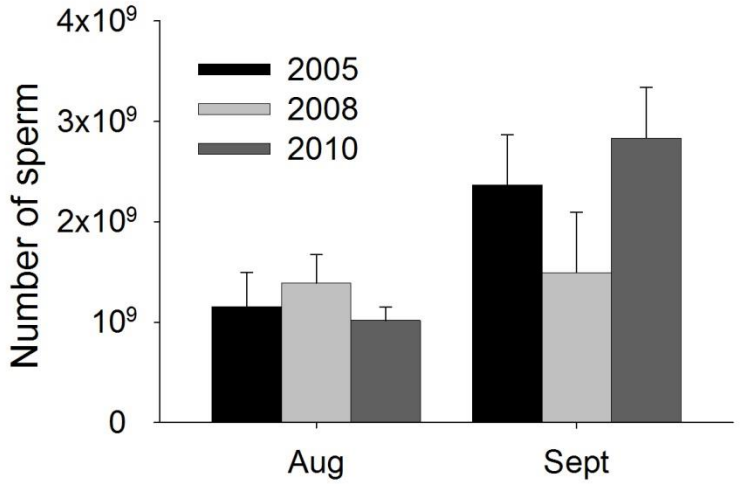

a
Number of sperm
3x10⁹
2x10⁹
10⁹
b
Spermathecae weight (g)
6
5
4
3
2
1
Jun
Jul
Aug
Sep
Oct
Nov

Figure 2.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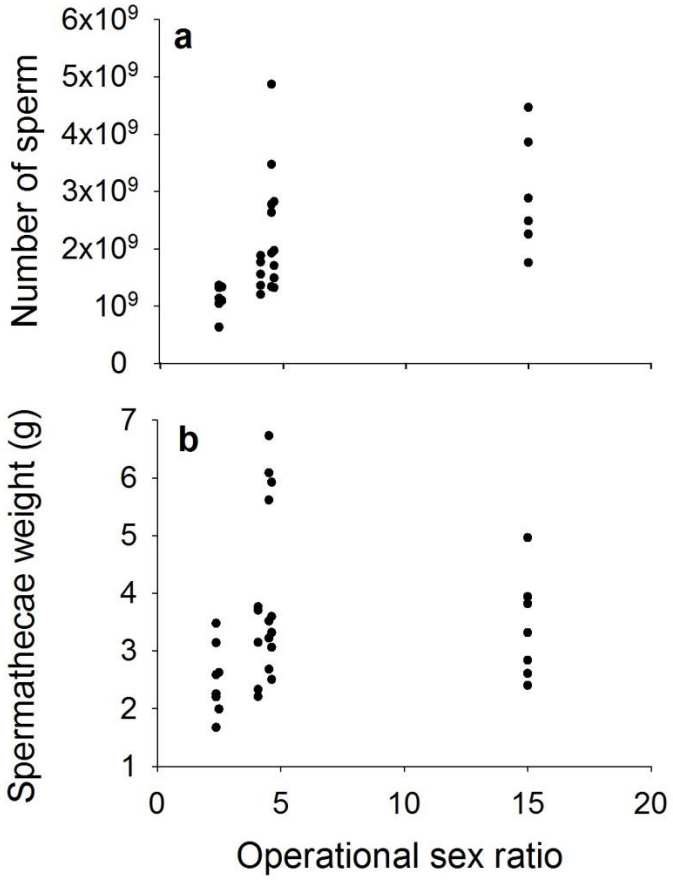
3x10⁹
2x10⁹
10⁹
0
Number of sperm
2005
2008
2010
Aug
Sept

Figure 3.


a
b
Number of sperm
Spermathecae weight (g)
Operational sex ratio
6x10⁹
5x10⁹
4x10⁹
3x10⁹
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0
5
10
15
20

Figure 4.

