

SPATIAL AND TEMPORAL OVIGERITY, EFFECT OF BENZO[α] PYRENE ON
REPRODUCTION, AND A K-12 CLASSROOM RESEARCH APPLICATION OF
THE DAGGERBLADE GRASS SHRIMP *PALAEMONETES PUGIO*

by

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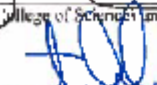


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DEDICATION

I dedicate this study to my supportive mother, Marguery, and loving family, Kayla, Adrian, and Alan. My family has provided me with invaluable support and guidance during the past few years.

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ABSTRACT

The daggerblade grass shrimp *Palaemonetes pugio* inhabits estuaries along the East and Gulf coasts of the United States, is a link between trophic levels, and is exposed to seasonal changes and pollution within these coastal habitats. The purpose of this thesis was to determine spatial and temporal ovigerity of the daggerblade grass shrimp *Palaemonetes pugio*, determine the effects of benzo[α]pyrene (BaP) on reproduction in the shrimp, and develop and implement a K-12 activity based on the data collected. Adult grass shrimp were collected twice a month from 3 sites in the Savannah, Georgia, U.S.A. region from August 2014 to August 2015. The greatest average weight, average length, and average number of eggs per shrimp were found at Country Club Creek ($p < 0.001$). Clutch sizes were larger early in the reproductive season (April, May, and June) than later in the reproductive season (July, August, September). The site with the highest concentration of polycyclic aromatic hydrocarbons in the sediment (108.5 $\mu\text{g}/\text{kg}$) was Country Club Creek. Adult grass shrimp were collected from Country Club Creek and exposed to 0, 3, or 6 $\mu\text{g}/\text{L}$ of BaP prior to and during spawning. After 7 d of exposure to clean seawater, the eggs were removed and the clutch size and embryonic stages were determined. The embryos were categorized into 3 stages and the eggs exposed to 0 $\mu\text{g}/\text{L}$ of BaP were more commonly more developed than eggs exposed to 3 or 6 $\mu\text{g}/\text{L}$ of BaP. Clutch size was not significantly different across the sites ($\alpha = 0.05$). Lastly, Shrimp Socktail was an activity created using size differences at different sites to teach students about modeling and effects of environmental conditions on growth.

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CHAPTER 1

Review: Crude oil on aquatic organisms and their environment and reproduction of the
daggerblade grass shrimp *Palaemonetes pugio*

INTRODUCTION

The daggerblade grass shrimp *Palaemonetes pugio* is common in salt marshes and estuaries along the East Coast of the U.S. (Morgan, 1980). These marshes protect the coast from offshore damage by receiving the initial impact of inclement weather (Shepard et al., 2011). This occurs because the vegetation in the marsh has a positive effect on wave attenuation thereby reducing either the height or energy of a wave (Shepard et al., 2011). Another benefit of the vegetation is an increase in accretion of sediment and a decrease in erosion in the marsh (Shepard et al., 2011). A final benefit is that dead vegetation can be recycled back into the environment. *Palaemonetes pugio* is a detritivore that feeds on such dead vegetation in the form of cordgrass *Spartina* and after digestion occurs, releases high amounts of ammonia and phosphate through fecal pellets (Welsh, 1975), which can be utilized in photosynthetic production. The grass shrimp is also a predator that eats meiofauna such as polychaetes, oligochaetes, and nematodes (Bell and Coull, 1978). Another role of the grass shrimp is being a prey item of numerous species including the mummichog *Fundulus heteroclitus* (Kneib, 1986), multiple species of heron (Post, 2008), and the red drum *Sciaenops ocellatus* (Humphries et al., 2011). Thus, the grass shrimp aids in the transfer of energy between the trophic levels (Anderson, 1985). Therefore, a change in the grass shrimp population can cause a shift in the food web because they can account for the majority of the macrofaunal community (Leight et al., 2005).

Palaemonetes pugio is also a host for parasites. Pung et al. (2002) determined that the parasitic trematode *Microphallus turgidus* is more prevalent in *P. pugio* than in *P. vulgaris*, making *P. pugio* an important intermediate host to this parasite. This parasite

has multiple hosts. The first intermediate host for the larval stage of this trematode is the hydrobiid snail *Spurwinkia salsa* (Heard and Overstreet, 1983; Pung et al., 2008). The trematode is present in *S. salsa* in marshes along the Skidaway River, GA (Pung et al., 2008). Aquatic birds are the definitive hosts of *M. turgidus* (Heard and Overstreet, 1983). The number of trematode cysts per shrimp in *P. pugio* ranged from 1-105 at sites in coastal Georgia and were found in the highest densities in areas with a salinity >19 ppt (Pung et al., 2002). Besides salinity, another factor that affected trematode abundance was shrimp size. Trematode cyst intensity increased with shrimp weight (Pung et al., 2002) and shrimp length (Sheehan et al., 2011). The number of trematode cysts per shrimp host may negatively affect the stamina of the shrimp. Kunz and Pung (2004) determined that shrimp with a heavy infection of trematode cysts (mean $\geq 41.5 \pm 1$ cysts/shrimp) had a lower swimming stamina than less heavily infected shrimp. In addition to the decrease in prolonged swimming stamina, the high infection of trematodes had an effect on the behavior of shrimp in the presence of a predator, causing grass shrimp to be more active in front of predators and were consumed more often (Kunz and Pung, 2004).

The daggerblade grass shrimp has been used in many studies because it is easy to maintain in a laboratory setting and its life history is well-known (Oberdörster et al., 2000). This species has a life span of approximately 1 yr (Welsh, 1975) and becomes mature when it reaches 15-18 mm in length (Anderson, 1985). Ovigerous *P. pugio* were collected in March through September in Vermillion, Louisiana, with the percentage of mature females with embryos increasing from 12.7% in March to 59.4% in April (Bauer and Abdalla, 2001). According to Welsh (1975), spawning occurred from May to June in

Bissel Cove, Rhode Island. More recent work identified an extended spawning period for grass shrimp that can last from February to October, based on the geographic location (Anderson, 1985). Gravid shrimp were present March through September at Country Club Creek and Moon River with the highest percentage of gravid females present in April, May, and August compared to March and June (Chaplin-Ebanks and Curran, 2007). The female daggerblade grass shrimp carries the clutch of eggs on the ventral surface of its abdomen (Anderson, 1985). The eggs hatch between 12-60 d after fertilization; and after spawning occurs, the female may produce another clutch of eggs (Anderson, 1985). If the eggs do not become fertilized, they are either dropped off the body of the female or are picked off by the mother (Burkenroad, 1947). Female grass shrimp monitored in the laboratory had a mean clutch size of 190 ± 5 (Romney and Reiber, 2013). Female length has a positive relationship with the number of eggs per clutch (Anderson, 1985), as was observed in Country Club Creek and Moon River (Modeste, 2009). The relationship can be defined by the following equation (Reinsel et al., 2001):

$$\text{number of eggs} = 12.28 \times \text{total length} - 85.3$$

Reproduction in the grass shrimp starts when the female molts. The mature female is receptive to a male only after molting occurs (Burkenroad, 1947). Berg and Sandifer (1984) determined that ecdysis in *Palaemonetes pugio* from Charleston, SC occurs at night between 2105 and 0715 h and shortly after molting, the shrimp is able to swim. The antennae of a male shrimp must make contact with the exoskeleton of the female for the male to identify that the female is ready for copulation (Burkenroad, 1947). Although the female shrimp remains physiologically receptive for 92-176 min,

copulation usually takes place 0-3 min after molting when the temperature is 27-29°C (Bauer and Abdalla, 2001). Copulation lasts for 5-10 s and is split into four events: 1) the lunge and capture, 2) the roll or dip, 3) pleopod lowering, and 4) disengagement (Berg and Sandifer, 1984). The male transfers the spermatophore into the sperm receptacle on the female using his first and second pleopods (Berg and Sandifer, 1984). The eggs are released into the brood chamber and adhere to each other and the female (Burkenroad, 1947). Once the eggs are in place, the pleopods on the female will move in a rhythm that fans the eggs (Burkenroad, 1947). The median incubation time for eggs of shrimp in Louisiana was 11 d, and the time from hatching of the eggs to the ecdysis of the female was 10 d (Bauer and Abdalla, 2000). Females are able to spawn successively a few days after the eggs hatch, if the female has a mature ovary and undergoes molting (Bauer and Abdalla, 2000). During one breeding season (April-September), a female grass shrimp in warm-temperate water (27-28°C) may produce 8 broods (Bauer and Abdalla, 2000).

Palaemonetes spp. have been studied as models for the possible effects of oil spills on marine organisms (Roth and Baltz, 2009; Moody et al., 2013) because they are bioindicators for potential toxins (Key et al., 2006). Estuaries are prone to receiving negative anthropogenic input. Savannah, GA is a port city where fuel oil, coal, and other chemicals are transported (Alexander et al., 1999). Sediment core samples dated back to 1948-1958 from the Savannah River Estuary were analyzed and there was an increase in PAH concentration, when the population and industries in Savannah increased (Alexander et al., 1999). Contaminated estuaries may affect many species in different ways. Weis et al. (2011) studied 5 species including the killifish *Fundulus heteroclitus* and *P. pugio* at two locations, a contaminated site and a “clean” site. Grass shrimp *P.*

pugio were larger and found in higher density at the polluted site, but this could be attributed to a decrease in top-down control (Weis et al., 2011). Growth rates were not different across the sites but the ability of the predator killifish to easily catch prey was negatively affected by the contamination, allowing the shrimp at the polluted site to live longer (Weis et al., 2011).

All life stages of the grass shrimp are sensitive to a wide range of contaminants (Key et al., 2006), including organic pollutants. Williamson et al. (2009) studied the effects of synthetic pyrethroid insecticides and determined that the presence of parasites affected the LC₁₀ to the toxicant. DeLorenzo and De Leon (2010) determined the 96-h LC₅₀ for the insecticide etofenprox on grass shrimp embryos, grass shrimp larvae, and adult grass shrimp and determined that it was higher for grass shrimp embryos (100 µg/L) compared to the larvae and adult grass shrimp (0.89 µg/L and 1.26 µg/L, respectively). It is essential to study the effects of the products created when an insecticide is degraded. The embryonic, larval, and adult stages of *P. pugio* were studied by Key et al. (2010) for the 96-h LC₅₀ for endosulfan sulfate, a product of the degradation of the insecticide endosulfan. The LC₅₀ for each stage was 45.85 µg/L, 1.64 µg/L, and 0.86 µg/L, respectively. Garner et al. (2009) studied adult grass shrimp and grass shrimp eggs from creeks in areas classified as forested, suburban, and urban and found that there was a positive relationship between the total amount of 16 polycyclic aromatic hydrocarbons (PAHs) in the sediment and the total amount of PAHs in shrimp tissue ($R^2 = 0.78$).

Oil spills are a consequence of oil exploration and production, and the results of a spill may have adverse influences on an ecosystem. The BP Deepwater Horizon oil spill

started on 20 April 2010 and consequentially over 207 million gallons of South Louisiana crude oil were released into the Gulf of Mexico (Venosa and Holder, 2013). Many studies arose following the Deepwater Horizon oil spill including ones that involved research on phytoplankton community structure and on the arthropod communities and vegetation. Gilde and Pinckney (2012) determined that crude oil affected the abundance of phytoplankton in the area. While phytoplankton abundance was affected, vegetation was not. McCall and Pennings (2012) found that there was not a significant impact on the vegetation when oil was introduced to the environment, which was demonstrated by the lack of significant difference in average height of the smooth cordgrass *Spartina alterniflora* in August 2011 (95.54 ± 6.27 cm) for oiled sites compared to that of control, non-oiled sites (89.46 ± 9.69 cm). By August 2010, for the same study, the arthropod community had decreased by 50% in oiled sites, but recovered in abundance by August 2011. Venosa and Holder (2013) determined the possible effects of 8 chemical dispersants that could have been used to break down South Louisiana crude oil, which was the same type of oil that was released in the Deepwater Horizon incident. Under their laboratory conditions, dispersant effectiveness was found to sometimes be influenced by temperature as was the case with SafRon Gold, Corexit 9500, Nokomis 3F4, and Nokomis 3AA (Venosa and Holder, 2013). For example, the effectiveness of SafRon Gold decreased from 30.95% effectiveness at 5°C to 16.25% effectiveness when the temperature was increased to 25°C (Venosa and Holder, 2013). Almeda et al. (2013) exposed zooplankton to oil concentrations ranging from 4.2-84.5 ppm to reflect the conditions of a possible oil spill. Crude oil had a significant effect on the survival of the

mesozooplankton communities with mortality ranging from 12-96% over the range of oil concentrations (Almeda et al., 2013).

The dispersion of oil in sea water is determined by physical factors including the spread of the plume by currents, the type of sediment in the area, and the agitation of the oil by waves (Blumer, 1971). Clay minerals and anaerobic sediment may contain components of oil months after a spill (Blumer, 1971). The components of oil may be subjected to biodegradation by fungi and bacteria (Arun et al., 2011). Oil breaks down at a rate that correlates with the amount of weathering it undergoes (Unger et al., 2008). Constant wave action and the absence of plant life can decrease the amount of time the oil is present in an area (Dicks et al., 1982). Thus, organisms in placid swamps and salt marsh ecosystems can be more affected by oil spills because these are relatively low-energy environments (Dicks et al., 1982). High-energy locations such as rocky shores are less affected by repetitive spills due to the physical weathering that degrades the oil (Dicks et al., 1982). Gundlach and Hayes (1978) developed a vulnerability index for various habitat types that may be exposed to oil spills, and determined that the primary producers in the salt marshes are vulnerable because oil may be present for years after a spill.

The farther seaward an oil spill occurs from the coast, the longer the oil may undergo weathering, which decreases the effects on the coastal ecosystem (McCall and Pennings, 2012). The toxicity of an oil spill on an organism is dependent on the proximity of the spill to the individual and the amount of time the organism is exposed (Harrison et al., 1975). Part of the weathering that the oil undergoes involves evaporation and dissolution. The rate of evaporation is faster in aromatics with low boiling points

(Harrison et al., 1975) and less than 15 carbons (UNEP/IOC/IAEA, 1992). Weathering can include natural dispersion of oil (Venosa and Holder, 2013), wave action causing fractionation of the components of oil, and the use of dispersants to degrade oil (Dicks et al., 1982). The temperature, type of dispersant, type of oil, weathering, and wave action can all change the effectiveness of oil dispersants (Chandrasekar et al., 2005). For example, the efficacy of dispersant “A” used on unweathered South Louisiana crude oil increased from 54.5% to 89.6% effectiveness when the speed of the orbital shaker, which served as a proxy for wave action, was increased from 150 to 250 rpm (Chandrasekar et al., 2005). When dispersants are used on weathered oil, effectiveness decreases compared to un-weathered oil. When dispersant “B” was used on un-weathered South Louisiana crude oil it was 83.1% effective, and only 77.7% effective on oil that was 20% weathered (Chandrasekar et al., 2005). This difference in dispersion and effectiveness could affect whether PAHs are deposited in the sediment and bioavailable to benthic organisms even after the use of a dispersant. Further studies are needed to determine the relationship between PAH exposure and its effects on the reproduction of the daggerblade grass shrimp in both the laboratory and in the field.

The goal of this thesis was to determine the spatial and temporal effects of polycyclic aromatic hydrocarbons on lethal and sublethal responses, including reproduction, of the daggerblade grass shrimp *Palaemonetes pugio*.

The objectives of this thesis were to:

1. Determine the effect of location, time within reproductive season, and concentration of polycyclic aromatic hydrocarbons on the ovigerity of the daggerblade grass shrimp *Palaemonetes pugio* in three creeks in Georgia, USA

2. Determine the effects of short-term exposure of non-ovigerous shrimp to benzo[*a*]pyrene on future clutch size and embryonic development of the daggerblade grass shrimp *Palaemonetes pugio*
3. Develop and implement a K-12 activity based on the data collected on the ovigerity of the daggerblade grass shrimp *Palaemonetes pugio*

CHAPTER 2

The effect of location, time in reproductive season, and polycyclic aromatic hydrocarbons on the ovigerity of the daggerblade grass shrimp *Palaemonetes pugio* in three creeks in Georgia, USA

ABSTRACT

The daggerblade grass shrimp *Palaemonetes pugio* inhabits estuaries along the East and Gulf coasts of the United States, is a link between trophic levels, and is exposed to changing environmental conditions including temperature and pollution within these coastal habitats. The purpose of this study was to determine the seasonal relationship between shrimp length, weight, and clutch size at 3 estuarine locations in the Savannah, Georgia, U.S.A. region. The study was conducted August 2014 - August 2015 at Country Club Creek (CC), Tom Thumb Creek (TT), and Moon River (MR). Male and female shrimp were collected by dip net twice a month during low tide. Length and weight were measured for all shrimp and additionally, for ovigerous shrimp, clutch size was recorded. Initial analyses led to segregating reproduction data into early (April-June) and late (July-September) in the reproductive season because of significant differences. Mean clutch size from April-June 2015 was 292.6 ± 70.86 , 282.6 ± 71.77 , and 255.9 ± 60.49 eggs/shrimp at CC, MR, and TT, respectively. Mean clutch size from July-September was 168.4 ± 53.43 , 136.6 ± 47.12 , and 140.0 ± 45.74 eggs/shrimp at CC, MR, and TT, respectively. Shrimp at CC had the greatest average length, weight, and clutch size, while shrimp at MR had the lowest length and weight, and TT had the lowest average clutch size. The concentration of polycyclic aromatic hydrocarbons (PAHs) varied among the sites as well. The concentration of PAHs in the sediment was significantly higher at CC, while the concentration of PAHs in the shrimp tissue was not significantly different among the sites. In the future, the grass shrimp tissue that is analyzed should come from male shrimp because female shrimp have the ability to pass on the polycyclic aromatic hydrocarbons to the eggs. Two cohorts were observed in this study; one that was hatched

in fall and one that hatched in early spring. This is the apparent cause of the temporal differences in reproduction. The sites are also different in the PAH pollution and this may have contributed to the observed spatial differences in reproduction. In conclusion, the life cycle of the grass shrimp could have contributed to the temporal differences in reproduction and differences in grain size composition at the sites could have led to the differences in the concentration of polycyclic aromatic hydrocarbons in sediment.

INTRODUCTION

Crude oil is mainly composed of polycyclic aromatic hydrocarbons (PAHs) that can be dispersed within an environment (Unger et al., 2008). PAHs can be introduced into aquatic environments by oil spills, refinery effluents (Canadian Council, 1999), river runoff, sewage discharge, fossil fuel combustion, or from an atmospheric input (Liu et al., 2000). The sources that cause the highest inputs of PAHs into the marine environment are municipal wastes with 0.7 million metric tonnes per annum and tanker accidents, with 0.4 million metric tonnes per annum (UNEP/IOC/IAEA, 1992). Due to the hydrophobic nature of PAHs, they attach to particulate matter and the sediment (Eadie et al., 1982). Polycyclic aromatic hydrocarbons and the compounds formed through the metabolism of PAHs can be carcinogenic, teratogenic, or mutagenic (Rand, 1995). The concentrations of 3 hydrocarbons, chrysene, fluoranthene, and phenanthrene, were tested after an experimental oil spill in the Wilmington River, GA and were found to have half-lives of 100 d in sediment, 70 d in mussels, and 30 d in oysters (Lee et al., 1981). The concentration of PAHs in a substrate is variable because of the associated half-lives, but there is also a relationship between the PAHs in an organism and in its environment (Garner et al, 2009). The ratio of PAHs in two benthic organisms, oligochaetes and chironomoids, in Lake Michigan was in equilibrium with the ratio of PAH concentration of the surrounding sediment (Eadie et al., 1982). The concentration of PAHs in the sediment is not uniform throughout the sediment column. Liu et al. (2000) determined that the total PAH concentrations were highest in the upper 33 cm of the sediment in the Yangtze estuary with a peak in concentration at 19 cm in depth (11.74 $\mu\text{g/g}$). The amount of PAHs in the sediment can have an effect on the survival of marine organisms. The 96-

h LC₅₀ for the daggerblade grass shrimp *Palaemonetes pugio* exposed to a mixture containing heavy molecular weight PAHs was 9.542 µg PAH/ g dry sediment (Wirth et al., 1998).

Marine organisms that are exposed to repeated oil spills or chronic discharges tend to be more negatively affected by the pollutant than areas subjected to single events (Dicks et al., 1982). Grass shrimp in areas with high runoff from land are exposed to oil and PAHs more frequently than those in more pristine areas, such as nature reserves (Leight et al., 2005; Roth and Baltz, 2009). Roth and Baltz (2009) determined the short-term effects of an oil spill on an assemblage of marsh inhabitants and found that the abundance of *Palaemonetes pugio* present was affected by the spill, with a decrease in the number of shrimp. Leight et al. (2005) studied 4 habitats of *P. pugio* with varying degrees of exposure to PAHs and agricultural runoff: one on a NOAA reserve, one bordered by a residential area, one adjacent to agricultural fields, and the most polluted one was located near a newly built golf course. The grass shrimp collected from the waters adjacent to the golf course tended to have significantly shorter average lengths (23.0-24.0 mm) when compared to those from the reserve (24.5 mm). The female grass shrimp (avg. length= 23 mm) from the polluted area had significantly smaller clutch sizes containing < 150 eggs, while females (avg. length= 24.5 mm) at the reserve had an average clutch size of 170 eggs (Leight et al., 2005). Therefore, sublethal effects should be studied because they may disrupt biological processes that are factors in the success and survival of an individual, such as the ability to find food, escape predation, and reproduce (Blumer, 1971). Whole oil is one form of pollution that can cause sublethal

effects. The oil fractions that may hinder these biological processes include the aromatic hydrocarbons with high boiling points (Blumer, 1971).

Polycyclic aromatic hydrocarbons are metabolized by some organisms, such as crustaceans, but remain intact in others such as bivalves (Perugini et al., 2007).

Pollutants, including PAHs, are sometimes passed from the adult organism to the offspring. PAH concentration in a crustacean, the Norway lobster *Nephrops norvegicus*, was higher in the summer, during gametogenesis when less elimination of pollutants is possible, compared to the winter, after eggs are produced, because pollutants may have been eliminated from the parent through egg production (Perugini et al., 2007). Watson et al. (2004) determined that PAH metabolites can be found in the urine and hemolymph of the shore crab *Carcinus maenas*. PAHs may also be found in the organs of a crustacean. Silva et al. (2013) found an increase in benzo[α]pyrene-type compounds in the digestive gland, eye, and muscle of the common prawn *Palaemon serratus* after exposure to BaP. Heavyweight molecular PAHs were found in higher concentrations in mysids and euphausiids that had come in contact with sediment when compared to concentrations in carnivorous crabs which has less contact with sediment (Baumard et al., 1998). One way contamination may affect reproduction is by reducing the feeding activity of an organism. Weis et al. (2011) determined that ovigerous grass shrimp from a contaminated site exhibited reduced feeding rates during the intermolt period.

The objectives of this study were to determine whether there is a correlation between total PAH concentration at a site and clutch size for ovigerous daggerblade grass shrimp *Palaemonetes pugio* and to determine the temporal and spatial relationship in the reproduction of the daggerblade grass shrimp *P. pugio*. I hypothesize that there will be an

inverse relationship between PAH concentration and shrimp length, weight, and clutch size.

MATERIALS AND METHODS

Three sites influenced by different land uses were sampled (Figure 2.1). A creek off Moon River (31.951214°N, 81.081649°W) is adjacent to conservation lands, Country Club Creek (32.020498°N, 81.057584°W) is located in land that is classified as urban area, and a creek off Tom Thumb Creek (32.031860°N, 81.014607°W) is located farther away from urbanization and is in a marshland. Temperature, salinity, and dissolved oxygen (DO) were measured with a YSI Pro2030 multimeter (YSI Incorporated, Cincinnati, OH), and pH was recorded for each site on the day of collection. Daggerblade grass shrimp *Palaemonetes pugio* were collected from each site within 2 hours of daytime low tide using dip nets. Shrimp samples were taken within 4 d of a spring tide and within 4 d of a neap tide, once a month during the reproductive season, August-October 2014 and February-August 2015. Ten ovigerous shrimp, if present, and 20 non-ovigerous shrimp from each creek were collected from multiple areas in the creeks, placed in buckets with site-water and transported to the laboratory. The shrimp were identified to the species level, according to Anderson (1985), by using the shape of the anterior carapace and rostrum when examined under a dissecting microscope. The number of trematode cysts in the shrimp abdomen were counted under a dissecting microscope. Shrimp with the bopyrid isopod were excluded from collection. The ovigerous shrimp were identified by the presence of eggs on the ventral surface. The male grass shrimp were identified by the presence of the *appendix masculina* on the endopod of the second set of pleopods as per Anderson (1985). The shrimp were weighed to the nearest mg using a digital scale and length was taken to the nearest mm. The eggs

were removed from the ovigerous shrimp using stainless steel forceps and counted under a dissecting microscope. The shrimp and eggs were stored at -30°C.

Sediment samples were collected from the aforementioned creeks within 2 hours of daytime low tide and on the same day as shrimp collection. The samples were taken within 4 d of a spring tide and within 4 d of a neap tide, once a month, from August-October 2014 and February-September 2015. Sediment was collected from the top 1-2 cm of the sediment on the creek bank in 5 haphazardly selected locations using a stainless steel scoop as per Garner et al. (2009). Large shells and trash were removed from the sediment sample by hand before it was homogenized in the field. The sediment (approximately 60 g) was placed in glass jars and kept on ice for transport to the laboratory and then stored in a freezer at -30°C prior to extraction and analysis. Sediment samples from August 2014 were analyzed by Test America in accordance with EPA SW846 method 8270D for polycyclic aromatic hydrocarbons (PAHs). Additional sediment samples and tissue samples were then analyzed at the NOAA Hollings Marine Laboratory by C. Thompson with the following methodology.

Extraction of Sediment:

In July 2016, frozen samples that were collected September 18 and 19, 2014, May 14 and 15, 2015, and August 13 and 14, 2015 were transported on dry ice to the Hollings Marine Laboratory in Charleston, SC and kept in an upright freezer at -40°C. Sediment samples were placed in a refrigerator at 4°C overnight, prior to extraction and allowed to thaw at room temperature the following morning. Each sample was homogenized with a steel spatula and a small subsample (ca. 4-7 g) was added to a tared aluminum weigh pan

and wet weight was recorded. After 24 h in a drying oven at 105-115°C, these samples were removed and the dry weight was recorded. The dry fraction was then calculated for the entire sample. Approximately 27 g of the drying agent sodium sulfate was added to a mortar bowl along with ca. 10 g of wet sediment. Initial mixing was performed with a stainless steel spatula. A pestle was used to further mix the sediment and to grind the sodium sulfate and sediment into a powder. This was added to a clean Accelerated Solvent Exchange (ASE) cell and spiked with a PAH internal standard mix. The quality control for the sediment samples included: 1) a blank cell filled with clean sodium sulfate, 2) a spike containing a PAH mix (8270D-EPA) added to sodium sulfate (Table 2.1), and 3) two matrix spikes composed of duplicate sediment samples with the PAH spike mix added. The samples were run on the Dionex 200 ASE and extracted with a solvent mixture of 50:50 acetone:dichloromethane (DCM). The extracts from the ASE were filtered through sodium sulfate, to remove any residual water, into a TurboVap tube and inserted into a Zymark TurboVap II concentration workstation. The extracts were processed through 2 solvent exchanges using dichloromethane in which extracts were concentrated to 0.5 mL and then were brought to a volume of ca. 10 mL. Each sample extract was then concentrated down to 0.5 mL again and removed from the TurboVap II workstation. The sample was pipetted out of the TurboVap tube into a culture tube and brought up to 2 mL with DCM and placed on the gel permeation chromatographer (GPC; Accu Prep Preparative LC System, J2 Scientific). The sample extract was cleaned up by the GPC and included less pigmentation. This extract was concentrated down to 0.25 mL with 2 solvent exchanges using hexane and then was transferred to an automated sampler vial (ASV). Then the extract was brought up to 1 mL with hexane and refrigerated until

the next step. The final step before analysis on the Gas Chromatograph-Mass Spectrometer (GC/MS, Agilent 6890/5973) was to run the sample through a Solid Phase Extraction (SPE) cleanup step with an alumina column. Glass cartridges were filled with 2.16-2.20 g of 5% activated alumina between two frits used for filtering. The column was prepared by running 1 column volume of 50:50 DCM:hexane followed by 1 column volume of 100% hexane through the cartridge. Then, collection vials/culture tubes were placed below the manifold and the sample (1 mL) in addition to a 1 mL hexane rinse of the vial were added to the column. Two column volumes of 35:65 DCM:hexane were added to each cartridge and allowed to drain into the culture tubes for collection. The alumina column cleaned up the sample by binding the non-target compounds and allowing the PAHs to pass through. This extract was concentrated down to 0.25 mL with 1 solvent exchange using hexane and brought up to 0.5 mL with hexane. A recovery standard (20 μ L) was added to each sample and then samples were run on the Agilent 6890/5973 GC/MS. The injection volume for each sample was 2 μ L. The samples were injected in splitless mode and the initial temperature of the column was 70°C. This temperature was held for 1 min before the first ramp, which increased the temperature by 45°C/min up to 150°C. Then the temperature was raised to 320°C at a rate of 10°C/min and held at this temperature for 41 min. The sample were run under constant pressure (23.20 psi) for the entire analysis on the GC/MS.

Extraction of Tissue:

Also in July 2016, extractions for select shrimp tissue samples that had been collected September 25 and 26, 2014, May 14 and 15, 2015, and August 13 and 14, 2015 as well as 2 additional sets collected July 9 and 10, 2015 and August 6 and 7, 2015 were analyzed.

The extractions were executed identically to the sediment samples with an exception of a few modifications. In the first drying and grinding step, 10 ovigerous shrimp (with eggs removed) from each site per collection date were added to 32.6 g of sodium sulfate. The shrimp were chopped with a stainless steel spatula and ground with a pestle before being placed into the ASE cell. The solvent used for the ASE was 100% DCM, unlike the solvents used for the sediment samples. Therefore, no solvent exchanges were made while concentrating the sample for extraction on the GPC, because the sample was in the desired solvent. The dry fractions from 3 non-ovigerous shrimp from each site were used to determine an average dry fraction per site.

Identification and Quantification performed in ChemStation (E.02):

Sediment and tissue samples were analyzed for 25 parent polycyclic aromatic hydrocarbons (PAHs), including the EPA 16 priority PAHs. These were identified by comparative analysis of a peak, if present. The retention time of the peak was compared to the internal standard (deuterated analytes) that behave similarly to the parent hydrocarbon. The next step was to examine the ions. The presence of the target and qualifying ion(s) in addition to the ion ratios of the target and qualifying ion(s) lead to the identification of the analytes. The area under the peak was used to determine the concentration when compared to the 11-point calibration curve. The quantified data were

imported into the NOAA Hollings Marine Laboratory database and the concentration of each analyte found in the blank was subtracted from every sample to remove any background in the analysis associated with possible residues that may be present on the column or that may have been introduced during the extraction process.

Additional Verification Procedure for Seasonal Trend in Ovigerous Shrimp

In September 2015, 50 ovigerous shrimp were collected to verify the trend present in the 10 shrimp collected during each tide. The shrimp were collected from Country Club Creek and transported back to the laboratory. The shrimp were identified to the species level by using the shape of the anterior carapace and rostrum examined under a dissecting microscope according to Anderson (1985). The ovigerous shrimp were identified by the presence of eggs on the ventral surface. The shrimp were weighed to the nearest mg using a digital scale and length was taken to the nearest mm. The eggs were removed from the ovigerous shrimp using stainless steel forceps and counted under a dissecting microscope.

Statistical Analyses

SAS was used for statistical analyses. ProcUnivariate was run to determine normality. Data were not normally distributed and thus a nonparametric analysis was performed to determine if there were differences among the sites. ANOVA and Tukey's multiple comparison tests were used to determine variance across sites. Comparative data for ovigerous shrimp were only analyzed for months when 10 ovigerous shrimp were collected at each site during each sampling effort. Linear regression was performed to

determine the relationship between shrimp length and clutch size, shrimp weight and clutch size, and PAH concentration and clutch size.

RESULTS

The water quality parameters (temperature, pH, salinity, and dissolved oxygen) were not significantly different among the sites ($\alpha = 0.05$). The mean temperatures among the sites were 24.5-24.7°C and the mean pH was 7.8 at all 3 sites, while average salinity was highest at Country Club Creek with 23.0 ± 3.00 ppt and lowest at Moon River with 21.6 ± 4.69 (Table 2.2). Temperature decreased at all sites from August 2014 to February 2015 for the months when measurements were taken and increased from February to August 2015 (Figure 2.2). Water temperature was highest in August 2014 (Figure 2.2). Salinity decreased until March and then increased until August 2015 during the spring tide (Figure 2.3A). Otherwise, salinity remained fairly constant except for during the neap tide in August 2014 at Moon River and at the September 2014 neap tide at Tom Thumb Creek (Figure 2.3B). The dissolved oxygen (DO) was lowest at Country Club Creek with 4.3 ± 1.77 mg/L (Table 2.2). Dissolved oxygen was variable at each site between spring and neap tides but was highest in February and lowest in June during spring tide and July during neap tide (Figure 2.4A and B). No measurement of DO was taken at Country Club Creek or Moon River (Figure 2.4B).

Site and month affected the size of the shrimp. Highest overall mean weight for all shrimp was significantly higher ($p < 0.0001$) at Country Club Creek with 223.4 ± 108.52 mg, followed by 200.5 ± 90.42 mg at Tom Thumb Creek, and 187.2 ± 96.53 mg at Moon River. Mean length followed a similar trend with 28.6 ± 4.49 , 27.8 ± 4.04 , and 26.9 ± 4.38 mm at Country Club Creek, Tom Thumb Creek, and Moon River, respectively with a significant difference among all 3 sites ($p < 0.0001$). Month had a

significant effect on the length of all shrimp at the sites ($p < 0.0001$) with shortest shrimp in February compared to other months ($p < 0.0001$).

There was a trend that the average lengths of male and female shrimp over time were shorter at Moon River (Figure 2.5B and C). Mean lengths of males and females were shortest during February 2015 for Country Club Creek and Tom Thumb Creek, but not for Moon River (Figure 2.5B and C). Average length of ovigerous shrimp decreased from June to July 2015 (Figure 2.5A). Mean weight over time followed a similar trend as length with the lowest male and female weights in February and a decrease in ovigerous shrimp weight from June to July 2015 (Figure 2.6).

The length, weight, clutch size, and presence of ovigerous shrimp varied with site and month. Length of ovigerous shrimp had wide ranges during June, July, August, and September. The largest range in length was found in August 2015 at Tom Thumb Creek (16 mm), April 2015 at Country Club Creek (11.5 mm), and during June at Moon River with a range of 10.5 mm. Mean clutch size was 217.4 ± 86.40 , 195.1 ± 90.88 , and 188.7 ± 77.68 eggs/female at Country Club Creek, Moon River, and Tom Thumb Creek, respectively. The average clutch size at Country Club Creek was significantly larger than at Tom Thumb Creek ($p = 0.0142$).

Clutch size was significantly larger earlier (April-June) in the season than later (July-September) in the season ($p < 0.0001$). The months were then divided into 3 month categories for 2 reasons: 1) 10 ovigerous shrimp were only collected from April through September (due to their absence at sites in other months); and 2) there were significant differences between clutch size data from April-June and July-September. Average clutch size was larger during April-June 2015 than July-October for all sites (Figure 2.7).

The length and clutch size of ovigerous shrimp were affected by both the reproductive season and site. There were no ovigerous shrimp present in the smallest size category of 20-25 mm in April, May, or June (Figure 2.8A). No shrimp for the largest size class were at Moon River (35.1-40 mm) late in the season (July-September) or at Country Club Creek in the smallest group size of 20-25 mm (Figure 2.8B). The average clutch size increased with size class in both the early months (April-June) and the later months of July, August, and September (Figure 2.8). The average clutch size decreased from the early season to the late season at all sites (Figure 2.9). The average clutch size at Moon River changed the most from 282.6 ± 71.77 to 136.6 ± 47.1 eggs/shrimp (Figure 2.9). The peaks of the length frequency distribution decreased at all sites from April-May to July-September (Figure 2.10). There were no significant differences in the length frequency distributions among the sites ($p=0.8915$), although there was a significant difference between the early and late reproductive months ($p=0.0029$). There were no ovigerous shrimp at Tom Thumb Creek shorter than 28 mm in length in April, May, or June (Figure 2.10A). From July-September, there were no shrimp longer than 36 mm at any of the sites (Figure 2.10B). A greater number of shrimp of longer lengths were at Country Club Creek from July-September (Figure 2.10B). The weight frequency distribution was similar to the length frequency distribution, with heavier shrimp in mid-spring/early summer and lighter shrimp in mid-summer/early fall (Figure 2.11). There were no shrimp smaller than 150 mg at any of the sites in mid-spring/early summer (Figure 2.11A). There were no shrimp larger than 450 mg during mid-summer/early fall (Figure 2.11B). There was no significant difference in the length frequency distributions among the sites ($p=0.99$), so the sites were combined. The length frequency distribution

of all shrimp decreased from June through September (Figure 2.12). There was a change from a bimodal distribution in April, May and June to a unimodal distribution in August and September (Figure 2.12). The modal lengths changed from 28 and 36 mm in July 2015 to 30 mm in August 2015 and finally to 28 and 26 mm in August and September 2014, respectively (Figure 2.12). The length of ovigerous shrimp increased from April to June and had a wider range from July to September (Figure 2.13). The peak in size shifted from 36 mm in June to 32 mm in July (Figure 2.13). There was a significant difference in seasonal clutch size among the sites in that Country Club Creek shrimp had higher clutch sizes than both Moon River and Tom Thumb Creek. There was a significant effect of month on clutch size ($p < 0.0001$), with the differences occurring most commonly between months early in the reproductive season (April, May, and June) and late in the reproductive season (July, August, September, and October).

Clutch size was then analyzed as the number of eggs per shrimp length for ovigerous shrimp. The number of eggs per mm of shrimp length was significantly higher ($p < 0.0001$) in May 2015 and decreased from June to July (Figure 2.14). Average clutch size (eggs per mm shrimp length) was smaller August-October 2014, and August 2015 (Figure 2.14). Data were similar for both August 2014 and 2015 (Figure 2.14). Country Club Creek had the highest averages during August-October 2014 and April-May 2015 (Figure 2.14). Month had a significant effect on the number of eggs per mm shrimp length ($p < 0.0001$) with the most difference occurring between the months early in the reproductive season (April-June) and late in the reproductive season (July-September).

Trematode cyst counts varied throughout the shrimp reproductive season ($p < 0.0001$). There was an increase in the average number of trematode cysts from

February through August at Country Club Creek (Figure 2.15), with month having a significant effect on the number of trematode cysts per shrimp ($p < 0.0001$). Trematode abundance was lowest at Tom Thumb Creek (10.9 ± 12.60 cysts/shrimp) and higher at Moon River with 17.5 ± 19.74 cysts/shrimp and Country Club Creek with 20.0 ± 18.15 cysts/shrimp. The highest average number of trematodes was found at Country Club Creek in September 2014 with 39.2 ± 17.75 cysts per shrimp, while the lowest was found at Tom Thumb Creek in February with 2.5 ± 3.98 cysts per shrimp (Figure 2.15). The number of cysts per shrimp length was significantly different ($p < 0.0001$) in August, September, and October (Figure 2.16). There were different trends for the monthly average number of eggs per mm shrimp length and the monthly average number of trematode cysts per mm shrimp length (Figure 2.16). The number of trematode cysts per mm shrimp length increased throughout the year, while the number of eggs per shrimp length decreased after June 2015 (Figure 2.16).

