Shifting Relationships Between Crayfish and Symbiotic Worms in Surface Water and Subterranean Environments

by

Catlin Waylon Ames

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 2013

Keywords: Crayfish, branchiobdellidan, cleaning symbiosis, context-dependence

Approved by

James A. Stoeckel, Associate Professor, Fisheries and Allied Aquacultures
Brian S. Helms, Invertebrate Collections Manager, Biological Sciences
Stephen A. Bullard, Assistant Professor of Fisheries and Allied Aquacultures

Abstract

Branchiobdellidans are small annelids (Clitellata) that have neutral, positive, and negative effects on stream crayfishes. Host effects are context dependent, with positive effects (increased survivorship and growth) observed under high fouling pressure. Branchiobdellidans may be particularly beneficial to burrowing crayfishes in subterranean water, which is characterized by reduced water quality relative to surface water. The red swamp crayfish (*Procambarus clarkii*) is an ideal organism for testing this model as this species spends significant portions of its life in both open water and burrows, and can host large numbers of branchiobdellidans (100+ worms per adult). I tested for effects of the branchiobdellidan *Cambarincola spp.* on survivorship, growth, and ecosystem-level processing of *P. clarkii* in subterranean burrows and surface water environments in laboratory experiments. I also evaluated survivorship of *Cambarincola spp.* in natural surface water and burrow environments and utilized acute (48 hr) dose response assays to test effects of pH and ammonia on worm mortality. Results from laboratory studies showed worms thrived and significantly increased the growth of *P. clarkii* in surface waters but not in underground burrows. Worms had significant impacts on some ecosystem-level processing in surface waters but not in burrows. Field study results supported the finding of poor worm survivorship in laboratory burrows, but showed variable worm survivorship in ponds. Dose response assays showed worms were tolerant of a broad range in ammonia (0-40 mg/L) and pH (6.5-9.5). My results suggest the symbiotic relationship between *C. spp.* and *P. clarkii* is context-dependent: mutualistic in open waters, and commensal to non-existent in subterranean burrows. The mechanisms behind differential survivorship of worms in burrows and among ponds remain unclear. Further investigation into the shift in outcomes between habitats requires a better understanding of the specific environmental factors limiting worm survivorship in burrow and pond environments.

Acknowledgments

This research was made possible by a grant funded by the National Science Foundation. First, I would like to thank my advisor Dr. James Stoeckel for his guidance during my writing, experimental setup, and contributions to burgeon my appreciation of international music. Secondly, I would like to thank everyone that contributed to facilitating my research: My committee members, Dr. Brian Helms (the statistical guru) and Dr. Ash Bullard (the parasite enthusiast). I am forever indebted to my fellow Crustacean and Molluscan Ecology Lab (CAMEL) mates: Michael Hart, Ian Palmer, Tyler Mosely, Andrew Gascho-Landis, and Brad Stanton for their manual labor and long hours into research that was only slightly interesting to them. I would like to thank the assistance of Bronwyn Williams for her help in identification of branchiobdellidans. Field collections and research were made possible with the help of Mark Peterman and Karen Veverica at the North Auburn Fisheries Unit.

.

Table of Contents

Abstract ii

Acknowledgments iii

List of Tables v

List of Figures vi

List of Abbreviations viii

Chapter 1: An introduction into cleaning symbioses, and the crayfish/branchiobdellidan relationship 1

Chapter 2: Habitat mediates the outcome of a cleaning symbiosis for a facultative burrowing crayfish 7

Introduction 7

Methods 10

Results 17

Discussion 21

Chapter 3: No worm's land: Mechanisms driving high mortality of branchiobdellidans in natural experiments remain elusive 26

Introduction 26

Methods 28

Results 32

Discussion 34

Chapter 4: Complexity in the conditional outcomes of the crayfish/branchiobdellidan symbiosis 38

Literature Cited 41

List of Tables

Table 1: Water Quality Comparison between Subterranean and Surface Water Habitats of Experiments in Chapter 2 47

Table 2: Water quality Comparisons between Subterranean and Surface Water Treatments in Chapter 3 48

List of Figures

Figure 1: Results of Ammonia, Dissolved Oxygen, and pH Between Subterranean and Surface Water Habitats over the 8 Week Experimental Period for Chapter 2 49

Figure 2: Results of Suspended Solids Between Subterranean and Surface Water Habitats for Chapter 2 50

Figure 3: Results of Initial versus Final Branchiobdellidan Densities Between Subterranean and Surface Water Habitats for Chapter 2 51

Figure 4: Results of Final Growth of Crayfish with/without Branchiobdellidans Between Subterranean and Surface Water Habitats for Chapter 2 52

Figure 5: Results of Total and Underwater Burrow Areas by Crayfish with/without Branchiobdellidans and by Gender for Chapter 2 53

Figure 6: Results of Detrital Processing Compared Between Crayfish with/without Branchiobdellidans and Controls for Chapter 2 54

Figure 7: Results of Water Quality Parameters Between Subterranean and Surface Water Ponds over the 10 Week Experimental Period for Chapter 3 55

Figure 8: Results of Suspended Solids Between Subterranean and Surface Water Ponds for Chapter 3 56

Figure 9: Results of Initial versus Final Branchiobdellidan Densities Between Subterranean and Surface Water Ponds for Chapter 3 57

Figure 10: Results of pH from Surface Water Ponds and Final Branchiobdellidan Intensity for Chapter 3 58

Figure 11: Results Survivorship of Branchiobdellidans to Ammonia and pH Dose Responses for Chapter 3 59

List of Abbreviations

BWM	Blotted Wet Mass (g)
CL	Carapace Length (mm)
DO	Dissolved Oxygen (mg/L)
TSS	Total Suspended Solids (mg/L)
ISS	Inorganic Suspended Solids (mg/L)
OSS	Organic Suspended Solids (mg/L)
I:O	Inorganic/Organic Ratio (mg/L)
ANOVA	Analysis of Variance
SD	Standard Deviation
SE	Standard Error
F	F-value
p	P-value

Ch. 1) An introduction into cleaning symbioses, and the crayfish-branchiobdellidan relationship

In 1875, the publication of Pierre van Beneden's article *Les Commensaux et les Parasites dans le Régne Animal* (*Commensal and Parasites in the Animal Kingdom*) described a new term of interspecific interaction, mutualism, and altered the previous simple dichotomy of commensals and parasites. In following years, the term "mutualism" was used to explain interspecific interactions observed in nature, (pollination and mycorrhizal root-nodules by Pound in 1893), and in society by Peter Kropotkin to protest Social Darwinism (1955). Mutualisms were viewed as a beneficial interaction to both species (+/+) on an interaction grid that includes parasitism (-/+), commensalism (0/+), and competition (-/-) (Boucher et al. 1982).

Contemporary ideas of interspecific relationships are not as static and are interpreted along a continuum of parasitism-mutualism based upon conditional outcomes (i.e. intensity of parasites on host, environmental factors, etc.) (Boucher et al. 1982, Bronstein 1994, Cushman and Beattie 1991). Conditional outcomes of mutualisms to parasitism and commensalism have been found to be context dependent (mycorrihzae and plants under a nutrient gradient, Johnson et al. 1997), and density dependent (herbivory protection correlates with increased density of ants, Bronstein 1994).

For ease of understanding cost/benefits to organisms, we can view mutualistic interactions as either obligate (partners cannot survive without each other) or facultative (partners able to survive and reproduce in absence of services provided by partners) (Bronstein 1994, Cushman and Beattie 1991, Boucher et al. 1982). In most definitions, mutualisms include symbioses and non-symbioses, which define the proximity of species in the relationship. The proximity is divided among direct physical association (symbiotic) and indirect association (non-

symbiotic) (Boucher et al. 1982, Cushman and Beattie 1991). Facultative interactions and obligate non-symbioses' cost/benefits are seldom studied due to the difficulty in ascertaining rewards between partners and observed variability in the outcome of the relationship (i.e. between plants and ants) (Bronstein 1994). In contrast, obligate symbiotic relationships have been extensively studied with documented benefits ranging from bioluminescence, to nutrient breakdown, to digestion, to ectoparasite removal (Cushman and Beattie 1991, Boucher 1982, Poulin and Grutter 1996, Cheney and Cote 2005). Recent studies have focused on the cost/benefits of obligate cleaning symbioses, primarily in marine reef systems using cleaner fish (Cheney and Cote 2005, Poulin and Grutter 1996, Grutter 2003, Losey 1979). Cleaning symbioses involve the removal of epibionts, ectoparasites, and necrotic tissue from a host 'client' by a 'cleaner' (Poulin and Gutter 1996). Studies of cleaning symbioses are complicated by the lack of empirical evidence of benefits from the cleaner to the client (Poulin and Gutter 1996). Possible explanations for the lack of benefits are 'cheating' where clients ingest cleaners and cleaners occasionally feed on client tissues, or that clients are 'hedonists' that will withstand parasitism for positive tactile stimulation (Poulin and Grutter1996).

Cleaning symbioses have evolved independently, multiple times, in many lineages (Poulin and Grutter 1996). These dynamic systems are formed on the basis of predator-prey interactions between cleaners and parasites on a host 'habitat patch' (Bshary and Grutter 2002). Studies of cleaner life history trait evolution suggest an initial free-living stage that has adapted to specific hosts due to predation and environmental pressure (fouling, food availability) on the cleaner (Bshary and Grutter 2002, Boucher et al. 1982). Cleaner diets are mainly composed of large multi-cellular eukaryotic parasites (copepods, gnathiids), but they can also benefit hosts by the ingestion or removal of microorganisms and inorganic debris, especially in aquatic systems

(Poulin and Grutter 1996, Bshary and Grutter 2002). Terrestrial hosts (particularly vertebrates) have greater abilities of self-cleaning, whereas aquatic hosts are prone to higher densities in parasite loads and fouling (Hart 1990, Bshary and Grutter 2002, Whal 1989). The density of water allows for greater motility of immersed organisms and inorganic material as compared to air (Whal 1989). Whal describes a temporal fouling sequence model in which four stages occur: biochemical conditioning, bacterial colonization, multicellular fouling (1989). Previous studies documenting cleaning symbioses have been primarily focused on multicellular parasite fouling and their subsequent removal by client cleaner wrasses in marine reef systems (Poulin and Grutter 1996, Bshary and Grutter 2002, Cheney and Cote' 2005, Losey 1979). Measuring ectoparasite density as an indicator of benefit to the host only accounts for larger parasite removal and may bias estimates of benefits in some systems (Cheney and Cote 2005, Bshary and Grutter 2002). Currently, evidence supporting density and context dependence of marine cleaning symbioses is only supported by ectoparasite density and observations of cleaners removing necrotic tissue, mucus, and scales, and this is complicated by the difficulty of completing long term controlled experiments (Bshary and Grutter 2002, Poulin and Grutter 1996).

Recently, research has focused on the cleaning symbiosis between stream crayfish and branchiobdellidans (crayfish worms). These studies empirically demonstrate the effect of cleaners (branchiobdellidans) on the growth and survivorship of the host client (crayfish) (Keller 1992, Brown et al. 2002, 2012, Lee et al. 2009). Branchiobdellidans are small ectosymbiotic (close proximity) annelids that live in association with aquatic crustaceans, and are found throughout the Holarctic region except for the Ural Mountains and Amur River drainage (Holt

and Opell 1993, Gelder 1999). Primarily associated with crayfish, branchiobdellidans have also been observed on isopods and freshwater crabs (Holt 1963, Hobbs and Figueroa 1958).

Branchiobdellidans attach to crayfish by using duo-gland adhesive organs where they subsequently reproduce and feed (Gelder and Rowe 1988, Keller 1992). Branchiobdellidans are thought of as obligate symbionts because successful reproduction of branchiobdellidans has only been reported on a live crustacean host, and worms are rarely found unassociated (Young 1966, but see Holt 1973). Branchiobdellidans prey on protozoans, algae, bacteria, diatoms, and other epibionts found on the exoskeleton and gill chamber of the crayfish host client (Holt 1973, Jennings and Gelder 1979, Gale and Procter 2011). Diets of branchiobdellidans have been observed to change with attachment site, maturation, and among congeneric species of worm in competition (Holt 1973, Grabda and Weizbecka 1969, Gale and Proctor 2011).

Crayfish are well known for their complex life history, with several species capable of switching from lotic or lentic surface waters, to subterranean burrows (Hobbs 1981). Subterranean burrows of crayfish are often anoxic, and contain high inorganic solids (Grow 1980). Crayfish are effective in removing inorganic particulates in the branchial chamber by the use of 'limb rocking' or the jostling of setobranch setae around gill filaments, but are ineffective in removing epibiotic fouling (Bauer 1998). The removal of particulate matter can increase colonization by bacilli (Bauer 1998), which are known prey of branchiobdellidans (Jennings and Gelder 1979, Gale and Procter 2011).

Some branchiobdellidan diets include host gill tissue indicating a parasitic relationship, typically by crayfish gill-dwelling specialists and select exoskeleton-dwelling individuals on stream crayfishes (Holt 1973). Juveniles of the species *Cambarincola chirocephala* were observed to ingest gill tissue of their host crayfish (Bishop 1968). In another study by Brown

(2002), parasitism by a surface dwelling worm (*Cambarincola sp.*) was experimentally evaluated by the use of ingested fluorescent paint in crayfish feed, where worms that were observed in a 'foraging behavior' in crayfish branchial chambers had ingested host gill tissue.

Not all cases of the branchiobdellidan-crayfish interaction are parasitic, and a few studies have demonstrated the nature of the relationship to commensal and mutualistic (Keller 1992, Lee et al. 2009, Brown et al. 2012). In mutualistic relationships, crayfish benefit by increased growth and reduced mortality, most likely due to gill cleaning (Brown et al. 2002, 2012, Lee et al. 2009). The exoskeleton and branchial chambers of crayfish are prone to fouling from inorganic and organic particulate matter, as well as copepods, protozoa, rotifers, and bacteria (Jennings and Gelder 1979, Bauer 1998, Gale and Procter 2011). Foraging by worms removes epibionts and other fouling agents, possibly increasing gill function in terms of ammonia excretion and gas exchange (Jennings and Gelder 1979, Brown et al. 2002, 2012).

The outcome of the symbiotic relationship between crayfish and worms is dependent on worm density and fouling pressure. Two studies from Brown et al. demonstrated the relationship between the worm *Cambarincola ingens* and crayfish *Cambarus chasmodactylus* varied with density (2002, 2012). In this case, lowest densities were found to be commensal, intermediate densities produced the highest growth indicating a mutualism, while the maximum densities of *C. ingens* were shown to be weakly parasitic to *C. chasmodactylus* (Brown 2012). The researchers observed crayfish gill scarring by branchiobdellidans at high densities, and hypothesized that limited resources of detritus and epibionts was the mechanism for a shift towards parasitism (Brown 2012). A laboratory study by Lee et al. in 2009 observed context-dependence by utilizing low and high fouling pressures of epibiosis, and demonstrated a shift from commensalism to mutualism respectively. Currently, field and lab studies have only been

conducted on relatively few species of crayfish and branchiobdellidan worms, all of which represent lotic systems.