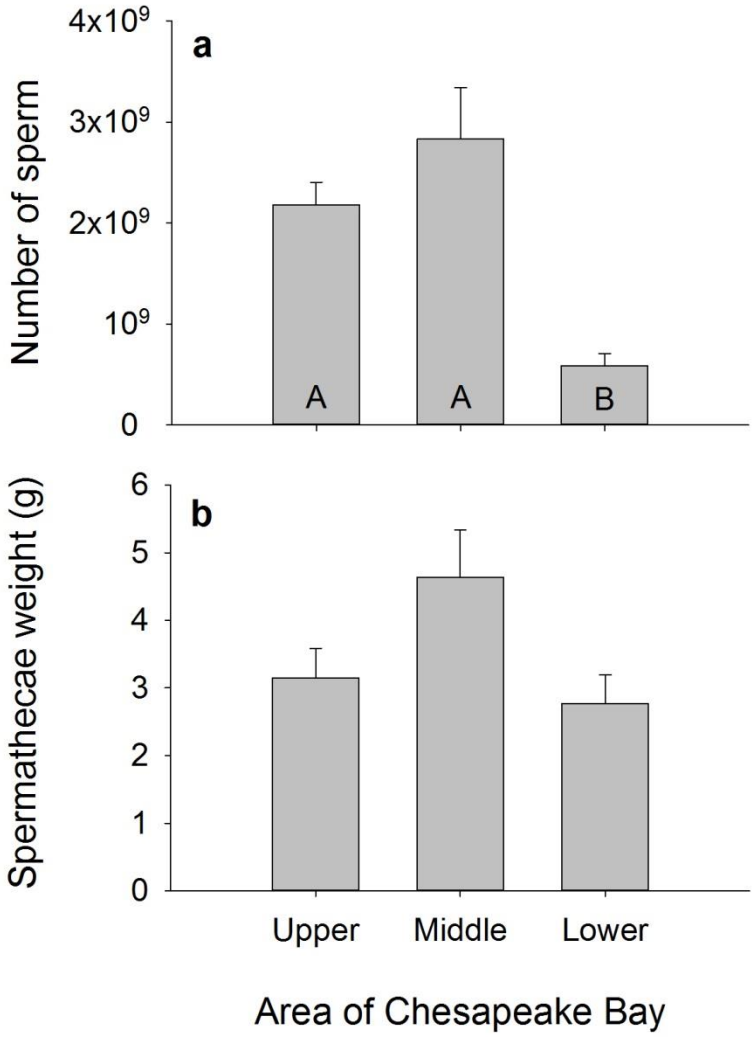

a
Number of sperm
4x10⁹
3x10⁹
2x10⁹
10⁹
0
A
A
B
b
Spermathecae weight (g)
6
5
4
3
2
1
0
Upper
Middle
Lower
Area of Chesapeake Bay

Figure 5.

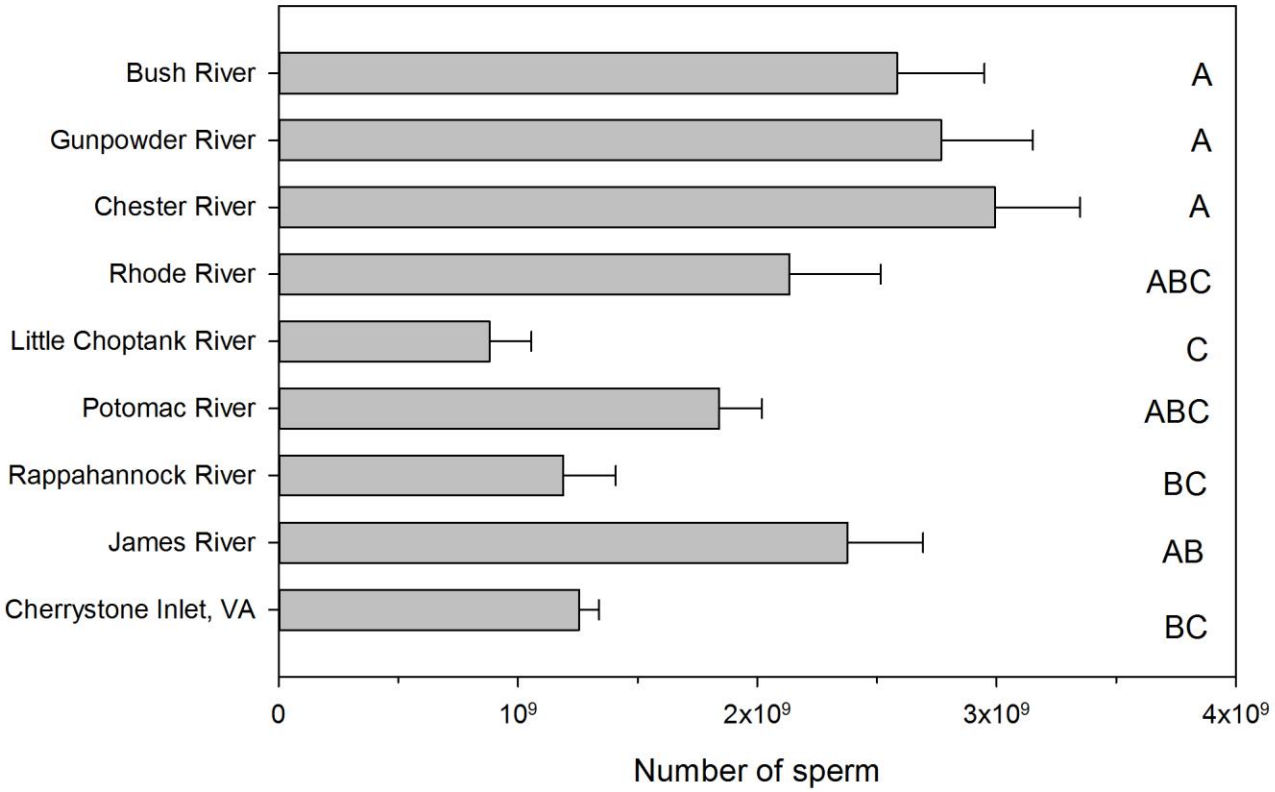

Bush River
Gunpowder River
Chester River
Rhode River
Little Choptank River
Potomac River
Rappahannock River
James River
Cherrystone Inlet, VA
A
A
A
ABC
C
ABC
BC
AB
BC
0
10⁹
2x10⁹
3x10⁹
4x10⁹
Number of sperm

Figure 6.