The presence and abundance of trematode cysts were also influenced by shrimp size. The number of trematode cysts increased with shrimp length (Figure 2.17). Length and weight had significant positive relationships with the number of trematode cysts per shrimp ($p < 0.0001$). The steepest trend line was at Country Club Creek when the number of trematode cysts was equal to 1.9 times the length in mm of the shrimp (Figure 2.17A). There were fewer trematode cysts and lower infections rates in shrimp at Tom Thumb Creek (Figure 2.17C) relative to Moon River and Country Club Creek. A similar trend was present for the number of cysts/mm shrimp length, except for at Moon River where there was not a significant effect ($p = 0.20$) of length on cysts/mm shrimp length (Figure 2.18).

Clutch size increased with weight (Figure 2.19) and length (Figure 2.20) at all sites. Length and weight had significant effects on clutch size ($p < 0.0001$). The eggs/mm shrimp length was also affected by length (Figure 2.21). The trend line was steeper during May, June, and July for the effect of length on clutch size, so the clutch size increased at a faster rate with an increase in length (Figure 2.22A). The strongest correlation ($R^2 = 0.75$) between length and clutch size was at Moon River during April, May, and June expressed by the equation: clutch size = $23.1(\text{length}) - 497.46$ (Figure 2.22B). The weakest correlation ($R^2 = 0.13$) and smallest length range of ovigerous shrimp was found at Country Club Creek from July-September (Figure 2.23A). Similar trends were present for the eggs/mm shrimp length for both early in the reproductive season and late in the reproductive season (Figures 2.24 and 2.25).

The 50 ovigerous shrimp collected in September 2015 at Country Club Creek were included to add more data to the present study by increasing the ovigerous shrimp analyzed for that month. There was the same trend of clutch size increasing with length (Figure 2.26). The correlation was stronger than July-September (Figures 2.23 and 2.26). The number of trematode cysts increased significantly ($p < 0.0001$) with length (Figure 2.27). All ovigerous shrimp in the additional sampling in September 2015 had trematode cysts and there was a positive correlation between length and the number of trematode cysts per shrimp (Figure 2.27). The length distribution was narrow in range, while the weight had a wide distribution (Figure 2.28). The modal length was 30 mm and the modal weight was 225 mg (Figure 2.28).

Sediment samples analyzed at Test America had the following results. Polycyclic aromatic hydrocarbons (PAHs) were found in the sediment at only CC site from the

August 2014 samples. Sediment from Tom Thumb and Moon River were analyzed at Test America in accordance with EPA SW846 Method 8270D but no PAHs were detected (Table 2.3). Country Club Creek had 7 PAHs detected when analyzed with a total concentration of 108.5 µg/kg (Table 2.3). All PAHs found at Country Club Creek contained 4-5 carbon rings.

The ovigerous shrimp used in the extraction for PAHs at the Hollings Marine Laboratory were not the same in length or wet weight. The greatest average length of the shrimp for all sites was used in the May 2015 extraction (Table 2.4). All samples were a composite of 10 ovigerous shrimp with eggs removed. The range in lengths was variable for every sample with the smallest shrimp being 23.5 mm and the largest shrimp being 39.0 mm (Table 2.4). The extracted weights ranged from 1.10 to 3.07 g (Table 2.4).

The results for contracted PAH analyses completed by Test America were different from the analyses completed by C. Thompson while at the NOAA Hollings Marine Laboratory. There were PAHs present in every sample except for the tissue sample from Tom Thumb Creek for the May 2015 collection (Table 2.5 and 2.6). Because of a power outage during extraction, a portion of the sample from Tom Thumb Creek was lost and this may have caused the lack of detectable PAHs. The highest total PAH concentrations were found in Country Club Creek sediment for September 2014, May 2015, and August 2015 with 1149.63, 660.09, and 569.23 ng/g, respectively (Table 2.5). Biphenyl was only detected in one sample of sediment and it was a sample from Country Club Creek during the May 2015 collection. In contrast, biphenyl was present in every composite tissue sample except for the May 2015 collection for Tom Thumb, which was the incomplete extraction (Table 2.6 and 2.7). The dry weight fractions were

not consistent for the sites, so the difference between the wet weight and dry weight PAH concentrations were highly variable among the sites (Table 2.6 and 2.7).

The PAHs present in sediment and shrimp tissue were different for all sites and samples. While most of the 25 parent PAHs were detected in the sediment, only 6 PAHs were present in the grass shrimp tissue: 2,6+2,7-Dimethylnaphthalene, benzo(α)pyrene, benzo(β)fluoranthene, biphenyl, naphthalene, and phenanthrene. The total concentration of PAHs in the tissue samples from September 2014 at Tom Thumb Creek was 100% biphenyl while the pyrene had the highest percentage with 29% in the sediment sample (Figure 2.29). For the Country Club Creek sample from September 2014, the tissue sample was made up of biphenyl and 2,6+2,7-Dimethylnaphthalene and the sediment sample had 9 PAHs that contributed $\geq 5\%$ to the composition (Figure 2.30). Biphenyl dominated was the only PAH present in the September tissue samples from Moon River, while 12 PAHs contributed $\geq 5\%$ to the composition of the sediment sample (Figure 2.31). There were no PAHs present in the tissue sample for Tom Thumb for the May 2015 sample (Figure 2.32). The tissue sample from May 2015 at Country Club Creek was composed of 3 PAHs (Figure 2.33A). Phenanthrene and biphenyl were the only 2 PAHs in the tissue sample from May 2015 at Moon River (Figure 2.34A). The sediment sample contained 11 PAHs contributing $\geq 5\%$ (Figure 2.34B). Only tissue samples were analyzed for July 2015. All 3 samples were dominated by biphenyl with a small proportion that was benzo(b)fluoranthene (Figure 2.35). The August 2015 samples followed the previous trend in which the tissue samples were composed of 1-3 PAHs and the sediment samples that were more heterogeneous, composed of smaller portions of many PAHs (Figures 2.36-2.38).

The PAH concentration in sediment and tissue were significantly different but neither affected reproduction of the grass shrimp. There was not a statistically significant difference in the PAH concentration for shrimp tissue samples from the sites at an α -level of 0.05 (Figure 2.39A). There was a significant difference in the average PAH concentration for sediment samples among the sites with a p-value of <0.0001 (Figure 2.39B). There was not a significant effect of the concentration of PAHs in the sediment on eggs/mm shrimp length (Figure 2.40). There was no trend present in the PAH concentration of the grass shrimp tissue in relation to the PAH concentration in the site sediment (Figure 2.41). The effect of PAH concentration in grass shrimp tissue on eggs/mm shrimp length was not significant with $p=0.3236$ (Figure 2.42).

DISCUSSION

The major finding of this study was that the length, weight, and clutch size of daggerblade grass shrimp *Palaemonetes pugio* were affected by month, site, and reproductive season and that the concentration of polycyclic aromatic hydrocarbons in the sediments at the sites were significantly different. The greatest length, weight, and clutch size were at Country Club Creek and lowest at Moon River and Tom Thumb Creek. The presence of biphenyl in the tissue samples may indicate that there are PCBs in the area or biphenyl in the water. Biphenyl is composed of 2 non-fused rings and has a low molecular weight. Due to this low molecular weight, if biphenyl is present in the environment it would dissipate quickly. Biphenyl does adhere to lipids in tissue and would persist in an organism longer than in the environment. The shorter length and lower weight of shrimp at the Moon River site could be because it is by a highway. It is located adjacent to a highway that may have runoff of substances other than PAHs not analyzed in this study, even though the concentration of PAHs was below the minimum detection level in August. A similar trend was present in *P. pugio* in South Carolina. Shrimp that were at sites contaminated with metals, PAHs, and pesticides were shorter in length than at other sites (Leight et al., 2005). Lee et al. (2004) found that grass shrimp in areas with higher PAH concentrations exhibited higher DNA strand breaks and decreased clutch size and hatching rates than those without PAHs. These effects could be the cause for the shorter lengths, lower weights, and smaller clutch sizes of shrimp at Moon River. The reason the concentration of PAHs may be lower in the sediment at Moon River is because of the sediment composition. PAHs, such as benzo[α]pyrene have an affinity for different sediment grain sizes (Xia and Wang, 2008). PAHs can also affect organisms.

Country Club Creek could have the highest measurements of length, weight, and clutch size because it is the most polluted. Weis et al. (2011) noticed a possible top-down control at a contaminated site in New Jersey. The predator in the area, the killifish, was affected by the contamination and was therefore smaller and a poorer predator (Weis et al., 2011). The decreased threat from a predator enabled shrimp to live long at this site. The grass shrimp were not growing at a faster rate, but had a higher survival rate than at the control site.

Clutch size increased with length at all three sites. The largest shrimp and the shrimp with the largest clutch sizes were both found at Country Club Creek with an average of 28.6 ± 4.49 mm and 217.4 ± 86.40 eggs/shrimp. Modeste (2009) found similar results of eggs per shrimp increasing with length at Country Club Creek and Moon River. In the present study, length explained a majority of the variability in clutch size at Tom Thumb Creek ($R^2=0.62$) and Moon River ($R^2=0.68$), but not at Country Club Creek ($R^2=0.13$). So other factors (e.g. weight, site) may be affecting the shrimp at Country Club Creek (Figure 2.23). Anderson (1985), Reinsel et al. (2001), and Modeste (2009) found a significant positive relationship between shrimp length and the number of embryos per female.

An inverse relationship between temperature and dissolved oxygen (DO) was seen at all three sites, when DO increased and temperature decreased from August to February (Figure 2.2 and 2.4). DO was not recorded for the neap tide in February because there was no YSI multi-meter available on that date (Figure 2.4). Salinity had minimal variability at the sites. Salinity decreased in Moon River in August during the neap tide

because the water measurement was taken at a different location than other dates (Figure 2.3).

Trematode abundance was highest at Country Club Creek and lowest at Tom Thumb Creek. This could be affected by the presence/absence of the initial or definitive host of the parasite as was noticed by Pung et al. (2008). It could also be influenced by the size of the shrimp at the different sites. Trematode intensity increases with shrimp weight (Pung et al., 2002) and shrimp length (Sheehan et al., 2011). The large size of the grass shrimp at Country Club Creek could explain why the highest trematode abundance was also present at this site. The number of cysts per mm shrimp length increased throughout the year.

Ovigerous shrimp were not found year round at the sites and were influenced by time in the reproductive season. Ovigerous shrimp were present March-September at Moon River and April-October at Tom Thumb and Country Club Creek. While ovigerous shrimp were present during those months, there were not always the 10 necessary for analysis. Chaplin-Ebanks and Curran (2007) determined that spawning occurred in Georgia from March through September. In the present study, ovigerous shrimp were not observed at Moon River in October and were not present again until March (Figure 2.7). Different cohorts may have been present throughout the year because larger ovigerous shrimp were present during May-June from the sites and smaller ovigerous shrimp were present July-September. Shrimp that hatched in late February and March could have begun reproducing in July and August. Juvenile shrimp become mature at 1.5-2 months (Anderson, 1985). The second cohort was likely composed of overwintering *Palaemonetes pugio* that hatched at the end of the previous year and began to spawn in

March. This could be why there is a large range of lengths in August at Tom Thumb Creek and the length varied the most during July, August, and September. Eggs hatch at different times of the season, creating cohorts. The shrimp that hatched the previous winter and spawn early during the reproductive season (March-May) may also be responsible for the larger clutch sizes, while the younger shrimp that spawn in the later months may have smaller clutch sizes.

Differences among the sites could be because of the different land uses. Highway runoff can decrease embryo production and hatching rate and increase the frequency of breaks in strands of DNA (Lee et al., 2004). Each site is surrounded by an area that is dominated by a different land use. Moon River and Country Club Creek are nearby highways and roads. Country Club Creek is also adjacent to residential and developed land with substantial amounts of impervious cover and vehicular traffic. Moon River and Tom Thumb are surrounded by marshland and hammocks. Water quality parameters could also lead to differences in sites, although there was not a significant difference in general water quality parameters during this study.

Reproductive season could affect the shrimp length, weight, and clutch size because of temperature and salinity. Shrimp grow the fastest at 25.4°C and 23.6 ppt (Vernberg and Piyatiratitivorakul, 1998). This can aid in the shrimp that hatch in the warmer months growing faster and becoming reproductively active in the fall.

Palaemonetes pugio are multiple brooders, being able to produce up to 8 broods in a season (Bauer and Abdalla, 2000) and both temperature and day length are important in initiating reproduction. Overall energy assimilation is also highest in adult grass shrimp (97.84%) when compared to larval and juvenile shrimp with the adult dedicating over

50% of the energy to reproduction (Vernberg and Piyatiratitivorakul, 1998). This could cause the differences in the size of the clutches of older and younger shrimp, because the older shrimp are able to expend more energy on reproduction and therefore have larger clutches.

The polycyclic aromatic hydrocarbons (PAHs) found at Country Club Creek in the sediment analyzed by Test America in accordance with EPA SW846 method 8270D for polycyclic aromatic hydrocarbons contained 4-5 rings. This could be due to the PAHs with lower molecular weights evaporating, at the same time the larger ringed structures (≥ 5) are difficult to detect. The PAHs found are carcinogenic, mutagenic, poisonous, and harmful if swallowed. Country Club Creek was the only site where PAHs were detected but that could be due to collection methods or the minimum detection level of the technique used.

Concentrations of PAHs in sediment and shrimp tissue were different in composition for all sites. The samples analyzed at the NOAA Hollings Marine Laboratory according to SOP: CCR-043 "Analysis of persistent organic pollutants by GC-MS" all contained PAHs, except for one sample in which some of the extract was lost. Country Club Creek sediment was significantly more polluted than the other 2 sites ($\alpha = 0.05$). This could be due to the differences in sediment grain composition. PAHs have certain affinities to different sediment types. The sediment should be analyzed at each site to determine the grain compositions.

Sediment contained many more PAH compounds than the shrimp tissue samples. The concentration of PAHs in shrimp tissue samples were not significantly different among the sites. The composite shrimp tissue samples were possibly made up of older

and younger shrimp and may be the reason why a correlation between PAH concentration and clutch size was not observed. The assimilation of PAHs in the sediment into the grass shrimp was not observed in this study and could be caused by the excretion of the toxicants into the eggs. Maternal transfer of pollutants would enable the adult female to pass on toxicants to the embryos during reproduction. PAH concentration in a crustacean, the Norway lobster *Nephrops norvegicus*, was higher in the summer, during gametogenesis, compared to the winter, after eggs are laid, because pollutants may have been eliminated from the parent through egg production (Perugini et al., 2007). Male shrimp should be analyzed in the future to determine if there is a correlation between PAHs in the sediment and tissue.

The major finding of the present study was that the length, weight, and clutch size of daggerblade grass shrimp *Palaemonetes pugio* were affected by month, site, and season. There was also a difference in the PAH concentrations in the sediment at the sites. Further research should be done to determine the other factors influencing each site including grain size, predator and prey abundance, and the concentrations of other pollutants. The differences at Country Club Creek could be due to top-down predator control because the predators of the shrimp are affected by the pollution. The shrimp are flourishing under the current conditions and the population is in no apparent danger at any of the sites. There are two distinguishable cohorts in the area and the differences in lengths and clutch sizes may be a way to determine relative age in the future.

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Table 2.1 The composition of the 8270D PAH spike mix with the initial concentrations before dilution. This mix was added to samples analyzed for polycyclic aromatic hydrocarbons (PAHs). The PAHs were extracted and analyzed from the samples according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS” and the spike was added to test the recovery and accuracy of the extraction.

Component	CAS Number	Units
benzo[k]fluoranthene	207-08-9	mg/L
1-methylnaphthalene	90-12-0	mg/L
2-methylnaphthalene	91-57-6	mg/L
acenaphthene	83-32-9	mg/L
acenaphthylene	208-96-8	mg/L
anthracene	120-12-7	mg/L
fluorene	86-73-7	mg/L
naphthalene	91-20-3	mg/L
phenanthrene	85-01-8	mg/L
Benzo(α)anthracene	56-55-3	mg/L
Benzo(α)pyrene	50-32-8	mg/L
chrysene	218-01-9	mg/L
fluoranthene	206-44-0	mg/L
Indeno(1,2,3-cd)pyrene	193-39-5	mg/L
pyrene	129-00-0	mg/L
Benzo(b)fluoranthene	205-99-2	mg/L
Benzo(g,h,i)perylene	191-24-2	mg/L
Dibenz(a,h)anthracene	53-70-3	mg/L
1,6,7-trimethylnaphthalene	2245-38-7	mg/L
1-methylphenanthrene	832-69-9	mg/L
2,6-dimethylnaphthalene	581-42-0	mg/L
biphenyl	92-52-4	mg/L
dibenzothiophene	132-65-0	mg/L
Benzo(e)pyrene	192-97-2	mg/L
perylene	198-55-0	mg/L

Table 2.2 Water measurements were taken twice monthly at three sites in the Savannah, GA region: Tom Thumb Creek, Country Club Creek, and Moon River from August-October 2014 and February-August 2015, once during spring tide and once during neap tide. The mean temperature, pH, salinity, and dissolved oxygen were determined ± 1 SD.

Site	Average temperature (°C) \pm SD	Average pH \pm SD	Average salinity (ppt) \pm SD	Average dissolved oxygen (mg/L) \pm SD
Tom Thumb Creek	24.5 \pm 6.92	7.8 \pm 0.37	22.3 \pm 3.66	5.0 \pm 1.99
Country Club Creek	24.7 \pm 6.11	7.8 \pm 0.42	23.0 \pm 3.00	4.3 \pm 1.77
Moon River	24.7 \pm 6.86	7.8 \pm 0.39	21.6 \pm 4.69	5.0 \pm 2.00

Table 2.3 Sediment samples were collected from Tom Thumb Creek, Country Club Creek, and Moon River in August 2014. Samples were analyzed at Test America in accordance with EPA SW846 method 8270D for polycyclic aromatic hydrocarbons (PAHs). The concentration of each PAH is listed. <MDL = Below minimum detection level.

	Tom Thumb	Country Club Creek	Moon River
PAH	Concentration (µg/kg)		
Benzo(α)anthracene	<MDL	13	<MDL
Benzo(α)pyrene	<MDL	12	<MDL
Benzo(b)fluoranthene	<MDL	18	<MDL
Benzo(k)fluoranthene	<MDL	9.5	<MDL
Chrysene	<MDL	13	<MDL
Fluoranthene	<MDL	23	<MDL
Pyrene	<MDL	20	<MDL
Total Concentration	<MDL	108.5	<MDL

Table 2.4 Tissue samples from daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) in September 2014, May 2015, July 2015 and August 2015. The polycyclic aromatic hydrocarbons (PAHs) were extracted and analyzed from the samples according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The average length (mm), minimum length, maximum length, and wet weight extracted (g) was measured for each composite sample. Ten ovigerous shrimp with eggs removed were used for each site for each month.

Month	Site	Avg. Length (mm)	Min. Length (mm)	Max Length (mm)	Wet Weight Extracted (g)
September	TT	28 ± 1.0	26.5	30.0	1.44
	CC	31 ± 1.6	29.5	35.0	2.24
	MR	27 ± 3.0	23.5	32.0	1.55
May	TT	35 ± 2.1	31.0	38.0	2.44
	CC	35 ± 2.1	31.0	39.0	2.55
	MR	34 ± 2.4	29.5	36.5	3.07
July	TT	29 ± 3.3	25.0	35.5	1.56
	CC	30 ± 2.7	27.5	35.5	2.25
	MR	32 ± 1.8	30.0	35.0	1.98
August	TT	28 ± 1.8	25.5	30.5	1.10
	CC	30 ± 2.4	26.5	35.0	2.17
	MR	30 ± 2.9	24.5	33.0	1.90

Table 2.5 Concentrations of PAHs (ng/g) in sediment samples collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) in September 2014, May 2015 and August 2015 as extracted and analyzed according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.”

Polycyclic Aromatic Hydrocarbons (PAH)	September			May			August		
	TT	CC	MR	TT	CC	MR	TT	CC	MR
1-Methylnaphthalene	<MDL	7.52	<MDL	<MDL	4.35	<MDL	<MDL	2.18	0.79
1-Methylphenanthrene	<MDL	16.53	<MDL	<MDL	4.93	<MDL	<MDL	5.06	<MDL
2,6+2,7-Dimethylnaphthalene	0.92	2.16	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
2-Methylnaphthalene	<MDL	8.27	<MDL	0.72	5.42	0.84	<MDL	3.04	0.90
Acenaphthene	<MDL	19.25	<MDL	0.33	2.26	<MDL	<MDL	2.77	<MDL
Acenaphthylene	<MDL	3.78	0.17	<MDL	6.99	<MDL	0.44	5.01	0.37
Anthracene	0.24	31.19	0.24	1.14	25.53	0.33	1.05	13.63	0.30
Benzo(α)anthracene	0.44	83.16	0.87	1.75	45.32	2.22	2.14	39.49	1.83
Benzo(α)pyrene	0.60	88.42	1.28	2.07	44.76	3.33	2.78	45.54	2.64
Benzo(b)fluoranthene	0.99	84.59	1.65	3.15	47.78	3.73	3.68	45.36	2.84
Benzo(e)pyrene	0.67	68.34	1.22	2.11	37.99	2.84	2.85	37.83	2.29
Benzo(g,h,i)perylene	0.62	46.98	1.18	1.44	23.99	2.20	2.11	24.78	1.77
Benzo(k)fluoranthene	0.37	41.78	0.71	1.18	25.17	1.63	1.58	22.38	1.19
Biphenyl	<MDL	<MDL	<MDL	<MDL	3.35	<MDL	<MDL	<MDL	<MDL
Chrysene+Triphenylene	0.75	102.14	1.58	2.73	105.60	3.70	3.39	59.31	3.00
Dibenz(a,h)anthracene	<MDL	14.52	<MDL	0.22	5.96	0.35	0.26	6.61	0.28
Dibenzothiophene	<MDL	4.70	<MDL	<MDL	1.94	<MDL	<MDL	1.46	<MDL
Fluoranthene	1.53	140.09	2.18	6.23	87.54	5.39	7.04	86.77	3.76
Fluorene	<MDL	15.15	<MDL	0.60	6.49	<MDL	0.33	5.29	<MDL
Indeno(1,2,3-cd)pyrene	0.46	56.73	1.00	1.37	29.22	2.38	2.18	29.53	1.84
Naphthalene	<MDL	12.20	<MDL	<MDL	11.17	1.40	<MDL	7.96	1.96
Perylene	1.11	26.53	2.29	2.98	21.67	1.98	3.98	20.14	1.63
Phenanthrene	0.61	118.71	0.97	2.55	36.72	1.71	2.45	31.10	1.58
Pyrene	4.26	152.70	1.56	7.66	68.91	4.85	15.74	68.11	3.71
Retene	0.92	4.18	0.99	2.55	7.04	3.32	2.61	5.87	12.93
Total PAH Con. (ng/g)	14.50	1149.63	17.90	40.77	660.09	42.21	54.60	569.23	45.62

Table 2.6 Tissue samples from daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) in September 2014, May 2015, July 2015 and August 2015. The polycyclic aromatic hydrocarbons (PAHs) were extracted and analyzed from the samples according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The concentrations of each PAH was calculated in relation to the dry fraction of shrimp from each site.

Polycyclic Aromatic Hydrocarbons	September 2014			May 2015			July 2015			August 2015		
	TT	CC	MR	TT	CC	MR	TT	CC	MR	TT	CC	MR
2,6+2,7-Dimethylnaphthalene	<MDL	5.82	<MDL	<MDL	6.66	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Benzo(α)pyrene	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.24	<MDL	16.48	2.66	5.62	25.98
Benzo(b)fluoranthene	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.50	0.61	1.57	2.66	<MDL	<MDL
Biphenyl	1.90	4.94	28.17	<MDL	<MDL	13.12	18.00	25.06	47.85	35.93	23.88	56.50
Naphthalene	<MDL	<MDL	<MDL	<MDL	7.19	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Phenanthrene	<MDL	<MDL	<MDL	<MDL	<MDL	5.08	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Total PAH Concentration (ng/g)	1.90	10.76	28.17	0.00	16.20	18.19	20.74	25.67	65.91	41.25	29.50	82.48

Table 2.7 Tissue samples from daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) in September 2014, May 2015, July 2015 and August 2015. The polycyclic aromatic hydrocarbons (PAHs) were extracted and analyzed from the samples according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The concentrations of each PAH was calculated in relation to the wet weight of shrimp from each site.

Polycyclic Aromatic Hydrocarbons	September 2014			May 2015			July 2015			August 2015		
	TT	CC	MR	TT	CC	MR	TT	CC	MR	TT	CC	MR
2,6+2,7-Dimethylnaphthalene	<MDL	3.00	<MDL	<MDL	3.42	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Benzo(α)pyrene	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.46	<MDL	4.08	0.99	2.89	6.43
Benzo(b)fluoranthene	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.56	0.32	0.39	0.99	<MDL	<MDL
Biphenyl	0.71	2.54	6.97	<MDL	1.21	3.25	6.70	12.89	11.85	13.37	12.29	13.99
Naphthalene	<MDL	<MDL	<MDL	<MDL	3.70	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Phenanthrene	<MDL	<MDL	<MDL	<MDL	<MDL	1.26	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Total PAH Concentration (ng/g)	0.71	5.54	6.97	0.00	8.33	4.50	7.72	13.20	16.32	15.35	15.18	20.42

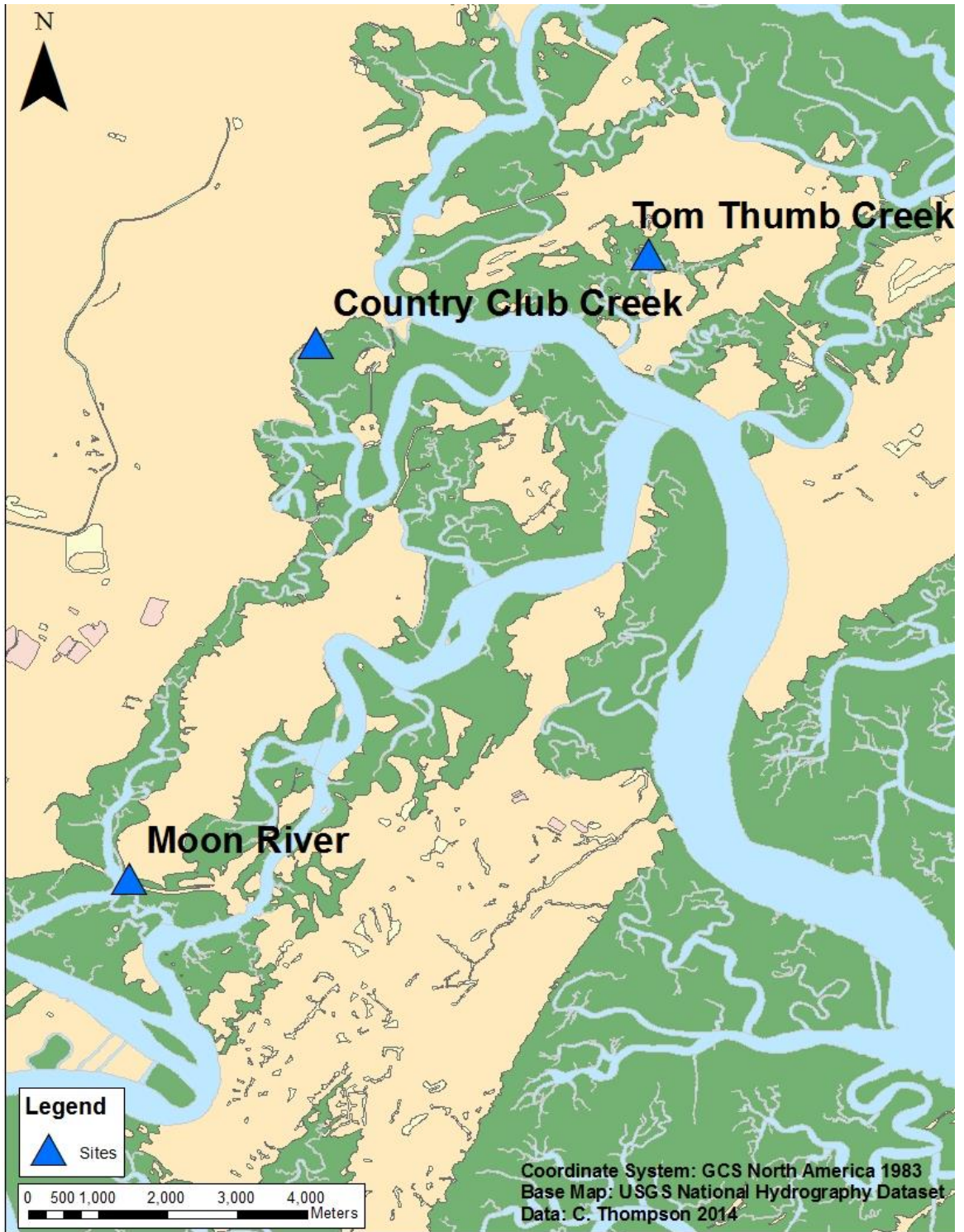


Figure 2.1. The sampling sites for daggerblade grass shrimp collection all located on the eastern part of the Savannah, Georgia region. Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR).

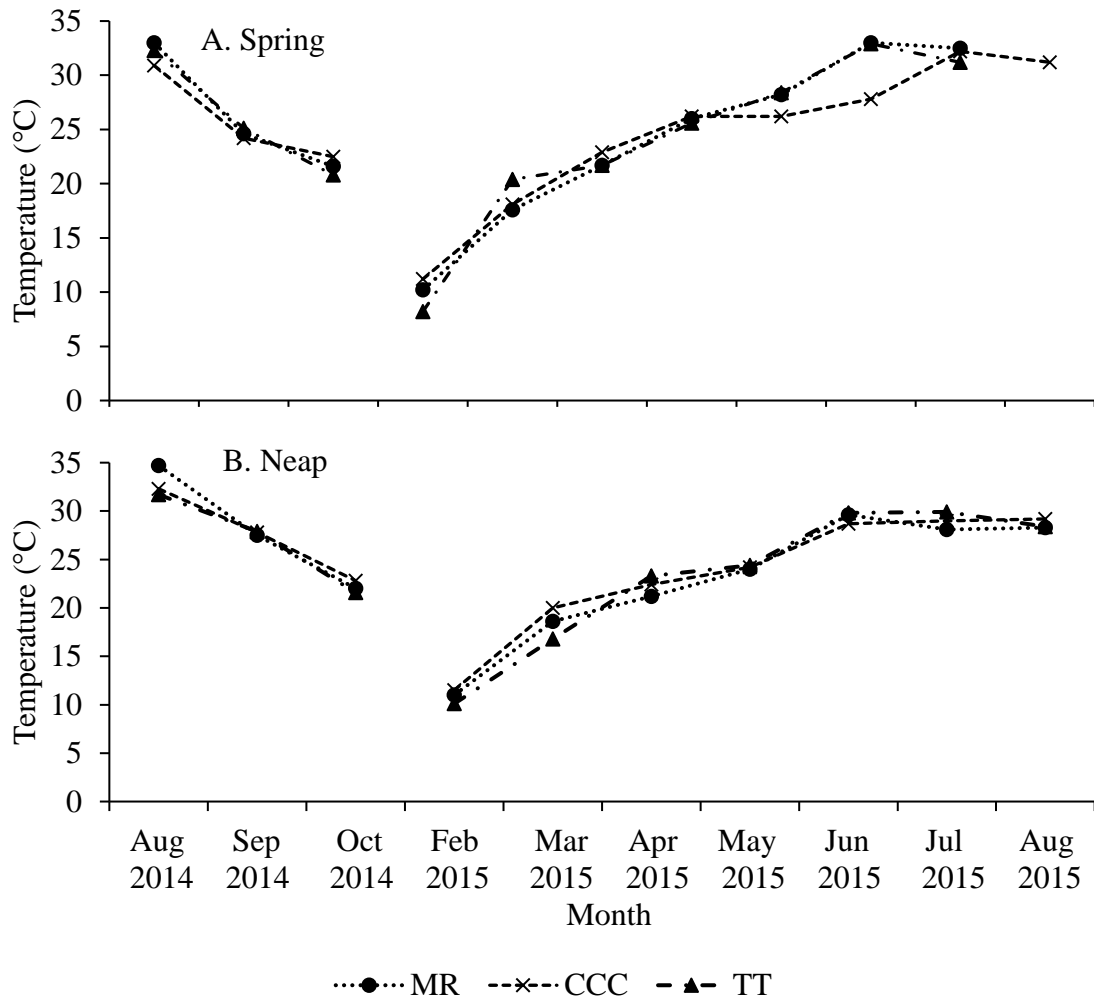


Figure 2.2. Temperature (°C) was measured at Tom Thumb Creek (▲), Country Club Creek (x), and Moon River (●) in Georgia, USA. Measurements were taken within 2 h of low tide monthly during (A.) spring and (B.) neap tide. Data were collected from August-October 2014 and February-August 2015.

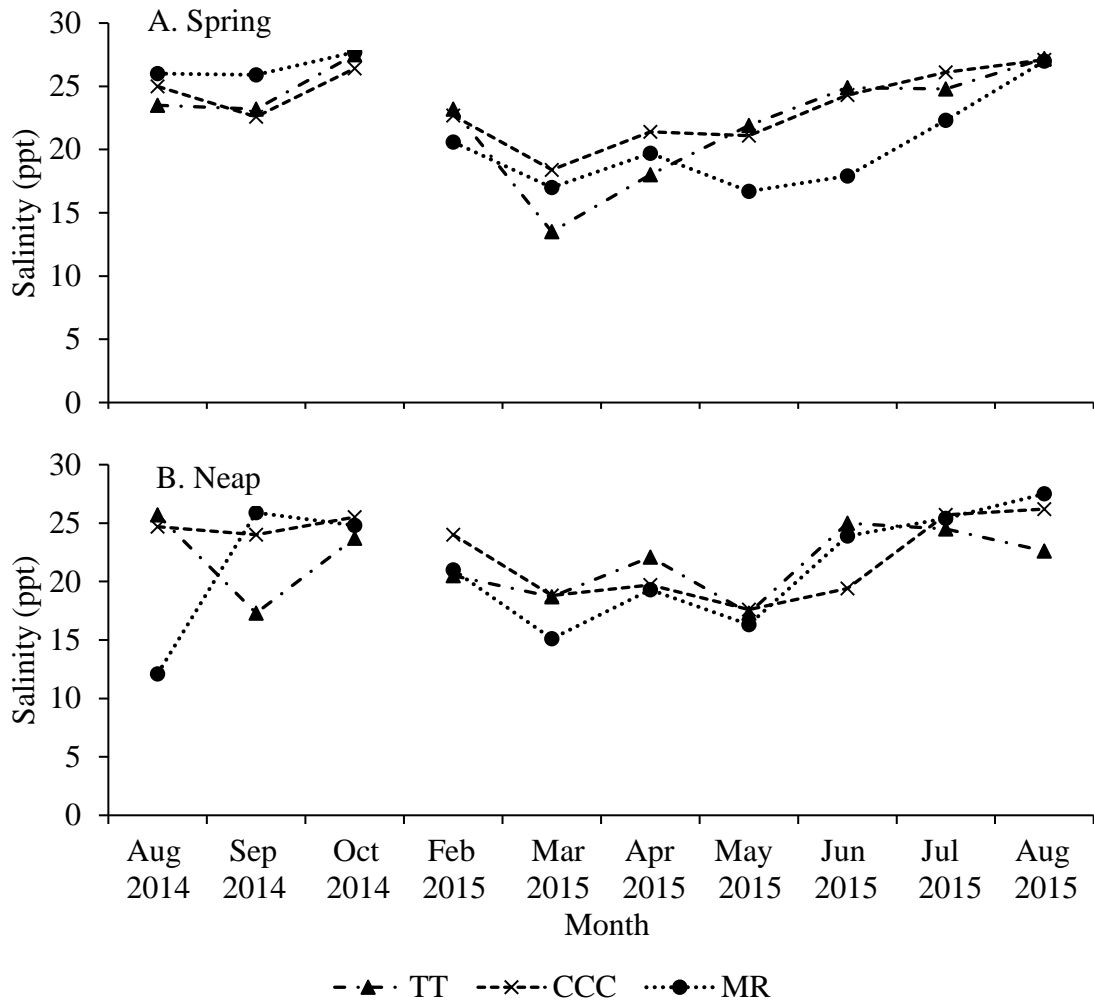


Figure 2.3. Salinity (ppt) was measured at Tom Thumb Creek (▲), Country Club Creek (x), and Moon River (●) in Georgia, USA. Measurements were taken within 2 h of low tide monthly during (A.) spring and (B.) neap tide. Data were collected from August-October 2014 and February-August 2015.

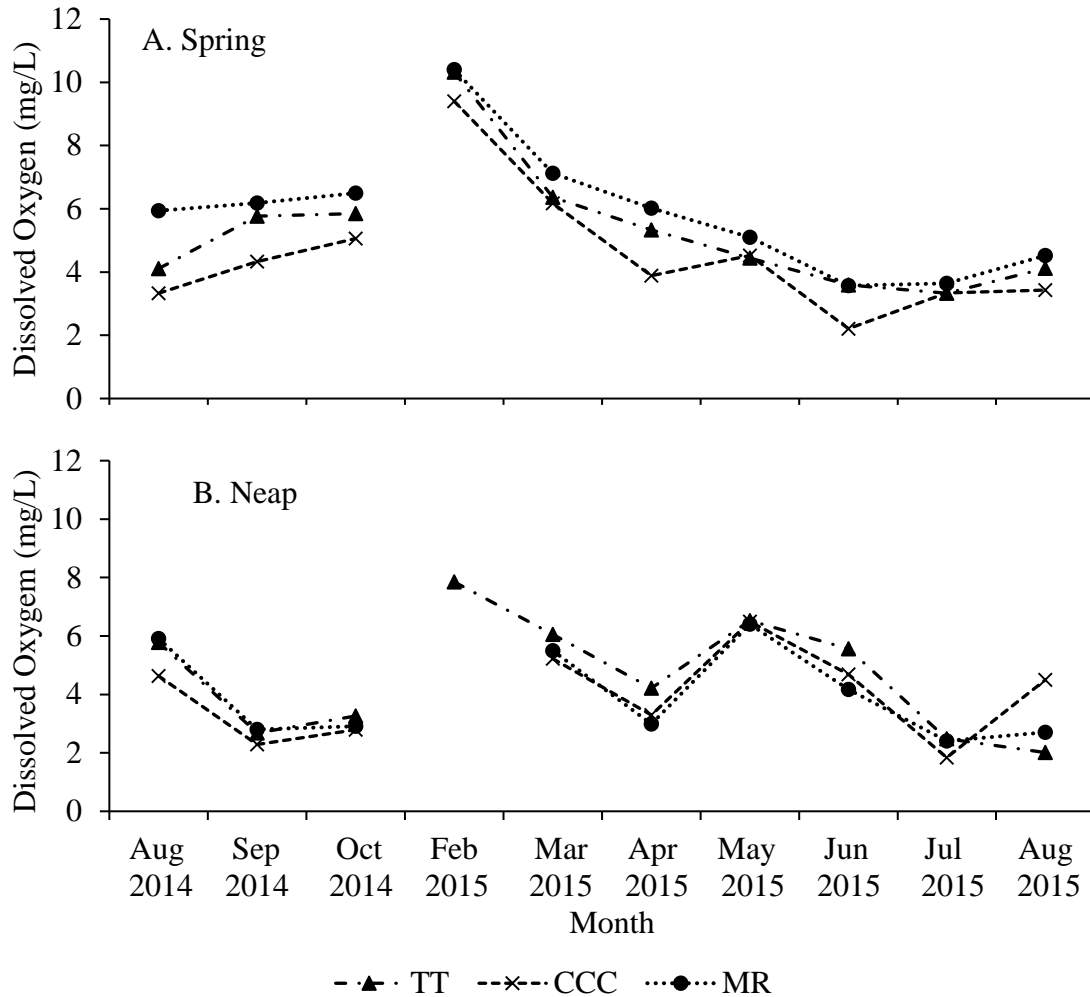


Figure 2.4. Dissolved oxygen (mg/L) was measured at Tom Thumb Creek (▲), Country Club Creek (x), and Moon River (●) in Georgia, USA. Measurements were taken within 2 h of low tide monthly during (A.) spring and (B.) neap tide. Data were collected from August-October 2014 and February-August 2015. There were no data collected for the February neap tide at Moon River nor Country Club Creek.