Mutualisms are an important factor in community structure and commonly support ecosystem defining foundation species (Hay et al. 2004). In reef systems, mutualisms alter fish diversity and trophic links (Hay et al. 2004 and references therein). Direct effects of branchiobdellidans on crayfish growth and survivorship could also translate into indirect effects on community structure and ecosystem-level processing. Crayfish are a strongly interacting species that facilitate multiple processes in aquatic and terrestrial environments; sediment and detrital processing (Creed and Reed 2004, Usio and Townsend 2001), community structure (Creed and Reed 2004, Usio and Townsend 2004), and pedoturbation/aeration (Stone 1993, Butler 2002, Richardson 1983). Presently, no studies have quantified the effect of branchiobdellidans on crayfish ecosystem-level processes.

The novelty of the crayfish-branchiobdellidan relationship model presents itself as a manipulative system to further describe cleaning symbioses. Complex effects of cleaners on their host client's growth, survivorship, as well as, ecosystem-level processes have currently not been evaluated in one model system. The content presented in this thesis observes the relationship between crayfish and branchiobdellidans between environmental context of habitat (surface water and burrows) and also indirect effects of a cleaner on host client behavior (ecosystem-level processing) in laboratory and field experiments.

Ch. 2) Habitat mediates the outcome of a cleaning symbiosis for a facultative burrowing crayfish

Introduction:

Symbiotic relationships are physiologically or morphologically integrated interactions between species that persist for the partners' lifespan (Bronstein 1994, Cushman and Beattie 1991). Conditional outcomes of symbiotic relationships are defined along a cost/benefit continuum between partners and are generally divided into four classes: mutualism (+/+), parasitism (+/-), commensalism (+/0), and competition (-/-). Symbiotic relationships can shift from one conditional outcome to another depending on the environmental context in which they occur (Heath and Tiffin 2007, Cheney and Cote 2005). For example, the presence/absence of herbivores determines the cost/benefit of the relationship between ants and the extra floral nectar-producing plants they protect (Bronstein et al. 2006) while nitrogen levels can mediate the outcome of plant/rhizobial interactions (Heath and Tiffin 2007).

Cleaning symbioses involve the removal of fouling agents (i.e. epibionts, ectoparasites, and necrotic tissue) from a host 'client' by a 'cleaner' (Poulin and Grutter 1996). The outcome of cleaning symbioses can be affected by the density of ectoparasites and degree of fouling. In coral reef systems, the density of host ectoparasites can mediate a shift from mutualism to parasitism between cleaning gobies and their client fish (Cheney and Cote 2005, Arnal et al. 2001). Shifts in the outcome of cleaning symbioses between crayfish and branchiobdellidan worms in freshwater systems can be mediated by organic fouling (Lee et al. 2009).

Branchiobedllidan worms (Annelida) are small ectosymbiotes that are found throughout the Holarctic region except, reportedly, in the Ural Mountains and Amur River drainage (Holt and Opell 1993, Gelder 1999). Branchiobdellidans are primarily associated with crayfish, but

have also been observed on freshwater isopods and crabs (Holt 1963, Hobbs and Figueroa 1958). Branchiobdellidans utilize crayfish as foraging habitat where they graze upon protozoans, algae, bacteria, diatoms, and other epibionts on the crayfish exoskeleton and gill chamber (Holt 1973b, Jennings and Gelder 1979, Gale and Procter 2011, Brown 2012). The relationship between branchiobdellidans and crayfish is generally considered obligate for branchiobdellidans as successful reproduction has only been reported on a live crustacean host, and worms are rarely found disassociated with a "host" (Young 1966, but see Holt 1973a).

The symbiotic relationship between crayfish and worms may shift from commensalism to mutualism as physical or biological fouling increases. In highly fouled environments, crayfish may benefit from the cleaning relationship via increased growth and reduced mortality (Brown et al. 2002, 2012, Lee et al. 2009), presumably due to the removal of epibionts and other fouling agents. The removal of fouling agents increases gill function in terms of ammonia excretion and gas exchange (Jennings and Gelder 1979, Brown et al. 2002, 2012).

Similar to other crustaceans (e.g. Bauer 1981), the environment a crayfish inhabits can influence the type and degree of fouling encountered. Many crayfish are capable of burrowing in terrestrial habitat and move between surface waters and subterranean habitat during different seasons and/or stages in their life (Hobbs 1981, Taylor 1983, Ilheu et al. 2003). Water quality in subterranean burrows is often anoxic, with high ammonia and little water exchange (Grow and Merchant 1980, C. Ames unpublished data). Thus burrows may represent a highly-fouled habitat where crayfish growth and survivorship are facilitated by branchiobdellidan worms, and those effects are dependent on the type, rather than degree, of environmental fouling.

Water in subterranean burrows is often high in inorganic solids (C. Ames unpublished data). While crayfish are ineffective in removing epibionts (Bauer 1998), that comprises the

main prey items of branchiobdellidans (Jennings and Gelder 1979, Gale and Proctor 2011), they are effective in removing inorganic particulates in the branchial chamber by the use of 'limb rocking' or the jostling of setobranch setae around gill filaments (Bauer 1998). Thus under conditions of high organic fouling, branchiobdellidans may play an important cleaner role in reducing epibiont build-up and increasing client crayfish growth and survivorship (Lee et al. 2009). However, under conditions of high inorganic fouling branchiobdellidans may have a reduced cleaner role, with client crayfish able to reduce inorganic fouling effects via physical behaviors.

Direct effects of branchiobdellidans on crayfish growth and mortality may also indirectly affect community structure and ecosystem-level processes. Symbiotic relationships have been cited as an important factor in community structure by facilitating ecosystem-defining foundation species (Hay et al. 2004). In marine reef systems, mutualisms can alter fish diversity and trophic links (Hay et al. 2004 and references therein). In freshwater and wetland systems, crayfish are strongly interacting species capable of influencing sediment and detrital processing (Creed and Reed 2004, Usio and Townsend 2001), community structure (Creed and Reed 2004, Usio and Townsend 2004), and soil disturbance and aeration (Stone 1993, Butler 2002, Richardson 1983). Costs/benefits of branchiobdellidans to crayfish may alter crayfish behavior and consequently, crayfish interactions on their environment. Presently, no studies have quantified indirect impacts of branchiobdellidans on ecosystem-level function via crayfish.

The primary objective of this study was to compare the nature of the branchiobdellidan-crayfish symbiosis in organically-fouled surface waters and inorganically-fouled underground burrows. We hypothesized that if branchiobdellidan worms increased crayfish growth and

survivorship, it would result in increased detrital processing (surface waters) or burrowing activity (subterranean environments).

Methods:

Experimental animals

We used Red Swamp Crayfish *Procambarus clarkii* (Girard, 1852) and the branchiobdellidan *Cambarincola spp.* as our model organisms. *Procambarus clarkii* is a common crayfish found throughout northern Mexico to Escambia County, Florida and north to southern Illinois and Ohio (Hobbs et al. 1989, Taylor et al. 2007). Red Swamp Crayfish typically inhabit lentic, swampy, surface water habitats with high organic content during wet seasons, and subterranean burrows during drought or summer months (Hobbs 1989, Correia and Ferreira 1995). As a strongly interacting species, *Procambarus clarkii* populations have induced significant changes in littoral communities in lakes and the disruption of farming equipment and levee operations during burrowing seasons (Gherardi and Acquistapace 2007, Correria and Ferreira 1995). *Cambarincola spp.* utilized in this study is a small (1.5 – 3mm) branchiobdellidan that was tentatively identified as *C. barbarae* (described hereafter as *Cambarincola spp.*), a worm only known to associate with *P. clarkii* only, and thought to be found throughout the host's range (Holt 1981). Characteristics used to identify the branchiobdellidan were body size and proportion, dental formula, prostate position/size, and penis shape (Holt 1981). Identification was also assisted by Bronwyn Williams, and specimens were deposited in the Auburn Museum of Natural History. *Cambarincola spp.* have only been recorded on external surfaces of *P. clarkii*, where they can occur at high densities (100+) on a single crayfish host (Holt 1981, C. Ames pers. obs.).

Experiment I: Subterranean habitat

Experimental animals were collected by trap and seine from earthen ponds in July 2011, at the E. W. Shell Fisheries Research Station of Auburn University, in Auburn, Alabama. Crayfish were immediately transported to the South Auburn Fisheries Research Station and held in an outdoor tank for 7 days until the initiation of the experiment. We removed all worms from 32 haphazardly-chosen crayfish (16 males and 16 females) by dipping crayfish in a 10% MgCl solution for 15 minutes (Brown et al. 2002). These worms were discarded and the crayfish retained for the experiment. Initial weight (blotted wet mass, BWM = 7-12.4 g) and length (carapace length, CL = 28.5-39.8 mm) were obtained for all crayfish. Crayfish of each gender were randomly assigned to one of two treatments: worms and no worms.

Branchiobdellidans for use in the experiments were collected from an additional 16 crayfish by the use of laboratory probes (Brown et al. 2012) and held in a common container. Within 24hrs of collection, branchiobdellidans were haphazardly chosen and placed on the ventral coxa of the walking legs of eight male and eight female crayfish (ten worms per crayfish). The number of worms placed on the experimental crayfish corresponded to the mean number of worms found on the body of field-collected crayfish (C. Ames, unpublished data). Crayfish in the no-worm treatment were given fake inoculations to mimic handling stress of crayfish in the worm treatment.

After inoculation, each crayfish was immediately transferred to an artificial burrowing chamber (ABC) modified from Stoeckel et al. (2011). ABCs were 66 cm L x 45.7 cm H x 4.4 cm W with adjustable groundwater levels, removable glass sides, and shades placed over the sides. A small surface arena was attached to the top of each burrowing chamber to provide room

for excavation of soil material and/or construction of chimneys while preventing escapement of the crayfish. All 32 chambers were filled with sandy loam (67.5% sand, 20% silt, and 12.5% clay) collected at the South Auburn facility, which was similar in composition to soils found near the *P. clarkii* collection ponds. Experimental groundwater consisted of artificial freshwater (AFW: 5 ml /L of 35 ppt saltwater, 0.05 g/L CaCO3, 0.05 g/L Na2CO3, 0.08 g/L CaCl) made up from reverse osmosis water to a final 0.5 ppt salinity and 12 ppm calcium**.** Groundwater was raised to just below the surface of the soil in each chamber for 24 hours to fully saturate the soil column. Crayfish were randomly assigned to chambers (one crayfish per chamber), and groundwater was gradually lowered over two weeks at a rate of ~1 cm / day to a depth of 15 cm below the soil surface. Preliminary runs showed that this gradual lowering of water was necessary to induce *P. clarkii* to burrow in the ABCs (C. Ames, pers. obs.). At the end of the two week drawdown period, we measured dissolved oxygen (DO), ammonia (total ammonia-N) and pH of the burrow water (Time 0) and then continued to monitor burrow water quality weekly for the next 8 weeks (9 measurement dates total) by the use of a YSI 55 DO probe (YSI Inc. Yellow Springs, OH), Symphony SP90M5 ammonia probe (VWR International LLC.) and pHTestr 30 for pH (Oakton Instruments Vernon Hills, Il). Total suspended solids (TSS), including inorganic (ISS) and organic (OSS) content, of burrow water were measured at the end of the study (week 8) following US EPA guidelines (US EPA 1993). Water for all measurements was collected by first penetrating the soil to the underwater burrow with a plastic 10 mL pipette, and then extracting burrow water by the use of 3/8”ID tubing connected to a syringe. Less than 50 ml of water was extracted from burrows at any given time to avoid depletion of burrow water (typically < 75%).

Burrow area within each chamber was measured weekly starting from Week 0. For each measuring event, shades were temporarily removed from the front and back and a red light was placed behind the chamber to light up the burrow. Tracing paper was taped to the front of the burrow chamber and the outline of the burrow traced. Tracings were scanned and imported into ImageJ 1.46 software for analysis of area. Total burrow area, and burrow area below the water line were recorded for each chamber.

The experiment was run for 8 weeks (Jul 26 – Sept 22, 2012) and all chambers were held at 25 ± 1.6^{o}C, the approximate water temperature of local burrow water (C. Ames, unpublished data). Crayfish were fed commercial sinking catfish feed (26 % protein; Cargill, Inc.) at a rate of 10% of body mass (approx. 3 pellets) three times a week. Pellets were dropped directly into the burrow water.

At the end of the experiment, crayfish were carefully removed from their burrows and immediately dipped in a 10% MgCl bath to collect worms. All crayfish were then individually rinsed above a 100µm filter to remove any remaining worms before taking measurements of BWM and CL. Following the dip, crayfish were sacrificed and the carapace removed to examine for worms in the branchial chamber.

To test for differences in BWM and TSS between treatments I used 1-way ANOVAs. Kruskal-Wallis tests were used for CL due to violations of normality. I tested for differences in burrow areas between treatments using repeated measures ANOVA. Any transformations of data are noted where applied. Minitab 16.1 was used for statistical analyses.

Experiment II: Surface water

In Oct-2011, *P. clarkii* individuals were collected, stored, worms removed, and infected with worms (worm treatment) or pseudo-infected (no-worm treatment) using the same methodology as for the subterranean habitat experiment. Eight males and eight female crayfish were randomly assigned to each treatment for a total of 32 crayfish.

After inoculation, each crayfish was randomly assigned to an experimental tank (one crayfish per tank). Eight additional tanks contained no crayfish, for a total of 40 experimental tanks. Experimental tanks were 16 quart containers (42.5 cm L x 30.2 cm W x 17.8 cm H) containing 3.6 L of AFW, 9 g of sand and 150 g (BWM) of organic material to facilitate organic epibiosis. Organic material consisted of vegetative detritus collected in August 2011 from a dried swamp that was a known habitat of *P. clarkii* at the E. W. Shell Fisheries Station, North Auburn Unit. Organic matter was then transported to the South Auburn Fisheries Research Station and placed in an outside 304.8 cm L x 60.96 cm W x 60.96 cm H trough containing aged tapwater. The organic material was soaked outdoors for a total of 4 weeks to allow for natural colonization by aquatic bacteria, microinvertebrates and macroinvertebrates.

All experimental tanks were assigned to random locations on a three-shelf rack and plumbed into a recirculation system. Water was slowly dripped into each chamber at a rate of 10 ml/hr. Out-flowing water from each tank drained into a common sump. Water was then pumped from the sump, through a 5 µm filter, and back through the tanks at the afore-mentioned rate. Container temperature was maintained at 22 ± 2.1 C for the 8 week trial period. Crayfish were fed the same ration as in the subterranean experiment.