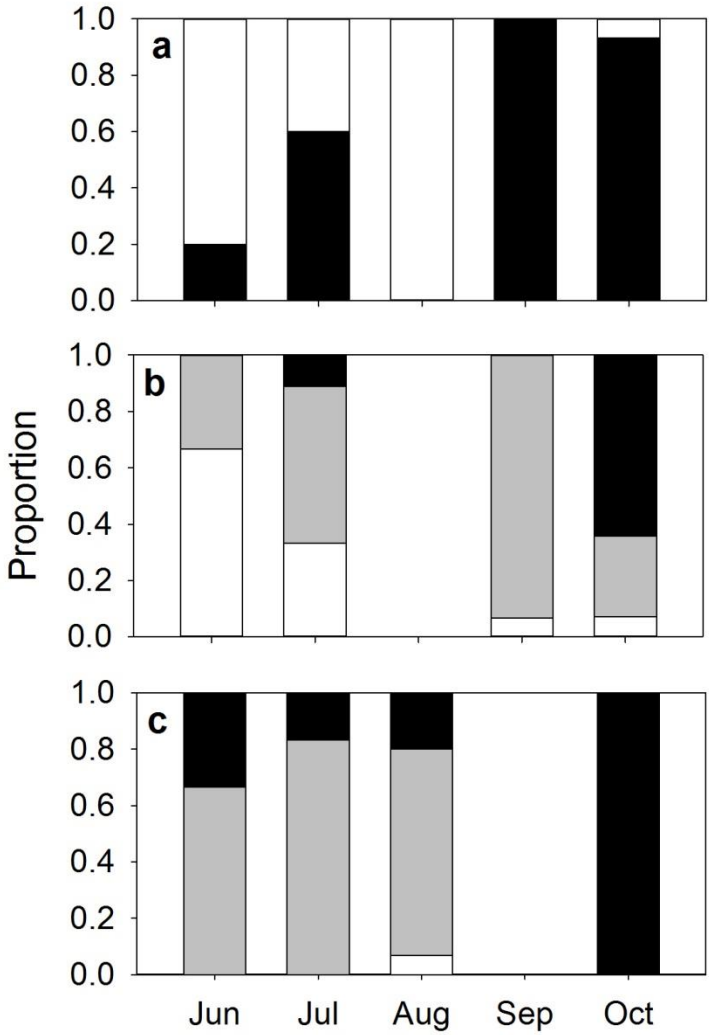


Figure 7.

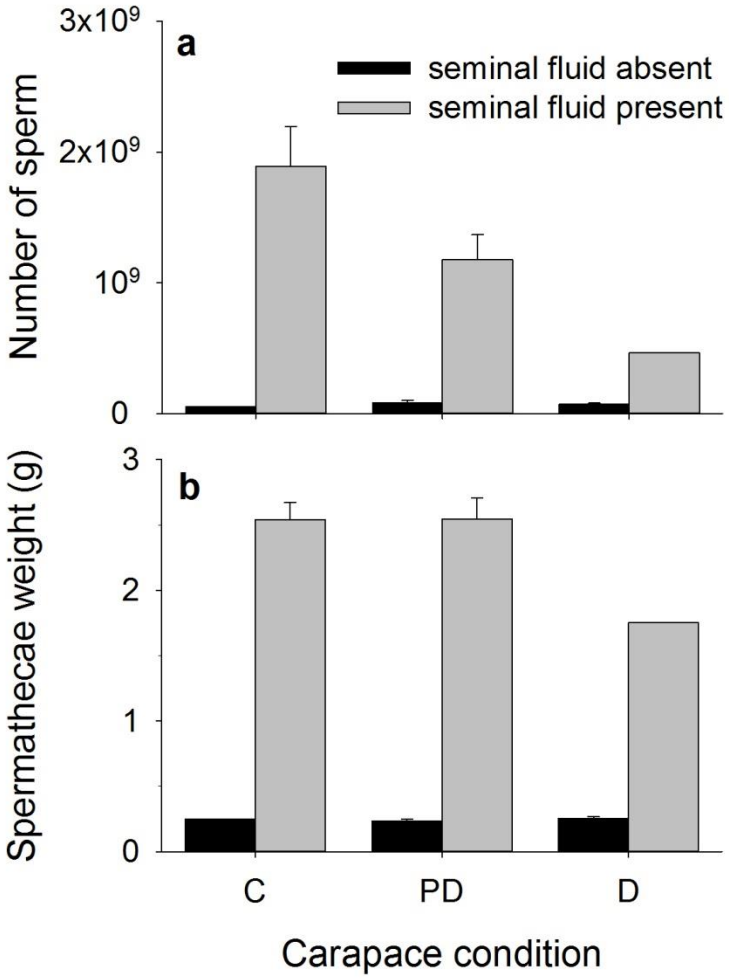

a
seminal fluid absent
seminal fluid present
Number of sperm
3x10^9
2x10^9
10^9
0
b
Spermathecae weight (g)
3
2
1
0
C
PD
D
Carapace condition

Figure 8.

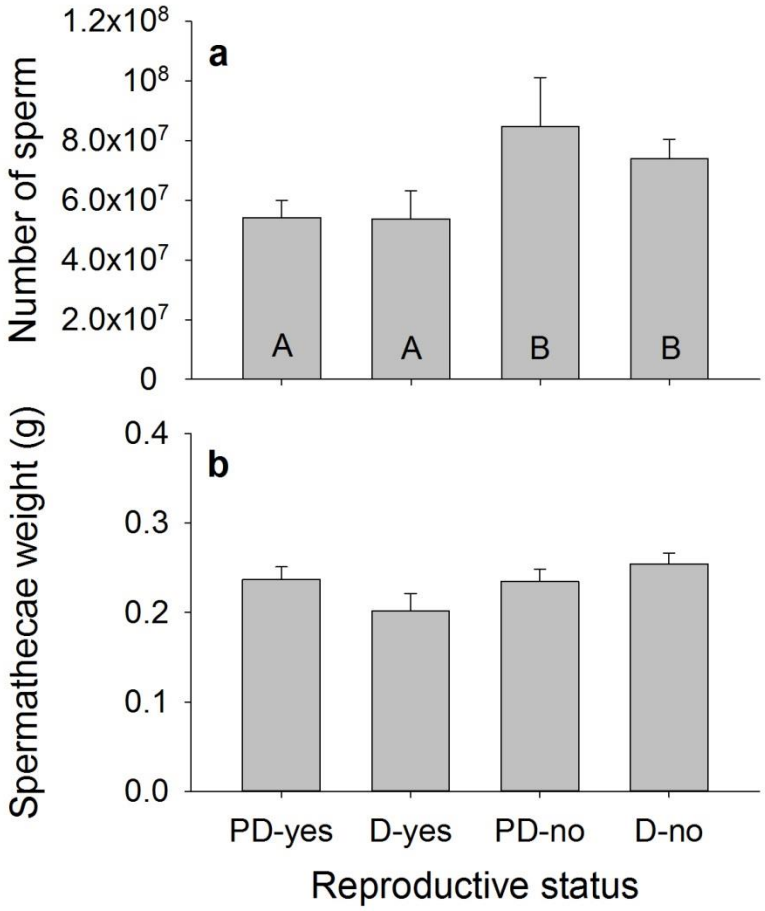

a
Number of sperm
1.2x10^8
10^8
8.0x10^7
6.0x10^7
4.0x10^7
2.0x10^7
0
A
A
B
B
b
Spermathecae weight (g)
0.4
0.3
0.2
0.1
0.0
PD-yes
D-yes
PD-no
D-no
Reproductive status

Figure 9.

