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**UNDERSTANDING THE ROLE OF *STYLOCHUS ELLIPTICUS*
AS A PREDATOR OF *CRASSOSTREA VIRGINICA*
IN CHESAPEAKE BAY TRIBUTARIES**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

by

M. KENSEY BARKER
B.S. Virginia Commonwealth University 2010

Director:
Bonnie L. Brown, PhD, Department of Biology

Committee:
Edward L. Boone, PhD, Department of Statistical Sciences and Operations Research
Alaina Hart Campbell, MS, Department of Biology
J. M. Clint Turbeville, PhD, Department of Biology
Karen M. Kester, PhD, Department of Biology

Virginia Commonwealth University
Richmond, Virginia
May 2014

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Abstract

UNDERSTANDING THE ROLE OF *STYLOCHUS ELLIPTICUS* AS A PREDATOR OF *CRASSOSTREA VIRGINICA* IN CHESAPEAKE BAY TRIBUTARIES USING A DNA-BASED METHOD

By M. Kensey Barker

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

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Director: Bonnie L. Brown, PhD, Department of Biology

Predation may be a key component of the unsuccessful restoration of the Eastern Oyster (*Crassostrea virginica*), a former keystone species in Chesapeake Bay. Here, I examine the polyclad flatworm *Stylochus ellipticus* and its potential role as an important predator of *C. virginica*. Using small-fragment size *C. virginica* specific DNA primers, oyster DNA was successfully detected in whole organisms homogenates of wild-caught *S. ellipticus* individuals. Of the 1,575 individuals tested, 68.1% tested positive, thus predation occurred. Predation did not appear to be affected by salinity or temperature; however, season did appear to have an effect on both predation and *S. ellipticus* abundance (p-value: <0.05). The findings also imply that *S. ellipticus* are highly mobile, entering the water column to reach hard substrate at various depths,

whereas previous studies suggest otherwise. These findings are useful in the planning and management of oyster cultivation and restoration. Furthermore, this study outlines a method of diet study that may be more sensitive than traditional DNA-based techniques.

INTRODUCTION

Decline of oysters in Chesapeake Bay

The Eastern oyster (*Crassostrea virginica*) was a keystone component of the Chesapeake Bay ecosystem that has rapidly decreased in number over the last century, chiefly due to overharvesting (Rothschild et al. 1994). Efforts to restore oyster populations have been largely unsuccessful (Hargis et al. 1999), but remain important because of the ecosystem services that oysters provide. Several categories of ecosystem services provided by oysters have been defined by researchers over the years and include oyster production for commercial fisheries, localized water quality improvement (water filtration), habitat creation for other aquatic organisms, nutrient sequestration (Higgins et al. 2011), eutrophication prevention (top-down algal bloom control) (Coen et al. 2007; Gillet et al. 2009; Grabowski and Peterson 2007).

Reasoning for lack of success in restoration varies, but may be attributable to disease, estuarine sediment and nutrient load (Gillet et al. 2009), competition, and predation (Newell et al. 2000, Campbell et al. 2011). Oysters are particularly susceptible to predation from crab and flatworm species 0-2 months post-settlement. *Stylochus ellipticus* is a species of flatworm known to feed on oysters during this vulnerable life stage, and this behavior has been addressed in several studies (Campbell et al. 2011; Landers and Rhodes 1970; Newell et al. 2000 and 2007).

Studies in S. ellipticus oyster predation

To examine the feeding habits and diet of *S. ellipticus*, Newell et al. (2000 & 2007) and Landers and Rhodes (1970) conducted a series of laboratory studies. Newell et al. (2007) examined the mortality rates of two species of oysters (native *C. virginica* and non-native *C. ariakensis*) from predatory flatworms (*S. ellipticus* and *Euplana gracilis*) from Choptank River, Maryland. Flatworms were placed in aquariums with oysters and oyster mortality rates were recorded (average of 44.2% for *C. virginica* over thirteen 30-day trials). Landers and Rhodes (1970) examined the behavior of *S. ellipticus* under varying saline conditions. Flatworms were simultaneously presented two food items (barnacle and oyster tissue) and meal preferences were recorded. The authors found that, under low salinity conditions, *S. ellipticus* preferred oyster meals, whereas at higher salinities, *S. ellipticus* preferred barnacle meals. It also has been suggested that oyster predation by *S. ellipticus* is linked to season, as inadvertently observed during a 2010 oyster spat study (Campbell et al. 2011). Oyster larvae were released in a tank containing biofouled porcelain tiles from Little Wicomico River, Virginia collected every three weeks from Spring to early Fall. They noted predation and presence of *S. ellipticus* in summer months, but not in spring months.

Methods of studying predator diet

Unfortunately, studies conducted in laboratory settings only offer inferences on the diet preferences and feeding behavior of predators. To accurately study predator diet, predators should be studied in their natural environment. Scientists have attempted this by utilizing various methods, each with their own set of pros and cons; however, *in situ* study efforts have

chiefly been limited to large mammalian predators, such as large cats and pinniped species. This is due both to the fact that they are charismatic megafauna (a.k.a., attractive to the general public for conservation), and because they are easily viewed, monitored, and studied (Davison et al. 2002). Direct observation, the simplest method of dietary study, offers the least impact on the environment and the predator; however, observation is often limited by the size of the predator and the location of its habitat. For example, cryptic organisms such as aquatic and soil-dwelling invertebrates are exceptionally difficult to study (Blankenship and Yayanos 2005).

Diets also may be studied by analyzing scat or gut content by: 1) visual inspection, which tends to overestimate the importance of vertebrate prey with hard-parts (beaks, bones, scales) and underestimate the importance of soft-bodied prey (invertebrates) (Dunshea 2009); 2) monoclonal antibody testing, which utilizes highly specific epitopes for organism identification, but requires intense optimization for every new organism analyzed (Dunshea 2009; Symondson et al. 2002; Zaidi et al. 1999), or 3) using a metagenomic approach, wherein genetic material obtained directly from environmental samples is subjected to PCR to detect and even quantify functional and taxonomic diversity (Dunshea 2009; Symondson et al. 2002; Zaidi et al. 1999). For small vertebrates and invertebrates, visual inspection of the scat and gut-content is inaccurate due to low concentration of undigested parts (Côté et al. 2013; Dunshea et al. 2009), which leaves monoclonal antibody or PCR-based testing the most reasonable methods for diet analysis. However, antibody testing is highly specific, analyses are time-consuming and often not ideal for non-model organisms (Dunshea 2009; Zaidi et al. 1999). Because of the challenges associated with dietary analysis, there has been a shift in the scientific community to optimize the DNA-based approaches.

DNA-based approaches to studying predator diet

Recent advances in DNA-based technologies have allowed scientists to examine the diet of predators in their natural environment. DNA-based approaches, such as PCR and next-generation sequencing, have been used to qualify (and sometimes quantify) diet composition. Depending on the digestion rate of different predators, DNA will generally remain intact long enough for extraction, amplification, and sequencing (Blankenship and Yayanos 2005). Because DNA in digested material can be highly degraded, high copy number, small fragments tend to be the most informative amplification targets (Dunshea 2009). Even with degraded DNA, taxonomic resolution is drastically improved from traditional diet-analyses and individual prey species can often be distinguished (Côté et al. 2013).

There are three approaches to using PCR to analyze gut contents. The first is species-specific amplification. Researchers have been successful in designing species-specific primers that amplify prey DNA from the gut of predators (Agusti et al. 2003; Blankenship and Yayanos 2005; Côté et al. 2013; Zaidi et al. 1999). DNA-based techniques have been shown to be sufficiently sensitive to detect DNA from even a single mosquito egg in a full-beetle homogenate (Zaidi et al. 1999). However, this approach is limited because it requires *a priori* diet knowledge, so for generalist predators that feed on many different species, it is not likely to provide a precise description of diet (Côté et al. 2013). A second, approach involves targeted amplification from material present in the gut using universal primers for rDNA and/or mitochondrial DNA (mtDNA), followed by sequencing the amplified fragments, and finally parsing the sequences into a “species profile.” This approach has been used successfully to provide a representative survey of organisms in environmental samples (Dollive et al. 2012, Pawlowski et al. 2012). A

third approach is shotgun metagenomic analysis wherein total DNA from the gut/organism is fragmented and sequenced without prior PCR (Pond et al. 2009). This latter approach allows comprehensive assessment of total microbial and eukaryotic diversity by detecting genomic differences within and across environments (Huson et al. 2011). However, it remains inordinately expensive to perform full metagenomic sequencing for more than one or two samples.

For the purposes of this study, I am interested in the role of *S. ellipticus* as a predator of *C. virginica*, therefore I adopted the PCR-based approach and utilized species-specific microsatellite loci to investigate diet. Results from this study will help us to better understand the impact of *S. ellipticus* on *C. virginica* populations in the Chesapeake Bay.

Study objectives

This study will: 1) examine seasonal, temporal and spatial variation in *Stylochus ellipticus* abundance; 2) demonstrate whether or not *S. ellipticus* in selected Chesapeake Bay tributaries consume *C. virginica*; and 2) investigate relationships among environmental variables that putatively affect *S. ellipticus* diet-preference in the Chesapeake Bay areas studied.

MATERIALS AND METHODS

Sampling

Specimens of *Stylochus ellipticus* (Girard 1850) were collected from several sites in Chesapeake Bay every three weeks for five months from June-September 2013, at three locations associated with the Virginia Institute of Marine Science (VIMS) annual spatfall survey at sites in the lower James, Great Wicomico, and Piankatank Rivers (Figure 1). Samples also were collected at an oyster farm site along Little Wicomico River, but for supplementary data only as sampling conditions were inconsistent with those from the three VIMS sites.

For each collection site, two sets of three ceramic tiles were suspended on “tile-hangers” fixed to a rope anchored by a cinder block at the base, and a crab buoy at the surface. For each rope, tile-hangers were suspended approximately 1 m below the water surface and 1 m above the bottom (Figure 2). At one Piankatank River site, the water level was insufficient for two sets of tile-hangers; therefore, only one tile-hanger was suspended in the water column, approximately 1 m below the water surface (Table 1).