Water quality was measured over the eight-week experiment from 4 randomly chosen tanks per treatment (inoculated, non-inoculation, no crayfish) each week. DO and pH levels were measured directly from the experimental tanks using the same instruments as for the

subterranean experiment (YSI 55 DO probe and pHTestr 30 probe).The ammonia probe used in the subterranean experiment malfunctioned at the beginning of the surface water experiment and I had to switch to using Tetra Easy Strips (Spectrum Brands, Inc.). I continued to use the Easy Strips throughout the remainder of the surface water experiment to keep methodology consistent within the experiment. TSS was analyzed at week 8from 250 mL samples extracted carefully from each chamber via syringe. Analyses of TSS, ISS, and OSS were conducted using the same protocols as in the subterranean experiment (EPA 1993).

Indirect effects of worms were evaluated in terms of detrital processing using three methods. First, prior to experiments, I collected recently senesced *Liquidambar styraciflua* (sweetgum) leaves, and soaked them in mesh bags for 2 weeks in the same outdoor troughs as the organic material (see previous description). Sweetgum is common around marshes and swamps in Alabama, and were abundant at the *P. clarkii* collection site. After the 2 week conditioning period, leafpacks (6.4 ± 0.1g BWM) were assembled by wrapping a rubber band around the stems of 5 leaves. One leafpack was placed in each experimental tank at week four, rather than at the beginning of the experiment, to ensure that they were not completely broken down by the end of the experiment. At week 8, leaf packs were removed from each tank, and weighed (BWM). Leaf pack processing was quantified in terms of material lost (initial - final BWM).

Secondly, I assessed detrital processing by adding small (60 D ×15 H mm) Petri dishes to each tank at week 0, to catch processed detrital material suspended by crayfish activity. Petri dishes were removed at week 8, and accumulated material washed into a crucible with reverse osmosis water. Material was dried for 24hrs at 60 C to determine dry weight and then ashed for 1

hr at 550 C to determine organic vs. inorganic content (US EPA 1993). Results were expressed in terms of mg of settled material / cm^2 Petri dish surface area.

Third, I assessed detrital processing in terms of the amount of material remaining on the bottom of each tank at the end of the experiment. All tanks were drained carefully through a 250 µm filter by the use of a siphon. Any detrital material caught in the filter was added back to the tank and all tank detritus was then washed through a 250µm filter. Material that was retained on the filter was measured for BWM.

Crayfish were removed from tanks just before draining (see previous sections) and CL and BWM recorded for each individual. The carapace (cephalothorax) was removed from each individual and dipped separately in a 10%MgCl solution to collect branchiobdellidan worms. All gills were then excised from the remaining cephalothorax and held in a 20µm filter cup sitting in a 10% MgCl bath for at least 10 minutes. The remaining abdomen was then dipped in the same MgCl solution as for the carapace in order to determine number of worms on the entire exoskeleton (cephalothorax plus abdomen, including chelipeds and periopods). Any worms collected from the exoskeleton were viewed under a dissecting scope, counted, and stored in a 90% ethanol solution. Gills in the filter cup were rinsed with 90% ethanol and all material remaining on the 20 µm filter was rinsed into a vial and stored for later worm counts using a dissecting microscope.

To test for differences between treatments for BWM and CL of crayfish, leaf packs, detrital processing, suspended solids, and amounts of material in Petri dishes, I used general linear models. Transformations for test assumption violations are noted where applied. All analyses were conducted with Minitab 16.1.

Habitat Comparisons

After the completion of the surface water experiment, I compared the water quality data between the two habitats (burrow water and surface water mesocosms). Ammonia concentrations were compared by individual Kruskal-Wallis tests due to violations in normality. Dissolved oxygen, pH, were compared by individual 1-way ANOVAs on each sample date over the 8 week trial period. Total suspended solids were compared by 1-way ANOVA.

Gender

Upon observations of crayfish in the first experiment, trends in gender were noticeable on burrowing activity. This was not an original part of the experimental design, but we felt that effects of gender should be examined to further explain trends between treatment and ecosystem-level processes of both studies.

Results:

Experiment I: Subterranean habitat

Over the 8-week trial period crayfish demonstrated two burrowing strategies, with 47% of crayfish burrowing down to the water table (deep burrows) while 53% constructed only a shallow depression or burrow that did not reach the water table (shallow burrows). A Chi-square goodness of fit test revealed no differences from random in burrowing strategies for worm treatments or gender (treatment: $X^2(1, N = 32) = 0.125$, $p = 0.72$; gender $X^2(1, N = 32) = 1.129$, $p = 0.29$).

Growth and survivorship

Survivorship of crayfish was high within both treatments (worms 81%; no worms 86%) and genders (males 86%; females 81%). One hundred percent of deeply burrowing crayfish survived compared to 77% of shallowly burrowing crayfish.

Branchiobdellidans experienced high mortality in the burrows. Only 7% of worms survived the experimental period, yielding a final intensity of 0.8± 2 worms/inoculated crayfish compared to an initial intensity of 10 worms / inoculated crayfish (Figure 3). No worms were observed in the branchial chamber of any crayfish.

There was no difference in initial BWM (mean ±SD) of crayfish between treatments (worms: 8.5g ± 1.2; no worms: 9.2g ± 1.7; $F_{1, 31} = 1.6$, $p = 0.22$) or gender (males: 8.9g ± 1.5; females: 8.7g ± 0.7; $F_{1, 31} = 0.14$, $p = 0.72$), nor did crayfish differ in initial CL between treatments (worm: 34.1mm ± 1.7; no worms: 34.3mm ± 2.5; $F_{1, 31} = 0.05$, $p = 0.82$) or gender (males: 34.5mm ± 1.9; females: 33.9mm ± 2.3; $F_{1, 31} = 0.14$, $p = 0.71$).

There was no significant difference in crayfish growth between treatments as measured by change in BWM (worms: -0.2 ± 0.4 g; no worms: 0.1 ± 0.7 g; $F_{1,26} = 1.23$ $p = 0.278$) (Fig. 4a) or CL (worms: 0.3 ± 0.5 mm; no worms: 0.4 ± 0.7 mm; Kruskal-Wallis: H = 0.2, 1 d.f., $p = 0.654$) (Fig. 4b). However, crayfish growth did differ significantly between burrowing strategies with deep burrowers exhibiting a greater positive change in BWM (deep: 0.4 ± 0.5 g; shallow: -0.5 ± 0.3 g; $F_{1,26} = 28.22$, $p< 0.0001$), and CL (deep: 0.6 ± 0.7 mm; shallow: 0.0 ± 0.1 mm; Kruskal-Wallis: H = 8.50, 1 d.f., $p = 0.004$) than shallow burrowers.

Burrow area

Throughout the experimental period, total burrow area of crayfish in the no-worm treatment was greater than that of crayfish in the worm treatment, but this difference was only marginally

significant ($F_{2,277}$ = 3.47 p = 0.06) (Fig. 5a). Underwater burrow area could only be quantified for deeply burrowing crayfish, and did not differ significantly between treatments ($F_{2,131}$ = 0.16 p = 0.69) (Fig. 5b).

Total burrow area did not vary significantly between genders ($F_{2,277}$= 0.10, p = 0.76; Fig. 5c), but underwater burrow area for deeply-burrowing crayfish was significantly greater for female than for male crayfish ($F_{2,131}$ = 39.84, p< 0.0001; Fig. 5d) with a significant interaction between gender and time ($F_{2,131}$ = 5.59, p< 0.0001) (Fig. 5d). Females initially produced significantly smaller underwater burrow areas than males during weeks 0-2, but subsequently produced larger underwater burrow areas (weeks 3-8) (Fig. 5d).

Experiment II: Surface water habitat

Survivorship and growth

Over the 8 week experimental period crayfish mortality was negligible with 98% of crayfish surviving the trial period. Two crayfish escaped from their respective tanks during the experiment. Final abundance of branchiobdellidans on inoculated crayfish (52 ± 37 worms / crayfish) was significantly higher than either initial abundance or final abundance in the burrows (Fig. 3). No branchiobdellidans were found on non-inoculated crayfish at the end of the experiment or in the branchial chambers of any crayfish.

No significant differences were observed in initial BWM between treatments (mean ± SD) (worms: 12 ± 1.2 g; no worms: 11.3 ± 2 g; $F_{1,31}$ = 0.91 p = 0.35) or gender (males: 11.7 ± 1.8 g; females: 11.7 ± 2.1 g; $F_{1,31}$ = 0.00 p = 0.99), nor did initial CL differ between treatment (worms: 37.3 ± 2 mm; no worms: 37.0 ± 2.2 mm; $F_{1,31}$ = 0.19 p = 0.67) or gender (males: 36.8 ± 2.1 mm; females: 37.5 ± 2 mm; $F_{1,31}$ = 0.88 p = 0.36).

There was a significant difference in crayfish growth between treatments as measured by change in BWM (worms: 9.4 ± 4.7 g; no-worms: 5.8 ± 4.2 g; $F_{1,28} = 4.81$, $p = 0.037$) (Fig. 4b), but not in terms of CL (worms: 7.3 ± 4.3 mm; no-worms: 4.5 ± 4.2 mm; $F_{1,28} = 3.12$, $p = 0.084$) (Fig. 4d). No correlation was observed between final branchiobdellidan intensity and final crayfish BWM across a final range of 6 – 125 branchiobdellidans per treatment crayfish (R^2 =7.8 $F_{1,10} = 0.72$, $p = 0.419$. Ninety percent of crayfish molted at least once during the experiment.

Detrital processing

There was no significant difference in final BWM of bottom detritus between worm and no-worm treatments ($F_{2,36} = 33.54$, $p = 0.155$; Fig. 6a). However, control tanks (crayfish absent) had significantly higher final BWM of detritus than either worm treatment ($F_{2,36} = 33.54$, $p< 0.0001$; Fig. 6a). Similarly, there was no significant difference in final BWM of detritus between male and female crayfish tanks ($F_{2,36} = 30.21$, $p = 0.545$), but control tanks (crayfish absent) had significantly higher final BWM of detritus than either crayfish gender ($F_{2,36} = 30.21$, $p< 0.0001$; Fig. 6b).

Crayfish with worms processed significantly more material than control tanks (no crayfish) in terms of leaf pack loss (rank-transformed: $F_{2,36} = 9.25$,$p = 0.001$), and marginally ($p = 0.07$) more than crayfish without worms (Fig. 6c). No significant differences in leaf pack processing were found between crayfish genders ($F_{2,36}= 0.36$, $p = 0.519$), but both genders processed significantly more leaf pack material than control tanks ($F_{2,36}= 0.006$; Fig. 6d).

Significantly more shredded material settled in crayfish tanks than in control (no crayfish) tanks ($F_{2,28} = 15.36$, $p< 0.0001$), but settled shredded material did not differ between worm and no-worm treatments ($F_{1,20} = 0.07$, $p = 0.801$) (Fig. 6e). Settled shredded material was

significantly higher in male tanks compared to females ($F_{1,20} = 5.05$, $p = 0.037$) and significantly lower in control chambers ($p = 0.001$) (Fig. 6f).

Water Quality Between Habitats

The subterranean habitat had significantly higher ammonia concentrations throughout the experimental period (Weeks 0-8) compared to surface water habitats, with a considerable increase in ammonia after week 2 (Table 1; Figure 1a). Dissolved oxygen was generally higher in the subterranean experiment with significant differences in concentrations in weeks 1, 4, and 5 (Table 1; Fig 1b). Potential hydrogen (pH) was variable for both experiments throughout the trial period, typically staying circumneutral (Table 1; Fig. 1c). Suspended solids were significantly higher in the subterranean habitat compared to surface water habitat in terms of TSS ($F_{1,50} = 326.33$ $p< 0.0001$), ISS ($F_{1.50} = 402.16$ $p< 0.0001$), and OSS ($F_{1.50} = 144.5$ $p< 0.0001$). There were significant differences in inorganic/ organic ratios between burrow water and surface water ($F_{1.50} = 74.21$ $p < 0.0001$). Burrow waters were dominated by ISS whereas surface waters were dominated by OSS (Fig. 2).

Discussion:

Conditional Outcomes

Similar to previous research (Lee et al. 2009), I observed a mutualistic relationship between crayfish and branchiobdellidan symbionts in the surface water mesocosms characterized by high organic fouling. Crayfish with worms grew significantly more, in terms of BWM, than crayfish without worms, and branchiobdellidan worms increased in intensity (# of worms per host) during the course of the experiment. Contrary to previous studies (Brown et al. 2002), there did not appear to be a shift from mutualism to parasitism at high branchiobdellidan intensities.

The mechanism(s) by which branchiobdellidans provide benefits to, or incur costs from, crayfish are not entirely clear and may vary among crayfish and branchiobdellidan taxa. Different species of branchiobdellidans occupy different micro-habitats on the crayfish (Brinkhurst and Gelder 2001). Previous studies have focused on branchiobdellidans (*Cambarincola ingens*, *Branchiobdella kobayashi*) that foraged in the branchial chamber and likely cleaned the gills at intermediate densities or consumed gill tissue at high densities (Brown et al. 2012, Lee et al. 2009). However, gill analysis of *P. clarkii* in our study revealed no worms in the branchial chamber; with worms primarily inhabiting the ventral base of the coxa and dorsal rostrum. The absence of worms in the gill chamber suggests that the model cleaner in this study benefits crayfish from a currently unknown mechanism.

The removal or reduction of epibionts by branchiobdellidans may reduce the costs associated with the anti-fouling mechanisms of the crayfish (e.g. molting, grooming) (Bauer 1998, 2002, Lee et al. 2009). Carapace dwelling branchiobdellidans foraging near the branchial chamber and removing epibionts near the entrance of the chamber might provide benefits (mutualism) by increasing water flow to the gills (Brown et al. 2002) while not feeding directly on the gills (parasitism) at high intensities.

Contrary to our expectations, results of this study showed that crayfish lost the benefits of a mutualistic relationship with branchiobdellidan worms when moving to burrows as water receded below the soil surface. Crayfish in the subterranean experiment exhibited little to no growth regardless of the initial presence/absence of worms. The differences in BWM between crayfish that burrowed deeply versus those that stayed near the soil surface was likely due to desiccation of shallow burrowers since all crayfish exhibited little to no change in CL during the experiment. Branchiobdellidans suffered high mortality in all burrowing chambers. Few

crayfish had retained any worms by the end of the experiment. Thus a shift to underground habitat resulted in a virtual lack of growth by crayfish, and subsequent loss of the symbiotic relationship by mortality of worms, rather than a behavioral shift in the outcome. Burrow water represented a different fouling environment compared to surface waters tested but the mechanism leading to high branchiobdellidan mortality is unclear. Ammonia levels were significantly higher in the burrows. However, even the high ammonia levels observed in this experiment were not likely to have caused the observed branchiobdellidan mortality. A dose response study with ammonia showed no significant differences in mortality between controls (0 mg/L) to concentrations as high as 40 mg/L (see Chapter 3). Branchiobdellidan mortality in subterranean burrows was also not likely to have been caused by low DO, since worms thrived in the surface waters even though DO levels were lower than in the burrow water. Branchiobdellidans were also found to be tolerant of low DO in a previous study (Berry and Holt 1959). Suspended solids were considerably higher in burrow water than surface waters and dominated by inorganic (non-food) particles. However, since branchiobdellidans are not filter feeders, mortality was not likely caused by the prevalence of inorganic particles.