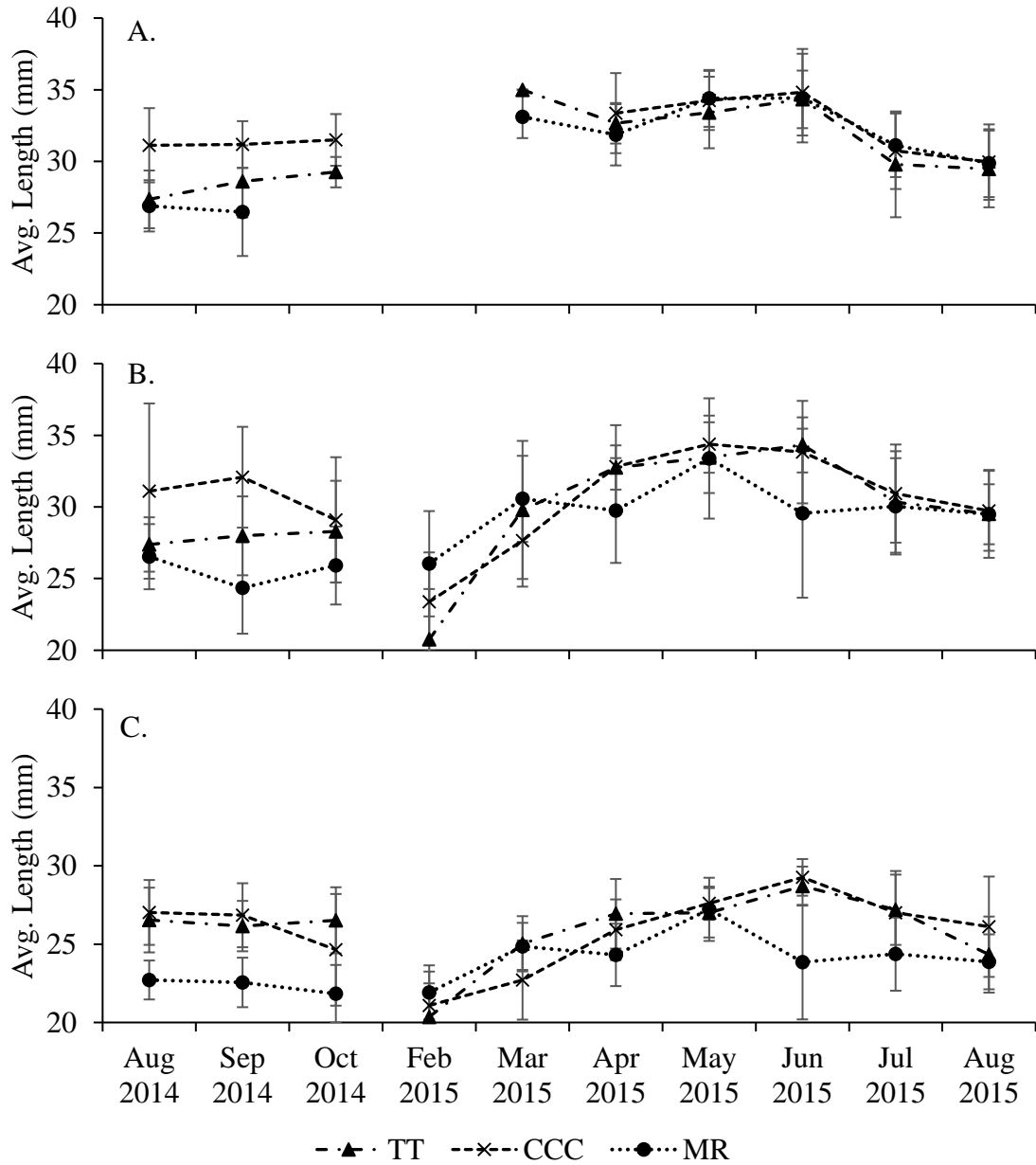


Figure 2.5. Average length (mm) of daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (▲), Country Club Creek (x), and Moon River (●) from August-October 2014 and February-August 2015. The average length for shrimp was measured for (A.) ovigerous females, (B.) non-ovigerous females, and (C.) males \pm SD. No ovigerous shrimp were collected during October at Moon River nor during March at Tom Thumb and Country Club Creek. No ovigerous shrimp were collected at any site during February.

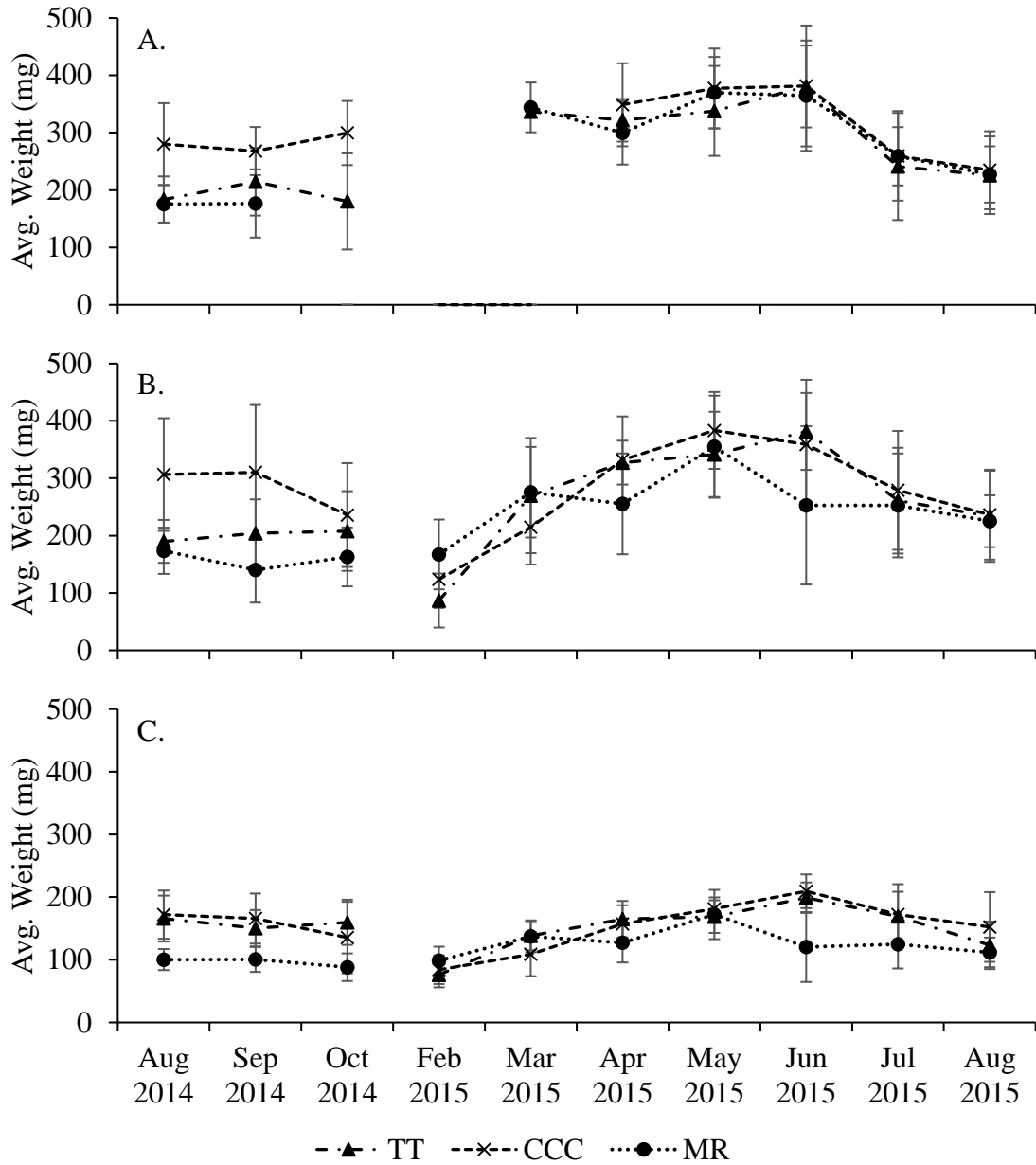


Figure 2.6. Average weight of daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (\blacktriangle), Country Club Creek (x), and Moon River (\bullet) from August-October 2014 and February-August 2015. The average weight for shrimp was measured for (A.) ovigerous females, (B.) non-ovigerous females, and (C.) males \pm SD. No ovigerous shrimp were collected during October at Moon River nor during March at Tom Thumb and Country Club Creek. No ovigerous shrimp were collected at any site during February.

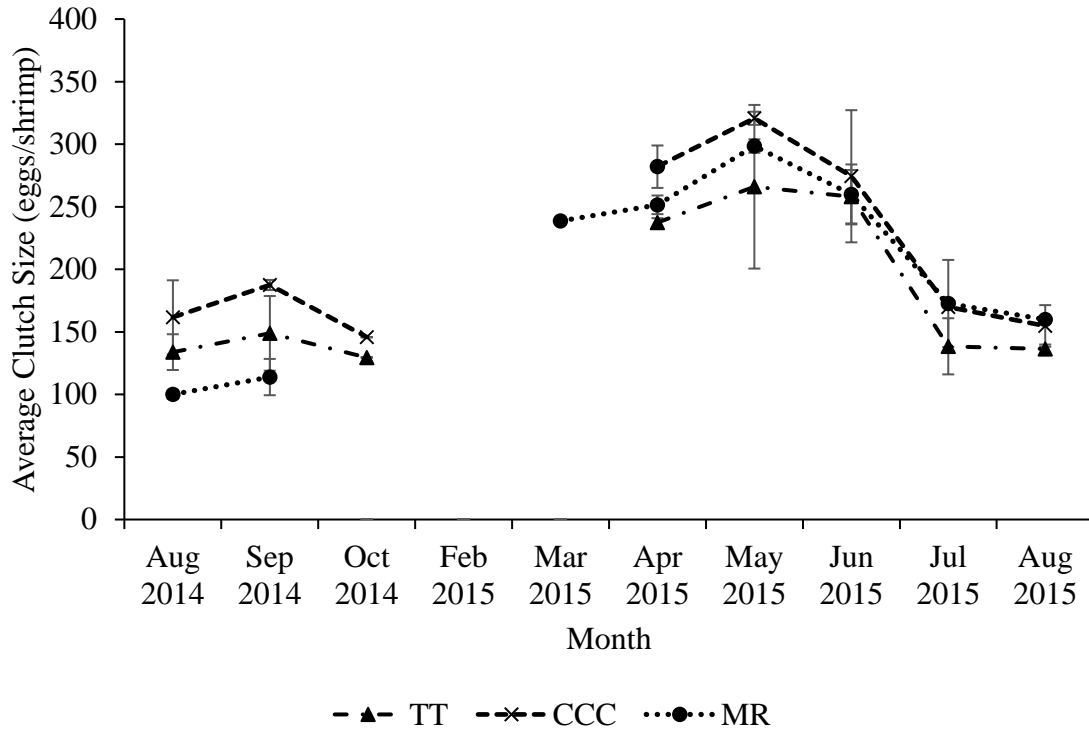


Figure 2.7. Average clutch size for ovigerous daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (▲), Country Club Creek (x), and Moon River (●) from August-October 2014 and February-August 2015. The monthly average clutch size (eggs/shrimp) for ovigerous shrimp was determined \pm SD. No ovigerous shrimp were collected during October at Moon River nor during March at Tom Thumb and Country Club Creek. No ovigerous shrimp were collected at any site during February.

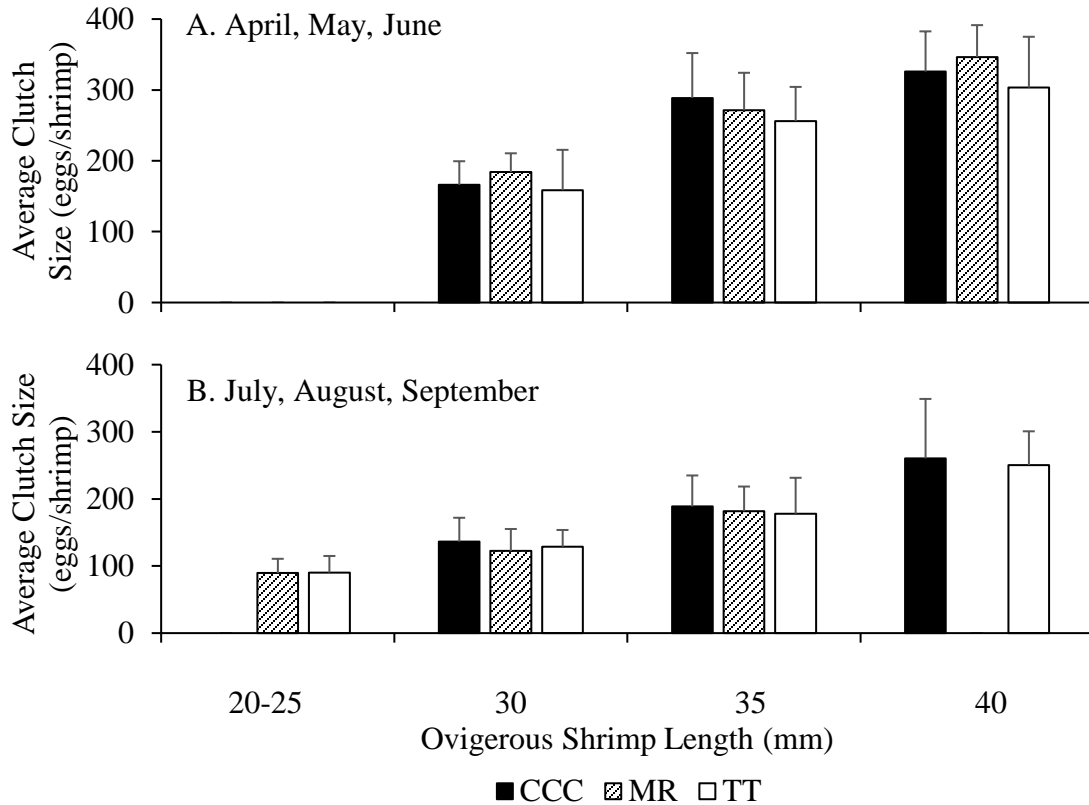


Figure 2.8. Average clutch size of ovigerous daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The clutch size for ovigerous shrimp was measured monthly for (A.) early months in the reproductive season (April, May, June) and (B.) the later months in the reproductive season (July, August, September). The average clutch size for each length category + 1 SD was calculated.

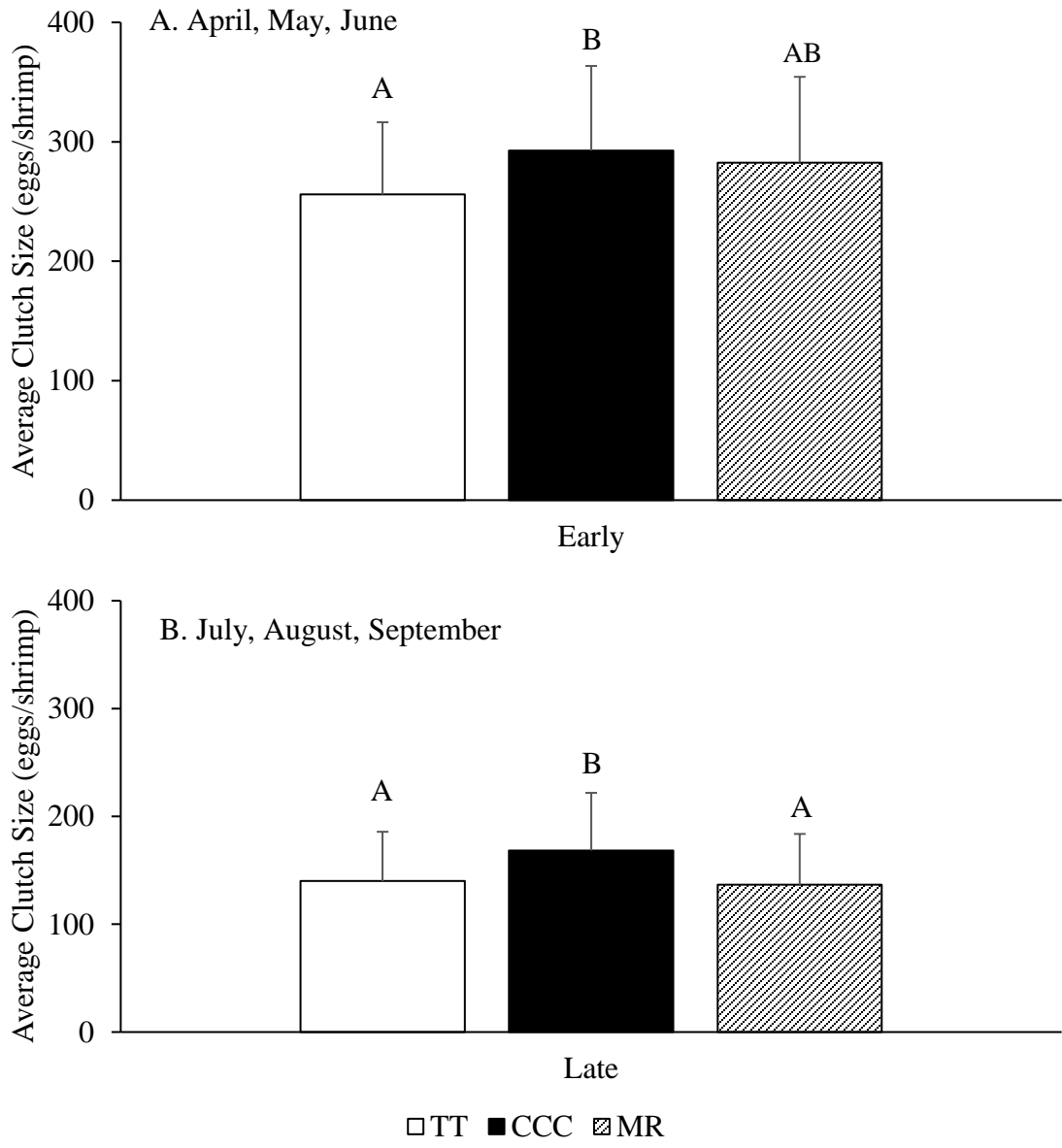


Figure 2.9. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The clutch size for ovigerous shrimp was measured monthly for (A.) early in the reproductive season in April, May, and June and (B.) late in the reproductive season in July, August, and September. The average clutch size for each site category + 1 SD was calculated for each season. Significant differences are indicated by different letters. ($\alpha=0.05$).

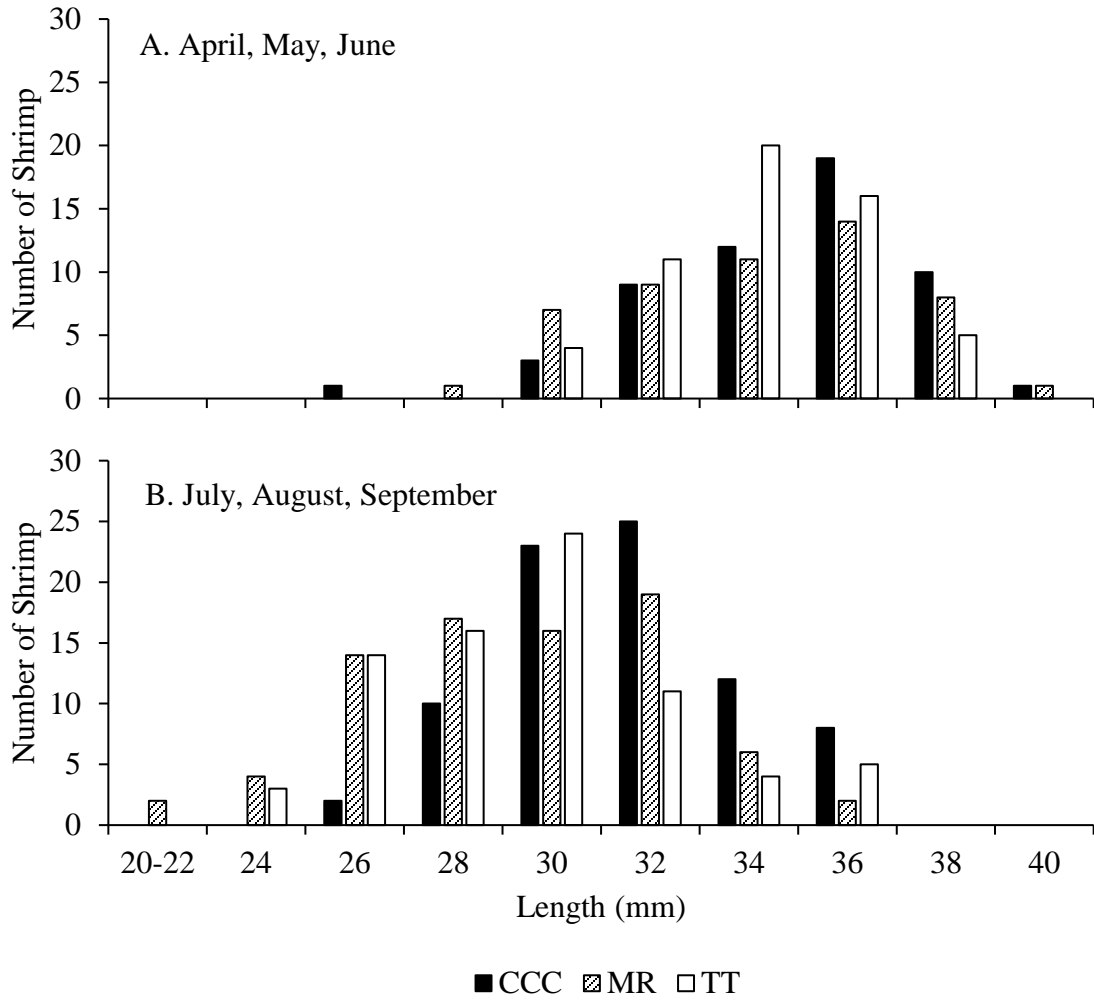


Figure 2.10. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The length frequency distribution for (A.) April, May, and June and (B.) July, August, and September.

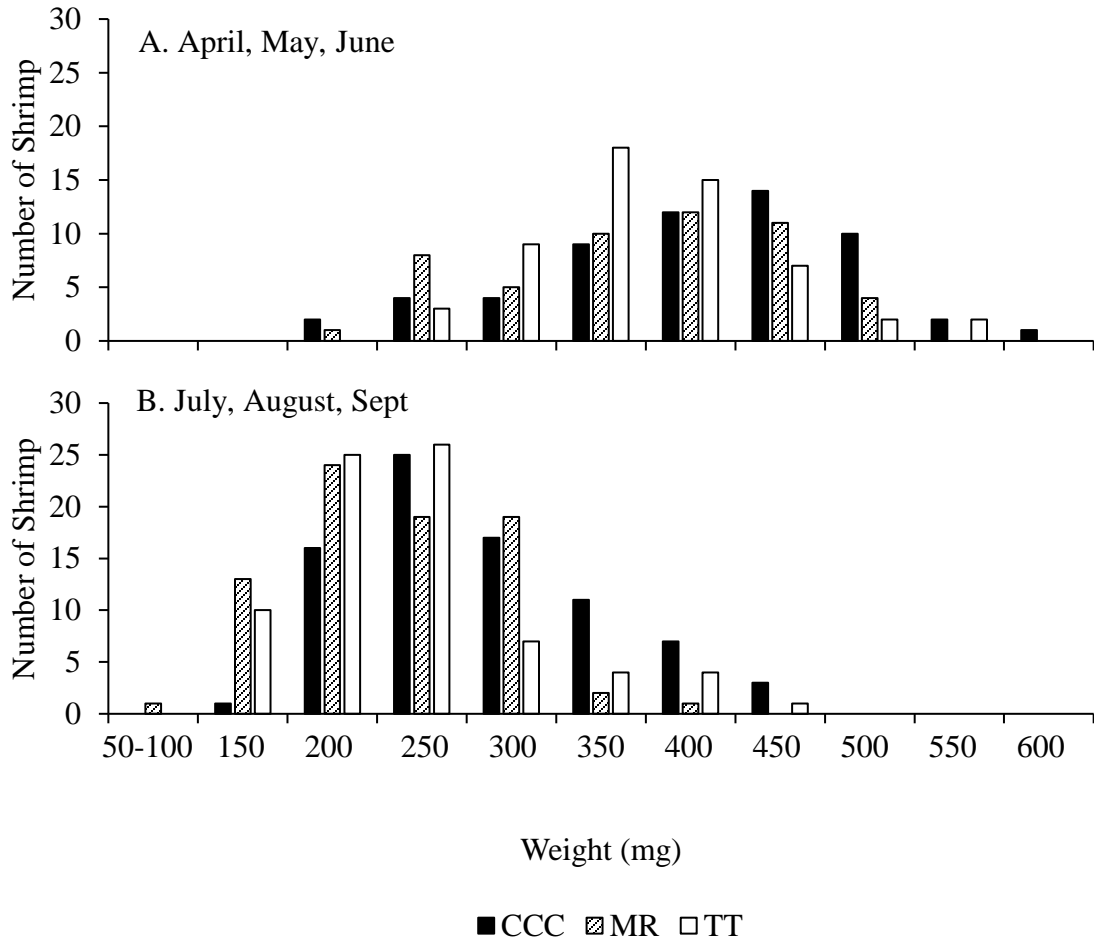


Figure 2.11. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The weight frequency distribution for (A.) April, May, and June and (B.) July, August, and September.

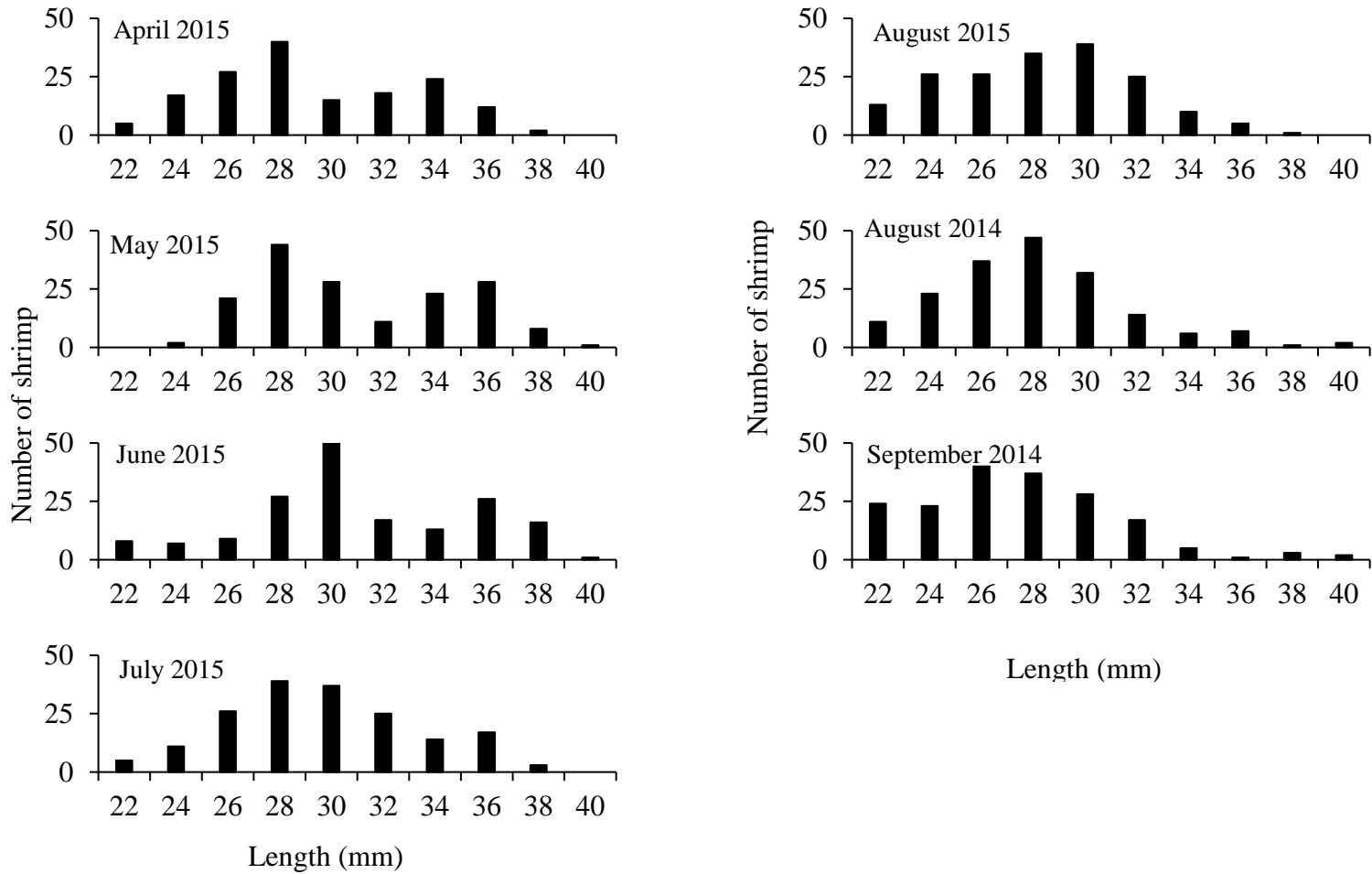


Figure 2.12. Daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The monthly length frequency distribution for April-September was calculated.

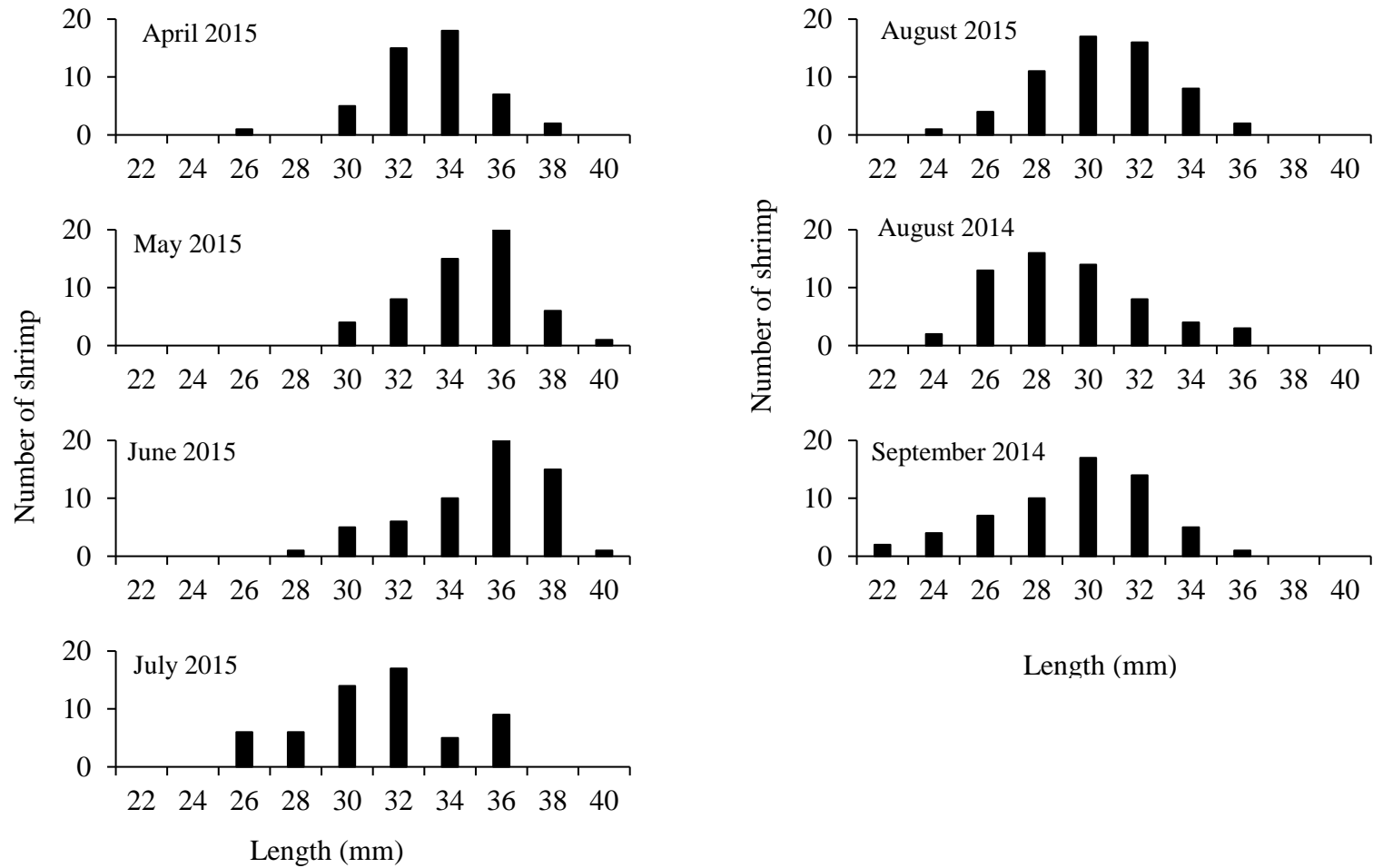


Figure 2.13. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-September 2014 and April-August 2015. The monthly length frequency distribution for April-September was calculated.

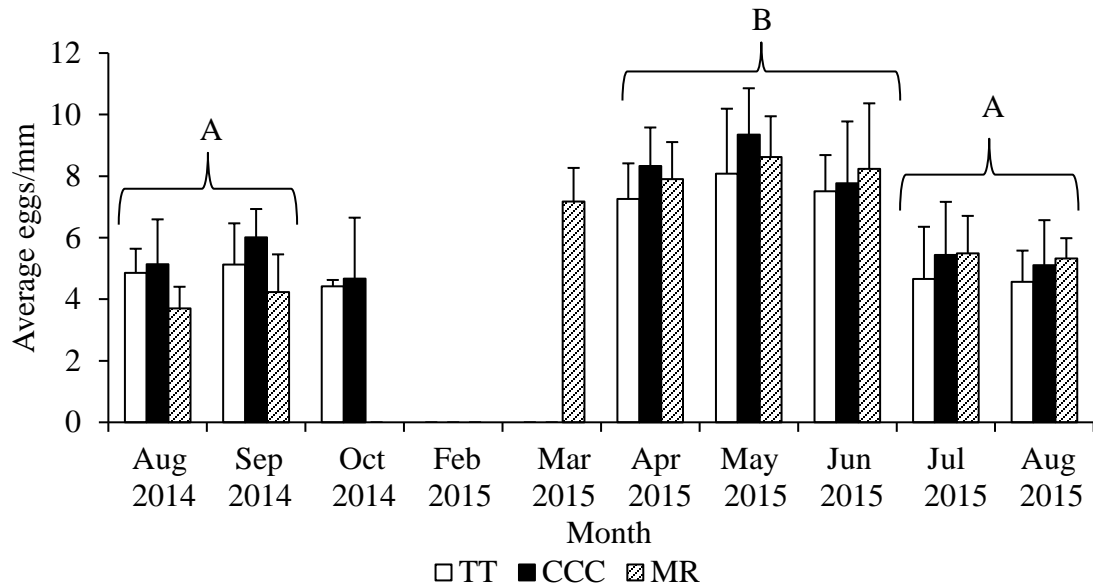


Figure 2.14. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected at Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR). The average number of eggs per mm shrimp length + 1 SD was calculated. Significant differences are indicated by different letters ($\alpha=0.05$).

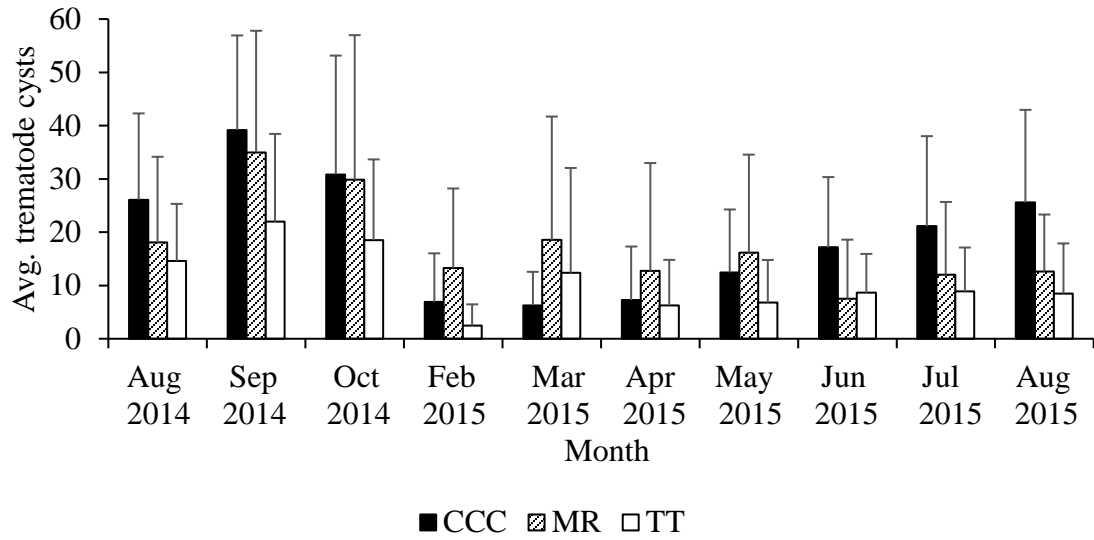


Figure 2.15. Daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek (TT), Country Club Creek (CC), and Moon River (MR) from August-October 2014 and February-August 2015. The number of trematode cysts in each shrimp was determined. Data are reported as average trematode abundance + 1 SD.

