

ABSTRACT

Title of thesis: THE ECOPHYSIOLOGY OF THE FISH ASSOCIATED
DINOFLAGELLATE *CREPIDOODINIUM CYPRINODONTUM*
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I assessed the occurrence of *Crepidoodinium cyprinodontum* on cyprinodontid and fundulid species in Maryland and Florida waters. Comparison of epibiont load across host taxa revealed *Fundulus majalis* as the preferred host of *C. cyprinodontum*. *Crepidoodinium cyprinodontum* infecting *Fun. majalis* reached a seasonal peak in epibiont load in summer in Maryland, and winter in Florida. Epibiont density decreased with increasing host (*Fun. majalis*) length, indicating smaller fish are more prone to colonization by *C. cyprinodontum*. Both numbers per fish and growth of *C. cyprinodontum* were influenced by light availability. Biomass based calculations of doubling time indicated that growth of *C. cyprinodontum* on fish gills appeared to below at optimum irradiances, suggesting refuge from predation may be a major factor in driving this dinoflagellate to colonize the opercular region of fish. Finally, I documented infections in two previously unknown host species (*Fun. similis c.f.* and *Floridichthys carpio*).

THE ECOPHYSIOLOGY OF THE FISH ASSOCIATED
DINOFLAGELLATE *CREPIDOODINIUM CYPRINODONTUM*

by

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DEDICATION

To my wife, Claudina, without whose patience, support,
understanding, and sacrifice, none of this would have been possible.

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CHAPTER 1

Introduction

Dinoflagellates inhabit pelagic and benthic communities in marine, estuarine, and freshwater systems around the world. Their ecological relevance is driven not only by their ubiquity, but also by their trophic diversity, as dinoflagellates are well known to play important roles as primary producers, predators, prey, and symbionts. Early botanists and zoologists divided dinoflagellates into two distinct clades; members of one branch possessed chloroplasts and were believed to survive solely by photosynthesis, while members of the other branch were colorless (i.e., lack plastids) and gained energy via heterotrophy. Doubt was cast on this parsimonious classification, as evidence of prey ingestion in plastid containing dinoflagellates was observed in the early 20th century (reviewed by Gaines and Elbrachter 1987). The evidence of feeding in photosynthetic species, termed mixotrophy, remained equivocal until the detailed description of prey ingestion in a hitherto photosynthetic freshwater dinoflagellate *Ceratium hirundella* (Hofendor 1930). Currently, it is believed that most photosynthetic dinoflagellates are capable of feeding, often in response to a decrease in resource availability (Schnepf and Elbrachter 1992, Jones 1994, Stoecker 1998). Today, dinoflagellates are recognized as one of the most trophically diverse groups of plankton organisms, with mixotrophic members represented in both free-living and parasitic lineages.

One role that photosynthetic dinoflagellates play in aquatic environments is that of symbionts. Examples of dinoflagellate symbiosis range from mutualistic species like *Symbiodinium* (zooxanthelle), where photosynthetic products are shared with the host

(Battey 1992, Loram et al. 2007), to lethal parasites capable of causing large scale mortality in host populations (Overstreet 1982, Shields 1994, Skovgaard and Saiz 2006). Mutualistic species have long been seen as important in host populations dynamics as loss of symbionts often results in host stress or death (Glynn 1996, Smith 2005). Parasitic dinoflagellates have only recently been recognized for the degree to which they can regulate host abundances (Coats and Heisler 1989, Coats et al. 1996, Messick and Shields 2000). It is estimated that approximately 7 % of extant species of dinoflagellates have evolved a parasitic lifestyle (Drebes 1984, Coats 1999). As a group, parasitic dinoflagellates infect a wide array of host taxa, including ciliates, other dinoflagellates, sarcodines, appendicularians, and fish (Cachon and Cachon 1987) and have impacts ranging from altering the structure and function of microbial food web to threatening fish and shellfish aquaculture operations (Coats 1999). The ecological footprint and ubiquitous distribution within a wide array of host organisms have prompted considerable interest in the biology, ecology, and population dynamics of parasitic dinoflagellates (Coats 1999, Park et al. 2004). While several studies have quantified the impacts on host species at both the population and individual level (Nishitani et al. 1985, Coats and Bocksthaler 1994, Coats et al. 1996, Coats and Park 2002, Park et al. 2002), few studies have examined factors that influence the distribution of parasitic dinoflagellates (Paperna 1980, Messick and Shields 2000, Steinfeld and Shields 2005).

Most parasitic dinoflagellates are obligate heterotrophs and several of these have been the focus of extensive ecological investigation (Coats 1999, Park and Coats 2004).

Some parasitic dinoflagellates, however, contain photosynthetic life-history stages and rely to some degree on photosynthesis for nutrition (Chatton 1920, Cachon and Cachon 1971, Pasternak et al. 1984, Skovgaard 2005). For instance, Coats (1999) estimated that eight genera of parasitic dinoflagellates, or roughly 22%, possess chloroplasts at some time during their life cycle. These mixotrophic parasites have been largely overlooked by microbial ecologists and fisheries biologists, yet may play a significant role in regulating host populations.

Several dinoflagellate genera across two orders exist in association with fish, and all but one of those genera are classified as ectoparasitic (Lom and Dykova 1992). These ectoparasitic forms share a similar life cycle (Fig. 1-1) consisting of a “feeding” or vegetative stage (trophont), a division stage (tomont), and bi-flagellated mobile stage (dinospore). The sessile trophont attaches to the host via a series of finger like projections referred to collectively as the holdfast, a structure presumably associated with feeding in heterotrophic species (Lom 1981). After growing to some maximal size, or when dislodged from the host, trophonts retract the holdfast, secrete a hyaline cyst wall, and undergo multiple nuclear and cytoplasmic divisions (palintomy) to produce hundreds of mobile dinospores that are then released to presumably begin the infection process anew.

The impact of ectoparasitic dinoflagellates on fish hosts varies from seemingly benign to erosion of host tissue leading to mortality. Some fish-associated dinoflagellates, such as *Amyloodinium ocellatum* and *Ichthyoodinium spp.* are clearly

parasitic, capable of causing large scale mortality of hosts (Lawler 1980, Paperna 1980, Shaharom-Harrison 1990, Kuperman et al. 1999, Martins et al. 2001). This lethality varies among genera with reports of epizootics of some occurring primarily in closed systems, such as aquaria or aquaculture facilities, while others are highly pathogenic in natural systems. Lawler (1980) in a survey of 46 fish species from Mississippi Sound reported 16 hosts lightly infected by *A. ocellatum*, with no apparent mortality. Yet in the same study, he found 73 of 79 species succumb to infection when challenged with dinospores of *A. ocellatum* in aquaria. Some species uninfected or lightly infected in field samples showed mortality within as little as 20 to 48 hours in the laboratory trials. Conversely prevalence of *Ichthyodinium chabelardi*, a lethal endoparasite occurring in the vitelline sac of fish eggs, may reach 50%, with epizootics occurring annually along the coast of Portugal. The parasitic nature of others such as *Crepidodinium spp* and *Piscinodinium pillulare*, however, is ambiguous, as they reportedly possess highly developed chloroplasts, and evidence of ingestion of host tissue is lacking (Lom and Dykova 1992, Cachon and Cachon 1987). While both trophonts and dinospores of some ectoparasites appear photosynthetic, survival time of these life-history stages ranges from hours to days independent of their host (Skovgaard and Saiz, 2006). To what degree these phototrophic symbionts rely on photosynthesis for survival in either the trophont or spore stage remains unknown.

Dinoflagellates within the genus *Crepidodinium* live on gill lamellae (Lom and Lawler 1973). Known hosts for this dinoflagellate genus belong to the families

Cyprinodontidae, Fundulidae, and Sillaginidae (Lawler 1967, 1968a,b, 1980, Williams 1972, Lom et al. 1993, Table 1-1). The type species for this genus, *C. cyprinodontum*, was described in the late 1960's from the York River, VA as an ectoparasite of fish, despite its clearly photosynthetic nature (Lawler 1967a, Lom 1981). Despite its large size (up to 673 μm) and obvious appearance on fish gills, *C. cyprinodontum* has been reported from only seven species of killifish in North America, *Adinia xenica*, *Cyprinodon variegatus*, *Fundulus majalis*, *Fun. heteroclitus*, *Fun. luciae*, *Fun. similis*, and *Lucania parva* (Dillon 1966, Lawler 1967a,b, Williams 1972, Lawler 1980), all of which inhabit primarily shallow, low energy estuarine habitats such as salt marshes and coastal lagoons. *Cyprinodon variegatus*, *Fundulus majalis*, *Fun. heteroclitus*, *Fun. luciae* occur along the East Coast of North America from Maine to Northeastern Florida, with the range of *Cyp. variegatus* extending into the Gulf of Mexico. *A. xenica* and *Fun. similis* are more Neotropical in distribution and occur in Florida coastal waters (Miller 1955, Brown 1957, Relyea 1983). More recently, a second species, *C. australe* inhabiting Sand Whiting (*Sillago ciliate*), was described from two sites along the southeastern coast of Australia (Lom et al 1993).

Crepidodinium has been considered by some investigators as a parasite (Lawler 1967, 1968a&b, Rogers & Gaines 1975) and by others as a commensal (Lom & Lawler 1973, Lom et al. 1993). This uncertainty regarding the relationship of *C. cyprinodontum* to its host stems from the complete lack of data regarding the epibiont's ecophysiology.

Furthermore, classification of *C. cyprinodontum* as a commensal is largely rooted in the fact that it possesses chloroplasts and the belief that it does not cause extensive damage to host gill tissue (Lom 1981, Lom and Dykova 1992, Lom et al. 1993). A previously unrecognized possibility is that *C. cyprinodontum* is a mixotrophic dinoflagellate capable of gaining nutrition from its host without causing severe damage to tissues. While mixotrophy is more common among free-living dinoflagellates, several parasitic species are known to employ this trophic strategy. Pasternak et al. (1984) reported *Blastodinium* sp. inhabiting the gut of its copepod host could satisfy up to 50% of its metabolic demands solely through photosynthesis. The fish-associated freshwater parasite, *Piscinoodinium* spp., contains plastids and clearly relies on photosynthesis to some extent (Lom 1981). Yet interestingly, despite obvious damage to gill tissue, no evidence of ingestion of host material has been observed in *Piscinoodinium* (Shaharom-Harrison et al. 1990, Lom & Dyková 1992). To what degree *Piscinoodinium* and other plastid containing fish-associated dinoflagellates rely on phototrophy and/or heterotrophy is unknown.

A variety of biotic and abiotic factors, including host habitat preference, season, host size, and host sex are known to influence the prevalence of metazoan fish parasite (Dogiel 1961, Rhode 1993, Barse 1998). While host habitat preference (macro-environment) undoubtedly influences all parasites, it is of particularly importance to ectoparasites, as they lack the more stable internal environment (micro-environment) a host must maintain to achieve homeostasis (Dogiel 1961). Of particular importance to

parasites are range of salinity and depth (shallow versus at depth) preferred by hosts, while the effects of host sex and size can be either biological or behavioral in nature. Structurally, sex specific differences in fish length may lead to increased substrate available for colonization, while sex and/or size based migration, schooling, and feeding choice may influence parasite success.

Generally, it is hypothesized that parasite intensity should increase as a function of host size (age), as larger (older) hosts possess greater surface area for colonization (Dogiel 1961, Rhode 1993). However, observational evidence in support of this hypothesis remains mixed and may depend on parasite and/or host taxa of interest. For instance, in a survey of gill parasites of *Fundulus heteroclitus* from small tributaries of Chesapeake Bay, Barse (1998) found load of parasitic flatworms to increase with increasing host size, while load of the copepod *Ergasilus manicatus* did not. Thus, comparing parasite absolute abundances across fish of differing lengths is problematic, as it may mask potential differences in host burden. This is particularly true for gill parasites, as the percentage of respiratory surface area lost is greater in smaller fish relative to larger hosts of equal parasite load. Parasite density, defined as the number of individuals per unit area of host tissue or surface, has been suggested to be a better indicator of the impacts of parasites on their hosts (Margolis et al. 1982). Most literature that exists regarding the interplay between biotic impacts on fish-associated symbionts involves heterotrophic species that are clearly detrimental to their hosts (Overstreet 1982, Barse 1998, Messick and Shields 2000, Stentiford and Shields 2005). The factors

controlling the abundance and distribution of fish-associated dinoflagellates, conversely, are not well understood. For a photosynthetic species like *C. cyprinodontum*, light may also play an important role in determining its distribution

The central goal of my thesis is to determine to what extent physical factors regulate growth/survival of *C. cyprinodontum*. Field observations were utilized to determine if *C. cyprinodontum* exhibits patterns of preference among and within hosts populations and to document its seasonal distribution in both temperate (Maryland) and neotropical (Florida) waters. Experimental manipulations were then used to determine the effect of irradiance on persistence and growth of epibionts. My specific objectives were to (1) relate occurrence of the *C. cyprinodontum* to host environment (salinity, temperature, dissolved oxygen, and solar irradiance), (2) examine correlations between colonization and host taxa, sex, and size, (3) assess seasonal patterns in distribution, and (4) evaluate the degree to which *C. cyprinodontum* is dependent on light for survival and growth. In addition, I examined other potential host taxa for susceptibility to infection by *C. cyprinodontum*. Answers to the questions above provide further insight into the factors controlling the distribution of *Crepidodinium* and therefore its relationship to its host.

Life cycle of *Crepidoodinium* spp.

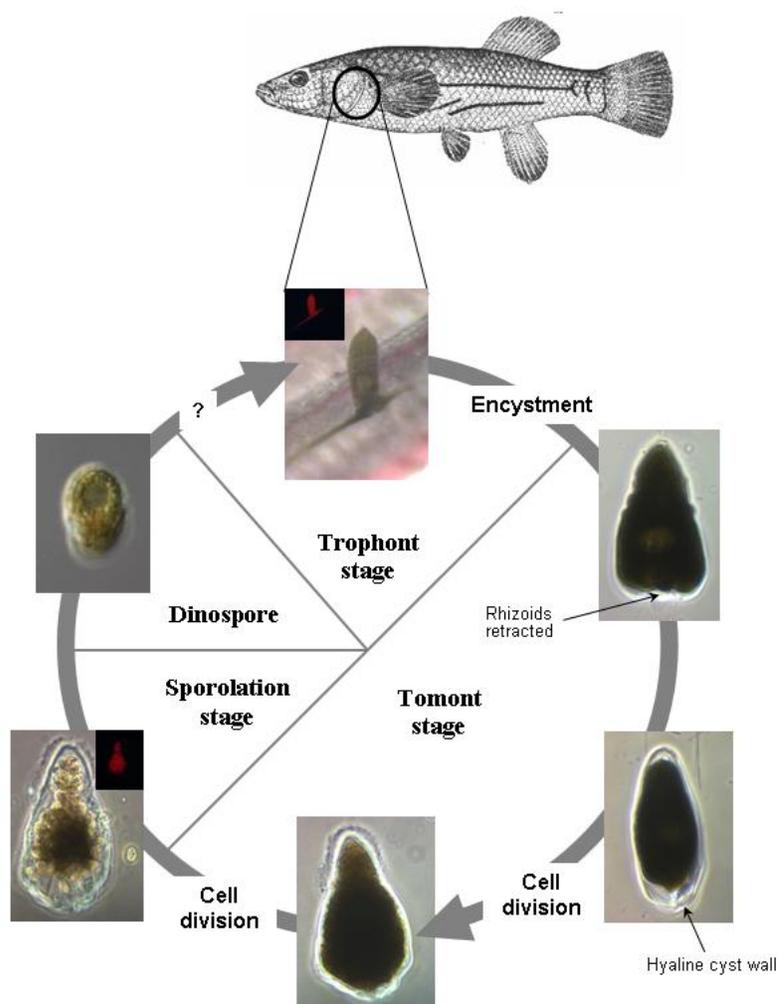


Fig. 1.1 Major transitional stages (i.e., trophont, tomont, and dinospore) occur in all fish-associated dinoflagellates

Table 1.1 Host record of fish colonized by dinoflagellates within the genus *Crepidodinium* (nd = no data reported).