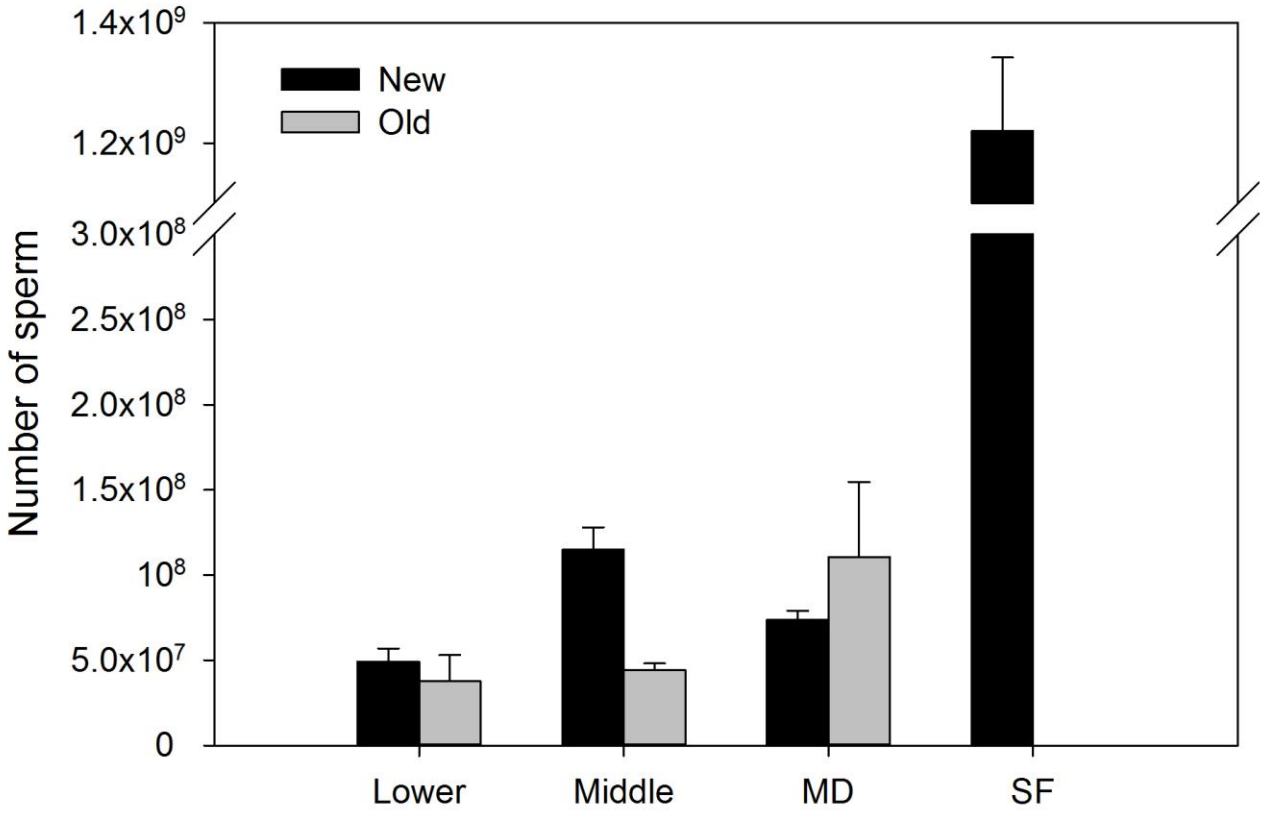
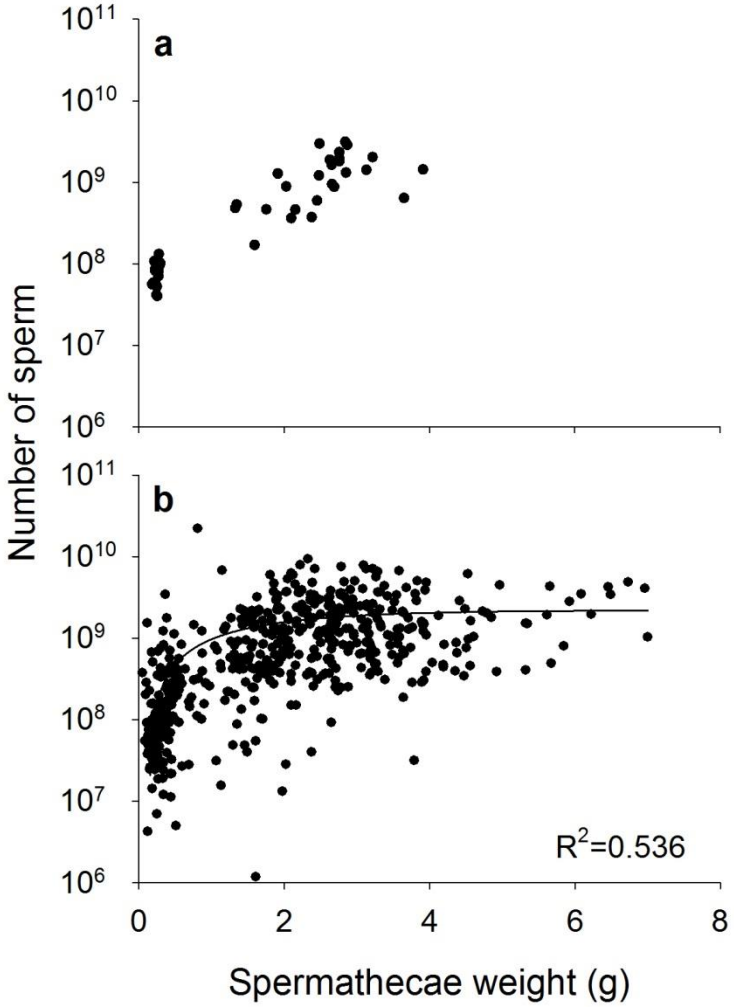