Tiles were suspended for three weeks before collection to allow for sufficient colonization of *S. ellipticus*. Collectors were deployed at three natural or artificial oyster reef sites within each of the three rivers. At the Little Wicomico River oyster farm site in Heathsville, Virginia, ropes were deployed at three locations: two within 1 m of an array of floating oyster trays and one approximately 5 m from the oyster floats. The water column at the Little

Wicomico River farm site is shallow at this sample area, restricting sampling to only one tile rack per sampling rig.

Environmental data for the VIMS sampling sites were generously made available to me by Melissa Southworth from VIMS for this study. Temperature (°C) and salinity (ppt) were measured using a handheld electronic probe (YSI Pro2030) at the time of sampling, approximately 0.5 m off the river bottom. *C. virginica* spat (newly settled oysters) counts were conducted at VIMS by Southworth and colleagues. Rigs of 10 oyster shells were deployed weekly to allow for oyster larvae recruitment. Dried shells were visualized under a dissecting microscope, and number of spat was recorded per shell. The total surface area of each 10 shell-string was equal to that of a tile-suspension rig with three tiles. Further detail is available in the published annual spatfall report for 2013 (Southworth and Mann 2014).

At the times of tile collection, hangers were pulled to the surface and tiles were removed and placed in separate labeled containers according to “top” and “bottom”, and covered with ambient water. After collection, barnacles and/or other biofouling organisms were removed from ropes as needed, then new tiles were applied to hangers and suspended on-site for the next sampling cycle. Tile containers were held in a cooler at ambient temperature and transferred to the laboratory within 12 hours where they were thereafter observed for 48-72 hours to retrieve *S. ellipticus* as they collected at the surface. Up to 48 *S. ellipticus* individuals were collected per site per sampling date and stored separately in 96-well plates in 80% ethanol to reduce incidence of DNA degradation. These 48 individuals were selected haphazardly upon collection to allow for random size selection (2-30 mm in length). In cases where counts exceeded 48 individuals, excess samples were pooled in RNAlater (Qiagen) and archived at -80°C.

Preparation of DNA and PCR amplification

As the gut of *S. ellipticus* extends throughout the body, removing the gut from the flatworm is impractical. Instead, whole-organism DNA extraction was performed for up to 48 individuals per sample using Denville Scientific DirectAmpTM. Prior to extraction, ethanol was removed by pipetting to remove as much co-occurring biota as possible. Primers targeting oyster microsatellite DNA were used to evaluate whether or not *C. virginica* was present in the gut (Table 2). Because oyster microsatellites are often affected by numerous null alleles (alleles that don't amplify, putatively because of mutations at the 3' end of one or both primers; Wang et al. 2010), the detection assay included two microsatellites targets RUCV60 and RUCV164, to reduce incidence of Type II error from the presence of null alleles. PCR amplifications were conducted using GoTaq® Green (Promega) and amplicons were resolved using high throughput E-Gel® Agarose Gel Electrophoresis (Life TechnologiesTM).

Scoring of presence-absence

Presence-absence of *C. virginica* was scored based on previously reported fragment size ranges for RUCV60 (~100-120 bp) and RUCV164 (~240-280 bp; *op. cit.*). If bands near the expected sizes appeared for either primer set, the sample was considered positive for oyster presence. To prevent scoring primer-dimers (PDs) as oyster DNA amplicons, any bands appearing <100 bp were scored as negative for oyster DNA. This served to decrease Type I error incidences (false-positives); however, it increased the probability of a Type II error (false-negatives). This is addressed further in the discussion below.

Statistical Analysis

To determine if *S. ellipticus* abundance was influenced by seasonal, temporal and spatial factors, Analysis of Variance (ANOVA), Kruskal-Wallis and Wilcoxon Rank Sum Tests were conducted in R (R Core Team 2014). Abundance data were analyzed using non-parametric tests as residual plots suggested parametric test assumptions were violated, although ANOVA data are presented in some cases. Scatter plots and box plots were generated in R, whereas additional plots displaying temperature, salinity and abundance data were generated in Microsoft Excel 2010.

To determine the potential for *S. ellipticus* as a predator of young oyster across sites and times, data were parsed to eliminate instances where *S. ellipticus* abundance was 0. Data were analyzed using the package “lme4” (Bates et al. 2013), and logistic regressions were performed to determine if *S. ellipticus* predation was different across sites, salinity, temperature, and time. Refer to Appendix A for the R scripts used in these analyses.

Additional linear regression multivariate test analyses were performed in the program PAST to determine the significance of *S. ellipticus* predation across ecological variables including water depth (surface/benthic), temperature, salinity, and site (Hammer et al. 2001). All tests were evaluated for significance at $\alpha=0.05$.

RESULTS

Seasonal patterns of S. ellipticus abundance

Over the course of this study, approximately 7,930 *S. ellipticus* were collected. More than half of these individuals were retrieved on the first collection date, June 20, 2013: 4,299 individuals (Figure 3). An Analysis of Variance (ANOVA) suggested that abundance was dependent on date sampled, thus season (overall p-value: 0.0486). However, upon analysis of the residuals, I determined that the data may violate some assumptions of ANOVA. Therefore, I performed non-parametric analyses of variance: a Kruskal-Wallis Rank Sum Test resulting in the finding that differences were nonsignificant using this test.

Temperature (Figure 4) and salinity (Figure 5) fluctuated over the course of the study. I found no significant relationship between *S. ellipticus* abundance and these factors independently and together. There was, however, a weak positive correlation between salinity and abundance ($r=0.14$, Figure 6). Salinity in this study ranged from 2.1 ppt to 19.6 ppt in this study. Furthermore, no *S. ellipticus* were found when salinity was below 7.6 ppt, and very few were found in waters of salinity below 11.3 ppt. All salinities below this point were observed in Deep Water Shoal and Point of Shoal of the James River. The highest abundance observations were found at 19.1 ppt (1527 individuals), 15.4 ppt (1430 individuals), and 14.7 ppt (828 individuals).

Temporal influences on S. ellipticus abundance

Distribution plots of *S. ellipticus* abundance across sampling sites suggested that there may be temporal variation in *S. ellipticus* abundance (Figure 7). Although Piankatank and Great Wicomico Rivers had greater overall *Stylochus* abundance than James River, analyses found no significant relationship between river sampled and abundance. Among sampling sites within river systems, however, I did find significant among-site variation when both benthic and surface tiles were considered together (Kruskal-Wallis Tests: p-values for surface tiles: 0.09, benthic tiles: 0.21, all tiles: 0.01).

Spatial influences on S. ellipticus abundance

Post hoc analyses of the influences of date, temperature and salinity, system, and site were performed three ways: with surface tiles only, benthic tiles only, and with both. I did this because I found significant differences between surface abundance and benthic abundance of *S. ellipticus*. In most sites and dates sampled, I found greater *S. ellipticus* abundance on surface tiles than on benthic tiles (Figure 8, Wilcoxon Test: p-value: 0.0006).

Spat and S. ellipticus abundance

I found a weak negative correlation between spat abundance and *S. ellipticus* abundance ($r = -0.146$), but no significant relationship was observed between VIMS spat counts and *S. ellipticus* abundance. However, visual interpretation showed a slight shift in abundances for the two (Figure 9). No spat were recorded until July, where it appeared *S. ellipticus* abundance was decreasing.

Predation of C. virginica by S. ellipticus

By subsampling from each site on each date, 1,575 *S. ellipticus* flatworms were analyzed for the presence of *C. virginica* DNA (1,318 surface, 257 bottom); 68.13% tested positive (68.40% surface; 66.93% bottom). The proportion of *S. ellipticus* testing positive for *C. virginica* did not vary significantly with salinity or temperature; however, there were some significant differences in proportion among dates (Table 3). We found the greatest proportion of *S. ellipticus* testing positive for *C. virginica* in June (76.2%) then in late July/early August (60.4%). However, further statistical tests in R and PAST suggested that some of this variation may have been due to variation among sites within tributaries (a random effect in this study).

Of the two microsatellites used to detect Eastern oyster DNA in the gut of *Stylochus*, the smaller locus, RUCV60, amplified 919 times. Of these, it amplified 624 times when the locus producing the larger amplicons, RUCV164, did not amplify. The larger locus, RUCV164, amplified 353 times. Of these, it amplified 58 times when RUCV60 did not amplify.

Results from Little Wicomico River

Data retrieved from the Little Wicomico River site were not used for statistical analyses, as sample collection methods were inconsistent with those from the other three river systems. Findings from these sampling events are presented separately here. Only four *S. ellipticus* individuals were retrieved during the sampling period, two on June 20 and two on July 11. Of these four flatworms, the June flatworms were negative for oyster DNA presence, and the July ones were positive. Salinity at this site ranged from 9.8-18.0 ppt, 12.5 ppt average over the sampling period (sd: 2.7 ppt). Temperature ranged from 5-21°C (average: 16.8°C; sd: 5.1°C).

DISCUSSION

*Seasonal, temporal, and spatial patterns of *S. ellipticus* abundance*

Understanding predator abundance across seasonal, temporal, and spatial gradients is an important component of oyster restoration and conservation success. Restoration efforts such as spat-on-shell and reef seeding may be thwarted by predation if predator abundance is too high. Long-term monitoring of predator abundance, or variables that may influence predator abundance, is therefore useful in management of these restoration and farming practices by establishing “high risk” criteria for oyster predation.

We found the highest abundance of *S. ellipticus* in June, particularly in Great Wicomico and Piankatank Rivers. The implication of this finding is that it may not be advisable to outplant smaller sized juvenile oysters during this time frame. Our data only cover May 30th - September 26th of 2013, but additional haphazard sampling conducted outside this study by Dr. Jon Allen (College of William & Mary, pers. comm.) suggested that *S. ellipticus* are present in many Chesapeake Bay tributaries even in the winter months.