Species	Host	Locality	Prev (%)	Load (mean)	Date	Reference
<i>C. cyprinodontum</i>	<i>F. majalis</i>	York River, VA	nd	nd	April-September	Lawler 1967
<i>C. cyprinodontum</i>	<i>L. parva</i>	York River, VA	nd	nd	April-September	Lawler 1967
<i>C. cyprinodontum</i>	<i>C. variegatus</i>	York River, VA	nd	nd	April-September	Lawler 1967
<i>C. cyprinodontum</i>	<i>F. heteroclitus</i>	York River, VA	nd	nd	April-September	Lawler 1967
<i>C. cyprinodontum</i>	<i>F. luciae</i>	Wachapreague, VA	8	1	24 May	Lawler 1968
<i>C. cyprinodontum</i>	<i>F. similis</i>	Santa Rosa Island, FL	nd	nd	30 July	Lawler 1968
<i>C. cyprinodontum</i>	<i>C. variegatus</i>	Mobile Bay, AL	nd	nd	nd	Williams 1972
<i>C. cyprinodontum</i>	<i>A. xenica</i>	Mississippi Sound, USA	33	1	nd	Lawler 1980
<i>C. cyprinodontum</i>	<i>C. variegatus</i>	Mississippi Sound, USA	66	25	nd	Lawler 1980
<i>C. cyprinodontum</i>	<i>F. similis</i>	Mississippi Sound, USA	33	5	nd	Lawler 1980
<i>C. australe</i>	<i>S. ciliata</i>	New South Wales, AU Arrawarra Creek Nambucca Head	83 50	Up to 50/arch 1-2/arch	September-October	Lom et al. 1993

CHAPTER 2

Ecology of the Fish-Associated Dinoflagellate *Crepidodinium cyprinodontum*

ABSTRACT

Crepidodinium cyprinodontum is a photosynthetic dinoflagellate that lives on gill lamellae of fish. Dinoflagellates within this genus have been considered by some investigators as parasites and by others as commensals. The uncertainty about the relationship of this dinoflagellate to its host stems from the complete lack of data about the epibiont's ecophysiology. This study assessed the occurrence of *C. cyprinodontum* on cyprinodontid and fundulid species in Maryland and Florida waters relative to season and selected environmental variables. When present, *C. cyprinodontum* showed high occurrence rates (prevalence) in host populations, with epibiont number (load) being highly variable among individual fish. *Fundulus majalis* and *Cyprinodon variegatus* exhibited highest epibiont prevalences among host taxa examined in both Maryland and Florida sites. *Fun. majalis* was found to harbor highest numbers of *C. cyprinodontum* with prevalence and load not significantly different among males and females. However, the number of *C. cyprinodontum* was negatively correlated with host size in males, but not females. When epibiont load was normalized to total gill surface area available for colonization, smaller fish in both sexes were found to harbor higher epibiont densities. The number of *C. cyprinodontum* per host varied seasonally on *Fun. majalis*, with peak values observed in summer months in Maryland. Conversely, seasonal maxima of *C. cyprinodontum* occurred in winter in Florida waters, with lowest values found in early summer. The proportion of infected hosts and number of *C. cyprinodontum* on *Fun. majalis* from Sinepuxent Bay appeared unaffected by any abiotic factors considered in this study. Two new host species were recorded for *C. cyprinodontum*, the longnose

killifish (*Fun. similis c. f.*) and goldspotted killifish (*Floridichthys carpio*), from Florida waters.

INTRODUCTION

Parasites have long been known to influence the ecology of organisms in aquatic environments (Rhode 1993). Parasitic dinoflagellates are no exception, as they represent approximately 7% of extant species within the phylum (Drebes 1984) and have impacts ranging from alteration of the structure and function of microbial food webs to threatening fish and shellfish aquaculture operations (Coats 1999). The influence of parasitic dinoflagellates is most evident during epizootics that cause mass mortality of host organisms (Lawler 1980, Overstreet 1982, Kimmerer and McKinnon 1990, Coats et al. 1996). Host taxa susceptible to infection by parasitic dinoflagellates include ciliates, other dinoflagellates, sarcodines, appendicularians, and fish (Cachon and Cachon 1987). The ubiquitous distribution of parasitic dinoflagellates within this wide array of host organisms has prompted considerable interest in their biology, ecology, and influence on host populations (Coats 1999, Park et al. 2004). While several investigations have addressed the impact of parasitic dinoflagellates on hosts at both the individual and population level (Nishitani et al. 1985, Coats and Bocksthaler 1993, Coats et al. 1996, Coats and Park 2002, Park et al. 2002, Park et al. 2004), few studies have examined the factors that influence the distribution of parasitic dinoflagellates (Paperna 1980, Messick and Shields 2000, Steinfeld and Shields 2005).

Most parasitic dinoflagellates are obligate heterotrophs, and several of these have been the focus of extensive ecological investigation (Coats 1999, Park et al. 2004). Some parasitic dinoflagellates, however, contain photosynthetic life-history stages and rely to some degree on photosynthesis for nutrition (Chatton 1920, Cachon and Cachon 1971, Pasternak et al. 1984, Skovgaard 2005). For instance, Coats (1999) estimated that eight

genera of parasitic dinoflagellates, or roughly 22%, possess chloroplasts at some time during their life cycle. These mixotrophic parasites have been largely overlooked by microbial ecologists and fisheries biologists, yet may play a significant role in regulating host populations.

Several dinoflagellate genera across two orders exist in association with fish, and all but two of those genera are classified as ectoparasitic (Lom and Dykova, 1992). These ectoparasitic forms share a similar life cycle, consisting of a “feeding” or vegetative stage (trophont), a division stage (tomont), and a mobile stage (dinospore). The sessile trophont attaches to its host by a series of finger like projections referred to collectively as the holdfast, a structure presumably associated with feeding in heterotrophic species (Lom 1981). The impact of ectoparasitic dinoflagellates on fish hosts varies from seemingly benign to erosion of host tissue leading to mortality. Some fish-associated dinoflagellates, such as *Amyloodinium ocellatum* and *Ichthyodinium spp.*, are clearly parasitic, capable of causing large scale mortality of hosts (Lawler 1980, Paperna 1980, Shaharom-Harrison 1990, Kuperman et al. 1999, Meneses et al. 2003). The relation of plastid containing members of the group to their host remains undetermined.

Crepidodinium cyprinodontum is a photosynthetic dinoflagellate that lives on gill lamellae of fish (Lom and Lawler 1973). Known hosts for this dinoflagellate genus belong to the families Cyprinodontidae, Fundulidae, and Sillaginidae (Lawler 1967, 1968a,b, 1980, Lom et al. 1993, Williams 1972). Despite its large size (up to 673 μm) and obvious appearance on fish gills, *C. cyprinodontum* has been reported from only seven species of killifish in North America, *Adinia xenica*, *Cyprinodon variegatus*,

Fundulus majalis, *Fun. heteroclitus*, *Fun. luciae*, *Fun. similis*, and *Lucania parva* (Dillon 1966, Lawler 1967a,b, Williams 1972, Lawler 1980). These species of killifish are small schooling fish (maximum length ~15 cm) that inhabit primarily shallow, low energy brackish or coastal waters, such as salt marshes, tidal creeks, and lagoons. *Cyp. variegatus* and *L. parva* occur from Cape Cod, MA around the tip of Florida and into the western Gulf of Mexico. *Fun. majalis*, *Fun. heteroclitus*, and *Fun. luciae* are found along the Eastern Seaboard of the U.S., with *Fun. majalis* and *Fun. heteroclitus* ranging from New England to North East Florida, while *Fun. luciae* shows a more limited range extending from Massachusetts to North Carolina. *A. xenica* and *Fun. similis* are tropical in distribution and occur primarily along the Eastern Gulf of Mexico coast. More recently, a second species, *C. australe*, was described as an ectocommensal from Sand Whiting (*Sillago ciliata*) at two sites on the southeastern coast of Australia (Lom et al 1993).

Crepidodinium cyprinodontum has been considered by some investigators as a parasite (Lawler 1967, 1968a,b, Rogers and Gaines 1975) and by others as a commensal (Lom and Lawler 1973, Lom et al. 1993). Uncertainty about the relationship of this dinoflagellate to its host stems from the complete lack of data regarding its ecophysiology. Furthermore, classification of *C. cyprinodontum* as a commensal is largely rooted in the fact that it possesses chloroplasts and the belief that it does not cause extensive damage to host gill tissue (Lom et al. 1993). A previously unrecognized possibility is that *C. cyprinodontum* is a mixotrophic dinoflagellate capable of gaining nutrition from its host without causing severe damage to tissues. While mixotrophy is more common among free-living dinoflagellates, several parasitic species are known to

employ this trophic strategy (Drebes 1984, Cachon and Cachon 1987, Pasternak et al 1984, Coats 1999, Skovgaard 2005).

A variety of biotic and abiotic factors, including host habitat preference, season, host size, and host sex are known to influence the prevalence of fish parasites (Dogiel 1961, Rhode 1993, Barse 1998). Unlike free-living species, parasites are completely reliant on their hosts to provide a suitable habitat for growth and therefore survival. While endoparasites enjoy a more regulated environment within their host, ectoparasitic forms are exposed to fluctuating external environmental conditions. Thus, physical factors, such as salinity and temperature of host preferred habitat, may play a more important role in determining suitability of host species for ectoparasites (Dogiel 1961, Kahn and Thulin 1991). Factors controlling the abundance and distribution of fish associated dinoflagellates, however, are not well understood. For a photosynthetic species like *C. cyprinodontum*, light may also play an important role in determining its distribution.

This field study was designed to assess the occurrence and ecophysiology of *C. cyprinodontum* on cyprinodontid and fundulid fishes. Comparisons were made between prevalence and load of known hosts of *C. cyprinodontum* in Maryland and Florida waters. I examined the relationship between epibiont prevalence and load with respect to host (*F. majalis*) size, sex, and gill surface area. Finally, I used correlation analysis to determine which, if any, abiotic factors may play a role in influencing the distribution of *C. cyprinodontum*. By documenting patterns in infection and examining the influence of abiotic factors on epibiont distribution, I provide further insight on the ecophysiology of *C. cyprinodontum* and its distribution in aquatic environments.

METHODS

Study Area and sample collections

Fundulus majalis, *Fun. heteroclitus*, *Cyprinodon variegatus*, *Floridichthys carpio*, and *Fun. similis* were collected from Maryland and/or Florida waters during the summer and autumn months of 2005 (Table 2-1). Additional samples of *Fun. majalis* were obtained from Maryland in spring to fall of 2006. Maryland sampling occurred in Sinepuxent Bay (38°12'30"N, 75°10'05"W), a back barrier coastal lagoon located within Assateague Island National Park, at monthly or bimonthly intervals from early summer to late fall. Fish were not collected in winter, as they were absent at the sampling site from December to March. Two sites in Florida, Tolomato River (29° 55.25'N, 81° 18.38'W) and Ft. Pierce Inlet (27° 27.94'N, 80° 19.09'W), were sampled every two to three months. Sinepuxent Bay and Tolomato River sites are both *Spartina* dominated temperate salt marshes, while Ft. Pierce inlet connects the Atlantic Ocean to the inter-coastal waterway and has a shoreline dominated by several species of mangroves. *Fun. similis* (*c. f.*) and *Flor. carpio* are tropical species (Duggins 1995, Hoese and Moore 1998) and were only encountered in Ft. Pierce inlet.

Fish collected from shallow water (<2 m) by hand held seine (at least 3 seines) were immediately sorted by species and distributed to separate containers filled with ~ 40 liters of site water. For each species, a maximum of 20 fish were selected to give equal numbers of small, medium and large size classes and equal number of males and females. Selected fish were placed in 10-liter buckets of site water (maximum of 10 fish per bucket), aerated, and transported to the laboratory (~ 3 hours drive) for processing within 24 hours. Fish were sacrificed by severing the spinal column according to AVMA 2000

guidelines, with host taxa, sex, total length (TL), and weight (for most specimens) recorded prior to dissection. Upon sacrifice of each specimen, the gill basket was immediately removed and placed in filtered (GF/C) site water for further dissection. Holobranches were removed with gill arches from right and left sides of the fish and kept in separate Petri dishes. Two gills from each side were arbitrarily chosen for determination of prevalence and load of *C. cyprinodontum*. All specimens present on all filaments of both the anterior and posterior hemibranches were counted within five minutes of host death using a stereomicroscope (10-50X total magnification). Preliminary studies indicated no significant differences in estimates of prevalence and load obtained by this method and by counting *C. cyprinodontum* present on all gills (see appendix A).

The number of *C. cyprinodontum* present on each fish was normalized to gill surface area to provide estimates for symbiont densities. Gill surface area was estimated as a function of fish mass, according to the allometric function $\text{Area} = 13.92\text{mass}^{0.85}$, with a scalar of 0.85 used for fish of intermediate activity (Gray 1954). When data for host weight were missing, fish mass was estimated using a length-weight relationship (Fig. 2-1) derived for specimens of known length and weight.

Data Analysis

Epibiont prevalence, load, and density used here follow classic parasitological terms as defined by Bush et al. (1997). Prevalence is defined as the percent of fish within a given population (sample) colonized by *C. cyprinodontum*. Epibiont load refers to the total number of individuals per fish, while epibiont density refers to the number of

individuals per unit gill surface area. Both load and density serve as estimates of infection intensity.

All statistical comparisons were made using SigmaStat 3.0 (SPSS), with data reported as mean \pm standard error of the mean (SEM), unless otherwise stated. Data for epibiont prevalence were analyzed by chi-squared tests of two-way contingency tables. In cases where greater than 20% of the calculated expected frequencies within contingency tables were less than five, Fisher's exact test was used, as chi-square tests are inaccurate when expected values are low (Sokal and Rohlf 1995). Epibiont prevalence was compared for host species encountered within each sampling region (i.e., Maryland or Florida), using data pooled across the year(s). To examine seasonal patterns of infection for host species in each region, prevalence data were pooled by month.

Epibiont load was also compared for host species encountered within each region, using data pooled across the year(s). For monthly comparisons of epibiont load, data were pooled by month for each host taxon and analyzed by One-way ANOVA. As most data failed to meet parametric assumptions, the non-parametric Kruskal-Wallis One-way ANOVA on ranks was used in the majority of analyses. For those datasets where transformation satisfied parametric assumptions, transformed data were analyzed by One-way ANOVA, with Tukey's test used for pair-wise mean comparisons. Means and standard errors of data requiring transformation for statistical analysis were back-transformed for presentation in the text, tables, and figures.

Comparisons of epibiont prevalence, load, and density relative to host sex were conducted for *F. majalis* only, using data pooled across regions and years. Relationships of male and female length with epibiont load and density were assessed by Spearman

rank order correlation after removing extreme outliers from non-transformed data.

Extreme outliers were identified by visual observations of box plots as recommended by Quinn and Keohough (2002).

Correlation analysis was used to gauge the association of epibiont prevalence and load on *F. majalis* with a suite of abiotic factors, including salinity, temperature, dissolved oxygen levels, solar irradiance, and Chlorophyll-a levels. Correlations were run on fish collected from Sinepuxent Bay with epibiont prevalence, epibiont load, and physical data pooled across years (2005 and 2006). Physical data for correlation analysis came from several sources. Temperature, dissolved oxygen levels and salinity were recorded at time of fish collection with a hand held YSI (model number 556 MPS). Chlorophyll *a* concentrations were determined monthly as part of larger, long-term water quality monitoring program conducted within the Maryland Coastal Bays by the National Park Service at Assateague Island. Whole-water samples for chlorophyll analysis were collected just below the surface and stored at 4°C in the dark until processed within four hours of collection (Wazniak et al. 2007). Solar irradiance (400-700 nm) was measured by an 18 channel multi-filter radiometer located at the Smithsonian Environmental Research Center, Edgewater, MD USA, with System for Transfer of Atmosphere Radiation software package used for calculations of spectral irradiances. Midday irradiances were summed within months to generate monthly mean irradiance values for months in which collections occurred (Neal et al. 2005).

RESULTS

Host Range

While *Crepidodinium cyprinodontum* occurred on all host species examined from Sinepuxent Bay, MD, contingency table analysis indicated prevalence (Fig. 2-2A) varied across host taxa. *Fundulus majalis* and *Cyprinodon variegatus* exhibited highest prevalences that did not differ significantly from each other ($\chi^2 = 3.724$, $P = 0.054$). However, prevalence on *Fundulus heteroclitus* was significantly lower than both *Fun. majalis* ($\chi^2 = 107.163$ test, $P < 0.001$) and *Cyp. variegatus* ($\chi^2 = 60.402$, $P < 0.001$). Infection intensities varied significantly across all host taxa (Kruskal-Wallis One-Way ANOVA on ranks, $P < 0.05$), with epibiont load highest on *Fun. majalis*, intermediate on *Cyp. variegatus*, and lowest on *Fun. heteroclitus* (Fig. 2-2B).

At Florida sites, prevalence (Fig. 2-3A) on *Fun. majalis* was significantly higher than *Fun. similis*, *Floridichthys carpio*, and *Fun. heteroclitus* (Fisher's exact test, $P < 0.5$), but not *Cyp. variegatus* (Fisher's exact test, $P = 0.153$). Prevalence on *Cyp. variegatus* was significantly higher than either *Flor. carpio* or *Fun. heteroclitus* (Fisher's exact test, $P < 0.001$), but not *F. similis* (Fisher's exact test $P = 0.236$). Prevalence on *Fun. similis* was significantly higher than *Fun. heteroclitus* (Fisher's exact test, $P < 0.001$) and *Flor. carpio* (Fisher's exact test, $P < 0.001$). Lowest prevalences occurred on *Flor. carpio* and *Fun. heteroclitus*, with no significant difference between the two host taxa (Fisher's exact test, $P = 0.553$). Epibiont load also varied among host taxa (Fig. 2-3B), with *Fun. majalis* having highest load, but not differing significantly from *Cyp. variegatus* (Kruskal-Wallis One-Way ANOVA on ranks, $P > 0.05$). *Fun. heteroclitus* had lowest load of Florida hosts, but did not differ significantly from *Flor. carpio* (K-W

One-Way ANOVA on ranks $P > 0.05$). Loads on *Fun. similis*, *Cyp. variegatus*, and *Fun. carpio* were not significantly different (K-W One-Way ANOVA on ranks $P > 0.05$)

Seasonality

C. cyprinodontum showed high prevalence on *Fun. majalis* in Sinepuxent Bay throughout sampling periods in 2005 and 2006 (Fig. 2-4A), with mean values not differing significantly between months in either year (Fisher's exact test, $P > 0.05$). By contrast, epibiont load varied seasonally, with highest values in summer (June to August) of both years. In 2005, load in fall (September and October) was significantly lower than high summer values in June and July (Fig. 2-4B; K-W ANOVA, $P < 0.05$). In 2006, epibiont load in spring (April) and autumn (October) were significantly lower than the summer peak in June, but not different from each other (One-way ANOVA $P < 0.05$). Conversely, *Cyp. variegatus* (Fig. 2-5) and *Fun. heteroclitus* (Fig. 2-6), showed no significant seasonal differences in either prevalence (Fisher's exact test ($P > 0.05$) or load (One-way ANOVA $P > 0.05$).