Another possible explanation for branchiobdellidan mortality in burrows is desiccation. Crayfish were frequently observed exiting the flooded portion of the burrows for extended periods of time during which branchiobdellidans were exposed on the carapace. Branchiobdellidans have been shown to survive > 45% water loss and are more tolerant to desiccation than other annelids (Yoder et al. 2007), thus they should have been somewhat tolerant of periodic emersion. However, I did not quantify the amount of time crayfish spent out of burrow water, and it is unknown how quickly exposed branchiobdellidans lost water in the humid burrows. Tolerance of desiccation appears to be size dependent and our species

(*Cambarincola spp.*) was a similar size to the smaller species (*C. fallax*) used by Yoder et al (2007). Unless branchiobdellidans took refuge in the branchial chamber (not observed from gill analysis in the surface water study), the small size of *Cambarincola spp.* may have led to desiccation during the burrowing process or while the crayfish temporarily left the flooded portion of the completed burrow. Further investigation into branchiobdellidan tolerance of desiccation, particularly in a humid environment, is needed.

Ecosystem-level effects

Many crayfish species are considered ecosystem engineers (Usio 2000, Creed and Reed 2004), and I hypothesized that a mutualistic relationship between crayfish and worms would increase the detrital processing and burrowing activity by crayfish. Interestingly, crayfish gender appeared to have a more consistent and stronger effect on burrowing activity than the crayfish/worm symbiosis, whereas in surface waters, branchiobdellidan infected crayfish significantly processed more detrital material when compared to controls. In surface waters, significantly higher amounts of detritus were processed when crayfish were present as compared to non-crayfish controls in two of three measured variables (container detritus and settled material). However, non-infected crayfish did not significantly process more leaf pack material than control containers. This suggests that without the presence of worms, the effect of crayfish leaf processing is no different than the complete absence of crayfish in the environment. Gender seemed to affect crayfish activities in both surface water and subterranean environments. In surface waters, significantly more material settled out of the water column in male tanks than in female tanks indicating males were foraging more than females. In the burrowing chambers, female crayfish excavated significantly larger burrows below the groundwater level than did

male crayfish. Greater excavation by females may be a behavior related to reproductive cycles in *P. clarkii*, as this species often produces broods in burrows.

Further directions

Facultative burrowing species such as *P. clarkii* do not spend the majority of their life span underground, and must make a choice at some point to forgo the benefits of food resources and mutualist symbionts in surface waters in order to reduce the risk of desiccation as surface waters recede. Once underground, they may slow their metabolic processes in a temporary state of hibernation until favorable conditions permit emergence and repopulation of surface waters (Powell and Watts 2010). This physiological adaptation to moist, but otherwise adverse underground conditions would compensate for the loss of the mutualistic relationship in surface waters.

An open question is whether or not the loss of the surface water mutualism is a selective pressure that favors reduced metabolic activity underground by facultative burrowing crayfish species. In contrast to *P. clarkii*, primary burrowing crayfish species spend the majority of their lives underground and remain active in that environment. Primary burrowing species such as *Cambarus striatus* and *Cambarus harti* collected directly from burrows have frequently been observed to host branchiobdellidan worms (Ames and Helms, pers. obs.). Future research is needed to determine whether some branchiobdellidan species are well-adapted to life underground and if primary burrowing crayfish species gain the same benefits of mutualistic symbiotic relationships that have been documented on secondary facultative burrowers in surface waters.

Ch. 3) No worm's land: Mechanisms driving high mortality of branchiobdellidans in natural environments remain elusive.

Introduction:

Branchiobdellidae (Annelida: Oligachaeta) is a family of small cleaning, symbiotic worms that live in association with crayfish and few other freshwater astacoid decapods (Bishop 1968, Brinkhurst and Gelder 2001). Branchiobdellids are found throughout North America, and the Holartic region of Europe and Asia (Holt and Opell 1993, Gelder 1999). Historically, branchiobdellidans were thought of as commensals or facultative parasites of crayfish, but are now known to be conditional mutualists (Bishop 1968, Keller 1992, Lee et al. 2009, Brown et al. 2012). Branchiobdellidans benefit by utilizing crayfish as sites for reproduction and by feeding on epibionts that accumulate on the exoskeleton and gills of crayfish (Young 1966, Holt 1973b, Jennings and Gelder 1979, Gale and Procter 2011, Brown 2012). Crayfish infested by branchiobdellidan worms may experience increased growth and survivorship, presumably due to the removal of epibionts, and other organic fouling agents (Lee et al. 2009, Brown et al. 2012). Similar to other cleaning symbioses (see Bronstein 1994, Cheney and Cote 2005), the nature of the relationship between crayfish and branchiobdellidans is mediated by environmental conditions (Lee et al. 2009). In stream crayfish, the relationship in some cases shifts from commensalism to mutualism as the environment becomes fouled by organic material (Lee et al. 2009). For burrowing crayfish, the mutualistic relationship may be sacrificed as crayfish move from surface waters to subterranean burrows. In a recent laboratory study, crayfish in surface water mesocosms were found to host > 100 branchiobdellidans on a single crayfish, whereas in subterranean burrows, branchiobdellidans experienced heavy mortality (Chapter 2). It is unknown whether this underground mortality was due to artificial conditions in the lab, or is

typical of a shifting relationship between the *P. clarkii* - *Cambarincola spp.* relationship in natural populations.

The subterranean habitat of crayfish is a characteristically harsh environment compared to surface waters. Water quality in subterranean burrows is often anoxic, with high ammonia, high suspended solids, and little water exchange (Grow and Merchant 1980, C. Ames unpublished data). In Chapter 2, water quality parameters were compared between subterranean and surface water environments. Dissolved oxygen concentrations were lowest in surface waters ($\geq$ 1 mg/L), but both environments remained above stressful limits (>1 mg/L) of the crayfish species tested (Bonvillain et al. 2012) (Chapter 2). Ammonia concentrations were significantly higher in burrow water (mean = 10 mg/L, max = 30 mg/L), whereas surface waters exhibited no detectable ammonia (> 0.01 mg/L) (Chapter 2). Suspended solids were also significantly higher (mean 895 mg/L) in burrows and dominated by inorganic suspended solids compared to surface waters (mean = 2.5 mg/L) which were dominated by organic material (Chapter 2).

High branchiobdellidan mortality in burrows may have been due to adverse water quality conditions. However, environmental tolerances of branchiobdellidans are poorly understood. Only one paper has been published regarding the oxygen and temperature tolerances of branchiobdellidans (Berry and Holt 1959). My primary objectives in this study were to (1 determine whether branchiobdellidans experience high mortality in natural crayfish burrows and (2 test branchiobdellidan tolerance of water quality parameters found to be different between natural burrows and surface waters. I hypothesized that differences in water quality and branchiobdellidan survivorship between natural habitats in this field study would support patterns of the laboratory study (Chapter 2), and that differences in water quality between burrows and surface waters could explain observed patterns of branchiobdellidan mortality.

Methods:

Experimental Animals

I used *Procambarus clarkii* as my model organism. Common throughout the Southeastern United States, *P. clarkii* typically inhabits lentic, swampy surface waters during the wetter seasons, and is a facultative burrower, retreating to subterranean burrows during drought or summer months (Hobbs 1989, Correia and Ferreira 1995). The branchiobdellidan, *Cambarincola barbarae*, is only known to associate with *P. clarkii*, and is thought to be found throughout the host's range (Holt 1981). Relatively small (1.5 – 3mm), *Cambarincola spp.* can occur at high densities (100+) on a single crayfish host (Holt 1981, C. Ames pers. obs.).

Experiment I: Field Study

Experimental animals (> 500) were collected by draining earthen ponds in June 2012, at the E. W. Shell Fisheries Research Station of Auburn University, in Auburn, Alabama. Crayfish were immediately transported in coolers and held in a large flow through tank for 7 days until the initiation of the experiment. Upon the initiation of the experiment, 30 crayfish were haphazardly subsampled for initial carapace length (CL ± SD; 34.5 ± 4.3mm), blotted wet mass (BWM ± SD; 9 ± 4g), and worm count (mean ± SD; 55 ± 31 worms/crayfish). Worms on the subsampled crayfish were counted by dipping crayfish into jars containing 10% MgCl solution for ten minutes, and the solution poured through a 200μm filter (Brown et al. 2012). Worms remaining on the filter were counted under a dissecting microscope at 20x magnification. The ratio of males to females was 1.5 M: 1 F. The remainder of the collected crayfish were then distributed 6 at a time to six earthen ponds, for a cumulative density of 87 crayfish/pond.

Previous to the start of the experiment, each earthen pond (~ 0.008 ha) had been drained and dried for two weeks and any resident crayfish removed. Ponds were refilled 24hrs prior to

the start of the experiment to a maximum depth of 90cm in preparation for crayfish stocking. After crayfish were stocked, 3 of the six ponds were slowly drained over a period of two weeks to induce burrowing. Preliminary laboratory runs showed that this gradual lowering of water was necessary to induce *P. clarkii* to burrow and reduce mortality (C. Ames, pers. obs.). After the drawdown period, I measured dissolved oxygen (DO), pH, temperature, and ammonia (NH_3) in the surface waters of the three filled ponds, and the burrow water of the three drained ponds every two weeks for 5 sample dates over 10 weeks. DO and ammonia were measured using a YSI 55 DO probe and YSI 9300 photometer (YSI Inc. Yellow Springs, OH) respectively. I used pHTestr 30 (Oakton Instruments Vernon Hills, IL) to measure pH, and an alarm digital thermometer (Pentair Aquatic-Ecosystems Apopka, Fla) to measure temperature. Total suspended solids (TSS), including inorganic (ISS) and organic (OSS) content, of water samples were measured at the end of the study (week 10) following US EPA guidelines (US EPA 1993).

To sample surface water ponds, a rope was run across the middle of the pond lengthwise and flags were hung at three equally spaced intervals. Water quality data was collected at each flag site from a kayak so as not to disturb the pond bottom. Measurements of DO and temperature were taken at ~ 2.5 cm from the pond bottoms. To sample ammonia, and suspended solids, 100 – 150 mL of water was collected at ~ 2.5 cm from the pond bottom using a plastic pipette and syringe. Ammonia was measured in sample water within 1 hr of collection. TSS samples were frozen for later analysis. To monitor burrow water quality in drained ponds, I first searched for three active burrows (indicated by signs of recent excavation) in different reaches of the pond at each sample date. After sampling, each burrow was flagged so it would not be re-sampled. Temperature was measured by inserting the temperature probe down a 3/8” tube inserted into the burrow until it reached the bottom. DO and pH were measured in the field by

inserting a 10mL pipette attached to 3/8" tubing and a syringe to just off the bottom of the burrow and extracting 100mL of water. After measuring DO and pH, the sample was returned to the lab for ammonia analysis within 1 hr of collection. TSS samples were frozen for later analysis.

The experiment ran for 10 weeks (Jun 28 – Aug 22, 2012). At the end of the experimental period, surface water ponds were drained and crayfish collected from all ponds (≥4 crayfish/pond) to obtain BWM, CL, and worm intensity. Subterranean ponds were sampled by excavating flagged crayfish burrows. Surface water ponds were sampled by hand as they drained. All crayfish were taken immediately to the lab for analysis.

To test for differences in BWM, CL and TSS between treatments I used 1-way ANOVA. I tested for differences in temperature, DO, ammonia, and pH between treatments using repeated measures ANOVA. Any transformations of data are noted where applied. Minitab 16.1 was used for statistical analyses.

Experiment II: Environmental Tolerances

Ammonia Dose Response

To obtain branchiobdellidans for the ammonia dose response assay, crayfish were collected in March 2011 by traps from ponds at the E. W. Shell Fisheries Research Station of Auburn University, in Auburn, Alabama. Crayfish were immediately transported to South Auburn Fisheries Research Station and held in a common tank. Branchiobdellidans for use in the experiments were collected from crayfish by the use of laboratory probes (Brown et al. 2012) and held in a common container. Within 24hrs, worms were placed randomly in 1 of 5 ammonia concentrations; 0, 10, 20, 30, and 40 mg/L based on the range observed in the laboratory burrow study. For consistency with previous lab experiments, treatment concentrations were made by

adding ammonia to artificial freshwater (AFW: 5ml of 35ppt saltwater, 0.05g CaCO3, 0.05g Na2CO3, and 0.08g CaCl per liter of reverse osmosis, deionized water for a final hardness of 50mg/LCaCO3). Based on previous pH concentrations observed in artificial burrows (~6.9 pH), assay water was adjusted to a pH of 7 with HCl. Ammonia concentrations were monitored using a Symphony SP90M5 ammonia probe (VWR International LLC).All cups were held at room temperature (22-24C). Each treatment was comprised of 5 replicate 150mL plastic cups that held 150 ml of assay water and 10 worms each. Experimental design and analysis were based on methods described for dose response studies by Newman and Unger (2003).

The assay was run for 48 hrs (Mar 24 – Mar 25, 2011), with assay cups randomly distributed on a table top. Mortality was monitored at 24 and 48hrs, and assay water was replaced at 24hrs to maintain consistent water quality and ammonia concentrations. Mortality was confirmed by a method similar to that employed by Berry and Holt (1959). A probe was inserted into the container and worms were considered dead if they did not become stimulated by prodding. Dead worms were immediately removed with tweezers (Berry and Holt 1959).

To test for differences in survivorship between ammonia concentrations I used 1- way ANOVA. Minitab 16.1 was used for statistical analyses.

pH Dose Response

Branchiobdellidans were obtained for the pH dose response assay by collecting crayfish in early May 2013 with traps in ponds at the E. W. Shell Fisheries Research Station. The methods for the storage of crayfish, collection of branchiobdellidans, and experimental design are similar to that mentioned in the ammonia dose response study. Worms were placed randomly in 1 of 4 pH treatments: 6.5, 7.5, 8.5, and 9.5. Potential hydrogen concentrations were adjusted by adding HCl or NaOH to AFW. I measured pH every 12hrs for the duration of the experiment using a HI

9813-6 pH probe (Hanna Instruments Woonsocket, RI). The treatment levels were based on the range of pH observed in burrows and surface waters of the field experiment (see results, Chapter 3). Treatments consisted of 3 replicate 200mL glass containers that each contained 250 ml of assay water and 10 worms.

The experiment ran for 48 hrs (May 14 – May 15, 2013) with assay cups randomly distributed on a lab table top and held at room temperature (20.8 – 25.5 C). Water was changed every 24 hrs, and survivorship was evaluated at 24 and 48hrs in the same manner as for the ammonia dose response study.

To test for differences in survivorship I used 1 – way ANOVA. Minitab 16.1 was used for statistical analyses.