1.4x10^9
1.2x10^9
3.0x10^8
2.5x10^8
2.0x10^8
1.5x10^8
10^8
5.0x10^7
0
Number of sperm
New
Old
Lower
Middle
MD
SF

Figure 10.


a
b
Number of sperm
Spermathecae weight (g)
R^2=0.536
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
2
4
6
8

2. Applications:

i. Outputs and management outcomes achieved.

Sperm stores in recently-mated female blue crabs were lower during summer in the Rhode River sub-estuary of Chesapeake Bay as compared to other seasons, corresponding to reductions in the operational sex ratio (OSR). Sperm quantities were highest in fall, nearly $3x10^9$, when there were as many as 15 mature males available to mate with each pre-pubertal female. These high sperm quantities, likely obtained from males with sufficient time between mating events to regenerate a full complement of sperm, were similar to the $3.4x10^9$ sperm observed for Chesapeake Bay females mated with "fully recovered" males in laboratory experiments (Kendall et al. 2002, Carver et al. 2005). These sperm stores were greater than the sperm stores of $1.2x10^9$ in laboratory experiments conducted in North Carolina (Wolcott et al. 2005) and in crabs collected from the field in Florida (Hines et al. 2003). The estimate from Florida appears to have been a substantial underestimate because sperm quantities decline with time after mating (Wolcott et al. 2005) and sampling was not restricted to recently-mated females as in this study. It is unclear why Wolcott et al. (2005) observed lower sperm stores in recently-mated females from North Carolina. In August, when OSR was only three mature males for each pre-pubertal female, sperm stores in recently-mated females declined to just 36% of the full complement observed in fall. The sperm stores of females in August were similar to the level of sperm transferred by recently-mated males in laboratory experiments (Kendall et al. 2002), indicating that these females likely mated with males with too short an interval between mating events to regenerate a full complement of sperm. The OSR may have been lowest in August because of intense fishing for mature males during summer and a late summer increase in the number of females molting to maturity (Hines et al. 1987), although we did not investigate the factors affecting OSR in this study. The seasonal pattern of sperm limitation was consistent across three years of sampling, indicating that it occurs annually in Chesapeake Bay.

The seasonal reduction in sperm stores described here is consistent with males encountering pre-pubertal females too frequently to regenerate a full supply of sperm and ejaculate between mating events (Kendall & Wolcott 1999, Kendall et al. 2002). In experimental manipulations with the unharvested crab *Hapalogaster dentata*, Sato and Goshima (2006) observed significant declines in the number of sperm ejaculated with increased male mating frequency. In heavily-fished areas of Chesapeake Bay, male blue crabs in pre-copulatory embrace have been observed with depleted sperm stores equivalent to males that have just mated indicating reduction in sperm stores due to frequent mating (Carver et al. 2005). Similar evidence for reduction in sperm stores in fished decapod populations has been observed for Dungeness crab *Cancer magister* (Smith & Jamieson 1991), snow crab *Chionocetes opilio* (Lovrich et al. 1995), Tanner crab *Chionocetes bairdi* (Stevens et al. 1993), and coconut crab *Birgus latro* (Sato 2011), suggesting that reduced sperm stores may be a common consequence of male-selective fishing for decapod crabs. All females in this study had mated, suggesting that the Chesapeake Bay blue crab population does not experience reproductive impairment due to an inability to find mates.

Spatial variation in sperm quantities in newly-mated females was indicative of reduced sperm stores received by females molting to maturity in some tributaries of Chesapeake

Bay during September 2010 and August 2013, although the spatial pattern was somewhat different among years. Female crabs from the middle and upper bay had stores of >$2x10^9$ sperm on average in September 2010, indicating that they received nearly a full load of sperm. In contrast, females from the lower bay had < 25% of the sperm load of middle bay crabs. In 2013, females were more likely to have high sperm counts in the upper bay tributaries, but some lower bay tributaries also had relatively high counts. For example, James River crabs had some of the lowest sperm counts in September 2010, but relatively high counts in August 2013. Based on the observed relationship between OSR and sperm counts in recently-mated females, we hypothesize that this spatial variation is due to differences in OSR. It is likely that variation in OSR is due to interactions between the male-biased blue crab fishery in summer and the large numbers of females molting to maturing in late summer. One potential mechanism driving observed spatial differences may be the intensity of fishing for males, but we do not have concurrent data from the same sites for evaluating this hypothesis.