Oysters may be more susceptible to predation in certain rivers and even, as suggested in this study, sites within river. Other studies have found varying amounts of *S. ellipticus* in different systems. For instance, one study found numerous flatworms in a cage-exclusion predator study in Choptank River (Newell et al. 2000), whereas another study found very few sites to be populated by *S. ellipticus* within York River (Sagasti et al. 2000). Thus, longer

sampling periods and standardized sampling methods should be considered. Other sites should be investigated over a relevant study period to determine what kind of long-term temporal pattern of *S. ellipticus* abundance exists. Such data would in turn provide impetus for refining management practices to address risk of *Stylochus* predation on oysters.

These data also suggest that *S. ellipticus* prefer, where available, habitat closer to the water surface. Although smaller *S. ellipticus* are recognized as mobile and free-swimming, little was previously known about the ability of these flatworms to access “off-bottom” habitat (Newell et al. 2000). The current findings strongly suggest that even larger *S. ellipticus* flatworms are highly mobile and not only *can* they access food suspended above the benthos, they *prefer* habitat above the benthos. These findings are further supported by a previous report on *S. ellipticus* by Brown et al. (2013). The implication is that oyster farms that utilize floating cages to hold very small oysters may be susceptible to high levels of *S. ellipticus* predation. A caveat is that very few *S. ellipticus* individuals were found on tiles suspended just below the surface at the oyster farm sampled in this study (4 individuals, 50% positive for oyster DNA).

It is reasonable to assume that seasonal, temporal, and spatial differences in *S. ellipticus* abundance may be influenced by environmental factors. For instance, the small positive correlation between salinity and abundance (Figure 8) suggests that *S. ellipticus* may prefer salinities above 10 ppt, and this pattern is being investigated further by Dr. Jon Allen (W&M). Identifying key environmental variables other than temperature and salinity also will be essential for establishing metrics to evaluate *S. ellipticus* predation risk. Particular parameters yet to be examined but which may be important include: time of day when active, dissolved oxygen, and flow rate.

Predation of S. ellipticus on C. virginica

Interestingly, the current data suggest that *S. ellipticus* abundance, and possibly *C. virginica* spat recruitment, may exhibit a Lotka-Volterra predator-prey dynamic. There was a weak negative correlation between abundances of both organisms; however, this may simply be a reflection of the fact that predator-prey dynamics in marine communities are highly variable among different seasonal, temporal, and spatial gradients (Connolly et al. 1990). Furthermore, metagenomic sequencing of *S. ellipticus* DNA preparations (Appendix B) suggested that *S. ellipticus* have a diverse diet; therefore, this putative model of predator-prey dynamics may not be appropriate for this relationship. As this study constituted a single ecological snapshot of these patterns, one cannot make inferences as to the long-term relationship between oyster spat recruitment and *S. ellipticus* abundance. However, with few exceptions, predation was relatively constant throughout the study period at approximately 68%, indicating that it is possible that this dynamic is important during the summer months. Ultimately, as 76% *S. ellipticus* tested in June were positive for oyster DNA at a time when the VIMS oyster spatfall survey revealed no oyster spat recruitment, it is reasonable to suspect that oyster spat were under heavy predation by *S. ellipticus*. Upon the elimination of oysters as a food source, *S. ellipticus* populations may have migrated or shifted prey, allowing for successful later recruitment of oyster spat.

Stylochus variation among tributaries in Chesapeake Bay

We did not find statistically significant evidence to support that *S. ellipticus* abundance varies among rivers; however, visual inspection of distribution data and statistical analyses among samples suggest that Piankatank and Great Wicomico Rivers have higher abundance (Figure 8),

and that the extreme variation among sites within rivers may have masked significant differences. With more replication and more sites sampled, some sites may prove to be outliers. A case representing this possibility is Glebe Point in Great Wicomico River, which had different characteristics than most of the other VIMS sites in this system. It was deeper (sometimes 6 m deep) with a greater width across the river, was prone to periods of hypoxia and anoxia during the summer (Southworth and Mann 2014), and was located beneath a large bridge with commuter traffic. These factors may have caused locally poor water quality and thus made it unsuitable for *S. ellipticus* colonization.

Although *S. ellipticus* abundance appeared to decrease from May-September, the proportion of *S. ellipticus* that tested positive for *C. virginica* differed only among a few sample dates (August predation appeared to be lower overall). This important finding contradicts previous reports (Landers and Rhodes 1970) suggesting that flatworm diet changes according to temperature and salinity. Further study should include fully annual data to more thoroughly study to the theory of variable ingestion based on environmental condition.

Notes on methods and implications on the field of DNA-based diet study

Scoring for the predation analyses employed a 240-280 bp (RUCV164) and a 100-120 bp (RUCV60) region of oyster genomic DNA and revealed, as suspected, that larger fragments were sparse in the highly degraded gut content of *S. ellipticus* flatworms. These two loci were used in conjunction because resulting PCR products were sufficiently different in length to resolve in a single lane of a high throughput 2% E-Gel. I did, however, encounter an issue associated with the RUCV60 locus. To detect oyster DNA present at low signal among high concentrations of

predator DNA, it was necessary to increase PCR amplification cycles to 50X, at which point primer dimers (PDs) were suspected to occur. High amplification cycles can increase the occurrence of PDs dramatically, even where no 3' complementary bps exist (Brownie et al. 1997). To assess the occurrence of PDs in selected RUCV60 amplicons, several fragments were processed with Exo-SAP-IT® (Affymetrix) and commercially Sanger sequenced (Amplicon Express). Among these sequences, some fragments as small as 85 bp were found to be microsatellite alleles with the expected tandem repeats, whereas other fragments as large as 80 bp were PDs characterized by primers closely linked with variable intervening nucleotide sequences. Eliminating RUCV60 from the study was rejected as an option due to an important observation: over two-thirds of samples confirmed positive for oyster DNA (several of these confirmed by sequencing) were only positive for RUCV60, and not the larger RUCV164 locus. Thus, it was determined that RUCV60 was necessary for greater understanding of *C. virginica* presence in *S. ellipticus* gut content and that to eliminate the bias associated with a Type II error (scoring a PD as an allele), all fragments < 100 bp would be scored as negative. Because of this approach, and knowing that some fragments < 100 bp were true alleles, the proportions of *S. ellipticus* with oyster presence in the gut are almost certainly underestimates.

Current DNA-based studies of predator diets typically utilize primers that target a 650 region of the mitochondrial COI gene, “the barcode of life” (Folmer et al. 1994; Hebert et al. 2003). Universal primers can be designed from this region that target broad groups of Metazoa, such as marine invertebrates (Geller et al. 2013, Leray et al. 2013), which may then be sequenced for further taxonomic resolution. Other studies use species-specific primers, like the study described herein, often targeting larger regions on the order of 1000 bp. Results of the current

study raise questions regarding the accuracy of diet coverage for such studies, as larger DNAs may be degraded quickly in guts of predators. The current findings suggest the need for design of smaller-sized amplicons, such as those designed by Leray et al. (2013) (~300 bp) or those utilized herein.

To eliminate PDs that interfere with the interpretation of small-fragment amplicons, blocking primers should be considered. Blocking primers are oligonucleotides designed as an extension of the prey-target primers that have a predator-specific extension that allows for annealing to predator DNA. A 3' spacer is included that blocks elongation by *Taq* Polymerase when bound to predator DNA. This eliminates predator DNA during the first few cycles of the PCR reaction, and therefore reduces primer competition for target DNA (Leray et al. 2013). This, in turn, allows for more stringent annealing temperatures as well as reduced numbers of amplification cycles, ultimately reducing the occurrence of PDs (Brownie et al. 1997). Blocking primers, however, were not used in this study, as reliable genomic data for *S. ellipticus* are not currently available.

CONCLUSIONS

Ultimately, this study provides data that aid understanding of the impact of *S. ellipticus* on *C. virginica* populations in Chesapeake Bay. This study suggests that there may be seasonal, temporal, and spatial aspects to patterns in *S. ellipticus* abundance. On the other hand, there appears to be no evidence that *S. ellipticus* predation on *C. virginica* is influenced by temperature or salinity. This is an important finding, as previous studies suggest that *S. ellipticus* have dietary preferences that vary based upon these environmental variables (Landers and Rhodes 1970). Perhaps the most profound finding was that the vast majority of *S. ellipticus* analyzed tested positive for oyster DNA (68.1% of 1,575 tested overall), and that this proportion may be underestimated due to the scoring triage. Finally, this study provides an outline for a method of DNA-based prey detection in predator gut using small fragment-size prey-specific target regions.

Table 1. Site information for each Chesapeake Bay tributary sampled. Depths listed are approximate because of tide fluctuations.

River	Site Name	Depth (m)	Approx. Location
Little Wicomico	Land Dock A	1-2 m	37.8983 N, -76.2998 W
	Land Dock B	1-2 m	37.8983 N, -76.2998 W
	Land Dock C	2-3 m	37.8983 N, -76.2998 W
James River	Day's Point	~3 m	37.0176 N, -76.5759 W
	Point of Shoal	2-3 m	37.1074 N, -76.6550 W
	Deep Water Shoal	2-3 m	37.1685 N, -76.6138 W
Piankatank	Ginney Point	3-4 m	37.5299 N, -76.4032 W
	Wilton Creek	<1.5 m	37.5198 N, -76.4149 W
	Burton Point	2-3 m	37.5102 N, -76.3499 W
Great Wicomico	Whaley's East	2-3 m	37.8164 N, -76.3077 W
	Rogue Point	1-3 m	37.8480 N, -76.3338 W
	Glebe Point	>5 m	37.8473 N, -76.3684 W

Table 2. Primers used in this study for presence-absence analysis (*C.virginica*) xxxx xxxxx xxx.

Name	Sequence (5' to 3')	Author
RUCV60	CAAGTTATGATAAGAGTGACAGG CACGACGTTGTAAAACGACCATACACAGAAACACACATACAG	Wang et al. 2007
RUCV164	GGAAGAGTGTTTTGAATTGACG CACGACGTTGTAAAACGACATATGTGATCCCCACACAAGG	Wang et al. 2009

Table 3. Significance values for proportion of *S. ellipticus* samples that tested positive for oyster DNA listed pairwise for each sample date. Significant p-values are shown in bold. Total proportion values (P) listed below.