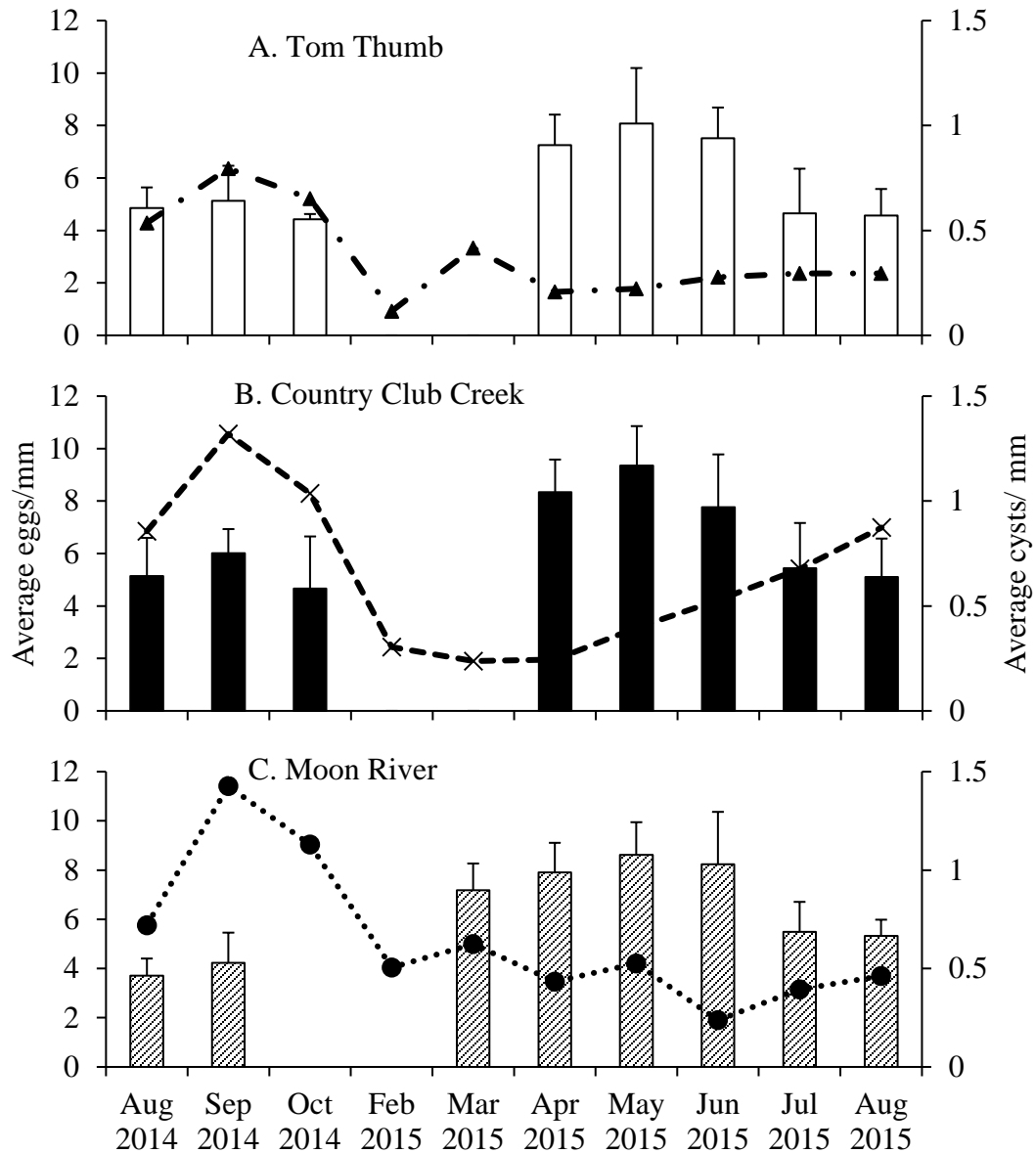


Figure 2.16. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek, (B.) Country Club Creek, and (C.) Moon River from August-October 2014 and February-August 2015. The average number of eggs per mm shrimp length + 1 SD and the average number of trematode cysts per mm shrimp length were determined for each month. Bars represent the average number of eggs/mm shrimp length. The lines represent the average number of trematode cysts/mm shrimp length.

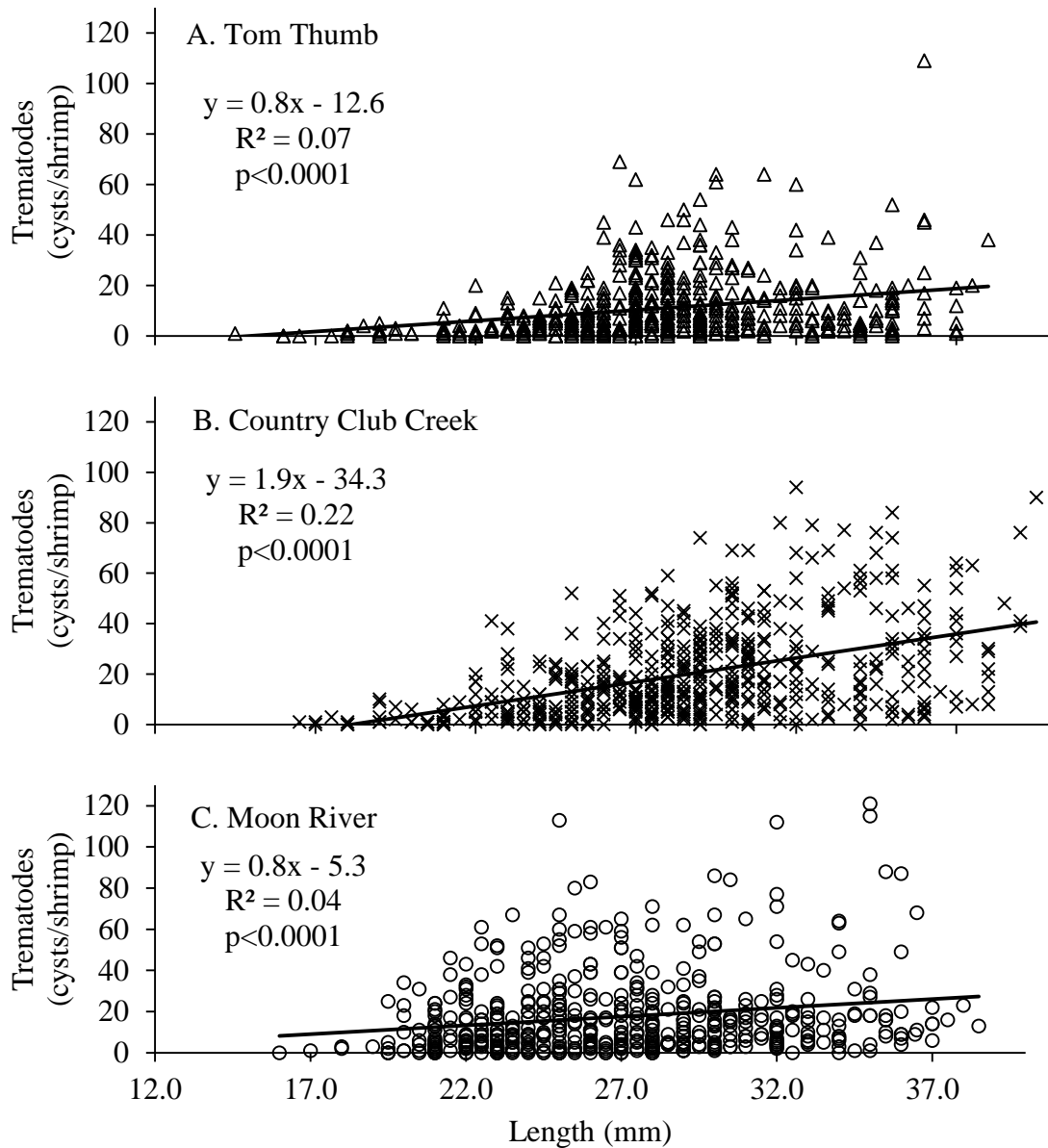


Figure 2.17. Daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from August-October 2014 and February-August 2015. The number of trematode cysts per shrimp was plotted against the length of each individual.

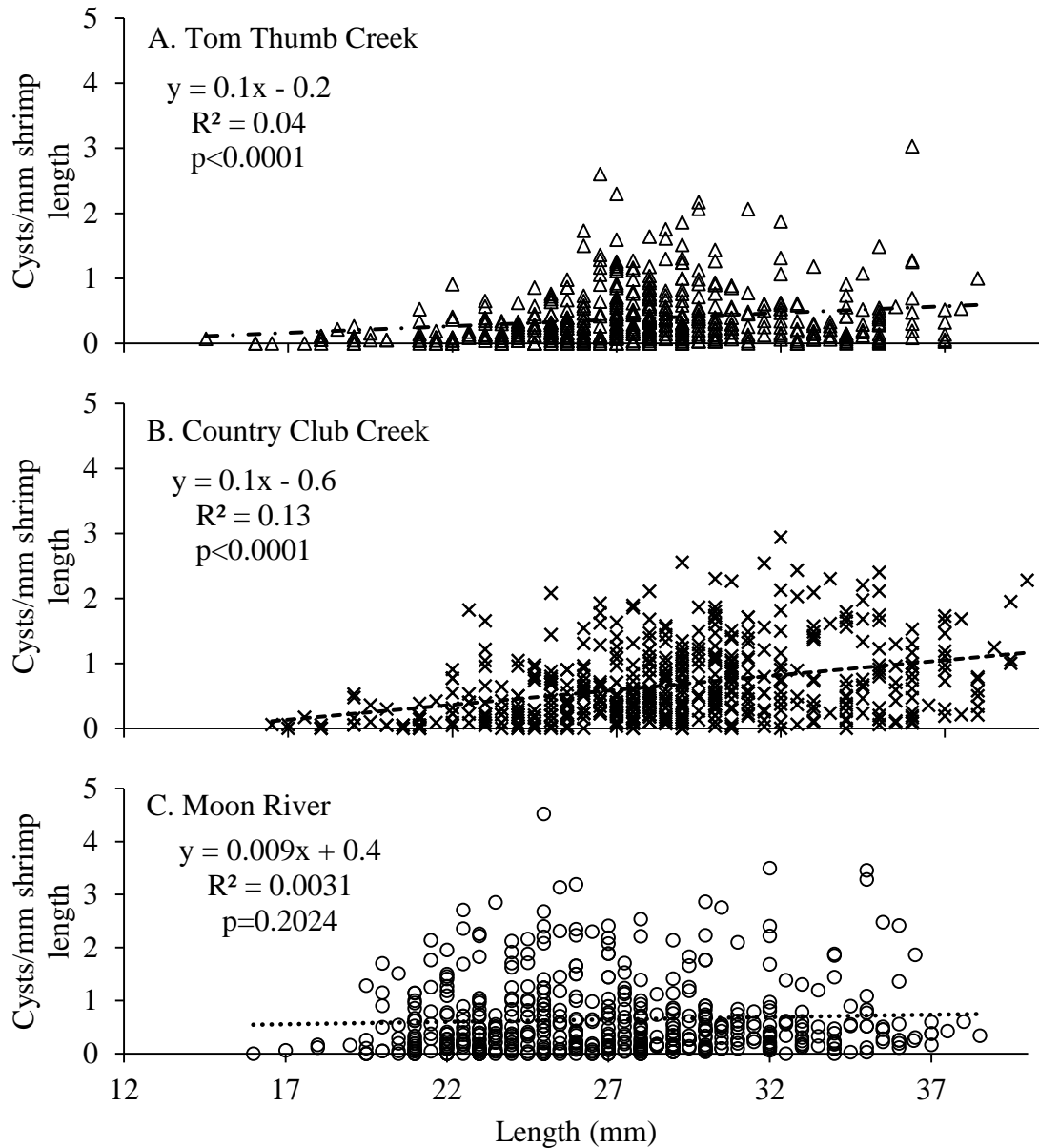


Figure 2.18. Daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from August-October 2014 and February-August 2015. The number of trematode cysts per mm shrimp length was plotted against the length of each individual.

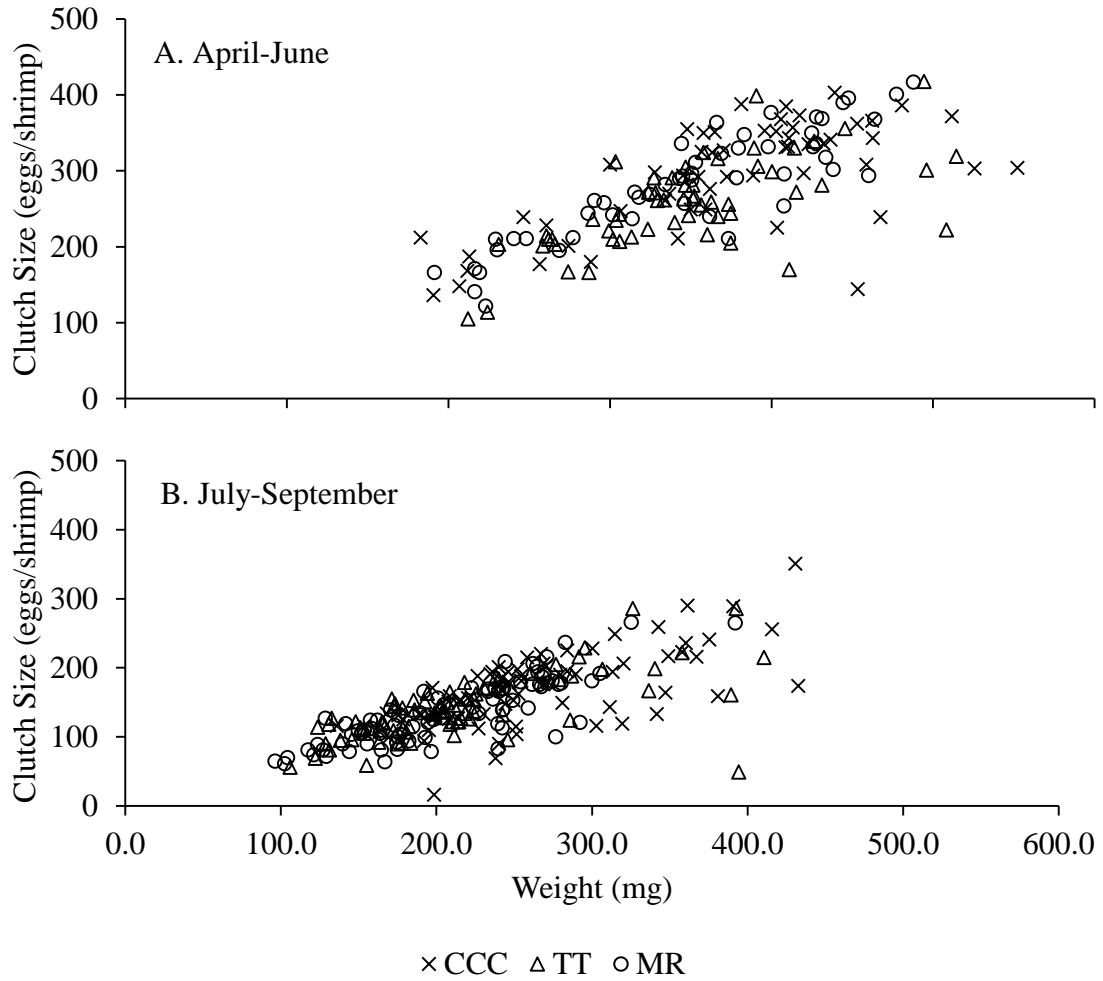


Figure 2.19. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Country Club Creek (x), Moon River (○), and Tom Thumb Creek (Δ) from August-September 2014 and April-August 2015. The number of eggs per shrimp was plotted against the weight of each individual for (A.) April, May, and June and (B.) July, August, September.

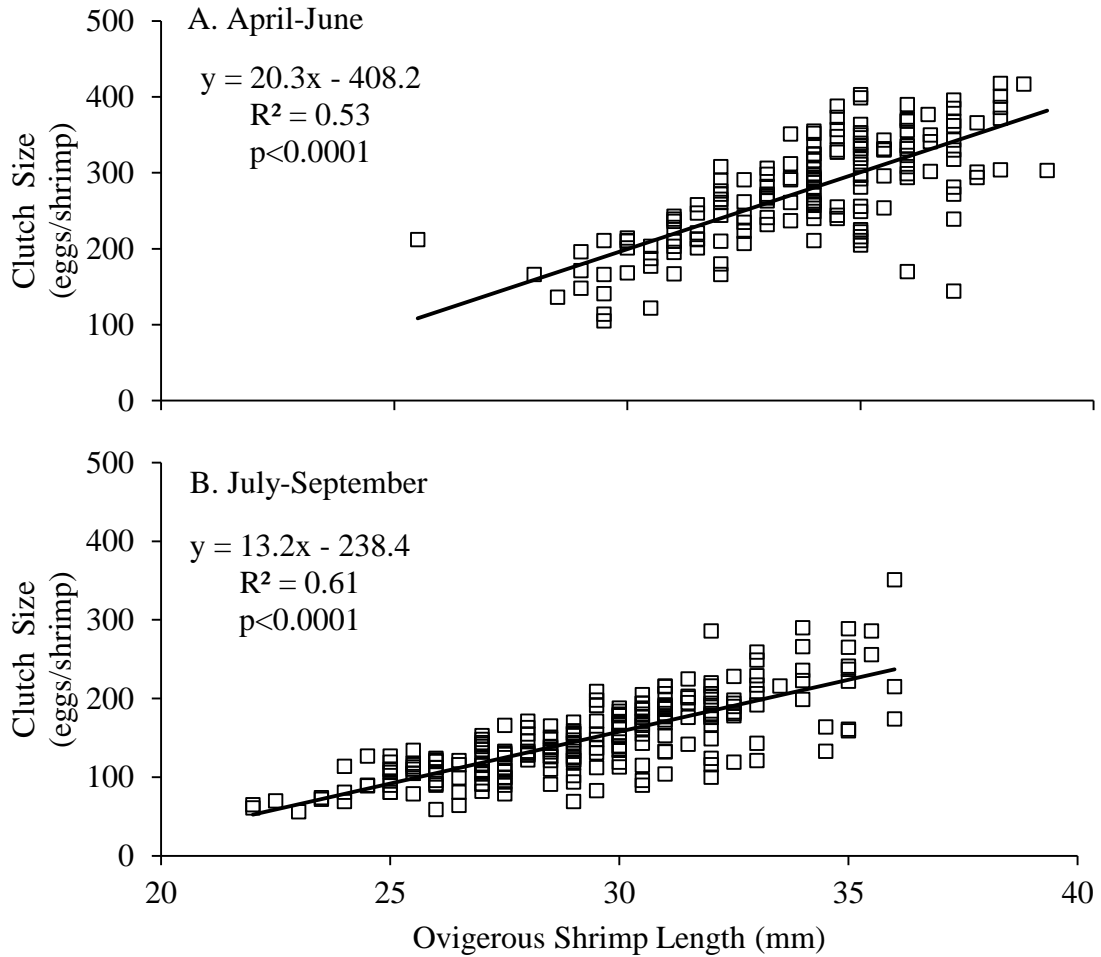


Figure 2.20. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek, Country Club Creek and Moon River combined from August-September 2014 and April-August 2015. The number of eggs per shrimp were counted and plotted against the length of each individual for (A.) April, May, and June and (B.) July, August, September. The solid lines represent the trend line from the combined locations.

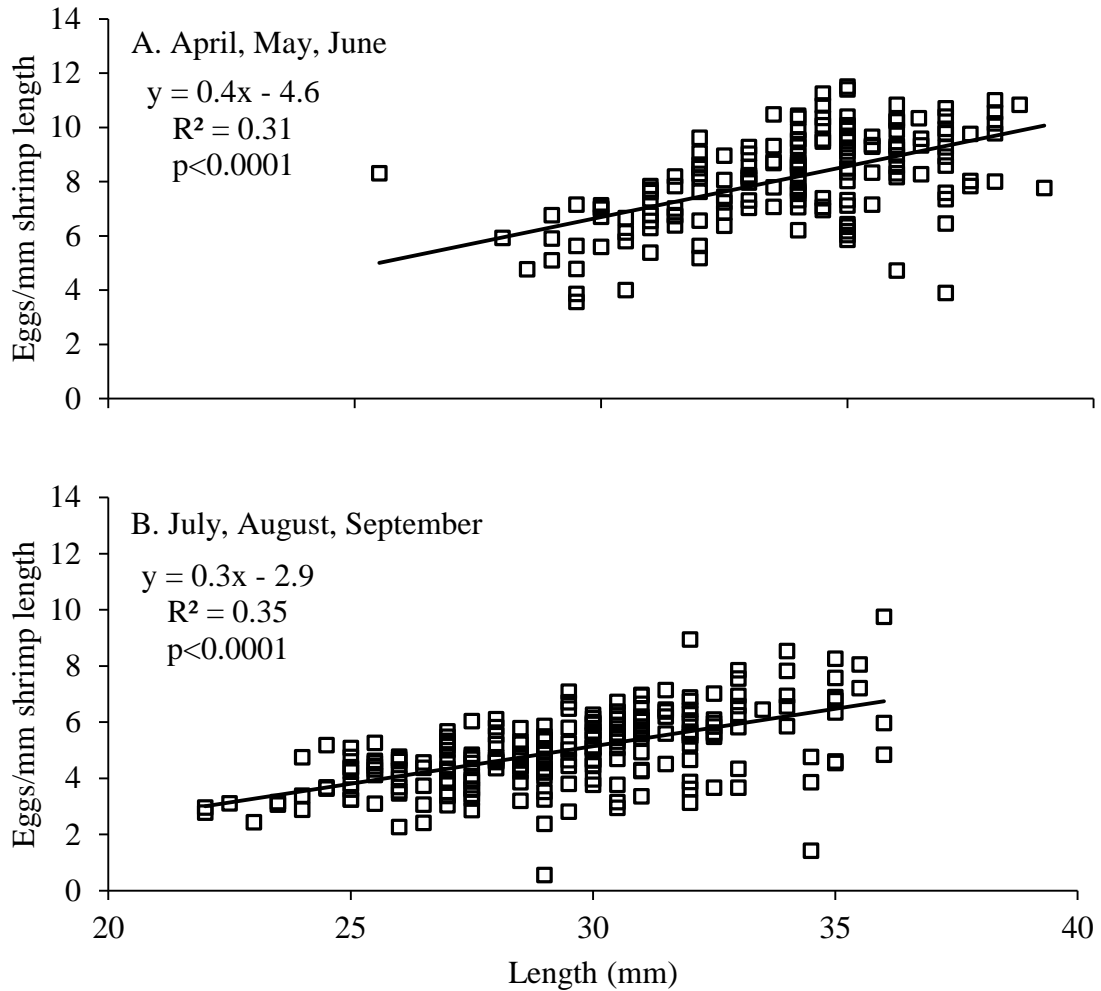


Figure 2.21. Oviparous daggerblade grass shrimp *Palaemonetes pugio* were collected from Tom Thumb Creek, Country Club Creek and Moon River combined from August-September 2014 and April-August 2015. The number of eggs per mm shrimp length was calculated and plotted against the length of each individual for (A.) April, May, and June and (B.) July, August, September. The solid lines represent the trend line from the combined locations.

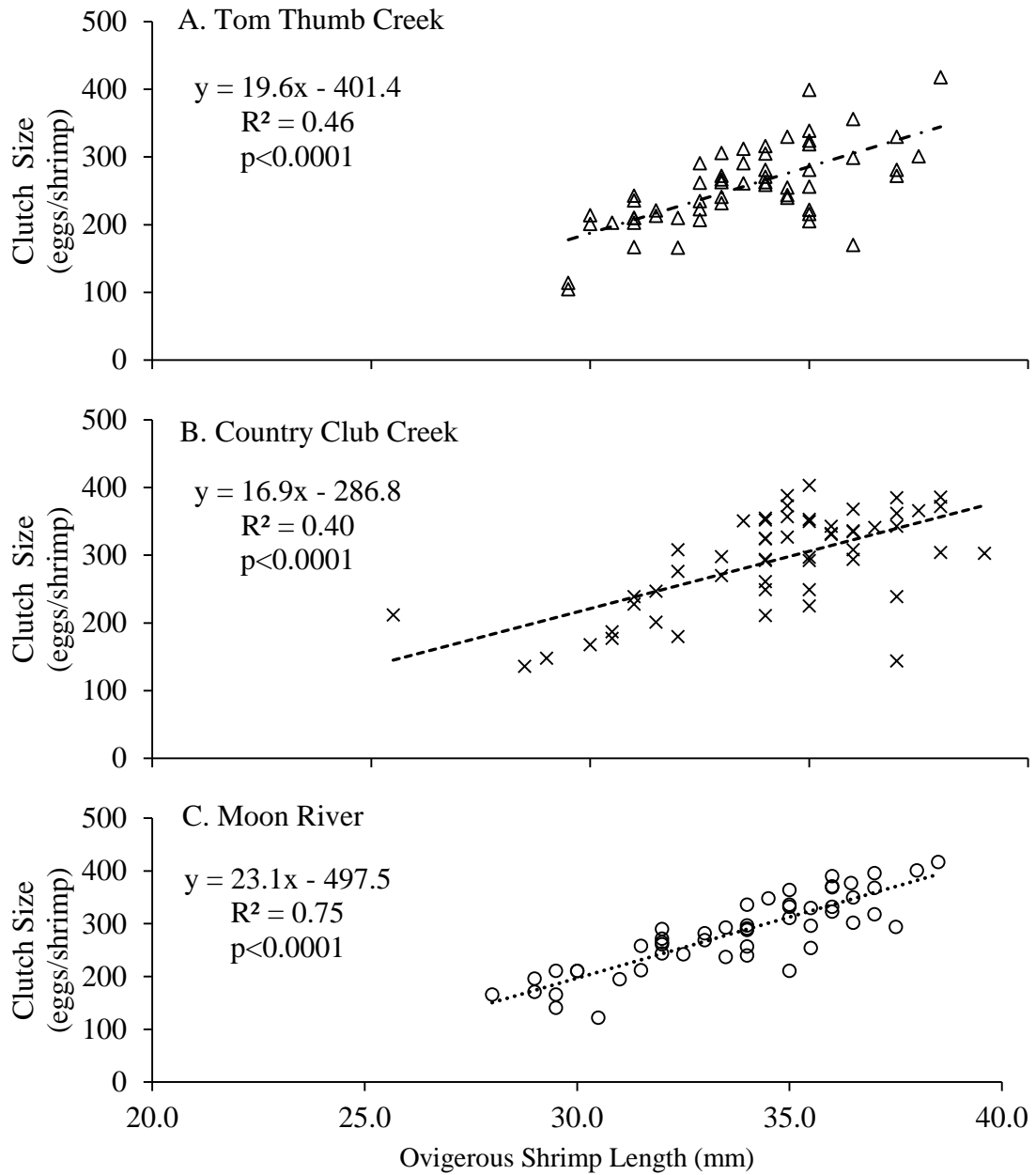


Figure 2.22. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from April, May, and June 2015. The number of eggs per shrimp were counted and plotted against the length of each individual. The dashed lines represent the trend line from each location. R^2 values were calculated for each site.

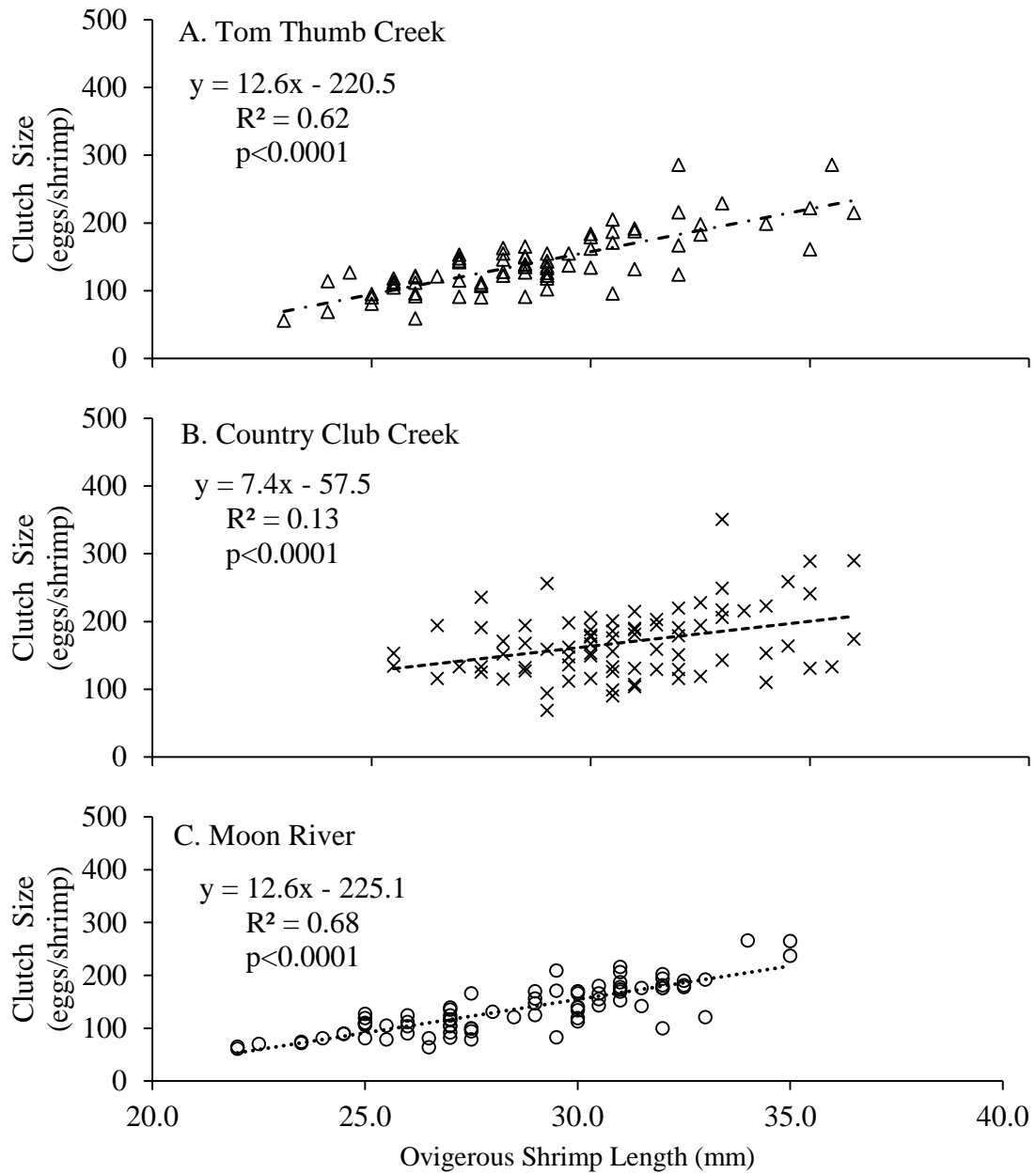


Figure 2.23. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from July 2015, August 2014 and 2015, and September 2014. The number of eggs per shrimp were counted and plotted against the length of each individual. The dashed lines represent the trend line from each location. R^2 was calculated for each site.

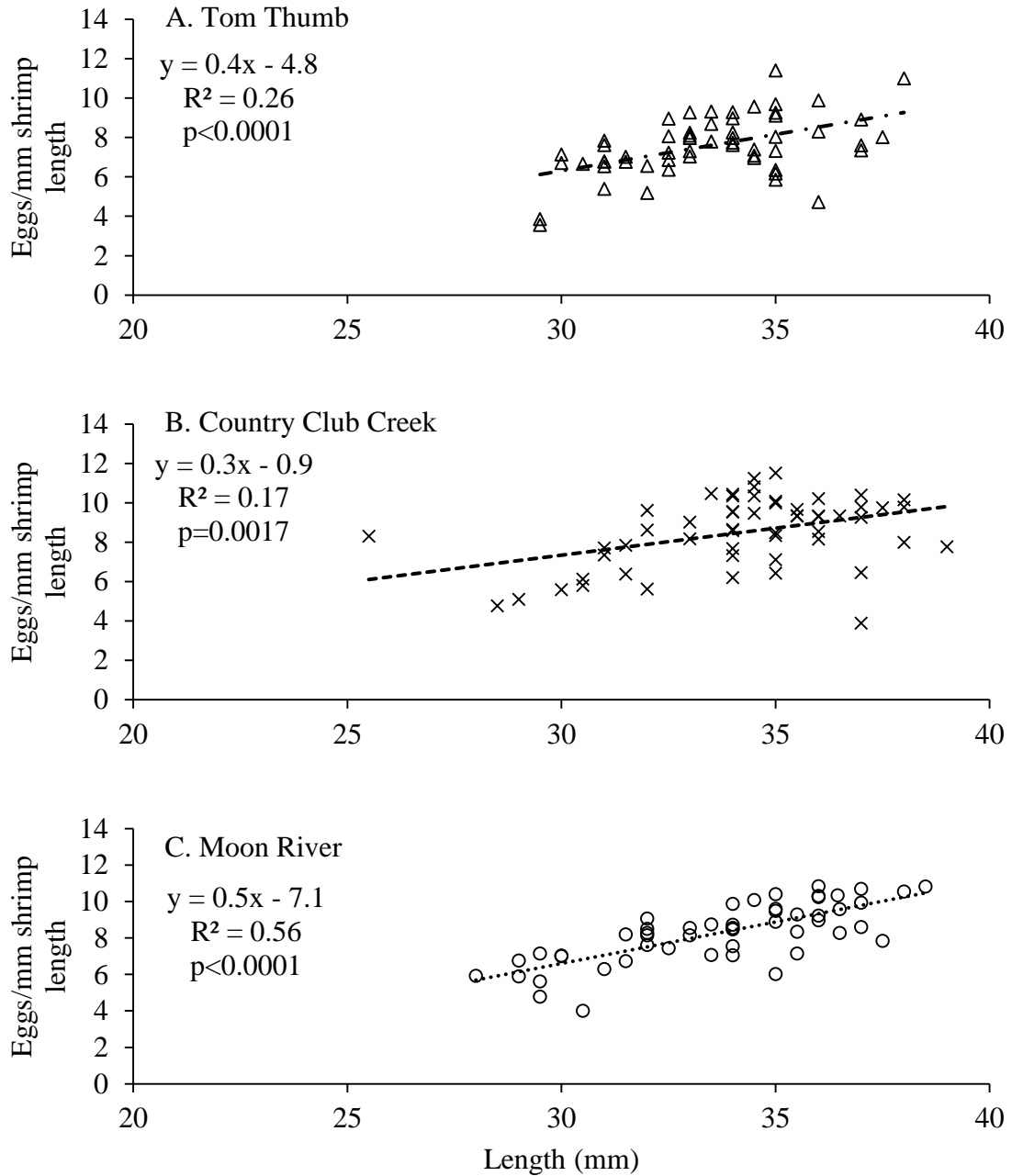


Figure 2.24. Oviparous daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from April, May, and June 2015. The number of eggs per mm shrimp length was calculated and plotted against the length of each individual. The dashed lines represent the trend line from each location. R^2 values were calculated for each site.

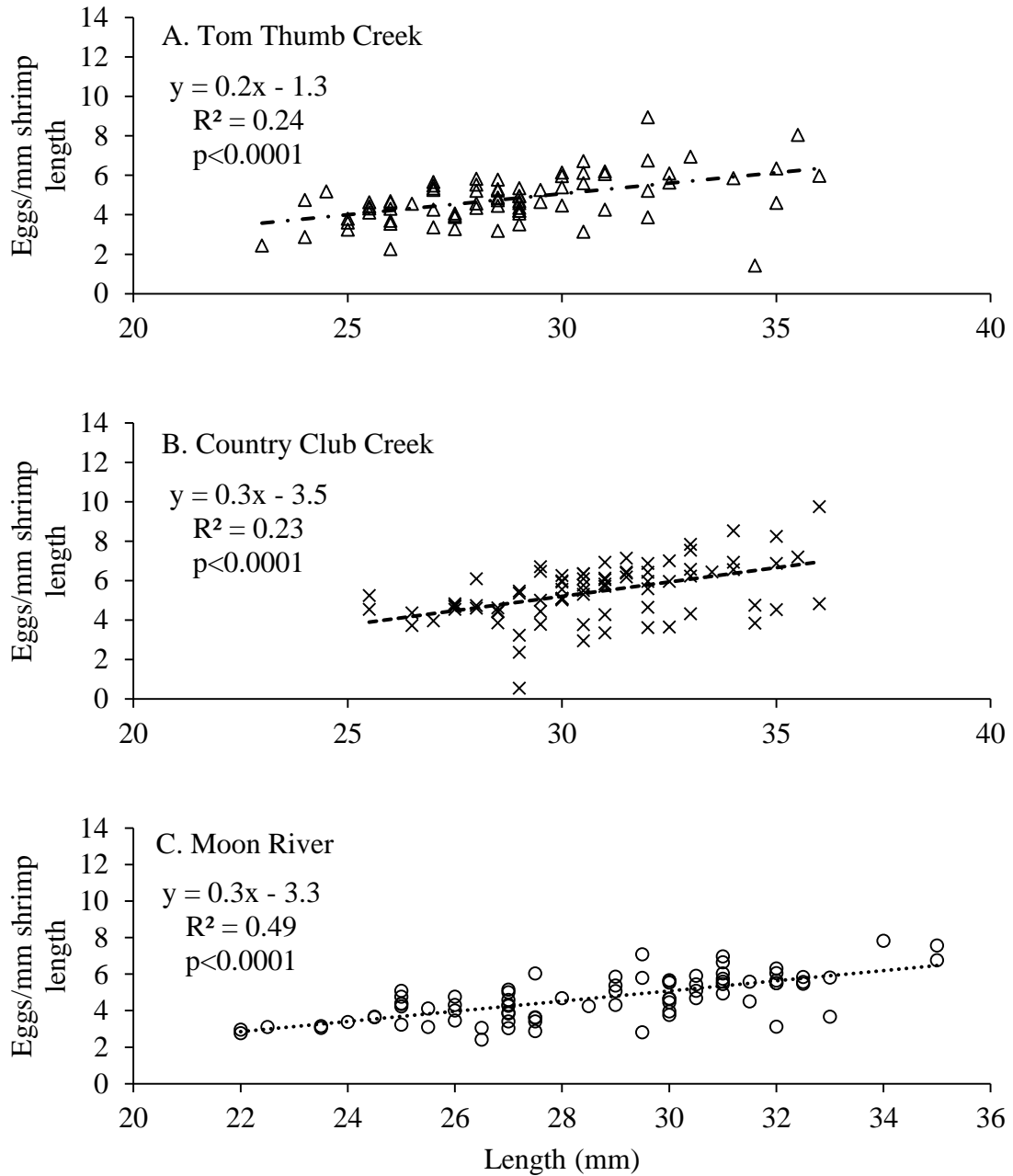


Figure 2.25. Oviparous daggerblade grass shrimp *Palaemonetes pugio* were collected from (A.) Tom Thumb Creek (Δ), (B.) Country Club Creek (x), and (C.) Moon River (\circ) from July 2015, August 2014 and 2015, and September 2014. The number of eggs per mm shrimp length was calculated and plotted against the length of each individual. The dashed lines represent the trend line from each location. R^2 was calculated for each site.

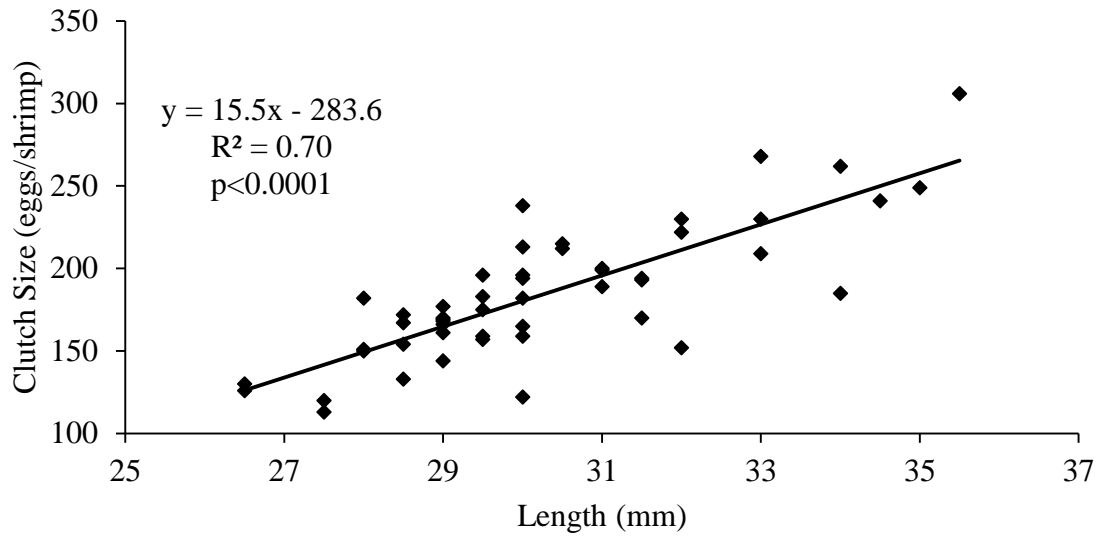


Figure 2.26. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* (n=50) were collected from Country Club Creek in September 2015. The number of eggs per shrimp were counted and plotted against the length of each individual. The solid line represents the trend line and the R^2 was calculated.