Prevalence of *C. cyprinodontum* on *Fun. majalis* collected from Tolomato River, Florida was also independent of month (Fig. 2-7A; Fisher's Exact test, $P > 0.05$), while load varied over time (Fig. 2-7B). Epibiont load was lowest in May and significantly higher in January (K-W ANOVA on ranks, $P < 0.05$). *C. cyprinodontum* on *Fun. similis* collected from Ft Pierce Inlet, however, showed seasonal oscillation in both prevalence and load (Fig. 2-8A&B). While *C. cyprinodontum* was absent in winter months, the portion of colonized hosts increased significantly from winter (January) to spring (March and May) with maximum values occurring in August. Epibiont load was generally low in

non-summer months, with no significant differences occurring in January, March, and May (One-way ANOVA, $P > 0.05$). Maximum infection intensity occurred in late summer, with August loads significantly higher than all other months (One-way ANOVA, $P < 0.001$).

Host Sex and Size

Pooled data for Maryland and Florida samples indicated high prevalence and load of *C. cyprinodontum* on both male and female *Fun. majalis* (Table 2-2), with no significant differences between the sexes ($\chi^2 = 2.114$, $P = 0.146$ for prevalence and $P = 0.075$ for load; K-W on ranks). Epibiont load on females showed no correlation with fish length, however, load on males was negatively correlated with host total length ($r = -0.246$, $P < 0.001$) for fish ranging from 4 to 18 cm (Fig. 2-9). Analysis of epibiont densities versus host length (Fig. 2-10) indicated smaller *Fun. majalis* supported higher numbers of *C. cyprinodontum* per unit gill surface area, with density on both male and female fish negatively correlated with total length (Females $r = -0.600$, $P = 0.001$, Males $r = -0.252$, $P = 0.001$).

Environmental Factors

In Sinepuxent Bay, pooled data from 2005 and 2006 of *C. cyprinodontum* prevalence and load on *Fun. majalis* appeared unaffected by any environmental variables used in this analysis.

DISCUSSION

All five fish taxa collected from Maryland and Florida examined in this study were susceptible to colonization by *C. cyprinodontum*. When present in an environment, *C. cyprinodontum* displayed high prevalence, with load highly variable among hosts of the same population. Highest prevalence of *C. cyprinodontum* occurred on *Cyp. variegatus* and *F. majalis* in both Sinepuxent Bay and Tolomato River, while maximum mean infection intensities occurred on Sinepuxent Bay populations of *Fun. majalis*. Prevalence of *C. cyprinodontum* on host populations did not vary at mid-latitudes, however, epibiont load on *Fun. majalis* varied seasonally with maximum loads occurring in summer months. In Florida waters, *C. cyprinodontum* showed seasonal variations in load on *Fun. majalis* and *Fun. similis*, with prevalence also varying on the latter. Density of epibionts decreased as a function of host (*Fun. majalis*) length, indicating smaller fish were more susceptible to colonization by *C. cyprinodontum*. Two new host species, *Flor. carpio* and *Fun. similis* (c.f.) from Florida waters, were recorded for *C. cyprinodontum*.

Prevalence of *C. cyprinodontum* among host taxa present in both Maryland and Florida sites showed a similar pattern. At both sites, highest prevalence occurred on *Fun. majalis* and *Cyp. variegatus* with lowest prevalence found on *Fun. heteroclitus*. Despite similarities in prevalence, *Fun. majalis* supported higher epibiont load, suggesting it is more susceptible to infection by *C. cyprinodontum*. Preference of particular host taxa within a host range is common among fish parasites although the degree of specificity varies among different parasites (Rhode 1993). In general, fish associated dinoflagellates show varying degrees of host preference in wild populations, with field surveys often

yielding contradictory results when compared to closed systems (i.e., aquaria). The heterotrophic dinoflagellate *Amyloodinium ocellatum* reportedly infected 16 of 43 fish species surveyed in Mississippi Sound, but only five species had loads greater than 20 per fish (Lawler 1980). Yet, 73 of 79 species succumb to infection when challenged with dinospores of *A. ocellatum* in aquaria, with some species that were uninfected or lightly infected in field samples showing mortality within as little as 20 to 48 hours. Other photosynthetic fish-associated dinoflagellates appear to exhibit preference with a range of host taxa as well. *Piscinoodinium*, a potentially lethal gill parasite of fresh water fish, has been reported from several fish species (Ferraz and Sommerville 1998, Martins et al. 2002, Carneiro et al. 2002). However, in epizootic outbreaks of *P. piscinoodinium* on three species of Cyprinids (carp) in Malaysian aquaculture ponds, only one species, lampam jawa (*Leptobarbus hoevenii*), proved highly susceptible to infestation and mass mortality (Shaharom-Harrison 1990).

The number of *C. cyprinodontum* per fish reported here are higher than those reported in previous studies (Lawler 1967a,b, Williams 1972, Lawler 1980, Lom et al. 1993). A maximum load of 657 *C. cyprinodontum* was recorded from a male *Fun. majalis* collected from Sinepuxent Bay in June 2005. A maximum mean load of 111 epibionts per fish occurred at the same site during the same month. Lawler (1980) reported a maximum mean load of 25 occurring on *Cyp. variegatus* from Mississippi sound. This discrepancy in maximum load is most likely due to a combination of smaller sample sizes of previous investigations and the longitudinal sampling approach taken in this study. Previous reports of load of *Crepidodinium* species constitute primarily

snapshots from single sampling periods of larger parasite surveys (Lawler 1967a,b, Williams 1972, Lawler 1980, Lom et al. 1993).

Load and prevalence of *C. cyprinodontum* on *Fun. majalis* report here are similar to values reported for *C. australe* on sand whiting collected from two estuaries located along the New South Wales coast, Australia (Lom et al. 1993). These authors report an approximate mean infection intensity of up to 50 trophonts per gill arch (400 epibionts per fish) on five of six specimens examined from Arrawarra Creek Estuary.

Interestingly, the authors report lower prevalence and infection intensities (50% and 1-2 trophonts per gill, respectively) in sand whiting collected from Nambucca Heads, an estuary located approximately 80 km to the south. The similar loads and prevalence of *Crepidodinium* on these two hosts could be linked to habitat of host species.

Sand whiting inhabit primarily shallow coastal waters and are reported to prefer sandy bottom regions of estuaries along the east coast of Australia, Tasmania, and Papua New Guinea (McKay 1992). In a comparison of shore-zone fish in Great South Bay on Long Island Sound, NY, *Fun. majalis* and *Cyp. variegatus* were more often encountered in sandy substrate environments, while *Fun. heteroclitus* occurred more often in muddy bottom environments (Briggs and O'Connor 1971). A decrease in light availability in muddy waters may drive the low prevalence and abundance on *Fun. heteroclitus*.

Although species co-occurred at time of capture, *Fun. heteroclitus* is known to move from sub-tidal waters at high tide to feed on inundated emergent marshes (Talbot and Able 1984, Kneib and Wagner 1994, Teo and Able 2003). *Fun. majalis* occurs primarily in sub-tidal areas and is rarely reported on emergent marshes or in salt marsh pools (Kneib 1984, Talbot and Able 1984, Able et al. 2005).

Prevalence of *C. cyprinodontum* on host taxa from Sinepuxent Bay was unaffected by season during months sampled. Of host taxa examined in Maryland, only epibiont load on *Fun. majalis* varied within months sampled, with load showing a strong seasonal peak in summer (June, July, August). Symbiont load, however, did not correlate with water column Chlorophyll-*a* values and appeared unaffected by incoming solar irradiance. Seasonal distribution of *C. cyprinodontum* in Sinepuxent Bay report here is similar to that of seasonal variation observed in *Amyloodinium ocellatum* infecting juvenile Tilapia (*Oreochromis mossambicus*) from the Salton Sea, a hyper-saline inland lake in California (Kupperman and Matey 1999). While infection intensity was reported qualitatively (i.e. few, dozens, hundreds of trophonts per fish), the authors observed a seasonal peak of *A. ocellatum* occurring in summer months (June to August) with lower loads occurring in spring (May) and fall (October).

Florida populations of *C. cyprinodontum* showed strong seasonal fluctuations in epibiont load on both *Fun. majalis* and *Fun. similis*, with prevalence varying seasonally on the latter. Epibiont load on *Fun. majalis* from Tolomato River was lowest in spring when salinity was highest (36). While load of *C. cyprinodontum* showed no relationship to salinity in Sinepuxent Bay, a salinity of 36 was well above the range of salinities used in correlation analysis (Table 2-1). The affect of high salinity on observed loads is unclear, as little is known regarding the salinity tolerance of *C. cyprinodontum*.

There was no difference in either epibiont load or prevalence between male and female *Fun. majalis*, however, load decreased significantly with female length. Generally, it is hypothesized that parasite intensity increases as a function of host age (size), as older (larger) fish increase the number of primary filaments or lamellae as they

increase in size (Roubal 1987). The increase in primary filaments or lamellae provides greater surface area for colonization (Dogiel 1961, Rhode 1993). However, evidence in support of this hypothesis remains mixed and relationships may depend on parasite and/or host taxa of interest. For instance, in a survey of gill parasites of *Fundulus heteroclitus* from small tributaries of Chesapeake Bay, Barse (1998) found load of parasitic flatworms to increase with increasing host size, while load of the parasitic copepod *Ergasilus manicatus* did not. A similar relationship between host sex and length as report here was found for the lethal parasitic dinoflagellate *Hematodinium* sp. and its host, the Norwegian lobster (*Nephrops norvegicus*), in which small females exhibited higher abundance of parasites (Field et al. 1998, Stentiford et al. 2001).

Parasite density expressed as the number of individuals per unit area of host tissue has been suggested to be a better metric of intensity of infection within hosts (Margolis et al. 1982). Normalization of epibiont load to gill surface area indicated density of *C. cyprinodontum* decreased as a function of host length in both male and female *Fun. majalis*, suggesting smaller fish are more susceptible to infection. Furthermore, this indicates colonization of *C. cyprinodontum* is not limited by substrate availability, as larger fish possess a greater gill surface area for settlement. The increased density of *C. cyprinodontum* occurring on smaller hosts suggest potential impacts, such as disruption of oxygen diffusion or ion regulation, may be greater in small fish. In a survey of six parasitic species infecting the gills of *Fun. kansae*, only the mobile peritrich ciliate, *Trichodina* sp, was reported to show preferential infection on small size-class hosts (Adams 1985). Among parasitic dinoflagellate genera, Messick and Shields (2000) report highest intensities of *Hematodinium* sp. occurred on juvenile blue crab

(*Callinectes sapidus*) in the Maryland Coastal Bays. The authors hypothesize increased molting of juveniles make them more susceptible to infection.

Possible factors that may be responsible for high epibiont densities observed in smaller fish in this study are increased host metabolic rates, shoaling choice, and light availability within the opercular cavity. Host metabolic rate has been suggested to be a key determinant of heterotrophic parasite biomass (Poulin and George-Nascimento 2006). In Teleostei, metabolic rates decrease with increasing fish size (length) (Jobling 1994, Kidder et al. 2006). Perhaps increased availability of excreted material concurrent with increased catabolism drives the higher density of *C. cyprinodontum* on the more metabolically active smaller fish. In marine fish, ammonium and urea are primarily excreted across host gill epithelial tissue (Wilke 2002). For an autotrophic species like *C. cyprinodontum*, this may be an important source of nitrogen for growth. Nothing is known regarding the potential uptake of host excretion products by plastid containing fish-associated dinoflagellates, however, preferential uptake of ammonium has been reported from a broad spectrum of phytoplankton taxa (Dugdale and Goering 1967, Goldman and Glibert 1982). Host schooling behavior and the affect of fish size may influence increased epibiont densities on small fish as well. Exposure to the motile bi-flagellated dinospore life-history stage of some fish-associated dinoflagellates, such as *A. ocellatum*, are known to propagate infections in naïve host (Lawler 1980, Noga and Bower 1987) and is the presumed mode of infection of *C. cyprinodontum*. For directly transmitted parasites, increased contact rates or higher host densities may increase the likelihood of infection (Begon et al 1996). Although little is known regarding the schooling behavior of *Fun. majalis*, shoal choice of the freshwater fundulid *Fun.*

diaphanous has been shown to be linked to fish size, with fish of similar lengths preferring to shoal together (Krause and Godin 1994). If *Fun. majalis* segregate spatially by size, higher epibiont densities on smaller fish may lead to increased exposure to the infective stage(s) of *C. cyprinodontum* in shoals of small fish. Finally thickness of the opercular flap may vary with fish size thereby altering the amount of light entering the opercular cavity. Larger fish may have thicker opercula thereby decreasing light transmitted to gills. This decrease in light availability for photosynthesis may result in lower growth rates driving decreased epibiont densities observed in larger hosts.

Physical factors, including salinity and temperature, are well known to influence fish parasites, particularly ectoparasitic forms (Rhode 1993). I observed no significant relationship between environmental parameters and epibiont prevalence or load on *Fun. majalis* in Sinepuxent Bay over months in which fish were encountered. However the failure to detect trends in load and/or prevalence in this study may be due to the limited range in fluctuations in physical factors experienced in Sinepuxent Bay. The effect of environmental factors, such as salinity and light environment, would be better assessed in controlled conditions, rather than field surveys.

In summary, *C. cyprinodontum* was widely distributed in host populations in Maryland and Florida sites, with infection intensity highly variable among host taxa. Prevalence and load of *C. cyprinodontum* reported here were much higher than previously observed (Lawler 1967, 1968a and b, 1980, Williams 1972, Rogers and Gaines 1975). Quantification of epibiont load across host taxa examined in this study revealed *Fun. majalis* as the preferred host of *C. cyprinodontum* at both Sinepuxent Bay and Tolomato River sites. This study is the first to document seasonal patterns in

infection intensity and prevalence of *C. cyprinodontum* across host species. In Sinepuxent Bay, *C. cyprinodontum* infecting *F. majalis* showed a seasonal peak in load in summer months. This seasonal pattern was reversed in Tolomato River where epibiont load reached a maximum in winter months. Symbiont density decreased with increasing fish length indicating smaller fish are more prone to colonization by *C. cyprinodontum*. Both prevalence and epibiont load of selected host taxa in Sinepuxent Bay appeared unaffected by any abiotic factors considered in this analysis, however, this may be a function of the limited range in variables during sample months. *C. cyprinodontum* appears well adapted to life in the opercular cavity of Cyprinodontid fish gills.

Table 2.1 Physical parameters of collection sites in Maryland and Florida waters.

Site	Date month/year	Salinity	Temp °C	DO mg l ⁻¹	Irradiance E mW m ⁻² nm ⁻¹	Chl-a ug l ⁻¹
Sinepuxent Bay, MD	Jun-05	25	30	6.6	1647	11.8
	Jul-05	24	31	5.8	1612	14.9
	Aug-05	26	31	10.8	1493	12.0
	Sep-05	30	25	8.7	1649	13.1
	Oct-05	27	27	8.9	1166	4.0
	Nov-05	28	8	8.4		
	Dec-05	28	5	11.5		
	Apr-06	31	19	7.3	1571	3.3
	May-06	30	23	8.3	1855	32.9
	Jun-06	31	29	7.6	1794	27.9
	Jul-06	33	33	8.0	1712	12.3
	Aug-06	29	21	8.7	1040	27.5
	Fort Pierce Inlet	Jan-05	33	21		
Mar-05		34	26			
May-05		36	25			
Aug-05		35	32			
Tolomato River	Jan-05	33	22			
	Mar-05	33	27			
	May-05	36	27			
	Aug-05	33	32			

Table 2.2 Prevalence and load of *C. cyprinodontum* on *F. majalis* pooled over 2005 and 2006. No significant differences were found.

Host sex	N	Prevalence (%)	Load (mean \pm SE)
Female	178	98	90.4 \pm 7.76
Male	227	95	95.9 \pm 7.38

FIGURE LEGENDS

Figure 2.1 Length-weight relationship of male and female *Fundulus majalis*. Data were pooled between Maryland and Florida during 2005 and 2006.

Figure 2.2 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* averaged across year on fish collected in Sinepuxent Bay, Maryland during 2005. Prevalence was analyzed by Chi-square tests and load by Kruskal-Wallis ANOVA on ranks. Bars with same letters are not significantly different ($P < 0.05$). Data presented as Mean \pm SE.