	20-Jun	11-Jul	1-Aug	22-Aug	12-Sep	26-Sep
20-Jun	1.000	0.974	0.001	0.000	0.080	0.174
11-Jul		1.000	0.034	0.750	0.513	0.786
1-Aug			1.000	0.034	0.137	0.005
22-Aug				1.000	0.390	0.330
12-Sep					1.000	0.588
26-Sep						1.000
P	0.762	0.740	0.604	0.625	0.680	0.719

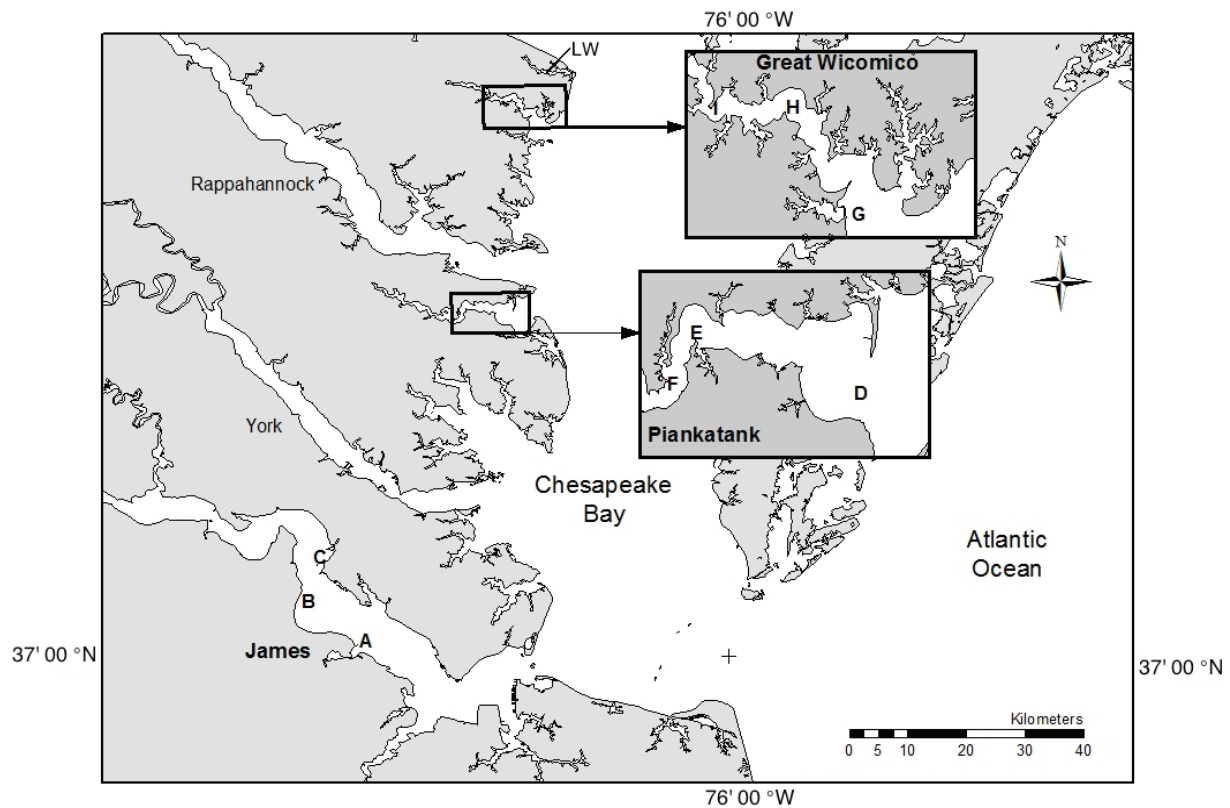


Figure 1. Map of tile sampling sites in Virginia tributaries of the Chesapeake Bay provided by Melissa Southworth from VIMS. James River: A) Day's Point, B) Point of Shoal, C) Deep Water Shoal. Piankatank River: D) Burton Point, E) Ginney Point, F) Wilton Creek. Great Wicomico River: G) Whaley's East, H) Rogue Point, I) Glebe Point. LW) Little Wicomico Sites.

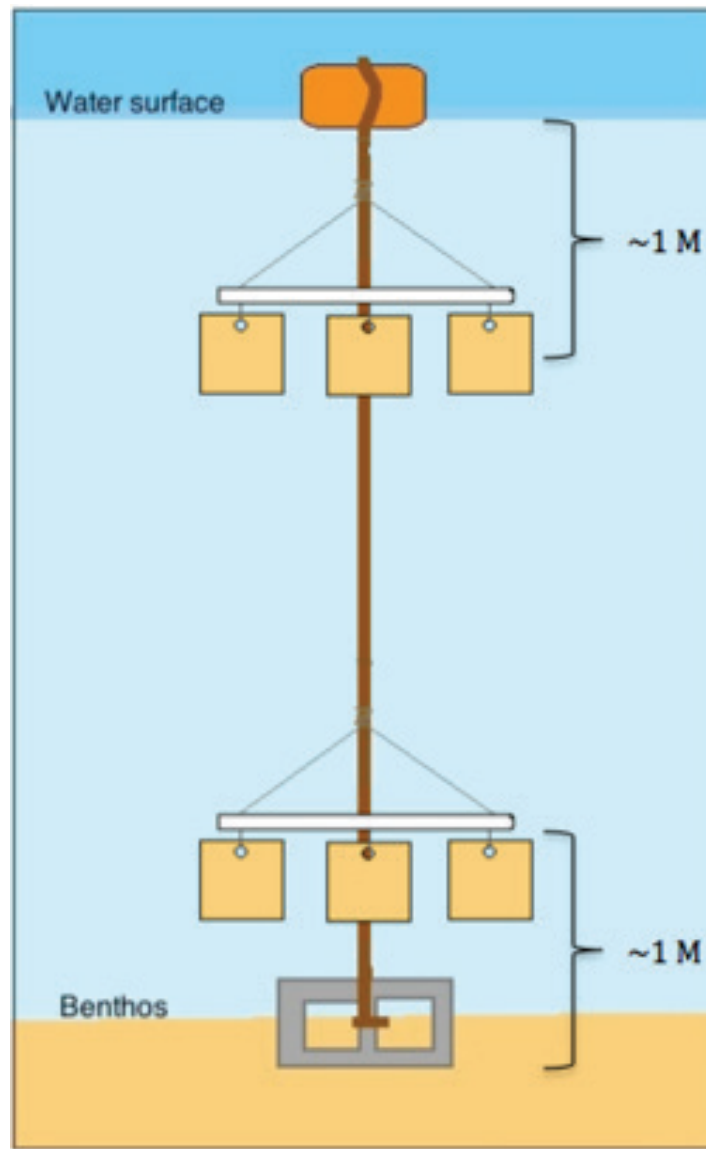


Figure 2. Tile suspension at VIMS spat collection sites, showing “surface” and “benthic” tiles.

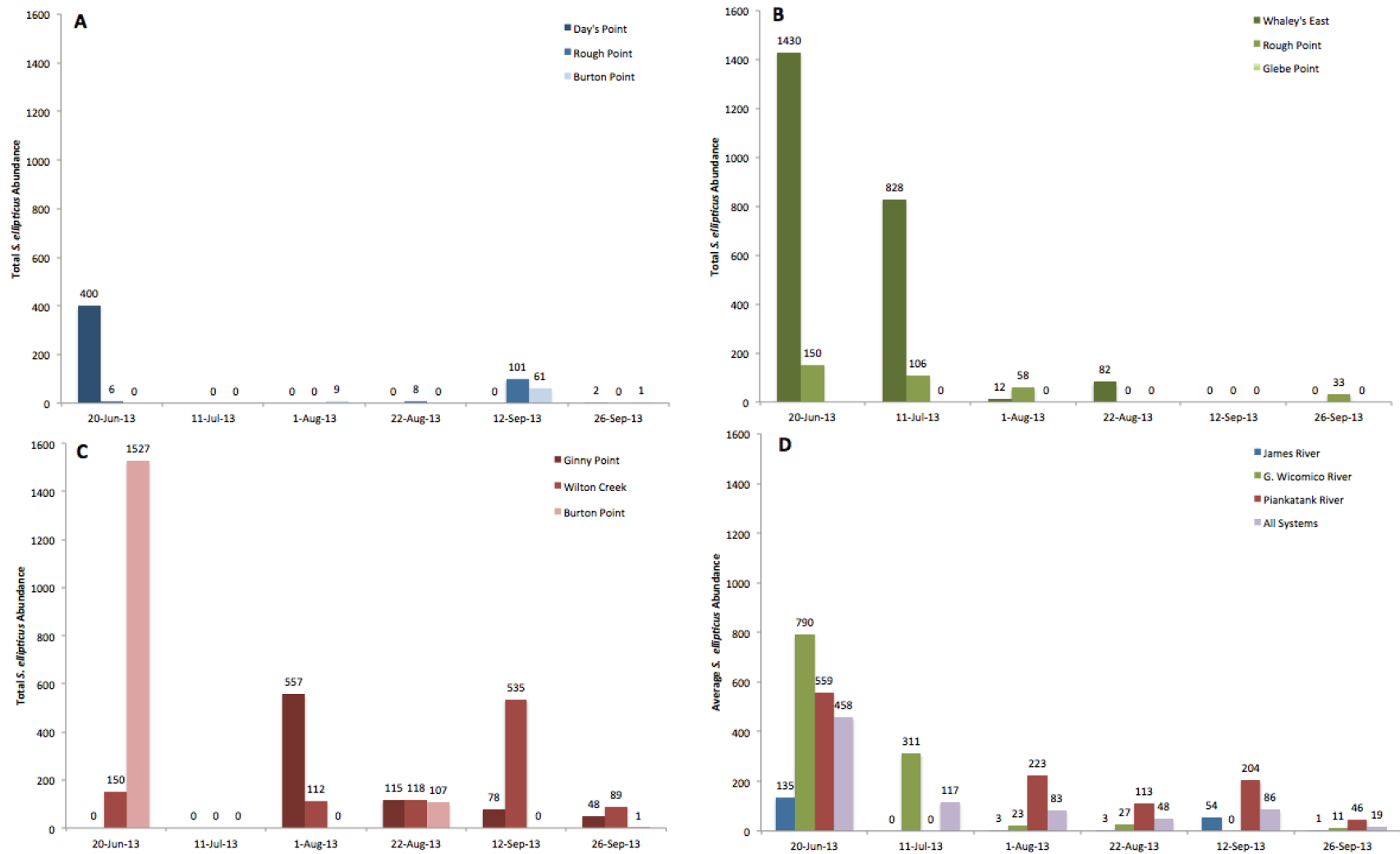


Figure 3. Bar plots of *S. ellipticus* sample abundance for sites within each river (James River: A, Great Wicomico River: B, and Piankatank River: C). Plot D contains average data across all sites for each river (J: blue, GW: green, P: red), and average across all rivers (purple), per date sampled.