Results:

Experiment I: Field Study

Water Quality

Significant differences ($p < 0.0001$) were found between DO, ammonia, pH, and temperature of surface water and burrow habitats for duration of the experiment. Daytime dissolved oxygen was significantly higher in surface waters than burrow water, typically staying 10 mg/L higher than subterranean burrows during the experiment (Table 2; Fig. 7a). Ammonia concentrations varied throughout the experimental period for both experiments (Table 2; Fig. 7b). Subterranean burrow ammonia concentrations rose over the first 3 sample periods (6 weeks) and declined over the next 2 sample dates (4 weeks) (Table 2; Fig. 7b), while surface water ponds had a single peak in ammonia at sample date 2 (week 4) (Table 2; Figure 7b). Average pH remained below 7.0 for subterranean burrows throughout the study while average

pH remained above 8.0 for surface waters and was variable among surface water ponds (Table 2; Fig.7c). Further analysis of surface water ponds showed significant differences in average pH values among ponds (1 – way ANOVA: $F_{2.42} = 33.35$ $p < 0.0001$). Subterranean burrow temperature was more stable and remained lower than surface water ponds (Table 2; Figure 7d). Suspended solids were significantly higher in subterranean burrows compared to surface waters in TSS (log transformed: $F_{1.17} = 222.52$ $p < 0.0001$), ISS (log transformed: $F_{1.17} = 211.86$ $p<0.0001$), and OSS (log transformed: $F_{1.17} = 98.61$ $p < 0.0001$) (Fig. 8). There were also significant differences in inorganic/ organic ratios between burrow water and surface water (log transformed: $F_{1.17} = 69.55$ $p < 0.0001$) (Fig. 8). Subterranean burrows were dominated by ISS whereas surface waters were dominated by OSS (Fig. 8).

Growth and Survivorship

Final size of surface water crayfish was significantly greater than burrow dwelling crayfish in terms of BWM (surface water: 26.2 ± 11.1 g; subterranean burrows: 12.0 ± 3.6 g; $F_{1.39} = 29.83$ $p < 0.0001$) and CL (surface water: 47.8 ± 6.4 mm; subterranean burrows: 37.6 ± 3.7 mm; $F_{1.39} = 37.46$ $p < 0.0001$).

As expected, worms in burrows experienced heavy mortality. Initial worm intensity (55 ± 31 worms per crayfish) had declined to 9 ± 7 worms per crayfish by the end of the 10 week study period: a decrease of approximately 84% in mean intensity (Fig. 9). However, contrary to our expectations, worms also experienced heavy, though more variable, mortality in surface waters with a final intensity of 9 ± 19 worms per crayfish (Fig. 9). Subsequent comparison of surface water ponds showed that the two ponds with average pH values between 8.5 and 9 had significantly higher mortality than the pond with an average pH of 7.8 (Kruskal-Wallis: H = 16.37, 2 d.f., $p < 0.0001$) (Fig. 10).

Experiment II: Environmental Tolerances

Ammonia dose response

No significant differences were found in survivorship among ammonia concentration levels after 48 hrs of exposure (Fig. 11a). Final survivorship was > 90% for all concentrations tested (Fig. 11a).

pH dose response

No significant differences were found in survivorship among pH concentration levels after 48hrs of exposure, with the only deaths occurring in the nominal 6.5 pH treatment (Fig.11b). Average pH values remained near the nominal values during the assay (6.6 ± 0.3, 7.5 ± 0.2, 8.26 ± 0.1, and 9.7 ± 0.1).

Discussion:

This study supports the idea that subterranean burrows are a poor environment for *P. clarkii* and burrows induce significantly high mortality for its symbiotic partner: *Cambarincola spp.*. Previous studies suggest that poor water quality of subterranean burrows and the metabolic costs of burrowing and hibernation during drought periods lead to a reduction in growth of secondary burrowing crayfish such as *P. clarkii* and *P. zonangulus* (Powell and Watts 2010). In my laboratory (Chapter 2) and field studies (Chapter 3), burrow water quality was consistently poor in terms of low DO, high ammonia, and high inorganic TSS. Burrowed crayfish showed little to no growth compared to their surface water counterparts.

Initially, I hypothesized that the cleaning activity of branchiobdellidan worms would increase growth and survivorship of crayfish in the low quality, fouled, burrow water

environment. However, results of the lab study (Chapter 2) indicated that in fact, crayfish lost their beneficial relationship with worms when they transitioned to a burrowing environment. This conclusion is supported by my field study (Chapter 3) in which branchiobdellidan worms consistently experienced high mortality (mean = 84%) in natural burrows.

The mechanism behind this decline remains unclear and does not seem related to DO, ammonia, or pH. Branchiobdellidans seem very tolerant of low DO. In my laboratory study, DO in surface water mesocosms (1.0 – 2.0 mg / L) was lower than or equal to DO in burrow waters of both the lab and field studies, yet worms exhibited high survivorship and reproductive rates in the low DO environment of the surface water mesocosms. Ammonia levels were consistently higher in burrows than in surface waters of both lab and field experiments. However, my 48 hour assay showed > 90% survivorship across a wide range of ammonia levels (0 – 40 mg /L total ammonia-N). Branchiobdellidan mortality was also not likely driven by low pH. In the laboratory study, burrow water pH remained between 7 and 7.5. In the field experiment, burrow water dropped below 7, but my 48 hour assay showed high survivorship (>90 %) at both low (6.6 ± 0.3) and neutral (7.5 ± 0.2) pH levels. Because the burrow experiments lasted for ~8 weeks, it is possible that the 48 assays were not long enough to reveal chronic mortality associated with the tested water quality parameters. However the assays showed branchiobdellidans were tolerant of a wide range of ammonia and pH levels at least in the short term.

Although branchiobdellidans thrived in the surface water mesocosms of the lab experiment, the field experiment showed that they do not thrive in all surface waters all the time. Branchiobdellidan mortality was variable and high (33 – 100%) on crayfish among the three flooded ponds. The general pattern of high mortality in the ponds may have been due to high

temperatures (30 – 36 C), but the upper thermal tolerance of *Cambarincola spp.* is unknown at this time and needs to be investigated further. Seasonal trends in branchiobdellidan abundance may also explain our observations. In a study in a Texas pond by Young (1966), a similar species of branchiobdellidan (*Cambarincola vitrea*) was found to have peak densities in early summer months, but decline in late summer. The decline in branchiobdellidan numbers was thought to be caused by lower reproduction rates and survival during adverse conditions and crayfish seasonal molting (Young 1966). *Procambarus clarkii* has been observed molting throughout the spring and summer months, and molt intervals decrease with increasing temperature up to 30C (Penn 1943, Chen et al. 1995). In the field study, several exuviae of crayfish were observed in surface water ponds, and temperatures observed were within the ranged studied by Chen et al. (1995). The combination of seasonal decline in branchiobdellidan reproduction and crayfish molting could be the mechanism behind low worm densities at the collection date.

More puzzling is the variability in worm mortality among ponds. Because the two ponds with the highest (100%) mortalities also exhibited unusually high pH (>8.5) levels for this region, I initially hypothesized that *Cambarincola spp.* was intolerant of high pH. However, worms exhibited 0% mortality over 48 hours at pH levels ranging from 7.5 to 9.7, indicating a tolerance of high pH conditions. The tolerance of branchiobdellidan worms to ranges in pH is most likely an adaptive feature when associating with a host that may commonly encounter these environmental contexts.

In summary, the relationship between *P. clarkii* and *Cambarincola spp.* is complex and seems dependent on a combination of habitat choice and water quality parameters. Results of this field study support our previous hypothesis that *P. clarkii* loses a potentially beneficial

symbiotic relationship with branchiobdellidan worms when it moves from surface waters to subterranean burrows, and also show that worm survivorship is not uniformly high in surface water environments. The mechanisms behind these patterns remain unclear. Assays examining effects of desiccation, high levels of inorganic TSS, and high temperatures on branchiobdellidan survivorship would be particularly useful in further investigations of the mechanisms behind high mortality events in burrow and surface water environments. Seasonal trends in surface water *Cambarincola spp.* population abundances would also be useful. As shown in previous research (Lee et al. 2009), the nature of the symbiotic relationship between crayfish and branchiobdellidan worms is context dependent – ranging from mutualism to parasitism to functional extinction (worm mortality). Crayfish and branchiobdellidans should be extremely useful model organisms for future studies examining mechanisms behind shifting symbiotic outcomes as well as the costs/benefits faced by host organisms when moving between two very different environments.

Ch. 4) Complexity in the conditional outcomes of the crayfish/branchiobdellidan symbiosis

Crayfish and branchiobdellidans are useful model organisms for evaluating shifts in parasitism, mutualism, and commensalism in cleaner symbioses, with the nature of the relationship a result of a) the species of branchiobdellidan, b) the intensity at which they occur, c) water quality and habitat choice (Holt 1973, Keller 1992, Lee et al. 2009, Brown et al. 2012). The diet and feeding location of branchiobdellidan species determine if their relationship with crayfish is parasitic, mutualistic, or commensal. Gill dwelling species are typically thought of as parasites (Holt 1973). These species (ex. *Bdellodrilus illuminatus*) ingest host gill tissue and/or feed on the host's blood (Holt 1973). Conversely, a carapace dwelling species was determined to be commensal across a range of densities (Keller1992). The proximity of the branchiobdellidan to the host's gill chamber may play affect the outcome of the relationship. In a study by Brown et al. (2012), branchiobdellidans inhabiting the crayfish exoskeleton were observed cleaning and feeding on crayfish gill filaments near the branchial chamber opening. The relationship followed a unimodal pattern with increasing worm intensity. Branchiobdellidans were found to be commensal at low densities, beneficial to crayfish growth and survival at medium densities, and parasitic at high densities (Brown et al. 2012). The outcome of the relationship can also change with changing fouling pressure. Under conditions of high organic fouling, crayfish were observed to benefit from branchiobdellidans, whereas under low fouling, the relationship was deemed commensal (Lee et al. 2009). Results observed from the research of this thesis support the idea that the crayfish/branchiobdellidan relationship is not static, but varies with environmental context.

Under laboratory conditions, branchiobdellidans benefited the crayfish by increased growth in surface water environments, compared to subterranean burrows. This was due to high

branchiobdellidan survival and reproduction in surface waters compared to subterranean burrows (see chapter 2). Factors limiting branchiobdellidan survivorship and reproduction in subterranean burrows are not clear, and further research into the differences found in this thesis are warranted (see chapters 2, 3).

Branchiobdellidans were never found to be parasitic even at high intensities (> 50 worms / crayfish) (see chapter 2). This finding supports previous studies where branchiobdellidans that did not infest in gill chambers or crayfish egg brood were commensal under low fouling conditions, and beneficial to crayfish under high fouling pressures (Holt 1973, Keller 1992, Lee et al. 2009).

What is truly puzzling from the results of this thesis is the mechanism(s) behind differential branchiobdellidan survivorship. Branchiobdellidan mortality was consistently high in subterranean burrows of both laboratory and field experiments, and variable between surface water experiments in the laboratory and field. Branchiobdellidans reproduced and flourished in the artificial surface water habitats, but experienced heavy mortality in the field experiment. Studies thus far on the crayfish/branchiobdellidan relationship have been focused on the benefit/harm to the crayfish host, and assume 'benefit' to the branchiobdellidan cleaner. The presence of the branchiobdellidan was beneficial to the crayfish host suggesting a beneficial relationship for crayfish (+), as well as an indirect ecosystem-level effect, though the heavy mortality of branchiobdellidans in burrows and field environments suggests a negative cost for the worm cleaner (-). This suggests that the relationship between *P. clarkii* and *Cambarincola spp.* is antagonistic (+/-) for most of the environments evaluated (Bronstein 2009).

If branchiobdellidans experience high mortality in subterranean and certain surface water environments inhabited by crayfish, how do they survive as a species? Reproduction and

feeding are thought to be the only factors pertaining to branchiobdellidan use of crayfish, though branchiobdellidans have been observed to live off of their crayfish host for extended periods of time (Young 1966, Holt 1973, Jennings and Gelder 1979, Gale and Procter 2011). In a study by Young, the branchiobdellidan *Cambarincola spp.* was observed to survive > 240 days off of the crayfish host, although the worms did not deposit cocoons except in the presence of a live crayfish host (1966). In the surface water experiment, branchiobdellidans were observed in high densities off of the crayfish host inhabiting the detritus of experimental containers. Young's and previous studies suggests that branchiobdellidans are nutritionally independent, but reproductively dependent upon crayfish for survival (1966, Holt 1973, Jennings and Gelder 1979, Gale and Procter 2011). Population estimates of branchiobdellidans have currently only been evaluated when observed on crayfish, and thus, may not represent all live worms in a given habitat (Young 1966). Based on this idea and previous observations, it could be deduced that branchiobdellidans may survive off of crayfish during crayfish inhabitation of unfavorable environments, or branchiobdellidans may maintain low densities until favorable conditions arise. Branchiobdellidans observed in the laboratory surface water study displayed remarkable population increases during the experimental period when under favorable environments (mean 400% increase). Though the population dynamics of branchiobdellidans are not well understood, it may be that these worms undergo a boom-crash seasonal cycle based on environmental conditions. Further investigation into the ability of branchiobdellidans to live off the crayfish host, branchiobdellidan population dynamics, and environmental factors mediating the survivorship of branchiobdellidans are warranted.