Figure 2.3 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* averaged across year on fish collected in Tolomato River and Fort Pierce Inlet, Florida during 2005. Prevalence was analyzed by Chi-square tests and load by Kruskal-Wallis ANOVA on ranks. Bars with same letters are not significantly different ($P < 0.05$). Data presented as Mean \pm SE.

Figure 2.4 Prevalence (A) and load (B) for *Crepidoodinium cyprinodontum* on *Fundulus majalis* averaged across month for samples from Sinepuxent Bay, Maryland during 2005 and 2006. For 2005, prevalence was analyzed by Fisher's exact test and load analyzed by Kruskal-Wallis ANOVA on ranks. For 2006, prevalence was analyzed by Chi-square tests and load by one way ANOVA on log transformed data. Data for load in

2006 are presented as back transformed mean \pm SE, with bars having same letters not significantly different ($P < 0.05$).

Figure 2.5 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* on *Cyprinodon variegatus* collected from Sinepuxent Bay, MD during 2005. Prevalence was analyzed by Fisher's Exact tests and load by One-way ANOVA on log transformed data. Values of prevalence and load do not differ significantly across months ($P > 0.05$). Data for load are presented as back transformed mean \pm SE.

Figure 2.6 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* on *Fundulus heteroclitus* collected from Sinepuxent Bay, Maryland during 2005. Prevalence was analyzed by Fisher's Exact test and load by Kruskal-Wallis ANOVA on ranks. Values of prevalence and load do not differ significantly across months ($P > 0.05$). Data presented as mean \pm SE.

Figure 2.7 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* on *F. majalis* collected from Tolomato River, Florida during 2005. Prevalence was analyzed by Fisher's Exact test and load by One-way ANOVA. Bars with same letters are not significantly different ($P < 0.05$). Data presented as mean \pm SE.

Figure 2.8 Prevalence (A) and load (B) of *Crepidoodinium cyprinodontum* on *Fundulus similis* collected from Fort Pierce Inlet, Florida during 2005. Prevalence was analyzed by Fisher's Exact test and load by One-way ANOVA on log transformed data.

Bars with same letters are not significantly different ($P < 0.05$). Load data presented as back transformed mean \pm SE.

Figure 2.9 Correlation of *Crepidodinium cyprinodontum* load on male and female *Fundulus majalis* versus total host length. Data were pooled between Maryland and Florida during 2005 and 2006.

Figure 2.10 Correlation of *Crepidodinium cyprinodontum* density on male and female *Fundulus majalis*. Data were pooled between Maryland and Florida during 2005 and 2006.