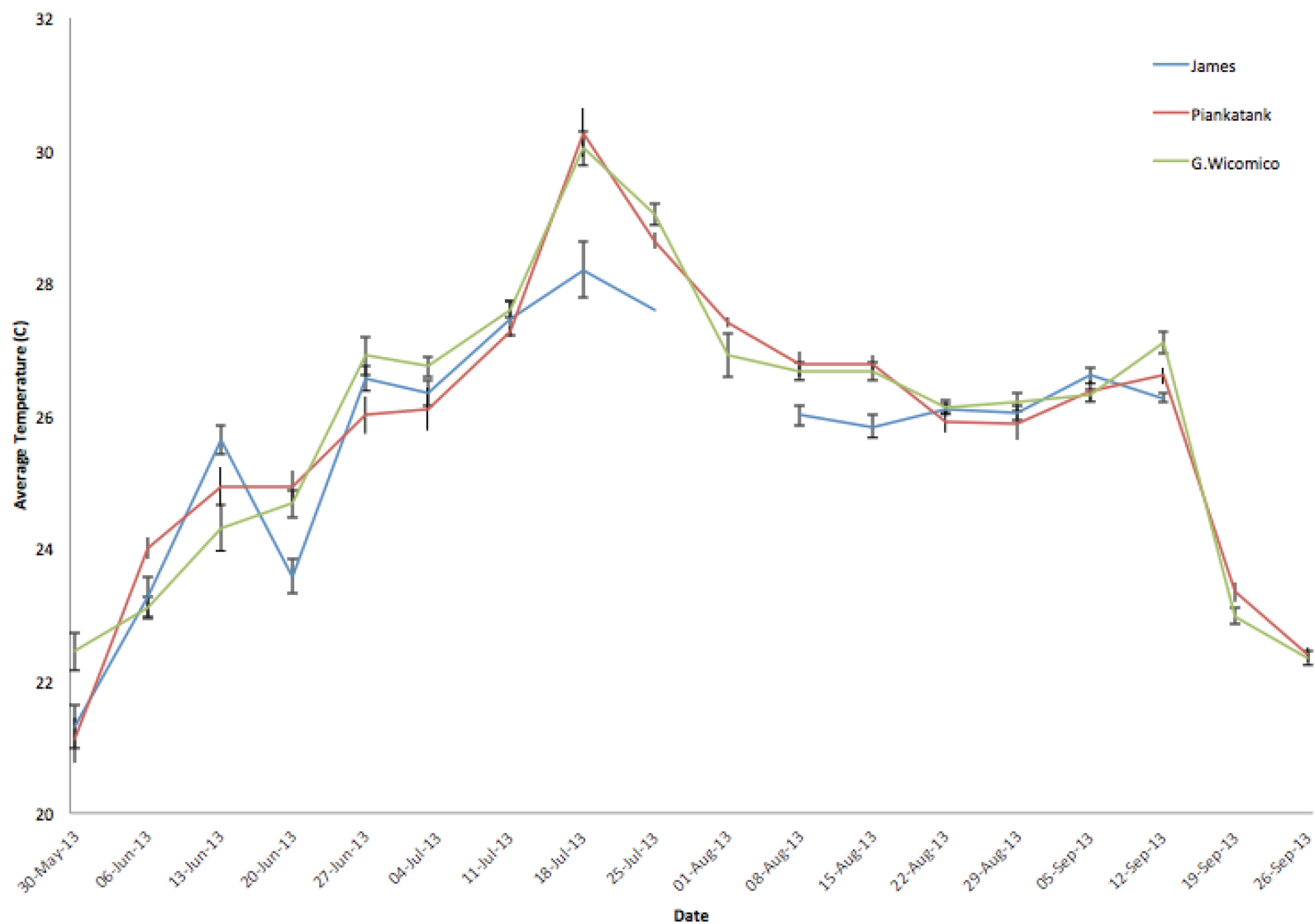


Figure 4. Line graph of average temperature of each for each river system over the course of the study with vertical standard deviation bars: Great Wicomico (green), Piankatank (red), James River (blue).

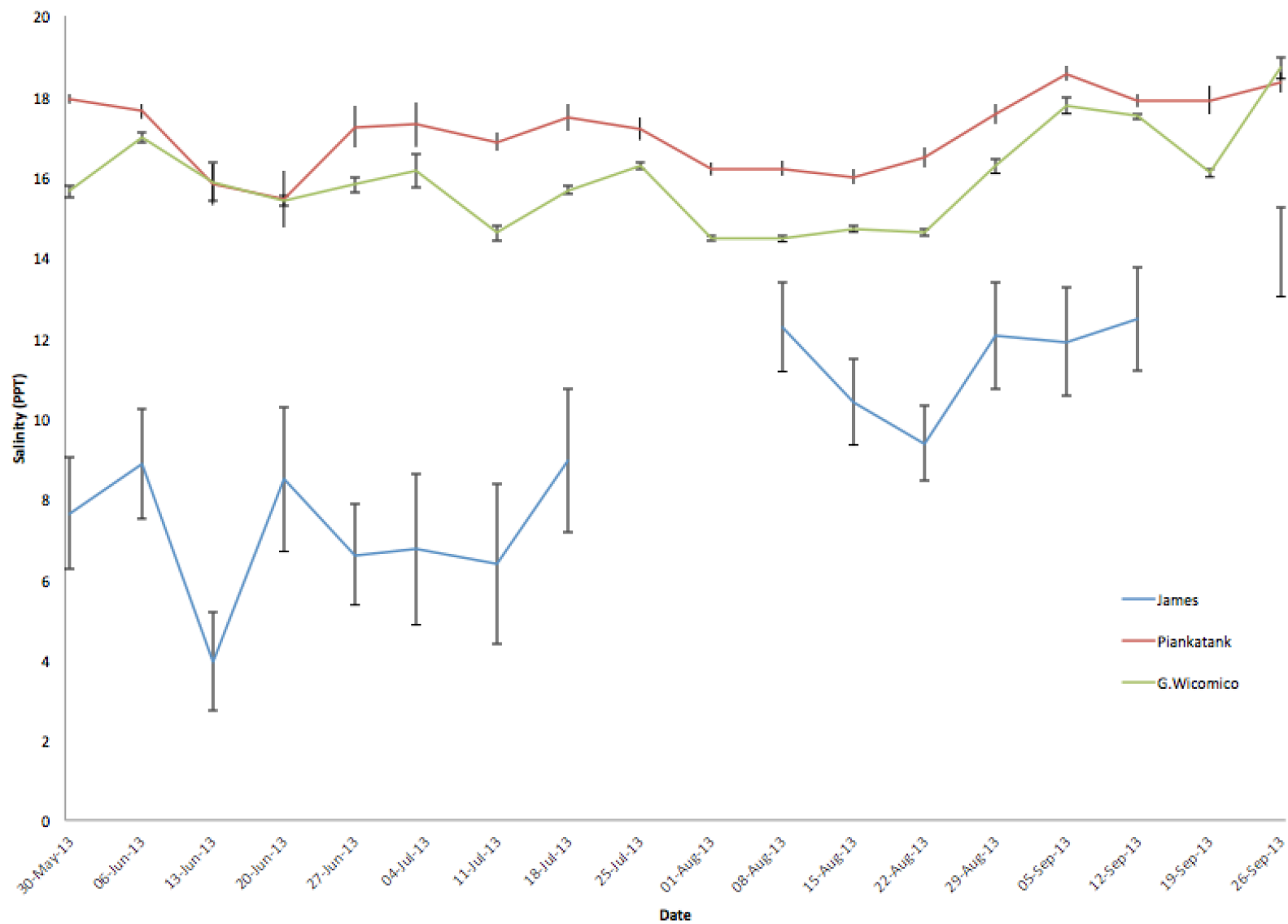


Figure 5. Line graph of average salinity of each for each river system over the course of the study with vertical standard deviation bars: Great Wicomico (green), Piankatank (red), James River (blue).