Unlike sperm quantity, the spermathecae weight of recently-mated females did not vary with season, OSR, or collection location. Variation in spermathecae weight is driven primarily by the quantity of seminal fluid transferred during mating, which is affected both by male size and male mating frequency (Jivoff 1997a, 1997b, 2003a, 2003b). Although small males transfer much less seminal fluid than large males during mating, the contribution of sperm is independent of male size (Kendall et al. 2001). Taken together, these results suggest that the variation we observed in sperm quantities was due to male mating frequency (which varies with OSR) and that male size, which was an unknown in our study, may have obscured the effect of mating frequency on spermathecae weight.

The quantity of stored sperm declined significantly with time since mating. Although there is not a precise method of determining time since mating, the hardened seminal fluid (sperm plug) present after mating is absorbed about one month later (Wolcott et al. 2005). In our study, females that no longer had a hardened sperm plug had sperm quantities that were one to two orders of magnitude less than the full complement of a recently-mated female. This decline was observed in females with and without evidence of spawning, suggesting that it was due to sperm loss and not simply the use of sperm for fertilization, which occurs during egg extrusion onto the abdomen. In North Carolina, Wolcott et al. (2005) observed 50% declines in sperm stores of captive females in three months prior to fertilization, consistent with our results from field collections. It remains unclear whether sperm loss occurs primarily during breakdown of the sperm plug or whether it occurs continuously following mating. Nor is it clear whether this attrition results from a process of leakage and sloughing or from deterioration - all independent of use during fertilization.

In the lower Chesapeake Bay spawning area, the composition of females shifted from primarily older, reproductively-active females in summer to younger, recently-mated females in fall. In summer, most females were either ovigerous, with external sponges of fertilized eggs, or had remnants of egg cases from previous broods. These reproductive females had significantly lower sperm stores ($5.4x10^7$) than females without evidence of spawning ($7.8x10^7$) as would be expected due to the use of sperm during fertilization. It should be noted that the females collected from the biologically relevant spawning area in lower Chesapeake Bay were collected by commercial fishers working 5 km outside the border of the spawning sanctuary

where fishery harvest of mature females is prohibited during summer. The presence of spawning females in this area is indicative of the sometimes substantial differences between biologically relevant spawning areas for blue crabs and spawning sanctuaries designated by fishery managers (Rittschof et al. 2009). Females collected during the winter dredge survey in 2012-2013 had sperm stores similar to those collected in the summer and fall.

The high prevalence of recently-mated females in the spawning area in fall, and to a lesser extent in early summer, is consistent with our understanding of the seasonal timing of mating and the spawning migration (Hines et al. 1987, Aguilar et al. 2005). Interestingly, we did not observe consistent differences in the quantity of stored sperm with respect to carapace condition except for recently-mated females with seminal fluid (a sperm plug) present (Fig. 7a). This result is indicative of substantial uncertainty in the value of carapace condition as a relative measure of time since mating in mature female blue crabs, as the mechanisms underlying changes in carapace condition have not been evaluated rigorously. A validated technique for determining time since mating will be needed to determine the pattern and rate of decline in sperm stores due to causes other than fertilization. Potential indicators of blue crab age include lipofuscin concentration in eye stalks (Ju et al. 1999) and growth rings in the eye stalk or gastric mill (Kilada et al. 2012), but additional validation is needed to determine whether these techniques are useful for determining time since mating.

Although reductions in sperm stores in recently-mated females were substantial in some locations and seasons, determining whether the blue crab spawning stock in Chesapeake Bay is sperm-limited at a level to affect population dynamics is much more challenging. We addressed this problem by modeling the lifetime brood production of females with various initial sperm quantities, patterns of sperm loss, and sperm:egg ratios required for fertilization. As in Hines et al. (2003), we assumed an average brood size of $3x10^6$ eggs and a reproductive season typical for Chesapeake Bay. In model results, the effect of different initial sperm quantities was difficult to assess due to the strong influence of 1) the timing of sperm loss, 2) the rate of sperm loss, and 3) the sperm:egg ratio. For example, the lifetime brood production of females in the spawning area without evidence of spawning (initial sperm quantity of $8x10^7$) was eight broods at a sperm:egg ratio of 1:1 and only two broods at 10:1 assuming fertilization was the only mechanism of sperm loss. When additional sperm loss occurred at 20% per month (based on observations in Wolcott et al. [2005]), females with no evidence of spawning were capable of producing five full broods at 1:1 and only one full brood at 10:1. The sperm:egg ratio needed for successful fertilization of a full brood in blue crabs has previously been estimated at 20:1 or 30:1 (Hines et al. 2003), although this estimate was likely too high because it did not take into account any sperm loss after mating. Our modeling results suggest that this number must be between 1:1 and 10:1 in order for females to fertilize the two to seven broods observed in brood production studies in North Carolina and Florida (Hines et al. 2003, Darnell et al. 2009, 2010).

In our field study, a difference of $2.4x10^7$ sperm was observed between females with and without evidence of spawning. Assuming 1) an average brood size of $3x10^6$, 2) that females with evidence of spawning had only produced one brood, and 3) that there was no sperm loss to mechanisms other than fertilization, this difference is indicative of eight sperm used per egg during fertilization of the first brood. If an equal number of sperm are used for each subsequent brood, only two additional full broods could be produced. This estimate is consistent with our

modeling results which suggested that only two full broods could be produced at a somewhat higher sperm:egg ratio of 10 assuming no sperm loss except during fertilization. However, it is inconsistent with observed brood production of up to seven fertilized broods (Hines et al. 2003, Dickinson et al. 2006, Darnell et al. 2009), suggesting that fertilization may be possible at even lower sperm:egg ratios. Snow crab *Chionocetes opilio* have been observed to cease egg extrusion at ratios below 7:1 (Sainte-Marie & Lovrich 1994). It is possible that decapod species such as blue crabs that store sperm from a single mating may be capable of fertilization at lower sperm:egg ratios than species such as snow crabs that mate multiple times. Very low sperm:egg ratios are not unheard of in the Arthropoda, as some Drosophila species have ratios as low as 1:1 (Bressac et al. 1994). Direct quantification of the number of sperm used during fertilization in the blue crab is needed.