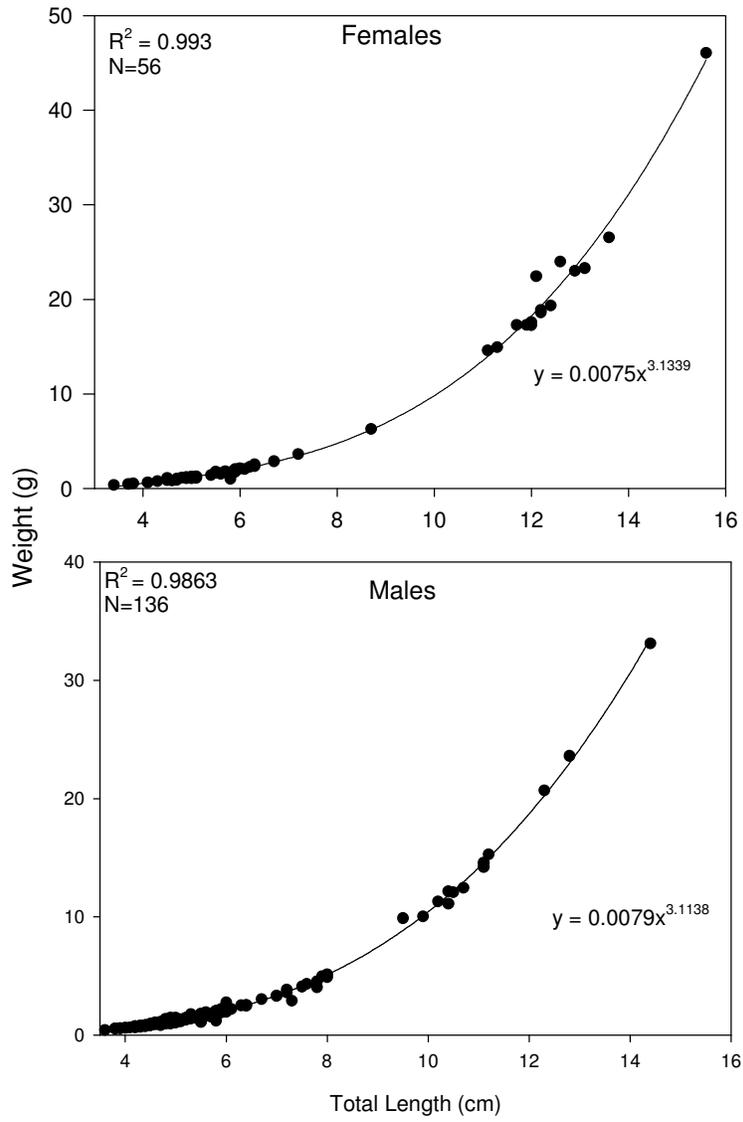


Figure 2.1

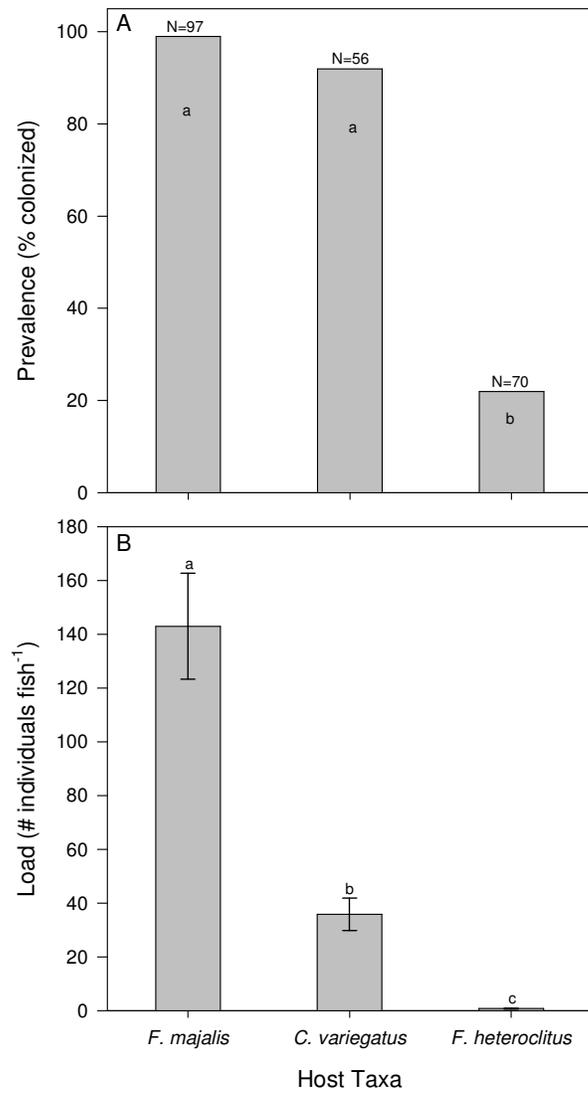


Figure 2.2

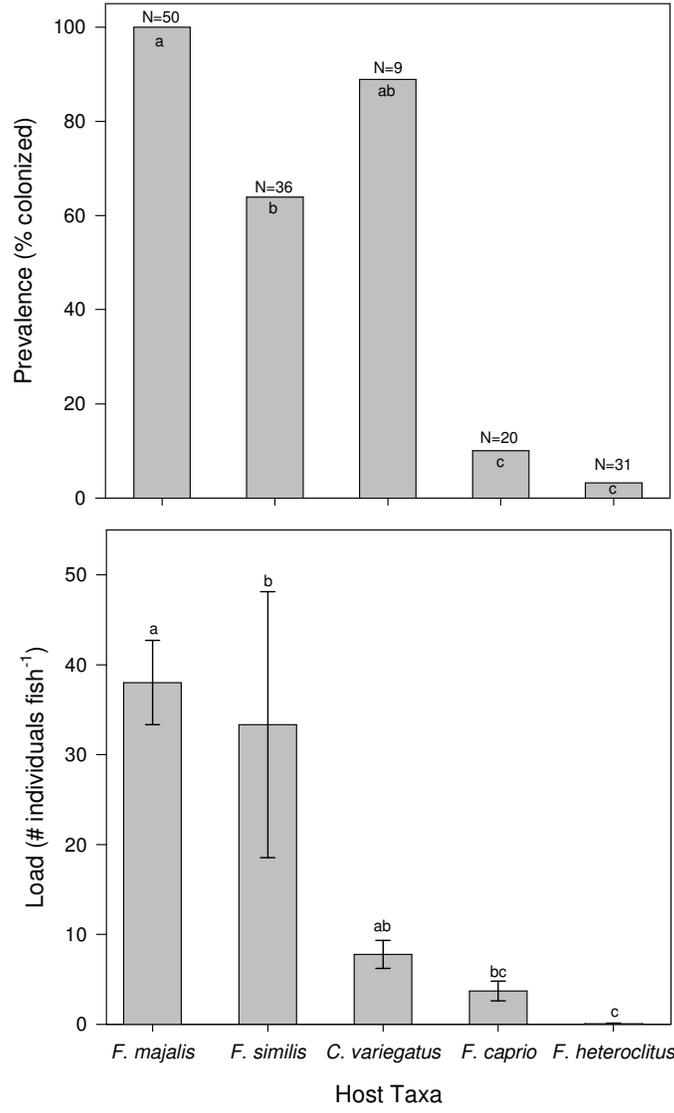


Figure 2.3

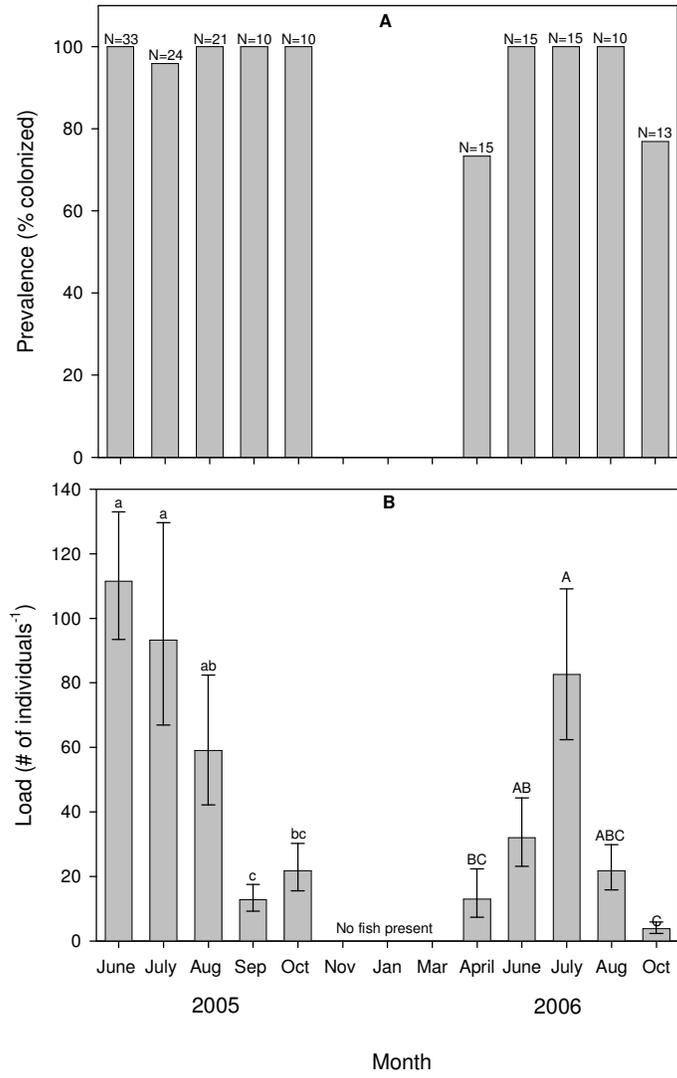


Figure 2.4

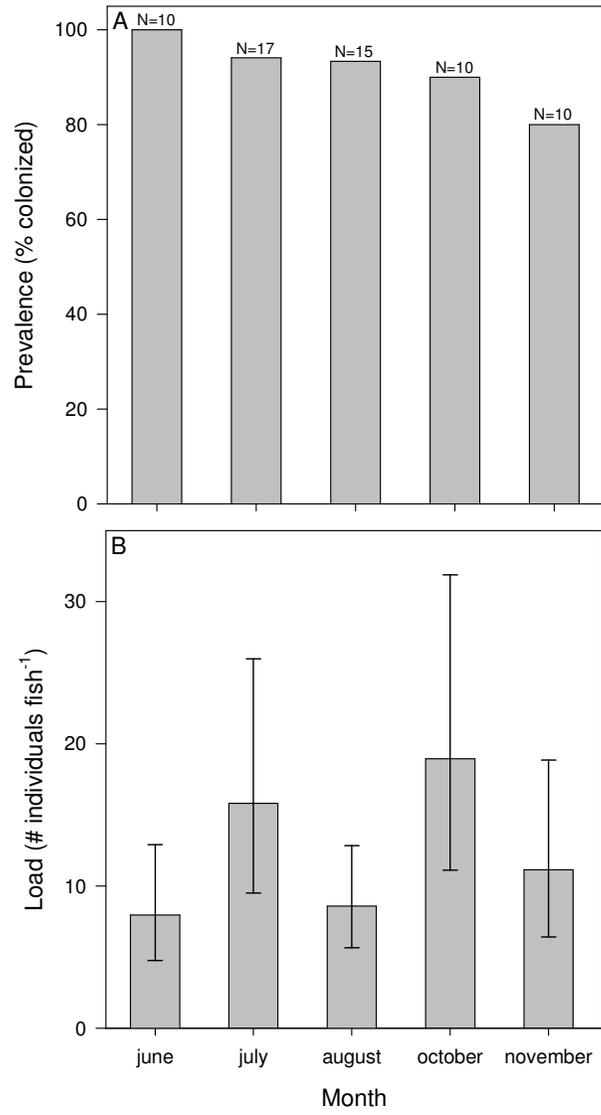


Figure 2.5

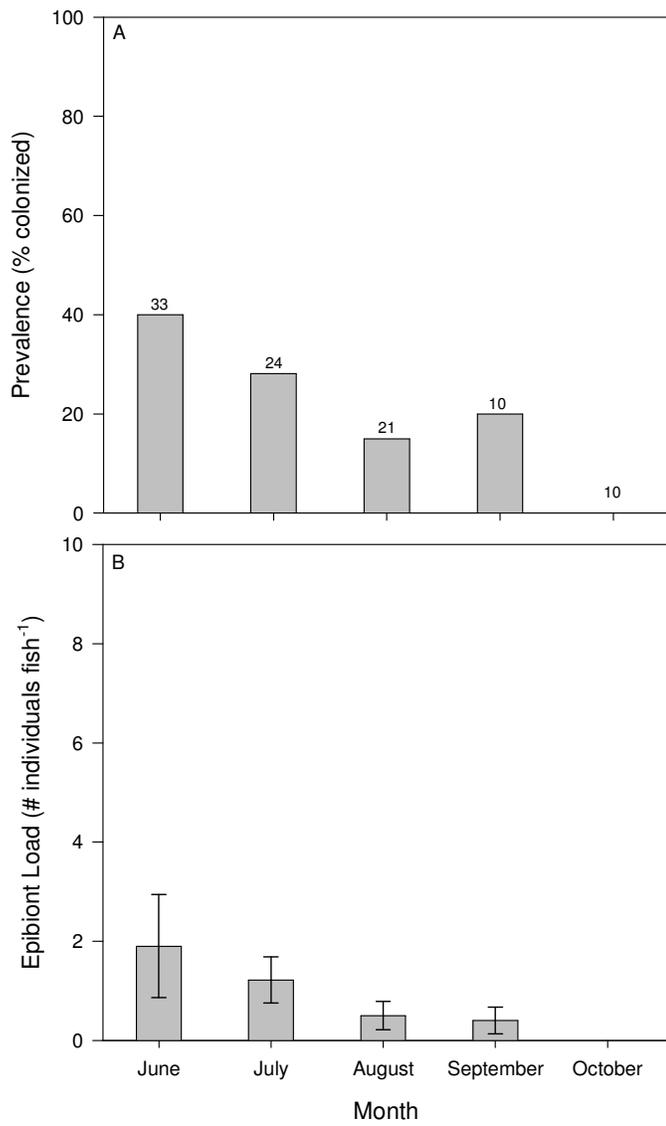


Figure 2.6

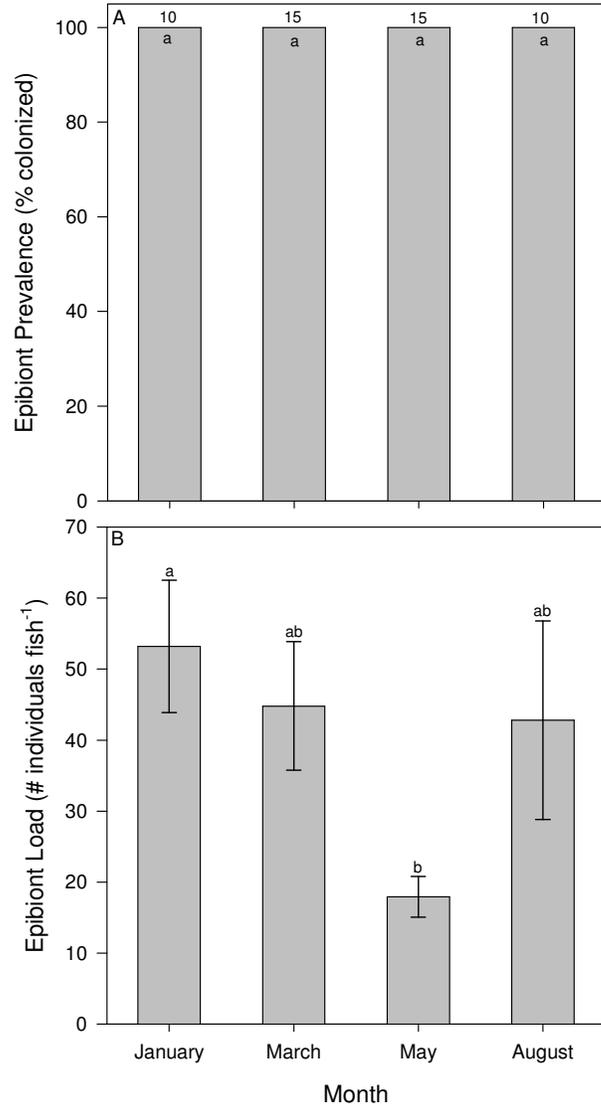


Figure 2.7

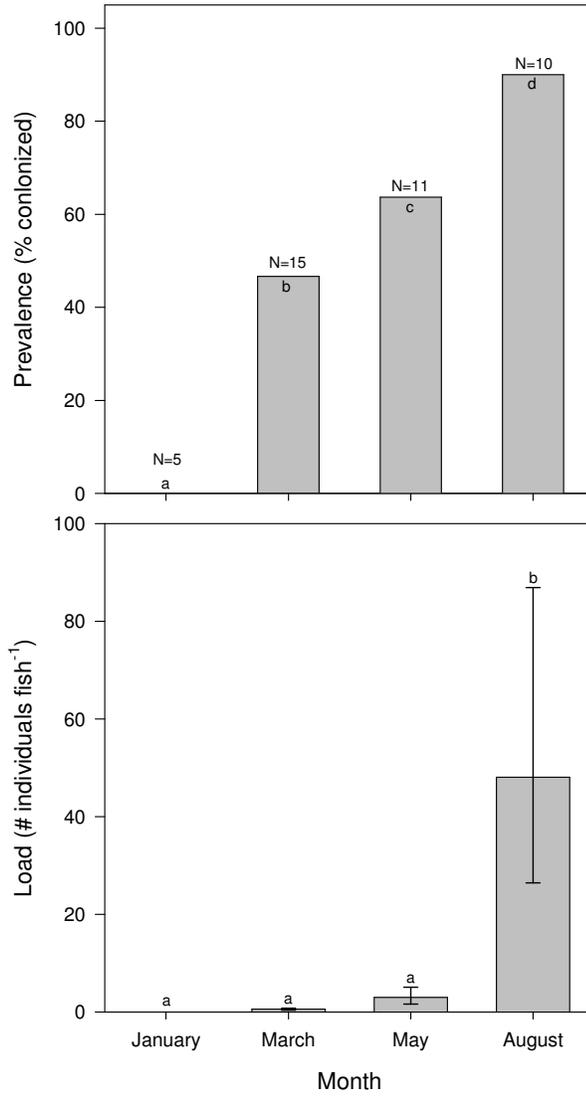


Figure 2.8

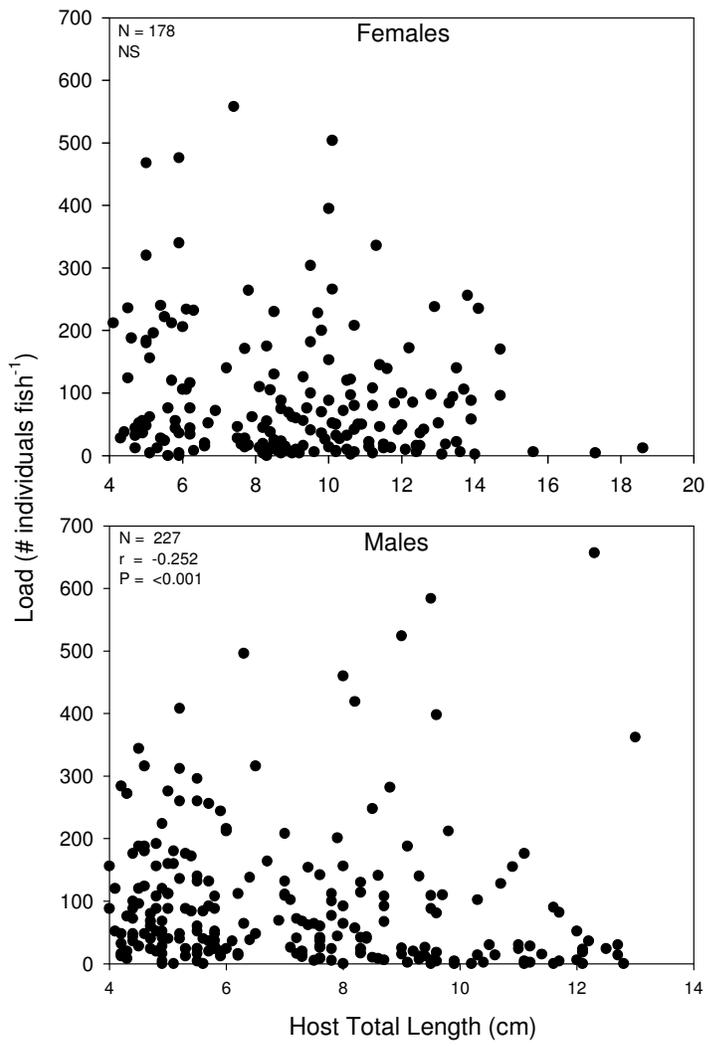


Figure 2.9

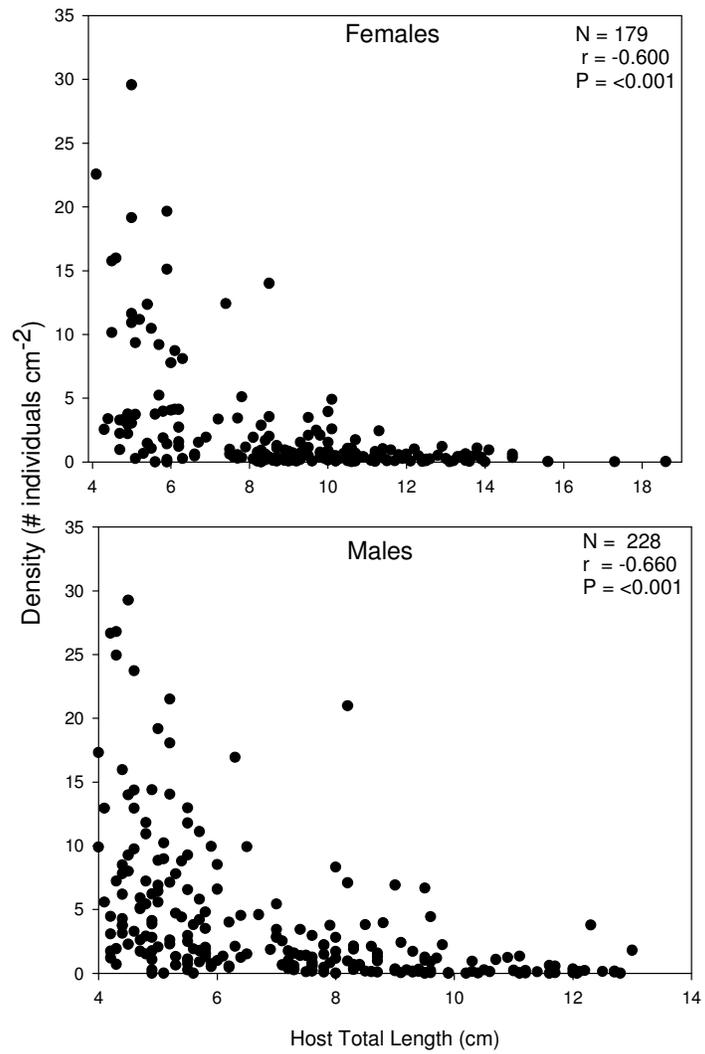


Figure 2.10

CHAPTER 2

The Effect of Light on *Crepidoodinium cyprinodontum* infecting *Fundulus majalis*

ABSTRACT

Crepidodinium cyprinodontum is a photosynthetic dinoflagellate that lives attached to the gills of several genera of small estuarine fish within the families Cyprinodontidae and Fundulidae. Considered by some a parasite and others a commensal, little is known regarding the trophic status of this dinoflagellate. Most photosynthetic dinoflagellates are believed to be capable feeding mixotrophically when faced with a decrease in resource availability, however, it is not known if *C. cyprinodontum* is mixotrophic. This study was undertaken to assess the importance of light on growth and the rate of change in epibiont load of *C. cyprinodontum* attached to the gills of fish (*Fundulus majalis*). A combination of outside incubations and laboratory experiments were conducted with fish and epibionts held at various irradiances to test the hypothesis that light availability would influence *C. cyprinodontum* numbers per fish and growth of attached trophonts. Comparisons across six light treatments in outside incubations indicated the rate of change in epibiont load in the dark decreased rapidly relative to all other treatments. In laboratory incubations, the same pattern was observed as detached epibionts occurred quickly in the dark. While cumulative epibiont biomass recovered over the 9-day incubation differed between light and dark treatments, neither differed significantly from trophont biomass at the beginning of the experiment (T_0). Biomass of tomonts formed during the experiment, however, was greater than that of trophonts at T_0 . Also, biomass of trophonts remaining attached to gills at the end of the experiment was greater than that at T_0 . Results of the field and laboratory studies indicated that *C. cyprinodontum* is an obligate phototroph, as it appears unable to acquire sufficient nutrition from its host to offset basic metabolic demands in

the dark. While *C. cyprinodontum* may not gain nutrition from its host at all, the possibility that it gains some small advantage at the expense of the host can not be eliminated. Thus, the possibility still remains that *C. cyprinodontum* is a mixotrophic dinoflagellate

INTRODUCTION

Dinoflagellates as a group inhabit pelagic and benthic communities in marine, estuarine, and freshwater systems around the world. Their ecological relevance is driven not only by their ubiquity, but also by their trophic diversity, as dinoflagellates are well known to play important roles as primary producers, predators, prey, and symbionts (Taylor 1987). Traditionally, dinoflagellates were divided into two distinct clades; members of one branch possessed chloroplasts and were believed to survive solely by photosynthesis, while members of the other branch lack pigments (e.g. lack plastids) and gained energy via heterotrophy. Doubt was cast on this parsimonious classification, as evidence of prey capture and ingestion in plastid containing dinoflagellates were observed in the early 20th century (reviewed by Gaines and Elbrachter 1987). Currently, it is believed that most photosynthetic dinoflagellates are capable of feeding when faced with a decrease in resource availability (Schnepf and Elbrachter 1992, Jones 1994, Stoecker 1998).. Today, dinoflagellates are recognized as one of the most trophically diverse groups of plankton organisms, with mixotrophic members represented in both free-living and symbiotic lineages (Coats 1999, Stoecker 1999, Jeong et al. 2005).

One role that photosynthetic dinoflagellates play in aquatic environments is that of symbionts (Taylor 1987, Larsen 1992). Examples of dinoflagellate symbiosis range from mutualistic species like *Symbiodinium* (zooxanthelle), where photosynthetic products are shared with the host (Battey 1992, Loram et al. 2007), to lethal parasites capable of causing large-scale mortality in host populations (Overstreet 1982, Shields

1994, Skovgaard 2006). Mutualistic species have long been seen as important in host populations dynamics, as loss of symbionts often results in host stress or death (Glynn 1996, Smith 2005). Parasitic dinoflagellates have only recently been recognized for the degree to which they can regulate host abundances (Coats and Heisler 1989, Coats 1996, Messick and Shields 2000, Coats and Park 2004).

While the majority of parasitic dinoflagellates are heterotrophic, several genera within the group possess chloroplasts at some life-history stage (Cachon and Cachon 1987, Coats 1999). Little is known regarding the extent to which these “photo-parasites” rely on photosynthesis for growth and survival. Estimates of the contribution of photosynthesis to the trophic demand of parasitic dinoflagellates exists for only one species. *Blastodinium* sp., an endoparasite inhabiting the gut of copepods, may satisfy up to 50% of its metabolic demands through photosynthesis (Pasternak et al. 1984).

Plastids, however, are most commonly found in ectoparasitic dinoflagellates with several genera, such as *Cystodinium* and *Stylodinium*, believed to be primarily photosynthetic (Chacon and Chacon 1987). Mixotrophy, however, may be hard to detect in photosynthetic dinoflagellates, as chloroplasts or other cellular inclusions may disguise evidence of ingestions (Stoecker 1999). To what degree these plastid containing dinoflagellate parasites rely on phototrophy and/or heterotrophy is an unknown.

Feeding in mixotrophs has been reported to result in several potential benefits, including acquisition of supplemental carbon, limiting macronutrients, or growth factors (Jones 1994, Stoecker 1999). Conceptually, mixotrophic species are thought to occupy points along a continuum of nutritional strategies (Jones 1994). On one end of this spectrum exists species that survive primarily by photosynthesis, while on the opposite

end are those mixotrophic species that are primarily heterotrophic. Despite their different trophic strategies for energy acquisition, organisms at both ends of the spectrum are hypothesized to switch trophic modes in response to a decrease in resource availability. Phagotrophic algae, as defined by Stoecker (1998), are those mixotrophs that rely primarily on photosynthesis, but are capable of acquiring dissolved inorganic nutrients or carbon via prey capture. Often environmental factors, such as inorganic nutrient concentrations and/or light availability, may influence phagotrophy in mixotrophs (Smalley et al. 2003, Caron et al. 1990, Li et al. 1999). For many phagotrophic algae, light intensity may play a key role in influencing feeding behavior (Hansen 1996, Skovgaard 1996, Stoecker et. al 1997, Legrand et al. 1998).

Crepidodinium cyprinodontum is a photosynthetic dinoflagellate that lives attached to the gills of several genera of small estuarine fish within the families Cyprinodontidae and Fundulidae (Lawler 1967, 1968a,b, 1980, Dillon Williams 1972, Lom & Lawler 1973). Unlike other fish-associated dinoflagellates, *C. cyprinodontum* appears not to damage host tissue and reportedly leads a strictly phototrophic existence. Lack of direct evidence regarding trophic status of *C. cyprinodontum* has led to this symbiont being classified as a parasite and as a commensal (Lawler 1967, 1968a,b, Rogers & Gaines 1975, Lom and Lawler 1973, Cachon and Cachon 1987). Known host species for *C. cyprinodontum* live primarily in shallow, high-light habitats, suggesting light availability may be a critical factor in determining distribution of the symbiont in the environment. Despite the photosynthetic nature of *C. cyprinodontum* throughout its entire life cycle (i.e., attached “feeding” stage (trophont), detached division stage (tomont), and dispersal

stage (dinospore) this species has never been reported independent of its host in field samples.

This study was undertaken to assess the importance of light to growth and the rate of change in epibiont load of *C. cyprinodontum* attached to the gills of fish (*Fundulus majalis*). I hypothesized that light availability would influence *C. cyprinodontum* numbers per fish and growth of attached trophonts. To evaluate the effect of light on the rate of change in epibiont load of *C. cyprinodontum* on gills, I measured the change in epibiont numbers per fish over a 9-day period at six different irradiances, ranging from full sunlight to complete darkness. To assess the impact of light on growth of *C. cyprinodontum*, I measured changes in epibiont biomass over a 9-day period in high light and complete darkness.

MATERIALS AND METHODS

Experimental Design

Outside incubations. To assess rate of change in epibiont load of *C. cyprinodontum* on the gills of *F. majalis* over various irradiances, outside incubations of fish and epibionts were conducted in a flow-through sea-water system supplied with water drawn from the Fort Pierce Inlet at the Smithsonian Marine Station (SMS), Ft. Pierce, FL. Fort Pierce Inlet is located on the Atlantic Coast of Florida and connects the Atlantic Ocean to the Indian River Lagoon of the Intercoastal Waterway. Fish and epibionts used in the incubations were collected from Tolomato River located just North of Saint Augustine Inlet. Fish captured by hand held seine were pooled to provide a single experimental population and transported to SMS in aerated site water. Fish were

then transferred to the flowing seawater system at SMS and allowed to acclimate for 24 hours. Following acclimation, fish were randomly assigned to one of six treatments, with each treatment consisting of one 1362-liter fiberglass raceway screened to provide a different irradiance; 100%, 75%, 48%, 28% 17% and 0% (dark) incidence PAR. Each treatment (tank) contained three replicate sub-tanks (60 liters each) to which 50 fish were randomly assigned for a total of 150 fish per treatment. Each sub-tank was equipped to receive its own flow-through water to ensure independence between replicates. Total weight of fish in each sub-tank was determined to compare biomass across replicates and treatments.