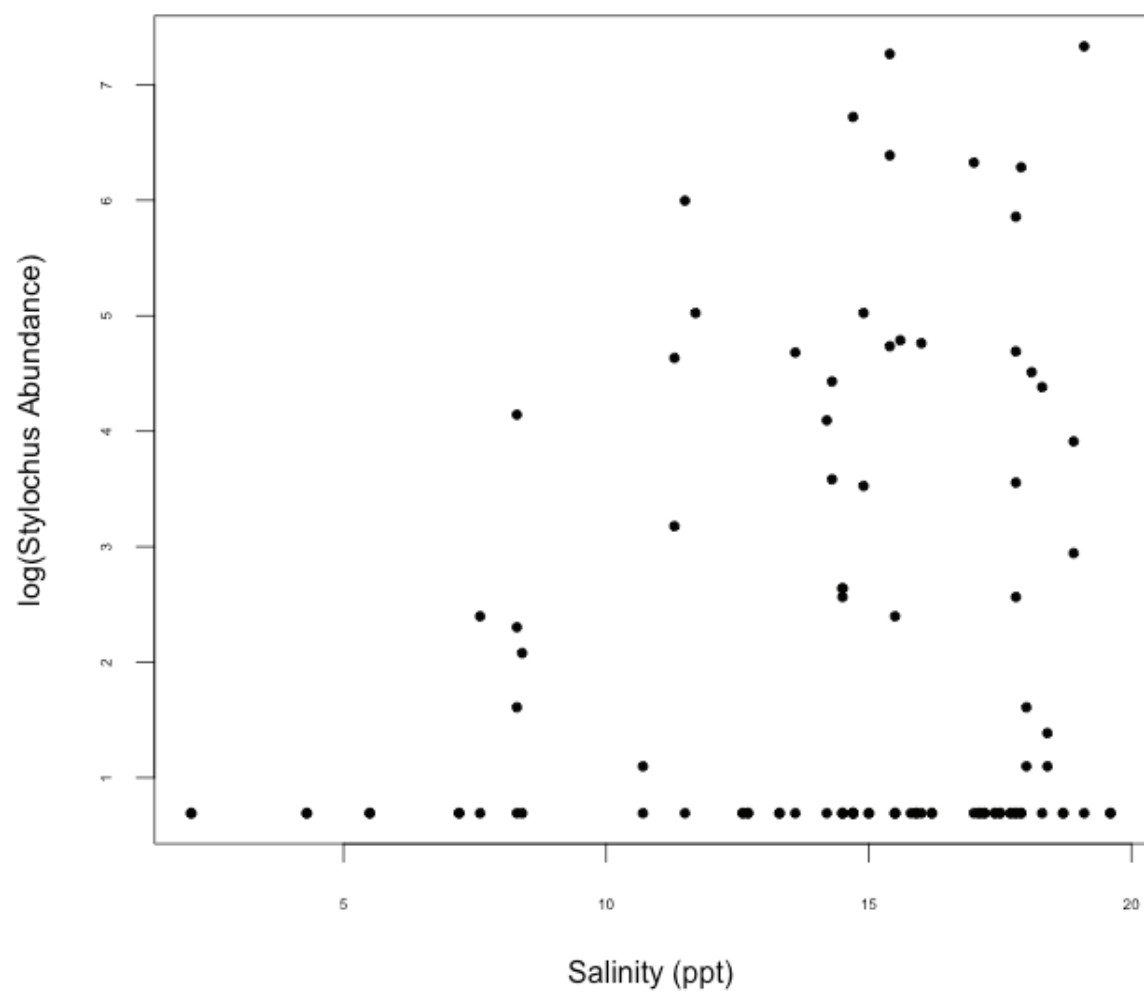


Figure 6. Scatter plot of overall *S. ellipticus* abundance as a function of measured salinity (ppt) using all data.

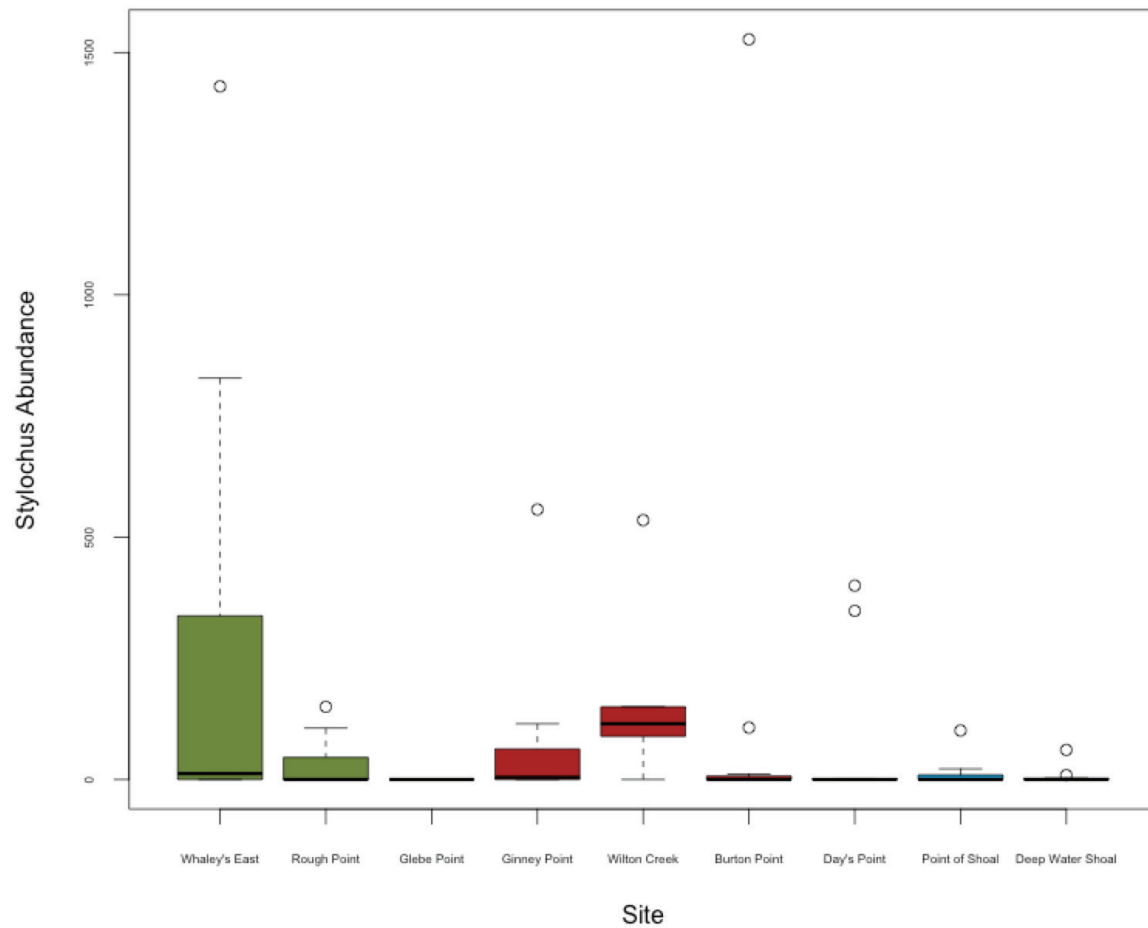


Figure 7. Overall distribution of *S. ellipticus* abundance for Great Wicomico sites (green), Piankatank River sites (red), and James River sites (blue) using all data.

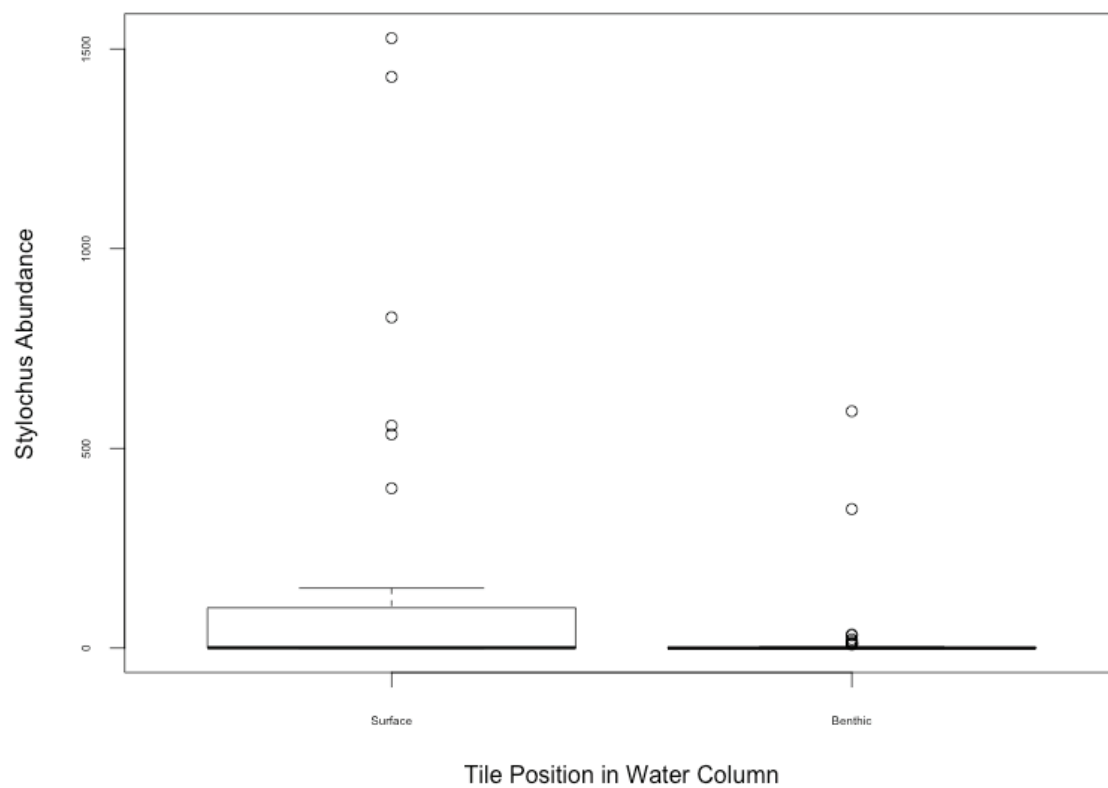


Figure 8. Distribution of *S. ellipticus* abundance according to tile suspension rack location (near surface or near bottom).