Two important limitations of our model were the assumptions that brood size and fertilization success are equal for all broods. In a study of captive crabs in North Carolina, brood size declined by 50% from brood 1-5, and the percentage of extruded embryos developing normally decreased 40% from brood 1-4 (Darnell et al. 2009). In Florida, Hines et al. (2003) occasionally observed that later broods (5th to 7th observed brood) were non-fertile. These combined findings provide strong evidence that both brood size and fertilization success decline over time in blue crabs, but these factors were left out of the model because mechanisms are unknown. Possible mechanisms include insufficient sperm stores, as indicated in our modeling and field-based estimates of brood production, or could be due to loss of sperm viability, declining female condition due to fouling or disease, or a combination of mechanisms. Understanding the extent to which insufficient sperm stores contribute to declines in the size and fertilization success of later broods is important for determining whether increasing the number of females surviving to spawn in a second spawning season is an effective management strategy for enhancing the reproductive output of the spawning stock. If sperm limitation is an important factor affecting brood production in an individual's second spawning season as indicated in this study, fishery managers could potentially increase the reproductive output and stability of the blue crab population in Chesapeake Bay through actions aimed at maintaining OSR in nursery habitats at the time of mating at sufficient levels to prevent sperm limitation.

Although this study refines our understanding of some aspects of blue crab reproductive biology, it also highlights data gaps critical to assessing the potential for population-level sperm limitation. 1) The pattern and rate of sperm loss following mating is important to determining whether the low sperm quantities we observed in spawning areas of the lower Chesapeake Bay are indicative of natural loss of sperm following mating or sperm reduction at the time of mating. 2) The sperm:egg ratio required for successful fertilization is unknown, yet it is a key factor in determining whether an individual female is sperm-limited. 3) Our model was focused on lifetime brood production and thus did not consider mortality. 4) Experiments are needed to determine whether reductions in the quantity of sperm acquired during mating result in actual reductions in the number or size of fertilized broods that can be produced. Management strategies in Chesapeake Bay are focused in part on increasing the reproductive output and stability of the spawning stock by increasing the number of females spawning for a second year (Miller et al. 2011), yet these second year females are most likely to be affected by sperm limitation. Mark-recapture experiments indicate that the annual survival rate of mature female blue crabs in Chesapeake Bay is 0.15±0.01 (mean ± SE) (Miller et al. 2011), however these

experiments were conducted in 2002-2006 prior to closure of Virginia's winter dredge fishery and the extent of any subsequent increase in survival rate is unknown. In this study, carapace condition did not appear to be a conclusive indicator of whether females were in their first or second spawning season. Therefore, developing a reliable method for distinguishing between first and second year spawners should be a priority.

In summary, we observed reduced sperm stores in recently-mated female blue crabs in Chesapeake Bay when OSR was lowest (August) and in lower bay tributaries in September. Sperm stores declined by at least an order of magnitude from the time of mating to fertilization of the first brood. The composition of the lower bay spawning stock matched expectations, with reproductively active females dominating in spring and summer and newly-arrived, recently-mated females dominating in fall. Reproductively active females in the lower bay had significantly lower sperm quantities than females without evidence of prior spawning, indicating that we were able to detect the use of sperm for fertilization. Modeling of lifetime brood production and estimates based on apparent sperm use during fertilization indicated that sperm limitation is likely for females that survive to a second spawning season. Information on the proportion of crabs surviving to a second spawning season and sperm:egg ratios required for successful fertilization is needed in order to better understand the degree to which the reductions in sperm observed in recently-mated females in this study may be affecting the total reproductive output of the blue crab population in Chesapeake Bay.

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a. Outputs

i. New fundamental or applied knowledge:

a) We observed substantial declines in the number of sperm stored by female blue crabs from the time of mating to the time females spawn. Previous studies in NC (Wolcott et al. 2005) had indicated a 50% decline in sperm prior to fertilization of the first brood, but this was for females that matured in early summer and produced the first brood three months later. In this study, we found that overwintering mature females collected by the winter dredge survey had sperm stores that were usually $<1x10^8$, whereas most females collected shortly after mating had sperm stores $>1x10^9$.
b) We estimated that most females appear to have enough sperm to support three full broods at a sperm:egg ratio of approximately 8:1. These findings are supported both by sperm counts of females collected in the spawning area and a brood production model. They suggest that females surviving to a second spawning season may be sperm-limited. The estimate of sperm:egg ratio at the time of fertilization is substantially lower than the previous best estimate of 20-30:1 from Hines et al. (2003).
c) This study highlighted important gaps in our understanding of blue crab reproductive biology including: 1) our poor understanding of the number of females surviving to spawn in a second year, 2) the minimum sperm:egg ratio required for successful fertilization, 3) the rate and pattern of sperm decline throughout a female's reproductive life, 4) mechanisms of sperm decline other than fertilization, 5) whether reduced sperm stores lead to actual reductions in brood production (sperm limitation) in the wild.

ii. Scientific publications:

Matthew B. Ogburn, Paige M. Roberts, Kimberly A. Richie, Eric G. Johnson, Anson H. Hines. Submitted. Temporal and spatial variation in sperm stores in mature female blue crabs *Callinectes sapidus* and potential effects on brood production in Chesapeake Bay. Marine Ecology Progress Series.

iii. Patents: None yet

New methods and technology

iv. New or advanced tools (e.g. models, biomarkers): We are comparing sperm levels in female crabs to variation in trace metals of shell chemistry as part of a NOAA Saltonstall-Kennedy grant that relates shell chemistry as biomarkers for subestuary of female. maturation and mating site. As part of the project, we will be examining the rate and pattern of decline of sperm stores with time after mating.