At 3-day intervals, five fish from each sub-tank were sacrificed and gill baskets removed for dissection. Sex, weight, and total length of each fish were recorded prior to being sacrificed. Gills from the right and left side of each fish were kept in separate Petri dishes containing GF/C filtered site water. One gill from each side was arbitrarily chosen for determination of prevalence and load of *C. cyprinodontum*. Counts per gill were then summed and multiplied by four to determine total number of epibionts per fish. All specimens present on all filaments of both the anterior and posterior hemibranches were counted within five minutes of host death, using a stereomicroscope (10-50X total magnification).

Laboratory incubations. To assess the fate of *C. cyprinodontum* on *F. majalis* in high light and darkness, symbionts and hosts were collected from shallow water of Sinepuxent Bay, Maryland, USA using a seine. Upon capture, fish were immediately placed in containers filled with site water and sorted by relative size classes (small, medium, large). Forty individuals from the small size class (≤ 6 cm) were then selected

and transported to the laboratory in a tank containing 50 liters of aerated site water. Upon returning to the lab, 11 fish were set aside for estimates of *C. cyprinodontum* starting abundance and biomass. Of the remaining fish, 20 were randomly assigned to each of two light treatments (400 $\mu\text{E m}^2\text{s}^{-1}$ on a 14:10 light:dark cycle; 24-h darkness) established in Percival incubators at 24°C. These light levels were chosen as they represent the extremes of irradiances from outside incubations described above. Each fish was held in a collection vessels consisting of two 800-ml plastic beakers, one placed inside the other, with the bottom of the inner beaker removed and replaced by ¼ inch netting. This allowed for separation of fish and detached tomons and easy transfer of fish.

Collection vessels were filled with 550 ml of artificial seawater formulate using sterile distilled water and Instant Ocean to provide salinity of 25, matching salinity of water at time of collection, with all water aerated for at least 4 hours before addition of fish. A 13 x 15 cm piece of clear Plexiglas was placed on top of collection vessels to prevent evaporation and fish escape. A small hole (~ 8 cm) was placed in the center of each piece of Plexiglas through which flexible air-line tubing was fed. Water in collection vessels was aerated using Tetra Whisper 20/60 Air Pumps connected to a 5 way air valve. Small air stones (2.5 cm) were placed just below the surface of the water to minimize possible re-suspension of detached tomons. A control beaker (one per treatment) was used to ensure that all *C. cyprinodontum* collected over the course of the incubation originated solely from host gills. For each control, a randomly selected fish was placed in a beaker containing one liter of aerated artificial seawater for five minutes

and then removed. Controls were then samples over the course of the incubations for presence/absence of *C. cyprinodontum*.

As emergence of dinospores from division cysts can occur within 22 hours after detachment of trophonts (Lawler 1967), sampling occurred every 12 hours to minimize possible recruitment of dinospore to host gills. At each time point, the inner beaker with fish was gently removed and placed into a new beaker containing artificial seawater. Water from which fish were removed was then gently swirled to dislodge any tomonts that may have adhered to the bottom of the beaker and dispensed to 600-ml sample bottles for fixation. Detached tomonts were preserved with CaCO₃- buffered formalin (1% final volume) and stored in the dark at 4°C until processing. For each fish, 12-h samples were combined to yield a sampling interval of 24 h. To enumerate tomonts that had detached during the 24 h intervals, a minimum of 500 milliliters of sample was filtered onto a 47-mm black nucleopore filter (3 µm pore-size) under low vacuum (< 10 mm Hg). Each filter was then placed on a large glass slide and the number of *C. cyprinodontum* counted by scanning the entire filter at 100X using an Olympus inverted microscope equipped with epifluorescence optics (450-490 nm excitation; 510 nm splitter; 520 nm barrier filter). Control beakers were sampled every 24 hours, with 100 milliliter aliquots of water removed after gentle stirring. Water was then processed as above and filters scanned for presence/absence of fluorescing cells. Digital image analysis (Zeiss Axiocam and Axiovision software) was used to determine biovolume of tomonts. Length and width of detached cells (≤ 20 cells/sample) were recorded and biovolume (μm^3) calculated assuming tomont shape as a prolate sphere.

Cell biovolume was then converted to biomass (pg C cell^{-1}) using the following conversion factor; $0.760 \times (\text{cell volume})^{0.819}$ after Menden-Deuer and Lessard (2000).

For estimates of starting biomass of trophonts, gills scored for initial epibiont load were preserved in CaCO_3 -buffered formalin (1% final concentration) and trophonts carefully dislodged from gills with a dissection needle. Cells were then settled in 10-ml settling chambers for one hour and cell carbon content determined as above. Fish were sacrificed at the end of the nine day incubation to determine final biomass and number of trophonts remaining on gills and processed as above.

Quantitative procedures

Outside incubations. Rate of change in epibiont load of *C. cyprinodontum* for each sub-tank within a treatment was determined as the slope of linear regression of natural log transformed data for load plotted against time (days). Slopes were then averaged across sub-tanks to generate a treatment mean and standard error. Mean treatment slopes were then plotted against incoming irradiance and compared by One-way ANOVA (SigmaStat 2.0).

Laboratory incubations. Percent cells detached from gills in light and dark treatments were determined for each fish over the 9-day incubation period. Percent cells detached per day for each fish was calculated by dividing the total number of cells recovered per day by the sum of tomons collected over course of the incubation plus the number of trophonts remaining on gills at final sampling point. Percents are reported as means using data for 10 replicate fish per treatment. Treatment release rates were determined as the slope of the linear regression of natural log transformed data plotted

against time, with the x intercept representing maximum trophont residence time on gills. Treatment slopes were then compared by One-way ANOVA (SigmaStat 2.0).

All data are reported as means \pm standard error of the mean (SE). Comparisons of mean cell numbers and biomass recovered per day in light and dark treatments were analyzed by paired student's T-test (SAS) on log-transformed data. Total biomass accumulated in light and dark treatments and that occurring on gills at time zero were compared by One-way ANOVA (SigmaStat 2.0).

RESULTS

Rate of change in epibiont load

Outside incubations. Linear regression of epibiont load versus incubation time showed significant negative slopes for three (i.e., 0, 17, 28 % incident irradiance) of the six treatments (Fig. 3-1). Comparisons among regression slopes across treatments showed significant differences in the rates of change in epibiont load (Fig. 3-2). Epibiont load in the dark decreased more rapidly than all treatments (ANOVA, $P < 0.05$). Epibiont load at 17 % incidence PAR decreased faster than in treatments having greater than or equal to 40 % incoming light. The remaining four treatments showed no significant difference in the rate of change of epibiont load.

Growth of epibionts in the light and dark

The mean number of tomons recovered from fish held in the dark was highest (~ 30 cells/fish) during the first two days of the experiment and decreased asymptotically thereafter (Fig. 3-3). By contrast, mean values for fish held at $400 \mu\text{E m}^{-2} \text{s}^{-1}$ increased to a peak on Day 3 (15 cells/fish) and then declined gradually over the remainder of the

incubation. Mean number of cells recovered per day differed significantly (paired t-test, $P < 0.05$) between treatments on all days except Day 3 (paired t-test, $P = 0.31$). Mean values for the dark treatment on Days 1 and 2 were significantly higher than for fish incubated in the light (paired t-test, $p < 0.05$). Following Day 3, this trend reversed, with mean values being significantly higher for the light treatment (t-test, $P < 0.05$). Mean load of *C. cyprinodontum* remaining on the gills at the end of the experiment (66 ± 21.53 and 0.8 ± 0.51 , in light and dark, respectively) differed significantly (Kruskal-Wallis One way ANOVA on ranks, $P < 0.001$).

The percent of *C. cyprinodontum* remaining on gills in both the light and the dark treatment decreased steadily over the course of the 9-day incubation (Fig. 3-4). Release rate of trophonts from gills (0.114 and 0.501, in light and dark, respectively) was significantly faster in the dark (One-way ANOVA, $P < 0.05$). Maximum residence time of trophonts on gills was estimated at 41 and 9 days in light and dark, respectively. No *C. cyprinodontum* were encountered in controls over the incubation.

Mean epibiont load at start of experiments (Fig. 3-5) was significantly greater than the mean number of *C. cyprinodontum* recorded as recovered tomonts plus trophonts remaining after nine days in the dark (One-way ANOVA on log transformed data, $P < 0.03$), but not mean epibiont load in the light (One-way ANOVA on log transformed data, $P > 0.05$). Mean values of trophonts plus tomonts for light and dark treatments did not differ.

Trophont biomass at start of experiment averaged $1.1 \times 10^5 \pm 1.5 \times 10^4$ pg C cell⁻¹. Mean tomont biomass released from fish each day varied during the incubation but showed no significant differences within or across treatments (Fig. 3-6). Biomass for

tomonts recovered for light and dark treatments, averaged $5.1 \times 10^5 \pm 1.1 \times 10^5$ and $4.2 \times 10^5 \pm 7.2 \times 10^4$ pg C cell⁻¹, respectively, with both values significantly higher than mean trophont biomass at T₀ (One-way ANOVA on log transformed data; P < 0.05). Biomass of trophonts remaining on gills at the end of the experiment averaged $1.1 \times 10^6 \pm 2.96 \times 10^5$ and $3.0 \times 10^4 \pm 2.03 \times 10^4$ pg C cell⁻¹ for light and dark treatments, respectively, and were statistically different (Kruskal-Wallis One-way ANOVA on Ranks, P < 0.0001). Furthermore, trophonts remaining on fish held in the light for nine days had significantly higher biomass than trophonts at T₀ (One-way ANOVA; P < 0.05).

Mean biomass of *C. cyprinodontum* present at the start of the experiment (T₀) did not differ significantly from that recorded over light or dark incubations as recovered tomonts, plus trophonts remaining after nine days (Fig 3-7; One-way ANOVA; P > 0.001). Mean biomass recorded for dark treated fish, however, was significantly lower than for fish held in the light (One-way ANOVA, P < 0.05).

DISCUSSION

Outside incubation of *Crepidodinium cyprinodontum* in flowing seawater baths screened to provide a range of irradiances show relatively rapid decline in epibiont load below 30% incident PAR, with no significant change at higher light levels. These observations suggest that *C. cyprinodontum* requires intermediate to high light to survive, a conclusion consistent with observations that *C. cyprinodontum* colonizes fish species that typically inhabit high light environments (Briggs and O'Connor 1971, Kneib and Wagner 1994, Teo and Able 2003, Chapter 2 of this thesis). An alternative interpretation, however, is that low light stimulated epibionts to transformation from trophonts to tomonts in order to produce dinospores capable of infecting new hosts. Such a response could be a survival strategy enabling *C. cyprinodontum* to abandon injured or dead hosts in favor of host individuals able to maintain a position near the surface. The failure of epibiont load to increase at higher light levels suggest that *C. cyprinodontum* has a very long generation time, although a balance between tomont formation and dinospore colonization of host can not be eliminated.

To explore alternative explanations for changes in epibiont load during the outdoor experiment, I conducted a laboratory study to follow the formation of tomonts and assess changes in epibiont biomass during incubation of fish under high light and in darkness. High numbers of tomonts formed quickly (within 2 days) when fish were held in the dark, but not when fish were held at high light. Rather, the daily formation of trophonts increased gradually in the light, with mean values for the first two days of the experiment being significantly lower than for the dark treatment. This observation

strongly indicates that prolonged darkness is a cue for *C. cyprinodontum* to abandon host organisms. Mean biomass of tomons recovered during light and dark treatments were comparable, but significantly higher than mean biomass of trophonts present on gills at the start of the experiment. Thus, low light does not appear to affect all trophonts equally. Rather, larger than average size trophonts appear to transform into tomons in either light or dark conditions. That primarily larger trophonts appear competent to abandon hosts suggest a life-history strategy to maximize tomont biomass and thus reproductive output in the form of dinospores capable of infecting new hosts.

The number of epibionts forming tomons and persisting as trophonts in the dark over the 9-day laboratory study was significantly lower relative to initial epibiont load, suggesting mortality of some *C. cyprinodontum* cells. Thus, patterns observed in the outdoor experiment probably reflect loss of *C. cyprinodontum* through a combination of host abandonment and epibiont death. The number of epibionts forming tomons and persisting as trophonts in high light treatment was not significantly different from initial epibiont load, suggesting that *C. cyprinodontum* either does not undergo binary fission when attached to gills, or has a very slow growth rate. Thus, persistence of *C. cyprinodontum* on fish at intermediate to high light during the field study probably reflects slow growth accompanied by low abandonment of host organisms.

Total epibiont biomass at the start of the laboratory experiment was not significantly different from biomass recovered from fish either as tomons or as trophonts in light and dark treatments. Total epibiont biomass, however, did differ between light and dark treatments, suggesting differences in growth of *C. cyprinodontum* in the two conditions. Interestingly, mean biomass of trophonts remaining on fish held at high light

for nine days, was significantly greater than biomass of trophonts at the start of the experiment. That observation, along with the larger than average per cell biomass of tomonts formed in high light, relative to T_0 trophonts, indicates some growth of epibionts during the incubation. That possibility must be viewed cautiously, for, as indicated above, total epibiont biomass recovered in light treatments did not differ from trophont biomass at T_0 .

The experimental design used to follow *C. cyprinodontum* numbers and biomass in light and dark treatments prevented recolonization of host and subsequent growth of epibionts. Thus, estimation of growth using an exponential model will underestimate growth rate and overestimate doubling time. Recognizing that short coming, biomass specific growth rates can be calculated using the following exponential growth equation;

$$\mu = \frac{(\ln \sum \text{pg } C_f - \ln \sum \text{pg } C_o)}{t_f - t_o} \quad (1)$$

where $\sum \text{pg } C_o$ is the total biomass at time zero (t_o) and $\sum \text{pg } C_f$ is the within treatment sum of biomass collected over the duration of incubations (t_f), including trophonts remaining on fish gills at final sampling period. Net growth rates for light and dark treatments calculate as 0.049 and - 0.055 pg C d^{-1} , respectively, resulting in a doubling time of approximately 14 d for *C. cyprinodontum* held at high light. Another approach to calculating growth of the epibiont is to utilize the rate of tomont formation, tomont biovolume, and dinospore biovolume to estimate epibiont generation time. Dividing mean tomont biovolume by average dinospore biovolume ($337 \pm 29.8 \text{ pg C cell}^{-1}$, unpublished data) gives an estimate for the number of dinospores that would be produced, and thereby an estimate for number of dinospore doublings. Dividing maximum trophont development time (i.e. maximum residence time on gills from Fig. 3-

4) by number of dinospore doublings required to form tomons yields an estimate of epibiont doubling time. Using this model provides an estimate of approximately 6 d for doubling time of *C. cyprinodontum* held in the light. A short coming of this approach is that it ignores possible growth of the photosynthetic dinospore. Were dinospores to increase in size prior to colonization of the host, then doubling time would be underestimated. While neither approach for calculation doubling time for *C. cyprinodontum* is ideal, the values likely represent upper and lower extremes and thus bracket actual values. Comparing calculations for epibiont growth in high light with doubling times of phototrophic, heterotrophic, or mixotrophic dinoflagellates (Fig. 3-8) indicates that *C. cyprinodontum* grows more slowly than its free-living relatives. This conclusion is supported by data from outside incubations where no net increase in *C. cyprinodontum* occurred in high light treatments. Interestingly, this lack of increase in epibiont load at optimal light levels in flow through tanks suggests release and recolonization rates of *C. cyprinodontum* were equal, assuming no binary fission on epibiont on gills.

Results of the field and laboratory studies indicated that *C. cyprinodontum* is an obligate phototroph, as it appears unable to acquire sufficient nutrition from its host to offset basic metabolic demands. Indeed, *C. cyprinodontum* may not gain nutrition from its host at all, however, the possibility that it gains some small advantage at the expense of the host can not be eliminated. Thus, the possibility still remains that *C. cyprinodontum* is a mixotrophic dinoflagellate.

Stoecker (1998) classified mixotrophic protists according to their reliance on phototrophy and heterotrophy, with changes in trophic modes driven by decreases in

resource availability. Ideal mixotrophs (Model I) are those species that are equally proficient at growing phototrophically or heterotrophically. Currently, few species appear to be model I mixotrophs. Model II mixotrophs are obligate phototrophs capable of phagotrophy to acquire macronutrients, growth factors, and/or to help meet carbon demands. Feeding in type II mixotrophs occurs when nutrients or light becomes limiting. Mixotrophs falling into Model II are further subdivided into three categories. Model IIA mixotrophs are those that feed to acquire macronutrients, usually as a response to nutrient limitation, while Model IIB mixotrophs are species that feed to acquire limiting growth factors. In Model IIC, ingestion of prey is a source of carbon and occurs due to light limitation and associated decrease in photosynthesis. Model III mixotrophs are primarily heterotrophic species that exhibit little or no growth in the absence of prey. They may have their “own” plastids and turn to photosynthesis to acquire carbon when prey are limiting, or they may have photosynthetic symbionts or plastids sequestered from prey, yet must feed to sustain growth. If *C. cyprinodontum* is a mixotroph, then it could only qualify as a Model II mixotroph, possibly gaining macronutrients, growth factor, or carbon from its host.