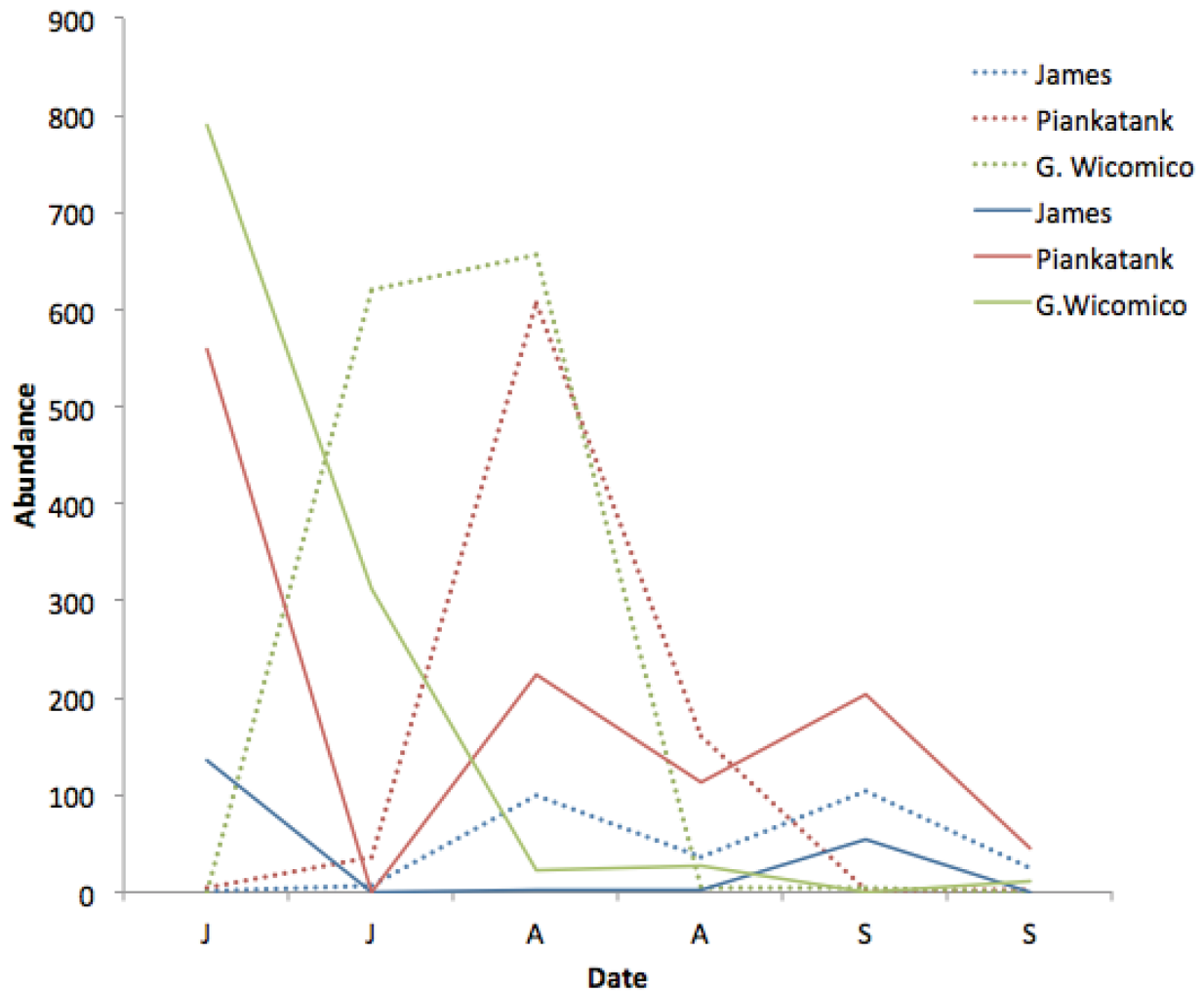


Figure 9. Total abundance of *C. virginica* spat (dotted lines) and *S. ellipticus* (solid lines) for each river: James River (blue), Great Wicomico River (green), and Piankatank River (red).

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Appendix A

R Scripts used for statistical analyses.

```
#Author: M.Kensey Barker
#Date last modified: 28Apr2014
#Statistical analyses for master's thesis (Stylochus ellipticus)
#####
#CITATIONS
citation(package = "lme4", lib.loc = NULL) #citation information for package
#Bates D, Maechler M, Bolker B, and Walker S. 2013. lme4: linear mixed-effects models
using Eigen and S4. R package version 1.1-6. <http://CRAN.R-project.org/package=lme4>.
citation() #citation for R
#R Core Team. 2014. A language and environment for statistical computing. R Foundation
for Statistical Computing. Vienna, Austria. <http://www.R-project.org/>
#set working directory
setwd("~/Desktop/Kenzrad Drive/Thesis/Data/R Statistics")
#packages
library(lme4) #for nonlinear regression (presence/absence)
```

```
#####
```

```
#-----#
#-----PT. 1-----#
#-----STYLOCHUS ABUNDANCE-----#
#-----#
#files for abundance (after reformatting pain and suffering)
a_all<-read.csv("pleasemore.csv") #field data, both top and bottom tiles
w/environmental
a_top<-read.csv("please_top.csv") #field data, top tiles w/environmental
a_bottom<-read.csv("please_bottom.csv") #field data, bottom tiles w/environmental
#for all figures, colors should be assigned by system:
  #James River: col = "deepskyblue3"
  #Piankatank River: col = "firebrick"
  #Great Wicomico River: col = "darkolivegreen4"
#tests used:
  #kruskal.test() #used for categorical
  #wilcox() #used for categorical w/two possibilities (top/bottom)
```

```
#-----#
#--ABUND~POSITION--#
#-----#
#Test for differences in tile position (top/bottom)
#parametric
oneway.test(a_all$abundance~a_all$Orientation) #p-value = 0.02605
#non-parametric
```

```

wilcox.test(a_all$abundance~a_all$Orientation) #p-value = 0.002784
#boxplot of distrubtions of abundance~position
boxplot(a_all$abundance~a_all$Orientation,
        names=c("Surface", "Benthic"),
        par(cex.axis=.5),
        xlab="Tile Position in Water Column",
        ylab="Stylochus Abundance")
#differences suggest that top tiles and bottom tiles should be analyzed separately

#-----#
#----ABUND~SITE----#
#-----#
#top tiles
#box plot (site, abund)
boxplot(a_top$abundance~a_top$code,
        names=c("Whaley's East", "Rough Point", "Glebe Point", "Ginney Point", "Wilton
Creek", "Burton Point", "Day's Point", "Point of Shoal", "Deep Water Shoal"),
        main="Site Stylochus Abundance of Surface Tiles",
        par(cex.axis=.5), #this changes the font in the x-axis
        xlab="Site",
        ylab="Stylochus Abundance",
        col=c("darkolivegreen4", "darkolivegreen4", "darkolivegreen4", "firebrick",
"firebrick", "firebrick", "deepskyblue3", "deepskyblue3", "deepskyblue3"))
#non-parametric test
kruskal.test(a_top$abundance~a_top$Site) #p-value = 0.0882
#bottom tiles
#box plot (site, abund)
boxplot(a_bottom$abundance~a_bottom$code,
        names=c("Whaley's East", "Rough Point", "Glebe Point", "Ginney Point", "Burton
Point", "Day's Point", "Point of Shoal", "Deep Water Shoal"),
        main="Site Stylochus Abundance in Benthic Tiles",
        par(cex.axis=.5),
        xlab="Site",
        ylab="Stylochus Abundance",
        col=c("darkolivegreen4", "darkolivegreen4", "darkolivegreen4", "firebrick",
"firebrick", "deepskyblue3", "deepskyblue3", "deepskyblue3"))
#non-parametric test
kruskal.test(a_bottom$abundance~a_bottom$Site) #p-value = 0.2095
#all tiles
#box plot (site, abund)
boxplot(a_all$abundance~a_all$code,
        names=c("Whaley's East", "Rough Point", "Glebe Point", "Ginney Point", "Wilton
Creek", "Burton Point", "Day's Point", "Point of Shoal", "Deep Water Shoal"),
        main="Site Stylochus Abundance",
        par(cex.axis=.5),
        xlab="Site",
        ylab="Stylochus Abundance",
        col=c("darkolivegreen4", "darkolivegreen4", "darkolivegreen4", "firebrick",
"firebrick", "firebrick", "deepskyblue3", "deepskyblue3", "deepskyblue3"))
#non-parametric test
kruskal.test(a_all$abundance~a_all$Site) #p-value = 0.01063

#-----#
#----ABUND~DATE----#
#-----#
#date top

```

```

#boxplot (date, abund)
boxplot(a_top$abundance~a_top$datecode,
        names=c("20Jun", "11Jul", "01Aug", "22Aug", "12Sep", "26Sep"),
        par(cex.axis=.5),
        xlab="Date",
        ylab="Stylochus Abundance")
#non-parametric test
kruskal.test(a_top$abundance~a_top$datecode) #p-value = 0.4429
#date bottom
#boxplot (date, abund)
boxplot(a_bottom$abundance~a_bottom$datecode,
        names=c("20Jun", "11Jul", "01Aug", "22Aug", "12Sep", "26Sep"),
        par(cex.axis=.5),
        xlab="Date",
        ylab="Stylochus Abundance")
#non-parametric test
kruskal.test(a_bottom$abundance~a_bottom$datecode) #p-value = 0.5529

```

```

#-----#
#----ABUND~TEMP----#
#-----#
#temp top
#scatter plot for (temp, abund)
plot(a_top$abundance~a_top$temp,
     par(cex.axis=.5),
     xlab="Temp",
     ylab="Stylochus Abundance",
     type="p",
     pch=20)
cor(a_top$temp, a_top$abundance) #r = 0.04302261
#scatter plot for (temp, log(abund+2))
plot(log(a_top$abundance+2)~a_top$temp,
     par(cex.axis=.5),
     xlab="Temp",
     ylab="log(Stylochus Abundance)",
     type="p",
     pch=20)
cor(a_top$temp, log(a_top$abundance+2)) #r = -0.01717795
#non-parametric test
kruskal.test(a_top$abundance~a_top$temp) #p-value = 0.3671
#temp bottom
#scatterplot (temp, abund)
plot(a_bottom$abundance~a_bottom$temp,
     par(cex.axis=.5),
     xlab="Temp",
     ylab="Stylochus Abundance",
     type="p",
     pch=20)
#scatterplot (temp, log(abund+2))
plot(log(a_bottom$abundance+2)~a_bottom$temp,
     par(cex.axis=.5),
     xlab="Temp",
     ylab="log(Stylochus Abundance)",
     type="p",
     pch=20)
#correlation
cor(a_bottom$temp, a_bottom$abund)
#non-parametric test
kruskal.test(a_bottom$abundance~a_bottom$temp) #p-value = 0.3141