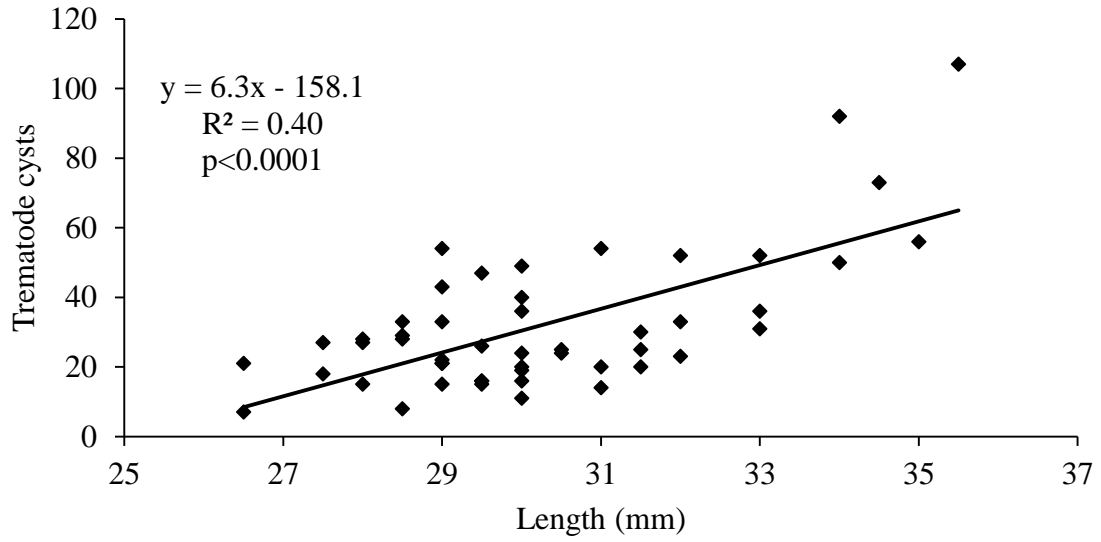


Figure 2.27. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* (n=50) were collected from Country Club Creek in September 2015. The number of trematode cysts per shrimp were counted and plotted against the length of each individual. The solid line represents the trend line and the R^2 was calculated.

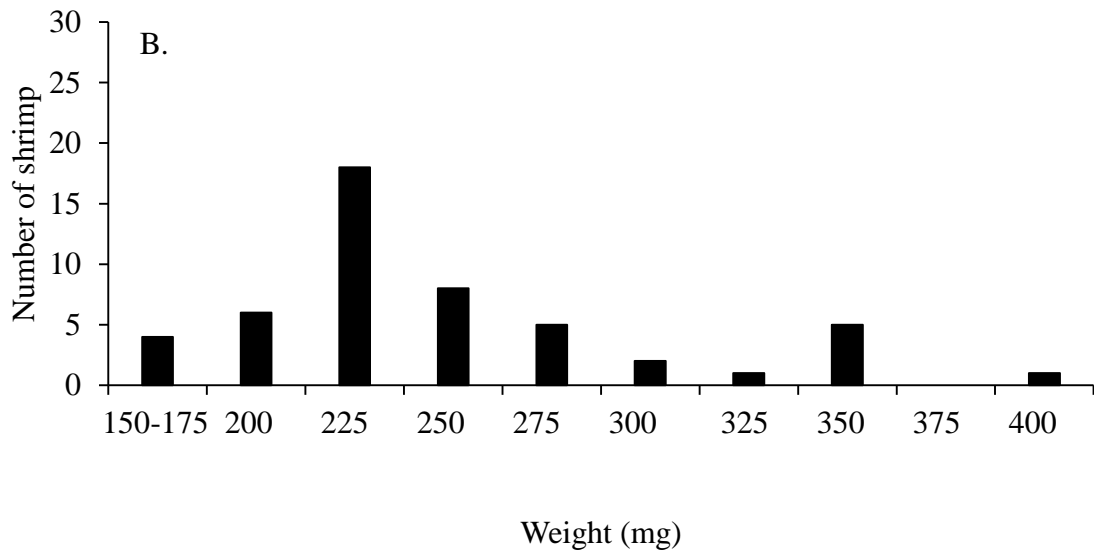
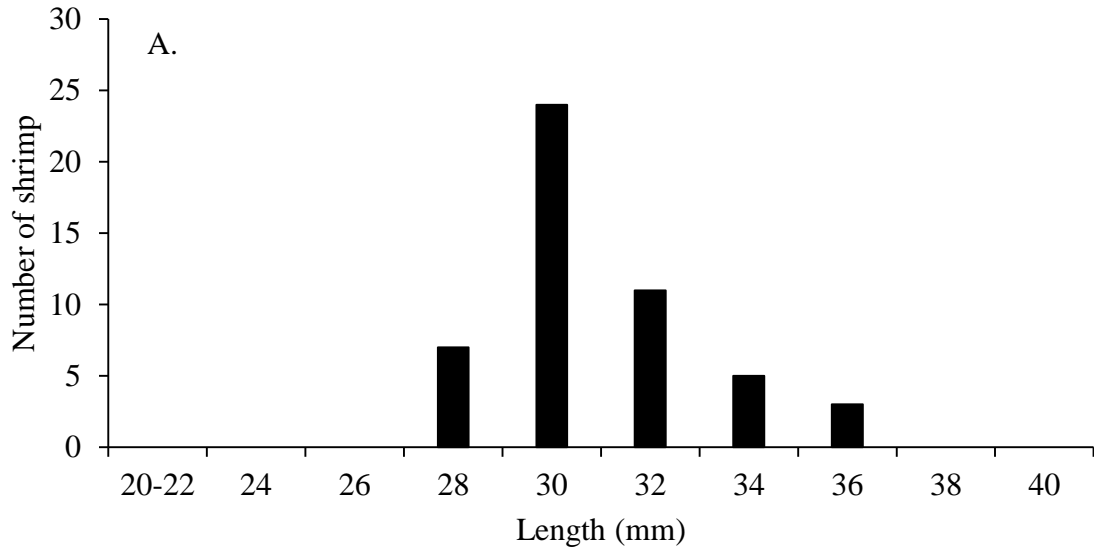
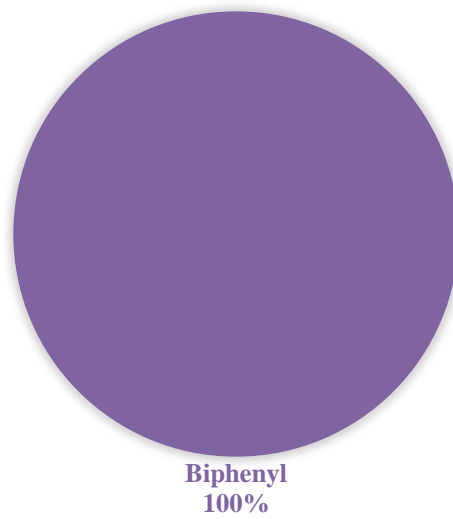


Figure 2.28. Ovigerous daggerblade grass shrimp *Palaemonetes pugio* (n=50) were collected from Country Club Creek in September 2015. The (A.) length and (B.) weight frequency distributions were calculated.

A.



B.

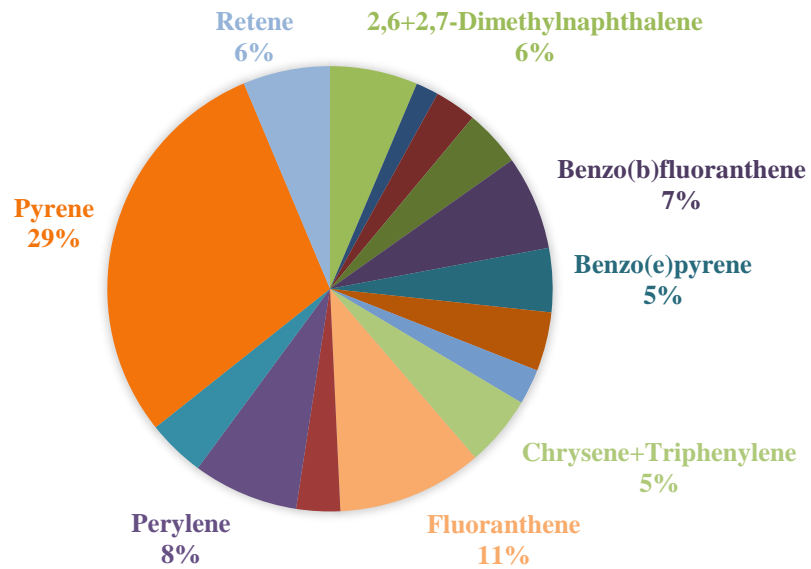


Figure 2.29. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Tom Thumb September 2014 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$ of the total PAHs.

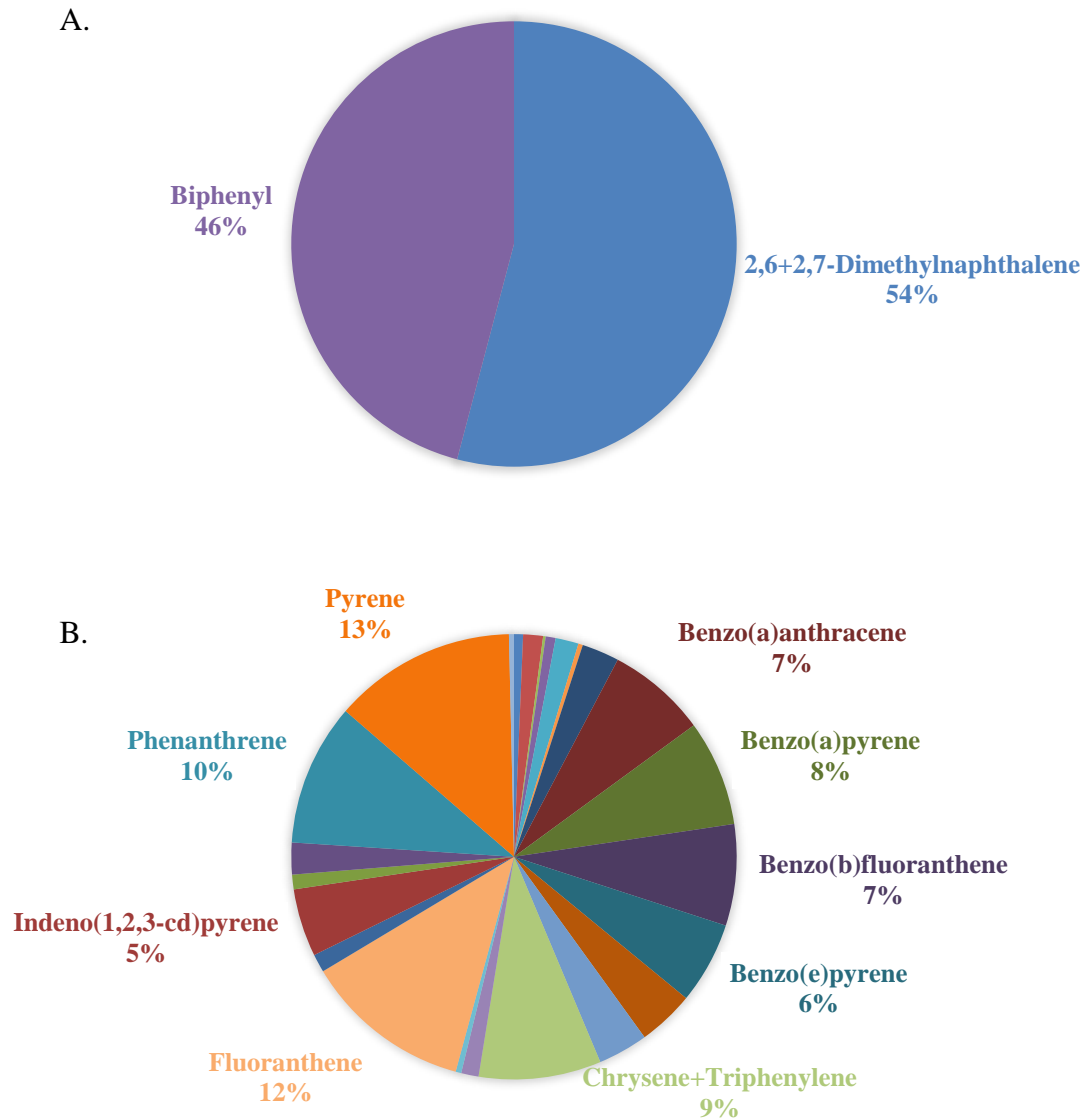


Figure 2.30. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Country Club Creek September 2014 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$ of the total PAHs.

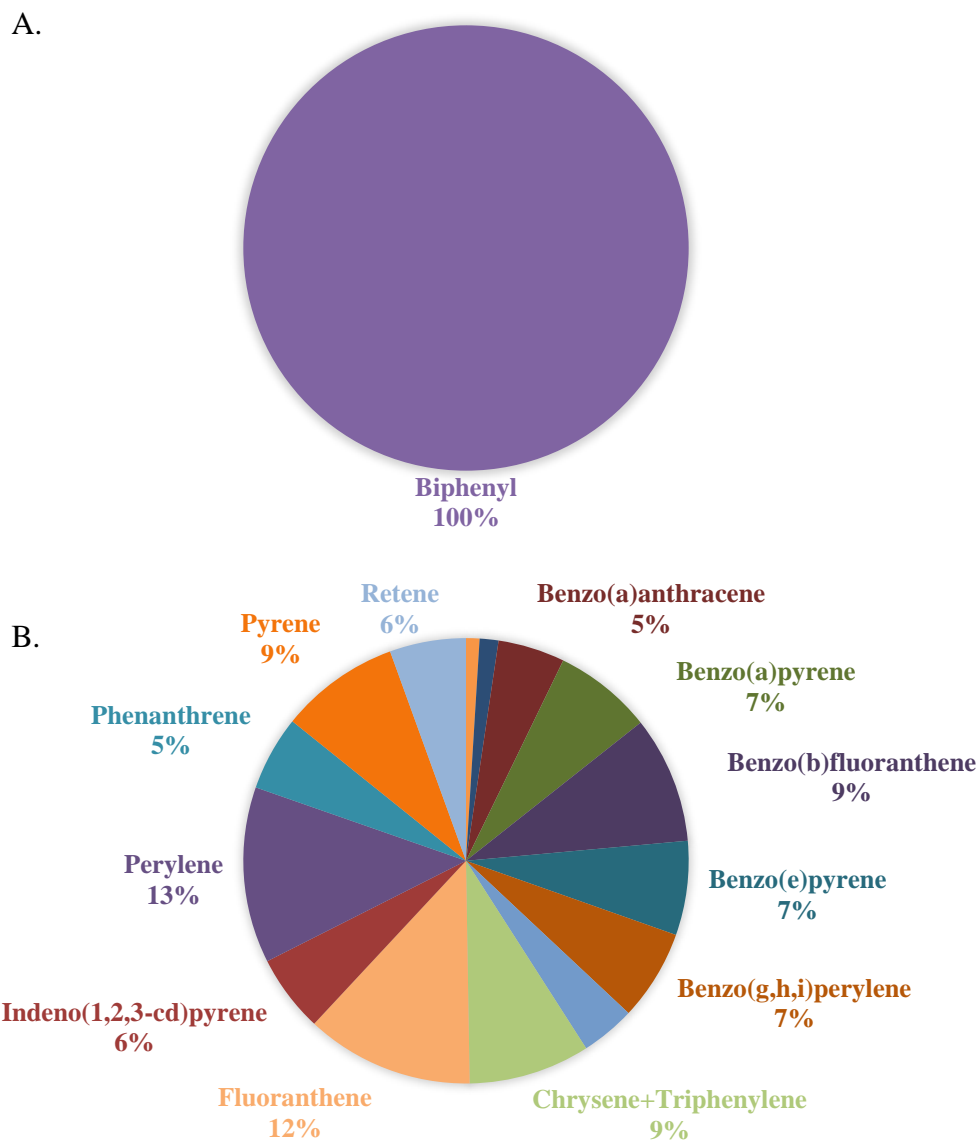


Figure 2.31. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Moon River September 2014 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$ of the total PAHs.

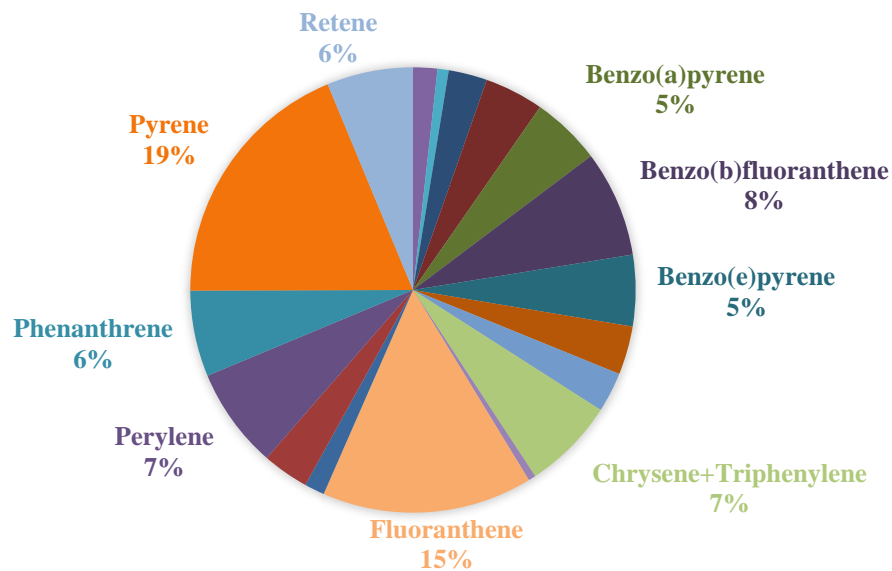


Figure 2.32. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Tom Thumb May 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for the sediment, while the concentration of PAHs in the tissue was below the minimum detection level. PAHs were labeled if $\geq 5\%$.

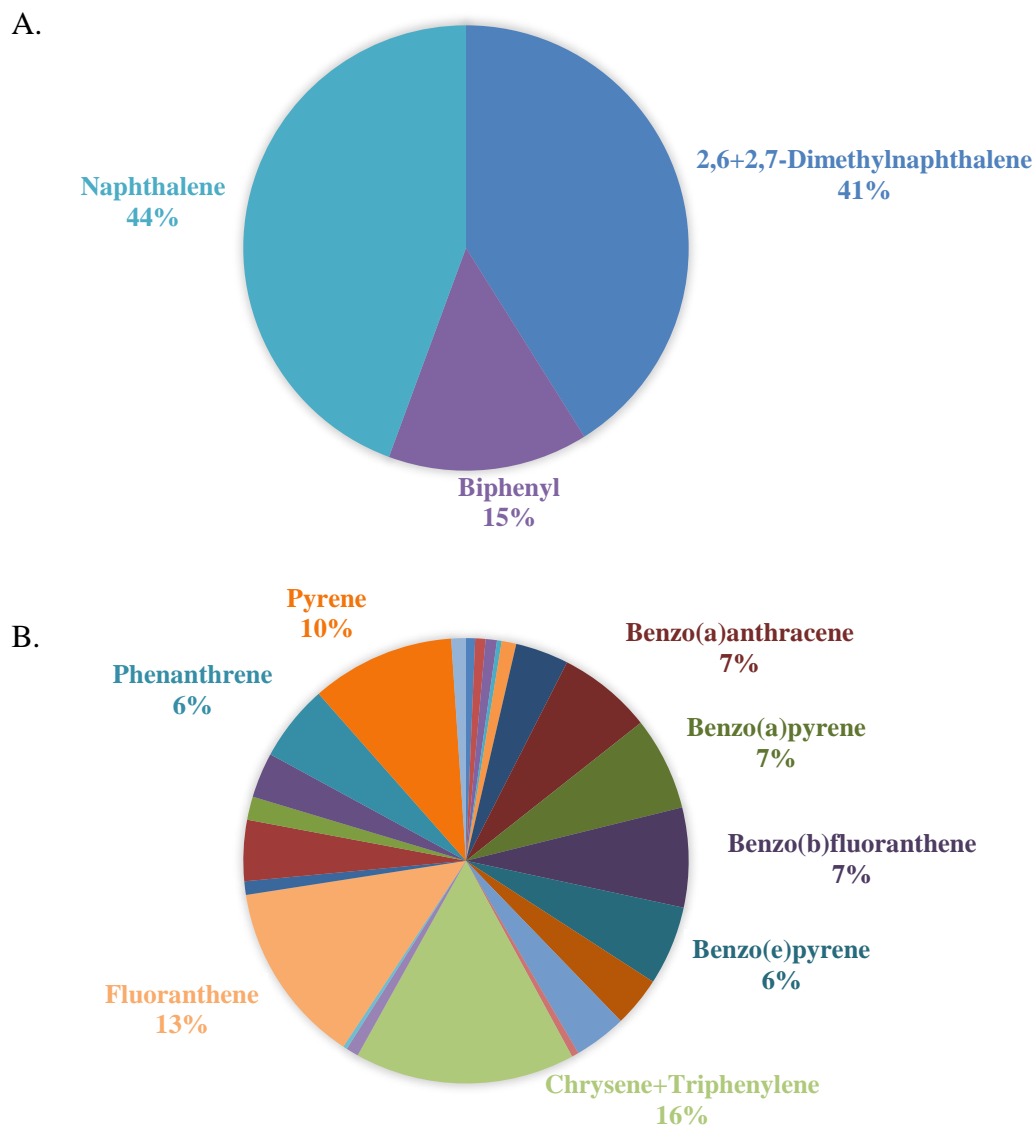


Figure 2.33. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Country Club Creek May 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$.

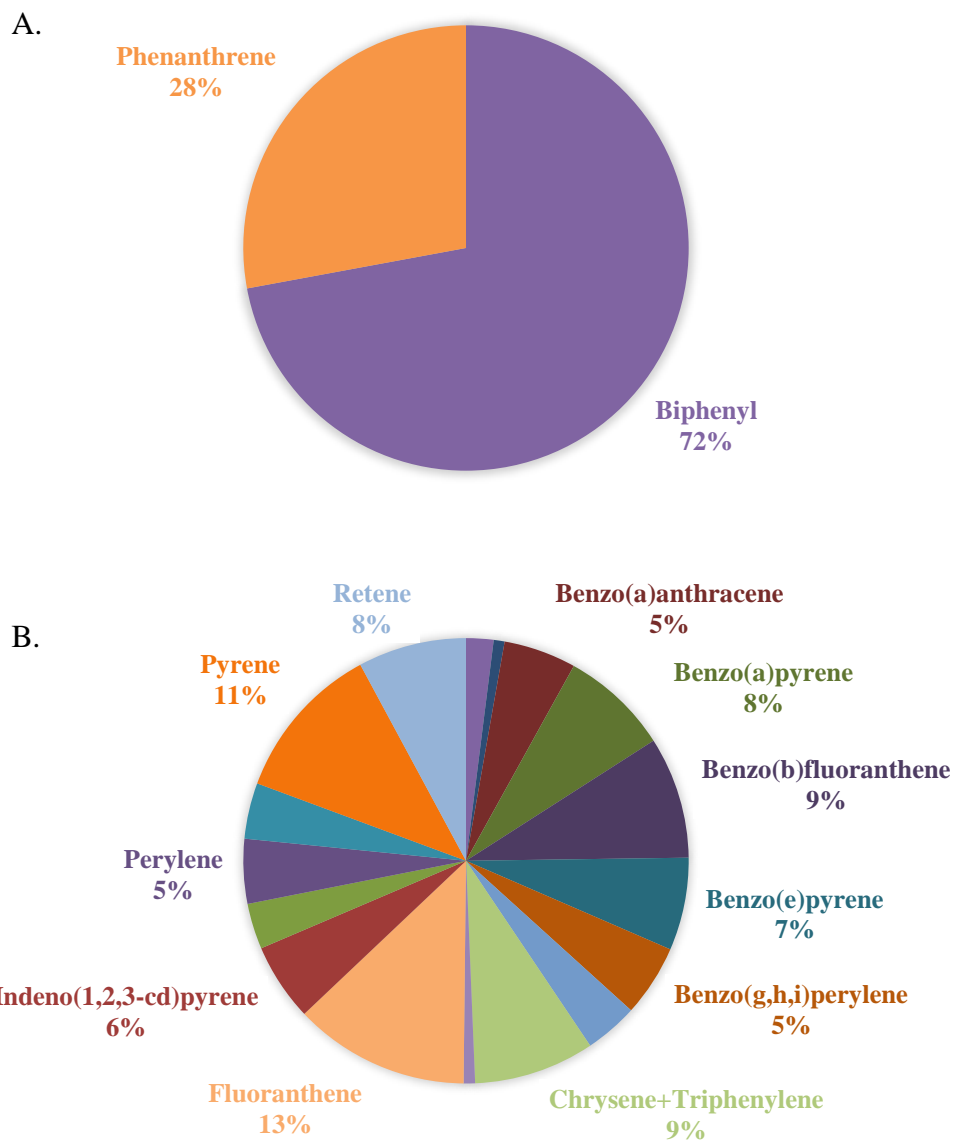


Figure 2.34. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Moon River May 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$.

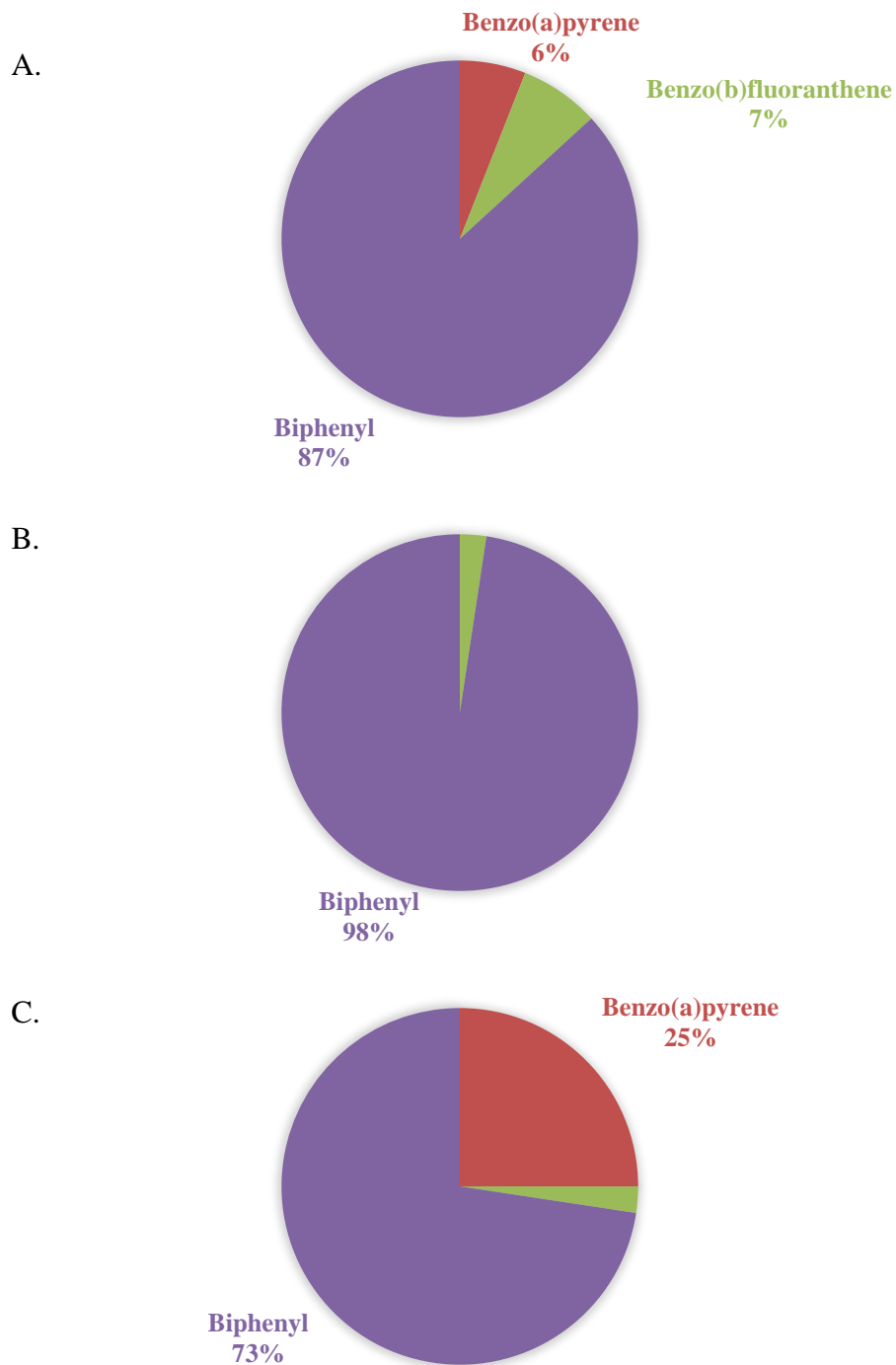


Figure 2.35. Tissue samples from daggerblade grass shrimp *Palaemonetes pugio* collected from (A.) Tom Thumb, (B.) Country Club Creek, and (C.) Moon River in July 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for the shrimp tissue. PAHs were labeled if $\geq 5\%$.

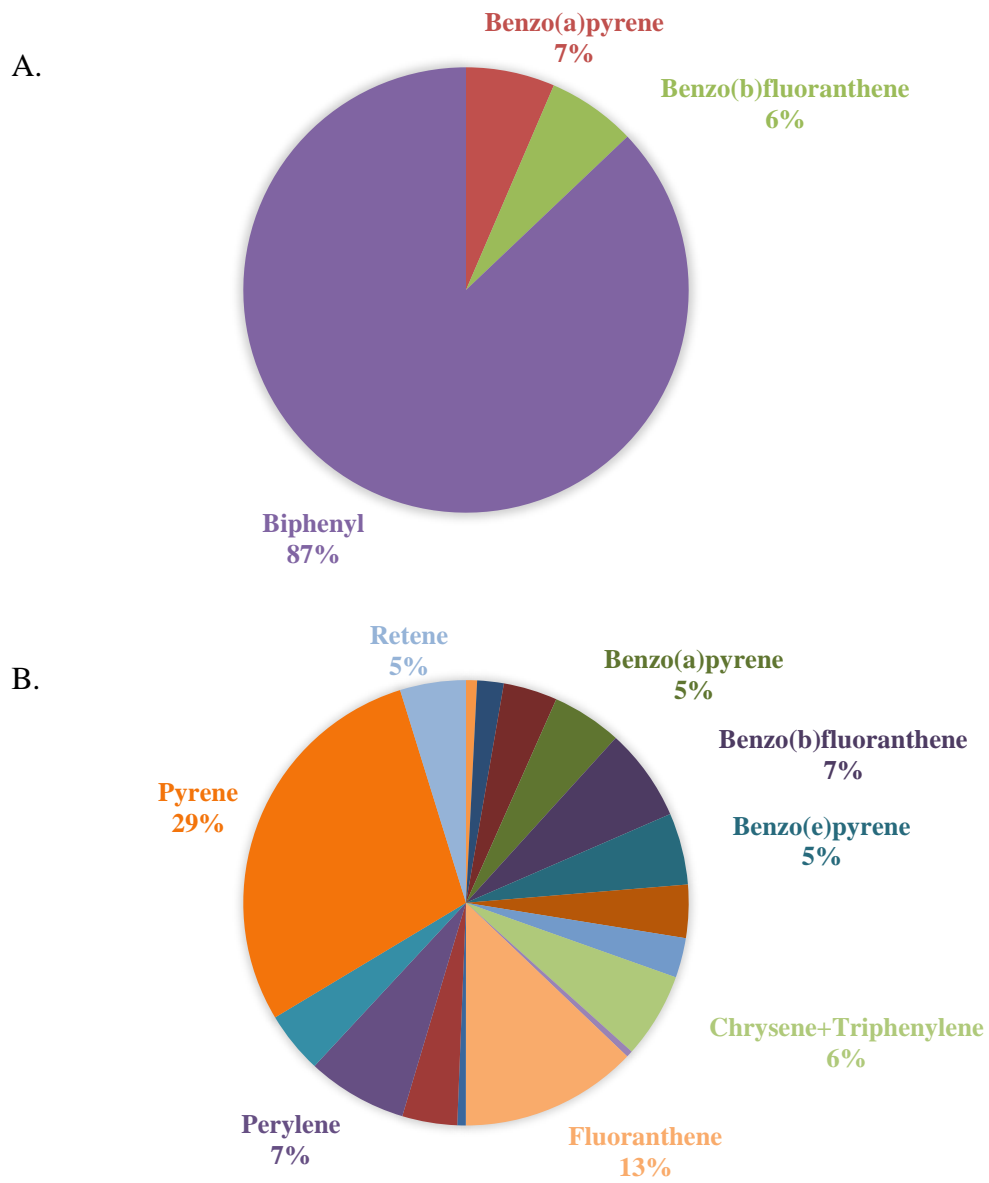


Figure 2.36. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Tom Thumb August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$.

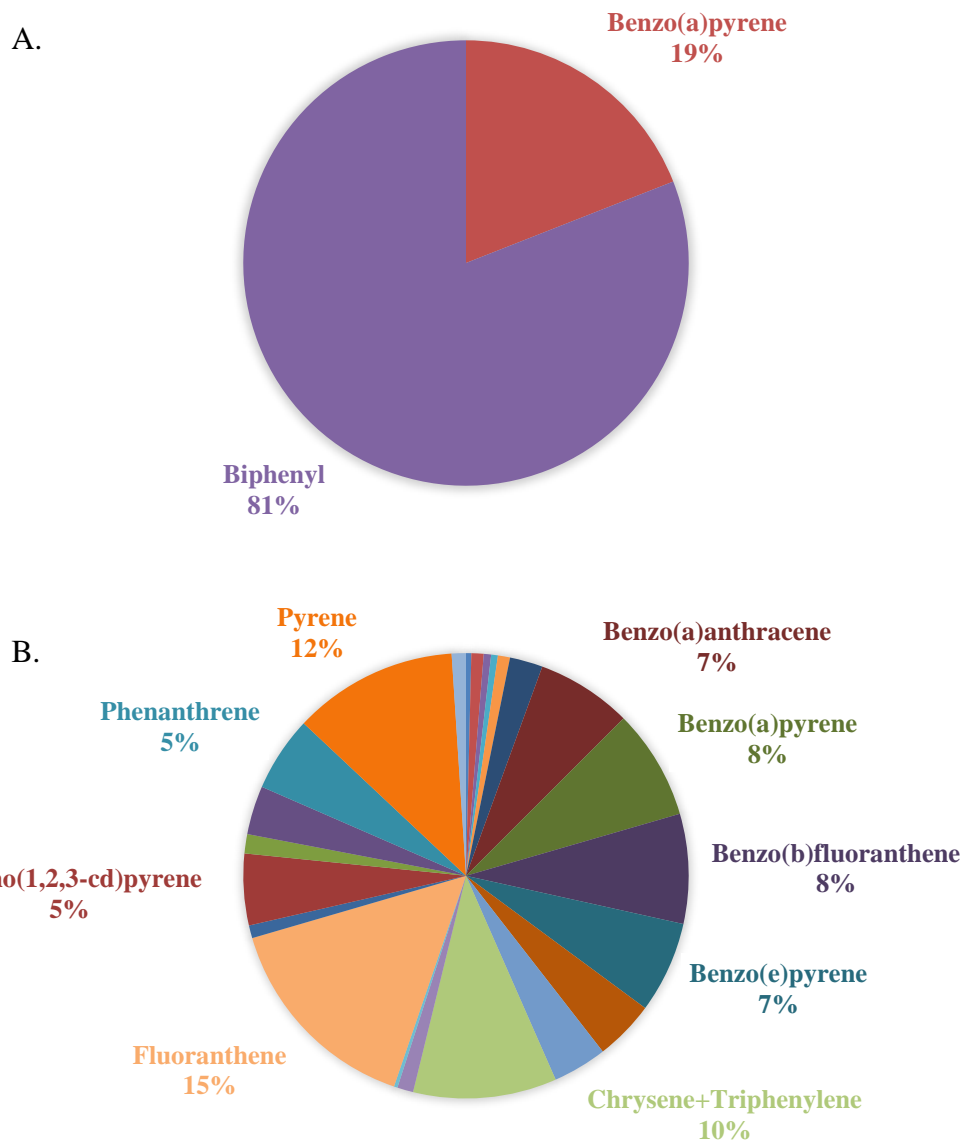


Figure 2.37. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Country Club Creek August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$.

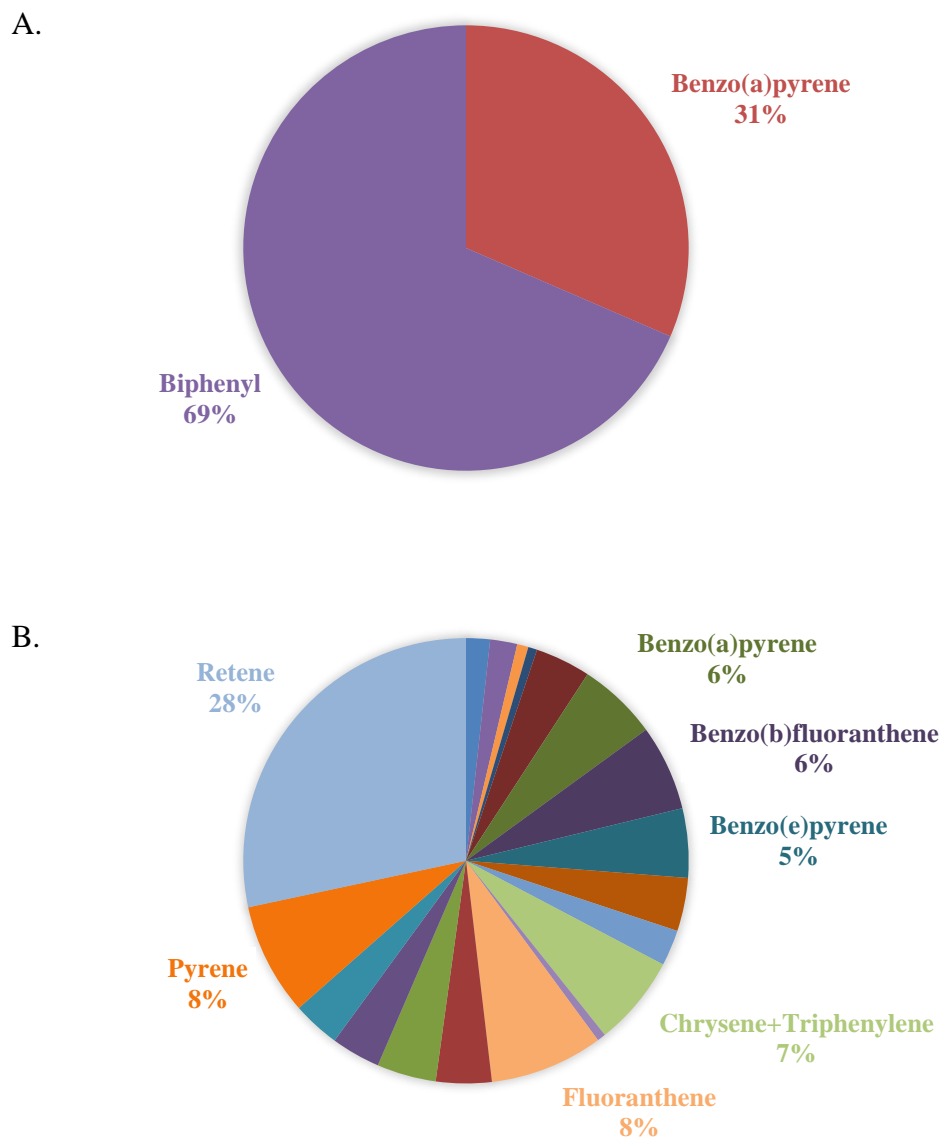


Figure 2.38. Tissue from daggerblade grass shrimp *Palaemonetes pugio* and sediment samples collected from Moon River August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The composition of the total concentration of PAHs was calculated for (A.) the shrimp tissue and (B.) sediment. PAHs were labeled if $\geq 5\%$.