Literature Cited

Arnal C, Côté IM, Morand S (2001) Why clean and be cleaned? The importance of client ectoparasites and mucus in a marine cleaning symbiosis. Behav Ecol Sociobiol 51:1-7

Bauer RT (1981) Grooming behavior and morphology in the decapods Crustacea. J Crustac Biol 1:153-173

Bauer RT (1998) Gill-cleaning mechanisms of the crayfish *Procambarus clarkii* (Astacidae: Cambaridae): experimental testing of the setobranch function. Invertebr Zool 117:129-143

Bauer RT (2002) The ineffectiveness of grooming in prevention of body fouling in the red swamp crayfish, *Procambarus clarkii*. Aquaculture 208:39-49

Beneden PJV (1875) Les Commensaux et les Parasites dans le Régne Animal. Bibliothèque Scientifique Internationale, 9:1-232

Berry JW, Holt PC (1959) Reactions of two species of Branchiobdellidae (Oligocheata) to high temperatures and low oxygen tensions. Virg Agri Exp Sta, Virg Poly Inst, Tech Bull 141:1-11

Bishop JE (1968) An ecological study of the branchiobdellid commensals (Annelida-Branchiobdellidae) of some mid-western Ontario crayfish. Can J Zool 46:835-843

Bonvillain CP, Rutherford DA, Kelso WE, Green CC (2012) Physiological biomarkers of hypoxic stress in red swamp crayfish *Procambarus clarkii* from field and laboratory experiments. Comp Biochem Physiol A Mol Integr Physiol 163:15-21

Boucher DS, James S, Keeler KH (1982) The ecology of mutualism. Ann Rev Ecol Syst 13:315-347

Brinkhurst RO, Gelder SR (2001) Annelida: Oligochaeta and Branchiobdellida. In: Thorp JH, Covich AP (eds) Ecology and classification of North American freshwater invertebrates, 2nd edn. Academic Press, San Diego

Bronstein JL (1994) Conditional outcomes in mutualistic interactions. Trends Ecol Evol9:214 217

Bronstein JL, Alarcón R, Gerber M (2006) The evolution of plant-insect mutualisms. New Phyt 172:412-428

Brown BL, Creed RP, Dobson WE (2002) Branchiobdellid annelids and their crayfish hosts: are they engaged in a cleaning symbiosis? Oecologia 132:250-255

Brown BL, Creed RP, Skelton J, Rollins MA, Farrell KJ (2012) The fine line between mutualism and parasitism: complex effects in a cleaning symbiosis by multiple field experiments. Oecologia 170:199-207

Bshary R, Grutter AS (2002) Parasite distribution on client reef fish determines cleaner fish foraging patterns. Mar Ecol 235:217-222

Butler DR (2002) The environmental impact of crayfish biopedoturbation on a floodplain: Roanoke River, North Carolina Coastal Plain, U.S.A.. Land Anal 3:35-40

Chen S, Wu J, Malone RF (1995) Effects of temperature on mean interval, molting and mortality of red swamp crawfish (*Procambarus clarkii*). Aquaculture 131:205-217

Cheney KL, Côté IM (2005) Mutualism or parasistim? The variable outcome of cleaning symbioses. Biol Lett 1:162-165

Correia AM, Ferreira Ó (1995) Burrowing behavior of the introduced Red Swamp Crayfish *Procambarus clarkii* (Decapoda: Cambaridae) in Portugal. J Crustac Biol 15:248-257

Creed RP, Reed JM (2004) Ecosystem engineering by crayfish in a headwater stream community. J North Am Benthol Soc 23:224-236

Cushman JH, Beattie AJ (1991) Mutualisms: assessing the benefits to hosts and visitors. Trends Ecol Evol 9:193-195

Gale KSP, Proctor HC (2011) Diets of two congeneric species of crayfish worm (annelida:clitellata:branchiobdellidae) from western Canada. Can J Zool 89:289-296

Gelder SR (1999) Zoogeography of branchiobdellidans (annelida) and temnocephalidans (platyhelminthes) ectosymbiotic on freshwater crustaceans, and their reactions to one another in vitro. Hydrobiologia 406:21-31

Gelder SR, Rowe JP (1988) Light microscopal and cytochemical study on the adhesive and epidermal gland cell secretions of the branchiobdellid *Cambarincola fallax* (Annelida: Clitellata). Can J Zool 66:2057–2064

Gheradi F, Acquistapace P (2007) Invasive crayfish in Europe: the impact of *Procambarus clarkii* on the littoral community of a Mediterranean lake. Freshw Biol 52:1249-1259

Grabda E, Wierzbicka J (1969) The problem of parasitism of the species of genus *Branchiobdellida* Odier, 1823. Pol Arch Hydrobiol16:93–104

Grow L, Merchant H (1980) The burrow habitat of the crayfish, *Cambarus diogenes diogenes* Girard. Am Midland Nat 103:231-237

Grutter AS, Murphy JM, Choat JH (2003) Cleaner fish drives local fish diversity on coral reefs. Cur Biol 13:64-67

Hay ME, Parker JD, Burkepile DE, Caudill CC, Wilson AE, Hallinan ZP, Chequer AD (2004) Mutualisms and aquatic community structure: the enemy of my enemy is my friend. Annu Rev Ecol Evol Syst 35:175-197

Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial mutualists. Proc R Soc B 274:1905-1912

Hobbs HH III, Jass JP, Huner JV (1989) A review of global crayfish introductions with particular emphasis on two North American species (Decapoda, Cambaridae) Crustaceana 56:299-316

Hobbs HH Jr (1981) The crayfishes of Georgia. Smithson Contrib Zool 318:1-549

Hobbs HH, Figueroa AV (1958) The exoskeleton of a freshwater crab as a microhabitat for several invertebrates. Virg J Sci (N.S.) 9:395-396

Holt PC (1963) A new branchiobdellid. J Tenn Acad Sci 38:97-100

Holt PC (1973a) A free living branchiobdellid (annelida:clitellata)? Trans Am Micros Soc 92:152-153

Holt PC (1973b) A summary of the branchiobdellid (annelida:clitellata) fauna of Mesoamerica. Smithson Contrib Zool 142:1-40

Holt PC (1981) A resumé of the members of the genus *Cambarincola* (Annelida: Branchiobdellida) from the Pacific drainage of the United States. Proc Biol Soc Wash 94:675-695

Holt PC, Opell BD (1993) A checklist of and illustrated key to the genera and species of the Central and North American cambarincolidae (clitella: branchiobdellida). Proc Biol Soc Wash 106:251-295

Iléu M, Acquistapace P, Benvenuto C, Gheradi F (2003) Shelter use of the Red Swamp Crayfish (*Procambarus clarkii*) in dry-season stream pools. Archiv für Hydrobiologie 157:535-546

Jennings JB, Gelder SR (1979) Gut structure, feeding and digestion in the branchiobdellid oligocheate *Cambarincola macrodonta* Ellis 1912, and ecotosymbionte of the crayfish *Procambarus clarkii*. Biol Bull 156:300-314

Johnson NC, Wedin DA (1997) Soil carbon, nutrients, and mycorrhizae during conversion of dry tropical forest to grassland. Ecol App 7:171-182

Keller, TA (1992) The effect of the branchiobdellid Annelid *Cambarincola fallax* on the growth rate and condition of the crayfish *Orconectes rusticus*. J Fresh Ecol 7:165-171

Kropotkin P (1955) Mutual aid: A factor in evolution. Extending Horizons Books, Boston

Lee J, Kim T, Choe J (2009) Commensalism or mutualism: conditional outcomes in a branchiobdellid-crayfish symbiosis. Oecologia 159:217-224

Losey GS (1979) Fish cleaning symbiosis: proximate causes of host behaviour. AnimBehav 27: 669-685

Penn GH (1943) A study of the life history of the red-crawfish, *Cambarus clarkii* Girard. Ecology 24:1-18

Poulin R, Grutter AS (1996) Cleaning symbioses: proximate and adaptive explanations. Bioscience 46:512-517

Pound R (1893) Symbiosis and mutualism. Am Nat 27:509-520

Powell ML, Watts SA (2010) Response to long-term nutrient deprivation and recovery in the crayfishes *Procambarus clarkii* and *Procambarus zonangulus*: oxygen consumption and metabolic enzymes. J Wor Aqua Soc 41:81-91

Richardson AMM (1983) The effect of the burrows of a crayfish on the respiration of the surrounding soil. Soil Biol Bio Chem 15:239-242

Stoeckel JA, Helms BS, Cash E (2011) Evaluation of a crayfish burrowing chamber design with simulated groundwater flow. J Crustac Biol 31:50-58

Stone EL (1993) Soil burrowing and mixing by a crayfish. Soil Sci Soc Am J 57:1096-1099

Taylor CA, Schuster GA, Cooper JE, DiStefano RJ, Eversole AG, Hamr P, Hobbs HH III, Robison HW, Skelton CE, Thoma RF (2007) A reassessment of the conservation status of crayfishes of the United States and Canada after 10+ years of increased awareness. Fisheries 32:372-389

Taylor RC (1983) Drought-induced changes in crayfish populations along a stream continuum. Am Midland Nat 110:286-298

US EPA (1993) ESS method 340.2 total suspended solids. Env Sci Sec 3:186-193

Usio N (2000) Effects of crayfish on leaf processing and invertebrate colonisation of leaves in a headwater stream: decoupling of a trophic cascade. Oecologia 124:608-614

Usio N, Townsend CR (2004) Roles of crayfish: consequences of predation and bioturbation for stream invertebrates. Ecology 85:807-822

Wahl M (1989) Marine epibiosis I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser 58:175-189

Yoder JA, Tank JL, Brown BL, Hobbs HH III (2007) Water exchange pertaining to host attachment sites and stream preference in crayfish-associated branchiobdellids, *Cambarincola fallax* and *Cambarincola ingens* (Annelida:Clitellata). Hydrobiologia 592:523-533

Young W (1966) Ecological studies of the branchiobdellidae (oligocheata). Ecology 47:571-578

Table 1. Listed are comparison values of water quality parameters from chapter 2 over the 8 week experimental period. Ammonia comparisons were conducted by Kruskal-Wallis, and H values are presented with p values. Given for DO and pH are one-way ANOVA and p values.

	Ammonia		DO		pH	
	H	p	F	p	F	p
Week 0	16.27	< 0.0001	0	0.977	38.02	< 0.0001
Week 1	12.25	0.002	30.53	< 0.0001	1.02	0.321
Week 2	24.98	< 0.0001	1.58	0.25	4.9	0.036
Week 3	25.6	< 0.0001	0	0.967	9.05	0.005
Week 4	24.34	< 0.0001	16.61	< 0.0001	6.88	0.014
Week 5	25.59	< 0.0001	11.18	0.002	6.34	0.018
Week 6	18.63	< 0.0001	0.27	0.607	0.27	0.61
Week 7	23.48	< 0.0001	1.34	0.257	32.6	< 0.0001
Week 8	20.45	< 0.0001	2.08	0.162	12.91	0.001

Table 2. Listed are comparison values of water quality parameters from chapter 3 over the 10 week experimental period. Given for temperature, DO, ammonia, and pH are the mean values for the duration of the experiment, and repeated measures ANOVA F and *p* values.

	Habitat	Mean	SE	F	*p*
Temperature (C)	Surface Waters	33.3	0.5	196.07	< 0.0001
	Subterranean Burrows	27.1	0.2		
Dissolved Oxygen (mg/L)	Surface Waters	10.2	0.4	854.59	< 0.0001
	Subterranean Burrows	1.3	0.2		
Ammonia (mg/L)	Surface Waters	2.7	1.6	116.7	< 0.0001
	Subterranean Burrows	40.5	4.9		
pH	Surface Waters	8.5	0.1	247.27	< 0.0001
	Subterranean Burrows	6.7	0.0		

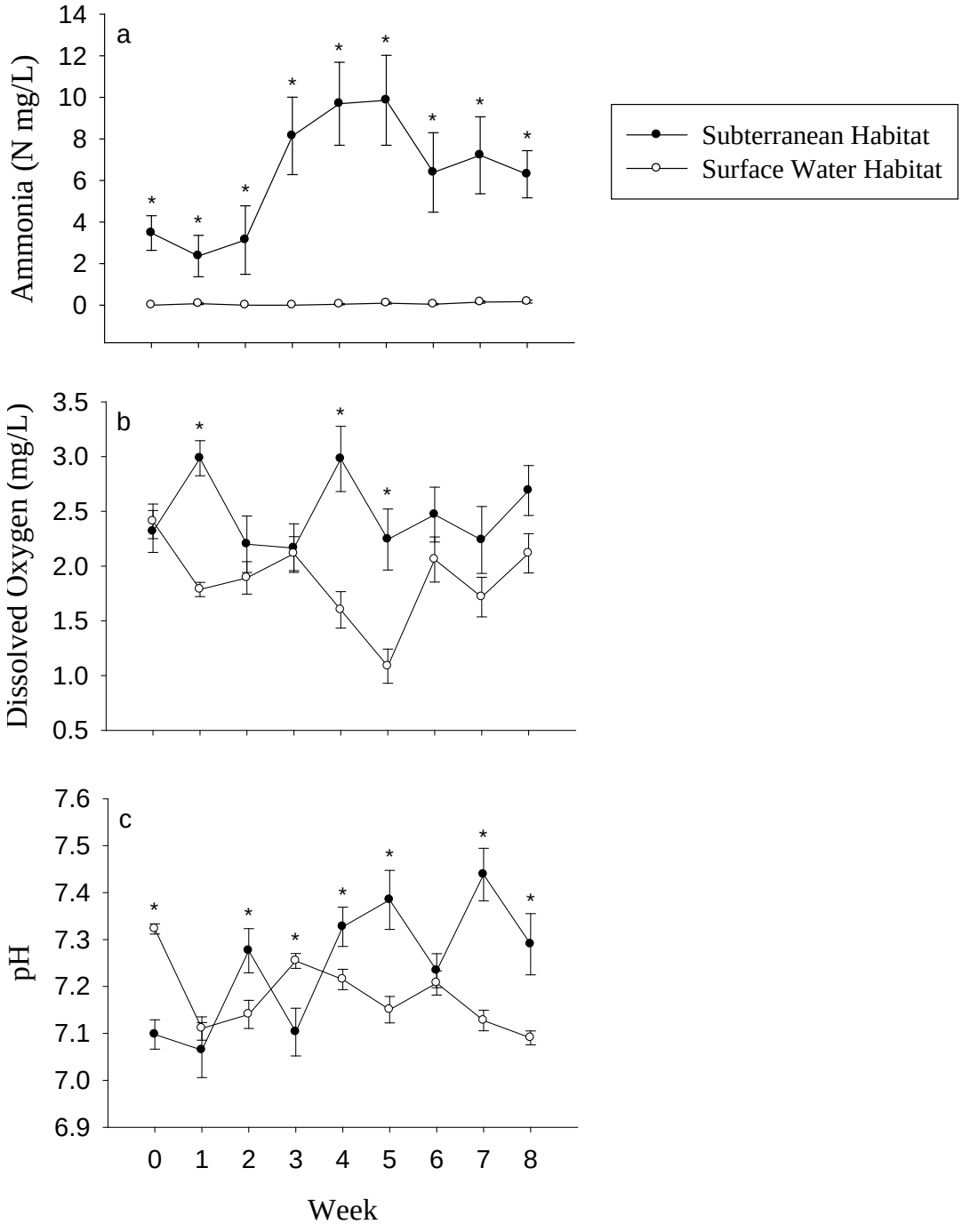

Figure 1. a) Ammonia, b) dissolved oxygen, and c) pH of surface water and burrow water habitats. Asterisks indicate significant differences between habitats on the sample date. Error bars are ± 1 SE.