Attenuation of light by the operculum of host organisms likely provides little light for photosynthetic gill epibionts like *C. cyprinodontum*, even when fish live in shallow, high-light environments. The heavy pigmentation and large, numerous plastids of *C. cyprinodontum* trophonts (Lom and Dykova 1993, Lawler 1967), suggests that this obligate phototroph is adapted to low light. The very slow growth rate observed for epibionts held at high light, however, indicates that *C. cyprinodontum* is living in a light limited environment. Why then does *C. cyprinodontum* live on fish gills? Obvious

possibilities include refuge from microbial grazers and macrozooplankton, access to a high nitrogen environment provided by excretion of ammonium across fish gills, and reduced competition with other microalgae for nutrient resources. These issues, as well as, definitive resolution of the parasitic or non-parasitic nature of *C. cyprinodontum* await further investigation.

FIGURE LEGENDS

Figure 3.1 Linear regression analysis of changes in epibiont load over 9-day incubation. Only slopes for the lower three light levels were significantly different from zero.

Figure 3.2 Rate of change in *Crepidoodinium cyprinodontum* load on *Fundulus majalis* incubated at different irradiances. Bars with same letters are not significantly different (One-way ANOVA, $P < 0.05$). Data presented as means \pm SE.

Figure 3.3 Mean number of *Crepidoodinium cyprinodontum* tomonts detached from gills per day. Open and closed circles represent light and dark treatments, respectively. Symbols (*) represent significant differences (t-test, $p < 0.05$) between treatments on each day. Data reported as mean \pm SE of the mean.

Figure 3.4 Linear regression of the natural log of percent of *Crepidoodinium cyprinodontum* tomonts collected per day over nine days. The regression line was extended to the x intercept to estimate maximum trophont residence time on gills. Slope values for light and dark differed significantly (One-way ANOVA, $P < 0.05$)

Figure 3.5 Mean load of *Crepidoodinium cyprinodontum* attached to hosts at T_0 compared with number of tomonts recovered during the incubation, plus the number of trophonts remained attached to fish at the end of the experiment. Bars

with different letters are significantly different (ANOVA, $p > 0.05$). Data are reported as mean \pm SE following back transformation.

Figure 3.6 Daily variation in cell biomass (pg C cell^{-1}) of detached *Crepidodinium cyprinodontum* tomonts in the light and dark. Open and closed symbols represent light and dark treatments, respectively. Values for light and dark treatments did not differ significantly on any day (t-test, $P > 0.5$). Data presented as means \pm SE.

Figure 3.7 Mean total biomass of *Crepidodinium cyprinodontum* attached to hosts at T_0 compared with mean total biomass recovered as tomonts during the incubation, plus that remained attached to fish as trophonts. Bars with different letters are significantly different (ANOVA, $p < 0.05$). Data are reported as mean \pm SE

Figure 3.8 Dinoflagellate doubling time as a function of cell volume. \circ from Strom and Morello 1998; \bullet from Banse 1982; \blacktriangledown from Rivkin and Seliger 1981; $+$ from Skovgaard 1998, Hansen & Neilsen 2000, Skovgaard 2000, Jeong et al. 2005, Li et al. 2005. Closed star is *Crepidodinium cyprinodontum* doubling time calculated using exponential model, while open star is doubling time calculated with dinospore volume and maximum residence time on gills. When necessary all rates were adjusted to 20°C using a Q_{10} of 2. For comparisons to Banse's and Strom and Morello's estimates, doubling times of all mixotrophic species and *Pyrocystis* spp. were calculated using maximal growth rate when ≥ 2 estimates were available.