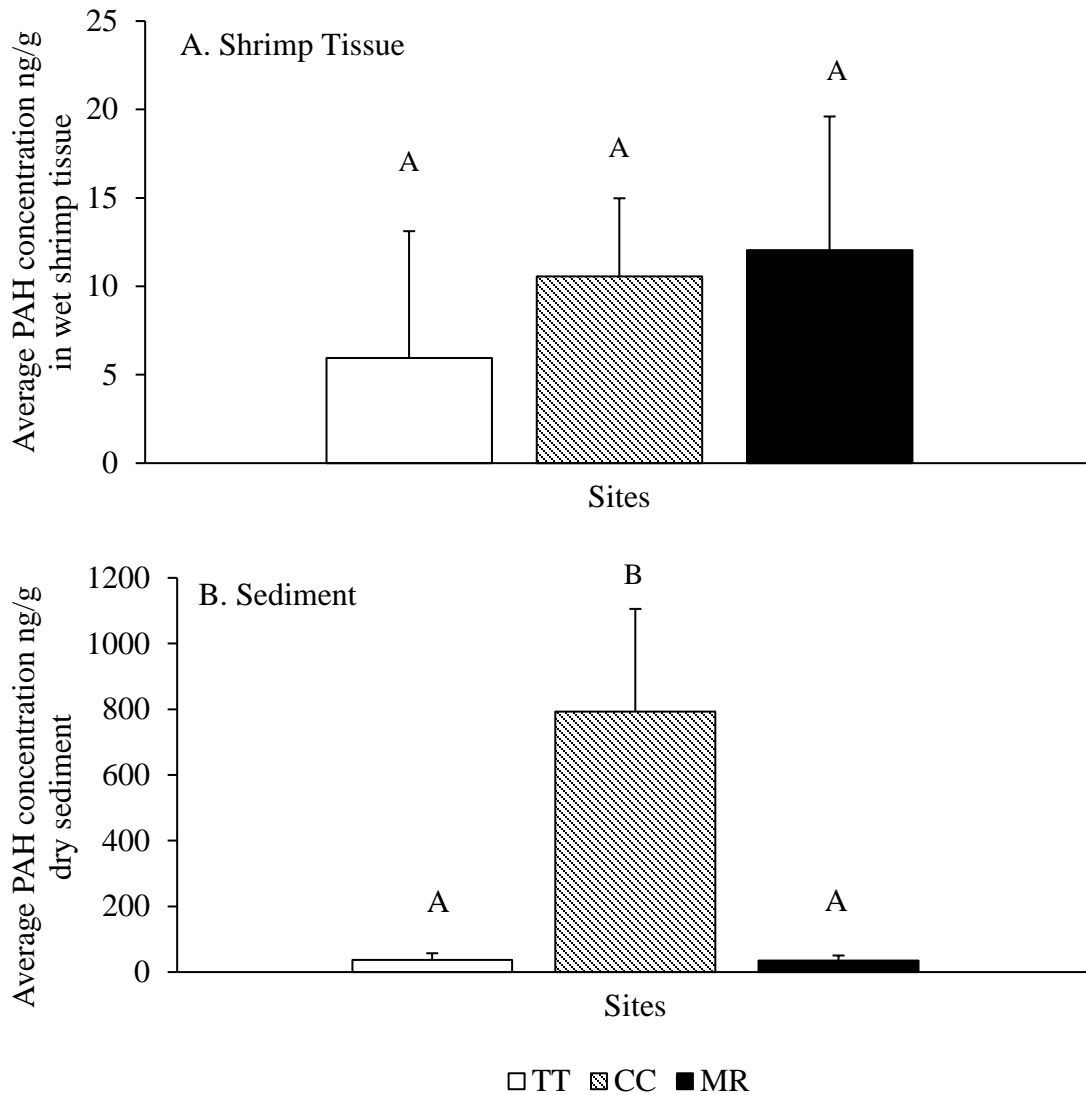


Figure 2.39. Tissue from daggerblade grass shrimp *Palaemonetes pugio* (September 2014, May 2015, July 2015, and August 2015) and sediment samples (September 2014, May 2015, and August 2015) collected from Tom Thumb, Country Club Creek, and Moon River were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The average concentration of PAHs was calculated for (A.) the wet weight shrimp tissue and (B.) the dry fraction of sediment. Significant differences are indicated by different letters. ($\alpha=0.05$).

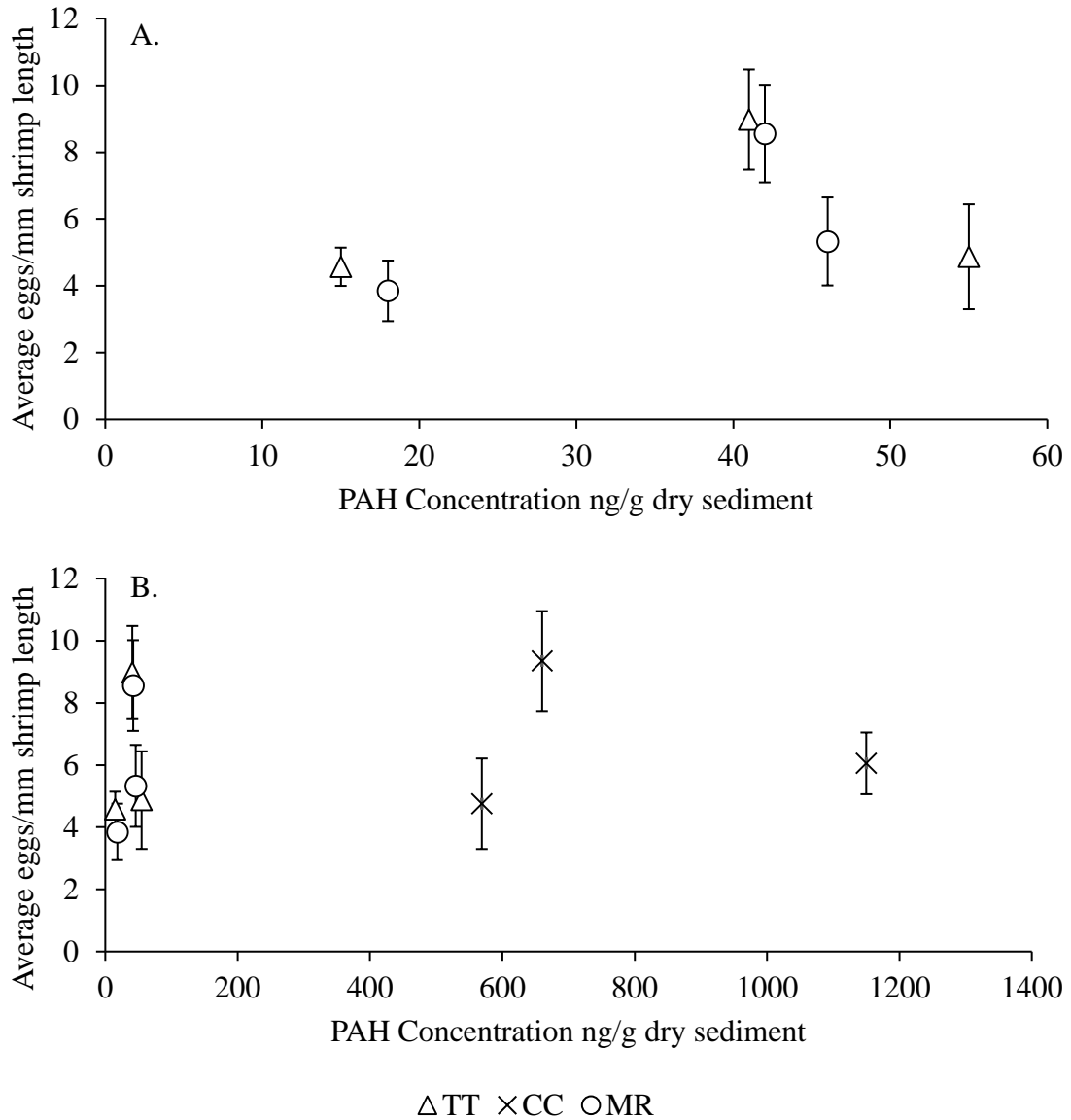


Figure 2.40. Sediment samples collected from Tom Thumb Creek (Δ), Country Club Creek (\times), Moon River (\circ) September 2014, May 2015, and August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The effect of PAH concentration on the average number of eggs per mm shrimp length \pm 1 SD was calculated with (A.) Country Club Creek removed for clarity because Country Club Creek had a greater concentration and masked the trends for the other sites and (B.) with Country Club Creek present.

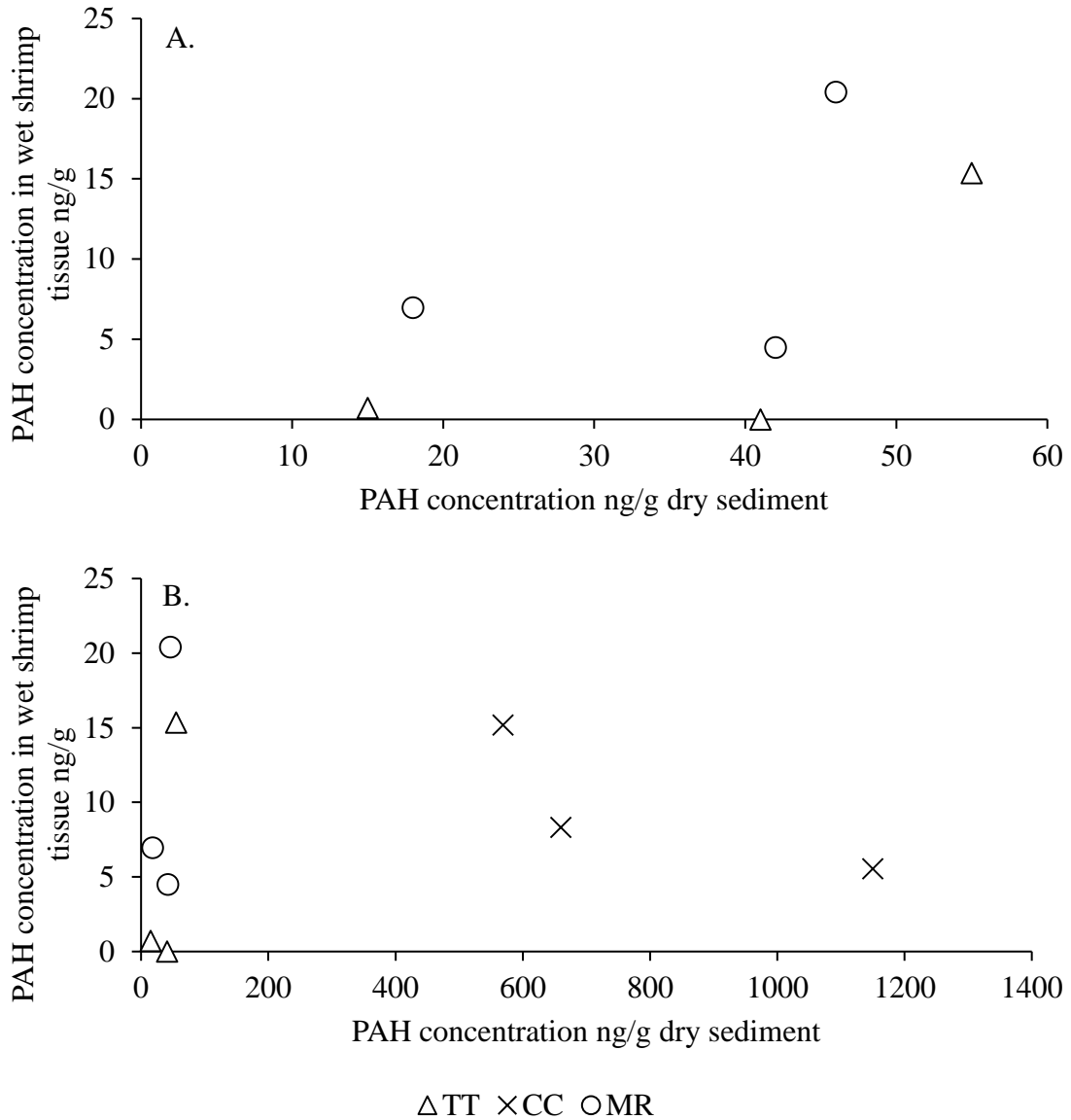


Figure 2.41. Sediment samples collected from Tom Thumb Creek (Δ), Country Club Creek (\times), Moon River (\circ) September 2014, May 2015, and August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 "Analysis of persistent organic pollutants by GC-MS." The effect of PAH concentration in the sediment on the PAH concentration in tissue was determined with (A.) Country Club Creek removed for clarity because Country Club Creek had a greater concentration and masked the trends for the other sites and (B.) with Country Club Creek present.

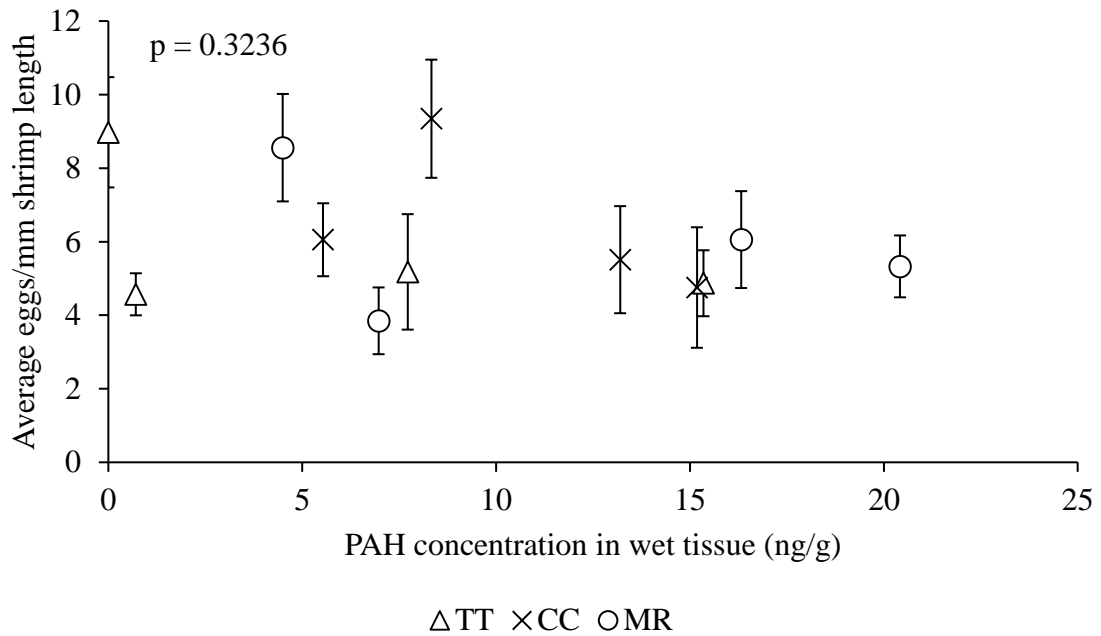


Figure 2.42. Tissue samples from daggerblade grass shrimp *Palaemonetes pugio* collected from Tom Thumb Creek (Δ), Country Club Creek (\times), Moon River (\circ) September 2014, May 2015, July 2015, and August 2015 were analyzed for polycyclic aromatic hydrocarbons (PAHs) according to SOP: CCR-043 “Analysis of persistent organic pollutants by GC-MS.” The effect of PAH concentration in the tissue on the average number of eggs per mm shrimp length \pm 1 SD was calculated. The p-value represents all data points combined.

CHAPTER 3

Effect of benzo[α]pyrene exposure on clutch size and embryonic development of the daggerblade grass shrimp *Palaemonetes pugio*

ABSTRACT

Benzo[α]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that is a fraction of crude oil. This and other PAHs enter estuaries by a variety of pathways and may have negative effects on the organisms, including the daggerblade grass shrimp *Palaemonetes pugio*. The purpose of this experiment was to determine the effect of benzo[α]pyrene on reproduction in the daggerblade grass shrimp *Palaemonetes pugio*. Male and female pairs were exposed to 0, 3, and 6 $\mu\text{g/L}$ of benzo[α]pyrene until the female became ovigerous, then the ovigerous shrimp was placed in clean artificial creekwater (~ 20 ppt). The greatest number of mortalities ($n=8$) occurred in the highest concentration and the fewest mortalities ($n=1$) occurred in the lowest concentration. Ten out of 45 shrimp became ovigerous in each concentration over 3 trials. There was not a significant difference in the length of the ovigerous shrimp across treatments. The average clutch size was greatest in 0 $\mu\text{g/L}$ (140 ± 80.9 eggs/shrimp), and eggs in the more advanced embryonic stages were present in 0 $\mu\text{g/L}$ (63%). The major conclusion of this experiment was that there was not a significant difference in clutch size or embryonic development when exposed to benzo[α]pyrene, but it could be due to higher concentrations of 12-88 ng/g or $\mu\text{g/L}$ of BaP in the marsh where the shrimp were collected. In the future, a study should be performed to determine if the eggs are viable and if the larval stages are affected by the short-term exposure to benzo[α]pyrene. If the short-term exposure did extend the length of embryonic development, a shrimp would produce fewer broods during the reproductive season, thus potentially impacting the abundance and growth of the population.

INTRODUCTION

Palaemonetes spp. are a large proportion of the resident members of the coastal marsh environment and have been studied for the possible effects of oil spills on marine organisms (Roth and Baltz, 2009; Moody et al., 2013) because they are bioindicators for potential toxins (Key et al., 2006). Oil spills are a consequence of oil exploration and production, and the results of a spill may have adverse influences on an ecosystem. Crude oil is partially composed of polycyclic aromatic hydrocarbons (PAHs) that can be dispersed within an environment (Unger et al., 2008). PAHs can be introduced into aquatic environments by oil spills, refinery effluents (Canadian Council, 1999), river runoff, sewage discharge, fossil fuel combustion, or from an atmospheric input (Liu et al., 2000). The sources that cause the highest input of PAHs into the marine environment are tanker accidents with 0.4 million metric tonnes per annum and municipal wastes with 0.7 million metric tonnes per annum (UNEP/IOC/IAEA, 1992A). Polycyclic aromatic hydrocarbons and the compounds created through the metabolism of PAHs can be carcinogenic, teratogenic, or mutagenic (Rand, 1995). Due to the hydrophobic nature of PAHs, they attach to particulate matter and the sediment (Eadie et al., 1982). The concentrations of three hydrocarbons, chrysene, fluoranthene, and phenanthrene, were tested after an experimental oil spill in the Wilmington River, GA and were found to have the following half-lives: 100 d in sediment, 70 d in mussels, and 30 d in oysters (Lee et al., 1981). The instantaneous concentration of PAHs is dependent on half-lives, but there is also a relationship between the concentration of PAHs in an organism and that of the surroundings. The ratio of PAH concentrations in two benthic infaunal organisms, oligochaetes and chironomids, in Lake Michigan was in equilibrium with the ratio of

PAH concentrations in surrounding sediment (Eadie et al., 1982). In general, the concentration of PAHs in the sediment is not uniform throughout the depths. Liu et al. (2000) determined that the total PAH concentrations were highest in the upper 33 cm of the sediment in the Yangtze estuary with a peak in concentration at 19 cm depth (11.74 $\mu\text{g/g}$). The amount of PAHs in the sediment can have an effect on the survival of marine organisms. The 96-h LC_{50} for *Palaemonetes pugio* exposed to a PAH mixture was 9542 ng PAH/ g dry sediment (Wirth et al., 1998).

One polycyclic aromatic hydrocarbon that is found in crude oil is benzo[α]pyrene (BaP), the first PAH identified as a chemical carcinogen (Hieger, 1937). In 1968, benzo[α]pyrene, also known as 3,4 benzopyrene, was found to be in crude oil ranging in amount from 450-1800 mg per ton of oil (Blumer, 1971). This PAH can also be found in cigarette smoke (Arun et al., 2011) and foods such as table margarine and peanut oil (Alomirah et al., 2010). Benzo[α]pyrene is found in higher quantities in the sediment than in the water column and the affinity of BaP to the sediment is dependent on grain size (Xia and Wang, 2008). The amount of BaP in the Yellow River, China was the highest in clay and fine silt with 97% and 96% of the PAH in the sediment, respectively, and lowest in coarse silt with 84% (Xia and Wang, 2008). BaP is a high-boiling fraction that is also a heavyweight molecule so it takes longer to be degraded after an oil spill occurs (Blumer, 1971). An oil pipeline in Texas leaked over 2,000 barrels of crude oil into the coastal area in 1994. After 83 h, BaP was the third most abundant PAH in the sediment with a concentration of 254,000 $\mu\text{g/L}$, after benzo(ghi)perylene and benzo(b)fluoranthene (Sharma et al., 2002). Rainbow trout *Oncorhynchus mykiss* eggs and alevins exposed to 0.08-0.21 $\mu\text{g/L}$ of BaP had chronic effects that included morphological abnormalities

(Canadian Council, 1999). Invertebrates, such as the water flea *Daphnia pulex*, are sensitive to BaP exposure with a 96-h LC₅₀ of 5µg/L (Canadian Council, 1999). In some organisms, behavior and activity are affected by exposure. The swimming velocity in the common prawn *Palaemon serratus* exposed to benzo[a]pyrene decreased with increased exposure to BaP with a decline of 43% at 128 µg/L (Silva et al., 2013).

Reproduction and behavior in the daggerblade grass shrimp *Palaemonetes pugio* are affected by factors including food availability and hypoxia. Female grass shrimp that were fed the smallest ration of food had the lowest reproductive score based on the presence/absence of eggs and visible oocytes (Reinsel et al., 2001). Total embryo production per tank was determined every 2 weeks in 40 L aquariums that contained 30 female grass shrimp each, and females that were fed the lowest ration of food (0.0062 g/shrimp) produced ≤1500 embryos per aquarium compared to 2000-5000 embryos per aquarium in which females were fed larger rations (Reinsel et al., 2001). Guadagnoli et al. (2005) found that the rate of pleopod fanning by ovigerous shrimp increased in hypoxic conditions from 48.5±3.5 beats per min at 150 mm Hg O₂ to 135±5.8 beats per min at 15 mm Hg O₂. Female grass shrimp are the least tolerant of hypoxic conditions when they are ovigerous with cardiac output beginning at 55.83±2.5 µL per min at normoxia (150 mm Hg O₂) and declining >15% when the oxygen was decreased below 75 mm Hg O₂ (Guadagnoli et al., 2005). The heart rate of gravid shrimp decreased from 266±7 beats per min under normoxic (153.8 mm Hg O₂) conditions to 229±16 beats per min under hypoxic (51.0 mm Hg O₂) conditions (Guadagnoli and Reiber, 2005).

Reproduction may be a pathway to decrease the concentration of pollutants in an organism because for some organisms, such as crustaceans, polycyclic aromatic

hydrocarbons are metabolized but the PAHs may remain intact in bivalves (Perugini et al., 2007). Maternal transfer of pollutants may occur in nature. One example is found in the Norway lobster *Nephrops norvegicus*. PAH concentration in the female parent was higher in the summer, during gametogenesis, compared to the winter, after eggs were laid, because pollutants may be eliminated from the parent through egg production (Perugini et al., 2007). PAHs may also be found in the organs of a crustacean. Silva et al. (2013) found an increase in BaP-type compounds in the digestive gland, eye, and muscle of the common prawn after exposure to benzo[α]pyrene. Heavyweight molecular PAHs, such as BaP, were found in higher concentrations in mysids and euphausiids that had come in contact with sediment than in a crab that did not often come in contact with sediment (Baumard et al., 1998). Wirth et al. (1998) exposed benthic copepods to a mixture of PAHs for 14 d and determined that this toxicant can negatively affect the gravidity and clutch size of the female depending on the concentration of the mixture. The relationship between PAH concentration and reproductive output should be studied for the daggerblade grass shrimp. The objectives of this chapter were to determine the sublethal effects of exposure to one PAH, benzo[α]pyrene, particularly on clutch size and embryonic development. I hypothesized that the daggerblade grass shrimp *Palaemonetes pugio* exposed to the higher concentrations of benzo[α]pyrene would have smaller clutch sizes and the eggs would have slower rates of embryonic development.

MATERIALS AND METHODS

Adult male and female daggerblade grass shrimp (>20 mm) *Palaemonetes pugio* were collected from Country Club Creek (32.031860°N, 81.014607°W) during low tide using dip nets. The shrimp were identified to the species level by using the shape of the anterior carapace and rostrum when examined under a dissecting microscope. The male grass shrimp were identified by the presence of the *appendix masculina* on the endopod of the second set of pleopods (Anderson, 1985). The cysts of the trematode *Microphallus turgidus* were counted using a dissecting microscope. Grass shrimp used for the experiment had <40 trematode cysts per shrimp and shrimp with the bopyrid isopod were excluded. The lengths of all females used in each trial were within 8 mm of each other. The male and female grass shrimp were placed in separate aerated tanks and kept in an environmental chamber at 25°C with a 16-h light/8-h dark photoperiod for a 24-h holding period. The shrimp were fed a pinch of TetraMin® until feeding had ceased for 2 min. There were a total of 4 trials, including a preliminary trial. The purpose of the first experimental trial was to determine if DMSO or the presence/absence of a substrate (sand) had a significant effect on behavior and reproduction of the shrimp. The preliminary trial consisted of 3 groups: control (0 µg/L DMSO and no sand), sand (0 µg/L DMSO with 500 g of play sand), and dosed (6 µg/L DMSO with no sand). Each tank was filled with 3 L of artificial creek water. There were 2 replicate aquaria for each treatment. Five male and 5 female shrimp were placed in each tank. The tanks were set up for 3 wks and the number of ovigerous shrimp, clutch size of each shrimp, and embryonic stage was determined. The remaining trials did not include a substrate (sand) because there was no difference found among the treatments. For the remaining trials,

benzo[α]pyrene (B α P) was obtained from Fisher Scientific and dissolved in DMSO for a 1:1 ratio. This solution has a half-life of 190 H according to Dąbrowska et al. (2008), so the water was changed weekly. After 4-6 wks, an adult female grass shrimp should become ovigerous, but due to the limited B α P and week-long half-life of the chemical, each trial could only last 3 wks. The shrimp were divided into 3 groups: control (0 μ g/L B α P), low concentration (3 μ g/L B α P), and high concentration (6 μ g/L B α P). There were 2 replicates of each treatment. The 2 concentrations of B α P were made up using serial dilutions in acid washed glassware instead of plastic to prevent adsorption to plastics. The serially diluted toxicant was added to tanks filled with 3 L of artificial creek water made from Instant Ocean[®]. The water and toxicant were allowed to equilibrate overnight before adding shrimp (Figure 3.1). Five male and female pairs were haphazardly placed in each tank. If shrimp molted, the exuviae were removed with stainless steel forceps. Dead shrimp were also removed and counted every day. The tanks were kept in the environmental chamber at 25°C with a 16-h light/8-h dark cycle.

Behavior of the shrimp was observed for the first trial. The behavior was scored as one of the following categories: Resting, foraging, walking, swimming, fanning, or darting. The behavior was recorded for every shrimp in every tank every 3 h for the first 24 h and every 6 h for the second 24 h. Observations began 5 minutes after entering the environmental chamber to allow time for the shrimp to return to their normal behavior after possible startling. The most prominent behavior was recorded at first glance after the 5-min wait period.

The females in each tank were allowed 3 wks to become ovigerous. Once a female was carrying eggs on her ventral surface, she was removed from the tank and

placed in an individual container with 300 mL clean seawater for 7 d. After 7 d, the eggs were removed using stainless steel forceps and counted to determine the clutch size. The eggs were examined under a dissecting microscope and the developmental stage was scored according to Romney and Reiber (2013). Embryos should fall into 1 of 2 stages at 7 d post-fertilization. Stage VIb is described as the late nauplius stage with cardiac contractions or VIIa, described as post-nauplius with eye pigmentation. The next stage VIIb (post-nauplius with eye condensation) was also observed in this study. The female grass shrimp was weighed to the nearest mg using a digital scale and length was taken to the nearest mm. The cumulative weight of the eggs for each female was taken. The clutch size for each female was recorded and an average clutch size was calculated. The average clutch size per shrimp length was also calculated and recorded.

Regression analysis was used to determine whether there was a stronger relationship between concentration level and clutch size or between the length of the female and clutch size. The stage of development at day 7 was used as an indicator of developmental progress. The difference in percent mortality and average molting was also analyzed. SAS was used for statistical analyses. After testing for normality, a nonparametric ANOVA was used because the data were not normally distributed.

RESULTS

For the preliminary trial, there was no significant difference in the clutch sizes of shrimp in the three conditions: sand, no sand, and DMSO ($p=0.9798$). The average clutch size was 149.63 ± 86.46 , 147.50 ± 88.99 , and 155.56 ± 81.63 eggs/shrimp for the tanks without sand, with sand, and with DMSO, respectively. As there were no significant differences, the remainder of the experiment proceeded with no sand and with DMSO as a solvent. DMSO was necessary because benzo[α]pyrene is hydrophobic. The benzo[α]pyrene was dissolved in DMSO in order to disperse the pollutant in the water column instead of having it float on top of the water.

In the remaining trials, there were more mortalities in the group with the highest concentration of benzo[α]pyrene (6 $\mu\text{g/L}$) with 2 or more deaths per trial (Table 3.1), but there was an inverse relationship between concentration and number of molting events. There was an average of 8.9 ± 3.85 mortalities in the highest concentration (Table 3.1). Average percent mortality increased with increased concentration from 1.1 ± 1.92 to 2.2 ± 3.85 and 8.9 ± 3.85 at 0, 3, and 6 $\mu\text{g/L}$ of benzo[α]pyrene, respectively. The control group had the most molting in every trial with 13, 12, and 9 exuviae in trials 1, 2, and 3, respectively (Table 3.1). There was not a significant difference in percent mortality or molting across the concentrations ($p= 0.057$ and $p= 0.571$, respectively). The average percent ovigerous was not significantly different ($\alpha=0.05$) across the concentrations (Table 3.1). The control group in the second trial was the only one for which a female did not become ovigerous (Table 3.1).

Ovigerity and clutch size in the shrimp changed over time and trials for the concentrations. Throughout the trials, the total number of ovigerous shrimp was 10 out of

45 female shrimp in each concentration. In trials 1 and 2, shrimp became ovigerous by day 2 of the experiment, followed by a period of no reproduction that lasted 7-9 d before reproduction occurred again (Figure 3.2). In trial 3, there was no reproduction until a week after the experiment started (Figure 3.2). There seemed to be synchronization of ovigerity in the shrimp, where shrimp across treatments became ovigerous within 3 d of one another (Figure 3.2). The overall average clutch size decreased with an increase in B α P concentration from 140 ± 80.9 to 123 ± 72.6 eggs/shrimp in $0 \mu\text{g/L}$ to $6 \mu\text{g/L}$, respectively, although the trend was not significant with a p-value of 0.8972 (Table 3.2). The average length, weight, and average clutch size decreased in all concentrations from trial 1 to trial 3 (Table 3.2). There were no significant length or weight changes in the tanks among trials or concentrations with an average difference of -0.76 to -0.34 mm and -0.02 to -0.01 g across the concentrations. (Table 3.3).

Overall, 3 of 11 embryonic stages described by Romney and Reiber (2013) were identified in this study (Figure 3.3) with variability among treatments. Stage VIb was the earliest stage found and there was no presence of eye pigmentation (Figure 3.4). The group with the lowest concentration of $0 \mu\text{g/L}$ had the greatest percentage of eggs in the later stage of VIIb (eye condensation) with $48.3 \pm 50.08\%$ (Table 3.4). The middle treatment ($3 \mu\text{g/L}$) had the greatest average percentage of eggs in the middle stage ($86.0 \pm 15.72\%$) with a p-value of 0.020 (Table 3.4). The group in the $6 \mu\text{g/L}$ concentration had the highest percentage of eggs in the early embryonic developmental stage (no visible eye-spot) after 7 d with $14.3 \pm 24.83\%$ in stage VIb (Table 3.4). The average number of eggs in the VIb stage was 159.0 ± 0.00 eggs/shrimp (n=1 shrimp) in the highest concentration of $6 \mu\text{g/L}$ (Figure 3.5). The average number of eggs in the middle stage

(VIIa) was greatest in the lowest concentration of 0 $\mu\text{g/L}$ ($n=2$ shrimp) with 256.0 ± 57.98 (Figure 3.5). Shrimp in the lowest concentration of 0 $\mu\text{g/L}$ ($n=8$ shrimp) had the greatest average number of eggs in the latest stage of VIIb with 110.4 ± 55.49 (Figure 3.5). The percent composition of embryonic stages differed among the concentrations. There was a shift from mostly late stage embryos (VIIa) in 0 $\mu\text{g/L}$, over 80% VIb stage embryos in 3 $\mu\text{g/L}$, and a mixture of all 3 stages in 6 $\mu\text{g/L}$ (Figure 3.6).

There was a positive relationship between length and clutch size exhibited in all 3 concentrations, but the correlation was the weakest in the group exposed to 3 $\mu\text{g/L}$ of B α P (Figure 3.7B). There was a significant relationship in both the control and 6 $\mu\text{g/L}$ with $p=0.0453$ and $p=0.0070$, respectively (Figure 3.7). The relationship between length and clutch size was not significant in the treatment of 3 $\mu\text{g/L}$ with $p=0.4271$. Highest average length and weight for ovigerous shrimp were both in 3 $\mu\text{g/L}$ (Table 3.2), but neither length nor weight was significantly different among the concentrations. There was not a significant difference in average clutch size of eggs per mm of shrimp length for each concentration (Figure 3.8).

The behavior of the grass shrimp was similar in all 3 concentrations and resting was the most common behavior followed by swimming (Figure 3.9). Darting and fanning behaviors were the least common behaviors (Figure 3.9). The most foraging behavior occurred in the group with 3 $\mu\text{g/L}$, but this difference was not significant (Figure 3.9). Shrimp in all concentrations exhibited a similar trend in frequency of the behaviors over the 48 h when behavior was recorded.

DISCUSSION

The major findings of this experiment were that embryos developed slower when exposed to benzo[α]pyrene and mortality rates appeared higher in shrimp exposed to benzo[α]pyrene, but not significantly. This could be due to the lethal and sublethal effects of the toxicant. Garcia et al. (2014) determined that there was an effect of caffeine and sulfamethoxazole on embryo development. Embryos in a mixture of the 2 pollutants took 8.9% longer to hatch than the control embryos (Garcia et al., 2014). Mortality was greater in the study by Garcia et al. (2014) with $42 \pm 7\%$ when shrimp were exposed to a mixture of caffeine and sulfamethoxazole. Williamson et al. (2009) also determined that percent mortality increased with an increase of resmethrin concentration, with 83.3% mortality after 24 h of exposure to 2.0 $\mu\text{g/L}$, compared to 0% mortality after 24 h exposure to 0.0 $\mu\text{g/L}$. The average percent mortality of the present study was 8.9% in the highest exposure of 6 $\mu\text{g/L}$ with a total of 8 shrimp that died in the greatest concentration of 6 $\mu\text{g/L}$ and only 1 shrimp overall died in the lowest concentration. While shrimp in all 3 concentrations had similar clutch sizes, a higher percentage of eggs were in the later stage in the lowest concentration of 0 $\mu\text{g/L}$. This provides support that the exposure caused the embryos to develop slower. So, embryos exposed to benzo[α]pyrene would take longer to hatch and a female would not be able to have a successive brood as quickly.

More molting occurred in the control tanks with 0 $\mu\text{g/L}$. The shrimp in the control group may have had more energy to exert on growth, while growth was slightly slower in the shrimp exposed to the toxicant. Growth was not significantly different across the concentrations though. The frequency of molting can also be an indicator of stress. Oberdörster et al. (2000) exposed shrimp to pyrene and male shrimp exhibited a similar

trend in molting to the present study. Male shrimp exposed to the highest concentration (63.4 $\mu\text{g/L}$) molted less often than the male shrimp in the control group and lower concentrations (Oberdörster et al., 2000). There was a negative correlation between molting and mortality. The mortality was significantly greater in the highest concentration of pyrene and the mortalities occurred during or immediately after molting (Oberdörster et al., 2000).

Length significantly affected the clutch size of the shrimp in two of the treatments (0 and 6 $\mu\text{g/L}$). This is similar to other studies. Anderson (2005) determined that there was a positive relationship between length and clutch size. Modeste (2009) also found that there was a positive relationship between the number of eggs per shrimp and length at Country Club Creek with an $R^2=0.42$, where the current shrimp were collected. Because of the low R^2 of 0.08 and high p-value of 0.427 in the present study, there is apparently another factor affecting the clutch size in the middle concentration of 3 $\mu\text{g/L}$. This could have been due to the short-term exposure to BaP.

The overall ovigerity was the same for all 3 concentrations with 10 out of 45 female shrimp in each treatment developing eggs, although the average ovigerity differed slightly with 22% in 3 $\mu\text{g/L}$ and 24.4% in 0 and 6 $\mu\text{g/L}$. Wirth et al. (1998) exposed the benthic copepod *Amphiascus tenuiremis* to a mixture of heavy molecular weight PAHs, including benzo[α]pyrene for 14 d and determined that this mixture can negatively affect the gravidity and clutch size of the female depending on the concentration of the mixture. The gravidity decreased from 76.2% in the control to 58.7% in copepods exposed to 5x treatment (Wirth et al., 1998). The clutch size in the 1x treatment (1211 ng/g) was

significantly less with only 83.7% of the control. The concentration of the exposure was much greater in that study in comparison with the present study.

No female became ovigerous in the control group during the second trial. This could be due to the length of the trial. Each trial lasted 3 wks but a female may take up to 4-6 wks to reproduce. The trials only lasted 3 wks because of the limitation set by the supply of benzo[α]pyrene. In the future, the trials should last longer to provide adequate time for reproduction to occur.

There was a difference of average length and average clutch size in all treatments among the trials. This could be due to the life cycle of the grass shrimp. The first trial began at the beginning of the summer, when shrimp are larger. (See chapter 2). The last trial began in fall, when the shrimp hatched in spring are becoming mature and reproducing. This can cause a difference in clutch size and length.

Shrimp at Country Club Creek are exposed to different polycyclic aromatic hydrocarbons in the environment compared to a single PAH in this experiment. A preliminary analysis of the sediment was performed and benzo[α]pyrene was present in the sediment at the collection site. The concentration of B α P ranged from 12 to 88.4 $\mu\text{g}/\text{kg}$, which is the equivalent of 12 to 88.4 $\mu\text{g}/\text{L}$. This could mean that the concentrations of 3 and 6 $\mu\text{g}/\text{L}$ used in this experiment may not have been high enough to significantly affect the reproduction, because of the higher concentrations in the environment.

The behavior was similar for the shrimp in all concentration groups. During the first 24 h, the behavior could have been influenced by the tidal cycle. Resting was the most common behavior in the present study. Chaplin-Ebanks and Curran (2006) also

found that resting was the most common behavior with $44\% \pm 4.0\text{SE}$ of shrimp exhibiting this behavior. They found that the number of shrimp at rest was significantly affected by the tidal stage because the behavior was most common during periods of flood tide. The behavior of the shrimp had tidal rhythmicity 30 h after collection (Chaplin-Ebanks and Curran, 2006). Shrimp in the current study were allowed to acclimate for 24 h prior to placement in treatment tanks and behavior observations. Silva et al. (2013) determined that behavior can be affected by exposure to benzo[α]pyrene and observed that the swimming velocity in the common prawn *Palaemon serratus* decreased with increased exposure to BaP with a decline of 43% at 128 $\mu\text{g/L}$. In this present study, behavioral observations were made for only a portion of the trial. Thus, there may have been insufficient behavioral data collected to determine a discernable difference.