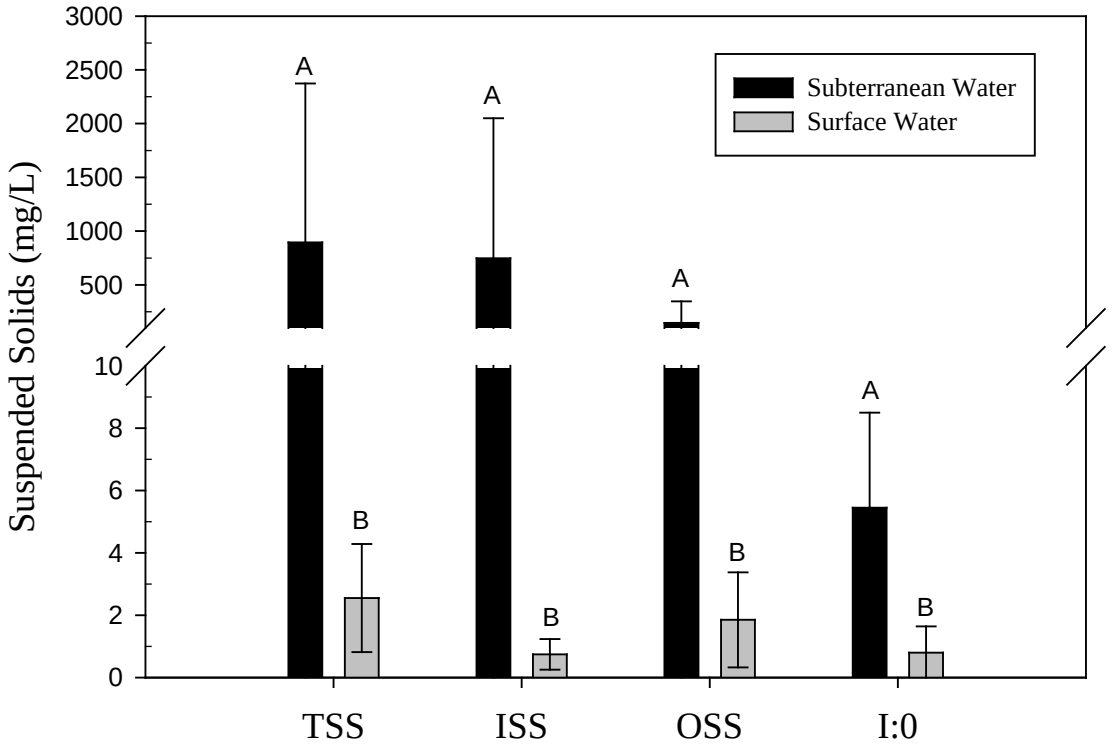

Figure 2. Total suspended solids (TSS), inorganic suspended solids (ISS), organic suspended solids (OSS), and inorganic to organic ratios (I:O) in surface and burrow water habitats at the end of experiments one and two (week 8). Error bars are ± 1 SD. Letters above bars indicate significant differences between habitats within each suspended solids category.

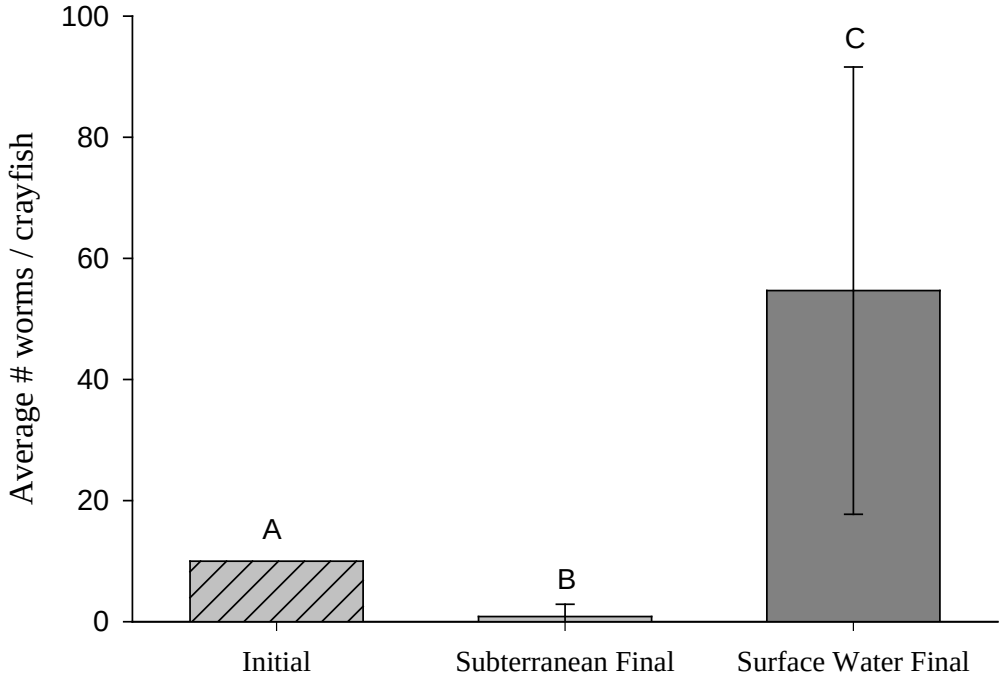

Figure 3. Average # worms/crayfish at the beginning and end (week 8)of each experiment in subterranean and surface water habitats. Error bars are ± 1 SD. Letters indicate significant differences between average worm densities.

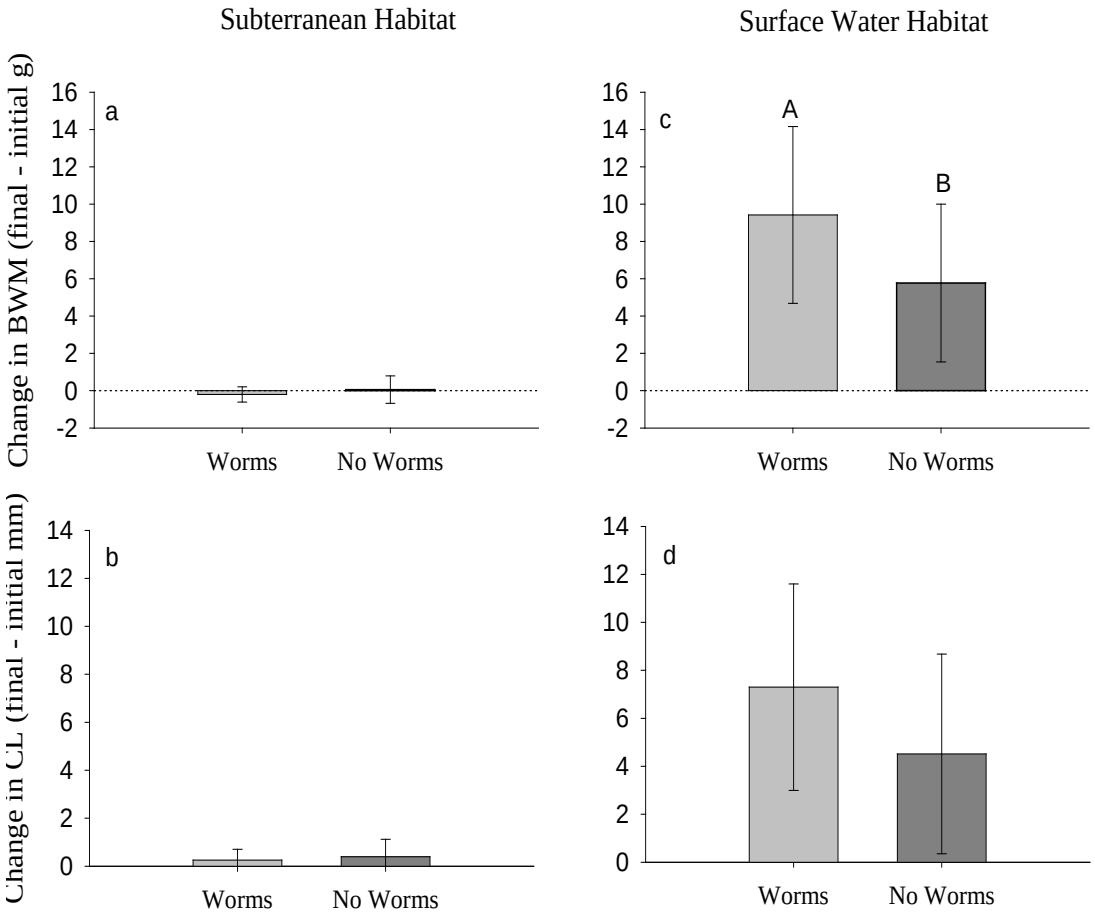

Figure 4.Change in blotted wet mass of crayfish with and without worms over an 8 week period in the a) subterranean and c) surface water habitats. Change in carapace length of crayfish with and without worms over the same 8 week period for b) subterranean and d) surface water habitats. Error bars are ± 1 SD. Capital letters above bars designate significant differences between treatments.

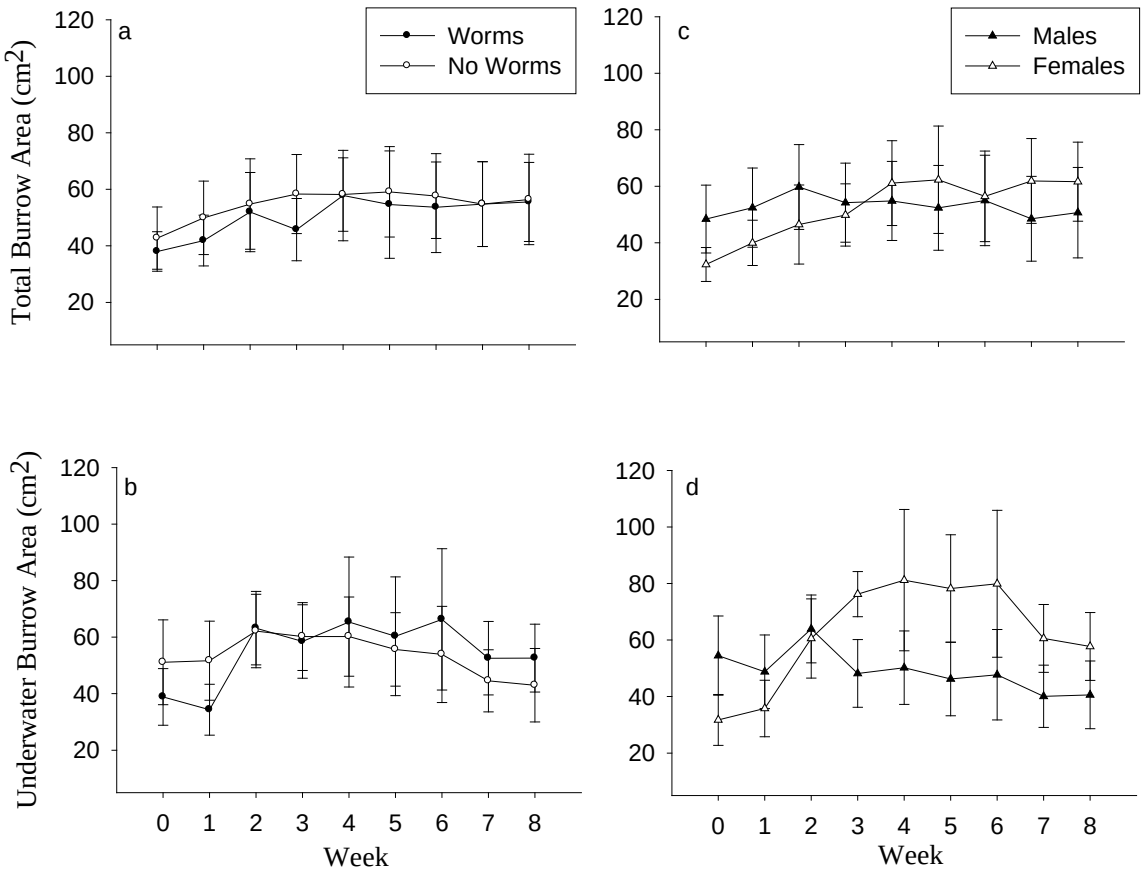

Figure 5.Comparisons of total burrow area between a) treatment and c) gender for all burrows. Comparisons of underwater burrow area between b) treatment and d) gender for those burrows that reached the groundwater. Error bars are ± 1 SE.

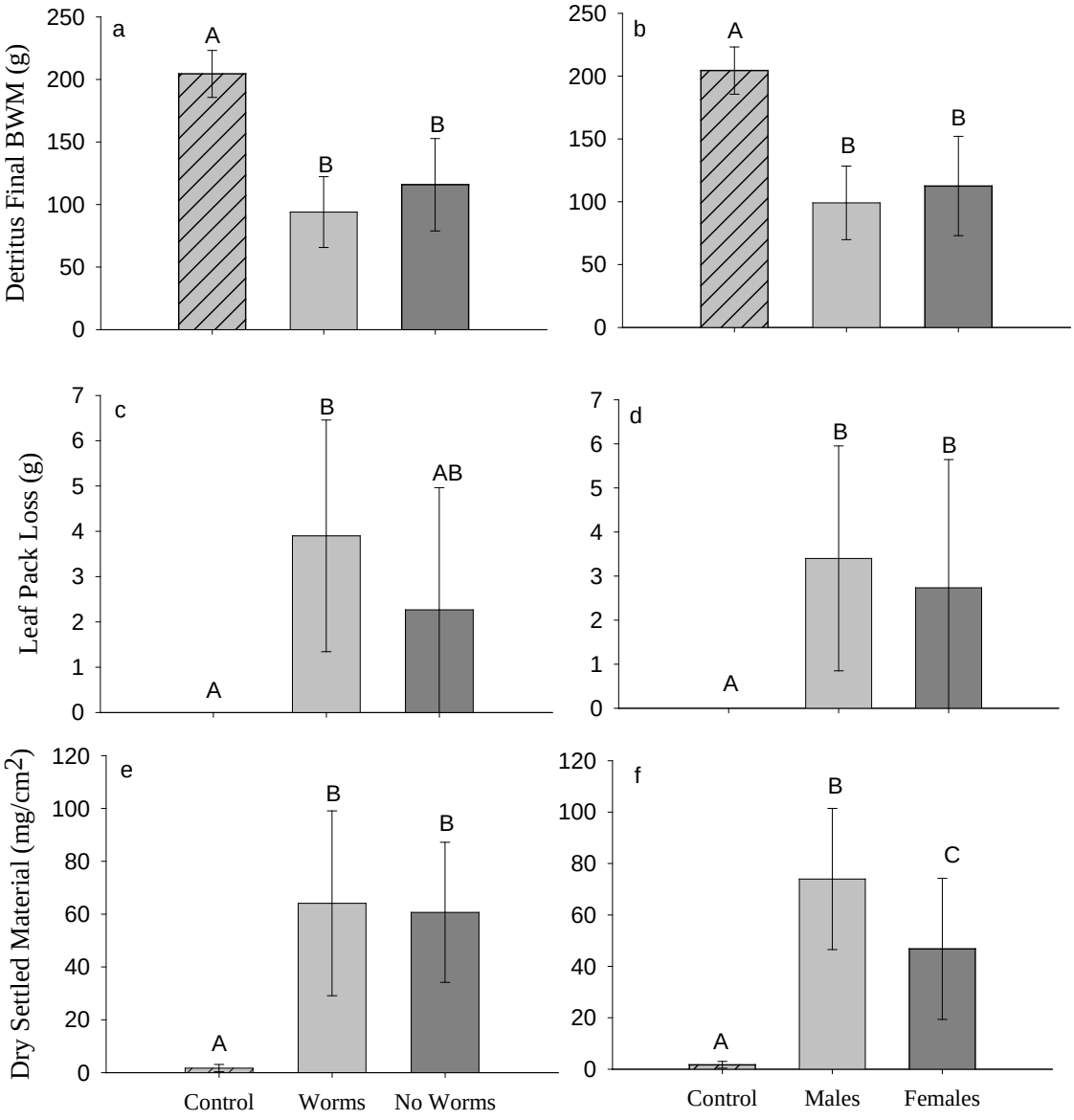

Figure 6. Comparisons of detrital processing between treatments (a, c, e) and between crayfish gender (b, d, f) in the surface water experiment. Error bars are ± 1 SD. Letters above bars designate significant differences between treatments or gender.