```



```

#-----#
#-----ABUND~SAL-----#
#-----#
#salinity top
#scatterplot (sal, abund)
plot(a_top$abundance~a_top$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="Stylochus Abundance",
      type="p",
      pch=20)
#scatterplot (sal, log(abund+2))
plot(log(a_top$abundance+2)~a_top$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="log(Stylochus Abundance)",
      type="p",
      pch=20)
#correlation
cor(a_top$salinity, a_top$abundance) #r = 0.1714807
#non-parametric test
kruskal.test(a_top$abundance~a_top$salinity) #p-value = 0.5155
#sal bottom
#scatterplot (sal, abund)
plot(a_bottom$abundance~a_bottom$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="Stylochus Abundance",
      type="p",
      pch=20)
#scatterplot (sal, log(abund+2))
plot(log(a_bottom$abundance+2)~a_bottom$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="log(Stylochus Abundance)",
      type="p",
      pch=20)
#correlation
cor(a_bottom$salinity, a_bottom$abundance) #r = 0.1023485
#non-parametric test
kruskal.test(a_bottom$abundance~a_bottom$salinity) #p-value = 0.5846
#sal all
#scatter plot (abund, sal)
plot(a_all$abund~a_all$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="Stylochus Abundance",
      main="Abundance of Stylochus as a function of Salinity (ppt)",
      type="p",
      pch=20)
#scatterplot (sal, log(abund+2))
plot(log(a_all$abundance+2)~a_all$salinity,
      par(cex.axis=.5),
      xlab="Salinity (ppt)",
      ylab="log(Stylochus Abundance)",
      main="Abundance of Stylochus as a function of Salinity (ppt)",
      type="p",
      pch=20)

```

```

#correlation
cor(a_all$salinity, a_all$abundance) #r = 0.1394279
#non-parametric test
kruskal.test(a_all$abundance~a_all$salinity) #p-value = 0.1013


#-----#
#----ABUND~MULTI----#
#-----#
#ANOVA with temp, salinity and temp + salinity
two.wayABUND <- lm(abundance~salinity+temp+salinity*temp, data=a_top)
anova(two.wayABUND)
#sal p-value = 0.2240
#temp p-value = 0.8687
#sal+temp p-value = 0.9293


#####
#####


#-----#
#-----PT. 2-----#
#-----STYLOCHUS PREDATION-----#
#-----#
#Read in files
pres<-read.csv("binary_presence.csv") #binary data with concatenated sites
#load package for nonlinear mixed models
library(lme4)
#-----#
#----POSITION----#
#-----#
#use function glmer() for generalize linear mixed-effect models with small-option
fixed effect (doesn't work with date/salinity)
#oy is "presence/absence", binomial value
#position is fixed effect
#sitecode is random effect
prop.big.pos <- glmer(oy~position+(1 | sitecode), data=bigdata,
family=binomial(link='logit'))
summary(prop.big.pos) #p-value = 0.343972


pres1.glmer <- glmer(result~orientation+(1 | site), data=pres,
family=binomial(link='logit'))
summary(pres1.glmer) #p-value = 0.414
#-----#
#-----SITE-----#
#-----#
#site is a random effect, but may affect outcome - tested here
prop.big1 <- glm(oy~position, data=bigdata, family=binomial(link='logit'))
summary(prop.big1)
prop.big.test1 <- -2*(logLik(prop.big1)-logLik(prop.big))
prop.big.pvalue <- pchisq(prop.big.test1, df=1, lower.tail=FALSE)
prop.big.pvalue #log Like. 4.096265e-06
#site has an effect - but randomly sampled, so not known
#site is a random effect, but may affect outcome - tested here

```

```

pres.glm <- glm(result~orientation, data=pres, family=binomial(link='logit'))
summary(pres.glm)
pres.test <- -2*(logLik(pres.glm)-logLik(pres1.glmer))
pres.pvalue <- pchisq(pres.test, df=1, lower.tail=FALSE)
pres.pvalue #log Like. 1.173718e-06
#site has an effect - but randomly sampled, so not known
#-----#
#-----DATE-----#
#-----#
unique(pres$date) #this function gives me all my unique classifications in the "date"
column, allows pairwise analysis
#for each dat pairwise
presdate1 <- pres[pres$date %in% c("20-Jun","11-Jul"),]
presdate1.glmer<- glmer(result~date+(1 | site), data=presdate1,
family=binomial(link='logit'))
summary(presdate1.glmer) #p-value = 0.974
presdate2 <- pres[pres$date %in% c("20-Jun","1-Aug"),]
presdate2.glmer<- glmer(result~date+(1 | site), data=presdate2,
family=binomial(link='logit'))
summary(presdate2.glmer) #p-value = 0.000808
presdate3 <- pres[pres$date %in% c("20-Jun","22-Aug"),]
presdate3.glmer<- glmer(result~date+(1 | site), data=presdate3,
family=binomial(link='logit'))
summary(presdate3.glmer) #p-value = 0.000149
presdate4 <- pres[pres$date %in% c("20-Jun","12-Sep"),]
presdate4.glmer<- glmer(result~date+(1 | site), data=presdate4,
family=binomial(link='logit'))
summary(presdate4.glmer) #p-value = 0.080320
presdate5 <- pres[pres$date %in% c("20-Jun","26-Sep"),]
presdate5.glmer<- glmer(result~date+(1 | site), data=presdate5,
family=binomial(link='logit'))
summary(presdate5.glmer) #p-value = 0.17417
presdate6 <- pres[pres$date %in% c("11-Jul","1-Aug"),]
presdate6.glmer<- glmer(result~date+(1 | site), data=presdate6,
family=binomial(link='logit'))
summary(presdate6.glmer) #p-value: 0.0338
presdate7 <- pres[pres$date %in% c("11-Jul","22-Aug"),]
presdate7.glmer<- glmer(result~date+(1 | site), data=presdate7,
family=binomial(link='logit'))
summary(presdate7.glmer) #p-value: 0.750
presdate8 <- pres[pres$date %in% c("11-Jul","12-Sep"),]
presdate8.glmer<- glmer(result~date+(1 | site), data=presdate8,
family=binomial(link='logit'))
summary(presdate8.glmer) #p-value: 0.5131
presdate9 <- pres[pres$date %in% c("11-Jul","26-Sep"),]
presdate9.glmer<- glmer(result~date+(1 | site), data=presdate9,
family=binomial(link='logit'))
summary(presdate9.glmer) #p-value: 0.7856
presdate10 <- pres[pres$date %in% c("1-Aug","22-Aug"),]
presdate10.glmer<- glmer(result~date+(1 | site), data=presdate10,
family=binomial(link='logit'))
summary(presdate10.glmer) #p-value: 0.0338
presdate11 <- pres[pres$date %in% c("1-Aug","12-Sep"),]
presdate11.glmer<- glmer(result~date+(1 | site), data=presdate11,
family=binomial(link='logit'))
summary(presdate11.glmer) #p-value: 0.1366
presdate12 <- pres[pres$date %in% c("1-Aug","26-Sep"),]
presdate12.glmer<- glmer(result~date+(1 | site), data=presdate12,
family=binomial(link='logit'))
summary(presdate12.glmer) #p-value: 0.00523
presdate13 <- pres[pres$date %in% c("22-Aug","12-Sep"),]

```

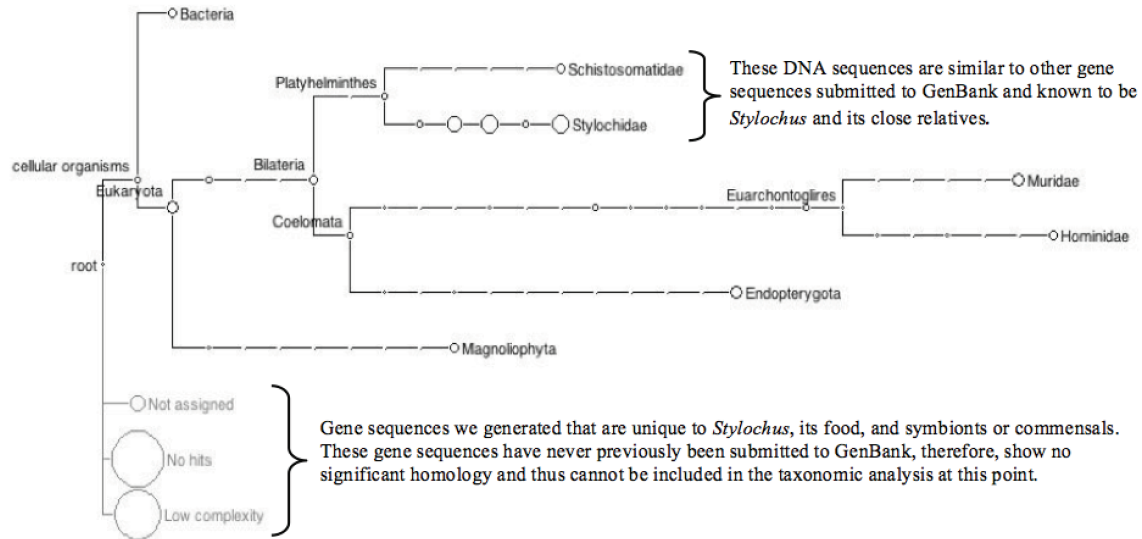
```

presdate13.glmer<- glmer(result~date+(1 | site), data=presdate13,
family=binomial(link='logit'))
summary(presdate13.glmer) #p-value: 0.39
presdate14 <- pres[pres$date %in% c("22-Aug","26-Sep"),]
presdate14.glmer<- glmer(result~date+(1 | site), data=presdate14,
family=binomial(link='logit'))
summary(presdate14.glmer) #p-value: 0.3301
presdate15 <- pres[pres$date %in% c("12-Sep","26-Sep"),]
presdate15.glmer<- glmer(result~date+(1 | site), data=presdate15,
family=binomial(link='logit'))
summary(presdate15.glmer) #p-value: 0.58804

```

Appendix B

Taxonomic breakdown of whole genome *S. ellipticus* sequences, courtesy of Tim King, USGS.



Vita

Marion Kensey Barker was born on September 11, 1986 in an Army Post in Heidelberg, Germany. Kensey, as called by her familiar, graduated from Woodbridge Senior High School, Woodbridge, VA in 2005. Kensey received her Bachelor of Science in Biology and Minor in Chemistry from Virginia Commonwealth University, Richmond, VA in 2010. She worked in vaccine research at Pharmaceutical Product Development, Inc. in Richmond, VA before joining the Master of Science in Biology program at VCU in 2012.