In the future, a study should be performed to determine if the eggs are viable and if larval development is affected by short-term exposure to benzo[α]pyrene. If the eggs and larvae develop at slower rates, the developmental progress of the grass shrimp could be affected. The decrease in embryo development could lead to the female producing fewer broods per season. Rainbow trout eggs *Oncorhynchus mykiss* and alevins exposed to 0.08-0.21 $\mu\text{g/L}$ of BaP had chronic effects that included morphological abnormalities and necrosis of the spine and brain (Canadian Council, 1999). Similar morphological abnormalities could also occur in grass shrimp. If the short-term effects cause abnormalities in the grass shrimp, the fitness of the organism could decrease and it could die before adulthood and reproduction. Considering all the data collected, the study could be improved by adding more replicates at the same time of the year to avoid comparing

different cohorts and by increasing the concentrations of BαP to which the shrimp are exposed to in the treatment groups.

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Table 3.1 Adult daggerblade grass shrimp *Palaemonetes pugio* were collected at Country Club Creek and exposed to a control (0 µg/L BaP), low concentration (3 µg/L BaP), and high concentration (6 µg/L BaP) of benzo[α]pyrene (n=45 males and females in concentration per trial). During the 3 wk exposure period, the number of dead shrimp, molts, and ovigerous shrimp in each concentration were recorded. There were no significant differences in mortality, molting, or percent ovigerous.

Trial	Concentration	% Mortality	Molting	% Ovigerous
1	0 µg/L	3.3	13	47
2	0 µg/L	0	12	0
3	0 µg/L	0	9	27
1	3 µg/L	0	13	33
2	3 µg/L	0	8	27
3	3 µg/L	6.7	4	7
1	6 µg/L	6.7	13	20
2	6 µg/L	6.7	8	20
3	6 µg/L	13.3	8	33
Average	0 µg/L	1.1 ± 1.92	11.3 ± 2.08	24.4 ± 23.41
Average	3 µg/L	2.2 ± 3.85	8.3 ± 4.51	22.2 ± 13.88
Average	6 µg/L	8.9 ± 3.85	9.7 ± 2.89	24.4 ± 7.70

Table 3.2 Ovigerous daggerblade grass shrimp *Palaemonetes pugio* that were exposed to a control (0 µg/L BaP), low concentration (3 µg/L BaP), and high concentration (6 µg/L BaP) of benzo[α]pyrene were placed in artificial creek water for 7 d. After 7 d, the female was measured after the eggs were removed (n=10 per concentration). The average clutch size, length, and weight \pm 1 SD were calculated. There was no significant difference in average clutch size, length, or weight.

Trial	Concentration	Avg. Clutch Size (Eggs/shrimp)	Avg. Length (mm)	Avg. Weight (g)
1	0 µg/L	155 \pm 99.5	34 \pm 2.8	0.37 \pm 0.090
2	0 µg/L	0	0	0
3	0 µg/L	116 \pm 43.3	32 \pm 1.7	0.29 \pm 0.049
1	3 µg/L	153 \pm 87.9	36 \pm 1.4	0.41 \pm 0.020
2	3 µg/L	107 \pm 28.2	34 \pm 1.7	0.34 \pm 0.074
3	3 µg/L	127 \pm 0.0	30 \pm 0.0	0.24 \pm 0.000
1	6 µg/L	170 \pm 60.1	34 \pm 2.5	0.35 \pm 0.035
2	6 µg/L	115 \pm 52.1	32 \pm 1.5	0.31 \pm 0.025
3	6 µg/L	93 \pm 89.7	33 \pm 3.4	0.32 \pm 0.106
Total	0 µg/L	140 \pm 80.9	33 \pm 2.7	0.33 \pm 0.083
Total	3 µg/L	132 \pm 65.2	35 \pm 2.3	0.37 \pm 0.071
Total	6 µg/L	123 \pm 72.6	33 \pm 2.6	0.31 \pm 0.076

Table 3.3 Ovigerous daggerblade grass shrimp *Palaemonetes pugio* that were exposed to a control (0 µg/L BaP), low concentration (3 µg/L BaP), and high concentration (6 µg/L BaP) of benzo[α]pyrene were placed in artificial creek water for 3 wk. After the 3 wk exposure period, the average length change and average weight change \pm 1 SD were calculated. There were no significant differences in average change in length or weight.

Trial	Concentration	Avg. Δ Length (mm)	Avg. Δ Weight (g)
1	0 µg/L	-1.21 \pm 0.955	-0.06 \pm 0.061
2	0 µg/L	0.23 \pm 0.283	0.00 \pm 0.010
3	0 µg/L	-0.03 \pm 0.822	0.01 \pm 0.031
1	3 µg/L	-0.73 \pm 1.584	-0.04 \pm 0.034
2	3 µg/L	-1.07 \pm 0.732	-0.02 \pm 0.018
3	3 µg/L	-0.48 \pm 1.050	-0.00 \pm 0.027
1	6 µg/L	-0.77 \pm 0.700	-0.01 \pm 0.029
2	6 µg/L	-0.36 \pm 0.324	-0.01 \pm 0.012
3	6 µg/L	-0.75 \pm 0.535	-0.01 \pm 0.024
Total	0 µg/L	-0.34 \pm 0.356	-0.02 \pm 0.026
Total	3 µg/L	-0.76 \pm 0.430	-0.02 \pm 0.008
Total	6 µg/L	-0.63 \pm 0.188	-0.01 \pm 0.009

Table 3.4 Ovigerous daggerblade grass shrimp *Palaemonetes pugio* that were exposed to a control (0 µg/L BaP), low concentration (3 µg/L BaP), and high concentration (6 µg/L BaP) of benzo[α]pyrene were placed in artificial creek water for 7 d. After 7 d, the eggs were removed, counted, and embryonic stage was determined. Significant differences are indicated by different letters beside the averages.

Trial	Concentration	% eggs in VIb Stage	% eggs in VIIa Stage	% eggs in VIIb Stage
1	0 µg/L	0	55	45
2	0 µg/L	0	0	0
3	0 µg/L	0	0	100
1	3 µg/L	0	89	11
2	3 µg/L	0	69	31
3	3 µg/L	0	100	0
1	6 µg/L	0	44	56
2	6 µg/L	0	54	46
3	6 µg/L	43	49	8
Average	0 µg/L	0 ± 0.00	18.3 ± 31.75 ^A	48.3 ± 50.08
Average	3 µg/L	0 ± 0.00	86.0 ± 15.72 ^B	14.0 ± 15.72
Average	6 µg/L	14.3 ± 24.83	49.0 ± 5.00 ^{AB}	36.3 ± 25.15

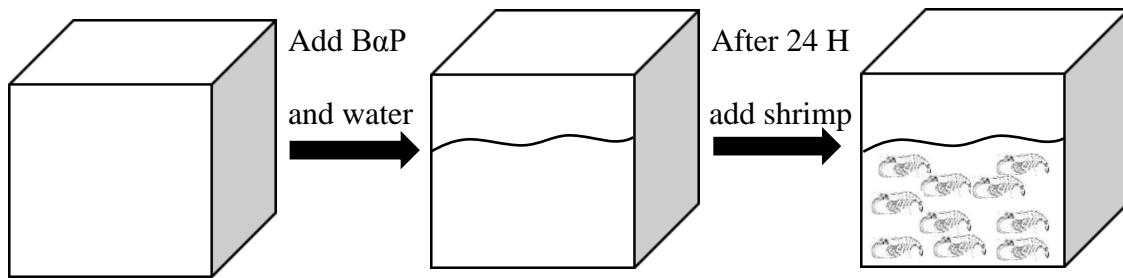


Figure 3.1. The schematic for preparing aquaria for reproduction experiments. The water and benzo[α]pyrene were added to the aerated tanks and allowed to mix overnight. After 24 h, male and female shrimp pairs (n=5) were added.

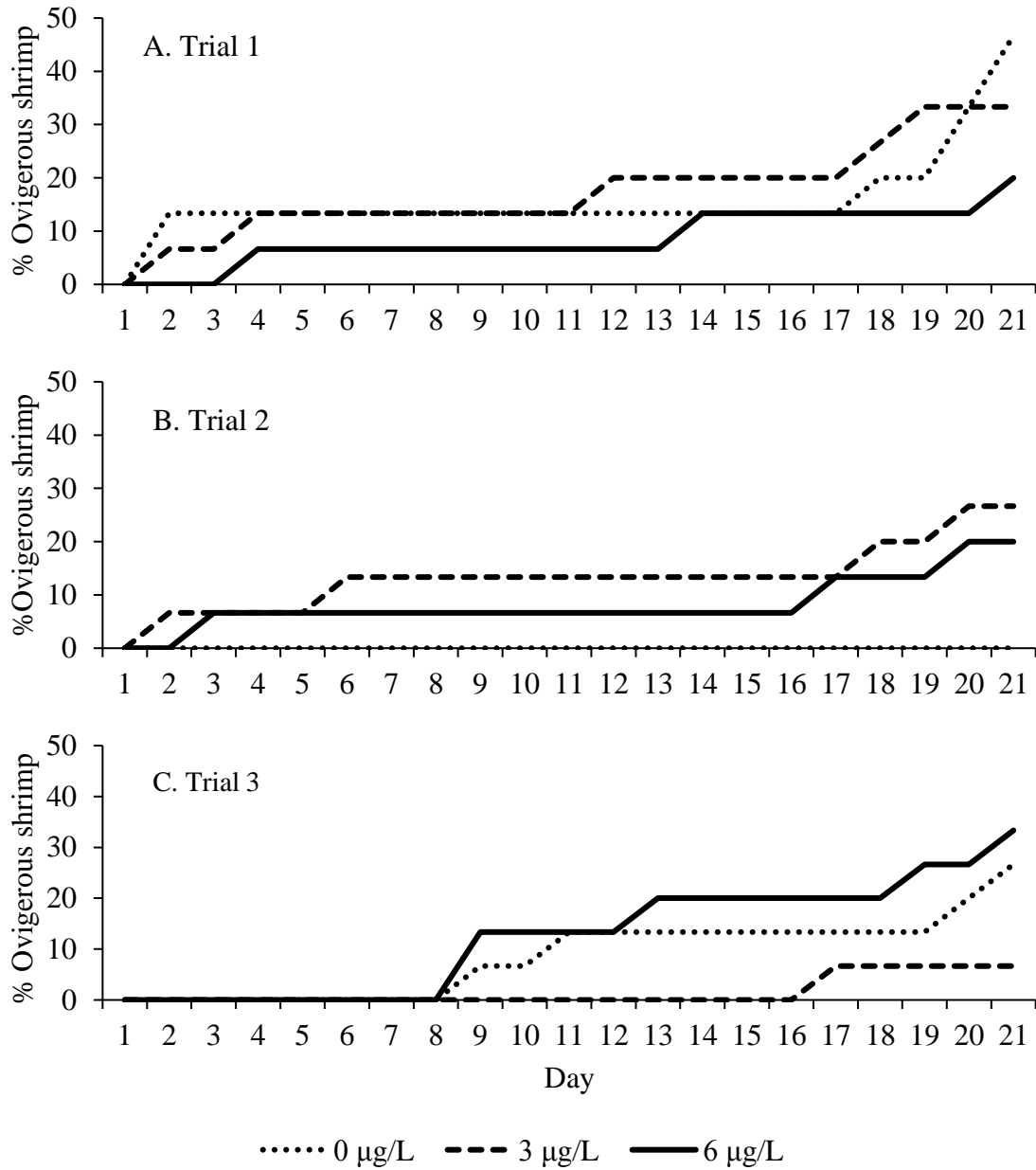


Figure 3.2. Adult daggerblade grass shrimp *Palaemonetes pugio* collected from Country Club Creek and exposed to 3 different concentrations of benzo[α]pyrene: 0 $\mu\text{g/L}$, 3 $\mu\text{g/L}$, and 6 $\mu\text{g/L}$. Shrimp that became ovigerous within 3 weeks were counted.

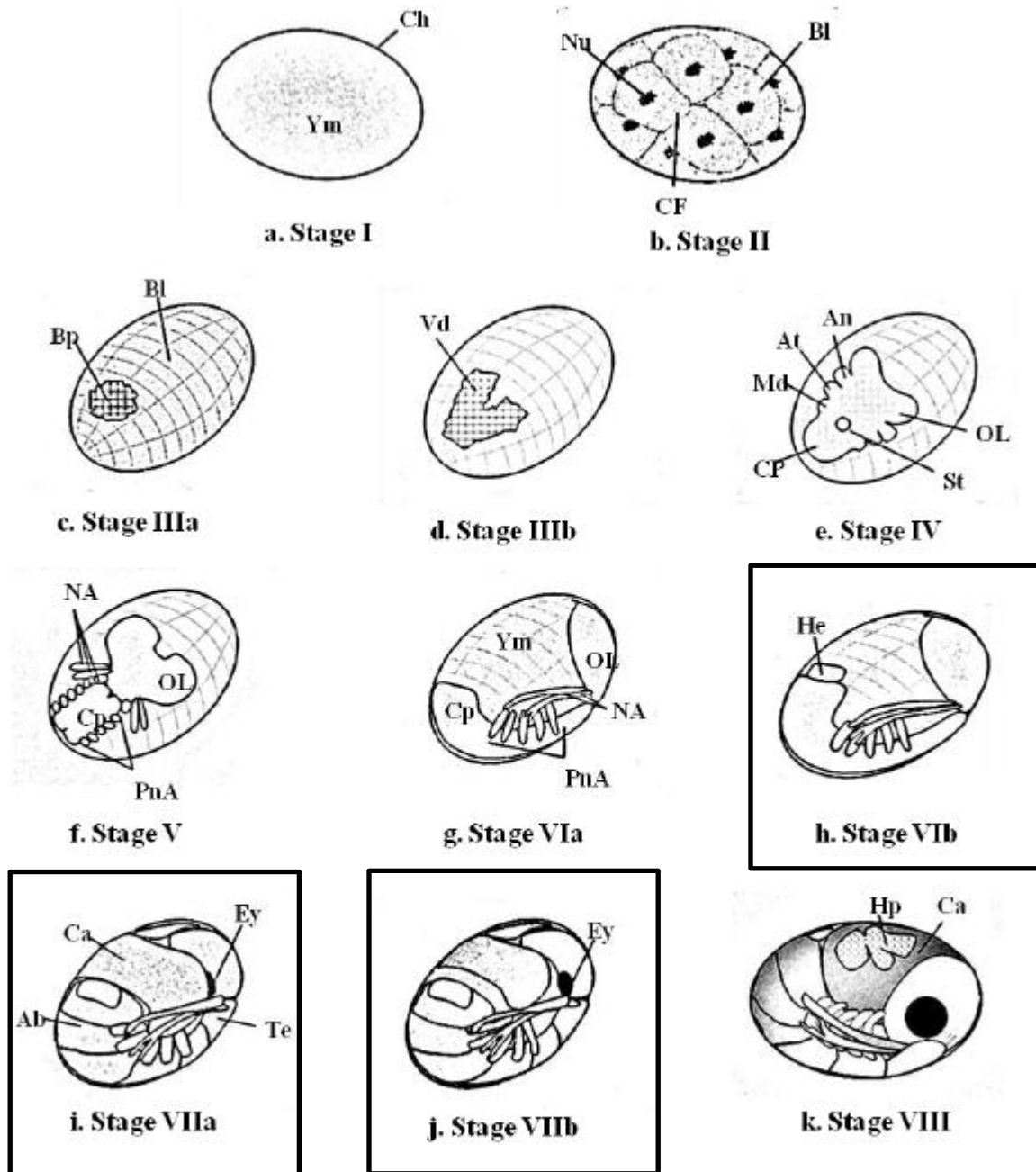
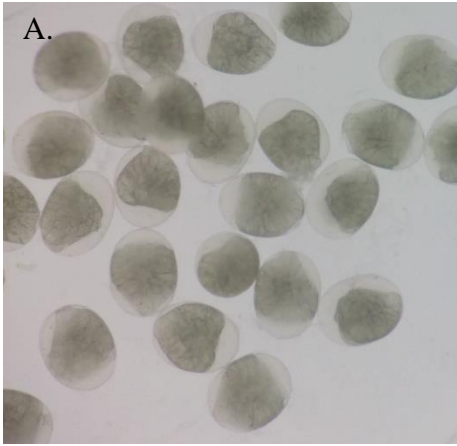
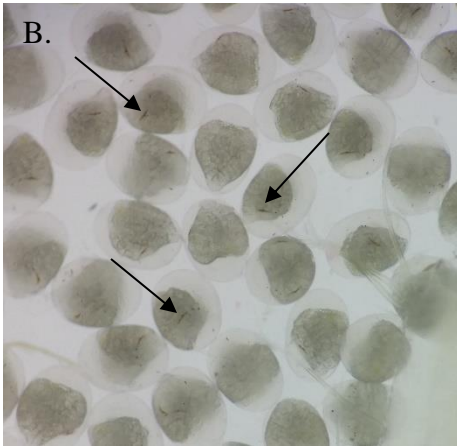


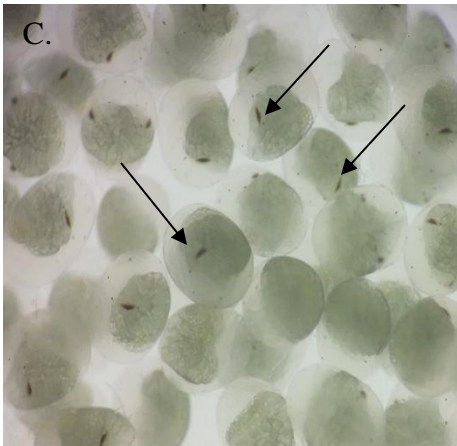
Figure 3.3. The stages of embryonic development as described and adapted from Romney and Reiber (2013). The black squares highlight the 3 stages that were identified in this experiment. Stage VIb is marked by the c-shaped embryo, stage VIIa is when the eye begins to have pigmentation, and stage VIIb is when the eye begins to condense.



Stage VIb



Stage VIIa



Stage VIIb

Figure 3.4. Pictures (by C. Thompson) of the stages of embryonic development as described and adapted from Romney and Reiber (2013). (A.) Stage VIb embryos lack an eye spot, (B.) stage VIIa is when the eye begins to have pigmentation, and (C.) stage VIIb is when the eye begins to condense. Arrows point to eye pigmentation and condensation in B. and C., respectively.

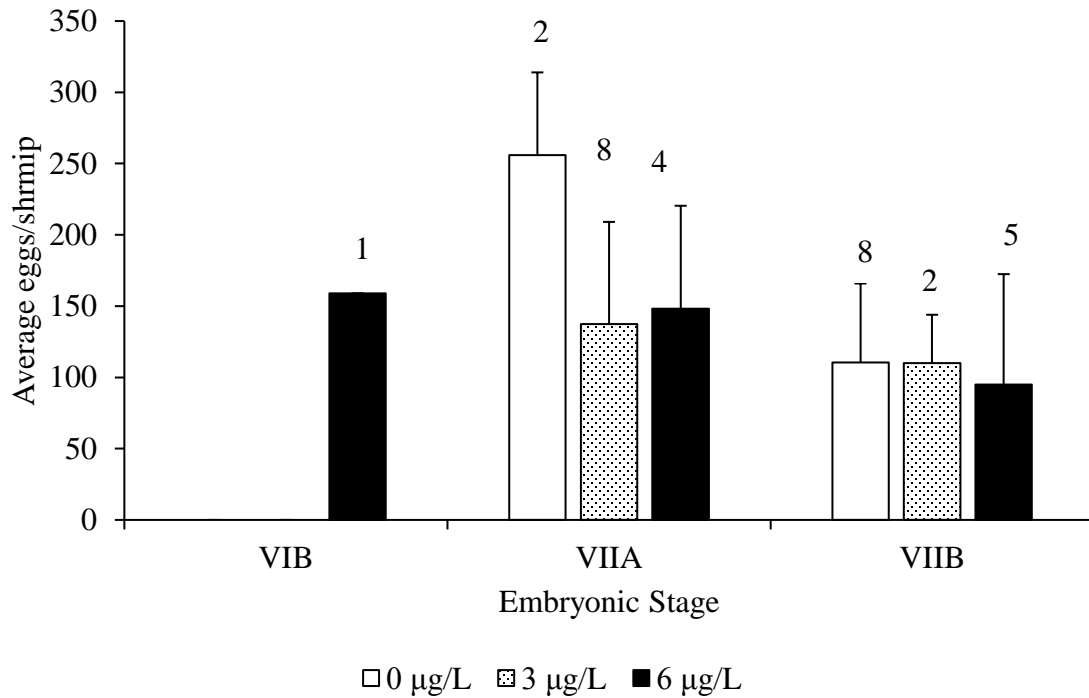


Figure 3.5. Adult daggerblade grass shrimp *Palaemonetes pugio* were collected at Country Club Creek and were exposed to 3 different concentrations of benzo[α]pyrene: 0 μg/L, 3 μg/L, and 6 μg/L. Shrimp that became ovigerous within 3 weeks were isolated for 7 d. The eggs were removed and the embryonic stage was determined. The average number of eggs in each stage + SD was calculated for each concentration. The number of shrimp with eggs in each stage is listed above the average. Significant differences are indicated by different letters.

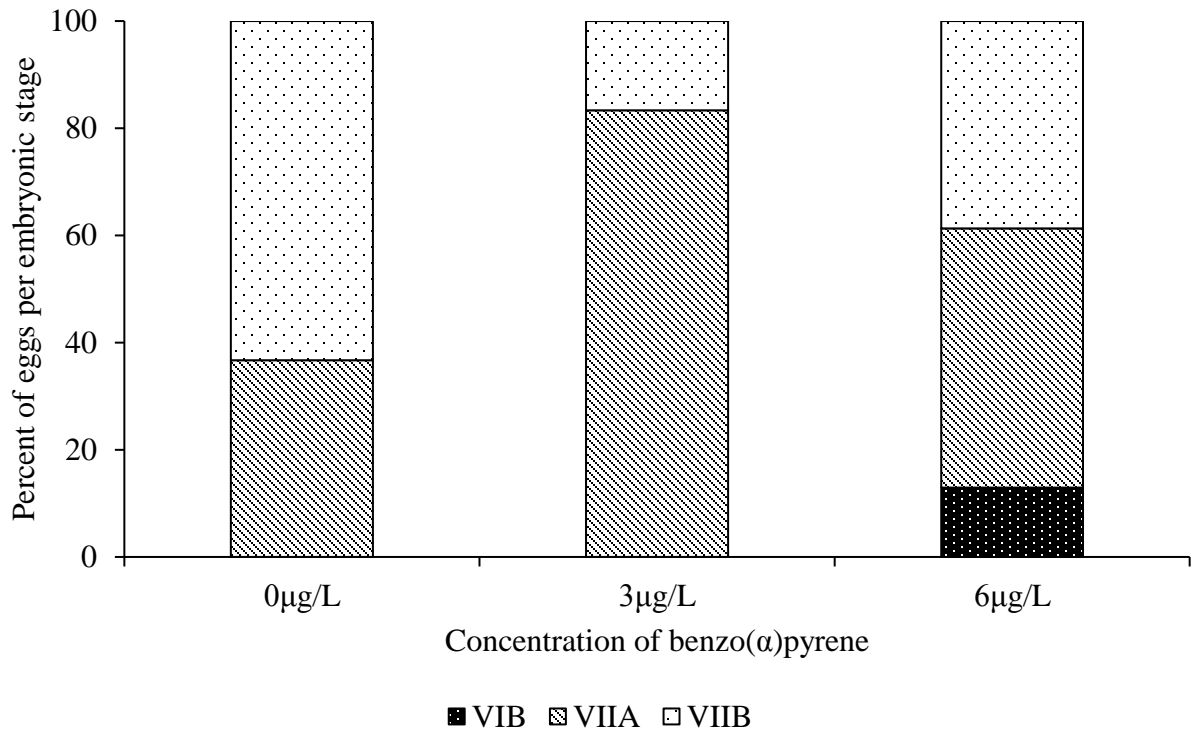


Figure 3.6. Adult daggerblade grass shrimp *Palaemonetes pugio* collected from Country Club Creek and exposed to 3 different concentrations of benzo[α]pyrene: 0 µg/L, 3 µg/L, and 6 µg/L. Shrimp that became ovigerous within 3 weeks were isolated for 7 d. The eggs were removed and the embryonic stage was determined. The percent composition of eggs in each stage was calculated for each concentration. Significant differences are indicated by different letters.

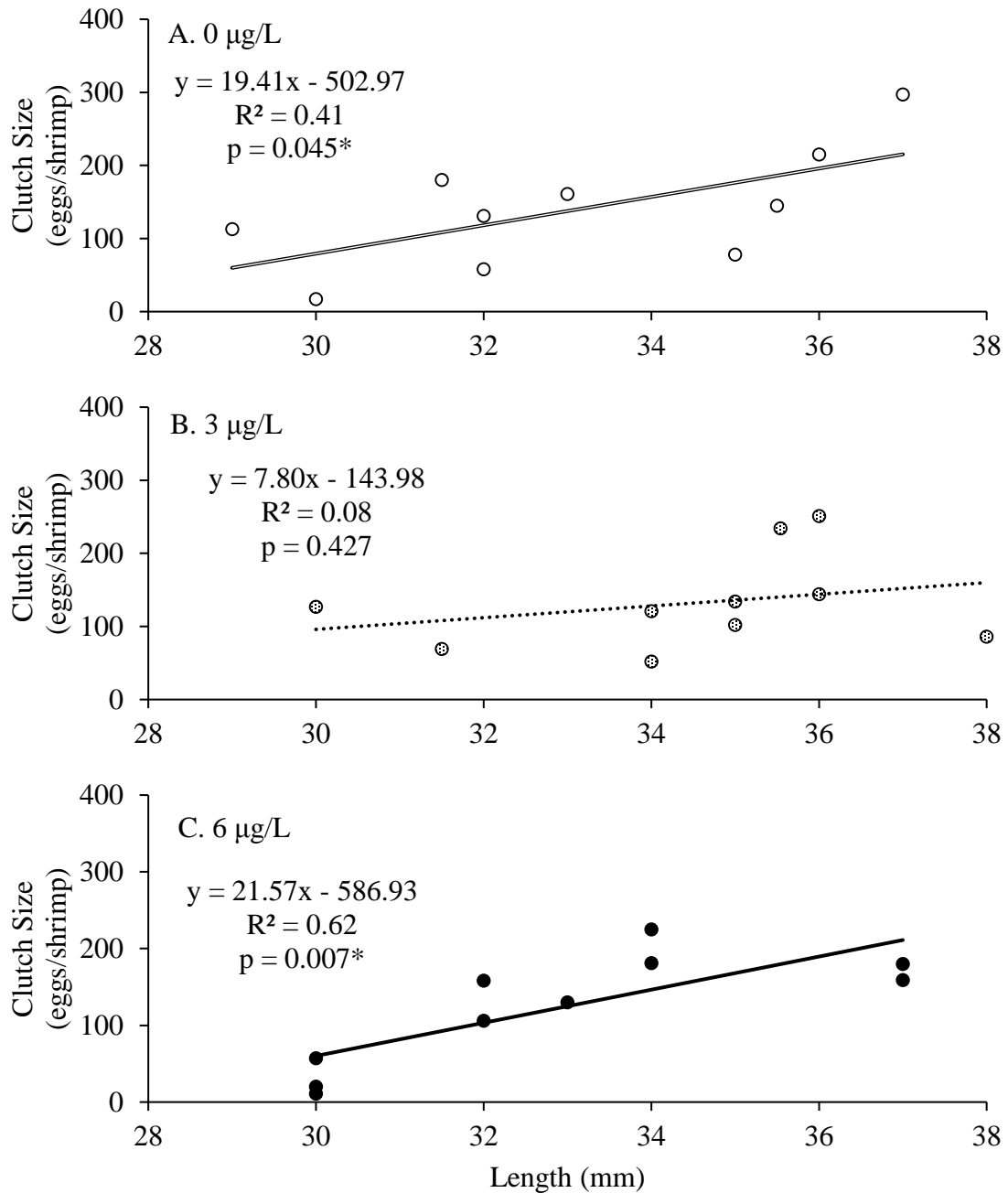


Figure 3.7. Adult daggerblade grass shrimp *Palaemonetes pugio* were collected at Country Club Creek and were exposed to 3 different concentrations of benzo[α]pyrene: (A.) 0 µg/L, (B.) 3 µg/L, and (C.) 6 µg/L. Shrimp that became ovigerous within 3 weeks were isolated for 7 d. The eggs were removed. Clutch size and length for each female shrimp (n=10 per concentration) was determined. Significant relationships are indicated by an asterisk (*) by the p-value.

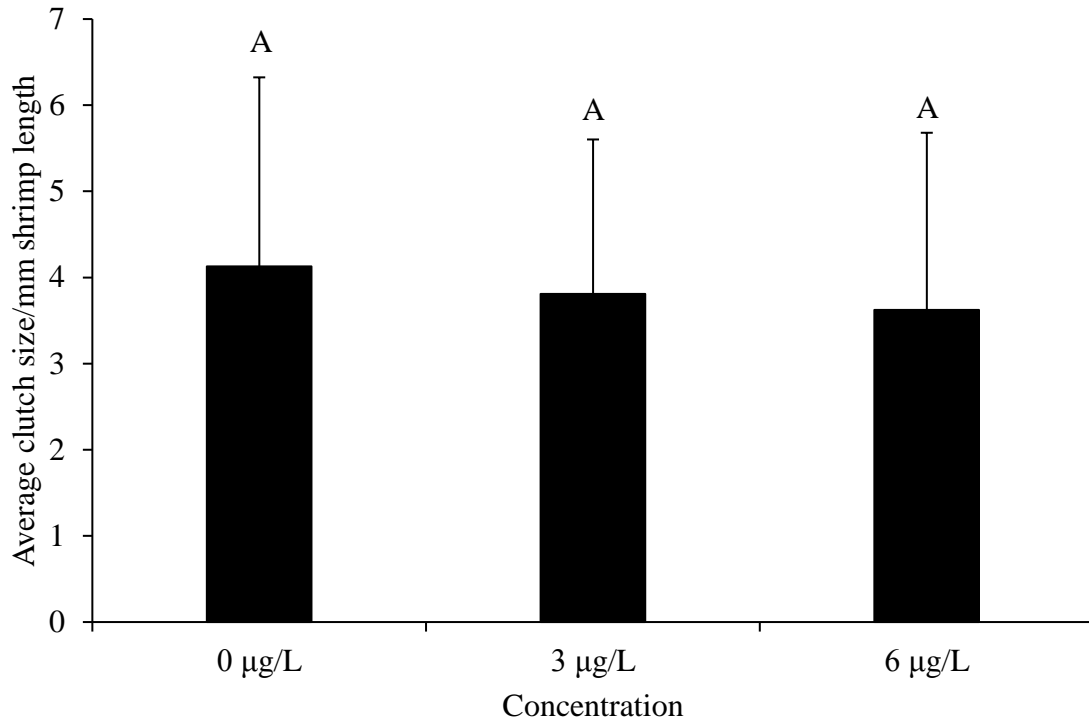


Figure 3.8. Adult daggerblade grass shrimp *Palaemonetes pugio* were collected at Country Club Creek and were exposed to 3 different concentrations of benzo[α]pyrene: 0 µg/L, 3 µg/L, and 6 µg/L. Shrimp that became ovigerous within 3 weeks were isolated for 7 d. The eggs were removed. Average number of eggs (clutch size) per mm length for each female shrimp (n=10 per concentration) was calculated + 1 SD. Significant differences are represented by different letters above the bars. ($\alpha=0.05$).

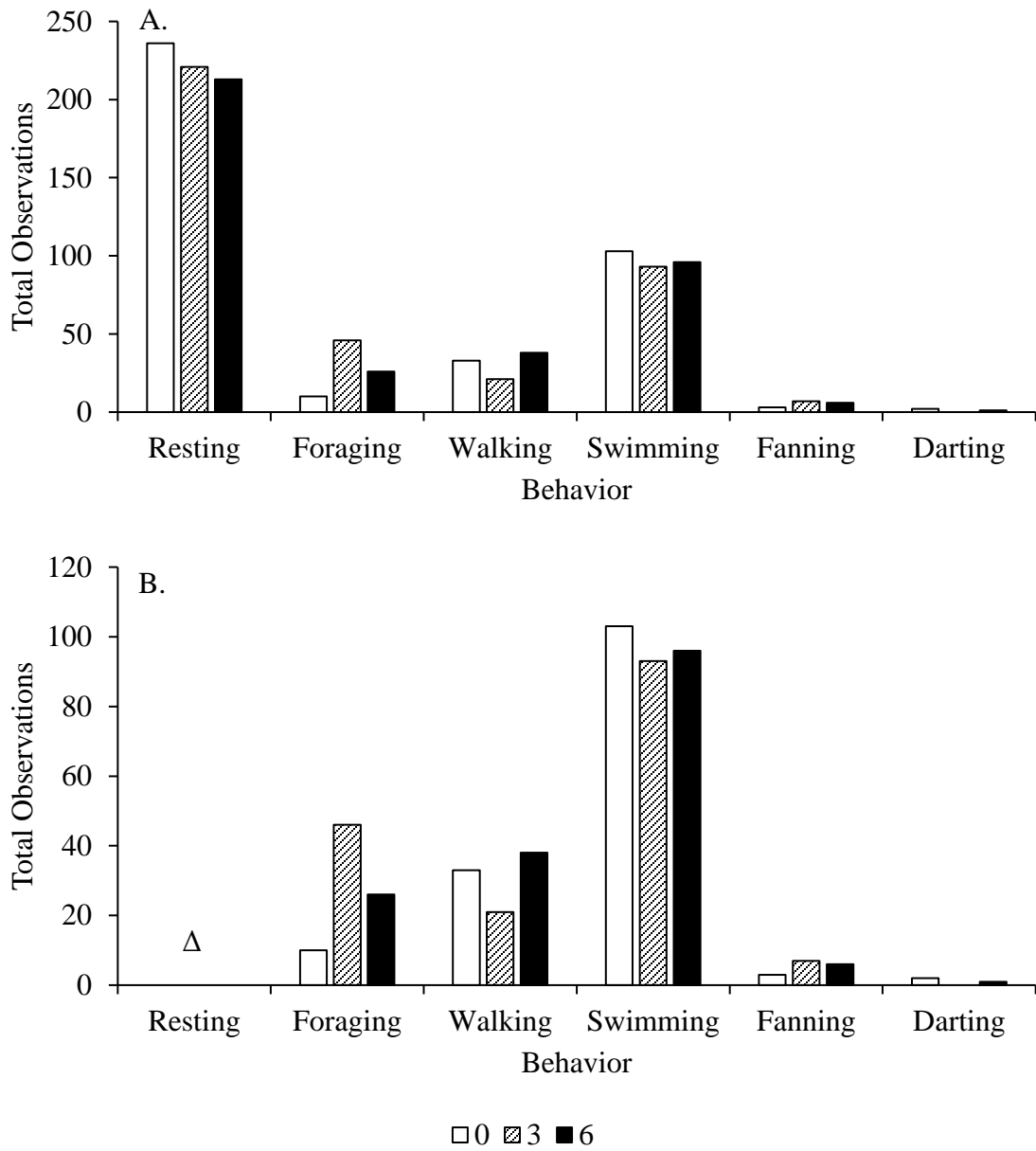


Figure 3.9. Adult daggerblade grass shrimp *Palaemonetes pugio* were collected at Country Club Creek and were exposed to 3 different concentrations of benzo[α]pyrene: 0 $\mu\text{g/L}$, 3 $\mu\text{g/L}$, and 6 $\mu\text{g/L}$. Behavior was observed every 3 h for the first 24 h and then every 6 h for the following 24 h. The total observations per concentration over the 48 h was recorded (A.). ^ΔResting dominated the graph, therefore the total observations, excluding resting, were calculated (B.).

CHAPTER 4

“Shrimp Socktail: The Shrimp You Feel Instead of Peel,” A K-12 Activity

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Shrimp Socktail: The Shrimp You Feel Instead of Peel

Abstract

Visual representations such as models increase student engagement in a lesson, stimulate linear thinking, and enhance quantitative skills involving mathematics, analysis, and graphing. In *Shrimp Socktail*, middle schoolers use socks and other easily accessible supplies to create physical models that represent parasitized female grass shrimp with eggs to learn about parasite/host relationships, reproduction, and shrimp populations. They count the eggs and learn about different factors that affect the reproductive output of shrimp. The students then analyze shrimp population data collected by a graduate student in Georgia and assess trends. This activity addresses Next Generation Science Standards, Common Core State Standards for Mathematics, and Ocean Literacy Principles.

Keywords: Shrimp, parasites, reproduction, modeling, NGSS

Grade Level: This activity is designed for middle school (grades 6-8) and is well-suited for students with visual impairments.

Introduction

Visualizations are any type of physical representation created to make intangible concepts tangible (Uttal and O’Doherty, 2008). Their use in teaching subjects such as science and mathematics has become universal (Uttal and O’Doherty, 2008). One type of visualization is a model, which can symbolize an object or a process. There are many

different types of models that range from mental to conceptual. A mental model is created in the mind of an individual, while a conceptual model is one that has an external form and is used by the public (Ornek, 2008). A physical model falls into the conceptual category and this representation can be touched or held (Ornek, 2008). The benefits of making such a model when teaching science may include increased student engagement in a lesson, stimulated linear thinking, and enhanced quantitative skills involving mathematics, analysis, and graphing (MacKay, 2015). Learning how to develop and use models is listed as 1 of the 8 Science and Engineering Practices in the Next Generation Science Standards (NGSS Lead States, 2013). Models can be used to link the microscopic and macroscopic worlds (Warren, 2015). In *Shrimp Socktail*, students create a physical model of a shrimp with parasites and eggs to replicate authentic research.

Science can be taught in a way that engages all 5 senses, but often visual observations dominate. There are few K-12 activities that incorporate multiple senses. This can limit the engagement of students with physical impairments. Sukkestad and Curran (2012) created an activity in which students imitated the fishing technique called noodling by feeling inside a covered container to retrieve mollusk shells. In *Shrimp Socktail*, students use their sense of touch to count parasites, which mimics the challenge of counting parasites embedded in a live shrimp. Students then incorporate sight when they visually count the eggs on the outside of the shrimp. Activities that focus on the sense of touch are particularly well-suited to engage the visually impaired.

In *Shrimp Socktail*, students also learn about variability in shrimp length and parasite numbers across populations. Students use a range of sock sizes to create shrimp of various lengths, which mimics differences among and within populations in nature. They also create variability by adding a different number of beads to their shrimp to represent the parasites embedded in the abdomen of the shrimp. Students then compare data across different populations.

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Background

Extensive salt marshes are found along the coast of Georgia, U.S.A. and provide a habitat for many species including a small crustacean called the daggerblade grass shrimp

Palaemonetes pugio. This shrimp is important to the estuarine food web because it is a decomposer, a predator, prey to other organisms, and host to parasites. The life cycle of the grass shrimp has several stages: egg, larva, juvenile, and adult. Spawning occurs from February to October (Anderson, 1985) and the eggs hatch 13 days after fertilization (Romney and Reiber, 2013). This organism has external fertilization and the female shrimp carries eggs underneath her abdomen (Anderson, 1985). This group of eggs is called a clutch. Grass shrimp have 5 pairs of swimming legs (pleopods), which surround the clutch (Figure 1). If scientists need to know the clutch size for a study, the eggs are gently removed from the shrimp and counted under a microscope. There is a positive relationship between the length of female shrimp and the number of eggs per clutch (Anderson, 1985).

Various environmental factors can influence the reproduction of grass shrimp. Temperature may affect the number of clutches a female may produce during a breeding season because the eggs develop more slowly in colder water (Anderson, 1985). The availability of oxygen affects health by causing a decrease in heart rate of a reproductively active female shrimp (Guadagnoli and Reiber, 2005). The availability of nutritional resources affects reproduction because female shrimp that receive less food have smaller clutch sizes (Reinsel et al., 2001). Clutch sizes are also smaller (< 150 eggs) in polluted environments than those from a less polluted area (170 eggs) (Leight et al., 2005).