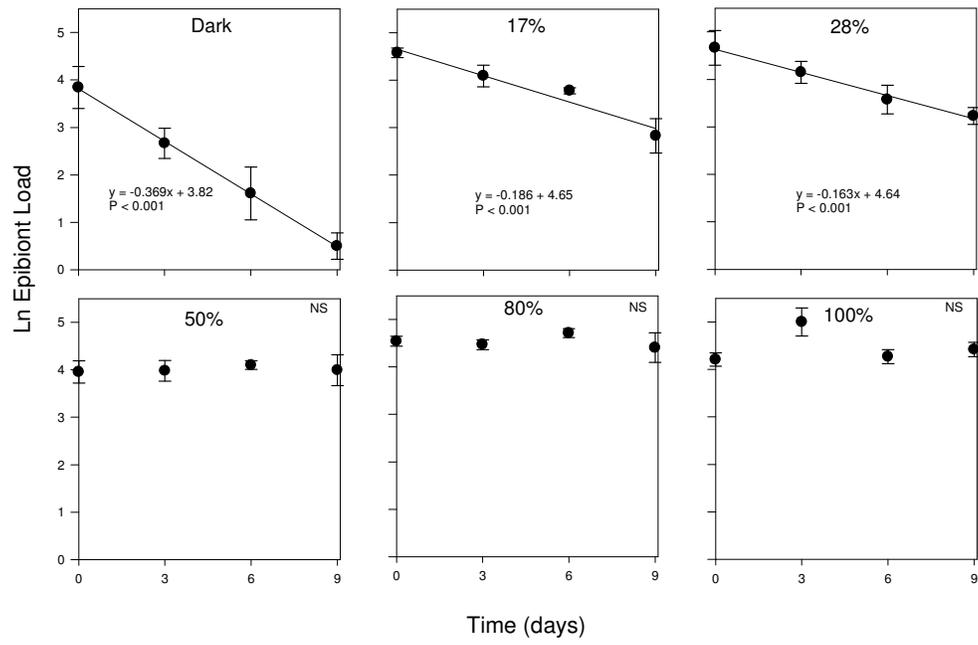


Figure 3.1

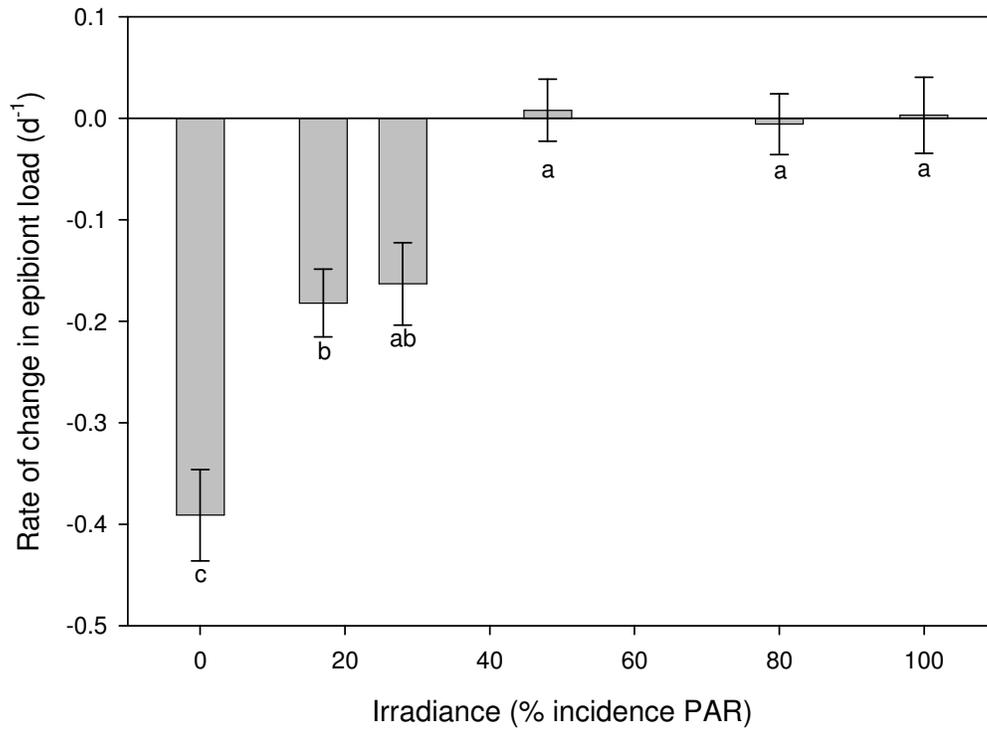


Figure 3.2

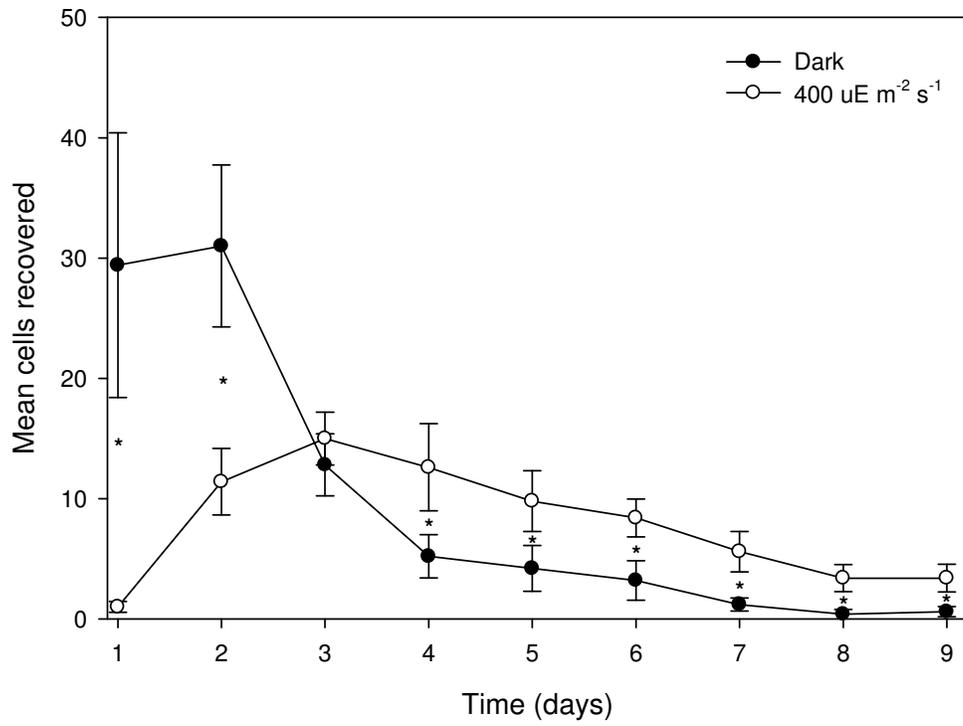


Figure 3.3

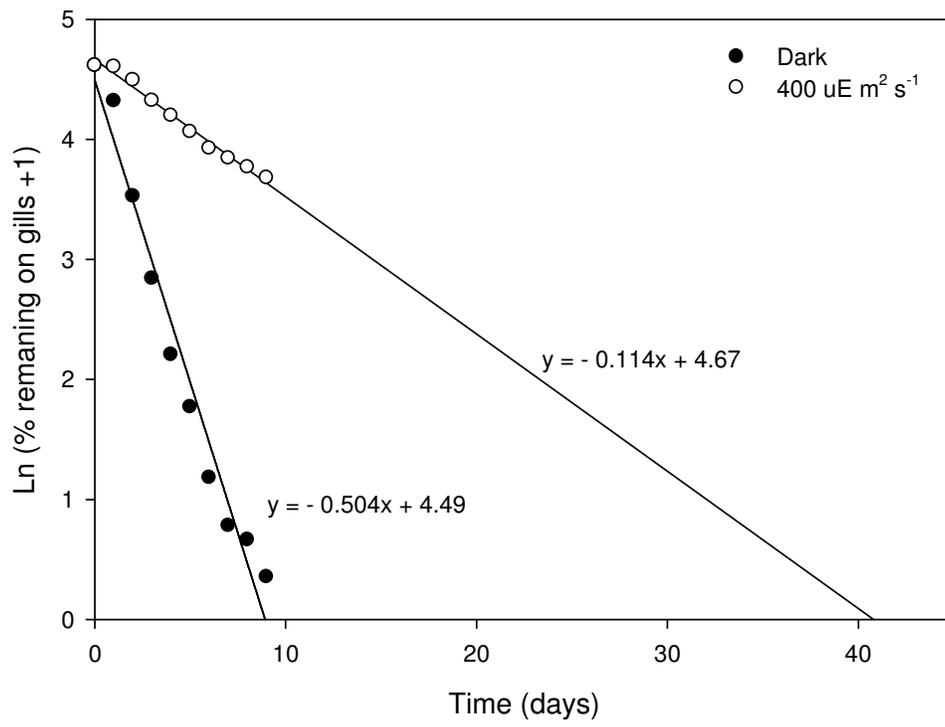


Figure 3.4

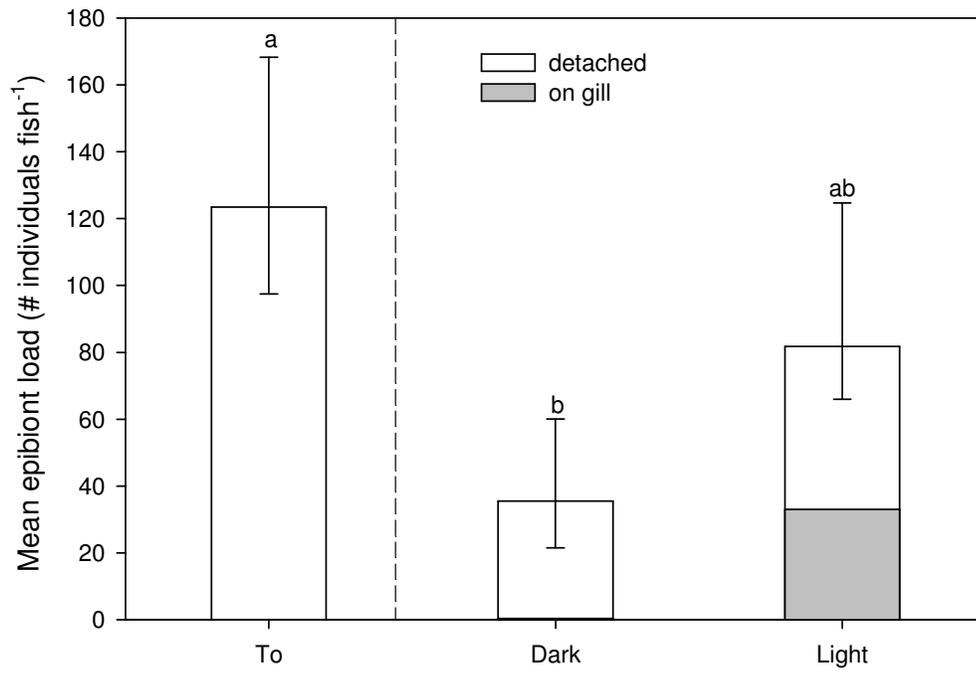


Figure 3.5

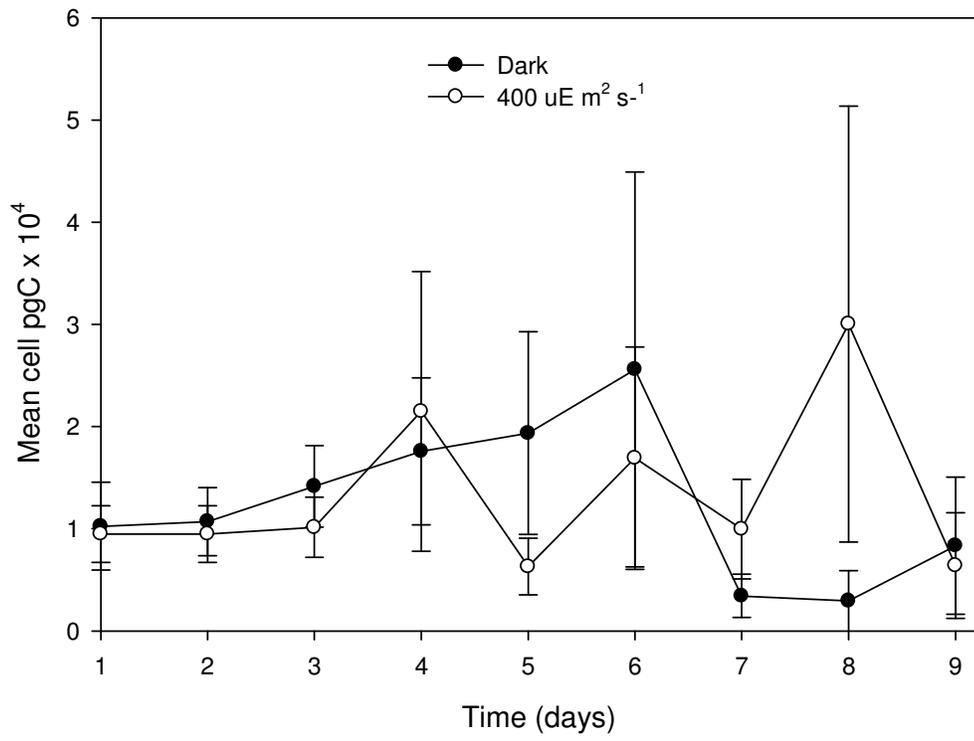


Figure 3.6

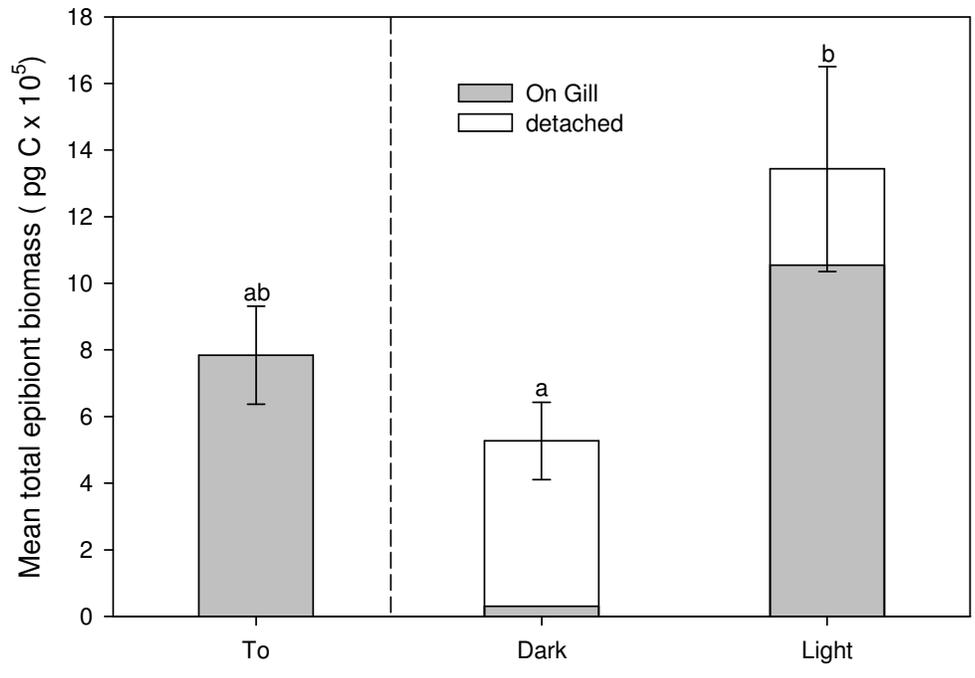


Figure 3.7

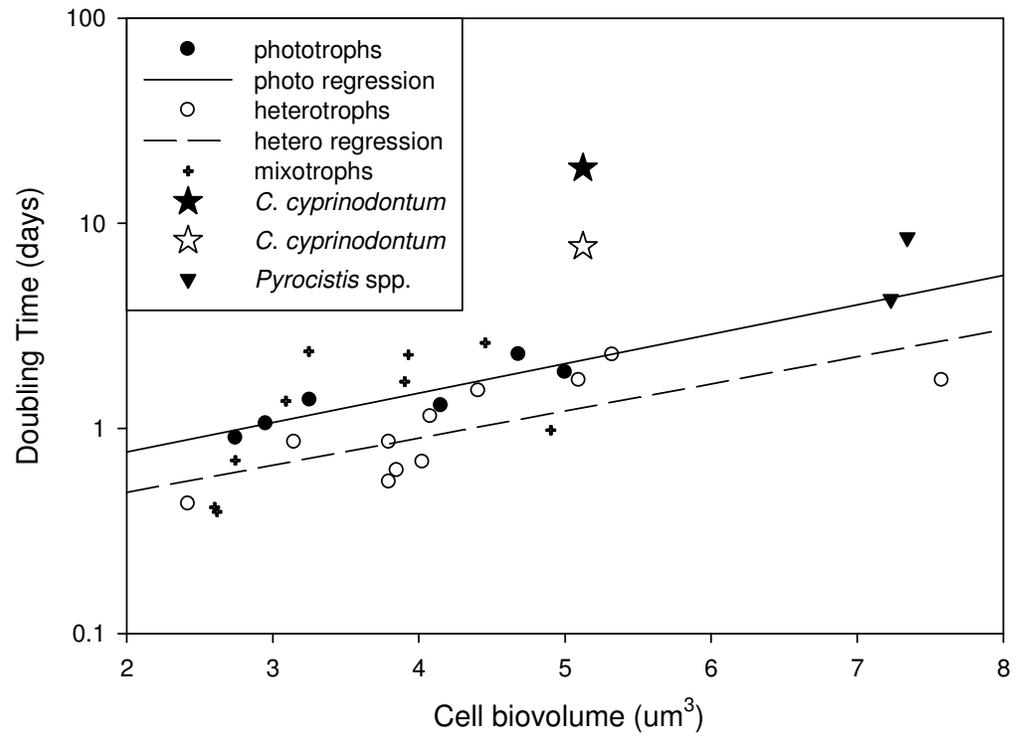


Figure 3.8

CHAPTER 4

CONCLUSIONS

The central goal of my thesis was to better characterize the relationship of *Crepidodinium cyprinodontum* to its host. When I began this project, little data existed on this photosynthetic fish-associated dinoflagellate. While *C. cyprinodontum* had been reported to occur on several host species, little was known regarding its prevalence, load, and seasonal occurrence in different host taxa. Confusions over the trophic status of *C. cyprinodontum* had led to its classification as a parasite by some researchers and a commensal by others. I utilized field observations to determine if *C. cyprinodontum* exhibited patterns of preference among and within host populations, as well as to document its seasonal distribution. I chose two distinct geographical study sites from which to sample, one located in Maryland (Sinepuxent Bay) and the other in Florida (Tolomato River). I applied standard parasitological techniques including determining prevalence, load, and density of *C. cyprinodontum* in host populations. Experimental manipulations were then used to determine the effect of irradiance on persistence and growth of *C. cyprinodontum* attached to fish.

Field observations indicated that *C. cyprinodontum* was widely distributed in host populations in Maryland and Florida sites, with infection intensity highly variable among host taxa. Prevalence and load of *C. cyprinodontum* reported in this study are much higher than values previously reported by other investigators. Comparison of epibiont load across host taxa revealed *Fun. majalis* as the preferred host of *C. cyprinodontum* at both Sinepuxent Bay and Tolomato River sites. *C. cyprinodontum* infecting *Fun. majalis* in Sinepuxent Bay, showed a seasonal peak in epibiont load in summer months. This

seasonal pattern was reversed in Tolomato River, where epibiont load reached a maximum in winter months. *Crepidodinium cyprinodontum* density decreased with increasing fish length indicating smaller fish are more prone to colonization by *C. cyprinodontum*. Prevalence and epibiont load of *C. cyprinodontum* in *Fun. majalis* in Sinepuxent Bay was not correlated with any abiotic factors used in my analysis. However, I suspected this was due to the limited range in variables occurring during sample months. Finally, I documented infections in two previously unknown host species.

Next, I investigated the importance of irradiance on the rate of change in epibiont load and growth of *C. cyprinodontum* occurring on the gills of its host (*Fundulus majalis*). While epibiont load appeared unrelated to solar irradiance in field analysis, the strong seasonal peak in load occurring in summer months led me to hypothesize that light availability would influence *C. cyprinodontum* numbers per fish and growth of attached trophonts. I used an experimental approach to test this hypothesis exposing fish and epibiont to various irradiances, including complete darkness, over a 9-day period. Results from experiments indicated that the number of *C. cyprinodontum* per fish was heavily influenced by light availability, with abandonment of hosts occurring rapidly in low light and darkness. Light appeared to have an effect on the growth of *C. cyprinodontum*, as biomass of trophonts and tomonts held at high light was greater than those epibionts held in complete darkness. However, this conclusion is tentative, as there was no statistical support for growth in the light, or loss of biomass in the dark when total biomass recovered during incubations was compared to that occurring at the start of the experiment. Biomass based calculations of doubling time indicated that

growth of *C. cyprinodontum* is slow relative to other photosynthetic, heterotrophic, and mixotrophic dinoflagellates.

Results from field observations indicate that *Crepidodinium cyprinodontum* shows a preference among host taxa. The extent to which the preference is driven by habitat selection of *Fun. majalis*, relative to other host species, should be further examined. Furthermore, experiments assessing host preference among host species should be conducted utilizing the method developed here for collecting tomons. Data from experimental incubations indicate that *C. cyprinodontum* is an obligate phototroph, highly dependent on light for survival and growth. Overall, the growth of *C. cyprinodontum* on fish gills appears to be low at optimum irradiances. This suggests refuge from predation may be a major factor in driving this dinoflagellate to colonize the opecular region of fish.

Further study is required to elucidate the mode of infection in *C. cyprinodontum*. A major question raised by the research presented here is how does *C. cyprinodontum* reach such high densities on fish gills with such low growth rates. Documentation of the infection process, and in particular whether sporulation occurs on host gills, may help explain not only the high densities occurring on smaller fish, but also fish to fish variation within a population . Also, the trophic status of *C. cyprinodontum* needs further examination. Quantification of the contribution of photosynthesis to cell energy budget and/or isotopic analysis would more accurately assess whether or not *C. cyprinodontum* is a mixotrophic dinoflagellate able to acquire carbon and/or growth factors from its host.

APPENDIX I

Comparison of *Crepidoodinium cyprinodontum* load estimated by different techniques.

Enumeration of all *C. cyprinodontum* present on gills of hosts is time consuming, requiring as long as 30 minutes when epibiont load is high (≥ 600 epibionts/fish). To reduce processing time and enable larger sample size for field and laboratory studies, I chose to enumerate epibionts present on a subset of gill arches from each fish as described in Chapters 2 and 3. Here, I present statistical justification for that approach.

Seventy *Fundulus majalis* collected from Sinepuxent Bay, Assateague Island, MD were sacrificed and dissected following standard protocol, with *C. cyprinodontum* present on each gill arch recorded separately. Data were then parsed in three ways: (1) all epibionts present per fish; (2) epibionts present on two randomly selected gill arches from the right and left side of each fish, and (3) epibionts present on one randomly selected gill arch from the right and left side of each fish. Data were then compared using statistical methods as explained below.

One-way ANOVA on log transformed data (Fig. I-1) showed no significant differences between mean epibiont load calculated from data obtained from counting *C. cyprinodontum* present on two, four, or eight gill arches per fish (One way ANOVA, $P = 0.863$, $P = 0.951$, respectively). Mean, standard error of the mean, and coefficient of variation for non-transformed data from the three approaches are provided in Table I-1. Plotting epibiont load from counts of two or four gill arches against that obtained from

counts of all eight arches (Fig I-2a,b) gave slopes approaching one (0.997 and 0.995, respectively).

For estimates of epibiont load in field samples, I enumerated the number of *Crepidoodinium cyprinodontum* on four gills per fish. This allowed for determination of epibiont load within 15 minutes of death of host. For experimental investigations, I determined epibiont load from two gills per fish. This allowed for the enumeration of epibiont load within approximately five minutes of death of host and for completion of sampling intervals within a 24 hour period.

Table I-1. Non-transformed mean epibiont load, standard error and coefficient of variation determined from counts of eight, four, and two gills.

	8 gills	4 gills	2 gills
Load	89.2	89.7	88.9
SE	13.65	13.60	13.62
CV	129.16	128.74	129.35

FIGURE LEGENDS

Fig. I.1 Mean epibiont load per fish estimated from scoring eight, four, or two gills. Values are not significantly different (One way ANOVA, $P > 0.5$). Data are reported as mean \pm SE following back transformation.

Fig. I.2 Plots of *Crepidodinium. cyprinodontum* load obtained from counts of eight gills versus epibiont load determined from of two (**A**) or four (**B**) randomly selected gill arches.

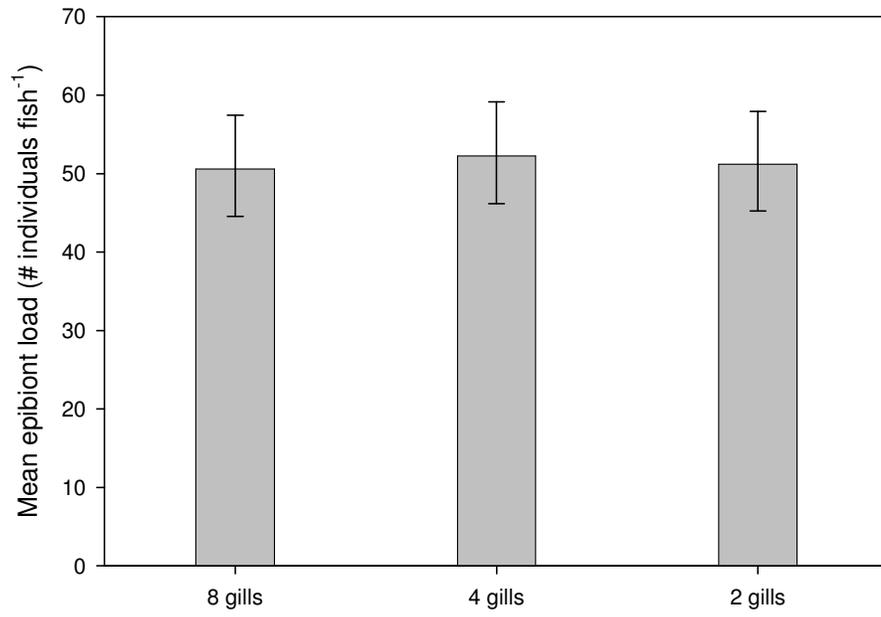


Figure I.1

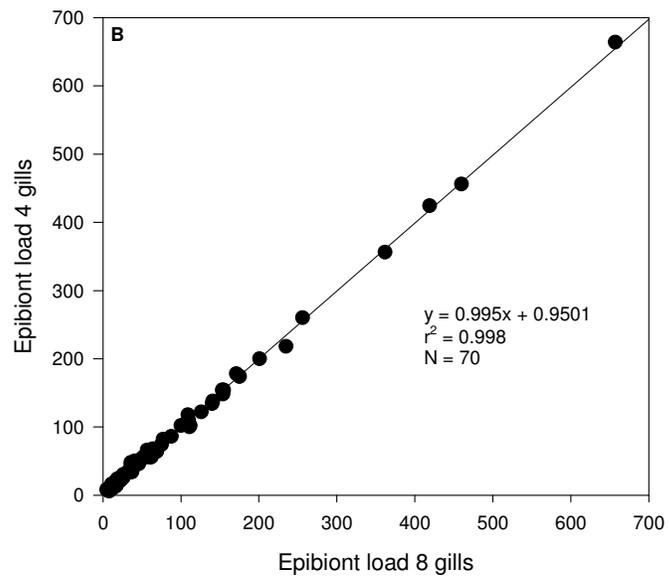
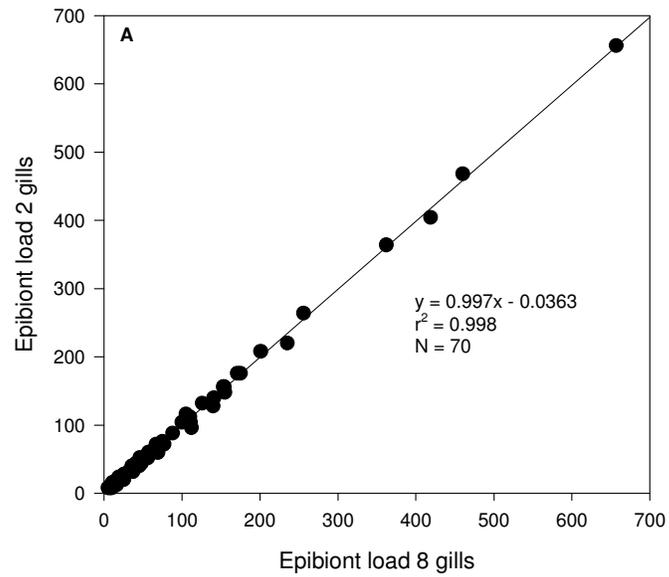


Figure I.2

APPENDIX II

Chlorophyll *a* concentration of *Crepidodinium cyprinodontum*

Here I present chlorophyll *a* (chl *a*) concentration of *Crepidodinium cyprinodontum* trophonts occurring on the gills of *Fundulus majalis*. Symbionts and hosts were collected from Sinepuxent Bay, Maryland and transported to the laboratory in aerated site water. Eleven fish were then randomly selected for estimates of epibiont load, biomass, and chlorophyll *a* content. For each fish, all gills were removed immediately upon sacrifice and placed in filtered site water, with gills from right and left side kept separate. Epibiont load was then determined from four (two per side) arbitrarily selected gills within 10 minutes of host death. Chl *a* was extracted from gills by placing all four scored gills in 90% acetone for 24 hours in the dark at 4°C and Chl *a* concentration determined using a Turner Designs 10-AU fluorometer.

To estimate biovolume of trophonts, remaining gills (four) were preserved in CaCO₃-buffered formalin (1% final concentration) and cells carefully dislodged from gills with a dissection needle. Cells were then settled in 10-ml settling chambers for one hour and length and width of at least 15 trophonts recorded and biovolume (μm³) calculated assuming trophont shape as a prolate sphere. Mean cell biovolume per fish was then multiplied by the epibiont load of four gills to determine total biovolume of *C. cyprinodontum* on gills. Chl *a* concentration was then normalized to cell volume by dividing the chl *a* concentration from four gills by total biovolume on those gills.

Chlorophyll *a* content of *C. cyprinodontum* averaged $1.07 \pm 0.2 \times 10^{-3}$ pg chl *a* (μm³)⁻¹.

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