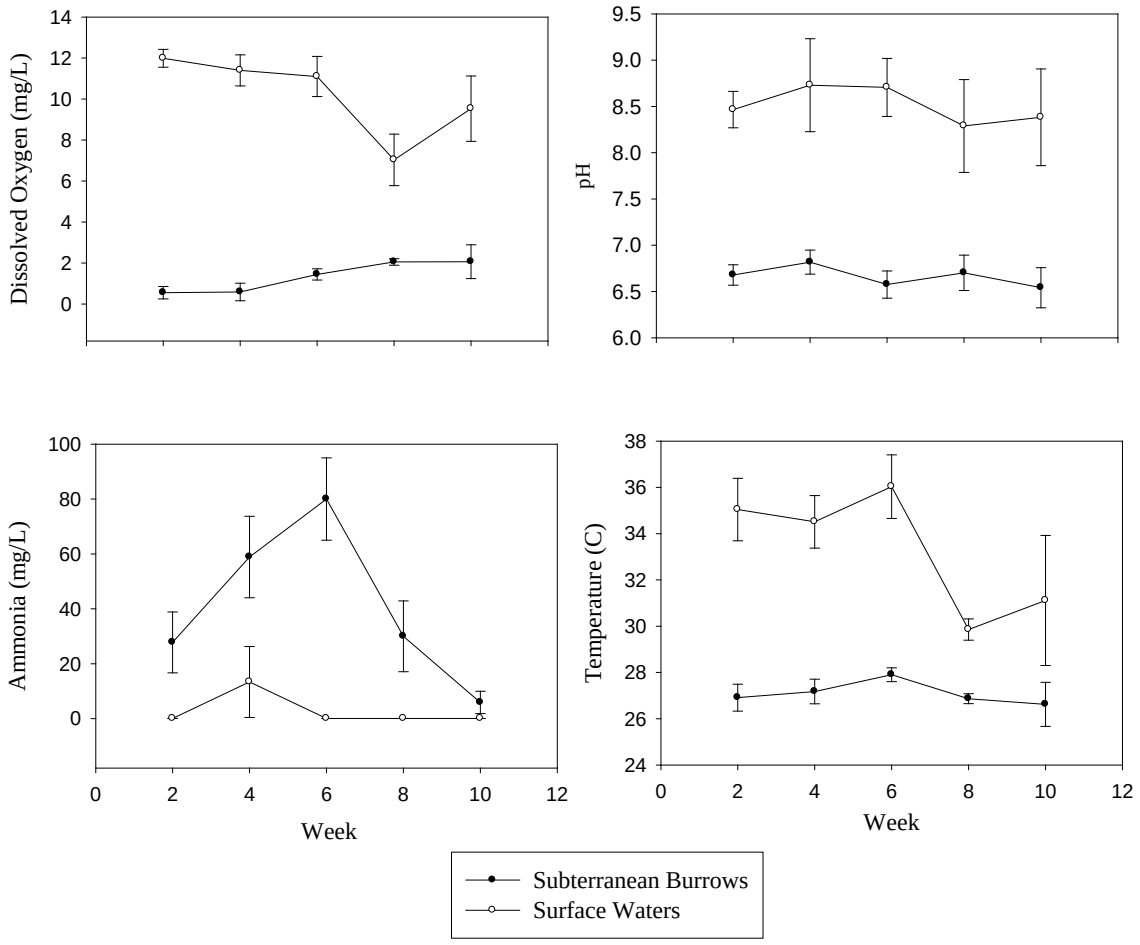

Figure 7. a) Dissolved oxygen, b) ammonia, c) pH, d) temperature of surface water and burrow water habitats over the 10 week experimental period. All data samples dates were significantly different ($p < 0.0001$), error bars are ± 1 SE.

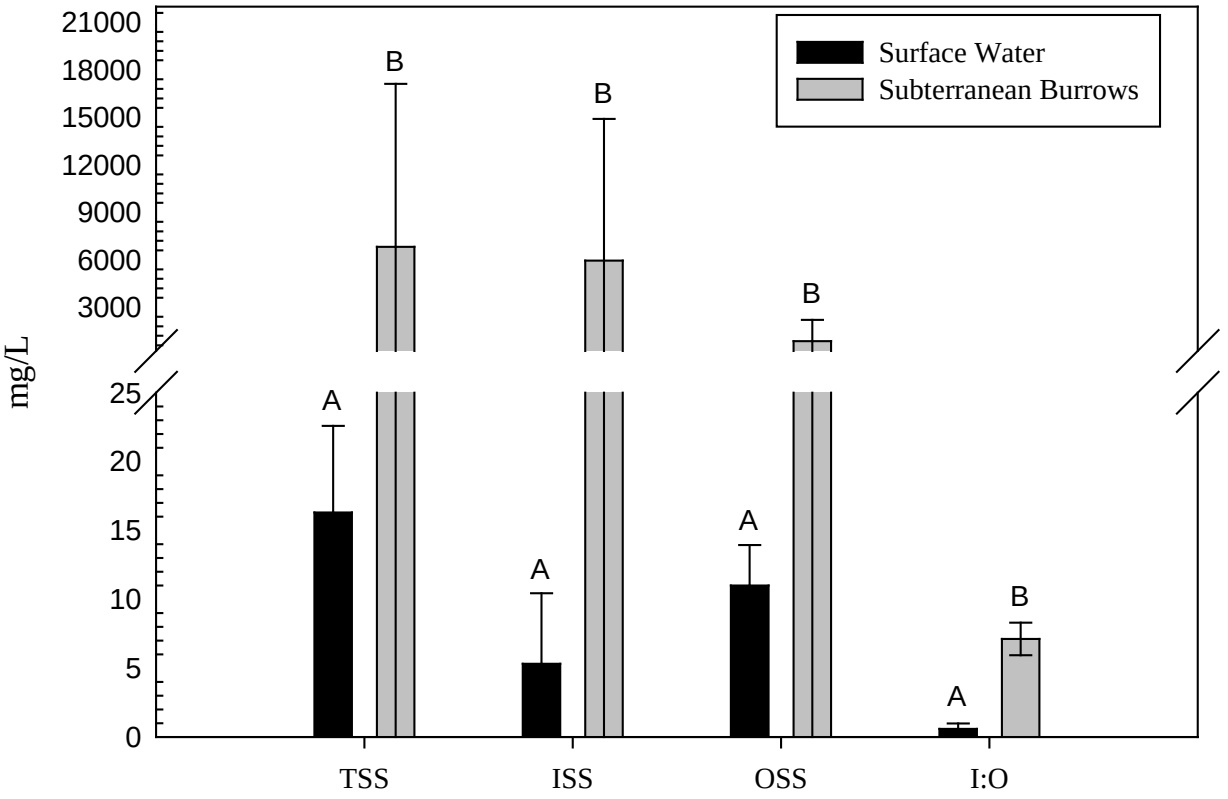

Figure 8. Total suspended solids (TSS), inorganic suspended solids (ISS), organic suspended solids (OSS), and inorganic to organic ratios (I:O) in surface and burrow water habitats at the end of the field experiment (week 10). Error bars are ± 1 SD. Letters above bars indicate significant differences between habitats within each suspended solids category.

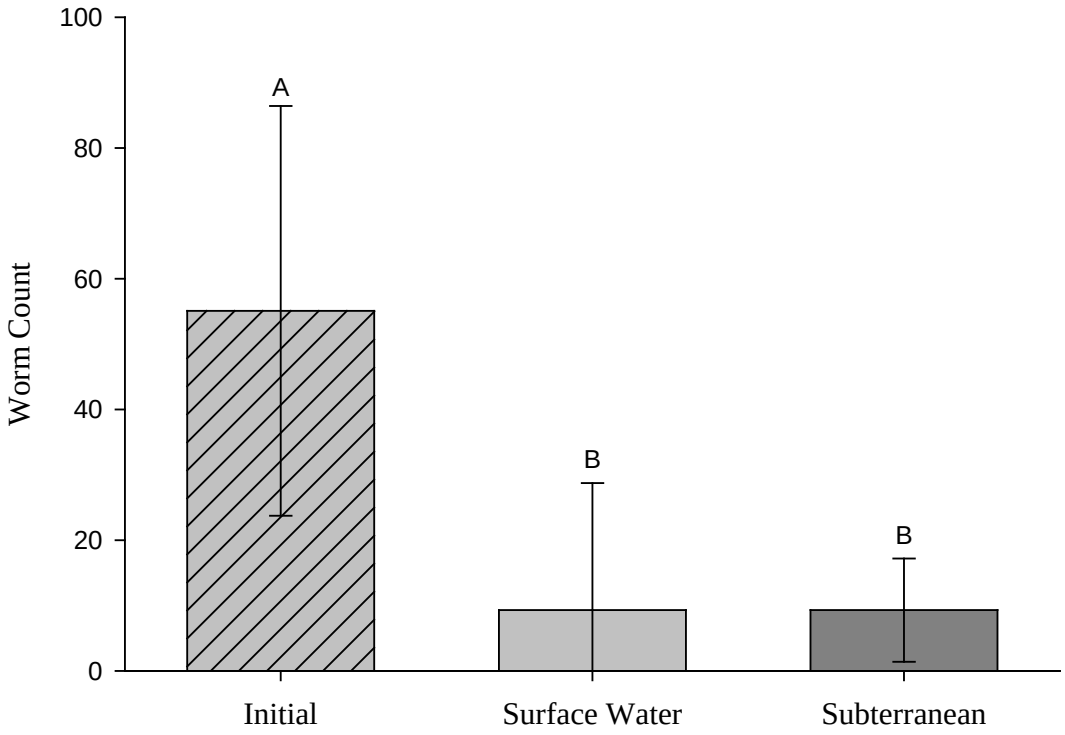

Figure 9. Average # worms/crayfish at initial and final sampling in subterranean and surface water habitats. Error bars are ± 1 SD. Letters indicate significant differences between average worm densities.

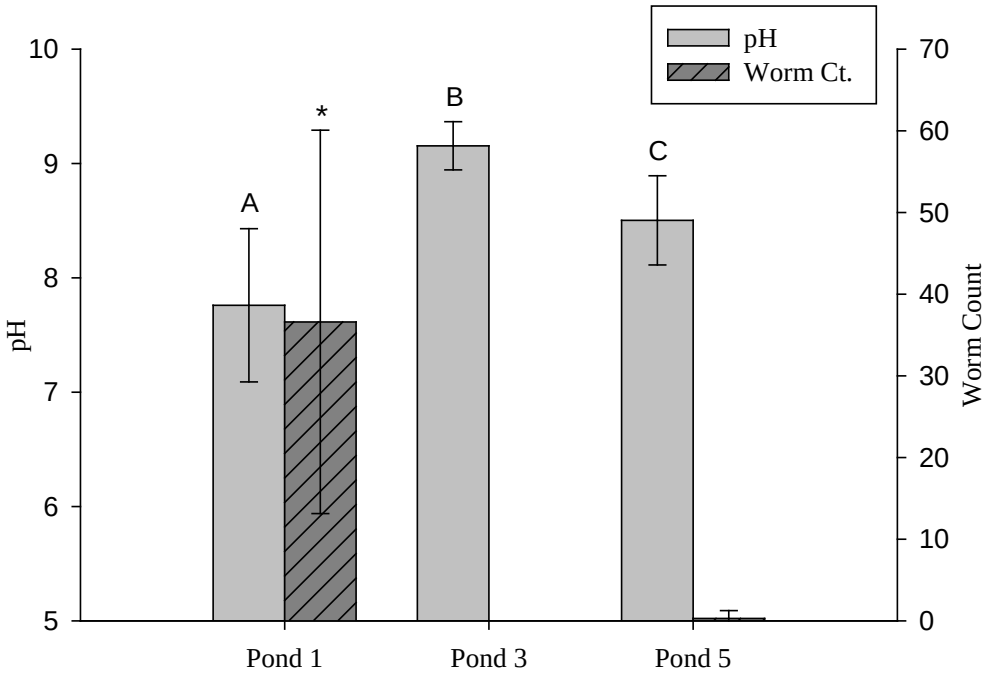

Figure 10. pH values of surface water ponds with final worm counts. Significant differences are denoted by letter for ponds, and asterisk for worm counts.

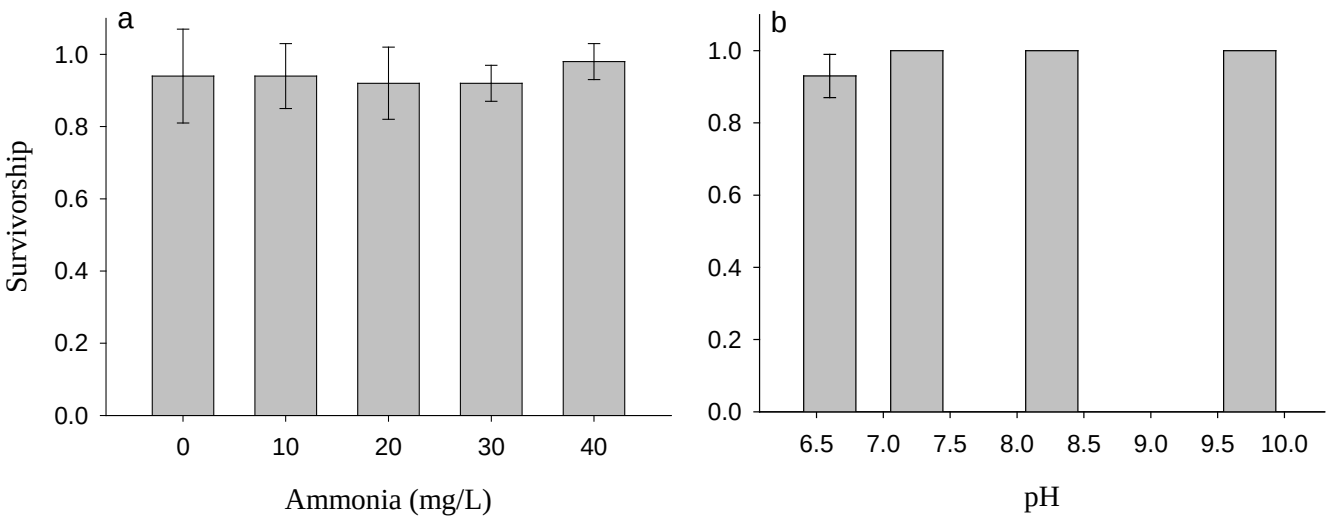

Figure 11. Survivorship of branchiobdellidans among different concentrations values of a) ammonia and b) pH.