Grass shrimp are hosts to several species of parasites, which are organisms that live on or in a host organism while gaining nutrition or other benefits at the expense of the host. The grass shrimp is a host to the parasitic trematode worm *Microphallus turgidus*. The trematode creates a cyst in the abdomen of the shrimp (Figure 2). When *P. pugio* were infected by the trematode, the number of cysts per shrimp ranged from 1-105 in coastal Georgia and were found in higher quantities in areas with higher salinities (>19 ppt) (Pung et al., 2002). Besides salinity, another factor that affected the number of trematode cysts was shrimp size. Trematode intensity or the number of trematode cysts per shrimp increased with shrimp length (Sheehan et al., 2011). The number of parasitic trematode cysts in the host shrimp affects the stamina of the shrimp. Trematode density or the number of cysts per shrimp length (mm) may range from 0.2-0.6 cysts/mm (Brinton and Curran, 2015). Kunz and Pung (2004) determined that shrimp infected with trematode cysts were consumed more often because they were more active in front of a predator despite having a lower overall swimming stamina for prolonged periods of time. When examining parasitized shrimp in the laboratory, it is often difficult to count all of the cysts under the microscope and it may take a long time to be accurate. This is due to the faint color of the cysts and the limited time that an aquatic organism may be kept out of water. The trematodes can also have high densities in some shrimp, which may cause some cysts to overlap. However, the number of parasites can instead be estimated by removing the shrimp briefly from the water in order to keep the shrimp alive for an experiment. This activity outlined here provides a biological model for estimation in contrast to the Gunzburger and Curran (2013) estimation activity that involved students using candy,

and included time restrictions to reflect the balance between counting accurately and keeping the shrimp alive.

Before beginning the activity students should be familiar with the terminology list below.

Clutch	a group of eggs produced at the same time
Cyst	a spherical growth found inside grass shrimp that is created by a trematode
Decomposer	breaks down dead organisms into usable products
Population	members of the same species living in a given/specified area
Relationship (positive vs. negative)	a connection between two factors that consists of an overall increase or decrease.
Salt Marsh	a wet land that contains a mixture of fresh and salt water
Spawn	to produce eggs or offspring
Trematode	a parasitic flatworm found as a cyst in grass shrimp

Next Generation Science Standards (NGSS Lead States, 2013)

Standard: MS-LS2 Ecosystems: Interactions, Energy, and Dynamics

MS-LS2-2. Construct an explanation that predicts patterns of interactions among organisms across multiple ecosystems.

Activity Objectives	Science and Engineering Practices	Disciplinary Core Ideas	Crosscutting Concepts
<p>Create models of parasitized female egg-bearing shrimp of varying sizes</p> <p>Interpret linear relationships in activity data and scientific data</p>	<p>Developing and Using Models</p> <ul style="list-style-type: none"> Develop a model to describe phenomena (MS-LS2-3) <p>Analyzing and Interpreting Data</p> <ul style="list-style-type: none"> Analyze and interpret data to provide evidence for phenomena (MS-LS2-1) 	<p>LS2.A: Interdependent Relationships in Ecosystems</p> <ul style="list-style-type: none"> Organisms, and populations of organisms, are dependent on their environmental interactions both with other living things and with non-living factors (MS-LS2-1) 	<p>Patterns</p> <ul style="list-style-type: none"> Patterns can be used to identify cause and effect relationships (MS-LS2-2) <p>Cause and Effect</p> <ul style="list-style-type: none"> Cause and effect relationships may be used to predict phenomena in natural or designed systems (MS-LS2-1)

Common Core State Standards for Mathematics (National Governors Association, 2010)

Standard: MCC8.EE Expressions and Equations

MCC8.EE.5: Graph proportional relationships, interpreting the unit rate as the slope of the graph. Compare two proportional relationships represented in different ways.

Standard: MCC8.SP Statistics and Probability

MCC8.SP.1: Construct and interpret scatter plots for bivariate measurement data to investigate patterns of association between two quantities. Describe patterns such as clustering, outliers, positive or negative association, linear association, and nonlinear association

MCC8.SP.2: Know that straight lines are widely used to model relationships between two quantitative variables. For scatter plots that suggest a linear association, informally fit a straight line, and informally assess the model fit by judging the closeness of the data points to the line.

MCC8.SP.3: Use the equation of a linear model to solve problems in the context of bivariate measurement data, interpreting the slope and intercept

Ocean Literacy Principles (National Marine Educators Association, 2013)

Principle 5: The ocean supports a great diversity of life and ecosystems.

I. Estuaries provide important and productive nursery areas for many marine and aquatic species.

Safety

Supervise students during this activity. Beads are a choking hazard, so advise the students to only use them as directed. Pipe cleaners have pointed ends, so have students handle

them with care. Rubber bands and all materials are only to be used for their intended purpose and not for horseplay.

Recommended Time for Activity

3 class periods lasting approximately 30 minutes on day 1 and 60 minutes on days 2 and

3. Total time is approximately 2.5 hours.

Materials

Sock of any size and color (1 per student; student can supply his/her own clean sock)

Clear pony-style beads (~60 per student) - Eggs and trematodes

Small cups to hold beads (1 per student)

Rubber bands (2 per student)

Pipe cleaners (8 per student) - Legs and clutch

Poly-fil or stuffing (Approx. 20 oz. bag per 20 students)

Markers or fabric marker (1 per group)

Metric ruler (1 per group)

Timer

Medium container for storing shrimp models (1 per group)

Shrimp Socktail Worksheet (1 per student; provided)

Table 4 (provided)

Shrimp Socktail Assessment (1 per student; provided)

Activity Lesson Plan

Day 1

1. Lead a discussion with the students about the provided background information on parasite/host relationships and shrimp reproduction. Begin the discussion by asking the students questions. Some example questions are: 1) Name abiotic and biotic factors that may be found in a marsh; 2) Name 3 animals that lay eggs. Do the organisms produce one or many eggs?; and 3) Explain in your own words the difference between a parasitic and a symbiotic relationship.

2. After the lesson, instruct students to bring in a spare sock of any size the following day. Have a few available if students forget or are unable to bring in a sock.

Day 2: Shrimp Socks

1. Divide students into groups of 4-5 based on the size of the sock each student brought. Try to make groups that would fall under 1 of the 3 categories: Small (ankle socks), medium (crew-length socks), or large (knee highs and longer). The number of groups assigned to each category does not have to be equal. Distribute the *Shrimp Socktail Worksheet* and inform each group how many eggs and trematodes a student can add to their sock. The small sock groups add 0-10 eggs and 0-10 trematodes, medium sock groups add 10-20 of each, and large sock groups add 20-30. Tell the groups these values quietly because this trend of smaller shrimp with fewer eggs and larger shrimp with more eggs will be discussed later. You may also write the ranges on a card and distribute the cards to the appropriate groups.

2. Each group chooses a name (students can be creative) and these groups represent different populations of shrimp. Have the students fill in the names of every group member in the first column of Table 1 on the *Shrimp Socktail Worksheet*. Then, have each student choose 2 numbers within the range given to his/her respective sock size group (small, medium, or large) and write them next to his/her name under trematodes and eggs, respectively (Table 1). Have the students quietly exchange data within their group and fill in all of Table 1 except for the length of the shrimp.

3. While the groups are working on calculating averages, pass out cups with clear beads (~60 beads) to each student along with a pile of poly-fil and one medium container for each group. Instruct students to take out their socks and begin filling them with poly-fil until the head of the shrimp is about the size of a golf ball (Figure 1). Students with larger socks may have a larger shrimp head. Once the head is formed, have students use a rubber band to securely separate the head of the shrimp from the abdomen.

4. Have each student count out the number of clear beads, or “trematodes,” that he/she listed in Table 1. Next, have each student fill his/her sock about $\frac{3}{4}$ full by alternating poly-fil with beads, then more poly-fil, etc. When this is complete, have each student add a rubber band to securely separate the body from the tail of the shrimp.

5. The legs and clutch are created by using pipe cleaners; 3 pipe cleaners hold the eggs and 5 become the legs (Figure 1). The 3 for the eggs are placed on the underside of the

sock, running lengthwise. The 5 for the legs are wrapped around the abdomen of the shrimp and twisted to stay in place. Instruct the students to count the number of clear beads (eggs) that they chose earlier. Then, have the student add beads to either end of the 3 pipe cleaners used for the clutch (Figure 1). The ends of the pipe cleaners used for holding the clutch are tied or twisted together to hold the eggs in place (Figure 1).

6. When the shrimp sock is complete, have each student write his/her name in marker on the tail of the shrimp and measure the length of the shrimp sock in centimeters. Next, have students record each shrimp length and then calculate the average length of the shrimp in their group to complete Table 1.

7. At the end of the second day, Table 1 should be complete and each student should have a finished shrimp sock resembling the model in Figure 4. Place the socks from each population in separate containers until the next activity day and collect the *Shrimp Socktail* Worksheets. Fill in Table 4 with the name of each group, the group members, trematode and egg counts associated with each student, and the 3 averages for each group. These data will be used during the next class meeting.

Day 3: Switching Populations

1. Return worksheets and give each group of students a container filled with shrimp from a different group. Instruct students to pick a shrimp and write the name of the student that created it in Table 2. Once this is written, have the students place the shrimp on the table and put their hands in the air. On “Go” have the students begin the first round of counting

trematodes by feeling the body of the shrimp for 5 seconds to count the beads. Once the 5 seconds have ended, have each student place the shrimp back on the table and record the number of trematodes counted in Table 2. Repeat the counts twice more with the time increased to 25 and 45 seconds in the second and third rounds, respectively. Remind students that they are trying to determine the total number of trematodes even for the short 5-second round. This is to represent that shrimp should not be out of water long.

2. Next, have the students visually count the eggs 3 times and record this in Table 2.

3. Display the numbers from Table 4 on the board, or hand out a copy to each student. Instruct the students to find the information for the sock they received and write this on Table 2 under “actual numbers.” Have the students find the absolute value for the difference between the actual number and the visual counts. Have each student fill in Table 3 at this time with the averages and differences from each group or population.

4. Give students time to complete the *Shrimp Socktail Assessment* by filling in the scatter plots and answering questions. Scatterplots are created using average length of the shrimp sock as the independent variable (x-axis) and average clutch size as the dependent variable (y-axis) (Graph 1, *Shrimp Socktail Assessment*). The second scatterplot is filled in using the group averages in Table 3 with clutch size as the independent variable (x-axis) and number of trematodes as the dependent variable (Graph 2). Have the groups determine the numbers to write on the x-axis because this depends on the range in the average lengths of the socks.

Modifications

This activity may be modified slightly if desired. Items other than beads could be used to represent the eggs or trematodes to help clarify the model and help the students distinguish between eggs and trematodes. Such substitutions could include beans, buttons, or other items that are small enough to fit in the socks but large enough to be felt through the sock and poly-fil. Instead of doing a visual count of the eggs on Day 3, visually impaired students may count the eggs by touch. Students may do a “dissection” of the shrimp sock to get an accurate count of the trematodes and eggs. The supplies could then be recycled and used another year. On Day 3 of this activity, students could complete and interpret the graphs on the *Shrimp Socktail* Worksheet as a group instead of independently and/or the teacher could demonstrate how to fill in points on a scatterplot and then allow the students to complete the remaining points on their own. The students could also use all of the individual length and egg counts from Table 4 instead of the averages if more work with plotting data is desired. Students could draw an anatomically correct grass shrimp and label the body parts discussed in this activity. A labeled grass shrimp diagram can be found in Aultman et al. (2010).

Discussion

Students built their own models of shrimp and it resulted in very diverse shrimp populations because of the different sock sizes, number of beads, and colorful pipe cleaners used as well as the decorations that some students drew on their models. These differences lead to a discussion of how the populations vary among groups in terms of

average shrimp length, clutch size, and the number of parasites, and how some of these variables were related. The different populations also provided the teacher with the opportunity to discuss diversity across natural populations and the source of these differences. Creating their own models also enabled students to associate the different materials as representations for the biological counterparts. For example, a student later recalled that parasites could be found inside an organism because there were beads in the shrimp sock. They also were able to see that the grass shrimp carried its eggs externally. Some comments that were made in our class included “there were more beads inside the bigger socks” and “the Shrimpette group made their shrimp look alike.” Some prompts to begin this discussion with your students include: 1) Which group had the longest shrimp?; 2) Which group had the greatest clutch size?; 3) Why might larger shrimp have more parasites?; 4) Was it difficult to count the trematodes?; and 5) Do you think it would be difficult to count parasites in a live shrimp? If so, why?

This activity is versatile because it can be used in both science and mathematics classrooms. On Day 3, students created scatterplots and used them to answer questions about concepts such as grass shrimp reproduction. We also discussed interpreting patterns. Students noted that data points that are grouped close together on a graph are more similar and sometimes part of a pattern or trend. Data points that did not follow the trend were called outliers.

The use of a visualization in this activity was effective for teaching students about grass shrimp and how populations of organisms differ. By creating their own models, students

were directly engaged in the lesson. Some of the correlations that were introduced to students in this activity, such as shrimp length and clutch size, may have been harder concepts to grasp had physical representations not been used.

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Photo Credits

Figure 1: Courtesy of Lawson Bruen (top) and Coral Thompson (middle and bottom)

Figure 2: Courtesy of Lawson Bruen

Figures 3-5: Courtesy of Coral Thompson

Figure Captions

Figure 1. The anatomy of a grass shrimp *Palaemonetes pugio* with eggs compared to a *Shrimp Socktail*.

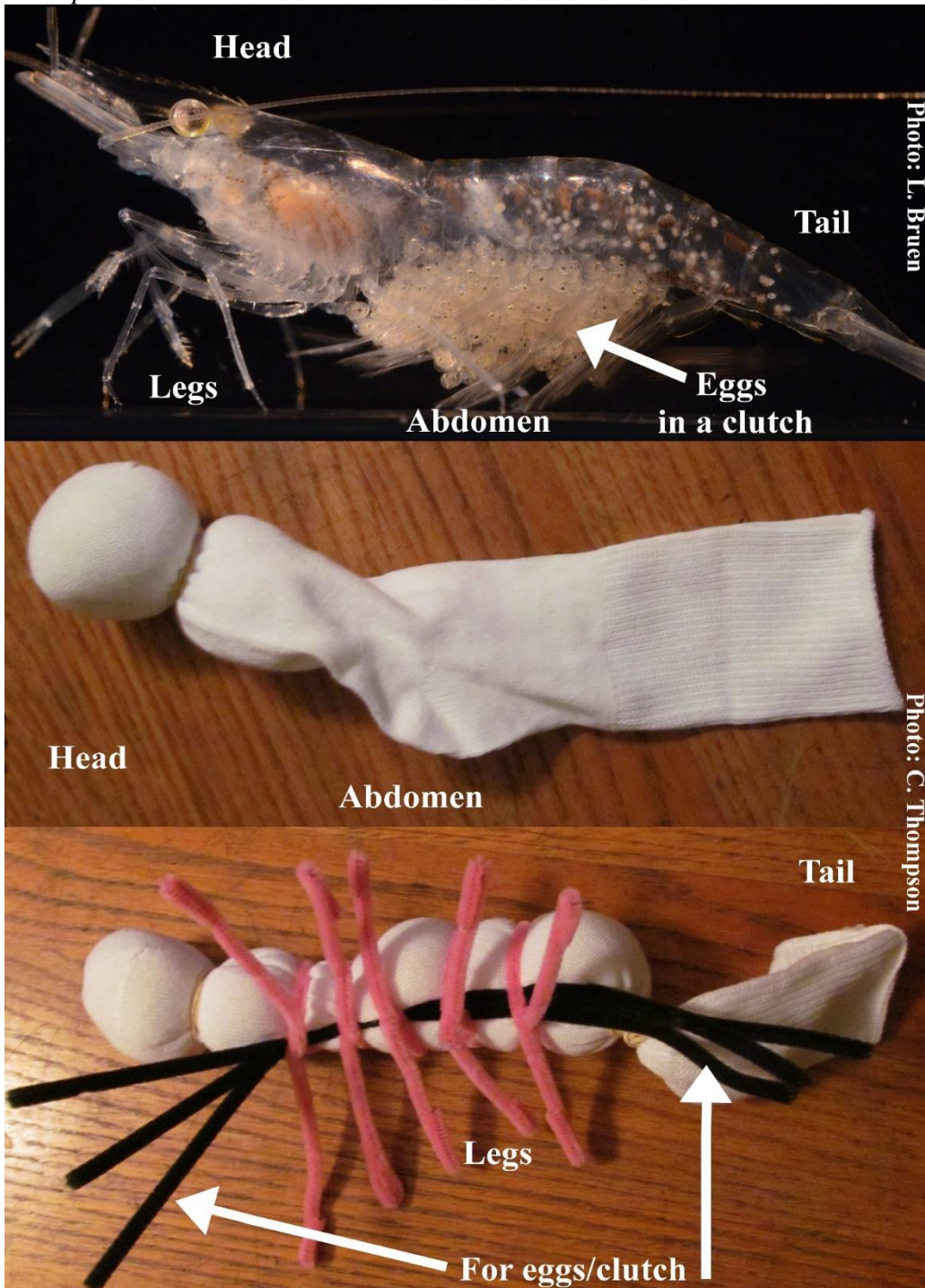


Figure 2. A daggerblade grass shrimp *Palaemonetes pugio* with trematode cysts inside the body.

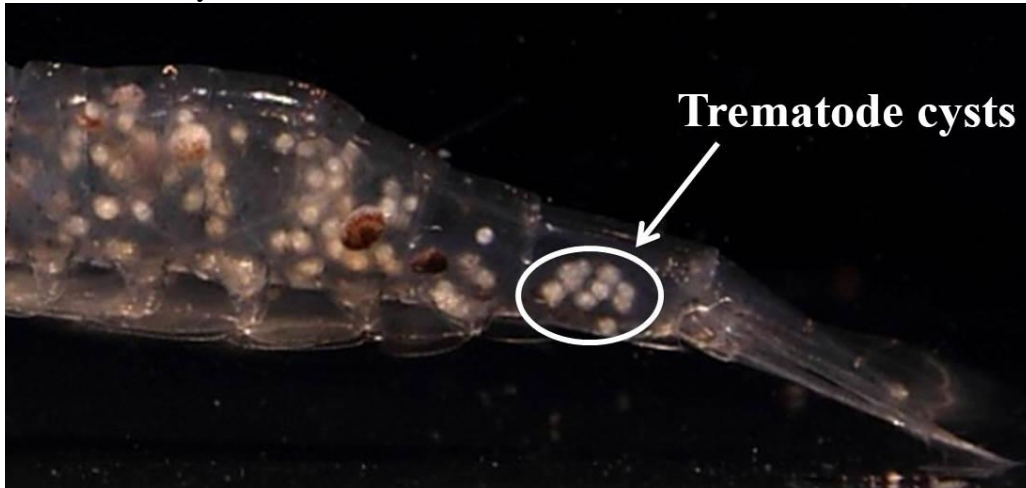


Figure 3. A student finished adding the eggs to a model of the grass shrimp.



Figure 4. A student holds his finished shrimp sock up to a picture displayed on the board. The *Shrimp Socktail* resembles a real grass shrimp.

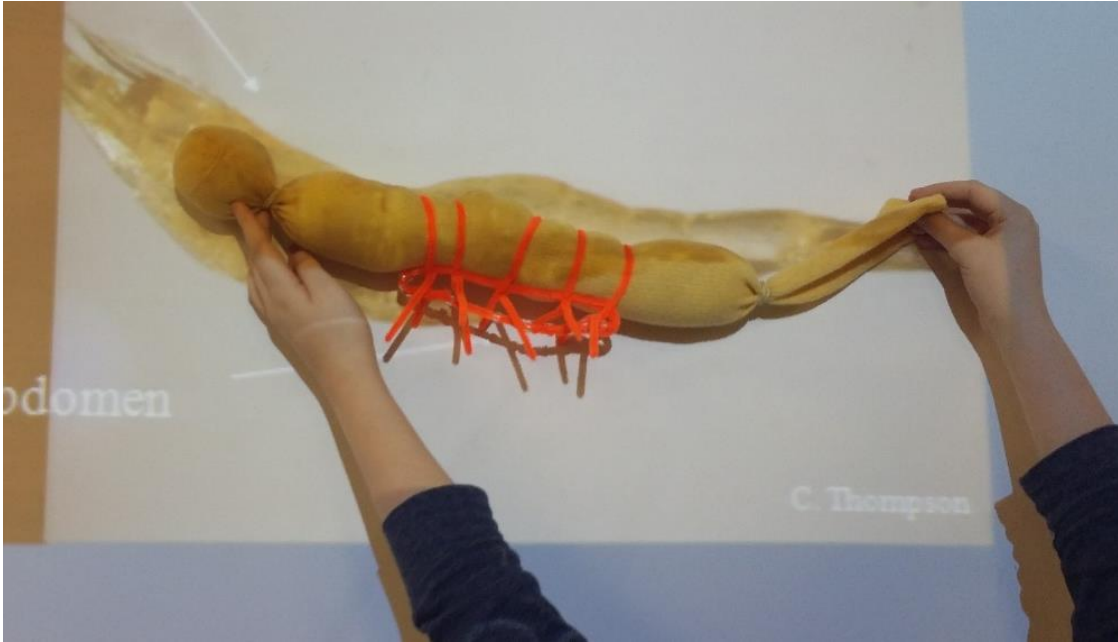


Figure 5. Two combined populations of shrimp socks that show the diversity among the shrimp.



Shrimp Socktail Worksheet - Days 2 & 3

Name: _____ Group Name: _____

Table 1. Initial group information.

Group Members	Trematodes	Eggs	Length
Total			
Average (Total/# shrimp)			

Table 2. Data collected from the shrimp received on day 2.

Shrimp Creator's Name	Trematodes Counted			Visual Egg Counts		
	5 s	25 s	45 s	1 st	2 nd	3 rd
Actual Numbers						
Difference (Actual-counted)						

Table 3. Group Averages.

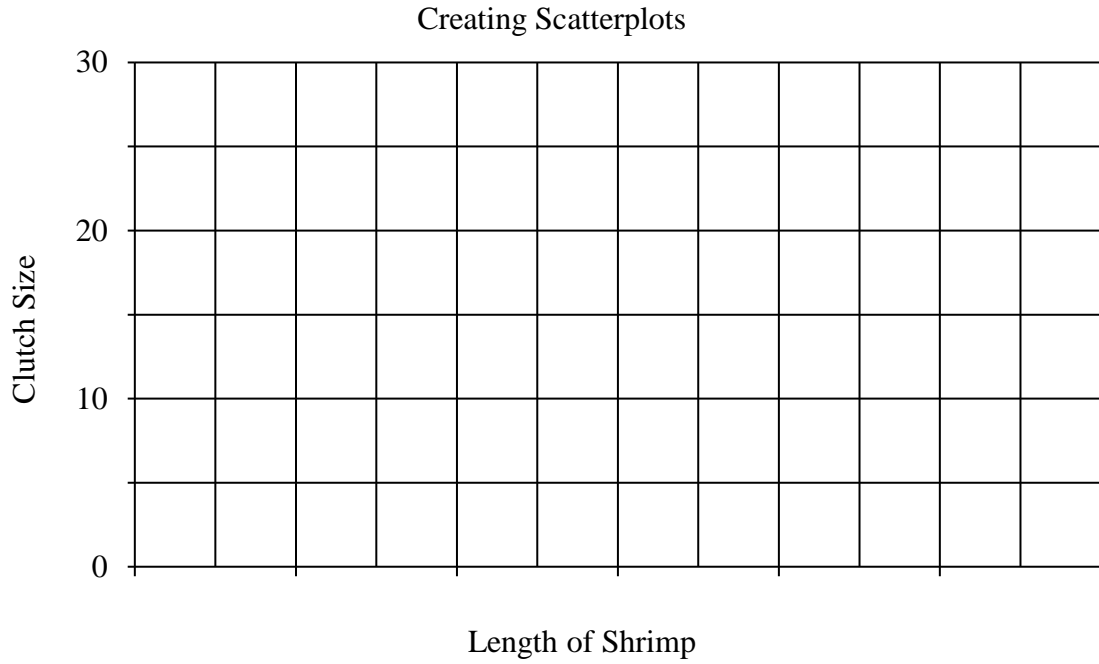
Group Name	Average Trematodes	Average Eggs	Average Length

Table 4. Table for the teacher to fill out after the first day of the activity.

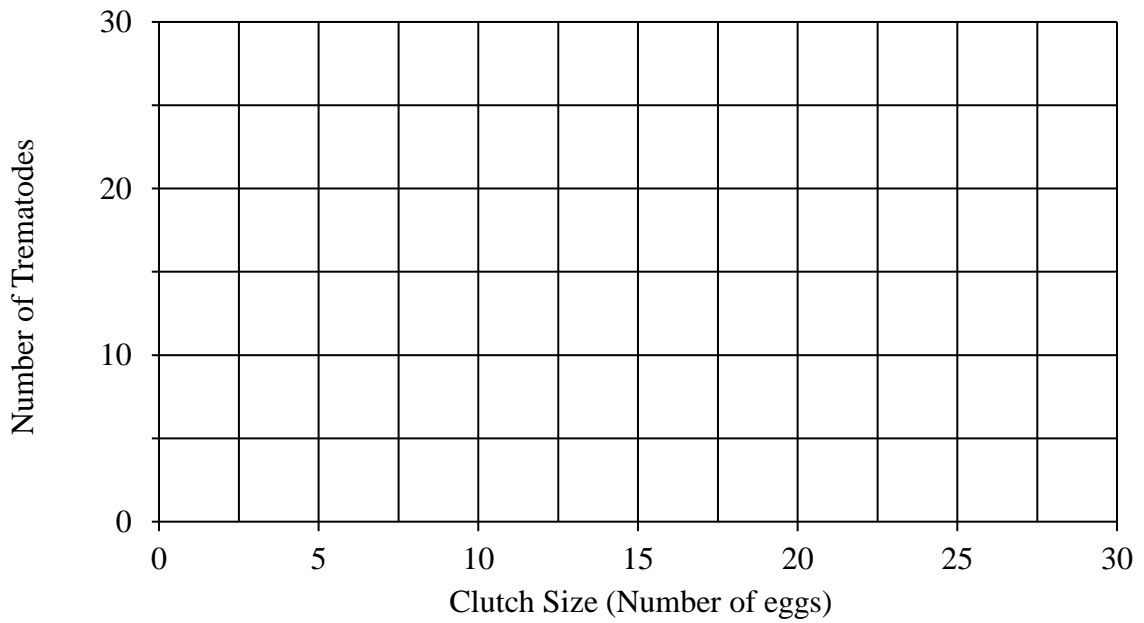
Group Name	Student Name	Actual Trematodes	Actual Eggs	Average Trematodes	Average Eggs	Average Length

Shrimp Socktail Assessment - Day 3

Name: _____ Date: _____



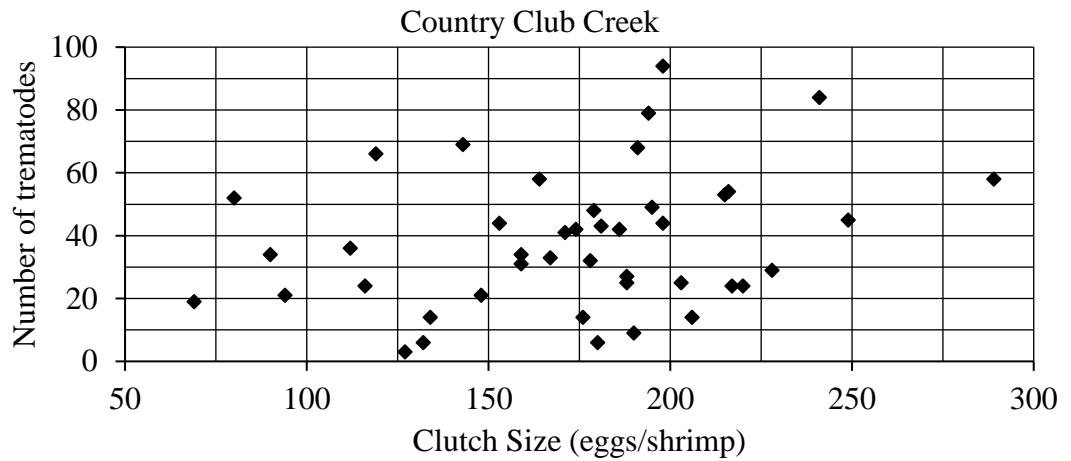
Graph 1. Plot the average length of each group of Shrimp Socktails and their average clutch size. (10 points)



Graph 2. Plot the clutch size and number of trematodes for Shrimp Socktail group averages that your class created. (10 points)

1. Complete Graphs 1 and 2 before answering the following question. Was there a pattern in either scatterplot that you created? Explain your answer. (5 points)

2. Use the graph below to answer the following questions.



- a. What was the smallest clutch size at Country Club Creek? (5 points)

- b. What was the highest number of trematodes at Country Club Creek? (5 points)

- c. Is there a pattern in the data for clutch size and number of trematodes? Why do you think there is or isn't a pattern in these data? (5 points)

3. You created a model of a grass shrimp with trematodes. Answer the following questions based on what was reviewed in class.

a. What animal is the parasite in this relationship? (5 points)

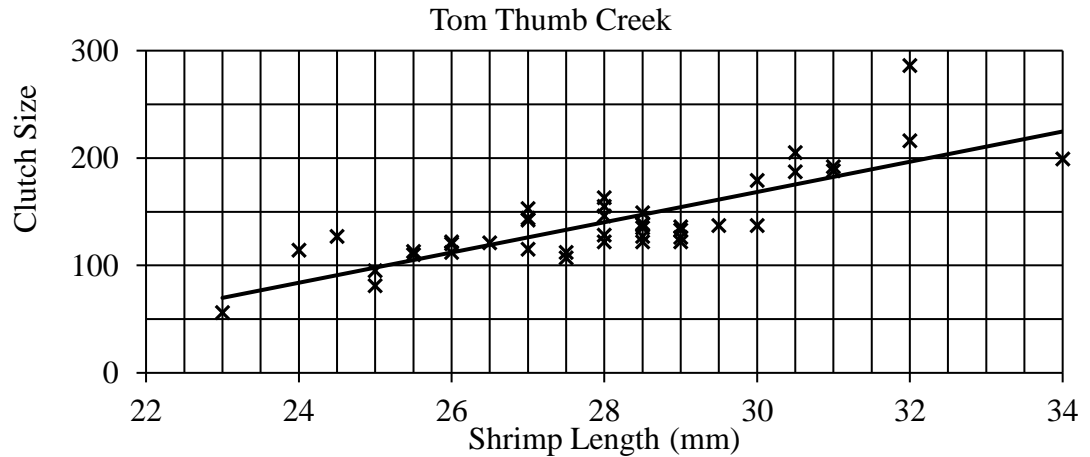
b. What animal is the host? (5 points)

c. Describe an effect that the parasite has on the host. (5 points)

4. Look at the graph below. If a shrimp collected from the Tom Thumb Creek was 30 mm long, what could be the clutch size? (5 points)

a. 50 eggs b. 160 eggs c. 400 eggs

5. Use the graph below to answer the following questions.



a. Is there a positive or negative relationship between length and clutch size? (5 points)

b. Are a majority of the data points clustered close to the trend line or scattered? (5 points)

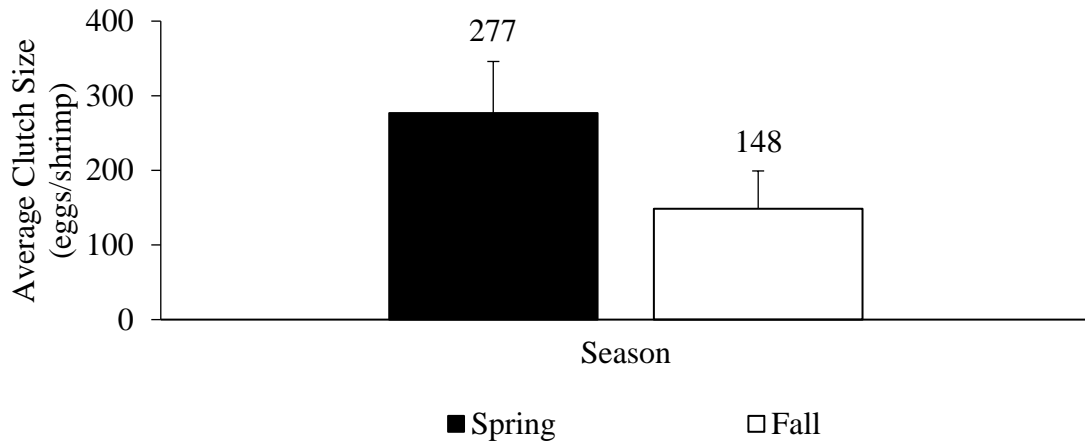
6. In the linear equation $y=mx + b$, the “m” represents the slope of the line. Interpret the meaning of a line with a slope of 20 eggs/millimeter. (5 points)

7. Length and clutch size were measured at Country Club Creek. The equation of a line is $y=23x-500$, where y equals the number of eggs and x equals the length of a shrimp in millimeters.

a. How many eggs would be in the clutch of a shrimp 30 millimeters long? (5 points)

b. Compare this clutch size to your answer for question 4. Do shrimp of the same length have larger clutch sizes at Tom Thumb Creek or Country Club Creek? (5 points)

8. Look at the graph below and answer the following questions.



a. What effect does season have on clutch size? (5 points)

b. Think about the environmental factors associated with season in a marsh. How might these influence clutch size? (5 points)

- c. What are other factors may influence shrimp length or reproduction? (5 points)

Shrimp Socktail Assessment - Answer Key

Possible responses are in italics

1. Complete Graphs 1 and 2 before answering the following question. Was there a pattern present in either scatterplot that you created? Explain your answer. (10 points)
Student must look at the scatterplots (Graphs 1-2) and determine if there is a pattern. Explanations may vary: "No, because the points are spaced out" or "Yes, because as length increases, the clutch size increases."
2. Use the graph below to answer the following questions.
 - a. What was the smallest clutch size at Country Club Creek? (5 points)
Any answer from 60 to 75
 - b. What was the highest number of trematodes at Country Club Creek? (5 points)
Any answer from 90 to 100
 - c. Is there a pattern in the data for clutch size and number of trematodes? Why do you think there is or isn't a pattern in these data? (5 points)
No. There is not a noticeable pattern because there are points all over the graph. No answer is incorrect if the student can justify their answer.
3. You created a model of a grass shrimp with trematodes. Answer the following questions based on what was reviewed in class.
 - a. What animal is the parasite in this relationship? (5 points)
Trematode
 - b. What animal is the host? (5 points)
Grass shrimp
 - c. Describe an effect that the parasite has on the host. (5 points)
Lower swimming stamina, more active in front of a predator.
4. Look at the graph below. If a shrimp collected from the Tom Thumb Creek was 30 mm long, what could be the clutch size? (5 points)
b. 160
5. Use the graph above to answer the following questions
 - a. Is there a positive or negative relationship between length and clutch size? (5 points)
Positive
 - b. Are a majority of the data points clustered close to the trend line or scattered? (5 points)
Clustered together
6. In the linear equation $y=mx + b$, the "m" represents the slope of the line. Interpret the meaning of a line with a slope of 20 eggs/millimeter. (5 points)
For every millimeter in length, there is an increase of 20 eggs.

7. Length and clutch size were measured at Country Club Creek. The equation of a line is $y=23x-500$, where y equals the number of eggs and x equals the length of a shrimp in millimeters.
- How many eggs would be in the clutch of a shrimp 30 millimeters long? (5 points)
190 eggs
 - Compare this clutch size to your answer for question 4. Do shrimp of the same length have larger clutch sizes at Tom Thumb Creek or Country Club Creek? (5 points)
Country Club Creek has the larger clutch size.
8. Look at the chart above and answer the following questions.
- What effect does season have on clutch size? (5 points)
Shrimp have larger clutch sizes during the spring.
 - Think about the environmental factors associated with season in an estuary. How might these influence clutch size? (5 points)
Answers may include temperature, salinity, parasites. Correct if the student can justify the answer. For example, the shrimp had larger clutch sizes in spring when the weather is warmer. Therefore clutch size increases with temperature.
 - What are other factors that may influence shrimp length or reproduction? (5 points)
Resources (food), month, location, age. Answers may vary, but should reflect abiotic or biotic factors.

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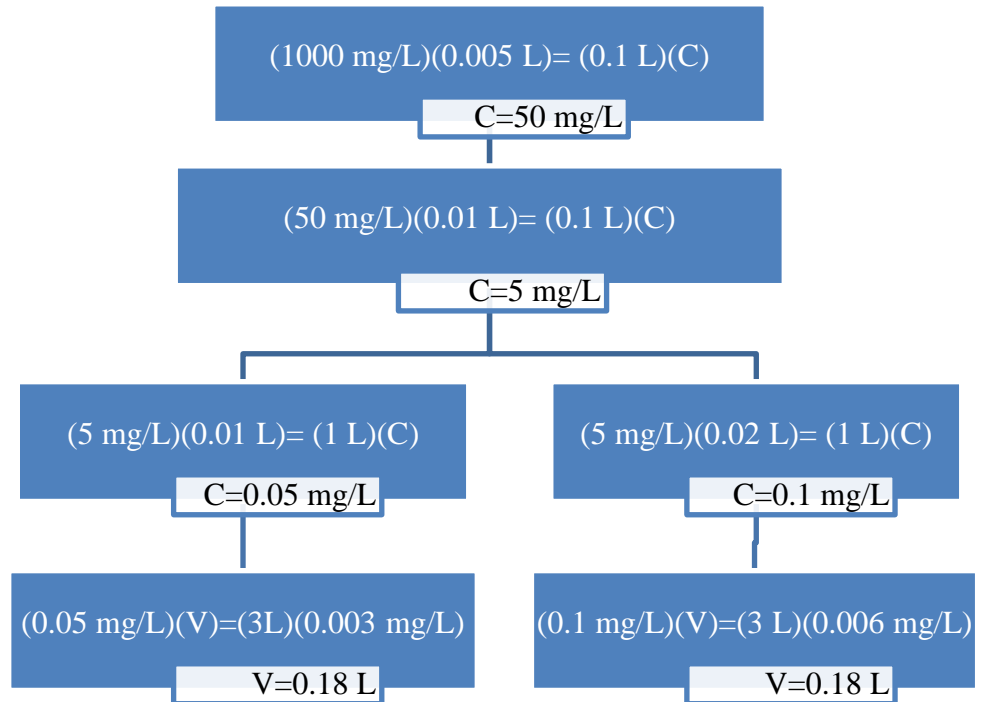
APPENDICIES

SOP: Preparation of benzo[α]pyrene for introduction into tanks

For each trial:

9 total tanks. 3 water changes. 3 concentrations (control= 0 $\mu\text{g/L}$, low= 3 $\mu\text{g/L}$, high= 6 $\mu\text{g/L}$) [n=3 tanks per trial]

3 concentrations x 3 water changes= 9 preps per trial



- 9 pre measured vials of benzo[α]pyrene for total experiment
- 5 mg of BaP dissolved in 5 mL of DMSO = 1000 mg/L concentration and 0.005 L
- Add this to 95 mL of artificial creek water (ACW). Concentration= 50 mg/L
- Transfer 10 mL of solution to 90 mL H₂O. Name: Solution B
- For 3 $\mu\text{g/L}$:
 - Add 10 mL of solution B to 990 mL of ACW.
 - Add 180 mL of this solution to tanks. Total 3 L in tank. (2820 mL ACW)
- For 6 $\mu\text{g/L}$:

- Add 20 mL of solution B to 980 mL of ACW.
- Add 180 mL of this solution to tanks. Total 3 L in tank. (2820 mL ACW)

180 mL added to each tank for each concentration.

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