Workshops

A NOAA press event was organized at the Smithsonian Environmental Research Center in January 2012. We presented the goals, methods, and results of our project for managers, scientists, and reporters. This resulted in media stories in Baltimore Sun, WJZ TV channel 13 Baltimore news, and Tom Pelton's blog.

This project was also presented at a workshop/ meeting of the Sustainable Fisheries Goal Implementation Team (GIT) of the Chesapeake Bay Program in Annapolis MD on June 11, 2012. We presented goals, methods and initial results to the committee of Chesapeake Bay state and federal fishery managers and reporters. This resulted in stories in the Bay Journal and WJZ TV channel 13 Baltimore news.

Presentations

Matthew B. Ogburn, Paige M. Roberts, Kimberly A. Richie, Eric G. Johnson, Anson H. Hines. 2014. National Shellfisheries Association meeting, March 30, Jacksonville, Florida.

Matthew B. Ogburn. What does the blue crab population look like throughout Chesapeake Bay. Science seminar at the East Coast Commercial Fishermen's and Aquaculture Trade Exposition, January 18, Ocean City, MD.

Anson H. Hines, Eric G. Johnson, Robert Aguilar, Margaret Kramer, Michael Goodison, Paige Roberts, and Kimberly Richie. 2012 Blue crab fishery stock dynamics: Managing for recruitment limitation. National Shellfisheries Association meeting, March 27, Seattle Washington.

Anson H. Hines, Eric G. Johnson, Robert Aguilar, Margaret Kramer, Michael Goodison, Paige Roberts, and Kimberly Richie. 2012. Evaluating Population Level Impacts of Sperm Limitation on the Chesapeake Blue Crab Stock. Sustainable Fisheries Goal Implementation Team (GIT) of the Chesapeake Bay Program, June 11.Annapolis, MD.

Anson H. Hines, Matthew Ogburn, Eric G. Johnson, Robert Aguilar, Margaret Kramer, Michael Goodison, Paige Roberts, and Kimberly Richie. 2012. Evaluating Population Level Impacts of Sperm Limitation on the Chesapeake Blue Crab Stock. Chesapeake Bay Stock Assessment Committee meeting, October 17, Solomons, MD.

Matthew B. Ogburn. Managing a Coastal Migrant: Why Ecology Data are Critical for Adaptive Management of Blue Crab Fisheries. Invited seminar. Department of Entomology and Wildlife Ecology, University of Delaware, October 8, Newark, DE.

Outreach activities/products (e.g. website, newsletter articles):

- Anson Hines, 2012. Blue crab sex – Males count! SERC Advisory Board meeting, April 28, Edgewater, MD.
- Anson H. Hines, Eric G. Johnson, Robert Aguilar, Margaret Kramer, Michael Goodison, Paige Roberts, and Kimberly Richie. 2012, Blue crab sex – the year after. Rotary Club, July 17, Annapolis, MD.
- Website: www.serc.si.edu|research\fishandinvertebratelab.
- Family Science Day on the Bay, open house at the Smithsonian Environmental Research Center, May 19, 2012 and May 18, 2013.
- Intern training: 2 undergraduate interns for blue crab biology.

b. Management outcomes - I. Management application or adoption of:

ii. New fundamental or applied knowledge:

iii. Sustainable Fisheries Goal Implementation Team (GIT) of the Chesapeake Bay Program has discussed reference points for male blue crab abundance based on operational sex ratio in Chesapeake Bay stock.

iv.

v. New or improved skills: None yet

vi. Information from publications, workshops, presentations, outreach products: None yet

vii. New or improved methods or technology: None yet

viii. New or advanced tools: None yet

c. Management outcomes - II. Societal condition improved due to management action resulting from output; examples:

i. Improved water quality: None yet

ii. Lower frequency of harmful algal blooms: None yet

iii. Reduced hypoxic zone area: None yet

iv. Improved sustainability of fisheries: Sustainable Fisheries Goal Implementation Team (GIT) of the Chesapeake Bay Program has considered setting reference points for male blue crab abundance based on operational sex ratio in Chesapeake Bay stock. It was determined that such reference points are not appropriate until there is a better understanding of the population level effects of sperm limitation.

d. Partnerships established with other federal, state, or local agencies, or other research institutions (other than those already described in the original proposal):
Collaborative work with University of North Florida Department of Biology, NOAA Chesapeake Bay Office, MD-Dept of Natural Resources, Virginia Marine Resources Commission, Potomac River Fisheries Commission, School of Aquatic and Fisheries Science of University of Washington, Department of Fisheries & Oceans Canada, Virginia Institute of Marine Science, University of Maryland Center for Environmental Science.

3. Expenditures:

b. Describe actual expenditures this period.

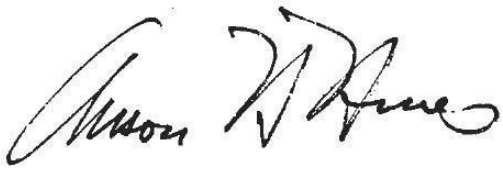
To date (February 2013) the project has spent $66,513 of the $66,513 total for the project.

c. Explain special problems that led to differences between scheduled and actual expenditures, etc.

Rate of spending was low initially because it took time to hire a Biological Technician to work on the project, and personnel costs are the majority of the budget. The Technician began in mid-May, and spending will be in line with the grant budget. A No-Cost-Extension for 1 year was granted, and the project ran through January 2014.

Prepared By: Signature of Principal Investigator; Date: 05/1/ 2014

Anson H. Hines



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